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The influence of olive leaf extract on the activity of antioxidant enzymes in the Hemolymph of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae)

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Keywords:
Olea europaea,
Galleria mellonella,
Catalase,
Superoxide dismutase,
Malondialdehyde

Abstract — The present study has aimed to examine the effects of olive leaf extract on the antioxidant enzyme activity of the invertebrate model organism *Galleria mellonella* L. (Lepidoptera: Pyralidae) hemolymph. For this purpose, after the olive leaves collected from Çanakkale province were dried at room temperature, the ethanolic extract was obtained with a soxhlet. After the ethanol was removed from the obtained extract in the rotary evaporator, the obtained dry matter was prepared in the phosphate buffer salt, 72 mg mL^{-1} as the highest dose and the lowest 0.010 mg mL^{-1} and injected into the larvae reared for the experiment. Hemolymph was collected at 4, 8, 12, 16, and 24-hours post-injection. Catalase (CAT) and superoxide dismutase (SOD) enzyme activities and malondialdehyde (MDA) formation levels were determined spectrophotometrically in the collected hemolymph. According to the results obtained, olive leaf extract decreased CAT and SOD activity in *G. mellonella* hemolymph at certain doses and increased the amount of MDA. For all enzymes, group, dose, and group-dose interactions were statistically significant. In addition, as a result of the examination made in terms of time, it was seen that the measurement of enzyme activities at the 24th hour was significant. The results of our study show that the antioxidant capacity of the olive leaf cannot be transferred to other living things and even reduces the antioxidant capacity of other organisms.

Subject Classification (2020): 62P10, 92C30

1. Introduction

Olive trees (*Olea europaea*), which naturally spread over the Mediterranean region, had a profound impact on the culture of the region, and the oil extracted from their fruits was utilized in a variety of applications. Ethnomedicine is one of these applications. The Mediterranean diet includes olive oil as well. Recent research indicates that the Mediterranean diet can help prevent cancer and heart disease [1-2]. Olive leaf has traditionally been used to treat malaria and accompanying fever [3]. According to numerous studies, olive leaf extract and its components, especially oleuropein and hydroxytyrosol, have antioxidant and antibacterial qualities that are beneficial to health [4]. The hydroxytyrosol, tyrosol, caffeic acid, p-coumaric acid, vanillic acid, vanillin, oleuropein, luteolin, diosmetin, rutin, verbascoside, luteolin-7 glucoside, apigenin-7-glucoside, and diosmetin-7-glucoside are the primary components of olive leaf [5-6]. Olive leaf extract has antimicrobial material that can help in the treatment of various infectious diseases [7]. At the same time, research has demonstrated that olive leaf extract can reduce

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blood pressure in animals [8], enhance blood flow in coronary arteries [9], relieve arrhythmia, and prevent intestinal muscular spasms [3]. Various compounds have been found in olive leaf extract, all of which are known as olive biphenols, which are primarily responsible for its therapeutic activities [10]. Oleuropein is the most abundant biphenol in olive leaf extract, while other biphenols are present in lower amounts [11]. In a study showing the significant and selective cytotoxic activity of olive leaf extract in hepatocellular carcinoma cell lines, olive leaf extract can be suggested as potential nutraceuticals for anserine inhibition [12].

Previous studies have shown that olive leaf has high antioxidant properties [13-16] and that these properties are due to its phenolic composition [7,17-19]. The immune systems of insects consist of both cellular and humoral defense mechanisms, with circulating hemocytes playing a crucial role in countering potential pathogens [20]. The immune functions of hemocytes are directly influenced by the secretion and release of endogenous enzymes through exocytosis and degranulation [21]. SOD, peroxidase, and CAT are three common antioxidants that play an important role in oxidative killing against infections but can also eliminate excess reactive oxygen species (ROS) that are dangerous to the host [22-23]. Activated hemocytes can go through an "oxidative burst" in which they release ROS while also retaining inorganic and organic oxygen ions, free radicals, and peroxides [24,21] Because of the existence of unpaired valence shell electrons, these molecules are often very tiny and very reactive [21]. ROS play an important role in cell signaling and induction of host immune related genes [25]. Recent research on the invertebrate immune system has discovered that immunity to ROS is critical for survival [26]. Although "oxidative burst" is effective against pathogens, it can cause tissue damage when ROS production is further increased due to an imbalance between oxidant and antioxidant status in the cell, a condition known as "oxidative stress," whereas antioxidants can remove excess ROS to protect hosts from oxidative damage [21]. *G. mellonella* larvae have been employed as model organisms in recent years to investigate the virulence mechanisms of human pathogens [27-29]. According to Pereira et al. [30], conducting immunological research on *G. mellonella* larvae is advantageous due to the large surface area of the larvae as well as the ease with which hemolymph can be isolated. Invertebrate model organisms provide a wide range of in vivo experiments without raising ethical problems. Furthermore, *G. mellonella* has the ability to endure a range of temperatures (25–37 °C) that are conducive to conducting infection studies, and this characteristic enables researchers to carry out experiments that closely resemble those conducted in mammalian systems [31]. There are many studies on *G. mellonella* hemolymph antioxidant enzymes and these studies show that *G. mellonella* hemolymph antioxidant enzymes are affected by plant extracts and other different substances [32-37]. Based on the results of studies investigating the effects of olive leaf extract on *G. mellonella* cell-mediated immunity [38] and phenoloxidase (PO) activity [39], it was observed that the administration of olive leaf extract at low doses led to a notable increase in cellular immune responses.

