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# In silico Prediction and In vitro Antioxidant Activities of Two Jujube Fruits from Different Regions

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**Keywords**
Antioxidant activity, DPPH, FRAP, CUPRAC, PASSonline, *Ziziphus jujuba*.

**Abstract:** Jujube is a fruit rich in antioxidant compounds and vitamin C. In this way, it can prevent cell damage by fighting free radicals. In the study, antioxidant activities, total phenolic and total flavonoid compound amounts of methanolic extracts of jujube fruits were determined. In addition, their biochemical compositions were determined using HPLC. Also, an in silico prediction study of the identified active ingredients was performed to evaluate antioxidant, antiradical, antibacterial, antifungal, anti-inflammatory, antimutagenic, and membrane integrity antagonist properties. For the determination of antioxidant capacity, ferric reducing power (FRAP) and cupric ion reducing capacity (CUPRAC), DPPH radical scavenging activity and β-carotene-linoleic acid assay were used. The amounts of total phenolic and the total flavonoid compounds of the extracts were determined as gallic acid equivalent (GAE) and as quercetin equivalent (QE). The methanolic extract of jujube collected from Antalya contains 580 µg GAE.mg$^{-1}$ and 240 µg QE.mg$^{-1}$, and the methanolic extract of jujube collected from Denizli contains 900 µg GA.mg$^{-1}$ and 380 µg QE.mg$^{-1}$. The $IC_{50}$ values of Antalya and Denizli methanolic extracts according to the DPPH scavenging assay were 10.34 and 9.82, respectively. Gallic acid, catechin, caffeic acid, coumaric acid, ferulic acid and cinnamic acid were detected by HPLC. In addition, the in silico effects of these molecules were estimated with the PASS online prediction program. As a result, it was seen that Denizli jujube had a higher antioxidant effect than Antalya jujube.



# Farklı Bölgelerden İki Hünnap Meyvesinin In silico Tahmin ve In vitro Antioksidan Aktiviteleri

**Anahtar Kelimeler**
Antioksidan aktivite, DPPH, FRAP, CUPRAC, PASSonline, *Ziziphus jujuba*.

**Öz:** Hünnap, antioksidan bileşikler ve C vitamini açısından zengin bir meyvedir. Bu sayede serbest radikallerle savaşarak hücre hasarını önleyebilir. Çalışmada hünnap meyvelerinin metanolik ekstraktlarının antioksidan aktiviteleri, toplam fenolik ve toplam flavonoid bileşik miktarları belirlendi. HPLC kullanılarak biyokimyasal bileşimleri belirlendi. Ayrıca, antioksidan, antiradikal, antibakteriyel, antifungal, antienflamatuar, antimutajenik ve membran bütünlüğü antagonist özelliklerini değerlendirmek için tanımlanan aktif bileşenlerin in silico tahmin çalışması yapıldı. Antioksidan kapasitenin belirlenmesi için ferrik indirgeme gücü (FRAP) ve bakır iyonu indirgeme kapasitesi (CUPRAC), DPPH radikal süpürme aktivitesi ve β-karoten-linoleik asit testi kullanıldı. Ekstraktların toplam fenolik ve toplam flavonoid bileşik miktarları gallik asit eşdeğeri (GAE) ve kersetin eşdeğeri (QE) olarak belirlendi. Antalya'dan toplanan hünnapın metanolik ekstraktı 580 µg GAE.mg$^{-1}$ ve 240 µg QE.mg$^{-1}$, Denizli'den toplanan hünnapın metanolik ekstraktı ise 900 µg GA.mg$^{-1}$ ve 380 µg QE.mg$^{-1}$ içerir. DPPH süpürme tahliline göre Antalya ve Denizli metanolik ekstraktlarının $IC_{50}$ değerleri sırasıyla 10.34 ve 9.82'dir. HPLC ile gallik asit, kateşin, kafeik asit, kumarik asit, ferulik asit ve sinnamik asit tespit edildi. Ayrıca bu moleküllerin in silico etkileri PASS online tahmin programı ile tahmin edilmiştir. Sonuç olarak Denizli hünnabının Antalya hünnabına göre daha yüksek antioksidan etkiye sahip olduğu görüldü.

## 1. INTRODUCTION

Free radicals are one of the most emphasized issues in recent years. Cellular sources of free radicals, the reactions they take place in, and the resolution of antioxidant mechanisms that prevent free radical damage are extremely important for many diseases. A free radical is an atom or molecule with an odd number of electrons in its outer orbit [1]. They exist as both organic and inorganic molecules. Free radicals cause the atom they affect, and accordingly, the substance in which that atom is located to not be able to function. Depending on the biological significance of the affected substance and whether it can be repaired, temporary or permanent effects are observed [2, 3].

The importance given to antioxidants is increasing day by day due to their positive contributions to health. Antioxidants, which are one of the most popular supplements of the last period, have become used by everyone thanks to their effects that prolong life, reduce the risk of diseases such as cancer and heart diseases, and delay aging. Oxygen, which is indispensable for our life, can harm our body in some cases. The reason for this possible damage of oxygen is the formation of reactive oxygen species as a result of metabolic reactions in our body using oxygen. These molecules, known as free radicals, can damage lipid, protein, DNA and similar cell components. Then, problems such as premature aging, cancer, cardiovascular diseases may be encountered [4]. Antioxidant defense systems developed in aerobic respiration organisms control the formation of free radicals that contain an unpaired electron in their structure and prevent the harmful effects of these molecules. Thus, the cell is not damaged and is protected from diseases. However, there may be cases where the existing antioxidant defense system can not completely prevent the effects of free radicals. That's when the condition called oxidative stress occurs due to the increase of free radicals. Antioxidants are widely used to treat brain damage caused by the brain's vulnerability to oxidative stress. It is known that antioxidant compounds that can be used in treatments prevent cell death and neurological damage by preventing oxidative stress in nerve cells [5].

