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Araştırma Makalesi

Zebularin'in Galleria mellonella (Lepidoptera: Pyralidae) Antioksidan Enzimleri ve Lipit Peroksidasyonu Üzerindeki Etkisi

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ÖZ

Zebularin (ZEB), kanser ve kist hücrelerinde hipermetile edilmiş genleri kanserli hücrelerde seçici bir etki gösteren DNA metiltransferazlarla yeniden aktive eder. Bu etkisi ile ZEB DNA metilasyon inhibitörü olarak yaygın olarak tercih edilir. Memeli modellerle olan fizyolojik benzerlikleri *Galleria mellonella* larvalarının bağışıklık çalışmalarında yaygın olarak tercih edilmesine yol açmaktadır. Çalışmamızda *G. mellonella* son evre larvalarına değişik konsantrasyonlarda (0.25-32 mg ml^{-1}) verilen ZEB'in antioksidan enzim aktivitesine ve lipit peroksidasyonuna etkileri analiz edildi. ZEB enjeksiyonu, sonrasında *G. mellonella* larvalarında katalaz (CAT) ve glutatyon S-transferaz (GST) aktivitelerinde kontrol gurubuna oranla bir değişiklik belirlenmedi. Bununla birlikte süperoksit dismutaz (SOD) aktivitesinde 1 ppm ve üstü dozlarda enzim aktivitesinde azalmaya neden olduğu görüldü. Lipid peroksidasyonu sonucu oluşan malondialdehit (MDA) miktarında 16 ppm ve üstü dozda artış belirlendi. Bu sonuçlar için iki sebep öngörülebilir: enzim aktivitelerindeki değişim yüksek oksidatif stress kaynaklı olması veya ZEB uygulanmasının DNA metilasyonunu inhibe ederken enzim aktivitesi üzerindeki genetik kontrolü azaltması. Sonuçlarımıza göre ZEB uygulanması *G. mellonella* larvalarında antioksidan savunmayı zayıflatmaktadır. Buna göre ZEB Birleşik Zararlı Organizma Denetimi programları için uygun bir aday gibi gözükmektedir..

Anahtar kelimeler: Antioksidan enzimler, DNA metilasyonu, Galleria mellonella, lipid peroksidasyonu, zebularin.

Influence of Zebularin on Antioxidant Enzymes and Lipid Peroxidation of *Galleria mellonella* (Lepidoptera: Pyralidae)

ABSTRACT

Zebularin (ZEB) reactivates hypermethylated genes in cancer and cyst cells with DNA methyltransferases that demonstrate a selective effect in cancer cells. With this influence, ZEB is commonly preferred as a DNA methylation inhibitor. Its physiological similarities with mammalian models have led to its widespread use in immunological studies of *Galleria mellonella* larvae. In the present study, the influence of different concentrations (0.25-32 mg ml^{-1}) of ZEB on antioxidant enzyme activity and lipid peroxidation in *G. mellonella* last instar larvae were analyzed. There was no alteration in catalase (CAT) and glutathione S-transferase (GST) activities in *G. mellonella* larvae after ZEB injection compared to the control group. However, it was observed that superoxide dioxidase (SOD) activity declined at doses of 1 ppm and above. The amount of malondialdehyde (MDA) which occurs as a result of lipid peroxidation increased at doses of 16 ppm and above. Two explanations for these results can be predicted: either the change in enzyme activities is due to high oxidative stress or ZEB treatment inhibits DNA methylation and reduces genetic control over enzyme activity. According to our results, ZEB treatment weakens antioxidant defense in *G. mellonella* larvae. Accordingly, ZEB seems to be a suitable candidate for Integrated Pest Management programs.

Key words: Antioxidant enzymes, DNA methylation, Galleria mellonella, lipid peroxidation, zebularine.

INTRODUCTION

The immunity of *Galleria mellonella* L. (Lepidoptera: Pyralidae) instars share a high degree of functional and structural homology to both cellular and humoral defenses of vertebrates and furthermore mammalians (Kavanagh and Reeves, 2004; Altincicek et al, 2007; Vogel et al, 2011). Due to this similarity and other biological properties that facilitate availability in laboratory conditions, using of *G. mellonella* as a model organism is becoming trend for studying human pathogen infection disease (Aperis et al, 2007; Seed and Dennis, 2008; Büyükgüzel et al, 2010; Uçkan et al, 2011; Mukherjee et al, 2010; Altuntaş et al, 2015). Researchers are still in progress using *G. mellonella* as an "intermediate model organism" before mammalian models especially in the production of new antimicrobial drugs. Besides that, for the measurement of the physiological state of insect internal environment, large hemolymph sample densities can be acquired from *G. mellonella* larvae. At this stage, all factors affecting the level of immune response of *G. mellonella* can easily be evaluated. One of these factors is the DNA methylation which is among the most important epigenetic modifications.

