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ASSESMENT OF THE ANTIOXIDANT ACTIVITY AND CONTENT OF POLYPHENOLIC COMPOUNDS IN GRAPEVINE SEEDS

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ABSTRACT
Our work was focused on the study of the antioxidant properties of grapevine seeds. We monitored the grapevine seeds of 6 cultivars of *Vitis vinifera*, L. (Nativa, Kofranka, Blaufränkish, Marlen, Cabernet Moravia and Italian Riesling). Antioxidant activity was determined by three principally different methods (DPPH, ABTS and FRAP), the content of the total polyphenolic compounds was determined by the Folin ciocalteu method, and the content of the total flavanols was determined by DMACA reagent (p-dimethylaminocinnamaldehyde). Results are presented as an equivalent of gallic acid in $g.L^{-1}$, respectively were expressed as $g.L^{-1}$ of catechin equivalents (DMACA method). The highest values of antioxidant activity were measured in the cultivar Nativa (DPPH – 7.75 $g.L^{-1}$, ABTS – 4.888 $g.L^{-1}$, FRAP – 4.25 $g.L^{-1}$). Conversely, the lowest values of antioxidant activity were detected in the cultivar Kofranka (DPPH – 7.08 $g.L^{-1}$, ABTS – 4.17 $g.L^{-1}$, FRAP – 4.55 $g.L^{-1}$). Cultivar Nativa also reached the highest content of flavonols (3.77 $g.L^{-1}$). The highest measured values of the content of total polyphenolic compounds were identified in the cultivar Cabernet Moravia (15.2 $g.L^{-1}$ of GAE). Conversely, the lowest values of the content of total polyphenolic compounds were detected in the cultivar Nativa (8.04 $g.L^{-1}$). Pearson correlation coefficients were calculated for the existing values between antioxidant activity (DPPH, ABTS, FRAP), contents of flavonols, and contents of total polyphenols. The highest correlation coefficient was found between the DPPH and ABTS methods; specifically, it was 0.857.

Keywords: antioxidant activity; flavonols; grapevine seeds; total polyphenolic compounds

INTRODUCTION
Worldwide, the grapevine (*Vitis vinifera*, L.) is one of the most frequently cultivated fruit species **(Vršič et al., 2011)**. In Europe, the total acreage of vineyards is approximately 4.5 million hectares (45,000 sq. km.), and the total volume of pomace produced is about 8 million tons. Recently, the attention of many researchers has been focused on the development of new technologies to enable a purposeful and efficient utilisation of this waste product.
At present, the potential spectrum of grapevine pomace use is relatively wide. So, for example, this waste may be used as a feedstuff for farm animals **(Besharati & Taghizadeh, 2009)**, to produce dietetic, top-quality grape seed oil **(Shinagawa et al., 2015)**, and for the production of energy **(Valente et al., 2015)**. The pomace may be also composted for application in horticulture **(Dominguez et al., 2014)**, for grappa production **(Da Porto, 2012)**, and for other purposes.

The aim of this study was to determine the antioxidant activity, the content of polyphenolic compounds, and the content of flavanols in grapevine seeds of 6 cultivars of *Vitis vinifera*, L. and discover connections among the values obtained.

MATERIAL AND METHODOLOGY
Biological samples
This experimental study was performed with the seeds of six grapevine (*Vitis vinifera* L.) cultivars, specifically Nativa, Kofranka, Blaufränkish, Marlen, Cabernet Moravia, and Italian Riesling.

Chemicals
Chemicals used in this study were supplied by the firm Sigma Aldrich (Germany). The chemicals were: Deionised water, stable free radical DPPH•, cation radical ABTS•, methanol, acetic acid (0.2%), liquid nitrogen, TPTZ (2,4,6-tripyridyl-s-triazin), hydrochloric acid, FeCl3, acetate buffer, sodium acetate, Folin-Ciocalteu reagent, sodium carbonate decahydrate ($NaCO_3$.10 H_2O), and p-dimethylaminocinnamaldehyde reagent (DMACA).

