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ORIGINAL ARTICLE / ÖZGÜN MAKALE

COMPARISON OF THE PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY IN PEPPERMINT PLANT ACCORDING TO THE DRYING METHOD

KURUTMA YÖNTEMİNE GÖRE NANE BİTKİSİNİN FENOLİK İÇERİĞİNİN VE ANTİOKSİDAN AKTİVİTESİNİN KARŞILAŞTIRILMASI

Ece MISER SALIHOĞLU[1,*], Bolkan SIMSEK[1], Erdoğan ÇAYIR[1], Sevgi AKAYDIN[1]

[1]Gazi University, Faculty of Pharmacy, Department of Biochemistry, 06330, Etiler, Ankara, Turkey

ABSTRACT

Objective: *Antioxidant activities of the plants were significantly affected by their drying methods. In the current study, total phenolic contents and antioxidant activity of aqueous extracts of peppermint (Mentha piperita L.) dried by two different methods, air-dried (AD) and freeze-dried (FD) were measured. The aim was to determine if there were any changes in the antioxidant activity according to the drying method.*

Material and Method: *In order to determine the total phenolic compounds and antioxidant capacity of air-dried (AD) and freeze-dried (FD) mint, were used Folin Ciocalteu, CUPRAC, DPPH, ABTS, DMPD methods.*

Result and Discussion: *Results were evaluated by comparing with the known antioxidant standards, such as gallic acid, trolox, ascorbic acid, and butylatedhydroxyanisole (BHA). The total phenolic contents (TPC) were calculated as 59.9 mg/g GAE for AD plant extract and 58.2 mg/g GAE for FD extract. According to the obtained results from all methods, radical scavenging activities were found to be slightly higher in AD samples than in FD samples. In conclusion, the results show that air-drying does not cause degradation in compounds that contribute to the antioxidant activity of the peppermint.*

Keywords: *Air-drying, antioxidant activity, freeze-drying, peppermint*

ÖZ

Amaç: *Bitkilerin antioksidan aktiviteleri kurutma yöntemlerinden önemli ölçüde etkilenir. Bu çalışmada, hava ile kurutulmuş (HK) ve dondurularak kurutulmuş (DK) nanenin (Mentha piperita L.) sulu ekstrelerinde toplam fenolik içeriği ve antioksidan aktivitesi ölçülmüştür. Amaç, kurutma yöntemine göre antioksidan aktivitesinde değişiklik olup olmadığını belirlemektir.*

Gereç ve Yöntem: *Bu bitkilerin toplam fenolik bileşiklerini ve antioksidan kapasitelerini belirlemek için Folin-Ciocalteu, CUPRAC, DPPH, ABTS, DMPD yöntemleri kullanılmıştır.*

[*] **Corresponding Author / Sorumlu Yazar:** Ece Miser Salihoğlu
e-mail / e-posta: ecemiser@hotmail.com, **Phone / Tel.:** +312 202 31 54

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Sonuç ve Tartışma: *Sonuçlar, gallik asit, trolox, askorbik asit ve butile hidroksianisol (BHA) gibi bilinen antioksidan standartlarla karşılaştırılarak değerlendirilmiştir. Total fenolik madde içeriği (TFM), hava ile kurutulmuş bitki ekstresi için 59.91 mg/g GAE (Gallik asit eşdeğeri), liyofilize bitki ekstresi için 58.2 mg/g GAE olarak hesaplanmıştır. Tüm yöntemlerden elde edilen sonuçlara göre, HK örneklerinde radikal süpürme aktivitesinin DK örneklerinden biraz daha yüksek olduğu bulunmuştur. Sonuç olarak, havayla kurutmanın, nanenin antioksidan aktivitesine katkıda bulunan bileşiklerde bozulmaya neden olmadığı anlaşılmıştır.*
Anahtar Kelimeler: *Hava ile kurutma, antioksidan aktivite, dondurarak kurutma, nane*

