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Research Article

Neurobehavioural and Histological Alterations in Lead Acetate-Exposed Rats Pretreated with Aqueous Leaf Extract of *Vernonia amygdalina*

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ABSTRACT

Lead is a systemic toxicant that affects virtually every organ system, primarily the central nervous system, through an increase in the production of reactive oxygen species (ROS). This increase in ROS overwhelms the natural antioxidant system of the body leading to oxidative stress. Some plants such as *Vernonia amygdalina* have been reported to possess antioxidant activity which counteracts the damage induced by free radicals. Accordingly, this study was designed to investigate the possible protective activity of aqueous *Vernonia amygdalina* leaf extract against lead-induced neurotoxicity in adult Wistar rats. Thirty Wistar rats were randomised into six groups (A-F) consisting of five rats each for a study period of 28 days. Group A served as the control group, Group B was administered with 100mg/kg body weight of lead only, Groups C and D were pretreated with 200mg/kg and 400mg/kg bodyweight of aqueous *Vernonia amygdalina* leaf extract and 100mg/kg body weight of lead respectively. Groups E and F were administered with 200mg/kg and 400mg/kg body weight of aqueous *Vernonia amygdalina* leaf extract only. The Open field and Novel Object Recognition tests were assessed and thereafter, the cerebellum, cerebrum and hippocampus were harvested for histological examination. Results showed a significant decrease in the body and brain weights, ambulation, rearing and discrimination index as well as a conversely significant increase in grooming and immobility in lead-treated rats. Histological examination of lead-alone treated groups showed loss of some Purkinje cells in the cerebellum, degenerating pyramidal cells in the cerebrum and altered morphology with pyknotic nuclei in the hippocampus. Pretreatment of rats with *Vernonia amygdalina* attenuated lead-induced alterations to body and brain weights, neurobehavioural activities and tissue histomorphology, thus indicating a potent protective activity. These findings provide the first research evidence that *Vernonia amygdalina* protects against lead-induced neurotoxicity in Wistar rats.

Keywords: *Vernonia amygdalina; neurotoxicity; neuroprotection; neurobehaviour; Lead acetate; Wistar rats*

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INTRODUCTION

Lead is an environmental pollutant often regarded as the most abundant non-essential element in humans due to its dispersion in ambient air, food, drinking water and dust (Highab et al., 2018). Lead toxicity is a common public health threat in developing countries due to human activities such as farming, mining and burning of fossil fuels (Sanders et al., 2009). Although several occupational and public health safety measures have been carried out to limit human exposure to a minimal level, several cases of lead poisoning are still recorded to date. The most sensitive organ to lead toxicity is the brain (Cleveland et al., 2008). Within the brain, lead induces toxicity in the prefrontal cerebral cortex, hippocampus and cerebellum, leading to a variety of neurological disorders. Some chelating agents (drugs) have been produced to reduce blood levels of lead, but these chelating agents are not without side effects (Flora and Pachauri, 2010). The presence of lead in the brain causes an increase in the production of free radicals such as reactive oxygen species (ROS) (Patra et al., 2011). This increase in free radicals overwhelms the natural antioxidant system of the body thus leading to oxidative stress. Antioxidants, endogenous and/or exogenous, are reported to prevent or attenuate oxidative injury or damage (Enogieru and Momodu, 2021; Sharifi-Rad et al., 2020). Some plants such as *Coriander sativum* and *Vernonia amygdalina* have been reported to be rich in antioxidants (Adesanoye and Farombi, 2014; Tang et al., 2013). *Vernonia amygdalina* is a small shrub that grows in tropical Africa and is commonly called 'bitter leaf' in English because of its bitter taste (Bonsi et al., 1995). *Vernonia amygdalina* is reportedly useful in the treatment of gastrointestinal ailments, intestinal parasites, diarrhoea and possesses antimicrobial as well as antiparasitic activities. Bioactive compounds responsible for its ethnobotanical uses

include alkaloids, flavonoids, phenolic acids, steroids, anthraquinone, saponins, terpenes, lignans, coumarins, sesquiterpenes and xanthones (Oyeyemi et al., 2018). Although previous reports show that leaf extracts of *Vernonia amygdalina* possess antioxidative, immunomodulatory, anticancer, and anti-tumour properties, there is little evidence to demonstrate the neuroprotective activity of *Vernonia amygdalina* against lead-induced brain damage or injury. Consequently, this research is designed to investigate such activity. Findings from this study will provide the first research evidence on the protective activity of *Vernonia amygdalina* against lead-induced neurotoxicity in adult Wistar rats.

MATERIALS AND METHODS

This study was submitted for review and approval was granted by the Research Ethics Committee of the College of Medical Sciences, University of Benin, with the number CMS|REC|2021|168.

