Traditionally made red wines produced from an autochthonous grapevine variety as a source of biologically active compounds and their antioxidant potential

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Summary

Wine samples, wine evaporated to dryness, dealcoholized wine and the original shape wine, made from autochthonous grapevine variety from Serbia and Montenegro, were examined in order to determine total phenols, flavonoids and proanthocyanins, as well as their antioxidant activity. Antimicrobial and anti-biofilm activities were evaluated. The grapevine variety Vranac had the best values for total phenols, flavonoids and proanthocyanins compounds. The original shape wine samples showed stronger antioxidative efficiency, with the effective concentration values at which 50 % of 2,2-diphenyl-1-picrylhydrazyl radicals were scavenged ranging from 12.98 $\mu l \cdot ml^{-1}$ to 132.27 $\mu l \cdot ml^{-1}$. The strongest antimicrobial activity of samples of wine evaporated to dryness was detected against *Bacillus cereus, B. subtilis* IP 5832 and *Lactobacillus plantarum*, minimum inhibitory concentration (*MIC*) being from < 0.08 $mg \cdot ml^{-1}$ to 2.50 $mg \cdot ml^{-1}$. Samples of dealcoholized wine and the original shape wine showed the strongest inhibitory activity against *B. cereus* (*MIC* from < 0.39 $\mu l \cdot ml^{-1}$ to 0.78 $\mu l \cdot ml^{-1}$). Dealcoholized wine samples showed biofilm-inhibitory activity at which 50 % of *Staphylococcus aureus* ATCC 6538 biofilm was reduced, with values ranging from 188 $\mu l \cdot ml^{-1}$ to 530 $\mu l \cdot ml^{-1}$. The results of the study contribute to the knowledge on the biological activities of Balkan red wines and the potential for developing a nutritive and health-promoting food product.

Keywords

antimicrobial activity; antioxidants; antibiofilm; grapevine variety; phenolics; red wine

The production of wines from Serbia and Montenegro is based on the cultivation of autochthonous grape varieties [1]. Jara-Palacios et al. [2] indicated that wine consists of various compounds with antioxidant activity. Phenolic compounds, which contribute to colour and taste of wine and determine its quality, are a result of the relations between biosynthesis and their transformation that occurs during grape maturation under the influence of internal (genetic) and external factors (climate, soil, terrain) [3].

The autochthonous Balkan grapevine variety Vranac is widely spread in Montenegro, Serbia and mostly in North Macedonia where it represents a very important variety from an economic point of view [4]. Serbian red wines, produced from autochthonous grape varieties, are a rich source of antioxidants [1]. Many other authors from different regions (Herzegovina, Turkey, Spain, Italia) indicated that there is a positive correlation between the antioxidant activity and the total phenol content [5–7]. Daudt and Fogaça [8] indicated that localization of the vineyard had more impact on wine characteristics than the type of maceration in wines. It was shown that antimicrobial properties of phenolic compounds from wine and wine extracts against some potential respiratory pathogens depend on the type of phenolic compounds [9]. Some authors indicated that the antimicrobial effects of wine depend on certain wine components, like polyphenols, on pH and on ethanol contents [10, 11]. Some phenolic acids showed the potential of growth inhibition agains certain pathogenic yeasts, such as *Candida albicans* strains [12, 13].

The aims of this study were to determine the

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total content of phenols, flavonoids, proanthocyanins as well as the antioxidant activity in three different types of wine samples made from six red wines originating from autochthonous grapevine variety from Serbia and Montenegro. Also, the aims were to investigate the in vitro antimicrobial activity of wine samples on selected species of bacteria and fungi, and to investigate the effects of partly evaporated wine samples on the inhibition of biofilm formation of staphylococci.

MATERIAL AND METHODS

Wines and sample preparation

Red wines, made from autochthonous grapevine variety, which are commonly consumed in Serbia and Montenegro, were purchased from local wineries during 2016 and stored at 5 °C until experiments, which were done during the same year. A listing of the wines used in this study and their origin is shown in Tab. 1.

Three types of samples were made from every red wine used in this study: wine evaporated to dryness (EW), dealcoholized (partially evaporated) wine (DW) and original shape wine (OW). For the preparation of EW and DW samples, a volume of wine of 500 ml was used separately. EW samples were obtained using evaporation to dryness by a rotary evaporator (IKA, Staufen, Germany) at 40 °C for 80 min. Since winemakers produced selected red wines with the addition of low concentration of SO_2 (< 10 $mg \cdot l^{-1}$, without other additives like ascorbic acid or glutathione), DW samples were obtained using evaporation of alcohol and SO_2 (30 °C for 40 min) from original wine. OW samples were not evaporated, so they contained alcohol and SO_2. Therefore, they presented a wine in its original shape.

