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Investigation of benzyl benzoate toxicity with anatomical, physiological, cytogenetic and biochemical parameters in *in vivo*

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Abstract. In this study, the toxic effects of benzyl benzoate, which is widely used in the food, cosmetics, agriculture and pharmaceutical sectors, have been investigated using *Allium cepa* L. test material. In the determination of toxicity, physiological parameters with determination of root lengths, weight gains and germination percentages; cytogenetic changes with determination of chromosomal abnormalities formation, micronucleus (MN) frequency and mitotic index ratio (MI); anatomical changes with determination of anatomical differentiations in root tip cells; biochemical changes with lipid peroxidation and antioxidant enzyme analysis were determined and the obtained data were evaluated statistically. The bulbs were divided into four groups consisting of one control and three application groups, bulbs of the control group were treated with tap water and the bulbs of the application groups were treated with Benzyl benzoate at doses of 10,000, 25,000 and 50,000 mg/L for 72 hours. At the end of the study, it was determined that germination percentage, weight gain and root length and MI ratio decreased, chromosomal abnormalities, MN formation, MDA, SOD and CAT levels increased dose-dependent in the application groups when compared with the control group. Depending on the application, it has been determined that root cells have chromosomal abnormalities such as fragments, sticky chromosomes, chromosome bridges, unequal distribution of chromatins and c-mitosis. Furthermore, when compared with the control group, it was determined that benzyl benzoate administration caused anatomical changes in root tip cells. It was determined that these changes were in the form of necrosis, cell deformation, flattened cell nuclei, cortex cell deformation, accumulation of certain substances in cortex cells, wall thickening in cortex cells and unclear vascular tissue. In conclusion, it was determined by physiological, anatomical, cytogenetic and biochemical parameters that benzyl benzoate showed a dose-dependent toxic effect in *Allium cepa* L. root cells. Also, the parameters used in the study were determined to be useful biomarkers for the determination of toxicity.

Keywords: benzyl benzoate, *Allium cepa* L., toxicity, chromosomal abnormalities, lipid peroxidation, antioxidant enzymes.

INTRODUCTION

With the increase in the world population, it is rapidly increasing in consumption in many areas such as food, medicine, clothing and cosmetics. Due to this increase, production quantities of foodstuffs, medicines and cosmetic products, that have an important place in human life, are increasing day by day. These increases in production and consumption cause competition among the manufacturers, that encourages the use of various chemicals to companies who want to gain more profit and find faster solutions to some problems occurring in production, marketing and storage. The negative effects caused by the chemicals used are not investigated sufficiently and the damages that can be given to the ecosystem, especially the human health and environment, are ignored (Searle, 1995).

Organisms are exposed to many foreign substances and are commonly referred to as xenobiotics. Exposure of people to xenobiotics can be caused by food additives, cosmetics and medicines that are directly consumed by people and used in the substances, while indirect exposure may be due to pesticides and agricultural drugs used in agricultural control (Akay, 2004; Alam and Jones, 2014).

The increasing use of chemical substances increases the importance of toxicological studies. It is possible to examine the possible effects of chemical substances that can be absorbed by respiration, nutrition or skin through toxicological researches, to develop strategies for control purposes and to prohibit use (Vural, 2005).

Benzyl benzoate is a chemical compound which is widely used in food, cosmetics, agriculture and pharmaceutical sectors. It is also widely used in combating mites and insects. The aim of this study was to investigate the physiological, cytogenetic, anatomical and biochemical effects of benzyl benzoate that is one of the most frequently used chemical substances, on *Allium cepa* L.

Benzyl benzoate is an ester of benzyl alcohol and benzoic acid, also known as benzoic acid phenylmethyl ester, benzoic acid benzyl ester, benzyl benzene carboxylate, benzyl phenyl formate and phenylmethyl benzoate. It is a chemical compound with the closed formula $C_{14}H_{12}O_2$, open formula $C_6H_5COOCH_2C_6H_5$, molecular weight 212.25 g/mol, density 1.118 g/cm^3, melting point 18-20 °C and boiling point 323 °C (Hassan and Mossa, 1981; Ash and Ash, 2004). It has a sharp burning taste and is colorless, oily and liquid. Benzyl benzoate is rapidly hydrolyzed to benzyl alcohol and benzoic acid in vivo, and benzyl alcohol is then oxidized to glycine-conjugated benzoic acid to form hippuric acid (Hassan and Mossa, 1981). Benzyl benzoate can be formed as a result of condensation of benzyl alcohol and benzoic acid and also from benzaldehyde by Tishchenko reaction (Kamm and Kamm, 1941). It is naturally found in various essential oils with Peru and Tolu balsams. In addition, it has been determined that benzyl benzoate was found to be 89.5% in the oils obtained from the leaves of the *Cinnamomum sulphuratum* Nees plant and 98.2% in the root crusts oils (Kar, 2003; Rameshkumar and George, 2006).

