Eurasian J. Sci. Eng., 2024, 10(2), 34-45
https://doi.org/10.23918/eajse.v10i2p03

Published by Tishk International University
Available at: https://eajse.tiu.edu.iq/submit/

RESEARCH ARTICLE Open Access

Flavonoids from *Aframomum melegueta* (Black Pepper) Improve Antioxidant Status and Modulate Aging Process in Lead-Induced Neurotoxic *drosophila melanogaster*

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Article History
Received: 29.12.2023
Revised: 13.03.2024
Accepted: 04.04.2024
Published: 02.05.2024
Communicated by: Dr. Orhan Tug
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Abstract:
There are insufficient shreds of evidence to support the longevity benefits of *Aframomum melegueta*-derived flavonoids. The present study focused on the investigation of the activity of flavonoid-rich extract in the neurotoxic *Drosophila melanogaster* model, where behavioral, biochemical, and molecular procedures were conducted to evaluate the activities and expression of phase II antioxidants and age-related enzymes. The extract was administered to fruit flies at doses of 3 and 5 mg/g over a period of seven days with subsequent exposure to 0.1 µg/g diet lead oxide for an equivalent number of days. The flies were homogenized for bioassays. The expression and activities of the phase II antioxidant and age-related enzymes were determined using quantitative polymerase chain reaction (qPCR) to assess the oxidative and longevity effects of the extract. Enhancement in locomotor function and flies' emergence were observed as the outcomes of behavioral assay. The extract also hindered the detrimental effects of Pb by increasing the activities and gene expression levels of catalase and superoxide dismutase with concurrent inhibition of AChE activity and expression in the toxic environment. These findings shed light on the mechanism through which the extract inhibits lead-induced toxicity in *D. Melanogaster*, which may have potential implications for human subjects.

Keywords: *Aframomum melegueta; Black Pepper; Drosophila melanogaster; Environmental Toxicant; Lead; Longevity; Neurotoxicity.*

1. Introduction

Prolonged subjection to heavy metals, including lead (Pb), damages numerous crucial organs and organ systems, most notably the central and peripheral nervous system [1]. The accumulation of Pb in the body may have fatal consequences for neurological functions, including learning disability, behavioural disorders, and irreversible injury to cognitive function [1]. Children are particularly susceptible to Pb toxicity as a result of the swift assimilation of the Pb into their developing brains. Pb exposure among children continues to be an issue in the home, workplace, and numerous places of worship. Pb inhibits presynaptic calcium channels and modifies the composition and function of N-methyl-D-aspartate (NMDA) receptors in the hippocampus [2]. Over the years, the most important preventive measure against cumulative Pb toxicity has been the avoidance of exposure to Pb [3]. Human exposure to Pb can arise through various means, all of which entail environmental contamination. Sources such as soil, food, lead dust, and contact with Pb in common items and occupational settings primarily introduce Pb into the body through ingestion or inhalation [4]. In the workplace, Pb and its compounds majorly enter the body through inhalation, although ingestion is also a possible route of absorption [5, 6]. In recent years, fruit flies also known as *Drosophila melanogaster* became an important model organism for age and nervous system-related diseases [4]. The aging process is believed to have evolved as a result of multiple processes that entail changes at the molecular and cellular levels of an organism which disrupt the central nervous system's equilibrium with a consequent increase in cellular senescence [7]. Metals accumulate in the brain, which makes it more vulnerable to neurotoxic insults [8]. This happens in many ways, such as by making mitochondrial dysfunction worse and throwing off the balance of calcium ions in neurons. Others include the

accumulation of damaged molecules, delayed DNA repair, reduced neurogenesis, and compromised energy metabolism [9]. These distinctive features have been determined to be among the causes of neuronal damage, leading to various neurological diseases [10]. A considerable body of research has identified robust associations between metal accumulation, aberrant protein expression, and the onset of neuronal disorders such as Parkinson's, Huntington's, and Alzheimer's disease [11].

