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Chemotypic variations and phytotoxic studies of essential oils of endemic medicinal plant, *Seriphidium kurramense*, from Pakistan

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Seriphidium kurramense is an endemic and economic medicinal plant growing in a small narrow valley of tribal region in Pakistan, Upper Kurram Agency, at Pakistan-Afghanistan border. GC-MS analysis of essential oils of *S. kurramense* identified 28 chemical constituents from 16 populations with two distinct chemotypes, A and B. Both chemotypes represented similar morphological features. Major chemical constituents of chemotype A comprising 12 populations, consisted of α -thujone (26.0 - 73.4 %), β -thujone (3.14 - 49.3 %), 1,8-cineole (10.2 - 22.3 %) and camphor (0 - 26.3 %), while the major chemical constituents of chemotype B from four populations were *p*-menthan-1,8-diol (23.1 - 35.2 %), 1,8-cineole (10.2 - 23.8 %) and an unknown chemical compound (12.5 - 25.9 %). Significant differences in toxicity levels were observed in all the 16 populations of *S. kurramense*. Chemotype B showed highest root and Hypocotyle inhibition of lettuce seeds as compared to Chemotype A.

Key words: *Seriphidium kurramense*, endemic, GC-MS, chemotypic variation, Kurram Agency, Pakistan, phytotoxic.

INTRODUCTION

Seriphidium kurramense is an endemic medicinal plant species of upper Kurram Agency and adjacent Afghanistan border (Ghafoor, 2002). Besides its importance as endemic species, it is also a medicinal and economic plant utilized by local communities of the Agency. It is also collected and transported to other parts of the country for santonin extraction. *S. kurramense* (Qazilb.) Y. R. Ling, is also reported with other synonyms, *Artemisia maritima* L. (Evans, 1989; Miraldi et al., 1998) and *Artemisia kurramensis* Qazilb. (Qazilbash, 1942).

The study was also conducted earlier to screen out 81 medicinal plants of Pakistan for their phytotoxic activities

(Gilani et al., 2009a). These results showed that *S. kurramense* remained among top three species with highest inhibitory effects on roots, Hypocotyle and seed germination of lettuce seeds (Gilani et al., 2009a). However, the phytotoxic studies on essential oils of *S. kurramense* are not reported yet. Earlier studies on GC-MS analysis of essential oils of *Artemisia verlotiorum* in France showed α -thujone, 1,8-cineole, β -caryophyllene and β -thujone as major chemical constituents (Juteau et al., 2005). It was also observed that *Artemisia vulgaris* collected from Croatia showed different chemical pattern with larger chemical variations in essential oil contents when these were subjected to GC-MS (Jerkovic et al., 2003). *S. kurramense* once was categorized in *Artemisia* genus. It was supposed that the species may also contain the same pattern of essential oil contents with chemical variations. The current study focuses on GC-MS analysis

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of essential oils of various populations of *S. kurramense* and phytotoxic studies of the essential oils.

MATERIALS AND METHODS

Plant collection

The plant materials were collected from 16 focal villages which were shade dried and packed in silica gel in zipped plastic bags. Voucher specimens were submitted at Department of Botany, Kohat University of Science and Technology (KUST), Kohat, Pakistan.

Essential oil extraction

The dried plant material was crushed into powder. The total amount of 30 - 50 g was used for essential oil extraction. Essential oil extraction was carried out through Clevenger type distillation apparatus for 5 h and stored at -20°C till further use.

GC-MS analysis

Essential oil sample was dissolved in n-hexane for GC-MS analysis. One thousand ppm (v/v) of 2 µl essential oil was injected into GC-MS QP-5050 (Shimadzu). EQUITY-5 (supelco) column (5% Phenyl, 95% Dimethylpolysiloxane) of 30 m, 0.25 mm i.d., with film thickness of 0.25 µm was used for essential oil analysis. The operating conditions of GC-MS were as follows: Time program 50 - 150°C with rise of 3°C/min, held for 10 min, then raised to 280°C with rise of 10°C/min. The compounds were identified with mass spectra of NIST/NBS and available literature (Adams, 1995; Masada, 1976).

