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Phenol composition, radical scavenging activity and antimicrobial activity of berry leaf extracts

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Berry leaves are recognized as potential medicaments which are rich in different phenolic compounds, and have been used in folk medicine for centuries. In order to evaluate phenol composition, berry leaf extracts were subjected to spectrophotometric and HPLC analysis. The radical scavenging activity was estimated using the DPPH test and the antimicrobial activity by the microwell dilution test. All extracts showed high phenol content but different compositions of phenol compounds. Flavonols, flavan-3-ols and phenolic acids were the main phenol classes found in the investigated leaf extracts. All extracts showed significant radical scavenging activity correlating with the total phenol content. Significant antimicrobial activity was found against Gram-positive, followed by Gram-negative strains, and yeast in all tested leaf extracts. All berry leaf extracts, rich in phenolic content, with significant antiradical and antimicrobial activity, can be used as food and medical supplements.

Keywords: Berry leaf extracts / phenolic compounds / radical scavenging activity / antimicrobial activity

INTRODUCTION

Phenolic compounds are produced by plants, both edible and inedible, as a response to the environmental stress and pathogens. They are present in all plant parts in different quantities, depending on the stage of plant development and the environment influence. These compounds are recognized as potential antioxidant agents with possible applications as food and medical ingredients. Berry fruits are recognized as plants which are rich in different phenolic compounds and have been used in folk medicine for centuries. Also, berry leaves are traditionally used for easing childbirth-related muscle spasms, morning sickness, for colds, sore throats, diarrhea, threat wounds, colic pain, uterine relaxant, etc. [1-3].

Berry fruits, such as grape, blueberry, chokeberry, bilberry, cranberry, blackberry, raspberry, blackcurrant, strawberry, etc. are a particularly rich source of antioxidants [4-8]. There have been many studies on antioxidant, anti-cancer, anti-inflationary, antimicrobial activities of berry extracts which are rich in polyphenol content [1-11]. There are also studies on the beneficial effects of these compounds on heart and other chronic diseases [12, 13]. However, there is less research of the antimicrobial activity [6] and the antioxidant activity and polyphenol content of berry fruit leaf extracts [8, 14, 15].

The objectives of this study were to identify the phenolic compounds from berry leaf extracts and to determine their radical scavenging activity and antimicrobial activity.

MATERIALS AND METHODS

Chemicals

Methanol, acetonitrile and formic acid of HPLC-grade were obtained from Merck (Darmstadt, Germany). The standard phenolic compounds, 2,2`-diphenyl-1-picrylhydrazyl (DPPH) free radical and all other chemicals were supplied from Sigma Chemical Co. (St. Louis, MO). The reagents used were of analytical quality.

Samples

The berry leaves were collected from the southern Serbia region after harvest. The collected samples of berry leaves from both domestic and wild species are shown in Table 1. Samples of berry leaves were washed and dried at 60 °C. Dried leaves were crushed in a grinder for 2 min and then used for extractions.

The samples of dry leaves (0.5 g DW, dry weight) were extracted with 40 mL of the solvent system methanol/acetone/water/acetic acid (30/42/27.5/0.5) by continuously stirring at room

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temperature in the dark for 30 min, and then centrifuged for 10 min at 2500 g. The extracts were evaporated in a vacuum rotary evaporator and diluted in 10 mL methanol. Extracts were filtered through a 0.45 μm syringe filter before analysis.

Spectrophotometric assay

Total phenols, hydroxycinnamoyl tartaric acids and flavonols in the tested extracts were determined according to the spectrophotometric method previously described [9]. Results were expressed as milligrams (mg) of gallic acid equivalents (GAE) for total phenols, mg of caffeic acid equivalents (CAE) for total hydroxycinnamoyl tartaric acids, and mg of quercetin equivalents (QE) for total flavonols per gram (g^{-1}) of extract dry matter (DM).

HPLC assay

Phenol composition of selected extracts was analyzed by high performance liquid chromatography (HPLC). The apparatus used for separation and determination of individual phenols from leaf extracts was an Agilent Technologies 1200 chromatographic system, equipped with a photodiode array detector (DAD) and fluorescence detectors (FD). The column was thermostated at 30 °C. The separation was performed on an Agilent-Eclipse XDB C-18 4.6 × 150 mm column. The HPLC grade solvents used were formic acid / water (5 : 95 v/v) as solvent A and acetonitrile / formic acid / water (80 : 5 : 15 v/v) as solvent B. The elution gradient was described previously [9]. The injection volume was 5 μL and the flow rate was 0.8 mL min^{-1}. The detection wavelengths were 280, 320 and 360 nm for UV, and 275/322 nm ($\lambda_{Ex}/\lambda_{Em}$) for fluorescence detection. The different phenolic compounds were identified by comparing their retention times and spectral characteristics with data of original reference standard compounds and with data given in the literature [16]. The calibration curves (five data points, n=2) were linear with $R^2 = 0.99$. Results were expressed as mg g^{-1}extract DM.

