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HPLC Fingerprint of Important Phenolic Compounds in Some *Salvia* L. Species from Iran

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Abstract: *Salvia* L. is a large genus of the Lamiaceae family with high medicinal value. Pharmaceutical properties of *Salvia* species are mainly due to their secondary metabolites, especially phenolic compounds. This study was focused on identification and determination of five bioactive phenolic compounds (rosmarinic acid, carnosic acid, caffeic acid, salvianolic acids A and B) in the 41 populations from 27 wild *Salvia* species of Iran using a simple and reliable HPLC-UV method. The principal component analysis (PCA) technique was used to study differentiation among species according to their phenolic compound profiles. Significant intra- and inter-specific variations were observed in the distribution patterns and contents of phenolic compounds in the studied *Salvia* species. As a result of this study, it was found that leaves had greater amounts of phenolic compounds as compared to the roots. The highest content of rosmarinic acid (41.53±0.88 mg/g DW) and salvianolic acid A (8.10±0.35 mg/g DW) were found in the leaves of *S. verticillata*. The leaves of *S. syriaca* and *S. sharifii* were rich in salvianolic acid B (54.47±2.00 mg/g DW) and carnosic acid (34.05±1.18 mg/g DW), respectively. The PCA results revealed chemical variations in the *Salvia* species collected from different regions and could fully distinguish between them based on the phenolic compounds concentrations. The present study demonstrated that apart from *S. officinalis,* some wild species such as *S. verticillata*, *S. hypoleuca*, *S. leriifolia* and *S. virgata* can be introduced as potent natural sources for medicinal and industrial purposes.

Keywords: *Salvia* species; rosmarinic acid; salvianolic acid A; salvianolic acid B; carnosic acid; HPLC.

1. Introduction

The genus *Salvia* L. (sage) consists of about 900 plant species and represents one of the most important and the largest genera of the Lamiaceae family [1-3]. Iran with 58 species, of which 17 are endemic [1-3], is one of the best countries for growing *Salvia* species in the world [1]. The name of *Salvia* comes from the Latin words *salvare*, s*alveo*, *salvus* or *salvere* meaning healing, non-harmful and safe and refers to the numerous medicinal applications of *Salvia* species [1,3-6].

Salvia species are known for their several therapeutic properties in folk medicine to treat tuberculosis, bronchitis, pyretic, rheumatoid arthritis, colds, wounds and skin infections, headache, cerebral ischemia and memory disorders, as well as hepatitis [1,7]. In Iranian traditional medicine, different parts of many indigenous *Salvia* species such as *S. aethiopis*, *S. aegyptiaca*, *S. officinalis*, *S. hydrangea*, *S. sclarea*, *S. macrosiphon* and *S. viridis* are used locally for treatment of some diseases

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including flatulence, eye disorders, antiseptic for wounds, diuretic and fever [8]. Furthermore, some pharmacological activities such as antioxidant, antibacterial, antifungal, antimicrobial, antitumor, antidiabetic, antituberculosis, antiplasmodial, antiinflammatory and anticholinesterase properties (treatment of Alzheimer's Disease) have been recently proven for the *Salvia* species through clinical studies in modern medicine [1,9,10]. *Salvia* species, specially *S. officinalis* have been traditionally used as herbal tea for the treatment of digestive and circulation disturbances, bronchitis, cough, asthma, angina, mouth and throat inflammations, depression, excessive sweating, skin ailments and as well as flavoring agents in the food industries, cosmetic, pharmaceutical and fragrance products [9,11].

Members of *Salvia* genus have been the subjects of extensive studies with the aim of identification and characterization of potential bioactive compounds. Terpenoids (di-and triterpenoids), phenolic acid derivatives and flavonoids are the predominant secondary metabolite constituents of *Salvia* species [12,13]. *Salvia* species mainly contain two major types of biologically active compounds: lipid-soluble abietane-type diterpenoid tanshinones [14] and carnosic acid [15] and water-soluble phenolic acids and flavonoids. Phenolic acids which are widely distributed in plant species are responsible for their various therapeutic effects [14].

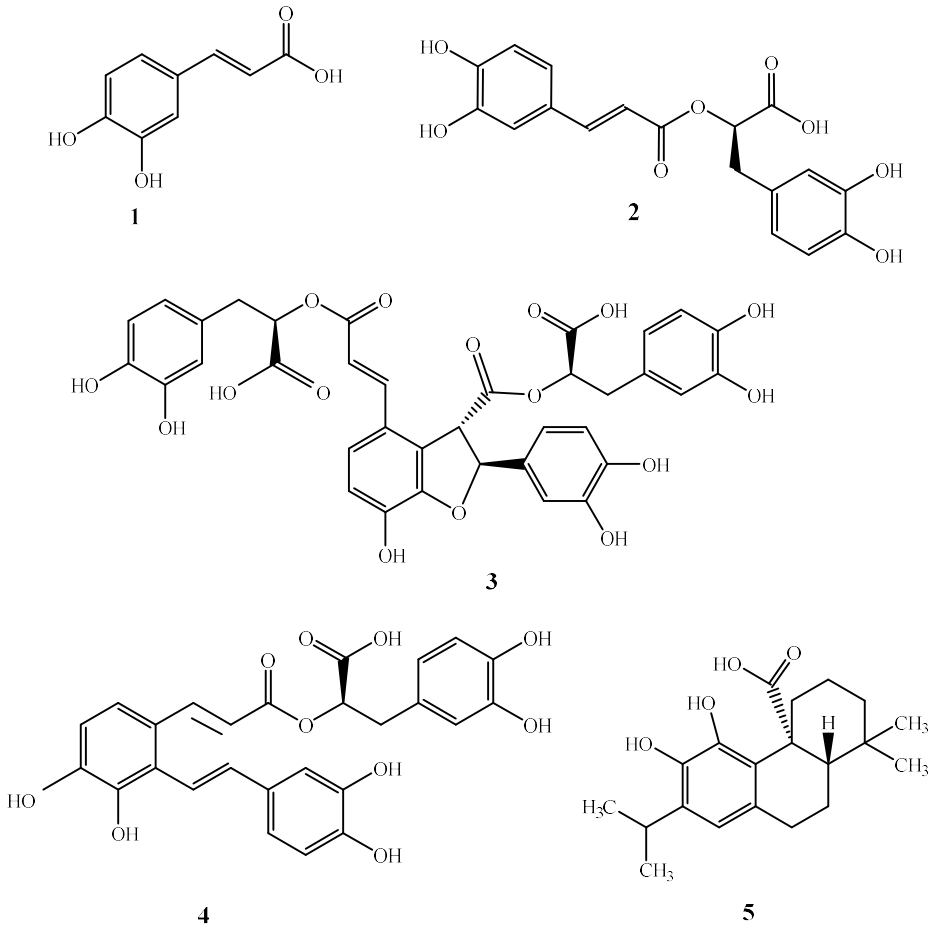

Figure 1. Typical phenolic compounds in *Salvia* genus
1: Caffeic acid, **2**: Rosmarinic acid, **3**: Salvianolic acid B, **4**: Salvianolic acid A, **5**: Carnosic acid

