Taxonomy and Biosystematics, 4th Year, No. 13, Winter 2012, Page: 59-72
Received: 8 July 2012 Accepted: 30 December 2012

Phenetic relationships among natural population accessions of *Glycyrrhiza glabra* L. (Fabaceae) in central Zagros region of Iran, based on quantitative morphology, flavonoids and glycyrrhizin contents data

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Abstract
Phenetic relationships among thirty five accessions from natural populations of two varieties of *Glycyrrhiza glabra* in central Zagros region of Iran were studied. Twenty one quantitative morphological characters were measured for twenty seven accessions. PCO, clustering, K-means and MDS analyses were performed on morphological dataset. Polar flavonoid constituents of twenty four accessions were extracted, purified using TLC and characterized at the skeleton class level. Glycyrrhizin contents of rhizomes in twenty four accessions were quantified using image processing methods. Results of multivariate analysis of both morphological and flavonoid spot profile data showed that accessions could be partitioned into two main groups based on geographical locality of the populations. The most variable morphological trait based on CV values, was seed area and the least variable one was Legume width in the widest portion. Accessions of both varieties produced various flavonoids of class flavones and flavonols. Seven flavonoid constituents from the two varieties were separated based on different R*f* values. The results revealed that there were moderate (not prominent) levels of variation between the studied accessions. Separation of the varieties based on the single qualitative character in the available literature, was confirmed. Rhizomes of both varieties showed similar amounts of glycyrrhizin and almost similar types of flavonoids in their TLC profiles, suggesting that both were equivalent as herbal drugs in folk medicine.
Key words: Flavonoid, Glycyrrhiza glabra, Glycyrrhizin, Iran, Morphology, Zagros

Introduction

Licorice refers to roots and rhizomes of *Glycyrrhiza glabra*; one of the about 18 accepted congeneric species in Leguminosae family (IPNI, 2008; WCSP, 2012), originated in Mediterranean region. They are distributed and grow wild throughout the northern hemisphere. Six species of the genus were reported for the Flora Iranica by Rechinger (1984): *G. bucharica* Regel, *G. aspera* Pall., *G. uralensis* Fisch. ex DC., *G. glabra* L., *G. echinata* L. and *G. macedonica* Boiss. & Orph. *Glycyrrhiza glabra* L. (Syn.: *G. violacea* Boiss.) is represented in Iran by two resembling varieties: *G. glabra* var. *glabra* (the autonym) and

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G. glabra var. *glandulifera* (WALDST. & KIT.) Boiss. (Rechinger, 1984).

Licorice species have been considered as the most important taxa in the genus; they have long been used as medicinal plants; their most important constituent (glycyrrhizin, $C_{42}H_{22}O_{16}$) is widely used as a natural sweetener and a pharmaceutical agent due to its anti-inflammatory and hepatoprotective properties (Hayashi and Sudo, 2009). Hayashi *et al.* (2005a) studied the relationships between 10 strains of so called economically important species of the genus *Glycyrrhiza* and divided them into two types (consisting GA and AT genotypes). The study failed to correlate chemotypes to genotypes, however, in other phylogenetic studies by Hayashi *et al.* (2000; 2005b), *G. glabra* was considered as a close relative to *G. inflata* and *G. uralensis*; a clade that produced glycyrrhizin as the major constituent. Other recent studies considered intra-specific variation of licorice *s. l.* species, quality control and authentication methods (Yao *et al.*, 2008; Khan *et al.*, 2009; Daei *et al.*, 2010; Zhang *et al.*, 2011). Variations in glycyrrhizin contents of licorice roots were evaluated by Haji-Mehdipour *et al.* (2008) in a number of wild populations of the species in Iran. Fars province population was reported among the three higher glycyrrhizin producing sites.

Flavonoids (also referred to as Vitamin P) are a class of plant secondary metabolites. The term is the general name of the compounds based upon a fifteen-carbon skeleton. At the simplest level, the skeleton consists of two phenyl rings (A- and B-rings) connected by a three-carbon bridge (C-ring). In general, plants alone possess the biosynthetic ability of the flavonoids (Mobh, 1939). This study was aimed to assess phenetic relationships between wild populations of the species *G. glabra* (licorice) in the central Zagros region of Iran (including Fars province), using quantitative morphological data, flavonoid spots and glycyrrhizin content profiles.

Material and Method

Plant material was collected from wild populations of G. glabra throughout Zagros Mountain. chain of Iran with the emphasis on central Zagros region. Locations of the studied specimens are shown on the map (Figure 1) and the corresponding list is presented in Table 1. Rhizome samples for flavonoid extraction and glycyrrhizin content analyses were selected from those rhizomes with 1-2 mm diameter collected in August-September, from the 0-300 mm soil layer (Douglas *et al.*, 2004; Bolouri-Moghaddam *et al.*, 2009).

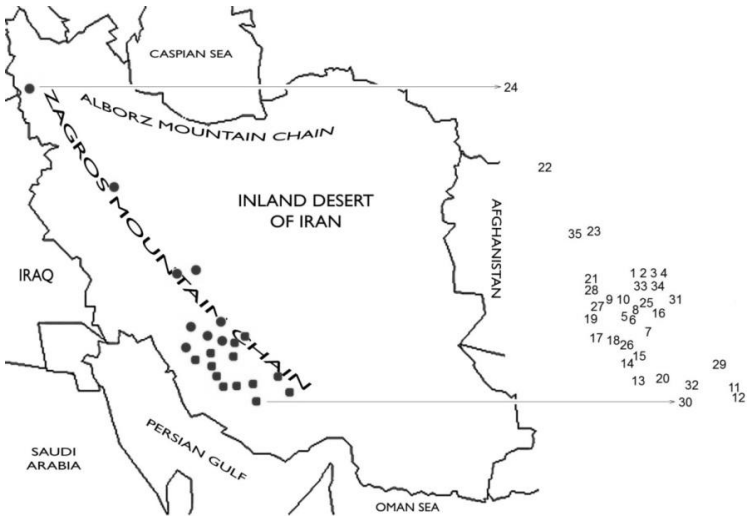

Figure 1. Locations of populations where licorice specimens were collected

Table 1. Sample code, scientific names, locality, soil texture and GPS coordinates of specimens were studied.

