Levels of phenolic compounds and their effects on antioxidant capacity of wild Vaccinium arctostaphylos L. (Qare-Qat) collected from different regions of Iran

Tahereh HASANLOO¹, Roshanak SEPEHRIFAR¹, Homa HAJIMEHDIPOOR²

¹Department of Molecular Physiology, Agricultural Biotechnology Research Institute of Iran, Karaj - IRAN
²Traditional Medicine and Materia Medica Research Center and School of Traditional Medicine, Shahid Beheshti University of Medical Sciences, Tehran - IRAN

Received: 29.09.2009

Abstract: Antioxidant activities in the leaves and fruits of wild Vaccinium arctostaphylos (locally named Qare-Qat) from different regions of Iran were evaluated using the stable 1, 1-diphenyl-2-picrylhydrazyl free radical and ferric reducing/antioxidant power methods. Variations in antioxidant activity, total phenolic, anthocyanin, and flavonoid content in fruit samples among genotypes was much greater than the variation observed between growing seasons, indicating that genetics plays a more important role than growing season in Vaccinium arctostaphylos fruits. The data indicated that the fruits of the Kelardasht genotype had the highest total phenolic content [42.73 ± 1.5 mg gallic acid equivalents (GAE) g⁻¹ DW], anthocyanin content (1.00 ± 0.76 mg g⁻¹), and antioxidant activity while the Masouleh genotype had the lowest antioxidant activity. Significant main effects for genotype, growing season, and genotype × growing season were observed for anthocyanin and antioxidant activity, while variation in polyphenolic content indicated that genotype plays more of a role than growing season in the leaves of Vaccinium arctostaphylos. Correlation analyses indicated that there was a linear relationship between antioxidant activity and the total phenolic and anthocyanin content in fruits and leaves. Over both growing seasons the flavonoids did not play an important role in the antioxidant potential of the leaves.

Key words: Anthocyanin, antioxidant activity, flavonoid, total phenolics, Vaccinium arctostaphylos

Introduction

Vaccinium arctostaphylos L. (Ericaceae), locally named Qare-Qat, is a medicinal plant which has been extensively used in Iranian folk medicine as an antidiabetic and antihypertensive agent for many years (1). V. arctostaphylos grows as a shrub or woody bush in the northern mountains of Iran. This plant has interesting medical properties mainly due to the presence of a significant amount of phenolic compounds (2).

Phenolics are a specific group of secondary metabolites that play the important role of protecting organisms against the harmful effects of oxygen radicals and other highly reactive oxygen species (ROS). ROS can initiate biomolecular oxidation which leads to cell injury and death. ROS can also create oxidative stress, which results in numerous diseases and disorders such as cancer, stroke, myocardial infarction, diabetes, septic and hemorrhagic shock, and Alzheimer and Parkinson diseases (3-7). Antioxidants that can quench reactive free radicals and prevent the oxidation of other molecules have health-promoting effects in the prevention of degenerative diseases (8). It has been well established with the DPPH and FRAP methods that a strong
Levels of phenolic compounds and their effects on antioxidant capacity of wild Vaccinium arctostaphylos L. (Qare-Qat) collected from different regions of Iran

and positive relationship exists between total phenolic and anthocyanin content and antioxidant activity (1,2,9), suggesting that breeders can select their intended characteristics in order to increase antioxidant capacity (10). These properties can be affected by different factors, including genotype and growing seasons (11,12).

The purpose of this study was to compare total phenolics and anthocyanin and flavonoid content with antioxidant activity in the leaves and fruits of V. arctostaphylos grown at 4 different locations over 2 growing seasons. This information is important since few data are currently available concerning the effect of different seasons on changes in antioxidant activity and total phenolics and the anthocyanin and flavonoid content of Iranian wild V. arctostaphylos.

Materials and methods

Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2, 4, 6-tripyridyl-s-triazine (TPTZ), Folin-Ciocalteu reagent, quercetin, gallic acid, and vitamin C were purchased from Sigma Chemicals Co. (Germany). All chemicals and reagents were analytical grade.

Plant material

Leaves and mature fruits of wild V. arctostaphylos were collected from 4 areas of 3 northern provinces in Iran: Masouleh (Gilan: 37°08’N, 48°57’E, 1550 m), Asalem (Gilan: 37°38’N, 48°49’E, 1250 m), Kelardasht (Mazandaran: 36°32’N, 51°07’E, 1704 m), and Hoor (Ardabil: 38°13’N, 48°41’N, 1550 m) in May and August of 2007 and stored at –80 °C before analysis. Plant materials were freeze dried for analysis. A voucher specimen was deposited in the Farabi Herbarium (FAR). The samples were identified by Zahra Tavakkoli. The herbarium numbers were 25726 (Masouleh), 31025 (Kelardasht), 33899 (Asalem), and 34164 (Hoor).

