Original article

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ANTIBACTERIAL ACTIVITIES OF FRUITS EXTRACTS OF THREE MULBERRY SPECIES (*MORUS ALBA L., MORUS RUBRA L. AND MORUS NIGRA L.*) AND BILBERRY (*VACCINIUM MYRTILLUS L.*)

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Delphinidin is a dominant anthocyanidin in bilberry. Antimicrobial activity of methanol extracts of the genus *Morus* showed that *M. nigra L.* extract was more active than extracts of other two species (*M. alba L.* and *M. rubra L.*). Minimal inhibitory and bactericidal concentration of *V. myrtillus* methanol extract was in the range of MIC/MBC = 15.75-252.00 mg/mL. Antimicrobial effect of the tested extracts was less potent against strains from wounds compared to ATCC strains as well Gram (-) bacteria compared to Gram (+) bacteria. The most sensitive strains were *S. epidermidis, S. pyogenes. P. mirabilis* and *S. aureus*.

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Key words: *Morus alba L., Morus rubra L., Morus nigra L., Vaccinium myrtillus L., antimicrobial activity*

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Introduction

Bilberry (*Vaccinium myrtillus L.*) is a shrub growing to 50 cm with elliptical leaves. Fruits are berries, globular, dark purple, juicy and sour (1). In some European countries, bilberry is one of the most economically important wild berry species (2). It is classified as a Class 1 herb by the American Herbal Products Association (3), which means that it can be safely consumed when it is used appropriately. *Morus alba L.* is a native tree in India, China, and Japan. It came to Europe a few centuries ago. The tree was introduced to America for silkworm cultivation in early colonial times and it was naturalized and hybridized with the local red mulberry. Red mulberry or American mulberry originates from the eastern part of the USA and black mulberry came from Asia. Red mulberry fruits arrived in Europe before Roman times. Black mulberry is distributed in Asia, Europe, North and South America and Africa (4, 5).

Various *Vaccinium species* (*V. myrtillus, V. vitisidaea, V. macrocarpon*) are used in phytomedicine and pharmacy. Fruits of these species may have additional health benefits because they are rich in phytochemicals such as anthocyanins responsible for their red, purple and blue colors. Bilberry fruits contain up to 10% tannins, anthocyanins, organic acids, and pectins. It contains 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) and stilbene (trans-resveratrol) (6, 7). Bilberry has higher anthocyanin content compared to other types of berries, such as strawberry, cranberry, elderberry, sour cherry, and raspberry (8-11). In traditional medicine, fruits of V. myrtillus are used as antidiarrheal (12).

There are papers on phytochemical analysis of leaves and fruits of bilberry plant (13, 14). There are, however, only a few reports on the effect of frozen storage on berry phenolics and their composition (15-17), although this knowledge is important because most Nordic berries are frozen due to the short harvesting season. Mulberry phenolics are investigated qualitatively (18), and our further investigations will be directed towards the quantitative definition of mulberry phenolics.

The control of human GI tract pathogens by diet or by natural medicinal components is actively examined (19-22). Minimal use of antibiotics is re-

commended due to the threat of the spread of antibiotic resistance among normal human GI tract microbial flora, and therefore alternative antimicrobial compounds are sought (21, 23). The antimicrobial activity of berry compounds drew the attention because of the recent studies which show that anthocyanins may protect against human pathogenic bacteria (24-30). Several mechanisms of action in the growth inhibition of bacteria are involved, such as destabilization of the cytoplasmic membrane, permeabilization of the plasma membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism and deprivation of the substrates required for microbial growth. Antimicrobial activities of berries may also be related to antiadherence of bacteria to epithelial cells, which is a prerequisite for colonization and infection of many pathogens (31). 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 (24).

Aims

The aims of our paper were investigations of anti-bacterial activities of fruits of three mulberry species (*Morus alba L., Morus rubra L. and Morus nigra L.*), and bilberry (*Vaccinium myrtillus L.*).

