Hacquetia 19/2 • 2020, 317–324 DOI: 10.2478/hacq-2020-0002 sciendo

Genetic and morphological variability in medicinal plant *Helichrysum oocephalum* Boiss. (Asteraceae) in Iran

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Key words: Gene flow, *Helichrysum oocephalum*, ISSR, morphology.

Ključne besede: genski tok, *Helichrysum oocephalum*, ISSR, morfologija.

Abstract

Helichrysum oocephalum is a medicinal plant of the genus *Helichrysum* that have limited distribution in Iran. Local geographical populations may differ in their genetic content and form different gene pools. Therefore, we carried out population genetic investigation and morphological studies in five geographical populations of *Helichrysum oocephalum* by using ISSR molecular markers. AMOVA produced the significant genetic differences. The mean Nm value revealed some degree of gene flow among *Helichrysum oocephalum 8*. Molecular and morphological analysis indicated that we have 2 groups in the studied populations. The present findings may be of use in the conservation of this medicinal plant in Iran.

Izvleček

Helichrysum oocephalum je zdravilna rastlina iz rodu *Helichrysum*, ki ima v Iranu omejeno razširjenost. Lokalne geografske populacije se lahko genetsko razlikujejo in vzpostavljajo različne genske nabore, zato smo zastavili populacijsko genetsko raziskavo in morfološko študijo petih geografskih populacij vrste *Helichrysum oocephalum* z ISSR molekularnimi markerji. Z analizo AMOVA smo dokazali značilne genetske razlike. Povprečna vrednost Nm je razkrila genski pretok med vrstami *Helichrysum oocephalum.* Z molekularno in morfološko analizo smo dokazali dve skupini med obravnavanimi populacijami. Predstavljeni rezultati bodo uporabni pri ohranjanju te zdravilne vrste v Iranu.

Received: 16. 9. 2018
Revision received: 24. 1. 2020
Accepted: 24. 2. 2020

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Introduction

The genus *Helichrysum* Mill., (Gnaphalieae) is a genus in the family Asteraceae that contains 500 to 600 annual, herbaceous perennials or shrub species (Galbany-Casals et al. 2009, Azizi et al. 2019). Some of these species have ornamental and medicinal values. For example, *Helichrysum italicum*, *H. leucocephalum* and *H. artemisioides* contain essential oils (Javidnia et al. 2009), while *H. compactum* and *H. italicum* contain flavonoids with antioxidant and antibacterial activity (Facino et al. 1990). There are 18 *Helichrysum* species in Iran (Azizi et al. 2019). According to this study *H. persicum* Ghahremani & Noori, is a member of *H. oocephalum* Boiss., species (Salehi et al. 2014).

Helichrysum oocephalum grows in limited regions located in the North-East of Iran, is a medicinal plant and extensively used by locals for its anti-inflammatory, antiallergic, antipsoriatic and diuretic effects (Firouznia et al. 2007, Azizi et al. 2014). To our knowledge there has been no detailed investigation on the genetic variability and population genetic structure of this rare medicinal plant in Iran and the present study is the first report on the subject. Such investigations can provide information about potential gene pools which then might be used in conservation and breeding of medicinal plants (Chen 2000, Ellis & Burke 2007, Sheidai et al. 2012).

It has been shown that many plant species which are distributed in different geographical regions, differ in their genetic structure and morphological characteristics too (see for example Sheidai et al. 2012; Azizi et al. 2014, Minaeifar et al. 2015, Azizi et al. 2019). Therefore, we may also encounter new infra-specific taxonomic forms like, varieties, or ecotypes within a single species (Sheidai et al. 2012, Koohdar et al. 2015).

Molecular markers have been used extensively in population genetic investigations (Sheidai et al. 2012, Koohdar et.al. 2015, Mosaferi et al. 2015). Multilocus molecular markers including simple sequence repeat markers (SSRs) and inter simple sequence repeat markers (ISSR) are good genetic markers to identify hybrid plants and plan genetic diversity (Gaskin & Kazmer 2009, Noormohammadi et al. 2012). These molecular markers are known to reveal genetic diversity in *Helichrysum* species and (Azizi et al. 2014, Taban et al. 2015). We used ISSR (Inter-simple sequence repeats) to study genetic diversity of populations in *Helichrysum oocephalum*, since these markers are reproducible, cheap, easy to work and are known to be efficient in population genetic diversity studies (Azizi et al. 2014, Sheidai et al. 2014).

