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ANTIMICROBIAL ACTIVITY AND THE QUANTITATIVE ANALYSES OF PHENOLIC COMPOUNDS AND HEAVY METALS OF RED MULBERRY EXTRACTS (*MORUS RUBRA* L.) FROM SERBIA

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ABSTRACT. All investigated extracts of red mulberry (*Morus rubra* L.) contain a high content of total phenols. The highest content of total phenols was shown by red mulberry in the ethanolic extract (50%). Certain extracts of the fruit of red mulberry (aqueous, ethanolic 50%, acetonic 50% and acetonic) contain very small quantities of anthocyanins. Four phenolic acids were identified in the extracts of red mulberry: chlorogenic, neochlorogenic, cryptochlorogenic acid and caffeic acid, and four flavonoids using HPLC analysis: quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, and kaempferol-3-*O*-rutinoside. Cyanidin-3-*O*-glucoside was identified in the methanolic extract of red mulberry. The content of heavy metals (Fe, Cu, Mn, Cd, Ni, Zn and Pb) was determined using atomic absorption spectroscopy in fruits and the extracts of red mulberry. Mulberry fruit has been shown the highest content of Fe and low content of toxic metals. The content of metals is the highest in the majority of cases in the extracts of acetone and acetone 50%. The methanolic extract of red mulberry shows the antimicrobial properties against all investigated bacteria except *Staphylococcus aureus.* Obtained results show that fresh fruits of red mulberry and its extracts can be used in nutrition and the preparation of pharmaceutical formulations.

Keywords: *Morus rubra L., heavy metals, antimicrobial activity.*

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INTRODUCTION

Morus, a genus of flowering plants in the family Moraceae, comprises 10–16 species of deciduous trees commonly known as mulberries. The most popular species of genus *Morus* are *Morus alba* L. (white mulberry), *Morus rubra* L. (red mulberry), and *Morus nigra* L. (black mulberry). Mulberry is found from temperate to subtropical regions of the Northern hemisphere to the Southern hemisphere's tropics. They can grow in a wide range of climatic, topographical, and soil conditions.

Mulberry fruit may be colored white, red, or black when ripe. Deep-colored fruits are good sources of phenolics [1-5]. Mulberry fruit has been used as a folk remedy to treat oral and dental diseases, diabetes, hypertension, arthritis, and anemia [6]. The primary use of mulberry globally is as feed for the silkworm, but depending on the location, it is also appreciated for its fruit (consumed fresh, in juice, or as preservatives), as a delicious vegetable (young leaves and stems), for its medicinal properties in infusions (mulberry leaf tea), for landscaping and as animal feed [7]. Mulberry fruit can be used for making jams, jelly, pulp, fruit drinks, fruit sauces, and cakes. Many desserts are made from Persian mulberries, sauces, piemaking, cakes and jelly, and fruit teas [8-10].

Until now, methanolic extracts of red mulberry were investigated from the territory of Turkey, and total phenols were determined (1035 mg GAE/100 g) and total flavonoids (219 mg CE/100 g) [11].

The content of total phenols (215 mgGAE/100 g) and anthocyanins (10.9 mg cyanidin-3-*O*-glucoside/100 g) was determined in the ethanolic solution, and the antioxidant activity of this solution was found (442 mg expressed as mg Trolox Eq/100 g) [12].

It was also investigated the aqueous acetonic extract, and it was determined the content of total phenols (160.3 mg GAE/100 g), and anthocyanins (9.88 mg cyanidin-3-*O*-glucoside/100 g) [9].

Based on the available literature, we noticed that incomplete data are present on the analyses of various extracts, so we decided to perform a systematic analysis of aqueous, methanolic, ethanolic, acetonic, aqueous-ethanolic, aqueous-methanolic, and aqueous-acetonic extracts of red mulberry regarding the content of total phenols, flavonoids, anthocyanins, and antioxidant activity. Also, we aimed to have a microbiological analysis of obtained extracts.