Neuronal degeneration is a condition of the nervous system associated with a decline in cognitive function and memory. Mammals and other model organisms naturally experience general bodily weakness as they age, as a result of a decline in functions by some vital organs [12]. Furthermore, cognitive decline that occurs with advancing age has been associated with a deficiency in central cholinergic transmission and an ineffective antioxidant system [4,13]. Studies have shown that blocking acetylcholinesterase (AChE) and increasing the amount of antioxidants in the body might be effective ways to treat and prevent dementia and cognitive decline that come with getting older [14]. AChE degrades acetylcholine, a neurotransmitter present in synaptic clefts throughout the CNS. Inhibition of AChE activity improves cholinergic neurotransmission within the system, as the decomposition of ACh into choline and acetate within the central nervous system decreases remarkably [15, 16]. It is impossible to overstate the significance of diet in shaping the aging process and cognitive development. Flavonoids and phenolics found in food have been linked to controlling signal transduction, keeping antioxidant regulation, and the expression of proteins and genes implicated in disease development [17]. Flavonoids derived from spices, such as Aframomum melegueta, purport to provide a multitude of health advantages, although the available scientific evidence is inadequate. Plants containing these molecules protect themselves against predators and viruses through their astringency. Flavonoids exhibit robust biological characteristics, such as antimicrobial, anti-inflammatory, and antioxidant activities [18]. Despite exhibiting pharmacological activity, the precise mechanism by which it promotes longevity extension and deceleration of the aging process remains unclear. This study was planned to investigate the mechanism through which flavonoids derived from *A. melegueta* mitigate Pb-induced neurotoxicity and aging in *D. melanogaster* model.

2. Methodology

2.1 Statement of Ethics

As a model organism, *Drosophila melanogaster* is used without restriction in fundamental scientific research. Since the flies serve as an invertebrate model system, their use does not necessitate ethical sanction by the laws governing the protection of animals.

2.2 Extract and Bioassay-Guided Chromatographic Fractionation

The dried and finely powdered sample of *Aframomum melegueta* was extracted in absolute ethanol and the rotary evaporated filtrate was completely dried in a water bath and stored under low temperature for further use. An ethanol diluted 20g of the dried extract was subjected to silica column chromatography with gradient elution of ethanol: water (1:0), ethanol: water (0.7/0.3), ethanol: water (0.5/0.5), ethanol: water (0.3/0.7) and water (1) ratio. The separation progress was monitored by thin-layer chromatography after collecting the fractions at unified time intervals. The p-Anisaldehyde reagent was used to visualize the compounds, under a UV lamp at 254 nm and 360 nm. The TLC output pattern was used to guide the combination of column fractions and the fractions were concentrated and dried using a rotary evaporator and water bath, respectively. Total phenolic contents and the *in vivo* modulatory action of the fractions on acetylcholinesterase (AchE), SOD, and glutathione-S-transferase were determined, where the fractions eluted from ethanol: water (1:0) had the highest total flavonoids contents, proved to have higher inhibitory activity against AchE and improved the activities of SOD and GST. The ethanol/ water (1:0) fraction was mass-produced, concentrated, dried, and used for the subsequent *in vivo* bioassay experiments.

2.3 Formulation of Diet And Culturing of Drosophila Melanogaster

D. melanogaster (Harwich strain), which was acquired from the Department of Clinical Biochemistry, Medical College of the University of Ibadan was cultivated in the *Drosophila* Research Laboratory, located in Kaduna State University, Department of Biochemistry. The experimental conditions were controlled at the specified standard pressure, temperature, and humidity (60–70%), under a light/dark cycle of 12 hours. The diet consisted of cornmeal containing yeast, methylparaben, and agar-agar in measurable proportions, as documented by [19].

2.4 Experimental Grouping And Treatment

We divided the 3-day-old male and female *D. melanogaster* into 5 distinct groups, each consisting of three biological replicates: normal control, vehicle (distilled water) control, negative (lead) control, treatment-1, and treatment-2 groups, respectively.

