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Original Research Article

Phenolic Content and Antioxidant Activities of Fruit Extracts of *Morus nigra L (Moraceae)* from Southeast Serbia

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Abstract

Purpose: *To evaluate the content of phenolic compounds (flavonoids and anthocyanins) of Morus nigra L. fruit (black mulberry) as well as the antioxidant activities of its extracts.*
Methods: *The contents of phenols, flavonoids and anthocyanins of the ethanol, ethanol-water (50/50,v/v) and water extracts of black mullbery were determined using spectrophotometric methods. Antioxidant assay was based on the measurement of 2,2-diphenyl-1-picrylhydrazyl (DPPH) absorbance at 517 nm caused by the reaction of DPPH with the test sample, and also on the measurement of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) absorbance at 734 nm caused by the reaction of ABTS with the test sample.*
Results: *Spectrophotometric analysis indicates that the content of total phenol compounds in the extracts varied from 90.26 to 118.84 mg GAE/100 g of fresh mulberry fruit. The content of anthocyanins ranged from 114.83 to 128.68 mg of cyanidin-3-O-glucoside/100 g of fresh fruit (ff). The fruit extract (0.1 ml) showed high antioxidant activity with DPPH radical transformation value of 71.41 % in ethanol-water (50/50, v/v)) extract and good antioxidant activity (relative to trolox equivalent as standard) with ABTS of 55.43 % in the water extract.*
Conclusion: *The high phenolic content and high antioxidant activity of black mulberry from Southeast Serbia underline the nutritive and phytomedicinal potentials of the fruit. Further studies are, however, required before the fruit extract can be exploited in the production of health foods and as an antioxidant carrier in the food and pharmaceutical industries.*

Keywords: *Morus nigra L., Natural phenolics, Antioxidant activity, Southeast Serbia*

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INTRODUCTION

Free radicals are atoms or molecules containing unpaired electrons. The chain reaction caused by free radicals can lead to cross-linking of atomic structures. In cases where the free radical-induced chain reaction involves base pair molecules in a strand of DNA, the DNA can become cross-linked. DNA cross-linking can in turn lead to various effects of aging, especially cancer. Other crosslinking can occur between fat and protein molecules, which leads to wrinkles. Free radicals can oxidize LDL, and this is a key event in the formation of plaque in arteries, leading to heart disease and stroke. These are examples of how the free-radical theory of aging has been used to neatly "explain" the origin of many chronic diseases [1].

Phenol compounds, as antioxidants, may affect in various ways: direct reaction with free radicals, scavenging of free radicals, growing dismutation of free radicals to the compounds with much lower reactivity, chelatation of pro-oxidant metals (mainly iron), delaying or strengthening many enzymes. Fresh fruit extracts are an excellent source of polyphenolic compounds which exhibit antioxidant activity.

Black mulberry, *Morus nigra* L. (Moraceae), is a deciduous tree growing to a height of 10 to 13 m. The leaves are from 10 to 20 cm long. The tree yields dark purple-black, edible fruits that are 2 - 3 cm long after they have matured. The genus Morus is widespread in Asia, Europe, North and South America and Africa. Mulberry grows in the temperate and sub tropical regions of the northern hemisphere and it can grow in a wide range of climatic, topographic and soil conditions. Black mulberry has a unique delicious fruit, sour and refreshing taste. It has been used as a folk remedy to treat oral and dental diseases, diabetes, hypertension, arthritis and anemia [2]. Mulberry leaves play a vital role in the cultivation of silkworms. The plant has high level of anthocyanins, hence it has a very important role in the food industry. The fruit colour has been attributed to the anthocyanins present in the fruit. This has contributed to the positive effects of the fruit on people's health. [3].

The total content and yield of these compounds are dependent on geographic location and soil on which the mulberry tree grows. Despite previous research on this plant, there are no data on the phenolic content of the fruit extract of Morus nigra L. grown in Southeast of Serbia.