This research was conducted as a continuation of studies on the effects of olive leaf and the model organism *G. mellonella* on cell-mediated immune response [38] and PO activity [39]. In this investigation, *G. mellonella* determined the effect of olive leaf extract on the hemolymph's antioxidant activity. As a consequence of this study, the data obtained from three studies were pooled and an attempt was made to provide a general assessment of the effect of olive leaf extract on immunity.

2. Materials and Methods

2.1. Insect Rearing

The *G. mellonella* specimens used in the experiment was obtained from a successive colony cultivated in the Department of Biology, Insect Physiology Laboratory at Çanakkale Onsekiz Mart University at 28±1°C, 65±5% relative humidity, and constant darkness. The four adult females and two adult males

were deposited in a one-liter glass jar with two grams of naturally darkened honeycomb in order for them to produce eggs and were maintained under the same conditions as the main colony. When the hatchlings were observed, artificial food developed by Bronskill for this species and composed of wheat cereal, glycerin, honeycomb, water, and feed additives was prepared and fed to all groups [40]. As the nutrients were consumed, they were replaced.

2.2. Extraction and Experimental Doses

The olive leaf was harvested for research purposes. After drying in the laboratory, the collected leaves were ground to the desired size using a grinder. The pulverized leaves were then dissolved in ethanol and extracted in a soxhlet apparatus. To obtain dry, the material dissolved in alcohol was removed with alcohol in rotary evaporator. The resultant dry matter was evaluated in appropriate solvents, which were prepared by combining Phosphate buffer salt (PBS), filtered water, and ethanol in appropriate quantities. The survival of the subjects for 72 hours and at least 50% survival at the end of 72 hours were examined in PBS extracts. As a result, the participants' LC_{50} value was determined to be 77.6 mg mL^{-1}. This value was calculated using test dosages of 72, 60, 50, 40, 30, 20, 10, 5, 2.5, 1, 0.5, 0.25, 0.10, 0.05, 0.025, and 0.010 mg mL^{-1}.

2.3. Injection

As control groups, the control (untreated) and PBS-injected groups were used. Regarding the dose groups, 0.010 - 72 mg mL^{-1} doses prepared in PBS were injected into the body cavity of last instar larvae using a 5 µl microinjector (Hamilton, United States). This procedure was carried out four times (four repetitions) with four larvae per repetition (n=16). To clearly monitor the post-injection state, hemolymph was collected and prepared from the individuals at 4, 8, 12, 16, and 24-hour intervals. Both control and injected larvae were tested during a 24-hour waiting period in glass petri dishes at the same temperature, relative humidity, and light levels as the primary colony-rearing conditions.

2.4. Enzyme Assays

A microplate reader (Thermo Scientific Multiskan GO Microplate Spectrophotometer, Finland) was used for all of the studies to determine the enzyme activities. In the present study, a total of 16 larval hemolymph samples were obtained across four different time periods for each dose group and the control group. Following the collection of each larva's hemolymph, the enzyme activity and total protein measurements were carried out four separate times.

Experiments were carried out according to the methods specified in [37]. According to this, the [41] method for determination of total protein, [42] for determining of SOD activity, [43] for CAT activity and [44] methods for MDA levels were adapted and used. Total protein was determined as mg protein mL^{-1} at an absorbance of 595 nm. SOD activity was determined as unit mg protein mL^{-1} at 560 nm absorbance. CAT activity was calculated as mmol min^{-1} mg $protein^{-1}$ with a 10-second interval kinetic reading at 240 nm absorbance. MDA level was determined as nmol mg $protein^{-1}$ at 532 nm absorbance.

