

Full Length Research Paper

Evaluation of antioxidant and antifungal potential of *Asphodelus aestivus* Brot. growing in Turkey

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***In vitro* antioxidant and antifungal activities of *Asphodelus aestivus* Brot. leaves were evaluated. Total flavonoid, chlorophyll and carotenoid contents, β -carotene bleaching effect, metal chelating ability, total antioxidant activity, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD), superoxide, hydroxyl and nitric oxide (NO) scavenging assays and antifungal activities of water and ethanol extracts were determined. The plant has a powerful antioxidant activity at various antioxidant systems *in vitro*. The metal chelating and radical scavenging activities of the water extract were higher than the ethanolic extract whereas the ethanolic extract exhibited higher β -carotene bleaching effect, total antioxidant activity and antifungal ability compared to water extract. Active extracts of *A. aestivus* Brot. might be an alternative to more toxic synthetic antioxidants as additives in food, pharmaceutical and cosmetic preparations.**

Key words: *Asphodelus aestivus* Brot., antioxidant activity, antifungal activity, free radical, scavenging activity.

INTRODUCTION

Free radicals are generated through normal reactions within the body during respiration in aerobic organisms. They can exert diverse functions like signalling roles and

providing defence against infections (Sarmadi et al., 2011). The free radicals can also be generated in biological systems in the form of reactive oxygen species (ROS), such as superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{\cdot}), the singlet oxygen (1O_2) and nitric acid (NO^3) radicals. In healthy individuals, there is equilibrium between the natural antioxidative defence system and ROS, generated from both living organisms and exogenous sources. When the equilibrium is disrupted, these ROS cause destructive and irreversible damage to the components of a cell, such as lipids, proteins and DNA, leading to age related degenerative diseases, cancer, diabetes, arthritis and cardiovascular diseases (Lee et al., 2004; Kannan et al., 2010; Liu et al., 2011).

Antioxidants are substances that when present in foods or body at low concentrations compared with that of an oxidizable substrate markedly delay or prevent the oxidation of that substrate and have function in the major signalling pathways of cells. Antioxidants may act by decreasing singlet oxygen concentration, intercepting singlet oxygen, preventing first chain initiation by scavenging initial radicals, binding metal ion catalysts,

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Abbreviations: **ABTS**, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; **BHA**, butylated hydroxyanisole; **BHT**, butylated hydroxytoluene; **DPPH**, 2,2-diphenyl-1-picryl-hydrazyl; **DTNB**, 5,5'-dithiobis(2-nitrobenzoic acid); ferrozine, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine; **NADH**, reduced nicotinamide adenine dinucleotide; **NBT**, nitroblue tetrazolium; **PMS**, phenazine methosulphate; **ROS**, reactive oxygen species; **TBA**, thiobarbituric acid; **TCA**, trichloroacetic acid; **Trolox**, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; **UV**, ultraviolet light; **PDA**, potato dextrose agar medium; **NO**, nitric oxide; **EDTA**, ethylenediaminetetraacetic acid; **DMPD**, N,N-dimethyl-p-phenylenediamine dihydrochloride.

decomposing primary products to non-radical compounds and chain breaking to prevent continued hydrogen abstraction from substrates. In order to protect foods and human beings against oxidative damage caused by free radicals, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary-butylhydroquinone (TBHQ) are used (Kannan et al., 2010; Liu et al., 2011; Tepe et al., 2011). However, many researchers reported the adverse effects of synthetic antioxidants such as toxicity, carcinogenicity and potential health hazards. Therefore, naturally occurring nutritive and non-nutritive antioxidants have recently become a major area of scientific research due to safety and limitation of synthetic antioxidant usage (Mariod et al., 2010; Tsaliki et al., 1999). Plants, including herbs and spices have many phytochemicals which are potential sources of natural antioxidants, for example, phenolic diterpenes, flavonoids, tannins and phenolic acids. Some studies have reported that extracts from natural products such as fruits, vegetables and medicinal herbs, have positive effects against cancer, the intake of plant flavonoids has been shown to be inversely related to the risk of cardiovascular disease and several flavonoids have been reported to quench active oxygen species and inhibit *in vitro* oxidation of low-density lipoproteins and therefore, reduce thrombotic tendency (Erkan et al., 2008; Skerget et al., 2005).

The genus *Asphodelus* (Asphodelaceae or Liliaceae) is a circum-Mediterranean genus, which includes five sections and it is presented by 16 species. *A. aestivus* Brot., is a common spring-flowering geophyte encountered on the Marmara, Aegean and Mediterranean coasts of Turkey, has been utilized traditionally for culinary and medicinal purposes (Matthews, 1984; Baytop, 1999). A new class of anthraquinone-anthrone-C-glycosides has been isolated from *A. ramosus*. Sesquiterpene lactones, flavonoids, and anthraquinones have been reported from *A. aestivus*, *A. globifera*, *A. anatolica* and *A. damascene*, whereas arylcoumarins, anthraquinones and glycosides have been isolated from *A. microcarpus*. The bulbs and roots of *A. microcarpus* are used as an antimicrobial agent (Adinolfi et al., 1991; van Wyk et al., 1995; Calis et al., 2006; El-Seedi, 2007). The leaves of *A. aestivus* Brot. are commonly consumed cooked as a vegetable dish in Turkey, where it is known as "ciris otu". In traditional medicine, the tuber and the roots of this plant are used against hemorrhoids, nephritis, burns and wounds (Tuzlacı and Aymaz-Eryasar, 2001; Ugurlu and Secmen, 2008). Extracts of *A. aestivus* roots were reported to have gastroprotective effect against ethanol-induced lesions (Gurbuz et al., 2002).

The objectives of this study were to access the antioxidant/antiradical activities of crude ethanolic and water extracts of leaves of *A. aestivus* Brot. on stable radicals using different chemical reaction based assays including the β -carotene bleaching effect, metal chelating

ability, total antioxidant and scavenging activities of these extracts. We also studied their total flavonoid, chlorophyll and carotenoid contents. These antioxidant activities were compared to those of standard synthetic antioxidants such as BHA, BHT, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), epicatechin, ascorbic acid and α -tocopherol. The antifungal activity of the extracts was also determined against a fungus (*Aspergillus niger*). The results were compared with Fluconazole as standard antifungal drug.

MATERIALS AND METHODS

Plant material and extraction procedure

The leaves of *A. aestivus* Brot. were purchased in May from a local market in Istanbul, Turkey and identified by Associate Prof. Dr. Tamer Ozcan (Institute of Botany, Department of Biology, Istanbul University). Plant materials were washed with deionized water and dried at room temperature. The dried parts were put in a plastic bag and stored at -20°C until used.

The water extract of leaves of *A. aestivus* Brot. was prepared by boiling 20 g plant material in 200 ml of distilled water in a flask due to infuse for 15 min while stirring. The ethanolic extract was prepared by refluxing 20 g of the dried leaves with 200 ml ethanol for 2 h at Soxhlet apparatus. After filtering through linen cloth, the filtrates were evaporated to dryness under reduced pressure using a rotary evaporator, then they were weighed to determine the total extractable compounds. This crude extracts were kept at -20°C and used for the assessment of antioxidant and antifungal activities.

