



Protective effects of anthocyanin-rich bilberry (*Vaccinium myrtillus* L.) extract against copper(II) chloride toxicity

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Received: 18 July 2019 / Accepted: 15 October 2019 / Published online: 20 November 2019
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Abstract

In this study, the protective effects of 50 mg/L and 100 mg/L doses of anthocyanin-rich bilberry extracts (ABE) against the toxicity caused by 20 μ M copper(II) chloride (CuCl_2) on *Allium cepa* L. were investigated. Alterations in weight gain, germination percentage, and root elongation were evaluated as physiological parameters while micronucleus (MN), mitotic index (MI), and chromosomal abnormality (CA) frequency were studied as cytogenetic parameters. Oxidative stress indicators such as malondialdehyde (MDA) formation, superoxide dismutase (SOD) activity, and catalase (CAT) activity were analyzed and also damages in root tip meristem cells were determined by cross sections. As a result, it was found that the percentage of germination, weight gain, root length, and MI decreased and the frequency of MN and CAs increased with CuCl_2 treatment. CuCl_2 exposure caused a significant increase in SOD and CAT activities and MDA levels. A number of anatomical abnormalities and damages were detected in the cross sections of CuCl_2 -treated roots. On the other hand, ABE applications ameliorated notably all copper-induced damages in a dose-dependent manner. Therefore, the powerful protective potential of ABE against copper-induced toxicity was proven through an extensive study in a popular plant model.

Keywords *Allium cepa* L. · Anthocyanin · Antioxidant · Bilberry · CuCl_2 · Genotoxicity

Introduction

Copper (Cu) and Cu compounds are widely used in industrial processes, agriculture, and daily life. As a result of its widespread usage in different areas, Cu can be present in high concentrations in the natural environment as well as in human living environments. As a heavy metal, Cu is a persistent environmental pollutant because it cannot be degraded or decomposed (Duruibe et al. 2007). It can be released into the environment by both natural events and anthropogenic activities such as mining, industrial and domestic wastes, agricultural practices, and use of pesticides and antifouling paints (Liu et al. 2009). As a cofactor, Cu is essential for

structural and catalytic functions of various enzymes within the biological processes required for growth, development, maintenance, and electron transport systems (Gaetke and Chow 2003; Jomova and Valko 2011). Although it is irreplaceable for many metabolic processes in prokaryotes and eukaryotes, it becomes toxic at high concentrations (Flemming and Trevors 1989). Excessive amounts of CuCl_2 may cause cytotoxicity and genotoxicity because it induces growth deceleration and inhibition, cell cycle disorders, oxidative stress, and degradation effects in many organisms, including plants (Francescon et al. 2018).

The negative and toxic effects of the chemicals that we are frequently exposed to in daily life can be reduced by the use of many natural and antioxidant compounds in the daily diet. Bilberry (*Vaccinium myrtillus* L.) is a berry of a shrubby plant which is grown in Europe and North America. Medicinal use of bilberry for many different illnesses dates back to early Renaissance (Valentova et al. 2007). Bilberry fruit is one of the richest sources of dietary anthocyanins, a group of water-soluble pigments, with antioxidant, anticancer, and anti-inflammatory properties (Takikawa et al. 2010; Seeram 2008). Nowadays, anthocyanin-rich bilberry extracts (ABE) of different brands can be found in the market for therapeutic

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purposes. There are a number of studies that focused on the health effects of bilberry and bilberry extracts (Valentova et al. 2007; Karlsen et al. 2010; Takikawa et al. 2010). Previous researchers studied antioxidative and cytoprotective roles of ABE against oxidative damage in several in vitro models (Martin-Aragon et al. 1998; Valentova et al. 2007). However, their protective effects against heavy metal-induced toxicity and genotoxicity have not been understood extensively.

