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Phenolics and anthocyanins in berries of European cranberry and their antimicrobial activity

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Key words: *anthocyanins; berry weight; clone; phenolic compounds.*

Summary. *European cranberry is a berry plant rich in biologically active substances, making it valued by both the phyto-pharmaceutical and food industries. The aim of this study was to examine the accumulation of phenolic compounds and anthocyanins in berries of European cranberry and to assess their antibacterial activity.*

Material and methods. Different wild clones of European cranberry were investigated according to berry weight and the amounts of total phenolics and anthocyanins. Anthocyanin profiles of extracts were evaluated by HPLC, whereas the antimicrobial properties were determined by the agar well diffusion method. A strong negative correlation between berry weight and the amount of anthocyanins was found. The amount of total phenolics among different cranberry clones in the field collection ranged from 224.0 mg/100 g to 498.0 mg/100 g, and the amount of total anthocyanins ranged from 40.7 mg/100 g to 207.3 mg/100 g. Quantitative HPLC-UV analysis revealed six anthocyanins in the berries of European cranberry, among which the anthocyanin peonidin-3-galactoside was most prevalent.

Conclusions. Investigation of the antimicrobial properties showed that European cranberry extracts inhibited the growth of wide range of human pathogenic bacteria, both gram-negative (Escherichia coli and Salmonella typhimurium) and gram-positive (Enterococcus faecalis, Listeria monocytogenes, Staphylococcus aureus, and Bacillus subtilis).

Introduction

The European cranberry *Vaccinium oxycoccos* L. is a common species in the oligotrophic and mesotrophic bogs. The largest *V. oxycoccos* fields of Lithuania are located in the strictly protected areas Žuvintas, Čepkeliai, and Kamanos. Sweet-and-sour berries ripen under Lithuanian climate conditions from the middle of August until the end of September (1). The unique collection of genetic resources of this species was established at Kaunas Botanical Garden of Vytautas Magnus University with the purpose to preserve valuable clones selected in the strictly protected areas Čepkeliai, Žuvintas, Kamanos, and in other bogs as well.

The consumption of these valuable berries is a long-lasting tradition in Lithuania, and *V. oxycoccos* was much appreciated in folk medicine as well. An increased interest in fruit with large amounts of biologically active substances has led to biochemical evaluations of cranberry. For example, the latest evaluations of the closely related American cranberry species *Vaccinium macrocarpon* Aiton have revealed a very valuable biochemical composition of these berries. Phenolic compounds in cranberries are a diverse group that includes anthocyanins, flavonoids, proanthocyanidins, and phenolic acids. These compounds have been identified as strong antioxidants, with the potential to prevent oxidative damage and protect against cardiovascular diseases and some cancers (2–5). Phenolic phytochemicals are secondary metabolites distinguished for their ability to protect plants against biological and environmental stresses, such as fungal or bacterial infections (6). The red color of cranberry fruit is also due to the presence of anthocyanins. The anti-inflammatory and antiulcer properties of these compounds are particularly important (7, 8).

In recent years, investigations of bioactive berry compounds have increased considerably. Different

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studies revealed that phenolic compounds of raspberry, blueberry, cloudberry, and American cranberry inhibit the growth of human pathogenic bacteria, such as *Salmonella*, *Staphylococcus*, and *Helicobacter* (9–11). An interest in the composition of European cranberry berries has been intensified because of the increased awareness of their possible positive health effects.

The aim of the present study was to examine the accumulation of phenolic compounds and anthocyanins in berries of European cranberry and to assess their antibacterial activity.

Material and methods

Plant material. The material for the evaluations was collected in the strictly protected reserve Žuvintas from 1996 to 1999 and other mesotrophic bogs in eastern Lithuania in 1995, where a great morphological variation had been noticed. The collected runners of 21 clones, conspicuous in different shape and berry size, were planted in the experimental plot of the Kaunas Botanical Garden of Vytautas Magnus University, were raised under the same growth conditions, and then were evaluated. Clones were transplanted to the field to avoid the influence of various ecological factors in natural habitats.

