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Antioxidant properties of different extracts of black mulberry (*Morus nigra* L.)

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Abstract: In vitro antioxidant properties of 3 different extracts of black mulberry (*Morus nigra* L.) were investigated. Acidified methanol, acidified water, and non-acidified methanol/water solutions were used to prepare extracts. Different solvents caused different protonation on black mulberry anthocyanin structures, which were predicted according to their UV-VIS spectrum. Extracts indicated 2 main peaks at about ~280 and ~520 nm with different peak areas in their UV-VIS spectrum. The rank of extracts' total phenolic content and reducing power values were both found to be in correlation with their absorbance at ~280 nm. Meanwhile the same relationship was observed between DPPH scavenging activity and absorbance values at ~520 nm. Acidified extract of black mulberry was higher in β-carotene prevention and DPPH radical scavenging activity than non-acidified extract. However, non-acidified extract represented a higher reducing power and metal chelating activity, and a higher content of total phenolics.

Key words: Antioxidant properties, black mulberry, phenolic, extract, metal chelating activity

Farklı karadut (*Morus nigra* L.) ekstraktlarının antioksidan özellikleri

Özet: Üç farklı karadut (*Morus nigra* L.) ekstraktının in vitro antioksidan özellikleri araştırılmıştır. Ekstraktları hazırlamak için asitlendirilmiş metanol, asitlendirilmiş su ve asitlendirilmemiş metanol/su karışımı kullanılmıştır. Farklı solventler karadut antosiyanın yapıları üzerinde UV-VIS spektrumlarından anlaşılan farklı protonlanmaya sebep olmuştur. Ekstraktlar UV-VIS spektrumlarında ~280 ve ~520 nm dalga boyu bölgelerinde başlıca iki pik vermiştir. Ekstraktların ~280 nm'de verdikleri absorbanslar ile toplam fenolik madde içeriği ve indirgeme gücü değerlerinin sıralaması arasında bir korelasyon görülmüştür. Aynı zamanda DPPH radikal süpürme gücü ile ~520 nm dalga boyu bölgesindeki absorbans değerleri arasında da benzer bir ilişki görülmüştür. Asitlendirilmiş ekstraktların β-karoten'i koruma aktiviteleri ve DPPH radikalini süpürme güçleri asitlendirilmemiş ekstraktlara oranla daha yüksek bulunmuştur. Ancak asitlendirilmemiş ekstrakt, daha yüksek indirgeme gücü, metal tutuklama aktivitesi ve toplam fenolik madde miktarı vermiştir.

Anahtar sözcükler: Antioksidan özellikler, kara dut, fenolik, ekstrakt, metal tutuklama aktivitesi

Introduction

Usage of food as health promoter beside its nutritional function is a wide issue in recent years. Especially fruits, vegetables, and herbs attract great attention due to their high content of bioactive compounds with antioxidant characteristics.

Black mulberry (*Morus nigra*) is one of the 3 common mulberry species along with white mulberry (*Morus alba*) and purple mulberry (*Morus rubra*) (1,2). Types of consumption of this fruit vary from fresh fruit, molasses, dried fruit, and fruit juice to alcoholic beverages (3-5). Black mulberry is a delicious fruit with a sugary-sour taste and refreshing flavor due to its aroma components (1) and sugar/acid ratio (3). In addition, it is used as a folk remedy in treatment of mouth lesions (6) and to strengthen the teeth (7).

Positive effects of fruits on health are supposed to originate mainly from their antioxidant compounds (8). Likewise, antioxidant activity of mulberry species is generally attributed to their phenolic compounds, especially anthocyanins (9-11). Major anthocyanins detected in mulberries are cyanidine-3-glucoside and cyanidine-3-rutinoside (12), which are reported to have an inhibitory effect on migration and invasion of lung cancer cells (13), cyanidine-3-sophoroside, pelargonidin-3-glucoside, and pelargonidin-3-rutinoside (14).

