

Antioxidant Activity and the Tocopherol and Phenol Contents of Grape Residues

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The antioxidant activity and the tocopherol and polyphenol contents of organic grapevines residue were analyzed after Soxhlet extraction of grape seeds and skin with ethanol and petroleum ether. The highest antioxidant activity was determined for the ethanol extract of the seeds and was 58.65 μM Trolox/mg, which was four times higher than for the ethanol extract of the skin at 14.24 μM Trolox/mg. It was comparable to that of the alpha-tocopherol (62.28 μM Trolox/mg) and butylated hydroxyanizol (69.55 μM Trolox/mg). A concentration of 29.5 $\mu\text{g/mL}$ ethanol seed extract was needed to decrease the initial DPPH radical concentration by 50%. The high antioxidant activity was because of the high tocopherol (402.28 mg/kg) and total phenols contents (113.7 gallic acid equivalent (g/kg)) in the seeds. The dominant polyphenol in the ethanol seed extract was coumaric acid (10.97 g/kg). The dominant polyphenols in the ethanol skin extracts were rutin (6.79 g/kg), quercetin (3.75 g/kg), and catechin (3.99 g/kg), which can be used in functional foods to reduce risk factors for several human diseases.

Keywords: Antioxidants; Rutin; Quercetin; Tocopherol; Grape skin; Grape seeds

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INTRODUCTION

Grapevine is one of the oldest cultivated fruit plants in the world. In the fruit crop industry, grapes have the second highest annual world production after oranges. Grapevine is an important plant from an economic point of view. Almost 75% of global grape production is devoted to wine production, approximately 25% of production is for table grapes, and a small portion of production is for dried grapes (raisins) and non-alcoholic grape juice. The residue after grape pressing makes up approximately 20% of the weight of the processed grapes and represents up to 10 million ton of by-products each year (Robinson and Harding 2015). Full utilization of this waste by-product is important for waste reduction. The utilization of the by-products from grape processing is important from economic and environmental points of view (Bail *et al.* 2008). Grape seeds contain 10% to 20% of oils, which are rich in unsaturated fatty acid. The content of fatty acids in grape seed oil is in range 7 to 10.24% for palmitic, 2 to 5% for stearic, 16 to 22% for oleic, 63 to 71% for linoleic, and 0.1 to 0.4% for linolenic acid (Beveridge *et al.* 2005; Fernandes *et al.* 2013; Lachman *et al.* 2015). The tocopherol content differs between varieties and is influenced by the climatic conditions (Beveridge *et al.* 2005). The dominant tocopherol is alpha-tocopherol at 128 mg/kg to 325 mg/kg, and there are lower values of gamma-

tocopherol (14 mg/kg to 45 mg/kg) and delta-tocopherol (0.5 mg/kg to 1.8 mg/kg) (Baydar *et al.* 2007).

Grape seeds are a good source of phenolic substances, such as catechin, epicatechin, gallic acid, vanillic acid, coumarin, ferulic acid, synergic acid, pyrogallol, and caffeic acids, which all have antioxidant properties. Rutin and quercetin, which are also found in grapes, have important biological activities. Rutin inhibits proliferation, decreases adhesion and migration of human cancerous cells, has an anticancer activity against colorectal carcinogenesis, acts against coronary circulation disease, and protects the vascular barrier integrity (Santos *et al.* 1999; Lee *et al.* 2012; Sghaier *et al.* 2016). Quercetin has an anti-carcinogenic potential, antiproliferative and anti-cancer properties, inhibits angiogenesis, and protects healthy cells against oxidative stress and mutagenesis as a chemo-preventive substance (Borska *et al.* 2012; Bulzomi *et al.* 2012). This phenolic compound are usable in the pharmaceutical industry because of its anti-inflammation, anti-aging, anti-mutagenic, and anti-carcinogenic properties and is associated with a reduced risk of cardiovascular disease (Teissedre *et al.* 1996; Catterall *et al.* 2000; Zern *et al.* 2005).

Tocopherols and phenols substances can be applied as functional compounds, which prevent health damage from free radicals. Free radicals can damage the proteins in cells, such as DNA, enzymes, and lipids. These damages may contribute to the development of diseases, such as cardiovascular disease and cancer. The consumption of foods with high antioxidant contents may contribute to an improvement in health. Grapes are a rich source of lipid-soluble tocopherol, which is a natural antioxidant (Tangolar *et al.* 2011).

For this reason, this study was focused on the extraction, separation, and identification of antioxidant active substances such as tocopherols and polyphenols from waste biomass of the organic grapevine.

