DETERMINANT EFFECTS OF ENVIRONMENTAL FACTORS ON MORPHOLOGY AND EPHEDRINE CONTENTS IN THE WILD POPULATIONS OF *Ephedra major* Host.

Somayyeh GHANBARI HAMEDANI[*], Younes ASRI[**], Iraj MEHREGAN[*]

[*]*Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.*
[**]*Department of Botany, Research Institute of Forest and Rangelands, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran.*
Correspondence author: Iraj Mehregan, Department of Biology, Science and Research Branch, Islamic Azad University, P.C. 1477893855, Tehran, Iran, Phone/Fax: +98-214-4865327, email: iraj@daad-alumni.de

Abstract. The genus *Ephedra* of the Ephedraceae contains more than 60 species of non-flowering seed plants (Gymnosperms) dispersed all through Asia, America, Europe, and North Africa. These *Ephedra* species have medicinal, ecological, and economic value. Ephedrine is an important active components of *ephedra* species. This study aimed to evaluate the content of Ephedrine alkaloid in 15 populations of *E. major* in Iran (NW to NE) covering the entire populations of *E. major* using the HPLC method. Our results showed the highest content of Ephedrine (EP) was from Chalous Road (CHL, N Iran; 14 mg/g) and Qorveh populations (QRV, W Iran; 13.6 mg/g). The lowest amounts of EP were detected in Ardebil (ARD, NW Iran; 0.9 mg/g) and Baraqan (BRQ; 0.2 mg/g) populations. No significant correlations were observed between the EP content and morphometric characters. The results showed that the amount of Ephedrine in populations grown in arid conditions was higher (high temperature) and negative correlation exists between the annual precipitation and EP content in different seasons (R2= -0.563. These results indicated a strong positive correlation between Ephedrine content and soil's pH (R2=0.598), and average temperature (R2=0.554). This study determined the important environmental and edaphic factors affecting the EP content in *E. major* populations and can be exploited for further domestication and ecological investigations.

Key words: alkaloid; ecological factors*; Ephedra;* ephedrine; HPLC; Iran.

INTRODUCTION

Ephedra L. has a place to the Ephedraceae which is the biggest of the three extant genera included within the Gnetales [20, 21, 28, 37, 38] is a taxon of approximately 50-60 species that are distributed in the Eurasian Continent, the northern part of the African Continent, and arid areas in the western American continent [21, 37]. *Ephedra* is native to central Asia, widely distributed throughout China, Tibet, India, Pakistan, Japan, and Southern Siberia, also cultivated extensively [8]. Classification of *Ephedra* plants has long been a matter of talk about since few morphological differences exist between them.

Ephedra major is dioecious and evergreen shrubs growing to 20-150 cm tall; woody stems well developed, erect or mostly procumbent, thick; herbaceous branchlets are slender, striate, smooth, bluish-green or grayish-green, 1-1.5 mm in diameter, internodes short, 1-3 cm × 1-1.5 mm, finely furrowed. Leaves are opposite, leathery, greenish or brownish (in mature plants), 1.5-3 mm, connate for ca. 3/4 their length, free part bluntly triangular. Female cones usually opposite at nodes, shortly pedunculate, elongate-ovoid or ovoid at maturity, 8-10 × 4-5 mm; bracts in 3 pairs, apical pair connate for ca. 2/3 their length, red and fleshy at maturity; integument tube to 2 mm, straight or slightly curved, slightly exerted. Seeds usually 1, elongate-ovoid, 5-7 × 2.5-3 mm and male cones are sessile, subglobose, 4-5 mm long [48].

Ephedra has long been utilized as a medicinal herb to initiate perspiration, reduce fever, treat coughing, and manage asthma [18, 20, 50]. It contains the following bioactive components: (–)-ephedrine (EP), (+) pseudoephedrine (PE), (–)-methylephedrine, (+)-methylpseudoephedrine, (–)-norephedrine, and (+)-norpseudoephedrine [9, 34, 46] is the central figure in the first era of scientific work on ephedrine, he separated ephedrine, which can exist in four shapes: l-ephedrine, which represents 40–90% of the total alkaloids [29]. Ephedrine (EP) alkaloids can activate the central nervous system through adrenaline and dopamine like activities, which are thought to be responsible for the adverse effects of *Ephedra* Herb, such as excitation, insomnia, and arrhythmia [35, 43, 47].

Rechinger introduced 10 species of *Ephedra* in his "Flora Iranica" [39, 40]. In some references reported 6 [40] to 8 species [4] from Iran. These species are distributed mostly in Irano-Turanian region and one of them (*E. foliata* Boiss.) the scientific names will be written in italics wherever they are in the text grows in lowland of Saharo-Sindian region in some part of Ilam, Khuzestan, Bushehr, Hormozgan and Baluchestan provinces and sometimes penetrate to the southern part of Irano-Turanian region in Fars province [33]. So far, little studies have been done on the *Ephedra* plant in Iran, available information shows among 9 species, the highest amount of Ephedrine was found in *Ephedra major* (0.8%-1.8% dry weight of plants) and the lowest amount belongs to *Ephedra brevifoliata* (0.05%-0.08% dry weight of plants) [5], Kashki, also showed it had the highest rate of EP among four species in Khorasan province (East of Iran) [22].

The different geological origins of the plants make the total content of main active alkaloids very diverse from plant to plant [24]. For this reason, different analytical methods have been reported for measurement of EP in plant extracts including high-performance liquid chromatography (HPLC) [49], high performance thin layer chromatography (HPTLC) [10], gas chromatography-mass spectrometry (GC-MS) [5], thin-layer chromatographic (TLC) [11, 19].

The amount and type of EP in the plant are highly

variable according to species, varieties, plant parts, harvest season, and geographic and altitude regions where the plant grows [14, 36, 37, 41].

The importance of understanding the impact of environmental conditions including soil properties and ecological variables is to find the most appropriate localities that can be selected for production of economical and medicinally important substances. Thus, secondary metabolites mainly responsive to the environment and cultivation of *E. major* first requires reliable data to comprehend its ecological and soil requirements. Therefore, we carried out this study to evaluate the content of EP in different populations of *Ephedra major* from northwestern and northeastern Iran and to determine any possible relationship between these amounts and different environmental or edaphic factors.

