Volume 13, Issue 3, Page 148-153, 2024 https://doi.org/10.46810/tdfd.1454074 Research Article

BİNGÖL UNIVERSITY

Türk Doğa ve Fen Dergisi
Turkish Journal of Nature and Science
www.dergipark.gov.tr/tdfd

Investigation of the Effects of Maleic Acid and Vanillic Acid on Copper Toxicity in the *Drosophila melanogaster* Model

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(Received: 16.03.2024, Accepted: 06.09.2024, Online Publication: 26.09.2024)

Keywords
Antioxidant, *Drosophila*, Maleic acid, Vanillic acid

Abstract: Copper is a metal that is necessary for the maintenance of biological functions in the all living organisms. Although copper is essential for the maintenance of cellular metabolism at low concentrations, at high concentrations it can cause toxic effects as it causes (Reactive Oxygen Species) ROS formation. In this study, toxicity was induced by $CuSO_4$ (1 mM) in larval and adult *Drosophila melanogaster*. The flies were then treated with maleic acid (MA) (2 mg) and vanillic acid (VA) (2 mg). The results showed that Cu toxicity caused a decrease in (Superoxide dismutase) SOD, Catalase (CAT), (Glutathione peroxidase) GPx, (Acetylcholinesterase) AChE and (Glutathione) GSH levels. There was a significant increase in (Malondialdehyde) MDA levels. However, it was found that treatment with MA and VA increased the amounts of SOD, CAT, GPx, AChE and GSH and decreased the amount of MDA. These results showed that MA and VA had ameliorative effects on ROS and oxidative stress caused by $CuSO_4$. In conclusion, the effects of natural compounds on different biological parameters against metal-induced toxicity should be evaluated in future studies.

Drosophila melanogaster Modelinde Maleik Asit ve Vanilik Asitin Bakır Toksisitesi Üzerine Etkilerinin Araştırılması

Anahtar Kelimeler
Antioksidan, *Drosophila*, Maleik asit, Vanilik asit

Öz: Bakır, tüm canlı organizmaların biyolojik fonksiyonlarının sürdürülmesi için gerekli olan bir metaldir. Bakır, düşük konsantrasyonlarda hücresel metabolizmanın sürdürülmesi için gerekli olmasına rağmen, yüksek konsantrasyonlarda (Reaktif Oksijen Türleri) ROS oluşumuna neden olduğundan toksik etkilere neden olabilir. Bu çalışmada larva ve yetişkin *Drosophila melanogaster*'de $CuSO_4$ (1 mM) ile toksisite oluşturuldu. Sinekler daha sonra maleik asit (MA) (2 mg) ve vanilik asit (VA) (2 mg) ile işlendi. Sonuçlar Cu toksisitesinin (Süperoksitdismutaz) SOD, (Katalaz) CAT, (Glutatyon peroksidaz) GPx, (Asetilkolinesteraz) AChE ve (Glutatyon) GSH düzeylerinde azalmaya neden olduğunu gösterdi. (Malondialdehit) MDA düzeylerinde önemli bir artış oldu. Ancak MA ve VA tedavisinin SOD, CAT, GPx, AChE ve GSH miktarlarını artırdığı, MDA miktarını ise azalttığı belirlendi. Bu sonuçlar MA ve VA'nın $CuSO_4$'ün neden olduğu ROS ve oksidatif stres üzerinde iyileştirici etkilere sahip olduğunu gösterdi. Sonuç olarak, gelecekteki çalışmalarda metal kaynaklı toksisiteye karşı doğal bileşiklerin farklı biyolojik parametreler üzerindeki etkileri değerlendirilmelidir.

1. INTRODUCTION

Heavy metals are elements that are frequently used in agriculture and industrial areas. Heavy metal pollution has reached dangerous levels in more than 5 million sites worldwide. Improper management of industrial wastes and widespread use of pesticides in agriculture are among the most important causes of heavy metal pollution [1]. Copper is a metal that is necessary for the maintenance of biological functions of all living organisms [2]. Although Cu, which is used in many areas from construction to transportation, from health to cosmetics, is not harmful at low concentrations, exposure to high concentrations of Cu can cause toxic effects on the living body [3]. Studies have indicated that copper concentrations in water and soil are

