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Full Length Research Paper

Analysis of phenolic compounds in wild populations of bilberry (*Vaccinium myrtillus* L.) from Montenegro

M. Jovančević[1], J. Balijagić[1], N. Menković[2], K. Šavikin[2]*, G. Zdunić[2], T. Janković[2] and M. Dekić-Ivanković[3]

[1]Biotechnical Faculty, Center for Continental Fruit-growing, Sandžadžka BB, 84000 Bijelo Polje, Montenegro.
[2]Institute for Medicinal Plants Research, Tadeuša Košćuška 1, 11000 Belgrade, Serbia.
[3]Institute for Medical Research, Tadeuša Košćuška 1, 11000 Belgrade, Serbia.

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Wild bilberry (*Vaccinium myrtillus* L.) is very important natural resource of Montenegro. In our study, bilberries were collected in the summer of 2009, from 11 different localities in mountain region of Montenegro (altitude ranged from 665 to 1700 m a.s.l.). The samples were freezed immediately and stored at -25°C until the analysis were done. The total phenolics were estimated by Folin-Ciocalteu method with slight modifications while the amounts of anthocyanins were analyzed according to the prescription of European Pharmacopoea 6.0. Anthocyanin aglycones were analyzed using high performance liquid chromatography (HPLC) after acid hydrolysis. Total phenolic content was determined in all analyzed samples and it ranged from 3.92 to 5.24 mgGAE/g fw. The amounts of total anthocyanins varied between 0.27 to 0.46%. Among eleven analyzed samples, 10 corresponded to the prescriptions of European Pharmacopoea 6.0. Significant correlation between total phenolics and total anthocyanins was noticed (r = 0.843, $P < 0.01$). Higher amounts of total phenolics and total anthocyanins were detected in samples harvested from localities more exposed to the sun in comparison with berries grown in shadow. Five cyanidin aglycones (delphinidin, cyanidin, petunidin, peonidin, and malvidin) were quantified after acid hydrolysis, where delphinidin was the most abundant in all samples (0.33 to 0.75 mg/g fw).

Key words: *Vaccinium myrtillus* L., Montenegro, phenolics, anthocyanins, anthocyanin aglycones.

INTRODUCTION

Phenolic compounds (phenolic acids, flavonoids, hydroxycinnamic acid derivatives) as widespread secondary metabolites in plants possess many potential health benefit effects. Flavonoids, including flavones, isoflavones, flavonones, anthocyanins, catechins, show various biological properties such as antibacterial, antioxidant, anti-inflammatory, anticarcenogenic activity (Shahidi and Naczk, 2004; Youdim et al., 2002; Rice-Evans et al., 1997). Bilberry (*Vaccinium myrtillus* L.) fruits are rich sources of dietary anthocyanins and antioxidants (Moyer et al., 2002). The anthocyanin composition of bilberries is caracterized by 15 anthocyanin glycosides in which five antocyanidins (delphinidin, cyanidin, petunidin, peonidin, malvidin) are combined with three types of sugars (galactose, glucose, arabinose) (Kalt et al., 1999). Plant anthocyanin levels can be affected by environmental conditions (e.g. growing location, intensity of light, photoperiod, temperature) (Macheix et al., 1990) and genetic differences (Moyer et al., 2002). Several studies have reported the influence of genotype and climatic conditions on total phenolics, total anthocyanins and antioxidant capacity of different cultivars of *Vaccinium* species (Prior et al., 1998; Moyer et al., 2002; Connor et al., 2002). The degree of anthocyanidin accumulation is primarily dependent on the light conditions, but a favorable impact of low temperatures and a limiting effect of high temperatures have often been reported (Macheix et al., 1990; Ortega-Regules et al., 2006). Due to these factors, the long summer day and

*Corresponding author. E-mail: ksavikin@mocbilja.rs. Tel: +38111 3031653. Fax: +38111 3031655.

