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Original article (Orijinal araştırma)

Toxicological and physiological effects of ethephon on the model organism, *Galleria mellonella* L. 1758 (Lepidoptera: Pyralidae)[1]

Etefonun model organizma *Galleria mellonella* L. 1758 (Lepidoptera: Pyralidae) üzerine toksikolojik ve fizyolojik etkileri

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Summary

Ethephon (ETF) has been used in agriculture as an ethylene releaser type of plant growth regulator. The aim of this work was to determine the ecotoxicological effects of ETF on the survival and the antioxidant metabolism of the insects using a model organism *Galleria mellonella* L. 1758. A toxicity test was performed to determine the lethal doses of ETF on larvae. According to probit assay, the LD_{50} and LD_{99} values for force fed larvae were 344 and 419 µg/5 µl, respectively, 30 d after treatment. Analyses performed with 10 doses ≤LD_{50} at 24 and 48 h upon feeding larvae revealed that the malondialdehyde level increased at 300 and 330 µg/5 µl doses, whereas glutathione-S-transferase activity increased only with a 360 µg/5µl dose of ETF at 24 h. However, an increase in glutathione-S-transferase activity was evident at all ETF doses at 48 h. An increase in glutathione peroxidases activity was determined at 250, 300 and 330 µg/5µl at 24 and 48 h. All ETF doses caused an important increase in catalase activity at 24 h but remained unchanged at 48 h. Superoxide dismutase activity also elevated at doses >250 µg/5µl at 24 h when compared to the control. Same changes in superoxide dismutase activity were also observed at all doses of ETF except for 360 µg/5µl at 48 h. These results showed that ETF induced oxidative stress resulted in toxic effects that affected on the survival of model organism *G. mellonella*.

Keywords: Antioxidant enzymes, ethephon, *Galleria mellonella*, malondialdehyde, toxicology

Özet

Etilen salınımına neden olan Etefon (ETF), bir bitki büyüme düzenleyicisi olarak tarımda kullanılmaktadır. Bu çalışmada bir model organizma olan *Galleria mellonella* L. 1758 (Lepidoptera: Pyralidae) türü kullanılarak, ETF'nin böceklerin antioksidan metabolizması ve canlılığı üzerindeki ekotoksikolojik etkilerinin araştırılması amaçlanmıştır. Larval dönemde ETF'nin letal dozunun belirlemesi amacıyla toksitite testi yapılmıştır. Larvalara zorla besleme (ağızdan besleme) yöntemi ile uygulanan ETF dozlarına göre, 30 günlük süreç içinde belirlenen LD_{50} ve LD_{99} değerleri sırasıyla 344 ve 419 µg/5µl olarak belirlenmiştir. LD_{50} ve daha düşük ETF dozlarıyla yapılan toksikolojik analizlerde ise iki zaman dilimi (24. ve 48. saat) tercih edilmiştir. 24. saatte, larval hemolenfteki malondialdehit seviyesi, 300 ve 330 µg/5µl ETF dozlarında artarken, glutatyon-S-transferaz aktivitesi sadece 360 µg/5µ'lik dozda yükselmiştir. Ancak 48. saatte kontrol ve tüm dozlarda glutatyon-S-transferaz aktivitesi yükselmiştir. Glutatyon peroksidaz aktivitesi ise hem 24. hem de 48. saatte, 330 ve 360 µg/5µl ETF dozlarında artmıştır. Tüm ETF dozları 24. saatte katalaz aktivitesinde artışa neden olurken, bu artış 48. saatte de aynı kalmıştır. Süperoksit dismutaz aktivitesi ise 24. saatte, 250 µg/5µl ve daha yüksek dozlarda yükselmiştir. 48. saatteki süperoksit dismutaz aktivitesinde de benzer değişimler meydana gelirken, 360 µg/5µl dozunda azalma belirlenmiştir. Bu sonuçlar, ETF'nin oksidatif stresi teşvik etmesi sonucunda model organizma *G. mellonella*'nın canlılığı üzerinde toksik etkisi olduğunu göstermiştir.

