

## ORIGINAL ARTICLE

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# Resveratrol protects against copper and iron toxicity in *Drosophila melanogaster*

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## ABSTRACT

## BACKGROUND

Copper (Cu) and iron (Fe) are essential trace elements that when in excess are capable of causing cytotoxic effects leading to lipid peroxidation and promoting oxidative stress. Resveratrol (RES) is a natural polyphenol with antioxidant and anti-inflammatory properties. This study was carried out to evaluate the protective role of RES in Fe and Cu sulphate-induced oxidative stress in *Drosophila melanogaster*.

## METHODS

Adult wild type flies were fed Cu<sup>2+</sup> and Fe<sup>2+</sup> (1 mM each) and/or RES (30 and 60 mg/kg diet) for 7 days. Survival, negative geotaxis and emergence rate were evaluated by daily recording of fruit fly mortality and final analysis. Fruit flies were anaesthetized using CO<sub>2</sub> gas, homogenized and centrifuged at 4,000 rpm for 10 minutes at 4°C. Aliquots of the supernatants were used for the estimation of biochemical markers using spectrophotometry.

## RESULTS

Fruit flies co-treated with FeSO<sub>4</sub> + CuSO<sub>4</sub> (1 mM each) + RES (30 and 60 mg/Kg) significantly elevated H<sub>2</sub>O<sub>2</sub>, NO, lipid peroxidation, acetylcholinesterase as well as GSH, GST, catalase and total thiols (p<0.05) compared with the Cu<sup>2+</sup> + Fe<sup>2+</sup> (1mM each) treated group. Flies co-treated with FeSO<sub>4</sub> + CuSO<sub>4</sub> (1mM each) + RES (30 and 60mg/Kg) also had significantly improved (p<0.05) eclosion and climbing rates compared with the Cu<sup>2+</sup> + Fe<sup>2+</sup> (1mM each) treated group.

## CONCLUSION

This study demonstrated that RES reduced Cu<sup>2+</sup> and Fe<sup>2+</sup>-induced radical generation in *D. melanogaster* and improved the antioxidant buffering capability of the flies. Therefore, RES could be used in management of disorders involving oxidative stress.

**Keywords:** Resveratrol, reactive oxygen species, antioxidant, *Drosophila melanogaster*

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## INTRODUCTION

Iron ( $\text{Fe}^{2+}$ ) and copper ( $\text{Cu}^{2+}$ ) are essential trace elements (ETE) for biological processes and vital functions such as cell respiration, maturation of erythrocytes, antioxidant defence, and enzyme cofactors.<sup>(1)</sup> Iron and copper are cofactors required by enzymes for structural and catalytic activities in oxidative phosphorylation, protein synthesis, and neurotransmission modulation.<sup>(2)</sup> The routes for copper and iron administration are through inhalation, consumption of contaminated food and water, and dermal contact with polluted air, water, and soil. Once inside the body, copper and iron are first deposited in the liver after which liver detoxification activities occur. These metals are present in trace amounts because a variety of homeostatic mechanisms in normal circumstances maintains a physiologically essential amount of these metals.<sup>(1)</sup> However, when in excess, dyshomeostatic mechanisms of iron and copper are capable of causing cytotoxic effects and production of highly damaging free radicals by Fenton or Haber-Weiss reactions, thus causing brain lipid peroxidation and protein oxidation which promotes oxidative stress.<sup>(3,4)</sup> Reactive oxygen species (ROS) are formed by the ability of copper to change states, from cupric ( $\text{Cu}^{2+}$ ) to cuprous ( $\text{Cu}^+$ ) by cuproenzymes which are involved in redox reactions. Cuprous ions can catalyse hydrogen peroxide decomposition, through the Fenton reaction, which forms hydroxyl radicals (OH) capable of reacting with biological molecules such as proteins, lipids and DNA, thereby damaging them.<sup>(5)</sup> The ability of iron to accept and donate electrons can lead to the formation of reactive nitrogen and oxygen species which trigger the oxidative attack of the brain, thereby contributing to brain disease and perhaps aging itself. The buildup of copper or iron in the liver, kidneys, brain, and eyes results in possible cell death, permanent nerve damage, oxidative stress, and reduced cell proliferation.<sup>(6,7)</sup>

Resveratrol (RES) is a polyphenol commonly found in grapes, wine, peanuts, and

soy, and is known to possess anti-oxidative, anti-carcinogenic, and anti-inflammatory properties.<sup>(8, 9)</sup> A study showed that RES can alleviate liver injury in iron-overloaded mice. The mechanism may be related to improving antioxidant capacity and reducing excess iron in the liver.<sup>(10)</sup>

