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Original Article

Cytotoxic protobassic acid saponins from the kernels of *Palaquium formosanum*

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ABSTRACT

Bioassay guided fractionation and separation of the EtOH extract of the kernels of *Palaquium formosanum* against PC-3 cells via Sephadex LH-20 and reverse phase C-18 columns led to the isolation of 13 protobassic saponins. One of these saponins is new and was characterized as 3‴-*O*-rhamnopyranosyl-arganin C, a bisdesmoside of 16α-hydroxyprotobassic acid at the C-3 and C-28 positions. The structures of these compounds were determined on the basis of 1D NMR (^{1}H, ^{13}C), 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY), and selectively excited 1D TOCSY spectroscopic analyses and MS data, and comparison with literature data. Bioassay of these compounds and five additional compounds, isolated from *Planchonella obovata* leaf, against PC-3 prostate cancer cells indicated arganin C to be the most potent one with the IC_{50} value of 13.8 μM. Some structure and activity relationships were also drawn.

1. Introduction

Several plants of the Sapotaceae family have been reported to contain protobassic acid saponins, which have been demonstrated to possess cytotoxicity [1] and antifungal activity [2]. Our recent study on *Planchonella obovata* (R. Br.) Pierre leaves also resulted the isolation and characterization of nine protobassic acid glycosides, of which 6β-hydroxy-conyzasaponin N and Mi-saponin A showed cytotoxicity against HL-60 leukemia cell line with the respective IC_{50} value of 16.88 and 15.50 μM [3]. As the increasing population suffered from prostate cancer, the demand of new drugs for the treatment of this disease is urgent. Platycodin D, a 16α-hydroxyprotobassic acid glycoside, was reported to possess cytotoxicity against PC-3 cell (IC_{50} 11.17 μM) [4]. A preliminary study indicated that the *n*-BuOH-soluble fraction of the ethanolic extract of the kernels of *Palaquium formosanum* Hayata (Sapotaceae) was active against this prostate cancer cell line. From the kernels of *Argania spinosa*, a related Sapotaceous plant, such

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16α-hydroxyprotobassic acid saponins have been reported [5]. These two points motivated us to investigate whether *P. formosanum*, a widely cultivated tree along the street and in the park in Taiwan and the Philippines [6], contained such type saponin for potential application in cancer therapy.

As the monosodium salt of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST -8) can be reduced by dehydrogenases in viable cells to produce a yellow-color formazan dye with absorption maximum at λ 450 nm. The generation of formazan is directly proportional to the number of living cells. Thus the Cell Counting Kit-8 (CCK-8) mainly containing WST-8 was used to determine the cytotoxic effect of the isolated compounds by comparison of the viable cell number between the treated and the controls after 72 h incubation [7].

This study led to the isolation of 13 oleanane-type saponins with either protobassic acid or 16α-hydroxyprotobassic acid as the common aglycon from the kernels of this plant. Among them, one compound is new. Herein the isolation and structural elucidation of these saponins and their inhibitory activity against the PC-3 cell lines are described.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were performed with a JASCO P-2000 polarimeter (Tokyo, Japan). MS spectra were obtained on Bruker Daltonik Esquire 2000 (ESIMS, ESIMS/MS) and micrOTOF orthogonal ESI-TOF (HRESIMS) mass spectrometers (Bruker Daltonics, Bremen, Germany). 1D and 2D NMR spectra were recorded in CD_3OD (δ_H 3.30 and δ_C 49.0) on a Bruker Avance III 600 spectrometer, equipped with a dual-cryo probe (Karlsruhe, Germany). Centrifugal partition chromatography (CPC; model LLB-M) was purchased from Sanki Engineering Co. (Kyoto, Japan). Column Chromatography (CC) was carried out on pre-packed Lobar columns (size A, 240 × 10 mm; size B, 310 × 25 mm; size C, 440 × 37 mm, LiChroprep RP-18, 40–63 μm, Merck, Darmstadt, Germany) and Sephadex LH-20 (Pharmacia Co., Stockholm, Sweden). TLC plate (aluminum sheets, Silica 60 F_{254}, 20 × 20 cm, 0.2 mm thick, Merck) was used analytically. HPLC system consisted of a Hitachi L-7000 pump, a Hitachi L-7000 UV detector monitored at 210 nm, and a Prodigy ODS3 100A column (semi-prep., 250 × 10 mm, 5 μm, Phenomenex, California, USA), delivered by a mixture of solvent A, 0.1% formic acid (Merck) in H_2O, and solvent B, 0.1% formic acid in CH_3CN (Merck), with flow rate 2.3 mL/min.

2.2. Plant material

Kernels of *P. formosanum* were collected in June 2014 at National Museum of Marine Biology and Aquarium, Ping-Tung, Taiwan. A voucher specimen (NTUSP201406) was stored in School of Pharmacy, National Taiwan University.

2.3. Extraction and isolation

Fresh kernels of *P. formosanum* (926 g) were ground and the formed paste was extracted by 95% EtOH (1.3 L × 1 and 1.0 L × 1, rt, each 1 d; 1.0 L, 50 °C, 1 h). Evaporation of solvents under reduced pressure at 50 °C gave an ethanolic extract (113.6 g). Most of this extract (100 g), suspended in H_2O (350 mL), was divided into fractions soluble in CH_2Cl_2 (2.8 g), EtOAc (7.8 g), *n*-BuOH (21.9 g), and H_2O (65.5 g) by successive liquid–liquid partitioning, each 350 mL × 3, and subsequent condensation.

