Turkish Journal of Agriculture - Food Science and Technology, 14(1): 117-126, 2026
DOI: https://doi.org/10.24925/turjaf.v14i1.117-126.8216

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology Publishing (TURSTEP)

Syzygium aromaticum (Cloves) on Ameliorates Lead-Iron Co-Induced Neurotoxicity in *Drosophila melanogaster* Model

Olufunto Omodele Adeleye[1,a,*], Esther Opeyemi Aworeni[1,b], Emmanuel Oluwagbenga Adejobi[1,c], Yasar Olalekan Sulaiman[1,d], Gladys Deborah Olorunlowu[1,e], Oluwatunmise Timothy Ajao[1,f], Blessing Oluwatimilehin Ayandokun[1,g], Oluwadara Ope Oluwasegun[1,h]

[1]*Department of Anatomy, Faculty of Basic Medical Sciences, Lautech, Ogbomoso. Nigeria. Eagles' Research Laboratory, Ogbomoso, Nigeria*
[*]*Corresponding author*

A R T I C L E I N F O

Research Article

Received : 04.08.2025
Accepted : 11.11.2025

Keywords:
Iron
Lead
Clove
Neurotoxicity
Antioxidant

A B S T R A C T

Lead hazards are prevalent globally, causing significant health issues by disrupting iron homeostasis and increasing the burden of diseases, Iron overload, causes oxidative damage to lipids, impairing mitochondrial and lysosomal functions, and compromising DNA, leading to severe health issues**.** To evaluate the neuroprotection of *Syzygium aromaticum* (also known as clove) against iron and lead co-exposure in Harwich strain *Drosophila melanogaster*. Adult *Drosophila melanogaster* were randomly assigned into six groups: control, clove only, iron only, lead only, co-exposure of iron-lead, and co-exposure with clove. Flies were treated via diet supplementation for seven days. Neurobehavioral tests such as negative geotaxis, phototaxis, and chemotaxis were carried out to qualitatively assess motor activity, sensory response, and feeding behavior of flies. Following the completion of tests, brain tissues were isolated and visually assessed histologically for evidence of neurodegenerative changes. *Drosophila melanogaster* exposed to iron and lead, relative to controls, exhibited significant deficits in climbing performance, light response, and food-seeking behavior following total co-exposure. This was coupled with histological evidence of neurodegeneration, such as neuronal vacuolization and evidence of tissue disorganization. Supplementation with clove extract significantly improved behavioral performance and preserved brain histoarchitecture, for instance, it reduced neuroinflammation and oxidative damage. *Syzygium aromaticum* extract has important neuroprotective characteristics against iron and lead-induced neurotoxicity in *Drosophila melanogaster*, likely operating through antioxidant, anti-inflammatory, and neurocellular protective actions.

[a] ooadeleye@lautech.edu.ng https://orcid.org/0000-0003-1398-9180
[b] opeyemiaworeni@gmail.com https://orcid.org/0009-0008-8297-3039
[c] emmanueladejobi217@gmail.com https://orcid.org/0009-0008-2648-8100
[d] sulaimanyasar99@gmail.com https://orcid.org/0009-0004-5962-6443
[e] olorunlowugladys@gmail.com https://orcid.org/0009-0005-0894-6380
[f] oluwatunmisea55@gmail.com https://orcid.org/0009-0003-4928-2205
[g] blessingayandokun5@gmail.com https://orcid.org/0009-0000-2718-8638
[h] oluwadaraope2487@gmail.com https://orcid.org/0009-0006-7462-2883

Introduction

Iron has a vital function in various key processes regulated in the body. Iron is an essential metal involved in several biological processes ranging from the transport of oxygen to energy production and DNA synthesis (Vogt et al., 2021). However, iron can be harmful and toxic when in excess via the generation of reactive oxygen species and oxidative damage (Gammella et al., 2016). Iron overload is common throughout the world and can lead to serious health problems by oxidatively damaging lipids, affecting lysosomal and mitochondrial processes, and harming DNA (Zhao et al., 2024).

Lead is the most important toxic heavy element in the environment. Due to its important physio-chemical properties, its use can be retraced to historical times (Ara & Usmani, 2015). Being a heavy metal, it embodies soft characteristic features, malleability features, and a relatively low melting point (Boldyrev, 2018). Globally it is an abundantly distributed, important yet dangerous environmental chemical. However, it is also known to be among the major toxicants (Ara & Usmani, 2015). The exposure to lead can be through air, dust, or lead-based paints and results in serious health effects. Therefore, there isn't a medication that works to prevent Pb from being absorbed (Finlay, 2019).

Cloves (*Syzygium aromaticum*) a precious spice, is a member of Mirtaceae family which has been employed for centuries as food preservative and medicine because of its antimicrobial, antifungal, antibacterial, anti-inflammatory effects and antioxidant properties (Otunola, 2022). Clove, scientifically, is one of the most valued spices and has been used for centuries not only as a food preservative but also for several pharmaceutical purposes (Idowu et al., 2021). It is considered one of the best sources of phenolic compounds such as gallic acid, eugenol, and eugenol acetate (da Silva et al., 2018). Cloves enhance memory retention. It is recommended for relieving brain fog, lethargy and depressive state of mind.

Drosophila melanogaster is a small fly typically pale yellow to reddish brown to black, with red eyes. The fruit fly has been used for genetic experiments since T.H. Morgan started his experiments in 1907 (Mohr, 2018). This species is commonly known to be found in environments like the kitchen, where rotting or fermented fruits especially banana (Rabha et al., 2024). *Drosophila melanogaster* has been a cornerstone in biological research, especially in genetics, physiology, and developmental biology (Victor et al., 2025), since its introduction as a model organism by Charles W. Woodworth in 1901 (Qu et al., 1901). It has been linked to having about 75% percentage of human disease-causing homologous genes (Casas-Tintó, 2024). Its short life span, large number of offspring, many genetic techniques, are a few of the numerous factors that make Drosophila melanogaster is an organism for biomedical research (Staats et al., 2018).

While much research has laid emphasis on the harmful impacts caused by heavy metals like iron overload on brain function, especially in humans and animals, there is limited understanding of how these metals specifically impact the brain of *Drosophila melanogaster* (fruit flies), which are widely used in scientific studies. The increasing prevalence of neurodegenerative disorders has been linked to environmental neurotoxins such as heavy metals, particularly iron (Fe) and lead (Pb). These metals have been implicated in oxidative stress, mitochondrial dysfunction, and neuronal apoptosis, contributing to neurodegeneration. Additionally, while cloves have known antioxidant and anti-inflammatory properties, there is not enough research on their potential neuroprotective effects against metal toxicity, particularly when both iron and lead are present together. This gap highlights the need for research to understand the combined impact of iron and lead on fruit fly brains and to explore whether cloves can provide a protective effect.

