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Original Scientific Paper

Phenolic profile and biological potential of wild blackberry (*Rubus discolor*) fruits

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ABSTRACT:
The berries of *Rubus discolor* are considered a rich source of phytochemicals which could play an important role in the prevention of prevalent contemporary chronic diseases. Thus, the goal of the presented study was to determine the profile of phenolic acids and anthocyanins of aqueous and ethanol extracts of *R. discolor* fruit, and their free radical scavenging, antiproliferative, antidiabetic and antimicrobial activities. LC-MS/MS analyses confirmed the presence of 11 phenolic acids with protocatechuic and gallic acids being the major compounds. Additionally, cyanidin-glucoside/galactoside was the most abundant among the five identified anthocyanins. The ethanol extract was more efficient in scavenging free radicals than the aqueous extract. The absence of antiproliferative activity was observed for both extracts. However, they inhibited carbohydrate hydrolysing digestive enzymes associated with type-2 diabetes. Furthermore, the obtained results for α-glucosidase inhibitory activity (IC_{50} values 44.52 and 80.72 μg/mL, for the aqueous and ethanol extracts, respectively) indicate significantly higher activity than the positive control, Glucobay® (233.38 μg/mL). The ethanol extract was more effective against all of the examined bacteria (*Bacillus cereus*, *Staphylococcus aureus* and *Enterobacter cloacae*) than the aqueous extract. On the contrary, the aqueous extract showed better antifungal properties, particularly against *Trihoderma viride* and *Penicillium verrucosum* var. *cyclopium*. The quantified phenolics and presented bioactivities of *R. discolor* fruit extracts candidate them as a potential source of bioactive compounds which might be used in the food, pharmacy and cosmetic industries.

Keywords:
Rosaceae, phenolic acids, anthocyanins, antioxidant, antimicrobial and enzyme-inhibitory activity

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INTRODUCTION

The Rosaceae Juss. family is of great economic importance since many species produce edible fruits. According to The Plant List (2013), the genus *Rubus* L., one of the most numerous genera of this family, encompasses 1568 species, while the possibility of a threefold higher number remains unresolved. Most *Rubus* species are perennial deciduous shrubs, growing in the wild or cultivated on all continents with the exception of Antarctica (Heslop-Harrison 1968; Alice & Campbell 1999; Krivošej *et al.* 2018). Archaeological and ethnobotanical studies indicate its centuries-old usage for food and medicinal purposes (Hummer 2010). The attractive colour and taste of *Rubus* fruits together with raised consumer awareness of their potential health benefits have recently increased the interest and demand for these fruits on the global market (Schulz & Chim 2019). The most significant bioactive

compounds found in blackberries are phenolics, characterized by the presence of an aromatic ring and at least one hydroxyl group. This structure allows phenols to be easily oxidized and to simultaneously form phenoxy radicals, which compared to other free radical species are more stable due to their resonant stabilization (Hidalgo & Almajano 2017). This is important because several recent studies indicate that those disorders which are the leading cause of deaths globally, such as cardiovascular and neurodegenerative diseases, diabetes and certain types of cancer, are usually triggered by free radicals and an imbalance in cell oxidative status (Lukyanets *et al.* 2020).

Phenolic acids along with flavonoids are the largest and the most studied groups of polyphenols which are widely distributed in fruits and vegetables, particularly in berry species, including blackberries (Wojdyło *et al.* 2007; Schulz *et al.* 2019; Schulz & Chim 2019; Samaniego *et al.* 2020). Phenolic acids are responsible for the bitterness and tartness of fruits. Similarly to other polyphenols, phenolic acids naturally occur more frequently in conjugated form (Staszowska-Karkut & Materska 2020). Anthocyanins are a large subclass of flavonoids characterized by the presence of flavylium cation. The bioactivity of specific anthocyanin compounds depends on the substitution pattern of flavylium cation (Samaniego *et al.* 2020; Staszowska-Karkut & Materska 2020). According to the literature data, the phenolic composition is highly dependent on a variety of factors such as the plant organ, weather conditions, cultivar, cultivation method etc.