INTRODUCTION

Peppermint (*Mentha piperita* L.) is one of the most widely used aromatic herbs. *Mentha piperita* L. is a hybrid species belonging to the Lamiaceae family and composed of two species, *Mentha aquatica* L. and *Mentha spicata* L. [1]. Peppermint has been used for a long time, both for therapeutic purposes and as flavoring agents. Several beneficial effects of peppermint include antioxidant, antitumor, antimicrobial, antibacterial, antimycotic, antiviral, antiallergenic, and immunomodulating actions [2-4]. It is used in the treatment of cold, headache, dysmenorrhea, gastrointestinal system disorders, and inflammation of the mouth, throat, sinuses, liver, bile duct, and intestines [5]. In addition, fresh and dried leaves of peppermint are widely used directly in meals, salads, drinks, or as aromatizing in various sauces.

Reactive oxygen/nitrogen species (ROS/RNS) are generated from exogenous and endogenous sources. Excessive and continuous ROS/RNS production leads to oxidative modification of lipids, proteins, and DNA bases, and stimulates many diseases, including cancer, cardiovascular diseases, diabetes mellitus, and inflammatory diseases. Natural antioxidants contained in plants protect cells from oxidative damage that may occur through reactive species and thus may have prophylactic effects against the risk of many diseases [7]. It has been reported that the antioxidant properties in plants are mostly due to phenolic compounds such as flavonoids, phenolic acids, and phenolic terpenes. Phenols give hydrogen ions (H+) from their hydroxyl group in the aromatic ring to prevent lipids and other macromolecules (nucleic acids, proteins, carbohydrates) from oxidative damage by reactive species [8].

Peppermint contains large amounts of different classes of phenolic compounds, including flavones, flavonols, flavanones, phenolic acids, terpenes, lignans, and stilbenes [9]. Several studies have reported that peppermint has antioxidant properties due to its phenolic compounds [10- 12].

Numerous *in vitro* methods have been developed to measure the antioxidant capacity in plant extracts. These methods have different test principles and experimental conditions, and thus the specific antioxidants in each method may have different contributions to the total antioxidant capacity [13]. It is claimed that the antioxidant activities of the plants were significantly affected by their drying methods [14].

Air-drying (AD) is a conventional and traditional method for drying of fruits and vegetables.

Several problems of air-drying, such as slow process, insect infestation, low quality in nutritional composition, and hygiene, have revealed the need to develop alternative drying methods [15,16]. Freeze-drying (FD) or lyophilization is the protection technique that takes place through a process called sublimation for the drying of the material [17]. The water contained in the sample is frozen first, and the iced water is removed by sublimation. The iced water evaporates directly in the low-pressure environment in the event of a slight increase in temperature. In this way, high temperatures are not needed for drying [18]. Due to low processing temperature and almost no oxygen during processing, freeze-drying/lyophilization does not change the molecular and cell structure of the materials and almost preserves their original state [19,20]. In lyophilization, the roasting effect caused by hot air in drying with hot air does not occur.

AD and FD are the most frequent use of drying methods, AD provides products which can have up to one year shelf life, while FD prevents spoilage and microbiological reactions as foods are dried under vacuum and at very low temperatures [14]. In this study was carried out to investigate which drying method has the better quality of the final product. For this purpose, we aimed to measure the total phenolic content and antioxidant capacity of peppermint in both AD and FD samples. We also aimed to compare antioxidant properties according to different *in vitro* methods, such as cupric reducing antioxidant capacity (CUPRAC), 2,2-diphenyl-1-picryl-hydrazyl-hydrate free radical scavenging method (DPPH), 2, 2'-azinobis (3-ethyl benzothiazoline-6-sulphonic acid diammonium salt) (ABTS), and N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) in the dried plants by using two different ways.

