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Investigation of the biochemical and apoptotic changes in breast cancer cells treated with leaf extract from tea (*Camellia sinensis* L.) grown with added boric acid

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Abstract: Tea obtained from the leaves of *Camellia sinensis* L., a medicinal plant, is a widely popular beverage. Deficiency in boron, a micronutrient for *C. sinensis*, affects the growth as well as the quality of tea. The aim of this study was to explore whether boric acid at various concentrations added to soil improves the quality of *C. sinensis* and also whether it changes the apoptotic, anti-proliferative, and anti-oxidative effects of *C. sinensis* leaf extract on breast cancer (MCF-7) cells. *C. sinensis* was grown in Rize-Turkey. Boric acid at concentrations of 100 (group B), 300 (group C), and 500 (group D) mg/m^2 in sodium tetraborate buffer was administered as a single dose to the soil; group A (no boric acid) was the control. Boron, glutathione (GSH), malondialdehyde and protein carbonyl levels in the *C. sinensis* leaves were measured. *C. sinensis* leaf extracts at different concentrations was applied to MCF-7 cells for 24 and 48h. Cytotoxicity, proliferation, and apoptosis were examined. The highest TUNEL+ cell percentage was in MCF-7 cells treated with D group leaf extract compared to the control group ($p<0.001$ at concentrations of 2.3, 2.6 and 3mg/mL). Moreover, the GSH level increased in the MCF-7 cells under the same conditions ($p<0.001$ for each concentration). Leaf extracts from *C. sinensis* grown in soil with boric acid have more anti-proliferative, apoptotic and anti-oxidative effects on the MCF 7 cells.

Keywords: Boric acid, *Camellia sinensis*, MCF-7, cell death, anti-oxidant.

INTRODUCTION

Cancer has been reported to be a serious disease and a threat to human health worldwide. Breast cancer is the most common cancer among women and also causes the largest number of cancer-related deaths (American Cancer Society, 2017). MCF-7, an epithelial cancer cell line derived from breast adenocarcinoma, is a cell line commonly used by multiple research groups. Furthermore, it has been reported that natural agents can be used to prevent breast cancer development (Comşa *et al*., 2015; Gomes *et al*., 2019).

Tea (*Camellia sinensis* L.) is the most commonly consumed drink after water. It contains abundant compounds which have presented medicinal properties and health benefits (Tahani & Sabzian, 2018; Ye *et al*., 2019). The consumption of tea, especially green tea, has been shown to reduce the risk of various conditions such as cancer and cardiovascular diseases. In addition, it has been reported that green tea has anti-inflammatory, anti-viral, and antioxidant activities (Serafini *et al*., 2011; Yang *et al*., 2018).

Boron is an essential micronutrient for plants and its compounds such as boric acid are used in agriculture. Excess boron can be toxic to plants and can negatively affect plant growth. However, its deficiency leads to the cessation of root elongation, loss of fertility, and reduced leaf expansion (Fukuda *et al*., 2018; Pommerrenig *et al*., 2019; Sakamoto *et al*., 2011). Boron has many important functions in various biological, physiological and metabolic processes in plants and animals. Studies have reported that boron may play a vital role in human and animal health in terms of its anti-inflammatory, antioxidant, anti-cancer and detoxifying properties (Abdelnour *et al*., 2018; Nielsen, 2009). According to a study by Yamada & Eckhert (2019), the intake of boron in food increases antioxidant levels and reduces cancer risk. Although there is some evidence of its benefit, there is no consensus on whether boron is an essential nutrient for humans.

The aims of the study were (i) to determine the boron concentration in *C. sinensis* leaves that were grown in soil to which boric acid had been added at different concentrations; (ii) to detect the oxidative stress response of the *C. Sinensis* leaves and (iii) to investigate the cytotoxic, anti-proliferative, apoptotic and antioxidant effects of *C. Sinensis* leaf extract grown in soil with added boric acid on MCF-7 cells.

MATERIALS AND METHODS

Samples collection

C. sinensis was collected in Rize-Turkey. The land was separated into four groups. Each group consists of five

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areas (10 m^2). Control (A) group is the first, there is no boric acid application to soil. Boric acid in sodium tetraborate buffer of 100 (B), 300 (C), 500 (D) mg/m^2 at concentration ranges were administered as only one dose on the second, third and fourth groups, respectively. At the end of first (May), third (July) and sixth (September) month, the tea leaves were collected. And then, the leaves were dried at room temperature.

