

Original Research Article

Antimicrobial Effectiveness of Selected Vranac Wines Against Six Gram-Positive and Six Gram-Negative Bacterial Strains

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Abstract

Purpose: The aim of this study was to assess potential antimicrobial effectiveness of selected red wines from Balkan region, made from autochthonic Vranac *V. vinifera* L. grape variety.

Methods: The antimicrobial activity of Vranac wines against Gram-positive: *C. perfringens*, *B. subtilis*, *S. aureus*, *L. innocua*, *S. Lutea*, and *M. flavus* and Gram-negative: *E. coli*, *P. aeruginosa*, *S. enteritidis*, *S. sonnei*, *K. pneumonia* and *P. vulgaris* bacteria strains were studied using the agar well diffusion and micro-well dilution methods. The concentrations of the wine phenolic compounds: gallic acid, caffeic acid, (+)-catechin, resveratrol, quercetin, quercetin-3-glucoside and malvidin-3-glucoside were determined using HPLC analysis.

Results: There was excellent correlation between the contents of gallic acid, caffeic acid, resveratrol, quercetin, quercetin-3-glucoside and malvidine-3-glucoside and the antimicrobial activity of the wines against Gram-positive - *C. perfringens* and *M. flavus* (from 0.936 to 0.999) and against Gram-negative bacteria strains - *E. coli*, *P. aeruginosa*, *S. enteritidis*, *S. sonnei* and *P. vulgaris* (from 0.904 to 0.999). Furthermore, the content of (+)-catechin has good correlation with the antimicrobial activity of the wines only against *L. innocua* and *P. vulgaris* with correlation coefficient of 0.996 and 0.999, respectively. All selected wine phenolic compounds, however, did not show correlation with antimicrobial activity against *K. pneumonia* strain.

Conclusion: The antimicrobial activity of selected Vranac wines indicates that some of the wine's phenolic constituents have the potential to inhibit the growth of certain bacterial strains.

Keywords: Red wine; Antimicrobial activity; Bacterial strains; Phenolic compounds.

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INTRODUCTION

The fact that microorganisms nowadays tend to develop resistance toward drugs, coupled to the undesirable side effects of certain antibiotics offer considerable potentials for the development of new effective antimicrobial agents [1-6]. It is well known that wine grape possesses therapeutically properties, attributed mainly to high polyphenolic content [7-10].

Recent studies indicate that consuming small amounts of red wine on a regular basis reduces the risk of coronary heart disease and arteriosclerosis, and this medical quality is connected to the antioxidant properties of phenolic compounds. Vascular diseases are comparatively rare in France, Portugal, and Italy, but are encountered most frequently in the

Scandinavian countries and the United Kingdom [11-15].

Moreover, wine polyphenolics manifest a wide range of beneficial health effects, such as antibacterial, antiviral, anti-carcinogenic and anti-atherogenic. In many instances, these effects can be attributed to plausible biochemical mechanisms including enhanced apoptosis, growth arrest at one point in the cell cycle, inhibition of DNA synthesis, and modulation of signal transduction pathways by altered expression of key enzymes such as cyclooxygenases and protein kinases activities [4].

The aim of this study was to evaluate the potential antimicrobial effectiveness against: *C. perfringens*, *B. subtilis*, *S. aureus*, *L. innocua*, *S. lutea* and *M. flavus* Gram-positive and *E. coli*, *P. aeruginosa*, *S. enteritidis*, *S. sonnei*, *K. pneumonia* and *P. vulgaris* Gram-negative bacteria strains of selected red wines from Balkan region, made from autochthonic *Vranac V. vinifera* L. grape variety grown in the Serbia and Montenegro.

EXPERIMENTAL

Chemicals

Acetonitrile, formic acid (HPLC-grade) and ethanol were obtained from Merck (Darmstadt, Germany); HPLC-grade methanol were purchased from Carlo Erba Reagent (Milan, Italy); gallic acid, (+)-catechin, quercetin, quercetin-3-glucoside, malvidin-3-glucoside and Folin - Ciocalteu's phenol reagent were supplied from Sigma Chemical Co. (St Louis, Mo, USA); used reagents were of analytical quality.

Wines samples

Selected *Vranac* wines from vintage 2009, made from autochthonic *Vranac V. vinifera* L. grape variety grown in Balkan region, were provided by different wineries: Plantaža, Podgorica, Montenegro (VW1), Rubin, Kruševac, Serbia (VW2) and Vino Župa, Aleksandrovac, Serbia (VW3).

