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# Protective role of caffeic acid phenethyl ester against tetramethrine-induced toxicity in mice

**Gokhan Nur[1], H.Ahmet Deveci[1], Yusuf Ersan[2], Oguz Merhan[3], Mumtaz Nazli[4], Ozlem Nur[1]**

[1]*Gaziantep University, Vocational School of Higher Education in Islahiye, Department of Veterinary, Gaziantep, Turkey*
[2]*Kafkas University, Faculty of Science and Arts, Department of Biology, Kars, Turkey*
[3]*Kafkas University, Faculty of Veterinary Medicine, Department of Biochemistry, Kars, Turkey*
[4]*Muğla University, Faculty of Medicine, Department of Histology-Embryology, Muğla, Turkey*

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**Abstract**
The purpose of this study was to determine the biochemical, histopathological and genotoxic effects of tetramethrine, which is widely used in domestic and agricultural activities as well as identify the protective effect of caffeic acid phenethyl ester (CAPE). 30 Swiss albino laboratory mice (*Mus musculus*) were used in the study. 10 μM/kg tetramethrine, dissolving in 10 mM DMSO (Dimethyl sulfoxide), was intraperitoneally injected to the Tetramethrine group (n = 10). To the CAPE-Tetramethrine group, 10 μM / $kg^{-1}$ CAPE, dissolving in 1% ethanol, was applied in three days before the experiment and then, CAPE and 10 μM / kg tetramethrine, dissolving in 10 mM DMSO, was intraperitoneally injected. No injection was made to the control group. At the end of the experiment, the rats anaesthetized with diethyl ether were killed by cervical dislocation and their livers and femur were removed for analysis. There was no statistical difference between three groups in terms of mitotic index (MI) and micronucleus frequency ($P > 0.05$). Chromosomal aberration frequency showed an increase in the other two groups compared to the control group ($P < 0.05$). Although severe degeneration and necrosis areas were identified in the liver, CAPE decreased the severity of degeneration in the Tetramethrine group. While Tetramethrine increased the malondialdehyde (MDA) level in the liver, CAPE decreased MDA and increased the GSH level. We think that CAPE may be used for therapeutic purposes in order to provide a limited protection against the tetramethrine-related detrimental effects in humans and other living organisms.

**Keywords:** Tetramethrine, CAPE, chromosomal aberration, micronucleus, *Mus musculus*

## Introduction

Chemically, pyrethroides (synthetic pyrethroides) are insecticides similar to the pyrethrins that are present in the natural pyrethrum obtained from the flowers of *Chrysanthemum*, insecticidal activity of which has been known for centuries [1]. Pyrethroides mainly carry cyclopropanecarboxylate or fenvalerate groups in their structures. Moreover, pyrethroides are chemically divided into two groups as those carrying alpha-cyano group (alphacypermethrin, cypermethrin, deltamethrin and fenvalerat etc.) and those not carrying alpha-cyano group (permethrine, allethrine, tetramethrine etc.) [1]. Tetramethrine is a synthetic pyrethroide and it is included in type-I pretroids class since it does not carry α-cyano group chemically in its structure. When compared to the mammals, its effect on insects is 5 times more. The effect of tetramethrine may cause ataxia, tremor, irritability, convulsion and ultimately stroke [2,3].

Caffeic acid phenethyl ester (CAPE) is a component of the propolis and has wide spectrum effects isolated. CAPE, a phenolic compound, is one of the active components of propolis, and a compound, whose protective effects in various systems were pointed out by several studies. CAPE has two annular structures. One of these annular structures has two $OH^-$ groups which are functional and display almost all the chemical properties of CAPE molecule. These hydroxyl groups actively accept and transmit the electrons, and thus they show an oxidizing and reductive feature [4,5]. Because CAPE has a lipophilic structure, it easily passes through the cell membrane and reaches the area where it displays effect [6]. It inhibits some enzymes (ornitine carboxylase, protease, 5-α reductase, cyclooxygenase, lipooxygenase, HIV-1 integrase) due to this property [7,8]. It has been used for alternative medicine in Middle Eastern countries for long years [9]. It has been shown in many studies that CAPE has antioxidant [10], antiinflammatory [11], anti carcinogenic [12], antiviral [13], immunomodulator [14] neuroprotective [15] and antiatherosclerotic [16] effects. No hazardous effect of CAPE on normal cells has been reported so far [15].