Caffeic acid (CAA) acts as a structural unit of a variety of the phenolic compounds from the simple monomers to condensed oligomer products (Figure 1) and plays a basic role in the biochemistry of the *Salvia* species [5,16]. Different CAA derivatives occur as the major hydrophilic components of the *Salvia* species and possess a variety of biological activities [13,16]. It is well known that anti-oxidative activities of *Salvia* species are mainly due to the presence of rosmarinic acid

(RA) (dimer of CAA) as the most abundant phenolic compound [5,16]. Salvianolic acids as derivatives of CAA are another groups of polyphenolic metabolites whose occurrence has only been approved in the *Salvia* genus [16-19]. Among these phenolic acids, salvianolic acid A (Sal A) a trimeric derivative of CAA, and salvianolic acid B (Sal B) a dimer derivative of RA, and thus a tetramer of CAA, are two of the most important ones [16,20,21]. It has been demonstrated that Sal A as the most effective salvianolic acid in *Salvia* genus has antioxidative, antitumor, biomembrane and cardiovascular protection effects [16,21]. Salvianolic acid B, as the most abundant phenolic compound of *S. miltiorrhiza* is widely used in clinical practice for the treatment of cardiovascular disorders and liver diseases in China [21, 22].

Carnosic acid (CA) is a phenolic diterpene of the abietane type compounds [23] that has received great attention as a strong antioxidant in food and biomedical sciences [24]. This phenolic diterpene is found frequently in *Rosmarinus officinalis* and *S. officinalis* [23,24]. The presence of CA has also been confirmed in other species of Lamiaceae including *Satureja montana*, *S. sclarea*, *S. glutinosa* [25], *S. mellifera* [19], *S. eremophila* and *S. santolinifolia* [26].

Since the pharmaceutical value of phenolic compounds is well known and *Salvia* species are widely distributed in Iran, the limited data available prompted the present investigation of the identification and determination of some phenolic compounds in some wild *Salvia* species from Iran.

2. Materials and Methods

2.1. Plant Materials

The 41 samples of 27 *Salvia* species were harvested at the flowering stage from different localities of Iran during the spring and summer 2012. The voucher specimens were deposited at the central herbarium of Bu-Ali Sina University (BASUH) and Herbarium of Biology Department, Hormozgan University (HAPH), Iran. The scientific names, collection sites and voucher numbers of the studied species are listed in Table 1.

2.2. Chemicals and Standards

All the analytical and HPLC-grade solvents were supplied from Merck Chemical Co. Ltd. (Darmstadt, Germany). Standards samples of carnosic acid, salvianolic acid B and caffeic acid were purchased from Sigma, rosmarinic acid and salvianolic acid A were supplied from Aldrich and Fluka, respectively.

2.3. Phenolic Compounds Extraction from Samples

One gram of the air-dried and finely powdered leaf and root samples of the examined *Salvia* species were separately extracted by maceration in methanol (2×10 mL for 24 h) at room temperature and dark place. After filtration, the solvents were removed on a rotary evaporator under vacuum at 40˚C to dryness. The crude extracts were stored in the dark at 4˚C until analysis. The dried extracts freshly were dissolved in 99% ethanol at 4mg/mL concentration for further studies [27].

2.4. HPLC Analysis

Phenolic compounds in the samples were identified and quantified using a Smartline HPLC instrument (Kenuer, Germany) equipped with a quaternary pump and a UV-VIS detector (D-14163 model). Reverse phase chromatography separation was performed with a C18 Eurospher-100 (5 µm particle, 125 mm × 4 mm). The chromatographic data were processed using ChromGate software (version 3.1). The flow rate used for column elution was 1 mL/min and peaks were monitored by UV detection at 280 nm. The sample injection volume was 20 µL. The solvent system was 0.2% (v/v) glacial acetic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution program was as follows: 90-75% A (v/v) at 0-15 min, 75-20% A (v/v) at 15-40 min, 20-0% A (v/v) at 40-45 min,

0% A (v/v) at 45-50 min, 0-90% A (v/v) at 50-55 min. Samples and standard solutions were filtered through 0.45 µm hydrophilic PTFE membrane filters before injection. Identification of the compounds in the chromatograms was performed by comparison of their retention times with those of reference standards. Determination of the each phenolic compound was performed using the corresponding calibration curve. Extract samples were injected three times to HPLC for analysis.

Table 1. Scientific name, locality and voucher number of the studied Iranian *Salvia* species