The process of DNA methylation in plant and animal systems has been widely researched but is relatively new in insects (Suzuki and Bird, 2008). DNA methylation in insects was first discovered in the mole cricket *Gryllotalpa fossor* (Scudder) (Orthoptera) about 27 years ago and has been shown quite changes among species (Sarkar et al, 1992). In mammals, there is an inverse relationship between DNA methylation and gene transcription (Jones and Baylin, 2002). The transformation of cytidine residues to 5-methylcytidine provides a mechanism for gene regulation by affecting the ability of DNA to interact with proteins (Özbek et al., 2020). However, the limited number of studies carried out in insect has shown the opposite, but they are still insufficient. There are many studies examining the effects of various substances on the antioxidant enzymes of the invertebrate model organism *G. mellonella*. In a study examining the effects of azadirachtin on the antioxidant enzymes of *G. mellonella*, it was found that it dose-dependently increased oxidative stress in this species (Dere et al. 2015). It was determined that titanium dioxide nanoparticules included in the diet had a dose-dependent toxic effect and increased resistance to oxidative stress at low concentrations (Zorlu et al. 2018). They reported that the antioxidant enzyme activity of *G. mellonella* was not affected by increasing concentrations of boric acid (Hyršl et al., 2007).

Because the conservation of CpG is very important in the evolutionary process of animals, its existence and methylation pattern are also of great importance to determine in *G. mellonella*. Therefore, previously researchers showed the existence of DNA methylation and its pattern in *G. mellonella* by using HpaII, MspI and ScrFI methylation-sensitive restriction endonucleases (Özyılmaz et al, 2019). Within the scope of our study, we aimed to define the effect of zebularine (ZEB) induced hypomethylation on the activity of antioxidant enzymes. For this purpose, we assayed whether ZEB, which is an inhibitory agent of DNA methylation and commonly used as a tumor suppressive in cancer treatments (Cheng et al, 2003) has any effects on malondialdehyde (MDA) density and activities of antioxidant enzymes, which can be affected by stress (Keloğlan et al, 2021); catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST) in the *G. mellonella* last instars hemolymph. The information provided here in our research will contribute to the development of *G. mellonella* as a "model organism" and will add upon the knowledge for a better understanding of the function of DNA methylation in insects. The acquired data will contribute to the evaluation of DNA methylation and epigenetic regulation in terms of evolutionary aspects upon comparing with that of vertebrates.

MATERIALS AND METHODS

Host cultures

G. mellonella laboratory colonies were reared at 25 ± 2°C, 60 ± 5% RH, and a photoperiod of 12:12 (L: D) h in Kocaeli University, Kocaeli, Turkey (Uçkan et al. 2015). First *G. mellonella* instars were maintained by breeding the insects on artificial diet (Sak et al, 2006).

Bioassays

ZEB, obtained from Sigma Aldrich – Merck, was dissolved in distilled water to prepare different concentrations of the solution (0.25, 1, 4, 8, 16, 32 mg ml^{-1}). A 10-µl solution of each ZEB concentration and distilled water (DW) as a control group were injected on the first hind right or left leg of last instars (Kaethner, 1992) by using a 10-µl Hamilton micro syringe (Hamilton Co., Reno, NV).

Hemolymph collection and storage

For purpose of collecting 10 µl hemolymph from larvae exposed to various doses of ZEB or distilled water at 24 h post treatments, thirty larvae were used in each analysis. For anesthesia, each larva was kept on ice for five minutes. After that, for sterilization of samples a cotton ball containing seventy percent ethyl

alcohol is used, afterwards hemolymph samples were gathered via glass microcapillary tube (Sigma, St. Louis, MO) into micro centrifuge tubes (2 ml) containing 0.001 mg 1-phenyl-2-thiourea (Sigma). All samples were stored at -20°C until used. To perform all analyses, the samples were first transferred in cold homogenization buffer (1:2 v v^{-1}) and then centrifuged at 7000 rpm for 10 min at +4 ˚C and the supernatant transferred to a new collection tube and keep on ice (Özyılmaz et al, 2019). All assays were repeated three times with 10 larvae in each replicate (n=30).

