



Determination of fruit chemical properties of *Morus nigra* L., *Morus alba* L. and *Morus rubra* L. by HPLC

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ABSTRACT

This study was carried out to determine the organic acid content, phenolic compound content, sugar content, vitamin C (ascorbic acid) content and total antioxidant capacity of white mulberry (*Morus alba* L.), black mulberry (*Morus nigra* L.) and red mulberry (*Morus rubra* L.) fruits grown in Van province of Turkey. It was determined that the chief organic acid in these mulberry species was malic acid ranging from 1.32 to 4.47 g 100 g⁻¹ fw, followed by citric acid ranging from 0.39 to 1.08 g 100 g⁻¹ fw. Looking at the contents of phenolic compound, chlorogenic acid and rutin had come to the fore ranging from 0.12 to 3.11 mg g⁻¹ fw and from 0.85 to 1.42 mg g⁻¹ fw, respectively. Fruit glucose contents of the studied species were higher than their fructose contents, varying between 6.07 and 7.75 g 100 g⁻¹ fw. Total antioxidant capacity and vitamin C contents of the mulberry species ranged from 4.49 to 13.99 μmol TE g⁻¹ fw and from 11.30 to 24.42 mg 100 g⁻¹ fw, respectively.

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1. Introduction

Tropical and subtropical fruit species, mulberry (*Morus* spp.) is involved in the genus *Morus*, the tribe *Moraceae* tribe, and the family *Moraceae* (Ozrenk et al., 2010). East, West and South East Asia, South Europe, South of North America, Northwest of South America and some parts of Africa are areas of distribution of this species. The most commonly grown species in the *Morus* genus are white mulberry, black mulberry, and red mulberry (Datta, 2002; Ozgen et al., 2009). The mulberry production in Turkey is significant (67,986 t) (Anonymous, 2010). There are wild as well as cultivated species of mulberry in Anatolia which has a significant variation (Ozrenk et al., 2010). Among these, there are white mulberry (*Morus alba* L.), black mulberry (*Morus nigra* L.), and red mulberry (*Morus rubra* L.). Compared to other species, white mulberry is more widely grown (95%) and produced mulberry species in Turkey (Ercisli, 2004). Today, due to its nutritive value, the mulberry fruit is consumed both fresh and processed forms. Mulberry fruit could be utilized in various forms such as syrup, jam, pulp, ice-cream, vinegar, concentrate, alcohol. Moreover, mulberry leaves and other plant organs is used as pharmacologically in the world, especially in China (Koyuncu, 2004).

Recently done studies have revealed that mulberry had essential effects in human diet and health with the help of its compounds

such as organic acids, phenolics, and sugar contents (Koyuncu, 2004; Zadernowski et al., 2005; Ercisli and Orhan, 2007; Pawlowski et al., 2008; Zhang et al., 2008; Ozgen et al., 2009). Organic acids are important sources of respiratory energy in fruits (Cemeroglu et al., 2004). The malic, citric, and tartaric acids are the most common organic acids in fruit (Cemeroglu et al., 2004). The main organic acids in most of fruit are either citric or malic acid. Phenolics are not present in live plant tissues, but they emerge by hydrolyze while fruit is being processed. Nitrosamines, which might be a cancerogenic substance, are inhibited by cinamic acids, which are sub-groups of phenolic acids. Chlorogenic acid, an ester obtained by caffeic and quinic acid, is the most common derivative of cinamic acids. Moreover, it has been determined that the common phenolics such as chlorogenic acids and catechins have anti-cancer and anti-mutagen effects (Cemeroglu et al., 2004). Carbohydrates are also the main parts of fruit. Carbohydrates present in the structure of plant tissues and acts as energy sources (Cemeroglu et al., 2004). The carbohydrates contents of fruit range from 3% to 30%. Among them, glucose and fructose are the main sugar having six carbons. Sucrose is the most common disaccharides in fruit. Plants produce carbohydrates by photosynthesis.