The preparation of C. sinensis leaf extract

Dried tea leaves (5 g) was brewed in 250 ml water at 30 ºC and 80 °C for 20, 40 and 60 minutes. The leaf extract was centrifuged at 4000 g and room temperature for 15 minutes and the clear supernatants were obtained.

Boron quantity analysis in C. sinensis leaf

Dried *C. sinensis* leaves were milled. NaOH was added on leaves and incubated at 70°C for 72h. Samples were burned in ash furnace at 550°C and treated with HNO_3. Finally, the mixture was filtered and obtained solvent was used for analysis. For boron analysis, azomethine-H buffer added on samples for 90 minutes at dark. Absorbance was measured at 420nm, spectrophotometrically (Mohammed *et al*., 2014).

Biochemical parameters of C. sinensis leaf extract

The levels of malondialdehyde (MDA), glutathione (GSH) and protein carbonyl (PC) were analyzed in *C. sinensis* leaf extract. The GSH levels were detected using metaphosphoric acid for protein precipitation and 5, 5'-dithiobis (2-nitrobenzoic acid) for the yellow colour development at 412nm. MDA was evaluated by trichloroacetic acid (TCA, 30%), Thiobarbituric acid (TBA, 0.75%) and hydrochloric acid (HCl). Pink colour in the samples was measured at 535 nm. PC groups were labelled by 2,4-dinitrophenylhydrazine (DNPH) precipitated with TCA (10%), washed with ethanol-ethyl acetate, and dissolved in guanidine-HCl. Results were calculated according to the amount of protein (Habashy *et al*., 2019; Yanar *et al*., 2019).

Cytotoxic effects of C. sinensis leaf extract on MCF-7 cells

MCF-7 cells (ATCC, USA) were cultured in DMEM-F12 medium (Sigma, Germany) supplemented with 10 % fetal bovine serum (FBS, Seromed, Turkey), penicillin (50 units/ml, Biological Industries, Israel) and streptomycin (0.05 mg/ml) (Biological Industries, Israel) in 75 cm^2 flask at 5% CO_2 and 37 °C environment.

MCF-7 cells were treated with A, B, C and D groups of *C. sinensis* leaf extract at 0-4 mg/mL concentrations for 24 h and 48 h. Physiological saline was given to control cells. Cell viability was performed according to the study of Mosmann MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) method was performed in accordance with the instructions of CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA). Each experimental condition was repeated 3 times. The percentage of relative cell viability was computed using the following formula: % Cell viability = (OD_{490} treated cells / OD_{490} control) x 100.

Anti-proliferative and apoptotic effects of C. sinensis leaf extract on MCF-7 cells

MCF-7 cells grown on coverslips were divided into 5 groups. I. group: physiological saline was given to MCF-7 cells. II., III., IV. and V. groups: A, B, C and D groups *C. sinensis* leaf extract was administered to MCF-7 cells, respectively. MCF-7 cells were incubated with *C. sinensis* leaf extract at 2, 2.3, 2.6 and 3 mg/mL doses for 48 h. Cell proliferation was detected using immunostaining of the proliferating cell nuclear antigen (PCNA) by using the streptavidin-biotin-peroxidase technique. For immunocytochemistry staining, the cells on coverslips were washed with PBS, and fixed with methanol. Histostain Plus Broad Spectrum Kit (Invitrogen, 859043) and PCNA primary antibody (1:300, 1h at room temperature, Neomarkers, USA) were used for labeling. Lastly, the detection step was performed with using a 3-amino-9-ethyl carbazole substrate kit (AEC, Invitrogen, 00-2007).

The transferase-mediated dUTP nick end-labeling (TUNEL) method was carried out to determine and quantitate apoptotic cell death using the *in situ* Cell Death Detection Kit (Millipore, USA) in accordance with the instructions. The reaction was visualized with the AEC substrate kit (Invitrogen, 00-2007). The cells were counterstained in Mayer's hematoxylin. Images were captured with an Olympus BX-50 bright-field microscope. Ten randomly selected areas in each slide were evaluated and the total cells were counted. The percentage of immune+ and TUNEL+ cells calculated using the following formula: [(the number of immune+ or TUNEL+ cells / total cells) x 100].

