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Full Length Research Paper

Comparative analysis of essential oil contents of *Juniperus excelsa* (M. Beib.) found in Balochistan, Pakistan

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Cones/berries of *Juniperus excelsa* from three provenances in Balochistan, Pakistan were collected and essential oil was extracted by solvent method. Oil contents were analyzed on gas chromatography mass spectrometry (GCMS). Identification and quantification was made by using Wiley and NIST spectral library and HP Chemstation software. Quantitatively the mean cone oil yield was 5.8, 6.5 and 4.5% for Ziarat, Zarghoon Ghar and Harboi District Kalat, respectively. Ziarat being the richest in number of compounds while Harboi in compound diversity. Quantitative and qualitative similarities were found among three locations. Ziarat was found to be rich in α-pinene, cedrol, camphene, copaene, phyllocladene, ferruginol, podocarp-7-en-3-one, and pimara-8(14) 15-dien. Zarghoon Ghar was found to be rich in α-pinene, limonene, germacrene d, widdrol, phyllocladene, ferruginol, androst-4-ene-3,6-dione and Harboi was represented by α-pinene, β-myrcene, limonene, cedrene, clovene, cadinene, patchoulene, cedrol, spathulenol, abietatriene, norkaur, pimaric acid and neoabietic acid. In all, 99 compounds were reported for the first time from the oil of cones of *J. excelsa* in Balochistan.

Key words: *Juniperus excelsa*, essential oils, GCMS, Balochistan.

INTRODUCTION

The genus Juniperus belongs to the family Cupressaceae consisting of 55 species, all of which occur throughout the northern hemisphere of the world (Farjon, 1998) except *Juniperus* procera which is the only species of the genus that grows naturally in the southern hemisphere (Adams et al., 1993). In Balochistan, *J. excelsa* has natural stands distributed between 20°9'N and 30°37'N and between 67°1'E, as well as in some isolated dry valleys (Rafi, 1965). There are three spatially segregated pockets of Juniper forests, namely Zarghoon, Ziarat and Harboi (in districts Quetta, Ziarat and Kalat respectively). Balochistan has approximately 141,000 hectares (ha) of J. excelsa forests, out of which about 86,000 ha are found in Ziarat and Loralai districts. *J. excelsa* trees typically grow as pure stands, and form characteristically open and multistoried forests between elevations of 2000 to 3000 m (Sheikh, 1985). Conifers are known as a renewable source of essential oil. Oil contents are believed to be genetically determined and little influenced by the environment. Besides having economic value, oil contents play an important role in plant defense system against fungus and insect attacks (Koukos et al., 2001, 2002). In addition to genetic makeup, the oil composition of junipers has also been reported to change due to inter-specific differences (Medini et al., 2010; Almaarri et al., 2010; Adams et al., 1993; Adams, 2001). The wood of juniper is used for fuel, beams and for pencil making. The wood and cone (berries) are also used as incense. Cone of *J. excelsa* is traditionally used as a medicine by the local people (WWF, 1998). Juniper cone oil is known to be an antiseptic, analgesic and sedative and has been

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reported to cure tuberculosis, jaundice, and eczema as well as for so many other ailments (Adams, 1987; Ucar and Balaban, 2002; Marina et al., 2004; Unlu et al., 2008; Derwich et al., 2010; Orav et al., 2010). Variations in essential oil contents of conifer species have been reported by many investigators; however, no work has been conducted to investigate the cone oil contents of *J. excelsa* in Balochistan. The purpose of this study was to investigate the composition of cone oil contents of *J. excelsa* and compare the quantitative and qualitative differences among the three different natural stands of juniper forests.

MATERIALS AND METHODS

Plant material and extraction

Cones were collected from the juniper forests of Ziarat valley, Zarghoon Ghar and Harboi, located in three districts of Balochistan, Pakistan namely Ziarat, Quetta, and Kalat respectively. Samples were collected from healthy mature trees having bluish-black ripe cones in fall October 2006. Ten trees were randomly selected in each location and approximately 200 cones were collected from all sides of the crown. Seeds were manually separated from the pulp. The oil was isolated from crushed dried juniper cones (100 g) by steam distillation and extraction (SDE) with n-hexane (Fluka >99.0%) as solvent (0.5 ml), using a Marcusson type micro-apparatus (Bicchi et al., 1990). The SDE process was carried out for 2 h. The oil amount (%) was determined using n-tetradecane (Reachim >99.9%) as the internal standard (2 µL). The reproducibility of three parallel SDE procedures with a single juniper sample showed the variation coefficients were below 20%.

