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RESEARCH ARTICLE

Oxidative Impact of Dietary Triclabendazole in *Galleria mellonella*

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Abstract

Recent studies have shown that anthelmintic and antifungal agents are recommended as alternative agents in chemical control of insect pests. In this study, first instar larvae of the greater wax moth, *Galleria mellonella,* were reared on artificial diets containing 0.001, 0.01, and 0.1 g/100 g of triclabendazole, an anthelmintic benzimidazole. The effects of these triclabendazole concentrations on the lipid peroxidation product, malondialdehyde (MDA) and glutathione-S-transferase (GST) activity were investigated in all G. mellonella developmental stages, as well as in the midgut tissue of the insect. Compared to controls, the highest triclabendazole concentration (0.1%) increased MDA content from 0.182±0.03 to 0.415±0.04 nmol/mg protein in the larvae, from 0.190±0.04 to 0.626±0.06 nmol/mg protein in the pupae, and from 0.354±0.06 to 0.451±0.04 nmol/mg protein in the adults. MDA content was determined to be 0.141±0.02 to 0.835±0.13 nmol/mg protein in the midgut. The highest concentration of triclabendazole was found to significantly increase GST activity in the larvae, pupae, adults, and in the midgut tissue of *G. mellonella* in comparison to controls. The three dietary concentrations of triclabendazole resulted in a high degree of oxidative stress and an increase in the activity of the detoxification enzyme GST following an increase in MDA levels in the midgut and in all developmental stages of G. mellonella.

Keywords: *Galleria mellonella, Oxidative stress, Triclabendazole, Malondialdehyde, Glutathione-S- transferase*

Triklabendazolün *Galleria mellonella*'daki Oksidatif Etkisi

Öz

Son çalışmalarda, antihelmintik ve antifungal ajanların, böcek zararlılarının kimyasal kontrolünde alternatif kimyasallar olarak önerildiğini görülmektedir. Bu çalışmada, büyük bal mumu güvesi, *Galleria mellonella,* birinci evre larvaları 0.001, 0.01 ve 0.1 g/100 g benzimidazol grubu bir antihelmintik olan triklabendazol içeren yapay besinler ile beslendi. Belirlenen konsantrasyonların böceğin farklı gelişme evrelerinde (7. evre larva, pup ve ergin) ve orta bağırsağında oksidatif stresin önemli bir belirteci olan lipid peroksidasyonu ürünü malondialdehit (MDA) miktarı ve bir detoksifikasyon enzimi olan glutatyon-S-transferaz (GST) aktivitesi üzerine etkileri araştırıldı. Kontrol grubu ile en yüksek triklabendazol konsantrasyonu (0.1%) karşılaştırıldığında MDA miktarını böceğin larval evresinde 0.182±0.03'ten 0.415±0.04 nmol/mg proteine, pupal evrede 0.190±0.04'ten 0.626±0.06 nmol/mg proteine, ergin bireylerde 0.354±0.06'dan 0.451±0.04 nmol/mg proteine yükseldi. Orta bağırsağında MDA miktarı 0.141±0.02'den 0.835±0.13 nmol/mg protein olarak belirlendi. Triklabendazolün en yüksek konsantrasyonu kontrol grubuna göre böceğin larva, pup, ergin evreleri ve orta bağırsağında GST aktivitesini arttırdığı tespit edildi. Elde edilen bu sonuçlar ile triklabendazolun üç farklı konsantrasyonunun bulunduğu yapay besinler ile beslenen *G. mellonella*'nın tüm gelişme evrelerinde ve orta bağırsağında oksidatif stres belirteci MDA miktarındaki artışa bağlı olarak yüksek derecede oksidatif stres meydana geldiği ve detoksifikasyon enziminin aktivitesinin arttığı belirlendi.

Anahtar sözcükler: *Galleria mellonella, Oksidatif stres, Triklabendazol, Malondialdehit, Glutatyon-S- transferaz*

INTRODUCTION

A number of physical, chemical, and biological techniques are used to protect crops from pest insects and increase crop yields [1,2]. However, the use of insecticides in agricultural fields poses a threat to other, non-target organisms, as well as to the environment. Therefore, the use of effective and environmentally sensitive chemicals as an alternative to traditional insecticides has increased. It has contributed to the growing interest in alternative pest control of

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clinically important drugs, including anthelmintics [3]. *Galleria mellonella* is a model organism, easy to rearing in laboratory conditions, used in many biological studies including physiological, genetic, and toxicological research areas in recent years [4,5]. Recent laboratory studies have shown that next-generation chemicals have negative effects on *G. mellonella* [6-10]. In a study by Çalık et al.[3], the sub-lethal effects of mebendazole, an anthelmintic in the benzimidazole group, on the biology of this model organism was investigated by observing survival, growth, adult longevity, fecundity, and hatchability and was found to have a deleterious effect on its physiology.

