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THE COMPARISON OF THE ANTIOXIDANT AND ANTIMICROBIAL POTENTIAL OF *VITIS VINIFERA* L., *VACCINIUM VITIS-IDAEA* L. AND *ROSA CANINA* L. FRUIT EXTRACTS

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The comparison of the antioxidant and antimicrobial potential of Vitis vinifera L., Vaccinium vitis-idaea L. and Rosa canina L. fruit extracts. – V. Sedlák[1], N. Chovancová[1], J. Poráčová[1], M. Mydlárová Blaščáková[1], Z. Gogaľová[1], M. Konečná[1], J. Porubská[2], Ľ. Tkáčiková[3], M. Majherová[4], V.S. Mirutenko[5], V.V. Mirutenko[6] – *We determined the antioxidant activity of aqueous and ethanol extracts of grapevine, lingonberry and rosehip fruits and their inhibitory effect on Gram-positive and Gram-negative bacteria. Based on the percentage of DPPH radicals inhibition (I %) we found better antioxidant activity in ethanol extracts. The highest average inhibition was observed in ethanol rosehip fruit extract (64.59%). Water extracts had significantly lower free radical scavenging activity. The best antimicrobial effects based on the agar diffusion test reached ethanol extracts of lingonberry and rosehip fruits.*
Keywords: *Agar diffusion method, antibacterial activity, antioxidants, antiradical activity, DPPH method, free radicals, oxidative stress.*
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Порівняння антиоксидантного та антимікробного потенціалу екстрактів Vitis vinifera L., Vaccinium vitis-idaea L. та Rosa canina L. – В. Седлак[1], Н. Цхованцова[1], Я. Порачева[1], М. Мидларова Блашчакова[1], З. Ґогалова[1], М. Конечна[1], Я. Порубська[2], Л. Ткачикова[3], М. Майгерова[4], В.С. Мірутенко[5], В.В. Мірутенко[6] *– Ми визначили антиоксидантну активність водних та спиртових екстрактів винограду, брусниці та плодів шипшини та їх інгібуючий вплив на грампозитивні та грамнегативні бактерії. Виходячи із відсоткового вмісту інгібіції радикалів DPPH (1%), ми виявили кращу антиоксидантну активність у спиртових екстрактах. Найбільше середнє пригнічення спостерігалося в спиртовому екстракті плодів шипшини (64,59%). Водні екстракти мають значно нижчу активність вільних радикалів. Найкращі антимікробні ефекти, виходячі з даних агар-дифузійного тесту, мають спиртові екстракти плодів брусниці та шипшини.*
Ключові слова: *метод дифузії агару, антибактеріальна активність, антиоксиданти, антирадикальна активність, метод DPPH, вільні радикали, оксідативний стрес.*
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Introduction

The plants and fruits present a rich source of pharmaceutically active substances with a wide range of biological activities, including antioxidant potential (Verma et al. 2009). A variety of natural substances found in fruits, vegetables and medicinal plants, especially phenols (e.g. anthocyanins), play an irreplaceable role in the prevention of many diseases (Kondakova et al. 2009). One of the most important and the most common plant antioxidants are polyphenols including plant pigments, phytosterols, vitamins, enzymes and minerals (Gupta and Sharma 2006). Compounds such as phenols, tannins and flavonoids have also rich antimicrobial effects (Konate et al. 2012), and the group of anthocyanins delivers a typical red and dark coloration to the fruits (Castaneda-Ovando et al. 2012). Anthocyanins refers to a group of water-soluble pigments responsible for red, pink, mauve, purple, blue, or violet colour of many flowers and fruits (Andersen and Jordheim 2006). In general, red and dark coloured fruits are a rich source of these substances and provide significant health benefits.

