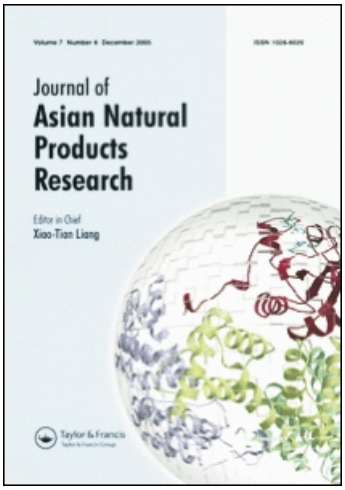

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C_{28} sterols with a cyclopentane ring at C-22 and 26 from cape gooseberry (berries of *Physalis pubeacens* L.)

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ORIGINAL ARTICLE

C_{28} sterols with a cyclopentane ring at C-22 and 26 from cape gooseberry (berries of *Physalis pubeacens* L.)

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Two new C_{28} sterols with a cyclopentane ring at C-22 and 26, alkesterol A (**1**) and alkesterol B (**2**), along with β-sitosterol (**3**), were isolated from the berries of *Physalis pubeacens* L. (cape gooseberry). The structures of the new compounds were established by HR-EI-MS, 1D and 2D (^{1}H–^{1}H COSY, HSQC, HMBC) NMR experiments. The known compound was identified by comparison of spectral data with published references. The two new compounds showed some cytotoxic activities by MTT assay.

Keywords: *Physalis pubeacens* L.; C_{28} sterols; alkesterol A; alkesterol B; cytotoxic activities

1. Introduction

Cape gooseberry (*Physalis pubeacens* L.) has long been a minor fruit of the northeast region of China and has also been grown in America [1]. The fruit of the *Physalis* genus is also extensively used as a herb medicine, and has the effects of detoxification and anti-inflammation in the traditional Chinese medicine [2]. Currently, the extracts of fruits of the *Physalis* genus have been reported to have potent antioxidant [3], anti-inflammatory [3], and cytotoxic activities [4]. During the course of our investigation for cytotoxic agents from the chloroform-soluble fraction of the EtOH extract of berries of *P. pubeacens* L., two new C_{28} sterols with a cyclopentane ring at C-22 and 26, alkesterol A (**1**) and alkesterol B (**2**), together with one known sterol, β-sitosterol (**3**) [5], were obtained. Cytotoxic activities of these sterols against human HeLa, SMMC-7721, and HL-60 tumor cell lines were evaluated. In this paper, we describe the isolation and structural elucidation of the two new compounds, as well as the evaluation of their cytotoxic activities.

2. Results and discussion

Compound **1** was isolated as colorless needles with a molecular formula of $C_{28}H_{44}O_2$ as indicated by the high-resolution mass measurement. Its ^{13}C NMR spectrum (Table 1) accounted for the 28 carbons in its molecular formula and presented four signals at δ 140.8 (s), 121.7 (d), 150.5 (s), and 109.5 (t) in the olefinic carbon region, indicating the presence of two double bonds, and two signals at δ 75.5 (s) and 71.8 (d) that were assigned to carbons bearing tertiary and secondary hydroxyl groups, respectively. The ^{1}H NMR spectrum (Table 1) presented four signals at δ 1.30 (3H, s), 1.03

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Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data of compounds **1** and **2** ($CDCl_3$, δ in ppm).

	1		**2**	
	δ_C	δ_H	δ_C	δ_H
1	37.3	1.84 m	37.2	1.85 m
2	31.7	1.84 m	31.6	1.85 m
3	71.8	3.51 m	71.8	3.52 m
4	42.3	2.27 m	42.3	2.27 m
5	140.8	–	140.8	–
6	121.7	5.35 dd (7.2, 5.5)[a]	121.7	5.35 dd (7.2, 5.5)
7	31.9	1.95 m	31.9	1.96 m
8	31.9	1.54 m	31.9	1.54 m
9	50.1	0.89 m	50.1	0.90 m
10	36.5	–	36.5	–
11	21.1	1.50 m	21.1	1.50 m
12	39.8	2.00 m	39.8	1.98 m
13	42.3	–	42.4	–
14	56.8	0.95 m	56.8	0.95 m
15	24.3	1.10 m	24.3	1.10 m
16	28.2	1.84 m	28.2	1.84 m
17	55.8	1.15 m	55.9	1.16 m
18	11.8	0.67 s	11.8	0.67 s
19	19.4	1.03 s	19.4	1.02 s
20	35.8	1.41 m	35.9	1.42 m
21	18.8	0.88 d (7.4)	18.8	0.88 d (7.4)
22	27.8	1.26 m	29.2	1.25 m
23	29.7	1.28 m, 1.45 m	27.5	1.84 m, 1.29 m
24	75.5	–	73.6	–
25	150.5	–	156.8	–
26	36.5	1.49 m	35.5	1.28 m
27	109.5	4.96 d, 4.82 d (2.0)	106.7	5.09 d, 4.76 d (2.0)
28	27.7	1.30 s	29.3	1.35 s