Species (population**)**	**Location (**province**)**	**Collection Time**	**Latitude (N)**	**Longitude (E)**	**Altitude (m)**	**Voucher number**
S. aegyptiaca L.	Hormozgan- Geno mountain	April	27° 26' 45"	56° 18' 17"	235	HAPH-92138
S. aethiopis L.	North Khorasan- Rooieen	June	37° 23' 11.66"	57° 02' 37.32"	1889	BASU 32969
S. aristata Aucher ex Benth.	Qazvin- Gardane Kaman	July	36° 27' 49"	50° 07' 59"	2034	BASU 34046
S. atropatana Bunge.	Alborz- Kandovan	June	36° 10' 20"	51° 18' 53"	2581	BASU 34027
S. ceratophylla L. (T)	Tehran- Damavand	June	35° 40' 56"	52° 01' 45"	2020	BASU 34058
S. ceratophylla L. (M)	Razavi Khorasan- Abghad	June	36° 30' 26.6"	59° 02' 18.41"	1450	BASU 33989
S. choloroleuca Rech. f. & Aell. (T1)	Tehran- Shemshak	June	35° 59' 27.7"	51° 28' 55.5"	2365	BASU 34022
S. choloroleuca Rech. f. & Aell. (T2)	Tehran- Darbandsar	July	36° 00' 00"	51° 29' 00"	2450	BASU 34038
S. choloroleuca Rech. f. & Aell. (M)	Razavi Khorasan- Ferizi	June	40° 37' 32"	40° 67' 45"	1965	BASU 34094
S. chorassanica Bunge.	Razavi Khorasan- Binalud mountain	July	36° 15' 57"	59° 07' 41.3"	2790	BASU 34047
S. hydrangea DC.	Qazvin- Razjerd village	July	36° 20' 43"	50° 09' 26"	1609	BASU 34510
S. hypoleuca Benth. (T1)	Alborz- Gajereh	July	36° 06' 08"	51° 20' 56"	2335	BASU 34037
S. hypoleuca Benth. (T2)	Tehran- Jajrud	July	35° 44' 52"	51° 43' 07"	1570	BASU 33080
S. hypoleuca Benth. (Q)	Qazvin- Khanjarbolagh village	July	36° 26' 16"	50° 13' 05"	2112	BASU 34512
S. leriifolia Benth.	Razavi Khorasan- Bojestan	April	34° 22' 31.81"	58° 41' 9.33"	1300	BASU 34050
S. limbata C.A. Mey.	Qazvin- Zereshk	July	36° 25' 59.39"	50° 06' 11.18"	2000	BASU 34511
S. macrosiphon Boiss.	Razavi Khorasan- Kalat road	July	36° 36' 4.56"	59° 54' 8.73"	1821	BASU 33990
S. mirzayanii Rech. f. & Esfand.	Hormozgan- Tangezagh	April	27° 37' 56"	56° 12' 11"	410	HAPH-91321
S. nemorosa L. (T)	Tehran- Firozkoh	July	35° 43' 39"	52° 40' 23"	1855	BASU 34072
S. nemorosa L. (M)	Razavi Khorasan- Tangalshur	June	36° 14' 17.93"	59° 40' 25.48"	973.5	BASU 34232
S. nemorosa L. (Q)	Qazvin- Aliabad forest	June	36° 26' 1"	49° 9' 53"	1940	BASU 33997
S. × *sylvestris* (IBRC)	Iranian Biological Resources Center	July	-	-	-	IBRC P1000875
S. officinalis L.	Razavi Khorasan- Tangalshur	June	36° 14' 17.93"	59° 40' 25.48"	937.5	BASU 33995
S. reuterana Boiss. (T1)	Tehran- Zaigan	July	35° 56' 49"	51° 32' 51"	2030	BASU 34070
S. reuterana Boiss. (T2)	Tehran- Damavand road	July	35° 42' 21"	51° 59' 14"	2032	BASU 33992
S. santolinifolia Boiss.	Hormozgan- Tangezagh	April	27° 36' 54"	56° 12' 45"	398	HAPH-91239
S. sclarea L. (T)	Tehran- Damavand	July	35° 44' 43"	52° 03' 39"	2160	BASU 34039
S. sclarea L. (M)	North Khorasan- Rooieen	July	37° 23' 11.66"	57° 2' 37.32"	1889	BASU 32965
S. sclarea L. (Q)	Qazvin- Joladak village	July	36° 22' 19"	50° 32' 10"	2200	BASU 34079
S. sharifii Rech. f. & Esfand.	Hormozgan- Geno mountain	April	27° 26' 29"	56° 18' 11"	198	HAPH-92054
S. staminea Montbr. & Auch. ex Benth.	Alborz- Kandovan	June	36° 10' 20"	51° 18' 53"	2581	BASU 34077
S. syriaca L.	Qazvin- Joladak village	July	36° 22' 19"	50° 32' 10"	2200	BASU 34052
S. tebesana Bunge.	South Khorasan- Bandar dare	July	32° 48' 59.68"	59° 12' 58.53"	1720	BUSU 34588
S. verticillata L.(T1)	Tehran- Shemshak	June	35° 52' 13"	51° 32' 25"	1826	BASU 34023
S. verticillata L. (T2)	Tehran- Darbandsar	July	36° 00' 23"	51° 29' 00"	2450	BASU 34034
S. verticillata L. (Q)	Qazvin-Joladak village	July	36° 22' 19"	50° 32' 10"	2200	BASU 33996
S. verticillata L. (IBRC)	Iranian Biological Resources Center	July	36° 28' 28.5"	50° 24' 57.5"	1536	IBRC P1003833
S. virgata Jacq. (Q)	Qazvin- Aghagir village	June	36° 23' 08"	50° 24' 08"	1405	BASU 34513
S. virgata Jacq. (M)	North Khorasan- Rooieen	July	37° 11' 41.61"	57° 29' 29.75"	1760	BASU 34044
S. viridis L.	Qazvin- Rajaee dasht	June	36° 27' 33"	50° 17' 01"	1135	BASU 34088
S. xanthocheila Boiss. ex Benth.	Qazvin- Pich bon village	July	36° 26' 16"	50° 13' 05"	2112	BASU 34041

2.5. Method Validation

2.5.1. Linearity

The standard stock solutions were separately prepared in pure ethanol and diluted to appropriate concentration range for the establishment of calibration curves. The calibration curves were plotted on the basis of linear regression analysis of the integrated peak areas (y) versus concentrations (x, µg/mL) of the five authentic compounds at different levels (Table 2). Correlation coefficients were considered to confirm the significant linear calibration for the standard samples.

Table 2. Validation parameters of HPLC method for the reported phenolic compounds

Compounds	Purity percentage	Stock concentration (mg/mL)	Linear range (µg/mL)	Regression equation	R^2	RSD (%)
Rosmarinic acid	96%	1	0-100	y = 42197x - 41350	0.9965	0.5
Salvianolic acid B	≥94%	1	0-1000	y = 2055.1x + 54.199	0.9938	0.4
Salvianolic acid A	≥95%	0.9	0-80	y = 20037x - 30584	0.9976	0.3
Carnosic acid	≥95%	1	0-600	y = 4519.9x + 62174	0.9683	1.5
Caffeic acid	≥98%	6	0-180	y = 7053.4x + 12476	0.9979	0.5

2.5.2. Precision and Recovery

The precision of the developed HPLC method was performed with different concentrations of five standard compounds. Twenty microliters of each standard solution was injected into the HPLC system continuously under the same chromatographic conditions for five times. The percent relative standard deviation (RSD%) for the peak area of each standard were calculated.

To evaluate of the accuracy of the applied method, recovery experiments were performed after adding three varying quantities of the standards to the samples (leaves of *S. officinalis*). Recovery values of the five components were measured from the corresponding calibration curve, and RSDs were calculated.

2.6. Statistical Analysis

Data were reported as means ± SE of three independent test. The means were compared using the one-way ANOVA test and multivariate analysis of variance followed by Duncan's multiple range tests (P<0.05). Graphs were drawn using Excel software. Statistical analysis was conducted with SPSS (version 22.0) software.