Preparation of extraction

Freeze-dried samples (0.25 g) were milled and extracted with 50 mL of 1% HCl in methanol. Extraction was carried out by stirring for 48 h. This was repeated in triplicate. The extracts were pooled, and this mixture was used for further procedures either immediately or after deep freezing (–80 °C) for no longer than 4 days.

DPPH radical scavenging activity

The scavenging effect of each extract was estimated according to the procedure established by Brand-Williams et al. (13,14). Each extract was prepared in 5 different dilutions in methanol, and the pH was adjusted to 2. An aliquot of 0.1 mL of the diluted extract was added to 3.9 mL of DPPH solution in methanol (60 μmol/mL), vortexed, and kept at room temperature in darkness for 20 min. The absorbance was then measured at 515 nm. The percentage of remaining DPPH (%DPPH REM) at the steady state was determined as follows:

\[ \% \text{DPPH}_{\text{REM}} = \frac{C_{\text{DPPH}, t=0}}{C_{\text{DPPH}}}, \]

where \( C_{\text{DPPH}, t=0} \) is the initial DPPH concentration, and \( C_{\text{DPPH}} \) is the DPPH concentration after 20 min. For each extract the percentage of remaining DPPH at the steady state for the 5 dilutions versus the ratio mg DPPH/mL of extract was plotted. The parameter IC \(_{50}\) was calculated graphically. A lower IC \(_{50}\) value indicates greater antioxidant activity, and vitamin C was used as reference for the potency of the radical scavenging effect.

FRAP assay

The antioxidant activities of all extracts were determined using a modified method of the ferric reducing/antioxidant power (FRAP) assay (15). The FRAP reagent contains 2.5 mL of a 10 mmol/L TPTZ solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L FeCl\(_3\) and 25 mL of 0.3 mol/L acetate buffer, pH 3.6. This was prepared fresh and heated to 37 °C. Then 80 μL of extract was mixed with 0.4 mL of distilled water and 3.6 mL of FRAP reagent, and the absorbance of the mixture at 593 nm was measured after incubation at 37 °C for 10 min. FeSO\(_4\) was used as the standard. The amounts are expressed as mmol of antioxidants as FeSO\(_4\)/g DW of plant. Vitamin C and quercetin was used for reference.

Total phenolic content

Total phenolics were determined using Folin-Ciocalteau reagent (16). Of each plant extract or gallic acid standard solution 0.5 mL was mixed with 5 mL Folin-Ciocalteau reagent (1:10 diluted with distilled water), and 4 mL aqueous Na\(_2\)CO\(_3\) 1M. After standing for 15 min at room temperature absorbance was measured at 765 nm. Results were expressed as milligrams of gallic acid equivalents (GAE)/g DW (17).
**Total anthocyanin content**

Total anthocyanin content was estimated using a pH different assay (18). An aliquot of plant extract (2 mL) was diluted up to 25 mL with pH 1 buffered solution (125 mL of 0.2 M KCl and 375 mL of 0.2 M HCl). A second solution was diluted with a pH 4.5 buffer solution (400 mL of 1 M CH<sub>3</sub>CO<sub>2</sub>Na, 240 mL of 1 M HCl, and 360 mL of H<sub>2</sub>O). Absorbance of the solutions was measured at 510 nm. Concentration of anthocyanin as cyaniding-3-glucoside was calculated using the equation

\[ C_{mg/L} = \frac{(Abs_{pH1} - Abs_{pH4.5}) \times 484.82 \times 1000}{24,825 \times DF}, \]

where the term in parentheses is the difference of the absorbance at 510 nm between the pH 1 and pH 4.5 solutions, 484.82 and 24,825 are the molecular mass and molar absorptive of cyanidin-3-glucoside at 510 nm in the different pH solution pH 1 and pH 4.5, and DF is the dilution factor.

**Total flavonoid content**

The total flavonoid content was determined according to the aluminum chloride colorimetric method described by Chang, et al. (2002) (19). An aliquot of plant extract (0.5 mL) was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride hexahydrate (AlCl<sub>3</sub>), 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. After incubation at room temperature for 30 min the absorbance of the solution was measured at 415 nm. The calibration curve was prepared by quercetin solutions at concentration 0-50 μg/mL in methanol. The data were expressed as milligram quercetin equivalents (QE)/g DW sample.

