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## NATURAL DRUGS

# COMPARATIVE STUDY OF ANTHOCYANIN COMPOSITION, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY IN BILBERRY (*VACCINIUM MYRTILLUS* L.) AND BLUEBERRY (*VACCINIUM CORYMBOSUM* L.) FRUITS

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**Abstract**: Simultaneous comparison of bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L) fruits for their anthocyanin composition, antimicrobial and antioxidant activity is reported. The aim of this study was to investigate and to compare anthocyanin composition, antimicrobial and antioxidant activity in bilberry and blueberry fruits and their skins. The investigations revealed that the highest amount of total anthocyanins was observed in fruits skins of blueberry cultivars. The results, obtained by chromatographic analysis, indicated that cyanidin is a dominant anthocyanidin in bilberry and malvidin in blueberry samples. Extracts of "Herbert", "Coville", "Toro" blueberry cultivars and bilberry fruits revealed antimicrobial properties. *Citrobacter freundii* (ATCC 8090) and *Enterococcus faecalis* (ATCC29212) were the most sensitive among eight tested Gram-negative and Gram-positive bacteria. Significant differences between berry and skin extracts were not established. Studies with fruits showed that the strongest antioxidant activity possesses blueberry cultivar "Berkeley" (82.13 ± 0.51%). Meanwhile, the amount of quenched free radicals in bilberry samples was 63.72 ± 1.11%, respectively. The lowest antioxidant activity was estimated in blueberry cultivar "Coville". Accordingly, the strongest antiradical properties were estimated in blueberry cultivar "Ama" fruit skins. Bilberry fruit skin samples possess strong antiradical activity as well (82.69 ± 0.37%).

**Keywords:** bilberries, blueberries, HPLC analysis, antioxidant, antimicrobial

Bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L., syn. *Vaccinium covilleanum* Butkus et Pliszka) are flowering plants belonging to the large genus of *Vaccinium*. Bilberry is one foot tall, thickly brached, has deciduous perennial shrub and is closely related to the blueberry (1). Differently from blueberry, *Vaccinium myrtillus* is native to Lithuanian forests (2).

These *Vaccinium* species (bilberry and blueberry) are noted for their high content of anthocyanin pigments. The constituents considered to be of primary importance in bilberry and blueberry are the anthocyanins. Anthocyanins, which belong to the flavonoid family, are natural water soluble pigments widely distributed in fruits and berries (3, 4). Anthocyanins are involved in a wide range of biological activities (5) that may affect positively the health. Many of the biological properties are closely associated with the antioxidant activity of anthocyanin pigments (6). These antioxidants help to neutralize free radicals (7-9) which are unstable molecules that are linked to the development of a number of degenerative diseases and conditions. Anthocyanins may reduce the risk of coronary heart disease (10), inhibit platelet aggregation (11) and protect arterial endothelial cells (12) In addition, these pigments could decrease the risk of cancer (13-15), reduce inflammatory insult (12) and modulate immune response (16).

Antimicrobial berry compounds, especially dietary flavonoids, may have important applications in the future as natural antimicrobial agents for the food industry as well as for the field of medicine (17). Among different berries and berry phenolics,

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cranberry, cloudberry, raspberry, strawberry and bilberry especially possess considerable antimicrobial effects against, e.g. *Salmonella* and *Staphylococcus* (18). Several mechanisms of action in the growth inhibition of bacteria are involved, such as destabilization of cytoplasmic membrane, permeabilization of plasma membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism and deprivation of the substrates required for microbial growth. Antimicrobial activity of berries may also be related to antiadherence of bacteria to epithelial cells, which is a prerequisite for colonization and infection of many pathogens. It was found that myricetin inhibited the growth of all lactic acid bacteria derived from the human gastrointestinal tract flora but did not affect the *Salmonella* strain (19). Extracts from common Finnish berries inhibited the growth of Gram-negative, but not Gram-positive bacteria. Other authors reported that there was no correlation between Gram-positive or Gram-negative bacterial status and susceptibility to the berries (20).

The aim of our study was to compare total anthocyanin and anthocyanidin content and their composition, antimicrobial and antioxidant activity in the bilberry and blueberry fruits and skins.

## MATERIALS AND METHODS

### Raw materials and extraction procedures

Wild bilberry (*Vaccinium myrtillus*) fruit samples were collected randomly in natural sampling sites in the territory of Lithuania and blueberry (*Vaccinium corymbosum*) were collected in the institute of botany in Vilnius. Fresh, ripe fruit samples were frozen and stored in the freezer at -19 ± 1°C until use.

