Research Article

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Behavioural cellular and neurochemical alterations in rat prefrontal cortex and hippocampus exposed to tigernut (*Cyperus esculentus)* treatment

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ABSTRACT

Investigating substances of plant origin for therapeutic advantages over subcellular mechanisms underlying a number of physiological dysfunctions could foster the development of potent therapeutic strategies for the treatment of these dysfunctions. We explored the effects of Tigernut (*Cyperus esculentus)* consumption on neurochemical, behavioural and cellular parameters in prefrontal and hippocampal regions of rat brain. Twenty-four (24) adult male Wistar rats with an average weight of 180g±10g were randomly split into 4 (A-D) groups (n=6); Groups A – C received 10mg/kg, 20mg/kg, and 30mg/kg bodyweight of Tigernut extract respectively for 14days, while Group D served as the control receiving distilled water. Animals were sacrificed 24hours after the last day of administration. Behavioural assessment of the cortico-hippocampal neural circuitry in Tigernut–treated rats showed increased memory function compared to control, evidenced by an increase in correct spontaneous alternation in the Tigernut-treated groups. Neural malondialdehyde (MDA) levels was significantly reduced in treated rats in order of increasing dose, while the concentrations of catalase (CAT) and superoxide dismutase (SOD) were significantly increased. These observations hinted at the antioxidant properties of Tigernut. Subsequent analysis of the total antioxidant capacity in animals revealed elevated antioxidant levels significantly in the 10mg/kg and 20mg/kg groups. Furthermore, the microarchitecture of the prefrontal cortex and hippocampus appeared normal and well-structured. Our results show that Tigernut has neurotherapeutic and antioxidant properties at moderate doses and can therefore, be used to augment the endogenous production of antioxidants in the different brain regions.

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1. INTRODUCTION

Tigernut (*Cyperus esculentus) is a plant* growing freely and consumed widely in Spain, Arabian Peninsula, east Africa, Tropics, Mediterranean areas and most parts of West Africa especially among most ethnic groups and across the geopolitical zones in Nigeria. It's a sweet almond-like tuber that are highly appreciated for their health benefits and nutritional value [19,21,24]. Two species has been identified (black and brown colored species), both species has a characteristic sweet taste and is commonly called by various names such as: "Aya'' in Hausa "Imumu'' in Yoruba, "Akiausa'' or: Offio" in Igbo, "chufa" in Spanish [16,19-21,23]. Other popularly known names include earth nut, yellow nut sedge, groundnut, rush nut, and edible galingale (19,21,23). Market survey revealed that several consumables has tigernut extract as part of their ingredients, some of the food and industrial use of tiger nut includes: beverage, milk or fermented milk product (such as yoghurt), flour, edible oil, honey, nougat ("*turron*" in Spanish), jam, beer, liqueur, chocolate, candies, a feed source and as soaps [6,15-16,23].

Tigernut extracts have been reported to be very rich in phosphorus, potassium, sodium, calcium,

iron, zinc, magnesium, manganese, starch, fat, sugars, Oleic acid, protein as well as vitamins: B_1, C and E [17,19-20,22]. Phytochemical screening showed that Tigernut contains beneficial amounts of alkaloid, tannins, saponins, glycoside, steroid, reducing sugar, flavonoid but lacks resin [3,10,14].

Tigernut has been associated with high level of Oleic acid with associated positive effect on cholesterol thereby preventing heart attacks, thrombosis and activates blood content of soluble glucose (2,19,). Pharmacological benefits of tiger nuts have been reported by several researchers to be high in dietary fiber content, which could be effective in the management of breast cancer [1,13,]. Also, its associated therapeutic potentials in the management of several pathophysiological conditions like: colon cancer, coronary heart disease, obesity, diabetes, gastrointestinal disorders, weight loss, urinary tract infections, constipation, infertility, dyspepsia, anaemia, diarrhea, dysentery, hypercholesterolemia and as anti-microbial agent have been well identified [4,8,11,13,24].

Cyperus esculentus is widely used by both humans and animals due to its various reported nutritional and functional properties. Current research and reviews on this plant have focused mainly on organoleptic properties [9], phytochemical compositions [2,7], oil content, biochemical activities and antioxidant properties [18], nutritional values [8,24], medicinal properties [19] and lots more.

