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Research Article

Antioxidant and Antiradical Activity of Beetroot (*Beta vulgaris* L. var. *conditiva* Alef.) Grown Using Different Fertilizers

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Fertilizers in different nitrogen forms (calcium ammonium nitrate (CAN), urea, ammonium sulfate (AS), and ammonium nitrate (AN)) and their doses (50, 100, and 150) for beetroot (BT) (*Beta vulgaris* L. var. *conditiva* Alef.) and the antioxidant and antiradical activities in the lyophilized water and alcohol extracts of BT were evaluated. In order to evaluate antioxidant and radical removing activities of BT roots, total phenolic compound amount assignment, total flavonoids amount assignment, method of Fe^{3+}-Fe^{2+} reduction activity using ferric cyanate reduction, cupric ions (Cu^{2+}) reducing capacity with CUPRAC method, Fe^{3+} reducing capacity according to FRAP method, ferrous ions (Fe^{2+}) chelating activity, superoxide anion radical ($O_2^{\bullet-}$) removing activity, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical removing activity were determined. In the study, BHA and α-tocopherol were used as standard antioxidants. It was determined that water and alcohol extracts obtained from BT roots indicated reduction activities, effectively. In addition, it was also determined that these reduction activities were indicated in most BT roots grown in fertilizer media at lower percentage and that they had higher antioxidative level than that of standard antioxidants.

1. Introduction

Beta vulgaris L. var. *conditiva* Alef. is used for the protection of the balance in the tissues and cells in all living creatures and performing of functions. Breakdown of the balance leads to the occurrence of oxidative stress and free radicals in living metabolism. Free radicals are generally reactive oxygen species (ROS), superoxide anion radical ($O_2^{\bullet-}$), and hydroxyl ($OH^{\bullet-}$) radicals. Oxygen types which are not free radicals include the derivatives which are not radical such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3), hypochlorous acid (HOCl), nitric oxide (NO^-), and peroxynitrite ($ONOO^-$) [1].

Occurring ROS components lead to a number of types of damage such as mutation and damage of DNA, breakdown of nucleotide structured coenzymes, changes in enzyme activities and lipid metabolism, mucopolysaccharides breakdown, occurring of structural damage in proteins, lipid peroxidation and, depending on this, breakdown of membrane structure, damage in membrane proteins, and breakdown of membrane transport systems and steroid, and accumulation of some substance called age pigment. These types of damage have played great role in process of biologic aging cancer or a lot of diseases such as hypertension and immune failure of senility. For this reason, the fact that living creatures should feed with the foods having high levelled antioxidative activity in order to fight against these negativeness has a great importance as regards their nutrition [2–5].

Beetroot (BT) having common usage among public (*Beta vulgaris* L. var. *conditiva* Alef.) is a vegetable, roots of which are traditional and popular. Having rich fiber structure, it facilitates digestion. It is very rich as regards B vitamins (B1, B2, B3, and B6) and folic acid [6]. BT roots include both dissolved and phenolics compounds taking place in the structure of cell wall and betalain compounds [7]. The pigments giving red colour to the BT roots are bioactive compounds and provide antioxidative activity for human health [8, 9]. It is revealed that BT roots may be useful

for improving blood sugar level and hypertension [10]. In addition, betalains have great importance for cardiovascular diseases by lowering the level of homocysteine [11–13].

According to the foodstuffs, not available sufficiently in the soil in which they are produced, in order to remove the lack and increase yield, the fertilization has been used [14, 15]. With the correct fertilization applied in agricultural application, yield increasing up to 60% has been obtained [16, 17]. In addition to the usefulness, fertilization has some negativeness as regards environment. In addition to excessive and wrong fertilization application, there are the accumulation of some substances in the plant and occurrence of some harmful gases as a result of evaporation with soil application of nitrogenous manure and especially occurrence of greenhouse effect as a result of participation of nitrogen oxides. Moreover, in some fertilizers' production, this leads to the formation of soil pollution of heavy metal ions such as Cd^{2+} and the increasing of the amount of nitrogen in waters as a result of excessive irrigation. As a result of the pollution in nature, it may increase risk of cancer in humans as well as the risk for plant and animal life. It is proved that the fertilizers led to ovarian and prostate cancers and there was mortality rate at 41%. It is detected that some fertilizers had effects on nervous system and even mutation may occur [18, 19]. Due to negativeness like these, excessive fertilizations should be avoided.

In this study, we tried to investigate the effect of fertilization with different nitrogen forms (CAN, urea, AS, and AN) and their doses (50, 100, and 150 kg ha^{-1}) on chemical structure and antioxidant or antiradical activities of beetroot (BT) (*Beta vulgaris* L. var. *conditiva* Alef.).

2. Materials and Methods

2.1. Chemicals. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), neocuproine (2,9-dimethyl-1,10-phenanthroline), riboflavin, methionine, nitroblue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), α-tocopherol, linoleic acid, gallic acid, quercetin, Folin-Ciocalteu reagent, and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). The other chemicals were obtained from Merck.

2.2. Beetroot (BT) (Beta vulgaris L. var. conditiva Alef.): The Growth of Plant Samples. The field experiment was conducted during May–October of 2015 in Erzurum, Turkey. Seeds for beetroot (*Beta vulgaris* L. var. *conditiva* Alef. cv. "Bikores") tested in this study were provided by the Metgen Seed Corporation (Istanbul, Turkey). The soil of the experimental area was clay loam texture (clay 35.46%, silt 34.99%, and sand 29.55%), ustorthent great soil group with neutral pH (7.56) and EC 315 μmhos cm^{-1}. It had 1.87% organic matter, 24.30 cmol kg^{-1} Ca, 2.35 cmol kg^{-1} Mg, 1.24 cmol kg^{-1} K, 0.27 cmol kg^{-1} Na, 40.67 mg kg^{-1} P, and 0.083% total N. Seeds were sown on plots of 5 m^2 in field, on 20 May in 2015, in rows 230 cm long, at a separation between rows of 40 cm and between plants of 20 cm. The plants were thinned after the emergence when they formed 4-5 true leaves. Irrigation was on a need basis, about twice a week after emergence. Experimental plots were kept weed-free using hand weeding.

