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RESEARCH ARTICLE

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Evaluation of the cytotoxic potential of a new pentacyclic triterpene from *Rhododendron arboreum* stem bark

Sajid Ali[a], Muhammad Nisar[b], Muhammad Qaisar[c], Aslam Khan[d] and Abid Ali Khan[e]

[a]Department of Chemistry, Bacha Khan University, Charsadda, Pakistan; [b]ORIC, University of Peshawar, Peshawar, Pakistan; [c]Medicinal Botanic Centre, PCSIR Laboratories Complex, Peshawar, Pakistan; [d]Department of Pathology, Hayatabad Medical Complex, Peshawar, Pakistan; [e]Institute of Integrative Bioscience, CECOS University of IT and Emerging Sciences, Peshawar, KP, Pakistan

ABSTRACT

Context: Traditionally, *Rhododendron arboreum* Sm. (Ericaceae) is a very important medicinal plant having oxytocic, estrogenic, anti-inflammatory, analgesic and hepatoprotective activities; it also inhibits the prostaglandin synthetase.

Objectives: This study determines the cytotoxic potential of 15-oxoursolic acid isolated from *R. arboreum* against selected human cancer cell lines.

Materials and methods: Extraction from stem bark (5 kg) of *R. arboreum* was performed with methanol, which was successively partitioned into hexane, dichloromethane and ethyl acetate fractions, respectively. The new antitumor agent [15-oxoursolic acid (**1**)] was isolated from ethyl acetate fraction through column chromatography. Structure elucidation of new compound was performed through extensive spectroscopy i.e., IR, MS and 1D and 2D NMR. Cytotoxicity of isolated compound was determined at doses 5–100 μM for a period of 72 h on specified human cancer cell lines [renal cell carcinoma (A498), non-small cell lung (NCI-H226), squamous cell carcinoma (H157) and human ovarian carcinoma (MDR-2780AD)].

Results: Structure of isolated compound was characterized as 15-oxoursolic acid on the basis of various extensive spectroscopic techniques. 15-Oxoursolic acid revealed considerable anticancer activity with IC_{50} values of 2.3 ± 0.1 μM, 4.9 ± 0.2 μM, 9.2 ± 0.2 μM and 10.3 ± 0.1 μM against MDR 2780AD, Hep G2, H157 and NCI-H226, respectively, while in the case of A498, the activity was good (IC_{50} 32.8 ± 1.2 μM).

Conclusions: This study highlighted the potential of 15-oxoursolic acid to be further explored as a new lead compound for cancer chemotherapy.

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Introduction

Curing and prevention of diseases is a growing issue which needs greater consideration. Plants produce a number of phytopharmaceuticals that provide health benefits including prevention and treatment of diseases. Triterpenoids are a very important class of biologically active organic compounds. A number of biological activities have been reported about these triterpenes. Among them anti-HIV (Mayaux et al. 1994; Kashiwada et al. 2000; Zhu et al. 2001), HIV protease inhibition (Ma et al. 1999), antibacterial (Wolska et al. 2010) and anticancer activities against different cancer cell lines (Lee et al. 1988; Lin et al. 1990; Liu 1995) are the most important. Triterpenes have been identified as a potent and selective growth inhibitor of human melanoma (Pisha et al.1995; Zuco et al. 2002), neuroectodermal (Fulda and Debatin 2000) and malignant tumour cell having the basic mechanism of inducing apoptosis in these cells (Yogeeswari and Sriram 2005). Some of the triterpenes have been reported to possess inhibitory potential against nitric oxide production. This ability of triterpenes suggest they are good chemopreventive drugs for cancer, as over production of nitric oxide (NO) is mechanistically the root cause for carcinogenesis which can destroy normal functional tissues (Finlay et al. 1997; Honda et al. 1997, 2000a, 2000b).

In the search for a new cytotoxic drug, 15-oxoursolic acid (**1**), a new pentacyclic triterpene, was isolated from the stem bark of *Rhododendron arboreum* Sm. (Ericaceae) and was screened for cytotoxic potential against five human cancer lines, i.e., squamous cell carcinoma (H157), renal cell carcinoma (A498), human hepatocellular carcinoma (Hep G2), non-small cell lung (NCI-H226) and human ovarian carcinoma (MDR 2780AD).

