Effects of Organophosphorus and Pyrethroid pesticides on antioxidant enzymes and reactivation effects of Pralidoxime: *In vitro* studies

Hatice Paluzar[1,*], Ayten Sagiroglu[2]

[1] *Dept. of Chemistry and Chemistry Process. Tech., Arda Vocational College*
[2] *Dept. of Chemistry, Faculty of Science*
Trakya University, Turkey

**Corresponding author: haticepaluzar@trakya.edu.tr*

Abstract

The present study aimed to assess the inhibition effects of organophosphate pesticides, malathion^R, dichlorvos^R; pyrethroid pesticides, deltamethrin^R, λ-cyhaloethrin^R on antioxidant enzymes and the reactivation ability of pralidoxime against pesticide inhibited-antioxidant enzymes. Oximes were reported by reactivation ability against organophosphate inhibited-acetylcholinesterase and we focused to investigate the reactivation effect of pralidoxime against organophosphate inhibited–antioxidant enzymes. IC_{50} values were determined by means of activity percentage diagrams. The concentrations of deltamethrin^R, malathion^R, dichlorvos^R, λ-cyhaloethrin^R that inhibited 50% of catalase were 5.2 μM, 158 μM, 133 μM, 320 μM, respectively, inhibited 50% of superoxide dismutase were 62 μM, 240 μM, 328 μM, 2320 μM, respectively and inhibited 50% of glutathione peroxidase were 0.7 μM, 1198 μM, 1638 μM, 98 μM, respectively. All pesticide doses showed an inhibition effect on antioxidant enzymes. Deltamethrin^R was found to be a more potent inhibitor for the antioxidant enzymes followed by the rest of the pesticides used in this study. The reactivation effect of pralidoxime was determined for organophosphate inhibited-enzymes. Reactivation results showed that only catalase is reactivated by pralidoxime against dichlorvos^R and malathion^R. Under the exposure of 50-800 μM malathion^R concentrations, the activities of catalase were calculated as 72-11%, respectively. After, inhibited catalase was incubated with 1 mM and 10 mM pralidoxime, the activities of catalase were calculated as 92-31% and 98-39%, respectively. Under the exposure of 100-1500 μM dichlorvos^R concentrations, the activities of catalase were calculated as 50-6%, respectively. After, inhibited catalase was incubated with 1 mM and 10 mM pralidoxime, the activities of catalase were calculated as 95-30% and 93-28%, respectively. When the results are examined, it is seen that increasing the pralidoxime concentration does not significantly affect the reactivation percentage of the catalase enzyme.

Keywords: Deltamethrin; dichlorvos; λ-cyhaloethrin; malathion; pralidoxime.

1. Introduction

Organophosphate (OP) and pyrethroid (PYR) pesticides are among the most commonly used insecticides in agriculture, home, gardening, and veterinary applications. They replaced organochlorine insecticides due to their higher instability to environmental degradation. OPs do not stay in the environment for more than a few days or weeks, unlike organochlorines (Soares *et. al*. 2019; Fernandes *et. al.* 2018). However, these were proven far more toxic than

organochlorines, as these inhibit the acetylcholinesterase enzyme vital to control body nerve signals beyond showing carcinogenic, genotoxic, cytotoxic, mutagenic, and immunotoxic effects in mammals (Sousa *et. al.* 2020). At the same time, many studies have shown that exposure to PYRs can cause adverse effects and increase the risk to human health, for example, it can be carcinogenic and mutagenic to organisms and can affect the immune system, reproductive system, and nervous system (Han *et. al.* 2017; Koureas *et. al*. 2012; Wongmaneepratip & Yang 2020). Due to their widespread use, residues of these compounds are found in food products, water, air, and house dust (Mercier *et. al.* 2011; Banarjee *et. al.* 2012; Coscollà *et. al.* 2017; Gibss *et. al.* 2017; Tang *et. al.* 2018; Van der Dries *et. al.* 2018; Manzoor *et. al.* 2016), and human exposure through diet (Tsatsakis *et. al.* 2003; Fortes *et. al.* 2003; Ciscato *et. al.* 2014), dermal contact or inhalation (Becker *et. al.* 2006; McKone *et. al.* 2007). However, these compounds represent potential risks to the environment and human health. Many chemicals, especially pesticides, at relatively low dosages affect the metabolism of organisms by altering the activities of enzymes (Isık *et. al*. 2004; Turan *et. al.* 2002). Looking at the widespread use of some OPs (malathion, dichlorvos) and PYRs (deltamethrin, λ-cyhalothrin) for agricultural purposes, the present study was aimed to evaluate the adverse effects of these pesticides on antioxidant enzymes namely catalase (CAT), speroxide dismutase (SOD) and glutathione peroxidase (GPx) *in vitro*.

On the other hand, when it comes to OP toxicity, the inhibition of acetylcholinesterase (AChE) comes to mind first. The inhibition process occurs mainly due to the formation of a P-O bond between the electrophilic center of OPs and the oxygen atom of the active serine residue, yielding a phosphylated AChE adduct (Jokanovic 2001). Until dealkylation occurs, the reaction is usually reversible. This removal of alkyl group is known as the 'aging' process (Worek *et. al*. 2004). The adduct formed after aging is no longer available for reactivation. Worldwide, efforts have focused for many years on finding ways to restore the function of aged AChE (Quinn *et. al.* 2017). However, if a nucleophile such as oxime attacks the phosphorous atom before aging of OP-AChE adduct, it can detach the OP from the serine group, and thus the enzyme can be regenerated (Kitagawa *et. al*. 2019). Oxime based cholinesterase reactivators such as pralidoxime (PAM) have been used as antidotes against OP-intoxication (Worek *et. al.* 2020; Antonijevic *et. al.* 2018; Savall *et. al.* 2020). No study has yet been reported on the reactivation effect of PAM on OP inhibited- antioxidant enzymes. Considering the OP inhibition with the mentioned pesticides, the *in vitro* reactivation effect of PAM on OP inhibited-antioxidant enzymes was first time experimentally investigated in this present study.

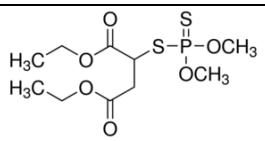
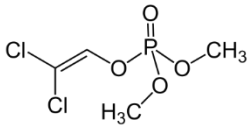
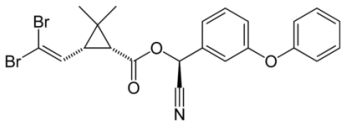
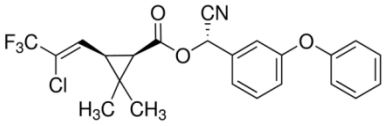
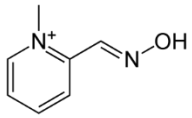
2. Materials and methods

2.1 Chemicals

All enzymes and all other reagents were purchased from Merck KGaA. Other chemicals were prepared analytically. Superoxide dismutase (from bovine erythrocytes, 300 KU, lyophilized powder, ≥3.000units/mg protein), catalase (from bovine liver, lyophilized powder, ≥10.000 units/mg protein), Glutation peroksidaz (from bovine erythrocytes, 300 KU, lyophilized powder, White, ≥100 units/mg), Glutathion reductase (from baker's yeast (*S. cerevisiae*), 2.5

