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ARTICLE

Antimicrobial activity of fruits from some cultivar varieties of *Rubus idaeus* and *Rubus occidentalis*

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Raspberries, derived from different cultivar varieties, are a popular ingredient of everyday diet, and their biological activity is a point of interest for researchers. The ethanol-water extracts from four varieties of red (*Rubus idaeus* 'Ljulin', 'Veten', 'Poranna Rosa') and black (*Rubus occidentalis* 'Litacz') raspberries were evaluated in the range of their antimicrobial properties as well as phenolic content – sanguin H-6, free ellagic acid and anthocyanins. The antimicrobial assay was performed with the use of fifteen strains of bacteria, both gram-negative and gram-positive. The antimicrobial activity of the extracts varied and depended on the analysed strain of bacteria and cultivar variety, with the exception of *Helicobacter pylori*, towards which the extracts displayed the same growth inhibiting activity. Two human pathogens *Corynebacterium diphtheriae* and *Moraxella catarrhalis* proved to be the most sensitive to raspberry extracts. Contrary to the extracts, sanguin H-6 and ellagic acid were only active against eight and nine bacterial strains, respectively. The determined MIC and MBC values of both compounds were several times lower than the tested extracts. The highest sensitivity of *Corynebacterium diphtheriae* to extracts from both black and red raspberries may be due to its sensitivity to sanguin H-6 and ellagic acid.

Keywords: *Rubus idaeus*, *Rubus occidentalis*, fruits, cultivar varieties, chemical composition, sanguin H-6, ellagic acid, antimicrobial activity

Introduction

Raspberries, originating from the genus *Rubus* (*Rosaceae*), are a popular ingredient of everyday diet. In addition to being food products, raspberries are also popular folk remedies in Eastern Europe. Red raspberries have been used for centuries to treat common cold, fever and flu-like infections¹. Nowadays, there is also a number of dietary supplements containing raw red raspberry extracts, which are used to support the immune system and reduce the duration of upper respiratory tract infections.

It has been shown that many secondary metabolites found in dietary and medicinal plants exhibit antimicrobial activities, and research of berry fruits points out the genus *Rubus* as a potential source of antibacterial agents²⁻¹¹.

Raspberries, derived from different cultivar varieties, are rich in phenolic compounds, mainly anthocyanins and ellagitannins^{10, 12}. Other phenols, such as flavonoids and phenolic acids, including free ellagic acid, occur in significantly lower concentrations³. The main group of raspberry chemical constituents are ellagic acid conjugates named ellagitannins^{3,}

¹³. The characteristic and predominant ellagitannin in *Rubus* berries is a dimeric HHDP (hexahydroxydiphenic) form called sanguin H-6, which is accompanied by a tetrameric HHDP form called lambertianin D¹⁴.

Antimicrobial properties of raspberries are being attributed mainly to the presence of ellagitannins^{6, 15}. Despite a few papers describing the antimicrobial properties of ellagitannin-rich fractions from raspberry fruits^{3, 4}, there is no research concerning raw extracts activity against human pathogenic bacteria responsible for respiratory tract, digestive tract and skin infections. There is also, to our knowledge, no research concerning the estimation of antimicrobial properties of raspberry extracts, with respect to their sanguin H-6 and free ellagic acid content. The antimicrobial activity of pure sanguin H-6 has also not been studied up to date.

The aim of the study was to evaluate and compare the antimicrobial activity of raw fruit extracts from three cultivar varieties of red raspberries – *Rubus idaeus* 'Ljulin', 'Veten', 'Poranna Rosa' and one variety of black raspberry – *Rubus occidentalis* 'Litacz' against sanguin H-6 and ellagic acid

activity towards fifteen strains of common human pathogenic bacteria.

Results and Discussion

Anthocyanin and sanguin H-6 content of the raw fruit extracts

The composition and content of anthocyanins, sanguin H-6 and ellagic acid in the investigated red and black raspberries were determined by validated HPLC-DAD-ESI-MS method (Online Supplement 1). In accordance with literature data it was confirmed that the yellow fruit 'Poranna Rosa' variety does not contain anthocyanins, and ellagitannins are its main group of biologically active compounds¹⁶. The chemical composition of three among the analysed cultivars, namely *R. idaeus* 'Ljulin' and *R. occidentalis* 'Litacz' has been recognized for the first time, and is in agreement with the anthocyanin set described in other varieties of red¹⁷⁻²⁰ and black raspberries¹⁷⁻²⁰.