Biochemical parameters of MCF-7 cells treated with C. sinensis leaf extract

MCF-7 cells grown on micro-wells (2 mL) were divided into 5 groups. I. group: physiological saline was administered to MCF-7 cells. II., III., IV and V. groups: A, B, C and D groups *C. sinensis* leaf extract was given to MCF-7 cells, respectively. The cells incubated with *C. sinensis* leaf extract at 2, 2.3, 2.6 and 3mg/mL doses for 48 h. MCF-7 cells were homogenized by ultra-sonicator. The homogenates were centrifuged and supernatants were collected for detection of MDA, GSH and PC levels. Results were calculated according to the amount of protein (Habashy *et al*., 2019; Yanar *et al*., 2019).

STATISTICAL ANALYSIS

Data analysis was performed by SPSS software (version 21.0, SPSS). The experimental values were presented as the mean ± standard error of the mean (SEM). One-way

analysis of variance (ANOVA) was used for statistical significance and then, comparisons between groups were analyzed by using Tukey's post hoc test. The p-value < 0.05 or less was considered statistically significant.

RESULTS

Boron levels in C. sinensis leave

The boron levels of *C. sinensis* leaves in B, C and D groups were increased at 1st and 3rd months with boric acid application to the soil as compared to a group ($p< 0.001$ for each). Boron level of the leaves in the groups showed a significant increase in parallel with the amount of boron supplied. At 6th month, boron levels of the leaves were high in C and D groups compared to A and B groups ($p<0.01$ and $p<0.001$ respectively, for each). Interestingly, the highest boron levels were determined in the *C. sinensis* leaves collected in the 3rd month (fig. 1a).

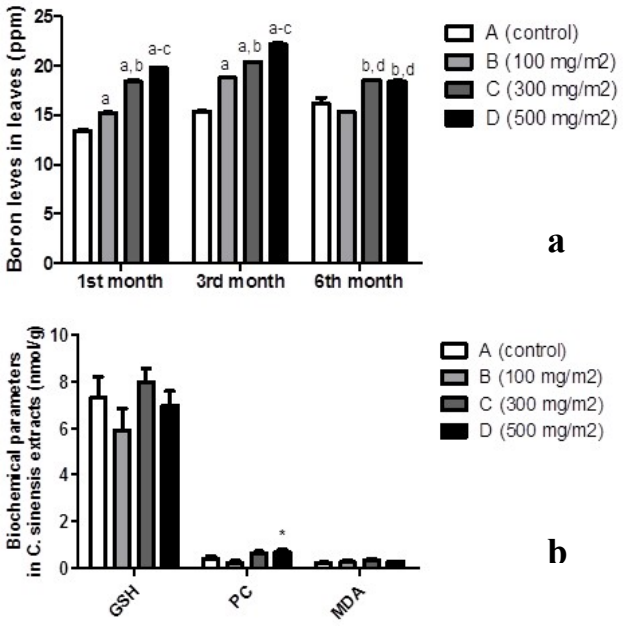

Fig. 1: Boron levels in leaves and biochemical parameters in *C. sinensis* leaf extract. Data are shown as the mean ± SEM. [a]$p<0.001$ vs. A group, [b]$p<0.001$ vs. B group, [c]$p< 0.001$ vs. C group, [d]$p<0.01$ vs. A group and *$p<0.05$ vs. B group.

Biochemical changes in C. sinensis leaf extract

The GSH, MDA and PC levels of tea extract brewed in various degrees and time were determined (data not shown). According to data, the *C. sinensis* leaf extract obtained by brewing the leaves collected in the 3rd month at 80°C for 20 minutes were used in the second part of the study. GSH and MDA levels in *C. sinensis* leaves extract, brewed at 80°C for 20 minutes, did not show a change with boric acid application to soil. However, the PC level in D group increased when compared to the B group ($p < 0.05$) (fig. 1b).

Cell viability of MCF-7 cells treated with C. sinensis leaf extract

According to the MTT test, the effect of tea extract on MCF-7 cells was more effective in 48h than 24h. So, half maximal inhibitory concentration (IC50) of MCF-7 cells at 48h were presented in this study. IC50 values of MCF-7 cells treated with A, B, C and D groups of *C. sinensis* leaves extracts at 48h were 2.79mg/mL, 2.69mg/mL, 2.66 mg/mL and 2.45mg/mL, respectively.

