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New cytotoxic withanolides from *Physalis peruviana*

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ABSTRACT

Investigation of the extracts of *Physalis peruviana* L. has led to the isolation of seven new withanolides, phyperunolide A (**1**), phyperunolide B (**2**), phyperunolide C (**3**), phyperunolide D (**4**), peruvianoxide (**5**), phyperunolide E (**16**), and phyperunolide F (**17**) together with ten known withanolides. The structure and absolute stereochemistry of all compounds were elucidated on the basis of CD and NMR spectral analysis, respectively. Compounds **1**, **6**, **7**, and **9** showed cytotoxicity against lung cancer (A549), breast cancer (MDA-MB-231 and MCF7), and liver cancer (Hep G2 and Hep 3B) cancer cell lines. The presence of 5β,6β-epoxy-2-en-1-ones in withanolides is greatly helpful in cytotoxicity, it is a key known important factor to cytotoxicity; however, interestingly, the unusual 5-chloride withanolide, **9**, displayed significant activity.

1. Introduction

The genus *Physalis* (Solanaceae) includes about 120 species mainly distributed in South and North America (Ahmad, Yasmin, & Malik, 1999). In previous studies, a series of C_{28} steroidal lactones, physalins and withanolides, had been isolated from this genus (Ahmad et al., 1999; Ahmad, Malik, Afza, & Yasmin, 1999; Dinan, Sarker, & Šik, 1997; Nagafuji, Okabe, Akahane, & Abe, 2004; Su et al., 2002). Withanolides are natural steroidal lactones, which have been isolated from the genera *Acnistus*, *Datura*, *Jaborosa*, *Lycium*, *Physalis*, *Withania*, and *Tubocapsicum* of the family Solanaceae (Glotter et al., 1991). In addition, they display significant pharmacological activities, including antimicrobial, antitumor, anti-inflammatory, hepatoprotective, immunomodulatory, antibacterial, insect–antifeedant, and insect–repellent activities (Gil, Misico, Sotes, & Oberti, 1997; Glotter et al., 1991; Veleiro, Oberti, & Burton, 2005). *Physalis peruviana* (Golden berry or Chinese lantern) is a shrubby herb distributed in South America. The fruit tastes like a sweet tomato and includes abundant vitamin C, vitamin A, and vitamin B-complex. In a previous report (Wu et al., 2006), the extracts of *P. peruviana* exhibited heightened antioxidant and anti-inflammatory activities.

In the current study, a crude ethanol extract from aerial parts of *P. peruviana* showed cytotoxicity against Hep G2, A549, and MDA-MB-231 cancer cell lines with IC_{50} values at the concentrations 9.91, 16.41, and 4.95 μg/ml, respectively. Seven new withanolides, phyperunolide A (**1**), phyperunolide B (**2**), phyperunolide C (**3**), phyperunolide D (**4**), peruvianoxide (**5**), phyperunolide E (**16**), and phyperunolide F (**17**) along with ten known 4β-hydroxywithanolide E (**6**) (Kirson, Abraham, Sethi, Subramanian, & Glotter, 1976), withanolide E (**7**) (Sakurai, Ishii, Kobayashi, & Iwao, 1976), withanolide S (**8**) (Glotter, 1991), withanolide C (**9**) (Bessalle & Lavie, 1992), withaperuvin (**10**) (Frolow et al., 1981), physalolactone (**11**) (Frolow et al., 1981), withaphysanolide (**12**) (Silva, Pacciaroni, Oberti, Veleiro, & Burton, 1993), physalactone (**13**) (Ray, Sahai, & Das, 1978), withaperuvin D (**14**) (Sahai, Ali, Ray, Slatkin, & Kirson, 1983), and loliolide (**15**) (Kimura & Maki, 2002) have been isolated from the aerial parts of *P. peruviana*. Of these, compounds **1**, **6**, **7**, and **9** showed cytotoxicity against A549, MDA-MB-231, MCF7, Hep 3B, and Hep G2 cell lines. In addition, the chemical origins of chloride withanolides and the cytotoxicity of isolated compounds are reported here.

2. Experimental

2.1. General experimental procedures

UV spectra were measured on a Jasco V-530 UV/VIS spectrophotometer. IR spectra were recorded on a Mattson Genesis II™ FT-IR

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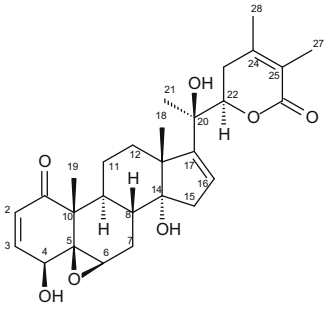
spectrophotometer. The optical rotations were taken on a Jasco-P-1020 polarimeter (cell length 10 mm). Circular dichroism was measured on a Jasco J-810 spectrophotometer. The NMR spectra were recorded on a Varian Unity-plus 400 MHz FT–NMR and a Varian Mercury-plus 400 MHz FT–NMR. The chemical shift (δ) values are reported in ppm (parts per million) with $CDCl_3$ and C_5D_5N as internal standard, and coupling constants (J) are in Hz. HRESI-MS and EI-MS measurement were performed on a Bruker Daltonics APEX II 30e mass spectrometer and VG Biotech Quattro 5022 mass spectrometer, respectively. TLC was performed on Kieselgel 60, F 254 (0.20 nm, Merck), spots were viewed under ultraviolet light at 254 and 365 nm and/or stained by spraying with 50% H_2SO_4 and heating on a hot plate. For column chromatography, silica gel (Kiesilgel 60, 70–230 and 230–400 mesh, Merck) and Sephadex LH-20 were used. Further purification of some compounds was achieved by preparative HPLC, Shimadzu LC-10AT and recycling HPLC, LC-918 (JAI) and Discovery column (250 × 10 mm, C_{18}), Hypersil ODS column (250 × 21.2 mm, C_{18}), and Develosil™ column (250 × 21.2 mm, C_{30}) were used.

