

Original Research

Effects of Guaiazulene on Lipid Peroxidation and Antioxidant Defense System in *Drosophila melanogaster*

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Abstract

Guaiazulene is a crystalline bicyclic sesquiterpene. Today, besides being used in herbal treatment, it is also used in skin - body care products, toothpaste, hair dyes, eye solutions and also in the treatment of nipple irritation in nursing mothers. In the study, it was aimed to evaluate the effects of Guaiazulene and its solvent dimethyl sulfoxide on the level of malondialdehyde, which is a lipid peroxidation marker, and superoxide dismutase and catalase enzyme activities, which belong to antioxidant defense systems, in *Drosophila melanogaster*. It was determined that dimethyl sulfoxide caused lipid peroxidation by increasing the level of malondialdehyde at all hour and dose concentrations. At the same time, it was determined that dimethyl sulfoxide caused an increase in catalase enzyme activity by increasing the radical formation, but did not affect superoxide dismutase enzyme activity. In addition, it was observed that increasing in the dose of guaiazulene or the exposure time to it decreased superoxide dismutase enzyme activity, and catalase enzyme activity at all doses and hours. It was concluded that these data support the fact that guaiazulene shows antioxidant properties. Malondialdehyde amount increased upon increasing in the exposure time to guaiazulene. And it was concluded that it caused lipid peroxidation.

Keywords: Guaiazulene, *Drosophila melanogaster*, antioxidative activity, MDA, SOD, CAT

Introduction

Guaiazulene, which is obtained from *Guaiaacum officinale* (Zygophyllaceae) and *Matricaria chumomilla* (Asteraceae), is a dark blue, crystalline bicyclic sesquiterpene. In addition to plants, it exists as a pigment in *Lactarius indigo* fungus and some invertebrates of the *Alcyonacea* group [1, 2]. Guaiazulene(Gua) is a chemical with antispasmodic and antimicrobial activity. With this

aspect, it is used in herbal therapy [3]. Gua, which is a cosmetic colorant approved by the FDA (Food and Drug Administration) in the USA, is used in skin - body care products, toothpaste, hair dyes, eye solutions, in the treatment of nipple irritation in nursing mothers and also to relieve itching caused by dry skin. In addition, photodynamic activation of Gua has been determined to suppress inflammation in peripheral blood mononuclear cells [4-6]. Azulene (AZ), a polycyclic aromatic hydrocarbon, and its derivatives, especially Gua, attract attention due to their various biological activities [7, 8]. Cao et al. (2016) also determined that Gua and its derivatives show anti-gastric ulcer activity [9].

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Oxygen (O_2) is essential for life. O_2 is consumed during metabolic reactions or respiration in cells. Meanwhile, free radicals are released and cause oxidative stress in cells [10, 11]. In addition, some reactive oxygen species (ROS) produced during normal metabolism in a healthy organism have the potential to cause intense harm to the organism. In addition, conditions such as inflammation, drugs, exogenous sources and exposure to radiation can cause an increase in ROS production. That is why, changes in the ROS signal transduction pathway can cause oxidative damage on both lipids and proteins [12, 13]. Oxidative stress has also been associated with diabetes mellitus, cardiovascular diseases, neurodegenerative diseases and many other pathologies [14-16]. Free radicals are atoms or molecules that are unstable because they have unshared electrons in their outer orbitals. They tend to gain electrons and react with biological materials to become chemically stable. That's why, they are chemicals with high reactivity [17]. Thus, hydroxyl radical and peroxynitrite cause damage especially to cell membranes and lipoproteins. This oxidative damage initiates chemical chain reactions that lead to the formation of malondialdehyde (MDA), which has a cytotoxic and mutagenic structure. In endogenous and exogenous antioxidants, it protects cells from damage caused by ROS in the long and short terms by neutralizing or inhibiting the effect of ROS [18]. However, when ROS is overproduced or there is a significant decrease in antioxidant defense, the antioxidant defense system is suppressed and oxidative stress occurs. This oxidant-antioxidant balance in the organism generally depends on internal and external factors acting together [19]. Oxidative stress can cause protein oxidation, DNA mutations and breaks, lipid peroxidation, cytotoxic effects and deterioration in signaling. Cell damage caused by free radicals is thought to play an important role in the aging process and the formation of degenerative diseases related to aging [20]. Antioxidants are substances that fight, prevent or reduce the oxidation of a substrate. Superoxide dismutase (SOD), one of the antioxidant defense enzymes, converts the superoxide anion to molecular O_2 and hydrogen peroxide (H_2O_2), catalase (CAT), catalyzes the conversion of H_2O_2 to water (H_2O) and O_2 [20, 21].

