Cost-effective Ultrasonic Extraction of Bioactive Polyphenols from Vine and Wine Waste in Serbia

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By using cost-effective ultrasonic extraction techniques, bioactive phenolic extracts were obtained from Merlot and Vranac (*Vitis vinifera* L.) vine and wine waste produced in Serbian wineries. These wastes include vine leaves, grape pomace, seeds, skins and stems, which can find further use in the food and pharmacology industries as preservatives against microbes. The extracts showed strong scavenging free radical activity (EC$_{50}$ from 0.37 to 2.02 mg/L), which was in very good correlation with the total polyphenol content. Strong antimicrobial activity was found against six Gram-positive and five Gram-negative bacterial strains, and against the yeast *Candida albicans*. The principal component and agglomerative hierarchical clustering analyses performed were shown to be good for grouping and distinguishing the results from the vine and wine by-products (leaves and seeds) from both investigated grape varieties based on the content of various polyphenolic classes, and antioxidant and antimicrobial activities. The valorisation of the grape by-products is consistent with the concept of a sustainable and environmentally oriented wine industry and provides an important economic advantage.

INTRODUCTION

Grape cultivation is one of the main extended agro-economic activities in the world. In 2014, a total surface area of 7 535 917 ha was under vineyards throughout the world, with global table grape production at 267 167.581 (in 1 000 quintels (ql)) and world wine production at 269 363.753 (in 1000 hL) (OIV, 2018). The Republic of Serbia is one of the major producers and consumers of grapes and wines in the Western Balkan. During wine production, significant quantities of grape by-products (seeds, skins, stems and residual pulp) are generated (Andjelković *et al*., 2013; Christ & Burrit, 2013; Brenes *et al*., 2016). The amounts of grape pomace generated from winemaking depend on the grape cultivar, the fermentation process and the pressing process (Abarghuei *et al*., 2010; Dwyer *et al*., 2014; Cuccia, 2015; Lingua et al., 2016a). Grape seeds represent 2% to 5% of the grape weight and constitute approximately 38% to 52% of solid grape pomace (Brenes *et al*., 2016). In recent years, it has been estimated that 3% of pomace produced is reused for animal feed, and for the production of brandy and oil (obtained from seeds);

other applications are fertiliser (obtained from the pomace) and a possibility of being used to improve thermal insulation in building construction (Dwyer *et al*., 2014; Muñoz *et al*., 2014; Santos *et al*., 2016).

Plants produce polyphenols as a response to the negative impacts of the environment (UV radiation, various pathogens, fungi, etc.). All plant parts contain phytochemicals such as phenols in different quantities, depending on the stage of plant development and the environmental influence. By using an ultrasonic extraction technique (Andjelković *et al*., 2014) for a short extraction period (15 min) at room temperature and with a small concentration of solvent, phenolic extracts were obtained from different parts of vine by-products.

It is known that grapes are rich in polyphenols, which are very important compounds for human health because of their antioxidant, anti-cancer, anti-inflammatory, antimicrobial and other biological properties (Jayaprakasha *et al*., 2003; Özkan *et al*., 2004; Baydar *et al*., 2006; Anastasiadi *et al*., 2009; Katalinić *et al*., 2010; Radovanović *et al*., 2012).

During the process of wine production from grapes, a significant amount of phenolic compounds pass into the wine, but a certain level of these compounds also remains in the pomace (Ky *et al*., 2014). Brenes *et al*. (2016) show that the composition of pomace can be different depending on the grape variety, location, fertilisation conditions, soil and harvest period, and that these differences give different application possibilities. The phenolic composition of the pomace (Özkan *et al*., 2004), stalks (Souquet *et al*., 2000; Spigno *et al*., 2007), seeds (Baydar *et al*., 2006; Baiano & Terracone, 2011), skins (Bartolome *et al*., 2004; Poudel *et al*., 2008) and leaves (Monagas *et al*., 2006; Dani *et al*., 2010) of different grape varieties have been well documented. The antimicrobial activity of their extracts has barely been studied (Jayaprakasha *et al*., 2003; Özkan *et al*., 2004; Baydar *et al*., 2006), although there are published reports on their antioxidant activity (Parry *et al*., 2011; Rockenbach *et al*., 2011; Lingua *et al*., 2016a, 2016b).

Because of the increased interest in the use of natural rather than synthetic compounds in the food industry, grape polyphenols are being investigated for use as a functional food (dietary fibre), in food processing (biosurfactants) and as supplements (grape pomace power) (Shinagawa *et al*., 2015). In this study, statistical analyses of correlations among the contents of various classes of polyphenolic compounds, determined using HPLC, and the antioxidant and antimicrobial activities of grape seeds, skins, stems and leaves from the Merlot and Vranac (*Vitis vinifera* L.) varieties, were investigated against six Gram-positive and five Gram-negative bacterial strains, and the yeast *Candida albicans*. Ultrasound extraction was used because it has been proven to require less time giving higher yields than conventional solvent extraction (Rostagno *et al*., 2003; Wang *et al*., 2008; Andjelković *et al*., 2014).

