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Diterpenes from the berries of *Juniperus excelsa*

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Abstract

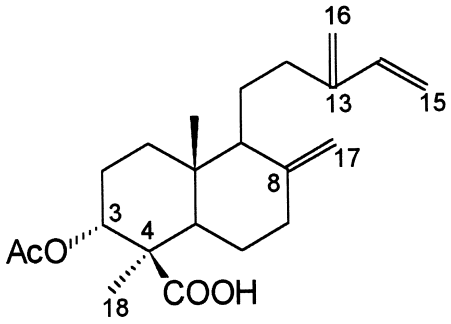
From the hexane extract of berries of *Juniperus excelsa*, one new and four known diterpenes were isolated besides a known sesquiterpene. The structures of the known diterpenes were identified as isopimaric, isocommunic, (−)*ent-trans* communic and sandracopimaric acids, along with the sesquiterpene 4a-hydroxycedrol and the new compound which was elucidated as 3α-acetoxylabda-8(17),13(16),14-trien-19-oic acid (juniperexcelsic acid). Cytotoxic activity of the hexane extract was investigated against a panel of cell line and found highyl active against LNCaP, KB-V (+VLB) and KB-V (−VLB) cell lines. Furthermore, the hexane and methanol extracts, and the new compound were found to be moderately active against *Mycobacterium tuberculosis*. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Juniperus excelsa; Cupressaceae; Berries; Diterpenes; Juniperexcelsic acid; Sesquiterpene; Cytotoxic; Antituberculosis and antibacterial activity

1. Introduction

Although 70 *Juniperus* species grow throughout the world (Krussmann, 1960), namely in the Balkan Peninsula, Saudi Arabia, Yemen, Oman, Iran, Afghanistan and Crimea (Kerfoot & Lavranos, 1984), only eight species are found in Turkey (Coode & Cullen, 1965; Baytop & Özocak, 1970). *Juniperus excelsa* is widely distributed in the Anatolian mountains. This plant is used locally as a traditional remedy for tuberculosis and jaundice in Saudi Arabia. In a recent study (Muhammad, Mossai, & El-Feraly, 1992), sandracopimaric acid isolated from *J. excelsa* was found to exhibit significant activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus durans*. An alcohol extract of *J. excelsa* showed acaricidal activity in plants (Tsitsin, Kovtunenko, Polyakov, & Khaidarov, 1973). Additional literature (Mishra & Agarwal, 1989) has revealed some pharmacological properties of *J. excelsa* extracts, including CNS depressant activity in albino rats.

In this study, we have isolated four known and one new diterpene in addition to a known sesquiterpene from the berries of *J. excelsa* growing in Anatolia. The new compound had a labdane skeleton which was elucidated as 3α-acetoxy-labda-8(17),13(16),14-trien-19-oic acid (**1**). The known diterpenes were identified as iso-



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communic acid (**2**) (Atkinson & Crow, 1970), (−)*ent-trans* communic acid (**3**) Blechschmidt & Becker, 1992), sandracopimaric acid (**4**) (Edwards, Nicholson, & Rodger, 1960; Wenkert & Buckwalter, 1972), isopimaric acid (**5**) (De Pascual Teresa, Barrero, Muriel, San Feliciano, & Grande, 1979) and the known sesquiterpene was identified as 4α-hydroxycedrol (Kuo, Wu, Cheng, & Wang, 1990; Joseph-Nathan, Santillan, & Gutierrez,

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1984) (**6**), by spectral methods including IR, UV, MS, and NMR. The hexane and methanol extracts and compound **1** were tested against *Mycobacterium tuberculosis* and found to be moderately active. The hexane extract was evaluated against a number of cell line and found highly active against KB-V (+VLB) (2.5 μg ml^{-1}), KB-V(−VLB) (2.5 μg ml^{-1}), and LNCaP (1.3 μg ml^{-1}) while methanol extract and compound **1** were only tested against cultured KB and P-388 cells and the latter indicated a week cytotoxic response with KB cells.