3. Results and Discussion

3.1. Optimizing of HPLC Condition

In this survey, different HPLC parameters were tested and compared. The phenolic compounds were detected at 260, 280, 290 and 330 nm wavelengths, tested mobile phases were consisted of acetonitrile–water system and methanol–water system and mobile phase flow rates were 0.5, 0.7, 0.8, 1 and 1.2 mL/min. The binary mixtures of the acetonitrile–water system were more effective for the separation of the detected compounds. Addition of 2% (v/v) acetic acid to water improved peaks shape and separation efficiency. Due to greater baseline stability, mobile phase system of acetonitrile–2% aqueous acetic acid was selected. Also, the results showed that the most suitable elution flow rate was 1 mL/min and all the examined compounds were well detected at wavelength of 280 nm. Under these experimental conditions, all the five compounds were eluted within a run time of 60 min and the separation was adequate.

3.2. Method Validation

The method was validated by the linearity, precision and reproducibility of the results. Regression equations were derived from the external standard method. The correlation coefficient of the equations (R^2) was over 0.9683, which indicated all the standard compounds showed good linearity in the relatively wide concentration. After determination, precision and repeatability (RSD of intra-day) of the five authentic substances was below 1.5% (Table 2). The RSDs were taken as a measure of

precision and their values indicated that the instrument was highly precise. The average recovery of the five components and RSDs were obtained (Table 3) and showed that the method had a good accuracy.

Table 3. A sample recovery data of the reported compounds in the leaves of *S. officinalis*

Compound	Sample contents (mg/g)	Added (mg/g)	Recovery (%)	Mean recovery (%)	RSD (%)
		7.50	99.84		
RA	15.02	11.30	100.10	100.08	1.48
		18.00	100.30		
		3.50	96.87		
Sal B	7.03	4.75	97.45	97.16	2.00
		9.20	97.15		
		2.30	98.20		
Sal A	4.55	3.35	97.65	98.45	2.80
		5.50	99.50		
		3.87	96.03		
CA	7.75	5.40	95.65	96.06	1.50
		9.00	96.50		
		0.95	99.52		
CAA	1.82	1.20	98.52	99.02	1.10
		2.20	99.03		

3.3. Quantitative Analysis of Samples

In the present study, the presence and the amount of some phenolic compounds (RA, CA, CAA, Sal A and Sal B) were simultaneously assessed in 41 populations from 27 wild *Salvia* species of Iran by HPLC. Based on the literature, this is the first report about the existence of the studied phenolic acids in some *Salvia* species, including *S. aegyptiaca*, *S. aristata*, *S. atropatana*, *S. ceratophylla*, *S. chorassanica*, *S. hydrangea*, *S. mirzayanii*, *S. reuterana*, *S. sharifii*, *S. tebesana*, *S. xanthocheila* and *S. syriaca.* Qualitative analysis of ethanolic extracts of the studied organs by HPLC confirmed the presence of the phenolic compounds. As shown in the Figure 2, HPLC chromatograms of the extracts exhibited five main peaks with the retention times of 20.5, 21.88, 23.05, 34.5 and 37.3 min, corresponding to RA, Sal B, Sal A, CA and CAA, respectively.

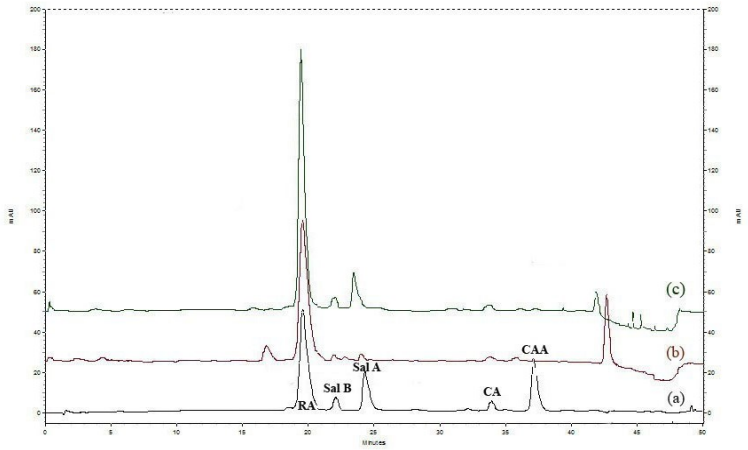

Figure 2. Representative HPLC chromatograms of the five phenolic components in the standard solution (a) and in the ethanolic extract of the roots (b) and leaves (c) of *S. verticillata* (IBRC); **RA**: rosmarinic acid, **Sal B**: salvianolic acid B, **Sal A**: salvianolic acid A, **CA**: carnosic acid, **CAA**: caffeic acid

Our findings showed the great variations in the contents of phenolic compounds among the studied *Salvia* species at both intra- and inter-species levels. The results showed that the leaves were rich in phenolic compounds with Sal B being the most abundant with descending amounts of RA, CA and Sal A in that order (Table 4).

Table 4. The content of determined phenolic compounds in the leaves and roots of 41 populations from 27 Iranian *Salvia* species