Insects are exposed to numerous stresses throughout their lives, especially physical (radiation, temperature, etc.) and chemical (pesticides, heavy metals, etc.) factors [11]. These factors may cause free radical formation, oxidative stress and, subsequently, cellular damage [11-13]. Malondialdehyde (MDA) is a widely used marker of oxidative stress [13,14], and an increase in free radicals causes the overproduction of MDA. However, there are antioxidant enzyme systems in insects that work against these free radicals [6,15-17]. In particular, glutathione S-transferases (GSTs) are multi-functional antioxidant enzymes involved in the detoxification process via a number of different mechanisms. They are evolutionarily conserved enzymes that are important in the detoxification of many xenobiotic compounds [15,18].

The midgut, where digestive enzymes are secreted and nutrients absorbed, is an important tissue of insects [19]. One study that investigated the effects of *Bacillus thuringiensis* (a bio-insecticide used for agricultural pest management) on antioxidant capacity and lipid peroxidation in *G. mellonella* found that it contributed to cell death by increasing the effect of oxidative stress in the midgut [20]. Recently, a number of other studies have indicated that anthelmintic and antifungal agents should be used as alternatives for insect management [3,6-10,21,22]. In one study, the effects of terbinafine (an antifungal agent) on MDA and protein carbonyl (PCO) contents, which are oxidative stress biomarkers in the midgut of *G. mellonella*, and glutathione S-transferase (GST) activity, a detoxification enzyme, were examined. It was determined that terbinafine increased the MDA content in the midgut tissue when compared to a control group, and similarly increased PCO content and GST enzyme activity with in a dose-dependent manner [22].

Triclabendazole is a benzimidazole anthelmintic known to be effective against the larval and adult stages of parasites *(Fasciola hepatica, Fasciola gigantica* and *Fascioloides magna)* that cause infections in humans and animals [23,24]. In a previous study that our laboratory conducted, we showed that triclabendazole also negatively affects the biology of *G. mellonella*. However, insect survival parameters are important criteria for investigating and understanding the appropriate use of triclabendazole as an insecticide [3]. Therefore, in this study, the oxidative effects of triclabendazole on the midgut tissue of *G. mellonella,* and at different development stages, was examined at increasing concentrations in an artificial diet.

Material and Methods

Galleria mellonella Culture

A stock culture was obtained by rearing *G. mellonella* (Lepidoptera: Pyralidae) pupae and adults at Zonguldak Bülent Ecevit University, Department of Molecular Biology and Genetics, Insect Culture Laboratory. Newly hatched first instar larvae were reared on an artificial diet consisting of 420 g wheat bran, 150 mL liquid honey, 150 mL glycerin, 20 g ground dark honeycombs, and 30 mL distilled water [25]. The insect culture was maintained in a Nüve FN 400 incubator (Nüve A.Ş., Ankara, Turkey) that was set to 28±2°C, 65±5% relative humidity, and in continuous darkness. The final larval stage, as well as the pupae, adults, and midguts were used for biochemical analysis.

Triclabendazole Concentrations

Triclabendazole [($C_{14}H_9Cl_3N_2OS$) (5-kloro-6-(2,3-dikloro-fenoksi)-2-(metil-tiyo)-1*H*-benzimidazol)] was obtained from Merck, Darmstadt, Germany. The triclabendazole concentrations used in this study were determined as gram quantity supplemented to 100 grams of diet. Three different concentrations of 0.001, 0.01 and 0.1% were used. A control group reared on a diet without triclabendazole was included. The experimental triclabendazole concentrations were based on our previous study investigating the effects of triclabendazole on the development and life parameters of *G. mellonella* [3].

Midgut Isolation

Last larval stage of *G. mellonella* were kept on ice for 5 min and disinfected with 95% ethanol. They were then fixed in a paraffin-filled petri dish and cut from the front of the first pair of thoracic legs to the third pair of abdominal legs along the mid-axis with dissection scissors. The midguts were retrieved using fine-tipped forceps under a stereo-microscope (Olympus SZ61; Olympus, Tokyo, Japan) and separated from the anterior and hindgut. The fat body, malpighian tubules, and gut contents were also removed. The midguts were placed in Eppendorf tubes containing cold homogenization buffer (w/v 1.15% KCl, 25 mM K_2HPO_4, 5 mM EDTA, 2 mM PMSF, 2 mM DTT, pH 7.4) and stored at -80°C.

