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INHIBITION OF THE GROWTH OF GRAM-NEGATIVE BACTERIA BY ANTHOCYANINS OF BERRIES FRUITS

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Inhibition of the growth of gram-negative bacteria by anthocyanins of berries fruits. – Konečná M., Sedlák V., Tkáčiková Ľ., Kšonžeková P., Mydlárová-Blaščáková M., Gruľová D., Gaľová J., Gogaľová Z., Babejová A., Vašková H., Avuková A., Mirutenko V.S., Nagy M., Poráčová J. – *Plants contain biologically active compounds useful in the prevention and therapy of different types of diseases. Polyphenol compounds, especially anthocyanins are the subject of extensive research in view of their biological activities. Anthocyanins are rich in their antimicrobial effects especially. The aim of this study was to assess the antimicrobial effect of extracts rich on anthocyanins prepared from the berry fruit (Vaccinium myrtillus L., Aronia melanocarpa L. and Sambucus nigra L.) by acetone and ethanol extraction. The effect of the extracts on the growth of Gram-negative bacteria (Escherichia coli, Salmonella enterica ser. Typhimurium, Pseudomonas aeruginosa, Enterobacter aerogenes) using the plate diffusion method was evaluated. Based on the results it can be stated that the anthocyanins isolated from berries reached some degree of antimicrobial activity. The bacterial strain of E. coli was the most sensitive to the action of tested extracts. The best antimicrobial activity was found in Vaccinium myrtillus ethanol extracts against gram-negative bacteria. Ethanol extracts were more effective compared to the acetone extracts. In conclusion, the berry fruits important in human nutrition as an important group of natural antioxidants. It can also acts as preventive and therapeutic agents for various bacterial infections.*
Keywords: *agar diffusion method, antimicrobial activity, bacterial strains, plant extracts.*
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Пригнічення росту грамнегативних бактерій антоціанами ягідних плодів. – Конечна М., Седлак В., Ткачікова Л., Кшонжекова П., Мидларова-Блашчакова М., Ґрульова Д., Ґальова Я., Ґоґальова Ж., Бабейова А., Вашкова Г., Авукова А., Мірутенко В.С., Нодь М., Порачова Я. – *Рослини містять біологічно активні сполуки, що можуть бути використання для профілактики та лікування різних видів захворювань. Поліфенольні сполуки, особливо антоціани, є предметом поглиблених досліджень з огляду на їх біологічну активність. Особливо важливим є в цьому відношенні є антимікробна дія антоціанів. Метою цього дослідження було оцінити антимікробну дію екстрактів, багатих на антоціани, які були виготовлені з плодів Vaccinium myrtillus L., Aronia melanocarpa L. та Sambucus nigra L. шляхом екстракції ацетоном та етанолом. Оцінено вплив екстрактів на ріст грамнегативних бактерій (Escherichia coli, Salmonella enterica ser. Typhimurium, Pseudomonas aeruginosa, Enterobacter aerogenes) методом дифузної пластинки. На підставі отриманих результатів можна стверджувати, що виділені з ягід антоціани проявляють, в певній мірі, антимікробну активність. Штам бактерій E. coli був найбільш чутливим до дії випробуваних екстрактів. Найбільша антимікробна активність виявлена в екстрактах етанолу Vaccinium myrtillus проти грамнегативних бактерій. Етанолові екстракти були більш ефективними в порівнянні з ацетоновими екстрактами. Слід відмітити, що плоди досліджених видів рослин є важливим компонентом у харчуванні*

людини, як важлива група природних антиоксидантів. Природні антоціани також діють як профілактичний та терапевтичний засіб при різних бактеріальних інфекціях.
Ключові слова: *метод дифузії в агар, антимікробна активність, штами бактерій, рослинні екстракти.*
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Introduction

