J. Entomol. Res. Soc., 13(3): 27-38, 2011 *ISSN:1302-0250*

Effects of Methidathion on Antioxidant Enzyme Activities and Malondialdehyde Level in Midgut Tissues of *Lymantria dispar* (Lepidoptera) larvae

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ABSTRACT

Lymantria dispar larvae are very hazardous to forests and they also cause allergic reactions in humans. In the present study, distinct concentrations of methidathion were applied to *L. dispar* larvae via oak leaves which were prepared by the dipping method. The data obtained was statistically appraised using probit analysis and a $LC_{50/48h}$ value for *L. dispar* larvae found to be 25,480 ppm. Antioxidant defense components protect insects by scavenging reactive oxygen species, leading to oxidative stress. The present study was investigated the effects of $LC_{50/48h}$ value of methidathion**,** on the oxidative stress indicator, malondialdehyde (MDA), and antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] activities in midgut tissues. There were statistically significant increases in the MDA level and SOD, CAT, GPx activities in the $LC_{50/48h}$ concentration of methidathion-treated larvae compared to the control larvae. These results indicated that methidathion which is an organophosphate pesticide, causes an increase in oxidative stress and we infered that increased oxidative stress induces antioxidant defence mechanisms.

Key words: Lymantria dispar, methidathion, LC_{50}, antioxidant enzymes, midgut.

INTRODUCTION

The gypsy moth, *Lymantria dispar* L., is a serious defoliator of deciduous trees, particularly oaks, and becomes an economically important pest during outbreaks (Goertz *et al.*, 2004). Although the caterpillars feed on over 300 species of trees and shrubs, they prefer oaks. These epidemics of the moth dermatitis and urticaria all happened during the spring of months of the years supporting large populations of first larval instars of the moth caterpillars (*L. dispar*) and were caused by the aerosolization of the urticating hairs and hemolymph of the caterpillars with subsequent human skin and mucosal contact (Diaz, 2005). Although there is an increasing demand for environmentally friendly alternative methods such as viruses, parasites, pheromones,

fungi, bacteria, chemical pesticides are still more often used to control this Lepidopteran insect (Rausell *et al.,* 1999; Kalender *et al.*, 2005; Ogutcu *et al.,* 2005).

Methidathion is an organophosphate (OP) pesticide and controls sucking and chewing insects and spider mites on many crops such as corn, fruits, vegetables, cotton, tobacco, sun flowers. (Hayes and Laws 1991). OP pesticides have been applied widely throughout the world for crop protection. In recent years, the development of resistance to pesticides has become an important problem in controlling pest (Cahill *et al.*, 1995; Fragoso *et al.*, 2002; Yang *et al.*, 2009). Therefore, understanding the biochemical aspects of the insect defence mechanisms that occur due to pesticide exposure may help us to develop effective agents for pest management. The well known generally accepted technique of evaluating the toxicity of pesticide is the determination of median lethal concentrations (LC_{50}values) (Shoba *et al*., 2010). Also estimation of LC_{50} is very precious. LC_{50} is indicator to the level of resistance of population response to pesticides (Bakr *et al*., 2010). So in this study we calculated the LC_{50} of methidathion.

