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Research Article

Carbohydrate Composition and Antioxidant Activity of Certain *Morus* Species

Maria P. Dimitrova[1*], Nadezhda Tr. Petkova[2], Panteley P. Denev[2], Iordanka N. Aleksieva[1]

[1]*Department of Catering and Tourism, University of Food Technologies, Plovdiv, Bulgaria*
[2]*Department of Organic Chemistry, University of Food Technologies, Plovdiv, Bulgaria*

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ABSTRACT
The aim of the current study was to investigate the carbohydrate composition, total phenolic content and *in vitro* antioxidant activities of 70 % (v/v) ethanol extracts obtained from fruits and leaves of three *Morus* species: white mulberry (*Morus alba*), black mulberry (*Morus nigra*) and red mulberry (*Morus rubra*) grown in Bulgaria. The carbohydrate content was determinated by spectrophotomeric, TLC and HPLC-RID methods. The total phenolic content was analyzed by Folin–Ciocalteau method. The antioxidant activities of above mentioned extracts were evaluated by DPPH and FRAP assays. From the obtained results, monosaccharides fructose and glucose were found to be the main sugars in all investigated extracts, as their content reached to 3.0 g/100 g fw in fruits and 0.6 g/100g fw in leaves. *M. alba* and *M. rubra* fruits were evaluated as a natural source of prebiotic, due to the presence of 1-kestose, nystose and inulin. The absence of sucrose in all mulberry fruit extracts was also established. The total phenolic content was reported to be the highest in black and red mulberry leaves, as their values reached up to 2 mg GAE/g fw. The extracts from *M. nigra* leaves demonstrated the highest antioxidant activity for both assays: for DPPH were 10.9 mM TE/g fw and for FRAP – 6.0 mM TE/g fw, respectively. The current research was the first comprehensive study for detailed analysis of carbohydrate composition and antioxidant properties of three mulberry species grown in Bulgaria. Therefore, mulberry fruits and leaves could be assumed as a rich source of biologically active substances with great importance for human nutrition.

Key words: mulberry, sugar content, prebiotics, inulin, antioxidant activity

INTRODUCTION

Mulberry is a traditional Chinese tree which belongs to genus *Morus* L., Moraceae family. It is economically important horticultural crop worldwide. The plant grows in diverse climatic, topographic and soil conditions and is widespread in Asia, Europe, North and South America, and Africa[1,2]. In the most countries mulberries are cultivated for fruit production[3,4], while its leaves play a vital role in the cultivation of silkworms[5]. Different vegetal parts of *Morus* species were used because of its nutritional and pharmacological properties. The mulberry leaves are known to possess hypoglycemic, hypotensive, diuretic effects[6] and anti-tumor activity of Epstein-Barr virus[7]. In folk medicines, *Morus* species fruits are used to treat fever, oral diseases, hypertension, arthritis, anemia, protect liver from damage[9,10]. In Asia different food grade products with mulberry extracts and leaf infusions are consumed by diabetes mellitus patients, because of antihyperglycemic properties of their polyhydroxyalkaloid[8]. Few *Morus* species were evaluated for their edible fruits (*M. alba*, *M. rubra*, *M. indica*, *M. nigra*, *M. laevigata*) and timber (*M. laevigata* and *M. serrata*)[11,12]. The most commonly grown and used in human diet are white (*M. alba*), black (*M. nigra*) and red mulberry (*M. rubra*)[10,13]. Its small, soft and nutritive fruits can be consumed fresh and also processed as jam, syrup, probiotic milk beverage[14], vinegar[15], ice-cream, concentrate[13]. Several studies have been reported on the chemical composition and nutritional potentials of some mulberry species worldwide[4,16-19]. The deep colored *Morus* fruits are rich source of phenolic compounds, including flavonoids, anthocyanins and carotenoids[13,20,21]. It has been found that mulberry fruit extracts exhibited antioxidant, antimicrobial and anti-inflammatory properties[23-25], as these activities were due to the above mentioned compounds. Carbohydrate content (including mono-, di- and oligosaccharides) in mulberry is usually calculated by difference or presented as total value[5,12]. Until now no data about inulin content in mulberry fruits were found. Some researches showed interesting results about the presence of iminosugars, inositol, myo-inositol, glycosyl-inositols[26] and oligosaccharides in white and black mulberries fruits[27]. Although several studies were conducted with local cultivars in different regions of Turkey[10], Pakistan[12] and Serbia[2], until now no information has been reported on the chemical composition and antioxidant properties of *Morus* species grown in Bulgaria. To the best of our knowledge, there

**Author for Correspondence*

were no comparative studies on carbohydrate content with emphasis of inulin and the antioxidant potential of the *Morus nigra*, *Morus rubra* and *Morus alba* grown under the same climatic condition. Therefore, the aim of this study was to determinate the carbohydrate composition, total phenolic content and to evaluate the *in vitro* antioxidant activities of extracts from black, red white mulberry different mulberry species grown on territory of Bulgaria.