Determination of MDA Content and GST Activity

Malondialdehyde content, which reacts to thiobarbituric acid at 532 nm, was measured based on the method by Jain and Levine [26] with a dial beam spectrophotometer (Shimadzu 1700 UV/Vis, Kyoto, Japan) and calculated using a coefficient of 1.56 x 105 $M^{-1}cm^{-1}$. Results are given as nmol/mg protein. GST (EC 2.5.1.18) activity was assayed

by measuring the formation of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene conjugate [27] with a dial beam spectrophotometer (Shimadzu 1700 UV/Vis, Kyoto, Japan). The specific activity of the enzyme is given as µmol/mg protein/min. Protein concentrations were determined according to Lowry et al.[28] and bovine serum albumin was used as a quantitative standard.

Data Analysis

One-way analysis of variance (ANOVA) was used to analyze MDA content and GST activity in the midgut, seventh instar larvae, pupae, and adult *G. mellonella* treated with different concentrations of triclabendazole in an artificial diet. To determine significant differences between mean values, the least significant differences test was used. Statistical significance was assessed at P<0.05. SPSS statistical software (version 15.0 for windows; SPSS Inc., Chicago, IL, USA) was used for the analyses [29].

Results

At the highest triclabendazole concentration (0.1%), MDA content was significantly higher than in the control group. It increased from 0.182±0.03 to 0.415±0.04 nmol/mg protein in the larvae, 0.190±0.04 to 0.626±0.06 nmol/mg protein in the pupae, and from 0.354±0.06 to 0.451±0.04 nmol/mg protein in the adult stage of the insect *(Fig. 1, Fig. 2, Fig. 3)*. Furthermore, when compared with the control group, the amount of MDA in the mid-intestinal tissue at the highest triclabendazole concentration increased approximately 6-fold, from 0.141±0.02 to 0.835±0.13 nmol/mg protein *(Fig. 4)*.

The effects of triclabendazole on GST activity showed statistically significant increases for all of the different triclabendazole concentrations at all developmental stages, and in the midgut tissue. At the larval stage, GST activity in the control group was 145.90±27.89 µmol/mg protein/min. However, this value was 329.76±32.51 µmol/mg protein/min at the highest triclabendazole concentration *(Fig. 5)*. While GST activity at the pupal stage was 36.25±6.82 in the control group, it was found to be 104.67±12.39 µmol/mg protein/min at 0.1% of triclabendazole *(Fig. 6)*.

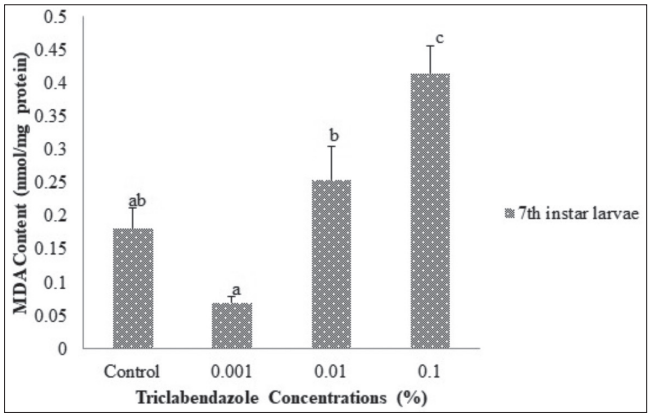

Fig 1. Effects of dietary triclabendazole on MDA content in 7th instar larvae of *G. mellonella*. Bars represent the means ± SE of four replicates. Means followed by different letter are significantly different (P<0.05, LSD test)

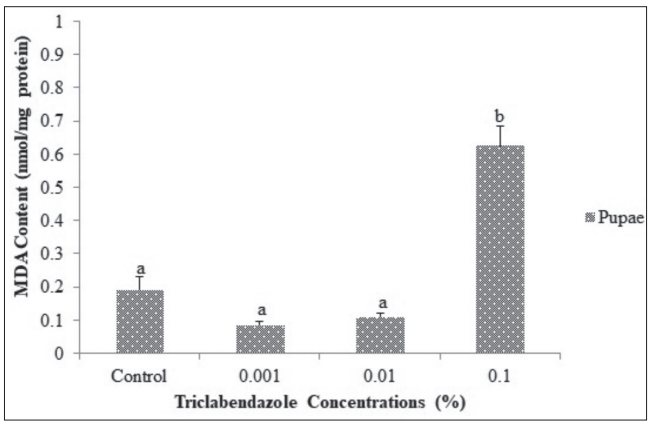

Fig 2. Effects of dietary triclabendazole on MDA content in pupae of *G. mellonella*. Bars represent the means ± SE of four replicates. Means followed by different letter are significantly different (P<0.05, LSD test)

