Pak. J. Bot., 43: 29-37, Special Issue, December, 2011 (Medicinal Plants: Conservation & Sustainable use)

ECOLOGICAL CONSEQUENCES, GENETIC AND CHEMICAL VARIATIONS IN FRAGMENTED POPULATIONS OF A MEDICINAL PLANT, *JUSTICIA ADHATODA* AND IMPLICATIONS FOR ITS CONSERVATION

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Abstract

Justicia adhatoda from Kohat Plateau was selected for genetic diversity studies, due to its fragmented habitat, importance in traditional and pharmaceutical medicine and a lack of population structure studies. We had two hypotheses: that habitat loss posed a greater threat to populations than loss of genetic diversity, and that chemical diversity would be higher among different populations than within populations. Genetic diversity within and among populations was evaluated using PBA (P450 based analogue) markers. AMOVA analysis revealed that there was higher genetic diversity within populations (90%) than among populations (10%). No genetic drift was observed, i.e., genetic diversity within populations was maintained despite fewer numbers of individuals in fragmented populations. Surveys of *J. adhatoda* populations revealed that they were growing in harsh conditions and were imperiled due to extensive harvesting for commercial and domestic purposes. Chemical diversity was evaluated by GC-MS (Gas Chromatography–Mass Spectrometry) analysis of 90% methanol and 1:2 chloroform:methanol extracts. GC-MS analysis of both the extracts showed nine and 18 chemical compounds, respectively, with higher chemical variations among populations. It is therefore recommended that efforts for the conservation of severely fragmented populations of *J. adhatoda* must be carried out along with sustainable harvesting.

Introduction

Justicia adhatoda is a member of family Acanthaceae, (synonyms of *Adhatoda vasica* , *Adhatoda zeylanica*) (Malik & Ghafoor, 1988). It is distributed in Indonesia, Malaya, Southeast Asia, India and Pakistan (Malik & Ghafoor, 1988). It has been used in Ayurvedic and Unani medicines and used locally for the last 2000 years in India (Atal, 1980). The roots and leaves of *J. adhatoda* are used to treat bronchitis, asthma, fever, and jaundice in Ayurvedic and Unani systems of medicine (Malik & Ghafoor, 1988). In remote areas of Pakistan, the local communities in Kaghan Valley use the leaves as an expectorant and antispasmodic, and to treat external wounds, inflammatory swellings, neuralgia, headache and nose bleeds (Jan *et al*., 2008). Several ethnomedicinal uses of different parts of *J. adhatoda* from Pakistan, India, Nepal, Sri Lanka, and Thailand have been reviewed by Claeson *et al*., (2000). Its high medicinal value and local use for fuel have fragmented populations of *J. adhatoda*. In different areas of Pakistan, it may be found in small patches with few individuals (Gilani *et al*., 2011).

The major chemical compounds of *J. adhatoda* are from the quinozoline alkaloidal group. These quinozoline alkaloids in *J. adhatoda* are vasicine (Sen & Ghose, 1925), and a bronchodilator alkaloid, vasicinone (Amin & Mehta, 1959). The leaves, stems, and roots of *J. adhatoda* also contain essential oils with major chemical constituents including α-phellandrene (Sarada & Rao, 2008). The essential oils have shown antimicrobial activities against several organisms (Sarada & Rao, 2008).

Due to the fragmented nature of populations in Kohat Plateau, the importance of the species in traditional and pharmaceutical remedies, and the lack of population structure studies, *J. adhatoda* was selected for genetic diversity studies. We had two hypotheses: that habitat loss posed a greater threat to populations than loss of genetic diversity, and that chemical diversity would be higher among different populations than within populations.

To test these hypotheses, genetic diversity studies within and among populations were studied using PBA (P450 based analogue) markers. Chemical diversity was evaluated by GC-MS analysis. Environmental factors such as slope, altitude, temperature, rainfall, relative humidity, soil characteristics, and geographical distances between populations were also analyzed. After obtaining data from genetic, chemical, and environmental studies, influences of genetic diversity and environmental factors on chemical diversity were also analyzed.

Materials and methods

Plant collection: During field surveys in the Kohat plateau, in districts Kohat and Karak, it was observed that *J. adhatoda* populations were rarely encountered. Sixteen localities were surveyed, but only four populations of *J. adhatoda* were found (Fig. 1). Leaves were collected from each of 16 individuals per population. Leaves were placed in labeled paper bags and were packed in a zipped plastic bag containing silica gel.

Environmental factors: Site characters including slope were observed in the field and recorded. Altitude, latitude and longitude were recorded using a global positioning system (GPS) unit. Soil samples from each locality of a *J. adhatoda* population were collected at 15-30 cm depth. The samples were tested at the Soil Testing Laboratories of Agricultural Research Institute, Tarnab, Peshawar, Pakistan and Barani Area Research Station (BARS), Kohat, Pakistan. The samples were tested for texture, pH, organic matter content, nitrogen, phosphorous, potassium and percentage of total soluble salts.

