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Potential Antimicrobial Activity of Red and White Wine Phenolic Extracts against Strains of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*

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Summary

The aim of this study was to assess antimicrobial activities of wine phenolic extracts. The potential antimicrobial activity of alcohol-free red and white wine extracts against pathogenic strains of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* was studied using the agar well diffusion method. Total phenolic content of wine extracts was determined by the Folin-Ciocalteu method, while their phenolic composition was specified by high performance liquid chromatography and diode array detector (HPLC-DAD). The antimicrobial activity of the tested extracts was related to their total phenolic content. Antimicrobial activity of the tested wine extracts was more effective against *S. aureus* and less effective against *E. coli* and *C. albicans*. Also, *C. albicans* was resistant to more wine extracts than the two bacterial species studied. The antimicrobial activity and the phenolic composition of the tested white and red wine extracts indicate that some phenolic acids have the potential to inhibit growth of certain pathogens such as *S. aureus*, *E. coli* and *C. albicans* strains.

Key words: wine, phenolics, antimicrobial, pathogens

Introduction

The increasing antimicrobial resistance of pathogens isolated from humans and animals, combined with the increasing awareness of the consumers on chemical substances used as food preservatives, necessitates research for more efficient antimicrobials with fewer side-effects on human health.

Recently, the antimicrobial effects of various plant extracts against certain pathogens have been reported by a number of researchers (*1–8*). Particularly, polyphenols of plant origin have been reported to have a variety of biological effects, including anti-oxidant, anti-carcinogenic, anti-inflammatory and anti-microbial activities. Specifically some phenolic compounds such as resveratrol, hydroxytyrosol, quercetin and a number of phenolic acids have been reported to inhibit various pathogenic microorganisms (*9–12*). Also, there are recent studies reporting the antimicrobial activities of wines and wine extracts against various pathogens (*13,14*).

Red wines have a higher content of total phenolics and contain a wider spectrum of phenolics than the white wines. Wine phenolics are divided into flavonoids and non-flavonoids. The family of flavonoids includes

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mainly flavonols, flavanols and anthocyanins, whereas the non-flavonoids include mainly phenolic acids (benzoic and hydroxycinnamic acids) and stilbenes. Red wines contain all the above phenolics, while white wines contain mainly phenolic acids and flavonols. The different wine phenolic content is attributed to the different phenolic composition of red and white grapes and to different wine making procedures. Red wine production process includes the procedure of maceration, which is not applied in the white wine production process as skins are removed during the vinification of white wine (*15*).

The objective of the present *in vitro* study was to screen a number of red and white wine phenolic extracts for potential antimicrobial activities against pathogenic strains of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans.*

Materials and Methods

Wine phenolic extracts

Wines

The wines examined were products of Greek vineyards, and were produced from the following grape varieties: Muscat, dry white wine; Debina, dry white wine; Sauvignon blanc, dry white wine; Agiorgitiko, dry red wine; Limnio, dry red wine; Xinomavro, dry red wine. White wines examined were one year old and red ones two years old.

Preparation of wine extracts

Liquid/liquid extractions of dealcoholated wines were performed to obtain three extracts containing different classes of polyphenols (*16*). Dealcoholated wines were obtained by evaporation; wine added to an equal volume of distilled water was concentrated to the original volume (25 °C, 80 mbar) in order to remove the alcohol without destroying the phenolic compounds (*17*).

The dealcoholated wine (pH=2.0) was first extracted with ethyl acetate and the aqueous phase was the first (1) fraction. The organic phase after evaporation was redissolved in water at pH=7.0, and extracted again with ethyl acetate. This organic phase was the second (2) fraction. The aqueous phase was adjusted to pH=2.0 and extracted again with ethyl acetate and this extract was the third (3) fraction.

Samples in 10 % ethanol were used for the phenolic analyses and in dimethyl sulphoxide (DMSO) for the screening of antimicrobial activities. The DMSO alone was tested for any bactericidal effect on the used microorganisms and was found that it did not inhibit growth of these microbial strains.

