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Research Article/Araştırma Makalesi

Assessment of The Effect of *Thymbra capitata* Ethanolic Extract on *Galleria mellonella* Hemolymph Antioxidant Enzymes

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Abstract: Conehead thyme (*Thymbra capitata*) is widely distributed in the countries of the Mediterranean region and used due to its medical properties. The antibacterial, antifungal, and strong antioxidant properties of *T. capitata* are known. The model organism *Galleria mellonella* is mostly preferred for immunological studies and for the study of human pathogens. The aim of the study was to determine the effect of the ethanolic extract of *T. capitata* on the antioxidant defense of the hemolymph in *G. mellonella* larva. Solutions prepared with Phosphate-Buffered Saline (PBS) from the dry matter obtained from ethanolic extract at doses between 2 mg mL^{-1} and 20 mg mL^{-1} were injected into *G. mellonella* larvae. According to our findings, *T. capitata* extract had no effect on malondialdehyde (MDA) levels. However, it was determined that all doses between 10 to 20 mg mL^{-1} significantly reduced superoxide dismutase (SOD) and catalase (CAT) activities compared to the control groups. According to the results of our study, high doses of *T. capitata* extract had negative effects on *G. mellonella* antioxidant defense.

Keywords: Malondialdehyde, superoxide dismutase, catalase, total protein.

Thymbra capitata Etanolik Ekstraktının *Galleria mellonella* Hemolenf Antioksidan Enzimleri Üzerine Etkisinin Değerlendirilmesi

Öz: Acı kekik (*Thymbra capitata*), Akdeniz bölgesi ülkelerinde geniş yayılış göstermektedir ve medikal özelliklerinden dolayı kullanımı yaygındır. *T. capitata*'nın antibakteriyel, antifungal ve güçlü antioksidan özellikleri bilinmektedir. Model organizma *Galleria mellonella* bağışıklık araştırmaları için ve insan patojenlerinin araştırılmasında çoğunlukla tercih edilmektedir. Çalışmanın amacı, *T. capitata*'nın etanolik ekstraktının *G. mellonella* larvasında hemolenf antioksidan savunması üzerindeki etkisinin belirlenmesidir. Etanolik ekstraktan elde edilen kuru maddeden Fosfat Tampon Tuzu (PBS) ile 2 mg mL^{-1} ile 20 mg mL^{-1} arasındaki dozlarda hazırlanan çözeltiler *G. mellonella* larvalarına enjekte edilmiştir. Bulgularımıza göre *T. capitata* ekstraktının malondialdehit (MDA) düzeylerine etkisi yoktur. Ancak 10 - 20 mg mL^{-1} arasındaki dozların hepsinin kontrol gruplarına kıyasla süperoksit dismutaz (SOD) ve katalaz (CAT) aktivitelerini önemli ölçüde azalttığı belirlendi. Çalışmamızın sonuçlarına göre *T. capitata* ekstrakt yüksek dozları *G. mellonella* antioksidan savunması üzerinde olumsuz etkilere sahiptir.

Anahtar kelimeler: Malondialdehit, süperoksit dismutaz, katalaz, toplam protein.

1. Introduction

Conehead thyme (*Thymbra capitata*) is a medicinal and aromatic plant species that grows in the Mediterranean region and has important pharmacological properties related to its essential oil (Gagliano Candela et al., 2019). It is used as a source of antioxidants in the food industry (Blanco-Salas et al., 2010). Some medicinal properties of *T. capitata* extracts is antifungal (Palmeira-de-Oliveira et al., 2012), antispasmodic (Al-Qura'n, 2009), anti-inflammatory (Albano & Miguel, 2011), antiprotozoan (Machado et al., 2010), antibacterial (Marinelli et al., 2018), and antioxidant (Hortigón-Vinagre et al., 2014) capacity. It is the result of another study in which essential oils of *T. capitata* showed nematicidal activity against Pinewood nematode, *Bursaphelenchus xylophilus* (Barbosa et al., 2010). In the study of Vila (2002), *T. capitata* contained over 70% carvacrol (Faleiro et al., 2005; Saija et al., 2016) and thymol as a secondary important component and its antioxidant capacity was mainly due to these two contents (Gagliano Candela et al., 2019). Carvacrol (5-isopropyl-2-methyl phenol) is also a natural compound that occurs in the leaves of many herbs and plants including wild bergamot, thyme, and black pepper (Marchese et al., 2018). Studies on broiler chickens revealed that carvacrol and thymol increased antioxidant activity and supported immunity and growth (Acamovic & Brooker, 2005; Hashemipour et al., 2013; Silveira et al., 2013; Du et al.,2016).