Plants, fruits, vegetables and other plant materials that are known to possess antioxidant activity have been investigated with the aim of finding new sources of natural antioxidants [3]. Many studies have investigated the contents of phenolics such as flavonoids and anthocyanins in mulberry extract [4-6]. Along with these compounds, black mulberry has been found to contain carotenoids [7,8]. Several studies have previously reported that anthocyanins display significant antioxidant activity [9, 10]. Phenolic compounds are a major source of antioxidant due to their ability to neutralize free radicals. Presently, there is growing interest in natural antioxidants, which prevent oxidation disorders in humans, in preference to synthetic antioxidants, some of which have been identified as carcinogens . Butylated hydroxy anisole (BHA) showed carcinogenic effects in F344 rats if their diet contain this artificial antioxidant [11].

EXPERIMENTAL

Preparation of the material

The plant material was collected in South-East Serbia in early July 2011. Fruit maturity was estimated on the basis of the color which was very black. The plant species were identified by Mirjana Milenkovic, Faculty of Biology, University of Belgrade. Voucher specimens (*Morus nigra* L No 2-1753, Bela Palanka, UTM 34TDR2 01, det.) were deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad.

The fresh plant material was stored in plastic bags and kept frozen until extraction.

Chemicals and reagents

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). Sodium chlorate buffer (pH 1.0) and acetate buffer (pH 4.5) were purchased from the same producer (Merck Chemical Suppliers (Darmstadt, Germany).

All other chemicals used, 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. pH measurements were made with Hanna Instruments pH- meter equipped with glass electrode.

Extraction of the fruits

The frozen fruits were thawed and cut into small pieces using a blender. A10 g portion of fresh fruit was extracted with either ethanol, ethanol-water (50/50,v/v) or water. Each solvent was acidified with 0.5 ml conc HCl prior to use. In each case, the extraction was performed three times with 30 ml of solvent in an ultrasonic bath for 15 min. The suspension was gravity filtered through a Buchner funnel with Whatman No. 1 filter paper and the liquid extracts stored in a refrigerator at 5 ^{o}C until they were analysed.

Determination of total phenolics

Total phenol contents of the extracts were determined by the modified Folin-Ciocalteu method [12]. An aliquot of the extracts (1 ml) was mixed with 0.5 ml of 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 one assay was obtained from three parallel determinations.

Determination of total flavonoid content

Total flavonoid content was determined using a spectrophotmetric method based on formation of flavonoid complex with aluminum [13]. The liquid extract (0.5 ml) or standard solution of catechin (50 - 500 mg l^{-1}) was mixed with 3 ml deionized water and 0.3 ml $NaNO_2$, for regulation of pH. After standing at room temperature for 5 min, 3 ml of aqueous solution of $AlCl_3$ (2 %) was added to the previously prepared solution, followed by 2 ml of 1M NaOH after another 5 min. The solution was then made up to mark with deionized water in a 10 ml flask. The absorbance of the solution was measured at 510 nm. Total flavonoid content was calculated as catechin (mg CE/100 g) based on a calibration curve for catechin (R^2=0,999). All samples were analysed in triplicate.

Determination of the total monomeric anthocyanins

Total monomeric anthocyanin content of the plant extracts was determined using pH-differential method as previously described [14]. Anthocyanins demonstrate maximum absorbance at 520 nm at pH 1.0 and also at 700 nm at pH 4.5. 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 producing oxonium forms. This alloved accurate and rapid measurement of total monomeric anthocyanins. Total monomeric anthocyanin pigment, is expressed as mg of cyanidin-3-O-glucoside. In this method, 1 ml of the liquid black mulberry extract prepared by a previously described procedure, was poured into two separate 10 ml volumetric flasks. One was made up to mark with a solution of potassium chloride (KCl) (pH 1), and the second with sodium acetate (CH_3COON_a) (pH 4.5). The two diluted solutions were left to stand for 15 min at room temperature in dark. Finally, the absorbance of each samples was measured at λ_{max} 520 nm and 700 nm, respectively. The absorbance of the investigated extracts was calculated from Eq 1.

$$A = (A_{\lambda vis\text{-}max}\text{-}A_{700})_{pH1.0} – (A_{\lambda vis\text{-}max} – A_{700})_{pH4.5} \quad \text{.....(1)}$$

Content of the monomeric anthocyanin pigment (MAP) was calculated from Eq 2.

