SUPPLEMENTARY MATERIAL

Cytotoxic cardiac glycosides from the fruit (pods) of *Adenium obesum* (Forssk.)

Roem. &Schult

Salman Khalid Ahmed^a, Muhammad Ali Versiani^{a,*}, Ambreen Ikram^a, Samina Abdul Sattar^a and Shaheen Faizi^b

^aDepartment of Chemistry, Federal Urdu University of Arts, Sciences and Technology, Gulshan-e Iqbal, Science Campus, Karachi-75300, Pakistan; ^bInternational Center for Chemical and Biological Sciences, HEJ Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan

Abstract:

Phytochemical investigation of methanolic extract of *Adenium obesum* led to the isolation of forty two (**1-42**) compounds belongs to cardiac glycosides, triterpenoids and steroids. The chemical structures of isolated compounds were elucidated by using spectral techniques UV, IR, NMR and FAB MS. The cardiac glycosides were tested against three human cell lines, 3T3 (normal cells), HeLa (Human cervical cancer cell lines) and PC-3 (Human prostate cancer cell lines). The cardiac glycoside, honghelin (**4**), obese B (**5**) and obese C (**6**) showed significant effects against cell lines HeLa, 3T3 and PC-3 compared to standard drug doxorubicin. **4**, **5** and **6** exhibited very low IC₅₀(μM) against the PC3 human cell line. Compounds **4** and **6** also showed least IC₅₀ against the HeLa human cell lines as compared to the standard drug doxorubicin whereas these three compounds showed effect on 3T3 cell line with high IC₅₀ values compared to drug cycloheximide.

Keywords: *Adenium obesum*, Apocynacea, cytotoxic, cardiac glycoside, triterpenoids, cancer cell lines.

Experimental

Plant material

The fruits (pods) of *A. obesum* were collected from the gardens of the University of Karachi. The plant was identified by Dr. Sahar Ansari, a taxonomist, Department of Botany,

University of Karachi. Voucher specimen (No. KUH 68671) was deposited in the herbarium of Botany department.

Extraction and isolation of compounds

Fruits (pods, 481g) of *A. obesum*, soaked into the methanol in three different time duration and after collecting the all three (3) extracts, evaporate under the reduced pressure to give a thick residue. The methanolic extract was partitioned between aqueous and ethyl acetate phase. The ethyl acetate phase was dried with sodium sulfate (anhydrous), charcoaled and evaporated under reduced pressure to give a residue. The residue was further fractioned by applying solvent separation technique, using petroleum ether (100%), petroleum ether: ethyl acetate (1:1), ethyl acetate (100%) and methanol (100%), which gave the soluble fractions. The ethyl acetate soluble sub-fraction of ethyl acetate was subjected to normal pressure column chromatography on gradient system and obtained 19 fractions, using petroleum ether, ethyl acetate and methanol as a solvent. Out of 19 fractions, Fraction no. 5 found to be mixture of three compounds, which was further purify with the help High Pressure Liquid Chromatography (HPLC, Dionex 300, C18 column, 250mm × 25mm, isocratic system, flow rate 0.2 ml/min, methanol:acetonitrile, 9:1, 254nm) and obtained three pure compound **3** (4 mg), **7**(2 mg) and **16** (10 mg), while Fraction no. 3 and 11 were identified as **21** (33 mg) and **22** (4 mg).

Fraction no. 8 was further fractioned into hexane and ethyl acetate soluble fraction. The ethyl acetate fraction was subjected to normal column chromatography, which gave six (6) fractions in which fraction no.1 found to be a mixture of five compounds. These compounds were purified by preparative thin layer chromatography (Prep. TLC)(C: M 9.5:0.5) which gave five bands in which band no. 3, 4 and 5 were identified as **20** (50 mg), **17**(10 mg) and **18** (12 mg) respectively. Petroleum ether, ethyl acetate soluble sub-fraction of ethyl acetate fraction was subjected to vacuum liquid chromatography (VLC) which afforded 103 fractions. Fraction 31 to 40 were combined on the basis of thin layer chromatography (TLC) and loaded to gel filtration column (Sephadex, LH-20) and got 30 fractions out of which fraction 6 to 10 and 20-21 were found to be pure and identified as a **19** (10 mg) and **20** (21 mg) respectively. Vacuum liquid chromatography (VLC) fraction nos. 51 to 100 were combined (500 mg) and subjected to normal pressure column chromatography by gradient solvent system using hexane, ethyl acetate and methanol by increasing 5% polarity which afforded 40 fractions in which fraction no. 12 found to be a mixture of compound **4** and **5** whereas fraction no 13 identified as **4** (11 mg.).