MATERIALS AND METHODS

All used reagents and solvents were of analytical grade scale. Carbohydrate standards fructose, sucrose, 1-kestose and nystose were purchased from Sigma-Aldrich (Steinheim, Germany). Fructooligosacchrides Frutafit®CLR (degree of polymerization 7-9), and inulin Frutafit®TEX were supplied by Sensus (Roosendaal, the Netherlands).

Plant material

The fruits and leaves of three mulberry species: white mulberry (*M. alba* L.), black mulberry (*M. nigra* L.), and red mulberry (*M. rubra* L.) were collected from Plovdiv region in the fully-ripen stage. The plant materials were stored at -18 °C for further studies. The moisture content of mulberry samples was analyzed by AOAC procedure[28].

Preparation of mulberry fruits and leaves extracts

Mulberry fruits and leaves were ground separately into pieces in an electric blender. Milled samples (1 g) were placed in 100 mL round bottom flask and were extracted in triplicate with 70 % (v/v) boiling ethanol under reflux. The duration of each extraction procedure was 30 min[29]. The extracts were passed through a paper filter and kept in the dark at 4°C for further analysis.

Total soluble carbohydrate content

The total soluble carbohydrate content in mulberry leaves and fruits extracts were estimated according to the reported method[30]. Briefly, 0.1 ml of each extract were mixed with 1 ml of 5 % phenol, 5 ml of sulphuric acid and placed in a water bath at 30° C for 20 minutes. The absorbance was measured at 490 nm against blank with d. H_2O. The amount of presented carbohydrates was determined from the calibration curve for glucose as a standard y = 0.0098x - 0.0465 (R^2=0.998) and the results were calculated as (g/100 g) of fresh weight (fw).

Reducing sugars content

The reducing sugars were estimated by PAHBAH method described by Lever[31].To 0.250 ml properly diluted extract, 0.750 ml of PAHBAH reagent was added. The mixture was boiled for 5 min in a water bath and then was cool in the ice bath for 5 min. The absorbance was measured at 410 nm against the blank, prepared with d. H_2O. The assay was set up by preparing glucose standard in the concentration range 5–100 μg/ml.

Identification of carbohydrate composition by thin layer chromatography

TLC analysis were used to elucidate the presence of mono-, di-, fructooligosaccharides (FOS) and inulin in 70 % (v/v) ethanol extracts from *Morus* species. Each sample (5 μl) were performed on silica gel 60 F_{254} plates (Merck, Germany) with mobile phase n-BuOH:i-Pro:H_2O:CH_3COOH (7:5:4:2) and the spots were detected with diphenylamine-aniline-H_3PO_4–acetone reagent, heated and scanned as previously described[32].

HPLC analysis of carbohydrates

Chromatographic separations of presented carbohydrates were carried out on HPLC Shimadzu, coupled with LC-20AD pump, refractive index detector and the software LC solution version 1.24 SP1 (Shimadzu Corporation, Kyoto, Japan)[33]. The analysis of mulberry leaves and fruits extracts were performed on a Shodex® Sugar SP0810 with Pb^{2+} a guard column (50 × 9.2 mm i.d.), an analytical column (300 mm × 8.0 mm i.d.) at 85 °C, mobile phase d. H_2O with flow rate 1.0 ml/min and the injection volume 20 μl.

Total phenolic contents

Total phenolic contents were measured using a Folin-Ciocalteu reagent according to the previously described procedure by Stintzing et al.[34] with some modifications. Briefly, 1 ml Folin-Ciocalteu reagent diluted five times was mixed with 0.2 ml sample and 0.8 ml 7.5% Na_2CO_3. The reaction was performed for 20 min at room temperature in darkness. Then the absorbance was measured at 765 nm against blank. The results were expressed as mg equivalent of gallic acid (GAE) per g fresh weight (fw), according to calibration curve, build in range of 0.02 - 0.10 mg gallic acid used as a standard[35].