Benzyl benzoate has a wide variety of application areas. Used as a diluent and solvent in solid aromatics, as a stabilizer in perfume compositions due to its low volatility, as solvent for substances such as cellulose acetate and nitrocellulose, instead of camphor in cellulose and plastic pyroxylin compounds and also in many different sectors and products such as confectionery and chewing gum products (Kar, 2003). Benzyl benzoate can also be used as an additive in foods. Benzyl benzoate use has been approved by the EU Food Processing Agents as a sweetening food additive (EU, 2012), and carrier solvent by the FAO/WHO Expert Committee on Food Additives (JECFA, 1996). It is also known that benzoic acid and its compounds are used in foods such as chocolates, beverages, oils, sauces, milk powders, fats, ketchup, mayonnaise, bakery products, dry yeasts, sugars, gums, salads and cookies (Erkmen and Bozoğlu, 2008). Benzyl benzoate is also known to be used in the cosmetic industry. In a study conducted in England, it was reported that benzyl benzoate was found in 23% of the cosmetics examined in research (Buckley, 2007). Benzoic acid and its species are generally used as pH adjuster and preservative in cosmetic products (Wenninger *et al.*, 2000). Benzyl benzoate is also used to increase agricultural yield by combating pests in agricultural production. Benzyl benzoate has been reported to be acaricidal (McDonald and Tovey, 1993) and insecticidal (Jantan *et al.*, 2005). One of the most common uses of benzyl benzoate is the health field. It is widely used to combat lice and to treat scabies. Benzyl benzoate is topically applied for the treatment of scabies. It should be applied to all skin surfaces from the scalp to the soles of the skin during the treatment process and should be avoided from contact with the eye and its use in inflamed or cracked skin. If used in children may show an irritant effect (Stuart *et al.*, 2009). Benzyl benzoate has been in use in the treatment of scabies since 1937. It is formulated as emulsions in concentrations from 20% to 35%. As a complaint after application, there is burning, irritation and tenderness in the case of intense contact. It may cause irritation dermatitis, especially in the genital area and on the face (Campbell and Rew, 1986; Habif, 2016).

The mechanism of action of benzyl benzoate against the disease agent *Sarcoptes scabiei* L. is not yet known (Micali and Lacarrubba, 2016). When used topically, it is a relatively toxic compound. Although its chronic effects are not known, it may cause mild allergic reactions which may be lost after the end of the exposure. When used as an acaricide, it may cause diarrhea, peristalsis, enterospasm, intestinal colic, spastic constipation, pylorospasm, hypertension, contraction of seminal vesicles and bronchospasm in the intestines (Wexler *et al.*, 2005). A 7-year-old male patient was reported to have died after marrow transplantation for aplastic bone marrow disease. The death cause could not be determined precisely, but the month before the diagnosis, his body was washed with ethyl alcohol, water, polysorbate and Ascabiol (containing 10% benzyl benzoate and 2% disulfiram) and death was probably caused by the chronic overdose of the scabicide (Hayes and Laws, 1991).

It was reported that oral LD 50 values of benzyl benzoate varied from 1700 mg/kg in rats to 22440 mg/kg in dogs. When applied to animals too often or over a large area of the skin, saliva can trigger signs of systemic toxicity such as pylorization, incoordination of muscles, tremors, the progression of hind limbs, severe convulsions, shortness of breath and death. When administered in high doses to laboratory animals, it may cause incoordination, hyperexcitation, convulsions, ataxia and respiratory paralysis (Wexler *et al.*, 2005). In addition, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has determined the ADI (Average Daily Intake) value of benzyl benzoate as 5 mg/kg, indicating the amount that a person can consume without a health risk throughout his or her life, based on the body weight of the individual (WHO, 2001).

In this study, *Allium cepa* L. test was used to investigate the effects of benzyl benzoate. *Allium cepa* L. test was considered to be suitable for evaluating chromosome damage and changes in the mitotic cycle because of the small number of chromosomes (2n = 16) and the large structure. Analysis of chromosomal changes allows the determination of structural changes, however, it is achievable to observe the numerical changes occurring in chromosomes. In addition, it has many advantages such as low cost, multiple roots, short test duration, storage and ease of use, ease of observing nucleus and abnormal events (Fiskesjö, 1985; Liu *et al.*, 1995). The data obtained as a result of the *Allium cepa* L. test provide accurate estimates of the effects of the agent investigated on other living biodiversity. Cytotoxicity tests using in vivo plant testing systems, such as *Allium cepa* L., have been approved by several researchers performing in vitro and in vivo animal testing and the results obtained are similar (Vicentini *et al.*, 2001; Teixeira *et al.*, 2003). In the evaluations, 76% of 148 chemicals evaluated with *Allium cepa* L. test gave positive results, this test was accepted as a standard test to determine the chromosomal damage caused by chemicals (Grant, 1982). Applying chemicals to *Allium cepa* L. root tips, cell progression can be blocked at one of the stages of the cell cycle or cell division. The applied chemical has a toxic effect by causing chromosomal abnormalities, bridge and MN formation in the root tip cells (Bonciu *et al.*, 2018).