Sample Code	Scientific Name	Locality	Alt. (m)	Soil Texture	GPS Coordinates
S01	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Eqlid-Dasht-e Bakian	2230	Clay	30°54'38.46"N 52°41'26.95"E
S02	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Eqlid-Dasht-e Bakian	2230	Sand	30°54'38.46"N 52°41'26.95"E
S03	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Eqlid- Dasht-e Namdan	2230	Clay	30°54'38.46"N 52°41'26.95"E
S04	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Eqlid- Dasht-e Namdan	2230	Sand	30°54'38.46"N 52°41'26.95"E
S05	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Beyza-Ali Abad	1520	Clay	30° 0'54.32"N 52°21'20.65"E
S06	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Beyza-Doshman Ziyari	1520	Clay	30° 4'23.09"N 52°21'54.62"E
S07	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Marvdasht- Dasht-e Miagh	1580	Clay	29°51'42.44"N 52°45'15.69"E
S08	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Marvdasht- Ramjerd	1620	Clay	30° 5'16.66"N 52°34'38.78"E
S09	*Glycyrrhiza glabra* var *glabra*	Sepidan- Kamhor	2240	Clay	30°26'18.26"N 51°52'44.81"E
S10	*Glycyrrhiza glabra* var *glabra*	Sepidan- Kamhor	2240	Sand	30°26'18.26"N 51°52'44.81"E
S11	*Glycyrrhiza glabra* var *glandulifera*	Fars prov.: Darab	1180	Clay	28°45'23.61"N 54°30'57.82"E
S12	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Darab	1180	Sand	28°45'23.61"N 54°30'57.82"E
S13	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Firooz Abad- Joukan	1600	Clay	28°52'43.07"N 52°33'13.94"E
S14	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Koohmarreh Sorkhi	1032	Clay	29°16'27.83"N 52° 9'4.98"E
S15	*Glycyrrhiza glabra* var *glandulifera*	Fars prov.: Koohmarreh Sorkhi	1032	Clay	29°16'27.83"N 52° 9'4.98"E
S16	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Pasargad	1700	Clay	30°12'41.04"N 53°12'9.70"E
S17	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Kazeroon	860	Clay	29°38'11.38"N 51°40'33.23"E
S18	*Glycyrrhiza glabra* var *glandulifera*	Fars prov.: Khaneh Zenian	1560	Sandy-Clay	29°40'37.41"N 52° 9'34.02"E
S19	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Nour Abad	920	Clay	30° 7'25.99"N 51°33'45.86"E
S20	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Khafr	1410	Clay	28°59'17.50"N 53°12'16.87"E
S21	*Glycyrrhiza glabra* var *glandulifera*	Kohgilouyeh & Boyerahmad prov.: Yasouj	2535	Clay	30°39'28.59"N 51°33'53.52"E
S22	Glycyrrhiza Spp.	Hamedan prov.: Hamedan	1813	n/a	34°49'9.32"N 48°33'26.87"E
S23	*Glycyrrhiza glabra* var *glabra*	Esfahan- Mobarakeh	1570	Clay	32°20'4.57"N 51°30'33.42"E
S24	Glycyrrhiza Spp.	Azerbayjan prov.: Orumiyeh	n/a	n/a	37°33'23.86"N 45° 7'3.76"E
S25	*Glycyrrhiza glabra* var *glandulifera*	Fars prov.: Marvdasht- Ramjerd	1620	Clay	30° 5'16.66"N 52°34'38.78"E
S26	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Khaneh Zenian	1560	Clay	29°40'37.41"N 52° 9'34.02"E
S27	*Glycyrrhiza glabra* var *glandulifera*	Fars prov.: Noor Abad	920	Clay	30° 7'25.99"N 51°33'45.86"E
S28	*Glycyrrhiza glabra* var *glabra*	Kohgilouyeh & Boyerahmad prov.: Yasouj	2535	Sand	30°39'28.59"N 51°33'53.52"E
S29	*Glycyrrhiza glabra* var *glandulifera*	Fars prov.: Neiriz	1795	Clay	29°11'37.51"N 54°20'57.71"E
S30	*Glycyrrhiza glabra* var *glandulifera*	Fars prov.: Jahrom	1050	Clay	28°28'54.43"N 53°33'13.68"E
S31	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Ghader Abad	1900	Clay	30°16'59.57"N 53°16'0.10"E
S32	*Glycyrrhiza glabra* var *glandulifera*	Fars prov.: Fasa	1370	Clay	28°56'11.38"N 53°43'4.37"E
S33	*Glycyrrhiza glabra* var *glabra*	Fars prov.: Eqlid	2150	Clay	30°54'38.46"N 52°41'26.95"E
S34	*Glycyrrhiza glabra* var *glandulifera*	Fars prov.: Eqlid	2354	Clay	30°54'38.46"N 52°41'26.95"E
S35	*Glycyrrhiza glabra* var *glandulifera*	Charmahal & Bakhtiari prov.: Shahrekord	2200	Clay	32°18'9.40"N 50°52'52.89"E

Morphological analysis

Twenty-one quantitative morphological characters (Table 2) were measured on 27 population accessions. Each character measured up to 6 times on collected materials. Data, including length, perimeter and seed area, legumes, leaflets, etc. were obtained from calibrated digital images and entered into a raw data matrix. Averages measurements of each character were calculated and P-value was defined as (Max-Min)/Average to determine variability of each character across all samples. P- and CV (coefficient of variation) values for each character are presented in Table 2. Then, data were encoded as qualitative data and new data matrix were formed on the basis of midpoints of distribution histograms. Qualitative data matrix was used for multivariate analyses (PCA, NMDS and Cluster analysis). Multivariate analyses were performed using NTSYS-pc software package (Rohlf, 2000). Cluster analysis based on SM similarity coefficient for qualitative data and dendrogram were constructed using ME sorting method of Tamura *et al.* (2007).

