Phytochemical and morphological diversity analysis of *Ziziphus nummularia* (Burm.f.) Wight & Arn. populations in South of Iran

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Abstract

Ziziphus nummularia is a multipurpose and tropical tree with medicinal, nutritional, industrial, and economic values. This tree, which belongs to the Rhamnaceae family, is originated from the South of Asia and North of Africa. This research was carried out to investigate the phytochemical and morphological diversity of 20 wild populations collected from different Southern regions of Iran. Statistical significant difference ranges between population were found in respect to saponin of the leaf (2.2-5.4 mg/g) and fruit (1.2-3.2 mg/g), phenol of the leaf (0.7-2.9 mg/g) and fruit (0.03-0.4 mg/g), tannin of the leaf (0.8-3.5 mg/g) and fruit (1.5-1.7 mg/g), and flavonoid of the leaf (3.3-4.3 mg/g) and fruit (1.5-2.4 mg/g). A factor analysis based on principal component analysis (PCA) revealed that the first three components (PC1-PC3) explain 79.04% of total variations. The first component (PC1) is explained by the most important traits of the PCA coefficient such as the leaf saponin, width of the end leaf, fruit saponin, length of the end leaf, leaf length and width, and leaf phenol with 42% of the total variation. Hierarchical cluster analysis divided the populations into four main groups with high diversity. In general, the Izeh Tarakab population had the highest content of leaf and fruit saponin. The content of leaf and fruit saponin as the major secondary metabolite could be a good determinant for detecting diversity in the wild population of *Z. nummularia*.

Keywords: flavonoid; phenol; principal component analysis (PCA); saponin; tannin

Introduction

The genus *Ziziphus* Mill., which belongs to the Rhamnaceae family, includes a large number of species all around the world. Of these species, five distinct species have been introduced and one hybrid species in the Flora Iranica (Iran plateau region) while four of them have been found in Iran (Rechinger, 1977). *Z. nummularia* (Burm.f.) Wight & Arn. is one of the Iranian species of the genus and is a tropical tree originated from South Asia and North Africa. This tree is also widely distributed in the south of Iran (Dinarvand and Zarinkamar, 2006). The plant extends from Iran to India and grows in dry and semi-arid regions of sandy and

rocky soils. The identification key of this species is introduced as follows, deciduous shrub, 1-3 m high, branches numerous, hardly curved and pubescent with two thorns one straight and the other curved. Leaves alternate, ovate roundish, 5-15 or rarely 30 mm long, 5-20 mm wide, pubescent in both surfaces especially on lower surfaces. Inflorescence pubescent racyme, calyx 3 mm long, corolla yellow, 3 mm long, stamens 5, style bifid, drupe spherical, red or dark brown (Dinarvand and Zarinkamar, 2006). *Z. nummularia* fruits are very nutritious and are usually eaten freshly. The fruits contain 81 to 97% of the pulp, 12-23% TSS, 0.13-1.42% acidity, 3.1-14.5% total sugars, 1.4-9.7% sodium, 5.6% sucrose, 1.5% glucose, 2.1% fructose, and 1.0% starch. This fruit, as a rich source of vitamin C (Akhtar *et al.*, 2017), is used as laxative and astringent. Moreover, its leaves are used in scabies, boils, and as an expectorant. Given its regrowth ability and its ability to function due to its root system, it is able to maintain its vitality against extreme events such as cold and dry (Mahishwar and Bhandari, 1963). The most suitable temperature for *Z. nummularia* growth is from 25 to 35 °C, exceeding which its formation will reduce (Pandey and Sing, 2010) although the species is evergreen, some of its leaves fall in the hot season (Mahishwar and Bhandari, 1963). Several factors, such as soil and climatic conditions, affect the content of biochemical compounds of plants.

The genotype of the population was revealed as an important factor in the phytochemicals content of the plant tree (Seta *et al.*, 2019). Studying the genetic diversity of any plant species provides important information for breeding programs as natural populations are a useful source of diversity (Awasthi *et al.*, 2009). The plants were selected based on morphological traits of their phenotypic appearances. The phenotype of each plant is composed of genetic and environmental factors. Thus, some of the apparent characters can be used as a marker for the identification, differentiation, and classification of species and visible differences such as flower colour and flower shape can be utilized as morphological features in the identification of different species (Paterson *et al.*, 1991). Furthermore, due to the presence of mutagenic agents and high risk of cancers, it is suggested using antioxidant compounds of the medicinal plant as an appropriate alternative or preservatives. These compounds can also be utilized as the available and rich source of antioxidant constituents in food industries and pharmacy. Furthermore, it has been found that the genetic variation of the cultivars plays an important role in the antioxidant capacity and chemical properties of all populations of jujuba (*Z. jujuba*) (Gao *et al.*, 2011).

The reasons for conducting the present study are as follows: The importance of *Z. nummularia* species, vast distribution and variation of species, the necessity of preserving of population, lack of a study on diversity evaluation in Iran, and introducing appropriate varieties for the supply of sustainable plant materials according to the needs of industries, and processing the herbal medicines in the country. The main objectives of the study were to identify biodiversity and the most important phytochemical traits of populations, determine the extent of population distribution and introduce populations of *Z. nummularia* with appropriate pharmaceutical value for sustainable cultivation and development in Iran and West Southern Asia. Moreover, comparing and determining the correlation between morphological and phytochemical traits of *Z. nummularia*, identifying its populations in Iran, and introducing of the superior population in qualitative and quantitative traits for reproduction and presentation to the manufacturing and processing industries are the major goals of this study.

Materials and Methods

Plant materials and morphological measurement
Natural habitats of *Z. nummularia* presence was identified after reviewing resources and referring to several areas in southern Iran. *Z. nummularia* leaves were sampled in late summer 2017 (as the maximum foliation period) from young (new one-year-old) and annual branches. Also, *Z. nummularia* fruits were harvested during two phenological stages in the fall. *Z. nummularia* fruits were sampled by the systematic-random method in the early stage of fruiting (from immature green fruits), as well as in the late stage of fruiting

(from fully ripe fruits). The treatments included 16 populations from different regions and natural habitats of Iran (in the provinces of Khuzestan, Kohgiluyeh-Boyer Ahmad, and Fars) with three replications from the same age trees in each region and 5 observations in every tree.

