Cytotoxic Principles of a Bangladeshi Crude Drug, Akond Mul (Roots of *Calotropis gigantea* L.)

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Three cardenolide glycosides, calotropin (1), frugoside (2), and 4'-*O*-β-D-glucopyranosylfrugoside (3), were obtained as the cytotoxic principles of "akond mul" (roots of *Calotropis gigantea* L.). The cytotoxicity of these compounds against various cell lines of human and mouse origin was tested. They showed similar cell line selectivity to those of cardiac glycosides such as digoxin and ouabain: they are toxic to cell lines of human origin, but not to those from mouse at 2 μg/ml.

Key words *Calotropis gigantea*; cytotoxicity; cardenolide; calotropin; frugoside; Asclepiadaceae

In our screening work on medicinal plants with cytotoxic activity, we found that a 30% aqueous acetone extract of the roots of *Calotropis gigantea* L. (Asclepiadaceae), purchased in a Bangladeshi market (called "akond mul"), showed strong cytotoxicity to human tumor cells (KB cells) but not to mouse leukemia cells (L1210). *Calotropis gigantea* L. is a large shrub which grows widely in South Asian countries. It is used as a tonic, expectorant, depurative and anthelmintic.[1] Some triterpenes[2] and flavonoid glycosides[3] have been reported in the latex and aerial parts of this plant, respectively. Steroidal saponins, named calotroposides and coroglaucigenin derivatives, were also isolated from the roots of this plant collected in Timor Island (Indonesia).[4] However, as yet, no cytotoxic principle has been reported for this plant.

Here, we report the isolation and identification of the cytotoxic principles of akond mul.

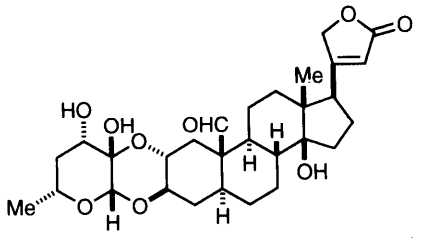
Results and Discussion

Isolation and Structures of the Cytotoxic Principles In order to test the cytotoxicity of each part of the plant, leaves, branches, and roots of *C. gigantea* collected in Nepal were separately extracted with MeOH and their cytotoxicity was tested. They showed similar toxicity against KB cells: leaves, $IC_{50}=2.8\,\mu g/ml$; branches, $IC_{50}=6.5\,\mu g/ml$; roots, $IC_{50}=2.2\,\mu g/ml$.

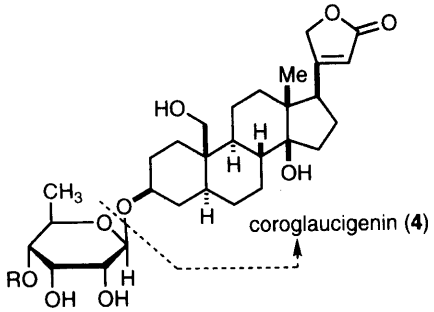
For the isolation of the active principles, roots of *C. gigantea* collected around Rajshahi University, Bangladesh, were used. The roots were extracted with MeOH and the extract was successively extracted with hexane, ether, $CHCl_3$, $CHCl_3$–MeOH, and MeOH in a Soxhlet extraction apparatus. The $CHCl_3$, $CHCl_3$–MeOH, and MeOH extracts,which showed strong cytotoxicity (IC_{50}s against KB cells were <0.8, <0.8, $1.35\,\mu g/ml$, respectively), were fractionated using normal and reversed-phase silica gel column chromatography, monitoring the cytotoxicity against KB cells as a guide to obtain three cytotoxic principles (**1**—**3**).

Compound **1** was obtained from the $CHCl_3$ extract as a colorless amorphous solid. Its ^{1}H-NMR spectrum showed the presence of an α,β-unsaturated γ-lactone moiety [δ 5.02 (dd, $J=18.1$, 1.0 Hz), 5.27 (dd, $J=18.1$, 1.0 Hz), 6.11 (br s)] and an aldehyde proton (δ 9.99). It also showed a singlet anomeric proton (δ 5.01), suggesting the presence of a 2-oxosugar moiety. The NMR data, together with the molecular weight of 532 [negative FAB-MS: *m/z*, 531 $(M-H)^-$], indicated that compound **1** is calotropin or calactin,[5] both of which have been isolated from some Asclepiadaceous plants. The ^{1}H-NMR data were in good agreement with those reported for calotropin **1**,[5a] and the identity was confirmed by direct comparison with an authentic sample.[5a]