MATERIAL AND METHODS
Reagents and chemicals
Solvents were obtained from Merck (Darmstadt, Germany). Standard phenolic compounds and 2,2`-diphenyl-1-picrylhydrazyl (DPPH) free radical were supplied by Sigma Chemical Co. (St. Louis, MO). Chloramphenicol, streptomycin and tetracycline were acquired from a local pharmacy. Nutrient agar and nutrient broth were purchased from Merck. The reagents used were of analytical quality.

Samples
The grape leaves of two varieties, *viz*. Merlot and Vranac *(Vitis vinifera* L.), were collected from the Serbia vineyard region in mid-October (2009 to 2012), after harvest. All samples were washed using distilled water at room temperature, and then frozen at -20°C. Before the extraction of seeds from the berries, the skin and pulp were separated by hand. Seeds and skins were dried at 60°C, and measured until achieving a constant mass. Dried seeds and skin were mixed in a blender, and then used for the extractions.

During the harvest season (mid-October, 2009 to 2012), healthy leaves were collected from Vranac and Merlot, dried at 60°C, mixed in the blender, and then used for the extraction. Pomace samples (side product after vinification of the investigated grape types) were also collected. Seeds,

skins and stems from the pomace were separated by hand. Pomace samples and the separated seeds, skins and stems were dried at 60°C, mixed in a blender, and then used for the extractions.

Ultrasound-assisted extraction
An ultrasound instrument (EI, Serbia) with a volume of 3 L, a frequency of 40 kHz and an input power of 500 W was used in the experiments. The samples (1 g) of dry grape leaves, stems and pomace, and the seeds and skins from the pomace, were ultrasonically extracted for 1 h with 40 mL of the solvent system, consisting of methanol:acetone:water:acetic acid (30:42:27.5:0.5), and then centrifuged (2 500 x *g*) for 10 min (Andjelković *et al*., 2014). After treatment, extracts were centrifuged for 10 min at 2 500 x *g* and evaporated to dryness under a vacuum rotary evaporator and diluted in methanol to a concentration of 0.1 g/mL. Dry extract/initial dry mass (%) was 9.64 ± 0.36 from the pomace, 16.14 ± 0.22 from the seeds, 12.03 ± 0.21 from the skins, 6.25 ± 0.14 from the stems, and 8.21 ± 0.12 from the leaf powder.

Spectrophotometric analysis
The total polyphenol content in the selected extract samples was determined according to the spectrophotometric method described previously (Mazza *et al*., 1999). The results are expressed as milligrams (mg) of gallic acid equivalents (GAE) per gram (g) of extract dry matter (DM).

HPLC analysis
The phenolic composition of the extracts was analysed by high-performance liquid chromatography (HPLC) after they were previously filtered through a 0.45 μm pore size membrane filter. An Agilent Technologies 1200 chromatographic system, equipped with an Agilent photodiode array detector (DAD) 1200 with RFID tracking technology for flow cells and a UV lamp, an automatic injector and ChemStation software, was used for the determination of individual phenolic compounds. The phenolic compounds in the selected extract samples were determined according to the HPLC method described previously (Radovanović *et al*., 2012, 2016). The wavelengths for detection were 280, 320, 360 and 520 nm for UV and 275/322 nm ($\lambda_{Ex}/\lambda_{Em}$) for fluorescence-detection analysis. The identification of compounds was achieved by comparison of their retention times and spectral characteristics to original reference standard compounds and data in the literature (Iacopini *et al*., 2008). The results are presented as mg/g DM.

Antioxidant activity
The antioxidant activity of all the extracts was estimated by determining the free radical-scavenging activity of extracts using the DPPH free radical test described previously (Radovanović *et al*., 2010). The antioxidant assay is based on the measurement of the loss of DPPH colour by change in the absorbance at 515 nm caused by the reaction of DPPH with the tested sample. The reaction was monitored by a UV/Vis spectrophotometer. The diluted extract and fresh 1×10^{-4} M DPPH methanolic solution were put into a cuvette at room temperature. After the 20 min incubation period at room temperature, the absorbance was read against a blank (the

absorbance of the diluted sample extract) at 515 nm. Radical scavenging activity (RSA, %) of each extract was calculated from the decrease in absorbance according to the following equation:

$$RSA\,(\%) = [(1 - A_{sample} - A_{blank})/(A_{control})]100 \qquad (1)$$

where $A_{control}$ is the absorbance of the control reaction, A_{blank} is the absorbance of the diluted extract, and A_{sample} is the absorbance of the extract with DPPH radical. The RSA was plotted against the extract concentration (mL g^{-1}) to determine the concentration of the extract that reduces the activity by 50% (EC$_{50}$).

Antimicrobial activity
The antimicrobial activity was determined against Gram (+) bacteria: *Clostridium perfringens* ATCC 19404, *Bacillus cereus* ATCC 8739, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 8538, *Sarcina lutea* ATCC 9341, and *Micrococcus flavus* ATCC 40240; Gram (-) bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076, *Shigella sonnei* ATCC 25931, *Klebsiella pneumoniae* ATCC 10031, and *Proteus vulgaris* ATCC 8427; and yeast: *Candida albicans* ATCC 10231. All of these were obtained from the American Type Culture Collection. The bacterial strain inocula were prepared from overnight broth cultures, and suspensions were adjusted to 0.5 McFarland standard turbidity (corresponding to 10^7 to 10^8 CFU/mL).

