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Proanthocyanidins-Rich Fraction of *Tamarindus Indica* Maintained Redox Status of Environmental Toxicant-Induced Genotoxicity in *Drosophila Melanogaster*

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Proanthocyanidins-Rich Fraction of *Tamarindus Indica* Maintained Redox Status of Environmental Toxicant-Induced Genotoxicity in *Drosophila Melanogaster*

Jaafaru Sani Mohammed[1]* and Jibril Habib Yusuf[2]
[1*]Medical Analysis Department, Tishk International University-Erbil, Kurdistan Region, Iraq.
[2]Medical Analysis Department, Tishk International University-Erbil, Kurdistan Region, Iraq.
[1]* jaafaru.sani@tiu.edu.iq

Abstract: Proanthocyanidin, a bioactive polyphenolic component of tamarin (*Tamarindus indica* L.), offers neuroprotective benefits with insufficient scientific evidence. This study evaluated the behavioral and molecular effect of proanthocyanidin-rich fraction on the gene expression level of antioxidant and aging enzymes in *Drosophila melanogaster*. The fruit flies were fed with the fraction at 1.5 and 2.5 mg/g diet for seven days, followed by exposure to 0.1 µg/g diet lead oxide for equivalent days. Negative geotaxis and survival assays were conducted on the flies. The expression level of phase II antioxidant enzymes and acetylcholinesterase were evaluated using rt-PCR to assess proanthocyanidin's protection level and mechanism in Pb-induced neurotoxic *D. melanogaster*. The results show that the proanthocyanidins-rich fraction enhanced locomotor activity and the rate of emergence of flies even in the presence of eco-toxicant. The fraction also mitigated the harmful effect of Pb via the activity of catalase and superoxide dismutase, as evidenced by the increase in the expression levels of the catalase and *SOD* genes in the noxious environment. Thus, the study provides insights into understanding the neuroprotection mechanism of proanthocyanidins-rich fraction against lead-induced neurotoxicity in *D. melanogaster*, which could be translated to humans when explored further.

1. Introduction

Long-term exposure to heavy metals such as lead (Pb) results in cumulative body concentration and causes damage to various vital organs and systems particularly the brain and the nervous system [1]. Cumulative Pb in the body could severely affect neurological functions through irreversible damage to cognitive function, resulting in learning disability and behavioral disorders with eventual death [1]. The most vulnerable group to Pb poisoning is children due to the rapid absorption of Pb in their brains during development. Pb exposure in children remains a problem domestically and in various places of work and worship. Pb inhibits presynaptic calcium channels and alters hippocampus NMDA receptor composition and function [2]. Avoidance of exposure to Pb has been the major preventive practice towards cumulative Pb poisoning over the years [3]. Over the past decade, *Drosophila melanogaster* has become a valuable model for studying nervous system disorders and aging [4]. Neurodegenerative disease (NDD) is a nervous system disorder characterized by memory loss and cognitive decline. They occur naturally at times with age depreciation in mammals and other model organisms [5]. Also, the lack of an effective antioxidant system and depreciation of central cholinergic transmission have been linked to cognitive decline as aging progresses [4,6]. Studies have shown that enhancing antioxidants' status and acetylcholinesterase (AChE) inhibitions have been proposed as therapeutic options for treating and preventing age-related cognitive decline and dementia [6]. AChE is an enzyme involved in the

breakdown of the neurotransmitter acetylcholine, which is present in many synapses of the central nervous system (CNS). Inhibiting the activity of AChE activity boosts the acetylcholine (ACh) effect on cholinergic neurotransmissions by preventing its breakdown into acetate and choline in the central nervous system [7].

The importance of diet in brain development and the aging process can never be overemphasized. Dietary phenolics and flavonoids such as proanthocyanidins have been implicated in regulating signal transduction, oxidative stress homeostasis, and expression of genes that play key roles in disease development [8]. Proanthocyanidins is a *Tamarindus indica* and other nuts-derived flavonoids that offer numerous health benefits with insufficient scientific shreds of evidence [9]. Most plants containing such molecules are shielded from viruses and predators by their astringency. Proanthocyanidins possess powerful biological properties, including antimicrobial, antioxidant, and anti-inflammatory activities [9]. Although it has demonstrated pharmacological action, its mechanism of enhancing survival and slowing aging is still unknown. Thus, this study aimed to explore the mechanism of neuroprotective activities of proanthocyanidins, a phenolic derived from *T. indica* against eco-toxicant-induced genotoxicity in the *D. melanogaster model*.