DPPH test

Antioxidant activity of all investigated extracts was estimated, determining the radical scavenging activity of extracts by the DPPH test previously described [10]. The antiradical activities of the investigated extracts were expressed as median efficient concentrations (EC_{50}) which represent the concentration of extract (mg L^{-1}) needed for a decrease in absorbance of DPPH solution by 50%.

Antimicrobial activity

The antimicrobial activity of the test samples was evaluated using the following laboratory control strains: *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 (Gram (-) bacteria) and *Candida albicans* ATCC 10231 (Yeast) obtained from the American Type Culture Collection. A broth microdilution method [9] was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). A serial doubling dilution of the testing samples were prepared in a 96/well microtiter plate over the range of 1500 - 0.25 μg mL^{-1} in inoculated nutrient broth (the final volume was 100 μL and the final bacterial concentration was 10^6 CFU mL^{-1} in each well). Two growth controls consisting of a medium with methanol (negative control) and a medium with tetracycline (positive control) were also included. The microbial growth was determined by measuring the absorbance at 620 nm using the universal microplate reader (ThermoLabsystems, Multiskan EX, Software for Multiscan ver.2.6.). MIC is defined as the lowest concentration of the test samples at which microorganisms showed no visible growth. The MBC is defined as the lowest concentration of the test samples at which 99.9 % of inoculated microorganisms were killed.

Statistical analysis

All experiments were performed in triplicate. Values are presented as mean ± standard deviation. Significant differences were determined by analysis of variance (ANOVA), followed by the Tukey test.

RESULTS AND DISCUSSION

Phenol content of extracts

The quantification of total phenols (TPC), hydroxycinnamoyl tartaric acids (HTAC) and flavonols (FC) in berry leaf extracts was performed by the spectrophotometric assay described in the experimental section. The results of the spectrophotometric assay of berry leaf extracts are shown in Table 1. The applied spectrophotometric assay is simple and provides fast information on TPC, HTAC and FC in the tested extracts. The

results showed high TPC in all tested berry leaf extracts, which ranged from 98.04 ± 0.20 to 119.14 ± 0.76 mg GAE g^{-1} extract DM. The TPC in the berry leaf extracts significantly differed between wild and domestic berries. The highest TPC were in the WBB extract, followed by extracts of DR, BT, HT, EC, BC, DBB, RC and RB. Wang and Lin [17] also reported high TPC in blackberry, raspberry and strawberry leaf extracts, which ranged from 47.2 ± 1.3 to 120.4 ± 2.8 mg GAE g^{-1} extract DM. They reported that TPC in those extracts mostly depends on berry variety and collecting date (young and old leaves).

Table 1. The species of collected domestic and wild berry fruit leaves.

Leaf code	Species	
	Domestic Species	
RC	Red Currant	*Ribes rubrum*
BC	Black Currant	*Ribes nigrum*
RB	Raspberry	*Rubus idaeus*
DBB	Blackberry	*Rubus fruticosus*
	Wild Species	
EC	European cornel	*Cornus mas*
DR	Dog rose	*Rosa canina*
HT	Hawthorn	*Crataegus L*
BT	Blackthorn	*Prunus spinosa*
WBB	Blackberry	*Rubus fruticosus*

Significant amounts of flavonols were found in all tested leaf extracts. Their content ranged from 30.74 ± 0.18 in HT to 39.14 ± 0.22 mg QE g^{-1} DM in the WBB extract. The hydroxycinnamoyl tartaric acids were also quantified by this method, but in lower amounts. As such compounds we consider all compounds that have strong absorbance at 320 nm such as hydroxycinnamoyl esters and also free hydroxycinnamoyl acids. The highest HTAC was in the RC extract, followed by WBB, DR, HT, DBB, BT, EC, RB and BC extracts.

In order to determine more precisely the phenolic content and composition of the investigated extracts, the HPLC assay was used. Results (Table 3) are in good agreement with those obtained by spectrophotometric determination of TPC, HTAC and FC (Table 2). The results showed quite different phenolic composition, which mainly includes the three phenolic classes: hydroxycinnamoyl acids, flavonols and flavan-3-ols (Table 3). Other authors also found the presence of these phenolic classes in some berry leaf extracts [7, 8, 14, 15].

