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Investigation of Antioxidant Activities of Fruit, Leaf, and Branche Extracts of White (*Morus alba* L.) and Black (*Morus nigra* L.) Mulberry Species from Diyarbakır*

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Abstract: This study aims to determine the antioxidant activities of fruit, leaf, and branche extracts of *Morus alba* L. and *Morus nigra* L. species collected from Diyarbakır. Different *in vitro* methods such as Total Phenol Content (TPC), Total Flavonoid Content (TFC), Copper (II) Ion Reducing Antioxidant Capacity (CUPRAC), 2,2´-diphenyl-1-pikrilhidrazil (DPPH), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging methods were used to evaluate the antioxidant activities of the two *Morus* species. The highest total phenolic amount belongs to acetone extract (257.5±0.05 µg gallic acid equivalent mg^{-1}) obtained from *M. nigra* branches, while the highest total flavonoid content was found in acetone extract (185.8±0.15 µg catechin equivalent mg^{-1}) obtained from *M. nigra* leaves. In the CUPRAC test, the highest activity was observed in methanol extracts from *M. alba* branches (IC_{50} values is 35.63±0.22 µg mg^{-1}). The IC_{50} values of the DPPH radical scavenging activity of the extracts are between 128.1±0.13 and 2494.7±0.30 µg mg^{-1}, the best radical scavenging activity was demonstrated by acetone extract (128.1±0.13 µg mg^{-1}) prepared from the *M. alba* branche. The highest ABTS radical scavenging activity belongs to the methanol extract (75.5±0.25 µg mg^{-1}) obtained from the *M. alba* branches. The results prove that both *Morus* species possess antioxidant properties, not only in leaves and fruits but also in the branches. At the end of the study, it was determined that *M. alba* and *M. nigra* have medicinal usage value which can be used as natural antioxidants.

Keywords: *Morus* sp., antioxidant, free radical, total phenol, total flavonoid, extract

1. Introduction

Unpaired electrons of free radicals are generally very reactive, high-energy, non-stable compounds. Free radicals can be positively charged (cation), negatively charged (anion), or electrically neutral. These molecules, which are also quite uncharacterized by these properties, can attack the other molecules that are stable in the body to become stable (Lobo et al., 2010). Antioxidants scavenge free radicals to protect the human body from oxidative stress, which is the main cause of body and heart ailments (Wang et al., 2012). Oxidative stress occurs the imbalance between oxidant formation and antioxidant defense is impaired in the direction of oxidants (Özyürek, 2009). Scientific studies in recent years have proved that antioxidants can protect the cells from free radical damage (Moure et al., 2001). Polyphenols have the ideal structural chemistry for free radical scavenging activities. Research in recent years has shown that many plant-derived polyphenol components derived from plants are a more effective antioxidant than vitamin E or C, and thus can be a significant contributor to preservation (Rice-Evans et al., 1997).

Mulberry is a fast-growing, leafy plant that can grow in various climatic conditions (Arabshahi-Delouee and Urooj, 2007). Mulberry is widely cultivated to feed silkworms used in commercial silk production. Its leaves are nutritious, tasty, and not toxic. The leaves can improve milk yield while feeding dairy animals. Apart from being used as animal and insect food, it also has medicinal properties such as diuretic, hypoglycemic, and

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hypotensive activities. Many berries such as mulberry fruit contain important health-protecting ingredients. These components have antimicrobial, antioxidant, antidiabetic, hypolipidemic, neuroprotective, hepatic, renal, antitumor, and antimetastatic, retinal damage, immunity and so on. It has also been determined that a polysaccharide (JS-MP-1) obtained from mulberry fruits influences the metabolism and reduces the number of fat cells (Geçer et al., 2016). The antioxidant activity of the plant material attributed to the phytochemicals, which are performing their actions through mechanisms such as radical scavenger, metal chelation, inhibition of lipid peroxidation, and cooling of singlet oxygen (Arabshahi-Delouee and Urooj, 2007).