Approximately 50 g of frozen berries were crushed in a pounder to produce a thick puree. A 5 g (accurate weight) aliquot was placed into a 100 mL flat-bottom flask and 95 mL of methanol was added. An ultrasonic bath BioSonic UC 100 (Coltene/Whaledent, Mahwah, NJ, USA) was used for sonication with occasional swirling. Ten min period of sonication was applied for extraction procedure (21). Three replicates of samples were prepared for all analytes.

### Chemicals

All solvents, reagents and standards, used in this study, were of analytical grade. Acetonitrile (HPLC grade) and orthophosphoric acid (85% purity, HPLC grade) were purchased from Sigma – Aldrich GmbH, (Buchs, Switzerland). The Folin-Ciocalteu reagent, sodium carbonate and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (95%) were purchased from Sigma-Aldrich Chemie GmbH, (Steinheim, Germany). Methanol was provided by Roth (Karlsruhe, Germany). Hydrochloric acid (35%) was purchased from Lachema (Neratovice, Czech Republic). Anthocyanidin standards (cyanidin chloride, delphinidin chloride, petunidin chloride, peonidin chloride and malvidin chloride) were of HPLC purity grade and purchased from Roth (Karlsruhe, Germany) and Chromadex (Santa Ana, USA). Water was deionized and filtered through a Millipore filter system (Millipore, USA) before use.

### Total phenolics measurement

The amount of total phenolics in the berry extracts was determined with the Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (22) using gallic acid as a standard. The reagent was prepared by diluting a stock solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with distilled water (1:10, v/v). Samples (1.0 mL, two replicates) were introduced into test cuvettes, and then 5.0 mL of Folin-Ciocalteu's phenol reagent, and 4.0 mL of $Na_2CO_3$ (7.5%) were added. The absorbance of all samples was measured at 765 nm using the Genesys10 UV/Vis spectrophotometer (Thermo Spectronic, Rochester, USA) after incubation at 20°C for 1.0 h.

### Total anthocyanins measurement

Total anthocyanin contents of berry extracts were measured using the spectrophotometric method. The samples were determined using a model Unicam Helios ALPHA spectrophotometer (Unicam, Cambridge, UK). The extract was filtrered through paper filter into a 100.0 mL volumetric flask and diluted with appropriate extractant to 100.0 mL. A 50-fold dilution of this solution in a 0.1% v/v of hydrochloric acid in methanol was prepared. The absorbance of the solution was measured at 528 nm wavelength, using a 0.1% v/v solution of hydrochloric acid in methanol as a blank. The results are expressed as a percentage of cyanidin 3-glucoside equivalents (23).

### Acid hydrolysis and chromatographic analysis

Twenty five mL of methanolic extract was mixed with 8.5 mL of concentrated hydrochloric acid. The mixture was heated under reflux condenser for 2 h. After acid hydrolysis, the anthocyanin content was converted to five major anthocyanidins. The hydrolyzed samples were passed through 0.22

μm pore size membrane filters (Carl Roth GmbH, Karlsruhe, Germany) prior to HPLC injection (24). Commercially available anthocyanidin standards were dissolved together in methanol to form a standard mixture of cyanidin chloride, delphinidin chloride, petunidin chloride, peonidin chloride, and malvidin chloride. The standard mixture was diluted in methanol to generate a five-point calibration curve for five anthocyanidin standard compounds separately. The peak areas of the anthocyanidins in fruit extracts were within the linear range of the calibration curves. The experiments were performed with Waters 2690 Alliance HPLC system, equipped with Waters 2487 Dual Absorbance Detector (UV/Vis), and Waters 996 Photodiode Array (PDA) Detector (Waters Corporation, Milford, MA, USA), coupled to a personal computer with Waters Millennium 2000w chromatographic manager system (Waters Corporation, Milford, MA, USA) for data storage and processing. Separation of anthocyanidins was carried out using a Supelco Hypersil ODS (C18) column (100 × 3 mm i.d.; particle size 3 mm), guarded with a Hypersil ODS (10 × 4.6 mm i.d.; particle size 5 mm) guard column (Supelco, Bellefonte USA). The column was kept at ambient temperature. The flow rate was kept constant at 1.0 mL/min for a total run time of 45 min. The mobile phases were: A – acetonitrile and B – 4% aqueous ortophosphoric acid (v/v). The system was run with a gradient program: 0–37 min, 7–25% A, 37–40 min, 25–7% A and 40–45 min 7% A. There was a 5 min post run at initial conditions for the column equilibration. The injection volume for all standard solutions and bilberry extracts was 10 μL. The UV/Vis detector was set at 520 nm, PDA 200-800 nm wavelength. Identification of anthocyanidins was achieved by comparing their retention times and UV-Vis spectra, obtained with a PDA detector, with those of the standards (21).

