Cumhuriyet Sci. J., 45(2) (2024) 256-262
DOI: https://doi.org/10.17776/csj.1425012

Cumhuriyet Science Journal

| csj.cumhuriyet.edu.tr | Founded: 2002 ISSN: 2587-2680 e-ISSN: 2587-246X Publisher: Sivas Cumhuriyet University

Influence of Selected Natural Antioxidants on Iron-Induced Enzymatic Alterations Related to Oxidative Stress

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Research Article

History
Received: 24/01/2024
Accepted: 13/06/2024

ABSTRACT

Iron is required in various biological processes of the cell, but excess iron causes oxidative stress. Oxidative stress can be prevented by antioxidants with free radical scavenging properties. Tannic acid and gallic acid are phenolic compounds with antioxidant properties found naturally in plants. In this study, the effects of gallic acid and tannic acid on iron-induced oxidative stress parameters were investigated in a fruit fly model. Effect of the compounds against iron-induced oxidative stress were evaluated by determining spectrophotometrically superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and acetylcholinesterase (AChE) enzyme activities, and levels of reduced glutathione (GSH) and malondialdehyde (MDA) in larvae (n: 10) and adults (n: 20) of wild type Oregon R strain of Drosophila melanogaster. Iron treatment decreased enzyme activities and GSH levels, but increased MDA levels. Co-treatment of these compounds with iron ameliorated iron-induced changes, especially in larvae. On the other hand, iron-induced decrease in AChE activity was increased in adults by treatment of these compounds with iron. The results showed that natural phenolic compounds have the potential to ameliorate iron-induced changes in oxidative stress parameters.

Keywords: Antioxidant, Drosophila, Phenolic compound.

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Introduction

Iron, which plays a role in normal cellular physiology such as oxygen transport and energy production, is an essential mineral for the growth, development and survival of most organisms [1]. However, excess iron intake has been associated with many diseases such as diabetes, hormonal abnormalities, immune system disorders, neurological disorders, certain types of cancer, liver and heart diseases. Due to its chemical structure and capacity to carry out single-electron reactions, iron plays an important role in the production and metabolism of free radicals in biological systems. Therefore, it has been reported that iron toxicity is caused by free radicals that cause tissue damage [2]. In this respect, antioxidants with metal chelating and/or free radical scavenging properties are being investigated to prevent free radical formation [3,4].

Polyphenols are bioactive compounds found in plants [5]. These compounds play a role in the prevention of various diseases such as degenerative diseases, cancer, hypercholesterolemia and hyperglycemia. They also have high antioxidant properties [5]. Polyphenols have the capacity to remove lipid peroxidation, scavenge hydroxyl radicals, and prevent oxidative damage. Tannic acid (TA), a polyphenol found in the fruit and peel of many plants, has been reported to have antimutagenic, anticancer, antioxidant, and antimicrobial activities [6–9]. Gallic acid (GA), found in various plants such as tea, hazelnut, grape and pomegranate peel, is a natural phenolic compound. Phenolic hydroxyl groups in GA can scavenge reactive oxygen species and prevent the formation of new radicals. This phenolic compound has anticancer, anti-inflammatory, antimicrobial, antioxidant and neuroprotective effects [10–12]. TA and GA are preferred to investigate their protective effects in toxicity studies due to their antioxidant properties [13–17].

Drosophila melanogaster is used as a model organism to identify novel therapeutics, evaluate anti-aging compounds, study drug addiction, and conduct toxicological and cancer studies, because it has a short life cycle and is cheap and easy to maintain and homologs approximately 75% of the genes associated with human diseases. model organism Drosophila is used as a model organism to identify novel therapeutics, evaluate anti-aging compounds, study drug addiction, and conduct toxicological and cancer studies, because it is cheap and easy to maintain, has a short life cycle and homologs approximately 75% of the genes associated with human diseases [18–23].

Since excess iron intake can lead to the formation of free radicals, the use of scavenging antioxidants can ameliorate iron-induced changes. This study was carried out to investigate the ability of TA and GA, which have antioxidant properties, to alleviate the harmful effects of iron (II) sulfate on lipid peroxidation and antioxidant enzymes in fruit flies.

Materials and Methods

Chemicals

In the study, iron (II) sulfate heptahydrate (AFG Scientific) were used as the iron sources. All chemicals including tannic and gallic acids were obtained from Sigma–Aldrich.