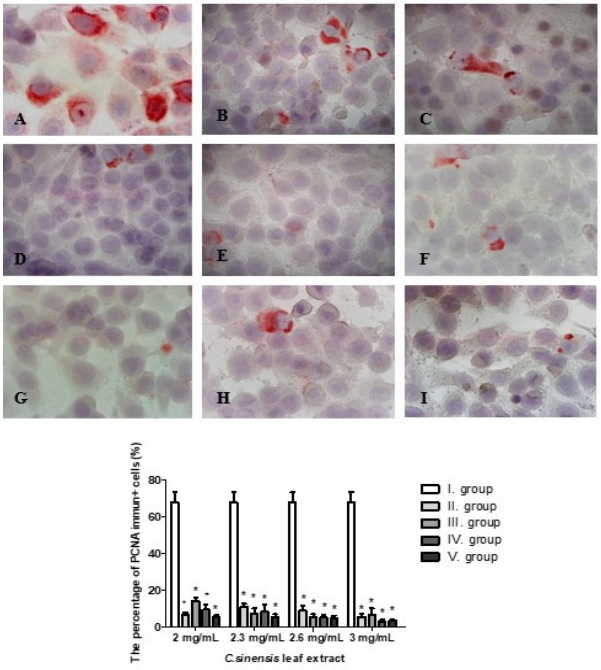

Fig. 2: Proliferating cell nuclear antigen (PCNA) immune+ cells (arrow) and the percentage of PCNA+ cells are seen by immunositochemistry in MCF-7 cells; I. group (A), II. Group at 2.3 (B) and 3 (C) mg/mL concentrations, III. Group at 2.3 (D) and 3 (E) mg/mL concentrations, IV. Group at 2.3 (F) and 3 (G) mg/mL concentrations, V. Group at 2.3 (H) and 3 (I) mg/mL concentrations. Streptavidin-biotin-peroxidase technique, counterstain hematoxylin (original magnification: X1000). Data are shown as the mean ± SEM. *$p<0.001$ I. group.

The anti-proliferative and apoptotic effects of C. sinensis leaf extract on MCF-7 cells

The percentage of PCNA immune+ cells of MCF-7 cells decreased applied in various concentrations (2, 2.3, 2.6 and 3mg/mL) of *C. sinensis* leaf extract in II. Group as compared to I. Group ($p<0.001$ for each). Similarly, administration of *C. sinensis* leaf extract to MCF-7 cells (III, IV and V. Groups) at 2, 2.3, 2.6 and 3mg/mL doses decreased the percentage of PCNA immune+ cells as compared to I. group. The lowest percentage of the PCNA immune+ cells was induced by the administration of the D group to the MCF-7 cells at a dose of 3mg/mL (V. group, fig. 2).