Note: [a] Data in parentheses are J values (in Hz).

(3H, s), 0.88 (3H, d, $J = 7.4$ Hz), and 0.67 (3H, s), which were assigned to four methyl groups. Three proton signals at δ 5.35 (1H, dd, $J = 7.2, 5.5$ Hz), 4.96 (1H, d, $J = 2.0$ Hz), and 4.82 (1H, d, $J = 2.0$ Hz) were assigned to two double bonds. This spectroscopic evidence suggested a pentacyclic steroidal for compound **1**. The analysis of the correlations observed in the COSY spectrum made us confirm the presence of the usual secondary hydroxyl at C-3 as well as a C-5, C-6 double bond in compound **1**, which was verified by the existence of the fragment ion peak at *m/z* 273 resulting from the cleavage of the side chain of the molecule of compound **1** in the EI-MS spectrum, as well as the fragment ion peak at *m/z* 327 resulting from the cleavage of ring A from the molecule [6].

Except for the four rings in the steroid nucleus, another ring appeared to be located at the side chain. The existence of two methyl groups and a terminal alkene in the side chain suggested a cyclopentane ring located at C-22 and 26, which was supported by the correlation between C-26 at δ 36.5 (t) and H-20 at δ 1.41 (1H, m), as well as the correlation between H-21 at δ 0.88 (3H, d, $J = 7.4$ Hz) and C-22 at δ 27.8 (d) in the HMBC spectrum (Figure 1). The tertiary hydroxyl group was located at C-24 as determined by the HMBC correlations between the

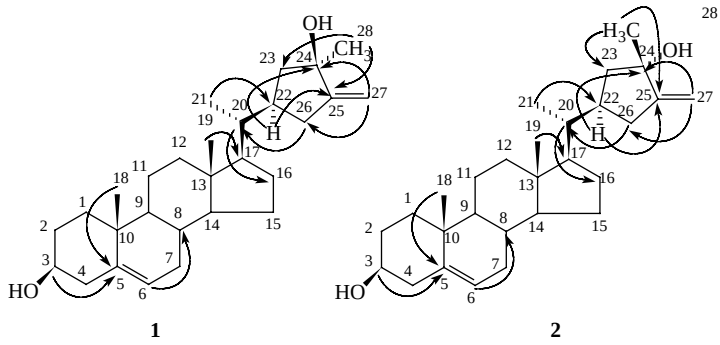

Figure 1. Key HMBC correlations of compounds **1** and **2**.

carbon signal at δ 75.5 (s) and the signals at δ 1.26 (1H, m) and 4.96, 4.82 (2H, d, $J = 2.0$ Hz), which were assigned to H-22 and H-27, respectively.

The relative stereochemistry of compound **1** was further established from its nuclear Overhauser effect spectroscopy (NOESY) spectrum (Figure 2). Interactions between H-22 (δ 1.26) and Me-28 (δ 1.30) indicated that they were on the same side of the cyclopentane ring in the side chain.

The absolute configuration of compound **1** was determined by the application of the circular dichroism (CD) spectrum. Compound **1a** was prepared by the oxidation reaction of the hydroxyl group at C-3 using Oppenauer oxidation. The benzoic acid ester (**1b**) of compound **1a** was prepared using the benzoyl chloride. The CD spectrum of compound **1b** exhibited a negative cotton effect at λ 228.5 (-10.1), indicating that the benzoxy group was oriented in an anticlockwise manner in accordance with the double bond at C-25 (Figure 3). Since the overall relative configuration was known, the absolute configuration of compound **1** was established as 22*R*, 24*R* [7].

