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PHENOLIC PROFILE, CHEMICAL RELATIONSHIP AND ANTIOXIDANT ACTIVITY OF IRANIAN *VERBASCUM* SPECIES

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Hydro-ethanolic extracts of flowers from four *Verbascum* species were evaluated for the phenolic content, composition, and antioxidant activity using Folin-Ciocalteu assay, HPLC-DAD analysis, and DPPH assay, respectively. The highest flavonoid content was detected in *V. sinuatum* extract from Khoramabad (19.91 mg RE/g DW). The extract of *V. pseudo-digitalis* from Maymand yielded the highest amount of total phenols, together with the highest apigenin and luteolin levels (55.62 mg GAE/g DW, 12.18 and 88.13 μg/mg DW, respectively), while that of *V. songaricum* from Ardekan showed the highest naringin content (12.44 μg/mg DW). The extract of *V. songaricum* from Shirmard exhibited the highest quercetin and rutin levels (1.0 and 24.24 μg/mg DW, respectively), whereas that of *V. sinuatum* from Ardekan had the highest caffeic acid content (7.78 μg/mg DW). The antioxidant activity of *Verbascum* samples showed IC_{50} values from 45.12 to 226.62 μg/mL.

Keywords: HPLC, Chemotaxonomy, *Verbascum* species

INTRODUCTION

Verbascum L. is one of the largest genera of the Scrophulariceae family encompassing almost 360 species. This genus includes 43 species and 3 hybrids in Iran, of which 20 species are endemic (Sotoodeh et al., 2015). Members of this genus are commonly called 'mullein'.

Verbascum species are used as mucolytic, expectorant, diuretic, and lenitive agents in the traditional medicine and in the treatment of respiratory diseases such as tuberculosis, bronchitis, dry coughs, and asthma (Boğa et al., 2016). They are valuable sources of various pharmaceutically active substances that have long been used due to antioxidant, antimicrobial, antibacterial, and anti-inflammatory activities (Turker and Camper, 2002; Tatli et al., 2003; Turker and Gurel, 2005; Tatli and Akdemir, 2006; Kupeli et al., 2007; Senatore et al., 2007; Kahraman et al., 2010; Moein et al., 2012; Karamian and Ghasemlou, 2013; Nofouzi, 2015; Shakeri and Farokh, 2015).

According to previous studies, more than 50 secondary bioactive metabolites belonging to iridoids, phenylethanoids, flavonoids and phenolic acids were detected in the methanol extract of *Verbascum ovalifolium* Donn ex Sims and its fractions (Luca et al., 2019). *Verbascum* flowers contain mainly flavonoids (tamarixetin 7-rutinoside, tamarixetin 7-glucoside, apigenin, luteolin, and their 7-glucosides, diosmin, chrysoeriol, eriodictyol, kaempferol, quercetin, and rutin) and phenolic acids (*p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids) (Bileflimi, 2004; Senatore

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et al., 2007; Klimek, et al. 2010; Gvazava and Kikoladze, 2012; Boğa et al., 2016; Selseleh et al., 2020).

Flavonoids and phenolic acids make up one of the most pervasive groups of plant phenolics and they are frequently used in pharmacy and medicine, because of their antioxidant, antibacterial, anti-cancer, cardio-protective, anti-inflammatory, as well as immune system promoting effects (Ghasemzadeh and Ghasemzadeh, 2011; Tungmunnithum et al., 2018). Therefore, according to a report provided on the presence of these compounds in some species of *Verbascum,* identification of new sources of flavonoids and phenolic acids plays a pivotal role in the pharmaceutical and food industries (Saleh et al., 2010). However, information about the phenolic contents in some *Verbascum* species is limited in Iran and the majority of it focuses on restricted geographical areas. This study was conducted to investigate the content of five flavonoids (apigenin, luteolin, naringin, quercetin, and rutin) and one phenolic acid (caffeic acid) as well as the antioxidant activity of four species of *Verbascum* (*V. songaricum*, *V. macrocarpum*, *V. pseudodigitalis* and *V. sinuatum*), with the aim to identify and quantify the phenolic compounds, occurring in different areas of Iran, in order to understand their bioactivities such as antioxidant potentialities for further applications.

MATERIALS AND METHODS

PLANT MATERIAL

Four *Verbascum* species, (*V. songaricum*, *V. macrocarpum*, *V. pseudodigitalis* and *V. sinuatum*) were collected at the full-blooming stage from their natural habitats in southwest Iran (Table 1). The identification was made by two expert botanists (Shirmardi, Hamzeh Ali, PhD., Research Center of Agriculture and Natural Resources, P.O. Box 415, Shahrekord, IRAN and Shahrokhi, Asghar; Education Organization, Chaharmahal and Bakhtiari Province, Shahrekord, Iran) by using information available in Flora Iranica (Sharifnia, 2011). The four species were represented by 13, 2,

TABLE 1. Geographical distribution *Verbascum* populations throughout southwest Iran.