Chemical analysis of wine samples

Total phenolic compounds of the wine samples were determined using the Folin-Ciocalteu's method [14] and expressed as total phenolic content (*TPC*) in grams per kilogram of dry matter and grams per litre of wine, expressed as gallic acid equivalents (GAE) using gallic acid (Sigma Aldrich, St. Louis, Missouri, USA) as a standard. According to Piljac Žegarac et al. [15], the low content of SO_2 does not interfere with the Folin-Ciocalteu test.

Total flavonoids content (*TFC*) of the wine samples were determined by the aluminium chloride colorimetric assay [16] and expressed as gram per kilogram of dry matter and gram of per litre of wine, expressed as rutin equivalents (RUE) using rutin (Sigma Aldrich) as a standard.

Total proanthocyanins were quantified by the method developed by Porter et al. [17] and expressed as gram per kilogram of dry matter and gram per litre of wine, expressed as cyanidin chloride equivalents (CChE) using cyanidin chloride (Sigma Aldrich) as a standard.

Determination of antioxidant activity

The ability of wine samples to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was investigated by using a method described by Takao et al. [18]. The tested concentrations of EW samples ranged from 15.63 $\mu g \cdot ml^{-1}$ to 500.00 $\mu g \cdot ml^{-1}$, while the activity of DW and OW samples was tested in dilutions made with methanol, from 15.63 $\mu l \cdot ml^{-1}$ to 500.00 $\mu l \cdot ml^{-1}$. Chlorogenic acid was used as a positive control. The ab-

Tab. 1. Red wines used in the study.

No.	Wine	Alcohol [%]	Dry matter [$g \cdot l^{-1}$]	pH	Manufacturer	Production year	Description	Grapes
1.	Montenegro Vranac	13.5	19	3.15	13 July – Plantaže, Podgorica, Montenegro	2012	Red wine	Vranac
2.	Vranac Pro Corde	14.0	21	3.01	13 July – Plantaže, Podgorica, Montenegro	2011	Red wine	Vranac
3.	Pecelj Vranac	14.0	19	2.93	Pecelj family, Svilajnac (Crkvenac), Serbia	2015	Red wine	Vranac
4.	Prokupac	13.5	24	3.09	Winery Ivanovic, Aleksandrovac, Serbia;	2014	Traditionally Serbian dark red wine	Prokupac
5.	Prokupac	13.0	23	3.42	Toplicki vineyards, Gojinovac, Prokuplje, Serbia	2011	Contains sulfites, aged in barrique French oak for 14 months	Prokupac
6.	Filigran – crna tamjanika	12.0	22	3.05	Winery of Monastery Bukovo, Negotin, Serbia	2014	Red wine, contains sulphites	Tamjanika

Prokupac – the homonymous autochthonous grape variety.

sorbance of samples was read in Jenway 6300 UV spectrometer (Cole-Parmer, Stone, United Kingdom), at 517 nm. Scavenging activity (*SA*) was expressed as the inhibition percentage calculated using the following equation:

$$SA = \frac{(A_C - A_S)}{A_C} \times 100 \quad (1)$$

where A_C is absorbance of the control and A_S is absorbance of the extract.

The effective concentration at which 50 % of DPPH radicals were scavenged (IC_{50}) was obtained from the graph of scavenging activity versus concentration of samples. A low IC_{50} value indicates strong ability of the extract to act as a DPPH scavenger. The antioxidant activity was expressed as antioxidant activity index (*AAI*), calculated using the following equation:

$$AAI = \frac{FC_{DPPH}}{IC_{50}} \quad (2)$$

where FC_{DPPH} is final concentration of DPPH.

The estimation of *AAI* was done in the following way: $AAI < 0.5$ indicated poor antioxidant activity, *AAI* from 0.5 to 1.0 indicated moderate antioxidant activity, *AAI* from 1.0 to 2.0 indicated strong antioxidant activity and $AAI > 2.0$ indicated very strong antioxidant activity [19].

Determination of antimicrobial activity

Antimicrobial activity of wine samples was tested against 20 microorganisms including 13 strains of bacteria. These comprised „probiotic“ strains *Lactobacillus plantarum, Bifidobacterium animalis* subsp. *lactis, Bacillus subtilis* IP 5832, “standard” strains *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 12453 and clinical isolates *Staph. aureus*, *B. cereus, E. coli, Salmonella enterica, S.* Typhimurium*, Proteus mirabilis*, together with 7 strains of fungi (four yeasts, namely, *Saccharomyces boulardii, Rhodotorula mucilaginosa, Candida albicans* ATCC 10231, *C. albicans* and three filamentous fungi, namely, *Penicillium italicum, Trichoderma viride* ATCC 13233, *Aspergillus flavus* ATCC 9170). Clinical isolates were a gift from the Institute of Public Health (Kragujevac, Serbia). Fungi and ATCC-strains were provided from a collection held by the University of Kragujevac (Kragujevac, Serbia). The bacterial strains were kept in glycerol stock at –80 °C and fungal strains in paraffin oil stock at 4 °C.