MATERIALS AND METHODS

Preparation of research materials and application groups

This study was carried out using benzyl benzoate at doses of 10,000 mg/L, 25,000 mg/L and 50,000 mg/L. Approximately equally large and healthy *Allium cepa* L. bulbs are used as research material. The bulbs were divided into four groups of one (1) control, three (3) administration groups and placed in glass beakers 85x100 mm in diameter and allowed to germinate at room temperature for 72 hours. During the application period, the bulbs in the control group were treated with tap water and the bulbs in the application groups were treated with benzyl benzoate at doses of 10,000 mg/L, 25,000 mg/L and 50,000 mg/L. At the end of the period, the root ends were washed with distilled water and prepared for cytogenetic analysis using standard crushing preparation techniques (Qian, 2004).

Measurement of physiological parameters

At the end of the application period, the root lengths were determined with the millimetric ruler based on the radicular formation of the bulbs germinating and the weight gains were determined by the precision scales by taking into consideration the weight differences obtained before and after the application. Germination percentages were determined using equation 1.

$$\text{Germination Percentage} = \frac{\text{Number of Germinated Bulbs}}{\text{Total Number of Bulbs}} \times 100 \quad (1)$$

Chromosomal abnormalities, micronucleus (mn) test and mitotic index (mi) determination

The root tips cut about 0.5 cm in length were fixed for two hours at the 'Clarke' fixator, washed for 15 min-

utes in 96% ethanol and stored in +4° C at 70% ethanol. In the next step, the root tips were hydrolyzed in 1N HCl for 17 minutes at 60 ° C, incubated in 45% acetic acid for 30 minutes and stained with Acetocarmine for 24 hours, then crushed with 45% acetic acid and photographed at X500 magnification in a binocular research microscope (Qian, 2004; Staykova *et al*., 2005). The criteria determined by Fenech *et al.* (2003; 2010) were taken into consideration in the determination of the existence of MN.

Prepared preparations were examined in a binocular research microscope to determine Mitotic Index (MI) ratio and the percentage of MI was determined by using equality 2.

$$\text{Mitotic Index (\%)} = \frac{\text{Number of Divided Cells}}{\text{Total Number of Analyzed Cells}} \times 100 \quad (2)$$

Anatomical damages

In order to determine the anatomical damages in the root tip meristematic cells, the *Allium cepa* L. root ends were washed with distilled water and the cross-sections were taken and stained with methylene blue.

Biochemical analysis

Determination of lipid peroxidation

Lipid peroxidation measured according to the method specified by Ünyayar *et al.* (2006) measuring the amount of malondialdehyde (MDA). The values of the MDA content are taken from the measurements of three independent samples and are expressed as mean + standard error (SE) µmol/g fresh weight (FW).

Antioxidant enzyme analysis

Approximately 0.5 g of the tissue sample taken from the control and administration group root tips were cut into small pieces by washing with deionized water and homogenized by trituration with 5 mL chilled sodium phosphate buffer. The homogenates were transferred to new tubes and centrifuged at 10,500 rpm for 20 minutes at room temperature, and the supernatant was stored at +4 ° C for antioxidant enzyme analysis.

Superoxide dismutase (SOD) determination

Superoxide Dismutase (SOD) activity was determined by making some modifications to the method of Beauchamp and Fridovich (1971). One unit SOD enzyme activity was determined as the amount of SOD enzyme required for 50% inhibition of NBT reduction under application conditions. The values of the SOD content were taken from the measurements of three independent samples and are expressed as mean + standard error (SE) U/mg fresh weight (FW).

Catalase (CAT) determination

Catalase activity (CAT) was determined according to the method determined by Beers and Sizer (1952). The CAT activity unit was defined as 0.1 unit change at 240 nm absorbance. The values of the CAT content were taken from the measurements of three independent samples and expressed as mean ± standard error (SE) OD_{240nm}/min.g fresh weight (FW).

Statistical analysis

SPSS Statistics V 23.0 (IBM Corp., USA, 2015) package program was used for the analysis of statistical data. The statistical differences between the groups were evaluated by using One-way ANOVA and Duncan tests. Data were given as mean ± SD values and P value was considered as statistically significant when less than 0.05.

RESULTS AND DISCUSSION

The effects of benzyl benzoate on germination percentage, root length and weight gain are shown in Table 1. Treatment of the *A. cepa* test material with different doses of benzyl benzoate showed a germination inhibitory effect. The germination percentage was 100% in Group I, 83% in Group II, 63% in Group III and 47% in Group IV. It was determined that the germination per-

Table 1. Effects of different doses of benzyl benzoate on germination percentage, root length and weight gain.

Groups	Germination Percentage (%)	Average Root Length ±SD	Weight gain (g)
Group I	100	8.25±0.81[a]	+7.71
Group II	83	5.58±0.75[b]	+4.88
Group III	63	3.13±0.60[c]	+2.96
Group IV	47	1.30±0.37[d]	+0.44

*Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate. Means with the different letters are statistically significant (P<0.05).

centage decreased with the increase in the benzyl benzoate doses. Although there is no comprehensive study investigating the effects of benzyl benzoate on root length in the scientific literature, there are similar studies conducted with other food additives and chemicals that support the results. For example, in a study conducted by Singh (2017), tartrazine, which is used as a coloring additive in food, medicine and cosmetic fields, was applied to the *Vigna radiata* (L.) R. Wilczek seeds at 0.05 ppm and 0.1 ppm doses, consequently 90% germination occurred in the 0,05 ppm tartrazine treated group, 0.1 ppm tartrazine treated group is not germinated and decrease in germination percentage due to application doses. Bidlan *et al.* (2004) investigated the effects of different doses of hexachlorocyclohexane on germination in radish and mung bean. As a result, the percentage of germination for both species was reported to decrease due to the application dose.