**Statistical analysis**

Data was analyzed using windows SAS software (Version 6.2). All experimental analyses were carried out on a minimum of 3 independent samples for each region, and 3 replications of each sample were assayed. Statistical significance was calculated using the Duncan test for unpaired data (P ≤ 0.05), and the ANOVA method was used for comparisons of means.

**Results and discussion**

Genotypic and seasonal effects on antioxidant activity and phenolic content and analysis of the variance in leaf samples showed that genotype and seasonal effects (G × S) for all variables were significant, with the exception of flavonoid and polyphenolic content. Variation in polyphenolic content between genotypes was much greater than variation due to the time of sample collection which indicates that genotype plays a more significant role than seasons in determining polyphenol content in the leaves of Qare-Qat. Significant effects of genotype, growing season, and G × S for anthocyanin and antioxidant activity (FRAP method) demonstrate that these factors can influence levels of anthocyanin and antioxidant activity. Connor et al. (12) reported that antioxidant activity and the phenolic content of blueberry cultivars were influenced more by genotype than by harvest year (Table 1). Moreover, analysis of variance in fruit samples showed that genotype effects were considerable in total phenolic, anthocyanin, and flavonoid content as well as the antioxidant activities of *V. arctostaphylos* methanolic extract (Table 2).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th></th>
<th>F</th>
<th></th>
<th>FRAP</th>
<th></th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>MS</td>
<td></td>
<td>DF</td>
<td>MS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype (G)</td>
<td>3</td>
<td>0.05</td>
<td>4.17*</td>
<td>3</td>
<td>355.78</td>
<td>1568.07**</td>
<td></td>
</tr>
<tr>
<td>Season (S)</td>
<td>1</td>
<td>0.11</td>
<td>9.59**</td>
<td>1</td>
<td>17.66</td>
<td>77.84**</td>
<td></td>
</tr>
<tr>
<td>G × S</td>
<td>3</td>
<td>0.15</td>
<td>13.02**</td>
<td>3</td>
<td>554.99</td>
<td>2446.07**</td>
<td></td>
</tr>
</tbody>
</table>

DF, degree of freedom; MS, mean square; *, Significant at P = 0.05; **, Significant at P = 0.01
Levels of phenolic compounds and their effects on antioxidant capacity of wild *Vaccinium arctostaphylos* L. (Qare-Qat) collected from different regions of Iran

Antioxidant activities using DPPH free radicals

Antioxidant activity of *V. arctostaphylos* methanolic extract was determined by DPPH assay. The DPPH stable free radical method is an easy, rapid, and sensitive way to evaluate an antioxidant's ability to scavenge free radicals (20) and the amount of each extract needed for 50% inhibition (IC$_{50}$). In simpler terms it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC$_{50}$). The radical scavenging activity for fruits ranged from a low of 0.14 mg/mL in Kelardasht to a high of 0.49 mg/mL in Masuleh. Assessment of the IC$_{50}$ of the leaves of *V. arctostaphylos* gave values that ranged from a low of 0.29 mg/mL in Masuleh to a high of 0.79 mg/mL in Kelardasht in May and ranged from a low of 0.28 mg/mL in Kelardasht to a high of 0.61 mg/mL in Masuleh in August, reflecting a 2.72 and 2.17-fold difference, respectively (Figure 1). IC$_{50}$ for ascorbic acid was $3.41 \times 10^{-3}$ mg/mL DPPH.

Antioxidant activity of the extracts by FRAP assay

The FRAP assay measures the ability of an antioxidant to reduce Fe$^{3+}$ to Fe$^{2+}$ in the presence of TPTZ and form an intense blue Fe$^{2+}$-TPTZ complex with maximum absorption at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant content (21). In the present study the results for ferric ion-reducing activities of the methanolic extract of *V. arctostaphylos* are shown in Figure 2. The highest reducing activities were observed in the Hoor and Kelardasht fruits (46.25 and 40.97 mmol/g). The antioxidant activity in leaves collected in May ranged from a low of 10.70 mmol/g in Kelardasht to a high of 49.41 mmol/g in Masuleh. The leaves of *V. arctostaphylos* in August gave values that ranged from a low of 21.96 mmol/g in Kelardasht to a high of 39.09 mmol/g in Asalem, reflecting a 4.6 and 1.8-fold difference, respectively (Figure 2). The reducing ability of methanolic extract of both *V. arctostaphylos* leaves and fruits and quercetin was considerably lower at 1167 mmol/g. As a reference, vitamin C was recorded at 906 mmol/g. A comparison of the 2 harvest seasons showed that the Kelardasht and Asalem genotypes had higher antioxidant activity in August. Surprisingly, the results showed that the FRAP value of the methanolic extract of the leaves and fruits was comparable to that of DPPH ($P > 0.05$). A direct correlation between the DPPH and FRAP methods was demonstrated by linear regression analysis. The strong correlation ($r > 0.857$) between the mean values of 2 kinds of IC$_{50}$ and FRAP values deserves detailed attention. This could be explained with the basic concept that antioxidants are reducing agents. Antioxidants are compounds capable of donating a single electron or hydrogen atom for reduction.
Total phenolic content of the extracts