Bacterial and yeast suspensions used in this study were prepared according to the method described by Andrews [20]. Antimicrobial activity was tested using the microdilution method [21], by determining the minimum inhibitory concentration (*MIC*), with modification described by Muruzović et al. [22]. Briefly, two-fold serial dilutions of the tested wine samples were made in sterile 96-well microtiter plates containing 0.1 ml per well of Mueller-Hinton broth (Torlak, Belgrade, Serbia) for bacteria and 0.1 ml per well of Sabouraud dextrose broth (Torlak) for fungi. The tested concentration range was from 0.08 mg·ml^{-1} to 10.00 mg·ml^{-1} for EW samples, while DW and OW samples were dissolved in the liquid medium, to reach concentrations from 50.00 μl·ml^{-1} to 0.39 μl·ml^{-1}. The microtitre plates were inoculated with the suspensions to obtain a final concentration of 5×10^5 CFU·ml^{-1} for bacteria and 5×10^3 CFU·ml^{-1} for fungi. The inoculated microtitre plates were incubated at 37 °C for 24 h for bacteria, at 28 °C for 48 h for yeasts, and at 28 °C for 72 h for filamentous fungi. Tetracycline (Sigma Aldrich) and fluconazole (Pfizer, New York, New York, USA), dissolved in the nutrient liquid medium, were used as reference compounds.

Determination of antibiofilm activity

We screened staphylococcal strains, including *Staph. aureus* ATCC 6538, for their ability to form biofilm by the tissue culture plate method (TCP) method [23] with some modifications described in detail by Muruzović et al. [22]. The concentrations required to reduce biofilm formation by 50 % (BIC_{50}) and 90 % (BIC_{90}) were defined as the lowest concentration of the extract that showed 50% and 90% inhibition on the biofilm formation, respectively. Only broth or broth with DW sample served as control to check sterility and non-specific binding of media. To compensate for background absorbance, absorbance readings from sterile medium, DW sample, fixative and dye were averaged and subtracted from all test values. All tests were performed in duplicate.

Data analysis

Data were presented as mean ± standard deviation. Antibacterial activity between groups of bacteria (Gram-positive, Gram-negative, “probiotic” bacteria), the difference between total content of phenolics, flavonoids and proanthocyanins between samples as well as Pearson correlation coefficients were analysed by one-way analysis of variance using SPSS 20 package (SPSS, Chicago, Delaware, USA). Principal component analysis (PCA) was used to reveal the associations of the different secondary metabolites content and wine samples using Statistica 13.0 package (TIBCO Software, Palo Alto, California, USA).

RESULTS AND DISCUSSION

Chemical composition of wine samples

It is well-known that red wines consist of secondary metabolites such as phenolics, flavonoids and proanthocyanins. EW sample of Vranac Montenegro had the highest phenol content (92.19 g·kg^{-1}, expressed as GAE). Between the DW and OW samples, Vranac Pro Corde (DW2 and OW2) had the highest concentration of total phenols (1.97–1.79 g·l^{-1}, expressed as GAE). When comparing the wines, Vranac Pro Corde was the richest in flavonoids in all samples (EW2, DW2, OW2). Grapevine variety Vranac showed a higher concentration of proanthocyanins. The highest concentration of proanthocyanins was in the DW sample of Vranac Montenegro (DW1) (Tab. 2, Tab. 3).

In our study, the EW sample of Vranac Montenegro (EW1) and DW and OW samples of Vranac Pro Corde (DW2, OW2) contained the highest number of phenolic compounds. When comparing all wines, total flavonoids were the highest in the wine Vranac Pro Corde, which was also emphasized by the higher total proanthocyanins. A statistically significant difference between EW, DW and OW samples in total phenolic compounds ($p < 0.05$), as well as the difference between EW and DW and EW and OW samples in total flavonoid and proanthocyanins

Tab. 2. Total phenolics, flavonoids and proanthocyanins contents, and antioxidant activity of the wine samples evaporated to dryness.