Based on the evidence from several literatures on the health benefits associated with Tigernut, not much is known on its effect on the nervous system, hence the need for further investigation into the several reported benefits and its associated effect on the structural integrity of the hippocampus and frontal cortex. Behavioural evaluations, brain biochemical redox, learning and cognition has not been exhaustively investigated. Therefore, this study was designed to expand the current knowledge and provide answers to the effect of Tigernut extract at varied doses on learning and memory, oxidative redox, enzymatic activities and cellular morphology through histomorphological and neurochemical appraisal of rat prefrontal cortex and hippocampus.

2. MATERIALS AND METHODS

2.1 Animal care and ethical approval

All protocols and treatment procedures were done according to the Institutional Animal Care and Use Committee (IACUC) guidelines and as approved by the Faculty of Basic Medical Sciences Ethics Review Committee, University of Ilorin, Nigeria. Twenty-four (24) adult male Wistar rats with an average weight of 180g±10g were purchased from a private animal holding in Ilorin and used for the research. These rats were kept in the animal house of the faculty of Basic Medical Sciences, University of Ilorin. The rats had liberal access to rat chow and water.

2.1.1 Animal grouping and treatment

The twenty-four (24) adult male rats were randomly assigned into 4 groups (A –D), each consisting of 6 rats (n = 6). The groups were treated as follows: Group A (received 10mg/kg of extract daily for 14 days); Group B (received 20mg/kg for 14 days); Group C (received 30mg/kg for 14 days), and Group D (received distilled water *ad libitum* for 14 days).

2.1.2 Purchase and preparation of extract

Fresh Tigernut (*Cyperus esculentus*) were purchased from Ipata Market, Ilorin, Nigeria. The tigernuts were identified and authenticated at the herbarium of the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria. The tiger nuts were screened and washed to remove sand and debris. They were dried and pulverized into fine powder with an electric blender. Three hundred grams (300g) of the Tigernut was soaked in 1000ml of distilled water; stirred and left for 72 hours in a refrigerator at 4°C. The mixture was sieved and filtered with Whatman No.1 filter paper. The resultant filtrate was dried in a water bath at 40°C for 96 hours to get the concentrate which was then diluted to stock.

2.1.3 Administration

The extract was administered via the oral route. Proper volume was ensured through the use of a calibrated syringe fitted with an oral cannula.

2.2 Y-maze spontaneous alternation test

This test was used to assess working and cognitive memory in rodents. Alternation behavior is based on the natural tendency in rodents to explore the maze systematically entering each arm in turn. Alternation behavior is then defined as consecutive entries into each of the three arms without repetition. This was examined using a Y- maze composed of three equally spaced arms (120°, 41cm long and 15cm high). The floor of each arm is made of plywood and is 5cm wide. Briefly, each rat was placed in one of the arm compartments and was allowed to move freely until its tail completely enters another arm. The sequence of arm entries was manually recorded with a video camera, the arms being labeled A, B, or C. An alternation is defined as entry into all three arms consecutively, for instance if the animal makes the following arm entries; A, C, B, C, A, B, C, A, C, A, B, C, A, in this example, the animal made 13 arm entries 3 of which are correct alternations. The number of maximum spontaneous alternations is then the total number of arms entered minus two, and the percentage alternation is calculated as {(actual alternations /maximum alternations) x 100}. For

each animal in a group the Y-maze testing was carried out for 5 minutes, three trials and recorded for later analyses. The videos were analyzed by a neutral observer to eliminate bias.

2.3 Sacrifice and sample collection

On completion of treatments, rats for histological analysis were euthanized using 20 mg/kg of ketamine (intraperitoneal). Transcardial perfusion was done by exposing the left ventricle and injecting 50 ml 0.1 M PBS (pH 7.4) followed by 400 ml 4% paraformaldehyde (PFA) while the rat was suspended in an inverted position (gravity). Excised brains were then rinsed in 0.25 M sucrose 3 times for 5 minutes each and then post fixed in 4% PFA for 24 hours before being stored in 30 % sucrose at 4 °C until further processing. Rats for enzymatic assays were sacrificed by separating the head from the trunk, to avoid the interference of ketamine with biochemical redox; brains were then excised, rinsed in 0.25 M sucrose 3 times for 5 minutes each and placed in 30 % sucrose in which they were stored at 4°C. Coronal sections of PFC and hippocampus were obtained stereotaxically (+4 mm) from each brain. Histological staining was carried out in paraffin wax embedded sections which were stained with Haematoxylin and Eosin using the methods described by Fischer et al 2008 (12). Histochemical demonstration of Nissl substances was done with slight modification to the method published by Kádár *et al* 2009 [5].