The plots were fertilized with different doses of nitrogen forms. Four forms of nitrogen fertilizer [urea (46% N), calcium ammonium nitrate (26% N), ammonium nitrate (33% N), and ammonium sulfate (21% N)] and three doses of them (50, 100, and 150 kg ha^{-1}) were applied. The same P dose (100 kg ha^{-1}, P_2O_5) was the accepted usual dose according to Swiader et al. [20] and Kaymak et al. [21] reports which applied all fertilized plots. All of the P_2O_5 and half of the nitrogen fertilizer were applied uniformly prior to planting onto soil surface by hand and incorporated. The remaining half of the nitrogen was given 20 days after emergence [21]. Plots not exposed to nitrogen fertilizer served as control. Beetroot plots were harvested on 9 October 2015 according to the suggestions of Seed Corporation for tested cultivar and they were stored at +4°C until use.

2.3. The Preparation of Lyophilized Water and Alcohol Extracts of Beetroot (BT) (Beta vulgaris L. var. conditiva Alef.) 100 g beetroot (BT) (*Beta vulgaris* L. var. *conditiva* Alef.) was weighted and split with blender device for each treatment. Then, samples were divided into two parts. In the first part, 100 mL purified water was added to the split sample. It was extracted at the room temperature for a night. Then, it was filtered and the operation was repeated. After the extracts were combined, they were filtered into filter paper; then the filtrates were taken to balloons and frozen in deepfreeze. The frozen extracts were lyophilized under the pressure of 50 mm-Hg until it dried in lyophilizer. After alcohol was added to the second part of split beetroot cells, extracted, it was filtered and removed by evaporator dissolver.

2.4. Assigning of Total Amount of Phenolic Compound. The amount of phenolic compounds available in lyophilized water and alcohol extracts of BT roots for all treatments (0, 50, 100, and 150 kg ha^{-1}) of all nitrogen sources was determined totally by using Folin-Ciocalteu reactivity [22]. Gallic acid was used as a standard, and standard graphic was prepared. 1000 μg extract was taken from stock solution and it was taken into a measure container and volume was completed to 23 mL by means of distilled water. 0.5 mL Folin-Ciocalteu reagent was added to the mixture and it was incubated for three minutes, and then 1.5 mL, 2% (w/v) Na_2CO_3 was added. Then, after the samples were stirred at the room temperature for two hours, absorbances at 760 nm were determined against the blend consisting of distilled water. Gallic acid equivalent amount accounting for obtained absorbance values was calculated by help of the equation obtained from standard graphic. The results obtained were given as gallic acid equivalent (GAE).

2.5. The Determination of the Amount of Total Flavonoid Compounds. Total flavonoid amounts in lyophilized water and alcohol extracts of BT roots for all sources of nitrogen doses were determined according to the method by Park et al. [23] for all doses (0, 50, 100, and 150 kg ha^{-1}) of all nitrogen sources. So, 4.3 mL ethanol solution including 1000 μg extract medium, 0,1 mL 1 M CH_3COOK, and 0,1 mL

10% $Al(NO_3)_3$ was added to an experiment tube and stirred with vortex. Then, after it was incubated for 40 minutes at the room temperature, its absorbances were read at 415 nm. By using the equation obtained from standard graphics prepared by using quercetin, total flavonoid concentrations of water and alcohol samples of all BT roots were determined as microgram quercetin equivalent (QE).

2.6. Fe^{3+}-Fe^{2+} Reducing Capacity. Total reducing assignment was done according to Oyaizu method [24]. So, firstly, stock solution at 1 mg/mL concentration was prepared. 10, 20, and 30 μg/mL samples from this stock solution were transported to the experiment tubes and the volume was completed up to 1 mL by distilled water. Then, 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL $K_3Fe(CN)_6$ (1% (w/v)) were added to each tube, and the mixture was incubated at 50°C for 20 minutes. After these processes, 2.5 mL TCA (10% (w/v)) was added to reaction mixture. 2.5 mL was taken from the upper phase of sediment. So, 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1% (w/v)) were added, and the absorbance of all samples was determined against the blank at 700 nm, and water was used instead of sample in the control.

2.7. Cu^{2+}-Cu^{+} Reducing Capacity (CUPRAC Method). Cu^{2+} reducing activities of lyophilized water and alcohol extracts of BT roots grown in different fertilization media were determined according to the reducing method of copper ions [25]. From lyophilized water and alcohol extracts of BT roots prepared with 10 and 30 μg/mL concentrations, 0.25 mL $CuCl_2$ solution (0.01 M), 0.25 mL ethanolic neocuproine solution (7.5×10^{-3} M), and 0.25 mL CH_3COONH_4 buffer solution (1 M) were added to the tubes, respectively, and at the room temperature for 30 min they were incubated, and their absorbances were determined against the blank occurring with pure water at 450 nm.

2.8. Ferrous Ions and (Fe^{2+}) Chelating Activity. Metal chelating activity of lyophilized water and alcohol extracts of BT roots were determined according to a method applied by Dinis et al. [26]. This process and solution medium included 0.05 mL $FeCl_2 \cdot 4H_2O$ (2 mM) and 0.35 mL distilled water; then the solution including BT root samples at 30 μg/mL concentration and 0.2 mL lyophilized water and alcohol extracts was added and the last volume was completed to the 4 mL by ethanol. Then, the solution of ferrozine (0.2 mL, 5 mM) was added to the reaction medium, and it was stirred strongly with vortex. After reaction mixtures were incubated at room temperature for 10 minutes, the absorbance was determined at 562 nm against the blank occurring with ethanol solution. As control, solution contents formed without BT root extracts were used.

2.9. Removing Activity of Super Oxide Anion Radicals (O^{2-}). Superoxide anion radicals removing activity of all BT roots water and alcohol extracts was determined by means of spectrophotometric measuring occurring in the medium as a result of reaction of nitroblue tetrazolium (NBT). For this aim, the method utilized by Zhishen et al. [27] was used. Stock solution which had been prepared before was used for this purpose. For this, different concentrations of BT root extracts and standards (BHA and α-tocopherol) were prepared with phosphate buffer (0.05 M and pH 7.8). To the reaction mixture including samples, the amounts of riboflavin, methionine, and NBT (1.33×10^{-5}, 4.46×10^{-5}, and 8.15×10^{-8} M concentrations) were stimulated at room temperature for 40 minutes with 20 W fluorescent light. The absorbances of reaction mixtures were recorded against the blend occurring at water at 560 nm.

