Bioactivity-guided isolation of new antiproliferative compounds from *Juniperus foetidissima* Willd.

Mahmood Rafieian-kopaei, Ibrahim Suleimani dehkordi, Mustafa Ghanadian, Ardeshr Shokrollahi, Mahmoud Aghaei, Ayatollahi Syed Majid, and M. Iqbal Choudhary

ABSTRACT

Based on a literature survey on cytotoxic medicinal plants, *Juniper* species were identified as interesting sources of antitumor compounds. Using bioassay-guided fractionation against Caov-4 cancer cells on *Juniperus foetidissima* led to the isolation of a new 3H-benzofuran-2-one: 4-methyl-3-methoxy-3H-benzofuran-2-one (1), a new sesquiterpene: 4,9(α)-dihydroxy-nardosin-6-en (2) and an already known labdane-type diterpene: 15-hydroxy-8(17),13(E)-labdadiene-19-carboxilic acid (3). Compounds 1–3 exhibited cytotoxic effects, with moderate cytotoxicity against the EJ-138 bladder and CAOV-4 ovary cancer cell lines.

CONTACT Mustafa Ghanadian  ghanadian@pharm.mui.ac.ir

This paper is part of the thesis of Ibrahim Suleimani submitted in partial fulfillment of the requirements for the degree of Masters of Science.

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/14786419.2015.1101106.

© 2015 Taylor & Francis
1. Introduction

Bladder and ovary cancers are common cancers in women. Discovery of safe and potent anti-cancer compounds is thus important to treat these cancers. Ethnobotanical knowledge and biological evaluation of medicinal plants can provide investigational new drugs to develop effective cancer therapies. *Cupressus semipervirens*, *Juniperus communis*, *Juniperus excelsa*, *Juniperus foetidissima*, *Juniperus oblonga*, *Juniperus sabina*, *Platycladus orientalis* and *Taxus baccata* are Iranian conifers. A few of them have exhibited interesting cytotoxic activities against a panel of cancer cells (Emami et al. 2005, 2007; Sadeghi-aliabadi et al. 2010). Among them, *J. foetidissima* (Cupressaceae), locally known as ‘Ardush’, is a tree with 5–15 m high, crown slender conical, branched to the ground. *J. foetidissima* has leaves of 2.0–3.4 mm, with branchlets of 1.2–2.0 mm diameter and 1–3 seeds per cone which differentiate it from *J. excelsa* with 0.6–1.4 mm leaves, 0.7–1.5 mm in diameter branchlets and 3–6 seeds per cone. It is grown in the mountains of Greece, Albania, Yugoslavia, Asia Minor to Transcaucasia and north parts of Iran (Riedl 1968; Emami et al. 2007; Marcysiak et al. 2007). In the Iranian traditional medicine, Rhazes (854 CE – 925 CE) in an old pharmacopoeias named Alhavi have suggested fruits and leaves of *Juniper* in combination with figs can be used for the treatment of a type of hyperplasia inside the nose (Al-rhazes & Al Havi 2005). In recent studies, a wide range of cytotoxic and antibacterial activities of the compounds of *Juniper* species stimulated our interest to study the chemical composition of *J. foetidissima* (El Sawi & Motawe 2008; Muto et al. 2008; Bahri et al. 2013; Robichaud et al. 2013). Previous studies on this plant described β-thujone, cedrol, sabinene and abietal as the main components of essential oil of its leaves and fruits (Tunialier et al. 2002), as well as cedrene-type sesquiterpene oxides such as 8,14-cedranoxide, 8,14-cedranediol, 8-cedren-13-ol and cedric acid in its nonpolar chloroform extract (Baggaley et al. 1968). We report here a cytotoxicity-guided fractionation and isolation of a new 3H-benzofuran-2-one, a new sesquiterpene and an known labdane-type diterpene from the acetone extract of *J. foetidissima* leaves and branchlets. These compounds exhibited cytotoxic activities against EJ-138 and Caov-4 Human bladder and ovary cancer cells.