2.4 Colorimetric assay for enzymatic studies

Determination of SOD, CAT, TAC, and MDA activities was carried out on whole brains of treated rats using spectrophotometric technique. Each of the assay kits were procured from Bio Legend Inc., San Diego, CA, USA. Whole brain (in sucrose at 4^{o} C) from rats across groups were weighed and pulverized in 0.25 M sucrose (Sigma) with the aid of an automated homogenizer at 4°C. Lysates from the brain were centrifuged for 10 minutes in a microfuge at 12,000 rpm to obtain the supernatant containing organelle fragments and synaptosomes. The supernatants were aspirated into plain labelled glass cuvette placed in ice. SOD, CAT, TAC, and MDA activities were assayed according to the manufacturer's instruction in the assay kit pack.

2.5 Light microscopy

Tissue sections on glass slides were captured using Olympus binocular research microscope (Olympus, New Jersey, USA) which was connected to a 5.0MP Amscope Camera (Amscope Inc, USA).

2.6 Statistical analysis

All quantitative data were analyzed using GraphPad Prism® (version 6) and SPSS (version 20) softwares. Spontaneous alternation, catalase (CAT), total antioxidant capacity (TAC), malondialdehyde (MDA), and superoxide dismutase (SOD) outcomes were plotted in ANOVA followed with Tukey's multiple comparisons test. Significance was set at $p<0.05^{*}$ and $p<0.01^{\#}$. The results were represented in bar charts with error bars to show the mean and standard error of mean (Mean±SEM) respectively.

3. RESULTS

3.1 Physical observation

Animals in the experimental groups were observed fed normally and hence were all seen to gain weight adequately across groups with demonstrated normal grooming pattern. No mortality was recorded over the period of administration.

3.1.1 Percentage incorrect spontaneous alternation directly proportional to tigernut dose

The y-maze test was used to assay for the working memory of the rats. It was observed that relative to the control group D, the low dose 10mg/kg did not result into any observable significant change ($p>0.05$, 0.01 and 0.005) in the percentage correct alternation of the rats in the Y-maze test. As the dose increased, the medium dose 20mg/kg resulted into a significant increase in the percentage correct alternation relative to the control group ($p<0.01$). Surprisingly, as the dose further increased to the high dose 30mg/kg, the % correct alternation of the animals reduced significantly relative to the control rats ($p<0.005$) as well as in relation to the low and medium doses (0.005) which suggests a role that high dose of the extract might play in neurobehavioral deficit.

3.1.2 Sod and cat profiles increases following tigernut administration

Differential expression of SOD and CAT were assessed in the brain of treated rats as shown in Fig. 2. Our results showed that the 20mg/kg body weight extract (group B) treated rats had very significant ($p< 0.05$, 0.01 and 0.005) increase in activities of catalase (CAT), and super oxide dismutase (SOD), when compared to the control group (group D). Rats in group A, treated with 10mg/kg body weight expressed averagely normal levels of these enzymes which was significant when compared to the control group and group C that received 30mg/kg body weight of the extract. The 30mg/kg body weight showed significantly decreased levels in the activities of these enzymes (Fig. 1).

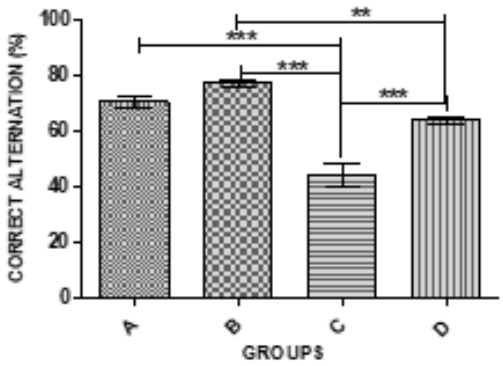

Figure 1. Chart showing the % correct alternation of animals in the Y-maze test. A=low dose, B=medium dose, C= high dose and D= control. *, ** and *** are p values less than 0.05, 0.01 and 0.005 respectively.