MATERIALS AND METHODS

Sample collection. Stems (branchlets) of *Ephedra major* were dug up from 15 different localities in northwestern, north and northeastern Iran where they grow wild in different ecological conditions (Figure 1 and Table 1). They were collected between June and October 2015. All of the samples were cleaned, cut into smaller pieces, at that point dried at the 25-degree centigrade (room temperature) and powdered utilizing electric process. All the samples were identified based on the morphological characters and were deposited in the herbarium of the Islamic Azad Science and research branch, Tehran, Iran.

Sample preparation. In this step, 1g dried plant material powder is extracted with 10 mL methanol/water (50:50, v/v) dissolved in 20 minutes at room temperature and then the extraction centrifuged at 15,000 rpm, for 5 minutes. This extraction was repeated three times, finally, it was diluted with methanol (50%) to a volume of 50 mL, then was transferred into 50 mL falcons and then filtered by a 0.45 µm syringe filters [6].

HPLC analysis. High-Performance Liquid Chromatography was performed on a Smartline® HPLC series (KNAUER, Germany) consisted of a Smartline® S-1000 pump, S-5000 manager with degasser and a S-2500 programmable UV detector with column C18 (150 × 4.6 mm). The mobile phase was methanol/water (70:30, v/v, containing 1% acetic acid). The flow rate was adjusted at 1.0 mL/min. After sample filtration with 0.45 µm diameter filter, 20 µL of each sample was injected (three times for each sample). Ephedrine Hydrochloride (Sigma-Aldrich) was used as the standard. After filtrations with 0.45 µm diameter filters, four different concentrations of Ephedrine hydrochloride (0, 55, 110, 220 and 440 ppm) were prepared and injected into HPLC apparatus.

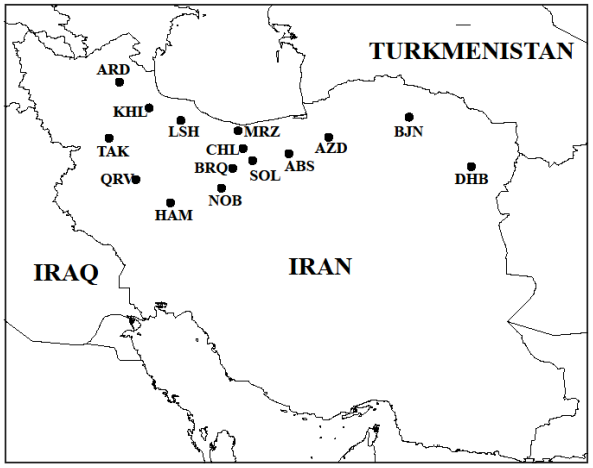

Figure 1. Localities of collected plant materials examined in this study (abbreviations and more details in Table 1)

Table 1. List and localities of collected plant material examined in this study

Accession numbers	Pop. code	Collection site	Locality	Longitude	Latitude	Altitude (m)
1	MRZ	Marzanabad	Iran: Mazandaran, 20Km from Chalous to Marzanabad	50° 21′	36°31′	316.17
2	BJN	Bojnord	Iran: Khorassan(N), Bojnord, 13 km from Hesar hoeini Road to Rakhtian	57° 12'	37°21'	1814
3	QRV	Ghorveh	Iran: Kordestan, 15 km from Ghorveh to songhor(Kermanshah)	47° 57'	35°6'	1829
4	LSH	Lowshan	Iran: Gilan 10km. from Lowshan to Amarloo	49° 44′	36° 46'	1800
5	ARD	Ardabil	Iran:Ardabil, Hir, 28 km from Hir to Ardebil	48° 32'	37°37'	1400
6	AZD	Azadshahr	Iran:Golestan, Azadshahr, 10 km from Azadshahr to Shahroud	55° 22′	36° 56'	1400
7	NOB	Nowbaran	Iran: Markazi, Saveh road, 85 km from nowbaran to Saveh	49° 33'	35° 07'	1755.65
8	KHL	Khalkhal	Iran: Ardebil, Khalkhal	48° 29'	37° 40'	1700
9	HMD	Abbasabad	Iran: Hamedan, Abbas Abad	48° 27'	34° 46'	2189
10	DHB	Dehbar	Iran:Khorasan razavi, 12Km from torghebeh to Dehbar	59° 29′	36° 5'	1578
11	CHL	Chalous R.	Iran: Alborz,Chalous Road ,shahrestanak, 15 km from Shahrestanak to Gachsar	51° 30′	36°02′	2015.49
12	TKB	Takab	Iran:West Azarbaijan, Takab	47° 11′	36°35′	1850
13	BRQ	Baraghan	Iran:Alborz, Baraghan, 10 km from Baraghan to karaj	57° 47′	35°055′	1625
14	SOL	Soleghan	Iran: Tehran, Soleghan, 10 km from Soleghan to Tehran	51° 16′	35°47'	1430
15	ABS	Damavand	Iran: Tehran, Damavand, Absard, 5 km from Absard to Khosravan	52° 13′	35° 39′	2273

Note: Pop.: Papulation, MRZ: Marzanabad, BJN: Bojnord, QRV: Qorveh, LSH: Lowshan, ARD: Hir, NOB: Nowbaran, KHL: Khalkhal, HMD:Abbasabad, DHB: Dehbar, CHL: Chalous Road, TKB: Takab, BRQ: Baraghan, SOL: Soleghan, ABS: Absard.

Standard curve preparation. Ephedrine Hydrochloride (Sigma-Aldrich) was used as the standard. After filtrations with 0.45 µm diameter filters.

Soil analysis. Soil samples from each population were collected at 20-30 cm profundity close the roots. Soil characters (Table 2) were determined using the following different methods. The soil texture was determined using Bouyoucos Hydrometer Method [15, 16]. The acidity rate (pH) and the electrical conductivity (EC) were measured using a portable CPD-65N multi-meter (ISTEK, South Korea). A modified Walkley and Black method was used to determine the amount of organic carbon (OC) content [1]. Chlorine in the soil was measured with the Ion chromatography (IC) method [23]. The amount of Lime percentage in soil (T.N.V.) as the total carbonates included in 100g of dry soil was determined using Calcimeter Bernard method. To determine the soil texture, the Textural Autolookup or TAL software was used, which is based on the soil texture triangle. The amount of Nitrogen (TN) was determined using Kjehdal method by converting the various nitrogen forms into NH_4^+ [7, 32].

Ecological data. The samples were taken from different localities (Table 3) and their climate data including precipitation, average, maximum and minimum temperatures were taken from the meteorological organization of Iran and the website https://en.climate-data.org/.

