Journal of Advances in Biology & Biotechnology

23(4): 39-47, 2020; Article no.JABB.58180
ISSN: 2394-1081

Antioxidant Activity of nHexane Extract of *Caryota no* Seed Using *Drosophila melanogaster* Model

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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.

Article Information

DOI: 10.9734/JABB/2020/v23i430151
Editor(s):
(1) Anil Kumar, Ph.D., Professor & Head (Chair), Devi Ahilya University, India.
Reviewers:
(1) Salahu D. Dine Marref, Université de Batna, Algeria.
(2) B. Premkumar, K. K. College of Pharmacy (Affiliated to The Tamilnadu Dr. M. G. R Medical University), India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/58180

Original Research Article

Received 15 April 2020
Accepted 20 June 2020
Published 03 July 2020

ABSTRACT

Aims: To investigate the anti-oxidant activity of the nhexane extracts of *Caryota no* seeds in *D. melanogaster*.
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: Total protein assays were carried out by exposing 50 flies in each vial to the following concentrations: 300 mg, 350 mg, 400 mg, 500 mg and 600 mg of nhexane extracts in 5 replicates for 7 days with daily recording of mortality. *In vivo* antioxidant activity study was conducted by measuring levels of Glutathione-S-Transferase (GST), catalase (CAT) and total thiol (TT) from supernatants of whole fly homogenates using a spectrophotometer at specific wavelenghts. The values were derived as part of the total protein value. The statistical difference among test groups was presumed at P <0.05.
Results: The nhexane extract of CN caused nonsignificant (P = .52) changes in total protein levels compared to the control. The antioxidant activity showed nonsignificant (P = .64) higher total thiol

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contents and GST (*P* = .76) activity in nhexane extract-treated flies and lower catalase (*P* = .30) activity was recorded in the nhexane extract-treated flies compared to the controls.
Conclusion: It can therefore be concluded that the nhexane extract of *Caryota no* possess appreciable concentrations of different types of antioxidants.

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

1. INTRODUCTION

Antioxidants are agents capable of effectively neutralizing free radicals by interfering with oxidation process, chelating catalytic metals and also by acting as oxygen scavengers. They play a vital role in keeping free radicals at normal physiological levels to prevent oxidative damage [1]. It is necessary to screen the antioxidant activities of nhexane extract of *Caryota no* seed since plants have proven to be reliable sources of potent phytochemicals. Free radicals can be produced by habits like smoking, drug abuse; chronic alcoholic consumption, radiation, bacterial and viral toxins [2]. Free radicals are overproduced under some disease conditions such as diabetes, inflammation, cancer, cardiovascular diseases, Alzheimer's disease, atherosclerosis, arthritis, neurodegenerative disease, and aging process [2,3]. These free radical scavengers prevent the cells and tissues from breakdown and apoptosis by reacting with free radicals and catalytic metals as oxygen scavengers and chelators [4]. Antioxidants can be endogenous; made by the body or exogenous in which case, the body relies on external sources, primarily the diet, to obtain the rest of the required antioxidants by the body [5]. It has been reported that some plants can serve as a reservoir of antioxidants that are capable of scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS) [6].

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 (7). In habitat, this palm can reach a height of 75 inches and the stems measure 18-20 inches in diameter [7]. 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 [8].

In the early 21st century, *Drosophila melanogaster* had been established as a model system for immune studies after analysis of its genome revealed unsuspected sophistication and similarity to the mammalian innate immune system [9,10]. For more than a century, the low cost, rapid generation time, and excellent genetic tools have made the drosophila fly indispensable for basic research [11].

The aim of this work is to screen crude nhexane extract of *C. no* for *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. nHexane 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 seed powder using analytical grade nhexane as a solvent following a method described by Virot, et al. [12]. 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 nhexane 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 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 [13] .

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 labeled accordingly. Adult flies of known age were then harvested from the newly hatched population.

2.4 *In vivo* Antioxidant Activities of n Hexane Seed Extract of CN -Treated Flies

50 flies were treated with 350 mg, 400 mg and 500 mg nhexane seed extract of *C. no* 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 Catalase, Glutathione-S-transferase and total thiol content.

2.5 Total Protein

The R1, Randox Total Protein Kit was diluted with distilled water in double dilution. It was then added to 11.765 µl of sample of homogenate supernatant and absorbence was read at 546nm using a UV–visible spectrophotometer (Jenway). From the total protein values, the levels of the antioxidants were derived by calculations.

2.5.1 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 [14] described by Abolaji et al. [15] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay reaction mixture contained 600uL of solution A (20 uL of 0.25 M potassium phosphate buffer, pH 7.0 with of 2.5 mM EDTA, and 510 uL of 0.1 M GSH at 25°C), 60 uL of sample (1:5 dilution) and 30uL of 25 mM CDNB. An increase in absorbance was measured at 340 nm for 2 min 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.