Materials and methods

General experimental procedures

Optical rotation was recorded on JASCO DIP-360 digital polarimeter. Melting points were determined in glass capillaries using Buchi 535 melting point apparatus (BÜCHI, Essen, Germany). Mass spectrometery was performed using various ionization techniques including electron impact mass spectrum (EIMS), fast atom bombardment positive/negative spectrum (FAB + ve/FAB-ve), high-resolution fast atom bombardment mass spectrum (HRFABMS) and GCMS spectrum measurements were performed on Varian Mat 312 and Jeol JMS-600H with GC and Jeol JMS HX 110 mass spectrometer (Joel Inc., Tokyo, Japan). UV–visible spectra were recorded on a Perkin Elmer λ-EZ 201 spectrophotometer (Perkin Elmer Inc., Tempe, AZ). IR spectra were recorded in KBr disk on a Jasco 320-A spectrophotometer (Jasco, Jackson, MS). The ^{1}H NMR (500 MHz, $CDCl_3$), ^{13}C NMR (125 MHz, $CDCl_3$) and related experiments were

CONTACT Sajid Ali sajidali_biochemist@yahoo.com Department of Chemistry, Bacha Khan University, Charsadda, Pakistan

conducted on Bruker instrument (Bruker Corp., Billerica, MA), chemical shift value was presented in δ (ppm) and coupling constant (f) in Hz.

Collection of plant material

Rhododendron arboreum bark was collected from Seran valley Khyber Pakhtoonkhwa, Pakistan, in February 2011 and was authenticated by Dr. Abdul Rasheed, Department of Botany, University of Peshawar. A specimen was deposited in the herbarium under voucher number 7212/Bot.

Extraction and isolation

The air-dried bark (5 kg) of *R. arboreum* was repeatedly percolated with methanol (3 × 10 L) at room temperature. The methanol extract was filtered and concentrated by rotary evaporator under vacuum at 40 °C, to obtain a brownish crude methanol extract (650 g, 13.0% w/w). The methanol extract (650 g) was redissolved in distilled water and was successively extracted with hexane (9.5% w/w), chloroform (17.46% w/w), ethyl acetate (36.5% w/w), butanol (17.46% w/w) and finally with water (22.22% w/w) in order of increasing polarity to afford the respective fractions. The ethyl acetate fraction (20 g) was subjected to column chromatography using silica gel column (800 g, 200–300 mesh). The column was eluted with *n*-hexane:ethyl acetate with increasing polarity and finally with methanol:chloroform which yielded a total of 20 sub-fractions EA-EU. Sub-fraction EN (403 mg) was subjected to flash column chromatography (silica gel, 5 g) and was eluted with *n*-hexane:chloroform and finally with methanol: chloroform; (1:99), which resulted in the isolation of compound **1** (15 mg).

Physical data of compound 1

White amorphous powder with molecular formula $C_{30}H_{46}O_4$ (15 mg), Rf value = 0.35 (methanol:chloroform; 2:8), m.p. = 288–290 °C, UV (λ_{max}) 280 nm. IR (KBr) λ_{max} cm^{-1}: 3430, 1740, 1720 and 1650 cm^{-1}. HREIMS m/z 470.0320 (calculated 470.3396 for $C_{30}H_{46}O_4$), for ^1H and ^{13}C NMR (Table 1).

Table 1. ^1H and ^{13}C NMR of 15-oxoursolic acid (**1**) [500 MHz (^1H NMR) and 125 MHz (^{13}C NMR), $CDCl_3$, δ in ppm, J in Hz].

C. No.	δ_C	δ_H/ppm (multi, integral, J/Hz)	HMBC
1	38.61	1.37, 1.62 m	C3
2	28.01	1.84, 0.92 m	
3	79.04	3.2 dd (5.0, 10.0)	C2, C24
4	38.75	–	
5	55.22	0.77 d (10.0)	
6	18.2	1.35.1.53 m	
7	30.6	1.65,1.71 m	
8	39.5	–	
9	47.5	1.49m	
10	37.1	–	
11	23.3	0.93 t (10), 1.91 dd (5,10)	C12, C13
12	125.9	5.24 s	C10, C11, C14
13	137.9	–	
14	42.0	–	
15	212.1	–	
16	36.7	1.65 m, 2.15 s	C15, C28
17	47.9	–	
18	52.7	2.18 d (15.0)	C13, C13, C28
19	38.8	0.84 d (5)	
20	39.0	1.63 m	
21	24.2	1.64, 1.89 m	
22	32.4	1.36, 1.46 m	
23	28.1	1.06 s	
24	15.6	0.72 s	C3, C4, C5
25	15.4	0.87 s	
26	17.1	0.80 s	
27	23.5	1.06 s	C8, C14
28	181.4	–	
29	17.0	0.77 d (5)	
30	21.1	0.84 d (10)	

