DRUG DISCOVERY

A pimarane diterpene from *Juniper servaschanica* inhibits growth of Ovarian and Breast Cancer Cells

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ABSTRACT

In our ongoing search for anticancer compounds from natural products we chromatographed cytotoxic active extracts from medicinal plants growing in Omanin order to isolate their active ingredients. We hereby report isolation of a cytotoxic pimarane diterpenoid, 3β-hydroxy-ent-pimara8(14),15-dien-19-oic acid (**1**) from hexane extract of *Juniperus servaschanica* collected from Oman. The anticancer property was evaluated against ovarian (MCAS) and breast (MDA MB231) cancer cell lines by Alamar-blue assay. Compound **1** was active against both ovarian and breast cancer cells (IC_{50} = 24.16 and 16.13 μg/ml, respectively). Additionally, the molecule showed little damaging effect against non-cancerous cells, fibroblast at its IC_{50} dosage. Further investigation on this metabolite and its derivatives might lead to discovery of new chemical entities for control of cancer.

Keywords: *Juniperus servaschanica*, Cupressaceae, diterpenes; ent-pimaradiene, anticancer

1. INTRODUCTION

Cancer continues to be among the major health burden of the world and is projected to increase due ageing and continued growth of the world's population. The world witnessed about 10 million cancer deaths in 2018. Furthermore, a total of 18 million new cases were diagnosed raising the number of people living with cancer to 43.8 million (IARC, 2018). Hence, new methods including availability of new and more potent chemotherapeutics are need for control of the enormous cancer burden. Bioactive secondary metabolites have long been recognized as a pool of novel and potent metabolites for discovery and development of new drugs including anticancer agents (Newman & Cragg, 2016). In this paper we report anticancer property of a pimarane diterpene isolated from *Juniperus servaschanica* collected from Oman against human breast and ovarian cancer cells.

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2. MATERIALS AND METHODS

2.1. General experimental procedures

All experiments used analytical grade consumables. All mixtures were chromatographed on Si gel 60 (230-400 mesh) obtained from Merck. Shimadzu instrument (IR-435) was used for IR analysis while Shimadzu UV1800 machine was used for recording UV spectra. All NMR data were recorded using Bruker Ascend 600 MHz machine (recording 1H and 13C at 600 and 150.9 MHz, respectively). NMR signals were referenced to the solvent used and reported in ppm. Mass Spectra were obtained at 70 eV using Varian VG 7070E system.

2.2. The plant sample

The leave sample of *J.servaschanica* was collected from Jabal Akhdhar, Oman on 16 May 2015. A voucher specimen; *Al-Farsi, A.* 599 is archived at the herbarium of Sultan Qaboos University.

2.3. Extraction and Isolation

Homogenized dried leaves were soaked twice in ethanol for 72 hours to giving crude ethanol extract. Fractionation of the ethanol extract by Kupchan's partitioning gave fractions of varying polarities viz. butanol, ethyl acetate chloroform and hexane extracts. Hexane extract was then flash chromatograph don silica gel by gradient elution using various mixtures of hexane/Ethyl acetate yielding 39 different fractions. Elution of the silica gel column using 5% EtOAc/hexane as mobile phase yielded greenish-white gum of the impure pimarane diterpene (**1**). Repeated column chromatography on the impure gum gave white crystals which were further purified by recrystallization from pure methanol to pure compound (**1**). Yield 23 mg = 0.14% of the dry leaves. M.p. 156 ^{o}C; $[\alpha]_D$ = + 18.06 ($CHCl_3$, c 0.053); IR (KBr): νmax3460, 3074,2930, 1694, 1631, 1463, 974, 910 cm^{-l}; EI-MS m/z 341 $[M+Na^+]$;NMR data Table 1.

2.4. Assay for anticancer activity

The anticancer property of compound **1** was measured using Alamar-blue assay on breast (MDA MB231) as well as ovarian (MCAS) cancer cell lines.

Cell culturing:

All cancer cell (MDA MB231 and MCAS) were purchased from ATCC and were cultured in DMEM media. They were then trypsinized and used for experiments after attaining 80-85 % confluency. The cells were then place in a 96 well plate and left for 24 hours to allow them to grow and attach. About 15,000 cells were seeded in each well (cell were counted using haemocytometer).