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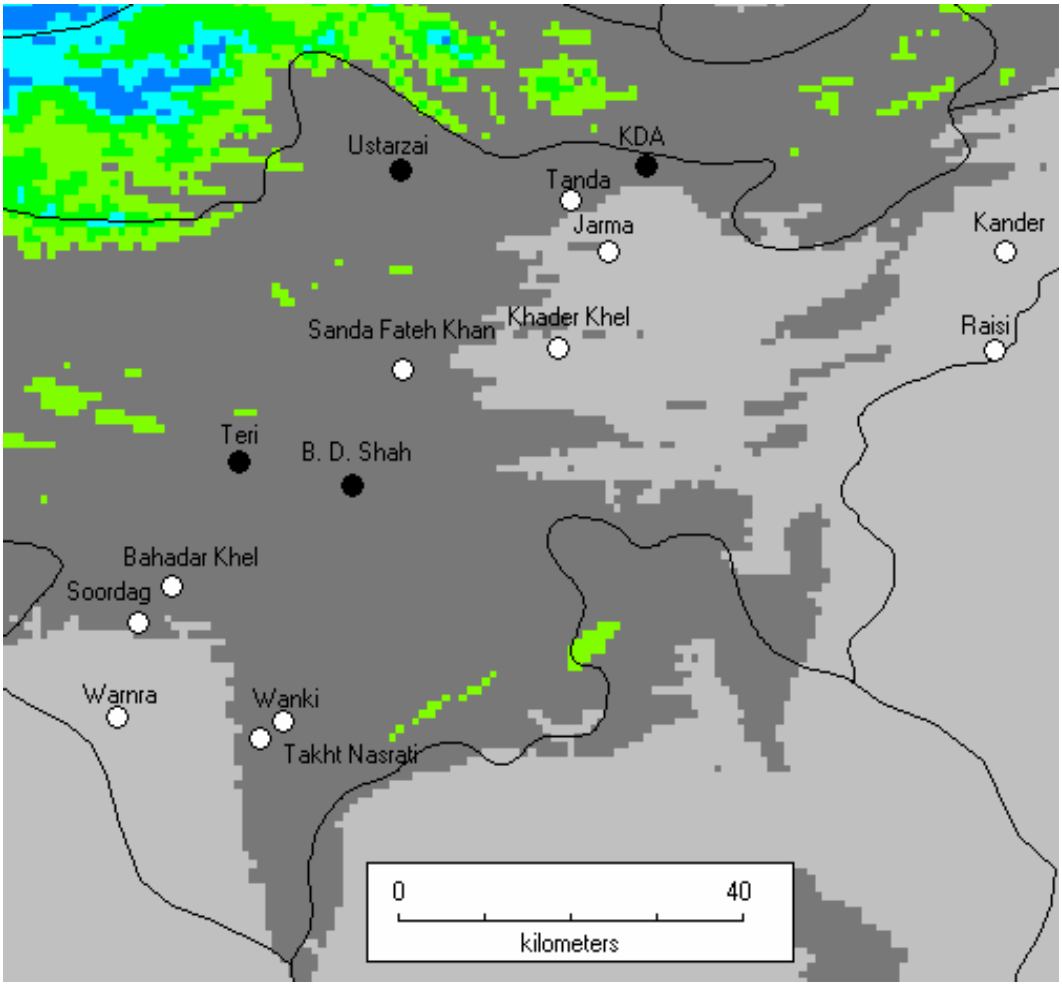

Fig. 1. Collection localities with (black circles) and without (white circles) *Justicia adhatoda* in Kohat Plateau.

Genetic diversity: The DNA extraction method of Doyle and Doyle (1990) was followed with minor modifications. Instead of using simple CTAB buffer (100 mM Tris base, 20 mM ethylenediaminetetraacetic acid (EDTA), 2% cetyltrimethyl ammonium bromide (CTAB), 1.4 M NaCl), the buffer cocktail was prepared by mixing extraction buffer (0.1 M Tris base, 5 mM EDTA-disodium salt, 0.35 M sorbitol), 5% sarkosyl, 1% sodium bisulphate, 2% PVP, lysis buffer (0.25M Tris base, 5 mM EDTA-disodium salt, 2% CTAB, 2 M NaCl), and 1% β-mercaptoethanol. Addition of chloroform-isoamyl alcohol (24:1) into plant-buffer mixture and centrifugation at 14,000 rpm was repeated twice. P-450 based analog (PBA) markers were applied to evaluate genetic structure of populations (Table 2) following protocol of Gilani *et al*., (2009). The bands were scored as '1' for present and '0' for absent.

Statistical analysis: For calculating genetic diversity within and among populations of *J. adhatoda*, AMOVA and F_{ST} were run using the software program GenAlEx 6 (Peakall & Smouse, 2006). Expected heterozygosity (*He*) of genetic diversity, effective number of alleles (Na), and Shannon's information index for binary dominant data were calculated according to the procedures of Lynch and Milligan (1994).

Chemical diversity: Two sets of dried leaves, of 100 g each from each population, were ground and soaked overnight in one of two different solvents, i.e., 90% methanol or 1:2 chloroform-methanol. The solvents were then filtered using Whatman filter paper No. 1. The filtered solution was transferred into glass vials and stored in a refrigerator until further use.

A sample of 2µl was injected into a GC-MS (Shimadzu, QP-5050) used with an equity-5 column (Supelco) with specifications of 30 m long, 0.25mm i.d. and thickness of 0.25µm. Each sample was run for 30 minutes with gradient temperature of 60-200°C. The mass spectra of visible and sharp peaks were compared with the spectra of NIST (National Institute of Standards and Technical) libraries and the literature. For cluster analysis, the data were analyzed in NTSYS using UPGMA cluster analysis and Principal Component Analysis (PCA).

