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Effect of Methanolic Extract of *Caryota no* Seed on Geotactic Behaviour and Fecundity in *Drosophila melanogaster*

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Authors' contributions

This work was carried out in collaboration among all authors. Author CAM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SO and MAE managed the analyses of the study. Author SSG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the effect of the methanolic extracts of *Caryota no* (CN) seeds negative geotaxis, fecundity and acetylcholinesterase (AChE) using *Drosophila melanogaster* (DM).
Study Design: Experimental design.
Place and Duration: Sample: African Centre of Excellence for Phytomedicine Research and Development, University of Jos, Jos Plateau State Nigeria between June 2018 and February 2019
Methodology: 50 flies were exposed in each vial to the following concentrations: 300 mg, 350 mg, 400 mg, 500 mg and 600 mg of methanolic extracts in 5 replicates for 7 days with daily recording of mortality. Total protein assays were carried out by Randox method from the supernatant from

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homogenized whole flies. *In vivo* antioxidant activity study was conducted by measuring level of acetylcholinesterase (AChE) enzyme activity from supernatants of whole fly homogenates using a spectrophotometer at specific wavelengths over a 2 minute duration. The values were derived as part of the total protein value. Negative geotaxis was done by the climbing assay and fecundity was examined by rate of emergence of larva after exposure of the flies to treatment. The statistical difference among test groups was presumed at $P < .05$.

Results: The methanolic extract of CN caused nonsignificant ($P = .33$) decrease in total protein levels compared to the control. There were also nonsignificant decreases in AChE ($P = .30$) activity, negative geotactic ($P = .80$) behaviour and nonsignificant increase in fecundity ($P = .17$) in the methanolic extract-treated flies compared to the controls.

Conclusion: It can therefore be concluded that the methanolic extract of *Caryota no* nonsignificantly improved fertitity and reduced negative geotaxis and AChE activity in DM.

Keywords: In vivo; caryota drosophila melanogaster; fecundity; geotaxis.

1. INTRODUCTION

Free radicals are involved in many pathological conditions such as different types of diabetes, neurodegenerative diseases, cardiovascular diseases, cancer, cataracts, asthma, rheumatoid arthritis, inflammation, burns, intestinal tract diseases, progerias and ischemic and post-ischemic pathologies [1]. When the production of damaging free radicals exceeds the capacity of the body's antioxidant defenses to detoxify them, oxidative stress (OS) occurs. Thus, oxidative/nitrosative stress is a disturbance in the balance between the production of reactive oxygen species/reactive nitrogen species (ROS/RNS) and antioxidant defenses, which may lead to tissue injury [2]. The resultant cellular injury caused by oxidative stress has been linked to several clinical disorders already enumerated [3].

The oxidative- stress state has been also implicated in several neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's, lateral amyotrophic sclerosis, and multiple Sclerosis [1]. Most importantly, the excess ROS can damage the integrity of various biomolecules including lipids [4], proteins [5] and DNA [6] leading to increased oxidative stress in various human diseases such as diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis, cataracts, cardiovascular diseases, respiratory diseases as well as in aging process [1]. Important neurodegenerative conditions such as Alzheimer, and Parkinson's diseases are associated with pathogenic oligomers due to misfolded proteins, ultimately resulting in gradual loss of neurons in the nervous system and brain [7]. The lipid peroxidation by ROS leads to progressive loss of membrane fluidity, decreases membrane potential, and increases permeability to ions such as Ca^{2+}. The regions of the brain such as hippocampus, substantia nigra, and the striatum are particularly susceptible to attack by free radicals [8].

The two main lines of defense against oxidative stress are endogenous antioxidant molecules [9] and repair enzymes that remove or repair oxidatively damaged macromolecules [10]. Paraquat (PQ) exposure has been previously linked to altered movement phenotypes in Drosophila Parkinson's disease (PD) models [11]. To evaluate whether reduction of Relish levels in dopaminergic (DA) neurons could rescue the mobility defects induced by PQ, negative geotaxis assay was employed, which is an indicator of the onset of PD pathogenesis-linked movement dysfunction. After ingestion of 5 mM PQ for 24 hour, both the control groups exhibited resting tremors and bradykinesia, which are characteristic clinical symptoms associated with PD in human patients [12].