Since cytogenotoxicity studies on experimental animals are expensive and time consuming, alternatives, including plant root experiments, have gained interest (Samuel et al. 2010). Onion (*Allium cepa* L.) bioassay is considered as an easy and reliable test model for assessing the genotoxicity mainly due to its fast growing roots, high percent of dividing cells, large sized monocentric chromosomes ($2n = 16$), and responsive genetic material to pollutants (Vesna et al. 1996; Fiskesjo 1997). Therefore, *Allium* has become a very common biological test material for cytogenetic researches since 1938 (Levan 1938). The validity of *A. cepa* assay has been well documented through the studies on a number of environmental contaminants such as heavy metals (Seth et al. 2008; Leme and Marin-Morales 2009; Yıldız et al. 2009; Çavuşoğlu et al. 2011; Yalçın et al. 2019). Furthermore, international organizations such as the United Nations Environment Programme (UNEP) and the International Programme on Chemical Safety (IPCS) accepted *A. cepa* assay as a well-established and functional test method for in situ monitoring of pollutants (Mangalampalli et al. 2018).

In the present study, the toxic effects of CuCl_2 and the protective effects of ABE against this toxicity were evaluated in physiological, cytogenetic, biochemical, and anatomical aspects using *A. cepa* assay in vivo. In order to assess physiological effects, germination percentage, root elongation, and weight gain of bulbs were determined. In addition to that, mitotic index (MI), micronucleus (MN) frequency, and chromosomal abnormalities were utilized to investigate the cytogenetic effects of CuCl_2 and ABE on root tips. Malondialdehyde (MDA) levels and the activities of SOD and CAT enzymes were used to screen the oxidative stress on *Allium* bulbs while the anatomical changes in CuCl_2 -treated bulbs were observed by microscopic examination of root tip cross sections.

Materials and methods

Materials

Copper(II) chloride dihydrate ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$) (Merck) was used for preparation of Cu solutions. *A. cepa* bulbs and ABE

with 25% anthocyanin content (Nexgen Pharma Inc., CA, USA) were obtained commercially from local markets.

Experimental design and treatments

Healthy and equally sized *A. cepa* bulbs were chosen for this study. The bulbs were divided into six groups and each group consisted of 25 bulbs. After the bulbs were placed in sterilized glass beakers, the groups were treated with the following solutions for 72 h at room temperature in the dark (Table 1).

Determination of germination percentage, root elongation, and weight gain

The germination percentage of the bulbs was calculated using the equation (Eq. 1) given by Atik et al. (2007).

$$\text{Germination (\%)} = \frac{\text{Germinated seeds}}{\text{total seeds}} \times 100 \quad (1)$$

Root elongations were measured with a ruler after germination. Differences between the weight of bulbs before and after 72 h of treatments were accepted as weight gain.

Micronucleus assay and chromosomal aberrations

After 72 h of treatment, *A. cepa* roots were thoroughly washed with distilled water. Root tips of onions were cut with a razor nearly 0.5 cm long and fixed with Clarke's solution (3: ethyl alcohol/1: glacial acetic acid) for 2 h. Then, they were washed for 15 min in 96% ethanol before hydrolyzed in 1 N HCl at a temperature of 60 °C for 11 min. Hydrolyzed root tips were stained with 1% acetocarmine solution for 24 h. The root tips were crushed in 45% acetic acid and photographed with a binocular research microscope (Staykova et al. 2005). The same slide was used for calculations of the MN and the MI and for determination of chromosomal aberrations. The total cell number for MN counting was identified according to the criteria set by Fenech et al. (2003). The MI was accepted as the number of cells in mitosis per 1000 observed cells and expressed as percentage.

Table 1 Experimental design and treatments

Groups	Treatments (72 h) ($n = 25$)
Group I	Tap water (negative control)
Group II	50 mg/L ABE
Group III	100 mg/L ABE
Group IV	20 μM CuCl_2 (positive control)
Group V	50 mg/L ABE and 20 μM CuCl_2
Group VI	100 mg/L ABE and 20 μM CuCl_2

Table 2 Effects of CuCl₂ and ABE applications on germination, root elongation, and weight gain

Groups	Germination percentage (%)	Mean root length (cm)	Mean weight gain (g)
Group I	100	15.51 ± 2.74 ^a	+ 8.57 ^a
Group II	100	15.77 ± 2.68 ^a	+ 8.30 ^a
Group III	100	15.90 ± 2.05 ^a	+ 8.45 ^a
Group IV	48	5.46 ± 1.60 ^d	+ 1.26 ^{c,d}
Group V	64	8.07 ± 1.70 ^c	+ 2.82 ^{b,c}
Group VI	84	11.73 ± 3.07 ^b	+ 4.05 ^b