Material and Methods

Obtaining and Reproducing *Drosophila melanogaster*

In our study, Oregon R wild strains of *D.melanogaster* obtained from the Biological Activity Laboratory of the Faculty of Science and Letters of Amasya University were used. The individuals were propagated and stored in standard media developed by Bozcuk (1976) [22]. The individuals reproduced in these nutrient media prepared in 250 ml culture

bottles were separated as male and female every 6 hours. The separated individuals were stored in different bottles and virgin individuals were obtained. The collection process lasted for 72 hours and at the end of this period, they were taken into media containing Gua.

Gua was obtained from Sigma-Aldrich Kimya and stored at room temperature. 1% Dimethyl sulfoxide (DMSO) was used as a solvent [23]. Gua, which was dissolved in DMSO, was added to the media as 100 mg/L, 200 mg/L, 400 mg/L [3]. In addition to these groups, 800 mg/L was added. The media were stored in 10 ml plastic tubes in the refrigerator at +4°C.

Individuals transferred to media containing Gua separately from male and female were starved for 6 hours before transfer [24]. Likewise, individuals were transferred to the medium containing DMSO and the control medium. At 12th, 24th, 48th hours, the individuals were removed from the medium and stored in glass bottles at -80°C. Each experimental group was repeated 3 times.

Preparation of Tissues for Biochemical Analysis

D. melanogaster samples were removed from -80°C and left to thaw at room temperature. They were then weighed on a precision balance (Ohaus Adventure Pro AV 264C) to determine their weight. Cold phosphate buffer (0.1 M pH: 7.4) solution was added to the tissue samples taken into glass bottles at a ratio of 1/30 (w/v). All tissue samples were crushed with baguette in phosphate buffer in a glass bottle. Then, the glass bottle containing the samples was taken into the ice container and subjected to ultrasonication and homogenization processes. The homogenates obtained were centrifuged (Kubota model 3500) for 15 mins at +4°C at 15,000 rpm and the supernatant was obtained. These supernatants were stored at -70°C to be used to determine the level of MDA and CAT, SOD activities. The level of MDA was determined according to the methods by Draper and Hadley (1990) and Hammouda et al. (1995) [25, 26]; the CAT activity was determined according to Luck (1963) method [27]; SOD activity determination was determined according to the methods by Mc Cord and Fridovich (1969) and Flohe and Otting (1984) [28, 29].

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) 20.0 program was used for the statistical analysis. The analyzes were evaluated using the Kruskal-Wallis Test, which is the non-parametric equivalent of one-way analysis of variance. Mann Whitney U test was used after Kruskal-Wallis to determine which groups differed from the others.

Results

Hour Dependent Dose Effect of Gua on Lipid Peroxidation (MDA) Level and SOD, CAT Enzyme Activities in *D. Melanogaster*

Lipid Peroxidation (MDA) Level

The effect of Gua on the level of MDA was observed to be highest in the group containing DMSO at the 12th hour. According to the control group; while the amount of MDA decreased in the groups containing 100 mg/L, 200 mg/L, 400 mg/L and 800 mg/L Gua, an increase was observed in the group containing DMSO. At the 24th hour, the effect of Gua on the amount of MDA was measured the most at a dose of 400 mg/L. Compared to the control group, while the effect of Gua on the amount of MDA decreased in the 100 mg/L Gua group, it continued to increase in the 800 mg/L Gua and 200 mg/L Gua groups of the DMSO group, respectively. At the 48th hour, the maximum amount of MDA was measured at a dose of 200 mg/L. Compared to the control group, while a decrease was observed in the group containing 100 mg/L Gua, an increase was observed in the groups containing 200 mg/L, 400 mg/L, 800 mg/L Gua and DMSO. The highest amount of MDA at all doses and hours was measured in the group containing 400mg/L Gua at the 24th hour (Fig. 1).

When evaluated statistically, it was observed that there was no statistically significant difference between hours on the level of MDA ($p>0.05$). However, there was a significant difference between the control and DMSO-containing groups ($p<0.05$), although there were significant differences between the 12th and 24th, 12th and 48th hours of these groups ($p<0.05$), there was no significant difference between the 24th and 48th hours ($p>0.05$).

SOD Enzyme Activity

The effect of Gua on SOD enzyme activity was measured to be the highest in the groups containing

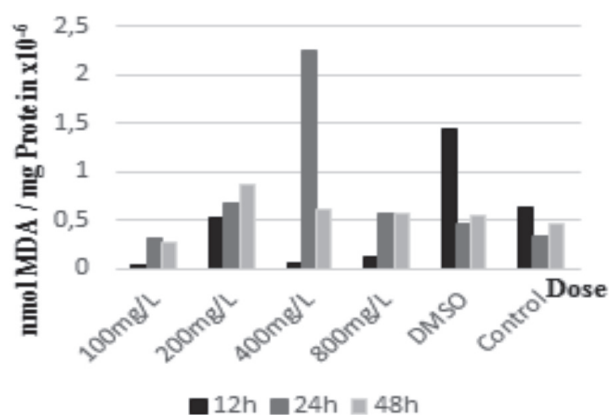


Fig. 1. Hour Dependent Dose Effect of Gua on MDA Level.