2.5 Dose Establishment

To determine the optimal concentrations (doses) of the extract and duration of exposure for the experiment, a flavonoid-rich extract-containing diet was administered to the experimental flies. A total of 200 flies of both sexes were maintained in four culture vessels, each containing 50 flies, and fed with the extract at a concentration ranging from 0.5 to 10 mg/g of diet for seven (7) days. Likewise, 0.1 µg/10g diet concentration was designated as the LD_{50} of Pb, which was adopted from [20] and was utilized for the duration of the study.

2.6 Flies' Survival Analysis

An experiment was carried out in three replicates to assess the survival rate of the flies in a noxious environment. Flies of both sexes were pre-fed with extracts containing diet at concentrations of 3.0 and 5.0 mg/g (most effective concentrations based on the findings from section 2.5) for seven (7) days and then exposed to a diet containing 0.1 µg/g of Pb for an additional 7 days, and the daily mortality of the flies was monitored and recorded. The data were collected, analyzed, and depicted as the percentage of survived flies after a period of 30 days of observation. Subsequently, essays on molecular, biochemical, and behavioral parameters were performed on the flies immediately after 7 days of extract feeding and pb exposure to determine the efficacy and durability of the extract containing flavonoids derived from *A. melegueta* under the harmful condition.

2.7 Climbing (Geotaxis) Assay

A flies' climbing assay was performed to evaluate the locomotor activity of the flies as outlined by [19]. A total of twenty (20) flies from each vial were rendered immobile and individually placed in vertically labeled tubes measuring 15 cm in length and 1.5 cm in diameter. The tally was taken for the flies that surpassed the 6 cm threshold on the column within 6 seconds, as well as those that stayed beneath the mark once the time had passed. Three readings were recorded for each vial at a 60-second gap, and the mean was reported as the count of the flies with high locomotor function.

2.8 Evaluation of Protein Concentration In The Fly Model

We determined the entire concentration of the in both the control and treated groups by using bovine serum albumin (BSA) as a standard, following the method previously described by [21] with minimal modifications.

2.9 Estimation of the Activity of Acetylcholinesterase (AChE)

The activity of AchE was estimated based on the reported methodology [22]. A fresh tube was used to combine 25 µL of the sample and 25 µL of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) with 500 µL of a 0.1M phosphate buffer of pH 7.4. Consequently, 10 µL of acetylthiocholine was added, and the mixture was shaken thoroughly. The contents were analyzed for three minutes spectrophotometrically

with spontaneous monitoring of changes in absorbance at 412 nm. The activity of AChE was quantified and expressed as nanomoles of substrate catalyzed per minute per milligram of protein thereafter.

2.10 Assessment of SOD and Catalase Activities

To measure the catalase activity, 0.1 µM phosphate buffer (pH 7.0) and 8.8 µM H_2O_2 (3%) were thoroughly mixed in a tube to make a 1 ml reaction mixture. The reaction began following the addition of 10 mg protein equivalent to the tube and was observed at 240 nm for 3 minutes. Additionally, the SOD activity was estimated using the inhibition approach, as described previously [23]. Briefly, a tube containing 4 mg protein, 8M N,N,N,N-tetramethyl ethylenediamine (TEMED), and 0.016 mM sodium phosphate buffer of pH 7.4 was supplemented with 0.15% quercetin and 10 µL homogenate sample. The mixture was shaken thoroughly and the optical density was taken at 406 nm for three minutes. The result was recorded as the amount of protein in nanomole per minute per mg protein required to inhibit half of the quercetin auto-oxidation.

2.11 Preparation of Sample For Gene Regulation

After the elapsed of the fourteen-day study duration, the experimental flies underwent anesthesia on ice and homogenized in a 0.1 M phosphate buffer of pH 7.0 after recording their weight. mRNA was extracted, and the purity was tested and recorded.