With the aim of safe use of fruits of red mulberry and its extracts, it was necessary to analyze the content of metals with special attention to toxic metals.

RESULTS AND DISCUSSION

The results of the determination of total phenolic compounds, flavonoids, anthocyanins, and antioxidant activity (RSC-Radical Scavenger Capacity) in different extracts of red mulberry fruit are presented in Table 1.

Table 1. Content of total phenols, flavonoids, and anthocyanins, and antioxidant activity of red mulberry fruit extracts (*Morus rubra* L.).

Solvent	Total phenols (mgGAE/100 g)	Flavonoids (mgCE/100 g)	Monomeric anthocyanins (mgCy-3-*O*-Glu/100 g)	DPPH (mgTE/100 g)	RSC (%)
Water	210.4±0.5	192±2	2.6±0.2	118±1	54.11±0.09
Ethanol 50%	320.8±0.8	99.83±0.09	5.3±0.1	183±1	78.4±0.7
Ethanol	109.8±0.4	112.43±0.06	-	175±3	79.6±0.4
Methanol 50%	56.40±0.05	38.4±1.5	-	43.7±0.4	85.6±2.4
Methanol	90.3±0.2	143±2	-	55.4±0.6	87.56±0.03
Acetone 50%	85.9±0.7	49.8±0.1	1.7±0.3	60.00±0.00	89.4±0.4
Acetone	71.4±2.4	45.0±0.7	5.3±0.6	62.2±0.1	86.80±0.09

The content of the total phenols of *Morus rubra* L. was found to be 56.40 to 320.8 mg GAE/100 g for the methanol-water (50/50 v/v%) and ethanol-water (50/50 v/v%) extract, respectively.

Total flavonoid contents in *Morus rubra* L. were ranging from 38.4 mg CE/100 g (methanol-water (50/50 v/v%)) to 192 mg CE/100 g fresh fruit (water extract). The extracts of *Morus rubra* L. showed anthocyanins content in water, ethanol-water (50/50 v/v%), acetone-water (50/50 v/v%) and acetone extracts (2.6 mg, 5.3 mg, 1.7 mg and 5.3 mg of cyanidin-3-*O*-glucoside/100 g, respectively).

Comparing the results on the content of total phenols in the fruit of red mulberry from Southeast Serbia with the results published by authors from other countries, we concluded that our results are lower than theirs. Methanol extract of red mulberry fruit from Turkey contains 1035 mg GAE/100 g [12], while ethanol extract contains 169 mgGAE/100 g [11]. Acetone extract 50% showed slightly higher values of phenol content compared to our results (160 mg GAE/100 g) [9]. The differences in the content of total phenols are most likely due to the different methods of extraction, the climatic area in which the tree grows, and the type of soil on which the wood is grown.

Ethanol 50% and acetone extracts have equal amounts of anthocyanins (5.3 mg cyanidin-3-*O*-glucoside/100 g). The aqueous extract contains 2.6 mg while acetone 50% extract contains 1.7 mg of cyanidin-3-*O*-glucoside per 100 g of fresh fruit. Acetone 50% red mulberry fruit extract from Turkey contains 9.88 mg cyanidin-3-*O*-glucoside/100 g [9]. Unlike the ethanol extract from this paper which did not show anthocyanin content, the ethanol extract of red mulberry fruit from Turkey contains 10.9 mg of cyanidin-3-*O*-glucoside/100 g [11].

Antioxidant activity, using the DPPH method, is relatively high and ranges from 54.11% in the aqueous extract to 89.4% in acetone 50% in red mulberry fruit extract. Ethanol extract of red mulberry from Turkey, in addition to the content of total phenols, showed higher antioxidant activity compared to our results (442 mgTE/100 g) [11].

Tables 2 and 3 show phenolic acids and flavonoids identified in methanolic and acetone extract of the red mulberry fruit.