Pesticides produce reactive oxygen species (ROS), leading to oxidative stress and alterations in radical scavenging enzymes in insects (Felton and Summers, 1995; Buyukguzel, 2006). ROS include oxygen ions, free radicals and peroxides, both inorganic and organic. These molecules are generally very small and highly reactive, because of the presence of unpaired electrons. ROS are formed as a natural by product of the normal metabolism of oxygen. They play an important role in cell signaling and the induction of host defense genes (Kamata and Hirata, 1999; Dalton *et al*., 1999). Besides, under environmental stress, ultraviolet irridation, bacterial infections, antibiotics and pesticides exposure, the ROS level may increase remarkably and result in oxidative stress in insects and human erythrocytes (Lopez-martinez *et al*., 2008; Buyukguzel and Kalender, 2009; Durak *et al*., 2009). To neutralize the toxicity of ROS, insects have developed a suite of antioxidant enzymes like other eukaryotes to overcome oxidative stress. Several antioxidant enzymes may decrease the level of lipid peroxidation in insects (Felton and Summers, 1995). In animals, including insects, various important components of the antioxidant system are identified. They are divided into enzymatic antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx) and non-enzymatic antioxidants-phenol containing compounds such as, vitamin E, vitamin C and molecular thiols (Dubovskii *et al*., 2005). GPx metabolizes H_2O_2 and detrimental lipid peroxides using reduced glutathione as a substrate (Ahmad *et al*., 1989). SOD catalyses the conversion of the superoxide radicals to H_2O_2 and oxygen and appears to be the main response to dietary pro-oxidant exposure (Ahmad and Pardini, 1990). CAT catalyze the degradation of H_2O_2 to water and oxygen (Ahmad *et al*., 1991).

A substantial amount of researchs has been done on antioxidant enzyme systems in insects (Yamamoto *et al*., 2011; Buyukguzel E., 2009), however, there is a paucity of reports exploring the oxidative stress to which gut tissues are subjected to and the relative antioxidant enzyme activities when insects feed on plants The value of an antioxidant enzyme system depends upon its location relative to where the oxygen radicals are generated. Thus it is in the herbivorous insects gut lumen and not tissues

that ingested allochemicals first encounter oxidizing or reducing conditions (Krishnan and Kodrik, 2006).

In the present study was investigated the hypothesis that methidathion-treatment (LC_{50} value) to *L. dispar* produces oxidative stress in the larval midgut tissue. This stress leads to paralyzed anti-oxidative defense systems. In this paper we presented on the outcomes of experiments intended to our hypothesis.

MATERIAL AND METHODS

Insects

Larvae of *Lymantria dispar* (Linne, 1758) (Lepidoptera: Lymantridae) were collected from Ankara-Kızılcahamam-Kargasekmez, Turkey. In the laboratory, the larvae were fed with *Quercus pubescens* leaves. Larvae were individually reared in the laboratory at 26°C±1 and relative humidity of 60-70 % under 12/12 light:dark photoperiod.

Chemical

Methidathion [*O*,*O*-dimethyl-*S*-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-yl-methyl)-phosphorodithioate], technical purity 95%, was obtained from Ministry of Agriculture and Rural Affairs Agricultural Struggle and Research Institute.

Toxicity Tests

Tests were done on fourth instar larvae (L_4) of *L. dispar*. Larvae were starved 2 days before the beginning of the experiments. Larvae were divided into control and test groups. Each group consisted of 20 larvae. Methidathion was diluted in distilled water in nine concentrations 12, 16, 20, 24, 28, 32, 36, 40 and 44 ppm. Fresh *Quercus pubescens* leaves were dipped in each concentration for approx. 30 seconds. Next, they were air dried for approx. 1 h and leaves were placed in plastic petri dishes. A single larva was put into each of the plastic dishes and their feeding was controlled. The mortality of larvae was determined after 48 h and the $LC_{50/48h}$ value was calculated. In the control group only distilled water was used.

Data Analysis

48 h after feeding, the total number of dead larvae were counted. The effect of methidathion on *L. dispar* was calculated using the probit analysis LC_{50} (lethal concentration) determination method (Finney, 1971). The LC_{50} software program, version 1.5 computer program was developed by EPA (US EPA, 1999).

Preparation of homogenates and determination of enzymatic activities and the levels of MDA

Tissue collection

For measurement of malondialdehyde levels and antioxidant enzyme activities a separate test was arranged by application of the $LC_{50/48h}$ value of methidathion. After 48 h, midguts from alive larvae were dissected.