Chemical analysis: GC and GC-MS Analysis

For GC and GC-MS, SPB-5 and DB-5 MS columns (30 m length, 0.32 mm ID and 0.22 µm df) were used respectively. Both GC and GC-MS analysis were performed with the identical gradient thermal ramping and temperatures as follows: the column was kept at 50°C for two min, and then ramped to 260°C at a rate of 5°C per sec. In case of GC, injector and detector were kept at 260°C. For GC-MS, injector was kept at 260°C, transfer line kept at 280°C and EIMS was operated at 70 eV, at 250°C. Helium (He) was used as carrier with a flow of 1.8 ml/min. Five microliter (5 µL) of filtered sample was injected at a split ratio of 1:40.

GC-FID was performed on Shimadzu (Japan) GC-17A coupled with Class GC-10 software while GC-MS was performed on an Agilent (USA) GC 6890 coupled with Jeol (Japan), 600H MS. For the spectral library search, NIST 2008 was used. The quantization was performed using area normalization method on Class GC-10 software. All chemicals used were of laboratory grade.

Statistical analysis

Pearson correlation tests were run to describe the degree of correlation between the essential oil chemical composition of cones from the three populations. IBM-SPSS 19 was used to run the tests.

RESULTS

The chemical composition of essential oils from cone of *J. excelsa* is listed in Table 1. Ninety nine compounds with their relative mean percentage at three different locations are shown in Table 1. The chemical compounds of oils have been grouped and the percentage of different groups of compounds in three spatially segregated populations is presented in Table 2. The cone oils of *J. excelsa* consist mainly of monoterpenes, sesquiterpenes, diterpenes and oxygenated hydrocarbons. Chemical content differed markedly between populations. The monoterpene hydrocarbons constituent of oils is 21.08, 3.02 and 19.4% at Ziarat, Zarghoon Ghar and Harboi populations, respectively. Among monoterpenes, α-pinene was 15.92% in the oil content of Ziarat and Harboi populations while its presence in the samples from Zarghoon Ghar was 1.70%. Sesquiterpenes were found to be 4.93% at Ziarat, 3.67% at Zarghoon Ghar and 5.12% at Harboi. Among sesquiterpenes, most abundant compound in the oils of Ziarat was α-cedrene which was (2.11%), while at Zarghoon Ghar it was germacrene D (1.24%) and at Harboi it was patchoulene (1.76%). The percentage content of diterpene oils was 17.79, 16.16 and 23.82 at areas of Ziarat, Zarghoon Ghar and Harboi, respectively. Most abundant compound among diterpenes was phyllocladene, which was noted 2.57, 3.97 and 10.73% at Ziarat, Zarghoon Ghar and Harboi areas, respectively.

Oxygenated hydrocarbons both oxygenated sesquiterpenes and oxygenated diterpenes contributed 33.84% in Ziarat, 24.64% in Zarghoon Ghar and 28.6% in Harboi. The compound cedrol was most abundant in Ziarat and Harboi sites with (8.50%) and (8.63%) respectively while androst-4-ene-3, 6-dione with (8.81%) was most abundant in cones from Zarghoon Ghar. Other quantitatively significant oxygenated hydrocarbons included bornyl acetate, 3-hexyloxyacetophenone, nonane, n-hexadecanoic acid, Ferruginol, podocarp-7en-3-one-13β-methyl-13-vinyl-, pimara-8(14), 15-dien, neoabietic acid and pimaric acid.