Assays of MDA level and antioxidant enzyme activities

Protein concentration of hemolymph samples were determined by using Bradford reagent (Sigma) according to 96 well plate method, and bovine serum albumin was used to create standard curve. SOD (706002), GST (703302) and MDA (700870) activities were evaluated by using commercial kits from CAYMAN (Cayman Chemical, Ann Arbor, MI).

CAT activity analysis was performed according to Chance and Maehly (1955). The reduction in absorbance over a 10 min period at 240 nm because of H2O2 decomposition was measured in this assay. Absorbance of CAT activity was read in microplate reader (BMG Labtech) and thus, the activity was defined as mmol ml^{-1} min^{-1} mg^{-1} per protein.

For SOD assay, 2-[4-iodophenyl]-3-[4-nitrophenol]-5-phenyltetrazolium chloride (INT) was reacted with superoxide radicals, using xanthine oxidase (XOD) and xanthine (Cayman Chemical, 706002) at 450 nm. SOD total activity per unit was measured by the amount of enzyme needed to cause fifty percent inhibition of the superoxide radicals in one mg protein. The activity was defined as U ml^{-1} mcg^{-1} per protein.

Total GST activity assay in larval hemolymph was performed by using kit protocol (Cayman Chemical, 703302) by following the principle of 1-chloro-2,4-dinitrobenzene (CDNB), a substrate, and GSH (glutathione) conjugate formation. The increment in absorbance activity was monitored at 340 nm for five minutes with a microplate reader and specific activity was described as conjugated 1.0 nmol CDNB with decreased GSH per minute per mg protein (µmol min^{-1} mg^{-1} per protein) at 25°C.

MDA (product of lipid peroxidation) amount in hemolymph samples were also determined using commercial available kit protocol (Cayman Chemical, Ann Arbor, MI). According to the protocol, MDA in hemolymph samples was incubated with thiobarbituric acid (TBA) at 95°C and thus absorbance was read at 530 nm in a microplate reader. The content of MDA was determined as the µM mg^{-1} per protein.

Statistics

All data was checked for normal distribution and represented as mean ± standard error (SE). ANOVA tests were performed. To describe significant differences between means, Tukey's Honestly Significant Difference (HSD) and Tamhane post hoc tests were used. Means were considered statistically significant when $P < 0.05$.

RESULTS

Effects to antioxidant enzyme activities

CAT activity in control was 0.032 mmol ml^{-1} min^{-1} mg^{-1} protein at 24 post treatments, however exposure to ZEB did not cause a significant change in the activity of CAT (F= 1.636; df= 6, 14; P=0.21, Tukey's HSD Test) although in particular an efficient reduction was observed at the highest dose of 32 ppm as 0.019 mmol ml^{-1} min^{-1} mg^{-1} (Table 1) (Fig.1).

SOD activity in DW was 7.635 U ml^{-1} mcg^{-1} protein at 24 h. Application with ZEB drastically resulted in nearly a more than two-fold decrease in the activity of SOD at 1.00 ppm at 24 h. Significant decreases were also evident at doses > 1.00 ppm (F= 8.098; df= 6, 14; P=0.001, Tamhane Test). Similarly, nearly a seven-fold reduction in SOD activity in larval hemolymph at 8, 16 and 32 ppm ZEB exposure was recorded at 24 h. However, these decreases among treatments in all ZEB doses were not significant (Table 1) (Fig. 2).

GST activity in DW group was 0.372 µmol min^{-1} mg^{-1} protein in larval hemolymph at 24 h. GST activities in hemolymph of larvae did not change in tested doses of ZEB except for that of at 8 ppm (F= 2.876; df= 6, 14; P=0.048, Tamhane Test) (Table 1) (Fig. 3).