Antioxidant activity (AOA)

The antioxidant activities of mulberry leaves and fruits extracts were evaluated by two methods: DPPH (1,1-diphenyl-2-picrylhydrazyl) radical based on mixed hydrogen atom transfer (HAT) and single electron transfer mechanisms and FRAP (ferric reducing antioxidant power) based only on single electron transfer mechanism.

The DPPH radical-scavenging ability

Each 70 % ethanol extract of mulberry leaves and fruits (0.15 ml) was mixed with 2.85 ml freshly prepared 0.1mM solution of DPPH in methanol. The sample was incubated for 15 min at 37 °C in darkness. The reduction of absorbance at 517 nm was measured by spectrophotometer in comparison to the blank containing methanol and % inhibition were calculated[36]. A standard curve was built with 6-hydroxy-2,5,7,8-tetramethylchroman- 2- carboxylic acid (Trolox) in concentration between 0.005 and 1.0 mM. The results were expressed in mM Trolox® equivalents (TE) per g fresh weight (fw).

Ferric reducing antioxidant power (FRAP) assay

The assay was performed according to Benzie and Strain[37] with slight modification. The FRAP reagent was freshly by mixing 10 parts 0.3 M acetate buffer (pH 3.6), 1 part 10 mM 2,4,6- tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 1 part 20 mM $FeCl_3.6H_2O$ in d. H_2O. The reaction was started by mixing 3.0 ml FRAP reagent with 0.1 ml of investigated extract. The reaction time was 10 min at 37 °C in darkness and the absorbance was measured at 593 nm against blank prepared with methanol. Antioxidant activity was expressed as mM Trolox® equivalents (TE) per g fresh weight (fw) by

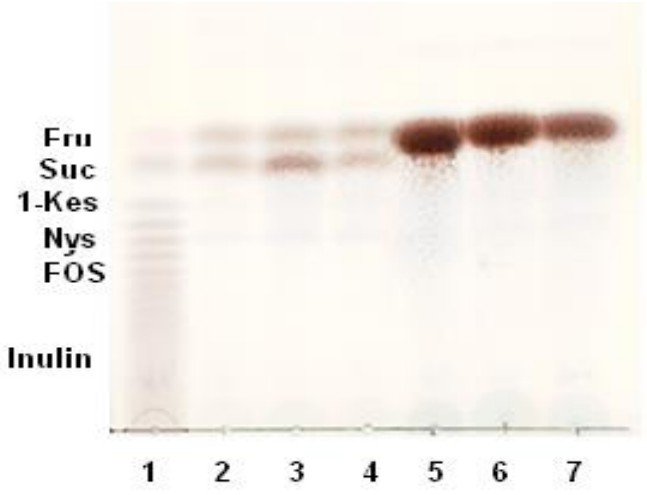

Fig. 1: Thin-layer chromatogram of 70 % ethanol extracts from *Morus* species, where (2, 3, 4) fruits and leaves (5, 6, 7) samples of black, red and white mulberry, respectively

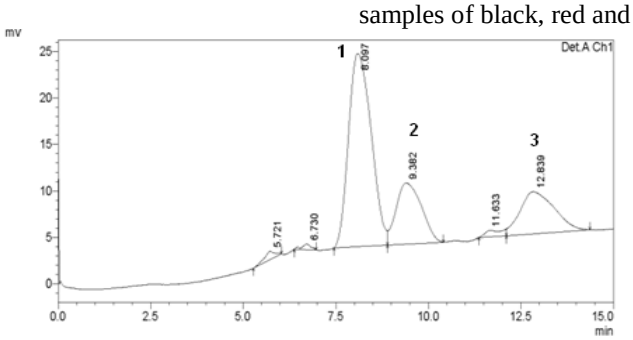

Fig. 2*:* HPL-RID chromatograms of extracts obtained from leaves of *Morus alba*, where 1. sucrose, 2. glucose, 3. fructose

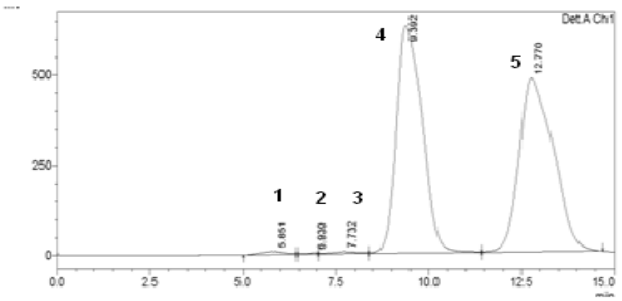

Fig.3: HPL-RID chromatograms of extracts obtained from fruits of *Morus rubra*, where 1. inulin, 2. nystose, 3. 1-kestose, 4. glucose and 5. fructose.

using calibration curve built in range of 0.05-0.5 mM Trolox[35]. All determinations were performed in triplicate($n = 3$) and the data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using MS Excel 2010. A difference was considered statistically significant, when $P < 0.05$.