400 mg/L Gua at the 12th hour and 100mg/L Gua at the 24th and 48th hours. Compared to the control group, while there was an increase in the SOD enzyme activity in the groups containing 100 mg/L, 400 mg/L, 800 mg/L Gua and DMSO at the 12th hour, a decrease was observed in the group containing 200 mg/L Gua. In addition, while an increase was observed in the SOD enzyme activity in the group containing 100 mg/L Gua at the 24th and 48th hours, a decrease was observed in the groups containing 200 mg/L, 400 mg/L, 800 mg/L Gua and DMSO (Fig. 2).

When evaluated statistically, it can be seen that there is a significant difference between hours on the SOD enzyme activity ($p<0.05$). It was determined that there was a significant difference between the control and DMSO containing groups ($p<0.05$), but there was no significant difference between the hours of these groups ($p>0.05$).

CAT Enzyme Activity

It was determined that the effect of Gua on CAT enzyme activity was highest in the group containing DMSO at 12th, 24th and 48th hours. It was observed that CAT activity increased with DMSO at all doses, but decreased with Gua (Fig. 3). Since CAT activity increased too much in the group containing DMSO, other doses cannot be observed in the graph. That's why, other doses are shown in fig.4 without the DMSO group (Fig. 4). Compared to the control group, an increase in CAT activity was observed at the 12th hour at all doses. On the other hand, in the 24th and 48th hour activity measurements, a decrease was observed in the groups containing 100 mg/L, 200 mg/L, 400 mg/L and 800 mg/L Gua. When evaluated as to the group containing DMSO, a decrease in CAT activity was observed again (Fig. 3). While DMSO increased CAT activity at all doses, Gua decreased it.

When evaluated statistically, it can be seen that there is a significant difference between hours on CAT enzyme activity ($p<0.05$). However, it was determined that there was no significant difference between neither

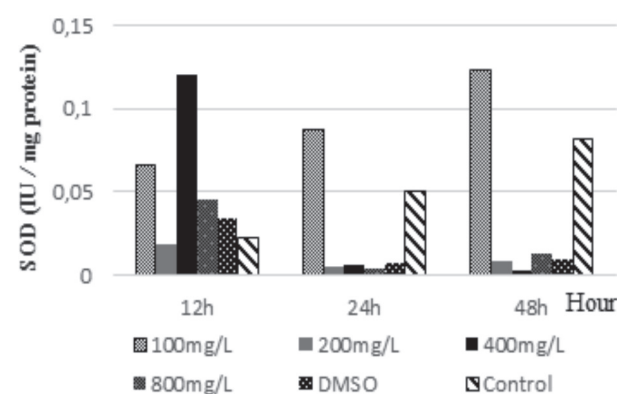


Fig. 2. Hour Dependent Dose Effect of Gua on SOD Enzyme Activity.

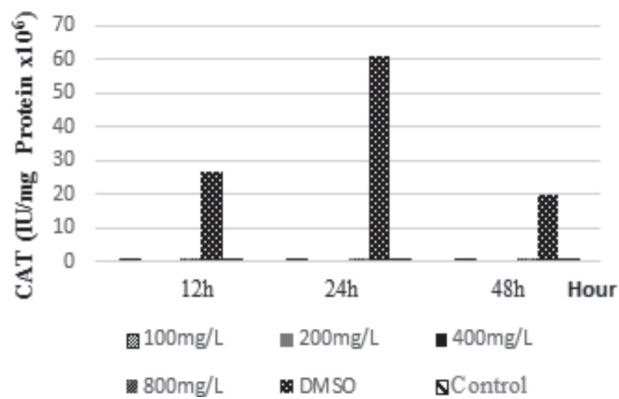


Fig. 3. Hour Dependent Dose Effect of Gua on CAT Enzyme Activity.

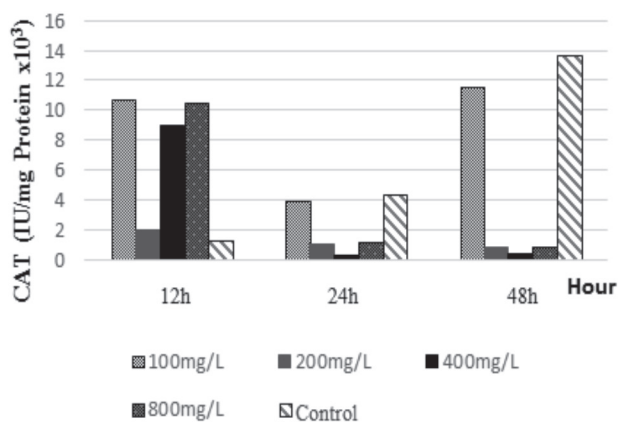


Fig. 4. Hour Dependent Dose Effect of Gua on CAT Enzyme Activity.

the control and the DMSO-containing groups ($p>0.05$) nor the hours of these groups ($p>0.05$).