Wine samples	*TPC* [g·kg^{-1}]	*TFC* [g·kg^{-1}]	*TPAC* [g·kg^{-1}]	IC_{50} [μg·ml^{-1}]	*AAI*
EW1	92.19 ± 0.04	7.72 ± 0.02	30.62 ± 0.08	63.70 ± 0.16	1.26
EW2	88.63 ± 0.14	9.57 ± 0.02	41.62 ± 0.05	59.30 ± 0.08	1.34
EW3	66.21 ± 0.08	9.03 ± 0.02	33.22 ± 0.12	114.80 ± 0.19	0.70
EW4	85.31 ± 0.11	8.00 ± 0.17	26.78 ± 0.04	57.80 ± 0.25	1.38
EW5	62.87 ± 0.04	6.27 ± 0.01	10.81 ± 0.06	51.03 ± 0.09	1.56
EW6	35.61 ± 0.08	1.53 ± 0.00	3.97 ± 0.07	62.80 ± 0.24	1.27
Chlorogenic acid	ni	ni	ni	11.65 ± 0.52	6.87

Each value shown is mean ± standard deviation.
TPC – total phenolics content is expressed as grams of gallic acid equivalents, *TFC* – total flavonoids content is expressed as grams of rutin equivalents, *TPAC* – total proanthocyanins content is expressed as grams of cyanidin chloride equivalents. Chlorogenic acid was used as a positive control for determination of antioxidant activity.
EW1–EW6 – wine samples evaporated to dryness, IC_{50} – concentration at which 50 % of DPPH radicals were scavenged, *AAI* – antioxidant activity index, ni – not investigated.

Tab. 3. Total phenolics, flavonoids and proanthocyanins concentration and antioxidant activity of dealcoholized and original shape wine samples.

Wine samples	*TPC* [g·l^{-1}]	*TFC* [g·l^{-1}]	*TPAC* [g·l^{-1}]	IC_{50} [μl·ml^{-1}]	*AAI*
DW1	1.81 ± 0.68	0.16 ± 0.06	62.34 ± 0.06	34.17 ± 0.47	2.34
DW2	1.97 ± 0.21	0.18 ± 0.07	55.23 ± 0.36	28.70 ± 0.10	2.79
DW3	1.37 ± 0.50	0.16 ± 0.13	50.31 ± 0.05	36.70 ± 0.12	2.18
DW4	1.79 ± 0.68	0.96 ± 0.05	62.14 ± 0.04	32.12 ± 0.20	2.49
DW5	1.20 ± 0.38	0.80 ± 0.04	28.53 ± 0.06	42.70 ± 0.11	1.87
DW6	0.72 ± 0.21	0.14 ± 0.02	11.43 ± 0.04	85.30 ± 0.15	0.94
OW1	1.56 ± 0.21	0.14 ± 0.05	56.01 ± 0.12	22.16 ± 0.05	3.61
OW2	1.80 ± 0.79	0.16 ± 0.10	60.77 ± 0.35	14.26 ± 0.05	5.61
OW3	1.26 ± 0.21	0.16 ± 0.05	35.48 ± 7.02	12.98 ± 0.06	6.16
OW4	1.66 ± 0.21	0.09 ± 0.04	47.87 ± 0.08	36.12 ± 0.13	2.21
OW5	1.07 ± 0.36	0.08 ± 0.04	24.50 ± 0.03	47.24 ± 0.08	1.69
OW6	0.61 ± 0.21	0.01 ± 0.04	9.46 ± 0.06	132.27 ± 0.97	0.60

Each value shown is mean ± standard deviation.
TPC – total phenolics concentration is expressed as grams of gallic acid equivalents, *TFC* – total flavonoids concentration is expressed as grams of rutin equivalents, *TPAC* – total proanthocyanins concentration is expressed as grams of cyanidin chloride equivalents.
DW1–DW6 – dealcoholized wine samples, OW1–OW6 – original shape wine samples, IC_{50} – concentration at which 50 % of DPPH radicals were scavenged, *AAI* – antioxidant activity index.

was shown. The difference between DW and OW in total flavonoids and proanthocyanins was not significant ($p > 0.05$).

Antioxidant activity

The antioxidant activity of three different types of wine samples was expressed in the form of IC_{50} values (Tab. 2, Tab. 3). EW samples showed the antioxidant activity ranging between 51.03 $\mu g \cdot ml^{-1}$ and 114.80 $\mu g \cdot ml^{-1}$, DW samples of wines showed antioxidant activity ranging between 28.70 $\mu l \cdot ml^{-1}$ and 85.30 $\mu l \cdot ml^{-1}$, while OW samples showed the antioxidant activity ranging from 12.98 $\mu l \cdot ml^{-1}$ and 132.27 $\mu l \cdot ml^{-1}$.