Increased interest in the chemical composition of berry fruit was due to the emergence of its positive effects on human health.Polyphenolic compounds including anthocyanins possess antimicrobial activity against a wide range of microorganisms, especially in inhibiting the growth of food-borne pathogens. Anthocyanins exhibit antimicrobial activity through several mechanisms, such as induced cell damage by destroying the cell wall, membrane, and intercellular matrix (Khoo et al. 2017). Berry fruit is a rich source of various bioactive substances that have an interesting biological activity. It is rich in fiber, vitamins, minerals, organic acids and various phenolic compounds, including anthocyanins. They can absorb visible light and as colored substances are responsible for the characteristic blue to violet coloring of berries(Puupponen-Pimiäet al. 2005). Anthocyanins as secondary metabolites play an important role in chemical protection against various pathogens. For example, blueberries have been used as medicinal plants in the treatment of diarrhea, urinary tract infections and also in night vision improvement (Escribano-Bailonet al. 2004). It is also known in folk medicine that the elderberry extract provides relief and can help in urinary tract diseases. This effect was due to the antibacterial effects of anthocyanins. Some studies show that regular consumption of elderberry juice has been able to reduce the incidence of bacteriuria and pyuria in the elderly patients (Padmavati et al. 1997). Proantocyanidines and anthocyanins are considered as active ingredients in the elderberry extract (Howell 2002).

A similar effect was found with blueberry extracts. Antimicrobial activity against *Staphylococcus aureus* was found in studies with fractions containing anthocyanins in blueberry juice. Recent studies suggest that the antimicrobial activity of berry fruits has an effect on the behavior and responses of human pathogenic bacteria. They point out those bioactive compounds in berry fruits can act as new antimicrobial agents that can affect a wide range of pathogens and thus overcome antibiotic resistance problems (Puupponen- Pimiä et al. 2005). Zhao et al. (2009) investigated the *in vitro* antimicrobial activity of *Mayszea purpurea* polyphenol extract and found its inhibitory effect on the growth of *Salmonella enteritis*, *Staphylococcus aureus*, and *Candida albicans*, but no effect on *E. coli*. The extract with the highest concentration of anthocyanins had the best inhibitory effect, with the antimicrobial effect mainly related to cyanidine derivatives. Despite the inhibition of the growth of various pathogenic bacteria, the phenolic extracts of berry fruits did not show antibacterial activity against prebiotic lactobacilli (Puupponen-Pimia et al. 2001; Puupponen-Pimia et al. 2005). Molan et al. (2009) in their study confirmed a significant increase in *Lactobacillus rhamnosus* and *Bifidobacterium breve* in human after blueberry extract application as well as in the intestinal content of rats fed with a feed enriched with blueberry extract. They pointed out the prebiotic effect of blueberry extracts enriched with anthocyanins.

In vitro experiments have shown antibacterial properties against gram-positive as well as gram-negative bacteria such as *E. coli*. However, anthocyanins failed to inhibit the growth of *Salmonella typhimurium* (Puupponen-Pimiä et al. 2005). As an mechanism of the antimicrobial effect of polyphenols, it has been suggested that after passing through the cell wall due to reactions with intracellular molecules, the metabolism of bacteria is affected (Leitao et al. 2005). In parallel, complex polyphenols such as proanthocyanidins with bacterial adhesion interact on the surface of the host cell, while bindings of polyphenols to proteins induce changes in cell wall structure and integrity (Foo et al. 2000; Puupponen-Pimiä et al. 2005). These positive effects of anthocyanins indicate the ability to cleave lipopolysaccharides on the outer bacterial membrane and thereby affect their vitality (Wuet al. 2004).

Anthocyanin-rich extracts, such as blueberryinhibit Gram-negative bacteria but not Gram-positive bacteria. This variation may be due to the different structures of cell wall between Gram-negative and Gram-positive bacteria, in which the outer membrane of Gram-negative bacteria acts as a preventive barrier against hydrophobic compounds but not on hydrophilic compounds. These antimicrobial activities of anthocyanin-containing extracts are possibly due to the multiple mechanisms and synergistic effects of various phytochemicals in the extracts, including anthocyanins, weak organic acids, phenolic acids, and their mixtures of different chemical forms. Thus, the antimicrobial effect of chemically complex compounds instead of solely anthocyanins should be extensively analyzed. Also, anthocyanins in purple, red, and blue-colored fruits and vegetables are the main bioactives in preventing microbial infection by several mechanisms (Khoo et al. 2017).

Material and Methods

Testing of antibacterial effects was performed using the plate agar diffusion method. Acetone and ethanol extracts of blueberry (*Vaccinium myrtillus* L.), elderberry (*Sambucus nigra* L.), black aronia (Aronia melanocarpa L.) and four gram-negative bacterial strains (*Escherichia coli*, *Salmonella enterica*s ser. Typhimurium, *Pseudomonas aeruginosa* and *Enterobacter aerogenes*) were used. After 24 hours cultivation at 37°C, the bacterial strains were diluted with phosphate buffered to a concentration of 0.5–1.0 of McFarland scale. A 0.1 ml of suspension was inoculated into 100 ml of tempered liquid agar (Standard plate count agar, Oxoid). It was then poured in a 20 ml volume onto Petri dishes (900 mm diameter). After solidification, wells (5 mm diameter) were cut into agar and filled with 50 µl of samples. Extracts of different concentrations (1, 2.5, and 5 mg/ml) were diluted in 0.01% acetic acid. Gentamycin sulfate was used as a positive control, 0.01% acetic acid was used as a negative control. Plates were then incubated at 37°C for 24 hours.