approximately 7 and 50 ppm, and Cu in the atmosphere is between 5 and 200 ng/m3. The maximum concentration of copper that can be ingested by humans has also been determined to be 1.5 mg/L serum [4]. Copper metal is essential for the continuity of biological reactions of organisms, but more than the permissible amount of free copper ions can damage cellular structures [5]. Exposure to high levels of copper causes oxidative stress, DNA damage and reduced cell proliferation [6]. Oxidative stress resulting from Cu exposure has also been associated with dysregulation of Cu metabolism and neurodegenerative disorders [7]. Cu toxicity can affect many other organs, especially the liver, as high amounts of Cu in the body accumulate in the liver after entering the bloodstream [8,9]. Phytochemicals may be effective compounds in improving oxidative stress [10]. Maleic acid (MA) is an organic compound and a dicarboxylic acid. It is found as a metabolite in plants [11]. Vanillic acid (VA) is a common monohydroxybenzoic acid found in many plants and presents antioxidant, anti-inflammatory, anti-allergy, and anti-diabetes activities [10]. *Drosophila melanogaster*, a fruit fly, is an important model organism that can be used to study the molecular mechanisms of metal toxicity. In this study, the ameliorative effects of maleic and vanillic acids, both phytochemicals, against copper toxicity in *D. melanogaster* were investigated.

2. MATERIAL AND METHOD

2.1. Chemicals

$CuSO_4$ was used as the copper source in this study. All chemicals, including maleic acid (MA) and vanillic acid (VA), were purchased from Sigma.

2.2. Animals and Experimental Design

The wild-type Oregon R strain *Drosophila* culture used in the study was purchased from Carolina (172100). Under laboratory conditions, flies were fed with standard *Drosophila* medium (SDB) consisting of corn flour, agar, sucrose, dry yeast and propionic acid. Flies were reared in rooms with a temperature of 25 ± 1 °C and 40-60% humidity and kept on a 12 h light/12 h dark cycle. Third instar larvae obtained from these flies were used in all treatments. The larvae were divided into six groups.

Group 1 (Control group): Larvae in this group were treated with distilled water.
Group 2 (Copper group): Larvae in this group were treated with 1 mM Cu^{+2} solution.
Group 3 (MA group): Larvae in this group were treated with 2 mg/mL MA solution.
Group 4 (VA group): Larvae in this group were treated with 2 mg/mL VA solution.
Group 5 (MA+ copper group): Larvae in this group were treated with 1 mM Cu^{+2} solution and 2 mg/mL MA solution.
Group 6 (VA+ copper group): Larvae in this group were treated with 1 mM Cu^{+2} solution and 2 mg/mL VA solution.

For each treatment, 1.5 g of Formula 4-24® Instant *Drosophila* Medium (Carolina) was wetted with 5 mL of test solution. Larvae were collected for analysis 24 hours after treatment. In addition, the heads of adult flies developed from larvae treated with the test solutions were dissected and used to analyze. The experimental design is schematized in Figure 1.

