Research Article

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Determination of Antioxidant Activity of Some Fresh Green Leafy Vegetables Using DPPH Free Radical Assay

Bazı Taze Yeşil Yapraklı Sebzelerin DPPH Serbest Radikal Testi Kullanarak Antioksidan Aktivitenin Belirlenmesi

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ABSTRACT:

The purpose of this study was to investigate the free radical scavenging capacity of the most frequently consumed fresh green leafy vegetables in the Black Sea region (such as *Brassica oleracea* var. Acephala (cabbage), *Spinacia oleracea* L. (spinach) and *Portulaca oleracea* L. (purslane). Antioxidant capacity test was determined by 2,2-diphenyl-1-picrylhydrazide (DPPH) free radical scavenging method using water extracts of fresh green leafy vegetables. For this purpose, methanol extracts of green leafy vegetables were prepared with 7 different concentrations of ascorbic acid (vitamin C, standard) prepared in the concentration range of 5-600 µg/ml. Inhibition rate (%) and EC50 values of DPPH radical scavenging activities corresponding to different concentrations of these extracts were calculated. In our study, EC50 values of P. oleracea, S. oleracea and B. oleracea water extracts were calculated as 2.124, 18.075 and 38.189 µg/ml, respectively. The EC50 values of ascorbic acid were 1.955 µg/ml. When the extracts and standards were compared, P. oleracea showed the highest antioxidant scavenging activity. The results obtained show that the green leafy vegetables we consume in our daily diet have varying levels of antioxidant capacity.

Keywords**:** Brassica oleracea, DPPH, EC50, Spinacia oleracea, Portulaca oleracea

ÖZ:

Bu çalışmanın amacı, Karadeniz Bölgesi'nde en sık tüketilen taze yeşil yapraklı sebzelerin (*Brassica oleracea* var. Acephala (beyaz lahana), *Spinacia oleracea* L. (ıspanak) ve *Portulaca oleracea* L. (semiz otu) gibi) serbest radikal kapasitesini incelemektir. Antioksidan aktivite testi, taze yeşil yapraklı sebzelerin su ekstraktlar kullanılarak 2,2-difenil-1-pikrilhidrazi (DPPH) serbest radikalini temizleme yöntemi ile belirlenmiştir. Bu amaçla 5-600 µg/ml konsantrasyon aralıklarında hazırlanan 7 farklı konsantrasyonda askorbik asit (vitamin C, standart) ile yeşil yapraklı sebzelerin su özütleri hazırlanmıştır. Bu özütlerin farklı konsantrasyonuna karşılık gelen DPPH radikalini temizleme aktivitelerinin inhibisyon değerleri (%) ile EC50 değerleri hesaplanmıştır. Çalışmamızda *P. oleracea*, *S. oleracea* ve *B.oleracea* su özütlerinin EC50 değerleri sırası ile 2.124, 18.075 ve 38. 189 µg/ml olarak hesaplanmıştır. Askorbik asitin EC50 değerleri ise 1.955 µg/ml'dir. Özütler ile standartlar karşılaştırıldığında en yüksek antioksidan süpürücü aktiviteyi *P. oleracea* göstermiştir. Elde edilen sonuçlar, günlük diyetimizde tükettiğimiz yeşil yapraklı sebzelerin değişen oranlarda antioksidan kapasiteye sahip olduğudur.

Anahtar Kelimeler: *Anahtar Kelime, Anahtar Kelime, Anahtar Kelime, Anahtar Kelime, Anahtar Kelime (en çok 5 anahtar kelime)*

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1. INTRODUCTION

Free radicals have unshared electrons in their outer orbitals, making them highly reactive. These free radicals consist of reactive nitrogen species, reactive sulfur species and oxygen reactive oxygen species (Halliwell and Gutteridge 2015). These reagents, especially reactive oxygen species (ROS), have been relationship metabolic disorders such as cancer, diabetes mellitus, obesity, insulin resistance, atherosclerosis, cardiovascular diseases, aging and chronic inflammation, and with protective mechanisms and physiological processes that living beings use to survive. These protective antioxidant mechanisms in living beings are antioxidant systems that help balance the levels of oxidative stress and molecular scavengers such as glutathione and thioredoxin, as well as enzymatic antioxidants such as peroxidase enzyme (POD), superoxide dismutase (SOD), glutathione peroxidases (GPXs) and catalase (CAT) (Halliwell and Lee, 2010). These antioxidants are naturally found in living beings or can be synthesized by chemical means. In cases where there is insufficient antioxidant in living beings, synthetic antioxidants such as tert butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), propyl gallate (PG) and butylated hydroxyanisole (BHA) is used, yet these synthetic antioxidants are not preferred because they do not have natural content. Instead, medicinal plants and their components (such as leaves, roots, bark), vegetables and fruits, seeds, grains, tea and oils with completely natural contents are preferred. These are natural antioxidant sources such as tocopherols, flavonoids (kaempferol, catechin, quercetin and naringenin derivatives), polyphenols, ascorbic acid, carotenoids, thiol (SH) compounds, gallic acid, which are found in large quantities (Anwar et al., 2018).