Animals and Treatment

The flies used in the study were wild-type Oregon R strain of *Drosophila melanogaster* and were obtained from Carolina Biological Supply Company. This strain is the standard winged fruit fly with red eyes and brown body. The flies The flies were cultured in vials including standard medium (corn flour, active dry yeast, sugar, agar, water and propionic acid) at 25 °C in an incubator. The third instar larvae obtained from these flies were used for all treatments. The larvae were divided into six groups: control group (treated with distilled water), iron group (treated with 1 mM Fe^{+2} solution), TA group (treated with 2 mg/mL TA solution), GA group (treated with 2 mg/mL GA solution), TA+iron group (treated with 1 mM Fe^{+2} solution and 2 mg/mL TA solution) and GA+iron group (treated with 1 mM Fe^{+2} solution and 2 mg/mL GA solution). For each treatment, 1.5 grams of and Formula 4-24® Instant *Drosophila* Medium (Carolina) was soaked with 5 mL of test solution. Larvae were harvested for analysis 24 hours after administration. In addition, the heads of adult flies developed from treated larvae with the test solutions were dissected and used for analysis.

Preparation of Homogenate

Ten larvae and twenty heads of adult fly belonging to each group were taken into tubes containing homogenate buffer (50 mM phosphate buffer with 1mM EDTA and 1 mM DTT, pH: 7.4). The larvae and heads were homogenized in the TissueLyser LT device (Qiagen) using stainless steel beads (5 mm diameter). The homogenates were centrifuged at 10000 rpm for 30 min at 4 °C. The obtained supernatants were used for biochemical analysis.

Determination of Enzyme Activities

Total protein contents of the supernatants were measured according to the method of [24] using a standard curve prepared with bovine serum albumin. The activity of Superoxide dismutase (SOD) was determined spectrophotometrically at 560 nm as described by [25]. The activity of Catalase (CAT) was measured in a spectrophotometer at 240 nm according to the method described by [26]. The of Glutathione peroxidase (GPx) was determined spectrophotometrically at 340 nm according to the method described by [27]. The activity of Acetylcholinesterase (AChE) was measured colorimetrically at 412 nm using the method described by [28].

Determination of the Reduced Glutathione Level

The reduced glutathione (GSH) concentrations in the supernatants of samples were determined in a spectrophotometer at 412 nm using GSH standard curve according to the method described by [29].

Determination of Lipid Peroxidation

Lipid peroxidation was assessed by the measurement of malondialdehyde (MDA) level. The MDA levels in the samples were measured spectrophotometrically at 532 nm using a standard curve of 1,1,3,3-tetramethoxypropane according to the method described by [30].

Statistical Analysis

All experimental results were expressed as mean ± standard deviation. The results were analyzed with one-way ANOVA and Tukey's post-hoc test using GraphPad Prism Software version 9.0. Differences were considered statistically significant at $p < 0.05$.

Results

The Enzyme Activities in the Larvae and Heads of Adults after the Treatments

The activities of enzymes (SOD, CAT, GPx and AChE) in larvae and heads of adults after treatment with gallic acid and tannic acid were shown in Figure 1 and Figure 2. The treatment of 1 mM Fe^{+2} caused a statistically significant decrease in enzyme activities compared to the control group. Gallic acid treatment did not alter CAT and GPx activities in larvae (Figure 1B and 1C), but increased SOD activity and decreased AChE activity (Figure 1A and 1D). After gallic acid treatment, no statistical change was observed in SOD, CAT and AChE activities in adults (Figure 1E, 1F and 1H), but a decrease in GPx activity was detected (Figure 1G). Gallic acid treatment with Fe^{+2} tolerated the Fe^{+2}-induced decrease in SOD and CAT activity in larvae (Figure 1A and 1B), and AChE activity in the adult (Figure 1G). It brought other enzyme activities closer to the control group. Tannic acid treatment increased SOD activity in both larvae and adults compared to the control group, but did not change the activity of other enzymes except GPx enzyme in larvae (Figure 2A-H). Tannic acid treatment in combination with Fe^{+2} ameliorated Fe^{+2}-induced changes in SOD, CAT and GPx activities (Figure 2A-C), except AChE activity (Figure 2D) in larvae. In adults, this treatment restored GPx and AChE activities (Figure 2G and 2H) to control group values, but increased SOD activity (Figure 2E).