Method of sample preparation
The experimental material originated from grape pomace. To eliminate undesirable water residues, the grapevine seeds were screened and purified. Thereafter the seeds were crushed with liquid nitrogen in a mortar.

Subsequently, 10 g of the homogenate were quantitatively transferred into a volumetric flask. The extraction was performed in a dark and cool environment with 100 mL of 75% methanol using the shaker IKA KS 260 Basic (manufacturer: Merci, France) for a period of 5 days. Final extracts were centrifuged and transferred into vials and micro test tubes (manufacturer: Eppendorf, Germany). All of the following measurements were executed 3 times successively.

Estimation of antioxidant activity

Spectrophotometric measurements of antioxidant activity were carried out using the BS-400 automated chemical analyser (manufacturer: Mindray, Shenzhen City, China). Transfer of samples and reagents was provided by a robotic arm equipped with a dosing needle (error of dosage did not exceed ±5% m/v). Immediately after the addition of reagents or samples, cuvette contents were mixed in an automatic mixer with a stirrer.

Determination of Antioxidant Activity by the DPPH Method

This procedure was performed according to **Sochor et al. (2010a)**. A 150 µL volume of reagent (0.095 mM 2,2-diphenyl-1-picrylhydrazyl – DPPH•) was incubated with 15 µL of the sample. Absorbance was measured at 505 nm for a period of 10 min.

Determination of Antioxidant Activity by the ABTS Test

The procedure for the determination of the antioxidant activity was performed according to **Pohanka et al. (2012)**. A 150 µL volume of the ABTS reagent (7 mM 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) and 4.95 mM potassium peroxodisulphate were mixed with 3 µL of the sample. Absorbance was measured at 660 nm for a period of 10 min.

Determination of Antioxidant Activity by the FRAP Method

The procedure for this determination was performed according to **(Sochor et al., 2010b)**. A 150 µL volume of reagent was injected into a plastic cuvette together with the subsequent addition of a 3 µL sample. Absorbance was also measured at 605 nm for a period of 10 min.

Estimation of the contents of total polyphenols

The Folin-Ciocalteu method, based on the reduction of a phosphotungsten-phosphomolybdate complex by phenols to blue reaction products, was used for the determination of phenolic compounds. A sample of 0.5 mL was pipetted into a cuvette and diluted with ACS water (1.5 mL). Subsequently, the Folin-Ciocalteu reagent (50 µL) was added, and the solution was incubated at 22 °C for 30 min. Absorbance was measured using a HELIOS Gama spectrometer at the wavelength $\lambda = 670$ nm against a blank sample. The absorbance was measured three times. Results were expressed as equivalents of gallic acid in $g \cdot 100\ g^{-1}$. The method was calibrated on the known phenolic compound (gallic acid).

Estimation of total flavanols

Total flavanols were estimated using the p-dimethylaminocinnamaldehyde (DMACA) method **(Li et al., 1996, McMurrough et al., 1996, Vivas, 1994)**. Compared to the widely-used vanillin method, a great advantage of this method is that there is no interference by anthocyanins. Furthermore, the method used provided higher sensitivity and better specificity. Wine (20 µL) was poured into a 1.5 mL Eppendorf tube, and 980 µL of DMACA solution (0.1% in 1 M HCl in MeOH) was added. The mixture was vortexed and allowed to react at room temperature for 12 min. The absorbance at 640 nm was then read against a blank sample prepared in a similar way but without DMACA. The concentration of total flavanols was then estimated from a calibration curve constructed by plotting known solutions of catechin (1 − 16 $mg \cdot L^{-1}$) against A640 (r = 0.998). The results were expressed as $g \cdot L^{-1}$ of catechin equivalents.

