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Flavonoid-rich extract of unripe *Terminalia catappa* Modulates Redox Status and Aging in Lead-InducedNeuro-genotoxic *Drosophila*

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Abstract

Terminalia catappa (Indian almond) fruit is an oval fruit in shape, growing on a large tree found mostly in the tropical region. The tree has wide health applications and benefits various African and Asian communities. However, there is little documented evidence of its effects on neuroprotection and longevity. This study explored the neuroprotective potential of the flavonoid content from the unripe fruit part of the plant against lead-induced neurogenotoxicity in a fly model (*Drosophila melanogaster).* Two to three days old male and female flies were harvested and used for the experiment. Negative geotaxis and flies' pupation assays were employed for the assessment of the flies' behavior. Phase II antioxidant enzymes' activities and their expression levels were evaluated to ascertain the redox status of the flies before and after the toxicants exposure. Following the effective dose determination assay, 3.0 and 5.0 mg/g diets were found to be highly effective, meanwhile a concentration of 0.1 µg/g diet of lead (Pb) successfully induced neuro and genotoxicity. There was a remarkable improvement in the emergence of new and the locomotor function of the matured flies fed with the extract before their exposure to the toxic environment compared to the control group. Significant increase in the activity of Catalase, Superoxide dismutase (SOD), and Glutathione-s-transferase (GST) with concurrent reduction of AChE activity in the extract pre-fed flies. A similar effect was also recorded in the mRNA expression level of the corresponding genes when evaluated using a polymerase chain reaction (qPCR). The collective findings of the present study revealed the potential neuro and gene protection effect of the studied extract through antioxidant and antiaging enzyme activities against Lead-induced toxicity in D. melanogaster. Showcasing the potential of the fruit as a source of nutraceuticals that could improve the wellness and quality of human lives when further studied.

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1.0 Introduction

Overexposure to heavy metals leads to neurotoxicity, which involves the degradation of neurons, neurodevelopmental abnormalities, oxidative stress, inflammation of microglial cells, and the occurrence of cell autophagy, apoptosis, or ferroptosis (Yu *et al.*, 2024). DNA methylation, RNA methylation, histone modification, or non-coding RNAs disrupt these processes (Jasim *et al.*, 2024). Heavy metals are frequently used in industrial applications, which poses a significant risk of toxic exposure for workers. While critical metals are crucial for the body's physiological

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functions, their excessive exposure, whether short-term or long-term, can pose significant health hazards (Prasad *et al.*, 2024). Metals pose a significant risk to the central nervous system (Prasad *et al.*, 2024). Metals easily accumulate in the brain and naturally integrate into vital metalloproteins, essential for neuronal health and energy balance maintenance (Fathima *et al.*, 2023). Excessive levels of essential metals or exposure to hazardous nonessential metals may lead to severe repercussions (Jomova *et al*., 2022). Occupational metal exposure continues to be the primary cause of hazardous substances in the human body (Jomova *et al.*, 2022). If the body fails to maintain metal homeostasis, it may become more vulnerable to the harmful effects of these metals, particularly in the central nervous system (Feng *et al.*, 2015). Heavy metals such as aluminum, mercury, arsenic, manganese, and lead significantly influence neurological illnesses, including Alzheimer's disease (Feng *et al.*, 2015). Researchers discovered that heavy metals negatively impact cognitive function, behavior, and the structure of neurons (Ijomone *et al.*, 2020; Mohammed & Yusuf, 2023). Furthermore, there was a correlation between exposure to heavy metals and alterations in the prevalence of certain bacterial phyla, such as Firmicutes and Proteobacteria, which are important for maintaining a healthy gut (Duan *et al.*, 2020).

Plants have been reliable sources of medication for various forms of ailments including disorders where oxidative stress is implicated (Abd Karim *et al.*, 2023; Abdulwanis Mohamed *et al.*, 2020). The application of such plants is widely accepted in numerous traditional settlements, especially among African and Asian populations (Jaafaru *et al.*, 2024). Flavonoid and polyphenol-rich plants, fruits, and vegetables have shown remarkable toxicant mitigating ability against a variety of eco-toxicants including Lead, a heavy metal found abundantly in domestic appliances such as batteries, paints, plastics, and some forms of cooking pots (Dwivedi *et al.*, 2022). *Terminalia catappa* is a large tropical tree belonging to the family *Combretaceae*, commonly found in tropical parts of the world including but not limited to Africa, Asia, and some parts of Europe (Jaafaru *et al.*, 2024). All the parts of the plant (fruits, leaves, bark, roots, and flowers) are used in the treatment of communicable and non-communicable diseases condition conditions including but not limited to asthma, rheumatism, cough, dysentery, diabetes, and some forms of cancer (Anand et al., 2015; Oyeniran *et al.*, 2021). Recently, the fruit fly known as *Drosophila melanogaster* has been engaged in biomedical research to address health burdens worldwide (Moraes & Montagne, 2021). The flies have many advantages that make them stand out among the many other models of health-related research such as the aging process, and brain disorders where oxidative stress is implicated (Mohr, 2018). Thus, the present study tends to evaluate the neuro and geno-protective potential of *T. catappa* unripe fruit extract against lead-induced toxicity and lifespan shortening in the *D. melanogaster* model.