2.10. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Free Radicals Removing Activity. In lyophilized water and alcohol extracts of all BT root samples, DPPH free radical removing activity was determined according to Blois method [28]. As free radical, the solution of $DPPH^{\bullet}$ (1 mM) was used. To the experiments tubes, at concentrations of 10, 20, and 30 μg/μL, water and alcohol extracts obtained from BT roots were transported and their total volumes were completed to 3 mL with ethanol. Then, to each sample tube, 1 mL from stock DPPH solution was added and incubated at room temperature for 30 minutes, and the absorbances were determined against the blank occurring with ethanol at 517 nm. As controls, 3 mL ethanol and 1 mL $DPPH^{\bullet}$ solution were used. Reduced absorbances gave remaining $DPPH^{\bullet}$ solution quantity, namely, free radical removing activity.

2.11. 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Removing Activity. In water and alcohol extracts of BT samples, ABTS radical removing activity was determined according to the method used by Re et al. [29]. By adding persulphate solution (2.45 nM) to the ABTS (7 mM) solution, the formation of ABTS radicals in reaction medium was provided. At 734 nm, the absorbance of control solution (0,1 M, pH 7.4) was adjusted to 0.700 ± 0,025 nm using phosphate buffer. 1 mL ABTS radical solution was added to the lyophilized water and alcohol extracts at 10–30 μg/mL of BT roots and incubated at room temperature for 30 minutes. The absorbances of all samples were determined against the blank occurring from ethanol at 734 nm.

3. Results and Discussion

In the fertilization process carried out for increasing the yield in agriculture, organic and inorganic fertilizers lead to soil and environmental pollution. As well as leading to death of living creatures in both soil and water, they cause some diseases in humans such as bronchitis, nervous system disorders, and some cancers (stomach cancers, gall bladder cancers, intestinal cancers, etc.). Due to having negative effects of fertilizers on a number of health subjects, antioxidative and antiradical activities of BT roots grown in different fertilization media have been examined.

3.1. Assignment of Total Flavonoid Amount. Flavonoids are polyphenolic compounds available in plants. It is known that these compounds have strong antioxidant features and they also have metal connection and keeping of free radical features [30]. Standard graphic was created using gallic acid (GAE) and total phenolic amounts available in lyophilized

Table 1: Total phenolic content and total flavonoids amounts of water extracts, alcohol extracts, α-tocopherol, and BHA.

	Total phenolics (μg of GAE[a]/mg dw)		Total flavonoids (μg of QE[b]/mg dw)	
	Water	Alcohol	Water	Alcohol
BHA	32.6 ± 5.7	66.5 ± 3.4	28.9 ± 8.5	62.6 ± 2.4
α-Tocopherol	25.4 ± 3.6	85.5 ± 4.8	21.5 ± 6.4	81.3 ± 4.8
CAN 50 kg ha^{-1}	27.3 ± 4.2	147.4 ± 3.8	23.6 ± 6.1	143.6 ± 8.9
CAN 100 kg ha^{-1}	37.6 ± 5.2	149.1 ± 0.6	33.9 ± 3.2	145.9 ± 1.1
CAN 150 kg ha^{-1}	17.9 ± 8.8	66.1 ± 1.8	14.3 ± 2.6	61.8 ± 2.6
Urea 50 kg ha^{-1}	24,5 ± 5.1	96.5 ± 4.4	20.8 ± 1.6	92.5 ± 4.6
Urea 100 kg ha^{-1}	31.4 ± 3.4	80.5 ± 1.4	27.7 ± 9.3	76.7 ± 7.3
Urea 150 kg ha^{-1}	25.1 ± 1.5	73.7 ± 6.9	21.3 ± 4.4	72.0 ± 9.7
AS 50 kg ha^{-1}	38.4 ± 3.9	109.3 ± 3.3	34.9 ± 2.7	105.7 ± 7.4
AS 100 kg ha^{-1}	31.9 ± 1.8	112.1 ± 0.8	29.2 ± 1.8	108.4 ± 3.6
AS 150 kg ha^{-1}	29.6 ± 2.4	106.4 ± 3.8	25.6 ± 5.7	103.1 ± 1.6
AN 50 kg ha^{-1}	21.4 ± 3.9	117.4 ± 3.2	17.9 ± 9.7	113.8 ± 7.7
AN 100 kg ha^{-1}	43.6 ± 1.8	104.8 ± 7.8	39.8 ± 8.4	101.2 ± 1.5
AN 150 kg ha^{-1}	32.6 ± 5.8	66.4 ± 3.5	28.9 ± 8.5	62.6 ± 6.6

[a]Determined as gallic acid equivalent (GAE). [b]Determined as quercetin equivalent (QE).

water and alcohol extracts of beetroot (*Beta vulgaris* L. var. *conditiva* Alef.) were calculated by means of standard graphic. Total phenolic matter amounts obtained by using all different fertilizes for BT samples were given in Table 1. From the results obtained, it was determined that all fertilizers had the highest phenolic matter rate at 1% concentration of fertilizers. It was also determined that the highest matter content was in beetroot's alcohol extracts at fertilization with 50 kg ha^{-1} CAN and urea, 150 kg ha^{-1} AS, and 100 kg ha^{-1} AN. It was found that fertilization with 100 kg ha^{-1} AN had maximum phenolic content of 43.6 ± 1.8 μg/mg GAE and 149.1 ± 0.6 μg/mg GAE for water and alcohol samples, respectively.

3.2. The Assignment of Total Phenolic Compound Amount. For assignment standard graphic was created using quercetin (QE) and total phenolic amounts in lyophilized water and alcohol extracts of BT roots (*Beta vulgaris* L. var. *conditiva* Alef.) were calculated using standard graphic and all results were given in Table 1.

