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MORUS SP. FOR REVIGORATING SILKWORM BREEDING IN ROMANIA AND PROMOTING HEALTH BENEFITS OF LEAVES AND FRUITS

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Abstract

Besides supplying food for silkworm rearing, initiation of a mulberry plantation is an important business, which may bring important income in rural areas. Plants of the genus Morus are known to be a rich source of bioactive compounds from flavonoids and phenolic acids, compounds with known antioxidant and other benefic properties. The aim of the present study was the characterization of mulberry extracts (from leaves and fruits) regarding their chemical composition and determination of some bioactive properties of the mentioned matrices. Sugar profile, total protein and lipid content, total phenolics and flavonoids, mineral composition and aminoacid profile were determined. Mulberry leaves are important protein sources (being the principal feed for silkworm) and also important source of free aminoacids, including the essential ones. 25 free aminoacids (aa), were identified and quantified by internal standard method using LC-MS and EZ: faast Phenomenex kit. The main aa were alanine (144 mg/100g) and Gama-aminobutiric acid (153 mg/100g). The main minerals from mulberry extracts were: iron, zinc and calcium. Total identified simple sugars ranged between 8 and 13% and lipid content between 0.5 to 1.5%.

Key words*: Morus sp., agriculture, mulberry leaves; bioactive properties, silkworm*

INTRODUCTION

Mulberry (*Morus* sp.) are grown worldwide for sericulture. Their leafs being the only natural food for silkworms, but also for other purposes, among them being fruit production. The most popular species are *Morus alba* and *Morus nigra*, and the intensive selection and mutation breeding have resulted in thousands of cultivars, hybrids and polyploids (Yongkang, 2000). Mulberry trees are valuable species not only for silkworm rearing, but also for gardening and landscaping, street shade and reduction of pollution level in the environment and soil (Rafati et al., 2011).

The fruits are important sources of bioactive compounds such as simple sugars (glucose and fructose) (Imran et al., 2010), alkaloids (Kim et al., 2014), carotenoids, polyphenols, anthocianins and minerals (Dimitrijevic et al., 2014; Okatan et al., 2016).

The bioactivity of mulberry fruits was determined in many studies, having antioxidant properties (Imran et al., 2010; Okatan et al., 2016), blood glucose reductor (Shin et al., 2016), antibacterial, anti-inflammatory and anticancer activity, as well as cardiovascular and cardioprotective activity (Gryn-Rynko et al., 2016). Even the phytochemical composition of leaves and fruits of mulberry are studied worldwide, new promising sources of natural antioxidants or other phytotherapeuticals can be found in this valuable matrix.

The aim of our study was to evaluate the chemical composition of extracts from fresh and dry leaves and fruits of *Morus nigra*, Ukraina variety.

MATERIALS AND METHODS

Chemicals and materials. All chemicals and reagents were analytical grade or chromategraphic grade and ultrapure water, purchased from Sigma, Merck and Fluka.

Mulberry leafs, belonging to *Morus nigra* specie, 'Ukraina' vartiety, were harvested in

2017, placed at -18°C or air dried in the dark and grounded in fine powder and kept at 4°C until analysis. Mulberry fruits were harvested in 2017, and kept at -18°C in plastic bags or dried in the oven at 60°C until constant weight. After drying, the fruits were grounded in fine powder and refrigerated in plastic bags until determinations.

Sample preparation. Mulberry fruits and leafs were prepared according to the parameter to be determined: water extracts, ethanol extracts or calcinations for mineral determination.

Sugar spectrum. Method used for determination of sugar spectrum is high performance liquid chromatography with refractive index detection (Bonta et al., 2008).

Operational parameters of chromatographic system were: column: modified Alltima Amino 100Å, 5 µm, 250 x 4.6 mm; mobile phase flow: 1.3 ml/min; mobile phase: acetonitrile/water (75/25; v/v); column temperature: 30°C; injection volume : 20 µl; separation time: 60 min. Sugar standard solutions are prepared like the analyzed sample. Standards are injected and analysed separately to determine the retention time of each sugar and in mixture for the calibration curve construction. Results are expressed in g/100 g sample.