Plant Material and Preparation of aqueous extract: The leaves of *Vernonia amygdalina* were collected and identified at the Department of Plant Biology and Biotechnology, University of Benin, Edo state with herbarium number UBH-V342. Leaves were rinsed to remove any extraneous material and air-dried at room temperature until a constant dry weight was attained. The dried leaves were pulverized into powder using the British milling machine (Christy and Norris Limited, LAB MILL, 47454). The fine powder was weighed at 700g and was macerated with 1.2 litres of distilled water in a chromatographic jar for 24 hours. The mixture was filtered using Whatman filtered paper, and the filtrate evaporated at 60°C using a vacuum rotary evaporator (Buchi, Switzerland). The residue was freeze-dried using a vacuum freeze-drier, stored in a desiccator and thereafter preserved in a refrigerator at 4°C until required. The crude extract was dissolved in distilled water to make a concentration of 100 mg/ml from which different doses of 200 and 400 mg/kg body weight by oral route were reconstituted.

Preliminary phytochemical screening: Phytochemical screenings were performed using standard procedures (Sofowora, 1993; Trease and Evans, 1983). Carbohydrates, flavonoids, tannins, phenols, saponins, steroids, alkaloids, phlobotannins, and terpenoids were screened for their presence in the plant material.

Care and management of Animals: A total of 30 adult Wistar rats, bred at the animal house, Department of Anatomy, School of Basic Medical Sciences, University of Benin, Benin City, Edo State, Nigeria, weighing between 120g and 180g were used for this study. The rats were fed with standard rat chow (Bendel livestock feed, Edo state, Nigeria) and water throughout the entire study period. They were weighed weekly before commencement and throughout the experiment using a digital weighing scale calibrated in grams and recorded to the nearest whole number.

Research design: They were randomised into six groups, (A, B, C, D, E, and F) with 5 rats in each group. All administrations were given orally, alongside the rat's feed, throughout the entire study period of twenty-eight days. Group A served as the control group and was given normal saline. Groups B, C, D, E, and F served as the treatment groups. Rats in group B (Pb) were administered with 100mg/kg body weight of lead only; rats in group C (Pb + VA1) were pretreated with 200mg/kg body weight of aqueous *Vernonia amygdalina* leaf extract 1 hour before administration of 100mg/kg body weight of lead; rats in group D (Pb + VA2) were pretreated with 400mg/kg body weight of aqueous *Vernonia amygdalina* leaf extract 1 hour before administration of 100mg/kg body weight of lead; rats in group E (VA1) and F (VA1) were administered with 200mg/kg and 400mg/kg body weight of aqueous *Vernonia amygdalina* leaf extract respectively. Animals were sacrificed on day 29 by cervical dislocation after anaesthesia.

Chemicals and reagents: Normal saline was manufactured by Unique Pharmaceuticals, Sango-Otta, Nigeria and lead acetate by Loba Chemie Pvt. Ltd, Mumbai, India. Other reagents were all of analytical grade.

Evaluation of neurobehavioural activity: Neurobehavioural activities that were performed include the Open field test (OFT) and the novel object recognition (NOR) test. This OFT was performed according to the method by Olopade and colleagues with each rat placed in a 72cm by 72 cm square box with lines on its floor dividing it into 18cm by 18 cm square (Olopade et al., 2012). Each animal was placed in the centre of the field and rearing, grooming, ambulation and immobility were evaluated. The NOR was performed as previously described (Malik et al., 2013). In a wooden open box apparatus measuring 80×60×40cm, the objects to be differentiated were of two different shapes and colours and were heavy enough to prevent displacement by the animals during the test. A first 5min sample trial test (T1) included two familiar objects (FO1 and FO2) in the box. Following a 1 hour delay after T1, a second 5min real test (T2) was performed with FO2 replaced by a new object (NO). To assess the effect on long term memory, animals were placed in the apparatus and time spent by rats in exploring FO1 and NO was recorded. The total times spent by rats in exploring two identical objects in T1 and two different objects in T2 were recorded separately. To eliminate any bias in general levels of exploration, a discrimination index (D) was thereafter calculated; $D = N-F/N + F$.

Determination of relative brain weight: Following the evaluation of neurobehavioral activity, rats were sacrificed by cervical dislocation. Thereafter, the brains were accessed through a longitudinal cranial incision, weighed and dissected into the cerebellum, cerebrum and hippocampus. To mitigate the individual bodyweight differences, the relative brain weight (%) was expressed as a percentage of the final body weight at sacrifice (Kim et al., 2008).

Histopathological studies: The cerebellum, cerebrum and hippocampus were processed for routine hematoxylin and

eosin (H and E) staining using standard procedures previously reported (Wallington and Drury, 1980). Briefly, the excised tissues were fixed in Bouin's fluid for 72 h and processed by paraffin embedding with sections cut at 5µ thickness. The sections were for H and E light microscopic demonstration of the cerebellum, cerebrum and hippocampus histoarchitecture. The processed slides were captured with a LABO® research trinocular microscope (Labo Microsystems GmbH, Germany) on which were mounted an Omax 9.0MP USB Digital Microscope Camera (made in Korea) domiciled in the Department of Anatomy, School of Basic Medical Sciences, University of Benin, Benin city.

