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Chemical composition and *in vitro* antimicrobial and cytotoxic activities of plum (*Prunus domestica* L.) wine

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A moderate intake of wine is associated with a positive impact on human health owing to the effects of important biologically active components present in the wine in large amounts. The aim of this study was to examine the chemical composition and to assess antimicrobial and cytotoxic activities of fruit wines produced from three plum varieties (Čačanska rana, Čačanska lepotica and Požegača) commonly grown in Serbia as an approach to assess the quality and acceptability of these wines as a functional food. Furthermore, the activity of a series of control samples was assessed in order to determine components from the wine that are responsible for its functional properties. The plum wines produced showed considerable antimicrobial activity against six bacterial and two yeast strains used in this study. In addition to antimicrobial activity, the plum wines showed a significant cytotoxic effect ($IC_{50} < 50\ \mu g\ mL^{-1}$) on the growth of three tested cancer cell lines (Hep2c, RD and L2OB). Regarding the determined activities, Čačanska rana plum wine achieved the best results. The results indicated that the antimicrobial activity of the plum wines was, in large part, based on the effects of the total acids and the pH value, while the contribution of ethanol and the content of the phenolic compounds were not significant. Similar conclusions were drawn regarding the cytotoxic activity of this fruit wine. The results can be seen as a contribution to the global acceptance of fruit wines as a functional food, with the accent placed on moderate consumption. An important advantage of fruit wines (in particular plum wine), compared with traditional grape wine, is their lower alcohol content.

Keywords: plum; wine; antimicrobial activity; cytotoxic activity; composition

Introduction

It is known that consumption of certain foods and beverages can have a positive impact on the health of the consumer. Such food is called a functional food because it has a favourable impact on human health in addition to its usual nutritional functions. Grapes and wines naturally contain large amounts of the important biologically active components such as phenolic compounds. In addition to grapes, fruits such as blueberries, blackberries, cranberries, plums etc. are considered to be equal or even better source of various phenolic compounds *(1–3)*. In order to assess the functional value of plum wine, it is necessary to examine its composition and, furthermore, to evaluate its potential antioxidant, antimicrobial and antiproliferative activity. An important advantage of fruit wines, compared with traditional grape wines, is usually a lower alcohol content. Alcohol content is the biggest obstacle to the acceptance of wine as a functional food owing to controversial opinions of the scientific community about its impact on health *(4,5)*.

Research on the antimicrobial properties of wine in the last two decades has confirmed its significant activity against a large number of bacteria and yeast species *(6–8)*. However, there are still inconsistent opinions about the components and mechanisms that are responsible for such effects. On the other hand, the antiproliferative activity of this alcoholic drink on cancer cell lines has been far less investigated.

The antibacterial activity of various commercial beverages such as soft drinks, beer, wine, milk and water has been evaluated *(9)*. *Salmonella* sp., *Shigella* sp. and enterotoxigenic *Escherichia coli* were used as the test microorganisms and the lowest survival rate among the pathogenic bacteria after 2 days of inoculation was recorded in those bacteria exposed to wine, while the most prominent cell growth was recorded in milk and water. Slightly lower antibacterial activity, in relation to wine, was observed in beer and soda. Strong antibacterial activity of white and red wines has been reported in numerous studies *(6,10)*. It was found that *E. coli*, *Salmonella enteritidis* and *Shigella sonnei* populations were reduced from 10^5–10^6 CFU mL^{-1} to none detected (<10 CFU mL^{-1}) *(6)*.

In order to determine the contribution of individual components (phenolic compounds, ethanol and pH) to antimicrobial activity of wine, various authors *(7,8,11)* simultaneously assessed the effect of different control samples such as dealcoholized wine, phenol-stripped wine, 10–15% ethanol solutions, low pH (3.0–3.5) buffer solutions and molecular sulphur dioxide concentration. Tested bacteria strains were differently sensitive to the bactericidal effect of evaluated wine parameters. The synergistic effect of individual parameters was emphasized since the control (untreated) wine samples generally demonstrated the highest antibacterial

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activity. It was shown that phenolic content and antioxidant capacity were not correlated to antibacterial activity, and the phenolic stripped wine was twice as effective at reducing bacterial counts as the dealcoholized wine *(8)*. On the other hand, there are reports that phenolic compounds are strong bactericidal agents *(12,13)*. *Serratia marcescens*, *Proteus mirabilis*, *E. coli*, *Klebsiella pneumonia*, *Flavobacterium* sp. and *Staphylococcus aureus* showed different sensitivity to various phenolic compounds characteristic for wine (gallic, vanillic, caffeic acids, rutin, catechin, quercetin etc.), as well as to grape and wine phenolic extracts. The combination of polyphenols can have a synergistic or an antagonistic effect on antibacterial properties *(14)*.

Significant antibacterial activity was also determined for different fruit wines *(15)*. Raspberry, blackberry, cherry and peach wines showed bactericidal effects against following pathogenic bacteria: *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, *Shigella dysenteriae* and *S. aureus*. Scanning and transmission electron microscopy revealed that the treatment of bacteria with fruit wines led to a reduction in cell size, damage to the cell wall and cell membrane disruption.

