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Determination of Total Phenolic Contents and Antioxidant Activities of Different Extracts Obtained from *Morus alba L.* (White Mulberry) Leaf

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ABSTRACT

Objective: The plant known as white mulberry, or *Morus alba L.*, has several uses and is employed in traditional medicine in many different cultures. The objectives of this study are to determine the total phenolic component and antioxidant levels of the methanol and ethanol extracts of leaves of *Morus alba L.* (White mulberry).

Methods: Using the techniques of DPPH (1,1-diphenyl-2-picrylhydrazyl), FRAP (Iron ion that decreases antioxidant power), and CUPRAC (Copper ion that reduces antioxidant capacity), the antioxidant activities of methanol and ethanol extracts of *Morus alba L.* leaves were examined. Additionally, the Folin-Ciocalteu Reagent (FCR) technique was used to evaluate the total phenolic content of the ethanol and methanol extracts. To determine the antioxidant capacity of extracts, reference samples were used to prepare various concentrations ranging from 1 to 100 µg/mL. The extract's equivalent antioxidant capacity was determined using sample concentrations of 125, 250, and 500 µg/mL.

Results: The methanol and ethanol extracts of the leaves of the *Morus alba L.* (White mulberry) plant were found to have the greatest level of phenolic compounds at the 500 µg/mL concentration of extracts. It was determined that the Trolox Eq value of methanol extract was higher in the DPPH, FRAP and FCR methods, and the Trolox Eq value of the ethanol extract was higher in the CUPRAC method.

Conclusion: The possible folk medicinal application of *Morus alba L.* is supported by this study. Determining the precise mechanisms of action of the extracts, the best extraction technique, the ideal dosage, and any possible adverse effects is needed.

Keywords: CUPRAC, DPPH, ethanol extract, FRAP, methanol extract, *Morus alba L.*

INTRODUCTION

The most significant compounds that aid in inhibiting the oxidation process are antioxidants. Endogenous antioxidant defense mechanisms struggle to destroy or diminish free radicals which are produced by metabolism, xenobiotics, or toxins. Many natural nutrients particularly fruits and vegetables are rich in antioxidants and can be considered exogenous antioxidants.[1-3]

Plants with antimutagenic and anticarcinogenic properties, rich in antioxidants and sources of physiologically active compounds, have gained popularity in recent years.[4] Numerous studies have examined pigments found in fruits and vegetables to determine whether they could improve human health or reduce the risks of diseases.[5] As a result, much research on plants has led to the creation of natural antioxidant formulations for use in food and cosmetics.

Morus alba L. (White mulberry), which belongs to the Morus genus of the Moraceae family, is a plant used in traditional medicine in many societies and has a wide range of uses. Various parts of the plant, such as leaves, fruits and seeds, are pharmacologically valuable. *Morus alba L.* contains a wide range of nutrients, including phenolic acids, flavonoids, flavonols, anthocyanins, macronutrients, vitamins, minerals and volatile aromatic compounds. It has some phytochemicals that have a wide range of pharmacological effects.[6]

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Morus alba L. folium contains neutral sugars such as arabinose, galactose, glucose, rhamnose, xylose, mannose, as well as large amounts of uronic acid in the form of galacturonic acid and glucuronic acid.[7-10] The most abundant amino acid in *Morus alba L*. leaf is glutamate, accounting for approximately 20%, followed by glycine and aspartate.[11] Chinese medicine has historically utilized mulberry leaves to treat fevers, strengthen joints, guard the liver, enhance vision, and regulate the maturation of endritic cells. The leaves have also been used in anti-obesity, anti-diabetic, antibacterial, and antioxidant treatments.[12-21] The aim of this study is to determine the antioxidant activities and total phenolic compounds of ethanol and methanol extracts of *Morus alba L*. leaves.

METHODS

Plant Material

The leaves of *Morus alba L*. were collected in August 2022 from the Uzundere district in the province of Erzurum, and they were dried according to the recommended methods (at room temperature, in a dry environment, away from sunlight). The plant was dried and then ground into a powder with a porcelain mortar and liquid nitrogen. Before the experiment started, the powdered mulberry leaves were kept in the proper storage.