Fourth-instars were used to determine MDA levels and antioxidant enzyme activities. Adhering fat body, Malpighian tubules and gut contents were then removed. 6 midguts were collected into a chilled Eppendorf tube charged with a cold homogenization buffer [w/v 1.15% KCl, 25 mM K_2HPO_4, 5 mM ethylen-diaminetetraacetic acid (EDTA), 2 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM dithiotreitol (DTT), pH 7.4] and stored at -80 °C. The cryotubes were kept at room temperature until the tissue began to thaw before using.

Sample Preparation

Extracts of midgut were prepared at 4 °C by a homogenizer (HEIDOLPH SilentCrusher M) at 10 seconds in the homogenization buffer and subsequent centrifugation (Minispin Plus Eppendorf) at 10,000g for 15 min at 4 °C. The resulting cell-free extracts were collected for biochemical analysis. Supernatants were centrifuged at either 1000g for 10 min at 4 °C (SOD and CAT assays), 16,000g for 20 min (GPx) or 2000g for 15 min (lipid peroxidation). MDA contents and antioxidant enzymes activities were determined by measuring the absorbance of the samples in a dual beam spectrophotometer (Shimadzu-1700, UV/vis, Kyoto, Japan). Essays were replicated six times with six midguts each. Protein concentrations were determined according to Lowry *et al.,* (1951) by using bovine serum albumin (BSA) as a standard. All chemicals used were analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Measurement of MDA levels

MDA is secondary product of lipid peroxidation (LPO). Midgut tissues were incubated at 95 °C with thiobarbituric acid under aerobic conditions (pH 3.4). The pink colour produced by these reactions was measured spectrophotometrically at 532 nm to measure MDA levels (Ohkawa *et al.*, 1979). MDA levels were defined as nanomole per gram tissue.

Measurement of SOD Activity

The total SOD (EC 1.15.1.1) activity was determined according to Marklund and Marklund, (1974) assaying the autooxidation and illumination of pyrogallol at 440 nm for 3 min. One unit total SOD activity was calculated as the amount of protein causing 50% inhibition of pyrogallol autooxidation. The total SOD activity was expressed as units per milligram of protein (U mg^{-1}). A blank without homogenate was used as a control for non-enzymatic oxidation of pyrogallol in Tris-EDTA buffer (50 mM Tris, 10 mM EDTA, pH 8.2).

Measurement of CAT Activity

Before the determination of CAT (EC 1.11.1.6) activity, samples were diluted with 1:9 with 1% v/v Triton X-100. The enzyme activity was measured according to Aebi (1984) assaying the hydrolysis of H_2O_2 and decreasing absorbance at 240 nm over a 3 min period at 25 °C. The CAT activity was expressed as millimoles of H_2O_2 reduced per minute per milligram of protein, using an extinction coefficient of 0.0394 mM^{-1} cm^{-1}.

A blank without homogenate was used as a control for non-enzymatic hydrolysis of peroxide in phosphate buffer (50 mM, pH 7.0).

Measurement of GPx Activity

The GPx (EC 1.11.1.9) activity was measured with H_2O_2 as substrate according to Paglia and Valentine (1987). This reaction was monitored indirectly as the oxidation rate of NADPH at 340 nm for 3 min. Enzyme activity was expressed as nmol of NADPH consumed per minute per milligram of protein, using an extinction coefficient of 6.220 $M^{-1}cm^{-1}$. A blank without homogenate was used as a control for the non-enzymatic oxidation of NADPH upon addition of hydrogen peroxide in Tris buffer (0.1 M, pH 8.0).

Statistical analysis

Data were analyzed using SPSS 11.0 for windows. Significance was calculated using Mann Whitney U test. $P < 0.05$ was considered statistically significant.

RESULTS

Toxicity Assay

In the present study, nine distinct concentrations of methidathion were administrated to *L. dispar* larvae. The mortality rates of larvae were calculated as a percentage after 48 h of methidathion treatment. The dose-response graph plotting in relation between the mortality rate and methidathion concentrations is given using a linear scale (Fig. 1). The mortality of *L. dispar* larvae increased depending on the dose of methidathion.