The black raspberry extract from *R. occidentalis* 'Litacz' was found to contain about five times more anthocyanins (5511.9 mg/100g of extract) than the fruit extracts of the red varieties 'Ljulin' and 'Veten'. In the fruits of *R. occidentalis* 'Litacz' cyanidin 3-O-rutinoside, cyanidin 3-O-sambubioside and cyanidin 3-O-(2^G-xylosylrutinoside) were the dominant compounds. The fruits of *R. idaeus* 'Ljulin' contained cyanidin 3-O-sophoroside as the main anthocyanin, while in the fruits of 'Veten' variety, cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside and cyanidin 3-O-sophoroside were the dominating cyanidin glycosides (Tab. 1).

Similar level of sanguin H-6 content were observed in the fruits of 'Litacz', 'Poranna Rosa' and 'Ljulin' varieties (1078.7-1482.5 mg/100 g), while it was three to five times higher in cultivar 'Veten' (5045.2 mg/100 g). The fruit extracts from 'Litacz', 'Poranna Rosa' and 'Ljulin' varieties contained from 23.9 to 29.5 mg/100 g of free ellagic acid, while the amount in the 'Veten' was approximately two times higher (49.8 mg/100 g) (Tab. 1).

Antimicrobial activity of pure compounds and fruit extracts

Antimicrobial properties were analysed against fifteen human pathogenic bacterial strains, both gram-positive and gram-negative. Sensitivity of 10 bacterial strains to raspberry extracts was investigated for the first time^{21,22}. Broth microdilution was used as a more accurate method to determine the antimicrobial activity of plant extracts²³.

Antimicrobial activity of the analysed fruit extracts was compared to that of sanguin H-6 and ellagic acid (Tab. 2). Both compounds proved to be active only against some of the tested bacteria in concentrations ranging from 0.015 mg/ml to 0.5 mg/ml. Sanguin H-6 was effective against eight bacterial strains, displaying bactericidal activity against *Streptococcus* group A, *Streptococcus pneumoniae*, *Corynebacterium diphtheriae* and *Staphylococcus epidermidis*, and inhibitory activity against *Bacillus subtilis*, *Clostridium sporogenes*, *Staphylococcus aureus* and *Moraxella catarrhalis* (Tab. 2). Ellagic acid showed activity towards the same strains as sanguin H-6, with the exception of no antimicrobial activity against *S. pneumoniae* and *S. epidermidis*, but displaying inhibitory activity against *Neisseria meningitidis* (MIC 0.06 mg/ml). Sanguin H-6 with MBC value of 0.03 mg/l was the most active against *C. diphtheriae* – a bacterium causing different types of diphtheria affecting respiratory tract and skin²⁴. Bactericidal activity of ellagic acid towards *C. diphtheriae* was at the same level as sanguin H-6 (MBC 0.03 mg/ml) (Tab.

2). *M. catarrhalis* – an exclusively human commensal and common respiratory tract pathogen²⁵, proved to be the most sensitive to ellagic acid (MBC 0.015 mg/ml). Our research confirms ellagic acid activity towards *Helicobacter pylori*, however only as an inhibiting agent (MIC 0.125 mg/ml). On the other hand, the dimeric sanguin H-6 did not exhibit any antimicrobial activity against *H. pylori*. According to Funatogawa et al., among hydrolysable tannins only monomeric forms are the most effective and promising *H. pylori* inhibitors²⁶.

Our results have shown varied sensitivities of the tested bacteria to raspberry fruit extracts, their MBC values ranging from 0.5 mg/ml to 128 mg/ml (Tab. 2). *R. idaeus* 'Poranna Rosa' and 'Ljulin' fruit extracts proved to be the most effective among the analysed extracts displaying the same MBC values towards seven studied microorganisms, despite the discrepancies in the presence of anthocyanins. One of the differences between the both extracts was a lack of *R. idaeus* 'Poranna Rosa' activity towards *N. meningitidis*. This can be probably explained by the fact that 'Poranna Rosa' contains sanguin H-6 as the main compound, which proved to be inactive against *N. meningitidis*. On the other hand, 'Poranna Rosa' extract possessed bactericidal activity against bacteria insensitive to sanguin H6, namely *Streptococcus* group B and *G. Streptococcus* group B, the β -hemolytic group which only member is *S. agalactiae* – causes pneumonia and meningitis in neonates and children, and in recent decades increasing cases of invasive infections among adults²⁷.