2.2. Plant Material

The plant material (10.8 kg) was collected in the Tainan District Agricultural Research and Extension Station, Taiwan, in April, 2005. The voucher specimen is deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

2.3. Extraction and isolation

The aerial parts (stems and leaves) of *P. peruviana* (10.8 kg) were extracted three times with EtOH at room temperature to ob-



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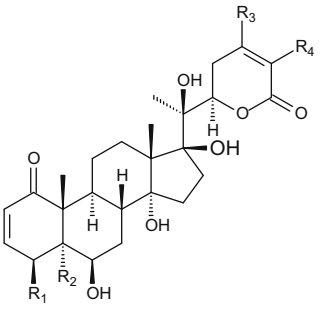
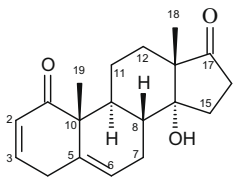
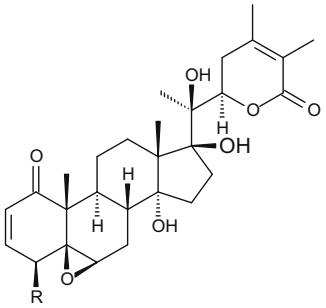


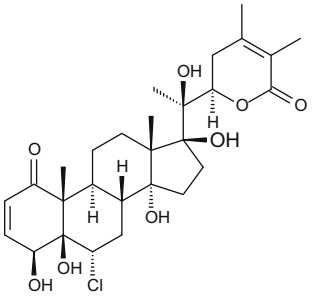
2 R_1=H, R_2=OH, R_3=CH_2OH, R_4=CH_3
3 R_1=H, R_2=Cl, R_3=CH_2OH, R_4=CH_3
4 R_1=H, R_2=OH, R_3=CH_3, R_4=CH_2OH
8 R_1=H, R_2=OH, R_3=CH_3, R_4=CH_3
9 R_1=H, R_2=Cl, R_3=CH_3, R_4=CH_3
10 R_1=OH, R_2=OH, R_3=CH_3, R_4=CH_3



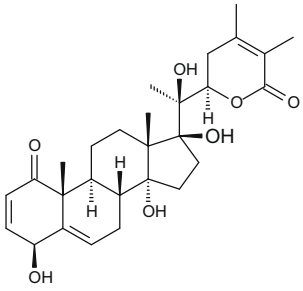
5



6 R=OH
7 R=H



11



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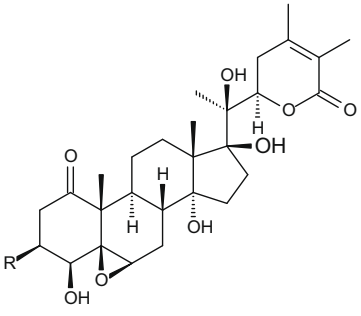
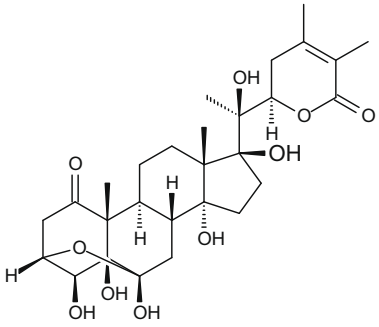


13 R=OCH_3
16 R=OH
17 R=OCH_2CH_3



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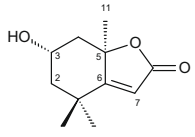

15

Fig. 1. Structures of compounds **1**–**17**.

tain a crude extract. The crude extract was partitioned with EtOAc and H_2O (1:1, v/v) to give an EtOAc layer. The EtOAc layer was then partitioned *n*-hexane and MeOH and H_2O (10:7:3, v/v/v) to give a MeOH/H_2O layer (65.2 g). The MeOH/water layer showed inhibitory activity against A549, MDA-MB-231, and HepG2 cells (IC_{50} < 20 μg/ml). The MeOH/water layer extract was chromatographed over silica gel column, using gradients of EtOAc/*n*-hexane–MeOH, and yielded twenty four fractions.

Fraction 9–11 (1.3 g) was chromatographed over a silica gel column ($CHCl_3$:*n*-hexane = 1:1-MeOH, gradient), yielding twenty one fractions. The eleventh fraction (fr. 9–11) was purified on preparative HPLC using a C_{18} column (250 × 21.2 mm) and H_2O–MeOH (40/60; flow rate: 3 ml/min) as the solvent system to give compound **5** (1.96 mg). The thirteenth fraction (fr. 9–13) was purified on preparative HPLC using a C_{18} column (250 × 21.2 mm) and H_2O–MeOH (40/60; flow rate: 3 ml/min) as the solvent system to give compound **15** (13.6 mg).

Fraction 15 (9154.4 mg) was subjected to Sephadex LH-20 (EtOAc:MeOH = 1:4). Subfraction (fr. 15–3–4) was purified by reverse-phase HPLC (250 × 21.2 mm, H_2O/MeOH = 40/60, flow rate: 3 ml/min) to give compound **8** (3.62 mg). Further subfraction (fr. 15–3–4–1) was purified by reverse-phase HPLC (250 × 21.2 mm, H_2O/MeCN = 30/70, flow rate: 3 ml/min) to give compound **12** (3.9 mg) and **17** (2.9 mg). Subfraction (fr. 15–3–5) was chromatographed over a silica gel column (EtOAc:*n*-hexane = 8:1-MeOH, gradient), yielding seven fractions. Further subfraction (fr. 15–3–5–3) was purified by reverse-phase HPLC (250 × 21.2 mm, H_2O/MeOH = 40/60, flow rate: 3 ml/min) to give compound **1** (18.3 mg), **10** (16.7 mg), **13** (16.7 mg), and **14** (14.4 mg). Subfraction (fr. 15–3–7) yielded crystals and the crystals were washed with acetone to give compound **6** (866.5 mg).