Results

Environmental factors: *Justicia adhatoda* was collected from four localities with a total number of 64 individuals. Although 16 localities were surveyed, *J. adhatoda* was found only at four localities and had fewer than 50 individuals per population (Fig. 1). Two populations, Ustarzai and KDA, were part of district Kohat and the

other two, Teri and B. D. Shah, were in district Karak. The Ustarzai population in district Kohat was the most isolated one among these four populations, at an altitude of 682 m and completely isolated from the other three populations. The plants were growing on south-facing slopes. In KDA, plants were growing at 541 m elevation in a small south-facing valley. The Teri population was growing on north-facing slopes at an altitude of 674 m while the B. D. Shah population was growing on southwestern-facing slopes at 586 m.

The Kohat Plateau has a minimum mean annual temperature of 6.80ºC while the maximum mean annual temperature is 29.05ºC (Anonymous, 1999, 2000). Annual precipitation in the area, based on 30 years' data, averages 545.72 mm, while relative humidity was recorded as 48.42% (Anonymous, 1999, 2000).

The soil analysis of localities of *J. adhatoda* showed that the populations were growing on sandy loam and silt clay loam soils (Table 1). However, negligible amounts (0.72-1.86%) of organic matter were found in these soil samples. The amount of nitrogen in these soils was 0.034-0.093 ppm, which is very low. The amounts of phosphorous and potassium were substantially high in the localities. Phosphorous amounts ranged from 4.95-59.15ppm. The lowest amount of phosphorous (4.95 ppm) was found in Ustarzai while highest amount (59.15ppm) was found in KDA. Similarly, potassium was lowest at Ustarzai (180 ppm) and highest at KDA (266 ppm). The pH of the soils of these localities, 8.1-8.8, showed their alkaline nature, and there were negligible amounts of salts (0.08-0.1 dSm^{-1}), and total soluble salts (0.029- 0.041%).

Table 1. Soil analysis of localities of districts of Karak and Kohat in NWFP Pakistan.

Locality	Texture	OM (%)	N (ppm)	P (ppm)	K (ppm)	pH	EC (dSm^{-1})	TSS (%)
KDA	Sandy loam	1.20	0.060	59.15	266.0	8.20	0.10	0.032
Ustarzai	Sandy loam	0.72	0.036	04.95	180.0	8.10	0.09	0.029
B. D. Shah	Sandy loam	1.86	0.093	49.68	237.0	8.50	0.13	0.041
Teri	Silty clay loam	0.69	0.034	23.66	228.0	8.80	0.08	0.026

OM = Organic matter, EC = Electrical conductivity with 1:5 ratio, N = Nitrogen, P = Phosphorus, K = Potassium, TSS = Total soluble salts

Table 2. Details of PBA primer-sets, amplified polymorphic bands in four populations of *J. adhatoda*.

No.	Primer – set (Forward/Reverse)*	*Tm* (°C)*	Total Loci	No. of PB	PPB (%)
P 01	CYP1A1F/CYP1A1R	46.5	10	8	80.0
P 02	CYP1A1F/CYP2B6R	52.0	12	11	91.7
P 03	CYP1A1F/CYP2C19R	46.5	5	3	60.0
P 04	CYP1A1F/heme2B6	56.0	13	12	92.3
P 05	CYPIAIF/heme2C19	56.0	8	0	0.00
P 06	CYP2B6F/CYP1A1R	52.0	7	6	85.7
P 07	CYP2B6F/CYP2B6R	52.0	15	8	53.3
P 08	CYP2B6F/CYP2C19R	46.5	11	9	81.8
P 09	CYP2B6F/heme2B6	52.0	12	12	100.0
P 10	CYP2B6F/heme2C19	52.0	10	7	70.0
P 12	CYP2C19F/ CYP2B6R	56.0	11	11	100.0
P 13	CYP2C19F/CYP2C19R	46.5	14	13	92.8
P 14	CYP2C19F/heme2B6	56.0	15	11	73.3
P 15	CYPI2C19F/heme2C19	56.0	13	13	100.0
Total			**156**	**124**	**79.5**

*****Yamanaka *et al*., (2003); Tm = Annealing temperature, PB = Polymorphic bands, PPB = Percentage of polymorphic bands

Genetic diversity: Several studies on genetic diversity on accessions/species basis have been carried out in Pakistan (Akbar *et al*., 2011; Mumtaz *et al*., 2011; Shah *et al*., 2011; Shinwari *et al*., 2011; Zeb *et al*., 2011), this was one of first comprehensive studies on population genetics of wild species, in addition to our earlier study on *Aleuropes lagopoides* (Ahmed *et al*., 2011). In this study, four populations with a total of 64 individuals were amplified by 15 primer combinations (Table 3). These primer combinations gave 5-15 fragments per primer with 156 total of loci (Table 3). The number of polymorphic loci/bands observed was 124, accounting for 79.5% of the polymorphisms. The CYP1A1F/ heme 2B6 primer set amplified eight fragments in all the individuals but all of them were monomorphic in nature. Polymorphic rates of 100% were observed with the CYP2B6F/ heme2B6, CYP2C19F/ CYP2B6R, and CYP2C19F/ heme 2C19 primer sets.