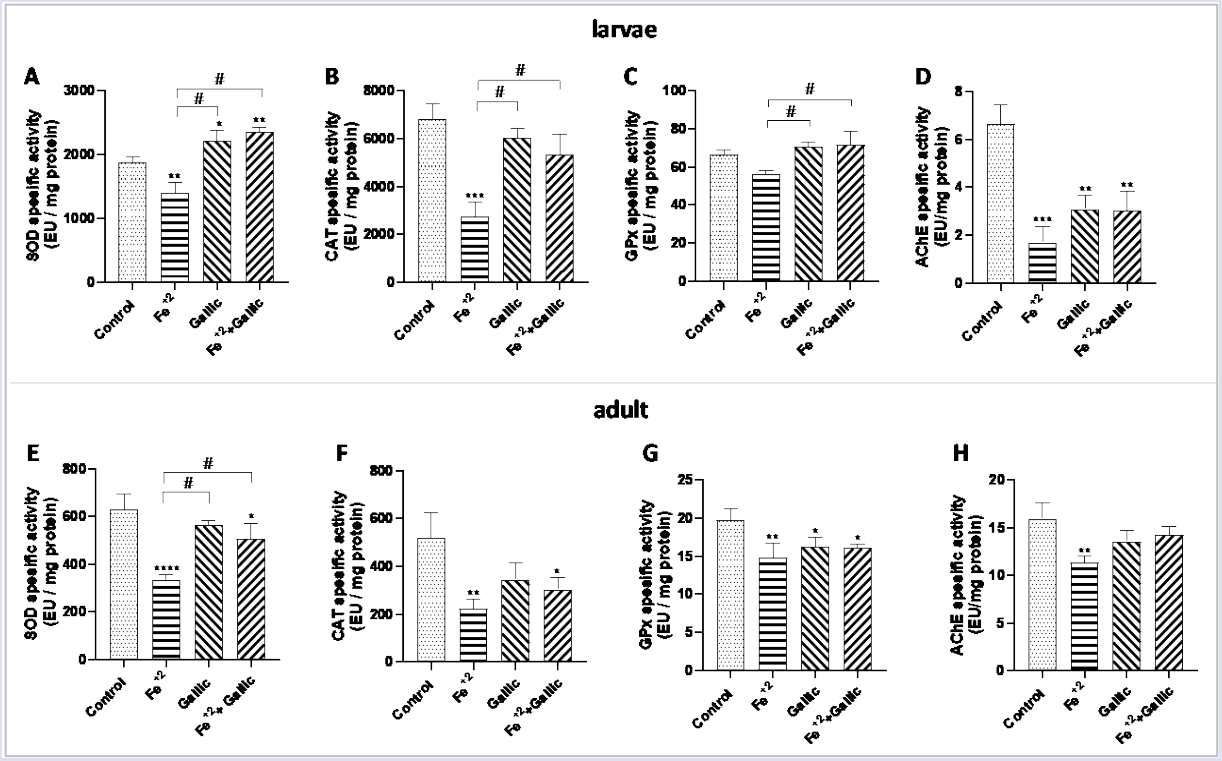

Figure 1. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity and acetylcholinesterase (AChE) activities in larvae (A-D) and heads of adults (E-H) after treatment with gallic acid. The bars represent the mean ± SD. Symbols indicate significance of differences between the groups: *p <0.05, **p < 0.01, ***p < 0.001 vs. control group; #p < 0.05 vs. other groups.

