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THE STUDY OF ANTIOXIDANTS IN GRAPEVINE SEEDS

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ABSTRACT
Grapevine seeds contain a large amount of antioxidant components, and are therefore recommended in the prevention and treatment of many diseases. For this research, we studied the antioxidant properties of grapevine seeds from the Marlen variety, as evidence suggests that these types have higher resistance against fungal diseases. Through high-performance liquid chromatography with UV/VIS detection, a total of 10 antioxidant components were selected for further investigation, specifically: catechin, epicatechin, rutin, quercitrin, quercetin, caftaric acid, caffeic acid, p-coumaric acid, ferulic acid, and gallic acid. The antioxidant activity was determinated spectrophotometrically through the adoption of three fundamentally different methods (the DPPH assay, the ABTS method, and the FRAP method). Using the Folin-Ciocalteu method, it was possible to determine the content of all the polyphenolic compounds. The results of the assessment antioxidant activity and the content of polyphenolic compounds were recalculated to gallic acid equivalents (GAE). The values of the antioxidant activity as determined by the DPPH test were 6643 (±154) mg of GAE; 1984 (±88) mg of GAE when using the FRAP method; and 812 (±31) mg of GAE when the ABTS method was utilised. The content of the total polyphenolic compounds came to 6982 (±221) mg of GAE. The most abundant antioxidant was catechin, with a content of 115 $mg.L^{-1}$, whilst the least represented compound was ferulic acid (0.139 $mg.L^{-1}$). Overall, this study showed a high antioxidant potential of grapevine seeds.

Keywords: grapevine seeds; antioxidants; HPLC-UV/VIS

INTRODUCTION
Antioxidants are potent scavengers of free radicals and serve as inhibitors of neoplastic processes **(Bagchi et al., 2000)**. *Vitis vinifera*, L. is one of the world's largest fruit crops, with approximately 38 million tonnes of grapes produced every year in Europe **(Vrsic et al., 2011)**. Grape seeds are rich sources of polyphenolic compounds, which are characterised by a variety of properties, such as antibacterial and antioxidant activities **(Berradre et al., 2013)**. Results of many studies indicate that grapevine seeds are a source of biologically active substances usable for pharmaceutical and other purposes **(Ali et al., 2010)**. Various research has indicated that the phenolics in grapes can be used to prevent atherosclerosis **(Pekić et al., 1998)**. Recognition of the health benefits of catechins and procyanidins has led to the use of grape seed extract as a dietary supplement **(Fuleki & Ricardo da Silva, 1997)**. In addition, the naturally occurring antioxidant of oligomeric proanthocyanidins has been reported to possess a broad spectrum of therapeutic benefits **(Bagchi et al., 2000)**. Furthermore, phenolics are largely responsible for a dietary anomaly known as the French paradox **(Catalgol et al., 2012)**. The antioxidant activity in grapevine seeds has been studied across a number of popular and hybrid varieties; however, further studies should start focusing more on interspecific varieties, such as Marlen, to determine their antioxidant activity and phenolic content **(Yilmaz et al., 2015)**.

The aim of this study is to determine the antioxidant activity, the content of polyphenolic compounds, and the content of concrete antioxidants in cultivar Marlen (*Vitis vinifera*, L.) Grapevine seeds contain great amounts of biologically active components **(Pascoa et al., 2015)**. This fact has been proven by many scientific studies dealing with the antioxidant potential of this waste material. It has also been suggested on numerous occasions that using grapevine seeds and derived products can protect humans against many diseases. Grape seed extract, in particular, has been reported to possess a broad spectrum of pharmacological and therapeutic effects, such as antioxidative, anti-inflammatory, and antimicrobial activities, as well as cardioprotective, hepatoprotective, and neuroprotective benefits **(Nassiri-Asl and Hosseinzadeh, 2009)**. Due to the influence of synergism, natural extracts from these seeds are more efficient than isolated material obtained from other identical substances.

Our work focuses on the study of antioxidant activity and polyfenolic compounds in grapevine seeds, with the main target being to determine 10 antioxidant components through high performance liquid chromatography.

MATERIAL AND METHODOLOGY

Biological samples

This experimental study was performed with grapevine seeds (*Vitis vinifera* L.) of the interspecific cultivar Marlen. The experimental material originated from the Department of Viticulture and Enology, Faculty of Horticulure, of Mendel University in Brno.

