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Protective role of hesperetin in *Drosophila melanogaster* model of ferrous sulphate-induced toxicity

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Abstract

The toxicological hazard of iron-containing products is a public health concern that inspires research in identifying and developing readily available, inexpensive antidotes. Natural products, like plant-sourced antioxidants, can be of great value in this regard. Hesperetin a flavonoid abundantly present in citrus fruits is known to possess a diverse pharmacological and antioxidant attribute. The present study investigated the alleviation of detrimental effects of ferrous sulphate ($FeSO_4$) by hesperetin in *Drosophila melanogaster*. Flies were exposed to $FeSO_4$ (10 µM) alone or supplemented with hesperetin (50 or 100 µM) via diet for 7 consecutive days. Antioxidant enzyme activities, non-enzymatic antioxidant levels, acetylcholinesterase activity and oxidative stress markers were then measured. Hesperetin supplementation significantly ($p < 0.05$) attenuated $FeSO_4$-induced oxidative stress by enhancement of enzymic antioxidants (catalase and glutathione-S-transferases) activities, preservation of non-enzymic antioxidants (total thiols and non-protein thiols), and reduction of other markers of oxidative stress (hydrogen peroxide, protein carbonyl and lipid peroxidation) in *D. melanogaster*. In addition, hesperetin supplementation decreased nitric oxide levels and enhanced acetylcholinesterase activity. Furthermore, hesperetin supplementation improved $FeSO_4$-induced locomotor deficit, while there was no significant difference in cell viability (mitochondrial metabolic rate) in the treatment groups. This study suggests that hesperetin might be a promising functional agent in preventing iron toxicity and similar metal-induced impairments.

Keywords Antioxidants, Hesperetin, Ferrous sulphate, Oxidative stress, *D. melanogaster*

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Introduction

Metal overload is usually associated with elevated levels of reactive oxygen species triggering a shift in the redox balance [1]. These reactive oxygen species (ROS) and free radicals' accumulation cause cellular alterations in the body, especially in the brain containing large amounts of unsaturated fatty acids prone to oxidative damage. Intracellular binding of metals such as Cu, Mn, Fe and Co can induce folding in proteins such as alpha-synuclein or amyloid beta thereby predisposing to Parkinson's disease, prion disease, or Alzheimer's disease [2].

Although iron is an essential trace element for life, iron overload has been implicated in the causation of oxidative stress-mediated diseases [3]. Total iron concentration in healthy individuals is maintained within a range of 4–5 g by strict control of its absorption, storage, recycling and mobilisation [4]. Approximately 1–2 milligrams of iron are assimilated from the food, with the remaining amount being compensated for by the macrophage-mediated recycling of aged red blood cells [5]. Reports show that values above 20 mg/kg weight intake of iron in humans lead to severe morbidity and mortality [6]. Exposure to iron through the ingestion of iron-containing products has been identified as a pathway of iron poisoning and death in children [7, 8]. Several household products including pharmaceuticals and packaged food items contain iron in varying quantities [9].

Ferrous sulphate ($FeSO_4$), a frequently used iron supplement has been described as the cheapest source of elemental iron and also, the most toxic iron supplement [7, 10]. Upon ingestion of ferrous sulphate, iron is assimilated in the ferrous form and then oxidized to the ferric form that binds to transferrin. The clinical manifestation of acute iron poisoning is divided into three stages; stage I lasts approximately 4 h and is characterised by manifestations associated with damage to the gastrointestinal system; stage II, the recovery stage, where the formation of toxic iron compounds continues; and stage III which commences about 24 h after ingestion and is characterised by multi-organ failure resulting in cerebral dysfunction and coma, ischemic bowel, myocardial depression, and hepatic and renal failure [7, 11, 12]. The time-lapse from exposure to death usually spans 6 to 42 h in children exposed to toxic doses of ferrous sulphate [7, 11, 13].

It is known that iron can undergo Fenton reactions to produce hydroxyl radicals from hydrogen peroxide resulting in oxidative damage to macromolecules [14, 15]. Consequently, the toxicological hazard of ferrous sulphate (and other iron-containing products) is a public health concern that requires the development of readily available, and inexpensive antidotes [16, 17]. Therefore, natural products, like plant-derived antioxidants are of great value.

Hesperetin (5, 7, 3′-trihydroxy-4-methoxyl flavanone) is a flavonoid abundantly present in citrus fruits [18, 19]. It is known to possess a variety of pharmacological and antioxidant properties [20–23]. It is also a good promoter of cellular antioxidant enzyme activity through its radical scavenging property [24, 25]. Hesperetin has been described as an active antioxidant and neuro-protectant with potential for the treatment of neurodegenerative diseases [20, 26]. Hesperetin possesses antioxidant and anti-inflammatory properties and protects brain disorder diseases [27]. It has been demonstrated that hesperetin elicits its antioxidant property by regulating gene expressions of antioxidant enzymes to suppress oxidative damage [28, 29]. Different experimental models including rats [30], cell lines [31] etc. have been used to study the protective properties of hesperetin. Hence, consumption of foods rich in hesperetin may confer benefits on humans.

Drosophila melanogaster is a versatile and reliable model organism for the elucidation of potential mechanisms of toxicity or mode of action of toxicants in humans due to its extensive genetic homology with humans [32]. It is easy to breed in the laboratory, inexpensive to house in large numbers and has a rapid life cycle [33–35].

There is a dearth of valuable scientific information on the preventive effect of hesperetin against $FeSO_4$-induced toxicity. This therefore informed the essence of this research to investigate the potential role that hesperetin plays in mitigating the negative effects caused by Fe using *Drosophila melanogaster* as an experimental model. By addressing this important gap in scientific data regarding the protective effects of hesperetin against $FeSO_4$-induced toxicity in *Drosophila melanogaster*, this study provides an advanced understanding of iron metabolism, toxicity mechanisms, and the pharmacological properties of hesperetin, with implications for human health and therapeutic interventions.