The observed number of alleles (Na) per population ranged from 1.526-1.583 with an average of 1.561 (Table 3). The highest observed numbers of alleles (1.583) and polymorphic bands (60.26%) were found in Teri (1.583) while lowest numbers of alleles (1.526) and polymorphic bands (56.41%) were seen in Ustarzai. Effective numbers of alleles (Ne) ranged from 1.394 to 1.461 with an average of 1.430 in all four populations. The highest and lowest effective numbers of alleles (Ne) were also observed in Teri (1.461) and Ustarzai (1.394). Shannon's information index (I) showed 0.327-0.363 diversity with an average of 0.347, while expected heterozygosity (*He*) was observed at 0.223-0.252 with an average expected heterozygosity (*He*) of 0.239. The current analysis of genetic variation within populations of *J. adhatoda* showed that the Teri population had higher genetic diversity than the other three populations while the Ustarzai population showed lower genetic diversity. It was also observed that genetic distance between Teri and

B. D. Shah was comparatively higher than between the rest of the populations, even though they were closer in distance (12.7 km) to each other than to the other populations. KDA and Ustarzai showed lower genetic distance and were situated closer to each other (24.7 km) than to the other populations.

AMOVA (Analysis of Molecular Variance) showed 90% genetic diversity within populations and 10% genetic diversity among populations (Table 4). F_{ST} value (Wright's Fixation Index as a measure of population differentiation) was also lowest within population at 0.096 while the gene flow (*Nm*) was higher among populations (Table 5). These results indicated that there was no genetic differentiation among populations while there was high genetic diversity within populations. These results suggest that there was no population structure though there were fewer than 50 individuals per population.

Cluster analysis found two major divisions, with B. D. Shah separated from the other three populations (Fig. 2). However, within this second cluster, the Teri population was separated from the other two populations. Principal Coordinate Analysis (PCA) also confirmed the same pattern of groupings with a cumulative average of 84.74% on the first two axes. The x-axis corresponded to 46.77% of the variation while the y-axis corresponded to 37.97% (Fig. 3).

Isolation by distance (Mantel test) also showed no correlation between genetic diversity and geographical distance. The populations were not classified as isolated geographically.

Table 3. Genetic variation within populations of *J. adhatoda* in NWFP Pakistan.

Population	n	na	ne	I	H	PPB
B. D. Shah	16	1.58 (0.042)	1.42 (0.033)	0.34 (0.025)	0.24 (0.017)	58.97
KDA	16	1.59 (0.045)	1.44 (0.033)	0.36 (0.025)	0.25 (0.017)	58.97
Teri	16	1.58 (0.043	1.46 (0.034)	0.36 (0.025)	0.25 (0.018)	60.26
Ustarzai	16	1.53 (0.046	1.39 (0.032)	0.33 (0.024)	0.22 (0.017)	56.41
Average	16	1.56 (0.022)	1.43 (0.016)	0.35 (0.012)	0.24 (0.009)	58.65

n= number of individuals; na = Observed number of alleles; ne = Effective number of alleles; I = Shannon's Information index; H = Nei's (1973) gene diversity; PPB = percentage of polymorphic bands

Table 4. Analysis of Molecular Variance (AMOVA) within and among populations of *J. adhatoda*.

Source	Df	SS	MS	Est. Var.	%	F_{ST}	*P* value
Among Pops	3	119.375	39.792	1.567	10		
Within Pops	60	883.625	14.727	14.727	90	0.096	0.001
Total	**63**	**1003.000**		**16.294**	**100**		

Df = degrees of freedom, SS = Sum of squares, MS = Mean square, Est. Var. = Estimate variance, % = percentage, .F_{ST} = Wright's Fixation Index, *P* value = probability value.

Table 5. Gene diversity within 4 populations of *J. adhatoda* using the Lynch and Milligan (1994) method.

N	Ht	Hw	Hb	F_{ST}
4	0.2089	0.1881	0.0208	0.0995
Standard Error	0.0102	0.0024	0.1272	
Variance	0.000102	0.000006	0.016197	

N= number of populations; Ht = total gene diversity; Hw = gene diversity within population, Hb = average gene diversity among population; F_{ST} = Wright's Fixation Index.

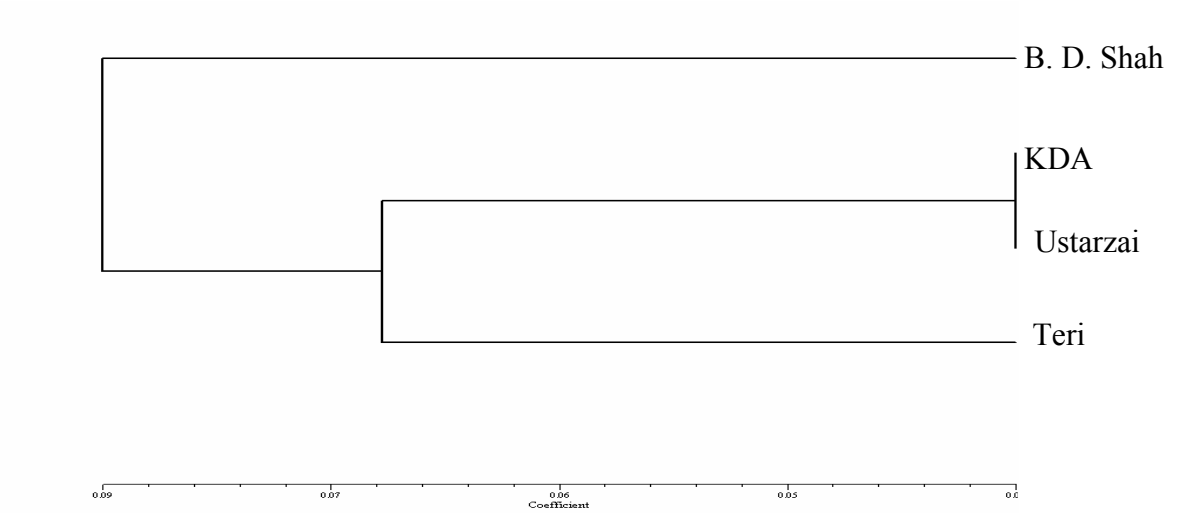

Fig. 2. UPGMA dendrogram illustrating genetic relationships of four populations of *J. adhatoda* based on Nei's genetic distance among different populations.