Toxicology has demonstrated the importance of DM as a model to screen several molecules for their potential to induce or ameliorate neurodegenerative diseases and also to assess oxidative stress and antioxidant markers. Each assay may require different approach depending on the levels of the markers of interest to the toxicologist. For instance, the procedure of homogenisation of the treated and control flies in appropriate buffers, centrifugation at appropriate speed and temperature, and using the separated supernatants to determine biochemical and molecular markers of interest to the toxicologist, can be employed [13].

The genome of *D. melanogaster* codes for plasma membrane receptors (PMRs) and nuclear receptors (NRs) that correspond to diverse subclasses of human receptors, including

those involved in neurotransmission [14]. The regulation of fruit fly development shares several chemical and biological features with vertebrates, however, the influence of classical developmental toxicants in mammals have been little explored in D. melanogaster [15]. The moult in D. melanogaster is preceded and controlled by Ecdysone (Ecy). The production of Juvenile Hormone (JH) ceases at the end of the third instar larva to allow for the peak in Ecy to initiate pupation, cell death, and the development of new cellular structure. JH later returns in the adult stage and regulates spermatogenesis, lifespan, locomotor behaviour, feeding, secondary sexual differentiation and courtship. It also interacts with Ecy to promote fertility [16].

Caryota no is native to Borneo rainforests located 1° north of the equator, so this is truly a tropical palm of the family Arecaceae (Palmae). It is considered endangered due to deforestation and harvest of edible palm hearts [17]. The palm dies after the last inflorescence closest to the ground is spent. The inflorescences are pendulous and can grow 6 inches long. Seeds are black and contain irritating calcium oxalate crystals. Handling fruit without the protection of gloves is not recommended. These palms are some of the fastest growing palms. Palms with large diameter stems usually take a lot of time to commence vertical growth, but these palms explode out of the ground [17]. Caryota species are mostly found in Asia, and are used traditionally in the treatment of gastric ulcer, migraine headaches, snakebite envenomation and also rheumatic swellings by preparing porridge from the flowers [18]. Additionally, in Ayurveda, *C. urens* is suggested to treat seminal weakness and urinary disorders [19].

Infertility and neurodegenerative diseases have for decades been linked to various sources of endogenous and exogenous stressors. Agents which can become useful in ameliorating these oxidative states would help in the management of these conditions.

In the course of this study, the following null hypothesis was formulated to be tested at 0.05 level of significance;

- **H_1:** Methanolic extract of *Caryota no* does not possess fecundity and negative geotactic activity.
- **H_2:** Methanolic extract of *Caryota no* possesses fecundity and negative geotactic activity.

The aim of this work is to screen methanolic extract of CN for effect on fecundity, negative geotaxis assay and *in vivo* reactive oxygen species scavenging activity in *D. melanogaster,* a sensitive model for studying oxidative stress.

2. MATERIALS AND METHODS

2.1 Reagents

All chemicals used were of analytical grade. Methanol and distilled water were obtained from Africa Centre of Excellence in Phytomedicine Research and Development, Jos, Plateau State, Nigeria. Randox Protein kit was purchased from Medicom, Jos Plateau State. 1-chloro-2, 4-dinitrobenzene (CDNB), 5,5′-dithiobis(2-nitro-benzoic acid) (DTNB) and acetylthiocholine iodide were purchased from Sigma Aldrich (St Louis, MO).

2.2 Plant Collection

The plant material was collected from Games Village (9.0166°N, 7.4475°E), Abuja, Nigeria. The plant was identified by a taxonomist in the herbarium of the Federal college of Forestry Jos. The seeds were sorted, air-dried for several days and then pulverized to powder using a commercial grinding machine. The soxhlet extractor was used for extraction of the seed powder using analytical grade methanolic as a solvent following a method described by Virot, et al., [20] A rotary evaporator was employed to recover the solvent. The extract was further dried in a water bath regulated at 40°c and further lyophilized into crystals kept in an airtight container. This yielded the methanolic extract from the CN seeds (2 %), which was used in the biological tests.

