Phytochemical attributes of endemic endangered primrose (*Primula heterochroma* Stapf.) accessions grown in Iran

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Abstract *Primula heterochroma* is indigenous to Caspian coast forests in the north of Iran. Total phenols, total flavonoids, total carotenoids and antioxidant capacity of 50 *P. heterochroma* accessions were investigated. The highest total phenol (1272.31 mg GA equivalent/100 g fresh weight) and total flavonoids (615 mg catechin/100 g fresh weight) were observed in G5 accession from Saravan. The highest antioxidant activity was observed in G14 accession (73.03 %) from Kacha, followed by G2 accession (69.75 %) and G5 accession (66.84 %) from Saravan. The results of HPLC analysis showed that quercetin-3-glucoside was the major phenolic compound widely found in these accessions followed by chlorogenic acid. There was a linear relationship between the antioxidant capacities and the total phenols and total flavonoid. Inversely, there was no statistically significant correlation between total carotenoid and antioxidant activity. Based on the path coefficient analysis, the maximum direct effect on antioxidant was observed in total phenols (0.908). In addition, the cluster analysis based on Euclidean distance with Unweighted pair-group method using arithmetic average (UPGMA) method separated the accessions into four main groups. Our results supported that Iranian wild primrose

accessions possess valuable antioxidant properties for therapeutic and potential medicinal use.

Keywords Antioxidant capacity · Correlation · Endangered plant · HPLC analysis · Path analysis

Introduction

Phenolics compounds have largely been in interest because of their significant bioactivities, such as chelating metals, regulating enzyme activity, scavenging free radicals and modulating cell proliferation. Health advantages of phenolic compounds such as antithrombotic, anticarcinogenic and vasodilatory activities have been demonstrated (Cook and Samman 1996; Wang et al. 2011). Vitamins, phenolic substances and carotenoids are the three major groups of natural antioxidants involved in defenses against several diseases (Thaipong et al. 2006). Therefore, natural antioxidants have become important to enhance the body's antioxidant defense system. Vegetables, fruits, cereals, medicinal plants and microalgae were widely evaluated to find natural antioxidants (Deng et al. 2012; Hassanpour et al. 2011; Li et al. 2009; Stangeland et al. 2009).

Primula L. is one of the most popular horticultural plants (Zhang and Kadereit 2004) belonging to the Primulaceae family. The genus *Primula* was traditionally used as a treatment for convulsions (Basbulbul et al. 2008). Different types of biological activities such as anxiolytic (Sufka et al. 2001), antimicrobial (Basbulbul et al. 2008), antiviral, and expectorant (Kati et al. 2001) have been reported for several species of *Primula*. Previous phytochemical studies on *Primula* species led to isolation of phenolic compounds and flavonoids (Vitalini et al. 2011), saponins (Okrslar et al. 2007), carotenoids (Yamamizo et al. 2011) and essential oil (Nan et al. 2002).

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Autochthonous plants have been more attended among the various medicinal herbs since they may be applied for the production of raw materials or preparations containing phytochemicals with remarkable antioxidant activities and health advantages (Exarchou et al. 2002). *Primula heterochroma* Stapf. is an endemic endangered plant to the north of Iran (Alinezhad et al. 2011) and covers low slopes and shady habitats of the Caspian coasts forests (Parsakhoo et al. 2009). Previous researches have shown that the extracts of *P. heterochroma* had high medicinal values: including antioxidants, antihemolytic and inhibitory effects of flavonoid-rich fractions against iron-induced oxidative stress and lipid peroxidation in brain tissues (Alinezhad et al. 2012), protective effect extracts against sodium fluoride-induced hemolysis in rat erythrocytes (Alinezhad et al. 2011) and interaction polyphenolic-, flavonoid-, polysaccharide-, or anthocyanin-rich fractions with red blood cell membrane lipids or proteins (Nabavi et al. 2012). Formerly, in vitro propagation and genetic diversity of *P. heterochroma* have been reported (Noroozisharaf et al. 2011; Noroozisharaf et al. 2015). However, there has been no standardized and comparative studies on antioxidant activities and phytochemical compositions of different *P. heterochroma* accessions in the north of Iran.