Interestingly, *Drosophila melanogaster* models have been used in vivo to investigate disease mechanisms.<sup>(8-10)</sup> *Drosophila* has a genetic makeup of about 60% homology to that of humans. Also, the fruit fly possesses 75% of the genes responsible for human diseases as homologues, thereby making it a very good model organism.<sup>(11)</sup> As a result of ROS production which links oxidative stress, resveratrol mitigates ROS generation through (i) the prevention of molecular oxygen and/or peroxide reactions and the chelation of iron and copper ion; (ii) the chelation of iron and copper to the redox state being kept, making the iron and copper incapable to reduce molecular oxygen; (iii) the trapping of formed radicals.<sup>(12)</sup> Previous studies have shown that RES has the potency to increase glutathione peroxidase, superoxide dismutase as well as glutathione levels. Furthermore, RES also decreases lipid peroxidation and protein oxidation. Thus, a decrease in the amount of lipid peroxides also confirms the decrease in oxidative stress.<sup>(8, 9)</sup>

One study showed that RES can alleviate liver injury in iron-overloaded mice. The mechanism may be related to improvement of antioxidant capacity and reduction of excess iron in the liver.<sup>(13)</sup> Studies on the use of *D. melanogaster* in iron and copper-induced oxidative stress and the preventive effects of resveratrol are scarce in the literature. Therefore, as an alternative to conventional rodent models, a study is needed to investigate the  $\text{Cu}^{2+}$  and  $\text{Fe}^{2+}$ -induced toxicity and the relevance of RES protective role in *D. melanogaster*. In this research, *D. melanogaster* was employed as a model to examine  $\text{Cu}^{2+}$  and  $\text{Fe}^{2+}$ -induced toxicity and the ameliorative impact of RES.

## METHODS

### Research design

The experimental study was carried out at the *Drosophila* laboratory, Department of Biochemistry, University of Ibadan, Nigeria, between January and August, 2021.

### *Drosophila melanogaster* stock and culture

*Drosophila melanogaster* (Harwich strain) of both genders (1-3 days old) were cultured and maintained in the *Drosophila melanogaster* Research Laboratory, Department of Biochemistry, College of Medicine, University of Ibadan, Oyo State, Nigeria. The flies were allowed to mate in vials monitored at regulated temperature and humidity (22–24°C; 60–70% relative humidity) until the eggs hatched into young adult fruit flies under 12 hours' light and 12 hours' dark daily cycles for the period of administration of the chemical compound under investigation.

### Chemical compounds

Resveratrol was procured from A K Scientific, 30023 Ahern Ave, Union City, CA 94587, United States of America at a percentage purity of 95%. Copper sulphate and ferrous sulphate were procured from Sigma Aldrich. All chemicals used were of analytical grade. 1-chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithiobis-2-nitro-benzoic acid (DTNB), acetylthiocholine iodide, and hydrogen peroxide (30% solution [w/w]) were purchased from Sigma USA. Other reagents were commercial products of high analytical grade.

### Treatment of *D. melanogaster* with CuSO<sub>4</sub> and FeSO<sub>4</sub> for oxidative stress inducement

The flies were fed on diet mixed with CuSO<sub>4</sub> and FeSO<sub>4</sub> at a final dose of FeSO<sub>4</sub> 0.5 mM, FeSO<sub>4</sub> 1 mM, FeSO<sub>4</sub> 0.5 mM + CuSO<sub>4</sub> 0.5 mM and FeSO<sub>4</sub> 1 mM + CuSO<sub>4</sub> 1 mM, respectively. The flies were monitored for 7 days, harvested and homogenized, after which free radical generation was determined.

### Treatment of *D. melanogaster* with CuSO<sub>4</sub>, FeSO<sub>4</sub> and resveratrol

The flies were fed on a diet mixed with CuSO<sub>4</sub>, FeSO<sub>4</sub>, and/or RES, while the final group (G5) received EDTA instead of RES, at a dose of 50mg/kg diet. The flies were divided into six groups, each containing five replicates/groups with 40 flies/vial: positive control: 50 mg EDTA/kg diet, G1: 30 mg RES/kg diet), G2: 60 mg RES/kg diet), G3: 1mM CuSO<sub>4</sub> + 1mM FeSO<sub>4</sub> + 30 mg RES/kg diet), G4: 1mM CuSO<sub>4</sub> + 1mM FeSO<sub>4</sub> + 60 mg RES/kg diet and G5: (1mM CuSO<sub>4</sub> + 1mM FeSO<sub>4</sub> + 50mg EDTA/kg diet). The flies were monitored for 7 days, harvested and homogenized, followed by determination of free radical generation.