2. Results and discussion

The HREIMS of compound **1** gave a molecular ion peak at m/z 360.229996 corresponding to a molecular formula $C_{22}H_{32}O_4$ (calcd 360.230060) The ^{13}C NMR spectrum displayed 22 signals indicated four methine, nine methylene, three methyl and six quaternary carbon atoms by APT experiments. The signals at δ 170.32 and δ 182.07 were attributed to the presence of acetyl and acid groups which were supported by the IR spectrum demonstrating absorptions at 1735 and 1250 cm^{-1} (acetyl), 1705 and 2500–2600 cm^{-1} (shoulder) (acid). The UV spectrum gave a maximum at 243 nm indicating some conjugation in the molecule. The presence of one exocyclic and two terminal methylene groups followed from the ^{13}C NMR spectrum observing the signals at δ 115.49, 113.19 and 106.79, as well as the three pairs of methylene signals in the ^{1}H NMR spectrum at δ 4.59, 4.89 (each 1H, br s, H-17, H-17′), δ 4.99, 5.00 (each 1H, br s, H-16, H-16′) and δ 5.06 (1H, brd, J=10.5 Hz, H-15), 5.21 (1H, brd, J=17.5 Hz, H-15′) (Table 1). The methyl signals were observed at δ 0.60, 1.21 and 2.09 as singlets, the latter one was indicative of acetyl methyl protons. All the ^{1}H NMR data suggested a labdane diterpene structure for **1**. For the location of the acid group in the structure, most plausible place is C-19, as expected biogenetically. As for the acetyl group, C-1, C-3, C-7 and C-12 should be considered based on the multiplicity, but, the most significant data obtained from the ^{13}C NMR chemical shift of one of the methyl carbons which was observed at δ 23. In the presence of an α-hydroxyl or acetyl group at C-3, the α-methyl group at C-4 would have resonated around δ 21–23 in the ^{13}C NMR spectrum as observed in similar diterpenes isolated from *Juniperus communis* (Kagawa et al., 1993) and isolated from some other plants (Gonzalez et al., 1990; Blechschmidt & Becker, 1992). Otherwise, only the presence of a substituent at C-4, either as C-18 or C-19, the methyl carbon at the same carbon (C-4) would not be shifted downfield less than δ 27. In labdane diterpenes, without a C-3 substituent and the presence of a β-carboxyl at C-4, the α-methyl group of C-4 can be observed at around δ 28 while in the case of no substituent either at C-3 or C-4, the α-methyl (C-18) should be observed at around δ 33 (Barrero & Altajeros, 1993). In fact, the chemical shifts of the six methylene carbons also indicated the location of the acetyl group. In the absence of a substituent at C-3, the chemical shift of C-1 should be observed at δ 38–41 while C-2 would be at 17–20 δ, but the presence of an acetyl group at C-3 caused an β-effect on C-2 and a γ-effect on C-1 thus giving the C-2 signal at δ 24.3 and the C-1 signal at δ 32.6. Therefore, all the ^{13}C NMR assignments of ring A revealed the presence of the acetyl group at C-3 and the narrow triplet at δ 5.30 was assigned the α-position of the acetyl group. The α-orientation of the acetyl group at C-3 was supported by NOE difference experiments, an irradiation at δ 5.30 caused an enhancement on the methyl signal at δ 1.21 while no NOE enhancement was observed between the δ 5.30 and δ 0.60 signals. A HETCOR experiment revealed direct correlations between protons and carbons. The COLOC experiment confirmed the location of the acetyl group at C-3 exhibiting three bond correlation between H-3 and C-19, as well as H-3 and C-18, another three bond correlation was also observed between H-3 and C-1, and a two bond correlation between H-3 and C-2. Thus, the COLOC experiment also supported the location of the acid group at C-4. All the spectral data indicated that compound **1** has the structure 3α-acetoxylabda-8(17),13(16),14-trien-

Table 1
^{1}H and ^{13}C NMR data of 1 in $CDCl_3$[a]