Compound **2** was obtained from the $CHCl_3$ and $CHCl_3$–MeOH extracts as a colorless amorphous solid. The IR (1737 cm^{-1}) and ^{1}H-NMR [δ 5.01 (dd, $J=18.1$, 1.5 Hz), 5.29 (d, $J=18.1$, 1.5 Hz), 6.10 (br s)] spectra showed the presence of an α,β-unsaturated γ-lactone moiety. The presence of 6-deoxyallose with a β-linkage



calotropin (**1**)



R = H: frugoside (**2**)
R= -β-D-Glc (**3**)

Chart 1

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Table 1. Cytotoxicity of the Cardenolides from Akond Mul against Various Cell Lines

Cell line	Origin	IC_{50} (μg/ml)			
		1	2	3	4
Human origin					
KB	Oral epidermoid carcinoma	0.015	0.15	0.075	1.0
Colo 320 DM	Colon adenocarcinoma	0.0086	0.061	0.062	0.71
DLD-1	Colon adenocarcinoma	0.052	0.51	0.29	2<
HT-1080	Fibrosarcoma	0.030	0.20	0.23	1.5
KKLS	Gastric adenocarcinoma	0.011	0.14	0.11	0.88
MKN-28	Gastric adenocarcinoma	0.022	0.23	0.08	1.1
MKN-45	Gastric adenocarcinoma	0.055	0.40	0.28	2<
HLE	Hepatoma	0.012	0.096	0.11	0.70
Hep 3B	Hepatoma	0.021	0.10	0.11	1.9
Hep G2	Hepatoma	0.094	1.1	1.1	2<
Hu H7	Hepatoma	0.030	0.22	0.25	2<
SK-Hep-1	Hepatoma	0.016	0.16	0.13	1.3
PC-9	Lung adenocarcinoma	0.040	0.32	0.31	2<
QG-56	Lung squamous carcinoma	0.025	0.26	0.24	1.7
G-292	Osteosarcoma	0.0072	0.030	0.035	0.31
KHOS/NP	Osteosarcoma	0.0065	0.034	0.034	0.27
MNNG/HOS	Osteosarcoma	0.011	0.088	0.088	0.73
OST	Osteosarcoma	0.044	0.44	0.44	2<
LNCap	Prostate adenocarcinoma	0.043	0.70	0.23	2<
PC-3	Prostate adenocarcinoma	0.011	0.11	0.058	0.74
Mouse origin					
Colon 26	Colon adenocarcinoma	2<	2<	2<	2<
L1210	Leukemia	2<	2<	2<	2<
L5178Y	Lymphoma	2<	2<	2<	2<
FM3A	Mammary tumor	2<	2<	2<	2<
B16-F10	Melanoma	2<	2<	2<	2<

was also deduced from the analysis of ^{1}H-, ^{13}C-NMR and correlated spectroscopy (COSY) spectra. The ^{13}C-NMR data for the aglycone moiety were almost identical with those reported for coroglaucigenin (**4**)[6] except at C-3, where a glycosylation shift (6.7 ppm) was observed. Thus, compound **2** was deduced to be a known cardenolide glycoside, frugoside (**2**), and was identified by direct comparison with an authentic sample.[7]

Compound **3** was obtained from the $CHCl_3$–MeOH and MeOH extracts as a colorless amorphous solid. It shared very similar spectral data with those of **2**, except that it has an additional sugar moiety. The additional sugar moiety was identified as a β-glucoside from the ^{1}H- and ^{13}C-NMR data. The position of the β-glucosyl moiety was determined at C-4′ of the 6-deoxyallose, where a large glycosylation shift of the carbon chemical shift (9.2 ppm) was observed compared with that of **2**. Thus, compound **3** was estimated to be 4′-*O*-β-D-glucopyranosylfrugoside (**3**) and was confirmed by direct comparison with an authentic sample.[7]