These accessions constitute the potential source of genes for primrose breeding programs through which polyphenols may be manipulated. The present study was carried out aimed at evaluating the antioxidant properties and phenolic profiles in fifty Iranian primrose accessions. Our research also displays a possible relationship between antioxidant activity and phenolic content. Path coefficient analysis was also performed to provide more information about the direct and the indirect effects of phytochemical variables.

Materials and methods

Plant materials

Fifty primrose accessions (*P. heterochroma* Stapf.) were collected in early spring from different regions of Guilan province, Iran (Table 1). Fresh leaf samples were taken from 3-month-old plants and kept at -80°C for phytochemical measurements.

Total phenolic content

Total phenolic content were determined colorimetrically using Folin-Ciocalteu reagent (Tavarini et al. 2008) modified as follows: Each sample (1 g) was extracted with 6 ml of extraction solvent containing methanol and acetic acid (85:15, v/v) and then 10 μl of the methanolic extract were mixed with 190 μl of distilled (DI) water in a test tube followed by addition of 1 ml

of 10 % Folin-Ciocalteu reagent and allowed to stand for 5 min. Then, 800 μl of 7.5 % sodium carbonate solution was added. Each sample was allowed to stand for 90 min(s) at room temperature in darkness and the absorbance was measured at 760 nm using an UV/Vis spectrophotometer (model PG Instrument +80, Leicester, United Kingdom). gallic acid (GA) was used as a standard and results were expressed as mg gallic acid equivalents per 100 g fresh weight (FW) basis (mg GA equivalent/100 g FW).

Total flavonoid content

Five gram of frozen tissue was ground to a fine powder under liquid nitrogen by cold mortar and pestle. One gram of the resultant powder was added to 6 ml of 85 % methanol (85 % methanol +15 % acetic acid) and held at room temperature for 24 h (Cordenunsi et al. 2003). The slurry was centrifuged at $10,000 \times g$ for 10 min at 4°C , and the supernatant was used. Total flavonoid contents were determined by a colorimetric assay (Shin et al. 2007) modified as follows. 50 μl of sample was added to a 10 ml tube containing 1.8 ml of methanol. Then 75 μl of 5 % NaNO_2 was added to this mixture, which was allowed to stand for 5 min(s) at room temperature, and 75 μl of 3 % $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ was added. The mixture was allowed to stand for 6 min(s) at room temperature, and 0.5 ml of 1 M NaOH was added and was allowed to stand for 15 min(s) at room temperature in darkness. The absorbance of the solution was measured immediately at 506 nm. The results are expressed as catechin equivalents using a standard curve prepared from authentic catechin.

Total carotenoid content

Pigment of carotenoid was determined by the spectrophotometer (Minguez-Mosquera and Perez-Galvez 1998). 100 mg of the resultant powder was added to 1200 μl of 80 % acetone and held at room temperature for 30 min(s). The slurry was centrifuged at $5000 \times g$ for 10 min at 4°C , and the supernatant was used. The absorbance of the solution was measured at 470 nm. The pigment concentrations were expressed in $\mu\text{g}/100 \text{ g FW}$.

Antioxidant activity

The antioxidant activity was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method (Sanchez-Moreno et al. 1999), with some modifications. Briefly, 10 μl of primrose extracts were added to 990 μl of DPPH. After vortexing, it was allowed to stand at room temperature in darkness. The absorbance of the samples was measured at 517 nm after 15 min(s) using the spectrophotometer. For each sample, three separate determinations were carried out. The antioxidant activity was expressed as the percentage of decline of the

Table 1 List of 50 germplasm accessions of *Primula heterochroma* collected from Guilan Province of Iran