Gallic, ellagic and chlorogenic acid were present in all tested leaf extracts, while caffeic acid was found only in RC, RB, DBB and DR. Vagiri*et et al*. [14] reported the presence of chlorogenic and neo chlorogenic acids in BC leaf extracts. Buricova *et al*. [8] reported the presence of gallic acid in some *Rubus* species.

In all tested extracts (-)-epicatechin was predominantly flavan-3-ol, followed by (+)-catechin, (-)-epicatechin gallate and procyanidin B2. The presence of these compounds was reported by Vagiri *et al*. [14] in BC leaf extracts and by Buricova *et al*. [8] in blackberry and raspberry leaf extracts.

Table 2. Total phenol, hydroxycinnamoyl tartaric acid and flavonol contents (mg g^{-1}DM), antioxidant activity of berry leaf extracts EC_{50} (mg mL^{-1}) and their correlation with EC_{50} (R^2).

Extract	Total phenols	Hydroxy-cinnamoyl tartaric acid	Flavonol	Antioxidant activity, EC_{50}
RC	101.14 ± 0.93a	9.75 ± 0.09c	36.71 ± 0.25b	0.50 ± 0.08b
BC	105.78 ± 0.89a	8.19 ± 0.13a	35.48 ± 0.29b	0.69 ± 0.07b
RB	98.04 ± 0.20a	8.24 ± 0.18a	31.54 ± 0.17a	0.72 ± 0.02b
DBB	104.72 ± 0.19a	8.68 ± 0.22a	32.92 ± 0.16a	0.67 ± 0.03b
EC	112.91 ± 0.40b	8.27 ± 0.08a	32.77 ± 0.19a	0.58 ± 0.03b
DR	117.34 ± 0.28b	8.98 ± 0.09b	33.51 ± 0.14b	0.39 ± 0.06a
HT	115.62 ± 0.31b	8.89 ± 0.08b	30.74 ± 0.18a	0.42 ± 0.01a
BT	115.76 ± 0.38b	8.34 ± 0.05a	34.82 ± 0.19b	0.44 ± 0.09a
WBB	119.14 ± 0.76b	9.18 ± 0.28b	39.14 ± 0.22b	0.35 ± 0.02a
R^2	0.6695	0.3280	0.1365	

Data are expressed as mean ± SD (n = 3); mean values with different letters within the same column are significantly different ($p<0.05$).

Table 3. Phenol composition (mg g^{-1} DM) of berry leaf extracts determined by HPLC analysis and their correlation with EC_{50} (R^2).

Phenolic compound	RC	BC	RB	DBB	EC	DR	HT	BT	WBB	R^2
Gallic acid	0.22 ±0.02	0.18 ±0.01	0.27 ±0.02	0.30 ±0.04	0.41 ±0.02	1.76 ±0.06	1.21 ±0.05	1.14 ±0.04	1.09 ±0.02	0.6883
Ellagic acid	4.30 ±0.04	4.15 ±0.01	4.38 ±0.02	4.48 ±0.04	3.55 ±0.04	3.31 ±0.03	4.09 ±0.02	4.36 ±0.03	4.24 ±0.04	0.3803
Caffeic acid	0.39 ±0.01	nd	0.27 ±0.02	0.13 ±0.01	nd	0.52 ±0.02	nd	nd	nd	0.0036
Chlorogenic acid	1.26 ±0.04	0.21 ±0.01	0.39 ±0.02	0.56 ±0.02	0.28 ±0.03	0.31 ±0.02	0.19 ±0.01	0.37 ±0.02	0.27 ±0.03	0.0030
Quercetin-3-glucoside	9.07 ±0.15	7.14 ±0.21	8.11 ±0.12	9.04 ±0.10	9.28 ±0.21	7.19 ±0.14	9.14 ±0.15	8.76 ±0.20	10.44 ±0.24	0.1467
Rutin	6.14 ±0.10	5.84 ±0.17	4.78 ±0.10	5.11 ±0.09	6.11 ±0.11	5.67 ±0.10	4.28 ±0.12	5.10 ±0.16	6.12 ±0.10	0.0147
Luteolin-3-glucoside	0.62 ±0.05	nd	nd	nd	0.11 ±0.01	1.10 ±0.03	0.25 ±0.03	0.95 ±0.02	nd	0.3146
Myricetin	2.74 ±0.05	nd	nd	nd	nd	1.18 ±0.06	nd	1.28 ±0.03	1.17 ±0.04	0.2761
Kaempferol-3-glucoside	3.11 ±0.09	4.10 ±0.06	2.78 ±0.02	2.14 ±0.05	4.27 ±0.10	3.11 ±0.07	4.13 ±0.09	2.95 ±0.05	4.78 ±0.10	0.1518
Quercetin	2.28 ±0.08	3.52 ±0.11	3.01 ±0.09	2.07 ±0.05	nd	3.57 ±0.09	nd	2.57 ±0.07	4.11 ±0.12	0.0030
(+)-Catechin	2.08 ±0.08	0.92 ±0.02	2.47 ±0.09	2.01 ±0.08	2.22 ±0.07	1.47 ±0.05	2.01 ±0.05	0.72 ±0.03	1.52 ±0.04	0.4558
(-)-Epi-catechin gallate	1.14 ±0.04	0.46 ±0.02	nd	1.17 ±0.04	nd	2.35 ±0.10	1.12 ±0.07	1.76 ±0.07	2.14 ±0.08	0.6584
(-)-Epi-catechin	3.76 ±0.11	1.27 ±0.05	3.78 ±0.13	3.45 ±0.10	4.07 ±0.15	3.03 ±0.09	1.37 ±0.05	3.12 ±0.09	3.76 ±0.10	0.3871
Procyanidin B2	nd	2.78 ±0.09	nd	1.87 ±0.06	nd	3.38 ±0.14	nd	2.14 ±0.12	2.11 ±0.09	0.0452
∑ Phenolic acids	6.17	4.54	5.31	5.47	4.24	5.90	5.49	5.87	5.60	0.2931
∑ Flavan-3-ols	6.98	5.43	6.25	8.50	6.29	10.23	4.50	7.74	9.53	0.5381
∑ Flavonols	23.96	20.60	18.68	18.36	19.77	21.82	17.80	21.61	26.62	0.3336
∑ Total phenols	37.11	30.57	30.24	32.33	30.30	37.95	27.79	35.22	41.75	0.5934