Table 2. Quantitative morphological characters

	Character Name	Character Code	P-value	CV
1	Number of ovule per legume	Lg-No	2.238	53.73
2	Number of seed per legume	Lg-Ns	2.477	60.77
3	Total length of legume	Lg-L1	1.716	35.15
4	Length of style	Lg-L2	2.928	55.73
5	Widest portion of legume	Lg-W	0.933	18.49*
6	Legume area	Lg-S	2.056	49.69
7	Legume perimeter	Lg-P	1.581	33.72
8	Length of terminal leaflet	Le-L1	0.771	20.5
9	Widest portion of lamina	Le-L2	0.793	22.8
10	Length of terminal leaflet petiolule	Le-C1	1.642	48.33
11	Length of sub-terminal leaflet at left	Le-L3	0.650*	20.82
12	Length of sub-terminal leaflet petiolule at left	Le-C2	1.410	34.09
13	Length of sub-terminal leaflet at right	Le-L4	0.679	21.81
14	Length of sub-terminal leaflet petiolule at right	Le-C3	1.046	29.1
15	Terminal leaflet area	Le-S1	0.706	19.71
16	Terminal leaflet perimeter	Le-P1	0.709	19.85
17	Seed max diameter	S-a	2.317	39.07
18	Seed min diameter	S-b	2.613	44.82
19	Seed area	S-S	6.146**	122.18**
20	Seed perimeter	S-P	2.611	39.27
21	Fuzz length	F	1.638	41.01

*minimum and **maximum values of P (see text) and cv.

Profile of flavonoid spots

Flavonoids were extracted from 5 gr of dried and grinded rhizomes of each sample using methanol (80%) according to Markham (1982). Flavonoids were separated from water-insolubles by vacuum drying then dissolved in water and then further extraction by dissolving in n-butanol. Extractions were vacuum-dried and dissolved in 5 ml pure methanol. Flavonoids were separated on UV254F silica-gel thin-layers using an optimized solvent system [water: 50, ethanol: 15, butanol: 20, acetic acid: 10, chloroform: 5], then visualized under UV254nm. Chromatograms were photographed digitally to inspect the spots and score. Presence or absence of each flavonoid spot was scored as 1/0; data were entered into a raw data matrix. Multivariate analyses (PCA and Cluster analysis) were performed in NTSYS-pc software package. Cluster analysis was performed using DICE similarity coefficient as the coefficient of choice for qualitative data (Duarte *et al.*, 1999)

and dendrogram was constructed using Tamura's ME sorting method (Tamura *et al.*, 2007).

Purification and identification of flavonoids

Close spots of total flavonoid were put on a horizontal line on a thin layer to separate constituent flavonoids. Skeleton of purified flavonoids were identified using UV-spectrophotometry (200-500 nm). Major substitutions on flavonoid skeleton were identified using NaOAc, H_3BO_3, HCl, $AlCl_3$ shift-reagents according to Markham (1982).

Extraction and quantitation of glycyrrhizin

Glycyrrhizin was extracted and purified from 2 gr of dried and grinded rhizomes of each accession using the method as described by Shabani *et al.* (Shabani *et al.*, 2009). Thickened roots (rhizomes) of each accession were dried and grinded to fine powder and 2gr of each material was used for extraction. Ten µl of each extract was placed on UV254F silica-gel and run using a solvent system consisting of [chloroform: 64, methanol: 50, water: 10]. Chromatogram was photographed digitally (Figure 11) and the image was processed using ImageJ software package (Rasband, 2011) to quantify the amount of glycyrrhizin. Pure glycyrrhizin standard (1 mg/ml) was used for calibration. Measures were reported as percent of dry weight.

Results and discussion

Morphology

Twenty-one quantitative morphological characters were measured across twenty-seven accessions of two varieties (*G. glabra* var. *glabra* and *G. glabra* var. *glandulifera*). Each character was measured up to 6 times to calculate the averages and coefficients of variation (CV). Most variable character was seed area (S-S) and least variable characters were i) Widest portion of legume (Lg-W) and ii) Length of sub-terminal leaflet at left (Le-L3) based on CV- and P-values, respectively.

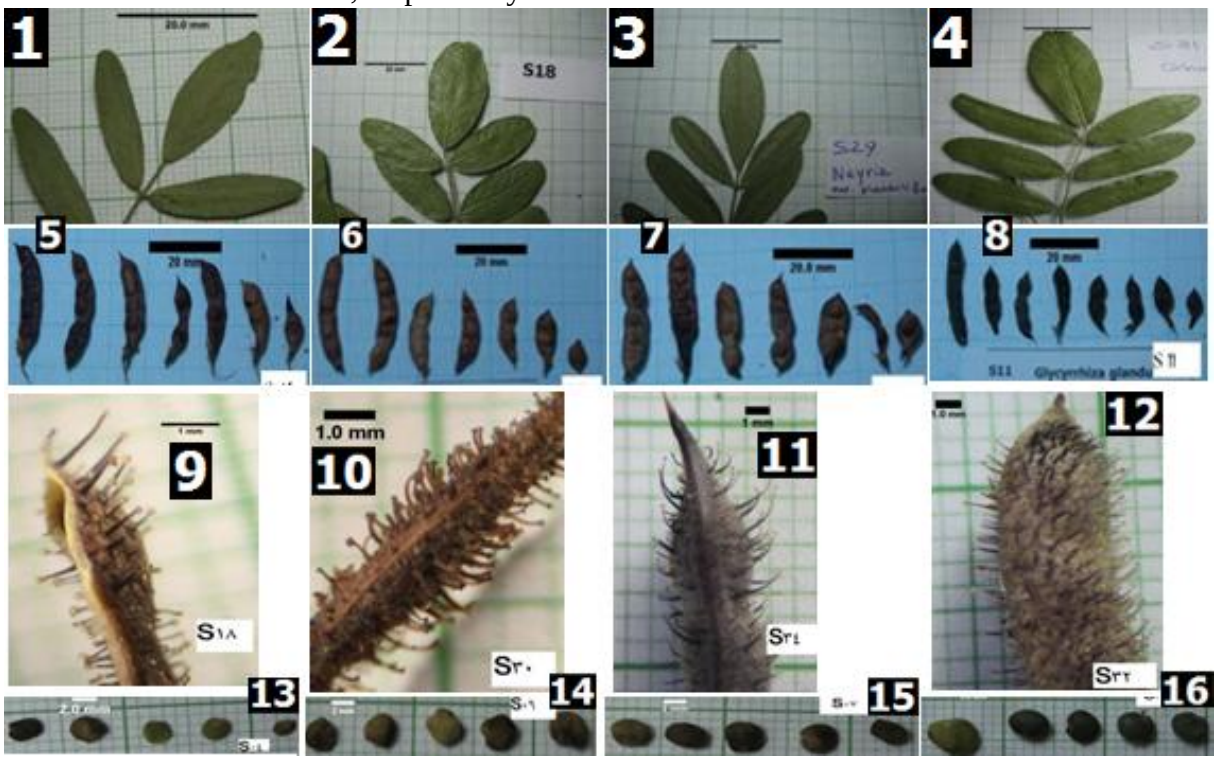

Figure 2. Exemplar photos in analysis of morphological variation in leaflets, legumes and seeds quantitative characters. Leaflets (1-4), Legumes (5-8), Fuzz on legumes (9-12) and Seeds (13-16).