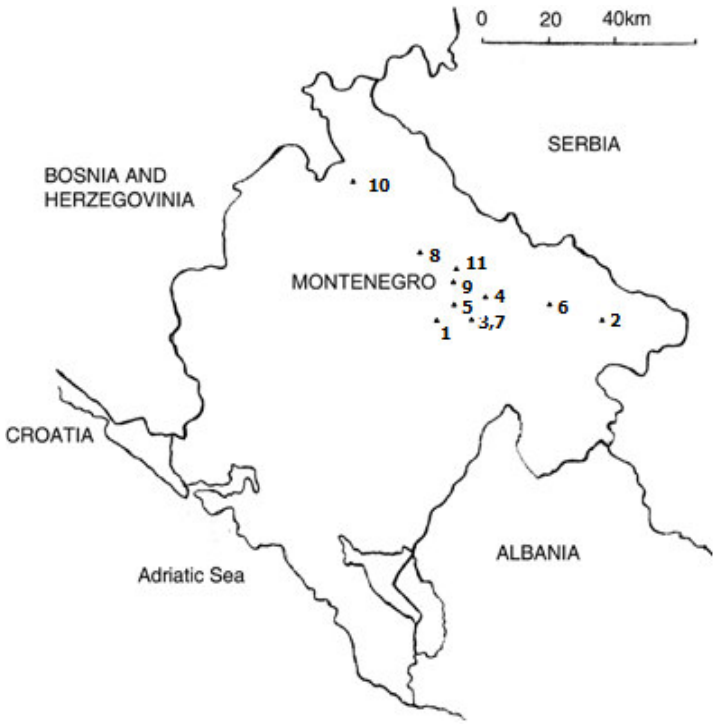

Figure 1. Map of localities in Montenegro. 1- Šiška, 2 – Lađevci, 3- Kordelj donji, 4 - Femića rupe, 5 - Bardov do, 6 – Krlje, 7 - Kordelj gornji, 8 – Lisa, 9 - Žute kose, 10 - Pljevlja – katun, 11 – Livadice.

lower night temperatures in mountain regions, ideal conditions for the formation of bilberry anthocyanins are provided.

Although there are few reports regarding the content of heavy metals and essential microelements in blueberry fruits from Montenegro (Jovančević et al., 2009; Antić – Mladenović et al., 2009), there is no information about the phenolic content in bilberries from Montenegro. The aim of the present study was to evaluate the effects of latitude and growing location on the level of total phenolics, total anthocyanins and anthocyanin aglycones in berries growing in mountain region of Montenegro.

MATERIALS AND METHODS

Berries

Riped bilberries (*V. myrtillus* L.), were manualy collected during summer time in 2009 from 11 locations different in altitude, exposition and type of habitat (sunny-H1 or shadow-H2) in Montenegro (Figure 1 and Table 1). Fresh undamaged berries were freezed immidiately and stored at -25°C. Analysis were performed after one month.

Total phenolic content

The total concentration of phenols was estimated by Folin-Ciocalteu method with slight modifications (Waterman, 1994). Fresh berries (5 g) were extracted with methanol for 30 min on ultrasonic bath and filtered. Two hundred microliters of the extracts were added to 1 ml of 1:10 diluted Folin-Ciocalteu reagent. After 4 min., 800 µl of sodium carbonate (75 g/L) were added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid (0 to 100 mg/L) was used for calibration of a standard curve. The results were expressed as gallic acid equivalents per gram of fresh weight (GAE/g fw). Triplicate measurements were taken and mean values were calculated.

Total anthocyanins content

Total anthocyanin content was investigated according to the procedure described in Ph. Eur. 6.0 (European Pharmacopoea 6.0., 2009). Briefly, 50 g of fresh berries were crushed extemporaneously. To 5 g of crushed, accurately weighed drug, 95 ml of methanol were added and mechanically stirred for 30 min then filtered into a 100 ml volumetric flask. Filter was rinsed and diluted to 100 ml with methanol. A 50-fold dilution of this solution in a 0.1% v/v solution of hydrochloric acid in methanol was prepared. The absorbance of the solution was measured at 528 nm, using a 0.1% v/v solution of hydrochloric acid in methanol as the compensation liquid. The percentage content of anthocyanins, expressed as cyanidin-3-glucoside chloride, was calculated from the expression: A x 5000/718 x m (A = absorbance at 528 nm; 718 = specific absorbance of cyanidin-3-glucoside chloride at 528 nm; m = mass of the substance to be examined in grams).

Quantitative analysis of anthocyanidins

Hydrolysis of the samples was performed according to Nyman and Kumpulainen (2001) with slight modifications. About 5 g of crushed,

Table 1. Localities, altitude, exposition and type of habitat of bilberries.