The disc diffusion method was carried out using 100 μL of bacterial suspension on Mueller-Hinton agar (MHA, Torlak) in Petri dishes (diameter 90 mm). The discs (HiMedia Laboratories Pvt. Limited) were covered with the test samples (50 μL) with a concentration of 0.1 g/mL and placed into the inoculated agar (20 mL). The inoculated plates were kept for 24 h at 37°C. Chloramphenicol (30 μg/disc), streptomycin (30 μg/disc) and tetracycline (30 μg/disc) were used as positive controls, and the solvent (methanol – 50 μL/ disc) was treated as a negative control (Radovanović *et al.*,

2017). As expected, methanol showed no inhibitory activity. All tests were performed in triplicate. Antibacterial activity was represented as the zone of inhibition (in mm) against bacterial strains.

Statistical analysis
All the experiments were performed in triplicate. Values are presented as means ± standard deviation. Principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) were performed using statistical applications available for Microsoft Excel® (XLSTAT 2018) (Addinsoft, 2018). XLSTAT 2018 was also used to perform the Pearson correlation analysis.

RESULTS AND DISCUSSION
Polyphenolic profile
The spectrophotometric analysis of the obtained seed, skin, stem, leaf and pomace extracts provides fast information on the total polyphenolic contents in the tested by-products (Table 1). The highest content of polyphenols was in the seed extracts obtained from the Merlot and Vranac grape pomace (105.16 and 113.25 mg/g, respectively), followed by that in the extracts of the leaves (93.31 and 88.42 mg/g, respectively), the stems (78.34 and 73.99 mg/g, respectively), the pomace (58.06 and 67.40 mg/g, respectively), and the skins (50.36 and 51.73 mg/g, respectively). A significantly higher polyphenol content was found in the seed extracts in relation to the other extracts, which agrees with the published data for other varieties (Revilla & Ryan, 2000; Jayaprakasha *et al.*, 2003; Baydar *et al.*, 2006; Poudel *et al.*, 2008).

To determine the polyphenolic content and composition of the investigated by-products more precisely, the HPLC method was used. The results (Table 2) agree well with those obtained by spectrophotometric determination of the total polyphenol content (Table 1).

The highest sum of phenolic acids, flavonols and flavan-3-ols determined by HPLC was in the Merlot and Vranac seed extracts (44.53 and 43.29 mg/g, respectively), followed by the leaves (35.41 and 34.71 mg/g, respectively), the stems

TABLE 1
Total phenols (mg GAE/g) and antioxidant activity (mg/L) of Merlot and Vranac waste

Grape waste	Variety	Total phenols	Antioxidant activity, EC$_{50}$
Seeds	Merlot	105.16 ± 0.93	0.41 ± 0.02
	Vranac	113.25 ± 0.89	0.37 ± 0.01
Skins	Merlot	50.36 ± 0.20	2.11 ± 0.09
	Vranac	51.73 ± 0.19	2.02 ± 0.07
Stems	Merlot	78.34 ± 0.40	0.81 ± 0.08
	Vranac	73.99 ± 0.28	0.73 ± 0.06
Pomace	Merlot	58.06 ± 0.31	1.20 ± 0.02
	Vranac	67.40 ± 0.38	1.16 ± 0.03
Leaves	Merlot	93.31 ± 0.76	0.72 ± 0.03
	Vranac	88.42 ± 0.58	0.66 ± 0.01

Data are expressed as mean ± SD (*n* = 3).

TABLE 2
Phenolic compounds (mg/g) of Merlot and Vranac waste, determined by HPLC analysis