Species (population)	Yield (%)		Rosmarinic acid (mg/g DW)		Salvianolic acid B (mg/g DW)		Salvianolic acid A (mg/g DW)		Carnosic acid (mg/g DW)		Caffeic acid (mg/g DW)	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
S. aegyptiaca	13.00	-	2.15±0.49^{p-r}	-	0.00±0.00^{r}	-	0.67±0.10^{l-q}	-	3.38±0.42^{i-n}	-	1.74±0.05^{n-q}	-
S. aethiopis	15.00	5.40	4.25±1.04^{k-n}	1.27±0.61^{k-o}	5.04±1.21^{o-q}	1.78±0.16^{kl}	0.70±0.29^{k-q}	0.04±0.01^{jk}	8.50±0.87^{hi}	0.00±0.00^{i}	7.83±0.48^{i}	5.65±0.38^{d}
S. aristata	16.50	21.80	0.45±0.03^{s}	0.94±0.36^{n-o}	6.38±0.66^{n-p}	5.21±0.18^{a}	0.31±0.01^{o-q}	2.45±0.14^{a}	1.15±0.03^{n-p}	0.00±0.00^{i}	3.78±0.16^{i-o}	13.29±0.64^{b}
S. atropatana	19.20	15.00	6.55±0.51^{g-i}	1.65±0.24^{j-n}	14.76±0.44^{jk}	3.18±0.14^{e-h}	1.55±0.53^{g-l}	0.46±0.06^{e-f}	18.18±2.53^{c}	1.47±0.06^{g}	8.38±0.83^{hi}	1.05±0.06^{f-k}
S. ceratophylla **(T)**	6.80	9.00	3.34±0.17^{n-p}	2.10±0.14^{h-l}	2.81±0.12^{qr}	3.33±0.16^{ef}	0.54±0.02^{m-q}	0.47±0.12^{e-e}	0.00±0.00^{p}	2.73±0.35^{f}	0.06±0.02^{q}	5.43±0.25^{d}
S. ceratophylla **(M)**	15.00	10.30	3.19±0.05^{n-p}	1.17±0.10^{l-o}	4.88±0.29^{o-q}	0.09±0.01^{pq}	1.62±0.11^{g-k}	0.14±0.03^{h-k}	3.38±0.41^{i-n}	2.64±0.30^{f}	2.10±0.21^{m-q}	7.94±0.15^{c}
S. choloroleuca **(T1)**	17.40	6.50	0.31±0.06^{rs}	1.92±0.03^{i-m}	15.49±0.86^{jk}	4.20±0.12^{bc}	0.33±0.04^{o-q}	0.16±0.02^{g-k}	1.15±0.06^{n-p}	2.26±0.43^{f}	1.94±0.57^{pq}	<LOD
S. choloroleuca **(T2)**	13.40	6.80	1.00±0.36^{rs}	1.99±0.11^{i-m}	13.13±0.65^{kl}	3.51±0.10^{de}	0.40±0.08^{n-q}	0.19±0.05^{g-k}	2.35±0.49^{m-o}	0.93±0.09^{h}	1.02±0.12^{m-q}	<LOD
S. choloroleuca **(M)**	18.00	5.50	0.92±0.46^{rs}	0.75±0.14^{n-o}	8.03±0.05^{m-o}	4.68±0.10^{b}	0.82±0.22^{k-q}	0.09±0.02^{i-k}	3.98±0.33^{k-m}	0.00±0.00^{i}	3.80±0.46^{i-o}	0.91±0.03^{g-k}
S. chorassanica	24.30	11.30	6.09±1.10^{g-j}	1.93±0.23^{i-m}	26.38±0.25^{e}	0.58±0.05^{n-q}	2.63±0.25^{ef}	0.05±0.01^{jk}	17.12±0.21^{cd}	0.00±0.00^{i}	29.60±1.21^{c}	1.60±0.03^{e-i}
S. hydrangea	9.60	-	5.51±0.74^{g-j}	-	20.35±0.78^{g-h}	-	0.24±0.02^{pq}	-	1.61±0.27^{n-p}	-	0.06±0.02^{q}	-
S. hypoleuca **(T1)**	17.80	19.80	8.52±0.28^{f}	1.92±0.09^{i-m}	30.08±1.69^{d}	1.22±0.11^{l-n}	6.79±0.91^{b}	0.28±0.05^{d-j}	10.98±0.18^{fg}	0.00±0.00^{i}	12.56±1.15^{g}	0.32±0.06^{i-k}
S. hypoleuca **(T2)**	18.80	3.50	5.05±0.11^{i-m}	0.91±0.18^{n-o}	20.83±1.05^{f-h}	1.28±0.23^{k-n}	3.36±0.14^{de}	0.12±0.02^{h-k}	10.49±0.29^{gh}	0.00±0.00^{i}	60.14±2.39^{a}	0.34±0.09^{i-k}
S. hypoleuca **(Q)**	24.30	11.10	12.48±0.67^{e}	1.93±0.30^{i-m}	21.36±0.87^{fg}	0.19±0.02^{o-q}	0.96±0.23^{i-q}	0.04±0.02^{jk}	10.29±0.70^{gh}	0.00±0.00^{i}	7.65±0.52^{i}	1.00±0.20^{f-k}
S. leriifolia	25.40	9.50	16.01±0.29^{d}	2.16±0.52^{h-k}	17.74±0.73^{h-j}	1.75±0.18^{kl}	4.10±0.35^{cd}	0.23±0.01^{e-k}	5.65±1.43^{k}	14.65±0.03^{b}	4.71±0.17^{j-l}	2.50±0.49^{e}
S. limbata **(Q)**	18.60	-	4.26±0.32^{k-n}	-	19.15±0.09^{g-i}	-	1.92±0.15^{f-h}	-	0.00±0.00^{p}	-	48.07±1.77^{b}	-
S. macrosiphon **(M)**	13.10	17.30	9.21±0.06^{f}	1.14±0.50^{l-o}	8.54±0.55^{mn}	0.19±0.02^{o-q}	2.11±0.28^{fg}	0.00±0.00^{k}	8.62±.58^{hi}	0.00±0.00^{i}	7.83±0.92^{i}	7.91±0.53^{c}
S. mirzayanii	22.50	-	4.79±0.55^{j-n}	-	23.86±1.65^{ef}	-	2.65±0.27^{ef}	-	8.49±0.10^{hi}	-	18.90±0.64^{e}	-
S. nemorosa **(M)**	15.80	-	3.55±0.17^{m-p}	-	7.12±0.16^{n-p}	-	0.30±0.03^{o-q}	-	1.66±0.08^{n-p}	-	1.15±0.09^{pq}	-
S. nemorosa **(Q)**	13.80	11.50	7.07±0.13^{g}	4.92±0.14^{d}	15.44±2.41^{jk}	2.79±0.23^{f-h}	1.94±0.25^{f-h}	0.67±0.10^{c}	3.26±0.15^{i-n}	10.02±0.20^{c}	4.25±0.14^{j-m}	<LOD