Benzyl benzoate also showed an inhibitory effect on another physiological parameter, root length. With the increase in the dose of benzyl benzoate, it was determined that the root length decreased. The average root lengths of the groups were measured as 8.25±0.81 cm in Group I, 5.58±0.75 cm in Group II, 3.13±0.60 in Group III and 1.30±0.37 in Group VI. The maximum root length was determined in the control group and the lowest root length was determined in Group IV treated with a 50,000 mg/L dose of benzyl benzoate. There are similar studies conducted with other food additives and preservatives by other researchers that support our findings. In a study conducted by Adeyemo and Farinmade (2013), monosodium glutamate (MSG), which is used as a flavor-enhancing additive in foods, is given to *A. cepa* test material with doses of 1.0 g/L, 3.0 g/L, 5.0 g/L and 7.0 g/L, changes in the root length were measured for five days. As a result, it was reported that MSG application inhibits root growth at all test concentrations and this is statistically significant at higher doses. In another study by Koç and Pandir (2018), *A. cepa* test material was treated with different doses of sunset yellow (E-110) and brilliant blue (E-133) additives, which were used as a coloring agent in foodstuffs. It was reported that root lengths decreased due to increasing application dose.

In the study, it was determined that benzyl benzoate application had an inhibitory effect on weight gain which is another physiological parameter. At the end of the 72 hours application period, the mean weight gains were measured as 7.71 g in Group I, 4.88 g in Group II, 2.96 g in Group III and 0.44 g in Group IV. It was determined that there was an inverse ratio between the dose of benzyl benzoate administered and weight gain. Although there is no comprehensive study investigating the effects of benzyl benzoate on weight gain, there are similar studies in the treatment of some diseases and chemical substances used in the fight against insects. In a study conducted by Arslanoglu (2011), *A. cepa* test material was administered Basudin 60 EM insecticide at doses of 600, 1200 and 1800 ppm, resulting in a reduction in weight gain due to increased dose. In another study, Çavuşoğlu *et al.* (2012b) applied 100 mg/kg, 250 mg/kg and 500 mg/kg doses of thiamethoxam insecticide to the *A. cepa* test material and reported that the weight gain was reduced depending on the application doses.

In our study, cytogenetic effects of benzyl benzoate application were investigated by determining chromosomal damages, MN formation and MI ratio. The effects of benzyl benzoate on MN formation are shown in Table 2 and Figure 1. Benzyl benzoate was found to promote the formation of MN in root tip cells of *A. cepa* depending on the dose of administration. As a result of microscopic examinations, there was an average of 0.30 ± 0.48 MN formation in the control group. In Group I, which is the control group, only a few MN formation was observed and it was determined that MN formation increased with increasing dose of benzyl benzoate. The mean MN formation was determined as 0.30±0.48 in Group I, 11.80±2.74 in Group II, 24.70±3.34 in Group III and 47.60±5.34 in Group IV. Similar studies have been carried out with other food additives and chemicals that support our findings. For example, Dönbak *et al.* (2002) investigated the cytogenetic effects of boric acid, which is used as a preservative additive in foods, on the *A. cepa* test material, resulting in boric acid causing the forma-

Table 2. Effects of different doses of benzyl benzoate on MN formation and Mitotic Index (MI).

Groups	MN Frequency Average±SD	Mitotic Index (MI)	Percent MI (%)
Group I	0.30±0.48^{d}	901.50±32.35^{a}	9.02
Group II	11.80±2.74^{c}	808.40±30.84^{b}	8.08
Group III	24.70±3.34^{b}	699.50±32.86^{c}	7.00
Group IV	47.60±5.34^{a}	534.70±16.67^{d}	5.35

*Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate. For the determination of the mitotic index, 1,000 cells at each root tip in each group and 10,000 cells in total were analyzed. For the formation of MN, 100 cells each root tip in each group and were 1,000 cells in total were analyzed. Data were shown as mean ± standard deviation (SD) (n = 10). The statistical significance between the means was determined by using "one-way" ANOVA analysis of variance following the Duncan's test. The averages indicated by different letters in the same column were statistically significant (P<0.05).