**Antimicrobial test**

Assessment of the antimicrobial activity of the extracts was performed on the following Gram-positive bacterial test cultures *Listeria monocytogenes* (ATCC 19117), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* ( ATCC 6633) (spores and vegetative cells), *Enterococcus faecalis* (ATCC 29212), and also on the Gram-negative bacterial test cultures *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Citrobacter freundii* (ATCC 8090), and *Salmonella typhimurium* (ATCC 14028). Eight strains of yeasts were also used: *Debaryomyces hansenii, Trichosporon cutaneum, Kluyveromyces marxianus var. lactis, Sacharomyces cerevisiae, Candida parapsilosis, Torulaspora delbrueckii, Pichia kluyveri,* and *Rhodotorula rubra*.

The antimicrobial properties were evaluated by the agar well diffusion method. The bacteria were grown in peptone-soy bouillon (LAB 04, LAB M) for 24 h at 37°C. After cultivation, test culture cells were mixed using a minishaker MS 1 (Wilmington. USA) and the cell suspensions were adjusted according to McFarland No. 0.5 standard (25). The suspension of bacteria cells was introduced into the dissolved media and cooled to 47°C; 20 mL was pipetted into a 90 mm diameter Petri plate. Wells eight-millimeters in diameter were pushed in the agar and filled with 50 μL of the ethanol extracts of berry or berry skin. The plates were incubated overnight at 37°C.

The yeasts were grown on a potato dextrose agar slant (LAB 98, LAB M) for 48 h at 25°C. After cultivation the yeast cells were washed with saline and the cell suspensions were treated as above. The media containing the yeast cells and oils were incubated overnight at 25°C. A 50 mL volume of ethanol extract of berry or berry cakes was added to the agar wells using an automatic pipettor. Ethanol was used as a control in blank samples for bacteria and yeast at the same conditions. Ceftazidime/clavulanic acid 30/10 μg sensi-disc (Becton, Dickinson and Company, USA) was used as positive control. After incubation the inhibition zones were measured with callipers to an accuracy of 0.1 mm and the effect was calculated as a mean of three replicate tests.

**Antioxidant activity assay**

The antioxidant activities of the extracts were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (26) to determine free radical-scavenging potential of each sample. The antiradical activity was calculated as a percentage of DPPH decoloration *versus* control (methanolic solution). Fifty μL of prepared extract was added to 2 mL of DPPH methanolic solution ($6 \times 10^{-5}$ M). The reaction was carried out at ambient temperature. Changes in the absorbance at 515 nm due to the scavenging of DPPH radical were measured with a spectrophotometer every 10 s for a period of 30 min, to attain reaction equilibrium (27). Antioxidant activity of berry extracts was expressed by percentage of inactivated DPPH reagent (28):

$$DDPH = \frac{A_b - A_a}{A_b} \times 100$$

where Ab = absorbtion of blank aliquot (t = 0 min.), Aa = absorbtion of solution with analyzed extract (after 30 min).

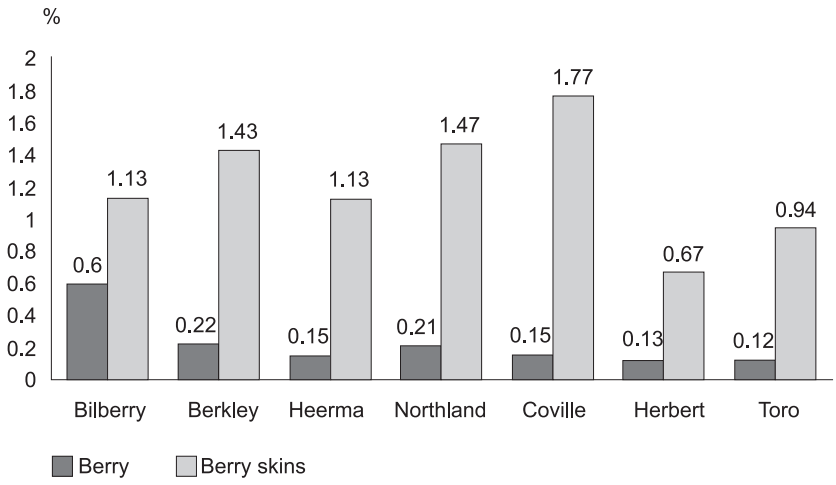


Figure 1. Total anthocyanins content in bilberry and blueberry cultivars, berry and berry skins

