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Effect of Methanolic Extract of *Caryota no* on Antioxidant Levels 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 with author MAE wrote the first draft of the manuscript. Authors SSG and SO managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the anti-oxidant activity of the methanolic extracts of *Caryota no* seeds in *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: These assays were conducted by exposing 50 flies per vial to the selected concentrations (350 mg, 400 mg and 500 mg) of the extract in 5 independent replicates for seven days while control group received distilled water. The total protein content was then determined from the supernatant of the fly homogenate. The antioxidant activity and levels of GST, CAT and total thiol were then measured. The statistical difference among test groups was presumed at $P < .05$.
Results: The methanolic extract of *Caryota no* caused nonsignificant ($P = .33$) decrease in total

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proteins in DM below basal levels in a dose-dependent pattern. The antioxidant activity showed nonsignificant (*P* = .28) lowering of the GST activity in DM below control levels. The methanolic extract of CN nonsignificantly (P > .05) increased the levels of catalase (*P* = .36) and total thiol levels (*P* = .22).

Conclusion: It can therefore be concluded that the methanolic extract of *Caryota no* contains appreciable concentrations of different types of antioxidants. This may provide perspectives for the evaluation and development of effective and safe phytomedicines created from the local biodiversity.

Keywords: In vivo; Caryota no; endogenous; exogenous; Drosophila melanogaster.

1. INTRODUCTION

Antioxidants play a major role in mediation of both physiological and pathological processes because they are free radical scavengers. A free radical is an atom containing one or more unpaired electrons in its outer shell or valence which is capable of independent existence. This unusual nature of the free radical makes it unstable, highly reactive and of a short life span. The free radical quickly attacks any biomolecule in sight extracting electrons from them in order to form stable molecules and in turn convert these biological molecules into free radicals themselves and thus initiating a chain reaction cascade that damages the living organism [1]. These free radicals, both the reactive oxygen species (ROS) and reactive nitrogen species (RNS), are derived from both endogenous sources (mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells) and exogenous sources such as pollution, alcohol, tobacco smoke, heavy metals, transition metals, industrial solvents, pesticides, certain drugs like halothane, paracetamol, and radiation [2]. These reactive species are harmful molecules, capable of inducing oxidative damage in amino acids, proteins, lipids, and DNA components of tissues at higher levels [3]. An imbalance in the redox status can tip the body's physiology to oxidative stress which would adversely affect a lot of biological molecules and lead to various forms of pathologies.

Drosophila melanogaster had been established as a model system for immune studies by the early 21st century, after analysis of its genome revealed unsuspected sophistication and similarity to the mammalian innate immune system [4]. For more than a century, the low cost, rapid generation time, and excellent genetic tools have made the drosophila fly indispensable for basic research [5]. Adaptive response, which is the ability of an organism to effectively counteract cellular damages induced by cytotoxic agents such as free radicals, has been identified in *D. melanogaster* [6]. Such elevation in activities of antioxidant enzymes as an adaptive response to cytotoxic agents has been previously reported in *D. melanogaster*, and they are often accompanied by impairments in cellular thiol levels [7,8]. In fact, experimental evidences have implicated oxidative stress in *D. melanogaster* in the pathophysiology of several disease conditions [9,10].

With the current global exposure to high degrees of radiations from telecommunication devices, medical therapies, industrial, environmental and automobile pollutants, it becomes pertinent that concerted efforts have to be made in the search for exogenous sources of antioxidants which would supplement the endogenous sources because of increased demand for them.

Caryota no palm is reported to be one of the largest species of the genus found in Borneo rainforests. The common name is the Giant Fishtail Palm [11]. In habitat, this palm can reach a height of 75 inches and the stems measure 18-20 inches in diameter [11]. 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 [12].

The aim of this work is to screen methanolic extract of *C. no* for *in vivo* scavenging activity of reactive oxygen species in *D. melanogaster*, a very sensitive model for studying oxidative stress. This may provide perspectives for the evaluation and development of effective and safe phytomedicines created from the Nigerian local biodiversity.

2. MATERIALS AND METHODS

2.1 Reagents

All chemicals used were of analytical grade absolute 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 and Preparation

The plant material was collected from Games Village, 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 plant compound using analytical grade methanol as a solvent following a method described by Virot et al., [13]. A rotary evaporator was employed to recover the solvent. The extract was further dried in a water bath regulated at 40 °C and further kept in a fume cupboard. This yielded the methanol extract from the seeds (2%), which was used in the biological tests.

2.3 Fly Strains and Diet

D. melanogaster Harwich strain was obtained from Africa Center 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 h 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 [14].

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 with pupae were strictly emptied of all flies and the date noted and labeled accordingly. Adult flies of known age were then harvested from the newly hatched population.