Preparation of Plant Extracts

The *Morus alba L*. plant's leaves were dried and then pulverized into a powder in a mortar with the use of liquid nitrogen. The leaves of *Morus alba L*. were extracted using ethanol and methanol in a shaking water bath at 50˚C for 72 hours. The filtrate was then filtered every 24 hours, condensed in an evaporator, and kept in a refrigerator at +4˚C until analysis day.

Determination of total phenolic content

A modified version of Slinkard and Singleton's method was used to evaluate the total phenolic compounds present in the ethanol and methanol extracts of *Morus alba L*.[22] The method was described in brief as follows: First, 50 milliliters of 7.5% Na_2CO_3 was prepared. Following 25 mg of gallic acid from the standard, methanol was added to a test tube until it reached 25 mL. Finally, Folin-Ciocalteu reagent was used to determine phenolic compounds. The necessary dilutions were created and stock solutions were made ready.

First, 200 µL Folin & Ciocalteu reagent and 40 µL sample were added to the plates and incubated for 5 minutes. Finally, 160 µL Na_2CO_3 was added and incubated for another 30 minutes. After incubation absorbance was measured at 765 nm. Data were expressed as mg gallic acid equivalent (GAE)/g using the standard graph with gallic acid.

Determination of Antioxidant Capacity

DPPH Radical Scavenging Capacity Assay

The Brand Williams method was utilized to determine the DPPH radical scavenging capabilities of ethanol and methanol extracts derived from *Morus alba L*.[23] Antioxidant capacity is determined by spectrophotometrically measuring the inhibitory response of materials to DPPH radical. In the presence of an antioxidant, the DPPH solution loses color during the reduction reaction; this reduction in color intensity facilitates measurement in the spectrophotometer. Following the DPPH solution's preparation, 210 µL of the extract sample was pipetted into each plate well, and 70 µL of the DPPH solution was then added. After one minute of stirring the plate, it was left in the dark for thirty minutes. The standard antioxidant for the control sample was trolox. The absorbance was then measured at 517 nm, and the percent inhibition was computed based on the data.

The ferric reducing antioxidant power (FRAP) assay

Huang et al. used the electron transfer method to determine the antioxidant capacity of extracts made from *Morus alba L*.[24] First, an acetate buffer with a pH of 3.6 (300 mmol/L) was made. A 100 mL-flask was filled with 10 mM TPTZ, 40 mM HCl, and more HCl to bring the total content to 100 mL. Lastly, a $FeCl_3$ solution containing 20 mmol/L was made. From these prepared solutions, 2.5 mL of TPTZ, 2.5 mL of $FeCl_3$, and 25 mL of acetate buffer were taken to make a total of 30 mL of FRAP solution. Following a 30-minute incubation period, 200 µL of FRAP solution and 10 µL of the extract sample were pipetted into the plate wells. The absorbance was then measured.

Cupric ions (Cu^{2+}) reducing-CUPRAC assay

According to Apak et al., this approach is based on the complex's absorbance at 450 nm wavelength and its conversion from Cu(II) Neocuproin complex to Cu(I) Neocuproin via antioxidant chemicals found in the environment.[25] $CuCl_2 \bullet 2H_2O$, weighing 0.4262 g, was dissolved in 250 mL of distilled water to prepare the CUPRAC reagent. (10 milligrams). NH_4Ac (19.27 g) was dissolved in 250 mL of water to create the acetate buffer. In a 25 mL flask, 0.039 g of the Neocuproin chemical was prepared with 96% pure ethanol to yield a 7.5 mM

neocuproin solution. Afterwards, solutions consisting of 60 µL $CuCl_2$, 60 µL acetate buffer, 60 µL neocuproin solution and 66 µL extracts were mixed and after 30 minutes of incubation, absorbances were measured at 450 nm wavelength. The standard antioxidant Trolox was used as a control sample. Calibration curves of the working range of 1-100 µg/mL, where the plot of absorbance versus concentration is linear, were derived.