Materials and methods

Drosophila melanogaster stock and culture

This study utilised the Harwich strain of *Drosophila melanogaster*. The fly stock was cultivated on cornmeal in Drosophila jars providing a mixture consisting of 1% weight/volume brewer's yeast, 2% weight/volume sucrose, 1% weight/volume milk in powdered form, 1% weight/volume agar, and 0.08% volume/weight nipagin. The cultivation was carried out at a temperature of 25±1 degrees Celsius and with a moisture content of 60%. The flies were kept and cared for at the Drosophila Research and Training Centre (DRTC) in Ibadan, Nigeria.

Chemicals

Each of the chemicals used was of analytical purity. Hesperetin (99%) and Ferrous Sulphate were purchased from

AK Scientific Inc. USA; while acetylthiocholine iodide, 1-chloro-2, 4-dinitrobenzene, 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB), and CDNB originated from Sigma Aldrich (St. Louis, MO).

Study on survival and lifespan

Survival and longevity/life span studies were conducted to determine the optimal duration of exposure, and doses of hesperetin and ferrous sulphate to be used for the experiment. The fly's feed was combined with ferrous sulphate and hesperetin at different concentrations for each group. Wild-type *D. melanogaster* flies, aged 1 to 3 days, of males and females, were separated into groups of 50 flies each. This was done in 5 replicates and 2 independent studies.

Determination of treatment dose and study duration

To establish the most effective length of $FeSO_4$ treatment for the experiment, the flies housed in a vial were assigned into four groups ($n=50$ flies), as listed: control (vehicle only), treated with 10, 20 and 30 μM $FeSO_4$ for 14 days.

To determine the optimal duration of hesperetin treatment for the study flies housed in a vial were assigned into four groups ($n=50$ flies) as follows: control (vehicle only), treated with 10, 50, 100 and 150 μM hesperetin for 14 days.

The number of dead flies was counted in each vial every day to calculate the percentage of mortality/survival per day. The percentage survival of flies in each group was recorded against the duration (number of days) of exposure and concentration of $FeSO_4$ and hesperetin respectively [36].

Furthermore, to determine the optimal dose of $FeSO_4$ for the study flies housed in a vial were assigned into four groups ($n=50$ flies) as listed: control (vehicle only), treated with 10, 20 and 30 μM $FeSO_4$ for 7 days. Biomarkers of toxicity such as glutathione-s-transferase (GST) activity, catalase activity, total thiol levels, nitric oxide levels, and hydrogen peroxide concentration were measured.

Ferrous sulphate exposure and hesperetin treatment

Based on the outcome of the survival studies, wild-type *D. melanogaster* flies, including both males and females, were separated into six groups in respective vials ($n=50$). Ferrous sulphate and/or hesperetin were meticulously matched with the fly's feed at different concentrations for each group. The 6 groups of 50 flies/vial are as follows: Group 1 was the control and received only the vehicle, Group 2 and 3 were administered 50 μM and 100 μM of hesperetin respectively, Group 4 was administered 10 μM $FeSO_4$, while Groups 5 and 6 were administered combined doses of 10 μM $FeSO_4$ with 50 μM and 100 μM of hesperetin respectively.

After the 7-day treatment period, each set of flies was anaesthetized using CO_2 gas. Subsequently, all the flies in the various groups were measured in weight and homogenized in a 0.1 M phosphate buffer with a pH of 7.0, using a ratio of 1 mg to 10 μL. The mixture was then subjected to centrifugation at 4000*g* for 10 min at a temperature of 4 °C. The liquid portion (supernatants) was subsequently isolated from the solid portion (pellets) and transferred into labelled Eppendorf tubes for the examination of oxidative stress, antioxidant, and inflammatory markers. The following parameters were evaluated: Catalase (CAT) activity, glutathione-S-transferase (GST) activity, non-protein thiol content, total thiol levels, hydrogen peroxide concentration, protein carbonyl levels, nitric oxide (NO) levels, cell viability, climbing rate, and acetylcholinesterase (AChE) activity.

Biochemical assays

Catalase (CAT) activity

The determination of catalase (CAT; EC 1.11.1.6) activity was conducted using the Aebi method. In summary, the elimination of H_2O_2 was detected at a wavelength of 240 nm, for 2 min with measurements taken every 10 s, and at a temperature of 25 °C. The reaction test comprised 1800 μL of 50 mM phosphate buffer (pH 7.0), 180 μL of 300 mM H_2O_2, and 20 μL of sample diluted at a ratio of 1:50. The reaction was observed for 2 min, with measurements taken at 10-s intervals, using a UV-visible spectrophotometer at a wavelength of 240 nm. The results were expressed as micromoles of H_2O_2 consumed per minute per milligramme of protein [37].

Glutathione-S-Transferase (GST) activity

The activity of Glutathione-S-Transferase (GST; EC 2.5.1.18) was measured using the method developed by Habig and Jakoby. The substrate used for this measurement was 1-chloro-2,4-dinitrobenzene (CDNB). The reaction mixture contained 270 μL of a solution made up of (20 μL of 0.25 M potassium phosphate buffer, pH 7.0, with 2.5 mM EDTA, 10.5 μL of distilled water and 500 μL of 0.1 M GSH at 25 °C), 10 μL of 25 mM CDNB and 20 μL of sample (1:5 dilution). Additionally, a small amount of the sample is added, after being diluted, along with a specific volume of CDNB. All these components were mixed at a controlled temperature of 25 °C. The reaction mixture was observed at 340 nm for 5 min with measurements taken every 10 s. Based on the data collected, the measurements were reported in μmol of CDNB/min/mg protein, using the GST molar extinction coefficient (ε) of 9.6 $mM^{-1}cm^{-1}$ [38].