Fruits of the 'Veten' variety displayed the most effective bactericidal properties against two studied microorganisms, namely *S. pneumoniae* (MBC 8.0 mg/ml) and *C. diphtheriae* (MBC 0.5 mg/ml). The strong bactericidal activity of the 'Veten' extract towards *C. diphtheriae* can be attributed to its high sanguin H-6 content as well as the presence of ellagic acid (Tab. 1), as both pure compounds proved to be strongly bactericidal to *C. diphtheriae* (Tab. 2). Similarly, the bactericidal activity of *R. idaeus* 'Veten' extract towards *S. pneumoniae* – one of the most common causes of pneumonia, bacterial meningitis and otitis media²⁸, can also be correlated with its high sanguin H-6 content, and sensitivity of the bacterium to sanguin H-6 (MBC 0.5 mg/ml).

Besides *C. diphtheriae*, *M. catarrhalis* seemed to be particularly sensitive to the raspberry extracts. However, despite the fact that *M. catarrhalis* was the most sensitive bacterium to pure ellagic acid (MBC 0.015 mg/ml), no clear association with free ellagic acid content and MIC and MBC values of the analysed extracts could be made.

R. occidentalis 'Litacz' was the least effective fruit extract, which showed the lowest MBC value for only *N. meningitidis* – bacterium which can cause life-threatening meningococcal diseases including cephalomeningitis and meningococemia²⁹.

Our results show a moderate growth inhibiting effect of raspberry fruit extracts against *H. pylori*, which was the same for all analysed varieties (MIC 8 mg/ml). Low pH (between 3 and 5) is a parameter considered in the antimicrobial action of fruit extracts against gram-negative bacteria [7, 24]. Compared to the *R. idaeus* 'Poranna Rosa' extract, the *R. occidentalis* 'Litacz' extract proved to be almost two times less acidic (2.3 and 1.3 respectively), and had higher pH (3.2 and 3.8 respectively) which remained without effect on MIC and MBC values towards *H. pylori* (Tab. 2). The inhibitory activity of the fruit extracts towards *H. pylori* may be connected to their content of free ellagic acid. Ellagic acid is mentioned among the compounds that could contribute to the antimicrobial

activity of raspberries. It has been proposed that by binding to bacterial membrane, ellagic acid can destabilize its structure, therefore disrupting cell functionality³⁰. Li et al. reports of raspberry-enriched alcoholic beverages having greater antimicrobial potential against *H. pylori* than pure alcohol³¹. The authors suggest that this effect is due to raspberry extracts damaging the bacterial cell membranes, therefore making them more sensitive to alcohol.

Puupponen-Pimia et al. stated, that the antimicrobial activity of raspberries may be due to their high ellagitannin content⁴. While we presented some cases where antimicrobial activity may be attributed to high sanguin H-6 content, as the dominant ellagitannin, our studies clearly show that it is difficult to attribute the antimicrobial effectiveness of the *Rubus* fruits to sanguin H-6 content alone. One such case is *Streptococcus* group A where the same bactericidal concentrations were observed for three cultivars, namely 'Ljulin', 'Veten' and 'Poranna Rosa', which significantly varied in sanguin H-6 content. The anthocyanin-rich *R. occidentalis* 'Litacz' extract proved to have the weakest antimicrobial properties towards *Streptococcus* group A, although it contained comparable levels of sanguin H-6 and ellagic acid to the more effective 'Ljulin' and 'Poranna Rosa' varieties. It can be explained by the fact that sanguin H-6 contribution to the complex of biologically active compounds is lower in comparison to the other extracts (Tab. 1).