Compound **2** was isolated as colorless needles with a molecular formula of $C_{28}H_{44}O_2$ as indicated by the high-resolution mass measurement. In comparison of the EI-MS, ^{1}H NMR and ^{13}C NMR spectral data with those of compound **1**, compound **2** was postulated to be a stereoisomer of compound **1**.

The relative stereochemistry of compound **2** was further established from its NOESY spectrum (Figure 2). Interactions between H-20 (δ 1.42) and Me-28 (δ 1.35) indicated that they were on the same side of the cyclopentane ring in the side chain.

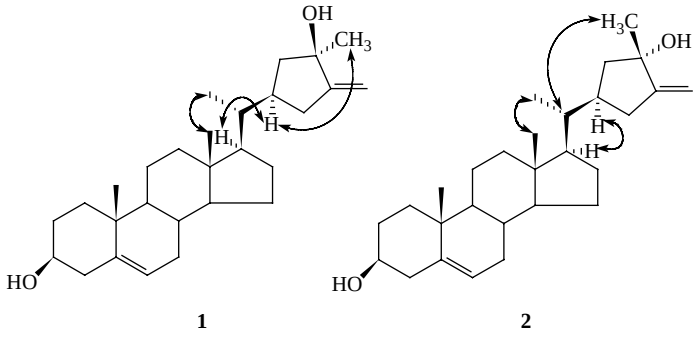

Figure 2. Key NOESY correlations of compounds **1** and **2**.

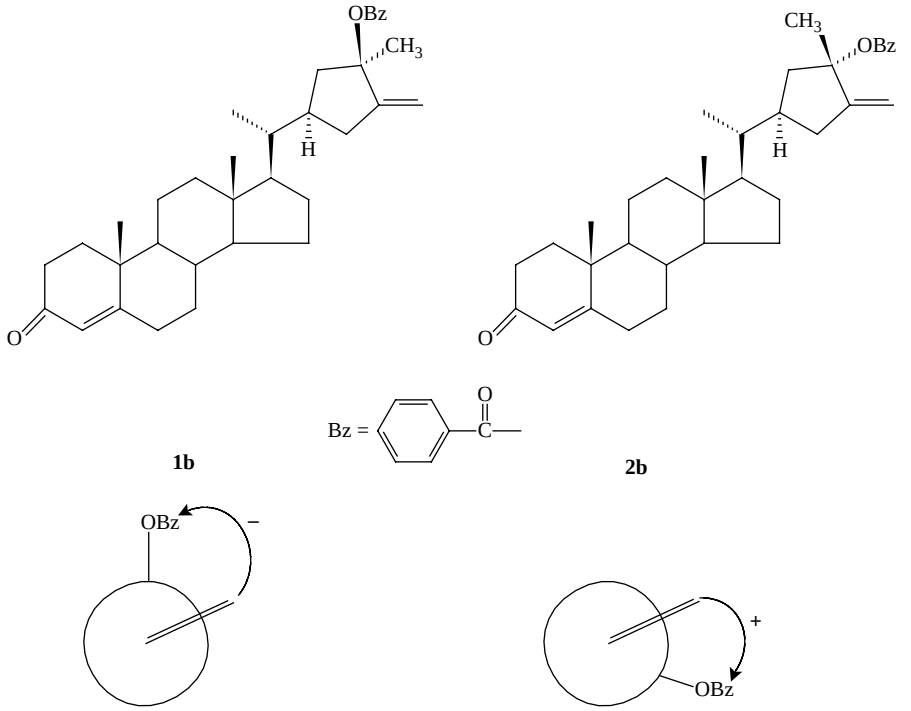

Figure 3. The orientation of dibenzoates according to the double bond in compounds **1** and **2**.

The absolute configuration of compound **2** was also determined by the application of the CD spectrum. Compound **2a** was prepared by the oxidation reaction of the hydroxyl group at C-3 using Oppenauer oxidation. The benzoic acid ester (**2b**) of compound **2a** was prepared using benzoyl chloride. The CD spectrum of compound **2b** exhibited a positive cotton effect at λ 227.5 (+8.6), indicating that the benzoxy group was oriented in a clockwise manner in accordance with the double bond at C-25 (Figure 3). Since the overall relative configuration was known, the absolute configuration of compound **2** was established as 22*R*, 24*S*.

