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Original Article

Phenolic Profile, Mineral Content and Antibacterial Activity of the Methanol Extract of *Vaccinium myrtillus* L.

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Abstract

Bilberry is considered as one of the most economically important wild berries. However, bilberry is not enough investigated, and there are only a few published works. Therefore, we performed in one place complete qualitative analysis, antimicrobial activity against different Gram-positive and Gram-negative bacteria and contents of metals. In our research, it was found that bilberry contains different active phenolic compounds, such as chlorogenic acid, delphinidin glycoside, delphinidin arabinoside, cyanidin glycoside, cyanidin arabinoside, malvidin glycoside, peonidin glycoside, and malvidin arabinoside. The content of metals was different in leaves and fruits. In our samples, the content of aluminum, boron, barium, calcium, cadmium, chromium, copper, iron, manganese, nickel, phosphorus, silicon and zinc was higher, and contents of potassium, magnesium, and sodium were lower than in bilberry samples investigated in Latvia**.** Antimicrobial activity of investigated extracts was evaluated against laboratory control strains from ATCC collection, Gram (+) bacteria: *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 6538, *Streptococcus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 19433, *Propionibacterium acnae* ATCC 11827, and Gram (-) bacteria: *Escherichia coli* ATCC 9863, *Pseudomonas aeruginosa* ATCC 9027, *Acinetobacter boumanii* ATCC 196060, *Proteus mirabilis* ATCC 12453, *Klebsiella pneumonia* ATCC 10031, and against related strains isolated from human wound swabs. *V. myrtillus* extract was less potent against strains from wounds compared to ATCC strains as well Gram (-) bacteria compared to Gram (+) bacteria. The most sensitive strains were *St. epidermidis*, *St. pyogenes*, *P. mirabilis* and *S. aureus*.

Keywords: antimicrobial activity, bilberry, element content, phenolic compounds, phytomedicine

Introduction

Bilberry is considered as one of the most economically important wild berries (Tomicevic *et al.*, 2011); *Vaccinium* species are used in pharmacy and phytomedicine, but also in juices and jams as colorants (Laaksonen *et al.*, 2010). It was determined that bilberry fruits contain up to 10 % tannins, organic acids, high quantity of anthocyanins (five anthocyanidins-delphinidin, cyanidin, petunidin, peonidin, and malvidin are combined with three sugar types-galactose, glucose, arabinose), flavonols (quercetin, myricetin, rutin), phenolic acids (chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, ellagic acid, gallic acid) (Zadernowski *et al.*, 2005; Colak *et al.*, 2016) and stilbene (*trans*-resveratrol) (Kalt *et al.*, 1999; Može *et al.*, 2011; Kahkonen *et al.*, 2003; Ogawa et al., 2008; Buchert *et al.*, 2005; Latti *et al.*, 2008), and pectins. Interestingly and surprisingly, bilberry has

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higher anthocyanin content compared to other berries (strawberry, cranberry, elderberry, sour cherry, and raspberry) (Kowalczyk *et al*., 2003; Bagchi *et al*., 2004; Yildirim, 2006; Cravotto *et al*., 2010). The content of proanthocyanidins in bilberries was determined by several research groups using different methods, but obtaining the same results (Latti *et al*., 2011; Hellstrom *et al*., 2009). Fruits of *V. myrtillus* are used as antidiarrheal (Saric, 1989). Many compounds present in bilberries, particularly myricetin-3-*O*-arabinoside, myricetin-3-*O*-glucoside, myricetin-3-*O*-galactoside and the unknown caffeoyl quinic acid, have been discovered as contributors to astringency and bitterness (Laaksonen *et al*., 2010). Also, it was proven as a valuable source for making wines (Yildirim, 2013).

Antimicrobial activity of berry compounds show that anthocyanins may protect against human pathogenic bacteria (Rauha *et al*., 2000; Puupponen-Pimia *et al*., 2001; Cavanagh *et al*., 2004; Kontiokari *et al*., 2003; Vattem *et al*., 2005; Nohynek *et al*., 2006). Finnish berries' extracts inhibited the growth of Gram-negative, and not Gram-positive bacteria. No correlation was observed between Gram-positive or Gram-negative bacterial status and susceptibility to the berries (Cavanagh *et al*., 2004).

Our aim was *de facto* to give in one place complete qualitative analysis, antimicrobial activity against different Gram-positive and Gram-negative bacteria, and contents of metals. To the best of our knowledge nobody before us performed this kind of the investigation.