Non-protein thiol content

The estimation of non-protein thiol content was conducted using Ellman's reagent (DTNB) following the colourimetric procedure outlined by Jollow et al. After the supernatant was obtained, it was treated with a solution of 4% sulphosalicyclic acid in a 1:1 ratio. The samples were stored at 4 °C for 1 h and then underwent centrifugation at 5000 rpm for 10 min at 4 °C. The assay mixture contained 550 µl of 0.1 M phosphate buffer, 100 µl of supernatant, and 100 µl of DTNB. The OD was measured at 412 nm and the findings were reported as moles of GSH per gramme of protein [39].

Total thiol content

The total thiol content was determined using the Ellman method. In the reaction mixture, there were 170 µL of 0.1 M potassium phosphate buffer (pH 7.4), 20 µL of the sample, and 10 µL of DTNB. Following a 30-min incubation period at room temperature, the absorbance was recorded at 412 nm. Each measurement utilised GSH as the standard, with the data being expressed in µmole/mg of protein [40].

Acetylcholinesterase (AChE) activity

A method developed by Ellman et al. was employed to assess the AChE activity. The reaction was carried out in 0.1 M potassium phosphate buffer of pH 7.4, 1 mM DTNB and 0.8 mM acetylthiocholine, the initiator. The reaction was observed for 5 min, with measurements taken every 15 s at a wavelength of 412 nm. The enzyme activity was calculated as the amount of acetylthiocholine hydrolyzed per minute per milligramme of protein [41].

Protein carbonyl

The Levine et al. method, with slight adjustments, was employed to determine the protein carbonyl (PC) content. Two identical portions of the supernatant fraction were collected. One portion was treated with an equal amount of 2,4-Dinitrophenyl hydrazine (10 mM dissolved in 2 M HCl) (referred to as the test sample). The other portion was mixed with a solution of 40% TCA and then extracted with a mixture of ethanol and ethyl acetate in a 1:1 ratio. The pellets were subsequently dissolved in a 1.0 mL solution of 6 M guanidine hydrochloride. The absorbance spectra of the sample treated with DNPH were compared to the blank solution containing HCl at a wavelength of 370 nm. The results were quantified in terms of the amount of DNPH integrated per milligramme of protein, using a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$ [42].

Thiobarbituric acid reactive substances (TBARS)

The lipid peroxidation end products of the flies were measured using thiobarbituric acid reactive substances (TBARS) as a quantification method [16]. The assay was conducted using the body region of the flies due to interference from the eye colour. In a nutshell, tissue samples from both control and treated flies were mixed in a cold 0.1 M potassium phosphate buffer (pH 7.4) at a ratio of 1:5. The 200 µL of the stock reagent (which consists of equal volumes of trichloroacetic acid (10%, w/v) and 2-thiobarbituric acid (0.75%, w/v) in 0.1 M HCl was incubated with 100 µL of tissue supernatant at 95 °C for one hour. Following the cooling and centrifugation process at 8000×*g* for 10 min, the absorbance of the supernatant was determined at 532 nm. The TBARS values were then adjusted based on the protein concentration.

Hydrogen peroxide

The hydrogen peroxide level was determined using the method described by Wolff. The assay mixture was prepared by combining 590 µl of FOX-1 reagent (Ferrous Oxidation-Xylenol orange, 10 ml of 100 mM xylenol orange, 50 ml of 250 mM ammonium ferrous sulfate, 10 ml of 100 mM sorbitol, 5 ml of 25 mM $H2SO_4$ and 30 ml of distilled water) with 10 µl of sample. After that, a 30-min incubation was done at room temperature and the absorbance was measured at 560 nm. The concentration of the hydrogen peroxide generated was determined by extrapolating from the standard curve [43].

Nitric oxide

The level of nitric oxide (NO) was determined using the Griess reaction method by incubating 250 µL of sample with 250 µL of Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride; 1% sulfanilamide in 5% phosphoric acid; 1:1 ratio] at room temperature for 20 min. Then the absorbance was measured at 550 nm. The concentration of NO in the sample was determined using the standard calibration curve of $NaNO_2$ [44].

Protein concentration

The Lowry method was replicated to determine protein concentration [45].

Cell viability (cell mitochondrial metabolic rate)

Cell viability was assessed by measuring dehydrogenase activity using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay. The analysis was conducted on the entire body of flies using enzymatic reduction of MTT to MTT-formazan at a final concentration of 5 mg/mL. The ratio values are expressed as percentages of the control [46].

Locomotor performance (negative geotaxis)

The flies' locomotor performance was assessed by manually conducting a negative geotaxis/locomotor assay [47] following treatment. The flies were carefully sorted while

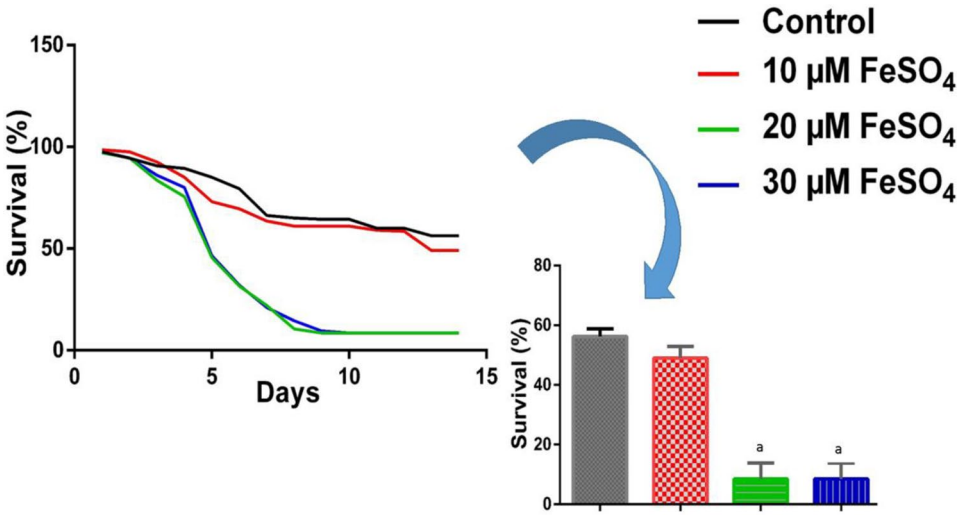

Fig. 1 Effects of $FeSO_4$ on the survival rate of *D. melanogaster* after treatment for 14 days. The data are presented as mean ± SEM of 50 flies/vial ($n = 5$). The *p* values (log-rank tests) for each group are control vs. 10, 20 and 30 μM of $FeSO_4$. Significant differences from the control group are indicated by [a] at $p < 0.05$