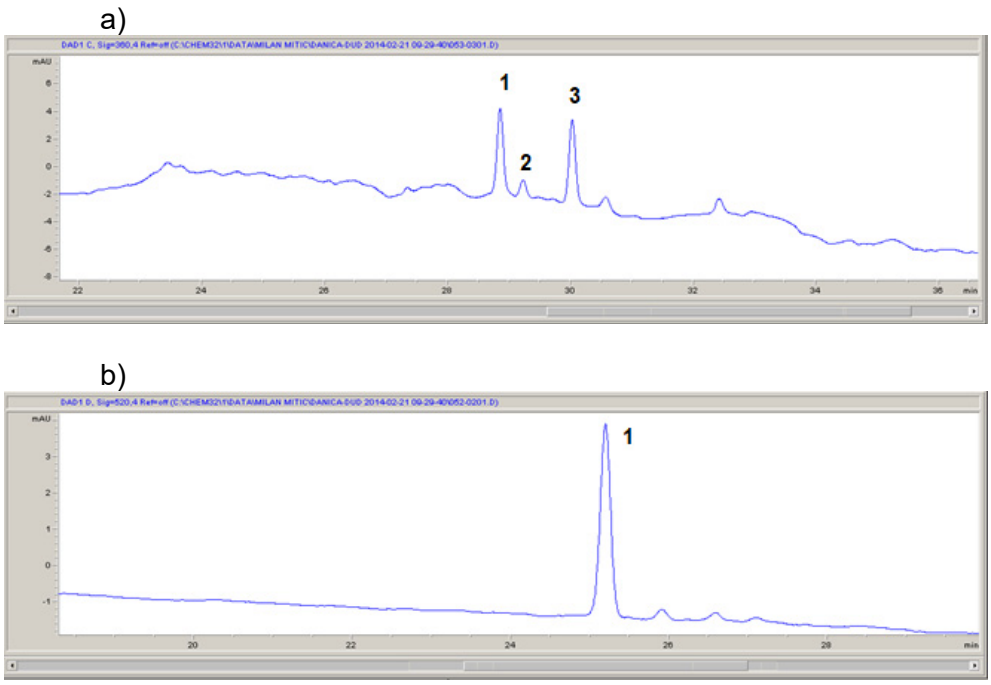

Figure 1. HPLC analysis of methanol extract of red mulberry fruit (*Morus rubra* L.) at a) 360 nm: quercetin-3-*O*-rutinoside (**1**), quercetin-3-*O*-glucoside (**2**), kaempferol-3-*O*-rhamnoside (**3**); b) 520 nm: cyanidin-3-*O*-glucoside (**1**).

Table 2. The identified phenolic acids content in red mulberry fruit extracts (mg/kg).

Phenolic acids (320 nm, mg/kg)	Red mulberry	
	Methanol	Acetone
Chlorogenic acid	9.22	-
Caffeic acid	3.87	-
Neochlorogenic acid	6.76	15.18
Cryprochlorogenic acid	-	8.57

HPLC analysis showed that the methanol extract contained the highest amount of chlorogenic acid and the lowest amount of caffeic acid. Cryptochlorogenic acid is not present in the methanolic extract of the red mulberry fruit. Acetone extract of red mulberry fruit contains two acids: neochlorogenic and cryptochlorogenic.

Table 3. Content of the flavonoids identified in red mulberry fruit extracts (mg/kg).

Anthocyanins (520 nm, mg/kg)	Red mulberry	
	Methanol	Acetone
Quercetin-3-*O*-rutinoside	12.59	13.94
Quercetin-3-*O*-glucoside	6.10	5.78
Quercetin-3-*O*-rhamnoside	-	22.85
Kaempferol-3-*O*-rutinoside	18.52	-

Methanol extract of red mulberry fruit contains the following flavonoids (Figure 1a): quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, and kaempferol-3-*O*-rutinoside. Of these three flavonoids, kaempferol-3-*O*-rutinoside was the most abundant, and quercetin-3-*O*-glucoside was the least abundant. Acetone extract does not only contain kaempferol-3-*O*-rutinoside. Among the flavonoids in the acetone extract, quercetin-3-*O*-rhamnoside was the most abundant, and the quercetin-3-*O*-glucoside was the least abundant.