Fraction 17–19 (3.0 g) was chromatographed over a silica gel column ($CHCl_3$–MeOH, gradient), yielding eleven fractions. Subfraction (fr. 17–8) was purified by reverse-phase HPLC (250 × 21.2 mm, H_2O/MeCN = 30/70, flow rate: 3 ml/min) to give compound **15** (106.14 mg) and **16** (39.4 mg). Subfraction (fr. 17–8–5) was further purified by reverse-phase HPLC (250 × 21.2 mm, H_2O/MeCN = 30/70, flow rate: 3 ml/min) to give compound **3** (5.7 mg). Fraction 21 got crystals and the crystals were washed with acetone to give compound **9** (94.5 mg).

Fraction 23 (3.1 g) was chromatographed over a silica gel column (EtOAc–MeOH, gradient), yielding fourteen fractions. Subfraction (fr. 23–6) was purified by reverse-phase HPLC (250 × 21.2 mm, H_2O/MeCN = 30/70, flow rate: 3 ml/min) to give compound **11** (2.19 mg). Subfraction (fr. 23–9) was purified by reverse-phase preparative HPLC (250 × 21.2 mm, H_2O/MeCN = 30/70, flow rate: 3 ml/min) to give compound **2** (6.7 mg) and compound **4** (9.0 mg).

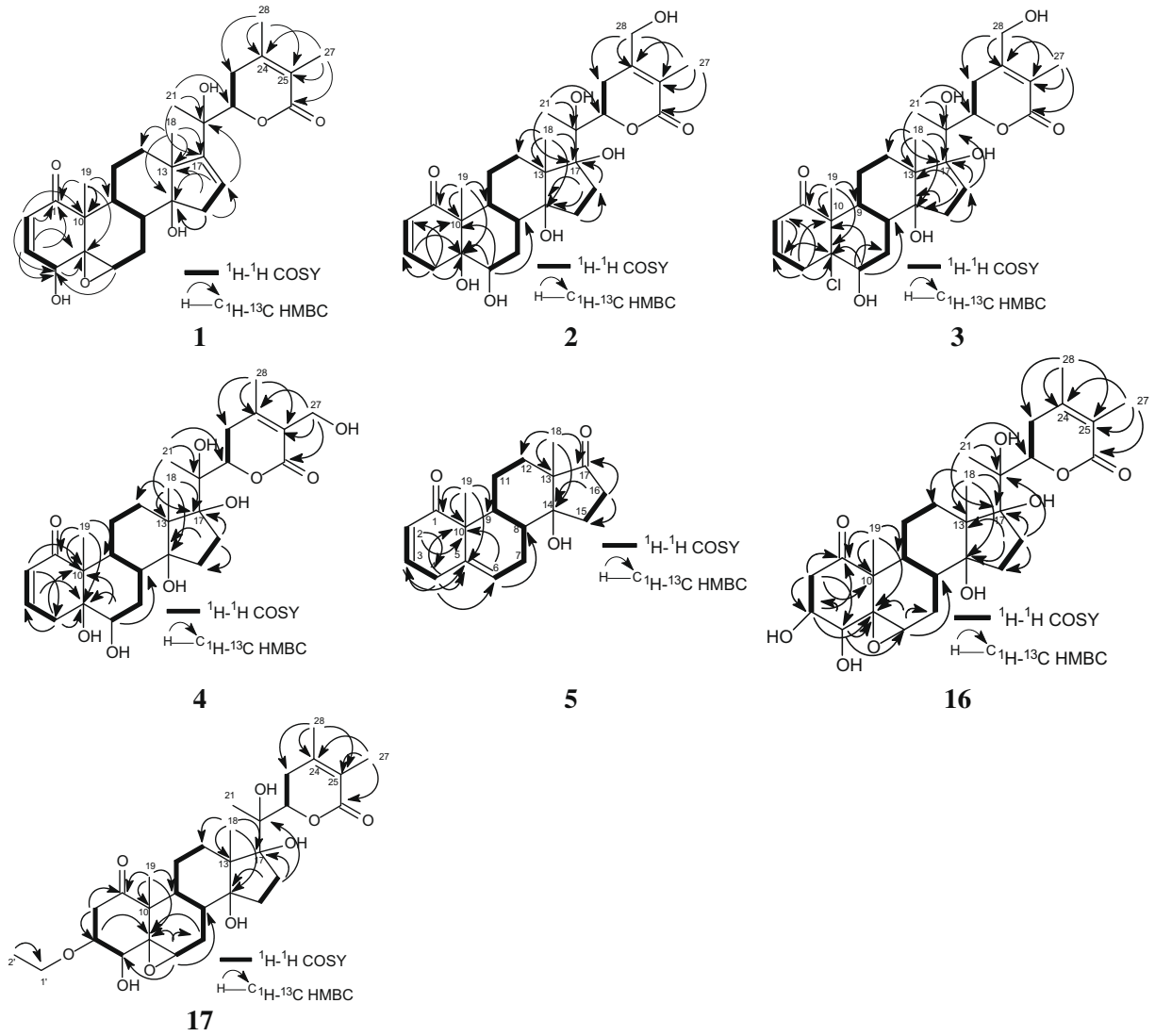

Fig. 2. COSY and HMBC correlations for compounds **1**–**5**, **16**, and **17**.

Table 1
^{1}H NMR data of compounds **1**–**5**, **16** and **17** in $CDCl_3$ and C_5D_5N (δ in ppm, J in Hz).[a]