RESULTS

Results of Total Phenolic Compound Quantification

Total phenolic compound amounts of ethanol and methanol extracts prepared from *Morus alba L*. were determined by Folin-Ciocalteu Reagent (FCR). Gallic acid was used as the standard phenolic compound and was calculated as gallic acid equivalent from the equations obtained from the calibration curves of gallic acid. (Figure 1)

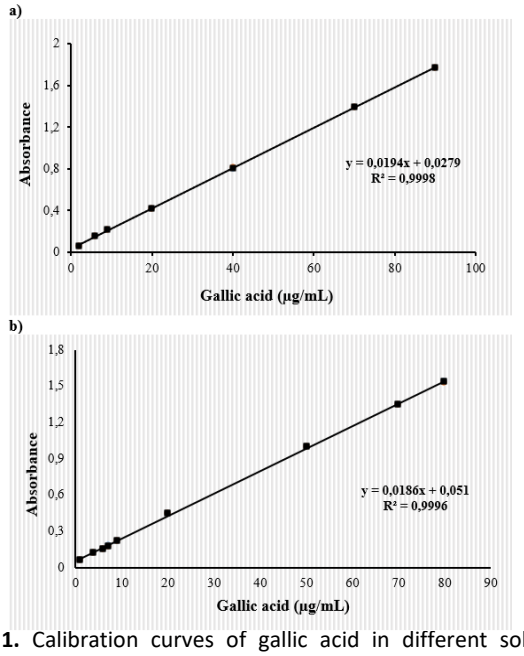


Figure 1. Calibration curves of gallic acid in different solvents (a. Methanol b. Ethanol)

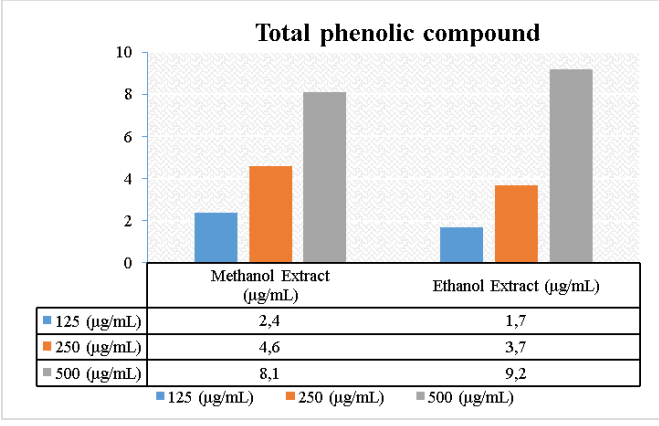


Figure 2. Comparison of total phenolic compound amounts of *Morus alba L*. extracts

The total phenolic content of ethanol and methanol extracts made from the fruit of *Morus alba L*. at different concentrations was measured. Consequently, the concentration of 500 µg/mL was shown to have the maximum total phenolic content.

Antioxidant Capacity Activity

Results of DPPH radical scavenging activity

DPPH radical scavenging activities of standard antioxidant compounds of ethanol and methanol extracts prepared from *Morus alba L*. were determined according to the Brand Williams method 23. The analyzed concentration range (1-100 µg/mL) was determined as a result of studies on standard antioxidant compounds.(Figure 3)