The aim of the present work is to investigate *in vitro* antioxidant activity of extracts prepared with solvents of different polarity from different parts of *Morus alba* L. and *Morus nigra* L. growing in Diyarbakır province of Turkey, to determine the best extract solvent and plant part that possesses the highest antioxidant potential.

2. Materials and Methods

2.1. Plant materials

White mulberry (*M. alba* L.) and black mulberry (*M. nigra* L.) leaves, fruits, and branches were collected from the village of İkiyüzoluk, which belongs to the Çınar district of Diyarbakır that located in the Southeastern Anatolia Region of Turkey. Mulberry fruits were collected in July 2017 from two Morus orchards and dried in a shady airy place for 4-6 days, the branches and leaves were collected in September 2017 from same places and dried for one week. The plant materials were characterized by Dr. Hülya Özpınar (at Department of Pharmaceutical Botany, Faculty of Pharmacy, Sivas Cumhuriyet University) and kept in the refrigerator at +4 °C until extraction.

2.2. Preparation of the extract

The dried leaves (10 g), fruits (10 g) and branches (10 g) of white and black mulberry were macerated for 24 hours with 50 mL of methanol, acetone, and water, separately. The extracts were then filtered through Whatman No. 1 filter paper. The filtrate was concentrated to dryness by rotary evaporator at 40 °C under low pressure. Aqueous extracts were dried completely using liofilizator. The yield percentage of each extract was calculated from the ratio of the weight of the extract residue obtained after solvent removal to the dry weight of the plant materials. The extracts were stored at -20 °C in the refrigerator until usage.

2.3. Antioxidant activity

2.3.1. Determination of total phenolic contents (TPC)

The TPC of the extracts was determined by the modified Folin-Ciocalteu method as gallic acid equivalent (GAE) according to the procedure (Zongo et al., 2010). Folin-Ciocalteu method was developed by Singleton and Rossi (1965) to measure total phenolic content, is based on the measurement of the absorbance of the complex-polymeric ions of phosphotungstic and phosphomolybdic acids in the Folin-Ciocalteu solution. The absorbance of the molybdenum-tungsten complex transformed from yellow to blue, which is the result of the oxidation of the phenolic compounds, the absorbance is measured at 750 nm. Twenty microliters of test solution in a concentration of 1000 µg mL^{-1} were dissolved in its solvent and Folin-Ciocalteu reagent (100 µL, 10%, v v^{-1}) were placed in a 96-well plate. After 5 minutes at room temperature, 80 µL of sodium carbonate (75 g L^{-1}) was added to each well and incubated in the dark for 30 minutes at 25 °C. Absorbance was measured at 735 nm using a microplate reader. Analyzes were made in three parallel and standard deviations were calculated. Total phenolic content of the extract was expressed as µg GAE mg of extract.

2.3.2. Estimation of total flavonoid content (TFC)

The TFC was determined by the $AlCl_3$ colorimetric method used by Molan and Mahdy (2014). 200 µL of the plant extract was mixed with 400 µL of distilled water and 28 µL of 5% $NaNO_2$ and left for 5 minutes. The mixture was then mixed with 28 µL of 10% $AlCl_3$. After 5 min of incubation, 200 µL of 1M NaOH and 240 µL of distilled water were added to the mixture. The absorbance of the mixture was read at 490 nm. Catechin was used as a standard at a concentration range of 0-1000 µg mL^{-1}. Calibration equation was found as;

y (Absorbance)= 1.9497x (mg of catechin) + 0.0887 (r^2= 0.9971)

Analyzes were done in three parallel and standard deviations were calculated. TFC in plant extracts was given as µg catechin equivalent (CE) per milligram of dry weight.

