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Original Article

Sugars, Total Acids and Physicochemical Characteristics of Black Mulberry (*Morus nigra*) Genotypes

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ABSTRACT

The aim of this study was to determine total acid and individual sugars in fruit of some black mulberry. The content of individual sugars in fruits was determined by HPLC. Total acidity (TA), total soluble solids (TSS) and pH value of juice were also evaluated. HPLC analysis of juice allowed the detection of 2 sugars. Glucose was the dominant sugar for all mulberries. The amount of total sugars ranged from 8.98 to 13.26 g/100g. The amount of total acids changed from 0.94 to 1.82%. The pH value ranged from 3.80 to 4.60. TSS content changed from 12.40 to 16.00 %. Juice content ranged from 50 to 70 %. Total flavonoid ranged from 0.94 to 1.26 mg/g DW. Among the three mulberries evaluated, genotype 3 demonstrated the maximum rate of sugars. As an outcome of our investigation, we can express that the genotypes can affect the amount of sugars and acids of fruit.

Abbreviations: HPLC, High performance liquid chromatography; DPPH, 2, 2-diphenyl-1-picryhydrazyl radical.

INTRODUCTION

Mulberry (*Morus* L.) belongs to the family Moraceae which comprises about 40 genera and more than 1000 species. Morus consists of 24 species and one subspecies, with more than 100 known varieties [1]. The most popular mulberry species with edible fruits grown in Iran are *M. alba*, *M. nigra*, *M. rubra* and *M. laevigata*.

Today, due to its nutritive value, the mulberry fruit is consumed both fresh and processed forms. Mulberry fruit can be utilized in various forms such as syrup, jam, ice-cream, juice, concentrate, alcohol, dried mulberry and etc [2].

Fructose and glucose are two major sugars of mulberry fruits. Glucose is known as the dominant sugar in mulberry fruit and is plentiful [3]. The most predominant organic acids in these mulberry species are malic acid and citric acid [4].

The ratio of sugars to acids affects the flavor of fruit and has been considered as a quality indicator by both fresh consumption group and juice factories [5]. Mulberry juice is a fantastic resource of sugars and acids. The amount of mulberry sugars is changeable and is dependent on the species [6], genotype [6] and etc. Several types of research have indicated that the genotypes can influence the physicochemical traits of black mulberry [7].

The objective of this study was to determine the physicochemical traits, organic acids, sugars, and phenolic acid levels, as well as the antioxidant capacities of some black mulberry for further breeding programs.

MATERIALS AND METHODS

Chemicals and Standards

Standards of fructose, glucose, gallic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), acetonitrile, methanol, Folin–Ciocaltaeu's reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide, Rutin and sodium carbonate were purchased from Merck (Darmstadt, Germany).

Plant Materials

Fruit samples of some black mulberry (*M. nigra* L.) at fully-ripened stage were collected from the

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orchards in the region of Roudehen, Iran during May-Jun, 2016. Fruits were collected from different parts of the same trees, early in the morning (6 to 8 am) and only during dry weather. The selection method was based on a completely randomized design. The fruit (2 kg for each category) were collected in polyethylene bags, transferred to the experimental lab and stored in a refrigerator at 5 °C overnight for further analyses.

Preparation of Juice Sample

Fruits juice was extracted using a juicer. Then, Juices were centrifuged at 15,000 rpm for 20 min at 4 °C. Three replicates were done for this research (n = 3).

Juice Analyses Technique

The total titratable acid was determined by titration with sodium hydroxide (0.1 N) and displayed as % citric acid. Total soluble solids were measured using a refractometer (Kruss, Germany). The pH value was determined using a digital pH meter (Jenway, Model: 3510). Ascorbic acid was determined by titration with Potassium iodide. Sugars were measured by HPLC.

Analysis of Sugars by HPLC

HPLC analysis was performed with a Platin blue system (Knauer, Berlin, Germany) equipped with a binary pump and a Refractive Index (RI) detector. The separation was carried out on a Shodex Asahipak NH2P-50 4E column (250 × 4.6 mm). The column temperature was maintained at 25 ºC, and the injection volume for all samples was 10 μL. Elution was performed isocratically with the mobile phase consisting of 75% (v/v) acetonitrile (eluent A) and 25% (v/v) water (eluent B) at a flow rate of 1 mL/min. The identification of sugars was based on retention times of unknown peaks in comparison with standards. The concentration of the sugars was calculated from peak area according to calibration curves. Standard solutions of sugars (fructose and glucose) were prepared by dissolving the required amount of each standard in deionized water. Calibration was performed by injecting the standard at three different concentrations (Fig. 1 to 2).