2. 0 Materials and Methods

2.1 Materials

The unripe fruit of *T. catappa* used in the present study was obtained within the Kaduna metropolis and was identified and authenticated by a botanist in the Department of Biological Science, Kaduna State University. A voucher number BCH 0073 was assigned to the identified fruit and documented in the department's herbarium collection unit. Chemicals including 5,5-dithiobis 2-ni-trobenzoic acid (DTNB), acetylthiocholine iodide, and 1-chloro-2, 4-dinitrobenzene (CDNB) were procured from Sigma Company (USA). Lead oxide and other pertinent reagents of analytical grades were outsourced from Inqaba Biotech Company Nigeria.

2.2 Preparation of *T. catappa* unripe fruit pulp extract.

To prepare the *T. catappa* fruit extract, the method reported by (Jaafaru et al., 2024) was employed. The fresh fruits were shed dried after the peel and seeds removal, and the pulp was reduced to fine particles on a mortar and pestle thereafter. A 500g of the resulting powder sample was soaked in 1.5 L warm water for 48 – 72 hours for effective phytochemical extraction. The extract was filtered using size no. 1 Whatman filter paper, and the filtrate was then reduced to dryness on a rotary evaporator facility and a moderate-size water bath, which was then kept at a low temperature for further use.

2.3 Diet Formulation and Flies' Culture

National Species Stock Center in Ohio, United States was the origin of the flies used in the present study, they were generously donated by the University of Ibadan, Medical College. The flies were grown based on 12 hours of lightness and darkness cycle, with 24 ± 2°C temperature and relative humidity of 60 – 70 % conditioning. They were fed a cornmeal diet comprising of 1% agar-agar, sucrose, and brewer's yeast each, with 0.08% preservative (methylparaben) in an appropriate amount of water.

2.4 Flies Grouping and Treatment

The 3-day-old flies of both sexes were divided into 3 different groups, with each group comprised of 3 replicates namely: normal control, placebo

(distilled water only) control, disease (lead only) control, 1st treatment, and 2nd treatment groups, respectively.

2.5 Effective Dose Determination

Effective doses of the extract and periods of exposure of the flies in the present study were determined through the administration of the extract in increasing order. Four culture vessels were used to maintain a total of 200 flies, consisting of both males and females. Each vessel contained 50 flies, and they were fed an extract at concentrations between 0.5 and 10 mg/g of diet for a period of seven days. The LD_{50} of Pb was found to be 0.1 µg/10 g diet which was adopted from (Shilpa et al., 2021) and was used throughout the study.

2.5.1 Flies Survival Assay

The assessment of flies' survival rate in a toxic environment was conducted using the newly hatched male and female flies. The flies were put on a diet containing 3.0 and 5.0 mg/g of flavonoid-rich T. catappa extract (the outcome of the above experiment) for seven days after which they were exposed to another diet integrated with 0.1 µg/g Pb metal for another seven days. The flies were monitored till the 30th day observational period, and the daily mortality was documented. The data collected was analyzed and presented as the percentage of survived flies under noxious conditions.

2.6 Climbing Assay

The climbing ability of flies was evaluated according to their locomotor activities as reported by (Abolaji et al., 2020). Twenty male and female flies from each of the replicate vials were selected, immobilized and placed in vertically labeled graduated tubes marked 15 cm in height and 1.5 cm in diameter. Flies that crossed 6cm in 6 seconds were considered highly active, meanwhile, those that remained below that after the elapsed of set seconds were recorded as impaired. The readings were taken three times at the interval of 60 seconds between each reading and the results were recorded as the number of flies that crossed a threshold of 6 cm/6 seconds.