Anahtar sözcükler: Antioksidan enzimler, etefon, *Galleria mellonella*, malondialdehit, toksikoloji

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Introduction

The plant hormones that regulate plant growth are collectively known as the growth hormones or plant growth regulators (PGRs). The commercial forms of PGRs are widely used for increasing agricultural productivity. However, several studies have recently reported that various PGRs also have toxic effects on insects causing survival, developmental, reproductive and biochemical disturbances (Kaur & Rup, 2003; Gupta et al., 2009; Uçkan et al., 2011a, b, 2014, 2015) and induce oxidative stress (Altuntaş, 2015a). For a considerable amount of time, entomologists have been investigating the overall effects of some PGRs on insects. Their results provide reliable data on the biological and biochemical effects of gibberellic acid (GA_3), and indol-3-acetic acid which belong to two major classes of PGRs; the gibberellins and auxins, respectively (Uçkan et al., 2008, 2011a, b, 2014, 2015; Altuntaş et al., 2012, 2014; Altuntaş, 2015a). Altuntaş (2015a) also reported that dietary GA_3 induced oxidative stress in *G. mellonella* larvae, in particularly, exposure of different doses of GA_3 into larval diet activated important antioxidant enzymes in animals.

Ethephon [(2-chloroethyl) phosphonic acid, ETF] is a synthetic growth regulator, and belongs to ethylene releasers, an important class of the PGRs. ETF is used in agricultural systems for promoting fruit ripening, abscission and flower induction by releasing ethylene gas, a natural plant hormone (Zhang et al., 2012; Bhadoria et al., 2015; Hussain et al., 2015). Several dietary studies have been conducted on ETF toxicity to rats, birds, and marine or freshwater invertebrates (Haux et al., 2000, 2002; Al-Twaty, 2006; Abd El Raouf & Girgis, 2011; Anant & Avinash, 2012). Previous studies revealed that ETF not only acts as a plant growth regulator agent but also has mutagenic, teratogenic and biochemical effects on higher animals, since it is an organophosphorus pesticide. Ethephon is also an eye and skin irritant, but not a skin sensitizer, and classified by International Agency for Research on Cancer as group D (not carcinogenic to humans) (Bui, 2007). Acute oral studies using rats have shown that ETF is slightly toxic to mammals (Haux et al., 2002).

Studies on the negative effects of various PGRs, including ETF, on antioxidant mechanism were largely related to higher animals. It has been observed that abscisic acid and GA_3 cause lipid peroxidation in some tissues of rats and they change the activities of the enzymes in the antioxidant defense system. ETF has been found to be an inhibitor of plasma cholinesterase in humans, dogs, rats and mice (Haux et al., 2000, 2002; Tuluce & Çelik, 2006). It is well known that lipid peroxidation of cell membranes, damage to DNA and proteins, and activation of enzymes are regulated by antioxidants (Felton & Summers, 1995). The effects of non-lethal doses of pesticides like ETF may induce defense mechanisms to protect the insect against environmental stressors, because the antioxidant mechanism is a metabolic process for detoxification of environmental pollutants and chemicals (Büyükgüzel et al., 2010, 2013; Aslantürk et al., 2011; Emre et al., 2013; Erdem & Büyükgüzel, 2015; Altuntaş, 2015a, b; Dere et al., 2015). However, the effect of ETF on the antioxidant system of insects is currently unknown. Therefore, this work will provide further information about the ecotoxicological characteristics of ETF on insects, using *Galleria mellonella* L. 1758 (Lepidoptera: Pyralidae) as a model organism for entomological studies.

Galleria mellonella is a well-known model organism and system for ecotoxicological, ecophysiological and immunological investigations (Uçkan et al., 2008, 2011a, b; Altuntaş et al., 2012; Büyükgüzel et al., 2010, 2013; Maguire et al., 2016). It is also known that *G. mellonella* is an excellent model organism which can be used instead of mammalian species for *in vivo* toxicity of environmental pollutants and pathogenicity studies (Maguire et al., 2016). In comparison to other mammalian model organism and invertebrate models, rearing *G. mellonella* larvae in the laboratory is easier and faster