Analysis of phenolics

The total phenol contents of the samples were determined by the Folin-Ciocalteau method (*18*) using gallic acid as a standard. The wine extracts were analysed by HPLC-DAD for individual phenolic compounds as described previously (*19*). Samples were filtered using syringe filter (PTFE 0.45, Altech) prior to the injection. A Waters 600E system with a 996-photodiode array detector and a 600E pump was used. Chromatograms were treated using the Millenium 32 program. The column used was a C18 reversed phase Spherisorb (4.0 x 250 mm) with 5-μm packing. The mobile phases were: A – water/glacial acetic acid (98:2), B – methanol/water/glacial acetic acid (60:38:2) and C – methanol/glacial acetic acid (98:2). The gradient used was: 0–30 min, 100 % A at 0.20 mL/min; 30–40 min, from 58.3 % A to 41.7 % B at 0.60 mL/min; 40–120 min, from 41.7 % A to 58.3 % B at 0.20 mL/min; 120–155 min, from 25 % A to 75 % B at 0.30 mL/min; 155–165 min, 100 % C at 0.60 mL/min and 165–180 min, 100 % C at 0.90 mL/min.

Some peaks were identified on the basis of the retention time and the UV-Vis spectra of several standards used. All peaks were classified using absorbance characteristics of the phenolic classes derived from the literature (*20,21*) and from our observations using several standards. The absorbance wavelengths of phenolic classes were as follows: benzoic acids at 250–280 nm; hydroxycinnamic acids at 305–330 nm, and several of them also at 290–300 nm; anthocyanins at 450–560 and 240–280 nm, and some of them at 315–325 nm; flavanols at 270–280 nm and at around 230 nm; flavonols at 350–380 and 250–270 nm, and some of them at around 300 nm; flavones and isoflavones at 300–350 and 245–270 nm; flavanones at 270–295 nm, and some of them at 300–320 nm. Peaks which exhibited maximum absorbance at 280–305 nm were expressed as unclassified 280–305 nm. Some of the standards used, like aldehydes and *trans*-resveratrol, exhibited these characteristics. Peaks which exhibited maximum absorbance at around 230 nm, and also absorbed at around 280 nm, were expressed as unclassified 230 nm. Subsequently, all peaks were classified into nine groups.

As main phenolic peaks were taken those exhibiting high area at 280, 255, 320, 360 or 520 nm.

Antimicrobial activity tests

Microbial strains

The tested microorganisms were clinical, animal and food isolates with known resistance pattern to common practice antimicrobial factors and were provided from the Culture Stock Collection of the Department of Medical Microbiology (Medical School, University of Ioannina). The bacterial species employed were different strains of *Staphylococcus aureus* (Gram+) and *Escherichia coli* (Gram–), while the selected yeast was *Candida albicans*. The isolates were biochemically and serologically characterized by standard methods (*22*). The selected species are common pathogens involved in a variety of human and animal infections; all 3 species are well recognized for resistance to a number of antimicrobials used in the medical and veterinary practice, they are safe for the researchers to handle and experiment with, their cultures are of low cost and easily maintained and they are supposed to be typical representatives of Gram+/Gram– bacteria and yeasts.

Culture media and inoculum

The strains of *S. aureus* and *E. coli* were maintained on trypticase soy agar (TSA Oxoid), while the strains of *C. albicans* were maintained on Sabouraud dextrose agar (SDA Oxoid). The microbial inoculum was prepared from 20 mL of overnight stock cultures in tryptone soy

broth (TSB Oxoid) at 37 °C and Sabouraud broth (SB Oxoid) at 30 °C The concentration of microbial inoculums was within the range of 10^6 cfu/mL determined by viable counts following serial dilutions. The Mueller-Hinton agar (MHA) for the bacterial strains and the SDA for the yeast strains were used for the sensitivity tests.

Antibiotic resistance of test strains

The bacterial strains used for the tests were selected based on their resistance to antibiotics, because it was thought that it would be essential to experiment with strains already exhibiting resistance mechanisms. All isolates of the selected strains exhibited resistance to certain antibiotics. The antibiotic susceptibility test of the selected strains was determined by the standard disk diffusion method of Bauer *et al.* (*23*). *S. aureus* strains were resistant to amoxycillin, cloxacillin, cefuroxime and nalidixic acid; *E. coli* strains were resistant to doxycycline, novobiocin and cloxacillin; *C. albicans* strains were sensitive to fluconazole and nystatin.

