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Phenolic Profile and Antioxidant Activity of Selected Premium Wines from Herzegovina Region

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Abstract: Red wines are rich in antioxidants, which have anti-inflammatory activity and beneficial effect on human health. The aim of this research was to determine the content of bioactive compounds, i.e. total phenols, anthocyanins and tannins, as well as antioxidant activity in red wines from the Herzegovina region. Four wines from two different harvest years, i.e. 2019 and 2021, were analysed. Phenolic compounds are important wine quality parameters that affect color, taste and aroma. The content of total phenols varied from 1733.87 ± 10.22 to 2382.09 ± 21.42 mg GAE/L. The content of total anthocyanins, which directly affect the color of red wines, was determined by the pH differential method and the results obtained ranged from 13.64 ± 0.19 to 21.76 ± 0.35 mg CGE/L. Total tannins were determined using spectrometric method, and values ranged from 9.00 ± 0.09 to 11.42 ± 0.21 g/L. The antioxidant activity was assessed using two different methods, FRAP and ABTS. The total phenolic content of the tested wines was positively correlated with their antioxidant activity. The content of total phenols, anthocyanins, tannins and antioxidant activity were influenced by the year of harvest as well as the aging methods used.

INTRODUCTION

The red wines of Herzegovina occupy a special place in the oenological tradition of Bosnia and Herzegovina, recognized for their specificity, richness of taste and unique character. This region, which stretches through the southern part of Bosnia and Herzegovina, is characterized by a Mediterranean climate and rich limestone soil, which is a key factor in the cultivation of vines (Kojić, Sefo and Delić, 2013). Blatina is an autochthonous Herzegovinian grape variety. The Blatina variety is special because it contains a functional female flower, therefore it is planted together with Trnjak or Merlot. In addition to enological and cultural aspects, red wines are also known for their positive effects on human health when consumed in small doses, reducing the risk of cancer, inflammatory and cardiovascular diseases (Hrelia, Di Renzo, Bavaresco et al., 2023). Numerous studies have shown that grapes and red wines are rich in bioactive compounds, and contain high levels of phenolic and antioxidant compounds, anthocyanins and tannins (Tutino, Gigante, Milella et al., 2020; Sabra, Netticadan, and Wijekoon, 2021). Polyphenols pass from grapes into must and wine, influencing organoleptic properties, ripening process, aging, wine stability , and nutritional efficacy (Zoričić, 1998). Anthocyanins, the most abundant flavonoids in grape berries, also exhibit antioxidant properties. Research shows that the antioxidant activity of berries is directly proportional to the concentration of anthocyanins in them (Heinone, Meyer and Frankel, 1998). Tannins in wine act as a natural preservative, allowing the product to age gracefully. During the aging process, tannins disperse, enhancing the wines complexity by contributing to its fullness of flavour, bitterness and astringency (Zhang, Wei, Han et al., 2023). The aim of this study was to determine the content of bioactive compounds and antioxidant activity in red wines of the Blatina variety from the Herzegovina region, produced from different harvest years (2019 and 2021) and aged using different methods.

EXPERIMENTAL

Chemicals

All solutions were prepared using analytical-reagent grade substances and distilled water.

Gallic acid, Folin-Ciocalteu reagent, sodium acetat-3-hydrate were purchased from Semikem (BiH). Compound 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) and 2,2'-azino-

bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (Germany). Potassium peroxysulfate and potassium chloride were purchased from Lach Ner (Czech Republic) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Acros Organics (USA). All other chemicals were purchased from Kefo d.o.o. (BiH).

Wine Samples

Four different red wines were selected for this study (Table 1). The wines were produced from grapes harvested in two different years (2019 and 2021). Two wines were stored in stainless steel tanks, while the other two wines aged for three years in barrique barrels. All four wines are from the winery Carski Vinogradi Mostar with a controlled geographic origin, commercially available and widely consumed. The winery maintains a temperature between 20°C and 22°C, with a relative humidity ranging from 65% to 75%, while the barrels, which are not new, have a medium toastiness. The wines were stored at room temperature in a dark environment until analysis. All wines were diluted with distilled water at a ratio 1:10. All analyses were performed in July 2024 in triplicates (three samples from the same bottle). Absorbance measurements were conducted using UV Shimadzu spectrophotometer.