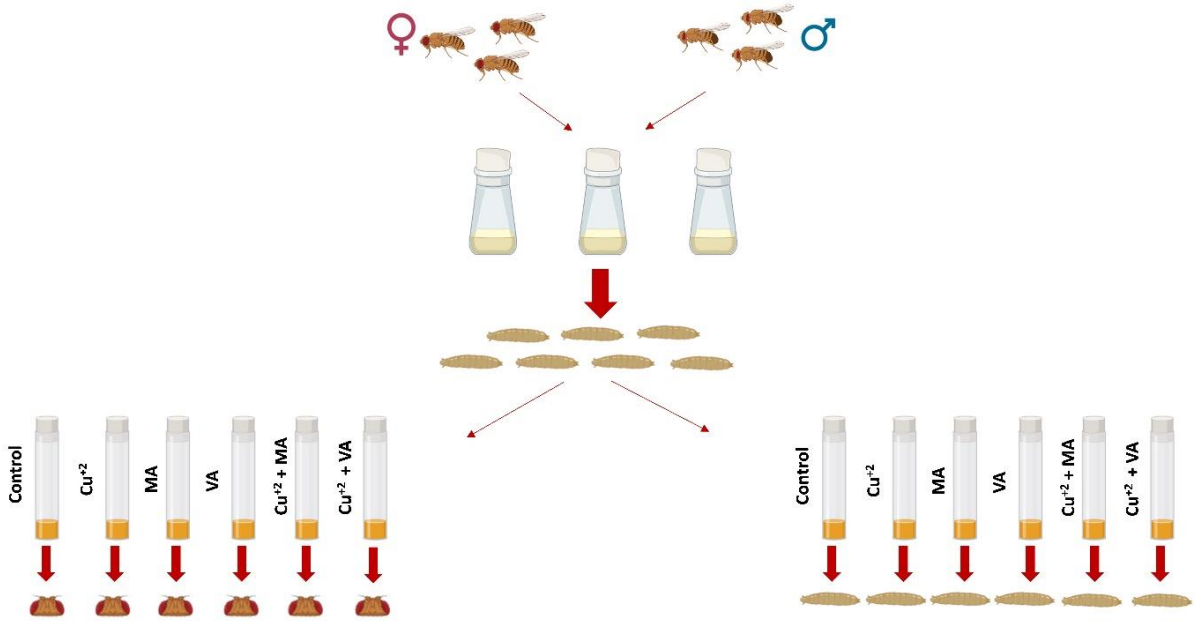

Figure 1. Schematization of the experimental design in adult flies and larvae

2.3. Determination of Total Glutathione (GSH)

The total GSH level was determined following the protocol suggested by Sedlak and Lindsay [12]. GSH (mM) level at 412 nm was measured spectrophotometrically using the supernatant of homogenized fly head and larval samples.

2.4. Malondialdehyde (MDA) Formation Determination

Lipid peroxidation (LPO) levels in fly head and larval homogenates were determined by measuring MDA by thiobarbituric acid (TBA) assay [13]. The amount of MDA was determined by measuring the absorbance at 532 nm of a pink colored product formed by reaction with TBA [13].

2.5. Enzyme Activity Assays

Protein content in fly head tissue and larval homogenate was measured according to the Bradford method [14]. The activities of SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), GPx (EC 1.11.1.9) and AChE (EC 3.1.1.7) enzymes were determined following the protocol suggested by Sun et al., 1988, Aebi et al., 1984, Beutler et al., 1975, and Ellman et al., 1961 respectively [15-18].

2.6. Statistical Analysis

Statistical analysis of the experimental results was performed using GraphPad Prism Software version 8.0 (GraphPad Software, San Diego, CA). Statistical comparisons were made by one-way ANOVA and Tukey's post-hoc tests. Symbol expressions are presented below: * $P < 0.05$ (significant); ** $P < 0.01$ (highly significant); *** $P < 0.001$ and **** $P < 0.0001$ (highly significant).

3. RESULTS AND DISCUSSION

Copper (Cu) is an essential metal required for the maintenance of physiological functions of living organisms. Cu is essential for the maintenance of metabolic processes and biological functions in living organisms, and high levels of Cu in the body can cause many adverse effects. Studies have shown that prolonged exposure to toxic levels of Cu can cause organ dysfunction in humans and animals [19,20]. The formation and accumulation of reactive oxygen species (ROS) is one of the most important consequences of heavy metal exposure [21. ROS interact with biological molecules and alter their structure or metabolic activity, leading to oxidation of proteins and nucleic acids and lipid peroxidation [22]. Antioxidant enzymes and compounds are agents that form the first line of cellular defense against oxidative damage [23]. Superoxide dismutases (SOD), which are antioxidant enzymes, provide the conversion of superoxide anions to dioxygen and hydrogen peroxide, while catalase (CAT) catalyzes the conversion of hydrogen peroxide to water. [24,7]. In a study, *Drosophila melanogaster* was exposed to 0.09 and 1.2 mg/mL dissolved copper for 7 days. The data obtained showed that Cu^{+2} increased mortality and decreased egg production and body size [25]. In a similar study, CAT and glutathione activity decreased while lipid peroxidation levels increased in flies treated with copper sulfate. After treatment with resveratrol (30 or 60 mg/kg), the antioxidant and anti-inflammatory capacities of the flies increased [26]. Copper toxicity can also cause locomotor dysfunction in living organisms. One study showed that exposure to 1 and 3 mM $CuSO_4$ inhibited total AChE activity in *D. melanogaster* and caused impaired climbing ability (negative geotaxis) in adult flies [4]. Our study investigated the ameliorating effect of maleic acid (MA) and vanillic acid (VA) on $CuSO_4$-induced copper toxicity in flies.

