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BIOACTIVE COMPOUNDS AND ANTIOXIDANT CAPACITY OF SOME *Morus* SPECIES

Mira-Elena IONICA[1,*], Violeta NOUR[1] and Ion TRANDAFIR[2]

1. University of Craiova, Faculty of Horticulture, Craiova, Romania.
2. University of Craiova, Faculty of Sciences, Craiova, Romania.
*Corresponding author, M.E. Ionica, E-mail: miraelena29@yahoo.com

ABSTRACT. *They were investigated the main phytochemicals, antioxidant capacity and the major phenolic compounds in white mulberry (Morus alba L.), black mulberry (Morus nigra L.) and red mulberry (Morus rubra L.). Dry matter (%), total soluble solids (%), titratable acidity, total phenolic and total flavonoid content, antioxidant activity and phenolic profile were determined on the fresh fruits of the studied species. The highest total dry matter content was found in red mulberry fruits (18.07%), followed by white mulberry (17.69%). The total soluble solids content varied in the selected mulberry fruits, the lowest content being recorded in the black mulberry fruits (5.97%). Red mulberry had the greatest antioxidant activity (505.502 mmol Trolox/100 g fw) followed by black mulberry and white mulberry. The content of flavonoid varied from one species to another from 72.285 mg QE/100 g fw in red mulberry to 241.215 mg QE/100 g fw in black mulberry. Mulberry fruits have an outstanding nutritional value due to their chemical composition with high levels of phenolic content as well as for the antioxidant activity. Mulberry fruits can be successfully used in obtaining of functional foods.*

KEY WORDS: *mulberry, phytochemicals, antioxidant capacity, total phenolic content.*

INTRODUCTION

Mulberry belongs to the *Morus* genus, of the *Moraceae* family, and it is found in East, West and South East Asia, Southern Europe, South America, Northwest South America and several areas of Africa. There are 24 species of *Morus* and a subspecies with at least 100 known varieties, the most common species of being: white mulberry (*Morus alba L.*), black mulberry (*Morus nigra L.*) and red mulberry (*Morus rubra L.*). Mulberry was

traditonal grown both for its leaves (used as food for silkworms - *Bombyx mori L.* and as animal feed), as well as ornamental trees (Awasthi et al. 2004). Mulberry is a unique delicious fruit with a refreshing acid taste (Kostić et al. 2013). Fresh mulberry is a very perishable fruit and since it softens very quickly, preservation during transportation and marketing is very difficult (Gerasopoulos and Stavroulakis 1997). However, due to its nutritional value, the fruit is consumed nowadays, both fresh and processed in the form of juices, syrups, jams, beverages, natural dyes or dried fruits. In addition, their fruits, roots and bark have been used in traditional medicine (especially in Chinese and Turkish medicine) to treat diabetes, hypertension, anemia and arthritis, these effects being mainly related to their phenolic composition. Also, black mulberry fruits are used for treating mouth lesions in Turkey (Özgen et al. 2009). The root of the mulberry tree is one of the constituents of a medicine that is used to treat high blood pressure. Root juice agglutinates blood and is very useful in killing worms from the digestive system (Khalid et al. 2011). Mulberry leaves have also promising biological effects. Recently, it was found that mulberries are a rich source of phenolic compounds, including flavonols, phenolic acids and anthocyanins, in the case of red and black mulberries. Additionally, multiple findings suggest that anthocyanin contents of mulberry and other red fruits may provide possible health benefits such as reduced risk of coronary heart disease, stroke, certain types of cancers and aging (Zafra-Stone et al. 2007). Identification and quantification of phenolic substances from mulberry is a starting point for assessing their specific nutritional and biological qualities (Sánchez-Salcedo 2015). The chemical constituents of white mulberry have been well studied by Taro Nomura (Nomura 1988) who has reported phenolic glycosides and prenylated flavonoids. The mulberry fruits extracts or their components, especially flavonoids (quercetin, routine and isoquercetin), reveal free radicals that have potential against oxidative stress. The presence of prenylated flavonoids has strengthened the beliefs about antioxidants, providing cardiovascular protection because it inhibits LDL oxidation and hence atherosclerosis (Butt et al., 2008). Different authors (Gunes & Cekin 2004; Ercisli & Orhan 2007, 2008; Katsube et al. 2006) studied the quality, nutritional potential and chemical composition of the fruits and leaves of some of the *Morus* species. However, studies on quantification of phytochemicals and antioxidant properties of mulberry fruits are very limited (Kostić et al. 2013). Given the above, the aim of this study was to examine the main

phytochemical, antioxidant capacity and the major phenolic compounds in white mulberry (*Morus alba L.),* black mulberry (*Morus nigra L.)* and red mulberry (*Morus rubra L.)* selected fruits grown in the spontaneous flora of Dolj county of Romania.