Antimicrobial assay

The antimicrobial effect of the wine extracts was tested using the agar well diffusion method following the well-established method of Deans and Ritchie (*24*). Overnight bacterial cultures were used for surface inoculation of Petri dishes containing 15 mL of MH agar or SD agar. Each Petri dish was spread on with 0.5 mL of strain inocula streaked thoroughly all over the surface of the MHA or SDA. Subsequently, four equidistant wells, 4 mm in diameter each, were punched into the inoculated medium with sterile glass Pasteur pipettes and were filled up with 25 μL of wine extract using a precise pipettor (Eppendorf). All plates were incubated at 37 °C and inhibition zones were measured after 24 h. Three different strains of each species (1 human isolate, 1 animal isolate, 1 food isolate) were tested in duplicate sets of plates, which were simultaneously processed for each strain. All the experiments were repeated twice, including two controls with plain DMSO and sterilized distilled water every time.

After incubation the inhibition zones were measured to an accuracy of 1 mm and the effect was calculated as a mean of the duplicate experiments for each triplicate strain test.

Results and Discussion

Antimicrobial activities of the alcohol-free extracts of white wines against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* strains are presented in Table 1. The values shown are the means from duplicate experiments performed for the three strains per species, since similar results were obtained on three strains used. The extracts M1, D1, M3 and D3 of Muscat and Debina wines inhibited the growth of *S. aureus*, *E. coli* and *C. albicans* strains, while the extracts Sb1 and Sb3 of Sauvignon blanc wine inhibited the growth of the bacterial strains but had no effect on the growth of *C. albicans* strains. The extracts 2 (M2, D2, Sb2) from the three white wines had no inhibitory effect on the growth of *S. aureus*, *E. coli* and *C. albicans* strains.

Table 1. Antimicrobial activities of white wine extracts against pathogenic *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* strains

Wine extract*	γ(total phenolics) (mg/L of gallic acid equivalents)	Diameter of inhibition zone/mm**		
		S. aureus	*E. coli*	*C. albicans*
M1	3260	29±4***	18±1	9±1
M1	1630	24±3	15±3	8±1
M2	4770	0±0	0±0	0±0
M2	2385	0±0	0±0	0±0
M3	2410	21±3	14±1	12±3
M3	1205	12±1	11±1	8±1
D1	3670	28±3	20±3	12±1
D1	1835	20±2	16±1	9±2
D2	5320	0±0	0±0	0±0
D2	2660	0±0	0±0	0±0
D3	4400	22±3	20±1	30±2
D3	2200	15±2	14±1	20±3
Sb1	3370	27±3	18±1	0±0
Sb1	1685	20±1	12±2	0±0
Sb2	2740	0±0	0±0	0±0
Sb2	1370	0±0	0±0	0±0
Sb3	2420	16±3	18±1	0±0
Sb3	1210	14±2	14±2	0±0

* M1, M2, M3: extracts 1, 2 and 3 of Muscat wine, respectively; D1, D2, D3: extracts 1, 2 and 3 of Debina wine, respectively; Sb1, Sb2, Sb3: extracts 1, 2 and 3 of Sauvignon blanc wine, respectively

** The values shown for the diameter of the inhibition zone are the means of duplicate experiments performed for three strains per species

***Standard deviation

The extracts 1, 2 and 3 from the tested three white wines exhibited similar phenolic composition. The extracts 1 (M1, D1 and Sb1) contained mainly benzoic acids, cinnamic acids and unclassified compounds at 230 nm, while the extracts 3 (M3, D3 and Sb3) contained almost exclusively benzoic and cinnamic acids. Since the extracts 1 and 3 of the tested white wines exhibited antimicrobial activity, it is assumed that some wine phenolic acids and probably unclassified compounds with maximum absorbance at 230 nm are most likely the compounds to exhibit the antimicrobial activity. The extracts 2 (M2, D2 and Sb2) contained mainly phenolic acids and tyrosol. The results of the sensitivity tests performed during our experimentations indicate that phenolic acids and tyrosol are least likely to exhibit any antimicrobial activity, at least towards the tested strains.

The antimicrobial activities of the alcohol-free extracts of red wines against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* strains are presented in Table 2. The values shown are the means of duplicate

experiments performed for the three strains per species, since similar results were obtained on the three strains used.