Accession name	Collected site	Latitude (N)	Longitude (E)	Average of Altitude (m)
G1 to G5	Saravan	37°8'	49°39'	58
G6 to G9	Kacha	37°5'	49°38'	226
G10 to G13	Qazian	37°5'	49°38'	172
G14 to G17	Kacha	37°4'	49°35'	178
G18 and G19	Oskoolak	37°0'	49°34'	191
G20	Siahrud	37°0'	49°35'	137
G21 to G27	Jokleh Bandan	37°6'	49°39'	98
G28 to G31	Imamzadeh Hashem	37°6'	49°38'	126
G32 to G35	Jokleh Bandan	37°7'	49°39'	156
G36 to G38	Mushangah	37°3'	49°37'	189
G39	Siyahkal	37°3'	49°53'	256
G40	Tutaki	37°1'	49°53'	234
G41 and G42	Lunab	37°0'	49°51'	543
G43	Qaleh Rudkhan	37°0'	49°16'	103
G44 to G46	Khararud	37°5'	49°47'	559
G47 and G48	Pirkoooh	36°57'	49°48'	601
G49 and G50	Deylaman	36°57'	49°51'	555

Table 2 Total phenolic (mg GAE/100 g FW) and total flavonoid (mg CAT/100 g FW) contents of 50 germplasm accessions of *Primula heterochroma*

Accession name	Total phenolic content	Total flavonoid content	Accession name	Total phenolic content	Total flavonoid content
G1	949.18	388.5	G26	727.93	411.5
G2	1244.81	501.5	G27	544.81	368
G3	861.68	419.5	G28	623.56	378
G4	646.68	496.5	G29	826.06	406.5
G5	1272.31	615	G30	1054.81	481.5
G6	692.31	384	G31	940.43	421
G7	674.18	427	G32	707.93	430.5
G8	1121.06	491.5	G33	582.31	382.5
G9	1079.81	495.5	G34	590.43	406.5
G10	1156.68	476.5	G35	541.68	379
G11	787.31	426	G36	628.56	394
G12	815.43	427.5	G37	852.93	459
G13	1080.43	490.5	G38	631.06	387.5
G14	1087.31	502.5	G39	831.06	449.5
G15	906.68	478	G40	849.18	405
G16	1164.81	489	G41	893.56	418.5
G17	854.18	421.5	G42	859.81	413
G18	644.18	356	G43	863.56	424.5
G19	825.43	398	G44	984.18	460
G20	1117.31	531	G45	790.43	395
G21	825.43	410	G46	776.68	386
G22	766.68	398.5	G47	814.81	437.5
G23	991.06	478	G48	1246.06	498
G24	614.81	415	G49	908.56	442.5
G25	761.06	436.5	G50	921.68	427
HSD _{1%}	574	166.41	HSD _{1%}	574	166.41

Mean differences with smaller amounts than Tukey's value (HSD) in each column, are not significantly different at $P < 0.01$

absorbance, relative to the control, corresponding to the percentage of DPPH that was scavenged. The percentage of DPPH, which was scavenged (%DPPHsc), was calculated using formula: $\%DPPHsc = \frac{A_{cont} - A_{samp}}{A_{cont}} \times 100$, where A_{cont} is the absorbance of the control, and A_{samp} the absorbance of the sample.

HPLC analysis of phenolic compounds

Phenolic compounds were determined using High-performance liquid chromatography (HPLC) as described by (Bakhshi and Arakawa 2006). Sixteen accessions based on genetic clustering (Noroozisharaf et al. 2015) have been selected for HPLC analysis. Each sample (1 g) was extracted with 6 ml of extraction solvent containing methanol and acetic acid (85:15, v/v). The primrose extracts were filtered by disposable 0.45 µm syringe filter. Fifty microliters of the filtered sample were injected in HPLC (Waters, 1525, Milford, USA) equipped with a UV-visible detector (Waters Dual λ Absorbance 2487), C18 column: Waters Symmetry C18

5 µm 4.6 mm × 150 mm (Waters, Dublin, Ireland), at 280, 320 and 350 nm. The flavonoids were identified by comparing their UV spectra and retention times with those of the corresponding standards and by the spiking of samples with the appropriate standard. Catechin and chlorogenic acid standards were purchased from Sigma-Aldrich (Canada Ltd) and quercetin-3-glucoside from Extrasynthase, France.