Morphological characterization. Berry weight and leaf area were quantified. The average weight of a berry was calculated by weighing 50 berries in three replications. The mean area of a leaf was determined by scanning 30 leaves with a scanner (HP Scan Jet 3600) in three replications and applying two noise removal filters. The image threshold was set with user-chosen values, and the amount of dark pixels representing the actual area of leaves was counted.

Reagents. Reagents used to estimate the amounts of total anthocyanins and phenolics were of analytical grade. Purified cyanidin-3-rutinoside was donated by the Danish Institute of Agricultural Science (Department of Fruit, Vegetable and Food Science, Tjele, Denmark). A gallic acid standard was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Biochemical evaluation. Berry samples used to evaluate the amounts of total phenolics and anthocyanins were gathered during the stage of mass ripening.

Analysis of total phenolic compounds. The amount of total phenolics in the cranberry extracts was determined with the Folin-Ciocalteu reagent according to the method of Slinkard and Singleton, using gallic acid as a standard (12). 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 h. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 grams of fresh weight.

Analysis of anthocyanins. Pigments were extracted from 5 g of frozen berries with acidified (0.1 N HCl, v/v), 95% (v/v) food-grade ethanol (13). The berries were ground with quartz sand, and the extraction was continued with 20-mL portions of solvent until the sample became colorless. The extract was diluted with acidified ethanol. The absorption was measured on a spectrophotometer Genesys-5 (Thermo Spectronic, Rochester, USA) at 544 nm. The concentration of anthocyanins was determined from the calibration curve, which was constructed by measuring the absorption of cyanidin-3-rutinoside (MW 595.2, ε=28.800) reference solutions. The concentration of anthocyanins (C) was calculated using the following formula and expressed in milligrams of cyanidin-3-rutinoside in 100 g of berries:

$$C = \frac{c \times V \times k}{m \times 10},$$

where c is the concentration of anthocyanins in mg/L obtained from the calibration curve, V is the volume of the extract in mL, k is the dilution factor, and m is the amount of berries used for the extraction in g.

HPLC-UV/MS analysis of anthocyanins. Anthocyanin profiles of extracts were characterized by HPLC using a reversed-phase C_{18} LiChrospher®100 RP 18e column (125×4 mm, 5 μm) (Merck, Darmstadt, Germany). The eluents were (A) 4% H_3PO_4 in water and (B) 100% HPLC-grade acetonitrile (Merck, Darmstadt, Germany). Chromatographic conditions were as follows: 10% B in A at the time of injection (20 μL), 14% B in A (4 min), 16% B in A (10 min), 30% B in A (25 min), initial conditions (26 min). Flow rate was 0.8 mL/min, and 20 μL was injected. Samples were filtered through a 0.45-μm cellulose syringe filter before analysis. Detection was performed using a UV detection system, L-7400 LaChrom Merck Hitachi (Merck KGaA, Darmstadt, Germany), at 520 nm.

The elution sequence of anthocyanins was confirmed by the HPLC-MS method. Mass spectra were registered by a Hewlett Packard 1100 MS (Agilent

Technologies), operating in nitrogen flow at atmospheric pressure, and by applying electrical ionization. The voltage in the capillary was 4500 V; the voltage of fragmentation was 100 V. The temperature was 250°C. The scanning range was 100–1000 m/z with an interval of 0.1 m/z.

Assessment of the antimicrobial activity. Antimicrobial activity of the extracts was performed on the following gram-positive bacterial test cultures: *Listeria monocytogenes* (ATCC 19117), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), and also on the gram-negative bacterial test cultures *Escherichia coli* (ATCC25922), *Salmonella typhimurium* (ATCC 14028). The antimicrobial properties were evaluated by the agar well diffusion method. Bacteria were grown in peptone-soy bouillon (LAB 04, LAB M) for 24 hours 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 (14). 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 8 mm in diameter were pushed in the agar and filled with 50 μL of the ethanol extracts of berries. The plates were incubated overnight at 37˚C.

Data analysis. The data were subjected to multivariate statistical analysis. Differences among the cranberry clones were tested by one-way analysis of variance (ANOVA).