The extensive overview of published papers provides insight into numerous biological activities of *Rubus* species including antioxidant, antimicrobial, anticancer and enzyme-inhibitory activities. However, there is little information about the biological activities of *R. discolor* Weihe & Nees. The blackberry is a deciduous perennial plant, with a high and shrubby habitus. It is widespread in warm and dry European pastures and along roadsides (Heslop-Harrison 1968). According to ethnobotanical studies, *R. discolor* is commonly used as a traditional remedy for diabetes, urogenital and gastrointestinal dysfunctions and illnesses (Kültür 2007; Cakilcioglu & Turkoglu 2010).

This research was designed to determine the composition of polyphenolic compounds in aqueous and ethanol fruit extracts, and their antioxidant, antibacterial, antifungal, antiproliferative and enzyme-inhibitory activities.

MATERIAL AND METHODS

Plant material. *Rubus discolor* fruits were collected in August 2012 from Mt. Cer (Serbia) and stored in a refrigerator at -18°C. The voucher specimen was deposited in the Herbarium of the Institute of Botany and Botanical Garden Jevremovac, Faculty of Biology, BEOU (Voucher No. 17081).

Chemicals and reagents. All chemicals and reagents used in the experiments were at least of the analytical grade of purity.

Extraction. The frozen blackberry fruits (10 g) were macerated for 5 minutes and subsequently extracted for 24 hours with 100 mL of solvent (distilled water or 96% ethanol) by ultrasound assisted extraction in order to prepare the ethanolic (FE) and water extracts (FW). The extracts were evaporated to dryness and kept in the fridge at 4°C before the experiments. The FW extract was dissolved in 50% MeOH and the FE in 80% MeOH to the final concentration of 5 mg/mL and these extracts were used for the analysis of the chemical composition. The dried fruit extracts (FE/FW) were also used for the preparation of the appropriate working solutions to estimate their potential bioactivity.

Quantitative LC-MS/MS analysis of the selected phenolic acids. The content of 11 phenolic acids (*p*-hydroxybenzoic, protocatechuic, 2.5-dihydroxybenzoic, vanilic, gallic, cinammic, *p*-coumaric, *o*-coumaric, caffeic, ferulic and chlorogenic acid) was investigated by LC-MS/MS according to the previously published method (Orčić *et al.* 2014). The standard compounds were purchased from Sigma Aldrich (Steinheim, Germany). The Agilent 1200 series liquid chromatograph, coupled with Agilent series 6410B electrospray ionization triple quadrupole mass spectrometer and controlled by MassHunter ver. B.03.01. software were used for the analysis. The analytes were separated using a Zorbax Eclipse XDB-C18 50mm × 4.6 mm ×1.8 μm (Agilent Technologies) reversed phase column. The compound-specific, optimized MS/MS parameters are given in Table 1. The compound peaks were determined using Agilent MassHunter Workstation Software – Qualitative Analysis (ver. B.06.01). The concentrations of particular compounds in the samples were calculated from standard calibration curves in Microsoft Excel software.

Semi-quantitative LC-MS/MS analysis of anthocyanins. The identification and semi-quantification of anthocyanins in the FW and FE extracts was done by an in-house developed LC-MS/MS method. Standard solutions of cyanidin, pelargonidin, delphinidin and malvidine (Sigma Aldrich, Steinheim, Germany) in a concentration range from 2.5 μg/mL to 200 μg/mL were used for calibration curve construction. The extracts were filtered through 25 mm, 45 μm regenerated cellulose membrane filters and analysed by the reversed phase LC-MS/MS technique, using Agilent Technologies series 1260 HPLC Agilent Technologies 6460 Triple-Quad mass detector, controlled by Agilent Technologies MassHunter Workstation software – Data Acquisition. A 5 μL aliquot was injected into the system, and the compounds were separated on a Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm × 5 μm) held at 30°C. The mobile phase consisted of 0.1% formic

Table 1. Optimised compound-specific dynamic SRM parameters.