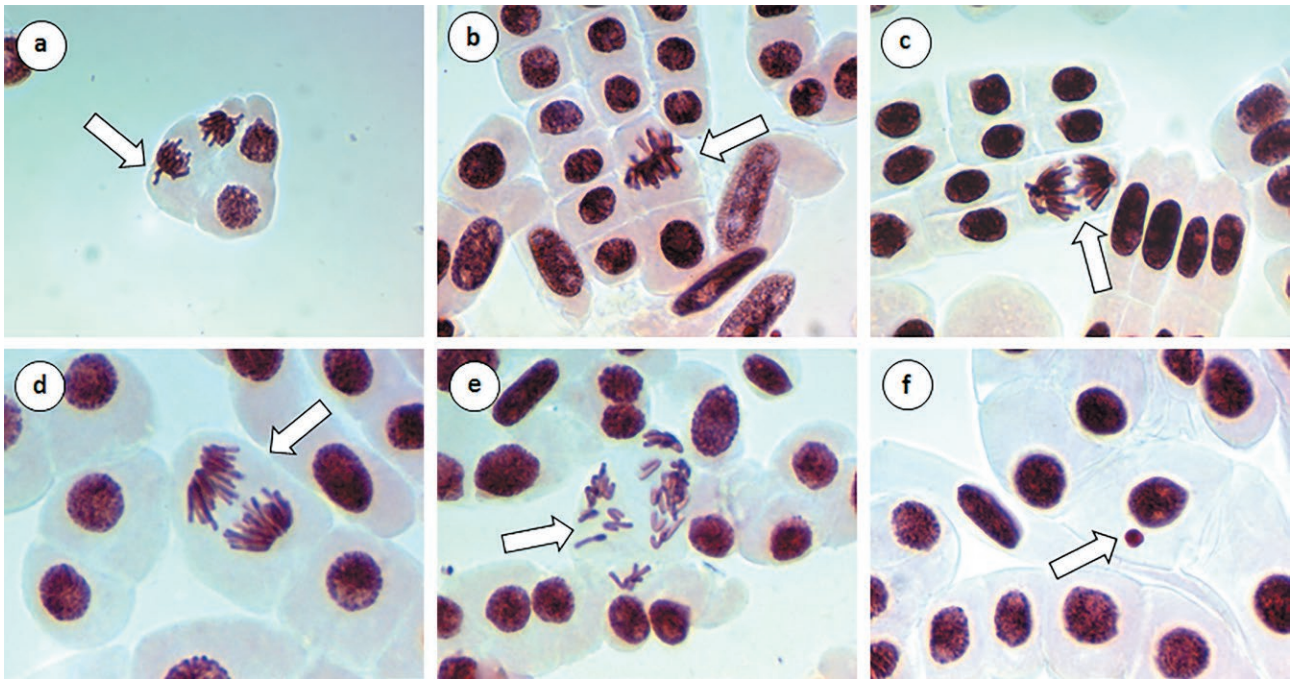

Figure 1. Chromosomal damages and formation of MN induced by benzyl benzoate (a: fragment, b: sticky chromosome, c: chromosome bridge, d: unequal distribution of chromatin dağılımı, e: c-mitosis, f: MN).

tion of MN. In another study, Gomes *et al.* (2013) investigated the cytogenetic effects of Bordeaux red (E-123), tartrazine yellow (E-102) and sunset yellow (E-110), which are used as colorant additives in foods, in *A. cepa* root tip cells. As a result, it has been reported that the application of these food additives causes the formation of MN.

The effects of benzyl benzoate on MN formation are shown in Table 3 and Figure 1. As a result of microscopic observations, it was determined that the frequency of chromosomal damages induced by benzyl benzoate application was fragment> sticky chromosome> chromosome bridge> unequal distribution of chromatin> c-mitosis. The highest effect of benzyl benzoate on chromosomes was determined as fragment formation. While no statistically significant chromosomal damages were observed in the control group (except several sticky chromosomes, chromosome bridge and c-mitosis), all chromosomal damages in the application groups increased due to the application dose and these increases were statistically significant ($P < 0.05$). Similar studies have been performed with other food additives and insecticides that support our findings. In a study conducted by Türkoğlu (2007), boric acid, sodium benzoate,

Table 3. Effects of different doses of benzyl benzoate on the frequency of chromosomal abnormalities

Groups	Number of root tips	Number of Mitotic Cells	FRG	SC	CB	UDC	CM
Group I	10	100	0.00 ± 0.00^{d}	0.30 ± 0.48^{d}	0.20 ± 0.42^{d}	0.00 ± 0.00^{d}	0.10 ± 0.32^{d}
Group II	10	100	17.10 ± 3.81^{c}	13.40 ± 2.91^{c}	10.80 ± 2.53^{c}	8.20 ± 2.20^{c}	3.60 ± 1.07^{c}
Group III	10	100	33.10 ± 4.77^{b}	27.60 ± 4.48^{b}	20.90 ± 5.15^{b}	14.80 ± 4.08^{b}	9.50 ± 2.07^{b}
Group IV	10	100	65.20 ± 7.36^{a}	46.70 ± 8.10^{a}	39.90 ± 8.12^{a}	30.20 ± 6.70^{a}	23.40 ± 5.62^{a}

*Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate. Data were shown as mean ± standard deviation (SD) (n = 10). For chromosomal abnormalities, 100 cells at each root tip in each group and 1000 cells in total were analyzed. The statistical significance between the means was determined by using "one-way" ANOVA analysis of variance following the Duncan's test. The averages indicated by different letters in the same column were statistically significant ($P<0.05$). (FRG: fragment, SC: sticky chromosome, CB: chromosome bridge, UDC: unequal distribution of chromatin, CM: c-mitosis).