Results and discussion

Morphological variability. Significant differences among clones of *V. oxycoccos* were ascertained in calculating the mean values of berry weight and leaf area (Table 1).

A high positive correlation between leaf area and berry weight was found with a coefficient of correlation of r=0.812. A regression analysis revealed that the interrelationship between leaf area and berry weight could be best expressed by the exponential equation y(x)=0.26708·e^ 3.18506x, with the coefficient of regression $R^2_{x/y}$=0.702.

Variability of phenolic and anthocyanin amounts. The differences in total phenolic and anthocyanin amounts among clones of European cranberry were ascertained. The clones accumulated from 224.1 mg/100 g to 498.2 mg/100 g of phenolic compounds. The most valuable clones V416, V411, V425, V407, and V413, which accumulated 498.2, 481.8, 485.9, 431.5, and 428.6 mg/100 g of phenolic compounds, respectively, were selected. Clone V425 was notable for its exceptionally large amount of anthocyanins, 207.3 mg/100 g. According to the results of the present study, anthocyanins comprised 18.3% to 42.7% of total phenolic content. As several authors have reported (15, 16), in bilberries, red raspberries, and lingonberries, anthocyanins accounted for 90%, 30%, and 22% of their phenolic profiles, respectively. In the high bush blueberry, anthocyanins make up on average 20–30% of total phenolic content (17). Berries of a related species *V. macrocarpon* accumulate on average 192.3–520.8 mg/100 g of phenolic compounds (18). *V. oxycoccos* berries have one of the richest sources of phenolic phytochemicals because they contain large amounts of total phenolic compounds including anthocyanins (Table 2).

Separation of cranberry anthocyanins by HPLC-UV revealed six anthocyanins. Mass spectra showed the elution sequence to be as follows: cyanidin-3-galactoside and cyanidin-3-glucoside (449 m/z), cyanidin-3-arabinoside (419 m/z), peonidin-3-galactoside and peonidin-3-glucoside (463 m/z), and peonidin-3-arabinoside (433 m/z). Masses for the aglycones are the following: cyanidin 287 m/z and peonidin 301 m/z. The order of elution of the glycosides from the C_{18} column is galactoside before glucoside, which is before arabinoside. The proportion of individual anthocyanins in berries depends on the clone of the cran-

Table 1. **Summary statistics of *Vaccinium oxycoccos* characteristics**

Characteristic	M	SD	M_{min}–M_{max}	*F*
Berry weight, g	0.93	0.17	0.56–1.22	44.07**
Leaf area, cm^2	0.39	0.05	0.28–0.49	20.09**
Total phenolic amount, mg/100 g	363.9	84.29	224.1–498.2	7.6**
Total anthocyanin amount, mg/100 g	81.5	32.41	40.7–207.3	22.2**

M – mean; SD – standard deviation; M_{min}–M_{max} – range of mean values; *F* – Fisher's criterion.
**P<0.01.

Table 2. **Contents of phenolics and anthocyanins in different fruit, as reported by other authors**

Species	Total amount of phenolic compounds, mg/100 g	Total amount of anthocyanins mg/100 g	References
Vaccinium macrocarpon (Aiton) Pursh	192.3–520.8 400.0	61.3–100.6 123.8–225.0	Sapers and Hargrave, 1987 (19) Povilaitytė et al., 1998 (18) Kalt et al., 2001 (17)
Rubus idaeus L.	228.0 282.0	 43.0	Apak et al., 2007 (20) Plessi et al., 2007 (21)
Ribes nigrum L.	763.0	262.0 233.5–450.0	Plessi et al., 2007 (21) Rubinskienė and Viškelis, 2002 (22)
Ribes rubrum L.	314.0	22.0	Plessi et al., 2007 (21)
Fragaria × *ananasa* Dusch.	330.0	 20.0–60.0 37.1–122.3	Apak et al., 2007 (20) Rivas-Gonzalo and Santos-Buelga, 2007 (23) Ngo et al., 2007 (24)
Hippophae rhamnoides L.	114.8–244.1		Gao et al., 2000 (25)

Table 3. **Qualitative and quantitative anthocyanin content of *Vaccinium oxycoccos* berries**