Sample	Altitude (m)	Habitat	Locality	Exposition	Area (ha)
B1	1700	H1	Šiška	NE	20
B2	1576	H1	Lađevci	NW	7
B3	1500	H1	Kordelj donji	N	6
B4	1250	H1	Femića rupe	N	6
B5	1700	H1	Bardov do	N	8
B6	1548	H2	Krlje	NE	12
B7	1550	H1	Kordelj gornji	N	6
B8	1050	H2	Lisa	NE	25
B9	665	H2	Žute kose	NW	4
B10	900	H2	Pljevlja - katun	N	6
B11	1057	H2	Livadice	SE	3

H1-sunny habitat; H2-shadow habitat.

Table 2. Content of total phenolics, total antocyanins and antocyanin aglycons in fresh bilberries.

Sample	Total phenolics (mg GAE/g fw)	Total anthocyanins (%)	Delphinidin (mg/g fw)	Cyanidin (mg/g fw)	Peonidin (mg/g fw)	Malvidin (mg/g fw)	Petunidin (mg/g fw)
B1	4.50 ± 0.05	0.38 ± 0.01	0.64 ± 0.03	0.41 ± 0.03	0.08 ± 0.01	0.25 ± 0.02	0.14 ± 0.01
B2	5.24 ± 0.05	0.46 ± 0.01	0.68 ± 0.04	0.66 ± 0.04	0.17 ± 0.01	0.43 ± 0.03	0.20 ± 0.02
B3	4.69 ± 0.07	0.42 ± 0.01	0.63 ± 0.04	0.44 ± 0.04	0.09 ± 0.01	0.27 ± 0.02	0.14 ± 0.02
B4	3.92 ± 0.08	0.33 ± 0.01	0.38 ± 0.03	0.33 ± 0.03	0.05 ± 0.01	0.11 ± 0.01	0.08 ± 0.03
B5	4.57 ± 0.11	0.42 ± 0.01	0.65 ± 0.05	0.52 ± 0.05	0.09 ± 0.01	0.24 ± 0.02	0.15 ± 0.01
B6	4.10 ± 0.15	0.38 ± 0.01	0.64 ± 0.05	0.52 ± 0.04	0.10 ± 0.02	0.29 ± 0.02	0.15 ± 0.02
B7	5.23 ± 0.12	0.46 ± 0.02	0.65 ± 0.04	0.44 ± 0.04	0.09 ± 0.01	0.31 ± 0.03	0.16 ± 0.01
B8	4.36 ± 0.09	0.36 ± 0.01	0.62 ± 0.04	0.55 ± 0.05	0.13 ± 0.02	0.37 ± 0.03	0.16 ± 0.01
B9	4.29 ± 0.03	0.36 ± 0.01	0.69 ± 0.03	0.75 ± 0.04	0.16 ± 0.02	0.37 ± 0.03	0.19 ± 0.02
B10	4.43 ± 0.03	0.44 ± 0.01	0.75 ± 0.06	0.63 ± 0.03	0.18 ± 0.02	0.52 ± 0.03	0.21 ± 0.03
B11	4.00 ± 0.04	0.27 ± 0.01	0.33 ± 0.04	0.25 ± 0.02	0.05 ± 0.01	0.15 ± 0.01	0.08 ± 0.02

accurately weighed drug was extracted with 40 ml of water/methanol solution containing 2 N HCl (50 ml of CH_3OH + 33 ml of H_2O + 17 ml of 37% HCl) on ultrasonic bath for 20 min. Each extract was filtered through 0.45 µm filter into teflon vial and hydrolysed for 60 min at 100°C. Hydrolyzed extracts were immediately cooled to room temperature and analyzed by high performance liquid chromatography (HPLC). Analyses were carried out on Agilent series 1200 with DAD detector, on reverse phase Lichrospher RP-18 analytical column 250 x 4 mm i.d., particle size 5 µm (Agilent). Mobile phase were A (H_2O containing 10% HCOOH) and B (CH_3CN). Extracts were separated by gradient elution according to the following scheme: start B 1%, 1 to 4 min B 7%, 7.5 min B 10%, 11.5 to 15.5 B 14%, 18.5 to 22 min B 18%. Flow was adjusted to 1 ml/min and detection at 290, 350 and 520 nm. Quantification was done using calibration curves of authentic standards (Fluka, Germany). All experiments were done in triplicate. The results are expressed as mean value ± standard deviation.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The Mann-Whitney U test was applied using the program SPSS11.5 for Windows for comparison of data between groups. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Total phenolic content

Content of total phenolics was analyzed in all samples and it ranged from 3.92 to 5.24 mgGAE/g fw (Table 2). The difference between total phenolics content was statistically significant in regard to the type of habitats ($P < 0.01$) as well as to the altitude ($P < 0.01$) (Figure 2A).