MATERIAL AND METHOD

Chemicals

1,1-diphenyl-2-picryl-hydrazyl (DPPH) (Aldrich), N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) (Fluka), ferric chloride (Aldrich), ammonium acetate (Fluka), 2, 2'-azinobis (3-ethyl benzothiazoline-6-sulphonic acid diammonium salt) (ABTS) (Sigma), ethanol (Merck), methanol (Merck), nicotinamide adenine dinucleotide (NADH) (Sigma), nitro blue tetrazolium chloride (NBT) (Sigma), phenazine methosulfate (PMS) (Sigma), Folin-Ciocalteu reagent (Sigma Aldrich), sodium carbonate (Na_2CO_3) (Sigma Aldrich), sodium hydroxide (NaOH) (Merck), copper (II) sulfate (CuSO4) (Sigma,Aldrich), potassium sodium tartrate ($NaKC_4H_4O_6$) (Sigma,Aldrich), dichloromethane (Sigma Aldrich), 2(3)-t-Butyl-4-hydroxyanisole, 2(3)-t-Butylhydroquinone monomethyl ether (BHA) (Aldrich), Trolox (Acros), L-Ascorbic acid (Vitamin C) (Sigma).

Plant Material and Drying Methods

The aerial parts of *Mentha x piperita* L. were collected from Ilgaz Mountain, Çankırı, Turkey, in May 2013. Botanical identification and authentication were conducted by Prof. Dr. Galip Akaydın from Hacettepe University. The plant materials were divided into two equal parts for drying processes. The first portion of fresh samples was dried at 27 °C of ambient air temperature for one week in the shadow. Mean relative humidity was 40-45%. The second portion of fresh peppermint was frozen at -80°C, then the frozen material was FD (lyophilized) under vacuum pressure at -55°C condenser temperature. The final vacuum pressure was 0.01 kPa. The air-drying process was continued until reaching a constant mass. FD was considered to be completed when a fixed pressure was reached. After drying, samples were weighed again, and mass loss of the sample was determined. It was found that AD samples weight were 0.1% lower than that of FD samples. Taking the differences between weights into account, an equal amount of samples were weighed.

Extraction was performed after air drying and FD (lyophlization) processes. Plant materials were pulverized in a grinder. One gram of the pulverized plant materials was extracted first with 15 mL of bidistilled water for 45 min, then added 5 mL of water and extracted for 45 min, at last added 5mL of water for 15 min at room temperature with a temperature-controlled ultrasonic bath. The extracts were filtered through pleated filter-paper and stored at -80 º C.

Folin-Ciocalteu Method for the Total Phenolic Content

The amount of total soluble phenolics was determined according to the Folin-Ciocalteu method [21]. The assay method has been adapted to work with 96-well plates [22]. This method was applied to plants extracts (1000-500-250-125-62.5-31.25-15.625 µg/mL). To 100 µL of herb extract, 125 µL Lowry C was added. The mixture was mixed on a microplate shaker at 250 rpm. Then, 12.5 µL Folin reagent was added, and for stabilization, 30 min was allowed to turn blue in colour. The absorbance against a reagent blank was measured at 750 nm. The samples were prepared in triplicate for each analysis, and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid, and the calibration line was construed. Based on the measured absorbance, the concentration of gallic acid equivalent is expressed in terms of (mg of Gallic acid (GA)/g of extract).

CUPRAC Assay

Cuprac assay is based on the reduction of Cu(II) to Cu(I) by antioxidants (reductants) which are present in the sample [23]. The method has been adapted for microplate analysis [22]. This method was applied to plants extracts (1000-500-250-125-62.5-31.25-15.625µg/mL). 50 µl $CuCl_2$ solution (10-2 M), 50 µL neocuproine alcoholic solution (7.5×10-3 M) and 50 µL NH_4Ac buffer solution, 27.5 µLsample,

and 27.5 µL water were added to wells. The mixture was then mixed well and incubated in the microwell strips at room temperature (18° to 25 °C) for about 30 min in dark. After that, absorbances of the reduced cupper were measured against a reagent blank at 450 nm. The same procedure was repeated for the standard solutions.