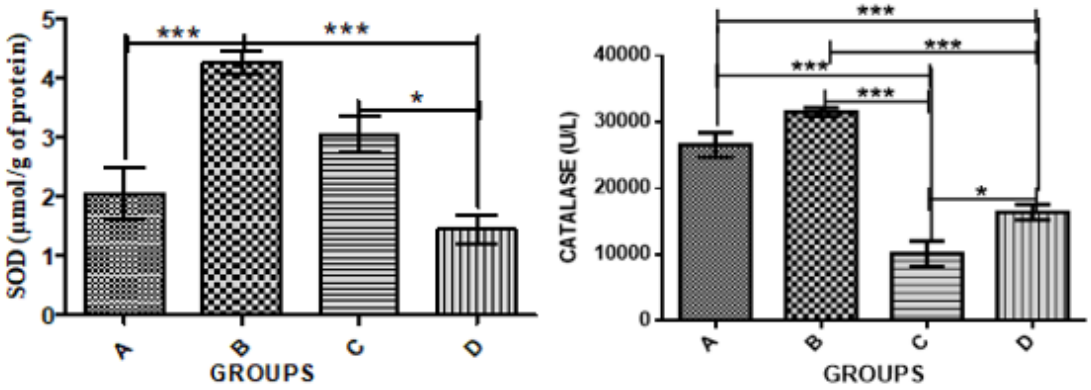

Figure 2. Chart showing the levels of super oxide dismutase (SOD) and catalase (CAT) in the brain of rats. A=low dose, B=medium dose, C= high dose and D= control. *, ** and *** are p values less than 0.05, 0.01 and 0.005 respectively.

3.1.3 Moderate dose of tigernut associated with increased tac

Total antioxidant capacity levels were significantly increased (P<0.05, 0.01 and 0.005) in group B (animals who received *Cyperus esculentus* extract at 20mg/kg body weight dose) when compared with the control. Its level in animals who received the extract at a low dose of 10mg/kg body weight also saw an increase, which was also statistically significant in comparison to the control group. These results showed some consistence with earlier findings of this study, buttressing the antioxidant properties of *Cyperus esculentus*. The levels of TAC in group C animals who received the high dose of 30mg/kg body weight of the extract was comparatively identical with the control animals as shown in Fig. 3.

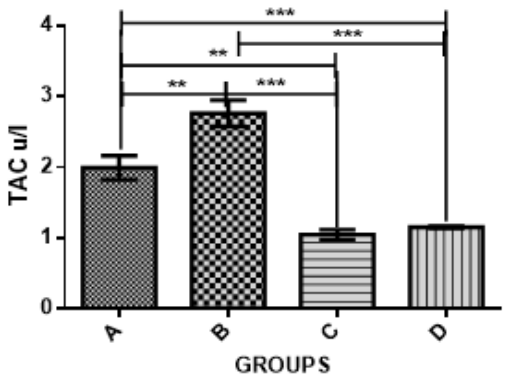

Figure 3. Chart showing the levels of total antioxidant capacity (TAC) in the brain of rats. A=low dose, B=medium dose, C= high dose and D= control. *, ** and *** are p values less than 0.05, 0.01 and 0.005 respectively.

3.1.4 MDA Level significantly drops upon exposure to tigernut treatment

MDA levels were significantly reduced (P<0.05, 0.01 and 0.005) in group B (animals who received *Cyperus esculentus* extract at 20mg/kg body weight dose) when compared with the control. Its level in animals who received the extract at a low dose of 10mg/kg body weight also saw a mild reduction relative to the control group. These results showed some consistence with earlier findings of this study, buttressing the antioxidant properties of *Cyperus esculentus* L. The levels of MDA in group C animals who received the high dose of 30mg/kg body weight of the extract was comparatively higher than the control animals as shown in Fig. 4.

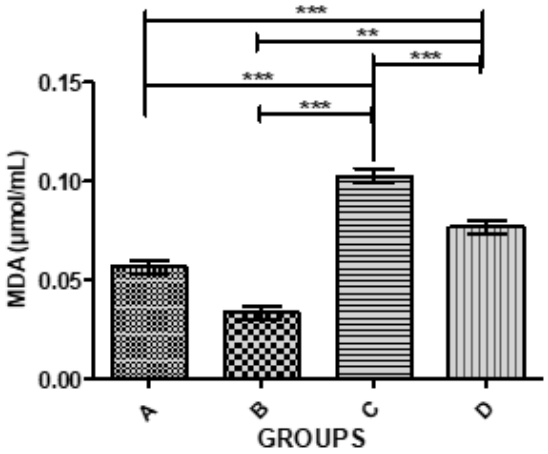

FIGURE 4. Chart showing the levels of malondealdehyde (MDA) in the brain of rats. A=low dose, B=medium dose, C= high dose and D= control. *, ** and *** are p values less than 0.05, 0.01 and 0.005 respectively.