Clone	Cyanidin-3-galactoside		Cyanidin-3-glucoside		Cyanidin-3-arabinoside		Peonidin-3-galactoside		Peonidin-3-glucoside		Peonidin-3-arabinoside	
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
V416	13.1	0.26	6.2	0.08	20.3	0.16	23.8	0.06	18.9	0.24	17.6	0.28
V485	17.5	1.22	0.3	0.48	22.7	0.93	34.3	2.59	1.9	1.05	23.3	0.69
V409	18.9	0.18	–	–	23.2	0.74	33.2	0.17	1.8	0.19	22.7	0.58
V411	15.1	0.22	0.4	0.12	17.1	0.07	39.3	4.97	4.8	4.33	23.1	0.07
V486	18.2	1.42	28.7	0.01	24.4	0.03	5.9	0.154	19.5	0.92	3.4	0.33
V426	18.0	1.21	0.09	0.06	16.5	0.42	42.8	0.81	1.68	0.24	21.0	0.63
V480	24.0	1.56	0.5	0.32	21.1	0.15	34.8	2.54	3.2	3.2	16.6	1.09
V475	17.9	1.54	–	–	21.2	0.17	36.4	1.35	3.0	1.03	21.4	0.89
V415	23.4	0.28	0.6	0.11	18.6	0.07	38.5	0.52	1.6	0.14	17.2	0.08
V425	23.7	0.50	1.2	0.77	21.6	1.49	31.9	0.31	5.1	2.97	16.4	0.53
V424	22.9	3.45	1.9	2.37	20.2	0.13	34.9	3.59	3.1	3.03	17.0	2.09
V481	17.4	2.00	5.9	1.46	17.8	2.56	27.8	2.89	17.1	2.3	14.0	5.9
V407	25.1	0.02	–	–	22.0	0.30	34.7	0.12	–	–	17.6	0.16
V413	22.7	0.48	1.0	0.18	20.1	0.53	32.2	0.48	6.9	1.21	17.2	0.31
V483	25.2	2.14	2.4	2.45	22.8	0.25	29.2	1.21	5.1	0.27	15.3	0.63
V408	26.8	0.13	1.0	0.03	22.8	0.65	31.1	3.44	3.4	2.46	14.9	0.17
V474	14.9	0.46	3.3	2.41	17.5	1.78	31.6	2.18	12.1	0.17	22.3	1.63
V487	14.2	0.89	13.4	0.72	40.5	0.52	8.2	1.19	18.7	0.47	5.2	0.47
V478	15.6	1.16	7.2	1.29	17.4	0.77	21.3	0.64	23.3	0.87	15.2	0.79
V423	24.6	0.61	–	–	27.0	0.22	30.2	0.13	1.4	0.01	16.8	0.27
V488	14.6	0.58	–	–	21.7	0.60	33.0	0.61	2.2	0.81	28.5	0.86

M – mean, expressed as percentage of total anthocyanin content; SD – standard deviation.

berry (Table 3). In some clones, cyanidin-3-glucoside was not detected (V409, V475, V407, V423, and V488), and in other clone, peonidin-3-glucoside was not detected (V407). The dominant anthocyanin was peonidin-3-galactoside; on average, it amounted to 30% of total anthocyanin content. The average composition of other anthocyanins was the following: cyanidin-3-galactoside, 19.8%; cyanidin-3-glucoside,

3.4%; cyanidin-3-arabinoside, 21.7%; peonidin-3-glucoside, 7.4%; and peonidin-3-arabinoside, 17.4%.