Materials and Methods

Chemicals

Lead (II) acetate and Iron sulphate was purchased from Laboratory Trade while the Cloves was purchased from a local market, Oja-Igbo, Oyo state, Nigeria.

Drosophila Strains and Rearing Conditions

D. melanogaster (Harwich strain) was obtained from Drosophila Research Laboratory; University of Ibadan, Nigeria were used in the present study. Diet preparation was performed following previously described (Abolaji et al., 2014) with some adaptations. They were maintained on a corn meal medium (3.08% w/v corn flour, 2% w/v brewer’s yeast, 1% w/v agar, 0.08% w/v nipagin and 93.92% distilled water) at constant temperature (22-24ºC) and relative humidity (60-70%) under 12- h dark/light cycle conditions at Eagles’ Research Laboratory, Ladoke Akintola University of Technology, Ogbomosho, Oyo state, Nigeria.

Experimental Design

Survival analysis

To ascertain the levels and length of exposure to iron, cloves, and lead. For seven-day survival tests, *D. melanogaster* (both genders, ranging from a 1–3 days old) were split up into six groups of 30 flies each, and administered lead (10, 20, and 30 mg/L) concentration, iron (200, 250, and 500 µM) concentration, cloves (100, 140, and 200 mg) concentration, lead and iron co-administration and lead and iron and cloves co-administration respectively. Data were analysed and presented as a percentage of live flies, and daily mortality was noted. Based on these data, 140 mg of cloves, 500 µM iron, and 30 mg/L lead were selected and subjected to be expose to flies for seven days.

Treatment of Drosophila melanogaster

D. melanogaster 1 to 3-day old, 30 flies per vial (n = 6) were exposed to control, only lead, only iron, only cloves (incorporated into the diet by solution), lead with iron, lead and iron with cloves of selected concentrations.

Flies in group A (control) received diet containing 1600 µM distilled water (vehicle).

Group B flies were fed with diet containing cloves (140mg/20g)

Group C flies were fed with diet containing Lead (30ppm)

Group D flies were fed with diet containing iron (500µM)

Group E flies were treated with diet mixed with Lead and iron (30ppm and 500µM respectively)

Group F flies were co-treated with lead (30ppm), iron (500µM) and cloves (200mg/20g diet).

Neurobehavioral Analysis

Determination of negative geotaxis

The locomotor performance of flies treated with lead, iron, cloves, and their co-exposure and co-treatment was examined using the negative geotaxis test method. Ten flies from each group were immobilised under light cold anaesthesia and put in separate vertical glass columns with labels (15 cm in length; 1.5 cm in diagonal). Following a 20-minute recovery time, the flies were gently tapped to the column's bottom. The number of flies that ascended to the column's 6 cm mark and those that stayed below it were counted after 20 seconds. Each group's score was calculated by averaging three trials for both the treated and control fly groups (Abolaji et al., 2014).

Phototaxis

This was carried out according to (Vang et al., 2012) with some modifications. The apparatus consisted of a vial of diameter 2.5 cm x 9.5 cm and 20 cm long put together by a connector. A visible light source (2.8 cm x 45.2 cm, 15 watt) established a gradient of light that acted as an attractant for the flies, about 3.000 lux at the nearest point of the apparatus and about 700 lux at the furthest point. Vial containing 10 flies plugged by cotton and the test tube were left separately for 30 minutes and kept in a dark room. This allowed adaptation of the flies to darkness. The vial was then gently pounded down to place the flies at the end away from the cotton, the cotton was removed, and the vial was attached to the test tube by a connector. This apparatus was horizontal and perpendicular to the horizontal light source 15 cm away. The vial was separated into a light exposed side and dark side (covered by opaque black substance). The light was then turned on and a timer was started. The flies on the light side were counted after 5 minutes. The side exposed to light was then covered by the opaque substance while the previous dark side was exposed to light. The number of flies that are able to crawl back to the recent light exposed area are recorded after another 5 minutes.

Determination of larvae crawling activity

The larvae's crawling speed was analysed by using five males and five females each in a vial, totalling ten flies, aged 1-3 days, were subjected for twenty-four hours to the specific concentrations of lead, iron, cloves, their co-exposure, co-treatment and control as shown above. After that, the larvae were gathered and all of the flies were taken out of the vials. The analysis was done in triplicate for each group, and the larvae were left to crawl around in a shaded petri dish for five minutes. Image J software was subsequently utilised to measure the crawling traces.

Chemotaxis (response to volatile chemicals smell)

The experiment was carried out in a dark room 15 cm away from a turned-on parallel visible-light source, which served for visibility throughout the duration of the experiment. (A lighted room also works but the light must be uniform, which may be difficult to achieve, because non-uniform light is itself a stimulus). 10 flies were placed into a vial of diameter 2.5 cm x 9.5 cm and 20 cm long with a screw cap and kept in the dark room for 30 minutes parallel to the light source. At that time the flies were randomly distributed. Then the screw cap was gently removed and Volatile repellent, benzaldehyde placed in the cover and replaced with a screw cap containing 1ml of 100 mM benzaldehyde dissolved in 1.5% agar. The number of flies in each third of the test tube was then counted every 2 minutes for 10 minutes (Vang et al., 2012).

Biochemical Analysis

Homogenization

Homogenization is the process in which a solid is converted to a liquid state (which mostly requires grinding with a vehicle (liquid)) (Kluge et al., 2012). The commonly used vehicle used for homogenizing *Drosophila melanogaster* is phosphate buffer as it helps to maintain the pH of the model. It should be noted that all homogenizing process should be done on or in ice (at 4°C temperature). *D. melanogaster* ranging from 1 to 3 days old, 50 flies per vial (n = 6) were grouped to control, iron, lead, cloves, co-exposed and co-treatment of selected concentrations for 7 days. The flies were collected into empty vials. Then, the flies were anesthetized in ice, weighed and homogenized in 0.1 M phosphate buffer, pH 7.4 (ratio of 1mg:10µL), and centrifuged at 10.000g for 10 min at 4 °C in a cold Mikro 220R centrifuge (CERID Laboratory, Ogbomoso, Oyo state). Then, the supernatants were transferred into labelled micro fugetube,and used for the evaluation of acetylcholinesterase (AChE), glutathione (GSH), nitric oxide (NO), Superoxide dismutase (SOD), malondialdehyde (MDA), and catalase, levels at Neuropharmacology Laboratory, University of Ibadan.