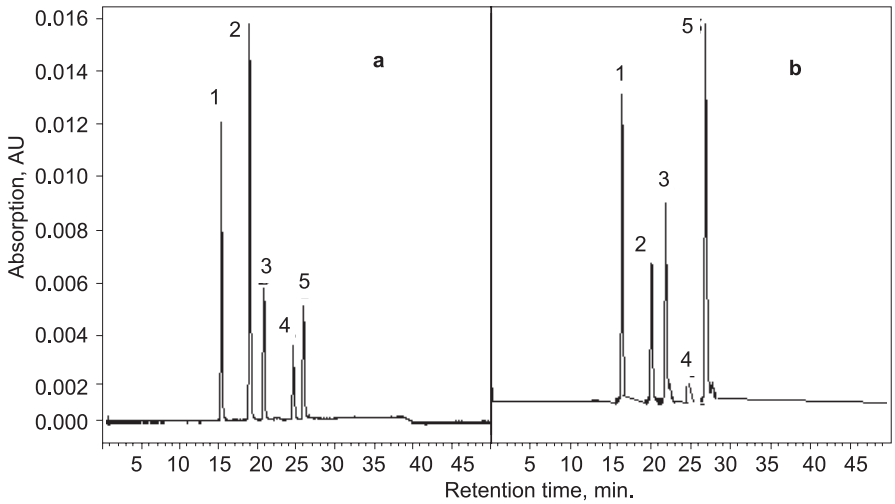


Figure 2. Representative chromatograms of anthocyanidins, a – bilberry, b – blueberry, 1 – delphinidin, 2 – cyanidin, 3 – petunidin, 4 – peonidin, 5 – malvidin.

**Statistical data analysis**

All of the assays were performed in triplicate. The mean values and standard deviations were obtained using the SPSS (Chicago, JAV) computer package.

## RESULTS AND DISCUSSION

**Anthocyanin and anthocyanidin composition**

For the quantification of total anthocyanins content in bilberry and blueberry fruits, the spectrophotometric assay, descripted in the European Pharmacopoeia, was performed. Spectrophotometric method is widely used for standardization of natural raw material. Total anthocyanin content in bilberry and blueberry fruits and their skins is presented in Figure 1. The highest amount of anthocyanins was observed in fruits skins of blueberry cultivars. The highest content of anthocyanins in blueberry skins was found in "Coville" cultivar skins (1.77%), the lowest – in "Toro" cultivar fruits skins. Though, identical amounts of total anthocyanins were determined in "Heerma" cultivar and bilberry skins samples (1.13 %). However the high-