Morphometrical analysis. Six morphological characters including the height of the plant, length of the leaves and internodes. Trunk, Stem and branch diameter were measured for at least six individuals from each population. The measurements were made using a digital Vernier caliper.

Resulted data were analyzed by SPSS v. 25 (IBM Inc., Chicago, IL). Kolmogorov-Smirnov test was performed to test the normality of frequency distributions. One-way ANOVA was utilized to compare the implies of typical conveyances. Duncan test was utilized to decide the contrasts in morphometric data. Pearson's correlation analysis was performed to decide the degree of relationship between distinctive factors. Populations were classified using Hierarchical cluster analysis (HCA) with the Average-linkage method and standard Euclidean coefficient. Principal component analysis (PCA) was carried out based on the relative contents of ecological and morphometry data from different populations as dependent variables [13].

Table 2. Results of analyses of soils collected from different localities

Pop. cod	Clay (%)	Silt (%)	Sand (%)	S.p. (%)	$CaCo_3$ (%)	OC (%)	Texture	EC (ds/m)	Sal.	pH	DO	TN (%)
MRZ	5	20	74	36.19	46.25	3.315	Sandy loam	864	0.6	7.24	6.3	0.33
BJN	12	28	60	32.59	33.25	2.125	Sandy loam	632	0.0	7.61	7.4	0.23
QRV	4	10	86	18.01	3.5	0.663	Loamy sand	382	0.0	7.28	6.8	0.14
LSH	16	14	70	31.09	32.5	1.258	Sandy loam	694	0.5	7.69	7.3	0.14
ARD	2	14	84	31.55	1.25	2.125	Loamy sand	480	0.0	7.72	6.8	0.23
AZD	2	10	88	18.46	10	1.275	sand	524	0.0	7.77	8	0.18
NOB	6	18	76	32.58	42.5	2.363	Sandy loam	707	0.6	7.07	6.6	0.18
KHL	2	8	90	23.77	1.25	0.884	sand	319	0.0	7.37	7.1	0.19
HMD	0	4	96	21.57	5	0.493	sand	218.3	0.0	7.34	6.5	0.23
DHB	8	30	62	31.57	0.408	10.5	Sandy loam	838	0.6	7.62	8	0.28
CHL	18	38	44	31.13	2.02	1.18	loam	648	0.5	7.45	6.5	0.11
TKB	10	18	72	25.16	3.75	1.241	Sandy loam	806	0.6	7.75	7.1	0.18
BRQ	6	8	86	30.64	1	2.21	Loamy sand	931	0.7	7.26	5.6	0.28
SOL	4	12	84	32.11	9.5	2.635	Loamy sand	763	0.6	7.76	7.2	0.48
ABS	6	14	80	38.66	25	3.655	Loamy sand	517	0.0	7.24	7.0	0.44

Abbreviations: Sp.: Saturation percentage, Sal.: Salinity, EC.: Electrical Conductivity, DO.: Dissolved Oxygen, TN.: Total Nitrogen.

Table 3. Environment data for the localities of collected samples of *E. major*

Pop. code	Collecting time	Sum. pptn. (mm)	Avg. temp. (ºC)	Max. temp. (ºC)	Min temp. (ºC)
MRZ	Sept.	20.25	19.10	30.00	18.00
BJN	Jun.	12.72	23.51	38.60	11.20
QRV	Oct.	1.63	14.85	26.09	2.90
LSH	Oct.	18.52	16.78	30.40	8.00
ARD	Oct.	42.00	8.96	29.00	-1.40
AZD	Aug.	9.00	30.25	44.10	17.10
NOB	Oct.	0.09	16.86	27.40	3.80
KHL	Oct.	30.00	10.25	25.10	-2.35
HMD	Sept.	1.00	19.88	34.50	-1.45
DHB	Jul.	3.51	28.83	40.00	15.80
CHL	Jul.	0.09	21.40	30.21	12.60
TKB	Jul.	6.00	11.82	37.40	10.80
BRQ	Jul.	0	30.58	40.30	22.60
SOL	Jul.	32.60	21.17	31.20	6.70
ABS	May	20.25	19.10	30.00	18.00

Abbreviations: Pop.: population, Sum pptn.: Sum precipitation, Avg. temp.: average temperature, Max. temp. : Maximum temperature, Min temp.: Minimum temperature.

RESULTS

As shown in Table 4, the amount of EP varied in different localities; the highest amount of EP was observed in CHL and QRV populations (14.0 mg/g, 13.6 mg/g) and the lowest amount was observed in BRQ population (<0.2 mg/g).

Results of soil analyses showed that the highest electrical conductivity (EC) amount belonged to BRQ populations (931 ds/m, Table 2) and the lowest one belonged to HMD population (218.3 ds/m). ABS, ARD, AZD and TKB populations demonstrated the highest pH amount (pH = 7.8) and BRQ population showed the lowest amount (pH = 6.8). Most of the localities had soil with sandy loam texture. ABS population was interesting to show the highest amounts of organic carbon (OC) (3.65%), total N (0.44%) and HMD showed the lowest amount of OC (0.49%), CHL showed lowest amounts of total N (0.11%).

The climate of localities is typically Semiarid, with a maximum and minimum annual temperature of 44.1 and -2.3°C respectively. The highest amount of average temperature was observed in AZD and SOL (> 30°C) and the lowest one observed in KHL (8.9°C). MRZ had the maximum annual precipitation (86.03 mm) and the lowest one observed in BRQ (13.14 mm) (Table 3).

Morphometric data including the means and standard deviations for the height of plant, trunk, stem and branch diameter length, and length of the leaf are shown in Table 5. Duncan test showed that the highest and the lowest height of plant diameter were observed in ABS (61.6667+20.29) and QRV (33.3333+3.06). SOL and TKB populations had the trunks with the highest diameters (7.2306+1.86) and the lowest diameters were recorded in MRZ (3.2267+0.42). The Highest stem diameters were observed in ABS population (2.1578+0.39) and the lowest ones were observed in HMD (1.6011+0.23). ARD population showed the lowest diameters of branches (0.7583+0.09a) and BRQ had the highest (1.1961+0.11) among others. The longest Internodes were observed in LSH (26.4828+6.87) and the shortest ones were recorded in ARD (16.3289+1.35). SOL population had the shortest leaf length (1.6961+0.28) and the tallest leaf length was recorded in MRZ population (2.2206+0.36).

