THE KINETICS OF ALCOHOLIC FERMENTATION, PHENOLIC CONTENT, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF THE WINE OBTAINED FROM PLOVDINA GRAPE WITH THE ADDITION OF AROMATIC HERBS

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The effects of the addition of aromatic herbs on the kinetics of alcoholic fermentation, as well as phenolic composition, antioxidant and antimicrobial activity of Plovdina red wine were studied. Aromatic herbs: anise seeds (*Pimpinella anisum* L.), cinnamon bark (*Cinnamomum zeylanicum*), wormwood leaf (*Artemisia absinthium*) and licorice root (*Glycyrrhiza glabra)* were added to Plovdina pomace at the beginning of fermentation. It was observed that the content of total phenolic compounds and flavonoids increased with the addition of aromatic herbs. The highest antioxidant activity was found in Plovdina wine with cinnamon (EC_{50}=0.023±0.001 mg/ml), where as Plovdina control wine (EC_{50}=0.067±0.0006 mg/ml) had the lowest antioxidant activity. All analysed wine samples expressed the antimicrobial activity against G (+) bacteria *Bacilussubtilis*. However, they did not showany activity against Gram(-) bacteria: *Escherichia coli*, *Salmonella typhimurium or, Candida albicans* yeast. Non-flavonoid compounds (benzoic acid and cinnamic acid derivatives), as well as flavonoids (flavan-3-ols, flavonols, flavanones and anthocyanins) were all identified by means of HPLC-DAD analysis.

Keywords: Plovdina wine, aromatic herbs, kinetics, HPLC-DAD, antioxidant activity, antimicrobial activity

Introduction

Phenolic compounds are wine components that affect not only sensoric characteristics of wine (color, astringency, bitterness) but also many physiological characteristics that have a positive effect on human health (reducing the risk of cardiovascular and neurodegenerative diseases, diabetics and other). Phenolic compounds are considered to be the strongest and most active antioxidant compounds of wine [1]. In wine phenols, there are two main groups: non-flavonoids (derivatives of hydroxybenzoic and hydroxycinnamic acids) and flavonoids (anthocyanins, flavanols, flavonons, and dihydroflavonols).

The antioxidant activity of polyphenols found in wine is the subject of many researchers because of its benign influence on human health and sensory characteristics of wine. Recently, there has been more and more insistence on the increased intake of antioxidants through food because they have a role in reducing free radicals, and that is how the oxidation of molecules in the series of reactions is prevented. Besides the importance in the pharmaceutical industry, the research shows that aromatic herbs have some important antioxidants. Natural products from aromatic herbs also have a wide range of use in food industry, especially for aromatization of spirit drinks and aromatized wines (vermouth, bermet) [2].

In the paper, we have examined the influence of aromatic herbs (anise seeds – *Pimpinella anisum*, the cinnamon bark-*Cinnamomum zeylanicum*, the leaf of wormwood *Artemisia absinthium* and the root of liquorice-*Glycyrrhiza glabra*) on the kinetics of fermentation, phenolic content, antioxidant and antimicrobial activity of red wine obtained from autochthonous variety of Plovdinagrape.

Experimental

Herbal material

Grapes of the grapevine variety Plovdina (*Vitis vinifera* L.) were grown in the Toplica region (Jug Bogdan vineyards, 43° 13' N/21° 42' E, at an altitude of 250- 400 m).In the pomace grape, anise seed (*Pimpinella anisum* L.), cinnamon bark (*Cinnamomum zeylanicum*), wormwood leaf (*Artemisia absinthium*), and liqoriceroot (*Glycyrrhi zaglabra*) were added (1% w/w). Herbs were purchased in a specialized herbal store from Adonis d.o.o. SokoBanja, (Serbia) and finely ground before use.Wines were labeled as follows: PL-W - Plovdina red wine; PL-AW - Plovdina wine with anis; PL-CW - Plovdina wine with cinnamon; PL-WW - Plovdina

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wine with wormwood; PL-LW - Plovdina wine with liquorice. Upon completion of the fermentation, wines were decanted from the lees, bottled and stored at 5-6 °C. After six months of bottle aging, wines were subjected to chemical analyses.

Chemicals

All the chemicals used were of analytical grade: Folin-Ciocalteu (FC) reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl), gallic acid, p-vanillin (Alfa Aesar, Germany). syringic acid, protocatechuic acid, ferulic acid, coumaric acid, sinapinic acid, caffeic acid, catechin, epicatechin, rutin, quercetin, hyperosid, naringin, naringenin, malvidin-3-glucoside, methanol, H_2SO_4, (Merck, Darmstadt, Germany). Starch, $NaNO_2$, NaOH, $K_2S_2O_5$, $KMnO_4$ and active coal were obtained from Centrohem (Stara Pazova, Serbia); $CuSO_4$ from mp Chemistry, doo, Belgrade, Serbia); iodine, from NRK Inženjering, Belgrade, Serbia). Standard substances for HPLC were purchased from Sigma chemicals (St. Louis, MO, USA); absolute ethanol and HCl from Merck (Darmstadt, Germany), Muller-Hinton agar, Sabouraud agar (Institute for immunology and virusology, Torlak, Belgrade, Serbia).