Phenolic compound		Seeds	Skins	Stems	Pomace	Leaves
Gallic acid	M[a]	3.21 ± 0.07	1.36 ± 0.03	1.58 ± 0.03	3.28 ± 0.08	0.65 ± 0.02
	V[b]	3.13 ± 0.03	1.40 ± 0.02	1.43 ± 0.04	3.33 ± 0.07	0.33 ± 0.01
t-Caftaric acid	M	nd	nd	nd	nd	9.34 ± 0.10
	V	nd	nd	nd	nd	7.78 ± 0.14
t-Coutaric acid	M	nd	0.31 ± 0.03	nd	0.37 ± 0.03	0.79 ± 0.06
	V	nd	0.44 ± 0.10	nd	0.41 ± 0.02	0.81 ± 0.10
Caffeic acid	M	nd	0.35 ± 0.03	nd	0.42 ± 0.04	0.75 ± 0.06
	V	nd	0.38 ± 0.02	nd	0.50 ± 0.04	0.68 ± 0.02
Chlorogenic acid	M	nd	nd	nd	nd	0.23 ± 0.03
	V	nd	nd	nd	nd	0.68 ± 0.02
Quercetin gl.	M	nd	0.11 ± 0.01	1.07 ± 0.03	0.09 ± 0.01	9.78 ± 0.11
	V	nd	0.13 ± 0.01	1.11 ± 0.03	0.11 ± 0.01	9.61 ± 0.13
Rutin	M	nd	0.15 ± 0.02	1.33 ± 0.04	0.11 ± 0.02	5.32 ± 0.09
	V	nd	0.14 ± 0.01	1.40 ± 0.07	0.15 ± 0.01	5.71 ± 0.10
Luteolin gl.	M	nd	0.09 ± 0.01	nd	0.04 ± 0.01	nd
	V	nd	0.11 ± 0.02	nd	0.06 ± 0.02	nd
Myricetin gl.	M	nd	0.09 ± 0.01	nd	nd	nd
	V	nd	0.08 ± 0.01	nd	nd	nd
Kaempferol gl.	M	nd	0.08 ± 0.01	0.91 ± 0.03	0.06 ± 0.01	2.44 ± 0.09
	V	nd	0.07 ± 0.01	0.88 ± 0.04	0.08 ± 0.01	2.77 ± 0.05
Quercetin	M	nd	0.04 ± 0.01	0.14 ± 0.01	0.04 ± 0.01	0.54 ± 0.01
	V	nd	0.05 ± 0.01	0.12 ± 0.01	0.05 ± 0.01	0.66 ± 0.03
(+)-Catechin	M	7.62 ± 0.09	1.89 ± 0.04	2.31 ± 0.06	3.64 ± 0.12	2.01 ± 0.04
	V	8.08 ± 0.11	2.02 ± 0.07	2.55 ± 0.05	3.84 ± 0.12	2.08 ± 0.03
(-)-Epicatechin gallate	M	15.50 ± 0.13	8.95 ± 0.09	7.27 ± 0.10	10.60 ± 0.13	nd
	V	13.88 ± 0.14	9.08 ± 0.11	6.55 ± 0.11	10.58 ± 0.12	nd
(-)-Epicatechin	M[a]	10.34 ± 0.12	nd	2.46 ± 0.03	1.17 ± 0.03	3.56 ± 0.03
	V[b]	10.60 ± 0.13	nd	2.60 ± 0.03	1.22 ± 0.05	3.60 ± 0.05
Procyanidin B$_2$	M	7.86 ± 0.10	4.64 ± 0.03	3.38 ± 0.03	nd	nd
	V	7.60 ± 0.09	4.71 ± 0.05	3.60 ± 0.02	nd	nd
Delphinidin-3-glucoside	M	nd	1.83 ± 0.03	nd	0.95 ± 0.03	nd
	V	nd	1.60 ± 0.01	nd	1.03 ± 0.02	nd
Cyanidin-3-glucoside	M	nd	1.28 ± 0.03	nd	0.20 ± 0.01	nd
	V	nd	1.44 ± 0.02	nd	0.27 ± 0.01	nd
Petunidin-3-glucoside	M	nd	0.60 ± 0.03	nd	0.28 ± 0.03	nd
	V	nd	0.58 ± 0.01	nd	0.24 ± 0.02	nd
Peonidin-3-glucoside	M	nd	2.73 ± 0.03	nd	0.13 ± 0.03	nd
	V	nd	2.60 ± 0.03	nd	0.11 ± 0.01	nd
Malvidin-3-glucoside	M	nd	24.47 ± 0.19	nd	1.12 ± 0.03	nd
	V	nd	25.02 ± 0.17	nd	1.36 ± 0.02	nd

[a] Merlot; [b] Vranac; Data are expressed as mean ± SD (*n* = 3); nd = not detected

and pomace (from 19.45 to 20.33 mg/g), and the skins (18.06 and 18.61 mg/g, respectively). The main compounds in the seeds were flavan-3-ols (41.32 and 40.16 mg/g, respectively) and gallic acid (3.21 and 3.13 mg/g, respectively).

The skin extracts were rich in anthocyanins (30.91 and 31.24 mg/g, respectively) and flavan-3-ols (15.48 and 15.81 mg/g, respectively). Malvidin-3-glucoside was the main anthocyanin found in the skins and grape pomace, followed by peonidin-, delphinidin-, cyanidin- and petunidin-3-glucosides. The grape pomace also showed a higher content of flavan-3-ols (15.41 and 15.64 mg/g, respectively) and significantly small contents of phenolic acids (4.07 and 4.24 mg/g, respectively) and anthocyanins (2.68 and 3.01 mg/g, respectively). A similar content of phenolic compounds in the seeds and skins has been found by other authors (Iacopini *et al.*, 2008; Anastasiadi *et al.*, 2009; Butkhup *et al.*, 2010; Katalinić *et al.*, 2010; Scola *et al.*, 2010).

HPLC analysis of the extracts of the stems showed that they were also rich in flavan-3-ols (14.42 and 15.30 mg/g, respectively) and contained small amounts of flavonols (3.45 and 3.51 mg/g, respectively). Souquet *et al.* (2000) reported a similar composition of an extract of Merlot stems in France, and Anastasiadi *et al.* (2009) in some Greek grape varieties.

