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Elderberry (*Sambucus nigra* L.) Wine: A Product Rich in Health Promoting Compounds

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Color components, antioxidative potential, and total phenolic content were monitored in elderberry must and wine. Among individual phenolic compounds, quercetin and kaempferol compounds, phenolic acids, and anthocyanins were detected with high performance liquid chromatography coupled with mass spectrometry. Conventional enological parameters were measured in elderberry wine and compared to grape and other fruit wines. Elderberry wine has a moderate ethanol concentration, intense red coloration, and higher pH value compared to most red wines. Total phenolic content of elderberry must and wine ranged up to 2004.13 GAE L^{-1}. Antioxidative potential of elderberry wine was in the range of red wine, and a tight correlation was detected between total phenolic content and antioxidative potential of elderberry wine. Anthocyanins were the most abundant phenolics in elderberry wine in tight correlation with color hue, and their content significantly decreased with aging. Similarly, a decrease in total phenolic content and antioxidative potential was determined after storage.

KEYWORDS: Elderberry wine; enological parameters; antioxidative potential; phenolics

INTRODUCTION

Among the black berry fruit of the moderate climate, elderberry (*Sambucus nigra* L.) is a widespread species that grows on sunlight-exposed locations in Europe, Asia, North Africa, and the USA. In the past years it has received attention due to large amounts of phytochemicals, particularly high contents of secondary metabolites, and many nutritional benefits of ripe berries, which can be used as a rich source of dietary anthocyanins and other phenolics. Antioxidant, anti-inflammatory, and immune-stimulating potential of elderberry fruit have been confirmed in model systems (*1*, *2*) and by several clinical studies (*3*−*5*).

Elderberry berries are mainly used for food colorants and components in pharmaceuticals, processed to concentrates, syrups, and juices; frequently they are consumed in preserves and alcoholic beverages such as elderberry wine. The juice pressed from black elderberry fruit contains various sugars and organic acids and, moreover, it is a rich source of secondary metabolites (*6*, *7*). Bioactive compounds, particularly anthocyanins, quercetins, and hydroxycinnamic acids, present in elderberry juice act as potent agents against oxidative stress, reducing oxidative damage to the human body (*7*). During the grape and fruit wine making process, significant changes take place in the composition and content of polyphenolic compounds resulting from fruit disintegration as well as fermentation and aging (*8*, *9*). Alcoholic fermentation of elderberry fruit yields an intensely purple−red colored elderberry wine with a high content of anthocyanic pigments and total phenolic content similar to red wines (*10*). A moderate consumption of red wines has been associated with reduced risk of heart diseases, and such findings have prompted research toward the evaluation of the antioxidant activity and polyphenol levels of red wines. In recent years, various fruit wines have also been evaluated for their health promoting effects, which may be related to their antioxidant activity. However, the phenolic profile of elderberry wine has not been evaluated to the present date and therefore the compositional analysis of elderberry wine phenolics can point out its health promoting properties. The beneficial compounds present in elderberry juice, such as quercetin-3-rutinoside, chlorogenic acid, and various cyanidins (*11*), are presumably also found in elderberry wine.

The aim of the study was to determine the content of secondary metabolites (anthocyanins, flavonols, and phenolic acids) as well as total phenolic content and antioxidative potential of elderberry wine. Moreover, the research aims to study the change of phenolic compounds, particularly anthocyanins, from elderberry must to wine and their relation to the color composition of elderberry products. Phenolic profiles will be assessed in young elderberry wine as well as aged wine after three-year bottle storage. Correlation between TPC and AP of elderberry wine will also be determined. The high content of phenolic antioxidants in elderberry wine makes it a potentially appealing home-produced alcoholic beverage with health promoting properties when moderately consumed.

MATERIALS AND METHODS

Samples. Elderberry fruit was collected in Aug 2006 from five different bushes grown in the eastern region of Slovenia (altitude 244 m; lat 46.15°N, long 15.15°E). Wine was produced by the standard procedure for elderberry wine; 1.5 kg berries of a single bush were destemmed

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and prewashed. Three L of water and 1 kg of sucrose were brought to boil and berries were added to the mixture. After cooling to 32 °C, 1.25 g of dry wine yeast (Uvaferm BDX; Lallemand, Langlois, Austria) was added and the mixture was transferred to a glass vessel affixed with an airlock. The wine was kept in the glass vessel for a period of 10 days, and after completion of maceration process, the elderberry fruit was discarded and the wine poured into a secondary vessel where the fermentation process was carried out at a maximum temperature of 20 ± 2 °C for two months. Finally, elderberry wine was bottled and stored in darkness at 20 °C until further analysis.

