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Short Communication

Phenolic composition and antioxidant properties of black mulberry (*Morus nigra* l.) fruits and leaves

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Abstract

Mulberry (*Morus* spp.) fruit is a very common species in the world and is used as a functional food. In this study, the phenolic composition and antioxidant properties of *Morus nigra* were examined. pH, Brix, acidity, and ascorbic acid values of ethanolic (60%) fruit and leaf extracts were measured. The total phenolic substance amount (TP) and total flavonoid substance amount (TF) of ethanolic extracts were to determine the phenolic substances measured. Ferric reducing antioxidant power (FRAP) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH)radical scavenging activity tests were used as antioxidant capacity tests. Phenolic compositions were analyzed by HPLC-PDA and validated against twenty-five phenolic standards. The average pH, acidity and ascorbic acid values in the fruit part were 3.49, 24.68, and 2737mg/100 g, respectively. The average pH and ascorbic acid values in the leaves were 4.04 and 156 mg/100g, respectively. The average amount of TP in the fruits and leaves was measured as 925.33 and 3.07 mg GAE/g, respectively. It was determined that the fruit part had a much higher antioxidant value than the leaves. It was determined that the fruit and leaves were rich in chlorogenic acid and protocatechuic acid. Additionally, it was determined that the leaves were rich in rutin and the fruits were rich in quercetin.

Keywords: *Morus,* mulberry, phenolic substance, samples, flavonoid

Introduction

Mulberry fruits, scientifically classified under the Morus genus, are succulent berries that grow on deciduous trees belonging to the Moraceae family. According to Kostić et al. (2013), white mulberry originates in China, red or American mulberry has its roots in the United States, and black mulberry traces back to western Asia. These sweet and flavorful fruits come in various colors, including red, white, and black, depending on the specific species. Mulberries are known for their nutritional richness, containing essential vitamins, minerals, and antioxidants. With a sweet-tart taste, mulberries are enjoyed both fresh and dried, and they are commonly used in various culinary applications, including jams, desserts, and beverages. Beyond their culinary appeal, mulberries have garnered attention for their potential health benefits, contributing to the

growing interest in incorporating these versatile berries into a balanced and nutritious diet (Zhang, et al. 2018; Khalifa, et al. 2018; Jiang, & Nie (2015).

Mulberry fruits (*Morus spp*.) exhibit a widespread distribution globally, thriving in diverse climates and geographical regions. Belonging to the *Moraceae* family, these deciduous trees are found on nearly every continent, with notable species including *Morus alba* (white mulberry), *Morus nigra* (black mulberry), and *Morus rubra* (red mulberry). White mulberries are native to East Asia but have been extensively cultivated in regions such as Europe and North America. Black mulberries are indigenous to Western Asia but are now cultivated worldwide. Red mulberries are primarily found in North America. The adaptability of mulberry trees to varying climates has facilitated their cultivation in countries ranging from China and India to Turkey and the United States (Zhang et al. 2018: Gündoğdu et al. 2018; Skrovankova et al. 2022).

In Turkey, four mulberry species - *Morus rubra*, *Morus nigra*, *Morus alba*, and *Morus laevigata* - have naturally grown for many years and exhibit significant diversity (Gundogdu et al. 2017). Morus nigra has become very popular lately and is consumed especially to strengthen the immune system (Jiang et al. 2017; Ma et al. 2022). The fruit is consumed as fresh and dried. Black mulberry is used especially in fruit juice and mulberry pestil production due to its dark color and rich in anthocyanins (Gündoğdu et al. 2018; Yıldız, 2013; Skrovankova et al. 2022).

The aim of this study was to investigate the phenolic composition and antioxidant properties of the fruit and leaves grown in the Black Sea region of Türkiye. Understanding the specific phenolic compounds present in the sampels from this region, as well as their antioxidant activity, can provide valuable information in terms if the development of functional foods and nutraceuticals capable of improving human health.

Materials and methods

Samples and extraction

Three different black mulberry fruits and their leaves were collected from the same gardens of Darica village of Trabzon city, Turkiye (45.0024" and 39° 31′ 56.0028") on June 15, 2023) in optimum maturity (Fig 1). The fruit samples were frozen at –18 °C until extraction, the leaves were dried at room temperature, and stored at +4°C.

Figure 1. Black mulberry (*Morus nigra*)

Extraction

For the extraction of the fruit, 50 g of fresh fruit was crushed in 250 ml of 60% ethanol, kept in an ultrasonic bath for 2 hours, and then macerated in a blender for 24 hours. After maceration, this mixture was first filtered through a fine cheesecloth and then filtered through Whatman filter paper. This resulting filtrate was stored in aliquots in the deep freezer for further studies. After the dried, the leaves were powdered, then extracted in 60% ethanol in a similar manner, first in an ultrasonic bath and then by maceration. The ethanolic filtrate was saved for study.