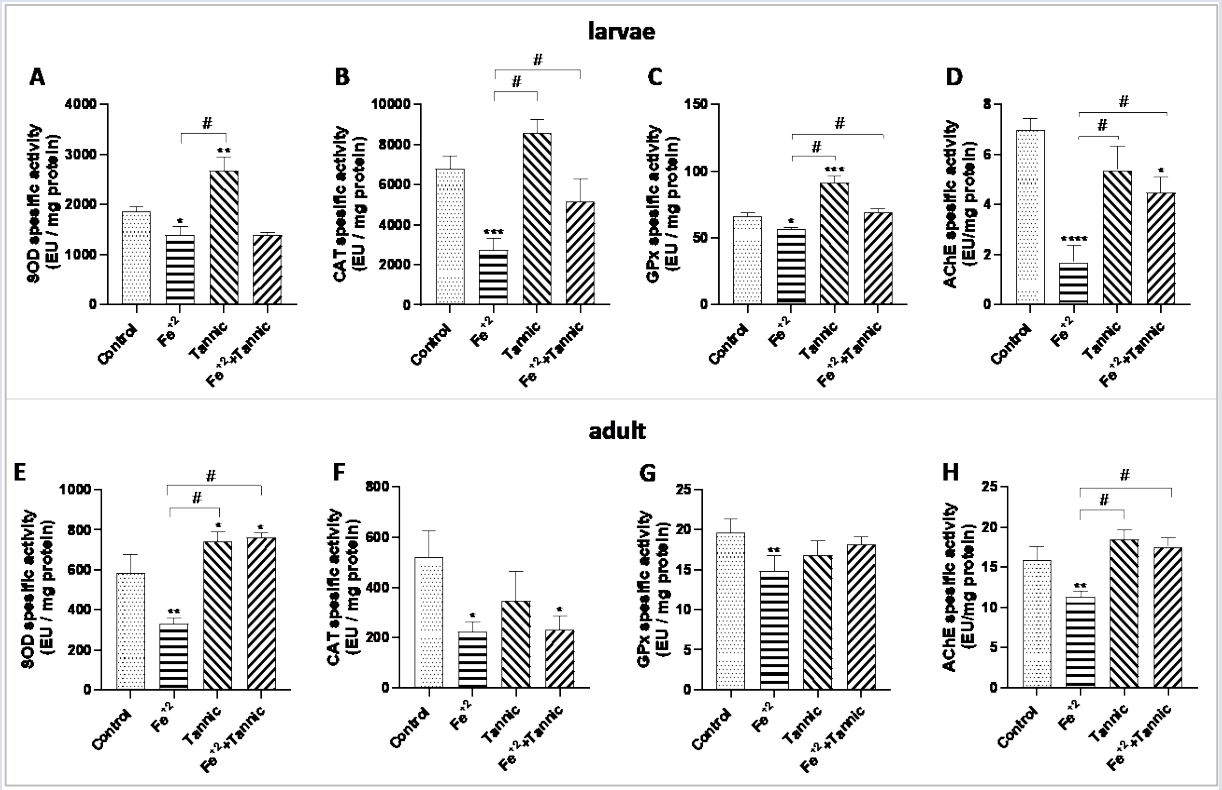

Figure 2. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity and acetylcholinesterase (AChE) activities in larvae (A-D) and heads of adults (E-H) after treatment with tannic acid. The bars represent the mean ± SD. Symbols indicate significance of differences between the groups: *p <0.05, **p < 0.01, ***p < 0.001 vs. control group; #p < 0.05 vs. other groups.

The Reduced Glutathione Levels in the Larvae and Heads of Adults after the Treatments

The reduced glutathione (GSH) levels in larvae and heads of adults after treatment with gallic acid and tannic acid were illustrated in Figure 3 and Figure 4. The Fe^{+2} treatment caused a significant decrease in GSH levels in both larvae and adults compared to the control group. However, gallic acid treatment did not change GSH levels in both larvae and adults (Figure 3A and 3B). Gallic acid treatment together with Fe^{+2} resulted in an increase in GSH level of larvae (Figure 3A). On the other hand, both alone and combined treatment of tannic acid with Fe^{+2} increased the GSH level of larvae compared to the control group (Figure 4A).

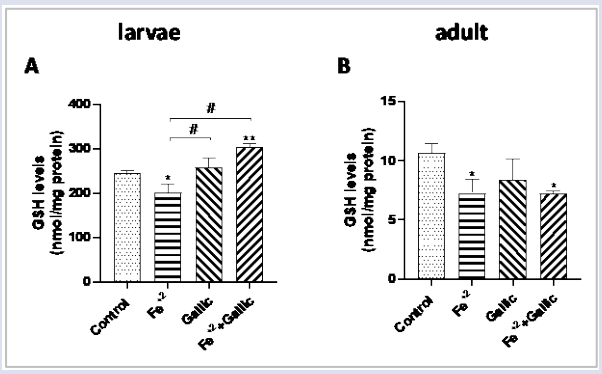

Figure 3. The levels of glutathione (GSH) in larvae (A) and heads of adults (B) after treatment with gallic acid. The bars represent the mean ± SD. Symbols indicate significance of differences between the groups: *p <0.05, **p < 0.01, ***p < 0.001 vs. control group; #p < 0.05 vs. other groups.