(Cook & McArthur, 2013; Maguire et al., 2016). In addition, large hemolymph sample volumes can be obtained from *G. mellonella* larvae for the measure of the physiological state of the internal environment of the insect. Any changes in the activity of antioxidant enzymes in the larval hemolymph profile resulting from ETF exposure would give us valuable information about insect physiology and biochemistry (Altuntaş et al., 2012, 2015a, b; Büyükgüzel et al., 2010, 2013). Thus, here we aimed to determine the ecotoxicological and ecophysiological effects of ETF on insects, using *G. mellonella* as a model organism, which is of great important for the risk assessment and management of ETF compounds. For this purpose, we determined if ETF had any toxicity affecting the survival of larvae and any effects on malondialdehyde (MDA) concentration and the activity of antioxidant enzymes, glutathione-S-transferase (GST), glutathione peroxidases (GPx), catalase (CAT) and superoxide dismutase (SOD), in the hemolymph of *G. mellonella* last instars.

Materials and Methods

Insects

Laboratory cultures of *G. mellonella* were maintained by feeding the insects with a modified Bronskill (1961) artificial diet including dark honeycomb (100 g), pollen (20 g), bran (340 g), glycerol (150 ml), honey (75 ml) and distilled water (75 ml). Colonies were kept at 25 ± 2°C, 60 ± 5% RH, and a photoperiod of 12L:12D h in Anadolu University, Eskişehir, Turkey.

Toxicity of ETF

ETF (Sigma, St. Louis, MO, USA) was dissolved in distilled water to prepare stock solution (1 mg/ml). ETF doses (250, 270, 300, 330, 350, 360, 370, 380, 400 and 430 µg/5 µl/larva) were prepared from the stock solution to determine the acute toxicity on larvae. Larvae of an approximate similar weight (0.14 ± 0.01 g) were selected from the insect culture for the force feeding treatment. Selected larvae were starved for 3 h, and then force fed with 5 µl of the ETF solution containing different doses or distilled water with a Hamilton syringe (22 gauges) (Dere et al., 2015). Each larva was exposed to a 2 g diet and observed daily to determine larval mortality in 30 d after treatment. Both experimental and control assays were performed with a total of 60 larvae (20 larvae in each of three replicates) for each dose. The lethal doses (LD_{10}, LD_{20}, LD_{30}, LD_{40}, LD_{50}, LD_{95} and LD_{99}) of ETF application were determined by probit analysis using the SPSS software (IBM IBM, Armonk, NY, USA) at 95% confidence levels.

Sample preparation

MDA concentration and antioxidant enzyme activities in hemolymph of last instars were carried out with doses below the upper limit (95% confidence levels) of LD_{50} that were determined for ETF (0, 250, 300, 330 and 360 µg/5 µl/larva) in toxicological studies. To collect hemolymph from force fed larvae exposed to different doses of ETF or distilled water at 24 and 48 h after treatment, 10 larvae (0.16 ± 0.01 g) were used in each analysis and 10 µl of hemolymph was collected. Each larvae was kept on ice for 5 min for anesthesia and were sterilized with a cotton ball containing 70% ethanol, subsequently hemolymph samples were collected into micro centrifuge tubes (0.5 ml) containing 1 µg 1-phenyl-2-thiourea and cold homogenization buffer (1:2 v/v) by removing the third pair proleg. All samples were frozen at -80°C until used. Before all analyses, samples were homogenized according to Altuntaş (2015a). All assays were repeated four times.

Assays of malondialdehyde concdentration and antioxidant enzyme activities

MDA analysis was performed according to kit protocol based on measuring the reaction of MDA with thiobarbituric acid at 95°C (Cayman Chemical, Ann Arbor, MI, USA) and this acidic reaction was monitored at 530 nm using a microtiter plate reader (Spectra Max M2). The concentration of MDA was calculated as the nmol/mg protein using the extinction coefficient 1.56 x 10^5 M^{-1} cm^{-1}.

Total GST activity in larval hemolymph was assayed using kit protocol (Cayman Chemical) by following the principle of 1-chloro-2,4-dinitrobenzene (CDNB), a substrate, and glutathione (GSH) conjugate formation. The increase in absorbance activity was monitored at 340 nm for 5 min with a microtiter plate reader and specific activity was defined as conjugated 1 nmol CDNB with reduced GSH/min/mg protein at 25°C according to the extinction coefficient of 0.00503 μM^{-1} cm^{-1}.