Anthocyanins are such compounds that their biological effects and appearance are quite dependent on pH (15). Although reports state that stability of anthocyanins is higher in strong acidic solutions (16), biological systems have variable and well-controlled pH dynamics that change from tissue to tissue. Thus, depending on the medium they exist in, anthocyanins face different pH conditions (17), which will change their protonation status and antioxidant power.

The effect of black mulberry methanol extract (18) and crude extract (19) on lipid peroxidation and free radical activity was studied. To the authors' best knowledge there is no study reporting antioxidant activity of black mulberry extracts prepared under different pH and solvent conditions. Thus, the aim of this study was to reveal that the antioxidant properties of black mulberry extracts in different solvents differ in acidification status.

Materials and methods

Materials

Black mulberries were collected at the commercially ripe stage (end of July 2007) in the campus area of İnönü University, Malatya, Turkey. The ripeness of the fruits was estimated according to their color, which was deep purple-black at their full mature stage. Fruits were sealed in plastic bags and stored at -70 °C until used. All chemicals were supplied by Sigma unless otherwise indicated.

Preparation of extracts

Ten grams of fruit was mixed into 100 mL of different solvents to obtain; acidified (0.5% HCl, pH: 2.8) methanol extract (AME), acidified (0.5% HCl, pH: 2.8) water extract (AWE), and methanol:water (70:30, pH: 3.9) extract (MWE). Mixtures were homogenized with an Ultra Turrax homogenizer (IKA Labortechnik, Staufen, Germany) for 5 min at 24,000 rpm in an ice cover and filtered through Whatman No:1 filter paper. Extracts were prepared daily and kept in a refrigerator at +4 °C until used.

UV-VIS spectrum

Spectral data were recorded for the 3 different extracts (AME, AWE, and MWE) using a double-beam spectrophotometer (Shimadzu model 1601, Tokyo, Japan) between 200 and 800 nm wavelengths in quartz cuvettes at 20 °C.

β-Carotene bleaching test

Miller's (20) method of β-carotene/linoleate system was used with slight modifications. Two milligrams of crystalline β-carotene was dissolved in 10 mL of chloroform and 1 mL of this solution was added to 40 µL of linoleic acid and 500 µL of Tween-20 (Merck) in a round-bottom flask. After removing the chloroform in a rotary evaporator under vacuum at 40 °C for 5 min, 100 mL demineralized water was added with vigorous stirring. Five milliliters of this emulsion was added to each tube containing different amounts of extracts in final volume of 1 mL. Mixtures were left in a water bath (Clifton, Ltd, North Somerset, England) at 50 °C and their absorbance was measured at 470 nm at intervals.

DPPH radical scavenging assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging activity was determined (21). One hundred microliters of mulberry extract was mixed into 1.9 mL of 0.025 g L^{-1} DPPH$^{\bullet}$ methanol solution in disposable spectrophotometer cuvettes. Remaining purple color was measured by using a spectrophotometer at 520 nm after 15 min of incubation in darkness. Radical scavenging power (RSP) was calculated by the following equation:

$$RSP = \left[1 - \left(\frac{A_{S:15}}{A_{B:15}}\right)\right] \times 100$$

where is absorbance of the sample andis absorbance of the blank at 15 min.

The kinetic behavior of the mulberry extracts and DPPH$^{\bullet}$ mixture was also observed using a spectrophotometer. For this purpose, the same amount of mulberry extract and DPPH$^{\bullet}$ solution (as described above) were mixed in quartz cuvettes and absorbance changes were monitored at 30 s intervals at 520 nm for 20 min during which the reaction almost reached a plateau.