2.3.3. DPPH free radical scavenging activity

A 96-well plate method was used to determine the DPPH (2,2´-diphenyl-1-pikrilhidrazil) free radical scavenging activity (Miser-Salihoglu et al., 2013). In this method, 50 µL of 0.1 mM DPPH solution was prepared freshly in methanol, then was

added to 150 µL of methanol, acetone and aqueous extracts of *M. alba* and *M. nigra* in different concentrations (0, 50, 100, 250, 500 and 1000 µg mL^{-1}). Gallic acid and butylated hydroxyanisole (BHA) were used as standards. The experiments were run in triplicate. After the mixture was gently shaken, it was left for 30 minutes. The absorbance was then read at 517 nm in the microplate reader. The % inhibition of the extracts was calculated by the DPPH method by the following Equation 1.

$$\text{Inhibition (\%)} = [(A_0 - A_t)/A_0] \times 100 \quad (1)$$

Where, A_0 is the absorbance of control without sample, A_t is the absorbance of the extract.

2.3.4. ABTS radical scavenging assay

The ABTS•+ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolorization assay was carried out to evaluate the radical scavenging ability of extracts using the method of Re et al. (1999) and modified by Yang et al. (2011). The stock solution of ABTS•+ was generated by mixing 246 µL of 140 mM potassium persulfate with 15 mL of 7 mM ABTS aqueous solution. The mixture was incubated in the dark for 12-16 h. The ABTS working solution was diluted with methanol and brought to equilibrium at room temperature to give an absorbance of 0.70 ± 0.02 at 734 nm. 50 µL of test samples was mixed with 100 µL of ABTS•+ working solution and incubated at 25 °C for 10 min. Absorbance was read at 734 nm in a microplate reader and all determinations were performed in triplicate. Trolox is used as a standard. The results were found by concentration against % inhibition.

2.3.5. Copper (II) ion reducing antioxidant capacity (CUPRAC) method

A modified 96-well plate method was used for the determination of CUPRAC (Miser-Salihoglu et al., 2013). Briefly, 50 µL of 10^{-2} M $CuCl_2$ solution, 50 µL alcoholic neocuproine solution (7×10^{-3}), 50 µL of 1M ammonium acetate aqueous solution, 27.5 µL of extract in various concentration and 27.5 µL of distilled water were added into 96-well plate. The mixture was stirred and incubated for about 30 minutes in the dark at 25 °C. Analyzes were done in three parallel. Eliza reader was used to read absorbance at 450 nm. Trolox in concentrations of 1000-500-250-100-50-0 µg mL^{-1} was used as the standard solution.

2.4. Statistical analysis

Statistical analyses were done using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). The experiments were conducted three parallel and results were given as mean ± standard deviation (SD).

3. Results and Discussion

Extraction yields are given in the range of 2.3-68.6% (Table 1). The *in vitro* antioxidant activities of all tested extracts were examined by various methods. It was observed that the antioxidant capacity of each extract was proportional to the concentration of extract. This is due to the increase in the amount of active substance such as phenolic compounds (phenolic acid, flavonoids, coumarins, etc.) in the extracts as the concentration of extract increases.

Table 1. Total phenol and flavonoid content and % yields of the extracts obtained from *M. alba* L. and *M. nigra* L. different parts

Plant material	Plant part	Extract	TPC (µgGAE mg^{-1})*	TFC (µgCE mg^{-1})*	Yield (%)
M. alba	Leaf	Methanol	53.7 ± 0.17	27.3 ± 0.09	22.7
	Branche		73.6 ± 0.15	15.5 ± 0.07	5.9
	Fruit		86.2 ± 0.04	49.9 ± 0.17	68.6
M. nigra	Leaf	Methanol	84.9 ± 0.09	20.2 ± 0.11	23.5
	Branche		242.4 ± 0.26	11.9 ± 0.19	6.5
	Fruit		92.6 ± 0.14	70.9 ± 0.16	42.5
M. alba	Leaf	Acetone	217.1 ± 0.04	109.4 ± 0.19	9.8
	Branche		182.3 ± 0.07	112.5 ± 0.07	3.0
	Fruit		149.3 ± 0.11	132.9 ± 0.27	21.5
M. nigra	Leaf	Acetone	256.1 ± 0.18	185.8 ± 0.15	12.9
	Branche		257.5 ± 0.05	117.1 ± 0.15	2.3
	Fruit		254.1 ± 0.33	83.2 ± 0.22	16.2
M. alba	Leaf	Water	72.3 ± 0.24	8.4 ± 0.04	19.1
	Branche		101.7 ± 0.19	39.1 ± 0.11	6.0
	Fruit		54.2 ± 0.03	13.5 ± 0.03	54.3
M. nigra	Leaf	Water	79.9 ± 0.31	11.4 ± 0.05	11.6
	Branche		47.3 ± 0.08	25.8 ± 0.09	6.3
	Fruit		93.6 ± 0.09	32.5 ± 0.04	32