2.3 Fly Strains

D. melanogaster Harwich strain was obtained from Africa Centre of Excellence in Phytomedicine Research and Development, University of Jos and maintained at constant temperature and humidity (23°C; 60 % relative humidity, respectively) under 12 hour dark/light cycle. The flies were cultured by feeding them with a standard medium of the following compositions; 1700 ml of water, 16 g agar agar, 20 g of baker's yeast, 100 g of corn flour, and 1 g of methyl paraben dissolved in 5 ml of absolute ethanol, 1700 ml of water [21].


A


B

Plate 1(A-B). *Caryota no* palm and seeds

(A) Caryota no palm inflorescence (www.rarepalmseeds.com) (B) Dried ripe seeds of Caryota no

The duration (days) of fly treatment for biochemical assays were pre-determined based on information from the literature, pilot studies and/or survival assays. Young flies 1-4 days old were preferred. To obtain the young flies of known age the culture bottles or vials with pupa were strictly emptied of all flies and the date noted and labelled accordingly. Adult flies of known age were then harvested from the newly hatched population.

2.4 7 Days Survival Assay of Methanolic Seed Extract of CN -treated Flies

50 flies of both genders (1–3 days old) were exposed to selected concentrations of methanolic extracts of CN seeds (300 mg 350 mg, 400 mg, 500 mg and 600 mg prepared in distilled water) in five replicates for 7 days as described [22,10].

The flies were divided into six groups containing 50 flies each. Control group was placed on normal diet alone while groups II–IV were placed on basal diet containing methanolic seed extract of CN at various concentrations of diet as shown thus;

Control group	Basal diet
300 mg group	Basal diet + 300 mg CN methanolic seed extract/10 g fly food
350 mg group	Basal diet + 350 mg CN methanolic seed extract/10 g fly food
400 mg group	Basal diet + 400 mg CN methanolic seed extract/10 g fly food
500 mg group	Basal diet + 500 mg CN methanolic seed extract/10 g fly food
600 mg group	Basal diet + 600 mg CN methanolic seed extract/10 g fly food

During the experimental period, flies were transferred onto new vials containing fresh food every 2 days. The flies were exposed to these treatments for 7 days, and the vials containing flies were maintained at room temperature. All experiments were carried out in triplicate (each experimental group was carried out in five independent vials). Mortality of flies was scored every 24 hours for 14 days and the survival rate was expressed as percentage of live flies. The data were subsequently analyzed and plotted as cumulative mortality and percentage survival after the treatment period. Survival analyses were calculated based on the number of deaths recorded and evaluated by the log-rank Mantel-Cox test.

2.5 *In vivo* Antioxidant Activities of Methanolic Seed Extract of CN - Treated Flies

50 flies were treated with 350 mg, 400 mg and 500 mg methanolic seed extract of CN for 7 days. Control flies were only treated with distilled water; each concentration was replicated five times. At the end of 7 days, the treated flies were anaesthetized in ice, weighed, homogenized in 0.1 M phosphate buffer, pH 7.0 (1 mg: 10 µL), and centrifuged for 10 min at 4000 rpm (temperature, 4°C). The supernatant obtained

was used to determine the level of total protein and hence the activities of acetylcholinesterase (AChE). From the total protein values, the levels of the antioxidants were derived by calculations.

2.6 Determination of Acetylcholinesterase Activity

0.0198 g of 10 mM DTNB was weighed and dissolved in 5 ml of 0.1M phosphate buffer pH 7.0. 0.0109 g of 8 mM acetylcholine iodide was weighed and dissolved in 5 ml of distilled water. Change in absorbance was read at 412 nm for 3 minutes at 15 seconds interval [23,10].

2.7 Negative Geotaxis Trial

The locomotor (climbing) performance of treated and control flies were evaluated using the negative geotaxis assay [24,12]. Ten flies per treatment group and control were immobilized under mild ice anaesthesia and placed separately in labelled vertical glass columns (length 15 cm; diameter, 1.5 cm). After the recovery period (about 20 minutes), the flies were gently tapped to the bottom of the column. Following 6 seconds, the number of flies that climbed up to the 6 cm mark of the column, as well as those that remained below this mark were recorded. Data were expressed as the percentage of flies that escaped beyond the 6 cm mark in 6 seconds. Each biological replicate was assayed three times at 1 min intervals. The score of each group was an average of the three trials for each group of treated and control flies.

