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Novel acylated steroidal glycosides from *Caralluma tuberculata* induce caspase-dependent apoptosis in cancer cells

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ABSTRACT

Aim of the study: Pregnane glycosides are potent cytotoxic agents which may represent new leads in the development of anti-tumour drugs, particularly in the treatment of breast cancer, because of the structural similarity to estrogenic agonists. *Caralluma* species are natural sources of a wide variety of pregnane glycosides. The aim of the study was to isolate, using an activity-guided fractionation approach, novel pregnane glycosides for testing on breast cancer and other tumour lines.

Materials and methods: The effect of crude extracts, specific organic fractions and isolated compounds from *Caralluma tuberculata* was tested on the growth and viability of MCF-7 estrogen-dependent, and MDA-MB-468 estrogen-independent breast cancer cells, Caco-2 human colonic cells, HUVECs and U937 cells. Neutral red uptake and MTT assays were used. Apoptosis was detected by Western blot of poly-(ADP ribose) polymerase (PARP) as were other markers of nuclear fragmentation (DNA ladder assay, staining of cells with nuclear dye DAPI). The involvement of caspases was investigated using the pan-caspase inhibitor Z-VAD-FMK.

Results: The ethyl acetate fraction of *Caralluma tuberculata* was found to be the most potent anti-proliferative fraction against all three cancer cell lines. Two novel steroidal glycosides were isolated from the active fraction after a series of chromatographic experiments. The structure of the isolated compounds was elucidated solely based on 2D-NMR (HMBC, HETCOR, DQF-COSY) and MS spectral analysis as compound **1**: 12-*O*-benzoyl-20-*O*-acetyl-3β,12β,14β,20β-tetrahydroxy-pregnan-3-yl*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-3-methoxy-β-D-ribopyranoside, and as compound **2**: 7-*O*-acetyl-12-*O*-benzoyl-3β,7β,12β,14β-tetrahydroxy-17β-(3-methylbutyl-*O*-acetyl-1-yl)-androstan-3-yl*O*-β-D-glucopyranosyl-(1 → 4)-6-deoxy-β-D-allopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymapyranosyl-(1 → 4)-β-D-cymaropyranoside. Compound **1** (pregnane glycoside) and compound **2** (androstan glycoside) induced apoptosis at <25 μM after 48 h as assessed by cell shrinkage, PARP cleavage, DNA fragmentation, and reversal with the caspase inhibitor.

Conclusions: Two novel steroid glycosides isolated from *Caralluma tuberculata* possess moderate, micromolar cytotoxic activity on breast cancer and other cells *in vitro*, which may indicate a source of activity *in vivo* of interest to future drug design.

1. Introduction

Caralluma tuberculata (N.E. Brown) a member of the *Asclepiadaceae* family, is a succulent, perennial herb growing in the wild in Pakistan and India (Andra Pradesh), United Arab Emirates, Saudia Arabia, the south east of Egypt (Täckholm, 1974; Baquar, 1989), and other countries. In the literature, the plant has also been reported as *Boucerosia aucheriana* (Ali, 1983).

The roots of *Caralluma tuberculata* are eaten raw, or cooked as a vegetable (Ali, 1983; Ahmad et al., 1988), and the plant is traditionally used for its anti-inflammatory and anti-tumour properties (Copra et al., 1956; Ahmad et al., 1983, 1988; Ahmed et al., 1993; Mahmood et al., 2010). Several pregnane glycosides isolated from organic extracts of *Caralluma tuberculata* showed cytotoxicity against the MRC5 human diploid embryonic cell line (Abdel-Sattar et al., 2008, 2009). Pregnanes are C_{21} steroids and often found in

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nature conjugated as glycosides. *Caralluma* and other members of the *Asclepiadaceae* family are rich in esterified polyhydroxypregnane glycosides, which have potentially important anti-cancer and anti-tumour effects (Deepak et al., 1989, 1997), and may provide interesting leads for the development of new drugs.

The aim of the present study was to isolate novel pregnane and related steroids from *Caralluma tuberculata* and to elucidate their structures and cytotoxicity. The next step was to use an activity-guided fractionation approach to identify those organic fractions of *Caralluma tuberculata* with the highest anti-proliferative activity against MCF-7 human breast cancer cells and Caco-2 human colon cells using the MTT and neutral red uptake assays. Following identification of any compounds isolated in the most active fractions, the cytotoxicity of these compounds would then be tested on the cell lines and the mode of cell death investigated using established assays of apoptosis and necrosis.

2. Materials and methods

2.1. Plant material

Caralluma tuberculata was collected from the surrounding areas of Mansehra Valley, Pakistan in February 2007, and identified by Dr. Manzoor Hussain, Department of Botany, Hazara University, Mansehra, Pakistan. A voucher specimen (HU-2761A-07) was deposited at the Herbarium of Department of Botany, Hazara University, Mansehra, Pakistan.

2.2. Extraction and fractionation

The whole fresh plant (10 kg) was air-dried, powdered and macerated in EtOH (3 × 1000 ml) at room temperature for 7 days. The extract was filtered under vacuum and evaporated under reduced pressure at 40 °C. This yielded a semi-solid residue, dark greenish in colour (200 g). The concentrated extract was dispersed in H_2O (1000 ml), partitioned into four organic fractions starting with hexane (3 × 300 ml), $CHCl_3$ (3 × 500 ml) and EtOAc (3 × 500 ml) respectively. The MeOH fraction (3 × 300 ml) was separated from the insoluble residue after evaporating the remaining aqueous layer. This procedure resulted in hexane fraction (5 g), $CHCl_3$ fraction (8 g), EtOAc fraction (20 g), MeOH fraction (22 g) and aqueous fraction (28 g).