Data are expressed as mean ± SD (n = 3); nd – not detected.

The quercetin-3-glucoside, rutin, kaempherol-3-glucoside and quercetin were the predominant flavonols while luteolin-3-glucoside and myricetin were less abundant. The high concentrations of quercetin (6.84 – 8.11 mg g^{-1} DM) and kaempherol (0.73 – 3.75 mg g^{-1} DM) in leaves of *Rosa* L. species was reported [7] which were similar to our results, and also for *Rubus* L. [8, 15] and BC leaves [14].

Radical scavenging activity of extracts

The results of the radical scavenging activity of extracts, expressed as EC_{50} values (mg mL^{-1}) are shown in Table 2. Lower EC_{50} values correspond to higher radical scavenging activity of the extracts. The highest radical scavenging activity was shown by the WBB leaf extract, followed by DR, HT, RB, BT, RC, EC, DBB, BC and RB leaf extracts. The radical scavenging activity in wild berry leaf extracts was stronger than in domestic berry leaf extracts. Strong radical scavenging activity of leaf extracts, corresponding to their high phenol content, suggests that the phenolic compounds are at least partially responsible for the strong radical scavenging activity of these extracts. A correlation (R^2 = 0.6695) was found between the radical scavenging activity and the total phenol content. Other authors also found a correlation between radical scavenging activity and total phenol content in some leaf extracts [2, 7, 17]. We also found a

correlation between the radical scavenging activities and the individual classes of phenols, but lower than with total phenol content (Table 3), which is in agreement with literature data [7]. The HPLC analysis showed that extracts of berry leaves are a mixture of phenolic and other compounds, e.g., ascorbic acid, not all identified in this study. It is possible that these constituents may interact to produce synergistic or antagonistic antioxidant effects with each other and with other compounds.

Antimicrobial activity of extracts

The antimicrobial activity data for all investigated extracts and an antibiotic against 13 microbial species are given in Table 4. Methanol did not show any inhibitory effects on the 13 microbial species. WBB leaf extracts showed the highest antimicrobial activity, followed by BC, DR, EC, RC, DBB, RB, BT and HT leaf extracts. The antimicrobial activity of these extracts can be connected with their high total phenol content. RC, BC, DR leaf extracts had the highest total phenol content and showed the strongest antimicrobial activity. The existing correlation between total phenol content and antimicrobial activity of plant extracts was also reported by others [3-6].

The investigated leaf extracts were in general more sensitive on Gram-positive strains compared to Gram-negative strains and yeast, which is in agreement with literature data [6]. *Sarcina lutea, Listeria monocytogenes* and *Staphylococcus aureus* were the most sensitive Gram-positive strains, and *Shigella sonnei* and *Pseudomonas aeruginosa* the most sensitive Gram-negative strains for the most investigated leaf extracts.