Herbarium specimens of the studied plants were prepared and the accurate identification of collected plants was carried out using valuable references. The voucher specimens were deposited at the Institute of Medicinal Plants Herbarium (IMPH). Detailed area information of collected plants is presented in Table 1. Plant specimens were collected from the longitude of 49º 50" 12' to 51º 60" 67', latitude of 30º 02" 41' to 31º 70" 94', altitude of 181 to 948 m, with average annual temperature of 22 to 35 °C. Also, the leaf mass samples were kept at the refrigerator (4±2 °C) until laboratory testing and analysis. In diversity studies, phytochemical and morphological traits are of particular importance for determining similarities and differences between groups or even in close species. In total, 25 traits were measured based on typical plant indicators with 17 morphological and developmental characteristics as well as 8 important phytochemical and pharmacological characteristics. Geographical regions and morphological characteristics for all populations were measured using laboratory equipment such as GPS and altimeter, millimetre ruler, Sartorius precision scale (with an accuracy of 0.001 g), and digital calliper (Table 2). In addition, the qualitative characters were coded in binary or multistate characters, clustered, and standardized (mean = 0 and variance = 1).

Phytochemical measurement

<u>Preparation of extracts for measurements</u>
The leaves and unripe and ripe fruits of *Z. nummularia* were dried in the shade at room temperature and then were ground to a fine powder. The powdered plant organs were extracted with 80% methanol using percolator for 48 h and then the extracts were dried by rotary apparatus. Finally, the extracts were enriched by complete drying at 60 °C. The prepared methanolic extract was kept at 4 °C until use. To prepare the aqueous extract, distilled water was used as a solvent. The extraction procedure was done at 25 °C for 24 h. During this time, the beaker containing the materials was placed on the rotator for better extraction. After centrifuging, the supernatant was removed and dried (Pourmorad *et al.*, 2006).

<u>Total flavonoids</u>
The amount of total flavonols was estimated by the aluminium chloride method (Ranjith, 2009). The extract was smoothed and placed in a 100 ml balloon with 70% methanol. Then, 2 ml of methanolic extracts of the plant were mixed with 1.5 ml of methanol, 0.1 ml of aluminium chloride (methanol 10%), 0.1 ml of potassium acetate (1 mol), and 2.8 ml of distilled water. The solutions were then placed at room temperature for 30 min. The absorbance of each composition was measured at 415 nm with a spectrophotometer (UV-Visible Model Cary 300). The standard curve was prepared with quercetin methanolic solutions at concentrations from 250 to 1000 μg/ml.

<u>Total phenolics</u>
To prepare the extract, 0.2 g of dried plant powder was used for soaking in methanol 70% at a 3:1 ratio (3 parts methanol and 1 part of herbal powder) for 24 h. After 1 h, the specimens were smoothed with filter paper and the solution reached 25 ml. To prepare the standard curve, 6 ml were poured standard gallic acid solution. Next, 5 ml of Folin-Denis reagent and 10 ml of 35% sodium carbonate were added and the distilled water was fed to a volume of 50 ml. The beakers were placed at room temperature for 45 min to maximize colour intensity. The solutions were absorbed by a spectrophotometer (UV-Visible Model Cary 300) apparatus at a wavelength of 760 nm and in the presence of a Blanc (5 ml Reagent Folin-Denis). Here, 10 ml sodium carbonate 35% was determined with a volume of 100 ml and its absorption curve was plotted relative to the concentration. To determine the absorbance of the specimens, 1 ml of the extract was mixed with 5 ml of Folin-Denis reagent. Afterward, 10 ml of sodium carbonate 35% in 100 ml bottles was mixed with distilled water in

a volume of 100 ml for 45 min at room temperature to maximize colour intensity. The absorbance of the specimen was read at 760 nm and, in the presence of a Blanc, the unobstructed sap was calculated according to the standard curve based on mg per 100 ml gallic acid) Wettasinghe and Shahid 1999).

Table 1. Information on collection area of *Z. nuummularia* populations

N o	Populati on no.	Region originated	Latitude (N)	Longitude (E)	Altitu de (m)	Average maximum temperature (°C)	Average minimum temperature (°C)	Average maximum moisture (%)	Average minimum moisture (%)
1	KH1	Khuzestan - Behbahan	30° 59" 68'	50° 35" 21'	328	35.2	17	88	34
2	KH2	Khouzestan - Ramhormoz	31° 25" 55'	49° 63" 36'	181	30.1	20.3	76	35
3	KH3	Khuzestan - Haftgol	31° 45 49'	49° 56" 55'	315	34	17.2	83	39
4	KH4	Khuzestan - Baghmalek	31° 52" 36'	49° 50" 12'	630	28	15	79	37
5	KH5	Khuzestan – Ghaleh tool Baghmalek	31° 65" 68'	49° 92" 19'	701	29	15.3	79	39
6	KH6	Khuzestan - Izeh Turkab	31° 69" 18'	49° 75" 05'	786	28	14	71	35
7	KH7	Khuzestan - Izeh Jaghband	31° 70" 94'	49° 81" 28'	791	29	13.5	68	33
8	KH8	Khuzestan - Izeh Koolfarh	31° 52" 01'	49° 52" 00'	790	29.3	13.4	64	40
9	F9	Fars - Qaemea	29° 84" 14'	51° 60" 67'	883	23	11	53	22
1 0	F10	Fars - Khomezar Noorabad	30° 02" 41'	51° 56" 10'	908	25.8	11.8	69	27
1 1	F11	Fars-Fahliyan Norabad	30° 15" 61'	51° 53" 12'	901	25	10	73	34
1 2	F12	Fars-Mosiry Rostam	30° 15" 56'	51° 53" 14'	920	22	10	56	27
1 3	F13	Fars - Tel pir Babaamidan	30° 26" 83'	51° 49" 10'	948	23	8	57	29
1 4	F14	Fars - Dehno Babaamidan	30° 34" 04'	51° 30" 98'	935	23	10	73	38
1 5	F15	Fars - Coupon	30° 33" 12'	51° 27" 84'	917	29.1	15.4	65	27
1 6	K16	Kohgiluyeh and Boyerahmad - Basht	30° 32" 93'	51° 17" 99'	781	25	11	64	29
1 7	K17	Kohgiluyeh and Boyerahmad - Gachsaran	30° 35" 12'	50° 80" 68'	719	26.5	12.4	62	20
1 8	K18	Kohgiluyeh and Boyer Ahmad-lick	30° 90" 22'	50° 09" 67'	673	25	9.1	51	24
1 9	K19	Kohgiluyeh and Boyer Ahmad -Dehdasht	30° 52" 32'	50° 45" 21'	803	26	8	53	22
2 0	K20	Kohgiluyeh and Boyer Ahmad – haft cheshmeh Gachsaran	30° 04" 71'	50° 61" 43'	735	27.5	13.8	68.75	32