Maleic acid is a dicarboxylic acid that acts as a fragrance agent and pH adjuster in cosmetics; it is used in low concentrations in several cosmetic product formulations [27]. In a study, the effect of MA on the toxicity caused by Cr stress in plants was investigated. The results showed that MA reduced oxidative stress by increasing the activities of antioxidant defense system enzymes inhibited by Cr stress [11]. In a similar study, MA was found to affect oxidative stress, lipid peroxidation and inflammatory response at the cellular level [28]. When the results obtained from our study were examined, it was observed that SOD, CAT and GPx activities increased significantly in the Cu-treated group, while the enzyme activities in the MA-treated group reached a level similar to the control. When compared with the Cu group, it was determined that the enzyme activities in the MA group were close to normal, but the enzyme activities in the Cu^{+2} + MA group were closer to the Cu^{+2} group (Figure 2a, 2b, 2c). When the enzyme activity changes in adults were examined, it was observed that there was a significant increase especially in SOD activity in the Cu^{+2} treated group. In the MA group, SOD, CAT and GPx activities approached the control (Figure 2e, 2f, 2g). These results showed that MA reduced Cu^{+2}-induced radical formation in *D. melanogaster* and improved the antioxidant buffering capacity of flies. Acetylcholinesterase enzyme activity is an effective and widely used mechanism to assess toxicological effects involving the nervous system. In a study, it was found that 1mM $CuSO_4$ treatment caused a significant inhibition of total AChE activity in flies and an impairment in the climbing ability (negative geotaxis) of adult flies [4]. In our study, it was observed that AChE enzyme activity decreased significantly in the Cu-treated group in both larval and adult flies, but the activity increased with MA treatment and approached the control (Figure 2d and 2h).

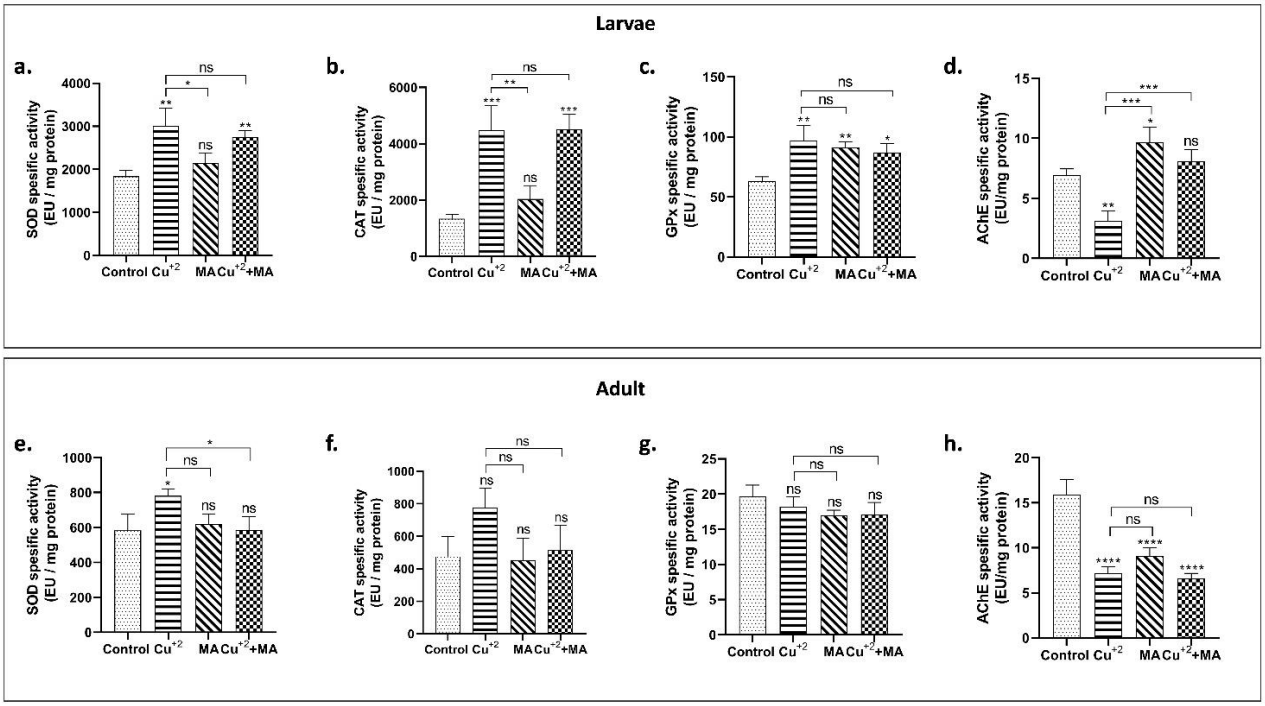

Figure 2. Changes in SOD, CAT, GPx and AChE enzyme activities in larval and adult D. melanogaster treated with maleic acid