### Preparation of samples for biochemical assays

For the determination of biochemical assays, 50 flies (of both genders) were exposed to final concentrations of FeSO<sub>4</sub> 0.5 mM, FeSO<sub>4</sub> 1 mM, FeSO<sub>4</sub> 0.5 mM + CuSO<sub>4</sub> 0.5 mM and FeSO<sub>4</sub> 1 mM + CuSO<sub>4</sub> 1mM and RES (30 and 60 mg/kg diet) for 7 days. At the end of the treatment period, flies were anaesthetized using CO<sub>2</sub>, weighed, homogenized in 0.1M phosphate buffer, pH 7.0 (ratio of 1 mg:10 mL), and centrifuged at 4000 g for 10 min at 4 °C in a Thermo Scientific Sorval Micro 17 R refrigerated centrifuge. Subsequently, the supernatants were separated from the pellets into labelled Eppendorf tubes, stored at -20 °C and used for the determination of acetylcholinesterase (AChE), GST and catalase activities as well as cell viability and TSH and H<sub>2</sub>O<sub>2</sub> levels. All the assays were carried out in duplicate for each of the five replicates of FeSO<sub>4</sub> + CuSO<sub>4</sub> and resveratrol concentrations.

## IN VIVO ASSAYS

### Survival and negative geotaxis (climbing) rate of flies

The flies were exposed to a diet mixed with CuSO<sub>4</sub> and FeSO<sub>4</sub>, namely FeSO<sub>4</sub> 0.5 mM,

FeSO<sub>4</sub> 1 mM, FeSO<sub>4</sub> 0.5 mM + CuSO<sub>4</sub> 0.5 mM and FeSO<sub>4</sub> 1 mM + CuSO<sub>4</sub> 1mM for 21 days. The survival rates were evaluated by daily recording of fly mortality after which the data were analysed.<sup>(14)</sup> We determined negative geotaxis and emergence rate of *D. melanogaster* offspring after being fed for a period of seven days on a diet mixed with CuSO<sub>4</sub>, FeSO<sub>4</sub>, and/or RES and EDTA, respectively, in the positive control group and groups G1–G5, as stated in a previous section.

## EX VIVO ASSAYS

### Sample preparation for biochemical assays

*Drosophila melanogaster* treated with CuSO<sub>4</sub>, FeSO<sub>4</sub>, and RES for 7 days were anaesthetized using CO<sub>2</sub> gas. The fruit flies were weighed and homogenized in 0.1 M potassium phosphate buffer (pH 7.4, 1 mg: 10 µL buffer). The homogenates were centrifuged at 4,000g for 10 minutes at 4 °C in a Thermo Scientific Sorval Legend Micro 7R centrifuge. The aliquots of the supernatants were separated from the pellets into Eppendorf tubes, kept at “20°C in a freezer and were used for the estimation of biochemical markers, ie. lipid peroxidation, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), total thiols (TSH), glutathione (GSH), protein, and nitric oxide (NO, nitrate and nitrite) levels as well as the activity of enzymes such as acetylcholinesterase, catalase and glutathione-S-transferase (GST). In addition, behavioural parameters such as negative geotaxis were also determined.

## BIOCHEMICAL ASSAYS

### Protein determination

The principle of the assay is based on the reaction of Cu<sup>+</sup>, produced by the oxidation of peptide bonds, with Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid). The reaction mechanism involves reduction of the Folin-Ciocalteu reagent and oxidation of aromatic residues (mainly tryptophan, also tyrosine). Cysteine residues in

protein probably also contribute to the absorbance seen in the Lowry assay. The concentration of the reduced Folin reagent was measured by absorbance at 750nm.

### Determination of catalase and acetylcholinesterase activity

Catalase activity was determined by the method described by Aebi.<sup>(15)</sup> The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 300 mM H<sub>2</sub>O<sub>2</sub> and sample (1:50 dilution). The loss in absorbance of H<sub>2</sub>O<sub>2</sub> was monitored for 2 min at 240 nm and thence used for the calculation of catalase activity expressed as µmol of H<sub>2</sub>O<sub>2</sub> consumed per minutes per milligram of protein.