DPPH Assay

The free radical scavenging activity of these plants was measured by 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\cdot}$) method [24] and adapted to work with 96 well plate [22]. With this method, it was possible to determine the antiradical power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm [24]. This method was applied to plant extracts (1000-500-250-125-62.5-31.25-15.625µg/mL). First, 0.1 mM solution of DPPH in MeOH was prepared. Then, 50 µL of this solution was added to 150 µL of extracts of plants. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in an ELISA reader. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity [25]. The DPPH. scavenging effect was calculated by using the following equation:

$$\text{DPPH Scavenging \%} = [Abs_{control}\text{-}Abs_{sample}/Abs_{control}]\times 100$$

ABTS Assay

ABTS assay [26] adapted for microplate analysis [22]. ABTS was dissolved in water to a 7 mM concentration. By allowing the mixture to stand in the dark at room temperature for 12-16 h before use, ABTS radical cation ($ABTS^{\cdot}$) was produced *via* the reaction of ABTS stock solution with 2.45 mM potassium persulphate (final concentration). The kept solution was diluted with 96% ethanolic at a ratio of 1: 30. This method was modified to work with 96 well plate and applied to plant extracts (1000-500-250-125-62.5-31.25-15.625µg/mL). Respectively, x µL sample, 60 µL $ABTS^{\cdot}$ and (240-x) µL solvent were added to wells. $ABTS^{\cdot}$ solution was used as control (A_0). Then, the change in absorbance during 6 min was recorded and measured at 734 nm by an ELISA reader. The $ABTS^{\cdot}$ scavenging effect was calculated by using the following equation:

$$\text{ABTS Scavenging \%} = [A_0\text{-}A_1/A_0]\times 100$$

DMPD Assay

DMPD assay [27] adapted for microplate analysis [22]. DMPD (100 mM) was prepared by dissolving 209 mg of DMPD in 10 mL of deionised water. This solution (1 mL) was added to 100 mL of 0.1 M acetate buffer, pH 5.25, and the colored radical cation ($DMPD^{+}$) was obtained by adding 0.2 mL of a 0.05 M solution of ferric chloride (the final concentration was 0.01 mM). This solution (225 µL) was directly transferred to the microwell, and its absorbance at 505 nm was measured (A_0). This method was applied to plant extracts (1000-500-250-125-62.5-31.25-15.625 µg/mL). Samples (20 µL)

were added to all wells. Then 280 µL of $DMPD^+$ was added to all samples, and stirred, and left to stand for about 30 min in the dark. After this period, a decrease in absorbance was measured (A_1). The $DMPD^+$ solution was used as control. The $DMPD^+$ scavenging effect was calculated by using the following equation:

DMPD Scavenging % = [A0-A1/A0]×100

RESULT AND DISCUSSION

The antioxidant activities of the plants were significantly affected by their drying methods. Phenolic compounds such as flavonoids and phenolic acids have been identified as responsible for the antioxidant effects of plant products [28, 29].

Peppermint leaves contain large amounts of different classes of phenolics, including flavones (e.g., luteolin derivatives), flavonols (e.g., catechin, quercetin), flavanones (e.g., eriocitrin derivatives), phenolic acids (e.g., rosmarinic and caffeic acids), lignans and stilbenes, and some antioxidant vitamins, including carotenoids and ascorbic acid [9]. Due to being a frequently consumed plant and having a high content of antioxidant compounds, mint has been interesting for antioxidant activity studies.

In our study, the total phenolic contents were expressed as mg/g gallic acid equivalent using the standard curve equation (y=0.5585 ln(x)–1.3345, R2=0.9815), where y: absorbance at 750 nm, x: total phenols in the extracts (Figure 1). By applying the absorbance values obtained from the plant extracts to the regression equation, the phenolic substance contents were expressed as µg gallic acid equivalent (GAE) (Table 1).