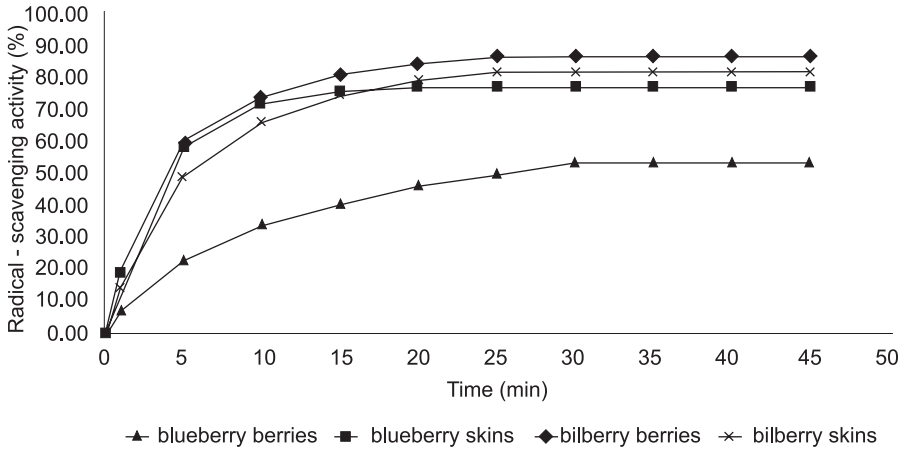


Figure 3. Radical scavenging activity dependence on time

Table 1. Anthocyanidin contents in bilberry and blueberry cultivars berry

| Sample raw material | Anthocyanidin contents in berry (%) | | | | | |
|---|---|---|---|---|---|---|
| | Delphinidin | Cyanidin | Petunidin | Peonidin | Malvidin | Sum |
| Bilberry | 0.12 | 0.25 | 0.10 | 0.05 | 0.06 | 0.58 |
| Berkeley | 0.04 | 0.04 | 0.05 | 0.01 | 0.05 | 0.17 |
| Heerma | 0.02 | 0.01 | 0.03 | 0.002 | 0.02 | 0.09 |
| Northland | 0.05 | 0.02 | 0.06 | 0.01 | 0.07 | 0.20 |
| Coville | 0.01 | 0.01 | 0.02 | 0.002 | 0.04 | 0.08 |
| Herbert | 0.01 | 0.002 | 0.02 | 0.002 | 0.03 | 0.07 |
| Toro | 0.03 | 0.01 | 0.04 | 0.002 | 0.05 | 0.13 |
| Mean | 0.04 | 0.05 | 0.05 | 0.01 | 0.05 | |
| Min. | 0.01 | 0.002 | 0.02 | 0.002 | 0.02 | |
| Max. | 0.12 | 0.25 | 0.10 | 0.05 | 0.07 | |
| SD | 0.03 | 0.08 | 0.02 | 0.02 | 0.02 | |

est content of anthocyanins in fruits was found in bilberry fruits (0.6%) compared with blueberry fruits. The higher amount of anthocyanins in blueberry fruits was found in "Berkeley" and "Northland" cultivars berries. The lowest one – in "Toro" cultivar berries (0.12%).

Acid mediated hydrolysis was performed and five major anthocyanidins (delphinidin, cyanidin, petunidin, peonidin and malvidin) were estimated by high performance liquid chromatography (HPLC) in bilberry and blueberry extracts, respectively. Numerous techniques are used for separation and quantification of anthocyanins. HPLC is a method of choice for the analysis of anthocyanins for it ensures efficient and simple identification, separation and quantification of individual anthocyanidins in bilberry and blueberry fruits and their products. The developed HPLC method, used in this study was optimized, validated and published previously (21). Representative HPLC chromatograms of bilberry and blueberry extracts are shown in Figure 2**.** Chromatographic analysis revealed that qualitative composition of anthocyanidins in bilberry and blueberry fruits and their skins is homogeneous. Quantitative results obtained for the amount of individual anthocyanidins in bilberry and blueberry fruits are presented in Table 1 and those in fruit skins in Table 2. The HPLC method was applied and variation of anthocyanidin composition in bilberry and blueberry extracts was estimated. The obtained

Table 2. Anthocyanidin contens in bilberry and blueberry cultivars berry skins

| Sample raw material | Anthocyanidin contents in berry (%) | | | | | |
|---|---|---|---|---|---|---|
| | Delphinidin | Cyanidin | Petunidin | Peonidin | Malvidin | Sum |
| Bilberry | 0.23 | 0.49 | 0.18 | 0.09 | 0.10 | 1.09 |
| Berkeley | 0.24 | 0.24 | 0.31 | 0.07 | 0.37 | 1.24 |
| Heerma | 0.24 | 0.24 | 0.31 | 0.07 | 0.37 | 1.24 |
| Northland | 0.24 | 0.24 | 0.31 | 0.07 | 0.37 | 1.24 |
| Coville | 0.24 | 0.24 | 0.31 | 0.07 | 0.37 | 1.24 |
| Herbert | 0.11 | 0.04 | 0.17 | 0.01 | 0.30 | 0.63 |
| Toro | 0.23 | 0.12 | 0.12 | 0.03 | 0.48 | 0.99 |
| Mean | 0.29 | 0.18 | 0.31 | 0.05 | 0.42 | |
| Min. | 0.11 | 0.04 | 0.12 | 0.01 | 0.10 | |
| Max. | 0.49 | 0.49 | 0.59 | 0.09 | 0.77 | |
| SD | 0.11 | 0.13 | 0.15 | 0.02 | 0.19 | |