Compound	t_R (min)	Precursor *m/z*	Product *m/z*	Vf (V)	Vc (V)
p-Hydroxybenzoic acid	2.20	137	93	80	10
Protocatechuic acid	1.62	153	109	105	9
2.5-Dihydroxybenzoic acid	2.06	153	109	100	9
Vanilic acid	2.45	167	108	100	15
Galic acid	1.28	169	125	90	10
Cinammic acid	7.28	147	103	100	5
p-Coumaric acid	3.26	163	119	90	9
o-Coumaric acid	4.90	163	119	100	5
Caffeic acid	2.29	179	135	100	10
Ferulic acid	3.57	193	134	90	11
Chlorogenic acid	1.67	353	191	100	10

tR –retention time; Vf – fragmentor voltage; Vc – collision voltage;

acid in deionized water (A) and 0.1% formic acid in acetonitrile (B) was delivered at a flow rate of 0.5 mL/min in gradient mode (0 min 5% B, 10 min 22% B, 35 min 42% B, 40-42 min 100% B, re-equilibration time 8 min). The eluted components were detected and identified by MS/MS, using the ion source parameters as follows: nebulization gas (N_2) pressure 30 psi, drying gas (N_2) flow 9 L/min and temperature 350°C, capillary voltage 4 kV, positive polarity. The data were acquired in the MS2Scan mode, in the mass range from 100-1200 Da and fragmentor voltage 135 V and 220 V. At the same time, the absorption spectra were recorded at 520 nm. The identification of compounds was done based on absorption spectra at 520 nm and mass spectra. The peak areas in the DAD chromatogram were determined using Agilent MassHunter Workstation Software – Qualitative Analysis (ver. B.06.01). The calibration curves of anthocyanin aglicones - cyanidin, pelargonidin, delphinidin and malvidine were plotted in Microsoft Excel software and used for the semi-quantification of the corresponding glycosides, and the results were expressed in mg of aglicon equivalents per 100 g of dry extract.

Free radical scavenging activity. The free radical scavenging activity of the extracts was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) free radicals according to the methods described by Blois (1958) and Miller & Rice-Evans (1997), respectively. The results were expressed as effective concentrations which reduce free radical concentrations by 50% (EC_{50}, μg/mL) and compared with known antioxidant compounds, *L*-ascorbic acid and 2(3)-*t*-butyl-4-hydroxyanisole (BHA). All of the measurements were performed in triplicate on a Jenway 6306 Uv/Vis spectrophotometer.

Antiproliferative activity. The antitumor activity was evaluated by the microculture tetrazolium test (MTT) assay according to Mosmann (1983) with modifications suggested by Ohno & Abe (1991). For that purpose, three adherent cell lines were seeded in 96 well plates for 24 h before the addition of extract dilutions, namely human cervical carcinoma (HeLa - 2500 cells per well), human breast cancer (MDA-MB-453 - 3000 cells per well) and non-cancerous human embryonic lung fibroblast (MRC-5, - 5000 cells per well). Nonadherent human chronic myelogenous leukaemia cells (K562 – 5000 cells per well) were seeded two hours prior to the addition of the investigated extracts.

The five working dilutions (0.125 – 2 mg/mL) for each extract were prepared in a complete nutrient medium (RPMI-1640 without phenol red) supplemented with 3 mM *L*-glutamine, 100 μg/mL streptomycin, 100 IU/mL penicillin, 10% heat-inactivated fetal bovine serum (FBS), and 25 mM Hepes (pH 7.2) and added to 96-well plates with previously seeded cells. After incubation for 72 hours at 37°C in a humidified atmosphere of 5% CO_2, cell survival was determined by the MTT assay. Briefly, 20μL of MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide, 5 mg/mL in phosphate buffered saline] was added to each well. The samples were incubated for a further four hours at 37°C in a humidified atmosphere of 5% CO_2. Then, 100μL of 10% SDS was added to the wells. The absorbance was measured at 570 nm the following day. In order to calculate the cell survival (S%), absorbance at 570 nm of a sample with cells grown in the presence of various concentrations of extracts was divided by the absorbance of the control sample (the absorbance of the cells grown only in the nutrient medium), implying that the absorbance of the blank was always subtracted from the absorbance of a corresponding sample with target cells.

