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Triterpenoid saponins from the buds of *Lonicera similis*

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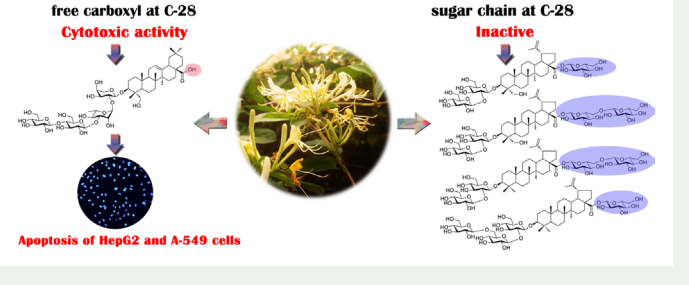
ABSTRACT

Four new lupane triterpenoid saponins, along with one known lupane and eight hederagenin saponins, were isolated from the EtOH extract of the buds of *Lonicera similis* Hemsl. The structures of the new compounds were established as 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl 23-hydroxybetulinic acid 28-*O*-*β*-D-glucopyranosyl ester (lonisimilioside A, **1**), 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl 23-hydroxybetulinic acid 28-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester (lonisimilioside B, **2**), 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl betulinic acid 28-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester (lonisimilioside C, **3**) and 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl betulinic acid 28-*O*-*β*-D-glucopyranosyl ester (lonisimilioside D, **4**), respectively. The cytotoxic activities of the isolates against human cancer cell lines HepG2, MCF-7 and A-549 were evaluated. Only the monodesmosidic saponin with a free carboxyl group at C-28 (**12**) exhibited significant cytotoxicities against HepG2, MCF-7 and A-549 cell lines with the IC_{50} values of 8.98 ± 0.19, 12.48 ± 0.45 and 11.62 ± 0.54 μM, respectively. Furthermore, Hoechst fluorescence 33342 staining was used to demonstrate that **12** could induce HepG2 and A-549 cells apoptosis significantly.



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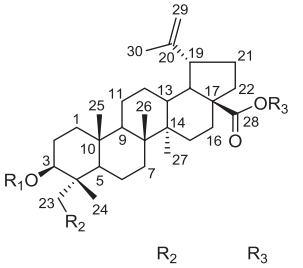
1. Introduction

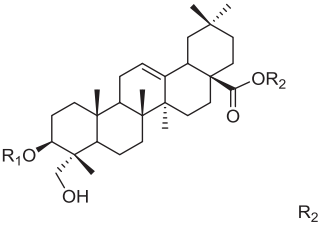
The buds of *Lonicera japonica* Thunb. (Caprifoliaceae), a well-known traditional Chinese medicine ('jin yin hua' in Chinese), have been used to treat encephalitis, fever, influenza, dysentery, enteritis and pneumonia for many years (Ko et al. 2006; Shang et al. 2011; Tian et al. 2012; Han et al. 2015). Previous investigations on *L. japonica* have led to the isolation of many secondary metabolites, such as triterpenoid saponins, flavonoids, organic acids and iridoids with a lot of bioactivities (Kumar et al. 2005; Choi et al. 2007; Lin et al. 2008; Yu et al. 2013; Li et al. 2015, 2017). Among them, triterpenoid saponins and chlorogenic acid derivatives are the main bioactive components of *L. japonica*. The buds of *L. similis* Hemsl. are used as a substitute for 'jin yin hua' in Sichaun province, but no triterpenoid saponins has been reported from *L. similis* so far (Li et al. 2001; Zheng et al. 2012; Zhang et al. 2015, 2016). In our search for bioactive saponins, the *n*-butanol soluble portion of the 95% EtOH extract of *L. similis* was phytochemically investigated, affording four new and one known lupane triterpenoid saponins (**1**–**5**) and seven hederagenin triterpenoid saponins (**6**–**12**) (Figure 1). In the *in vitro* assay, compound **12** displayed significant cytotoxic activities against HepG2, MCF-7 and A-549 cell lines, whereas other saponins were inactive. Furthermore, the apoptosis of HepG2 and A-549 cells induced by **12** was studied using Hoechst fluorescence 33342 staining.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. Its molecular formula was determined to be $C_{48}H_{78}O_{19}$ on the basis of a HRESIMS peak at *m/z* 981.5040 $[M + Na]^+$ (calcd for $C_{48}H_{78}O_{19}Na$, 981.5035), indicating 10 degrees of unsaturation for **1**. The ^1H and ^{13}C NMR spectra of **1** showed resonances attributable to five tertiary methyl groups (δ_H 1.70, 1.01, 0.96, 0.89 and 0.70; δ_C 19.5, 17.2, 16.7, 15.1 and 13.1), an isolated oxymethylene [δ_H 3.74 (d, J = 11.4 Hz) and 3.26 (d, J = 11.4 Hz); δ_C 64.7], an oxymethine [δ_H 3.63 (dd, J = 12.0, 6.0 Hz); δ_C 84.1], an exocyclic double bond [δ_H 4.72 (brs) and 4.60 (brs); δ_C 151.8 and 110.3] and a carboxylic carbon (δ_C 176.1) (Table S1, Supplementary Material). The above spectroscopic features suggested the presence of a $\Delta^{20(29)}$-lupane-type aglycone with a hydroxymethyl and a carboxylic group (Ye et al. 1996; Yang et al. 2010). In addition, the ^1H NMR spectrum