Table 3. The antimicrobial influence of 50 mL berry extracts on Gram-negative test cultures

| Sample raw material | Inhibition zone size, mm | | | |
|---|---|---|---|---|
| | *E. coli* | *P. aeruginosa* | *S. typhimurium* | *C. freundii* |
| "Herbert" berry | 13.66 ± 0.47 | 14.00 ± 0.00 | 20.00 ± 0.00 | 21.66 ± 1.24 |
| "Coville" berry | 14.66 ± 0.47 | 12.66 ± 0.47 | 18.00 ± 0.00 | 21.66 ± 0.47 |
| "Toro" berry | 15.66 ± 0.47 | 12.66 ± 0.47 | 15.00 ± 0.00 | 21.66 ± 0.94 |
| "Herbert" skin | 13.66 ± 0.47 | 14.66 ± 0.47 | 16.33 ± 0.47 | 22.66 ± 0.47 |
| "Coville" skin | 13.33 ± 0.47 | 13.00 ± 0.00 | 16.00 ± 0.00 | 23.00 ± 0.00 |
| "Toro" skin | 13.66 ± 0.47 | 14.33 ± 0.47 | 14.00 ± 0.00 | 25.00 ± 0.00 |
| Bilberry skin | 17.33 ± 0.47 | 14.66 ± 1.90 | 11.00 ± 0.01 | Not investigated |
| Bilberry berry | 15.39 ± 1.24 | 12.30 ± 0.40 | 13.33 ± 1.24 | Not investigated |
| Ceftazidime/ Clavulanic acid 30/10 µg | 38.0 ± 0.00 | 36.10 ± 0.47 | 34.30 ± 0.06 | 34.00 ± 0.00 |

results indicated that cyanidin is a dominant anthocyanidin in bilberry and malvidin in blueberry samples. It was determined that bigger amounts of anthocyanidins are accumulated in skins of blueberry and bilberry fruits than in entire fruits. The biggest amounts of anthocyanidins were estimated in blueberry cultivars skins, where total athocyanidins reached 2% ("Coville" cultivar), whereas the smallest amount of total anthocyanidins was determined in "Herbert" cultivar skins (0.63%), however, differently from skins, the biggest amounts of anthocyanidins were found in bilberry fruit samples. The amounts of total anthocyanidins in bilberry fruits exceeded 0.5% and the smallest amounts of total anthocyanidins were assessed in blueberry "Herbert" cultivar (0.07%). Variation of individual anthocyanidin composition in bilberry and blueberry cultivar fruits and fruit skins was evaluated as well. It was found that the biggest amounts of individual anthocyanidins (delphinidin, cianidin, petunidin, peonidin) are accumulated in bilberry fruits, except of malvidin. The biggest amounts of malvidin were estimated in blueberry "Northland" cultivar fruits (0.07%).

The highest content of delphinidin, petunidin and malvidin (0.49%, 0.59% and 0.77%, respectively) were determined in "Coville" cultivar fruit skins, however, the biggest amounts of cyanidin and peoni-

Table 4. The antimicrobial influence of 50 mL blueberry extracts on Gram-positive test cultures

| Sample raw material | Inhibition zone size, mm | | | | |
|---|---|---|---|---|---|
| | *B. subtilis* vegetative cells | *B. subtilis* spores | *L. monocytogenes* | *S. aureus* | *E. faecalis* |
| 'Herbert' berry | 17.33 ± 0.47 | 17.66 ± 0.47 | 19.00 ± 0.00 | 18.66 ± 0.47 | 26.00 ± 0.00 |
| 'Coville' berry | 15.00 ± 0.00 | 13.66 ± 0.47 | 17.66 ± 0.47 | 18.00 ± 0.81 | 26.00 ± 0.00 |
| 'Toro' berry | 19.33 ± 0.47 | 14.33 ± 0.47 | 20.33 ± 0.47 | 17.00 ± 0.00 | 30.66 ± 0.94 |
| 'Herbert' skin | 17.00 ± 0.00 | 13.33 ± 0.47 | 18.00 ± 0.00 | 15.66 ± 0.47 | 22.66 ± 0.47 |
| 'Coville' skin | 14.66 ± 0.47 | 13.33 ± 0.47 | 19.00 ± 0.00 | 16.00 ± 0.00 | 23.00 ± 0.00 |
| 'Toro' skin | 17.66 ± 0.47 | 14.00 ± 0.00 | 22.33 ± 0.47 | 17.66 ± 0.47 | 25.00 ± 0.00 |
| Bilberry skin | 18.00 ± 0.00 | 12.00 ± 0.00 | 15.00 ± 0.00 | 13.00 ± 0.00 | Not investigated |
| Bilberry berry | 18.00 ± 0.00 | 14.66 ± 0.47 | 18.00 ± 1.60 | 15.00 ± 0.81 | Not investigated |
| Ceftazidime/ Clavulanic acid 30/10µg | 31.00 ± 0.00 | 31.00 ± 0.00 | 32.00±0.00 | 27.00 ± 0.00 | 36.00 ± 0.00 |