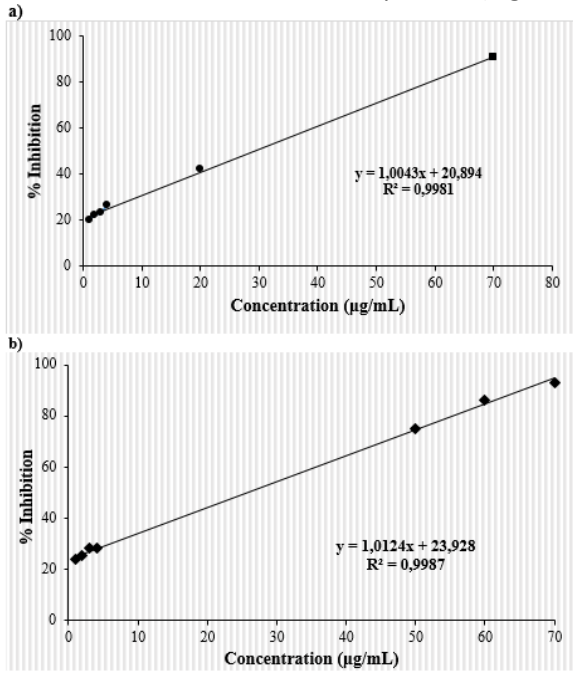


Figure 3. Concentration-% Inhibition graph of Trolox (a.Methanol b. Ethanol)

The DPPH radical scavenging capacities of methanol and ethanol extracts of *Morus alba L*. leaves in the range of (125-500 µg/mL) are shown in Figure 4 as % inhibition.

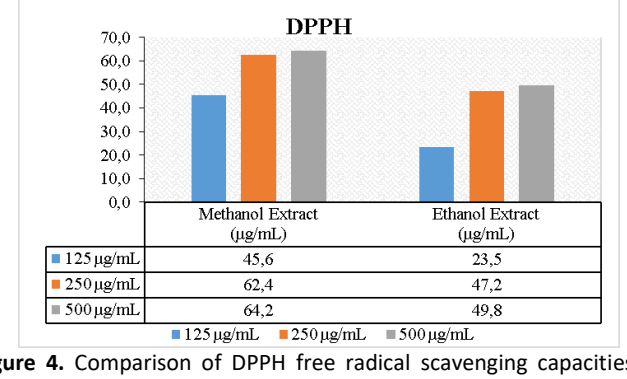
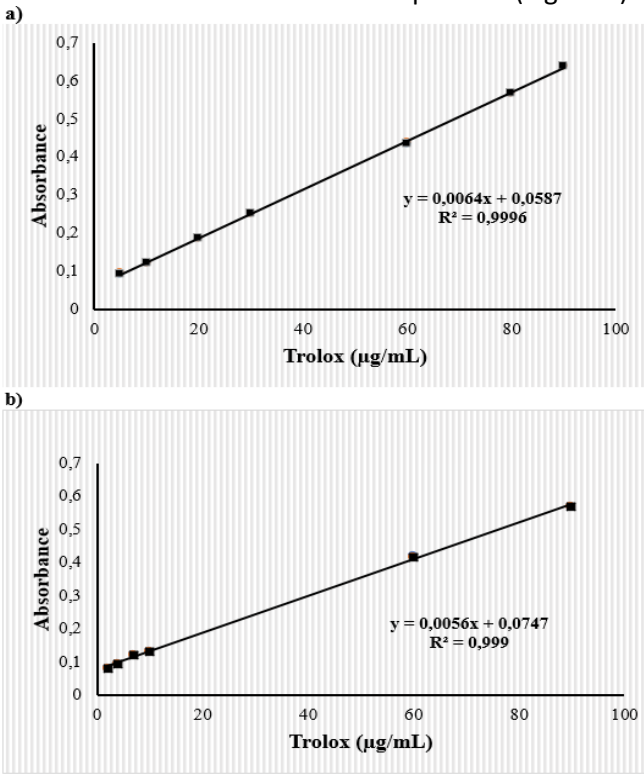


Figure 4. Comparison of DPPH free radical scavenging capacities of extracts at different concentrations

Morus alba L. fruit methanol extract showed the highest DPPH free radical scavenging effect at 500 µg/mL concentration.

