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RESEARCH ARTICLE

Short Period Storage Impact on Bioactive Constituents from Bilberries and Blueberries

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Objectives: The aim of this study was to assess storage effects on anthocyanin and total polyphenol content in different bilberry and blueberry extracts and to evaluate the antioxidant and antibacterial activity of these extracts. **Materials and methods:** Total phenolic content, total monomeric anthocyanin content and antioxidant activity were determined in the first month and after three months storage of berries at either -20 °C or -50 °C. Two different solvents were used (methanol and 50% ethanol). Antibacterial activity was determined for the 3 months stored fruits using a microdilution method and was expressed as the minimum inhibitory concentration. **Results**: There were significant differences between the concentration in the first month and after three months storage in both types of fruit extracts. Regarding the extracting solvent, we noticed that total phenols were better extracted with 50% ethanol, while the total monomeric anthocyanin content was higher in the methanolic extracts. No significant or slightly significant differences were observed between the fruits stored at -20 °C or -50 °C. Ethanolic extracts showed the highest scavenging activity. Good antibacterial activity was observed on gram-positive bacteria. **Conclusions**: Storage conditions are an important factor that can influence chemical composition of fruits. Although freezing is a good option for preservation, our study showed a high decrease in the concentration of total phenols and anthocyanins after only three months. The fruits have shown a high antioxidant activity and a good antibacterial effect. Further studies are needed for better understanding the changes that can appear during the storage.

Keywords: blueberry, bilberry, storage conditions, antioxidants, antibacterial

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1. Introduction

Vaccinium berries are widely consumed worldwide because of their nutritional properties. As main components they contain polyphenolic compunds, among them polyphenolcarboxilic acids, flavonoids and anthocyanins, mainly in their glycosylated form. These berries have been studied for the last ten years because of their complex composition and multiple health-beneficial effects [1]. Researches conducted in different geographical areas have revealed great differences among berries collected from various areas, and also great differences have been noticed among different cultivars [2, 3]. This is why it is quite important to evaluate each regional fruit before carrying out any other determination. Bilberries are European wild berries collected from *Vaccinium myrtillus* L., they contain a bigger amount of anthocyanins comparative with the cultivated species. Blueberries are the fruits of a cultivated species, *Vaccinium corymbosum*, also called highbush blueberry. Researches conducted on the effects of storage conditions on active constituents from fruits are still unclear. There are many studies conducted on the storage conditions of different types of processed berries, but only few of them evaluated the changes during freezing of unprocessed berries [4, 5].

The aim of this study is to assess whether the freezing temperature influences the composition and the free radical scavenging activity of two types of fruits from *Vaccinium* species collected from Mureș county area, comparative with the composition and antioxidant activity determined on the freshly harvested fruits. Antibacterial activity on six bacterial strains was determined for the stored fruits.

2. Materials and methods

2.1. Plant material

Bilberries (*Vaccinium myrtillus* L.) were collected from their natural habitat in Lunca Bradului area, Mureș county, Romania. Blueberries (*Vaccinium corymbosum* L.) were collected from a culture near Trei Sate village area, Mureș county, Romania. The fruits were collected at their commercial harvest maturity according to ground color, in July 2016. After harvesting, the fruits from each species, were divided in three samples: the first sample was immediately prepared for analysis (VC 1; VM 1), the second and the third were stored for three months at either -20 °C (VC 3; VM 3), or at -50 °C (VCF 3; VMF 3).

2.2. Chemicals

Gallic acid, Folin-Ciocâlteu reagent, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) tablets were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH,

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Steinheim, Germany). Other chemicals used were of analytical grade.

2.3. Extraction procedure

Berries were crushed extemporaneously, and the crushed fruits were mixed with quartz sand (1:1 w/w) at room temperature for 5 minutes. This procedure was based on the sea sand disruption method (SSDM) [6]. The mixture was extracted with either methanol (MeOH) or 50% ethanol (EtOH) with a solid to solvent ratio of 1:10 (w/v, on a fresh weight basis). The extractions were carried out on an ultrasonic water bath at 40°C for 30 minutes, after which they were vortexed at high speed for 10 minutes and the extract was separated from the residue by filtration through a 0.45 μm filter, giving a number of 12 samples as they appear in Table I.