All extracts (1, 2 and 3) of Agiorgitiko (A1, A2, A3), Limnio (L1, L2, L3) and Xinomavro (X1, X2, X3) wines inhibited the growth of *S. aureus* and *E. coli* strains. However, only extract A1 and extracts 2 (A2, L2 and X2) and 3 (A3, L3 and X3) inhibited the growth of *C. albicans* strains.

Table 2. Antimicrobial activities of red wine extracts against pathogenic *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* strains

Wine extract*	γ(total phenolics) (mg/L of gallic acid equivalents)	Diameter of inhibition zone/mm**		
		S. aureus	*E. coli*	*C. albicans*
A1	21200	25±2***	16±1	12±1
A1	10600	20±1	12±1	11±1
A1	5300	15±2	8±1	0±0
A1	2650	12±2	0±0	0±0
A2	10020	18±3	12±1	18±2
A2	5010	12±1	11±1	16±1
A2	2505	8±1	10±2	8±1
A3	14300	21±2	15±2	15±2
A3	7150	19±2	12±2	14±1
A3	3575	14±2	9±1	13±1
L1	17100	31±3	21±2	0±0
L1	8550	26±2	15±1	0±0
L1	4275	20±3	13±1	0±0
L1	2138	10±1	11±1	0±0
L2	9380	18±2	12±1	15±1
L2	4690	14±2	8±1	12±2
L2	2345	8±1	0±0	10±2
L3	10200	26±3	22±2	15±1
L3	5100	22±1	16±2	14±1
L3	2550	18±1	13±2	12±1
X1	23880	19±2	12±1	0±0
X1	11940	17±1	9±1	0±0
X1	5970	15±2	8±1	0±0
X1	2985	12±2	0±0	0±0
X2	22960	17±1	12±1	13±1
X2	11480	14±1	12±2	0±0
X2	5740	0±0	0±0	0±0
X3	24980	22±3	18±3	10±1
X3	12490	20±1	12±1	8±1
X3	6245	14±1	0±0	0±0

* A1, A2, A3: extracts 1, 2 and 3 of Agiorgitico wine, respectively;
L1, L2, L3: extracts 1, 2 and 3 of Limnio wine, respectively;
X1, X2, X3: extracts 1, 2 and 3 of Xinomavro wine, respectively
** The values shown for the diameter of the inhibition zone are the means of duplicate experiments performed for three strains per species
*** Standard deviation

The extracts 1 (A1, L1 and X1) of red wines were rich in anthocyanins and flavanols and also contained unclassified compounds with maximum absorbance at 230 nm, phenolic acids, flavones and flavonols. The main phenolics of the extracts 2 (A2, L2 and X2) were flavanols, tyrosol, phenolic acids and unclassified compounds with maximum absorbance at 280 nm. The extracts 3 (A3, L3 and X3) were rich in cinnamic and benzoic acids.

The antimicrobial activity pattern of red wine extracts indicates that their different classes of phenolics are most likely to be the active substances in inhibiting the growth of *S. aureus*, *E. coli* and *C. albicans* strains. Among them, there are definitely phenolic acids, since all red wine extracts contained phenolic acids and also the extracts 3 contained almost exclusively phenolic acids. Therefore, it is assumed that phenolic acids are presumably the main compounds that exhibit inhibitory effects against the tested pathogens.

It should be pointed out that our results suggest that the extracts 2 of red wines exhibited antimicrobial activity, whereas the respective extracts from the white wines were inactive. The major difference in the phenolic composition among the extracts 2 (ethyl acetate extracts at pH=2.0 and pH=7.0) of red and white wines was the presence of flavanols and unclassified compounds with maximum absorbance at 280 nm. So, the antimicrobial effect of these red wine extracts may be attributed to such phenolic compounds. Moreover, the extract 2 of Agiorgitiko wine exhibited a main peak of *trans*-resveratrol. HPLC-DAD chromatograms of the extract 2 of Agiorgitiko wine are presented in Fig. 1.

The inhibition zone of each wine extract against *S. aureus*, *E. coli* or *C. albicans* strains increased whenever the total phenolic content of the extract was increased (Table 1). Accordingly, it appears that the antimicrobial activities of all phenolic extracts from white and red wines were dose dependent.