The highest flavonol content was found in the extract of leaves (18.08 and 18.75 mg/g, respectively), especially a significant amount of quercetin glucoside (9.78 and 9.61 mg/g, respectively), rutin (5.32 and 5.71 mg/g, respectively) and kaempferol glucoside (2.44 and 2.77 mg/g, respectively), which is in good agreement with the data in the literature (Monagas *et al.*, 2006; Dani *et al.*, 2010). The investigated leaves also had a high content of phenolic acids (11.76 and 10.28 mg/g, respectively), especially *trans*-caftaric acid (9.34 and 7.78 mg/g, respectively).

Antioxidant activity

All the investigated extracts showed strong antioxidant activity (Table 1). Extracts of Vranac were slightly stronger antioxidants (EC_{50} from 0.37 to 2.02 mg/L) than extracts from Merlot (EC_{50} from 0.41 to 2.11 mg/L). The highest antioxidant activity was shown in the seed extracts (0.37 and 0.41 mg/L, respectively), followed by extracts of the leaf (0.66 and 0.72 mg/L, respectively), stem (0.81 and 0.73 mg/L, respectively), pomace (1.16 and 1.20 mg/L, respectively) and skin (2.11 and 2.02 mg/L, respectively). The strong antioxidant activity of seed extracts corresponds to the highest polyphenol content and suggests that the phenolic compounds are partially responsible for the strong antioxidant activity of these extracts. The pomace and skin extracts showed slightly weaker antioxidant activity compared to the rest of the extracts, which has also been observed by others (Baydar *et al.*, 2006; Poudel *et al.*, 2008).

The investigated extracts showed scavenging free radical activity, which was in very good correlation with the content of total polyphenols as determined by spectrophotometric analysis (0.9239 ± 0.2789 and 0.9258 ± 0.2657, respectively), and with the concentration of total flavonoids determined by HPLC analysis (0.7795 ± 0.4402 and 0.7804 ± 0.4559, respectively). The data in the literature also confirm the antioxidant activity of the seed, leaf and stem extracts and the correlation with polyphenols (Jayaprakasha *et al.*, 2003; Bartolome *et al.*, 2004; Spigno & De Faveri, 2007).

Antimicrobial activity

The data on the antimicrobial activity of all the investigated extracts and three antibiotics (positive control) against *Clostridium perfringens*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Sarcina lutea* and *Micrococcus flavus* (Gram-positive strains), *Escherichia*

TABLE 3

Antimicrobial activities (inhibition zone diameters, mm) of Merlot and Vranac by-products (50 µL/disc) and reference antibiotics (30 µg/disc) against Gram-positive strains

Grape by-product		*C. perfringens*	*B. cereus*	*S. aureus*	*L. monocytogenes*	*S. lutea*	*M. flavus*
Seeds	M[a]	17.7 ± 1.3	17.1 ± 1.0	18.3 ± 1.2	18.5 ± 0.9	19.4 ± 1.3	16.7 ± 1.2
	V[b]	17.5 ± 1.2	17.1 ± 1.2	18.4 ± 1.3	18.5 ± 1.3	19.6 ± 1.0	16.9 ± 1.1
Skins	M	16.7 ± 1.3	16.2 ± 1.0	17.2 ± 1.0	17.3 ± 1.0	18.1 ± 1.2	15.7 ± 1.1
	V	16.9 ± 1.0	16.3 ± 1.2	17.4 ± 1.0	17.2 ± 1.0	18.5 ± 1.2	15.6 ± 1.3
Stems	M	17.2 ± 1.3	16.8 ± 1.3	18.2 ± 1.2	18.1 ± 1.3	19.0 ± 1.4	16.2 ± 1.1
	V	17.4 ± 1.3	16.9 ± 1.3	18.5 ± 1.2	18.2 ± 1.2	19.3 ± 1.1	16.4 ± 1.1
Pomace	M	15.9 ± 1.2	15.2 ± 1.2	16.3 ± 1.3	16.4 ± 1.3	17.3 ± 1.3	14.8 ± 1.0
	V	15.9 ± 1.1	15.3 ± 1.0	16.5 ± 1.1	16.4 ± 1.2	17.3 ± 1.3	15.1 ± 1.3
Leaves	M	17.4 ± 1.2	16.7 ± 1.1	18.1 ± 1.1	18.2 ± 1.2	19.5 ± 1.1	16.3 ± 0.9
	V	17.6 ± 1.3	16.8 ± 0.9	18.3 ± 1.3	18.1 ± 1.0	19.7 ± 1.3	16.5 ± 0.8
Chloramphenicol		nt	26.0 ± 1.1	25.0 ± 1.2	18.0 ± 2.0	38.0 ± 2.0	35.0 ± 2.1
Tetracycline		29.0 ± 2.0	23.9 ± 1.0	18.5 ± 1.3	18.7 ± 1.2	20.0 ± 1.2	23.6 ± 0.7

[a] Merlot; [b] Vranac; Data are expressed as mean ± SD (*n* = 3); nt = not tested

TABLE 4

Antimicrobial activities (inhibition zone diameters, mm) of Merlot and Vranac by-products (50 µL/disc) and reference antibiotics (30 µg/disc) against Gram-negative strains and yeast

