

ORIGINAL RESEARCH

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The effect of caffeic acid phenethyl ester (CAPE) on hepatic histopathology and oxidative stress in rats treated with malathion**Gokhan Nur¹, Mehmet Tahir Husunet², Izzettin Guler¹, Ayla Devenci¹, Evren Koc³, Ozlem Nur¹, Pinar Aksu Kilic⁴**¹Gaziantep University, Islahiye Vocational School, Department of Veterinary, Gaziantep, Turkey²Çukurova University, Faculty of Science and Literature, Department of Biology, Adana, Turkey³Kafkas University, Faculty of Architecture and Engineering, Department of Bioengineering, Kars, Turkey⁴Kafkas University, Faculty of Science and Literature, Department of Biology, Kars, Turkey

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Abstract

Malathion is a broad-spectrum organophosphate insecticide used in the agricultural industry. Our study aimed to investigate the protective effects of caffeic acid phenethyl ester (CAPE) against malathion by examining histopathological and biochemical parameters. Forty Sprague-Dawley male rats were used in this study. Groups formed; group I (control), only 5 ml kg⁻¹ oral corn oil; group II (malathion), 40 mg kg⁻¹ malathion by gavage; group III (malathion+CAPE), intraperitoneal (i.p.) CAPE (10 µmol kg⁻¹) followed by intragastric malathion (40 mg kg⁻¹) after 1 hour; group IV (CAPE), intraperitoneal CAPE (10 µmol kg⁻¹). At the end of the administrations, rats were anesthetised with ketamine/xylazine intraperitoneally and euthanasia was performed with cervical dislocation. Their intracardiac blood was drawn under anaesthesia, and after the euthanasia, their liver tissues were removed for histopathological analysis. In group II that was treated with malathion, cell infiltrations around the central and portal vein, degeneration and focal necrosis areas in the liver were detected. Although the histopathological findings observed in the malathion group were also encountered in group III (malathion+CAPE), lesions were less severe. In the malathion group II, total oxidant status (TOS) and total sialic acid (TSA) levels in plasma increased compared with the control group, while total antioxidant status (TAS) decreased compared with the control group. In the Malathion+CAPE group III, it was observed that biochemical parameters were closer to the control group. In malathion-treated rats, it was determined that CAPE reduced the degeneration in the liver and it could have a protective effect by partially correcting oxidative stress parameters. It was concluded based on our findings that malathion, which is an organophosphate compound, makes toxic effects in the liver, but that CAPE has a protective potential because it partially reduces the severity and frequency of histopathological lesions and it brings biochemical values closer to normal.

Keywords: CAPE, malathion, histopathology, total sialic acid, total oxidant/total antioxidant level**Introduction**

The rapid increase of the world population and the fact that agricultural production fails to reach the sufficient production capacity have significantly increased the use of biological and chemical substances against the pests which cause a loss of production in this industry [1]. Those most commonly used pesticides include organophosphates, carbamates and chlorinated hydrocarbons. Organochlorines were used around the world very extensively. However, they have been prohibited because their biodegradation time in nature is longer than that of organophosphates [2]. The easy accessibility of pesticides and their low controllability in terms of use is indicated as the primary cause of poisonings. Chronic toxicity is caused by the effect of pesticide residues that lead to environmental pollution [3].

Malathion was first commercialised in the 1950s, and it has been among the world's best-selling broad spectrum organophosphate (OP) pesticides since the 1980s. Like other OPs, the main mode of actions of this insecticide is the inhibition of the acetylcholinesterase (AChE) activity in target tissues [4-7]. Misuse of organophosphate pesticides that affect the nervous system causes hundreds of thousands of poisonings and even deaths every year. The main treatments for such poisonings are based on immediate administration of atropine and acetylcholinesterase activators that are currently represented by mono- or bis-pyridinium aldoximes [8].

In a study with rats treated with acute malathion, this substance was found to have adverse effects on the biochemical parameters in the liver and other tissues of rats and cause oxidative stress, whereas CAPE was found to significantly reduce the harmful effects of malathion [9]. Measurement of the substances in the urine and amniotic fluids of Wistar rats orally treated with binary mixtures of 5 different insecticides including malathion showed a significant exposure of foetus not only to the insecticides but also to their

metabolites [10]. In a study investigating the effects of malathion and green tea on liver tissue and biochemical parameters, it was observed that the antioxidant content of the green tea reduced the malathion's damage to liver tissue. It was also found that green tea significantly lower aspartate transaminase, alanine transaminase, alkaline phosphatase, total oxidant capacity and malondialdehyde concentrations and significantly increase the total antioxidant capacity [11].