2.5.2 Total thiol determination

Total thiol content was determined using the Ellman method [16] described by Etuh, et al., [13]. The reaction mixture contained 510uL potassium phosphate buffer (0.1 M, PH 7.4), 25 uL of sample as well as 30uL 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 35ul of reduced glutathione (GSH) as standard.

2.5.3 Catalase (CAT) activity

The measurement of catalase (CAT; EC 1.11.1.6) activity was followed by a procedure described by Aebi [17]. The reaction mixture containing 100 mL of potassium phosphate buffer, pH 7.0, 194 mL of 300 mM H_2O_2 to form solution A. 10 uL of sample was reacted with 590ul of solution A and monitoring the clearance of hydrogen peroxide (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 presence of the nhexane extracts initially caused a nonsignificant (P = .52) rise in total protein above basal level at the lowest extract

dose. As the doses of the extracts were increased, the total protein levels dropped below basal levels in a rather irregular pattern (Fig. 1). The one-way ANOVA summary revealed a *P* value of .5181. On further analysis with Turkey's multiple comparison test, it was further shown that there was no difference between the control and test groups and no difference between the different treatment group means when compared to one another.

The total protein value was highest in the lowest nhexane extract concentration and appeared to have an indirect dose dependent relationship with the extract concentrations but for the middle dose where there was a dip. It can therefore be inferred that lowest dose of the extract increased protein above basal level while higher doses decreased protein below basal level.

3.2 *In vivo* Antioxidant Activities

3.2.1 Glutathione-S-transferase (GST) activity

There was sharp elevation in the GST activity of *C. no*nhexane extract-treated flies but not significantly different (*P* = .76) from the control group (Fig. 2). GST is a major free radical species scavenger. This result suggests the high antioxidant activity of *C. no*nhexane extract in *D. melanogaster.* It can therefore be inferred that doses of the extract increased GST activity above basal level with lowest dose having the highest GST activity level and vice versa.

3.2.2 Total thiol level

There was also an elevation in the TT content of *C. no*nhexane extract-treated flies but not significantly different (*P* = .64) from the control group (Fig. 3). Thiol is also another major free radical species scavenger. This result also implies that there is high antioxidant activity of *C. no*nhexane extract in *D. melanogaster.* It can therefore be inferred that doses of the extract increased total thiol content in a direct dose-dependent pattern with lowest dose having the least thiol content and for other doses, thiol levels rising above basal level with increasing extract dose.

3.2.3 Catalase (CAT) activity

*C. no*nhexane extract nonsignificantly decreased (*P* = .30) catalase activity in the treated groups compared to the control group (Fig. 4). Catalase activity is always high at the instance of reactive oxygen species but of low activity when the reverse becomes the case. The result suggests that *C. no*nhexane extract has the ability to prevent oxidative stress. It can therefore be inferred that doses of the extract increased CAT in a near direct dose-dependent pattern but all of them are below basal level with the highest dose having the highest CAT activity level.

Antioxidants are biochemical substances that prevent the living cells and tissues from oxidative damage by scavenging on free radicals. Free radicals are recognized as highly reactive and toxic oxygen moieties due to the presence of unpaired electrons [18]. The accumulation of free radicals in the living system could lead to oxidative stress and eventually cell death, the oxidative stress can be defined in terms of an imbalance between the oxidant and antioxidant systems [19] and can lead to the depletion of endogenous antioxidant agents such as glutathione, vitamins E and C and antioxidative enzymes such as superoxide dismutase, Catalase, Glutathione-S-transferase and Glutathione peroxidase [19] Antioxidants have been reported to prevent oxidative damage caused by ROS by interacting with free radicals, chelating, and catalytic metals and also by acting as oxygen scavengers [20].

Glutathione exists in two forms, the thiol-reduced (GSH) and disulfideoxidized (GSSG) [21]. Eukaryotic cells have three major reservoirs of GSH, cytosol (90%), mitochondria (10%) and small percentage in the endoplasmic reticulum [22]. This is a ubiquitous tripeptide, γ-glutamyl-cysteinyl glycine, found in most plants, microorganisms, and all mammalian tissues [23]. In the human body, glutathione has diverse important functions such as storage and transport of cysteine, maintaining the reduced state of proteins and thiols, and protecting cells from toxic compounds such as reactive oxygen species, drugs, or heavy metal ions [24] and to protect against or delay apoptosis triggered by many different stimuli [25].