*: Results were presented as mean (n= 3) ± standard deviation

3.1. Total phenolic content

The total phenol content of mulberry extracts was calculated using the curve equation obtained from the gallic acid serial solution and was shown in Table 1. As can be seen from the results, it was found for TPC of acetone extracts ranging from 149.3±0.11 to 257.5±0.05 µg GAE mg^{-1}, in methanol extract ranging from 53.7±0.17 to 242.4±0.26 µg GAE mg^{-1}, and in water extract ranging from 47.3±0.08 to 101.7±0.19 µg GAE mg^{-1}. Generally, the amounts of phenolic compounds in black mulberry extracts were detected as higher than white mulberry extracts. In other studies, the TPC of black mulberries was defined as 14.22 µg GAE mg^{-1} (Ercisli and Orhan, 2007). TPC of methanol and 80% methanol extract from black mulberry was found 1172 and 1091 µg GAE mg^{-1} from Afyonkarahisar, 1416 and 1169 µg GAE mg^{-1} from the Anamur district of Mersin, respectively (Uygur-Yalgı, 2015). The results we have obtained are among the results of these two studies.

In our study, it was determined that the methanol and acetone extract of black mulberry branches (242.4±0.26 and 257.5±0.05 µg mg^{-1}, respectively) and water extracts of white mulberry branches (101.7±0.19 µg mg^{-1}) was found as the highest phenolic content. These results were also supported by another study in the literature (Bayır, 2011). In addition, it has been determined that the phenolic content changes depending on many factors such as climate, geological structure, precipitation amount, and agricultural applications (Ghasemi et al., 2011). The differences between our results and other studies may have been due to these factors.

3.2. Total flavonoid content

The TFC of the extracts was determined using the equation obtained from the standard catechin graph. The values of flavonoids, which were expressed catechin equivalents of methanol, acetone and water extracts of leaves, branches, and fruit parts of white mulberry and black mulberry plants, were given in Table 1. The acetone extract from white and black mulberry were identified have the higher amount of catechin equivalent flavonoid than other solvent extracts. The highest TFC content belongs to acetone extract of *M. nigra* leaf (185.8±0.15 µg CE mg^{-1}), and it was followed by the acetone extract of *M. alba* fruits (132.9±0.27 µg CE mg^{-1}). The water extract of white mulberry leaves (8.4±0.04 µg CE mg^{-1}) has the lowest flavonoid content (Table 1). In a previous work, the antioxidant activity of *M. indica* (Chinese mulberry) leaves was investigated by extracting them with methanol, acetone, and water. The methanol extract has showed the highest antioxidant activity due to the high phenolic compound content (Arabshahi-Delouee and Urooj, 2007). In our previous study, the flavonoid content was found as 142.2 and 43.6 mg quercetin equivalent per g of dry ethanol extract from *M. alba* and *M. nigra*, respectively (Eruygur and Dural, 2019).

3.3. DPPH radical scavenging activity

DPPH is a stable radical that is frequently used to determine the radical scavenging activity of plant materials. In all extracts of leaf, branches, and fruit parts of white and black mulberry, it was observed that the inhibition rate increased depending on the extract concentration. The percent inhibitions of methanol extract were detected higher than acetone and water extract. The highest DPPH radical scavenging activity was observed in acetone and methanol extract of *M. alba* branches (The IC_{50} value were 128.1±0.13 and 172.6±0.02 µg mg^{-1}, respectively). However, they were lower than the standard BHA (70.5±0.02 µg mg^{-1}) and gallic acid (109.7±0.01 µg mg^{-1}) (Table 2). In a similar study, it was determined that the radical scavenging activity of fruit and leaf methanol extracts was higher than that of water extract (Yiğit et al., 2008). This result was correlated with our study.