*Lipid content***.** Total lipids were determined by extraction with organic solvents using Soxhlet method. Two grams of sample were weighted on filter paper, which will be packed and placed in the paper cartridge. Dry and clean extraction glasses containing 2 boiling stones, will be weighted and together with the cartridge and the solvent (70 ml n-hexane) will be fixed in PTFE cylinders. The method is set from the multistat: extraction temperature 140°C, extraction time 3 h, 25 min, washing 30 min, solvent evaporation in hot air flow 10 min. Extraction glasses, dried in the oven at 60°C and cooling, are weighted and the result were expressed as percent.

Protein content. From the homogenized sample, 1g is weighted in paper bags handled with a tweezer so that they are not contaminated with different substances that may contain nitrogen. Paper bags are placed in digestion unit vialse (Buchi Digesion Unit K-424), with 2 Kjeldahl tablets and 20 ml concentrated sulphuric acid (95-98%). Digestion lasts 2 h, until the solution turns to green. Distilation is made with Büchi, KjelFlex K-360 unit, every determination is made with a mixture of reagents (50 ml H_2O: 90 ml NaOH: 60 ml H_3BO_3). Titration is made with automatic titrator TitroLine Eeasy (Schott), using 0.05M sulphuric acid for low protein matrices, until pH of 4.65.

Determination of mineral content. To determine the levels of micro and macroelements: Na, Mg, K, Ca, Fe, Pb from studied plant matrices, the atomic absorption spectrometry method was used. The mineralization of the samples was performed in a microwave furnace, Berghof digestion system MWS-2. Approximately 0.3 grams of the homogenized samples were placed in special Teflon tubes, 2 ml of 65% HNO_3 was added and let to react for 15 minutes, after which 3 ml of H_2O_2 was added before the container was sealed (Quinn et al., 1994; Finger et al., 2014). At the end of the initiated program, the solution is transferred into plastic containers and the sample is diluted with ultrapure water to a volume of 125 ml.

An Aanalyst 800 Atomic Absorption Spectrometer from Perkin-Elmer was used, equipped with a cross-linked graphite furnace. It is electrically heated and the voltage will be applied transversely to the tube, perpendicular to the light beam, and finally the electromagnet will generate a magnetic field parallel to the radiation beam emitted by the lamp (Farrukh, 2012).

Free aminoacid profile. Aminoacid profile was determined by liquid chromatography – mass detection (LC-MS), as a method with high selectivity and sensitivity. Determinations were made on a Shimadzu LC-MS (Japan), with electrospray ionization, opedrating in positive mode. Operational parameters of the method were: EZ: faast AAA-MS, 250 x 3.0 mm chromatographic column, mobile phases: ammonium formate 10 mM in water (A) and ammonium formate 10 mM in methanol (B), 0.3 ml/min flow, column temperature 35ºC, injection volume 1 µL, detector tension 1.7 KV, acquisition time 33 min.

Polyphenolic and Flavones Contents. For the total polyphenol content determination, the Folin-Ciocâlteu method was used (Folin and Ciocâlteu, 1927), modified by various authors and adapted to all types of matrices (Duda et al., 2015; Mihaylova et al., 2013; Singleton and

Rossi, 1965; Kim et al., 2003). A volume of 25 µl of each ethanolic extract was mixed for 5 minutes with 125 µl of 0.2 N Folin-Ciocâlteu. The samples were incubated in the dark for 120 min. The absorbance was measured at 760 nm, using a Sinergy 2 Biotek Multichannel spectrophotometer. The standard curve was prepared by using different concentrations of gallic acid and the results were expressed as gallic acid equivalents/100 g).

The quantification of flavones in the samples was made using Dowd method (1959) based on the reaction of aluminium chloride, as specific reagent, with the flavonoids present in the sample giving a yellow color, proportionally with the concentration of the compounds, determined spectrophotometrically at 415 nm.