This fact suggests that, in some cases, high anthocyanin content may adversely affect antimicrobial activity (Tab. 2). Participation of anthocyanins in antimicrobial activity of raspberry extracts is not clear, however Vuorela et al. reports of anthocyanins inhibiting the growth of *Lactobacillus acidophilus*, a gram-positive bacterium³.

Puupponen-Pimia et al. also suggest that berry extracts inhibit gram-negative bacteria but not gram-positive bacteria, which may be attributed to the differences in cellular wall structures⁴. This is not in agreement with our findings as we found gram-positive bacteria sensitive to the raspberry extracts, including *C. diphtheriae* – the most sensitive strain in our study (Tab. 2). All four tested extracts revealed the strongest bactericidal activity towards *C. diphtheriae* (MBC 0.5-4.0 mg/ml), which can suggest synergistic action of sanguin H-6 and ellagic acid.

Experimental

Plant Material

The fruits from *R. idaeus* 'Ljulin' and 'Veten' (red varieties) were obtained from the Experimental Station of Variety Assessment in Masłowice and the Research Institute of Pomology and Floriculture in Skierniewice (Poland), respectively. The fruits of *R. idaeus* 'Poranna Rosa' (yellow variety) and *R. occidentalis* 'Litacz' (black variety) were obtained from the Experimental Fruit-Growing Station of the Research Institute of Pomology and Floriculture in Brzezna (Poland).

Dry extract preparation

The frozen fruits were lyophilised and powdered using an electric mill. The powdered plant material (2g) was extracted three times using 30 ml mixture of ethanol : water (80:20 v/v), in a sonic bath for 10 min (Sanorex Digitec, Bandelin), followed by a centrifugation period (20 min, 5000 RPM, MPW-2 Centrifuge). Ethanol was evaporated from the combined extracts (Unipan Water Bath type 356P) and the dried residue

was dissolved in water and lyophilized. The extracts were stored in sealed containers at 9°C.

Acidity and pH

Titrate acidity was determined according to a Polish Norm PN – EN 12147:2000, by titrating a known volume of fruit juice with 0.1 M NaOH until pH reached 8.1. Total acidity was expressed in g/100g of juice calculated per malic acid equivalents. The pH was determined using a refractometer ATAGO PR 101 α ³².

HPLC-DAD-ESI-MS analysis

To evaluate anthocyanin, sanguin H6 and ellagic acid content a HPLC-DAD-ESI-MS analysis was performed using Shimadzu HPLC system consisting of CBM-20 system controller, steel wash pump LC-20AD, online degasser DGU-20A5, auto-sampler SIL 20AC, column thermostat CT0-20AC, DAD and ESI-MS detectors. Constituents of the samples were separated using a Discovery HS C18 column (150 mm x 2,1 mm, 3 μ m) (Supelco, Bellefonte, USA). Column temperature was set at 32°C. The mobile phases consisted of 0.1% aqueous TFA solution (A) and 0.1% TFA solution in a mixture of water:acetonitrile (50:50 v/v) (B). The gradient elution programme was as follows: 0 min, 12% B; 10 min, 20% B; 30 min, 43% B; 40 min, 100% B; 55 min, 100% B; 60 min, 12% B; 75 min, 12% B. Flow rate was 0.3 ml/min. The injection volume was 1 μ l. ESI-MS analysis was conducted in both positive and negative mode using SIM technique and following parameters of analysis: nebulizing gas flow 1.5 l/min, drying gas flow 16 l/min, detector voltage 1.6 kV, interface voltage 3.5 kV were used. The chromatograms were registered at λ =280 nm for anthocyanins and at λ =520 nm for sanguin H-6. Phenolic compounds were identified by comparing their retention time values (t_R) to that of the standard compounds and by comparison of their mass spectra with literature data¹⁷⁻²⁰. The standard compounds were obtained from Extrasynthese (France). Sanguin H6 was isolated from the leaves of *Rubus fruticosus* in accordance with previously described method¹⁸.

Test microorganisms

Gram positive bacteria. β -hemolytic *Streptococcus* group A,B,G, *Streptococcus pneumoniae* (clinical isolates), *Corynebacterium diphtheriae*, *Enterococcus faecalis* (collection of the Department of Pharmaceutical Microbiology, Medical University of Gdańsk), *Staphylococcus aureus* ATCC9027, *Staphylococcus epidermidis* ATCC14990, *Bacillus subtilis* ATCC6633, *Clostridium sporogenes* PCM2486.