Principal coordinate analysis of qualitative (0/1) morphological data was implemented using Jaccard coefficient (Jaccard, 1908), scattered accessions on the PCO 3-D plot. Relationships between accessions were evaluated by superimposition of a minimum-length spanning tree (Rohlf, 1975) and rotating the plot (Figure 3A). Cluster analysis of qualitative (0/1) morphological data using ME sorting method (Tamura *et al.*, 2007) of Simple Matching coefficient (SM) similarities, grouped 27 accessions based on geographical location of populations from which the accession was collected (Figure 3B). Resultant groupings were not clear cut; however, meaningful clusters were obtained from both analyses. Two main clusters were observed on the unrooted tree (dendrogram). One consisted of accessions S15 (Kuhmareh), S16 (Pasargad), S07, S08 (Marvdasht), S09 (Sepidan), S11 (Darab), S12 (Darab) and S13 (Firuzabad) and S14 (Kuhmareh). The other cluster consisted of the rest of accessions. Members of this cluster belonged to the variety *glabra*, except S11 and S15. Intermix of accessions belonging to the two varieties studied were also observed in other clusters on the phenogram.

Close phenetic relationship between accessions S05, S06 (collected from Beyza), S04, S02 (collected from Eghlid) and a sub cluster consisted of accessions S27 (Nourabad), S28 (Yasouj), S34 (Eghlid) and S35 (Shahrekord), all belonging to the variety *glandulifera* is shown on the phenogram. Cluster analyses of both quantitative and qualitative (0/1) morphological data failed to separate accessions based on taxonomic rank, nor "clearly" grouped accessions based on their population geographic location, although, interpretable clusters were obtained. A North-South partitioning of populations may be deduced. Populations in the first cluster were located in southern part of study area (except for S08 and S09) and populations in the second cluster were located in northern part (except for S29, S30 and S32).

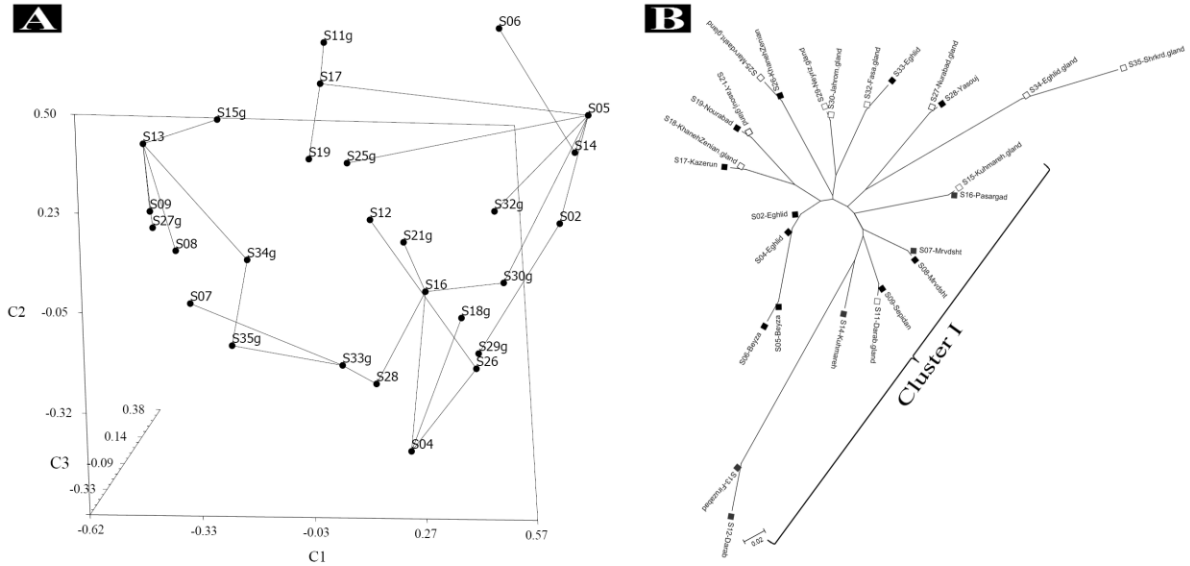

Figure 3. Multivariate analysis of qualitative (0/1) morphological data showing phenetic relationships between 27 accessions. A: Principal Coordinate Analysis using Jaccard coefficient (Jaccard, 1908). A minimum-length spanning tree (Rohlf, 1975) is superimposed on PCO plot, showing the relationships between objects (accessions) . B: Unrooted tree obtained from cluster analysis based on ME sorting method (Tamura *et al*., 2007) of Simple Matching coefficient similarities calculated by using NTSYS-pc software package (Rohlf, 2000). Open boxes are var. *glandulifera* and solid boxes are var. *glabra* (branch lengths proportional to distances).

Results of K-means-Clustering and MDS (Multi-Dimensional Scaling) showed that six clusters of accessions could be defined. Internal similarity tended to be reduced for K>6 or

K<6. Each obtained cluster for K=6 consisted of from 2 to 8 accessions. Results were compatible to those of cluster analysis using SM coefficient and ME sorting method (Figure 3). Clusters obtained in K-means-MDS analysis (K=6) were identical to corresponding clusters in the unrooted tree (Figure 3), confirming the robustness of analyses and further confirmed the partitioning of accessions into two major groups consisting of a total of six subgroups.

The first cluster consisted of accessions S02, S04-S06, S17-S19, S21, S25-S30 and S32-S35. The rest of accessions fell into the second cluster. Membership of S29, S30 and S32 in the first cluster and the membership of S08 and S09 in the second cluster were inconsistence with partitioning of accessions along NW-SE. However, the grouping of the rest of accessions was consistence to geographical partitioning.