Determination of acetylcholinesterase (AChE) activity

The activity of acetylcholinesterase was determined with the method of (Ellman et al., 1961). The reaction was carried out in 0.1 M potassium phosphate buffer of pH 7.4, 1 mM Dithiols-nitrobenzoic acid and 0.8 mM acetylthiocholine, the initiator. The reaction was monitored for 2 minutes (30 s interval) at 412nm. The enzyme activity was estimated as µmol of acetylthiocholine hydrolysed/minute/mg protein.

Determination of nitrite levels

Nitrite was measured as an indicator of nitric oxide (NO) production according the Griess method as described by (Green et al., 1982). Griess reagent was freshly prepared by mixing equal volumes of 0.1% N-(1-napththyl) ethylene diamine dihydrochloride and 1% sulphanilamide (in 5% phosphoric acid). 50µL of 2X diluted supernatant was added into microtitre plate and diluted with 50µL of distilled water before incubation with 100µL of Griess reagent for 10 min at room temperature in the dark. The concentration of nitrite was determined from sodium nitrite standard curve and expressed as µM nitrite/mg protein.

Determination of glutathione level

Reduced glutathione (GSH) as non-enzymic antioxidant maker was measured in tissue homogenate supernatant (Palsamy et al., 2010). Briefly, 100µL of supernatant was diluted twenty times in 0.15 M Tris-KCl buffer, and deproteinized with 500µL trichloroacetic acid (30%). The mixture was centrifuged in a bench top centrifuge at 4000 rpm for 10 min at room temperature. 100µL of the deproteinized supernatant was mixed 100µL of 5’, 5’-Dithiols-nitrobenzoic acid (DTNB, 0.0006M) in a microplate plate.

Determination of lipid peroxidation level

Malondialdehyde was measured as an index of lipid peroxidation using the assay of thiobarbituric reacting substances (TBARs) by the method of (Nagababu et al., 2010). 100µL of supernatant was diluted twenty times in 0.15M Tris-KCl buffer, and deproteinized with 500µL trichloroacetic acid (30%). The mixture was centrifuged in a bench top centrifuge at 4000rpm for 10min at room temperature. 200µL of the supernatant was removed into microtubes, followed by addition of 200µL thiobarbituric acid (0.75%), and the mixture was heated at 80°C for an hour. The tubes were cooled by placing on ice, 200µL was removed into microtitre plate and absorbance measured at 532nm. The concentration of TBARs in the tissues were expressed as ηmol MDA/mg protein

Determination of catalase enzyme activity

Catalase activity in the supernatants of the flies was determined using the colorimetric assay based on the yellow complex with molybdate and H_2O_2, which was described by (Goth et al., 1991). Briefly, 50µL of 2X diluted supernatant was added into a microtiter plate, and then 50µL of a reaction mixture containing 65mmol/mL of H2O2 in sodium-potassium phosphate buffer (60mM, pH 7.4) was added. The enzymatic reaction was incubated for 3 minutes and stopped with 100µL of ammonium molybdate (64.8mM) in sulfuric acid. The absorbance at 405nm was measured. The catalase enzyme activity unit was expressed as µ/mg protein

Histology of brain of Drosophila melanogaster

D. melanogaster was kept in 10% neutral buffered formalin for histological examination under a light microscope. The brains were prepped for haematoxylin and eosin (H&E) and immunostaining, following deparaffinization. A light microscope to inspect the slides and gave their interpretation (Gregor et al., 2022)

Statistical Analysis

All the experiments were replicated at least three times. The data are presented as the Mean ± SEM. A One-way Analysis of variance (ANOVA) was used to assess the significant differences among multiple groups under various treatments, followed by Turkey's and post-hoc and Fischer's LSD test where appropriate. In all the groups, differences with a p-values < 0.05 were considered statistically significant.

Results

Survival Analysis Results

The effect of lead, iron, and clove on the survival of *D. melanogaster* was assessed as mentioned in the methodology. Lead exposure resulted in a concentration-dependent decline in survival, with the most significant reduction observed at 30mg/L, followed by 20mg/L and 10mg/L as shown in Figure 1A. Similarly, iron exposure produced a dose-dependent decrease in survival, with the highest concentration (500µM) showing the most pronounced effect. Co-exposure to both lead (30mg/L) and iron (500µM) led to a significant synergistic decline in survival compared to individual metal exposures (Figure 1B). As depicted in Figure 1C, clove administration at 100mg/20g slightly improved survival relative to the control group, while 140mg/20g further enhanced survival, suggesting a protective effect. However, exposure to 200mg/20g of clove resulted in a reduction in survival, indicating potential toxicity at higher doses.

Neurobehavioral Results

Locomotion activity

Climbing activity and crawling

As presented in Figure 2A (a), as shown in the crawling assay, all other groups show a significant decreased when compared with control except the co-treatment group. The co-treatment shows a significant increase when compared to iron and the co-exposed flies. Notably, the group co-treated exhibited a significant improvement in crawling ability when compared to the iron-only group, suggesting a potential mitigating effect of clove on iron-induced locomotor deficits. Figure 2A (b) Illustrates the graphical representation of adult climbing activity (negative geotaxis), iron has a significant decrease when compared to control and cloves. Also, the co-treatment shows a significant increase when compared to the flies co-exposed to lead and iron, backing up the crawling assay result which shows that cloves offer protective roles in flies exposed to heavy metals like lead and iron overload. Statistical significance is indicated as follows: "*" denotes a significant difference compared to the control group (p < 0.05), and "@" indicates a significant difference compared to the cloves group, and "&" represents a significant difference compared to the iron group.

Phototaxis

Figure 2 Illustrates that lead causes a significant decrease in the response of flies to light on both the right and left side. On the left side, lead and iron causes a significant decrease in the flies' response when compared to the control and cloves. On the right side, lead and iron has a significant decrease when compared to control and cloves. Also, lead and iron co-exposure has a significant decrease when compared to cloves.