Different solvent extracts have been prepared according to increasing polarity as in many studies. The highest DPPH radical scavenging activity in methanol extracts was obtained in the order of as follows: BHA > Gallic acid > *M. alba* branche > *M. nigra* branche > *M. nigra* leaf > *M. alba* leaf > *M. nigra* fruit > *M. alba* fruit. For acetone extracts it was in the order of: BHA > Gallic acid > *M. alba* branches > *M. nigra* branches > *M. alba* fruit > *M. nigra* fruit > *M. alba* leaf > *M. nigra* leaf. In water extracts the order was as follows: *M. alba* leaf > *M. alba* fruit > *M. nigra* fruit > *M. nigra* leaf > *M. alba* branches > *M. nigra* branches. In this study, the IC_{50} values of aqueous extracts from *M. alba* leaf, branches, and fruits were found between 305.2±0.1 and 665.8±0.05 µg mg^{-1}. In the literature on *M. alba*, the IC_{50} values of aqueous extracts from leaves, roots and fruits were found to be 7.11±1.45, 86.78±3.21 and 14.38±2.83 mg mg^{-1}, respectively (Wang et al., 2012). Both studies support that the antioxidant effect of the leaf is higher than fruits. At the same time, it was revealed that the antioxidant activity of the *M. alba* L. used in the study we conducted was higher than the previous study.

3.4. ABTS radical scavenging activity

Another antiradical test is ABTS•+ radical scavenging activity. According to the results, all extracts showed strongly inhibition of ABTS•+

Table 2. The IC_{50} values of extracts obtained from *M. alba* L. and *M. nigra* L. different parts in DPPH, ABTS and CUPRAC method

Plant material	Plant part	Extract	DPPH* (IC_{50} µg mg^{-1})	ABTS* (IC_{50} µg mg^{-1})	CUPRAC* (IC_{50} µg mg^{-1})
M. alba	Leaf	Methanol	837.4 ± 0.27	471.9 ± 0.18	62.33 ± 0.18
	Branche		158.3 ± 0.33	75.5 ± 0.25	35.63 ± 0.22
	Fruit		1395.7 ± 0.17	366.7 ± 0.27	71.22 ± 0.33
M. nigra	Leaf	Methanol	779.2 ± 0.19	3825.7 ± 0.11	49.81 ± 0.08
	Branche		443.0 ± 0.20	3126.8 ± 0.33	99.72 ± 0.17
	Fruit		877.4 ± 0.09	2521.5 ± 0.33	124.64 ± 0.19
M. alba	Leaf	Acetone	1972.3 ± 0.11	3546.3 ± 0.61	81.68 ± 0.07
	Branche		128.1 ± 0.13	449.7 ± 0.02	55.33 ± 0.01
	Fruit		1295.6 ± 0.18	549.8 ± 0.52	166.16 ± 0.05
M. nigra	Leaf	Acetone	2494.7 ± 0.30	694.2 ± 0.72	83.11 ± 0.02
	Branche		387.3 ± 0.22	496.4 ± 0.11	71.18 ± 0.44
	Fruit		1386.1 ± 0.31	172.6 ± 0.02	124.60 ± 0.77
M. alba	Leaf	Water	305.2 ± 0.10	309.5 ± 0.04	99.741 ± 0.38
	Branche		665.8 ± 0.05	366.2 ± 0.07	124.66 ± 0.36
	Fruit		507.1 ± 0.06	307.7 ± 0.05	249.32 ± 0.19
M. nigra	Leaf	Water	586.8 ± 0.07	394.6 ± 0.21	99752.2 ± 0.45
	Branche		808.9 ± 0.09	549.8 ± 0.12	249260.5 ± 0.12
	Fruit		549.1 ± 0.16	473.6 ± 0.03	124719.3 ± 0.17
Trolox				1.8 ± 0.01	14.99 ± 0.66
GA			109.7 ± 0.01		
BHA			70.5 ± 0.02		

*: Results were presented as mean (n= 3) ± standard deviation

radicals, and the highest ABTS•+ scavenging activity was observed in *M. alba* branches methanol extract (IC_{50}= 75.5±0.25 µg mg^{-1}), Following this, *M. nigra* fruit acetone extract (IC_{50}= 172.6±0.02 µg mg^{-1}) and *M. alba* leaf water extract (IC_{50}= 309.5±0.04 µg mg^{-1}) were detected in high ABTS•+ scavenging activity. The results showed that the antioxidant activity of leaf and fruit extract was found as lower than the branch extracts (Table 2).