Cod	Species	Origin	Elevation (m a.s.l.)	Latitude	Longitude
VS1	*V. songaricum*	Naghneh	2302	31° 55′	51° 19′
VS2	*V. songaricum*	Tumanak 1	2427	32° 31′	50° 36′
VS3	*V. songaricum*	Semirom 2	2204	31° 19′	51° 27′
VS4	*V. songaricum*	Fathabad	2405	31° 35′	51° 32′
VS5	*V. songaricum*	Sendegan	1988	31° 12′	51° 18′
VS6	*V. songaricum*	Semirom 2	2320	31° 22′	51° 32′
VS7	*V. songaricum*	Semirom 3	2492	31° 34′	51° 38′
VS8	*V. songaricum*	Ardekan	2427	30° 18′	51° 54′
VS9	*V. songaricum*	Kallar	2344	31° 49′	51° 04′
VS10	*V. songaricum*	Karsanak	2356	32° 37′	50° 33′
VS11	*V. songaricum*	Shirmard	2206	31° 24′	51° 12′
VS12	*V. songaricum*	Bardeh	2466	32° 32′	50° 34′
VS13	*V. songaricum*	Tumanak 2	2282	32° 30′	50° 40′
VM1	*V. macrocarpum*	Tumanak	2204	32° 29′	50° 42′
VM2	*V. macrocarpum*	Bardeh	2383	32° 33′	50° 32′
VPS1	*V. pseudo-digitalis*	Kharaji	2020	31° 04′	50° 49′
VPS2	*V. pseudo-digitalis*	Maymand	1801	31° 08′	51° 15′
VSI1	*V. sinuatum*	Yasuj	1734	30° 39′	51° 32′
VSI2	*V. sinuatum*	Khoramabad	1147	33° 26′	48° 17′
VSI3	*V. sinuatum*	Ardekan	2253	30° 16′	51° 58′

2 and 3 specimens, respectively. Herbarium specimens (*V. songaricum* 1427; *V. macrocarpum* 3443; *V. pseudodigitalis* 1553 and *V. sinuatum* 3571) were deposited at the Research Center of Agriculture and Natural Resources, Shahrekord, Iran.

PREPARATION OF EXTRACTS

Dried flowers, 0.5 g, were extracted with 70% ethanol at room temperature for 72 h. The filtered extracts were concentrated using a rotary evaporator (IKA RV 10) at 40°C under reduced pressure to remove the solvent and finally dried in an incubator (Memmert, Germany) at 37°C. Finally, 50 mg of the dried extract was gathered to be used in the next steps.

CHEMICALS AND REAGENTS

The standards of apigenin, luteolin, naringin, quercetin, rutin, and caffeic acid were obtained from Sigma (St. Louis, MO, USA). HPLC-grade solvents, gallic acid, folin-ciocalteu reagent, sodium bicarbonate, aluminum chloride and potassium acetate were provided by Merck (Darmstadt, Germany).

DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC)

The total phenolic content of the samples was measured using the Folin-Ciocalteu method (Singleton and Rossi, 1965). At the beginning, 0.01 g of the extract was dissolved in 60% methanol and then brought to a volume of 10 mL. Briefly, the reaction was carried out using 2 mL of crude extract (10 mg/mL) mixed with 0.5 mL of Folin reagent 10% and, after 3-8 min, with 0.4 mL of sodium carbonate (7.5% w/v) solution. The samples were incubated at room temperature for 30 min in the dark and the absorbance was measured using a UV-Vis Spectrophotometer (Unico 2100) at 765 nm. Gallic acid was used as the standard and the results were calculated as mg gallic acid equivalent (mg GAE/g DW).

DETERMINATION OF TOTAL FLAVONOID CONTENT (TFC)

Flavonoids were measured based on the method of Ling et al. (2010) with some changes. Briefly, 0.01 g of the dried extract was dissolved in 60% methanol and brought to a volume of 10 mL. Then, 0.1 mL of this solution was transferred to a test tube and 0.5 mL of aluminum chloride 2% and 3 mL of potassium acetate 5% were added. After 40 min, the absorption of the samples was read at 415 nm. The total flavonoid content was expressed as mg of rutin equivalents (mg RE/g DW) (Dan-ling, 2010).

HPLC ANALYSIS OF FLAVONOIDS AND PHENOLIC ACIDS

The qualitative analysis of the phenolic acid and flavonoids were performed using a HPLC system (Knauer, Germany) coupled with a C18 column (5 μm particle size, i.d. 250 mm × 4.6 mm) and UV–visible detector (PDA Detector 2800). The binary mobile phase, including solvent A (methanol and trifluoroacetic acid 0.05%) and solvent B (deionized water and trifluoroacetic acid 0.05%) was eluted as follows: 0-10 min, 20% A and 80% B; 10-40 min, 30% A and 70% B; 40-45 min, 60% A and 40% B; 45-50 min, 80% A and 20% B; 50-55 min, 100% A and 0% B. The flow rate was 0.5 mL/min and the injection volume was 20 μL. Detection was performed by scanning from 190 to 800 nm and reading in the range of 280-372 nm. Identification of phenolic acid and flavonoids was carried out by comparing their UV spectra and retention times (Rt) with those of analytical standards (Figure 1). 2.5 mg of dry extract of each sample was dissolved in 1 ml methanol and water in a ratio of 1 : 4 to provide a concentration of 2500 ppm and the injection volume was 20 μL. The content of each flavonoid and phenolic acid was calculated using the calibration curve equation of analytical standards of phenolic acid (caffeic acid) and flavonoids (apigenin, luteolin, naringin, quercetin, rutin). The results were expressed as μg/mg DW of extract (Tarnawski et al., 2006).