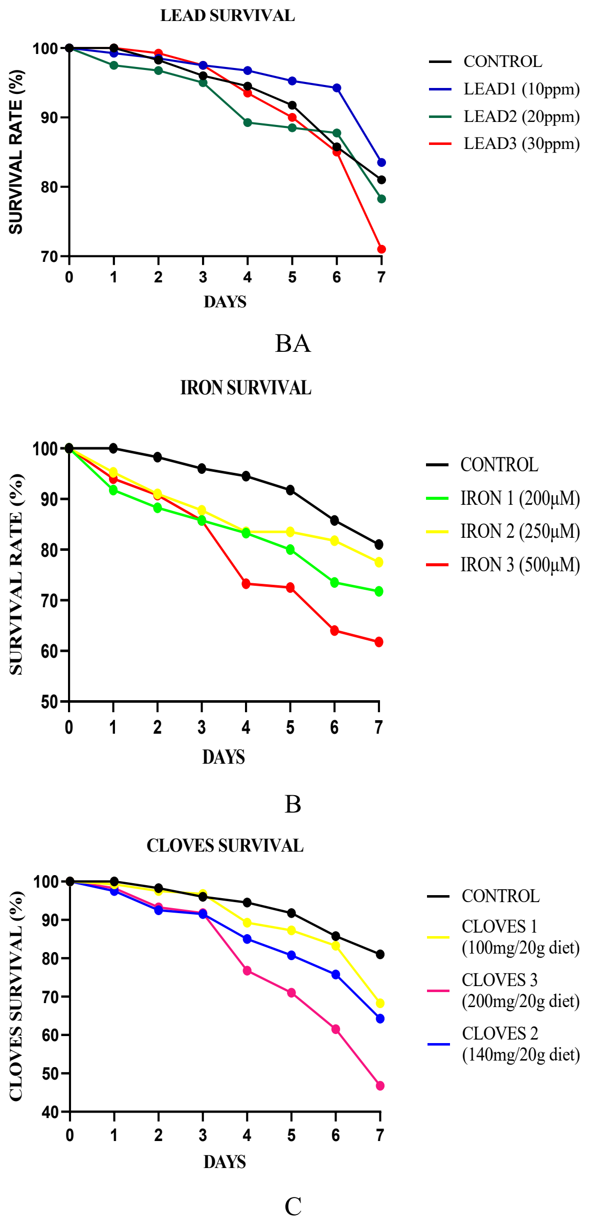


Figure 1. A graphical representation illustrating the survival rate of Drosophila melanogaster when exposed to lead, iron and cloves for 7 days.

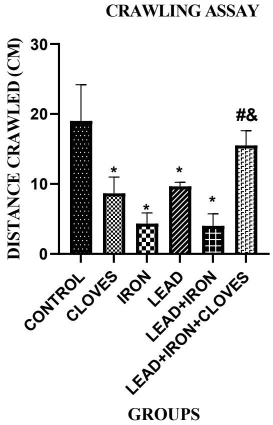
Biochemical Results

Oxidative stress markers

Biomarkers of oxidative stress, such as nitric oxide (NO) and malondialdehyde (MDA), were measured in *D. melanogaster* (Figure 3). Exposure to lead (4.68nmol/mg protein) iron (2.76nmol/mg protein), and the co-exposure (3.49 nmol/mg protein) led to a significant elevation in MDA levels in comparison to the control group (1.69nmol/mg protein). A notable reduction in MDA levels (2.10 nmol/mg protein) was recorded in the co-treatment flies, in contrast to the co-exposed flies (3.49nmol/mg protein). The co-exposed flies exhibited a notable elevation in NO levels (7.17µmol/mg protein) relative to those co-treated flies (6.45 µmol/mg protein).

Enzymatic markers

As illustrated in Figure 3, acetylcholinesterase (AChE) and catalase represent anti-inflammatory and antioxidant enzymes, respectively.



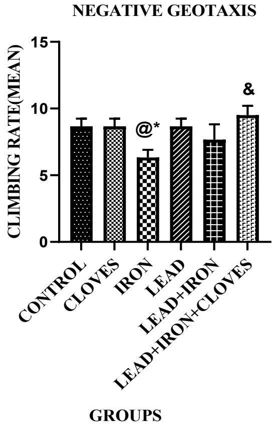
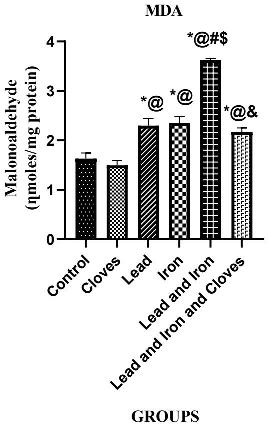
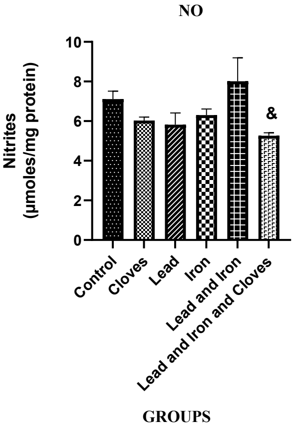


Figure 2.1 Illustrating the crawling and climbing activity (negative geotaxis) of third instar larvae and adults Drosophila melanogaster exposed to lead, iron, cloves for 7 days respectively.

[1]Figure 2.2 A graphical representation showing the phototaxis response of *Drosophila melanogaster* on both the right and left side after 7-days of exposure to lead, iron, cloves and their mixtures.

[1]Data is presented as Mean ± SEM. "*" denotes a significant difference compared to the control group ($p < 0.05$), and "@" indicates a significant difference compared to the cloves group. Pb: Lead; Fe: Iron.





[1]Figure 3.1 Effect of Pb, Fe and Cloves on oxidative stress biomarkers including nitric oxide (NO) and malondialdehyde (MDA), following a 7-Day Exposure to Lead and Iron-induced toxicity.

[2]Figure 3.2 Effect of Pb, Fe and Cloves on Acetylcholinesterase (AChE) (A) and Catalase Enzyme Activity (B) in *D. melanogaster* Following a 7-Day Exposure to Lead and Iron-induced toxicity.

[1]Data are presented as mean ± SEM from 50 flies per vial, with three independent replicates per treatment group. Statistical significance is indicated as follows: "*" denotes a significant difference compared to the control group ($p < 0.05$), "@" indicates a significant difference compared to the cloves group, "#" represents a significant difference compared to the iron group, "$" represents a significant difference compared to the lead group, and "&" represents a significant difference compared to the iron group. Pb: Lead; Fe: Iron. [2]Data are presented as mean ± standard error of the mean (SEM) from 50 flies per vial, with three independent replicates per treatment group. Statistical significance is indicated as follows: "@" denotes a significant difference compared to the clove group ($p < 0.05$). Pb: Lead; Fe: Iron.

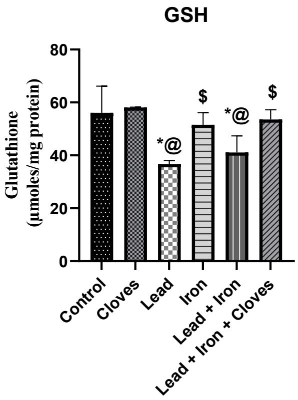
No significant change was observed in AChE activity across the experimental groups, nonetheless the cloves group showed an increase in AChE activity compared to other groups. Also, the co-exposed flies showed an increase in AChE activity when compared to the co-treated flies. The catalase activity was significantly reduced in the lead-treated (16.19µ/mg protein) group compared to the clove (22.82µ/mg protein) group. Similarly, the cloves group showed an increase in catalase activity compared to other groups and the co-exposed flies showed an increase in catalase activity when compared to the co-treated flies.