This study determined the biochemical, histopathological and genotoxic effects of tetramethrine commonly used in domestic and agricultural activities and identified protective effect of CAPE against tetramethrine.

## Materials And Methods

**Experimental groups:** Kafkas University Animal Experiments Ethics Board (Decision No: 12.02.2010/05 number 14) approved by this study, used in any study 30 mice (male) were used in each group including 10. The Swiss albino type laboratory mice (*Mus musculus)* to be

***Coresponding Author:** Gokhan Nur, Gaziantep University, Vocational School of Higher Education in Islahiye, Department of Veterinary, Gaziantep, Türkiye
**E-mail:** gokhannur@gantep.edu.tr

used in the study were 10-12 week-old and 30-35 grams in weight and they were kept in standard cages in 22 ± 2°C ambient temperature, in light of 12 hours and in darkness of 12 hours and were ad libitium. The studies conducted by Natarajan and Motomuro were taken into consideration to determine dosage of the tetramethrine [17,18]. In this study totally 30 mice were used in 3 groups, each of which included 10 mice (Swiss albino). No injection was made mice in the control group (n = 10). 10 μM/kg tetramethrine (Riedel) dissolving in 10 mM DMSO (Dimethyl sulfoxide) was injected intraperitoneally to the mice in the tetramethrine group (n = 10) on daily basis for 15 days. 10 μM / $kg^{-1}$ CAPE (Sigma) dissolving in 1% ethanol was applied to the mice in the CAPE and tetramethrine group (n = 10) in 3 days before the experiment, and then 10 μM / kg tetramethrine dissolving in 10 mM DMSO (Merck) together with CAPE was intraperitoneally injected to those mice for 12 days. 2 mg/kg colchicine dissolved in distilled water was given intraperitonally two hours before the mice were sacrified.

**Histological analysis:** At the end of the experiment period, the liver tissue of the mice killed by cervical dislocation was determined in bouin and 10% formol solutions. After determination, the tissues taken following the routine tissue follow up (graduated alcohols, methyl benzoate and benzol follow-up) were immersed in paraffin and 5 μ sections were obtained using a microtome. Histological staining by means of hematoxylen-eosinstaining method, and finally histopathological changes were monitored under the light microscope [19].

**Genotoxic analysis:** The mouse was killed by cervical dislocation (under anesthesia). Then, both femur were disjointed as a whole. The muscle fibers around the bones were cleaned by using fingers and gauze bandage. The proximal end of the femur was shortened using a scissor carefully until the medullary canal became visible. The centrifuge tube was filled with fetal calf serum (Sigma) up to the neck. 0.2 ml of the serum in this centrifuge tube was taken into the injector. The injector was immersed a few mm into the medullary canal through the proximal part of the femur and the inside of the bone marrow was emptied. This procedure was carried out 3-4 times. The suspension obtained was centrifuged at 1000 rpm for 10 minutes. After the centrifugation, the supernatant in the upper part was removed and 0.075 M. of hypotonic solution was added. It was kept for 30 minutes in order for the hypotonic to display effect and then it was centrifuged at 1000 rpm for 10 minutes and the hypotonic was separated from the cells. After the centrifuge, the supernatant in the upper part was removed and the Carnoy fixative prepared from 3-part methanol and 1-part glacial acetic acid was added and it was mixed to make the fixed. This solution was centrifuged again at 1000 rpm for 10 minutes and then the supernatant in the upper part was removed. This procedure was repeated twice and the residue of last fixative was eliminated and the 2 ml part of it in the test tube was mixed and laid on the slide. After the lamina dried, the preparates were kept in giemsa's stain for 15-20 minutes and they were stained. Then the stained preparates were examined using the light microscope. The micronucleus test was performed according to method of Schmid and Matter [20,21]. At the studies carried out with low dose, the peripheral blood displayed less variation, because MNPCEs of the spleen in the mice were removed selectively, bone marrow was preferred instead of circulating blood when mice were used at the micronucleus studies. The preparates obtained as a result of the procedures were examined at 1000 magnification under the light microscope of brand ‘‘Leica DM500’’, on each preparate 200 polychromatic erythrocyte (PCE) were randomly counted and the number of the micronucleated polychromatic erythrocyte (MNPCE) among these were determined. The ratio between PCE and NCE counted in a total of 1000 erythrocyte is an important index in terms of displaying the toxicity of the chemical substance affecting the bone marrow cells [22]. The fact that PCE/NCE ratio at the experimental group decreased compared to the control group showed that the chemical substance affected the bone marrow and decreased the erythrocyte formation by preventing the reproduction and maturation of the nucleated erythrocyte precursor cells [23]. Therefore, 1000 erythrocyte (PCE and NCE) were randomly counted from each animal and the PCE/NCE ratio was determined.