The reaction was carried out in 0.1 M potassium phosphate buffer of pH 7.4, 1 mM DTNB and 0.8 mM acetylthiocholine as the initiator. The reaction was monitored for 2 min (at 30 s intervals) at 412 nm. The enzyme activity was determined as µmol of acetylthiocholine hydrolysed/minute/mg protein. The activity of acetylcholinesterase was determined according to the method described by Ellman et al.<sup>(16)</sup>

### Determination of total thiols, hydrogen peroxide, and nitric oxide (nitrate/nitrite) level

The reaction mixture contained 510 µL of 0.1 M phosphate buffer (pH 7.4), 20 µL of sample, 35 µL of 1 mM DTNB and 35 µL of distilled water. Then, incubation was carried out at room temperature for 30 min. The absorbance was measured at 412 nm. Hydrogen peroxide level was determined using the method of Wolff.<sup>(17)</sup> The reaction mixture consisted of FOX 1 (10 ml of 100 mM xylenol orange, 50 ml of 250 mM ammonium ferrous sulphate, 10 ml of 100 mM sorbitol, 5 ml of 25 mM H<sub>2</sub>SO<sub>4</sub> and 30 ml of distilled water) and was reacted with the sample. After 30 min incubation at room temperature, the absorbance was measured at 560 nm and the values were extrapolated from the standard curve and expressed in micromole per milligram (µm/mg) protein.

Nitric oxide (nitrate and nitrite) level was estimated according to the method of the Griess reaction. 250  $\mu$ L of sample was incubated with 250  $\mu$ L 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. The absorbance was measured spectrophotometrically at 550 nm (OD 550) and nitrite concentration was quantified by comparison with the OD 550 of a standard sodium nitrite solution of known concentration.

#### **Determination of glutathione S-transferase activity**

The activity of GST was quantified based on the method of Habig and Jakoby<sup>(18)</sup> where 1-chloro-2, 4-dinitrobenzene (CDNB) was used as substrate. The reaction mixture contained 270  $\mu$ L of a solution made up of 20  $\mu$ L of 0.25 M potassium phosphate buffer, pH 7.0, with 2.5 mM EDTA, 10.5  $\mu$ L of distilled water and 500  $\mu$ L of 0.1 M GSH at 25 °C), 10  $\mu$ L of 25 mM CDNB and 20  $\mu$ L of sample (1:5 dilution). The mixture was monitored for 5 min (at 10 s intervals) and absorbance was read at 340 nm using a spectrophotometer.

#### **Determination of lipid peroxidation**

Lipid peroxidation was estimated by measuring the formation of thiobarbituric acid reactive substances (TBARS) following the method of Varshney and Kale.<sup>(19)</sup> Under acidic condition, malondialdehyde (MDA) produced from peroxidation of membrane fatty acids and food products react with the chromogenic reagent, 2-thiobarbituric acid (TBA). A pink coloured complex is generated and absorbance is read at 532 nm.

#### **Determination of reduced glutathione level**

The reduced glutathione (GSH) level was measured according to the method of Beutler et al.<sup>(20)</sup> The reduced form of glutathione comprises in most instances the bulk of cellular non-protein sulphhydryl groups. This technique depends on the development of a relatively stable (yellow)

colour when 5', 5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) is added to sulphhydryl compounds. The chromophoric product resulting from the reaction of Ellman's reagent with the reduced glutathione is measured at 412nm.

#### **Estimation of glutathione (GSH) content**

Glutathione (GSH) content was determined colorimetrically using Ellman's reagent (DTNB) according to the procedure described by Jollow et al.<sup>(21)</sup> The supernatant was precipitated with 4% sulphosalicylic acid (4%) in the ratio of 1:1. The samples were preserved at 4 °C for 1 h and then subjected to centrifugation at 5000 rpm for 10 min at 4 °C. The assay mixtures consisted of 550  $\mu$ L of 0.1 M phosphate buffer, 100  $\mu$ L of supernatant and 100  $\mu$ L of DTNB. The OD was read at 412 nm and the results were expressed as moles of GSH/gram tissue.