Phytotoxic studies

For phytotoxic studies, the bioassay method of Sekine et al. (2007) for essential oils of the test plant, *S. kurramense*, was followed. Fifty (50) µl of an essential oil of each sample was added to a 0.25 ml sample cup (11.0 x 13.5 x 16.3 mm). The sample cup was placed in the lower left well of the 6-well multi-dish plastic plate (0 mm distance well), while filter papers were placed in rest of the five wells. 0.7 ml water was added to these 5 wells and seven seeds were added into each well. The plates were sealed with plastic tape and incubated for 72 h in dark at 24°C. After 72 h, the length of roots and Hypocotyle were recorded. Each treatment was replicated thrice. The standard deviations of each treatment were also measured.

Statistical analysis

Cluster analysis and principal component analysis which were performed in NTSYSpc version 2.1 was used (Rohlf, 2000).

RESULTS AND DISCUSSION

Chemotypic variation

GC-MS analysis of 16 populations of *S. kurramense* identified 28 essential oils (Table 1). It was observed that 12 populations of *S. kurramense* showed a similar pattern

of chemicals, while four populations exhibited different pattern of chemical compounds. Morphological studies of all the 16 populations showed no differences in their appearances. The leaves were hoary and tomentose in appearance in both the chemotypes with same tomentose and hoary floral morphology.

Two different chemotypes were interestingly found in the 16 populations, which were named as 'chemotype A' and 'chemotype B'. It was also observed that fragrances of 12 populations of *S. kurramense* comprising of chemotype A were bitter while 4 populations of Malikheil plains, Karakheila, Shaheed Abad and Luqman Kheil, were sweet. The details of chemotype A and chemotype B are as follows:

Chemotype A

Major constituents of Chemotype A were α-thujone (26.0 - 73.4%), β-thujone (3.14 - 49.3%), 1,8-cineole (10.2 - 22.3%) and camphor (0 - 26.3%) (Table 1). Cluster analysis of essential oils also separated two chemotypes A and B into two different clusters (Figure 1). Populations from Kirman, Kharlachi and Bughday were grouped at the same cluster distance. Principal Component Analysis also distinctly separated chemotype A and chemotype B.

The current results were based on detailed essential oil analysis of populations using GC-MS but not single individual. The current analysis also separated the populations into two different chemotypes, Chemotype A and Chemotype B. Two terpenoids, thujones and cineole, are used in perfumeries (Goryaev et al., 1959) and drinks, however, thujones in higher doses cause confusion, convulsions and coma (Watt and Breyer, 1962). It is necessary to keep the safety and efficacy before preparing any medicinal drug from the cloned or cultivated plants (Viljoen et al., 2006). Other prominent chemical constituents included camphene, β-phellandrene, γ-terpinene, 1-methyl-4-(1-methylethyl)-trans-2-cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-cis-2-cyclohexen-1-ol, 6,6-dimethyl-bicyclo[3,1,1]-hept-2-ene-2-methanol and two unknown chemical compounds, unknown 2 and 3 which were absent in chemotype B.

Chemotype B

Major chemical constituents of four populations of Karakheila, Luqman Kheil, Shaheed Abad and Malikheil plains were *p*-menthan-1,8-diol (23.1 - 35.2%), 1,8-cineole (10.2 - 23.8%) and an unknown chemical compound 5 (12.5 - 25.9%) and most probably the new chemical compound (Table 1). GC-MS analysis of these 4 samples from the total of 16 samples, gave different chemical composition among which, eight terpenoids such as *p*-menthan-1,8-diol; 7-methyl-3-methylene-6-octen-1-ol; (+)-α-terpineol; 1,2,3,4,4a,5, 6,7- octahydro

Table 1. GC-MS analysis of essential oils of *S. kurramense* from various localities of Kurram Agency, Pakistan.