Chemicals

The chemicals used in this study were supplied by the firm Sigma Aldrich (Germany). Antioxidant standards were products of the company Extrasynthese (France).

Chemicals: Deionised water, stable free radical DPPH$^{\bullet}$, cation radical ABTS$^{\bullet}$, methanol, acetic acid (0.2%), liquid nitrogen, TPTZ (2,4,6-tripyridyl-s-triazin), hydrochloric acid, $FeCl_3$, acetate buffer, sodium acetate, Folin-Ciocalteu reagent, sodium carbonate decahydrate ($NaCO_3.10\ H_2O$ standard antioxidants: catechin, epicatechin, cis-resveratrol, rutin, quercitrin, quercetin, tyrosol, vanillic acid, syringic acid, caftaric acid, caffeic acid, p-coumaric acid, ferulic acid, and gallic acid.

Method of sample preparation

The experimental material originated from grape pomace. Seeds were cleaned, and dried for 24 hours (55 °C). The seeds were then crushed using a laboratory mill (MF 10 basic, IKA, Germany). The mixture was extracted with ethanol (75%), 1:10 (seeds: EtOH). The extraction was performed under dark conditions for 72 hours and at 15 °C using the shaker (IKA KS 260 Basic, Germany). The samples were subsequently centrifuged (CompactStar CS4, Company Manek, Czech Republic) and transferred into vials, ready for spectrometric and chromatographic analysis.

Assessment of antioxidant activity

Antioxidant activity was determinated by three fundamentally different methods. Spectrophotometric measurements of antioxidant activity were carried out using the UV-Vis Spekol 1300 (Analytikjena, Germany). All samples were measured three times. The result value was obtained as an average of these measurements, with the results of these analyses expressed as gallic acid equivalents.

Determination of Antioxidant Activity by the ABTS Test

Preparation of the solution

The solution for the determination of the antioxidant activity was prepared by mixing two solutions. Solution 1: 7 mmol.L^{-1} solution ABTS (2,2'-azinobis 3 ethylbenzothiazoline-6-sulfonic acid) was prepared by weighing m = 9.60 mg per 5 ml of distilled water. Solution 2: 4.95 mmol.L^{-1} solution of potassium peroxodisulfate used m = 1.67 mg per 5 ml of distilled water. These two solutions were then mixed. The resulting solution was diluted with distilled water at the ratio 1:10. The solution was left for 12 hours in a dark and cold environment.

Spectrometric analysis

A 1500 µL volume of the ABTS reagent (7 mM 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) and 30 µL of the sample (extract from the grapevine seeds) was pipetted into the cuvette (3 mL). The mixture was incubated at ambient room temperature for 30 minutes. After this time, the absorbance was measured at 734 nm.

Figure 1 Grape seeds (*Vitis vinifera* L., cultivar Marlen).

Determination of Antioxidant Activity by the DPPH Method

Preparation of the solution

9.35 mg of DPPH• radical was weighed. This amount was transferred to a 250 mL volumetric flask and filled with methanol.

Spectrometric analysis

A 2000 µL volume of the DPPH• solution was pipetted into the cuvette (3 mL). 40 µL of the sample (extract from the grapevine seeds) was then pipetted into the same cuvette. The mixture was incubated at ambient room temperature for 25 minutes. After the incubation, the absorbance was measured at 505 nm.

Determination of Antioxidant Activity by the FRAP Method

Preparation of the solution

Three solutions were used to determine the antioxidant activity using the FRAP method. 1. The solution TPTZ: 10 mM TPTZ (m = 78.02 mg) was dissolved in 25 mL of 40 mM hydrochloric acid (HCl); 2. The solution of 20 mM $FeCl_3$: m = 135.13 mg of $FeCl_3$ was dissolved in 25 mL of distilled water; 3. The solution of acetate buffer: 0.02 M acetate buffer pH 3.6 (m = 775 mg of sodium acetate) was dissolved in 250 mL of distilled water, upon which the pH was adjusted with acetic acid. These three solutions were mixed at a ratio TPTZ: $FeCl_3$: acetate buffer – 1: 1: 10.