Total anthocyanins content

The amounts of total anthocyanins varied between samples from 0.27 to 0.46% (Table 2) and it was shown that difference was statistically significant in regard to the altitude ($P < 0.01$) and type of habits ($P < 0.05$) (Figure 2B). Significant correlation between total phenolics and total anthocyanins was also noticed ($r = 0.843$, $P < 0.01$).

Content of anthocyanidins

Quantification of five characteristic anthocyanin

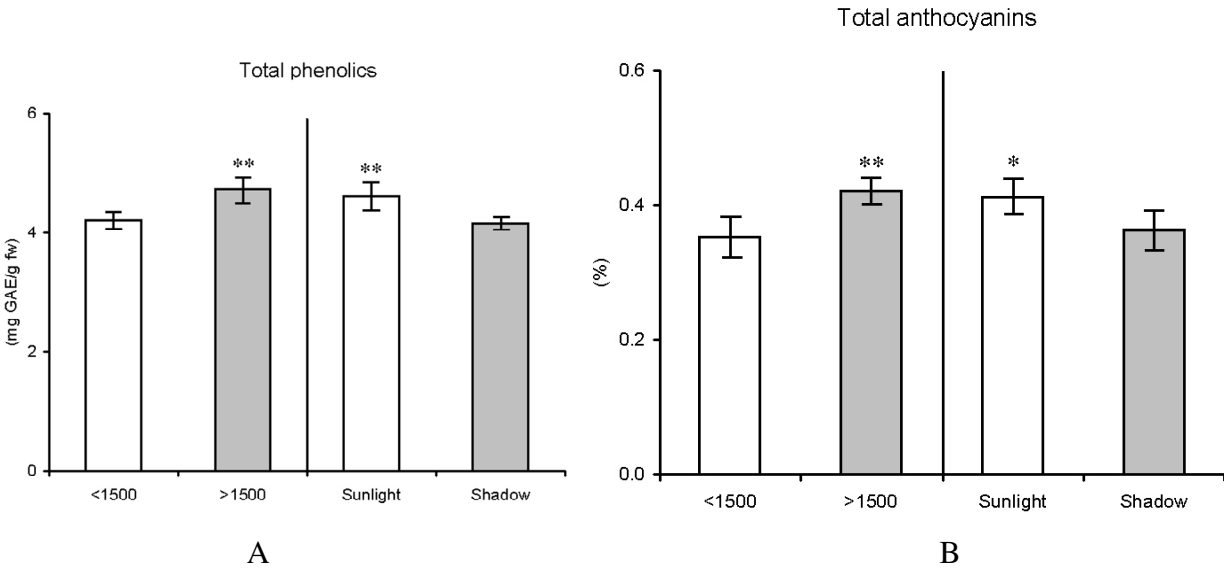

Figure 2. Content of total phenolics (mg GAE/g fw) (A), and content of total anthocyanins (%) in bilberries (B) measured in the samples collected from localities different in altitude and type of habitats (sunlight or shadow). Data are presented as mean ± standard deviation of triplicate measurement. *P < 0.05; **P < 0.01.