Thiols contain sulfhydryl groups and play a very major role in the mop up of ROS [23]. Albumin makes up the major portion of the protein bound thiols, which binds to sufhydryl group at its cysteine-34 portion [26]. Thiols share significant role in detoxification, signal transduction, apoptosis and various other functions at molecular level. Serum levels of thiols which can be easily measured reveal the thiol status of the body. Decreased levels of thiols has been noted

in various medical disorders including chronic renal failure and other disorders related to kidney, cardiovascular disorders, stroke and other neurological disorders, diabetes mellitus, alcoholic cirrhosis and various other disorders [27]. Therapy using thiols has been under investigation for certain disorders.

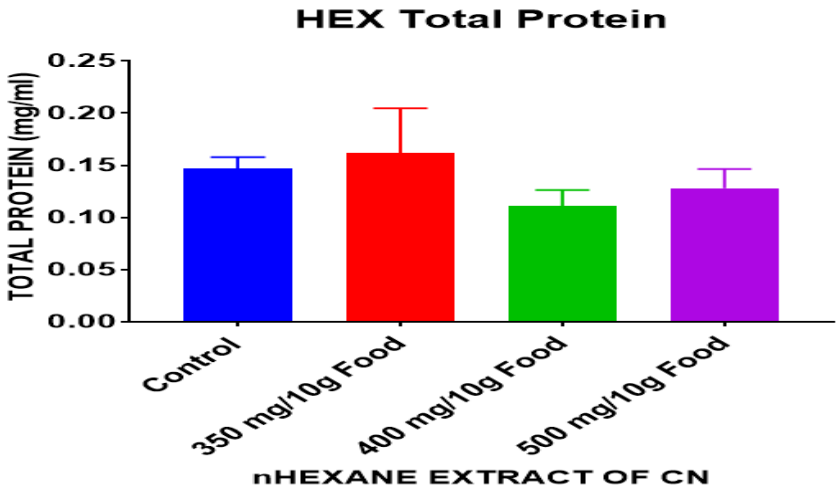

Fig. 1. Total Protein in whole fly homogenate of n Hexane Extract of CN-treated flies after 7 days. Exposure to nhexane extract of CN seeds nonsignificantly (*P* =.05) decreased total protein levels in *D. melanogaster*

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)

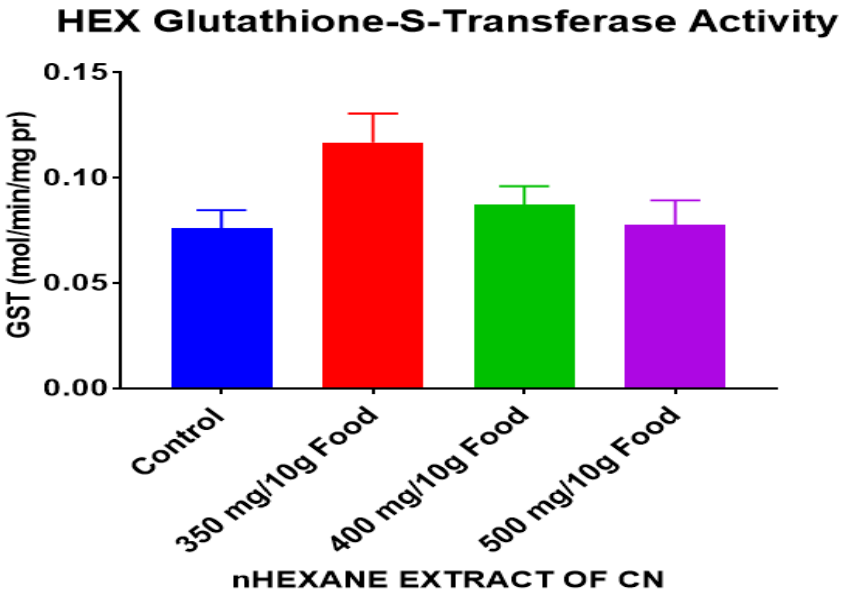

Fig. 2. GST activity in whole fly homogenate of n Hexane Extract of CN-treated flies after 7 days. Exposure to nhexane extract of CN seeds caused nonsignificant (*P* = .76) increase in GST activity in *D. melanogaster*

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)