2.5. Statistics

The data processing and analysis were performed using the R package software. The data set's descriptive statistics included mean, standard deviation, standard error, range, and 95% confidence intervals. Histogram, Q-Q, and violin charts were used for data visualization. The type I error in statistical inferences was assumed to be 0.05 in probability. A two-way analysis of variance based on

the hypotheses of normality and homogeneity was used to assess the effects of group and time on enzymes as well as the effects of their interactions. A non-parametric two-way analysis of variance, which produced statistics over the rank rather than the actual values, was used since there were violations of the assumptions. To identify the time and groups that contributed to the difference in enzyme values, the Games-Howell test, one of the non-parametric post-hoc tests, was performed.

3. Results and Discussions

Table 1 displays the descriptive statistics and lower - higher limits of 95% confidence for the CAT, SOD enzymes and MDA.

Table 1. Descriptive statistics of enzymes

Enzyme	N	Mean	SE	95% Confidence Interval		SD	Range
				Lower	Upper		
CAT	1440	6.61e-4	8.03e-6	6.45e-4	6.76e-4	3.05e-4	0.00338
SOD	1440	5.08	0.06399	4.96	5.21	2.428	22.12340
MDA	1440	3.00	0.01603	2.97	3.04	0.608	4.37710

N: Sample Size, SE: Standard Error, SD: Standard Deviation

Additionally, the results of enzyme activity for different time and dose groups are graphically presented in Figures 1-3, for more details about supplementary data (SD), see [45].

SD 1 and SD 2 contain enzyme statistics that were investigated individually based on time and groups, respectively. In terms of CAT enzyme, the 24th hour value showed a statistically significant difference compared to the 4th and 12th hours. The 4th hour has a greater SOD enzyme value than the 8th, 12th, and 16th hours, but is lower than the 24th hour. Moreover, the SOD enzyme value rose after 24 hours compared to all other times. In terms of MDA, an increase in enzyme value is observed after the 4th hour (SD 1). The 0.025 mg mg mL^{-1} dose group had a higher level of CAT enzyme activity than the 0.5, 2.5, and 5 mg mL^{-1} dose groups. Additionally, the PBS and control groups' CAT activity was higher than that of the 5 mg mL^{-1} dosage group. The PBS and control groups had higher SOD enzyme activity than the 40 mg mL^{-1} dosage group. The MDA level of the 0.025 mg mL^{-1} dose group was higher than the 0.05, 0.01, 0.25, 1.72 and control groups (SD 2).

To examine the combined effects of various time and dose groups on enzyme activity, two-way analysis of variance was utilized. However, the presumption of normality was not supported by the CAT and SAD enzymes or MDA. SD 3 provides the Shapiro-Wilk tests for the assumption of normality. The homogeneity of group variances was examined at each analysis by Levene's test. Moreover, the heterogeneity of variances according to time and dose groups is clearly seen in Figures 1 and 2. Since the assumptions were not provided, non-parametric two-way analysis of variance was applied. Non-parametric tests are based on rank values. For this reason, a rank transformation was applied to the data, and then two-way analysis of variance was applied. In SD 4, it displays the Q-Q plot that was produced using the findings of both parametric and non-parametric two-way ANOVA. It is clearly seen that the original values of the enzymes are quite far from the normality assumption but approach normal with the rank transformation (SD 4).

Table 2. The results of two-way ANOVA

Enzyme	Cases	Sum of Squares	df	Mean Square	F	p	η^2
CAT	Group	$7.852\times10^{+6}$	17	461899.501	4.091	< .001	0.032
	Time	$4.595\times10^{+7}$	4	$1.149\times10^{+7}$	101.745	< .001	0.185
	Group×Time	$4.262\times10^{+7}$	68	626769.429	5.552	< .001	0.171
SOD	Group	$1.013\times10^{+7}$	17	595672.882	10.882	< .001	0.041
	Time	$1.192\times10^{+8}$	4	$2.981\times10^{+7}$	544.572	< .001	0.479
	Group×Time	$4.558\times10^{+7}$	68	670258.916	12.245	< .001	0.183
MDA	Group	$1.481\times10^{+7}$	17	871211.776	7.719	< .001	0.060
	Time	$4.968\times10^{+7}$	4	$1.242\times10^{+7}$	110.033	< .001	0.200
	Group×Time	$3.196\times10^{+7}$	68	470051.831	4.164	< .001	0.128

The following are the two-way ANOVA results for different types of enzymes that demonstrate the effects of time, dose, and time-dose interactions. The Games-Howell test findings are provided in SDs 5-7, which identify the groups responsible for the difference. However, since it takes a long time to read the tables and is difficult, in Figures 3-5, it is also shown visually.

