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# Determination of Antioxidant Activity of The Leaves of Hawthorn (*Crataegus Monogyna*)

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**Highlights:**

- Hawthorn as a natural source of antioxidants
- The role of free radicals in degenerative diseases such as cancer, aging and cardiovascular disorders

**Keywords:**

- Hawthorn
- *Crataegus monogyna*
- Antioxidant activity
- Metal reduction

**ABSTRACT:**

This study was carried out to make decision the antioxidant activity of the leaves of hawthorn (*Crataegus monogyna*), which is a natural source of antioxidants. For this purpose, the leaves of hawthorn were collected and dried, and an ethanol extract was prepared. Total antioxidant activity (with respect to the ferric thiocyanate method), ion reduction capacity of $Cu^{2+}$ (copper method), and $Fe^{3+}$ (potassium ferricyanide reduction method) were determined on the prepared ethanol extracts. Additionally, scavenging activity of DPPH (1,1-diphenyl-2-picrylhydrazyl) and determination of the total phenolic-flavonoid compound content were carry out. The extracts obtained from Hawthorn (*Crataegus monogyna*) leaves were found to inhibit lipid peroxidation (of linoleic acid emulsion) by 40.9%. This inhibition occurred at a concentration of 10 µg/mL of the extracts. At the similar concentration, BHT exhibited 68.3% inhibition, while trolox exhibited 29.9% inhibition. Hawthorn (*Crataegus monogyna*), which was found to have a higher total phenolic content than trolox, exhibited significant DPPH scavenging and metal reduction activities. During the investigation of the antioxidant activity of the ethanol extract obtained from *Crataegus monogyna*, α-tocopherol, its water-soluble analogue trolox and BHT (butylated hydroxytoluene) were utilized as reference antioxidant compounds.

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## INTRODUCTION

According to recent studies on degenerative diseases (i.e. aging, cancer, cataracts and cardiovascular diseases) it has been revealed that free radicals play a key role in the body (Atoui et al., 2005). It is widely accepted that the research on free radicals began with Gomberg's proof of the existence of the triphenylmethyl radical (Ph3C•) in 1900 (Gomberg, 1900).

Free radicals, contain one or more unpaired electrons in an orbital. Despite variations in their activities, radicals are generally less stable than non-radical species. Almost every radical species can interact with another radical or molecule through different mechanisms. The selectivity of these interactions depends on the concentration of radicals, the localization of unpaired electrons in the radical, and the presence of weak bonds in the molecules that radicals interact with (Perkins et al., 1996; Demir et al., 2016).

Oxygen and its derivatives are highly reactive molecules that can cause functional impairment or destruction of proteins, lipids, and nucleic acids. This can lead to cell death, tissue damage, and various diseases (i.e. neural disorders, inflammations, cancer, cardiovascular diseases, atherosclerosis and skin irritations). Due to these effects, they are referred to as reactive oxygen species (ROS) (Bar-Or, 2015; Aggul et al., 2020). In addition to ROS, certain reactive nitrogen species (RNS) are also formed in the body, and both RO and RN species are significant contributors to oxidative stress. Reactive species can occur due to both internal and external sources (i.e. air pollution, smoke, radiation, free transition metal ions and pesticides) (Gulcin et al., 2003; Koksal, 2007).

The reaction between reactive oxygen species and biomolecules occurs in a radical chain reaction manner, and oxidative damage also follows a chain mechanism. This chain reaction generates new reactive species that can further damage other biomolecules. This process becomes more prominent in the organism as time progresses (Szweda, 2002; Grune et al., 2004).

In response to the detrimental effects of reactive species mentioned above, organisms have developed systems to prevent or tolerate these effects. The most significant barrier against oxidative damage is the stark difference between the oxygen concentration in the atmosphere (150 mmHg) and the oxygen concentration in tissues (30 mmHg) (Mavi, 2005).

Living organisms need to protect themselves against free radicals and the toxic effects of normal oxygen metabolism. Especially aerobes have various defense systems against reactive oxygen and nitrogen species. These systems are generally referred to as the "Antioxidant System" (Bursal, 2009). Antioxidants serve as radical scavengers in our body by inhibiting lipid peroxidation and other free radical reactions. In this way, they help prevent various diseases caused by radicals (Gencaslan, 2007).