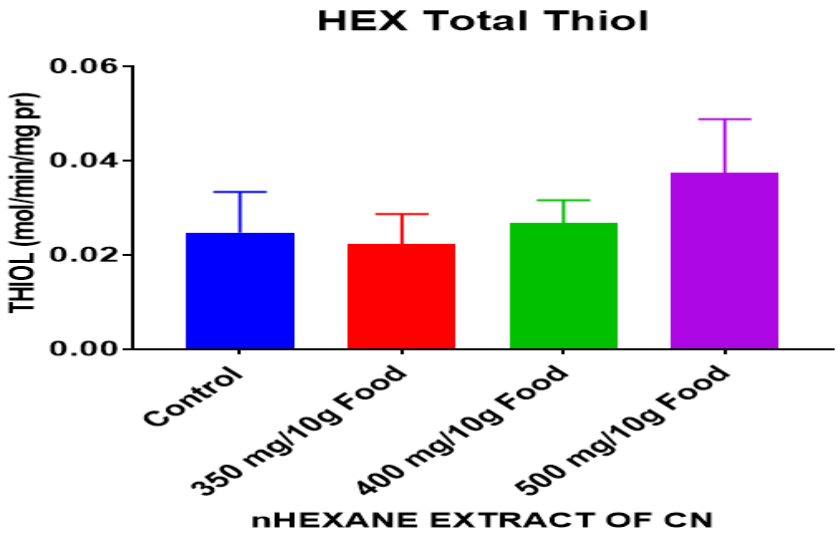

Fig. 3. Total thiol content in whole fly homogenate of n Hexane Extract of CN-treated flies after 7 days. Exposure to nhexane extract of CN seeds nonsignifgficantly (*P* = .64) increased total thiol levels in *D. melanogaster*

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)

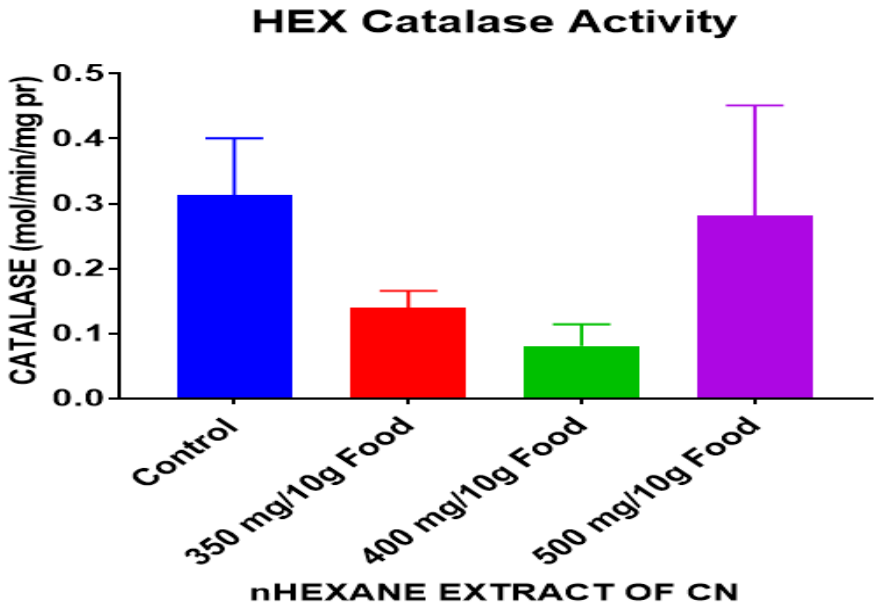

Fig. 4. CAT activity in whole fly homogenate of n Hexane Extract of CN-treated flies after 7 days. Exposure to nhexane extract of CN seeds nonsignificantly (*P* = .30) decreased CAT activity in *D. melanogaster*

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)

Hydrogen peroxide (H_2O_2) is broken down to O_2 and water by the antioxidant enzyme catalase [28]. Although, H_2O_2 is not a free radical by definition, it is a biologically important oxidant

because it selectively participates in hydroxyl radical generation, an extremely potent radical [28]. Also, because of its nonionized and low charged state, H_2O_2 has a long diffusion distance, since it readily diffuses through hydrophobic membranes as seen with the leakage of H_2O_2 from mitochondria [29]. ROS play some important roles in a number of physiological processes, including the intracellular killing of bacteria by neutrophil granulocytes [30], detoxification by the liver [31], prostaglandin production and certain cell signaling processes [32]. 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 [33].

Under normal condition total thiol content and GST activity is high but very low under disease or toxic condition while Catalase activity under toxic condition has been reported to be high [34]. Therefore, the increase in these antioxidant enzymes could be associated with induction of ROS production in the flies. The high levels of GST and total thiol (Fig. 2 and Fig. 3) and concurrently lowered level of catalase (Fig. 4) also imply that the extract is not major sources of oxidative stress to the flies since situations of stress would cause a reversal in levels of these antioxidants. This result suggests that nhexane extract *C. no* seeds might possess appreciable in vivo anti-oxidant activity against reactive oxygen species.

4. CONCLUSION

The antioxidant activity showed nonsignificant higher total thiol contents and GST activity in nhexane extract-treated flies and lower catalase activity was recorded in the nhexane extract-treated flies compared to the controls and can serve as exogenous antioxidant to supplement the system against oxidative stress.

It is recommended that a more detailed assessment of drosophila fly antioxidant system treated with nhexane extract of *C. no* seeds should be carried out to fully understand the dynamics of oxidative stress in relation to this plant extract.

ACKNOWLEDGEMENTS

The authors are grateful to TETFUND for the financial support obtained for part of this work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/58180