Determination of total flavonoid

The total flavonoid contents of extracts were determined according to colorimetric method (Zhishen et al., 1999). The amount of total flavonoids was calculated as μg of (+)-catechin equivalents from the calibration curve of (+)-catechin standard solution (covering the concentration range between 2-20 $\mu\text{g}/\text{ml}$) and expressed as μg catechin/mg of extract. Data were presented as the average of triplicate analyses.

Determination of total chlorophyll and total carotenoid

Total chlorophyll and total carotenoid content were calculated using the modified equations of Lichtenthaler and Wellburn (1983). 10 mg of each extract of leaves was dissolved with 10 ml distilled water. The absorbance of the sample was measured at 450, 645 and 663 nm in the ultraviolet (UV)-Vis light spectrophotometer. The total chlorophyll and total carotenoid content were calculated using the equations as followed:

$$\text{Chlorophyll a} = 12.7A_{663} - 2.69A_{645}$$

$$\text{Chlorophyll b} = 22.9A_{645} - 4.68A_{663}$$

$$\text{Total Chlorophyll} = 20.2A_{645} + 8.02A_{663}$$

$$\text{Total Carotenoid} = 4.07A_{450} - [(0.0435 \times \text{Chlorophyll a}) + (0.367 \times \text{Chlorophyll b})]$$

β -Carotene bleaching test

Approximately 10 mg of trans- β -carotene was dissolved in 10 ml of

chloroform and 0.2 ml of the solution was placed in a boiling flask containing 20 mg linoleic acid and 200 mg of Tween-40. After removal of chloroform, 50 ml of distilled water was added to the flask and vigorously shaken. 5 ml of this emulsion were added to tubes containing the 0.2 ml of *A. aestivus* Brot. leaf extracts. The extracts were dissolved in distilled water (2 mg/ml). The tubes were stopped and placed in a water bath at 50°C. Spectrophotometric readings at 470 nm were taken after 60 and 120 min of incubation. BHA (2 mg/ml) was used for comparative purposes. A control solution containing 0.2 ml of distilled water and 5 ml of the above emulsion was prepared. Relative antioxidant activities (RAA) were calculated with the following formula (Bruni et al., 2004):

$$\text{RAA} = \text{Absorbance of sample} / \text{Absorbance of BHA}$$

Chelating activity on ferrous ion

The chelating activity of the aqueous and ethanolic extract on ferrous ions (Fe^{2+}) was measured according to the method of Decker and Welch (1990). Aliquots of 1 ml of different concentrations (0.25, 0.50, 0.75 and 1.0 mg/ml) of the samples were mixed with 3.7 ml of deionized water. The mixture was incubated with FeCl_2 (2 mM, 0.1 ml) for 5, 10, 30 and 60 min. After incubation the reaction was initiated by addition of ferrozine (5 mM and 0.2 ml) for 10 min at room temperature, and then the absorbance was measured at 562 nm in a spectrophotometer. A lower absorbance indicates a higher chelating power. The chelating activity of the extract on Fe^{2+} was compared with that of ethylenediaminetetraacetic acid (EDTA) at a level of 0.1 mM. Chelating activity was calculated using the following formula:

$$\text{Chelating activity (\%)} = [1 - (\text{Absorbance of sample} / \text{Absorbance of control})] \times 100$$

Control test was performed without addition of the sample. Extract concentration providing 50% inhibition (IC_{50}) was obtained plotting inhibition percentage versus extract solutions concentrations.

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) radical-scavenging assay

The free radical scavenging activity of the aqueous and ethanolic extracts were measured with DPPH $^{\cdot}$ using the slightly modified method (Brand-Williams et al., 1995). Briefly, 20 mg/L DPPH $^{\cdot}$ solution in methanol was prepared and 1.5 ml of this solution was added to 0.75 ml of the sample, BHA, BHT, α -tocopherol and ascorbic acid solution (20-100 $\mu\text{g/ml}$). The mixture was shaken vigorously and kept at room temperature for 30 min. Then the absorbance of the mixture was measured at 517 nm. Water (0.75 ml) in place of the plant extract was used as control. The decrease in the absorbance indicates an increase in DPPH $^{\cdot}$ radical scavenging activity. This activity was calculated by the equation below:

$$\text{DPPH Scavenging Effect (\%)} = [(A_0 - A_1)/A_0 \times 100],$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the extracts or standards. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration.

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS $^{+\cdot}$) scavenging assay

For ABTS cation radical scavenging assay, the procedure followed the method of Arnao et al. (2001) with some modifications. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and incubating them for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS $^{+\cdot}$ solution with 60 ml methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using the spectrophotometer. Fresh ABTS $^{+\cdot}$ was prepared for each assay. Plant extracts (150 μl) were allowed to react with 2850 μL of the ABTS $^{+\cdot}$ solution for 2 h in the dark. Then the absorbance was measured at 734 nm using the spectrophotometer. The standard curve was linear between 100 and 500 μM Trolox. Results are showed as μM Trolox equivalents (TE)/ μg extract.

N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD $^{+\cdot}$) scavenging assay

DMPD radical scavenging ability of the extracts were performed according to the method of Fogliano et al. (1999). 100 mM DMPD was prepared in deionized water and 1 ml of this solution was added to 100 ml of 0.1 M acetate buffer (pH 5.3), and the coloured radical cation (DMPD $^{+\cdot}$) was obtained by adding 0.2 ml of a solution of 0.05 M FeCl_3 . The absorbance of this solution, which is freshly prepared daily, is constant up to 12 h at room temperature. Different concentrations (20 to 100 $\mu\text{g/ml}$) of extracts or standard antioxidants were added in a test tubes and the total volume was adjusted with distilled water to 0.5 ml. After 10 min, the absorbance was measured at 505 nm. 1 ml of DMPD $^{+\cdot}$ solution was directly added to the reaction mixture and its absorbance at 505 nm was measured. The buffer solution was used as a blank sample. The scavenging capability of DMPD $^{+\cdot}$ radical was calculated using the following equation:

$$\text{DMPD}^{+\cdot} \text{ scavenging effect (\%)} = [(A_0 - A_1)/A_0 \times 100],$$

where A_0 is the absorbance of the initial concentration of DMPD $^{+\cdot}$ and A_1 is the absorbance of the remaining concentration of DMPD $^{+\cdot}$ in the presence of sample solution.

Inhibition on linoleic acid peroxidation

The antioxidant activity was determined according to the thiocyanate method with slight modifications (Osawa and Namiki, 1981). For the stock solution, 10 mg of extracts were dissolved in 10 ml water. Then, the different amounts of stock solution or standards samples (20-100 μg) prepared in 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 ml of linoleic acid emulsion. Linoleic acid emulsion contains Tween-20 (175 μg), linoleic acid (155 μl) and potassium phosphate buffer (0.04 M, pH 7.0). In addition, a solution containing 2.5 ml of linoleic acid emulsion and 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.0) was prepared as control. Each solution was then incubated at 37°C in a glass flask in the dark. At 24 h intervals during incubation, 0.1 ml of this incubation solution was added to 4.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. Precisely 3 min after addition of 0.1 ml of 0.02 M FeCl_2 in 3.5% (w/v) HCl to the reaction mixture, the absorbance of the red colour was measured at 500 nm in a spectrophotometer. The solutions without added extracts or standards were used as a control. The inhibition

of lipid peroxidation in percentage was calculated by the following equation:

$$\text{Inhibition \%} = [(A_0 - A_1)/A_0 \times 100],$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the extracts or standards.