HPLC analysis of red mulberry fruit extracts for anthocyanin content revealed that the methanol extract contained cyanidin-3-*O*-glucoside (Figure 1b) (6.10 mg/kg).

Thabti et al. [13] isolated neochlorogenic, cryptochlorogenic, and caffeic acids from the red mulberry fruit. They also identified flavonoids: quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, and kaempferol-7-*O*-glucoside, which largely agrees with our results. Several authors have identified cyanidin-3-*O*-glucoside in red mulberry fruit extracts.

The content of heavy metals such as iron, zinc, copper, manganese, nickel, lead, and cadmium in fruit and extracts of red mulberry fruit is shown in Table 4.

Table 4. Content of heavy metals in fruit and extracts of the red mulberry fruit.

	Red mulberry						
System	Fe (mg/100 g)	Cu (mg/100 g)	Mn (mg/100 g)	Cd (μg/100 g)	Ni (mg/100 g)	Zn (mg/100 g)	Pb (mg/100 g)
Fruit	57±6	1.5±0.2	2.0±0.2	1.8±0.2	0.37±0.03	5.04±0.08	0.20±0.02
Water extract	1.0±0.2	0.01±0.00	0.09±0.01	-	0.05±0.01	0.43±0.03	-
Ethanol 50% extract	0.70±0.01	0.02±0.00	0.15±0.01	-	0.04±0.00	0.43±0.03	-
Ethanol extract	0.32±0.04	0.01±0.00	0.18±0.01	-	0.02±0.00	0.57±0.02	-
Acetone 50% extract	5.8±0.2	0.05±0.01	0.39±0.02	0.29±0.04	0.06±0.01	0.55±0.03	-
Acetone extract	5.7±0.4	0.28±0.01	0.59±0.07	0.27±0.06	0.05±0.01	1.04±0.02	-
Methanol 50% extract	0.31±0.04	0.09±0.01	0.09±0.01	-	0.04±0.00	0.08±0.01	-
Methanol extract	0.32±0.08	0.11±0.01	0.25±0.03	-	0.02±0.00	0.12±0.01	-

Red mulberry fruit contains the highest amount of iron (57 mg/100 g) and the lowest amount of cadmium (1.8 μg/100 g). The content of heavy metals in the fruit of red mulberry decreases in the following order: iron> zinc> manganese> copper> nickel> lead> cadmium (Table 4).

Ercisli et al. [11] published a significantly lower iron content in red mulberry fruit (5.00 mg/100 g). The manganese content was 5.00 mg/100 g which is higher than our results (2 mg/100 g). The amount of zinc confirmed in red mulberry fruit was 3.00 mg/100 g [11] which is slightly less than our results. Authors from Turkey have published lower iron, zinc, and copper values in red mulberry fruit (4.5, 3.2 and 0.4 mg/100 g, respectively) [12]. The amount of manganese was slightly higher than our values (4.00 mg/100 g).

The amount of iron in the extracts ranges from 0.31 to 5.8 mg/100 g in 50% methanol and 50% acetone extract, respectively. Aqueous and ethanolic extracts of red mulberry fruit contain the least amount of copper

(0.01 mg/100 g), while acetone extract contains 0.28 mg/100 g. The manganese content ranges from 0.09 mg/100 g in methanol 50% and aqueous extract to 0.59 mg/100 g in acetone extract. The presence of cadmium was detected only in acetone and acetone 50% extract (0.27 and 0.29 μg/100 g, respectively). The amount of nickel in red mulberry fruit extracts ranges from 0.02 in methanol and ethanol extract to 0.06 mg/100 g in acetone 50% extract. Methanol 50% extract contained the lowest amount of zinc (0.08 mg/100 g), while acetone extract showed the highest amount of zinc (1.04 mg /100 g). Lead content was not detected in any extract except the fruit (0.20 mg/100 g).