Available scientific data about anti-proliferative and cytotoxic activities of wine is rather meagre. In this regard, there is no data about the contribution of individual components to overall effects of wine on the inhibition of cancer cell growth. The potential anticancer effects of wine, based on current available scientific data, are mainly associated with the presence and influence of various phenolic compounds, especially flavonoids (quercetin, catechin), trihydroxystilbene (resveratrol) and phenolic acids (gallic acid) *(16)*. Anthocyanins have also shown anticancer activity against several types of cancer cell lines *in vitro*, as well as against cancers of gastrointestinal tract and skin *in vivo (17)*. It was shown that phenolic compounds interfere with the initiation, promotion and progression of cancer *(18)*. Polyphenols prevent the transition of benign to malignant cancer caused by the activity of free radical species and protect the links in the DNA from endogenous oxidative damage and termination caused by γ-rays, which can lead to the appearance of mutations and carcinogenesis *(19)*. These compounds have also been demonstrated to act on multiple key elements in signal transduction pathways related to cellular proliferation, differentiation, apoptosis, inflammation, angiogenesis and metastasis but the molecular mechanisms of their action are still not completely clear *(18)*.

However, according to some authors *(20,21)* there is no clear relationship between total phenolic/flavonoid contents and inhibition of cell proliferation. The inhibition of cancer cell proliferation *in vitro* therefore cannot be explained by the concentration of phenolic/flavonoid compounds alone. It was suggested that concentration of specific phytochemicals may play a major role in the antiproliferative activity of plant extracts and products. Among some of the most important phenolic compounds isolated from red wine (quercetin, (+)-catechin, *trans*-resveratrol and gallic acid), quercetin was shown as the most effective ($ED_{50} < 1$ μmol) against mice with skin cancer (CD-1). Since it has been shown that *trans*-resveratrol is absorbed much more efficiently in human plasma when used in oral form than quercetin and (+)-catechin, as well as taking into account the relative concentrations of these polyphenols in red wine, the authors concluded that *trans*-resveratrol may be considered the most effective anticancer phenolic compound of red wine *(16)*.

The highest antiproliferative activity among different fruit extracts against a cell line of human liver cancer HepG2 was determined for blackcurrants (IC_{50} 14.5 mg mL^{-1} after 96 h of incubation), followed by lemons, apples, strawberries, red grapes, bananas, grapefruit and, finally, peaches *(3)*.

Plum (*Prunus domestica* L.) is the most important and most commonly grown fruit species in Serbia. Based on the data of the Food and Agriculture Organization of the United Nations *(22)* for the period 2007–2014, Serbia is ranked as second largest producer of plums, with an average annual yield around 600,000 tons. In terms of chemical composition, plums, as a potential raw material for fruit wine production, are characterized by a favourable balance of sugars and fruit acids, a high content of pectin and significant amounts of phenolic compounds, vitamins and minerals *(23,24)*. However, extensive characterization and evaluation of functional properties of this alcoholic beverage have still not been the subject of published scientific research. For this reason, the aim of this study was to examine the chemical composition and to assess the antimicrobial and cytotoxic activities of fruit wines produced from three plum varieties commonly grown in Serbia, as an approach to assessing the quality and acceptability of the produced wines as a functional food.

Material and methods

Plum wine production

Plum varieties Čačanska rana, Čačanska lepotica and Požegača (*P. domestica* L.) were used as raw materials for fruit wine production in this study. Varieties Čačanska rana and Čačanska lepotica resulted from the cross of Wangenheims Fruhzwetsche and Požegača and they were released in 1975 by the Fruit Research Institute, Čačak, Serbia. These plum varieties grown in Serbia possess good qualities for processing and they are characterized by different fruit harvest (ripening) times. Čačanska rana is an early ripening variety, Čačanska lepotica is a mid-early ripening variety, and Požegača is a late ripening plum variety. The plums for this research were procured at technological maturity from the local registered producers (Srem region, Serbia). Plums were halved and stones were removed by hand, after which the plums were subjected to crushing. In order to prevent contamination and oxidation, the pomace was treated with $K_2S_2O_5$ (SO_2 level was set to 50 mg SO_2 kg^{-1} pomace). The values of the plum wine fermentation conditions were applied as follows: temperature, 25 °C; pH value, 3.5; and dosage of pectolytic enzyme (Lallzyme EX-V, Lallemand S.A., St. Simon, France), 0.005 g kg^{-1}.

Pomace was divided into 3 L glass jars (2 kg of pomace in each) fitted with a fermentation bung for CO_2 release. Inoculation was performed with 0.25 g kg^{-1} of a previously rehydrated *Saccharomyces cerevisiae* strain sold under the commercial name Spriferm (Erbslöh, Geisenheim, Germany). After the completion of the fermentation, wine was separated from the pomace, the level of free SO_2 was adjusted to 20 mg L^{-1} and the wine was poured into 330 mL bottles, closed with screw caps and kept at 12–13 °C in the absence of light. After 2 months, during which clarification and stabilization processes took place, young plum wines were subjected to chemical analysis and to determination of antimicrobial and antiproliferative activity.

