DOI: https://doi.org/10.4314/njbmb.v38i4.5 NJBMB (2023) 38(4) 197-205

Nigerian Journal of Biochemistry and Molecular Biology

The Official Publication of the Nigerian Society of Biochemistry & Molecular Biology (NSBMB). Journal homepage: https://www.nsbmb.org.ng/journals

e-ISSN: 2659-0042

Research Article

Antioxidant and Protective Activities of Aqueous *Theobroma cacao* Seed Extract against Aluminium-induced Hippocampal Toxicity in Wistar Rats

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OPEN ACCESS

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ARTICLE HISTORY
Received: 23/10/2023
Reviewed: 15/12/2023
Revised: 26/12/2023
Accepted: 08/01/2024
Published: 10/01/2024

CITATION
Okhah, A.A. and Enogieru, A.B. (2023). Antioxidant and protective activities of aqueous *Theobroma cacao* seed extract against aluminium induced hippocampal toxicity in wistar rats. *Nigerian Journal of Biochemistry and Molecular Biology*. 38(4), 197-205
https://doi.org/10.4314/njbmb.v38i4.5

ABSTRACT

Aluminium (Al) is a known toxic metal linked to oxidative damage and neurodegeneration. Hence, the antioxidant and neuroprotective effects of aqueous *Theobroma cacao* seed extract (CO) in aluminium chloride-exposed Wistar rats was examined. The study consisted of six (6) groups containing six (6) Wistar rats each. Group A (Control) received 1 ml of distilled water/day. Group B (Al) received 100 mg/kg body weight (BW) of Al only. Group C received 250 mg/kg BW/day of CO and 100 mg/kg BW of Al. Group D received 500 mg/kg BW of CO and 100 mg/kg BW of Al. Group E received 250 mg/kg BW of CO only. Group F received 500 mg/kg BW of CO only. The administration was via an orogastric tube for 28 days after which the Novel Object Recognition (NOR) neurobehavioral test was conducted. Thereafter, the rats were sacrificed and the hippocampi were harvested for antioxidant and histological evaluations. A significant decrease in the antioxidant enzymes activities, final body weight and neurobehavioural functions were observed in the rats treated with Al only following comparison with the control and CO-treated groups. However, CO protected against these Al-induced effects. Also, several histological alterations were observed in the Al groups, whereas control and CO-treated groups were relatively normal. Taken together, CO had potent antioxidant and protective activities against Al-induced toxicity in the experimental rats.

Keywords: *Aluminium; Neurotoxicity; Neuroprotection; Hippocampus; Theobroma cacao*

INTRODUCTION

Aluminium, a universal element, is often considered as the third most frequent element on Earth (Mitkus *et al*., 2011). Human contact with aluminium occurs via medication, utensils, food, water as well as air and its widespread presence, both in foodstuffs and the environment, makes it virtually impossible to avoid (Rahimzadeh *et al*., 2022). Aluminium is neurotoxic to humans causing neuronal degeneration and apoptosis which impedes recognition and lowers the capacity for learning and memory (Dey *et al*., 2022). Reports indicate that the targets of Aluminium toxicity include the hippocampus, cerebellum and cerebrum (Yuan *et al*., 2012). A strong association exists between aluminium exposure, Alzheimer's disease and neurodegeneration (Kandimalla *et al*., 2016; Maya *et al*., 2016). A primary mechanism underlying aluminium-induced neurotoxicity is oxidative stress which is typified by a pro-oxidant/antioxidant imbalance. Aluminium's pro-oxidant activity induces protein and lipid oxidation, excessive free radical production and oxidative stress, thus resulting in its toxic effects (Exley, 2013). Antioxidants are reported to enhance cellular defence and mitigate oxidative stress (Enogieru *et al*., 2018; Enogieru and Momodu, 2021). Recently, *Theobroma cacao* (Cocoa) has become a focus of

investigation due to its diverse oxidant-attenuating and anti-cancer activities (Baharum *et al*., 2016).