Enzyme-inhibitory activity. The ability of the extracts to inhibit α-amylase (α-AIA) was assessed as previously described by Zengin *et al.* (2014). The appropriate concentrations of the working extract dilutions (25μL) were pre-incubated for 15 min at 37°C with 0.5 mg/mL of α-amylase solution (50 μL) in 6mM NaCl phosphate buffer (pH 6.8). A 0.2% starch solution was added to the mixture and incubated for a further 20 min at 37°C. Then, 25μL of 1 M HCl was added to stop the reaction and 100 μL of iodine-potassium iodide solution (IKI reagent) to visualize the reaction. Afterwards, absorbance was read at 630 nm by a Multiscan Sky ThermoScientific Plate Reader.

The α-glucosidase inhibitory activity (α-GIA) was evaluated according to Wan *et al.* (2013). The enzyme (0.6 U/mL α-glucosidase) was pre-incubated with the appropriate concentration of extract solution in a final volume of 140 μL for 15 min at 37°C. Then, the mixture was incubated for 20 min at 37°C with 20 μL of the substrate [3.5 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG)]. The reaction was stopped with 0.2 M sodium-carbonate and the absorbance was read at 405 nm.

The results for both assays were expressed as the concentration of the extract (mg/mL) which causes 50% inhibition (IC_{50} value) of enzyme activity. The commercial medicine Glucobay® was used as the positive control.

Antimicrobial activity. Antimicrobial activity was assessed by the microdilution method according to Soković *et al.* (2010) and Kostić *et al.* (2017). The microdilution method was carried out using serial dilutions of *R. discolor* aqueous and ethanol fruit extracts in Tryptic soy broth to estimate their antibacterial properties and in Malt broth for their antifungal properties. Then, the appropriate concentrations of microbial cultures (1×10^5 CFU / mL) in sterile saline solutions were added to each well with the exception of the negative control. Antimicrobial activity was evaluated against three bacteria: *Bacillus cereus* (clinical isolate), *Staphylococcus aureus* ATCC 6538 and *Enterobacter cloacae* ATCC 35030, and eight fungi: *Aspergillus fumigates* (human isolate), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC12066), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum* var. *cyclopium* (food isolate). The results were expressed as minimum inhibitory (MICs) and minimum bactericidal/fungicidal concentrations (MBCs/MFCs). Commercially available drugs, ampicillin and ketoconazole, were used as the positive control.

RESULTS

Phenolic acid content. In the present study, the presence of 11 phenolic acids was confirmed in the examined extracts (Table 2). The four hydroxybenzoic acids – *p*-hydroxybenzoic, protocatechuic, vanillic and gallic acid were quantified in the extracts, while the concentration of 2.5-dihydroxybenzoic acid was beyond the limit of quantification (LOQ). Among the hydroxycinnamic acids, *p*-coumaric, caffeic and ferulic acid were found in small quantities in both extracts, cinnamic and *o*-coumaric acids were not detected, while chlorogenic acid was present only in the aqueous extract. In both extracts, the total amount of hydroxybenzoic acids was higher than the total amount of hydroxycinnamic acids, with protocatechuic (14.6 and 4.4 mg/100 g in the FW and FE extracts, respectively) and gallic acid (15.5 and 6.2 mg/100 g in the FW and FE extracts, respectively) being the most dominant.

Anthocyanin content. Four cyanidin derivatives were detected and semi-quantified in the examined extracts by LC-DAD/MS (Table 3). The dominant compound in both extracts was cyanidin-glucoside/galactoside (671 and 2680 mg of cyanidin equivalents/100 g in the FW and FE extracts, respectively). The total amount of anthocyanins detected in the ethanol extract (3131 mg of cyanidin equivalents/100 g of dry extract) was much higher than in the aqueous extract (745 mg of cyanidin equivalents/100 g), and was also much higher than the amount of detected phenolic acids.

Free radical scavenging activity. The radical scavenging activity results for the examined extracts in the DPPH and ABTS assays are presented in Table 4. The FE extract expressed higher free radical scavenging activity than the FW in both the DPPH and ABTS assays (EC_{50} values). The activity was significantly lower in comparison with well-known antioxidants – BHA and ascorbic acid.

Antiproliferative activity. The examined extracts were ineffective in inhibiting the growth of cancerous cells (Table 4). The IC_{50} values towards all three malignant cell lines, HeLa, K562, MDA-MB-453, as well as the control cell line, MRC-5, were higher than 2000 μg/mL, indicating the lack of activity and selectivity for both the examined extracts.