Twelve compounds were present exclusively in cone oils from Ziarat area. These compounds were β-pinene, camphene, α-cedrene, copaene, 3-hexyloxyacetophenone, dodecane, 8βH-cedran-8-ol, nonane, tretinoin, verticiol, 6-methoxy-benzo[c] phenanthrene, indan, and 2-butyl-5-hexyl (Table 1). While 13 compounds were unique to the cone oil of Zarghoon Ghar which included calarene, cubenol, guaiene, elemene, germacrene D, thujopsene-(12), guaia-1(5),7(11)-diene, cyprene, cyclosativene, undec-1-ene, octahydrophenanthrene, 4-epiabietal dehydro, cholest-7en-3-ol-15-one,14-methyl. Whereas ten compounds were unique to cone oil from Harboi area, these included terpinene α, β-myrcene, clovene, cadinene, patchoulene, spathulenol, chrysene, pimaric acid, neoabietic acid and α -phenanthrene.

Finally, there were nine compounds found on all the three locations these compounds were: α-Pinene, limonene, n-hexadecanoic acid, phyllocladene(-)-, norkaur-15-ene,13,methyl-, (8β,13β), ferruginol, Podocarp-7en-3-one-13β-methyl-13-vinyl-, 9(1H)-phenanthrenone, and pimara-8(14),15-dien.

Table 1. Composition of essential oil *Juniperus excelsa* cone extracts as percentage of total peak area.

S/N	Group	Compound	R.T	Population		
				Ziarat	Zarghoon Ghar	Harboi
1		α-Pinene	4.43	15.92	1.70	15.92
2		β-Pinene	5.38	1.48	n. d.	n. d.
3		Camphene	5.85	2.96	n. d.	n. d.
4	Monoterpenes	Limonene	6.77	0.60	1.32	1.46
5		α-Terpinene	5.38	n. d.	n. d.	0.81
6		β-Myrcene	5.80	n. d.	n. d.	1.09
7		n-Butyl butyrate	10.18	0.12	n. d.	0.12
8		Cedrene	17.17	0.92	n. d.	1.73
9		Himachala-2,4-diene	17.37	0.46	0.17	n. d.
10	Sesquiterpenes	Copaene	22.97	0.06	n. d.	n. d.
11		α-Cedrene	18.93	2.11	n. d.	n. d.
12		Calarene	16.72	n. d.	0.07	n. d.
13		Elemene	17.75	n. d.	0.28	n. d.
14		Germacrene D	18.9	n. d.	1.24	n. d.
15		Thujopsene-(12)	20.73	n. d.	0.92	n. d.
16		Guaia-1(5),7(11)-diene	21.20	n. d.	0.32	n. d.
17		Cycloisolongifolene	23.02	0.38	0.11	n. d.
18		Cyprene	20.70	n. d.	0.45	n. d.
19		Cyclosativene	19.93	n. d.	0.11	n. d.
20		Clovene	17.37	n. d.	n. d.	0.47
21		Cadinene	18.90	n. d.	n. d.	1.16
22		Patchoulene	20.78	n. d.	n. d.	1.76
23		8βH-cedran-8-ol	21.78	1.00	n. d.	n. d.
24		14-Pentaene	29.27	1.83	n. d.	0.85
25		Cupressene	30.38	0.27	2.60	n. d.
26		Phyllocladene,(-)-	31.62	2.57	3.97	10.73
27		Norkaur-15-ene,13,methyl-,(8β,13β)-	32.77	1.00	1.21	2.94
28		Abietatriene	31.12	n. d.	0.80	2.21
29		Octahydrophenanthrene	31.12	n. d.	0.44	n. d.
30		Indan,2-butyl-5-hexyl-	31.27	1.70	n. d.	n. d.
31		Preg-17(20)-ene	33.42	0.49	0.18	n. d.
32		10-Methyldotriacontane	54.03	n. d.	0.35	0.46
33		a-Phenanthrene	28.15	n. d.	n. d.	0.17
34		Chrysene	36.95	n. d.	n. d.	0.55
35		Abieta-8,11,13-trien-	40.98	n. d.	0.61	0.50
36		Unidentified Diterpene (MW=220)	24.43	n. d.	0.12	n. d.
37	Diterpenes	Unidentified Diterpene (MW = 275)	29.88	n. d.	n. d.	0.39
38		Unidentified Diterpene (MW = 316)	38.22	1.95	n. d.	n. d.
39		Unidentified Diterpene (MW = 316)	38.75	1.79	n. d.	n. d.
40		Unidentified Diterpene (MW = 316)	38.85	1.79	n. d.	n. d.
41		Unidentified Diterpene (MW = 329)	39.90	n. d.	n. d.	0.60
42		Unidentified Diterpene (MW = 329)	41.02	0.33	n. d.	n. d.
43		Unidentified Diterpene (MW = 330)	38.57	1.08	n. d.	n. d.
44		Unidentified Diterpene (MW = 330)	39.57	0.65	n. d.	n. d.
45		Unidentified Diterpene (MW = 330)	38.37	0.09	n. d.	n. d.
46		Unidentified Diterpene (MW = 330)	38.63	n. d.	1.60	n. d.
47		Unidentified Diterpene (MW = 330)	40.98	n. d.	0.61	0.50
48		Unidentified Diterpene (MW = 330)	39.55	n. d.	2.12	n. d.
49		Unidentified Diterpene (MW = 330)	38.52	n. d.	n. d.	1.49
50		Unidentified Diterpene (MW = 330)	38.33	n. d.	n. d.	0.06
51		Unidentified Diterpene (MW = 393)	54.08	0.42	n. d.	n. d.