Extraction of tannin

To measure tannin, to 1 ml of methanolic extract, 1 ml of methanol and 20 mg of polyvinylpyrrolidone was added and then vortexed for 10 seconds and then placed in ice for 30 min. The specimens were then centrifuged for 6 min a day for 10 min. Then, 1.5 ml of the centrifuge solution was poured into a micro-tube, to which 10 mg of polyvinylpyrrolidone was added and re-extracted. After centrifuging, 1 ml of the extract was removed, and the tannin was measured. To prepare a standard curve in 6 balloons, 50 ml of 1, 2, 3, 4, 5, and 6 ml of standard gallic acid solution was poured. Next, 5 ml of reagent Folin-Denis and 10 ml of 35% sodium carbonate were added, and distilled water was fed to a volume of 50 ml. The balloons were placed at room

temperature for 45 min to maximize colour intensity. Absorption of solutions was determined by a spectrophotometer at 760 nm and in the presence of a blender (5 ml Reagent Folin-Denis and 10 ml of 35% sodium carbonate in the volume of 100 ml), and its adsorption curve was plotted relative to the concentration. To determine the absorbance of the specimen, 1 ml of the extract was mixed with 5 ml of Folin-Denis and 10 ml of 35% sodium carbonate in a 100 ml bottle with distilled water in a volume of 100 ml and was placed for 45 min at room temperature to maximize colour intensity. The absorbance of the specimen was read at 760 nm in the presence of blanc, and then the unknown concentration was calculated according to the standard curve based on mg per 100 ml of gallic acid. According to this method, tannin compounds were deposited with polyvinylpyrrolidone and their value was calculated based on the difference in absorption (Makkar, 2000).

Extraction of saponin

To extract and identify saponin, the extract from alcoholic extract (infiltration) was used. For this purpose, a vacuum distillation apparatus was utilized to separate the solvent extract. Also, to identify the saponin and its contents, 1 g of ethanol extract was added to the test tube and added to the distilled water, then the tube was shaken vigorously for 5 min. Finally, the saponin level was measured after settling for 30 min (Salehi Surmaghi *et al.*, 1992).

Statistical analysis

The treatments included 20 different wild populations from natural habitats with 3 sampling replications in each region. The statistical analysis of the data is obtained from this experiment in a complete randomized design (CRD) with three replications. An analysis of variance (ANOVA) appropriate to the experimental design was performed using SPSS software (ver. 24). Mean comparison of the traits was made using Duncan Multiple Range Test at $p \leq 0.05$ significance level. A hierarchical cluster analysis (HCA) of the data was performed based on the Euclidean distances of morphological and phytochemical traits, using the Wards method. To determine the most variable characters among the populations, factor analysis (FA) based on principal component analysis (PCA) was performed.

Results and Discussion

The ANOVA results showed that most of the morphological and phytochemical traits have a significant difference at the 5% level, except for the sub-stem length at a 1% level (Table 2). Moreover, a significant difference was observed between the mean values of leaf and fruit characteristics.

Table 2. Analysis of variance for morphological and phytochemical trials in *Ziziphus nummularia* populations

S.O.V	df	Mean of square (MS)									
		Leaf width	Leaf length	Number of thorns	Thorns	Lateral stem length	Flower stem length	Intermediate length	Annual branches length	The length of the end leaf	The width of the end leaf
Replication	2	5.994[*]	3.556[*]	8.15[**]	5.162[*]	1.071[*]	0.059[ns]	0.017[*]	1163.6[ns]	0.079[**]	0.009[ns]
Treatment	19	14.207[*]	12.137[*]	361.354[*]	8.632[*]	0.156[*]	1.79[*]	0.059[*]	1185.7[**]	8/786[*]	5.074[*]
Error	38	0.458	0.419	18	0.256	0.025	0.02	0.928	542.687	0.046	0.087
C.V. (%)	-	6.062	4.847	9.741	4.59	8.774	3.863	2.07	8.802	2.201	4.238
S.O.V	df	Mean of square (MS)									
		Unripe fruit length	Unripe fruit width	Ripe fruit length	Ripe fruit width	Weight of fruit	Weight of the	Core weight of the ripe fruit	Fruit saponin	Fruit tannin	Fruit flavonoids

						premature	ripe fruit				
Replication	2	0.005^{ns}	0.004^{ns}	0.036^{ns}	0.006^{**}	0.01^{*}	0.087^{*}	0.00029^{ns}	0.141^{**}	0.0001^{**}	0.002^{**}
Treatment	19	0.935^{*}	1.505^{*}	3.335^{*}	3.937^{*}	0.079^{*}	0.473^{*}	0.022^{*}	0.846^{*}	0.069^{*}	0.212^{*}
Error	38	0.033	0.026	0.013	0.005	0.035	0.005	0.0011	0.1	0.00004	0.002
C.V. (%)	-	2.45	2.563	0.961	0.572	4.099	4.576	8.001	18.813	0.113	2.186

S.O.V	df	Mean of square (MS)				
		Fruit phenol	Leaf saponin	Leaf tannin	Leaf flavonoids	Leaf phenol
Replication	2	0.0002^{**}	0.117^{*}	0.005^{**}	0.0005^{**}	0.0009^{*}
Treatment	19	0.035^{*}	2.834^{*}	1.483^{*}	0.285^{*}	1.18^{*}
Error	38	0.004	0.021	0.002	0.0003	0.008
C.V. (%)	-	3.643	4.441	2.733	0.435	4.229

*: significant at $p \leq 0.05$; **: significant at $p \leq 0.01$; CV: Coefficient of variation; df: Degree of freedom; S.O.V: Source of variation.