For the antimicrobial analysis, the following microorganism strains were used: *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Clostridium perfringens* ATCC 19404, *Enterococcus faecalis* ATCC 19433, *Sarcina lutea* ATCC 9341, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076, *Escherichia coli* ATCC 11775, *Proteus mirabilis* ATCC 12453, *Enterobacter aerogenes* ATCC 13048.

Methods

Fermentation

The alcoholic fermentation of the grape pomace was carried out with the addition of yeast *Saccharomyces cerevisiae* Lalvin 1116 (0.30 g/L), at the temperature of 25 °C (5 kg of the grape pomace in each separate Erlenmeyer flask of 5 L volume). Sulphitation of pomace was done by adding of 5% solution of potassium metabisulfite (0.10 g/L). Kinetics of alcoholic fermentation of the grape pomace was monitored by measuring the quantity of released carbon dioxide. The total weight of the samples was determined daily, and the weight loss resulting from CO_2 release was measured using a technical scale EK-1200 A (A&D Japan: accuracy ± 0,1 g; maximum capacity: 1200 g).

Determination of total phenols

The total content of phenols was determined spectrophotometrically by the Folin–Ciocalteu method using Gallic acid as a standard substance [3]. The concentration of total phenolic compounds was shown in mg/l as the of gallic acid equivalent (GAE).

Determination of total flavonoids

The total flavonoids content was determined spectrophotometrically according to aluminum–chloride method [4], and was expressed as catechin equivalent (CE/l).

Determination of total anthocyanins

The total anthocyanins content was determined spectrophotometrically at 520 nm according to Somers and Evans-method [5].Anthocyanins content was expressed in mg/L using malvidin-3-glucoside as a standard.

Determination of antioxidant activity

The scavenging activity of the wine was determined by the DPPH method described elsewhere [6].The capability to scavenge the DPPH free radical (%) was calculated by the equation:

Capacity of neutralizing of DPPH radicals (%)=100- [(As-Ab) 100/Ac]

As- absorbance of 'sample' at 517 nm (2.5 cm^3 of wine of different diluteness treated with 1 cm^3 of the solution of DPPH radicals).

Ab – absorbance of 'blank' at 517 nm (2.5 cm^3 of wine of different diluteness in 1cm^3 of methanol)

Ac – absorbance of 'control' at 517 nm (1 cm^3 of the solution of DPPH radicals of the concentration 3x10-4 mol/dm^3 in 2.5 cm^3 of methanol).

Efficient concentration values (EC_{50}) were calculated according to experimental data by using a nonlinear regression model and Sigma Plot 2000 Software(trial version).

HPLC-DAD chromatography

The analysis of phenolic compounds of wine was done by HPLC (High-Performance Liquid Chromatography, Agilent 1200 Series with UV-DAD Detector). The analysis was carried out by the original method [7]. Zorbax – Eclipse XDB-C 18 column was used at the volume of an injected sample of 20 µl, while chromatograms were recorded at the wavelength of 360 nm. As a mobile phase, a system of solvents was used: A- 1% formic acid in the water solution and B- methanol at the flow of 0.5 ml/min and by applying the following elution profile: 0-5 min: 70% A and 30% B; 5-20 min 30% A and 70% B; 20-25 min 10% A and 90% B.

Determination of the antimicrobial activity of wine

The antimicrobial activity of wine was determined by disc-diffusion method [8] and microdilution method [9].

Disc-diffusion method

Wine samples were tested in vitro on panel strains of microorganisms which belong to the collection of microorganisms: ATTC (American Type of Culture Collection). Standard strains of microorganisms were obtained from the Institute of Immunology and Virology, Torlak, Belgrade. The examination of antimicrobial activity of wine was done with the following strains of microorganisms: a Gram (+) bacterium: *Bacillus subtilis* ATCC 6633, Gram (-) bacteria: *Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC 14028 and yeast: *Candida albicans* ATCC 10231. The obtained results were compared to the activity of antibiotics (Chloramphenicol and Streptomycin). Chloramphenicol (30 µg/disc) and Streptomycin (10 µg/disc) and Nystatin (30 µg/disc) were used as a control of the sensitivity of the tested microorganism.