Plants are viable and unlimited source of bioactive compounds, including antimicrobial agents protecting against microorganism, insects, and predators (Pinho et al. 2014). Many plant phenols, including phenolic acids, flavonoids and tannins, are synthesized by plants as a reaction to microbial infection. These substances demonstrate a broad spectrum of antimicrobial effects against many species of microorganisms (Ignat et al. 2013). The increase in bacterial resistance to antibiotics encourages the discovery of new sources of antibiotics (Hafidh et al. 2011, Subedi et al. 2012). Some studies have suggested the use of antimicrobial effects of berry fruits for storing and preserving foods as a new source of antimicrobial activity capable of acting on a number of pathogens, but especially on the most common food spoilage agents – *Salmonella* and *Staphylococcus* (Česonienė, Jasutienė and Šarkinas 2009). The most virulent microorganisms are considered methicillin-resistant *Staphylococcus aureus, Escherichia coli, Mycobacterium tuberculosis* a *Pseudomonas aeruginosa* (Hafidh et al. 2011).

The accumulation of free radicals causes an oxidative stress in organism. This is one of the primary reasons of diseases such as neurodegenerative diseases, cardiovascular diseases, cancer, etc. An increased amount of free radicals in the organism and their destructive effects can be eliminated by an intake of antioxidants (Diaconeasa et al. 2015, Kris-Etherton et al. 2002). Antiradical activity of antioxidants represents one of the defence mechanisms regarding the elimination of harmful processes in an organism (Jirovský 2007).

The increased accumulation of free radicals in the human organism is involved in the pathogenesis of many civilization diseases. Oxidative stress occurs when free radicals exceed the antioxidant defences of the organism (Yung et al. 2006). Increased (excess) production of reactive oxygen species play an important role in the damage of different tissues and in reducing or completely loses their function (Verma et al. 2009). Reactive oxygen species negatively affect biomolecules in the human and animal organisms, e.g. lipids, proteins and DNA, which results in different serious diseases such as oncogenic diseases, diabetes, arteriosclerosis, arthritis, inflammatory processes, genotoxicity and nervous diseases (Shukla et al. 2009).

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body. There is also preference for antioxidants from natural rather than from synthetic sources (Molyneux 2004). The antioxidants are natural substances that can protect cells against the harmful effects of free radicals and can help to eliminate oxidative damage of biomolecules (Poracova et al. 2011). International studies show the biological active properties of polyphenols: anti-oxidant, anti-inflammatory, anti-platelet aggregation, hypocholesterolemic, vasorelaxation, (Kaur, Geetha 2006), antimicrobial and antiviral effects (Hafidh et al. 2011; Konate et al. 2012, Pinho et al. 2014). High content of polyphenols is an indicator of potential antioxidant activity. The fruits are used as part of a diet, nutritional supplements and as drugs (Abdel-Hameed 2009).

Currently, antimicrobial and antioxidant activity of plant-based substances, specifically polyphenols, which exhibit antibacterial effects and can also suppress antibiotic resistance of microorganisms, is in the interest of research. They are also able to scavenge free radicals and act as antioxidants (Guardado Yordi et al. 2012, Ignat et al. 2013). The main task of this study was to measure and compare the antioxidant and antimicrobial potential of aqueous and ethanol extracts of the grapevine, lingonberry and rosehip fruits using DPPH method and agar diffusion test.

Materials and methods

The investigated plant material were mature fruits of dark grapevine (*Vitis vinifera* L.), lingonberry (*Vaccinium vitis-idaea* L.) and rosehip fruits (*Rosa canina* L.) in order to find out and compare their antioxidant properties. Dark grapes

were harvested in September 2015 from locality in Petkovce village, Slovakia (N 49°00'06", E 21°35'53"), alt. 205 m, annual average temperature 7 – 8 °C, annual average rainfall 500 – 600 mm. Lingonberry fruits were harvested in September 2015 on Minčol hill (N49°13′50″, E 21°0′27″), alt. 1157 m, annual average temperature 4 °C, annual average rainfall 800 mm. Rosehip fruits were harvested in October 2015 in Teriakovce village, Slovakia (N 48°59′22″, E 21°18′35″), alt.365 m, annual average temperature was 6 °C, annual average rainfall 600 – 700 mm. After harvest, all fruits samples were stored in the dark and preserved at -20°C.