2. Methods

2.1. Ethical Statement

There is no restriction on the use of *Drosophila melanogaster* as a model for basic science research. The flies are invertebrates model system, as such ethical approval is not required to use them according to animal protection laws.

2.2 Diet formulation and culturing of D. melanogaster

D. melanogaster (Harwich strain), originally obtained from the National Species Stock Center, Bowling Green, Ohio, United States was obtained from the College of Medicine, University of Ibadan, Nigeria. The flies were grown in the *Drosophila* Research Laboratory (KASU-Dros-Research Lab), Department of Biochemistry, Kaduna State University. They were maintained at the respective standard temperature and pressure (24 ± 2 °C) and relative humidity (60 – 70%) under 12 hours of light/dark cycle conditions on a cornmeal diet containing Methylparaben, agar-agar, brewer's yeast, sucrose, and powdered in ratios in appropriate ratios as reported by [10].

2.3 Grouping, treatment, and exposure to environmental toxicant

Three days-old *D. melanogaster* of different sexes were split accordingly into five different groups as follows: standard control, vehicle (ethanol only) control, negative (Pb only) control, treatment (fraction "x mg/g diet" + Pb), and treatment (fraction "y mg/g diet" + Pb) groups respectively, using three biological replicates in each group.

2.4 Establishment of working concentration(s)

To establish the appropriate concentrations of proanthocyanidins-rich fraction and the period of exposure for the experiment, an initial cohort of flies was subjected to a proanthocyanidins-rich fraction-feeding experiment. The primary objective of this experiment was to observe the cumulative number of deceased flies throughout their entire lifespan. However, the specific data collected from this experiment is not presented in this context. A population of 200 flies was cultivated in four vials at varying concentrations (0.1 – 5 mg/g of diet) of the fraction, with each vial containing a density of 50 flies. Meanwhile, a 0.1 µg/10g diet was considered LD_{50} of Pb, used throughout the experiment.

2.5 Analysis of Survival Rate

To evaluate the rate of survival of the flies in a toxic environment, the experiment was conducted in three replicates, where flies were pretreated with proanthocyanidins-rich fractions at 1.5 and 2.5 mg/10g diets for seven days and exposed to 0.1 µg/10g diet for additional seven days. The survival rate was assessed by monitoring the daily mortality of flies across different concentrations of proanthocyanidins-rich fraction. After 30 days, the collected data were analyzed and presented graphically as the percentage of surviving flies. Further similar experiments were conducted in three replicates, each vial containing

200 flies. After 14 days experimental period, the flies were subjected to behavioral, biochemical, and molecular assays to ascertain the protection level of the *T. indica* fraction in a noxious environment.

2.6 Negative Geotaxis Assay

The negative geotaxis assay was carried out to examine the locomotor function of the experimental flies according to the method reported by [10]. Twenty (20) flies from each group were immobilized and placed separately in labeled vertical glass columns with a length of 15 cm and a diameter of 1.5 cm. The number of flies that crossed the 8 cm mark of the column in 8 s and those that remained below the mark after the time elapsed were recorded. The reading was taken three times per vial at one-minute intervals, and the average was expressed as the number of flies with functional locomotor.

2.7 Sample Preparations for Gene Expression Assay

Following the elapse of 14 days experimental period, the flies were anesthetized on ice, weighed, and homogenized in 0.1 M phosphate (pH 7.0) buffer. The homogenates remained on the ice and committed to mRNA extraction thereafter.

2.8 Isolation and analysis of mRNA by quantitative real time-PCR

Approximately, about 3 µg of total mRNA was extracted from each group of vial-containing flies. The extraction was done using a TRIZOL reagent by the manufacturer's instructions. Primer sequences for the three enzymes (Catalase, SOD, and ACh.E) used in this study (Table 1) were obtained from NCBI GenBank accordingly. The primers were designed on Primer 3 program v 0.4.0 and synthesized by Invitrogen company. Quantifying the extracted mRNA was done spectrophotometrically on NanoDrop 2000, where 1.9 – 2.0 appeared to be the OD260/OD280 ratio, indicating high purity. A total of 20 µL volumes comprising of 1X PCR buffer, 2.0 mM MgCl2, 1 U Taq DNA polymerase, 0.1X SYBR Green I (probes), and 1 µL RT product (cDNA) template was mixed vigorously and subjected to quantitative real-time PCR. The thermal cycle was performed on StepOne Plus real-time PCR machine from Applied Biosystems by the stipulated steps as follows: activation (95 °C for 5 min), then 40 cycles of 95 °C for 15 min, 60 °C for 15 min, and finally 72 °C for 25 min. The following StepOne software v2.0 of Applied Biosystems was used to determine the reaction's baselines and threshold. It was also used to analyze the SYBR fluorescence. The samples' cycle threshold (CT) values were calculated accordingly and recorded as $2^{-\Delta\Delta CT}$. The analysis for each well was conducted in triplicates, and the value for change in cycle threshold (ΔCT) was obtained by subtracting the CT values for the housekeeping gene (GAPDH) from that of the gene of interest. GAPDH was employed in normalizing the mRNA levels of the genes of interest as reported by [11]. Meanwhile, specific primers for the GAPDH were also utilized in normalizing the quantity of the samples with no changes in response to either proanthocyanidins-rich fraction pretreated and or Pb-exposed flies.