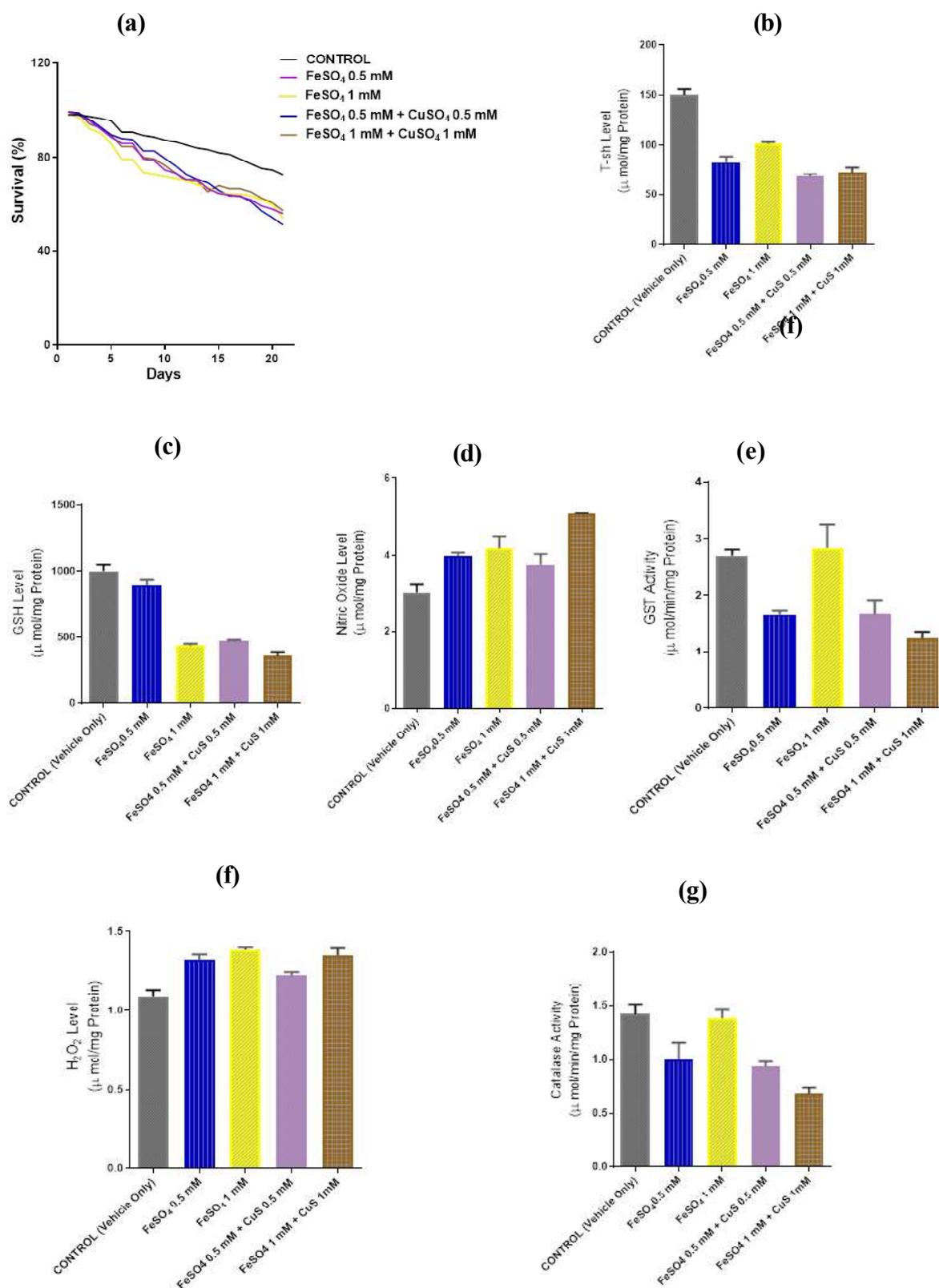
#### **Statistical Analysis**

Data were expressed as mean  $\pm$  standard error of mean. Treated and control groups were compared using One-way ANOVA followed by Tukey's post hoc test. In all the groups, differences with a p-value < 0.05 were considered statistically significant.

## **RESULTS**

### **Survival rate and selected biochemical markers after exposure to ferrous and copper sulphate induced oxidative stress in *Drosophila melanogaster***

Figure 1 shows the effects of ferrous and copper sulphate on the survival biochemical markers in of *D. melanogaster*. All doses of FeSO<sub>4</sub> and CuSO<sub>4</sub> induced a toxic effect thus, reducing the survival of flies (Figure 1). At varied concentration, FeSO<sub>4</sub> and CuSO<sub>4</sub> significantly (p<0.05) depleted T-SH, GST, GSH levels and catalase activities except 1mM FeSO<sub>4</sub> where there was no significant difference for both GST and catalase after 7 days of treatment when compared to the control group (Figure. 1B, 1C, 1E and 1G) (p>0.05). Furthermore, significant



**Figure.1.** Effects of  $FeSO_4$  and  $CuSO_4$  on survival rate and biochemical parameters in *D. melanogaster*. Data are presented as Mean  $\pm$  SE of 40 flies/ vial with 5 replicates per treatment group. Significant differences when compared with the control group are indicated by ( $p < 0.05$ )

Table 1. Comparison of ameliorative impact of resveratrol on ferrous and copper sulphate-induced oxidative stress in *Drosophila melanogaster*

