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Phenolic composition and DPPH radical scavenging activity of plum wine produced from three plum cultivars

MARKO LJEKOČEVIĆ¹, MILKA JADRANIN², JOVANA STANKOVIĆ², BRANKO POPOVIĆ³, NINOSLAV NIKIĆEVIĆ⁴, ALEKSANDAR PETROVIĆ⁴ and VELE TEŠEVIĆ^{5*}

¹Distillery Zarić, Maksima Markovića 42, 31260 Kosjerić, Serbia, ²Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Njegoševa 12, 11000 Belgrade, Serbia, ³Fruit Research Institute, Kralja Petra 1 9, 32000 Čačak, Serbia, ⁴Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade-Zemun, Serbia and ⁵University of Belgrade – Faculty of Chemistry, Studentski trg 12–16, 11000 Belgrade, Serbia

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Abstract: Plum wines made from two cultivars of *Prunus domestica* L. (Pože-gaća and Crvena ranka) and one cultivar of *Prunus insititia* L. (Trnovača) were evaluated for their total phenolic and anthocyanin contents. LC–MS/MS analysis based on specific MS transitions in the multiple reaction monitoring (MRM) mode was used for the identification and quantification of selected phenolic compounds. Catechin, chlorogenic and caffeic acids, as well as quercetin, were identified as the main polyphenols in plum wines. The total amount of phenolic compounds ranged from 1.24 to 1.58 g gallic acid equivalent per L. Among the examined wines, the Crvena ranka wine had a higher content of anthocyanins (12.31 mg cyanidin-3-glucoside equivalent per L). The antioxidant capacity of the wines was determined using the DPPH assay. The variations in the physicochemical characteristics, phenolic composition and DPPH radical scavenging activity of these wines are related to differences due to the different plum cultivars used in the preparation of each wine.

Keywords: plum wine; cultivars; polyphenols; anthocyanins.

INTRODUCTION

In recent years, because of increased interest in human health, nutrition and disease prevention, consumers have increased their demand for functional foods, including fruits and their products such as wine.¹

The process of winemaking is the result of biochemical transformations brought about by the action of several enzymes from various microorganisms, including yeasts, especially *Saccharomyces cerevisiae*, resulting in alcoholic fermentation producing ethanol as the main ingredient of alcoholic beverages such

*Corresponding author E-mail: vtesevic@chem.bg.ac.rs
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as wines.² In the winemaking process, different strains of *Saccharomyces cerevisiae*, as well as non-*Saccharomyces* yeast genera, transform sugars to ethanol, CO₂ and numerous fermentation by-products. Additionally, lactic acid bacteria and acetic acid bacteria could be active during and after completed alcoholic fermentation and affect the wine composition.^{3,4} Fruit wines (derived from fruits other than grapes) include cider, made from apples, perry, produced from pears, plum and cherry wines, as well as wines made from various berries.

Despite recent increased production, consumption and popularity of fruit wines, their chemical composition are less studied compared to grape wines.

Plums are one of the most misunderstood stone fruits crops around the world. They are members of the *Prunus* family, alongside apricot, cherry and peach. There are over 2000 cultivars of plums available throughout the world. The European plum, *Prunus domestica*, is commercially cultivated mainly in China, India, USA, Serbia and other European countries.⁵

Prunus domestica, the common or European plum, is known as *Šljiva* in Serbia. Plums represent one of the most important fruit crops in Serbia. Plum fruits may be consumed fresh, dried, or prepared into preserves, compotes, mousse, pulp, candied fruit, frozen fruit, jams and jelly products. They are used in production of traditional Serbian plum spirit called Rakija or Šljivovica.⁶

Plums are an important source of compounds influencing human health and preventing the occurrence of many diseases.⁷ Plums contain an abundance of bio-active compounds, such as phenolic acids, anthocyanins, carotenoids, flavanols, organic acids, fibre, tannins, aromatic substances, enzymes, minerals and vitamin A, B, C and K.⁸ The predominant phenolic compounds in plums are caffeic, 3-*O*-caffeicquinic, 5-*O*-caffeicquinic and 4-*O*-caffeicquinic acids.⁵

In Serbia, the production of fruit wines does not have a significant place in the overall wine production, but in the last decade different types of fruit wines have been available on the market. To our knowledge, several studies have been carried out in order to analyse chemical composition and biological activity of plum wines from Serbia.