Statistical analysis showed different values for the correlation between EP content and soil components were observed (Table 6). Based on the results, there was significant correlation between pH from soil compounds and with the content of Ephedrine ($P \leq 0.05$ and R2 =0.598). Additionally, the correlation relationship between EP and other soil properties was mainly insignificant. The negative correlation between EP and sand, Sp, CaCos and Salt was noticeable. The correlation between EP and EC was positive (R2 =0.410) but not significant. The same applies to silt and EP (R2 =0.369), texture and EP (R2 =0.331), and T.N.V percentage and EP (R2 =0.385).

As seen in Table 7, internode length showed positively correlated with soil salinity, EC and T.N.V percentage ($CaCo_3$) ($P \leq 0.05$ and R =0.580, 0.548, 0.560); and leaf length showed significant correlation with soil gravel ($P \leq 0.01$ and R= 0.652) and the other morphological characters didn't show any correlation with soil factors. Table 8 represents the correlation between morphometric data and ecological factors.

Table 4. The amount of EP in different localities (mg/g).

Pop.	MRZ	BJN	QRV	LSH	ARD	AZD	NOB	KHL	HMD	DHB	CHL	TKB	BRQ	SOL	ABS
Ephedrine	6.7	6.8	13.6	2.2	0.9	12.4	8.3	7.9	3.0	11.0	14.0	1.9	<0.2	11.9	4.2

Table 5. Morphometric features of different populations of *E.major* (The population code is according to Table 1)

Pop.	Plant Height (PH)	Trunk Diameter (TD)	Stem Diameter (SD)	Branch Diameter (BD)	Inter node Length (LIN)	Leaf Length (LL)
MRZ	46.5000±9.46 ^{c,d}	3.2267±0.42^{a}	1.6400±0.28^{a,b,c}	0.9828±0.17^{b,c}	24.2083+4.97^{f,g}	2.2206±0.36^{g}
BJN	42.1667±1.82 ^{b,c}	4.7967±0.66^{c,d,e}	2.0244±0.51^{d,e,f}	1.1778±0.19^{f}	23.3383+5.52^{f}	2.1617±0.36^{f,g}
QRV	33.3333±3.06 ^{a}	4.1344±1.35^{b,c}	1.6644±0.32 ^{a,b,c}	1.0383±0.19^{c,d}	19.1350+3.33 ^{b,c}	2.0194±0.29^{d,e,f,g}
LSH	55.1667±6.07 ^{e,f}	6.3911±1.01 ^{h}	2.0411±0.18^{d,e,f}	1.0656±0.16 ^{c,d,e}	26.4828+6.87 ^{g}	1.7728±0.13 ^{b,c}
ARD	37.8333±2.54 ^{a,b}	3.8883±0.77^{a,b}	1.4756±0.23^{a}	0.7583±0.09^{a}	16.3289+1.35 ^{a}	1.9828±0.22^{c,d,e}
AZD	59.3333±3.69 ^{f,g}	5.8406±0.38^{f,g,h}	1.9778±0.17^{d,e,f}	1.0456±0.17^{c,d}	16.9850+3.17^{a,b}	1.8317±0.28^{b,c,d}
NOB	36.3333±5.30 ^{a}	4.9133±1.28^{c,d,e}	1.4550±0.28^{a}	0.9900±0.11^{c}	24.5078±2.44^{f,g}	1.7411±0.26^{b}
KHL	45.6667±9.71 ^{c,d}	4.9639±0.71^{c,d,e,f}	2.0150±0.37^{d,e,f}	0.8800±0.08^{b}	18.3400±3.56^{a,b,c}	2.1406±0.22 ^{f,g}
HMD	42.5000±5.23 ^{b,c}	5.0867±1.43^{d,e,f}	1.6011±0.23^{a,b}	0.9850±0.08 ^{b,c}	22.6606±1.35 ^{e,f}	1.9050±0.37 ^{b,c,d,e}
DHB	50.0000± 4.56 ^{d,e}	6.1517±0.73^{g,h}	1.9544±0.40 ^{d,e,f}	1.0039±0.14 ^{c,d}	22.5350±3.28 ^{e,f}	2.1547±0.27 ^{f,g}
CHL	43.0000±5.60 ^{b,c}	4.7000±1.51 ^{b,c,d}	1.8156±0.32 ^{b,c}	0.9578±0.11 ^{b,c}	24.2794±2.97 ^{f,g}	2.0539±0.26 ^{e,f,g}
TKB	47.1667±6.09 ^{c,d}	7.2306±1.86 ^{i}	1.9928±0.25 ^{d,e,f}	1.1156±0.12 ^{d,e,f}	20.0150±3.05 ^{c,d,e}	1.7378±0.28 ^{b}
BRQ	46.1667±8.84 ^{c,d}	5.3156±1.92 ^{d,e,f,g}	1.8672±0.24 ^{c,d,e}	1.1961±0.11 ^{f}	22.4989±3.03 ^{e,f}	2.0883±0.45 ^{e,f,g}
SOL	44.0000±5.14 ^{c}	7.2306±0.86 ^{i}	2.0567±0.16 ^{e,f}	1.1583±0.09 ^{c,f}	19.6535±1.60 ^{b,c,d}	1.6961±0.28 ^{b}
ABS	61.6667±20.29 ^{g}	5.9961±2.16 ^{g,h}	2.1578±0.39 ^{f}	1.1533±0.13 ^{e,f}	20.2067±2.85 ^{c,d,e}	0.0000

Note: The descriptive statistics are presented in terms of the mean ± SD. Mean values with the same letters indicate homogeneous subsets for $P \leq 0.05$ according to Duncan test. a,b,c,d,e,f,g,h,i Means in the same column with different superscripts differ ($P \leq 0.05$)

Table 6. Correlation between soil compounds and the amount of EP (Abbreviation of parameters are described in Table 2)