MATERIALS AND METHODS

Plant material

Mulberry fruits were harvested from selected white mulberry (*Morus alba L.*), black mulberry (*Morus nigra L.*) and red mulberry (*Morus rubra L.*) from spontaneous flora of Dolj County, Romania. Each clone includes 3 trees of the same age. All berries were picked at the commercially ripe stage. The berries were selected according to uniformity of shape and color. Mulberry fruits (15 kg for each species) were harvested in July (commercially ripe stage) and sorted to eliminate damaged, shriveled and unripened fruits. The mulberries were selected according to uniformity of shape and color and reflect the attributes of the studied species. Dry matter (%), total soluble solids and titratable acidity were determined on the same day from the fresh fruits of the studied species. For determination of the total phenolic and total flavonoid content, antioxidant activity and phenolic profile the samples were stored (three hours after harvest) into a freezer at -20°C for a month until analysis.

Analytical methods

The total dry matter content (TDW%) was determined by drying in an oven at 105°C until constant weight. Total soluble solids (TSS%) were measured with a digital refractometer (Hanna Instruments, Woonsocket, USA) from the juice pressed from the fruit at 20°C. The results were expressed in percentages. Titratable acidity (TAC) was measured by the titrimetric method (AOAC, 1984) and the results were expressed as % citric acid.

Antioxidant activity

The antioxidant activity was measured in the methanolic extract using the DPPH (2,2-diphenyl-1-picrylhydrazil) assay. Methanol (Merck, Germany), DPPH (2,2-diphenyl-1-picrylhydrazil) (SigmaAldrich, Germany) and Trolox (Merck, Germany) were employed. The extraction of samples was made according to the same protocol described for total phenolic content. The free radical scavenging ability of the extracts against DPPH free radical was evaluated as described by Oliveira et al. (2008), with some modifications. Each ethanol mulberry extract (50 µL) was mixed with 3 mL of a 0.004% (v/v) DPPH methanolic solution. The mixture was incubated for 30 min at room temperature in the dark and the absorbance was

measured at 517 nm on Varian Cary 50 UV-VIS spectrophotometer. The DPPH free radical scavenging ability was calculated in reference to Trolox (6-hydroxy-2,3,7,8-tetramethylchroman-2-carboxylic acid), which was used as standard reference to convert the inhibition capability of each extract solution to the mmol Trolox equivalent antioxidant activity/L. The radical was freshly prepared and protected from light. A blank control of methanol/water was used in each assay. All assays were conducted in triplicate and results were expressed in mmol Trolox/100 g fresh weight (fw).

Total phenolic content

Total phenolic content was assessed by using the Folin-Ciocalteu phenol reagent method (Singelton and Rossi, 1965). Folin-Ciocalteu reagent (2 N, Merck), gallic acid (99% purity, Sigma-Aldrich) and anhydrous sodium carbonate (99% Sigma-Aldrich) were used. One gram of mulberry homogenate was extracted with 15 mL methanol in an ultrasonic bath for 60 min at ambient temperature. After extraction, the samples were centrifuged for 5 min at 4200 rpm and supernatants were filtered through polyamide membranes with pore diameter of 0.45 µm and stored at a temperature of -20 °C. 100 µL of each mulberry methanolic extract were mixed with 5 mL of distilled water and 500 µL of Folin-Ciocalteu reagent. After 30 sec to 8 min, 1.5 mL of sodium carbonate (20% v/v) was added. The reaction mixture was diluted with distilled water to a final volume of 10 mL. The preparation of the standard solution of gallic acid followed the same procedure. The absorbance at 765 nm of each mixture was measured on a Varian Cary 50 UV spectrophotometer (Varian Co., USA) after incubation for 30 min at 40 °C and results were expressed in mg gallic acid (GAE)/100 fresh weight (fw).

Total flavonoid content

Total flavonoid content was assessed by the spectrophotometric method described by Mohammadzadeh et al. (2007) based on the color reaction of this class of compounds with the ions of Al (III). Mulberry homogenate (1 g fresh matter) was extracted with 15 mL methanol in an ultrasonic bath for 60 min at ambient temperature. After extraction, the samples were centrifuged for 5 min at 4200 rpm and supernatants were filtered through polyamide membranes with a pore diameter of 0.45 µm and stored at -20 °C. 0.5 mL filtrate was placed in a polyethylene test tube, together with 0.1 mL 10% aqueous solution of aluminum nitrate, 0.1 mL aqueous 1 M sodium acetate, 4.3 mL methanol, mixed well and left to react for 40 min at room temperature. After completion of the reaction, the absorbance of the mixture was measured at 415 nm on a Varian Cary 50 UV-VIS spectrophotometer (Varian Co., USA). The quantification of flavonoids was carried out on the basis of a calibration curve using quercetin as standard reference in the range of 0-100 mg/L. The results were expressed as mg of quercetin equivalents/100 g fw.