**Glutathione (GSH), malondialdehyde (MDA) and sialic acid analysis (TSA):** After the liver tissue samples were washed with normal saline, they were homogenized under iced water at phosphate buffer (A :50 mM, $KH_2PO_4$ and B : 50 mM $Na_2HPO_4.2H_2O$ A:B (v/v) = 1 :1.5). After the homogenates were centrifuged for 15 minutes at 4 °C and 2400 g , the supernatants obtained were kept at -25 °C until the analysis was made. The reduced GSH, was measured spectrophotometrically according to the method stated by Beutler et al [24], and on the other hand MDA concentrations were measured spectrophotometrically according to the method stated by Yoshoiko et al. [25] (UV-1201, Shimadzu, Japan). Plasma TSA analysis was performed according to the method Sydow [26].

**Statistical analysis:** SPSS software program [27] was used to conduct statistical analysis of the data obtained from the study. The one way analysis of variance (ANOVA) was performed to determine differences between means of the experimental groups and if there was a difference between means of the experimental groups, the “Anova-Duncan” test was applied to means of the groups to determine which group or groups cause this difference observed, and $p< 0.05$ value was statistically accepted as significant.

## Results

**Mitotic Activity:** In the bone marrow preparates 500 cells were randomly counted from each animal and the cells in the metaphase were counted and the mitotic activity was determined. In order to investigate the effect

of tetramethrine on the mitotic activities of the cells in the bone marrow *of Mus musculus albino*, the cell counts of the preparates obtained from control, tetramethrine and tetramethrine+CAPE groups and the means in percentage of the cells in which mitosis was observed were determined (Table 1).

**Table 1.** Mitotic index ratios of the groups.

| Groups | Total number of cells | Number of cells in the interphase | Number of cells in the metaphase | MI±SD |
|---|---|---|---|---|
| Control | 5000 | 4860 | 140 | 2.8±0,9189$^{ns}$ |
| Tetramethrine | 5000 | 4895 | 105 | 2.1±0,8756$^{ns}$ |
| Tetramethrine+CAPE | 5000 | 4885 | 115 | 2.3±0,9487$^{ns}$ |

*p<0,05 (SD: Standard deviation), ns: non significant (p > 0,05)

The tetramethrine, decreased the mitotic index with respect to the control group. As a result of comparing the groups statistically, there was no significant difference between the control group, the tetramethrine and tetramethrine+CAPE groups on the basis of p<0.05. Thus, although tetramethrine decreased the number of cells under mitosis, this was not found to be statistically significant in comparison with the other groups.

**Micronucleus Test:** In comparison of the groups in terms of the micronucleus test, a total of 2000 polychromatic erythrocytes were counted from the preparations obtained from each group and the number of micronucleated polychromatic erythrocyte was identified. Again on the preparations obtained from each group, a total of 1000 polychromatic and normochromatic erythrocyte (NCE) were counted and it was determined whether or not the chemical used affected the bone marrow cells (Table 2).

**Table 2.** Polychromatic and normochromatic erythrocyte ratios of the groups and the micronucleus frequency.

| Groups | Total PCE | MNPCE±SD | PCE+NCE | Number of PCE | Number of NCE | PCE/NCE |
|---|---|---|---|---|---|---|
| Control | 2000 | 5.8± 1,033$^{ns}$ | 1000 | 625 | 375 | 1.66 |
| Tetramethrine | 2000 | 6.7± 1,174$^{ns}$ | 1000 | 598 | 402 | 1.48 |
| Tetramethrine+CAPE | 2000 | 6.4± 1,252$^{ns}$ | 1000 | 629 | 371 | 1.69 |

*p<0,05 (SD: Standard deviation), ns (non significant) (p>0,05), PCE: Polychromatic Erythrocyte, MNPCE: micronucleated polychromatic erythrocyte, NCE: Normochromatic Erythrocyte.