In the researches on mulberry in Turkey, pomological and chemical properties have been usually examined. Studies on organic acids, phenolic compounds, sugar and antioxidant content of mulberry fruit are not sufficient. Ozgen et al. (2009) stated that mulberry had essential effects in human health with the help of its antioxidant contents.

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These researchers identified the antioxidant capacities of *M. nigra*, on average, 11.4 mmol TE g⁻¹ fw, while species *M. rubra* 6.6 mmol TE g⁻¹ fw according to the method of the TEAC. Akbulut et al. (2006) determined the chemicals, physicochemical properties and minerals of four different varieties of mulberry obtained from Gaziantep, Konya and Malatya provinces in Turkey. The high anthocyanin content (184.3–227.0 mg 100 g⁻¹) was determined in black and red mulberry varieties, but it was not found in white mulberry varieties. All of mulberry varieties have potential for the ascorbic acid and phenolics. The highest ascorbic acid content were determined as 124.5 µg g⁻¹ fw in red mulberry. Total phenolic contents of the mulberry varieties were found between 114.3 mg kg⁻¹ and 354.5 mg kg⁻¹. The highest minerals were determined as K, Ca, P, Mg, S, Na and Fe, respectively.

In the present study, the organic acid content (citric, malic, tartaric, succinic, lactic, fumaric, and acetic acids), phenolic compounds content (gallic, catechin, caffeic, chlorogenic, o-coumaric, p-coumaric, ferulic, syringic, vanillic, phlorodizin, quercetin, rutin, and pyrocatechin), sugar content (glucose, fructose, and sucrose), vitamin C (ascorbic acid) content and total antioxidant capacity (TEAC) of white mulberry (*M. alba*), black mulberry (*M. nigra*) and red mulberry (*M. rubra*) fruits grown in Van province of Turkey were determined by HPLC.

2. Materials and methods

2.1. Plant materials

Homogeneous fruits samples were collected at the harvest time of the determined white mulberry (*M. alba* L.), black mulberry (*M. nigra* L.) and red mulberry (*M. rubra* L.) fruits grown in Van province. Approximately 200 g fruit samples from each species were maintained at -20 °C before analysis.

2.2. Chemicals

In the present study, chemicals with analytical purity were used. Organic acid standards (citric acid, tartaric acid, malic acid, succinic acid, lactic acid, fumaric acid, and acetic acid), phenolic acid standards (gallic, catechin, caffeic, chlorogenic, o-coumaric, p-coumaric, ferulic, syringic, vanillic, phlorodizin, quercetin, rutin, and pyrocatechin), sugar standards (glucose, fructose, and sucrose), and vitamin C standards (L-ascorbic acid) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals were obtained from Merck (Darmstadt, Germany).

2.3. Extraction and determination of organic acids

For organic acid extraction, the method by Bevilacqua and Califano (1989) was modified. About 200 g of samples was fragmented and 5 g from each sample was transferred to centrifuge tubes. The 10 ml of 0.009 N H₂SO₄ was added to the samples and the samples were homogenized with Heidolph Silent Crusher M, Germany. Then, the samples were mixed for an hour with a shaker (Heidolph Unimax 1010, Germany) and centrifuged at 15,000 × g for 15 min. The supernatant were passed through coarse filter paper, then twice in 0.45 µm membrane filter (Millipore Millex-HV Hydrophilic PVDF, Millipore, USA), and last in the SEP-PAK C₁₈ cartridge. The concentration of organic acids was determined by HPLC using an Aminex column (HPX-87H, 300 mm × 7.8 mm, Bio-Rad) fitted on an Agilent 1100 series HPLC G 1322 A, Germany) (Bevilacqua and Califano, 1989). Organic acids were detected at 214 and 280 nm wavelengths. As the mobile phase, 0.009 N H₂SO₄ were passed through 0.45 µm filter membrane.