S. nemorosa **(T)**	15.70	11.60	5.65±0.40^{g-i}	7.15±0.78^{b}	5.07±0.54^{o-q}	1.36±0.21^{k-m}	0.36±0.03^{n-q}	0.21±0.01^{g-k}	1.83±0.10^{n-p}	1.47±0.12^{g}	1.47±0.03^{o-q}	<LOD
S.× sylvesteris	16.00	-	2.64±0.06^{o-q}	-	2.17±0.09^{qr}	-	1.36±0.48^{g-m}	-	0.00±0.00^{p}	-	3.94±0.43^{j-n}	-
S. officinalis	20.00	6.90	15.02±0.53^{d}	2.31±0.25^{h-j}	7.03±1.30^{n-p}	1.35±0.09^{k-m}	4.55±0.77^{c}	0.35±0.12^{d-h}	7.75±0.82^{ij}	4.41±0.10^{d}	1.82±0.12^{n-q}	31.77±1.60^{a}
S. reuterana **(T1)**	10.80	16.60	6.73±0.58^{gh}	3.02±0.14^{e-h}	19.07±0.62^{g-i}	3.08±0.07^{e-h}	1.02±0.09^{h-p}	0.24±0.03^{d-k}	15.92±0.94^{de}	0.00±0.00^{i}	15.70±0.98^{f}	1.71±0.17^{e-h}
S. reuterana **(T2)**	11.70	9.60	2.35±0.20^{p-r}	1.08±0.06^{m-o}	17.05±0.55^{ij}	0.62±0.14^{n-q}	0.73±0.13^{k-q}	0.02±0.01^{k}	31.96±0.27^{b}	1±0.06^{gh}	22.11±1.22^{d}	0.43±0.10^{h-k}
S. santolinifolia	12.40	-	5.77±0.44^{g-k}	-	30.77±0.63^{d}	-	1.25±0.15^{g-o}	-	5.03±0.56^{kl}	-	1.12±0.02^{pq}	-
S. sclarea **(M)**	15.50	12.30	5.43±0.12^{h-i}	1.38±0.21^{j-o}	14.85±2.34^{jk}	0.00±0.00^{q}	1.09±0.12^{h-p}	0.28±0.13^{d-j}	4.31±0.79^{k-m}	0.00±0.00^{i}	5.31±0.46^{j}	0.67±0.19^{g-k}
S. sclarea **(Q)**	19.80	20.50	4.10±0.06^{l-o}	1.36±0.04^{j-o}	50.92±2.26^{b}	0.75±0.46^{m-p}	1.89±0.12^{f-i}	0.22±0.06^{f-k}	15.05±0.05^{e}	0.00±0.00^{i}	10.12±0.65^{h}	1.25±0.50^{e-k}
S. sclarea **(T)**	13.60	10.50	5.18±0.05^{h-i}	1.40±0.16^{j-o}	21.13±1.23^{fg}	0.84±0.08^{m-o}	0.22±0.05^{pq}	0.10±0.05^{i-k}	5.62±0.17^{k}	0.00±0.00^{i}	1.31±0.28^{pq}	1.95±0.03^{e-g}
S. sharifii	13.50	-	5.74±0.33^{g-i}	-	6.77±0.27^{n-p}	-	1.07±0.04^{h-p}	-	34.05±1.18^{a}	-	19.37±0.65^{e}	-
S. staminea	13.40	5.80	1.54±0.27^{q-s}	1.15±0.09^{l-o}	4.84±0.31^{o-q}	1.70±0.16^{kl}	0.18±0.02^{pq}	0.16±0.08^{g-k}	0.39±0.03^{op}	3.38±0.43^{e}	2.19±0.47^{m-q}	1.68±0.18^{e-h}
S. syriaca **(Q)**	14.90	13.90	5.17±0.46^{h-i}	2.68±0.18^{j-o}	54.47±2.00^{a}	2.60±0.27^{g-i}	1.20±0.19^{g-o}	0.30±0.12^{d-i}	12.66±1.35^{f}	3.80±0.28^{e}	4.69±0.35^{j-l}	2.27±0.61^{ef}
S. tebesana	16.80	2.80	9.05±0.59^{f}	0.62±0.13^{o}	35.04±1.76^{c}	1.87±0.03^{j-l}	1.28±0.13^{g-n}	0.23±0.05^{f-k}	5.69±0.40^{k}	0.00±0.00^{i}	2.93±0.25^{k-p}	0.03±0.00^{k}
S. verticillata **(T1)**	18.80	19.50	22.40±0.40^{b}	11.56±0.35^{a}	0.80±0.02^{r}	4.05±0.55^{b-d}	0.04±0.00^{q}	1.25±0.14^{b}	0.10±0.02^{p}	2.27±0.13^{f}	0.07±.02^{q}	1.59±.32^{e-j}
S. verticillata **(T2)**	23.70	8.20	41.07±0.80^{a}	3.89±0.33^{e}	6.74±0.29^{n-p}	1.63±0.15^{kl}	3.03±0.02^{e}	0.11±0.01^{h-k}	1.73±0.13^{n-p}	0.00±0.00^{i}	2.44±0.13^{i-q}	0.04±0.01^{k}
S. verticillata **(Q)**	19.40	15.20	41.53±0.88^{a}	5.99±0.19^{c}	10.91±0.35^{lm}	2.50±0.23^{h-j}	1.60±0.15^{g-l}	0.07±0.03^{i-k}	4.58±0.43^{k-l}	0.00±0.00^{i}	5.17±0.25^{jk}	0.28±0.13^{jk}
S. verticillata **(IBRC)**	20.20	17.40	18.85±0.15^{c}	3.79±0.17^{ef}	4.36±0.21^{pq}	3.28±0.47^{e-g}	8.10±0.35^{a}	0.39±0.03^{d-g}	6.04±0.76^{jk}	0.21±0.12^{i}	3.20±0.58^{i-p}	0.52±0.16^{h-k}
S. virgata **(M)**	17.40	7.80	5.15±0.66^{h-m}	2.95±0.23^{f-h}	8.72±0.42^{mn}	1.44±0.12^{k-m}	0.44±0.03^{m-q}	0.15±0.06^{g-k}	1.54±0.27^{n-p}	3.37±0.21^{e}	2.06±0.15^{m-q}	0.18±0.00^{k}
S. virgata **(Q)**	15.90	10.00	11.41±0.84^{e}	3.62±0.20^{e-g}	20.77±0.56^{f-h}	3.66±0.34^{e-e}	1.80±0.29^{f-j}	0.47±0.12^{cd}	5.94±0.65^{jk}	0.00±0.00^{i}	4.93±0.04^{jk}	1.13±0.13^{f-k}
S. viridis	20.90	-	1.15±0.61^{q-s}	-	21.50±0.42^{fg}	-	0.91±0.06^{j-q}	-	0.00±0.00^{p}	-	3.20±0.18^{i-p}	-
S. xanthocheila	15.30	13.40	4.65±0.45^{j-n}	2.81±0.21^{g-i}	4.85±0.18^{o-q}	1.99±0.40^{i-k}	2.13±0.41^{fg}	0.39±0.06^{d-g}	0.00±0.00^{p}	18.51±0.29^{a}	3.80±0.12^{i-o}	13.36±0.44^{b}
Total	37.43	11.33	7.93±0.81	2.61±0.23	15.35±1.11	2.08±0.15	1.66±0.16	0.32±0.05	6.84±0.70	2.29±0.44	8.35±1.12	3.34±0.64