When the groups were compared in terms of the micronucleus formation frequency, there was no statistical difference among the three groups (p>0,05). Tetramethrine increased slightly the micronucleus frequency compared to the control group and it is not statistically significant difference. The decrease of PCE/NCE ratio in the tetramethrine group proved that the chemical affected the bone marrow cells. In the control and tetramethrine groups, normal PCE and NCE and MNPCEs were observed (Figure 1A, B, C). Although tetramethrine increased the amount of chromosomal aberration, such an increase was not observed in the micronucleus frequency. The most important factor behind this was considered that the micronucleus formation in the cell took a longer period compared to formation of the chromosomal aberration and that it occurred at higher administration dosages of the substance.

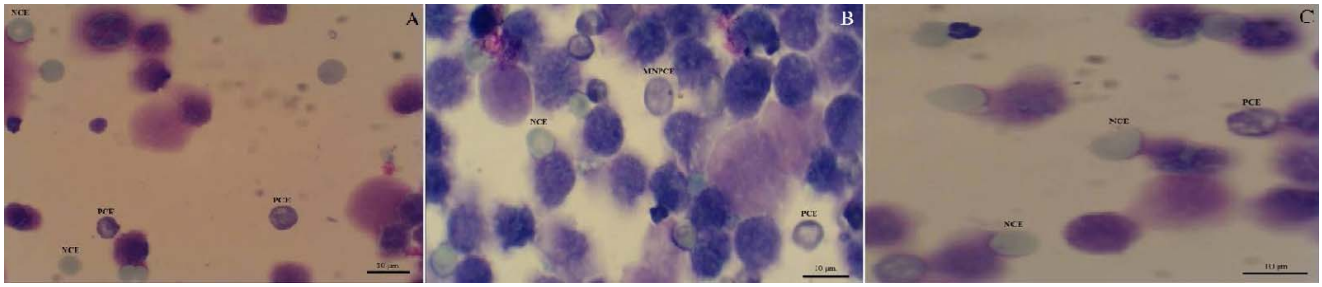


**Figure 1. A.** The PCE and NCE without any micronucleus in the bone marrow of mice in the control group **B.** The MNPCE and the normal PCE and NCE in the bone marrow of the mice in the tetramethrine group **C.** The PCE and NCE without any micronucleus in the bone marrow of the mice in the tetramethrine+CAPE group.

**Chromosomal Aberrations:** 10 µM/kg tetramethrine injection was intraperitoneally (i.p) administered to the experimental group and 10 µM / $kg^{-1}$ CAPE and 10 µM / kg tetramethrine were intraperitoneally (i.p) administered to the tetramethrine+CAPE group. When the numbers of chromosomal aberration, mitotic index and the micronucleated polychromatic erythrocyte of the groups were taken into consideration, it was determined that in the mitotic index and the micronucleus frequency there was statistically no significant difference (p>0.05), but when it was considered in terms of the chromosomal aberration, there was a statistical difference. Comparing the groups in terms of abnormal cell percentage, a statistically significant difference (p<0.05) was observed between the control group and the other two groups (tetramethrine and tetramethrine+CAPE groups) (Table 3, Figure 2). Considering the abnormal cell percentage and the chromosomal aberration, as a result of

determining a significant difference between the control group and the other two groups, it was observed that tetramethrine increased the chromosomal aberration frequency in the cell and the protective effect of CAPE against tetramethrine remained restricted.

**Table 3.** Comparison of the groups in terms of chromosomal aberrations.

| Groups | Chromosomal aberrations | | | | Abnormal Cell±SD (%) | KA/Cell±SD |
|---|---|---|---|---|---|---|
| | SSU | B' | SU | F | | |
| Control | - | 1,8±0,83 | 0,6±0,548 | - | 0,528±0,131 $^{a*b*}$ | 0,00624±0,0008$^{a*b*}$ |
| Tetramethrine | 1,80±1,483 | 6,0±1,0 | 2,6±1,140 | 1,8±0,83 | 3,6±1,140 $^{a*}$ | 0,0324±0,0124$^{a*}$ |
| Tetramethrine+CAPE | 1,2±0,8367 | 4,8±0,83 | 2,6±1,342 | - | 2,6±0,894 $^{b*}$ | 0,0288±0,0084$^{b*}$ |

*p<0,05 (SD: Standard deviation), ns (non significant) (p>0,05), SSU: Sister strand union, B': Chromatid fracture, SU: Sister chromatid union, F: Fragment, a*: Significant difference between the control group and tetramethrine group, b*: Significant difference between the control group and tetramethrine+CAPE group.