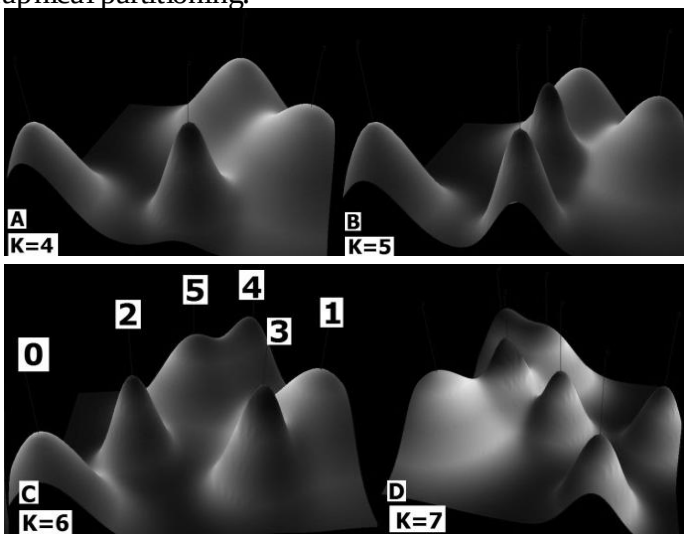

Figure 4. K-means-MDS clustering. Six clusters were defined based on maximized internal similarities in groups. For membership of each accession in each cluster refer to text and table 3.

Table 3. Membership of accessions in each cluster (K=4 to K=7). K=6 was the best solution based on maximized internal similarities in defined groups.

Cluster	0	1	2	3	4	5	6
K=4	29, 30, 32, 33	27, 28, 34, 35, 07, 08, 09, 11, 14, 12, 13	15, 16	05, 06, 04, 02, 17, 18, 19, 21, 25, 26			
K=5	29, 30, 32, 33	27, 28, 34, 35, 07, 08, 09, 11, 14, 12, 13	15, 16	25, 26	05, 06, 04, 02, 17, 18, 19, 21		
K=6	29, 30, 32, 33	05, 06, 04, 02, 17, 18, 19, 21	15, 16	25, 26	27, 28, 34, 35	07, 08, 09, 11, 14, 12, 13	
K=7	07, 08, 09, 11, 14, 12, 13	05, 06, 04, 02, 17, 18, 19, 21	15, 16	25, 26	27, 28, 34, 35	29, 30	32, 33

Flavonoids spot profiles

A total of 95 spots (bands) were scored for 25 accessions (TLC-33 using solvent system [chloroform (25%), acetic acid (25%), butanol (25%), methanol (25%)]). Number of scored bands in TLC-34 (for the 24 accessions analyzed using solvent system [water (20%) ethanol (20%) butanol (10%) chloroform (5%) acetic acid (10%) acetonitryl (10%) metanol (20%) aceton (5%)] was 89 bands, while the number of scored bands in TLC-35 (for 24 accessions analyzed using solvent system [water (50%) ethanol (15%) butanol (20%) acetic Acid (10%) chloroform (5%)] was 135 bands. TLCs were visualized under UV254nm and UV366nm. Data obtained from TLC-35 were adopted and profiles for 2-dimensional TLC for selected accessions was checked to make sure that all the possible bands were separated in TLC-35 (Figure 5).

The solvent system used for TLC-35 was a polar system, lacking non-polar components used in TLC-34. It also had increased proportions for polar solvents compared to the solvent system used for TLC-33. Therefore, it was expected that TLC-35 effectively separate the polar flavonoid constituents.

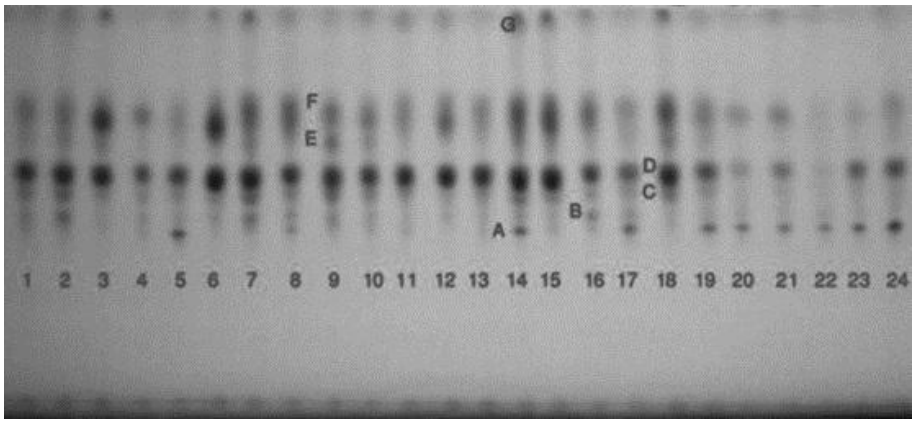

Figure 5. Flavonoid spots on chromatogram of TLC-35. Numbers beneath chromatogram correspond to accessions. For accession names and the solvent system used, refer to text.

Figure 6 shows phenetic relationships between accessions based on flavonoids spots. Four main clusters (groups1-4) of accessions were identified in the resultant dendrogram. Group 1 and group 2 consisted of a mix of 19 accessions from both varieties. Two subgroups were identified in group 1 while members of group 2 were chained. Members of the second subgroup in group 1 were arranged based on their population distance. Populations of accessions S11 and S12 (both from Darab), S13 and S14 (from Firouzabad and Kuhmareh) were close together with similar soil textures. Accessions S17 and S18 made in small cluster that was also observed in the dendrogram of morphological data. They were geographically located in close distances and were related to different varieties. The fourth group consisted of accessions S01 to 04; all located in Eghlid (NE of Shiraz). Members of this group were also closely related in the dendrogram obtained from morphological data.

Clustering of accessions in groups 1 and 2 were not exactly based on the geographical locations of their populations, however, analyses were robust when different qualitative similarity coefficients were utilized (only results of DICE similarity coefficient are presented).

Accessions studied here could be assigned to two groups; NE populations in Eghlid, Marvdasht and Sepidan and the rest of populations in NW and South of the studied area.