In the organism, RO and RN species are continuously generated, and the unwanted effects of these species are counteracted by the antioxidant system. This situation maintains a balance in a continuous manner. The disruption of this balance in favor of oxidants is referred to as "Oxidative Stress" (Köksal, 2007; Aggul et al.,2022). Antioxidants may put a limit on the effects of ROS by giving a hydrogen atom or forming a metal chelate complex. (Prakash et al., 2007; Kavaz et al., 2021). Antioxidants require the presence of other acceptors alongside them to be able to donate the electrons they receive. Therefore, naturally occurring antioxidants that are similar to each other and coexist possess greater value compared to synthetic uniformly antioxidants (Calıskan, 2006).

Synthetic antioxidants (i.e. BHT and butylated hydroxyanisole (BHA)) are utilized as food additives to prevent lipid oxidation. However, due to their toxic and potentially carcinogenic effects, the usage of BHA and BHT is restricted by legal regulations. Therefore, consumer trends indicate an increasing demand for natural and trustworthy antioxidants (Koksal et al., 2011; Aras et al., 2018). This

highlights the importance of researching natural sources of antioxidants for human health (Stoilova et al., 2007; Aras et al., 2016). According to Baytop, Hawthorn leaves are the leaf parts of the tree, which can reach up to 10 meters in height and belong to the Hawthorn species (*Crataegus monogyna*). The tree is thorny and bears white or pink flowers. There are around 20 *Crataegus monogyna* species in Turkey. The fruit is 6-10 mm in diameter, 1-3-seeded, and has a brownish-red or red color. It is a common plant in western and southern Anatolia (Baytop, 1999).

The leaf and flower of Hawthorn (*Crataegus monogyna*) were recognized as a modern herbal medicine and published in the official German gazette on July 19, 1994 (Heftnummer:133, ATC-code:c01EF). They are also available as ready-made medications (Craegium, Crataegutt, Crataegysat, Faros, Orthangin) in Europe. Additionally, Hawthorn leaf and flower are included in the monographs of ESCOP (European Scientific Cooperative on Phytotherapy) and WHO (World Health Organization). There are numerous studies in the literature related to Hawthorn (*Crataegus monogyna*). Among these, the antioxidant effects in in vivo and in vitro settings and the preventive effect against selenite-induced cataracts are prominent research areas (Wang et al., 2011a).

In another experimental study conducted by Wang et al., the antioxidant capacity of Hawthorn (*Crataegus monogyna*) fruit was determined (Wang et al., 2011b). Furthermore, the microwave-assisted extraction of Hawthorn (*Crataegus monogyna*) fruit was investigated for its antioxidant activity and surface methodology of polyphenols (Liu et al., 2010). Park et al. investigated the changes in phenolic ingredient and antioxidant activity of Hawthorn (*Crataegus monogyna*) fruit throughout different stages of ripening (Park et al., 2010). Dalli et al., carried out a study on the inhibitory effect of extracts of Hawthorn (*Crataegus monogyna*) on neutrophil functions in humans. (Dalli et al., 2008). In a study conducted by Kilic and Sengun in 2023, the bioactive properties of Kombucha beverages produced from Hawthorn (*Crataegus monogyna*) leaves were determined (Kilic & Sengun, 2023).

Here, it was aimed to compare the antioxidant features of extracts obtained from Hawthorn (*Crataegus monogyna*) leaves with standard antioxidants like trolox, α-tocopherol, and BHT.

## MATERIALS AND METHODS

### Chemicals

Neocuprine (2,9-dimethyl-1,10-phenanthroline), 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozin), linoleic acid, α-tocopherol, polyoxyethylene sorbitan monolaurate (Tween-20), and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich GmbH, Sternheim, Germany. Potassium ferricyanide ($K_3Fe(CN)_6$), potassium dihydrogen phosphate ($KH_2PO_4$), gallic acid, FCR, quercetin, aluminum nitrate ($Al(NO)_3$), and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich GmbH, Sternheim, Germany. Ammonium thiocyanate was purchased from Merck.

### Collection of Hawthorn (*Crataegus monogyna*) Leaves and Preparation of Sample Extracts

Plant samples were gathered in Bağıştaş, İliç, Erzincan. The identities of plants were confirmed by Assoc. Prof. Dr. Mustafa Korkmaz from Erzincan University. The plant leaves were subsequently divided into small pieces and dried (at room temperature). After this process, the dried samples were kept in the dark until they were used in the experiments For ethanol extraction, 20 g of powdered hawthorn (*Crataegus monogyna*) leaves were blended and pulverized. The sample was then mixed with twenty times its volume of ethanol (400 ml) in a closed-neck flask and stirred on a magnetic stirrer for 24 h. The obtained ethanol extract was filtered through filter paper. The filtered extracts were combined and the ethanol was evaporated at 40°C using an evaporator (Heidolph 94200, Bioblock Scientific, Germany). The sample was put in to fridge (-20°C).