Materials and Methods

Extraction

Bilberries (*Vaccinium myrtillus* L.) were used from the period of the fully ripe stage in July 2015 in woods from Lake Vlasina, Serbia. Fresh fruits were stored at -20 °C for one week when extracts were prepared. The extraction method was modified and improved according to the already reported method (Može *et al*., 2011). Frozen samples (150 g) were firstly homogenized in 0.5 L ice-cold deoxygenated methanol that had previously been flushed for a few minutes with nitrogen. The homogenate was extracted for 1 h by shaking on magnetic stirrer IKA REO Basic C (Königswinter, Germany) at room temperature. The extract was filtered by vacuum through the technical filter paper. The residue was extracted again in 0.25 L ice-cold deoxygenated methanol for 0.5 h and the suspension was filtered as before. The third time the residue was extracted as described before. Finally, all three filtrates samples were pooled, flushed with nitrogen for a few minutes, and then stored at -20 °C until analysis.

Ultra high-performance liquid chromatography – diode array – electrospray ionization mass spectrometry analysis

The liquid chromatography (UHPLC) runs were carried out using Dionex Ultimate 3000 UHPLC+ system equipped with a diode array (DAD) detector and also connected with LCQ Fleet Ion Trap Mass Spectrometer (Thermo Fisher Scientific, USA). The separations were performed on the Hypersil gold C18 column (50×2.1 mm, 1.9 μm) of the same producer, at 25 °C. The mobile phase was made from (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. A next linear gradient program at flow rate of 0.250 ml/min has been applied: Method-I: 10% to 30% (B) for first two minutes, then 40% to 50% (B) for 5-7 min and 80% to 90% (B) from 9 to 11 min, followed by isocratic run at 90% (B) from 11-12 min and from 90–10% (B) from 12 to 12.1 min, and finally the isocratic run with 10% (B) to 20th min; Method-II: 20% to 50% (B) for first five minutes, then 70% to 90% (B) for 5-7 min, followed by isocratic run at 90% (B) from 7-9 min and from 90-20% (B) from 9 to 9.1 min, and finally the isocratic run with 20% (B) to 15th min.

Absorption UV-VIS spectra were recorded on DAD-detector (with a total spectral range between 200 nm and 800 nm). MS analysis was performed using LCQ 3D-ion trap mass spectrometer with electrospray ionization (ESI) in the negative, and in positive ion mode. The ESI source parameters for negative mode were set as follows: source voltage 4.5 kV, capillary voltage -41 V, tube lens voltage -95 V, capillary temperature 350 °C, sheath and auxiliary gas flow (N_2) 32 and 8 (arbitrary units), respectively. On the other hand, the ESI source parameters for positive ion mode were: source voltage 4.5 kV, capillary voltage 19 V, tube lens voltage 95 V, capillary temperature 275 °C, sheath and auxiliary gas flow (N_2) 32 and 8 (arbitrary units), respectively. MS spectra (both modes) were obtained by full range acquisition of *m/z* 130-900. For fragmentation study (MS/MS), a data dependent scan was performed by deploying the collision-induced dissociation (CID). The normalized collision energy of the CID cell was set at 15 and 25 eV, for the negative and positive mode, respectively.

Determination of the mineral content

Wet digestion of fruits

To 0.5 g of dried sample 5 mL conc. HNO_3 was added and placed on the hot plate for 1 h, and semi-dried; again, 5 mL of conc. HNO_3 were added and 2 ml of H_2O_2 and kept on the hot plate for 1 h and after getting semi-dried cooled and filtered using Whatman filter paper, and the volume of the residue was made up to 25 mL with 2N HNO_3 and taken to the Atomic absorption spectrophotometer for mineral analysis using Aluka standards (AOAC, 1990).

Dry method for leaves

The sample of dried leaves (2 g) was placed into a furnace for 2 h at 250 °C. Afterwards, the temperature was gradually increased to 550 °C, and calcined for another 12 h. The ash was covered with 5 mL conc. HNO_3, filtered through quantitative filter paper, the pot was rinsed a couple of times using 0.5% HNO_3 and made up to 25 mL using 0.5% HNO_3. Results were expressed in mg/kg of dry residue (Azcue and Mudroch, 1994; Mateos-Aparicio *et al*., 2010).