Table 1

This is gradient elution program for the determination of phenolic compounds in mulberry fruit.

| Time (min) | Dissolvent A (%) | Dissolvent B (%) |
|------------|------------------|------------------|
| 0 | 100 | 0 |
| 15 | 85 | 15 |
| 25 | 50 | 50 |
| 35 | 15 | 85 |
| 45 | 0 | 100 |

2.4. Extraction and determination of phenolic compounds

The phenolic compounds were determined using the HPLC separation method described by Rodriguez-Delgado et al. (2001). About 100 g of samples were fragmented and 5 g from each sample was transferred to centrifuge tubes. The samples were mixed homogeneously then diluted 1:1 with distilled water and centrifuged at 15,000 × g for 15 min. The supernatant was passed through 0.45 µm membrane filter (Millipore Millex-HV Hydrophilic PVDF, Millipore, USA), then injected into HPLC system (gradient). The chromatographic separation in Agilent 1100 series HPLC took place in DAD detector (Agilent, USA) with 250 mm × 4.6 mm, 4 µm ODS column (HiChrom, USA). The following solvents in water with a flow rate of 1 ml/min and 20 µl injection volume were used for spectral measurements at 254, and 280 nm: as mobile phase solvent A, methanol-acetic acid-water (10:2:88) and Solvent B, methanol-acetic acid-water (90:2:8) (Table 1.).

2.5. Content of sugar

The samples were prepared according to the method described by Melgarejo et al. (2000) with minor modifications. Briefly, the samples of 5 g fruit was centrifuged at 12,000 rpm for 2 min at 4 °C. Then the supernatant was filtrated with SEP-PAK C₁₈ cartridges and transferred into a vial and used for analysis. Analysis of sugars was performed by HPLC (isocratic program) with µbondapak-NH₂ column and refractive index (RI) detector using 85% acetonitrile as a mobile phase. The calculation of concentrations was based on standards prepared in the laboratory.

2.6. Extraction and determination of total antioxidant activity

For the standard trolox equivalent antioxidant capacity (TEAC) assay, TEAC extract was prepared: ABTS was dissolved in acetate buffer and prepared with potassium persulfate, as described by Rice-Evans et al. (1995) and Ozgen et al. (2006). The mixture was diluted in acidic medium of 20 mM sodium acetate buffer (pH 4.5) to an absorbance of 0.700 ± 0.01 at 734 nm for longer stability (Ozgen et al., 2006). For the spectrophotometric assay, 3 ml of the ABTS⁺ solution and 20 µl of fruit extract were mixed and incubated for 10 min and the absorbance was determined at 734 nm determined after 6 min from mixing.

2.7. Extraction and determination of ascorbic acid (vitamin C)

Ascorbic acid content was determined following the modified HPLC (isocratic program) (Agilent 1100 series HPLC G 1322 A, Germany) analytical procedure outlined by Cemeroglu (2007). The 5 g of sample was transferred to a 50 ml volumetric flask including 10 ml 6% (w/v) metaphosphoric acid (Sigma, M6285, 33.5%). The sample was then homogenized at 24,000 rpm for 15 s, and centrifuged at 14,000 rpm for 10 min at 1 °C. 5 ml of the supernatant was filtered through 0.45 µm PTFE syringe filters (Phenomenex, UK) and placed in an amber colored vial (AIM, Screw vial, SV-15A). Quantification of ascorbic acid was made by an external standard method using an L-ascorbic acid Standard (Sigma A5960). Samples

Table 2
Fruit organic acid contents of mulberry (*Morus* spp.) species.