EXPERIMENTAL

Methods

Grapevine biomass characterization

Grapevine pressing residues (skin and seeds) from the Cabernet Sauvignon grape variety were obtained from Natural Wine Domin & Kušický (Veľký Krtíš, Slovak Republic), which is a producer of organic grapevine products. The whole territory of vineyard lies on the borders of the Ipel' valley and Krupina plateau (south Slovakia) that is of volcanic origin. Vineyards soils are rich in medium heavy and heavy brown soil (this type of soil is fertile, has a favorable water regime, and good aeration). Only copper and sulfur-based insecticides are used to vine protection, but with the limited use. Grapes are harvested manually; it is immediately processed and carefully pressed. Grape skin and seeds are waste biomass and by-products from processing grapes into wine; therefore, the biomass residue obtained was classified into skin and seeds.

Grapevine biomass extraction

The grape skins and seeds were extracted by Soxhlet extraction. The ratio of the biomass and extraction solvent was 1:50. The extraction solvents were ethanol and petroleum ether (Centralchem s.r.o., Bratislava, Slovakia). The extraction time was 6 h. Petroleum ether was used for extraction of fat-soluble antioxidants such as tocopherols. Ethanol was used for extraction of polyphenols according to the studies of Franco *et al.* (2018) and Pinelo *et al.* (2005), in which ethanol was the best extraction solution for

extraction of grape antioxidants. The extraction solvents were then evaporated, and the dry extracts were stored at -20 °C until analysis.

Tocopherol determination

The tocopherol content was analyzed by high performance liquid chromatography-ultraviolet (HPLC-UV) and calculated from a calibration curve of tocopherol standards. The column was a Nucleosil 100 Si (Macherey-Nagel Ltd., Düren, Nemecko) with a grain size of 5.10^{-6} m and the dimensions 250 nm × 4 nm. The mobile phase was n-hexane-isopropanol with a ratio of 98.8:1.2 (Centralchem s.r.o., Bratislava, Slovakia). The flow rate was 1 mL/min and the injection volume was 20 µL. The concentration of the injected sample was 70 mg/mL of n-hexane at a wavelength of 292 nm.

Polyphenol determination

The content of selected phenolic/polyphenol compounds was determined by HPLC-UV according to the modified method by Vallverdú-Queralt *et al.* (2015) and calculated with a calibration curve of standards (quercetin, rutin, catechin, caffeic acid, and coumaric acid). The column was a two times Watrex Reprosil 100 C18 (5 µm) (Watrex, Praha, Czech Republic). The composition of mobile phase A was a 95:5 ratio of deionized water:acetonitrile with a pH of 2.9. Mobile phase B was composed of a 50:50 ratio of deionized water:acetonitrile with a pH of 2.9. For the flow rate, the mobile phases ratio from 10 min to 50 min changed from 70:30 (A:B) to 50:50, and then the mobile phases ratio from 50 min to 55 min changed from 50:50 to 70:30. The flow rate of mobile phase was 1 mL/min. The injection volume was 5 µL, and the concentration of the injected sample was 50 mg/mL n-hexane at a wavelength of 375 nm.

Determination of antioxidant activity

This assay is based on electron donation of antioxidants to neutralize the DPPH radical, which is accompanied by a color change that is measured at 517 nm (Yhong *et al.* 2015). Antioxidant activity was performed according to modified method by Mareček *et al.* 2017. To conduct the DPPH method, 75 µL of DPPH solution with a concentration 500.10^{-3} mM were added to 200 µL of all of the extracts in ethanol UV with a concentration 1 mg/mL. The control used 200 µL of the prepared sample with 75 µL of ethanol. The calibration solutions with concentrations of 1 µM, 2 µM, 3 µM, 4 µM, 5 µM, 6 µM, 7 µM, 8 µM, 9 µM, 10 µM, 11 µM, 12 µM, 13 µM, 20 µM, 30 µM, 50 µM, and 60 µM were prepared by diluting of the Trolox solution with concentration 400 µM.