**Determination of Total Antioxidant Activity**

The total antioxidant activity was made decision by utilizing the ferric thiocyanate method (Mitsuda et al., 1966). Firstly, stock solutions were prepared. To this end, 20 mg of ethanol extracts were thawed in 20 ml of ethanol. The stock solutions were pipetted into weighing boats using automatic pipettes to achieve the desired concentration, and the volume was arranged to 2.5 ml with a buffer solution.

Subsequently, linoleic acid emulsion (2.5 ml) was added to each weighing dish (2.5 ml of linoleic acid emulsion and 2.5 ml of buffer solution were utilized as a control). The incubation temperature was set at 37°C. A 100 µl sample were received from each weighing dish and transferred to separate test tubes including amount of 4.7 ml ethanol (this was done every 6 hours). Then, $Fe^{2+}$ solution (100 µl) and $SCN^-$(100 µl) solution were added. A blind sample was made ready by adding $Fe^{2+}$ (100 µl) and $SCN^-$ (100 µl) solutions to a tube including 4.8 ml of ethanol. The samples were measured at 500 nm absorbance value.

**Ferric Reducing Antioxidant Power (FRAP)**

The determination of total reducing power was performed utilizing the Oyaizu method (Yen & Chen 1995). Freshly prepared stock solutions were taken from each at concentrations of 10, 20, and 30 µg/ml, and taken into tubes. The volume was adjusted to 1 ml with pure water. Subsequently, phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%) were transferred to each tube. The mixture was then waited (50°C, 20 min). Subsequently these steps, 2.5 ml of TCA (trichloroacetic acid, 10%) was added to the reaction mixture. Afterwards, 2.5 ml of the solution was taken from the upper phase, and 2.5 ml of pure water and $FeCl_3$ (0.5 ml, 0.1%) were added. The absorbance value was measured at 700 nm wavelength. Pure water was utilized as the blank sample. Water was utilized as a control instead of the sample.

**Cupric Ions Reducing Assay (CUPRAC)**

The copper ion ($Cu^{2+}$) reduction capacities of the ethanol extract from hawthorn (*Crataegus monogyna*) leaves were made decision by using a slight modification of the CUPRAC method, as employed by Apak et al. (Apak et al., 2006). First of all, $CuCl_2$ (0.25 ml, 0.01 M) solution was taken into the tubes. Then, ethanolic solution (0.25 ml, $7.5x10^{-3}$ M) of neocuprine and ammonium acetate buffer (0.25 ml,1 M) were added. After mixing the solution, the ethanol extract of hawthorn (*Crataegus monogyna*) leaves and standards were added at different concentrations (10-30 µg/ml). Subsequently incubation (30 min.), absorbance measurements were carried out and recorded (at a wavelength of 450 nm). The increasing absorbance of the reaction mixture shows the incremented copper ion ($Cu^{2+}$) reduction capacity.

**DPPH· Scavenging Activity**

DPPH· free radical scavengting test was execute following the method defined by Blois (Blois, 1958). A 2,2-diphenyl-1-picrylhydrazyl (DPPH, 1 mM) was utilized as the free radical. Stock solutions were transferred to the tubes to create solutions at concentrations of 10, 20, and 30 µg/µl, and the total volume was arranged to 3 ml with pured ethanol. Then, the stock DPPH• solution (1 ml) was transferred to each sample tube. After waiting (in the dark and room temperature, 30 min) the absorbances at 517 nm were recorded against the ethanol blank. As controls, 3 ml of ethanol and 1 ml of DPPH• solution were utilized. The decrease in absorbance indicated the remaining amount of DPPH• solution, thus representing the free radical scavenging activity.