Physico-Chemical Analysis

The total soluble solid content (TSS) was assessed by analyzing the juice of fresh fruits with a digital handheld refractometer, presenting the outcome as a percentage value (Atago, Pr-32A, Japan). Titratable acidity (TA) in the juice filtrate obtained from blender-shredded fruits was determined in accordance with the protocols established by the Association of Official Analytical Chemists (Mettler–Toledo Inc., Columbus, OH, USA) utilizing an automated titrator (Bayrak et al. 2023).

Determination of ascorbic acid

The analysis of ascorbic acid (vitamin C) in the ethanolic extracts was conducted following the (AOAC, 1995) method, employing titration with 2,6-dichlorphenolindophenol sodium salt solution in chloroform for extracts displaying intense coloration. Titrations were repeated three times, and average values were taken.

Total phenolic contents

The phenolic content of the fruit extracts was determined using the Folin–Ciocalteu method and gallic acid was used as standard (Singleton et al., 1999). In the method, 680 µL of distilled water was mixed with 400 µL of 0.2 N Folin reagent. Then, 20 µL of the ethanolic extract was added, followed by the addition of 400 µL of 10% Na_2CO_3. The mixture was incubated in a dark environment for 2 hours. After the incubation, absorbance readings were taken at 760 nm. To determine of total phenolic content, a linear standard graph was prepared by different concentrations of gallic acid standards ranging from 0.50 to 0.0156 mg/mL. The results were calculated as mg GAE/g fresh fruit.

Total Flavonoid Content (TFC)

The total flavonoid content was determined using a colorimetric assay of Fukumoto and Mazza (1999). 250 µL of the ethanolic extract solution, 0.05 mL of a 10% $Al(NO_3)_3$ solution, and 0.05 mL of a 1M $NH_4.CH_3COO$ solution was mixed in a test tube. The mixture was then incubated at room temperature for 40 minutes. After the incubation period, the absorbance was measured at a wavelength of 415 nm using a spectrophotometer. The results were expressed as milligrams of quercetin equivalents (mg QUE) per gram of the fresh fruits ample (mg QUE/g).

Total antioxidant capacity (FRAP)

FRAP (Ferric Reducing Antioxidant Power) assay is used to determine the total antioxidant activity of the samples (Benzie and Strain in 1996). The FRAP assay measures the ability of an antioxidant to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in the presence of a suitable reducing agent. To prepare the FRAP reagent, a mixture was made by combining 2.5 ml of a 10 mM solution of TPTZ, 2.5 ml of a 20 mM solution of $FeCl_3$, and 25 ml of a 0.3 M acetate buffer at pH 3.6. Next, 50 µl of the sample and 1.5 ml of the FRAP reagent were mixed. After 4 minutes, the absorbance was measured at a wavelength of 595 nm. For the assessment of antioxidant activity, the study utilized $FeSO_47H_2O$. The FRAP results were expressed as µmol $FeSO_47H_2O$/ g fresh fruits.

DPPH radical scavenging activity

Free radical scavenging activity was of DPPH measured using spectrophotometric assay (Molyneux, 2004). The result was calculated as SC_{50}, defined as the value of the sample concentration causing a 50% reduction in the concentration of DPPH• radicals, with lower SC_{50} values indicating higher radical scavenging activity. To calculate the SC_{50} (µg/mL) value, a working curve was prepared with Excell with at least 6 different concentrations of samples. The sample concentration, the SC_{50} value, which halved the maximum absorbance, was calculated from the graph.

RP-HPLC-PDA Analysis

The phenolic composition was analyzed using high-performance liquid chromatography (HPLC) (Shimadzu Liquid Corporation LC 20AT HPLC, Japan), with a photodiode array (PDA) detector. A C18 column with dimensions of 250 mm × 4.6 mm and a particle size of 5 µm, was provided by GL Sciences (Kara et al. 2022). Twenty-five phenolic compounds were analyzed according to the validated device and method. The elution process was conducted using a gradient program: mobile phase A, which consisted of a 70% acetonitrile-ultra-pure water solution, and mobile phase B, which was a 2% acetic acid in water solution. The flow rate during the analysis was set at 1 ml/min, with an injection volume of 20 µl. The column temperature was maintained at 30 ºC throughout the analysis. Before to analysis, all prepared samples were filtered through 0.45 µm membranes before being injected into the device.