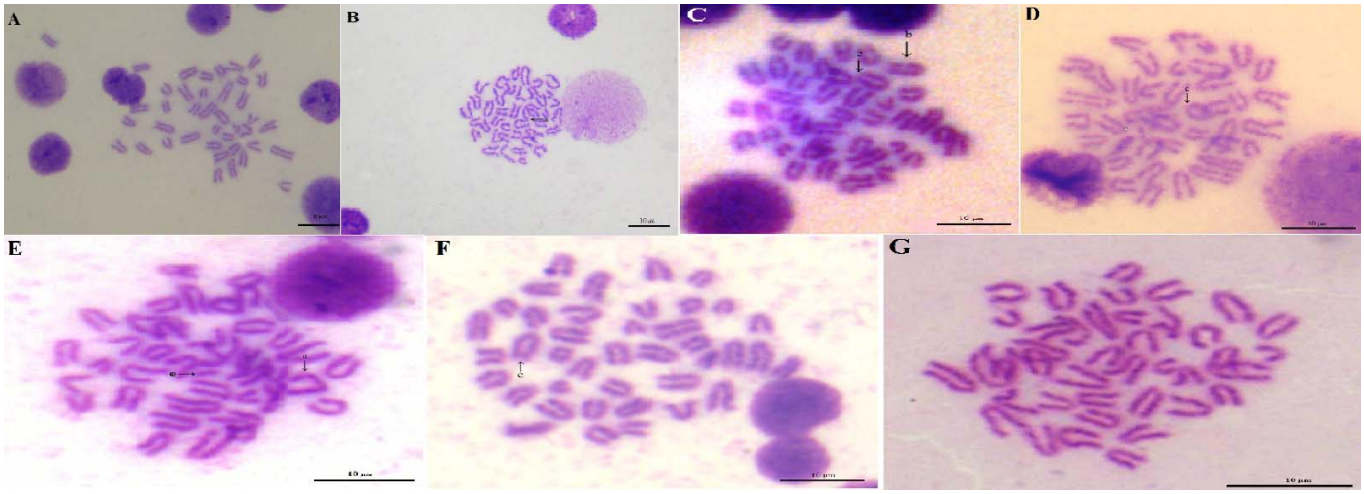


**Figure 2. A.** Metaphase sample of the control group. **B, C, D, E.** Metaphase samples of tetramethrine group **a.** Sister strand union (SSU), **b**. Chromatid fracture (B'), **c.** Sister chromatid union (SU), **d.** Gap (G), **e.** Fragment (F). **F, G.** Metaphase samples of Cape+Tetramethrine group. **c.** Sister chromatid union (SU)

**Biochemical Findings:** In the biochemical analysis of the three groups. a significant difference was found between the control, tetramethrine, and tetramethrine+CAPE groups in the comparison performed in terms of the MDA values. It was determined that the chemical substance significantly increased the MDA level in the liver compared to the control group. CAPE, an antioxidant matter, limited this increase and caused a decrease in the MDA level. In terms of the GSH levels, tetramethrine significantly decreased the GSH level compared to the control group and CAPE had a therapeutical effect against tetramethrine by increasing the GSH level. Although CAPE increased the GSH level in comparison with the tetramethrine group, no statistically significant difference was observed between the CAPE and the tetramethrine group. Sialic acid levels were significantly increased (p<0,05) (Table 4).