Statistical Analysis: Statistical analysis was performed using GraphPad Prism statistical package (version 7) with data expressed as the standard error of mean (SEM) using a one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison post-hoc test

RESULTS

Phytochemical screening

Results of the phytochemical screening are shown in Table I. The leaf extract of *Vernonia amygdalina* was found to contain carbohydrates, flavonoids, tannins, phenols, saponins, reducing sugars, steroids, alkaloids and cyanogenetic glycosides. However, phylobotanin was observed to be absent.

Table 1:
Qualitative phytochemical analysis of *Vernonia amygdalina* leaves

Phytochemicals	Results
Carbohydrates	+
Flavonoids	+
Tannins	+
Phenolic compounds	+
Saponins	+
Reducing sugar	+
Steroids	+
Cyanogenetic glycosides	+
Alkaloids	+
Phylobotanin	-

Effect of treatment on body and brain weights: Table 2 shows the changes in body and brain weight of the different experimental groups. There was a significant ($p< 0.05$) decrease in final body weight and absolute whole brain weight of rats treated with lead (Pb) alone when compared to control. Also, there was a significant ($p< 0.05$) increase in absolute whole brain weight of rats pretreated with 400mg/kg *Vernonia amygdalina* (Pb + VA2) when compared to those treated with lead alone.

Effect of treatment on Neurobehavioural activity: The findings from the OFT evaluation are presented in Fig. 1. There was a significant decrease ($p< 0.05$) in rearing activity in rats treated with lead alone (Pb) when compared to control and a significant increase ($p< 0.05$) in rearing activity in pretreated rats (Pb + VA1 & Pb + VA2) when compared to Pb. For grooming, a significant increase ($p< 0.05$) was observed in rats treated with lead alone (Pb) when compared to control and a significant decrease ($p< 0.05$) in pretreated rats (Pb + VA1 & Pb + VA2) when compared to Pb. For ambulation, a significant decrease ($p< 0.05$) was observed in rats treated with lead alone (Pb) when compared to control and a significant increase ($p< 0.05$) in rats pretreated with 400mg/kg *Vernonia amygdalina* (Pb + VA2) when compared to Pb. For immobility, a significant increase ($p< 0.05$) was observed in rats treated with lead alone (Pb) when compared to control and a significant decrease ($p< 0.05$) in pretreated rats (Pb + VA1 & Pb + VA2) when compared to Pb.

Table 2:
Bodyweight, absolute whole brain and relative brain weights of control and treatment groups after 28 days.

Groups	Initial BW (g)	Final BW (g)	Absolute whole brain weight (g)	Relative brain weight (%)
Control	165.8 ± 3.816	182.5 ± 4.664	1.725 ± 0.075	0.933 ± 0.034
Pb	153.8 ± 3.664	151.3 ± 3.351#	1.320 ± 0.080#	0.838 ± 0.038
Pb + VA1	156.8 ± 5.276	162.6 ± 4.411	1.540 ± 0.060	0.965 ± 0.040
Pb + VA2	161.8 ± 4.188	170.5 ± 1.848	1.625 ± 0.062*	0.950 ± 0.027
VA1	164.5 ± 8.261	179.3 ± 9.961	1.650 ± 0.065	0.925 ± 0.022
VA2	159.8 ± 5.138	174.0 ±5.986	1.725 ± 0.048	0.995 ± 0.032

*Values are given as mean ± SEM of each group. # $p<0.05$ compared with the control group; * $p<0.05$ compared with Pb-alone group.*

The findings from the NOR evaluation are presented in Fig. 2-5. For the trial test T1, a significant decrease ($p< 0.05$) in mean exploration times (FO1 and FO2) was observed in rats treated with lead alone (Pb) when compared to control and a significant increase ($p< 0.05$) for FO2 was observed in pretreated rats (Pb + VA1 & Pb + VA2) when compared to Pb (Fig. 2). For the real test T2, a significant decrease ($p< 0.05$) in mean exploration times for the novel object (NO) was observed in rats treated with lead alone (Pb) when compared to control and a significant increase ($p< 0.05$) was observed in pretreated rats (Pb + VA1 & Pb + VA2) when compared to Pb (Fig. 3). For the total exploration times (T1 and T2), a significant decrease ($p< 0.05$) was observed in rats treated with lead alone (Pb) when compared to control, however, a significant increase ($p< 0.05$) was observed in pretreated rats (Pb + VA2 for T1) and (Pb + VA1 & Pb + VA2 for T2) when compared to Pb (Fig. 4). The improved discrimination capacity (by *Vernonia amygdalina* at 200 and 400 mg/kg), in contrast to lead, was further apparent from the discrimination index of the animals (Fig. 5). Here, a significant decrease ($p< 0.05$) was observed in rats treated with lead alone (Pb) when compared to control and a significant increase ($p< 0.05$) was observed in pretreated rats (Pb + VA1 & Pb + VA2) when compared to Pb.