Cell viability assay:

Each cell was tested against eleven different concentrations of compound **1** including 50, and 100 – 1000 ug/ml. Cells treated with only DMSO were used as negative controls while control blank were untreated cells in used media. Vehicle control contained only DMSO mixed with media. Each experiment was repeated thrice. The cell death was then observed under microscope after incubating the plate for 24 hours.

Alamar blue assay:

The media was refreshed after 24-hours and each well was then treated with and alamar blue reagent. Optical densities were then recorded at λ = 570nm after incubating the plate at 37°C for 1-4 hours.

2.5. Data Analysis

Cell viability was calculated as:

Percent Cell viability= (sample absorbance/control absorbance) x 100%.

IC_{50} values against each of the studied cells were obtained from the cell viability data by using MS-Excel

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3. RESULTS AND DISCUSSION

Molecular formula of the diterpene **1,** $C_{20}H_{28}O_3$ deduced from its NMR and MS spectral data contained one extra oxygen atom compared to compound **2**; a molecule previously isolated from a Mexican plant species *Gochnatiaglutinosa* (García, Guerreiro, & Joseph-Nathan, 1985).

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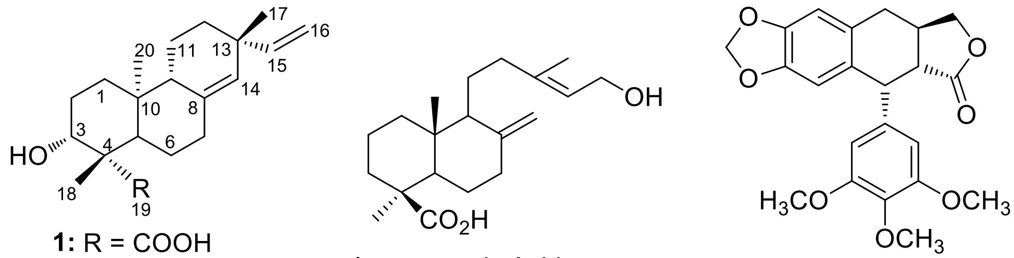

1: R = COOH
2: R = CH_2OH

Isocupressic Acid

Deoxypodophyllotoxin

Figure 1: The pimaranediterpne (**1**)

1H- and 13C-NMR spectral absorption values of the isolated pmaranediterpene **1** (Table 1) matched well with those of compound **2** and other pimaranediterpenoids (Carvalho et al. 2006; Reveglia et al. 2018; Secaa, Pinto & Silvab 2008). However, 13C spectrum of compound **1** missed a signal for the primary alcohol carbon at δ 66.0 and instead contained a peak for a carbonyl carbon at δ 180 ppm (C-19), indicating that the hydroxymethylene group in **2** has been oxidized to carboxylic acid in compound **1**. Likewise, two doublet signals between δ 3.5 – 4 ppm for the two diastereotopic hydroxymethylene protons H-19 were also missing in 1HNMR spectrum of the isolated compound **1**. The presence of the carbonyl functionality in compound **1** was further confirmed by the considerable deshielding effect on C-4 that absorbed at δ = 54.7 and shielding effect on C-18 (δ = 11.8). Furthermore, the Infrared (IR) spectrum of **1** contained most of the expected absorptions for a carboxyl group.