RESULTS AND DISCUSIONS

The moisture contents in mulberry fruits were in the range of 85.9 ± 0.4 to 80.6 ± 0.6 %. The results for Bulgarian species were near to the reported data for the moisture contents in *Morus* species (Pakistan[12] and Macedonian[27] origin) 78 - 82 % and higher from these from Turkish origin[18]- 71.5% to 74.6%. The moisture content in leaves were 68 - 74 %. The lowest values for leaves and fruits were obtained for *M. rubra* – 68 % and 81 %, respectively.

Carbohydrate composition in Morus species

The detailed information about carbohydrate composition in 70 % (v/v) ethanol mulberry extracts with special emphasis on prebiotic inulin type fructan were provided (Figure 1). TLC analysis showed that all investigated mulberry fruits (2, 3 and 4) were characterized with a presence of monosaccharides glucose and fructose (R_f = 0.50), fructooligosaccharides 1-kestose (R_f= 0.37), nystose (R_f=0.32) and polysaccharide inulin. Only fructose and sucrose (R_f= 0.44) were found in mulberry leaves extracts (Figure 1). HPLC-RID method was used for more detailed analysis and quantitative determination of individual carbohydrate composition in mulberry leaves and fruits. The separation of the presented carbohydrates was shown on HPLC chromatograms (Figure 2 and Figure 3). Mulberry leaves extracts characterized only with the presence of glucose, fructose and sucrose (Figure 2), while the fruits of investigated red and white mulberry revealed the presence of inulin, 1-kestose, nystose, glucose and fructose (Figure 3). The results from carbohydrate composition in *Morus* leaves and fruits extracts were summarized in Table 1 and Table 2, respectively. The total carbohydrate contents showed slight variations among the studied plant materials. It was found to be in the range from 3-4 g/100 g fw for leaves (Table 1) and 3.4 to 9.8 g/100 g fw for fruits (Table 2). The mulberry fruits contained more carbohydrates than the leaves. The level of reducing sugars were higher in fruits. From the investigated samples, the highest reducing sugars content was found in black mulberry leaves - 2.6 g/100 g fw. In general, the fruits and leaves of *M. rubra* were evaluated as a rich source of carbohydrates and sugars. The lower quantity of total soluble carbohydrates were found in *M. alba* (white fruits) - 3.4±0.4 g/100 g fw and the highest value was recorded for *M. rubra* (red fruit) - 9.8 ± 0.9 g/100 g fw (Table 2). A similar trend was also observed for the

Table 1: Carbohydrate composition of leaves from three *Morus* species (g/100 g fw)[1]

Mulberry species	Reducing sugars	Total soluble carbohydrates	Fru[2]	Glc[3]	Suc[4]	Total (Fru, Glc, Suc)
Black (*M. nigra*)	2.6 ± 0.2	3.7 ± 0.7	0.4±0.2	0.4 ±0.1	0.9 ±0.2	1.7 ± 0.2
Red (*M. rubra)*	1.9 ± 0.1	4.1 ± 1.0	0.5±0.1	0.6±0.1	2.7 ±0.1	3.9 ± 0.1
White (*M. alba*)	1.5 ± 0.2	3.1 ± 0.6	0.3±0.1	0.3 ±0.1	1.1 ±0.1	1.7 ± 0.1

[1]Values are means ± SD of 3 measurements, [2]Fru – fructose, [3]Glc – glucose, [4]Suc – sucrose

Table 2: Carbohydrate composition in fruits of three *Morus* species (g/100 g fw)[1]

Mulberry species	Reducing sugars	Total soluble carbohydrates	Fru	Glc	1-Kes[2]	Nys[3]	Inulin
Black (*Morus nigra*)	5.4± 0.2	6.2 ± 1.1	1.5	1.4	n.d[4]	n.d	n.d
Red *(Morus rubra)*	5.6 ± 0.3	9.8 ± 0.9	3.2	3.3	0.1	0.01	0.04
White *(Morus alba)*	1.7 ± 0.3	3.4 ± 0.4	3.0	3.1	0.1	0.01	0.04