2.12 mRNA Isolation, Purification, and rtPCR Quantitative Analysis

Each vial of flies produced approximately 3 µg of mRNA upon isolation. The isolation process was conducted using TRIZOL reagent according to the guidelines provided by the manufacturer. The sequences of the primers used were extracted from the NCBI website as displayed in Table 1. The primers were designed on version 0.4 of Primer 3 and manufactured by Invitrogen. The isolated mRNA was quantified using NanoDrop 2000; the OD260/OD280 ratio was found to be between 1.9 and 2.0, signifying high purity. A mixture of 20 µL, including 1 µL of RT product (cDNA) template, 1 fold PCR buffer, 1 U Taq DNA polymerase, 2.0 mM MgCl2, and 0.1 fold SYBR Green probes, was prepared and used for the quantification process on real-time PCR. Applied Biosystems StepOne Plus real-time PCR machine was used for the thermal cycling according to 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. Version 2.0 of StepOne software was employed for the assessment of thresholds and baselines of the reactions. The values of the threshold cycle (CT) for the samples were estimated as 2-CT and recorded accordingly. Each sample was run three times on the system and the average was calculated, and the estimation of changes in the value of cycle threshold (ΔCT) was done via subtraction of CT value of GAPDH CT from that of genes of interest. The mRNA levels of the genes of interest were normalized using that of GAPDH, as highlighted previously [24]. Additionally, customized primers for GAPDH were used to normalize the sample quantities, which remained unchanged when exposed to Pb or extracts rich 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

2.13 Statistical Analysis

The data obtained in the present study were analyzed on GraphPad Prism version 9, using one-way Analysis of variance (ANOVA), employing post hoc Tukey's option. The findings were depicted graphically as mean ± SD. Each experiment was performed in triplicate, and $p < 0.05$ was considered statistically significant in both analyses.

3. Results

3.1 Flavonoids, Phenolics, And Ascorbic Acid Content of A. Melegueta Ethanolic Extract.

Figure 1 revealed the total flavonoids, total phenolics, and ascorbic acid content found in various fractions of *Aframomum melegueta* Seeds in different solvent combinations. Total flavonoid content expressed in mg GAE/ g of the fraction appeared to be significantly ($p<0.05$) the highest in the fraction of 100% ethanol when a comparison was made with the other fractions of different solvent combinations. Similarly, the same fraction possessed the highest content of total phenolics when compared with the content of other fractions of different solvent ratios. There wasn't a significant ($p>0.05$) difference in ascorbic acid content across the fractions of varying solvent ratios. This indicated that 100% ethanolic fraction is richer in the flavonoids and, as such regarded as the working fraction in the present studies.

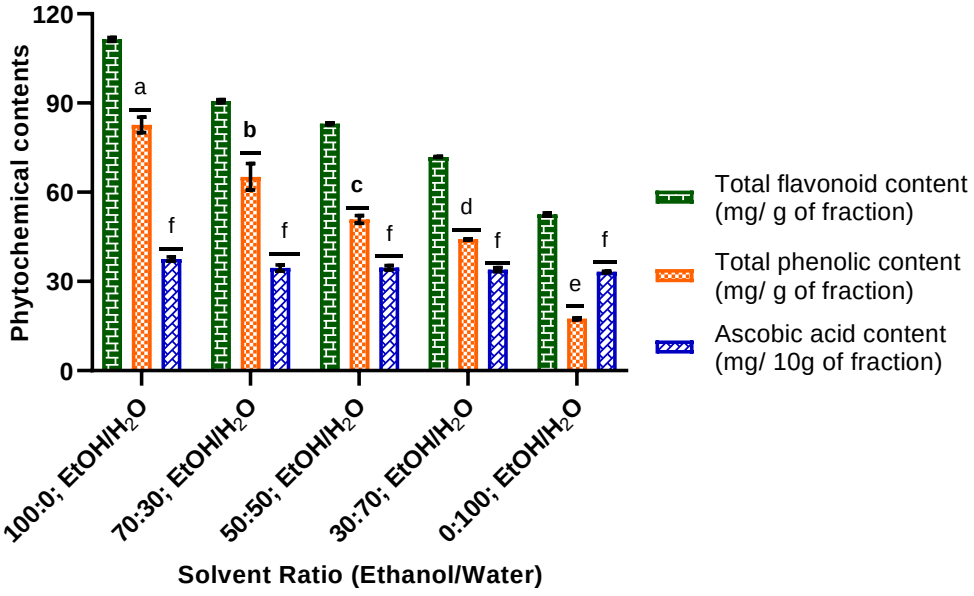

Figure 1: Phytochemical contents of various fractions of *A. digitata* seeds using different ratios of ethanol and water solvent combinations. The experiment was conducted in triplicate and $p<0.05$ was considered statistically significant.