3.1. CAT Activity

When the CAT enzyme activity was compared across dosage groups, the 0.025 mg mL^{-1} dose group outperformed the 0.5, 2.5, 5, and 40 mg mL^{-1} dose groups. The PBS dosage group had greater CAT enzyme activity than the 5 and 40 mg mL^{-1} dose groups. Furthermore, the control group's CAT enzyme activity was greater than the 0.05, 0.5, 1, 2.5, and 40 mg mL^{-1} dosage groups (SD 5.1). While the CAT enzyme activity at the 4th hour was significantly higher than the enzyme activity at the 12th hour, it was significantly lower than the CAT enzyme activity at the 8th, 16th, and 24th hours. In addition, CAT activity at 8 hours is higher than at 12 and 16 hours (Figure 1). While the CAT enzyme activity at the 24th hour was higher than the enzyme activity at the 12th and 16th hours, there was no significant difference between the enzyme activity at the 8th hour (SD 5.2).

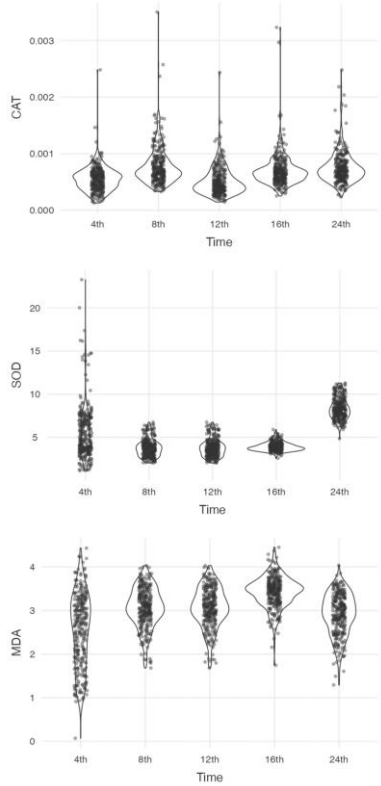

Figure 1. Violin graph of enzyme activities over time

There was no statistically significant difference between CAT enzyme activities in terms of different dose groups at the 4th hour (SD 5.3).

A comparison of doses according to groups and hours is presented in Figure 3. Accordingly, the CAT enzyme activity at the 8th hour of the 50 mg mL^{-1} dose group was higher than the 8th hour CAT enzyme activity of the 0.1, 0.25, 0.5, 1, 2.5, and 5 mg mL^{-1} doses and the control group. The CAT enzyme activity at the 8th hour of the 30 mg mL^{-1} dose group was higher than the 8th hour CAT enzyme activity in the 0.1, 0.25, 0.5, and 2.5 mg mL^{-1} doses. The CAT enzyme activity at the hour of 8 of the 72 mg mL^{-1} dose group was higher than the 8th hour CAT enzyme activity at the 0.1, 0.25, and 0.5 mg mL^{-1} dose groups. At the 8th hour, the CAT enzyme value in the 40 mg mL^{-1} dose group was higher than that in the 0.25 and 0.5 mg mL^{-1} dose groups. In addition, CAT enzyme activity for the 60 mg mL^{-1} dose group at the 8th hour was higher than the 0.25 mg mL^{-1} dose group. The CAT enzyme activity at the 12th hour of the 0.01 and 0.05 mg mL^{-1} dose groups was higher than the 12th hour CAT enzyme activity in the 20, 30, 40, 50, 60, and 72 mg mL^{-1} doses. The CAT enzyme activity at the 12th hour in the 0.025 mg mL^{-1} dose group was higher than the 12th hour CAT enzyme activity in the 40, 50, and 72 mg mL^{-1} doses. At the 12th hour, the CAT enzyme value in the control dose group was higher than that in the 0.1, 0.25, 0.5, 1, 5 and 10 mg mL^{-1} dose groups. At the 12th hour, the CAT enzyme value in the control and PBS dose groups was higher than that in the 20, 30, 40, 50, 60, and 72 mg mL^{-1} dose groups. There were no significant differences between the CAT enzyme activities of the dose groups at 16 and 24 hours (SD 5.3).