2.8 Fecundity Assay

Flies were exposed to different doses of the extract and control [25]. After about seven days, five male and five female flies were selected and placed into an empty vial for 24 hours in order to encourage mating. They were removed after 24 hours and their eggs monitored for emergence for a period of 14 days.

2.9 Statistical Analysis

The data was expressed as mean ± SEM (standard error of mean) of five parallel measurements, and the statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Turkey's post-hoc test with the software, Graphpad prism version 7.0 (GraphPad Software, San Diego, CA, USA). The results were considered statistically significant at P <0.05.

3. RESULTS

3.1 Survival Assay

The results (Fig. 1) reveal nonsignificant changes between the treated groups and the control. The Log-Rank test give P = .123. Although it appears that the control improved survival better than the test groups by days 1 and 2, but by the end of the 7 days, it only did better than the highest extract dose. By the 7th day, the survival proportions for the control, 300, 350, 400, 500, and 600 mg/10g diet groups were 79.5, 89.5, 85.7, 82.0, 87.1 and 74.4 percent respectively. This result agrees with an earlier toxicology work on same extract [25] although it lasted for 28 days and forms the basis for the choice of the 3 middle doses (350, 400, and 500 mg/10g) for the rest of the assays.

3.2 *In vivo* Antioxidant Activities

3.2.1 Acetylcholinesterase assay

The results (Fig. 2) of the kinetics of the enzyme, acetylcholinesterase revealed nonsignificant difference (P = .304) between the groups and the control. The methanolic extract of CN was observed to cause general decreases in the levels of AchE all below basal level in a near regular indirect pattern. The least extract dose of the extract caused the least decrease in enzyme level.

3.3 Negative Geotaxis

This was also carried out on both extracts after a seven-day exposure of the flies to the extracts. This result (Fig. 4) revealed no statistically significant difference (P = .80) between the groups and the control. The methanolic extract also showed little observable effect in negative geotaxis assay when compared to the control. There is slight decrease in percent escape which dips more with increasing extract dose. This result however revealed no statistically significant difference between the groups and the control. The methanolic extract of CN produced barely discernible decreases that were indeed very mild at the 400 mg/10g fly food and 500 mg/10g fly food levels.

3.4 Fecundity

The rate of emergence of the larva, pupa and the adult fly was observed for the methanolic extract of CN after seven days treatment and one day mating grace as shown graphically below. This

result (Fig. 4) revealed no statistically significant difference ($P > 0.05$) between the groups and the control. The one-way ANOVA summary revealed $P = 0.17$. The methanolic extract improved fertility better than the control. However, the difference was not statistically significant but rather the extract concentration was indirectly proportional to fertility. The higher the concentration of the extract, the lower the positive effect on fertility and vice versa.

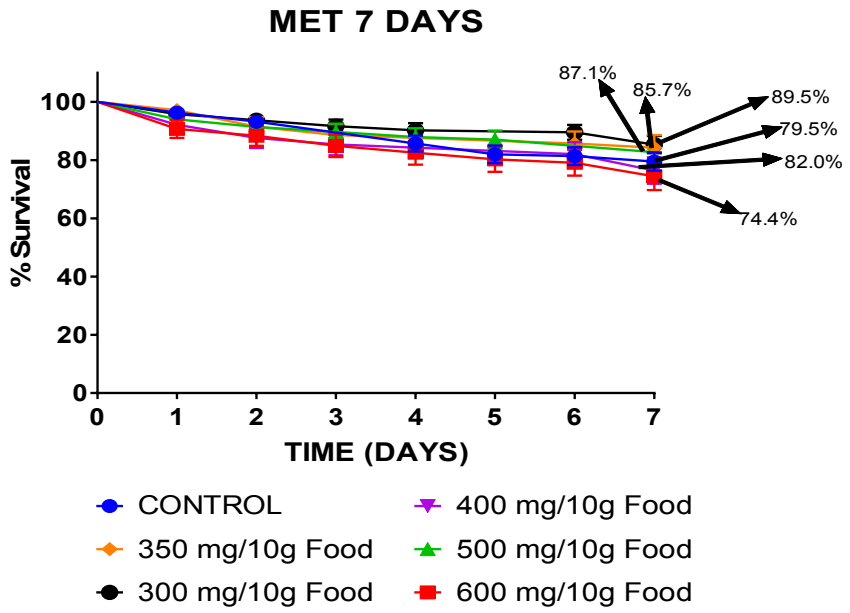

Fig. 1. Kaplan-Meier 7 days survival curves of methanolic extract of CN on DM.