Group I, control group; group II, 50 mg/L ABE; group III, 100 mg/L ABE; group IV, 20 μM CuCl₂; group V, 50 mg/L ABE+20 μM CuCl₂; group VI, 100 mg/L ABE+20 μM CuCl₂ (*n* = 25). The mean values indicated by different superscript letters (^{a–d}) in the same column are significant at *p* < 0.05

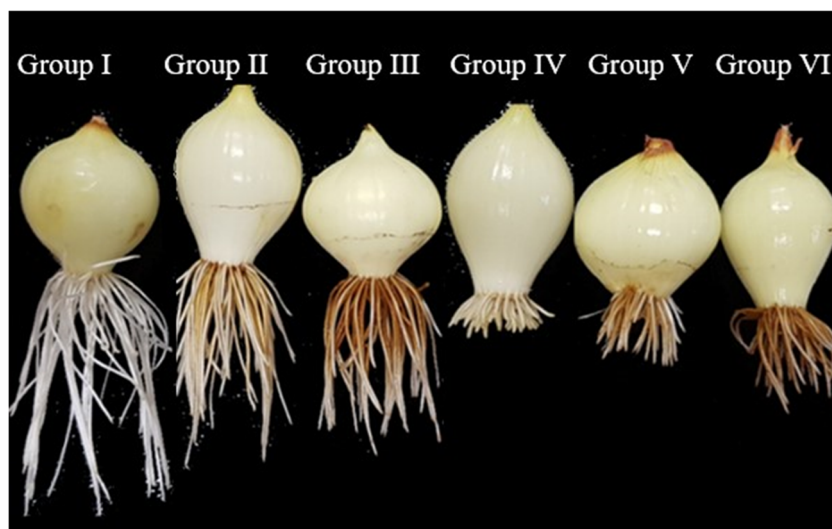
Anatomical alterations

In order to determine the anatomical alterations, cross sections of the root tips of *A. cepa* bulbs were taken. Slides were stained using 1% methylene blue and photographed at × 500 magnification with a binocular research microscope.

MDA analysis

At the end of the treatments, MDA contents of *A. cepa* roots were evaluated with the method suggested by Unyayar et al. (2006). 0.5 g of root material was homogenized by means of an ULTRA-TURRAX homogenizer in 5% trichloroacetic acid (TCA). Collected homogenates were centrifuged at 12,000 rpm for 15 min at room temperature. Equal amounts of 20% TCA–0.5% thiobarbituric acid (TBA) solution and supernatant were mixed in a glass tube. Glass tubes with reaction mixture were boiled at 98 °C for 25 min. After 25 min, the tubes were placed in an ice bath to interrupt the reactions. Chilled mixtures were centrifuged at 10,000 rpm for 5 min. The absorbance of mixtures was measured spectrophotometrically at 532 nm and 600 nm.

Fig. 1 Effects of CuCl₂ and ABE on root elongation. Group I, control group; group II, 50 mg/L ABE; group III, 100 mg/L ABE; group IV, 20 μM CuCl₂; group V, 50 mg/L ABE+20 μM CuCl₂; group VI, 100 mg/L ABE+20 μM CuCl₂



SOD and CAT analyses

0.2 g of fresh onion roots was weighted and homogenized with 5 mL of cooled sodium phosphate buffer (50 mM, pH 7.8) for determination of SOD and CAT activities. Homogenates of roots were centrifuged at 10,000 rpm at 4 °C for 20 min. The supernatants were used for SOD and CAT enzyme activity analyses.

The method mentioned by Beauchamp and Fridovich (1971) was used for determination of SOD activity. SOD activity analysis results were presented as units per milligram fresh weight (U/mg FW). The method mentioned by Beers and Sizer (1952) was used for determination of CAT activity. CAT activity was expressed as OD_{240nm}/min/g FW.