The activity of GPx was determined using a commercially kit protocol (Cayman Chemical). This kit measures the oxidation rate of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm for 5 min in a microtiter plate reader. Specific activity of GPx was calculated as nmol/min/mg protein according to 0.00622 μM^{-1} cm^{-1} extinction coefficient value.

The assay of CAT activity is based on determining the H_2O_2 decomposition at 240 nm for 3 min (Chance & Maehly, 1995). Decreasing absorbance was recorded in ultraviolet-visible spectrophotometer (Shimadzu UV-1601, Tokyo, Japan) and results were expressed as hydrolysis of 1 mmol H_2O_2/minute/mg protein using e240 = 0.0394 $mM^{-1}cm^{-1}$.

SOD assay was performed by 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride reacting with superoxide radicals, using xanthine and xanthine oxidase at 450 nm (Cayman Chemical). One unit of total activity of SOD (U/mg protein) was calculated by the quantity of enzyme needed to cause 50% inhibition of the superoxide radicals in one mg protein.

To determine MDA amount and antioxidant enzyme activities, Bradford (1976) method was performed to measure protein concentration in homogenates. Bovine serum albumin was also used to prepare the standard curve. All analyses were repeated four times using 10 larvae per treatment.

Statistics

All data was normally distributed. Therefore, one-way analysis of variance tests was performed to compare the normally distributed means for MDA level and antioxidant enzyme activities. To define significant differences among means, Tukey's Honestly Significant Difference (HSD) post hoc tests were used. Furthermore, time related changes in enzyme activities and MDA level (24 and 48 h) were determined with t-tests. SPSS program was carried out for all statistical analyses (SPSS, 2010). Means were considered statistically significant when $P \leq 0.05$.

Results

Toxicity of ETF

The ETF-treated larvae of *G. mellonella* exhibited toxic symptoms in a dose-dependent manner (Table 1). The ETF-treatment significantly decreased larval survival ≥50% beyond 360 µg/5 µl. LD_{50} and LD_{90} doses of ETF-treated larvae were determined as 344 (95% confidence limits, 331-361) and 419 (95% confidence limits, 392-458) µg/5 µl/larva, respectively (X^2 = 24.1, df = 8, P = 0.002). A 100% mortality was recorded at the highest concentration tested of 430 µg/5 µl. According to probability doses of ETF obtained from probit analysis, we used the doses ≤LD_{50} (250, 300, 330 and 360 µg/5 µl/larva) for analysis of the MDA level and antioxidant enzyme activities in larval hemolymph.

Table 1. Mortality and lethal doses of ETF (µg/5 µl/larva) on force fed *Galleria mellonella* larvae

ETF Doses (µg/5 µl)	*No. of exposed larvae (n=60)	No. of dead larvae	Lethal Doses (µg/5 µl/larva)			
Control	60	0	Lethal Doses	Probability Doses	95 % Confidence limits**	
250	60	2			Upper	Lower
270	60	6				
300	60	14	LD_{10}	283	257	300
330	60	20	LD_{20}	303	281	317
350	60	24	LD_{30}	318	300	331
360	60	30	LD_{40}	331	316	344
370	60	36	LD_{50}	344	331	361
380	60	50	LD_{70}	373	359	393
400	60	54	LD_{90}	419	397	458
430	60	60	LD_{99}	443	415	495

* All assays were designed with a total of 60 larvae (20 larvae in each of three replicates) for each dose.
** Values are displayed with lower and upper confidence limits, Probit = -38.294 + 15.095 X doses (doses are transformed using the base 10 logarithm).

Effects on MDA level

MDA levels in larval hemolymph of the ETF force fed larvae (doses ≤LD_{50}) differed depending on dose and time (Figure 1). MDA levels of controls were 2.77 and 2.39 nmol/mg protein at 24 and 48 h, respectively. The ETF treatment had the most significant effect on MDA level with more than 80% increase in doses >250 µg/5 µl when compared to the control at 24 h ($F = 28.9$; $df = 4, 15$; $P < 0.001$). Similar changes were also detected at 300 and 330 µg/5 µl except for 360 µg/5 µl at 48 h following treatments ($F = 26.3$; $df = 4, 15$; $P < 0.001$). The exposure to ETF in diet did not significantly change the level of MDA in larval hemolymph at doses 250 and 300 µg/5 µl (t-test, $P>0.05$). However, it decreased considerably from 24 to 48 h at doses of 330 and 360 µg/5 µl (t-test, $P<0.05$).