Grape by-product		*E. coli*	*P. aeruginosa*	*S. enteritidis*	*S. sonnei*	*K. pneumoniae*	*C. albicans*
Seeds	M[a]	14.8 ± 0.7	15.6 ± 0.9	15.1 ± 1.0	17.4 ± 1.2	16.1 ± 1.0	15.2 ± 1.1
	V[b]	15.7 ± 1.2	15.9 ± 1.2	15.4 ± 1.1	17.5 ± 1.2	16.1 ± 1.3	15.5 ± 0.9
Skins	M	13.0 ± 1.1	14.4 ± 1.0	14.0 ± 1.2	16.3 ± 1.3	15.7 ± 1.2	14.3 ± 1.0
	V	13.3 ± 0.7	14.3 ± 0.9	14.0 ± 0.8	16.6 ± 0.8	15.2 ± 1.4	14.3 ± 0.9
Stems	M	14.1 ± 1.2	15.6 ± 1.3	15.2 ± 1.1	17.6 ± 1.3	16.6 ± 1.1	15.1 ± 1.0
	V	14.0 ± 1.0	15.4 ± 1.0	15.1 ± 1.2	17.7 ± 1.0	16.5 ± 1.1	15.2 ± 1.0
Pomace	M	12.1 ± 0.8	13.5 ± 1.1	13.0 ± 0.9	15.8 ± 1.3	15.0 ± 1.2	13.1 ± 0.7
	V	12.7 ± 1.2	13.7 ± 1.2	13.3 ± 1.0	15.6 ± 1.3	15.1 ± 1.3	13.3 ± 1.3
Leaves	M	14.3 ± 1.0	15.6 ± 1.0	15.0 ± 1.2	17.3 ± 1.2	16.5 ± 1.1	15.4 ± 1.0
	V	14.7 ± 1.1	15.9 ± 1.2	15.1 ± 1.1	17.7 ± 1.2	16.6 ± 1.0	15.5 ± 1.2
Streptomycin		16.0 ± 1.2	23.0 ± 1.0	18.0 ± 1.0	19.0 ± 2.0	nt	nt
Tetracycline		23.2 ± 1.2	20.8 ± 1.5	23.3 ± 1.3	31.1 ± 0.8	23.6 ± 0.6	19.2 ± 0.5

[a] Merlot; [b] Vranac; Data are expressed as mean ± SD (*n* = 3); nt = not tested

coli, Pseudomonas aeruginosa, Salmonella enteritidis, Shigella sonnei, Klebsiella pneumoniae and *Proteus vulgaris* (Gram-negative strains) and *Candida albicans* (yeast) are given in Tables 3 and 4 (inhibition zones).

It was found that the investigated extracts were on average more sensitive to Gram-positive strains compared to Gram-negative strains and yeast, which is in agreement with the data in the literature (Jayaprakasha *et al*., 2003; Özkan *et al*., 2004; Scola *et al*., 2010). We assume that the difference in activity is caused by the different structure of bacterial walls in Gram-positive and Gram-negative bacteria. The values of antimicrobial activity were in agreement with the results of the HPLC analysis (Table 2) (Radovanović *et al*., 2017). The analysis of the Merlot and Vranac by-products shows that seeds have the highest antimicrobial activity – in the range of 16.7 to 19.6 mm against Gram-positive and 14.8 to 17.5 mm against Gram-negative strains. The extracts of leaves also show high antimicrobial activity in the range of 16.3 to 19.7 mm against Gram-positive and 14.3 to 17.7 mm against Gram-negative strains, followed by extracts of the stems (16.2 to 19.3 mm against Gram-positive and 14.0 to 17.7 mm against Gram-negative strains), the extracts of skins (15.6 to 18.5 mm against Gram-positive and 13.0 to 16.6 mm against Gram-negative strains), and the extracts of pomace (14.8 to 17.3 mm against Gram-positive and 12.1 to 15.8 mm against Gram-negative strains). All of the tested extracts exhibited satisfactory antimicrobial activity against *Candida albicans* (yeast) – in the range of 13.1 to 15.5 mm.

The highest antimicrobial activity of the investigated extracts was shown against Gram-positive strains: *Sarcina lutea, Listeria monocytogenes* and *Staphylococcus aureus* (inhibition zones in the range of 16.3 to 19.7 mm), and against the Gram-negative strains *Shigella sonnei* and *Pseudomonas aeruginosa* (inhibition zones in the range of 15.0 to 17.7 mm). The obtained results are similar to the results of other authors, who perform investigations on different grape varieties against some bacterial strains (Özkan *et al*., 2004; Baydar *et al*., 2006; Anastasidi *et al*., 2009; Butkhup *et al*., 2010; Katalinić *et al*., 2010).

Statistical analyses

Statistical analyses were performed of the correlations among the contents of various classes of polyphenolic compounds in the extracts of both the Merlot and Vranac by-products, determined by HPLC analysis, and antioxidant (AA) and antimicrobial activities against Gram-positive and Gram-negative bacteria and *Candida albicans* yeast.