The chemical composition of plum wines was determined along with their *in vitro* antimicrobial and cytotoxic activities by Miljić *et al.* The plum wines produced from Čačanska rana, Čačanska leptotica and Požegača cultivars showed considerable antimicrobial activity against six bacterial and two yeast strains. In addition to antimicrobial activity, the plum wines showed a significant cytotoxic effect on the growth of three cancer cell lines, *i.e.*, Hep2c, RD and L2OB.⁹

The work reported by Miljić *et al.*¹⁰ showed that there are significant differences in the fermentative characteristics and the content of certain volatile compounds as a result of the metabolic activity of various indigenous and/or selected yeasts during fermentation of plum pomace. 2-Methylpropan-1-ol, 3-methyl-but-

anol, 1-heptanol and 1-octanol are the most prevalent higher alcohols and ethyl acetate the predominant ester in plum wines.

Plums contain a large amount of different phenolic compounds, mostly in the skin, and these are the main compounds responsible for the colour and taste of plum wine. Miljić *et al.*¹¹ studied the phenolic compounds, chromatic characteristics and antiradical activity of plum wines made from three plum cultivars (Čačanska rana, Čačanska lepotica and Požegača). Čačanska lepotica wine is characterised by the highest content of total phenols, total monomeric anthocyanins and flavan-3-ols, the highest colour intensity and the strongest antiradical activity. Peonidin-3-glucoside, cyanidin-3-rutinoside, peonidin-3-rutinoside, chlorogenic and caffeic acids and rutin were identified as the main polyphenols in plum wines.

In another work, Miljić *et al.*¹² investigated the influence of temperature, pH and duration of fermentation on plum wine composition and quality. The optimal conditions for plum wine production were 18.3 °C, pH 3.0 and 7 days.

In Serbia, the most commonly grown plum cultivars of European plum (*P. domestica* L.) are Požegača, Čačanska rodna, Stanley and Čačanska lepotica.¹³ Požegača is the predominant cultivar in some regions of western Serbia. Additionally, there is a number of old native (autochthonous) cultivars used for brandy production, some of which proved suitable for high quality brandies, such as the cultivars Crvena ranka (*P. domestica* L.) and Trnovača (*Prunus insititia* L.). Crvena Ranka plum (also known as Darosavka, Šumadinka, Crvenjača or Ranošljiva) is an autochthonous variety of Serbia. It is believed to have originated in vicinity of village Darosava near Arandelovac. Trnovača originated in western Serbia.¹⁴ Although they are important cultivars, the phenolic compositions and antioxidant activities of Crvena ranka and Trnovača wines have not hitherto been investigated. Thence, the present research was undertaken to determine the phenolic composition and antioxidant capacity of wines obtained from the cultivars of Požegača, Crvena ranka and Trnovača.

EXPERIMENTAL

Samples of wine

The plum fruits of three cultivars Požegača, Crvena ranka and Trnovača, were collected near town Kosijerić, West Serbia, in 2016. The plums were harvested at the stage of technological maturity, Crvena ranka and Trnovača in the middle of August and Požegača in the first half of September. A stone-removal machine was used for simultaneous fruit pitting and crushing. Then, commercial pectinase (Lalzyme Cuvée Blanc, Lallemand) was added to the plum mash at a rate of 30 mg L⁻¹. Subsequently, the pomace was inoculated with the pure strain of selected wine yeast Lalvin QA23 (Lallemand) at a dose of 10 g 100 kg⁻¹. Sulphur dioxide was not used during the winemaking process.