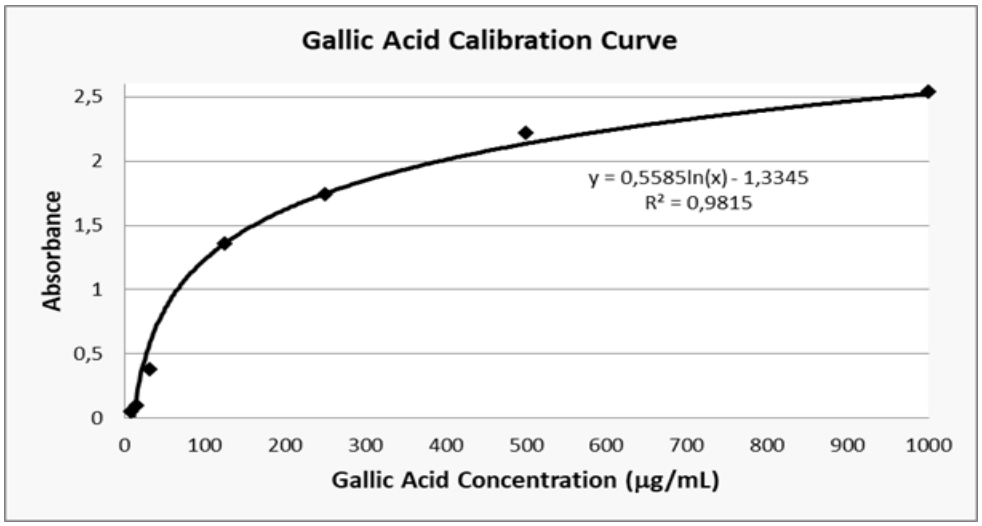

Figure 1. Standard calibration curve of Gallic Acid

Table 1. Total phenolic contents in aqueous extracts.

Aquaeous extracts of *M. piperita*	Total phenolic content (mg GA/g) ± SD	Aquaeous extracts of *M. piperita*
Air-dried	58.20 ±2.34	Air-dried
Areeze-dried	59.91±24.97	Freeze-dried

No significant difference was observed between the total phenolic contents (TPC) values obtained according to the drying method. In a previous study, Papageorgiou et al. found lower total phenolic content in the FD samples rather than AD samples [30]. In another previous study, TPC was found as 75.31 ± 3.58 mg GAE $g^{-1}$for aqueous extract of leaf of *Mentha piperita* [31]. Uribe et al. obtained TPC values ranging from 11.56 to 24.4 mg/g GAE on vacuum-dried mint leaves and 12.43 mg/g GAE on fresh mint leaves [32]. Safaiee et al. found maximum TPC values of *Mentha aquatica* (0.2451 mg/g) by freeze-drying method and they suggested that in FD samples, phenolics could be better preserved in the substrate [33].

The CUPRAC method is based on the reduction of Cu(II), which is used as an oxidant, to Cu(I) by both hydrophilic and lipophilic antioxidant compounds present in the sample. GA, BHA, and vitamin C were used as standard solutions. According to the results of the CUPRAC study, peppermint samples had lower antioxidant capacity than those of all standard compounds. The AD and FD samples had similar absorbance values up to 500 µg/mL, whereas the AD samples had higher values than the FD samples at the 1000 µg/mL concentration (Figure 2).

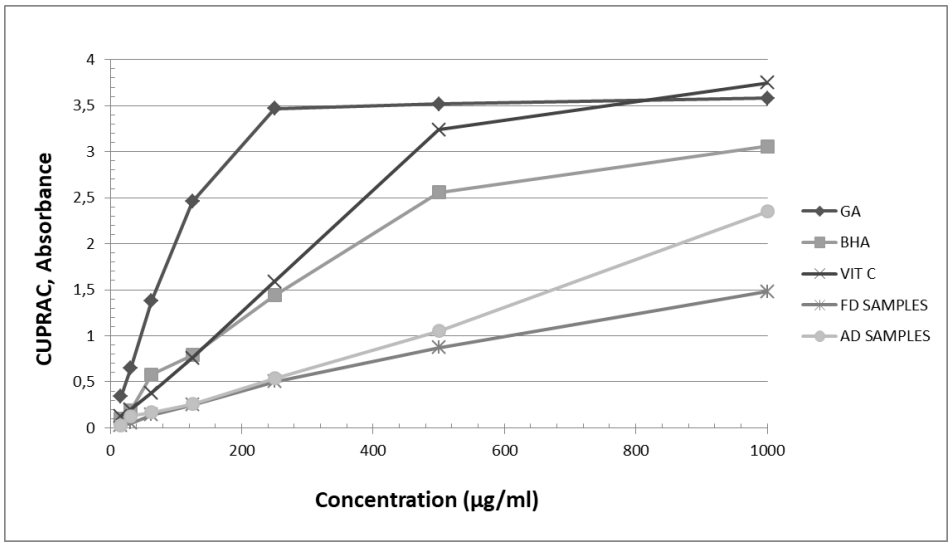

Figure 2. The determined antioxidant activities of plant extracts by using CUPRAC method