AAI is a number that indicates the success of a compound in the effects of antioxidation. EW samples showed strong antioxidant activity in most cases (*AAI* from 1.0 to 2.0). DW and OW samples of Vranac Montenegro, Vranac Pro Corde, Pecelj Vranac and Prokupac (Winery Ivanovic) showed very strong antioxidant activity (*AAI* > 2.0). Prokupac (Toplički vineyards, Prokuplje) showed strong antioxidant activity (*AAI* from 1.0 to 2.0), while Filigran – crna tamnjanika showed moderate antioxidant activity (*AAI* from 0.5 to 1.0)

It is well-known that wines present a great source of natural compounds, which can be treated as antioxidants. Wine grapes, which were collected from a southern Serbian vineyard, were evaluated for their phenolic profile and antioxidant properties. Among the varieties, the highest total phenolic content, total flavonoid, and proanthocyanin content were found in "Cabernet Sauvignon", a red wine grape variety that showed the strongest DPPH radical-scavenging activity [24]. Piljac Žegarac et al. [15] indicated that Croatian wines had a high phenolic content. Among them, red wines showed 10× higher levels of antioxidants in comparison with white wines. Proestos et al. [25] also showed that red wines contained higher amounts of phenolic substances than white ones. According to the results presented previously [15, 24, 25], it could be concluded that red wines are rich sources of natural antioxidants, which was confirmed by our study. Jiang and Zhang [26] indicated that the contents of phenolic compounds and the levels of antioxidant activity in the wine samples greatly varied with cultivar and environmental factors of vine growth. They also showed a significant correlation between concentration of phenolic compounds and antioxidant capacity ($p < 0.05$). Our results indicated that correlation between the total phenolic, flavonoids and proanthocyanin content and DPPH radical-scavenging activity in EW samples was not significant ($p > 0.05$), while for DW samples, the correlation was significant between DPPH radical-scavenging activity and the total phenolics and proanthocyanin concentrations ($p = 0.15$ and $p = 0.18$, respectively). A significant correlation was also shown between the total phenolics, flavonoids and proanthocyanin concentration and DPPH radical-scavenging activity in OW samples ($p = 0.30$, 0.03, 0.47, respectively). Radovanović et al. [27] showed the correlation between the contents of quercetin-3-glucoside and quercetin and DPPH of the red wines. Our results are in correlation with authors who indicated a significant correlation between the concentration of phenolic compounds and antioxidant capacity of wine in its original shape. Milutinović et al. [28] showed that wine extract of "Oligo Grapes" supplement from Bionys Plus (Krnjevo, Serbia) showed *AAI* value of 0.58, which indicated moderate antioxidant activity, like EW3 sample from our study. The rest of EW samples showed strong antioxidant activity. Our results indicated that wine of Vranac Montenegro, Vranac Pro Corde, and Pecelj Vranac in its original shape showed very strong antioxidant activity, suggesting that red wines present good sources of natural antioxidants. Also, mentioned wines are a good source of active compounds, which is important from biological and health point of view.

Determination of antimicrobial activity

The results of in vitro antimicrobial activities of wine samples made from six different red wines, expressed as *MIC*, are shown in Tab. 4 and Tab. 5. In this study, *MIC* values were in the range from < 0.08 $mg \cdot ml^{-1}$ to > 10.00 $mg \cdot ml^{-1}$ for EW samples and from < 0.39 $\mu l \cdot ml^{-1}$ to > 50.00 $\mu l \cdot ml^{-1}$ for DW and OW samples. It was noticeable that all tested wine samples generally acted weaker on Gram-negative bacteria than on Gram-positive bacteria ($p < 0.05$; Tab. 4, Tab. 5). EW samples showed a stronger effect on *Lb. plantarum, B. subtilis* IP 5832 and *B. cereus* (*MIC* from < 0.08 $mg \cdot ml^{-1}$ to 2.50 $mg \cdot ml^{-1}$). DW and OW samples showed strong inhibitory activity on *B. cereus* (*MIC* from < 0.39 $\mu l \cdot ml^{-1}$ to 0.78 $\mu l \cdot ml^{-1}$). Regarding the tested Gram-negative bacteria, the most sensitive to EW samples was *P. mirabilis* (*MIC* at 5.00–10.00 $mg \cdot ml^{-1}$). *P. mirabilis* was the most sensitive to DW and OW samples (*MIC* at 12.50–50.00 $\mu l \cdot ml^{-1}$).

The strongest antifungal activity showed EW1, EW2 and EW3 samples, with *MIC* values ranged from 2.50 $mg \cdot ml^{-1}$ to 5.00 $mg \cdot ml^{-1}$ for the *R. mucilaginosa* (Tab. 4). Between DW samples, Prokupac (winery Ivanović) gave *MIC* for *C. albicans* 6.25 $\mu l \cdot ml^{-1}$ and for *T. viride* ATCC 13233 12.50 $\mu l \cdot ml^{-1}$; Prokupac (Toplički vineyards, Pro-

Tab. 4. Antimicrobial activities of tested wine samples evaporated to dryness.