Gender Dependent Dose Effect of Gayazulen (Gua) on Lipid Peroxidation (MDA) Level and SOD, CAT Activity in *D. Melanogaster*

Lipid Peroxidation (MDA) Level

The effect of Gua on the level of MDA was determined to be highest in the group containing DMSO in both males and females at 12th hours. Amount of MDA; It was measured more in female individuals of groups containing 100 mg/L, 200 mg/L and 800 mg/L gua compared to male individuals, and in male individuals of groups containing 400 mg/L gua and DMSO compared to female individuals. When evaluated as to the control group, in the male individuals, while an increase in the level of MDA was observed in the group containing DMSO, a decrease was observed in the other experimental groups. In the female individuals, compared to the control group, while the level of MDA increased in the groups

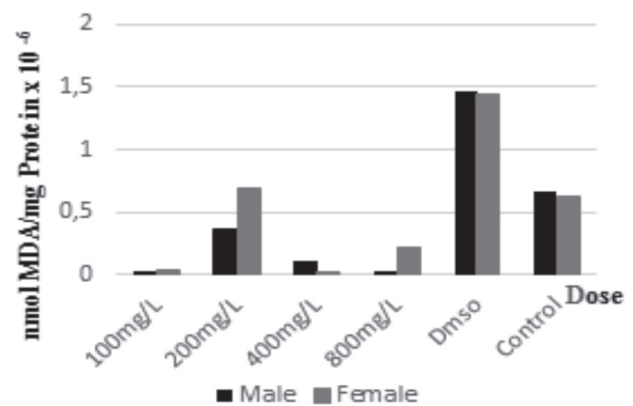


Fig. 5. Gender-Related Dose Effect of Gua (MDA/12).

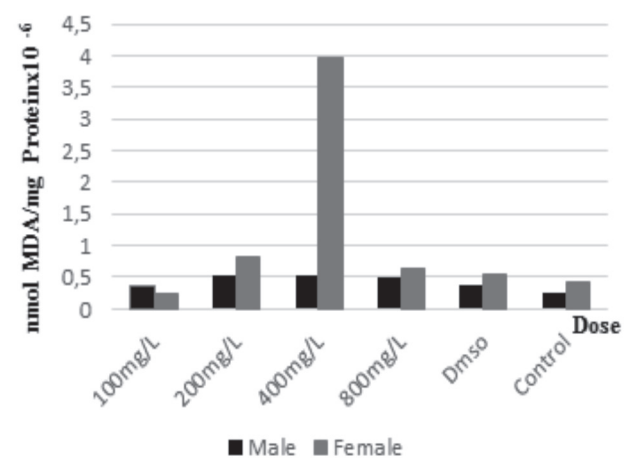


Fig. 6. Gender-Related Dose Effect of Gua (MDA/24).

containing 200 mg/L Gua and DMSO, a decrease was observed in the groups containing 100 mg/L, 400 mg/L and 800 mg/L Gua (Fig. 5).

The effect of Gua on the amount of MDA was determined to be greatest in the group containing 400 mg/L Gua in both the males and the females at the 24 hours. While the amount of MDA in the groups containing 200 mg/L, 400 mg/L, 800 mg/L Gua and DMSO was measured more in the female individuals than in the male individuals, it was measured more in the males than in the females in the group containing 100 mg/L Gua. When evaluated compared to the control group, an increase in the amount of MDA was observed in all groups of the male individuals. In the female individuals, compared to the control group, while the amount of MDA decreased in the group containing 100 mg/L Gua, an increase was observed in the groups containing 200 mg/L, 400 mg/L and 800 mg/L Gua and DMSO (Fig. 6).

The effect of Gua on the amount of MDA; At 48th hour, it was determined that it was highest in the group containing 200 mg/L Gua in males and highest in the group containing DMSO in females. While the amount of MDA in the groups containing 100 mg/L

Gua, 800 mg/L Gua and DMSO was measured more in the female individuals than in the male individuals, it was measured more in the males than in the females in the group containing 100 mg/L Gua and 400 mg/L Gua. When evaluated compared to the control group, in the male individuals, while the amount of MDA decreased in the group containing 100 mg/L Gua, an increase was observed in the groups containing 200 mg/L Gua, 400 mg/L Gua, 800 mg/L Gua and DMSO. In the females, compared to the control group, there was a decrease in the amount of MDA in the groups containing 100 mg/L Gua, 200 mg/L Gua, 400 mg/L Gua, 800 mg/L Gua, and an increase in the group containing DMSO (Fig. 7).