| | Black mulberry (<i>Morus nigra</i>) | White mulberry (<i>Morus alba</i>) | Red mulberry (<i>Morus rubra</i>) |
|--|---------------------------------------|--------------------------------------|-------------------------------------|
| Citric acid (g 100 g ⁻¹ fw) | 1.084 ± 0.003 a [*] | 0.393 ± 0.002 c | 0.762 ± 0.002 b |
| Tartaric acid (g 100 g ⁻¹ fw) | 0.123 ± 0.002 c | 0.223 ± 0.001 b | 0.336 ± 0.001 a |
| Malic acid (g 100 g ⁻¹ fw) | 1.323 ± 0.001 c | 3.095 ± 0.001 b | 4.467 ± 0.525 a |
| Succinic acid (g 100 g ⁻¹ fw) | 0.342 ± 0.001 a | 0.168 ± 0.001 b | 0.132 ± 0.002 c |
| Lactic acid (g 100 g ⁻¹ fw) | 0.049 ± 0.000 b | 0.074 ± 0.001 a | 0.074 ± 0.001 a |
| Fumaric acid (g 100 g ⁻¹ fw) | 0.011 ± 0.001 c | 0.024 ± 0.001 b | 0.028 ± 0.001 a |
| Acetic acid (g 100 g ⁻¹ fw) | 0.019 ± 0.000 a | 0.008 ± 0.001 c | 0.015 ± 0.001 b |

^{*} There were significant ($P < 0.01$) differences among the different letters in the same lines.

were separated on a Luna C18 column (250 mm × 4.60 mm, 5 μm from Phenomenex) at 25 °C by an HPLC. The mobile phase was 25 mM KH₂PO₄ (adjusted to pH 2.2 with phosphoric acid) with a flow rate of 1 ml/min. L-Ascorbic acid was detected at 254 nm.

2.8. Statistical analysis

Statistical analysis was conducted using the program SPSS11.0 (www.spss.com). One-way analysis of variance was used to compare the organic and phenolic acids concentrations in mulberry cultivars. Statistical differences between organic and phenolic acids concentrations of the mulberry cultivars were estimated using Duncan's multiple range test.

3. Results and discussion

3.1. Organic acid content

Organic acids are water soluble materials found in the cytoplasm of fruit and vegetable at various amounts. Accompanied with the sugars, they contribute to taste of fruit and vegetables (Cemeroglu et al., 2004). In the present study, there were significant differences among the fruit organic acid contents of mulberry species (Table 2).

As seen from these results, the most predominant organic acids in these mulberry species was malic acid generally followed by citric, tartaric, succinic, lactic, fumaric, and acetic acid. On the other hand, in black mulberry, succinic acid content was higher than tartaric acid content. The differences between species in terms of organic acid content might be caused by genetic factors as well as cultural practices and ecological factors (temperature, light, humidity, etc.). Koyuncu (2004) reported that malic, citric, tartaric, and fumaric acid contents of black mulberry ranged from 35.4 to 198.5 mg g⁻¹, 5.5 to 23.4 mg g⁻¹, 2.95 to 5.55 mg g⁻¹, 0.015 to 0.033 mg g⁻¹, respectively. In another study, Ozgen et al. (2009) stated that malic acid and citric acid contents in black mulberry were determined as 0.16 g 100 ml⁻¹ and 1.87 g 100 ml⁻¹, respectively, whereas malic acid and citric acid contents in red mulberry were determined as 0.18 g 100 ml⁻¹ and 0.59 g 100 ml⁻¹, respectively. The values for organic acid contents of the red, white, and black mulberry species grown in Lake Van Basin, Turkey in the present study were in line with the data obtained by these researchers (Koyuncu, 2004; Ozgen et al., 2009).

Acids found in fruits have no negative effects in the body, as they are rapidly oxidized in the metabolism. Their salts have an alkaline effect; therefore, they have an important effect on diet (Schobinger,

1988; Savran, 1999). Organic acids constitute a complex with heavy metal ions, and their oxidation prevents their catalyzing effect (Balci, 1996; Savran, 1999). Ratio of total acid content to the amount of sugar in fruits is a criterion for maturity. Organic acids have an impact on taste because of their reducing effect on sweetness and their favoring effect sourness. Moreover, the type and amount of acidity could be a criterion of food decay. If fruit is molded during wait, there is an increase in amount of some organic acids. Organic acids also have been reported to have a significant impact in the control of purity (Ozkaya, 1988; Savran, 1999).