Material and methods

Extraction

Bilberries (*Vaccinium myrtillus L.*) were sampled at the fully ripe stage in July 2011 from woods from Koroska and Skofja Loka. They were stored at -20 0C for one week when extracts were prepared. The extraction method was modified and improved according to the already reported method (6). Frozen samples (600 g) were firstly homogenized in 2 L ice-cold deoxygenated methanol that had previously been flushed for a few minutes with nitrogen. The homogenate was extracted for 1h by shaking on mag netic stirrer IKA REO Basic C (Konigswinter, Germany) at room temperature. The extract was filtered by vacuum through the technical filter paper. The residue was extracted again in 1 L ice-cold deoxygenated methanol for 0.5h and the suspension was filtered as before. The third time the residue was extracted as described before. Finally, all three filtrates were pooled, flushed with nitrogen for a few minutes, and then stored at -20^{0}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 diode array (DAD) detector and also connected with LCQ Fleet Ion Trap Mass Spectrometer (Thermo Fisher Scientific, USA). The separations were performed on Hypersil gold C18 column (50 x 2.1 mm, 1.9 µm) of the same producer, at 25^{0}C. The mobile phase consisted of (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in acetonitrile. The next linear gradient program at flow rate of 0.250 ml/min has been applied: Method I: 10 % to 30 % (B) for the 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 90-100 % (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 the 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, as well as 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^{0}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^{0}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.

Micro-well dilution assay

Bacterial strains

Antimicrobial activity of investigated extracts was evaluated against laboratory control strains from ATCC collection,

Gram (+) bacteria:
- Staphylococcus aureus ATCC 6538,
- Staphylococcus epidermidis ATCC 12228,
- Streptococcus pyogenes ATCC 19615,
- Enterococcus faecalis ATCC 19433,
- Propionibacteri-um acnes ATCC 11827,

Gram (-) bacteria:
- Escherichia coli ATCC 9863,
- Pseudomonas aerugino-sa ATCC 9027,
- Acinetobacter baumannii ATCC 196060,
- Proteus mirabilis ATCC 12453,
- Klebsiella pneumoniae ATCC10031,

and against related strains isolated from human wound swabs.

Micro-well dilution method

Minimum inhibitory (MIC) and minimum bacteri cidal concentrations (MBC) of extracts were deter-

mined by employing the broth micro-well dilution method with some modifications (32). 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 0.02-100.00 mg/mL (*Morus alba L.*), 0.13 - 270.50 mg/mL (*Morus rubra L.*), 0.12 - 251.00 mg/mL (*Morus nigra L.*), and 0.06-126.00 mg/*mL (Vaccinium myrtillus L.*). The final volume was 100 µL and the final concentration of bacterial suspensions was 106 CFU/mL in each well. The plates were incubated for 24h at 37^0C. Metronidazole, Doxycycline, Ciprofloxacin, and Gentamicin were used as positive control (Sigma Aldrich, St Louis, MO, USA), and dilutions were prepared at concentrations ranging from 0.01 to 100 mg/mL. All determinations were performed in triplicates. Microbial growth was determined by adding 20 µL of 0.5 % triphenyl tetrazolium chloride (TTC) aqueous solution in microtitar plates. MIC was defined as the lowest concentration of the extracts at which the microorganisms showed no visible growth. In order to determine MBC, the broth was taken from each well and inoculated on Mueller Hinton agar (MHA) for 24h at 37^0C. The MBC is defined as the lowest concentration of the extracts at which 99.9 % of inoculated bacteria were killed.

Results and Discussion

The content of extracted anthocyanins was determined, and their quantities are presented in Table 1. The obtained results indicated that delphinidin is a dominant anthocyanidin in bilberry. Burdulis et al. (31) found cyanidin as a dominant anthocyanidin in their bilberry samples. Meanwhile, Moze et al. (6) identified 15 anthocyanins using LC - MS/MS from seven different locations in Slovenia, which contents were 1210.3 ± 111.5 mg CGE/100 g FW. The content of total anthocyanins in the Slovak Republic in bilberry was in the range from 5578 mg/kg to 2887.75 mg/kg (33).