Model organisms are generally defined as non-human species that have been extensively studied to understand a range of biological phenomena (Leonelli & Ankeny, 2013). The model organism is selected according to the biological phenomenon and the characteristics to be studied. In recent years, the importance of invertebrates among model organisms has increased when ethical concerns, the cost of growing specimens, the growth rate of specimens, and the physical conditions required for the cultivation of specimens are evaluated. Invertebrate model organisms include *G. mellonella* (Lepidoptera): among other things, its ability to withstand high temperatures offers unique opportunities for the evaluation of human pathogens. This strain is also the most preferred strain for immune studies (Eguchi & Iwabuchi, 2006; Mukherjee et al., 2010; Cook & McArthur, 2013).

The aim of the study was to determine the effect of *T. capitata* leaf extract on the antioxidant enzymes of the

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model organism *G. mellonella* hemolymph. *T. capitata* is consumed as a spice and for its therapeutic effects. The role of *T. capitata* in the regulation of antioxidant defense system, which is an important part of natural immunity in animals, was clarified with this study.

2. Material and Methods

2.1. Insect Rearing

Galleria mellonella specimens were grown in the Department of Biology, Faculty of Sciences, Çanakkale Onsekiz Mart University, Türkiye under equal photoperiod (12:12 light:dark) conditions under 25±2 °C and 65±5% relative humidity conditions. When the first larvae were seen in the jars created for the experiment, 10 gr of artificial food (Sak et al., 2006) was added to the medium and fed. The last stage larvae (0.18±0.02 gr) grown in these jars were chosen.

2.2. Plant material and extraction

The aerial parts of the *T. capitata* specimens, which grow naturally around the central district of Çanakkale province, were collected during the flowering season and dried naturally in a dark and airy environment in the Insect Physiology Laboratory of the Department of Biology of Çanakkale Onsekiz Mart University. 50 gr of these collected dry leaves were extracted by soxhlet extractor. At the end of this period, the ethanol was removed by rotary evaporator (OMNILab, China). The remaining 2 gr dry matter was dissolved in 100 mL phosphate buffer saline (PBS - Sigma, Germany pH: 7.4). The highest solubility levels of the dry material were determined as a 2 gr 100 mL^{-1}.

2.3. Injection

The Control (Untreated) and PBS injected groups were used as the control groups. As for the dose groups, 2-20 mg mL^{-1} doses prepared in PBS were injected into the body cavity from the last proleg of the *G. mellonella* larvae with the help of a 5 µl microinjector (Hamilton, USA). This procedure was performed at four different times (four repetitions) and using 4 larvae in each repetition (n=16). Both control and injected larvae were subjected to a 24-hour waiting period in glass petri dishes under the same temperature, humidity, and light conditions as the main colony-rearing conditions.

2.4. Hemolymph collection

24 hours after the injection, 20 µl of the hemolymph leaking from the larvae was taken and placed in microcentrifuge tubes containing 180 µl of phosphate buffer solution. This prepared hemolymph/phosphate buffer mixture was centrifuged for five minutes at 12 000 rpm at +4 °C. Afterwards, the cell-free supernatant was collected and stored at -20 °C until the experiments were performed.

2.5. Enzyme assays

All the studies for the determination of enzyme activities were carried out in a microplate reader (Thermo Scientific Multiskan GO Microplate Spectrophotometer, Finland) at the appropriate wavelength. In our study, for each dose and the control groups, a total of 16 larval hemolymph were collected in four independent time periods. The enzyme activity and total protein (TP) measurement were performed in four independent times after all four larval hemolymph was collected each time.