RESULTS

The antioxidant activity and contents of flavanols and total polyphenols were assessed by spectrophotometry. The results of the performed analyses were expressed as arithmetic means and as the standard deviations of three measurements. Correlations existing between the antioxidant parameters were expressed by means of Pearson's correlation coefficients.

Assessment of antioxidant activity

The assessment of antioxidant activity represents one of the possibilities how to determine contents of total antioxidant compounds **(Rop et al., 2010, Sochor et al., 2010c)**. Unfortunately, there is no simple and universal method that can be used for a proper quantitative estimation of this activity **(Rop et al., 2011, Sochor et al., 2011)**. For that reason, three principally different spectrophotometric methods were used to assess this parameter – DPPH, FRAP and ABTS. The results obtained were converted to equivalents of gallic acid that was used as a standard. These values are presented in $g.L^{-1}$ of GAE (gallic acid equivalent).

Determination of Antioxidant Activity by the DPPH Method

As shown in Figure 1, the antioxidant activity was fairly uniform in all cultivars under study. The average value of antioxidant activity, as assessed by this method, was 7.75 $g.L^{-1}$. The highest and the lowest values were recorded in the cultivars Nativa and Kofranka, respectively. The difference was 18.8%.

Assessment of antioxidant activity by the ABTS method

As shown in Figure 2, the antioxidant potential was also relatively uniform, with the highest value being recorded in the cultivar Nativa. The average value of antioxidant activity, as assessed by this method, was 4.888 $g.L^{-1}$. The lowest value was recorded in the cultivar Marlen. In this case, the difference between both values was 20.6%.

Assessment of antioxidant activity by the FRAP method

As shown in Figure 3, the antioxidant activity was again relatively uniform in all cultivars under study. The average

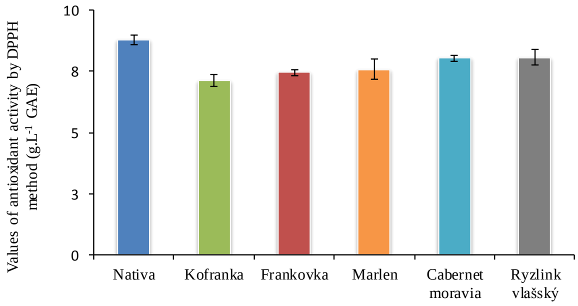

Figure 1 Results of the assessment of the antioxidant activity by the DPPH method in cultivars Nativa, Kofranka, Blaufränkish, Marlen, Cabernet Moravia and Italian Riesling. Results are presented as GAE in g.L^{-1}.

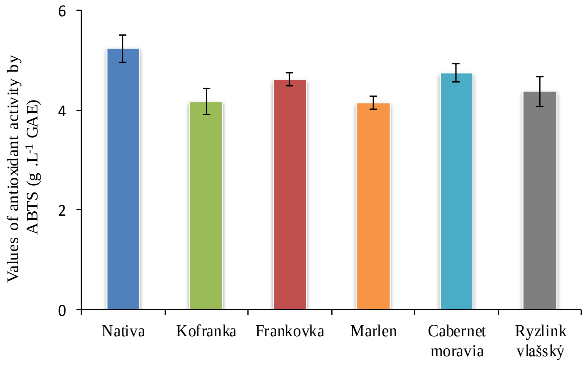

Figure 2 Results of the assessment of the antioxidant activity by the ABTS method in cultivars Nativa, Kofranka, Blaufränkish, Marlen, Cabernet Moravia and Italian Riesling. Results are presented as GAE in g.L^{-1}.

value of antioxidant activity, as assessed by this method, was 4.54 g.L^{-1}. The highest and the lowest values were recorded in the cultivars Cabernet Moravia and Marlen, respectively. The difference, 26.6%, was even greater.

Assessment of flavonols

Concentrations of total flavonols were assessed by means of the method based on the reaction of DMACA. In contradistinction to a widely-used method based on the reaction of flavonols with s vanillin, there was no interference by anthocyanins. Moreover, this method provided higher sensitivity and also better selectivity. Quercetin, myricetin, rutin and kaempferol are considered to be the most important flavonols that usually occur in wine.