Species	Minimum inhibitory concentration [mg·ml^{-1}]							
	EW1	EW2	EW3	EW4	EW5	EW6	Tetracycline	Fluconazole
Lactobacillus plantarum	0.31	0.16	0.16	0.31	0.31	0.16	0.13	ni
Bacillus animalis subsp. *lactis*	< 0.08	< 0.08	< 0.08	< 0.08	0.62	< 0.08	4.00	ni
Bacillus subtilis IP 5832	0.62	0.62	0.62	0.16	0.62	0.62	< 0.06	ni
Bacillus subtilis ATCC 6633	> 10.00	> 10.00	> 10.00	> 10.00	> 10.00	> 10.00	0.25	ni
Bacillus cereus	< 0.08	< 0.08	0.31	< 0.08	0.31	0.31	0.25	ni
Staphylococcus aureus ATCC 25923	0.62	2.50	2.50	0.31	1.25	1.25	1.50	ni
Staphylococcus aureus	0.08	2.50	0.62	0.08	0.31	0.31	< 0.06	ni
Proteus mirabilis ATCC 12453	10.00	5.00	5.00	5.00	5.00	5.00	> 128.00	ni
Proteus mirabilis	10.00	10.00	5.00	5.00	10.00	10.00	> 128.00	ni
Escherichia coli	> 10.00	> 10.00	> 10.00	> 10.00	> 10.00	> 10.00	2.00	ni
Escherichia coli ATCC 25922	10.00	10.00	10.00	10.00	10.00	10.00	4.00	ni
Salmonella enterica	> 10.00	> 10.00	> 10.00	> 10.00	> 10.00	> 10.00	2.00	ni
Salmonella Typhimurium	> 10.00	> 10.00	> 10.00	> 10.00	> 10.00	> 10.00	2.00	ni
Rhodotorula mucilaginosa	5.00	2.50	2.50	5.00	5.00	10.00	ni	31.25
Saccharomyces boulardii	> 10.00	> 10.00	10.00	> 10.00	> 10.00	> 10.00	ni	7.81
Candida albicans ATCC 10231	> 10.00	10.00	10.00	10.00	> 10.00	> 10.00	ni	31.25
Candida albicans	2.50	2.50	2.50	> 10.00	> 10.00	> 10.00	ni	62.50
Penicillium italicum	> 10.00	> 10.00	> 10.00	> 10.00	> 10.00	5.00	ni	250.00
Trichoderma viride ATCC 13233	0.62	> 10.00	> 10.00	10.00	10.00	10.00	ni	500.00
Aspergillus flavus ATCC 9170	10.00	10.00	10.00	10.00	10.00	> 10.00	ni	500.00

EW1–EW6 – wine samples evaporated to dryness, ni – not investigated.

kuplje) gave *MIC* < 0.39 μl·ml^{-1} for *T. viride* ATCC 13233. Filigran – crna tamnjanika gave *MIC* for *T. viride* ATCC 13233, 6.25 μl·ml^{-1}. The *MIC* for the OW samples was in the range from 25.00 μl·ml^{-1} to > 50.00 μl·ml^{-1} for all species, except for *T. viride* ATCC 13233 (Tab. 5).

According to Darra et al. [29], phenolic compounds extracted from the red grapes extract demonstrated stronger antimicrobial effects against Gram-positive bacteria than Gram-negative bacteria and yeasts, which was confirmed in our study. EW samples showed better antifungal activity than DW and OW samples, which showed a limited and selective antifungal activity. Results of our study are in accordance with Milutinović et al. [28], who indicated that "Oligo Grapes" supplement had no influence on *Saccharomyces boulardii*, while some activity against *Candida albicans* and *Rhodotorula mucilaginosa* was determined. Papadopoulou et al. [12] indicated that some phenolic acids from white and red wine extracts had the potential to inhibit the growth of certain pathogens such as *Staph. aureus, E. coli* and *C. albicans* strains. In our study, a clinical isolate of *E. coli* showed resistance to all tested samples of wine, while their effect on *C. albicans* was only weak. The strongest antifungal activity showed EW samples of grapevine variety Vranac, on the growth of *R. mucilaginosa*. Serbian red wines showed significant antimicrobial activity against *Staph. aureus, B. subtilis, E. coli, P. aeruginosa* and *S. enteritidis* strains [27]. White grape juice extract showed in vitro inhibition of *Staph. aureus* ATCC 6538P and *E. coli*. No effect on the growth of *Candida* sp. and *A. niger* was previously detected [30]. Møretrø and Daeschel [10] indicated that wine had an antibacterial effect

against *Staph. aureus* (*MIC* at 35 % (v/v)), which was conformed in our study. Vaz et al. [31] investigated the antimicrobial activity of wine against *B. cereus* vegetative cells and showed that wine exerts a strong inactivation effect. After threatment, the number of *B. cereus* vegetative cells decreased to undetectable levels. Wines tested in our study showed strong effect on *B. cereus* (*MIC* values ranging from < 0.39 $\mu l \cdot ml^{-1}$ to 0.78 $\mu l \cdot ml^{-1}$). Overall, samples of wines from Serbia and Montenegro tested in our study showed a moderate impact on tested bacteria and a limited and selective antifungal activity.