Parameter	EP	Clay	Silt	Sand	Sp	$CaCo_3$	OC	Texture	EC	Sal.	pH	DO	TN
EP	1	0.068	0.369	-0.278	-0.258	-0.096	0.151	0.331	-0.296	-0.035	0.598*	0.385	-0.092
Clay	0.068	1	0.730**	-0.881**	0.361	0.208	0.051	-0.146	0.195	0.415	0.231	0.054	-0.335
Silt	0.369	0.730**	1	-0.966**	0.447	0.150	0.434	-0.108	0.172	0.333	0.393	0.188	-0.164
Sand	-0.278	-.881**	-0.966**	1	-0.450	-0.193	-0.318	0.135	-0.198	-0.392	-0.353	-0.144	0.237
Sp	-0.258	0.361	0.447	-0.450	1	0.524*	0.431	-0.410	0.334	0.407	-0.157	-0.197	.545*
$CaCo_3$	-0.096	0.208	0.150	-0.193	0.524*	1	-0.006	-0.556*	0.167	0.160	-0.289	-0.054	0.142
OC	0.151	0.051	0.434	-0.318	0.431	-0.006	1	-0.410	0.333	0.340	0.154	0.372	0.384
Texture	0.331	-0.146	-0.108	0.135	-0.410	-0.556*	-0.410	1	-0.362	-0.384	0.203	-0.137	-0.194
EC	0.410	0.617*	0.548*	-0.617*	0.132	0.246	-0.181	0.146	-0.075	0.022	0.349	-0.100	-0.216
Sal.	-0.296	0.195	0.172	-0.198	0.334	0.167	0.333	-0.362	1	0.747**	-0.306	-0.302	0.261
pH	-0.035	0.415	0.333	-0.392	0.407	0.160	0.340	-0.384	0.747**	1	-0.209	-0.265	0.124
DO	0.598*	0.231	0.393	-0.353	-0.157	-0.289	0.154	0.203	-0.306	-0.209	1	0.581*	0.084
TN	0.385	0.054	0.188	-0.144	-0.197	-0.054	0.372	-0.137	-0.302	-0.265	0.581*	1	-0.020

Note: *Significant difference in α = 5%, **Significant difference in α = 1%, minus sign shows the negative correlation between data's and plus sign shows positive correlation

Table 7. Correlation coefficient between some morphometry elements and soil factors

Factors	Texture	Do	Ec.	pH	Sal	Sp.	Gravel	T.N.V	OC	TN
Plant Height	0.021	0.417	0.061	0.142	-0.019	0.181	-0.039	0.111	0.228	0.293
Trunk Diameter	-0.119	0.431	0.249	0.376	0.319	0.000	-0.317	-0.213	0.153	0.346
Stem Diameter	-0.011	0.488	-0.063	0.485	0.003	0.108	0.079	-0.096	0.158	0.310
Branch Diameter	-0.297	0.009	0.397	0.145	0.274	0.156	0.154	0.196	0.041	0.379
Inter node Length	-0.326	-0.272	0.580*	-0.251	0.548*	0.437	0.373	0.560*	0.136	-0.143
Leaf Length	-0.041	0.063	0.239	0.364	0.064	-0.196	0.652**	0.211	-0.199	-0.183

Note: Pearson's correlation coefficient is indicated with level of significance ($P \leq 0.05$ and $P \leq 0.01$). Negative and positive correlation between factors are shown by minus and plus sign. **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).

Table 8. Correlation coefficient between some morphometry elements and climatic factors (Abbreviation of the climatic factors are described in Table 3)

Factors	Altitude	Collecting time	Ann. pptn.	Avg. Temp.	Min temp.	Max temp.
Plant Height	-0.171	-0.627*	0.265	0.351*	0.365	0.313
Leaf length	0.213	-0.013	-.580*	0.393	0.420	0.394
Trunk Diameter	0.127	0.551*	-0.305	0.289	0.378	0.264
Stem Diameter	0.158	-0.765**	0.068	0.289	0.453	0.457
Branch Diameter	0.209	-0.732*	-0.407	0.548*	0.265	0.307
Inter node Length	-0.360	0.428	-0.361	-0.125	-0.046	0.086

Note: Pearson's correlation coefficient is indicated with level of significance ($P \leq 0.05$ and $P \leq 0.01$). Negative and positive correlation between factors are shown by minus and plus sign. **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed)

Based on achieved results, plant height showed a positive correlation with average temperature ($P \leq 0.05$ and $R = 0.351$) and negative correlation with collecting time of plant ($P \leq 0.05$ and $R = -0.627$). Branch and Stem Diameter showed correlation with average temperature positively ($P \leq 0.05$, $P \leq 0.01$ and $R = 0.548, 0.351$).

The dendrogram achieved from Hierarchical cluster analysis (HCA) of the morphometric data (Figure 2) showed that 15 populations of *E. major* were divided into two main clusters in average distance value (ADV) of 25: cluster A which is then divided into two sub-clusters at ADV 7, A1 divided into two groups at ADV 5 which including TKB and SOL, LSH, DHB and BRQ and A2 including two groups of populations AZD and ABS to gather and KHL. The cluster B, also divided into two sub-clusters at ADV 11. These are: sub-cluster B1 including HMD, CHL, MRZ, QRV and BJN in one group and ARD and NOB in other group.

The graph obtained from principal components analysis (PCA) of the morphometric data (Figure 3) indicated a Comparison of morphometric data among 15 populations of *E. major* was almost similar to the groups that separated according to Amount of Ephedrine. Based on Table 9, four factors are effective in separating different populations of *E. major* (stem, trunk, branch Diameter, and plant height).

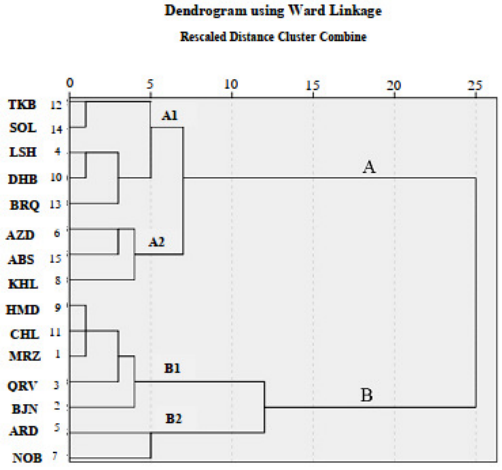

Figure 2. Dendrogram obtained by the hierarchical cluster analysis Morphometric data (Abbreviation of the names are described in Table 1)