The data were obtained from the toxicity tests was evaluated using the Probit Analysis Method. The $LC_{50/48h}$ value for the *L. dispar* larvae was found to be 25,480 ppm. %95 confidence limits were between 23,401-27,519 ppm (Table 1).

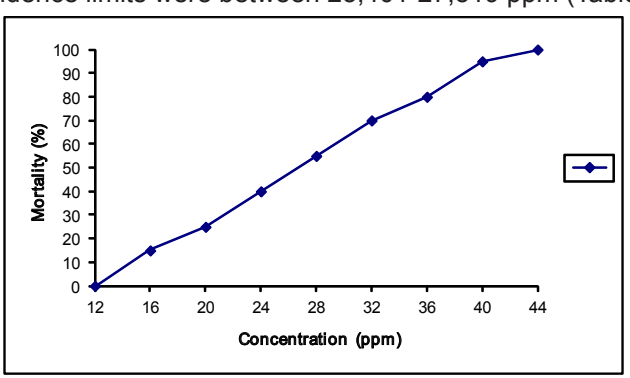

Fig.1. The relationship between methidathion concentration and the mortality rate of *L. dispar* larvae throught the 48h period.

Malondialdehyde Level

MDA level is an indicator of lipid peroxidation. MDA level in midgut tissue of the control *L. dispar* larvae was found to be 2,61±0,29 nmol/g tissue. There was

statistically significant increase in the malondialdehyde level in midgut tissue of methidathion-treated *L. dispar* larvae in comparison with the control larvae (Fig. 2).

Table 1. 48 h toxicity results of the methidathion bioassay on *L. dispar* larvae.

Points of Lethal Concentration	Concentration (ppm)	% 95 Confidences limits (ppm)	Intercept±SE**	Slope±SE
*LC 1.00	11,631	8,677-13,951	-4,604±1,245	6,830±0,865
LC 5.00	14,634	11,723-16,848		
LC 10.00	16,541	13,743-18,658		
LC 15.00	17,966	15,285-20,009		
LC 50.00	**25,480**	**23,401-27,519**		
LC 85.00	36,135	32,990-41,104		
LC 90.00	39,249	35,451-45,619		
LC 95.00	44,363	39,336-53,376		
LC 99.00	55,818	47,589-71,987		

Note: Each experimental group consisted of 20 larvae. * LC: Lethal concentration, **SE:Standard error.

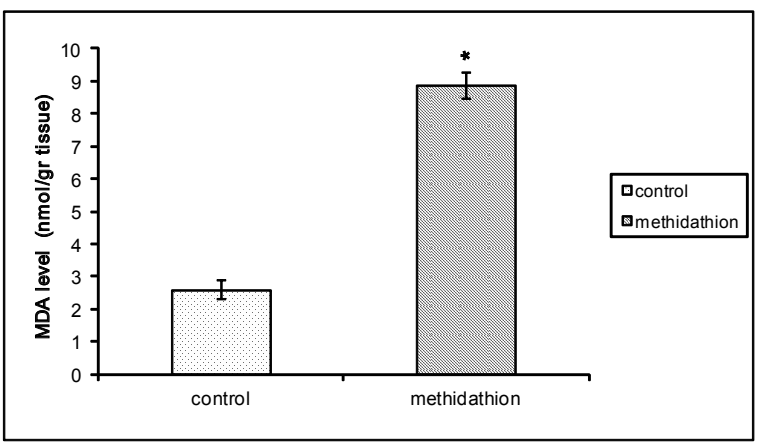

Fig. 2. MDA levels in midgut tissues of control and methidathion-treated *L. dispar* larvae.

*Comparison of control larvae with methidathion-treated larvae ($P < 0.05$). Bars represent the means ±SD of six replicates with 6 midguts per replicate.

Antioxidant Enzyme Activities

SOD, CAT and GPx activities were determined to be 0,59±0,05 U/mg protein, 283±11,6 mmol/mg protein and 0,54±0,05 mmol/mg protein in midgut tissue of control *L. dispar* larvae, respectively (Figures 3-5).