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

where (ε) is molar absorptivity (26.900), MW is the molecular weight of cyaniding-3-O-glucoside (449.2), DF is the dilution factor ($DF = V_{extract}/V_{solution}$), *l* is the length of cuvette. Monomeric anthocyanin pigment (MAP) was expressed as mg of cyanidin-3-O-glucoside dm^{-3} (Table 1).

Determination of the total polymeric anthocyanins

Polymeric anthocyanins are resistant to the effects of bisulphate, while monomeric anthocyanins completely react with bisulphate, this caused blencing,. The black mulberry extract (1 ml) was poured into two separate 10 ml volumetric flasks. The first solution was made up to the mark with potassium chloride (KCl) solution (pH = 1), while to the second, 0.5 ml potassiumbisulphite ($K_2S_2O_3$) 0.4g$ml^{-1}$was added and the solution was made up to mark with KCl buffer pH=1. After 15 min standing at room temperature, the absorbance of each solution was measured at 420, 520 and 700 nm. Color intensity was determined from the sample without bisulphate (Eq 3).

$$Color = \{(A_{420} – A_{700}) + (A_{520} – A_{700})\} \bullet Df \quad \text{……(3)}$$

Polymeric color was determined from the sample with bisulphate (Eq 4.).

$$Polymeric\ color = ((A_{420} – A_{700}) + (A_{520} – A_{700})) \bullet Df \quad \text{……..(4)}$$

Polymeric color data were expressed as a percentage of color intensity (Table 1).

The free radical scavenging activity of the plant extracts was analyzed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [15-19]. The antioxidant assay is based on the measurement of the loss of colour of DPPH solution by change of absorbance at 517 nm caused by the reaction of DPPH with the test sample. The reaction was monitored with a UV-VIS spectrophotometer. The plant extract (0.1 ml), 1.8 ml of freshly prepared DPPH in methanol and 0.1 ml methanol were p;aced in a cuvette at room temperature. After 30 min of reaction, the absorbance was read against a blank at 517 nm. All measurements were performed in triplicate. The ability of the extracts

to inhibit DPPH (% RSC) was computed from the decrease in absorbance as in Eq 5.

$RSC\ (\%) = (1-A_{sample}/A_{blank}) \times 100$……(5)

where A_{blank} is the absorbance of control ($1 \cdot 10^{-4}$ mol dm^{-3} DPPH methanol solution) and A_{sample} is the absorbance of the test sample. The data were expressed as milligram of Trolox equivalent (TE) per 100 g of fresh sample (mgTE/100 g f.f.) and are presented in Table 1. Methanolic solution of Trolox (10 - 30 mM) and 1.8 ml of freshly prepared DPPH in methanol (20 mg period at room temperature, the absorbance was read against a blank at 515 nm. The determinations were performed in triplicate. Total antioxidant capacity was calculated as Trolox (mmol/ /100g f.f.) using the equation based on the calibration curve ($R_2 = 0.996$).

ABTS radical scavenging activity was measured using a modification of the method of Re *et al* [20]. ABTS was dissolved in methanol to a concentration of $7 \cdot 10^{-3}$ mol L^{-1}. ABTS radical cation was produced by reaction of ABTS stock solution with $2.4 \cdot 10^{-3}$ mol L^{-1} potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12 - 16 h before use. The ABTS radical cation solution was diluted with methanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. An aliquot of each extract (0.1 mL) was mixed with 3.9 mL of diluted ABTS radical cation solution. After reaction at room temperature for 6 min, the reduction in absorbance at 734 nm was measured. Trolox calibration curve was plotted as a function of the decrease in absorbance ($\Delta A = A_{blank} - A$) of ABTS radical cation scavenging activity. The results are expressed as milligrams of Trolox equivalents (TE) per 100 g of fresh sample (mgTE/100 g).