Jujube (Ziziphus jujuba Mill.) is a fragrant, 4-5 m high thorny tree, red bark, hard core, large olive-shaped plant that blooms yellow flowers between April and May. The outermost wall is leathery and thin, the pulp is yellow, sweet and delicious. The trunks of the tree are cylindrical, with brown bark and many branches. The leaves are in two opposite rows, with a short stem, and two small leaflets in the form of thorns. The sepals are five-part and green in color. The petals are yellow, curved and have five parts. It is thought to have spread from North Africa and Syria to India and China. Although the tree is adaptable to many climates, it needs hot summers to bear fruit well. It is located in Marmara, Western and Southern Anatolia in Turkey. In addition, different species are seen in the Eastern Black Sea Region and especially in the Coruh Valley Basin. The plant has been used as an emollient, expectorant and diuretic since ancient times. It is very useful in diseases such as cardiovascular stiffness and shortness of breath. It is also used as a cholesterol and lipid lowering agent [6, 7].

In the study, ferric ion reducing power (FRAP), cupric ion reducing capacity (CUPRAC), DPPH radical scavenging activity and β-carotene-linoleic acid emulsion methods were used to determine the antioxidant activity of jujube plant. Total phenolic and flavonoid substance amounts of the extracts were determined as equivalent to gallic acid and quercetin, respectively. In addition, the phenolic compounds of the methanolic extract of the fruit were determined by HPLC. Also, in silico some biologic avtivity prediction of the determined active substances was applied with the PASS online program.

The aim of this study is to evaluate the phytochemical structure and antioxidant activity of the methanolic extract of jujube fruit in order to determine the beneficial effects and the components responsible for these effects. In addition, it is to perform in silico study of the identified active ingredients to understand their antioxidant, antiradical, antibacterial, antifungal, anti-inflammatory, antimutagenic, and membrane integrity antagonist properties.

## 2. MATERIAL AND METHOD

### 2.1. Materials



#### 2.1.1. Chemicals

The Folin reagent and 1,1-diphenyl-2-picryl-hydrazil (DPPH•) used in the study were from Sigma-Aldrich and $Na_2CO_3$, methanol, gallic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), chloroform ($CHCl_3$), β-carotene, lineolic acid, Tween 20, $FeCl_3$, potassium ferrocyanate, trichloroacetic acid (TCA), $CuCl_2.2H_2O$, ammonium acetate and trolox were obtained from Merck.

#### 2.1.2. Fruits

The two jujube fruits used in the study were collected from two different regions of Turkey. One was collected from Alanya, Antalya, located in the Mediterranean region, from 36°30'07" north latitude and 31°38'40" east longitude coordinates (ZJA). The other was collected from Çivril, Denizli, which is located in the Aegean region and from 38° 18′ 5″ North latitude and 29° 44′ 19″ east longitude (ZJD). The fruits were identified as Ziziphus jujuba Mill based on the book "Flora of Turkey and the East Aegean Islands" by PH Davis.

#### 2.1.3. Devices

Devices used in the analysis: Shimadzu UV 1700 spectrophotometer for absorption measurements, Inolap brand pH meter for pH measurements, Precisa XB 220A precision balance for weighing, Nüve brand incubator for heating and drying processes, Heidholph brand

evaporator and Lancome brand lyophilizer to remove the solvent after extraction.

### 2.2. Methods

#### 2.2.1. Jujube fruit extraction

Jujube fruits were collected, cut into small pieces and dried. Then approximately 50 g of each was taken and placed in the soxlet cartridge. In the literature review, it was decided to use 100% methanol. It was extracted in methanol solvent at 30ºC for 6 hours. In order to remove the solvent of the obtained extract, it was subjected to 40ºC under vacuum in the evaporator. After evaporation, it was taken into vials and stored at 4ºC for analysis [8].

#### 2.2.2. Determination of total phenolic and total flavonoid contents

The amount of phenolic compounds in the methanolic extract prepared from the jujube fruits used in the study was determined by the Folin Ciocalteu method [9]. Gallic acid was used as the equivalent standard phenolic compound. A calibration graph of gallic acid was drawn. Using the equation obtained from this graph, the total phenolic compound amounts of the jujube samples were determined. In the study, absorbances were read at 760 nm. Results were calculated in µg in gallic acid equivalent (GAE).

The amount of flavonoid compounds in the methanolic extract prepared from jujube fruits was determined by the aluminum nitrate method [10]. Quercetin was used as the standard flavonoid compound. A calibration graph of Quercetin was drawn. The total flavonoid content of the jujube samples was calculated using this calibration graph. Absorbances were read at 415 nm. Results were calculated in µg in quercetin equivalent (QE).

2.2.3. DPPH free radical scavenging activity

DPPH free radical scavenging activities of extracts prepared from jujube fruits used in the study were carried out according to the method developed by Blois [11]. DPPH was used as a free radical. Absorbance measurements were read at 517 nm. Ethanol was used as a control.

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample})/A_{control}]x100$$

The plant extract concentration ($IC_{50}$) values at the time of scavenging half of the DPPH free radical were calculated for each sample. It was compared with the synthetic antioxidants BHT and BHA.

#### 2.2.4. β-Carotene/linoleic acid bleaching method

Lipid peroxidation was carried out according to the method developed by Miller [12]. A solution of β-carotene in chloroform was used. Synthetic antioxidants BHA and BHT were used as standard. Absorbances were read at 470 nm. One measurement was read at the first time and the other measurements were read every 15 minutes for 120 minutes. Based on this absorbance, the rate of change in absorbance and, accordingly, the % oxidation inhibition coefficients were calculated.

$$R = \ln(a/b)$$

Here; In is the natural logarithm, a is the initial absorbance, b is the absorbance after 120 minutes of incubation.

$$\text{Inhibition (\%)} = [(R_{control} - R_{sample})/R_{control}]x100$$

#### 2.2.5. Ferric ion reducing antioxidant power (FRAP)

Ferric ion reducing antioxidant power of extracts prepared from jujube fruits used in the study was applied according to Benzie and Strain [13]. The FRAP reagent was used and freshly prepared before use. Absorbances were read at 593 nm. Ethanol was used as a control. BHA and BHT were used as positive standards.