Reducing power

The assay was performed according to the method of Oyaizu (22). Black mulberry extracts were diluted to 1 mL with distilled water and 2.5 mL of the 0.2 M phosphate buffer (pH: 6.6) and 2.5 mL of 1% potassium ferricyanide solution were added and vortexed. The mixtures were left to incubate at 50 °C for 20 min in a water bath. The tubes were cooled to room temperature and 2.5 mL of 10% trichloroacetic acid was added and centrifuged (MSE Mistral 1000, Sanyo, Japan) at 6000 rpm for 10 min. Two and a half milliliters of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% aqueous ferric chloride. Absorbance of the final solution was recorded at 700 nm.

Total phenolic content

Total phenolic content of extracts was determined using the Folin & Ciocalteu phenol reagent method (23). One hundred microliters of the black mulberry extract was mixed with 1.7 mL of Folin & Ciocalteu reagent and after 3 min with 1.2 mL of 2% aqueous sodium carbonate solution. Spectrophotometric reading (760 nm) was taken after 15 min of incubation at room temperature performed with shaking. The calibration curve was obtained with gallic acid, and the results were expressed as equivalents micrograms of gallic acid per milliliter of black mulberry extracts.

Iron chelating activity

Determination of chelation of iron (II) ions by different black mulberry extracts in comparison with EDTA was carried out as described by Dinis et al. (24). One hundred microliters of different extracts and 1.7 mL of water was added to 75 µL of 1 mM $FeCl_2 \cdot 4H_2O$ solution. After 5 min of incubation, the reaction was initiated by adding 200 µL of 5.0 mM ferrozine. Absorbance of the solutions was recorded at 562 nm after vigorous shaking for 3 min.

Statistical analysis

Experimental data were evaluated by using analysis of variance (ANOVA) and significant differences among means from triplicate analysis at $P < 0.05$ were determined by Duncan's multiple range test, using SPSS 9.0 for Windows.

Results and discussion

Antioxidant actions of bioactive compounds in biological systems and foods take place in very complex media (25). Two of the main important parameters that determine the characteristics of these media are the solubility of antioxidant compounds in this environment and the pH of the medium itself. The efficiency of an antioxidant compound or extract should be dependent on these parameters (26).

Different extraction systems were used to estimate the antioxidant characteristics of black mulberry fruit. Acidified alcohols are widely used to extract anthocyanins due to their protective role on anthocyanins in their protonated forms (15) and we chose methanol as it is more commonly used than ethanol in the literature. Acidified water, which may imitate biological conditions, was used to extract more polar constitutes, and a methanol:water mixture (70:30) was used to understand the effect of relatively alkaline medium on extractability of antioxidant compounds in the presence of both methanol and water as solvent.

UV-VIS spectrum

The spectrum of the 3 extracts obtained by UV-VIS spectrophotometer indicated 2 main peak points observed at about ~280 and ~520 nm (Figure 1). This pattern has similar characteristics with known spectrum of anthocyanins (27). While MWE did not show any peak near 520 nm, it gave its highest peak near 280 nm, which indicates peak migration from 520 to 280 nm caused by deprotonation. The probable mechanism for this shift is deprotonation of flavylium cation to form a quinonoidal base, which has a bluish color (15). At 520 nm, AME showed a higher peak and AWE showed a peak between AME and MWE. With the presence of acid in the solution, anthocyanin structures were protonated and reddish colored flavylium cations were formed (28). The height of peak of AWE was smaller than that of AME. Therefore, we can say that compared to AME, cationic structures in AWE were not effectively protonated due to their smaller peak at ~520 nm, although both solvents included the same amount of acid. This could be the result of the lower solubility of anthocyanins in water rather than methanol (29) or probable lower dissociation of acid.

β-Carotene bleaching test

Results gained from the β-carotene/linoleate model system reflect antioxidant efficiency of the extracts in an emulsion during accelerated lipid oxidation conditions. Initial absorbance of each tube, containing samples or control plus β-carotene emulsion, is presumed as 100% and the following readings calculated as percentage of initial absorbance and plotted against incubation time (Figure 2). The reaction was stopped at 90 min when control sample bleached almost completely.