2.7 Total Protein Estimation

The total protein concentration in both the control and treated groups was quantified by utilizing bovine serum albumin from bovine as a standard sample, adhering to the method previously outlined by (Lundblad & Price, 2004) with minor changes.

2.8 Evaluation of Acetylcholinesterase Activity

The technique reported by (Abolaji et al., 2020) was used to estimate AChE's activity. 500 µL of a 0.1M phosphate buffer with a pH of 7.4 was combined with 25 µL of the sample and 25 µL of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in a new tube. As a result, 10 µL of acetylthiocholine was introduced to the mixture, which was then vigorously shaken. The contents were analyzed spectrophotometrically for three minutes, with continuous monitoring of alterations in absorbance at 412 nm. The activity of AChE was estimated and presented as nanomoles of substrate converted to product per unit time in minutes per amount of protein in milligrams of protein after that.

2.9 Evaluation of Catalase, SOD, and GST Activities

An 8.8 µM H_2O_2 (3%) and 0.1 µM phosphate buffer (pH 7.0) were mixed well in a tube to make a 1 ml reaction mixture. This was done to assess the catalase's activity. After adding 10 mg of protein equivalent to the tube, we observed the reaction at 240 nm for 3 minutes. Furthermore, we used the inhibition approach, as previously outlined in the method (Jaafaru *et al.*, 2024), to calculate the SOD activity. In summary, 0.15% quercetin and 10 µL of the homogenate sample were added to a tube that contained 4 mg of protein, 8M N, N, N, N-tetramethyl ethylenediamine (TEMED), and 0.016 mM sodium phosphate buffer of pH 7.4. After thoroughly shaking the mixture for three minutes, we measured the optical density at 406 nm. We quantified the outcome as the amount of protein, measured in nanomoles per minute per mg of protein, required to impede half of the quercetin auto-oxidation.

2.10 Sample Preparation for Gene Expression Study

Following the completion of the fourteen-day study period, the experimental flies were anesthetized on ice and subsequently homogenized in a 0.1 M phosphate buffer at pH 7.0 after their weight was recorded. mRNA was extracted, and its purity was assessed and documented.

2.11 mRNA Extraction and Quantitative PCR

Upon isolation, each vial of flies generated approximately 3 µg of mRNA. The TRIZOL reagent was used to carry out the isolation process in accordance with the manufacturer's instructions. The NCBI website was consulted to extract the primer sequences, as illustrated in Table 1. Meanwhile, the primers were manufactured and provided by Invitrogen and were designed using Primer 3 version 0.4. The isolated mRNA was quantified on NanoDrop

2000, and the OD260/OD280 ratio was determined to be between 1.9 and 2.0, indicating a high level of purity. A 20 µL mixture was prepared and utilized for the quantification process in real-time PCR. This mixture contained 1 µL of RT product (cDNA) template, 1x PCR buffer, 1 U Taq DNA polymerase, 2.0 mM MgCl2, and 0.1-fold SYBR Green probes. The thermal cycling was conducted using the standard procedure of 5 minutes of activation at 95 °C, followed by 15 minutes of 40 cycles at 60 °C, 95 °C, and 25 minutes at 72 °C, conducted on an Applied Biosystems StepOne Plus real-time PCR machine. The thresholds and baselines of the reactions were evaluated using StepOne software version 2.0. The threshold cycle (CT) values for the samples were estimated as 2-CT and recorded accordingly. The system was used to run each sample three times, and the average was calculated. The estimates of changes in the cycle threshold (ΔCT) were obtained by subtracting the CT value of GAPDH CT from that of the genes of interest. As previously mentioned in (Valenti *et al.,* 2006), the mRNA levels of the genes of interest were normalised using GAPDH. Furthermore, the sample quantities were normalised using customised primers for GAPDH, which remained unaltered in the presence of Pb or extracts high in flavonoids.

Table 1: Gene name, accession number, and sequences of the gene of interest and the housekeeping,used in the study.

SN	Gene	Accession No.	Direction	Primer Sequence
1	*Mn-SOD*	NM_057577.3	Forward	CACATCAACCACACCATCTTC
			Reverse	CGTCTTCCACTGCGACTC
2	*Catalase*	NM_080483.3	Forward	TGAACTTCCTGGATGAGATGTC
			Reverse	TCTTGGCGGCACAATACTG
3	*AChE*	NM_001275601.1	Forward	TGAAGACCAATCCCGCTCAC
			Reverse	GGCCACATGGGTTATGTGCT
4	*GAPDH*	JF_915526	Forward	GTCTGATGACAACAGTGCAT
			Reverse	GTCCATCACGCCACAACT TTC

2.12 Statistical Analysis

The data acquired in this study were analysed using GraphPad Prism version 9, utilizing one-way analysis of variance (ANOVA) with post hoc Tukey's test. The results were illustrated graphically as mean ± standard deviation (SD). All experiments were conducted in triplicate, with $p < 0.05$ deemed statistically significant in both analyses.