After the Covid-19 pandemic, nutrition has gained importance all over the world, as it has in our country. Because nutrition is the basis for overcoming diseases or increasing our immunity against viruses. In addition, protecting the body against nanoparticle toxicity, which we have been exposed to in recent years, has become increasingly important. (Dağlıoğlu et al., 2016; Dağlıoğlu & Öztürk, 2016; Dağlıoğlu & Yılmaz, 2018; Dağlıoğlu et al., 2023). For this reason, our awareness of natural vegetables, fruits and medicinal plants with high antioxidant content has increased. For this purpose, considering the geographical region we are in, easily accessible, seasonal vegetables that are consumed daily have attracted attention. In living things, including plants, complex antioxidant defense systems consisting of endogenous and exogenous antioxidants protect cells from oxidative processes caused by RSS, ROS and RNS and protect them from the damage of reactive species (Finkel and Holbrook, 2000). There are many bioanalytical methods to estimate the effectiveness of antioxidants, and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging test is the most common method used to determine the antioxidant ability. When the DPPH solution is mixed with the antioxidant solution that can donate hydrogen atoms, the violet color disappears and hydrazine (DPPH-H) is formed, which is the reduced form of the DPPH radical (Yapıcı et al., 2021). Formation of hydrazine changes the purple color of the mixture to yellow, and the intensity of this color is easily measured by spectroscopy at 517 nm. In this study, the antioxidant capacity of vegetables (green leafy) consumed in daily nutrition in the Black Sea region was evaluated and compared with the DPPH test. Green leafy vegetables contain antioxidant minerals and vitamins and therefore form a very important part of a balanced daily diet (Subhasree et al., 2009). In many studies, the medicinal properties of these plants such as antibacterial, anticarcinogenic, antihistamine and antidiabetic effects have been attributed to their antioxidant activity (Kesari et al., 2005; Khanna et al., 2002; Kubo et al., 2004). Because antioxidants prevent oxidation caused by free radicals, it is assumed that adequate intake of antioxidants provides protection against diseases (Yesil-Celiktas et al., 2007). Antioxidants, which act by scavenging and preventing the formation of reactive oxygen species (phagocyte activation), preventing the emergence of OH, repairing damage, the breakdown of lipid hydroperoxides any combination of these (Niwa vd., 2001). For this purpose, the antioxidant capacity of green vegetables *Spinacia oleracea* L. (Spinach), *Portulaca oleracea* (Purslane pirpirim) and *Brassica oleracea* var. Acephala (Cabbage) freshly collected from the same region was evaluated. *P. oleracea* contains omega-3 fatty acids, gallotannins, quercetin, kaempferol and apigenin (Lewis and Elvin-Lewis, 2003; Radhakrishnan et al., 2001). These rich contents are known to prevent heart diseases and asthma, strengthen the immune system, heal wounds, relax skeletal muscles and reduce locomotor activity (Chan et al., 2000; Simopoulos, 2004; Malek et al., 2004; Lim and Quah, 2007; Parry et al., 1993). Spinacia oleracea vegetable contains plenty of vitamins (contains high amount of vitamins E, C, A and K), flavonoids (glucuronide, patuletin, neoxanthin and spinacetin), polyphenols (ortho-coumaric, para-coumaric and ferulic acids) and carotenoids (lutein, neoxanthin, violaxanthin and β-carotene), and these contents increase their antioxidant capacity (Joseph et al., 1998; Kaur et al., 2016; Hedges and Lister 2007). *Brassica oleracea* has

anticancer, antioxidant and anti-obesity effects thanks to the phytochemicals it contains, such as glucosinolates, polyphenolics, carotenoids and vitamin C (Samec et al., 2014).
As mentioned above, although there are studies on the bioactive contents of P. oleracea, *S. oleracea* and *B. oleracea* vegetables, there are very few studies on their antioxidant capacity. Comparative evaluation of antioxidant activity of different green vegetables grown in the same geographical area has not been recorded. This article goal to complete this gap in knowledge for daily consumed vegetables.

