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, 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 acnes* ATCC 11827, and Gram (-) bacteria: *Escherichia coli* ATCC 9863, *Pseudomonas aeruginosa* ATCC 9027, *Acinetobacter baumannii* 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, peonidin, 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...
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 antidiarreal (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 
with nitrogen 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 Ultiimate 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 (50x2.1 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 (N2) 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 (N2) 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. HNO3 was added 
and placed on the hot plate for 1 h, and semi-dried; again, 5 
ml of conc. HNO3 were added and 2 ml of H2O2 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 HNO3 and 
taken to the Atomic absorption spectrophotometer for 

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. HNO3, filtered 
through qualitative filter paper, the obtained ash was filtered 
and made up to 25 mL using 0.5% HNO3. 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 acnes ATCC 11827, and
C. The MBC is defined as the lowest concentration of the extracts at which 99.9% of inoculated bacteria were killed.

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 10⁶ CFU mL⁻¹ in each well. The plates were incubated for 24 h at 37 °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>
<thead>
<tr>
<th>Compound Number</th>
<th>UHPLC Method-I (min)</th>
<th>Name</th>
<th>Negative ESI-MS [(M-H)/m/z]</th>
<th>MS/MS</th>
<th>Confirmation</th>
<th>UV/Vis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.  (1.90)</td>
<td>chlorogenic acid</td>
<td>353</td>
<td>-</td>
<td></td>
<td>standard</td>
<td>326 nm</td>
</tr>
<tr>
<td>2.  (5.56)</td>
<td>resveratrol-rutinoside (tentative)</td>
<td>535</td>
<td>227 (100%)</td>
<td>Hu et al., 2008; Hu et al., 2010</td>
<td>314, 230 nm</td>
<td></td>
</tr>
<tr>
<td>3.  (1.62)</td>
<td>delphinidin-glycoside</td>
<td>465</td>
<td>303 (100%)</td>
<td></td>
<td></td>
<td>525, 278 nm</td>
</tr>
<tr>
<td>4.  (1.76)</td>
<td>delphinidin-arabinoside</td>
<td>435</td>
<td>-</td>
<td></td>
<td></td>
<td>516, 281 nm</td>
</tr>
<tr>
<td>5.  (1.80)</td>
<td>cyanidin-glycoside</td>
<td>449</td>
<td>287 (100%), 317</td>
<td></td>
<td></td>
<td>517, 279 nm</td>
</tr>
<tr>
<td>6.  (1.93)</td>
<td>cyanidin-arabinoside</td>
<td>419</td>
<td>287 (100%)</td>
<td></td>
<td></td>
<td>522, 280 nm</td>
</tr>
<tr>
<td>7.  (2.09)</td>
<td>malvidin-glycoside</td>
<td>493</td>
<td>331 (100%)</td>
<td></td>
<td></td>
<td>527, 279 nm</td>
</tr>
<tr>
<td>8.  (2.25)</td>
<td>peonidin-glycoside</td>
<td>463</td>
<td>270 (100%), 301</td>
<td></td>
<td></td>
<td>≈520, ≈300 nm</td>
</tr>
<tr>
<td>9.  (2.28)</td>
<td>malvidin-arabinoside</td>
<td>463</td>
<td>331 (100%)</td>
<td></td>
<td></td>
<td>≈520, ≈300 nm</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Element</th>
<th>Sample 1/Dried leaves</th>
<th>Sample 2/Fresh fruit</th>
<th>Sample 1/Dried leaves</th>
<th>Sample 2/Fresh fruit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>0.0719±0.0002</td>
<td>0.0162±0.0001</td>
<td>0.28</td>
<td>0.62</td>
</tr>
<tr>
<td>As</td>
<td>9.7·10⁻²±3·10⁻²</td>
<td>4.2·10⁻²±2·10⁻²</td>
<td>3.09</td>
<td>4.76</td>
</tr>
<tr>
<td>B</td>
<td>0.0135±4·10⁻⁴</td>
<td>0.0121±4·10⁻⁴</td>
<td>2.96</td>
<td>0.83</td>
</tr>
<tr>
<td>Ba</td>
<td>0.0370±5·10⁻⁷</td>
<td>0.0099±1·10⁻⁶</td>
<td>1.35</td>
<td>1.01</td>