Berry weight was found to be negatively correlated with anthocyanin content (r=–0.744). A regression analysis revealed the interrelationship between berry weight and amount of anthocyanins is expressed by the linear equation (Fig. 1). It shows that berry size was an important factor for determining the amount of total anthocyanins, with a regression coefficient of $R^2_{x/y}$=0.554. An analogous trend was also detected for the amount of total phenolics, the interrelationship of which with berry weight could be best expressed by the hyperbolic equation (Fig. 2) with a regression coefficient of $R^2_{x/y}$=0.22. The same trend was established for *V. macrocarpon*; i.e., the levels of phenolic compounds were negatively correlated with hor-

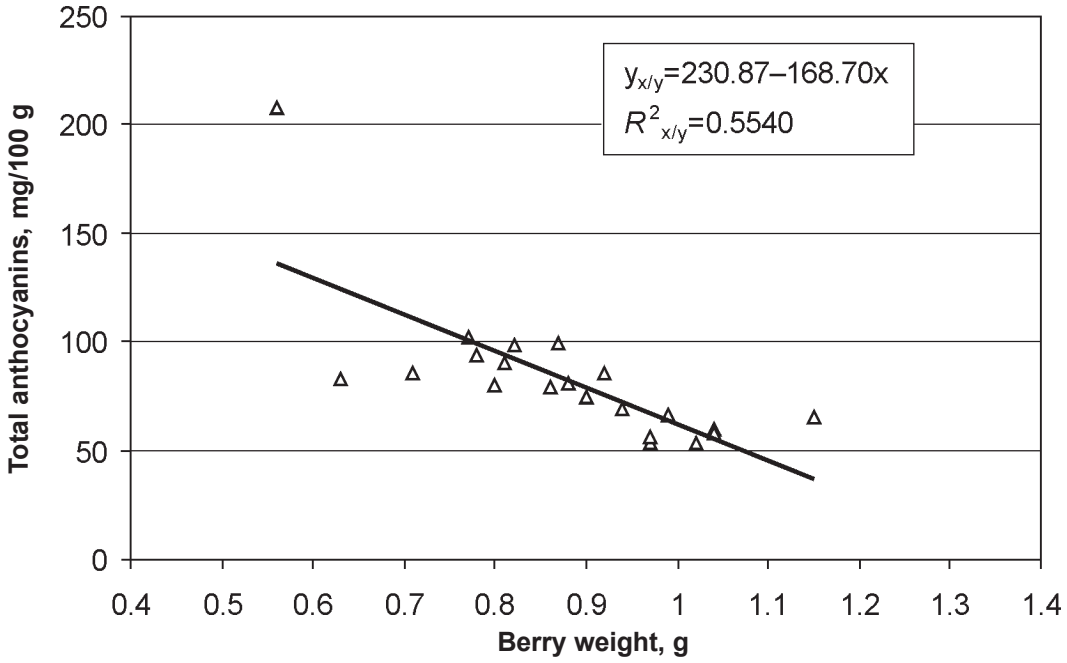

Fig. 1. **Relation between the total amount of anthocyanins and berry weight of *Vaccinium oxycoccos***