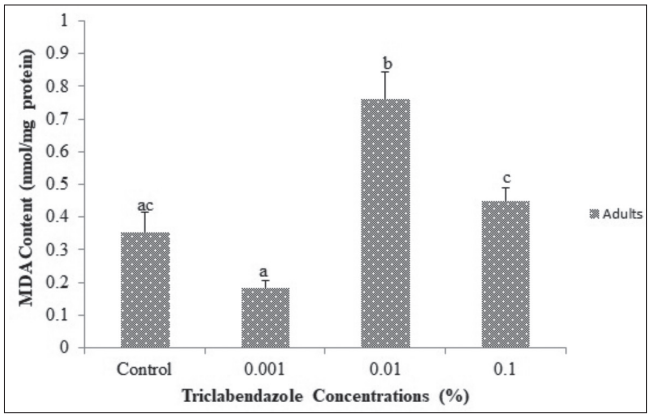

Fig 3. Effects of dietary triclabendazole on MDA content in adults of *G. mellonella*. Bars represent the means ± SE of four replicates. Means followed by different letter are significantly different (P<0.05, LSD test)

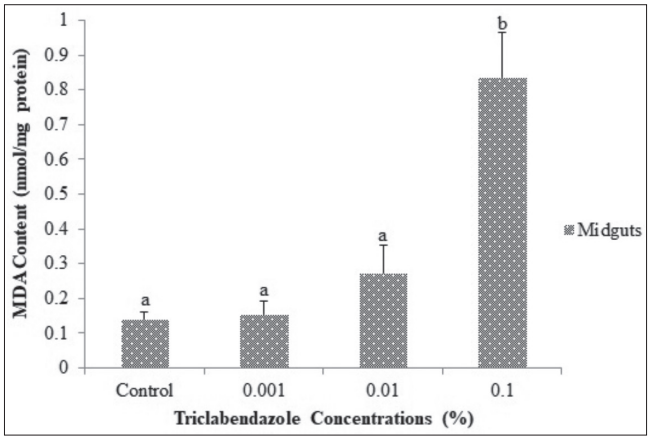

Fig 4. Effects of dietary triclabendazole on MDA content in midguts of *G. mellonella*. Bars represent the means ± SE of four replicates. Means followed by different letter are significantly different (P<0.05, LSD test)

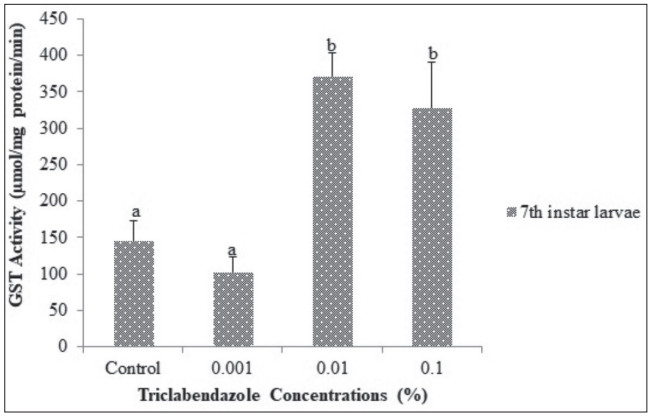

Fig 5. Effects of dietary triclabendazole on GST activity in 7th instar larvae of *G. mellonella*. Bars represent the means ± SE of four replicates. Means followed by different letter are significantly different (P<0.05, LSD test)

