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A CYTOTOXIC STEROID FROM BERRIES OF *SOLANUM INCANUM* GROWING IN OMAN

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ABSTRACT: In pursuit of anticancer metabolites from natural sources, we examined organic extracts of *Solanum incanum* berries collected in Oman. The ethyl acetate fraction demonstrated significant inhibition of Carbonic Anhydrase (CA) activity, with an IC_{50} value of 20.20 ± 1.2 µg/ml. From this fraction, a β-sitosterol derivative was isolated and shown to suppress the proliferation of MCF-7 breast cancer cells by 71.5% in the MTT assay. These findings suggest that *S. incanum* represents a promising source of potential anticancer agents.

INTRODUCTION: *Solanum incanum* L is a shrub growing in dry tropical biome and is native to Africa, Arabian peninsula and SW India [1]. Numerous biological activities have been recorded for plant species in the genus Solanum. This plant species have analgesic, anthelminthic, antianemic, antibacterial, antifungal, antileishmanial, antitrypanosomal, antiprotozoa, antiplasmodial, schistosomicidal, anti-allergic, anti-inflammatory, anti-nociceptive, antihistaminic, anti-asthmatic, anti-diabetic, anti-cancer, cardio-vascular, hypolipidemic, antihypertensive, diuretic, anti-convulsant, antimelanogenetic, anti- anti-psoriatic, molluscicidal, antiurolithiatic, antiviral, mosquito larvicidal, nephrotoxic, spasmolytic, and vasorelaxant properties [2]. Traditional medicine uses Solanum species to treat a variety of conditions, including angina, sore throat, stomach discomfort, toothache, headache, pain from pleurisy, liver pain, painful menstruation, pneumonia, rheumatism, and river blindness [3-8]. Natural products that have been isolated from *S. incanum* include alkaloids, flavonoids, saponins, reducing sugars, proteins, phenols, glycosides, terpenoids, anthraquinone, resins, steroids, tannins & cardiac glycosides [2].

Our investigation on some medicinal plants collected from Oman showed organic extracts from *S. incanum* to be active against some of the tested cancer cell lines [9].

This paper report isolation of a cytotoxic steroid 1 (**Fig. 1)** from berries of *S. incanum* collected from Jabal Akhdhar, Oman.

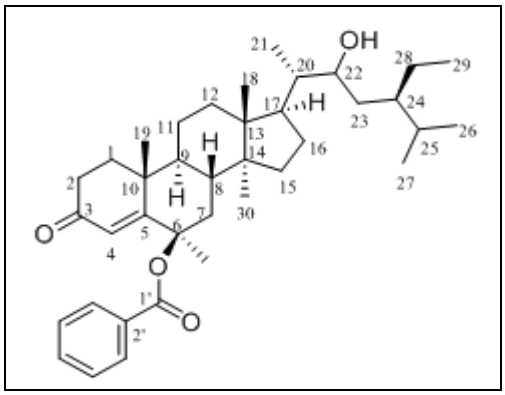

FIG. 1: STRUCTURE OF COMPOUND 1

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MATERIALS AND METHODS:

General Experimental Procedures: All of the solvents were of analytical grade and were used without additional purification. Methanol, hexane, chloroform, dichloromethane, and ethyl acetate were purchased from J.T. BAKER, India. Silica gel used was 63 -200 micron and was obtained from Merck Germany. Nuclear Magnetic Resonance (NMR) experiments were conducted with a Bruker Ascend 600 MHz machine. Protons (1H) were recorded at 600 MHz, while carbons (13C) were measured at: 150.9 MHz). Signals are described in parts per million (δ) in reference to the experimental solvent. Infra-Red (IR) spectra were taken on Shimadzu IR-435 Spectrophotometer. The Mass spectra were recorded using the Agilent Technologies 6530 Accurate Mass Q-TOF-LC/MS.

Sample Collection and Preparation of Extracts: Berries of *S. incanum* were obtained from Jabal Akhdhar, Oman. The species was identified by Dr. Amna Al Farsy at Sultan Qaboos University (SQU). A voucher specimen no. Al-Farsi, A. 567 is deposited at SQU herbarium. The sample was dried in the shade for two weeks and pulverized to give coarse powder. About 300 g of the powder was macerated in methanol for 48 hours and then filtered to give clear solution. The experiment was repeated two times. Methanol was then removed at low pressure and the resulting crude extract was Kupchan's partitioned to give extracts of varying polarities including hexane, chloroform, ethyl acetate and butanol.