	1			**2**			**3**	**4**	**5**	**16**			**17**
	δ_H ($CDCl_3$)	δ_H (C_5D_5N)		δ_H ($CDCl_3$)	δ_H (C_5D_5N)		δ_H (C_5D_5N)	δ_H (C_5D_5N)	δ_H (C_5D_5N)	δ_H ($CDCl_3$)	δ_H (C_5D_5N)		δ_H (C_5D_5N)
2	6.22 d (10.0)	6.40 d (10.0)	−0.18	5.76 dd (2.4, 10.0)	6.11 dd (2.4, 10.0)	−0.35	6.11 dd (2.4, 10.0)	6.14 dd (2.4, 10.0)	1.40 dd (3.6, 14.2)	2.62 dd (4.0, 15.8)	3.32 dd (3.6, 15.6)	−0.70	3.11 dd (4.4, 15.6)
									2.02 d (14.2)	2.89 dd (8.0, 15.8)	3.36 dd (7.2, 15.6)	−0.47	3.17 dd (7.6, 15.6)
3	6.92 dd (6.0, 10.0)	7.21 dd (6.4, 10.0)	−0.29	6.55 ddd (2.4, 5.0, 10.0)	6.64 ddd (2.4, 5.0, 10.0)	−0.09	6.67 ddd (2.4, 5.2, 10.0)	6.67 ddd (2.4, 5.0, 10.0)	4.36 m	4.13 m	4.74 m	−0.61	4.05 m
4	3.73 d (6.0)	4.01 m	−0.28	2.00 dd (5.0, 19.6)	2.45 br dd (5.0, 19.4)	−0.45	2.67 dd (5.2, 19.6)	2.45 dd (5.0, 19.6)	1.74 dd (3.6, 13.2)	3.37 d (3.2)	4.03 br d (2.4)	−0.66	3.93 br s
				3.13 dt (2.4, 19.6)	3.80 m	−0.67	3.92 dt (2.4, 20.0)	3.80 dt (2.4, 19.6)	2.58 d (13.2)				
6	3.27 br s	3.33 br s	−0.06	3.56 br s	4.28 br s	−0.72	4.41 m	4.29 br s		3.34 br s	3.81 br s	−0.47	3.53 br s
7	2.02 m	2.07 m	−0.05	1.20 m	2.06 m	−0.86	1.98 m	2.07 m		2.03 m	2.32 m	−0.29	2.30 m
	2.19 m	2.34 m	−0.15	2.36 m	3.07 m	−0.71	3.09 m	3.09 m		2.03 m	2.78 m	−0.75	2.70 m
8	1.94 m	2.16 m	0.22	2.12 m	2.77 m	−0.65	2.72 m	2.80 m	5.84 s	1.77 m	2.13 m	−0.36	2.08 m
9	1.75 m	2.03 m	−0.28	2.37 m	3.55 n	−1.18	3.46 m	3.56 m	1.13 s	1.80 m	2.50 m	−0.70	2.35 m
10									1.51 s				
11	1.55 m	1.79 m	−0.24	2.21 m	1.71 m		1.64 m	1.86 m	1.91 s	1.28 m	1.48 m	−0.20	1.35 m
	1.72 m	1.92 m	−0.20		2.85 m		2.80 m	2.86 m		1.44 m	1.69 m	−0.25	
12	1.45 m	1.68 m	−0.23	1.29 m	1.67 m	−0.38	1.68 m	1.53 m		1.26 m	1.40 m	−0.14	1.42 m
	2.11 m	2.42 m	−0.31	2.34 m	2.98 m	−0.64	2.97 m	2.89 m		2.24 m	2.62 m	−0.38	2.61 m
15	2.24 br d (3.2)	2.27 d (3.2)	−0.03	1.52 m	1.91 m	−0.39	1.88 m	1.91 m		1.57 m	1.81 m	−0.24	1.84 m
	2.34 br dd (1.2, 16.0)	2.30 m	+0.04	1.65 m	1.91 m	−0.26	1.88 m	1.91 m		1.65 m	1.81 m	−0.24	1.84 m
16	5.81 br dd (1.2, 3.2)	6.19 dd (1.6, 3.2)	−0.38	1.47 m	1.99 m	−0.52	1.95 m	1.97 m		1.48 m	1.98 m	−0.50	
				2.57 m	3.17 m	−0.60	3.15 m	3.12 m		2.66 m	3.07 m	−0.41	1.97 m 3.07 m
18	1.11 s	1.17 s	−0.06	1.08 s	1.49 s	−0.41	1.45 s	1.51 s		0.99 s	1.34 s	−0.35	1.32 s
19	1.41 s	1.91 s	−0.50	1.25 s	1.73 s	−0.48	1.72 s	1.80 s		1.24 s	1.85 s	−0.61	1.80 s
21	1.29 s	1.50 s	−0.21	1.34 s	1.84 s	−0.50	1.85 s	1.79 s		1.37 s	1.78 s	−0.41	1.77 s
22	4.39 dd (3.6, 12.8)	4.56 dd (3.6, 12.6)	−0.17	4.84 dd (2.8, 13.4)	5.39 dd (2.8, 14.8)	−0.55	5.41 dd (2.8, 13.6)	5.30 dd (3.2, 13.2)		4.80 dd (5.2, 11.4)	5.24 dd (2.8, 13.2)	−0.44	5.23 dd (3.2, 13.0)
23	2.20 m	2.38 m	−0.18	1.43 m	2.79 m	−1.36	2.73 m	2.71 m		2.45 m	2.66 m	−0.21	2.64 m
	2.80 m	3.04 m	−0.24	2.87 m	3.72 m	−0.85	3.76 m	3.00 m		2.48 m	2.93 m	−0.45	2.93 m
27	1.85 s	1.84 s	+0.01	1.81 s	2.02 s	−0.21	2.04 s	4.77 br d (12.0)		1.83 s	1.95 s	−0.12	1.95 s
28	1.96 s	1.73 s	+0.03	4.15 d (14.4)	4.38 d (14.4)	−0.23	4.40 br d (14.4)	4.91 br d (12.0)		1.91 s	1.77 s	+0.14	1.77 s
				4.30 d (14.4)	4.66 d (14.4)	−0.36	4.74 br d (14.4)						
1′													3.44 m 3.44 m
2′													1.09t (6.8)

[a] All assignments were confirmed from 2D NMR spectra. Coupling constants in Hz are in parentheses, chemical shifts are in δ values.

The fundamental data of the new natural chemicals are summarised in Table 4.