Pearson correlation analysis

Phenolic acid (PA) and flavonols (FOS) are in a strong positive correlation (0.909) with each other. On the other hand, antioxidant activity (AA) has a medium-negative correlation with the antimicrobial activities of Gram-positive bacteria – *L. monocytogenes* (LM) (-0.646) and *M. flavus* (MF) (-0.655), and Gram-negative bacteria – *E. coli* (EC) (-0.709), *P. aeruginosa* (PA) (-0.689), *S. enteritidis* (SE) (-0.650) and *S. sonnei* (SS) (-0.632). The antimicrobial activity of *C. perfringens* (CP) has a strong, positive correlation with the antimicrobial activities of all the investigated bacteria [*B. cereus* (BC) (0.982), *S. aureus* (SA) (0.972), LM (0.974), SL (0.983), MF (0.962), EC (0.901), PA (0.954), SE (0.956), SS (0.951) and *K. pneumoniae* (KP) (0.902)]. Similar, strong positive correlations were observed among other Gram-positive and Gram-negative bacteria.

Principal component analysis (PCA)

In the first step of the statistical evaluation, the Kolmogorov-Smirnov test (the significance level, α, was 0.05) was used to test the normality of the data. The data included total phenolic acids, total flavonols, total flavan 3-ols, antioxidant activities and antimicrobial activities (expressed as inhibition zones) obtained for all the investigated Gram-positive and Gram-negative bacteria. Before PCA analysis, the data matrix was tested to detect outliers. Grubb's test was used

with the experimental data, and no outliers were found. From the shape of the scree plot, shown in Fig. 1, the number of important components that were used in further calculations can be seen.

The PCA of the dataset revealed the presence of two components with characteristic (Eigen) values (11.224 and 2.381) exceeding 1. This two-component solution explained a total of 90.697% of the variance, with 74.826% contributed by the first component, and 15.871% by the second compo-
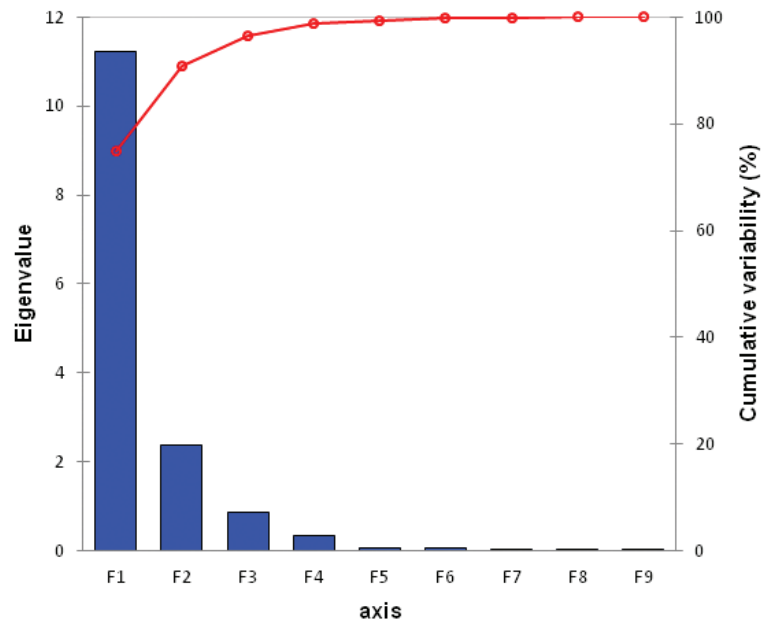

FIGURE 1

Scree plot. In this plot, the eigenvalues are sorted from the largest to the smallest.