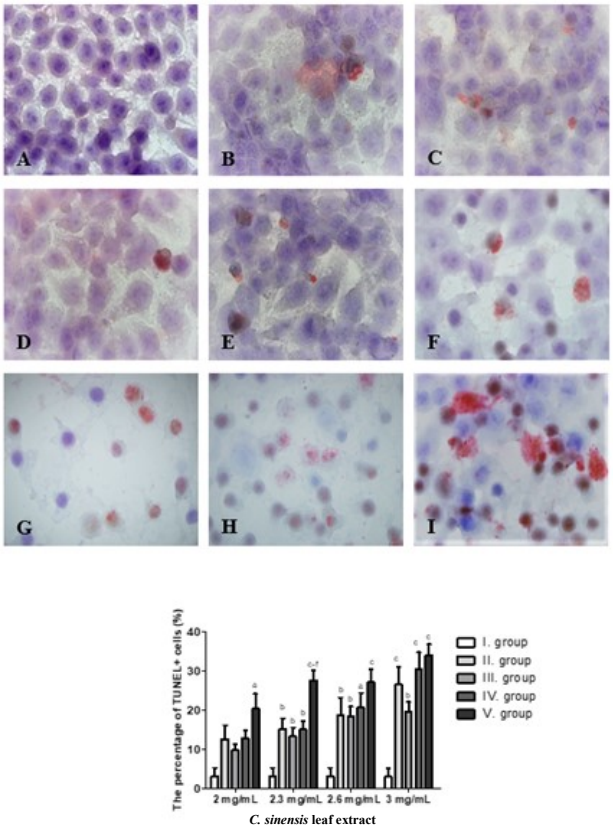

Fig. 3: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of MCF-7 cells (arrows) and percentage of TUNEL+ cells in the groups treated with the *C. sinensis* leaf extract for 48 h. TUNEL staining in I. group (A), II. group at 2.3 (B) and 3 (C) mg/mL concentrations, III. group at 2.3 (D) and 3 (E) mg/mL concentrations, IV. group at 2.3 (F) and 3 (G) mg/mL concentrations, V. group at 2.3 (H) and 3 (I) mg/mL concentrations. Counterstain hematoxylin (original magnification: X1000). Data are shown as the mean ± SEM. [a]p<0.01 vs. I. group, [b]p<0.05 vs. I. group, [c]p< 0.001 vs. I. group, [d]p<0.01 vs. II. group, [e]p<0.01 vs. III. group, [f]p<0.01 vs. IV. group.

Unlike proliferation, the percentage of TUNEL+ cells in MCF-7 cells increased in II. group with the administration of *C. sinensis* leaf extract at doses of 2.3, 2.6 and 3 mg/mL as compared to I. group (p<0.05, p<0.05 and p< 0.001, respectively). Furthermore, the highest TUNEL+ cells percentage of MCF-7 cells was in V. group when compared to I. group (p<0.01 at 2mg/mL concentration, p < 0.001 at 2.3, 2.6 and 3mg/mL concentrations) (fig. 3).

The biochemical effects of C. sinensis leaf extract on MCF-7 cells

C. sinensis leaf extract obtained from soil without boric acid (II. group) at 2, 2.6 and 3 mg/mL concentrations increased the GSH levels in MCF-7 cells compared to I. group (p < 0.001 for each). The GSH levels in IV. group were higher than I., II. and III. groups at 2, 2.3 and 3 mg/mL concentrations (p<0.001 for each). Similarly, the GSH level increased in the V. group as compared to I., II., III. and IV. groups at 2.3, 2.6 and 3mg/mL concentrations (p < 0.001 for each) (fig. 4).

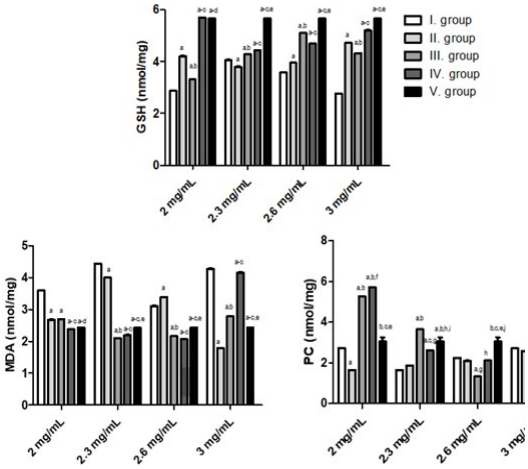

Fig. 4: Glutathione (GSH), malondialdehyde (MDA) and protein carbonyl (PC) levels in MCF-7 cells treated with *C. sinensis* leaf extract at 2, 2.3, 2.6 and 3mg/mL concentrations. Data are shown as the mean ± SEM. [a]p< 0.001 vs. I. group, [b]p<0.001 vs. II group, [c]p<0.001 vs. III. group, [d]p<0.01 vs. IV. group, [e]p<0.001 vs. IV. group, [f]p< 0.05 vs. III. group, [g]p<0.01 vs. II. group, [h]p<0.01 vs. III. group, [i]p<0.05 vs. IV. group, [j]p<0.01 vs. I. group, [k]p< 0.05 vs. II. group.

MDA levels in MCF-7 cells decreased with *C. sinensis* leaf extract (II. group) at 2, 2.3 and 3mg/mL concentrations compared to I. group (p<0.001 for each). A significant decrease in the MDA level was shown in III. group at 2.3, 2.6 and 3mg/mL concentrations as compared to I. and II. groups (p<0.001 for each). Also, MDA level was reduced in the IV. group at 2, 2.3 and 2.6mg/mL concentrations as compared with I., II. and III. groups (p <0.001 for each). The MDA level in the last group (V. group) was lower than III and IV. groups at 3mg/mL concentration (p<0.001 for each) (fig. 4).