Cocoa and its constituent parts have been used in the treatment of diseases and have been reported for pharmacological properties including anti-malarial, anti-hypertensive, anti-diabetic, and anti-influenza effects (Kim *et al*., 2011; Ishaq and Jafri, 2017). Catechins and oligomeric procyanidins, found in Cocoa, have noteworthy inflammation-attenuating activity and polyphenols from cocoa exhibit strong radical scavenging effects (Andújar *et al*., 2012). Additionally, flavanols, from Cocoa, are reported to be useful in the management of dementia and stroke in elderly humans (Sorond *et al*., 2008). However, there is a dearth of literature evidence on the protective role of *Theobroma cacao* seed against aluminium-induced hippocampal toxicity in animal models. Consequently, this study aimed to investigate such activity in Wistar rats.

MATERIALS AND METHODS

Extract preparation

Purchase of *Theobroma cacao* seeds were from the Isinkan market in Akure, Ondo state, and authenticated at the University of Benin (Department of Plant Biology and Biotechnology), with UBH-T562 as the authentication number. The seeds (dried) were powdered using an industrial grinding machine and thereafter 2 kg was soaked in 10 litres of distilled water and was extracted for Twenty-four hours. Thereafter, filtration of the extract was via a Whatman's filter paper before freeze-drying (LGJ-10, SearchEquipment, UK) of the filtrate to get a dried powder.

Phytochemical investigation

Qualitative phytochemistry of Cocoa seeds was carried out using standard methods (Trease and Evans, 1983; Sofowora, 1993). Compounds such as tannins, phenols, flavonoids, steroids, saponins, terpenoids, carbohydrates, phylobotannins, and alkaloids were investigated.

Acute toxicity study

The acute toxicity study followed previously reported methods (Lorke, 1983; Enogieru and Inegbedion, 2022). Examination for behavioural deviations and death was done for seventy-two hours.

Experimental rats

Thirty-six (36) Wistar rats, weighing between 150-180 g were bought and accommodated in Anatomy Department, University of Benin, Nigeria. The rats were nourished with standard rat chow (Bendel livestock feed, Edo state, Nigeria) and water freely. Acclimatization was for fourteen days prior to the start of the study.

Experimental design

Group A (Control) received 1 ml of distilled water/day. Group B (Al) received 100 mg/kg body weight (BW) of Aluminium (Al) only. Group C (Al + CO1) received 250 mg/kg BW/day of aqueous *Theobroma cacao* seed extract and 100 mg/kg BW of Al. Group D (Al + CO2) received 500 mg/kg BW of aqueous *Theobroma cacao* seed extract and 100 mg/kg BW of Al. Group E (CO1) received 250 mg/kg BW of aqueous *Theobroma cacao* seed extract only. Group F (CO2) received 500 mg/kg BW of aqueous *Theobroma cacao* seed extract only. Pretreatment with aqueous *Theobroma cacao* seed extract was done sixty minutes before the rats received Al daily for twenty-eight days.

Evaluation of neurobehavioural activity

The novel object recognition test was carried out in a piece of open wooden equipment (80 × 60 × 40 cm) as previously reported (Pitsikas *et al*., 2001; Enogieru and Omoruyi, 2022; Enogieru and Egbon, 2022). Briefly, a two-minute exploration of the apparatus was permitted on the 27th day of the experiment. Twenty-four hours later (test day), a five-minute test (T1) was done, with 2 familiar objects (FO1 & FO2). Another five-minute test (T2) was done and FO2 was substituted with a new object (NO) and FO1 and NO exploration times were documented. The discrimination between FO1 and NO was calculated as follows: DI = NO−FO1/NO + FO1.

Evaluation of weights

Following the sacrifice of rats under low-level ether anaesthesia, the brains of experimental rats were removed and weighed. The relative weight of the brain (%) was calculated as previously reported (Kim *et al*., 2008). Thereafter, the hippocampus was dissected out and each half of the hippocampus contained in the hemispheres of the brain was used for biochemical and histological investigations, respectively.