Samples were taken at the beginning of fermentation: elderberry must (when the mixture was transferred into the glass vessel), at the end of fermentation process (two months of fermentation), elderberry wine, and after three-year bottle storage (aged elderberry wine). Five replicates were measured at each treatment.

Chemicals. The following standards were used to determine the chemical compounds in elderberry juice and wine: malic acid, citric acid, fumaric acid, shikimic acid, cyanidin-3-glucoside, and quercetin-3-glucoside from Fluka Chemie (Buchs, Switzerland), chlorogenic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and quercetin-3-rutinoside from Sigma-Aldrich Chemie (Steinheim, Germany). The chemicals for mobile phases were HPLC grade acetonitrile and formic acid from Fluka Chemie. The water used in sample preparation, solutions, and analysis was twice-distilled and purified with a Milli-Q water purification system by Millipore (Bedford, MA).

pH, Ethanol Concentration, Total Acidity, and Color Measurements. Elderberry must and wine pH level was measured with a pH meter (S20 SevenEasy; Mettler Toledo, Columbus, OH). Ethanol concentration in elderberry wine was determined with a steam-distillatory (Polydest; Gravitech, Rodgau, Germany). Individual organic acids were analyzed according to the method described by Sturm, Koron, and Stampar (*12*) using high-performance liquid chromatography (HPLC; Thermo Scientific, Finnigan Spectra System, Waltham, MA), and total acidity was expressed as the sum of all organic acids in elderberry must and wine in g L^{-1}. For each analysis, 20 μL of sample was used. Organic acids were analyzed with the Rezex organic acid column (300 mm × 7.8 mm; Phenomenex, Torrance, CA), and a UV detector set at 210 nm with a flow of 0.6 mL min^{-1}, maintaining the column temperature at 65 °C. For the mobile phase, 4 mM sulphuric acid (H_2SO_4) was used. The concentrations of organic acids were calculated with the use of corresponding external standards.

For the determination of color intensity (CI) and hue (H) of elderberry must and wine, a buffer solution of distilled water was used and pH was adjusted to 4.0 with sulphuric acid. Color parameters were determined spectrophotometrically on a Lambda Bio 20 UV/vis spectrophotometer (Perkin-Elmer, Waltham, MA) in 2 mm pathway cuvettes after 1:10 dilution with the buffer solution. CI and H were calculated from absorbance measurements obtained at 420, 520, and 620 nm (*13*).

HPLC-MS Identification of Phenolics. HPLC analysis was performed using a Surveyor HPLC system with a diode array detector (DAD), controlled by CromQuest 4.0 software (Thermo Finnigan, San Jose, CA). A mass spectrometer (LCQ Deca XP MAX, Thermo Scientific) with an electroscopy ionization (ESI) operating in positive/negative ion mode using MS^2 scanning mode from m/z 115 to 1000 was used for the identification of anthocyanins and other phenolics in elderberry must and wine. The injection volume was 10 μL and the flow rate maintained at 1 mL min^{-1}. Capillary temperature was 250 °C, and the sheath gas and auxiliary gas were 20 and 8 units respectively; the capillary voltage was 26 V and spray voltage 4 V. Multipole R_f amplitude was 550 V_{p-p}. Spectral data were elaborated using the Excalibur software (Thermo Scientific). The peak identification was confirmed by injecting the corresponding standards or comparing the retention time and fragmentation with literature data. All compounds were expressed as mg L^{-1}. Kaempferol-3-rutinoside was expressed as kaempferol and neochlorogenic acid as chlorogenic acid. All cyanidin compounds were expressed in equivalents of cyanidin-3-glucoside.

Determination of Total Phenolic Content. The determination of total phenolics (TPC) in elderberry must and wine was assessed with the Folin−Ciocalteu phenol reagent method (*14*). Six mL of twice-distilled water and 500 μL of Folin−Ciocalteu reagent were added to 100 μL of diluted sample (1:2 v/v methanol), and after waiting for between 8 s and 8 min at room temperature, 1.5 mL of sodium carbonate (20% w/v) and 1.9 mL of twice-distilled water were added. The extracts were mixed and allowed to stand for 30 min at 40 °C before measuring absorbance at 765 nm on a Lambda Bio 20 UV/vis spectrophotometer (Perkin-Elmer). A mixture of water and reagents was used as a blank. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg L^{-1}. Absorptions were measured in three replicates.