It was observed that *C. sinensis* leaf extract (II. group) at 2 mg/mL concentration significantly decreased PC level in MCF-7 cells when compared with I group (p<0.001). PC level in MCF-7 cells was reduced in III. group at 2.6 and 3mg/mL concentrations when compared with I. group (p<0.001 for each). Interestingly, the 2.6 and 3mg/mL concentrations of V. group were elevated PC levels in MCF-7 cells compared to III. and IV. groups (p<0.001 for each) (fig. 4).

DISCUSSION

Tea has economic and social importance for the many countries in the world. It is reported that 3.27 million ha of land is covered by tea plant (*C. sinensis*) in the world. Moreover, it has been shown that the application of micronutrients such as boron, manganese, and zinc are important to increase the productivity of tea (Kumar, 2017; Karak *et al*., 2017). Baruah *et al*., (2011) suggested boron deficiency affects the growth as wells as the quality of tea. According to Gohain *et al*., (2000), foliar boric acid application had a slight effect on the quality of tea. Similarly, Hajiboland *et al*., (2013) suggested that tea plant had a high tolerance to boron deficiency. According to our data, when the boron levels of *C. sinensis* leaves in all groups were compared, the highest boron levels in leaves were determined during the 2nd harvest period in the 3rd month, which might have been due to seasonal rainfall. Since the amount of boron in the soil decreases with rainfall, a higher level of boron in the soil can be detected in July.

According to GSH, MDA and PC levels of *C. sinensis* leaf extracts, we preferred *C. sinensis* leaves collected in the 3rd month. Likewise, the brewing temperature and time were determined as 80ºC and 20 minutes, respectively. Similar to the study by Gohain *et al*., (2000), the application of boric acid to the soil slightly affect biochemical activities of the *C. sinensis* leaf extracts.

Boron can cause several great metabolic and inflammatory changes (Hasbahceci *et al*., 2013; Cetin *et al*., 2017). Turkez *et al*., (2012) showed that boron compounds can provide serious prevention against metal toxicities by their antioxidant capacity. In addition, authors suggested that boron compounds may be useful in the development of functional foods and raw materials of medicine. Karak *et al*. (2017) suggested that a wide range of micronutrients in tea could be a source of micronutrients for human, with boron being one of them. In our study, even the slight influence of boric acid on the *C. sinensis* leaf extracts led to significant results on their effects on MCF-7 cell cultures.

C. sinensis leaf extracts inhibited the cell proliferation of the MCF-7 cells and the percentage of apoptotic cells increased, both of which were enhanced when boric acid had been added to the soil of the growing plants. Thus, *C. sinensis* leaf extract showed both anti-proliferative and increased apoptotic activities in the MCF-7 cells, and boric acid addition to the soil of the growing plants enhanced these activities.

The depletion of antioxidants or the overproduction of ROS leads to oxidative damage of biomolecules such as protein, lipid, and DNA. Oxidative stress has been known to be a major component of several pathological and biological processes such as inflammation, carcinogenesis, aging and several other metabolic diseases (Zou *et al*. 2005; Terzioglu *et al*., 2016; do Nascimento Kaut, 2018). According to a study on lipopolysaccharide-induced liver oxidative stress in rats, oxidative stress induces a reduction in GSH level and an increase in MDA levels (Sha *et al*. 2019). Terzioglu *et al*., (2016) reported that MDA (a lipid peroxidation marker) and PC levels increased with oxidative stress. In the present study, GSH levels increased and MDA levels decreased in MCF-7 cells when boric acid was added to the soil of the *C. sinensis* plants from which the leaf extracts were obtained. Meanwhile, the PC levels in the MCF-7 cells changed in a dose-dependent manner: the PC levels were particularly low at leaf extract doses of 2.6 and 3 mg/mL from groups C and D. Therefore, we can say that boric acid addition to the soil in which *C. sinensis* is grown increases the anti-oxidative activity against oxidative stress in MCF-7 cells.

CONCLUSION

According to our findings, the quality of *C. sinensis* leaf extract depends upon various parameters, including harvest time, and brewing temperature and time. Although *C. sinensis* is tolerant toward boron deficiency, boric acid application to tea plant growing soil may affect the quality of the plants. *C. sinensis* leaf extract has anti-proliferative, apoptotic and anti-oxidative effects which increased in the leaf extracts obtained from *C. sinensis* grown when boric acid had been applied to the soil. Further studies are needed to determine the therapeutic effects of the consumption of food grown in soil treated with boric acid.

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