Tab. 5. Antimicrobial activities of tested dealcoholized and original shape wine samples.

Species	Minimum inhibitory concentration [$\mu l \cdot ml^{-1}$]											
	DW1	DW2	DW3	DW4	DW5	DW6	OW1	OW2	OW3	OW4	OW5	OW6
Lactobacillus plantarum	0.78	0.78	0.78	1.56	1.56	3.13	6.25	0.78	1.56	1.56	1.56	1.56
Bacillus animalis subsp. *lactis*	< 0.39	3.13	< 0.39	< 0.39	< 0.39	< 0.39	< 0.39	< 0.39	< 0.39	< 0.39	< 0.39	< 0.39
Bacillus subtilis IP 5832	0.78	3.13	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	< 0.39	0.78
Bacillus subtilis ATCC 6633	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00
Bacillus cereus	< 0.39	< 0.39	< 0.39	< 0.39	< 0.39	0.78	< 0.39	0.78	< 0.39	0.78	< 0.39	< 0.39
Staphylococcus aureus ATCC 25923	1.56	1.56	1.56	3.13	6.25	6.25	3.13	3.13	3.13	3.13	6.25	6.25
Staphylococcus aureus	1.56	0.78	1.56	3.13	6.25	6.25	3.13	3.13	3.13	3.13	3.13	6.25
Proteus mirabilis ATCC 12453	12.50	12.50	25.00	12.50	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	25.00
Proteus mirabilis	12.50	12.50	25.00	12.50	50.00	25.00	25.00	12.50	25.00	12.5	25.00	25.00
Escherichia coli	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00
Escherichia coli ATCC 25922	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	50.00
Salmonella enterica	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00
Salmonella Typhimurium	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00
Rhodotorula mucilaginosa	25.00	25.00	50.00	25.00	25.00	> 50.00	25.00	25.00	50.00	25.00	25.00	> 50.00
Saccharomyces boulardii	> 50.00	> 50.00	50.00	> 50.00	> 50.00	> 50.00	> 50.00	50.00	50.00	> 50.00	> 50.00	> 50.00
Candida albicans ATCC 10231	50.00	50.00	25.00	> 50.00	> 50.00	> 50.00	> 50.00	50.00	50.00	50.00	> 50.00	> 50.00
Candida albicans	50.00	50.00	50.00	25.00	> 50.00	> 50.00	50.00	50.00	> 50.00	25.00	> 50.00	> 50.00
Penicillium italicum	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00
Trichoderma viride ATCC 13233	50.00	> 50.00	> 50.00	12.50	< 0.39	6.25	6.25	50.00	50.00	50.00	> 50.00	25.00
Aspergillus flavus ATCC 9170	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00	> 50.00

DW1–DW6 – dealcoholized wine samples, OW1–OW6 – original shape wine samples.

Antibiofilm activity

All the wines stimulated additional growth of pre-formed staphylococcal biofilms, so the influence was positive on all tested strains. For the biofilm formation, there was a suppression of growth in the case of *Staph. aureus* ATCC 6538 (Tab. 6). The other two staphylococcal strains were not influenced.

In this study, the in vitro activity of DW samples on biofilm formation was examined for the first time. Biofilm formation is the main staphylococcal survival strategy. The triggers for biofilm formation or disassembling are a part of a lot of investigations. All the red wines used in the study by Cho et al. [32] inhibited *Staph. aureus* biofilm formation and hemolysis unaffecting bacterial growth. Antibiofilm activity of tannic acid along with quercetin was a part of testing on *Staph. aureus* strains conducted by Lee et al. [33]. Biofilm formation was inhibited by a lot of extracts they used, while the planktonic form was not influenced. These studies suggest that components present in wine and its products interact with *Staph. aureus* biofilms. The influence on biofilm formation

Tab. 6. Antibiofilm activity of dealcoholized wine samples.

Wine sample	Biofilm-inhibitory concentration [μl·ml^{-1}]	
	BIC_{50}	BIC_{90}
DW1	188	238
DW2	253	462
DW3	256	496
DW4	213	334
DW5	530	> 1 000
DW6	506	915

Inhibition of *Staphylococcus aureus* ATCC 6538 biofilm was examined.
BIC_{50} – concentration of the extract that showed 50% inhibition on the biofilm formation, BIC_{90} – concentration of the extract that showed 90% inhibition on the biofilm formation.

and preformed biofilm in this study were different. *Staph. aureus* ATCC 6538 was the only strain inhibited by DW samples during the biofilm formation, but there was no influence on pre-formed biofilm. The antibiofilm activity was the strongest by Montenegro Vranac (DW1).