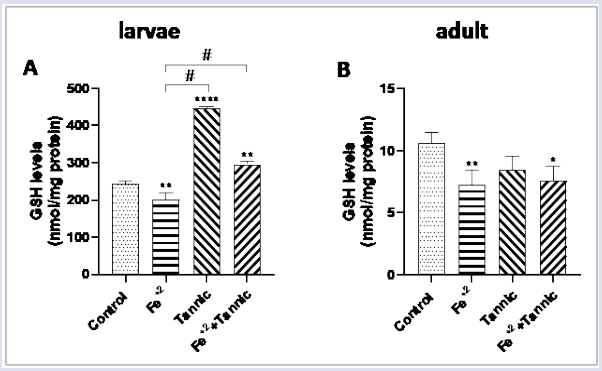

Figure 4. The levels of glutathione (GSH) in larvae (A) and heads of adults (B) after treatment with tannic acid. The bars represent the mean ± SD. Symbols indicate significance of differences between the groups: *p <0.05, **p < 0.01, ***p < 0.001 vs. control group; #p < 0.05 vs. other groups.

The Malondialdehyde Levels in the Larvae and Heads of Adults after the Treatments

The MDA levels in larvae and heads of adults after treatment with gallic acid and tannic acid were shown in Figure 5 and Figure 6. Fe^{+2} treatment increased MDA levels in larvae and adults compared to the control group. Gallic acid treatment caused a decrease in MDA levels in adults. In addition, co-treatment of gallic acid with Fe^{+2} attenuated the Fe^{+2}-induced increase in MDA levels in adults (Figure 5B). Tannic acid treatment did not change

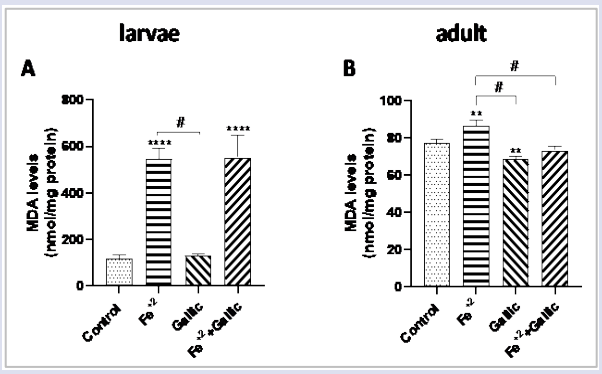

Figure 5. The level of malondialdehyde (MDA) in larvae (A) and heads of adults (B) after treatment with gallic acid. The bars represent the mean ± SD. Symbols indicate significance of differences between the groups: *p <0.05, **p < 0.01, ***p < 0.001 vs. control group; #p < 0.05 vs. other groups.

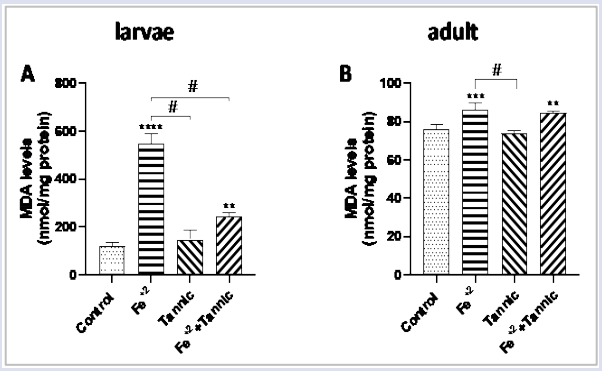

Figure 6. The level of malondialdehyde (MDA) in larvae (A) and heads of adults (B) after treatment with tannic acid. The bars represent the mean ± SD. Symbols indicate significance of differences between the groups: *p <0.05, **p < 0.01, ***p < 0.001 vs. control group; #p < 0.05 vs. other groups.

MDA levels in larvae and adults, whereas co-treatment with Fe^{+2} brought the Fe^{+2}-induced increase slightly closer to the values of the control group (Figure 6A and 6B).

Discussion

Heavy metals have entered the ecosystem as a result of natural and industrial activities. When exposed to metals through water, food and air, metals can bioaccumulate and cause toxicity in living organisms [31].