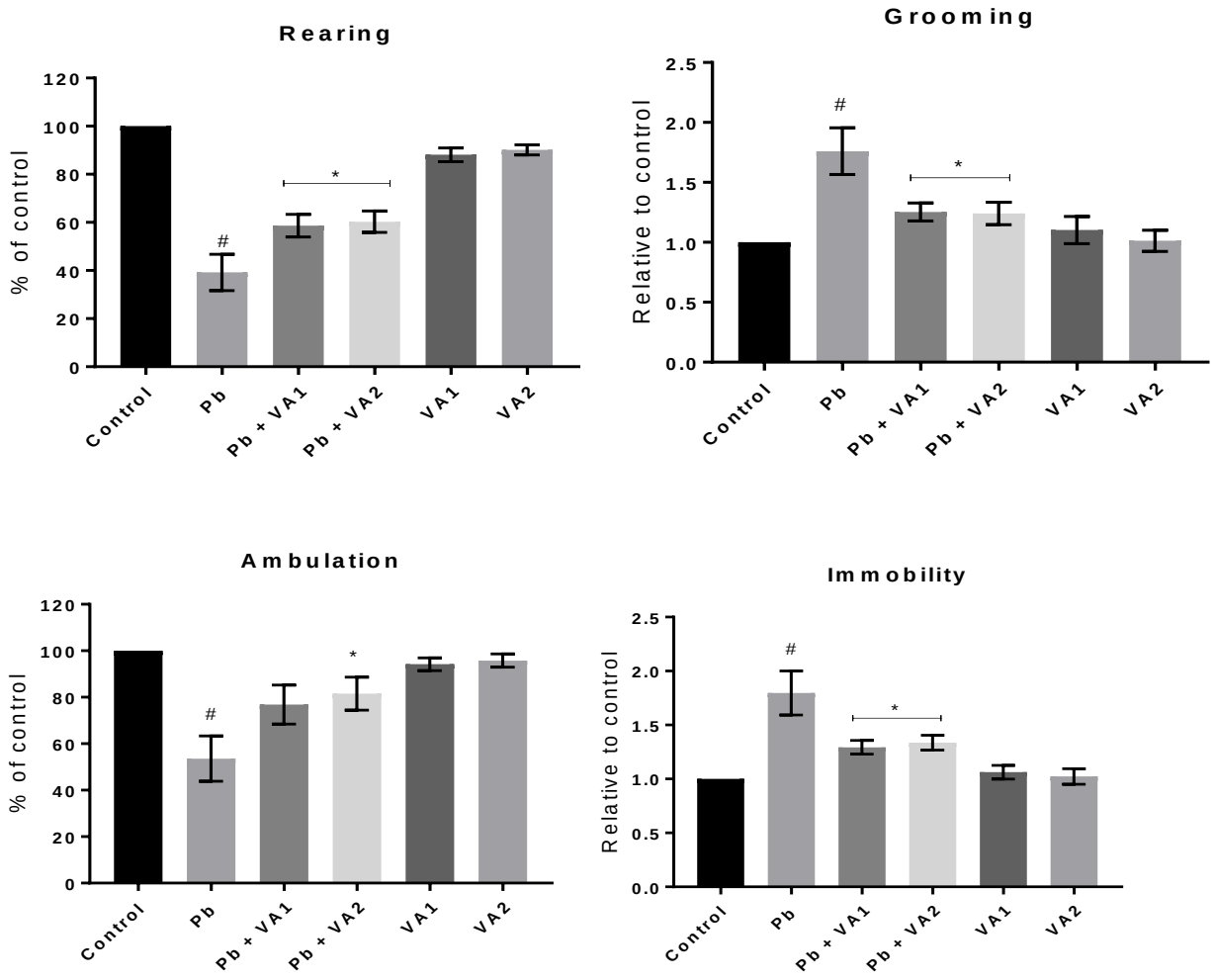

Fig. 1:
Open field test of control and treatment groups after 28 days. Values are given as mean ± SEM. # $p< 0.05$ compared with the control group; * $p< 0.05$ compared with the Pb-alone group