3.0 Results and Discussion

3.1. Flavonoid-rich Extract of *T. catappa* Improves Flies' Locomotor Function and Survival

After the experiment, it was found that the flies that were given two concentrations of flavonoid-rich extract (3.0 and 5.0 mg/g diet) before being exposed to Pb (0.1 µg/g diet) had significantly better locomotor function (Figure 3.1a). We observed this increase when comparing the extract-pre-fed groups to the control groups (normal and diseased). Similarly, our results show that flies that were given the flavonoid-rich extract before the exposure to Pb had a much higher survival rate ($p < 0.05$) than flies that were not provided with the extract before the exposure to Pb (Figure 3.1b).

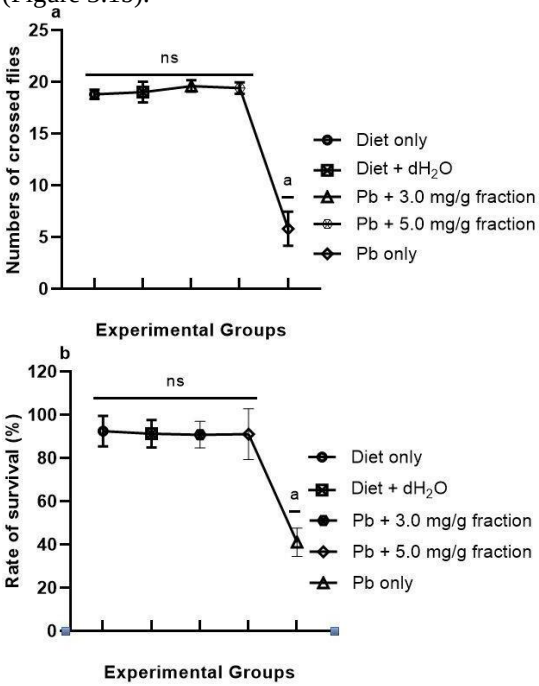
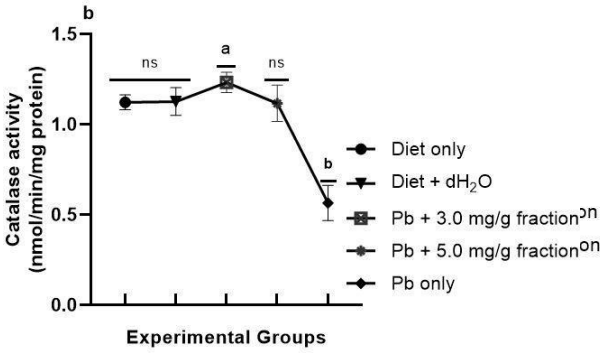


Figure 3.1: Illustration of the influence of a flavonoid-rich extract on the survival rate (b) and locomotive activity (a) of neurotoxic and normal flies.

The data were reported as mean ± SD standard deviation, and the experiments were conducted in triplicate. Statistical significance was defined as $p < 0.05$.

3.2. Flavonoid-rich Extract Regulates Antioxidant and Aging Markers in *D. melanogaster*

The extract influenced particular indicators of Pb-induced oxidative stress, as indicated in Figure 3.2. Acetylcholinesterase activity was significantly lower ($p < 0.05$) in the flies fed the extract for seven days before exposure to lead, as shown in Figure 3a. This effect differed from both the untreated group and the disease control group. Moreover, the extract impeded the inhibitory effect of Pb on the activity of a particular subset of antioxidant enzymes. It was found that the flies that were fed the extract had significantly higher levels of catalase (Figure 3.2b) and superoxide dismutase (SOD) (Figure 3c) activities compared to the normal control group ($p < 0.05$). Conversely, the enzymatic activities were significantly diminished ($p < 0.05$) in the Pb-only fed group relative to the normal control group. Therefore, it was evident that the flavonoid-rich extract from *T. catappa* could shield *D. melanogaster* neurons from lead-induced damage.