**Table 4.** MDA (nmol/g Protein) level and reduced GSH (µg/g Protein) level in liver tissue and plasma TSA (mg/dl) in the Control, Tetramethrine and Tetramethrine+CAPE groups

| Groups | Sample quantity | MDA±SD (nmol/g Protein) | GSH±SD (µg/g Protein) | TSA±SD (mg/dl) |
|---|---|---|---|---|
| Control | 10 | 19,428± 3,246$^{a*}$ | 17,940±4,185$^{a*}$ | 56,51±4,27 $^{a*c*}$ |
| Tetramethrine | 10 | 27,975± 4,903$^{a*b*}$ | 13,786±2,573$^{a*}$ | 68,71±7,12 $^{a*b*}$ |
| Tetramethrine+CAPE | 10 | 21,698±3,804$^{b*}$ | 14,984±2,566 | 61,15±5,25 $^{b*c*}$ |

*p<0,05 (SD: Standard deviation), a*: Significant difference between the control group and tetramethrine group, b*: Significant difference between the Tetramethrine group and Tetramethrine+CAPE group, c*: Significant difference between the control group and Tetramethrine+CAPE group.

**Histological Findings:** In the light microscope examinations of the slices obtained, the hepatocytes and sinusoidal structure in the preparates obtained from the control group were in normal view (Fig. 3A). In the animals of the group to which only tetramethrine was applied, severe degeneration, widespread necrosis areas along with hydropic and vacuolar degeneration was observed (Figure 3B). In the group to which CAPE was applied together with tetramethrine, the severity of these degenerations decreased (Figure 3C).

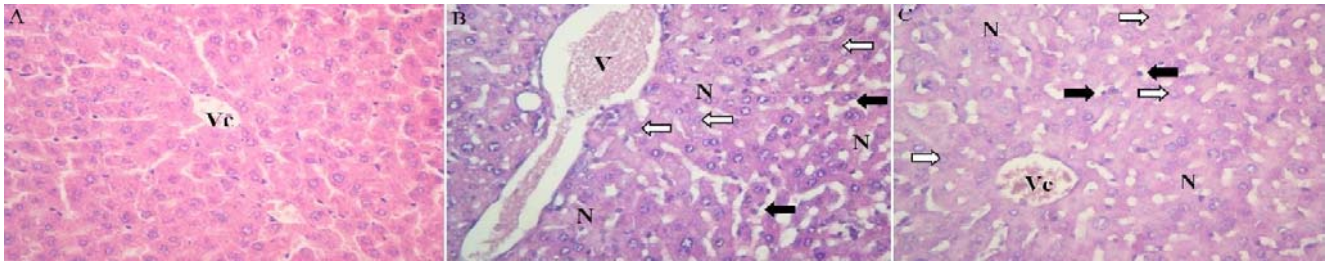


**Figure 3. A.** The liver tissue obtained from the animals in the control group. Hepatocytes and sinusoidal structure are in normal view. (Vc: Vena centralis). **B.** The liver tissue obtained from the animals in the group to which 10 μM / kg tetramethrine was applied. Severe degeneration, widespread necrosis areas (N) and hydropic (white arrows), and vacuolar degeneration (black arrows) areas. (V: Interlobular vein). **C.** The liver tissue obtained from the animals in the group to which 10 μM / $kg^{-1}$ CAPE was applied together with 10 μM / kg tetramethrine. It is observed that although the severity of degeneration caused by tetramethrine decreased, there were still focal necrosis areas (N) as well as hydropic (white arrows) and vacuolar degeneration (black arrows) areas in some regions. (Vc: Vena centralis). H-E x40

## Discussion

In the studies conducted on the genotoxicity of pesticides, Kocaman observed that acetamiprid (Acm) and Alpha-cypermethrin (A-cyp) statistically increased significantly the formation of Sister Chromatid Exchange (SCE), and chromosome abnormalities (CA) in all periods and doses, and formation of micronucleus (MN) in higher three doses; decreased significantly mitotic index (MI), nuclear division index (NDI) in both periods and all doses; and decreased significantly proliferation index (PI) in 35-40 μg/ml for 24-hour treatment, and in 40 μg/ml for 48-hour treatment. Acm and A-cyp had a genotoxic potential [28].