Results of the copper ion reducing antioxidant capacity determination method (CUPRAC)

The conversion of ethanol and methanol extracts prepared from *Morus alba L*. and standard antioxidant compounds of Cu(II) neocuproin complex at 450 nm to Cu(I) neocuproin by means of compounds with antioxidant effect in the medium was done by measuring the absorbance at 450 nm. The concentration range to be analyzed (1-100 µg/mL) was determined as a result of studies on standard antioxidant compounds. (Figure 5)



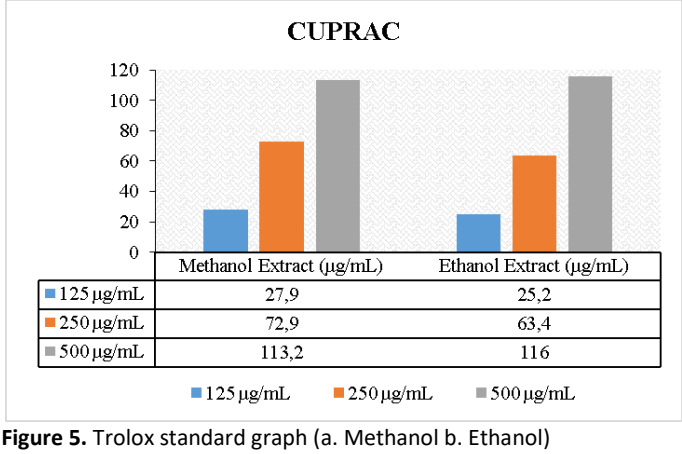


Figure 5. Trolox standard graph (a. Methanol b. Ethanol)

Figure 6. Comparison of the conversion of the extracts from Cu (II) neocuproin complex to Cu (I) neocuproin at different concentrations in terms of µg TEAC

Results of iron ion reducing antioxidant power (FRAP)

Spectrophotometric measurements of iron (III) reduction/antioxidant equivalent absorbances of *Morus alba L*. leaves, ethanol, methanol extract and standard antioxidant compounds were made at 593 nm. The antioxidant power capacity of trolox, one of the standard antioxidant compounds, was studied in ethanol and methanol extract at concentrations between 1 and 100 µg/mL. (Figure 7)

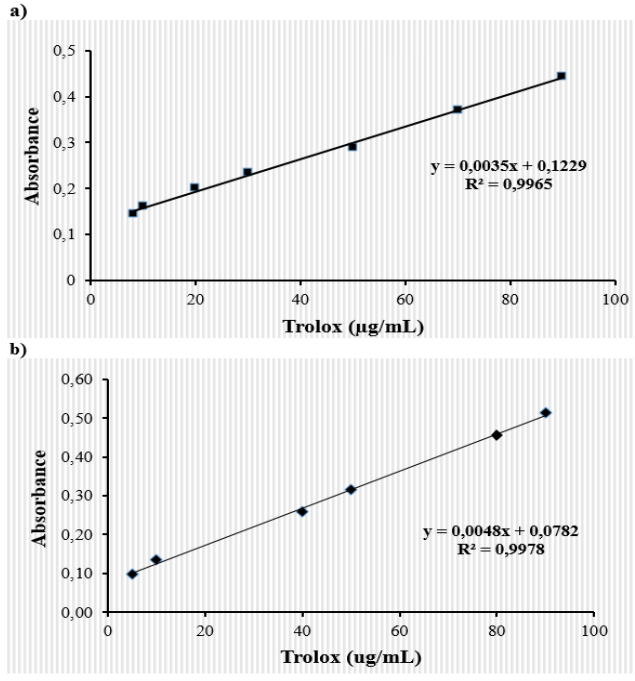


Figure 7. Trolox standard graph (a.Methanol b. Ethanol)

Comparison of the iron (III) reduction/antioxidant powers of *Morus alba L*. ethanol and methanol extracts in terms of µg/mL Trolox equivalent Antioxidant Capacity (TEAC) using the spectrophotometric method at 593 nm is given in Figure 8.