Statistical analysis

The statistical analysis of data was performed with SPSS software (SPSS Inc., Chicago, IL, USA). Results were analyzed using Duncan's test and analysis of variance (ANOVA) test. The data were presented as means and standard deviation (SD), and the statistical significance of difference was taken as less than 0.05.

Table 3 Effects of CuCl₂ and ABE applications on MI and MN frequency

Groups	MI	MN
Group I	888.00 ± 19.59 ^a	0.00 ± 0.00 ^d
Group II	900.10 ± 20.67 ^a	0.00 ± 0.00 ^d
Group III	910.50 ± 19.98 ^a	0.00 ± 0.00 ^d
Group IV	518.00 ± 31.32 ^d	60.00 ± 7.83 ^a
Group V	582.20 ± 33.74 ^c	44.00 ± 7.07 ^b
Group VI	650.70 ± 27.69 ^b	25.00 ± 5.93 ^c

Results and discussion

The effects of CuCl₂ and ABE treatments on physiological parameters of *A. cepa* are presented in Table 2 and Fig. 1. It was clearly seen that CuCl₂ negatively changed the physiological parameters and caused a decrease in weight gain, root elongation, and germination percentage. The most remarkable toxic effect was observed in the weight gain of the 20 μM CuCl₂-treated group, which gained only 1.26 g of total weight at the end of the experimental period.

It was determined that CuCl₂ treatment led to statistically marked reductions in both root elongation and germination percentage (*p* < 0.05). The shortest roots were measured as 5.46 ± 1.60 cm in group IV while the maximum root elongation was determined in the control group as 15.51 ± 2.74 cm. Also, germination percentage decreased upon CuCl₂ treatment and the lowest germination was obtained as 48% in group IV.

Doğanlar and Atmaca (2011) reported that uptake and accumulation of heavy metals led to a morphological and a physiological retardation in plants. According to previous studies, negative effect of Cu on root growth is due to its inhibitory function on cell division (Kahle 1993; Doncheva et al. 1996; Posmyk et al. 2009). Furthermore, Geremias et al. (2010) and Yıldız et al. (2009) mentioned a reduction in root

elongation in copper-exposed onion bulbs similar to our study. Our results in group V and group VI clearly demonstrated that when ABE are applied together with CuCl₂ solution, they ameliorate the negative effects of CuCl₂ on growth parameters.

ABE can contain very high anthocyanin amounts along with other phenols, and the healing power of the extract may be associated with its high anthocyanin content (Valentova et al. 2007). Indeed, anthocyanins, as strong antioxidants, are crucial members of defense systems against stresses in different parts of plants including fruits.

The mitotic index is considered as an important parameter since it reflects the frequency of cell division (Liu et al. 2009). MI values of group I, group II, and group III were found as 888.00 ± 19.59, 900.10 ± 20.67, and 910.50 ± 19.98, respectively, without statistical difference (Table 3). MI frequency of CuCl₂-treated group IV (518.00 ± 31.32) decreased drastically while the values of group V (582.20 ± 33.74) and group VI (650.70 ± 27.69) improved progressively as a function of increased ABE concentrations. It has already been reported by other researchers that copper and copper salts significantly suppress mitosis and reduce mitotic index of *A. cepa* (İnceer and Beyazoğlu 2000; Srivastava et al. 2005; Yıldız et al. 2009; Liu et al. 2009). Inhibition of the mitosis in meristem cells of CuCl₂-treated *A. cepa* roots clarified the decrease in root elongation and the weight gain.

Group I, control group; group II, 50 mg/L ABE; group III, 100 mg/L ABE; group IV, 20 μM CuCl₂; group V, 50 mg/L ABE+20 μM CuCl₂; group VI, 100 mg/L ABE+20 μM CuCl₂. 1000 cells were counted for MN frequency. 10,000 cells were counted for MI analysis. The mean values indicated by different superscript letters (^{a-d}) in the same column are significant at *p* < 0.05