SOD Enzyme Activity

The effect of Gua on SOD Activity was determined to be greatest in the group containing 400 mg/L Gua in both the males and the females at the 12th hour. While the SOD activity in the group containing 100 mg/L Gua was measured more in the males than in the females, it was measured more in the females than in the males in groups containing 200 mg/L Gua, 400 mg/L Gua, 800 mg/L Gua and DMSO. When evaluated as to the

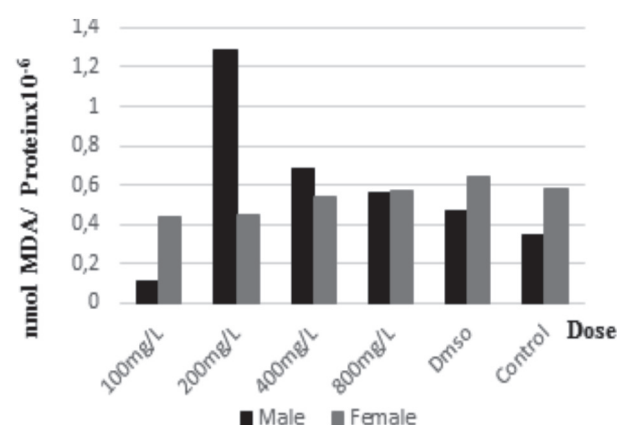


Fig. 7. Gender-Related Dose Effect of Gua (MDA/48).

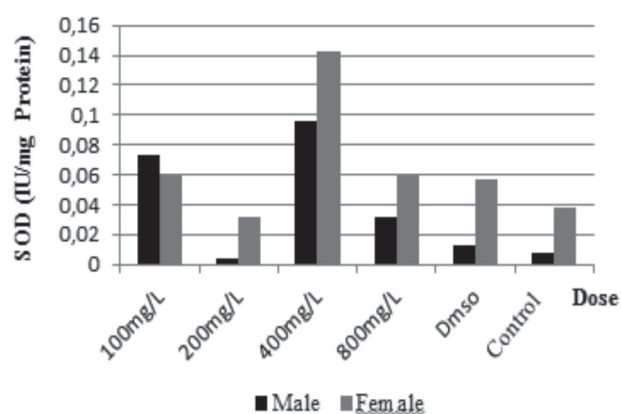


Fig. 8. Gender-Related Dose Effect of Gua (SOD/12).

control group, while the level of enzyme decreased in the group containing 200 mg/L Gua in both the male and the female individuals, it increased in the groups containing 100 mg/L Gua, 400 mg/L Gua, 800 mg/L Gua and DMSO (Fig. 8).

In the 24th hour measurements, the highest SOD activity was measured in both the males and the females at a dose of 100 mg/L Gua. When the male and female individuals are compared, while it was observed that the SOD activity increased in male individuals in the groups containing 100 mg/L Gua, 400 mg/L Gua, 800 mg/L Gua and in the females in the group containing DMSO, it was measured the same in both the male and the female individuals in the group containing 200 mg/L Gua. When evaluated compared to the control group, in the males, while the enzyme activity increased in the group containing 100 mg/L Gua, it decreased in the groups containing 200 mg/L Gua, 400 mg/L Gua, 800 mg/L Gua and DMSO. In female individuals, there was a decrease in the enzyme activity at all doses compared to the control group (Fig. 9).

In the 48th hour activity measurements, while the highest SOD Activity was measured in the group containing 100 mg/L Gua in males, it was measured in the group containing DMSO in females. When the male and female individuals are compared, it was observed that SOD activity increased both in the male individuals of groups containing 100 mg/L Gua, 200 mg/L Gua, 800 mg/L Gua and in the female individuals of the groups containing 400 mg/L Gua and DMSO. When evaluated compared to the control group, in the male individuals, while an increase in enzyme activity was observed in the group containing 100 mg/L Gua, a decrease was observed in the groups containing 200 mg/L Gua, 400 mg/L Gua, 800 mg/L Gua and DMSO. In the female individuals, there was a decrease in the enzyme activity at all doses compared to the control group (Fig. 10).

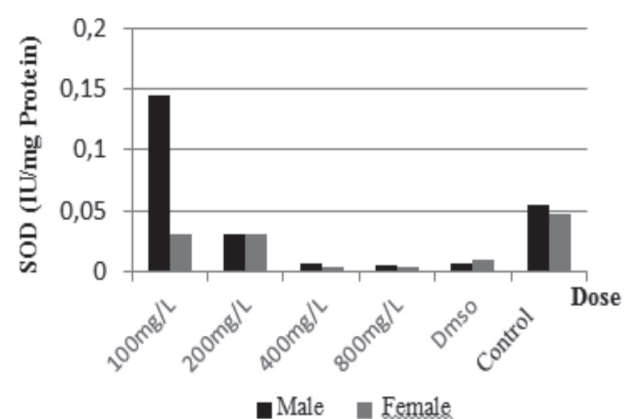


Fig. 9. Gender-Related Dose Effect of Gua (SOD/24).