**Determination of Total Phenolic Content**

The total phenolic compound ingredient in the extract of hawthorn (*Crataegus monogyna*) leaves was made decision by using the FCR (Folin-Ciocalteu reagent) method (Singleton et al., 1999). A standard curve was first plotted utilizing gallic acid as the standard phenolic compound. For this, gallic acid (25 mg) was dissolved in pure water (25 ml), resulting in a stock solution with a concentration of 1 mg/ml. From this stock solution, different concentrations (100, 200, 400, 500, and 600 µg of gallic acid) were transferred to separate tubes. The volumes of the tubes were adjusted to 23 ml with pure water. To each flask, Folin-Ciocalteu reagent (0.5 ml, FCR) were supplemented, and after waiting for 3 minutes, $Na_2CO_3$ (1.5 ml, 2%) solution was transferred. The mixture was mixed (at room temperature, 2 h), and the absorbance of the samples was recorded at 760 nm against the blank sample, which consisted of distilled water. Distilled water was used as a control in place of the sample. The total phenolic compound content in the ethanol extract of hawthorn (*Crataegus monogyna*) leaves was appraised with the method carried out by Gulcin et al. (Gulcin, 2005).

From each previously prepared stock solution, 1000 µl was taken and transferred to weighing boats, and the sum was arranged to 23 ml with pure water. To the mixture, 0.5 ml of FCR (Folin-Ciocalteu reagent) was added, followed by the addition of 1.5 ml of 2% $Na_2CO_3$ solution after 3 minutes. The samples were mixed at room temperature for two hours. After this process, the absorbance of the samples were measured at 760 nm. These measuring was repeated three times. The amount of gallic acid equivalent (GAE) corresponding to the absorbance values of the samples was determined using the following equation ( The equation was obtained from the standard curve). The results were calculated based on the equation with an equality of 1, and the gallic acid equivalent (GAE) amounts were presented in micrograms (µg) ($r^2$: 0.9884).

$$Absorbance_{(\lambda 760nm)} = 0{,}002 \times [\text{Gallic acid}] + 0{,}0025 \quad (1)$$

**Determination of Total Flavonoid Content**

The total flavonoid ingredient in the extracts of hawthorn (*Crataegus monogyna*) leaves was determined using the method developed by Park et al. Quercetin was utilized as the standard. For this purpose, a standard curve was first plotted. The stock solution was prepared by dissolving the standard in pure water at a concentration of 1 mg/mL (quercetin-25 mg were thawed in pure water-25 ml). From this stock solution, different concentrations (10, 20, 30, 40, and 50 µg of quercetin) were transferred to test tubes. Then, to each tube, sequentially, a solution of $CH_3COO^-$ (1 M, 0.1 ml) prepared in water and $Al(NO_3)_3$ solution (10%, 0.1 ml) were added. The volume of each tube was completed with 4.3 ml of ethanol solution and vortexed. After incubating (at room temperature, 40 min.), the absorbances were recorded at 415 nm against the ethanol blank. The concentration of flavonoids was calculated in quercetin equivalents (QE) using the following equation derived from the standard curve (with quercetin as the standard compound).

$$Absorbance_{(\lambda 415nm)} = 0{,}0147 \times [\text{Quercetin}] \quad (2)$$

To determine the total flavonoid ingredient in the ethanol extracts of hawthorn (*Crataegus monogyna*) leaves, stock solutions utilized for the determination of total phenolic compounds were transferred to test tubes containing 1000 µg of extract in each. Subsequently, the various amounts of extracts added to the tubes were diluted with ethanol solution(4.3 ml) including potassium acetate (0.1 ml, 1 M) prepared in water and aluminum nitrate solution (0.1 ml, 10%). The mixtures were vortexed. After incubating (at room temperature) for 40 minutes, the absorbances were recorded at 415 nm in the same manner.

## RESULTS AND DISCUSSION

### Total Antioxidant Activity

The total antioxidant activity of ethanol extracts obtained from hawthorn (*Crataegus monogyna*) leaves was determined using concentrations of 10 µg/ml, as shown in Figure 1. As depicted in the figure, the total antioxidant activity of the ethanol extract from hawthorn leaves increased proportionally with the increasing concentration.