2. MATERIAL AND METHOD

2.1 Green vegetables Extraction

Freshly obtained *S. oleracea, P. oleracea* and *B. oleracea* vegetables were cleaned with pure water, dried and chopped into small pieces with a blender.30 g of ground vegetables were extracted with methanol. Extraction procedure was carried out at 40 ^{o}C for 48 h. Then, methanol was removed in the evaporator and lyophilized.

2.2 DPPH radical-scavenging assay

DPPH radical scavenging analysis was determined spectrophotometrically at 517 nm with minor modifications to the procedure of Brand-Williams et al. (1995). Briefly, 1 ml of freshly prepared 4 M DPPH solution in methanol and 3 ml of plant extracts prepared at different concentrations (5, 10, 50, 100, 200, 400 and 600 µg/ml) were mixed and vortexed. After incubation for 25 minutes at 21°C in the dark, the diminish in absorbance was determined in a spectrophotometer at a wavelength of 517 nm. Pure methanol (3 ml) was added to the control (blank). Measurements were made 3 times. Absorbance was determined against a blank (methanol) solution. The percentage of inhibition of radicals resulting from the antioxidant properties of the extracts was calculated using the equation [% Inhibition = [$A_{Control}$ - A_{Sample} / $A_{Control}$] x 100].

2.3 Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 26.0. Differences between groups showing normal distribution were measured by one-way analysis of variance (ANOVA) and groups that created differences were appointed by Tukey's test. *P* values less than 0.05 were accepted as significant. Antioxidant concentrations corresponding to 50% inhibition were acquired by linear regression from the graph drawn with the calculated % inhibition values against different concentrations of vegetable extracts and the results were explained as EC50 (µg/ml).

3. RESULT AND DISCUSSION

DPPH, a stable free radical, takes a hydrogen or electron radical. In the DPPH method, the lower the absorbance values read at 517 nm, the further the free radical scavenging capacity. The decrease in the amount of DPPH and the decrease in the absorbance values of the antioxidant amount up to a certain concentration are directly proportional (Ndhlala et al., 2010). The reason for the decrease in absorbance is the destruction, i.e. scavenging, of the free radical after the hydrogen exchange between free radical and antioxidant. In our study, the DPPH radical scavenging activities calculated against different concentrations (5-600 µg/ml) of *Spinacia oleracea* L. (Spinach), *Portulaca oleracea* (Purslane pirpirim) and *Brassica oleracea* (Cabbage) leaf extracts are shown in the graph below with their % inhibition values (Figure 1). This study investigated the concentration-dependent antioxidant activities of freshly picked green colored leafy vegetables grown in the same experimental conditions and in the same environments (geographical region). Percentage inhibition and EC50 amount of the extracts were estimated for all tested vegetable extract concentrations. Statistical analysis of the data estimated by analysis of variance (ANOVA) showed that there is a statistically significant coaction between the types of vegetable extracts and the concentration of these extracts at the $p < 0.05$ level ($p < 0.05$). Furthermore, statistical tests were used to calculate the mean value of the concentrations for each test so that the free radical scavenging capacity of the three vegetable extracts could be easily compared. Additionally, the free radical scavenging capacity of vegetable leaf extracts increased in a concentration dependent manner. However, Tukey test was used for multiple comparison analysis of the tested extracts. The results of this test and comparison of the data showed that DPPH free radicals increased with increasing vegetable extract concentration. However, all concentration groups indicated significant separation with each other ($p < 0.05$).