Table 1: Description of analysed red wine samples

Sample	Harvest year	Aging
1	2019	stainless steel
2	2021	stainless steel
3	2019	barrique barrels
4	2021	barrique barrels

Determination of content of total phenols

The content of total phenols was determined by the Folin-Ciocalteu method described by Singelton and Rossi (1965) and later modified by Keskin-Šašić, Tahirović and Topčagić (2012). 0.4 mL of sample was added to 2 mL (1/10 dilution) of Folin-Ciocalteu reagent. After 10 minutes, 1.6 mL of 7.5% sodium carbonate (Na_2CO_3) was added. The samples were left at room temperature for 30 minutes. After that, the absorbances were read at 743 nm. The calibration curve was created using different concentrations of gallic acid (50, 100, 150, 250 and 350 mg/L). The content of total phenols was expressed as mg of gallic acid equivalent (GAE) per liter of sample.

Determination of total anthocyanin content

Total anthocyanin content was determined using the pH differential method described by Zhishen, Mengcheng and Jianming (1999). This method is based on the structural difference of anthocyanins at different pH values of the medium. Two test tubes were used for each sample. 1 mL of the prepared sample was added to each test tube. Then, 4 mL of pH 1.0 buffer (potassium chloride, 0.025 M) was added to one tube, and 4 mL of pH 4.5 buffer (sodium acetate, 0.4 M) to the other. The content of total anthocyanins was expressed as mg of cyanidin-3-*O*-glucoside equivalent (CGE) per liter of sample.

Determination of antioxidant activity using Ferric Reducing Antioxidant Power method

Determination of the antioxidant activity with FRAP reagent was carried out according to Benzie and Strain (1996). This method is based on the reduction of the colourless Fe^{3+}-TPTZ to Fe^{2+}-TPTZ of intense blue colour. The FRAP reagent was prepared by mixing 200 mL of acetate buffer pH 3.6 (300 mM), 20 mL of TPTZ (2,4,6-tris(2-pyridyl)-*s*-triazine) solution (10 mM) in 40 mM hydrochloric acid, 20 mL of $FeCl_3$ (20 mM) and 24 mL of distilled water. The calibration curve was created using different concentrations of $FeSO_4$ (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM). 200 µL of sample was added to 3800 µL of freshly prepared FRAP reagent. The mixture was left for 4 minutes at room temperature. After that, the absorbances at 593 nm were read. The results were expressed as mM $FeSO_4$ equivalent per liter of sample (mmol Fe^{2+}/L).

Determination of antioxidant activity using ABTS method

The determination of antioxidant activity using ABTS radical was carried out according to Re, Pellegrini, Proteggente et al. (1999). This method is based on the reduction of ABTS radicals, which are formed either by chemical or enzymatic oxidation of the solution of ABTS. ABTS cation radical was prepared by dissolving 19.5 mg of ABTS and 3.3 mg of potassium persulfate in 7 mL of distilled water. The prepared solution was left to stand in the dark for 12-16 hours at room temperature before use. This solution was then diluted with ethanol (96%) to a final concentration of 1%, resulting in an absorbance of 0.70 ± 0.02 at 734 nm. A calibration curve was constructed using different concentrations of Trolox (0.1, 0.2, 0.4 and 0.6 mg/mL). 40 µL of sample was mixed with 4 mL of the ABTS radical solution and the absorbance was measured after 6 minutes at 734 nm. The results were expressed as mM of Trolox equivalent (TE) per liter of sample.

Determination of total tannin content

Total tannin content was determined using the method described by Ribéreau-Gayon, Glories, Maujean and Dubourdieu (2006). Two test tubes (A and B) were prepared for each sample. 1 mL of distilled water, 3 mL of hydrochloric acid (12 M) and 2 mL of sample were added to each test tube. One test tube (A) was heated in a water bath at 100°C for 30 minutes. 0.5 mL of ethanol (96%) was added to the other test tube (B) and kept in the dark at room temperature. After 30 min, test tube A was also placed in the dark until it had partially cooled, and then 0.5 mL of ethanol was added. After cooling, the absorbance for each prepared sample (A and B) was measured at 470, 520 and 570 nm. The concentration of total tannins was calculated according to the following formulas (1-3):

$$\Delta A_{520} = 1.1 \times \Delta A_{470} \quad (1)$$

$$\Delta A_{520} = 1.54 \times \Delta A_{470} \quad (2)$$

$$\gamma_{tannins} = 15.7 \times minimum_{\Delta A_{520}} \quad (3)$$

The content of total tannins was expressed as g/L.

Statistical analysis

All measurements are expressed as mean ± standard deviations. Statistical analysis was performed using analysis of variance (ANOVA). The statistical differences are considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The results of the content of total phenols, anthocyanins and tannins are presented in Table 2.