under a brief CO_2 anaesthesia and gently placed in a vertical glass column. The column had a length of 10 cm and a diameter of 1.5 cm, allowing for a comfortable arrangement of 10 flies in each section. Once the anaesthesia wore off, the flies were delicately tapped to the bottom of the column. Flies that made it to the top of the column, measuring 6 cm, were observed and counted for 6 s. The scores were calculated using a specific equation that considers the number of flies at the top, at the bottom, and the total number of flies. The final score was determined by the equation:

$$1/2[(n\text{tot} + n\text{top} - n\text{bot})/n\text{tot}] \times 100$$

*n*top represents the number of flies at the top, *n*bot is the number of flies at the bottom, and *n*tot represents the total flies' number.

Quantitative analysis of data using statistical methods

The results are reported as the mean ± standard error of the mean (SEM). The data was analysed using a one-way analysis of variance (ANOVA) and then followed by Tukey's post hoc test. The results were deemed statistically significant with a *p*-value of less than 0.05 ($p < 0.05$).

Results

Analysis of survival rate and certain biological indicators

The survival study results are presented in Fig. 1. Results indicated that the flies tolerated 10 μM $FeSO_4$ better than 20 μM and 30 μM $FeSO_4$ respectively, as demonstrated by a higher survival percentage (comparable to the naïve group) through the course of treatment. All treatment groups and the naïve group attained above 60% survival

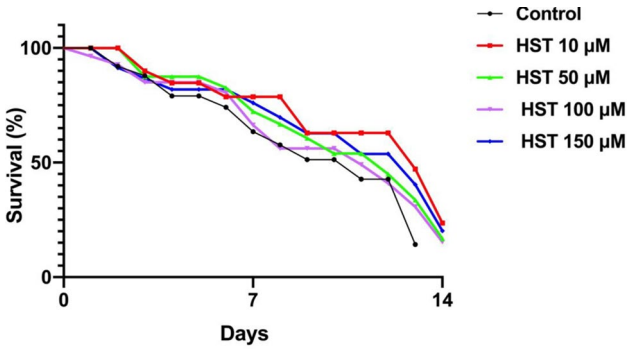

Fig. 2 Effects of hesperetin on the survival rate of *D. melanogaster* after treatment for 14 days

within about the first 5 days of the experiment before the decline in survival set in at about the 6th day.

The 10 μM $FeSO_4$ and naïve groups maintained slightly above 50% survival from about the 6th day through the remaining course of exposure, while the survival percentage of the 20 μM and 30 μM $FeSO_4$ groups dipped sharply from about the 6th day to almost zero through the remaining course of the experiment.

On the other hand, from the survival study results presented in Fig. 2, hesperetin conferred longevity on the flies because the hesperetin-treated flies survived better than the naïve flies. The group treated with 50 μM hesperetin had a higher survival rate than all other hesperetin-treated groups and the naïve group. The observed survival percentage relative to days of exposure is in the order 50 μM > 100 μM > 150 μM > 10 μM > Naïve flies. A survival of about 80% was observed at about 8–10 days for all groups before survival started diminishing steadily. Therefore, 50 μM and 100 μM hesperetin were adopted as the optimal concentration for the study to investigate

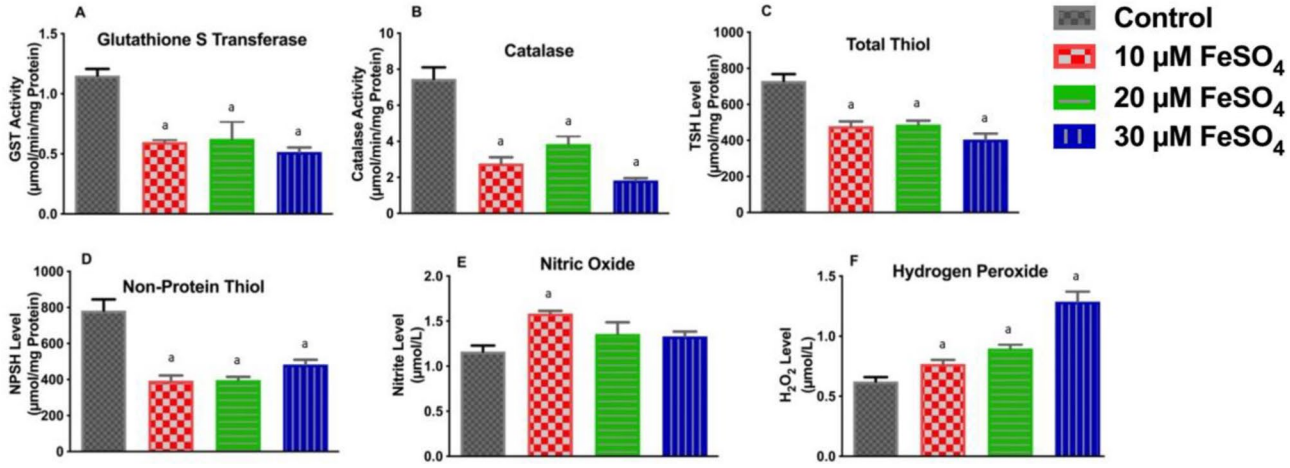

Fig. 3 FeSO4-induced toxicity in *D. melanogaster* after 7 days of exposure. Glutathione-S-transferase activity (**A**), catalase activity (**B**), total thiol level (**C**), non-protein thiol level (**D**), nitric oxide (nitrate/nitrite) level (**E**), and hydrogen peroxide level (**F**). Data are presented as mean ± SEM of 50 flies/vial ($n = 5$). Significant differences from the control group are indicated by [a] at $p < 0.05$