For the microbiological analysis, the following panels

were used: Muller-Hinton agar for cultivating G (+) and G (-) bacteria and Sabouraud agar for cultivating *Candida albicans.*

All agar panels were prepared in Petri dish (90 mm) with agar (10 ml). Disk agar panels were inoculated with the suspension of tested microorganisms (100 µl). Sterile disks of filter papers (Antibiotica Test Blattchen", Schleicher and Schuell, Dassel, Germany, of 12.7 mm diameter) were soaked into each wine sample (50 µl) and placed on the inoculated panels. Petri dish with inoculated agar and wine samples were incubated (at 37 °C for 24 h for bacteria and at 28 °C for 48 h for fungi).

Standard disks (6 mm diameter) on Chloramphenicol, Streptomycin and Nystatin (30, 10 and 30 µg of active components, respectively), were used as positive controls. The diameters (in mm) of the zones of sample inhibitions were measured and compared to the diameters of the zones of standard inhibitions (antibiotics).

Microdilution method

The antimicrobial activity of the wine sample was determined by microdilution methodby using microtiter plates with 96 conoidal cavities. By microdilution method minimal inhibitor (MIC) the concentration was determined and the minimal bacterial concentration (MBC) according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003).

For the analysis, the following microorganisms were used:

Gram (+): *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Clostridium perfringens* ATCC 19404, *Enterococcus faecalis* ATCC 19433, *Sarcinalutea* ATCC 9341,

Gram (-): *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076, *Escherichia coli* ATCC 11775, *Proteus mirabilis* ATCC 12453, *Enterobacte raerogenes* ATCC 13048

The cavities were filled with inoculated liquid nutritional medium into which the series of diluted samples were added. The wine was diluted in the liquid panel and added to microtitar plates in Mueller- Hinton broth (the volume of 100 ml and the concentration of 10^6 CFU/ml) and the plates were incubated for 24 h at 37 °C. After the incubation, the microbiological growth was determined by a universal reader for microtiter plates at the absorbance of 620 nm. The microbiological growth was determined by adding triphenyltetrazolium chloride (20 ml, 0.5%). MIC represents the lowest concentration at which microorganisms show a visible growth. For MBC determination, broth from each plate was taken and it was inoculated on Mueller-Hinton agar (37 °C, 24h). MBC is the lowest concentration of the extracts at which 99.9% of inoculated bacterial cultures were killed.

Statistical analysis

All the experiments were carried out in triplicate, and the results were shown as the average value±standard deviation. The processing of experimental data was done by Origin 8.0, Microsoft Excel 2007 and Sigma Plot Trial 2000. Significant differences were obtained by the analysis of variance ANOVA.

Result and discussion

Kinetics of alcoholic fermentation

Kinetics of alcoholic fermentation of the grape pomace Plovdina, with the addition of aromatic herbs at 25 °C is shown in Figure 1. In all samples a lag phase lasted for two days, and maximal CO_2 productivity was obtained four days after yeast inoculation. The exponential phase lasted from the second to the fourth day after which CO_2 production rate strongly decreased.

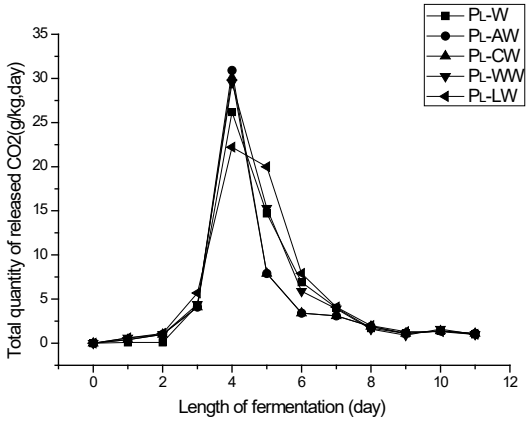

Figure 1. Kinetics of alcoholic fermentation of the grape pomace of Plovdina at 25 °C

The values of CO_2 production rate during the exponential phase are shown in Table1.

Table 1. The value of CO_2 production rate during the exponential phase

Wine sample	Production rate, g/dm^{3}day
Plovdina, (P_L-W)	10.2±0.94
Plovdina-anise, (P_LA-W)	12.0 ±0.84
Plovdina-cinnamon,(P_LC-W)	11.7±0.86
Plovdina-wormwood, (P_LW-W)	11.6±0.95
Plovdina-licorice, (P_LL-C)	9.7±0.96

CO_2 production rate is used indirectly as a measure of the fermentable sugars rate consumption. On the assumption that the correlation between the yeast growth rate and the sugar consumption rate is established, CO_2 production rate of the yeast growth rate can be measured.