	R_1	R_2	R_3
1	Glc-(1→2)-Glc	OH	Glc
2	Glc-(1→2)-Glc	OH	Glc-(1→6)-Glc
3	Glc-(1→2)-Glc	H	Glc-(1→6)-Glc
4	Glc-(1→6)-Glc-(1→2)-Glc	H	Glc
5	Glc	H	Glc-(1→6)-Glc



	R_1	R_2
6	Glc	Glc
7	Ara	Glc-(1→6)-Glc
8	Glc-(1→2)-Glc	Glc
9	Rha-(1→2)-Ara	Glc-(1→6)-Glc
10	Glc-(1→2)-Glc	Glc-(1→6)-Glc
11	Glc-(1→2)-Rha-(1→2)-Ara	Glc-(1→6)-Glc
12	Glc-(1→4)-Glc-(1→3)-Rha-(1→2)-Ara	H

Figure 1. Chemical structures of compounds **1**–**12**.

of **1** exhibited diagnostic signals corresponding to three anomeric protons of sugar units at δ_H 5.49, 4.68 and 4.51, which were correlated with the anomeric carbon signals at δ_C 95.2, 104.5 and 104.5 in the HSQC spectrum, respectively (Table S2, Supplementary Material). The three sugar units were further deduced to be *β*-glucose residues based on the coupling constants of the anomeric protons (J = 7.2, 7.8 and 8.4 Hz), together with the ^{13}C signals for 12 oxymethines (δ_C 71.7–81.4) and three oxymethylenes (δ_C 62.4, 62.8 and 63.0) in the sugar units (Table S2, Supplementary Material). This conjecture was verified by an enzyme hydrolysis that afforded *β*-D-glucose as the sole sugar, identified by TLC comparison and the positive optical rotation (Zi et al. 2008; Li et al. 2016).

Detailed analysis of 2D NMR data (HSQC, ^1H-^1H COSY and HMBC) revealed that the aglycone in **1** was 23-hydroxybetulinic acid (Ye et al. 1996), which was confirmed by the enzymatic hydrolysis. However, the resonances for H-3 and C-3 in **1** were deshielded significantly, while the resonance for C-28 in **1** was shielded, as compared to 23-hydroxybetulinic acid. Thus, compound **1** was a bisdesmosidic saponin of 23-hydroxybetulinic acid containing two *β*-D-glucose chains at C-3 and C-28. Furthermore, HMBC correlations of H-1′ (δ_H 4.51) with C-3 (δ_C 84.1) and of H-1‴ (δ_H 5.49) with C-28 (δ_C 176.1) confirmed the above attachments. The location of another *β*-D-glucose residue at C-2′ position was determined by HMBC correlation of H-1″ (δ_H 4.68) with C-2′ (δ_H 81.4) and the significant glycosidation shift of C-2′. Thus, compound **1** was established to be 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl 23-hydroxybetulinic acid 28-*O*-*β*-D-glucopyranosyl ester and named lonisimilioside A.

