Free radical scavenging activity and total phenolic and flavonoid contents of mulberry (*Morus* spp. L., *Moraceae*) extracts

Marija M. Radojković[1], Zoran P. Zeković[1], Senka S. Vidović[1], Drago D. Kočar[2], Pavle Z. Mašković[3]

[1]*University of Novi Sad, Faculty of Technology, Novi Sad, Serbia*
[2]*University of Ljubljana, Faculty of Chemistry and Chemical Technology, Ljubljana, Slovenia*
[3]*University of Kragujevac, Faculty of Agronomy, Čačak, Serbia*

Abstract

Mulberry (*Morus* spp. L., *Moraceae*) fruit, leaves, bark and branches have been used in traditional medicine as diuretic, hypoglycemic and hypotensive medicaments. The mechanism of their effects is correlated with the content of active components. The objective of this work was to evaluate and compare antioxidant properties of different extracts of two *Morus* species growing in Serbia: *Morus alba* L. (white mulberry) and *Morus nigra* L. (black mulberry). Potential antioxidant activity, content of antioxidant compounds (phenolics and flavonoids) and radical scavenging capacity, tested by the DPPH method, were evaluated. The phenolic and flavonoid composition of different *Morus* extracts was determined by the HPLC method. The extracts prepared from fruits, leaves and roots of *M. alba* and *M. nigra* exhibited different characteristics. The highest extraction yield was achieved by *M. alba* leaves extraction (23.40%). *M. nigra* roots extract showed the highest total phenolics (186.30 mg CAE/g), while the highest total flavonoids content (67.37 mg RE/g) was determined for *M. nigra* leaves extracts. In addition, black mulberry leaf extracts with the highest antioxidant activity had the highest phenolic acids contents. The dominant phenolic components in the samples were rutin and chlorogenic acid. All investigated mulberry dry extracts showed high content of phenolic compounds and significant antioxidant activity. This work contributes to knowledge of the antioxidant properties of *Morus* species. The obtained results may be useful in the evaluation of new dietary supplements and food products.

Keywords: *Morus nigra*, *Morus alba*, phenols, flavonoids, HPLC.

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Plants have received a lot of attention as a source of biologically active substances, including antioxidants, antimutagens and anticarcinogens [1]. Many herbs, cereals, fruits, vegetables, oils, spices and other plant materials possess antioxidant properties, mainly because of the content of phenolic compounds. The compounds from these materials are indeed useful as alternative therapy agents, or as models for new synthetic substances. Phenolic compounds: flavonoids, phenolic acids, anthocyanins, stilbenes, tannins, lignans, and lignins, are important for normal plant growth and development as well as a defense against infection and injury. These compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity [2]. However, data on antioxidant properties of various plants, particularly those that are not used in nutrition and medicine are still deficient. Therefore, investigation of such properties has been of interest, mainly for finding new sources for natural antioxidants, functional foods and nutraceuticals [3,4].

Mulberry (*Morus spp.* L., Moraceae) has been domesticated over thousands of years and adapted to the wide area of tropical, subtropical, and temperate zones of Asia, Europe, North and South America, and Africa. Mulberry trees have historically been used for leaf yield in sericulture. In additional, their fruit, roots and bark have been used in folk medicine (especially in Chinese medicine) to treat diabetes, hypotension, anemia and arthritis [5]. Different researchers investigated certain properties of whole mulberry fruit, leaves, bark, root and extracts of part of mulberry trees. Few species of mulberry were evaluated for their edible fruits. Previously, some autors [5–9] studied the quality, nutritional potentials and chemical composition of some of the *Morus* species. Lee *et al.* [10] and Du *et al.* [11] found that mulberries have cyanidin-based anthocyanins. In recent studies, Naderi *et al.* [12] found that extract of *M. nigra* fruits have protective action against peroxidative damage to biomembranes and biomolecules. Arabshahi-Delouee and Urooj [4] and Memon *et al*. [13] studied antioxidant properties of various extracts of mulberry leaves. Gecgel *et al*. [14] investigated phytochemical properties and fatty acid composition of

Correspondence: M.M. Radojković, Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia.
E-mail: ramarija@uns.ac.rs
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black mulberry seed oil. As mulberry can grow in a wide range of climatic, topographical, and soil conditions, this may affect the chemical composition and nutritional status of plant. Mulberry grown in Serbia has not been a subject of previous studies. Therefore, the current study was undertaken to investigate the phenolic content and antioxidant potentials of fruit, leaves and roots extracts of two *Morus* species.