Most of the *n*-BuOH-soluble fraction (20.4 g) was subjected to Sephadex LH-20 CC (7.0 (O.D.) × 62.0 cm, MeOH) to give eight fractions (frs. 1–8). An aliquot of fraction 2 (2.3 g out of 5.0 g) was chromatographed over an RP-18 Lobar column (size C), eluted with a gradient of MeOH–0.1% formic $acid_{(aq)}$ (50%–65%), to yield five fractions (frs. 2.1–5). Fraction 2.2 (203.5 mg) was chromatographed over a Sephadex LH-20 column (1.5 (O.D.) × 69.0 cm, MeOH–H_2O 1:1), followed by semi-prep. HPLC (4 mg × 14; A–B 24:76) to give **2** (11.1 mg, *R*t 20.7 min), **3** (3.7 mg, *R*t 23.7 min), **10** (2.6 mg, *R*t 34.7 min), **1** (4.2 mg, *R*t 38.6 min), and **9** (7.6 mg, *R*t 43.4 min). Fraction 2.4 (282.4 mg) was chromatographed on a Sephadex LH-20 column (2.0 (O.D.) × 61.0 cm, MeOH–H_2O 1:1) to give four fractions (frs. 2.4.1–4). Fraction 2.4.2 (75.1 mg) was chromatographed on a semi-prep. HPLC column (5 mg × 15; A–B 26:74) to afford **13** (37.9 mg, *R*t 18.3 min), **6** (6.6 mg, *R*t 21.1 min), and **12** (2.1 mg, *R*t 24.0 min). Fraction 2.4.3 (72.5 mg) yielded **11** (24.6 mg) via Sephadex LH-20 CC (1.5 (O.D.) × 69.0 cm, MeOH–H_2O 1:1).

Fraction 3 (965.7 mg) was fractionated via CPC, using the lower and upper layers of $CHCl_3$–MeOH–0.1% formic $acid_{(aq)}$ (2:2:1) as the stationary and mobile phases, respectively, to yield three fractions (frs. 3.1–3). Fraction 3.1 (517.4 mg) was chromatographed over a RP-18 Lobar column (size B; MeOH–0.1% formic $acid_{(aq)}$ 45%–70%) to yield five fractions (frs. 3.1.1–5). Fraction 3.1.4 was **5** (47.4 mg). Fraction 3.1.2 was chromatographed over a RP-18 Lobar column (size B; CH_3CN–0.1% formic $acid_{(aq)}$ 25:75) to afford **8** (18.3 mg) and **7** (15.2 mg). Fraction 3.1.3 (230.2 mg) was chromatographed in sequence on a Lobar column (size B; MeOH–0.1% $HOAc_{(aq)}$ 50%–60%), Sephadex LH-20 column (MeOH–H_2O 1:1), and a Lobar column (size A; CH_3CN–0.1% formic $acid_{(aq)}$ 25:75), to afford **4** (38.3 mg).

2.4. 3‴-O-rhamnopyranosyl-arganin C (1)

White powder, $[\alpha]_D^{25}$ − 48.8 (*c* 0.42, MeOH); for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 3 and Fig. S1 and 2, Supplementary data; 1D TOCSY, see Fig. 2; COSY, NOESY, HSQC, and HMBC, see Fig. S3–9, Supplementary data; ESIMS *m/z* (rel. int.): 1407 $[M + Na]^+$ (100); ESIMS/MS (1407) *m/z* (rel. int.): 725 $[M + Na - 520 - 162]^+$ (100), 705 $[M + Na - 132 - 132 - 146 - 146 - 146]^+$ (27), 593 $[M + Na - 520 - 162 - 132]^+$ (10); HRESIMS$^-$: *m/z* 1383.6490 $[M - H]^-$ (Calc. for $C_{64}H_{103}O_{32}$: 1383.6432), see Fig. S13, Supplementary data.

2.5. Bioassay

2.5.1. Chemicals and cell line

PC-3 cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), glutamine, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Paclitaxel, DMSO, and Cell

Table 1 – ^{1}H and ^{13}C NMR data of the aglycon moiety of compounds 1, 6 and 10[a] in methanol-d_4.

No.	1				6	
	δ_C	δ_H (m, J/Hz)[b]	HMBC corr. (C#)	NOESY corr. (H#)	δ_C	δ_H (m, J/Hz)
1	46.7, CH_2	1.18[b] (ax)	10, 25	1 (eq), 2, 3, 5, 9	46.7, CH_2	1.16[d]
		2.05 (br d, 14.3) (eq)	2, 3, 5, 10, 25	1 (ax), 2		2.02 (dd, 14.0, 2.2)
2	71.4, CH	4.33 (q-like, 3.1)	10	1 (ax), 1 (eq), 3	71.4, CH	4.29 (q-like, 2.6)
3	83.7, CH	3.57 (d, 3.8)	2, 4, 23, 24, 1 (Glc-I)	1 (ax), 2, 5, 1 (Glc-I)	83.5, CH	3.55 (d, 3.5)
4	44.1, C				44.1, C	
5	49.0, CH	1.32 (br s)	6, 10, 24, 25	1 (ax), 3, 6, 7 (ax), 9	49.1, CH	1.31 (br s)
6	68.6, CH	4.45 (m)	4, 5, 8, 10	5, 7 (ax), 7 (eq), 23 (a), 23 (b)	68.6, CH	4.46 (m)
7	41.4, CH_2	1.52 (dd, 14.0, 1.6) (eq)	5, 6, 8, 9	6, 7 (ax)	41.3, CH_2	1.51 (dd, 15.1, 1.4)
		1.81 (dd, 14.0, 4.7) (ax)	8, 14, 26	5, 6, 7 (eq), 27		1.77[e]
8	40.0, C				39.9, C	
9	48.8, CH	1.65 (dd, 11.5, 6.0)	1, 8, 10, 11, 14, 25, 26	1 (ax), 5, 11 (α), 27	49.7, CH	1.59[f]
10	37.2, C				37.2, C	
11	24.6, CH_2	1.97 (dt, 17.9, 4.7) (α)	8, 9, 12, 13	9, 11 (β), 12	24.7, CH_2	1.96 (dt, 18.1, 4.6)
		2.11 (ddd, 17.9, 11.5, 2.3) (β)	9, 12, 13	11 (α), 12, 25, 26		2.11 (ddd, 18.1, 11.5, 2.8)
12	124.2, CH	5.42 (br t, 3.5)	9, 14	11 (α), 11 (β), 18, 26	124.3, CH	5.34 (br t, 3.2)
13	143.9, C				144.2, C	
14	43.4, C				43.7, C	
15	36.2, CH_2	1.39 (dd, 14.6, 3.3) (eq)	13, 16, 17	15 (ax), 16	29.0, CH_2	1.14[d]
		1.84[c] (ax)	8, 14, 27	15 (eq), 16, 26		1.72[e]
16	74.7, CH	4.48 (br t 3.0)	14, 15, 17, 18	15 (ax), 15 (eq), 22 (eq)	23.7, CH_2	1.65 (br d, 13.1), 2.01 (m)
17	50.4, C				48.4, C	
18	42.2, CH	3.09 (dd, 14.3, 4.0)	12, 13, 14, 16, 17, 19, 28	12, 19 (eq), 30	42.8, CH	2.93 (dd, 13.6, 3.7)
19	47.6, CH_2	1.05 (eq)	17, 20, 21, 30	18, 19 (ax)	47.1, CH_2	1.15[d]
		2.27 (t, 13.5) (ax)	13, 17, 18, 20, 29, 30	19 (eq), 21 (ax), 27		1.72[e]
20	31.3, C				31.6, C	
21	36.4, CH_2	1.15[b] (eq)	20	21 (ax), 22 (eq), 29	34.9, CH_2	1.21 (br d, 13.6)
		1.89[c] (ax)	20, 30	19 (ax), 21 (eq), 29		1.39 (td, 13.6, 3.5)
22	31.7, CH_2	1.80[c] (ax)	16, 17, 20, 21, 28	30	33.4, CH_2	1.55[f]
		1.88[c] (eq)	16, 17, 18, 20, 21	16, 21 (eq)		1.76[e]
23	65.3, CH_2	3.41 (d, 11.3) (a)	3, 4, 5, 24	6, 23 (b), 24	65.3, CH_2	3.41 (d, 12.5)
		3.72 (d, 11.3) (b)		6, 23 (a), 24		3.73 (d, 12.5)
24	16.3, CH_3	1.30 (s)	3, 4, 5, 23	23 (a), 23 (b), 25	16.3, CH_3	1.30 (s)
25	19.3, CH_3	1.62 (s)	1, 5, 9, 10	11 (β), 24, 26	19.2, CH_3	1.60 (s)
26	19.1, CH_3	1.06 (s)	8, 9, 14	11 (β), 12, 15 (ax), 25	18.9, CH_3	1.05 (s)
27	27.4, CH_3	1.33 (s)	8, 13, 14, 15	7 (ax), 9, 19 (ax)	26.3, CH_3	1.13 (s)
28	177.1, C				177.9, C	
29	33.4, CH_3	0.88 (s)	18, 19, 20, 21, 30	21 (ax), 21 (eq)	33.5, CH_3	0.90 (s)
30	25.2, CH_3	0.98 (s)	19, 20, 21, 29	18, 22 (ax)	24.0, CH_3	0.94 (s)