The microbiological tests show that methanol 50% mulberry extract shows antibacterial effect only on the bacterium *S. typhimurium*. Methanol extract of red mulberry shows the antibacterial effect on all tested bacteria except *Staphylococcus aureus*. At given concentrations, aqueous extracts of red mulberry fruit did not show antimicrobial activity.

In Table 5, the antimicrobial activity of red mulberry fruit extracts was given.

Table 5. Antimicrobial activity of red mulberry fruit extracts (*Morus rubra* L.).

Test microorganisms	Water		Methanol		Methanol 50%		DMSO	Tetracycline
	20 μl	50 μl	20 μl	50 μl	20 μl	50 μl	50 μl	30 μg
Salmonella typhimurium NCTC 6017	-	-	-	16 mm	16 mm	-	-	28 mm
Pseudomonas aeruginosa ATCC 9027	-	-	12 mm	16 mm	-	-	-	19 mm
Bacillus subtilis ATCC 6633	-	-	-	17 mm	-	-	-	36 mm
Staphylococcus aureus ATCC 6538	-	-	-	-	-	-	-	34 mm
Escherichia coli ATCC 8739	-	-	12 mm	16 mm	-	-	-	30 mm

CONCLUSIONS

In this paper, quantitative determination of phenolic compounds was performed in different fruit extracts of red mulberry (*Morus rubra* L.) from the Southeast region of Serbia, as well as the determination of the antioxidant

activity of tested extracts using the spectrophotometric methods. The content of heavy metals in the fruit and extracts was determined by the AAS method. Also, the antimicrobial activity of the tested extracts was determined. The results indicate red mulberry's fresh fruits and extracts are rich in phenolic compounds and have high antioxidant activity. Also, the tested samples did not show the presence of toxic metals, exhibit antibacterial activity, and can be used in the diet and the preparation of pharmaceutical formulations.

EXPERIMENTAL SECTION

Preparation of the fresh fruit extracts

Plant material was collected in Southeast Serbia in early July 2011. Voucher specimens (*Morus rubra* L. No 2-1753, Bela Palanka, UTM 34TDR2 01, determined by Mirjana Milenkovic, Faculty of Biology, University of Belgrade, Belgrade, Serbia) were deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad, Novi Sad, Serbia. Fresh fruit maturity was estimated based on the color [14]. Samples were stored in plastic bags and kept frozen until extraction. The frozen fresh fruit material was homogenized using a blender. Black, red, and white mulberry fresh fruits (10 g) were extracted with water, methanol-water (50/50, v/v%), methanol, ethanol-water (50/50, v/v%), and ethanol. All solvents were acidified with 1 ml conc. HCl. The extraction was performed with 100 ml of solvents using the ultrasonic bath for 30 minutes. The suspension was gravity-filtered through a Buchner funnel and Whatman No. 1 filter paper. Extracts were stored in the fridge at 5ºC until their analyses.

Chemicals and apparatus

1,1-Diphenyl-2-picrylhydrazyl (DPPH), catechin, and $AlCl_3$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Acros Organics (New Jersey, USA). Folin-Ciocalteu's phenol reagent and sodium carbonate were purchased from Merck Chemical Suppliers (Darmstadt, Germany). Potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5) were purchased from the same producer. The other used chemicals, including solvents, were of analytical grade. An Agilent 8453 UV/Vis spectrophotometer was used for absorbance measurements and spectra recording, using optical or quartz cuvettes of 1 cm optical path. The pH measurements were made with Hanna Instruments pH-meter equipped with a glass electrode.

Determination of the total phenolic compounds

Total phenol contents of the extracts were determined by the modified Folin-Ciocalteu method [15]. An aliquot of the extracts (1 ml) was mixed with 0.5 ml Folin-Ciocalteu reagent and 2 ml of sodium carbonate (20%). Absorbance was measured after 10 min incubation at room temperature at 760 nm. Total phenolic content was expressed as mg/100 g gallic acid equivalent (GAE). The result of each assay was obtained from three parallel determinations.