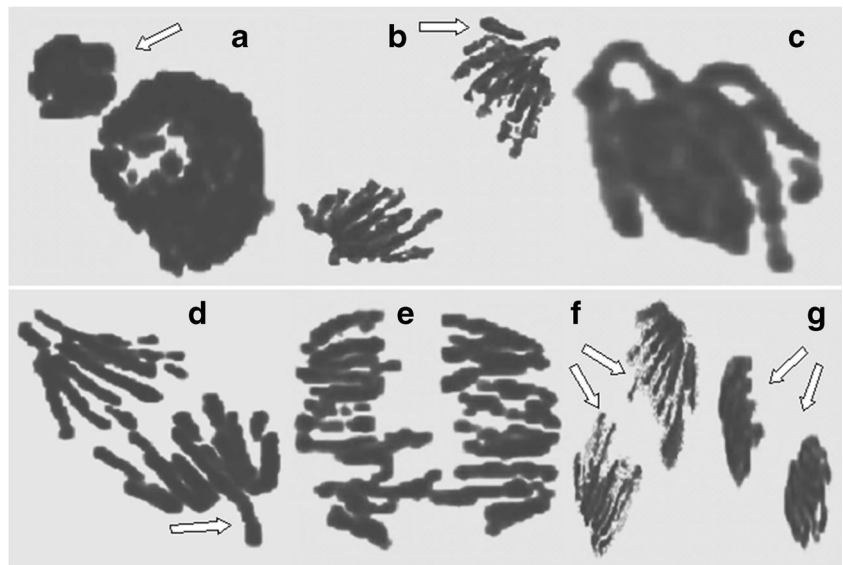
The roots are the first and the most sensitive part of the plants that absorb Cu from soil. Rapid proliferation of meristematic tissues, as apical root tips, offers a highly sensitive way for monitoring the genotoxicity of chemical agents. The MN frequency test and the chromosomal aberration analysis

Table 4 Effects of CuCl₂ and ABE applications on CA frequency

Groups	FRG	SC	VC	B	UCD	AP
Group I	0.00 ± 0.00 ^d	0.30 ± 0.48 ^d	0.20 ± 0.42 ^d	0.00 ± 0.00 ^d	0.30 ± 0.48 ^d	0.20 ± 0.42 ^d
Group II	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.20 ± 0.42 ^d	0.00 ± 0.00 ^d
Group III	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d
Group IV	79.10 ± 9.01 ^a	71.00 ± 8.51 ^a	58.30 ± 8.26 ^a	39.20 ± 8.65 ^a	47.60 ± 8.28 ^a	30.70 ± 6.95 ^a
Group V	68.20 ± 7.58 ^b	59.10 ± 8.63 ^b	48.50 ± 8.00 ^b	30.40 ± 6.64 ^b	34.60 ± 7.88 ^b	21.80 ± 5.92 ^b
Group VI	56.00 ± 8.25 ^c	48.40 ± 8.11 ^c	41.70 ± 5.29 ^c	22.40 ± 4.03 ^c	27.60 ± 6.42 ^c	12.70 ± 3.37 ^c

Group I, control group; group II, 50 mg/L ABE; group III, 100 mg/L ABE; group IV, 20 μM CuCl₂; group V, 50 mg/L ABE+20 μM CuCl₂; group VI, 100 mg/L ABE+20 μM CuCl₂ (*n* = 10). Chromosomal aberrations were calculated by analyzing 100 cells from each root end and a total of 1000 cells in each group. The mean values indicated by different superscript letters (^{a-d}) in the same column are significant at *p* < 0.05. FRG, fragment; SC, sticky chromosome; VC, vagrant chromosome; B, bridge; UCD, unequal chromatin distribution; AP, abnormal polarization