The average content of the flavonols was 3.04 g.L^{-1}. The lowest and the highest contents of these compounds were found in the cultivars Blaufränkish (2.09 g.L^{-1}) and Nativa, (3.77 g.L^{-1}), respectively. In Blaufränkish, the content of flavonols was lower than in Nativa (by 44.6%).

Assessment of total polyphenols

Polyphenolic compounds represent one of the most abundant groups of antioxidants occurring in fruit **(Paixao et al., 2007)**. Red wine is rich in polyphenols; their total contents range from 1,000 to 4,000 g.L^{-1} **(Li et al., 2009)**. As usual, the content of polyphenolic compounds can be quantified by colorimetric assays based on using the Folin-

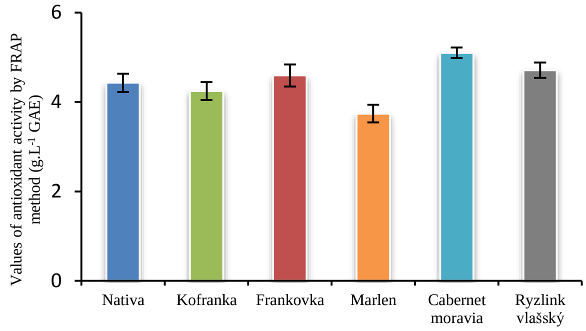

Figure 3 Results of the assessment of the antioxidant activity by the FRAP method in cultivars Nativa, Kofranka, Blaufränkish, Marlen, Cabernet Moravia and Italian Riesling. Results are presented as GAE in g.L^{-1}.

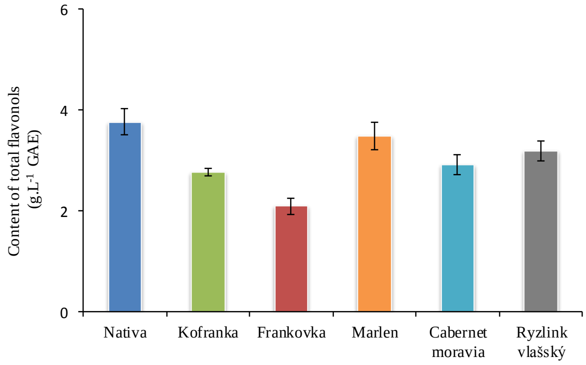

Figure 4 Results of the assessment of total flavonols in cultivars Nativa, Kofranka, Blaufränkish, Marlen, Cabernet Moravia and Italian Riesling. Results are presented as GAE in g.L^{-1}.

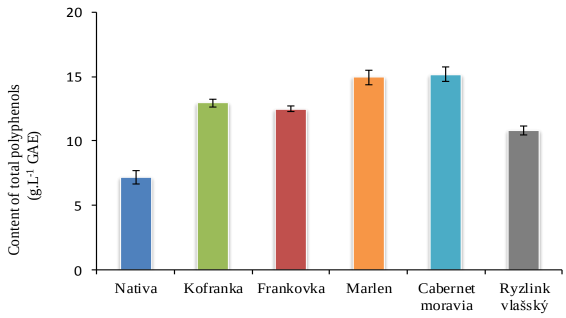

Figure 5 Results of the assessment of total polyphenols in cultivars Nativa, Kofranka, Blaufränkish, Marlen, Cabernet Moravia and Italian Riesling. Results are presented as GAE in mg.L^{-1}.

Ciocalteu reagent. This method is simple and is well reproducible, so it is widely used in biochemistry **(Huang et al., 2005, Singleton et al., 1999)**.