Parameters	Control	EDTA (50mg/Kg)	REV (30 mg/Kg)	REV (60 mg/Kg)	FeSO <sub>4</sub> + CuSO <sub>4</sub> (1mM each)	FeSO <sub>4</sub> + CuSO <sub>4</sub> (1mM each) + REV (30 mg/Kg)	FeSO <sub>4</sub> + CuSO <sub>4</sub> (1mM each) + REV (60 mg/Kg)	FeSO <sub>4</sub> + CuSO <sub>4</sub> (1mM each) + EDTA (50mg/Kg)	p-value
H <sub>2</sub> O <sub>2</sub> (µmol/mg)	1.93 ± 0.03	1.93 ± 0.04	1.84 ± 0.05	1.86 ± 0.02	2.36 ± 0.03	1.92 ± 0.06	1.87 ± 0.08	1.99 ± 0.05	<0.001
Nitric Oxide (µmol/mg)	5.15 ± 0.20	4.95 ± 0.28	4.67 ± 0.50	4.39 ± 0.19	7.81 ± 0.36	6.98 ± 0.41	5.61 ± 0.80	6.41 ± 0.28	<0.001
TBARS (nmol/h)	0.18 ± 0.02	0.25 ± 0.01	0.19 ± 0.02	0.18 ± 0.01	0.32 ± 0.02	0.19 ± 0.02	0.19 ± 0.02	0.23 ± 0.02	0.004
T-SH (µmol/mg)	781.60±36.82	634.80±41.69	793.90±16.36	740.40±23.15	578.90±9.46	711.10±34.61	745.80±43.14	609.30±24.75	0.003
GSH (µmol/mg)	6.43 ± 0.39	5.89 ± 0.35	6.76 ± 0.55	6.20 ± 0.13	4.33 ± 0.31	5.39 ± 0.45	5.44 ± 0.53	5.37 ± 0.302	0.017
GST(µmol/min/mg)	2.33 ± 0.14	1.75 ± 0.13	2.27 ± 0.13	2.23 ± 0.05	1.39 ± 0.06	2.01 ± 0.14	2.20 ± 0.19	1.20 ± 0.18	0.002
Catalase (µmol/min/mg)	3.08 ± 0.04	2.60±0.19	3.09±0.17	3.08±0.09	1.93±0.16	2.90±0.33	2.79±0.21	2.40±0.23	0.008

\*Significant difference compared with control group (p<0.05)

Table 2: Comparison of ameliorative impact of resveratrol on climbing rate, eclosion rate, and selected biochemical markers after exposure of *D. melanogaster* to varied concentrations of ferrous and copper sulphate

Parameters	Control	EDTA (50mg/Kg)	RES (30 mg/Kg)	RES (60 mg/Kg)	FeSO <sub>4</sub> + CuSO <sub>4</sub> (1mM each)	FeSO <sub>4</sub> + CuSO <sub>4</sub> (1mM each) + RSV (30 mg/Kg)	FeSO <sub>4</sub> + CuSO <sub>4</sub> (1mM each) + RSV (60 mg/Kg)	FeSO <sub>4</sub> + CuSO <sub>4</sub> (1mM each) + EDTA (50mg/Kg)	p-value
Climbing Rate (%)	100.0±2.30	82.67±1.76	103.3±1.76	102.7±2.40	58.67±1.76	90.67±3.52	90.0±1.155	74.67±5.20	<0.001
Eclosion Rate (%)	100±7.63	111.2±2.88	139.2±14.47	142.4±7.11	33.6±5.54	86.4±13.22	86.4±9.99	68.8±14.15	<0.001
AChE(µmol/min/mg)	0.28±0.03	0.33±0.02	0.29±0.01	0.27±0.01	0.43±0.02	0.25±0.03	0.22±0.020	0.33±0.05	0.001

Key: Values expressed in Mean±Standard Error of Mean (n = 5), p<0.05=significant, FeSO<sub>4</sub> = ferrous sulphate, CuSO<sub>4</sub> =copper sulphate, RES = resveratrol, EDTA= ethylenediaminetetraacetic acid, AChE = acetylcholinesterase

increase was observed in NO (nitrate and nitrite, Figure. 1D) and H<sub>2</sub>O<sub>2</sub> (Figure 1F) levels at varied concentration of FeSO<sub>4</sub> and CuSO<sub>4</sub> when compared to the control group (p<0.05).