#### 2.2.6. Cupric ion reducing antioxidant capacity (CUPRAC)

The copper II ion-reducing antioxidant capacities of the extracts prepared from the jujube fruits used in the study were determined according to the method developed by Apak et al. [14]. Absorbances were read at 450 nm. Ethanol was used as a control. BHA and BHT were used as positive standards.

#### 2.2.7. HPLC analysis

For HPLC analysis of phenolic compounds in fruit extracts, an analysis method was developed by drawing the calibration graph of 15 different phenolic compound standards separately [15]. After 20 mg of the samples were weighed and dissolved in 1 mL of methanol, 20 µL of the solutions were injected into the HPLC. First of all, standard phenolic substances were injected. Results are given as µg.g-1 with 95% confidence interval.

#### 2.2.8. In silico toxic risks prediction by PASSonline

A computer-based program PASS (Prediction of Activity Spectra for Substances) was used to screen the antimicrobial and antioxidant potential of the phenolic compounds that the fruit's methanolic extract contains at the highest rate. The software is used to predict the biological activities of chemical structures, including phytochemicals, based on the structure-activity relationship with a known chemical entity. Besides the desired pharmacological effect, it predicts molecular mechanisms of action and undesirable side effects such as mutagenicity, carcinogenicity, teratogenicity and embryotoxicity [16]. It compares the structure by utilizing a library of molecules containing more than 205,000 compounds exhibiting more than 3750 biological activities. Activity is estimated in terms of Pa (probable activity) and Pi (probable inactivity). Structures where Pa is greater than Pi denote compounds considered for a particular pharmacological activity [17].

### 2.2.9. Statistical analysis

Statistical differences between groups were determined by Tukey post-hoc test and one-way ANOVA. Data were expressed as a mean of 7 standard deviations for three independent determinations. In ANOVA tests, $p<0.05$ values were accepted as the limit of significance. Analyzes were performed using GraphPad Prism 9.0 for Windows (GraphPad Software, San Diego, CA, USA).

## 3. RESULTS

In recent years, many studies have been carried out on the therapeutic properties of plants. This therapeutic feature of plants is due to the fact that they contain antioxidant substances. Antioxidants provide the removal of radicals formed in normal ways in human metabolism. The most important antioxidants found in plants are flavonoid, carotenoid and phenolic compounds. Various spectroscopic and chromatographic methods have been developed for the identification of these compounds, and many antioxidant substances such as catechin, gallic acid, rutin, quercetin can be determined in plant and food samples. However, these methods are both expensive and it can not be fully commented on whether they can give antioxidant properties after the molecules are defined. For antioxidant determination, total antioxidant activity determination, total reducing capacity, DPPH scavenging, β-carotene and CUPRAC method, total flavonoid substance determination and similar methods are frequently used [18].

The increasing availability of life-saving drugs such as antibiotics has led to advances in the world of advanced medicine. However, the widespread use of these synthetic drugs has led to life-threatening side effects and the development of resistant strains of deadly pathogenic microorganisms [19, 20]. The development of new antimicrobial drugs that overcome these problems is therefore a major imperative for the pharmaceutical industries. Plant-derived antimicrobials and antioxidants have a long history of providing much needed safe and new therapeutics [21–23]. Plants constantly interact with rapidly changing and potentially harmful external environmental factors such as microbial attack and oxidative stress. This interaction involves alternative defense strategies, including the synthesis of a wide variety of chemical metabolites that counteract these stress factors. According to the World Health Organization, plants are the best source of various biologically active drugs [20]. To date, only 10-15% of plant species have been studied for their therapeutic potential for various ailments [17].

### 3.1. Total Phenolic and Total Flavonoid Substance Results

The most important antioxidant compounds are phenolic compounds. Phenols can donate electrons and hydrogens due to the functional groups they carry in terms of their structure. These groups eliminate radicals and oxidizing groups. Phenolic groups are rich in OH groups. These groups add polarity to them and increase their antioxidant properties [24].

**Table 1.** Total phenolic and flavonoid contents of *Ziziphus spp.* and statistic analysis[a]

| Samples | Total Phenolic Contents (µg GA.mg$^{-1}$ extract)[b] | Total Flavonoid Contents (µg QE.mg$^{-1}$ extract)[c] |
|---|---|---|
| ZJA[d] | 580 ± 0,014 | 240 ± 0,27 |
| ZJD[e] | 900 ± 0,068 | 380 ± 0,55 |

[a]Statistical analyzes were performed using One Way Anova test and Tukey test in GraphPad Prism 9.0 program. According to the results, significant differences were found between four groups ($p<0.05$, $F=58.17$). [b]GA, gallic acid equivalents. [c]QE, quercetin equivalents. [d]ZJA: *Z. jujuba* extract collected from Antalya. [e]ZJD: *Z. jujuba* extract collected from Denizli.

In this study, the total phenolic substance determination was made according to the Folin-Ciocaltaeu method. The total amount of flavonoid substances was determined by a method based on aluminum chelation. For the calibration curves of gallic acid and quercetin, which are used as standards, solutions of these substances in methanol at five different concentrations were prepared.

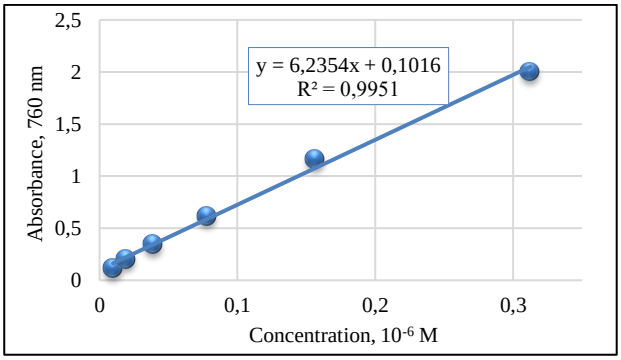


**Figure 1.** Calibration curve of gallic acid

The concentrations of total phenolic compounds in *Z. jujuba* extracts were calculated as gallic acid equivalent from the graphic equation obtained from the calibration curves of gallic acid methanol solutions given in Figure 1. The resulting graphic equation was found to be y = 6,235x + 0,101. According to the results given in Table 1, it was observed that the methanolic extract of ZJD contained a higher amount of phenolic substances than the methanolic extract collected from ZJA.