In the case of bioactivities of compounds **1**–**3**, their cytotoxic activities were expressed as IC_{50} values. As determined by MTT assay, the IC_{50} values of compounds **1**–**3** for HeLa carcinoma cell lines were 22, 19, and 71 μg/ml, respectively, for SMMC-7721 carcinoma cell lines were 30, 36, and 88 μg/ml, and for HL-60 carcinoma cell lines were 16, 21, and 95 μg/ml.

3. Experimental

3.1 General experimental procedures

Optical rotations were obtained on a Perkin-Elmer model 241 polarimeter. IR spectra were taken on a Perkin-Elmer 983 G spectrometer. ^{1}H, ^{13}C NMR and 2D NMR spectra were recorded on a Varian Inova 600 spectrometer in $CDCl_3$ using tetramethylsilane (TMS) as the internal standard. EI-MS and HR-EI-MS spectra were determined on a Micromass Zabspec spectrometer. HR-ESI-MS spectra were determined on a Q-TOF2 spectrometer. CD spectra were determined on a JASCO-715 spectrometer. Semi-preparative HPLC was carried out on a column of ODS (250 × 9.4 mm i.d.; Agilent Zorbax SB-Phenyl, Palo Alto, CA, USA) with a Waters 2996 detector, and the flow rate

was 2 ml/min and the wavelength for detection was 210 nm. Medium pressure liquid chromatography (MPLC) was carried out on a column of silica gel H (460 × 26 mm i.d.; Buchi Borosilikat 4.6, Flawil, Switzerland). Silica gel (200–300 mesh) for column chromatography was obtained from Qingdao Marine Chemical Factory, Qingdao, China. Precoated plates of silica gel used for TLC were obtained from Qingdao Marine Chemical Factory. Compounds on the TLC were colored by 10% sulfuric acid–alcohol solution. 4-Dimethylaminopyridine (DMAP), aluminum isopropoxide, cyclohexanone, and benzoyl chloride were purchased from Sinopharm Chemical Reagent Corporation Limited, Shanghai, China.

3.2 Plant material

The berries of *P. pubeacens* L. were collected in Qiqihaer, Helongjiang Province of China in November 2006, and identified by Prof. Jian-Wen Wang of our college. A voucher sample (S 2006-11-1) is deposited in the Herbarium of the College of Pharmacy, Soochow University.

3.3 Extraction and isolation

The dried plant material (10 kg) was extracted three times with 95% EtOH (120 liters) under reflux. The solvent was subsequently dried under reduced pressure to give the residue (1.12 kg), which was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$-soluble fraction (51.5 g) was further partitioned between petroleum ether and 90% MeOH (v/v). The 90% MeOH fraction (32.2 g) was chromatographed over a silica gel column (60.0 cm × 5.0 cm i.d.), which was eluted with a petroleum ether–EtOAc gradient (0–100%), to afford 20 fractions. Subfraction 6 (3.28 g) was isolated by MPLC eluted with petroleum ether–EtOAc (80:20) to give β-sitosterol (**3**, 825 mg, yield 82.5 mg/kg), and subfraction 10 (0.16 g) was subjected to semi-preparative RP-HPLC with 80% MeOH to give alkesterol A (**1**, 62.8 mg, yield 6.28 mg/kg) and alkesterol B (**2**, 23.0 mg, yield 2.30 mg/kg).

3.3.1 Alkesterol A (*1*)

Colorless needles, $[\alpha]_D^{25} = -19.86$ ($c = 0.18$, CH_3OH). IR (KBr) ν_{max}: 3400, 3082, 3015, 2951, 2925, 2863, 1640, 1435, 1376, 1300, 1190, 993, 910 cm^{-1}. ^1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) spectral data: see Table 1. HR-EI-MS *m/z*: 412.3328 $[M]^+$ (calcd for $C_{28}H_{44}O_2$, 412.3341). CD($CHCl_3$): $\Delta\varepsilon_{212.5\,nm} + 0.31$, $\Delta_{\varepsilon 219.0\,nm} - 4.93$, $\Delta\varepsilon_{220.5\,nm} - 3.89$, $\Delta\varepsilon_{223.5\,nm} - 7.55$, $\Delta\varepsilon_{224.5\,nm} - 7.04$, $\Delta\varepsilon_{228.5\,nm} - 10.10$, $\Delta\varepsilon_{239.5\,nm} - 6.00$, $\Delta\varepsilon_{251.5\,nm} + 2.13$.