50 g of defrosted fruits were homogenized using mortar and pestle and 100 ml of 96.3% p.a. ethanol (Centralchem, Slovakia) was added. Mixtures in sealed glass containers were stored in dark at room temperature for 24 hours. Similarly, water extracts were prepared using 50 g of defrosted homogenized fruits and 500 ml of boiled distilled water. All solutions were filtered after 24 hours using KA3 filter (filtration speed: fast; Papírna Perštejn s.r.o. Czech Republic) to obtain ethanol and water fruit extracts. Subsequently, the extracts were stored in dark place at 8 °C.

The antioxidant activity of fruit extracts was determined spectrophotometrically using modified DPPH radical (2,2-diphenyl-1-picrylhydrazyl) method (Brand-Williams et al. 1995; Sanchéz-Moreno et al. 1998; Espin et al. 2000; Burda and Oleszek 2001; Šeršeň and Grančai 2008). DPPH method allows simple and quick way of evaluating the presence of antioxidants (Mensor 2001). This method is based on the interaction of the antioxidant substances with a stable radical DPPH. The elimination of DPPH radical in the presence of an antioxidant leads to a decolourization of the observed solution. Decrease in absorbance referred to the reaction of present antioxidants with DPPH radical (Marxen et al. 2007; Melendéz 2014). This means that, the lower the value of the absorbance was measured the better antioxidant activity of the extract was observed. The decolonization was monitored spectrophotometrically (λ = 517 nm), using spectrophotometer UV-1800 (Shimadzu, Japan).

The DPPH solution (0.06 mM) was prepared with ethanol, 96.3% p.a. (Centralchem, Slovakia) and DPPH radical (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich, Germany). The DPPH ethanol solution was prepared daily, and during analyses was stored in a cool and dark place. Pure ethanol 96.3 % was used as a blank. The reaction mixture was prepared by following way, 10 µl of particular non- diluted fruit extract was added into 5 ml of DPPH solution. The reaction (DPPH solution and active substances in the fruit extract) ran at room temperature for 35 minutes in the dark. This reaction will incorporate the colour changes of monitored solution from purple to yellow colour (Sung-Kun et al. 2004). It was observed as a continuous decrease in measured absorbance, which was recorded at regular time intervals (8 times in 5 minute intervals). Five parallel measurements were performed for each analysed extract; subsequently the average values were used for the evaluation.

Using the obtained values of the decreasing absorbance, the percentage of inhibition (%) was calculated by the following formula: $I\ (\%) = [(A_{C0} - A_{At}) / A_{C0}] \times 100$ (Cipak et al. 2006; Habanova and Haban 2008). The equation expresses the percentage of inhibition of DPPH activity, where I (%) is the per cent (%) of DPPH activity inhibition, A_{C0} is the absorbance of the control solution (DPPH solution only) and A_{At} is absorbance of the tested sample. A_{C0} absorbance is measured before each measurement of tested sample of extract due to the slight in-time decreasing during storing.

Antimicrobial activity was observed using agar diffusion assays according to Jimenez et al. (2011) on Standard plate count agar CM0463 type (OXOID, Great Britain) by determining the minimum inhibitory concentration using six selected bacterial species divided into Gram-negative group (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica* ser. Typhimurium) and Gram-positive group (*Staphylococcus aureus*, *Bacillus subtilis*, a *Listeria monocytogenes*). Cultivation of bacteria in the broth lasted 20 hours at 35 ± 2 °C. The broth cultures were diluted with sterile broth to value 0.5 of McFarland scale, corresponding to value 3.0 at densitometer DEN-1 SIA (BioSan, Lithuania). Liquid tempered agar (maximum of 50 °C) was inoculated with bacteria culture resuspended in broth with final concentration of 5×10^5 CFU/mL (colony forming unit). 20 ml of the inoculated agar was poured into a Petri dish (diameter 90 mm), and allowed to solidify for 15 minutes at room temperature and then for 30 minutes in a refrigerator at 8 °C. The procedure was repeated for each type of bacteria in triplicate, so in total 18 Petri dishes were prepared.