Gram negative bacteria. *Klebsiella pneumoniae* (clinical isolate), *Neisseria meningitidis* PCM2586, *Moraxella catarrhalis* PCM2340, *Haemophilus influenzae* PCM2340, *Helicobacter pylori* ATCC10231. Clinical isolates were obtained from St. Adalbert Specialist Hospital in Gdańsk (Independent Public Health Care Facility in Gdańsk, Poland).

Antibacterial assay

Bacterial cultures were prepared by transferring cells from the stock cultures to a tube with adequate broth as described in the literature³³⁻³⁶, and were incubated for 24-48 hours at 37°C. The cultures were diluted to achieve an optical density corresponding to 10⁵ colony forming units per ml (CFU/ml) for all bacteria species, with the exception of *H. pylori*. For *H.*

pylori, the inoculum was prepared from colonies grown on TSA blood agar plates with final concentration of approximately 10^5 CFU/ml³⁷.

Minimum inhibitory concentration (MIC) was determined by broth microdilution technique using 96-well plates. After filling each well with 100 μ l of broth, dry fruit extract samples from four raspberry varieties were dissolved in water to a final concentration of 512 mg/ml. 100 μ l of each extract solution were added to the first well of each microtiter line. Then the dilutions proceeded in geometric progression by transferring 100 μ l of the solution to the next well. The microbial suspensions (100 μ l) of the tested bacterial strains were added to each well. The final concentrations of the extracts used to the antimicrobial activity ranged from 128 to 0.5 mg/ml. Sanguin H6 and ellagic acid were also diluted in a geometric progression (concentrations from 1 to 0.015 mg/ml). The plates were incubated in the conditions adequate for each bacterium as described in the literature³³⁻³⁶.

After incubation a visual observation of growth was performed. The MIC was established as the lowest sample concentration that prevented visible growth³⁸. In addition 100 μ l of suspension from each well without visible growth were inoculated (48 hours) on an agar plate to check bacterial viability. MBC (minimal bactericidal concentration) was defined as the minimum concentration of extract required to kill the bacteria in the medium. For determining *Helicobacter pylori* viability Christiansen broth (urease test) was used.

Conclusions

Raspberry fruits proved to be active against all analysed bacteria, both gram-positive and gram-negative. Sensitivity of the bacterial strains to the raspberry extracts varied greatly with *C. diphtheriae* and *M. catarrhalis* being the most sensitive. However, the obtained results suggest that the antimicrobial activity of raspberry fruits cannot be attributed exclusively to sanguin H-6 – which is the main ellagitannin in raspberries. The participation of other compounds present in raspberries, possibly anthocyanins, and their contribution to antimicrobial activity should also be explained.

Possible limitations of this study are associated with the bioavailability of raspberry compounds. Anthocyanins have been reported in lung³⁹ and brain tissue⁴⁰, while other literature data suggest that they are poorly absorbed and excreted unmetabolised⁴¹⁻⁴³. Ellagitannins are also reported not to be absorbed in the small intestine, but extensively metabolised by colonic microflora to form urolithins^{44, 45}. Therefore it is difficult to predict and estimate if the compounds will be able to reach the infection sites at a desired concentration. The usefulness of the present research refers to local administration of raspberry extract (in the form of lozenges for example), which could prevent or limit pathogens from reaching infection sites.

Red and black raspberry fruits seem to be valuable dietary constituents that could be used in the prophylaxis of infective diseases of the respiratory and digestive tract. Therefore they may be of interest to supplement markets and pharmaceutical industries.