Micro-well dilution assay

Bacterial strains

Antimicrobial activity of investigated extracts was evaluated against laboratory control strains from ATCC collection, Gram (+) bacteria: *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 6538, *Streptococcus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 19433, *Propionibacterium acnae* ATCC 11827, and

Gram (-) bacteria: *Escherichia coli* ATCC 9863, *Pseudomonas aeruginosa* ATCC 9027, *Acinetobacter boumanii* ATCC 196060, *Proteus mirabilis* ATCC 12453, *Klebsiella pneumonia* ATCC 10031, and against related strains isolated from human wound swabs.

Micro-well dilution method

Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of extracts were determined by employing the broth micro-well dilution method with some modifications (Cockerill *et al.*, 2012). An overnight culture of tested bacterial strains was used for the preparation of suspensions (0.5 McFarland standard turbidity). A serial doubling dilution of the extracts (in 10% aqueous dimethyl sulfoxide-DMSO) was prepared in a 96 well microtiter plate with inoculated Mueller Hinton broth (MHB) at concentrations ranging from 0.06-126 mg mL^{-1}. The final volume was 100 μL, and the final concentration of bacterial suspensions was 10^6 CFU mL^{-1} in each well. The plates were incubated for 24 h at 37 ^{0}C. Metronidazole, doxycycline, ciprofloxacin, and gentamicin were used as positive control (Sigma Aldrich, St Louis, MO, USA), and dilutions were at concentrations ranging from 0.01 to 100 mg mL^{-1}. All determinations were performed in triplicate. Microbial growth was determined by adding 20 μL of 0.5% triphenyl tetrazolium chloride (TTC) aqueous solution in microtiter plates. In order to determine MBC, the broth was taken from each well and inoculated on Mueller Hinton agar (MHA) for 24 h at 37 °C. The MBC is defined as the lowest concentration of the extracts at which 99.9% of inoculated bacteria were killed.

Results and Discussion

Qualitative analysis of phenolic compounds

Although we followed the experimental procedure of Može *et al.* (2011), fewer compounds in the investigated extracts were found in our case. Isolated compounds using UHPLC method were identified using literature by the comparison of the mass spectra (Table 1). Our results were in accordance with Hokkanen *et al.* (2009) and Laaksonen *et al.* (2010), too. Latti *et al.* (2008) observed the high variation in the content of anthocyanins in bilberries from different parts of Finland (northern berries have higher contents than southern). The same explanation can be prescribed for our sample and results for berries north from Serbia. Riihinen *et al.* (2008) claim that most of the phenolic acids and flavonols can be found in the leaves rather in berries of bilberry.

Determination of the content of metals

The content of metals was found to be different in leaves and fruits. The performed investigation showed that the content of aluminium, boron, barium, calcium, cadmium, chromium, copper, iron, manganese, nickel, phosphorus, silicon and zinc is higher than those found by Skesters *et al.* (2014) (Table 2). On the other hand, contents of potassium, magnesium, and sodium were lower than in bilberry found from the same research group (Skesters *et al.*, 2014) (Table 2).

Table 1. Isolated compounds from bilberry, their characteristic ions from mass spectra and UV/VIS adsorption characteristics

Compound Number UHPLC Method-I ($t_{ret.}$/min)	Name	Negative mode ESI-MS $[M-H]^-$/m/z	MS/MS	Confirmation	UV/Vis
1. (1.90)	chlorogenic acid	353	-	standard	326 nm
2. (5.56)	resveratrol-rutinoside (tentative)	535	227 (100%)	(Hu *et al.*, 2008; Hu *et al.*, 2010)	314, 230 nm
UHPLC Method-II		Positive mode ESI-MS $[M]^+$/m/z	MS/MS		
2. (3.12)	resveratrol-rutinoside (tentative)	559 $[M+Na]^+$	-		314, 230 nm
3. (1.62)	delphinidin-glycoside	465	303 (100%)	(Nakajima *et al.*, 2004; Xianli and Prior, 2005)	525, 278 nm
4. (1.76)	delphinidin-arabinoside	435	-	(Nakajima *et al.*, 2004; Xianli and Prior, 2005)	516, 281 nm
5. (1.80)	cyanidin-glycoside	449	287 (100%), 317	(Nakajima *et al.*, 2004; Xianli and Prior, 2005)	517, 279 nm
6. (1.93)	cyanidin-arabinoside	419	287 (100%)	(Nakajima *et al.*, 2004; Xianli and Prior, 2005)	522, 280 nm
7. (2.09)	malvidin-glycoside	493	331 (100%)	(Nakajima *et al.*, 2004; Xianli and Prior, 2005)	527, 279 nm
8. (2.25)	peonidin-glycoside	463	270 (100%), 301	(Nakajima *et al.*, 2004; Xianli and Prior, 2005)	≈520, ≈300 nm
9. (2.28)	malvidin-arabinoside	463	331 (100%)	(Nakajima *et al.*, 2004; Xianli and Prior, 2005)	≈520, ≈300 nm