Data analysis

The experiment was conducted in a one-way analysis of variance with three replications. Data were subjected to analysis of variance and means were separated by Tukey's HSD test at $P < 0.01$ significance level in SAS (Software Version 9.1 SAS). Correlation and path analysis were performed in SPSS (IBM SPSS Software Version 22) and Path2 software, respectively. Path analysis was carried out using the procedure suggested by Dewey and Lu (1959), which has been used to quantify a perceived biological relationship through partitioning of correlation coefficients into direct and indirect

Table 3 Total carotenoid content (µg/100 g FW) and antioxidant activity (%DPPH) of 50 germplasm accessions of *Primula heterochroma*

Accession name	Total carotenoid content	Antioxidant activity	Accession name	Total carotenoid content	Antioxidant activity
G1	2.29	62.88	G26	2.33	47.02
G2	1.79	69.75	G27	2.92	37.26
G3	1.85	59.09	G28	2.54	39.63
G4	1.98	43.75	G29	2.06	49.48
G5	2.07	66.84	G30	2.74	59.93
G6	2.39	44.83	G31	2.79	53.09
G7	2.51	47.68	G32	2.47	42.79
G8	2.25	63.81	G33	2.54	35.82
G9	2.92	66.33	G34	2.65	43.06
G10	2.38	65.01	G35	2.28	38.19
G11	2.36	39.93	G36	2.65	39.30
G12	2.45	55.94	G37	2.79	50.93
G13	2.14	63.75	G38	3.02	39.90
G14	2.22	73.03	G39	2.89	55.07
G15	2.93	56.90	G40	2.80	49.27
G16	2.42	66.33	G41	2.41	52.43
G17	2.43	52.49	G42	2.82	50.81
G18	2.59	39.06	G43	2.49	50.90
G19	2.16	50.63	G44	2.95	53.00
G20	2.80	64.02	G45	2.04	50.48
G21	2.31	51.53	G46	2.55	45.88
G22	2.24	50.30	G47	2.53	48.61
G23	2.63	59.27	G48	2.70	63.78
G24	2.40	40.30	G49	2.40	50.51
G25	2.68	52.64	G50	2.77	56.15
HSD _{1%}	1.11	21.26	HSD _{1%}	1.11	21.26

Mean differences with smaller amounts than Tukey's value (HSD) in each column, are not significantly different at $P < 0.01$

effects. Similarity matrix based on Euclidean distance was constructed from phytochemical data. It was used for the cluster analysis and construction of dendrogram through Unweighted pair-group method using arithmetic average (UPGMA), performed by the PAST (Version 1.97) software package (Hammer et al. 2001). The cophenetic correlation coefficient was calculated to check the fitness of the cluster.

Results

The differences in all parameters among *P. heterochroma* accessions were statistically significant ($P < 0.01$). As shown in Table 2, total phenolic contents of primrose accessions were in 541.68–1272.31 mg GA equivalent/100 g FW range. The total flavonoid contents of primrose accessions were in 356–615 mg catechin/100 g FW range (Table 2). The highest amount of total flavonoids was observed in G5 accession, while the lowest one was observed in G18 accession.

A low variation was found among primrose accessions in terms of total carotenoid content (Table 3), ranging from 1.79 μg to 3.14 $\mu\text{g}/100\text{ g FW}$ basis. The accession G13 had the highest total carotenoid content.

As shown in Table 3, the antioxidant activity was significantly different ($P < 0.01$) among all *P. heterochroma* accessions. However, most of the accessions showed a high antioxidant activity. The highest antioxidant activity was observed in G14 accession (73.03 %) from Kacha, followed by G2 accession (69.75 %) from Saravan and G5 accession (66.84 %) from Saravan. The lowest antioxidant activity was observed in G33 accession (35.82 %) from Jokleh Bandan.

Chlorogenic acid, quercetin-3-glucoside and catechin were identified in primrose accessions (Fig. 1). Variations in the phenolic compounds (Table 4) among *P. heterochroma* accessions were statistically significant ($P < 0.01$). The level of quercetin-3-glucoside ranged from 166.11 in G46 from Khararud to 980.46 $\mu\text{g/g FW}$ in G19 from Oskoolak. The highest and lowest catechin were observed in G2 from Saravan (135.46 $\mu\text{g/g FW}$) and G19 from Oskoolak (11.59 $\mu\text{g/g FW}$), respectively. The highest chlorogenic acid was observed G34 from Jokleh Bandan (744.36 $\mu\text{g/g FW}$), followed by G1 from Saravan (583.32 $\mu\text{g/g FW}$) and G36 from Mushangah (534.93 $\mu\text{g/g FW}$).