The addition of anise and cinnamon decreased the total quantity of the released CO_2. The total quantity of the released CO_2 was 1.6, 15.6 and 16.7% lower when wormwood, anise and cinnamon were added, respectively. The addition of cinnamon and anise reduced the speed of alcoholic fermentation as the result of the antifungal effect on the yeast *Sacharomyces cerevisiae*, and reduced the rate of CO_2 extraction. [10]

During the first two days of fermentation (lag phase), only a small quantity of alcohol was produced, and after that, in the exponential phase of growth, the highest quantity of alcohol was produced. In that phase, the yeast cells divide intensively which leads to the extreme increase of the yeast biomass and to the dissolution of sugar form of the grape pomace to carbon dioxide and ethanol, with releasing the energy needed for their growth.The exponential phase lasts from the second to the fourth day. After that, the stationary phase appears and in the end, the phase of dying out of yeast when the quantity of the produced alcohol also decreases. The obtained alcohol has the inhibitory effect on the yeast cells thus stopping them to grow and they start to dye out. In the end, this leads to finishing the alcoholic fermentation.

Total phenolic compounds, total flavonoids, and total anthocyanins

The content of total phenolic compounds, total flavonoids, and total anthocyanins in Plovdina wine samples is presented in Table 2.

Table 2. The content of total phenolic compounds, total flavonoids, and total anthocyanins in Plovdina wine samples.

	Total phenolic compounds mg GAE/L	Total flavonoids mgCTE/L	Total antocyanins mg/L
P_L-W	225.7±2.45	150.3±0.74	14.3±0.26
P_LA-W	266.1±2.85	168.7±0.36	26.4±0.30
P_LC-W	370.4±2.11	263.9±0.30	25.6±0.25
P_LW-W	282.7±2.03	161.1±1.05	15.6±0.21
P_LL-W	304.1±2.31	162.4±0.62	33.9±0.21

The total phenolic compounds content was in the range from 225.7 mgGAE/L (Plovdina wine) to 304.1 mgGAE/L (Plovdina wine with cinnamon), total content flavonoids was in the range from 150.3 mgCTE/L (Plovdina wine) to 263.9 mgCTE/L showed Plovdina wine with cinnamon while the highest content of antocyanins showed Plovdina wine with liquorice (33.9 mg/L).

Antioxidant activity of wine

Neutralizing power of wine against DPPH radicals has been shown as the EC_{50} value that represents the concentration of the wine that neutralizes 50% of DPPH radicals [11]. The content of total phenolic compounds and antioxidant activities (EC_{50}) of Plovdina wine with aromatic herbs is given in Table 3.

The highest antioxidant activity was shown in Plovdina wine with cinnamon (EC_{50}=0.023±0.0011ml/ml) and with liqorice (EC_{50}=0.024±0.0017ml/ml), whereas the lowest antioxidant activity was shown in Plovdina wine without aromatic herbs(EC_{50}=0.067±0.0006 mg/ml).

Table 3. Total phenolic content (TPC) and antioxidant activities (EC_{50})

	TPC,mg GAE/l	EC_{50}, mg/ml
P_L-W	225.7±2.45	0.067±0.0006
P_LA-W	266.1±2.85	0.058±0.0036
P_LC-W	370.4±2.11	0.023±0.0011
P_LW-W	282.7±2.03	0.040±0.0013
P_LL-W	304.1±2.31	0,024±0,0017

Correlation of the content of total phenolic compounds in wine and antioxidant activities

In order to establish the relation between the content of total phenolic compounds and the antioxidant activity, a correlation analysis was done. In the series of experiments [12,13], a high level of positive correlation of the content of phenolic compounds and antioxidant activities of wine were observed. The influence of adding aromatic herbs, i.e., the content of total phenolic compounds on the antioxidant activity was presented by positive correlation. A regression model was shown by a negative sign with parameter b which shows that with the increase of phenolic matters, the EC_{50} value decreases, meaning that with the increase of the content of total phenolics,the antioxidant activity increases as well.The coefficient of linear regression of Plovdina wine with aromatic herbs is R=0.86(Figure 2). That is why there is a relatively high correlation between total phenols and the antioxidant activity.