3.2 Histological observation

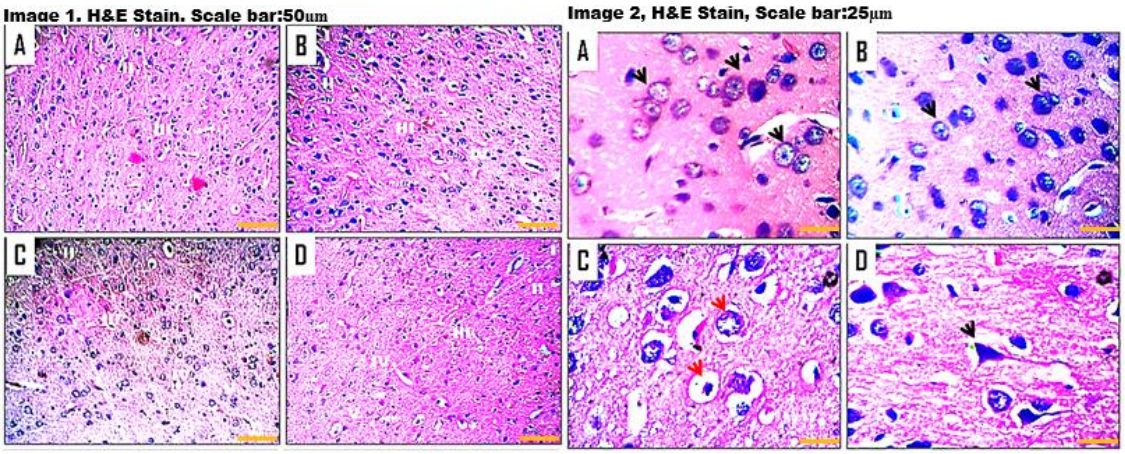

Figure 6. Photomicrographs showing panoramic views of prefrontal cortex (PFC) general histomorphological presentations in Wistar rats across the study groups. Hematoxylin and Eosin stain (Images 1&2 Scale bars -50µm and 25µm respectively). The molecular layer (I), External granular layer (II), External pyramidal layer (III), Internal granular layer (IV), Internal pyramidal layer (V) and the multiform layer (VI) are demonstrated across study groups however layers II -IV are well captured.

Representative micrographs of H&E staining (Fig. 6) showing the general cytoarchitecture of the PFC in Wistar rats. Images 1 & 2. Normal histological features of the PFC in groups A, B and D (10mg/kg, 20mg/kg and control respectively) is characterized by large pyramidal neurons (black arrows) with relatively appreciable long axons that extend well from the soma to adjacent neurons within the neuropil (black arrow heads). Apical and basal dendrites extend from the well delineated soma of the pyramidal neurons in this groups appears normal with group B showing better cellular morphological presentation relative to other treatments. Group C treatment (30mg/kg) caused some observable degenerative changes in the PFC that was characterized by clustered pyknotic pyramidal neurons that appear with fragmented cytoplasm and condensed nuclei within soma (red arrow). Perineural spaces can be seen surrounding degenerating neurons (red arrows) Axons and dendrites are scarcely appreciable around neurons in this group. C treatment shows toxic effect on the PFC neuronal health (red arrows).