There were statistically significant increases in the SOD, CAT and GPx activities in the $LC_{50/48h}$ concentration of methidathion treated larvae compared with the control larvae (Figs. 3-5).

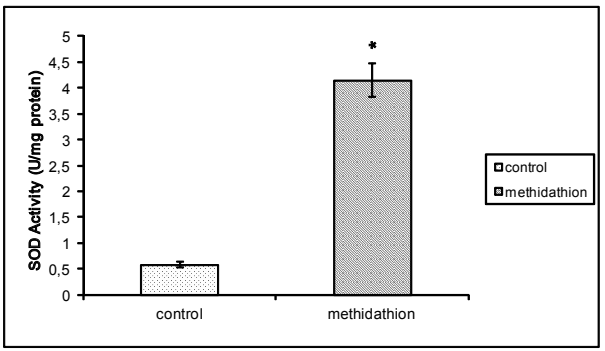

Fig. 3. SOD activities in midgut tissues of control and methidathion-treated *L. dispar* larvae.

*Comparison of control larvae with methidathion-treated larvae ($P < 0.05$). Bars represent the means ±SD of six replicates with 6 midguts per replicate.

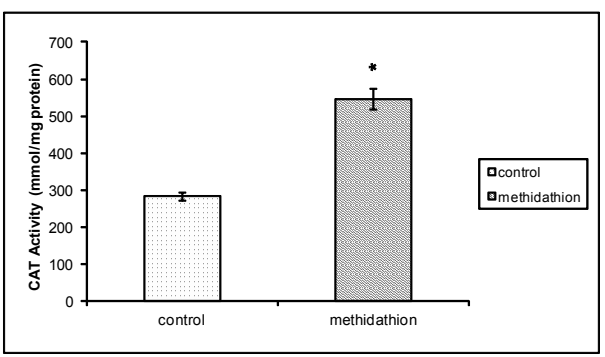

Fig. 4. CAT activities in midgut tissues of control and methidathion-treated *L. dispar* larvae. *Comparison of control larvae with methidathion-treated larvae ($P < 0.05$). Bars represent the means ±SD of six replicates with 6 midguts per replicate.

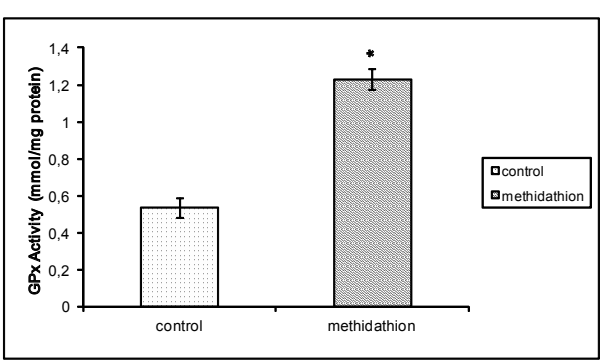

Fig.5. GPx activities in midgut tissues of control and methidathion-treated *L. dispar* larvae. *Comparison of control larvae with methidathion-treated larvae ($P < 0.05$). Bars represent the means ±SD of six replicates with 6 midguts per replicate.

DISCUSSION AND CONCLUSIONS

The caterpillars of *Lymantria dispar* (L.), are very harmful in deciduous forests, urban environments, orchards, and parks (Liebhold *et al*., 2000). In humans, the hairs of most lymantriid caterpillars may cause strong urticarial dermatitis and conjunctivitis on contact, or wheezing and bronchospasm if inhaled, especially in patients with histories of asthma or atopic allergies (Diaz, 2005). For this reason this larvae should be controlled.