Data presented as Mean ± S.E.M = Mean values ± Standard error of means of five independent biological replicates for each extract concentration (n = 50). Extracts: significant from control. Control group: Basal diet; 350 mg group: Basal diet + 350mg methanolic seed extract/10g fly food; 400 mg group: Basal diet + 400mg methanolic seed extract/10g fly food; 500 mg group: Basal diet + 500mg methanolic seed extract/10g fly food

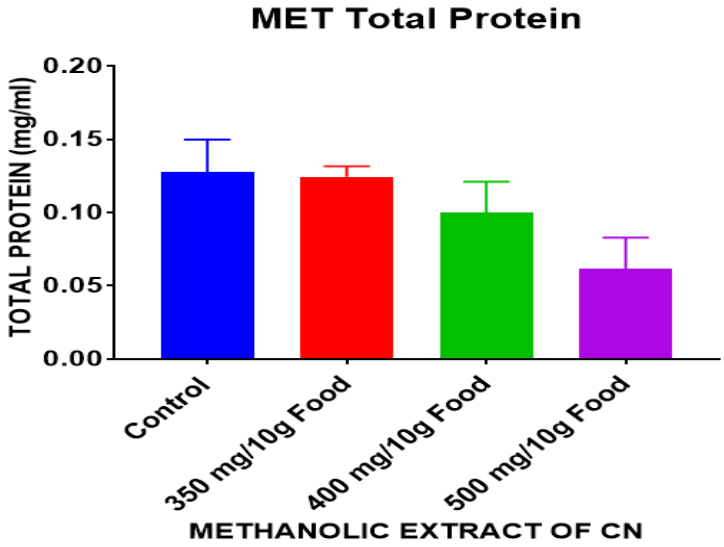

Fig. 2. Effect of dietary inclusions of Methanolic extract of Caryota no (CN) seed on Total Protein (TP) level in Drosophila melanogaster

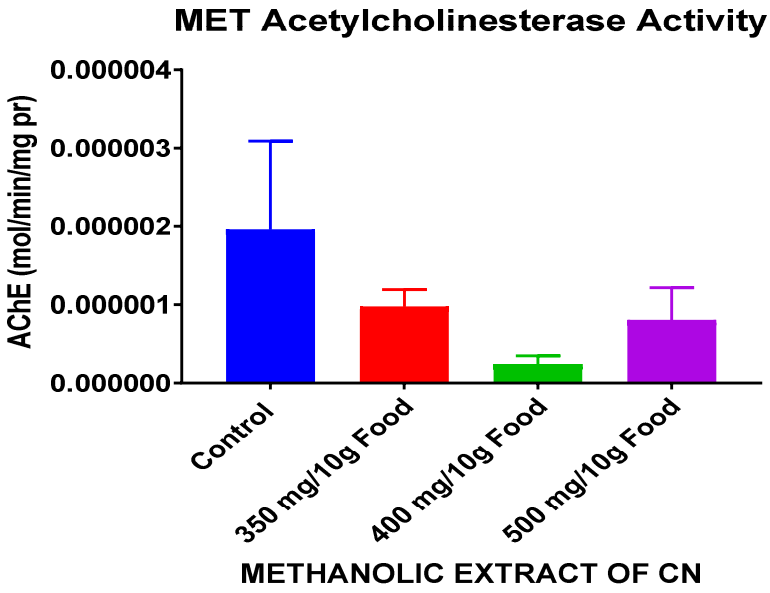

Fig. 3. Effect of dietary inclusions of Methanolic extract of *Caryota no* (CN) seed on Acetylcholinesterase (AChE) activity in *Drosophila melanogaster*