KU, ammonium sulphate suspension, 100-300 units/mg protein (biuret)), Malathion [Diethyl 2-[(dimethoxy phosphorothioyl) sulfanyl] butanedioate], dichlorvos [2,2-dichlorovinyl dimethyl phosphate], deltamethrin [(S)-Cyano-(3-phenoxyphenyl)-methyl] (1R,3R)-3-(2,2-dibromoethenyl)-2,2-dimethyl-cyclopropane-1-carboxylate] and λ-cyhalothrin [(R) - cyano - (3-phenoxyphenyl) methyl] (1S, 3S)-3-[(Z)-2-chloro-3, 3, 3 - trifluoroprop – 1 - enyl]- 2, 2 dimethyl cyclopropane - 1 - carboxylate], Pralidoxime [2-pyridine aldoxime methyl chloride] were purchased form Merck KGaA. All pesticides were prepared in ethanol. The chemical structures of the pesticides and Pralidoxime (PAM) were shown at Table 1.

Table 1. Structures of investigated pesticides and pralidoxime reactivator

Malathion	
Dichlorvos	
Deltamethrin	
λ-cyhalothrin	
Pralidoxime	

2.2 Aparatus

Ultrasonic water bath (Wise Clean DAIHAN, WUC-AO3H, Korea), microplate reader (Thermo Scientific, Type 1510, Thermo fisher scientific Oy, Rotastie 2, FI-01620, Vantaa, Finland), precision scale (Precisa XB 220A, Precisa Gravimetrics AG/Switzerland), 8-channel dispenser pipette (Eppendorff from Research Plus, from 10 to 100 µL), the dispenser pipette (Eppendorff the Multipette plus), micropipettes (20 µL, 200 µL, 1000 µL Eppendorff micropipette),

circulating water bath (core IC 302, Turkey), magnetic stirrer (Biosan magnetic stirrer MSH 300) was used. Glass and disposable polypropylene or polystyrene plastics tend to absorb light in the UV range so that 96-well quartz plates were used in the UV range. For obtaining good results, using the appropriate microplate for UV region is more important (Held 2001).

2.3 Methods

2.3.1 CAT activity assay: inhibition and reactivation

CAT enzymatic activity was determined by using a method by Aebi (Aebi 1984). The principle of the assay is based on the determination of the rate constant (s^{-1}, k) of hydrogen peroxide decomposition by CAT enzyme. H_2O_2 consumption was measured at 240 nm for 180 s using a Thermo Scientific, Type 1510 microplate reader. Quartz 96 well plates are used for the assay. CAT activity was determined using a specific absorption coefficient at 0.040 cm^2 μmol^{-1} H_2O_2. For the preparation of Catalase solution, 26.6 U/mL of CAT (from bovine liver, lyophilized powder, ≥10.000 units/mg protein) was prepared by dissolving in phosphate buffer (50 mM, pH: 7.0). For substrate solution, approximately 9.375 mM stock H_2O_2 solution is prepared by 50 mM phosphate buffer (pH: 7.0). 20 µL CAT enzyme solution and 200 µL 50 mM phosphate buffer (pH: 7.0) were added to the plate. Then, 80 µL H_2O_2 stock solutions were added to the reaction medium, and absorbance was rapidly measured at this time at 240 nm (Abs^0). After the quartz plate was placed into the microplate reader, absorbances were measured for 180 s at 240nm (Abs^1). One unit of CAT activity corresponds to the amount of enzyme, decomposing the reaction of 1µmol H_2O_2 per 1 min.

For the inhibition assay of CAT, PYR pesticides (deltamethrin^R, λ-cyhaloethrin^R) and OP pesticides (malathion^R, dichlorvos^R) were used as inhibitors. 10 µL of different concentrations of these pesticides were added to enzyme activity determination medium and incubated for 15 min before adding substrate solution. After 15 min, 80 µL H_2O_2 was added to the reaction medium. Abs^0 and Abs^1 were read, and CAT activities were determined for different concentrations of the pesticides. Thus, minimum and maximum pesticide concentrations that affect the activity of CAT were determined; for deltamethrin^R 2 µM – 40 µM; for λ-cyhaloethrin^R 250 µM - 2250 µM; for malathion^R 50 µM - 500 µM; for dichlorvos^R 100 µM - 1500 µM. Graphs of the percent of activity versus the pesticide concentration were drawn by using these pesticide concentrations range. The values of IC_{50} were drawn by regression analysis graphs with a GraphPad Prism 8.0 program and calculated from activity (%) - pesticides concentration graphs. CAT activity without pesticides (0 mM) was accepted as 100% and this measurement was called control. All the runs (n=5) were carried out in specified conditions.

For reactivation assays, the activity of enzyme in the control remained constant during the experiment. Oximes were reported by reactivation ability against organophosphate inhibited-AChE (Kobrlova *et. al.* 2019) and in this work, we focused to investigate the reactivation effect of pralidoxime against OP inhibited – antioxidant enzymes. So that, in order to determine the

reactivation effect of PAM, OP pesticides (malathion^R, dichlorvos^R) solutions were added to the enzyme solutions and incubated for 15 min at 37°C.

For the reactivation assay of CAT, the concentrations of organophosphates used were based on the concentrations determined at the inhibition assay pesticides (for malathion^R between 50 µM - 500 µM; for dichlorvos^R between 2 µM - 1500 µM). Two different concentrations of PAM (final concentrations of tested reactivator were 1 and 10 mM) were used for the reactivation assay, for this, stock-PAM solutions were prepared. 10 µL of stated concentrations of these pesticides were added to enzyme activity determination medium and incubated for 15 min before adding PAM solution. After 15 min, 10 µL of stock-PAM solutions was added to the reaction medium and again incubated for 15 min at 37°C. 80 µL H_2O_2 was added to the reaction medium. Abs^0 and Abs^1 were read. CAT activities were measured and calculated with a constant substrate and different inhibitor concentrations. The reactivation percentage of the inhibited enzyme was calculated as the ratio of the recovered enzyme activity and activity in the control. The reactivation analysis graph for CAT was drawn using activity % values.

2.3.2 SOD activity assay: inhibition and reactivation

Determination of SOD enzyme activity was performed according to the method of NitroBlue Tetrazolium/Riboflavin (NBT/RF) (Cakmak and Marschner 1992). In SOD activity assay, Iodonitrotetrazolium chloride (INT) which forms a red dye in the presence of free radical was used instead of NBT. The principle of SOD activity measurement is based on the reduction of INT by O_2^- under light and measurement, these are reduced at 560 nm. SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of INT using the method of Dhindsa (Dhindsa and Plumb-Dhindsa 1981). For the preparation of SOD solution, 379.75 U/mL of SOD (from bovine erythrocytes, 300 KU, lyophilized powder, ≥3.000units/mg protein) was prepared by dissolving in phosphate buffer (50 mM, pH: 7.0).