2. Results and discussion

MTT assay was performed on different fractions against CAOV-4 cells. Fractions 3, 5 and 6 showed cytotoxic activities with IC₅₀ values of 24 ± 4.2, 36 ± 5.1 and 24 ± 2.1 μg/mL against CAOV-4, which were chromatographed on normal column and purified by HPLC on YMC-silica column to yield F3d2 (1, 8 mg), Fr5 h (2, 15 mg) and Fr6 h (3, 32 mg) as pure compounds.

Compound 1 was obtained as a white solid with positive reaction to methanolic ferric sulphate and exhibited HREI-MS molecular ion [M]+ at m/z 178.0618, suggesting its molecular formula as C₁₀H₁₀O₃ (calcd. 178.0630, Δ 6.7 ppm). The IR indicated absorptions for a broad peak of carbonyl (1722 cm⁻¹), aromatic C=C (1614, 1562 and 1458) and C–O bonds (1051, 1086 and 1140 cm⁻¹) with no free hydroxyl absorption. The ¹H-NMR spectrum showed AMX spin pattern of a 1,2,3 trisubstituted aromatic ring at δ_H 7.06 (d, J = 8.0 Hz, H-5), 7.40 (t, J = 8.0 Hz, H-6) and, 7.22 (d, J = 8.0 Hz, H-7) and also a methyl δ_H 2.69 (s) attached to the aryl group. In addition, two deshielded protons, an oxymethine proton at δ_H 5.69 (s) and a singlet methoxy group at δ_H 3.99 (s) was observed in the ¹H-NMR spectrum of compound 3. The ¹³C NMR spectrum supported the presence of the aromatic ring showing six carbon...
peaks $\delta_C$ 114.4 (s, C-3a), 137.1 (d, C-4), 127.6 (d, C-5), 131.6 (d, C-6), 115.3 (d, C-7) and 154.7 (s, C-7a) at the aromatic region along with one carbonyl carbon at $\delta_C$ 169.5, an oxymethine ($\delta_C$ 90.1), a methoxy ($\delta_C$ 56.1) and a methyl carbon ($\delta_C$ 23.4). DQF H–1H-COSY showed connectivities between atom connectors 5–7. HMBC indicated correlations of aromatic carbons H-6/C-4, C-7a, H-5/C-4, H-7/C-5, singlet methyl ($\delta_H$ 2.69, H-8) with C-4 and C-3a, oxymethine proton ($\delta_H$ 5.69, H-3) with bond connector C-3a and carbonyl carbon. Singlet methoxy protons ($\delta_H$ 3.99) showed weak HMBC with both oxymethine carbon ($\delta_C$ 90.1, C-3) and carbonyl carbon ($\delta_C$ 169.5, C-2). But due to the lack of free hydroxyl absorption in IR and EI MS fragments at m/z 178 [M], OCH$_3$ was located on C-3 and structure was confirmed as 4-methyl-3-methoxy-3H-benzofuran-2-one.

Compound 2 was obtained as a colourless crystal. The HREI-MS showed the M$^+$ at m/z 238.1928 corresponded to the molecular formula C$_{15}$H$_{25}$O$_1$ (calculated: 238.1933, $\Delta$ −2.2 ppm). IR absorptions indicated the presence of broad hydroxyl group (3429 cm$^{-1}$), double bonds (1645, 1471, 1456 and 889 cm$^{-1}$), and C–O bonds (1178–1080 cm$^{-1}$). The $^1$H-NMR spectrum