2.4. Cytotoxicity assay

Fractions and isolates were tested against lung (A549), breast (MEA-MB-231 and MCF7), and liver (HepG2 and 3B) cancer cell lines using established colorimetric MTT assay protocols (Mosmann, 1983). Doxorubicin was used as a positive control. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000–10,000 cells per well with tested compounds added from DMSO stock solution. After 3 days in culture, attached cells were incubated with MTT (0.5 mg/ml, 2 h) and subsequently solubilised in DMSO. The absorbance was measured at 550 nm using a microplate reader. The IC_{50} is the concentration of agent that reduced cell growth by 50% under the experimental conditions.

3. Results and discussion

The EtOH extract of aerial parts of *P. peruviana* was partitioned with EtOAc and water (1:1, v/v), and the EtOAc layer was then partitioned with *n*-hexane and MeOH and H_2O (10:7:3, v/v/v). Further fractionation of the MeOH/H_2O extract was performed by column chromatographic separation, which yielded 24 fractions. Chromatographic fractionation on these subfractions yielded fifteen compounds with two artifact compounds **16** and **17** (Fig. 1).

The molecular formula of **1** was established as $C_{28}H_{36}O_7$ by HRESIMS. The IR spectrum showed absorptions for an α,β-unsaturated carbonyl group (1697 cm^{-1}) and a hydroxyl group (3530 cm^{-1}). The mass fragments at *m/z* 125 and 165 were characteristic of cleavages between C-20/C-22 and C-17/C-20 with 3,4-dimethyl-5,6-dihydropyran-2-one side chain moiety, respectively (Ahmad, Yasmin, et al., 1999). The ^{1}H NMR spectrum showed five methyl protons, three oxymethine protons, and three olefinic protons. The COSY spectrum clearly showed the correlations from H-2 to H-4, H-6 to H-12, H-15 to H-16, and H-22 to H-23 (Fig. 2). The ^{13}C NMR and DEPT spectrum showed the presence of 28 carbon resonances including ten quaternary carbons, eight methines, five methylenes, and five methyls. Comparison of the ^{1}H and ^{13}C NMR spectra data with 4β-hydroxywithanolide E (**6**) (Kirson et al., 1976) and withanolide E (**7**) (Sakurai et al., 1976) indicated **1** is a withanolide derivative. The HMBC spectrum suggested a hydroxyl group at C-14 and a double bond at C-16/C-17 (Fig. 2). To determine the orientation of OH-4, the 1D and 2D NMR spectra of **1** were detected in both $CDCl_3$ and C_5D_5N. Pyridine and hydroxyl groups can form weak hydrogen bonds and collision complexes, influencing the chemical shift of the neighbouring protons (Su et al., 2002). The solvent shifts ($\Delta = \delta CDCl_3 - \delta C_5D_5N$) were reported in Table 1, and pyridine-induced shift for CH_3-19 ($\Delta = -0.50$) suggested the orientation of OH-4 and CH_3-19 were β form. In addition, the observed NOESY correlation between H-4α and H-6α and the ^{1}H–1H coupling constants of H-6 and H-7 proved the *cis*-A/B ring junction (Kirson and Glotter, 1981). The absolute configuration of C-22 was established as *S* form through the positive Cotton effect ($\Delta\varepsilon$ + 2492) at 254 nm in the CD spectrum (Kuroyanagi, Shibata, and Umehara, 1999). Thus, compound **1** was clarified to be 4β,14α,20β-trihydroxy-5β,6β-epoxy-1-oxowitha-2,16,24-trinenolide and named phyperunolide A.

The molecular formula of compound **2** was deduced to be $C_{28}H_{40}O_9$ by HRESIMS. Its IR spectrum displayed the presence of an α,β-unsaturated carbonyl group (1676 cm^{-1}) and a hydroxyl group (3275 cm^{-1}). Four methyl protons, two oxymethine protons, one hydroxymethyl group, and two *cis*-olefinic protons were displayed in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum disclosed ten quaternary carbons, six methines, eight methylenes, and four methyls. The NMR assignments were found to be similar to withanolide S (**8**) (Glotter et al., 1991), and the only exception in **2** is one methyl group in **8** was replaced by the hydroxymethylene

Table 2
^{13}C NMR data of compounds **1**–**5**, **16**, and **17** in C5D5 N (δ in ppm).

Carbons	**1** δ_C (C_5D_5N)	**2** δ_C (C_5D_5N)	**3** δ_C (C_5D_5N)	**4** δ_C (C_5D_5N)	**5** δ_C (C_5D_5N)	**16** δ_C (C_5D_5N)	**17** δ_C (C_5D_5N)
1	202.4 s	205.2 s	201.8 s	205.2 s	36.2 s	210.7 s	210.0 s
2	132.6 d	129.1 d	128.8 d	129.2 d	47.6 t	44.1 t	41.5 t
3	144.7 d	142.0 d	142.2 d	142.0 d	65.9 d	69.2 d	76.8 d
4	70.4 d	36.9 t	38.1 t	36.9 t	46.4 t	78.9 d	75.2 d
5	64.7 s	77.7 s	83.1 s	77.8 s	87.0 s	65.4 s	65.1 s
6	60.7 d	75.4 d	74.9 d	75.5 d	182.9 s	59.7 d	59.2 d
7	26.5 t	30.5 t	30.3 t	30.5 t	113.1 d	26.7 t	26.6 t
8	32.1 d	35.2 d	35.3 d	35.3 d	171.8 s	34.8 d	34.7 d
9	38.0 d	34.7 d	35.7 d	34.7 d	30.6 q	36.8 d	36.6 d
10	48.6 s	53.1 s	53.8 s	53.1 s	26.5 q	51.1 s	51.0 s
11	20.5 t	23.4 t	23.3 t	23.4 t	27.3 q	21.8 t	21.7 t
12	28.0 t	31.8 t	31.6 t	31.9 t		30.3 t	30.3 t
13	52.5 s	55.6 s	55.5 s	55.5 s		55.0 s	55.0 s
14	84.4 s	83.3 s	82.7 s	83.2 s		81.9 s	81.8 s
15	40.2 t	33.3 t	33.3 t	33.3 t		33.0 t	33.0 t
16	124.5 d	37.3 t	37.3 t	37.3 t		37.2 t	37.2 t
17	157.4 s	88.7 s	88.6 s	88.6 s		88.2 s	88.2 s
18	22.0 q	21.7 q	21.7 q	21.8 q		20.8 q	20.7 q
19	16.7 q	16.1 q	16.8 q	16.1 q		15.2 q	15.1 q
20	74.6 s	79.5 s	79.5 s	79.3 s		79.3 s	79.3 s
21	24.4 q	19.9 q	19.9 q	19.8 q		19.6 q	20.2 q
22	81.3 d	83.2 d	83.1 d	82.0 d		81.6 d	81.6 d
23	30.3 t	30.1 t	30.1 t	35.6 t		35.1 t	35.1 t
24	150.7 s	154.5 s	154.5 s	155.1 s		151.0 s	151.0 s
25	121.0 s	121.0 s	121.0 s	127.1 s		121.4 s	121.4 s
26	165.8 s	167.4 s	167.3 s	166.6 s		166.9 s	166.9 s
27	12.5 q	12.0 q	12.0 q	56.3 t		12.5 q	12.5 q
28	20.2 q	60.9 t	61.0 t	20.2 q		20.2 q	19.6 q
1′							64.3 t
2′							15.6 q