In mice treated with subchronic malathion, acetylcholinesterase activity and testosterone levels decreased, apoptosis and necrosis were induced in spermatozoa, the reproductive performance of male mice diminished and their semen parameters changed [12]. An *in vivo* study investigating the effects of malathion on glucose metabolism regulation showed that malathion increased the biomarkers of insulin resistance and reduced insulin sensitivity indices [13]. In a study investigating the protective effect of N-acetyl-L-cysteine (NAC) against the toxic effects of malathion exposure in Wistar rats, leucocytosis and reduced hemoglobin (Hgb) content were determined in malathion treated rats when compared with the control group. In addition, with malathion administration, there was a significant increase in liver enzymes such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase and creatinine kinase. A decrease in acid phosphatase activity, protein and globulin levels was also observed. In the same study, it was reported that there was a defect in calcium, magnesium, phosphorus and iron contents due to malathion administration. NAC showed therapeutic effects against malathion toxicity, allowing HGB content and all liver enzymes to return to normal values [14].

In this study, CAPE was used as a protective agent against malathion. In studies conducted, CAPE was shown to exhibit antioxidant, antimicrobial, antiinflammatory, antiproliferative, cytostatic, antiviral, antibacterial, antifungal, antineoplastic, anticancerogenic, apoptosis inducing and immunomodulatory properties [7,15-24]. It was reported that CAPE is an active polyphenol in versatile therapeutic terms and an effective tumour suppressor agent in chemotherapy to reduce toxicities caused by chemotherapy [18,19]. In another study with rats, CAPE was shown to improve memory by influencing cholinergic signals. CAPE was therefore recommended as a potential therapeutic agent in disorders involving the cholinergic system [25].

The aim of this study is to investigate with histopathological and biochemical methods the protective effects of CAPE in rats against the harmful effects of malathion, which is widely used around the world and cause high toxicity in living organisms.

Material and Methods

Chemicals

All chemical substances obtained were trademarked. Malathion was purchased from Sigma-Aldrich (CAS No. 121-75-5). CAPE with 97% purity was purchased from Sigma-Aldrich (CAS No: 104594-70-9). Ketamine 10% (Inj. Deutch Farm Int.) and Xylazina 10% (Pro-Ser S.A.) were purchased commercially. Ethanol (CAS No. 64-17-5) used as solvent for CAPE was purchased from Merck.

Animals

In the study, 40 adult male Sprague Dawley rats weighing 200-

240 gr on average obtained from the Experimental Animals Implementation and Research Centre, Kafkas University, were used. Rats were selected at random and 4 groups were formed with 10 rats in each group (n=10). Throughout the experiment, the animals were kept at 25±2°C, a relative humidity of 55±10% and a 12-hour dark cycle. They were given food and drinking water *ad libitum*.

Experimental design

This study was carried out using laboratory facilities after obtaining ethics committee certificate numbered KAÜ-HADYEK/2017-093. For the groups formed with rats, administration of substances was carried out for 15 days according to the protocols. At the end of the study, rats were subjected to general anaesthesia by giving intraperitoneal ketamine 90 mg kg⁻¹ i.p. xylazine 10 mg kg⁻¹ and euthanasia was performed with cervical dislocation.

Group I (Control): Only 5 ml kg⁻¹ of oral corn oil (malathion's solvent) was administered.

Group II (Malathion): 40 mg kg⁻¹ malathion was administered via gavage.

Group III (Malathion+CAPE): Intraperitoneal administration of CAPE (10 µmol kg⁻¹), followed by intragastric malathion (40 mg kg⁻¹) 1 hour later.

Group IV (CAPE): Intraperitoneal administration of CAPE (10 µmol kg⁻¹).

Intracardiac blood of the rats, which were sacrificed by euthanasia at the end of the treatment, was drawn. Liver tissues were taken in fixation solution for histological analysis.

Histological Analysis

At the end of the experiment period, liver tissues of rats sacrificed by cervical dislocation under general anaesthesia were fixed in 10% buffered formalin solution. After the fixation and routine tissue processing (graded alcohols, methyl benzoate and benzol processing), the tissues taken were embedded in paraffin and 5 µm serial sections were taken with microtome from the blocks to slides pre-coated with chrome alum gelatine (CAG). Histopathological changes were examined at light microscopic level by treating the sections with haematoxylin-eosin as a histological staining method [26].