The TP determination in the study was carried out using the Bradford (1976) method. A 5 µl sample taken from the prepared hemolymph-phosphate buffer mixture was placed in the well of the 96-well F base microplate. A 155 µl of distilled water and 40 µl of Bradford reagent were added to the sample. After the prepared microplate was incubated for 30 minutes at room temperature, measurement was performed at 595 nm absorbance in the microplate reader. The results were determined as mg protein mL^{-1}.

While determining the Superoxide Dismutase (SOD) activity, the method of Flöhe and Ötting (1984) was used. The prepared hemolymph/phosphate buffer mixture was incubated for 20 minutes under bright light at room temperature by adding 3.5 µl of xanthine oxidase and 190 µl of SOD reagent on the 6.5 µl sample. After incubation, 6.5 µl of $CuCl_2$ (0.8 mM) was added to each well. The microplate prepared after the procedures was measured at 560 nm absorbance. The results obtained were calculated as unit mg $protein^{-1}$.

The Aebi (1984) method was used to determine the Catalase (CAT) activity. According to this, 60 µl of phosphate buffer (50 mM, pH=7.2) and 133.5 µl of H_2O_2 (30 mM) were added to the 6.5 µl sample of hemolymph/phosphate buffer mixture and kinetic measurements were taken at 240 nm absorbance for two minutes with 10-second intervals. The results were determined as mmol min^{-1} mg $protein^{-1}$.

While determining malondialdehyde (MDA) levels, the Buege and Aust (1978) method was used. While applying the method, 150 µl of TBA-TCA mixture was added to the 75 µl sample of hemolymph/phosphate buffer mixture and incubated at 90 °C for 20 minutes. After incubation, the measurement was made at 532 nm absorbance. The results were presented as nmol mg $protein^{-1}$.

2.6. Statistics

The obtained data were evaluated with one-way-ANOVA ($p<0.05$) and Tukey's HSD test in the SPSS statistical program.

3. Results

3.1. Hemolymph TP

The TP levels determined in the *G. mellonella* larval hemolymph after extract injection are presented in Figure 1. Accordingly, there was no significant difference between the groups in terms of TP. The highest TP value was determined in the 20 mg mL^{-1} dose group as 1.07 mg protein mL^{-1}. In the control group, it was determined as 1.025 mg protein mL^{-1}.

3.2. Hemolymph SOD activity

The data obtained as a result of the experiments of the study for the determination of SOD activity are shown in Figure 2. Accordingly, no significant difference was found between the control and PBS injection groups and the 2-8 mg mL^{-1} injection groups ($p>0.05$). However, a significant difference was determined between these groups and the 10-20 mg mL^{-1} injection groups ($p<0.05$). At the same time,

the difference between the 10-20 mg mL^{-1} injection groups was found to be insignificant (p>0.05).

3.3. Hemolymph CAT activity

The changes in CAT activity as a result of the injection of *T. capitata* doses in *G. mellonella* larval hemolymph are presented in Figure 3. The results obtained are similar to the results of SOD activity. No significant difference was found between the control groups and the 2-8 mg mL^{-1} injection groups (p>0.05). At the same time, the difference between the 10-20 mg mL^{-1} injection groups was found to be insignificant (p>0.05). However, a significant difference was found between these groups and the 10-20 mg mL^{-1} injection groups (p<0.05).

3.4. Hemolymph MDA levels

The MDA level results of the study are shown in Figure 4. According to the experimental results, the injection of *T. capitata* doses did not cause significant changes in the MDA level in *G. mellonella* larval hemolymph (p>0.05).