Fermentations were conducted in 1000 L stainless steel tanks with an airlock. After five days, the wine was separated from the pomace and the fermentation process was continued for three weeks. The alcoholic fermentation was conducted at 23–25 °C until the residual reduced

sugar reached 4 g L⁻¹. After two months, during which clarification and stabilization processes occurred, the young plum wines were subjected to chemical analysis and to determination of their DPPH radical scavenging activity.

Standards and solvents

Methanol and formic acid of HPLC grade were purchased from Merck (Darmstadt, Germany). DPPH and acetonitrile (HPLC grade) were obtained from Sigma–Aldrich (Steinheim, Germany). Folin–Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Standards of catechin, quercetin, protocatechuic, chlorogenic, vanillic, *p*-coumaric, ferulic and *p*-hydroxy benzoic acid were purchased from Fluka (Buch, Switzerland). Gallic and caffeic acids, and kaempherol and naringenin were supplied from Sigma–Aldrich (Steinheim, Germany). Ultrapure water (TKA Germany MicroPure water purification system, 0.055 μS cm⁻¹) was used in the liquid chromatography/mass spectrometry (LC/MS) analyses. The 0.45 μm Captiva premium syringe regenerated cellulose filters were purchased from Agilent Technologies (Santa Clara, CA, USA).

Physicochemical analyses

The standard chemical parameters of wine, total acidity (expressed as g malic acid per L), volatile acidity (expressed as g acetic acid per L), ash, ethanol content and pH, were determined according to the standard methods.¹⁵

Spectrophotometric analysis of the total phenolic content

The total phenolic content was determined by UV–Vis spectrophotometry using modified Folin–Ciocalteu procedure.¹⁶ All wine samples were filtered through a 0.45 μm Captiva filters before analysis. An aliquot (0.25 mL) of an appropriately diluted sample and a standard solution of gallic acid were mixed with 6 mL deionised water and 1.25 mL of Folin–Ciocalteu reagent. After 5 min, 1.0 mL of 7.5 % sodium carbonate was added with mixing. The absorbance was measured at 765 nm using a UV–Vis spectrophotometer (GBC Cintra 40). This analysis was performed in triplicate. The content (calculated using gallic acid as standard) is expressed as gallic acid equivalents per L (mg GAE L⁻¹).

Determination of the DPPH radical scavenging activity

The assay was performed according to Silva *et al.*¹⁷ 0.2 mL of the samples (0.100, 0.050, 0.020, 0.005 and 0.001 mg mL⁻¹) was mixed with 1.8 mL of DPPH solution (0.04 mg L⁻¹ in ethanol) followed by incubation in the dark for 30 min. The absorbance of each sample was read at 517 nm. Trolox (0.01, 0.025, 0.05, 0.075 and 0.1 mg mL⁻¹) was used as positive reference. The percentage of scavenged DPPH was calculated using the following equation:

$$\text{DDPH scavenging activity, \%} = 100 \times \frac{(A_c - A_s)}{A_c}$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample. The calculated IC_{50} values denote the concentration of the sample required to decrease the absorbance at 517 nm by 50 %. All experiments were performed in triplicate. The DPPH data are expressed as mg Trolox L⁻¹.

Determination of anthocyanin content

The total content of monomeric anthocyanin was determined using the pH-differential method, by measuring the absorbance of the sample at pH 1 (KCl, 0.025 mol L⁻¹) and pH 4.5 (NaOAc/HOAc, 0.4 mol L⁻¹).¹⁸ The measurements were performed at two wavelengths, at 520

and 700 nm, against a blank cell filled with distilled water. All analyses were repeated three times and anthocyanin content was calculated as mg of cyanidin-3-glucoside per 1 L of sample.

Solid-phase extraction (SPE)

This extraction was performed on a vacuum device (SPE Vacuum Manifold Baker SPE-12G) using Oasis HLB bcc/200 μm cartridges. The cartridge was conditioned with 5 mL of methanol followed by 5 mL of distilled water. A wine sample (5 mL) was passed through the cartridge, washed with 2 mL of water and eluted with 2 mL of methanol. The samples were collected and analysed by LC-MS/MS.