Physicochemical analyses

Total sugars, total acidity (expressed as malic acid), volatile acidity, ethanol content and pH of produced wines were determined using official methods *(25)*. The pH was measured directly in the

pomace by the laboratory multi-parameter analyser Consort C860 (Consort, Turnhout, Belgium).

The contents of glycerol, malic and citric acid were determined using commercial enzyme tests (Megazyme Co., Wicklow, Republic of Ireland). Sample preparation and analysis procedure were performed in accordance with the manufacturer's recommendations. Contents of ethanol (using a pycnometer) and volatile acids were determined using official methods *(25)*. The methanol content was determined by a colorimetric method *(25)*. The mineral composition (micro- and macro-elements) of the wine samples was determined by atomic absorption spectrophotometry *(25)* using a Varian (USA) model SpectrAA-10 instrument. The content of total phenolic compounds in wine was determined by the Folin–Ciocalteu method, using gallic acid as the standard *(26)*. Prior to analysis, all wine samples were filtered through a membrane filter (0.45 μm) and diluted 1:5 with distilled water. The results were expressed as grams of gallic acid equivalents (g GAE L^{-1}).

Antimicrobial activity

Samples for testing the antimicrobial activity were as follows: plum wine samples (U_1, U_2, U_3), dealcoholized wine samples from which alcohol and SO_2 were removed by vacuum evaporation (30 °C, 40 min; A_1, A_2, A_3), phenol-stripped plum wine, from which phenolic compounds were removed using polyvinylpolypyrolidone (FB_1, FB_2, FB_3). To compensate for the loss of volume owing to evaporation, distilled water was added up to the initial volume before dealcoholization. The control samples tested included 8% ethanol solution (K_{et}), a solution of malic and acetic acids buffered to pH 3.6 (K_{pH}), $K_2S_2O_5$ solution (SO_2 concentration 20 mg L^{-1}; K_{SO2}) and phosphate buffer pH 7.0 (buffer), which was used for preparation of sample K_{pH}. Subscripts 1–3 indicate samples of wines originating from varieties Čačanska lepotica, Požegača and Čačanska rana, respectively. All samples were filtered through a membrane filter with pore diameter 0.2 μm (Chromafil® CA-20/25S).

Test organisms for determination of antimicrobial activity were reference and wild strains of gram-negative and gram-positive bacteria: *S. typhimurium* (ATCC 14028), *E. coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC27853), *S. aureus* (ATCC 11632), *Bacillus cereus* (ATCC 10876), *Listeria monocytogenes* (ATCC 19111). Yeast strains were *S. cerevisiae* (112, Hefebank Weihenstephan) and *Candida albicans* (ATCC 10231).

Antimicrobial activity was determined by the disc diffusion and agar-well diffusion method. Bacterial strains were grown on Müeller–Hinton agar (MHA; Himedia, Mumbai, India) for 24 h at 37 °C and yeasts at 25 °C on Sabouraud maltose agar (SMA; Himedia, Mumbai, India). Cells were suspended in a sterile 0.9% NaCl solution and 2 mL of the suspension for inoculation (1×10^6 cells mL^{-1}, estimated by McFarland nephelometer) was homogenized with 18 mL of melted (45 °C) MHA or SMA and poured into Petri dishes. After desiccation of the agar surface (2 h at room temperature), for the disc diffusion method, sterile 6 mm discs (Himedia, Mumbai, India) were placed onto the inoculated agar plates and impregnated with 15 μL of sample. For the agar-well diffusion method, wells of 9 mm diameter were made with a sterile metal tube by means of a vacuum pump. Wine and control samples (100 μL) were then transferred into the wells of inoculated agar plates. For both methods, the test plates were refrigerated at 8 °C for 1 h to allow the samples to diffuse into the medium, and then were incubated at 37 °C (bacteria) and 25 °C (yeasts) for 24 h. After incubation, the diameters of the inhibition zones (clear zones or zones with reduced growth) were measured and recorded in millimetres (mm). The evaluation of antibacterial activity was carried out in three repetitions.