DPPH radical-scavenging activity

All of the extracts in 100 µL of ethanol UV with concentrations of 5 mg/mL⁻³, 10 mg/mL, 20 mg/mL, 50 mg/mL, 80 mg/ml, 100 mg/mL, and 300 mg/mL were added to 75 µL of ethanolic DPPH solution (500.10^{-3} mM). The mixtures were shaken at 26 °C for 60 min. After this, the absorbance was measured at 517 nm using a microplate reader (EPOCH 2, BioTek Instruments, VT, USA). The scavenging activity (IC50) of the extracts was calculated according to Rezaei-Sadabady *et al.* (2016) with Eqs. 1 through 3,

$$IC50 (\%) = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100\% \quad (1)$$

$$A_{\text{control}} = A_{\text{DPPH+ethanol}} - A_{\text{ethanol}} \quad (2)$$

$$A_{\text{sample}} = A_{\text{extract+DPPH}} - A_{\text{extract+ethanol}} \quad (3)$$

where IC_{50} is the amount of antioxidant needed to decrease the initial DPPH radical concentration by 50% (Brand-Williams *et al.* 1995).

Determination of the total phenols

The total phenols were determined according to the modified method by Singleton *et al.* (1999). A Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to 200 μ L of the extract. After 10 min, 3 mL of Na_2CO_3 were added and after 30 min, the absorbance was measured at 765 nm. Gallic acid was used as the standard.

Statistical analysis

All analysis were performed three times. Mean value of experimental data was determined with confidence interval (95%), calculated with single-variable analysis of data.

RESULTS AND DISCUSSION

Grape seeds and skins, as agricultural by-products, are attractive potential sources of natural antioxidants, which consist of tocopherols and polyphenols. The grape seeds had a higher tocopherol content in this study, as is shown in Fig. 1.

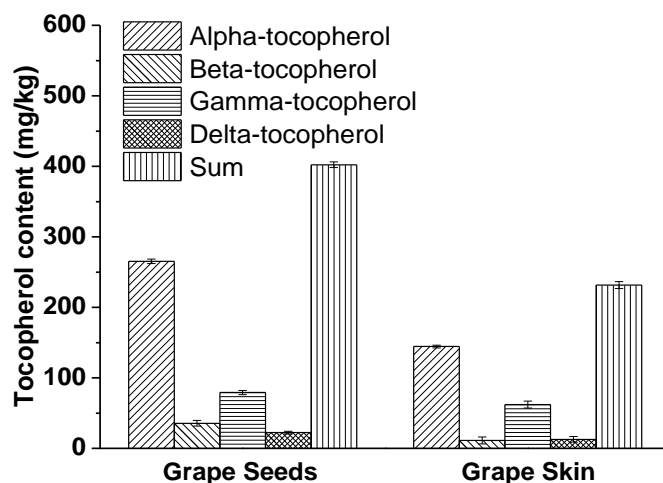


Fig. 1. Comparison of the tocopherol content in the grape seeds and skin (Cabernet sauvignon)

The total tocopherol content in the grape seeds was 402.28 mg/kg, which was 1.7 times higher than in the grape skin. According to Juhaimi and Özcan (2018), the total tocopherol content in petroleum ether seed extracts was 444.01 mg/kg. Baydar and Akkurt (2001) stated that the total tocopherol content ranged from 328 mg/kg to 578 mg/kg. The tocopherol content in the seeds decreased in the following order: alpha-tocopherol (265.25 mg/kg) > gamma-tocopherol (79.15 mg/kg) > beta-tocopherol (35.59 mg/kg) > delta-tocopherol (22.29 mg/kg). The same order of tocopherol content is presented in study Gliszczynska-Świgło and Sikorska (2004), where they found α , γ , β , and δ -tocopherols to be 100.55 mg/kg, 17.14 mg/kg and 3.89 mg/kg, respectively. Baydar *et al.* (2007) determined the content of alpha-tocopherol 128 to 325 mg/kg, 14 to 31 mg/kg for gamma-tocopherol, and 0.6 to 1.6 mg/kg for delta-tocopherol. The variance of the values is

dependent on the grape variety. Several studies confirm that alpha-tocopherol is the dominant type of tocopherol in seeds, and its content varies depending on climatic conditions, the growing area and grape cultivars (Crews *et al.* 2006; Sabir *et al.* 2012).

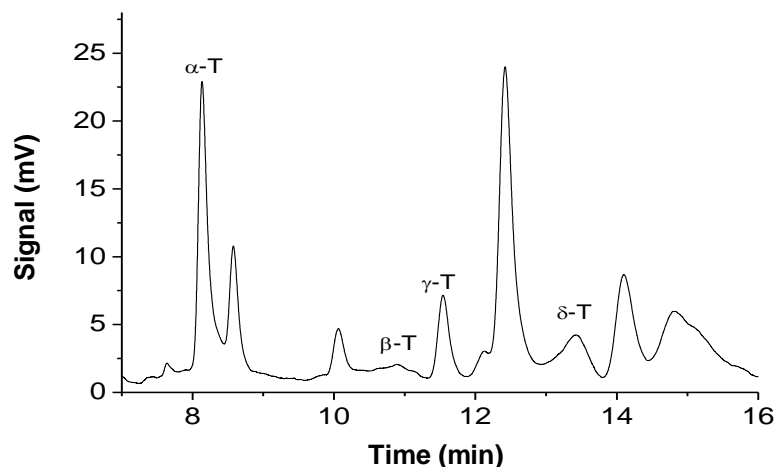


Fig. 2. HPLC chromatogram of tocopherols in petroleum ether extract of grape seed