Determination of Antioxidant Potential by the DPPH Radical Scavenging Method. The free radical scavenging activity of elderberry must and wine was measured according to the DPPH (2,2-diphenyl-2-picrylhydrazyl) method (*15*), with slight modifications. First, 50 μL of diluted sample (1:4 v/v methanol) was placed in 96-well microplates, and 200 μL of 0.1 mM methanolic solution of DPPH was added and allowed to react in the dark at room temperature. Then the decrease in absorbance of DPPH at 520 nm was measured at 5 min intervals by a spectrophotometer (Perkin-Elmer) until the absorbance stabilized (30 min). Methanol was used as blank. All samples were prepared in triplicate. Determination of antioxidant potential (AP) of the samples at various concentrations was made using the trolox standard curve. The DPPH radical scavenging activity of elderberry must and wine was expressed as mM trolox equivalents per L.

Statistical Analysis. The results were statistically analyzed with the Statgraphics Plus program for Windows 5.0 (Herndon, VA) using a one-way analysis of variance (ANOVA). Significant differences among treatments for each of the phenolic constituents, TPC and antioxidative activity were determined by one-way analysis of variance and LSD test. P values of less than 0.05 were considered statistically significant. Linear regression (r^2) was calculated between color hue (H) and total anthocyanins and between TPC and AP at $P < 0.05$.

RESULTS AND DISCUSSION

pH, Ethanol Concentration, Total Acidity, and Color Measurements. The pH of elderberry must was significantly higher than that measured in elderberry wine (**Table 1**). The pH of red wines has been reported to range between 3.0 and 3.7 (*16*, *17*), making elderberry wine less acidic; however, some authors measured higher pH values for grape and other fruit wines (*18*, *19*). Similar to reports on red wine and kiwi wine aging and storage (*18*, *20*), pH of elderberry wine was significantly increased during three years of aging in bottles. Guadalupe and Ayestarán (*18*) and Chung et al. (*16*) linked the increase of pH level during red wine aging to esterification of organic acids and a decrease in total acidity, which was also evident for elderberry wine (**Table 1**). The compositional analysis of organic acids revealed malic and citric acids as the predominant organic acids in must and wine, which is in accordance with the reports of Veberic et al. (*7*).

Ethanol concentration in elderberry wine was 13.20% v/v (**Table 1**); compared to other fruit wines such as kiwi wine, elderberry wine has a higher ethanol concentration (*20*). In enology, the color intensity (CI) is defined as the sum of the wine absorbance at 420, 520, and 620 nm. The hue (H) or nuance is defined as the ratio between the absorbances at 420 and 520 nm, thus a high value means an oxidized hue of the wine (*13*).

The evolution of color intensity and hue value from elderberry must to wine revealed a significant increase in both parameters (**Table 1**). CI was significantly decreased after three years of bottle aging; however, H was increased, suggesting the development of color toward brown−orange (*21*). Chung et al. (*16*) associated color changes during red wine aging with various reactions including oxidation, condensation, and polymerization of phenolic compounds, particularly anthocyanins. According to Rommel, Heatherbell, and Wrolstad (*22*), the color components of red raspberry wine were similarly unstable after storage. This can be partly explained with the oxidation of anthocyanins at higher pH levels of aged elderberry wine (**Table 1**). Some authors correlated color components of red wine with the content of anthocyanic pigments and copigments (*13*) and, similarly, the hue value of elderberry wine was negatively correlated ($R^2 = 0.94$, $P = 0.000$)

Table 1. Conventional Enological Parameters of Elderberry Must and Wine

			enological parameters		
treatment	ethanol % v/v[a]	pH	TA[b]	CI	hue
elderberry must		4.00 ± 0.09 ab	3.17 ± 0.06 b	8.24 ± 0.93 b	0.63 ± 0.03 a
elderberry wine	13.20 ± 0.06 a[c]	3.90 ± 0.06 a	3.26 ± 0.09 b	14.02 ± 1.01 c	0.72 ± 0.03 b
aged wine	13.04 ± 0.05 a	4.17 ± 0.02 b	1.12 ± 0.08 a	4.16 ± 0.20 a	1.13 ± 0.01 c

[a] % v/v mL of ethanol for 100 mL of wine at 20° C. [b] TA total acids in g L^{-1}. [c] Different letters (a−c) in columns denote statistically significant differences in parameters among individual treatments by LSD multiple range test at $P < 0.05$.