For activity assay, the 300 µL reaction mixture contained final concentrations of 50 mM phosphate buffer (pH 7.5), 13 mM methionine, 75 M INT, 2 M riboflavin, 0.1 mM EDTA was prepared and pipetted into a plate, and absorbance was read at 560 nm (A^0). After reading A^0, 15 µL of SOD solution was added to this medium. The plate was shaken and placed 30 cm below a light bank consisting of two 15 W fluorescent lamps for 10 min. The absorbance (A^1) by the reaction mixture was read at 560 nm using a Thermo Scientific, Type 1510 microplate reader. One unit of SOD activity represents a 50% inhibition of the production of free radicals.

To determine the inhibition effects of pesticides on SOD activity, pyrethroid pesticides (deltamethrin^R, λ-cyhaloethrin^R) and organophosphate pesticides (malathion^R, dichlorvos^R) were used as inhibitors. 10 µL of different concentrations of these pesticides were added to stock SOD enzyme solution and incubated for 15 min before adding substrate solution. After 15 min, 15 µL of SOD enzyme solution inhibited by the pesticides was added to the reaction medium. Abs^0 and Abs^1 were read, and SOD activities were determined for different concentrations of the pesticides. Thus, minimum and maximum pesticide concentrations that affect the activity of SOD were determined; for deltamethrin^R 2 µM – 40 µM; for λ-

cyhaloethrin^{R} 250 µM - 2250 µM; for malathion^{R} 50 µM - 500 µM; for dichlorvos^{R} 100 µM - 1500 µM. Graphs of the percent of activity versus the pesticide concentration were drawn by using these pesticide concentrations range. The values of IC_{50} were drawn by regression analysis graphs with a GraphPad Prism 8.0 program and calculated from activity (%) – pesticides concentration graphs. SOD activity without pesticides (0 mM) was accepted as 100% and this measurement was called control. All the runs (n=5) were carried out in specified conditions.

For reactivation assays, only organopohosphate inhibited – SOD enzymes were examined (organopohosphate pesticides; malathion^{R}, and dichlorvos^{R}). For the reactivation assay of SOD, the concentrations of organophosphates used were based on the concentrations determined at the inhibition assay pesticides (for malathion^{R} between 50 µM - 500 µM; for dichlorvos^{R} between 100 µM - 1500 µM). Two different concentrations of PAM (final concentrations of the tested reactivator were 1 and 10 mM) were used for the reactivation assay. For this, stock-PAM solutions were prepared. 10 µL of stated concentrations of these pesticides were added to stock SOD solution and incubated for 15 min before adding PAM solution. After 15 min, 10 µL of stock-PAM solutions was added to the inhibited SOD solutions and again incubated for 15 min at 37°C. 15 µL of SOD enzyme solution inhibited by the pesticides and reactivated by PAM added to the reaction medium for determination of the activity. Abs^{0} and Abs^{1} were read. SOD activities were measured and calculated with a constant substrate and different inhibitor concentrations. The reactivation percentage of the inhibited enzyme was calculated as the ratio of the recovered enzyme activity and activity in the control. Reactivation analysis graph for SOD was drawn using activity % values.

2.3.3 GPx activity assay: inhibition and reactivation

The enzymatic activity of GPx was measured by Beutler's method (Beutler 1971). The assay recording of NADPH loss measures H_2O_2 reduction by GPx to alcohol at 340 nm (Christine and Joseph 2010). To determine the GPx activity within a sample, given that 1 unit=1 µmole NADPH oxidized min^{-1} at the specified GSH concentrations or more correctly, µmoles GSH produced min^{-1}. For the preparation of GPx solution, 0.5 U/mL of GPx (from bovine erythrocytes, 300 KU, lyophilized powder, White, ≥100 units/mg), was prepared by dissolving in phosphate buffer (50 mM, pH: 7.0).

GPx activity was assayed by measuring the decrease in absorbance at 340 nm due to the oxidation of NADPH. The 300 µL reaction mixture contained final concentrations of 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM NADPH, and 1mM GSSG was prepared and pipetted into a quartz plate and absorbance was read at 340 nm (A^{0}) at 30 °C. After reading absorbance, 15 µL of enzyme solution was added to this medium and after the incubation time (1 min), absorbances were read by using Thermo Scientific, Type 1510 microplate reader. Quartz 96 well plates are used for the assay. One enzyme unit was defined as the enzyme amount reducing 1 µmole $NADP^{+}$ per 1 min at optimum pH.

For inhibition assays of GPx, pyrethroid pesticides (deltamethrin^R, λ-cyhaloethrin^R) and organophosphate pesticides (malathion^R, dichlorvos^R) were used as inhibitors. 10 µL of different concentrations of these pesticides were added to stock GPx enzyme solution and incubated for 15 min before adding substrate solution. After 15 min, 15 µL of GPx enzyme solution inhibited by the pesticides was added to the reaction medium. Abs^0 and Abs^1 were read, and GPx activities were determined for different concentrations of the pesticides. Thus, minimum and maximum pesticide concentrations that affect the activity of GPx were determined; for deltamethrin^R 2 µM – 40 µM; for λ-cyhaloethrin^R 250 µM - 2250 µM; for malathion^R 50 µM - 500 µM; for dichlorvos^R 100 µM - 1500 µM. Graphs of the percent of activity versus the pesticide concentration were drawn by using these pesticide concentrations range. The values of IC_{50} were drawn by regression analysis graphs with a GraphPad Prism 8.0 program and calculated from activity (%) – pesticides concentration graphs. GPx activity without pesticides (0 mM) was accepted as 100% and this measurement was called control. All the runs (n=5) were carried out in specified conditions.

For reactivation assays, only organopohosphate inhibited – GPx enzymes were examined (organopohosphate pesticides; malathion^R and dichlorvos^R). For the reactivation assay of GPx, the concentrations of organophosphates used were based on the concentrations determined at the inhibition assay pesticides (for malathion^R between 50 µM - 500 µM; for dichlorvos^R between 2 µM - 1500 µM). Two different concentrations of PAM (final concentrations of the tested reactivator were 1 and 10 mM) were used for the reactivation assay. For this, stock-PAM solutions were prepared. 10 µL of stated concentrations of these pesticides were added to stock GPx solution and incubated for 15 min before adding PAM solution. After 15 min, 10 µL of stock-PAM solutions was added to the inhibited GPx solutions and again incubated for 15 min at 30°C. 15 µL of GPx enzyme solution inhibited by the pesticides and reactivated by PAM added to the reaction medium for determination of the activity. Abs^0 and Abs^1 were read. GPx activities were measured and calculated with a constant substrate and different inhibitor concentrations. The reactivation percentage of the inhibited enzyme was calculated as the ratio of the recovered enzyme activity and activity in the control. Reactivation analysis graph for GPx was drawn using activity % values.

2.3.4 Total protein assay

The protein concentration was measured spectrophotometrically at 750 nm by the method of Lowry (Lowry *et. al.* 1951). Bovine serum albumin was used as a standard for the determination of protein concentration. For this purpose, four solutions were prepared: 1. Solution (A): 0.5 g $CuSO_4.5H_2O$ and 1 g sodium citrate dehydrate were dissolved in distilled water and completed to 100 ml; 2. Solution (B): 20 g Na_2CO_3 and 4 g NaOH was dissolved in distilled water and completed to 1000 ml; 3. Solution (C): 1ml solution A was added to 50 ml solution B; 4. Solution (D): 10 ml Folin Ciocalteu was added to 10 ml distilled water. After that, 2.5 ml solution C was added to 0.5 ml of prepared sample solution, vortexed, waited for 10 minutes at room temperature, mixed with 0.25 ml solution D, vortexed, waited for 30 minutes, and read at 750 nm for protein concentration determination.