Evaluation of biochemical parameters

Homogenization of the hippocampus was done in ice-cold 20 Mm Tris-HCl buffer (pH 7.4), and centrifuged at 10,000g for ten minutes at 4°C (Montilla *et al*., 2005). The resulting supernatant was utilized for the evaluation of malondialdehyde (Buege and Aust, 1978), catalase (Cohen *et al*., 1970), superoxide dismutase (Misra and Fridovich, 1972) and glutathione peroxidase (Nyman, 1959).

Histological evaluation

Following appropriate fixation (10% buffered formal saline) of the hippocampus for seventy-two hours, processing through the paraffin wax embedding and the hematoxylin

and eosin staining methods were carried out as previously described (Drury and Wallington, 1980).

Statistical analysis

A version 7 GraphPad Prism Software was utilized for data statistics and is represented by the mean ± standard error of mean (SEM). ANOVA and Tukey's multiple comparisons post hoc test defined significance ($p \leq 0.05$).

RESULTS

Phytochemistry composition

Findings showed that aqueous *Theobroma cacao* seed extract contained glycosides, saponins, phenolic, terpenoids, eugenols, alkaloids, flavonoids and reducing sugar (Table 1). However, steroids and tannins were absent.

Acute toxicity

Results showed the absence of behavioural alterations and death at doses from 10 mg/kg to 5000 mg/kg body weight.

Body and brain weights

Results showed a significant ($p<0.05$) reduction in the final body and whole brain weights of group B rats (Al) following comparison to control. Although an increase was noticed in the final body weight of groups C (Al + CO1) and D (Al + CO2) following comparison to group B, it was insignificant ($P>0.05$).

Neurobehavioural findings

Figure 1 illustrates the trial test for FO1 and FO2. In Figure 2, a significant ($p<0.05$) reduction was seen in the mean exploration times of NO in group B following comparison to control, but, a significant ($p<0.05$) improvement was noticed in groups C (Al + CO1) and D (Al + CO2) when compared to the aluminium only treated group. In Figure 3, a significant ($p<0.05$) reduction was seen in T1 in group B (Al) following comparison to control. Figure 4 shows the discrimination index across experimental groups. Here, a significant ($p<0.05$) reduction was observed in group B following comparison to control. However, a significant ($p<0.05$) rise was seen in groups C (Al + CO1) and D (Al + CO2) following comparison to group B.

Table 1. Qualitative Phytochemical Analysis of Aqueous *Theobroma Cacao* Seed Extract

Phytochemicals	Results
Glycoside	+
Saponins	+
Phenolics	+
Terpenoids	+
Eugenols	+
Alkaloids	+
Flavonoids	+
Reducing sugar	+
Steroids	-
Tannins	-

(+) present; (-) absent

Table 2. Weights across Experimental Groups.

Groups	Initial BW (g)	Final BW (g)	Weight Change (g)	Absolute whole brain weight (g)	Relative brain weight (%)
Control	151.2 ± 8.261	182.2 ± 11.280	31.0 ± 5.52	1.720 ± 0.037	1.077 ± 0.076
Al	156.8 ± 4.748	142.6 ± 8.152#	-14.2 ± 9.62#	1.680 ± 0.037#	1.030 ± 0.036
Al + CO1	150.4 ± 5.183	169.2 ± 5.267	18.8 ± 4.14*	1.740 ± 0.040	1.027 ± 0.032
Al + CO2	151.2 ± 4.532	175.0 ± 3.962	23.8 ± 4.58*	1.740 ± 0.060	1.013 ± 0.024
CO1	152.0 ± 7.477	173.8 ± 8.303	21.8 ± 6.73	1.740 ± 0.060	1.057 ± 0.029
CO2	153.0 ± 2.025	186.8 ± 12.640	33.8 ± 11.32	1.720 ± 0.058	1.053 ± 0.050

and * represents $p< 0.05$ following comparison to control and A1 respectively.