In the cultivars under study, the average content of the total polyphenolic compounds was as follows: Nativa, 7.2 g.L^{-1} of GAE; Kofranka, 12.9 g.L^{-1} of GAE; Blaufränkish, 12.5 g.L^{-1} of GAE; Marlen, 14.9 g.L^{-1} of GAE, Cabernet Moravia, 15.2 g.L^{-1} of GAE, and Italian Riesling, 10.8 g.L^{-1} of GAE. The contents of the polyphenols were markedly different, and the highest and the lowest ones were found in Cabernet Moravia and in Nativa, respectively. The difference was 52.5%.

Table 1 Correlation coefficients among the values of antioxidant activity (DPPH, ABTS, FRAP), the contents of the flavonols, and the contents of total polyphenols.

	DPPH	ABTS	Flavanols	Polyphenols
FRAP	0.177	0.077	0.393	0.108
DPPH	x	0.857	0.311	0.724
ABTS	x	x	0.579	0.649
Flavanols	x	x	x	0.695

Note: The highest correlation coefficient was found between the DPPH and ABTS methods (0.857).

Correlations existing between antioxidant activity, the content of total polyphenols, and the contents of flavonols are presented in Table 1. For this determination was used Pearson correlation coefficient.

DISCUSSION

Songsermsakul et al. (2013) used the DPPH test in their study of antioxidant activities of extracts from grapevine seeds and from other fruits containing high amounts of caroten and vitamins C and E. The results obtained indicated that the antioxidant activity of a capsule containing 20 mg of grapevine extract was approximately 10 to 20btimes higher than that of antioxidants contained in 1 g of tomato fruit, custard apple, banana, or the common mango. In spite of this, the consumption of fruit and vegetables seems to be the cheapest and most efficient way to supply antioxidants within the framework of healthy nutrition, primarily because the prices of dietary supplements are relatively high.

Using the DPPH method, **Soto et al. (2012)** estimated the antioxidant activity in flour from grapevine seeds (cereal sticks, pancakes, and noodles) of the cultivars Merlot and Cabernet Sauvignon. The highest value of antioxidant activity was determined in pancakes that contained flour made of Cabernet Sauvignon seeds. The second place was occupied by cereal sticks also containing Cabernet Sauvigno flour, and the third place by noodles made from Merlot seeds. During sensory evaluation, however, consumers preferred cereal sticks containing Merlot flour. From the general point of view, it is possible to conclude that in these cereals sticks, the ratio between high antioxidant activity and their acceptability for consumers was well balanced **(Soto et al., 2012)**.

Values of antioxidant activity were assessed by DPPH and FRAP with Trolox as a standard antioxidant **(Rockenbach et al., 2011)**. In 100 g of seeds, the cultivar Pinot Noir contained 16,925 mmol of Trolox equivalent (DPPH) and 21,492 mmol Fe^{2+} (FRAP), while the skins of the cultivar Isabel contained only 3,640 µmol TE·100 g^{-1} and 4,362 µmol Fe2·100 g^{-1}. In the skins of the cultivars Cabernet Sauvignon and Primitivo, the highest contents of anthocyans were 935 and 832 mg·100 g^{-1}, respectively. The grapevine seed extract was rich both in oligomeric and polymeric flavanols.

Jakubcová et al. (2015) studied effect of phytogenic additives. Three additives (grapevine seeds, grape and rosehip pressings) were selected to be monitored. The extracts about concentrations of 1: 3 and 1: 5 were prepared from them. The monitoring of antimicrobial properties was focused on the pathogenic bacteria Clostridium perfringens and Escherichia coli causing a serious disease in avian species. Within the antioxidant analysis, the highest antioxidant activity was found out in grapevine seeds (7.021 g.L^{-1} GAE), which also contained the highest content of flavanols (3000 times higher than the rosehip pressings and 300 times higher than grapevine seeds pressings), hydroxycinnamic acids (1000 times higher than in grape pressings and 7600 times higher than in rosehip pressings) and the total content of polyphenolic compounds (580 times higher than grape pressings and 2000 times higher than the rosehip pressings) of the monitored additives.