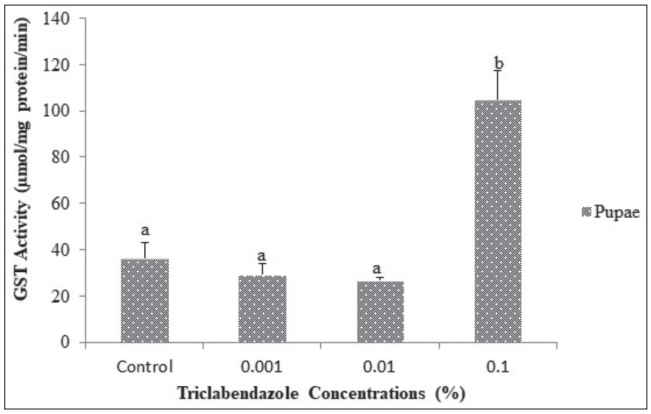

Fig 6. Effects of dietary triclabendazole on GST activity in pupae of *G. mellonella*. Bars represent the means ± SE of four replicates. Means followed by different letter are significantly different (P<0.05, LSD test)

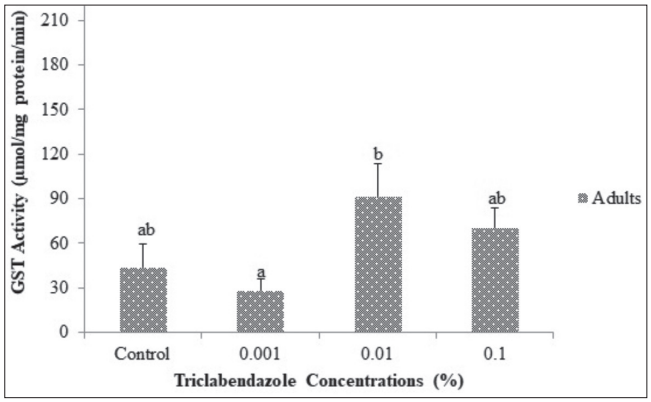

Fig 7. Effects of dietary triclabendazole on GST activity in adults of *G. mellonella*. Bars represent the means ± SE of four replicates. Means followed by different letter are significantly different (P<0.05, LSD test)

The GST activity of the adults was 44.13±15.27 in the control group and 91.62±21.57 µmol/mg protein/min at 0.01% of triclabendazole *(Fig. 7)*. A statistically significant increase was recorded in the GST activity in the midgut tissue as well, which increased approximately 9-fold

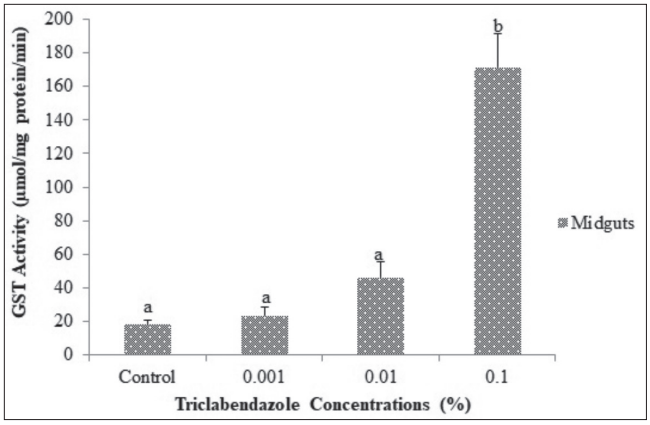

Fig 8. Effects of dietary triclabendazole on GST activity in midguts of *G. mellonella*. Bars represent the means ± SE of four replicates. Means followed by different letter are significantly different (P<0.05, LSD test)

(from 18.31±2.34 µmol/mg protein/min in the control group to 171.29±20.33 µmol/mg protein/min at 0.1% of triclabendazole) *(Fig. 8)*.

Discussion

Insect lipid reserves are affected by a number of factors including nutritional status, developmental stage, environmental variables, the diaphragm, migration flight, and energy metabolism [30]. Diet quality during the larval stage is especially important for the other development stages [7,31]. The effect of triclabendazole on the biology of *G. mellonella* has also been investigated by Kılıç et al.[7] who determined that the chemical negatively affected survival rates and developmental time. Our results support those of Kılıç et al.[7] and further show a statistically significant increase in MDA content and GST activity at all developmental stages, as well as in the midgut tissue of *G. mellonella,* at the highest dietary concentration of triclabendazole. This indicates that increased MDA content at high triclabendazole concentrations induces oxidative stress in response to free radical formation. However, additional studies are needed to determine the mechanisms (other than dietary interaction, life parameter) that underlie how triclabendazole affect on *G. melloenlla*.