Spectrometric analysis

A 980 µL volume of reagent (a mixture of TPTZ: $FeCl_3$: acetate buffer) together with 20 µl of the sample was injected into a plastic cuvette and incubated at 37 °C in a thermoblock. 1000 µL of 100 mM of Na_2SO_4 in 50 mN HCl was the added and agitated; after 10 minutes, the absorbance was measured at 620 nm against a blank. The reducing power was calculated from a calibration curve using gallic acid as a standard. Results are expressed as mg.L^{-1} equivalents of gallic acid.

Assessment of content of total polyphenols

A 40 µL volume of the sample was pipetted into the cuvette (3 mL) and diluted with 1960 µL of distilled water. 50 µL of Folin-Ciocalteu reagent was added into the cuvette and the mixture was shaken. After 3 minutes, 300 µL of 20% solution of $NaCO_3$ decahydrate was added. The mixture was shaken and incubated at 22 °C for 120 minutes. After this time, absorbance was measured at λ = 750 nm against a blank.

Assessment of antioxidant components by HPLC-UV-VIS

To determine the HPLC profiles of the individual cultivars, high performance liquid chromatography (HPLC) with UV-VIS detection was used. The system consisted of two Model 582 ESA chromatographic pumps (ESA Inc., Chelmsford, MA, USA) with a working range from 0.001 to 9.999 mL.min^{-1} and a Zorbax SB C18 (150 × 4.6; size of particles 5 µm, Agilent Technologies, USA) reverse phase chromatographic column. For UV detection, a Model 528 ESA UV detector was used.

RESULTS

To determine the antioxidant characteristics, chromatographic and spectrometric techniques were used. Spectrophotometric methods (determination of the total polyphenolic compounds and antioxidant activity) was assessed by monitoring the number of antioxidants in the sample. Through chromatography, the 10 determined antioxidant components were particularly interesting due to their content in grapes of *Vitis Vinifera*, L.

Assessment of antioxidant activity and polyphenolic compounds

The Folin-Ciocalteu method was used spectrometrically, with the content of the total polyphenolic compounds being determined in the grape seed extract. Such an approach was used because it is a good, simple, and inexpensive way to determine the polyphenols in various fruits and vegetables. A wide range of methods to determine antioxidant activity can be found in existing literature, highlighting how low molecular weight antioxidants act differently depending on the mechanism used. Consequently, antioxidant activity was explored through three fundamentally different methods (ABTS, FRAP, and DPPH). The results are shown in Figure 2. Results are present as a mg.L^{-1} of gallic acid equivalents.

6,643 (± 154) mg of gallic acid equivalents (GAE) of antioxidant activity was determined using the DPPH test,

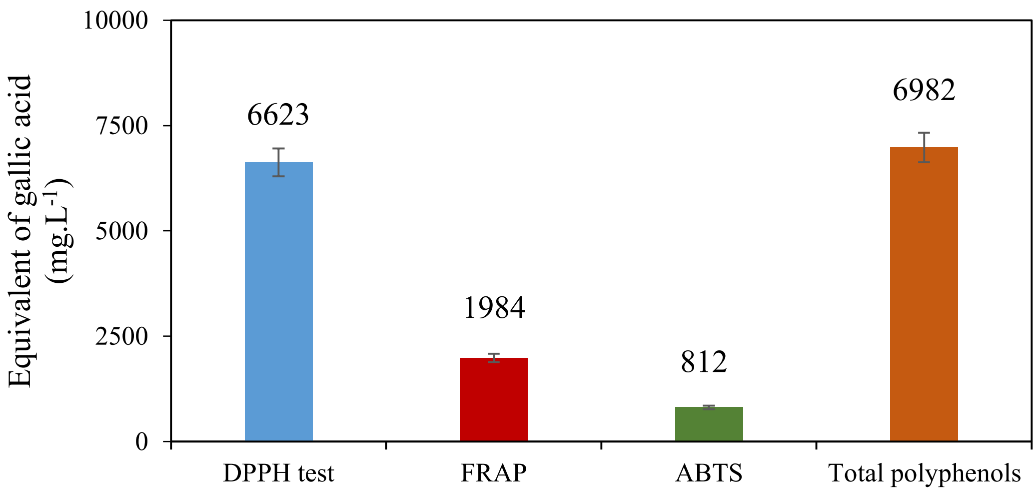

Figure 2 Antioxidant activity of the grape seeds (*Vitis vinifera* L., cultivar Marlen).