[b-f] Overlapped signals are reported without designated multiplicity.
[a] ^{1}H and ^{13}C NMR data of the aglycon moiety of **10** were almost superimposable with those of **1**.

Table 2 – HRESIMS data and molecular formula of 1–13.

Cpd.	Molecular formula	HRESIMS		
		Parent ion	m/z observed (Calc.)	Error (ppm)
1	$C_{64}H_{104}O_{32}$	$[M-H]^-$	1383.6490 ($C_{64}H_{103}O_{32}$, 1383.6432)	−4.2
2	$C_{64}H_{104}O_{33}$	$[M-H]^-$	1399.6372 ($C_{64}H_{103}O_{33}$, 1399.6382)	0.7
3	$C_{63}H_{102}O_{33}$	$[M-H]^-$	1385.6272 ($C_{63}H_{101}O_{33}$, 1385.6225)	−3.4
4	$C_{58}H_{94}O_{28}$	$[M-H]^-$	1237.5855 ($C_{58}H_{93}O_{28}$, 1237.5853)	−0.1
5	$C_{57}H_{92}O_{28}$	$[M-H]^-$	1223.5722 ($C_{57}H_{91}O_{28}$, 1223.5697)	−2.1
6	$C_{58}H_{91}NaO_{28}$	$[M+Na]^+$	1281.5493 ($C_{58}H_{91}Na_2O_{28}$, 1281.5492)	−0.0
7	$C_{57}H_{89}NaO_{29}$	$[M+Na]^+$	1283.5271 ($C_{57}H_{89}Na_2O_{29}$, 1283.5285)	1.1
8	$C_{58}H_{91}NaO_{29}$	$[M+Na]^+$	1297.5437 ($C_{58}H_{91}Na_2O_{29}$, 1297.5441)	0.3
9	$C_{64}H_{104}O_{33}$	$[M-H]^-$	1399.6375 ($C_{64}H_{103}O_{33}$, 1399.6382)	0.5
10	$C_{70}H_{114}O_{37}$	$[M-H]^-$	1545.6968 ($C_{70}H_{113}O_{37}$, 1545.6961)	−0.5
11	$C_{58}H_{94}O_{27}$	$[M-H]^-$	1221.5908 ($C_{58}H_{93}O_{27}$, 1221.5904)	−0.3
12	$C_{57}H_{89}NaO_{28}$	$[M+Na]^+$	1267.5335 ($C_{57}H_{89}Na_2O_{28}$, 1267.5336)	0.1
13	$C_{64}H_{104}O_{32}$	$[M-H]^-$	1383.6439 ($C_{64}H_{103}O_{32}$, 1383.6432)	−0.5

Table 3 – ^{1}H and ^{13}C NMR data of the glycon moiety of compounds 1 and 10[a] in methanol-d_4.