LC-MS/MS profiling of the phenolic compounds

After SPE purification, samples were directly injected into analysing system including liquid chromatograph (Waters Acquity UPLC H-Class; WAT-176015007; Milford, MA USA) interfaced to a mass detector (Waters TQ (Tandem Quadropole), WAT-176001263)). Separation of phenolic compounds was realised on an Acquity UPLC BEH C18 column (50 mm \times 2.1 mm; 1.7 μm) using 0.2 vol. % formic acid in deionised water (solvent A) and acetonitrile (solvent B). The following gradient program was used: 0–0.5 min 5 % B, 0.5–8 min 5–50 % B, 8–10 min 5 % B. The mobile phase flow rate was 0.40 mL min⁻¹, the column temperature was 40 °C and the injection volume of standard solutions and samples was 10 μL . After separation, the phenolics were analysed and quantified using a mass detector. The parameters for the quantification of the components by the mass detector, including ionization mode, cone voltage, collision energy, and characteristic transitions (MRM, multiple reaction monitoring), were obtained using the IntelliStart feature of MassLynx V4.1 by direct injection of methanolic solutions of the authentic standards into the mass spectrometer. The electrospray source operated under the following conditions: capillary voltage, 3.90 kV (positive mode of ionization) or 4.00 kV (negative ionization mode), source temperature, 150 °C, desolvation gas temperature, 450 °C, desolvation gas flow (nitrogen), 650 L h⁻¹. A personal computer system running MassLynx V4.1 software (Waters, Milford, MA, USA, 2005) was used for data acquisition and processing.

Identification and quantification of phenolic compounds was conducted according to retention times and multiple reaction monitoring of transitions in the ESI⁻ or ESI⁺ mode. All experiments were performed in triplicate and the concentration of the determined compounds is expressed in mg L⁻¹.

RESULTS AND DISCUSSION

Basic physicochemical characteristics

The physicochemical characteristics of the fruit wines produced from plum cultivars used in this study are shown in Table I.

The main components of the wine are ethanol and water, with a series of volatile and non-volatile substances that comprise a smaller portion. Ethanol is present in wine as a consequence of the fermentation of carbohydrates with the yeast. The yield of ethanol is dependent upon the initial concentration of the total sugar present in the fruit. Of the examined wines, highest content of ethanol was found in the Trnovača wine (10.6 vol. %) and the smallest content Crvena ranka wine (7.0 vol. %).

Acidity is one of the most important organoleptic parameters of fruit wine, due to the presence of weak organic acids. It has significant effect on the aesthetic character of a wine and also on the microbial stability of the wine. Among organic acids, malic acid accounts for more than 70 % of the total titratable acidity in ripe plums.¹⁹

TABLE I. Physicochemical characteristics of the examined plum wines

Parameter	Crvena ranka	Trnovača	Požegača
Content of alcohol, vol. %	7.0	10.6	8.4
Content of volatile acids ^a , g L ⁻¹	2.46	1.58	1.74
Content of total acids ^b , g L ⁻¹	6.26	5.38	5.16
Dry extract, g L ⁻¹	59.28	75.26	58.19
Content of ash, g L ⁻¹	4.17	6.22	5.69
pH	3.8	3.9	4.1

^ag L⁻¹ of acetic acid; ^bg L⁻¹ of malic acid

The highest total acid content was determined in the Crvena ranka wine (6.26 g L⁻¹), while the lowest value of this parameter was found in Požegača wine (5.16 g L⁻¹). The pH values for all plum wines produced were in a narrow range (3.8–4.1).

Acetic acid is the main volatile acid in fermented beverages and constitutes more than 90 % of the volatile acidity in wine.²⁰ Its presence at high concentrations gives off a vinegar odour and a disagreeable sensation in the mouth. Acetic acid may be produced by yeast, lactic acid bacteria and acetic acid bacteria. However, *Saccharomyces cerevisiae* normally only produce small quantities (0.1–0.3 g L⁻¹). The high production of volatile acids – acetic acid (1.5–5.5 g L⁻¹) is almost certainly a consequence of acetic acid activity (high pH, no SO₂ addition).