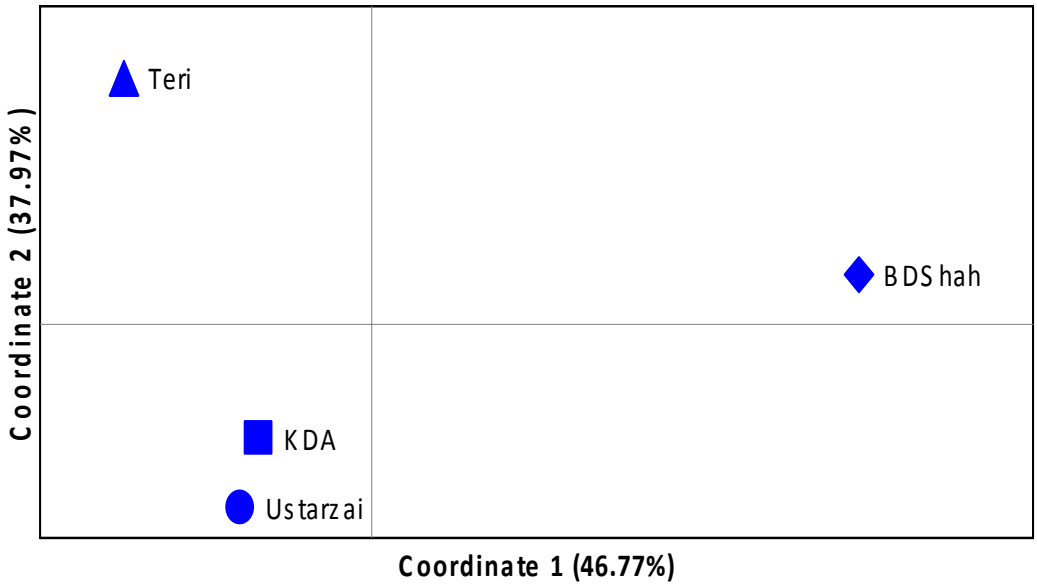

Fig. 3. Principal Coordinate Analysis illustrating genetic differences among four populations of *J. adhatoda*.

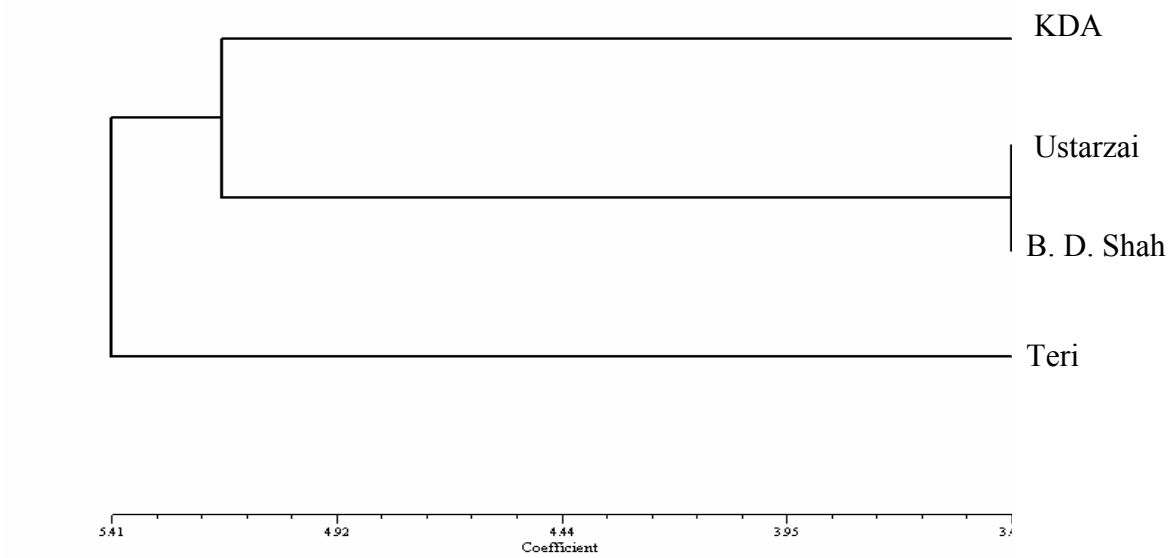

Fig. 4. Cluster analysis of *J. adhatoda* populations is based on chemical diversity data of 90% methanol and 1:2 chloroform – methanol extracts.

Chemical diversity: GC-MS analysis of four populations of *J. adhatoda* exhibited 23 different known chemical compounds from the two different extraction methods (Table 6).