</tr>
<tr>
<td>Ca</td>
<td>2.36±0.03</td>
<td>0.75±0.01</td>
<td>1.27</td>
<td>1.33</td>
</tr>
<tr>
<td>Cd</td>
<td>3.9·10⁻²±2·10⁻⁶</td>
<td>3.6·10⁻²±2·10⁻⁶</td>
<td>5.13</td>
<td>5.56</td>
</tr>
<tr>
<td>Co</td>
<td>1.21·10⁻⁴±6·10⁻⁷</td>
<td>1.45·10⁻⁴±5·10⁻⁷</td>
<td>4.96</td>
<td>3.45</td>
</tr>
<tr>
<td>Cr</td>
<td>2.98·10⁻⁵±4·10⁻⁶</td>
<td>2.54·10⁻⁵±3·10⁻⁶</td>
<td>1.34</td>
<td>1.18</td>
</tr>
<tr>
<td>Cu</td>
<td>0.0093±2·10⁻⁶</td>
<td>6.1·10⁻³±2·10⁻⁴</td>
<td>2.15</td>
<td>3.28</td>
</tr>
<tr>
<td>Fe</td>
<td>0.0386±4·10⁻⁶</td>
<td>0.0198±1·10⁻⁴</td>
<td>1.04</td>
<td>0.51</td>
</tr>
<tr>
<td>K</td>
<td>13.3±0.2</td>
<td>2.86±0.01</td>
<td>1.50</td>
<td>0.35</td>
</tr>
<tr>
<td>Mg</td>
<td>0.40±2.10⁻⁵</td>
<td>0.352±1.10⁻⁵</td>
<td>0.48</td>
<td>0.26</td>
</tr>
<tr>
<td>Mn</td>
<td>0.290±0.003</td>
<td>0.24±0.003</td>
<td>1.03</td>
<td>1.23</td>
</tr>
<tr>
<td>Na</td>
<td>0.0027±1·10⁻⁷</td>
<td>0.0012±1·10⁻⁷</td>
<td>3.70</td>
<td>8.33</td>
</tr>
<tr>
<td>Ni</td>
<td>5.1·10⁻³±2·10⁻⁶</td>
<td>6.2·10⁻⁵±1·10⁻⁶</td>
<td>3.92</td>
<td>1.61</td>
</tr>
<tr>
<td>P</td>
<td>0.65±0.002</td>
<td>1.37±0.03</td>
<td>3.08</td>
<td>2.18</td>
</tr>
<tr>
<td>Pb</td>
<td>6.2·10⁻³±2·10⁻⁶</td>
<td>2.5·10⁻³±1·10⁻⁶</td>
<td>3.23</td>
<td>4.0</td>
</tr>
<tr>
<td>Sc</td>
<td>3.58·10⁻³±3·10⁻⁷</td>
<td>2.1·10⁻⁵±1·10⁻⁷</td>
<td>0.84</td>
<td>4.76</td>
</tr>
<tr>
<td>Sr</td>
<td>0.114±0.0002</td>
<td>0.0096±0.0002</td>
<td>1.75</td>
<td>2.08</td>
</tr>
<tr>
<td>V</td>
<td>9.8·10⁻⁶±3·10⁻⁷</td>
<td>7.5·10⁻⁷±3·10⁻⁷</td>
<td>3.06</td>
<td>4.0</td>
</tr>
<tr>
<td>Zn</td>
<td>0.059±0.001</td>
<td>0.055±0.003</td>
<td>1.69</td>
<td>5.45</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gram (+) bacterial strains</th>
<th>Methanol extract of <em>Vaccinium myrtillus</em> L.</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Source</td>
<td>Metronidazole</td>
</tr>
<tr>
<td>Wound swabs</td>
<td>63.00/63.00</td>
<td>3.91/15.62</td>
</tr>
<tr>
<td>ATCC 6538</td>
<td>63.00/63.00</td>
<td>15.62/31.25</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Wound swabs</td>
<td>15.75/31.50</td>
</tr>
<tr>
<td>ATCC 12228</td>
<td>15.75/15.75</td>
<td>7.81/62.50</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>Wound swabs</td>
<td>31.50/126.00</td>
</tr>
<tr>
<td>ATCC 19615</td>
<td>31.50/63.00</td>
<td>0.08/15.62</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>Wound swabs</td>
<td>31.50/31.50</td>
</tr>
<tr>
<td>ATCC 19433</td>
<td>31.50/63.00</td>
<td>3.91/62.50</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em></td>
<td>ATCC 11827</td>
<td>126.00/126.00</td>
</tr>
<tr>
<td>Gram (-) bacterial strains</td>
<td>Source</td>
<td>Metronidazole</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Wound swabs</td>
<td>63.00/126.00</td>
</tr>
<tr>
<td>ATCC 9865</td>
<td>31.50/126.00</td>
<td>31.25/31.25</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Wound swabs</td>
<td>31.50/252.00</td>
</tr>
<tr>
<td>ATCC 9027</td>
<td>31.50/126.00</td>
<td>31.25/31.25</td>
</tr>
<tr>
<td><em>Acinetobacter baumanii</em></td>
<td>Wound swabs</td>
<td>252.00/252.00</td>
</tr>
<tr>
<td>ATCC 196060</td>
<td>63.00/63.00</td>
<td>15.62/15.62</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>Wound swabs</td>
<td>63.00/126.00</td>
</tr>
<tr>
<td>ATCC 12453</td>
<td>31.50/63.00</td>
<td>62.50/125.00</td>
</tr>
<tr>
<td><em>Klebsiella sp.</em></td>
<td>Wound swabs</td>
<td>126.00/252.00</td>
</tr>
<tr>
<td>ATCC 10031</td>
<td>126.00/126.00</td>
<td>31.25/62.50</td>
</tr>
</tbody>
</table>
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.

Acknowledgements

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