Table 1. Individual anthocyanins in bilberries and their extract.

	Anthocyanins	[1]Extract concentration mg/L	[2]Mass concentration g/100 g	Percentage %
1.	delphinidin 3-galactoside	692.0 ± 1.1	153.4 ± 0.3	15.6
2.	delphinidin 3-glucoside	654.8 ± 1.5	145.1 ± 0.3	14.8
3.	cyanidin 3-galactoside	506.7 ± 1.6	112.3 ± 0.4	11.5
4.	delphinidin 3-arabinoside	595.6 ± 1.7	132.0 ± 0.4	13.5
5.	cyanidin 3-glucoside	495.5 ± 0.8	109.8 ± 0.2	11.2
6.	petunidin 3-galactoside	153.0 ± 1.0	33.9 ± 0.2	3.5
7.	cyanidin 3-arabinoside	298.3 ± 0.4	66.1 ± 0.1	6.7
8.	petunidin 3-glucoside	298.4 ± 7.7	66.1 ± 1.7	6.7
9.	peonidin 3-galactoside	38.6 ± 0.3	8.6 ± 0.1	0.9
10.	petunidin 3-arabinoside	95.5 ± 0.4	21.2 ± 0.1	2.2
11.	peonidin 3-glucoside	158.2 ± 0.3	35.1 ± 0.1	3.6
12.	malvidin 3-galactoside	88.2 ± 0.2	19.5 ± 0.0	2.0
13.	peonidin 3-arabinoside	14.7 ± 0.1	3.3 ± 0.0	0.3
14.	malvidin 3-glucoside	269.9 ± 0.8	59.8 ± 0.2	6.1
15.	malvidin 3-arabinoside	63.5 ± 1.5	14.1 ± 0.3	1.4

Data were quantified as mg of standard cyanidin 3-glucoside equivalents per [1]L of bilberry extract or [2]100 g of fresh bilberries.
Data were expressed as mean ± SEM, a number of independent measurements was n=3.

The obtained results (Table 2a, 2b) for antimicrobial activity of methanol extracts of the genus *Morus* showed that M. nigra extract was more active than extracts of other two species. This extract had MIC = 31.26-125.05 mg/mL, and MBC = 125.05-251.00 mg/mL. The highest tested concentration of the extract showed no bactericidal activity against most strains isolated from wounds such as *S. aureus, S. pyogenes, E. faecalis, E. coli, P. aeruginosa* and *K. pneumoniae*, as well as against the ATCC strains, *P. acnes* and *K. pneumoniae*. The best activity of the extract was observed against strains *S. epidermidis* ATCC 12228 and *P. mirabilis* from swabs of wounds, MIC / MBC = 62.52/125.05 mg /mL, and against *S. pyogenes* ATCC 19615, MIC / MBC = 31.26/251.00 mg/mL. *M. alba* extract has a weak inhibitory activity at the highest tested concentration against 73 % of investigated strains. The bactericidal activity was present only against *S. aureus, S. epidermidis* and *S. pyogenes* (MIC = MBC = 100

mg/mL). Extract of *M. rubra* had no inhibitory or bactericidal effect against all tested bacterial strains.

Minimal inhibitory and bactericidal concentration of *V. myrtillus* methanol extract (Table 2a, 2b) was in the range of MIC/MBC = 15.75 - 252.00 mg/mL. The best activity was against *S. epidermidis* ATCC 12228, (MIC = MBC = 15.75 mg/mL) and *S. Epidermidis* isolated from wound swabs (MIC/MBC = 15.75/31.50 mg/ml), against *E. faecalis* from wound (MIC = MBC = 31.50 mg/mL) and against *S. Pyogenes* ATCC 19615 and *P. mirabilis* ATCC 12453 (MIC / MBC = 31.50/63.00 mg/mL, respectively).