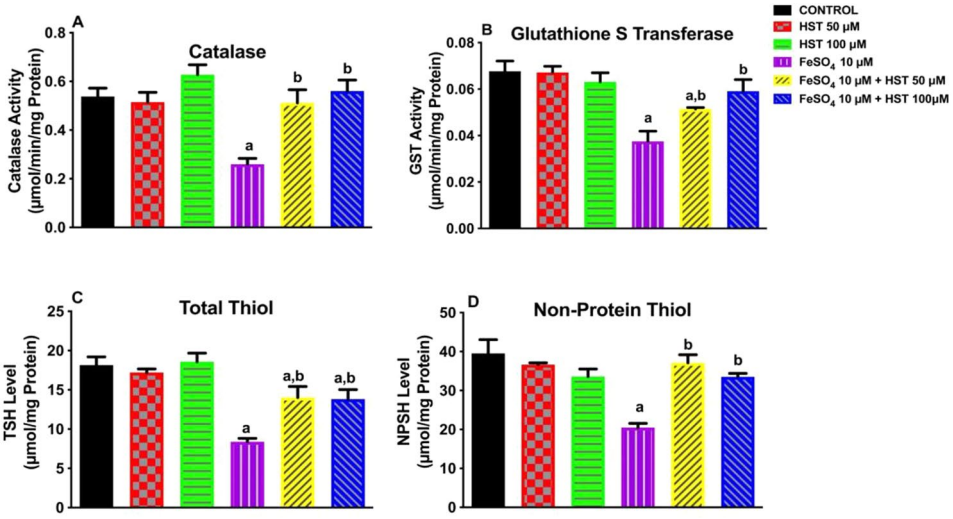

Fig. 4 Effects of hesperetin and/or $FeSO_4$ on oxidative stress and inflammatory markers in *D. melanogaster*. Catalase activity (**A**), glutathione-S-transferase activity (**B**), total thiol level (**C**), and non-protein thiols (**D**). Data are presented as mean ± standard error of the mean of 50 flies/vial ($n = 5$). Significant differences from the control group are represented by [a] and from the $FeSO_4$ group by [b] at $p < 0.05$

if hesperetin protects in a dose-dependent fashion after 7 days of treatment.

$FeSO_4$-induced toxicity in *D. melanogaster*

The effects of $FeSO_4$ on selected oxidative stress and antioxidant biomarkers in *Drosophila melanogaster* are presented in Fig. 3. There was a significant reduction of glutathione-s-transferase (GST) activity (3 A), catalase (CAT) activity (3B), non-protein thiol (NPSH) levels (3 C) and total thiol levels (3D) in the 10 µM, 20 µM and 30 µM $FeSO_4$ groups respectively when compared to the control group. On the converse, hydrogen peroxide (3E) and nitric oxide (3 F) levels were increased in the 10 µM, 20 µM and 30 µM FeSO4 groups respectively when compared to the control group.

Given the results above, all doses of $FeSO_4$ elicited a toxic response in the flies. However, the flies survived better with 10 µM $FeSO_4$ concentration. Therefore, 10 µM $FeSO_4$ was adopted as the optimal exposure concentration for the study.

Efficacy of hesperetin on FeSO4-induced oxidative stress and inflammation

Figures 4 and 5 display the outcome of a 7-day experiment where *D. melanogaster* was exposed to $FeSO_4$ and hesperetin.

The 10 µM $FeSO_4$ group showed a significant reduction in catalase (4 A) and GST (4B) activities by 2.0-fold and 1.8-fold respectively when compared to the control group flies. However, respective treatments with 50 µM and 100

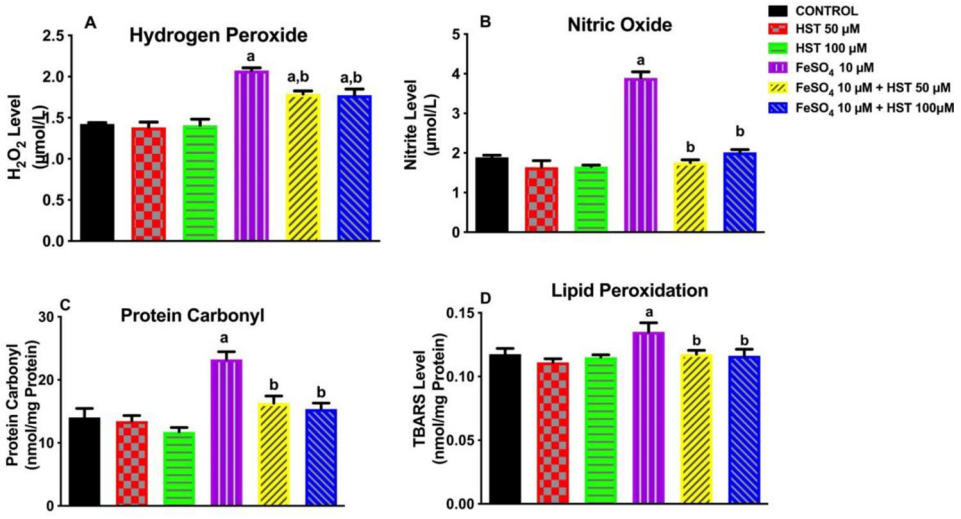

Fig. 5 Effects of hesperetin and/or $FeSO_4$ on oxidative stress and inflammatory markers in *D. melanogaster*. Hydrogen peroxide level (**A**), nitric oxide (nitrite/nitrate) level (**B**), protein carbonyl level (**C**), and lipid peroxidation status (**D**) in *D. melanogaster* exposed to hesperetin and/or $FeSO_4$ for 7 days. Data are presented as mean ± standard error of the mean of 50 flies/vial ($n = 5$). Significant differences from the control group are represented by [a] and from the $FeSO_4$ group by [b] at $p < 0.05$

µM hesperetin significantly reversed this $FeSO_4$-induced reduction of catalase and GST activities by 1.9-fold and 1.2-fold when compared with the 10 µM $FeSO_4$ group. The activities of catalase and GST were restored to about the control group status in the 50 µM hesperetin/10 µM $FeSO_4$ and 100 µM hesperetin/10 µM $FeSO_4$ groups. A 46% reduction in thiol level (4 C) and 52% reduction in non-protein thiol level (4D) was observed in the 10 µM $FeSO_4$ group when compared to the naïve flies, However, the respective treatments with 50 µM and 100 µM hesperetin significantly reversed the observed $FeSO_4$ induced reductions of total thiol and non-protein thiol levels.