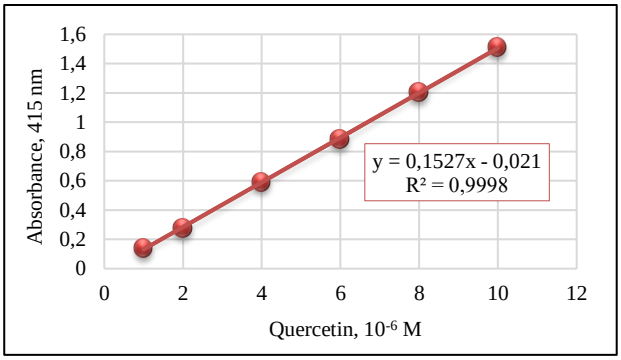


**Figure 2.** Calibration curve of quercetin

The concentrations of total flavonoid compounds in Z. jujuba extracts were calculated as equivalent to the total amount of flavonoid substance quercetin from the graphic equation obtained from the calibration curves of quercetin methanol solutions given in Figure 2. The

obtained graphic equation was found as y = 0,152x – 0,021. According to the results given in Table 2, it was observed that the methanolic extract of ZJD contained a higher amount of flavonoid substances than the methanolic extract collected from ZJA. As a result of the examinations, it has been determined that the total phenolic and total flavonoid substance amounts of the ZJD are higher than the ZJA.

### 3.2. DPPH Free Radical Scavenging Results

The radical scavenging activities of antioxidants are very important for the biological system and the food industry. Excessive formation of free radicals accelerates lipid peroxidation. This is an undesirable situation in foods and causes some diseases in humans. These diseases include premature aging, cancer, and forgetfulness [18].

In this method, DPPH• radical scavenging activity assay of methanol extract of *Z. jujuba* and standard antioxidant compounds which is BHA and BHT were determined. Calibration curve of DPPH in methanol was drawn. From the graph equation obtained (y = 0.013x + 0.006), $IC_{50}$ values of extracts and standarts were calculated.

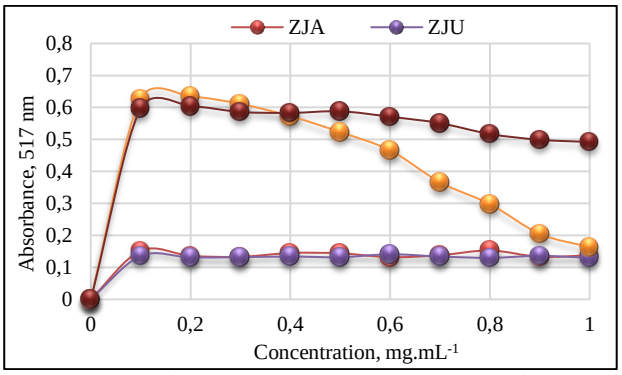


**Figure 3.** DPPH free radical scvenging activity graph of *Z. jujuba* methanolic extract collected from Antalya (ZJA), *Z. jujuba* methanolic extract collected from Denizli (ZJD), standart BHA and standart BHT.

The DPPH radical scavenging activity was determined for different concentrations of Z. jujuba methanolic extract, BHA and BHT. For this purpose, inhibition values at each concentration were calculated from the absorption values obtained and plotted against the concentration (Figure 3). As seen in Figure 3, there is no significant increase or decrease in the DPPH radical scavenging activities of methanolic extracs of ZJA and ZJD. In addition, it was observed that there was not a very large concentration difference between the two extracts.

### 3.3. β - Carotene Lineolic Acid Emulsion Assay Results

When free radicals attack the fat molecules in the cell membrane, the fat molecule undergoes a change. When fats are changed in the body, the structure and functions of the cell membrane are damaged. Therefore, the cell membrane cannot transfer nutrients, oxygen and water for a long time. It also cannot regulate the disposal of spent products. Continuing the attacks of free radicals for a long time causes the breakdown of the oils in the structure of the cell membrane, the rupture of the plant membrane and the disintegration of the cell components [25].

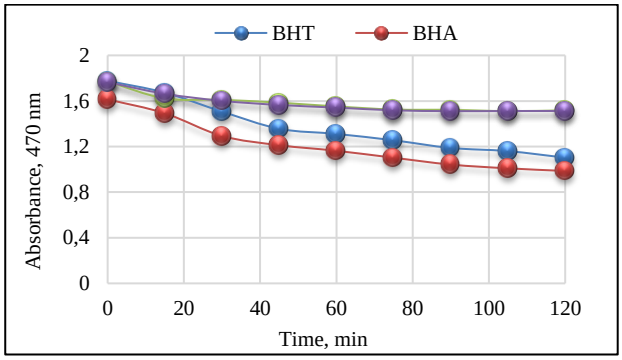


**Figure 4.** β-Carotene linoleic acid emulsion assay graph of *Z. jujuba* methanolic extract collected from Antalya (ZJA), *Z. jujuba* methanolic extract collected from Denizli (ZJD), standart BHA and standart BHT.

β-Carotene-lineolic acid emulsion method is based on the disappearance of the yellow color over time, which is formed by the reaction of the radicals formed as a result of the oxidation of lineoic acid in the emulsion with β-carotene. The presence of antioxidants prevents the color from lightening. In the β-carotene lineolic acid system, the fact that the yellow color did not fade immediately during the test period (120 min) indicates the presence of a high potential antioxidant (Figure 4).

In this method, antioxidant activities of ZJA extract and ZJD extract were measured and compared with the antioxidant activities of synthetic antioxidants BHA and BHT. As a result of the analyzes, it was found that the methanolic extract of ZJD showed higher antioxidant activity than the methanolic extract of ZJA according to the β-carotene lineoleic acid emulsion method. In addition, it was observed that two extracts showed higher activity than the standards.