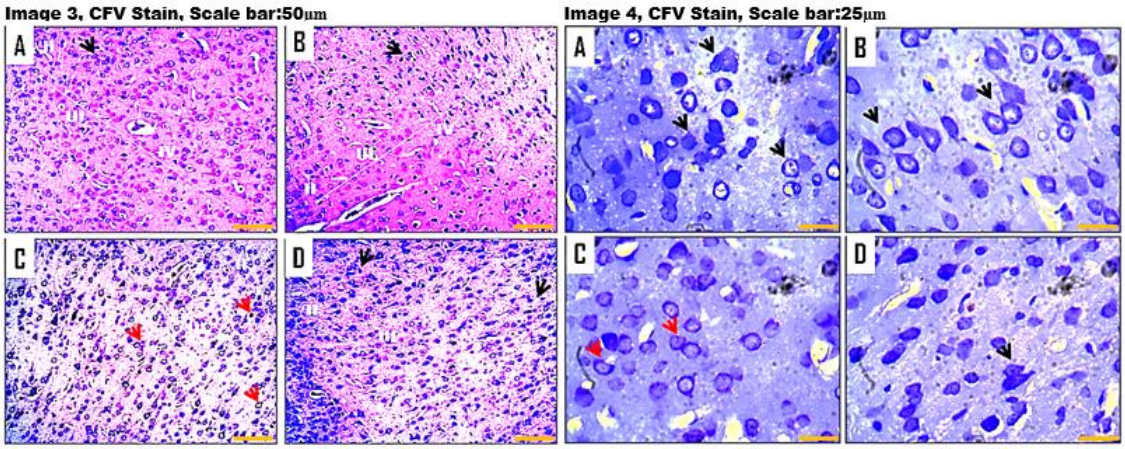

Figure 7. Photomicrographs showing panoramic views of prefrontal cortex (PFC) general histomorphological presentations in Wistar rats across the study groups. Cresyl Fast Violet stain (Images 3&4 scale bars -50µm and 25µm respectively). The molecular layer (I), External granular layer (II), External pyramidal layer (III), Internal granular layer (IV), Internal pyramidal layer (V) and the multiform layer (VI) are demonstrated across study groups however layers II -IV are well captured across study groups.

Image A, B and D (10mg/kg, 20mg/kg and control respectively) (Fig. 7) did not show any observable significant altered panoramic morphological presentation of the cerebral cortical layers from this study. In these groups, the fine array of cells within the cerebral cortex can be seen distinctly arranged, cellular density appears normal across all cortical layers, although the cell layers are less conspicuous at 50µm, they show proper cellular organization, staining intensity appears normal with no signs of chromatolysis with intact Nissl profiles and cellular processes (black arrows). Group C (30mg/kg) dosage on the other hand, induced degenerative changes characterized by comparatively sparsely distributed cellular density across the cortical granular and pyramidal layers with increased severity of chromatolysis and poorly outlined Nissl profiles (red arrows). Tiger nut treatments had no neurodegenerative effects on the cerebral histology at low and medium dosage (10mg/kg and 20mg/kg respectively) as the general cortical morphology in groups A and B relative to D presents no observable pathological features but depleted cellular changes are observable in group C treated with higher dose of tiger nut.

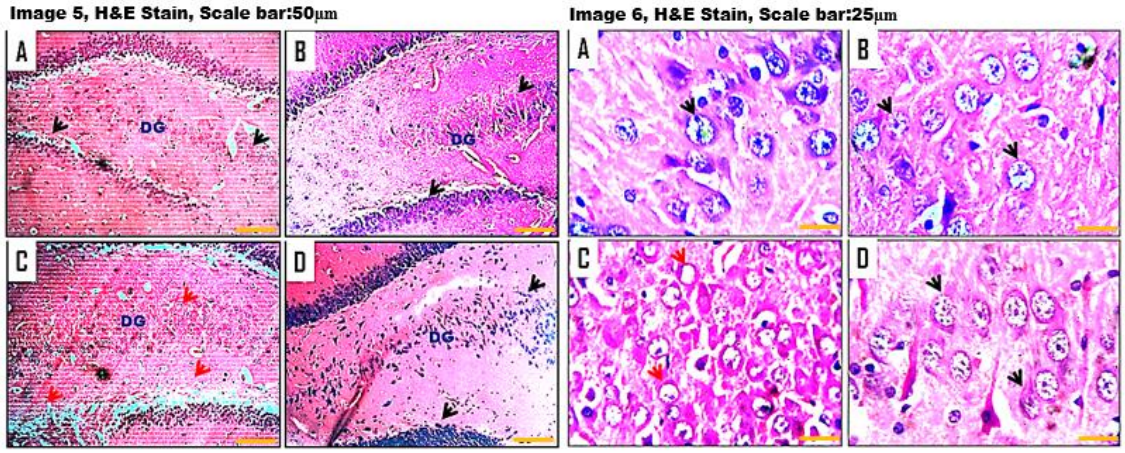

Figure 8. Photomicrographs showing panoramic views of hippocampus general morphological presentations in Wistar rats across the various study groups. Hematoxylin and Eosin stain (Images 5&6, Scale bars -50µm and 25µm respectively). The Dentate gyrus (DG) composed of granule cells, Cornu amonus containing pyramidal cells, are well demonstrated across the study groups.