Compound **2** had spectroscopic data very similar to those of **1**. The molecular formula of **2** was determined to be $C_{54}H_{88}O_{24}$ by a HRESIMS peak at *m/z* 1143.5559 $[M + Na]^+$ (calcd for $C_{54}H_{88}O_{24}Na$, 1143.5563), with one more glucose residue than **1**. Both the enzymatic hydrolysis and the NMR data confirmed that compound **2** was also a 23-hydroxybetulinic acid *β*-D-glucoside. However, the resonances for an additional *β*-D-glucose residue were observed in the ^1H and ^{13}C NMR spectra of **2** [δ_H 4.34 (d, J = 7.8 Hz, anomeric proton); δ_C 104.7 (anomeric carbon)] (Table S2, Supplementary Material). In addition, the resonances for H_2-6‴ and C-6‴ in **2** were deshielded by $\Delta\delta_H$ + 0.30 and +0.08, and $\Delta\delta_C$ + 7.1, respectively, when compared to those of **1**. These downfield shifts indicated that the additional *β*-D-glucose residue was linked to the 28-*O*-*β*-D-glucopyranosyl through 1→6 glucosidic band. Finally, the structure of **2** was further confirmed by 2D NMR data analysis. Particularly, HMBC correlations of H-1′ (δ_H 4.51) with C-3 (δ_C 84.1), of H-1″ (δ_H 4.68) with C-2′ (δ_H 81.4), of H-1‴ (δ_H 5.48) with C-28 (δ_C 176.2) and of H-1⁗ (δ_H 4.34) with C-6‴ (δ_C 69.5) revealed the linkage of *β*-D-glucose residues. Therefore, compound **2** was determined to be 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl 23-hydroxybetulinic acid 28-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester and named lonisimilioside B.

Compound **3** possessed the molecular formula $C_{54}H_{88}O_{23}$ with one less oxygen atom than **2**, as indicated by a HRESIMS peak at *m/z* 1127.5616 $[M + Na]^+$ (calcd for $C_{54}H_{88}O_{23}Na$, 1127.5614). The spectroscopic features of **3** were similar to those of **2**, except that the NMR resonances of the isolated oxymethylene group in **2** were replaced by those of a tertiary methyl group [δ_H 1.02 (s) and δ_C 28.3] in **3** (Table S1, Supplementary Material). In addition, the resonances for C-3 and C-5 in **3** were deshielded significantly by $\Delta\delta_C$ + 7.4 and + 8.6 ppm, respectively. In contrast, the resonance for C-4 in **3** was shielded by $\Delta\delta_C$ −3.7 ppm. The above spectroscopic data revealed that the aglycone of **3** was betulinic acid (Gossan et al. 2016), which was confirmed by 2D NMR experiments and enzymatic hydrolysis. Detailed analysis

of HMBC correlations of the anomeric protons in four *β*-D-glucose residues (δ_H 5.45, 4.64, 4.40 and 4.32) indicated that **3** had the same glucosidic bands as **2**. Therefore, compound **3** (lonisimilioside C) was determined to be 3-*O*-*β*-D-Glucopyranosyl-(1→2)-*β*-D-glucopyranosyl betulinic acid 28-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester.

The spectroscopic data of **4** showed that it was another betulinic acid *β*-D-glucoside with the same molecular formula as **3**. The main difference between **4** and **3** was the linkage of the four *β*-D-glucose residues. Detailed analysis of 2D NMR data indicated that the sugar chain at C-3 position possessed three *β*-D-glucose residues, while the sugar chain at C-28 position had only one *β*-D-glucose residue. In particular, the interglycosidic linkages were established by HMBC correlations from H-1′ (δ_H 4.40) to C-3 (δ_C 91.4), from H-1′′ (δ_H 4.64) to C-2′ (δ_H 81.2) and from H-1′′′′ (δ_H 4.49) to C-6′′ (δ_C 69.7). Thus, compound **4** was elucidated to be 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl betulinic acid 28-*O*-*β*-D-glucopyranosyl ester and named lonisimilioside D.

