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Induction of Antioxidant Enzyme Activity by Sandy Everlasting (*Helichrysum Arenarium*) in *Galleria mellonella*

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Abstract

Helichrysum arenarium (L.), which spreads in a wide geography between European and Asia, is a medicinal plant mostly used in detoxification and digestive system diseases in that region. The greater wax moth *Galleria mellonella* is preferred as an invertebrate model organism in many studies to understand the effects of many disease factors, including human pathogens, treatments developed against them, and innate immunity. The aim of this study is to determine the effects of *H. arenarium* extracts, which were previously determined to be effective on *G. mellonella* cell-mediated immune responses, on *G. mellonella* hemolymph antioxidant enzymes. For this purpose, catalase, superoxide dismutase, glutathione-s-transferase activities and malondialdehyde levels in hemolymph collected after injection of *H. arenarium* extracts into *G. mellonella* hemocoel were determined. As a result of the study, it was determined that a 0.5% dose increased catalase, superoxide dismutase and glutathione-s-transferase activities compared to the control. The determined dose had no effect on malondialdehyde formation. The findings showed that *H. arenarium* extracts had a positive effect on *G. mellonella* hemolymph antioxidant enzymes at a dose of a 0.5%, and that the same dose supported cell-mediated immunity in the previous study. Accordingly, it was concluded that 0.5% dose of *H. arenarium* extracts had a immunomodulatory effects on immunity.

Keywords: *Helichrysum arenarium*, *Galleria mellonella*, catalase, superoxide dismutase, glutathione-s-transferase, malondialdehyde.

1. Introduction

Today, plants with antioxidant properties have reached an increasing popularity as health support products. The perennial herbaceous plant known as Sandy everlasting (*Helichrysum arenarium*) is classified under the Asteraceae family and is indigenous to Europe, Central Asia, and China [1]. It is prevalent in Europe, particularly in nations located in the middle and eastern regions, and is recognised in traditional medicine for its application in treating many diseases [2]. The primary bioactive constituents of *H. arenarium* are flavonoids, containing chalcone isosalipuroside, and flavanone salipurposide, prunin and naringenin as the most important constituents [3]. The other compounds found in significant amounts in *H. arenarium* are phthalides, carotenoids, essential oil, and yellow pigments [3]. In traditional medicine, *H. arenarium* (L.) is utilised as a cholagogue for the treatment of dyspeptic illnesses [4]. Additionally, it is utilised as a choleretic, diuretic, moderate spasmolytic, hepatoprotective

agent, and detoxification agent in tradetional medicine [5]. Although the importance of the effect of phalavonoids on antioxidants in Helichrysum species has been emphasized, there are no clinical data on testing *H. arenarium*-based extracts or preparations [6]. However, it is considered that *H. arenarium* flavonoids may show choleretic, hepatoprotective and antimicrobial properties [7].

The evolutionary achievements of insects can be attributed, in part, to their capacity to develop a complex, efficient, and flexible defence mechanism against a wide range of pathogens, including pathogenic fungus [8]. Due to its structural and functional similarities to the mammalian innate immune system, the insect immune response is often used to test the virulence of microbial pathogens or the efficacy of antimicrobial agents [9]. Compared to traditional mammalian models, the larvae of *Galleria mellonella*, an invertebrate species, have significant advantages. These benefits include their large-scale availability, affordability, and ease of use and maintenance, which do not require specialised laboratory equipment [10]. Additionally, their usage does not necessitate ethical permission, and the fact that they have a relatively short life cycle makes them an excellent choice for conducting investigations on a big scale [11-13].

Antioxidant enzymes are vital to protect against the effects of oxygen radicals, which are formed as a result of immune responses. The effect of antioxidant properties of *H. arenarium* [14-16], which showed high antioxidant properties in previous studies, is a phenomenon that has not been revealed yet.