Cytotoxic activity

The impact of the produced plum wines on the growth of malignant transformed cell lines was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay. The following cell lines were used: Hep2c (cell line derived from human cervix carcinoma – HeLa derivative), RD (cell line derived from human rhabdomyosarcoma) and L2OB (cell line derived from murine fibroblast). Cells were seeded (2×10^4 cell $well^{-1}$) into a 96-well cell culture plates (NUNC) in nutrient medium [minimum essential Eagle medium supplemented with 5% (for Hep2c) or 10% (for RD and L2OB) of fetal calf serum] and grown at 37 °C in a humidified atmosphere for 24 h. Then, the corresponding wine samples and control (distilled water) were diluted with nutrient medium to the desired concentrations (5, 10, 20, 40, 60, 80 and 100 μg mL^{-1}). Diluted samples and control were added to cells (100 μL per well). The cells were incubated at 37 °C in a humidified atmosphere for 48 h. After the incubation period, supernatants were discarded, 100 μL of MTT (dissolved in Dulbecco's modification of Eagle's medium to a concentration of 500 μg L^{-1}) was added to each well and plates were incubated at 37 °C in a humidified atmosphere for 4 h. Reactions were halted by the addition of 100 μL of sodium dodecyl sulphate (SDS; 10% in 10 mM HCl). After overnight incubation at 37 °C, absorbance was measured at 580 nm using a spectrophotometer (Ascent 6-384 -Suomi, MTX Lab Systems Inc., Vienna, VA, USA). Cell viability (%) was calculated using the following formula:

$$\%\ cell\ viability = A/A_0 \times 100$$

where A is absorbance at 580 nm of a sample with cells grown in the presence of various concentrations of plum wine and A_0 is the absorbance at 580 nm of a sample, where instead of wine samples, only nutrient medium was added (negative control) and which represents 100% cell growth.

The cytotoxic activity of the tested plum wine samples was expressed as the IC_{50} value, which represents the concentration of an agent inhibiting cell survival by 50% compared with a negative control. The results of the measurements were compared with a control for cytotoxic effect (positive control) obtained using *cis*-diammine–dichloroplatinum (*cis*-DDP) instead of wine samples (at the same concentration) as given in a previously described procedure for the MTT assay. All experiments were performed in triplicate.

Statistical analysis

Statistical analysis in the present study was performed using Statistica 12.0 (Statsoft). The statistical difference between mean values of parameters was estimated by analyses of variance (ANOVA), at the 95% confidence level. Values detected as significantly different by use of Duncan's test are marked with different letters.

Results and discussion

Chemical composition of plum wines

Determination of the chemical characteristics of a beverage is very important for investigation and assessment of its functional properties and capability to induce beneficial effects on human health. The chemical composition of the fruit wines produced from plum varieties commonly grown in Serbia is shown in Table 1. The ethanol content in the tested plum wines was in the range 6.32–7.85% v v^{-1}, with the maximal amount determined in the wine from Požegača variety. The highest total acid content was determined in the Čačanska rana wine (8.5 g L^{-1}), while the lowest value of this parameter was characteristic of Požegača wine (6.7 g L^{-1}). Based on the different fruit ripening times of the tested plum varieties, the results obtained were as expected. Regarding the pH values, these differences were not as pronounced since the values for all plum wines produced were in a narrow range (3.4–3.5). Malic acid was the most dominant acid in the investigated plum wines, and its share of the total acid content ranged from 60% with Čačanska rana and Požegača to up to 70% with Čačanska lepotica. In addition to malic acid, a considerable concentration of citric acid (1–2 g L^{-1}) was seen.

The greatest concentration of volatile acids was determined in wine from the Požegača variety (0.8 g L^{-1}), whereas in the case of other two wines, this value was significantly ($p < 0.05$) lower (around 0.4 g L^{-1}). The yeasts during alcoholic fermentation of grape pomace or must normally produce 0.3–0.4 g L^{-1} of volatile acids, as by-products *(27)*. Slightly higher amounts of these acids in Požegača wine may be due to the degradation of residual sugar and glycerol by activity of anaerobic lactic acids or ethanol oxidation under the action of acetic acid bacteria. It was pointed out that acetic acid is the most dominant (>90%) volatile acid, and that the threshold of sensory detection of this acid is 0.7–1.1 g L^{-1} *(28)*. The determined content of 0.8 g L^{-1} did not exhibit a negative effect on the sensory properties of the Požegača wine.

Regarding total phenolic compounds, the highest content was determined in Čačanska lepotica wine, followed with Požegača, and lastly, Čačanska rana wine. The concentration of methanol in the tested wines differed significantly ($p < 0.05$) depending on the plum variety used and ranged from 658 to 735 mg L^{-1}. In addition to the activity of pectin methyl esterase, the content and the degree of esterification of pectin in the raw material directly affect the concentration of methanol in the wines. The highest concentration of methanol was, as expected, recorded in the wine from Čačanska rana, the variety characterized by the highest content of pectin and its degree of esterification (data not shown).

Glycerol is a non-volatile ingredient of wines, which significantly contributes to wine quality by providing sweetness and smooth mouth-feel *(29)*. The content of this compound is particularly important for the taste fullness and harmony of fruit wines considering, in general, their significantly lower extract and alcohol content as well as higher total acid content, compared with wines made from grapes. Statistically significant differences in the content of glycerol were determined in wines from different plums varieties. Maximal content of this compound was determined in Čačanska lepotica wine (6.2 g L^{-1}). The amount of glycerol in plum wines is mostly somewhat lower than in wines produced from grapes (4–9 g L^{-1}) *(30)*, mango (6.0–8.4 g L^{-1}) *(31)* and raspberries (6–10 g L^{-1}) *(32)*. Higher concentrations of glycerol are formed in the substrates with higher glucose content *(33)*.