2.3.5 Statistical analysis

Experimental data are presented as mean ±standard deviation (mean ± SD). The direct linear plot method was used for the determination of the kinetic constants of enzyme reactions (Eisenthal and Cornish 1974), for that the GraphPad prism software version 8 (GraphPad Inc. La Jolla, CA, USA) was used. IC_{50} values were obtained when the fitting was made by a non-linear least-squares method (Snedecor and Cochran 1980). For the statistical analyses, ANOVA (one-way analysis of variance) was used, followed by the Student Newman-Keul's test using the SPSS version 21 statistical software (SPSS Inc., Chicago, IL, USA). Differences were considered significant if $p<0.05$.

3. Results

3.1 Effect of organophosphate and pyrethroid pesticides on CAT Activity

Different pesticide concentrations with CAT enzyme solutions were prepared, then, the activities of CAT were measured. Minimum and maximum pesticide concentrations that affect the activity of CAT were determined; for deltamethrin^R 2 µM - 40 µM; for λ-cyhaloethrin^R 250 µM - 2250 µM; for malathion^R 50 µM - 500 µM; for dichlorvos^R 100 µM - 1500 µM. Graphs of the percent of activity versus the pesticide concentration were drawn by using these pesticide concentrations range and are shown in Figure 1. Under the exposure of 50, 100, 200, 400, 600 and 800 µM malathion concentrations, CAT activity % were calculated as 72; 63; 38; 10; 12 and 11 %, respectively. Under the exposure of 100, 200, 400, 1000, 1200 and 1500 µM dichlorvos concentrations, % CAT activity were calculated as 50; 38; 22; 8; 7 and 6 %, respectively. Under the exposure of 2, 4, 8, 20, 30 and 40 µM deltamethrin concentrations, CAT activity % were calculated as 80; 72; 56; 20; 22 and 18 %, respectively. Under the exposure of 250, 500, 1200, 1500, 2000 and 2500 µM λ-cyhaloethrin concentrations, CAT activity % were calculated as 70; 55; 36; 24; 22 and 23 %, respectively. Compared to the control activity, there are statistical differences between all CAT activities which interacted with all pesticides ($p<0.05$, $n=5$) (Table 2). The percentages of both CAT activities were decreased under the exposure of determined concentrations of malathion, dichlorvos, deltametrhrin, and λ-cyhaloethrin. But, decreasing CAT activity with dichlorvos and malathion (OP pesticides) was higher compared to deltamethrin and λ-cyhaloethrin (PYR pesticides). However, it is also seen that deltamethrin, which is a pyrethroid class pesticide, has high inhibition % even at very low concentrations. IC_{50} values (the most suitable parameters for seeing inhibitory effects) of these pesticides for CAT were determined and shown in Table 2. The IC_{50} values of deltamethrin, malathion, dichlorvos and λ-cyhalothrin for CAT were found as 5.2 µM, 158 µM, 133 µM, and 320 µM, respectively.

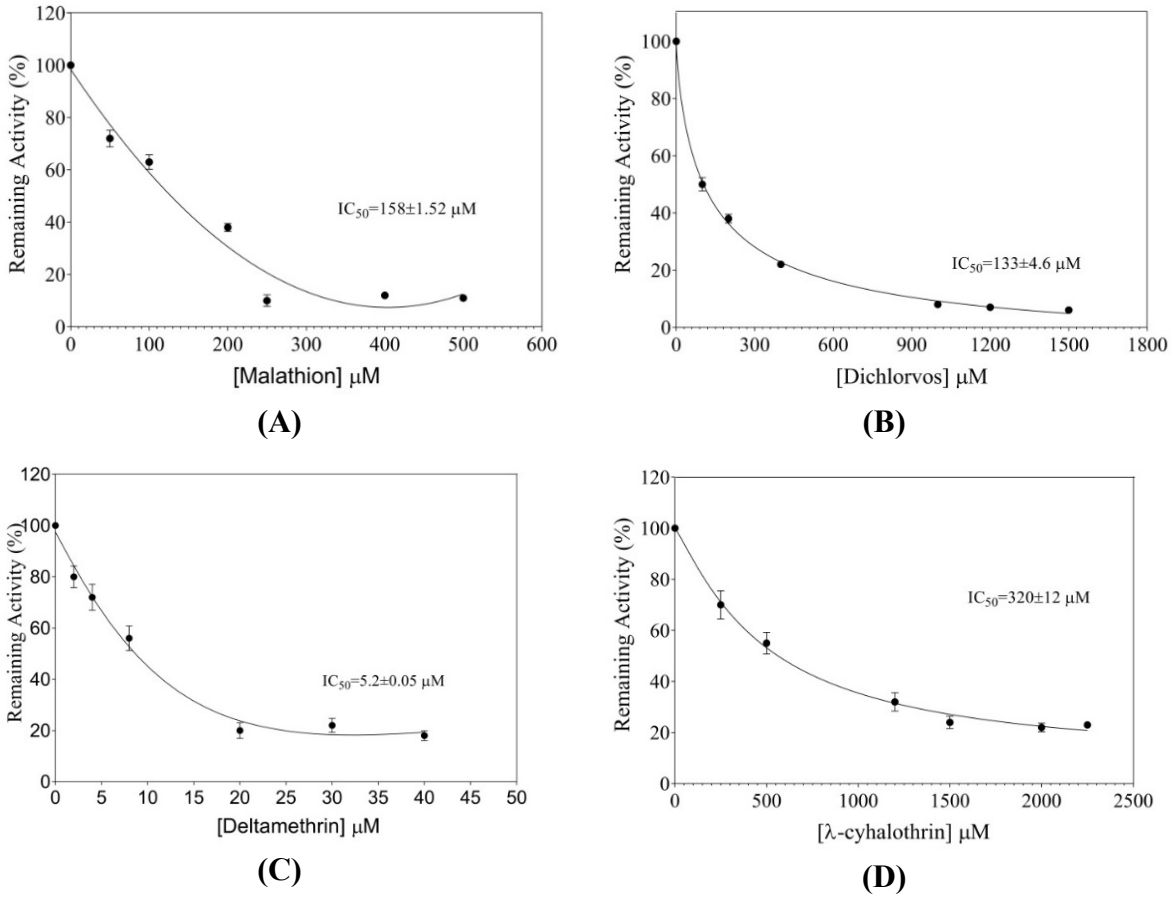


Fig. 1. The activity of catalase in the presence of various concentrations of malathion (A) and dichlorvos (B), deltamethrin (C), and λ-cyhalothrin (D). IC_{50} values were determined from these graphs. (n=5)