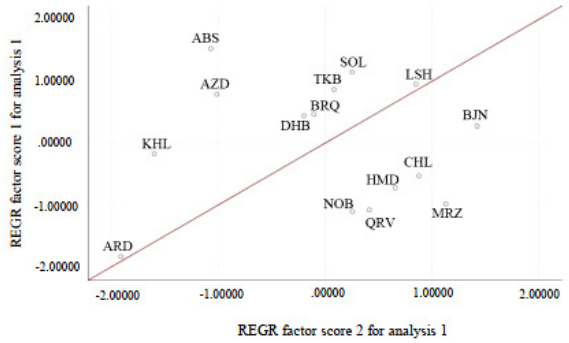

Figure 3. Principal component analysis (PCA) of the morphometric data. (Abbreviation of the names are described in Table 1)

Table 9. Component matrix of principal components analysis (PCA) of the morphometric data from different populations of *E. major*

Factors	Component	
	1	2
Stem Diameter	0.921	-0.051
Trunk Diameter	0.813	-0.041
Plant Height	0.771	-0.226
Branch Diameter	0.745	0.437
Length of Internode	0.036	0.786
Length of leaf	-0.133	0.801

DISCUSSION

Identification of *Ephedra* species can be troublesome because of their simple morphological characteristics and simple adjustment to the changes in their environment [25]. The distinctive extents of the alkaloids vary between different species of the genus *Ephedra* [6]. Quantitative data on the chemical composition of the alkaloids in *Ephedra* species are extremely variable, depending on factors such as the plant species, the amount of rainfall, soil characteristics, altitude, harvesting, storage conditions of the plant, and the analytical quantification method [15, 17, 19]. As mentioned the harvesting timing is critical in many species as *E. major* should be collected in the autumn since the amount of alkaloid shows considerable variation at different seasons [12]. Analyses of *Ephedra major* populations, collected from 15 locations in Iran showed a significant variation regarding EP content. The highest EP content was from CHL population while BRQ population showed the lowest amount. Overall, the concentration of EP among populations did not follow a stable pattern according to the geographical location, for instance, QRV population indicated a high level of EP whereas a physically close population, HMD, showed a considerably lower level of EP. Among ecological factors, altitude found out to have a positive correlation with EP content similar to collecting time. Among populations collected in autumn (MRZ, ARD, QRV, KHL, and NOB) QRV showed the highest amount EP was related to the location's altitude. The investigated populations that were harvested in different seasons (summer, spring), CHL stands out as a population with the highest amount of EP which altitude could be the main reason. The influence of altitude on the content of secondary metabolites has been frequently addressed in the literature. The nature of the plant species is also an important factor in determining the content of secondary metabolites [30]. In previous study Ibragic and Sofic [19] reported 16.3 mg/gr EP in *E. major* which to some extent was similar to our results in some of the populations. While, Aghdasi *et al.* [2] indicated a significantly lower level of EP in *E.major* (1.5 to 2.12 mg/gr) compare to our results, however, their result obtained from populations of *E.major* in only one location. Therefore, the outcome of their study may not be conclusive whereas our study covers almost all the populations of *E. major* in Iran. Spitaler *et al.* [44] and Parsaeimehr *et al.* [37] indicated that by increasing in altitude enhances the exposure to UV which imposes a stress on plants and triggers the defensive mechanisms that secondary metabolites are an important part of. Therefore, such stressors conditions lead to increase in secondary metabolites particularly alkaloids. We observed some of the population growing in destroyed places as result of human activity, the levels of EP were higher (CHL). The level of light that plants receive is another key factor in determining the concentration of secondary metabolism. The photosynthetic activity directly influenced by light intensity [45]. Thus, plants collected in CHL population where disruption by human was significant, it could be speculated that removing the upper layer of forest conferred *E. major* a higher level of light that results in increasing photosynthesis and ultimately secondary metabolites (EP). The amount of EP contents showed a positive correlation with pH (Table 6). The strong influence of soil pH has been reported on the content of secondary metabolites of medicinal plants. Shah *et al.* [42] indicated the positive effect of pH on paeonol and paeoniflorin concentrations. In consistent with our results Mikage *et al.* [26, 31] reported that *Ephedra* plants grown in soil with high pH (alkaline soil) contain more alkaloids. The soil properties including pH value, fertility and trace elements can have direct and indirect influence on the secondary metabolites of the plants [3]

Based on our results, the amount of EP content is negatively related to annual precipitation (rainfall) and a positive correlation with average temperature that means EP content increases with an increase in temperature, whereas the alkaloids are washed out by rain. Similar to our results, a positive correlation between annual precipitation and EP content in *E. sinica* was reported from China and Mongolia [27, 48]. It seems that annual precipitation and light intensity play important roles in determining the EP content of *E. major*.

According to the morphometric analysis, there were no significant relationship between amount of EP and morphologic characters (Table 10), whereas the results achieved by Mikage *et al.* [26, 31] reported that the amount of alkaloids content in the plant growing at the upper part of a slope had rather shorter internodes were higher than those growing at the bottom of a slope.

Table 10. Correlation coefficient between climatic conditions and EP (Abbreviation of parameters are described in Table 3 and 5)

Parameter	Altitude	HP	TD	SD	BD	LIN	LL	Sum pptn	Avg. Temp	Max Temp	Min Temp	EP
Alt	1	-0.171	0.213	0.127	0.165	0.208	-0.182	-0.102	-0.269	-0.290	-0.511	-0.221
HP	-0.171	1	0.434	0.707**	0.371	-0.008	-0.280	0.265	0.401	0.424	0.357	-0.138
TD	0.213	0.434	1	0.669**	0.591*	-0.063	-0.113	-0.305	0.416	0.584*	0.420	0.003
SD	0.127	0.707**	0.669**	1	0.640*	-0.051	-0.085	0.068	0.396	0.454	0.417	0.083
BD	0.165	0.371	0.591*	0.640*	1	0.254	0.349	-0.407	0.536*	0.514	0.537*	0.134
LIN	0.208	-0.008	-0.063	-0.051	0.254	1	0.403	-0.361	0.125	-0.133	0.173	-0.055
LL	-0.182	-0.280	-0.113	-0.085	0.349	0.403	1	-0.580*	0.274	0.257	0.428	0.264
Sumpptn	-0.102	0.265	-0.305	0.068	-0.407	-0.361	-0.580*	1	-0.482	-0.361	-0.385	-.563*
AvgTem	-0.269	0.401	0.416	0.396	0.536*	0.125	0.274	-0.482	1	0.766**	0.763**	.554*
MaxTem	-0.290	0.424	0.584*	0.454	0.514	-0.133	0.257	-0.361	0.766**	1	0.674**	0.163
MinTem	-0.511	0.357	0.420	0.417	0.537*	0.173	0.428	-0.385	0.763**	0.674**	1	0.451
EP	-0.221	-0.138	0.003	0.083	0.134	-0.055	0.264	-0.563*	0.554*	0.163	0.451	1