Table 5. The antimicrobial influence of 50 mL blueberry extracts on yeast test cultures

| Sample raw material | Inhibition zone size, mm | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Debaryomyces hansenii* | *Trichosporon cataneum* | *Kliuveromyces marxianus var. lactis* | *Torulaspora delbrueckii* | *Saccharomyces cerevisiae* | *Candida parapsilosis* | *Pichia kluyveri* | *Rhodotorula rubra* |
| "Herbert" berry | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| "Coville" berry | 0 | 0 | 0 | 9.33 ± 0.47 | 10.00 ± 0.0 | 0 | 0 | 0 |
| "Toro" berry | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| "Herbert" skin | 0 | 0 | 0 | 13.33 ± 0.47 | 17.00 ± 0.00 | 0 | 0 | 0 |
| "Coville" skin | 0 | 0 | 0 | 13.00 ± 0.00 | 17.00 ± 0.00 | 0 | 0 | 0 |
| "Toro" skin | 0 | 0 | 0 | 10.33 ± 0.81 | 16.33 ± 0.81 | 0 | 0 | 0 |
| Bilberry skin | 0 | 0 | 0 | 13.00 ± 0.00 | 11.50 ± 0.07 | 12.00 ± 0.47 | 13.00 ± 0.00 | 0 |
| Bilberry berry | 0 | 0 | 0 | 11.05 ± 0.33 | 15.00 ± 0.00 | 9.66 ± 0.47 | 9.66 ± 0.47 | 0 |

din were assayed in bilberry skin samples. The lowest content of all individual anthocyanidins was determined in "Herbert" cultivar fruit skins.

**Antimicrobial assay**

Investigation of antimicrobial properties of berry extracts by the diffusion to agar method showed that among Gram-negative test cultures *C. freundii* were the most sensitive – average zones of inhibition were 21.66 mm for skin, and 23.55 mm for berry extracts (Table 3). *E. coli* showed largest resistance to berry extracts; the average zone of inhibition was 13.6 mm for all investigated samples. Though phenolic compounds and anthocyanin content was higher in the extracts of skin, significant differences between extracts of berries and berry

Table 6. Antioxidant activity of bilberry and blueberry cultivars

| | Radical – scavenging activity (%) | |
|---|---|---|
| | Berry | Berry skins |
| Bilberry | 63.72 ± 1.11 | 82.69 ± 0.37 |
| Blueberry cultivars | | |
| Berkeley | 82.13 ± 0.51 | 87.25 ± 0.23 |
| Heerma | 50.40 ± 0.57 | 92.10 ± 1.10 |
| Ama | 81.65 ± 0.68 | 93.15 ± 0.11 |
| Northland | 53.34 ± 0.72 | 76.68 ± 3.15 |
| Coville | 51.30 ± 0.72 | 59.88 ± 0.45 |
| Herbert | 76.22 ± 0.36 | 82.19 ± 0.34 |
| Toro | 71.27 ± 0.23 | 77.73 ± 0.24 |

Each value is the mean ± standart devation of 3 replicate assays

skin were not established. All investigated cultivars showed the same antimicrobial activity. The extracts of bilberry and bilberry skin had the strongest influence on *E. coli*. Unfortunately, the antimicrobial influence to *C. freundii* was not investigated.

The ethanol extracts of berry and berry skins showed inhibitory effects on growing of Gram-positive test cultures *L. monocytogenes* and *B. subtilis* (Table 4). More sensitive was *L. monocytogenes* test culture. After comparison of antimicrobial influence on spores and vegetative cells, it was found that *B. subtilis* vegetative cells were more sensitive and made large inhibition zones (16.83 mm). When spores graduate to the vegetative form or during germination process, *B. subtilis* spores made transparent inhibitory zones with the average diameter of 14.38 mm. The test cultures in coccus form showed sensitivity to the blueberry extracts of investigated cultivars: *S. aureus* and *E. faecalis* made large inhibition zone in diameter 15.66 – 18.66 mm and 22.66 – 30.66 mm, respectively. The differences between extracts of the investigated blueberry cultivars and bilberry were not established. All investigated test cultures were more sensitive than the ceftazidime/clavulanic acid 30/10 µg sensi-disc.