Pos.	δ_C	δ_H (m, J/Hz)	1	
			HMBC corr. (C#)	NOESY corr. (H#)
Ara				
1	93.8, CH	5.67 (d, 3.1)	2, 3, 5, 28 (agly.)	2, 3, 5 (α)
2	76.0, CH	3.78 (dd, 4.9, 3.1)	3, 1 (Rha-I)	1, 1 (Rha-I)
3	70.8, CH	3.89 (dd, 4.9, 3.4)	2	1, 5 (α)
4	66.6, CH	3.83 (m)	3	5 (α)
5	63.3, CH_2	3.49 (dd, 10.9, 3.8) (α)	1, 3	1, 3, 4, 5 (β)
		3.92 (dd, 10.9, 6.9)[b] (β)	1, 3, 4	5 (α)
Rha-I				
1	101.5, CH	4.97 (d, 1.6)	3, 5, 2 (Ara)	2, 2 (Ara)
2	72.3, CH	3.94 (dd, 3.7, 1.6)	3, 4	1
3	81.3, CH	3.89 (dd, 9.3, 3.7)[b]	4, 1 (Rha-II)	5, 1 (Rha-II)
4	77.7, CH	3.74 (t, 9.3)	5, 6, 1 (Xyl)	6, 1 (Xyl)
5	69.4, CH	3.75 (m)	3, 4	3, 6
6	18.3, CH_3	1.26 (d, 6.2)	4, 5	4, 5
Rha-II				
1	104.4, CH	4.98 (d, 1.3)	3, 5, 3 (Rha-I)	2, 3 (Rha-I)
2	72.1, CH	4.04 (dd, 3.1, 1.3)	2, 4	1, 3, 1 (Xyl)
3	72.2, CH	3.73 (dd, 9.4, 3.1)	4	2
4	74.0, CH	3.39 (t, 9.4)	2, 3, 6	6
5	70.3, CH	3.82 (dt, 9.4, 6.2)[b]	4, 6	6
6	18.0, CH_3	1.24 (d, 6.2)	4, 5	4, 5
Xyl				
1	105.1, CH	4.50 (d, 7.7)	2, 3, 5, 4 (Rha-I)	3, 5 (ax), 2 (Rha-II), 4 (Rha-I)
2	75.5, CH	3.30 (dd, 8.9, 8.1)[b]	3	4
3	84.1, CH	3.42 (t, 8.9)	2, 4, 1 (Rha-III)	1, 5 (ax), 1 (Rha-III)
4	70.1, CH	3.54 (ddd, 10.4, 8.9, 5.4)[b]	3, 5	2, 5 (eq)
5	67.0, CH_2	3.16 (dd, 11.4, 10.4) (ax)	1, 3, 4	1, 3, 5 (eq)
		3.87 (dd, 11.4, 5.4) (eq)	1, 3, 4	4, 5 (ax)
Rha-III				
1	102.5, CH	5.15 (d, 1.5)	3, 5, 3 (Xyl)	2, 3 (Xyl)
2	72.2, CH	3.94 (dd, 3.0, 1.5)	3, 4	1
3	72.3, CH	3.70 (dd, 9.6, 3.0)	4	5
4	74.0, CH	3.38 (t, 9.6)[b]	3, 5, 6	6
5	70.1, CH	3.99 (dq, 9.6, 6.2)	4, 6	3, 6
6	17.9, CH_3	1.24 (d, 6.2)	4, 5	4, 5
Glc				
1	105.3, CH	4.43 (d, 7.7)	5, 3 (agly.)	3, 5, 3 (agly.)
2	75.4, CH	3.29 (dd, 7.7, 9.6)[b]	1	
3	78.2, CH	3.35 (t, 9.6)[b]	2, 4	1
4	71.1, CH	3.36 (t, 9.8)[b]	2, 3	
5	77.7, CH	3.27 (m)	3	1, 6
6	62.3, CH_2	3.69 (dd, 11.9, 4.7) (a)	5	5
		3.80 (br d, 11.9) (b)	4, 5	5

[a] ^{1}H and ^{13}C NMR data of the 28-*O*-glycosyl moiety of **10** were almost superimposable with those of **1**; ^{1}H and ^{13}C NMR data of 3-*O*-diglycosyl moiety of **10**: Glc-I δ_H 4.50 (H-1, d, 7.7), 3.48 (H-2, dd, 8.8, 7.7),[b] 3.55 (H-3, dd, 9.2, 8.8),[b] 3.48 (H-4, t, 9.2),[b] 3.30 (H-5, m); 3.71 (dd, 12.1, 6.0) & 3.81 (dd, 12.1, 2.4) (H_2-6); δ_C 105.0 (C-1), 74.8 (C-2), 88.0 (C-3), 69.6 (C-4), 77.4 (C-5), 62.2 (C-6); Glc-II δ_H 4.56 (H-1, d, 7.9),[b] 3.30 (H-2, dd, 9.3, 7.9),[b] 3.38 (H-3, t, 9.3),[b] 3.27 (H-4, t, 9.3),[b] 3.32 (H-5, m); 3.63 (dd, 11.8, 6.2) & 3.87 (dd, 11.8, 2.4)[b] (H_2-6); δ_C 105.3 (C-1), 75.5 (C-2), 77.8 (C-3), 71.6 (C-4), 78.2 (C-5), 62.6 (C-6).

[b] The coupling constants were obtained from the 1D TOCSY spectroscopic analysis.

Counting Kit-8 (CCK-8) were obtained from Sigma–Aldrich (St Louis, MO, USA).

2.5.2. *Sample preparation*

The *n*-BuOH, H_2O-soluble fractions, and tested compounds were dissolved in water. Paclitaxel (positive control), CH_2Cl_2- and EtOAc-soluble fractions were dissolved in DMSO and further diluted in culture medium. The final concentration of test compounds was ranging from 3 μM to 30 μM and the final concentrations of DMSO for all treatments were less than 0.1%.

2.5.3. *Cell culture and cell viability assay (CCK-8 assay)*

PC-3 cells were cultured in RPMI-1640 medium, supplemented with 10% FBS, glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL), and were maintained at 37 °C in a 5% CO_2 atmosphere.

Cell viability of PC-3 cells was analyzed using the Cell Counting Kit-8 (CCK8). According to the manufacturer's instructions, PC-3 cells (8×10^3) were seeded in 96-well plates, then different concentrations of individual fraction or compound were added to each well. H_2O and DMSO served as the

control and solvent control respectively, and paclitaxel served as the positive control. After 72 h incubation at 37° C, CCK-8 reagent (10 μL) was added and incubated for 1 h at 37 °C. Then, the absorbance was measured using the microplate reader (Biotek Instruments, EPOCH, VT, USA) at a wavelength of 450 nm. The cell viability (% of control) is expressed as the percentage of $(OD_{test} - OD_{blank})/(OD_{control} - OD_{blank})$, where OD_{test} is the optical density of the cells exposed to the test sample, $OD_{control}$ is the optical density of the control sample and OD_{blank} is the optical density of the wells without PC-3 cells [7].