2.4 *In vivo* Antioxidant Activities

The flies (both gender, 3–5 days old) were divided into four groups containing 50 flies each [15]. 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
350 mg group Basal diet + 350 mg CN methanolic seed extract/10g fly food
400 mg group Basal diet + 400 mg CN methanolic seed extract/10g fly food
500 mg group Basal diet + 500 mg CN methanolic seed extract/10g fly food

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.

2.4.1 Preparation of tissue homogenate

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 at temperature, 4°C. Subsequently, the supernatant was separated from the pellet into labeled Eppendorf tubes and used for the various biochemical assays. The level of total protein was first determined before the activities of Catalase (CAT), Glutathione-S-transferase (GST) and total thiol content were also determined.

2.5 Biochemical Tests

2.5.1 Total protein determination

The R1, Randox Total Protein Kit (Randox Laboratories, UK) was diluted with distilled water in double dilution. It was then added to 11.765 µl of homogenate supernatant sample and absorbence was read at 546 nm using a UV–visible spectrophotometer (Jenway).

2.5.2 Glutathione-s-transferase (GST) activity

The activity of glutathione-S-transferase (GST; EC 2.5.1.18) was determined by the method of Habig and Jakoby [16] described by Abolaji et al. [17] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay reaction mixture contained 600 µL of solution A (20 µL of 0.25 M potassium phosphate buffer, pH 7.0 with of 2.5 mM EDTA, and 510 µL of 0.1 M GSH at 25°C), 60 µL of sample (1:5 dilution) and 30µL of 25 mM CDNB. An increase in absorbance was measured at 340 nm for 2min at 10 s interval

using spectrophotometer (Jenway). The data were expressed in mmol/min/mg of protein using the molar extinction coefficient (ε) of 9.6 mM1 cm1 for the coloured GS–DNB conjugate formed by GST.

2.5.3 Total thiol determination

Total thiol content was determined using the method of Ellman [18] described by Etuh, et al., [14]. The reaction mixture contained 510µL potassium phosphate buffer (0.1 M, PH 7.4), 25 µL of sample as well as 30 µL of DTNB (10 mM). After incubation for 30 min at room temperature, the absorbance was measured at 412 nm and used to calculate the sample total thiol levels (in mmol/mg protein) using 35µl of GSH as standard.

2.5.4 Catalase (CAT) activity

The measurement of catalase (CAT; EC 1.11.1.6) activity was followed by a procedure described by Aebi [19]. The reaction mixture containing 100 mL of potassium phosphate buffer, pH 7.0, 194mL of 300 Mm H_2O_2 to form solution A. 10µL of sample was reacted with 590µl of solution A and monitoring the clearance of H_2O_2 at 240 nm at 25°C. The decrease in H_2O_2 was monitored for 2 min (10 s intervals), at 240 nm using a UV–visible spectrophotometer (Jenway) and expressed as mmol of H_2O_2 consumed/min/mg of protein.

2.6 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 AND DISCUSSION

3.1 Total Protein

The methanolic extract of CN caused nonsignificant ($P = .33$) decrease in total proteins in DM below basal levels in a dose-dependent pattern (Fig. 1). The higher the extract dose, the greater the decrease in protein levels with the lowest extract dose causing the least decrease in total protein level. There was an obvious inverse relationship between protein concentration in the fly and the dose of the administered extract with least extract dose having the highest protein level while the highest extract dose presents the least protein concentration. This pattern was also seen with the nhexane extract of CN [20].

3.2 Antioxidant Assays

3.2.1 Glutathione-s-transferase (GST) activity

For the methanolic extract, the GST levels initially decreased below basal levels (Fig. 2) as the extract concentration rises, the levels sharply rose with the highest extract concentration. Contrary to observations with the nhexane extract of CN [20], there was a nonsignificant ($P = .28$) lowering of the GST levels in DM below basal or control levels and also in an inverse dose-dependent manner. The higher the methanolic extract dose, the smaller the GST concentration and vice versa. It can be inferred that the methanolic extract of CN at high dose is a veritable source of exogenous GST antioxidant.

3.2.2 Total thiol content

In the case of the methanolic extract of CN, the thiol levels decreased nonsignificantly ($P = .22$) below basal levels at the lowest extract dose before rising sharply above control level at the highest treatment dose (Fig. 3). The pattern of effect of the methanolic extract of CN on thiol is exactly as observed with the nhexane extract of CN [20]. It can be inferred that the methanolic extract of CN at high dose is a veritable source of exogenous thiol antioxidant.