The plum wines produced in this study were also characterized by a significant mineral content. The content of some macro- and micro-elements in wine is important for its nutritional value and health impact, and for its role in the wine stability, as well as for possible toxicological risk *(34)*. The most dominant macro-element in these fruit wines is potassium while, to a lesser extent, calcium, magnesium and, especially, sodium were also present. The highest

Table 1. Chemical composition of plum wines

	Variety		
Parameter	Čačanska rana	Čačanska lepotica	Požegača
Ethanol (% v v^{-1})	6.32 ± 0.13^{A}	7.64 ± 0.19^{B}	7.85 ± 0.11^{B}
Total sugar (g L^{-1})	0.8 ± 0.1^{A}	1.0 ± 0.1^{A}	2.1 ± 0.2^{B}
pH	3.45 ± 0.00^{A}	3.4 ± 0.00^{A}	3.5 ± 0.00^{A}
Total acids (g L^{-1})	8.6 ± 0.2^{C}	7.9 ± 0.1^{B}	6.7 ± 0.3^{A}
Malic acid (g L^{-1})	5.0 ± 0.4^{B}	5.3 ± 0.1^{B}	3.8 ± 0.2^{A}
Citric acid (g L^{-1})	2.0 ± 0.3^{B}	1.1 ± 0.2^{A}	1.1 ± 0.3^{A}
Volatile acids (g L^{-1})	0.41 ± 0.07^{A}	0.37 ± 0.04^{A}	0.8 ± 0.00^{B}
Total phenols[a] (g L^{-1})	1.65 ± 0.07^{A}	2.18 ± 0.04^{C}	1.88 ± 0.05^{B}
Methanol (mg L^{-1})	735 ± 7^{C}	658 ± 10^{A}	694 ± 6^{B}
Glycerol (g L^{-1})	4.6 ± 0.4^{A}	6.2 ± 0.3^{C}	5.4 ± 0.1^{B}
Mineral compounds (mg L^{-1})			
K	2451	1589	2100
Na	5.41	9.35	4.74
Ca	68.2	74.8	96.3
Mg	95.7	86.7	79.3
Fe	1.35	6.52	3.96
Zn	0.85	1.36	0.52
Cu	<0.1	0.13	<0.1

[A, B, C] Different letters in the same row indicate statistically significant differences among values ($p < 0.05$).

[a] Expressed as g L^{-1} of chlorogenic acid.

content of K and Mg was in the Čačanska rana wine. Čačanska lepotica wine was characterized by the highest content of Na, as well as by considerably lower amounts of K, compared with the other two wines. Also, the wine from the Požegača variety was distinguished by a slightly higher content of Ca (96 mg L^{-1}). The most common trace elements in plum wines tested were iron, zinc and copper, and their content significantly differed depending on the variety used. Average values of the mineral content (mg L^{-1}) reported in plum wines from the Santa Rosa (*P. domestica*) variety were as follows: K, 2000; Ca, 160; Mg, 36; Na, 40; Fe, 24; Zn, 2; and Cu, 0.4 *(35)*. It should be noted that the aforementioned plum wines are characterized by similar contents of K, Zn and Cu, higher contents of Ca, Na, Fe, and a lower content of Mg compared with the plum wines examined in this study. Furthermore, compared with the composition of mineral elements in wines made from grapes (Cabernet sauvignon and Chardonnay), blueberries, raspberries, blackcurrants, cranberries, cherries and apples *(36)*, it can be seen that wines produced from the plum varieties were characterized by a significantly higher content of K, similar contents of Ca and Mg and a considerably lower content of Na.

Antimicrobial activity of plum wines

The disc diffusion method demonstrated the absence of activity of plum wine against all tested bacterial strains. These results are not unexpected because the volume of the investigated samples was 15 μL, unlike the 100 μL sample that was used in the agar-well diffusion method. The antimicrobial activity of the plum wines and control samples, expressed as the diameter of inhibition zone (mm) obtained by agar-well diffusion method, is shown in Table 2.

The tested untreated wines (U_{1-3}), the dealcoholized wine samples (A_{1-3}) and phenol-stripped wines showed similar antibacterial activities against all tested bacterial strains. Clear zones of inhibition appeared around the wells, indicating the bactericidal action of the tested samples. Generally, inhibition zones of wines produced from different plum varieties toward bacteria were similar (average diameter of inhibition zone was 12–13 mm), except in the case of the untreated Čačanska rana wine, which demonstrated more pronounced antibacterial effect against *P. aeruginosa*, *S. aureus* and *B. cereus* (diameter of inhibition zone 15–16 mm).