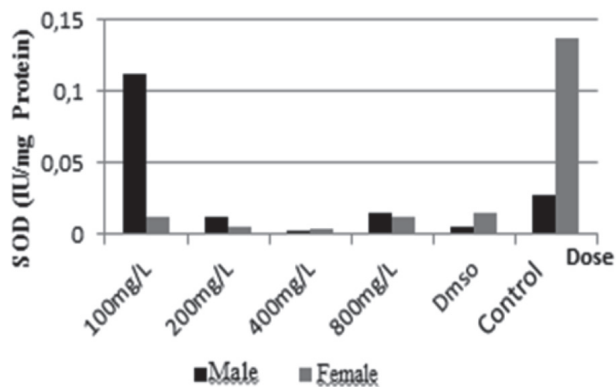


Fig. 10. Gender-Related Dose Effect of Gua (SOD /48).

CAT Enzyme Activity

In the 12th hour activity measurements, the highest CAT activity was measured in the group containing DMSO in the male individuals (Fig. 11). Since CAT activity increased too much in this group, the other doses cannot be observed in the graph. That's why, the other doses are shown in Fig. 12 without DMSO. In the females, the CAT activity was highest in the group containing 400 mg/L Gua (Fig. 12). In the groups containing 100 mg/L Gua, 800 mg/L Gua and DMSO, the CAT activity was found to be higher in the males than in the females. On the other hand, in the groups containing 200 mg/L Gua and 400 mg/L Gua, it was determined that it was higher in the female individuals than in the male. When evaluated compared to the control group, while a decrease was observed in the enzyme activity at 200 mg/L dose in the male individuals, an increase was observed in the enzyme activity in all other groups. In the female individuals, there was an increase in the amount of enzyme in all groups compared to the control group (Fig. 12).

In the 24th hour measurements, the CAT activity was measured the highest in the DMSO-containing group in both the males and females (Fig. 13). Since CAT activity

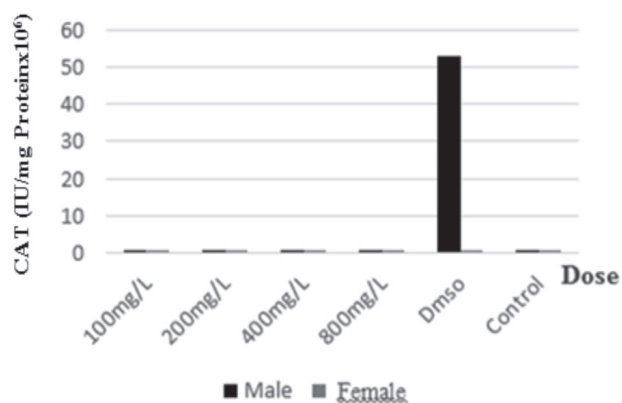


Fig. 11. Gender-Related Dose Effect of Gua (CAT/12).

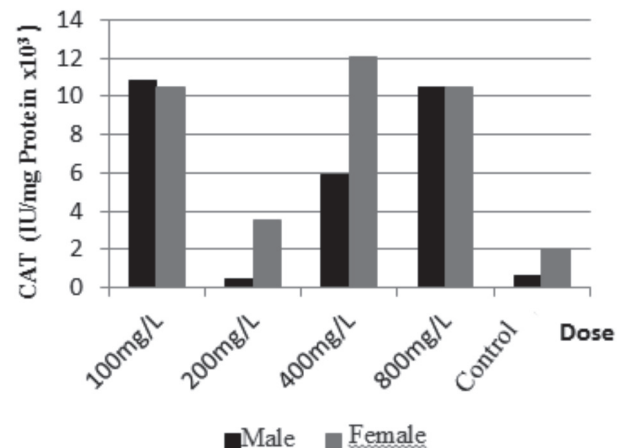


Fig. 12. Gender-Related Dose Effect of Gua (CAT/12).

increased too much in this group, other doses could not be observed in the graph. That's why, the other doses are shown in Fig. 14 without DMSO. When the male and female individuals are compared, it was observed that CAT activity increased in the male individuals of the groups containing 100 mg/L Gua and DMSO. In addition, it was observed that it increased in the females in the groups containing 200 mg/L Gua, 400 mg/L Gua and 800 mg/L Gua. When it was evaluated compared to the control group, while an increase in the enzyme activity was observed in the group containing DMSO in both the male and the female individuals, a decrease was observed in the groups containing 100 mg/L Gua, 200mg/L Gua, 400 mg/L Gua and 800 mg/L Gua (Fig. 14).