Sugars concentration was estimated from the calibration curve and the result was expressed as gram of compound per 100 grams (g/100g).

Identification of Sugars

Identification of sugars was based on retention times of unknown peaks in comparison with standards.

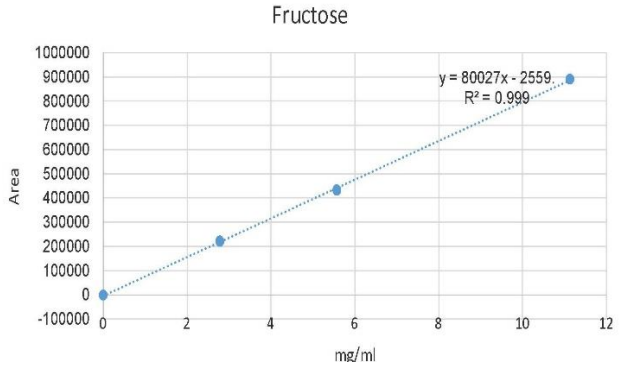

Fig. 1 The standard curve of fructose

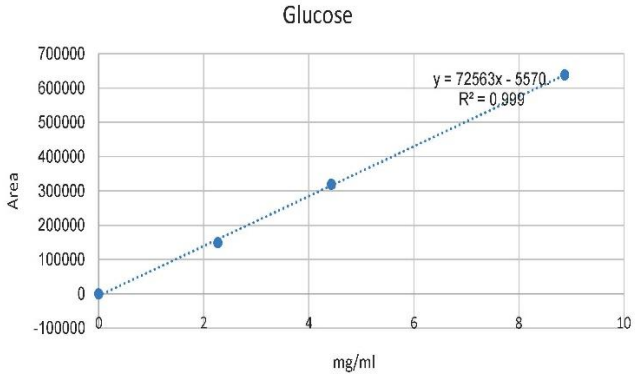

Fig. 2 The standard curve of glucose

Physical Traits of Fruit

Fifty fruits were randomly sampled and evaluated for each tree. Fruit physical traits were presented in Table 1. Total dry matter was determined by dehumidifying of fruits in an oven at 80 °C. Ash was measured by placing the weighed fruits in a furnace at 560 °C. The weight of fresh fruit was determined using a scale. The weight of dried fruit evaluated with the oven. Fruit length and fruit diameter were determined using a caliper. Fruit shape index was explained as the ratio of fruit length to diameter.

Fruit Extraction Technique

The fruit was extracted according to the method of Chen *et al.* [8] with slight modifications. In order to obtain the phenolic compounds from samples, 0.2 g of dried fruit (powder) were placed in a 200 ml spherical flask, along with 20 mL of methanol. The flask was covered and then placed in an ultrasonic water bath for 20 min. Extraction were performed with an ultrasound cleaning bath-Fisatom Scientific-FS14H (Frequency of 40 KHz, nominal power 90 W

and 24 × 14 × 10 cm internal dimensions water bath). The temperature of the ultrasonic bath was held constant at 40 °C. The extract was subsequently filtered through 0.45 mm filter paper. The concentration of the extract was finally reduced to 40 ml using methanol and placed in a vial. Vial sealed and was kept in the refrigerator at 4 °C until the future analysis.

Determination of Total Flavonoid Content

The total flavonoid content was determined by the aluminum chloride colorimetric method. Standard solutions of rutin were prepared by dissolving 16.2 mg rutin with 70% ethanol into 100 ml after shaking evenly and used to obtain a standard curve at concentrations of 0, 75, 100 and 125 mg/L. Sodium nitrite solution (5%, 0.5 ml) was added to the standards and maintained for 5 min. Then, 0.5 ml of aluminium chloride (10%) was added. It remained at room temperature for 6 min. Finally, 5 ml of sodium hydroxide (1 M) was added. The mixture was diluted to 10 ml with distilled water.

The absorbance of all the samples was measured using a spectrophotometer (UV 1600 PC, Shimadzu, Tokyo, Japan) at 415 nm. The total flavonoid content was calculated from calibration curve and the result was expressed as mg rutin equivalent per g dry weight [8].