Determination of total phenolic content

The concentration of total phenolics in selected *Vranac* wines was determined by the Folin-Ciocalteu method described by Singleton and Rossi [16], using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The concentration was calculated using gallic acid as standard and the

results were expressed as mg of gallic acid equivalent/L wine.

High performance liquid chromatography (HPLC) analysis of wine phenolics

Phenolic compounds contents were determined by using HPLC, by direct injection of each wine sample into an Agilent 1200 chromatographic system - photodiode array detector (DAD) with radiofrequency identification tracking technology for flow cells and fluorescence detector for multiwavelength detection with online acquisition of excitation (Ex) and emission (Em) spectra, and a Chem-Station software. Elution was carried out in gradient mode using two solvent mixtures: (A) formic acid/water (5:95 v/v) and (B) acetonitrile/formic acid/water (80:5:15 v/v). The elution profile was as follows: from 0 to 28 min, 0-10.0 % B, from 28 to 35 min, 10-25 % B, from 35 to 40 min, 25 – 50 % B, from 40 to 45 min, 50 - 80 % B, and for last 10 min again 0 % B. Aliquots of 5 µl were injected into a 4.6 × 250 mm RPC-18 column (Zorbax Eclipse XDB-C18) with 5 µm particle size. The flow rate was 0.8 mL/min. The detection wavelengths were 280, 320, 360 nm for UV, and 275/322 nm ($\lambda_{Ex}/\lambda_{Em}$) for fluorescence-detection. Identification and quantification of various phenolic compounds were made by using calibration curves obtained with the standard solutions of pure phenolic in the same conditions as the wine samples. The results are expressed in mg per l of sample (mg/L).

Microorganisms

The antimicrobial activity of wine samples were tested against a panel which included laboratory control strains obtained from the American Type Culture Collection (Rockville, Md., USA): Six Gram-positive bacteria: *Clostridium perfringens* ATCC 19404, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 8538, *Listeria innocua* ATCC 13076, *Sarcina lutea* ATCC 9341 and *Micrococcus flavus* ATCC 40240 and six Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076, *Shigella sonnei* ATCC 25931, *Klebsiella pneumonia* ATCC 10031 and *Proteus vulgaris* ATCC 8427). The strains were grown at 37 °C for 24 h on Mueller-Hinton agar (MHA, Torlak, Serbia).

Disc diffusion assay

Preliminary antimicrobial tests were carried out by disc diffusion method using 100 µl of bacterial suspension spread on Mueller-Hinton agar (MHA, Torlak, Serbia) in sterilized Petri dishes (90 mm in diameter). The discs (9 mm in

diameter, HiMedia Laboratories Pvt Ltd, Mumbai, India) were impregnated with 50 µl of the testing samples and placed on the inoculated agar (20 ml). The inoculated plates were incubated for 24 h at 37 °C. Reference antibiotics, Chloramphenicol (30 µg/disc), Streptomycin (30 µg/disc) and Tetracyclin (30 µg/disc) served as a positive control, while the solvent (10% ethanol - 50 µl/disc) was used as a negative control. It was found that the solvent showed no inhibitory activity. All the tests were performed in triplicate. Antibacterial activity was evaluated by measuring the zone of inhibition (in mm) against the test bacterial strains.

Broth microdilution assay

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003). The inocula of the bacterial strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. A serial doubling dilutions of the testing samples were prepared in a 96/well microtiter plate over the range of 500 - 0.25 µl/ml in inoculated nutrient broth (the final volume - 100 µl and the final bacterial concentration was 10⁶ CFU/ml in each well). The plate was incubated for 24 h at 37 °C. All experiments were performed in triplicate. Two growth controls consisting of medium with water (negative control) and medium with Chloramphenicol, Streptomycin and Tetracyclin (positive control) were also included. The microbial growth was determined by absorbance at 620 nm using the universal microplate reader (Thermo Lab systems, Multiskan EX, Software for Multiscan ver. 2.6, Vantos, Finland). MIC was defined as the lowest concentration of test samples at which microorganisms showed no visible growth. In order to determine MBC, broth was taken from each well without visible growth and inoculated on Mueller Hinton agar (MHA) for 24 h at 37 °C. The MBC is defined as the lowest concentration of the wine samples at which 99.9 % of inoculated microorganisms were killed.