Most wine extracts exhibited some kind of antibacterial activity against both Gram-positive and Gram-negative strains. In almost all extracts, the diameter of the inhibition zone for *S. aureus* was greater than the zone for *E. coli* strain, indicating that the Gram-positive strain was more sensitive than the Gram-negative one. This observation can be attributed to differences in the structure of bacteria cell wall. The less complex structure of the cell wall in the Gram-positive bacteria makes it more permeable to the antimicrobial compounds.

A considerable number of white and red wine phenolic extracts inhibited the growth of *C. albicans*. However, in all tests the diameter of the inhibition zone for *C. albicans* was smaller than the diameter measured for *S. aureus* and *E. coli* inhibition zones. It appears that the yeast strains were more resistant to wine phenolics than the bacterial strains, which has been observed by other researchers too (*25*). These different resistant patterns are likely to be related to differences in yeast and bacteria cell wall structures and protein synthesis.

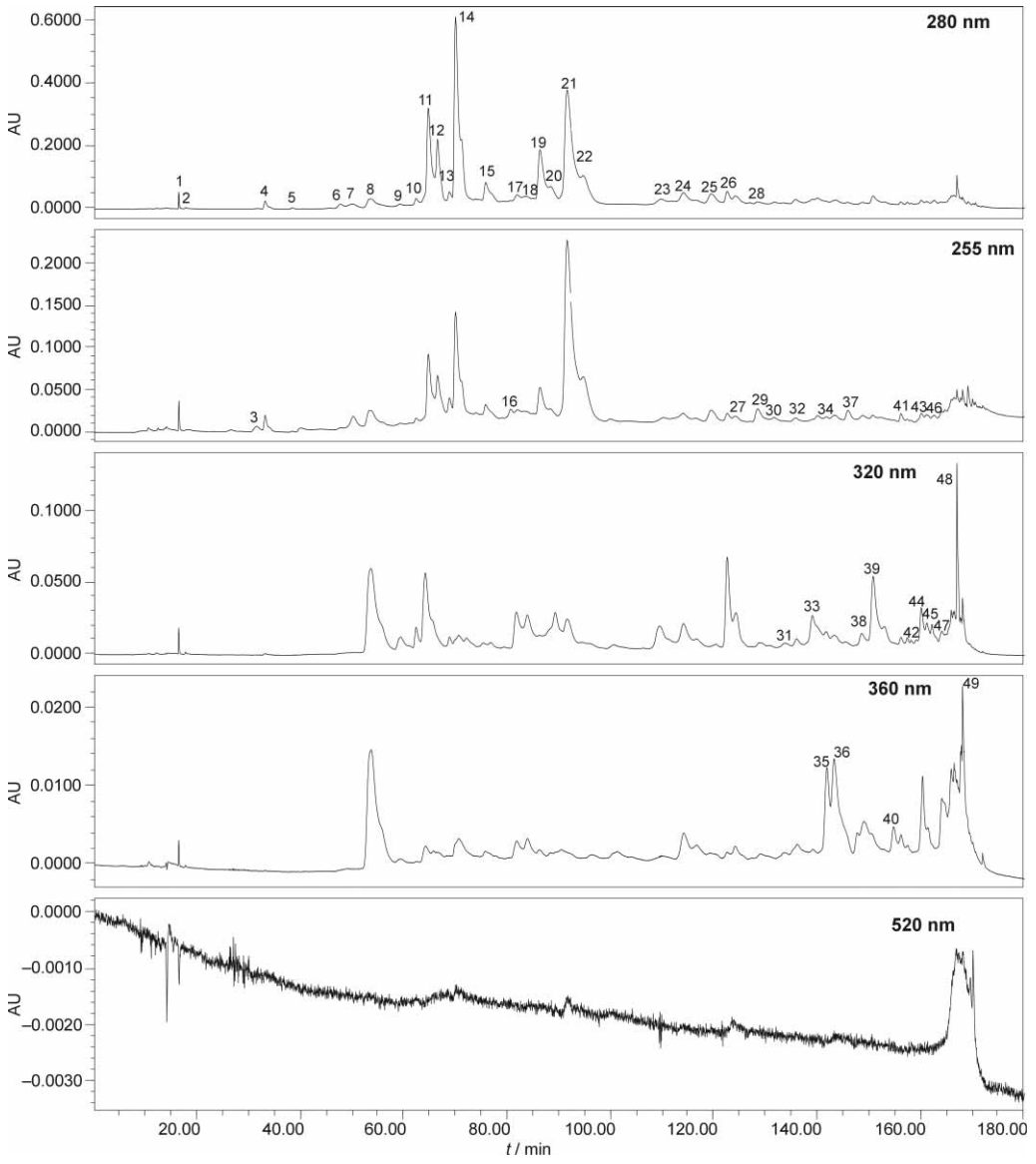

Fig.1. HPLC chromatograms of A2 extract at 280, 255, 320, 360 and 520 nm. Peaks: 1–2, unclassified 280 nm; 3–5, benzoic acids; 6, flavanol; 7, benzoic acid; 8–9, hydroxycinnamic acid; 10, flavanone; 11, tyrosol; 12, flavanol; 13, unclassified 280 nm; 14, catechin; 15, flavanol; 16, benzoic acid; 17–18, hydroxycinnamic acids; 19, benzoic acid; 20, flavanone; 21, syringic acid; 22, benzoic acid; 23, hydroxycinnamic acid; 24, unclassified 280 nm; 25, flavanol; 26–27, unclassified 280 nm; 28, flavanol; 29, protocatechuic acid ethyl ester; 30, unclassified 280 nm; 31, hydroxycinnamic acid; 32, unclassified 280 nm; 33, hydroxycinnamic acid; 34, unclassified 280 nm; 35–36, flavonols; 37, benzoic acid; 38, flavone; 39, *trans*-resveratrol; 40, flavonol; 41, benzoic acid; 42–43, unclassified 280 nm; 44–45, hydroxycinnamic acids; 46, flavanol; 47, flavone; 48, hydroxycinnamic acid; 49, flavonol; 50, anthocyanin