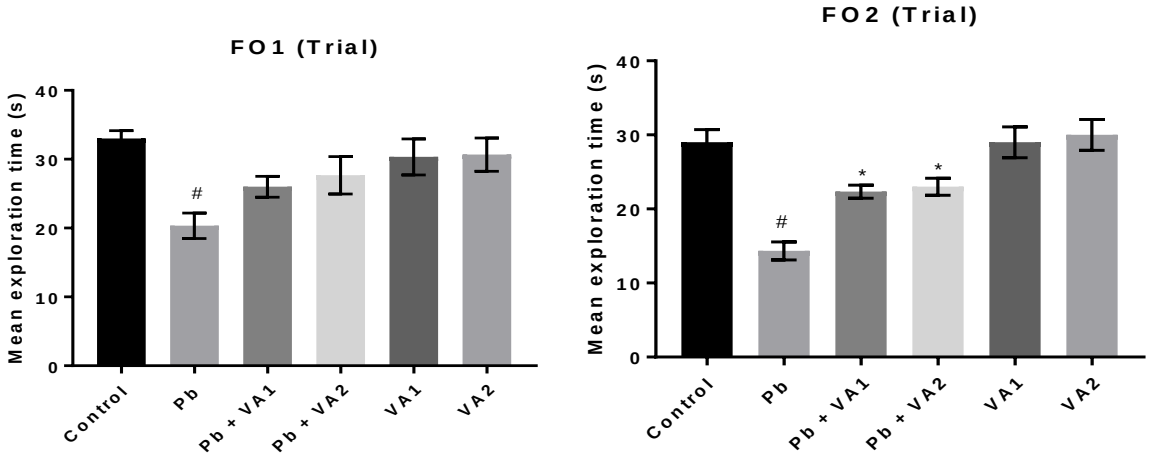

Fig. 2:
Trial novel object recognition test of control and treatment groups after 28 days. (FO1 – Familiar object 1; FO2 – Familiar object 2). Bars represent the mean ± SEM. # $p< 0.05$ compared with the control group; * $p< 0.05$ compared with the Pb-alone group.

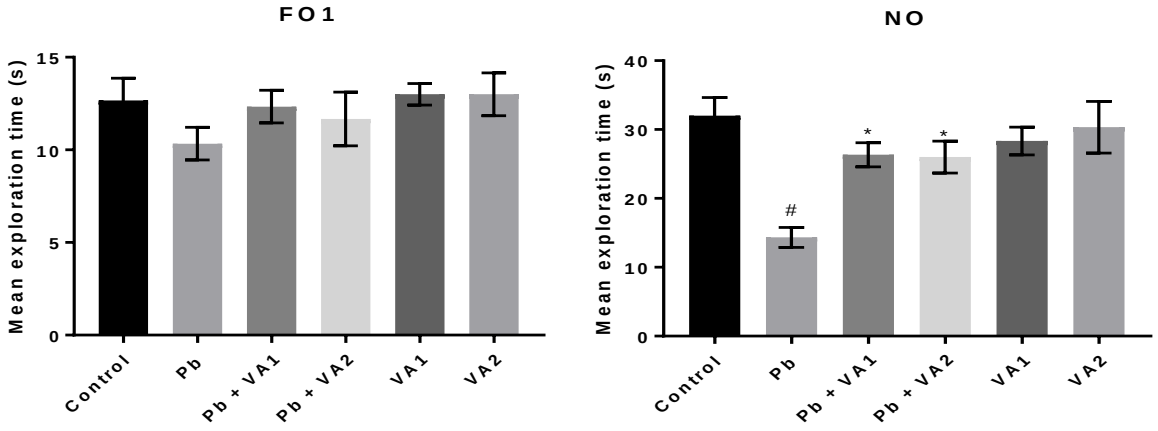

Fig. 3:
Real novel object recognition test of control and treatment groups after 28 days (FO1 – Familiar object 1; NO – Novel object). Bars represent the mean ± SEM. # $p< 0.05$ compared with the control group; * $p< 0.05$ compared with the Pb-alone group.

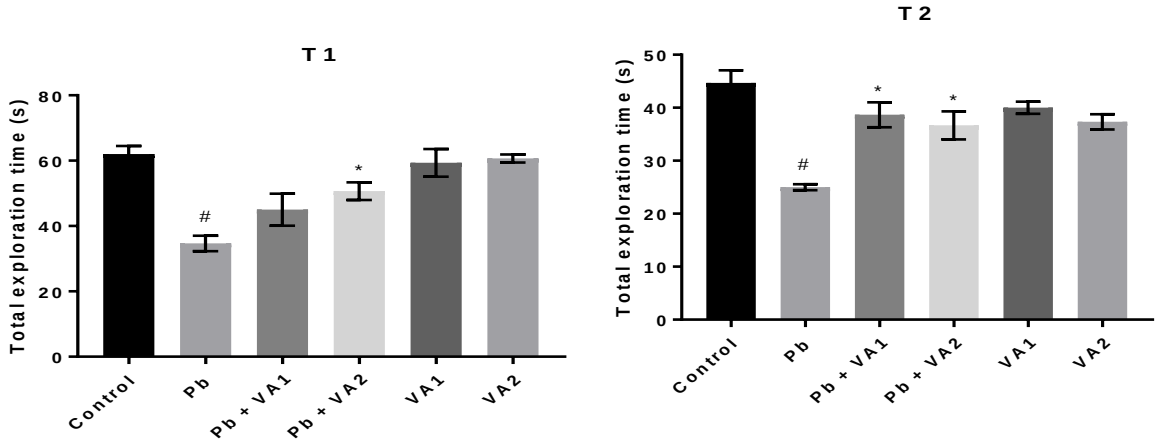

Fig. 4:
Total exploration times (NOR) of control and treatment groups after 28 days. Bars represent the mean ± SEM. # $p< 0.05$ compared with the control group; * $p< 0.05$ compared with the Pb-alone group