3.2 Effect of organophosphate and pyrethroid pesticides on SOD Activity

Inhibitory effects of malathion, dichlorvos, deltamethrin, λ-cyhalothrin pesticides were examined on SOD enzyme activity. For this purpose, firstly, activity assays in varying concentrations of inhibitors were done and half-maximal inhibitory concentrations (IC_{50}) were calculated by drawing [I]-% activity graphs. The maximum enzyme activity of SOD was assumed to be 100% and the inhibition activity of SOD was assumed to be 0% in the absence of any pesticide. Different pesticide concentrations with SOD enzyme solutions were prepared, then, activities of SOD were measured. Minimum and maximum pesticide concentrations that affect the activity of SOD were determined; for deltamethrin^R 2 µM – 40 µM; for λ-cyhaloethrin^R 250 µM - 2250 µM; for malathion^R 50 µM - 500 µM; for dichlorvos^R 100 µM - 1500 µM. Graphs of the percent of activity versus the pesticide concentration were drawn by using these pesticide concentrations range and are shown in Figure 2. Under the exposure of 50, 100, 200, 400, 600 and 800 µM malathion concentrations, SOD activity % were calculated as 82; 76; 61; 37; 38 and 36 %, respectively. Under the exposure of 100, 200, 400, 1000, 1200 and 1500 µM dichlorvos concentrations, SOD activity % were calculated as 78; 62; 42; 42; 43

and 41 %, respectively. Under the exposure of 2, 4, 8, 20, 30 and 40 µM deltamethrin concentrations, SOD activity % were calculated as 98; 85; 70; 62; 61 and 60 %, respectively. Under the exposure of 250, 500, 1200, 1500, 2000 and 2500 µM λ-cyhaloethrin concentrations, SOD activity % were calculated as 90; 78; 57; 55; 54 and 53 %, respectively.

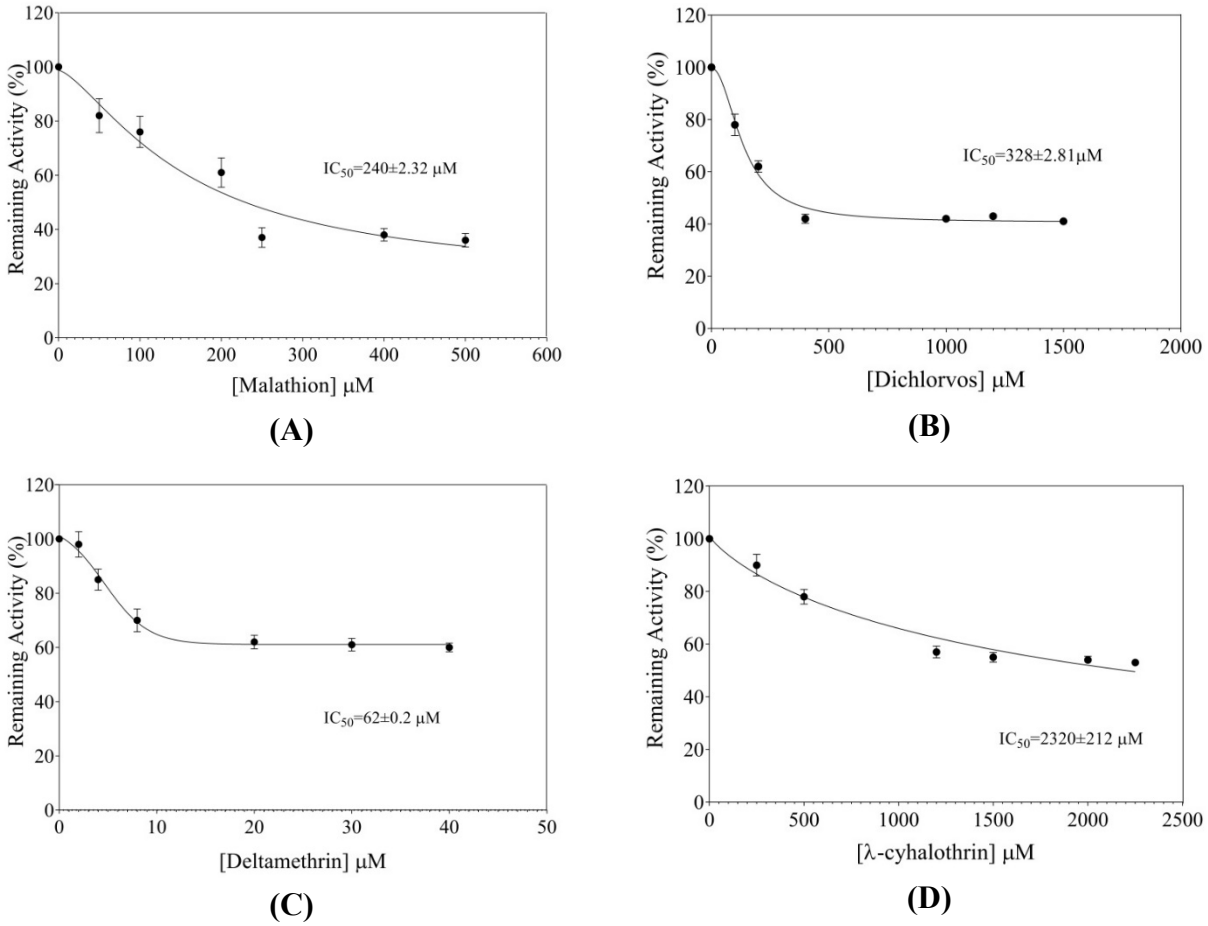


Fig. 2. The activity of superoxide dismutase in the presence of various concentrations of malathion (A) and dichlorvos (B), deltamethrin (C), and λ-cyhalothrin (D). IC_{50} values were determined from these graphs. (n=5)

Compared to the control activity, there are statistical differences between all SOD activities which interacted with all pesticides ($p<0.05$, n=5) (Table 2). The percentages of both SOD activities were decreased under the exposure of determining concentrations of malathion, dichlorvos, deltamethrin, and λ-cyhaloethrin. But, decreasing SOD activity with dichlorvos and malathion (OP pesticides) was higher compared to deltametrhrin and λ-cyhaloethrin (PYR pesticides). However, it is also seen that deltamethrin, which is a pyrethroid class pesticide, has high inhibition % even at very low concentrations. IC_{50} values (the most suitable parameters for seeing inhibitory effects) of these pesticides for SOD were determined and shown in Table 2. The IC_{50} values of deltamethrin, malathion, dichlorvos, and λ-cyhalothrin for SOD were found as 62 µM, 240 µM, 328 µM, and 2320 µM, respectively.