Bounded inhibition zones with a diameter proportional to the concentration of the tested extract were observed on inoculated agar. The antibacterial effect of the extracts was recalculated according to the formula:

$$\%\mathbf{RIZD} = (\mathbf{IZD}_{\mathbf{sample}} - \mathbf{IZD}_{\mathbf{negative\ control}}) / \mathbf{IZD}_{\mathbf{positiveconrol}} \mathbf{x\ 100}$$

RIZD (*Relative Inhibition Zone Diameter*) – % of average relative inhibition zone
IZD (*Inhibition Zone Diameter*) – diameter of the inhibition zone, mm.

All bacterial inhibitory tests were conducted in triplicate and repeated three times. Data were collected and the significant differences were assessed with the probability associated with a one-tailed Student's t-test. Statistical analyses were performed using Microsoft Excel and Statistica ver.12.

Results

Table 1 shows the results of the antimicrobial effects (RIZD %) of the tested extracts.The best antibacterial effect of the extracts was reported in the gram-negative *E. coli* bacterium.Ethanol extract of blueberry, aronia and elderberry was effective at all three concentrations compared to acetone extract of elderberry, whose inhibitory effect was not observed.The same results were also observed with bacteria *Salmonella typhimurium*. Growth inhibitory activity has not been demonstrated in the acetone extracts of *A*ronia *melanocarpa* and *Sambucus nigra*. The growth of *Enterobacter aerogenes* was negatively affected by all ethanol extracts, with the highest antibacterial activity exhibited by aronia extract. The inhibitory effects of acetone extracts were not observed with *Enterobacter aerogenes*. The exception was the acetone extract of aronia, in which we observed an inhibitory effect at a 5 $mg.ml^{-1}$ concentration.

The moderate antimicrobial effect of acetone extracts (% RIZD = 50 – 100) and a weak (% RIZD < 50) antibacterial effect was showed only in extract of *Vaccinium myrtillus* and *Sambucus nigra* applied to all gram-negative bacteria (*E. coli*, *Salmonella typhimurium*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*). The results are shown in Table 1. The strongest antimicrobial effect of all extracts was observed in ethanol extract of aronia at a concentration of 5 $mg.ml^{-1}$ (% RIZD = 123.3). In contrast, the antimicrobial effect of the acetone extract from the elderberry was not confirmed in any of the three concentrations on the growth of tested Gram-positive bacteria strains.

Discussion

The antimicrobial activity of phenol compound plant extracts against human pathogens is intensively studied to characterize and develop modern healthy food ingredients as well as medical and pharmaceutical products.

Table 1. Evaluation of antimicrobial effects (RIZD %) of tested berry fruit extracts

Sample	Concentration, mg.ml^{-1}	*Escherichia coli*	*Salmonella enterica* ser. Typhimurium	*Enterobacter aerogenes*	*Pseudomonas aeruginosa*
	1	0	0	0	0
VMA	2.5	24.1	7.4	0	15.6
	5	52.6	24.1	0	34.4
	1	32.9	24.6	13.3	14.1
VME	2.5	70.2	53.7	46.7	39.1
	5	103.1	91.7	75.6	64.2
	1	0	0	0	0
ARA	2.5	28.5	0	0	23.5
	5	72.4	14.8	23.3	32.9
	1	41.7	11.1	0	32.9
ARE	2.5	78.9	48.1	73.3	58.7
	5	109.6	66.7	123.3	70.4
	1	0	0	0	0
BCA	2.5	0	0	0	0
	5	0	0	0	0
	1	24.1	17.9	0	0
BCE	2.5	61.4	44.7	13.3	23.5
	5	81.1	78.3	26.7	36

VMA –*Vaccinium myrtillus* acetone extract, VME-*Vaccinium myrtillus* ethanol extract, ARA-*Aronia melanocarpa* acetone extract, ARE – *Aronia melanocarpa* ethanol extract, BCA – *Sambucus nigra* acetone extract, BCE –*Sambucus nigra* ethanol extract. RIZD% < 50%: weak effect; RIZD% 50% – 100%: moderate effect ;RIZD% > 100%: strong effect.