Data are expressed as mean ± SE. Mean values within the same column sharing a common letter are not significantly different (P <0.05).
LOD:(≤ 0.001 mg/g DW)

Rosmarinic acid was detected in all of the investigated *Salvia* species. Based on the results, the leaves of four populations of *S. verticillata* were rich in RA, with the highest amount obtained for the leaves of *S. verticillata* (41.53 mg/g DW) from Qazvin location. Among the studied species, leaves of *S. aristata* and *S. choloroleuca* had the lowest contents of RA (≤1 mg/g DW).

The highest content of RA with the value of 11.56 mg/g DW was expressed in the roots of *S. verticillata* (T1), while the lowest content of this phenolic acid was found in the roots of *S. tebesana* (0.62 mg/g DW).

The presence of RA in the members of the genus *Salvia* is well known [28-31]. Previous reports indicated that the RA content of the aerial parts and leaves of *S. officinalis* ranged from 5.5 to 39.3 mg/g DW depending on the extraction method and collection site [13,28,32-36]. In the present study, we obtained a value of 15.02 mg/g DW for RA in the leaves of this species. In recent years, the presence of RA has been investigated in the other *Salvia* species [26,28,29, 32,34,37-42]. Rosmarinic acid was found in 39 wild growing populations of 35 *Salvia* species in China, ranging from trace amounts to 26.31 mg/g DW in the roots of *S. maximowicziana* [13]. As far as we know, the highest RA contents have been reported in the leaves of *S. glutinosa* (47.3 mg/g DW) and *S. sclarea* (41.1 mg/g DW) from Botanical Garden in Lithuania [32]. According to the results of Zengin et al. [43], aerial parts of *S. verticillata* subsp. *amasiaca* of Turkey were rich in RA (67 ± 2 mg/g DW). Similarly, among the studied species in this research, leaves of three populations (Q, T2 and T1) of *S. verticillata* with the values of 41.53, 41.07 and 22.40 mg/g DW were rich in RA, respectively. Also, the highest content of RA (11.56 mg/g DW) in the roots was reported for *S. verticillata*.

The content of Sal B, as the most abundant phenolic compound, varied from 0.80 mg/g DW in the leaves of *S. verticillata* (T1) to 54.47mg/g DW in the leaves of *S. syriaca*. Salvianolic acid B was not detected in the leaf extract of *S. aegyptiaca.* The amount of this phenolic acid ranged from 0.09 to 5.21 mg/g DW in the roots of *S. ceratophylla* (M) and *S. aristata*, respectively, although this compound was not identified in the root of *S. sclarea* (M). The amount of Sal A ranged from 0.18 mg/g DW in the leaves of *S. staminea* to 8.10 mg/g DW in the leaves of *S. verticillata* (IBRC). On the other hand, except for the roots of *S. aristata* (2.45 mg/g DW) and *S. verticillata* (T1) (1.25 mg/g DW), Sal A content in the roots of the other species was less than 1mg/g DW.

There are a few reports about the presence of salvianolic acids in *Salvia* species, but these are mostly limited to *S. miltiorrhiza* [16, 19, 44, 45]. According to the results of Min-hui et al. [13], the content of Sal B varied from trace amounts to 82.52 mg/g DW in the roots of *S. bowleyana;* while only trace amounts of Sal A were determined in the *S. miltiorrhiza* root samples. Unlike their results, we identified the presence of Sal A and Sal B in the leaf and root extracts of *S. officinalis*. The highest amounts for the Sal B have been reported for the roots of *S. miltiorrhiza* as 130.00 mg/g DW [46]. Among the examined Iranian *Salvia* species in this research, the leaves of *S. syriaca* with the value of 54.47 mg/g DW were the richest source of Sal B. According to our results, the content of Sal B in the leaves (17.74 mg/g DW) and roots (1.75 mg/g DW) of a wild population of *S. leriifolia* were much more higher than those values reported by Modarres et al. [38] in the leaves (0.13 mg/g DW) and roots (0.11 mg/g DW) of another wild population of the same species. In accordance with the Chinese Pharmacopoeia (2010), Sal B is an index and chemical marker for the quality control of Danshen, dried roots of *S. miltiorrhiza*, and its content should not be less than 30 mg/g DW (3%) [47]. Based on the extraction solvent, the highest amount for Sal A has been reported as 0.41 mg/g DW in the roots of commercially available *S. miltiorrhiza* plants [48]. Inconsistent with Min-hui et al. [13] results, Sal A was also found in all of the investigated *Salvia* species of Iran, except for the roots of *S. macrosiphon.* Furthermore, in our study the maximum value of Sal A (8.10 mg/g DW) was obtained in the leaves of *S. verticillata* (IBRC), which was approximately twenty times higher than that previously reported for the commercial roots of *S. miltiorrhiza* by Ho et al. [48].

Among the tested samples, the content of CA ranged from trace amounts to 34.05 mg/g DW in the leaves of *S. sharifii*. Also, results of the content assessment of this abietane compound in 32 populations of 19 species of *Salvia* showed that most of the studied species did not contain this phenolic compound in their roots. However, the roots of *S. xanthocheila*, *S. leriifolia* and *S. nemorosa* (Q) with the values of 18.51, 14.65 and 10.02 mg/g DW, respectively, were the richest sources of CA.

Most of the previous reports have been focused on the production of CA in calli, suspension cultures and shoot cultures of *R. officinalis* and *S. officinalis* [23, 33, 49]. According to the published reports, CA is highly unstable and its content might be changed depending on some factors such as environmental growing conditions, season of harvest [25, 50, 51], extraction solvent and extraction methods [49, 52-54]. The contents of CA in the leaves of *S. officinalis* and *S. sclarea* in this study were significantly greater than those found in the aerial parts of the same species native to Slovenia and Croatia [25]. In an investigation on *S. eremophila* and *S. santolinifolia* from Iran, the highest contents of CA in the aerial parts have been reported as 39.05 and 9.35 mg/g DW, respectively [26]. Among the studied *Salvia* species in this survey, the leaves of *S. sharifii* (34.05 mg/g DW) and *S. reuterana* (T2) (31.96 mg/g DW) and the roots *S. xanthocheila* (18.51mg/g DW) and *S. leriifolia* (14.65mg/g DW) could be considered as the richest natural sources of CA. As far as we know, this is the first report on the presence of this valuable abietanic compound in the roots of plant species.

The CAA content of the leaves varied from 0.06 mg/g DW in *S. ceratophylla* (T) and *S. hydrangea* to 60.14 mg/g DW in *S. hypoleuca* (T2). As compared to the leaves, the amount of this phenolic acid was relatively low in the roots of *Salvia* species, with the highest value of 31.77 mg/g DW in the *S. officinalis*.

3.4. PCA Analysis

The PCA assay was performed to determine the importance of the chemical variables for discrimination and screening of the examined *Salvia* species. The quantitative data obtained for the five compounds examined were subjected to PCA to describe the differences in the distribution of them across various geographic locations through 41 populations from 27 *Salvia* species. The PCA results revealed that three principal components with eigenvalues greater than 1 explained 57.26% of the total variance (Table 5).