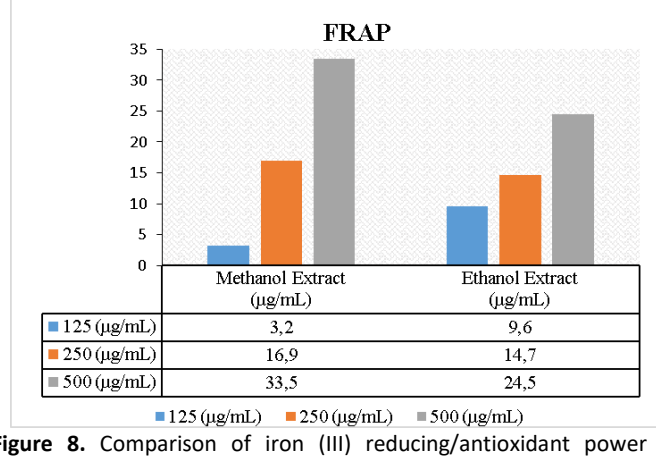


Figure 8. Comparison of iron (III) reducing/antioxidant power of extracts at different concentrations in µg TEAC

It was determined that the ethanol and methanol extracts prepared from *Morus alba L*. had iron ion-reducing antioxidant power capacity, the concentration of 500 µg/mL was high in both extracts and the results were close to each other.

DISCUSSION

The activities of antioxidant compounds arise from basic antioxidative properties such as preventing radical chain reactions, chelating metal ions, preventing peroxide formation, radical scavenging or reducing power. In this study, which we conducted with the ethanol and methanol extracts of *Morus alba L*.(mulberry) leaves within the framework of the study on antioxidant activity, the total antioxidant capacity was determined separately at different concentrations using different antioxidant methods and additionally determination of the amount of total phenolic compounds.

The antioxidant activity of all the ethanol and methanol extracts made from *Morus alba L*. leaves was assessed in this study. The ethanol extract was shown to have high levels of copper ion reducing antioxidant capacity (CUPRAC) and total phenolic compounds. In terms of DPPH radical scavenging activity and ferric ion-reducing antioxidant power (FRAP), the methanol extract showed significant antioxidant activity. We believe that the compounds found in the leaves of *Morus alba L*. are responsible for this property. It is believed that differences in the concentrations and molecular composition of the polyphenolic chemicals transferred to the solvent of choice account for the different variations in antioxidant activity seen in the extracts. Mulberry is a plant traditionally used among the public for various diseases. The wide range of biological activities exhibited by plants is due to the components they contain, and the phytochemical composition and biological potential of the plant varies depending on the region where it grows.[26] White mulberry leaves contain many components such as flavones, steroids, triterpenes, amino acids, vitamins and minerals. As a result of the antidiabetic activity studies conducted on the plant, it was determined that the leaves showed antidiabetic activity thanks to the phytosterol glycosides and scopoletin they contain.[27,28]

As a result of the studies, it can be remarked that the contents of mulberry leaves varies as follows: total amount of phenolic substances 24.12-39.38 mg/g, chlorogenic acid 3.10-10.05 mg/g, flavonoid substance 38.32-76.42 mg/g, rutin 0.96-3.49 mg/g, 1.17-6.91 mg/g of alkaloids, deoxynojirimycin 0.40–5.31 mg/g. Additionally, 153.1-309.1 mg/g protein, 80.1-134.2 mg/g carbohydrate, 8.1-22.7 mg/g mineral, 6.4-15.1 mg/g fat and 276.0–366.6 mg/g dietary fiber content were detected in mulberry leaves.[29-31] It has been determined that the compound called deoxynojirimycin found in mulberry leaves regulates the inhibition of enzymes such as glycosidase, sucrase and maltase. Thus, mulberry leaves can be used in the treatment of diabetes.[32, 33] Mulberry fruit is a functional food rich in anthocyanins that has attracted the attention of researchers and consumers due to its potential pharmacological activities on health.[34,35] Although there are many studies examining the pharmacological activities of mulberry leaves,[36,37] there are a limited number of studies on the pharmacological properties of its fruits.[34]