Antioxidant markers

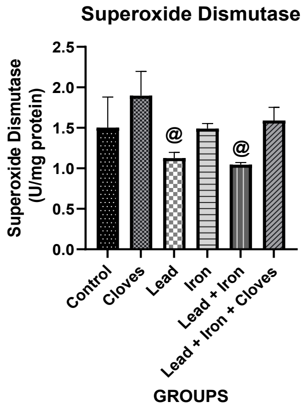
Biomarkers of antioxidants such as glutathione (GSH), and superoxide dismutase (SOD), were measured in *D. melanogaster* (Figure 3). In GSH, flies exposed to lead (42.15µmol/mg protein), iron (61.71µmol/mg protein) and lead and iron co-exposure (45.83µmol/mg protein) showed a significant reduction in GSH levels when compared to the control group and clove group flies. Also, there is a significant increase in the GSH levels of the flies exposed to Iron (61.71µmol/mg protein) and the co-treatment of lead, iron and cloves (79.32µmol/mg protein) when compared to the flies exposed to lead only (42.15µmol/mg protein). No substantial change was observed in SOD levels among flies subjected to lead, iron, or its co-exposure as compared to the control group or the co-treated flies. A significant decrease in SOD levels was detected in flies subjected to lead (1.48µ/mg protein) and lead and iron co-exposed group (0.97µ/mg protein) compared to those exposed to cloves (1.66µ/mg protein).

Histological Results

Photomicrograph (Haematoxylin and eosin) *Drosophila melanogaster* brain exposed to lead, iron and cloves for 7 days at X400 magnification.



[1]Figure 3.1 Effect of Pb, Fe and Cloves on oxidative stress biomarkers including nitric oxide (NO) and malondialdehyde (MDA), following a 7-Day Exposure to Lead and Iron-induced toxicity.



[2]Figure 3.2 Effect of Pb, Fe and Cloves on Acetylcholinesterase (AChE) (A) and Catalase Enzyme Activity (B) in D. melanogaster Following a 7-Day Exposure to Lead and Iron-induced toxicity.

[1]Data are presented as mean ± SEM from 50 flies per vial, with three independent replicates per treatment group. Statistical significance is indicated as follows: "*" denotes a significant difference compared to the control group ($p < 0.05$), "@" indicates a significant difference compared to the cloves group, "#" represents a significant difference compared to the iron group, "$" represents a significant difference compared to the lead group, and "&" represents a significant difference compared to the iron group. Pb: Lead; Fe: Iron. [2]Data are presented as mean ± standard error of the mean (SEM) from 50 flies per vial, with three independent replicates per treatment group. Statistical significance is indicated as follows: "@" denotes a significant difference compared to the clove group ($p < 0.05$). Pb: Lead; Fe: Iron.

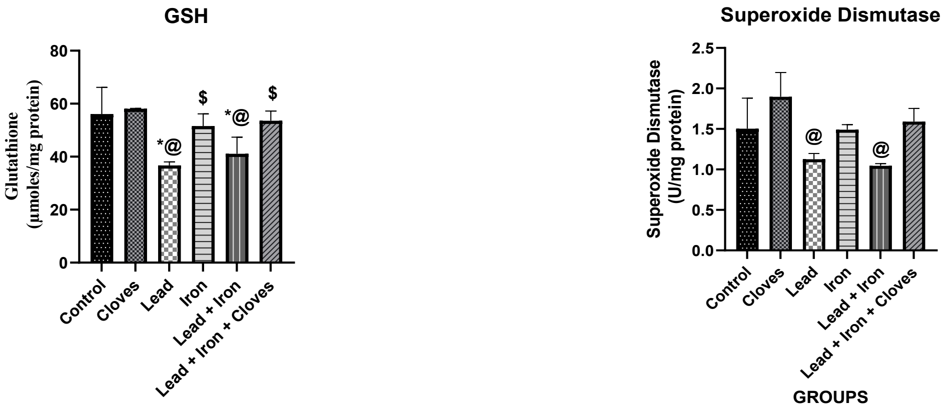
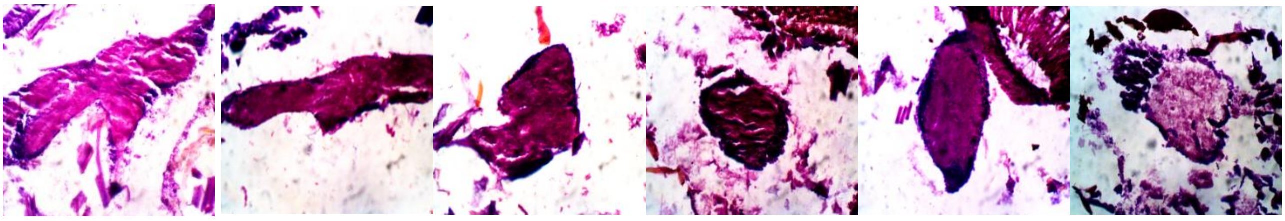


Figure 3.3 Effect of Pb, Fe and Cloves on Glutathione (GSH) activity, in D. melanogaster Following a 7-Day Exposure to Lead and Iron-induced toxicity.

Data are presented as mean ± standard error of the mean (SEM) from 50 flies per vial, with three independent replicates per treatment group. Statistical significance is indicated as follows: "*" denotes a significant difference compared to the control group ($p < 0.05$), "@" indicates a significant difference compared to the cloves group, and "$" represents a significant difference compared to the lead group. Pb: Lead; Fe: Iron.



Control | Clove | Lead + Iron + Clove | Lead | Iron | Lead + Iron

Figure 4 Hematoxylin and Eosin (H&E) photomicrograph of the brain region of D. melanogaster brain following 7-day exposure to lead, iron and cloves.

The control group exhibits normal cellular morphology and cytoplasmic density. Mild tissue lesions and the presence of pyknotic cells (Yellow Arrows) are observed in the iron and cloves treated groups. The lead exposed group shows a severe tissue rarefaction with pyknotic cells. Co-exposure to lead and iron results in a significant level of tissue damage and nuclear pyknosis. However, co-treatment with cloves displays only mild tissue lesions and pyknotic cells, suggesting a potential neuroprotective effect of cloves against heavy metal-induced toxicity.