**Figure 1.** Cytotoxicity effect of compounds 1–3 against EJ-138, Caov-4 cancer cell lines.

Notes: Cells were treated with different concentrations (1, 10, 20, 40, 80 and 100 μM) of compounds 1–3 in three replicates. Results (mean ± SD) were calculated as percent of corresponding control values; (* $p < 0.5$; ** $p < 0.01$ vs. control).
showed an olefinic proton $[\delta_H 5.28 (1H, dd, J = 2.4, 3.2 \text{ Hz})]$, an oxygenated methine group $[\delta_H 3.25 (1H, bdd, J = 11.6, 3.6 \text{ Hz})]$, two teritary methyls $[\delta_H 0.96 (3H, d, J = 6.8 \text{ Hz})$ and 0.95 $(3H, d, J = 6.8 \text{ Hz})]$, and two singlet methyls $[\delta_H 1.12$ and 0.89]. The $^{13}$C-NMR and DEPT spectra demonstrated fifteen carbons comprised of four methyls, four aliphatic methylenes, two aliphatic methines (one oxygenated), two quaternary aliphatic carbons (one oxygenated), one olefinic methine and one quaternary olefinic carbon. Three degrees of unsaturation and one double bond indicated the presence of two rings in the structure. The $^1$H-1H-COSY showed correlations between H-1 to H-3, H-7 to H-10, H-11 to H-13 and H-14. From these structural characteristic, and HMBC correlations of H-3/C-4, H-10/C-5, H-7/C-6, H-11/C-6, Me-14/C-4 and Me-15/C-5, the structure of compound 2 was deduced as 4,9-dihydroxy-nardosin-6-en. The relative stereochemistry was determined using NOEs spectra. Taking H-15 in $\beta$ configuration, NOE cross-peaks of H-15/H-14, and H-8b indicated $\beta$ orientation for H-14 while NOEs of H-8a/H-9 and H-9/H-10 supported $\alpha$ position for H-10 and H-9.

Compound 3 was obtained as a colourless powder. HREI-MS showed the M$^+$ at $m/z$ 320.2325 corresponded to the formula C$_{20}$H$_{32}$O$_3$ (calcd. 320.2351, $\Delta$ –8.2 ppm). NMR data assigned it as 15-hydroxy-8(17),13-labdadiene-19-carboxilic acid which is reported earlier by Cambie and coworkers from Phyllocladus trichomanoides (Cambie et al. 1981). However, several assignments of $^1$H- and $^{13}$C-NMR signals were revised.

To assess the anticancer activities of the isolated compounds, standard MTT assays were performed on EJ-138 and CAOV-4 human cancer cells, in vitro (Zarei et al. 2013). As it is shown in Figure 1, cytotoxicity of compounds 1–3 with different concentrations (1, 10, 20, 40, 80 and 100 $\mu$M) were evaluated against EJ-138, Caov-4 cancer cell lines. Compounds 1–3 exhibited cytotoxic effects in a dose-dependent manner with IC$_{50}$ values of 43.26 ± 3.21, 44.27 ± 4.25 and 26.17 ± 2.96 $\mu$M against the EJ-138 cell line, and IC$_{50}$ values of 25.72 ± 3.13, 57.38 ± 4.56 and 37.35 ± 4.13 $\mu$M against the CAOV-4 cell line, respectively.

3. Experimental

3.1. General

Isolation of compounds were carried out with open column chromatography using silica gel (60–200 $\mu$m, Merck, Germany) and normal preparative HPLC using a YMC-Pack-Sil column (250 × 20 mm i.d., YMC, Japan). The structures of the compounds were elucidated by $^1$H-NMR, BB $^{13}$C-NMR, DEPT, COSY, HMBC, NOESY, FT-IR and HREI-MS. The NMR spectra were acquired with Bruker AV-400 using CDCl$_3$ as solvent. The Infrared spectra were acquired by Rayleigh WQF-510 FTIR spectrophotometer, with NaCl discs. The HREI-MS spectra were obtained with Waters Q-TOF Micro YA019 mass spectrometer in $m/z$ and EI-MS spectra with Varian MAT 312 spectrometer.