### 3.4. CUPRAC Results

In the method, Cu (II) chloride solution, neocuprine solution and ammonium acetate (pH=7 buffer) solution are mixed. Antioxidant solution to be determined is added to the solution, and after 30 minutes, absorbance values are measured at 450 nm against the reference without antioxidant.

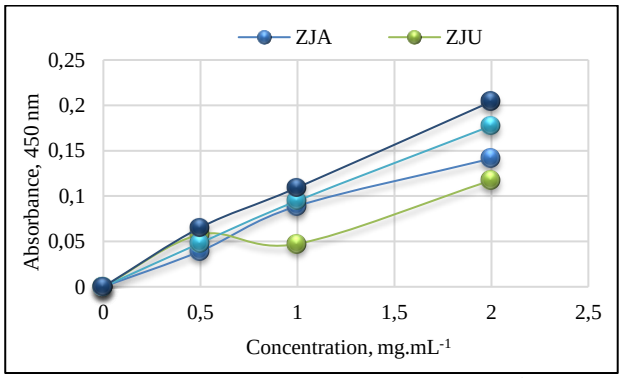


**Figure 5.** Cupric ions reducing capacity assay graph of *Z. jujuba* methanolic extract collected from Antalya (ZJA), *Z. jujuba* methanolic extract collected from Denizli (ZJD), standart BHA and standart BHT.

The cupric ion reduction potential results of Z. jujuba extracts according to the CUPRAC method are given in Figure 5. The results were obtained by measuring the absorbance of methanol solutions of the samples at 450 nm. When Figure 5 is examined, it has been observed that the sample of ZJA reduces more cupric ions to cuprous than the sample of ZJD. It was determined that both extracts had similar reducing power with standard BHA and BHT.

### 3.5. FRAP Results

In the method, the yellow color of the solution turns into green in different shades due to the reduction activities of the antioxidant substances in the solution. In the presence of antioxidants, the ferricyanide ($Fe^{3+}$) complex is reduced to its ferrous form ($Fe^{2+}$). The $K_3Fe(CN)_6$ complex gives a maximum absorbance at 593 nm by forming a complex of $Fe_4[Fe(CN)_6]_3$ with added FeCl3 and Perl's prussian blue (Ak Tuba, 2006).

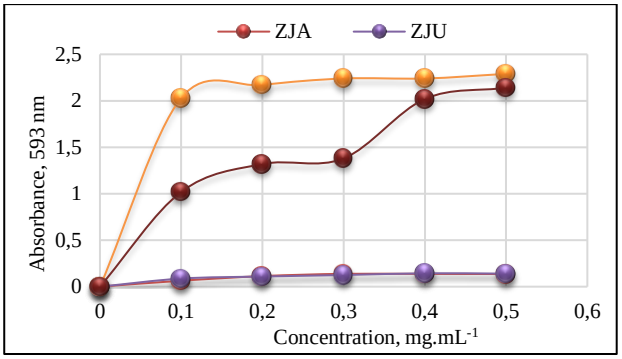


**Figure 6.** Ferric reducing antioxidant potential assay graph of *Z. jujuba* methanolic extract collected from Antalya (ZJA), *Z. jujuba* methanolic extract collected from Denizli (ZJD), standart BHA and standart BHT.

The ferric ion reducing power results of *Z. jujuba* methanol extracts according to the FRAP method are given in Figure 6. As seen in figure, *Z. jujuba* extracts showed a lower antioxidant effect than the standard antioxidants BHT and BHA. It was found that there was not much difference in value between the two extracts at a concentration of 1 $mg.mL^{-1}$, but the methanolic extract of ZJD showed slightly higher antioxidant activity than the methanolic extract of ZJA.

### 3.6. HPLC Results

Some of the phenolic and flavonoid compounds that provide antioxidant properties to *Z. jujuba* extracts were determined qualitatively and quantitatively by high performance liquid chromatography. These analyzed components and the amount of these components in each of the methanolic extract are given in Table 2. The chromatogram for the analysis of all standards is given in Figure 7(A). Also, Figure 7(B) shows the chromatogram of ZJA and Figure 7(C) shows the chromatogram of ZJD. As a result, all antioxidant molecules analyzed in Z. jujuba extracts contributed to their antioxidant capacity. The greatest contribution was made by the high levels of gallic acid, catechin, p-coumaric acid, cinnamic acid and ferulic acid molecules. However, caffeic acid from these molecules was found only in the ZJA extract, while quercetin was not found in any of the extracts. However, it is possible that there are some unanalyzable antioxidant substances in each extract.

**Table 2.** HPLC results of phenolic compounds from two *Z. jujuba* species*

| Samples | ZJA[a] | ZJD[b] |
|---|---|---|
| Gallic acid | 6,39 | 31,20 |
| Catechin | 3,90 | 20,75 |
| Caffeic acid | 2,15 | ND[c] |
| p-Coumaric acid | 1,36 | 1,76 |
| Ferulic acid | 2,56 | 5,01 |
| Cinnamic acid | 0,43 | 0,98 |
| Quercetin | ND[c] | ND[c] |

*Results are given in $\mu g.g^{-1}$ with 95% confidence interval. [a]*Z. jujuba* extract collected from Antalya. [b]ZJD: *Z. jujuba* extract collected from Denizli. [c]ND, Not detected.

### 3.7. In Silico Prediction Analysis Results

The hydroxyl and ketone groups in the structures of the molecules enable them to be interactive. The number of these groups and their bonding angles affect reaction rates and activities. The structural formulas of the active ingredients determined by HPLC in the ziziphus sample collected from both places are given in Figure 8.