Cytotoxicity of the Active Principles The three cardenolide glycosides obtained from akond mul, **1**—**3**, showed strong cytotoxicity against KB cells (Table 1). Among the three compounds, **1** showed the strongest activity with an IC_{50} of 15 ng/ml against KB cells. Furgoside (**2**) and its 4′-*O*-β-glucoside **3** showed moderate activity, whereas the activity of their aglycone, **4** was much weaker (Table 1). It is known that cardiac glycosides such as digoxin and ouabain, which have a 5β configuration, show strong cytotoxicity[8a] and structure-activity relationships have been reported for this type of compound.[9] Recently, some cytotoxic cardenolides with a 5β configuration were also isolated from *Euonymus* spp.[10] The cytotoxic principles of akond mul obtained above have a 5α configuration, but showed comparable cytotoxicity with those with a 5β configuration. This was in accordance with the results obtained for the inotropic effects of such cardenolides.[11]

The cytotoxic principles **1**—**3** did not show any toxic effects on mouse leukemia cells (L1210) up to 2 μg/ml. Such species selectivity of cytotoxicity has been reported for cardiac glycosides such as digoxin and ouabain: they are about two orders more toxic to cultured human and monkey cell lines compared with cell lines of mouse origin.[8a] To determine the selective toxicity of the above compounds to cultured cells of human and mouse origin, 20 human cell lines and 5 murine cell lines were tested for cytotoxicity (Table 1). The cardenolide glycosides **1**—**3** showed toxicity to all the 20 human cell lines tested, whereas they were not toxic to any of the five cell lines of mouse origin at a concentration of 2 μg/ml. These results are in good agreement with previous reports[8,9b] and indicate that the mechanism of the toxicity of the above compounds is the same as that of digoxin and ouabain.[8a]

Experimental

General Unless otherwise noted, the following procedures were adopted. Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. IR spectra were recorded with a Shimadzu IR-460 spectrometer and the data are given in cm^{-1}. NMR spectra were measured on a JEOL JNM-GSX500 (500 MHz for ^{1}H, 125 MHz for ^{13}C) spectrometer with tetramethylsilane as an internal standard and chemical shifts are given in δ values. Mass spectra were recorded with a JEOL JMS-SX102 spectrometer and major peaks are indicated as *m/z* (%). Column chromatography was performed on LiChroprep® RP-18 (octadecyl silica (ODS), 40—63 μm, Merck) for reversed-phase operation and Micro Bead Silica Gel 4B (100—200 mesh, Fuji Silysia Chemical) for normal phase operation. Recycling HPLC was performed with a JAI LC-908 HPLC system (Japan Analytical Industry) on an Inertsil Prep-ODS column (20 × 250 mm, Gasukuro Kogyo). For thin-layer chromatography (TLC), Kieselgel 60 F_{254} and RP-18 $F_{254}S$ precoated plates (Merck) were used and the spots were developed by spraying with 10% H_2SO_4 and heating the plates until colors developed.

The tetrazolium-based semiautomated colorimetric assay (MTT assay) was used for the *in vitro* cytotoxicity assay as described previously.[12]

Isolation of Cytotoxic Principles Roots of *C. gigantea* L. (akond mul) collected around Rajshahi University in Bangladesh were cut into pieces and dried in the sun for several days. The dried roots (1 kg) were pulverized and exhaustively extracted with MeOH in a Soxhlet apparatus and the solvent was removed under reduced pressure to give 35 g extract. Part of the extract (10 g) was adsorbed on celite (100 ml) and successively extracted with hexane (15 h), ether (8 h), $CHCl_3$ (17 h), $CHCl_3$: MeOH = 19 : 1 (8.5 h) and MeOH (8 h) in a Soxhlet apparatus to give hexane (2.41 g), ether (0.96 g), $CHCl_3$ (2.51 g), $CHCl_3$–MeOH (0.35 g) and MeOH (0.48 g) extracts, respectively. The cytotoxic activity (IC_{50}, μg/ml) of each fraction against KB cells was 32, 3.0, <0.8, <0.8, 1.35, respectively.

The $CHCl_3$ extract (2.5 g) was fractionated by silica gel column chromatography with $CHCl_3$–MeOH into nine fractions (solvent ratio, yield, IC_{50} in μg/ml): C-I (19 : 1, 378 mg, >4), C-II (19 : 1, 213 mg, 2.8), C-III (19 : 1, 125 mg, 0.13), C-IV (19 : 1, 60 mg, 0.12), C-V (19 : 1, 72 mg, 2.0), C-VI (9 : 1, 398 mg, 2.4), C-VII (4 : 1, 458 mg, 0.55), C-VIII (2 : 1, 210 mg, 0.64), C-IX (MeOH, 193 mg, 2.7).