EXPERIMENTAL

Chemicals

1,1-Diphenyl-2-picrylhydrazyl hydrate (DPPH), Folin-Ciocalteu reagent and standard substances including gallic acid, sinapic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid and dihydroxy benzoic acid were purchased from Sigma (St. Louis, USA), vanillic acid, ferrulic acid, rutin and quercetin were purchased from Fluka (St. Gallen, Switzerland). All reagents and standards were prepared using Milli-Q deionized water (Millipore, Bedford, USA). All other chemicals and reagents were of analytical reagent grade.

Plant material

In this research dried plant material was used. Voucher specimens (*Morus alba* L. N^{o} 2-1794, Kać, UTM 34TDR211, and *Morus nigra* L. N^{o} 2-1753, Novi Sad, Rimski šančevi, UTM 34TDR2 01, det.: Goran Anačkov) were identified and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad, Serbia [15].

Sample preparation

The samples of mulberry leaves and roots were dried naturally (in the shade, on a draft) during one month. Prior to extraction samples were ground in a blender. Mulberry samples of leaves and roots were extracted with 70% ethanol, at a temperature of 30 °C while the liquid-solid ratio was 15 mL/g (solvent volume per g of sample). Samples of mulberry fruits were stored in polyethylene bags at –20 °C (up to 1 month) until undertaking of the analysis. Mulberry fruits samples were extracted by ethanol:water:acetic acid (70:29.5:0.5, v/v/v) mixture, at the temperature of 30 °C with the same liquid–solid ratio as in prior extraction.

The extraction process was carried out in a shaker (3015 GFL, Germany) over 24 h. Extractions under the same conditions were repeated three times. After filtration, 50 mL of liquid extract was used for extraction yield determination. The solvent was removed by a rotary vacuum evaporator (Devarot, Elektromedicina, Ljubljana, Slovenia) and dried at 60 °C until constant mass was achieved. Dry extracts were stored in glass bottles at 4 °C for prevention from oxidative damage until the analysis.

Spectrophotometric determination of total phenolic and flavonoids content

Total phenolic contents (TPC) were determined spectrophotometrically by using Folin-Ciocalteu reagent [2,16]. The absorbance was measured at 765 nm and results were expressed as mg of chlorogenic acid equivalents (CAE) per gram of dry extracts. Determination of total flavonoids content (TFC) was performed using a modified colorimetric method [17]. The absorbance was measured at 510 nm and results were expressed as mg of rutin equivalents (RE) per gram of dry extract. Triplicate tests were conducted for each sample. Spectrophotometric measurements were performed using a Vis spectrophotometer (Janwey 6300, Germany).

Determination of DPPH free radical scavenging activity

The free-radical scavenging activity of mulberry extracts was determined as described by Espin *et al.* [18]. Radical scavenging capacity (*%RSC*) was calculated using Eq. (1), where A_{sample} is the absorbance of sample solution and A_{blank} is the absorbance of a blank sample.

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

This activity was also expressed as IC_{50}, which was the concentration of the solution tested required to obtain 50% of radical scavenging capacity.

HPLC/MS/MS Analysis

The HPLC system Perkin Elmer PE200 was composed of a binary pump, a column thermostat and an autosampler. The mass spectrometer used was a 3200 QTRAP MS/MS with ESI ionization (Applied Biosystems/MDS Sciex, Foster City, USA). The experimental conditions were: mobile phase A: 50% acetonitrile, 50% acetic acid (0.5%); mobile phase B: 2% acetic acid; gradient elution: 0 min 30% A, 70% B; 10 min 30% A, 70% B; 30 min 100% A, 0% B; 35 min 100% A, 0% B; 40 min 30% A, 70% B for reconditioning of the system; flow rate: 0.7 mL/min; injection volume: 20 µL; ionisation: ESI negative; dwell time 50 ms; multiple reaction monitoring (MRM) transitions: gallic acid 169/125, dihydroxy benzoic acid 153/109, sinapic acid 223/164, vanillic acid 167/123, caffeic acid 179/135, quercetin 301/151, chlorogenic acid 353/191, ferullic acid 193/ /134, *p*-coumaric acid 163/119. Stock solutions of standards were diluted in the mobile phase to obtain working standard solutions. Concentrations of the compounds were calculated from chromatogram peak areas on the basis of calibration curves. The method linearity was assessed by means of linear regression of the mass of compounds injected vs. its peak area. All

solvents were of HPLC grade and were filtered and degassed before use.