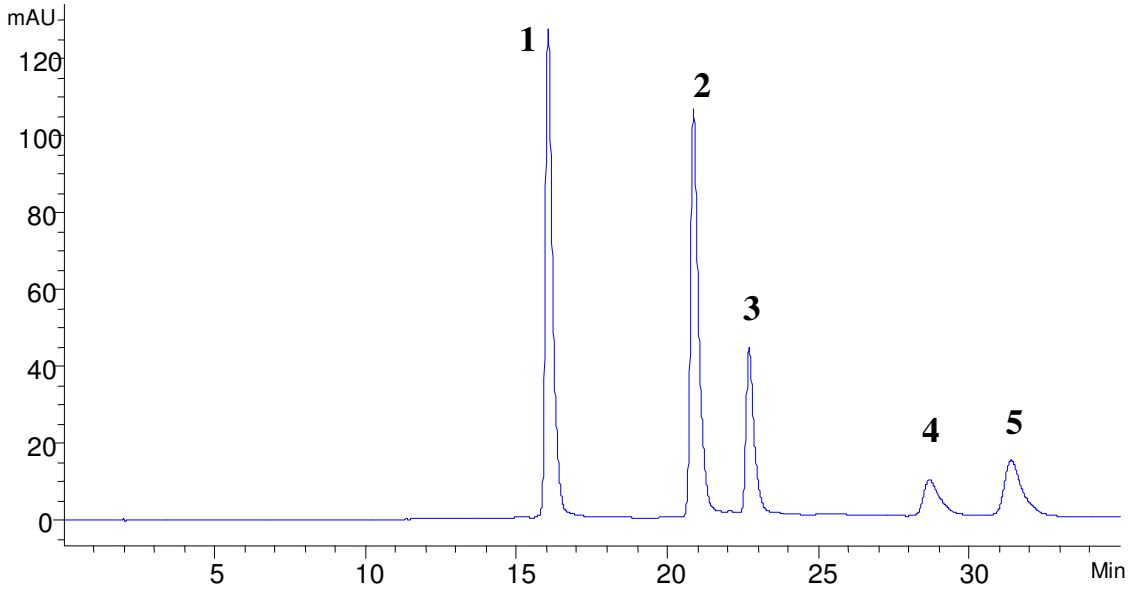

Figure 3. HPLC chromatogram of anthocyanin aglycones profile of bilberries. 1- delphinidin, 2-cyanidin, 3-petunidin, 4- peonidin, 5- malvidin.

aglycones that is cyanidin, delphinidin, peonidin, malvidin and petunidin, was done after acid hydrolysis and HPLC chromatogram is presented in Figure 3. The amounts of anthocyanin aglycones varied between samples (Table 2), delphinidin from 0.33 to 0.75 mg/g fw, cyanidin (0.25 to 0.75 mg/g fw), peonidin (0.05 to 0.18 mg/g fw), malvidin (0.11 to 0.52 mg/g fw) and petunidin (0.08 to 0.21 mg/g fw).

DISCUSSION

Berries are known as a rich source of phenolics (Macheix, 1990). Eleven bilberries samples were collected from localities different in altitude, exposition and type of habitat (sunny-H1 or shadow-H2) (Table 1) and the amount of phenolic compounds were analyzed. Total phenolic content was determined in all analyzed

samples and it ranged from 3.92 to 5.24 mgGAE/g fw (Table 2). Similar amount of total phenolics in our bilberry samples were detected as it was earlier shown by Prior et al. (1998). Higher amounts of total phenolics were detected in samples harvested from localities exposed to the sun in comparison with berries grown in shadow.

It has been reported earlier that light intensity, photoperiod and temperature influence the biosynthesis of many secondary metabolites (Hohtola, 2007). Jaakola et al. (2004) found that the concentration of anthocyanins, catechins, flavonols and hydroxycinnamic acids were higher in the bilberry leaves exposed to the direct sunlight. It was also noticed that at altitude higher than 1500 m, higher amounts of total phenolics was observed.

The highest amounts of total anthocyanins were noticed in samples B2 and B7 which were also the samples containing the highest amounts of total phenolics, while the lowest amount was found in B11. Fresh bilberry fruit is official in Ph. Eur. 6.0. which demands minimum 0.3% of anthocyanins, expressed as cyanidin-3-glucoside chloride. Among eleven analyzed samples, 10 corresponded to the prescriptions of Ph Eur 6.0 suggesting good quality of fresh bilberries from investigated area. Similarly to the total phenolics, higher amounts of total anthocyanins were detected in samples harvested from localities exposed to the sun in comparison with berries grown in shadow. Several reports have shown that anthocyanins are directly affected by exposition to the sunlight (Downey et al., 2006). Dokoozlian and Kliewer (1996) suggested that light exposure of the grapes fruit prior to pigment accumulation could increase the activity of enzymes responsible for anthocyanins synthesis. Also, it could be seen that total anthocyanins amount slightly increase with the increase of altitude. Quantification of five characteristic anthocyanin aglycones that is cyanidin, delphinidin, peonidin, malvidin and petunidin, was done after acid hydrolysis. Delphinidin was found to be the most abundant aglycone, followed by cyanidin. It is in accordance with the results of Määttä-Riihinen et al. (2004) and Latti et al. (2008) who also detected delphinidin and cyanidine as the most abundant anthocyanin aglycones.

In conclusion, the quality of tested samples was in accordance with official standard values for bilberries, thus enabling promotion of the idea of sustainable use of the natural resources. According to our knowledge, this potential is not sufficiently used. Through economic development and commercial use of available natural resources populations living in those regions could achieve a better standard of living.

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