The results obtained also showed that a clear difference between gram-positive and gram-negative bacteria, in terms of their susceptibility to the activity of plum wines, was not evident. The absence of differences between the effects of A_{1-3} and FB_{1-3} against bacteria and their corresponding untreated wines (U_{1-3}), as well as the lack of activity of the control samples (K_{et} and K_{SO2}), suggest that the phenolic compounds and the contents of ethanol and SO_2 should not be considered as the main components responsible for the antibacterial activity of plum wines. The Čačanska lepotica wine showed weaker antimicrobial activity than the Čačanska rana toward *P. aeruginosa*, *S. aureus* and *B. cereus* than the Čačanska rana, despite a significantly higher content of total phenolic compounds (Table 2). A weak contribution of the phenolic compounds occurring in wine to the antimicrobial activity has also been reported in previous studies *(8,37)*. However, as already mentioned, there are studies that are not consistent with these findings *(12,13)*. In this regard, a significant linear correlation between antibacterial activity towards *S. aureus* and *E. coli* and the total phenolic content ($r = 0.83–0.91$) and the monomeric anthocyanin content ($r = 0.82$-0.88) was pointed out *(38)*.

Table 2. Antimicrobial activity of plum wines and control samples [diameter of the inhibition $zone_{mean}$ (mm) including well (9 mm) ± standard deviation]

Test microorganism	Samples										
	U_1	U_2	U_3	A_1	A_2	A_3	FB_1	FB_2	FB_3	K_{et}	K_{pH}	K_{SO2}	Buffer
Salmonella typhimurium	12.0 ± 0.0	12.0 ± 0.0	13.0 ± 0.0	12.0 ± 0.0	13.0 ± 0.0	11.0 ± 0.0	12.3 ± 0.6	13.3 ± 0.6	12.3 ± 1.2	n.d.	17.0 ± 0.0	n.d.	n.d.
Escherichia coli	11.7 ± 0.6	14.7 ± 0.6	13.3 ± 0.6	13.0 ± 0.0	17.0 ± 1.0	15.0 ± 0.0	12.0 ± 0.0	13.0 ± 0.0	12.5 ± 0.8	n.d.	17.0 ± 1.0	n.d.	n.d.
Pseudomonas aeruginosa	14.0 ± 0.0	12.7 ± 0.6	15.0 ± 0.0	14.0 ± 1.0	13.7 ± 0.6	13.0 ± 0.0	13.7 ± 0.6	14.7 ± 0.6	15.0 ± 1.0	n.d.	18.0 ± 0.0	n.d.	n.d.
Staphylococcus aureus	12.0 ± 0.0	13.7 ± 0.6	16.0 ± 1.0	11.67 ± 0.58	12.7 ± 1.5	14.7 ± 0.6	12.3 ± 1.2	14.3 ± 1.2	17.0 ± 1.0	n.d.	12.0 ± 0.0	n.d.	n.d.
Bacillus cereus	13.7 ± 0.6	13.7 ± 0.6	15.0 ± 0.0	13.33 ± 0.58	14.0 ± 1.0	14.0 ± 1.0	13.0 ± 0.0	14.0 ± 1.0	12.3 ± 0.6	n.d.	12.0 ± 0.0	n.d.	n.d.
Listeria monocytogenes	13.0 ± 0.0	11.0 ± 0.0	13.0 ± 0.0	11.0 ± 0.0	12.0 ± 0.0	11.0 ± 0.0	12.7 ± 0.6	12.0 ± 0.0	13.7 ± 0.6	n.d.	15.0 ± 0.0	n.d.	n.d.
Saccharomyces cerevisiae	n.d.	n.d.	n.d.	15.33 ± 0.58	11.7[a] ± 0.6	14.7 ± 0.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Candida albicans	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., Not detected.
[a]Zone of reduced growth.

When considering the antimicrobial activity of polyphenols, the fact that these compounds are very sensitive to physical and chemical conditions of the medium in which they are present must be taken into account. These conditions and their changes could significantly affect their solubility, precipitation and interactions with proteins, and thus affect their biochemical properties and biological activity. For these reasons, it is possible that fractions of phenolic compounds isolated from wines, in most cases, do not exhibit the antimicrobial activity that they would demonstrate in the physico-chemical environment of untreated wine.

Control sample K_{pH} showed significant antibacterial activity, but no effect on the growth of yeasts. The antibacterial activity of K_{pH} was especially pronounced against gram-negative bacteria (diameter of inhibition zone 17–18 mm). The activity of K_{pH} against the tested bacteria (except for *S. aureus* and *B. cereus*) was more pronounced than in case of the untreated wines and other control samples. This indicates that the acidity and pH value could be considered as main factors responsible for the plum wine's antibacterial activity. This conclusion is supported by the fact that Čačanska rana wine, which was characterized by the highest total acid content, showed stronger antimicrobial activity than the wines from the other two cultivars. Čačanska rana also contained the highest amount of malic acid, which has been reported to possess the most pronounced antimicrobial effect amongst the organic acids *(39)*. However, some authors have reported weak antibacterial activity of the buffer solution with appropriate pH value (as in the tested wine) *(10,37)*. Comparing the above-mentioned literature data with the results obtained in this study, it can be concluded that the antimicrobial activity of the wine was largely derived from the effects of both total acids and pH value.