Statistical analysis

Statistic analysis was carried out using Statistica 6.0 (StatSoft Inc, Tulsa, OK, US). All experiments were performed at least in triplicate unless specified otherwise. Results are presented as a value ± standard deviation (*SD*). Significant levels were defined at $p < 0.05$.

RESULTS AND DISCUSSION

Total phenolic and flavonoids content and DPPH radical scavenging activity

Many reports have revealed that the physiological function of natural foods can be attributed to the antioxidative capacity of their phenolic components [19,20]. Table 1 shows the extraction yield, total phenolic (TPC), total flavonoid content (TFC), total flavonoids and total phenolics ratio (TFC/TPC), as well radical scavenging activity of different extracts of mulberry species grown in Serbia. The yield of extractable components, expressed as % (w/w) was ranged from 16.32% (*M. alba* root) to 23.40% (*M. alba* leaves). The mulberry extracts were a rich source of phenolics, with the highest level of these compounds detected in root extracts of *M. nigra* (186.30 mg CAE/g) and *M. alba* (170.20 mg CAE/g). Also, high TPC shown extracts of *M. nigra* leaves (115.23 mg CAE/g). The lowest TPC has been detected in mulberry fruit extracts in all species. All investigated extracts of tree parts of *M. alba* showed lower content of total phenols then extracts of tree parts of *M. nigra.* Flavonoids are considered as phenol compounds with highest antioxidant activity due to their chemical structure [2]. The TFC in investigated samples was in the range of 0.8948 to 67.3690 mg RE/g of dry extracts. The highest TFC has been detected in extracts of *M. nigra* (leaves and roots). The lowest content of TFC in the mulberry extracts of the same species determinate in the mulberry fruits extracts. Also, the highest total flavonoids and total phenolics ratio (TFC/ /TPC) shown extracts prepared of *M. nigra* leaves (Table 1).

In Table 2, the available data for phenolic content in mulberry plants grown in other regions of the world are presented. In mulberry fruit extracts from Serbia the total phenol content was lower than in other regions. However, mulberry leaves grown in Serbia showed higher content of phenolic compounds than mulberry leaves grown in Pakistan and India. Also, mulberry root extracts have higher content of phenolic compounds than samples in other regions. Phenols posses a wide spectrum of biological activities [21] and the results show that mulberry extracts could be good sources of these natural constituents.

The method of scavenging of stable $DPPH^{\cdot}$ radicals is a method widely used to evaluate antioxidant activity in relatively short time compared to other methods. The effect of antioxidants on $DPPH^{\cdot}$ radical scavenging was attributed to their hydrogen donating ability [22]. $DPPH^{\cdot}$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [16]. Decrease in absorbance of $DPPH^{\cdot}$ radical is caused by a reaction between antioxidant molecules and the radical, which results in the scavenging of radical by hydrogen donation, what is visually noticeable as a discoloration from purple to yellow. Table 1 shows antioxidant activity of extracts prepared from different parts of *Morus* species expressed as IC_{50} values (lower values indicate more powerful antioxidant capacity). The effectiveness in reducing powers was in a descending order extracts of leaves (*M. nigra*)> leaves (*M. alba*)> root (*M. nigra*)> root (*M. alba*)> fruits (*M. nigra*)> fruits (*M. alba*). Radical scavenging activity was found to exhibit 50% of inhibition (IC_{50} value) at extract concentration lower than 0.006 mg/ml for extracts of *M. nigra* leaves.

HPLC Analysis

The content of plant phenolics in *Morus* extracts, expressed as mg/100 g of dry extract are shown in Table 3. In crude extracts of *Morus* species, the following compounds were identified and quantified: gallic acid, chlorogenic acid, ferulic acid, sinapic acid, rutin and quercetin. Rutin is predominant flavonoid in the mulberry fruit species (43.5 mg/100 g for *M. alba* fruit and 72.6 mg/100 g for *M. nigra* fruit). Ferulic and sinapic acids were not detected in mulberry fruit extracts.