### **Resveratrol reverses ferrous and copper sulphate induced oxidative stress in *Drosophila melanogaster***

We assessed hydrogen peroxide and nitric oxide levels in *D. melanogaster* after 7 days of ferrous and copper sulphate exposure and observed that RES restored ferrous and copper sulphate -induced elevations of H<sub>2</sub>O<sub>2</sub> (p < 0.05), and NO levels (p<0.05). We also evaluated selected markers of antioxidants such as TBARS, TSH, GSH, GST, and catalase (Table 1). The levels of total thiols (TSH) in Cu + Fe and RES-exposed flies were not significantly different when compared with the controls (p>0.05) as shown in Table 1. In addition, RES reversed Cu + Fe-induced inhibition of catalase activity and improved GST activity that was inhibited by Cu + Fe in *D. melanogaster* (p<0.05). For TBARS, a significant increase (p<0.05) was observed in the flies when comparisons between the control group and the various treatment groups were made. However, flies that were treated with 60mg RES /kg diet were closer in values to that of the control group. It could be inferred that resveratrol restored ferrous and copper sulphate-induced elevated H<sub>2</sub>O<sub>2</sub>, NO and TBARS levels in *Drosophila melanogaster*.

### **Comparison of ameliorative impact of resveratrol on negative geotaxis (climbing rate), eclosion and selected biochemical markers after exposure of *D. melanogaster***

Table 2 shows the comparison of ameliorative impact of RES on climbing rate, eclosion and selected biochemical marker after exposure of *D. melanogaster* to varied concentration of ferrous and copper sulphate after seven days of treatment. There was a significant reduction in the climbing rate (p<0.05) in Cu + Fe challenged flies when compared with other groups; however, resveratrol significantly

increased the climbing rate (p<0.05). A significant reduction in the eclosion rate (Table 2, p<0.05) in Cu + Fe challenged flies when compared with the control and other groups was also observed. However, resveratrol significantly increased the eclosion rate of flies as shown in Table 2.

For acetylcholinesterase enzyme, a significant increase (p<0.05) was observed when comparison between control and Cu + Fe challenged flies were made. Interestingly, a significant decrease (p<0.05) was observed in the acetylcholinesterase activities of flies treated with resveratrol. Therefore, resveratrol significantly reduced the activities of acetylcholinesterase after co-treatment with toxicants to a level comparable to the control group as well as improved the climbing rate and the eclosion rate in *Drosophila melanogaster*.

### **Effects of ferrous sulphate (FeSO<sub>4</sub>) and copper sulphate (CuSO<sub>4</sub>) on survival rate of *Drosophila melanogaster***

The effect of ferrous sulphate (FeSO<sub>4</sub>) and copper sulphate (CuSO<sub>4</sub>) on survival of *Drosophila melanogaster* is shown in Figure 2 below. Selected concentrations of FeSO<sub>4</sub> and combination of FeSO<sub>4</sub> and CuSO<sub>4</sub> doses were used as shown in Figure 2., 7 days exposure duration was carried out and daily mortality was recorded in the survival study. It was observed that FeSO<sub>4</sub> and CuSO<sub>4</sub> induced toxic effect at all doses used when compared with the control. Hence, FeSO<sub>4</sub> and CuSO<sub>4</sub> reduced the survival rate of the flies.

## **DISCUSSION**

This study showed that ferrous and copper sulphate reduced the survival rate of *D. melanogaster*, suggesting that these metal ions are harmful at 1mM. Excessive ROS formation, which is an unavoidable by-product of cellular breakdown, leads to oxidative damage to macromolecules, increasing the susceptibility to degenerative disorders.<sup>(22)</sup> The significant increase in the levels of H<sub>2</sub>O<sub>2</sub>, nitric oxide, lipid peroxidation (TBARS) with reduced total thiols

and glutathione as well as catalase inhibition across flies treated with ferrous and copper sulphates (1mM each) in this study suggests that the formation of oxidants in the metal-treated flies outpaced the antioxidant buffering capability of the flies, resulting in ineffective oxidant detoxification. Elevated nitric oxide (NO) serves as a pro-inflammatory mediator.<sup>(14,23)</sup> Therefore, both the ineffective oxidant detoxification and inflammation directly reduced the survival rate of the flies in the face of oxidative stress.