Biochemical analysis

The total antioxidant level (TAS) measurement was determined by an automated measurement method based on the decolourization of the characteristic colour formed by the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical with antioxidants in the sample added to the medium [27]. The results were calculated as mmol Trolox equivalent/L. The total oxidant status (TOS) measurement was made by the automated measurement method [28]. The oxidants in the sample have the task of converting the ferrous ion complex to ferric ion. Ferric ion (Fe³⁺) formed by oxidation of iron (Fe²⁺) to more stabilised form (Fe₂O₃) forms colour with xylenol orange in acidic environment. The intensity of the spectrophotometrically measured colour is related to the total amount of oxidant molecules present in the sample. The measurement was calibrated with hydrogen peroxide (H₂O₂) and the results were calculated as micromolar H₂O₂ equivalent (µmol H₂O₂ equiv./L) per litre. Total sialic acid (TSA) analysis was carried out colorimetrically using a spectrophotometer

(PowerWave XS, BioTek, USA) according to the method of Sydow [29] and given as mg dl-1.

Statistical analysis

Statistical analysis of the data obtained from the study was carried out in the SPSS package program (IBM SPSS Statistic 22). One-way analysis of variance (ANOVA) was used to determine whether there was a difference between the means of the experimental group and, if there was a difference between the means of the experimental group, the “Anova-Duncan” test was performed on the group means in order to determine the group or groups from which this difference was derived, and the value $p<0.05$ was considered statistically significant.

Results

Histological findings

After the fixation and tissue processing stages in the end of the study, the hepatic tissues taken were embedded in paraffin, and

the 5 μm serial sections taken with a microtome from these blocks were examined at light microscopic level after staining with haematoxylin-eosin. Histopathological examinations of the sections obtained from the groups showed that the vena centralis and portal area appeared normal and that the hepatocyte sequence was regular in the control and CAPE groups. In the sections obtained from the malathion-treated group, hyperaemia was identified around the vein (central and portal), and cell infiltrations, necrotic and degenerative regions were determined around the central and portal vein.

In the group in which CAPE was administered as a protection against malathion, the same lesions as in the malathion group (central and portal vein congestion and surrounding infiltrations) were detected although the severity and frequency of lesions were diminished, (Table 1, Figure 1a, b, c, d, e, f).