3.2. Plant material

The leaves and branchlets of J. foetidissima were collected from Azadshahr (Golestan Province, north of Iran), in July 2011. The plant was authenticated by Dr Iraj Mehregan and a voucher specimen (No.1419) deposited at the Herbarium of the Pharmacognosy Department, School of Pharmacy, Isfahan University of Medical Sciences, Isfahanl, I.R. Iran.
3.3. Extraction and isolation

The plant material was chopped into small pieces and dried in shade at room temperature. It was crushed in electrical blender (3.5 kg) and then macerated with acetone (20 L x 3), at room temperature for three days. Evaporation to dryness gave 350 g of residue which was subjected to liquid/liquid partitioning between methanol and hexane. Evaporated methanol part (152 g) was column chromatographed (7 x 100 cm) on silica gel (60–200 μm, 1300 g) using hexane/acetone (5→100%) following with increasing the polarity by adding the methanol to afford ten fractions: Fr.1–10. After MTT cytotoxicity assay against CAOV-4 ovary cancer cells, fractions Fr.3, Fr.5 and Fr.6 with more cytotoxic activities were selected and column chromatographed on silica gel (45–60 μm, 50 g) using hexane/ethyl acetate (0→50%). Fr.3d, Fr.5 h and Fr.6 h were more purified by preparative HPLC using YMC-Pak-Sil column (250 x 20 mm) and hexane/acetone (70:30) as mobile phase to yield compound 1 (Fr.3d1, 12 mg), compound 2 (Fr.5h1, 18 mg) and compound 3 (Fr.6h1, 28 mg) as pure compounds (Figure 2).

3.3.1. 4-Methyl-3-methoxy-3H-benzofuran-2-one (1)

Amorphous white powder, [α]D20: –39.3 (c 0.1, CHCl3). IR (NaCl) v max: 2958, 2929, 2856, 1722, 1614, 1562, 1458, 1377, 1255, 1140, 1086, 1051 and 962, 771 cm−1. 1H-NMR (CDCl3 400 MHz in CDCl3) δH (mult., J in Hz): 5.69 (s, H-3), 7.06 (d, 7.2, H-5), 7.40 (t, 8.0, H-6), 7.22 (d, 8.0, H-7), 2.69 (s, H-8), 3.99 (s, 3-OMe); 13C-NMR (100 MHz, CDCl3) δC: 169.5 (C-2), 90.1 (C-3), 114.4 (C-3a), 137.1 (C-4), 127.6 (C-5), 131.6 (C-6), 115.3 (C-7), 154.7 (C-7a), 23.4 (C-8), 56.1 (3-OMe). HREI-MS m/z 178.0618 (calcd. for C10H10O3, 178.0630, Δ 6.7 ppm). EI-MS m/z 178 (5), 163 (10), 151 (5), 147 (10), 135 (10), 119 (25), 109 (20), 107 (30), 105 (28).

Figure 2. Compounds 1–3 isolated from J. feotidissima.
3.3.2. 4,9(α)-Dihydroxy-nardosin-6-en (2)

Amorphous white powder, [α]D: −74.0 (c 0.1, CHCl3). IR (NaCl) \(\nu_{\text{max}}\): 3429, 2964, 2935, 2850, 1695, 1645, 1471, 1456, 1394, 1265, 1219, 1178, 1030, 889 and 771 cm\(^{-1}\). 1H-NMR (CDCl3 400 MHz) \(\delta^H\) (mult., J in Hz): 1.98 (m, H-1a); 2.03 (m, H-1b); 1.54 (dd, 13.2, 7.2, 4.0, H-2a); 1.81 (ddd, 13.2, 3.6, 0.8, H-2b); 1.47 (bbd, 13.2, 4.0, H-3a); 1.67 (dd, 13.2, 3.6, 2.4, H-3b); 5.28 (dd, 3.6, 2.4, H-7); 1.78 (m, H-8a); 2.02 (m, H-8b); 3.25 (bbd, 11.6, 3.6, H-9); 1.25 (bbd, 11.6, 5.6, H-10); 2.11 (sept, 6.2, H-11); 0.96 (d, 6.2, H-12); 0.95 (d, 6.2, H-13); 1.12 (s, H-14); 0.89 (s, H-15). 13C-NMR (100 MHz, CDCl3) \(\delta^C\): 23.0 (C-1); 26.8 (C-2); 39.5 (C-3); 71.0 (C-4); 37.7 (C-5); 141.9 (C-6); 116.1 (C-7); 40.7 (C-8); 79.9 (C-9); 46.2 (C-10); 35.0 (C-11); 21.8 (C-12); 21.2 (C-13); 29.9 (C-14); 21.2 (C-15). HREI-MS \(m/z\) 238.1928 (calcd. for C15H26O2, 238.1933, Δ −2.2 ppm).