Superoxide radical scavenging activity

Measurement of superoxide anion scavenging activity of water and ethanolic extracts of *A. aestivus* Brot. were based on the method described by Liu et al. (1997). Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide anion was generated in 3 ml of Tris-HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 μ M) solution, 1 ml of NADH (78 μ M) solution and different concentrations (20-100 μ g/ml) of sample solution. The reaction was started by adding 1 ml of PMS solution (10 μ M) to the mixture. The reaction mixture was incubated at 25°C for 5 min and absorbance at 560 nm was recorded against blank samples in a spectrophotometer. Epicatechin, BHA and Trolox were used as standard samples (20-100 μ g/ml). The inhibition of superoxide radical generation (%) was calculated by the following equation:

$$\text{Inhibition \%} = [(A_0 - A_1)/A_0 \times 100],$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the extracts or standards.

Hydroxyl radical scavenging activity

The effect of hydroxyl radical was assayed by using the 2-deoxyribose oxidation method (Chung et al., 1997). 2-Deoxyribose is oxidized by the hydroxyl radical that is formed by the Fenton reaction and degraded to malondialdehyde. The reaction mixture contained 0.45 ml of 0.2 M sodium phosphate (pH 7.4), 0.15 ml of 10 mM 2-deoxyribose, 0.15 ml of 10 mM FeSO₄-EDTA, 0.15 ml of 10 mM hydrogen peroxide, 0.525 ml of distilled water and 0.075 ml (20-100 μ g/ml) of sample solution in a tube. The reaction was started by the addition of hydrogen peroxide. After incubation at 37°C for 4 h, the reaction was stopped by adding 0.75 ml of 2.8% (w/v) trichloroacetic acid (TCA) and 0.75 ml of 1.0% (w/v) of thiobarbituric acid (TBA). The mixture was boiled for 10 min, cooled in ice and then measured at 520 nm. The reaction mixture not containing test sample was used as control. The inhibition of superoxide radical generation (%) was calculated as:

$$\text{Inhibition (\%)} = [(A_0 - A_1)/A_0 \times 100],$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the extract sample. The extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting extract solution concentration versus inhibition percentage.

Nitric oxide (NO) scavenging activity

The scavenging effect of extracts on NO was measured according to the slightly modified method of Marcocci et al (1994). 4 ml of water or ethanolic extract solutions of different concentrations were

then added, in the test tubes, to 1 ml sodium nitroprusside solution (10 mM), in phosphate-buffered saline, and the test tubes incubated at 37°C for 3 h. The same reaction mixture, without the extracts but with an equivalent amount of water, served as control. An aliquot (0.5 ml) of the incubation solution was removed diluted with 0.5 ml Griess reagent (1% sulfanilamide in 5% H₃PO₄ and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 570 nm and referred to the absorbance of standard solutions of sodium nitrite treated in the same way with Griess reagent.

Antifungal activity against *Aspergillus niger*

The antifungal activity of water and ethanolic extracts were determined on *A. niger* using agar well diffusion method as performed by Schillinger and Luke (1989). The stock solution of the extracts was prepared at 1 mg/ml concentration and was suitably diluted with sterilized distilled water to get dilution of 0.25 and 0.50 mg/ml. Control for each dilution was prepared with sterilized distilled water. The fungus was subcultured in potato dextrose agar medium (PDA). PDA plates were inoculated with the freshly grown culture by spreading method. Wells (6 mm diameter) were punched in the agar and loaded with the 150 μ L samples of the samples. Standard antifungal drug (Fluconazole) was used for comparison. The plates were incubated at 37°C for 24 h. Activity was determined by measuring the diameter of the zone showing complete inhibition (cm). Antifungal activity was calculated as a mean of three replicates.

Statistical analysis

The experimental results were given as mean \pm standard deviation of three parallel measurements. Statistical comparisons were performed using the Student's t-test. Differences were considered significant at $p < 0.05$.

RESULTS

Determination of total flavonoid

Total flavonoid contents of water and ethanolic extracts from *A. aestivus* Brot. leaves were shown in Table 1. The results were expressed as catechin equivalent of flavonoids per mg of sample extract. The total flavonoid content of water extract (3.57 ± 0.03 μ g catechin/mg of extract) was nearly two fold higher than ethanolic extract (1.46 ± 0.01 μ g catechin/mg of extract). The method using water showed a greater efficiency in the extraction of flavonoids than that with ethanol. However, the extraction with water resulted in lower yields of extractable compounds. The yield for ethanol and water extraction was 1.80 g (36%) and 1.34 g (26%), respectively.

Determination of total chlorophyll and total carotenoid

Total chlorophyll and carotenoid contents of extracts of *A.*

Table 1. The total flavonoid, chlorophyll and carotenoid contents of extracts from *A. aestivus* Brot. leaves.

Extract	Chlorophyll a ($\mu\text{g/ml}$) ^a	Chlorophyll b ($\mu\text{g/ml}$) ^a	Total Chlorophyll ($\mu\text{g/ml}$) ^a	Total Carotenoid ($\mu\text{g/ml}$) ^a	Total Flavonoid Content ($\mu\text{g catechin/mg of extract}$) ^a
Ethanol	1.02 \pm 0.02	1.96 \pm 0.04	2.98 \pm 0.06	0.16 \pm 0.02	1.46 \pm 0.01
Water	1.06 \pm 0.06	2.14 \pm 0.12	3.20 \pm 0.06	0.40 \pm 0.01	3.57 \pm 0.03

^aValues were the means of three replicates \pm standard deviation (SD).

Table 2. β -Carotene bleaching test^a.

Sample	RAA ^b 60 min	RAA 120 min
Ethanol extract	4.53 \pm 0.02	4.23 \pm 0.04
Water extract	1.56 \pm 0.06	1.39 \pm 0.02
BHA	1	1
Negative control ^c	0,58 \pm 0,02	0,36 \pm 0,05

BHA was used as reference antioxidant. ^aValues were the means of three replicates \pm standard deviation (SD). ^bRelative antioxidant activities. ^cNegative control: Linoleic acid and β -carotene emulsion.

aestivus Brot. were shown in Table 1. Water extract of the plant exhibited significantly higher total chlorophyll and carotenoid content compared to ethanolic extract.

β -Carotene bleaching test

In the β -carotene bleaching test, the ethanol and water extracts of *A. aestivus* Brot. were exhibited higher activity than the positive control BHA at 60 min (4.53 \pm 0.02 and 1.56 \pm 0.06, respectively) and this activity was only slightly reduced after 120 min (4.23 \pm 0.04 and 1.39 \pm 0.02) of incubation (Table 2).

Chelating activity on ferrous ion

The ferrous ion (Fe^{2+}) chelating effect of *A. aestivus* Brot. extracts is presented in Figure 1. The chelating activity of the extracts was concentration dependent. At the same amounts, water extract exhibited higher chelating activity on Fe^{2+} than ethanolic extract. The water extract at 1.0 mg/ml concentration showed 62.83 \pm 1.86% chelating effect on ferrous ions at an incubation time of 60 min. According to our results, the chelating activity of *A. aestivus* water extract of 1.0 mg/ml was higher than EDTA at 0.1 mM concentration (60.97 \pm 1.14%) for an incubation time of 60 min. IC₅₀ values (the inhibitory concentration at which ferrous ions were chelated by 50%) were obtained for the ethanol (4.61 \pm 0.22 mg/ml) and water (2.97 \pm 0.10 mg/ml) extracts.