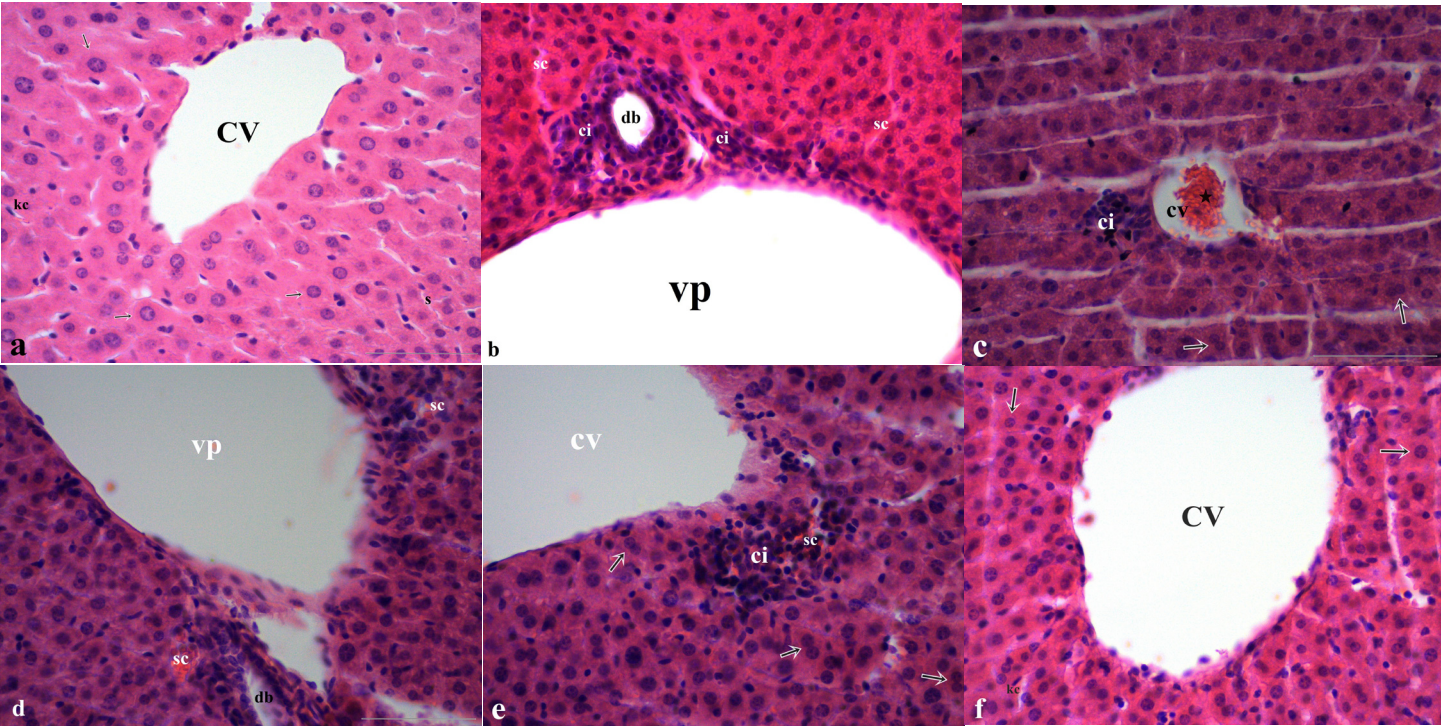


Figure 1. The effect of CAPE (10 $\mu\text{mol/kg}$), corn oil (5 ml/kg) and malathion (40 mg/kg) either separately or 1 h ago malathion histology of liver section by using hematoxylin and staining (H&E): (a) control group: hepatocytes (arrows), s: sinusoid, kc: kupffer cells, cv: central veins, (b, c) Malathion group: regenerative binuclear hepatocytes (arrows), ci: cell infiltrations, sc: sinusoidal congestion, vp: portal veins, db: ductus biliferi, (d, e) Malathion+CAPE group: sc: sinusoidal congestion, regenerative binuclear hepatocytes (arrows), ci: cell infiltrations, cv: central veins, vp: portal veins, db: ductus biliferi, (f) CAPE group: hepatocytes (arrows), kc: kupffer cells, cv: central veins, (bar: 50 μm).

Table 1. Evaluation of histopathological findings in liver tissue

Groups	Histopathology	Inflammatory cell infiltrations	Vascular congestions	Regenerative binuclear hepatocytes	Sinusoidal congestion	Focal necrosis areas
Control group (corn oil, 5 ml kg ⁻¹)		-	-	+	-	-
Malathion group (40 mg kg ⁻¹ malathion)		+++	++	++	++	++
Malathion+CAPE group (40 mg kg ⁻¹ dichlorvos+10 $\mu\text{mol kg}^{-1}$ CAPE)		++	+	+	++	+
CAPE group (10 $\mu\text{mol kg}^{-1}$ CAPE)		-	-	-	-	-

Scoring values: (-) none, (+) low, (++) moderate, (+++) severe.

Biochemical Findings

Total antioxidant, total oxidant and total sialic acid levels were measured in the obtained plasma samples. As a result of statistical comparison of biochemical data of the groups, there was no statistically significant difference between the control and CAPE groups in terms of total antioxidant capacity ($p>0.05$). But the statistical difference between the malathion group and all other groups was significant ($p<0.05$). Again, the difference between the group in which malathion and CAPE were administered together and all other groups was significant ($p<0.05$). In the evaluation of total oxidant capacity, the difference between the control and CAPE groups was insignificant ($p>0.05$) whereas the difference between the malathion group and all other groups was significant ($p<0.05$). Similarly, the difference between the malathion+CAPE group and all other groups was significant ($p<0.05$). Finally, when the groups were compared in terms of total sialic acid levels, no statistically significant difference was observed between malathion

and CAPE groups ($p>0.05$), while the malathion group and the malathion+CAPE group were statistically different both from each other and also from all other groups ($p<0.05$) (Table 2, Figure 2a, b, and c).