group. The HMBC correlations between CH_2-28 and C-23, C-24 and C-25 also proved this assumption (Fig. 2). Further analyses of HMBC spectrum suggested two hydroxyl groups were located at C-14 and C-17, respectively (Fig. 2). The small couplings of both H-7 protons to H-6 pointed to an α-orientation of H-6. The difference in ^{13}C NMR chemical shifts allows the determination of the stereo compression effect between CH_3-19 and 5-OH. Because of gauche-interaction, the presence of 5β-OH will cause a 10 ppm upfield shift of CH_3-19 around δ 9. In compound **2**, the chemical shift of CH_3-19 appeared at δ 16.1 and no upfield shift effect was observed (Ahmad, Yasmin, et al., 1999). Further, the NOE correlation between H-4ax and CH_3-19 was noted. Therefore, the stereochem-

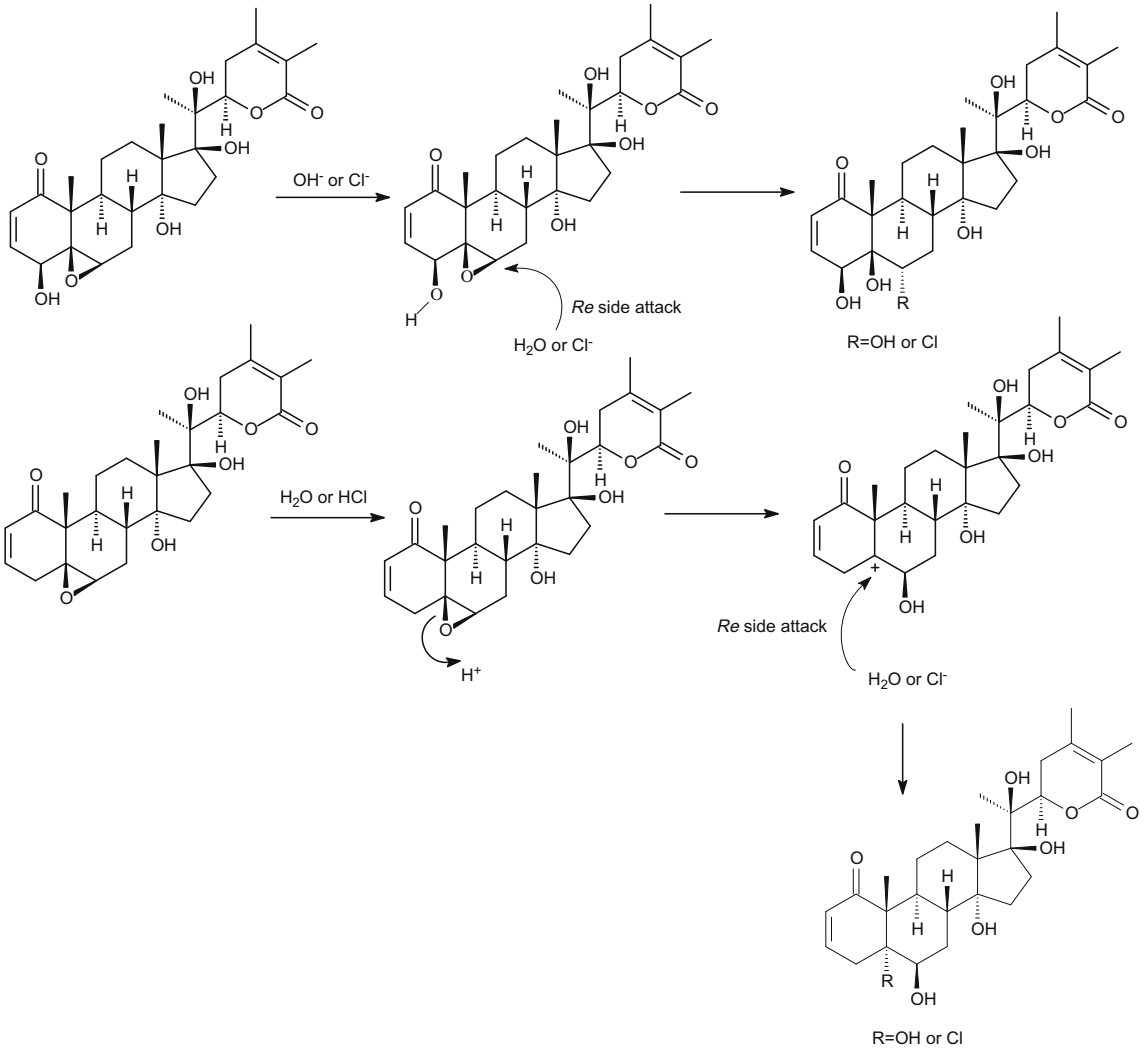

Fig. 3. Proposed mechanism of the formation of withanolides.