Effects on MDA level

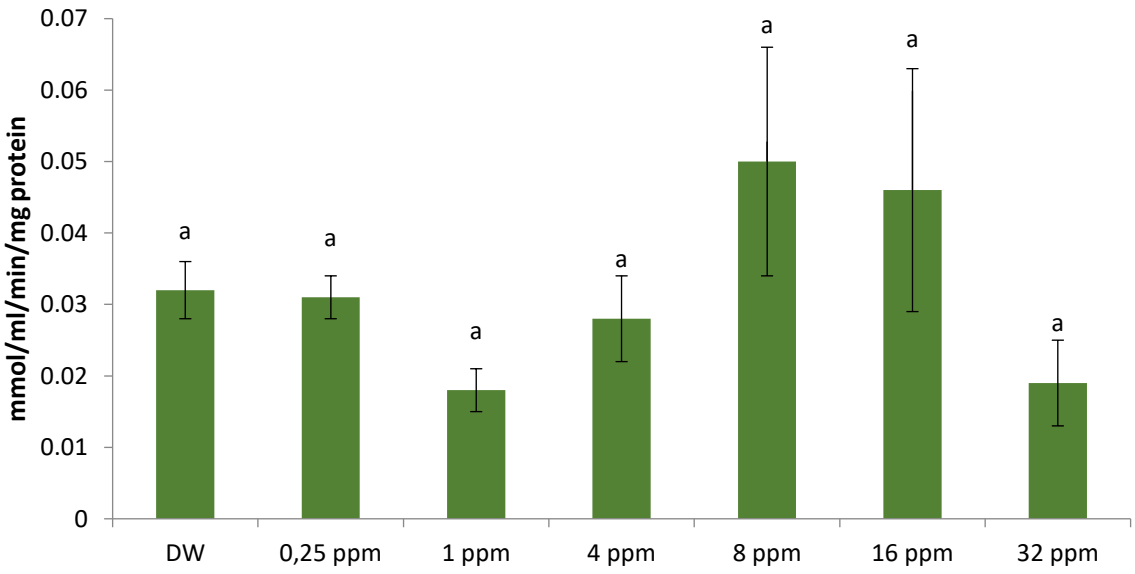
Larval hemolymph MDA levels of the ZEB treated larvae differed depending on dose (F= 38.92; df= 6, 14; P=0.00) (Table 1). MDA level of controls was 23.834 µM mg^{-1} per protein at 24 h post treatment. ZEB showed the greatest increase on MDA level with more than ten- and twenty-fold increase at doses > 8 ppm at 24 h.

However, exposure to ZEB did not significantly change the larval hemolymph MDA level at the other doses ($p > 0.05$) (Fig.4).

Table 1. Changes in antioxidant enzyme activities and malondialdehyde amount in the *G. mellonella* larval hemolymph exposed to Zebularine.

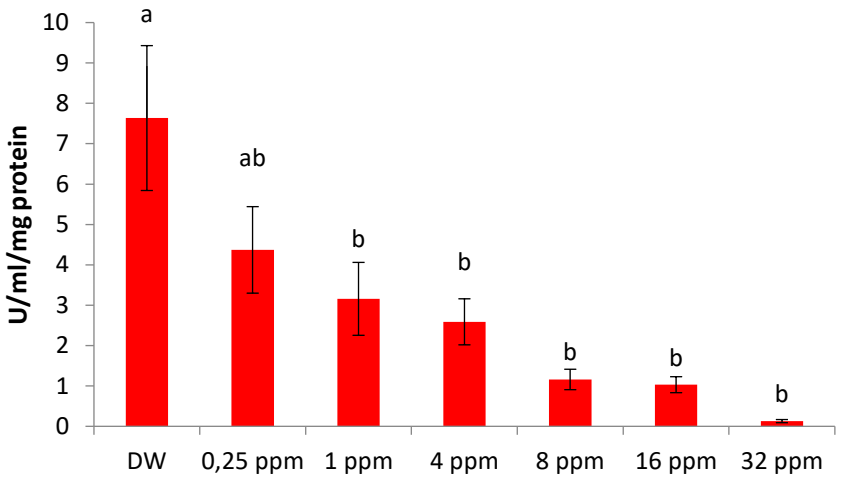
Zebularine Dose (ppm)	CAT (± SE**)* mmol ml^{-1} min^{-1} mg^{-1} protein	SOD (± SE**)* U ml^{-1} mcg^{-1} protein	GST (± SE**)* µmol min^{-1} mg^{-1} protein	MDA (± SE**)* µM mg^{-1} protein
DW	0,032 ± 0.044[a]	7.635±1.794[a]	0.372± 0.014 [ab]	23.834±5.548[a]
0.25	0.031± 0.003[a]	4.370±1.071[ab]	0.266 ±0.052 [ab]	75.653±7.748[a]
1.00	0.018 ±0.003[a]	3.157±0.903[b]	0.361± 0.016 [ab]	36.352±1.511[a]
4.00	0.028 ±0.006[a]	2.588±0.570[b]	0.156± 0.032 [a]	68.253±9.265[a]
8.00	0.050 ±0.016[a]	1.161±0.253[b]	0.391± 0.081[b]	143.014±19.794[a]
16.00	0.046 ±0.017[a]	1.031±0.198[b]	0.302± 0.036[ab]	279.124±59.691[b]
32.00	0.019± 0.006[a]	0.129± 0.039[b]	0.313± 0.060[ab]	452.099±18.170[c]
Sigma	0.210	0.001	0.048	0.000
F	1.636	8.098	2.876	38.920
df1, df2	6.140	6.140	6.140	6.140
SPSS	ANOVA-Tukey HSD	ANOVA-Tamhane	ANOVA-Tamhane	ANOVA-Tukey HSD