Table 2. Element concentrations (mg/g wet weight±standard deviations, n=3) in bilberry. Sample 1 was prepared from dried leaves, and sample 2 from fresh fruit

Element	Element concentrations c_{st}±SD (mg g^{-1})		RSD (Relative standard deviation) (%)	
	Sample 1/Dried leaves	Sample 2/Fresh fruit	Sample 1/Dried leaves	Sample 2/Fresh fruit
Al	0.0719±0.0002	0.0162±0.0001	0.28	0.62
As	$9.7 \cdot 10^{-5} \pm 3 \cdot 10^{-6}$	$4.20 \cdot 10^{-5} \pm 2 \cdot 10^{-6}$	3.09	4.76
B	$0.0135 \pm 4 \cdot 10^{-4}$	$0.0121 \pm 1 \cdot 10^{-4}$	2.96	0.83
Ba	$0.0370 \pm 5 \cdot 10^{-4}$	$0.0099 \pm 1 \cdot 10^{-4}$	1.35	1.01
Ca	2.36±0.03	0.75±0.01	1.27	1.33
Cd	$3.9 \cdot 10^{-5} \pm 2 \cdot 10^{-6}$	$3.6 \cdot 10^{-5} \pm 2 \cdot 10^{-6}$	5.13	5.56
Co	$1.21 \cdot 10^{-5} \pm 6 \cdot 10^{-7}$	$1.45 \cdot 10^{-5} \pm 5 \cdot 10^{-7}$	4.96	3.45
Cr	$2.98 \cdot 10^{-4} \pm 4 \cdot 10^{-6}$	$2.54 \cdot 10^{-4} \pm 3 \cdot 10^{-6}$	1.34	1.18
Cu	$0.0093 \pm 2 \cdot 10^{-4}$	$6.1 \cdot 10^{-3} \pm 2 \cdot 10^{-4}$	2.15	3.28
Fe	$0.0386 \pm 4 \cdot 10^{-4}$	$0.0198 \pm 1 \cdot 10^{-4}$	1.04	0.51
K	13.3±0.2	2.86±0.01	1.50	0.35
Mg	$0.410 \pm 2 \cdot 10^{-3}$	$0.352 \pm 1 \cdot 10^{-3}$	0.48	0.26
Mn	0.290±0.003	0.244±0.003	1.03	1.23
Na	$0.0027 \pm 1 \cdot 10^{-4}$	$0.0012 \pm 1 \cdot 10^{-4}$	3.70	8.33
Ni	$5.1 \cdot 10^{-5} \pm 2 \cdot 10^{-6}$	$6.2 \cdot 10^{-5} \pm 1 \cdot 10^{-6}$	3.92	1.61
P	0.65±0.002	1.37±0.03	3.08	2.18
Pb	$6.2 \cdot 10^{-5} \pm 2 \cdot 10^{-6}$	$2.5 \cdot 10^{-5} \pm 1 \cdot 10^{-6}$	3.23	4.0
Se	$3.58 \cdot 10^{-5} \pm 3 \cdot 10^{-7}$	$2.1 \cdot 10^{-6} \pm 1 \cdot 10^{-7}$	0.84	4.76
Si	0.114±0.0002	0.0096±0.0002	1.75	2.08
V	$9.8 \cdot 10^{-4} \pm 3 \cdot 10^{-5}$	$7.5 \cdot 10^{-4} \pm 3 \cdot 10^{-5}$	3.06	4.0
Zn	0.059±0.001	0.055±0.003	1.69	5.45

Table 3. Antimicrobial activity (MIC/MBC in mg/mL) of *Vaccinium myrtillus* L. methanol extract and referent antibiotics against pathogenic bacterial strains