Name of location	Alam Sher	Borki	Bughday	Karakheila	Kharlachi	Kirman	Lalmay	Luqman Kheil	Malikheil Hospital	Malikheil plains	Pewar	Shaheed Abad	Shingak	Sports Complex	Zeran	Zeran Sehra	
Chemotype	A	A	A	B	A	A	A	B	A	B	A	B	A	A	A	A	
Compounds	RT (min)	% of each compounds															
Camphene	8.2	-	1.14	-	-	-	-	1.97	-	-	-	-	-	-	1.23	0.82	1.42
β -Phellandrene	9.1	2.30	-	1.05	-	1.64	0.63	0.00	-	1.17	-	-	-	1.35	1.29	0.90	1.58
Unknown 1	10.1	-	-	-	0.98	-	-	-	0.9	-	1.16	-	-	-	-	-	-
p-cymene	11.2	1.89	1.23	1.69	1.01	1.16	1.39	1.06	-	1.77	-	1.03	-	1.41	1.70	1.98	1.51
1,8-Cineole	11.4	10.15	16.67	15.34	23.79	14.78	16.84	18.73	16.02	14.02	10.23	14.97	21.30	15.85	22.30	12.70	16.45
p-Menthan-1,8-diol	11.6	-	0.96	-	23.14	-	-	-	24.06	-	35.19	-	23.27	-	-	-	-
Unknown 2	11.8	-	-	1.54	-	0.84	1.14	0.96	-	0.94	-	0.80	-	1.16	-	1.10	-
γ -Terpinene	12.7	-	-	-	-	-	-	-	-	-	-	0.40	-	0.53	0.82	0.54	-
β -Thujone	14.7	49.34	27.60	11.93	0.92	15.99	11.44	3.54	-	5.28	-	17.51	-	3.25	4.21	3.14	19.29
α -Thujone	15.3	26.01	20.76	56.67	5.66	48.23	56.24	40.72	3.55	73.42	3.35	30.12	3.97	63.22	36.45	51.40	39.20
1-methyl-4-(1-methylethyl)-trans-2-cyclohexen-1-ol	15.5	1.16	-	0.95	-	0.89	-	-	-	-	-	0.55	-	0.75	2.24	1.15	2.83
1-methyl-4-(1-methylethyl)-cis-2-cyclohexen-1-ol	16.3	0.85	-	-	-	-	-	-	-	0.75	-	0.68	-	0.62	1.45	1.00	1.48
Camphor	16.5	-	25.76	5.85	2.18	6.75	3.90	32.05	3.15	-	1.61	26.33	-	6.15	19.14	10.34	12.57
Unknown 3	17.2	0.98	-	-	-	0.83	-	-	-	-	-	0.81	-	0.74	0.71	0.67	-
Unknown 4	17.3	-	-	-	3.18	-	-	-	-	-	-	-	4.8	-	-	-	-
Borneol	17.5	-	0.96	-	1.06	-	-	-	1.07	-	1.63	0.49	-	-	1.08	-	-
7-Methyl-3-methylene-6-octen-1-ol	17.6	-	-	-	0.99	-	-	-	0.91	-	1.24	-	1.65	-	-	-	-
Unknown 5	17.8	-	-	-	17.46	-	-	-	25.94	-	12.52	-	21.74	-	-	-	-
4-Methyl-1-(1-methylethyl)-4-terpineol (3-cyclohexen-1-ol	18.1	1.61	1.33	1.42	1.11	1.35	0.85	0.00	0.89	1.03	1.30	1.44	1.4	1.06	1.97	1.18	1.58
(+)-alpha-Terpineol	18.7	-	-	-	-	-	-	-	-	-	0.64	-	-	-	-	-	-
6,6-Dimethyl-bicyclo[3,1,1]hept-2-ene-2-methanol	19.0	-	-	0.95	-	0.98	0.64	0.97	-	0.86	-	1.01	-	0.89	1.33	-	-