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) radical-scavenging assay

The DPPH radical scavenging effects of *A. aestivus* extracts were presented in Figure 2. The free radical scavenging activity of the water extract was higher than ethanolic extract at all concentrations. The ethanolic extract exhibited activity of 33.92 \pm 0.12% at a concentration of 100 $\mu\text{g/ml}$. The water extract showed an inhibition of 48.91 \pm 0.42% at a concentration of 100 $\mu\text{g/ml}$. However when compared to reference antioxidants, BHA, BHT, α -tocopherol and ascorbic acid, all of the tested extracts showed significantly ($p < 0.05$) lower DPPH radical scavenging activity. IC₅₀ value of water extract (4.58 \pm 0.04 mg/ml) was higher than that of ethanol (9.54 \pm 0.14 mg/ml) extract. A higher DPPH radical scavenging activity is associated with a lower IC₅₀ value.

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{•+}) scavenging assay

The antioxidant activity measurements of ethanolic and water extracts from *A. aestivus* Brot. are expressed as Trolox equivalent antioxidant capacity was presented in Table 3. All extracts showed antioxidant activities proving their capacity to scavenge the ABTS^{•+} radical cation. ABTS^{•+} scavenging ability of ethanolic and water extracts of *A. aestivus* Brot. leaves were 320.20 \pm 2.25 and 320.00 \pm 1.68 μmol Trolox equivalent at 100 $\mu\text{g/ml}$, respectively.

N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD^{•+}) scavenging assay

The DMPD^{•+} radical inhibitions of water and ethanolic extracts of *A. aestivus* Brot. and standard antioxidants decreased in the order of ascorbic acid (97.98%)>Trolox (82.71%)>BHA (47.12%)>water extract (27.09%)>ethanolic extract (17.01%), in the presence of 100 $\mu\text{g/ml}$ test sample (Figure 3).

Inhibition on linoleic acid peroxidation

The effect of *A. aestivus* extracts at different

Table 3. ABTS^{•+} scavenging ability of the extracts from *A. aestivus* Brot. leaves.

Extract	ABTS ^{•+} scavenging ability ($\mu\text{mol Trolox} / \mu\text{g of extract}$) ^a				
	20 $\mu\text{g/ml}$	40 $\mu\text{g/ml}$	60 $\mu\text{g/ml}$	80 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$
Ethanol	301.73 \pm 2.24	306.45 \pm 1.91	308.25 \pm 1.02	314.42 \pm 1.98	320.20 \pm 2.25
Water	299.43 \pm 2.44	304.06 \pm 2.17	310.08 \pm 2.13	314.55 \pm 1.26	320.00 \pm 1.68

^aValues were the means of three replicates \pm standard deviation (SD). The results were expressed as $\mu\text{mol Trolox}$ equivalent at 20, 40, 60, 80, 100 $\mu\text{g/ml}$ of extract, respectively.

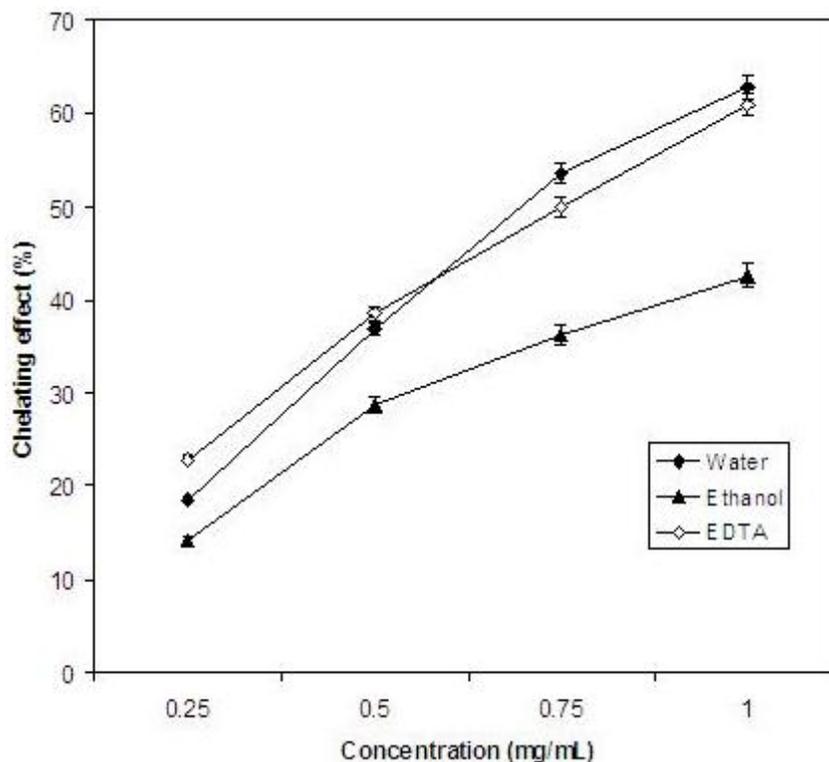


Figure 1. Chelating effects of different concentrations of ethanol and water extracts on Fe²⁺ ion at different incubation times. Values are means \pm SD. (n = 5).

concentration on peroxidation of linoleic acid emulsion was shown in Figure 4. The antioxidant activity of *A. aestivus* ethanol and water extract was compared with commercial antioxidants such as α -tocopherol, BHT and BHA. The extracts showed maximum antioxidant activity at 100 $\mu\text{g/ml}$ concentration. The ethanol extract exhibited higher activity when compared with the water extract. Total antioxidant activity of the ethanol extract (65.06 \pm 0.05%) at 100 $\mu\text{g/ml}$ did not show any significant difference ($p > 0.05$) from that of BHA (70.82 \pm 0.08%) and BHT (69.98 \pm 0.56%) at the same concentration. The ethanol extract, at higher concentration (100 $\mu\text{g/ml}$), exhibited higher activity than the well-known standard α -tocopherol at 100 $\mu\text{g/ml}$ (57.17 \pm 0.23%). These results

showed that the *A. aestivus* Brot. ethanol extract has a strong total antioxidant activity compared to α -tocopherol.

Superoxide radical scavenging activity

Superoxide radical scavenging effect of the extracts was represented in Figure 5. The water extract had a higher superoxide radical scavenging ability than the ethanol extract. At a concentration of 100 $\mu\text{g/ml}$, the inhibition activities were 83.08 \pm 1.21% and 73.85 \pm 1.36% for the water and ethanol extract, respectively. The superoxide radical scavenging activity by 100 $\mu\text{g/ml}$ of ascorbic acid, Trolox, epicatechin and BHA was found to be 92.31 \pm 1.35%,