3.2. Trolox equivalent antioxidant capacity (TEAC), sugar and vitamin C content

There were significant differences in terms of total antioxidant capacity (TEAC), sugar and vitamin C contents of examined mulberry species in the present study (Table 3).

It is remarkable that, light colored mulberry species have higher vitamin C content than dark colored ones. On the other hand dark colored mulberry species have higher total antioxidant capacity (TEAC), than light colored ones. Black mulberry had the TEAC (13.999 μmol TE g⁻¹ fw), but white mulberry had the lowest TEAC (4.494 μmol TE g⁻¹ fw). Different researchers have studied on chemical composition of fruits (Sun et al., 2002; Bae and Suh, 2007; Celik et al., 2008; Ozgen et al., 2009). Ozgen et al. (2009) reported that total antioxidant capacity (TAC), fructose and glucose contents of black mulberry were 11.4 μmol TE g⁻¹, 5.77 g 100 g⁻¹, and 6.39 g 100 g⁻¹, respectively. The same researchers determined that total antioxidant capacity (TAC), fructose and glucose contents of red mulberry were 6.6 μmol TE g⁻¹, 3.78 g 100 g⁻¹, and 4.07 g 100 g⁻¹, respectively. Moreover, they detected ascorbic acid contents (vitamin C) of black mulberry and red mulberry as 0.005 g 100 g⁻¹ fw and 0.004 g 100 g⁻¹ fw, respectively. Gungor (2007) determined that antioxidant activity of mulberry fruits ranged from 33.96% to 38.96%. Our findings in the present study are in line with the results of the mentioned researchers.

3.3. Phenolic matter content

Phenolic matters are the compounds allowing some chemical activities in vegetables and particularly in fruits such as taste, color formation, and browning, and their positive effects on human health are more emphasized in recent years (Cemeroglu et al., 2004).

Table 3
Total antioxidant capacity (TEAC), sugar and vitamin C contents of examined mulberry species.

| | Black mulberry (<i>Morus nigra</i>) | White mulberry (<i>Morus alba</i>) | Red mulberry (<i>Morus rubra</i>) |
|---------------------------------------|---------------------------------------|--------------------------------------|-------------------------------------|
| Fructose (g 100 g ⁻¹ fw) | 5.634 ± 0.009 b [*] | 6.269 ± 0.007 a | 5.407 ± 0.007 c |
| Glucose (g 100 g ⁻¹ fw) | 7.748 ± 0.002 a | 6.864 ± 0.098 b | 6.068 ± 0.004 c |
| Vitamin C (mg 100 g ⁻¹ fw) | 11.302 ± 0.241 c | 24.422 ± 0.067 a | 16.166 ± 0.521 b |
| TEAC (μmol TE g ⁻¹ fw) | 13.999 ± 0.008 a | 4.494 ± 0.067 c | 5.497 ± 0.002 b |

^{*} There were significant ($P < 0.01$) differences among the different letters in the same lines.

Table 4
Fruit phenolic matter contents of mulberry (*Morus* spp.) species.