Table 2. Content Levels of Individual Phenolic Compounds, Total Phenolic Content (TPC), and Antioxidative Potential (AP) in Elderberry Must and Wine

	treatment		
phenolic compd[a]	elderberry must	elderberry wine	aged wine
neochlorogenic acid[b]	15.46 ± 2.18 NS[e]	21.83 ± 5.29 NS	15.48 ± 0.41 NS
chlorogenic acid	42.76 ± 4.51 a	66.10 ± 10.23 b	31.69 ± 0.79 a
Q-rutinoside	109.57 ± 1.28 b	125.45 ± 2.18 c	92.80 ± 4.05 a
Q-glucoside	7.79 ± 0.78 a	15.95 ± 2.44 b	9.44 ± 0.92 a
K-rutinoside	0.74 ± 0.05 a	1.10 ± 0.10 b	1.10 ± 0.12 b
Cy 3-sam-5-glu	58.99 ± 8.52 b	65.93 ± 3.14 b	6.13 ± 1.19 a
Cy 3,5-diglu	139.46 ± 17.03 b	149.09 ± 22.25 b	18.49 ± 1.73 a
Cy 3-sam	397.55 ± 34.20 b	29.63 ± 2.59 a	4.92 ± 0.26 a
Cy 3-glu	350.07 ± 26.45 b	538.19 ± 41.16 c	20.88 ± 2.31 a
Cy-3-rut	19.22 ± 1.94 ab	34.76 ± 3.78 b	5.28 ± 0.68 a
sum anthocyanins	941.61 ± 57.51 b	865.06 ± 61.83 b	55.69 ± 5.80 a
TPC[c]	1714.53 ± 71.40 b	2004.13 ± 49.44 b	1584.99 ± 24.26 a
AP[d]	8.15. ± 0.43 b	9.95 ± 0.36 c	6.13 ± 0.25 a

[a] Phenolic compound: Q-rutinoside, quercetin-3-rutinoside; Q-glucoside, quercetin-3-glucoside; K-rutinoside, kaempferol-3-rutinoside; Cy 3-sam-5-glu, cyanidin 3-sambubioside-5-glucoside; Cy 3,5-diglu, cyanidin 3,5-diglucoside; Cy 3-sam, cyanidin 3-sambubioside; Cy 3-glu, cyanidin 3-glucoside; Cy 3-rut, cyanidin 3-rutinoside. Average values ± standard error are presented. [b] Individual phenolic compounds are presented in mg L^{-1}. [c] TPC is expressed as mg GAE L^{-1}. [d] AP is expressed as mM trolox equivalents per L. [e] Different letters (a−d) in columns mean statistically significant differences between individual anthocyanins among treatments by LSD multiple range test at $P < 0.05$.

with total anthocyanins. These pigments are predominately responsible for the red color of wines (*18*, *22*).

Individual Phenolic Compounds. Ten phenolic compounds were identified in elderberry must and wine: chlorogenic acid and neochlorogenic acid, quercetin-3-rutinoside, quercetin-3-glucoside, kaempferol-3-rutinoside, and five cyanidin based anthocyanins. Anthocyanins were the predominant polyphenols in elderberry must and wine (**Table 2**), similar to data obtained for red wines (*23*). Compared to some red wines, which contain from 431 to 506 mg L^{-1} total anthocyanins (*21*), a 2-fold amount of anthocyanins was measured in elderberry wine. Cyanidin-3-sambubioside was the predominant anthocyanin in elderberry must; however, in elderberry wine, cyanidin-3-glucoside was measured in highest concentrations (**Table 2**). Both anthocyanins were also reported as the most abundant pigments in elderberry fruit and juices, with their proportions varying among cultivars (*7*, *11*, *24*). During the elderberry wine making process, significant transformations in phenolics take place and the composition of anthocyanic pigments is altered, similar to the reports on blackcurrant and cherry wine (*8*), where condensation and polymerization of some polyphenols and anthocyanins is reported. Like in our study, the major anthocyanin in blackcurrant fruit, cyanidin-3-rutinoside, is reportedly only the second major pigment in blackcurrant wine. Vinification resulted in a minor decrease of anthocyanin content, similar to that reported for blackcurrant and cherry wine (*8*). During storage and aging, elderberry wine color changed from bright-red to brown hues, which can be attributed to the formation of new, more stable, polymeric pigments (*21*) as well as degradation reactions (*18*). The content of total anthocyanic pigments after three years of storage was significantly reduced to only 6% of the content measured in young elderberry wine (**Table 2**). Previous data on red wine aging showed 5-fold decrease in the content of total anthocyanic pigments after 16 months of storage (*21*). As TPC in aged elderberry wine only decreased by 20%, reactions between anthocyanins and other phenolic compounds occurred during elderberry wine aging as reported for red wine (*21*).