[1]Values are means ± SD of 3 measurements, [2]1-Kes – 1-kestose, [3]Nys – nystose, [4]n.d. – not detected

Table 3: Total phenolic content (mg GAE/g fw) and *in vitro* antioxidant activities (mM TE/g fw) in 70 % ethanol extracts from fruits and leaves of three mulberry species

Sample		Total phenolic content	DPPH	FRAP
Leaves	*Morus nigra*	1.2±0.1	10.9±0.1	6.0±0.2
	Morus rubra	2.2±0.2	3.2±0.1	3.9±0.1
	Morus alba	0.3±0.1	3.9±0.2	4.5±0.1
Fruits	*Morus nigra*	0.8±0.1	2.6±0.4	3.8±0.4
	Morus rubra	0.9±0.1	1.0±0.2	1.8±0.2
	Morus alba	0.4±0.1	0.3±0.1	0.8±0.2

reducing sugar contents. The reported values for total carbohydrate content in fruits were in accordance with data for purple mulberry[38] 7.8 % and black mulberry 6 – 9 %[12,39-41]. Similar to our results were reported by Mahmood et al.[42] for fruits of *M. nigra* and *M. alba*. Our finding that the deep coloured mulberry fruits contained higher level of sugars was in agreement with early report. It explained the relationship between the sugar content and the intensity of berry coloration, resulting in elevated anthocyanin[38]. The results obtained for carbohydrate content in mulberry leaves were near to reported values for total and reducing sugars in four *Morus* spices[43] (2.74 – 3.02 g/100 g and 0.59 – 0.71 g/100 g, respectively). The dominant sugars found in mulberry extracts were fructose and glucose (Table 1 and Table 2). Their values were in range 0.3-0.6 g/100 g fw in the leaves and 1.4 –3.3 g/100 g fw in the fruits of investigated *Morus* species. The levels of fructose and glucose in mulberry fruits were near to reported by Ozgen et al.[10] results for fructose in *M. nigra* and *M. rubra* contained in the range of 4.86 – 6.41 and 2.77 – 4.66 g/100 ml, glucose (5.50 – 7.12, 2.85 –4.96 g/100 ml). The results showed that the sugar contents of *Morus* species of Bulgarian origin were higher than reported for Pakistani mulberry and lower than the sugars levels found in fruits of Turkish black mulberry (11.3% – 16.2%) cultivars from Aegean region of Turkey[16]. Nevertheless, the presence of sucrose in fruits of three investigated *Morus* spices grown in Bulgaria were not detected (Figure 3). Generally the levels of this disaccharide in mulberry fruits were quite low in range 0.01–0.1 g/100 ml[27,42]. Sucrose were found only in leaves of three investigated *Morus* species (Figure 2), as its content varied in range from 0.9 to 2.7 g/100 g fw (Table 1). The highest values were reported for red mulberry leaves 2.7 ±0.1 g/100 g fw. It could be suggested that these variations in sugars content were due to the differences in species and cultivars, environmental, geological, agroclimatic conditions of harvest time[12,42]. Our investigation revealed also the presence of the prebiotics fructooligosacharides 1-kestose, nystose only in fruits of *M. alba* and *M. rubra* (Table 2) in quantity 0.1 and 0.01 g/100 g fw, respectively. It is known that fructooligosaccharides and inulin are mostly accumulated in the roots and tubers of plants belonging to Compositae family, and some of its representatives were investigated for sugars and inulin content in our previous researches[32,33]. However, for the first time this study evaluated *M. rubra* as a source of fructooligosacharides 1-kestose, nystose and inulin as their total content was higher than 0.1 g/100 g fw (Figure 3). Until now only one report mentioned about the content of fructooligosacharides found in Macedonian white and black mulberry fruits. Our results for 1-kestose and nystose values in *M. alba* were near to the reported by Malinovska et al.[27] Additionally, our research enriched the information about carbohydrate content for white mulberry fruits, as revealed the presence of polysaccharide inulin 0.04 g/100g fw. Surprisingly, our investigation detected 1-kestose, nystose and inulin in fruits of *M. nigra* (Figure 1), but could not be quantified. The possible explanation of this evidence would be lower values of these compounds in black mulberry below the detection limit.

In this investigation detailed analysis of carbohydrate composition of three mulberry grown on territory of Bulgaria were done. For the first time red mulberry fruit was evaluated as a rich source of carbohydrates, especially of inulin 0.04 g/100g fw and fructooligsaccharides. Therefore, the absence of sucrose and presence of inulin-type fructan (1-kestose, nystose

and inulin) may encourage the use of mulberry fruits as source of prebiotics for production of functional foods with dietary and improved healthy effect.