*All assays were designed with a 30 larvae for each dose.
**Mean ± SE (standard error) and significant differences between groups considered as $p < 0.05$.



*All assays were designed with a 30 larvae for each dose.
**Mean ± SE (standard error) and significant differences among groups considered as $p < 0.05$.
Figure 1. Changes in the catalase activities in the *G. mellonella* larval hemolymph exposed to Zebularine.

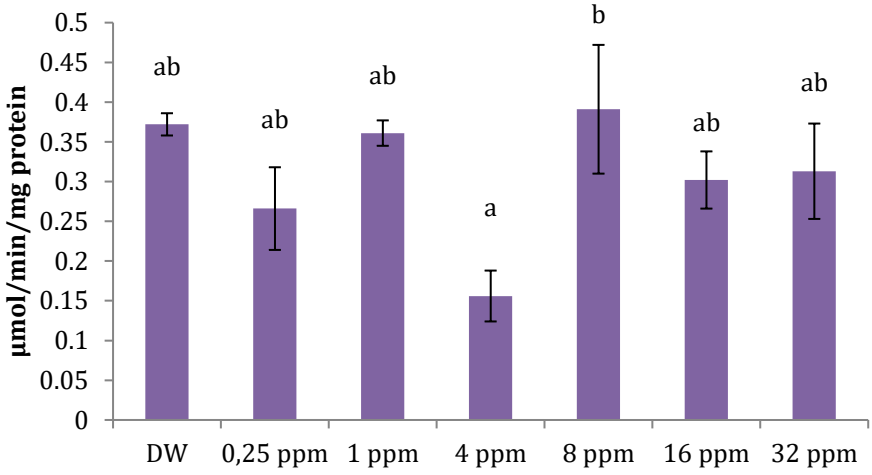
SOD activity in DW was 7.635 U ml^{-1} mcg^{-1} protein at 24 h. Application with ZEB drastically resulted in nearly a more than two-fold decrease in the activity of SOD at 1.00 ppm at 24 h. Significant decreases were also evident at doses > 1.00 ppm (F= 8.098; df= 6, 14; P=0.001, Tamhane Test). Similarly, nearly a seven-fold reduction in SOD activity in larval hemolymph at 8, 16 and 32 ppm ZEB exposure was recorded at 24 h. However, these decreases among treatments in all ZEB doses were not significant (Table 1) (Fig. 2).



*All assays were designed with a 30 larvae for each dose.
**Mean ± SE (standard error) and significant differences among groups considered as p < 0.05.

Figure 2. Changes in the superoxide dismutase activities in the *G. mellonella* larval hemolymph exposed to Zebularine.

GST activity in DW group was 0.372 µmol min^{-1} mg^{-1} protein in larval hemolymph at 24 h. GST activities in hemolymph of larvae did not change in tested doses of ZEB except for that of at 8 ppm (F= 2.876; df= 6, 14; P=0.048, Tamhane Test) (Table 1) (Fig. 3).



*All assays were designed with a 30 larvae for each dose.
**Mean ± SE (standard error) and significant differences among groups considered as p < 0.05.

Figure 3. Changes in the glutathione-S-transferase activities in the *G. mellonella* larval hemolymph exposed to Zebularine.

Effects on MDA level

Larval hemolymph MDA levels of the ZEB treated larvae differed depending on dose (F= 38.92; df= 6, 14; P=0.00) (Table 1). MDA level of controls was 23.834 µM mg^{-1} per protein at 24 h post treatment. ZEB showed the greatest increase on MDA level with more than ten- and twenty-fold increase at doses > 8 ppm at 24 h.