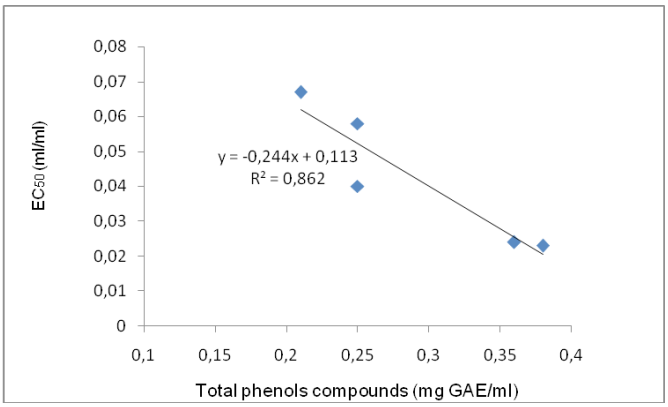

Figure 2. Correlation between total phenolic compounds and antioxidative activity of wine Plovdina with the addition of aromatic herbs

Microbiological assays

The wine obtained from the autochthon sort of Plovdina wine with cinnamon showed the highest bactericidal activity to Gram (+) bacterium *Bacillus subtilis* (50.0% of activities of Chloramphenicol, that is, 42.9% of Streptomycin). Plovdina with wormwood showed a bacteriostatic activity to Gram (+) bacterium *Bacillus subtilis* (46.7% of activities of Chloramphenicol, that is, 40.0% of Streptomycin). Plovdina with anise showed a bactericidal activity to *Bacillus subtilis* (43.3% of activities of Cholamphenicol, and 37.1% of Streptomycin).

The obtained results indicated that wines Plovdina

and Plovdina with aromatic herbs only showed the antimicrobial activity to G (+) *Bacillus subtilis* ATCC 6633, and they did not show the activity to Gram (-): *Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028 and yeast *Candida abicans* 10231.

Antibiotics Streptomycin and Chloramphenicol were used as the positive control. Streptomycin had a bactericidal activity tothe bacterial strain *Bacillus subtilis* with the inhibition zone of 35 mm, to *Escherichia coli* ATCC 8739 with the inhibition zone of 19 mm, tothe yeast *Candida albicanis* ATCC 10231 with the inhibition zone of 19 mm, while it did not have the antimicrobial activity to the bacterial strain *Salmonella typhimurium* ATCC 14028.

Chloramphenicol had the bactericidal influence on *Bacillus subtilis* with the inhibition zone of 30mm, on *Escherichia coli* ATCC 8739 with the inhibition zone of 30mm, and also on yeast *Candida albicans* ATCC 10231 with the inhibition zone of 33mm. It did not show the antimicrobial activity on Gram (-) bacterial strain *Salmonella typhimurium*. The results of microbiological analysis by Disk-diffusion method of Plovdina with aromatic herbs are shown in Table 4.

Table 4. The results of microbiological analysis by Disk-diffusionmethod of Plovdina with aromatic herbs

Microorganism	*B. subtilis* ATCC 6633		*E. coli* ATCC 8739		*S. typhimurium* ATCC 14028		*C. albicans* ATCC 10231	
Effect	BC	BS	BC	BS	BC	BS	BC	BS
P_L-W	/	/	/	/	/	/	/	/
P_LA-W	/	13	/	/	/	/	/	/
P_LC-W	15	/	/	/	/	/	/	/
P_LW-W	/	14	/	/	/	/	/	/
P_LL-W	/	/	/	/	/	/	/	/
Streptomycin	35	/	19	/	/	/	19	/
Chloramphenicol	30	/	30	/	/	/	33	/
Nystatin	Nd.	Nd.	Nd.	Nd.	Nd.	Nd.	18	Nd.

BC- bactericidal, BS- bacteriostatic, Nd.- not defined

Table 5. The results of microbiological analysis by microdilution method of Plovdina with aromatic herbs

SAMPLES	P_L		P_LA-W		P_LC-W		PLW-W		P_LL-W		Chloramphenicol		Streptomycin	
Strains	µL/mL										µg/mL			
S. aureus ATCC 25923	250	500	250	500	250	500	250	500	250	250	1.0	8.0	1.0	1.0
B. cereus ATCC 11778	125	>500	125	>500	125	500	125	500	125	250	1.0	4.0	0.5	0.5
C. perfringens ATCC 19404	125	125	125	125	125	250	125	125	125	250				
E. faecalis ATCC 19433	31	62	31	62	31	62	15	62	31	62	2.0	4.0	4.0	4.0
S. lutea ATCC 9341	125	250	125	250	125	250	125	250	125	250				
Gram (-)														
P. aeruginosa ATCC 9027	125	125	125	125	125	125	125	125	125	125	1.0	4.0	8.0	8,0
S. enteritidis ATCC 13076	125	250	250	250	125	250	125	250	125	250	4.0	8.0	4.0	4.0
E. coli ATCC 11775	250	250	250	250	250	250	250	250	125	125	128	512	8.0	8.0
P. mirabilis ATCC 12453	125	250	125	250	125	250	125	250	62	250	4.0	64	128	256
E. aerogenes ATCC 13048	125	500	125	500	125	500	125	500	125	250	1.0	1.0	0.5	0.5

Microdilution method

The results of microbiological analysis by microdilution method of Plovdina with aromatic herbs are shown in Table 5.