MW= Molecular Weight; n. d. = Not detected; R.T= Retention Time.

Table 1. Contd.

52		Unidentified Diterpene (MW = 406)	48.67	1.83	n. d.	n. d.
53		Unidentified Diterpene (MW = 406)	48.62	n. d.	1.55	n. d.
54		Unidentified Diterpene (MW = 406)	48.70	n. d.	n. d.	2.37
55		Bornyl acetate	13.92	1.00	n. d.	0.84
56		Dodecane	19.82	0.79	n. d.	n. d.
57		Cubenol	17.13	n. d.	0.15	n. d.
58		Guaiene	17.32	n. d.	0.17	n. d.
59		Widdrol	21.82	n. d.	1.94	0.57
60		3-Hexyloxyacetophenone	20.75	1.56	n. d.	n. d.
61		Cedrol	21.98	8.50	n. d.	8.63
62		Spathulenol	22.52	n. d.	n. d.	0.41
63		Undec-1-ene	25.47	n. d.	0.18	n. d.
64		Lanceol,cis	22.52	0.54	0.20	n. d.
65		Selina-6-en-4-ol	23.82	n. d.	0.44	0.66
66		Nonane	24.9	1.51	n. d.	n. d.
67		n-Hexadecanoic acid	29.70	0.79	0.20	1.35
68	Oxygenated hydrocarbons	6-Methoxy-benzo[c] Phenanthrene	29.97	0.93	n. d.	n. d.
69		Ferruginol	34.33	1.79	1.68	1.46
70		Verticiol	35.00	0.44	n. d.	n. d.
71		Podocarp-7en-3-one-13β-methyl-13-vinyl-	35.65	2.03	3.44	3.51
72		Androst-4-ene-3,6-dione	36.65	8.18	8.81	n. d.
73		Pimara-8(14),15-dien	37.8	4.31	3.92	5.85
74		Kaur-16-ene,(8β,13β)-	31.53	0.50	n. d.	0.50
75		Androstadien-17β-ol-3-one	34.15	0.25	0.78	n. d.
76		Tretinoin	39.55	0.09	n. d.	n. d.
77		9(1H)-Phenanthrenone	40.78	0.63	1.35	0.23
78		Totarol	33.42	n. d.	0.61	0.60
79		4-epiabietal,dehydro	34.83	n. d.	0.67	n. d.
80		Cholest-7en-3-ol-15-one,14-methyl-	53.42	n. d.	0.10	n. d.
81		Neoabietic acid	38.22	n. d.	n. d.	2.77
82		Pimaric acid	35.95	n. d.	n. d.	1.22
83		Unidentified (MW = 286)	35.33	3.72	14.28	n. d.
84		Unidentified (MW = 286)	35.53	0.31	n. d.	n. d.
85		Unidentified (MW = 286)	35.53	n. d.	4.17	n. d.
86		Unidentified (MW = 286)	36.52	n. d.	1.61	n. d.
87		Unidentified (MW = 286)	36.15	n. d.	0.16	n. d.
88		Unidentified (MW = 286)	35.25	n. d.	n. d.	0.84
89		Unidentified (MW = 286)	36.16	n. d.	n. d.	0.61
90	Unidentified compounds	Unidentified (MW = 288)	35.92	0.73	n. d.	n. d.
91		Unidentified (MW = 302)	36.18	0.55	n. d.	n. d.
92		Unidentified (MW = 302)	37.18	4.58	n. d.	n. d.
93		Unidentified (MW = 302)	36.95	0.90	n. d.	n. d.
94		Unidentified (MW = 302)	38.07	0.31	n. d.	n. d.
95		Unidentified (MW = 302)	38.33	n. d.	4.15	n. d.
96		Unidentified (MW = 302)	35.98	n. d.	1.50	n. d.
97		Unidentified (MW = 302)	36.25	n. d.	1.34	n. d.
98		Unidentified (MW = 302)	37.37	n. d.	8.42	n. d.
99		Unidentified (MW = 302)	38.33	n. d.	4.15	n. d.
			Total	88.74	87.27	78.39