The previous study [17] determined that *H. arenarium* increased encapsulation and melanization immune response, total hemocyte count and phenoloxidase enzyme activity in *G. mellonella* larvae at certain doses. This study is built on the results of the previous study. The purpose of this research is to assess the impact of *H. aranerium* extract on antioxidant enzymes in *G. mellonella* larvae. The findings gathered from this investigation will provide to reveal basic information about the potential impacts of *H. aranerium* extract on other living things.

2. Material and Methods

2.1. Insect Rearing

As in the previous study (Kaya et al. 2021), the larvae in this study were obtained using adult female and male moths obtained from the successive culture in Çanakkale Onsekiz Mart University Insect Physiology Laboratory. Both the mother colony and the subjects were grown at 29±1 °C under conditions of 65% relative humidity and constant darkness. The artificial food [18] was used for the rearing of the larvae. The last instar larvae (0.18 ± 0.02 g) were selected and used for injection.

2.2. Plant Materials and Doses

After *H. arenarium* specimens were obtained from local markets, botanist Dr. Ersin Karabacak was diagnosed them. The plant samples were ground in the laboratory and the extract was obtained with 70% ethanol in a soxhlet device (Isolab, Germany). The dry matter was obtained by removing ethanol from the obtained extract with a rotary evaporator (Omnilab, China). The obtained dry matter was prepared by dissolving it in 10% Dimethyl Sulfaoxide (DMSO) at the rates of 0.1%, 0.25%, 0.5% and 1%, which were the doses used in the previous study [17]. In our previous study, only the data of the DMSO group could be compared with the data of the other dose injection groups due to the statistical difference between the untreated sample and the DMSO injection group. Therefore, only the DMSO injection group was determined as the control group in this study.

2.3. Injection

For each repetition of these prepared doses, 4 larvae in each group were injected with a microinjector

(Hamilton, USA). Before injection, the larvae were surface sterilized with 70% ethanol. A total of 16 samples were used in 4 replicates for each dose (n=16) and waited for 24 hours after injection. At the end of the waiting period, 30 µl of hemolymph was taken from the anterior segment of the first proleg of each larva with a sterile needle and added to the microcentrifuge tube containing 270 µl of phosphate buffer. The resulting mixture was centrifuged at 10000 rpm (IKA, Germany). After centrifugation, 290 µl of supernatant was taken and transferred to a microcentrifuge tube containing 0.001 mg of N-Phenylthiourea (1-phenyl-2-thiourea) [19] crystal and stored at -80ºC. After all repetitions were completed, the determination of the antioxidant enzyme activities was carried out.

2.4. Antioxidant Enzyme Acitivity

Catalase (CAT), Superoxide Dismutase (SOD), glutathione-s-transferase (GST) and Lipid Peroxidation (MDA) kits were used to determine the antioxidant enzyme activities. All kits used were manufactured by Sigma Aldrich (Germany). In these kits, experiments were carried out using the manufacturer's prescribed procedure. All experiments were carried out by determining absorbances at appropriate wavelengths in a microplate reader (ThermoFisher MultiscanGo, Finland) in Çanakkale Onsekiz Mart University Experimental Research Application and Research Center (ÇOMÜDAM).

Total Protein (TP). The Bradford [20] method was used to determine the total protein amount. Accordingly, 5 µl of hemolymph mixed with Phosphate buffer was placed on the F base microplate, and 155 µl of sterile distilled water and 40 µl of Bradford's reagent (Sigma Aldrich, Germany) were added and incubated for 30 minutes at room temperature. After incubation, absorbance reading was performed at 595 nm wavelength in the microplate reader. By using bovine serum albumin (BSA) as protein standard in the same device, Bradford curve (Figure 1) was extracted and the formula to be used for protein calculation in hemolymph was determined.