2.5.4. *Statistical analysis*

Values are reported as means ± standard error of the mean.

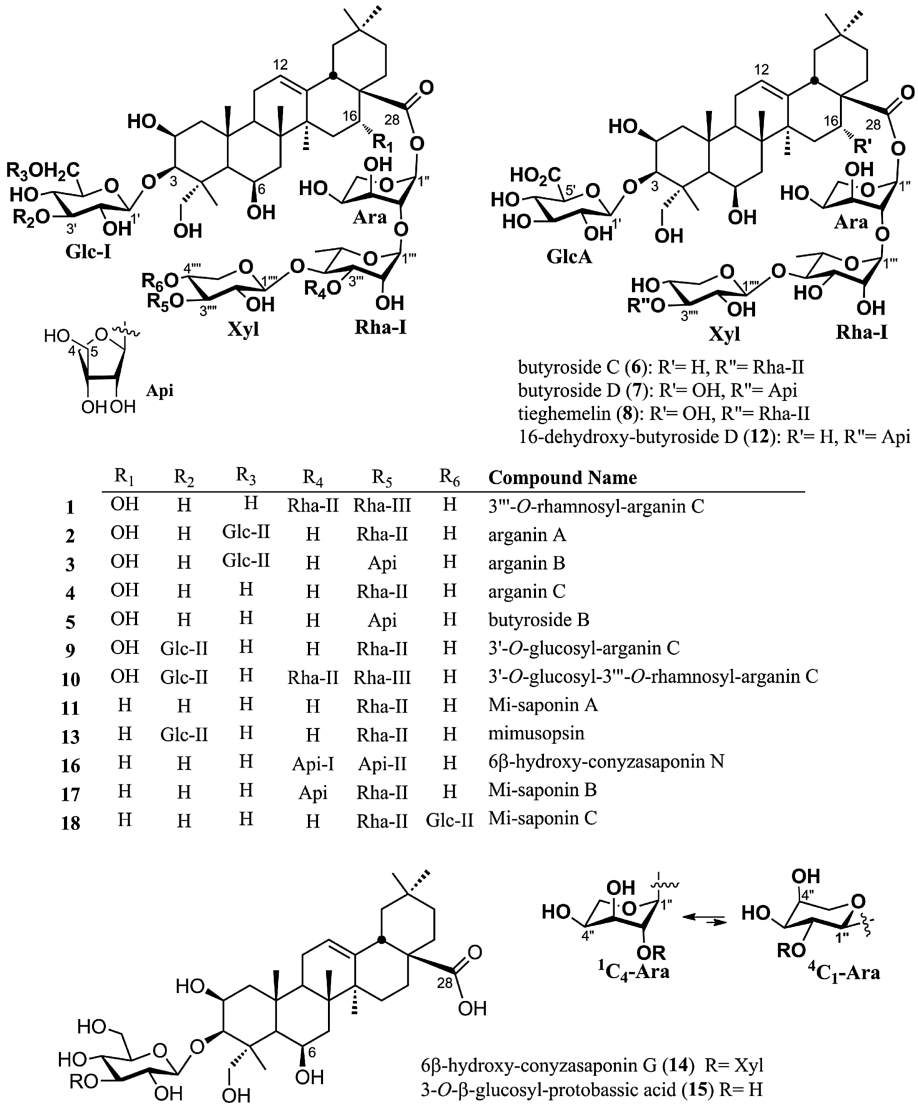
3. Results and discussion

The 95% ethanolic extract of *P. formosanum* kernels was divided into fractions soluble in dichloromethane, ethyl acetate, *n*-butanol, and water by liquid–liquid partitioning process. The *n*-butanol-soluble fraction being cytotoxic against PC-3 prostate cancer cell line (Table S1; Supplementary data) was further fractionated by Sephadex LH-20 to focus the bioactive saponins, e.g. fraction 2 (IC_{50} 30.8 μg/mL). These highly polar saponins possessed great similarity in polarity. Attempt to separate them by centrifugal partition chromatography (CPC), however, was not successful ascribable to their surfactant property, which disturbed the equilibrium between mobile and stationary phases. They were then separated by repeated chromatography on Sephadex LH-20 and reverse phase C-18 columns (Lobar and semi-preparative HPLC). These efforts led to the isolation and characterization of one new (**1**) and 12 known oleanane-type saponins (**2**–**13**).

Compounds **6** and **11**–**13** (Fig. 1) possess the protobassic acid residue, i.e., 2β,6β,23-trihydroxy-oleanolic acid, in common as aglycon, verified by their ^{1}H NMR spectra, showing characteristic six methyl singlets (δ 0.90, 0.94, 1.05, 1.13, 1.30, 1.60; **6**), H-12 (δ 5.34, br t, J = 3.2 Hz; **6**), three oxymethine signals ($\delta_{H\text{-}2}$ 4.29, q-like, J = 2.6 Hz; $\delta_{H\text{-}3}$ 3.55, d, J = 3.5 Hz; $\delta_{H\text{-}6}$ 4.46, m; **6**), an AX system for one oxymethylene ($\delta_{H\text{-}23}$ 3.41 and 3.73, J_{AX} = 12.5 Hz; **6**), and a double doublet for H-18 (δ 2.93, dd, J = 13.6, 3.7 Hz; **6**) (Table 1). The presence of a protobassic acid residue in these compounds was further supported by their ^{13}C NMR spectra, showing typical signals for the corresponding six methyls (δ 16.3, 18.9, 19.2, 24.0, 26.3, and 33.5; **6**), three oxymethines (δ 68.6, 71.4, and 83.5; **6**), one oxymethylene ($\delta_{C\text{-}23}$ 65.3; **6**), and two olefinic carbons ($\delta_{C\text{-}12}$ 124.3, CH and $\delta_{C\text{-}13}$ 144.2, C; **6**) in addition to an ester carbonyl ($\delta_{C\text{-}28}$ 177.9; **6**) (Table 1) [8]. These four compounds belong to protobassic acid pentaosides (**6**, **11** & **12**) and hexaoside (**13**) as deduced from the molecular formula (Table 2), obtained from HRESIMS and NMR (^{1}H and ^{13}C) data. Among them, compounds **6** and **12** contained a sodium glucuronyl residue as exemplified by characteristic NMR signals, $\delta_{GlcA\ H\text{-}5}$ 3.72, d, J = 9.6 Hz and $\delta_{GlcA\ C\text{-}6}$ 174.2 (**6**, Table S2; Supplementary data), and HRESIMS$^+$, in which $[M + Na]^+$ ion of **6** had *m/z* 1281.5493, affording a molecular formula of $C_{58}H_{91}NaO_{28}$ (Table 2). On comparison with the spectroscopic data with those reported in the literature, they were identified as butyroside C (**6**) [9], Mi-saponin A (**11**) [3], 16-dehydroxy-butyroside D [i.e., 3-*O*-β-D-glucuronopyranosyl-28-*O*-(β-D-apiofuranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-protobassic acid] (**12**) [10], and mimusopsin (**13**) [9].