Hayashi *et al.* (2000) in their phylogenetic study on licorice species using *rbc*L sequence claimed that It was difficult to distinguish the variation in GL-producing species by *rbc*L sequence, since they were very similar in all of the *Glycyrrhiza* species. Our results from morphological and flavonoids spots profiles showed that although a clear-cut grouping was not achieved, variations at the infra-specific level could be elucidated. The variation of flavonoids in leaves of *Glycyrrhiza* species was reported to be higher than that of rhizomes (Hayashi *et al.*, 2003a; 2003b), which makes them proper markers for further investigation of phenetic relationships in central Zagros region populations. However, flavonoid spot profiles of rhizomes of the studied accessions in this study were also good enough to reveal phenetic relationships among them.

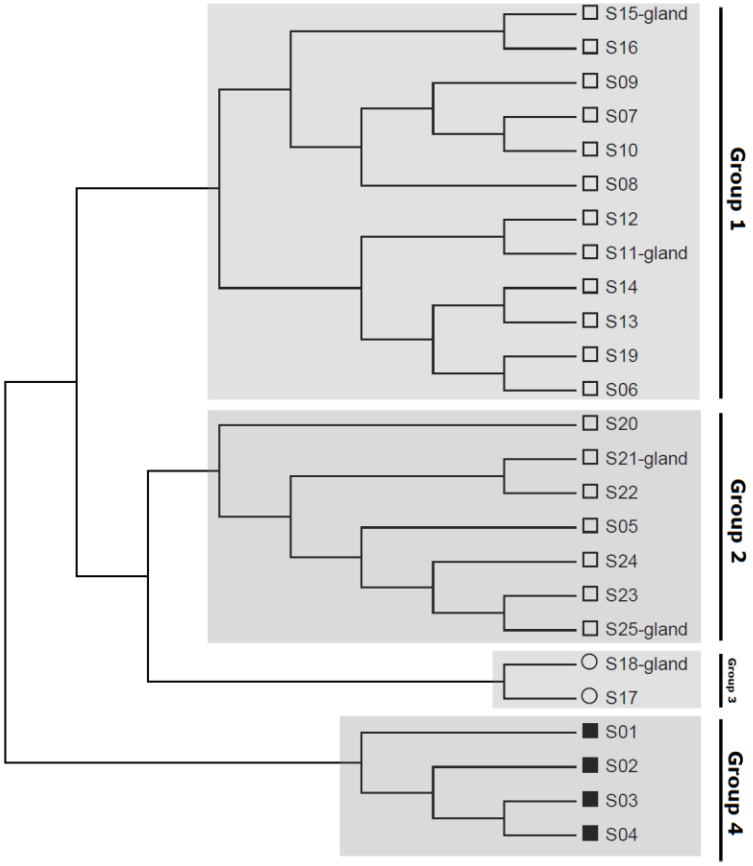

Figure 6. Phenetic relationships between licorice accessions based on flavonoid spots profile. Dendrogram based on data from TLC-35 visualized by UV 254nm and UV 366nm.

Flavonoid Identification

Flavonoid constituents of rhizomes were separated by using TLC chromatography. The solvent system used for separation of flavonoid spots (see material and method) was also used for purification of each flavonoid. Inspection of chromatograms under UV254nm showed that six bands could be extracted and purified for each variety. Chromatograms of flavonoid constituents of each variety (A: variety *glandulifera*, B: variety *glabra*) are presented in Figure 7 and R*f* values are reported in Table 4. Rf values ranged from 0.454 to 0.735 for mentioned solvent system and TLC type (refer to Material and Method) and 25^{o}C Temperature.

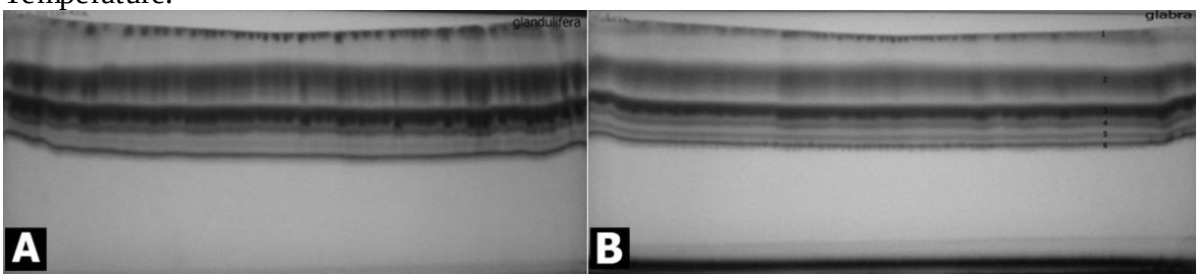

Figure 7. Chromatograms for separation and identification of major flavonoids in A: var. g*landulifera* and B: var. *glabra*. R*f* values are reported in Table 4. Twelve bands were recovered from two TLC plates from which seven different flavonoids were identified (Table 7 and Figure 9).

UV spectrophotometry of each constituent in the range of 200-500 nm before and after

applying shift reagents (Markham, 1982) showed that three flavonoid skeletons were involved in the separated flavonoids. Variety *glandulifera* consisted of 3 flavonoids, while variety *glabra* consisted of four flavonoids. Properties of each skeleton are presented in Table 4 and skeletons are themselves shown in Figure 10.

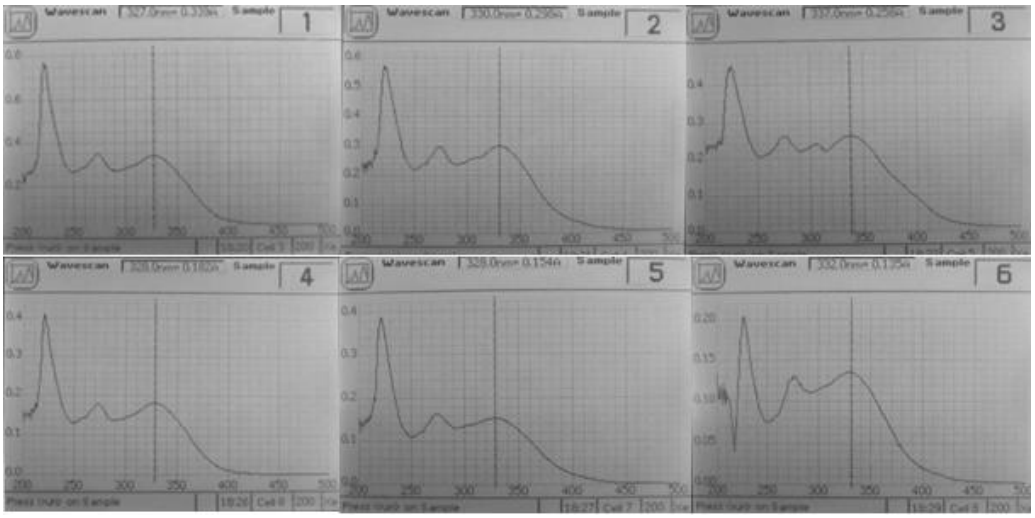

Figure 8. Exemplar UV spectra of methanolic and shift reagents for band#4 in *G. glabra* var. *glabra*. 1: MeOH spectrum, 2: $AlCl_3$ spectrum, 3: MeOH spectrum, 4: MeOH spectrum, 5: NaOAc spectrum, 6: H_3BO_3 spectrum.