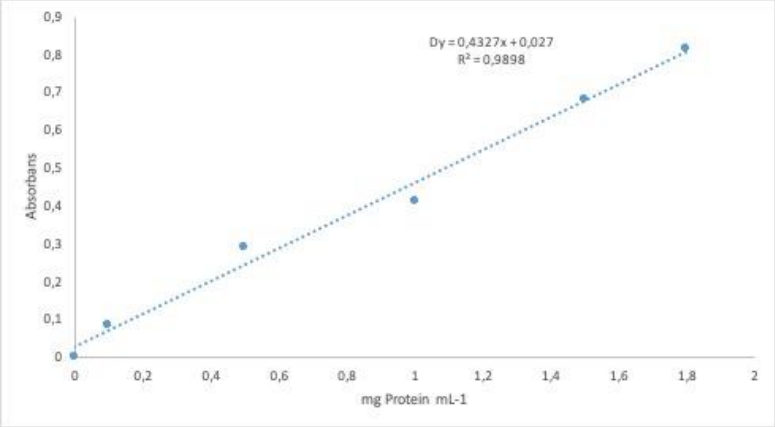

Figure 1. Bradford curve and formulation.

2.5. Statistics

The data obtained in the study were evaluated with the Tukey HSD test and the SPSS v22 (IBM, USA) program with a confidence interval of 95% by one-way analysis of variance (ANOVA).

3. Results

3.1. Catalase (CAT)

According to the results of the experiment to determine the CAT activity of the study, the 0.5% dose difference compared to the control group was statistically significant ($p<0.05$). The results of the study

are presented in Figure 2. According to the data obtained, the control group (DMSO) CAT activity was 0.00191 mmol min^{-1} mg protein mL^{-1}, while the mean 0.5% *H. arenarium* dose injection group was 0.00243 mmol min^{-1} mg protein mL^{-1}. The difference between the other dose injection groups with the DMSO group and the 0.5% *H. arenarium* dose injection group was insignificant ($p>0.05$).