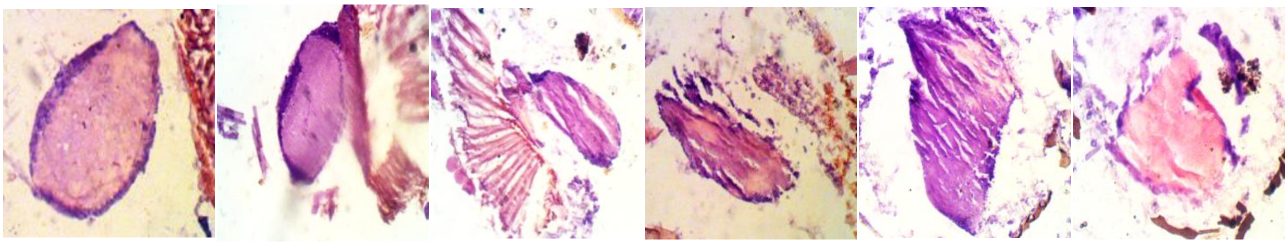


Figure 3.5 Caspase-3 immuno-stained photomicrograph of the brain region of D. melanogaster brain following 7-day exposure to lead, iron and cloves.

The control and cloves treated groups exhibit no caspase-3 immunoreactivity, indicating the absence of apoptosis in the fly's brain. In contrast, flies exposed to lead and iron show significant caspase-3 expression, reflecting elevated apoptotic activity. The co-exposed group results in a pronounced increase in apoptosis, as evidenced by strong caspase-3 staining. Similarly, the group co-treated with cloves also demonstrates a slight level of caspase-3 activation, suggesting that cloves did not completely prevent metal-induced apoptotic cell death but offered some protective roles in reversing the effects to some extent.

Immunostaining Results (caspase-3)

Immunostaining (Caspase-3) photomicrograph of *Drosophila melanogaster* brain exposed to lead, iron and cloves for 7 days at X400 magnification.

Discussion

This study aims to evaluate the neuroprotective potential of clove in mitigating iron and lead co-exposure-induced neurotoxicity in Harwich strain *Drosophila melanogaster*, with a focus on oxidative stress reduction, preservation of neuronal integrity, and behavioural recovery.

This study demonstrated that clove significantly enhanced the survival rate and prolonged the lifespan of *D. melanogaster*. While the specific anti-aging mechanisms of clove were not directly investigated, the observed increase in survival suggests potential anti-aging properties. Aging is a progressive biological process characterized by physiological decline and reduced cellular function, leading to increased susceptibility to age-related diseases such as Alzheimer's and Parkinson's disease (Dharmarajan, 2021). Although multifactorial, aging has been closely associated with the accumulation of reactive oxygen species (ROS), which contribute to oxidative stress and cellular damage (Zeng et al., 2024). In this study, exposure to lead and excessive iron markedly decreased the survival rate of *D. melanogaster*, consistent with previous findings indicating their toxic effects (Zhou et al., 2016). Conversely, clove treatment improved survival and lifespan, suggesting its potential role in mitigating metal-induced oxidative stress and supporting longevity (Elsawy et al., 2022).

Progressive deterioration of learning, memory, and motor function is a hallmark of \parkinsonism, with neurodegenerative diseases frequently manifesting as impaired locomotor activity (Pathak et al., 2022). In this study, the potential synergistic and neuroprotective effects of clove on lead and iron co-exposure were evaluated using neurobehavioral assays, including larval crawling and adult climbing tests. The locomotor performance measured in third instar larvae exposed to iron overload exhibited a significant reduction in crawling distance compared to the control group, although no significant differences were observed among other iron-treated groups. Similarly, lead exposure significantly reduced larval locomotor activity relative to controls. Notably, co-treatment with lead, iron, and clove resulted in a significant enhancement in crawling ability compared to both the iron only and lead and iron co-exposed groups, indicating a protective effect of clove against metal-induced neurotoxicity. Furthermore, adult flies exposed to iron alone displayed a significant reduction in climbing ability compared to both the control and clove-treated groups. In contrast, the group co-treated with lead, iron, and clove demonstrated a significant improvement in climbing activity compared to the lead and iron co-exposure group. These findings reinforce the hypothesis that clove exerts neuroprotective and ameliorative effects in mitigating locomotor deficits induced by lead and iron co-exposure.

However, acetylcholine, a neurotransmitter essential for the regulation of motor function and movement, is hydrolysed by the enzyme acetylcholinesterase (AChE) (Sam & Bordoni, 2022). In this study, AChE activity was elevated in the clove treated group compared to the control. In contrast, both the lead and iron exposed groups exhibited reduced AChE activity relative to the control and clove groups. The co-exposure to lead and iron resulted in the most pronounced reduction in AChE activity among all groups. Interestingly, the group co-treated with lead, iron, and clove demonstrated higher AChE activity than the lead and iron co-exposed group alone, highlighting a potential ameliorative effect of clove. However, this biochemical finding contrasts with the neurobehavioral outcomes observed in both third instar larval crawling and adult climbing assays. This discrepancy may be attributable to the duration of exposure (7 days) or other underlying factors, warranting further investigation in future studies (Alaraby, 2016).

Phototaxis measures the movement of flies in response to light (Kim et al., 2019). The phototaxis analysis on both sides, showed that flies exposed to lead and iron overload showed low sensitivity to light compared to that of the control and cloves group. The low sensitivity to light of the flies exposed to heavy metals can be attributed to oxidative stress and damage to cells in the retina, including photoreceptors (rods and cones), which are responsible for detecting light (Kim et al., 2019). The H&E photomicrographs and immunostaining, Caspase-3 clearly illustrated tissue lesions and cell apoptosis, this further explains the possible cause of low sensitivity of the flies exposed to heavy metals to light. Also, the oxidative stress markers, MDA and NO shows a very high level of oxidative stress in the flies exposed to lead and iron further supporting the phototaxis analysis

Chemotaxis measures the sensitivity of flies to odorants (Fishilevich et al*.,* 2005). The chemotaxis analysis showed that flies exposed to lead, iron, and their co-exposure exhibited significantly enhanced sensitivity to benzaldehyde, with the co-exposure group showing the highest olfactory response compared to the control and clove-treated groups. High sensitivity to odorants, also known as heightened olfactory sensitivity, can be influenced by exposure to heavy metals which can generate reactive oxygen species (ROS), leading to oxidative stress (Al-Gubory 2014). Oxidative stress can damage olfactory receptor cells and other components of the olfactory system, potentially increasing sensitivity to odours (Zheng et al*.,* 2020*)*. The oxidative stress markers, MDA and NO shows a very high level of oxidative stress in the flies exposed to lead and iron further supporting this chemotaxis analysis. Also, excessive exposure to heavy metals like lead and iron overload can deplete antioxidants activity increasing the risk of olfactory dysfunction (Jan et al*.,*2015). The flies exposed to lead and iron show a low level of GSH activity compared to the group co-treated with clove which shows a higher level further backing up the chemotaxis analysis results.