S. cerevisiae and *C. albicans* have been shown to be more resistant to the effect of the samples compared with the bacteria. The yeast *S. cerevisiae* was susceptible only to the activity of the dealcoholized wine samples (A_{1-3}). The effect was probably a result of the concentration of the samples and lowering of the pH after removal of the alcohol. Furthermore, the *S. cerevisiae* strain used as the test microorganism was reference strain 112 from the Weihenstephan Hefebank. This yeast is known to be more susceptible than the commercial winemaking strain Spiriferm (Erbslöh, Germany), used for inoculation of the plum pomace prior to fermentation. The activity of sample A_2 was fungistatic according to the zone of reduced growth around wells, while samples A_1 and A_3 showed clear zones, which indicated fungicidal activity. The yeast *C. albicans* showed the greatest resistance to the effect of the investigated samples, since inhibition zones were not detected in any of the test sets. The lack of antifungal activity can be partially explained by the fact that yeasts are acid-tolerant organisms. Yeast resistance towards organic acids, which are dominant active components in the determination of antimicrobial activity of different extracts and beverages, has been reported by other authors *(12,40)*.

Cytotoxic activity of plum wine

The cytotoxic activity of fruit wines from native plum varieties was tested *in vitro* against three histologically different cancer cell lines: Hep2c, RD and L2OB. The cytotoxic effect of the produced wines was expressed as an IC_{50} value (the concentration which inhibits 50% of cell growth) and is shown in Table 3.

Experimental plum wines showed significant activity against the cell lines and all tested wine samples manifested cytotoxic effects at concentrations below 50 $\mu g\,mL^{-1}$. The most dominant inhibitor of cell line growth was the plum wine produced from the Čačanska rana variety. The cells proven to be the most sensitive to the effect of this wine were the Hep2c. On the other hand, the effect of the Čačanska rana plum wine was the least pronounced regarding the L2OB cells. Since the US National Cancer Institute criteria for the cytotoxic activity of plant extracts is $IC_{50} < 30\ \mu g\,mL^{-1}$ *(41)*, it can be concluded that Čačanska rana plum wine satisfied this condition for Hep2c and RD cell lines, whereas in the case of L2OB cells the determined IC_{50} value was slightly above the set limit. On the other hand, the effect of the Čačanska lepotica wine was the weakest among the tested wines. This plum wine reduced survival of respondent cancer cells by 50% at concentrations of 35–50 $\mu g\,mL^{-1}$. It should be noted that the concentration of wine from the Čačanska rana variety, which caused 50% inhibition of cell growth of RD and L2OB lines, was almost 2-fold lower than in the case of the Čačanska lepotica wine. Plum wine produced from the Požegača variety was characterized by a considerable cytotoxic effect against the Hep2c cells ($IC_{50} = 27.29\ \mu g\,mL^{-1}$), whereas in the case of the other two cell lines, activity was less pronounced (especially for RD line). Significant differences ($p < 0.05$) in the cytotoxic activity of wines from Čačanska lepotica and Požegača were not present only in terms of the RD cell growth inhibition. The results obtained in this part of the study indicated that the large contribution to the cytotoxic activity of plum wines could be attributed to the synergistic effects of acidity and pH value. In support of this, and similar as with the antimicrobial activity, the highest cytotoxic activity was recorded for the wine (Čačanska rana) characterized by the highest total acid content and the lowest content of phenolics. Also, despite the lowest total organic acid content, the Požegača plum wine manifested similar activity against the Hep2c cells to the Čačanska rana, possibly owing to the significantly higher content of volatile acids (primarily acetic acid). It was previously demonstrated that malic acid exhibits an anti-proliferative effect in human keratinocyte cell lines (HaCaT) through the inhibition of cell cycle progression at G0/G1, and the induction of programmed cell death through endoplasmic reticulum stress- and mitochondria-dependent pathways *(42)*. The occurrence of apoptosis was proven by the increased expressions of FasL, Fas, Bax, Bid, caspases-3, -8, -9, cytochrome c, and the declined expressions of Bcl-2, PARP. Furthermore, significant *in vitro* cytotoxic activity of acetic acid has been reported previously *(43)*.

Unlike many other studies that showed a high positive correlation between the content of total phenolic compounds (in grape extracts and wine) and antiproliferative and cytotoxic activities *(16,44)*, in the case of the plum wines tested in this work, this dependence was not noted. Despite the lowest content of total phenols, Čačanska rana wine exerted the strongest cytotoxic effect against the cancer cell lines tested. This could perhaps be attributed to the fact that the plum wine from Čačanska rana variety was characterized by a significantly higher concentration of cyanidin-3-rutinoside and peonidin-3-glucoside (data not shown), anthocyanins reported to exhibit strong anti-cancer effects *(45–47)*. The cyanidin-3-rutinoside has shown a cytotoxic effect against several leukaemia and lymphoma cell lines by induction of apoptosis. In addition, the cyanidin-3-rutinoside treatment resulted in reactive oxygen species (ROS)-dependent activation of p38 MAPK and JNK, which contributed to cell death by activating the mitochondrial pathway mediated by Bim. Notably, cyanidin-3-rutinoside treatment did not lead to increased ROS accumulation in normal human peripheral blood mononuclear cells and had no cytotoxic effects on these cells *(47)*. Furthermore, an