Table 2: Content of total phenols, anthocyanins and tannins in wine samples

Sample	Total phenols (mg GAE/L)	Total anthocyanins (mg CGE/L)	Total tannins (g/L)
1	1733.87 ± 10.22^{a}	15.47 ± 0.59^{c}	9.46 ± 0.11^{b}
2	1828.19 ± 87.87^{b}	21.76 ± 0.35^{d}	9.00 ± 0.09^{a}
3	2382.09 ± 21.42^{d}	13.64 ± 0.19^{a}	11.42 ± 0.21^{d}
4	2051.60 ± 16.06^{c}	14.08 ± 0.54^{b}	9.57 ± 0.15^{c}

Different letters within the same column represent a statistically significant difference by ANOVA with Tukey's test ($p<0.05$)

The results of this study on premium red wines indicate that the content of total phenols ranged from 1733.87 ± 10.22 to 2382.09 ± 21.42 mg GAE/L. The highest value was observed in sample 3, a wine aged in barrique barrels, while the lowest was observed in sample 1, a wine that was not aged and was directly bottled from stainless steel vessels. The statistically significant difference between the results indicates that oak barrels influence the increase in the content of total phenols. Marković and Talić (2013) analysed nine wines from the Herzegovina region, from different vintage years, using the same method as us for determining the content of total phenols. Blatina from the Stolac region showed lower values compared to ours. Similarly, Radeka, Rossi, Bestulić et al. (2022) analysed red wines from Croatia, comparing young wines with those aged in barrique barrels using the Folin-Ciocalteu method. Their content of total phenols varied between 1527.12 and 3936.21 mg GAE/L, with wines aged in barrique barrels exhibiting significantly higher concentrations than young wines, which is consistent with our results. The increased concentration of phenols is attributed to the release of hydrolysable tannins from oak barrels during aging, as confirmed by Waterhouse, Sacks and Jeffery (2016). According to Table 2., the total anthocyanin content ranged from 13.64 ± 0.19 to 21.76 ± 0.35 mg CGE/L. The highest content was found in sample 2, a wine stored in a stainless steel vessel from the 2021 vintage, while sample 3 had the lowest value. These results confirm that the total anthocyanin content decreases with aging due to copigmentation and polymerization reactions (Boulton, 2001). Wines aged in barrique barrels exhibited lower anthocyanin levels, supporting the notion that aging in oak barrels leads to anthocyanin degradation. A study by Aleixandre-Tudo and du Toit (2020) on 82 red wines from South Africa supports these findings. They proved that the content of total anthocyanins decreases with time in both barrique barrels and bottled wines. The differences between their results and ours may be attributed to variations in aging periods, geographical regions, climatic conditions, grape varieties and harvest years. Similarly, Guld, Racz, Tima et al. (2019) investigated three grape varieties typical of Hungary, monitoring changes in anthocyanin content during aging in barrique barrels. They also applied the pH differential method for determination, reporting a significant decrease in anthocyanin concentration with time, which is consistent with the findings of this study.

The determination of the total tannins content yielded values ranging from 9.00 ± 0.09 to 11.42 ± 0.21 g/L. Sample 2, representing a young wine, exhibited the lowest values, while sample 3, aged in barrique barrels, displayed the highest content. Both samples aged in barrique barrels (samples 3 and 4) exhibited a higher total tannins content compared to samples 1 and 2. These results confirm that aging in barrique barrels leads to an increase in total tannins content. American oak was used for aging the tested wines, which, due to its lower porosity, allows for lower oxygen permeability compared to French oak (Martínez-Gil, Del Alamo-Sanza and Nevares, 2022). It was shown that the concentration of tannins released from barrique barrels ranges from 2 to 4 g/L, and that these tannins are hydrolysable. Such increased concentrations contribute significantly to the total tannin content, particularly when the aging period is extended to several years (Smith, McRea and Bindon, 2015).