Enzyme-inhibitory activity. The examined extracts were active in inhibiting carbohydrate-hydrolysing enzymes whose role is linked with diabetes mellitus type 2 (Table 4).

Table 2. The content of phenolic acids in *R. discolor* aqueous and ethanol fruit extracts determined by LC-MS/MS.

Compound	Mw	t_R (min)	FW[a] (mg/100 g)	FE[a] (mg/100 g)
p-Hydroxybenzoic acid	138	2.20	1.2±0.04	1.1±0.04
Protocatechuic acid	154	1.62	14.6±0.68	4.4±0.20
2.5-Dihydroxybenzoic acid	154	2.06	<LoQ	<LoQ
Vanilic acid	168	2.45	2.1±0.08	1.3±0.05
Galic acid	170	1.28	15.5±0.33	6.2±0.13
Cinammic acid	148	7.28	<LoQ	<LoQ
p-Coumaric acid	164	3.26	0.6±0.02	0.6±0.02
o-Coumaric acid	164	4.90	<LoQ	<LoQ
Caffeic acid	180	2.29	1.3±0.04	1.5±0.04
Ferulic acid	194	3.57	0.9±0.04	0.9±0.04
Chlorogenic acid	354	1.67	0.5±0.04	<LoQ
Sum of hydroxybenzoic acids (TBA)	/	/	33.4	13
Sum of hydroxycinammic acids (TCA)	/	/	3.3	3.0

Mw – molecular weight of compound; tR – retention time; FW – aqueous fruit extract; FE – ethanol fruit extract; The results are given as the concentration mg/100 g of extract dry weight ± standard error of repeatability (as determined by method validation).

Table 3. The content of anthocyanins in *R. discolor* aqueous and ethanol fruit extracts as determined by LC-DAD-MS and expressed in cyanidin equivalents.

Compound	Mw	t_R (min)	FW (mg/100 g)	FE (mg/100 g)
Cyanidin-glucoside/ galactoside	448	10.21	671±24	2680±74
Cyanidin-arabinoside	418	11.66	29±5	349±35
Cyanidin derivatives 1	534	12.10	23±0	41±5
Cyanidin derivatives 2	592	12.45	22±1	61±5
Sum of anthocyanins (TA)	/	/	745	3131

Mw – molecular weight of compound; tR – retention time; FW – aqueous fruit extract; FE – ethanol fruit extract; The results are given as the mean ± SD of three measurements (three extractions).

Table 4. Free radical scavenging (DPPH, ABTS), antitumor and enzyme-inhibitory (α-amylase inhibitory activity (α-AIA), α-glucosidase inhibitory activity (α-GIA)) activity of *R. discolor* aqueous (FW) and ethanol (FE) fruit extracts.

Activity	Free radical scavenging		Antitumor activity				Enzyme-inhibitory activity	
	DPPH	ABTS	HeLa	K562	MDA-MB-453	MRC-5	α-AIA	α-GIA
Units	EC_{50}(μg/mL)	EC_{50}(μg/mL)	IC_{50} (μg/mL)				IC_{50}(mg/mL)	IC_{50} (μg/mL)
Extract/Control								
FW	179.33±4.31	68.12±0.01	>2000	>2000	>2000	>2000	275.93±1.35	44.52±6.66
FE	162.76±1.03	41.38±1.02	>2000	>2000	>2000	>2000	65.18±0.65	80.72±3.97
***L*-ascorbic acid**	3.74±0.12	2.27±0.10	/	/	/	/	/	/
BHA	5.43±0.02	nt	/	/	/	/	/	/
Glucobay	/	/	/	/	/	/	0.20±0.01	233.38±34.44

The ethanol extract showed more than four-fold higher α-amylase inhibitory activity in comparison to the aqueous extract (IC_{50} 65.18 mg/mL *vs.* 275.93 mg/mL). On the contrary, the aqueous extract was shown to be more efficient in inhibiting α-glucosidase. However, both extracts were more active in inhibiting α-glucosidase than α-amylase with IC_{50} values lower than the positive control, Glucobay®.