Statistics

The results were analyzed on SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA and Tukey's test were used to compare TPC, TFC, FRAP, and DPPH parameters among the fruit samples.

Results and discussion

In this study, some classical analyzes of *Morus nigra* fruits and leaves, their phenolic composition and antioxidant properties were examined and compared. The classical fruit analyzes are given in Table 1. The average pH value was measured as 3.49 in the fruit and 4.04 in the leaves. It was determined that fruits with an average brix value of 13 contained approximately 87% water. Similar to our study, the humidity amount in *Morus nigra* was reported to be 87.20% (Kamiloglu et al. 2013; Thaipitakwong et al. 2018).

Table 1. The black mulberry fruits and leaves

	pH	Acidity	Brix	Ascorbic acid (mg/100g)
Mulberry fruits				
Mean±Std	3.49±0.01	24.68±0.32	13±1	27.37±80.67
Min and Max	3.40-3.55	24.40-25.04	12-14	26.58-28.30
Mulberry leaves				
Mean±Std	4.04±0.06	-	-	0.16±0.06
Min and Max	3.98-4.10	-	-	0.14-0.16

The average amount of ascorbic acid in the fruit part is 27.37 g/100g, and it was determined that this value varied between 26.58 and 28.30 g/100g. The average amount of ascorbic acid in the leaves is 0.16 g /100 g, and it was noted that this value is very low compared to the fruit. In a study conducted in Turkey, the amount of ascorbic acid in *Morus nigra* genetics was reported as 17 to 28 g/100 g of fatty fruit. Compared to many other fruits, the M. nigra was found to have rich content in ascorbic acid (Ercisli et al. 2010). When compared with studies conducted on *M. nigra* from different regions, it was found to have similar ascorbic acid content (Gundoğdu et al. 2017; Skrovankova et al. 2022). It has been shown in the literature that the amount of ascorbic acid generally found is 15 to 30 mg/100 g. The amount of ascorbic acid in the leaves is quite low compared to the fruit, and we did not have the opportunity to compare it with the literature.

Most of the secondary metabolites of black mulberry samples consist of polyphenols. Total phenolic and flavonoid substance amounts are summarized in Table 2. Total phenolic substance amounts were found to be 925.33 mg GAE/g on average and vary between 910 and 936 mg GAE/g. The average TPC value detected in the leaves was 3.07 mg GAE/g. Similar to our study, a study conducted on Morus nigra showed that the amount of TPC was 1451.4 ± 124.2 mg GAE/100 g dw (Kamiloglu et al. 2013). In a different *Morus nigra* study, the TPC value was reported to vary between 182.6 and 248.3 mg GAE/100 g (Ercisli et al. 2010). The TPC values obtained in this study were found to be lower. The reason for the lower TPC value is thought to be measurement or calculation error. The resemblance between the study conducted by Kamiloglu et al. (2013) and our findings suggests that there should not exist such a substantial discrepancy of 100-fold with respect to the other study. It is thought that a large part of the total phenolic substance amount comes from anthocyanins. As a matter of fact, studies have shown that the total amount of anthocyanin in *M.nigra* is 1221 mg C3Cy 3-glu/100 g (Kamiloglu et al. 2013). In another study, it was shown that the total anthocyanidin amount in *M.nigra* was 674 to 787 mg/100 g (Ercisli et al. 2010). The dark color of *Morus nigra* fruit is attributed to the presence of polyphenols, including anthocyanins (Qin et al. 2010; Ma et al. 2022).

Table 2. Amount of total phenolic and flavonoid substances

	Total Phenolic mg (GAE/ 100g)	Total Flavonoid (mg QUE/ 100g)	Total antioxidant FRAP (µmol $FeSO_4.7H_2O$/ g)	DPPH (mg/ml)
Mulberry fruits				
Mean±Std	925.33±13.62	164.33±6.12	169.00±4.58	13.92±1.62
Intervals	910-936	158-170	164-170	12.08-15.09
Mulberry leaves				
Mean±Std	3.07±0.14	0.82±0.09	63.33±4.16	143.30±10.60
Intervals	2.98-3.24	0.75-0.92	62.00-68.00	132.00-153.00

It was determined that the total amount of flavonoid substance varied between 158 and 170 mg QUE/100 g. In a study, the amount of TFC in *Morus nigra* was reported to be 768.7 mg QUE/100 g, and it was determined to be higher than our values (Kamiloglu et al 2013). It is understood from the literature that the amount of total flavonoid substance in *M.nigra* is lower than the amount of total anthocyanins (Kamiloğlu et al. 2013; Ercisli et al. 2010).