Compound 1 - Benzoyl β-Sitosterol: The extract of ethyl acetate was tested using vacuum liquid chromatography on silica gel, eluting with hexane and increasing concentrations of ethyl acetate, due to its strong action against carbonic anhydrase. Evaporation of solvent from a fraction obtained while eluting the column with 30% EtOAc/hexane gave brownish-white gum containing one major compound based on TLC. Gel filtration of the gum using Sephadex LH-20 eluting with methanol gave pure compound 1. 15.4 mg, HRMS ESI^+ m/z 563.4121 $[M+H]^+$ (calcd for $C_{37}H_{55}O_4$ 563.4100), 1H-&13C-NMR data **Table 1.**

Carbonic Anhydrase (CA) Assay: Inhibition of CA was done by a method described by Supuran and coworkers [10]. In a typical experiment: test sample (in DMSO 0.5 mM) 20 µL, the HEPES-tris 140 µL, the enzyme (0.1 mg/mL) 20 µL and the substrate 4-NPA (0.7 mM) 20 µL were placed in a well in a 96 well plate. The experiment was started by incubating a mixture of the test samples and the enzyme in the buffer for 15 min. After adding 20 µL of substrate, the SpectraMax M2, USA was used to monitor the rate of product formation at 400 nm at 1-minute intervals for 30 minutes. DMSO was used as a negative control while Acetazolamide (Standard inhibitor) was a positive control.

Cytotoxicity Assay (MTT Assay): Cytotoxicity assay used Cancer Cells (Human Breast Cells) – MCF-7 and Normal Cells (Human Fibroblast Cells) – BJ-HTERT. The cells were purchased from ATCC®. The experiment was done using MTT Assay Kit (BI-2004-5) from Sigma Aldrich.

Protocol of MTT Assay: Each well contained 7000 cells and was kept for 24 hours to make sure the growth and distribution are good. To minimize error, 97 µL of cell medium and 3 µL of 3% pure compound were added to each experiment's cells. The experiment was duplicated and monitored for activity over a 24-hour period. The cell medium was taken out and 100 µL of the Ready-To-Use RPIN1640 (MTT media) was added after the 24-hour period. Each well received 10 µL of the MTT stock solution (MTT Reagent). The plate was incubated at 37°C for three hours. Following the incubation period, the MTT reagent and medium were withdrawn. Each well was then filled with 50 µL of the DMSO. After that, it was incubated at 37°C for 5 minutes. At 570 nm, the absorbance was measured.

Data Analysis of MTT Assay: The absorbance data were used to calculate the percent inhibition of the cells by using the following equation:

$$\% \text{ Cell inhibition} = 100 - (A1-Ao) / (A2-Ao) \times 100$$

Where, A_1: absorbance of tested compound, A_0: absorbance of the blank (without any cells), $A_{2:}$ absorbance of control (only cells).

RESULTS AND DISCUSSION:

Structure of Compound 1: The molecular formula of compound **1** deduced from spectroscopic data is $C_{37}H_{54}O_4$. **Table 1** displays the 1D and 2D-NMR

data of compound 1. The isolated compound contains a monosubstituted benzene ring, as evidenced by the ^{1}H and ^{13}CNMR data. Absorption values and the HMBC correlations of the phenyl protons suggest the ring to be part of benzoate functionality. Apart from the benzoate system, the rest of the protons and carbon-13 signals correspond to those of β-sitosterol [11]. As a result, the seven methyl protons of the β-sitosterol were found to have characteristic absorption at δ 0.62 (3H, br, s), 0.83 (3H, m), 0.91 (3H, m), 0.93 (3H, m), 0.96 (3H, d, J = 6.72), 1.10 (3H, d, J = 5.91 Hz), and 1.31 (3H, m) in the 1H-NMR spectrum.