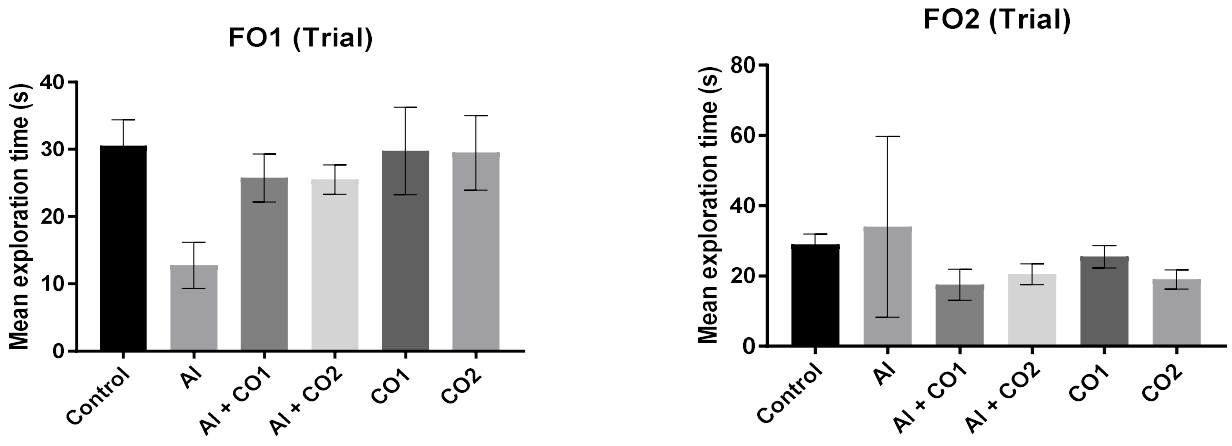

Figure 1. Mean Exploration Times (trial test) across Experimental Groups. (FO1 – Familiar object 1; FO2 – Familiar object 2)

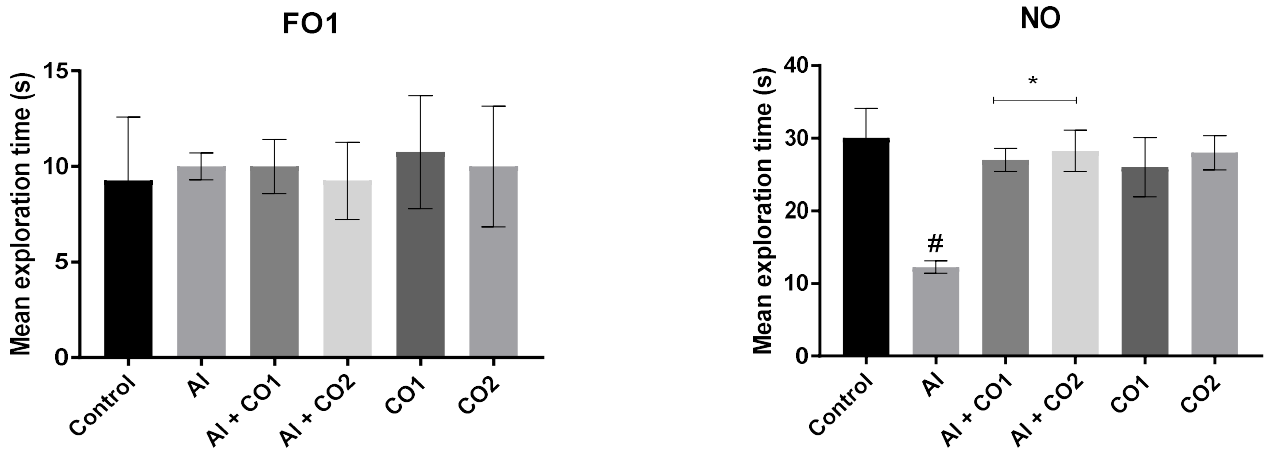

Figure 2. Mean Exploration Times (real test) across Experimental Groups.
[#] and * represent $p < 0.05$ following comparison to control and Al respectively. (FO1 – Familiar object 1; NO – Novel object)