potassium citrate and citric acid used as preservatives in foods were applied to *A. cepa* bulbs in 20, 40, 60, 80 and 100 ppm doses, as a result, it was reported that chromosome damage occurred in anaphase bridge, c-mitosis, laggard chromosome and sticky chromosome, chromosome damages occurred due to the increase in application time and doses. In another study, Singh *et al.* (2007) treated *Hordeum vulgare* L. seeds with 0.01%, 0.1% and 0.5% concentrations of prophenophos insecticide and mancozeb fungicide. As a result, it was reported that chromosomal abnormalities occurred due to the application doses and chromosomal damages were reported as disturb metaphase, bridge, laggards, chromosomal breakage and diagonal anaphase.

In this study, it was determined that benzyl benzoate had an inhibitory effect on Mitotic Index (MI) in *A. cepa* root tip cells.

The effect of benzyl benzoate application on Mitotic Index (MI) in root cells of *A. cepa* is shown in Table 2. When the data were analyzed, MI was 9.02% in the control group, 10.85% in Group II, 7.00% in Group III and in Group IV it was determined as 5.35%. Depending on these findings, it was determined that MI was decreased with increasing doses of benzyl benzoate, in other words, the MI and benzyl benzoate administration dose showed inverse proportions. In addition, the differences between the groups were determined to be statistically significant ($P < 0.05$). Similar studies have been carried out with other food additives and insecticides that support our findings. Njagi and Gopalan (1982) applied different doses of sodium benzoate and sodium sulfite used as preservative additives in foods to the root tip cells of *Vicia faba* L., as a result, a reduction in MI was reported due to the administration dose. In another study, Rencüzoğulları *et al.* (2001), treated *A. cepa* test material with sodium metabisulphite used as a coloring additive in foods at doses of 7.5 mg/L, 15 mg/L and 30 mg/L for 10 and 20 hours, consequently, it was determined that MI decreased in both applications duration due to application doses.

As a result of microscopic observations, it was observed that the application of benzyl benzoate caused significant anatomic damages in *A. cepa* root tip cells. Anatomical examinations of root tips of the control and application groups application group are shown in Figures 2 and 3. These damages are in the form of necrosis, cell deformation, flattened cell nucleus, accumulation of some substances in cortex cells, cortex cell deformation, cortex cell wall thickening and unclear transmission tissue (Figure 2). As a result of the investigations, it was found that there was no anatomical change in the root

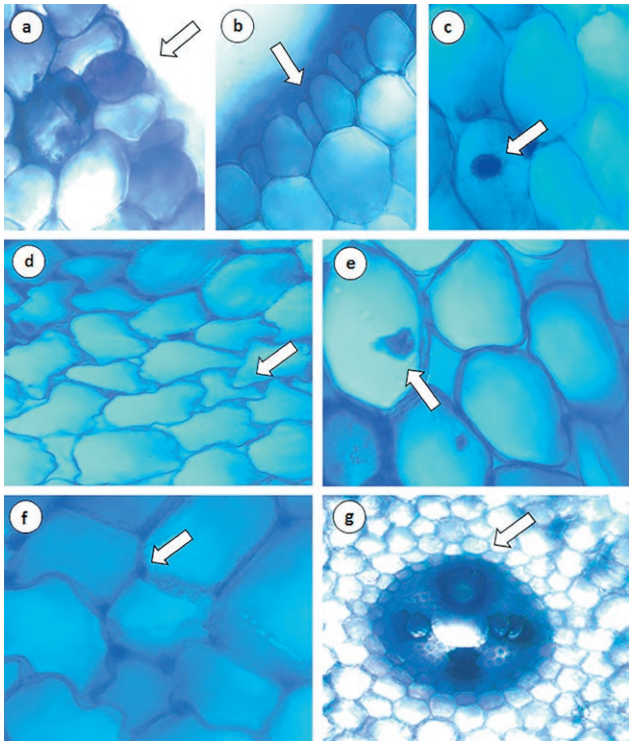

Figure 2. Anatomical changes induced by benzyl benzoate in root cell meristematic cells of *A.cepa* (a: necrosis, b: cell deformation, c: flattened cell nucleus, d: cortex cell deformation, e: accumulation of some substances in cortex cells, f: cortex cell wall thickening, g: unclear transmission tissue).