Position	δH	δC
1	–	32.7 t
2	–	24.3 t[b]
3	5.29 t (2.5 Hz)	73.2 d
4	–	47.1 s
5	–	50.0 d
6	–	25.4 t[b]
7	–	38.5 t
8	–	147.6 s
9	–	55.6 d
10	–	40.1 s
11	–	22.6 t
12	–	30.3 t
13	–	147.0 s
14	6.36 dd (17.5, 10.5 Hz)	139.0 d
15	5.21 br d (17.5 Hz)	115.5 t
15′	5.06 br d (10.5 Hz)	–
16	5.00 br s	113.2 t
16′	4.99 br d	–
17	4.89 br s	106.8 t
17′	4.59 br s	–
18	–	23.8 t
19	1.21 s	182.1 q
20	0.60 s	12.4 q
C=O	–	170.3 s
CH_3	2.09 s	21.2 q

[a] J values are given in parentheses.
[b] Assignments are interchangeable.

Table 2
Cytotoxic activity results of the extracts and compound **1** (μg ml^{-1})

	P_{388}	KB	KB-V (+VLB)	KB-V (−VLB)	LNCaP	BCI	LUI	COL2	ASK9
Hexane extract	>5	1.9	2.5	2.5	1.3	>20	>20	5.0	+
Methanol extract	>5	10.5	NT[a]	NT	NT	NT	NT	NT	NT
Juniperexcelsic acid (**1**)	>5	>20	NT	NT	NT	NT	NT	NT	NT

[a]NT means not tested.

19-oic acid and was given the trivial name juniperexcelsic acid.

The hexane extract was evaluated with a number of cancer cell lines and found to be particularly active against human colon cancer cell line (LNCaP) ($ED_{50}=1.3$ μg ml^{-1} as well as KB-V(+VLB) ($ED_{50}=2.5$ μg ml^{-1}) and KB-V(−VLB) ($ED_{50}=2.5$ μg ml^{-1}), while compound **1** and methanol extract were only tested with cultured KB and P388 cells, and the latter indicated a week cytotoxic response with KB cells ($ED_{50}=10.5$ μg ml^{-1}). The hexane extract also showed a positive response to ASK 9 glioma cell (Table 2).

On the other hand, the hexane and methanol extracts, as well as the new compound, juniperexcelsic acid, were tested against *M. tuberculosis* and found to be moderately active with MIC values 15.5, 17.0 and 14.4 μg ml^{-1}, respectively (Table 3). In the same study, the known pimarane diterpene sandracopimaric acid (**4**) and a well known labdane diterpene sclareol were also tested against *M. tuberculosis*, and their MIC values were found as 15.0 and 6.0 μg ml^{-1}, respectively. Furthermore, the hexane and methanol extract were tested against a number of standard bacteria and methanol extract was found to be more or less active against all bacteria cell lines, particularly *B. subtilis*, *S. aureus* and *S. epidermidis* while the hexane extract showed some activity against only *B. subtilis* and *S. aureus* (Table 4).

3. Experimental

3.1. General

UV: Varian Techtron 685 in EtOH. IR: Perkin Elmer 980 in $CHCl_3$, ^{1}H and ^{13}C NMR: Bruker AC 200 L instrument 200 and 50.32 MHz, respectively with TMS as int standard; HRMS: VG Zab Spec GC–MS spectrometer.

3.2. Plant material

The berries of *J. excelsa* M. Bieb. were collected from Isparta, south-western Turkey. A voucher specimen (AEF 19787) was deposited at the Herbarium of the Faculty of Pharmacy, University of Ankara.

3.3. Extraction and isolation

Crushed plant material (270 g) was extracted with hexane, the extract was evapd in vacuo to give 15 g of a residue. The latter was fractionated on a silica gel column (5.5×70 cm) eluting with hexane, followed by a gradient of $CHCl_3$ up to %100 and then EtOH. After TLC application, similar frs. were combined and further separated on smaller silica gel columns and purified on prep. TLC plates. The following compounds were obtained: **1** (25 mg), **2** (8 mg), **3** (6 mg), **4** (41 mg), **5** (17 mg) and **6** (12 mg).