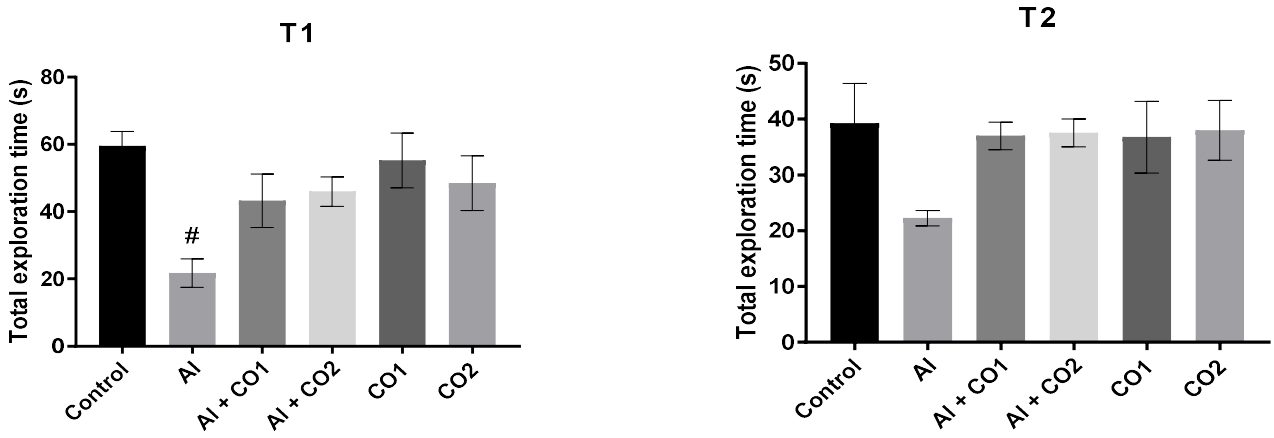

Figure 3. Total Exploration Times across Experimental Groups.
[#] and * represent $p < 0.05$ following comparison to control and Al respectively. (T1 – Total exploration time 1; T1 – Total exploration time 2)

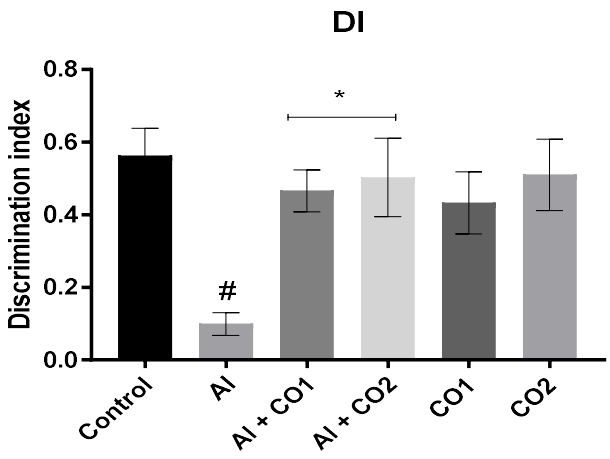

Figure 4. Discrimination Index (DI) across Experimental Groups.
[#] and * represent p< 0.05 following comparison to control and Al respectively.

Findings on antioxidants activity

A significant (p<0.05) decrease in CAT, SOD and GPx was observed in aluminium-only treated group B following comparison to control. However, a significant (p<0.05) improvement in CAT, SOD and GPx was observed in groups C (Al + CO1) and D (Al + CO2) when compared to the aluminium-only treated group B (Figure 5).

Findings on lipid peroxidation

Aluminium-only treatment resulted in a significant (p<0.05) elevation in lipid peroxidation (MDA) in the hippocampus of group B rats following comparison to control. However, the decrease in lipid peroxidation in the hippocampus was significant (p<0.05) in group C (Al + CO1) and group D (Al + CO2) following comparison to Aluminium-only treated group (Figure 6)