Furthermore, increases in hydrogen peroxide (5 A), nitric oxide (5B), protein carbonyl (5 C) and lipid peroxidation (TBARS) levels (5D) were observed in the 10 µM FeSO4 group when compared to the naïve flies. The respective treatments with 50 µM and 100 µM hesperetin significantly ($p < 0.05$) reversed this $FeSO_4$-induced increase of H_2O_2, NO, protein carbonyl and TBARS levels when compared with the $FeSO_4$-treated group. However, respective treatment with 50 µM hesperetin and 100 µM hesperetin did not reverse the $FeSO_4$-induced oxidative stress and inflammation to the naïve flies status when the hesperetin and $FeSO_4$ co-administered groups were compared with the control group.

Action of hesperetin on $FeSO_4$-induced reduction in acetylcholinesterase (AChE) activity

The 10 µM $FeSO_4$ group demonstrated a notable reduction in AChE activity when compared to the naïve flies as presented in Fig. 6A. The respective treatments with 50 µM and 100 µM hesperetin significantly reversed this $FeSO_4$-induced reduction of AChE activity when compared with the 10 µM $FeSO_4$ group. However, the treatment with 100 µM hesperetin significantly increased the AChE activity to the naïve flies status when the 100 µM hesperetin/10 µM $FeSO_4$ group is compared with the untreated control group. Furthermore, treatment with 100 µM hesperetin alone, significantly increased AChE activity compared to the control group.

Action of hesperetin on FeSO4-induced reduction in locomotor performance (negative geotaxis)

$FeSO_4$ (10 µM) decreased negative geotaxis. A normal climbing rate (6B) was observed in the untreated control, 50 µM hesperetin, and 100 µM hesperetin groups, while there was a significant decrease in the climbing rate of the 10 µM $FeSO_4$ group. This lethargy was however significantly reversed when the 50 µM hesperetin/10 µM $FeSO_4$ and 100 µM hesperetin/10 µM $FeSO_4$ treated groups were compared to the 10 µM $FeSO_4$ group.

Action of hesperetin FeSO4-induced reduction mitochondrial metabolic rate (cell viability)

The 10 µM $FeSO_4$ group showed a non-significant reduction in the number of viable cells (6 C) when compared to the naïve flies. The respective treatments with 50 µM and 100 µM hesperetin significantly reversed this $FeSO_4$-induced reduction in cell viability when compared with the 10 µM $FeSO_4$ group.

Discussion

Drosophila melanogaster is widely recognized as a valuable model organism for studies in neurotoxicology and genetics. Its suitability stems from its uncomplicated

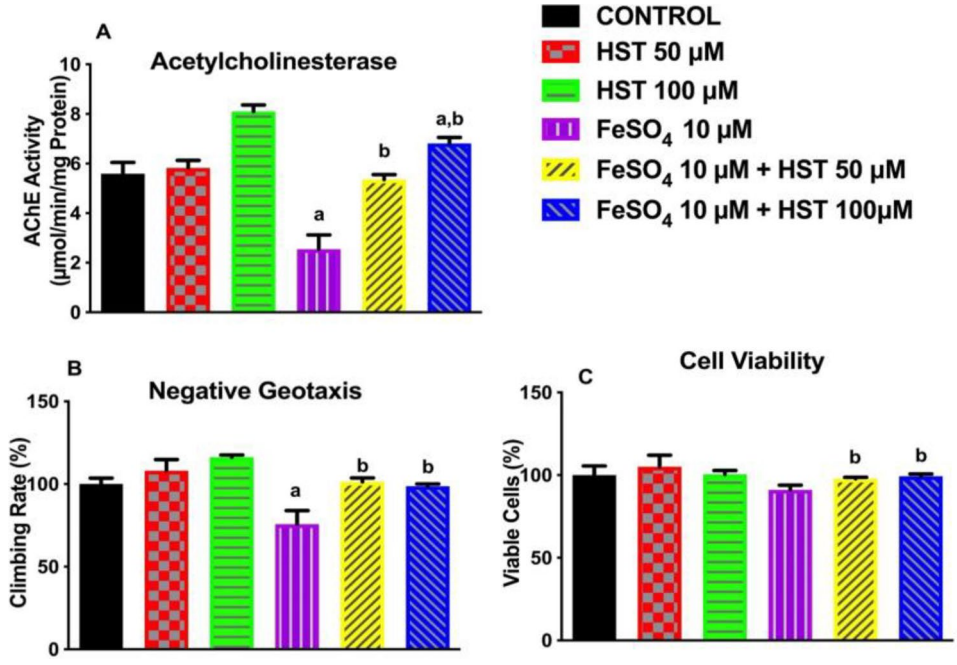

Fig. 6 Effects of hesperetin and/or $FeSO_4$ on acetylcholinesterase activity, negative geotaxis and cell viability in *D. melanogaster*. Acetylcholinesterase activity (**A**), negative geotaxis (**B**) and cell viability (**C**) in *D. melanogaster* exposed to hesperetin and/or $FeSO_4$ for 7 days. Data are presented as mean ± standard error of the mean of 50 flies/vial ($n = 5$). Significant differences from the control group are represented by [a] and from the $FeSO_4$ group by [b] at $p < 0.05$

nervous system, short lifespan, and ease of genetic manipulation [48]. Moreover, approximately 75% of genes implicated in human diseases have counterparts in *D. melanogaster*, underscoring its relevance for investigating human ailments [49, 50]. Furthermore, *D. melanogaster* has been utilized as an alternative and complementary model in exploring conditions potentially linked to metal dyshomeostasis, as noted in previous studies [32, 51–53].