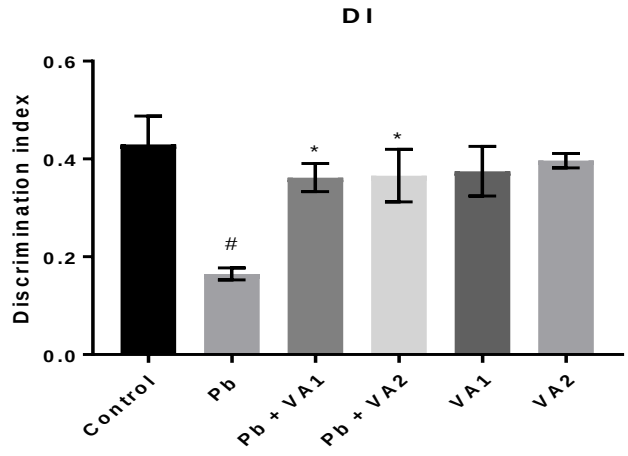

Fig. 5:
Discrimination index (NOR) of control and treatment groups after 28 days. Bars represent the mean ± SEM. # $p< 0.05$ compared with the control group; * $p< 0.05$ compared with the Pb-alone group.

Effect of treatment on the histology of the cerebellum, cerebrum and hippocampus: Fig. 6 shows the histological findings from the experimental groups following appropriate treatments. For the cerebellum, Group A (control) presents normal outer grey matter and an inner white matter. The grey matter shows three distinct layers; an outer molecular layer, a middle Purkinje layer and an inner granular layer. Group B (lead treated group) shows a dissociation of the Purkinje cell layer from the granular layer and loss of some Purkinje cells (black arrows). Groups C and D (pretreated groups) show molecular, Purkinje and granular layers similar to that of control while groups E and F also show similar histological architecture to that of control.

Fig. 7 shows the histology of the cerebrum following appropriate treatments. Group A shows the control group with normal morphology. Group B shows a disordered architecture of the cerebrum, fewer cells in the pyramidal and inner granular layer and degenerating pyramidal cells (black arrows) following treatment with lead. Pretreated groups C and D as well as groups E and F show similar morphology to that of the control.

For the hippocampus (Fig. 8), Group A shows normal hippocampal histology. Group B shows altered morphology with the presence of vacuoles (black arrows) and pyknotic nuclei (yellow arrows) in the CA1 region following administration of lead. Pretreated group D shows a healthier tissue architecture when compared to lead only treated group. Groups E and F show normal hippocampal histology with similar tissue morphology when compared to control.

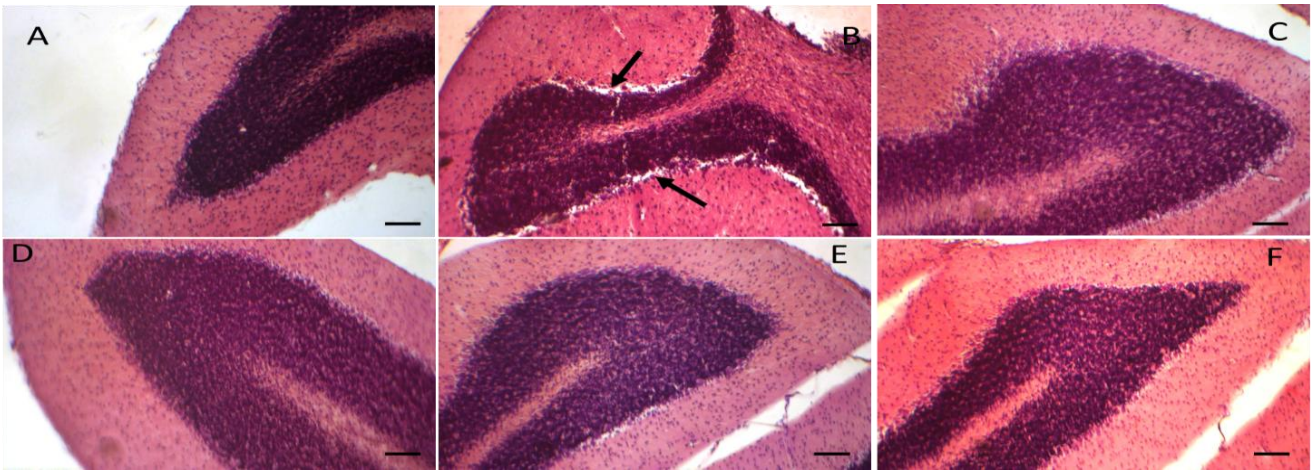

Figure 6:
Effect of VA on Pb-induced changes in the cerebellum of control and experimental rats. (A) Control. (B) Pb-induced group 100mg/kg (C) Pb + 200mg/kg VA (D) Pb + 400mg/kg VA (E) 200mg/kg VA (F) 400mg/kg VA (H&E x100). Scale bar: 100µm