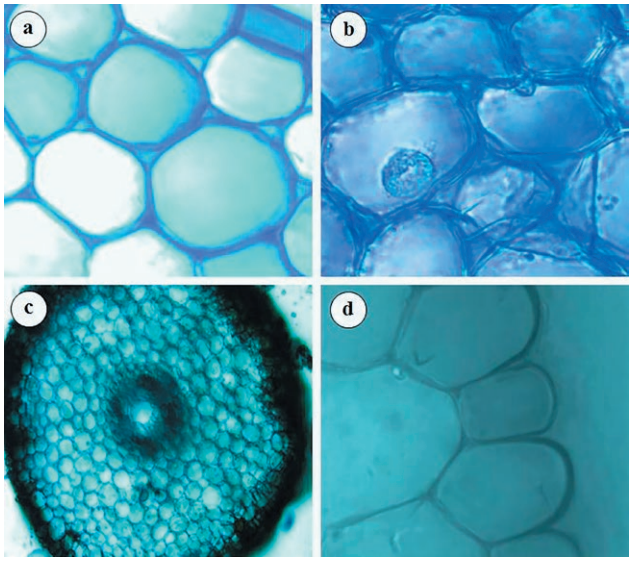

Figure 3. The appearance of the control group root tip meristematic cells (a: normal appearance of cortex cells, b: the usual shape of the cell nucleus, c: the usual appearance of the transmission tissue, d: normal appearance of epithelial cells).

tip cells and tissues of the control group (Figure 3), but an increase in anatomic damage rates was also observed in the application groups due to the increasing doses of benzyl benzoate. Since there is no comprehensive study on the anatomic damage caused by benzyl benzoate in plant root tip cells, the results are discussed with data from other chemical agents. Bıçakcı *et al.* (2017) reported that the application of diazinon cause anatomic damages in the root tip cells of *A. cepa* as unclear conduction tissue, flattened cell nucleus, cell deformation, thickening of cortex cell walls, necrosis and accumulation of some substances in the tissue of transmission, and the frequency of these damages increased due to application dose. Çavuşoğlu *et al.* (2011) reported that the application of glyphosate causes anatomic changes in the cell-root cell of *A. cepa*, unclear vascular tissue, cell deformation, unclear epidermis layer, binuclear cell and abnormal cell nucleus.

In our study, the effects of benzyl benzoate on lipid peroxidation in *A. cepa* root tip cells were also investigated. Lipid peroxidation is a metabolic process that causes oxidative degradation of reactive oxygen species (ROS) and lipids, especially polyunsaturated fatty acids and MDA production (Odjegba and Adeniran, 2015). MDA is an oxidative product of membrane lipids and a biological marker that indicates the level of oxidative stress (Janero, 1990). ROS damage the peroxidation of biological molecules including lipids, proteins, RNA and DNA (Shah *et al.*, 2001; Dinakar *et al.*, 2010). Free oxygen groups can act on DNA and cause mutations in nucleic acids and changes in chromosomes (Yarsan, 2014). The effects of benzyl benzoate application on root MDA levels are shown in Figure 4. When MDA levels were examined, the lowest MDA level was 10.00 µmol/g FW in the control group. SOD levels were found to be 1.45 times higher in Group II, 1.83 times higher in Group III and 2.21 times higher in Group IV compared to the control group. It was determined that the level of MDA increased with the increase in the benzyl benzoate doses. In addition, it was observed that the differences in the MDA level between the groups were statistically significant ($P < 0.05$). In the literature, because of the lack of a comprehensive study investigating the effect of benzyl benzoate on lipid peroxidation in plant test materials, our findings are discussed with similar studies examining the effects of other chemicals on lipid peroxidation in plants test materials. In the investigations chromium (IV) application of *A. cepa* root tip tissues (Patnaik *et al.* 2013), high-dose lead application on *Allium sativum* L. (Liu *et al.* 2009), bentazone herbicide application in rice (Wang *et al.* 2008) reported an increase in MDA levels in tissues according to applications.

Various defense mechanisms have been developed by aerobic organisms against free radicals. One of the so-called protective antioxidants CAT decomposes hydroperoxides or hydrogen peroxide and SOD reduces the formation of free radicals and active oxygen by quenching and modifying active oxygen (Comporti, 1993). The effects of benzyl benzoate application on root SOD and CAT activity are shown in Figure 5 and 6 respectively. The lowest SOD levels were measured as 75.00 U/mg FW in the control group. SOD levels in benzyl benzoate application groups were 94.00 U/mg in Group II, 157.00 U/mg in Group III and 180.70 U/mg in Group IV. It was determined that the level of SOD increased with the increase in benzyl benzoate dose. In addition, it was observed that the SOD level differences between the groups were statistically significant ($P < 0.05$). The level of SOD is thought to be increased as a result of ROS formation in the form of superoxide radi-

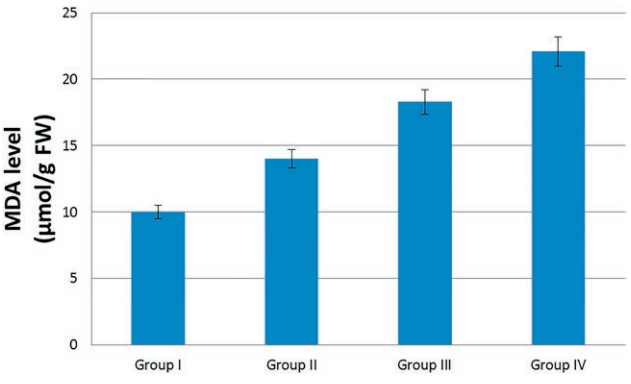

Figure 4. Effects of benzyl benzoate application on MDA levels. (Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate). Vertical bars denote Standard Error (SE).