Determination of the total flavonoid content

Total flavonoid content was determined using a spectrophotometric method based on the formation of the flavonoid complex with aluminum [16]. Red mulberry extract (1 ml) was mixed with 3 ml deionized water and 0.3 ml $NaNO_2$. After standing at room temperature for 5 minutes, 3 ml $AlCl_3$ was added to the solution, followed by the addition of 2 ml of NaOH after another 5 minutes of standing. The solution was then filled up with deionized water in a 10 ml flask. The absorbance of the prepared solution was measured at 510 nm. Total flavonoid content was calculated as catechin (mgCE/100 g) using the equation based on the calibration curve.

Determination of the total monomeric anthocyanins

The total monomeric anthocyanin content in the plant extracts was determined using the previously described pH-differential method [17]. Anthocyanins demonstrate a maximum absorbance at 520 nm at pH 1.0. The colored oxonium form of anthocyanin predominates at pH 1.0, and the colorless hemiketal form at pH 4.5. The pH-differential method is based on the reaction of the formation of oxonium forms. This allowed accurate and rapid measurement of total monomeric anthocyanins. Total monomeric anthocyanin pigment is expressed as mg of cyanidin-3-*O*-glucoside, using molar absorptivity (ε) of 26900 and a molecular weight of 449.2. For this method, 1 ml of the red mulberry extract, prepared by the previously described procedure, was poured into two separate 10 ml volumetric flasks. Then, one was filled up to the line with a solution of potassium chloride (KCl) (pH=1), and the second with sodium acetate (CH_3COONa) (pH=4.5). The two diluted solutions were left to stand for 15 minutes at room temperature in the dark.

Finally, the absorbance of both samples was measured at λ_{max} 520 nm and 700 nm. Absorbances (A) of the investigated extracts were calculated by Eq 1:

$$A = (A_{\lambda vis\text{-}max}\text{-}A_{700})pH_{1.0}–(A_{\lambda vis\text{-}max}–A_{700})pH_{4.5} \quad (1)$$

Content of the monomeric anthocyanin pigment (MAP) was calculated by Eq 2:

$$MAP(mg\ l^{-1}) = (A \cdot MW \cdot DF \cdot 1000)/(\varepsilon \cdot l) \quad (2)$$

where A is absorbance calculated by equation 1, ε is the molar absorptivity (26900 $dm^3mol^{-1}cm^{-1}$), MW is the molecular weight (449.2 $gmol^{-1}$), and DF is the dilution factor, *l* is the path length (1 cm). The result, taken as the monomeric anthocyanin pigment (MAP), was expressed as mg of cyanidin-3-*O*-glucoside dm^{-3}.

Free radical scavenging activity

The free radical scavenging activity of the plant extracts was analyzed by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [18-22]. The antioxidant assay is based on the measurement of the loss of color of DPPH solution by the change of absorbance at 517 nm caused by the reaction of DPPH with the tested sample. The reaction was monitored using a UV-VIS spectrophotometer. Plant extracts (1 ml), 5 ml of freshly prepared DPPH in methanol, and 4 ml of water were put into a cuvette at room temperature. After 30 minutes of incubation at room temperature, the absorbance was read against a blank at 517 nm. All measurements were performed in triplicate at a final concentration. The ability of extracts to inhibit DPPH in percents (RSC %) was calculated from the decrease of absorbance according to the relationship (Eq 3):

$$RSC\ (\%) = (1 - A_{sample}/A_{blank}) \cdot 100 \quad (3)$$

where A_{blank} is the absorbance of the control ($1 \cdot 10^{-4}$ mol dm^{-3} DPPH methanol solution), and A_{sample} is the absorbance of the test sample. The results were expressed as milligrams of Trolox equivalents (TE) per 100 g of fresh sample (mgTE/100 g).

HPLC method

High-Performance Liquid Chromatography (HPLC) with UV/Vis and a high-resolution fluorescence detector was applied to determine and quantify the phenolic compounds in the prepared samples. The HPLC method was developed, with the following parameters showing the best results. Chromatographic separation was performed on an Eclipse XDB-C18 column (4.6 mm x 150 mm) using a solvent system:

A - (H_2O + 5% HCOOH) and B - (80% ACN + 5% HCOOH + H_2O).