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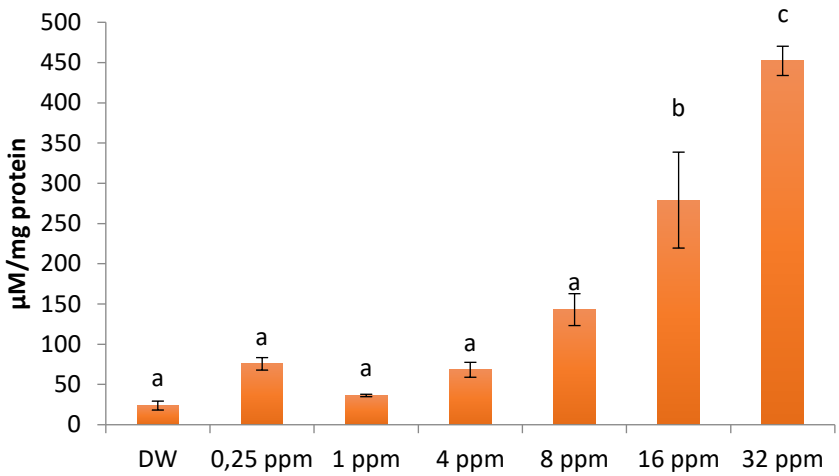

*All assays were designed with a 30 larvae for each dose.
**Mean ± SE (standard errors) and significant differences among groups considered as $p < 0.05$.
Figure 4. Changes in the malondialdehyde levels in the *G. mellonella* larval hemolymph exposed to Zebularine.

DISCUSSION

Reactive oxygen species (ROS), which is a natural side product of oxygen metabolism, play a crucial role in cell signaling and activation of the genes involved in immunity (Kamata and Hirata, 1999; Dalton et al, 1999). Under normal circumstances, there appears a balance in the production and degradation of ROS. Dubovskii et al (2005) reported an increase in both ROS production and antioxidant defense in *G. mellonella* exposed to bacterial infection.

SOD, CAT, GST and POX were defined as antioxidant enzymes in insects. Our results showed that the ZEB application did not reveal a significant change in both the CAT and GST activities concerning control whereas an important decrement in the activity of SOD. MDA level, which is a lipid peroxidation product used as a biological indicator of oxidative stress, also increased in larval hemolymph of ZEB-exposed *G. mellonella* larvae This increment in the density of malondialdehyde indicates that the repair mechanisms of *G. mellonella* are inadequate and the resulting stress exceeds the limits of larval physiological adaptability to increased level of lipid peroxidation (Ahmad et al, 1995; Mano et al, 2005; Krishnan et al, 2009). Therefore, the oxidative stress revealed as a result of the degradation and production imbalance of ROS may lead to cell structure corruption by interacting with DNA and essential molecules such as proteins and lipids (Cnubben et al, 2001; Kohen and Nyska, 2002). A chain of reactions defined as lipid peroxidation occurs due to the oxidative stress with a degradation of cell membrane and other cellular lipid structures, resulting with the formation of aldehyde derivatives, especially MDA. The increase in the amount of MDA is a strong indicator of the rise in lipid peroxidation level as an end product of oxidative stress (Ahmad et al, 1995; Mano et al, 2005; Krishnan et al, 2009). Furthermore, this oxidative stress should also be associated with the increasing activity of antioxidant enzymes (Altuntaş, 2015). However, this considerable increase on the other hand might affect the structures of these enzymes resulting in a decrease in their activity. The change in the level of DNA methylation may also be effective on the transcription level of these enzymes. Further studies should be conducted to indicate the transcription levels of antioxidant enzymes by RT-PCR. In addition to the increase in MDA level revealing an increase in oxidative stress, consideration is also needed clarifying the effect of ZEB on necrosis or apoptosis since this increase in oxidative stress may lead to cell death (Zamzami et al, 1995; Tan et al, 1998; Kannan and Jain, 2000; James and Xu, 2012). Such consideration would help to evaluate enzyme activity results more reliably.

According to the results of the study, ZEB application did not cause changes in CAT and GST levels in *G. mellonella* larvae, while SOD activity decreased at doses of 1 ppm and above. Doses of 16 ppm and above caused an increase in MDA formation. These results indicate that ZEB treatment decreased antioxidant defense in *G. mellonella* larvae. The reduction of these antioxidant enzymes, which are responsible for the decrease of DNA damage and methylation in living organisms, means that the organism is vulnerable to pathogens and

external factors. Therefore, it is thought that ZEB application should be evaluated in the Integrated Pest Management program.

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