Metal dyshomeostasis, which refers to the imbalance or abnormal regulation of metals within the body, is associated with various disorders across different organ systems. Metal dyshomeostasis is an important factor that leads to neurodegenerative disorders. Alzheimer's disease (AD) stands as the prevailing neurodegenerative condition, distinguished by pathological features such as amyloid aggregation and the accumulation of phosphorylated Tau protein. These hallmark abnormalities contribute to cognitive decline, a defining aspect of the disease [54]. Parkinson's disease is the most common movement disorder pathologically characterized by loss of dopamine in the substantia nigra. Growing evidence indicates that disruptions in the homeostasis of metals like copper, zinc, and iron may significantly contribute to the development of Alzheimer's and Parkinson's diseases. These disturbances could potentially exacerbate oxidative stress, leading to further damage within the brain and exacerbating the progression of these neurodegenerative conditions [55, 56]. Aside from these two diseases, metal dyshomeostasis is also associated with Wilson's disease, hemochromatosis, amyotrophic lateral sclerosis (ALS), atherosclerosis, Huntington's disease, celiac disease, and thalassemia among others [57]. These disorders underscore the importance of maintaining proper metal homeostasis for overall health and highlight the detrimental effects of metal dyshomeostasis on various physiological processes.

In the present study, we determined the beneficial effect of hesperetin on ferrous sulphate-induced toxicity. Results from our study show a reduced survival rate in flies exposed to ferrous sulphate. This may be associated with the reports that heavy metal overload plays a role in neuronal death, as they generate free radicals which induce oxidative damage to macromolecules and cellular toxicity [58–60]. The reduced survival rate observed in this present study implies that the toxic biological effect of ferrous sulphate is detrimental to the lifespan and survival of flies. On the other hand, our results indicated an increased survival rate in the flies exposed to hesperetin. Previous studies have shown that supplementation of diets with antioxidants increases the survival and lifespan of *D. melanogaster* [61].

Furthermore, we measured the activities of enzymatic antioxidants including CAT and GST during ferrous sulphate exposure. Hesperetin, a flavonoid found in citrus fruits, has shown promising antioxidant and cytoprotective properties in various experimental models [62, 63]. Our data regarding the activities of these enzymatic antioxidants during $FeSO_4$ exposure suggests that hesperetin

supplementation ameliorated the $FeSO_4$-induced diminution in the expression of the antioxidant enzymes. The significant decrease in CAT and GST activities in response to $FeSO_4$ exposure indicates a disruption in antioxidant defence mechanisms and cellular detoxification processes, which may contribute to oxidative damage and toxicity [64, 65], while supplementation of diet with hesperetin potentiated a significant increase in CAT and GST activities; with 100 μM hesperetin giving better improvement. The findings imply that hesperetin supplementation has a beneficial effect on antioxidant defence mechanisms and cellular detoxification processes. The significant increase in CAT and GST activities indicates enhanced protection against oxidative stress and improved cellular resilience. Additionally, the dose-dependent response suggests that higher concentrations of hesperetin may be more effective in promoting antioxidant enzyme activity [66]. The data lay more credence that hesperetin supplementation can enhance enzymatic antioxidant activities and protect against $FeSO_4$-induced reduction in these activities. This highlights the potential of hesperetin as a therapeutic agent for conditions involving oxidative stress and iron toxicity [67].

Our result also demonstrated that co-administration with 100 μM hesperetin reversed $FeSO_4$-induced loss of CAT and GST activities when compared to control naïve flies which were not exposed to $FeSO_4$. This suggests that hesperetin tends to improve CAT and GST activities in a dose-dependent manner. Antioxidant enzymes play their protective role by scavenging cellular reactive oxygen species (ROS) and other free radicals. Therefore, the results suggest that hesperetin may offer protection from damage caused by $FeSO_4$ via induction of enzymatic antioxidant activity.

Evidence abounds that thiols are potent intracellular antioxidants that provide antioxidant defence by acting as chelators that mop up reactive species and thus prevent their deleterious oxidant reactions [68–70]. The thiols through their sulfhydryl group form complexes with the reactive species. This scavenging reaction results in the depletion of GSH and total thiol during oxidative stress. In the present study, the levels of total thiols reduced significantly under $FeSO_4$ assault, while the level of total thiols was maintained by supplementation of diet with hesperetin. Our results imply that the thiols are used to combat the production of free radicals in the presence of $FeSO_4$ and that hesperetin may be eliciting its antioxidant property by scavenging free radicals and thus reducing the consumption of the thiols. Our observation is consistent with the report of Yang et al., wherein they demonstrated that hesperetin displayed antioxidant activity in vitro by scavenging superoxide anion, improving reducing power, and chelating metals [22].

In the present study, we observed a $FeSO_4$–induced inhibition of AChE activity which was prevented by hesperetin supplementation of diet. The ability of $FeSO_4$ to cause oxidative stress is associated with the generation of free radicals [3]. It is well-known that free radical production is implicated in the inhibition of AChE activity [69, 71]. Similarly, this observed impact of $FeSO_4$ on AChE activity in this present study may be linked to the reported neurologic symptoms observed during the advanced stage of $FeSO_4$ exposure in children [7, 11, 72]. The treatment with 100 μM hesperetin appears to be more efficacious in preserving AChE activity under $FeSO_4$ assault. This effect of hesperetin on AChE activity observed in the present study is consistent with the report of previous investigators [69, 73, 74]. This hesperetin-mediated preservation of AChE activity is an indication that hesperetin may possess anticholinergic properties, and thus prevent the accumulation of acetylcholine at cholinergic synapses and the resultant or associated neuro disorders [69, 73].