Table 3
Cytotoxicity of compounds **1**–**16** (IC_{50}:μg/ml).

Compound \ cell line	Hep G2	Hep 3B	A549	MDA-MB-231	MCF 7
Phyperunolide A (**1**)	2.11	3.04	4.03	3.93	1.94
Phyperunolide B (**2**)	–	–	–	–	–
Peruvinaolide C (**3**)	–	–	–	–	–
Phyperunolide D (**4**)	–	–	–	–	–
Peruvianoxide (**5**)	–	–	–	–	–
4β-Hydroxywithanolide E (**6**)	0.53	0.10	1.48	0.18	0.90
Withanolide E (**7**)	0.31	1.77	3.20	0.47	1.96
Withanolide S (**8**)	–	–	–	–	–
Withanolide C (**9**)	0.07	0.06	0.65	0.27	0.80
Withaperuvin (**10**)	–	–	–	–	–
Physalolactone (**11**)	17.10	3.89	–	6.99	–
Withaphysanolide (**12**)	3.21	13.37	14.35	11.82	12.78
Physalactone (**13**)	4.05	10.68	–	–	12.24
Withaperuvin D (**14**)	–	–	–	–	–
Loliolide (**15**)	–	–	–	–	–
Phyperunolide E (**16**)	18.61	14.16	–	–	–
Doxorubicin	0.33	0.04	0.32		0.18

istry of 5α-OH and 6β-OH was determined. Similarly, the β configuration of OH-17 was confirmed by the chemical shifts of the H-22, CH_3-18, and CH_3-20 in the pyridine-induced shift experiment (Su et al., 2002). EIMS spectra displayed the fragments at *m/z* 185 and 141, cleavage between C-17/C-20 and C-20/C-22 with δ-lactone side chain, characteristic of the presence of a withanolide with a 20-hydroxyl and an α,β-unsaturated δ-lactone moiety (Ahmad, Yasmin, et al., 1999). The positive Cotton effects at 255 nm in the CD spectrum indicated the 22*R* configuration. Therefore, the structure of **2** was established as 5α,6β,14α,17β,20β -pentahydroxy-1-oxowitha-2,24-dienolide, and named phyperunolide B.

Compound **3** showed an $[M+Na]^+$ ion at *m/z* 561.2228 ($C_{24}H_{39}ClO_7$) in HRESIMS. The IR spectrum indicated the presence of hydroxyl groups (3253 cm^{-1}) and α,β-unsaturated carbonyl groups (1674 cm^{-1}). Comparing the NMR data with those of **2** showed they were similar, except for the signals around C-5. Further, a downfield signal (δ 83.1, C-5) presumed the chlorine substitution instead of the hydroxyl group, and NMR data were consistent with 5α-chloro-6β-hydroxy assignment (Nicota et al., 2006; Nicotra, Gil, Oberti, & Burton, 2007). The axial orientation of the β OH-6 was also confirmed by the coupling analysis method (Ahmad, Yasmin, et al., 1999). Acetylation of **3** results in downfield shift of H-6 from δ 4.41 to 5.56 indicated the location of the hydroxyl group at C-6. All assignments were supported by the COSY, HMQC, and HMBC spectra, and the stereochemistry of **3** was determined by comparing the data of **2** and other related withanolides. The α,β-unsaturated δ-lactone having a 22*R* configuration gave a positive Cotton effect at 256 nm in the CD spectrum. Therefore, compound **3** was identified as 6β,14α,17β,20β-tetrahydroxy-5α-chloro-1-oxowitha-2,24-dienolide, and named phyperunolide C (**3**).

Compound **4**, an isomer of **2**, showed the same molecular formula ($C_{28}H_{40}O_9$) and similar characteristic NMR signals. The only difference in NMR assignments between compounds **2** and **4** is the data of the hydroxymethyl group substituted at C-28 and C-27, respectively. All structural assignments were supported by the 2D NMR techniques, the NMR assignments of CH_2OH-27 at δ_H 4.77 (*d*, *J* = 12.0 Hz, H-27a) and δ 4.91 (*d*, *J* = 12.0 Hz, H-27b) as well as δ_C 56.3 were confirmed. The HMBC correlations between CH_2-27/C-24, C-25, and C-26 were further supported for the location of CH_2OH-27. The configuration at the C-22 was defined as *R* based on the CD spectrum, where a strong positive Cotton effect was found at 253 nm. Therefore, compound **4** was clarified to be 5α,6β,14α,17β,20β,27-hexahydroxy-1-oxowitha-2,24-dienolide and named phyperunolide D.

Compound **5**, $C_{19}H_{24}O_3$, displayed characteristic absorption bands at 1733 and 3479 cm^{-1} in the IR spectrum. The ^1H NMR spectra indicated the presence of two tertiary methyl groups (δ 1.03 and 1.24), and three olefinic protons (δ 5.56, 5.96, and 6.67). The ^{13}C NMR spectrum confirmed the presence of two ketone groups (δ 203.8 and 218.4), four quaternary carbons, five methines, six methylenes, and two methyls. The correlation sequences from H-2 to H-4, from H-6 to H-12, and from H-15 to H-16 were confirmed based on the COSY spectrum. Further, the HMBC correlations between CH_3-19/ C-1, C-9, and C-10, CH_3-18/C-12, C-13, and C-17 affirmed the positions of the methyl groups. Based on the comparison with the NMR spectral data of **5** with those of cinedione (Maldonado, Alvarado, Torres, Martinez, & Pérez-Castorena, 2005), compound **5** was determined and named peruvianoxide.

Two presumed artifacts, compounds **16** (phyperunolide E) and **17** (phyperunolide F), were also isolated during this study (Tables 1 and 2, Figs. 1 and 2). These two compounds were possibly generated by Michael-type additions of solvents. The structures were determined by spectroscopic methods and the absolute configurations were established by CD analysis.

The mechanism of formation of 5- or 6-chlorinated/hydroxyl withanolides was proposed in Fig. 3. According to a previous report

Table 4
The fundamental data of compounds **1-5**, **16**, and **17**.