Methidathion is one of the most widely used OP pesticides in pest control (Altuntas *et al*., 2002). A variety of methods are used in pest control, the most effective and most widely used method is chemical insecticides. Unconsciously used these chemical insecticides cause deterioration of ecological balance, these insecticides also constitute a serious threat for non- target organisms. It is observed that pesticides caused pathological changes in mammalian tissues (Kalender S. *et al*., 2004, 2005, 2007; Kalender *et al*., 2006; Uzunhisarcikli *et al*., 2007; Ogutcu *et al*., 2006, 2008; Uzun *et al*., 2009). For this reason, determination of LC_{50} values of chemical insecticides is very important as scientific for target organisms. In this study, the $LC_{50/48h}$ value of methidathion for *L. dispar* larvae was found to be 25.480 ppm in laboratory conditions.

Some studies have also shown that oxidative stress could be an important component of the mechanism of toxicity of OP insecticides. OP insecticides may induce oxidative stress leading to a generation of free radicals and alterations in antioxidants or reactive oxygen species (ROS)-scavenging enzymes in vivo and in vitro (Bagchi *et al*., 1995; Gultekin *et al.,* 2000). It was reported that pesticides effected on malondialdehyde level and antioxidant enzyme activities in gut tissue of insects (Dubovskii *et al*., 2005; Dubovskiy *et al*., 2008). In this study a change in SOD, CAT, GPx activities and MDA level was found in midgut tissue after application of the LC_{50} value of methidathion. This suggested that methidathion caused oxidative damage in *L. dispar*, possibly by producing ROS in midgut tissue. Other studies reported that OP pesticides caused lipid peroxidation and the alterations in the antioxidant defence enzymes of insect (Gupta *et al*., 2010; Wu *et al.*, 2011).

MDA, a major oxidation product of peroxidized polyunsaturated fatty acids, has been used to determine the degree of lipid peroxidation and as a biological marker of oxidative stress (Rael *et al*.,2004; Del rio *et al*., 2005). It has been shown previously that pesticides increase MDA level in human erythrocytes and insects (Durak *et al*., 2009; Buyukguzel, 2009). In our study, MDA level was increased in midgut tissues of $LC_{50/48h}$ value of methidathion-treated *L .dispar* larvae, which suggest that MDA levels could be used as a marker of methidathion injury.

Under physiological conditions, intracellular antioxidant enzymes, such as SOD, CAT, and GPx, eliminate ROS, thereby playing an integral role in the oxidative stress defenses of the cell (Bukowska, 2004).

SOD plays an important role as an antioxidant enzyme by reducing high level of intracellular SOD activity suggested that methidathion induces the superoxide radical in midgut tissue of *L. dispar* larvae. One study concerning SOD activity in the midgut

L. dispar larvae fed on unfavorable plants has shown similar results (Peric-mataruga *et al*., 1997). SOD activity significantly increased when the insects were exposure to methidathion, suggesting that SOD was stimulated by scavenging superoxide radical to protect the larvae from methidathion stress. It has been reported that an increase in SOD activity is probably a response towards increased ROS generation in rat erythrocytes (John *et al*., 2001). In present study, CAT activity significantly increased in response to methidathion-induced oxidative stress in midgut tissue of *L. dispar* larvae. CAT is perfectly suited for reducing the high amount of H_2O_2 and regulated by the concentration of H_2O_2 (Fridovich, 1978). We infer that increased SOD activity would result in an increased H_2O_2 concentration and consequently a further increase in CAT activity. Previous studies have shown that CAT can protect against oxidative stress and extend the lifespan of insects (Orr and Sohal 1994). GPx catalyzes the glutathione-dependent reduction of lipid hydroperoxides and hydrogen peroxide for detoxification. In this study, GPx activity significantly increased in midgut tissue of *L. dispar* larvae.

In present study, we observed that the $LC_{50/48h}$ value of methidathion caused lipid peroxidation and increasing of antioxidant enzyme activity. As a result of this study, we could say that chemical insecticides effect the antioxidant defense system of insects when chemical insecticides are received orally.

ACKNOWLEDGEMENTS

The authors thank the TUBITAK for financial provided through the research project TBAG-106T278.

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Received: March 15, 2011 Accepted: September 07, 2011