DETERMINATION OF DPPH RADICAL SCAVENGING ACTIVITY

In the DPPH assay, the free radical 2,2-diphenyl-1-picrylhydroazyl (DPPH) was used to find the antioxidant activity of flower extracts. In a modified assay, 2 mL of 0.1 mM solution of DPPH radical in methanol was mixed with 2 mL of the extract. After mixing, it was left for 15 min at room temperature. The DPPH radical inhibition was measured at 517 nm by using a spectrophotometer (Unico 2100). The antioxidant activity was given by:

$$\text{Percent (\%) Inhibition} = 100 - \left[\frac{(A)sample - (A)blank}{(A)control} \right] \times 100$$

where A stands for the absorbance of the color formed in a spectrophotometer cell, using DPPH as

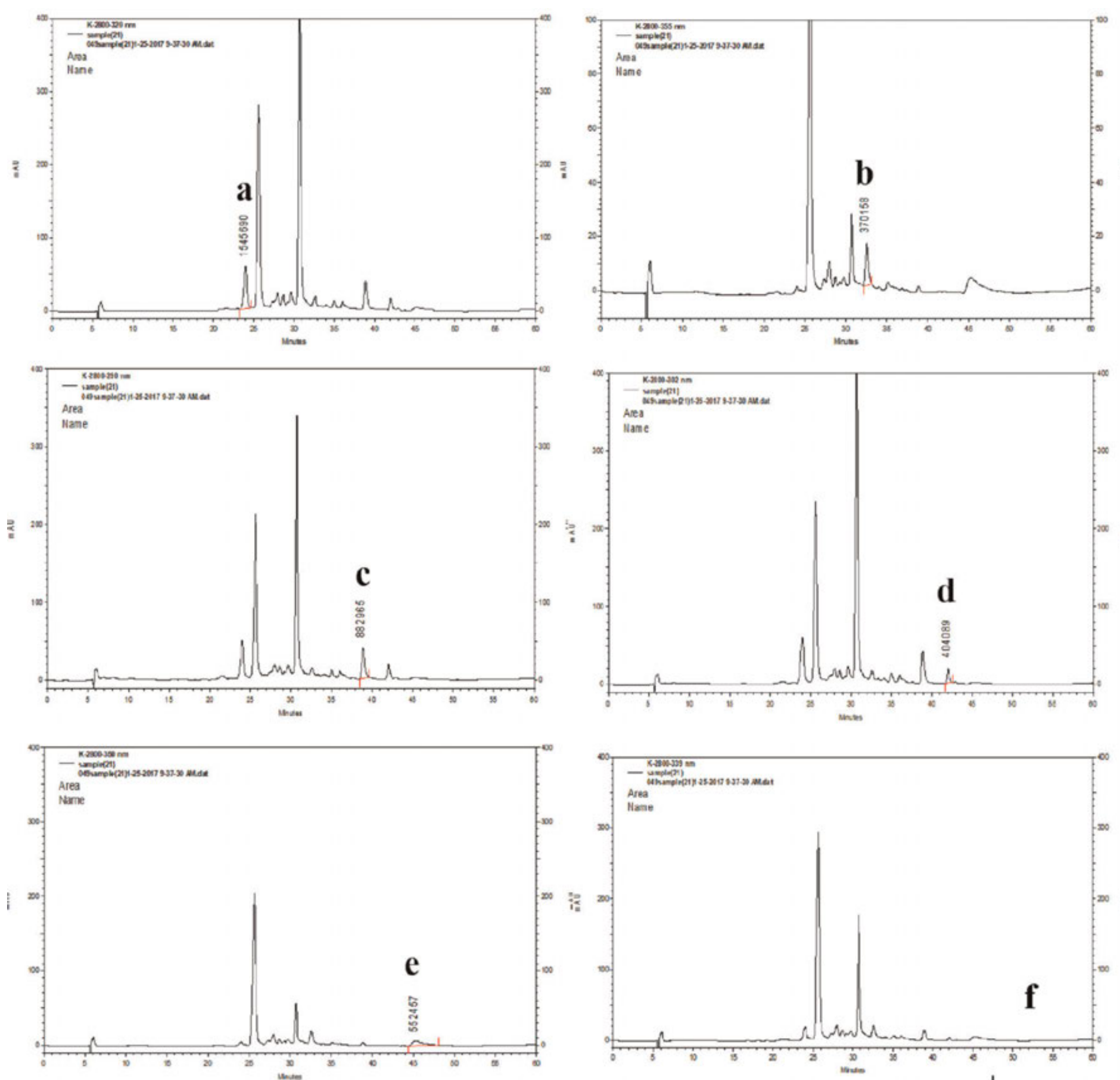

Fig. 1. Representative chromatogram of flavonoids and phenolic acid of extracts (sample VSI3) (**a**) Caffeic acid (Rt= 22.63 min) (**b**) Rutin (Rt= 33.16 min) (**c**) Naringin (Rt= 39.34 min) (**d**) Apigenin (Rt= 42.7 min) (**e**) Luteolin (Rt= 46.66 min) (**f**) Quercetin (Rt= 53.9 min).

control (no extract) and blank consisting of methanol only. The IC_{50} (μg/mL) of each sample (concentration in required to inhibit DPPH radical formation by 50%) was calculated. This was obtained by interpolation and using linear regression analysis. All experiments were conducted in triplicate, and the measurements were reported as means ± SD (Moein et al., 2007).