Table 2a. Antimicrobial activity of methanol extracts of *Morus species* and *Vaccinium myrtillus L*. against pathogenic bacterial strains (MIC/MBC in mg/mL)

Bacterial strains		**Methanol extracts**			
Isolated and ATCC strains		*Morus nigra* L.	*Morus alba* L.	*Morus rubra* L.	*Vaccinium myrtillus* L.
Gram (+)	**Source**				
Staphylococcus aureus	Wound swabs	125.05/>251.00	100.00/>100.00	>270.50/>270.50	63.00/63.00
Staphylococcus aureus	ATCC6538	62.52/251.00	100.00/100.00	>270.50/>270.50	63.00/63.00
Staphylococcus epidermidis	Wound swabs	62.52/251.00	100.00/100.00	>270.50/>270.50	15.75/31.50
Staphylococcus epidermidis	ATCC12228	62.52/125.05	100.00/>100.00	>270.50/>270.50	15.75/15.75
Streptococcus pyogenes	Wound swabs	31.26/>251.00	>100.00/>100.00	>270.50/>270.50	31.50/126.00
Streptococcus pyogenes	ATCC19615	31.26/251.00	100.00/100.00	>270.50/>270.50	31.50/63.00
Enterococcus faecalis	Wound swabs	125.05/>251.00	100.00/>100.00	>270.50/>270.50	31.50/31.50
Enterococcus faecalis	ATCC19433	125.05/251.00	>100.00/>100.00	>270.50/>270.50	63.00/126.00
Propionibacterium acnes	ATCC11827	125.05/>251.00	100.00/>100.00	>270.50/>270.50	126.00/126.00
Gram (-)	**Source**				
Escherichia coli	Wound swabs	125.05/>251.00	100.00/>100.00	>270.50/>270.50	63.00/126.00
Escherichia coli	ATCC9863	125.05/251.00	>100.00/>100.00	>270.50/>270.50	31.50/126.00
Pseudomonas aeruginosa	Wound swabs	125.05/>251.00	>100.00/>100.00	>270.50/>270.50	31.50/252.00
Pseudomonas aeruginosa	ATCC9027	125.05/251.00	>100.00/>100.00	>270.50/>270.50	31.50/126.00
Acinetobacter spp.	Wound swabs	125.05/125.05	100.00/>100.00	>270.50/>270.50	252.00/252.00
Acinetobacter baumannii	ATCC196060	125.05/251.00	100.00/>100.00	>270.50/>270.50	63.00/63.00
Proteus mirabilis	Wound swabs	62.52/125.05	>100.00/>100.00	>270.50/>270.50	63.00/63.00
Proteus mirabilis	ATCC12453	125.05/251.00	>100.00/>100.00	>270.50/>270.50	31.50/63.00
Klebsiella spp.	Wound swabs	125.05/>251.00	100.00/>100.00	>270.50/>270.50	126.00/252.00
Klebsiella pneumoniae	ATCC10031	125.05/>251.00	>100.00/>100.00	>270.50/>270.50	126.00/126.00

Table 2b. Referent antibiotics against pathogenic bacterial strains (MIC/MBC in mg/mL)