It was determined that water extracts of BT roots had the highest flavonoid matter amount as 39.8 ± 8.4 μg/mg QE in fertilization with 100 kg CAN, 50 kg urea, 100 kg AS, and 50 kg AN ha^{-1}. When alcohol extracts were compared as regards flavonoid matter amount, it was seen that CAN (100 kg ha^{-1}), urea (50 kg ha^{-1}), AS (100 kg ha^{-1}), and AN (100 kg ha^{-1}) had the highest flavonoid matter amount. In all samples, it was detected that fertilization with 100 kg CAN ha^{-1} had maximum amount of flavonoid matter at the value of 143.9 ± 1.1 μg/mg QE. From the results obtained, the fact that BT roots have high phenolic and flavonoid content in lower fertilizer concentrations was evaluated as a positive result [31].

3.3. Superoxide Anion Radical Removing Activities. Superoxide radicals from free radicals in both enzymatic and nonenzymatic reactions are most easily produced. These radicals lead to lipid peroxidation and depend on the breakdown of the structure of membrane [32]. In addition, superoxide anion radicals can reduce Fe^{3+} ions to Fe^{2+}. In addition, it is known that Fe^{2+} ions by using hydrogen peroxide with Fenton reaction led to formation of OH radicals which are very highly reactive in a number of disorders. For these reasons, removing superoxide anion radicals in medium is needed.

It is found that, in the study in which BT roots were used with lyophilized water extract at 30 μg/mL concentrations, superoxide anion radical removing activity became the highest at fertilization with 150 kg ha^{-1}, *urea* > *BHA* > *AN* > *CAN* > α-*tocopherol* > *AS*, respectively. These values were shown in a way as 77.1 ± 3.4 > 66.4 ± 1.2 > 64.1 ± 9.8 > 59.3 ± 3.3 > 55.4 ± 7.3 > 48.0 ± 3.2, respectively. In alcohol extracts of BT roots, the highest superoxide anion radical removing activities at fertilization with 150 kg ha^{-1} are *urea* > *AN* > *BHA* ≈ *CAN%* > *AS* > α-*tocopherol*, respectively. These values were 84.6 ± 3.5 > 70.3 ± 9.1 > 66.4 ± 1.2 ≈ 65.7 ± 6.9 ≈ 64.3 ± 6.8 > 55.4 ± 7.3, respectively. As is seen in Table 1, when the results were compared to standard, it was observed that BT roots removed effectively superoxide anion radicals in both water and alcohol extracts at fertilization with 150 kg of all nitrogen sources, ha^{-1}.

3.4. Ferrous Ions Chelating Capacity. The activities of ferrous ions chelating of lyophilized water and alcohol extracts of BT plants grown in different media and comparison with BHA and α-tocopherol being a standard antioxidant were given in Table 2. When chelating activities of ferrous ions (Fe^{2+}) for α-tocopherol, BHA, and water and alcohol extracts of BT plant at 30 μg/mL concentration were measured, it was observed that high ferrous ions chelating activities were observed for *E-Urea 150 kg ha^{-1}, W-AS-50 kg ha^{-1}, and E-CAN 150 kg ha^{-1}* according to standards. These values were determined as

Table 2: The results of superoxide anion radical scavenging, ferrous ion chelating, and hydrogen peroxide scavenging activity of water extracts, alcohol extracts, α-tocopherol, and BHA.

	Superoxide scavenging activity (%)		Ferrous ion chelating activity (%)		H_2O_2 scavenging activity (%)	
	Water	Alcohol	Water	Alcohol	Water	Alcohol
BHA	66.4 ± 1.2		61.4 ± 3.6		38.6 ± 1.5	
α-Tocopherol	55.4 ± 7.3		48.5 ± 4.9		41.6 ± 5.6	
CAN 50 kg ha^{-1}	21.3 ± 2.9	33.7 ± 6.3	30.9 ± 9.6	55.9 ± 2.3	38,6 ± 3.8	52.3 ± 2.2
CAN 100 kg ha^{-1}	46.9 ± 8.8	58.6 ± 5.5	21.9 ± 1.9	51.2 ± 1.9	42.6 ± 9.5	58.6 ± 1.9
CAN 150 kg ha^{-1}	59.3 ± 3.3	65.7 ± 6.9	21.2 ± 1.6	83.1 ± 2.6	40.1 ± 3.3	53.6 ± 2.8
Urea 50 kg ha^{-1}	27.0 ± 3.0	35.8 ± 4.7	61.4 ± 1.3	33.9 ± 7.1	55.4 ± 3.6	63.2 ± 5.7
Urea 100 kg ha^{-1}	49.2 ± 2.1	58.2 ± 1.2	57.8 ± 7.7	11.6 ± 4.0	58.3 ± 4.4	66.2 ± 1.1
Urea 150 kg ha^{-1}	77.1 ± 3.4	84.6 ± 3.5	28.6 ± 2.7	92.8 ± 8.4	56.3 ± 0.5	65.1 ± 5.3
AS 50 kg ha^{-1}	21.3 ± 2.9	33.6 ± 3.3	88.3 ± 3.2	60.7 ± 3.6	65.3 ± 7.6	72.3 ± 4.2
AS 100 kg ha^{-1}	32.4 ± 3.9	56.6 ± 2.5	63.0 ± 0.2	20.6 ± 5.9	69.2 ± 2.5	71.5 ± 1.4
AS 150 kg ha^{-1}	48.0 ± 3.2	64.3 ± 6.8	58.3 ± 2.3	8.9 ± 8.6	63.1 ± 8.1	70.3 ± 3.3
AN 50 kg ha^{-1}	48.8 ± 7.6	53.2 ± 2.7	52.4 ± 3.6	13.3 ± 2.7	68.1 ± 2.3	75.6 ± 5.1
AN 100 kg ha^{-1}	58.4 ± 3.5	62.3 ± 3.6	46.1 ± 9.4	29.4 ± 3.9	76.6 ± 4.6	78.2 ± 6.9
AN 150 kg ha^{-1}	64.1 ± 9.8	70.3 ± 9.1	27.2 ± 4.1	23.6 ± 2.4	77.2 ± 8.9	74,3 ± 7.8