Calculations using PASS software are widely used to verify and correlate the biological activities of chemical molecules [26, 27]. The results are confirmed by a structural study of the same molecules using software that works on the principle of structure-based comparison of key molecules of jujube fruit with existing antioxidant, antimicrobial, anti-radical, anti-inflammatory, anti-mutagenic and antifungal compounds. Gallic acid showed the highest Pa value for antibacterial activity, cinnamic acid showed the highest Pa value for anti-inflammatory potential, as catechin showed antioxidant activity, antifungal activity, antimutagenic activity, the highest scavenging effect against free radicals and the highest membrane integration antagonist. Significant inhibition effects of these molecules against oxidation, bacteria, fungi, inflammation and mutation have been detected. Catechin was found to be the molecule with the most extensive action. Catechin can be shown as the general factor of biological activities in experimental studies.

## 4. DISCUSSION AND CONCLUSION

In the study, the total phenolic compound content, total flavonoid compound content, scavenging effect of DPPH• radical, β-carotene lineolic acid emulsion system, copper ion reducing capacity, iron ion reducing power of methanolic extracts of ZJA and ZJD were determined. The results obtained were compared with the synthetic antioxidants BHA and BHT for both extracts separately.

The first study on the determination of total phenolic substances was done in 1965 by Singleton et al [28]. They used the Folin-Ciocalteu Method for determination. Atanassova and colleagues examined the

total phenolic content of *Melissa officinalis*, *Salvia officinalis* and *Mentha piperita*. They found 48.86 mg GAE.100g$^{-1}$ for *Melissa officinalis*, 27.94 mg GAE.100g$^{-1}$ for *Salvia officinalis* and 45.25 mg GAE.100g$^{-1}$ for *Mentha piperita* [29]. Javanmardi et al. studied the total phenolic content of different species of the plant called *Ocimum basilicum*. As a result, they found values ranging from 22.9 mg GAE.g$^{-1}$ to 65.5 mg GAE.g$^{-1}$ [30]. In the study, the amount of phenolic substances contained in 2 mg.mL$^{-1}$ methanol extracts of *Z. jujuba* was calculated as equivalent to gallic acid. The phenolic contents of the ZJD and ZJA were found to be 0.90 mg GAE.g$^{-1}$ and 0.58 mg GAE.g$^{-1}$, respectively.

The first study on the determination of total flavonoid compound was done by Moreno et al. in 2000 [10]. They applied the calorimetric method for the determination. Atanassova et al. examined the total phenolic content as well as the total flavonoid content of *M. officinalis*, *S. officinalis* and *M. piperita*. They found the total flavonoid contents of 45.06 mg CE.100g$^{-1}$ for M. officinalis, 27.54 mg CE.100g$^{-1}$ for *S. officinalis* and 25.17 mg CE.100g-1 for *M. piperita*, respectively [29]. Chang et al. examined the total flavonoid compound content in propolis, as a result, they found values ranging from 10.38 mg QE.g$^{-1}$ to 24.91 mg QE.g$^{-1}$ [31]. In the study, the amount of flavonoids contained in *Z. jujuba* extracts was calculated as equivalent to quercetin. The flavonoid contents of ZJD and ZJA was found to be 0.38 mg GAE.g$^{-1}$ and 0.24 mg GAE.g$^{-1}$, respectively.

The determination of DPPH free radical scavenging activity was first made by Blois in 1958 [11]. After Blois, the method was frequently applied because the process was short-lived and easy. Bencheraiet et al. applied the DPPH method to determine the antioxidant activity of *Ammi visnaga*. They found the $IC_{50}$ value of the plant as 8.77±0.2 µg.mL$^{-1}$ and the $IC_{50}$ value of Rutin, a standard, as 3.01±0.2 µg.mL$^{-1}$ and compared with each other. Accordingly, they detected the scavenging activity of DPPH radical [32]. In the study, $IC_{50}$ values of the methanolic extract of *Z. jujuba* were calculated at a concentration of 1 mg.mL$^{-1}$ (ZJA = 5.17 µg.mL$^{-1}$, ZJD = 4.91 µg.mL$^{-1}$, BHA = 3 .5 µg.mL$^{-1}$, BHT = 1.5 µg.mL$^{-1}$). The DPPH radical scavenging activities of the extracts and synthetic antioxidants were listed as BHT > BHA > ZJD > ZJA.

The β-carotene lineolic acid emulsion method was first performed by Miller in 1971 [12]. In this method, antioxidant activity is determined by measuring the oxidation of linoleic acid in the presence of β-carotene. Türkoğlu et al. applied the β-carotene lineolic acid emulsion method for the determination of the antioxidant activity of *Laetiporus sulphureus* and found the inhibition value of the extract to be 82.2% [33]. In the study, the inhibition value of 1 mg.mL$^{-1}$ *Z. jujuba* methanolic extract was found to be 94.49% for the sample collected from Denizli, and 88.83% for the sample collected from Antalya. In addition, inhibition values of BHT and BHA, which are synthetic antioxidants, were calculated with this method. Inhibition values for BHT and BHA were obtained as 84.2% and 81.4%, respectively.

Benzie and Strain applied the ferric ion reducing power method for the first time in 1996 [13]. They used the reduction calorimetric method. They determined by measuring the absorption of 100 µmol.L$^{-1}$ bilirubin and 280 µmol.L$^{-1}$ albumin at 593 nm. In the study, the ferric reducing power was determined according to the Oyaizu [34] method. The reducing capacity of the methanolic extract of *Z. jujuba* at 1 mg.mL$^{-1}$ was determined as 20.16 mg TEAC for ZJD and 19.63 mg TEAC for ZJA.

Apak et al. applied the CUPRAC method for the first time in 2004 [14]. The method is based on the reduction of cupric ions to cuprous ions in the presence of antioxidants in the compounds. Cikrikci et al. applied the CUPRAC method to determine the antioxidant activity of *Curcuma longa* [35]. Curcumin and turmeric were used in the determination. The CUPRAC.TEAC$^{-1}$ value for curcumin is 0.8 mg TEAC.g$^{-1}$, while the CUPRAC.TEAC$^{-1}$ value for turmeric is 0.7 mg TEAC.g$^{-1}$. In the study, CUPRAC.TEAC$^{-1}$ values of 1 mg.mL$^{-1}$ ZJA methanolic exract and 1 mg.mL$^{-1}$ ZJD methanolic extract were calculated for the determination of antioxidant capacity by CUPRAC method. According to this method, trolox equivalent amounts of ZJA extract and ZJD extract were obtained as 9.15 mg TEAC.g$^{-1}$ and 6.95 mg TEAC.g$^{-1}$, respectively.