3.3.2 Alkesterol B (*2*)

Colorless needles, $[\alpha]_D^{25} = -12.02$ ($c = 0.18$, CH_3OH). IR (KBr) ν_{max}: 3402, 3080, 3012, 2953, 2922, 2862, 1644, 1438, 1372, 1295, 1187, 992, 912 cm^{-1}. ^1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) spectral data: see Table 1. HR-EI-MS *m/z*: 412.3332 $[M]^+$ (calcd for $C_{28}H_{44}O_2$, 412.3341). CD($CHCl_3$): $\Delta\varepsilon_{210.5\,nm} - 0.02$, $\Delta\varepsilon_{214.0\,nm} - 0.54$, $\Delta\varepsilon_{219.0\,nm} + 2.01$, $\Delta\varepsilon_{227.5\,nm} + 8.60$, $\Delta\varepsilon_{241.5\,nm} - 0.72$, $\Delta\varepsilon_{250.5\,nm} + 1.44$, $\Delta\varepsilon_{254.5\,nm} + 2.36$.

3.4 Oppenauer oxidative product of 1 and 2

To a solution of cyclohexanone (150 μl) and aluminum isopropoxide (8.0 mg) in methylbenzene (2.0 ml), compound **1** (10.0 mg) was added, and the mixture was allowed to react for 1.5 h under reflux. After the reaction system became light blue and cool, 2.0 ml of saturated NaCl aqueous solution was added, and then

Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data of compounds **1a** and **2a** ($CDCl_3$, δ in ppm).

	1a		**2a**	
	δ_C	δ_H	δ_C	δ_H
1	35.8	1.21 t (6.2)[a]	35.6	1.20 t (6.4)
2	33.9	2.89 t (6.2)	33.8	2.88 t (6.4)
3	199.2	–	199.1	–
4	124.1	5.75 s	124.1	5.73 s
5	170.4	–	170.3	–
6	32.7	2.35 t (6.0)	32.6	2.34 t (6.1)
7	31.9	1.85 m	31.9	1.83 m
8	35.9	1.32 m	35.9	1.32 m
9	53.7	1.40 m	53.6	1.41 m
10	38.6	–	38.5	–
11	20.9	1.53 m	21.0	1.55 m
12	39.9	2.02 m, 1.16 m	39.8	2.00 m, 1.16 m
13	42.3	–	42.4	–
14	56.8	0.96 m	56.8	0.96 m
15	24.3	1.10 m, 1.50 m	24.4	1.11 m, 1.50 m
16	28.1	1.85 m, 1.26 m	28.1	1.85 m, 1.24 m
17	55.7	1.14 m	55.8	1.15 m
18	13.2	0.92 s	13.1	0.92 s
19	19.2	1.02 s	19.3	1.03 s
20	35.7	1.40 m	35.9	1.42 m
21	18.6	0.89 d (7.2)	18.8	0.89 d (7.2)
22	27.6	1.26 m	29.1	1.26 m
23	29.8	1.29 m, 1.48 m	27.4	1.82 m, 1.31 m
24	75.7	–	73.5	–
25	150.3	–	156.6	–
26	36.8	1.50 m, 1.43 m	35.6	1.25 m, 1.61 m
27	109.2	4.95 d, 4.81 d (2.0)	106.5	5.08 d, 4.76 d (2.0)
28	27.8	1.31 s	29.2	1.34 s

Note: [a] Data in parentheses are *J* values (in Hz).

stirred for 0.5 h. The reaction was quenched by the addition of 2.0 ml water, and the mixture was subsequently extracted with CH_2Cl_2 (3 × 1.0 ml). The CH_2Cl_2-soluble layers were combined, dried over anhydrous $MgSO_4$, and evaporated. The residue was subjected to short silica gel column chromatography using *n*-hexane–EtOAc (85:15) to yield the Oppenauer oxidative product of **1** (5.0 mg, **1a**) [8]. HR-ESI-MS *m/z*: 411.3269 $[M + H]^+$ (calcd for $C_{28}H_{43}O_2$, 411.3263); ^{1}H and ^{13}C NMR ($CDCl_3$) spectral data: see Table 2. The Oppenauer oxidative product of **2** (4.7 mg, **2a**) was prepared by the same procedure. HR-ESI-MS *m/z*: 411.3275 $[M + H]^+$ (calcd for $C_{28}H_{43}O_2$, 411.3263); ^{1}H and ^{13}C NMR ($CDCl_3$) spectral data: see Table 2.