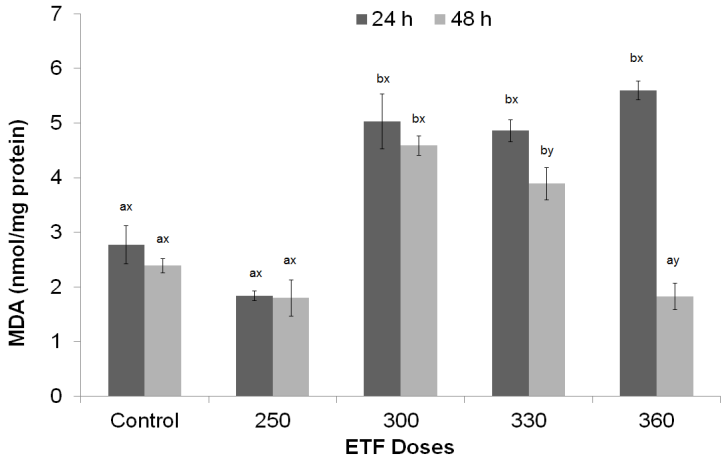

Figure 1. MDA content in the hemolymph of control and ETF-treated last instars. Vertical bars represent the mean ± standard error per replicate (n = 40). Statistically significant differences are indicated with letters a-b among groups at 24 or 48 h ($P < 0.05$, Tukey-HSD test) and x-y between two time points at the same dose ($P < 0.05$, t-test).

Effects on antioxidant enzyme activities

GST activity was 4.55 (24 h) and 5.31 (48 h) nmol/min/mg protein in larval hemolymph of the control group. GST activities in hemolymph of larvae did not change in tested doses of ETF except for 360 µg/5 µl 24 h when compared to the control ($F= 51.5$; $df= 4, 15$; $P<0.001$). On the other hand, GST activity increased nearly two-fold at all ETF doses at 48 h after treatment when compared to the control ($F = 19.759$; $df = 4, 15$; $P < 0.001$). Data indicated that GST activity in control and all assay groups were significantly higher at 48 h than at 24 h, except for 360 µg/5 µl (t-test, $P < 0.05$, Figure 2).

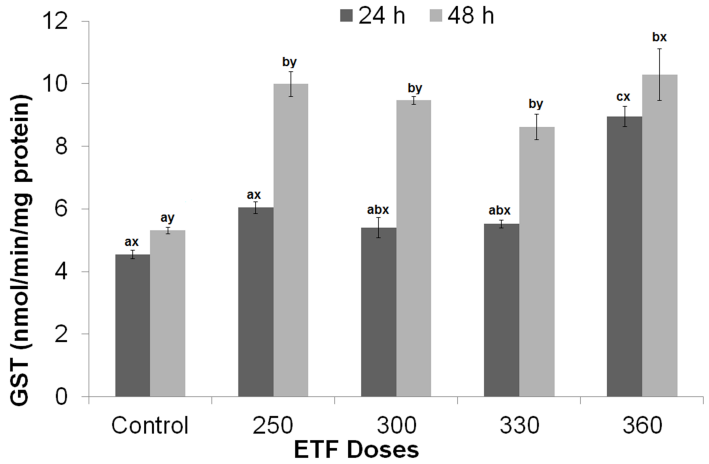

Figure 2. GST activity of larval hemolymph of *Galleria mellonella* force fed with different concentrations of ETF. Vertical bars represent the mean ± standard error per replicate (n = 40). Statistically significant differences are indicated with letters a-c among groups at 24 or 48 h ($P < 0.05$, Tukey-HSD test) and x-y between two time points at the same dose ($P < 0.05$, t-test).

EFT exposure significantly increased GPx activity in larval hemolymph at 330 and 360 µg/5 µl doses at 24 ($F = 7.69$; $df = 4, 15$; $P<0.001$) and 48 h ($F = 7.88$; $df = 4, 15$; $P < 0.001$) after treatment when compared to the control and other doses (Figure 3). In particular, the highest activity occurred at 330 µg/5 µl ETF (0.96 and 0.57 nmol/mg protein/min, respectively) at 24 and 48 h. Furthermore, GPx activities of all groups nearly halved at 48 h compared to the activities at 24 h after treatment (t tests, $P < 0.05$, Figure3).