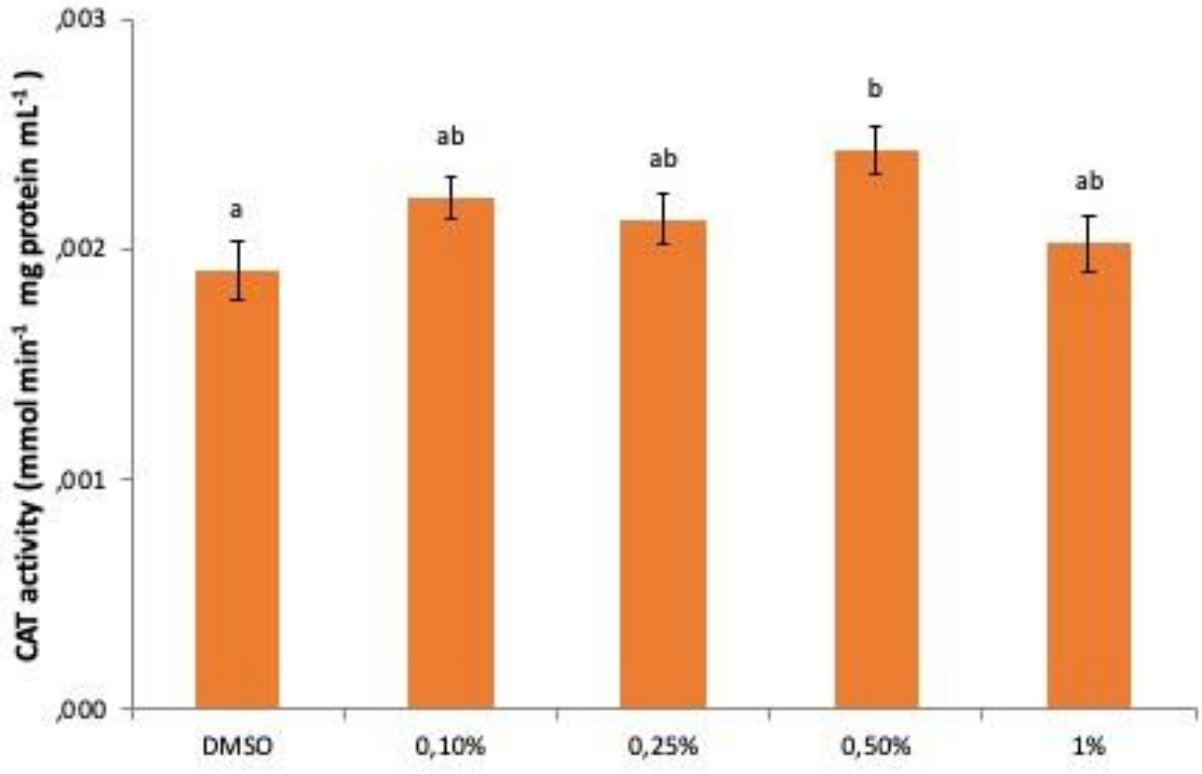

Figure 2. Changes in CAT activity in *G. mellonella* larval hemolymph after injection of *H. arenarium* extract. Each data was the mean of 16 larvae in four replicates. The difference between bars with different letters is significant (a-b) ($p<0.05$).

3.2. Superoxide dismutase (SOD)

The changes in SOD activity in *G. mellonella* larval hemolymph after *H. arenarium* injection are presented in Figure 3. According to the results obtained, the highest SOD activity was determined in the 0.5% *H. arenarium* dose injection group (0.313 u mg $protein^{-1}$). The difference between this group and the injection groups of DMSO (0.223 u mg $protein^{-1}$), 0.1% and 0.25% *H. arenarium* doses was significant ($p<0.05$). Similarly, while the difference between the 1% injection group and the DMSO group and the 0.1% injection group was significant ($p<0.05$), the difference between the other groups was insignificant ($p>0.05$).

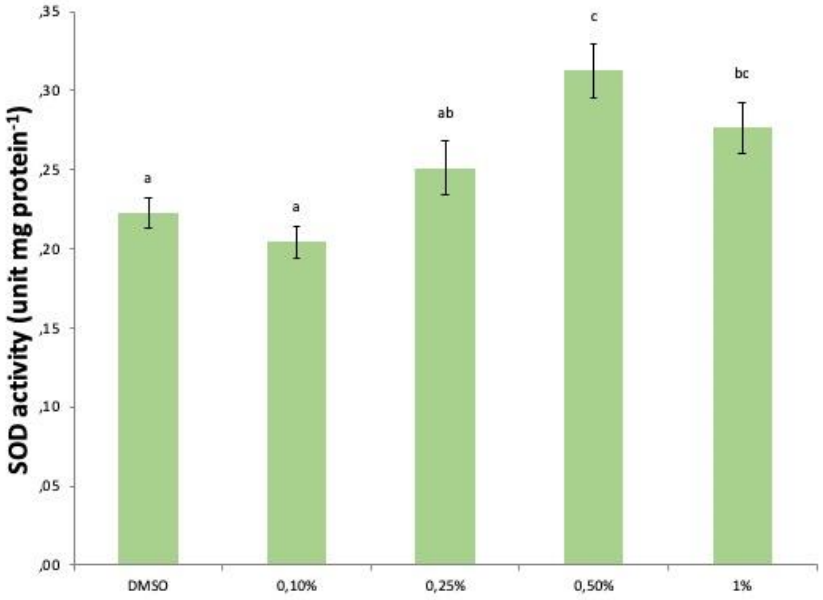

Figure 3. Changes in SOD activity in *G. mellonella* larval hemolymph after *H. arenarium* extract injection. Each data was the mean of 16 larvae in four replicates. The difference between bars with different letters is significant (a-c) (p<0.05).

3.3. Glutatyon-S-Transferaz (GST)

The results of the studies to determine GST activity in larval hemolymph are shown in Figure 4. According to the results obtained, the highest GST activity was in the 0.5% (2,133 nmol min^{-1} mg $protein^{-1}$) dose injection group. The difference between this group and the DMSO (1,753 nmol min^{-1} mg $protein^{-1}$) injection group was significant. In addition, the difference between the 0.25% dose injection group and the DMSO group was also significant ($p<0.05$). The difference between the other injection groups and the DMSO group was found to be insignificant.