Therefore, the aim of present study was population genetic analysis of 5 geographical populations in *Helichrysum oocephalum* by using ISSR molecular markers for the first time. These informations may be of use for future conservation and breeding of this medicinally important plant species.

Material and Methods

Plant material

Sixty-eight plant specimens were collected from 5 populations of *Helichrysum oocephalum*. Details of the studied populations are provided in Table 1 and Figure 1.

Figure 1: Loctions of the studied populations in *Helichrysum oocephalum*.
Slika 1: Lokacije obravnavanih populacij vrste *Helichrysum oocephalum*.

Table 1: Populations studied, their locality and ecological features.
Tabela 1: Obravnavane populacije, njihove lokacije in ekološke lastnosti.

Pop	Province	Locality	Altitude (m)	Longitude	Latitude	Voucher number
1	North Khorasan	Gholaman village	1226	3803	5708	HSBU68
2	North Khorasan	Raz city	1294	3756	5706	HSBU69
3	Razavi Khorasan	Khanroodvillage	1656	3608	5924	HSBU70
4	Razavi Khorasan	Boghmech village	1740	3650	5914	HSBU71
5	Razavi khorasan	Abqad village	1660	3629	5959	HSBU72

Morphological study

At first, 50 morphological characters were studied in the randomly selected plants of these 5 populations. Preliminary analysis revealed that 9 morphological characters differ among the studied populations. These characters are involucre length, sporangia width, stem length, involucre color, involucre form, involucre overlap, leaf color and, stem color. They were coded as binary (1 = presence, or 2 = absence), or multistate characters and used for further multivariate analysis (Podani 2000).

DNA extraction and ISSR assay

Fresh leaves were collected randomly in each of the studied populations and dried in silica gel powder. Genomic DNA was extracted using CTAB with activated charcoal protocol (Sheidai et al. 2014, Koohdar et al. 2015).

The quality of extracted DNA was examined by running on 0.8% agarose gel. Ten ISSR primers; (AGC)5GT, (CA)7GT, (AGC)5GG, UBC810, (CA)7AT, (GA)9C, UBC807, UBC811, (GA)9A and (GT)7CA commercialized by UBC (the University of British Columbia) were used (Sheidai et al. 2014, Koohdar et al. 2015).

PCR reactions were carried out according to our previous reports (Sheidai et al. 2014, Koohdar et al. 2015). For this we used 25µl volume mixture containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany).

The amplifications' reactions used also were according to our previous reports (Sheidai et al. 2014, Koohdar et al. 2015). They were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94 °C, 30 S at 94 °C; 1 min at 50 °C and 1min at 72 °C. The reaction was completed by final extension step of 7 min at 72 °C. The amplification products were visualized by running on 2% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Morphological analysis

For morphological grouping of the studied populations, coded characters were used to determine Gower distance. This was then used in UPGMA (Unweighted paired group method using average) and Ward (minimum spherical cluster) clustering (Podani 2000, Koohdar et al. 2015). Principal components analysis (PCA) biplot was performed to identify the most variable morphological characters differentiating the studied populations.

Genetic diversity and population structure

For genetic diversity analysis we used ISSR bands. These bands were coded as binary characters (presence = 1, absence = 0). Data obtained were analyzed for the genetic diversity parameters like,, Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Weising et al. 2005, Freeland et al. 2011).

For grouping of the studied populations, we used Nei's genetic distance (Weising 2005, Freeland et al. 2011). Neighbor Joining (NJ) clustering and principal coordinate analyses (PCoA) were performed after 100 times bootstrapping/ permutations (Sheidai et al. 2016, Koohdar et al. 2015).

The correlation between genetic and geographical distance in the studied populations was determined by using Mantel test as performed in PAST ver. 2.17 (Podani 2000, Hammer et al. 2001). DARwin ver. 5 (2012) programs were used for cluster analyses.