TABLE 1: NMR DATA FOR COMPOUND 1 IN $CDCL_3$*

Carbon	δ13C	Multp.	δ1H	HMBC
1	38.8	CH_2	2.14; 1.47 (2H, m)	C-3, C-2
2	36.2	CH_2	1.87; 1.62 (2H, m)	C-2, C-4
3	200.1	C	–	C-10, C-13
4	123.6	CH	5.71 (1H, d, J = 2.34 Hz)	
5	161.0	C	–	
6	78.9	CH	4.69 (1H, ddd, J1 = 10.81 Hz; J2 = 10.70 Hz; J3 = 4.71 Hz)	C-5, C-1’
7	41.4	CH	1.28 (1H, m)	
8	42.6	CH	1.73 (1H, m)	
9	54.9	CH	2.06 (H, m)	
10	39.3	C	–	
11	21.7	CH_2	1.80; 1.63 (2H, m)	
12	42.8	CH	1.73 (1H, m)	
13	45.1	C	–	
14	53.1	C	–	
15	23.6	CH_2	1.47 (1H, m); 1.28 (1H, t, J1 = J2 = 3.82 Hz)	
16	30.0	CH_2	1.25; 1.04 (2H, m)	
17	60.0	CH	2.27 (1H, d, J = 10.89 Hz)	C-10, C-24, C-30
18	11.8	CH_3	0.91 (3H, m)	
19	20.5	CH_3	0.93 (3H, m)	
20	31.8	CH	1.73 (1H, brs) (d, J = 10.30 Hz)	
21	17.6	CH_3	0.83(3H, s),	
22	71.0	CH	3.75 (1H, d, J = 10.29 Hz)	C-20, C-21
23	26.1	CH_2	2.01; 1.23 (2H, m)	
24	51.0	CH	2.26 (1H, m)	
25	28.7	CH	1.70 (1H, m)	
26	17.4	CH_3	0.85 (3H, m)	
27	14.7	CH_3	0.96 (3H, d, J = 6.72 Hz)	
28	22.5	CH_2	1.62; 1.54 (2H, m)	
29	12.5	CH_3	0.97 (3H, m)	
30	12.3	CH_3	0.62(3H, br, s)	
1’	166.4	C		–
2’	130	C		–
3’&7’	129.6	CH	8.06 (2H, dd, J1 = 8.10 Hz; J2 = 1.37 Hz)	C-1’, C-5’, C-3’/7’
4’ & 6’	128.4	CH	7.45 (2H, t, J1 = J2 = 7.73 Hz)	C-2’, C-4’/6’
5’	132.8	CH	7.56 (1H, m)	C-3’/7’

The characteristic olefinic proton H-4, of this steroid resonated as doublet at δ 5.70 (1H, d, J = 2.34 Hz). Based on HMBC correlations, the downfield methine signals at δ 4.69 and δ 3.75 were attributed to the oxygenated protons H-6 and H-22. A total of 37 carbon signals were identified in the 13C-NMR spectrum of compound 1.

Seven of these signals were assigned to the benzoate substituent at position 6 while the remaining 30 account for the β-sitosterol skeleton. The 13C-NMR signal at δ 200.1ppm C-3 suggested oxidation of the characteristic hydroxyl group of the sterol to a keto system. The benzoate group was placed at position 6 based on HMBC cross peaks between H-6 absorbing at δ 4.69 and C-1’ resonating at δ 166.4.

Assignment of stereochemistry was based on the NOESY spectrum and the reported inherent biogenetic configuration of the β-sitosterol skeleton.

On basis of this information the isolated compound is identified as 17 - (5 – ethyl – 3 - hydroxy – 6 - methylheptan-2-yl)-2, 3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17-tetradecahydro-10,13,14-trimethyl-3-oxo-1H-cyclopenta[a]phenanthren-6b-yl benzoate (1) **Fig. 1.**

Isolation of this compound from S. *incanum* is reported for the first time. However, the same compound was previously isolated from berries of another Solanum species, *S. aculeastrum* collected from Kenya [12]. Hence, the isolated derivative of β-sitosterol could be used as a biomarker for chemotaxonomic identification of Solanum species.

Inhibition of Carbonic Anhydrase by Extracts of *S. Incanum:* In the initial testing of the extracts at higher concentration (0.2 mg/mL) only ethyl acetate extract inhibited CA at 71.5% which was greater than the cut-off value (70%) for active sample. This extract gave IC_{50} value 20.20 ± 1.20 µg/ml in further experiment at six diluted concentrations ranging from 62.5 – 1000 µg/ml.

***In-vitro* Cytotoxic Activity of Compound 1:** Compound was active against the tested cancer cell it inhibited the growth of MFC-7 by 76.02% **Table 2.** However, it also inhibited the growth of the normal cells (Fibroblast, BJ-HTERT) at higher percentage (82.12%). These preliminary results suggest that while the isolated compound has demonstrated potent *in-vitro* activity against human breast cancer cell line (MCF-7), it could also be toxic as it inhibits the normal cell growth almost at the same level.

TABLE 2: % GROWTH INHIBITION OF MFC-7 AND BJ-HTERT BY COMPOUND 1

Cells type	Inhibition %
MCF-7	76.02
BJ-HTERT	82.12

CONCLUSION: In conclusion *S. incanum* is a potential source of bioactive metabolites for development of anticancer agents but some of its products need optimization for practical application.

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CONFLICTS OF INTEREST: Nil

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