Determination of Total Phenol Content

The total phenol content was determined by Folin-Ciocalteu's reagent. Standard solutions of gallic acid were prepared by dissolving 6.2 mg gallic acid with 25 ml distilled water and used to obtain a standard curve at concentrations of 0, 62.5, 125 and 250 mg/L. Then Folin-Ciocalteau reagent (0.5 ml) was added. It remained at room temperature for 2 min. Finally, sodium carbonate (5%, 0.5 ml) was added. It remained at room temperature for 3 h.

Absorbance was measured using a spectrophotometer (UV 1600 PC, Shimadzu, Tokyo, Japan) at 760 nm. The total phenol content was calculated from the calibration curve and the results were expressed as mg of gallic acid equivalent per g dry weight [8].

DPPH Free Radical Scavenging Activity

The free radical scavenging activity was measured according to the method of Umamaheswari and Asokkumar [9] with slight modification. Briefly, 0.2 ml of extract was mixed with 2 ml DPPH (2, 2-diphenyl-1-picryl-hydrazyl). It remained at room temperature for 30 min. Absorbance was measured at 517 nm. DPPH expressed as (%).

Data analysis

SPSS 18 was used for the analysis of the data obtained from the experiments. Analysis of variations was based on the measurements of 21 traits. Comparisons were made using a one-way analysis of variance (ANOVA) and Duncan's multiple range tests. Differences were considered to be significant at $P< 0.01$. The correlation between pairs of characters was evaluated using Pearson's correlation coefficient.

RESULTS

Result of the HPLC Analyses

HPLC analyses of juice allowed the identification of 2 sugars (fructose and glucose) (Fig. 3, Table 1).

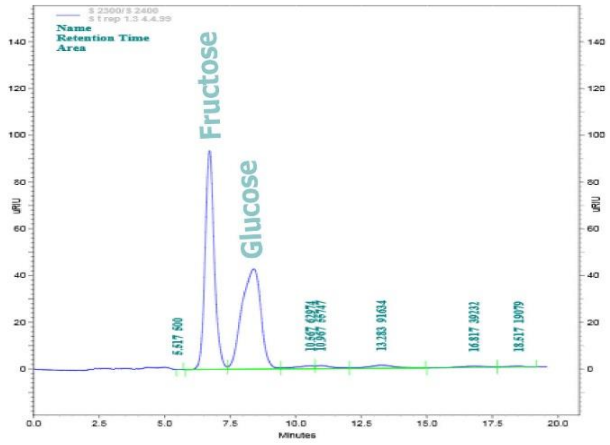

Fig. 3 HPLC chromatogram of sugars of black mulberry

Determinations of Sugars

Fructose and glucose were two sugars recognized in this study. Moreover, the amount of total sugars ranged from 8.98 to 13.26 g/100g. Glucose was the dominant sugar in this study. For all the sugars, the differences among mulberries were found significant on the 1% level. Fruits of genotype 3 showed a significant increase in fructose (6.15 g/100g) and glucose (7.11 g/100g). Among three mulberries evaluated, genotype 3 indicated the maximum level of sugars (Table 1).

Results of Total Titratable Acid (TA)

The amount of total titratable acid ranged from 0.94 to 1.82%. There was a statistically significant difference on the 1% level in total titratable acid. The highest percentage of total acids (TA) was in fruits

from trees of genotype 1, followed by genotype 3, whereas the lowest TA was detected in fruits from trees of genotype 2. The fruits from trees of genotype 1 showed ascorbic acid content significantly lower than those of genotype 3 (Table 1).

Results of pH, TSS, TSS/TA and Juice Content

The amount of pH, TSS, TSS/TA and juice content were given in Table 1. There was a significant difference on the 1% level in the content of pH, TSS, TSS/TA and juice. Among three mulberries evaluated, genotype 3 indicated the maximum level of pH, TSS and TSS/TA. Fruits from genotype 1 gave the highest juice percentage (70 %) while those from genotype 3 gave the least juice percentage (50 %).

Results of Fruit Physical Traits

The amounts of fruit physical traits were given in Table 1. For more physical traits, the differences among three mulberries were found significant. The results indicated that trees of genotype 1 significantly gave the heaviest fruit (4.20 g), while trees of genotype 2 gave the lightest fruit (3.00 g). With respect to fruit length and diameter, fruits from the trees of genotype 1 significantly gave the longest fruit (24.32 mm), while the least values were recorded for those on genotype 2 (17.42 mm). Although no significant differences for fruit shape index (Fl/Fd) were observed among the three mulberries, fruits from trees of genotype 3 gave the highest content. In addition, fruits from the trees on genotype 2 significantly gave the longest stalk (4.40 mm) followed by those from genotype 3 (4.00 mm) and genotype 1 (3.40 mm).