The PCA scores plots based on the two principal components are shown in Figure 3. The situation of each *Salvia* species on the scores plots can be explained with reference to the loadings plots for PC1 and PC2. The first axis (PC1), representing 27.45% of the total variance, and mainly correlated positively to Sal B (loading, 0.61), Sal A (loading, 0.60) and RA (loading, 0.59) in the roots, but negatively correlated with CA (loading, -0.76), CAA (loading, -0.65) and Sal B (loading, -0.61) in the leaves. The second axis (PC2) accounted for 15.53% of the total variation and showed a high positive correlation with the RA (loading, 0.86) in the leaves. Also, the PCA results clearly showed the absence of marked variances among populations of a species. On the one hand, the PC1 separated *S. aristata*, which was characterized by the highest content of Sal B in the roots and very low concentrations of RA in the both of leaves and roots, to the remaining species. Moreover, the PC2 could easily distinguish the leaves and roots of different populations of *S. verticillata* rich in RA. Other species, which were distributed in a larger region, based on the concentrations of phenolic compounds and their position on PC1 and PC2 were separated as the third group. The PCA results reported by Ben Farhat et al. [55], revealed that the studied *Salvia* species characterized based on the quantitative distribution of nine phenolic compounds in their leaves. Similarly, PCA plots confirmed that antioxidant capacities of *S. officinalis* plants, which were collected from four wild locations in Croatia, were significantly correlated with the content of polyphenols, especially RA in their leaves [36]. Liang et al. [56] based on PCA analysis of HPLC and HPLC-MS^n fingerprints data of forty-nine compounds from twenty-five root samples of *S. miltiorrhiza* which were harvested from various geographical areas in China and were processed with different methods, classified and screened the examined samples into three groups with different contents of phenolic acids and tanshinones. In a similar PCA analysis, chemical profiles of phenolic acids and tanshinones were used as suitable markers for pharmacological evaluation of wild-grown *S. miltiorrhiza* plants in China [57].

Table 5. Principal components data based on the reported phenolic compounds of *Salvia* species

Compounds	Component		
	PC1	PC2	PC3
RA-L	0.281	**0.862**	0.005
RA-R	**0.587**	0.538	-0.284
Sal B-L	**-0.608**	-0.049	-0.209
Sal B-R	**0.614**	-0.206	-0.391
Sal A-L	-0.164	0.500	0.501
Sal A-R	**0.598**	-0.364	-0.180
CA-L	**-0.765**	0.018	-0.081
CA-R	0.321	-0.091	**0.594**
CAA-L	**-0.655**	0.065	-0.037
CAA-R	0.270	-0.283	**0.711**
Eigenvalue	2.745	1.553	1.428
% of variance	27.451	15.526	14.284
Cumulative%	27.451	42.977	57.261

The values higher than 0.55 are presented as bold significant.
L: leaf; R: root.

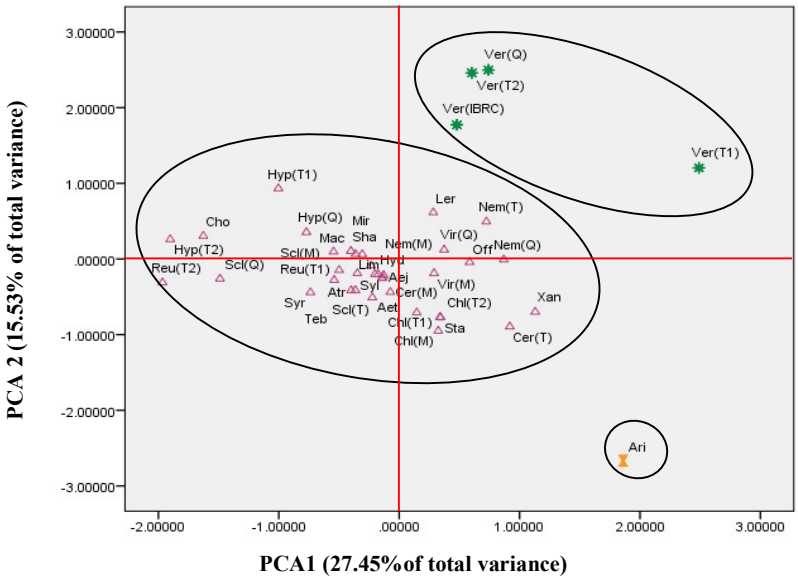

Figure 3. Scores plots of PC1 and PC2 of the PCA results obtained from distribution of 5 phenolic compounds in the leaves and roots of 41 populations from 27 *Salvia* species. Aeg: *S. aegyptiaca*, Aet: *S. aethiopis*, Ari: *S. aristata*, Atr: *S. atropatana*, Cer: *S. ceratophylla*, Chl: *S. choloroleuca*, Cho: *S. chorassanica*, Hyd: *S. hydrangea*, Hyp: *S. hypoleuca*, Ler: *S. leriifolia*, Lim: *S. limbata*, Mac: *S. macrosiphon*, Mir: *S. mirzayanii*, Nem: *S. nemorosa*, Off: *S. officinalis*, Reu: *S. reuterana*, San: *S. santolinifolia*, Scl: *S. sclarea*, Sha: *S. sharifii*, Sta: *S. staminea*, Syl: *S.* × *sylvesteris*, Syr: *S. syriaca*, Teb: *S. tebesana*, Ver: *S. verticillata*, Vir: *S. virgata*, Vi: *S. viridis*, Xan: *S. xanthocheila*

In Conclusion, according to our research significant differences were observed in the distribution patterns of phenolic compounds of the studied *Salvia* species at both of intra- and inter-species levels. It seems that the variations in contents may be due to the environmental conditions and genetic factors. Based on the collective results from phytochemical studies, *S. verticillata*, *S. hypoleuca*, *S. leriifolia* and *S. virgata* were particularly rich in rosmarinic acid. The leaves of *S. hypoleuca, S. sclarea, S. tebesana*, *S. santolinifolia* and *S. syriaca* had valuable amounts of salvianolic acid B. The higher amounts of salvianolic A were achieved in the leaves of *S. hypoleuca* and *S. verticillata*. The leaves of *S. sharifii* and *S. reuterana* had high capacity for biosynthesis and storage of carnosic acid. It

was noticeable that plant samples of *S. verticillata*, *S. hypoleuca*, *S. virgata* and *S. sclarea* which were harvested from Qazvin province possessed the highest contents of phenolic compounds, as compared to the other studied populations. Due to the widespread distribution of the wild *Salvia* species in Iran and also according to the findings of this research and similar data from the literature, some of these species might be used as new resources of the studied compounds, especially RA, with a wide range of strong biological activities in food and medicinal industries. In general, the results of this research approved that the mentioned species can be used as potent natural sources of the studied valuable phenolic compounds for pharmaceutical, industrial, breeding, domestication and cultivation purposes, beside the *S. officinalis* which is currently known as the most important medicinal plant of the *Salvia* genus.

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