Table 1. Extraction yield, total phenolic (TPC), total flavonoid content (TFC), total flavonoids and total phenolics ratio (TFC/TPC), and radical scavenging activity (IC_{50}, mg/ml) of Morus extracts

Sample	Extract	Extraction yield, mass%	*TPC* / mg CAE g^{-1}	TFC / mg RE g^{-1}	100*TFC*/*TPC*, %	IC_{50} / mg ml^{-1}
Morus alba L.	Fruit	21.03	4.133±0.120	0.899±0.014	21.65	0.1469
	Leaf	23.40	66.766±0.749	33.303±0.059	49.88	0.0124
	Root	16.32	170.200±1.414	52.285±0.033	30.72	0.0274
Morus nigra L.	Fruit	19.22	6.368±0.1853	1.508±0.015	23.67	0.0450
	Leaf	22.42	115.232±0.514	67.369±0.098	58.46	0.0055
	Root	22.48	186.300±0.424	67.105±0.012	36.02	0.0153

Table 2. Comparison of phenolic content data from mulberry plants grown in other regions of the world

Sample	Part of plant (origin)	Phenolic content, mg/g of fresh material	Reference
Morus nigra L.	Fruit (Turkey)	14.22	8
Morus alba L.	Fruit (Turkey)	1.81	
Morus rubra L.	Fruit (Turkey)	10.35	
Morus nigra L.	Fruit (Turkey)	19.43–22.37	9
Morus alba L	Fruit (Korea)	2.23–2.57[a]	24
Morus alba	Fruit (Taiwan)	15.16	25
Morus atropurpurea Roxb.	Fruit (southern China)	4.41–17.10[a]	26
Morus nigra L.	Fruit (Pakistan)	13.80	13
Morus alba L.	Fruit (Pakistan)	22.30	
Morus laevigata W.	Fruit (Pakistan)	21.30	
Morus nigra L.	Fruit (Turkey)	17.66–34.88	5
Morus rubra L.	Fruit (Turkey)	10.05–23.88	
Morus alba	Fruit (Pakistan)	16.50	23
Morus nigra	Fruit (Pakistan)	8.80	
Morus leavigata	Fruit (Pakistan)	13.00	
Morus leavigata	Fruit (Pakistan)	11.00	
Morus nigra L.	Fruit (Serbia)	1.22	Current work
Morus alba L.	Fruit (Serbia)	0.86	
Morus indica L	Leaves (India)	93.20[a]	4
Morus nigra L.	Leaves (Pakistan)	10.20	13
Morus alba L.	Leaves (Pakistan)	15.07	
Morus laevigata W.	Leaves (Pakistan)	12.60	
Morus nigra L.	Leaves (Serbia)	25.83	Current work
Morus alba L.	Leaves (Serbia)	15.62	
Morus alba L.	Root (Taiwan)	60.65[a]	27
Morus nigra L.	Root (Serbia)	186.30[a]	Current work
Morus alba L.	Root (Serbia)	170.20[a]	

[a]mg/g of dry extract

Table 3. Content of plant phenolics in Morus extracts, expressed as mg/100g of dry extractsm (nd – not detected)

Sample	*Morus alba* L.			*Morus nigra* L.		
	Fruit	Leaf	Root	Fruit	Leaf	Root
Gallic acid	14.50±0.05	91.50±0.11	28.40±0.03	23.10±0.01	85.00±0.02	25.60±0.04
Chlorogenic acid	33.00±0.02	265.80±1.22	13.70±0.07	20.00±0.04	1957.10±4.76	118.80±1.00
Ferulic acid	nd	510.20±3.45	142.90±0.99	nd	113.30±0.87	357.50±2.12
Sinapic acid	nd	271.30±2.43	574.90±2.03	nd	167.20±1.02	872.20±2.71
Rutin	43.50±0.04	289.30±2.04	26.20±0.81	72.60±1.09	2534.60±9.75	19.00±0.98
Quercetin	3.70±0.09	nd	nd	13.30±0.02	nd	nd