The percentage inhibition rates of all extracts of leaf, branches, and fruit parts of white mulberry and black mulberry, increase with increasing concentration. The percentage inhibition rates of water extracts were higher than acetone and methanol. While the ABTS method can be applied to both hydrophilic and lipophilic antioxidant systems, the DPPH method is used for hydrophobic systems due to its dissolution in the organic medium (Uygur-Yalgı, 2015). Therefore, the percentage inhibition of aqueous extracts by the ABTS method is higher than methanol and water extracts and the percentage inhibition rate of methanol extracts is higher than others in the DPPH method.

3.5. CUPRAC method

In all extracts of leaves, branches, and fruits of white and black mulberry plants, it was observed that the cupric reducing antioxidant activity increased with the increase in the concentration of extracts. Trolox was used as a standard, and the results were compared with this reference standard. Generally, the highest copper (II) ion reducing power was observed in methanol extracts. This was followed by acetone extracts and least in water extracts. The IC_{50} value of CUPRAC that the concentrations providing 50% inhibition are given in Table 2. The highest cupric reducing activity was observed in the methanol extract (35.63±0.22 µg mg^{-1}) obtained from the *M. alba* branches. The lower cupric reducing activity was observed in the water extract (249.260±0.12 µg mg^{-1}) prepared from *M. nigra* branches. In a study, the copper (II) ion reducing power of the water and methanol extracts obtained from white mulberry leaves were 0.188±0.006 mg mg^{-1} and 0.236±0.007 mg mg^{-1} (Uysal, 2013). The results of this study overlap with the results of our work.

Antioxidant compounds have a very important role in defending the body against oxidative stress induced by reactive oxygen species, chronic and degenerative diseases associated with oxidative stress. Due to the importance of antioxidant compounds, the identification of new antioxidant sources has become one of the most popular topics in natural drug research. It has been seen that studies on antioxidant activity in herbal products have increased in recent years, but the most important thing is that whether it shows the same antioxidant activity even after it is taken into the body. Therefore, larger phytochemical and *in vivo* studies are recommended for precise determination of biological activities. Experimental conditions such as extraction solvent, extraction method and storage conditions, differences in methodology, various

operations applied, cause different research results made by different researchers (Azwanida, 2015). For this reason, the identification of new natural antioxidant sources from plants has gained speed in recent years.

In this work, the leaves, fruits, and branches of white and black mulberry species grown in Diyarbakır were used to investigate their phenolic, flavonoid content and antioxidant activity. Various solvent extracts from white and black mulberry fruits, leaves and branches showed an increase in activity with the increase of the extract concentration in different antioxidant methods. It was observed that the phenolic and flavonoid contents of branches were higher than those of leaves and fruits.

4. Conclusions

The results obtained from the present work prove that the different extracts prepared from *M. alba* and *M. nigra* have potential antioxidant activity. When the obtained results considering, it shows that the leaves and branches can be considered as a source of potential natural antioxidants besides the fruits as it used. In this way, it is indicated that the leaves and branches of white and black mulberry can be important in terms of the country's economy by using them as a natural antioxidant raw material. For this reason, our work will be a horizon for new research in this regard. Optimum technological conditions and production factors are important for the productivity and bioavailability of herbal antioxidants used in food and biological systems. In addition, the beneficial effects and safety of antioxidants obtained from plants must be proven by clinical studies. Consideration of these questions will guide future research work. In addition, the present study is the basis for the use of mulberry as an important antioxidant source in the food and pharmaceutical industries.

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