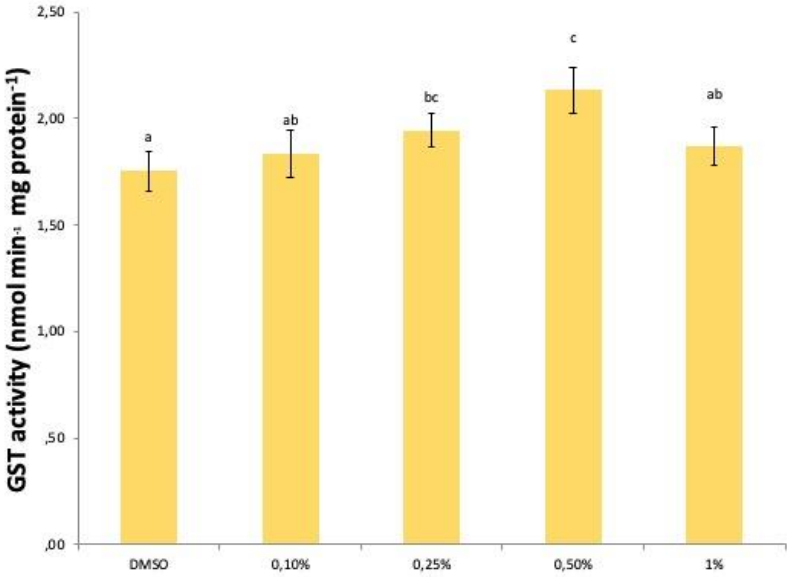

Figure 4. Changes in GST activity in *G. mellonella* larval hemolymph after *H. arenarium* extract injection. Each data was the mean of 16 larvae in four replicates. The difference between bars with different letters is significant (a-c) (p<0.05).

3.4. Malondialdehyde (MDA)

The results of the experiment to determine the MDA level formed as a result of lipid peroxidation in *G. mellonel*la hemolymph are given in Figure 5. Accordingly, only the 0.1% (9.480 nmol mg protein^{-1}) *H. arenarium* dose injection group showed a significant increase in MDA formation compared to DMSO (7.984 nmol mg protein^{-1}) ($p<0.05$). The difference between the other groups with DMSO was found to be insignificant. Accordingly, 0.1% dose of *H. aranerium* caused an increase in lipid peroxidation. The difference between the other dose injections with each other and with these groups was found to be insignificant ($p>0.05$).

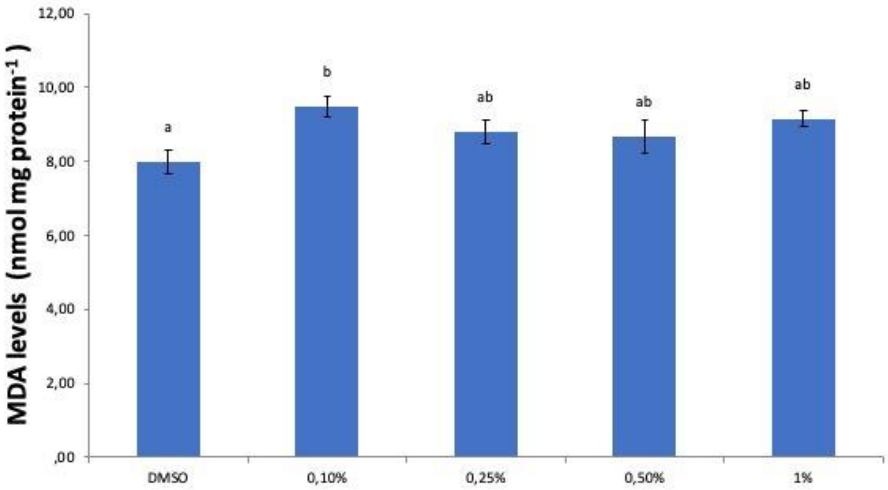

Figure 5. Changes in MDA activity in *G. mellonella* larval hemolymph after *H. arenarium* extract injection. Each data was the mean of 16 larvae in four replicates. The difference between bars with different letters is significant (a-b) ($p<0.05$).