The extracts of mulberry leaves and roots have all tested phenolic compounds, except quercetin. *Morus* root extracts were the richest with sinapic acid (574.9 mg/100 g for *M. alba* and 872.2 mg/100 g for *M. nigra*). Quantitative analysis of phenolic compounds in *M. alba* leaves extracts has shown the presence of ferulic acid as the most dominant compound (510.2 mg/100 g). In all tested extracts the highest content showed rutin and chlorogenic acid were the most abundant in *M. nigra* leaves (2534.6 mg/100 g and 1957.1 mg/100 g). Obtained results of chlorogenic acid contents in black mulberry extracts were in accordance with literature data [13]. Comparable the results of our study and previously published, there are certain differences in the content of phenolcarboxylic acid in *Morus* species (4,9,13,25). Katsube *et al.* [28] have reported that the main flavonol glycoside of *M. alba* was rutin, which is in accordance with our results. The variation of phenolic compounds in the extract depends on many factors, such as degree of maturity at harvest, genetic diffe-

rences and environmental conditions during fruit development [29,30].

CONCLUSIONS

In this study, we compared and characterized phenolic, flavonoids content and antioxidant activities of different extracts of selected *Morus* species of Serbia. *M. alba* and *M. nigra* exhibited different activities and chemical composition. The mulberry roots showed higher contents of phenolic and flavonoids than mulberry fruits and leaves. In addition to the highest antioxidant activity, black mulberry leaves extracts had the highest phenolic acids contents. The dominant phenolic components in the samples were rutin and chlorogenic acid. High phenolic content and high antioxidant activity increase nutritive and phytomedicinal potentials of mulberry. These results may also provide a basis for planning breeding strategies as well as selecting cultivars with high phytonutrients profiles and antioxidant capacities as functional foods for consumers. The present study is a step to the potential use of mulberry as an important antioxidant carrier in food and pharmaceutical industries.

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IZVOD

ANTIOKSIDATIVNA AKTIVNOST I UKUPAN SADRŽAJ FENOLA I FLAVONOIDA U EKSTRAKTIMA DUDA (*Morus* SPP. L., *Moraceae*)

Marija M. Radojković[1], Zoran P. Zeković[1], Senka S. Vidović[1], Drago D. Kočar[2], Pavle Z. Mašković[3]

[1]*Univerzitet u Novom Sadu, Tehnološki fakultet, Novi Sad, Srbija*
[2]*University of Ljubljana, Faculty of Chemistry and Chemical Technology, Ljubljana, Slovenia*
[3]*Univerzitet u Kragujevcu, Agronomski faklutet, Čačak, Srbija*

(Naučni rad)

Plod, list, kora i koren vrste *Morus* imaju dugu upotrebu u tradicionalnoj medicini kao diuretik, hipoglikemik i za snižavanje krvnog pritiska. Mehanizmi pomenutih aktivnosti su u vezi sa sadržajem aktivnih komponenata. Cilj ovog rada je određivanje i upoređivanje antioksidativnih osobina dve različite vrste roda *Morus* koje rastu u Srbiji: *Morus alba* L. (beli dud) i *Morus nigra* L. (crni dud). Određen je sadržaj antioksidativnih komponenata (fenola i flavonoida) i antioksidativna aktivnost ekstrakata *Morus* vrsta. Sadržaj pojedinačnih fenolnih komponenata u ekstraktima različitih delova duda određen je HPLC metodom. Ekstrakti plodova, listova i korena belog i crnog duda pokazali su različite karakteristike. Najveći prinos ekstrakcije pokazao je list *M. alba* (23,40%). Ekstrakt korena *M. nigra* ima najveći sadržaj ukupnih fenola (186,30 mg CAE/g), a najviši sadržaj ukupnih flavonoida (67,37 mg RE/g) određen je u ekstraktu lista *M. nigra*. Najveću antioksidativnu aktivnost i najveći sadržaj pojedinačnih fenolnih i flavonoidnih komponenata pokazao je ekstrakt lista *M. nigra*. Svi ispitani uzorci ekstrakata duda pokazali su visok sadržaj polifenolnih jedinjenja i izraženu antioksidativnu aktivnost. Ovi podaci doprinose upoznavanju osobina *Morus* vrsta i predstavljaju osnovu za koncipiranje novih dijetetskih suplemenata i prehrambenih proizvoda.

Ključne reči: *Morus nigra* • *Morus alba* • Fenoli • Flavonoidi • HPLC