The greatest concentration of volatile acids was determined in the wine from the Crvena ranka cultivar (2.46 g L⁻¹), whereas in the case of the other two wines, this value was significantly lower, 1.58 g L⁻¹ for Trnovača wine and 1.74 g L⁻¹ for Požegača wine. The plum wine prepared in the study of Miljić *et al.* had slightly increased total acid content (6.7–8.6 g L⁻¹) and lower pH (3.4–3.5).⁹ Miljić *et al.* also reported that the most dominant macro-element in plum wines is potassium (1589–2451 mg L⁻¹) with calcium, magnesium and sodium also being present.⁹ A high concentration of potassium may result in a higher pH of the fruit but, more importantly, a higher pH of the wine. In addition, the volatile acid content was significantly lower (0.37–0.80 g L⁻¹) compared to the present samples. The different results among studies could be due to the fact that the acidity and ethanol content of wine depend on several factors, including type of cultivar, type of yeast used and methods of wine production.

The total dry extract represents all non-volatile matter that under specific physical conditions do not volatilize. From the chemical point of view, the matter

is represented by: fixed organic acids (tartaric, malic, succinic and lactic acid), glycerol, 2,3-butane-diol, sugars, tannins and dyes, pectin, gums, *etc.*²¹ The total dry extract varied from 58.19 to 75.26 g L⁻¹, and ash from 4.17 to 6.22 g L⁻¹. The highest content of total dry extract and ash were recorded in wines from Trnovača cultivar.

Phenolic and anthocyanins compositions of the plum wines

Phenolic composition and antioxidant activity in a fruit wine are very important and directly influence the quality of the wine. The phenolic compounds present in fruit wines are known for their positive effects on inflammation and cardiovascular diseases,²² besides having antimicrobial, cytotoxic and antioxidant activities.^{9,11} Phenolic compounds greatly contribute to the organoleptic properties by affecting the colour, astringency and aroma.²³ The concentrations of total phenolic compounds and anthocyanins, and the DPPH radical scavenging activity of the plum wines are given in Table II. The content of total phenolic compounds in the plum wines produced from three plum cultivars Čačanska rana, Čačanska leptotica and Požegača analysed in the work of Miljić and Puškaš⁹ (0.87–1.16 g L⁻¹, as GAE) was significantly lower compared with the results obtained for the plum wines in the present study (1.24 to 1.58 g L⁻¹). It may be assumed that the differences in these results among the wines are caused by differences in the wine-making processes and by the use of different plum cultivars for wine production.

TABLE II. Total phenolic and anthocyanin content and DPPH radical scavenging activity of plum wines

Sample	Total phenolic content ^a , g L ⁻¹	Total monomeric anthocyanin content ^b , mg L ⁻¹	DPPH radical scavenging activity, mg Trolox L ⁻¹
Crvena ranka	1.24±0.04	12.31±1.14	0.94±0.02
Požegača	1.45±0.12	6.55±0.34	1.33±0.03
Trnovača	1.58±0.13	7.48±0.30	1.40±0.04

^aGallic acid equivalent (mg GAE L⁻¹); ^bcyanidin-3-glucoside equivalent

Anthocyanins are responsible for the basic colour of a wine. Anthocyanin contents of fruits wines depend on the concentrations of these molecules in the fruits and are modified by the employed vinification techniques. In fruit wine-making, these compounds are extracted from the fruit skins during maceration and transferred to the must. In turn, the participation of anthocyanins in oxidation, hydrolysis, cycloaddition, condensation and polymerisation reactions produces important changes in the pigment composition of musts and wines.²⁴

The anthocyanin contents of the studied plum wines are given in Table II. It could be seen that the anthocyanin contents (12.31±1.14, 6.55±0.34 and 7.48±0.3 mg L⁻¹, respectively) of the three plum wines were significantly lower than

the plum wines investigated by Miljić *et al.* (168.0–194.6 mg/L).¹¹ The reduced anthocyanin content is probably due to the shorter time of maceration.