	Appearance	Optical rotation value	UV λ_{max}^{MeOH} nm (λ_{max} (log ε))	IR (neat) ν_{max}	MS	CD data
Phyperunolide A (**1**)	White powder	$[\alpha]_D^{24.3}$ 71.1° (*c* 0.045; MeOH)	229 (3.80)	3530, 2923, 1697, 1555, 1447, 1379, 1321, 1214, 1124, 1092, 1034, 952 cm^{-1}	HRESIMS *m/z* 507.2359 (calcd for $C_{28}H_{36}O_7$ + Na, 507.2461)	$\Delta\varepsilon_{254}$ + 2492 (MeOH; *c* 0.1 mg/ml)
Phyperunolide B (**2**)	Yellow powder	$[\alpha]_D^{22.9}$ + 26.0° (*c* 0.114; MeOH)	206 (4.34)	3275, 2934, 2862, 2347, 1737, 1676, 1375, 1538, 1452, 1416, 1384, 1340, 1218, 1139, 1027, 955b cm^{-1}	HRESIMS *m/z* 543.2568 (calcd for $C_{28}H_{40}O_9$ + Na, 543.2672)	$\Delta\varepsilon_{255}$ +1151 (MeOH; *c* 0.1 mg/ml)
Phyperunolide C (**3**)	White powder	$[\alpha]_D^{24.3}$+38.8° (*c* 0.091; MeOH)	206 (4.39)	3253, 2920, 2854, 2347, 1674, 1567, 1384, 1258, 1214, 1124, 1085, 1016, 959 cm^{-1}	HRESIMS *m/z* 561.2228 (calcd for $C_{28}H_{39}ClO_8$ + Na, 561.2333)	$\Delta\varepsilon_{256}$ + 2532 (MeOH; *c* 0.1 mg/ml)
Phyperunolide D (**4**)	Yellow powder	$[\alpha]_D^{24.3}$ + 47.4° (*c* 0.102; MeOH)	206 (4.39)	3222, 2927, 2870, 2352, 1742, 1676, 1602, 1552, 1383, 1319, 218, 1140, 1079, 1018, 950 cm^{-1}	HRESIMS *m/z* 543.2572 (calcd for $C_{28}H_{40}O_9$ + Na, 543.2672)	$\Delta\varepsilon_{253}$ + 1697 (MeOH; *c* 0.1 mg/ml)
Peruvianoxide (**5**)	White powder	$[\alpha]_D^{22.9}$ + 19.1° (*c* 0.187; MeOH)	207 (4.06)	3479, 1733 cm^{-1}	HRESIMS *m/z* 323.1622 (calcd for $C_{19}H_{24}O_3$ + Na, 323.1725)	
Phyperunolide E (**16**)	White powder	$[\alpha]_D^{23.9}$ − 3.5° (*c* 0.087; MeOH)	233 (3.82)	3401, 2926, 2861, 1668, 1551, 1384, 1318, 1239, 1205, 1129, 1092, 1012, 950, 903 cm^{-1}	HRESIMS *m/z* 543.2572 (calcd for $C_{28}H_{40}O_9$ + Na, 543.2672)	$\Delta\varepsilon_{250}$ + 1377 (MeOH; *c* 0.1 mg/ml)
Phyperunolide F (**17**)	White powder	$[\alpha]_D^{23.9}$ + 7.2° (*c* 0.286; MeOH)	229 (4.05)	3357, 2915, 2578, 1690, 1568, 1454, 1384, 1317, 1247, 1214, 1129, 1089, 1015, 952, 919 cm^{-1}	HRESIMS *m/z* 571.2882 (calcd. for $C_{30}H_{44}O_9$ + Na, 571.2985)	$\Delta\varepsilon_{248}$ + 1489 (MeOH; *c* 0.1 mg/ml)

(Nittala, Velde, Frolow, & Lavie, 1981), 6-chlorine substituted withanolides with the hydroxyl group at the 4-position, such as compound **11**, could be generated by 5,6-epoxide ring opening reaction by chloride. The chloride attacks the less hindered side because the hydroxyl group at 4-position would destabilize the β-carbocation. In the presence of water or hydroxide, 6-hydroxyl withanolides, such as **10**, would be obtained in a similar manner. However, 5,6-epoxide withanolides without a hydroxyl group at 4-position, such as compound **7**, could undergo the nucleophilic attack preferred at the more steric hindered side because the tertiary carbocation is more stable. 5α-chloro-withanolides **3** and **9** could be formed by this reaction pathway. Up until now, the hypothesis is consistent with the structural features of withanolides, where no 4-hydroxyl-5-chloro-withanolide has been found in nature.

All isolates were screened in a cytotoxicity assay (Table 3). Among them, compounds **1**, **6**, **7**, and **9** showed significant cytotoxicity against lung cancer (A549), breast cancer (MEA-MB-231 and MCF7), and liver cancer (Hep G2 and Hep 3B) cell lines. Doxorubicin was used as a positive control. Cytotoxic tests were performed on a series of withanolides from previous investigation and our experimental findings (Budhiraja, Krishan, & Sudhir, 2000). In this study, compounds **1**, **6** and **7** with 5,6-epoxy-2-en-1-one moiety showed significant cytotoxicity as the same SAR observation in the past study (Falsey et al., 2006). While cleavage happened on the 5,6-epoxide ring such as **2**, **3**, **4**, **8**, **10**, and **11** resulted in less cytotoxicity. In addition, compounds **13** and **17** with 5,6-epoxide function but no double 2,3-bond showed weak cytotoxicity. Further, the structural differences between **6** and **7** showed the substitution of the hydroxyl group at C-4 did not cause any significant change in biological activity. Interestingly, the unusual known 5-chloro-withanolide, **9**, displayed significant activity, whereas compound **3** is inactive. For this special case, the cytotoxicity of **9** was repeatedly measured and confirmed for several times; however, an insufficient amount of **3** led to one assay only. The cytotoxicity of 5-chloro-withanolides needs further investigation.

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