Principal component analysis

The results obtained for the total content of secondary metabolites in six different wine samples from the territory of Serbia and Montenegro were examined by statistical testing of multivariate components. Fig. 1 shows a bi-plot analysis of *TPC, TFC, TPAC* and antioxidant activity in wine samples. The two principal components (PC1 and PC2) separate the concentration of test values with a total variation of 90.3 %. The concentrations of the compounds varied widely in the tested wine samples. Factor loadings for *TPC* (0.88), *TFC* (0.87) and antioxidant activity (0.71) had positive values, while *TPAC* had a negative value (–0.63). For PC1, the *TPAC* and *TFC* had the highest factors loading, while for PC2 the highest factors loading had *TPC* (0.66).

The dependence between wine samples, related to the total content of secondary metabolites and antioxidant activity, is shown in Fig. 2. The results indicated that the wine samples EW6, DW6 and OW6 were different from other wine samples based on antioxidant activity, while the higher *TPC* and *TFC* was determined in EW samples of all six tested wines. Based on the performed PCA, it was demonstrated that Filigran – crna tamjanika and Prokupac (Toplicki vineyards) were the wines that contributed to the higher variability along the PC1 axis. The wine Filigran – crna tamjanika differed from the other samples by the lowest antioxidant activity, while samples DW5 and OW5 also showed a weak antioxidant activity.

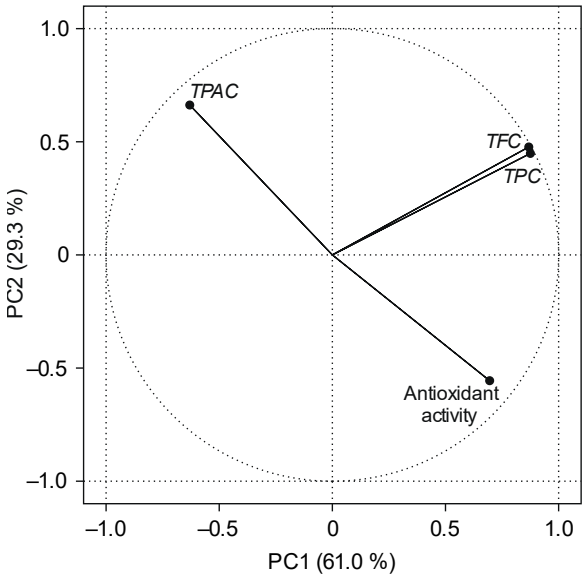

Fig. 1. Mutual dependence of secondary metabolites and antioxidant activity in different wine samples.

TPC – total phenolics concentration, *TFC* – total flavonoids concentration, *TPAC* – total proanthocyanins concentration.

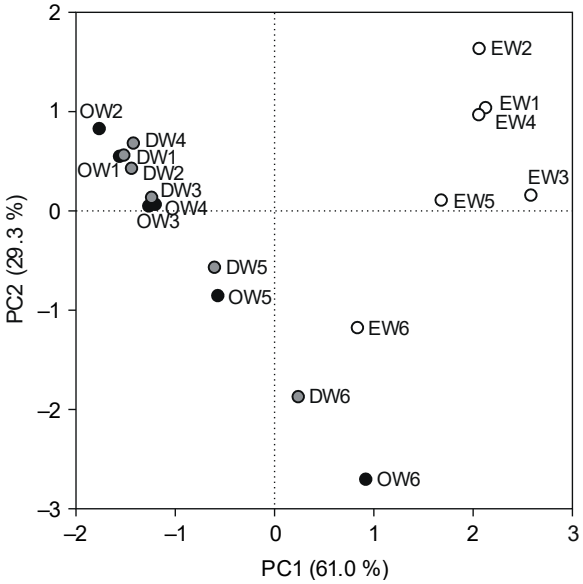

Fig. 2. Principal components based on secondary metabolites and antioxidative activity of wine samples.

EW1–EW6 – wine samples evaporated to dryness, DW1–DW6 – dealcoholized wine samples, OW1–OW6 – original shape wine samples.

CONCLUSION

Based on the examinations of wine samples used in this study, it could be concluded that all samples of red wine, made from autochthonous grapes varieties from Serbia and Montenegro, are a source of biologically active compounds. Tested wines showed a significant concentration of total phenols and a good antioxidant activity. Wine samples, in particular EW samples, showed moderate antibacterial effects on tested Gram-positive bacteria, while the strongest antifungal activity showed EW1, EW2 and EW3 samples. The inhibition of biofilm formation of dealcoholized wine samples was significant. Based on the results, it could be concluded that original shape wine samples showed strong antioxidant activity, but dry matter from evaporated wine samples showed a better antimicrobial activity. Also, wines made from grapevine variety Vranac are a better source of biologically active compounds, compared with other tested grapevine varieties. The results indicated a potential for further investigation of wines made from autochthonous grapes varieties and ability for using wines for developing a nutritive and health-promoting food product.

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