Table 2. TAS, TOS and TSA activities as a result of biochemical data obtained from the groups

Parameters	Groups (n:10)				p
	Control (oil) (Mean \pm SD)	Malathion (Mean \pm SD)	Malathion+CAPE (Mean \pm SD)	CAPE (Mean \pm SD)	
TAS (mmol Trolox Eqv./L)	1.44 \pm 0.08 ^a	0.82 \pm 0.01 ^c	1.05 \pm 0.02 ^b	1.40 \pm 0.07 ^a	*
TOS (μ mol H ₂ O ₂ Eqv./L)	8.29 \pm 0.61 ^c	15.76 \pm 0.95 ^a	11.50 \pm 0.89 ^b	8.95 \pm 0.7 ^c	*
TSA (mg dL ⁻¹)	54.25 \pm 3.21 ^c	69.10 \pm 4.05 ^a	60.75 \pm 4.88 ^b	53.43 \pm 4.23 ^c	*

* $p<0.05$: Statistically significant difference, ^{a, b, c}: Horizontally, values with different letter indicate significant differences, SD: Standard deviation.

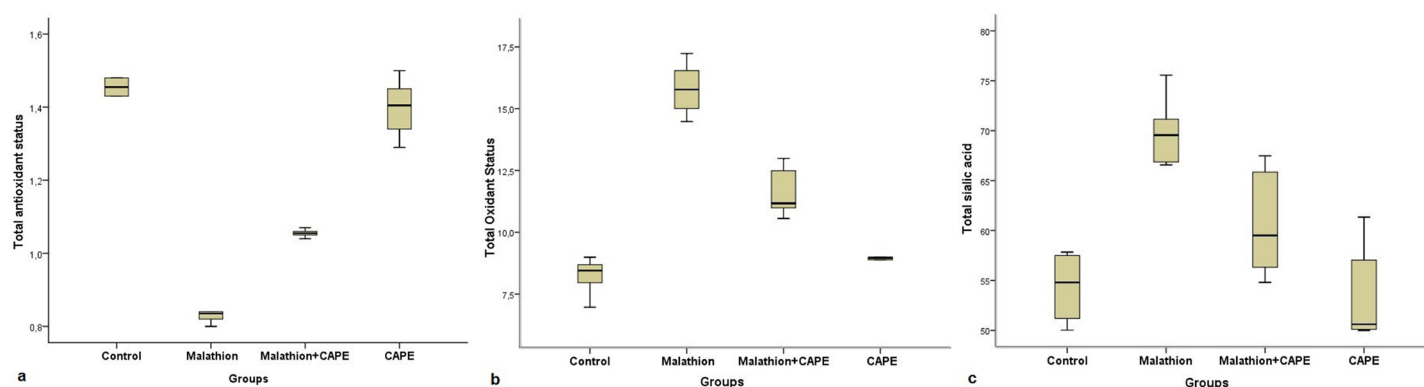


Figure 2. Box plot display of CAPE on TAS, TOS and TSA values in rats treated with subacute malathion a. TAS, b. TOS, c. TSA levels

Discussion

The widespread use of organophosphate insecticides can lead to poisoning and even death in humans and many other living beings. Among pesticides, organophosphates are the most commonly used group around the world and cause the greatest number of poisoning cases globally.

The reason organophosphate pesticides are preferred over organochlorine compounds is because they are degraded faster [30]. However, it is known that these chemicals used are dangerous for the environment and public health [31]. Organophosphate compounds constitute 50% of the total poisoning rate among the pesticides [32]. Many of those used as insecticides are dimethoxy and diethoxy compounds. This group of pesticides contains the best known insecticides of malathion and diazinon. Because of this prevalence of use, malathion was chosen as the pesticide material of our study. Malathion toxicity is associated with acetylcholinesterase inhibition, oxidative stress induction, liver damage, and impaired renal function. In particular, detoxification of toxic substances or drugs entering the body via the oral route is performed in the liver [33]. Based on the histopathological examinations in our study, it was observed that the vena centralis and portal area appeared normal and that the hepatocyte sequence was regular in the control and CAPE groups. In the sections obtained from the malathion-treated group, hyperaemia was identified around the vein (central and portal), and cell infiltrations, necrotic

and degenerative regions were determined around the central and portal vein. In a study investigating the effects of malathion in rats, it was reported that a high dose of malathion caused damage to the liver tissues of rats [34]. Another study reported that malathion given for one month resulted in deterioration and damage of liver structure, and displayed histology of necrosis in many cells, and these findings are similar to our results. It was reported that malathion-exposed rats have increased vacuole formation in the liver, enlarged sinusoids, leukocytic infiltrates, and vaso-dilatation due to occlusion [35]. In another study, the livers of malathion-treated animals were reported to have mononuclear cell infiltration, bleeding, calcification, vacuolar degeneration, enlargement of sinusoids, vascular occlusion and necrosis [36]. All of the findings based on the malathion used in these studies are in parallel with the results of our study.