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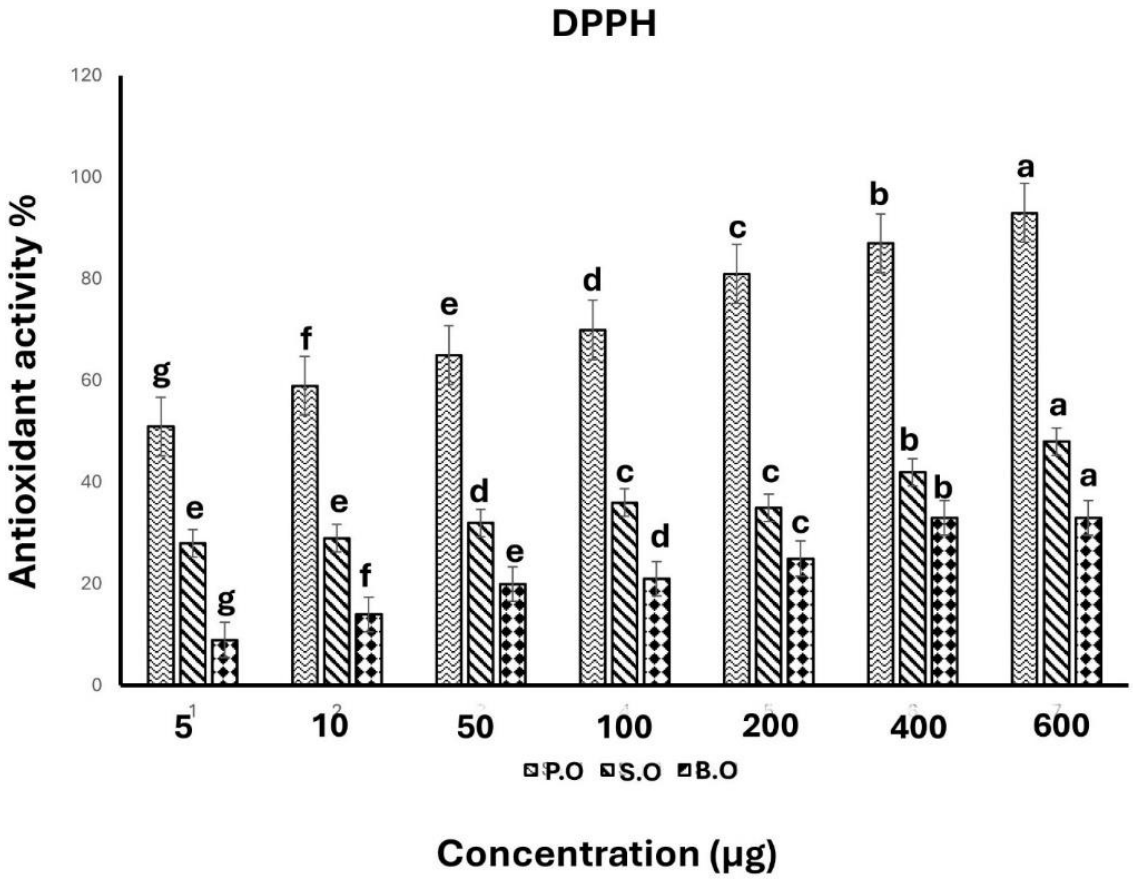

Figure 1. Determination of antioxidant activity of fresh green leafy vegetables by DPPH method. Different lower-case letters show that the concentrations for each vegetable extract are significantly different from each other according to Tukey's HSD test ($p < 0,05$).

The percentage inhibition of DPPH radical originating from green leafy fresh vegetable extracts ranged from 8.9% to 92.6%. Among the green leafy vegetables evaluated, *P. oleracea* showed further DPPH radical scavenging activity regarding other studied vegetable leaf extracts.

Table 1. EC50 values of fresh green leaf vegetable extracts investigated for DPPH radical scavenging activities.

Vegetable extracts with antioxidant content	**EC50 (µg/ml)**
Portulaca oleracea	2.124
Spinacia oleracea	18.075
Brassica oleracea	38.189
Ascorbic acid	1.955

Gelecek Araştırmalar:

The lower the calculated EC50 values, the further the DPPH radical scavenging capacity. Accordingly, the EC50 values of green leafy fresh vegetable extracts are shown (Table 1). In our study, ascorbic acid was used as the