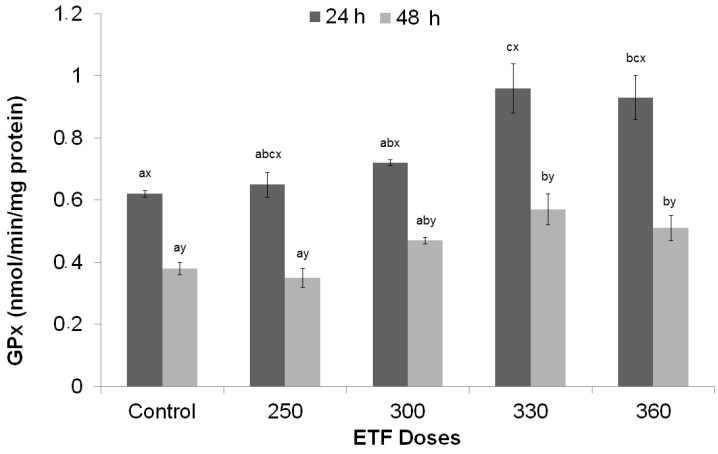

Figure 3. GPx activity of larval hemolymph of *G. mellonella* force fed with different concentrations of ETF. Vertical bars represent the mean ± standard error per replicate (n=40). Statistically significant differences are indicated with letters a-c among groups at 24 h or 48 h ($P<0.05$, Tukey-HSD test) and x-y between two time points at the same dose ($P<0.05$, t-test).

CAT activity in control was 0.45 and 0.49 mmol/min/mg protein at 24 and 48 h, respectively, however exposure to ETF caused nearly twice the activity of CAT in comparison with that of control at 24 ($F = 13.3$; $df = 4, 15$; $P < 0.001$) and 48 h ($F = 46.5$; $df = 4, 15$; $P < 0.001$). Time dependent changes between 24 and 48 h in CAT activities were not significant in the control and all experimental groups, except for 250 µg/5 µl (t-tests, $P > 0.05$, Figure 4).

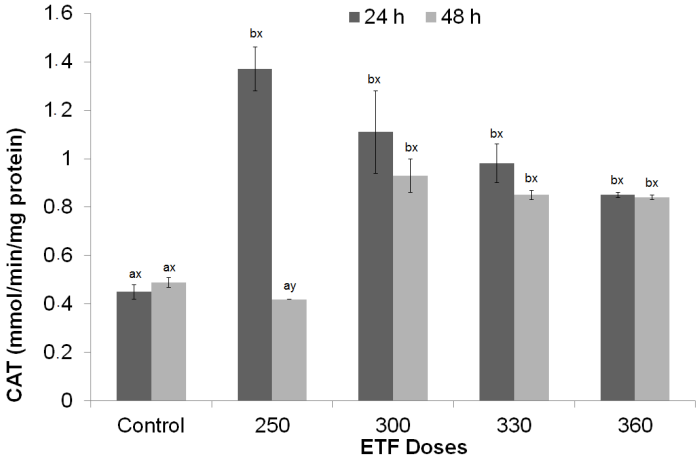

Figure 4. CAT activity of larval hemolymph of *Galleria mellonella* force fed with different concentrations of ETF. Vertical bars represent the mean ± standard error of per replicate (n = 40). Statistically significant differences are indicated with letters a-b among groups at 24 or 48 h ($P < 0.05$, Tukey-HSD test) and x-y between two time points at the same dose ($P < 0.05$, t-test).

SOD activities of control were 0.54 and 0.15 U/mg protein at 24 and 48 h, respectively. Force feeding with ETF increased the activity of SOD five-fold at 250 µg/5 µl at 24 h compared to the control. Significant elevations were also evident at doses >250 µg/5 µl ($F = 44.6$; $df = 4, 15$; $P < 0.001$). Similarly, a three-fold elevation of SOD activity in larval hemolymph at 300 µg/5 µl ETF exposure was recorded at 48 h compared to the control ($F = 142$; $df = 4, 15$; $P < 0.001$). However, these increased activities in all ETF doses did not exhibit a dose-dependent response at 24 and 48 h, because SOD activity decreased again at higher doses of ETF compared to other doses. SOD activities in larval hemolymph were considerably lower for both untreated and ETF-treated groups at 48 h after treatment compared to those at 24 h (t tests, $P < 0.05$, Figure 5).