Antimicrobial activity. Both extracts showed antibacterial activity with MICs and MBCs ranging from 2.19 to 22.73 mg/mL and 4.38 to 45.45 mg/mL, respectively (Table 5). The tested pathogenic bacteria were more sensitive to the ethanol than the aqueous extract. The ethanol extract was particularly effective against *E. cloacae*. The officially used antibiotic (ampicillin) showed better antibacterial features than the examined extracts (the MIC values varied between 0.10 and 0.17 mg/mL and the MBCs were 0.20 mg/mL). The results of antifungal activity are presented in Table 6. The MICs were among 1.09 and 11.26 mg/mL, while the MFCs varied from 2.17 to 22.52 mg/mL. The positive control, ketoconazole, exhibits lower MIC (0.20 to 1.33 mg/mL) and MFC (0.27 to 2.00 mg/mL) values. In contrary to the tested bacteria, the growth of the examined fungi was more affected by the aqueous extract. *Aspergillus* species were particularly susceptible.

Table 5. Antibacterial activity of *R. discolor* aqueous (FW) and ethanol (FW) fruit extracts.

Extract/Control	FW		FE		Ampicillin	
	MIC	MBC	MIC	MBC	MIC	MBC
Units	mg/mL	mg/mL	mg/mL	mg/mL	mg/mL	mg/mL
Tested bacteria						
Bacillus cereus (clinical isolate)	5.68	11.36	4.38	8.77	0.17	0.20
Staphylococcus aureus ATCC 6538	22.73	45.45	4.38	8.77	0.10	0.20
Enterobacter cloacae ATCC 35030	11.36	22.73	2.19	4.38	0.17	0.20

DISCUSSION

Previous publications lack data concerning the chemical composition of *R. discolor*. However, Schulz *et al.* (2019) recently examined the content of polyphenols in *R. ulmifolius* Schott fruits and also found that the most dominant phenolic acids are protocatechuic and gallic acid, the same as we found for *R. discolor* in our study. Additionally, they confirmed the presence of caffeic, *p*-coumaric, ferulic, chlorogenic and vanillic acids in the *R. ulmifolius* extract, which were also found in our study of the fruits of *R. discolor*. On the other hand, Oszmiański *et al.* (2015) reported significantly higher concentrations of phenolic acids (862 to 4314 mg/100g) in the leaves of wild *Rubus* species.

Anthocyanins are the major group of flavonoids found in blackberry fruits. Their composition is usually influenced by many different factors (light exposure, temperature, nitrogen and phosphate deficiencies, genetic factors etc.) and is therefore highly variable (Chaves-Silva *et al.* 2018). Nevertheless, the most commonly found anthocyanins are cyanidin derivatives, particularly glycosides, which is in line with our results (Sultana 2018; Schulz & Chim 2019). On the other hand, the leaf extracts of *Rubus* species generally contain insignificant quantities of anthocyanins.

According to recent epidemiological studies, less than 10% of the population consume 400 g of fruits and vegetables per day, which is the daily intake recommended by the FAO/WHO (Radhika *et al.* 2011). Thus, the presented results are significant since they indicate that far lower amounts of *R. discolor* are needed to achieve health-promoting effects. This would be of particular interest in the post-Covid era due to growing awareness of the beneficial effects of plant foods (Galanakis *et al.* 2021).

The free radical scavenging activity of various *Rubus* species has been previously reported by many authors (Muniyandi *et al.* 2019; Shamsudin *et al.* 2019; Veljković *et al.* 2019; Zengin *et al.* 2019; Abdel-Hamid *et al.* 2020; Samaniego *et al.* 2020). Muniyandi *et al.* (2019) examined the antioxidant activity of *R. niveus* Thunb., *R. ellipticus* Smith and *R. fairholmianus* Gard. fruit extracts of different polarity and obtained lower EC_{50} values for the alcoholic than the aqueous extracts which are consistent with the results presented here. Similarly, Shamsudin *et al.* (2019) proved higher antioxidant activity of ethanol

Table 6. Antifungal activity of *R. discolor* aqueous (FW) and ethanol (FE) fruit extracts