Two approaches were used to determine genetic differentiation of the studied populations, 1 – AMOVA (Analysis of molecular variance) (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall & Smouse 2006), and 2 – Nei's Gst analysis of GenoDive ver. 2 (2013) (Meirmans & Van tienderen 2004, Sheidai et al. 2016). The new genetic differentiation parameters like G'st_est = standardized measure of genetic differentiation (Hedrick 2005), and D_ est = Jost measure of differentiation (Jost 2008), were also determined.

The potential problems caused by the dominance of ISSR markers, were resolved by using the Bayesian program, Hickory (ver. 1.0) (Holsinger & Lewis 2003). It was used to estimate parameters related to genetic structure (theta B value) (Tero et al. 2003).

The genetic structure of the studied populations was also determined by two approaches: 1 – Bayesian based model STRUCTURE analysis (Pritchard et al. 2000), and 2 – maximum likelihood-based method of K-Means clustering (Sheidai et al. 2014).

For STRUCTURE analysis of dominant ISSR molecular markers, we followed the instructions of Falush et al. (2007). This was followed by performing the Evanno test to find out the proper number of genetic groups (K) by using delta K value (Evanno et al. 2005). We performed K-Means clustering according to GenoDive ver. 2. (2013), which produces two summary statistics of 1 – pseudo-F and 2 – Bayesian Information Criterion (BIC). These statistics provide the best fit for k (Çaliskan 2012). Finally, the correlation coefficient was determined between gene diversity/ genetic polymorphism and the studied envi-

ronmental features i.e. altitude, longitude, and, latitude (Sheidai et al. 2014, 2016).

Gene flow

Gene flow among populations was determined by two different approaches: 1 – Calculating Nm an estimate of gene flow from Gst by PopGen ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. This approach considers equal amount of gene flow among all populations. 2 – Population assignment test based on maximum likelihood as performed in Genodive ver. in Genodive ver. 2. (Sheidai et al. 2014, 2016).

Results

Genetic diversity

The Genetic diversity parameters estimated are provided in Table 2. The highest value for gene diversity occurred in populations 1 and 4 (0.133 and 0.129, respectively). The highest level of genetic polymorphism (15.79) occurred in population 2, while the lowest value of the same parameter occurred in population 4 (35.62).

Table 2: Genetic diversity parameters in the studied populations of *Helichrysum oocephalum*. (Population numbers are according to Table 1).

Tabela 2: Genetska raznolikost obravnavanih populacij vrste *Helichrysum oocephalum*. (Oznake populacij so enake kot v Tabeli 1).

Pop	N	Na	Ne	I	He	UHe	%P
Pop1	9.0	0.895	1.214	0.206	0.133	0.141	44.74
Pop2	3.0	0.421	1.109	0.091	0.062	0.075	15.79
Pop3	5.0	0.763	1.167	0.172	0.109	0.122	36.84
Pop4	8.0	1.053	1.184	0.212	0.129	0.138	52.63
Pop5	9.0	1.053	1.154	0.184	0.108	0.114	52.63

Abbreviations : N = Number of populations, Na = No. of Different Alleles, Ne = No. of Effective Alleles, I = Shannon's Information Index, He = Gene diversity, UHe = Unbiased gene diversity, and %P = Percentage of polymorphism.

Population genetic structure

AMOVA revealed significant genetic difference among the studied population (PhiPT = 0.196, P = 0.010). It also revealed that, 80% of total genetic variation was due to within population diversity, while 20% was due to among population genetic differentiation. Moreover, Hickory test also produced high Theta B value (0.40) supporting AMOVA. The new genetic differentiation parameters also support AMOVA results, as Gst (0.218, P = 0.001), Hedrick' standardised fixation index (G'st = 0.233, P = 0.001) and Jost' differentiation index (D-est = 0.06, P = 0.001), revealed that the studied populations are genetically differentiated.

Neighbor Joining (NJ) tree and PCoA plot produced similar results. Therefore, only PCoA plot is presented (Figure 2). The PCoA plot separated some of the studied populations from each other due to their genetic difference. This is in agreement with AMOVA. However, in some cases, plants of different populations were intermixed. This happened due to within-population genetic variability and gene flow/ shared alleles in those populations.