Note: *Significant difference in α = 5%, ** Significant difference in α = 1%, minus sign shows the negative correlation between data and plus sign shows positive correlation

It appears that the contents of Ephedrine among populations determine by soil composition and environmental factors. Total Ephedrine content responded positively to high pH in addition to collecting time and annual precipitation. Higher temperatures emphatically affected the amount of Ephedrine content. In general, temperature and precipitation were the most important climatic factors. Besides, according to our results there is no correlation between the morphometric elements and the amount of Ephedrine content. These results are firsthand and can be exploited for further domestication and understand the edaphic and ecological requirements of *E. major*. It's highly recommended that in the future studies other compositions of secondary metabolites also be analyzed to pinpoint the effect of environmental variables in a comprehensive manner. Additionally, the phytochemical potential (e.g., antibacterial and fungicide) of *E. major* may vary from population to another therefore further investigation could take into account the influence of environmental condition on this aspects as well.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article.

REFERENCES

[1] Allison, L.E., Moodie, C.D., (1965): Carbonate. In C.A. Black *et al.*, Eds., Methods of Soil Analysis, Part 2 - Chemical and Microbiological Properties 1st ed. American Society for Agronomy, Madison, WI, pp. 1379-1396.

[2] Aghdasi, M., Mofid Bojnoordi, M., Mianabadi, M., & Nadaf, M. (2016). Chemical components of the *Ephedra major* from Iran. Natural product research, 30(3), 369-371.

[3] Antoniadis, V., Levizou, E., Shaheen, S. M., Ok, Y. S., Sebastian, A., Baum, C., Rinklebe, J., (2017). Trace elements in the soil-plant interface: Phytoavailability, translocation, and phytoremediation–A review. Earth-Science Reviews, 171: 621-645.

[4] Assadi, M., (1998): Flora of Iran: Nos. 19-22 Pinaceae, Taxaceae, Cupressaceae and Ephedraceae Ministry of Agriculture, Islamic Republic of Iran, pp. 58.

[5] Bahernik, Z., Ahmadi, L., Babakhanlou, P., (2000): Comparison of ephedrine and pseuduephedrine contents in ephedra species in Iran. Iranian Journal of Medicinal and Aromatic Plants, 6: 48-65.

[6] Blechman, K.M., Karch, S.B., Stephens, B.G., (2004): Demographic, pathologic, and toxicological profiles of 127 decedents testing positive for ephedrine alkaloids. Forensic science international, 139(1): 61-69.

[7] Bremner, J.M., (1996): Nitrogen-total. In D.L. Sparks *et al.*, Eds. Methods of Soil Analysis, Part 3-Chemical Methods. Soil Science Society of America, American Society of Agronomy, Madison, WI, pp. 1085-1121.

[8] Bruneton, J., (1995): Pharmacognosy, phytochemistry, medicinal plants. Lavoisier publishing, pp. 711-715.

[9] Caveney, S., Charlet, D.A., Freitag, H., Maier-Stolte, M., Starratt, A.N., (2001): New observations on the secondary chemistry of world *Ephedra* (Ephedraceae). American journal of botany, 88: 1199-1208.

[10] Chin, F.-S., Chong, K.-P., Markus, A., Wong, N.K., (2013): Tea polyphenols and alkaloids content using soxhlet and direct extraction methods. World Journal of Agricultural Sciences, 9: 266-270.

[11] Djilani, A., Legseir, B., Soulimani, R., Dicko, A., Younos, C., (2006): New extraction technique for alkaloids. Journal of the Brazilian Chemical Society, 17: 518-520.

[12] Evans, W.C., (2009): Trease and Evans Pharmacognosy 16th ed. Saunders. Elsevier Ltd. China, 603 p.

[13] Farag, M.A., Porzel, A., Wessjohann, L.A., (2012): Comparative metabolite profiling and fingerprinting of medicinal licorice roots using a multiplex approach of GC–MS, LC–MS and 1D NMR techniques. Phytochemistry, 76: 60-72.

[14] Fukushima, K., (2004): Bioactivity of *Ephedra*: integrating cytotoxicity assessment with real-time biosensing. MSc Thesis, University of Maryland, 67 p.

[15] Gee, G., Bauder, J.W., (1979): Particle size analysis by hydrometer: a simplified method for routine textural analysis and a sensitivity test of measurement parameters. Soil Science Society of America Journal, 43: 1004-1007.

[16] Grue-Sørensen, G., Spenser, I.D., (1989): The biosynthesis of ephedrine. Canadian Journal of Chemistry, 67: 998-1009.

[17] Haller, C.A., Duan, M., Benowitz, N.L., Jacob Iii, P., (2004): Concentrations of ephedra alkaloids and caffeine in commercial dietary supplements. Journal of analytical toxicology, 28: 145-151.

[18] Hsu, H., Chen, Y., Sheu, S., Hsu, C., Chen, C., Chang, H., (1996): Chinese materia medica: A concise guide modern drug. Weekly Press, Taipei, pp. 31-32.

[19] Ibragic, S., Sofić, E., (2015): Chemical composition of various *Ephedra* species. Bosnian journal of basic