Compounds **1**–**5** and **7**–**10** (Fig. 1) possess the 16α-hydroxyprotobassic acid residue, i.e., 2β,6β,16α,23-tetrahydroxy-oleanolic acid, in common as aglycon, verified by the NMR spectra, showing signals for an additional oxymethine, $\delta_{H\text{-}16\beta}$ 4.48 and $\delta_{C\text{-}16}$ 74.7 (**1**) (Table 1), besides those typical signals for the protobassic acid residue as indicated above [11]. These nine compounds belong to 16α-hydroxyprotobassic acid pentaosides (**4**, **5**, **7**, **8**), hexaosides (**1**–**3**, **9**), and heptaoside (**10**) as deduced from the molecular formula, obtained from HRESIMS (Table 2), ^{1}H and ^{13}C NMR data. Among them, compounds **7** and **8** contained a glucuronic acid residue and were also present as the sodium salt, exemplified by the characteristic NMR signals, $\delta_{GlcA\ H\text{-}5}$ 3.78, d, J = 9.7 Hz and $\delta_{GlcA\ C\text{-}6}$ 173.5 (**7**, Table S2; Supplementary data) and HRESIMS$^+$ data (Table 2). On comparison with the ^{1}H and ^{13}C NMR data, which were assigned comprehensively by 1D and 2D NMR spectroscopic analyses (to be described later), with those reported in the literature, eight of them were identified as arganins A (**2**), B (**3**) and C (**4**) [5,9], butyrosides B (**5**) & D (**7**) [12,13], tieghemelin (**8**) [9], 3′-*O*-glucosyl-arganin C [i.e., 3-*O*-(β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl)-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid] (**9**) [14], and 3′-*O*-glucosyl-3‴-*O*-rhamnosyl-arganin C [i.e., 3-*O*-(β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl)-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid] (**10**) [15].

Compound **1**, obtained as a white powder, had a molecular formula of $C_{64}H_{104}O_{32}$ as established by ^{13}C NMR data and HRESIMS. The ^{1}H NMR spectrum showed proton signals for the aglycon 16α-hydroxyprotobassic acid residue, six methyl singlets (δ 0.88, 0.98, 1.06, 1.30, 1.33, and 1.62), one triplet for the olefinic H-12 (δ 5.42), an AX system (δ 3.41 and 3.72, d, J = 11.3 Hz) for an oxymethylene (H_2-23), and typical signals for four oxymethine protons ($\delta_{H\text{-}2}$ 4.33, q-like, J = 3.1 Hz; $\delta_{H\text{-}3}$ 3.57, d, J = 3.8 Hz; $\delta_{H\text{-}6}$ 4.45, m; $\delta_{H\text{-}16\beta}$ 4.48, br t, J = 3.0 Hz), which were closely resemblance to those of the aglycon moiety in **10** (Table 1). Compared with those of 6β-hydroxy-conyzasaponin G (**14**) [3], both proton and carbon signals of the Me-27 in **1** were downfield shifted (δ_H 1.33 vs 1.13; δ_C 27.4 vs 26.3) due to the respective polarization (^{1}H) and δ-syn (^{13}C) effect to this methyl by the α-oriented (i.e. axially oriented) 16-hydroxy group. The ^{13}C NMR spectrum showed the presence of six methyl groups (δ 16.3, 19.1, 19.3, 25.2, 27.4, and 33.4), two olefinic carbons ($\delta_{C\text{-}12}$ 124.2 and $\delta_{C\text{-}13}$ 143.9), and an ester carbonyl carbon ($\delta_{C\text{-}28}$ 177.1) (Table 1). These analyses confirmed the aglycon of **1** as 16α-hydroxyprotobassic acid [11]. Subtraction of the molecular formula of **1** from that of 16α-hydroxyprotobassic acid (C_{30}) left a C_{34} residue, corresponding to four hexosyl and two pentosyl residues. Using ESIMS/MS, bombardment on the quasi-molecular ion of **1** ($[M + Na]^+$, *m/z* 1407) gave three daughter ions at *m/z* 725 $[M + Na - 520 - 162]^+$, 705 $[M + Na - (2 \times 132) - (3 \times 146)]^+$, and 593 $[M + Na - 520 - 162 - 132]^+$, indicating the presence of two pentosyl (132 amu, each), three deoxyhexosyl (146 amu, each), and one hexosyl residue



	R_1	R_2	R_3	R_4	R_5	R_6	Compound Name
1	OH	H	H	Rha-II	Rha-III	H	3'''-*O*-rhamnosyl-arganin C
2	OH	H	Glc-II	H	Rha-II	H	arganin A
3	OH	H	Glc-II	H	Api	H	arganin B
4	OH	H	H	H	Rha-II	H	arganin C
5	OH	H	H	H	Api	H	butyroside B
9	OH	Glc-II	H	H	Rha-II	H	3'-*O*-glucosyl-arganin C
10	OH	Glc-II	H	Rha-II	Rha-III	H	3'-*O*-glucosyl-3'''-*O*-rhamnosyl-arganin C
11	H	H	H	H	Rha-II	H	Mi-saponin A
13	H	Glc-II	H	H	Rha-II	H	mimusopsin
16	H	H	H	Api-I	Api-II	H	6β-hydroxy-conyzasaponin N
17	H	H	H	Api	Rha-II	H	Mi-saponin B
18	H	H	H	H	Rha-II	Glc-II	Mi-saponin C

Fig. 1 – Structures of compounds 1–18 isolated from *Palaquium formosanum* kernels (1–13) and *Planchonella obovata* leaf (14–18).