Both varieties consisted of Flavones and Flavonols. Spots number 1, 5, 6 in variety *glandulifera* and spots number 1, 6 in variety *glabra* were not flavonoids, but phenolic compounds. Those spots were excluded from further identification. The flavonoid skeletons of spots number 2 and 4 in variety *glandulifera* were flavones. Spots number 2, 4 and 5 in variety *glabra* were also flavones. Spot number 3 in variety *glandulifera* and the same number in variety *glabra* were flavonols, although they were not the same. The flavonol in variety glandulifera was characterized by an additional hydroxyl group on the carbon number 7 of aromatic ring A.

Table 4. Major flavonoid skeletons identified from licorice rhizomes.

Variety	Spot	Skeleton	Rf	detail	detail	Fig#
G. glabra var *glandulifera*	2	Flavone	0.735	5-OH, 6-prenyl		1
	3	Flavonol	0.604	5-OH, 6-prenyl	7-OH	3
	4	Flavone	0.542	5-OH, 6-prenyl		1
G. glabra var *glabra*	2	Flavone	0.695	5-OH, 6-prenyl		1
	3	Flavonol	0.561	5-OH, 6-prenyl		2
	4	Flavone	0.500	5-OH, 6-prenyl		1
	5	Flavone	0.454	5-OH, 6-prenyl		1

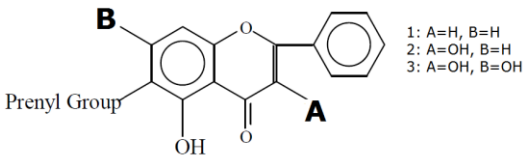

Figure 9. Major flavonoid skeletons identified from licorice. Three flavonoids with skleton 1, 3 were identified from var. *glandulifera* and four flavonoids with skeleton 1, 2 were identified from var. *glabra*.

Hayashi *et al* (2003b) reported Glabridin as the major flavonoid in underground parts of *G. glabra* collected from Kazakhstan. They also reported Rutin (RT), Isoquercitrin (IQ), Pinocembrin (PN), Licoflavanone (LF) as the four major flavonoids identified from the leaves of same specimens.

In this study, seven more flavonoids with different R*f* values were separated in this species; three flavonoids in rhizomes of the var. *glandulifera* and four flavonoids in rhizomes of the var. *glabra*.

Glycyrrhizin contents of rhizomes

Glycyrrhizin contents of rhizomes in 24 accessions belonging to the two studied varieties were measured using image processing technique. Results showed that accessions were highly variable (from 0.03 to 0.23 percent of dry weight), so that the most glycyrrhizin rich accession (S15, Kuhmareh, 0.23% DW) had more than seven folds glycyrrhizin than the glycyrrhizin-poor accession (S20, Khafr, 0.03% DW). Both the richest and poorest accessions belonged to variety *glandulifera* and both were collected from locations of similar soil texture (clay). Quantities of glycyrrhizin in rhizomes of accessions collected from sandy soils (S4, S10, S12, S17, S18, S21 and S28) were also diverse, suggesting that neither soil texture nor variety (taxonomic rank) were main factors affecting the glycyrrhizin amount in rhizomes. Hayashi and co-workers claimed that glycyrrhizin contents of rhizomes of *Glycyrrhiza glabra* were 10.5% of dry weight (Hayashi *et al.*, 2000). In another report by the same author, glycyrrhizin contents in the underground parts of 3-years-old cultivated *G. uralensis* (China type) was 2.08 to 5.12% of dry weight; relatively higher than those of the Kazakhstan type (0.75 - 2.55% of dry weight) and both were much higher than those of Iranian natural populations studied here (Hayashi *et al.*, 2005a).

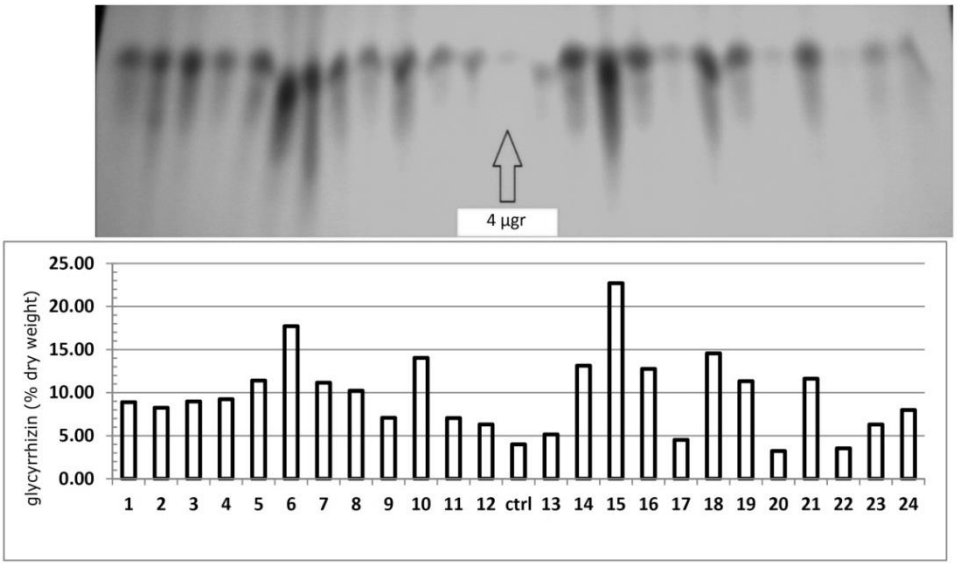

Figure 10. Quantification of glycyrrhizin contents of rhizomes using image analysis. Control lane contained 4µg of glycyrrhizin standard (pure).