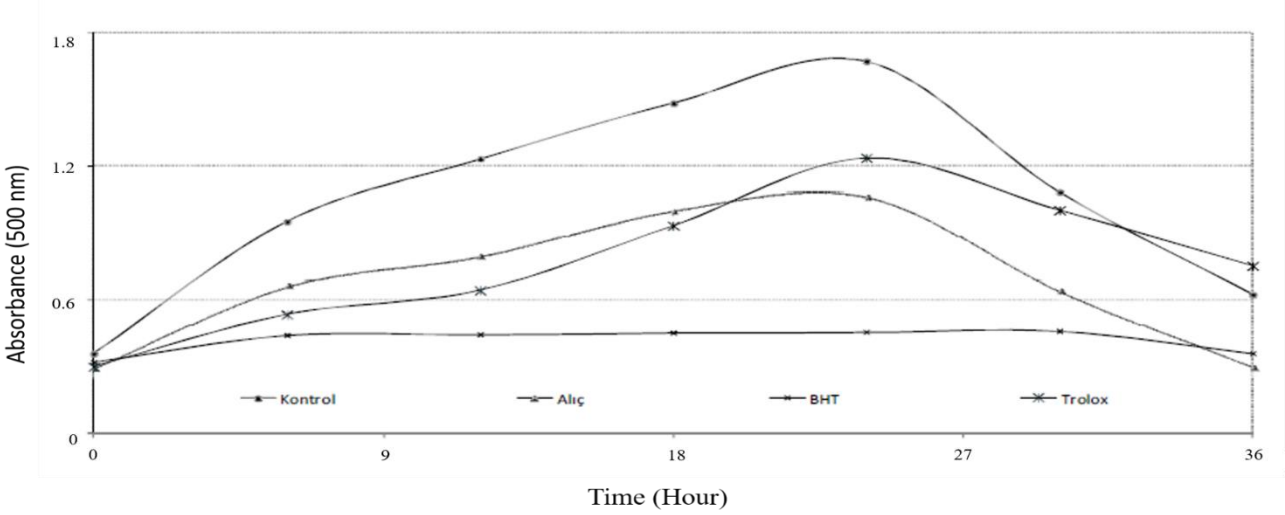


**Figure 1**. Comparison of the total antioxidant activity of the ethanol extract obtained from hawthorn (*Crataegus monogyna*) with two standard antioxidants, BHT and trolox, was performed at a concentration of 10 µg/ml

According to the determined 24-h maximum incubation period, the inhibition percentages of standard antioxidants on linoleic acid emulsion were calculated. The calculations were performed according to the following equation (3).

Inhibition of LP (lipid peroxidation, %) = 100-($A_{sample}/A_{control}$ x 100) (3)

Here, $A_{sample}$ represents the absorbance value of the ethanol extract at the incubation moment when the values at different concentrations reached their maximum. $A_{control}$ represents the absorbance value at the incubation moment when reached the maxımum control value. Trolox and BHT were used as positive controls (Figure 1). When compared, it was observed that the ethanol extract obtained from the leaves of hawthorn (*Crataegus monogyna*) at a concentration of 10 µg/ml inhibited linoleic acid emulsion peroxidation by 40.9%. Also, at the same concentration, trolox and BHT inhibited linoleic acid peroxidation by 68.3% and 29.9%, respectively.

### FRAP Assay

The reduction capacity of the ethanol extract of hawthorn (*Crataegus monogyna*) leaves used in the study also increased proportionally with the increasing extract concentration, similar to the total antioxidant activity. The decrease potential of this extract was determined by measuring the absorbances of its solutions at different concentrations (10-30 µg/ml) at 700 nm (Figure 2). As can be seen from the figure, the reduction capacity was determined to be in the following order: Trolox > BHT > Hawthorn> α-tocopherol.