3.5. Total Protein (TP)

The TP results of our study are presented in Figure 7. Accordingly, the difference between the 0.5% (0.934 mg protein mL^{-1}) and 1% (0.966 mg protein mL^{-1}) injection groups was significant ($p<0.05$). The mean TP of the DMSO injection group was determined as 0.951 mg protein mL^{-1}.

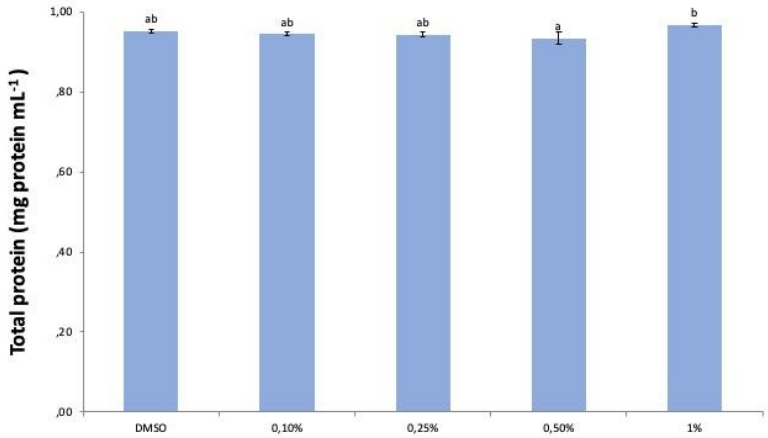

Figure 6. Changes in TP activity in *G. mellonella* larval hemolymph after *H. arenarium* extract injection. Each data was the mean of 16 larvae in four replicates. The difference between bars with different letters is significant (a-b) ($p<0.05$).

4. Discussion

According to the results of our study, the CAT, SOD and GST activities increased in the 0.5% injection group compared to the DMSO injection group, which was the control group, in *G. mellonella* larval hemolymph after *H. arenarium* extract injection. However, the 0.5% injection group did not differ with the control group in MDA formation. The difference between the control group and the 1% injection group in terms of SOD activity was also significant, but the other injection groups did not show any difference. In terms of GST activity, the 0.25% injection group made a significant difference compared to the control. Only 0.10% injection group had an effect on MDA formation.

Helichrysum plicatum ssp. plicatum was determined to provide the expected benefit from its use in folk medicine by showing antihypoglycemic and antioxidant properties in rats made diabetic by induction of streptozotocin [21]. According to another study conducted with *H. plicatum*, it was determined that the ethanolic extract of this plant had high antimicrobial activity [22]. It was determined that Helichrysum species distributed in Turkey had strong antioxidant properties [14]. It showed strong antioxidant properties in *H. aranerium*, and it was thought that this property was due to the flavonides it contains [15]. It was also thought that *H. aranerium* flavonoids might show choleretic, hepatoprotective and antimicrobial properties [7]. In another investigation, *H. aranerium* extract shown antibacterial efficacy against clinical isolates of methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae* and ampicillin-resistant *Moraxella catarrhalis* [23].

Most of the studies are aimed at determining the antioxidant properties of plant extracts in vitro. However, studies show that plants do not always reveal their antioxidant properties in other living things [24].

Infusions of *Helichrysum italicum* subsp. (HI) or *H. arenarium* (HA) should be given to people who have at least two of the signs of metabolic syndrome, such as high blood pressure, extra body fat, or an irregular lipid or glucose profile [16]. HI infusion consumption showed a beneficial influence on anthropometric parameters [16]. In the same study findings revealed notable reductions in body weight, body mass index, as well as visceral and total body fat [16]. Additionally, it was shown that the group that received the HA infusion experienced a more significant reduction in serum glucose levels and an enhancement in the lipid profile, and furthermore, both groups exhibited increased serum antioxidant characteristics [16].