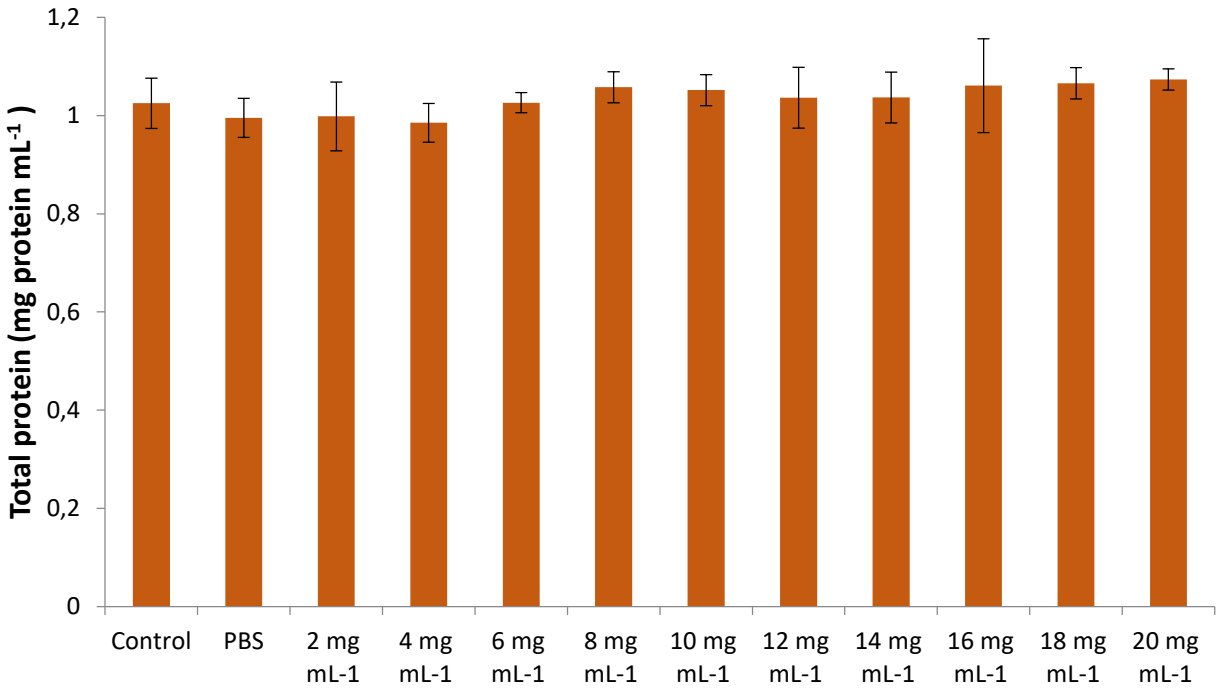

Figure 1. Total protein levels in *G. mellonella* larval hemolymph exposed to *T. capitata* extract. Each column represents the mean of 16 samples.