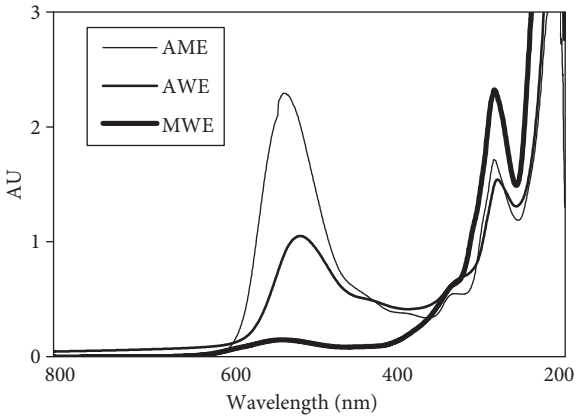

Figure 1. UV-VIS spectrum of acidified methanol (AME), acidified water (AWE), and methanol + water (MWE) extracts of black mulberry.

AME and AWE showed better protective action against bleaching of β-carotene at lower concentrations when 2.5-25 μL of extract was used ($P < 0.05$) (Figure 2a). However, at higher concentrations (50-100 μL of extract used) AWE showed less activity in comparison with AME and MWE (Figure 2b, 2c). This is due to the probable pro-oxidant effect that was observed with more than 25 μL of extract volume for AWE in the reaction mixture. The limiting factor for this test was found to be water as solvent. Although the antioxidant activity of both methanol containing extracts increased with increasing concentration, the protective action to β-carotene of the extract that lacked methanol (AWE) paused at the level of 85% even for higher concentrations.

DPPH radical scavenging activity

DPPH radical scavenging activity of the 3 different black mulberry extracts are shown in Figure 3. MWE showed the poorest and AME the best activity in the DPPH assay ($P < 0.05$) when we consider the lower concentrations (10 and 25 μL of extract in reaction mixture). The order of radical scavenging power (RSP) values of extracts could only be seen clearly in relatively low concentrations. At higher portions of extracts in the reaction mixture, the medium was probably saturated with antioxidant compounds coming from AME and the other extracts' RSP reached the AME level with increasing concentrations. Although AWE was a better antioxidant at lower concentrations in the DPPH system, at higher portion of extracts in the reaction medium, methanol containing MWE showed better results probably due to the higher interactions of methanol soluble antioxidant compounds with methanol soluble $DPPH^{\bullet}$ molecules. While the increase in radical scavenging capacity versus extract concentration is close to linear for AWE and MWE ($R^2 = 0.95$ and 0.94, respectively), it was nonlinear for acidified methanol ($R^2 = 0.82$).

Kinetic behavior of extracts towards DPPH solution was also observed and at the end of the measurement period the order of RSP of extracts was