9 holes with a diameter of 5 mm were cut out into the agar in Petri dishes. We marked prepared holes and we added 50 µl of plant extracts into prepared holes as follows: hole 1 – grapevine ethanol extract, hole 2 – rosehip fruit ethanol extract, hole 3 – lingonberry ethanol extract, hole 4 grapevine water extract, hole 5 – rosehip fruit water extract, 6 – lingonberry water extract. We added Gentamicin (0.1%) into hole 7, Gentamicin (0.05%) into hole 8 and ethanol (96.3% p.a.) into hole 9 as control (Figure 1). After the incubation at 35 ± 2 °C for 24 h, the inhibitory growth zones of bacteria (mm) corresponding to the halo formed from the well edge to the beginning of the zone of microbial growth was measured. The tests were performed in triplicate and the final results were presented as the arithmetic average.

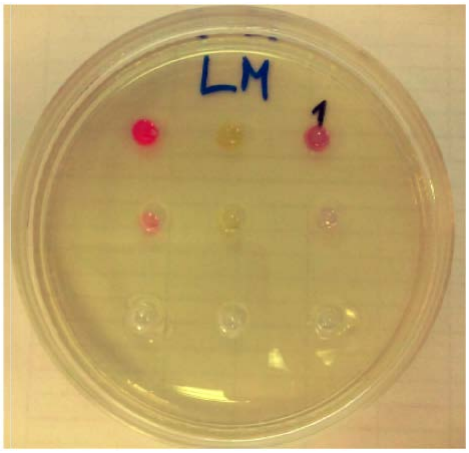

Figure 1 Agar diffusion assay

Results and discussions

We determined the antioxidant activity expressed as average percentage of DPPH radical inhibition (I %) of ethanol and water rosehip, lingonberry and grapevine fruits extracts. The ethanol rosehip fruits extract showed the highest average inhibition of 64.59% and the water rosehip fruits extracts showed average inhibition of 34.82%. The sample was quickly decolourized and the absorbance values were decreasing rapidly. The results show, that both, ethanol and water rosehip fruits extracts reached significantly higher (approx. twofold higher) antioxidant potential in scavenging DPPH free radical comparing the other ethanol and water lingonberry and grapevine fruit extracts. The lowest antioxidant potential was found in water grapevine fruit extract (2.28%), ethanol grapevine fruits extracts reach average percentage of DPPH radical inhibition (14.26%). Extracts of lingonberry fruits showed average values of DPPH radical inhibition: ethanol extracts (30.50%), water extracts respectively (15.05%) (Table 1).

Table 1. Comparison of the average antioxidant activity of ethanol and aqueous extracts of the rosehip, lingonberry and grapevine fruits

Antioxidant potential in DPPH• inhibition (%)			
Sample	***Rosa canina* L.**	***Vaccinium vitis-idaea* L.**	***Vitis vinifera* L.**
Ethanol extract	64.59	30.50	14.26
Water extract	34.82	15.08	2.28

Polyphenol compounds of red and dark coloured fruits are generally extracted using methanol, ethanol, acetone, or an aqueous solution (Hohnová at al. 2008). The maximum antiradical activity was observed in the extracts prepared using ethanol, followed by methanol and water extracting solution (Xia et al. 2010). Molyneux (2004) also mentioned that ethanol and methanol are more effective solutions for extraction compared to water solutions.