Reactive oxygen species (ROS) are produced in the cell in normal metabolic processes and transduction conditions, but also play an important role in pathophysiological processes. Biomolecules such as lipids, proteins and nucleic acids undergo degeneration by reactive oxygen species [37,38]. Total antioxidant, total oxidant and total sialic acid levels were measured in the plasma samples obtained from our study. In terms of total antioxidant capacity, there was no statistical difference between control and CAPE groups ($p>0.05$). The statistical difference between the malathion group and all other groups is significant ($p<0.05$). Again, the difference between the group in which malathion and CAPE were administered

together and all other groups was significant ($p < 0.05$). Similarly, total oxidant capacity and total sialic profiles were parallel to total antioxidant capacity. Ekremoglu reported that the total oxidant level increased due to increasing doses when malathion was administered acutely (24 hours) at different doses (100 mg kg⁻¹, 200 mg kg⁻¹, 400 mg kg⁻¹) [39]. In a study investigating the in vivo effects of malathion, Glutathion-S-transferase (GST) and acetylcholinesterase (AChE) activities were evaluated in different organs of newborn rats. Accordingly, in adult female rats fed malathion, liver GST activity was increased about 2-fold, while AChE activity was reduced by more than 20% compared with the control. Similarly, liver and heart GST activities (0.14 and 0.035 U mg⁻¹ protein, respectively) increased approximately 2-fold in neonatal rats, but a decrease was observed in brain GST activity (0.074 U mg⁻¹ protein). Significant decreases were also observed in liver, brain, kidney and lung AChE activities (0.002, 0.035, 0.046, and 0.018 U/mg protein, respectively) compared with the control [40]. In an in vivo study with dichlorvos, the total antioxidant levels in the dichlorvos group were significantly lower than the other groups ($p < 0.05$), whereas the total antioxidant values in the CAPE groups were significantly higher than the dichlorvos+CAPE group ($p < 0.05$). In the same study, total oxidant values were significantly lower in the control and CAPE groups than in the dichlorvos group. No significant difference was observed between the other groups [9]. In a study investigating the protective effect of CAPE in malathion-treated rats, it was reported that malathion disrupts the enzyme activity of AChE, GP (glycogen phosphorylase) and HK (hexokinase) but improves AChE, GP and HK activities of the group in which malathion was given with CAPE [41]. In another study investigating the acute protective effect of CAPE against poisoning by chlorpyrifos-ethyl (CPF), an organophosphate compound, paraoxonase (PON1) activity and TAS levels were reduced while oxidative stress index and TOS levels were found to increase in the context of a CPF-induced intoxication. CAPE was reported to have a protective effect by reducing the oxidative stress caused by CPF [16]. In one study, malathion was reported to reduce the expression of catalase and superoxide dismutase-2 from antioxidant enzymes, thus suppressing the antioxidant system [42]. One study reported that the amount of malondialdehyde (MDA) increased and glutathione (GSH) levels decreased with increasing reactive oxygen species after tetramethrin administration. In the same study, reduction of MDA and increase in GSH levels were observed due to the free radical scavenging properties of CAPE by administration of CAPE with tetramethrin [15].

Conclusion

In conclusion the current study determined that malathion caused cell infiltrations, degeneration and focal necrosis in liver tissue, while CAPE reduced the severity of histopathological lesions caused by malathion. At the same time, it was observed that malathion increased total oxidant and sialic acid level and decreased total antioxidant compared with the control group. On the other hand, CAPE was observed to reduce the harmful effects of malathion and bring biochemical parameters closer to the control group. It was found that in rats administered CAPE together with malathion, CAPE reduced the degeneration in the liver and could have a protective effect by partially correcting the oxidative stress parameters. These data show that malathion, an organophosphate compound, has a toxic effect on the liver and

CAPE, an important antioxidant molecule, may have a protective effect on the antioxidant system of the organism against the oxidative stress caused by organophosphate pesticides, and that intake of CAPE and other antioxidant molecules in advance has a protective potential against cases of poisoning among those people who are engaged in agriculture.

Competing interests

The authors declare that they have no competing interest

Financial Disclosure

The financial support for this study was provided by the investigators themselves.

Ethical approval

Before the study, permissions were obtained from local ethical committee.

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