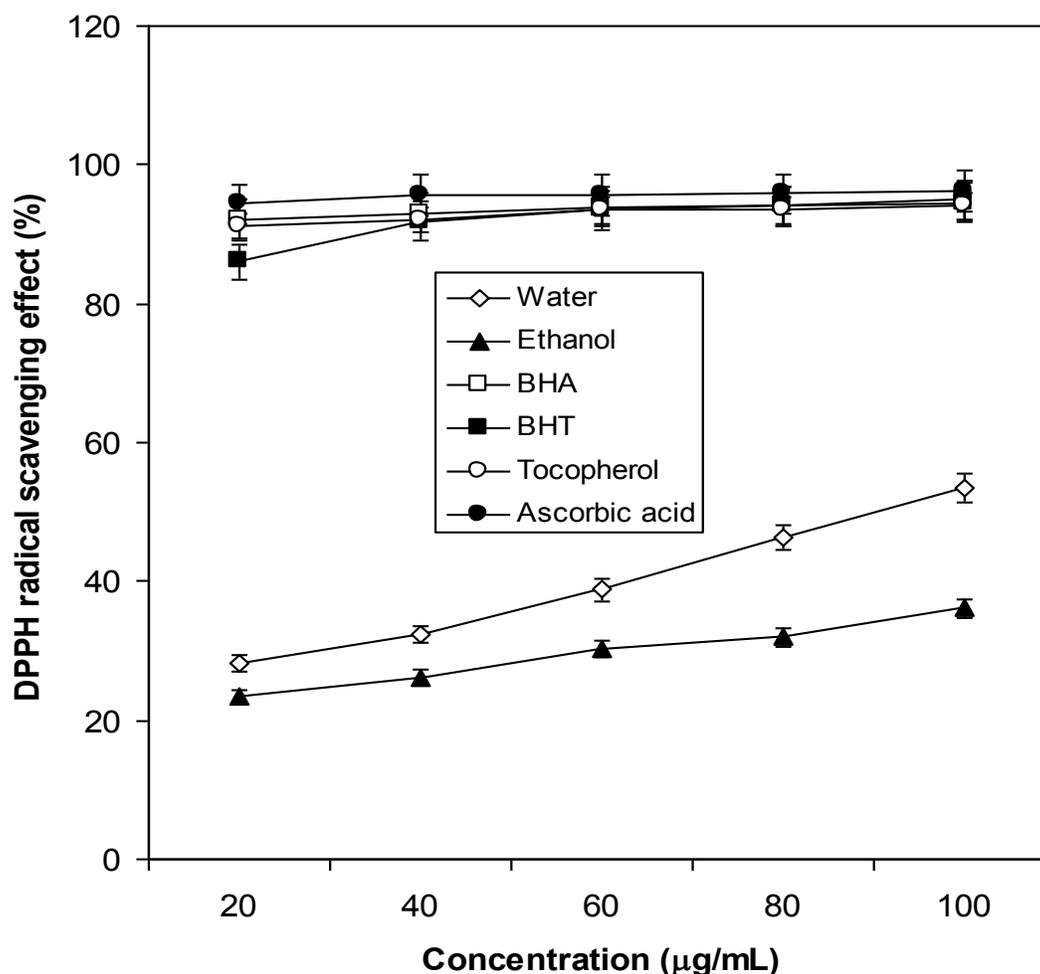


Figure 2. DPPH radical scavenging activity of the extracts from *A. aestivus* Brot. leaves. α -Tocopherol, ascorbic acid, BHA and BHT were used as reference antioxidants. Values are means \pm SD, (n = 3).

87.69 \pm 1.11%, 84.62 \pm 0.52%, and 67.69 \pm 1.22%, respectively. Water and ethanolic extract exhibited significantly higher superoxide anion scavenging activity than BHA ($p < 0.05$).

Hydroxyl radical scavenging activity

Figure 6 shows the hydroxyl radical scavenging effects determined by the 2-deoxyribose oxidation method. At 20 to 100 μ g/ml concentrations, hydroxyl radical scavenging activity of the water extract of *A. aestivus* Brot. leaves were higher than BHA, BHT and α -tocopherol. At 100 μ g/ml concentration, ethanol extract of *A. aestivus* Brot. leaves showed a higher hydroxyl radical scavenging activity when compared to the BHT and α -tocopherol. IC_{50} values in scavenging abilities on hydroxyl radicals were 5.70 \pm 0.02 μ g/ml and 6.20 \pm 0.12 for water and ethanol extracts, respectively.

Nitric oxide (NO) scavenging activity

The NO scavenging effect of the extracts of *A. aestivus* Brot. leaves were shown in Figure 7. Sodium nitroprusside is known to decompose in PBS solution and then to produce NO. NO, under aerobic conditions, reacts with oxygen to form nitrate and nitrite, which can be determined using Griess reagent. Our results showed that the extracts generated a dose-dependent inhibition of nitrite production. The nitrite levels of the water and ethanol extracts at a concentration of 50 μ g/ml were 55.30 \pm 1.57 and 60.33 \pm 1.32 mmol, respectively, indicating that the extracts had a similar high level of scavenging activity on NO.

Antifungal activity against *Aspergillus niger*

All the tested extracts showed a remarkable antifungal

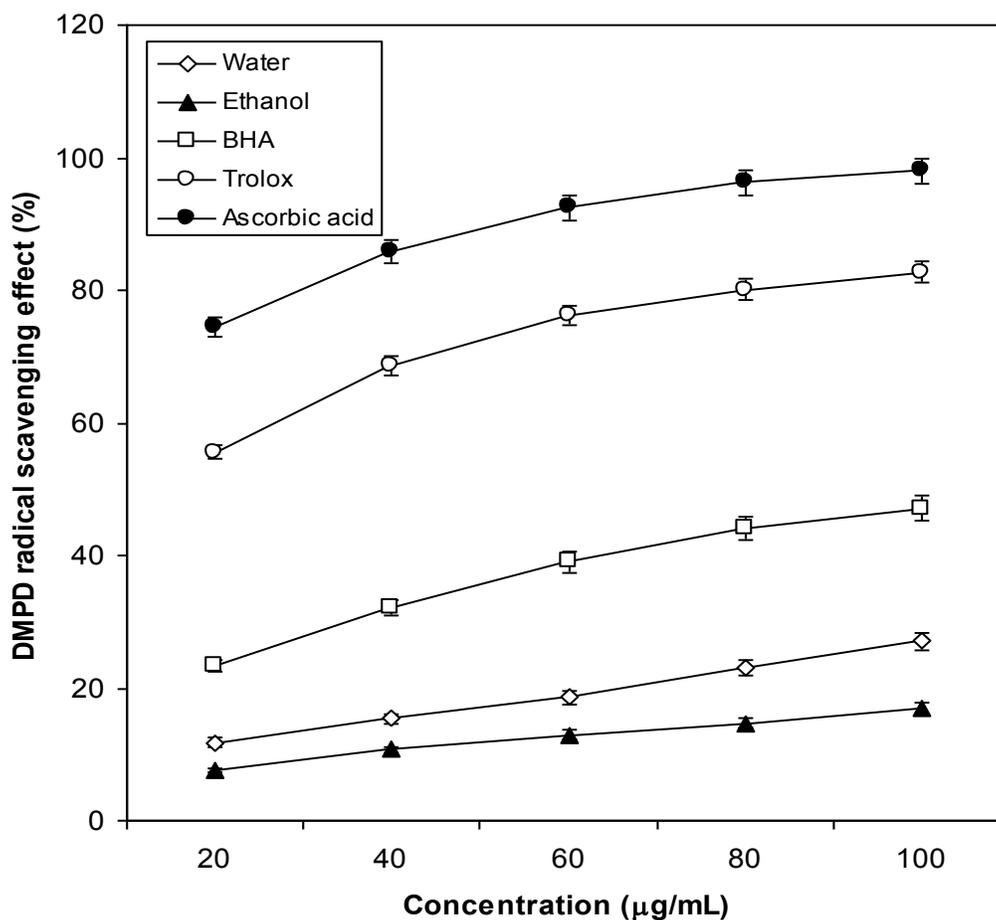


Figure 3. DMPD radical scavenging activity of the extracts from *A. aestivus* Brot. leaves. Trolox, ascorbic acid and BHA were used as reference antioxidants. Values are means \pm SD (n = 3).

activity against *A. niger*. The results were shown in Figure 8. The ethanol extract showed higher activity at the concentrations of 0.25 and 0.50 mg/ml when compared with water extract. However, ethanol and water extract exhibited similar activities at the concentration of 1 mg/ml. Standard antifungal drug Fluconazole exhibited higher activity than the extracts tested (2.5 cm of inhibition zone at a 100 µg/ml concentration).