Montazeri et al. (2011) also reported similar results of various extracts of rosehip fruits, which showed significant DPPH free radical scavenging activity. Ethanol extracts seem to be more concentrated in active substances content, which contribute to their antioxidant properties significantly. This could be due to more effective ethanol extraction of bioactive substances (especially anthocyanins) in samples which showed high antioxidant activity, because ethanol increases extraction as reported also by Angela and Meireles (2008). Better results in antioxidant activity were found also in ethanol extracts compared to water extracts (Bidchol et al. 2011). Peschel et al. (2007) studied antioxidant potential of blackcurrant residue after juice separation by DPPH method. It also showed some antioxidant activity, where the ethanol residues extracts had approx. twofold higher antioxidant activity than aqueous residues extracts. Wu et al. (2006) determined the total anthocyanins (as significant group of polyphenols) of blackcurrant and redcurrant. Authors reported higher concentrations of anthocyanins in blackcurrant compared with redcurrant (37-fold). The highest antioxidant activity was found in the plants, which contain anthocyanins. Vollmannová et al. (2009) compared the antioxidant activity of selected blueberry cultivars using DPPH method. The average antioxidant activity ranged from 70.70% to 77.08%.

The result values of antioxidant activity may differ, since the content of bioactive compounds in fruits is influenced by natural conditions of particular locality (temperature, soil conditions, rainfall, etc.) and cultivation conditions. Based on former studies it is obvious that the content of bioactive substances in berries depends on many factors (e.g. genotype, location and year) (Vagiri et al. 2013). Dragović-Uzelac et al. (2010) found a definite impact of climatic conditions on the growth of cultivars and content of phenolic compounds, which is reflected in their antioxidant activity. Krížová et al. (2010) compared the content of total polyphenols and antioxidant activity of wild blueberries from four different locations in Slovakia and confirmed, that the antioxidant activity can be affected by different environmental conditions (e.g. rainfall and climate). As we reported previously, the antioxidant activity was expressed as average percentage of DPPH radical inhibition (I %) of non-purified (35.36%) and purified (65.40%) blackcurrant ethanol extracts. Purified extracts seem to be more concentrated in active substances content as a result of removing inert constituents, which do not contribute to antioxidant activity (Sedlák et al. 2016).

We determined antimicrobial activity using agar diffusion assay. We did not observe any antimicrobial effect using water fruit extracts (0 mm inhibition zones). Ethanol fruit extracts showed antimicrobial activity in the agar-well diffusion method as follows: the best antimicrobial effect showed ethanol extract of lingonberry, followed by ethanol extract of rosehip (both antimicrobial effect against *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes* bacteria strains). Ethanol fruit extract of grapevine showed antimicrobial effect only against *Bacillus subtilis.* The strongest inhibitory effects of the ethanol fruit extracts were observed on Gram-positive bacteria *Listeria monocytogenes* followed by *Bacillus subtilis* and *Staphylococcus aureus* (Table 2)

Table 2. The average inhibitory growth zones of bacteria after activity of ethanol extracts

Bacteria		Fruit ethanol extracts		
		Grapevine	Rosehip	Lingonberry
G -	*Pseudomonas aeruginosa*	0.0 mm	7.5 mm	7.8 mm
	Escherichia coli	0.0 mm	0.0 mm	0.0 mm
	Salmonella enterica ser. Typhimurium	0.0 mm	0.0 mm	0.0 mm
G +	*Staphylococcus aureus*	0.0 mm	7.8 mm	9.8 mm
	Bacillus subtilis	6.0 mm	8.7 mm	9.7 mm
	Listeria monocytogenes	0.0 mm	13.7 mm	14.3 mm

The results of the studies show that the Gram-positive and Gram-negative bacteria strains have different levels of sensitivity to extracts, as the result of the structural differences of the anthocyanins. Gram-negative bacteria also have an outer membrane which limits the spread of hydrophobic components through their lipopolysaccharide membrane (Jimenez et al. 2011). We found similar results compared to the study of Molyneux (2004), aqueous extracts have a minimal inhibitory effect on bacteria, while ethanol extracts have been effective in all three fruit species extracts.