Isolated and ATCC bacterial strains		Methanol extract of *Vaccinium myrtillus* L.	Antibiotics			
			Metronidazole	Doxycyclin	Ciprofloxacin	Gentamicin
Gram (+)	Source					
Staphylococcus aureus	Wound swabs	63.00/63.00	3.91/15.62	7.81/7.81	1.26/1.26	0.60/0.60
Staphylococcus aureus	ATCC 6538	63.00/63.00	15.62/31.25	7.81/15.61	1.26/2.52	0.60/0.60
Staphylococcus epidermidis	Wound swabs	15.75/31.50	7.81/31.25	3.91/3.91	0.63/0.63	0.30/0.30
Staphylococcus epidermidis	ATCC 12228	15.75/15.75	7.81/62.50	3.91/7.81	0.63/0.63	0.30/0.30
Streptococcus pyogenes	Wound swabs	31.50/126.00	7.81/7.81	0.06/0.12	0.16/0.16	0.30/0.30
Streptococcus pyogenes	ATCC 19615	31.50/63.00	0.98/15.62	0.06/0.12	0.16/0.16	0.30/0.30
Enterococcus faecalis	Wound swabs	31.50/31.50	7.81/62.50	0.25/0.25	0.30/0.30	0.08/0.08
Enterococcus faecalis	ATCC 19433	63.00/126.00	3.91/62.50	0.25/0.49	0.30/0.30	0.16/0.16
Propionibacterium acnae	ATCC 11827	126.00/126.00	0.98/15.62	15.61/15.61	2.50/ >20.00	2.50/ >20.00
Gram (-)	Source					
Escherichia coli	Wound swabs	63.00/126.00	15.62/31.25	7.81/7.81	2.50/2.50	2.50/20.00
Escherichia coli	ATCC 9863	31.50/126.00	31.25/31.25	7.81/15.61	2.50/2.50	2.50/20.00
Pseudomonas aeruginosa	Wound swabs	31.50/252.00	15.62/62.50	7.81/15.61	0.02/0.63	2.50/10.00
Pseudomonas aeruginosa	ATCC 9027	31.50/126.00	31.25/31.25	15.61/15.61	0.02/0.63	2.50/10.00
Acinetobacter sp.	Wound swabs	252.00/252.00	1.95/62.50	15.61/15.61	10.00/20.00	10.00/20.00
Acinetobacter boumanii	ATCC 196060	63.00/63.00	15.62/15.62	15.61/15.61	10.00/20.00	10.00/20.00
Proteus mirabilis	Wound swabs	63.00/63.00	7.81/7.81	7.81/15.61	10.00/20.00	1.25/5.00
Proteus mirabilis	ATCC 12453	31.50/63.00	62.50/125.00	7.81/15.61	10.00/20.00	5.00/10.00
Klebsiella sp.	Wound swabs	126.00/252.00	31.25/125.00	15.61/15.61	0.63/20.00	2.50/10.00
Klebsiella pneumoniae	ATCC 10031	126.00/126.00	31.25/62.50	15.61/15.61	10.00/20.00	10.00/20.00

Investigation of the anti-bacterial activity of the investigated extracts

Minimal inhibitory and bactericidal concentration of *V. myrtillus* methanol extract (Table 3) was in the range of MIC/MBC=15.75-252.00 mg mL^{-1}. The best activity was against *St. epidermidis* ATCC 12228, (MIC=MBC=15.75 mg mL^{-1}) and *St. epidermidis* isolated from wound swabs (MIC/MBC=15.75/31.50 mg mL^{-1}), against *E. faecalis* from wound (MIC=MBC=31.50 mg mL^{-1}) and against *St. pyogenes* ATCC 19615 and *P. mirabilis* ATCC 12453 (MIC/MBC=31.50/63.00 mg mL^{-1}, respectively).

Conclusions

Bilberry as a source of bioactive agents is not investigated enough, so we performed the complete analysis of metals, complete qualitative analysis and determined the anti-bacterial activity of the extract. We found that it contains different active phenolic compounds, such as chlorogenic acid, delphinidin glycoside, delphinidin arabinoside, cyanidin glycoside, cyanidin arabinoside, malvidin glycoside, peonidin glycoside, and malvidin arabinoside. The contents of metals in leaves and fruits vary, and in some cases they are higher, and sometimes lower than found by others. Antimicrobial effect of the *V. myrtillus* extract was less potent against strains from wounds compared to ATCC strains as well Gram (-) bacteria compared to Gram (+) bacteria. The most sensitive strains were *St. epidermidis*, *St. pyogenes*, *P. mirabilis,* and *S. aureus* and therefore the fruit extract of the investigated plant may find use as an additive in creams for skin care and protection.

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