Table 1 Statistical analysis of variation in juice compositions and fruit physical traits of black mulberry genotypes

	Black mulberry (genotype 1)		Black mulberry (genotype 2)		Black mulberry (genotype 3)		
Compounds	Mean	SD	Mean	SD	Mean	SD	F value
Sugars							
1) Fructose (g/100g)	5.60 ab	0.24	4.13 b	0.29	6.15 a	0.27	**
2) Glucose (g/100g)	6.79 ab	0.31	4.85 b	0.36	7.11 a	0.34	**
Total	12.39	0.55	8.98	0.65	13.2 6	0.61	
Organic acids							
Total titratable acid (%)	1.82 a	0.11	0.94 b	0.13	1.14 b	0.10	**
Ascorbic acid (%)	6.00 d	0.20	8.85 b	0.24	10.80 a	0.29	**
pH	3.80 b	0.10	4.10 b	0.13	4.60 a	0.11	**
TSS (%)	15.30 b	0.10	12.40 d	0.10	16.00 a	0.13	**
TSS/TA	8.40 d	0.16	13.19 b	0.19	14.03 a	0.20	**
Juice (%)	70 a	1.00	58 b	1.00	50 d	1.11	**
Moisture (%)	86.50 a	0.52	85.00 a	0.60	83.00 b	0.48	**
Total dry matter (%)	13.50 d	0.14	15.00 b	0.15	17.00 a	0.11	**
Ash (%)	4.00 b	0.19	4.00 b	0.11	5.00 a	0.14	**
Fresh fruit weight (g)	4.20 a	0.22	3.00 b	0.27	3.62 ab	0.28	**
Dry fruit weight [z](g)	0.80 b	0.04	0.92 ab	0.08	1.00 a	0.06	*
Fruit length (mm)	24.32 a	1.00	17.42 d	0.97	21.13 b	1.00	**
Fruit diameter (mm)	16.90 a	0.64	11.48 d	0.53	13.75 b	0.80	**
Fruit shape index (Fl/Fd)	1.43 a	0.10	1.51 a	0.10	1.53 a	0.13	NS
Fruit stalk length (mm)	3.40 d	0.10	4.40 a	0.15	4.00 b	0.12	**
Fruit stalk diameter (mm)	1.40 a	0.10	1.00 b	0.10	1.27 ab	0.10	**
Total flavonoid (mg/gr DW)	0.94 d	0.05	1.26 a	0.06	1.11 b	0.08	**
Total phenol (mg/gr DW)	2.83 d	0.13	3.97 a	0.12	3.70 b	0.14	**
DPPH %	23 d	1.00	29 a	1.14	25 b	1.10	**

The mean is the average of traits applied with three replicates. SD = standard deviation. Results of analysis of variance: NS = not significant, *the significant difference at $P \leq 0.05$, ** significant difference at $P \leq 0.01$. Any two means within a row not followed by the same letter are significantly different at $P \leq 0.01$ or $P \leq 0.05$.

[z] For 7.00g fruit.

Table 2 Interco relations between 6 traits in a correlation matrix

	Fructose	Glucose	Total acid	Ascorbic acid	pH	TSS
Glucose	0.79 *	-	-	-	-	-
Total acid	-0.88 **	-0.76 *	-	-	-	-
Ascorbic acid	-0.86 **	-0.91 **	0.95 **	-	-	-
pH	0.96 **	0.91 **	-0.83 **	-0.89 **	-	-
TSS	0.95 **	0.87 **	-0.96 **	-0.96 **	0.95 **	-

Results of Total Flavonoid, Total Phenol and DPPH

There was significant difference on the 1% level in the content of total flavonoid, total phenol and DPPH. Not only genotype 2 indicated the maximum level of total flavonoid (1.26 mg/g DW) but also indicated the maximum level of total phenol (2.97 mg/g DW) and DPPH (29 %) (Table 1).

Results of Correlation

PH showed a high positive correlation with fructose at about 0.96. TSS also showed a high positive correlation with fructose and pH about 0.95 (Table 2).