EI-MS \(m/z\) 238 (10), 220 (37), 187 (57), 161 (48), 159 (100), 145 (25), 119 (45), 107 (22).

3.3.3. 15-Hydroxy-8(17),13(E)-labdadiene-19-carboxilic acid (3)

Amorphous white powder. IR (NaCl) \(\nu_{\text{max}}\): 3471, 3086, 2852, 1722, 1695, 1645, 1471, 1456, 1385, 1269, 1219, 1180, 1030, 987, 893, 771 and 667 cm\(^{-1}\). 1H-NMR (CDCl3 400 MHz) \(\delta^H\) (mult., J in Hz): 1.06 (m, H-1a), 1.80 (overlapped, H-1b), 1.53 (m, H-2a), 1.88 (overlapped, H-2b), 1.02 (m, H-3a), 2.14 (m, H-3b), 1.32 dd (12.0, 2.8, H-5), 1.88 (overlapped, H-6a), 1.98 (m, H-6b), 1.89 (overlapped, H-7a), 2.39 (dd, 8.0, 2.8, H-7b), 1.56 (bd, 11.2, H-9), 1.52 (m, H-11a), 1.63 (m, H-11b), 1.78 (overlapped, H-12a), 2.17 (m, H-12b), 5.38 (t, 6.8, H-14), 4.15 (d, 6.8, H-15), 1.67 (s, H-16), 4.53 (bs, H-17a), 4.86 (bs, H-17b), 1.24 (H-18s), 0.60 (s, H-20); 13C-NMR (100 MHz, CDCl3) \(\delta^C\): 38.7 (C-1); 38.0 (C-2); 19.9 (C-3); 44.2 (C-4); 55.6 (C-5); 26.1 (C-6); 39.1 (C-7); 147.9 (C-8); 56.3 (C-9); 40.4 (C-10); 22.0 (C-11); 38.4 (C-12); 140.4 (C-13); 122.8 (C-14); 59.2 (C-15); 16.4 (C-16); 106.5 (C-17); 29.0 (C-18); 183.5 (C-19); 12.8 (C-20). HREI-MS \(m/z\) 320.2325 (calcd. for C20H32O3, 320.2351, Δ −8.2 ppm). EI-MS \(m/z\) 320 (10), 305 (20), 302 (21), 287 (23), 259 (17), 241 (15), 189 (28), 135 (21), 133 (27) 121 (68), 109 (28), 105 (26).

3.4. Cytotoxicity MTT assay

EJ-138 and CAOV-4 cancer cell lines from Pasteur Institute, Iran, were grown adherently in RPMI-1640 media, supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO2/95% air. Cells were seeded at 5000 cells per well in 5% CO2 at 37 °C in RPMI medium containing 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin, in 96-well plates. After overnight incubation, breast cancer cells were treated with different concentrations (1, 10, 20, 40, 80 and 100 μM) of compounds 1–3 for 48 h. Doxorubicin hydrochloride (Ebewe Pharma, Unterach, Austria) as standard drug was applied as a positive control at concentrations of 0.01, 0.10, 1.00 and 10.00 μM. MTT was added to the wells following with incubation for another 4 h at 37 °C. Each experiment was independently carried out in triplicates and the absorbance was read by the microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. Cell viability percentages using the formula: (mean OD of treated cells /mean OD of control cells) \times 100 were expressed as percent of control cells which were not treated (Zarei et al. 2013).