Conclusions

The two varieties of *G. glabra* studied here, grew together forming mixed populations. Intermixture of closely related taxa in licorice plants was already reported for *G. glabra* and *G. uralensis* in a previous study by Hayashi *et al.* (2003b). Cluster analysis of morphological

data neither separated the two varieties nor accessions separated based on their geographical location of populations, although some meaningful groups were obtained. K-means clustering and NMDS analyses confirmed the groupings. Populations from which the accessions were obtained, could be grouped into two Northern and Southern groups in central Zagros region, with a weak support from morphological data.

Hayashi *et al.* (2005a) divided strains of *G. uralensis* into two types based on their cp-*rbc*L sequences (the GA type and AT type). He reported that there were no correlations between the chemotype and the *rbc*L genotype. Accessions of the two varieties of *G. glabra* in our study were not separate in cluster analysis as both produced similar flavonoid spot profiles.

Separation of major polar flavonoids of bulked extractions from each variety resulted in identification of seven flavonoids which had not been reported before. The major non-polar flavonoid of this species without considering the variety of samples was reported as glabridin (Hayashi *et al.*, 2003b).

Our solvent systems efficiently extracted and purified seven flavonoid skeletons which were not reported before for roots and rhizomes of *G. glabra* varieties. These flavonoids shared the prenyl group on aromatic ring-A (position at carbon 6) and differed in R*f* values and other substitution properties.

Glabridin which was a major non-polar constituent of underground parts of licorice was not a major flavonoid constituent in our results.

Both varieties consisted of flavones and flavonols. It is expected that *G. glabra* var. *glandulifera* exhibit more pharmacological properties due to the presence of an extra hydroxyl group on carbon number 7 of skeleton number 3 (Figure 10), although this claim must be examined at the variety level.

Closest relatives of *G. glabra* which was studied by Hayashi *et al.* (2005b) based on *rbc*L sequences, were *G. inflata* and *G. uralensis*. These taxa represented a clade that produced glycyrrhizin (an oleanane-type triterpene saponin) as the major constituents in rhizomes. Their sister group to *G. glabra* clade consisted of *G. echinata*, *G. macedonica* and *G. pallidiflora*; which did not produced glycyrrhizin as the major constituent (they produced macedonoside C as the major constituent in rhizomes). Our results provided more detailed information about *G. glabra* populations in Fars province at the variety level and refined the results of a previous study by Haji-Mehdipour at al. which reported Fars populations among top three populations regarding glycyrrhizin contents of rhizomes (Haji-Mehdipour *et al.*, 2008).

Finally, while the phylogenetic relationship between *glycyrrhiza* spp. at species level is clear, the infra-specific relationships at population level are still not known. Phenetic relationships between varieties of *G. glabra* which was studied here claimed that their classification under the putative species was accurate, as they shared similarities in amounts of glycyrrhizin produced in rhizomes, morphological characters and flavonoid constituents. However, the varietal rank of these taxa may be changed to forma according to suggestions made by Brummitt (1990). Results of this study showed intermix of both populations and their characteristics regarding morphology, flavonoids and glycyrrhizin contents, support this suggestion.

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مطالعه روابط فنتیک میان جمعیت‌های طبیعی گیاه شیرین‌بیان (تیره باقلائیان) در ناحیه زاگرس مرکزی ایران بر اساس داده‌های ریخت‌شناسی کمّی، ترکیبات فلاونوئیدی و محتوای گلیسیریزین

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چکیده

روابط فنتیک میان ۳۵ نمونه جمعیتی جمع‌آوری شده از جمعیت‌های طبیعی دو واریته از گونه *Glycyrrhiza glabra* در ناحیه زاگرس مرکزی ایران مورد مطالعه قرار گرفته است. ۲۱ صفت ریخت‌شناسی کمّی روی ۲۷ نمونه جمعیتی اندازه‌گیری شد و آنالیزهای مختصات اصلی، خوشه‌بندی، K-means و MDS روی داده‌های ریخت‌شناسی صورت گرفت. محتوای فلاونوئیدی ۲۴ نمونه جمعیتی استخراج و با روش کروماتوگرافی لایه‌نازک خالص‌سازی و بررسی در سطح تعیین اسکلت و کلاس فلاونوئیدی صورت گرفت. محتوای گلیسیریزین ریزوم‌ها با استفاده از روش پردازش تصویر در ۲۴ نمونه به صورت کمّی اندازه‌گیری شد. نتایج آنالیزهای چند متغیره روی داده‌های ریخت‌شناسی و داده‌های پروفایل لکه‌های فلاونوئیدی نشان داد که می‌توان نمونه‌های جمعیتی مطالعه شده را بر اساس موقعیت جغرافیایی محل جمع‌آوری نمونه‌ها به دو گروه عمده تقسیم نمود. اندازه سطح بذر، متغیرترین صفت ریخت‌شناسی کمّی بر اساس مقادیر CV تشخیص داده شد و عرض نیام در عریض‌ترین بخش، کمترین تغییرات را نشان داد. نمونه‌های هر دو واریته، فلاونوئیدهای متنوعی را از کلاس‌های فلاون و فلاونول تولید کرده بودند. در مجموع، تعداد ۷ ترکیب فلاونوئیدی مختلف با *Rf*های متمایز از دو واریته بدست آمد. نتایج، سطوح متوسطی از تنوع میان نمونه‌های مورد مطالعه را نشان داد. نتایج این مطالعه، جداسازی دو واریته در منابع موجود بر اساس یک تک صفت کیفی را تأیید می‌کند. ریزوم‌های هر دو واریته، مقادیر مشابهی از گلیسیریزین و انواع مشابهی از فلاونوئیدها را در پروفایل کرواتوگرام‌های لایه نازک ارایه نمودند که اهمیت و کاربرد یکسان ریزوم‌های هردو واریته را از نظر کاربرد به عنوان دارویی گیاهی در طب سنتی نشان می‌دهد.

واژه‌های کلیدی: ایران، زاگرس، شیرین بیان، فلاونوئید، گلیسیریزین، ریخت‌شناسی

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