3.2 Flavonoid-Rich Extract of A. Melegueta Enhances Flies' Locomotor Function And Survival

Upon completion of the experiment, a significant ($p < 0.05$) rise in the locomotor function (Figure 2a) was observed in the flies that received the two concentrations of flavonoid-rich extract (3.0 and 5.0 mg/g diet) before being exposed to Pb (0.1 µg/g diet). The increase was noticed when a comparison was done between the extracted pre-fed groups and the control groups (normal and disease). In the same way, our results show that flies that had been fed the flavonoid-rich extract before being exposed to Pb had a much higher survival rate ($p < 0.05$) than flies that had not been fed the extract before being exposed to Pb (Figure 2b).

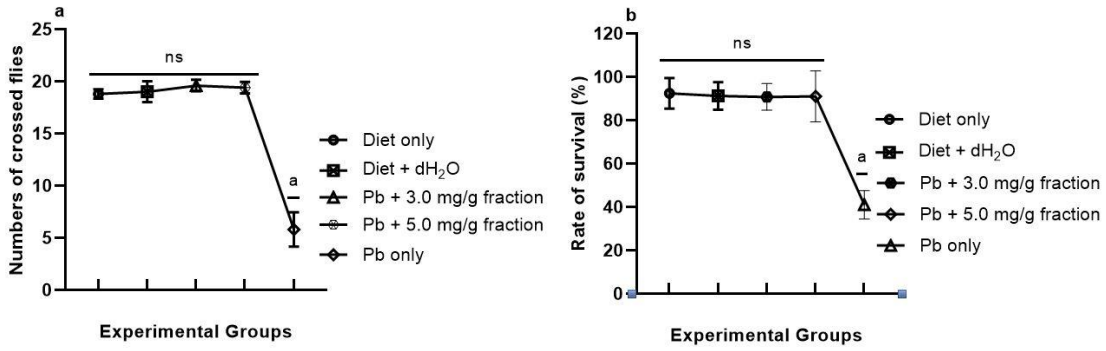

Figure 2: Impact of flavonoid-rich extract on locomotive activity (a) and survival rate (b) in normal and neurotoxic flies. The experiments were conducted in triplicates, and data were reported as mean ± SD standard deviation, with statistical significance defined as $p < 0.05$.

3.3 A. Melegueta-Derived Flavonoids Improve Antioxidant And Aging Activities In D. Melanogaster

The extract had an impact on specific markers of Pb-induced oxidative stress, as shown in Figure 3. When the experimental flies were pre-fed with the extract for seven days before being exposed to lead, the activity of acetylcholinesterase was reduced significantly ($p < 0.05$), as seen in Figure 3a. This effect was different from both the normal (untreated) group and the disease control group. Furthermore, the extract hindered the inhibitory action of Pb on the activity of a specific subset of antioxidant enzymes. The results also indicated that the extract-pre-fed flies exhibited a significant ($p < 0.05$) increase in the activity of catalase (Figure 3b) and that of SOD (Figure 3c) compared to the normal control group. On the contrary, the enzymatic activities reduced significantly ($p < 0.05$) in the Pb-only fed group compared to the normal control group. Thus, it was evident that the flavonoid-rich extract from *A. melegueta* could prevent Pb-induced neurotoxic damage in *D. melanogaster*.