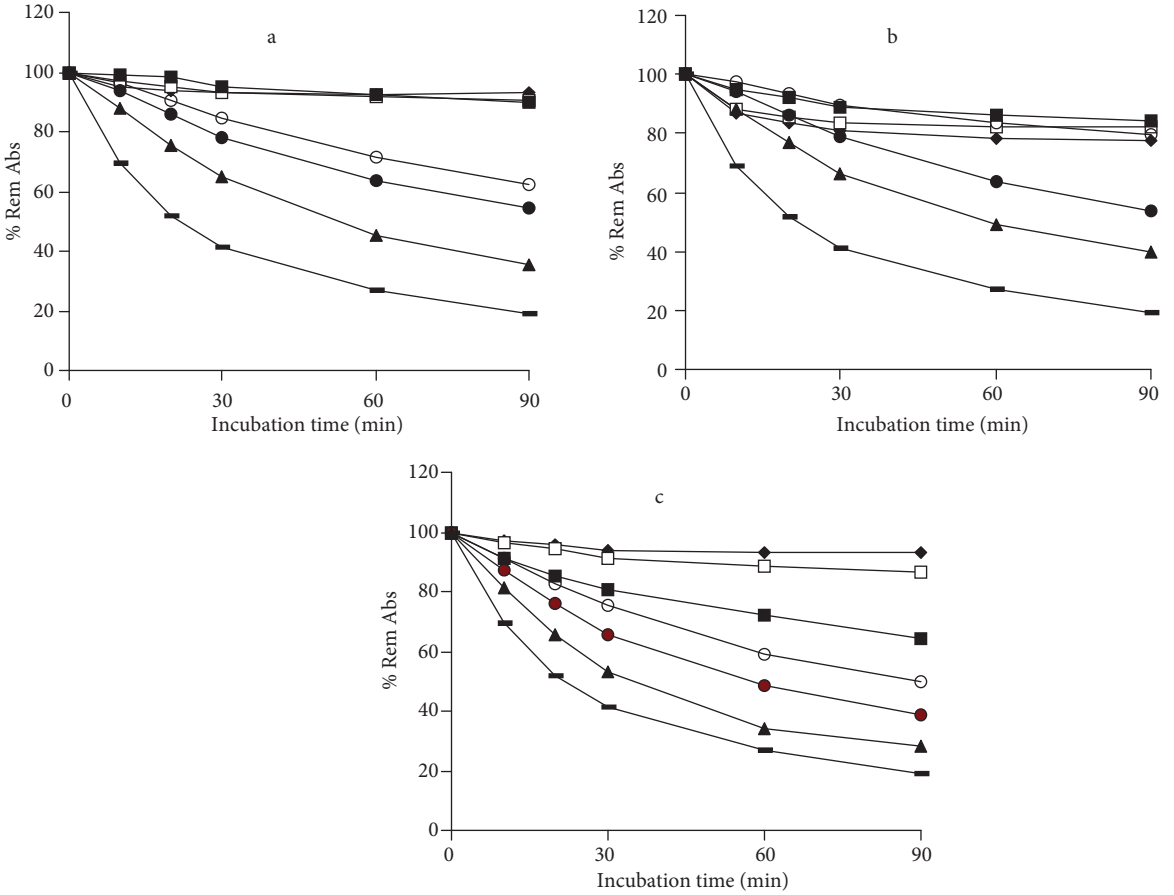

Figure 2. Preventive activity of control (♦) and 2.5 µL (●), 5 µL (○), 10 µL (▼), 25 µL (▽), 50 µL (▪), 100 µL (▫) of different black mulberry extracts on b-carotene in accelerated lipid oxidation conditions. (a: acidified methanol extract, b: acidified water extract, and c: methanol-water extract.)

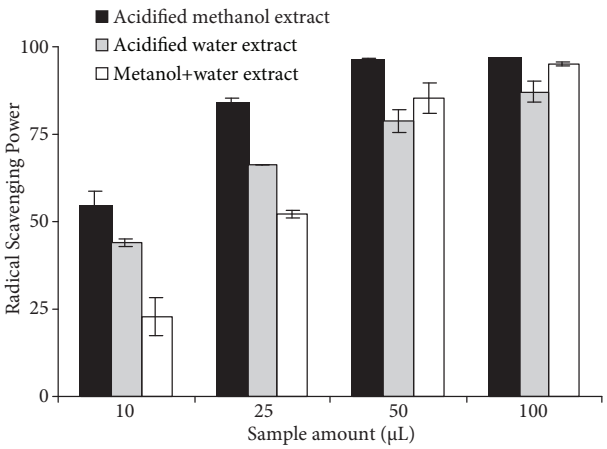

Figure 3. DPPH• radical scavenging power of acidified methanol (AME), acidified water (AWE), and methanol + water (MWE) extracts of black mulberry.

similar to that of the incubation method (Figure 4). In kinetic monitoring, we were also able to observe interaction rates of different extracts with DPPH radical. MWE scavenged DPPH better than AWE until the initial absorbance of the DPPH decreased to its 50% level. This is an important finding that can help researchers to design their experimental settings for DPPH or some of other antioxidant assessment tests that have time dependent dynamic characteristics. Until the 8th min of the kinetic monitoring, AWE showed less scavenging in comparison to MWE, whereas the opposite is true after the 8th min. Therefore, for the final absorbance measurements for such tests in which a certain incubation period is applied, the interaction rates of every single sample with DPPH• should be taken into account.