Table 2. Percentage of different groups of compounds in cone oil of *Juniperus excelsa* at three locations.

Group	Ziarat	Zarghoon Ghar	Harboi
Monoterpenes	21.08	3.02	19.4
Sesquiterpenes	4.93	3.67	5.12
Diterpenes	17.79	16.16	23.82
Oxygenated hydrocarbons	33.84	24.64	28.6
Unidentified	11.1	39.78	1.45

Pearson correlation tests show that Ziarat and Harboi populations were most similar in cone oil chemical composition ($r=0.635$, $p<0.001$). Ziarat and Zarghoon Ghar were also significantly correlated but with a lower correlation coefficient ($r=0.270$, $p=0.007$). Finally Harboi and Zarghoon Ghar cone oil chemical compositions were not significantly correlated ($r=0.066$; $p=0.515$).

DISCUSSION

The chemical composition of cone oil contents of *J. excelsa* revealed that α-pinene and limonene coexisted in all the three sites. Nevertheless in the oil of Zarghoon Ghar, α-pinene content was much lower β-pinene and camphene were found in Ziarat and missing in the samples of other two sites. Monoterpene content of oils of Zarghoon Ghar was very low as compared to the other two sites (Table 2). Adams (1990) reported that α-pinene and limonene were the dominant essential oils in *J. excelsa* collected from Greece. Again Adams (1999) reported almost similar quantities of α-pinene (15.5%) in *J. excelsa* samples of Ziarat area; however these oils were extracted from the leaves. It is worth mentioning that there is a great diversity of pure sesquiterpenes and out of sixteen compounds only three compounds are common to two sites. Another pattern which is evident from the results of oils of *Juniperus excelsa* is that oxygenated hydrocarbons are dominating at all the three sites (Table 2). Adams et al. (1993) also reported similar results while comparing the terpenoids of *J. excelsa* and *Juniperus procera*. It is interesting to mention that nine compounds common to all the three sites also possessed remarkable quantitative differences. Adams et al. (2003) have also reported quantitative variation in the leaf oils of *Juniperus thurifera* populations from Morocco and Europe. Quantitative and qualitative differences in the cone oil of *J. excelsa* found at three different locations in Balochistan Pakistan suggest that there has been a long-time held effective geographic isolation between populations.

Ziarat and Zarghoon Ghar juniper forests are located adjacent to each other; however, there is more compound similarity between Ziarat and Harboi which are almost 210 km apart from each other. This suggests a closer genetic relationship between these distant populations than between the closer ones. Particularly intriguing is the dissimilarity in essential oil composition between the adjacent populations of Ziarat and Zarghoon Ghar, which suggests genetic isolation between the populations.

Compound diversity among the three locations and the compounds unique to each location call for further investigation and determination of phylogenetic relationship of the different populations on the genomic level and on the possible ecological causes of these differences. Different chemical compositions could respond to different chemical defense needs against a different array of insects, which calls for further investigation on differences between populations on the insect community feeding on juniper cones. Given the economic and medicinal significance of essential oils of *J. excelsa*, there is potential for future exploitation of this forest resource.

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