In a recent study, Ferhat et al. studied the antioxidant activity in different extracts of *Mentha aquatica*. Similar to our results, they showed that AD samples of *Mentha aquatica* roots had lower

activity than the BHA standard [34]. We could not find any studies to compare our results according to the drying method.

DPPH assay is a rapid, simple, and widely used method which has been used to evaluate the antioxidant activity of plants. In this method, the antioxidant activity of a compound is determined by scavenging the DPPH radical as a hydrogen donor and is expressed as percent inhibition. Gallic acid, Trolox, BHA, and Vitamin C were used as the reference standards. In our study, we found that vitamin C had the highest activity in the DPPH radical scavenging. At the same time, we observed that AD samples had moderately higher DPPH radical-scavenging activity than FD samples at all concentrations (Figure 3). DPPH radical scavenging activity in AD samples was found to be higher than in FD samples, and both samples had lower antioxidant capacity than those of standard compounds.

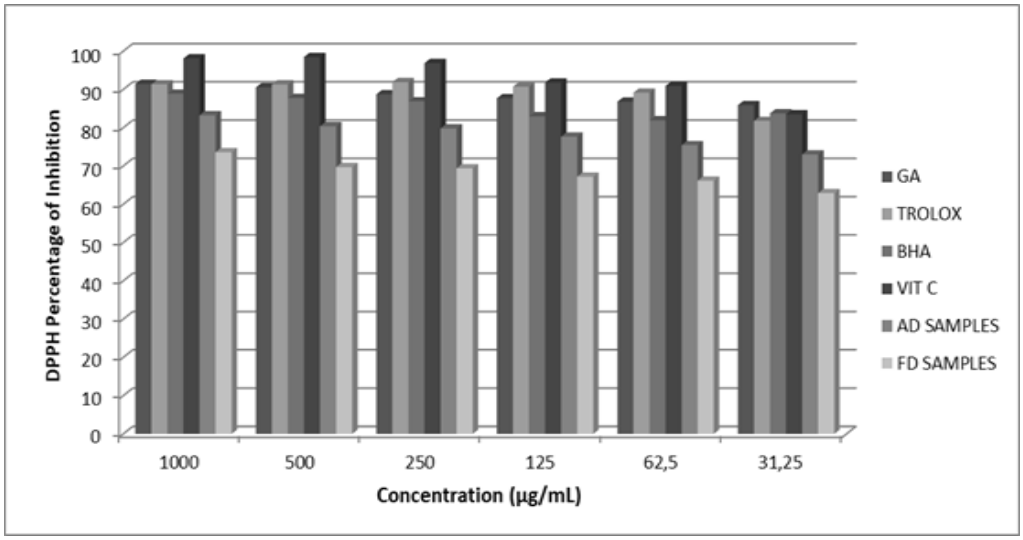

Figure 3. The ability of peppermint extracts to inhibit DPPH radical

Orphanides et al. found significantly higher phenolic content and DPPH radical-scavenging activity in FD samples of spearmint than those in AD samples [35]. According to DPPH antioxidant assay results, they reported that FD was the best effective drying method. In a previous study conducted with collected peppermint at different periods, DPPH radical scavenging activity was significantly lower in all of the FD materials than AD plant materials [30]. Studies showed that extraction yield and DPPH scavenging activity increased when the temperature is increased from 30 °C to 60 °C [33,36].

The ABTS method is based on the inhibition of the ABTS radical, which is formed during the reaction by a molecule with antioxidant activity. In this study, antioxidant activity was determined by decolorizing the blue-green ABTS radical cation with antioxidants in peppermint extracts. Gallic acid, Trolox, BHA, and Vitamin C were used as the reference standards. Although plant extracts had lower antioxidant capacity than those in all standard compounds until 500 µg/mL concentration, all antioxidant

capacities were similar at 1000 µg/mL. Taking the results of phenolic composition and ABTS assay in our study into consideration, although there was a slight difference (Figure 4), AD peppermint had higher ABTS radical scavenging activity than lyophilized peppermint.