3.2. SOD Activity

Comparison of dose groups with each other is shown in Figure 2. According to these results, the SOD enzyme activity of the 0.01 and 0.05 mg mL^{-1} dose groups was higher than the 0.25, 40 and 50 mg mL^{-1} dose groups. While the SOD enzyme activity of the 0.025, 0.1, 1.5 mg mL^{-1} dose group was higher than the 50 mg ml^{-1} dose group, it was lower than the 72 mg mL^{-1} dose and the control groups. SOD enzyme activity of 10 mg mL^{-1} dose groups was lower than 2.5, 20, 72, the control and PBS groups. SOD enzyme activity of 2.5 and 20 mg mL^{-1} dose groups was higher than 40, 50 and 60 mg mL^{-1} dose groups. SOD enzyme activity of 72 mg mL^{-1} dose, the control and PBS groups was higher than 30, 40, 50, and 60 mg mL^{-1} dose groups (SD 6.1).

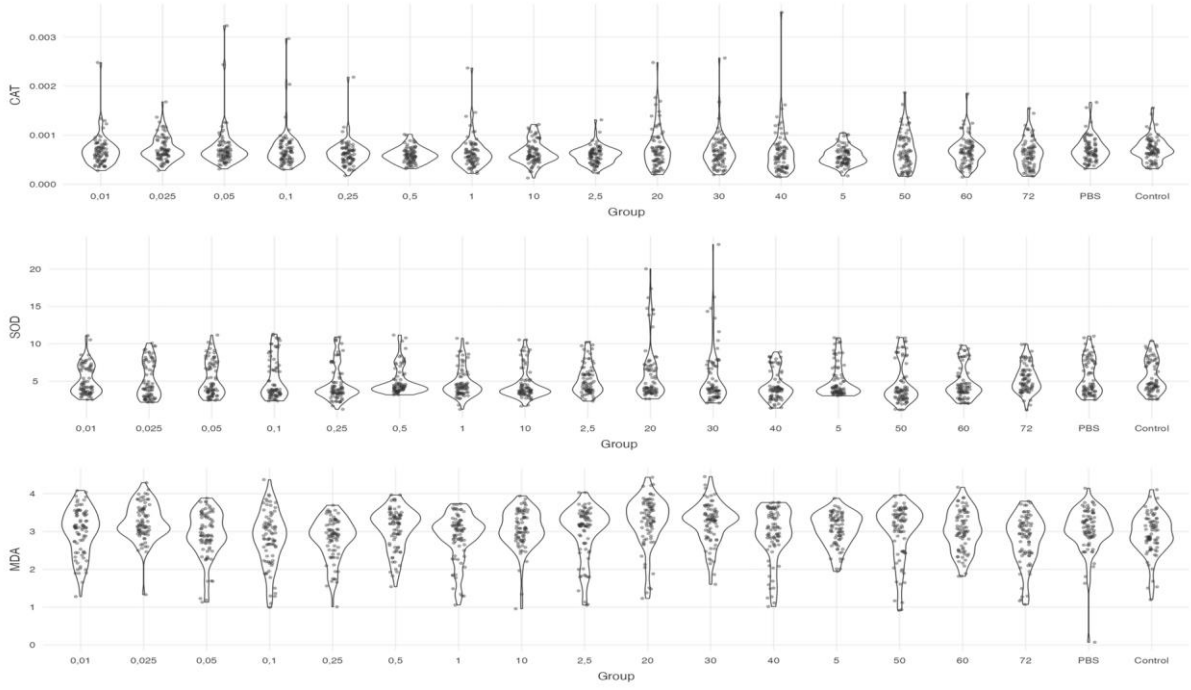

Figure 2. Violin graph of enzyme activities according to dose groups

SOD enzyme activity at 24 hours is higher than at 4, 8, 12 and 16 hours (Figure 1). SOD enzyme activity at the 4th hour is higher than at the 8th, 12th, and 16th hours. In addition, the SOD enzyme activity at the 8th and 12th hours was lower than at the 16th hour (SD 6.2).