(162 amu), and a 520 amu aglycon, the latter being consistent with that of 16α-hydroxyprotobassic acid. This MS fragmentation pattern revealed a pentosyl and a hexosyl residue as terminal glycon. The ^{1}H NMR spectrum of **1** showed signals for six anomeric protons [δ 4.43 (d, J = 7.7 Hz), 4.50 (d, J = 7.7 Hz), 4.97 (d, J = 1.6 Hz), 4.98 (d, J = 1.3 Hz), 5.15 (d, J = 1.5 Hz), and 5.67 (d, J = 3.1 Hz)] and three methyl doublets, two of which overlapped at δ 1.24 (d, J = 6.2 Hz, 6H) and the other at δ 1.26 (d, J = 6.2 Hz, 3H), suggesting **1** to be a three rhamnosyl containing hexaoside. The HSQC spectrum of **1** showed correlation of δ_H 4.43/δ_C 105.3, δ_H 4.50/δ_C 105.1, δ_H 4.97/δ_C 101.5, and δ_H 4.98/δ_C 104.4, δ_H 5.15/δ_C 102.5, and δ_H 5.67/δ_C 93.8. Complete ^{1}H and ^{13}C NMR assignments for each sugar residue were achieved from 1D and 2D NMR spectroscopic analyses (1D TOCSY, 2D COSY, HSQC, HMBC, and NOESY) (Table 3). Selective excitation at the respective anomeric proton and the Rha Me-6 using 1D TOCSY pulse program yielded the relayed ^{1}H NMR spectrum of each monosaccharide residue (Fig. 2). Analysis of these spectra designated these six sugar residues as β-Glc ($\delta_{H\text{-}1}$ 4.43), β-Xyl ($\delta_{H\text{-}1}$ 4.50), α-Rha ($\delta_{H\text{-}1}$ 4.97, 4.98, 5.15), and α-Arap ($\delta_{H\text{-}1}$ 5.67). The HMBC spectrum of **1** showed correlations of aglycon H-3 (δ 3.57)/Glc C-1 (δ 105.3), Ara H-1 (δ 5.67)/aglycon C-28 (δ 177.1), Rha-I H-1 (δ 4.97)/Ara C-2 (δ 76.0), Rha-II H-1 (δ 4.98)/Rha-I C-3 (δ 81.3), Xyl H-1 (δ 4.50)/Rha-I C-4 (δ 77.7), Rha-III H-1 (δ 5.15)/Xyl C-3 (δ 84.1), as shown in Table 3, designating the following glycosidic linkages, Glc-(1 → 3)-aglycon, Ara-(1 → 28)-aglycon, Rha-I-(1 → 2)-Ara, Rha-II-(1 → 3)-Rha-I, Xyl-(1 → 4)-Rha-I, and Rha-III-(1 → 3)-Xyl. The monosaccharide compositions in these glycosides were almost identical as what had been isolated from *P. obovata*, a related species to *P. formosanum*. They had been determined as L-arabinose, D-xylose, D-glucose, and L-rhamnose by GC analysis of the respective trimethylsilyl L-cysteine derivative of the acid hydrolysates of Mi-saponins B (**17**) and C (**18**) [8]. Accordingly, compound **1** was elucidated as 3-*O*-β-D-glucopyranosyl-28-*O*-(α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 3)]-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)-16α-hydroxyprotobassic acid (i.e., 3‴-*O*-rhamnopyranosyl-arganin C).