Nitric oxide is a signalling molecule involved in various physiological processes, including vasodilation, neurotransmission, and immune response. However, excessive production of NO, often in response to oxidative stress or inflammation, can contribute to tissue damage and inflammation. In the present study, the observed $FeSO_4$-induced elevation of NO was markedly reversed by hesperetin supplementation of diet. We hypothesized that this effect of hesperetin on NO production may be connected to the activity of iNOS. Other investigators have demonstrated that treatment with hesperetin suppressed the toxicant-induced expression of iNOS in a dose-dependent fashion [22]. Furthermore, the reversal of $FeSO_4$-induced elevation of NO by hesperetin supplementation suggests that hesperetin could modulate NO levels and counteract the detrimental effects associated with NO overproduction [75]. This highlights the potential therapeutic benefit of hesperetin in attenuating oxidative stress and inflammation-related pathology.

Protein carbonylation is a marker of protein oxidation, where reactive oxygen species (ROS) or reactive nitrogen species (RNS) react with proteins, leading to the formation of carbonyl groups while hydrogen peroxide is a reactive oxygen species that can cause oxidative damage to various cellular components, including DNA, proteins, and lipids. Additionally, Thiols, including glutathione, are important antioxidants that help protect cells from oxidative damage by scavenging free radicals and reactive oxygen species. The data generated from the present study is consistent with the fact that $FeSO_4$ induces oxidative stress in animal models [3]. In the present study, treatment with $FeSO_4$ caused oxidative stress as exemplified by a significant elevation of protein carbonyl and hydrogen peroxide levels and a decline of total thiol

levels. This oxidative stress likely contributes to cellular damage and dysfunction, highlighting the need for interventions to mitigate oxidative damage and restore redox balance [76]. However, hesperetin treatment reversed this observed oxidative stress as indicated by a significant decline in protein carbonyl and hydrogen peroxide levels and elevation of total thiol levels. The data indicate that hesperetin treatment effectively counteracted the oxidative stress induced by $FeSO_4$. Hesperetin's antioxidant properties likely contributed to this reversal by scavenging free radicals, reducing ROS production, and enhancing the cellular antioxidant defence system, highlighting its potential as a therapeutic intervention for oxidative stress-related conditions [77]. This suggests that hesperetin has the potential to protect against oxidative damage and promote cellular health in the context of $FeSO_4$-induced oxidative stress.

The two oxidative forms of iron, ferrous (Fe^{2+}) and ferric (Fe^{3+}), mediate cellular oxidative stress via the Fenton and Haber Weiss reactions by the generation of free radicals [3, 78, 79]. These free radicals generated by iron then react with biomolecules (proteins, DNA, lipids etc.) causing the cellular damage seen during oxidative stress [3, 80]. The lipid peroxidation and protein oxidation metabolites generated in response to oxidative stress may further compound cellular injury by generating diffusible and stable cytotoxic agents [73, 81]. In the present study, the administration of hesperetin significantly attenuated the oxidative stress observed in $FeSO_4$-challenged flies. Our result suggests that hesperetin may have free radical scavenging ability as evidenced by the improvement in total thiol levels; and the reduction in protein carbonyl, lipid peroxidation, and hydrogen peroxide level. This observation is consistent with the report of previous investigators that hesperetin has hydroxyl radical scavenging properties [20, 22, 82].

Furthermore, the effect of $FeSO_4$ on the locomotor behaviour of the flies was studied. Normal climbing rate was observed in the untreated control, 50 µM hesperetin, and 100 µM hesperetin groups. However, a decline was observed in the locomotor activity of the $FeSO_4$ alone flies. It is known that the extent of cellular oxidative stress determines the extent of ageing and age-related functional declines in *D. melanogaster* [83–85]. The lethargy observed in the flies in this present study indicates that *D. melanogaster* locomotor activity is sensitive to $FeSO_4$ exposure. Hesperetin supplementation of diet appears to improve the negative geotaxis score of the flies in the present study. The finding suggests a potential beneficial effect of hesperetin supplementation on the locomotor performance of the flies, which could be attributed to its antioxidant or other physiological effects. This observation may have implications for understanding the potential protective role of hesperetin against the toxicity induced by ferrous sulphate or other stressors in the Drosophila model. This behaviour change is consistent with previous reports that dietary antioxidants confer resistance to oxidative stressors and thus protect against age-related behavioural decline in rats [86, 87], and flies [88–90].

Conclusion

All the results from the present study taken together indicate that hesperetin supplementation significantly ameliorated biochemical defects caused by $FeSO_4$ in *D. melanogaster*. On the evidence of these findings, it can be posited that hesperetin possesses a protective ability against $FeSO_4$-induced stress in *D. melanogaster* through inhibiting protein carbonyl formation and lipid peroxidation, the maintenance of thiol levels, and the restoration of the activities of antioxidant enzymes and AChE.

In conclusion, our results pointed to the involvement of oxidative stress (demonstrated by disruptions of the antioxidant system), and neurotoxicity (demonstrated by alteration of AChE activity) in $FeSO_4$ toxicity. In addition, our results indicated that hesperetin may rescue $FeSO_4$ toxicity by ameliorating oxidative stress, and neurotoxicity. Therefore, further investigations should be focused on understanding the mechanism of action by which hesperetin rescues this $FeSO_4$-induced oxidative stress and neurotoxicity. This will further provide novel insight into mechanisms by which antioxidants protect against metal toxicity.

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Author contributions
Conceptualization and study design, FOA, GIA and AOA; Investigation, FOA and AOA; Methodology, analysis and interpretation of data, GIA, AOA and AOA; Drafting the manuscript, GIA and OBO; Revising for intellectual content, FOA, GIA, OBO, and AOA. Final approval, FOA, GIA, OBO, AOA and AOA. All authors read and agreed for the manuscript to be published.

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Declarations

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