Radical Scavenging Activity Assay (DPPH).

The antioxidant activity is the primary step in determining the biological activity of any natural matrix. DPPH free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving a discoloration of solution, proportionally with the amount of antioxidant present in the sample (Paşca et al., 2016; Duda et al., 2015). The free radical scavenging ability of the ethanolic extracts was measured in terms of hydrogen donation or radical scavenging ability using this method. Thus, 5 µl of 1% plant alcoholic extract was mixed with 295 µl of 0.02 mg/ml DPPH solution in methanol, stirred and incubated in the dark for 20 minutes. The absorbance changes were monitored at 517 nm using a Sinergy 2 Biotek multichanel spectrophotometer. The percent inhibition of DPPH free radical was calculated by the formula: Percentage inhibition (% I) = $[(A_{blank} - A_{sample})/A_{blank}] \times 100$, where, A_{blank} is the absorbance of the control reaction (DPPH alone) and A_{sample} is the absorbance of DPPH solution in the presence of the test compound.

RESULTS AND DISCUSSIONS

Mulberry fresh leaves and fresh fruits have a high content of water (62.07% in leaves and 40.89% in fruits).

Chemical composition of leaves and fruits of *Morus* sp. are presented in Table 1.

Table 1. Sugar spectrum, total lipids and total nitrogen (%) of fruits and leaves of *Morus nigra*

Specification	Fresh leaves	Dry leaves	Fresh fruits	Dry fruits
Glucose	0.56	1.48	5.96	27.09
Fructose	0.89	1.77	6.16	29.65
Zaharoza	1.43	3.06	-	-
Galactoza	1.02	2.03	-	-
Total lipids	2.41	3.40	1.51	3.65
Total nitrogen	11.56	19.70	0.96	1.57

Leaves of mulberry contain fructose and glucose in small amounts, but a higher content of sucrose. Also galactose is present in this matrix. Instead, the fruits contain only fructose (in higher amounts) and glucose. The amounts determined in our study were in accordance with other studies of *Morus nigra* fruits (Okatan et al., 2016). Dry fruits are important simple sugar sources, as seen in Table 1.

Leaves of mulberry contain high amounts of lipids, several times higher than determined by different authors (Gryn-Rynko et al., 2016; Imran et al. 2010), but smaller than those of Iqubal et al. (2012). However, the presence of an appreciable content of lipids demonstrates the potential of these leaves to have dietary purposes with promising nutritional attributes.

Higher amounts of lipids were obtained in the fruits of *Morus nigra*, compared with literature studies (Imran et al., 2010).

The contents of some minerals and lead from mulberry leaves and fruits are given in Table 2.

Table 2. Mineral content of leaves and fruits of *Morus nigra*

Specification	Fresh leaves	Dry leaves	Fresh fruits	Dry fruits
Na (mg/kg)	23.41	50.51	71.65	81.51
Mg (mg/kg)	2.54	4.03	41.80	56.76
Ca (mg/kg)	542.25	908.79	2693.82	7102.44
Fe (mg/kg)	50.41	76.21	67.52	86.86
K (mg/kg)	1756.22	3308.46	3333.33	7009.18
Pb (mg/kg)	0.00	0.00	0.00	0.00

The distribution of the elements among the leaves and fruits of mulberry were in favour of fruits, much higher amounts being determined in fruits compared to leaves.

No lead was determined in leaves and fruits of mulberry, indicating no pollution in the area of mulberry plantation.

Dimitrijevic et al. (2014) found in mulberry fruits different heavy metals in fruits of mulberry grown in Serbia (9 mg/kg lead and 36 mg/kg nichel).

Very high amounts of potassium were determined both in leaves and in fruits of mulberry. If the leaves are good source of pota-

ssium for silkworm, the fruits are very good candidates for potassium supplementation in human diet. High amounts of calcium (7102.44 mg/kg) were determined in dry fruits. This mineral is very important in diet, and having such a high content in the fruits of *Morus nigra*, makes this fruit even more valuable.