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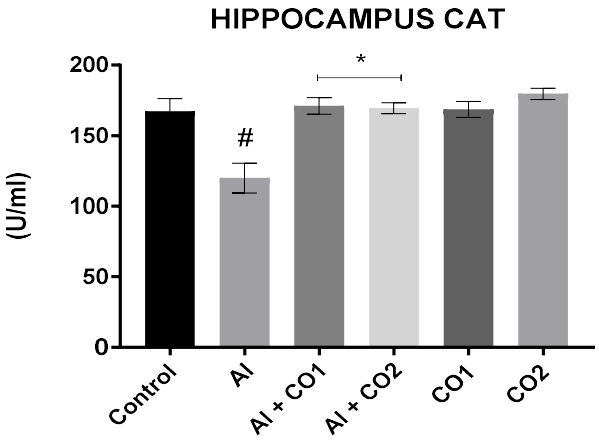

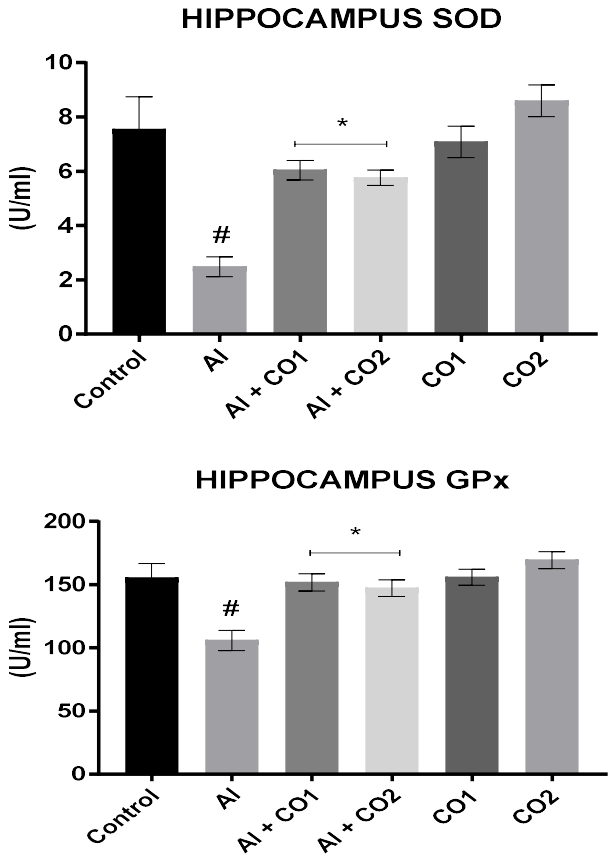

Figure 5. Antioxidants activity in the hippocampus.
[#] and * represent p< 0.05 following comparison to control and Al respectively.

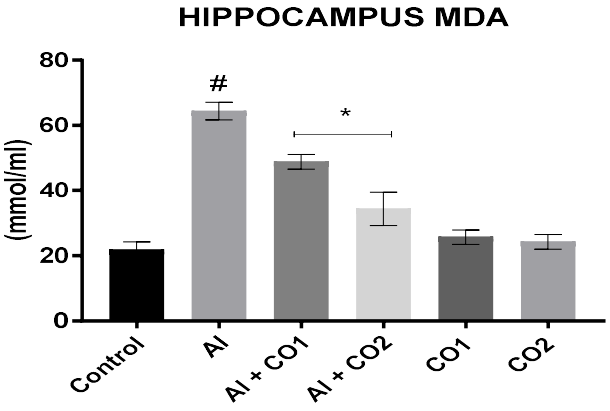

Figure 6. Activity of Lipid Peroxidation in the hippocampus across experimental groups.

[#] and * represent p< 0.05 following comparison to control and Al respectively.

Histological findings in the hippocampus

Histological findings in the hippocampus are shown in Figure 6. Photomicrographs of control rats revealed normal hippocampus histology of pyramidal cells and astrocytes (Figure 6A). However, atrophy and vacuolation of astrocytes and pyramidal cells were seen in group B rats treated with aluminium-only (Figure 6B). For rats in groups C (Al + CO1) and D (Al + CO2), the histological appearance was similar to control, and displayed significantly reduced atrophy and vacuolation of the astrocytes and pyramidal cells, thus demonstrating a protective effect of Cocoa (Figure 6C-D). It was also observed that rats treated with aqueous *Theobroma cacao* seed extract only showed normal architecture of the hippocampus (Figures 6E-F).