Vanillic acid (VA, 4-hydroxy-3-methoxybenzoic acid) is an intermediate used in the production of vanillin from ferulic acid and is a phenolic derivative of edible plants. VA has antimicrobial activity and is an effective component in clearing free radicals [23]. When we examined the effect of vanillic acid against copper toxicity, it was found that SOD, CAT and GPx activities increased significantly in the Cu-treated group in larvae. In the VA-treated group, these enzyme activities were found to be similar to the control. In the Cu^{+2} and VA groups, enzyme activities approached the control (Figure 3a, 3b and 3c). When the results in adult flies were examined, it was found that SOD activity increased significantly in the Cu-treated group. It was observed that all enzyme activities in VA and Cu^{+2} + VA group reached similar levels with the control. When all the results were evaluated together, it was determined that VA showed ameliorative effect against Cu toxicity by reducing oxidative stress (Figure 3e, 3f, 3g). When the change in AChE enzyme activity was examined, it was found that AChE activity decreased significantly in both larvae and adults in the Cu-treated group and increased as a result of VA treatment (Figure 3d and 3h).

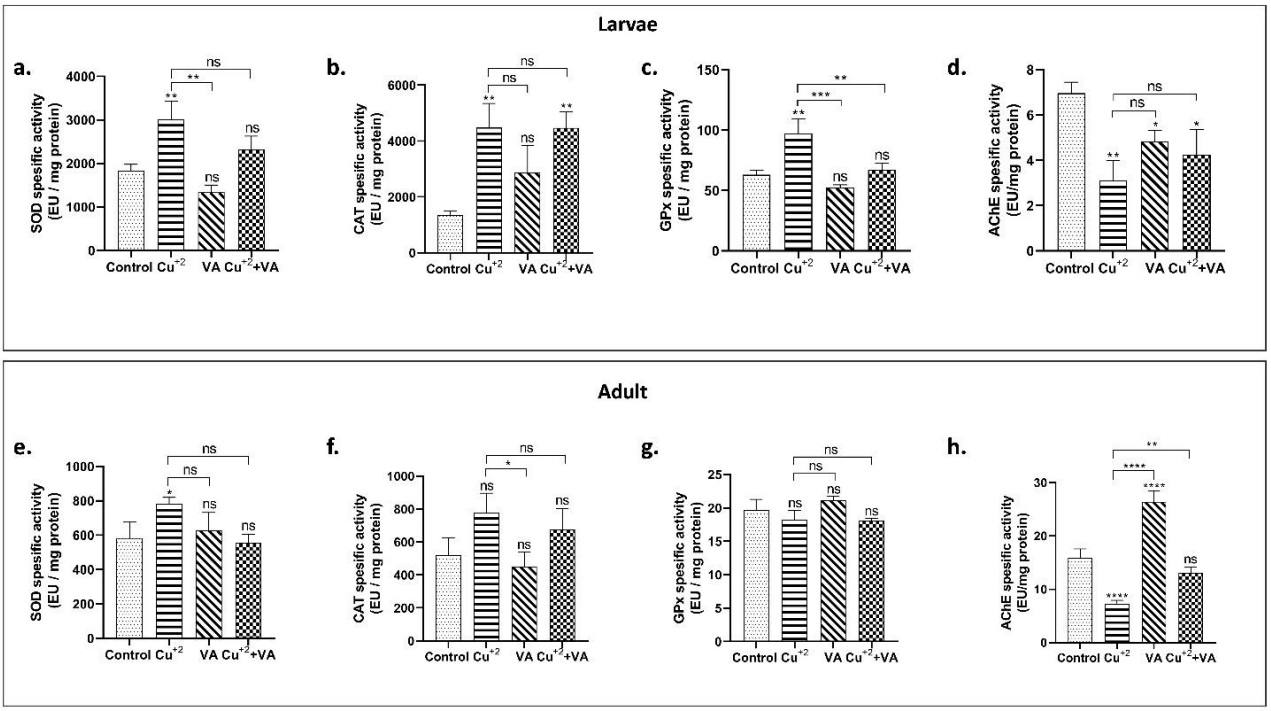

Figure 3. Changes in SOD, CAT, GPx and AChE enzyme activities in larval and adult *D. melanogaster* treated with vanillic acid