2.4. Phytochemical analysis

2.4.1 Total phenolic content

The total phenolic content (TPC) of the extracts has been measured spectrophotometrically according to a previously described protocol, using Folin-Ciocalteu reagent and gallic acid as reference. All samples were analyzed in three replicates. The results were expressed as mg gallic acid equivalents / 100 g fresh weight (R = 0,99714) [7,8].

2.4.2. Total monomeric anthocyanin content

Total monomeric anthocyanins (TMAC) were determined using the pH-differential method [9]. The extracts were diluted with 0.025 M potassium chloride (adjusted with HCl to pH 1.0) and 0.4 M sodium acetate (pH 4.5) and the absorbance was measured at 520 and 700 nm. The absorbance values were calculated as follows: $A = (A_{520} - A_{700})_{pH\ 1} - (A_{520} - A_{700})_{pH\ 4,5}$. TMAC was calculated with the formula:

$$TMAC = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

The results were expressed as mg cyanidin-3-glucoside equivalents / 100 g fresh weight, using the molecular weight (MW) of 449.2 for cyandin 3-glucoside, the dilution factor (DF), molar extinction coefficient (ε) of 26,900 L x mol^{-1} x cm^{-1} and the pathlength (1, in this case). All samples were analyzed in three replicates.

Table I. List of tested samples

	Sample	Solvent	Abbreviation
1.	Blueberries freshly harvested	50% Ethanol	VCE1
2.	Blueberries freshly harvested	Methanol	VCM1
3.	Blueberries stored at -20 °C	50% Ethanol	VCE3
4.	Blueberries stored at -20 °C	Methanol	VCM3
5.	Blueberries stored at -50 °C	50% Ethanol	VCEF3
6.	Blueberries stored at -50 °C	Methanol	VCMF3
7.	Bilberries freshly harvested	50% Ethanol	VME1
8.	Bilberries freshly harvested	Methanol	VMM1
9.	Bilberries stored at -20 °C	50% Ethanol	VME3
10.	Bilberries stored at -20 °C	Methanol	VMM3
11.	Bilberries stored at -50 °C	50% Ethanol	VMEF3
12.	Bilberries stored at -50 °C	Methanol	VMMF3

2.5. Antioxidant activity

2.5.1. DPPH Radical Scavenging Activity

The antiradical capacity of each sample was tested using a series of dilutions. Briefly, an aliquot of 150 μL of sample solution at different concentrations was mixed with 3000 μL DPPH solution (0,1 mmol/L in methanol) [10]. The reaction mixture was incubated for 30 minutes in the dark at room temperature and the absorbance was measured at 517 nm. The percentage of DPPH inhibition at different concentrations of each sample was plotted and the concentration of extract required to inhibit oxidation by 50% (IC_{50}) was obtained by interpolation. Results were expressed as mg/ml and all samples were analyzed in three replicates. Ascorbic acid was used as positive control.

2.5.2. Scavenging effect on ABTS• radical

The scavenging effect of all extracts against the radical cation $ABTS^{\bullet+}$ was determined according to a procedure described previously [11, 12]. Briefly, a stock solution of 2,45 mM potassium persulfate was prepared as well as an ABTS stock solution of 7 mM, which was left to react at room temperature in the dark for 12 hours. The ABTS solution was diluted with methanol to the appropriate absorbance. A volume of 100 μL for different concentrations of samples was added to 2500 μL diluted ABTS solution and then, the mixture was allowed to react in the dark at room temperature for 30 minutes and the absorbance was read at 734 nm. The controls contained the extraction solvent instead of the test sample. The percentage of ABTS inhibition at different concentrations of each sample was plotted and the concentration of extract required to inhibit oxidation by 50% (IC_{50}) was obtained by interpolation. Results were expressed as mg/ml and all samples were analyzed in three replicates.

2.6. Antibacterial activity

For this determination, ethanolic extracts of the fruits stored for three months, were used after a prior evaporation of the ethanol, at 40°C using an Ika rotary evaporator RV 05-ST with a vacuum controller, giving a final concentration of 200 mg fresh fruits/mL. The antibacterial activity of the extracts was tested on six bacterial strains: *Staphylococcus aureus* ATCC 25923, MRSA ATCC 43300, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853.