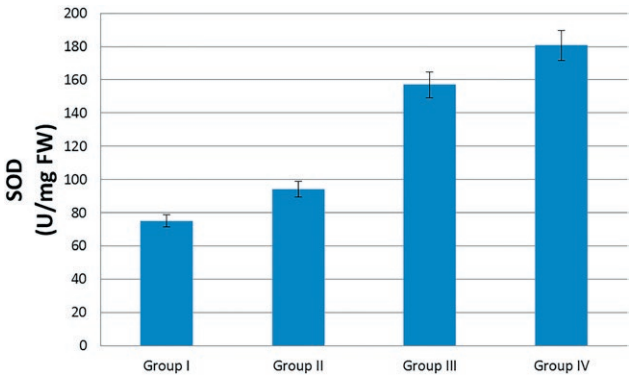

Figure 5. Effects of benzyl benzoate application on SOD activity (Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate). Vertical bars denote Standard Error (SE).

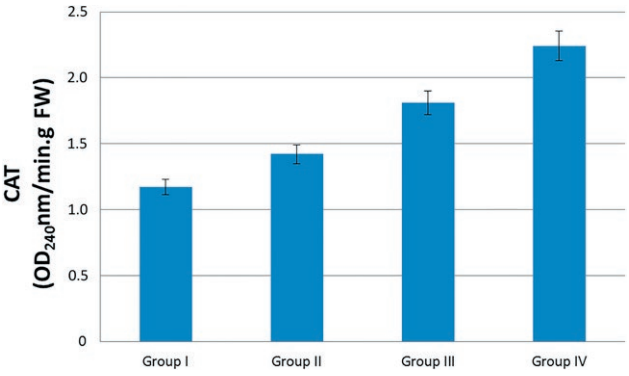

Figure 6. Effects of benzyl benzoate application on CAT activity (Group I: Control, Grup II: 10,000 mg/L benzyl benzoate, Grup III: 25,000 mg/L benzyl benzoate, Grup IV: 50,000 mg/L benzyl benzoate). Vertical bars denote Standard Error (SE).

cals by exposure to benzyl benzoate. Superoxide is a key component of signal transduction triggers genes responsible for antioxidant enzymes, including SOD (Alavarez and Lamb, 1997). The lowest CAT levels were measured as 1.17 OD_{240nm}/min.g FW in the control group. CAT levels in benzyl benzoate application groups were 1.42 OD_{240nm}/min.g FW in Group II, 1.81 OD_{240nm}/min.g FW in Group III and 2.24 OD_{240nm}/min.g FW in Group IV. Increased levels of SOD and CAT were determined by increasing benzyl benzoate dose. Similarly, in many studies, antioxidant enzyme activities have been reported to increase in *Allium* species due to stress-induced by other chemicals. Application of different doses of chlorpyrifos and mancozeb insecticides caused an increase in CAT and SOD levels in *A. cepa* leaves (Fatma *et al.* 2018), the increase in SOD and CAT levels due to the application doses and duration of cypermethrin insecticide on *A. cepa* root tips (Çavuşoğlu *et al.* 2012a), the application of cadmium inhibited SOD and CAT levels in *Allium sativum* L. leaves but when the application was continued the increase in SOD and CAT levels was reported (Zhang *et al.* 2005).

CONCLUSIONS

When all the data obtained in the study were examined; benzyl benzoate application showed inhibitory effects on physiological parameters investigated in *A. cepa* test material. This is thought to be due to the inhibitory effect of benzyl benzoate on the cell cycle. Inhibition in physiological parameters is considered as an indicator of benzoate toxicity. Benzyl benzoate increases the occurrence of chromosomal damage with the frequency of MN and decreases in the rate of MI suggests that it may be caused by the production of more ROS that can be detoxified by the cellular defense mechanisms and by causing the damage to DNA by being tolerated. Increases in MDA, SOD and CAT levels also support this situation. In addition, due to the application of benzyl benzoate, the anatomical changes occurring in the transmission tissue and root tip cells may be due to the defense mechanisms developed to inhibit the cellular uptake of the benzyl benzoate developed by the plant. Despite these mechanisms, high benzyl benzoate doses may be caused the penetration of the substance into the plant.

As a result; benzyl benzoate, which is used in many different fields, such as food, health, cosmetics and agricultural production, has been identified using *A. cepa* test material, which can show toxic effects if it reaches certain concentrations. For this reason, the use of benzyl benzoate exposure should be avoided considering the damages that can be caused to living things. In cases such as disease treatments where the use is essential, the appropriate dosage and duration range should be determined and the areas of use should be limited.

In this study conducted using *A. cepa* test material, it was found that physiological parameters such as germination percentage, root length and weight gain are important precursors in the rapid detection of toxicity. Cytogenetic parameters such as chromosomal abnormalities, MN formation and MI ratio are sensitive biomarkers in the biological monitoring of toxicity. In addition, it was determined that the determination of MDA, SOD and CAT levels contributed to explaining the causes and effect mechanisms of toxicity, the anatomical changes occurring in the root tip meristematic cells were found to contribute to the understanding of the cellular responses of the plant during the incorporation of the chemical agent into the plant.

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