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Notes and references

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Table 1. Determined anthocyanin, sanguin H6 and ellagic acid content in the analysed raspberry extracts (mg/100g dry weight) by HPLC/DAD/ESI-MS method

	<i>R. occidentalis</i> 'Litacz'	<i>R. idaeus</i> 'Ljulin'	<i>R. idaeus</i> 'Veten'	<i>R. idaeus</i> 'Poranna Rosa'
cyanidin 3,5-di-O-glucoside	35.7 ± 0.8	-	-	-
cyanidin 3-O-sophoroside	80 ± 4.2	989.7 ± 25.3	252.3 ± 5.4	-
cyanidin 3-O-(2 ^G -glucosylrutinoside)	-	x	88.5 ± 4.7	-
cyanidin 3-O-sambubioside	576.9 ± 20.2	63 ± 17.8	39 ± 10.7	-
cyanidin 3-O-glucoside	399.9 ± 12.8	184.4 ± 24.8	244.5 ± 26.6	-
cyanidin 3-O-rutinoside	2538.6 ± 214.3	x	264.8 ± 20.6	-
pelargonidin 3-O-glucoside	36.9 ± 4.8	21.8 ± 8.6	x	-
pelargonidin 3-O-rutinoside	87.8 ± 3.7	-	x	-
cyanidin	2.6 ± 0.4	-	x	-
pelargonidin 3-O-sophoroside	-	69.3 ± 5.4	x	-
cyanidin 3-O-(2 ^G -xylosylrutinoside)	826.4 ± 68.7	-	-	-
total anthocyanins	5511.9 ± 329.1	1328.2 ± 81.8	889.1 ± 67.9	-
sanguin H6	1482.5 ± 70.5	1366.8 ± 18.5	5045.2 ± 470.4	1078.7 ± 16.6
ellagic acid	29.5 ± 3.1	27.9 ± 2.2	49.8 ± 2.5	23.9 ± 4.2

'x' - concentration of the compound present below the limit of detection

'-' - compound not detected

Table 2. Antimicrobial activity of raspberry extracts, sanguin H6 and ellagic acid (mg/ml)

	<i>R. occidentalis</i> 'Litacz'		<i>R. idaeus</i> 'Ljulin'		<i>R. idaeus</i> 'Veten'		<i>R. idaeus</i> 'Poranna Rosa'		Sanguin H6		Ellagic acid	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Streptococcus</i> group A	32.0	32.0	16.0	16.0	16.0	16.0	16.0	16.0	0.5	0.5	1.0	1.0
<i>Streptococcus</i> group B	>128	>128	32.0	32.0	64.0	128.0	32.0	32.0	>1	>1	>1	>1
<i>Streptococcus</i> group G	64.0	64.0	16.0	16.0	16.0	16.0	16.0	16.0	>1	>1	>1	>1
<i>Streptococcus pneumoniae</i>	32.0	32.0	4.0	16.0	4.0	8.0	16.0	16.0	0.5	0.5	>1	>1
<i>Enterococcus faecalis</i>	64.0	64.0	128.0	128.0	128.0	128.0	16.0	16.0	>1	>1	>1	>1
<i>Corynebacterium diphtheriae</i>	1.0	1.0	4.0	4.0	0.5	0.5	4.0	4.0	0.03	0.03	0.03	0.03
<i>Bacillus subtilis</i>	32.0	>128	16.0	16.0	32.0	>128	32.0	>128	0.5	>1	0.06	>1
<i>Clostridium sporogenes</i>	4.0	>128	16.0	16.0	4.0	>128	16.0	>128	0.06	>1	0.06	>1
<i>Staphylococcus aureus</i>	8.0	16.0	8.0	8.0	4.0	8.0	8.0	8.0	0.25	>1	0.5	>1
<i>Staphylococcus epidermidis</i>	4.0	8.0	4.0	8.0	4.0	8.0	8.0	8.0	0.125	0.125	>1	>1
<i>Neisseria meningitidis</i>	16.0	16.0	16.0	32.0	32.0	32.0	>128	>128	>1	>1	0.06	>1
<i>Moraxella catarrhalis</i>	8.0	8.0	4.0	4.0	4.0	8.0	2.0	4.0	0.5	>1	0.015	0.03
<i>Haemophilus influenzae</i>	32.0	32.0	32.0	32.0	32.0	32.0	16.0	16.0	>1	>1	>1	>1
<i>Helicobacter pylori</i>	32.0	32.0	8.0	16.0	8.0	16.0	8.0	32.0	>1	>1	0.125	>1
<i>Klebsiella pneumoniae</i>	128.0	>128	32.0	>128	32.0	128.0	32.0	64.0	>1	>1	>1	>1