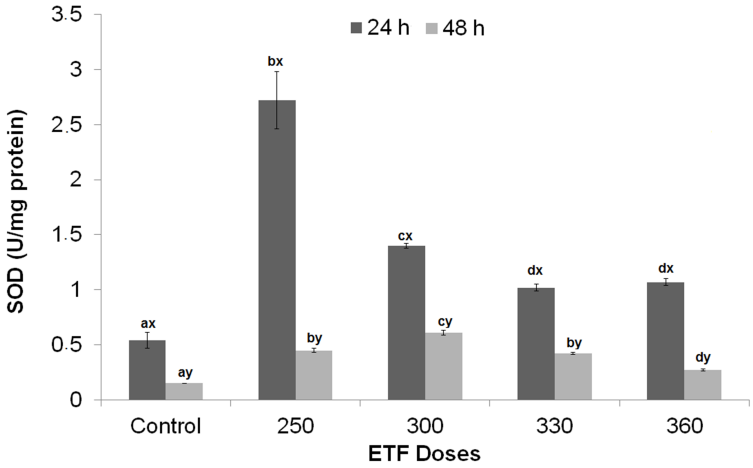

Figure 5. SOD activity of larval hemolymph of *Galleria mellonella* force fed with different concentrations of ETF. Vertical bars represent the mean ± standard error per replicate (n = 40). Statistically significant differences are indicated with letters a-d among groups at 24 or 48 h ($P < 0.05$, Tukey-HSD test) and x-y between two time points at the same dose ($P < 0.05$, t-test).

Discussion

Several studies have reported that exposure of ETF to plants indirectly induces plant defense responses and increase resistance or tolerance of plants against insect pests (Henneberry et al., 1988; Stotz et al., 2000). ETF has been shown to be nontoxic to shrimp, and slightly toxic to estuarine/marine mollusks. It is also practically nontoxic to cold water fish, and nontoxic to slightly toxic to warm water fish and freshwater invertebrates. Other studies on rats showed that ETF has slight acute toxicity to mammals when applied orally (Haux et al., 2000, 2002; Al-Twaty, 2006; Abd El Raouf & Girgis, 2011; Anant & Avinash, 2012). Wang et al. (2011) reported that acute toxicity of ETF to the *Daphnia magna* Straus, 1820 embryos had an EC_{50} range of 125-130.5 mg/l. Despite previous studies, the results presented here are the first detailed report of the toxicological effects of ETF on insects. Mortality data, obtained from the toxicity test and probit analysis showed that ETF has considerable acute toxicity to *G. mellonella* larvae. Due to the high insecticidal potential (LD_{50} dose = 72 µg/µl) of ETF, we investigated the potential effects of ETF on the fundamental physiological processes of *G. mellonella.* It is of great importance to discover the effects of environmental chemicals, such as ETF, on the biochemical and physiological response mechanisms of insects. It is also known that activities of antioxidant enzymes can be stimulated by oxidative stress state and these adaptation mechanisms are more important for organophosphate pesticide or xenobiotic-induced stress conditions in insects (İçen et al., 2005; Dere et al., 2015; Erdem & Büyükgüzel, 2015).