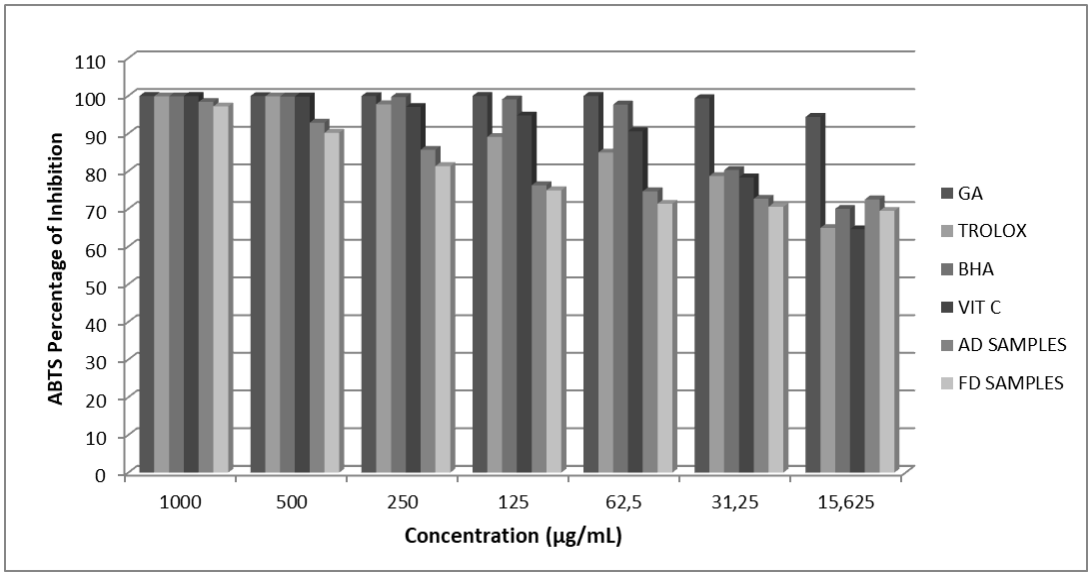

Figure 4. ABTS radical scavenging activity of the plant extracts

Similar results to ours were reported in AD *Mentha piperita* by Okmen et al. [37]. Previous studies have shown that the antioxidant activities of AD samples were significantly lower than in FD plant materials, and this is attributed to a decrease of the phenolic compounds in the plant extracts [35]. Antal et al. reported that when the convectively dried spearmint and lyophilized samples were compared, lyophilized samples were kept with more volatile component [38]. Also, Abascal et al. reported that air-drying preserved more volatiles compared with freeze-drying [39]. Additionally, in a study on the investigation of the antioxidant behavior of AD and FD aromatic plant materials, it was shown that the antioxidant activities of all the FD samples were significantly lower than AD plant materials [30].

The DMPD method has been applied to determine the antioxidant activity of hydrophilic compounds in peppermint extract. Gallic acid, Trolox, BHA, and Vitamin C were used as the reference standards. The antioxidant activity in mint extracts was calculated according to the inhibition percentage of the DMPD radical formed in the experimental environment. DMPD radical scavenging activity is found to be more effective in AD plant sample (Figure 5). DMPD radical scavenging effect is higher than BHA for each of the samples. Among standards, the highest DMPD radical scavenging activity was obtained with vitamin C, and the lowest with BHA.

A study on the influence of drying on the flavor quality of *Mentha spicata* showed that AD samples had a stronger mentholated aroma than FD samples, and they indicated that AD samples

released higher quantities of monoterpenes which had antioxidant properties [40].