STATISTICAL ANALYSIS

The data were statistically analyzed using one-way ANOVA ($p \leq 0.05$), and comparison of the means was performed by Duncan's multiple range tests using Statistical Package for the Social Science (SPSS 16.0, SPSS Inc., USA) computer software. Specimen homogeneity was analyzed by hierarchical cluster analysis using the Ward method based on the squared Euclidean distance criterion.

In order to understand the relationships among different *Verbascum* samples and to determine the main constituents influencing the chemical variability, a covariance data matrix composed of twenty samples and 6 variables was prepared and subjected to Principal Component Analysis using STATISTICA 7.1 (Stat Soft Italia S.r.l.,

Vigonza, Italy). Eigenvalues were calculated and score- and loading plots including both *Verbascum* samples and phenolic compounds were generated.

RESULTS AND DISCUSSION

TOTAL PHENOLIC AND FLAVONOID CONTENT

The TPC and TFC of flower extracts from 20 Iranian mullein populations are shown in Table 2. High variation was observed within and among the four *Verbascum* species. A statistical analysis showed that there were significant differences ($p \leq 0.05$) among populations of all species. Among the four species, the highest TPC was found in the extract of *V. pseudodigitalis* (population–VPS2, collected from Maymand, 55.62 mg GAE/g DW). The lowest TPC was obtained in the flower extract of *V. macrocarpum* (population-VM2 collected in Tumanak, 27.35 mg GAE/g DW). The production of phenolics in plants is regulated by genetics, the development stage and geographic factors (Dutta et al., 2005; Mamati et al., 2006; Bajalan et al., 2016). TFC of the flower extracts varied from 10.01 mg RE/g DW in *V. songaricum* (population-VS9 collected in Kallar) to 19.88 mg RE/g DW in *V. sinuatum* (population-VSI2 collected in Khoramabad) (Table 2). The lowest TFC value was obtained from the VS9, VS8, VS12 and VS2 populations (10.01, 10.11, 10.11, and 10.14 mg RE/g DW, respectively), all belonging to *V. songaricum*. Comparing the four spices, *V. sinuatum* showed the highest value of TFC, while *V. songaricum* had the lowest one. TFC values showed quite a variation among the *V. songaricum* ecotypes connected with the geographical origin of the samples.

Tatli and Akdemir (2004) indicated that the genus *Verbascum* is a good natural source of phenols. The different accumulation of phenolic compounds in flowers of various *Verbascum* species should be explained by substantial variability in genetics and environmental conditions of the populations. According to previous studies,

Table 2. Total phenol and flavonoid content and antioxidant activity of *Verbascum* populations.

Species	Population	TPC (mg GAE/g DW)	TFC (mg RE /g DW)	IC50 (μg/mL)
V. songaricum	VS1	28.18 ± 0.06 [hij]	13.20 ± 0.058 [j]	226.62 ± 0.70 [q]
	VS2	28.55 ± 0.05 [gh]	10.14 ± 0.032 [k]	175.02 ± 0.30 [m]
	VS3	28.04 ± 0.07 [jk]	13.27 ± 0.006 [i]	147.83 ± 0.90 [h]
	VS4	29.26 ± 0.04 [e]	19.71 ± 0.075 [c]	129.92 ± 0.60 [f]
	VS5	27.68 ± 0.04 [klm]	13.32 ± 0.005 [h]	170.26 ± 0.20 [l]
	VS6	29.10 ± 0.02 [ef]	13.31 ± 0.00 [hi]	118.81 ± 0.70 [e]
	VS7	28.67 ± 0.18 [fg]	19.73 ± 0.00 [c]	155.72 ± 0.50 [i]
	VS8	29.17 ± 0.05 [e]	10.11 ± 0.015 [k]	131.80 ± 0.40 [g]
	VS9	28.38 ± 0.08[ghi]	10.10 ± 0.04 [k]	195.85 ± 0.80 [o]
	VS10	28.53 ± 0.03 [gh]	13.19 ± 0.005 [j]	165.72 ± 0.20 [k]
	VS11	27.86 ± 0.59 [jk]	13.42 ± 0.01 [g]	115.32 ± 0.60 [d]
	VS12	28.13 ± 0.16 [hijk]	10.11 ± 0.005 [k]	196.44 ± 0.70 [o]
	VS13	27.83 ± 0.13 [jkl]	19.80 ± 0.005 [b]	223.06 ± 0.45 [p]
V. macrocarpum	VM1	27.35 ± 0.04 [m]	13.30 ± 0.0 [hi]	165.65 ± 0.60 [k]
	VM2	27.38 ± 0.01 [lm]	13.16 ± 0.01 [j]	165.44 ± 0.90 [j]
V. pseudo-digitalis	VPS1	29.78 ± 0.02 [d]	13.36 ± 0.02 [e]	176.76 ± 0.40 [n]
	VPS2	55.62 ± 0.09 [a]	13.54 ±0.00 [f]	99.59 ± 0.40 [c]
V. sinuatum	VSI1	53.42 ± 0.91 [c]	19.82 ± 0.04 [b]	75.40 ± 0.62 [b]
	VSI2	29.03 ± 0.18 [ef]	19.91 ± 0.00 [a]	175.46 ± 0.25 [m]
	VSI3	54.77 ± 0.08 [b]	13.72 ± 0.02 [d]	45.12 ± 0.16 [a]