We consider that exposure to doses of ETF ≤LD_{50} caused an oxidative stress in force fed larvae because MDA level in hemolymph of last instars increased at all doses of ETF except for 360 µg/5 µl at 48 h (Figure 1). It is also known that MDA is a lipid peroxidation product and used for as biological indicator of oxidative stress in insects (Ahmad, 1995; Hyrsl et al., 2007). On the other hand, the MDA level decreased to control level again at higher doses of ETF after 48 h. In addition, a considerable decrease was recorded between 24 and 48 h at 330 and 360 µg/5 µl. This decrease may be associated with the increasing activity of GST with time and ETF doses (Figure 2), because, GST, a phase II detoxifying enzyme, has an important role in the cellular detoxification of stressors in insects (Hyrsl et al., 2007; Oruc, 2011; Erdem & Büyükgüzel, 2015; Altuntaş, 2015a). Therefore, the increase in GST activity may be related to an inhibition of the lipid peroxidation process and physiological response mechanism against ETF toxicity for cellular detoxification. These results are consistent with the findings of Altuntaş (2015a). In that study, the author reported that GST activity in hemolymph of *G. mellonella* larvae increased at low doses of GA_3 treatment. Similar results have also been reported with GST activity by organophosphate insecticides and other PGRs in vertebrate animals and insects (Yu, 2004; Hyrsl et al., 2007; Oruc, 2011; Tuluce & Çelik, 2006). We assume that GST activity can be used as a biomarker to evaluate ecotoxicological properties of ETF for insects.

Considerable elevation in the activity of GPx at 330 and 360 µg/5 µl (LD_{50}) of ETF at both times tested (Figure 3) may be an attempt to counteract the elevation of MDA level as a defense mechanism against the accumulation of lipid peroxidation products in the cells (Hemming & Lindroth, 2000; Fahmy, 2012). This is because, GPx regulates hydrogen peroxides and lipid hydroperoxides using reduced glutathione (Peric-Mataruga et al., 1997). However, previous studies revealed low GPx activity in insects including lepidopteran species (Ahmad et al., 2005; Erdem & Büyükgüzel, 2015). Furthermore, it is reported that this deficiency in the activity of GPx is supplemented by peroxidase activity (GSTPx) of GST (Peric-Mataruga et al., 1997) and higher CAT activity. Therefore, these previous findings are consistent with our data on to this higher activity at GST and CAT.

In this study, the important finding was a substantial increase in CAT and SOD activities in the hemolymph of last instars at all ETF doses at 24 h after treatment (Figures 4 and 5). This result was also similar to that of 48 h except at the lower doses of ETF for CAT activity. However, no change was detected in CAT activity at 24 and 48 h while SOD activity decreased in control and at all doses of ETF at 48 h. In particular, an important decline was observed at LD_{50} dose at 48 h in respect of other doses. These substantial changes in the activities of SOD and CAT, the primary enzymes against ROS-mediated toxicity in all living organisms, may be attributed to several reasons. Firstly, the elevated CAT activity may be associated with scavenging of hydrogen peroxides by CAT. The results obtained are in agreement

with previous findings reported for some lepidopteran species treated with various pesticides and PGRs (Krishnan & Kodrik, 2006; Aslantürk et al., 2011; Büyükgüzel et al., 2013; Altuntaş, 2015a, b). The second reason may be that the increased SOD activity in ETF treated larvae at 24 h caused an increase in hydrogen peroxide concentration and a further elevation in CAT activity in response. It has been found that CAT activity is normally higher in insects than mammals (Ahmad & Pardini, 1990). Therefore, the third assumption is that the relative low levels of SOD increase with respect to treatment time and higher activity of GPx at high doses of EFT may affect hydrogen peroxide concentration. Collectively, the results presented here indicated that the elevated SOD activity at lower doses of ETF treatment may be derived from increasing hydrogen peroxide concentration, and as a result, CAT activity increased in larval hemolymph. Altuntaş (2015b) also determined that the total lipid, protein and glucose amount in hemolymph of *G. mellonella* last instars decreased following ETF treatment. ETF-induced stress conditions may cause the lipids and glucose to be used for cell repair, lipoprotein formation (Riberio et al., 2001) and the increased protein catabolism. This is because protein catabolism may be stimulated due to high energy demand under stress conditions (Sancho et al., 1998). Therefore, the current study demonstrated that treatment of larvae with ≤LD_{50} ETF doses caused peroxidation of cellular lipids and increases in the activities of antioxidant enzymes.

In conclusion, this study showed that ETF has toxic effects on insects via biochemical and physiological alterations in a dose and time dependent manner when received orally. These results obtained from model organism *G. mellonella,* provide reliable data which can be used as an index of the ecotoxicological and physiological significance of ETF in the context for insects. Consequently, consideration is needed to avoid the reckless use of this type of chemicals at high concentrations without evaluating technical procedures as such use might cause disruption of the ecological balance.

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