Table 2. Effect of malathion, dichlorvos, deltamethrin, and λ-cyhalothrin concentrations on CAT, SOD, and GPx activity. (Differences were considered significant if $p<0.05$)

		Percentage of remaining activity ± SD			
Pesticides	**[pesticide] µM**	**CAT**	**SOD**	**GPx**	**p**
Malathion	50	72±3.2	82±6.3	95±5.4	<0.05
	100	63±2.8	76±5.7	90±4.3	<0.05
	200	38±1.5	61±5.4	88±2.7	<0.05
	250	10±2.2	37±3.6	86±3.8	<0.05
	400	12±0.8	38±2.3	85±3.4	<0.05
	500	11±1.3	36±2.5	87±3.1	<0.05
		<0.05	<0.05	<0.05	**p**
Dichlorvos	100	50±2.3	78±4.1	98±4.5	<0.05
	200	38±1.5	62±2.2	96±5.3	<0.05
	400	22±1.2	42±1.7	85±5.1	<0.05
	1000	8±0.4	42±1.2	83±3.4	<0.05
	1200	7±0.2	43±1.3	85±3.2	<0.05
	1500	6±0.5	41±0.9	84±2.7	<0.05
		<0.05	<0.05	<0.05	**p**
Deltamethrin	2	80±4.2	98±4.7	18±1.2	<0.05
	4	72±5.1	85±3.9	16±0.7	<0.05
	8	56±4.8	70±4.2	17±0.9	<0.05
	20	20±3.1	62±2.5	15±1.2	<0.05
	30	22±2.7	61±2.3	14±0.7	<0.05
	40	18±1.9	60±1.6	13±1.3	<0.05
		<0.05	<0.05	<0.05	**p**
λ-cyhalothrin	250	70±5.5	90±4.1	22±1.7	<0.05
	500	55±4.2	78±2.8	20±1.4	<0.05
	1200	32±3.6	57±2.2	18±0.6	<0.05
	1500	24±2.4	55±1.8	16±0.4	<0.05
	2000	22±1.7	54±1.4	17±1.7	<0.05
	2250	23±1.1	53±0.5	16±0.2	<0.05
		<0.05	<0.05	<0.05	**p**

Table 3. Concentrations of the pesticides caused to 50% inhibition of CAT, SOD, and GPx activity

	IC_{50} (µM)±SD		
PESTICIDES	**CAT**	**SOD**	**GPx**
Deltamethrin	5.2±0.05	62±0.2	0.7±0.06
λ -cyhalothrin	320±12	2320±212	98±2.1
Malathion	158±1.52	240±2.32	1198±118
Dichlorvos	133±4.6	328±2.81	1638±17

Table 4. Effect of PAM on pesticide inhibited – CAT, SOD, and GPx activity.

			Percentage of remaining activity ± SD		
	Pesticides	**[pesticide] µM**	**After being inhibited by pesticide**	**After reactivated by 1 mM PAM**	**After reactivated by 10 mM PAM**
CAT	**Malathion**	50	72±3.2	92±4.3	98±5.2
		100	63±2.8	78±3.1	89±4.3
		200	38±1.5	58±2.7	72±2.8
		250	10±2.2	40±2.1	57±1.7
		400	12±0.8	35±1.8	45±1.1
		500	11±1.3	31±2.4	39±0.9
	Dichlorvos	100	50±2.3	95±2.7	93±3.7
		200	38±1.5	89±3.5	90±3.9
		400	22±1.2	62±2.4	65±3.1
		1000	8±0.4	40±1.9	43±1.2
		1200	7±0.2	35±1.7	32±0.1
		1500	6±0.5	30±1.3	28±1.4
SOD	**Malathion**	50	82±6.3	83±5.1	81±4.2
		100	76±5.7	74±4.2	76±4.7
		200	61±5.4	63±4.7	63±5.1
		250	37±3.6	38±3.2	35±3.8
		400	38±2.3	34±2.1	37±3.2
		500	36±2.5	35±1.8	35±2.7
	Dichlorvos	100	78±4.1	76±3.7	74±3.1
		200	62±2.2	60±0.9	65±2.0
		400	42±1.7	43±1.1	40±1.3
		1000	42±1.2	43±1.5	40±0.7
		1200	43±1.3	41±0.7	43±0.9
		1500	41±0.9	43±1.4	41±1.2
GPx	**Malathion**	50	95±5.4	93±5.1	97±4.3
		100	90±4.3	91±3.7	89±4.1
		200	88±2.7	87±2.2	90±3.3
		250	86±3.8	86±4.1	85±3.5
		400	85±3.4	86±4.4	86±3.2
		500	87±3.1	86±3.8	86±2.7
	Dichlorvos	100	98±4.5	96±2.3	95±4.1
		200	96±5.3	98±4.7	96±4.2
		400	85±5.1	83±5.5	82±4.8
		1000	83±3.4	83±2.8	85±2.3
		1200	85±3.2	82±2.2	83±1.8
		1500	84±2.7	86±1.9	86±1.2

3.3 Effect of organophosphate and pyrethroid pesticides on GPx Activity

GPx activity assays in varying concentrations of pesticides were done and the values of IC_{50} were calculated by drawing [I]-% activity graphs. GPx activity without pesticides was accepted as 100% activity. Different pesticide concentrations with GPx enzyme solutions were prepared,

then, activities of GPx were measured. Minimum and maximum pesticide concentrations that affect the activity of GPx were determined; for deltamethrin^R 2 µM – 40 µM; for λ-cyhaloethrin^R 250 µM - 2250 µM; for malathion^R 50 µM - 500 µM; for dichlorvos^R 100 µM - 1500 µM. Graphs of the percent of activity versus the pesticide concentration were drawn by using these pesticide concentrations range and are shown in Figure 3. Under the exposure of 50, 100, 200, 400, 600 and 800 µM malathion concentrations, GPx activity % were calculated as 95; 90; 88; 86; 85 and 87 %, respectively. Under the exposure of 100, 200, 400, 1000, 1200 and 1500 µM dichlorvos concentrations, GPx activity % were calculated as 98; 96; 85; 83; 85 and 84 %, respectively. Under the exposure of 2, 4, 8, 20, 30 and 40 µM deltamethrin concentrations, GPx activity % were calculated as 18; 16; 17; 15; 14 and 13 %, respectively. Under the exposure of 250, 500, 1200, 1500, 2000 and 2500 µM λ-cyhaloethrin concentrations, % GPx activity were calculated as 22; 20; 18; 16; 17 and 16 %, respectively. Compared to the control activity, there are statistical differences between all GPx activities which interacted with all pesticides ($p<0.05$, $n=5$) (Table 2). The percentages of both GPx activities were decreased under the exposure of determining concentrations of malathion, dichlorvos, deltamethrin, and λ-cyhaloethrin. But, decreasing GPx activity with deltametrhrin and λ-cyhaloethrin (PYR class pesticides) were higher compared to dichlorvos and malathion (OP pesticides). However, it is also seen that deltamethrin, which is a pyrethroid class pesticide, has high inhibition % even at very low concentrations. IC_{50} values (the most suitable parameters for seeing inhibitory effects) of these pesticides for GPx were determined and shown in Table 2. The IC_{50} values of deltamethrin, dichlorvos, malathion, and λ-cyhalothrin for GPx were found as 0.7 µM, 1198 µM, 1638 µM, and 98 µM, respectively.