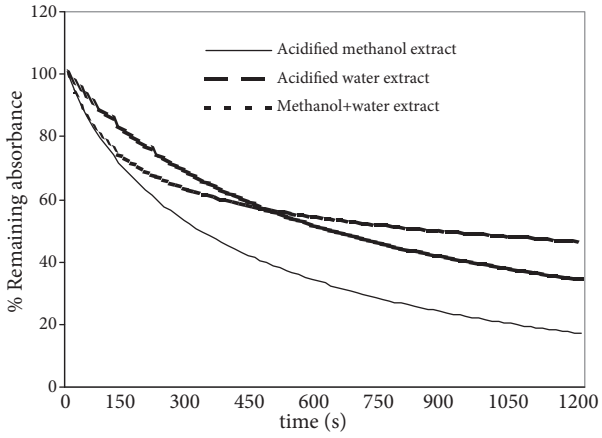

Figure 4. Spectrophotometric monitoring of the disappearance of DPPH$^{\bullet}$ in the presence of 3 different black mulberry extracts.

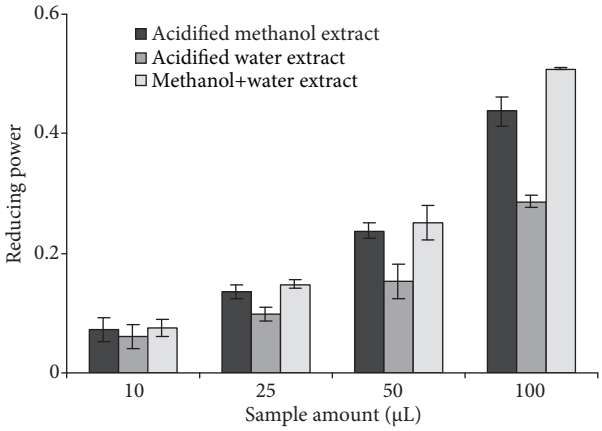

Figure 5. Reducing power of acidified methanol (AME), acidified water (AWE), and methanol + water (MWE) extracts of black mulberry at different volumes.

UV-VIS absorbance values (Figure 1) of the 3 extracts at ~520 nm showed similarity with their DPPH scavenging capacities. The better DPPH scavenger extract had the highest absorption at this region. At lower pH, anthocyanin structures present as (AH^+) form, which is a good electron acceptor. We found that radical scavenging power of flavylium cation rich extract better than poor ones, though it is reported that this form of anthocyanins is supposed to be pro-oxidant (30).

Reducing power

Reducing power of the samples is summarized in Figure 5. MWE was found to be the most powerful one among the tested extracts when 100 µL extract volume was used ($P < 0.05$). At lower concentrations the difference in reducing power values of AME and MWE was statistically insignificant. Except when 10 µL of extract volume was used, AWE showed the weakest reducing power at all concentrations ($P < 0.05$). The increase was exponential ($R^2 > 0.99$, equations are not shown) in reducing power for all extracts against increasing extract volume. The order of reducing power of black mulberry extracts correlated to total phenolic contents and absorption values at 280 nm.