Table 3: Content of antioxidant activity in wine samples

Sample	ABTS (mmol TE/L)	FRAP (mmol Fe^{2+}/L)
1	16.97 ± 0.23^{a}	20.65 ± 0.84^{a}
2	18.14 ± 0.35^{b}	20.94 ± 0.20^{c}
3	19.81 ± 0.62^{d}	25.04 ± 0.70^{d}
4	19.07 ± 2.54^{c}	20.87 ± 0.70^{b}

Different letters within the same column represent a statistically significant difference by ANOVA with Tukey's test ($p<0.05$)

FRAP and ABTS methods were used to determine the antioxidant activity. According to the results shown in Table 3, the values obtained by the FRAP method ranged from 20.65 ± 0.84 mM Fe^{2+}/L to 25.04 ± 0.70 mM Fe^{2+}/L. The results obtained by the ABTS method ranged from 16.97 ± 0.23 to 19.81 ± 0.62 mM TE/L. Both methods confirm that the red wine from 2019, which was aged in barrique barrels for three years, had the highest antioxidant activity, while the wine from the same year from stainless steel vessels showed the lowest activity. Kesić, Zaimović, Ibrišimović-Mehmedović et al. (2018) conducted a study on 5 red wines, including Blatina from the Čapljina area. Using FRAP method, Blatina showed a value of 2427 μM Fe^{2+}/L. The significant difference between the results of our study and those reported by Kesić, Zaimović, Ibrišimović-Mehmedović et al. (2018) can be attributed to the development of various Blatina varieties over the years in different regions of Herzegovina, which significantly affects the yield and quality of the grapes (Kojić, Blesić, Delić et al., 2010). Stasko, Brezova, Mazur et al. (2008) determined the antioxidant capacity of red wines from Austria and Slovakia using the ABTS method. The results ranged from 7.84 to 13.59 mmol TE/L. Stratil, Kuban and Fojtova (2008) also analysed red and white wines from the

Czech Republic using the ABTS method. Their results for red wines ranged from 4.92 to 13.94 mmol TE/L. Based on these results, we can conclude that the geographical area and grape varieties significantly influence the results of the analyses. Crippen and Morrison (1986) explained in their study that grapes that are more exposed to sunlight and higher temperatures contain higher concentrations of phenolic compounds and monomeric anthocyanins, which ultimately affects the antioxidant activity of red wines.

The correlation coefficient between the total phenolic content and antioxidant activity was determined. The results show a strong positive correlation between total phenols content and the antioxidant activity determined by FRAP ($r = 0.995$) and ABTS method ($r = 0.994$). Statistical analysis was performed using analysis of variance (ANOVA) revealing a statistically significant difference between harvest years.

CONCLUSIONS

The red wines analysed in this study showed high levels of total phenols, anthocyanins, tannins and antioxidant activity. A statistically significant difference in the concentrations of bioactive compounds was found between wines from two different harvest years. The results obtained in this study also demonstrate that the content of bioactive compounds in the wines is significantly influenced by the technological process used. The wine samples aged in oak barrels showed higher concentrations of total phenols, total tannins and antioxidant activity compared to the wines from stainless steel vessels from the same harvest year. At the same time, we showed that the total anthocyanin content decreased over time in oak-aged wine. These findings suggest that wine aged in oak barrels may be more beneficial for health-related applications due to its stronger antioxidant properties. However, this study had a limited number of samples and focused on a single grape variety. Future studies should include a larger and more diverse sample set, explore different aging techniques, and assess sensory characteristics and bioavailability to better understand the health and quality implications of wine.

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Summary/Sažetak

Crna vina bogata su antioksidansima koji imaju protuupalno djelovanje i blagotvoran efekat na ljudsko zdravlje. Cilj ovog istraživanja bio je utvrditi sadržaj bioaktivnih spojeva, tj. ukupnih fenola, antocijanina i tanina, kao i antioksidativnu aktivnost u crnim vinima sa područja Hercegovine. Analizirana su četiri vina iz dvije različite godine berbe, tj. 2019. i 2021. Fenolni spojevi važni su parametri kvalitete vina koji utječu na boju, okus i aromu. Sadržaj ukupnih fenola varirao je od 1733.87 ± 10.22 do 2382.09 ± 21.42 mg GAE/L. Sadržaj ukupnih antocijanina, koji direktno utječu na boju crnih vina, određen je pH diferencijalnom metodom pH, a dobiveni rezultati kretali su se od 13.64 ± 0.19 do 21.76 ± 0.35 mg CGE/L. Ukupni tanini određeni su spektrometrijskom metodom, a dobivene su vrijednosti u rasponu od 9.00 ± 0.09 do 11.42 ± 0.21 g/L. Antioksidativna aktivnost procijenjena je dvjema različitim metodama, FRAP i ABTS. Ukupni sadržaj fenola u ispitivanim vinima pozitivno je korelirao s njihovom antioksidativnom aktivnošću. Na sadržaj ukupnih fenola, antocijanina, tanina i antioksidativnu aktivnost utječu godina berbe kao i korištene metode odležavanja vina.