A and B relative to D treatments (10mg/kg, 20mg/kg and control) (Fig. 8) did not show altered panoramic morphological presentation of the hippocampal layers from this study. In these groups, the fine array of cells within the hippocampus can be seen distinctly arranged from the Cornu amonus (CA) to the dentate gyrus (DG). In addition, cellular density within groups A, B and D appears normal across all hippocampal layers, although the granule cell layers are more conspicuous at 25µm (black arrows). C treatment (30mg/kg) on the other hand, induced degenerative changes in the hippocampus and was characterised by fragmented pyramidal and granule cell layer. Also, there appeared to be a comparative increased cell density in the hippocampal granular layer of group C.

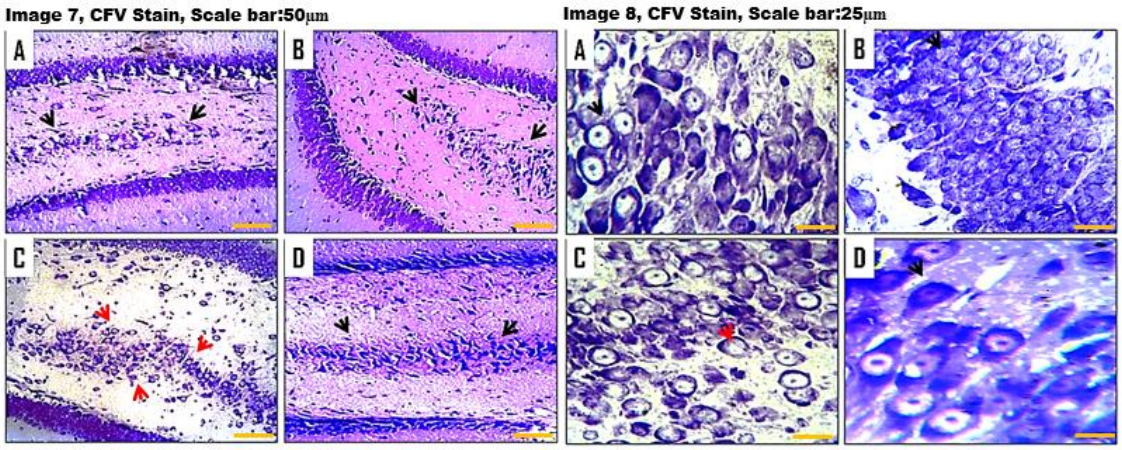

Figure 9. Photomicrographs showing panoramic views of hippocampus general morphological presentations in Wistar rats across the various study groups. Cresyl Fast Violet stain (Images 5&6, Scale bars -50µm and 25µm respectively). The Dentate gyrus (DG) composed of granule cells, Cornu amonus containing pyramidal cells, are well demonstrated across the study groups.

A and B relative to D treatments (10mg/kg, 20mg/kg and control) (Fig. 9) did not show altered morphology, they show distinct cellular layering, staining intensity appears normal with no signs of chromatolysis with intact Nissl profiles and cellular processes (black arrows). Group C (30mg/kg) dosage on the other hand, induced degenerative changes characterized by comparatively increased cellular density with loss of cellular processes across the cortical granular and pyramidal layers with increased severity of chromatolysis and poorly outlined Nissl content (red arrows). Tiger nut treatments had no neurodegenerative effects on the cerebral histology at low and medium dosage (10mg/kg and 20mg/kg respectively) as the general cortical morphology in groups A and B relative to D presents no observable pathological features but depleted cellular changes are observable in group C treated with higher dose of tiger nut.

4. DISCUSSION

Natural products derived from plants, have been used to help mankind sustain its health since the dawn of medicine, and as well serve as phytomedicine providing cure for various illness and as well providing templates for the development of new drugs. Measuring behavioural outcomes represent an important means of evaluating treatment effectiveness. As a correlative test for cellular, molecular and neuropathological changes within the prefrontal cortex and hippocampus in this study, we assessed alternation behavior in treated rats. Y maze spontaneous alternation test for behavioural assessment is based on the natural tendency in rodents to explore the maze systematically entering each arm in turn. The result of the test revealed a significant reduction in the correct percentage alternation in Group C animals which received the extract at 30mg/kg body weight when compared with the control (Group D). Group B which received the extract at 20mg/kg body weight had a higher correct percentage alternation in comparison to the control, but was not statistically significant. Group A which received the extract at 10mg/kg body weight was relatively at par with the control group (group D). The results gotten instigated that at a relatively low to moderate dose (10mg/kg and 20mg/kg), of tiger nuts was somewhat beneficial to cortico-hippocampal dependent cognition and working memory. But, at a high dose of 30mg/kg it impacts negatively. It therefore suggests tiger nut as a "double-edged sword". The possibility that alteration of normal mitochondrial redox and glucose bioenergetics dysfunction, initiated by high dose is responsible for impaired performance of treated rats in the Y-maze recorded in this study is high. The biochemical indices monitored in the brain are useful "markers" for assessment of tissue integrity. The measurement of activities of various enzymes in the tissues and body fluids plays significant role in disease investigation and diagnosis.