Table 1: 1D- & 2D-NMR spectral data of diterpene **1**

	δ_C	δ_H(multi., J Hz)	$^{2,3}J_{CH}$
1	38.3	1.85 (1H, m, obs) 1.32 (1H, m)	C3, C5, C10, C20
2	19.8	1.60 (1H, m, obs) 1.65 (1H, m)	C3, C4, C10
3	76.4	4.02 (1H, dd, *J*= 4.56 & 11.7 Hz)	C18, C19
4	54.7		
5	51.5	1.82 (1H, obs)	C3, C10, CC18, C19, C20
6	25.5	1.55 (1H, mult.) and 1.25 (1H, m)	C5, C7, C10
7	36.6	2.27 (1H, ddd,*J* = 1.62, 4.38, 14.04 Hz) and 2.09 (1H, m, obs)	C8, C9, C14
8	137.7		
9	51.8	1.77 (1H, t, *J* = 7.8 Hz)	C8, C14, C5, C10, C20
10	38.5		
11	28.0	1.68 (1H, m) and 1.33 (1H, m)	C10, C13
12	35.7	1.48 (1H, m, obs) and 1.39 (1H, m)	C9, C13
13	38.6		
14	130.4	5.26 (1H, brd, s)	C15, C9, C13, C7
15	149.9	5.76 (1H, dd, *J* = 10.62& 17.46 Hz)	C14, C13, C12, C17
16	110.8	4.87 (2H, m, obs)	C15, C13
17	26.6	1.06 (3H, s)	C12, C13, C14, C15
18	11.8	1.14 (3H, s)	C3, C4, C5, C19
19	180		
20	15.7	0.87 (3H, s)	C5, C9

The chemical structure elucidation of the pimarane **1** was further assisted by the HMBC cross-peak correlation shown in Table 1. Key HMBC correlations that helped to build the main skeleton of compound **1** are $^{2,3}J$ cross-peaks displayed by protons resonating at δ 4.02 H-3, δ 1.82 H-5, δ 1.06 H-17and δ 5.26 H-14 (Table 1). The stereochemistry was assigned based on NOE cross-peaks. For example, methyl group 18 resonating at δ 1.14 was placed at axial position due to presence of NOE cross-peak between this group and a proton resonating at δ 4.02, H-3. In addition to NOE the chemical shift values of some of the group also assisted to identify

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configuration of some of the streogenic centers in compound **1**. Methyl-17 (δ 26.6) was assigned axial orientation rather than equatorial which should usually resonate atδ value > 30 ppm (Reveglia et al. 2018; Secaa, Pinto & Silvab, 2008).

3.1. In vitro anticancer activity of compound 1

The isolated diterpenoid (**1**) showed in vitro anticancer activity against both MDA MB231 and MCAS (IC_{50} = 16.13and 24.16μg/ml, respectively) Table 2. Moreover, in vitro laboratory experiment to determine toxicity of compound **1** at its IC_{50} dosage showed little damaging effect against on-cancerous cells, fibroblast.

Table 2: Anticancer activity of the diterpenoid **1**

IC50 (ug/ml)		Cell viability %	
MDA MB231	MCAS	normal cell line	cancer cell line
16.13	24.16	100.27	13.51

J. servaschanica is a corrected botanical name for juniper growing at Al Hajar mountain of Oman that was previously identified as J. excelsa (Adams, Al-Farsi & Schwarzbach, 2014). Junipers species have a history of being used as remedy for several ailments in traditional medicine of the Arabian Peninsula (Sadeghi-aliabadi, Emami, Sadeghi & Jafarian, 2009; Ghazanfar, 2011). We have isolated a pimaranediterpenoid, 3β-hydroxy-ent-pimara8(14),15-dien-19-oic acid (1) which inhibits the growth of MCAS and MDA MB231 from J. servaschanica. This is the first report on isolation of a cytotoxic pimaranediterpenoid from Juniperus species. However, isocupressic acid, a labdanediterpene, the bicyclic isomers of the pimaranes has been isolated from *J. communis* is growing in America (Benzina et al. 2015). Isocupressic acid and deoxypodophyllotoxin obtained from this species induced apoptosis in MDA MB231 cancer cells (Benzina et al. 2015).

4. CONCLUSION

In conclusion a secondary metabolite isolated from *J. servaschanica* exhibits anticancer activity against MCAS and MDA MB231 cancer cell lines. It will be interesting to pursue further investigation on this compound and its derivatives with the aim of discovering and developing New Chemical Entities for treatment of cancer.

Conflict of interest

We hereby confirm that there is no conflict of interest in the content of this article.

Ethical approval

The ethical guidelines for plants & plant materials are followed in the study for experimentation. Moreover, ethical guidelines for cell lines are followed in the study for experimentation

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Data and materials availability

All related data have been presented in this paper.

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