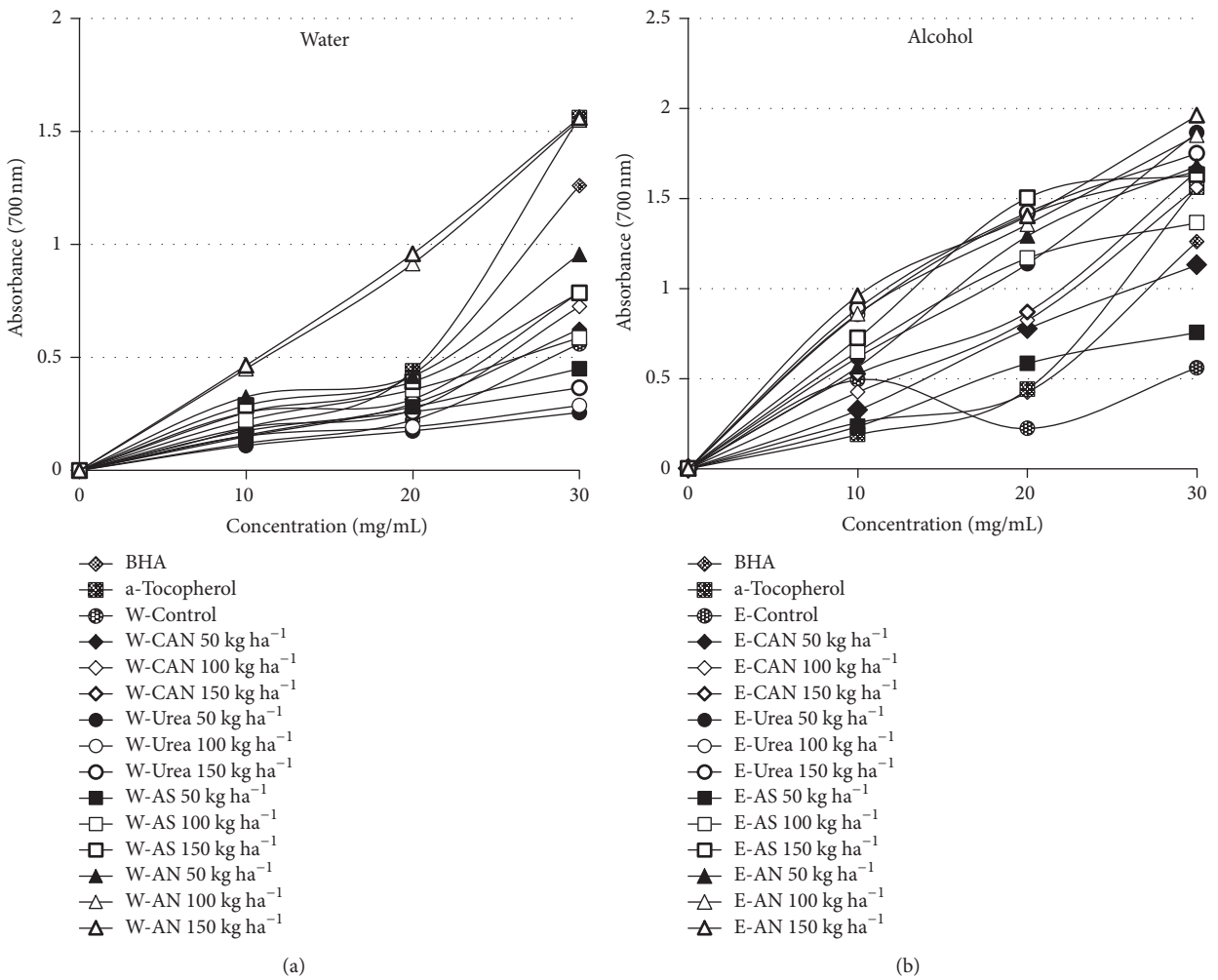

Figure 1: The Fe^{3+}-Fe^{2+} reducing activity of different concentrations (10–30 μg/mL) of water extracts, alcohol extracts, α-tocopherol, and BHA.