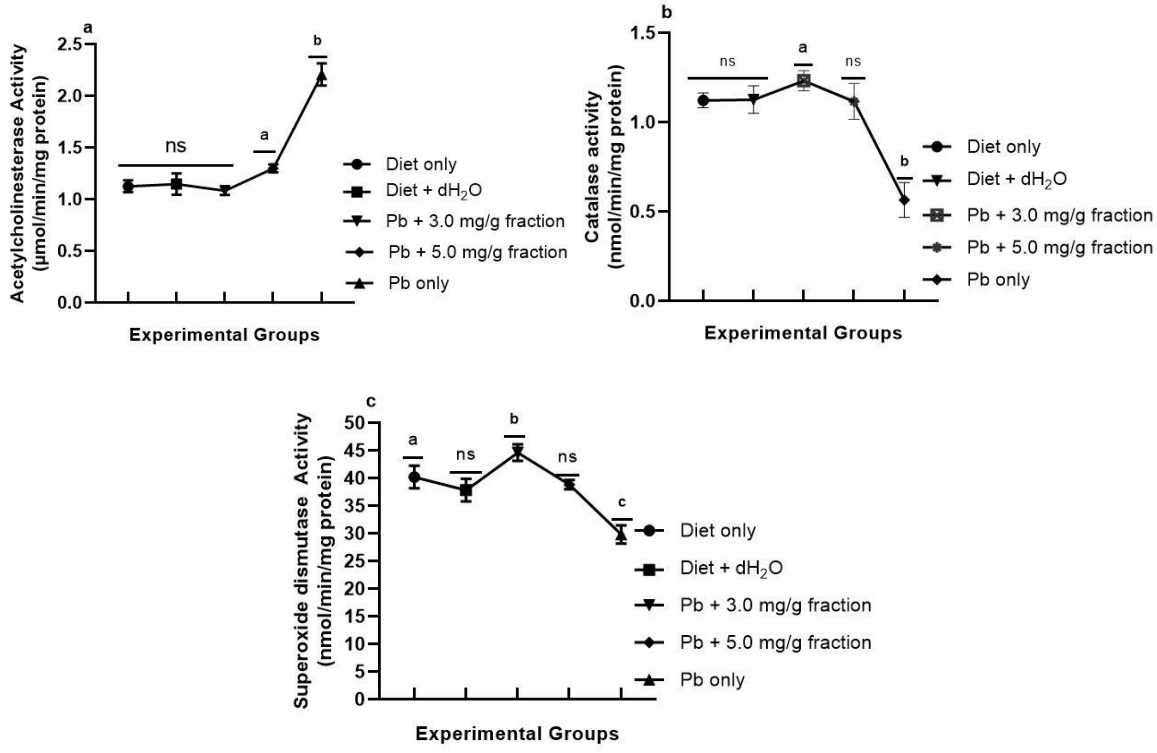

Figure 3: The impact of flavonoid-rich extract on the activity of aging and antioxidant enzymes including (a) acetylcholinesterase (AChE), (b) catalase, and (c) superoxide dismutase in Pb-induced

toxic and normal fly model. The experiments were performed in triplicate, and the results were depicted as mean ± SD, with statistical significance defined as $p < 0.05$.

3.4 A. melegueta Derived-Flavonoids Influence Gene Regulation of Phase II Antioxidant And Age-Related Enzymes

Figure 4 presents the assessment of the impact of *A. Melegueta*-derived flavonoid-rich aqueous extract on the regulation of catalase, *AchE*, and *SOD* genes in the neurotoxic fly model. The findings indicate that pre-feeding with the extract resulted in a significant ($p < 0.05$) upregulation of the catalase gene (Fig. 4a) and *SOD* gene (Fig. 4b) at both concentrations (3.0 and 5.0 µg/g diet) in the presence of the environmental toxicant called Pb. Furthermore, there was also significant a remarkable downregulation in the expression of *AChE* gene at the two concentrations compared to the disease group.