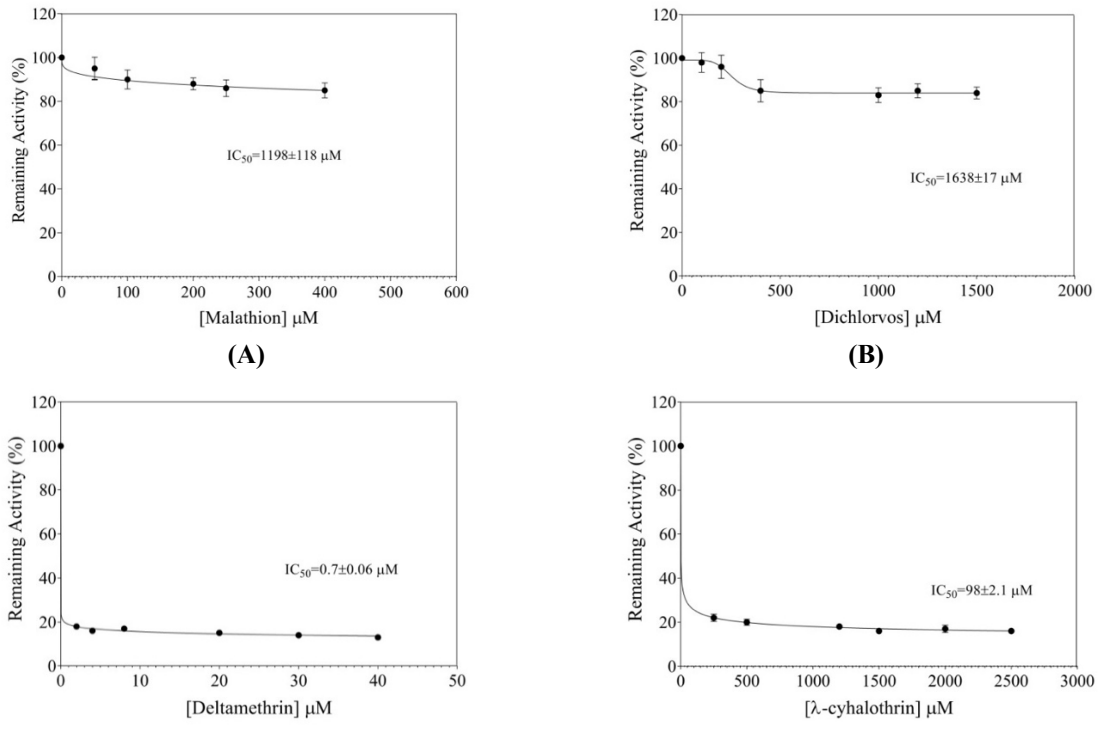


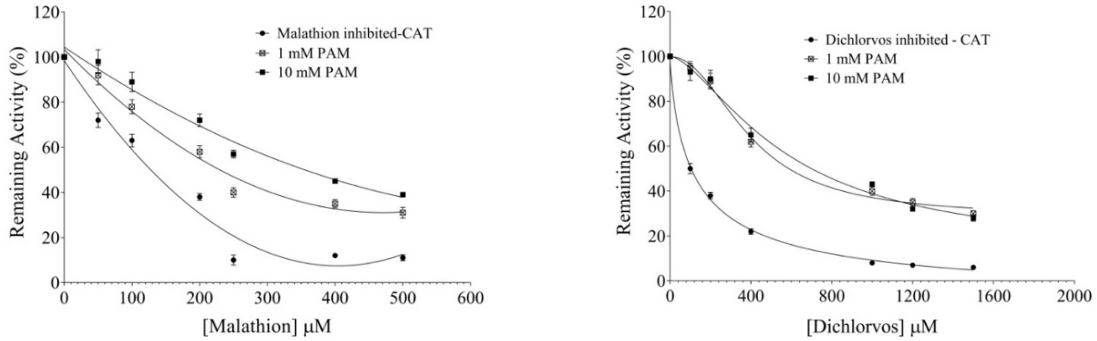
Fig. 3. The activity of glutation peroxidase in the presence of various concentrations of malathion (A) and dichlorvos (B), deltamethrin (C), and λ-cyhalothrin (D). IC_{50} values were determined from these graphs. (n=5)

3.4 Reactivation studies: effect of PAM on the enzymes

The activity of an enzyme in the control remained constant during the experiment. The reactivation percentage of the inhibited enzyme was calculated as the ratio of the recovered enzyme activity and activity in the control. For reactivation assays, only OP inhibited – enzymes were examined (OP pesticides; malathion^{R} and dichlorvos^{R}). Different pesticide concentrations with CAT, SOD, and GPx solutions were prepared and incubated for 15 min, then, two different concentrations of PAM (final concentrations of tested reactivator were 1 and 10 mM) were added to inhibited enzyme solutions and again incubated for 15 min for reactivation. Then, the activities of enzymes were measured. The reactivation percentage of the inhibited enzyme was calculated as the ratio of the recovered enzyme activity and activity in the control.

The results of the reactivation experiment show that only CAT, which is inhibited by organophosphate pesticides, can be reactivated by PAM. GPx and SOD are not reactivated by PAM. Reactivation analysis graph for CAT was drawn using activity % values (Figure 4). As seen in Figure 4, under the exposure of 50, 100, 200, 400, 600 and 800 µM malathion concentrations, CAT activity % was calculated as 72; 63; 38; 10; 12 and 11 %, respectively. After, inhibited CAT incubated with 1 mM PAM, CAT activity % was calculated as 92; 78; 58; 40; 35 and 31 %, respectively. When inhibited CAT incubated with 10 mM PAM, CAT activity % was calculated as 98; 89; 72; 57; 45 and 39 %, respectively.

Under the exposure of 100, 200, 400, 1000, 1200 and 1500 µM dichlorvos concentrations, % CAT activity were calculated as 50; 38; 22; 8; 7 and 6 %, respectively. After, inhibited CAT incubated with 1 mM PAM, CAT activity % was calculated as 95; 89; 62; 40; 35 and 30 %, respectively. When inhibited CAT incubated with 10 mM PAM, CAT activity % was calculated as 93; 90; 65; 43; 32 and 28 %, respectively. When the results are examined, it is seen that increasing the PAM concentration does not significantly affect the reactivation percentage of the CAT enzyme.



(A) **(B)**

Fig. 4. Remaining Activity % vs (A) [Malathion] and (B) [Dichlorvos] reactivation analysis graphs for CAT in the presence of 1 mM and 10 mM PAM

4. Discussion

When the results obtained in the present study were examined, the concentration range of OP pesticides (malathion and dichlorvos) inhibited CAT, SOD, and GPx enzymes were determined

as 50-500 µM and 100-1500 µM, respectively. For PYR pesticides (deltamethrin and λ-cyhaloethrin), this concentration range was determined as 2-40 µM and 250-2250 µM, respectively. No change in enzyme activities was detected at low and high amounts of these pesticide concentrations (Figure 5). It was determined that deltamethrin and λ-cyhaloethrin, which are PYR class pesticides, inhibited CAT activity maximum~80% -~75%, SOD activity ~25% -~40%, GPx activity ~85% -~80%, respectively, in the determined concentration range (Figure 5). When the IC_{50} values given in Table 3 are examined, it could be concluded that deltamethrin is inhibited CAT, SOD, and GPx in very low concentrations in comparison with the other pesticides.