The antibacterial activity was determined by a microdilution method and the minimum inhibitory concentration (MIC) was interpreted as the last dilution without bacterial growth.

2.7. Statistical analysis

All the data were statistically evaluated and the significance of various treatments was calculated. The results were expressed as mean ± standard deviation (SD) and comparison between the groups was made by Analysis of variance (one-way ANOVA), followed by a Tukey-Kramer test, using GraphPad Prism (version 7.0b, 2016). Pearson's linear correlation was calculated using StatPlus:mac (statistical analysis program for Mac OS®, Version v6 AnalystSoft Inc.).

3. Results

The results of total phenolic content, total monomeric anthocyanin, DPPH free radical scavenging activity and ABTS scavenging activity are presented in Table II and Table III.

The highest total phenolic content was found in 50% ethanolic extracts for both bilberries and blueberries, but the difference between 50% EtOH and MeOH is not statistically significant. The highest total monomeric anthocyanin content was found in the methanolic extracts.

All extracts have shown a high scavenging activity, which decreases in the following order: VME1 > VMM1 > VME3 > VMEF3 > VMMF3 > VMM3 > VCE1 > VCM1 > VCE3; VCEF3 > VCM3 > VCMF3.

The susceptibility of bacteria against tested samples are presented in Table IV. Of the tested gram-negative microorganisms, bilberries were active on *Escherichia coli* and *Klebsiella pneumoniae*, while blueberries were active only on *Klebsiella pneumoniae*. *Pseudomonas aeruginosa* was not susceptible to any of the samples at the given concentration. Both berries had an inhibitory effect on all tested gram-positive microorganisms.

4. Discussion

As it is known, bilberries contain higher amounts of phenolic compounds than blueberries. Regarding the extracting solvent there can be observed that in both types of fruits there is no or just a small statistically significant difference in TPC between methanolic and ethanolic extracts, but there are significant differences in methanolic and ethanolic extracts regarding total monomeric anthocyanin content, which leads us to the conclusion that anthocyanins are better extracted with methanol. This is a long debate between different studies, some being in accordance with our results. This could be explained by the different chemical profile and concentration of phytoconstituents between fruits collected from different area or from different cultivars. Compared with freshly harvested fruits, the fruits that have been stored for three months have lost a great percent of compounds. Similar results have been reported by Giovanelli G. et al [13]. In case of further use of the extracts for animal or human studies, ethanolic extracts should be used due to the high toxicity of methanol.

Our results (determined per 100 g fresh weight) show a higher TP and TA content than in other reported data

Table IV. Assessment of MICs for bilberry and blueberry ethanolic extracts

Microorganisms	MIC (mg/ml)	
	Bilberry	Blueberry
Gram-positive		
Staphylococcus aureus	25	25
MRSA	25	50
Enterococcus faecalis	25	50
Gram-negative		
Escherichia coli	100	-
Klebsiella pneumoniae	50	100
Pseudomonas aeruginosa	-	-

Table II. Bioactive compounds and antioxidant activity of Vaccinium corymbosum

Sample	TPC mg GAE / 100 g FW	TMAC mg C3GE / 100g FW	DPPH IC50	ABTS IC50
VC-E1	427.32 ± 14.4^{a}	262.08 ± 14.29^{a}	1.47±0.12	0.139±0.002^{b}
VC-M1	392.73 ± 8.7^{a}	323.12 ± 11.58^{b}	1.85±0.01^{a}	0.207±0.003^{acd}
VC-E3	311.94 ± 4.8^{b}	170.46 ± 2.75^{c}	1.91±0.04^{a}	0.172±0.021^{abc}
VC-M3	251.26 ± 0.5^{c}	267.83 ± 13.2^{a}	1.99±0.09^{a}	0.216±0.02^{ad}
VC-EF3	343.27 ± 14.7^{b}	172.43 ± 13.77^{c}	1.91±0.06^{a}	0.153±0.008^{bc}
VC-MF3	306.27 ± 14.^{b}	226.66 ± 10.37^{a}	2.05±0.1^{a}	0.236±0.014^{d}

Means sharing the same superscript are not significantly different from each other (Tukey's HSD, P<0.05).