Fig. 2 Chromosome aberrations caused by CuCl_2 application. **a**, MN; **b**, fragment; **c**, sticky chromosome; **d**, vagrant chromosome; **e**, bridge; **f**, unequal chromatin distribution; **g**, abnormal polarization



in *Allium* root meristems have been widely used to detect DNA damage associated with hazardous contaminants (Gupta et al. 2018; Bolsunovsky et al. 2019). The absence of MN and chromosomal aberration in group I, group II, and group III is an undeniable proof that ABE are not genotoxic (Table 3 and Table 4). As expected, the members of CuCl_2 -treated group IV exhibited a sharp rise in MN abundance, while the increasing doses of ABE in CuCl_2 solutions depressed the MN formation gradually in group V and group VI. According to Silveira et al. (2017), the rise in MN and chromosomal aberrations are markers of chromosomal instabilities in cells. Here we proved that CuCl_2 triggered a number of chromosomal aberrations in *Allium* roots, including fragment, sticky chromosome, vagrant chromosome, bridge, unequal chromatin distribution, and abnormal polarization (Fig. 2 and Table 4). These abnormalities, together with an increased MN incidence, are strong evidence for clastogenic and aneugenic damage caused by CuCl_2 . The most observed chromosomal aberrations were fragment (79.10 ± 9.01) and sticky chromosome (71.00 ± 8.51) formation in group IV. Similar to our results, Gupta et al. (2018) reported that fragment and sticky chromosome were among the most abundant

chromosomal aberration types induced by heavy metal exposure in the *Allium* root meristem. Moreover, sticky chromosomes are accepted as critical signs for cell death risk (Gupta et al. 2018). For all chromosomal damage types, incidence decreased significantly with enhanced ABE doses as seen in group V and group VI. Owing to its mobility and redox potential, Cu has a central function in the generation of active oxygen radicals that attack DNA molecules rapidly and lead to breaks in DNA strands. These breaks are accompanied with modifications of the bases and deoxyriboses of the nucleic acids which then induces severe carcinogenesis in cells (Theophanides and Anastassopoulou 2002). Genoprotective and anticancer effects of bilberry fruits are powerful but very complex. Smeriglio et al. (2014) summarized that these effects are mainly results from anthocyanins that scavenge oxidants, induce the antioxidant response element and phase II detoxification enzymes, and interact directly forming a DNA copigmentation complex. Indeed, our results of MN and chromosomal abnormalities verify the protective role of ABE on genetic material.

Cross sections of CuCl_2 -treated *A. cepa* roots were photographed with a research microscope (Fig. 3).

Fig. 3 Anatomical damages caused by CuCl_2 exposure. **a** Thickened cortex cell wall. **b** Flattened cell nucleus. **c** Cell deformation

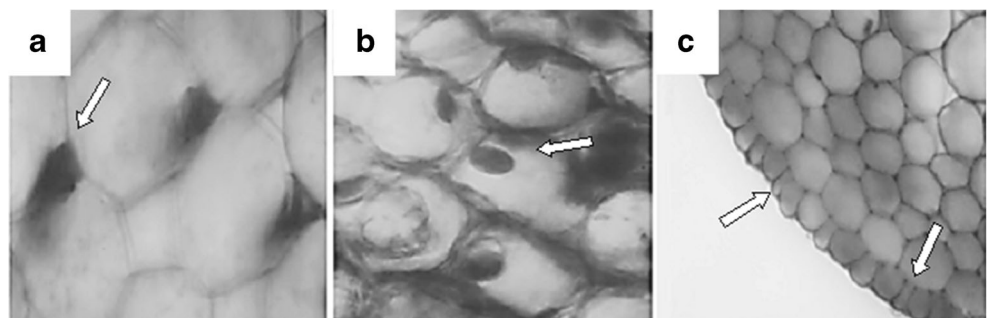
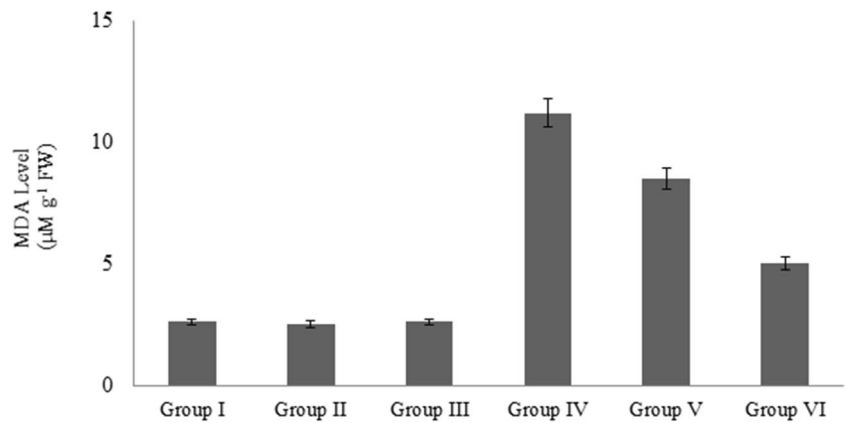