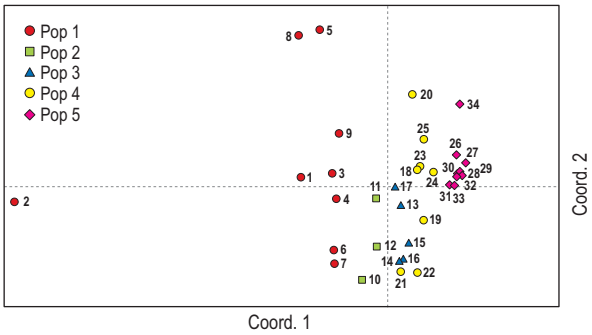

Figure 2: PCoA plot in *Helichrysum oocephalum* populations based on ISSR marker.

Slika 2: Graf PCoA populacij vrste *Helichrysum oocephalum* na podlagi ISSR markerjev.

Evanno test performed on STRUCTURE analysis and pseudo-F index of K-Means clustering produced optimum number of k = 2. These results indicated that we have 2 genetic groups in the studied populations. STRUCTURE plot (Figure 3) based on k = 2, identified these two genetic groups (potential gene pools).

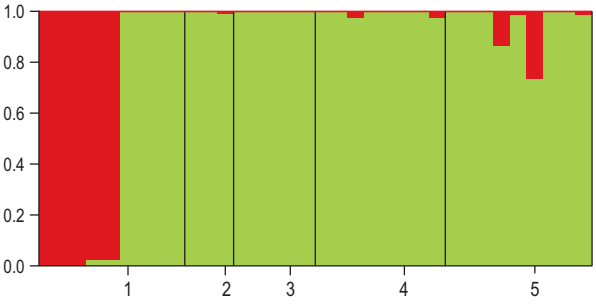

Figure 3: STRUCTURE plot based on k = 2 in *Helichrysum oocephalum* populations.

Slika 3: Graf STRUCTURE populacij vrste *Helichrysum oocephalum* s k = 2.

The STRUCTURE plot revealed that some plants in the population 1 are genetically different from the other plants within this population. They also differed greatly from the other studied populations. Plants in population 2–5 also had some alleles shared with these plants (red colored segments). Therefore, limited gene flow among these plants may explain genetic difference observed in population 1.

Correlation coefficient determined between gene diversity and environmental features was not significant (r = 0.29 with altitude, r = 0.32 with latitude, and, r = -0.10 with longitude, P .0.1). Similarly, genetic polymorphism was not also correlated with latitude (r= -0.3974), altitude (r = 0.56), or the longitude (r = -0.3974) as well.

Gene flow and genetic admixture

The reticulogram obtained revealed some degree of genetic admixture in the studied populations (Figure 4). For example, plant No. 17 of population 3 had shared alleles with plants in population 5. Similarly, plants in population 1, had shared alleles with plants in population 5.

More detailed information was obtained by population assignment test (Table 3). It revealed the occurrence of gene flow or ancestral shared alleles between plants in populations 1 and 4, 3 and 4, as well as 1 and 6.