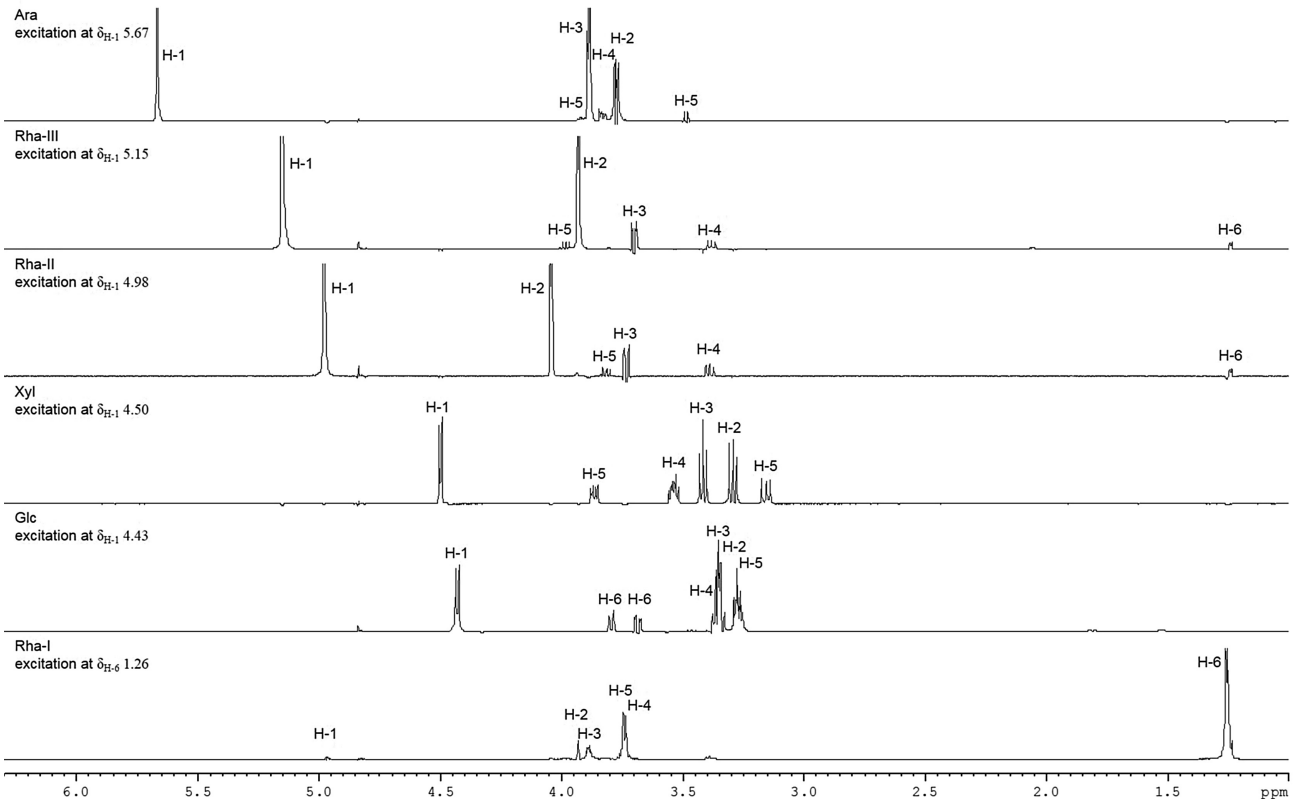


Fig. 2 – 1D TOCSY NMR spectroscopic spectra of 1 (CD_3OD, 600 MHz) with the selectively excited signals shown in insets.

While examining the ^1H and ^{13}C NMR assignments, a $^1C_4$ conformation was assigned for the α-L-arabinopyranosyl moiety in common for these compounds (Fig. 1). This designation was based on the small coupling constant for Ara H-1 ($J = 3.1$ Hz), for which di-equatorial coupling accounted. While one report for **10** was assigned $^4C_1$ conformation to this moiety, based on a diaxial coupling between H-1 and H-2 ($J = 7.5$ Hz) and between H-2 and H-3 ($J = 9.6$ Hz) [15]. Analysis of a NOESY spectrum of **1**, the 3′-deglucosylated **10**, however, did show the correlation of $\delta_{Ara\ H\text{-}1}$ to $\delta_{Ara\ H\text{-}3}$ and $\delta_{Ara\ H\text{-}5}$ (Table 3 and Fig. S5; Supplementary data), indicating a dynamic equilibrium between both $^1C_4$ and $^4C_1$ conformations (Fig. 1). Through extensive examination of the 1D TOCSY, 2D HSQC, and HMBC, more precise ^{13}C NMR assignment of **10** as marked in Figs. S10–12 (Supplementary data) were made (Table 3), leading to the revision for three pairs, Ara C-3 and C-4, Rha-I C-1 and Rha-III C-1, Xyl C-1 and Glc-II C-1, from the reported data [15].

Compounds **1**–**13** together with five protobassic acid glycosides (**14**–**18**), isolated from *P. obovata* leaf, were evaluated for *in vitro* cytotoxic activity against prostate cancer cell line PC-3. The viability of this cell line after culturing respectively with these compounds at 30 μM and the IC_{50} values of the more potent ones are shown in Table 4. Among them, the 16α-hydroxyprotobassic acid glycosides arganin C (**4**) and butyroside B (**5**) showed better inhibitory activity with the IC_{50} values of 13.8 and 17.2 μM, respectively. From this assay result, some structure-activity relationships of protobassic acid glycosides were also drawn. Absence of the glycon moiety at the C-28 position led to almost complete loss of cytotoxicity, as shown for **14** and **15**. If C-3 is *O*-glucuronylated, the compound will be either inactive (**12**) or decrease activity to some extent (**6**–**8**) relative to those *O*-glucosylated. While the aglycon is 16α-hydroxylated, the glycosides containing this aglycon are much more active than those without this substitution, e.g. **7** vs. **12**, **8** vs. **6**, and **4** vs. **18**. If C-3 was *O*-diglucosylated, sugar linkage played an important role in cytotoxicity. For those possessing 3-*O*-Glc-(6 → 1)-Glc substitution (**2** and **3**), they are almost inactive relative to those having 3-*O*-Glc-(3 → 1)-Glc substitution (**9**, **10**, and **13**). In addition, the latter 3-*O*-diglucosides and the corresponding 3-*O*-glucosides (**4**, **1**, and **11**) possessed similar activity with the IC_{50} values around 25 μM.

Table 4 – The cytotoxicity of 1–18 against PC-3 cells after incubation for 72 h.

Cpd.	Cell viability (% of control)/IC_{50} (μM)[a]	Cpd.	Cell viability (% of control)/IC_{50} (μM)[a]
1	50.5 ± 0.4/29.7	**10**	46.0 ± 1.1/27.1
2	81.4 ± 6.2/-	**11**	33.7 ± 3.1/22.2
3	80.7 ± 3.8/-	**12**	125.1 ± 8.8/-
4	34.4 ± 1.1/13.8	**13**	43.4 ± 1.8/25.4
5	36.7 ± 0.5/17.2	**14**	100.2 ± 16.0/-
6	86.9 ± 3.4/-	**15**	86.3 ± 11.1/-
7	59.9 ± 4.6/-	**16**	38.8 ± 0.4/24.1
8	59.8 ± 1.9/-	**17**	42.8 ± 2.6/26.4
9	36.8 ± 0.4/21.9	**18**	48.6 ± 1.3/29.2

Paclitaxel was used as a positive control which the cell viability was 48.6 ± 1.7% at 5 μM.

[a] % of cell viability at 30 μM were calculated from the dose response of each test sample (n = 4).

Replacement of the deoxyhexosyl Rha at C-3‴′ (R_5, Fig. 1) by the pentosyl Api seems not affect the activity as shown for **4** (vs. **5**) and **17** (vs. **16**).

4. Conclusion

Thirteen protobassic saponins were isolated and characterized from the *n*-BuOH-soluble fraction of *P. formosanum* kernels. Of these, 3‴-*O*-rhamnopyranosyl-arganin C (**1**) is a new bisdesmoside of 16α-hydroxyprotobassic acid and its ^{1}H and ^{13}C NMR data were completely assigned. Bioassay against PC-3 prostate cancer cells indicated arganin C (**4**) to be the most potent one with the IC_{50} value of 13.8 μM. Some structure and activity relationships drawn in this study will be useful for the development of anti-cancer drugs.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfda.2017.06.004.

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