Statistical analysis

Three analytical replicates were carried out on each sample wine. Measurements were averaged and results are given as mean ± standard deviation. The standard deviation was calculated by ANOVA using the Minitab statistical package (Minitab Inc, State College, PA, USA).

RESULTS

All tested Vranac wines exhibited antibacterial activity. Inhibition zone diameters against Gram positive: *C. perfringens*, *B. subtilis*, *S. aureus*, *L. innocua*, *S. Lutea*, and *M. flavus* were 12.0 – 14.9 mm and Gram-negative: *E. coli*, *P. aeruginosa*, *S. enteritidis*, *S. sonnei*, *K. pneumonia* and *P. vulgaris* bacteria stains were 12.1 – 16.8 mm, which is very significant in comparison to the reference antibiotics: chloramphenicol, streptomycin and tetracyclin:

The data in Table 1 show that the antimicrobial activity of investigated Vranac wines was followed: VW1 > VW2 > VW3. All tested Vranac wines show the highest activity against *S. aureus* and *S. lutea* Gram-positive and against *E. coli* and *S. sonnei* Gram-negative strains, then the lowest activity against *K. pneumoniae*.

Antibacterial activity of selected red wines was affected by their biochemical composition, expectedly by their phenolic constituents [1-15]. The concentrations of some wine phenolic compounds as well as gallic acid, caffeic acid, (+)-catechin, resveratrol, quercetin, quercetin-3-glucoside and malvidin-3-glucoside, determined using HPLC analysis and total phenolic content, determined by UV/VIS spectroscopic analysis are reported in Table 2.

The changes in total phenolic contents and the concentrations of selected phenolic compounds of the wine samples were closely followed with the changes in their antibacterial activity.

The results obtained by microdilution method confirmed disc diffusion results in most cases. Tested red wines showed antimicrobial activity against all tested strains in the range from 62.5 – 250 µL/mL (Table 3).

DISCUSSION

The concentrations of wine phenolic compounds can vary significantly within grape cultivars according to environmental conditions of viticulture regions. In addition, during the process of wine preparation, significant changes take place in the composition and content of phenolic compounds, as a result of different winemaking techniques as well as wine fermentation and aging [17-20].

It is reported about the important antimicrobial role played by organic acids in wine, immediately followed by phenolic acids and then by flavonoids [1-16]. Also, antimicrobial efficacy of the wine may be also enhanced by other components of the inherent present in wine, as low pH.

Table 1: Antimicrobial activities, expressed as diameters of inhibition zone (mm) of selected Vranac wines and referents antibiotics: streptomycin (Strep), tetracycline (Tet) and chlorphenicol (Chlor) against six Gram (+) - and six Gram (-) strains

Microorganisms	VW1	VW2	VW3	Strep	Tet	Chlor
Gram (+) strain						
<i>C. perfringens</i>	13.6±0.2	12.4±0.5	12.1±0.9	nt	29.0±0.7	nt
<i>B. subtilis</i>	13.8±0.7	14.2±0.7	13.5±0.5	nt	23.9±0.9	26.0±0.8
<i>S. aureus</i>	14.5±0.9	14.9±0.4	14.2±0.7	nt	18.5±0.3	25.0±0.4
<i>L. innocua</i>	13.0±0.8	12.9±0.3	12.0±0.6	nt	18.7±0.2	18.0±0.9
<i>S. lutea</i>	14.4±0.2	15.2±0.4	14.5±0.5	nt	20.0±0.2	38.0±0.8
<i>M. flavus</i>	13.4±0.8	12.7±0.2	12.3±0.6	nt	23.6±0.7	35.0±0.6
Gram (-) strain						
<i>E. coli</i>	16.8±0.8	13.5±0.9	12.8±0.5	16.0±0.4	23.2±0.2	nt
<i>P. aeruginosa</i>	14.4±0.3	13.2±0.8	12.6±0.6	23.0±0.7	20.8±0.5	nt
<i>S. enteritidis</i>	16.0±0.7	13.6±0.9	12.2±0.3	18.0±0.9	23.3±0.3	nt
<i>S. sonnei</i>	14.9±0.5	14.0±0.8	13.7±0.6	19.0±0.8	31.1±0.8	nt
<i>K. pneumoniae</i>	12.2±0.2	12.8±0.9	12.1±0.2	nt	23.6±0.6	nt
<i>P. vulgaris</i>	14.7±0.4	13.6±0.3	12.1±0.2	nt	19.2±0.5	nt