The total phenolic contents and in vitro antioxidant properties

The total phenolic contents and *in vitro* antioxidant properties of 70 % (v/v) ethanol extracts of mulberry fruits and leaves were evaluated (Table 3). The results showed that the total phenolic content in the investigated mulberry extracts ranged from 0.3 to 2.2 mg/g fw. Among the investigated *Morus* species the leaves of red mulberry (*M. rubra)* contained predominantly levels – 2.2 ±0.2 mg GAE/ g fw (Table 3). The total phenolic content varied with the type of the mulberry species and the matrix (leaves or fruits) analyzed. For fruits the high level of phenolic compounds were observed in the black and red mulberries extracts, which was in accordance with results noted by Arfan et al.[44] Overall, the lowest values for total phenolic content were found in *M. alba* fruits and leaves extracts below 0.4 mg GAE/g fw. The obtained results in current investigation for *Morus* species were lower than early reports[10,18,19,45]. Total phenolic content of *M. nigra* fruits were 17.66-34.88 in Turkey[10], 14.22 in Turkey[18] , 19.43-22.23 in Turkey[19] and 8.80 in Pakistan[12] (mg phenolic content/g fresh fruits material), while these values for *M. alba* fruits were 1.81 mg in Turkey[18], 15.16 mg in Taiwan[45], and 16.50 mg in Pakistan[12] as (mg phenolic content/g fresh fruits material). The total phenolic content for *M. nigra* fruits found in our study were in agreement with the reported results for black mulberry fruits grown in Serbia: 90.26 mg GAE/100 g in ethanol extracts and up to 118.84 mg GAE/100 g for water extracts[2]. The obtained results for total phenolic content in mulberry leaves were in range from 0.3 to 2.2 mg GAE/g fw and were near to the levels found in *M. alba* - 14.2 mg GAE/100 g[46,47] and other *Morus* species[5,43] – 145.24 - 160.86 mg %. These differences in total phenol content were dependent on the extraction method used and polarity of the extracting solvents[48]. The variation of phenolic compounds in the fruits depends on many factors, such as degree of maturity at harvest, genetic differences, and environmental conditions[12].

The fruits and leaves extracts of *M. nigra* showed the highest antioxidant evaluated by both AOA methods (Table 3). The antioxidant activity of black mulberry leaves were10.9±0.1 mM TE/g fw (DPPH assay) and 6.0±0.2 (FRAP assay) mM TE/g fw, and for fruits were 2.6 ± 0.4 mM TE/g fw (DPPH assay) and 3.8±0.4 mM TE/g fw (FRAP assay) respectively. The results were close to the reported data for black[2] and purple mulberries[19] from 283.10 ± 3.61 to 168.71 ± 9.91 mg TE/100g fw. The lowest level of antioxidant activities were exhibited by 70 % ethanol extracts from white mulberry fruits – DPPH 0.3 ± 0.1 mM TE/g fw, which was in accordance to those reported by Memon et al.[47] for *M. alba*, grown in Pakistan and water extract from *M. alba* leaves obtained by Flaczyk et al.[46]

Our results showed that the mulberry leaves extracts, contained higher values of total phenolic compounds, and also exhibited higher antioxidant activity, as measured by DPPH and FRAP assays. Similar results were reported by *M. alba* (Polish origin)[46] and Arabshahi-Delouee and Urooj[17] for *M. indica* L. (Indian origin) leaves. According to the last authors, a strong correlation between free radical scavenging and the phenolic contents has been reported for mulberry. However, our results coincided with statement of Khan et al.[49] that no correlation was found between radical scavenging activity and the total phenols in mulberry extracts.

CONCLUSION

The three mulberry species grown in Bulgaria were evaluated as a potential source of prebiotics, total phenols and antioxidants. In the carried research, leaves possessed comparatively high values of total phenolic content, while fruits contained higher total carbohydrates. Red mulberry was characterized as plant with higher biological activity among the investigated *Morus* species. Especially, mulberry fruits were shown the future nutritional potential for preparation of dietetic foods, because of absence of sucrose and presence of inulin-type prebiotics. *Morus* species due to its carbohydrate composition and the total phenolic compounds were evaluated also as an antioxidant carrier and prebiotics in the food and pharmaceutical industries. The current study showed the efficacy of extracts from mulberry fruits to be considered as food additive and natural antioxidant preservatives for functional food production.

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