The correlation between total phenolic contents, total flavonoid contents, total carotenoid content and antioxidant activity in *P. heterochroma* accessions are shown in Table 5. There was a significant correlation between most indices,

Fig. 1 HPLC chromatogram: **a** chlorogenic acid in G19, **b** quercetin-3-glucoside in G12 and **c** catechin in G13

Table 4 Different phenolic compounds ($\mu\text{g/g}$ FW) of 16 germplasm accessions of *Primula heterochroma*

Accession name	Quercetin-3-glucoside	Catechin	Chlorogenic acid
G1	673.55	28.58	582.99
G2	354.07	135.46	307.44
G3	662.49	70.41	105.59
G10	360.30	87.71	272.85
G12	338.49	13.29	365.33
G13	222.78	18.40	132.70
G14	239.28	23.93	387.37
G19	979.97	11.59	503.37
G26	391.22	39.73	176.35
G27	311.53	97.85	117.62
G34	660.42	12.97	744.03
G36	477.94	65.65	534.59
G38	445.97	49.31	474.51
G41	902.84	41.86	165.43
G46	165.45	39.02	217.17
G47	304.85	14.12	238.70
HSD _{1%}	36.37	3.93	28.77

Mean differences with smaller amounts than Tukey's value (HSD) in each column, are not significantly different at $P < 0.01$

but no statistically significant correlation was observed between total carotenoid content and other parameters.

The path coefficient analysis results are shown in Table 6. Among the characters, total phenol had the higher positive direct effect on antioxidant activity (0.908). The indirect effect of total phenol through total carotenoid was positive but scarce (0.003) and through total flavonoid was positive but low (0.020). The indirect effect of total flavonoid via total phenol (0.724) was higher than the direct effect of total flavonoid (0.025) on antioxidant activity. The direct and indirect effects of total carotenoid were negative but estimated as low. A residual effect in path coefficient analysis was 0.359.

Cluster analysis was performed to develop a UPGMA dendrogram based on phytochemical data (Fig. 2). *P. heterochroma* accessions were divided into four main groups based on the values of the Euclidean distance. Groups 1 to 4 consisted of 25, 14, 8 and 3 accessions,

respectively. The cophenetic correlation coefficient ($r=0.79$) was calculated to evaluate the usefulness of the UPGMA method in clustering plant accessions.

Discussion

The total phenolic contents of 50 accessions of wild primrose were assessed. As shown in Table 2, there was a wide range of total phenolic contents of primrose accessions. The amount of phenolic contents was in a considerable range in G5 from Saravan, while it was negligible in G35 from Jokleh Bandan. Previously, 117.5 mg GA equivalent/100 g FW basis was observed in a *P. heterochroma* accession from Darabkola in Mazandaran- a province in Iran (Alinezhad et al. 2012). In our study, total phenolic results were higher than those described elsewhere. The phenolic compounds were used in plant defense mechanisms to scavenge reactive oxygen species as well as to prevent molecular damage, and damaging by microorganisms, insects and herbivores (Yilmaz et al. 2009). *P. heterochroma* accessions revealed the presence of considerable amounts of flavonoids. Previously, the 16 mg/g FW basis of total flavonoid was observed in a *P. heterochroma* accession from Darabkola (Alinezhad et al. 2012). However, our results showed that the total flavonoid contents were in 356–615 mg catechin/100 g FW range. Therefore, the results of our study confirmed the antioxidant potential in G5 from Saravan, followed by G20 from Siahrud and G14 from Kacha, since they have been shown to contain the highest amounts of total flavonoid contents among considered accessions.

The antioxidant activity assay was carried out to assess the ability of the phytochemical extracts to scavenge free radicals. In this study, high antioxidant activity was observed. Among all the accessions, G14 from Kacha, G2 from Saravan and G5 from Saravan had the highest antioxidant activity. The similarity between our results with the previous results obtained from *Primula elatior* (Mostafa et al. 2014) and *Primula vulgaris* (Demir et al. 2014) showed an equivalent or higher antioxidant activity.