CONCLUSIONS

Both methods, spectrophotometric and HPLC, confirmed the high phenol content in all investigated leaf extracts from both domestic and wild berries. These compounds are responsible for the significant antioxidant and antimicrobial activities of all leaf extracts. Simple extraction procedure of these compounds from leaves opens the possibility for their application in food and pharmaceutical industry.

Table 4. Antibacterial (MIC)/bactericidal (MBC) activities (μg mL^{-1}) of berry leaf extracts and reference antibiotic against Gram-positive strains, Gram-negative strains and yeast

	RC	BC	RB	DBB	EC	DR	HT	BT	WBB	Te.
Gram-positive strains										
Clostridium perfringens	63/ 125	31/ 31	125/ 250	125/ 250	31/ 31	16/ 16	250/ 500	250/ 500	16/ 16	0.9/ 0.9
Bacillus cereus	63/ 63	31/ 63	125/ 250	125/ 125	31/ 63	16/ 63	250/ 250	125/ 250	16/ 63	0.9/ 0.9
Staphylococcus aureus	31/ 63	16/ 16	63/ 125	63/ 125	16/ 31	16/ 31	125/ 250	125/ 125	8/ 16	0.12/ 0.9
Listeria monocytogenes	31/ 63	16/ 16	63/ 125	31/ 63	16/ 16	16/ 31	125/ 250	63/ 125	8/ 16	0.46/ 0.9
Sarcina lutea	31/ 63	8/ 16	63/ 125	63/ 125	16/ 31	16/ 16	125/ 250	63/ 125	8/ 8	0.06/ 0.06
Micrococcus flavus	125/ 250	31/ 63	250/ 500	125/ 250	63/ 63	31/ 63	500/ 750	250/ 500	31/ 63	0.4/ 0.9
Gram-negative strains										
Escherichia coli	187/ 375	63/ 125	250/ 500	187/ 375	125/ 250	125/ 250	500/ 750	250/ 500	63/ 125	3.8/ 7.5
Pseudomonas aeruginosa	94/ 187	63/ 63	125/ 250	94/ 187	63/ 125	31/ 125	250/ 500	125/ 250	31/ 63	7.5/ 7.5
Salmonella enteritidis	94/ 187	63/ 63	125/ 375	94/ 187	63/ 125	63/ 125	250/ 500	125/ 250	63/ 63	0.9/ 1.9
Shigella sonnei	63/ 187	31/ 63	125/ 250	63/ 187	31/ 125	16/ 31	250/ 375	125/ 187	16/ 63	0.06/ 0.12
Klebsiella pneumoniae	94/ 250	31/ 31	187/ 500	125/ 250	63/ 125	63/ 63	250/ 500	250/ 375	31/ 31	0.9/ 1.9
Proteus vulgaris	94/ 187	63/ 63	125/ 375	94/ 187	63/ 125	63/ 125	250/ 500	125/ 250	63/ 63	0.9/ 1.9
Yeast										
Candida albicans	250/ 500	250/ 500	500/ 750	250/ 500	125/ 250	125/ 250	750/ 1500	500/ 750	250/ 375	16/ 16

Te. – Tetracyclin; nt – not tested.

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СЪДЪРЖАНИЕ НА ФЕНОЛИ, СПОСОБНОСТ ЗА ПРЕМАХВАНЕ НА СВОБОДНИ РАДИКАЛИ И АНТИМИКРОБНА АКТИВНОСТ НА ЕКСТРАКТИ ОТ ЛИСТА НА ГОРСКИ ПЛОДОВЕ

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(Резюме)

Листата на горските плодове са признати като потенциални медикаменти, богати на фенолни съединения. Те са използвани в народната медицина от векове. За определянето състава на фенолните съединения екстрактите от листата на горските плодове са анализирани с високо-ефективна течна хроматография и спектофотометрия. Способността им да премахват свободни радикали е оценена с помощта на DPPH-тест, а антимикробната активност - чрез метода на последователно микроразреждане на средата. Всички екстракти показват високо съдържание на феноли. Главните групи феноли, намерени в изследваните екстракти са флавоноли, флавон-3-оли и фенолови киселини. Всички екстракти показват значителна активност спрямо свободните радикали, която се корелира с общото съдържание на феноли. За всички екстракти е установена значителна антимикробна активност спрямо Грам-положителни, следвана от активност спрямо Грам-отрицателни щамове и дрожди. Всички екстракти, показали антирадикална и антимикробна активност могат да се използват като хранителни добавки и лекарствени средства.