According to Table 3, the highest value of annual branches length was related to the plants collected from the Khuzestan-Haftkel population (KH3) while its lowest value was found in the specimens related to Khuzestan-Behbahan (KH1). Intermediate length ranged from a low length in Kohgiluyeh and Boyerahmad-Basht (K16) to a high length in Fars- Coupon (F15). As shown in Table 3, the highest flower stem length was obtained from plants in the Fars-Qaemea (F9) location, while the lowest peduncle length was recorded from the Kohgiluyeh and Boyerahmad-Basht (K16) location. The samples collected from Khuzestan-Haftkel (KH3) showed the highest lateral stem length while the lowest lateral stem length was observed in the plants from Kohgiluyeh and Boyer Ahmad-Dehdasht (K19). The maximum and minimum thorn lengths were reported from Khuzestan-Ghale Tol Baghmalek (KH5) and Fars-Dehno Baba Meydan (F14; 7.188 mm), respectively. The number of thorns ranged from a low value (33.3) in the population of Kohgiluyeh and Boyer Ahmad-Lick (K18) to a high value (64) in the population of Kohgiluyeh and Boyer Ahmad-Dehdasht (K19). The highest lengths of leaf, the end leaf, unripe fruit and ripe fruit were respectively observed in the samples collected from population of Khuzestan-Baghmalek (KH4), Khuzestan-Ramhormoz (KH2), Fars-Coupen (F15), and Khuzestan-Baghmalek (KH4). On the other hand, their lowest lengths were respectively obtained from populations of Kohgiluyeh and Boyer-Ahmad-Basht (K16), Khuzestan-Izeh Kul-e Farah (KH8), Fars-Fahliyan Nourabad (F11), and Fars-Qaemea (F9). In addition, the highest widths of leaf, the end leaf, unripe fruit, and ripe fruit were respectively detected in the samples collected from populations of Khuzestan-Baghmalek (KH4), Khuzestan-Izeh Tarakab (KH6), Khuzestan-Ramhormoz (KH2), and Khuzestan-Ghale Tol Baghmalek (KH5); meanwhile, their lowest widths were respectively observed from populations of Kohgiluyeh and Boyer Ahmad-Dehdasht (K19), Khuzestan-Behbahan (KH1), Fars-Tel Pir Babamaidan (F13), and Fars-Kopen (F15). The highest value of the weight of premature fruit (1.07 g), the weight of the ripe fruit (2.33 g), and core weight of the ripe fruit (0.54 g) were related to the population of Fars-Fahliyan Nourabad (F11), Khuzestan-Baghmalek (KH4), and Kohgiluyeh and Boyer Ahmad-Haft Cheshmeh Goshsaran, respectively; meanwhile, the lowest values of these traits were observed in a population of Kohgiluyeh and Boyer-Ahmad-Gachsaran (K17), Fars-Kopen (F15), and Fars-Khoome Zar Nourabad (F10), respectively.

Mean comparison results showed that the highest and lowest values of leaf phenol were related to Kohgiluyeh and Boyer Ahmad-Haft Cheshmeh Goshsaran (K20), and Fars-Dehno Baba Meydan (F14), respectively. The highest value of phenol leaf was K20, which was not significantly different from KH3, KH4, KH5, K18, and K19 populations. In this study, the highest amount of leaf flavonoids was found in the population of Fars-Tel Pir Baba Meydan (F13), which had a significant difference with Khuzestan-Baghmalek (KH4), and Fars-Khoome Zar Nourabad (F10) populations as the lowest values of populations. The maximum

and minimum content of leaf tannin were obtained from Khuzestan-Baghmalek (KH4) and Fars-Dehno Baba Meydan (F14), respectively. The highest leaf tannin content was recorded in KH4, which had a significant difference with all populations. The highest amount of leaf saponin was found in the Khuzestan-Izeh Tarakab (KH6) population while its lowest amount was determined in the Fars-Kopen (F15) population (Table 4). The highest amount of fruit phenol, flavonoids, and tannins was observed in the KH2 population while the highest amount of fruit saponin was obtained in the KH6. The lowest value of fruit phenol, flavonoids, tannins, and saponin was obtained in the K18, KH6, K20, and F12 population, respectively (Table 4).

However, plants from Khuzestan-Ramhormoz (KH2) location showed the highest length of the end leaf, unripe fruit width, fruit phenol, fruit flavonoid, and fruit tannin compared with other populations. Furthermore, the population of Khuzestan-Baghmalek (KH4) had the highest values in characteristics of leaf length, leaf width, ripe fruit length, weight of the ripe fruit, and leaf tannin (3.5 mg/g) (Table 3 and 4). Finally, the amount of leaf and fruit saponin as the most important bioactive ingredient was highest in Khuzestan-Izeh Tarakab (KH6) population (Table 4).

Table 3. Comparison between the means of morphological traits in the studied of *Z. nummularia* populations

Population no.	Annual branches length (mm)	Intermediate length (mm)	Flower stem length (mm)	Lateral stem length (mm)	Thorns length (mm)	Number of thorns	Leaf length (mm)	Leaf width (mm)	Length of the end leaf (mm)
KH1	220.33 c	14.34 f	3.35 h	1.68 de	12.205 bc	35.00 ef	9.95 i	7.815 j	7.34 m
KH2	277 b	18.46 b	4.35 cd	1.82 cde	10.81 fgh	35.67 ef	14.53 bcd	12.67 bcde	12.73 a
KH3	321.67 a	16.51 d	3.67 g	2.26 a	12.07 bcd	43 c	12.06 gh	10.19 hi	10.56 ed
KH4	242.67 bc	12.64 h	3.15 hij	1.74 de	10.37 igh	59 a	16.44 a	14.91 a	10.01 f
KH5	271.67 b	13.69 g	3.97 ef	1.79 ed	14.16 a	54 a	15.35 abc	9.25 i	10.83 cd
KH6	271.67 b	17.44 c	4.09 ef	1.82 cde	11.15 defgh	33.67 ef	15.35 abc	13.52 b	11.87 b
KH7	266.67 b	16.67 de	4.14 ed	1.78 ed	12.65 b	53.33 b	14.43 bcde	12.67 bcde	9.51 g
KH8	270.67 b	15.63 e	4.14 ed	1.72 de	10.26 ih	50.67 b	14.42 bcde	12.40 bcde	6.95 n
F9	269.33 b	13.57 g	5.64 a	2.15 abc	11.04 efgh	44.33 c	11.66 h	9.35 i	11.12 c
F10	270.33 b	14.63 f	3.26 hi	1.58 e	11.72 cdef	27.33 g	13.54 def	11.10 fgh	10.68 d
F11	256 bc	13.43 g	4.43 c	2.19 ab	11.35 cdef	43.33 c	13.13 gf	10.89 gh	9.09 h
F12	266 b	14.76 f	4.45 c	1.86 cde	11.94 bcde	33.33 gf	15.68 ab	13.37 bc	12.45 a
F13	277.67 b	10.6 i	3.12 hij	1.85 cde	9.76 i	37.33 de	14.62 bcd	12.83 bcd	8.57 ji
F14	255 bc	13.52 g	3.89 gf	1.74 de	7.18 k	41.67 cd	12.12 gh	9.95 hi	8.32 jk
F15	275.33 b	19.56 a	3.82 g	1.78 de	8.29 j	31.33 gf	14.36 cde	12.66 bcde	8.11 lk
K16	255 bc	12.35 h	2.59 k	1.70 de	10.28 ih	52 b	8.94 i	16.84 j	8.55 ji
K17	260.67 bc	14.73 f	3.89 g	1.93 bcd	13.69 a	61.33 a	13.81 edf	11.53 efg	8.96 hi
K18	264 bc	13.62 g	3.69 g	1.63 de	11.47 cdef	33.33 ef	14.55 bcd	11.71 defg	10.27 ef
K19	234.67 bc	14.76 f	3.07 ij	1.16 f	11.22 defg	64 a	14.34 cde	7.45 j	7.86 l
K20	266.67 b	13.36 g	4.86 b	1.93 bcd	8.71 j	34 ef	14.35 cde	12.19 cdef	11.77 b