3.5. Statistical analysis

All data are reported as mean ± SD of the mean and the IC50 values were calculated using Excel based program. One-way ANOVA; post-hoc Dunnett test was used and \(p < 0.05\) was considered to indicate a statistically significant difference.
Acknowledgements
We are grateful to the Isfahan Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran and Shahrekord University of Medical Sciences, Shahrekord, Iran for their support.

Disclosure statement
No potential conflict of interest was reported by the authors.

ORCID
Mustafa Ghanadian http://orcid.org/0000-0001-6446-4734

References
Al-rhazes (Rhazes) M. 2005. Al Havi (Continents). Persian translation by Afsharipour S. Tehran: Academy of Medical Sciences Publication.

Baggerly KH, Erdtman H, Norin T. 1968. Some new cedrane derivatives from Juniperus foetidissima Willd.: configuration of cedrolic acid. Tetrahedron. 24:3399–3405.

Bahri F, Harrak R, Achak N, Romane A. 2013. Chemical composition and antibacterial activities of the essential oils isolated from Juniperus thurifera L. var Africana. Nat Prod Res. 27:1789–1794.

Cambie RC, Grigor BA, Mee-Ing T. 1981. Chemistry of the Podocarpaceae. LVI. Resin acids from Phyllocladus trichomanoides. Aust J Chem. 34:1073–1078.

El Sawi SA, Motawe HM. 2008. Labdane, pimarane and abietane diterpenes from the fruits of Juniperus phoenicea L. grown in Egypt and their activities against human liver carcinoma. Can J Pure Appl Sci. 2:115–122.

Emami SA, Asili J, Mohaghghiz Z, Hassanzadeh MK. 2007. Antioxidant activity of leaves and fruits of Iranian conifers. eCAM. 4:313–319.

Emami SA, Sadeghi-aliabadi H, Saeidi M, Jafarian A. 2005. Cytotoxic evaluations of Iranian conifers on cancer cells. Pharm Biol. 43:299–304.

Marcysiak K, Mazur M, Romo A, Montserrat JM, Didukh Y, Boratynska K, Jasinska A, Konsinski P, Boratynska, A. 2007. Numerical taxonomy of Juniperus thurifera, J. excelsa and J. foetidissima (Cupressaceae) based on morphological characters. Bot J Linn Soc. 155:483–495.

Muto N, Tomokuni T, Haramoto M, Tatemoto H, Nakanishi T, Inatomi Y, Murata H, Inada A. 2008. Isolation of apoptosis- and differentiation-inducing substances toward human promyelocytic leukemia HL-60 cells from leaves of Juniperus taxifolia. Biosci Biotechnol Biochem. 72:477–484.

Riedl H. 1968. Cupressaceae. In: Rechinger KH, editor. Flora Iranica. Graz: Akademische Druck-u. Verlagsanstalt; 50. p. 1–10.

Robichaud GA, Picot N, Jean S, Carpenter C, Gray CA. 2013. Abstract 2266: the characterization of anti-breast cancer compounds isolated from the Juniperus communis. Cancer Res. 73:2266–2266.

Sadeghi-aliabadi H, Emami A, Saidi M, Sadeghi B, Jafarian A. 2010. Evaluation of in vitro cytotoxic effects of Juniperus foetidissima and Juniperus sabina extracts against a panel of cancer cells. Iran J Pharm Res. 8:281–286.

Tunali Z, Kirimer N, Baser KHC. 2002. The composition of essential oils from various parts of Juniperus foetidissima. Chem Nat Compd. 38:43–47.

Zarei SM, Ayatollahi AM, Ghanadian M, Aghaei M, Choudhary MI, Fallahian F. 2013. Unusual ingenoids from Euphorbia erythradenia Bioss. with pro-apoptotic effects. Fitoterapia 91:87–94.