At the 4th hour, the SOD enzyme activity of the 0.01, 0.025, and 0.05 mg mL^{-1} dose groups was higher than that of the 0.25, 1, 2.5, 5, 10, 40, 50, 60, and 72 mg mL^{-1} dose groups. SOD enzyme activities at the 4th hour of the 0.1 and 0.5 mg mL^{-1} dose groups were higher than those of the 1 and 40 mg mL^{-1} dose groups. In addition, the SOD enzyme activity at the 4th hour of the 0.1 mg mL^{-1} dose group was higher than the 0.25, 5, 10, 50, and 72 mg mL^{-1} dose groups. The SOD enzyme activity at the 4th hour of the 0.25 mg mL^{-1} dose group was lower than the 0.5, 20, and 30 mg mL^{-1} dose groups and the control and PBS groups. 20 mg mL^{-1} dose group and the control, PBS groups had lower SOD enzyme activity at the 4th hour compared to the 0.5, 1, and 2.5 mg mL^{-1} dose groups. In addition, the SOD enzyme activities at the 4th hour of the 1, 2.5, and 10 mg mL^{-1} dose groups were lower than the 30 mg mL^{-1} dose group. The 4th hour SOD enzyme activities of the 20 and 30 mg mL^{-1} dose groups were higher than the 5, 40, 50, 60 and 72 mg mL^{-1} dose groups. SOD enzyme activities at the 4th hour of the control and PBS groups were higher than in the 5, 50, 60, and 72 mg mL^{-1} dose groups. SOD enzyme activities at the 8th hour of the 0.01, 0.25, 5, 10, 30, 40, 50, and 60 mg mL^{-1} dose groups were lower than the 72 mg mL^{-1} dose group. The 8th-hour SOD enzyme activities of the 2.5 and 72 mg mL^{-1} dose groups were higher than those of the 0.025, 0.05, and 0.1 mg mL^{-1} dose groups. 8th hour SOD enzyme activities of 0.5, 1 and 2.5 mg mL^{-1} dose groups are higher than 30 and 50 mg mL^{-1} dose groups, and 1, and 2.5 mg mL^{-1} dose groups are higher than 60 mg mL^{-1} dose groups. In addition, the 8th hour enzyme activities of the control and PBS groups were lower than the 72 mg mL^{-1} dose group. SOD enzyme activity at the 12thhour of the 72 mg mL^{-1} dose group was higher than the 0.05, 0.1, 0.25, 10, 20, 30, 40, 50, and 60 mg mL^{-1} dose groups. The 12-hour SOD enzyme activity of the 1 mg mL^{-1} dose group was higher than the 0.025, 0.05 and 0., 30, 50, and 60 mg mL^{-1} dose groups. The SOD enzyme activity in the 2.5 mg mL^{-1} dose group at 12 hours was higher than that of the 0.25, 30, 50 and 60 mg mL^{-1} dose groups. In addition, the 12-hour SOD activities of the control and PBS dose groups were lower than those of the 72 mg mL^{-1} dose group. The SOD activity of the control group at the 16th hour was higher than the 1, 10, 20, 50 and 60 mg mL^{-1} dose groups. No significant difference was observed between the SOD enzyme activities of the dose groups at 24 hours (SD 6.3).