The known triterpene saponins were identified as 3-*O*-*β*-D-glucopyranosyl betulinic acid 28-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester (**5**) (Wang et al. 1996), 3-*O*-*β*-D-glucopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester (**6**) (Liu et al. 2013), 3-*O*-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside (**7**) (Choi and Woo 1987), 3-*O*-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl] hederagenin 28-*O*-*β*-D-glucopyranosyl ester (**8**) (Zhang et al. 2013), 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester (**9**) (Chen et al. 2008), helixoside A (**10**) (Bedir et al. 2000), 3-*O*-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl ester (**11**) (Yoshimitsu et al. 2007), hederagenin 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranoside (**12**) (Bang et al. 2005) by spectroscopic analysis and comparison with the literature data.

Since triterpenoid saponins have remarkable anticancer activity (Jeong et al. 2008; Xu et al. 2016), the cytotoxic activities of all compounds were determined by an MTT colorimetric assay using HepG2, MCF-7 and A-549 human cancer cell lines. Interestingly, only the monodesmosidic saponin (**12**) showed cytotoxic effects against HepG2, MCF-7 and A-549 cell lines with IC_{50} values of 8.98 ± 0.19, 12.48 ± 0.45 and 11.62 ± 0.54 μM, respectively, while all bisdesmosidic saponins (**1**–**11**) were inactive (IC_{50} > 50 μM). It can be inferred that glycosylation of COOH in 23-hydroxybetulinic acid, betulinic acid and hederagenin saponins resulted in a significant loss of the inhibitory effects on the growth of HepG2, MCF-7 and A-549 cell lines. This deduction was consistent with the previous literature conclusion (Podolak et al. 2010). Furthermore, Hoechst fluorescence 33342 staining was used for investigating the apoptosis-inducing effect of **12** on HepG2 and A-549 cells. The nuclei membranes of the intact cells were clear and complete, and the nuclei of the intact cells were regular in shape. At concentrations of 2.5, 5 and 10 μM, compound **12** caused obvious apoptotic morphological changes of HepG2 cells, particularly the nuclei fragmentation and condensed chromatin (Figure 2(A)). The apoptosis effect of **12** on HepG2 cells enhanced with increasing dosage. Similarly, compound **12** could induce apoptosis of A549 cells at concentrations of 5, 10 and 15 μM (Figure 2(B)). The significant induction of apoptosis confirmed the cytotoxic activities of **12** against HepG2 and A-549 cells.

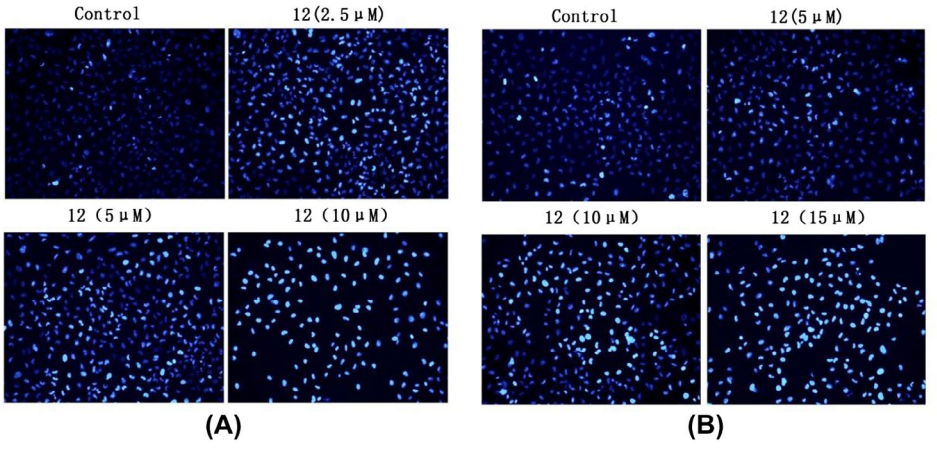


Figure 2. Nuclear DNA staining of HepG2 cells (A) and A-549 cells (B). Cell apoptosis was assayed by Hoechst 33342 fluorescence staining to detect chromosomal condensation and nuclei fragmentation (magnification × 400).