3.3.1. 3α-acetoxylabda-8(17),13(16),14-trien-19-oic acid (juniperexcelsic acid) (*1*)

$[\alpha]_D^{25}$ −21.5°C (MeOH; *c* 0.5). IR $\nu_{max}^{CHCl_3}$, cm^{-1}: 3420, 2920, 2500–2600 (sh), 1735, 1705, 1640, 1595, 1465, 1450, 1380, 1250, 1035, 900, 760. UV λ_{max}^{MeOH} (log ε) nm: 243(3.7). ^{1}H and ^{13}C NMR: Table 1. HREIMS *m/z*: 360.229996 $[M]^+$ (calcd 360.230060 for $C_{22}H_{32}O_4$); EI-MS *m/z* (rel.int): 360 $[M]^+$ ($C_{22}H_{32}O_4$) (0.1), 300 $[M–AcOH]^+$ (24), 285 $[M–AcOH–Me]^+$ (16), 255 [300-COOH]$^+$ (9), 121 (30), 105 (25), 9 (29), 87 (43), 85(89), 83(100).

3.4. Cytotoxic activity test

The MeOH extract and compound **1** were evaluated with cultured KB cells as described previously (Likhitwitayawuid, Angerhofer, Ruangrungsi, Cordell,

Table 3
Antituberculous activity test against *Mycobacterium tuberculosis*

	Hexane extract	Methanol extract	Juniperexcelsic acid	Sandracopimaric acid	Sclareol
Mycobacterium tuberculosis (μg ml^{-1})	15.5	17.0	14.4	15.0	6.0

Table 4
Antimicrobial activity of the extracts and sandracopimaric acid[a]

	B. subtilis	*S. aureus*	*S. epidermidis*	*B-hem. Streptococcus*	*Enterococcus*	*E. coli*	*P. mirabilis*	*Kl. pneumonia*	*Ps. aureginosa*	*C. albicans*
Hexane extract	22	23	16	22	17	20	24	22	26	19
Methanol extract	32	32	33	25	18	25	25	27	26	27
Sandraco-pimaric acid	20	25	20	27	22	22	25	26	23	27
Chloroform[b]	0	13	17	23	20	22	23	22	20	22
Methanol[b]	23	20	22	20	19	24	24	22	21	22

[a]The results are given in mm as zone diameter by disc-diffusion method.
[b]Hexane extract and sandracopimaric acid were dissolved in chloroform while methanol extract was dissolved in methanol.

& Pezzuto, 1993) while the hexane extract was evaluated with BC1, LU1, COL-2, KB-V (+VLB), KB-V (−VLB), P388, LNCaP and ASK cell lines.

3.5. Antituberculosis activity test

The broth microdilution method was used (Wallace, Nash, & Steele, 1986; Cooksey, Crawford, Jacobs, & Shinnick, 1993).

M. tuberculosis H37Rv std. strain was passaged into screw capped tubes in broth medium 7H9 middlebrook. They were left at room temperature for 3 weeks. After observation of the growth, they were vortexed and the additional suspension was transferred to tubes and adjusted according to Mac Farland turbidity standards with in 96-well middlebrook 7H9 broth medium. This suspension was used for MIC determinations; 50 ml H37Rv std. strain was added to all wells. Microplates were left for three weeks and MIC values were determined for the lowest concentration where growth was not observed. The same test was carried out with chloroform as control.

3.6. Antimicrobial activity test

The disc-diffusion method (Colee, 1976; Sleigh & Timburg, 1981) was used to determine the inhibition zones of the hexane and methanol extracts and sandracopimaric acid. The plates were kept overnight in the incubator at 37°C and the sizes of the inhibiton zones were measured. Samples that had inhibition zones greater than 7 mm compared to control solvent were considered to select for tube dilution tests (Kavanagh, 1963), MeOH and $CHCl_3$ were used as control. But, the tube dilution test could not be realized due to an unexpected accident. Standard bacterial strains *S. aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Proteus mirabilis* ATCC 14153, *Klebsiella pneumonia* ATCC 4352, *Pseudomonas aeruginosa* ATCC 1539, beta-hemolytic *Streptococcus* (clinical isolate), *Enterococcus* (clinical isolate), and a yeast *Candida albicans* (ATCC 10231) were used.

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