<u>*HPLC analysis of phenolic acids and flavonoids*</u>

The phenolic compounds for chromatographic analysis were extracted from 0.5 g of mulberry fruit in 10 mL of 70% methanol and 10 mL of 4 N HCl for 30 min at 80°C under reflux conditions. The chromatographic separation of the individual phenolic compounds was carried out using a Surveyor Thermo Electron system (Thermo Electron Corporation, San Jose, CA, USA) including vacuum degasser, Surveyor Plus LCPMPP pump, Surveyor Plus ASP autosampler, PDA5P diode array detector and Chrom Quest 4.2 system manager as data processor. Separation was achieved by a reversed-phase Hypersil Gold C18 column (5 m particle size, 250 mm × 4.6 mm) provided by Thermo Electron Corporation (USA). HPLC analysis was performed according to our previously reported method (Nour et al., 2013). The mobile phase consisted of 1.5% aqueous acetic acid solution (A) and methanol (B). The samples were eluted with the following gradient: 90% (A) from 0 to 27 min, from 90 to 60% (A) in 28 min, 60% (A) for 5 min, from 60 to 56% (A) in 2 min, 56% (A) for 8 min, from 56 to 90% (A) in 1 min and 4 min 90% (A) to re-establish the initial conditions, before the injection of another sample. All gradients were linear. The flow rate was 1 mL/min, the injection volume was 5 μL while column temperature was maintained at 20 °C. UV detection was carried out at 254 nm for rutin, myricetin and quercetin, 278 nm for gallic acid and 300 nm for chlorogenic, caffeic, *p*-coumaric, ferulic, sinapic and salicylic acids. Identification was based on the comparison of the spectra obtained between 220 and 450 nm and the retention time of the unknown substances in relation to that of pure standards. Phenolic compounds quantification was achieved by regression analysis of compound peak area against concentration. The results regarding the content of phenolic compounds were expressed in mg/100 g fw.

RESULTS AND DISCUSSIONS

The results of the total dry matter content (TDW), total soluble solids content (TSS) and titratable acidity (TA) in the selected black, white and red mulberry fruits are presented in Table 1.

The highest total dry matter content was found in red mulberry fruits (18.07%), followed by white mulberry (17.69%), with no significant differences between the two species. In contrast, a lower amount of total dry matter was found in the black mulberry fruits (12.29%). Imran et al. (2010) found a total dry matter content of 18.88% in white mulberry fruits and 17.60% in black mulberry fruits, while higher values were found by Ercisli & Orhan (2007), namely 29.5% in white mulberry fruits and 27.4% in black mulberry fruits. The total soluble solids content varied in the selected

Table 1. Dry matter content (TDW), total soluble solids content (TSS) and titratable acidity (TAC) in the selected black, white, and red mulberry fruits.

Compositional characteristics	White mulberry	Red mulberry	Black mulberry
TDW (%)	17.69±0.903	18.07±0.910	12.29±0.618
TSS (%)	12.7±0.711	14.14±0.709	5.97±0.300
TAC (%, as citric acid)	0.096±0.005	0.128±0.005	0.352±0.019

mulberry fruits, the lowest content being recorded in the black mulberry fruits (5.97%), and the highest in the red mulberry fruits (14.14%). Linghong et al. (2012) found similar values of TSS (12.5 - 14.7%) in white mulberry fruits. Regarding the titratable acidity, amounts ranging from 0.096% citric acid in white mulberry fruits to 0.352% citric acid in black mulberry fruits were found. These values are lower than those found by Linghong et al. (2012) (0.73%) in white mulberry fruits and by Ercisli & Orhan (2007) in white (0.25%), and black (1.37%) an red (1.37%) mulberry fruits. The total phenolic content, total flavonoid content and antioxidant activity in the selected mulberry fruits are given in Table 2.

Table 2. The total phenolic content, total flavonoid content and antioxidant activity in the mulberry fruits.