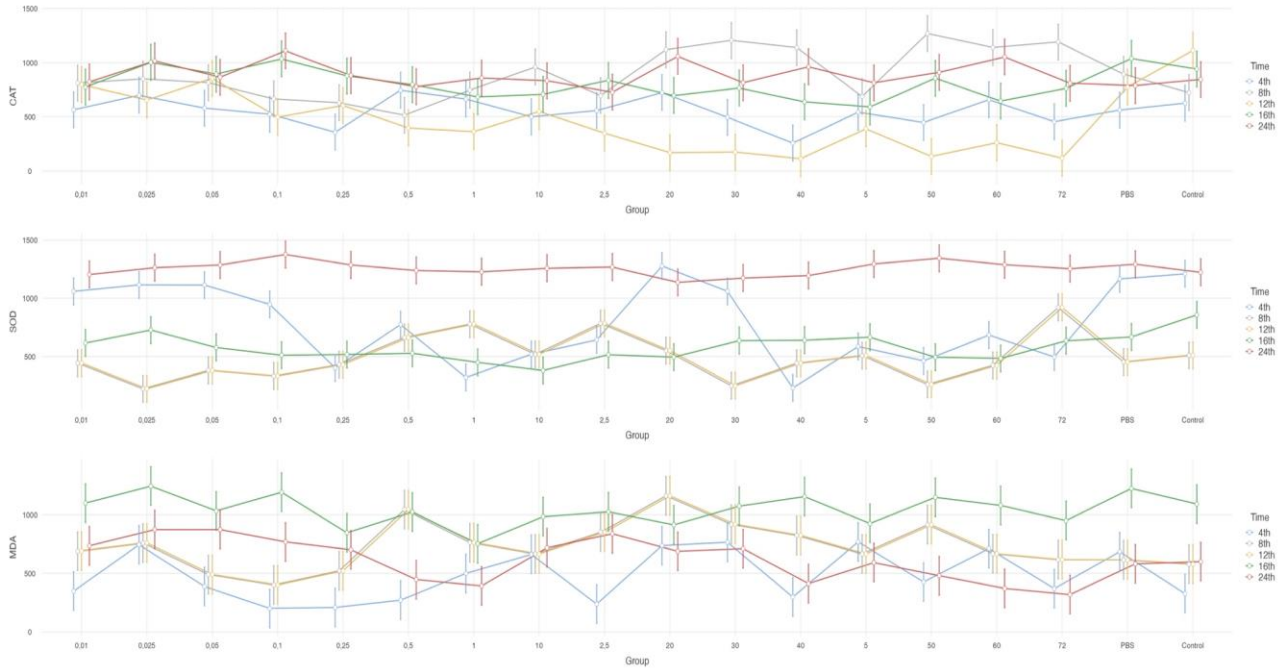

Figure 3. Plot of estimated marginal means for group-time interaction

3.3. MDA Levels

MDA levels of 0.025, 0.5, 2.5, 20, 30, and 50 mg mL^{-1} dose groups were higher than 72 mg mL^{-1} dose groups (Figure 2). MDA levels of 20 mg mL^{-1} dose group is higher than 0.01, 0.05, 0.1, 0.25, 1, 5, and 10 mg mL^{-1} dose groups. MDA levels of 0.025 mg mL^{-1} dose group is higher than 0.05, 0.1, 0.25, 1 mg mL^{-1} dose groups and control group. MDA levels of 20 and 30 mg mL^{-1} dose groups are higher than 60 mg mL^{-1} dose group and control group is high. In addition, the MDA levels of the 20 mg mL^{-1} dose group is higher than the 40 mg mL^{-1} dose group and the PBS group. The MDA levels of the 30 mg mL^{-1} dose group are higher than those of the 0.1 and 1 mg mL^{-1} dose groups, and at 50 mg mL^{-1}, the levels of the dose group were higher than those of the 0.1 mg mL^{-1} dose group (SD 7.1).

While the MDA levels at the 8th and 12th hours are lower than the 16th hour, they are higher than the 24th hour. MDA levels at the 4th hour is lower than at the 8th, 12th, 16th, and 24th hours. In addition, MDA levels at the 24th hour are lower than at the 16th hour (SD 7.2).

The 4th hour MDA levels of the 5, 20, and 30 mg mL^{-1} dose groups were higher than the 0.1, 0.25, and 2.5 mg mL^{-1} dose groups. In addition, the 4th hour MDA levels of the 60 mg mL^{-1} dose group were higher than the 0.1 and 0.25 mg mL^{-1} dose groups. The 4th hour MDA levels of the 0.025 mg mL^{-1} dose group were higher than those of the 0.1, 0.025, and 2.5 mg mL^{-1} dose groups (Figure 3).

The MDA level at the 8th hour of the 20 mg mL^{-1} dose group was higher than the 0.05, 0.1, 0.25, 5, and 72 mg mL^{-1} dose groups and the control and PBS groups. While the 8th hour MDA level in the 0.05 mg mL^{-1} dose group was higher than the 0.1 and 0.25 mg mL^{-1} dose groups, the 8th hour MDA level in the 30 and 50 mg mL^{-1} dose groups was higher than the 0.1 mg mL^{-1} dose group (Figure 3).

The 12th hour MDA levels of the 0.05 and 20 mg mL^{-1} dose groups were higher than the 0.05, 0.1, and 0.25 mg mL^{-1} dose groups. The 12th hour MDA level of the 20 mg mL^{-1} dose group was higher than the 5, 10, 60, and 72 mg mL^{-1} dose groups and the PBS group. In addition, the 12th hour MDA levels of the 30 and 50 mg mL^{-1} dose groups are significantly greater than 0.1 mg mL^{-1}.

There were no statistically significant differences between the MDA levels of the dose groups according to the 16th hour. While the 24th hour MDA level of the 72 mg mL^{-1} dose group was higher than the 0.05 and 2.5 mg mL^{-1} dose groups, the 24th hour MDA level of the 60 mg mL^{-1} dose group was also higher than the 0.05 mg mL^{-1} dose group (SD 7.3).