In another work 3 Kg of the fruits (pods) were soaked in MeOH (5L) for 30 days at room temperature. Solvent was evaporated under vacuum to get the extract (150 g) which was partitioned in aqueous and ethyl acetate phase. The ethyl acetate phase (27 g) was further fractionated by using hexane and methanol. The later fraction (21.5 g) was subjected to flash column chromatography to get 40 fractions (start polarity with 1:1 H: EA to 100% MeOH). Fraction 1 to 5 were merged (1.5 g) and purified by normal pressure column chromatography and got the pure compounds **19** (30 mg), **20** (25 mg), **21** (15 mg), **22** (8 mg), **23** (3 mg), **24** (5 mg), **25** (8 mg), **5** (10 mg) and **6** (7 mg). Remaining fraction nos. 6 to 15 were combined and subjected to normal pressure column chromatography followed by reversed phase High Pressure Liquid Chromatography (HPLC, Dionex 300, C18 column, 250mm × 25mm, isocratic system, flow rate 0.2 ml/min, 100 % acetonitrile, 254nm) afforded compound **1** (4 mg), **2** (9 mg), **3** (11 mg) and **7** (9 mg). Fraction 22 to 40 were also combined and got the cyanidin **26** (3 mg), **11** (3 mg), **12** (6 mg) and **13** (5 mg), **16** (10 mg), **27** (7 mg), **28** (4 mg), **22** (9 mg) by normal pressure column chromatography. Fraction no. 16 (125 mg) dissolved in water and partitioned by using chloroform, ethyl acetate, butanol and water fraction. Butanol fraction found to be mixture of 3 compounds and they were purified by the HPLC (Dionex 300, C18 column, 250mm × 25mm, isocratic system, flow rate 0.2 ml/min, acetonitrile: Water, 1:1, 254nm) and identified as **8** (5mg), **9** (2mg) and **10** (7mg). Water soluble fraction found to be mixture of 3 compounds and they were purified by the HPLC (Dionex 300, C18 column, 250mm × 25mm, isocratic system, flow rate 0.2 ml/min, methanol: Water, 4:6, 254nm) and identified as **14** (2mg), **15** (5mg). Powder Flower (500 g) of *A. obesum* extracted in methanol and the crude extract (100 g) was partitioned between aqueous and hexane (10g), chloroform (2.4g), ethyl acetate (20g), and butanol (5g) phase. The chloroform phase was dried with sodium sulfate (anhydrous), charcoaled and evaporated under reduced pressure to give a thick residue which was subjected to normal pressure column chromatography on gradient system and obtained one hundred and twenty-nine (129) fractions, using hexane, chloroform and methanol as a solvent which afforded the pure compounds **11** (6 mg), **12** (5 mg), **29** (12 mg), **30** (10 mg), **31** (5 mg), **32** (19 mg), **33** (22 mg), **34** (5 mg), **35** (15 mg), **36** (9 mg), **37** (6 mg), **38** (8 mg), **39** (3 mg) and **40** (10 mg).

The ethyl acetate phase was also dried and the residue was further fractionated by solvent separation technique, by using hexane (45 mg), chloroform (350 mg), ethyl acetate (16.4 g) and methanol (3.2 g) soluble fractions. The ethyl acetate soluble sub-fraction (13.0 g) was purified by vacuum liquid chromatography (VLC) by using solvent hexane, ethyl acetate and methanol (volume of each eluant 500 ml) by increasing gradient polarity followed by normal

pressure column chromatography which afforded the pure compounds **41** (10 mg) and **42** (16 mg).

Honghelin (Gitoxigenin- β -D-thevetoside, **4**)

White crystalline solid, FAB Mass (–ve) 533 (M^+ -H), molecular formula $C_{30}H_{46}O_8(M^+)$, IR $V_{\max}$ KBr cm^{-1} : 3413, 1705, 1735, 1627 cm^{-1} . UV $\lambda_{\max}$ MeOH nm 216nm.

1H NMR (300MHz, $CDCl_3$) δ ppm: 0.848(H-18), δ 0.914(H-19), 1.294 (d, $J=6.0$ Hz, H-6'), 2.98(m, H-17), 4.012(bris, H-3), 4.265 (d, $J= 8.4$ Hz, H-1'), 3.430 (t, $J=8.4$, Hz, H-2'), δ 3.192 (t, $J=8.4$ Hz, H-4'), 3.430 (t, $J=8.4$ Hz, H-2'), 3.126 (d, $J= 8.4$ Hz, H-5'), 3.325 (d, $J= 8.4$, H-2'), 3.631 (bris, OCH_3), 4.976, 4.800 (dd, $J=18.6,2.0$ Hz, H-21a,b), 5.848 (bris, H-22).

Obeside B (Oleandrogenin- β -D-thevetoside, **5**)

White crystalline solid, FAB Mass (–ve) 549 (M^+ -H), molecular formula $C_{30}H_{46}O_9(M^+)$, IR $V_{\max}$ KBr cm^{-1} : 3419, 1716, 1739, and 1609. UV $\lambda_{\max}$ MeOH nm 216nm.