In vitro cytotoxicity assay

Human cancer cell lines, i.e., HepG2, A498, NCI-H226, H157 and MDR-2780AD were purchased from American Type Culture Collection (ATCC). The cells were grown and maintained in RPMI 1640 medium supplemented with foetal bovine serum at 37 °C, 5% CO_2 and 90% humidity.

The assay was performed in 96-well microtitre plates. The cells were seeded separately to 96-wells plates at a concentration of 1×10^5 cells/well. These cells were then treated with different concentrations of compound **1** (5–100 µM) with three replicates of each concentration. The control contained only the cells without test sample. The culture plates were then incubated at 37 °C for 72 h in a humidified incubator. After 72 h of incubation, the fraction of surviving cells was measured relative to the cell population in the control by colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Mossmann 1983). A volume of 20 µL of MTT (5 mg/mL) in phosphate buffer solution was added to each well and incubated for 3–4 h. After this incubation, 100 µL of DMSO was added to dissolve the resulting MTT formazan crystals by pipetting up and down 10–20 times. The plates were left at room temperature for 15–30 min then the optical density (OD) was measured on an ELISA microplate reader at 570 nm. The percentage of cell viability was calculated using the following equation:

$$\%\text{Viability} = \frac{\text{Optical density of the sample}}{\text{Optical density of control}} \times 100$$

A plot of cell viability against the concentration of the sample gave a measure of cytotoxicity. The IC_{50} was then calculated from the plot.

Results and discussion

Compound **1** (15-oxoursolic acid) was obtained as white amorphous powder from the ethyl acetate fraction of bark. The structure of the title compound was elucidated by developing molecular formula $C_{30}H_{46}O_4$ via high-resolution electron impact mass spectrometry, which showed molecular ion peak at m/z 470.0320 $[M+H^+]$ (calculated 470.3396). The IR spectrum showed strong absorption bands at 3430 cm^{-1}, 1740 cm^{-1} and 1720 cm^{-1} indicating the presence of free hydroxyl, carboxyl and ketonic functionalities respectively, whereas three absorptions at 2998, 1650 and 815 cm^{-1} indicated the presence of trisubstituted double bond (Figure 1).

The ^1H NMR spectra ($CDCl_3$, 500 MHz) of compound **1** distinctly showed the presence of five singlet resonating at δ 1.06, δ 0.72, δ 0.87, δ 0.80 and δ 1.06 which were assigned to C-23, C-24, C-25, C-26 and C-27 methyl protons, respectively. Two doublets resonating at δ 0.77 (d, J = 5 Hz) and δ 0.84 (d, J = 10 Hz) were assigned to C-29 and C-30 methyl protons which were indicative of ursane skeleton. A downfield doublet resonating at 3.2 (dd, J = 5.0 Hz, J = 10.0 Hz) was assigned to the H-3 methine proton indicating the presence of a geminal hydroxyl group. The chemical shift and the coupling constant of this proton suggested

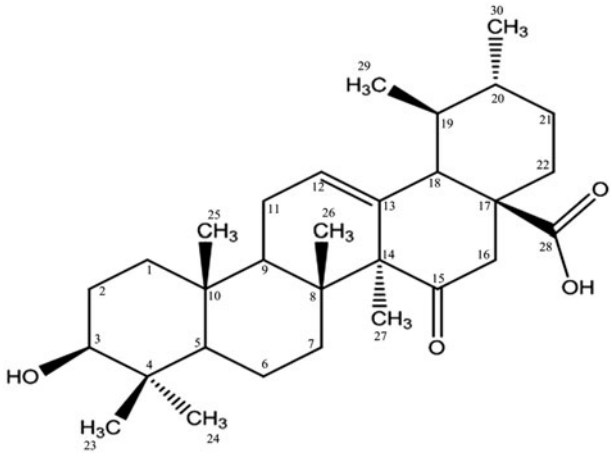

Figure 1. Structure of compound **1**.