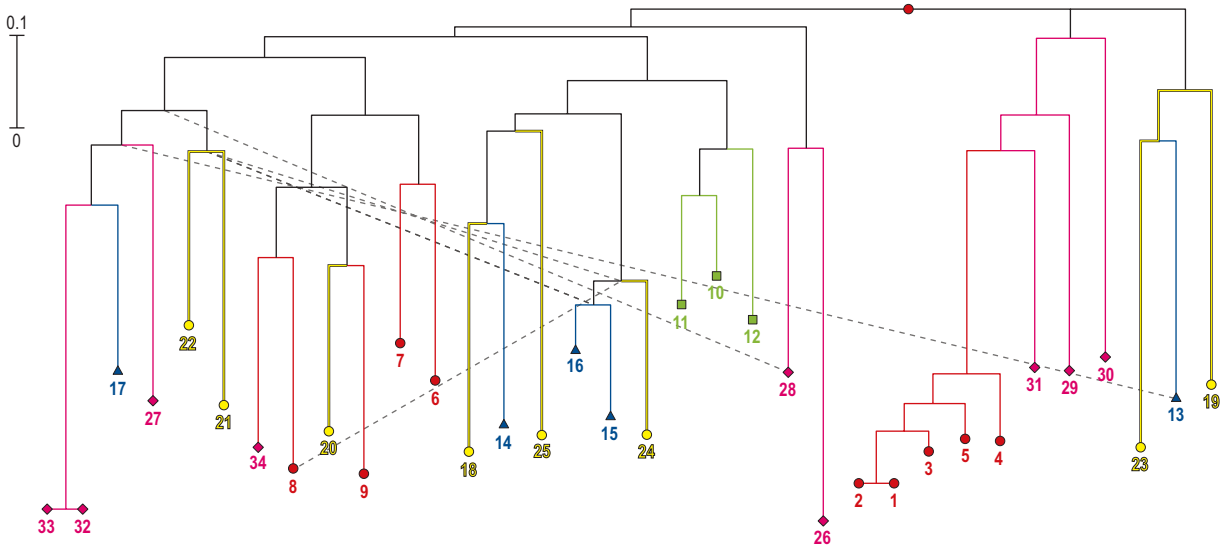

Figure 4: The reticulogram in *Helichrysum oocephalum* populations.
Slika 4: Retikulogram populacij vrste *Helichrysum oocephalum*.

Table 3: Population assignment test result showing plants inferred to be from another population.
Tabela 3: Določitev pripadnosti posameznih primerkov določeni populaciji.

Individual	Current	Inferred	Lik_max	Lik_home	Lik_ratio
6	Pop001	Pop004	-18.283	-20.724	4.881
7	Pop001	Pop004	-11.304	-20.765	18.922
8	Pop001	Pop004	-9.869	-12.016	4.295
9	Pop001	Pop004	-14.315	-18.057	7.484
13	Pop003	Pop005	-19.529	-25.196	11.335
14	Pop003	Pop004	-15.490	-18.106	5.232
17	Pop003	Pop005	-12.022	-15.868	7.691
22	Pop004	Pop003	-8.554	-12.833	8.558
24	Pop004	Pop003	-12.055	-16.591	9.071
29	Pop005	Pop001	-8.624	-13.213	9.177
30	Pop005	Pop004	-15.239	-15.67	0.861
34	Pop005	Pop001	-10.59	-11.632	2.082

The mean Nm value = 1.25 was obtained for these populations that is showing high degree of gene flow among them. Therefore, all these results revealed some degree of gene flow among *Helichrysum oocephalum*.

The Mantel test produced significant correlation between genetic distance and geographical distance of the studied populations (r = 0.22, P = 0.01). This indicated the occurrence of isolation by distance (IBD) in *Helichrysum oocephalum* populations. Therefore, gene flow mainly occurred between the neighboring populations.

Morphological variability

UPGMA and Ward clustering of morphological characters produced similar results. Therefore, only Ward dendrogram is presented (Figure 5). This dendrogram grouped the studied populations in two major clusters. The populations 1 and 2 formed the first cluster, while populations 3–5 comprised the second.

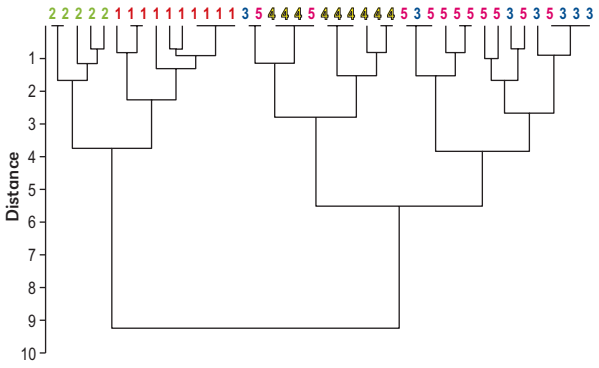

Figure 5: WARD dendrogram of *Helichrysum oocephalum* populations based on morphological data.
Slika 5: Dendrogram WARD populacij vrste *Helichrysum oocephalum* na podlagi morfoloških podatkov.

Representative plants of each population were almost grouped together and formed a separate cluster. This is particularly true for populations 1 and 2. This result indicated that each population differed in its morphological features from the other populations.