Bacterial strains		**Antibiotics**			
Isolated and ATCC strains		Metronidazole	Doxycyclin	Ciprofloxacin	Gentamicin
Gram (+)	**Source**				
Staphylococcus aureus	Wound swabs	3.91/15.62	7.81/7.81	1.26/1.26	0.60/0.60
Staphylococcus aureus	ATCC6538	15.62/31.25	7.81/15.61	1.26/2.52	0.60/0.60
Staphylococcus epidermidis	Wound swabs	7.81/31.25	3.91/3.91	0.63/0.63	0.30/0.30
Staphylococcus epidermidis	ATCC12228	7.81/62.50	3.91/7.81	0.63/0.63	0.30/0.30
Streptococcus pyogenes	Wound swabs	7.81/7.81	0.06/0.12	0.16/0.16	0.30/0.30
Streptococcus pyogenes	ATCC19615	0.98/15.62	0.06/0.12	0.16/0.16	0.30/0.30
Enterococcus faecalis	Wound swabs	7.81/62.50	0.25/0.25	0.30/0.30	0.08/0.08
Enterococcus faecalis	ATCC19433	3.91/62.50	0.25/0.49	0.30/0.30	0.16/0.16
Propionibacterium acnes	ATCC11827	0.98/15.62	15.61/15.61	2.50/ >20.00	2.50/ >20.00
Gram (-)	**Source**				
Escherichia coli	Wound swabs	15.62/31.25	7.81/7.81	2.50/2.50	2.50/20.00
Escherichia coli	ATCC9863	31.25/31.25	7.81/15.61	2.50/2.50	2.50/20.00
Pseudomonas aeruginosa	Wound swabs	15.62/62.50	7.81/15.61	0.02/0.63	2.50/10.00
Pseudomonas aeruginosa	ATCC9027	31.25/31.25	15.61/15.61	0.02/0.63	2.50/10.00
Acinetobacter spp.	Wound swabs	1.95/62.50	15.61/15.61	10.00/20.00	10.00/20.00
Acinetobacter baumannii	ATCC196060	15.62/15.62	15.61/15.61	10.00/20.00	10.00/20.00
Proteus mirabilis	Wound swabs	7.81/7.81	7.81/15.61	10.00/20.00	1.25/5.00
Proteus mirabilis	ATCC12453	62.50/125.00	7.81/15.61	10.00/20.00	5.00/10.00
Klebsiella spp.	Wound swabs	31.25/125.00	15.61/15.61	0.63/20.00	2.50/10.00
Klebsiella pneumoniae	ATCC10031	31.25/62.50	15.61/15.61	10.00/20.00	10.00/20.00

Minimal inhibitory and bactericidal concentration of *V. myrtillus* methanol extract (Table 2a, 2b) was in the range of MIC/MBC = 15.75-252.00 mg/mL. The best activity was against *S. epidermidis* ATCC 12228, (MIC = MBC = 15.75 mg/mL) and *S. Epidermidis* isolated from wound swabs (MIC/MBC = 15.75 / 31.50 mg/ml), against *E. faecalis* from wound (MIC = MBC = 31.50 mg/mL) and against *S. pyogenes* ATCC 19615 and *P. mirabilis* ATCC 12453 (MIC / MBC = 31.50/63.00 mg/mL, respectively).

Conclusion

Methanol extracts of *V. myrtillus* exhibited better antimicrobial activity compared to the methanolic extract of *M. nigra*, whereas the extracts of *M. alba* and *M. rubra* had weak or no effect. In general, the antimicrobial effect of the tested extracts was less potent against strains from wounds compared to ATCC strains as well Gram (-) bacteria compared to Gram (+) bacteria. The most sensitive strains were *S. epidermidis, S. pyogenes. P. mirabilis* and *S. aureus* and therefore the fruit extracts of the investigated plant species may find use as additives in creams for skin care and protection.

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ANTIBAKTERIJSKE AKTIVNOSTI EKSTRAKATA PLODOVA TRI VRSTE DUDA (*MORUS ALBA L., MORUS RUBRA L.* I *MORUS NIGRA L.*) I BOROVNICE (*VACCINIUM MYRTILLUS L.*)

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Delfinidin je dominantni antocijanidin u borovnici. Antimikrobna aktivnost metanolnih ekstrakata roda *Morus* je pokazala da je *M. nigra L*. ekstrakt aktivniji nego ekstrakti druge dve vrste (*M. alba L.* i *M. rubra L*.). Minimalna inhibitorna i baktericidna koncentracija *V. myrtillus L*. metanolnog ekstrakta bila je u rasponu MIC/MBC = 15,75-252,00 mg/mL. Antimikrobni efekat testiranih ekstrakata je bio slabiji prema sojevima iz rana u poređenju sa ATCC sojevima, kao i Gram (-) bakterijama u poređenju sa Gram (+) bakterijama. Najsenzitivniji sojevi su bili *S. epidermidis, S. pyogenes., P. mirabilis* i *S. aureus*.

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Ključne reči: *Morus alba L, Morus rubra L, Morus nigra L, Vaccinium myrtillus L, antimikrobna aktivnost*