Statistical analysis

The experimental results were expressed as mean ± standard error of mean (SEM) of three determinations. Significant differences among mean values, where applicable, were determined by one-way analysis of variance (ANOVA), while differences among samples were determined by Duncan's Multiple Range test using Statistical Analysis System (SAS 1999) software [21].

RESULTS

Total phenols, anthocyanin and flavonoid contents, as well as antioxidant activity of *Morus nigra* L. fruit extracts are given in Table 1.

The results (Table 1) show that the content of total phenols in the investigated extracts ranged from 90.26 mg GAE/100 g to 118.84 mg GAE/100 g. Water extract displayed the highest content of phenol compounds. Total flavonoids was in the range 141.70 mg (water extract) to 183.90 mg CE/100 g (ethanol/extract) while anthocyanin content was between 114.83 mg cyanidin 3-glucoside/100g for water extract and 128.68 mg cyanidin 3-O-glucoside/100g for ethanol-water extract. All the extracts exhibited good scavenging activity (40.55 to 71.41 %) against DPPH radicals. Based on ABTS test, the activity ranged from 41.80 to 55.43 %. Ethanol extract exhibited the highest level of polymeric color (37.67 %) followed by the water and ethanol/water extracts with 28.26 and 22.80 %, respectively.

Table 1: Total phenols, anthocyanin and flavonoid contents, and antioxidant activity of Morus nigra L. extracts

Parameter	Extract		
	Water	***Ethanol/water (50/50 v/v)***	***Ethanol***
Total phenols [a]	118.84±0.93	109.80±2.76	90.26±0.92
Flavonoids [b]	141.70±6.60	144.90±8.50	183.90±11.50
Monomeric anthocyanin[c]	114.83±2.14	128.68±0.93	125.84±2.22
Polymeric colour (%)	28.26±0.97	22.80±0.68	37.67±1.02
RSC (%)	52.05±3.78	71.41±1.47	40.55±2.62
DPPH[d]	197.50±10.83	283.10±3.61	168.71±9.91
ABTS (%)	55.43±4.30	41.80±0.50	44.93±2.00
ABTS[e]	156.35±11.53	115.00±7.28	127.80±5.37

[a]Expressed as mg of GAE/100g f.f.; [b]Expressed as mg of CE/100g f.f.t [c]Expressed as mg of cyanidin-3-O-glukoside/100g f.f.; [d,e]Expressed as mg of TE/100g f.f.;

The correlation coefficients between the e are shown in Table 2. The correlation is very high ($0.9 < |r| \leq 1$), high ($0.7 < |r| \leq 0.9$), substantial ($0.5 < |r| \leq 0.7$) , insignificant ($0.2 < |r| \leq 0.5$) and low ($|r| \leq 0.2$).

As Table 2 shows, there was a very high coefficient of correlation between the content of phenols and flavonoids (0.9405) and between flavonoids and polymeric color (0,9137). Substantial correlation also occurred between the total phenol content and polymeric color (0.5987), while there is a non significant correlation between the content of total phenols and monomeric anthocyanins (0.3594).

Additionally, a high degree of correlation exists between monmeric anthocyanin content and antioxidant activity based on ABTS assay (0.9993) and between the content of polymeric color and antioxidant activity based on DPPH assay (0.9137). Some correlation exists between

Table 2: Correlation of total phenol, flavonoid and anthocyanin contents, and their correlation with antioxidant activity of mullbery (*Morus nigra* L.) fruit

		Total phenols	***Flavonoids***	***Monomeric anthocyanin***	***Polymeric colour (%)***	***RSC (%)***	***ABTS (%)***
***Morus nigra* L.**	*Total phenols*	1.0000	0.9405	0.3594	0.5987	0.3064	0.3349
	Flavonoids		1.0000	0.1491	0.8189	0.5476	0.1313
	Monomeric anthocyanin			1.0000	0.0019	0.1121	0.9993
	Polymeric colour (%)				1.0000	0.9137	0.0047
	RSC(%)					1.0000	0.1288
	ABTS (%)						1.0000

the content of flavonoids and RSC (%) (0.5476). There is a no correlation between total phenols and RSC (0.3064), and betwwen phenol and ABTS (0.3349). In all other cases, there was also no correlation.