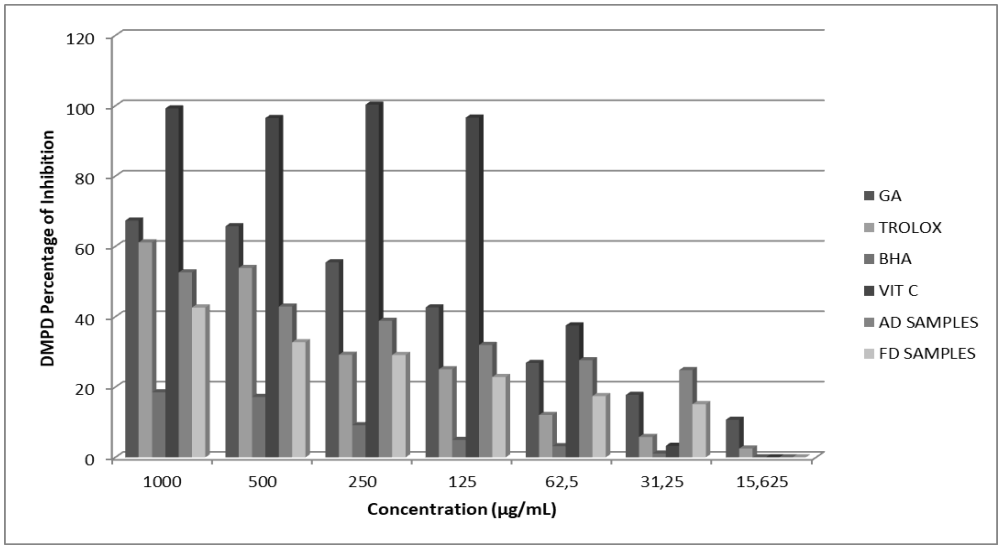

Figure 5. DMPD radical scavenging activity of the plant extracts

The results distribution suggested a good correlation between TPC and CUPRAC (r = 0.995, p=0.0001 and r = 0.986, p=0.0001 for FD and AD samples, respectively), ABTS radical-scavenging activity (r = 0.954, p=0.001 and r = 0.948, p=0.001 for FD and AD samples, respectively), DMPD radical-scavenging activity (r = 0.950, p=0.001 and r = 0.941, p=0.002 for FD and AD samples, respectively), and DPPH radical-scavenging activity (r = 0.852, p=0.015 and r = 0.825, p=0.022 for FD and AD samples, respectively). Similarly, positive correlations were also observed among the antioxidant activities obtained by different methods (data not shown). Correlations between polyphenol content and antioxidant capacity may suggest that the antioxidant activity of the peppermint may be related to the total phenolic content. Similar correlations obtained by both drying methods support the accuracy of our study results.

In conclusion, dried mint, both in Turkey and in the world, is a plant widely used in four seasons. However, it is an important question whether the nutritional values are preserved in dried samples or not. Air drying with/without sun, vacuum, and freeze-drying have been widely used for the drying process of plants. Since FD is carried out at very low temperatures, it is considered the best method for drying products that are sensitive to heat treatment. In addition, FD is a very good method to maintain the color, taste, aroma, and nutrient quality of the products. Lyophilized samples are suggested to be the closest to the fresh form of the plant. However, FD is an expensive preservation method and difficult to apply in daily life. The results of the current study showing that the AD version of the plant has a slightly higher antioxidant activity concluded that air-drying, most commonly used to extend the shelf life of the

peppermint, does not cause a degradation in compounds that contribute to its antioxidant activity.

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AUTHOR CONTRIBUTIONS

Concept: *S.A., B.S.*; Design: *S.A., B.S*; Control: *S.A.*; Sources: *S.A., B.S.*; Materials: *E.Ç.*; Data Collection and/or processing: *E.M.S, E.Ç,* Analysis and/or interpretation: *S.A, E.M.S.*; Literature review: *S.A., E.M.S.*; Manuscript writing: *S.A., E.M.S.;* Critical review: *S.A.*; Other: -

CONFLICT OF INTEREST

The authors declare that for this article they have no actual, potential or perceived conflict of interests.

ETHICS COMMITTEE APPROVAL

The authors declare that this study does not include any experiments with human or animal subjects.

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