Numerous investigations have shown that different anti-fungal and antibacterial agents have deleterious effects on the survival parameters of *G. mellonella,* and that high concentrations of these substances reduce the quality of artificial diets and cause free radical formation [10,18,32-34]. In a study using niclosamide, an anthelmintic in the salicylanilide group, it was found that a concentration of 0.1% increased MDA content and GST activity in the midgut of *G. mellonella* by 4-fold and 2-fold, respectively [6]. These results are similar to ours, where high concentrations of triclabendazole increased MDA content and GST activity in the larva, pupa, and adult stages of *G. mellonella*, as well as in the midgut tissue. Antioxidant enzymes are known

to be highly sensitive to reactive molecules that occur under oxidative stress. GST is a detoxifying and antioxidant enzyme that removes lipid peroxidation products or the hydroperoxides of cells [35]; therefore, the free radicals that occurred with high concentrations of triclobendazole may have been removed by increasing GST activity.

Insects require essential biomolecules such as proteins, carbohydrates, lipids, enzymes, and vitamins for growth, development, and reproduction [36]. In a study investigating the antihelmintic oxyclozanide that was added to the artificial diet of *G. mellonella*, the authors found that it increased the amount of total protein; although, it had a negative effect on the survival rate in the larvae, pupae, and adult stages. The researchers suggested that the chemical reduced diet quality and consequently decreased the consumption of diet. On the other hand, they also suggested that the chemical increased the amount of total protein, which may have been caused by the developing tolerance of insect by using this substance in an effective way [10]. In another study, triclabendazole was added to the artificial diet of *G. mellonella*. The anthelmintic affected the chemical and physical components of the diet and possibly altered the feeding behavior of the larvae, which may have led to the biological characteristics of the insect being adversely affected [7]. In our study, we believe that high concentrations of triclabendazole had a negative effect on the diet quality of the insect, which led to increased MDA content and GST activity. In a similar study, terbinafine was provided to *G. mellonella* at concentrations of 0.001, 0.01, 0.1, and 1% and was found to adversely affect survival and development. Moreover, MDA and PCO content in the midgut tissue increased, and consequently, GST enzyme activity increased [22]. In another study, the effects of gemifloxacin (at concentrations of 150, 300, 600, and 900 mg/L) on *Drosophila melanogaster* was investigated. The authors found that the survival and development of the insect was adversely affected in a dose-dependent manner, and both MDA content and GST enzyme activity were increased, especially at a dose of 300 mg/L, as a result of oxidative damage in the eggs of the insect [18]. In a study by Güneş and Büyükgüzel [17] four different concentrations of boric acid (10, 100, 200 and 300 mg/L), was added to the artificial diet of *D. melanogaster*, which significantly increased MDA content in the final larval stage of the insect. The authors also reported that MDA and GST activity significantly increased in eggs collected from female individuals.

The larval midgut epithelium of *G. mellonella* is an area that is constantly renewed by via apoptosis, and a new epithelium is formed during the transition from the larval stage to the pupal stage [19,32,37,38]. The intestines of Lepidoptera insects are alkaline, with wide reduction-oxidation potential, which are sensitive to oxidative injury during digestion [34]. Hence, the effect of reactive oxygen species -stimulated oxidative stress in the midgut can occur by disrupting the antioxidant defense system [38]. In this study, it appears that the increase in MDA content in the midgut tissue of *G. mellonella* initiated increased GST activity.

Numerous studies have been conducted to evaluate the effect of xenobiotics, including triclabendazole, on the survival, development, and other biological aspects of different insect species [6-10,18,21,34]. In addition to how chemicals with different mechanisms of action and clinical significance effect these parameters, information regarding the stress mechanisms and antioxidant enzyme capacities in insects have been investigated. According to the literature review, this is the new study to investigate the oxidative effects of different concentrations of triclabendazole fed to *G. mellonella* at three different developmental stages, as well as its effects on the midgut tissue. Our results demonstrate that oxidative stress occurs in *G. mellonella* at high concentrations of triclabendazole and affects its detoxification capacity. According to our results, we may be recommended the 0.1% triclabendazole concentration for use in the field. However, before applying in the field, its effects on other non-target creatures should also be considered. We believe these results will contribute to bodies of research that investigate new, environmentally-friendly, alternative chemicals in insect pest management and on its use as antimicrobial agent in artificial diet conducted under laboratory conditions.

Conflict of Interest

The authors have declared that no competition interests.

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Author Contributions

EB and KB planned and designed the research procedure. EB performed the biochemical analysis and the *Galleria mellonella* culture. EB and KB carried out the statistical analysis. The manuscript was written by EB and KB. KB contributed to the language editing of the final manuscript. Both authors have interpreted the data, revised the manuscript for contents, and approved the final version.

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