The antimicrobial effects of the tested wine phenolic extracts, especially of the red wines, indicate that there are phenolic classes which have important bactericidal or bacteriostatic activities. Also, the observed antimicrobial activity and the analysis of the phenolic composition of the tested white and red wine extracts indicate that there are phenolic acids encompassing inhibitory properties towards *S. aureus*, *E. coli* and *C. albicans* strains. According to the existing literature there are several phenolic acids, such as chlorogenic, caffeic, *p*-coumaric, ferulic, *p*-hydroxy-benzoic, vanillic, protocatechuic, syringic (*11*,*12*), as well as some other phenolic compounds like quercetin, hydroxytyrosol, resveratrol (*9*–*11*) identified to have antimicrobial activities.

Conclusion

According to the results of the present screening study the white and red wine alcohol-free extracts appear to contain compounds responsible for antimicrobial effects against Gram-positive and Gram-negative bacteria and yeasts. The phenolic composition of the tested wine extracts indicates that some phenolic acids are probably the most active components in inhibiting the growth of pathogens.

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Antimikrobna aktivnost fenolnih ekstrakata bijelih i crnih vina prema sojevima *Staphylococcus aureus*, *Escherichia coli* i *Candida albicans*

Sažetak

Svrha je rada bila utvrditi antimikrobnu aktivnost fenolnih ekstrakata vina. Jačina antimikrobne aktivnosti u bezalkoholnim ekstraktima bijelih i crnih vina prema patogenim sojevima *Staphylococcus aureus*, *Escherichia coli* i *Candida albicans* ispitana je difuzijom u jažicama agara. Ukupni udjel fenola u vinskim ekstraktima određen je Folin-Ciocalteuovim postupkom, dok je sastav fenola utvrđen HPLC-om i detektorom diodnih zraka (HPLC-DAD). Antimikrobna aktivnost ispitanih ekstrakata uspoređena je s ukupnim udjelom fenolnih spojeva. Antimikrobna aktivnost ispitanih vinskih ekstrakata bila je znatna prema *Staphylococcus aureus*, a manje djelotvorna prema *E. coli* i *Candida albicans.* Pokazalo se da je soj *Candida albicans* otporniji prema većem broju vinskih ekstrakata od preostala dva ispitana soja. Antimikrobna aktivnost i sastav fenola u ispitanim ekstraktima bijelih i crnih vina pokazuje da neke fenolne kiseline mogu inhibirati rast određenih patogena, kao što su *Staphylococcus aureus*, *Escherichia coli* i *Candida albicans*.