The antioxidant capacity of the *M. nigra* samples was measured according to two different methods. FRAP. The FRAP method is an indicator of the reducing capacity of the Fe (III) TPTZ complex, and a high FRAP value indicates high antioxidant capacity. It was found that the measured FRAP value varied between 164 and 170 and the average FRAP value was 169.00 µmol

$FeSO_4.7H_2O$/ g. The average amount of TFC detected in the leaves was found to be 0.82 mg QUE/g100. The antioxidant activity detected in the Mulberry leaves was almost half of that of the fruit, and an average of 63.33 μmol FeSO4.7H2O/g was detected. The antioxidant activity observed in mulberry leaves and fruits is attributed to various bioactive compounds, including phenolic compounds and ascorbic acid. These constituents act as antioxidants and contribute to the overall free radical scavenging ability of mulberry. Phenolic compounds, such as flavonoids and other polyphenols, are known for their strong antioxidant properties, which help neutralize reactive oxygen species (ROS) and protect cells from oxidative damage. Ascorbic acid (vitamin C) is another important antioxidant present in mulberry, playing a key role in reducing oxidative stress and promoting health benefits. Together, these vitamins and phenolic compounds contribute to the potent antioxidant activity observed in mulberry leaves and fruits (Skrovankova et al. 2022; Gundogdu et al. 2017). The higher levels of total phenolic content (TPC), total flavonoid content (TFC), and ascorbic acid in mulberry fruits compared to its leaves substantiate this observation. Indeed, previous studies support our findings (Ercisli et al. 2010*;* Kamiloglu et al 2013; Gundogdu et al. 2017).

Phenolic components of the samples were analyzed by HPLC-PDA. Phenolic component analyzes obtained using 25 phenolic standards are given in Table 3. In this table, phenolic components are grouped into two classes: phenolic acids and flavonoids. While chlorogenic acid, one of the phenolic acids, was detected as the major component in both samples, its amount in the leaves was determined to be higher. Protocatechuic acid was detected in both samples, but gallic acid was detected only in the fruit. Additionally, t-Cinnamic acid was determined only in the leaves but in low amounts. While quercetin and pinocembrin were detected as major flavonoids only in the fruit part, Rutin was detected only in the leaves. Chrysin was detected as a common flavonoid in leaves and fruit. A study shows that gallic acid and chlorogenic acid are the major phenolic acids in fresh mulberry fruit (Kamiloglu et al. 2013; Zhang et al. 2018). In the same study, low amounts of syringic acid and caffeic acid were detected in Mulberry fruit. In a different Mulberry study, it was reported that the highest organic acid component after ascorbic acid was chlorogenic acid, followed by gallic acid, caffeic acid and ellagic acids. In the same study, it was reported that Routine, quercetin and catechin were detected as flavonoids in Mulberry (Skrovankova et al. 2022). Similar phenolic acids and flavonoids were detected in our current study, but it was noted that the amounts in the leaf and fruit parts were different. These changes may be due to differences in climate and soil characteristics in the regions where fruits grow. The amount and types of polyphenols, called secondary metabolites, vary according to the physiological needs of the plant (Sharma & Padwad, 2020; Zhang et al. 2018; Thaipitakwong et al. 2018). It may also be caused by differences in extract preparation and sensitivity of HPLC devices.

Table 3. Phenolic composition of the Mulberry fruits and leaves

	Mulberry fruits	**Mulberry leaves**
	Phenolic acids	
Gallic acid	10.28	-
Protocatechuic acid	93.00	8.70
Chlorogenic acid	345.73	587.39
p-OH Benzoic acid	-	-
Caffeic acid	-	-
Syringic acid	-	-
Vanillic acid	-	-
p-Coumaric acid	-	-
Ferulic acid	-	-
t-Cinnamic acid	-	4.79

Ellagic acid	-	-
	Flavonoids	
Rutin	-	47.01
Epicatechin	-	-
Myricetin	-	-
Resveratrol	-	-
Daidzein	-	-
Luteolin	-	-
Kuersetin	76.54	-
Apigenin	-	-
Hesperetin	-	-
Rhamnetin	-	-
Krisin	2.84	1.70
Pinocembrin	8.46	-
CAPE	-	-
Galangin	-	-

(-): The value is below detection (<LOD)

In conclusion, *Mullberry nigra* is a natural product rich in leaves and fruit rich in ascorbic acid and polyphenols. The composition and bioactive properties of this antioxidant-rich fruit vary depending on the geographical conditions of the region where it is cultivated, exhibiting high levels of chlorogenic acid, rutin, quercetin, and pinocembrin.

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