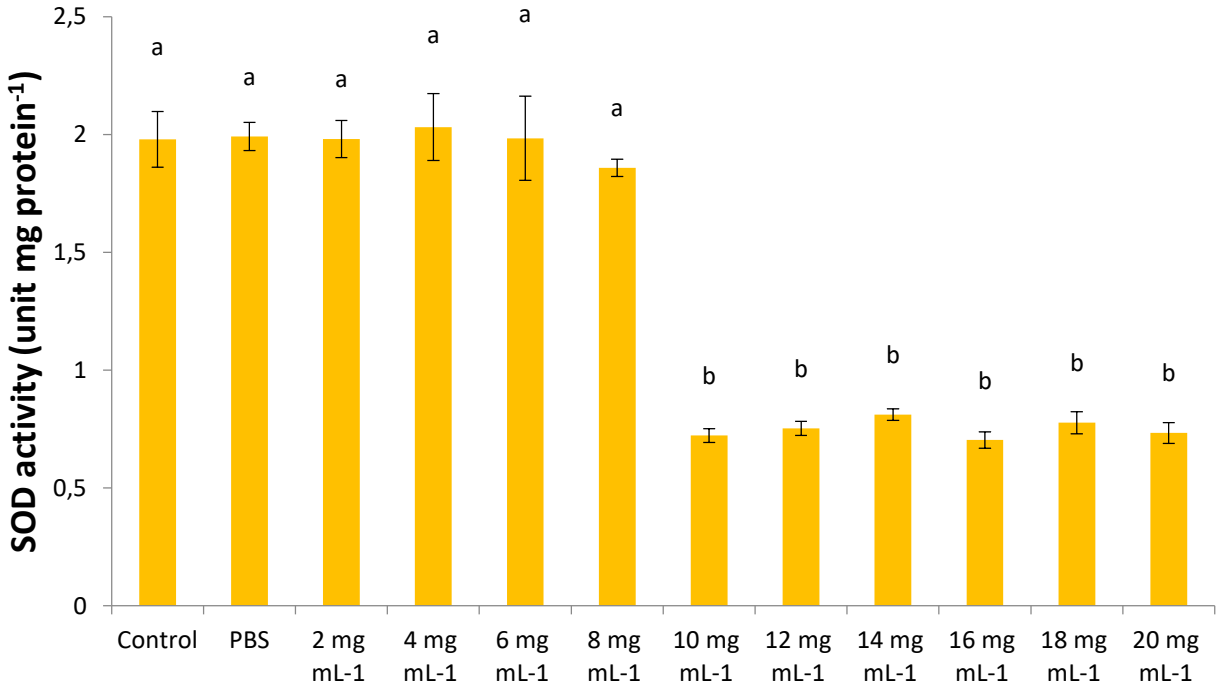

Figure 2. SOD activity changes in *G. mellonella* larval hemolymph exposed to *T. capitata* extract. Each column represents the mean of 16 samples. The difference between groups with different letters (a-b) was statistically significant (p<0.05).

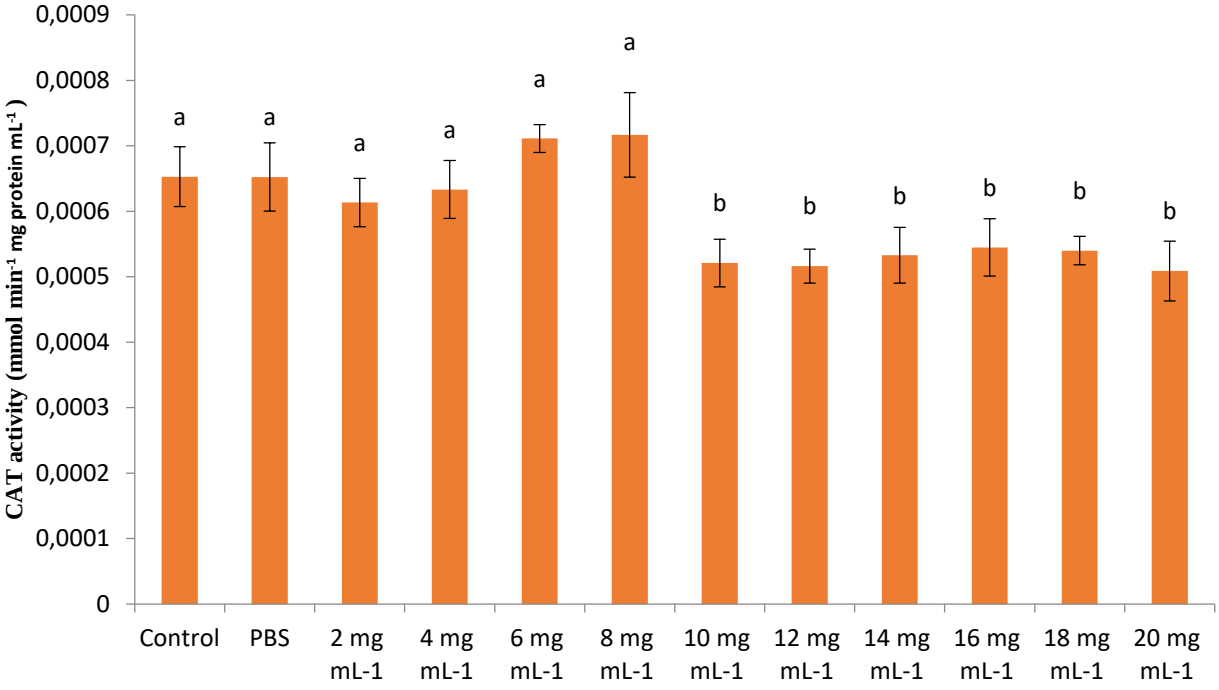

Figure 3. CAT activity changes in *G. mellonella* larval hemolymph exposed to *T. capitata* extract. Each column represents the mean of 16 samples. The difference between groups with different letters (a-b) was statistically significant ($p<0.05$).