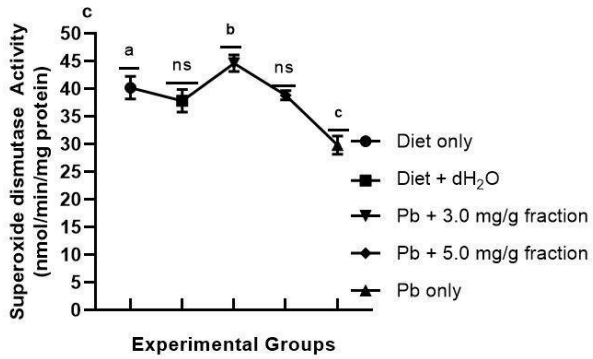
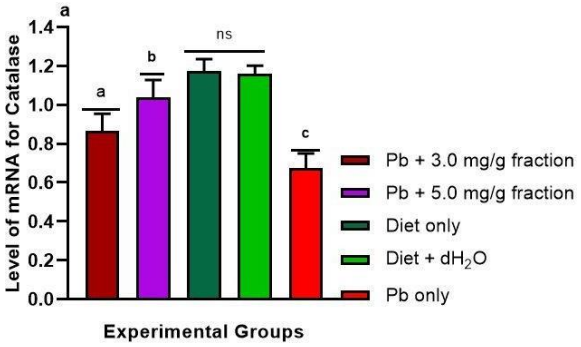


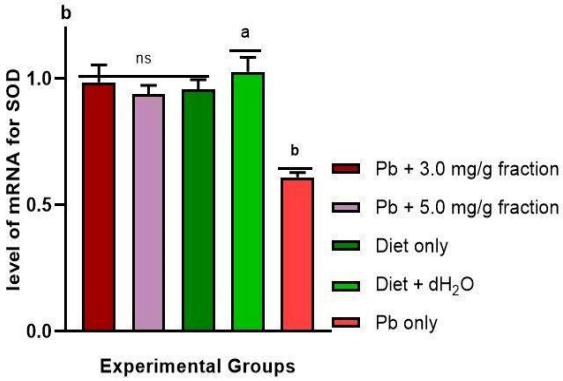
Figure 3.2 illustrates the impact of a flavonoid-rich extract on the activity of aging and antioxidant enzymes, specifically (a) acetylcholinesterase (AChE), (b) catalase, and (c) superoxide dismutase, in both lead-induced toxic and normal fly models.

The experiments were conducted in triplicate, and the results were presented as mean ± SD, with statistical significance established at $p < 0.05$.

3.3. *T. catappa* Based-Flavonoids Regulate the Expression of Phase II Antioxidant and Age-Related Enzymes

The impact of *T. catappa*-derived flavonoid-rich extract on the regulation of *catalase, AChE, and SOD* genes in the neurotoxic fly model is evaluated and depicted in Figure 4. The results suggest that the catalase gene (Fig. 3.4a) and SOD gene (Fig. 3.x4b) were significantly upregulated ($p < 0.05$) by pre-feeding with the extract at two concentrations (3.0 and 5.0 µg/g diet) in a Pb contaminated environment. Additionally, the expression of the AChE gene was significantly and remarkably downregulated under both concentrations in comparison to the disease group.





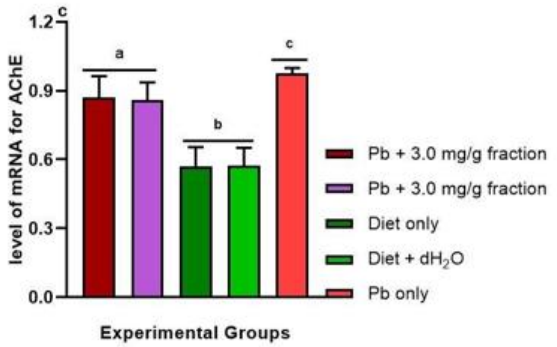


Figure 3.4: Showing an illustration of neuroprotective effects of a flavonoid-rich fraction via the regulation of catalase (a), Mn-SOD (b), and AChE (c) genes in both Pb-contaminated and clean environments.
The outcomes were presented as the mean ± standard deviation, with statistical significance defined as $p < 0.05$, and the experiments were conducted in triplicate.