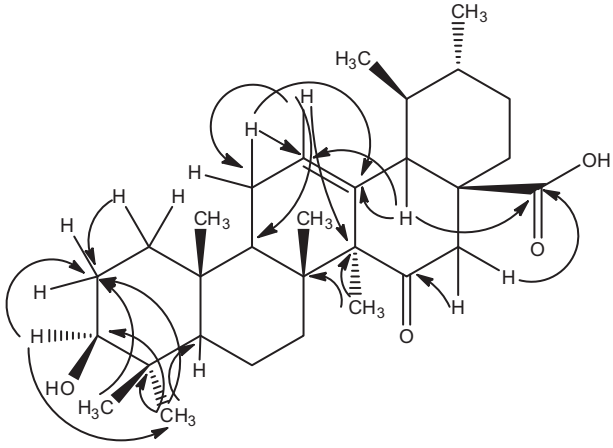

Figure 2. Important HMBC correlation of compound **1**.

the configuration of hydroxyl group as β and equatorial at C-3. In addition, a downfield singlet resonating at δ 5.24 was assigned to C-12 olefinic methine proton. The detailed ^{1}H NMR assignment of compound **1** is summarized in Table 1.

The ^{13}C NMR spectra ($CDCl_3$, 125 MHz) broad band decoupled and DEPT of compound **1** revealed the presence of 7 methyl, 8 methylene, 7 methine and 8 quaternary carbon atoms. The ^{13}C chemical shift resonating at δ 212.1 was assigned to C-15 quaternary carbon atom having the ketonic group. Similarly, signal at δ 79.04 was ascribed to the C-3 methine carbon having the hydroxyl group. The resonances at δ 28.1, δ 15.6, δ 15.4, δ 17.1, δ 23.5, δ 17.0 and δ 21.1 were assigned to C-23, C-24, C-25, C-26, C-27, C-29 and C-30 methyl carbons, respectively. In addition, the resonances at δ 125.9 and δ 137.9 were ascribed to the olefinic carbons at C-12 and C-13, respectively. The detailed ^{13}C NMR data of compound **1** are presented in Table 1.

The structure of compound **1** was further confirmed from 2D-NMR techniques (HMBC, COSY and NOESY). The important HMBC, COSY and NOESY interactions of compound **1** are presented in Figures 2 and 3, respectively.

In the HMBC spectrum, the C-3 methine proton (δ 3.2) showed relation with C-2 (δ 28.01) and C-24 (δ 15.6) methyl carbons. Similarly, the C-23 methyl protons (δ 1.06) exhibited interaction with C-2 (δ 28.01) methylene carbon. Furthermore, C-24 methyl protons (δ 0.72) exhibited interaction with C-4 (δ 38.75), C-3 (δ 79.04) and C-2 (δ 28.01), thus, confirming the position of the hydroxyl group at C-3. In addition, the C-12 olefinic methine proton (δ 3.2) exhibited heteronuclear interaction with C-11

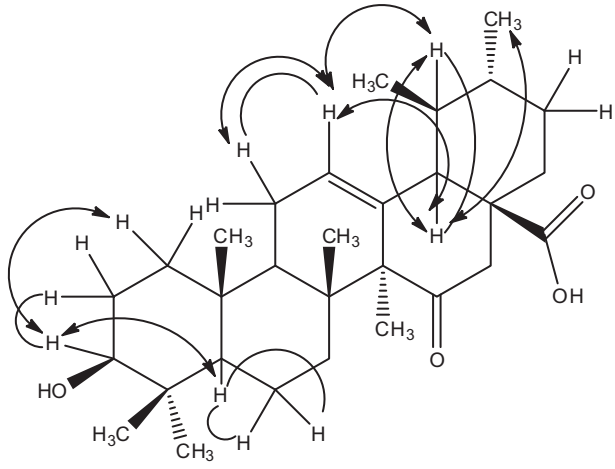

Figure 3. NOESY and COSY correlation.