GC-MS analysis of the 90% methanol extracts showed nine chemical compounds (Table 6). The percentage composition of these chemicals varied or was even absent from some populations. The major chemical compound was 7-(1,1-dimethylethyl)-3,4-dihydro 1(2H)-naphthalenone, with a range of 79.55-88.49%. The highest percentage of this chemical constituent was observed from the B. D. Shah population while the lowest was observed from the Teri population. The second major chemical compound in the methanol extracts was 1,2-diethenyl-*cis*-cyclohexane, with a percentage composition of 0.17-10.95 %. The highest percentage composition of cyclohexane was found in the B. D. Shah population while in the other three populations it was less than 2%. (Z) 6-Pentadecen-1-ol was observed in only the Teri population (0.64%). Benzene acetaldehyde was absent from the KDA population, 3-benzyl-1-(trimethylsilyloxy)-cyclohexene were not found from the Teri and Ustarzai populations, and palmitic acid was absent from the Ustarzai population.

When 1:2 chloroform-methanol extracts of *J. adhatoda* populations were subjected to GC-MS, 18 chemical constituents were identified (Table 6). In 1:2 chloroform-methanol extract, four chemical compounds, benzene acetaldehyde, 7-(1,1-dimethylethyl)-3,4-dihydro 1(2H)-naphthalenone, palmitic acid, and phytol were also identified in 90% methanol extracts from the same plant populations. As in the 90% methanol extracts, 7-(1,1-

dimethylethyl)-3,4-dihydro 1(2H)-naphthalenone was the major chemical compound with 28.04-46.72% composition. The lowest percentage of this chemical was observed from the KDA population (28.04%), while the highest percentage was observed from the B. D. Shah population (41.64%). The second major chemical constituent was palmitic acid, which ranged from 6.31% to 19.02%. The lowest percentages of palmitic acid were observed in Teri (6.31%) and KDA (6.32%) while the highest was observed in B. D. Shah (19.02%). Other interesting compounds were indole alkaloids, methyl ester, and (2β, 3α, 12β, 19α)-aspidospermidine-3-carboxylic acid and quebrachamine, which were found only in the KDA population at 11.78% and 8.39%, respectively. The KDA population contained indole alkaloids but had no (Z,Z)-9,12-octadecadienoic acid [cis, cis-linolic acid] or (Z,Z,Z)-9,12,15-octadecatrien-1-ol. No 2,2,2-trifluoro-N [2-(hexahydro-1(2H)-azocinyl)ethyl]-acetamide was present from B. D. Shah, but it was present at 6.27-9.50% in the other three populations.

Table 6. GC-MS analysis of *J. adhatoda* using 90% methanol and 1:2 chloroform:methanol extracts. Values are expressed in percentage composition.

No.	Chemical constituents	Rt	B.D. Shah	Teri	KDA	Ustarzai
	90% Methanol extract					
A1	Benzene acetaldehyde	7.424	1.08	0.35	0.00	0.20
A2	2,3-Dihydro-3,5-dihydroxy-6-methyl 4H-pyranone	9.491	1.14	0.95	1.18	0.45
A3	4-Methyl-2,5-dimethoxybenzaldehyde	16.853	0.48	2.27	1.52	0.69
A4	7-(1,1-Dimethylethyl)-3,4-dihydro 1(2H)-naphthalenone	22.651	88.49	79.55	84.16	88.27
A5	3-Benzyl-1-(trimethylsilyloxy)-cyclohexene	23.262	1.93	0.00	4.90	0.00
A6	Palmitic acid	23.668	1.18	6.44	4.15	0.00
A7	Phytol	28.121	3.50	5.34	2.88	4.01
A8	(Z)-6-Pentadecen-1-ol	28.987	0.00	0.64	0.00	0.00
A9	1,2-Diethenyl-cis-cyclohexane	29.238	10.95	1.97	0.38	0.17
	1:2 Chloroform: Methanol extract					
B1	Benzene acetaldehyde	7.264	0.23	0.00	0.00	0.00
B2	Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl ester	13.227	0.24	0.00	0.00	0.00
B3	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	13.587	0.29	0.00	0.00	0.00
B4	2,2-Diethyl-N-ethylpyrrolidine	17.695	0.00	2.41	1.48	2.00
B5	2,2,2-Trifluoro-N[2-(hexahydro-1(2H)-azocinyl)ethyl]-acetamide	18.188	0.00	9.50	8.15	6.27
B6	Clindamycin	18.413	0.27	5.61	4.49	0.68
B7	Octadecanoic acid	19.723	0.31	0.27	0.75	0.00
B8	4,9,13,17-Tetramethyl-4,8,12,16-octadecatetraenal	20.909	2.83	2.47	0.89	0.68
B9	Squaline	21.236	2.75	0.00	0.00	21.35
B10	2,2,4-Trimethyl-3(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	21.699	4.71	1.24	0.00	0.00
B11	7-(1,1-Dimethylethyl)-3,4-dihydro-1(2H)-naphthalenone	22.692	41.64	35.07	28.04	46.72
B12	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	23.515	0.73	0.00	1.55	0.00
B13	Palmitic acid	23.699	19.02	6.31	6.32	9.32
B14	Methyl ester, (2β, 3α, 12β , 19α)-aspidospermidine-3- carboxylic acid		0.00	0.00	11.78	0.00
B15	Phytol	28.004	4.45	2.25	1.35	2.51
B16	Quebrachamine		0.00	0.00	8.39	0.00
B17	(Z,Z)-9,12-Octadecadienoic acid [Syn: cis, cis-linolic acid]	28.979	2.10	0.57	0.00	0.00
B18	(Z,Z,Z)-9,12,15-Octadecatrien-1-ol	29.292	8.09	0.96	0.00	0.58

Rt = Retention time

Cluster analysis on the basis of both the methanol and 1:2 chloroform-methanol extracts gave two divisions, with the Teri population separated from the other three populations (Fig. 4). The KDA population was again divided from the Ustarzai and B. D. Shah populations. Principal Component Analysis also separated the Teri population from the rest of the three populations (Fig. 5). Eigenvectors and components as well as communalities and an un-rotated component matrix did not show clearly which specific chemical compound was responsible for separating Teri from the rest of the populations. Oblique (non-orthogonal) rotation of the component matrix using Promax with Kaiser Normalization exhibited a significant difference between variables (Table 7). These results showed that squaline had a major contribution in separating the Teri population from the rest of the populations (Table 7; Fig. 6).