Table 1. Contd

Unknown 6	20.9				2.77				-		3.88		1.84				
Piperitone	21.7	1.75	1.19	1.66	3.41	1.03	0.58	-	3.49	-	1.94	0.84	4.51	0.87	1.48	2.43	2.08
β -Caryophyllene	29.3	1.18	-	-	1.86	1.60	0.99	-	5.35	0.76	5.87	0.45	6.45	0.69	1.02	0.69	-
D-Germacrene	32.0	1.90	-	-	-	-	1.20	-	2.61	-	1.59	-	-	-	-	-	-
Spathulenol	36.3	-	-	-	-	-	-	-	-	-	0.6	-	-	-	-	0.57	-
β -Caryophyllene oxide	36.6	-	-	-	1.87	-	-	-	2.53	-	2.72	0.54	2.7	0.59	-	1.19	-
1,2,3,4,4a,5,6,7-Octahydro- α , α ,4a,8-tetramethy-2-naphthalene methanol	39.4	-	-	-	1.08	-	-	-	1.68	-	2.27	-	-	-	-	-	-
Total (%)		99.12	97.6	99.05	92.47	96.07	95.84	100	94.4	100	91.13	97.97	93.63	99.13	98.42	91.8	99.99

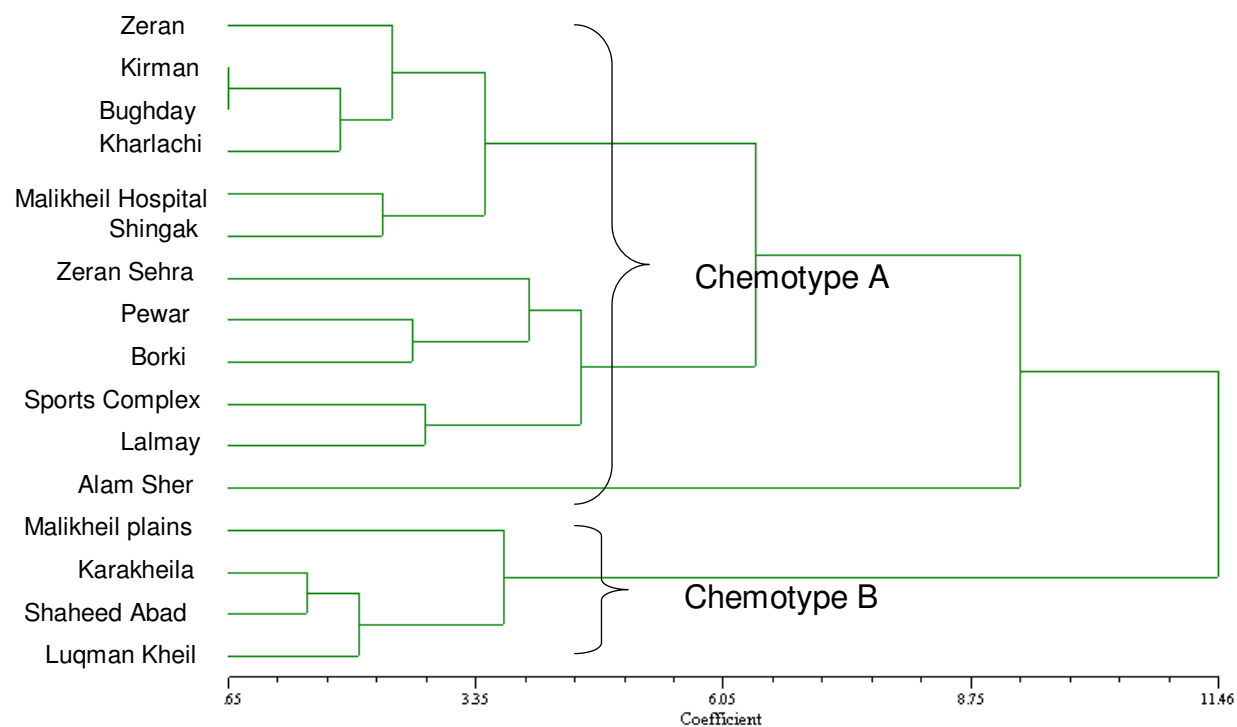


Figure 1. Hierarchical cluster analysis based on GC-MS analysis of essential oils for 16 samples of *S. kurramense* from various localities.