DPPH radical scavenging activity

The DPPH scavenging method has been used to evaluate the antioxidant activity of plum wine samples due to the simple, rapid, sensitive, and reproducible procedure. The total phenolic contents (*TPC*) and antioxidant capacities as measured by the DPPH assay were compared. All the analysed plum wines showed significant DPPH radical scavenging activity (IC_{50} from 0.94 to 1.40 mg L⁻¹). In addition, the obtained results show strong correlations both between the DPPH radical scavenging activities and the total phenolic contents (Fig. 1).

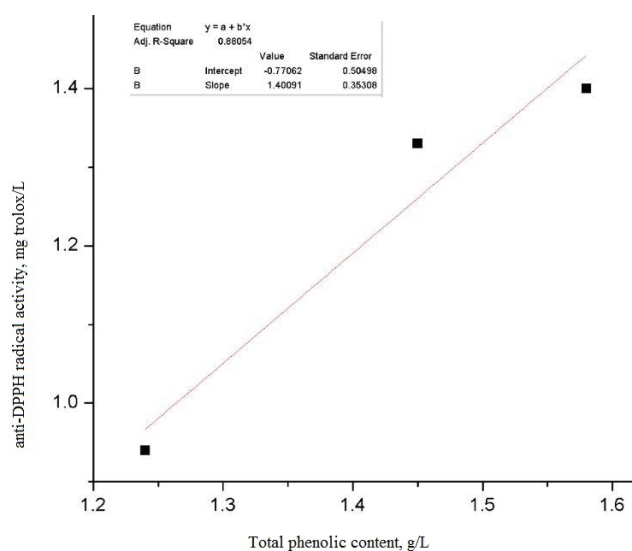


Fig. 1. Correlation between DPPH radical scavenging activity and *TPC*.

The anthocyan content showed non-significant correlation with the results obtained by the DPPH method, similar to the results previously published by Giovanelli²⁵ and Granato *et al.*²⁶ These authors also found that there was no correlation between the contents of anthocyanins and the antioxidant activity measured by the DPPH assay.

Individual phenolic compounds of fruit wines

A total of 12 phenolic compounds were identified and quantified in the plum wines, including hydroxybenzoic acids (4), hydroxycinnamic acids (4) and flavanoids (4), Table III.

Four different hydroxybenzoic acids (gallic, protocatechuic, *p*-hydroxybenzoic and vanillic acid) were detected and quantified in the plum wines (Table III).

Protocatechuic acid accounted for the largest proportion of the total hydroxybenzoic acid contents. The highest level of this acid was detected in Požegača wine (1.18 mg L⁻¹), followed by Trnovača wine (1.03 mg L⁻¹) and lastly Crvena ranka wine (0.84 mg L⁻¹).

TABLE III. The contents of the phenolic compounds in the plum wine samples determined by LC-MS/MS

Phenolic compound	Retention time min	Sample			MRM parameters			
		Crvena ranka	Požegača	Trnovača	ESI mode	Quantification transition	Cone voltage V	Collision energy eV
		Content, mg L ⁻¹						
Gallic acid	0.7	< 0.01	< 0.01	< 0.01	-	169→125	30	20
Protocatechuic acid	1.1	0.84	1.18	1.03	-	153→109	30	20
<i>p</i> -Hydroxybenzoic acid	1.9	0.48	0.48	0.80	-	137→93	30	20
Catechin	2.4	7.99	7.11	7.41	+	291→139	26	20
Chlorogenic acid	2.5	15.03	11.66	18.17	+	355→163	20	12
Vanillic acid	2.6	0.60	0.76	0.59	+	169→93	15	30
Caffeic acid	2.7	3.01	8.27	3.22	-	179→135	30	20
<i>p</i> -Coumaric acid	3.4	0.12	0.15	0.16	-	163→119	15	30
Ferulic acid	3.7	< 0.01	0.75	0.13	+	195→145	20	16
Quercetin	5.4	1.47	3.91	4.66	-	301→179	30	20
Naringenin	6.0	0.01	0.02	0.12	-	271→151	24	24
Kaempferol	6.1	0.07	0.16	0.24	+	287→153	56	36