3. Experimental

3.1. General

Optical rotations were measured using a SGW®-2 automatic polarimeter (Shanghai Precision Scientific Instrument Corporation, Shanghai, China). NMR spectra were obtained using an AVIIIHD-600 NMR spectrometer (Bruker Corporation, Billerica, MA, USA). Solvent peaks were used as references. HRESIMS were measured using a Synapt G2 HDMS instrument (Waters Corporation Milford, MA, USA). Column chromatography was performed using silica gel (200–300 mesh; Yantai Institute of Chemical Technology, Yantai, China), Macroporous resin (D-101; Chengdu Kelon Chemical Reagent Factory, Chengdu, China) and Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). HPLC separations were performed using a CoMetro instrument (CoMetro Technology Ltd., South Plainfield, NJ, USA) equipped with a CoMetro 6000 LDS pump, a CoMetro 6000 PVW UV/vis detector and an Ultimate (250 × 10 mm^2) preparative column packed with C_{18} (5 μm). TLC was performed using glass plates precoated with silica gel (GF254; Qingdao Marine Chemical Inc., Qingdao, China). Cell apoptosis was assayed by Hoechst 33342 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and imaged by Leica DMI 3000B.

3.2. Plant material

The buds of *L. similis* were collected in Nanjiang City (Sichuan, China). The plant was authenticated by Prof. Min Li (Chengdu University of TCM, Sichuan, China). A voucher specimen (XZMRD-1) was deposited at State Key Laboratory Breeding Base of Systematic Research, Development and Utilisation of Chinese Medicine Resources, Chengdu University of TCM.

3.3. Extraction and isolation

The buds of *L. similis* (9.5 kg) were macerated in 95% EtOH (3 × 60 L) at room temperature for 3 × 24 h. The EtOH extract was evaporated under reduced pressure to obtain a dark brown

residue (2.4 kg). The residue was suspended in H_2O and successively partitioned into EtOAc and *n*-BuOH fractions. The *n*-BuOH fraction (650 g) was chromatographed over a D-101 macroporous resin column. Successive elution with 10, 30, 50, 70 and 95% EtOH in H_2O afforded five portions (A-E). Portion C (125 g) was subjected to polyamide column chromatography with a gradient elution of increasing EtOH (10–95%) in H_2O to afford five fractions (C_1–C_5). Fraction C_3 (15.6 g) was subjected to RP-MPLC eluted with a gradient of MeOH-H_2O (10:90–100:0) to provide 11 subfractions ($C_{3\text{-}1}$-$C_{3\text{-}11}$). Subfraction $C_{3\text{-}10}$ was separated by silica gel chromatography column using a gradient elution of CH_2Cl_2-MeOH (20:1–0:1) to afford 15 subfractions ($C_{3\text{-}10\text{-}1}$–$C_{3\text{-}10\text{-}15}$). Subfraction $C_{3\text{-}10\text{-}3}$ was further purified via PTLC (CH_2Cl_2-MeOH-H_2O, 7:3:0.5) followed by RP semi-preparative HPLC (83% MeOH in H_2O) to yield **6** (5 mg). Subfractions $C_{3\text{-}10\text{-}5}$ and $C_{3\text{-}10\text{-}6}$ were purified by preparative TLC (CH_2Cl_2-MeOH-H_2O, 7:3:0.5) followed by RP semi-preparative HPLC (72% MeOH in H_2O) to yield **12** (5 mg) and **4** (9 mg), respectively. Successive purification of $C_{3\text{-}10\text{-}15}$ using preparative TLC (CH_2Cl_2-MeOH-H_2O, 7:3:0.5) and RP semi-preparative HPLC (60% MeOH in H_2O) afforded **1** (8 mg), **5** (10 mg), **7** (9 mg) and **8** (8 mg). Separation of $C_{3\text{-}11}$ using repeated Sephadex LH-20 (MeOH-H_2O, 8:1) and RP semi-preparative HPLC (55% MeOH in H_2O) successively yielded **2** (9 mg), **3** (10 mg), **9** (9 mg), **10** (9 mg) and **11** (9 mg).