Metal-induced toxicity is associated with increased lipid peroxidation and alteration of cell homeostasis, with emphasis on the formation of reactive oxygen species and free radicals. Antioxidants play a protective role against free radical-induced attacks [32]. There are studies showing that antioxidants may provide protection against iron-related toxicity. It has been reported that vitamin E, an important antioxidant, can prevent most iron-induced damage in both in vitro and in vivo systems [33–37]. On the other hand, plant extracts and their components have been shown to have protective potential on iron-induced toxicity. For example, it has been found that baru nut and the phytic acid obtained from it have a protective effect against iron-induced oxidative stress [38]. Extracts of *Terminalia chebula* (Retz.) and *Drosera burmannii* Vahl have been reported to have iron chelation activity, which can reduce toxicity caused by iron overload [39,40]. Rutin, a natural flavonoid, was able to improve antioxidant defense systems against iron-induced hepatic oxidative stress [41]. Gallic acid (GA) and tannic acid (TA) suppressed cisplatin-induced ROS formation, lipid peroxidation and oxidative stress in rat kidney tissue [14]. TA reduced iron overload-induced liver damage in mice through ROS regulation [42]. *Molinga oleifera* leaf extract and its bioactive compound GA reduced metal-induced intracellular reactive oxygen species (ROS) accumulation in *Saccharomyces cerevisiae* [43]. It is emphasized that carboxyl and hydroxyl groups of GA and TA are important in the chelation of metals [42,43]. Therefore, since natural flavonoids and phenolic compounds are well-known antioxidants, they can be effective protective agents against oxidative stress. In this study, the preventive effect of natural phenolic compounds GA and TA against iron-induced oxidative stress in larvae and adult flies was investigated. Iron treatment reduced SOD, CAT, and GPx activities and GSH levels in larvae and adults. Since SOD, CAT and GPx are antioxidant enzymes that fight against oxidative stress, a decrease in their activities is an indicator of oxidative stress. Co-treatment of GA and TA with iron had a better effect on larvae to tolerate the decrease in enzyme activities. The compounds may have been more effective in the larvae because the organism is in the developmental stage and is in a more feeding state. The iron-induced decrease in GSH levels indicates that the primary intracellular antioxidant function is impaired. On the other hand, MDA level, which is an indicator of lipid peroxidation caused by oxidative stress, increased with iron treatment. The increase in MDA level may have been observed because the increase in iron-induced ROS formation causes membrane biochemical and functional changes [44].

AChE is an important enzyme involved in the termination of neurotransmission in cholinergic nerves. The cholinergic system and activity of AChE can be affected by metals such as copper, iron and aluminum [45]. Excessive iron intake is associated with dysfunction in cholinergic neurotransmission as a result of a decrease in acetylcholinesterase pathways involved in neurodegenerative diseases [46]. In this study, we found that iron treatment decreased AChE activity in both larvae and adults. GA and TA treatment with iron brought the iron-induced decrease closer to the control group only in adults. In a study examining the effect of copper on AChE activity at different stages of *D. melanogaster* development, it was determined that there were different changes in enzymatic parameters in larvae and adults [47]. Differences in enzyme activities in larvae and adults may be related to responses to endogenous or exogenous stressors due to complex neurochemical, metabolic and hormonal differences during development.

The fruit fly is used as a model because of its cheap and easy maintenance, short life span, homology with human genes, observable variations and large number of offspring [48]. But it is important to keep in mind that no model organism is exactly the same as a human being. The fly's body size and organization are different from that of a human, which limits its use. However biomedical and toxicological studies, epigenetics, human genetics and disease research are conducted with *D. melanogaster*, and useful results have been obtained [49,50]. Fruit flies and humans have similar metabolic pathways, such as superoxide metabolism and DNA repair, which play a role in pharmaceutical modulation [51]. In the present study, the ameliorative properties of GA and TA against iron-induced changes on antioxidant system elements were studied in a fly model. TA and GA helped to reverse iron-induced changes. The results may pave the way for further studies in this field.

Conclusion

In conclusion, this study showed that gallic acid and tannic acid can affect changes in iron-induced oxidative stress parameters. These natural compounds may be useful for applications to eliminate the negative effects of metal-induced toxicity. Therefore, the concentrations, interactions and effects of natural polyphenols on different biological parameters against the toxicity should be evaluated in further studies.

Conflicts of interest

There are no conflicts of interest in this work.

Acknowledgement

The authors acknowledge with thanks Ataturk University technical support.

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