Population no.	Width of the end leaf (mm)	Unripe fruit length (mm)	Unripe fruit width (mm)	Ripe fruit length (mm)	Ripe fruit width (mm)	Weight of premature fruit (g)	Weight of the ripe fruit (g)	Core weight of the ripe fruit (g)
KH1	5.33 i	6.96 i	5.45 kj	13.37 b	13.49 d	0.75 d	1.90 cd	0.46 c
KH2	8.83 ab	8.07 abc	8.29 a	12.14 f	12.83 f	0.88 c	2.16 b	0.53 a
KH3	7.67 c	7.07 i	6.02 gh	13.40 b	14.55 b	0.63 g	1.98 cd	0.48 ac
KH4	7.06 de	7.16 hi	5.88 hi	13.87 a	13.92 c	0.87 c	2.33 a	0.45 ce
KH5	5.61 hi	7.54 efg	6.24 fg	12.33 ef	14.67 a	0.83 c	1.89 cd	0.39 e
KH6	9.14 a	7.98 cde	6.85 c	13.06 c	13.9 c	0.69 ef	1.93 cd	0.44 ce
KH7	7.97 c	7.26 ghi	6.27 fg	12.83 d	13.32 e	0.83 c	2.02 cb	0.44 ce
KH8	5.66 hi	7.01 i	6.49 def	12.75 d	12.87 f	0.86 c	1.91 cd	0.42 e
F9	7.57 cd	7.46 fgh	5.97 hi	10.55 j	11.33 k	0.43 i	1.49 f	0.37 f
F10	7.67 c	6.54 j	5.86 hi	11.25 h	11.71 i	0.53 h	1.32 gh	0.24 h
F11	7.56 cd	6.21 k	5.67 ji	10.68 j	11.82 i	1.07 a	1.42 gf	0.31 g
F12	9.12 a	7.80 cde	6.60 ced	11.28 h	12.04 h	0.53 hi	1.45 gf	0.33 g
F13	6.41 f	8.23 ab	5.28 k	10.94 i	12.03 h	0.65 gf	1.35 gh	0.44 ce

F14	5.89 gh	7.64 ef	6.73 cd	12.13 f	12.28 g	0.73 ed	1.06 i	0.37 f
F15	6.37 fg	8.38 a	7.19 b	11.33 h	11.12 l	0.63 g	1 i	0.27 g
K16	5.49 hi	7.99 bcd	7.26 b	11.53 g	11.42 jk	0.62 g	1.25 h	0.443 ce
K17	5.87 hi	7.24 ghi	5.66 ji	10.70 j	11.48 j	0.49 i	0.98 i	0.52 a
K18	6.83 ef	7.83 cde	5.81 hi	12.38 e	12.18 g	0.73 d	1.74 e	0.45 ce
K19	5.36 hi	7.42 fgh	6.60 cde	13.75 a	14.44 b	0.95 b	1.85 ed	0.49 ac
K20	8.52 b	7.71 def	6.36 efg	12.45 e	12.87 f	0.83 c	1.83 ed	0.54 a

KH1= Behbahan, KH2 = Ramhormoz, KH3= Haftgol, KH4= Baghmalek, KH5= Ghaleh tool, KH6= Izeh Turkab KH7= Izeh Jaghband, KH8= Izeh Koolfarh, F9= Qaemea, F10= Khomezar Noorabad, F11= Fahliyan Norabad F12= Mosiry Rostam ,F13= Tel pir Babaamidan, F14= Dehno Babaamidan, F15= Coupon, K16= Basht, K17= Gachsaran, K18= lick, K19= Dehdasht, K20=haft cheshmeh Gachsaran

Table 4. Comparison between the averages of phytochemical traits of fruit in the studied *z. nummularia* populations

Population no.	Leaf phenol (mg/g)	Leaf flavonoids (mg/g)	Leaf tannin (mg/g)	Leaf saponin (mg/g)	Fruit phenol (mg/g)	Fruit flavonoid (mg/g)	Fruit tannin (mg/g)	Fruit saponin (mg/g)
KH1	1.34 g	4.13 b	1.67 fg	2.38 ij	0.38 b	2.45 a	1.68 b	1.36 f
KH2	2.44 c	4.07 c	2.63 cd	3.53 e	0.41 a	2.45 a	1.73 a	2.33 bc
KH3	2.84 ab	4.01 d	2.62 c	2.5 hij	0.40 ab	2.33 bc	1.62 e	1.43 ef
KH4	2.84 ab	3.41 j	3.57 a	2.81 fg	0.38 bc	2.11 fg	1.65 c	1.73 cdef
KH5	2.86 ab	3.94 e	1.51 h	3.3 e	0.40 ab	2.23 de	1.45 k	2.3 bc
KH6	2.47 c	3.87 f	1.72 f	5.4 a	0.34 def	1.58 j	1.33 n	3.23 a
KH7	2.73 b	4.33 a	1.92 e	2.93 f	0.32 ghi	1.97 h	1.59 f	1.43 ef
KH8	2.27 ed	4.34 a	1.62 g	3.33 e	0.35 de	2.05 g	1.63 d	1.5 ef
F9	2.14 e	3.76 g	1.32 ij	4.13 d	0.30 i	1.74 i	1.50 j	2 bcde
F10	1.23 g	3.39 j	1.16 k	4.06 d	0.31 hi	1.73 i	1.51 i	2 bcde
F11	2.39 cd	4.01 c	1.24 jk	4.4 c	0.37 efg	1.69 i	1.53 h	2.23 bcd
F12	1.77 f	3.85 f	1.23 jk	2.76 fgh	0.36 efgh	1.88 h	1.54 g	1.2 f
F13	2.14 e	4.35 a	1.39 i	2.73 fgh	0.33 fgh	1.86 h	1.62 e	1.66 def
F14	0.79 h	3.50 i	0.83 l	2.7 fgh	0.34 def	1.91 h	1.52 f	2.03 bcde
F15	1.73 f	3.73 g	1.61 g	2.28 j	0.34 def	2.12 fg	1.43 l	2.2 bcd
K16	1.71 f	3.65 h	1.21 k	2.38 ij	0.36 cd	2.23 de	1.34 m	1.3 f
K17	2.12 e	3.87 f	1.85 e	2.36 ij	0.05 l	2.16 ef	1.51 i	1.53 ef
K18	2.9 ab	4.08 c	2.56 d	4.6 c	0.03 m	2.32 bc	1.26 o	2.4 b
K19	2.84 ab	3.65 h	2.95 b	2.58 ghi	0.15 k	2.27 cd	1.26 p	1.26 f
K20	2.96 a	4.34 a	1.90 e	5.1 b	0.27 j	2.40 ab	1.214 q	2.53 b