Discussion

Justicia adhatoda was found growing on south-facing slopes at Ustarzai and KDA in the north of Kohat Plateau while populations at Teri and B. D. Shah in the south of Kohat Plateau were growing on south and southwestern-facing slopes. The vegetation pattern of *J. adhatoda* reflects that valleys run east-west in the northern part and northwest-southeast in the southern part. The soil analysis shows that the plant grows on sandy loam and alkaline soils with negligible organic matter. Generally, the localities of *J. adhatoda*, except Ustarzai, are in an arid region, which suggests that harsh climatic conditions have no or little effect on the survival of *J. adhatoda* populations.

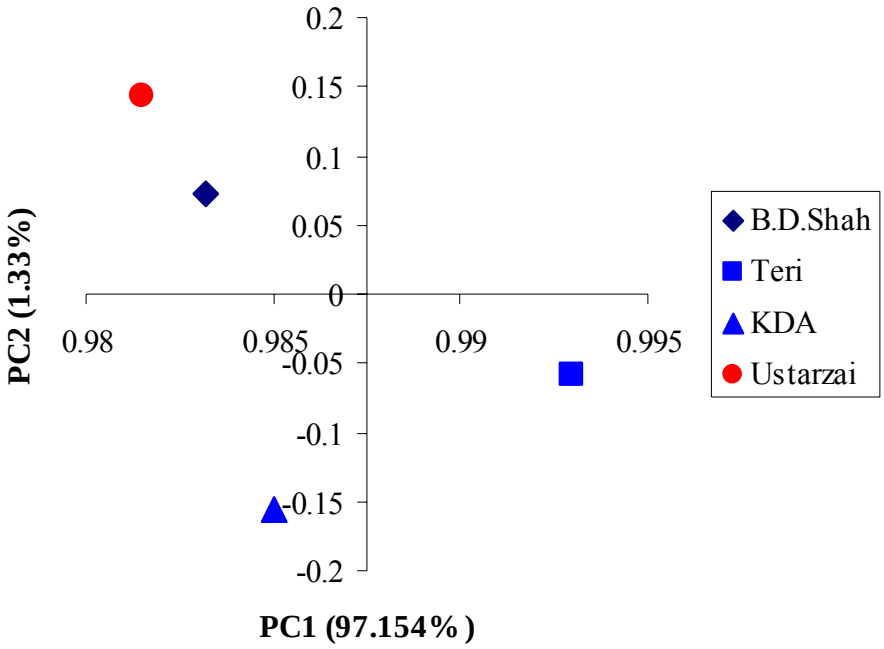

Fig. 5. Principal component analysis (PCA) is based on GC-MS data of four populations of *J. adhatoda*.

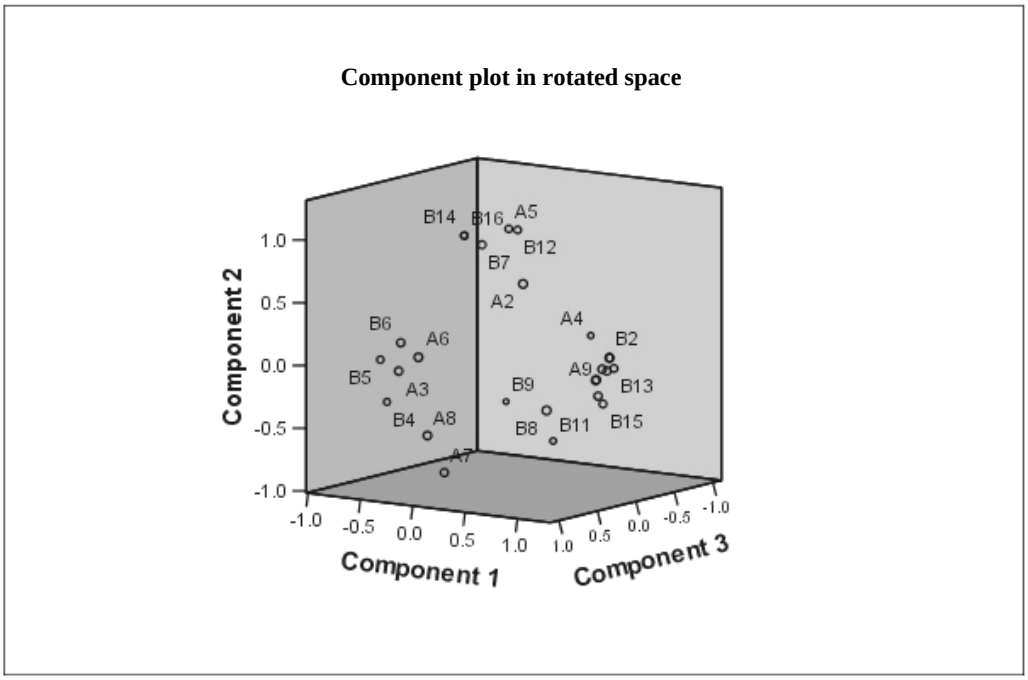

Fig. 6. 3-D Component plot of chemical compounds 27 chemical compounds of *Justicia adhatoda*. Key for point labels is in Tables 6 and 7.