In the 48th hour measurements; the CAT Activity was also measured the highest in the females of the group containing DMSO (Fig. 15). Also in this group, since the amount of CAT increased too much, other doses could not be observed in the graph. That is why, other doses are shown in Fig. 16 without DMSO. Among male individuals, the CAT activity was measured the highest in the group containing 100 mg/L Gua. When

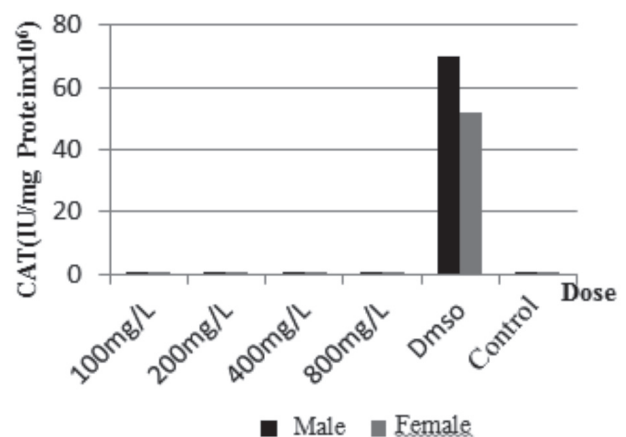


Fig. 13. Gender-Related Dose Effect of Gua (CAT/24).

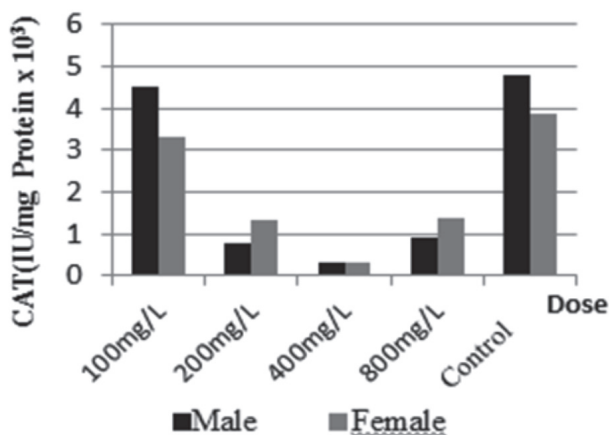


Fig. 14. Gender-Related Dose Effect of Gua (CAT/24).

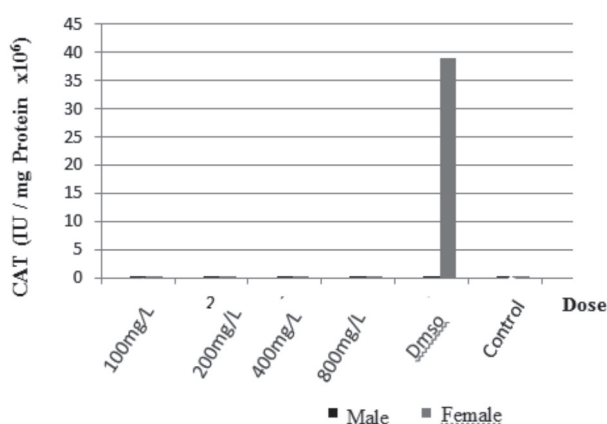


Fig. 15. Gender-Related Dose Effect of Gua (CAT/48).

the male and female individuals are compared, it was observed that, while the CAT activity increased in the male individuals of the group containing 200 mg/L Gua, it increased in the female individuals in groups containing 100 mg/L Gua, 400 mg/L Gua, 800 mg/L Gua and DMSO (Fig. 16). When it was evaluated compared to the control group, a decrease in enzyme activity was observed in the male individuals of all groups. On the other hand, in the females, while

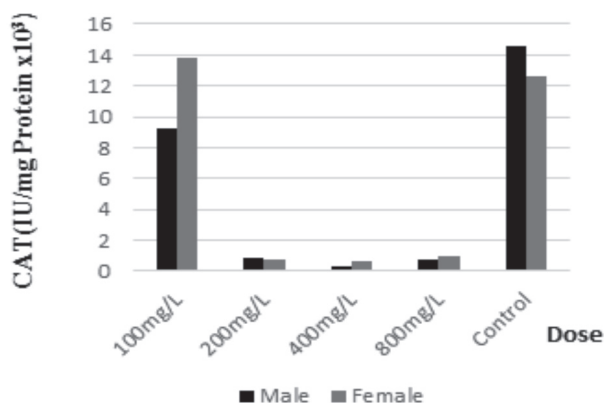


Fig. 16. Gender-Related Dose Effect of Gua (CAT/48).

an increase was observed in the groups containing 100 mg/L Gua and DMSO, a decrease was observed in the groups containing 200 mg/L Gua, 400 mg/L Gua and 800 mg/L Gua (Fig. 16).

When statistically evaluated, it was seen that, although there was no significant difference in the amount of MDA and SOD enzyme activity between the and male individuals ($p > 0.05$), there was a significant difference ($p < 0.05$) between the doses given. However, it was seen that there was no significant difference between the control and DMSO-containing groups in both the MDA amount and the SOD enzyme activity ($p > 0.05$). It was seen that, although there was no significant difference ($p > 0.05$) between the male and female individuals in the CAT enzyme activity, besides a significant difference ($p < 0.05$) between the doses given, there was a significant difference ($p < 0.05$) between the control and DMSO-containing groups.