KH1= Behbahan, KH2 = Ramhormoz, KH3= Haftgol, KH4= Baghmalek, KH5= Ghaleh tool, KH6= Izeh Turkab KH7= Izeh Jaghband, KH8= Izeh Koolfarh, F9= Qaemea, F10= Khomezar Noorabad, F11= Fahliyan Norabad F12= Mosiry Rostam ,F13= Tel pir Babaamidan, F14= Dehno Babaamidan, F15= Coupon, K16= Basht, K17= Gachsaran, K18= lick, K19= Dehdasht, K20=haft cheshmeh Gachsaran

To identify the biodiversity and different chemotypes of *Z. nummularia* populations, their morphological and phytochemical traits were subjected to principal component analysis (PCA) and cluster analysis (CA). The study indicated five components explaining 91.8% of the total variance in the PCA, of which the first three components (PC1-PC3) comprised more than 79.04% of the variations (Table 5). The contribution of the first component (PC1) was 41.05% of the total variance. In PC1, some characteristics such as leaf saponin, the width of the end leaf, fruit saponin, length of the end leaf, leaf length, leaf width, leaf phenol, the weight of the ripe fruit, and leaf peduncle length, and sub-stem length had the highest variance. Besides, in the second component (PC2) with 20.05% of the total variance, ripe fruit length and width, leaf tannin, core weigh of the ripe fruit, weight of the ripe fruit, leaf saponin, number of thorns, fruit flavonoids, fruit saponin, leaf phenol, and weight of the premature (unripe) fruit had the highest variance. Moreover, the highest variance for the length and width of the premature (unripe) fruit, and fruit tannin was obtained from PC3 (Table 5).

Table 5. Eigenvectors of the first three principal component axes from PCA analysis of fruit and leaf variables in studied *Z. nummolaria* populations

Character	Component		
	1	2	3
Annual branches	.475	-.321	.149
intermediate length	.394	-.074	-.319
Flower stem length	.624	-.198	.003
Lateral stem length	.336	-.403	.535
Thorns	.064	.292	.539
Number of thorns	-.295	.632	.197
Leaf length	.663	-.257	.146
Leaf width	.643	-.263	.118
Length of the end leaf	.744	-.274	-.038
Width of the end leaf	.797	-.386	.058
Unripe fruit length	.179	-.164	-.697
Unripe fruit width	.252	-.053	-.693
Ripe fruit length	.251	.790	-.146
Ripe fruit width	.362	.749	.099
Weight of the fruit premature	.252	.548	.011
Weight of the ripe fruit	.625	.664	.142
Core weight of the ripe fruit	.238	.677	-.115
Leaf phenol	.630	.553	.108
Leaf flavonoids	.370	.149	.262
Leaf tannin	.388	.731	-.080
Leaf saponin	.814	-.653	-.058
Fruit phenol	.179	-.142	.170
Fruit flavonoids	-.008	.628	-.308
Fruit tannin	-.015	-.053	.544
Fruit saponin	.783	-.615	-.310
% of Variance	41.053	20.054	9.666
Cumulative %	41.053	67.205	79.035

However, a highly significant variation was detected in some morphological and phytochemical characters. Six morphological traits including width of the end leaf, length of the end leaf, leaf length, leaf width, weight of the ripe fruit, and leaf peduncle length and three phytochemical traits including content of leaf saponin, fruit saponin, and leaf phenol revealed the highest variation and PCA coefficient according to the results of PCA (Tables 5 and 6). The lowest content of leaf saponin was detected in F15 population, whereas its highest content was recorded in KH6 population. In addition, fruit saponin content varied from F12 to KH6 (3.23 mg/g) in the studied populations. The lowest and highest content of leaf phenol was measured in F14 and K20 populations, respectively. In general, total saponin, phenol, and tannin contents of leaf and fruit in PC1 and PC2 were most important for identifying the chemotypes and populations.

The wide range of variation across the *Z. nummularia* populations in the content of saponin and phenol could be exploited for selecting suitable genotypes and also for the improvement and commercial exploitation of this plant. Therefore, the saponin content was the main variable in chemical constituents for the effective detection of original populations of *Z. nummularia*. Based on PC1 and PC2 of these traits, *Z. nummularia* populations were divided into four main groups with similar clustering results.

A cluster analysis of studied populations was performed for grouping populations based on the Euclidean distances of all morphological and phytochemical traits, using the Wards method. In general, hierarchical cluster analysis divided the populations into four main groups with high diversity (Figure 1). The first main group (I) was divided into six populations. The first group consisted of populations from Behbahan

(KH1), Haftkel (KH3), Baghmalek (KH4), Ghale Tol Baghmalek (KH5), Izeh Jughband (KH7), and Izeh Kul-e Farah (KH8) with similar characteristics. The second main group (II) was divided into four populations comprised of Ramhormoz (KH2), Izeh Tarakab (KH6), Lick (KH18), and Haft Cheshmeh Gachsaran (KH20) population. The third group (III) consisted of nine populations of Qaemea (F9), Khoome Zar Nourabad (F10), Fahliyan Nourabad (F11), Masiri Rostam (F12), Tel Pir Baba Meydan (F13), Dehno Baba Meydan (F14), Kopen (F15), Basht (K16), and Gachsaran (K17). At last, the fourth group (IV) comprised only one population of Dehdasht (K19). Some of the prominent characteristics of these populations are saponin and phenol contents in leaf and fruit and weight of the ripe fruit, which made them distinct among the other populations (Table 6).

Table 6. Morphological and phytochemical characteristics for categorized groups of *Z. nummolaria* populations in Iran

Character	Group cluster (Mean±SD)			
	I	II	III	IV
Population	KH1, KH3, KH4, KH5, KH7, and KH8	KH2, KH6, K18, and K20	F9, F10, F11, F12, F13, F14, F15, K16, and K17	K19
Flower stem length	3.76± 0.4	3.99±0.372	3.88±0.852	3.05±0.027
Leaf length	13.93±2.425	14.80±0.440	13.09±1.874	14.35±0.12
Leaf width	11.20±2.593	12.64±0.936	12.06±2.123	7.46±0.025
Length of the end leaf	9.27±1.61	11.62±1.25	9.54±1.43	7.86±0.393
Width of the end leaf	6.55±1.159	8.26±1.254	6.88±1.112	5.36±0.132
Weight of the ripe fruit	2.09±0.168	1.93±0.195	1.27±0.194	1.85±0.393
Leaf phenol	2.48±0.6	2.60±0.255	1.78±0.475	2.84±0.382
Leaf saponin	2.87±0.396	4.51±0.937	3.09±0.804	2.58±0.246
Fruit saponin	1.68±0.353	2.65±0.501	1.79±0.362	1.26±0.323
Average maximum temperature (˚C)	31.3	27.5	24.6	26
Average minimum temperature (˚C)	15.7	13.85	12.2	8
Average maximum moisture (%)	76.8	68.75	63	53
Average minimum moisture (%)	39.13	32	28	22