*Data presented as Mean ± S.E.M = Mean values ± Standard error of means of five independent biological replicates of for each extract concentration (n = 50) Extracts: significant from control, * P < 0.05; ** P < 0.001. Key: as described for Fig. 1*

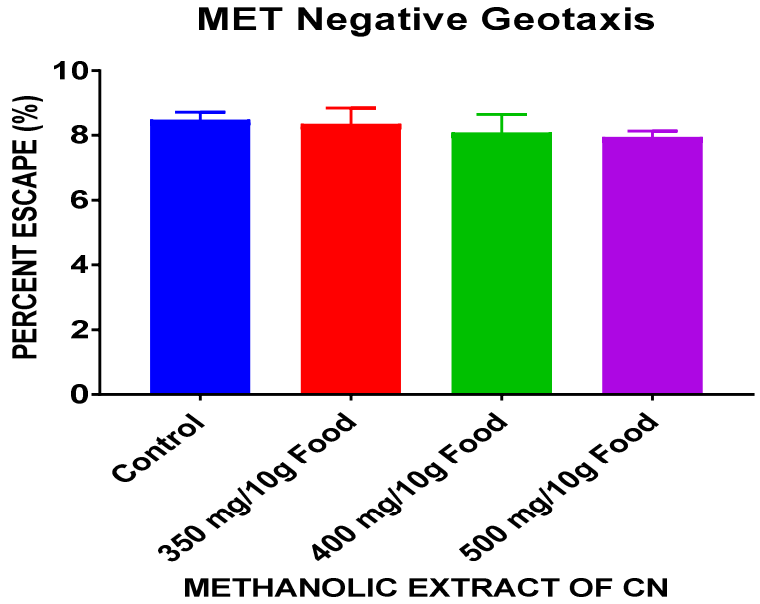

Fig. 4. Effect of dietary inclusions of Methanolic extract of *Caryota no* (CN) seed on Negative Geotaxis in *Drosophila melanogaster*

*Data presented as Mean ± S.E.M = Mean values ± Standard error of means of five independent biological replicates of for each extract concentration (n = 50) Extracts: significant from control, * P < 0.05; ** P < 0.001. Key: as described for Fig. 1*

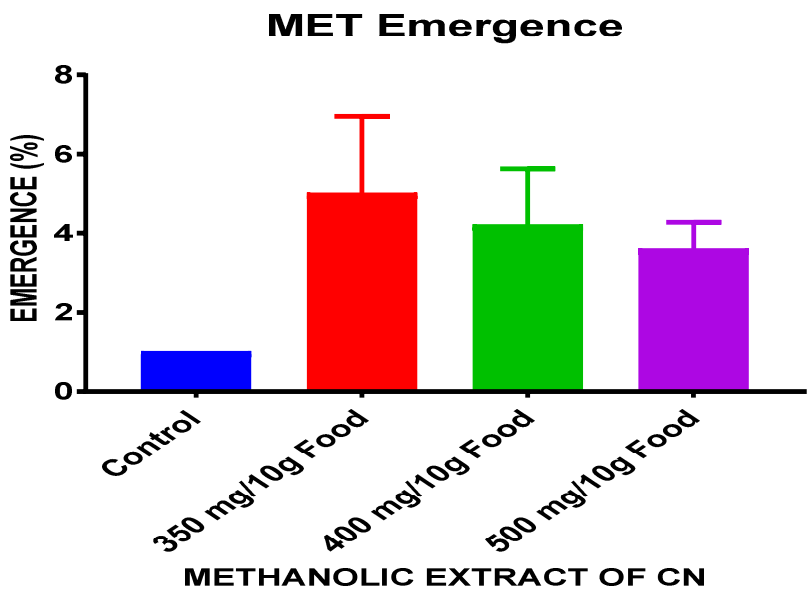

Fig. 5. Effect of dietary inclusions of Methanolic extract of *Caryota no* (CN) seed on fly emergence in *Drosophila melanogaster*

*Data presented as Mean ± S.E.M = Mean values ± Standard error of means of five independent biological replicates of for each extract concentration (n = 50) Extracts: significant from control, * P < 0.05; ** P < 0.001. Key: as described for Fig. 1.*

4. DISCUSSION

Higher dietary inclusions of GK seeds (0.5 % and 1.0 %) showed a significant decrease in survival rate and also induced a decrease in AChE activity [10]. This could be an additional reason for the observed decreased longevity observed with methanolic extract [25] But the information is not strong enough to establish any point in survival since the effect of the extract on longevity was not significant as the reduction in AChE (Fig. 2) was also insignificant compared to the control. Paraquat (PQ) treatment mediates nuclear translocation of active Relish showing that targeted knockdown of relish specifically in the dopaminergic neurons significantly improves survival, climbing performance and rescues DA neuron loss following PQ exposure. Our results suggest that activation of Relish contributes to environmental toxin-induced neurodegeneration [12].