Table 1. Primers for *Catalase*, *SOD*, and *AChE* genes.

SN	Gene	Direction	Primer Sequence	Product Size
1	Catalase	Forward	GCAGATACCTGTGAACTCTC	203
		Reverse	GTAGAATGTCCGCACCTGAG	
2	SOD	Forward	GTTCGGTGACAACACCAATG	159
		Reverse	GGAGTCGGTGATGTTGACCT	
3	AChE	Forward	GCCGTGGGCAATGTAATAGT	185
		Reverse	CGACTCTCCGAACAGTGTCA	
4	GAPDH	Forward	ATGGAGATGATTCGCTTCGT	160
		Reverse	GCTCCTCAATGGTTTTTCCA	

2.9 Statistical Analysis

This study employed a one-way ANOVA and post hoc Tukey's test for data analysis. The results were presented graphically using mean and standard deviation. All the experiments were conducted in triplicates, considering $p<0.05$ as a significant difference in both analyses.

3. Results and Discussion

3.1 Role of proanthocyanidins-rich fraction on locomotor activity on survival rate

After 14 days of the experiment, we observed that there was a significant ($p<0.05$) increase in locomotor activities (Fig. 1 A) in the flies pretreated with both concentrations of proanthocyanidins-rich fraction (1.5 and 2.5 mg/g diet) prior to their exposure to Pb (0.1 µg/g diet) upon comparison with normal and Pb only control groups. Similarly, the present findings revealed that the rate of survival of the proanthocyanidins-rich fraction pretreated flies was significantly ($p<0.05$) higher compared to those exposed to Pb only without pretreatment (Fig. 1 B). Phenolics and flavonoids from various fruits and vegetables have shown improvement in the quality of life and extended longevity of *D. melanogaster,* though their holistic mechanisms of action were not fully explored [8,12].

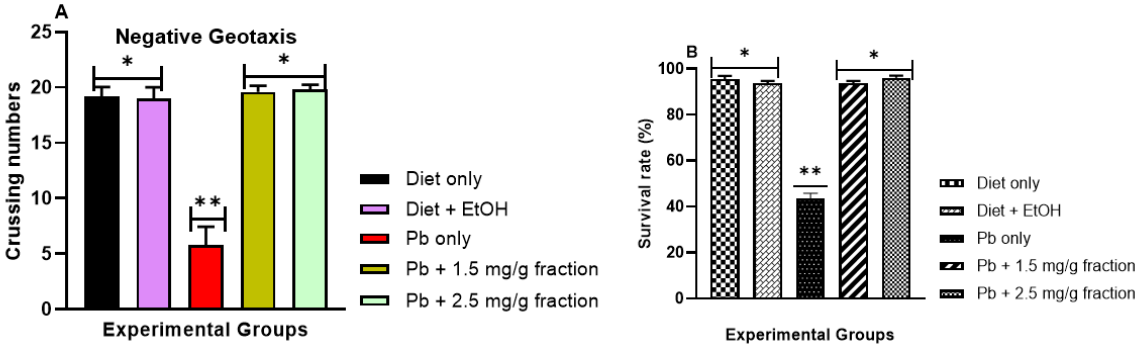

Figure 1. Effect of proanthocyanidins-rich fraction on rate of survival (A) and locomotor activities (B) in Pb-induced neurotoxic *D. melanogaster model.* The assays were conducted in three biological replicates, and the results are presented as the mean ± SD considering $p<0.05$ statistically significant.