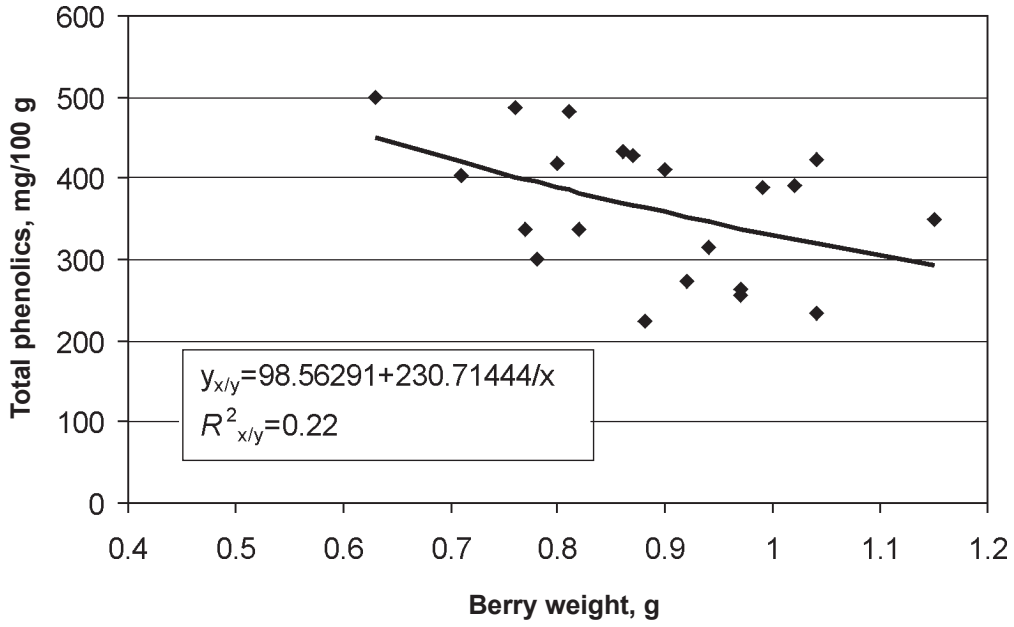

Fig. 2. **Relation between the total amount of phenolics and berry weight of *Vaccinium oxycoccos***

ticultural traits such as yield and fruit size (4). Meanwhile, the results obtained by Moyer et al. showed that there was a strong positive correlation between fruit size and amount of total anthocyanins in *V. corymbosum*, but no correlation was found across other *Vaccinium* species investigated (26, 27).

Antibacterial activity. Investigation of the antimicrobial properties showed that European cranberry extracts inhibited the growth of wide range of human pathogenic bacteria, both gram-negative and gram-positive. *Listeria monocytogenes* and *Enterococcus faecalis* were the most sensitive (average zones of inhibition were 20.35 and 19.71 mm, respectively), *Salmonella typhimurium* and *Staphylococcus aureus* were found to be of moderate resistance, and *Escherichia coli* was the least sensitive (Table 4). A comparison of results from the different clones showed that the largest zones of inhibition were made by the extract of the clones V409 (16.00–23.66 mm), V481 (15.00–26 mm), and V488 (16.00–22.00 mm). Extracts of less active clones V487 and V478 showed zones of 14.00–17.00 mm and 13.00–19.00 mm in diameter, respectively.

Several authors have reported that phenolic berry extracts inhibited the growth of *Salmonella*, *Escherichia*, and *Staphylococcus* species (10, 11, 28). Berry phenolics affect the growth of bacterial species in different mechanisms. There seem to be complex interactions between pH of the growth media and antimicrobial effects of the berry phenolics varying in different bacterial species (10). It is suggested that the antimicrobial activity of berries may enhance shelf life of food products and act as new type antimicrobials, which may control a wide range of pathogens. Natural food preservatives targeted to foods that are easily contaminated by *Salmonella* and *Staphylococcus* are highly desired (28, 29). Cranberry-derived antimicrobials could be included as effective supplements to traditional antimicrobial preparations and treatment measures.

Conclusions

The results of these analyses indicate a significant variability in amounts of biologically active substances in the berries of European cranberry. Accumulation of a large amount of phenolic compounds enables their use as strong antioxidants. The data comprising morphological and chemical diversity should motivate