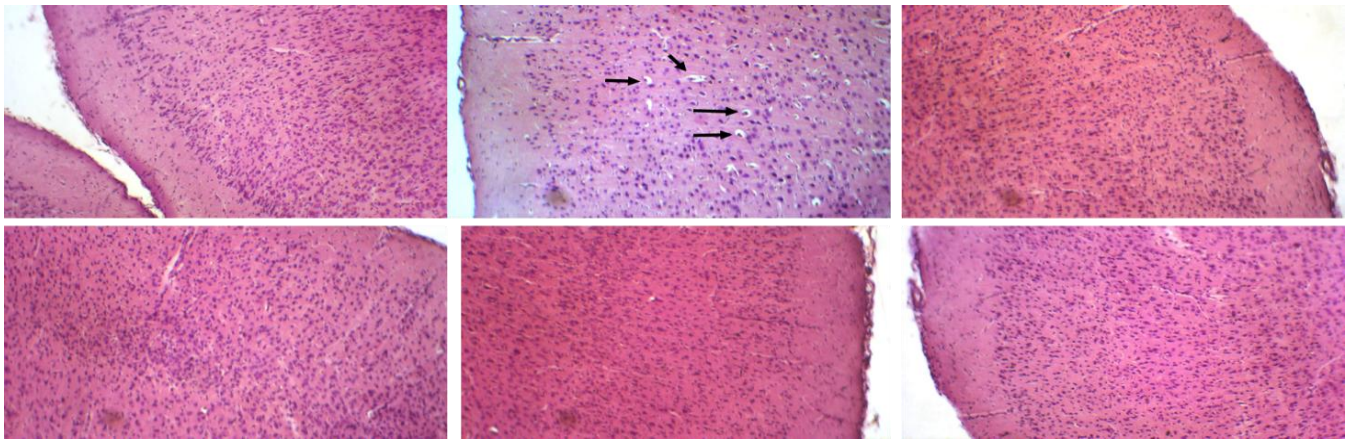

Figure 7:
Effect of VA on Pb-induced changes in the cerebral cortex of control and experimental rats. (A) Control. (B) Pb-induced group 100mg/kg (C) Pb + 100mg/kg VA (D) Pb + 200mg/kg VA (E) 100mg/kg VA (F) 200mg/kg VA (H&E x100). Scale bar 100µm

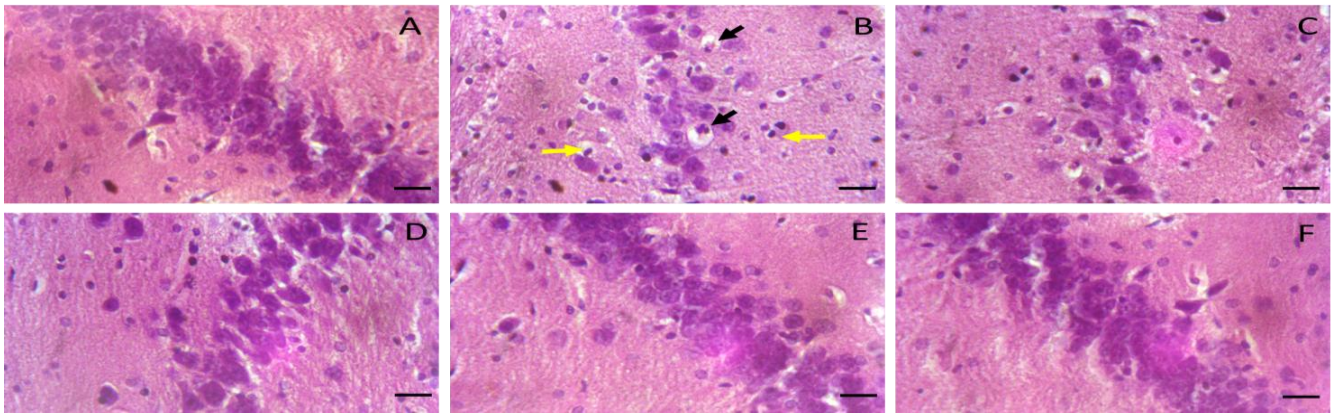

Figure 8:
Effect of VA on Pb-induced changes in the hippocampus of control and experimental rats. (A) Control. (B) Pb-induced group 100mg/kg (C) Pb + 100mg/kg VA (D) Pb + 200mg/kg VA (E) 100mg/kg VA (F) 200mg/kg VA (H&E x400). Scale bar 25µm