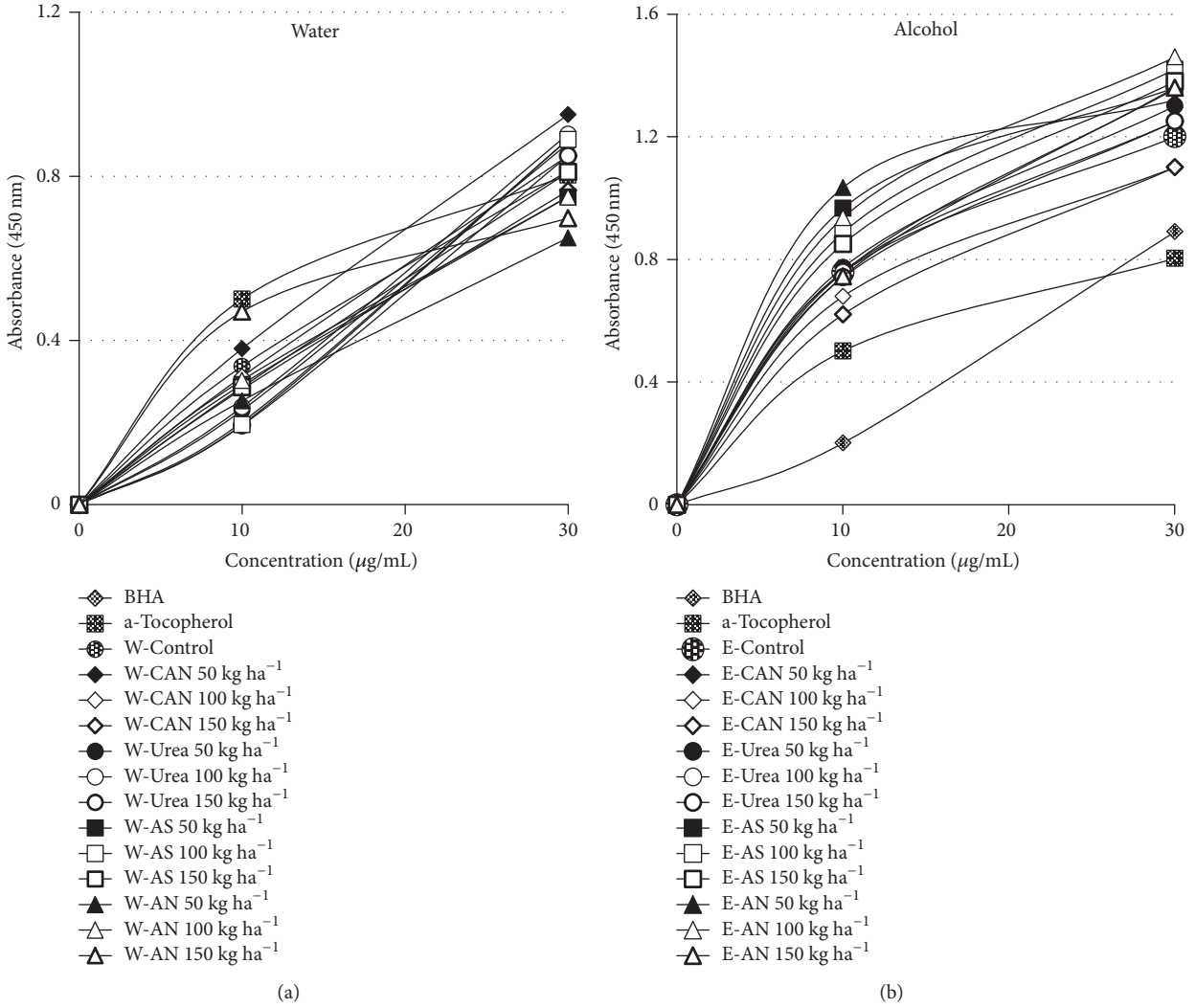

Figure 2: The Cu^{2+}-Cu^{+} reducing activity of water extracts, alcohol extracts, BHA, and α-tocopherol at different concentrations (10–30 μg/mL).

61.4% for BHA and 48.5% for *α-tocopherol*, and also for *E-Urea 150 kg ha^{-1}, W-AS 50 kg ha^{-1}, and E-CAN 150 kg ha^{-1}* values were 92.8%, 88.3%, and 83.1%, respectively.

3.5. Hydrogen Peroxide Scavenging Activity. During the reaction, when oxygen is reduced in the cell by taking electron, in the case complete reducing is not obtained, the formation of H_2O_2 and $OH^{\bullet -}$ which is very reactive is done true. The reaction of reduction from oxygen to water is shown as follows:

$$O_2 \xrightarrow{e^-} O_2^{\bullet -} \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} OH^{\bullet -} \xrightarrow{e^-} H_2O \quad (1)$$

For this reason, H_2O_2 should be removed by means of antioxidative substances. In both water and alcohol extracts of BT roots grown at different fertilizer media, hydrogen peroxide scavenging activity was carried out according to Ruch et al. [33] and the results were compared with standard BHA and α-tocopherol, and they were given in Table 2. At 15 μg/mL concentration, while hydrogen peroxide scavenging activity was 38.6% for BHA and 41.6% for α-tocopherol, in E-AN-150 kg ha^{-1}, for example, the highest activity was observed with 78.2% (Table 2). It was determined that both water and alcohol extracts plant samples grown in all fertilizers media indicated much higher hydrogen peroxide scavenging activity than BHA and α-tocopherol standards. These results showed that BT samples which grew with all used fertilizers had an effective hydrogen peroxide scavenging activity.

3.6. The Fe^{3+}-Fe^{2+} Reducing (FRAP) Activity. The reducing power of a compound is known as the capacity of giving electron of that compound and can be measured with different methods. The Fe^{3+}-Fe^{2+} reducing method is the one in which antioxidants give electrons and indicate antioxidant activity. It was found that ferrous ions (Fe^{3+}) reducing capacity was increased with increasing fertilizer concentrations (50, 100, and 150 kg ha^{-1}) according to FRAP method. It was also observed that alcohol extracts had higher FRAP activity in all samples. At 30 μg/mL concentrations, the samples of W-AN