Previous research on the antioxidant properties of olive leaf solely looked at the plant's antioxidant capacity. The majority of olive leaf research is done in vitro [46-47]. Edgecombe et al. [48]'s study is the most important source on the antioxidant impact of this plant in other living things. According to this study, oleuropein increased broiler chickens' overall antioxidant capacity by preserving other antioxidant substances in the gut. In their study, (see [48]) stated that when consumed as food, oleuropein contributes to total antioxidant capacity by supporting the antioxidants already present in the intestine, which can be interpreted as olive leaf extract's inability to transfer its antioxidant capacity to the living thing. Aside from the study demonstrating this indirect impact, no research has been conducted on how olive leaf extract increases antioxidant capacity in animals. In this manner, the findings of our investigation support the literature.

In the study examining the effect of olive leaf methanolic extract on *Penaeus vannamei* immunity, it was determined that it caused a decrease in SOD and PO activities [49]. In this study, it was determined that the activity of the control group for SOD was statistically higher than the activity of 0.025, 0.1, 0.25, 1, 5, 10, 30, 40, 50, and 60 mg mL^{-1} doses (Figures 2 and 3). The findings are consistent with the results presented by Gholamhosseini et al. [49].

It was determined that the low antioxidant enzyme levels in the blood of diabetic rabbits subjected to a diet enriched with olive leaf extract increased and were similar to the control [50].

The findings of a separate investigation involving diabetic rats indicated that administering oleuropein and hydroxytyrosol-rich extracts through drinking water, together with a four-week treatment regimen, effectively mitigated the impact of oxidative stress in these animals [51].

The study revealed that feeding Wistar rats a diet high in cholesterol for a duration of 16 weeks resulted in an elevation in the activity of antioxidant enzymes, thereby decelerating the process of lipid peroxidation [52].

Lockyer et al. [53] stated in their research that although previous studies [50–52] have demonstrated that antioxidant activity is the primary mechanism via which olive polyphenols exert their effects in organisms, it is now recognized that this is not the exclusive or primary mode of action in the body.

Furthermore, it was found that the administration of olive leaf extract through diet had a regulatory effect on the antioxidant gene expression of common carp (*Cyprinus carpio*) and resulted in a decrease in oxidative stress [54].

In another study found that administering olive leaves orally at a dosage of 1000 mg/kg effectively protected lead-induced brain damage in mice by enhancing their antioxidant capacity [55].

The aforementioned trials demonstrate that olive leaf extract was incorporated into the individuals' diets and administered to them as part of the treatment. However, Edgecombe et al. [48] as stated in study, olive leaf extract applied in the diet has an indirect effect by increasing the antioxidant enzymes produced by beneficial organisms in the intestines of living things. The content of olive leaf extract taken through food has poor absorption from the intestines [56]. Our study differs from studies conducted with feeding as it simulates the content of olive leaves entering the body.

According to the study conducted to determine the *G. mellonella* cell-mediated immune responses of olive leaf extract, olive leaf increases cell-mediated immune responses at a dose of 0.1 mg mL^{-1} [38]. In another study related to PO activity, which plays a key role in the melanization process from *G. mellonella* humoral immune responses, it was found to increase at a dose of 0.1 mg mL^{-1} [39]. The most important result of our study is that olive leaf extract supports cellular immunity but causes a decrease in antioxidant enzyme activity. When the results of these three studies are evaluated together, it is thought that olive leaf extract supports cell-mediated immunity at low doses but has a negative effect on oxidative stress.

4. Conclusion

According to the findings of our study, CAT and SOD enzymes are more active in the 24th hour compared to the other hours. Furthermore, in terms of MDA level, the 24th hour is lower than the previous hours' values. While CAT and SOD activity increased, MDA levels decreased, indicating that the 24th hour will provide a healthy result when testing antioxidant enzyme activity. In terms of dose groups, the control group's CAT enzyme activity was shown to be greater than the 0.05, 0.5, 1, 2.5, and 40 mg mL^{-1} dose groups. SOD activity was greater in the 72 mg mL^{-1}, control, and PBS groups than in the 30, 40, 50, and 60 mg mL^{-1} dose groups. Another finding from our investigation was that the level of MDA in the control groups was lower than at the other dosages. According to the researchers of this study, these results mean that olive leaf extract was unable to spread its antioxidant properties to other species, resulting in decreased animal antioxidant capacity.

Author Contributions

All the authors equally contributed to this work. They all read and approved the final version of the paper.

Conflict of Interest

All the authors declare no conflict of interest.

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