The antimicrobial effects can vary according to the type of observed microorganism; our results are consistent with the study of Konate et al. (2012), which states that Gram-positive bacteria are generally more sensitive to the extracts containing phenolic substances, compared to Gram-negative bacteria. Examined extracts do not show activity against Gram-negative bacteria *Escherichia coli* and *Salmonella enterica* ser. Typhimurium, antimicrobial activity was detected only against *Pseudomonas aeruginosa* (rosehip fruit ethanol extract – 7.5 mm inhibitory zone; lingonberry fruit ethanol extract 7.8 mm inhibitory zone). Conversely, in the case of Gram-positive bacteria of *Bacillus subtilis*, all three ethanol extracts were effective and in the case of Gram-positive strains of *Listeria monocytogenes* and *Staphylococcus aureus* we found no antimicrobial effect only in ethanol extract of grapevine fruit.

Gram-negative bacteria permeability is less potent, presumably for the presence of a greater amount of phospholipids in the cell wall compared to Gram-positive bacteria. The resistance of Gram-negative bacteria to antimicrobial components is associated with the hydrophilic surface of their outer membrane, which is rich in lipopolysaccharide molecules that represent a barrier to the penetration of many antibiotic molecules, and is also associated with the presence of enzymes involved in the periplasmic space that are capable to disturb the molecules from the outside environment (Konate et al. 2012). The *E. coli* and *Pseudomonas aeruginosa* bacteria showed also greater resistance in a study by Ignat et al (2013). Our results are consistent with the results of Taguri, Tanaka a Kouno (2004), which showed that *S. aureus* bacteria are considerably more sensitive to the presence of polyphenols than *Salmonella enterica* ser. Typhimurium and *E. coli*. Krumina et al. (2015) reported the antimicrobial activity of 70% ethanol extracts of rosehip fruits against *Streptococcus mutans* estimated by the agar-

well diffusion method with inhibition zone of 18.5 mm. We found 7.8 mm inhibition zone of ethanol rosehip fruit extract against *Staphylococcus aureus*, *Bacillus subtilis* (8.7 mm) and *Listeria monocytogenes* (13.7 mm).

Conclusion

The ethanol fruits extracts of rosehip showed the highest antioxidant potential; however there was confirmed antioxidant potential also in extracts of lingonberry and grapevine fruits. We did not find any antimicrobial effect of tested water fruit extracts. The highest antimicrobial effect was found in ethanol extract of lingonberry, followed by ethanol extract of rosehip. Ethanol fruit extract of grapevine showed antimicrobial effect only against one bacteria strain of *Bacillus subtilis*. The strongest inhibitory effects of the ethanol fruit extracts were observed on Gram-positive bacteria *Listeria monocytogenes* followed by *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* as the only one Gram-negative bacteria strain. The rosehip, lingonberry and grapevine fruits are a rich source of biologically active substances, which have a wide potential application in the food, pharmaceutical and cosmetic industries and other technologies. Fruits extracts are presented as valuable source of natural antioxidants and may offset negative effects of free radicals and help protect human health. Apparently, the extraction processes can affect the presence of active substances in fruit extracts. Thus the appropriate method of processing of extracts is important for the final antioxidant and antimicrobial properties.

Natural medication is becoming still a more popular way of a treatment and a promising substitute for antibiotics and antifungal drugs to which more and more bacteria strains are becoming resistant. Antioxidant activity depends on the capability of present compounds to scavenge free radicals in an organism. Phytochemical compounds, which can be found especially in red and dark coloured berry fruit, prove positive effects in the prevention of illnesses caused by high free radical production linked to antioxidant activity.

The active phytochemicals present in the fruit may be used in the new drug and alternative antibiotic production and also can be applied in the prevention of illnesses linked with free radical influence in the organism.

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