In general, anthocyanins are effective against various microbes, but Gram-positive bacteria are usually more sensitive to anthocyanins effects compared to Gram-negative bacteria. The mechanisms underlying the activity of anthocyanins include both membrane and intracellular interactions of these compounds. The antimicrobial activity of anthocyanin-containing fruits is probably due to many mechanisms and synergies because they contain various compounds including anthocyanins, weak organic acids, phenolic acids and mixtures of different chemical forms. *In vitro* and *in vivo* studies have shown that anthocyanins have beneficial biological properties that play an important role in human health protection. We found the best antibacterial effect of the extracts in the Gram-negative bacterium of *E. coli*. However, the results of the study by Cisowska et al. (2011) showed that in the Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa a Salmonella enterica* ser. *Typhimurium*), *E. coli* showed the greatest resistance among the tested bacteria. Activity against Gram-positive bacteria was higher compared to Gram-negative bacteria due to the outer membrane of Gram-negative bacteria, which acts as a barrier to permeability and reduces the uptake of compounds in the cell. Despite higher antimicrobial activity in Gram-positive bacteria, based on our findings and findings by Puuppon-Pimiä et al. (2001) we can state that our selected Gram-negative bacteria reacted positively to the inhibitory effects of ethanol berry fruit extracts. The acetone extract of elderberries was the exception; this extract could not inhibit the growth of bacteria in any of the three tested concentrations.

The antimicrobial effect of ethanol extracts from *Vaccinium corymbosum* L. was investigated by Pervin et al. (2013). They evaluated the antibacterial effect by determining RIZD % in two Gram-positive and five Gram-negative bacteria. The tested extracts showed a broad spectrum of activity against all sensitive microorganisms, but a stronger antimicrobial effect was observed against Gram-negative bacteria compared to Gram-positive bacteria.

The best antimicrobial effect of all tested extracts was observed in blueberry extract

(*Vaccinium myrtillus* L.).Positive antibacterial effects of *Vaccinium myrtillus* L. have been shown also by research results of Vučić et al. (2013), which confirmed a potential source of antibacterial agents in a set of thirty Gram-negative bacterial strains.

In our study, acetone extracts of *Aronia melanocarpa* and *Sambucus nigra* were unable to inhibit bacterial growth at a concentration of $1 mg.ml^{-1}$.The effect of *Aronia melanocarpa* ethanol extract on the growth of *E. coli*was confirmed by Bräunlich et al. (2013), who investigated the inhibitory effect of the extract on biofilm formation.In their study, they found that exposure of *Aronia melanocarpa* extract to a biofilm-forming bacterial strain of *E. coli* reduced the ability of a bacterium to produce a biofilm, thereby reducing its ability to resist.

The study by Baydari et al. (2004), pointed out the advantages of acetone extraction over ethanol extraction, which makes it possible to avoid problems with pectins. Shipp and Abdel Aal et al. (2010) reported in their study that alcohol extraction causes acylation of the sugar constituent of anthocyanins, which does not occur during acetone extraction. The process of acylation stabilizes anthocyanins and at the same time protects anthocyanins from degradation by microbial glycosides. Based on the results, we conclude that ethanol extracts had a higher degree of antimicrobial activity compared to acetone extracts. We assume that the different effect of anthocyanin compounds is influenced by different extraction methods.

Conclusions

The study showed that ethane extracts of anthocyanins from berries show strong antimicrobial activity against gram-positive bacteria (*E. coli, Salmonella enterica* ser. Typhimurium, *Enterobacter aerogenes, Pseudomonas aeruginosa*). The results confirmed the antimicrobial activity of anthocyanins by inhibitory effects on the growth of gram-negative bacteria, while the lowest resistance was observed in *E. coli*. *Enterobacter aerogenes* showed the highest resistance to ethanol extracts compared to acetone extracts. We found that the efficiency of the extracts also affects the method of extraction of anthocyanins from berries. This resulted in a higher efficiency of ethanol extracts compared to acetone extracts. Anthocyanins can become very important and promising natural tools in the prevention of various diseases, mainly due to their evident antimicrobial, but also antioxidant, antimutagenic and immunomodulatory potential.

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