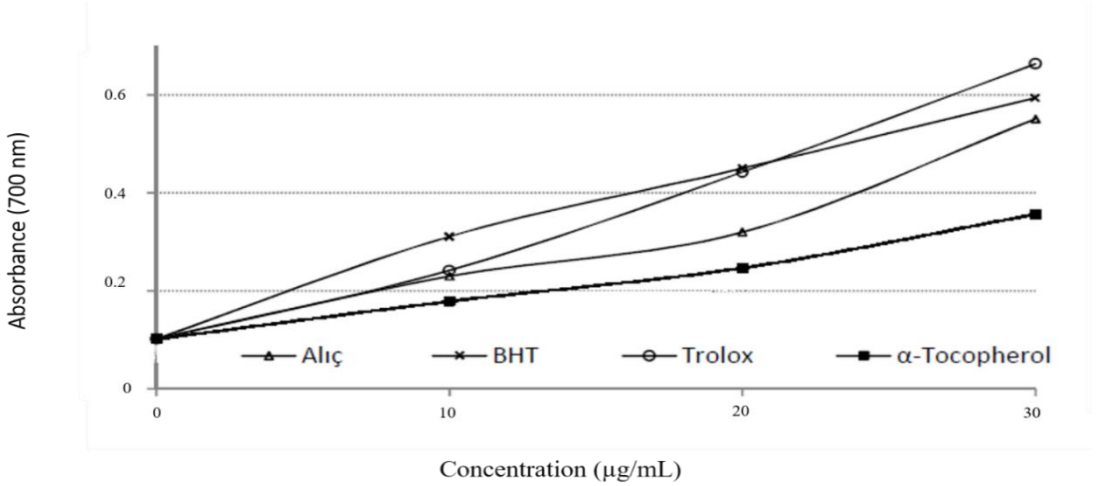


**Figure 2**. Comparison of the reduction capacities of the ethanol extract of hawthorn (*Crataegus monogyna*) leaves at different concentrations (10-30 µg/ml) with the standard antioxidants α-tocopherol, trolox, and BHT

## CUPRAC Assay

The reduction capacity of the ethanol extract of hawthorn (*Crataegus monogyna*) leaves for copper ions ($Cu^{2+}$) as well as its ability to reduce ferric ions ($Fe^{3+}$) to ferrous ions ($Fe^{2+}$) increases proportionally with the concentration of the extract. Moreover, the reduction capacity of hawthorn leaves for copper ions is higher than that of the standard antioxidant, trolox. The reduction capacity of the extract for copper ions ($Cu^{2+}$) was determined by measuring the absorbances of its solutions at different concentrations (10-30 µg/ml) at 450 nm (Figure 3).

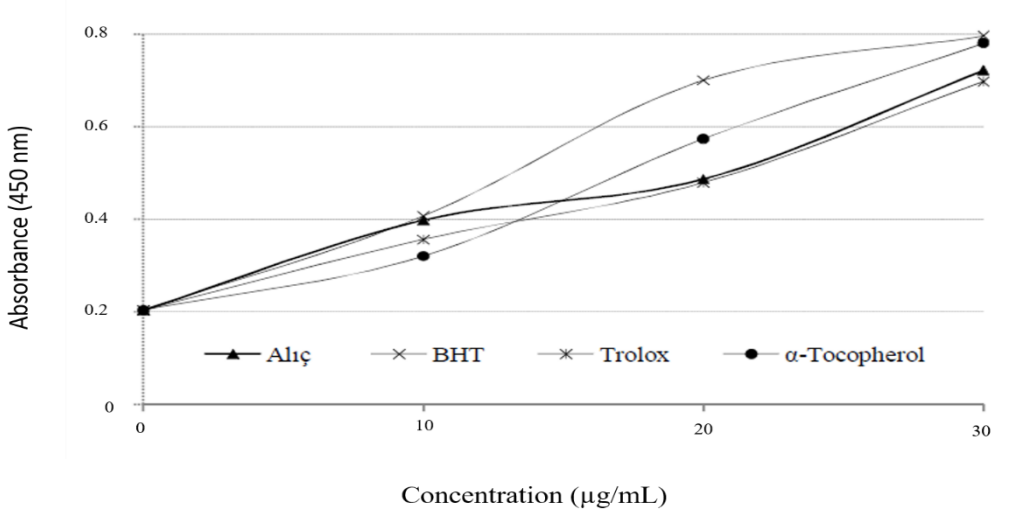


**Figure 3.** Comparison of the reduction capacities of copper ions ($Cu^{2+}$) by the ethanol extract obtained from hawthorn (*Crataegus monogyna*) leaves at different concentrations (10-30 µg/ml) with the standard antioxidants α-tocopherol, trolox, and BHT

## DPPH Assay

Hawthorn (*Crataegus monogyna*) leaves exhibited different effects in scavenging the DPPH free radical. As observed from Figure 4, the scavenging activity of the DPPH· free radical was determined to be in the following order: α-tocopherol > Trolox > Hawthorn > BHT. The ethanol extract of hawthorn leaves showed lower but comparable scavenging activity compared to the standards. Additionally, it was determined that hawthorn leaves had a higher capability to scavenge DPPH free radicals compared to BHT.