KH1= Behbahan, KH2 = Ramhormoz, KH3= Haftgol, KH4= Baghmalek, KH5= Ghaleh tool, KH6= Izeh Turkab KH7= Izeh Jaghband, KH8= Izeh Koolfarh, F9= Qaemea, F10= Khomezar Noorabad, F11= Fahliyan Norabad F12= Mosiry Rostam ,F13= Tel pir Babaamidan, F14= Dehno Babaamidan, F15= Coupon, K16= Basht, K17= Gachsaran, K18= lick, K19= Dehdasht, K20=haft cheshmeh Gachsaran
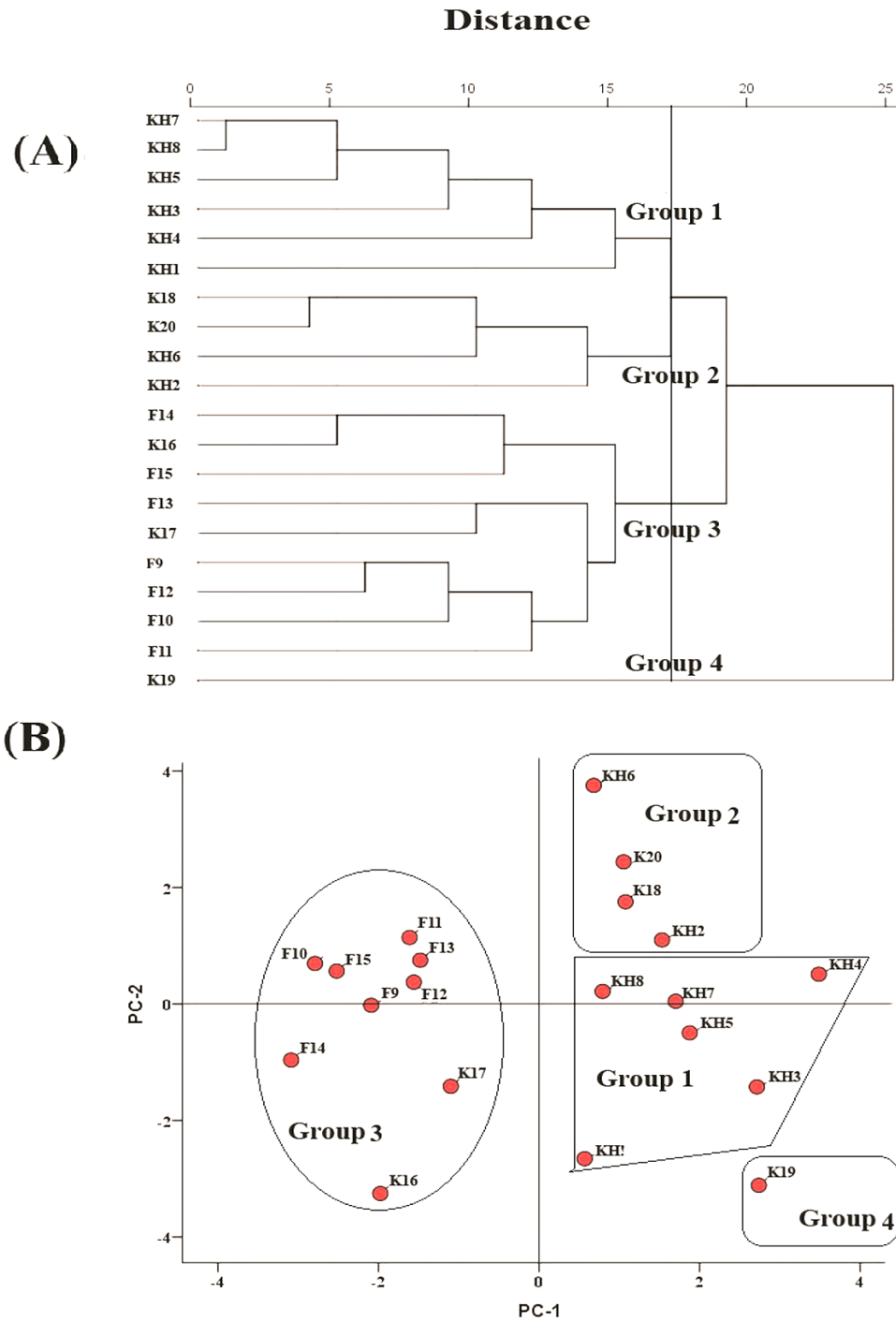
Figure 1. Cluster (A) and PCA (B) analyses of *Z. nummolaria* populations based on the first and second components (PC1 and PC2)

KH1= Behbahan, KH2 = Ramhormoz, KH3= Haftgol, KH4= Baghmalek, KH5= Ghaleh tool, KH6= Izeh Turkab KH7= Izeh Jaghband, KH8= Izeh Koolfarh, F9= Qaemea, F10= Khomezar Noorabad, F11= Fahliyan Norabad F12= Mosiry Rostam ,F13= Tel pir Babaamidan, F14= Dehno Babaamidan, F15= Coupon, K16= Basht, K17= Gachsaran, K18= lick, K19= Dehdasht, K20=haft cheshmeh Gachsaran

Phytochemical and morphological traits are affected by the climatic, geographic and soil conditions of the growth site. Therefore, the division of populations into four different clusters could be due to differences in environmental and genetic factors. Thus, this group classification can interpret the results of differences between populations. In different ecosystems, performance and yield of plants were affected by the number of factors such as plant species, climate height of area from sea level, temperature, and relative humidity (Bernath, 2002; Islam *et al.*, 2019). In a recent study, according to the results of environmental factors, the climatic changes significantly affected the qualitative and quantitative yields in populations under study (Rajeswara *et al.*, 1990). Considering the results of cluster analysis, the most resemblance in the individual's first occupied cluster was due to the saponin content. In comparison, the resemblance between individuals in clusters II and III came from the fruit size and the leaf size in the fourth cluster. The highest saponin content was estimated in KH6 in cluster II. The highest mature fruit weight was measured in KH2 in cluster II and K16 in cluster III. The lowest temperature and relative humidity were measured in the fourth cluster. Therefore, it was deduced that the leaf size was probability affected by the lowest temperature and relative humidity. Furthermore, the temperature and relative humidity seemed very close in clusters II and III, leading to similar fruit weight in these clusters. The highest temperature and relative humidity were measured in cluster I and saponin content. It was concluded that high temperature and relative humidity may lead to high saponin content in individuals.