1H NMR (300MHz, $CDCl_3$) δ ppm: 0.763(H-18), 0.824(H-19), 1.198 (d, $J=6.0$ Hz, H-6'), 2.98 (m, H-17), 4.61 (bris, H-16), 3.926(bris, H-3), 4.183 (d, $J= 8.0$ Hz, H-1'), 3.228 (t, $J=8.0$, Hz, H-3'), δ 3.092 (t, $J= 8.0$ Hz, H-4'), δ 3.045 (brd, $J= 8.0$ Hz, H-5'), 3.325 (d, $J= 8.0$, H-2'), 3.452 (bris, OCH_3), 4.934 and 4.743 (dd, $J=18.6,2.0$ Hz, H-21a,b), 5.764 (bris, H-22).

Obeside C (Gitoxigenin- β -D-thevetoside, **6**)

White crystalline solid, FAB Mass (–ve) 591 (M^+ -H), molecular formula $C_{32}H_{48}O_{10}(M^+)$, IR $V_{\max}$ KBr cm^{-1} : 3417, 1711, 1742, and 1621. UV $\lambda_{\max}$ MeOH nm 216nm.

1H NMR (300MHz, $CDCl_3$) δ ppm: 0.848(H-18), 0.914 (H-19), 1.294 (d, $J=6.0$ Hz, H-6'), 1.89 (bris, $OCOCH_3$), 4.012 (bris, H-3), 4.265 (d, $J= 8.0$ Hz, H-1'), 3.430 (t, $J=8.0$, Hz, H-2'), δ 3.192 (t, $J=8.0$ Hz, H-4'), 3.430 (t, $J=8.0$ Hz, H-2'), 3.126 (brd, $J= 8.0$ Hz, H-5'), 3.325 (d, $J= 8.0$ Hz, H-2'), 3.631(bris, OCH_3), 4.976 and 4.800 (dd, $J=18.6,2.0$ Hz, H-21a,b), 5.459 (m, H-16), 5.848 (bris, H-22).

Cytotoxic activity

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell growth inhibition effect of extracts and compounds on two cancer cell lines [i.e HeLa (Cervical Cancer Cell) and PC-3 (Prostrate Cancer Cell)] using the same protocol

as mentioned in our recent report (Ikram *et al*, 2015). Cells were grown in DMEM (Dulbecco's Modified Eagle Medium), and MEM (Modified Eagle's medium) containing 10% FBS (Fetal Bovine Serum) and 2% Antibiotic (Penicillin and streptomycin), and maintained at 37°C with 5% CO₂ level for 24 hours in flask. Cells (1x10⁵ cells mL⁻¹) were plated in a 96-well flat bottom plates for 24 hours incubation to allow for cell attachment. Various concentrations (200-10 µg mL⁻¹) of sample were added into the well and incubated for 48 hours. A 50 µL MTT (2 mg mL⁻¹) was added to the well, 4 hours before the end of incubation. Medium and reagents were aspirated and 100 µL DMSO was added and mixed thoroughly for 15 minutes to dissolve the formazan crystals. The absorbance was measured at 570 nm using microplate Spectra Max 340 (Molecular Devices, CA, USA). At the end, IC₅₀ values were calculated and at least three independent experiments were carried out for each sample. Doxorubicin used in this assay, as a positive control for prostate and cervical cancer cell lines whereas cycloheximide used as positive control for normal cell line (Mosmann T. 1983; Yeskaliyeva *et al.* 2006).

References

- Ikram A, Versiani MA, Khatoon A, Salman KA, Samina AS and Shaheen F. 2015. New dammarane and ursane type triterpenoids from the flower of *Ixora coccinea* Linn. *Nat Prod Res.* 30:768-775.
- Mosmann T, 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 65:55–63.
- Yeskaliyeva B, Mesaik MA, Abbaskhan A, Kulsoom A, Burasheva GS, Abilov ZA, Choudhary MI, Atta-ur-Rahman. 2006. Bioactive flavonoids and saponins from *Climacoptera Obtusifolia* *Phytochem.* 67:2392-97.

Captions

Table S1. *In-vitro* cytotoxic activity of cardinolides **4**, **5** and **6** with IC₅₀ (µM).

Figure S1. Structures of honghelin (**4**), obese B (**5**) and obese C (**6**) isolated from fruit (Pods) of *Adenium obesum*

Table S1. *In-vitro* cytotoxic activity of cardinolides **4**, **5** and **6** with IC₅₀ (μM).

Compounds	Human cell lines		
	Hela	PC3	3T3
Honghelin (4)	0.2020±0.0420	0.0180±0.0008	18.7360±0.0900
Obeside B (5)	10.2480±0.031	0.1044±0.0084	24.8400±0.0310
Obeside C (6)	0.0580±0.0030	0.1439±0.0030	27.8760±0.0150
Doxorubicin	0.2480±0.1500	0.9120±0.0900	-
Cycloheximide	-	-	0.2600±0.1000

Figure S1. Structures of honghelin (**4**), obeside B (**5**) and obeside C (**6**) isolated from fruit (Pods) of *Adenium obesum*

Compound	R
(4)	H
(5)	OH
(6)	OCOCH ₃