The minimal inhibitory concentrations of Plovdina and Plovdina with aromatic herbs to Gram (+) *Staphylococcusaureus* ATCC 25923 are 250 ml/mL for all wine samples, while the minimal bactericidal concentration was between 250-500 mL/mL. Wine samples had a stronger antimicrobial influence on *Bacillus cereus* ATCC 11778, where MIC was 125ml/mL and MBC was between 250-500 mL/mL. For the samples of Plovdina and Plovdina with anise, MBC had bigger values than 500 mL/mL.

ATCC 19404 MIC was determined with the influence of wine samples on *Clostridium perfringen sand* it was 125 mL/mL, while MBC was between 125-250 mL/mL.

The most sensible G(+) bacterium was *Enterococcus faecalis* ATCC 19433. MIC was between 15 mL/mL for Plovdina with wormwood and 31 mL/mL for all other wine samples, while MBC was 62mL/mL.

For G(+) *Sarcina lutea* ATCC 9341 MIC was obtained for all wine samples (Plovdina with aromatic herbs) and had the value of 125 mL/mL, while MBC had the value of 250 mL/mL.

G (-) bacteria also showed higher resistance in comparison to G (+) bacteria at the influence on Plovdina with aromatic herbs.

Under the influence of the wine samples' on G (-) bacterial strain *Pseudomonas aeruginosa* ATCC 9027, MIC with the value of 125 mL/mL was obtained. *Escherichia coli* ATCC 11775 was more resistant and had MIC of 125-250 mL/mL, and MBC was between these values as well. The bacteria with MIC of 62.5-125 ml/mL had the highest antimicrobial value for G (-), while the values for MBC were between 125-250 mL/mL. For *Enterobacter aerogenes*, ATTC 13048 values for MIC were 125 mL/mL while the values for MBC were between 250-500 mL/mL

HPLC-DAD analysis of Plovdina wine

Based on retention times and the maximum absorbance of standard compounds,16 phenolic compounds were identified in Plovdina wine by means of HPLC-DAD analysis. Benzoic acid and cinnamic acid derivatives (non-flavonoid compounds), as well as flavonoids (flavan-3-ols, flavonols, flavanones and anthocyanins), were identified. As for hydroxybenzoic acids, the following ones were also identified: gallic acid, syringic acid, protocatechuic acid and dihydroxybenzoic acid. With the addition of aromatic herbs, the content of total hydroxybenzoic acids increased. The lowest content of total benzoic acids was found in Plovdina wine (8.68% out of total identified phenolic compounds), then Plovdina wine with the addition of anise (6.93%), Plovdina wine with the addition of liquorice (8.58%) and the wine to which wormwood was added (8.73%),where as Plovdina wine to which cinnamon was added had the highest content of total hydroxybenzoic acids (14.68%).

Table 6. HPLC-DAD profile of Plovdina wine

PHENOLIC COMPOUNDS, (%)	P_L-W	P_LA-W	P_LC-W	P_LW-W	P_LL-W
Nonflavonoidphenolics					
Hydroxybenzoic acids					
Gallic acid	1.99	1.45	6.60	1.90	1.32
Syringic acid	1.09	0.99	1.28	1.09	1.97
Protocatechuic acid	3.43	2.20	4.10	3.31	2.62
Dihydroxy-benzoic acid	2.17	2.29	2.70	2.43	2.67
Total hydroxybenzoic acids	**8.68**	**6.93**	**14.68**	**8.73**	**8.58**
Hydroxycinnamic acids					
Ferulic acid	3.06	1.17	1.02	1.19	1.53
Coumaric acid	4.53	3.17	3.89	2.76	1.92
Sinapinic acid	1.77	1.73	1.42	1.69	1.39
Caffeic acid	3.08	3.06	2.68	2.94	2.61
Total hydroxycinnamic acids	**12.44**	**9.13**	**9.01**	**8.58**	**6.94**
Sum of individual nonflavonoidphenolics	21.12	16.06	23.69	17.31	15.52
Flavonoids					
Flavan-3-ols					
(+)-Catechin	2.33	2.58	2.97	2.70	1.94
(-)-Epicatechin	2.15	2.15	2.76	2.41	2.56
Total flavan-3-ols	**4.48**	**4.73**	**5.73**	**5.11**	**4.50**
Flavonols					
Rutin	1.86	0.61	0.93	0.86	1.98
Hiperozid	2.52	1.93	1.60	2.00	2.14
Myricetin	0.73	0.55	0.55	0.49	1.64
Total flavonols	**5.11**	**3.09**	**3.08**	**3.35**	**5.76**
Flavanones					
Naringin	2.92	2.97	2.64	3.08	4.64
Naringenin	0.00	0.21	0.15	0.30	1.43
Total flavanons	**2.92**	**3.18**	**2.79**	**3.38**	**6.07**
Anthocyanin					
Malvidin-3-glucoside	**0.00**	**0.91**	**0.00**	**0.00**	**0.6**
Sum of individual phenolic compounds	**33.63**	**27.97**	**35.29**	**29.15**	**32.45**