Mean values within columns followed by the same letters were not significantly different at 5% level according to LSD test ($p < 0.05$).

the phenol and flavonoid compositions in *Verbascum* are highly variable due to various factors such as genetics and environmental conditions of the samples (Bodor et al., 2007; Karamian and Ghasemlou, 2013). In general, the potential to synthesize phenolic acids and flavonoids in different species is the result of the interaction between climate and genetics (Diaz et al., 2012; Medina-Medrano et al., 2015).

ANTIOXIDANT ACTIVITY

The antioxidant capacity of the *Verbascum* flower extracts was measured by a spectrophotometric assay using the DPPH radical as a reagent. A statistical analysis showed that there was a significant difference ($p \leq 0.05$) among populations with respect to their antioxidant activity. The IC_{50} values varied from 45.12 to 226.62 μg/mL, whereas the synthetic antioxidant BHT showed an IC_{50} of 62.6 μg/mL.

The highest radical scavenging activity was obtained for *V. sinuatum* from Ardekan population (IC_{50} = 45.12 μg/mL), whereas the lowest (IC_{50} = 226.62 μg/mL) for *V. songaricum* from Naghneh population (Table 2). Notably, the antioxidant activity of *V. sinuatum* was higher than that of BHT. Other populations showed weaker activity than that of the positive control. The present results demonstrated that, except for the samples from VSI3 and VSI1 populations (*V. sinuatum*), which showed significant radical scavenging values, samples from the other populations displayed moderate to weak activity. Our results are consistent with those of Moein et al. (2012) who concluded that *V. sinuatum* extract may be a good candidate for a natural antioxidant.

In this study we found out that TPC, apigenin, naringin, and caffeic acid content had a significant negative correlation with IC_{50} (-0.746**, -.0592**, -0.497* and -0.635**, respectively) and a positive correlation with the antioxidant activity. Previous studies showed that antioxidant activities of plants can be attributed to their high levels of phenolic compounds (Alothman et al., 2009; Dai and Mumper, 2010; Bajalan et al., 2016; Tan et al., 2016). The highest TPC was found in the extracts of *V. pseudodigitalis* from Mayman population (VPS2, 55.62 mg GAE/g DW) and of *V. sinuatum* from Ardekan (VSI3) and Yasuj (VSI1) populations (54.77 and 53.42 mg GAE/g DW, respectively). Although VPS2 extract showed a higher TPC than VSI3 and VSI1, it displayed lower antioxidant activity. This can be explained by a much lower amount of caffeic acid in VPS2 (0.90 μg/mg DW) compared with VSI3 (7.78 μg/mg DW) and VSI1 (6.34 μg/mg DW). A previous study reported that caffeic acid is a potent antioxidant (Gülçin, 2006). Another study indicated that caffeic acid and its derivatives are good substrates for polyphenol oxidase, and under appropriate conditions may undergo oxidation (Bassil et al., 2005).

In addition, the results of the present study revealed that there was a negative correlation between TPC and IC_{50} values (-0.748) which is in agreement with previous reports (Corral-Aguayo et al., 2008; Kumar et al., 2014; Bajalan et al., 2016; Tewari et al., 2017; Zheng et al., 2019). It is known that the plant phenols have the ability of scavenging free radicals, thus producing antioxidant effects (Shahidi and Ambigaipalan, 2015). Structure-activity relationship studies also demonstrated that compounds with phenolic hydroxyl groups have a stronger antioxidant effect (Kahraman et al., 2010). Identifying natural products with radical scavenging activities is very important for the pharmaceutical and food industries to find potential alternatives to synthetic antioxidant preservatives such as BHT and others. Several factors such as genetics, geographical origin, plant part, harvesting time, may overall influence the antioxidant properties (Alothman et al., 2009; Teixeira et al., 2013; Shahidi and Ambigaipalan, 2015).

The distribution of phenols and flavonoids revealed both intra- and inter-specific variability among all *Verbascum* species. The results obtained from this study highlight the use of metabolite composition as an important marker for studying variations and relationships within and among *Verbascum* species.

HPLC ANALYSIS

In this study, five flavonoids, i.e., apigenin, luteolin, naringin, quercetin and rutin, and one phenolic acid, i.e., caffeic acid, were quantified in flower extracts from four *Verbascum* species using a HPLC method. *V. pseudodigitalis* flowers from Maymand population (VPS2) yielded the highest amount of apigenin and luteolin (12.18 and 88.13 μg/mg DW, respectively). The lowest content of apigenin (0.37 μg/mg DW) and luteolin (2.18 μg/mg DW) was detected in *V. songaricum* collected from Naghneh (VS1) and *V. sinuatum* collected in Khoramabad (VSI2), respectively. Naringin levels ranged from 0.65 μg/mg DW for *V. songaricum* collected in

TABLE 3. Amount of flavonoids and phenolic acids (µg/mg DW) in flower extracts of *Verbascum* populations