2.3. Isolation of compounds

The EtOAc fraction was loaded (12 g) on silica gel 60 (2 kg) for column (100 cm × 12 cm) adsorption chromatography packed in $CHCl_3$. Stepwise elution was carried out using $CHCl_3$–MeOH gradient solvent system (1:0, 20:1, 10:1, 8:1, 5:1, 3:1, 2:1, and 0:1; 2.0 L for each step). Fractions of 200 ml were collected and concentrated; TLC was carried out for all the eluted fractions and visualised under UV illumination (254 nm). Fractions possessing similar R_f values were combined together and this resulted in 25 major fractions. Fraction 18 (352 mg) showed a mixture of two compounds on TLC. The fraction was chromatographed on a silica gel column (500 g, 3.5 cm × 60 cm) and eluted with EtOAc–MeOH (5:1, 4:1, 1 L each eluent). Thin layer chromatography was carried out on silica gel 60 F_{254}, precoated aluminium cards (0.2 mm thickness) from Merck Ltd., Germany. Repeated adsorption column chromatography of fraction 18 resulted in three sub-fractions. Sub-fraction I yielded compound **1** (62 mg) while sub-fractions II and III were further purified over RP-C18 column (100 g, 40 cm × 3 cm) with MeOH–H_2O (80:20, 0.5 L) to give compound **2** (75 mg). Silica gel 90 C18-reversed phase (60757) for column chromatography was purchased from Fluka Analytical, Switzerland. To determine the steroidal nature of the compounds, the EtOAc fraction and isolated compounds **1** and **2** were analysed by Liebermann–Burchard test (Burke et al., 1974; Halim and Khalil, 1996). Melting points were determined on a Gallenkamp (Sanyo) melting point instrument and are uncorrected. Optical rotations were measured in MeOH solutions with a Optical Activity AA-10 automatic polarimeter. FT-IR spectra were recorded on PerkinElmer Spectrum 100FT-IR spectrometer with spectrum express sodium chloride discs.

2.4. NMR and mass spectrometry (MS) analysis

^1H, ^{13}C-NMR, DEPT, edited DEPT and two-dimensional HETCOR, DQF-COSY, HMBC NMR spectra were recorded in methanol-D_3 (CD_3OD) using JEOL Eclipse 400 MHz NMR spectrometer with Jeol Delta version 7.2 control and processing software. Analysis was carried out on GC–MS (Agilent Technologies) 5890 GC with 5973 mass spectrophotometer, operating with electron ionisation with helium gas as carrier. HR Mass was recorded on WATER's LCT mass spectrophotometer with time-of-flight (TOF) using electron spray ionisation (ESI) connected to Alliance auto-sampler injection system. ESI-MS fragmentation pattern was recorded on LC MS/MS TSQ Quantum Access (Thermo Electronic Corporation, UK) with ACCELA auto-sampler.

2.5. Cell culture

MCF-7 (human breast estrogen-dependent adenocarcinoma) and MDA-MB-468 (human breast estrogen-independent adenocarcinoma) cells were obtained from LGC Standards, Teddington, UK. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat inactivated foetal bovine serum (FBS) and gentamycin (40 μg/ml), penicillin (100 units/ml) and streptomycin (1040 μg/ml). The Caco-2 (human colon adenocarcinoma) cell line was obtained from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, UK) and grown in complete growth medium: Dulbecco's modified Eagle's medium (DMEM) containing 10% v/v FBS, 2 mM L-glutamine (all materials from Sigma Aldrich, UK). U937 cells were from LGC Standards (Teddington, UK) and were grown in RPMI-1640 medium supplemented with FBS to a final concentration of 10% and gentamycin (40 μg/ml), penicillin (100 units/ml) and streptomycin (1040 μg/ml). HUVEC (human umbilical vein endothelial cells) were isolated from umbilical cords (kindly donated by Dr. Anshuman Ghosh, School of Life Sciences, Kingston University, UK) by enzymatic detachment using collagenase, as previously described (Bernhard et al., 2003). HUVEC Cells were routinely passaged in 0.2% gelatine-coated (Sigma, Steinheim, Germany) polysterene culture flasks (Becton Dickinson, Meylan Cedex, France) in MCDB 131 growth media (Invitrogen Ltd. Paisley UK) supplemented with EGM Single Quots Supplements and growth factors (Invitrogen Ltd., Paisley, UK).

All cell lines were grown in a humidified incubator at 37 °C in an atmosphere containing 5% CO_2 and were in the logarithmic phase of growth at the time of cytotoxicity assays. Cells were harvested and seeded into 96-well tissue culture plates at a density of 1×10^4 cells per well in 200 μl aliquots of medium. The cells were allowed to attach for 24 h at 37 °C, 5% CO_2 in air in a humidified atmosphere. The next day, the plant extract in serial dilution or desired final concentration of organic fraction and isolated glycosides dissolved in DMSO (maximum: 0.01%) were added to the desired final concentrations and after a 24 h exposure period, the toxic endpoints were determined. Control groups received the same amount of DMSO. Actinomycin-D (4 μM), tamoxifen (5 μM) and anastrozole (5 μM) were used as positive controls in 200 μl media as a final concentration in the well.