Fraction C-III (115 mg) was fractionated by ODS column chromatography (80% MeOH) to give six fractions (yield, IC_{50} in μg/ml): C-III-1 (23 mg, 0.078), C-III-2 (15 mg, 0.24), C-III-3 (20 mg, 2.2), C-III-4 (5 mg, 1.1), C-III-5 (21 mg, >4), C-III-6 (31 mg, 2.9). Fraction C-III-1 was further fractionated by ODS column chromatography (60% MeOH) and preparative TLC (benzene : acetone = 2 : 1) to give compound **1** (3 mg). Fraction C-IV (55 mg) also gave compound **1** (1 mg) after fractionation

by silica gel column chromatography ($CHCl_3$: MeOH = 9 : 1), preparative TLC (benzene:acetone = 1 : 1) and HPLC (ODS, 60% MeOH).

Fraction C-VII (402 mg) was fractionated on a Lobar® RP-8 (M) column with 80% MeOH to give seven fractions: C-VII-1 (35 mg, 2.0), C-VII-2 (42 mg, <0.8), C-VII-3 (5 mg, not tested), C-VII-4 (13 mg, <0.8), C-VII-5 (218 mg, 64), C-VII-6 (12 mg, not tested), C-VII-7 (15 mg, 41). Fraction C-VII-2 was purified on a Lobar® RP-8 (M) column with 60% MeOH to give compound **2** (24 mg). TLC examination showed the presence of compound **2** in fractions C-VII-3, C-VII-4 and C-VIII.

The $CHCl_3$–MeOH extract (349 mg) was fractionated by silica gel column chromatography with $CHCl_3$–MeOH into four fractions (solvent ratio, yield, IC_{50} in μg/ml): CM-I (9 : 1, 76 mg, 1.15), CM-II (9 : 1, 62 mg, 2.2), CM-III (4 : 1, 113 mg, not tested), CM-IV (2 : 1, 82 mg, 0.84). Fraction CM-III was fractionated on a Lobar® RP-8 (M) column with 80% and 60% MeOH as described above to afford compound **2** (9.2 mg) as the cytotoxic principle. Fraction CM-IV was fractionated by ODS column chromatography (80% MeOH) to give four fractions, and the active fraction (52 mg, $IC_{50} = 0.55\,\mu$g/ml) was further purified by Sephadex LH-20 column chromatography (MeOH) and preparative TLC (benzene : acetone = 2 : 1) to give compound **3** (8 mg) as the active principle.

The MeOH extract (960 mg) was fractionated using ODS (60% MeOH), Sephadex LH-20 (MeOH), and silica gel ($CHCl_3$: MeOH = 2 : 1) column chromatography, and the active fraction (30 mg) was purified by HPLC (ODS, 60% MeOH) to give compound **3** as the active principle.

Compound **1** (Calotropin): Colorless amorphous solid, mp 160—165 °C (lit. 202—205 °C).[5d] FAB-MS (neg.) *m/z*: 531 ($[M-H]^-$, 11). ^{1}H-NMR was in good agreement with the literature data.[5a]

Compound **2** (Frugoside): Colorless amorphous solid, mp 163—168 °C (lit. 162—170 °C).[13] IR (KBr): 3435, 2935, 1737. FAB-MS (pos.) *m/z* (%): 559 ($[M+Na]^+$, 8), 537 ($[M+H]^+$, 4). ^{1}H-NMR (pyridine-d_5) δ: 0.86 (1H, td, J = 13.2, 3.4 Hz), 1.04 (3H, s, H-18), 1.64 (3H, d, J = 6.4 Hz, H-6′), 2.27 (1H, m), 2.35 (1H, m), 2.64 (1H, td, J = 13.2, 3.4 Hz), 2.78 (1H, m), 3.69 (1H, dd, J = 9.3, 2.9 Hz, H-4′), 3.93 (2H, overlapped, H-19, H-2′), 4.08 (2H, overlapped, H-3, H-19), 4.37 (1H, dq, J = 9.8, 6.4 Hz, H-5′), 4.68 (1H, t, J = 2.9 Hz, H-3′), 5.01 (1H, dd, J = 18.1, 1.5 Hz, H-21), 5.25 (1H, s, OH), 5.29 (1H, dd, J = 18.1, 1.5 Hz, H-21), 5.44 (1H, d, J = 7.8 Hz, H-1′), 5.61 (1H, br s, OH), 6.08 (1H, br s, OH), 6.10 (1H, br s, H-22), 6.48 (1H, br s, OH), 6.88 (1H, br s, OH). ^{13}C-NMR (pyridine-d_5) δ: 32.5 (C-1), 30.7 (C-2), 77.4 (C-3), 35.4 (C-4), 44.7 (C-5), 28.1, 28.7 (C-6, 7), 42.3 (C-8), 50.6 (C-9), 39.8 (C-10), 23.3 (C-11), 40.4 (C-12), 50.2 (C-13), 84.8 (C-14), 33.1 (C-15), 27.3 (C-16), 51.5 (C-17), 16.3 (C-18), 59.0 (C-19), 176.1 (C-20), 73.7 (C-21), 117.6 (C-22), 174.6 (C-23), 99.5 (C-1′), 72.5 (C-2′), 72.9 (C-3′), 74.5 (C-4′), 70.3 (C-5′), 18.8 (C-6′).