Total phenolic content

Total phenolic contents of the 3 different extracts of black mulberry are shown in Figure 6. Mean total phenolic contents of MWE, AME, and AWE were

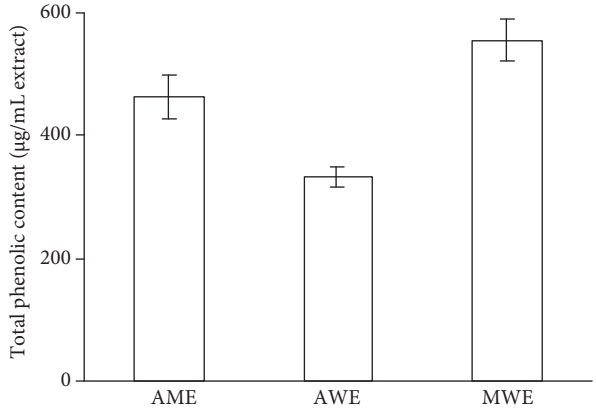

Figure 6. Total phenolic content as equivalent µg of gallic acid per mL of acidified methanol (AME), acidified water (AWE), and methanol + water (MWE) extracts of black mulberry.

found to be significantly different ($P < 0.05$) and 555, 462 and 332 µg gallic acid equivalent/mL extract, respectively. Acidification in AME and AWE did not increase the efficiency of extraction of phenols. The presence of methanol and water together in the extraction solvent increased the total phenolic yield as seen for MWE. Comparison between acidified extracts (AWE and AME) shows that methanol better extracted phenolic constitutes than water. In both the reducing power assay and total phenolic determination assays, the pH of the reaction medium was strongly predetermined by buffers, acids, or bases (22,23). Considering that all extracts have closer

protonation status in these tests, the difference in reducing power and total phenolic content could be the result of different polarity of the solvents and solubility of antioxidative compounds in these solvents. MWE, which had the highest scores in these tests, included both alcohol and water soluble compounds, while the other 2 extraction solvents had limited solvent capacity.

Metal chelating activity

Metal chelating activity of the black mulberry extracts and EDTA is presented in Figure 7. MWE, AWE, and AME showed the highest to lowest chelating activity, while the difference between MWE and AWE was statistically insignificant ($P < 0.05$). According to these results it can be stated that water is better than methanol in extracting chelators from black mulberry. Anthocyanins are reported to be good chelators for metal ions, which are well known catalyzers for free radical forming reactions (16). Although this kind of activity is not directly related to the antioxidant mechanism, due to its importance in biological systems it can be assumed to be an indirect antioxidant effect. It is reported that metal chelating of anthocyanin structures strongly depends on the pH of the medium (31).

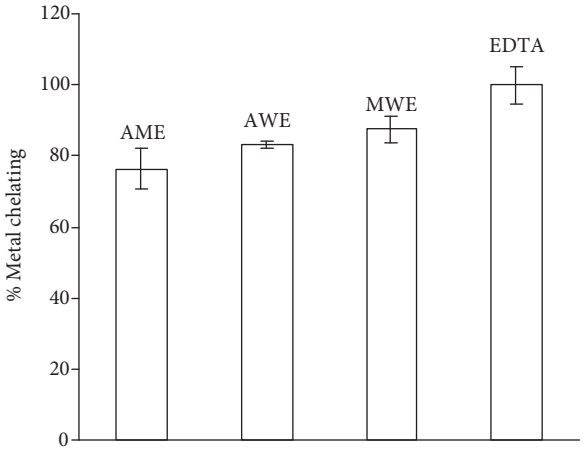

Figure 7. Iron chelating activity of acidified methanol (AME), acidified water (AWE), and methanol + water (MWE) extracts of black mulberry in comparison with EDTA.

In conclusion, it was shown that usage of methanol alone or with water in the preparation of black mulberry extracts for antioxidant tests increases phenolic yield, reducing power, and protective effect on β-carotene, in comparison to usage of water alone. On the other hand, acidification caused an increase in the radical scavenging power of extracts in the DPPH test. The antioxidant behavior of different extracts showed an interconnection with their UV-VIS spectrum. Total phenolic content and reducing power were correlated with absorbance values at ~280 nm, and radical scavenging power with the values at ~520 nm. In further studies, individual anthocyanins of black mulberry could be isolated and pH and solvent dependent antioxidant behaviors could be determined.

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