Quercetin-3-rutinoside was the most abundant quercetin, followed by quercetin-3-glucoside, which is comparable to the compositional analysis of elderberry fruit (*2*, *7*) as well as elderberry juice (*11*). Statistically highest content of both compounds were measured in elderberry wine and were decreased after three years of storage (**Table 2**). Similar to compositional analysis of elderberry juice (*11*), neochlorogenic acid and chlorogenic acid were detected in elderberry must and wine; the latter was significantly higher in elderberry wine compared to must and aged wine. This is in accordance with reports on long-term bottle aging of red wines, where a considerable decrease was detected for this group of phenolics (*18*).

Total Phenolic Content and Antioxidative Potential. As a result of chemical reactions of monomeric and dimeric phenolics during processing from musts to wine as well as aging, many polymeric compounds are formed, causing differences in both TPC and AP (*9*). Both parameters were significantly higher in elderberry wine compared to elderberry must and were again decreased after aging (**Table 2**). Similarly, vinification increased TPC in red wine "Tempranillo" (*18*) and, in a research on blackcurrants and cherries fruit wines, a decrease in the content of polyphenols in the process of wine aging was detected (*8*). TPC of elderberry wine was 2004.13 mg GAE L^{-1}, comparable to TPC measured in different red wines, which on average contain 2000 mg GAE L^{-1} (*23*). This is also in accordance with previously published data on fruit wine phenolics, where TPC value of 1753 mg GAE L^{-1} was reported for elderberry wine (*10*). Based on TPC, elderberry wine is particularly rich in phenolic compounds, exceeding blueberry, currant, and raspberry wines (*19*) as well as some red and rosé wines (*25*). According to previously reported data on various fruit and grape wines, phenolic content expressed as TPC determines their antioxidant properties (*8*, *20*, *23*). Antioxidative potential of elderberry must was 8.18 mM trolox L^{-1} and increased significantly in elderberry wine to 9.95 mM trolox L^{-1} (**Table 2**). A decrease to 6.13 mM trolox L^{-1} was detected after three years of storage. Mulero et al. (*17*) reported values of antioxidant activity for red wines in the range from 5.03 to 7.73 mM trolox L^{-1}. Some authors demonstrated a significant decrease in red wine antioxidant potential caused by aging (*26*).

Correlation analysis revealed a close relationship between TPC and antioxidant potential in elderberry wine ($R^2 = 0.92$, $P = 0.000$), thus demonstrating that polyphenols are, in vitro, significant antioxidants in elderberry must and wine. A high correlation coefficient between TPC and total antioxidant capacity was reported for various fruit wines (*10*) as well as red and rosé wines (*25*).

Elderberry wine is an appealing alcoholic beverage not only because of its intense red coloration but mostly due to its high total phenolic content. Specifically, anthocyanins were the most

abundant phenolics in elderberry wine and in tight correlation with color hue. Therefore, elderberry wine could be used as a potential additive to other alcoholic beverages with inadequate coloration. During elderberry wine aging, the content of many phenolic compounds was decreased and the color shift was noticeable to brown−red hues. As a direct correlation between phenolics and antioxidative potential of elderberry wine was detected, we do not recommend elderberry wine for storage for a longer period. The compositional analysis of elderberry wine revealed a similar phenolic profile and antioxidative potential to red wines, and as beneficial effects of grape wine have been extensively studied, we can conclude that elderberry wine exhibits similar health promoting benefits. High contents of phenolics from the groups of quercetins, hydroxycinnamic acids, and anthocyanins indicate that the consumption of elderberry wine may contribute to prevention of several degenerative diseases as these are potent chemopreventive agents. Additional investigations on the antioxidative potential would be advantageous to identify biologically relevant properties of elderberry wine and compare them to grape wines, which will be the focus of our further studies.

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