Species	Population	Apigenin	Luteolin	Naringin	Quercetin	Rutin	Caffeic acid
V. songaricum	VS1	0.48 ± 0.01 ^{t}	7.14 ± 0.01 ^{n}	1.75 ± 0.01 ^{p}	0.79 ± 0.01 ^{b}	3.35 ± 0.01 ^{q}	0.62 ± 0.01 ^{n}
	VS2	4.84 ± 0.03 ^{j}	4.56 ± 0.01 ^{r}	4.84 ± 0.00 ^{i}	0.65 ± 0.01 ^{e}	4.70 ± 0.00 ^{o}	0.78 ± 0.08 ^{m}
	VS3	4.39 ± 0.04 ^{l}	8.9 ±0.00 ^{l}	4.62 ± 0.01 ^{j}	0.65 ± 0.02 ^{e}	6.34 ± 0.01 ^{k}	0.85 ± 0.40 ^{kl}
	VS4	2.09 ± 0.03 ^{n}	5.33 ±0.01 ^{p}	2.31 ± 0.00 ^{o}	0.69 ± 0.03 ^{d}	3.65 ± 0.03 ^{p}	0.81 ± 0.00 ^{lm}
	VS5	5.16 ± 0.01 ^{i}	8.53 ±0.00 ^{m}	6.35 ± 0.01 ^{g}	n.d	8.02 ± 0.01 ^{h}	0.81 ± 0.00 ^{lm}
	VS6	4.71 ± 0.00 ^{k}	13.55 ±0.00 ^{h}	2.42 ± 0.02 ^{n}	n.d	9.35 ± 0.01 ^{d}	2.51 ± 0.00 ^{e}
	VS7	5.25 ± 0.04 ^{h}	12.89 ± 0.00 ^{j}	5.11 ± 0.01 ^{h}	0.69 ± 0.00 ^{d}	8.81 ± 0.03 ^{f}	2.53 ± 0.01 ^{e}
	VS8	15.17 ±0.00 ^{b}	19.54 ± 0.00 ^{c}	12.44 ± 0.00 ^{a}	0.71 ± 0.02 ^{cd}	9.22 ± 0.00 ^{e}	4.65 ± 0.00 ^{d}
	VS9	2.46 ± 0.02 ^{m}	19.36 ± 0.00 ^{d}	2.46 ± 0.02 ^{n}	n.d	10.66 ± 0.00 ^{c}	2.35 ± 0.00 ^{f}
	VS10	0.68 ± 0.00 ^{r}	6.01 ±0.00 ^{o}	0.74 ± 0.02 ^{r}	n.d	2.70 ± 0.04 ^{s}	1.11 ± 0.01 ^{h}
	VS11	13.74 ± 0.00 ^{d}	43.55 ±0.00 ^{b}	10.63 ± 0.00 ^{d}	1.00 ± 0.01 ^{a}	24.24 ± 0.01 ^{a}	1.45 ± 0.01 ^{g}
	VS12	0.74 ± 0.00 ^{q}	4.42 ± 0.00 ^{s}	0.65 ± 0.04 ^{s}	0.63 ± 0.01 ^{f}	3.00 ± 0.01 ^{r}	0.87 ± 0.00 ^{l}
	VS13	1.20 ± 0.04 ^{o}	19.04 ± 0.00 ^{e}	1.46 ± 0.00 ^{q}	0.65 ± 0.01 ^{ef}	7.56 ± 0.01 ^{i}	0.85 ± 0.00 ^{kl}
V. macrocarpum	VM1	0.56 ± 0.01 ^{s}	13.52 ± 0.01 ^{i}	4.31 ± 0.07 ^{l}	0.72 ± 0.03 ^{c}	6.25 ± 0.01 ^{l}	0.80 ± 0.07 ^{m}
	VM2	0.84 ± 0.01 ^{p}	5.3 ±0.01 ^{q}	4.57 ± 0.01 ^{k}	0.65 ± 0.00 ^{e}	7.34 ± 0.16 ^{j}	0.92 ± 0.03 ^{j}
V. pseudo-digitalis	VPS1	10.69 ± 0.00 ^{e}	12.53 ± 0.00 ^{k}	11.66 ± 0.00 ^{c}	0.66 ± 0.01 ^{e}	8.14 ± 0.00 ^{g}	0.98 ± 0.02 ^{i}
	VPS2	33.18 ± 0.00 ^{a}	88.12 ±0.01 ^{a}	2.67 ± 0.02 ^{m}	n.d	13.38 ± 0.00 ^{b}	0.90 ± 0.01 ^{jk}
V. sinuatum	VSI1	9.02 ± 0.01 ^{f}	16.98 ± 0.01 ^{f}	7.26 ± 0.00 ^{e}	n.d	5.55 ± 0.00 ^{m}	6.34 ± 0.01 ^{b}
	VSI2	5.85 ± 0.00 ^{g}	3.18 ±0.03 ^{t}	7.10 ± 0.00 ^{f}	n.d	4.90 ± 0.00 ^{n}	4.98 ± 0.02 ^{c}
	VSI3	13.82 ± 0.01 ^{c}	16.28 ± 0.00 ^{g}	12.32 ± 0.0 ^{b}	n.d	5.51 ± 0.01 ^{m}	7.78 ± 0.00 ^{a}

Mean values within columns followed by the same letters were not significantly different at 5% level according to LSD test ($P < 0.05$). n.d: not detected.