Fig. 4 Effect of CuCl₂ and ABE on root MDA levels. Group I, control group; group II, 50 mg/L ABE; group III, 100 mg/L ABE; group IV, 20 μM CuCl₂; group V, 50 mg/L ABE+20 μM CuCl₂; group VI, 100 mg/L ABE+20 μM CuCl₂ (n = 3)



Thickened cortex cell wall (Fig. 3a), flattened cell nucleus (Fig. 3b), and cell deformations (Fig. 3c) were the anatomical disorders observed in root meristematic tissues. Thickening of the cortex and squeezed epidermal cell deformations were probably the adaptations by the plant to limit the absorption of excess CuCl₂ from the growth medium. Yalçın et al. (2019) stated that plants developed some anatomical adaptations to prevent the transfer of an external toxic agent to inner tissues. On the other hand, flattened cell nucleus may be an irreversible damage caused by toxic CuCl₂ doses that plants were unable to overcome.

As a product of membrane lipid peroxidation, MDA content increases when plants experience oxidative stress (Meng et al. 2007). While the MDA contents of *Allium* roots in group I, group II, and group III were statistically identical, the MDA content in CuCl₂-treated group IV was significantly increased compared with the control group (group I) (Fig. 4). On the other hand, as the doses of ABE increased, MDA concentrations of group V and group VI gradually decreased. Our results in MDA levels showed that CuCl₂ application causes oxidative stress in *Allium* plants. Previous studies on copper toxicity showed that this heavy metal resulted in an increase in MDA levels of onion and garlic root membranes (Meng et al.

2007; Vargas et al. 2017). Meanwhile, ABE application remediated oxidative stress originated from CuCl₂-oriented. Aly et al. (2019) suggested that various berries had a great level of metal-chelating and radical scavenging potential to prevent oxidative damages. The ability of bilberry fruits to suppress the membrane-destructive oxidative stress is mainly due to their superior phenolic and anthocyanin contents (Sellappan et al. 2002).

Results of SOD and CAT levels on *Allium* roots were highly correlated with each other (Figs. 5 and 6). SOD and CAT contents of group II and group III were statistically equal to group I. Levels of both enzymes peaked sharply in group IV and reduced with increasing ABE concentrations in group V and group VI. CuCl₂, as a crucial cofactor of various metalloproteins including SOD enzyme, plays an important role in radical scavenging in cells. However, in elevated doses, it induces oxidative stress and triggers a series of protective responses in plants (Qin et al. 2016). Enzymatic antioxidant system including the induced activity of SOD and CAT is a vital process in order to scavenge free radicals such as superoxide (O₂⁻) and hydrogen peroxide (H₂O₂).

Qin et al. (2016) indicated that the antioxidant enzyme acted as natural guards in ROS defense at low-dosed CuCl₂

Fig. 5 Effect of CuCl₂ and ABE on root SOD activities. Group I, control group; group II, 50 mg/L ABE; group III, 100 mg/L ABE; group IV, 20 μM CuCl₂; group V, 50 mg/L ABE+20 μM CuCl₂; group VI, 100 mg/L ABE+20 μM CuCl₂ (n = 3)