3.2 Role of proanthocyanidins-rich fraction on Catalase, SOD, and AChE genes expression

The role of proanthocyanidins-rich fraction of Tamarindus ethanolic extract on the expression level of *catalase*, *SOD*, and *AChE* genes in the Pb-induced genotoxic *D. melanogaster* model was evaluated and findings are presented graphically in Fig 2. The results showed that the fraction's pretreatment caused an increase in the expression level of *Catalase* (Fig 2A) and *SOD* (Fig. 2B) genes significantly ($p<0.05$) at both concentrations in the presence of an eco-toxicant known as Pb. There was no significant ($p>0.0$) difference in the activities of AChE in both the control and fraction pretreated groups when the comparison was made. Our findings on the gene expressions agree with the previous report [13], where phenolics from the curcuminoid plant altered the gene expression level of both *SOD* and *Catalase* genes which also reflects on their respective enzymatic activities. However, pretreatment of the flies with proanthocyanidins-rich fraction did not stop the upregulation of *AChE* gene due to exposure to a noxious environment. This contradicts findings reported by [14], where phenolics such as curcumin appeared to upregulate the expression level of AChE in fruit flies. The variation in our findings may be due to genetic diversity and other factors, such as nutritional and environmental factors [15,16] within the fruit flies' population that could influence gene expression.

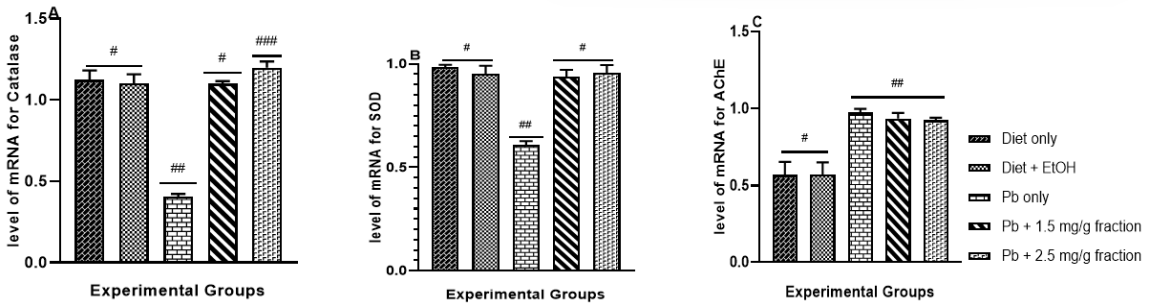

Figure 2. Effect of proanthocyanidins-rich fraction on catalase [A] superoxide dismutase (SOD) [B] and Acetylcholinesterase (AChE) [C] activities Pb-induced neurotoxic *D. melanogaster model.* Results are presented as mean ± SD. Experiment was conducted in three biological replicates considering $P < 0.05$ in comparison with the Control.

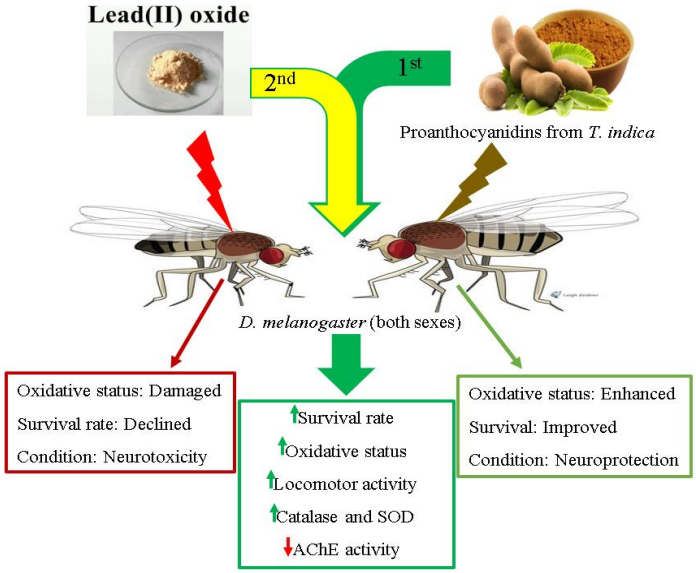

Figure 3. Proposed mechanistic route for proanthocyanidins-rich fraction neuroprotection in *D. melanogaster*.

4. Conclusion

Proanthocyanin-rich fraction enhanced the neuromuscular strength and survival rate of *D. melanogaster*. The fraction also upregulated the expression level of Catalase and Superoxide dismutase while having no effect on the acetylcholinesterase gene expression in the experimental flies. As such, the research has provided evidence that the presence of phenolic and or flavonoid compounds, especially proanthocyanidin from *T. indica* in living organisms such as fruit flies could favor redox status toward mitigating the effect of oxidative stress due to exposure to environmental toxicants, including lead (Pb), one of the heavy metals that affect nervous system upon accumulation in the body.

5. References

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