The eight yeast species *Debaryomyces hansenii, Trichosporon cutaneum, Kluyveromyces marxianus var. Laktis, Sacharomyces cerevisiae, Candida parapsilosis, Torulaspora delbrueckii, Pichia kluyveri,* and *Rhodotorula rubra* showed complete resistance to the blueberry extracts (Table 5). Most of inhibition zones were 0.00 mm. Minimal sensitivity was demonstrated only by *Torulaspora delbrueckii* and *Saccharomyces cerevisiae*. Similar results were obtained after investigation of black currant, cranberry and bilberry extracts (29). Minimal sensitivity showed only the bilberry extract. Such resistance is explained by the wide occurrence of yeasts on the plants and berries.

**Antioxidant activity**

Anthocyanins are efficient free radical scavengers, due to their ability to inactivate free radicals. Photometric DPPH bleaching method was applied for measuring radical scavenging activity of bilberry and blueberry extracts. This method is unsophisticated, cheap and sufficiently accurate and shows the radical scavenging activities of berry extracts, contributed by both anthocyanins and other compounds. However, as it is well known and confirmed by our study, berries contain a large amount of phenolic compounds (Table 3), which has antioxidant activity as well. Since the blueberry and bilberry extracts are very complex, it is difficult to distinguish which compounds contribute the most to antioxidant activity. The bilberry and blueberry fruits, besides anthocyanins, are rich in flavonoids (catechin, epikatechin, myrcetin, quercetin, and kempferol), phenolic acids, chlorogenic acid and ascorbic acid, which possess antiradical properties as well (30, 31).

Anthocyanins and other polyphenolics, major contributors to antioxidant activity in berries, have similar patterns of behavior in response to time. The DPPH radical inactivation dependence from time was evaluated. With an increase of time the amount of quenched free radical was increased proportionally. It was found that equilibrium, when amount of inactivated free radical is stable, was attained after 30 min. The analysis was carried out with bilberry and blueberry fruits and fruit skins. The free radical inactivation dependence on time is presented in Figure 3. The antioxidant activity was assayed for bilberry and blueberry (“Ama”, “Berkeley”,

"Coville", "Heerma", "Herbert", "Toro" cultivars) fruits and fruit skins. On the basis of the obtained data, bilberry and blueberry skins were distinguished by stronger antioxidant activity. This may be due to the estimated higher content of anthocyanins and phenolic compounds in the investigated samples. Our studies with fruits revealed that the strongest antioxidant activity possess blueberry cultivar "Berkeley", in which the amount of quenched free radicals was 82.13 ± 0.51%. Meanwhile, the amount of quenched free radicals in bilberry samples was 63.72 ± 1.11%, respectively. The lowest antioxidant activity was estimated in blueberry cultivar "Coville" (51.30 ± 0.72%). Accordingly, the strongest antiradical properties were estimated in blueberry cultivar "Ama" fruit skins where the amount of quenched free radical was 93.15 ± 0.11%. The bilberry fruit samples possess strong antiradical activity as well (82.69 ± 0.37%). The lowest antioxidant activity was found in blueberry cultivar "Coville" fruit skins (59.88 ± 0.45%). The amounts (in percentage) of the inactivated DPPH radical are presented in Table 6.

## CONCLUSIONS

Our study revealed that the highest amount of total anthocyanins was observed in fruits skins of blueberry cultivars. The highest content of anthocyanins in blueberry skins was found in "Coville" cultivar skins (1.77%), the lowest – in "Toro" cultivar fruits skins.

Chromatographic results indicated that cyanidin is a dominant anthocyanidin in bilberry and malvidin in blueberry samples. It was determined that bigger amounts of anthocyanidins are accumulated in skins of blueberry and bilberry fruits than in entire fruits.

The extracts of "Herbert", "Coville", "Toro" blueberry cultivars and bilberry revealed antimicrobial properties. *Citrobacter freundii* (ATCC 8090) and *Enterococcus faecalis* (ATCC29212) were the most sensitive among eight tested Gram-negative and Gram-positive bacteria: inhibitory zones were 22.6 and 25.6 mm, respectively. Significant differences between berry and skin extracts were not established. The eight yeast species showed resistance to the blueberry and bilberry extracts.

Our studies with fruits revealed that the strongest antioxidant activity posseses blueberry cultivar "Berkeley" (82.13 ± 0.51%). Meanwhile, the amount of quenched free radical in bilberry samples was 63.72 ± 1.11%, respectively. The lowest antioxidant activity was estimated in blueberry cultivar "Coville" whereas the strongest antradical properties were estimated in blueberry cultivar "Ama" fruit skins. The amount of quenched free radical was 93.15 ± 0.11%. The bilberry fruit skin samples possess strong antiradical activity as well (82.69 ± 0.37%).

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