It was observed that the reducing capacity of *Z. jujuba* extracts increased with increasing concentration as well as antioxidant activity. The reducing power may be an important factor in the antioxidant activity of a compound, but the antioxidant property of any pure substance can be explained by different mechanisms. In summary, antioxidant compounds can exhibit their antioxidative properties by different mechanisms such as binding transition metals, breaking down peroxides, and removing radicals.

The β-carotene lineolic acid method is based on hydrogen atom transfer. Electron transfer-based methods measure the capacity of antioxidants as a result of reduction of oxidants that change color when reduced. This event can be in the form of an increase or decrease in absorbance. The degree of color change is related to the total antioxidant concentration of the sample. In fact, reactions based on hydrogen atom transfer and electron transfer are intertwined in a sense and there are no insurmountable boundaries between them.

In this study, gallic acid, catechin, p-coumaric acid, cinnamic acid, ferulic acid, caffeic acid and quercetin were determined qualitatively and quantitatively in the extracts by HPLC analysis. From the obtained chromatograms, it was seen that the HPLC values of the methanolic extract of ZJD were higher than the methanolic extract of ZJA. Gallic acid is the highest in both extracts, followed by catechin. While the gallic acid value in the extract obtained from ZJD is 31.20 µg.g$^{-1}$, it is 6.39 µg.g$^{-1}$ in the ZJA extract. When the catechin values are examined, ZJD extract is 20.75 µg.g$^{-1}$ and

ZJA extract is 3.90 $\mu g.g^{-1}$. Likewise, it was found that the extract obtained from ZJD was higher than that obtained from ZJA in other values.

Gallic acid is an antioxidant and phenolic acid found in varying amounts in most plants. As an antioxidant, gallic acid can defend the body against free radicals and oxidative damage. When cells are exposed to free radicals, they damage their proteins and cell membranes and die faster than normal. The anti-inflammatory property of gallic acid is effective in curing inflammation. The use of ointments containing it for skin diseases such as arthritis and psoriasis significantly reduces inflammation [36, 37]. Caffeic acid is an organic compound belonging to the hydroxycinnamic acid class [38]. Caffeic acid is widely available in the human diet [39]. p-Coumaric acid is an organic compound belonging to the hydroxycinnamic acid class. It has three isomers. 1) ortho-coumaric acid, 2) meta-coumaric acid, 3) para-coumaric acid [40]. p-Coumaric acid is found in esterified or free acid form in the cell wall of grasses, cereals, fruits and vegetables (40, 41). Cinnamic acid is found naturally in most plant sources [42]. It is usually encountered in balms from cinnamon or storax tree [43]. These molecules are phenolic compounds detected in high amounts in methanolic extracts of *Z. jujuba*. Thanks to these molecules, the extract acts against oxidation. In addition to in vitro analyses, some biological effects of these molecules were investigated in silico. As a result of in silico prediction analysis, it was determined that gallic acid and catechin had the highest effect, while the others had potential at certain levels.

Antioxidant molecules have been reported to hold a growing attention owing to their defensive functionalities in food products, fruits, vegetables, bee products and drug products against oxidative damage. Investigation of antioxidant activities of plants and plant derivated substances demand appropriate procedures that discourse the machineries of antioxidant oroperties. Several studies evaluating the antioxidant activity of various samples of research interest using different methods in food and human health have been carried out (44, 45, 46, 46, 47).

Consequently, the biochemical composition of the methanolic extracts of ZJA and ZJD was determined by HPLC analysis. The total amount of phenolic substances and total flavonoid substances were determined in the same extracts. As a result of both HPLC and phenolic and flavonoid substance determination methods, it was found that the amount of phenolic substance in plants was higher than the amount of flavonoid substance. For the determination of antioxidant capacity, β-carotene lineolic acid emulsion method and CUPRAC method were applied to the methanolic extracts of ZJA and ZJD, and then the iron ion reducing power and DPPH radical scavenging activities of the extracts were determined. The biological activities of the phenolic compounds contained in the extract were estimated with the PASS online program. When the results obtained are compared with standards and in silico prediction analysis data, it can be said that Z. jujuba methanolic extract is effective against oxidation, free radicals and lipid peroxidation, as well as metal reduction potentials and its fruits can be used as a potential antioxidant in medicine, pharmaceutical and food industries.