Antioxidant activity, phenolic content and colour of the Slovak cabernet sauvignon wines were measured by **Bajcan et al. (2016)**. Twenty-eight Cabernet Sauvignon wine samples, originated from different Slovak vineyard regions, were analyzed using spectrophotometry for the content of total polyphenols, content of total anthocyanins, antioxidant activity and wine colour density. Determined values of antioxidant activity in observed wines were within the interval 69.0 – 84.2% inhibition of DPPH (average value was 78.8% inhibition of DPPH) and total polyphenol content ranged from 1,218 to 3,444 mg gallic acid per liter (average content was 2,424 mg gallic acid.L^{-1}). Determined total anthocyanin contents were from 68.6 to 430.7 mg.L^{-1} (average content was 220.6 mg.L^{-1}) and values of wine colour density ranged from 0.756 to 2.782 (average value was 1.399).

Liang et al. (2016) studied beneficial effects of grape seed proanthocyanidin extract on arterial remodeling in spontaneously hypertensive rats via protecting against oxidative stress. Grape seed proanthocyanidin extract (GSPE) has been reported to exhibit a protective effect on cardiovascular disease. In the present study, the effects of GSPE on arterial remodeling were analyzed by treating spontaneously hypertensive rats (SHRs) with GSPE (250 mg.kg^{-1}.day^{-1}). Arterial remodeling was quantified through morphological methods; thoracic aortas were stained with hematoxylin-eosin or sirius red-victoria blue. The arterial ultrastructure was imaged using transmission electron microscopy. The content of nitric oxide (NO) and endothelin-1 (ET-1) were examined to determine endothelial function. Oxidative stress was assessed by malondialdehyde (MDA) levels and the activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). Administration of GSPE markedly alleviated hypertension-induced arterial remodeling, which was not associated with blood pressure control. ET-1 production was reduced, while NO production was increased in the GSPE group, which exhibited improved endothelial function. In addition, treatment with GSPE significantly ameliorated oxidative stress by improving SOD and CAT activities and reducing MDA formation.

Glyphosate impacts on polyphenolic composition in grapevine (*Vitis vinifera* L.) berries and wine studied **Donnini et al. (2016)**. In this study they tested, under field conditions, the effects of glyphosate applications on the quality of berry and wine, from cv. Ancellotta (*Vitis vinifera* L.), with particular regard to anthocyanin concentration and composition. Datas suggest that treatment with glyphosate did not change the concentration of anthocyanins, other flavonoids and phenolic acids in the wine.

Identification of Vitis vinifera L. grape berry skin color mutants and polyphenolic profile was examined by Ferreira et al. **(Ferreira et al., 2016**). A germplasm set of twenty-five grapevine accessions, forming eleven groups of possible berry skin color mutants, were genotyped with twelve microsatellite loci, being eleven of them identified as true color mutants. Results showed differences in the contribution of malvidin-3-0-glucoside to the characteristic Pinot Noir anthocyanins profile. Regarding the two Pique-Poul colored variants, the lighter variant was richer than the darker one in all classes of compounds, excepting anthocyanins. In Moscatel Galego Roxo the F3'H pathway seems to be more active than F3'5'H, resulting in higher amounts of cyanidin, precursor of the cyanidin derivatives.

CONCLUSION

Seeds and their products are recommended for the prevention of many diseases. The issue of dealing with the analysis and comparison of antioxidant components in the seeds of interspecific varieties of *Vitis vinifera*, L. has not yet been sufficiently studied. Grape seeds have shown potential for the prevention and treatment of many diseases. This study showed a high antioxidant potential for grapevine seeds. The results presented corroborate that the content of antioxidant components in grapevine seeds was very high. Differences among the values of antioxidant activity in specific cultivars were not significant. Significant differences were found for the content of flavanols (Blaufränkish was lower than in Nativa by 44.6%) and polyphenolic compounds (Nativa was lower than Cabernet by 52.5%).

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