In a study in which the mutagenic effect of tetramethrine was examined by the Salmonella / microsome test system, tetramethrine (10-1000 μg / plaque) was not found to be mutagenic in *Salmonella typhimurium* TA 98 and TA 100 strains [29]. In the study conducted by Tisch et al., to investigate the effect of transfluthrin in the mucosa cells of human nasal cavity, they determined that transfluthrin had a genotoxic effect [30]. Institoris et al., examined the genotoxic effects of permethrin on the bone marrow cells of male winstar rats and identified that numerical chromosome abnormalities increased in all the dosages used [31]. In our study, although tetramethrine decreased MI, this had no statistical importance. It was observed that chromosome aberration ratio increased, which created a statistical difference in the other two groups compared to the control group. Tetramethrine increased chromosomal aberration frequency; on the other hand, the protective effect of CAPE against tetramethrine was limited. In terms of formation frequency of MN; no statistical difference was observed between all the three groups. Although the chromosome aberration frequency increased, the reason behind why there was no significant increase in the MN frequency may be that the micronucleus formation within the cell takes a longer period of time compared to the formation of chromosome aberration and forms in higher administration dosages of the substance. The fact that PCE/NCE ratio decreased in tetramethrine group showed that the chemical affected the bone marrow cells. In parallel to results of the study conducted on the genotoxicity of pesticides in pyrethroide group [28,30-32]. This study concluded that tetramethrine had a genotoxic effect; however, it had a limited effect depending on dosage.

The protective role of CAPE on the antioxidant system and different tissues was revealed by various studies. In another study conducted with malathion application, it was determined that MDA levels increased, which caused oxidative stress and in return, CAPE decreased the toxic effect of malathion and significantly decreased the MDA levels [33]. A study using clorprifos indicated that, MDA levels increased but there was therapeutic effect of antioxidant enzymes by decreasing the MDA ratio [34]. In another study conducted by researchers with cisplatin, they reported that no change occurred in the MDA levels and the reason for this was that MDA secreted in the liver tissue was rapidly metabolized [35]. A study investigated the protective role of CAPE against the oxidative stress forming in rats as a result of over iron loading, and indicated that the increase in MDA level due to the iron application decreased depending on the CAPE application [36]. In an application investigating the protective effect of CAPE against the oxidative damage formed by carbon tetrachloride, it was determined that MDA level increased, GSH level decreased in the carbon tetrachloride group, the effect of this oxidative damage together with the CAPE application decreased and in the histopathological examinations, focal necrosis areas developed with degenerations in the hepatocytes and hepatic cords with carbon tetrachloride in liver tissues and the CAPE application provided to decrease the strength of these effects again [37]. In another study, it was determined vacuolization, neuroglial cell infiltration and pyknotic cells in the molecular layer in the prefrontal cortex samples of the rats exposed to cigarette smoke; on the other hand, the structural changes caused by the cigarette smoke exposure decreased to a large extent in the rats which CAPE was injected together with cigarette smoke exposure [38]. It was indicated that the cadmium application increased the MDA level and decreased the nitrite oxide (NO) level; however, when CAPE was applied together with cadmium, NO level started to increase and MDA level started to decrease [39]. A similar study determined that gentamicin (GEN) application caused an increase in MDA and NO levels and a decrease in SOD, CAT and GSH levels; when CAPE was applied together with GEN, MDA and NO

levels decreased and the SOD, CAT and GSH levels increased. In the same study, upon histopathological examination of kidneys, it was specified that tubular necrosis formed prominently and when CAPE was applied together with GEN, the severity of the necrosis were reduced to a less extent [40]. In this study, it was observed that the tetramethrine application caused an increase to create a significant difference in the MDA levels in parallel to the other studies [33,34,36,37]; on the other hand, CAPE limited this increase, increased the GSH level and caused a decrease in the MDA levels. In terms of the histological appearance, it was determined that the severe degeneration and necrosis areas formed by tetramethrine decreased in the group to which CAPE was applied together with tetramethrine in parallel to results of the study using the other antioxidants [37,40]. It is thought that this protective effect was performed as a result of the fact that CAPE decreased the oxidative damage by inducing the antioxidant system.

**Conclusion:** It is understood that tetramethrine, found widely in the environment, has a limited genotoxicity at 10 μM / kg dosage, CAPE has a limited protective effect against tetramethrine, tetramethrine is toxic for the liver and CAPE shows the protective effect against this toxication. In line with these data, we think that CAPE may be used for therapeutic purposes in order to provide a limited protection against the tetramethrine-related detrimental effects in humans and other living organisms.

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