Separation of components was performed using the following linear gradient: 0-28 min, 0.0% B; 28-35 min, 25% B; 35-40 min, 50% B; 40-45 min, 80% B, and finally 0% B again for the last 10 min. The mobile phase flow

was 0.8 cm^3/min. The sample solution (5 µl) was injected automatically using autosamplers. The column was thermostatically controlled at 30 ° C.

The phenolic components present in the samples were identified by comparing their retention times and spectra with the retention time and spectrum of standards for each component. The following standards were used: malvidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, chlorogenic acids, *p*-coumaric, caffeic and ferulic acids, catechin, epicatechin, quercetin, and kaempferol. The quantitative determination of the components was performed by the external standard method.

For each individual standard, a stock solution of a mass concentration standard of 1.0 mg/cm^3 was prepared by dissolving in 10% methanol solution. A calibration curve for each standard was constructed based on the obtained surfaces, depending on the mass concentration of the standard. From the obtained linear dependence equation, mass concentrations of the components in the samples were calculated. For components in samples for which no standard was available, quantification was performed based on a calibration curve by the structure of the corresponding standard. All analyses were performed in triplicate.

Determination of the content of heavy metals

The standard procedure described by the Association of Official Analytical Chemists (AOAC) was followed for the preparation of the samples for the analysis of heavy metals [23]. Accurately weighed (2 g) sample was transferred into a silica crucible and kept in a muffle furnace for ashing at 450˚C for 3 h and then 5 ml of 6 M hydrochloric acid was added to the crucible. Care was taken to ensure that all the ash came into contact with acid. Further, the crucible containing acid solution was kept on a hot plate and digested to obtain a clean solution. The final residue was dissolved in 0.1 M nitric acid solution and made up to 50 ml. Working standard solutions were prepared by diluting the stock solution with 0.1 M nitric acid to check the linearity. Red mulberry fresh fruits (10 g) were extracted with water, ethanol-water (50/50, v/v%), ethanol, acetone-water (50/50, v/v%), acetone, methanol-water (50/50, v/v%), and methanol. All solvents were acidified with 1 ml concentrated HCl. The extraction was performed with 100 ml of solvents using the ultrasonic bath for 30 minutes. The suspension was gravity filtered through a Buchner funnel and Whatman No. 1 filter paper. Extracts were stored in the fridge until their analyses.

Determination of antimicrobial activity

In vitro antimicrobial activity of the extracts was tested on a panel of laboratory control strains belonging to the American Type Culture Collection Maryland, USA (except one, which belongs to the National Collection Type

Cultures). Antibacterial activity was assessed against two Gram-positive and three Gram-negative bacteria. The Gram-positive bacteria used were *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538. The Gram-negative bacteria used in the experiment were *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella typhimurium* NCTC 6017. The disk diffusion method was used.

Inoculated bacterial strains were prepared from overnight broth culture, and suspensions were adjusted to a turbidity standard of 0.5 McFarland. 100 µl of a suspension containing 1.0×10^8 CFU/cm^3 of spore bacteria was seeded on Mueller-Hinton agar (MHA, Torlak) and Sabouraud dextrose agar (SDA, Torlak), respectively, in sterilized Petri dishes (90 mm in diameter). Discs (diameter 9 mm, Macherey-Nagel, Düren) (Germany) were impregnated with 20 µl and 50 µl of extracts (conc. 30 mg/ml) and placed on inoculated agar. Negative control samples were prepared using the same solvent (ethanol).

Tetracycline (30 µg, Torlak) was used as a positive reference standard for determining the susceptibility of strains of each tested microorganism species. Inoculated plates were stored at 4 °C for 2 h and incubated at 37 °C (24 h) for bacterial strains. Antimicrobial activity was assessed by measuring the zone of inhibition for the tested microorganisms. Each trial of this experiment was repeated three times.

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