Bardeh to 12.44 µg/mg DW for *V. songaricum* collected in Ardekan. Quercetin and rutin reached the highest levels (1.0 and 24.24 µg/mg DW, respectively) in the extract of *V. songaricum* (WS11) from Shirmard. On the other hand, quercetin was not detected in four population of *V. songaricum* (VS4, VS6, VS9, and VS10), one population of *V. pseudodigitalis* (VPS2), and any populations of *V. sinuatum* (VSI1, VSI2 and VSI3). Among the twenty *Verbascum* populations, *V. songaricum* (VS11) was found to be the richest in rutin (24.24 µg/mg DW), while the VS10 population possessed the lowest quantity of this compound (2.7 µg/mg DW). The *V. sinuatum* populations showed the highest level of caffeic acid (4.98-7.78 µg/mg DW). The lowest content of this metabolite was detected in *V. songaricum* (VS1, 0.62 µg/mg DW) (Table 3).

Previous studies reported the presence of several flavonoids and phenolic acids in flower extracts from *Verbascum* species (Serdyuk et al., 1976; Naumov et al., 1998; Klimek et al., 2010; Grigore et al., 2013). However, little information is available about the phenolic profiles of Iranian *Verbascum* species. Our data revealed that *V. pseudodigitalis* flowers from Maymand population (VPS2) yielded the highest amount of apigenin (33.18 µg/mg DW). Quercetin reached the highest levels in the extract of *V. songaricum* from Shirmard population (1.0 µg/mg DW). Grigore et al. (2013) reported that the content of quercetin, apigenin and caffeic acid of *V. phlomoides* L. growing in Romania was 17.29, 0.358 and 39.96 mg/g DW, respectively. These differences may be explained by variability in the geographic origin, genetics and environmental conditions.

CLASSIFICATION OF *VERBASCUM* POPULATIONS

Cluster analysis (CA), principal component analysis (PCA) and heat-map visualization were performed to explore the chemotaxonomic affinity and relationship among 20 populations of four *Verbascum* species according to their chemical compositions. The results showed similar patterns among the constructed dendrogram in CA and bidimensional biplots in PCA (Figures 2 and 3). The obtained dendrograms showed that the samples were divided into four main groups. The results showed an obvious separation between the two populations of *V. pseudodigitalis* (VPS2) and *V. songaricum* (VS11) and the other populations. *V. pseudodigitalis* from Maymand (VPS2) yielded

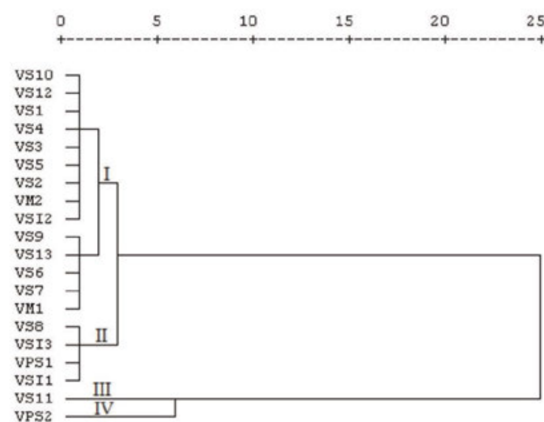

Fig. 2. Average-linkage dendrogram of four *Verbascum* species (20 populations) resulting from cluster analysis (based on Euclidean distances). Chemotype II (caffeic acid/naringin), Chemotype III (of quercetin/rutin), Chemotype IV (apigenin/ luteolin) with a detailed description of populations.

the highest amount of apigenin and luteolin, while *V. songaricum* from Shirmard (VS11) showed the highest amounts of quercetin and rutin. Fourteen populations (i.e., VS10, VS12, VS1, VS4, VS3, VS5, VS2, VM2, VSI2, VS9, VS13, VS6, VS7, and VM1) formed chemotype I that was the largest one and enclosed all species. Four populations (VSI1, VSI3, VPS1 and VS8) formed chemotype II that was rich in caffeic acid and naringin. Chemotype III included only one population of *V. songaricum* (VS11), which was characterized by high concentrations of luteolin (43.55 µg/mg DW), quercetin (1 µg/mg DW) and rutin (24.24 µg/mg DW). High values of apigenin (33.18 µg/mg DW) and luteolin (88.12 µg/mg DW) differentiated VPS2 population (*V. pseudodigitalis*, collected in Maymand region) as a distinct chemotype (chemotype IV). The heat-map summarizes the quantitative data on the phytochemical components and antioxidant activity in flower extracts from the four *Verbascum* species (Figure 4). A color was associated with the content of each compound, with blue meaning a low content and red alluding to a high content. The results obtained from HCA and PCA showed that genotypes from *V. sinuatum* and *V. pseudodigitalis* species had notable antioxidant potential due to the presence of phenolic compounds and individual flavonoid and phenolic acids (i.e., apigenin, naringin and caffeic acid). Overall, the present study showed that *Verbascum* flowers are extremely variable in phenolic constituents. This variability