It has been reported that antioxidant activity of plants might be attributed to their phenolic compounds (Cook and Samman 1996). Quercetin, a major representative of the flavonoids,

Table 5 Correlation between quantitative determinations in *Primula heterochroma* accessions

Variables	Total phenolic	Total flavonoids	Total carotenoid	Antioxidant activity
Total phenolic	1	0.802**	- 0.074 ^{ns}	0.931**
Total flavonoids		1	- 0.095 ^{ns}	0.758**
Total carotenoid			1	- 0.113 ^{ns}
Antioxidant activity				1

** Correlation is significant at the 0.01 level; ns correlation is not significant

Table 6 Path analysis of antioxidant components in *Primula heterochroma* accessions

Character	Total phenolic	Total flavonoids	Total carotenoid	Pooled effects
Total phenolic	0.908	0.020	0.003	0.931
Total flavonoids	0.728	0.025	0.004	0.758
Total carotenoid	- 0.068	- 0.003	- 0.044	- 0.113
Residual effects ($\sqrt{1 - R^2}$) = 0.359				

Bold values are direct effect

prevents the oxidation of low-density lipoprotein by scavenging free radicals (Cartea et al. 2010). The results suggested that among phenolic compound, quercetin-3-glucoside, followed by chlorogenic acid were frequently detectable in *P. heterochroma* accessions. The same result was also reported in *Primula denticulate* (Tokalov et al. 2004). A high concentration of quercetin may be attributed to its function as a photosynthetic apparatus protectant (Edreva 2005). It was shown that flavonoids might act as filters of UV radiation, since they strongly absorb radiation, within the range 280–315 nm, that is UVB (Harborne and Williams 2000).

In this study, there was no statistically significant correlation between total carotenoid contents and other parameters. A high correlation between the total phenolic and flavonoid contents and the antioxidant capacity suggested that antioxidant components in these accessions could reduce oxidants and scavenge free radicals. In some literatures, the correlation between antioxidant activity and total phenolic content has been

reported (Cai et al. 2004; Orak 2007; Tulipani et al. 2008). The antioxidant activity of phenolics results, to a great extent, from their singlet oxygen quenchers and hydrogen donors properties. They may also have a metal chelating potential (Javanmardi et al. 2003). Path coefficient analysis helps in partitioning of correlation coefficient into direct and indirect effects of various traits on antioxidant or any other attributes (Khan et al. 2009). The path coefficient analysis results indicated that total phenolic content is an effective major factor on antioxidant activities.

Cluster analysis was performed to grouping of accessions. The similarity matrix based on Euclidean distance showed that the lowest distance (4.10) was between G21 from Jokleh Bandan and G29 from Imamzadeh Hashem and the highest one (595.91) was between G20 from Siahrud and G35 from Jokleh Bandan. Accessions from the same regions generally were clustered into the same group or subgroups. For example, G45 and G46 from Khararud with the same subgroup

Fig. 2 UPGMA dendrogram of phytochemical data based on Euclidean distance in 50 *Primula heterochroma* accessions

were clustered in the first group. The same manner occurred for G49 and G50 from Deylaman. While G33 and G34 from Jokleh Bandan with the same subgroup were clustered in the second group, and G8 and G9 from Kach in the third group, indicating the relative consistency of the phytochemical profile and the influence of the accessions geographical origin.

In all, the present study demonstrated the potential value of *P. heterochroma* accessions containing rich source of phenolic compounds and total flavonoids. Among the accessions, antioxidant activity was high and varied. Therefore wild primrose accessions could be considered as a rich source of natural antioxidants. These results could be considerable for the efficient use of these accessions as breeding materials in advanced biotechnology studies or future traditional breeding.

Conclusion

In this study, antioxidant capacities and total phenolic and flavonoid content of fifty accessions of Iranian primrose were evaluated. These accessions had high antioxidant capacities and total phenolic contents. A high correlation between total phenolic content and antioxidant capacity showed that phenolic compounds could be main contributors of antioxidant capacities in these accessions. Flavonoids such as quercetin-3-glucoside and chlorogenic acid were widely found in these accessions.

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