Remarkably, treating flies with just resveratrol (30 or 60 mg/kg) increased their antioxidant and anti-inflammatory capacity, as seen by the lowering of hydrogen peroxide, nitric oxide, and lipid peroxidation, while increasing catalase activity and elevating glutathione, glutathione-S-transferase and total thiols. Our study agrees with a previous study done by Konyalioglu et al.,<sup>(24)</sup> which suggested that resveratrol partially protected embryonic neural stem cells from hydrogen peroxide-induced toxicity. These findings also agreed with a previous study carried out by Rege et al.,<sup>(25)</sup> which postulated that resveratrol inhibited membrane lipid peroxidation and decreased the toxic effects produced by ROS. In living organisms, glutathione-S-transferases (GSTs) as detoxifying enzymes catalyse the conjugation of GSH to exogenous and endogenous electrophilic sites.<sup>(26)</sup> Glutathione-S-transferase is a component of the first line of antioxidant defence. It aids in the prevention of free radical chain reactions and the detoxification of harmful ROS when used in conjunction with GSH-dependent enzymes.<sup>(27)</sup> Indeed, resveratrol corrected the depletion of GSH and total thiols caused by copper and ferrous sulphate in the flies. Total thiols (including non-protein and protein thiols) protect the body from oxidative stress.<sup>(28)</sup> In addition, resveratrol shielded GST activity from copper and iron-induced reduction, and the flies' response to oxidative stress and heavy metal detoxification was activated. This finding led us to believe that resveratrol's protective mechanism against copper and iron-induced toxicity is improved

further by the enhancement of thiol-containing proteins other than the GSH molecule alone.

These findings agree with a previous report that RES restored cigarette smoke extract (CSE)-depleted GSH levels by up regulation of antioxidant genes such as glutamate-cysteine ligase (GCL) through activation of nuclear erythroid-related factor 2 (Nrf2) and also quenched CSE-induced release of reactive oxygen species.<sup>(29)</sup> The antioxidative effects of RSV determined in the current study were consistent with the results of a study by Li et al.,<sup>(30)</sup> which indicate the role of RSV in protecting against oxidative stress by decreasing ROS levels and increasing the expression of CAT, GSH-Px, GSH and T-SOD in obese-asthmatic rat models. Increased catalase by resveratrol catalyses the dismutation of H<sub>2</sub>O<sub>2</sub> to molecular oxygen and water to reduce oxidative stress-induced toxicity.<sup>(31)</sup> The fact that resveratrol significantly decreases ferrous and copper sulphate-induced nitric oxide increase to levels comparable to the control group, shows that the flies' basal level of NO, which is essential for physiological functions, was restored. This finding is in line with Man et al.,<sup>(32)</sup> who suggested that resveratrol inhibits the acetylation of endogenous eNOS on lysine residues *in vitro* and postulated that SIRT 1 gene activation may play a fundamental role in regulating endothelial NO and endothelium-dependent vascular tone by deacetylating nitric oxide synthase (eNOS). Also, Oh and Yun<sup>(33)</sup> state that activation of SIRT 1 through resveratrol in RINm5F cells (a pancreatic islet cell line) or isolated rat islets also prevented the pro-inflammatory stress induced by IL-1 $\beta$  and IFN- $\gamma$  by inhibiting iNOS and nitric oxide production likely through the inhibition of the NF $\kappa$ B signalling pathway. This observation also agrees with a previous report that resveratrol is effective in reducing the inflammatory status *in vitro* and *in vivo* settings of neuroinflammation.<sup>(34,35)</sup>

The levels of TBARs, the end result of lipid peroxidation, were also measured to support the idea that ferrous and copper sulphate cause oxidative stress. The copper- and ferrous-sulphate

treated groups had significantly higher levels of TBARS, indicating oxidative stress, but resveratrol treatment was able to ameliorate this impact. This observation is in agreement with a previous study, in that resveratrol prevents iron-driven mitochondrial dysfunction by inhibiting glycogen synthase kinase-3 beta activity (a mechanism useful also to prevent tau hyperphosphorylation),<sup>(36)</sup> and by reducing peroxidation of lipoproteins and lipids through its activity as scavenger. Therefore, the protective effects of resveratrol on ferrous and copper sulphate-induced oxidative stress can be related to its ability to scavenge free radicals and restore the cellular redox balance as well as control physiological activities of the flies. Due to certain constraints, elucidation of the possible mechanism by which these metals induce their oxidative effects on *Drosophila melanogaster* could not be ascertained. However, the ability of resveratrol in ameliorating these effects may present it as a suitable antioxidant agent in the prevention of heavy metal-associated diseases.

## CONCLUSION

We demonstrated that  $\text{FeSO}_4$  and  $\text{CuSO}_4$  were able to induce oxidative stress. Interestingly, resveratrol was able to ameliorate free radical generation. As a result, resveratrol protects against copper and iron oxidant toxicity in vivo when studied in combination. Because of the greatly complex chemical composition and multiple pharmacological effects of RES, further research on its protective mechanism is required.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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## CONTRIBUTORS

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