It was also inferred that high temperature and relative humidity may lead to high saponin content in individuals. Obeed *et al.* (2008) examined the morphological traits and the fruition of some populations of trees and reported large variations in tree height, leaf length, and branch and fruit traits. They identified these traits as the most reliable traits for the classification of cultivars and populations. In a study on Fars populations of *Z. nummularia* in Iran, a significant difference was obtained between various regions in terms of the morphological traits and the phenological stages (Jahromi and Zandi, 2012). They also concluded that *Z. nummularia* belonging to arid regions are more and longer than wet areas. These results are close to those of the present study. Tatari *et al.* (2016) reported that Jujube in humid and cold regions has fewer shoot thorns, thinner and smaller thorns, smaller shoots, and taller trees with wider canopies, while in arid and semi-arid regions it has more shoot thorns, thicker and higher thorns, longer shoots, and shorter trees with denser canopies. Climatic conditions have high effects on the morphological traits of Jujube ecotypes and their vegetative characteristics. Isfahan province of Iran is experiencing climate change such as reduced rainfall, drought, and warmer temperatures. An effective factor in justifying the greater diversity within the population is the number of cases such as plant grown-ness, seed germination, number of allele locations, allele position and population genotype, type of crosses, and population size. Saeedi *et al.* (2016), based on the cluster analysis of different populations of *jujube* in Iran, divided the studied specimens into eight groups. Here, the lack of compliance in cluster analysis and the placement of ecotypes together could be due to genetic differences.

In another paper on leaf variation of *Z. nummularia* (*Zizyphus christi-spina*) in Abolfars, Khuzestan province of Iran, it was reported that except for the leaf thickness trait, the remaining traits in the three habitats have significant differences. The results indicate that the high morphological diversity of *Z. nummularia* leaves in the studied habitats. According to the results, the petiole and thigh lengths are most affected by environmental conditions (Alidadi *et al.*, 2014). Another study showed that altitude above sea level is quite effective in determining the morphological characteristics of *Z. nummularia*. As this species is deciduous, it can grow and extend at altitudes up to 1,200 meters above sea level. In the evaluation of phytochemical traits, the mean comparison results showed that most of the populations had a significant difference in these traits (Arndt *et al.*, 2001).

In a study on the hydro-alcoholic extract of leaf and fruit of *Z. nummularia*, Gupta *et al.* (2011) observed that leaf flavonoid content in the extract was higher than that of fruit. They reported that the hydro-alcoholic extract of *Z. nummularia* fruit had the highest phenol and its leaf extract had the highest flavonoid content. In this study, the highest amount of tannin of fruit belonged to KH2, which had a significant difference with all populations. The highest level of saponin in the fruit was also in KH6, in which the population was significantly

different from other populations. Saponin is one of the several important constituents of *Z. nummularia* leaves and fruits. The high content of saponin has emerged as a desirable factor in the selection of sea buckthorn suitable genotypes. In a study carried out on different *Z. nummularia* masses to investigate the number of phytochemicals present in the tree, it was shown that the amount of minerals in the fruit of *Z. nummularia* depends on the climate, soil elements, harvest time, altitude, and genetics of the mass (Bouis, 1996). The results showed that the highest levels of saponin were in populations KH6 and K20, while the lowest was among populations F15 and K17. In a study by Bagdi and Rathore (2016) on *Z. nummularia*, it was concluded that saponins are present in the leaf extract, but not in its fruit; these results are inconsistent with those of the present study. In this study, saponin was present in leaf and fruit, but the content of saponin was greater than the content of saponin in the fruit. Moreover, the highest flavonoid content of the whole leaf belonged to the population of F13 and K20, and the low content was seen in F10. This result is in agreement with the results of the research conducted by Gupta *et al.* (2011) on *Z. nummularia*. The results showed that leaf and fruits of *Z. nummularia* have high phenolic content, and populations K20 and KH2 are higher in phenol compared to the rest of the populations.

In another work, it was reported that planting of different populations in environmental and geographical conditions increased the environmental effects of vegetative and reproductive traits; moreover, the variety resulted in bigger genetic differences among populations. This variety will pave the way for introducing optimal ecotypes from the perspective of vegetation. Thus, by comparing environmental conditions, it is possible to considered and compare morphological and phytochemical traits (Bahari *et al.*, 2015). Li *et al.* (2009) evaluated the genetic diversity among 117 jujubes collected from different areas. The results showed high genetic diversity in the samples and, therefore, the high potential for correcting these species. In their study, there was no relationship between geographic regions and genetic distances. Elsewhere, Saeedi *et al.* (2016) studied the *Ziziphus* species genetic variation and reported that the cultivars' mass collections of the region were genetically different, probably due to the climatic conditions. In the present research, the most important characteristics of populations of *Z. nummularia* were phytochemical traits, especially saponins in fruits, leaves, and phenols in leaves. Among the populations evaluated, the KH6 population was the highest in the fruit saponin and leaf saponin while K20 was the highest in phenol leaf.

According to the results, the fluctuations in temperature, altitude, and geographic coordinates of the population have created a variety of them. It is suggested prioritizing the importance of the medicinal and nutritional value of these species and their importance for soil stabilization, desertification, crop management and stabilization of its developmental sands. Moreover, it is recommended conducting similar research projects to determine the populations.

Conclusions

In this study, the important morphological and phytochemical traits of *Z. nummularia* populations in the main regions of Iran were evaluated. There was wide variability in morphological and phytochemical of different *Z. nummularia* populations in the study areas. The results of this research revealed valuable information about the morphological and phytochemical evaluated from wild populations of *Z. nummularia* in Iran. In general, hierarchical cluster analysis and principal component analysis (PCA) divided the wild populations of *Z. nummularia* into four main groups with high diversity. The wide range of variation across the *Z. nummularia* populations in the content of saponin and phenol as the major compounds could be exploited for selecting suitable chemotypes for the improvement and commercial exploitation of this plant. Therefore, the saponin content was the main variable in chemical constituents for the effective detection of original populations of *Z. nummularia*. Generally, Izeh Tarakab population had the highest amount of leaf and

fruit saponin. The high saponin content emerged as a desirable factor in selecting *Z. nummularia* suitable genotypes.

Authors' Contributions

The first author carried out the experiment and collected available literature and prepared the first draft of the manuscript with support from the second and third authors. The second author analysed the statistical data and verified the accuracy of the tests. The third author designed the model and the computational framework and he was also responsible for the correspondence. The fourth author edited the manuscript as a plant science consultant.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article, and the authors are responsible for the content of the paper.

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