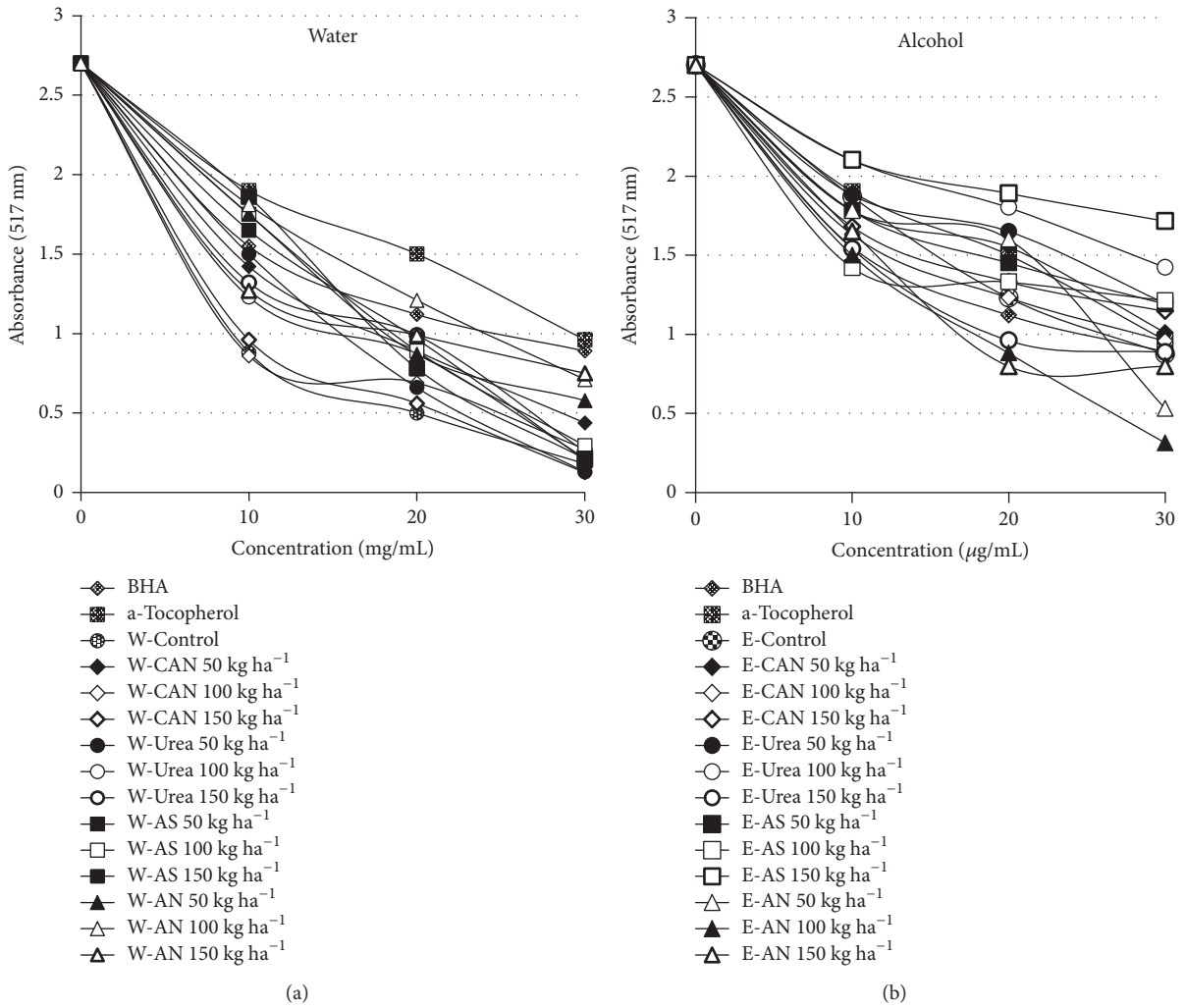

Figure 3: The DPPH• scavenging effect of water extracts, alcohol extracts, BHA, and α-tocopherol at different concentrations (10–30 μg/mL^{-1}).

150 kg ha^{-1} and E-AN-150 kg ha^{-1} had the highest FRAP activity. In addition, according to BHA and α-tocopherol used standardly, it was detected that all samples indicated higher FRAP activity (Figure 1).

3.7. The Cu^{2+}-Cu^{+} Reducing Activity. Another method used for determining the reducing capacity is CUPRAC method. Reducing capacity of cupric ions of lyophilized water and alcohol extracts of BT roots (Cu^{2+}) was determined by means of spectrophotometric method at different concentrations in the samples which contain extract (10–30 μg/mL). Reducing capacity of cupric ions of water and alcohol extracts obtained from red beetroots growth at different fertilizer and concentrations media (Cu^{2+}) was compared with BHA and α-tocopherol, a standard antioxidant. Related results were shown in Figure 2. As seen in Figure 2, both water and alcohol extracts samples exhibited higher reducing capacity than both BHA and α-tocopherol antioxidant standards. At 30 μg/mL concentration, when compared with the standards of reducing capacity of cupric ions (Cu^{2+}), the highest ones of them were E-AN-100 kg ha^{-1} > W-CAN-50 kg ha^{-1} > BHA > α-tocopherol, respectively.

3.8. The DPPH• Scavenging Activity. DPPH• (1,1-diphenyl-2-picrylhydrazyl) is an organic structured radical giving absorbance at 517 nm. In our study, as to removing of DPPH• radical activity, absorbance reducing at 517 nm, and residing in DPPH• solution amount by measuring, namely, free radical removing activity, were determined. In order to assign of DPPH• radical removing activity, firstly standard graphic was formed and used for calculations. It is clearly seen from Figure 3 that lyophilized water and alcohol extracts of BT roots grown in different fertilizer media exhibited higher DPPH• radical removing activity than standard antioxidant compounds such as BHA and α-tocopherol. At 30 μg/mL concentrations, the highest activities in water and alcohol extracts of BT roots were compared with standard antioxidants; they are exhibited in the way of W-AN-100 kg ha^{-1} > E-AN-100 kg ha^{-1} > BHA > a-tocopherol DPPH• radical removing activity. These values were calculated as 84.4%,