Notice: the term nt deals with not detected

Table 2: Concentrations of phenolic constituents - gallic acid, caffeic acid, (+)-catechin, resveratrol, quercetin and quercetin-3-glucoside - determined by HPLC analysis, and total phenolic content, determined by UV/VIS analysis (mg/L), of selected Vranac wines

Wine code	Gallic acid	Caffeic acid	Catechin	Resveratrol	Quercetin	Quercetin-3-glucoside	Malvidin-3-glucoside	Total phenolics
VW1	79.67±0.47	6.59±0.91	27.66±0.13	0.82±0.12	2.54±0.12	9.39±0.32	232.96±0.12	3710.18±0.72
VW2	64.33±0.67	3.03±0.55	27.61±0.97	0.55±0.11	1.47±0.12	5.36±0.15	91.35±0.17	3605.02±0.27
VW3	47.53±0.34	2.95±0.88	18.35±0.76	0.41±0.09	1.12±0.13	2.88±0.57	53.98±0.11	3476.10±0.21

Table 3: Antimicrobial activity, expressed as MIC/MBC (µl/mL) of selected Vranac wines against six Gram (+) - and six Gram (-) strains

Microorganism	VW1	VW2	VW3	Strep	Tet	Chlor
Gram (+) strain						
<i>C. perfringens</i>	125/125	125/125	125/125	nt	0.9/0.9	1/8
<i>B. subtilis</i>	250/250	250/250	250/250	nt	0.9/0.9	8/8
<i>S. aureus</i>	125/125	125/125	125/125	nt	0.12/0.9	2/16
<i>L. innocua</i>	62.5/62.5	125/125	125/125	nt	0.46/0.9	8/18
<i>S. lutea</i>	125/125	250/250	125/125	nt	0.06/0.06	0.5/2
<i>M. flavus</i>	125/125	125/125	125/125	nt	0.4/0.9	1/1
Gram (-) strain						
<i>E. coli</i>	250/250	250/250	250/250	16/16	3.8/7.5	nt
<i>P. aeruginosa</i>	125/125	250/250	125/125	8/8	7.5/7.5	nt
<i>S. enteritidis</i>	125/125	250/250	250/250	4/4	0.9/1.9	nt
<i>S. sonnei</i>	125/125	250/250	250/250	16/16	0.06/0.12	nt
<i>K. pneumoniae</i>	125/125	250/250	250/250	nt	0.9/1.9	nt
<i>P. vulgaris</i>	62.5/62.5	125/125	125/125	nt	1.9/1.9	nt

SD ± 0.2

In this study, we examined the relative importance of separated wine phenolic components as well as: gallic acid (main and referent component of hydroxybenzoic acids), caffeic acid (main component of hydroxycinnamic acids), (+)-catechin (main and referent component of flavan-3-ols), resveratrol (main component of stilbens), quercetin and quercetin-3-glucoside (main components of flavonols and flavonols glucosides) and malvidin-3-glucoside (main and referent component of anthocyanins) to the antimicrobial activity of same variety wines from Balkan region.

As compared dates presented of Tables 1 and 2, it is clear that VW3 sample, which contained the lowest concentrations of phenolic compounds shows the lowest inhibition zones against all tested bacteria.

In Table 4 are given the correlation coefficients between concentrations of gallic acid, (+)-catechin, resveratrol, quercetin, quercetin-3-glucoside and malvidin-3-glucoside, determined by HPLC analysis and antimicrobial activity in tested red wine samples.

Table 4: Correlation coefficients ($r \pm sd + p$) between concentrations of gallic acid, caffeic acid, (+)-catechin, resveratrol, quercetin, quercetin-3-glucoside and malvidin-3-glucoside (mg/L) determined by HPLC-analysis and total phenolic content, determined y UV/VIS with antimicrobial activities (mm) in selected Vranac wines