Numerous research have been conducted to investigate the impact of diverse organic and inorganic chemicals on the antioxidant enzymes of *G. mellonella*. These findings unequivocally demonstrated that the antioxidant defence mechanism of *G. mellonella* was impacted by both organic and inorganic substances. According to the stages of microsporidiosis, it was determined that they had different effects on antioxidant enzymes in *G. mellonella* larvae [25]. It was demonstrated that *E. coli* infection causes oxidative stress and damage in *G. mellonella* larvae [26]. It has been shown that *G. mellonella* larvae infected with a fungal species, *Conidiobolus coronatus*, did not cause a change in MDA level, while GPx level increased and CAT and SOD activity decreased [8]. As a result of the same study, Kazek et al. [8] was suggested that the glutathione repair mechanism had a strong effect on *G. mellonella* larvae after fungal infection.

It was determined that the *Bacillus thuringiensis*, one of the most widely used sources of biorational pesticides, infection caused an increase in SOD and GST in *G. mellonella* midgut and a decrease in CAT level [27]. It was determined that subletal doses of Dichlorvos, an organophosphate type of pesticide, caused oxidative stress and lipid peroxidation [28].

In the study of the effects of chromium (Cr) and lead (Pb) on the immune and antioxidant systems of *G. melonella*, the activities of antioxidant enzymes (SOD, POD, CAT) showed significant increases with increasing dietary Cr and Pb concentrations, and that activity was found to be significantly higher than the control [29]. According to the results of the same study, it was shown that the use of certain amounts of Cr and Pb resulted in significant oxidative stressors [29]. It was determined that low doses of titanium dioxide (TiO2) nanoparticles caused significant increases in *G. melonella* hemolymph total protein, MDA and GST activities, that all doses increased SOD activity, and that high doses caused an increase in CAT [30]. According to the results of the study examining the effects of zinc (Zn) and copper (Cu) in *G. melonella* larvae, while all Cu concentrations increased SOD and CAT activities, only the highest Zn concentrations caused an increase in SOD and CAT activities [31].

It was determined that juglone, which is concentrated in walnut leaves and green shells, caused a change in the antioxidant enzyme activities of *G. melonella* and an increase in lipid peroxidation depending on the increasing dose [32]. In the same study, it was determined that juglone increased SOD and GST activity in LC_{10} and LC_{30} doses compared to the control, increased CAT activity and lipid peroxidation in LC_{30} and LC_{50} compared to the control, and decreased glutathione peroxidase level in LC_{10} and LC_{30} doses compared to the control, and increased it in LC_{50} dose [32].

There are few studies on the effect of plant extracts on the antioxidant properties of *G. mellonella*. In a study evaluating the influence of *Thymbra capitata* ethanolic extract, a plant consumed for its medicinal properties, on the antioxidant properties of *G. mellonella*, it was determined that this plant extract inhibited antioxidant enzyme activity above a certain dose [33]. Kaya [33] study revealed that plants with medicinal properties did not always show the expected effect.

The results of the present study show that *H. arenarium* extract increases the antioxidant enzyme activity of *G. mellonella* at a certain rate (0.5%). In the study examining the *G. mellonella* cell-mediated immune responses of *H. arenarium* extract, it was determined that the plant extract caused an increase in hemocyte count, encapsulation-melanization immune responses and phenoloxidase activity at a certain rate (0.25% and 0.5%) [17].

The results of our study showed that 0.5% use of *H. arenarium* extract increased antioxidant enzyme activity. These results support the results of the studies of Kenig et al. [16] and Kaya et al. [17]. Accordingly, it is thought that *H. arenarium* is a potentially important medicinal plant and that the importance of this plant will be revealed with future studies.

5. ACKNOWLEDGMENTS

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