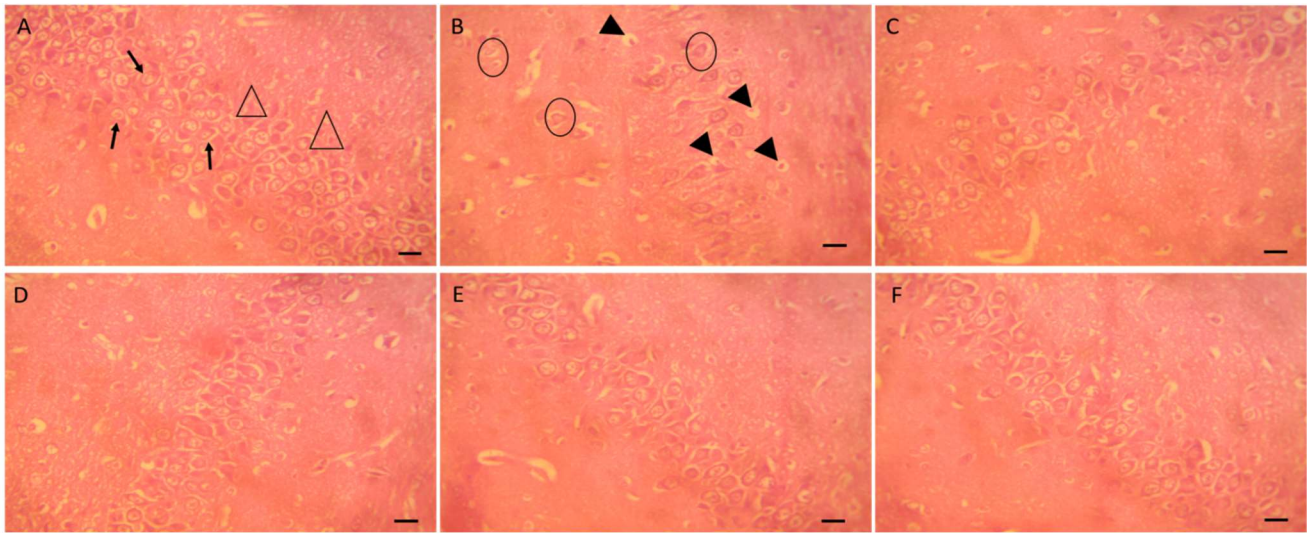

Figure 7. Representative Histology of the Hippocampus.

(A) Control rats revealed the normal pyramidal cells (arrows) and astrocytes (triangles). (B) Group B rats showed atrophy and vacuolated astrocytes (arrowheads) and pyramidal cells (circles) (C-D) Group C and D rats revealed relatively pyramidal cells and few astrocytes vacuolation (E-F) Group E and F rats revealed normal pyramidal cells and astrocytes. H&E, Scale bar: 25µm.

DISCUSSION

Aluminium (Al) is ubiquitous in the environment and exposure to it is unavoidable (Saiyed *et al*., 2005). It is neurotoxic to humans and is reported to accumulate throughout the brain especially the hippocampus (Julka *et al*., 1996). Consequently, this study investigated whether aqueous *Theobroma cacao* seed extract will protect against hippocampal toxicity in Al-exposed Wistar rats. Our findings showed that in rats treated with Al-only, there was significant weight loss following comparison to control, however, Al-exposure, in the present dose and duration, did not affect relative brain weights in agreement with previous studies (Nayak and Chatterjee, 2001; Usen and Enogieru, 2023). Reports indicate that body weight is a sensitive gauge of toxicity (Ogueche *et al*., 2014). The observed reduction in body weight might be a result of the possible meddling of Al with normal metabolic processes; this Al-induced decrease in body weight agrees with the previous findings (Salam *et al.,* 2005; Azzaoui *et al*., 2008; Tair *et al*., 2016). However, pretreatment with aqueous *Theobroma cacao* seed extract was able to mitigate the adverse effect of Al on the body weight of rats, thus highlighting a protective activity.