Exposure to toxic levels of Cu also causes peroxidation of lipids in membranes, which can damage cellular components [3]. In a study, it was found that GSH level decreased and MDA level increased in serum and liver in rats overloaded with Cu [29]. Reduced glutathione (GSH) is a non-enzymatic antioxidant. Decreased GSH levels in cells are an indicator of ROS accumulation and therefore oxidative stress [30]. In rats exposed to Cu^{+2} and $CuSO_4$ for 21 and 42 days, GSH levels decreased significantly and triggered oxidative stress [9]. In our study, Cu toxicity significantly decreased GSH levels and increased MDA levels in both larval and adult flies.

When the post-treatment results were analyzed, it was observed that MA treatment increased GSH levels and decreased MDA levels in both larval and adult flies (Figure 4).

When we examined the effect of VA on Cu toxicity, it was observed that VA treatment increased GSH levels and decreased MDA levels in both larval and adult flies (Figure 5). The increase in GSH level and decrease in MDA level after VA treatment showed that VA may reduce the oxidative stress caused by Cu.

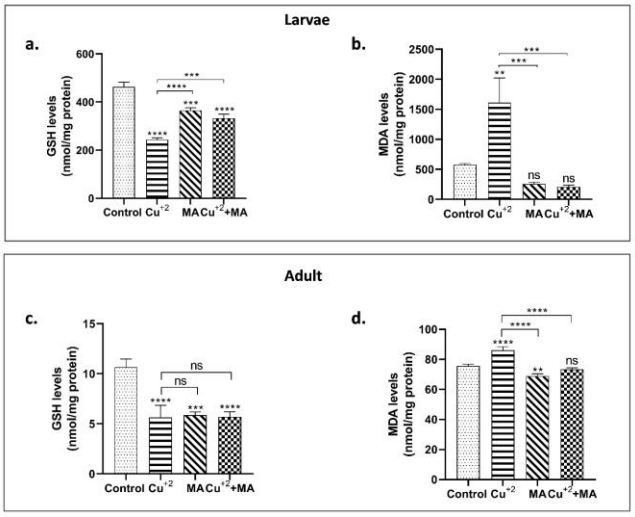

Figure 4. Changes in GSH and MDA enzyme activities in larval and adult D. melanogaster treated with maleic acid

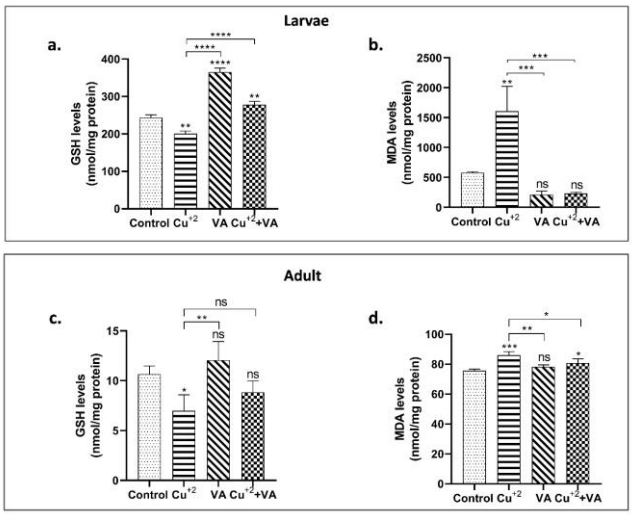

Figure 5. Changes in GSH and MDA enzyme activities in larval and adult D. melanogaster treated with vanillic acid

4. CONCLUSION

Although Cu metal is essential for the maintenance of many biological functions, exposure to high levels of Cu can damage cellular components and cause oxidative stress. This study showed that MA and VA reduced Cu^{+2}-induced radical formation in *D. melanogaster* and improved the antioxidant buffering capacity of flies. MA and VA can therefore be used to treat disorders involving oxidative stress. However, the concentrations of natural compounds, their interactions and their effects on different biological parameters against toxicity should be evaluated in future studies.

Acknowledgement

The authors acknowledge with thanks Ataturk University technical support.

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