DISCUSSION

Data obtained from this experiment revealed that the sugars and organic acids in mulberry significantly impressed by genotypes that was in accordance with previous studies [6]. However, it should be kept in mind that the environmental factors and extraction methods also may influence the results. It was observed that the application of fertilizer and irrigation affected the content of sugars present in crops [10]. Fertilization, irrigation and other operations were carried out uniform in this study so we did not believe that this variability was a result of these factors.

The discovery of sucrose -6- phosphate, as an intermediate between UDP- Glucose and sucrose, led to a rapid description of the biosynthetic pathway of sugar compounds. The biosynthetic pathway of sugar compounds in higher plants is as follows:

Photosynthesis→Triose-P → Fructose- 6-phosphate→ Glucose- 6- phosphate→Glucose- 1-phosphate→UDP- Glucose→ Sucrose -6-phosphate→Sucrose → Glucose and Fructose [11].

Reaction pathway catalyzed by sucrose-6-phosphate synthase and sucrose-6-phosphate phosphatase respectively [12]. An increase in the amount of sugars, when genotype 3, used as the sample, showed that either the synthesis of Triose-P was enhanced or the activities of both enzymes increased. Considering that Triose-P is necessary for the synthesis of sugars, it can be assumed that there is a specialized function for this molecule and it may be better served by genotype 3.

Kenan-Gecer *et al* [13] stated that the values of fructose and glucose in black mulberry were about 8.15 and 9.55 g /100g respectively. Akin *et al* [4] reported that the values of fructose and glucose in black mulberry were about 8.61 and 9.18 g /100g respectively. Okatan [14] reported fructose from 5.68 to 8.85 and glucose from 6.99 to 10.33 g /100g in black mulberry. Okatan *et al* [15] reported fructose from 5.79 to 8.89 and glucose from 7.22 to 10.39 g /100g in black mulberry. Eyduran *et al* [3] reported fructose from 4.16 to 7.16 and glucose from 6.17 to 8.57 g /100g in black mulberry. These values were not similar for the genotypes under study. In our study, the average concentrations of fructose and glucose were determined as 4.13 to 6.15 and 4.85 to 7.11 g/100g, respectively. Also, in our research the fructose and glucose content were shown to be lower when compared to those reports by Kenan-Gecer *et al* [13], Akin *et al* [4], Okatan [14], Okatan *et al* [15] and Eyduran *et al* [3]. The lower values in this study may due to the genotype, environmental conditions or analytical methods used. In addition, in fruits, sugars and organic acids vary according to species, varieties, and also environmental and horticultural conditions such as climate, Fertilization and irrigation [16].

Based on the results obtained in the present study, total flavonoids ranged from 0.94 to 1.26 mg/g DW and total phenol ranged from 2.83 to 3.97 mg/g DW. Wang *et al* [17] reported total flavonoids from 1.21 to 2.86 mg/g DW and total phenol from 10.82 to 27.29 mg/g DW. Radojkovic *et al* [18] reported total flavonoids about 1.50 mg/g DW and total phenol about 6.36 mg/g DW. Bajpai *et al* [19] reported total flavonoids about 0.59 mg/g DW and total phenol about 3.70 mg/g DW respectively. The amount of

total flavonoids and total phenol obtained in presented investigation was lower than previously published data. It might be related to genotype, environmental factors and extraction method that could influence the compositions.

As stated in the present study, content of DPPH ranged from 23 to 29 %. Okatan [14] reported content of DPPH from 16.87 to 26.80 %. Okatan *et al* [15] reported DPPH from 18.34 to 26.93 %. These findings agreed with the results of this study.

According to the results obtained in the present study, Total titratable acid (TA) ranged from 0.94 to 1.82 % and TSS ranged from 12.40 to 16.00%. Gozlekci e*t al* [20] reported TA about 1.73 % and TSS about 20.10%. Okatan [14] reported TA from 1.47 to 1.97 % and TSS from 14.23 to 19.43%. Okatan *et al* [15] reported TA from 1.77 to 1.98 % and TSS from 17.48 to 19.55%. The amount of TA and TSS obtained in presented investigation was slightly lower than previously published data.

CONCLUSION

In the present study we found that the amount of sugars and total acid were significantly impressed by genotypes and there was a great variation in most of the measured characters among three mulberries. Among three mulberries examined, genotype 3 showed the highest content of fructose and glucose. The lowest of fructose and glucose were produced by genotype 2. Further research on the relationship between mulberry genotypes and sugars is necessary.

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