Extract/Control	FW		FE		Ketoconazole	
	MIC	MFC	MIC	MFC	MIC	MFC
Units	mg/mL	mg/mL	mg/mL	mg/mL	mg/mL	mg/mL
Tested fungi						
Aspergillus fumigatus (human isolate)	2.89	5.79	4.47	8.94	0.23	0.67
Aspergillus versicolor ATCC 11730	1.38	2.76	2.13	4.27	0.20	0.47
Aspergillus ochraceus ATCC 12066	1.43	2.87	1.11	2.21	0.20	0.27
Aspergillus niger ATCC 6275	1.40	2.82	1.09	2.17	0.27	0.42
Trichoderma viride IAM 5061	1.43	2.87	11.26	22.52	0.83	2.00
Penicillium funiculosum ATCC 36839	2.84	5.68	2.19	4.39	0.23	0.67
Penicillium ochrochloron ATCC 9112	2.74	5.48	2.12	4.23	1.33	1.67
Penicillium verrucosum var. *cyclopium* (food isolate)	2.79	5.58	8.62	17.23	0.27	0.40

and methanol *R. fraxinifolius* Poir. fruit extracts than the aqueous extracts when using the same assays. On the other hand, in another study, *R. sanctus* Schreb. and *R. ibericus* Juz. aqueous leaf extracts showed a higher scavenging capacity for DPPH and ABTS free radicals than methanol extracts (Zengin *et al*. 2019). The variations in phenolic profiles among fruits and leaves, particularly the higher content of phenolic acids in leaves, could serve as the explanation for the differences in its bioactivity which has also been noted by some authors (Sultana 2018; Shamsudin *et al*. 2019; Veljković *et al*. 2019).

Our free radical scavenging activity results could be associated with anthocyanins since their content is higher in the ethanol extract, whilst phenolic acids were more abundant in the aqueous extract. These findings are in accordance with previous publications (Muniyandi *et al*. 2019; Schulz *et al*. 2019; Veljković *et al*. 2019).

Up to now, there has been no data about the antiproliferative activity of *R. discolor* fruit extracts. However, the antiproliferative activity of various *Rubus* species has been previously evaluated (Skupień *et al*. 2006; Durgo *et al*. 2012; Muniyandi *et al*. 2019; Veljković *et al*. 2019; Abdel-Hamid *et al*. 2020). Muniyandi *et al*. (2019) proved weak antiproliferative activity (IC_{50} ranged from 9.6 to 10 mg/mL) of methanol fruit extracts of *R. ellipticus*, *R. niveus* and *R. fairholmianus* on human colon cancer cells (CaCo-2). Similarly, Veljković *et al*. (2019) evaluated the antitumor properties of leaf and fruit extracts of wild-growing *R. idaeus* L. and found very low or no activity for the fruit extracts, which supports our results.

The digestive enzymes α-amylase and α-glucosidase hydrolyze α-1,4-glucosidic bonds in starch causing post-prandial hyperglycaemia in patients with diabetes mellitus type 2 (Spínola *et al*. 2019; Tian *et al*. 2021). Despite this being the first report of the enzyme – inhibitory activity of *R. discolor* fruits, the α-amylase and α-glucosidase inhibitory activity of different *Rubus* species has been reported in several recently published papers (Salehi *et al*. 2013; Grochowski *et al*. 2019; Spínola *et al*. 2019; Zengin *et al*. 2019; Tian *et al*. 2021). In the present study the examined extracts of *R. discolor* fruits showed low efficacy in inhibiting α-amylase activity. However, they were shown to be more potent in α-glucosidase inhibition even when compared to commercially available medicines. The previously published results of the antidiabetic potential of *R. fruticosus* L., *R. caesius* L. and *R. grandifolius* L. extracts also indicate significantly higher enzyme-inhibitory activity for α-amylase than for α-glucosidase (Salehi *et al*. 2013; Grochowski *et al*. 2019; Spínola *et al*. 2019). Those findings are of particular interest because the strong inhibition of α-amylase leads to digestive disconformities due to the fermentation of undigested starch which are the main side effects of officially used drugs in the treatment of diabetes mellitus type 2. Therefore, the use of pharmaceuticals with high α-glucosidase and moderate or weak α-amylase inhibitory activity is highly recommended to control postprandial hyperglycaemia (Spínola *et al*. 2019; Zengin *et al*. 2019; Tian *et al*. 2021).