***Table 4*. The antibacterial influence of *Vaccinium oxycoccos* extracts on test cultures**

Cranberry clone	Inhibition zone size, mm					
	Escherichia coli	*Salmonella typhimurium*	*Bacillus subtilis*	*Listeria monocytogenes*	*Staphylococcus aureus*	*Enterococcus faecalis*
V416	15.00±0.00	18.66±0.47	16.00±0.81	20.66±0.47	18.00±0.00	21.66±0.47
V485	14.66±0.47	22.00±1.41	16.66±0.47	21.66±0.47	19.66±0.47	21.66±0.47
V409	17.00±0.00	18.00±0.00	16.00±0.00	21.00±0.00	19.00±0.00	23.66±0.47
V411	13.33±0.47	14.00±0.00	14.66±0.47	21.00±0.00	10.33±0.47	14.33±0.47
V486	17.00±0.00	14.33±0.47	19.00±0.00	21.33±0.47	12.00±0.00	13.66±0.47
V426	14.00±0.81	16.00±0.00	17.00±0.81	23.00±0.00	19.00±0.81	23.66±0.47
V480	13.33±0.47	16.33±0.47	18.33±0.47	22.00±0.00	18.33±0.47	20.66±0.47
V475	12.66±0.47	16.00±0.00	15.66±0.47	19.00±0.00	16.66±0.47	22.00±0.00
V415	15.00±0.00	13.33±0.47	23.00±0.00	21.00±0.00	22.00±0.00	22.66±0.47
V425	15.00±0.00	17.00±0.00	15.00±0.00	19.00±0.00	17.00±0.00	19.66±0.47
V424	14.00±0.00	19.33±0.47	14.00±0.00	20.33±0.47	16.00±0.00	20.66±0.47
V481	15.00±0.00	22.00±0.00	18.00±0.00	19.00±0.00	15.00±0.00	26.00±0.00
V407	13.00±0.00	21.00±0.00	17.00±0.00	18.00±0.00	16.00±0.00	21.33±0.47
V413	16.00±0.00	18.33±0.47	16.00±0.00	23.33±0.47	15.33±0.47	21.66±0.47
V483	14.00±0.00	17.00±0.00	18.00±0.00	21.00±0.00	18.66±0.47	23.00±0.00
V408	13.00±0.00	14.00±0.00	17.00±0.00	20.00±0.00	15.00±0.00	17.33±0.47
V474	13.00±0.00	22.33±0.47	13.00±0.00	22.33±0.47	17.00±0.00	16.66±0.47
V487	15.00±0.00	14.00±0.00	16.00±0.00	17.00±0.00	16.00±0.00	16.00±0.00
V478	13.00±0.00	18.00±0.00	15.00±0.00	19.00±0.00	18.00±0.00	15.33±0.47
V423	13.00±0.00	22.00±0.00	14.00±0.00	19.00±0.00	20.00±0.00	15.66±0.47
V488	22.00±0.00	18.66±0.47	16.00±0.00	18.66±0.47	19.00±0.00	16.66±0.47

Data are expressed as means ± standard errors.

the selection of clones with putative large amounts of phenolics and anthocyanins. Selected clones of European cranberry with large amounts of phenolic compounds could be used for breeding new cultivars with desired biochemical characteristics. Our study indicates that European cranberry may act as antimicrobials, which control a wide range of pathogens. Antimicrobial berry compounds could have important applications in the future as natural antimicrobial agents for the food industry as well as for the field of medicine.

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Paprastosios spanguolės uogų fenoliniai junginiai ir antocianinai bei jų antimikrobinis aktyvumas

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Raktažodžiai: antocianinai, uogos masė, klonas, fenoliniai junginiai.

Santrauka. Paprastosios spanguolės uogos sukaupia didelius biologiškai aktyvių medžiagų kiekius, jos yra vertinamos tiek farmacijoje, tiek maisto pramonėje. *Tyrimo tikslas* – nustatyti fenolinių junginių ir antocianinų kiekius paprastosios spanguolės uogose ir įvertinti jų ekstraktų antibakterinį aktyvumą.

Medžiaga ir metodai. Natūraliose augimvietėse surinkti įvairūs paprastosios spanguolės klonai lyginti lapų dydžio, vidutinės vienos uogos masės ir fenolinių junginių bei antocianinų kiekių atžvilgiu. Antocianinų kiekybinė ir kokybinė sudėtis ekstraktuose nustatyta efektyviosios skysčių chromatografijos metodu. Antimikrobinės uogų savybės įvertintos difuzijos į agarą metodu.

Rezultatai. Didelė neigiama koreliacija nustatyta tarp vidutinės uogos masės ir antocianinų kiekio. Bendras fenolinių junginių kiekis skirtingų klonų uogose siekė nuo 224,0 mg/100 g iki 498,0 mg/100 g, o bendras antocianinų kiekis svyravo nuo 40,7 mg/100 g iki 207,3 mg/100 g. Paprastosios spanguolės uogose išskirti šeši antocianinai, o antocianinas peonidin-3-galaktozidas buvo vyraujantis.

Išvados. Antimikrobinių paprastosios spanguolės uogų savybių tyrimai parodė, kad jų ekstraktas inhibuoja tiek gramteigiamų (*Enterococcus faecalis*, *Listeria monocytogenes, Staphylococcus aureus*, *Bacillus subtilis*), tiek gramneigiamų (*Escherichia coli* ir *Salmonella typhimurium)* bakterijų augimą.

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