standard antioxidant. The EC50 value of ascorbic acid was calculated as 1.955 µg/ml. The order of DPPH radical scavenging activity of the analyzed fresh green leafy vegetable extracts was calculated as *P. oleracea* (2.124 µg/ml) > *S. oleracea* (18.075µg/ml) > *B. oleracea* (38.189 µg/ml). Up to now, there are many studies that have evaluated the antioxidant capacity of vegetables. One of them was conducted in Sri Lanka, where researchers evaluated the antioxidant features of 34 leafy vegetables commonly consumed in Sri Lanka by several different methods (total antioxidant acticity, lipid peroxidation, DPPH and reducing power). As a result of their studies, they noted that vegetables contain different antioxidants. They found that the percentage of inhibiting the DPPH radical varied between 3.70% (*Asteracantha longifolia* L) and 52.2% (*Olax zeylanica*) (Gunathilake and Ranaweera, 2016). The antioxidative effect of the extract (methanolic) of *Spinacia oleracea* L. leaves was appraised using the DPPH free radical scavenging capacity method and it was recorded that it had no effect (Shaheen et al., 2017). Again, the antioxidant activities of n-hexane, methanol, aqueous extracts and ethanol of *S. oleracea* leaves were evaluated and it was recorded that the highest antioxidant content was in the methanol extract (Hussain and Bashir, 2022). Water and methanol extracts of *S. oleracea* showed scavenging activity of 16.625 µg/g and 17.25 µg/g and gallic acid equivalent of 20.7% and 18.83% at 100 g/ml concentration, respectively (Sekar et al., 2012). In the study in which the antioxidant contents and antioxidant activities of the methanol extract of *Portulaca oleracea* L. (purslane) collected from 13 different regions were evaluated, it was recorded that the antioxidant content of *Ornamental purslane* was higher and the DPPH scavenging capacity activities varied between 2.52-3.29mg/mL (Alam et al., 2014). The antioxidant capacity of extracts of 6 varieties of *P. oleracea* was evaluated. The evaluations showed that ornamental plant varieties have higher antioxidant activities than the common vegetable plant *Portulaca oleracea*. (Lim and Quah, 2007). *P. oleracea* fractions acquired from the rude extract of the plant by reverse phase separation method were researched for their antioxidant capacities and phenolic compound contents. The IC50 amount of the crude extract was 511.8 µg/ml, which was higher than other fractions, indicating that it had the lowest antioxidant activity (Erkan, 2012). Among the different solvent (methanolic, aqueous, ethanolic, hexane and acetone) extracts of P. oleracea L. grown in Kashmir Valley, acetone extract showed the highest antioxidant activity (Khursheed and Jain, 2021). Fresh and dried *P. oleracea* leaves were extracted with MeOH/H_2O and EtOH solvents and the fresh hydroalcoholic extract indicated the most radical scavenging potential in the DPPH test (IC50 66.98 µg/mL) (Sicari et al., 2018). The antioxidant activity of ethanol and water extracts obtained from *P. oleracea* was compared and it was found that the ethanol extract had better antioxidant activity than the water extract (Kim et al., 2018). In the study where the potential antioxidant activity of *Brassica oleracea* (Cauliflower) water and ethanol extracts was evaluated, the measured ethanol extract and water extract neutralized the activity of DPPH radical. In short, water and ethanol extracts have effective DPPH radical scavenging activity (Köksal and Gülçin, 2008). Antioxidant activities of 17 colored cabbages (*Brassica oleracea* var. capitata) grown in Korea were evaluated and DPPH radical scavenging activities were found to be significantly further in colored cabbages ($p < 0.0001$) (Ha et al., 2023). One study researched the antioxidant activity and content of fat- and water-soluble antioxidants in savoy, red, Brussels sprouts and white cabbage. Brussels and Red sprouts were richest in antioxidants, while white cabbage was lowest. However, phenolic extracts have the talent to scavenge DPPH radicals and inhibit lipid peroxidation in linoleic acid emulsion (Podse et al., 2006). In a study investigating the talent of the water extract of outer leaves of *Tronchuda cabbage* to act as a DPPH radical scavenger, *T. cabbage* extracts demonstrated antioxidant activity in a concentration dependent manner (Vrchovská et al., 2006).

3. CONCLUSION

The results of the estimation of antioxidant capacity of green leafy fresh vegetables with DPPH test showed that *Spinacia oleracea*, *Portulaca oleracea* and *Brassica oleracea* leaf extracts have antioxidant activity. However, antioxidant capacity raised with rising concentration of the extracts. Again, it was investigated that *P. oleracea* has a very high antioxidant activity. The components in the leaf tissues may also alteration with maturity; for example, while anthocyanins, phenolic content decreases and flavonoids increase. The antioxidant content and antioxidant activities of vegetables vary according to both their geographical location and their ripeness or type. For this reason, antioxidant activity evaluations vary and do not show a certain standard. However, there is an undeniable fact that both in this study and in previous studies, the presence of general antioxidant status.

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ETHICAL STANDARDS

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethics Committee Approval: No ethics committee approval is required.

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