3.5 Benzoylation of 1a and 2a

Benzoyl chloride (1.2 ml) was added drop by drop to a solution of compound **1a** (4.0 mg) and catalytic amount of DMAP (2.0 mg) in dry pyridine (2.0 ml) at room temperature during a period of 20 min. Stirring was continued for another 2 h, and then saturated aqueous $NaHCO_3$ (1.0 ml) was added to the reaction mixture to quench the reaction. The mixture was extracted with CH_2Cl_2 (3 × 1.0 ml),

Table 3. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data of compounds **1b** and **2b** ($CDCl_3$, δ in ppm).

	1b		**2b**	
	δ_C	δ_H	δ_C	δ_H
1	35.8	1.21 t (6.2)[a]	35.6	1.20 t (6.4)
2	33.9	2.89 t (6.2)	33.8	2.88 t (6.4)
3	199.2	–	199.1	–
4	124.1	5.75 s	124.1	5.73 s
5	170.4	–	170.3	–
6	32.7	2.35 t (6.0)	32.6	2.34 t (6.1)
7	31.9	1.85 m	31.9	1.83 m
8	35.9	1.32 m	35.9	1.32 m
9	53.7	1.40 m	53.6	1.41 m
10	38.6	–	38.5	–
11	20.9	1.53 m	21.0	1.55 m
12	39.9	2.02 m, 1.16 m	39.8	2.00 m, 1.16 m
13	42.3	–	42.4	–
14	56.8	0.96 m	56.8	0.96 m
15	24.3	1.10 m, 1.50 m	24.4	1.11 m, 1.50 m
16	28.1	1.85 m, 1.26 m	28.1	1.85 m, 1.24 m
17	55.7	1.14 m	55.8	1.15 m
18	13.2	0.92 s	13.1	0.92 s
19	19.2	1.02 s	19.3	1.03 s
20	35.7	1.40 m	35.9	1.42 m
21	18.6	0.89 d (7.2)	18.8	0.89 d (7.2)
22	27.6	1.26 m	29.1	1.26 m
23	29.8	1.29 m, 1.48 m	27.4	1.82 m, 1.31 m
24	75.7	–	73.5	–
25	150.3	–	156.6	–
26	36.8	1.50 m, 1.43 m	35.6	1.25 m, 1.61 m
27	109.2	4.95 d, 4.81 d (2.0)	106.5	5.08 d, 4.76 d (2.0)
28	27.8	1.31 s	29.2	1.34 s
Carbonyl	166.7	–	166.9	–
Ar	120–140	7.3–8.1 m	120–140	7.3–8.1 m

Note: [a] Data in parentheses are *J* values (in Hz).

washed with brine (1.0 ml), and dried ($MgSO_4$). The solvent was removed, and the residue was subjected to RP-HPLC preparation using acetonitrile–H_2O (90:10) as the eluent, to yield the benzoylation product of **1a** (3.2 mg, **1b**) [9]. HR-ESI-MS *m/z*: 515.3532 $[M + H]^+$ (calcd for $C_{35}H_{47}O_3$, 515.3525); ^{1}H and ^{13}C NMR ($CDCl_3$) spectral data: see Table 3. The benzoylation product of **2a** (2.8 mg, **2b**) was prepared by the same procedure. HR-ESI-MS *m/z*: 515.3538 $[M + H]^+$ (calcd for $C_{35}H_{47}O_3$, 515.3525); ^{1}H and ^{13}C NMR ($CDCl_3$) spectral data: see Table 3.

3.6 Cytotoxic activity

To evaluate the cytotoxic activities of sterols from the fruits of *P. pubeacens* L. against human HeLa, SMMC-7721, and HL-60 tumor cell lines, the MTT colorimetric assay was performed [10]. The amount of formazan was determined by a photometer at 570 nm. Cells were plated into 96-well flat-bottomed cultured plates at a concentration of 5×10^4 cells per well in a complete RPMI-1640 culture medium. Twenty-four hours after plating, the medium containing fetal calf serum was removed and test solutions were given to

cells in various final concentrations such as 2.5, 5, 10, 20, 50, and 100 μg/ml. After incubation with drugs for 24 h, MTT solution was added to the wells and plates were incubated at 37°C for 4 h. Results were expressed as the percentage of the absorbance in control cells compared to that in drug-treated cells.

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