Table 3. Cytotoxic activity of wines from investigated native plum varieties

	IC_{50} ($\mu g\ mL^{-1}$)		
Sample	Hep2c cells[a]	RD cells[b]	L2OB cells[c]
Čačanska rana	23.39 ± 0.46^{A}	24.53 ± 0.94^{A}	32.28 ± 3.10^{C}
Čačanska lepotica	36.77 ± 5.91^{CD}	40.59 ± 2.34^{D}	50.34 ± 0.90^{E}
Požegača	27.29 ± 0.72^{AB}	42.57 ± 2.29^{D}	33.35 ± 1.29^{C}
cis-DDP	0.94 ± 0.55	1.4 ± 0.97	0.72 ± 0.64

Data given in the table represent mean values ± 2SD. *cis*-DDP, *cis*-diammine–dichloroplatinum.
[a]Cell line derived from human cervix carcinoma.
[b]Cell line derived from human rhabdomyosarcoma.
[c]Cell line derived from murine fibroblast.

inhibitory effect of cyanidin-3-rutinoside on the migration and invasion of highly metastatic A549 human lung carcinoma has been confirmed *(46)*. The effect of peonidin-3-glucoside on the growth of several cancer cells lines has also been tested *(45)*. Strong inhibitory effects on cell growth via G2/M arrest were the consequence of peonidin-3-glucoside application. Regarding cell cycle-related proteins, peonidin-3-glucoside treatment resulted in a decrease of protein levels of cyclin-dependent kinase (CDK)-1, CDK-2, cyclin B1 and cyclin E. Peonidin-3-glucoside also induced caspase-3 activation, chromatin condensation, and cell death. The inhibition on the growth of Lewis lung carcinoma cells *in vivo* by peonidin 3-glucoside was also reported in the same study.

It was reported that some fractions of phenolic compounds (especially flavonoids) extracted from plum have a pronounced cytotoxic effect against breast cancer cells *(48)*. Furthermore, a significant inhibitory effect of plum extracts on the growth of three cell lines of human breast cancer (MCF-7, MDA-MB-453 and MCF-10A), two cell lines of human colon carcinoma (HT-29 and Caco-2) and a human prostate cancer cell line (PC-3) has been reported *(49)*. For comparison, Čačanska rana, Čačanska lepotica and Požegača plum wines possess stronger anticancer effects compared with raspberry wine *(50)*, which was characterized by IC_{50} values above 100 $\mu g\ mL^{-1}$.

The cytotoxic activity of plum wines was evaluated by comparing with the results obtained for the activity of *cis*-DDP. The compound *cis*-DDP is an inorganic agent with prominent antineoplastic activity. Antineoplastic (cytostatic) agents are medicines whose mechanism of action is based on the ability to interfere with the metabolism or reproductive cycle of cancer cells, and consequently to destroy them. It is used as reference substance for the expression of cytotoxic action, owing to its strong inhibition effect on cancer cell growth. It was observed in this study that the cytotoxic activity of *cis*-DDP was 25–40 times stronger than the activity of the plum wines investigated. However, it should be borne in mind that, owing to their unselective nature, antineoplastic agents (including *cis*-DDP) often do not discriminate between cancer and normal cells, which may clinically manifest through various undesirable actions *(51)*.

Conclusions

The results of physicochemical analyses indicated a uniform quality for the Čačanska lepotica and Požegača wines. On the other hand, the Čačanska rana wines were, above all, characterized by having a lower ethanol content and a higher content of total acids and methanol, in comparison with the wines from the other two varieties. The determined differences in wine quality could be related to the different fruit ripening times of the tested plum varieties. The concentration of methanol in the tested plum wines was in the range 658–735 $mg\ L^{-1}$, which is significantly higher compared with normal values for wine from grapes (<200 $mg\ L^{-1}$).

Wines produced from the three plum varieties commonly grown in Serbia showed antibacterial activity against all tested bacterial strains. Significant differences in susceptibility/resistance of the bacteria to the effects of plum wines were not registered, except in the case of the Čačanska rana wine, whose antibacterial activity against *P. aeruginosa*, *S. aureus* and *B. cereus* was more pronounced. *S. cerevisiae* and *C. albicans* yeasts were shown to be more resistant than the bacteria to the activity of the tested wine samples. Based on the results obtained, it can be concluded that the antimicrobial activity of plum wines was, in large part, derived from the effect of the total acids and the pH value.

In addition to antimicrobial effects, plum wines showed significant cytotoxic effects on the growth of three tested cancer cell lines (Hep2c, RD and L2OB). The Čačanska rana wine, characterized by the highest total acid content and the lowest total phenolics content, was the most potent inhibitor of growth with all three cell lines.

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