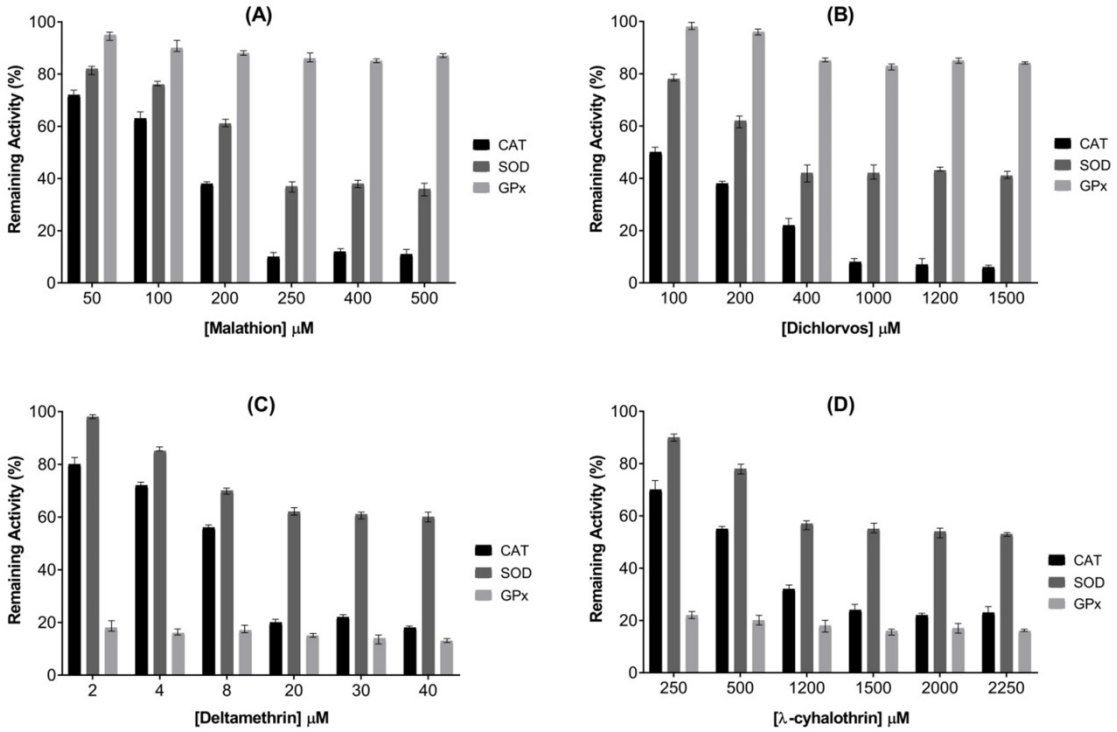


Fig. 5. Effects of malathion (A), dichlorvos (B), deltamethrin (C), λ-cyhalothrin (D) on CAT, SOD, and GPx activities (n=5)

When the literature was examined, it was observed that the inhibition effect of pesticides as environmental pollutants was investigated on some enzymes. Al-Ghanim *et al*. have reported that CAT and SOD were inhibited by fenvalerate (PYR pesticide) *in vivo* (Al-Ghanim *et al* 2020). Gultekin *et al.* have shown the *in vitro* inhibition effect of chlorpyrifos - ethyl (OP pesticide) on the antioxidant enzymes; CAT, GPx, and SOD and they observed that the activity of all three enzymes was decreased significantly at low (0.01-0.1 g/L) and high concentration (0.4-100 g/L) of chlorpyrifos-ethyl (Gultekin *et. al.* 2000). Sadowska-Woda *et al.* reported that a significant reduction in the activities of CAT was observed at all β-cyfluthrin (PYR pesticide) (43-1075 ppm) concentrations, while SOD activities were significantly decreased only in erythrocytes incubated with the highest β-cyfluthrin concentration (Sadowska-Woda *et. al.* 2010). Vasuki *et al.* reported that cypermethrin (PYR pesticide) caused a decrease in CAT

activity and an increase in SOD activity *in vivo* (Vasuki *et. al.* 2016). Kale *et al.* reported that a single dose of cypermethrin (PYR pesticide) (2500 mg/kg body wt) was administered orally to rats and increased oxidative stress resulted in an increase in the activity of antioxidant enzymes such as CAT and SOD *in vivo* (Kale *et. al.* 1999). Also, Nwamba *et al.* reported an *in vivo* study about the effect of pesticides on antioxidant enzymes and it was observed that dichlorvos (OP pesticide) (43 mg/L) increased the activity of SOD and CAT (Nwamba *et. al.* 2018). Decreased SOD, CAT, and GPx activity in *in vitro* studies may be attributed to direct damage to protein structure and increased production of H_2O_2. Similar to the results obtained in the literature studies, it was found that the OP and PYR class pesticides that we used in our study inhibited antioxidant enzymes.

When the literature was examined, it was observed that the number of *in vivo* studies on pesticides was higher than *in vitro* studies but more investigation of *in vitro* systems for pesticides is necessary to verify their applicability to the estimation of pesticide metabolism in live (Katagi 2020). On the other hand, *in vitro* enzyme activity studies with pesticides will lead to biosensor studies that can be designed for pesticide determination (Goçalves *et al.* 2021; Zhai *et al.* 2021; Paluzar *et al.* 2017).

On the other hand, oxime based cholinesterase reactivators such pralidoxime (2-PAM), trimedoxime (TMB-4), obidoxime (LüH-6), asoxime (HI-6), and other agents like anticonvulsants (e.g. benzodiazepine) and anticholinergics (e.g. atropine) have been used as antidotes against OP-intoxication (Worek *et. al.* 2020; Antonijevic *et. al.* 2018; Savall *et. al.* 2020). But, no study has yet been reported on the reactivation effect of PAM on OP inhibited-antioxidant enzymes. Considering the OP inhibition with the mentioned pesticides, the *in vitro* reactivation effect of PAM on OP inhibited-antioxidant enzymes was first time experimentally investigated in this present study. For this, the reactivation effect of PAM was determined for only OP inhibited-enzymes. It was found that only inhibited CAT recovered its activity with PAM, although SOD and GPx were inhibited. Reactivation results of CAT were shown in Figure 4. When the results are examined, it is seen that increasing the PAM concentration does not significantly affect the reactivation percentage of the CAT enzyme. When the results are examined, it is seen that increasing the PAM concentration does not significantly affect the reactivation percentage of CAT enzyme.

5. Conclusion

This work has determined activity changes of CAT, SOD, and GPx during the incubation period to OP and PYR pesticides widely used in agriculture. The results showed that deltamethrin, a PYR class pesticide, was found to be a more potent inhibitor for the antioxidant enzymes followed by the rest of the pesticides used in this study. Additionally, only CAT is reactivated by PAM against OP pesticides, dichlorvos^R, and malathion^R. In this context, the potential doses of pesticides, which can create a risk for live life, have been identified. This study showed that malathion, dichlorvos, deltamethrin, and λ-cyhalothrin pesticides are potent inhibitors for CAT, SOD, and GPx enzymes and PAM can be used as a reactivator for organophosphate inhibited-CAT enzyme. Accordingly, our findings confirmed the essential importance of conscious and

inspected usage of pesticides. Additionally, this *in vitro* enzyme activity study with the pesticides will lead to biosensor studies that can be designed for pesticide determination.

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