In a study, mulberry powder was used as a natural sweetener in ice cream production, and a decrease in caloric value was observed.[38] In a clinical study, diabetic mice were fed a diet consisting of a combination of mulberry leaf flour and oat bran in a 1:1 ratio for 28 days, inhibiting α-glycosidase activity, insulin effect. It has been found to have antidiabetic effects.[31] Considering the role of oxidative stress in the pathogenesis of diabetes, we can conclude that mulberry fruit may be rich source to reduce oxidative stress in diabetes. A study from Turkey was conducted to evaluate the antidiabetic and antioxidant properties of water and ethanol extracts made from the leaves of white and black mulberry trees cultivated in the province of Edirne by means of in vitro enzyme inhibition experiments. It was found that only water extracts of mulberry leaves have the power to chelate metal ions. In the antidiabetic activity study, water extracts of the leaves showed varying degrees of inhibition of α-amylase and α-glucosidase. The fact that water extracts have a potential inhibitory effect on carbohydrate digestive enzymes indicates that mulberry leaves, which are not consumed as food in our country, can be considered as a source of pharmaceutical raw materials. Again, black mulberry leaves can be brought into the economy to be used in cosmetic applications as antioxidant additives.[26]

Turkoglu et al.[39] were examined the antioxidant and antiradical capacity of Morus alba collected from Elazığ. The leaves and fruits of the plant were dried in the shade and water and ethanol extracts were prepared separately. Antioxidant and antiradical tests of these extracts were performed using different antioxidant methods such as total antioxidant capacity, reducing oxidant capacity, metal chelating activity, DPPH free radical scavenging activity, ABTS + radical scavenging

activity, superoxide anion radical scavenging activity, H_2O_2 scavenging activity and FRAP test. In addition, the total phenolic compound amount was determined separately at different concentrations using quantification. Considering the results obtained, it has been determined that mulberry leaves and fruits are a good free radical scavenger and can be used as a natural antioxidant.

The methanol and ethanol extracts prepared from the *Morus alba L*. leaves, which were examined in support of other studies, were shown to be high in antioxidant activity and phenolic content in our study. When the FCR, DPPH, FRAP, and CUPRAC results of *Morus alba L*. leaves extract from ethanol and methanol extracts were analyzed, the efficiency of different extracts in different ways was usually determined. In this work, antioxidant activity was determined using 125, 250, and 500 g/mL quantities of each produced extract. In the findings obtained at the end of the study, it was observed that there was a correlation in antioxidant capacity proportional to the increasing amount of each extract, depending on the amount of extract. This is due to the fact that as the amount of extract increases, the amount of active ingredients in the extracts also increases. The reason for this correlation may be many free radical scavenger groups contained in plants, such as phenolic compounds with antioxidant effects (phenolic acid, flavonoids, coumarins, etc.), nitrogenous compounds (alkaloids, amines, etc.), vitamins and terpenoids.

CONCLUSION

As a result of this study, it was found that ethanol and methanol extracts of *Morus alba L*. leaves have a strong antioxidant capacity in various antioxidant systems in inanimate environments. Plants can be used as an easily available source of natural antioxidants for the pharmaceutical industry and food additive industry. However, the components of the sample extract responsible for the antioxidant activities are not fully clear. In future studies, the active compounds contained in plants can be chemically analyzed using different methods and techniques and their pharmacological properties can be examined. The active compounds contained in the plant can be isolated and its antioxidant properties in living systems can be investigated. Additionally, other therapeutic properties along with its antioxidant activity can be investigated by taking into account the active substances contained in the plant.

Ethics Committee Approval: Ethical approval was not required as this study was conducted in vitro.
Author Contributions: Concept –N.K.B., L.D.; Design – N.K.B., L.D; Supervision – N.K.B.; Resources – N.K.B..; Materials – N.K.B., L.D; Data Collection and/or Processing – N.K.B., L.D., S.K.; Analysis and/or Interpretation – L.D., S.K.; Literature Search – S.K.; Writing Manuscript – L.D., N.K.B., S.K.; Critical Review – N.K.B., L.D, S.K.
Conflict of Interest: The authors have no conflicts of interest to declare.
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