Table III. Bioactive compounds and antioxidant activity of Vaccinium myrtillus

Sample	TPC mg GAE / 100 g FW	TMAC mg C3GE / 100g FW	DPPH IC50	ABTS IC50
VM-E1	636.63 ± 7.45^{a}	520.81 ± 9.57^{a}	0.175±0.03^{a}	0.106±0.004^{a}
VM-M1	534.34 ± 3.17^{b}	576.86 ± 65.26^{a}	0.33±0.03^{a}	0.129±0.005^{ac}
VM-E3	484.87 ± 23.6^{c}	277.86 ± 26.73^{b}	1.03±0.08^{b}	0.138±0.003^{bc}
VM-M3	443.53 ± 13.7^{cd}	396.43 ± 8.94^{c}	1.19±0.01^{b}	0.161±0.002^{bd}
VM-EF3	465.98 ± 3.92^{cd}	293.37 ± 6.13^{b}	1.09±0.12^{b}	0.134±0.003^{c}
VM-MF3	426.32 ± 6.98^{d}	367.18 ± 10.89^{bc}	1.18±0.09^{b}	0.166±0.013^{d}

Means sharing the same superscript are not significantly different from each other (Tukey's HSD, P<0.05).

from Romanian bilberries [14]. The high difference between our results and the other results reported could be explained by the extraction using quartz sand. The extraction method using quartz sand also known as sea sand disruption method (SSDM) is a simple and cheap process, which due to the abrasive character of the sand, facilitates the extraction [15].

Other factors that could have influenced the chemical content are the storage period and the time of harvest. Our own results indicate that the content may decrease by almost 27% for TP and by 46% for TMA in only three months. It has been previously reported that increased maturity of fruits influences their chemical profile and their antioxidant activity [16]. Several researches concluded that the higher the content of anthocyanins in fruits, the higher the stability of the extract [17]. In the case of frozen fruits we recorded the higher lost of anthocyanins in the fruits with the highest content. The difference in the stability between extracts and unprocessed fruits could be explained by the antioxidant activity of the total polyphenols from the extract.

The concentration determined in our study for IC_{50} is lower than in other studies [18], which shows a higher antioxidant activity even in the fruits stored for 3 months. Using Pearson's correlation we noticed a highly negative correlation between DPPH IC_{50} and TPC ($r = -0,8247$, $p < 0,05$) in fruit extracts of *Vaccinium corymbosum*. For *Vaccinium myrtillus* fruit extracts, DPPH IC_{50} is highly correlated with TPC ($r = -0,9149$, $p < 0,05$) and TMAC ($r = -0,868$, $p < 0,05$) while for the ABTS IC_{50} the correlation coefficient shows a highly negative correlation with TPC ($r = -0,9474$, $p < 0,05$). The antioxidant activity of plant extracts containing phenolic compounds is attributed to their ability to donate hydrogen atoms or electrons, and therefore scavenging free radicals.

Regarding the microbiological study, a good antibacterial activity was observed on Gram-positive bacteria, with a higher activity for bilberry extracts, as it was expected. Previous studies have shown that phenolic compounds posses antibacterial activity and it is believed that these compounds suppress virulence factors of bacterial pathogens or have a direct antimicrobial effect [19, 20]. This antibacterial activity needs to be further tested with more concentrated extracts for a better evaluation, and also the bactericidal and bacteriostatic effect should be assessed.

5. Conclusions

This study shows important data regarding the impact of short time storage conditions on active constituents from *Vaccinium* berries. Our results demonstrated that freezing is not sufficient for anthocyanin preservation; other treatments could be needed to avoid degradation. Regarding the overall results we may conclude that both types of berries collected from our area contain a high amount of polyphenolic compounds, and, although the level of main active constituents decreased during the storage period, we could record a high antioxidant activity, and an antibacterial activity. These are important findings for the future use of our local fruits as nutraceuticals in prevention and treatment of different illnesses.

Conflict of interest

None to declare

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