DISCUSSION

Many studies have shown that the physiological functions of natural ingredients linked usually to the antioxidant activity of phenolic compounds. In a previous work, methanol was used as the extracting solvent. However, in this study, ethanol was used instead of methanol due to the toxicity of the latter in humans. Ethanol is good solvent for polyphenol extraction and is safe for human consumption. In present work, all the solvents used were acidified [22].

Previous studies have examined the total content of phenols, flavonids, anthocyanins and antioxidant activity of *M. nigra* from different countries and regions . For example, Kutlu *et al* used acidified methanol, acidified water and non acidified methanol-water (70/30 v/v %) for the extraction of phenolic compounds. The highest content of phenolic compounds was found in non-acidified methanol-water (70/30 v/v) (580 mg GAE/100 g) extract, while water extract exhibited the lowest amount of phenols (330 mg GAE/100 g) [6]. The methanol extract had 440 mg GAE/100 g fresh fruit. The authors concluded that increase in the acidity of the solvents does not increase the amount of extracted phenols [6]. Ercisli *et al* analyzed the phenolic content of the black mulberry collected in Turkey using Folin Ciocalteu method [23]. Their results indicate that the plant contained 215 mgGAE/100 g phenols, which was higher than the contents of phenolics (90.26-118.84 mgGAE/100g fresh fruit) obtained for Southeast Serbia in the present study.

Also, Ozgen *et al* [2] found higher phenol content (270 mg GAE/100 g fruit) than that found in this work. Ercisli *et al* discovered significantly higher values of total phenol content of 1422 mg GAE per 100g fruit in the plant [24]. The phenol content was high (1943 - 2237 mg GAE/100 g fruit and 1515 – 2570 mg GAE/100 g) probably because the extraction with methanol was carried out in a Soxhlet apparatus, [26]. The ethanol extract of black mulberry from Korea gave 867 mgGAE/100g [27].

Differences in total phenol content are dependent on the extraction medium used and polarity of the extracting solvents. Extraction duration, method and the condition of the plant material can also influence the content of phenols.[22].

Previously reported flavonoids content of 0.56 to 6.54 mg CE/100 g [26] is lower than that found in the present work. Anthocyanins analysis of black mulberry from Turkey showed 720 mg Cy-3-O-glucoside/100 g fruit [23]. which is much higher than the 114.83 to 128.68 mg Cy-3-O glucoside/100 g of fruit obtained in our studies. On the other hand, black mulberry from Korea contained 571 mg Cy-3-O-glucoside/100 g dry fruit [26].

Furthermore, the methanol, aqueous and methanol/water (70/30 v/v) extracts of black mulberry from Turkey had antioxidant activity of 95, 80 and 85 % [5]. Other studies have shown that antioxidant activity, expressed as Trolox equivalent, 0.48 mgTE/100 g which is considerably less than that observed in the present studies [23].

Extracts of fresh mulberry fruit from South East Serbia, contain high levels of total phenols, flavonoids and anthocyanins, > 100 mg/100 g of fruit. The highest content of phenols were found in aqueous extract, flavonoids in ethanol extract and anthocyanins in ethanol/water extract. Extracts of mulberry have a high content of polymeric anthocyans. All investigated extracts showed high antioxidant activity. The highest activity against DPPH was by ethanol/water extract. All the tested extracts being high in phenolic content, and antioxidant activity means that they can possibly be used as effective antioxidant agents.

CONCLUSION

The high phenolic content and high antioxidant activity of black mulberry from Southeast Serbia underline the nutritive and phytomedicinal potentials of the fruit. Further studies are, however, required before the fruit extract can be exploited in the production of health foods and as an antioxidant carrier in the food and pharmaceutical industries.

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