Discussion and Conclusions

Gua is an azulene-derived sesquiterpene commercially obtained from plants and has been widely used in cosmetic products such as hair dyes, face and body care creams, eye drops, and cleansers such as toothpaste in recent years [30]. In addition, Gua and other azulene derivatives are interesting biochemicals that also show anti-inflammatory with peptic ulcer, antineoplastic with leukemia, anticancer, antidiabetes, antispasmodic and antimicrobial and antifungal effects [3, 7, 31-34]. In our studies, the model organism *D. melanogaster* was used. Gua was added to the diet of this model organism as described in the material and method. Thus, the effects of Gua, which is used in many products, on the amount of MDA, which is an oxidative stress indicator, and SOD and CAT activities, which are enzymes that show the defense response against oxidative stress, were investigated. These effects were also studied depending on time, dose, and gender.

SOD and CAT, enzymes responsible for cellular antioxidant defense mechanisms, not only eliminate superoxide anions and hydrogen peroxides, but also inhibit free radical production [35]. SOD is a front-line antioxidant enzyme that catalyzes the breakdown of superoxide as well as acting as a first defense against oxygen-induced free radical generation and is important for most forms of eukaryotic life [36]. Thus, it converts O_2^- into less harmful reactive H_2O_2 radical. When oxidative stress increases in cells, SOD enzyme activity increases. SOD activity indicates the presence and scavenging activity of oxygen radicals. The CAT enzyme prevents cell damage by converting the formed H_2O_2 to H_2O [37, 38]. Increased CAT enzyme activity results in increased scavenging activity of hydrogen peroxide, so binding of oxygen radicals to the cell membrane may be less likely to be damaged.

DMSO is often used as a solvent for organic compounds in both *in vivo* and *in vitro* studies. Nazir

et al. (2003), recommended that DMSO should be used very carefully and evaluated separately in order not to cause false data in the scan results in studies with *D. melanogaster* [39]. Peet et al. (2016), DMSO was used as a solvent for Gua and the results were evaluated compared to both the control group and the control group containing DMSO [23, 40]. Based on the literature review, in our study, the control group and the control group containing DMSO were evaluated separately and it was observed that the amount of CAT increased in the groups containing DMSO in all hours. For this reason, in the CAT activity studies, it is suggested that organic solvents other than DMSO can be used. Cvetković et al. (2015), in order to determine the effects of DMSO in *D. melanogaster*, found that the ineffective concentration was <0.04%, the minimum effective concentration was 0.04%, and the LC_{50} was 0.42% as a result of their study with larvae [41]. These data revealed that the toxic effect of DMSO for *D. melanogaster* may be more than thought. The data we obtained in our study also support these findings. For CAT enzyme studies in *D. melanogaster*, contribution to studies can be made by finding ineffective concentration, minimum effective concentration and LC_{50} doses. The SOD activity increased at the 12th hours and decreased at 24th and 48th hours. Although a general conclusion cannot be reached for this, it has been suggested that the separate evaluation of the control group and the group containing DMSO is important for the reliability of the results.

In the studies carried out, it has been observed that Gua has radical scavenging properties, can protect cells against oxidative stress, can be used as an antioxidant and antiproliferative agent, and heals cells with hepatotoxicity [42, 43]. In addition, it has been reported that Gua has antiretroviral activity against HIV 1 virus and can be used in anticancer drugs in another study [40, 44, 45]. Park et al. (2021), also stated that Gua showed the significant ABTS+ radical scavenging activity similar to ascorbic acid [46]. Our studies support that Gua exhibits antioxidant properties by decreasing both the SOD enzyme activity in the experimental groups containing 200 mg/L Gua, 400 mg/L Gua, 800 mg/L Gua at the 24th and 48th hours and the CAT enzyme activity in all doses and at all hours. In addition, SOD activity was increased in all doses of the 12th hour experimental groups and in the dose group containing 100 mg/L Gua of the 24th and 48th hour experimental groups.

These results reveal that Gua exhibits antioxidant properties with increasing dose or exposure time. In our study; In addition, it was determined that the effect of Gua did not change according to gender. However, in the study of Uysal et al. (2020), it was observed that the number of both male and female individuals decreased due to the increasing concentration of Gua [23]. In our study, when the concentrations of 100 mg/L Gua and 200 mg/L Gua were compared, an increase was observed in the level of MDA, which is a marker of cell

damage. This explains the decrease in life expectancy due to Gua observed in the study of Uysal et al. (2020) [23]. While the free radicals formed in 100 mg/L Gua in our study were swept away by increased SOD and CAT activity, the radicals formed by 200 mg/L Gua suppressed SOD and CAT activity. MDA levels corresponding to 100 mg/L and 200 mg/L Gua support to explain the activity of these enzymes.

Acknowledgments

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Conflict of Interest

We declare that there is no conflict of interest in the planning, execution and writing of the article.

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