Table 2. Load coefficient of each chemical compound with respect to its first three principal components (PCs).

Chemical compounds	PC1	PC2	PC3
Camphene	0.351	0.157	0.516
β -Phellandrene	0.624	-0.580	-0.218
Unknown 1	-0.852	-0.074	-0.124
<i>p</i> -cymene	0.871	-0.204	-0.064
1,8-Cineole	-0.112	0.385	0.812
<i>P</i> -Menthan-1,8-diol	-0.992	-0.048	0.002
Unknown 2	0.550	0.453	-0.446
γ -Terpinene	0.360	-0.315	0.392
β -Thujone	0.435	-0.422	-0.206
α -Thujone	0.819	0.174	-0.316
1-methyl-4-(1-methylethyl)-,trans-2-cyclohexen-1-ol	0.503	-0.633	0.364
1-methyl-4-(1-methylethyl)-,cis-2-cyclohexen-1-ol	0.515	-0.626	0.323
Camphor	0.413	0.333	0.439
Unknown 3	0.465	-0.535	-0.043
Unknown 4	-0.929	0.015	0.173
Borneol	-0.638	-0.177	0.191
7-Methyl-3-methylene-6-octen-1-ol	-0.945	0.004	0.144
Unknown 5	-0.910	0.053	0.178
4-Terpineol	0.082	-0.799	0.264
(+)- α -Terpineol	-0.582	-0.225	-0.379
6,6-Dimethyl bicyclo[3,1,1]hept-2-ene-2-methanol	0.595	0.306	0.035
Unknown 6	-0.822	-0.083	-0.024
Piperitone	-0.696	-0.285	0.369
β -Caryophyllene	-0.892	-0.124	-0.037
Germacrene D	-0.477	-0.285	-0.457
Spathulenol	-0.341	-0.305	-0.319
β -Caryophyllene oxide	-0.935	-0.067	0.054
1,2,3,4,4 α ,5,6,7-Octahydro- α , α , 4 α , 8-tetramethyl 2-naphthalenemethanol	-0.851	-0.118	-0.218
Eigen value	12.78	3.37	2.75
Percent	45.65	12.04	9.82
Cumulative	45.65	57.69	67.51

α , α , 4 α , 8-tetramethyl-2-naphthalenemethanol; and four unknown compounds, unknown 1, 4, 5, and 6 were the characteristics of the new chemotype B present in a considerable amount but were absent in chemotype A. UPGMA cluster analysis also separated chemotype B into a separate major cluster (Figure 1). It was also observed that α -thujone, *p*-cymene and 1,8-cineole played an important role in clustering and had major contribution towards chemical variations (Table 2).

p-Menthan-1,8-diol is reported as < 0.01% in *Freesia hybrida* flowers, 0.41% in *Agastache mexicana*, 20 - 31% in edible mushroom, *Cinnamomum camphora* and *Magnolia* species (Harada and Mihara 1984; Jeannot et al., 2007; Kitzberger et al., 2007; Reyes et al., 2004; Zufa et al., 1993). The plant materials from Karakheila, Luqman Kheil and Shaheed Abad were grouped in one sub-cluster with same cluster distance while the plant material of Malikheil plains stood alone in a separate sub-cluster.