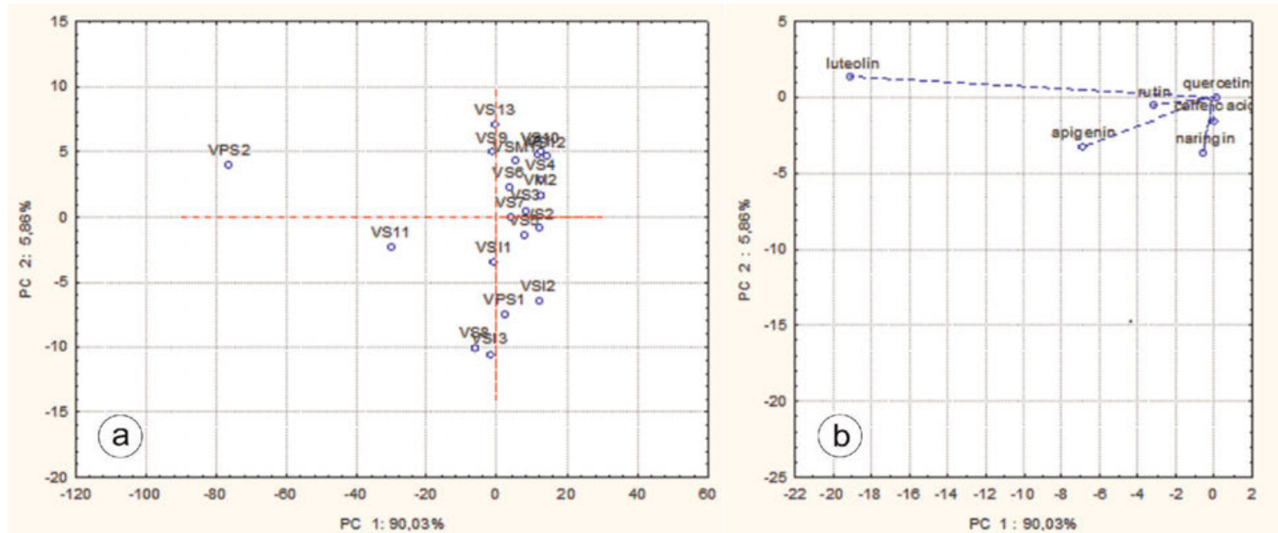

Fig. 3. (**a**) PCA score plot for main variation of phenolic compositions among Iranian *Verbascum* populations (**b**) Loading plot for phenolic constituents explaining 95.89% of the variation on PC1 and PC2 axes.

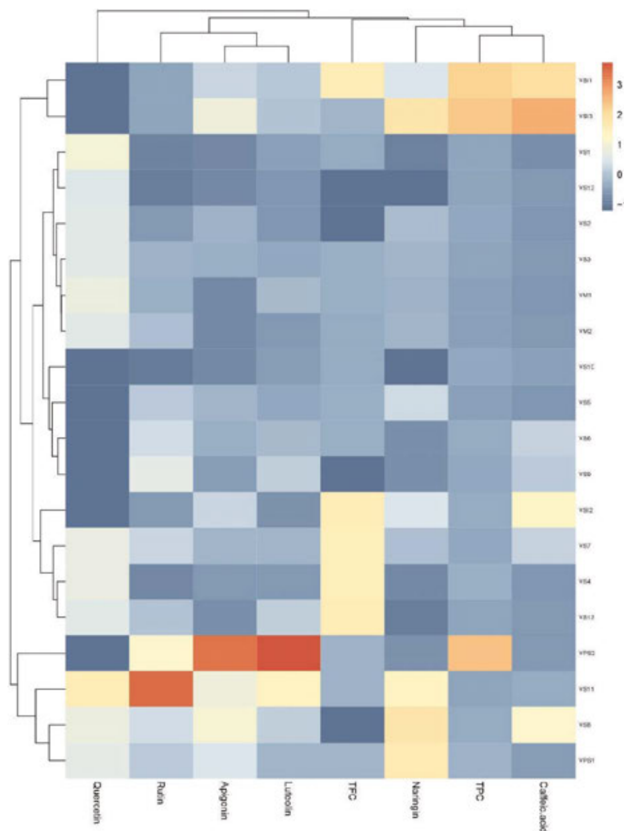

Fig. 4. Distribution of biochemical compounds in flowers of *Verbascum* genotypes with heat map visualization from blue for low contents to red for high contents.

might be due to differences in the climate, habitat and genetics.

CONCLUSION

The present investigation was aimed at studying the variation in phenolic composition and antioxidant activity in 20 *Verbascum* accessions sampled in Iran and belonging to four different species. In this study, the *Verbascum* samples showed a great rate of phytochemical variation and seemed to have a high potential for exploitation in cultivation and breeding. Exploring the level of phytochemical diversity in the *Verbascum* species growing in Iran provided important information for exploiting their bioactivity potentials such as antioxidant activity. The results showed that the geographic origin and environmental conditions could significantly affect the phenolic composition in *Verbascum* species.

AUTHORS' CONTRIBUTIONS

ZL contributed to the design of the study, supervised the research and manuscript editing, FJ and FM helped with the supervision and preparation of the manuscript. FJ, KS and FM performed the experiments and data collection, and prepared manuscript drafting. All authors have read and

confirmed the final version of the manuscript for publication. The authors declare that there is no conflict of interest.

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