medical sciences, 15: 21-27.
[20] Ickert-Bond, S.M., Skvarla, J.J., Chissoe, W.F., (2003): Pollen dimorphism in *Ephedra* L. (Ephedraceae). Review of Palaeobotany and Palynology, 124: 325-334.
[21] Ickert-Bond, S.M., Wojciechowski, M.F., (2004): Phylogenetic relationships in *Ephedra* (Gnetales): evidence from nuclear and chloroplast DNA sequence data. Systematic Botany, 29: 834-849.
[22] Kashki, M., (2000): Comparison content of alkaloids in endemic species of *Ephedra* in Khorasan province. Final report of research project, Khorasan agriculture and natural resources research center, 26 p. (In Persian).
[23] Khym, J.X., (1974): Analytical ion-exchange procedures in chemistry and biology: Theory, equipment, techniques. Prentice-Hall, Inc., Englewood Cliffs, NJ., 257 p.
[24] Kim, H.K., Choi, Y.H., Chang, W.-T., Verpoorte, R., (2003): Quantitative analysis of ephedrine analogues from Ephedra species using 1H-NMR. Chemical and Harmaceutical bulletin, 51: 1382-1385.
[25] Kitani, Y., Zhu, S., Omote, T., Tanaka, K., Batkhuu, J., Sanchir, C., Fushimi, H., Mikage, M., Komatsu, K., (2009): Molecular analysis and chemical evaluation of Ephedra plants in Mongolia. Biological and pharmaceutical bulletin, 32: 1235-1243.
[26] Kondo, N., Mikage, M., Idaka, K., (1999): Medico-botanical studies of *Ephedra* plants from the Himalayan region, part III: Causative factors of variation of alkaloid content in herbal stems. Natural medicines, 53: 194-200.
[27] Krizevski, R., Bar, E., Shalit, O., Sitrit, Y., Ben-Shabat, S., Lewinsohn, E., (2010): Composition and stereochemistry of ephedrine alkaloids accumulation in *Ephedra sinica* Stapf. Phytochemistry, 71: 895-903.
[28] Kubitzki, K., (1990): Ephedraceae, The families and genera of vascular plants (Vol. 1). Pteridophytes and Gymnosperms, Springer-Verlag, Berlin, pp. 379-382.
[29] Leung, A.Y., (1980): Encyclopedia of common natural ingredients used in food, drugs, and cosmetics. New York, John Wiley & Sons, pp. 266-267.
[30] Mafakheri, M., Kordrostami, M., (2020): Recent advances toward exploiting medicinal plants as phytoremediators. In: Hasanuzzaman M., Prasad M. N. V. (eds.): Handbook of Bioremediation: Cambridge, USA: Academic Press, pp. 371-383.
[31] Mikage, M., Takano, A., Jin, H., Tomimori, T., Namba, T., (1987): Studies on the Nepalese Crude Drugs (VII). On the Variation in the Morphological Appearances and the Alkaloid Contents of the Herbal Stem of *Ephedra gerardiana* Wall. according to the Differences of Habitats. The Japanese journal of pharmacognosy, 41: 209-214.
[32] Miller, D.K., Segert, I.L., (2005): Mecamylamine attenuates ephedrine-induced hyperactivity in rats. Pharmacology Biochemistry and Behavior, 81: 165-169.
[33] Mozaffarian, V., (2010): Three new species and two species records from Iran, Ilam Province. The Iranian Journal of Botany, 16: 204-212.
[34] Nagai, W., Kanao, S., (1928): Constituents of Chinese drug "Ma Huang". The Pharmaceutical Society of Japan, 48: 845-851.
[35] O'Dowd, N., McCauley, P., Wilson, G., Parnell, J., Kavanagh, T., Mc Connell, D., (1998): In Vitro culture, micropropagation and the production of ephedrine and the production of ephedrine and other alkaloids. In Biotechnology in agriculture and forestry 41. Medicinal and aromatic plants Springer-Verlag, Berlin, pp. 154-193.
[36] O'Neil, M.J., (2013): The Merck index: an encyclopedia of chemicals, drugs, and biologicals. RSC Publishing, 666 p.
[37] Parsaeimehr, A., Sargsyan, E., Javidnia, K., (2010): A comparative study of the antibacterial, antifungal and antioxidant activity and total content of phenolic compounds of cell cultures and wild plants of three endemic species of *Ephedra*. Molecules, 15: 1668-1678.
[38] Price, R.A., (1996): Systematics of the Gnetales: a review of morphological and molecular evidence. International Journal of Plant Sciences, 157: S40-S49.
[39] Riedl, H., (1963): *Ephedra* (Ephedraceae). pp. 476-479. In: Rechinger K.H. (ed.): Flora Iranica. – Graz: Akademische Druck- und Verlagsanstalt.
[40] Riedl, R., (1967): Die Gattung *Ephedra* in Europa. – Sci. Pharm. 36: 225-228.
[41] Schaneberg, B.T., Crockett, S., Bedir, E., Khan, I.A., (2003): The role of chemical fingerprinting: application to *Ephedra.* Phytochemistry, 62: 911-918.
[42] Shah, F. A., Ren, Y. U. A. N., Yuan, Y. J., Fu, S., Wang, Y., Chen, H., (2018). Effect of plant age and geographical location on active paeonol and paeoniflorin accumulation in the roots of *Paeonia ostii*. Pakistan Journal of Botany 50: 1785-1790.
[43] Shaw, D., (2010): Toxicological risks of Chinese herbs. Planta medica, 76: 2012-2018.
[44] Spitaler, R., Winkler, A., Lins, I., Yanar, S., Stuppner, H., Zidorn, C., (2008): Altitudinal variation of phenolic contents in flowering heads of *Arnica montana* cv. ARBO: a 3-year comparison. Journal of chemical ecology, 34: 369-375.
[45] Thoma, F., Somborn-Schulz, A., Schlehuber, D., Keuter, V., Deerberg, G., (2020). Effects of Light on Secondary Metabolites in Selected Leafy Greens: A Review. Frontiers in Plant Science, 11, 497-510.
[46] Vaidya, V.S., Mehendale, H.M., (2014): *Ephedra*. In: Wexler P. (ed.): Encyclopedia of Toxicology (3rd edition): Oxford, UK: Elsevier, pp. 426-430.
[47] Vidakovic, M., (1991): Conifers morphology and variation, Translated from Croatian by Maja Soljan. Graficki Zavod Hrvatske, Croatia. (from the Gymnosperm Database), 3(13): 775.
[48] Wang, J., Van Der Heijden, R., Spruit, S., Hankermeier, T., Chan, K., Van Der Greef, J., Xu, G., Wang, M., (2009): Quality and safety of Chinese herbal medicines guided by a systems biology perspective. Journal of ethnopharmacology, 126: 31-41.
[49] Wang, L.L., Kakiuchi, N., Mikage, M., (2010): Studies of *Ephedra* plants in Asia. Part 6: Geographical changes of anatomical features and alkaloids content of *Ephedra sinica*. Journal of natural medicines, 64: 63-69.
[50] Yamazaki, K., (1985): Chemical components of Ma-Huang. Wakan Iyakan Gakkaishi, 2: 93-94.

Received: February 25, 2020
Accepted: January 19, 2021
Published Online: January 28, 2021
Analele Universităţii din Oradea, Fascicula Biologie
http://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
University of Oradea Publishing House