Biochemical analysis was conducted to evaluate the neuroprotective role of clove on major enzymatic, oxidative and antioxidant markers. Both oxidative stress markers, NO and MDA showed an increased level of oxidative stress activity in the heavy metal exposed group especially the co-exposed group when compared to the control, cloves and co-treatment group. Oxidative stress in fruit flies can be linked to developmental problems, reduced lifespan, accelerated aging, physiological and behavioural changes (Deepashree et al*.,*2019). The high rate of death of flies exposed to lead and iron supports this oxidative stress results as it proves that the increased level of oxidative stress in the flies resulted in a reduction of lifespan which resulted in the deaths of these flies. Alternatively, the low level of oxidative stress observed in the flies treated with cloves and the co-treatment flies supports the survival analysis results which showed that cloves have anti-aging properties. Also, the locomotion activity further supports these results as the flies co-treated with cloves showed better locomotion activity (in both larvae crawling and adult climbing) due to lower oxidative stress compared to the flies exposed to lead and iron, and its co-exposure, which showed a reduced locomotion activity due to high oxidative stress as expressed in the oxidative stress results.

The two antioxidants' markers, GSH, and SOD, in the flies showed a reduced level of antioxidant in the lead and iron exposed group compared to the control and co-treated with cloves group. Low antioxidant activity in fruit flies can be linked to increased oxidative stress, reduced lifespan, and impaired locomotor function (Ajagun-Ogunleye et al*.,*2021). The results of oxidative stress, survival analysis, and locomotion shows that the low level of antioxidant resulted in increased oxidative stress, reduced lifespan, and impaired locomotor function in the flies exposed to lead and iron, compared to the flies co-treated with cloves which showed a better result i.e. lower oxidative stress, improved lifespan, and better locomotor function once again showcasing clove protective role.

The two enzymatic markers, AChE and Catalase shows lead and iron toxicity increases when typically, according to previous studies, their activity ought to decrease (Khedr & Talkan, 2022). This increase can be due to oxidative damage and enzyme inhibition, short-term or sublethal exposure can trigger adaptive upregulation of these enzymes. This reflects a compensatory response aimed at mitigating cellular stress. Elevated levels at 7 days may indicate that the flies are still actively combating oxidative damage. However, prolonged or high-dose exposure would likely lead to enzyme suppression and irreversible neurotoxicity. The enzymatic markers in the AChE and Catalase of cloves treated group show an increase in enzymatic activity compared to the control group showing that cloves increase enzymatic activity.

Collectively, these biochemical findings indicate that cloves offer antioxidant and neuroprotective role in lead and iron co-exposure, but its efficacy may be a function of concentration and time. Further research is recommended to ensure the greatest therapeutic effect by optimizing the concentration and time of exposure.

Hematoxylin and Eosin (H&E) and Caspase- 3 photomicrograph of the brain region of *D. melanogaster* brain shows mild tissue lesions and the presence of pyknotic cells are observed in the co-treated with cloves flies, compared to the groups exposed to lead and iron which shows a severe tissue rarefaction with pyknotic cells. High oxidative stress and low antioxidant activity can cause tissue lesions and cell apoptosis in *D. melanogaster* brain (Yang et al*.,* 2022). The oxidative stress and antioxidant markers results provides an explanation or the cause of cell apoptosis in the lead and iron exposed group as the high level of oxidative stress and low level of antioxidant can be attributed to cause tissue lesions and cell apoptosis. Also, the flies co-treated with cloves had a lower level of oxidative stress and higher level of antioxidant compared to the co-exposed flies which reduced the rate of cell apoptosis and tissue lesions showing that cloves offer protective roles in reducing the rate of cell apoptosis and tissue lesions caused by lead and iron overload.

Conclusion

Briefly, this study confirms that co-exposure with lead and iron produces potent neurotoxicity in *D. melanogaster* via obvious reduced locomotor activity, lowered enzymatic antioxidant defence, heightened oxidative stress indicators, histopathological brain damage, and caspase-3-mediated apoptosis. Cloves treatment showed exemplary neuroprotection with enhanced survival, recovery of locomotor function, and inhibition of oxidative and apoptotic damage. Even though the effectiveness of clove would then appear to be a matter of concentration and duration of exposure, as with excessive or short treatment possibly reducing its protective action, clove does have potential as a natural therapeutic agent for the prevention of heavy metal-induced neurotoxicity and further research is needed for optimal application and identification of its specific mechanisms of action.

References

Abolaji, A. O., Kamdem, J. P., Lugokenski, T. H., Nascimento, T. K., Waczuk, E. P., Farombi, E. O., ... & Rocha, J. B. T. (2014). Involvement of oxidative stress in 4-vinylcyclohexene-induced toxicity in *Drosophila melanogaster*. *Free Radical Biology and Medicine*, *71*, 99-108.

Ajagun-Ogunleye, O. M., Adedeji, A. A., & Vicente-Crespo, M. (2021). Pineapple fruit extract prolonged lifespan and endogenous antioxidant response in *Drosophila melanogaster* exposed to stress. *African J Biomed Res*, *24*(1), 99-108.

Alaraby, M., Annangi, B., Marcos, R., & Hernández, A. (2016). *Drosophila melanogaster* as a suitable in vivo model to determine potential side effects of nanomaterials: A review. *Journal of Toxicology and Environmental Health, Part B*, *19*(2), 65-104.

Al-Gubory K. H. (2014). Environmental pollutants and lifestyle factors induce oxidative stress and poor prenatal development. *Reproductive biomedicine online*, *29*(1), 17–31.

Ara, A., & Usmani, J. A. (2015). Lead toxicity: a review. Interdisciplinary toxicology, 8(2), 55.

Boldyrev, M. (2018). Lead: properties, history, and applications. WikiJournal of Science, 1(2), 1-23.

Casas-Tintó, S. (2024). Drosophila as a Model for Human Disease: Insights into Rare and Ultra-Rare Diseases. *Insects*, *15*(11), 870.

da Silva, F. F. M., Monte, F. J. Q., de Lemos, T. L. G., do Nascimento, P. G. G., de Medeiros Costa, A. K., & de Paiva, L. M. M. (2018). Eugenol derivatives: synthesis, characterization, and evaluation of antibacterial and antioxidant activities. Chemistry Central Journal, 12(1), 34.

Deepashree, S., Niveditha, S., Shivanandappa, T., & Ramesh, S. R. (2019). Oxidative stress resistance as a factor in aging: evidence from an extended longevity phenotype of *Drosophila melanogaster*. *Biogerontology*, *20*(4), 497-513.

Dharmarajan, T. S. (2021). Physiology of aging. In *Geriatric gastroenterology* (pp. 101-153). Cham: Springer International Publishing.

Ellman, G. L., Courtney, K. D., Andres Jr, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical pharmacology*, *7*(2), 88-95.