Polyphenolic antioxidants are predominantly polar substances. For this reason, ethanol extracted more of these substances than petroleum ether. The solvent types, extraction time, and temperature had important effects on the polyphenol concentration and antioxidant activity of the grapes (Spigno *et al.* 2007). Jayaprakasha *et al.* (2001) confirmed that substances with a high antioxidant content affect the ethanol extraction of grapevines, which was consistent with the results of this study (Table 1). The total phenol content was determined to be 113.7 gallic acid equivalents (GAE, g/kg) and 7.1 g/kg for the seeds and skin, respectively. In previous studies, the total phenol content in the seeds ranged from 70 g/kg to 350 g/kg (Bail *et al.* 2008; Maier *et al.* 2009; Rombaut *et al.* 2015). Iacopini *et al.* (2008) determined total phenol content in skin of Cabernet sauvignon 53.04 g/kg and in seed it was 87.08 g/kg.

Table 1. Polyphenols Content in the Grape Biomass and Total Phenols Content

		GAE (g/kg)				
Seed Et-OH		113.71±0.03				
Skin Et-OH		7.1±0.01				
		Polyphenols Content (g/kg)				
		Quercetin	Catechin	Caffeic Acid	Coumaric Acid	Rutin
Seed Et-OH		1.41±0.02	2.66±0.01	0.63±0.04	10.97±0.05	0.07±0.01
Skin Et-OH		3.75±0.03	3.99±0.02	0.68±0.02		6.79±0.06
Et-OH – Ethanol extract						

Only the grape seeds contained coumaric acid (10.97 g/kg). HPLC chromatograms of grape seed ethanol extract are shown in Fig. 3. Quercetin (3.75 g/kg) and glycoside rutin (6.79 g/kg) were found in higher amounts in the grape skin. Several studies confirm the presence of rutin and quercetin in grape skin (Romero-Pérez *et al.* 2001; Careri *et al.* 2003; Sun *et al.* 2006; Iacopini *et al.* 2008; Mazza *et al.* 2018). Rutin (0.4 mg/kg), quercetin (0.63

g/kg), and catechin (1.15 g/kg) were also found in variety Syrah grape skin (Mazza *et al.* 2018). Iacopini *et al.* (2008) confirmed the presence of rutin and quercetin in grape skin in the amounts 0.88 to 1.58 g/kg for rutin and 0.6 to 1.5 g/kg for quercetin. On the other hand, the grape seeds contained 2.5-times lower content of quercetin (1.41 g/kg) and 6-times lower amount of rutin (0.07 g/kg). Grape seeds are good sources of catechin (2.66 g/kg). It is in good correlation with Iacopini *et al.* (2008), where catechin were determined in range 0.67 to 2.05 mg/kg depending on the grape variety. Previous studies confirmed that grape seeds contain only small amounts of quercetin and rutin (Iacopini *et al.* 2008; Maier *et al.* 2009). The high amount of quercetin and rutin in the grape skin makes this waste material potentially interesting for the food, pharmaceutical, and cosmetic industries. The consumption of functional food with high antioxidant contents reduces the risk factors for human diseases (Udenigwe *et al.* 2009; Chen *et al.* 2017).