a)   b)
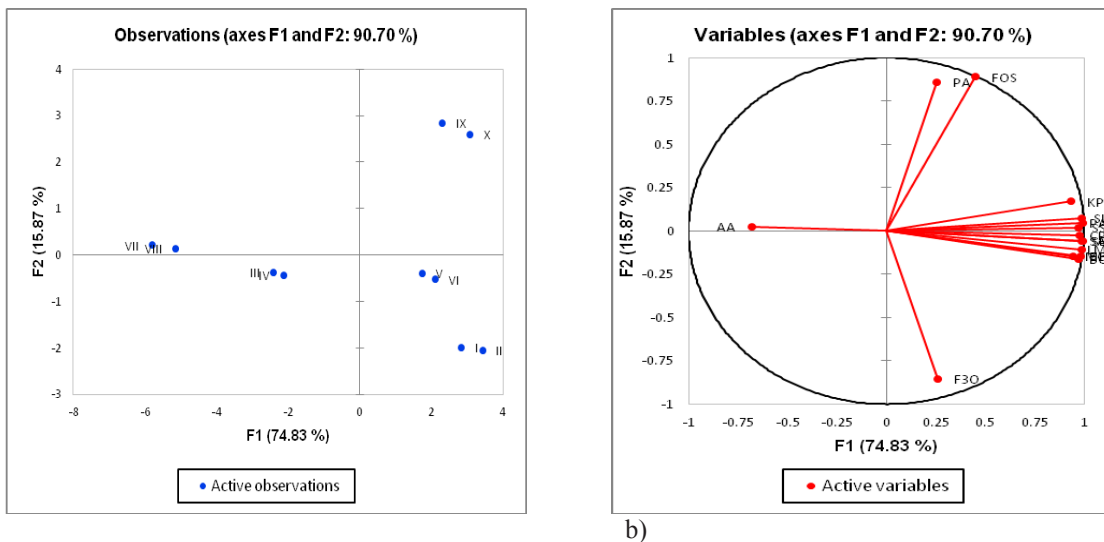
FIGURE 2

Principal component analysis (PCA). a) PCA scree plot of the first major component 1 (PCA1) versus the second component (PC2); b) Loading plot of the first main component (PC1) versus the second component (PC2). Variables: PA - phenolic acids, FOS - flavonols, F3O - flavan-3-ols, AA - antioxidant activity, CP - *C. perfringens*, BC - *B. cereus*, SA - *S. aureus*, LM - *L. monocytogenes*, SL - *S. lutea*, MF - *M. flavus*, EC - *E. coli*, PA - *P. aeruginosa*, SE - *S. enteritidis*, SS - *S. sonnei*, KP - *K. pneumoniae*; Samples: I - seeds (Merlot), II - seeds (Vranac), III - skins (Merlot), IV - skins (Vranac), V - stems (Merlot), VI - stems (Vranac), VII - pomace (Merlot), VIII - pomace (Vranac), IX - leaves (Merlot), X - leaves (Vranac).
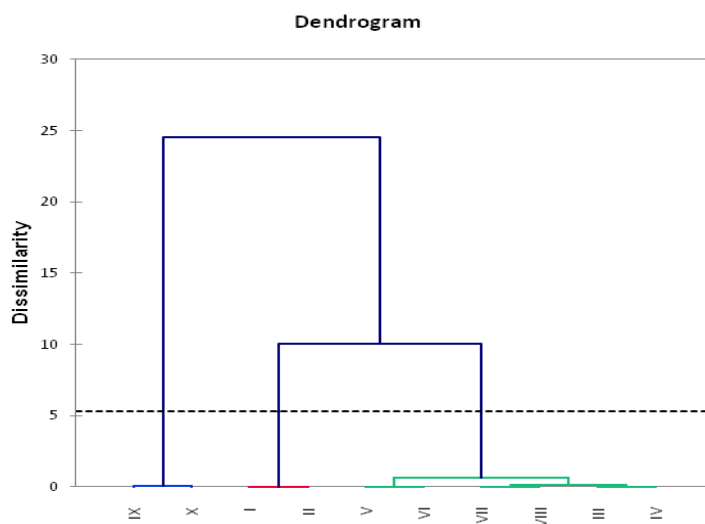
FIGURE 3

Dendrogram derived from the results obtained on the basis of the extracts of Merlot and Vranac grape varieties. Samples: I - seeds (Merlot), II - seeds (Vranac), III - skins (Merlot), IV - skins (Vranac), V - stems (Merlot), VI - stems (Vranac), VII - pomace (Merlot), VIII - pomace (Vranac), IX - leaves (Merlot), X - leaves (Vranac).

nent. The PCA scree plot shows that samples I, II, V, VI, IX and X (the extracts of seeds, stems and leaves) contained higher concentrations of phenolic acids – the first variable (positive values in PC1), and samples III, IV, VII and VIII (the extracts of skins and pomace) contained lower concentrations of total phenolic acids (negative values in PC1). On the other side, VII to X (pomace and leaf extracts) contained higher concentrations of flavonols, and samples I to VI (seed, skin and stem extracts) contained lower concentrations of flavonols (negative values in PC2) (Fig. 2a). The loading plot shows very similar values for both Gram-positive and Gram-negative bacteria, with AA as the only parameter with a negative value in F1 (Fig. 2b).

Agglomerative hierarchical clustering (AHC)
Agglomerative hierarchical clustering (AHC) of the standardised variables using the Ward method as an amalgamation rule was performed with the squared Euclidean distance as a measure of the proximity between the samples. The obtained dendrogram presenting the clustering of the analysed samples is presented in Fig. 3.

The dendrogram in Fig. 3 shows that all the monitored samples could be grouped into three main clusters, which are presented using different colours. Cluster I includes samples with concentrations of phenolic acids of more than 10 mg g^{-1}: IX and X (the extracts of leaves from Merlot and Vranac); cluster II includes samples with no detected flavonols: I and II (the extracts of seeds from Merlot and Vranac), and cluster III includes samples III to VIII (the extracts of skins, stems and pomace from both Merlot and Vranac). Thus, this cluster analysis was not able to distinguish samples based on the grape variety, but it was successful in differentiating among parts of the vine – leaves and seeds show different properties compared to skins and stems, which apparently were the major ingredients in the pomace.

CONCLUSIONS
The grapevine is a powerful plant that is rich in polyphenol compounds – even in the by-products after the vinification process. Those compounds are responsible for the strong antioxidant and antimicrobial activities of grapes, wines and their by-products. The seeds and leaves had the highest total phenol content and showed the strongest antioxidant and antimicrobial activities. The other extracts – of the stems, skins and pomace – also showed significant antioxidant and antimicrobial activities.

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