It has been recognized that polyphenolics show antioxidant activity, and their effects on human nutrition and health are considerable (22). As shown in Figure 3 the total phenolics in fruits varied from a low of 9.48 mg GAE/g DW in Asalem to a high of 42.73 mg GAE/g DW in Kelardasht, reflecting a 4.51-fold difference. The highest phenolics content in May was obtained from the extract of *V. arctostaphylos* leaves from Masuleh (42.69 mg GAE/g DW). The total phenolics in harvested leaves varied from a low of 11.48 mg GAE/g DW in Hoor to a high of 20.56 mg GAE/g DW in Kelardasht in August, reflecting a 1.79-fold difference.

Total anthocyanin content of the extracts

Changes in anthocyanin content varied widely in collected leaves and fruits. The fruits were rich in anthocyanin content in all 4 genotypes: Kelardasht (1.003 mg/g) > Asalem (0.962 mg/g) > Hoor (0.356 mg/g) > Masouleh (0.211 mg/g).

The total anthocyanin in harvested leaves varied from a low of 0.010 mg/g in Kelardasht to a high of 0.106 mg/g in Asalem in May, reflecting a 10.45-fold difference. The total anthocyanin of leaves harvested in August varied from a low of 0.0159 mg/g in Kelardasht to a high of 0.0366 mg/g in Masuleh, reflecting a 2.29-fold difference (Figure 4).
Levels of phenolic compounds and their effects on antioxidant capacity of wild *Vaccinium arctostaphylos* L. (Qare-Qat) collected from different regions of Iran

Total flavonoid content of the extracts

This study showed that total flavonoid content in the fruits of selected regions varied: Kelardasht (0.757 mg QE/g) > Asalem (0.6765 mg QE/g) > Hoor (0.4754 mg QE/g) > Masouleh (0.33 mg QE/g) DW. Total flavonoid content in the collected leaves ranged from a low of 2.04 in Asalem to a high of 2.93 mg QE/g DW in Kelardasht in May, reflecting a 1.44-fold difference. In August the total flavonoid content in harvested leaves ranged from a low of 2.27 in Kelardasht to a high of 2.91 mg QE/g DW in Masuleh, reflecting a 1.28-fold difference (Figure 5).

Correlations of antioxidant activity with the total polyphenol, anthocyanin, and flavonoid content

A correlation analysis was performed for all results. The correlation between IC\textsubscript{50} and total phenolic content was 0.992, which is highly significant (P < 0.01). There was a positive correlation (r = 0.624) between anthocyanin and phenolic content (P < 0.05) for leaf samples that were collected in May. Our results showed that there was not any correlation between assayed factors in leaves that were collected in August. A correlation was also exhibited between flavonoid and the IC\textsubscript{50} of fruits, since r = 0.856 (P = 0.01). The regression analysis of IC\textsubscript{50} on flavonoid, phenol, and anthocyanin content showed the total phenolic content influence of IC\textsubscript{50} since R\textsuperscript{2} = 0.73. Therefore, the antioxidant capacity of fruits can be related to anthocyanin and phenolic content.

The antioxidant activities of Iranian Qare-Qat were determined and presented in this paper. The leaves and fruits of 4 selected genotypes of *V. arctostaphylos* were examined for antioxidant activity and phenolic, anthocyanin, and flavonoid content in 2 growing seasons with different colorimetric methods. The fruits of the Kelardasht genotype had the highest antioxidant activity that can be related to total phenolics and anthocyanin content. It has also been established that phenolic and anthocyanin content plays an important role in the antioxidant potential of leaves in all 4 regions over 1 growing season (May).

In conclusion, the leaves and fruits of *V. arctostaphylos* possess antioxidant activity. Therefore, this plant may be a potential source of antioxidant compounds for the food and pharmaceutical industries.

Acknowledgements

The authors gratefully acknowledge the financial support of the Agricultural Biotechnology Research Institute of Iran (ABRII) (Grant No. 2-05-05-86006). This research has also been supported by Iran National Science Foundation (INSF).

Corresponding autor:

Tahereh HASANLOO
Department of Molecular Physiology,
Agricultural Biotechnology
Research Institute of Iran,
Karaj - IRAN
E-mail: thanasloo@abrii.ac.ir

References