However, in populations 3–5, some of the plants were intermixed with plants of the other populations due to morphological similarities. These morphological similarities might be consequence of gene flow or genetic admixture of these populations as presented before.

PCA analysis of morphological characters revealed that the first 3 PCA axes comprised about 70% of total morphological variations. It also showed that morphological characters like, Involucre length, involucre color, involucre form, involucre overlap, leaf color and stem color, are the most variable morphological characters among the studied *Helichrysum oocephalum* populations. PCA biplot showed that the involucre form separated populations 1 and 2 from the others.

Discussion

Medicinal plants such as *Helichrysum oocephalum* are extensively used by locals and therefore are subject to negative selection pressure which reduces the in number of plants or their complete elimination from the natural habitat. We encountered high degree of population genetic differentiation. Environmental disturbances causing disappearance and fragmentation of natural populations reduce the rate of gene flow among populations, which in turn increases the population genetic differentiation. In this situation, the genetic drift acts strongly and reduces within population genetic variability (Setsuko et al. 2007, Hou & Lou 2011). However, we obtained a high degree of within population genetic variability in the studied populations of *Helichrysum oocephalum*. AMOVA indicated that, out of total genetic variation, 80% was due to within population. This was also indicated by high Nm values obtained. The observed within population genetic variability may be related to out crossing nature of this species. The presence of high within population genetic variability helps the population to cope with local environmental changes.

Both STRUCTURE analysis and population assignment test, along with reticulogram obtained revealed the occurrence of some degree of gene flow among *Helichrysum oocephalum* populations. Gene flow brings about adequate genetic diversity for the studied populations. Genetic diversity is important for continuity of plant species and adaptation to environmental conditions (Çalişkan 2012). The occurrence of gene flow between different geographical populations introduces new genes to the local populations and adds to the genetic variability of these populations (Hou & Lou 2011, Sheidai et al. 2014).

Significant AMOVA, Gst and differentiation parameters obtained for the studied populations, indicate that, in spite of limited gene flow, the local populations have acquired their specific genetic structure. This may be due to genetic drift, isolation by distance or local adaptations (Hou & Lou 2011, Sheidai et al. 2014). This has also been reported by Galbany-Casals et al. (2011) in the Mediterranean *Helichrysum italicum.*

Sabetta et al. (2006) investigated the genetic diversity in populations of *H. italicum* from Corsica and Italy by AFLP (Amplified fragments length polymorphism) molecular markers and obtained a dendrogram that grouped these populations in three primary clusters without any cases of homonymy. The plants collected from different geographical regions showed different genetic structure.

Smissen et al. (2006) investigated the genetic diversity of the endemic complex species of *H. lanceolatum* in New Zealand and reported a weak geographic structure. However, they observed that the populations followed isolation by distance model. Azizi et al. (2014) reported within-population genetic variability and the occurrence of gene flow among geographical populations in *Helicrysum leucocephalum*. These populations did not reveal isolation by distance.

Mantel test revealed a pattern of isolation-by distance across the distribution range of the studied *Helichrysum oocephalum* populations. This means that the dispersal of populations might be constrained by distance, and gene flow occurs mostly between neighboring populations (Sheidai et al. 2014). As a result, more closely situated populations tend to be more genetically similar to one another (Slatkin 1993, Hutchison & Templeton 1999, Medrano & Herrera 2008).

Morphometric studies also revealed that the studied populations differed from each other. Therefore, geographical populations in *Helichrysum oocephalum* differed in both genetic and morphological features. Azizi et al. (2014) reported morphological, cytological and genetic differences among geographical populations of *Helicrysum leucocephalum* and considered different forms as potential ecotypes within this species. Therefore, we conclude that, a combination of genetic and morphological divergence, limited gene flow and local adaptation have played role in diversification of *Helichrysum oocephalum* populations in Iran. The use of molecular markers has revolutionized the techniques for characterizing genetic variation and validates genetic selection. However, molecular markers are the most reliable source for the analysis of genome structure and behavior in medicinal plant (Chen et al. 2016). Therefore, these findings may be of use in conservation this medicinal plant in the country

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