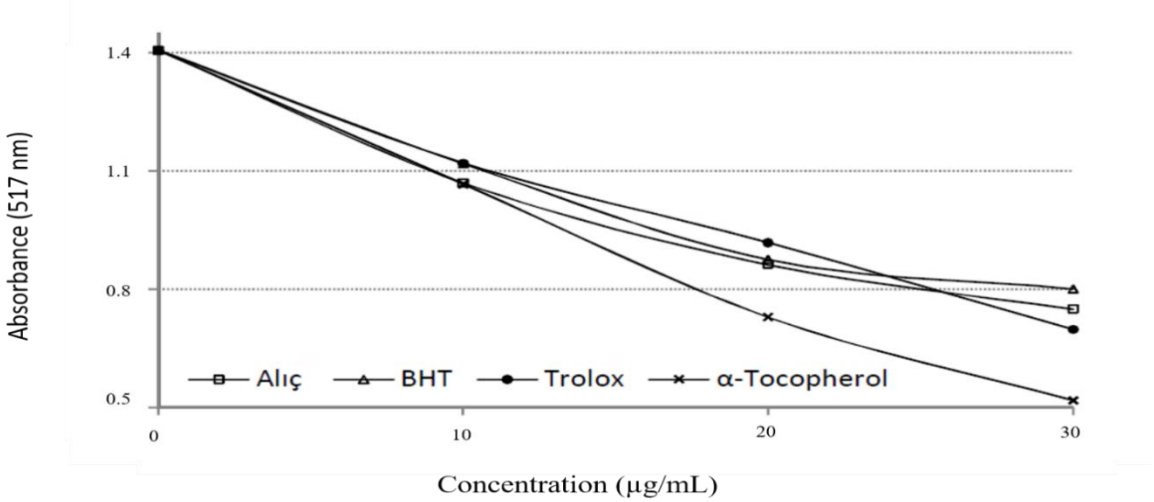


**Figure 4.** Comparison of the scavenging activities of DPPH free radicals by the ethanol extract of hawthorn (*Crataegus monogyna*) leaves at different concentrations (10-30 µg/ml) with the standard antioxidants BHT, α-tocopherol, and trolox

## Total Phenolic and Flavonoid Content

According to the standard curve of gallic acid, the ethanol extract obtained from hawthorn (*Crataegus monogyna*) leaves contained 161 µg of phenolic compounds per 1 mg It was found that the total flavonoid compound content in 1 mg of ethanol extract was 55.6 µg quercetin equivalent (QE) (The standard curve created with quercetin was used for the calculation) (Table 1).

**Table 1.** Equivalent amount of total phenolic and flavonoid compounds in 1 mg of ethanol extract obtained from hawthorn (*Crataegus monogyna*) leaves

| Radicals | Total phenolic compound (µg GAE /mg extract) | Total flavonoid compound (µg QE /mg extract) |
|---|---|---|
| Hawthorn (*Crataegus monogyna*) | 161 | 55.6 |

## CONCLUSION

The antioxidant properties of Hawthorn (*Crataegus monogyna*) leaf extracts were determined using various in vitro tests. These tests included total antioxidant activity using the ferric thiocyanate method, scavenging of DPPH· free radicals, CUPRAC test, FRAP test, determination of total flavonoid content, and determination of total phenolic compound content.

An increase in total antioxidant activity, reduction capacity, and DPPH· radical scavenging activity was observed in the ethanol extract of hawthorn (*Crataegus monogyna*) leaves at the tested concentrations (10-30 µg/ml), indicating a positive correlation with increasing concentration.

In the comparison of antioxidant and antiradical studies, standard substances such as BHT, α-tocopherol, and trolox were used. It was observed that in some antioxidant analyses, the activity of the ethanol extract of hawthorn (*Crataegus monogyna*) leaves was higher than that of the standards at the same concentrations. There appears to be a correlation between the metal-reducing and free radical scavenging capacities of hawthorn ethanol extracts and their phenolic and flavonoid contents. While the ethanol extracts of hawthorn (*Crataegus monogyna*) leaf parts exhibited high activity in metal reduction and DPPH· free radical scavenging measurements, the situation was reversed in total antioxidant activity assays. This can be easily explained by the fact that the ethanol extracts of hawthorn leaf parts contain higher amounts of phenolic and flavonoid compounds compared to the standards, indicating their superior reducing and free radical scavenging abilities.

In conclusion, hawthorn (*Crataegus monogyna*) leaves can be considered as a natural source of antioxidants that can be utilized in both pharmaceutical and dietary products.

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### Conflict of Interest

The article authors declare that there is no conflict of interest between them.

### Author's Contributions

The authors declare that they have contributed equally to the article.

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