Compound **3** (4′-*O*-β-D-Glucopyranosylfrugoside): Colorless amorphous solid, mp 186—188 °C (lit. 187—190 °C).[14] IR (KBr): 3410, 1733. FAB-MS (neg.) *m/z*: 697 ($[M-H]^-$, 10). ^{1}H-NMR (pyridine-d_5) δ: 0.85 (1H, td, J = 13.3, 3.4 Hz), 1.04 (3H, s), 1.63 (1H, q, J = 11.2 Hz), 1.72 (3H, d, J = 6.4 Hz), 2.64 (1H, dt, J = 13.2, 4.5 Hz), 2.77 (1H, dd, J = 8.8, 5.4 Hz), 3.83 (1H, dd, J = 9.5, 2.7 Hz), 4.24 (2H, m), 4.34 (1H, dd, J = 11.5, 5.1 Hz), 4.44 (1H, dd, J = 11.5, 2.5 Hz), 4.52 (1H, dq, J = 9.8, 6.4 Hz), 5.30 (1H, s), 5.31 (1H, dd, J = 18.1, 1.5 Hz), 5.42 (1H, d, J = 7.8 Hz), 6.11 (1H, s). ^{1}H-NMR (pyridine-d_5 + D_2O) δ: 0.84 (1H, td, J = 8.6, 3.4 Hz), 1.05 (3H, s), 1.43 (1H, d, J = 13.2 Hz), 1.62 (1H, q, J = 11.7 Hz), 1.72 (3H, d, J = 6.4 Hz), 2.65 (1H, d, J = 13.2 Hz), 2.78 (1H, dd, J = 9.4, 5.4 Hz), 3.84 (1H, dd, J = 2.7, 9.5 Hz), 3.92 (1H, m), 3.94 (2H, m), 4.01 (1H, t, J = 8.3 Hz), 4.05 (1H, m), 4.22 (1H, t, J = 9.0 Hz), 4.26 (1H, t, J = 8.8 Hz), 4.32 (1H, dd, J = 4.9, 11.7 Hz), 4.44 (1H, dd, J = 2.4, 11.7 Hz), 4.52 (1H, dq, J = 9.3, 6.4 Hz), 5.04 (1H, dd, J = 18.1, 1.5 Hz), 5.04 (1H, d, J = 7.8 Hz), 5.07 (1H, t, J = 2.6 Hz), 5.31 (1H, dd, J = 18.1, 1.5 Hz), 5.44 (1H, d, J = 8.0 Hz), 6.12 (1H, br s). ^{13}C-NMR (pyridine-d_5) δ: 32.7 (C-1), 30.7 (C-2), 77.6 (C-3), 35.5 (C-4), 44.7 (C-5), 28.8 (C-6), 28.2 (C-7), 42.3 (C-8), 50.7 (C-9), 39.8 (C-10), 23.3 (C-11), 40.5 (C-12), 50.2 (C-13), 84.8 (C-14), 33.1 (C-15), 27.4 (C-16), 51.5 (C-17), 16.3 (C-18), 59.1 (C-19), 176.2 (C-20), 73.7 (C-21), 117.5 (C-22), 174.7 (C-23), 99.4 (C-1′), 72.1 (C-2′), 72.4 (C-3′), 83.6 (C-4′), 68.8 (C-5′), 18.5 (C-6′), 106.3 (C-1″), 75.2 (C-2″), 78.7 (C-3″), 71.6 (C-4″), 78.2 (C-5″), 62.5 (C-6″).

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