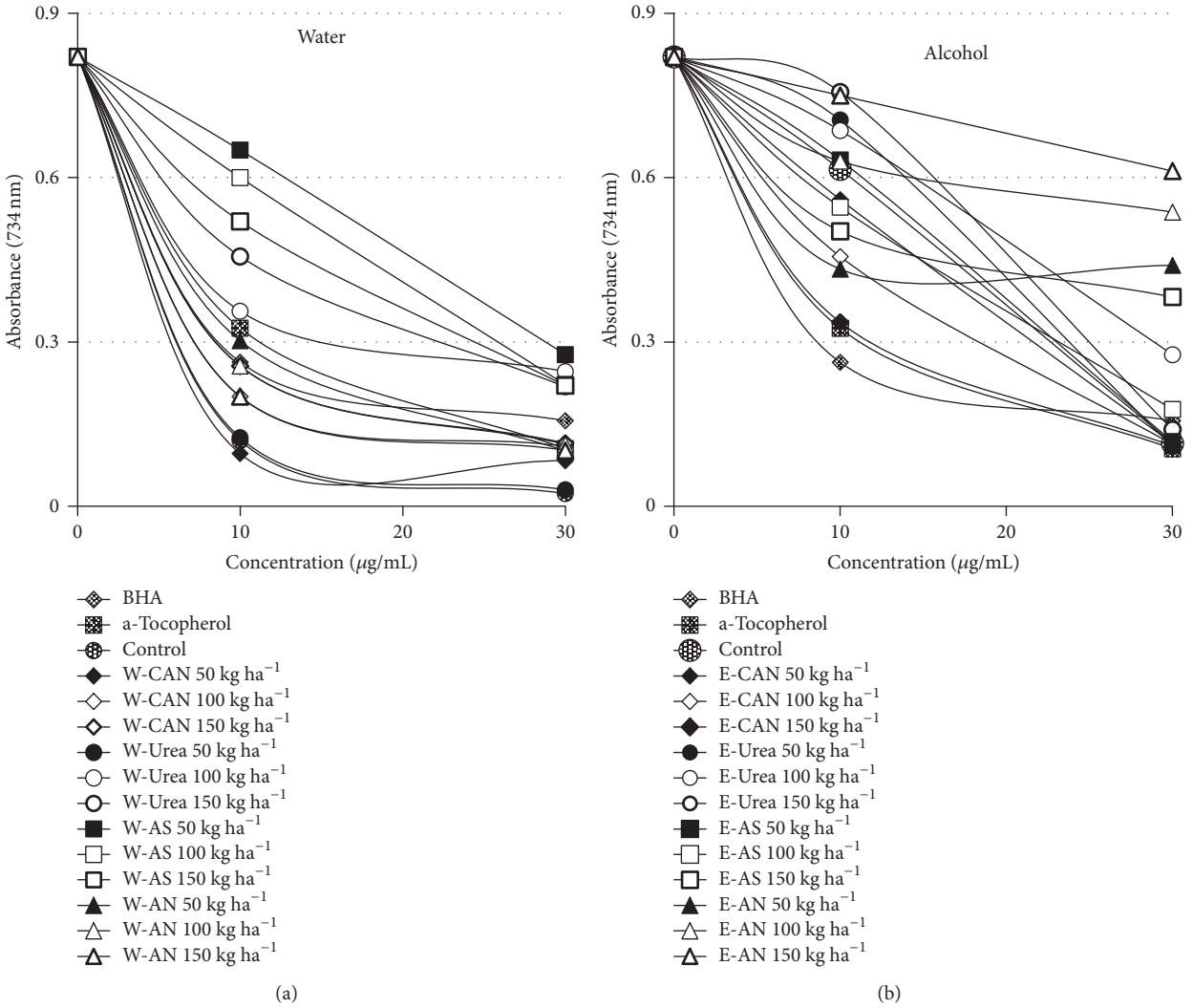

Figure 4: The stable $ABTS^{\bullet+}$ scavenging effect of water extracts, alcohol extracts, BHA, and α-tocopherol at different concentrations (10–30 $\mu g/mL^{-1}$).

52.8%, and 16.7%, respectively. That water and alcohol extracts of BT roots grown in all different fertilizer media indicated higher DPPH radical removing activity than that of control samples which was shown in Figure 3.

When the previous studies were examined it was found that, in the extracts whose contents of C vitamin and polyhydroxy aromatic compounds are high, excessive $DPPH^{\bullet}$ scavenging activity was high. We could say that these studies supported our results. Also, BT roots obtained from fertilization with 150 kg ha^{-1} of nitrogen sources giving high yield indicate that there is no need for excessive fertilization application.

3.9. The $ABTS^{\bullet+}$ Scavenging Activity. $ABTS^{\bullet+}$ radical is a coloured compound giving absorbance at 734 nm. $ABTS^{\bullet+}$ radical participates in chemical reaction with antioxidant substances, transfers one electron, and turns into a unradical ABTS substance. Related reaction was given as follows:

$$ABTS^{+\bullet} + A \longrightarrow ABTS^{+\bullet} + A^{+\bullet} \quad (2)$$

In the study carried out, first spectrophotometric measuring was conducted and then was followed by reducing the absorbance value at 734 nm, and $ABTS^{\bullet+}$ radical removing activity was calculated.

$ABTS^{\bullet+}$ removing activity has been commonly used in the radical removing activities from watered mixtures, beverages, and extracts and pure substances [34]. Firstly standard graphic was formed to assign the ABTS removing activities of lyophilized water and alcohol extracts of BT roots grown at different fertilizer media and standard antioxidant compounds such as BHA α-tocopherol; this standard graphic was used for $ABTS^{\bullet+}$ removing activity calculation in all samples. According to the results obtained, at 30 μg/mL concentrations, it was detected that BHA indicated ABTS radical removing at the rate of 81.0% and α-tocopherol at the rate of 87.6% (Figure 4). In this study, it was found that lyophilized water and alcohol extracts of BT roots removed ABTS radical stronger than standard antioxidants.

Nitrogen is an indispensable component of proteins used to form cell materials and plant tissues, but high nitrogen

levels are toxic to plant growth. NH_4^+ toxicity probably indicates that excessive production of ROS can cause an amount of oxidative damage to proteins, lipids, and DNA, resulting in lipid peroxidation, cell damage, and cell death [35]. In this study, urea, CAN, AN, and AS fertilizers were utilized as the most used organic nitrogen source in the cultivation of BT.

Although urea, CAN, AN, and AS are generally known to have low toxicity to organisms, they have indirect and long-term harm to ecosystems such as eutrophication, groundwater pollution, and soil acidification [36, 37]. Ammonium formed as a result of the hydrolysis of the urine, CAN, AN, and AS is more toxic to plants [38]. Higher amounts of urea cause decreased biological efficiency of the plants and cause physiological disorders [39, 40]. However, the effects of plant-induced oxidative stress on plants are not clear [41].

In low concentration urea application (100 mg L-1) in plant, oxidative stress has been reduced due to decreased ROS (superoxide and hydrogen peroxide) formation and lipid peroxidation. At high concentration, urea leads to the depletion of a low molecular weight antioxidant pool. It is thought to be associated with increased oxidative stress and increased antioxidative protection of the plant [41, 42]. Similar results were obtained in the application of fertilizers of nitrogen origin such as CAN, AN, and AS to the plant. In low doses of nitrogen fertilizers, good growth was observed in the plant, while high-dose plant growth caused unnecessary and lethal outcomes.

Also, nitrogen application at higher rates negatively affected the antioxidant activities such as ferric cyanate reduction, cupric ions (Cu^{2+}) reducing capacity with CUPRAC method, Fe^{3+} reducing capacity according to FRAP method, ferrous ions (Fe^{2+}) chelating activity, superoxide anion radical, and 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) radical removing activity. Therefore, based on these results, it had been concluded that low nitrogen was effective in plant growth and high antioxidative activity and it also caused decrease of oxidative stress in BT.

4. Conclusion

On the basis of the results of this study, it is clearly indicated that BT roots growth by using low doses of CAN, urea, AS, and AN fertilizers has a powerful antioxidant activity against various oxidative systems *in vitro*. For this reason, as the concentration of applied fertilizer lowers, environmental pollution and threat factors of human health also lower, and so the rate of cost of the products will lower.

Disclosure

This work was previously submitted at 4th International ISEKI-Food Conference (6–8 July 2016, Vienna, Austria) as a poster presentation.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this study.

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