The Teri population showed the highest genetic diversity while the Ustarzai population showed the lowest. Both populations grow in the vicinities of villages and are equally subjected to human pressures for medicinal or fuel purposes. Therefore, both populations should be given consideration for conservation efforts. All four populations had fewer than 50 individuals, leading us to hypothesize that there would be lower genetic diversity within populations than among populations, but our results did not support this hypothesis. There was higher genetic diversity within populations than among *J. adhatoda* populations. These kinds of results would be expected in larger undisturbed populations. *Justicia adhatoda* is a long-lived, perennial, out-crossing species, which gives it an advantage over short-lived, out-crossing perennials and self-pollinated species. Long-lived perennials show higher genetic diversity within populations than among populations, and the opposite is true for short-lived, out-crossing and self-pollinated species (Hamrick & Godt, 1996). Even though the *J. adhatoda* populations have been recently fragmented, they have maintained genetic diversity. Another reason for

these results may be that the recent loss of *J. adhatoda* vegetation from the surrounding area has left population islands that have not yet become different. Local-level fragmentation of plant species results from past disturbances (Silverton & Charlesworth, 2001) and especially due to habitat destruction (Channel & Lomolino, 2000). However, as time passes, it is possible that certain genetic differences will accumulate among the populations, if these populations remain isolated for a long time with low numbers of surviving individuals.

Table 7. Rotated Component Matrix[a] of chemical compounds of *Justicia adhatoda* populations. Principal component analysis was used as an extraction method while Promax with Kaiser Normalization was applied as orthogonal rotation. Significant loadings are shown in bold.

No.	Chemical compound	PC1	PC2	PC3
A9	1,2-Diethenyl-cis-cyclohexane	1.000		-0.226
B18	(Z,Z,Z)-9,12,15-Octadecatrien-1-ol	0.996		-0.324
B10	2,2,4-Trimethyl-3(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	0.993		-0.140
B17	(Z,Z)- 9,12-Octadecadienoic acid	0.992		-0.133
B1	Benzene acetaldehyde	0.989		-0.364
B3	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	0.989		-0.364
B2	Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl ester	0.989		-0.364
A1	Benzene acetaldehyde	0.979	-0.250	-0.227
B13	Palmitic acid	0.943	-0.130	-0.544
B15	Phytol	0.930	-0.370	-0.415
B5	2,2,2-Trifluoro-N[2-(hexahydro-1(2H)-azocinyl)ethyl]-acetamide	-0.899		0.638
B4	2,2-Diethyl-N-ethylpyrrolidine	-0.878	-0.313	0.484
B8	4,9,13,17-Tetramethyl-4,8,12,16-octadecatetraenal	0.782	-0.211	0.375
A5	3-Benzyl-1-(trimethylsilyloxy)-cyclohexene		0.992	
B12	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester		0.984	
B7	Octadecanoic acid		0.968	0.356
B14	Methyl ester, (2 beta, 3 alpha, 12 beta, 19 alpha)- aspidospermidine-3- carboxylic acid	-0.397	0.933	0.129
B16	Quebrachamine	-0.397	0.933	0.129
B11	7-(1,1-Dimethylethyl)-3,4-dihydro-1(2H)-naphthalenone	0.284	-0.807	-0.666
A7	Phytol	-0.147	-0.747	0.577
A2	2,3-Dihydro-3,5-dihydroxy-6-methyl 4H-pyranone	0.444	0.744	0.421
A6	Palmitic acid	-0.291	0.249	0.989
A4	7-(1,1-Dimethylethyl)-3,4-dihydro 1(2H)-naphthalenone	0.415		-0.981
A3	4-Methyl-2,5-dimethoxybenzaldehyde	-0.509	0.107	0.957
B6	Clindamycin	-0.531	0.314	0.925
A8	(Z)-6-Pentadecen-1-ol	-0.189	-0.378	0.878
B9	Squaline	-0.285	-0.584	-0.715

[a] Rotation converged in 7 iterations

Chemical analysis showed that certain chemical compounds were absent in some populations but were present in high amounts in other populations. Similarly, high diversity in quantities of chemical compounds was found among populations. These kinds of variations are not uncommon in plants (Bottin *et al*., 2007), but it would be interesting to study what controls variation in chemical compounds within the same plant species. However, the mechanism behind chemical variations within and among species is still unknown (Pichersky & Gang, 2000). There are other factors, in addition to genetic and environmental factors, that affect the chemical diversity and need to be explored (Kumar *et al*., 2009). Therefore, future detailed greenhouse studies are suggested to assess correlations between genetic and chemical diversities.

Acknowledgements

This research work was conducted at University of Tsukuba, Tsukuba, Japan as a part of the doctoral degree research of the principal author. The principal author was financially supported by a Japanese government MEXT scholarship during his whole stay in Japan. All the travel and field expenses were sponsored by Kohat University of Science and Technology (KUST) under the joint collaborative project titled, "Establishment of Linkages with University of Tsukuba (Japan) for Joint Research and Education Program", between KUST and University of Tsukuba, which was funded by Higher Education Commission (HEC) Pakistan.

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(Received for publication 10 October 2011)