As for hydroxycinnamic acids, the following ones were identified: ferulic acid, coumaric acid, sinapinic acid and also caffeic acid. The addition of aromatic herbs led to the alteration of the content of hydroxycinnamic acids. Plovdina wine with the addition of cinnamon contained 9.01% of total hydroxycinnamic acids, whereas Plovdina wine (control sample) contained 12.44% of total hydroxycinnamic acids.

As for flavan-3-ols, catechin and epicatechin were identified. The lowest content of flavan-3-olswas found in Plovdina wine (4.48%), whereas the highest content of flavan-3-ols was found in Plovdina wine to which cinnamon was added (5.73%). The addition of aromatic herbs led to the alteration of the content of (+)-catechins compared to (-)-epicatechin. InPlovdina wine, this ratio was2.33: 2.15% in favor of catechin, whereas in the wine with the addition of licorice, this ratio was 1.94:2.56% in favor ofepicatechin.

The alteration of the content of flavanones (naringin and naringenin) was observed in Plovdina wines

with the addition of aromatic herbs. The lowest content was found in Plovdina wine to which anise was added (2.79%), whereas the highest content was found in Plovdinawine to which licorice was added (6.07%).

Plovdina wine to which cinnamon was added contained the highest percentage of identified phenolic compounds (35.29%), whereas Plovdina wine with the addition of anise contained the lowest percentage of identified total phenolic compounds (27.97%).

Conclusion

The addition of anise, cinnamon and liqorice in Plovdina wine leads to the increase of the content of total phenolic compounds, flavonoids, and anthocyanins. Plovdina wine with the addition of liquorice had the lowest content of total phenols and flavonoids, while the highest content of total phenols and flavonoids was determined in Plovdina with the addition of cinnamon 382.3 (mg GAE/l) and 274.4 (mg CTE/l), respectively. Anthocyaninsshowed the same trend, Plovdina control wine had the lowest value (16.1 mg/l), and Plovdina with the addition of liquorice had highest value (35.6 mg/l).

All wines with added aromatic herbs had a higher antioxidant value when compared to Plovdina control wine. The content of total phenolic compounds and the antioxidant activity were positively correlated.

The wine obtained from the autochthonous variety Plovdina with the addition of cinnamon showed the highest bactericidal activity to Gram (+) bacterium *Bacillus subtilis* (50% of activities of Chloramphenicol, that is, 42.9% of activities of Streptomycin). Plovdina with the addition of wormwood showed the bacteriostatic activity towards Gram (+) bacterium *Bacillus subtilis* (46.7% of activities of Chloramphenicol, that is, 40.0% of Streptomycin). Plovdinawine with anise had the bactericidal activity towards *Bacillus subtilis* (43.4% of activities of Chloramphenicol and 37.1% of activities of Streptomycin). The obtained results indicated that wines Plovdina and Plovdina with aromatic herbs showed theantimicrobial activity only to Gram (+) bacterium *Bacillus subtilis* ATCC 6633 and did not show the activity to Gram (-) bacteria: *Escherichia coli* ATCC 8739, *Salmonella typhimurim* ATCC 14028 and yeast *Candida albicans* ATCC 10231.

HPLC assay in Plovdina wine with aromatic herbs was applied to identify the difference in polyphenolic profiles of the studied wines.The wine with the addition of aromatic herbs showedthe increased content of phenolic compounds and a higher antioxidant activity, compared to the control Plovdina wine.

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References

[1] C. Katya, L. Fiordiponti, Colour Evaluation, Bioactive Compound Content, Phenolic Acid Profiles and in Vitro Biological Activity of Passerinadel Frusinate White Wines: Influence of Pre-Fermentative Skin Contact Times. Molecules, 21(2016) 960.

[2] Pokorny, J., Yanishlieva, N. Gordon, M. (2001) Antioxidants in Food. Practical Applications, Woodhead Publishing Limited, Abington Hall, Abington, Cambridge, England.

[3] V.L. Singleton, J. Rossi,Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. American Journal of Enology and Viticulture, 16 (1965) 144-158.

[4] J.Zhishen, T. Mengchneg, W. Jianming, The determination of flavonoids content in mulberry and their scavenging effects on superoxide radicals, Food Chemistry, 64 (1999) 555-559.

[5] G.J. Soleas, G.Tomlinson, M.D. Goldberg, Kinetics of poliphenol Release into WineMust During Fermentation of Different Cultivars, Journal of Wine Research, 9(1) (1998) 27 -41.