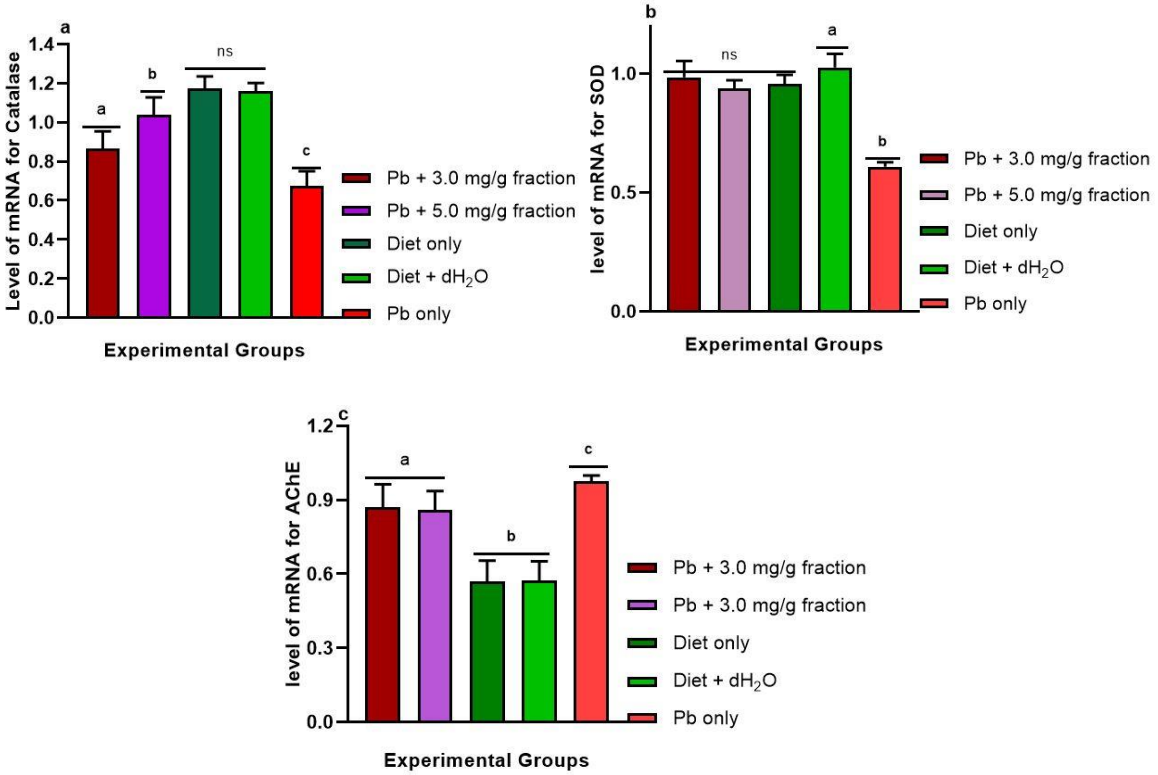

Figure 4: The impact of A. melegueta-derived flavonoid-rich extract on the regulation of catalase (a), Mn-SOD (b), and AChE (c) genes in Pb-influenced neurotoxic and normal flies. The experiments were performed in triplicate, and the results were depicted as mean ± SD, with statistical significance defined as $p < 0.05$.

4. Discussion

Aging is considered one of the most complex biological processes as it involves changes in various biochemical molecules and pathways, yet the main cause of aging is still under investigation [25]. A growing number of studies revealed the clear links between oxidative damage inducement and environmental impurities including heavy metals and other noxious chemicals [12, 26]. The neurotoxicity was successfully induced in the experimental flies of both sexes as seen in all the disease control groups named Pb only group. Studies have demonstrated the role of heavy metals in the induction of neuro or genotoxicity, which has been associated with neurodegeneration in rodents [27], flies [28], and other models of biomedical research [29]. Plant-derived bioactive compounds especially flavonoids, phenolics, and glycosides hold promises of enhancing antioxidant enzymes' synthesis with a resulting increase in the activity of the corresponding enzymes [30, 31]. Flavonoids