DISCUSSION

This paper provides the first documentation on the antioxidant and antifungal capacities of *A. aestivus* Brot. leaves extract growing in Turkey as well as their ethanolic and water extractions. The total flavonoids content is reported as catechin equivalents by reference to standard curve ($Y = 0.0686X + 0.0128$ and $r^2 = 0.09998$). It was found that leaves had the highest flavonoid contents. Flavonoids are a main class of polyphenols in plants.

They are known as antioxidants and free radical scavengers. The antioxidant activity of plants has been correlated to the total flavonoid content. Our results are higher than the work of Sulaiman et al. (2011) who reported that the ethanolic extract (1.0 ± 0.3 mg quercetin/g dry weight) of *Ocimum basilicum* and water extract (1.4 ± 0.2 mg quercetin/g dry weight) of *Lactuca sativa* also had flavonoid contents. The major group of the pigments, chlorophyll a and b, and carotenoids are the non-polyphenolic compounds of plants. It has been known that all these pigments exhibit significant antioxidant activities. Therefore, flavonoid, chlorophyll a and b, and carotenoid as non-polyphenolic compounds might also contribute to antioxidant activities of the water and ethanolic extract. β -Carotene has a strong biological activity and it is a physiologically important compound. Two important properties of β -carotene are its ability to trap certain organic free radicals and to deactivate the excited molecules, particularly excited or singlet oxygen.

A. aestivus Brot. leaf extracts exhibited antioxidant

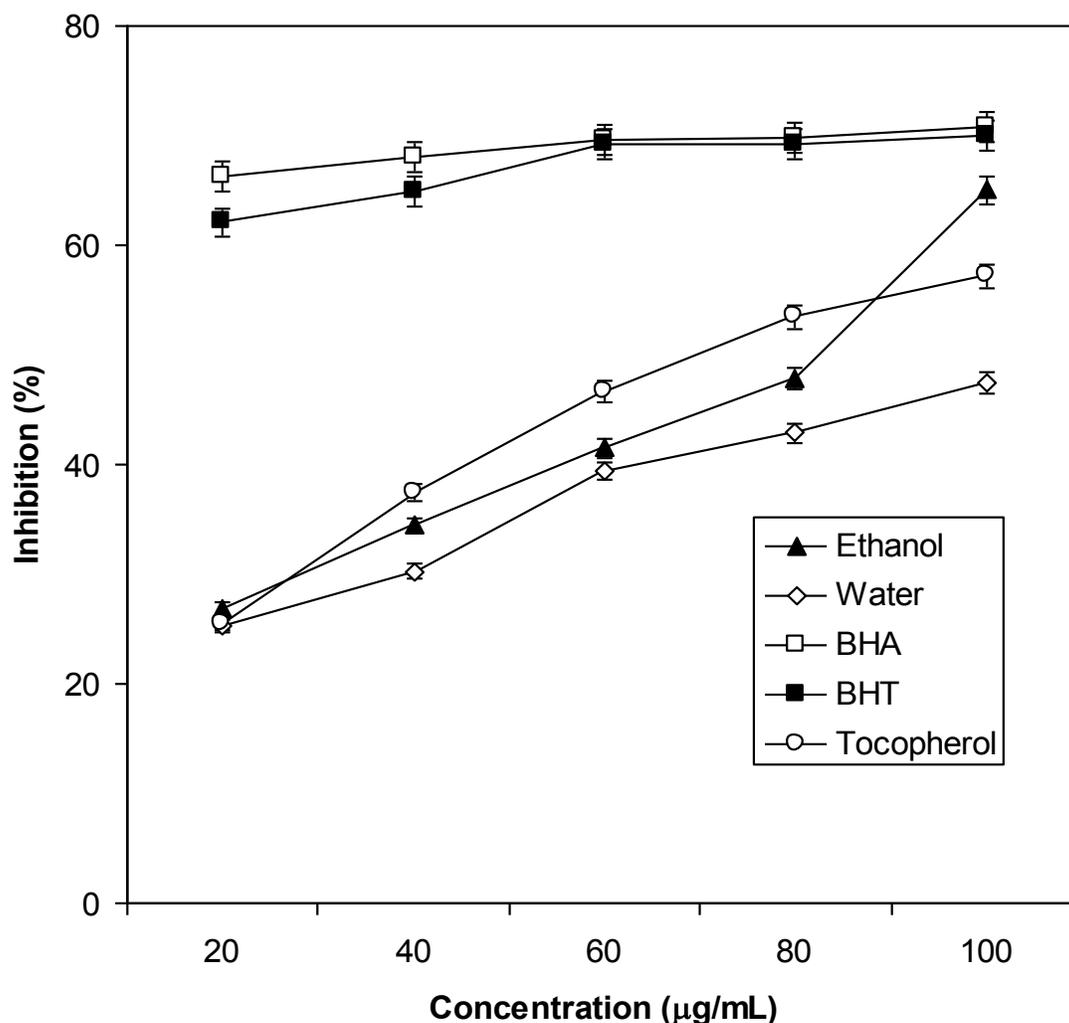


Figure 4. Inhibition on linoleic acid peroxidation of ethanolic and water extracts from *Asphodelus aestivus* Brot. leaves measured by ferric thiocyanate method. α -Tocopherol, BHA and BHT were used as reference antioxidants. Values are means \pm SD (n = 3).

activity in the β -caroten linoleate model system. In this test, β -carotene underwent rapid discoloration in the absence of an antioxidants. During oxidation, a hydrogen atom was abstracted from the active bis-allylic methylene group of linoleic acid located on carbon-11 between two double bonds. The pentadienyl free radical so formed then attacked highly unsaturated β -carotene molecules in an effort to reacquire a hydrogen atom. As the β -carotene molecules lost their conjugation, the carotenoids lost their characteristic orange colour; this process was monitored spectrophotometrically. Our result obtained with the ethanolic and water extract of *A. aestivus* Brot. leaves was higher than that obtained from the methanolic extracts of *Cistus ladanifer* (0.66 ± 0.01), and *Cupressus lusitanica* leaves (0.44 ± 0.04) at 120 min (Guimaraes et al., 2010). Thus, it is apparent that *A. aestivus* extracts

have strong effects against the discoloration of β -carotene. Chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. The data obtained from our chelating activity on ferrous ion study reveals that *A. aestivus* Brot. extracts have effective capacity for iron binding, suggesting that its action as an antioxidant might be related to its iron binding capacity.

Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in biological systems. Hydrogen-donating ability is an index of the primary antioxidants. These antioxidants donate hydrogen to free radicals, leading to non-toxic species and therefore to inhibition of the propagation phase of lipid oxidation. DPPH assay

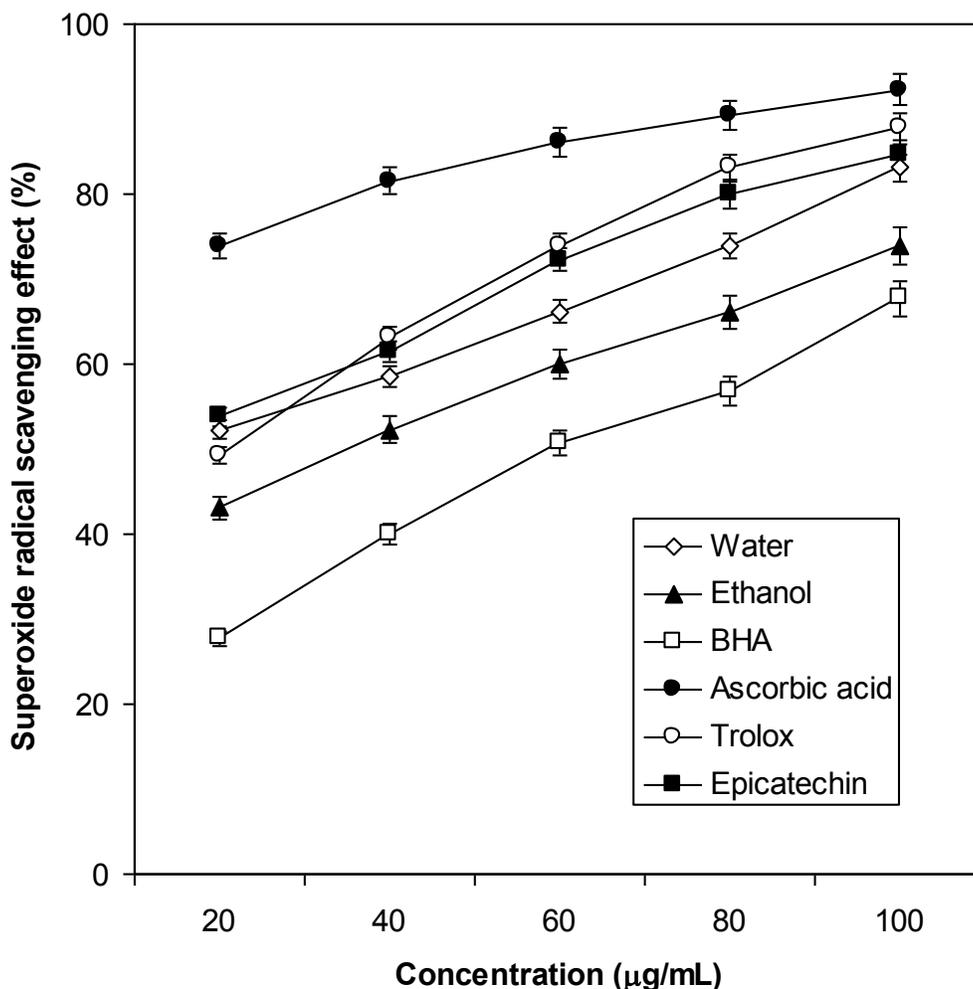


Figure 5. Superoxide radical scavenging activity of the extracts from *Asphodelus aestivus* Brot. leaves. BHA, ascorbic acid, Trolox and epicatechin were used as reference antioxidants. Values are means \pm SD (n = 3).

evaluates the ability of antioxidants to scavenge free radicals. All the extracts showed antiradical activity by inhibiting DPPH radical in a concentration dependent manner. The DPPH scavenging activities of the extracts, expressed as an IC_{50} value (the inhibitory concentration at which the DPPH radicals were scavenged by 50%). It was evident that the extracts did show the hydrogen donating ability to act as antioxidants. One of the most commonly used organic radicals for the evaluation of antioxidant efficiency of pure compounds and complex mixtures is the radical cation derived from ABTS. These radical cations could be generated by enzymatic, chemical, and electrochemical means. The extend and the rapidity with which polyphenols quench the ABTS radical cation chromophores are the criteria that are used to assess their relative antioxidant capacity compared to a standard antioxidant, Trolox. In these ABTS^{•+}-based

methods it is assumed that the antioxidants simply reduce the radicals back to the parent substrate, ABTS (Osman et al., 2006). Our results showed that the ethanolic and water extracts of the plant exhibited a higher ABTS^{•+} scavenging activity. DMPD^{•+} scavenging assay focuses on the ability of the antioxidant compounds to transfer a hydrogen atom to the coloured radical DMPD^{•+} turning it into an uncoloured DMPD⁺ compound. Dark colour of DMPD^{•+} radical cation solution becomes lighter and absorbance of solution becomes lower, in the presence of an antioxidant compound. The DMPD^{•+} radical cation solution shows a maximum absorbance at 505 nm. Antioxidant compounds that are hydrogen donors to DMPD^{•+} quench the colour of DMPD^{•+} solution. According to data of the present study, DMPD^{•+} radical scavenging of the extracts and standard antioxidants were positively correlated with increasing concentration

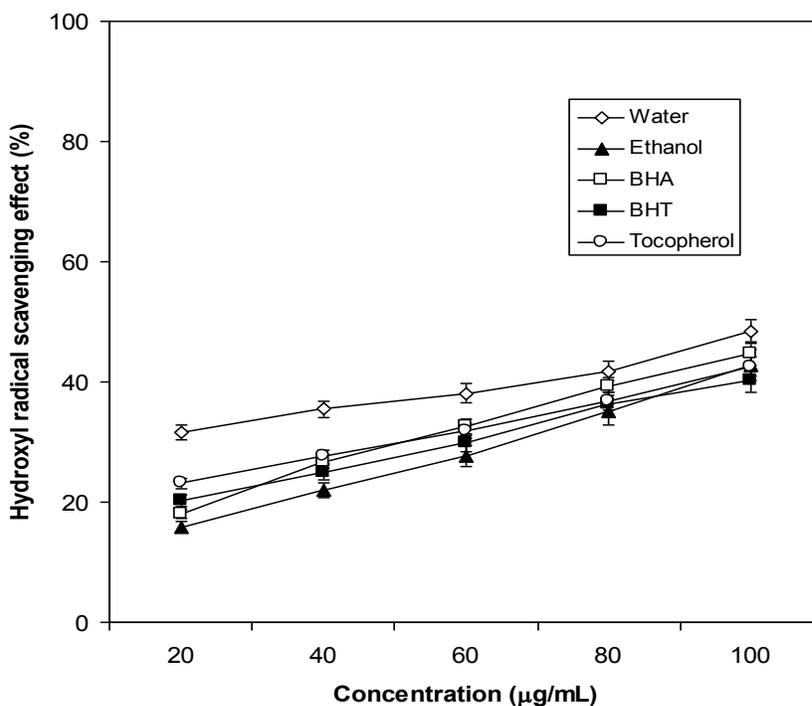


Figure 6. Hydroxyl radical scavenging activity of the extracts from *Asphodelus aestivus* Brot. leaves. BHA, BHT and Trolox were used as reference antioxidants. Values are means \pm SD (n = 3).