Table 2. Anti-cancer activity of 15-oxoursolic acid (**1**).

Cancer cell line	IC_{50} (μM)
Squamous cell carcinoma (H157)	9.2 ± 0.23
Renal cell carcinoma (A498)	32.8 ± 1.54
Human hepatocellular carcinoma (Hep G2)	4.9 ± 0.02
Non-small cell lung (NCI-H226)	10.3 ± 0.01
Human ovarian carcinoma (MDR 2780AD)	2.3 ± 0.04

SEM: standard error mean of three experiments.

(δ 23.3), C-9 (δ 47.5) and C-14 (δ 42.0). Likewise, C-11 methylene proton resonating at δ 1.91 showed interactions with C-12 (δ 125.9) and C-13 (δ 137.1), thus, confirming the position of double bond. Furthermore, of C-16 methylene proton, the one resonating at δ 2.15 (H-16) showed interaction with C-15 (δ 212.1) ketonic carbon, while the other one resonating at δ 1.65 (H-16) exhibited relationship with C-28 (δ 181.4) carboxylic carbon. Similarly, C-18 methine proton (δ 52.7) exhibited interaction with C-13 (δ 137.9), C-12 (δ 125.9) and C-28 (δ 181.4). In addition, the C-27 methyl protons (δ 1.06) showed interaction with C-14 (δ 42.0) and C-8 (δ 39.5), thus, confirming the position of different functionalities.

The magnitude of the coupling constant of proton geminal to hydroxyl group at C-3 suggested the assignment of β configuration to the hydroxyl group. The stereochemistry of the compound **1** was further confirmed from the NOESY experiment which showed the interaction of C-3 methine proton with C-1 methylene proton and C-5 methine proton. Similarly, the C-12 olefinic methine proton exhibited interaction with C-11 methylene proton, on one hand, and C-19 methine proton on the other hand. In addition, C-18 methine proton showed coupling interaction with C-19 methine proton and C-30 methyl protons. The important NOESY and COSY interactions are presented in Figure 3.

15-Oxoursolic acid **1** was screened against five human cancer cell lines: MDR 2780AD, Hep G2, H157, NCI-H226 and A498 cell lines for analyzing their cytotoxic potential. The results are expressed as the concentration inhibiting 50% of cells growth (IC_{50}) (Table 2). Based on the IC_{50} values, interestingly, compound (**1**) exhibited considerable cytotoxic activity against MDR 2780AD, Hep G2, H157 and NCI-H226 cell line with IC_{50} values being 2.3 ± 0.1 μM, 4.9 ± 0.2 μM, 9.2 ± 0.2 μM and 10.3 ± 0.1 μM, respectively, while the effect was good against A498 cancer cell line with an IC_{50} value being 32.8 ± 1.2 μM. Taxol was used as a standard drug. The IC_{50} value of taxol against MDR 2780AD, Hep G2, H157, NCI-H226 and A498 cancer cell lines was

0.19 ± 0.08, 7.4 ± 0.31, 8.4 ± 0.31, 63.01 ± 0.03 and 95.98 ± 0.09 μM, respectively.

It is evident from the literature that the presence of hydroxyl group at C-3 and a carbonyl group at C-17 in triterpenoid is necessary for the cytotoxicity towards cancer cells. So the presence of both groups on triterpenes was essential for activity. Similarly, the configuration at C-3 was found to be much more important for cytotoxic activity. The compounds with a β-orientated hydrogen-bond forming group manifested significant cytotoxic activity than α-counterpart. The potential mechanisms behind the cytotoxicity of ursolic acid derivative may be either induction of apoptosis in cancer cell or blocking of the cell cycle progression in the G1 phase resulting in the triggering of apoptosis as determined by a DNA fragmentation assay (Hsu et al., 2004).

Conclusions

Phytochemical investigation of the bark of *R. arboreum* resulted in the isolation of a new potent triterpene. Cytotoxicity assays of the title compound **1** showed interesting results against different human cancer cell lines. Based on the cytotoxicity results, the compound **1** may be used as an important cytotoxic agent for the management of cancer; however, further investigation are needed to ascertain its efficacy, potency and safety for lead compound of long-term clinical utility.

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Disclosure statement

The authors of this article have no declaration of interest.

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