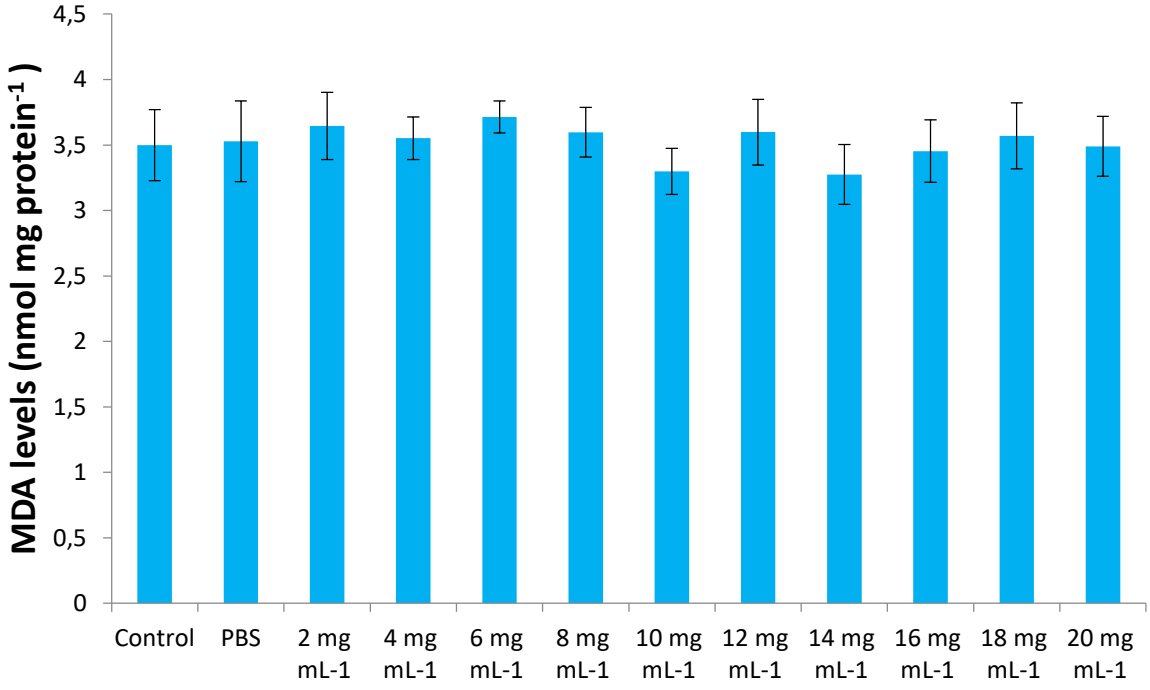

Figure 4. MDA levels changes in *G. mellonella* larval hemolymph exposed to *T. capitata* extract. Each column represents the mean of 16 samples.

4. Discussion

It is of vital importance to remove the oxygen radicals that occur as a result of normal body functions from the body. Hemolymph antioxidants are considered an important marker in immune studies as they support immunity in insects.

Studies reported that extracts of *T. capitata* showed strong antioxidant properties (Faleiro et al., 2005; Saija et al., 2016). In a study with Gilthead seabream (*Sparus aurata* L. - Sparidae), it was determined that the addition of *T. capitata* extract to the food caused lower indices of oxidation (Álvarez et al., 2012). In a study conducted with New Zealand white rabbits, it was determined that thymol reduced oxidative stress by showing antioxidant activity and could suppress the progression of hyperlipidemia and atherosclerosis due to high-fat diet by attenuating inflammatory responses (Yu et al., 2016).