4.0 Discussion

The primary cause of aging is still under investigation, even though aging is considered one of the most intricate biological processes, as it involves changes in a variety of biochemical molecules and pathways (Rudzińska *et al.,* 2020). The clear links between oxidative damage inducement and environmental contaminants, such as heavy metals and other noxious chemicals, were revealed by an increasing number of studies (Mishra *et al.,* 2019; Mitra *et al.,* 2022). The experimental flies of both sexes were successfully induced with neurotoxicity, as evidenced by the Pb-only group, which served as the disease control group. In rodents (Vellingiri *et al.*, 2022), flies (Shilpa *et al.*, 2021), and other biomedical research models (Fasae & Abolaji, 2022), studies have shown that heavy metals induce neuro or genotoxicity, which is associated with neurodegeneration. Plant-derived bioactive compounds, particularly flavonoids, phenolics, and glycosides, have the potential to improve the synthesis of antioxidant enzymes, thereby increasing the activity of the corresponding enzymes (Jaafaru *et al.,* 2018; Sarkar *et al.,* 2020). Flavonoids have also shown a beneficial effect on the synthesis and activities of a diverse array of enzymes, which are involved in a variety of metabolic pathways that promote longevity. Even in a noxious environment, the current research demonstrates that flavonoid pretreatment increases the flies' lifespan. As a result, the modulators increased the phase II antioxidant enzyme while simultaneously inhibiting age-related enzyme activities, which directly influence the longevity of the flies. Researchers have demonstrated that flavonoids and phenolics, derived from a diverse array of fruits, vegetables, and spices, enhance the quality of life, neuromuscular strength, and longevity of flies (Iorjiim *et al.,* 2020; Jaafaru, 2024). However, the precise mechanisms by which the enzymes exert their effects are not thoroughly examined. Additionally, the current results on gene expression are in line with a recent study that looked at how curcumin-derived flavonoids affect the expression level of SOD and catalase genes (Hu *et al.,* 2023), which in turn affects their overall functions. Administration of a flavonoid-rich extract to the fly before exposure to a harmful environment inhibited the expression of the AChE gene. This reflects previous reports, indicating that phenolic compounds, including curcumin and glycosides, can decrease AChE levels in both laboratory and biological contexts (Rahman *et al.,* 2021). This study employed the use of GAPDH for normalization. GAPDH also known as glyceraldehyde-3-phosphate dehydrogenase, is used as a housekeeping gene due to its stable expression, essential cellular function, unrelated to experimental variables, and widely validated in most cell types and under various physiological conditions, making it a reference gene for comparison in expression. The observed variations in our results can be ascribed to genomic heterogeneity and additional variables, such as environmental and nutritional factors, within the fruit fly population, which may influence gene regulation. Figure 4.1 illustrates the proposed protective and lifespan-related mechanisms of *T. catappa* flavonoid-rich extracts in flies, derived from the integrated results of the current study.

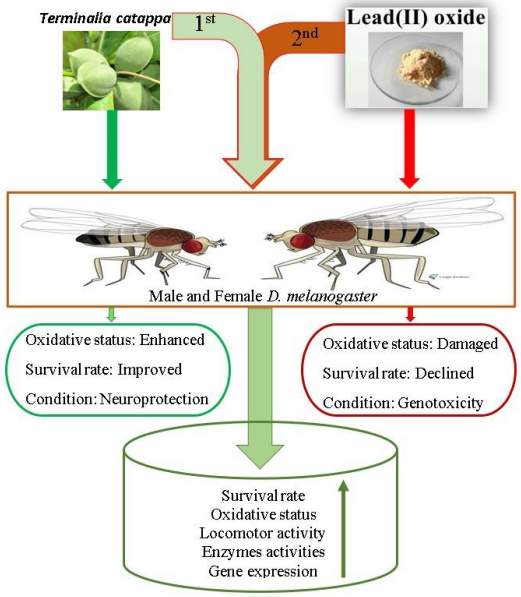


Figure 4.1: The proposed neuroprotective and longevity effects of a flavonoid-rich extract from *T. catappa* in *D. Melanogaster.*

5.0 Conclusion

Fruit flies exposed to lead oxide showed significant changes in neuromuscular activity and longevity after receiving the flavonoid-rich extract from T. catappa. It was found that the extract decreased the activity and expression of AChE while increasing the activity and regulation of catalase and superoxide dismutase. The study confirmed, to a greater extent, that flavonoids derived from T. catappa enhance redux status in fruit flies by mitigating the effects of oxidative stress induced by environmental pollutants, including lead, a heavy metal that accumulates in the body and adversely affects the nervous system.

Declarations

Ethics approval and consent to participate

Not Applicable.

Consent for publication

All authors have read and consented to the submission of the manuscript.

Availability of data and material

Not Applicable.

Competing interests

All authors declare no competing interests.

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