The decrease in the AChE activity following dietary inclusions of GK seeds at 0.5 % and 1.0 % could be due to an increase in acetylcholine levels in the synaptic cleft and as a result induce cholinergic toxicity which could impair neuromuscular activities such as climbing abilities of flies [26] It should also be noted that prolonged reduction in the activity of AChE in the flies could lead to oxidative stress which could also contribute to their reduced survival rate [27]. This is further corroborated by the strong correlation between the survival rate of flies and their AChE activities among the treated groups [10]. In spite of the overall decrease in the activity of AChE in the AD brain, current AD therapy is mostly based on inhibitors of acetylcholinesterase (AChE-I), which enhance cholinergic transmission, but which have modest and transient therapeutic effects [28].

Altered plasma levels of AChE might have potential as an indicator of disease progress and prognosis in Alzheimier's dementia (AD) where increased levels were measured in patients compared to age and gender-matched controls [28]. Decreased levels could be observed in conditions like organophosphate poisoning, where acetylcholinesterase inhibitor is administered like in myasthenia gravis and other acute or chronic inflammatory states. This plant extract may have a protective effect over these different neurodegenerative conditions since it prevents fluctuations in plasma levels of AChE (Fig. 2).

The results by Abolaji et al., indicated that 4-vinylcyclohexene (VCH) toxicity is associated with oxidative damage, as evidenced by the

alteration in the oxidative stress–antioxidant balance, and possible neurotoxic consequences due to decreased AChE activity, and impairments in negative geotactic behavior [29]. Another group also showed impairments in climbing behavior and disruptions in antioxidant balance and redox status in the flies exposed to 4-vinylcyclohexene 1,2, monoepoxide (VCM) and 4-vinylcyclohexene diepoxide (VCD) [9]. Oboh et al., also reported that higher dietary levels of GK caused reduced AChE activity and reduced locomotion [10]. Once again, these two parameters of AChE (Fig. 2) and negative geotaxis (Fig. 3) were not significantly affected on treatment with this extract implying that it may be neuroprotective especially knowing that negative geotaxis is an indicator of the onset of PD pathogenesis-linked movement dysfunction.

Several reports from current research have implicated oxidative/nitrosative stress in male reproductive dysfunction [30,31]. Impairment of normal spermatogenesis and sperm function are the most common causes of male factor infertility [32] Several conditions have been identified to promote oxidative stress in the testis which lead to infertility including aging, pathological states and exposures to some toxicants [3]. Testicular receptors 4 (TR4) mutants also display reproductive defects consistent with their abundant expression in the testes and ovary. These include defects in spermatogenesis and reduced male fertility, as well as reduced female fertility, smaller ovaries, and defects in follicle development [33] Other major up-regulated gene categories correspond to defense response pathways suggesting that DHR78 mutants have an active stress response [34] The methanolic extract of CN have clearly shown an indirect dose dependent improvement in fertility (Fig. 4).

5. CONCLUSION

From the findings it can be concluded that the methanolic extract of *Caryota no* nonsignificantly improved fertitity and reduced negative geotaxis and AChE activity in DM. This implies that there are indeed bioactive substances present in this extract requiring further pharmacological evaluation of the extract which would help to validate or abrogate the claims of the folkloric usage of the plant. It is recommended that a more detailed assessment of drosophila fly neuronal, locomotor and endocrine systems treated with methanolic extract of *C. no* seeds should be carried out to fully understand the dynamics of oxidative stress in relation to this plant extract. This extract may be extrapolated to the clinical management of infertility and neurodegenerative illnesses in the future after detailed studies.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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