[6] W. Brand-Williams, M.E.Cuvelier, C.Berset, Use of a free radical method to evaluate antioxidant activity, LWT - Food Science and Technology, 28 (1995) 25-30.

[7] M.Veit, C. Beckert, C. Hohne, K. Bauer, H. Geiger, Interspecific and intraspecific variation of phenolics in the Equisetum subgenus Equisetum. Phytochemistry, 38 (1995) 881– 891.

[8] J.F. Acar, F.W. Goldstein, Disk susceptibility test. In: Lorian V. (Ed.). Antibiotics in Laboratory Medicine, 4th ed ., Williams § Wilkins, Baltimore (1996) 1-51.

[9] F. Candan, M. Unli, tepe, B. Daferera, D. Polission, Sokmen M, Akpulat A. Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *Mullefolium* Afan. (Asteraceae). Journal of Etheropharmacology, 87 (2003) 215-220.

[10] S.Felšöciová, M. Kačániová, E. Horská, N. Vukovič, L. Hleba, J. Petrová, K. Rovná, M. Stričík, Z. Hajduová, Antifungal activity of essential oils against selected terverticillatepenicillia.Ann Agric Environ Med., 22(1) (2015) 38-42.

[11] D.Villano, M.S. Fernandez-Pachon, M.L. Moya, A.M. Troncoso, M.C. Garcia- Parilla, Radical scavenging ability of phenolic compounds towards DPPH free radical, Talanta, 71 (2007)230–235.

[12] D.Braga de Lima,B.C. Agustini,E. Guimarães da Silva, F.Gaensly, R.B.Cordeiro, M.L.D.Fávero, D. Brand, M.Maraschin, T.M.B.Bonfim, Evaluation of phenolic compounds content and in vitro antioxidant activity of red wines produced from Vitislabruscagrapes.Ciênc.Tecnol. Aliment,Campinas, 31(3)(2011) 793-800.

[13] C.R. Minussi, M. Rossi, L. Bologna, L. Cordi, D. Rotilio, M.G. Patore, N. Duran, Phenolic compounds and total antioxidant potential of comercial wiens. Food Chem. 82 (2003) 409-416.

Izvod

KINETIKA ALKOHOLNE FERMENTACIJE, FENOLNI SASTAV, ANTIOKSIDATIVNA I ANTIMIKROBNA AKTIVNOST VINA PLOVDINE SA DODATKOM AROMATIČNOG BILJA

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U radu je analiziran uticaj dodavanja aromatičnog bilja na kinetiku alkoholne fermentacije, fenolni sastav, antioksidativnu i antimikrobnu aktivnost crvenog vina napravljenog od sorte grožđa Plovdina. Na početku alkoholne fermentacije, u kljuk Plovdine dodato je semeanisa (*Pimpinella anisum* L.), koracimeta (*Cinnamomum zeylanicum*), list pelina (*Artemisia absinthium*) i koren sladića (*Glycyrrhiz aglabra*), u količiniod 1% (m/m). Kinetika alkoholne fermentacije praćena je merenjem izdvojene količine oslobođenog CO_2. Sadržaj ukupnih fenolnih jedinjenja, flavonoida i antocijana određene su spektrofotometrijskim metodama. Antioksidativna aktivnost određena je DPPH metodom a fenolnisastav je određen HPLC-DAD metodom. Dobijeni rezultati ukazuju na povećanje sadržaja ukupnih fenolnih jedinjenja i ukupnih flavonoida u uzorcima vina sa dodatkom aromatičnog bilja. Najveću antioksidativnu aktivnost pokazalo je vino Plovdina sa dodatkom cimeta (EC_{50}=0,023±0,0011 mg/ml) a najmanju vino Plovdina uzeto kao kontrola EC_{50}=0,067±0,0006 mg/ml. Vino Plovdina (kontrola) i vino Plovdina sa dodatkom aromatičnog bilja pokazali su antimikrobnu aktivnost samo prema G(+) bakteriji *Bacilussubtilis*, dokprema Gram (-) bakterijama (*Escherichia coli, Salmonella typhimurium*) i kvasacu *Candida albicans* nije ispoljena antimikrobna aktivnost. HPLC-DAD analizom identifikovana su neflavonoidna jedinjenja (derivati benzoeve i cimetne kiseline) i flavonoidi (flavan-3-oli, flavonoli, flavanoni i antocijani). Dodatak aromatičnog bilja u vino Plovdina doveo je do povećane antioksidativne i antimikrobne aktivnosti vina.

Ključne reči: vino Plovdina, aromatično bilje, kinetika, HPLC-DAD, antioksidativna aktivnost, antimikrobna aktivnost