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## Appendices

## Appendix A.

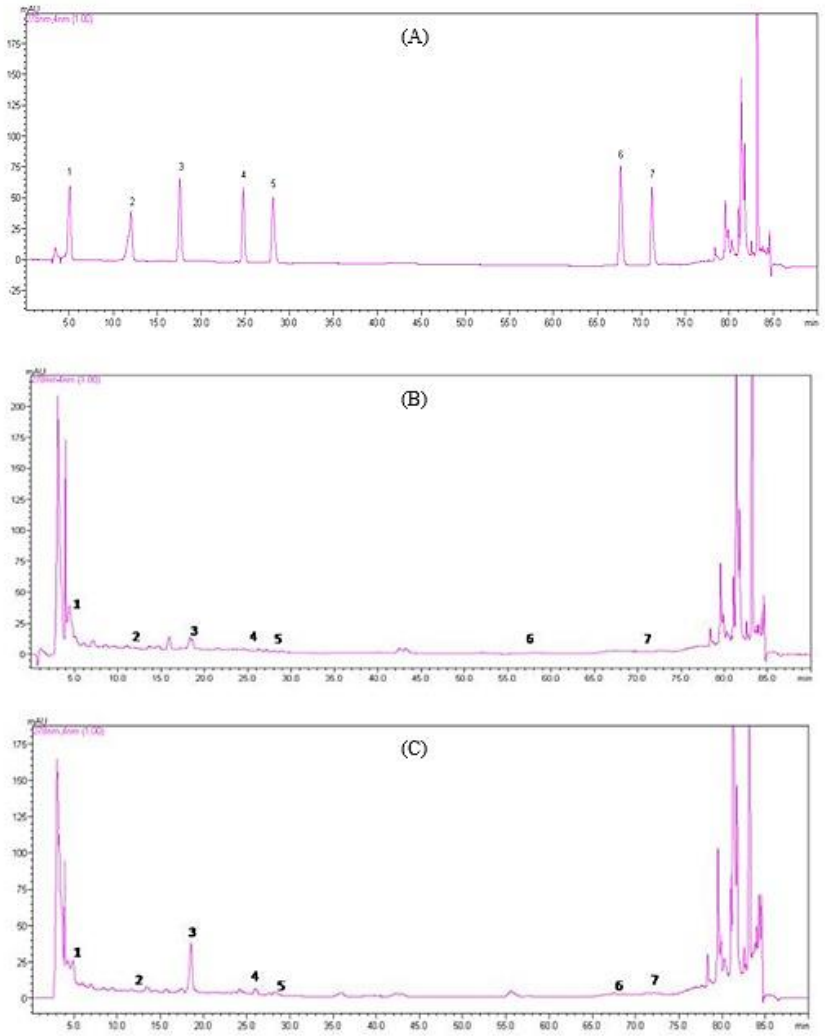


**Figure 7.** A) Standard chromatogram, B) Chromatogram of *Z. jujuba* collected from Antalya, C) Chromatogram of *Z. jujuba* collected from Denizli. *Molecular numbers given in the chromatogram; 1: Gallik acid, 2: Catechin, 3: Caffeic acid, 4: p-Coumaric acid, 5: Ferulic asid, 6: Cinnamic acid, 7: Quercetin.

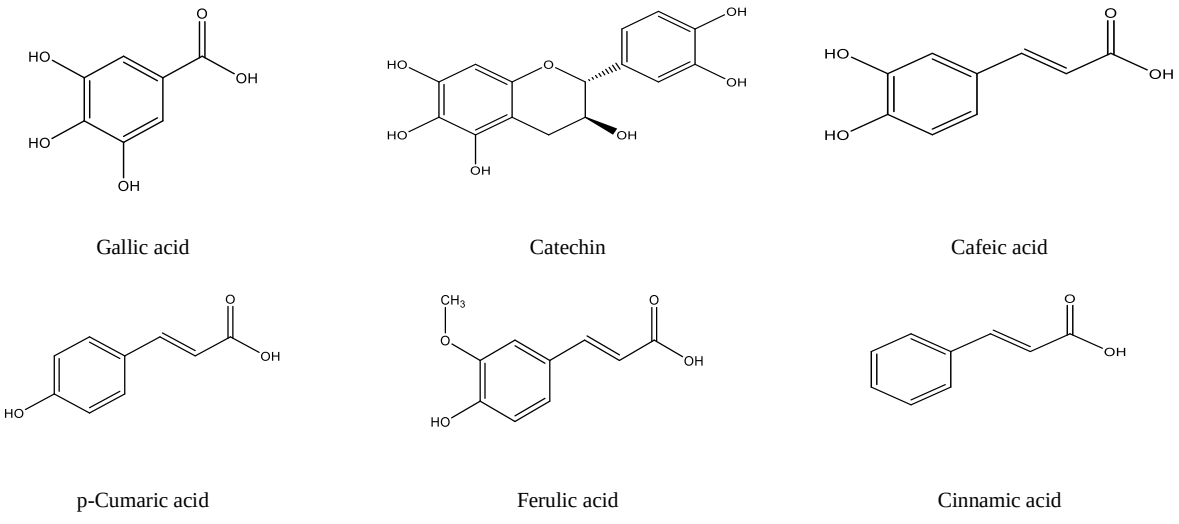


**Figure 8.** Molecular structures of the major components of both jujube fruits

**Table 4.** Pharmacological activities predicted for major phenolic compounds of *Z. jujuba* methanolic extracts

| Activity | Gallic acid | | Catechin | | Cafeic acid | | p-Cumaric acid | | Ferulic acid | | Cinnamic acid | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pa* | Pi** | Pa* | Pi** | Pa* | Pi** | Pa* | Pi** | Pa* | Pi** | Pa* | Pi** |
| **Antioxidant** | 0,520 | 0,006 | 0,847 | 0,003 | 0,603 | 0,005 | 0,553 | 0,005 | 0,540 | 0,005 | 0,489 | 0,007 |
| **Free radical scavenger** | 0,570 | 0,007 | 0,827 | 0,002 | 0,647 | 0,005 | 0,627 | 0,005 | 0,731 | 0,004 | 0,497 | 0,010 |
| **Antibacterial** | 0,418 | 0,026 | 0,360 | 0,041 | 0,358 | 0,041 | 0,343 | 0,045 | 0,333 | 0,048 | 0,312 | 0,056 |
| **Antifungal** | 0,398 | 0,050 | 0,561 | 0,002 | 0,450 | 0,039 | 0,451 | 0,039 | 0,430 | 0,044 | 0,401 | 0,049 |
| **Antiinflammatory** | 0,548 | 0,044 | 0,616 | 0,028 | 0,651 | 0,023 | 0,641 | 0,024 | 0,604 | 0,031 | 0,656 | 0,022 |
| **Antimutagenic** | 0,597 | 0,010 | 0,917 | 0,002 | 0,845 | 0,003 | 0,886 | 0,002 | 0,900 | 0,002 | 0,817 | 0,004 |
| **Membran integrity antagonis** | 0,543 | 0,035 | 0,971 | 0,002 | 0,955 | 0,003 | 0,954 | 0,003 | 0,276 | 0,130 | 0,774 | 0,014 |

*Pa: Probable activity; **Pi: Probable inactivity. Pa > 700: probable activity greater than 70%. The PASS prediction results were interpreted and used as follows: (i) only activities with Pa > Pi are considered as possible for a particular compound; (ii) if Pa > 0.7, the chance to find the activity experimentally is high.