Santonin-containing plants in Lalmay, Bughday and adjoining areas and santonin-free *S. kurramense* in Malikheil plains are also growing in the Kurram Agency with the similar morphological characters (Qazilbash, 1948). These two areas are also distinct geographically, Lalmay, Bughday and adjoining areas and upper regions of Upper Kurram Agency are lush green, while the Malikheil plains and adjoining areas are arid zones. In other words, the santonin-containing populations are growing on one side of the Kurram River (*cis*-Kurram) while santonin-free populations are growing on the other side (*trans*-Kurram) (Krishna and Varma, 1933). From these two distinct chemotypes, it is now clear that one with Chemotype A may be santonin containing and the other with chemotype B may be santonin-free plant population. However, genetic studies are also required to confirm the status of chemotype A and chemotype B using various molecular markers such as P450 based analog markers, PBA (Gilani et al., 2009b), AFLP

Table 3. Phytotoxic effects of essential oils of *S. kurramense* from various localities using dish pack method.

Locality	Germination percentage	Radicle	Hypocotyle
Alam Sher	85.7	71.8 ± 1.99	84.3 ± 0.32
Borki	100	72.9 ± 0.97	82.5 ± 0.10
Bughday	85.7	76.5 ± 1.89	84.3 ± 0.32
Karakheila	0	100 ± 0.00	100 ± 0.00
Kharlachi	100	66.5 ± 1.34	86.0 ± 0.10
Kirman	100	57.1 ± 0.95	75.5 ± 0.52
Lalmay	100	80.6 ± 0.48	82.5 ± 0.10
Luqman Kheil	0	100 ± 0.00	100 ± 0.00
Malikheil hospital	100	55.3 ± 1.35	82.6 ± 0.10
Malikheil plains	85.7	87.1 ± 2.81	84.3 ± 0.32
Pewar	100	82.5 ± 0.48	80.6 ± 0.10
Shaheed Abad	0	100 ± 0.00	100 ± 0.00
Shingak	100	71.8 ± 0.79	82.5 ± 0.10
Sports Complex	100	80.0 ± 0.84	79.0 ± 0.42
Zeran	85.7	64.1 ± 2.81	68.5 ± 0.63
Zeran Sehra	100	70.0 ± 1.60	82.5 ± 0.10

All the values are shown as percentages comparable to the control (water). Means are represented with standard deviations. Roots and hypocotyle values of lettuce seeds are growth inhibition percentages.

(Amplified fragment length polymorphism etc., and SSR (Single sequence repeats) markers.

Phytotoxic studies

Significant differences in toxicity levels were observed in all the 16 populations of *S. kurramense* when tested against roots considering locality as a major factor (Table 3). Chemotype A exhibited variable toxicity levels ranging from 55.3 - 80.6% root growth inhibition and 68.5 - 86.0% Hypocotyle growth inhibition. Essential oils of chemotype B (Karakheila, Luqman Kheil, Malikheil plains and Shaheed Abad) showed highest inhibitory rates against both the radical and Hypocotyle growth ranging from 87.1 - 100% and 84.3 - 100%, respectively (Table 3). Chemotype B contains *p*-menthan-1,8-diol and the compound Unknown 5, which may be an important inhibitory chemical in chemotype B. The biological activities of *p*-menthan-1,8-diol are still unknown but the ethyl-acetate and dichloromethane fractions containing *p*-methan-1,8-diol as 2nd major constituent have shown 64.83 and 92.93% antioxidant activities, respectively (Kitzberger et al., 2007).

CONCLUSION AND RECOMMENDATIONS

Two clearly distinct chemotypes of *S. kurramense* have been identified through GC-MS analysis while morphologically they were similar in appearances. Phytotoxic studies showed significant differences in both the chemo-

types. Based on these conclusions, further study may be carried out with the following recommendations:

1. Studies on santonin presence and absence through HPLC analysis is recommended to confirm santonin-containing and santonin-free *S. kurramense*.
2. Both chemotypes may be subjected for further isolation of chemical compounds, as currently, little study has been carried out on chemical analysis of chemotype A while no phytochemical study is reported from Chemotype B.
3. Genetic diversity studies of these chemotypes using various molecular markers are also recommended.
4. Targeted isolation and identification of highly significant pharmacologically and pharmaceutically important chemicals are recommended.

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