Micro-organisms	$r \pm sd + p$ vs Gallic acid	$r \pm sd + p$ vs Caffeic acid	$r \pm sd + p$ vs Catechin	$r \pm sd + p$ vs Resveratrol	$r \pm sd + p$ vs Quercetin	$r \pm sd + p$ vs Quercetin-3-glucoside	$r \pm sd + p$ vs Malvidin-3-glucoside	$r \pm sd + p$ vs Total phenolics
Gram (+)								
<i>C. perfringens</i>	0.936±0.395	0.985±0.191	0.658±0.845	0.988±0.170	0.999±0.055	0.981±0.219	0.999±0.010	0.924±0.428
<i>B. subtilis</i>	0.451±0.443	0.063±0.495	0.819±0.284	0.257±0.479	0.156±0.491	0.300±0.473	0.117±0.493	0.479±0.435
<i>S. aureus</i>	0.451±0.443	0.063±0.495	0.819±0.285	0.257±0.479	0.156±0.491	0.300±0.473	0.116±0.492	0.479±0.435
<i>L. innocua</i>	0.919±0.308	0.592±0.627	0.996±0.067	0.816±0.448	0.753±0.512	0.842±0.420	0.727±0.535	0.930±0.284
<i>S. lutea</i>	0.101±0.614	0.580±0.502	0.393±0.566	0.292±0.589	0.389±0.567	0.249±0.597	0.142±0.557	0.056±0.615
<i>M. flavus</i>	0.983±0.143	0.940±0.268	0.781±0.492	0.999±0.019	0.992±0.101	0.999±0.015	0.986±0.132	0.977±0.167
Gram (-)								
<i>E. coli</i>	0.927±1.135	0.989±0.437	0.639±2.324	0.984±0.534	0.997±0.224	0.975±0.666	0.999±1.105	0.914±1.225
<i>P. aeruginosa</i>	0.977±0.278	0.951±0.401	0.759±0.843	0.999±0.011	0.996±0.122	0.996±0.071	0.991±0.173	0.969±0.319
<i>S. enteritidis</i>	0.984±0.478	0.938±0.941	0.784±1.687	0.999±0.082	0.991±0.363	0.999±0.038	0.985±0.469	0.970±0.564
<i>S. sonnei</i>	0.953±0.267	0.975±0.195	0.697±0.633	0.995±0.088	0.999±0.003	0.999±0.127	0.999±0.038	0.943±0.294
<i>K. pneumonia</i>	0.158±0.528	0.363±0.498	0.606±0.425	0.049±0.534	0.152±0.529	0.004±0.535	0.191±0.525	0.190±0.525
<i>P. vulgaris</i>	0.904±0.542	0.829±1.031	0.999±0.065	0.964±0.491	0.730±0.866	0.823±0.720	0.916±0.740	0.999±0.055

* $p < 0.005$

It was found the excellent correlation coefficients between contents of gallic acid, caffeic acid, resveratrol, quercetin, quercetin-3-glucoside and malvidin-3-glucoside and the antimicrobial activity of wine samples against *C. perfringens* and *M. flavus* (from 0.936 to 0.999) and between contents of gallic acid and (+)-catechin against *L. innocua* (0.919 – 0.996). Also, contents of gallic acid, caffeic acid, resveratrol, quercetin, quercetin-3-glucoside and malvidin-3-glucoside showed excellent correlation with antimicrobial activity of wine samples against all Gram-negative bacteria stains: *E. coli*, *P. aeruginosa*, *S. enteritidis*, *S. sonnei* and *P. vulgaris* (from 0.904 to 0.999), except against *P. pneumonia*. Then, the content of (+)-catechin has good correlation with antimicrobial activity of tested wines only against *P. vulgaris*. All selected wine phenolic compounds do not show correlation with the antimicrobial effectiveness against *K. pneumonia* strain.

Total phenolic content of investigated Vranac wines, also shown very good correlation with Gram (-) - bacterial strains: *C. perfringens*, *L. innocua* and *M. flavus* (from 0.924 to 0.977) and with all Gram (+) - bacterial strains (from 0.914 to 0.999), except against *K. pneumonia* strain.

CONCLUSION

The concentrations of phenolic compounds are very important for understanding biological potency of red wines. This study confirmed that high levels of phenolic compounds in Vranac

wines contribute to their high antimicrobial effectiveness. It can be concluded that the very good correlations exhibited between the contents of some wine phenolic constituents and the antimicrobial activities of Vranac wines against six Gram-positive and six-Gram negative pathogenic bacterial strains. Development of alternative regimen using natural compounds as well as grape/wine phenolic compounds for the prevention and control of infections caused by bacteria resistant to antibiotics is very important for human health.

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