Elsawy, M. A., & Mostafa, M. H. (2022). Antimicrobial Applications of Nanoparticles. *Nanomaterials and Nanotechnology in Medicine*, 517-552.

Finlay, J. T. (2019). Perceptions of Child Lead Poisoning Among Educational Psychologists. University of Johannesburg (South Africa).

Fishilevich, E., Domingos, A. I., Asahina, K., Naef, F., Vosshall, L. B., & Louis, M. (2005). Chemotaxis behavior mediated by single larval olfactory neurons in Drosophila. *Current biology*, *15*(23), 2086-2096.

Gammella, E., Recalcati, S., & Cairo, G. (2016). Dual role of ROS as signal and stress agents: iron tips the balance in favor of toxic effects. Oxidative medicine and cellular longevity, 2016(1), 8629024.

Goth, L. 1991. A simple method for determination of serum catalase activity and revision of reference range. Clinica chimica acta, 196(2-3), 143-151.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. 1982. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Analytical Biochem. 126(1), 131-138.

Gregor, K. M., Becker, S. C., Hellhammer, F., Schön, K., Baumgärtner, W., & Puff, C. (2022). Histochemical staining techniques in Culex pipiens and *Drosophila melanogaster* (Diptera) with a comparison to mammals. Veterinary pathology, 59(5), 836-849.

Idowu, S., Adekoya, A. E., Igiehon, O. O., & Idowu, A. T. (2021). Clove (Syzygium aromaticum) spices: A review on their bioactivities, current use, and potential application in dairy products. Journal of Food Measurement and Characterization, 15(4), 3419-3435.

Jan, A. T., Azam, M., Siddiqui, K., Ali, A., Choi, I., & Haq, Q. M. (2015). Heavy Metals and Human Health: Mechanistic Insight into Toxicity and Counter Defense System of Antioxidants. *International journal of molecular sciences*, *16*(12), 29592–29630.

Khedr, N. F., & Talkan, O. F. (2022). New insights into arsenic, lead, and iron neurotoxicity: Activation of MAPK signaling pathway and oxidative stress. *Journal of biochemical and molecular toxicology*, *36*(6), e23040.

Kim, K. N., Huang, Q. Y., & Lei, C. L. (2019). Advances in insect phototaxis and application to pest management: A review. *Pest Management Science*, *75*(12), 3135-3143.

Kluge, J., Muhrer, G., & Mazzotti, M. (2012). High pressure homogenization of pharmaceutical solids. *The Journal of Supercritical Fluids*, *66*, 380-388.

Mohr, S. E. (2018). First in fly: Drosophila research and biological discovery. Harvard University Press.

Nagababu, E., Rifkind, J. M., Boindala, S., & Nakka, L. (2010). Assessment of antioxidant activity of eugenol in vitro and in vivo. In Free Radicals and Antioxidant Protocols (pp. 165-180). Humana Press.

Otunola, G. A. (2022). Culinary spices in food and medicine: an overview of *Syzygium aromaticum* (L.) Merr. and LM Perry [Myrtaceae]. Frontiers in Pharmacology, 12, 793200.

Palsamy, P., Sivakumar, S., & Subramanian, S. (2010). Resveratrol attenuates hyperglycemia-mediated oxidative stress, proinflammatory cytokines and protects hepatocytes ultrastructure in streptozotocin–nicotinamide-induced experimental diabetic rats. *Chemico-biological interactions*, *186*(2), 200-210.

Pathak, N., Vimal, S. K., Tandon, I., Agrawal, L., Hongyi, C., & Bhattacharyya, S. (2022). Neurodegenerative disorders of alzheimer, parkinsonism, amyotrophic lateral sclerosis and multiple sclerosis: an early diagnostic approach for precision treatment. *Metabolic Brain Disease*, *37*(1), 67-104.

Qu, X., Lai, X., He, M., Zhang, J., Xiang, B., Liu, C., ... & Qiao, J (1901). Investigation of epilepsy-related genes in a Drosophila model. *Neural Regeneration Research*, 10-4103.

Rabha, A., Sharma, D. K., Baruah, C., & Das, A. N. (2024). Impact of wild yeast added to culture media on Drosophila abundance in and around a banana market, Daranggiri, Assam, India. International Journal of Agriculture and Biosciences, 13(1), 18-29.

Salceda, R. (2024). Light pollution and oxidative stress: Effects on retina and human health. *Antioxidants*, *13*(3), 362.

Sam, C., & Bordoni, B. (2020). Physiology, acetylcholine.

Staats, S., Lüersen, K., Wagner, A. E., & Rimbach, G. (2018). *Drosophila melanogaster* as a versatile model organism in food and nutrition research. Journal of agricultural and food chemistry, 66(15), 3737-3753.

Vang, L. L., Medvedev, A. V., & Adler, J. (2012). Simple ways to measure behavioral responses of Drosophila to stimuli and use of these methods to characterize a novel mutant. PloS one, 7(5), e37495.

Victor Atoki, A., Aja, P. M., Shinkafi, T. S., Ondari, E. N., Adeniyi, A. I., Fasogbon, I. V., ... & Akin-Adewumi, A. (2025). Exploring the versatility of *Drosophila melanogaster* as a model organism in biomedical research: a comprehensive review. Fly, 19(1), 2420453.

Vogt, A. C. S., Arsiwala, T., Mohsen, M., Vogel, M., Manolova, V., & Bachmann, M. F. (2021). On iron metabolism and its regulation. International journal of molecular sciences, 22(9), 4591.

Yang, P., Yang, X., Sun, L., Han, X., Xu, L., Gu, W., & Zhang, M. (2022). Effects of cadmium on oxidative stress and cell apoptosis in *Drosophila melanogaster* larvae. *Scientific Reports*, *12*(1), 4762.

Zeng, Y., Buonfiglio, F., Li, J., Pfeiffer, N., & Gericke, A. (2024). Mechanisms Underlying Vascular Inflammaging: Current Insights and Potential Treatment Approaches. *Aging and Disease*, *16*(4), 1889.

Zhao, Y., Yang, M., & Liang, X. (2024). The role of mitochondria in iron overload-induced damage. Journal of Translational Medicine, 22(1), 1057.

Zheng, F., Gonçalves, F. M., Abiko, Y., Li, H., Kumagai, Y., & Aschner, M. (2020). Redox toxicology of environmental chemicals causing oxidative stress. *Redox Biology, 34*, 101475.

Zhou, S., Morozova, T. V., Hussain, Y. N., Luoma, S. E., McCoy, L., Yamamoto, A., ... & Anholt, R. R. (2016). The genetic basis for variation in sensitivity to lead toxicity in *Drosophila melanogaster*. *Environmental Health Perspectives*, *124*(7), 1062-1070.