Iron and magnesium determined in our study was similar with other studies (Imran et al., 2010). As can be seen in Table 2, mulberry fruits may be considered as good sources of magnesium.

Very different amounts were determined by Yigit et al. (2010) in some varieties of mulberry (leaves and fruits) drom Turkey. Similar results were obtained only for calcium and sodium.

Taking into consideration the high amount of protein from mulberry leaves, we conducted a study of determining the free aminoacid profile from this matrix (Table 3).

Table 3. Aminoacid profile of mulberry leaves

Aminoacid	Leaf (base)	Leaf (middle)	Leaf (top)
Arginine	33.46	20.48	45.54
Serine	164.43	16.25	17.05
Asparagine	75.67	43.33	46.63
1-metil-histidine	0.18	0.14	0.21
4-hidroxiproline	46.29	27.25	28.82
Glicine	13.21	15.07	8.85
Glicina-proline (dipeptide)	0.28	0.23	0.27
Treonine	29.80	31.10	23.61
Alanine	144.01	128.96	66.54
Gama-aminobutiric acid	153.30	129.07	105.78
Alfa-aminobutiric acid	0.32	0.51	0.00
Ornitine	0.55	0.38	0.48
Metionine	0.21	0.13	0.17
Proline	60.66	47.63	42.90
Lizine	23.20	21.49	23.46
Aspartic acid	11.39	15.36	12.52
Histidine	6.72	6.14	5.64
Thiaproline	0.06	0.08	0.02
Valine	25.69	26.93	12.80
Glutamic acid	27.29	33.07	20.18
Triptophan	13.58	12.57	12.60
Leucine	24.94	22.75	21.07
Phenilalanine	18.71	16.00	20.12
Izoleucine	11.45	12.29	8.71
Tyrozine	17.71	16.07	17.64

Twenty five free aminoacids were detedrmined in the leaves of mulberry, harvested from three different parts of the tree. No significant differences were observed in the three locations of the tree in respect of free aminoacid profile, but the total amount of free aminoacids were determined in leaves from the base of the tree (903.1 mg/100 g), followed by the leaves from middle (643.2 mg/100 g) and the leaves from the top of the tree (541.6 mg/100 g).

High amount of alanine and Gama-aminobutiric acid were determined (Table 3), followed by asparagine and proline.

Phenolic compounds are secondary metabolites of plants and contribute to different flavours (sweet, bitter, astringent), and determine the antioxidant activity of the matrix (Thomas-Barberan and Espin, 2001).

The amount of total polyphenols and total flavonoids are presented in Figure 1.

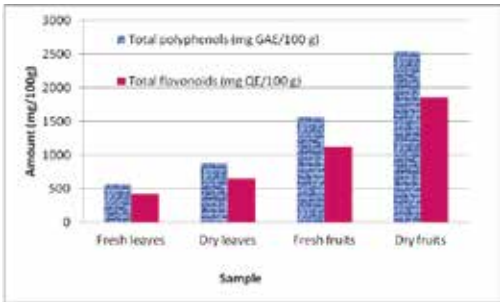

Figure 1. Polyphenolic and flavonoid content in mulberry leaves and fruits

Higher amounts of phenolics (total polyphenols and flavonoids) were determined in fruits, compared to leaves.

The obtained results were in accordance with literature studies (Dimitrijevic et al., 2014; Popescu et al., 2014; Okatan et al., 2016)

Radical scavenging activity of the ethanolic extracts from leaves and fruits of mulberry ranged between 45 - 67% inhibition percent, similar to literature studies (Iqbal et al., 2012; Dimitrijevic et al., 2014; Okatan et al., 2016).

CONCLUSIONS

The high polyphenolic content, together with the high amount of simple sugars, total lipids and total proteins, underline the nutritive and phytopharmaceutical potential of mulberry.

This study suggested that mulberry fruits, but not only, may be used as a potential healthy food, or an important antioxidant carrier for different pharmaceutical applications and food supplements.

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