The four hydroxycinnamic acids identified in the analysis were caffeic, chlorogenic, *p*-coumaric and ferulic acid. Chlorogenic acid was the most dominant hydroxycinnamic acid in the plum wines (11.66–18.70 mg L⁻¹). In the previous study, the chlorogenic acid content varied with the variety of plum wine: Čačanska leptotica wine had the highest (12.92 mg L⁻¹), while Požegača wine had the lowest (2.09–3.22 mg L⁻¹) content.¹¹ Caffeic acid was the second most abundant hydroxycinnamic acid, followed by *p*-coumaric and ferulic acid. The caffeic acid contents in the plum wine were in agreement with the previously reported data from Miljić *et al.* (*i.e.*, 1.25–11.86 mg L⁻¹).¹¹ The concentration of gallic acid in the produced plum wines was below the quantification limit of the applied method.

Catechin is the major flavonoid in plum wines. The levels of catechin reported here (7.11–7.99 mg L⁻¹) are lower than that found by Miljić *et al.*¹¹ These authors reported that level of catechin content in Požegača wine ranged from 14.67 to 19.65 mg L⁻¹ in different growing sites. Quercetin was the second most abundant flavanone in each of the samples, ranging from 1.47 mg L⁻¹ in the Crvena ranka wine to 4.66 mg L⁻¹ in the Trnovača wine.

CONCLUSIONS

The wines analysed in this study, produced from three plum cultivars of which two had not previously examined, had concentrations of phenolic compounds higher than those reported in other studies. These values resulted in higher antioxidant capacities of these wines as assessed using the DPPH method. Based on the level of phenolic compounds present in plum wine and their anti-oxidative activity, all three cultivars are promising raw materials for wine production.

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ИЗВОД

ФЕНОЛНИ САСТАВ И АКТИВНОСТ ЗА ВЕЗИВАЊЕ DPPH ВИНА ПРОИЗВЕДЕНИХ ОД ТРИ СОРТЕ ШЉИВА

МАРКО ЛЈЕКОЧЕВИЋ¹, МИЛКА ЈАДРАНИН², ЈОВАНА СТАНКОВИЋ², БРАНКО ПОПОВИЋ³,
НИНОСЛАВ НИКИЋЕВИЋ⁴, АЛЕКСАНДАР ПЕТРОВИЋ⁴ и ВЕЛЕ ТЕШЕВИЋ⁵

¹Десџилерија Зарић, Максима Марковића 42, 31260 Кошерић, ²Институт за хемију, технологију и металургију, Универзитет у Београду, Њешићева 12, 11000 Београд, ³Институт за воћарство, Краља Петра I 9, 32000 Чачак, ⁴Пољопривредни факултет, Универзитет у Београду, Немањина 6, 11080 Београд-Земун и ⁵Универзитет у Београду – Хемијски факултет, Студентски бр 12–16, 11000 Београд

Вина произведена из две сорте шљиве врсте *Prunus domestica* L. (Пожегача и Црвена ранка) и једне сорте врсте *Prunus insititia* L. (Трновача) су испитиване на садржај укупних фенолних једињења и антоцијанина. LC–MS/MS анализа заснована на специфичним масеноспектрометријским прелазима у тзв. режиму за праћење вишеструких реакција (MRM) је коришћена за идентификацију и квантификацију одабраних фенолних једињења. Утврђено је да су главни полифеноли у винима шљиве катехин, хлорогенска и кафена киселина и кверцетин. Садржај укупних фенолних једињења кретао се од 1,24 до 1,58 g еквивалената галне киселине/L. Од три испитивана вина, вино од Црвене ранке одликовало се већим садржајем антоцијанина (12,31 mg еквивалената цијанидин-3-глукозида/L). Антиоксидативни капацитет вина одређен је DPPH тестом. Различитост у физикохемијским карактеристикама, фенолном саставу и активности за везивање DPPH ових вина повезана је са разликом у коришћену различитих сорти у добијању вина.

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