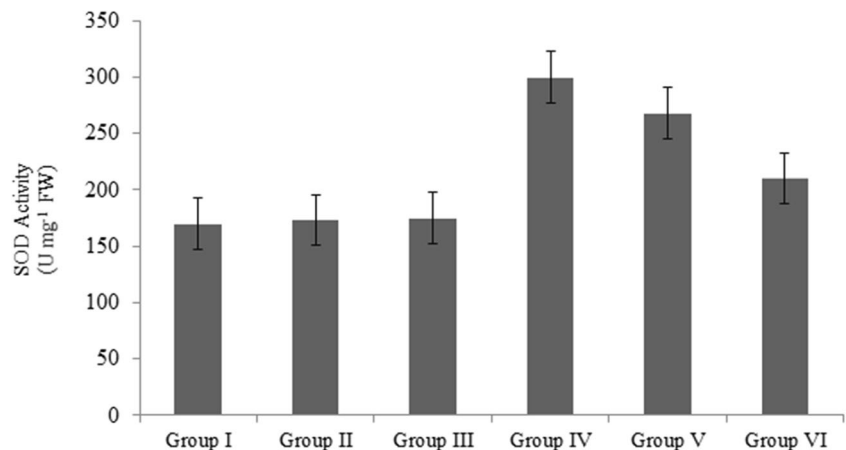
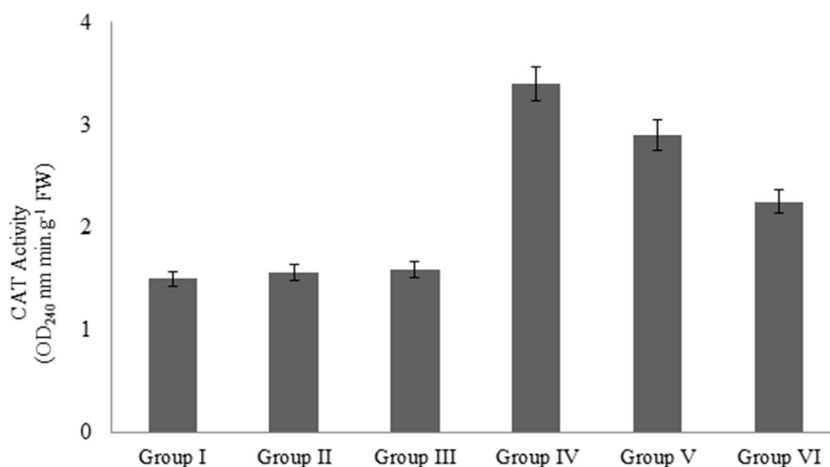


Fig. 6 Effect of CuCl_2 and ABE on CAT activity. Group I, control group; group II, 50 mg/L ABE; group III, 100 mg/L ABE; group IV, 20 μM CuCl_2 ; group V, 50 mg/L ABE+20 μM CuCl_2 ; group VI, 100 mg/L ABE+20 μM CuCl_2 ($n = 3$)



stress in *A. cepa* roots. Coordinated function of ABE in *Allium* roots with regulated enzyme activities reduced the oxidative stress to some extent. Yalçın et al. (2019) stated that antioxidant enzyme activities increased in the case of oxidative stress. In our study, elevated levels of these oxidative stress indicators were in accordance with the frequency of CA which might be among the results of oxidative stress.

Conclusion

In conclusion, restorative and protective abilities of ABE against CuCl_2 -induced toxicity were investigated comprehensively by using *A. cepa* bioassay. Although the deleterious effects of CuCl_2 stress on organisms have already been known, this is the first study which demonstrated the therapeutic and preventive potential of ABE against such a hazardous and common pollutant in onion roots. Anatomical cross sections of roots clearly exhibited the “struggle” of the plant to block the entrance of CuCl_2 to inner tissues. In any case, the data obtained from the other parameters of CuCl_2 -treated groups proved that the plant could not prevent the intake of the chemical completely. On the other hand, ABE acted as a vital protector to overcome the physiological and genotoxic harms of CuCl_2 . Indeed, the restorative effects of ABE increased gradually with its increasing doses. Therefore, *Allium* assay was shown once again to be a reliable method for not only screening the carcinogenic roles of the pollutants but also monitoring the protective potentials of antioxidant agents. Additionally, the functional mechanism of ABE, a popular supplement with a range of health benefits, was revealed efficiently. Our findings provided a reliable basis for further studies that focus on the use of ABE for the prevention of cancer and heavy metal-associated diseases. These kinds of studies on ABE will gain value as the heavy metal pollution increases.

Funding information The present study was supported financially by the Giresun University Scientific Research Unit (project no. FEN-BAP-A-150219-21).

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