The hippocampus is vital for spatial memory development and is extremely susceptible to oxidative damage. The preservation of the integrity of the hippocampus is essential for the inhibition of cognitive dysfunction (Marosi *et al*., 2012). Al exposure has been linked to confusion, memory deficiencies and dementia (Parkinson *et al*., 1981). NOR test is a non-spatial working memory test, used to measure the retention and discrimination ability of animals (Malik *et al*., 2013). Our results showed that rats in the aluminium-only group had a decrease in the mean and total exploration times as well as a lower discrimination index following comparison to control. Nevertheless, in rats pretreated with aqueous *Theobroma cacao* seed extract, a significantly higher discrimination index was observed which suggests that Cocoa improves memory and discrimination ability. This agrees with a study demonstrating that cocoa flavonol improves overall cognitive function and performance in elderly subjects (Mastroiacovo *et al*., 2015).

The body's endogenous antioxidant system cooperatively fights against and scavenges free radicals. This is achieved through first-line defence antioxidant enzymes including Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx), all playing an essential role in the whole antioxidant defence scheme (Ighodaroab *et al*., 2018). A significant reduction in SOD, CAT and GPx in the

hippocampus of Al-treated rats suggests that Al can adversely alter antioxidant activities. Compromised antioxidant defences could arise from the inhibitory activities of Al on these enzymes, thus making the cells more vulnerable to oxidative damage. The attenuation of the impairment of antioxidant enzymes activity in rats pretreated with Cocoa indicates its potent antioxidant properties. This agrees with earlier reports demonstrating that Cocoa is a beneficial natural antioxidant source with antioxidative and free radical scavenging activities (Lamuela *et al*., 2005; Fernandez *et al*., 2015). Malondialdehyde (MDA) is often utilized as a marker of lipid peroxidation. The significant elevation in hippocampal MDA level in Al-exposed rats indicates high lipid peroxidation activity. This agrees with earlier reports (Exley, 2004; Yuan *et al*., 2012). Conversely, the significant reduction in the hippocampal MDA level of rats pretreated with aqueous *Theobroma cacao* seed extract signifies its ability to protect against lipid peroxidation.

The histological observations show atrophy and vacuolated astrocytes in the hippocampus of rats treated with aluminium only. This is in agreement with Bittencourt and colleagues in a report that aluminium causes hippocampal neurodegeneration and atrophy (Bittencourt *et al*., 2022). Hippocampal neurodegeneration and atrophy are reported to cause learning and memory deficits (Anand and Dhikav, 2012; Gemmell *et al*., 2012). Pretreatment of rats with Cocoa mitigated the histological alterations observed in the aluminium-only group of rats, thus highlighting a protective effect. Also, the treatment of rats with Cocoa-only did not cause any alteration to the hippocampus and displayed a normal histological appearance which suggests that Cocoa was not toxic to the hippocampus of the Wistar rats. This agrees with earlier reports demonstrating that *Theobroma cacao* does not affect the brain (Kebe *et al*., 2019; Martín *et al*., 2020).

CONCLUSION

Findings from this study highlight the antioxidant and protective activities of aqueous *Theobroma cacao* seed extract against aluminium-induced hippocampal toxicity in adult Wistar rats. Consequently, efforts should be made to harness and further develop this seed as a potential neuroprotective agent that might be advantageous in the search and development of novel therapeutic options relevant to the treatment of Al toxicity and its related neurodegenerative disorders.

AUTHORS' CONTRIBUTIONS

Author ABE conceptualize the study, including compilation of methodology; formal analysis; writing—review and editing; and supervision. Author AAO participated in investigation of the study and writing of original draft preparation.

FUNDING STATEMENT

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

ACKNOWLEDGEMENT

The authors are grateful to Mr. David Odili and Miss Osariemen Aghedo for their technical assistance throughout the study duration.

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