also showed positive impact on the synthesis and activities of numerous enzymes involved in a variety of metabolic pathways of longevity [32, 33]. The present studies demonstrated enhancement in the lifespan of the flies due to flavonoid pretreatment even in a noxious environment. As such, the modulators enhanced the phase II antioxidant enzyme with concurrent inhibition of age-related enzyme activities which directly modulate the flies' longevity. Flavonoids and phenolics derived from a variety of fruits, vegetables, and spices have been shown to improve the longevity, neuromuscular strength, and quality of life of the flies [34, 35]. However, the specific processes through which they exert their effects have not been thoroughly investigated [12, 36]. Additionally, The current findings on gene expression align with a recent study [37], which demonstrated that flavonoids and phenolics derived from the curcumin plant can regulate the expression of superoxide dismutase and catalase genes, thereby affecting their respective enzymatic activity. Interestingly, when a flavonoid-rich extract was administered to the flies before exposing them to a harmful environment, it hindered the expression of the *AChE* gene. This is a reflection of what was reported previously [38], which revealed that phenolic compounds such as curcumin and glycosides could reduce the amount of AChE in both laboratory settings and living organisms [39]. The slight differences in our results may be attributed to the genome heterogeneity and some other variables, including environmental and nutritional factors [40, 41], present in the population of fruit flies, which could affect gene regulation. Figure 4 below reveals the suggested protective and lifespan mechanistic impact of *A. melegueta* in the flies, based on the combined findings of the present study.

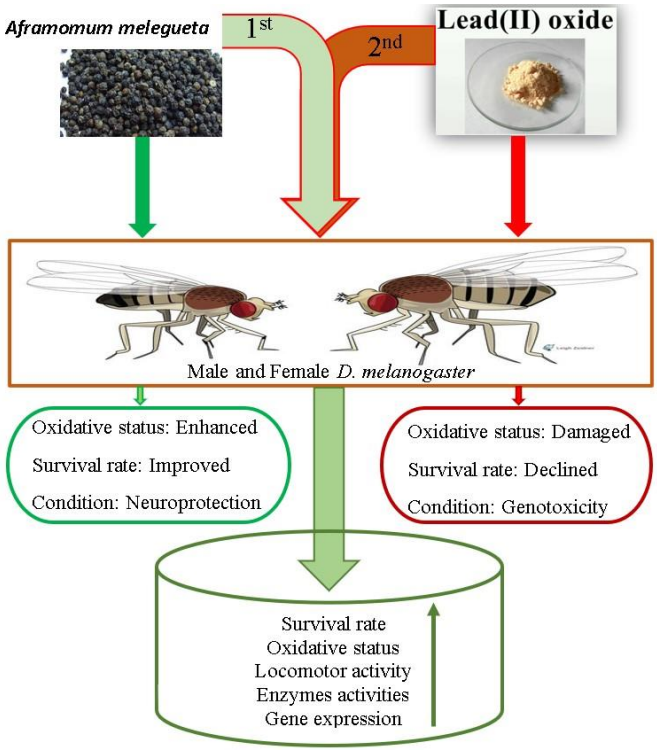

Figure 5: The suggested mechanism of neuronal protection and longevity effects of *A. melegueta* derived flavonoid-rich extract in *D. melanogaster*.

5. Conclusion

A. melegueta-derived flavonoid-rich extract demonstrated a remarkable impact on the activity of neuromuscular and longevity of the fruit flies exposed to lead oxide. The extract also decreased the activity and expression of *AChE*, with a concurrent rise in the activity and regulation of catalase and superoxide dismutase in the present study. The study, therefore, verified at a certain level the promotion of redux status in fruit flies by the *A. melegueta*-derived flavonoids, by deterring the impact

of oxidative stress damage brought about by eco-pollutants, including Pb, a heavy metal that affects the nervous system when it builds up in the body.

6. Author's Contribution

The author conceived the idea, conducted the research, and drafted the manuscript for publication.

7. Conflict of interest

The is no conflict of interest associated with this paper.

8. Acknowledgment

I gratefully appreciate the support of all laboratory assistants in the *Drosophila* research laboratory and the chief technologist of the Department of Biochemistry, Kaduna State University for their immense support in conducting the experiments. I also recognized the support of the entire medical analysis team, the faculty of applied science, and the TIU at large for providing an enabling platform and unwavering support to the success of this paper.

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