3.4. *Lonisimilioside A* (1)

White amorphous powder; $[\alpha]_D^{25}$ −17.7 (*c* 0.13, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data see Tables S1 and S2 in Supplementary Material; (+)-HRESIMS *m/z* 981.5040 $[M + Na]^+$ (calcd. for $C_{48}H_{78}O_{19}Na$, 981.5035).

3.5. *Lonisimilioside B* (2)

White amorphous powder; $[\alpha]_D^{25}$ −10.4 (*c* 0.38, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data see Tables S1 and S2 in Supplementary Material; (+)-HRESIMS *m/z* 1143.5559 $[M + Na]^+$ (calcd. for $C_{54}H_{88}O_{24}Na$, 1143.5563).

3.6. *Lonisimilioside C* (3)

White amorphous powder; $[\alpha]_D^{25}$ −15.1 (*c* 0.09, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data see Tables S1 and S2 in Supplementary Material; (+)-HRESIMS *m/z* 1127.5616 $[M + Na]^+$ (calcd. for $C_{54}H_{88}O_{23}Na$, 1127.5614).

3.7. *Lonisimilioside D* (4)

White amorphous powder; $[\alpha]_D^{25}$ −13.5 (*c* 0.05, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data see Tables S1 and S2 in Supplementary Material; (+)-HRESIMS *m/z* 1127.5613 $[M + Na]^+$ (calcd. for $C_{54}H_{88}O_{23}Na$, 1127.5614).

3.7. *Enzymatic hydrolysis of saponins* 1–4

A solution of each saponin (5 mg) in 1% HOAc (4 mL) was individually hydrolysed with cellulase (5 mg) in a sealed tube at 37°C for 3 d. Each reaction mixture was diluted with H_2O

and extracted with EtOAc to yield the individual EtOAc extract and H_2O residuum after removing the solvents. The EtOAc extracts were separately purified via preparative TLC (CH_2Cl_2-MeOH, 25:1) to afford aglycones **1a** (1.4 mg), **2a** (1.3 mg), **3a** (1.5 mg) and **4a** (1.1 mg). Compounds **1a** and **2a** were identified as 23-hydroxybetulinic acid (Ye et al. 1996), **3a** and **4a** were identified as betulinic acid (Gossan et al. 2016) by spectroscopic analysis and comparison with literature data. The aqueous residua were separately subjected to chromatography column over silica gel (MeCN-H_2O, 8:1) to yield the glucose with a positive optical rotation. The glucose was also identified by TLC analysis (MeCN-H_2O, 5:1) with a D-glucose authentic sample.

3.8. Cytotoxic activity assay

Cytotoxic activity evaluations were performed for three human tumour cell lines (HepG2, MCF-7, and A-549) by the MTT method described in previous literatures (He et al. 2015; Peng et al. 2016). Taxol was used as the positive control. The IC_{50} values represented the mean of three independent replicates, and $IC_{50} > 50$ μM was considered to be inactive.

3.9. Hoechst 33342 fluorescence staining

Hoechst 33342 fluorescence staining was used to visualise nuclear DNA in live and dead cells. At the end of the treatment of each compound on cancer cells, the medium containing the test sample was replaced by a fresh blank medium. Then an aliquot of 100 μL of counterstain/fixation solution (6 μL of Hoechst 33342 and 3 mL of 16% paraformaldehyde in 9 mL PBS) was added. The wells were incubated for 15 min at room temperature, after which the counterstain/fixation solution was removed and the wells were washed with PBS. After adding PBS to each well, the cells were observed under a microscope (ImagExpress micro XLS).

Disclosure statement

No potential conflict of interest was reported by the authors.

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