The antimicrobial effects of *Rubus* species are rarely evaluated, especially with regard to fungi strains. Thus, the results which have been published to date refer predominantly to *R. idaeus*, *R. occidentalis* L., *R. fruticosus* and *R. ulmifolius* (da Silva *et al*. 2019; Schulz & Chim 2019), indicating moderate antibacterial activity (MICs from 5 to 20 mg/mL and MBCs higher than 20 mg/mL) against *Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Listeria monocytogenes* and *S. aureus* including methicillin-resistant strains. The aforementioned MIC/MBC values are close to those presented here for *R. discolor* fruit extracts. Riaz *et al*. (2011) proved no antifungal activity of *R. fruticosus* methanolic extract on 9 pathogenic fungal strains, while we have found moderate antifungal activity of *R. discolor* against all the investigated fungal strains, especially against *A. versicolor*, *A. ochraceus* and *A. niger*. The antimicrobial properties of *Rubus* species have previously been associated with polyphenol compounds and their synergistic effects (da Silva *et al*. 2019; Schulz & Chim 2019; Veljković *et al*. 2019; Staszowska-Karkut & Materska 2020). According to our results, the higher antibacterial activity of the ethanol extract could be attributed to the higher content of anthocyanins. Conversely, the

water extract showed higher antifungal activity in most of the cases which could be ascribed to phenolic acids, since their concentration was higher in the FW extract.

CONCLUSION

The results of our study point out that *R. discolor* fruit extracts are a good source of phenolic acids and anthocyanins known for their beneficial effects on human health. The examined extracts revealed diverse bioactivities such as free radical scavenging, enzyme-inhibitory, antibacterial and antifungal activities, but also the absence of antitumor activity. The presented results highlight the effectiveness of both extracts in inhibiting α-glucosidase, the enzyme linked with type-2 diabetes, where the effect was even stronger than the positive control. However, α-amylase inhibitory activity was significantly weaker than the positive control. These findings justify the use of blackberries in traditional medicine and recommend it as a potentially valuable source for food supplements, and the prevention and control of diabetes mellitus type 2.

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REZIME

Botanica SERBICA

Fenolni profil i biološki potencijal plodova divlje kupine (*Rubus discolor*)

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Plodovi divlje kupine (*Rubus discolor*) se smatraju bogatim izvorom fitohemikalija sa potencijalno važnom ulogom u prevenciji najzastupljenijih savremenih bolesti. U vezi sa tim, cilj ove studije je bilo određivanje sastava fenolnih kiselina i antocijanina u vodenim i etanolnim ekstraktima plodova *R. discolor*, utvrđivanje njihove aktivnosti u neutralisanju slobodnih radikala, kao i antiproliferativne, antidijabetične i antimikrobne aktivnosti. LC-MS/MS analizom je kvantifikovano 11 fenolnih kiselina, među kojima su se protokatehinska i galna kiselina izdvojile kao dominantne komponente. Pored toga, urađena je semikvantifikacija pet antocijanina među kojima je dominirao cijanidin-glukozid/galaktozid. Etanolni ekstrakt je bio efikasniji u neutralisanju slobodnih radikala u odnosu na vodeni. Odsustvo antiproliferativne aktivnosti je utvrđeno za oba ekstrakta. Nasuprot tome, oni su uspešno inhibirali digestivne hidrolizujuće enzime ugljenih hidrata čije je dejstvo u vezi sa dijabetesom tipa II. Štaviše, dobijene IC_{50} vrednosti (44.52 i 80.72 μg/mL, za vodeni i etanolni ekstrakt, respektivno) ukazuju da su testirani ekstrakti efikasnije inhibirali α-glukozidaza enzim nego pozitivna kontrola (233.38 μg/mL). Etanolni ekstrakt je pokazao bolje antibakterijsko dejstvo, a vodeni antifungalno. Kvantifikovana fenolna jedinjenja, kao i prezentovane bioaktivnosti plodova kandiduju ovu vrstu divljih kupina kao potencijalni resurs bioaktivnih komponenti koje mogu naći primenu u prehrambenoj, farmaceutskoj i kozmetičkoj industriji.

Ključne reči: Rosaceae, fenolne kiseline, antocijanini, antioksidantna aktivnost, antimikrobna aktivnost, enzim-inhibirajuća aktivnost