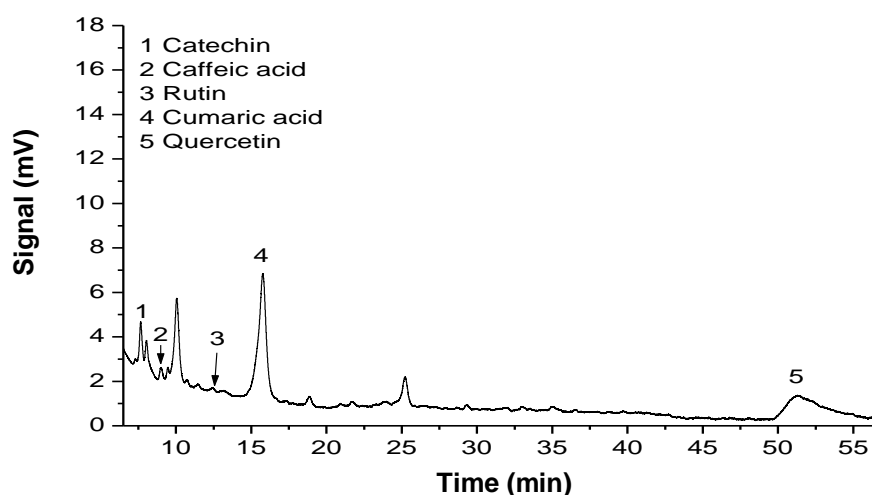


Fig. 3. HPLC chromatograms of grape seed ethanol extract; analysis at 375 nm

Table 2. Antioxidant Activity and IC₅₀ of the Grape Seeds, Skins, and Selected Antioxidants

Extracts/antioxidants	Antioxidant activity	IC ₅₀
	μM TE/mg extract	μg/mL
Skin PEE	2.03 ± 0.01	200.21±0.06
Seed PEE	4.42 ± 0.51	528.10±0.08
Skin Et-OH	14.24 ± 0.51	66.78±0.04
Seed Et-OH	58.65 ± 0.02	29.50±0.02
Alfa-tocopherol	62.28 ± 0.03	27.10±0.01
Delta-tocopherol	61.39 ± 0.06	28.41±0.03
Quercetin	57.49 ± 0.01	31.20±0.02
Butylated Hydroxytoluene	41.76 ± 0.02	39.15±0.03
Butylated Hydroxyanisole	69.55 ± 0.03	19.78±0.01

PEE – Petroleum ether extract; Et-OH – ethanol extract

The solvent type has a major influence on the extraction of active antioxidant substances from grapevine biomass (Spigno *et al.* 2007; Zhong *et al.* 2015). Ethanol extracted polyphenolic substances, which have a high antioxidant activity (Table 2). Petroleum ether extracted lipophilic antioxidants include substances such as tocopherols.

Secen (2017) stated that the content of active antioxidant phenolic substances in grapes can be affected by environmental factors and processes applied during grape growing. Results of antioxidant activity showed that the ethanol seed extract had a high value of 58.65 $\mu\text{M TE/mg}$. This value was comparable to that of polyphenol quercetin, as well as the antioxidant alpha-tocopherol and synthetic antioxidant butylated hydroxyanisole (BHA), which can be applied as additives in the food industry. The high antioxidant activity is due to the presence of polyphenols (Table 1) and tocopherol (Fig. 1) in grape seed. According to several studies, there is a presumption of a synergic effect of antioxidants (polyphenols, tocopherol, anthocyanins, and carotenoids) (Guendez *et al.* 2005; Bozan *et al.* 2008). The result is excellent in terms of applicability in the food and cosmetic industries. The possibility of using antioxidant active substances from grapes is confirmed in the studies Lorenzo *et al.* (2018) and Trošt *et al.* 2016. Jayaprakasha *et al.* (2001) confirmed that substances with a high antioxidant content affect the ethanol extraction of grape seeds. The ethanol skin extract had a value four times lower than the ethanol seed extract 14.25 $\mu\text{M TE/mg}$. The lower antioxidant effect of the ethanol skin extract could have been because of the fermentation process, where most of the biologically active substances, such as tannins and anthocyanins, are extracted (Su *et al.* 2006). The IC50 values of the alpha-tocopherol, butylated hydroxytoluene (BHT), and BHA standards had the same values as reported by Gülçin (2006).

Antioxidants are useful for reducing autooxidation and are used as stabilizers and supplements in food. Both synthetic and natural antioxidants are used in food and cosmetic products. Synthetic antioxidants, such as BHT and BHA, are controversial because of their potentially adverse effects on human health (Shahidi and Zhong 2010). For this reason, interest in the application of natural antioxidants as a substitute for synthetic antioxidants has increased (Szeto *et al.* 2002; Shahidi and Zhong 2010).

CONCLUSIONS

1. The antioxidant activity of the seeds was 58.65 $\mu\text{M Trolox/mg}$ extract. This value was only 5.8% lower than the antioxidant activity of the alpha-tocopherol. The seed and skin by-products from grape processing can be used as natural stabilizers and supplements in food.
2. The grape seeds contained 402.3 mg/kg total tocopherol and the grape skin contained 231.6 mg/kg. Tocopherol has a beneficial effect on human health.
3. The grape skin was a rich source of antioxidant active rutin (6.79 g/kg) and quercetin (3.75 g/kg), which have many types of biological properties.

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