Table 1 The content of antioxidant components in grape seeds (*Vitis vinifera* L., cultivar Marlen) determinated by HPLC-UV/VIS. Results are expresed in the $mg.L^{-1}$ of extract.

compounds	content ($mg.L^{-1}$)
catechin	114.51 (±7.1)
epicatechin	79.81 (±4.1)
rutin	1.98 (±0.19)
quercitrin	1.09 (±0.14)
quercetin	1.15 (±0.17)
caftaric acid	2.87 (±0.20)
caffeic acid	2.09 (±0.22)
p-coumaric acid	0.687 (±0.11)
ferulic acid	0.139 (±0.05)
gallic acid	27.54 (±1.9)

1,984 (± 88) mg of GAE was obtained using the FRAP method, and 812 (± 31) mg of GAE was found using the ABTS method. Overall, the content of the total number of polyphenolic compounds was 6,982 (± 221) mg of GAE.

Determination of individual antioxidant components

HPLC with UV/VIS detection was used to determine the content of 10 interesting antioxidant components (Table 1). Although the experiment focused on both flavonoids and non-flavonoids, we deliberately devoted more time to analysing hydroxycinnamic acids, as these are an important part of grapes and grapevine seeds. Large quantities of caftaric acid and caffeic acid substances are also contained within the grapes.

The most abundant antioxidan was catechin, with a content of 115 $mg.L^{-1}$, whilst the least represented compound was ferulic acid (0.139 $mg.L^{-1}$). This study showed a high antioxidant potential of grapevine seeds.

DISCUSSION

Berradre et al. (2013) analysed the varieties of Malvasia and Tempranillo. They determinated the total polyphenol content using the Folin-Ciocalteu method, and the antioxidant activity with the ABTS method, using Trolox as standard. Antioxidant activity was TEAC 54.5 mmol.100 g^{-1} of the Malvasia sample, and TEAC 48.5 mmol.100 g^{-1} with the Tempranillo variety **(Berradre et al., 2013)**.

Brazilian researchers **(Rockenbach et al. 2011)** studied the antioxidant activity and the content of phenolic compounds from the seeds and skin of *Vitis vinifera* and *Vitis labrusca*. The concentration of phenolic compounds in the seeds ranged from 2,128 to 16,518 mg catechin per 100 g, and from 660 to 1,839 mg catechin per 100 g in the skin. The antioxidant activities were determined using DPPH and FRAP, with Trolox as a standard. Seeds of Pinot Noir contained 16,925 mmol Trolox equivalent per 100 g (DPPH), and 21,492 mmol Fe $^{2+}$.100 g^{-1} (FRAP). The skin of the Isabel varieties had 3,640 umol TE.100 g^{-1} and 4,362 umol Fe^{2+}.100 g^{-1}, whilst Cabernet Sauvignon and Primitivo had the highest content of anthocyanins (935 and 832 mg.100 g^{-1}) **(Rockenbach et al., 2011)**.

Polish scientists **(Samoticha et al., 2017)** used the ABTS, FRAP and ORAC methods to study fruit quality parameters and chemical properties (soluble solids, pH, total acidity and total sugar content, phenolic compounds, and antioxidant activity) of 30 white, red and pink grapes; 28 interspecific hybrids, and 2 *Vitis vinifera L.* commonly grown in Poland. A total of 49 polyphenolic compounds were identified through LC-PDA-QTOF/MS, and 26 anthocyanins, 9 flavonols and fiavons, 7 phenolic acids, 6 flavan-3-ols, and 1 stilbene were quantified using UPLC-PDA-FL. The content of all the polyphenols ranged from 1037.0 (Cascade cv.) to 5759.1 mg.100 g^{-1} dm (Roesler cv.). Red grape cultivars such as Roesler, Rothay and Swenson Red were characterised as having the highest content of bioactive compounds and antioxidant activity (significantly more than 24, 12 and 53 mmol TE.100 g^{-1} dm, by ABTS, FRAP and ORAC, respectively).

Burg et al. (2017) studied the physical properties and level of oil extraction from grape seeds from three white (Welschriesling, Green Veltliner, Hibernal) and two red (Zweigelt and Saint Laurent) must varieties of grapevine, using cold screw pressing as the appropriate extraction process. The results show that the density ranged from 602.7 to 606.3 $kg.m^{-3}$, the weight of 1,000 seeds was between 21.9 – 26.6 g, the humidity of dry matter and seed oil content was between 5.6 – 7.1%, depending on the variety and the extracting; it reached 15.3 – 17.5% in dry conditions.