3.2.3 Catalase (CAT) activity

All the doses of the methanolic extract of CN nonsignificantly ($P > .05$) increased the levels of catalase above basal levels (Fig. 4). The increase is in direct proportionality with extract doses with the lowest extract dose causing the least increase while the highest extract dose brought about the greatest increase in catalase levels in DM. The one-way ANOVA summary revealed $P = .36$. The methanolic extract of CN produced an opposite constellation of effects in catalase levels from the observations with the nhexane extract of CN [20]; causing slight increases above basal levels in a direct dose-dependent pattern. It can be inferred that the methanolic extract of CN at high dose is a

veritable source of exogenous catalase antioxidant.

4. DISCUSSION

Halliway [21] stated that radicals are the species which contain at least one unpaired electron in the shells around the atomic nucleus and are capable of independent existence. The oxygen molecule itself is a radical, and because of the presence of two unpaired electrons it is referred as biradical. The examples for radicals include superoxide, oxygen radical, hydroxyl, alkoxy-radical, peroxyl radical, nitric oxide

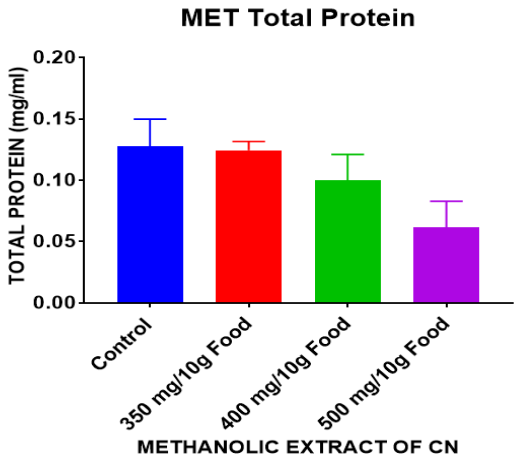

Fig. 1. Effect of dietary inclusions of methanolic extract of *Caryota no* (CN) seed on Total Protein (TP) level in *Drosophila melanogaster*

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

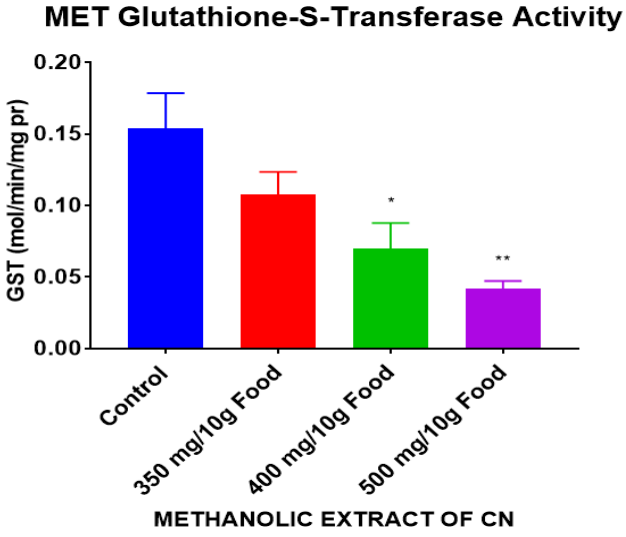

Fig. 2. Effect of dietary inclusions of methanolic extract of *Caryota no* (CN) seed on Glutathione-S-Transferase (GST) activity in *Drosophila melanogaster*

*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*

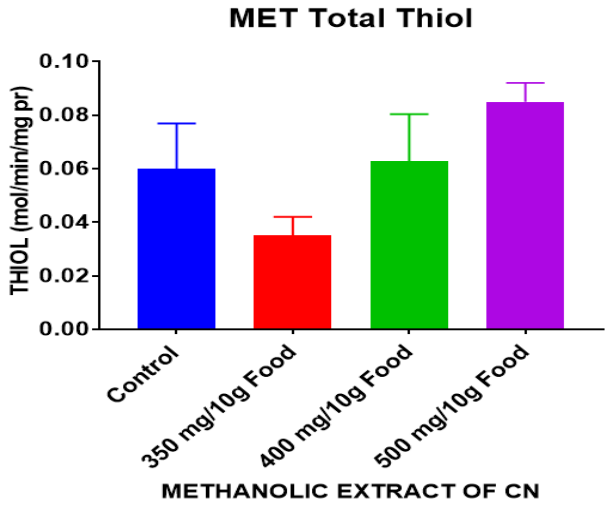

Fig. 3. Effect of dietary inclusions of methanolic extract of *Caryota no* (CN) seed on Total thiol (TT) content in *Drosophila melanogaster*

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. Key: as described for Fig. 1