| | Black mulberry (<i>Morus nigra</i>) | White mulberry (<i>Morus alba</i>) | Red mulberry (<i>Morus rubra</i>) |
|---|---------------------------------------|--------------------------------------|-------------------------------------|
| Gallic acid (mg g ⁻¹ fw) | 0.150 ± 0.003 c [*] | 0.215 ± 0.001 b | 0.504 ± 0.004 a |
| Catechin (mg g ⁻¹ fw) | 0.075 ± 0.000 b | 0.037 ± 0.001 c | 0.086 ± 0.002 a |
| Chlorogenic acid (mg g ⁻¹ fw) | 3.106 ± 0.004 a | 0.119 ± 0.001 c | 0.569 ± 0.001 b |
| Caffeic acid (mg g ⁻¹ fw) | 0.131 ± 0.001 a | 0.133 ± 0.000 a | 0.116 ± 0.002 b |
| Syringic acid (mg g ⁻¹ fw) | 0.103 ± 0.000 a | 0.049 ± 0.002 c | 0.083 ± 0.001 b |
| p-Coumaric acid (mg g ⁻¹ fw) | 0.129 ± 0.002 a | 0.047 ± 0.001 b | 0.129 ± 0.003 a |
| Ferulic acid (mg g ⁻¹ fw) | 0.064 ± 0.001 b | 0.033 ± 0.002 c | 0.155 ± 0.005 a |
| O-coumaric acid (mg g ⁻¹ fw) | 0.134 ± 0.001 a | 0.015 ± 0.001 c | 0.084 ± 0.003 b |
| Phloridzin (mg g ⁻¹ fw) | 0.031 ± 0.001 a | 0.011 ± 0.003 b | 0.031 ± 0.004 a |
| Protocatechuic acid (mg g ⁻¹ fw) | 0.017 ± 0.000 a | 0.015 ± 0.001 a | 0.012 ± 0.006 b |
| Vanilic acid (mg g ⁻¹ fw) | 0.036 ± 0.002 a | 0.008 ± 0.003 c | 0.024 ± 0.009 b |
| Rutin (mg g ⁻¹ fw) | 1.423 ± 0.036 a | 1.111 ± 0.002 b | 0.851 ± 0.001 c |
| Quercetin (mg g ⁻¹ fw) | 0.113 ± 0.021 a | 0.015 ± 0.002 c | 0.048 ± 0.001 b |

* There were significant ($P < 0.01$) differences among the different letters in the same lines.

There were significant differences in terms of phenolic matter contents of examined mulberry species grown in the Van region in the present study (Table 4).

Among the studied phenolic compounds, while dominating phenolic in black mulberry was chlorogenic acid, dominating one in white and red mulberries was rutin. Except for gallic acid, caffeic acid, and rutin, other phenolic compounds of black and red mulberries had much more than those of white mulberry. The differences in phenolic compounds might be influenced by genetic and environmental factors (temperature, humidity, light, etc.), and cultural practices. In a study on some berry fruits, the total phenol and anthocyanin contents were determined by spectrometric method, and gallic acid of black mulberry genotype was measured as 340 mg 100 g⁻¹ (Kafkas et al., 2006). Gungor (2007) determined that the total phenolic content of mulberry ranged as 18.16–19.24 μg mg⁻¹ in gallic acid equivalent. Zadernowski et al. (2005) reported that the gallic acid, pyrocatechonic, vanillic acid, caffeic acid, o-coumaric acid, and p-coumaric acid, and ferulic acid acids in black mulberry fruits were as 27.3 mg kg⁻¹, 121.8 mg kg⁻¹, 6.5 mg kg⁻¹, 117.2 mg kg⁻¹, 212.7 mg kg⁻¹, 761.8 mg kg⁻¹, and 34.1 mg kg⁻¹, respectively. Similar findings were found by other researchers (Zhisen et al., 1999; Yu et al., 2007; Zhang et al., 2008). The findings of the present study were in line with the literature.

4. Conclusion

The demand for fruit species containing anthocyanins and anthocyanidins has been increasing due to the identification of flavonoids having anticarcinogenic effects in the studies in recent years (Tosun and Artik, 1998). Mulberry is also included in this fruit species. In the present study it was determined that chlorogenic acid and antioxidant contents in fruit of *M. nigra* were found to be quite higher than those of *M. alba* and *M. rubra*. It has been reported that phenolic compounds such as widely prevailing the chlorogenic acid and catechins have antimutagenic and anticarcinogenic features (Cemeroglu et al., 2004). Moreover, nitrosamines suspected to cause cancer are blocked with cinnamic acids, a sub-group of phenolic acids (Cemeroglu et al., 2004). This situation increases the importance of black mulberry fruit for human health. The region of the study is one of Turkey's most important regions in terms of production of mulberry. Mostly pomological studies have been conducted to date on mulberry species examined in this region. The present study that might lighten the future studies is also important and has a separate value on this aspect. Such studies need to be developed widely in order to determine and protect important mulberry germplasm and for a better understanding of the importance of this kind of fruit species.

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