Weidner et al. (2013) studied the phenolic compound obtained from the seeds of European and Japanese species of grapevine (*Vitis vinifera* and *Vitis coignetiae*) using 80% methanol and 80% acetone. The total content of phenolic compounds was determined using the Folin-Ciocalteu reagent, which also monitored the content of tannins. The methods of DPPH and ABTS were used to determine their antiradical activities. The HPLC method was used to determine the phenolic compounds, such as phenolic acids and catechins. The seeds contained large quantities of tannins and an observable quantity of catechins, p-coumaric, ferulic, and caffeic acid. The content of total phenolic compounds was higher in European grapes than in those from Japan.

Iraqi scientist **Dalaram (2017)** determined the total polyphenol content and antioxidant activity in four varieties of lupin. The content for all the polyphenols was determined using the Folin-Ciocalteu reagent (FCR). Antioxidant activity was measured using a compound $DPPH^{\cdot}$ (2.2-diphenyl-1-picrylhydrazyl). Based on the measured values of total antioxidant capacity (TAC), the lupin samples can be classified as follows: L. Albus (white) lupin (43.44%) >L. Angustifolius (blue) lupin

(38.27%) >L. Luteus (yellow) lupin (22.29%) >L. Mutabilis (Pearl) lupin (20.80%).

Li et al. (2008) studied the content of phenolic substances and the antioxidant activity of a powder made of grapevine seeds. The antioxidant potential of the seed extract was assessed by the following tests: CUPRAC, DPPH, ABTS, and OH quenching of electronically excited radicals. A physiological *in vitro* method enabled higher levels of phenolic compounds to be reached, and a greater antioxidant capacity than would have been possible using chemical methods. A mixture of aceton:water (70: 30) was used as a solvent to maximise the yield of phenols and to enhance the antioxidant capacity. Our results indicate that biological properties of natural antioxidants treated physiologically under *in vitro* conditions may be useful in the field of healthy nutrition **(Li et al., 2008)**.

Farhadi et al. (2016) determinated the content of phenolic compounds and antioxidant activity in the skin, pulp, seed, cane and leaf of one international (Muscat) and five native (Hosseini, Ghara Shira, Agh Shani, Ghara Shani and Ghara Ghandome) grapes cultivated in West Azerbaijan, Iran. The skin of the Ghara Shani grape was found to contain the highest total content of phenolics and anthocyanin, and its cane contained the highest amount of flavonoid. Due to a remarkable DPPH, where radical scavenging activity rose to 95%, the lowest IC50 was found in the skin of the Ghara Shani.

Scientists from China **(Wen et al., 2016)** explored the potential of the large amount of grape pomace in wineries of China; the oils of three Eurasian grape cultivars (Chardonnay, Merlot and Carbernet Sauvignon), and two traditional Chinese grape cultivars (Vitis amurensis and Vitis davidii). Grape seed oils proved to be good sources of polyunsaturated fatty acid (PUFA) (63.88 – 77.12%), sterols (227.99 – 338.83 mg.100 g^{-1} oil) and tocotrienols (320.08 – 679.24 $mg.kg^{-1}$ oil). Seed oil of V. amurensis exhibited the highest values for polyunsaturated fatty acid, total tocotrienols, and total tocols, as well as for the DPPH centre dot scavenging capacity. Seed oil of the Carbernet Sauvignon had the highest content of squalene, total sterols, total tocopherols, and total phenolics.

CONCLUSION

Studying the antioxidant components in grape seeds and their potential benefit to human health is the greatest contribution in this field of research. From the obtained results, we can say that grapevine seeds display a very high content of antioxidative components, which provide a lot of benefits for human health. Exploring the functional components of grapes is interesting from several aspects. In addition to being an important factor in the organoleptic properties of wine (as well as other products such as oil and flour), what is more interesting is how they benefit human health, as demonstrated by countless studies. As they come from agro-industrial waste, grapes do not represent a significant economic burden in the production of final products. Therefore, they have the potential to be used in the production of natural supplements and products for people directly.

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