	Antioxidant activity (mmol Trolox/100g fw)	Total phenolic content (mg GAE/100 g fw)	Total flavonoid content (mg QE/100 g fw)
White mulberry	454.97±21.72	458.42±23.11	78.04±4.03
Red mulberry	505.50±24.96	436.93±22.01	72.29±3.86
Black mulberry	480.89±24.13	924.55±46.39	241.22±12.15

Among the selected mulberries, red mulberry had the greatest averages of antioxidant activity (505.502 mmol Trolox/100 g fw) followed by black mulberry and white mulberry. Ercisli & Orhan (2008) and Özgen et al. (2009) found moderate antioxidant activity in selected black mulberries using different antioxidant methods, while Linghong et al. (2012) found a high radical scanvenging activity (92-97%). It is known that mulberries have high contents of phenolics and flavonoids. The total phenolic content (TPC) of mulberry fruits varied from 436.93 mg GAE/100 g fw to 924.55 mg GAE/100 g fw. White mulberry had the lowest whereas black mulberry had the highest level which is according with the results found by Sánchez-

Salcedo et al. (2015). Scalzo et al. (2005) and Ercisli & Orhan (2007) reported that total phenolic and flavonoids content are affected by plant genotype and cultivation. Different values of total phenolics content have been reported by Kostić et al. (2013) who found 118.84 mg EGA/100 g in the aqueous extract of black mulberry while Ercisli & Orhan (2007) found 1422 mg EGA/100 g. The content of flavonoids varied from one species to another from 72.29 mg QE/100 g fw in red mulberry to 241.22 mg QE/100 g fw in black mulberry. Similar values were found by Ercisli & Orhan (2007) in black mulberry fruits (276 mg QE/100 g fw) and Memon et al. (2010) in white mulberry (48.13 mg QE/100 g fw). The phenolic compounds were determined in black mulberry fruits by high performance liquid

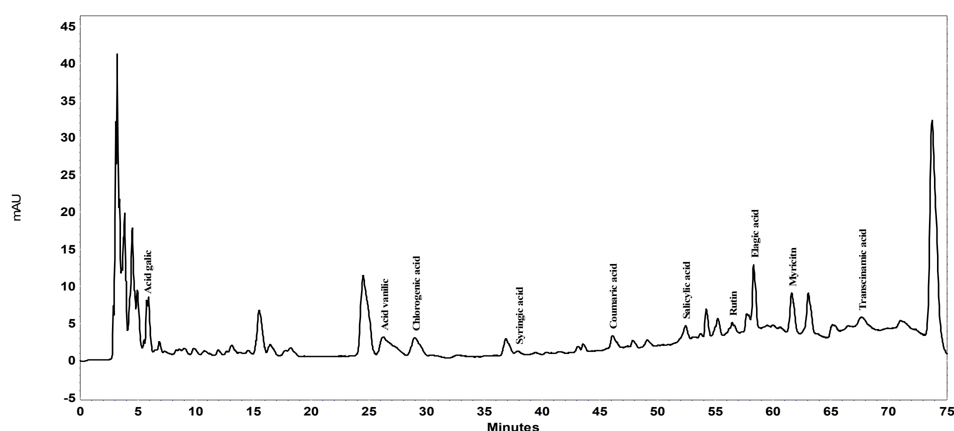

Figure 1. HPLC chromatogram at 278 nm of the black mulberry methanolic extract.

chromatography (Figure 1) and the results are given in Table 3. Ellagic acid (9.56 mg/100 g fw), followed by chlorogenic acid (5.08 mg/100 g fw), and then salicylic acid (4.46 mg/100 g fw) content had the largest ratio in black mulberries, while the siringic acid had the lowest value (0.14 mg/100 g fw). Memon et al. (2010) found similar values for some phenolic compounds in the mulberry fruits grown in Pakistan (4.41 mg chlorogenic acid/100 g dw and 5.81 mg gallic acid/100 g dw) while Gundogdu et al. (2011) reported contents of 3.016 mg chlorogenic acid /100 g dw and 0.150 mg gallic acid/100 g dw.

Table 3. Phenolic compounds in black mulberry fruits.

Phenolic compound	Content (mg/100 g fw)
Gallic acid	1.14±0.01
Catechinhidrate	Not detected
Vanillinic acid	1.79±0.09
Chlorogenic acid	5.08±0.26
Caffeic acid	Not detected
Syringic acid	0.14±0.01
Epicatechin	Not detected
Coumaric acid	0.76±0.04
Ferulic acid	Not detected
Sinapic acid	Not detected
Salicylic acid	4.460±0.24
Rutin	1.94±0.10
Ellagic acid	9.56±0.52
Myricitn	4.24±0.23
Transcinamic acid	Not detected
Quercitin	Not detected

CONCLUSIONS

Mulberry fruits are distinguished by a significant amount of nutrients and bioactive compounds, which have a special role in human health. However, in Romania, the mulberry tree is cultivated on small surfaces for its fruit because the benefits of the mulberry fruit have not been sufficiently highlighted over the years. In the past, the mulberry tree was cultivated on vast areas in our country, as a feed for silkworms (leaves). White mulberries are distinguished by a higher dry matter and total soluble solids content as against black mulberries, the latter having a higher titratable acidity. Mulberry fruits posses an outstanding nutritional value due to their high levels of phenolic compounds as well as their high antioxidant activity and they could be successfully used in obtaining functional foods.

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