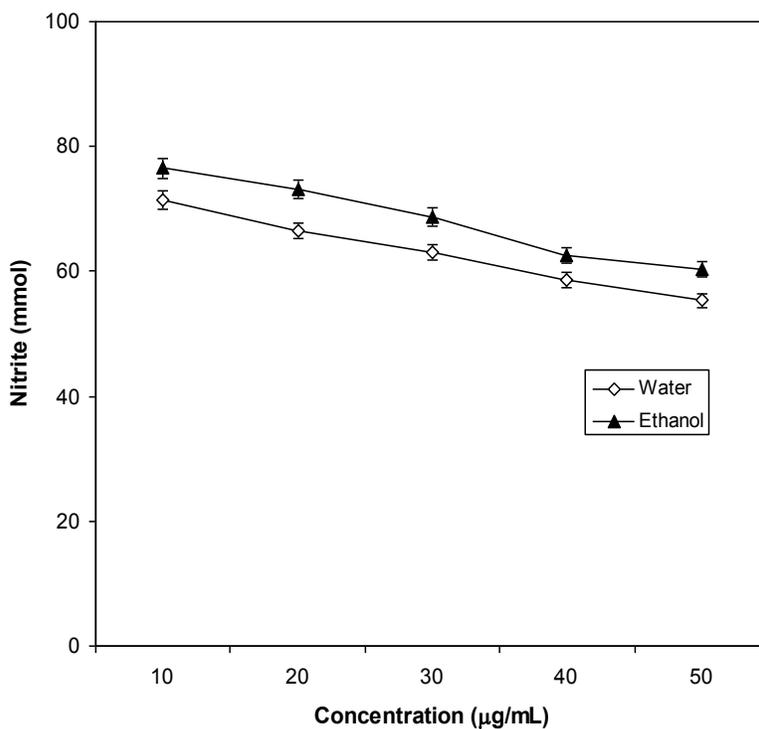


Figure 7. Nitric oxide (NO) scavenging effect of water and ethanol extracts from *Asphodelus aestivus* Brot. leaves. NO was generated with sodium nitroprusside during incubation. Values are means \pm SD (n = 3).

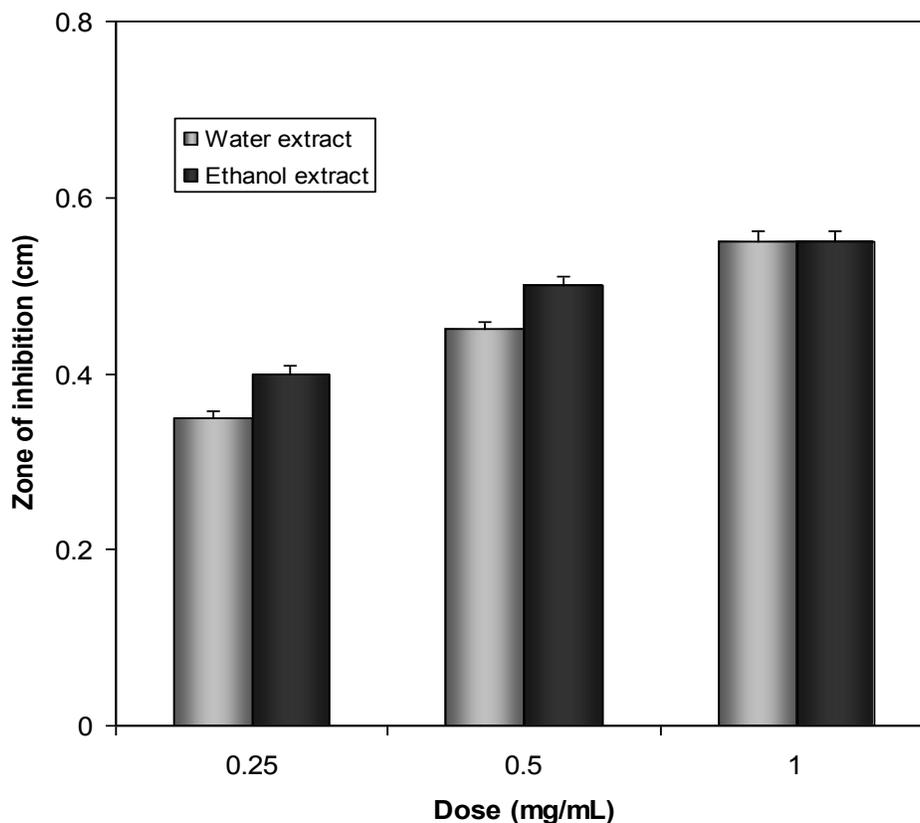


Figure 8. Antifungal activity of water and ethanol extracts against *Aspergillus niger* at different concentrations. Values are means \pm SD (n = 3).

of samples. The analyses indicated that there was a significant correlation between the DMPD cation radical scavenging ability for the extracts. Our results are higher than the work of Bursal and Koksall (2011) who reported that the ethanolic extract (2.6%) of *Rhus coriaria* L. also had DMPD radical inhibition. Total antioxidant activity of *A. aestivus* Brot. extracts were determined by the thiocyanate method in linoleic acid emulsion. The amount of peroxide in the initial stages of lipid oxidation was measured every 24 h, over a period of 6 days. The extracts showed maximum antioxidant activity at 100 μ g/ml concentration. The superoxide anions are well-recognized free radical species and they are generated continuously by several cellular processes, including the microsomal and mitochondrial electron transport systems. Although the superoxide anion is limited in activity, its combination with other reactive species, such as NO that is produced by macrophages, might yield more reactive species. Our results showed that water and ethanolic extract from the leaves has a scavenging effect on superoxide radicals. The superoxide radical scavenging activity of those samples were in the following order ascorbic acid > Trolox > epicatechin > water extract >

ethanol extract > BHA. The hydroxyl radical is a highly reactive free radical species and capable of damaging almost every molecule found in living cells. This damage causes aging, cancer and several diseases. The hydroxyl radical scavenging effects of ethanolic and water extracts were determined at the concentration of 20-100 μ g/ml.

Our results suggested that *A. aestivus* Brot. extracts have a strong hydroxyl radical scavenging activity. The results is higher to the work of Peksel et al (2006) who reported that the extract of *Portulaca oleracea* subsp *sativa* L. also had hydroxyl radical scavenging activity. NO is synthesized in many different mammalian cells types such as endothelial cells, vascular smooth muscular cells, neurons, platelets, macrophages and neutrophils. NO or some related reactive nitrogen species act as a neurotransmitter, prevent platelet aggregation and they are one of the defence molecules of immune system against tumor cells, parasites and bacteria. However, large amounts of NO, peroxy nitrite and other reactive nitrogen oxide species are considered to be potentially cytotoxic and capable of injuring the surrounding cells. According to the results, the extracts had a similar high level of scavenging activity on NO.

The antifungal activity of ethanol and water extracts was screened by *in vitro* (agar well diffusion method) using the stock culture of *A. niger*. The extracts caused different inhibition zones on the tested microorganism. The activity was confirmed for all the extracts. In other words, *A. aestivus* Brot. leaves extracts exhibited moderate antifungal activity against the fungus. El-Seedi (2007) reported that the arylcoumarins from *A. microcarpus* also had antimicrobial activity.

Our results showed that the plant has a powerful antioxidant activity at various antioxidant systems *in vitro*. Both of the extracts were potent radical scavengers, and their antioxidant capacities seem to be related to their chemical compositions. The metal chelating and radical scavenging activities of the water extract were higher than the ethanolic extract whereas the ethanol extract exhibited higher β -carotene bleaching effect, total antioxidant activity and antifungal ability compared to water extract. Therefore, active extracts of *A. aestivus* Brot. might be an alternative to more toxic synthetic antioxidants as additives in food, pharmaceutical and cosmetic preparations.

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