The biochemical indices monitored in the brain are useful "markers" for assessment of tissue integrity. The measurement of activities of various enzymes in the tissues and body fluids plays significant role in disease investigation and diagnosis. Tissue enzymes can also indicate tissue cellular damage caused by chemical compounds in the extract long before structural damages that can be picked by conventional histological techniques. Superoxide Dismutase (SOD) serves a key antioxidant role. SOD decrease reactive oxygen species generation and oxidative stress and inhibits endothelial activation and indicate that modulation of factors that govern adhesion molecule expression and leukocyte-endothelial interactions. Superoxide dismutase levels in the medium (20mg/kg body weight) dose increased in comparison to the control, and this increase was statistically significant. SOD level in the high dose group of 30mg/kg body weight was very well reduced. The former (20mg/kg) gives truth to the claim that tiger nut has antioxidative properties (18). This result however, further suggests that tiger nut exhibits some degree of

paradoxical functional facilitation i.e. it is detrimental at a high dose but is potentially beneficial at medium dose. There was also significant increase in the level of catalase activity in the prefrontal cortices of rats treated with 10mg/kg body weight and 20mg/kg body weight of tiger nut when compared to the 30mg/kg body weight group. Malondialdehyde (MDA) is a lipid peroxidation index. Lipid peroxidation is the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. MDA levels increased in the treated groups with increasing dose of tiger nuts (10mg/kg, 20mg/kg, and 30mg/kg), when compared with the control group. This increase was however significant only in the 30mg/kg body weight group. These antioxidant findings correspond with the total antioxidant capacity (TAC) test carried out in this study.

Cytomorphological appraisal show that distilled water treatment (Group D) did not alter the panoramic morphological presentation of the prefrontal and hippocampal layers from this study. In these group, the fine array of cells within the cerebral cortex and hippocampus can be seen distinctly arranged the cellular layers of the two brain regions examined. In addition, cellular density appears normal across all cortical layers, they show proper cellular outline with a distinct layering. Treated groups (A and B) also showed similar cellular integrity in these brain regions relative to distilled water treatment. The 30mg/kg body weight treatment, induced degenerative changes in the cortical and hippocampal layers and was characterised by fragmented granule cells, pyknosis, mild fibrosis across the neuronal profiles, degenerated granule and pyramidal cells and major parts of the cellular projections showed total depletion. Also, there appear to be a comparatively reduced cellular density in the cortical granular layer as well as some chromatolytic changes and reduced Nissl substance was observed within this group.

CONCLUSION

Interestingly, our study showed that Tigernut interference with normal neurochemical redox and cellular presentation at 30mg/kg dosage within the mitochondrial milieu of cortical and hippocampal cells resulted in an increased production of free radicals, which overwhelmed the intracellular antioxidant system. The highly reactive and unstable excess neuro-cytotoxic molecules then oxidized cellular components including DNA and lipids, thereby causing damage to the neuronal membrane. These degenerative changes compromised neuronal biochemical homeostasis and led to the aggregation of excess production of these cytotoxic molecules that subsequently initiated neuronal hypertrophy and loss of Nissl substance. We conclude that 30mg/kg of Tigernut altered behavioural, biochemical, enzymatic and structural profiles of rat prefrontal cortex and hippocampus. In addition, 10mg/kg and 20mg/kg of Tiger Nut treatment exhibited appreciable neuroprotective and excess production of fee radical inhibitory potentials and this neuromodulatory potentials should be explored more. It is important to note that Tiger Nut extract is more efficacious when given at low and medium doses (10mg/kg and 20mg/kg) than high dose (30mg/kg) from this study.

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AUTHOR CONTRIBUTIONS

EOY, TTA, ITG and ETL initiated the research, participated in the design and implementation of the experiments as well as manuscript writing. OST participated in the analysis of results and manuscript writing and also proof read the article for final corrections. ASA took part in research initiation.

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