There are many studies examining the effects of various natural or chemical substances on the antioxidant enzymes of the model organism *G. mellonella*. Lozinskaya

et al. (2004) found that free radical formation decreased due to suppression of the prophenoloxidase system and increased antioxidant activity in *G. mellonella* hemolymph in the sprogonium stage of microspores. It was determined that the antioxidant enzyme activity of *G. mellonella* larvae was not affected by increasing concentrations of boric acid (Hyršl et al., 2007). Dubovskiy et al. (2008) reported that *Bacillus thuringiensis* ssp. *galleriae* infection of *G. mellonella* increased the oxidative stress level in the larval midgut and that oxidative damage contributed to cell death in the midgut during infection in their study. Dubovskii et al. (2010) revealed a statistically significant increase in reactive oxygen species (ROS) formation in lymph and a decrease in enzymatic antioxidant activities in insect hemocytes after foreign body injection. In another study, researchers found that eicosanoids mediated enzymatic responses to insect antioxidants and dietary pro-oxidants (Büyükgüzel et al., 2010). In another study on *G. mellonella* with boric acid, it was found that catalase, superoxide dismutase, glutathione S-transferase, and glutathione peroxidase activities increased in relation to lipid peroxidation (Büyükgüzel et al., 2013). Büyükgüzel and Kayaoğlu (2014) found in their study that niclosamide had negative effects on the antioxidant enzymes of *G. mellonella*. Dere et al. (2015) examined the effects of azadirachtin on the antioxidant enzymes of *G. mellonella* in their study and found that it increased oxidative stress in this species depending on the dose. It was found that heavy metals increased the oxidative stress of *G. mellonella* (Wu & Yi, 2015). Zorlu et al. (2018) found that titanium dioxide nanoparticles included in the *G. mellonella* diet had a dose-dependent toxic effect and increased resistance to oxidative stress at low concentrations. Increasing doses of Cucurbitacin-E essential oil caused decreasing in SOD, CAT, GST GPx, GR, and AChE activities and increasing in MDA levels (Ercan et al., 2022).

As a result of a study examining the toxic effects of different plant extracts on *G. mellonella* larvae, the high variability of Lethal Dose (LD) values from one plant to another indicates that *G. mellonella* is highly sensitive to plant extracts and that *G. mellonella* can be used as a reliable system model for the evaluation of the toxicity of medicinal plants (Mbarga et al., 2021).

When evaluated in terms of the results of our study, it is seen that *T. capitata* extract does not affect lipid peroxidation and does not change the amount of TP in *G. mellonella* larval hemolymph. In the literature, it is seen that some substances have no effect on *G. mellonella* antioxidant enzymes, and some of them decrease antioxidant enzyme activities depending on the dose. In the results of our study, it was determined that a high rate (10 mg mL^{-1}) of *T. capitata* extract decreased SOD and CAT activities depending on the dose. These results are compatible with the literature.

Different effects on immunity are expected as different plants have different toxic effects on *G. mellonella* larvae (Mbarga et al., 2021). There are limited studies on the effects of medicinal plant extracts on *G. mellonella* immunity. Some plant extracts cause a decrease in the antioxidant enzyme activity of *G. mellonella* (Ercan et al., 2022), and this decrease is probably due to the effect of plant secondary metabolites. It was determined that *Helichrysum arenarium* increased cell-mediated immunity at low doses in *G. mellonella* larvae at certain doses and had no effect outside this range (Kaya et al., 2021). According to the results of the study with *Olea europea* leaf extract, immune responses were strengthened at the lowest dose in *G. mellonella* larvae (Kaya & Demir, 2020).

It is thought that the most important reason why *T. capitata*, which has strong antioxidant properties, cannot show these effects in the living body is the dilution of the enzyme rate when it enters the living body and the lack of optimum enzyme activity environment. It was also determined that Carvacrol nanoemulsion caused apoptosis in human lung adenocarcinoma (A549) cells by inducing the production of ROS (Khan et al., 2018).

According to our findings, the injection of *T. capitata* significantly reduced antioxidant enzyme activity in the model organism above a certain dose (10 mg mL^{-1}). This result shows that the use or consumption of this plant species, which is also preferred as a spice, in high doses reduces antioxidant enzyme activities which are critical for immunity. The results of our study support the results of Khan et al. (2018) study. Therefore, it is thought that the consumption of *T. capitata* should be within certain limits.

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Conflict of interest: The author declared that there is no conflict of interest.

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