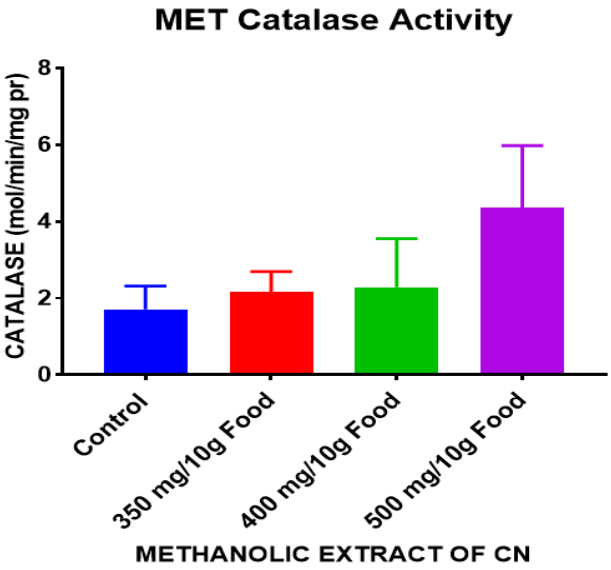

Fig. 4. Effect of dietary inclusions of methanolic extract of *Caryota no* (CN) seed on Catalase (CAT) activity in *Drosophila melanogaster*

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. Key: as described for Fig. 1

(nitrogen monoxide) and nitrogen dioxide [22]. The high reactivity of these radicals is due to the presence of one unpaired electron which tends to donate it or to obtain another electron to attain stability. The non-radical species include hydrogen peroxide, hypochlorous acid, hypobromous acid, ozone, singlet oxygen, nitrous acid, nitrosyl cation, nitroxyl anion, dinitrogen trioxide, dinitrogen tetraoxide, nitronium (nitryl) cation, organic peroxides, aldehydes and peroxynitrite [21]. These non-radical species are not free radicals but can easily lead to free radical reactions in living organisms [23].

Dietary inclusions of GK seed (0.5% and 1.0%) increased significantly the activities of Catalase and Glutathione S- transferase [15]. Indeed, cellular macromolecules are protected primarily from the insult of free radical species by endogenous antioxidant molecules including catalase, glutathione peroxidase, glutathione- S-transferase, and superoxide dismutase [24]. There was sharp elevation in the GST activity of C. no nhexane extract-treated flies [20].

Epidemiological studies reveal that low levels of antioxidants are associated with an increased risk of cancer. Significant increase in total oxidant status levels and decrease in total antioxidant status were observed in patients with bladder cancer [25]. Significantly lower levels of plasma protein, total thiol groups and protein-bound thiol groups and elevated levels of Protein carbonyl groups were observed in bladder cancer patients than in healthy controls [26]. This methanolic extract of CN has proven to be a source of exogenous thiol especially at high doses and may serve some protective function against oxidative stress.

In order to combat endogenous as well as exogenous oxidative stress, organisms have developed an intricate system of interacting enzymes. This array of enzymes works in a stepwise manner to maintain a low level of ROS in the body. Catalase and myeloperoxidase are two of the most important enzymes involved in this function. Catalase is a ubiquitous enzyme that catalyzes the decomposition of hydrogen peroxide, a reactive oxygen species, which is a toxic product of both normal aerobic metabolism and pathogenic ROS production [22]. Banerjee and colleagues [27] reported that catalase and superoxide dismutase activity was significantly increased in candidates chronically exposed to arsenic but in the present study, catalase increase was not significant proving that CN was not a source of oxidative stress but is rather a source of exogenous antioxidant. Catalase activity under toxic condition has been reported to be high [28]. Kohen et al., [22] reported in their *in vivo* antioxidant assay that the extract of *Aframomum melegueta* increased the activity of serum superoxide dismutase (SOD) and catalase. The increased serum activities of catalase and SOD as observed in that study suggest that the extract has an in vivo antioxidant activity and is capable of ameliorating the effect of ROS in biologic system [29]. In the present study, all the doses of the extract also increased catalase activity above basal levels albeit nonsignificantly. This implies that the methanolic extract of CN may be a good source of exogenous catalase.

Under normal condition total thiol content and GST activity are high but very low under disease or toxic condition [28]. Catalase mediates the reduction of H_2O_2 to H_2O and O_2 and thus protects biological tissues from the deleterious peroxidative effect of H_2O_2 [6]. Therefore, the increase in these antioxidant enzymes could be associated with protection in the flies. The high levels of total thiol (Fig. 3) imply that the extract has some protective antioxidant effect although not significant. The significantly ($P < .05$) reduced levels of GST activity (Fig. 2) and concurrently increased levels of catalase (Fig. 4) with increasing extract doses show some form of induction of oxidative stress by the methanolic extract of CN at increased dose levels. This may explain the reason for the shortened life span of the methanolic extract treated flies observed during survival studies (unpublished).

5. CONCLUSION

From the findings it can be concluded that methanolic extract of *Caryota no* seed caused insignificant increases in total thiol and catalase activity but significantly lowered GST activity in DM and therefore can serve as exogenous antioxidant to supplement the system against oxidative stress.

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 partially funded by an independent research body.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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