DISCUSSION

Lead is a ubiquitous environmental pollutant that induces a broad range of toxic manifestations within biological systems and several reports indicate that the brain is the most vulnerable organ (Wani and Usmani, 2015). Lead toxicity is induced by the over-production of reactive oxygen species and depletion of cellular antioxidant capacity. An imbalance in the free radical/antioxidant ratio in tissues and cellular components is known to harm membranes, proteins, and DNA, thus leading to tissue damage (Hsu and Guo, 2002). Consequently, exogenous supplementation of antioxidants would be advantageous to the cell's antioxidant defence system and would help mitigate the damaging effects of lead.

In this study, lead administration caused a significant decrease in body and absolute whole-brain weights when compared to control. This is in agreement with previous studies reporting that lead toxicity causes body and organ weight losses (Amjad et al., 2013; Ibrahim et al., 2012). The observed loss in body and brain weight may be associated with nausea, vomiting, anorexia or due to oxidative stress which promotes catabolic states in skeletal muscles, thus leading to muscle wasting (Rafique et al., 2008; Reid and Li, 2001). Rats pretreated with *Vernonia amygdalina* showed an increase in absolute whole brain and body weights when compared to those treated with lead alone. This increase in body and brain weights suggest that *Vernonia amygdalina* protected against lead-induced reduction in body and brain weight. These findings correspond to that of previous studies reporting that plant extracts are capable of ameliorating and protecting against lead-induced weight loss in rats (Amjad et al., 2013; Shaban et al., 2021).

The OFT is used to evaluate anxiety, general locomotor activity and willingness to explore in rats (Kraeuter et al., 2019). In this study, rearing, grooming, ambulation and immobility were evaluated. Reduction in rearing, a measure of stress and anxiety in rodent models; is an indication of elevated stress and anxiety (Borta and Schwarting, 2005). Ambulation, often measured by the number of lines crossed by the animal, is a direct measure of motor activity (movement), with increased ambulation denoting improved motor activity (Ewalds-Kvist et al., 1999). Findings from this study show a significant reduction in rearing and ambulation in rats treated with lead alone when compared to normal control rats, thus signifying elevated stress, anxiety and impaired motor activity. However, pretreatment of rats with *Vernonia amygdalina* attenuated the effects of lead on rearing and ambulation, thus demonstrating a protective activity against lead. This is in line with previous studies that reported a lead-induced reduction in rearing and ambulation as well as its attenuation with honey (Abdulmajeed et al., 2016; Moreira et al., 2001). An increase in grooming is indicative of stress and anxiety (Kalueff and Tuohimaa, 2005). Immobility, also regarded as freezing, shows a lack of movement and its increase is a direct indication of impaired motor activity (Roelofs, 2017). Findings from this study show a significant increase in grooming and immobility in rats treated with lead alone when compared to normal control rats, thus demonstrating elevated anxiety and impaired motor activity in rats treated with lead alone. However, pretreatment of rats with *Vernonia amygdalina* mitigated the effects of lead on grooming and immobility, therefore demonstrating a protective activity against lead. This is in line with previous studies that reported a lead-induced increase in grooming and immobility as well as its attenuation with *Nigella sativa* and olive (Bauchi et al., 2016; Moreira et al., 2001; Seddik et al., 2010). The NOR test evaluates the normal tendency of a rat to explore a novel versus familiar object. Findings from this study showed that exploration of the novel object was significantly decreased in animals treated with lead. This test comprises exploratory behaviour and memory retention components such that an animal must have adequately explored the familiar object during T1 in order to distinguish it from a novel object in T2. In this study, lead-treated rats demonstrated lower total exploration time during T1 than control animals. This is in agreement with previous studies showing that lead-treated rats demonstrate reduced exploratory behaviour and significantly decreased discrimination index in the NOR task (Azzaoui et al., 2009; Mansouri et al., 2012). Following pre-treatment by *Vernonia amygdalina*, a significant protective effect was observed on the total amount of time spent exploring the novel objects as well as on the discrimination index when compared to the lead-alone group, hence signifying that the extract protects memory formation in rats with lead-induced neurotoxicity.

Histological assessment of the cerebellum, cerebrum and hippocampus of the control group showed normal histological architecture. Evaluation of lead-treated rats showed dissociation of Purkinje cell layer from the granular layer and loss of some Purkinje cells in the cerebellum, degenerating pyramidal cells in the cerebrum and altered morphology with vacuoles as well as pyknotic nuclei in the CA1 region of the hippocampus. These alterations in the cerebellum, cerebrum and hippocampus ultimately disrupt normal functioning such as impairment of equilibrium, dysregulation of temperature and muscle tone, loss of grasping and cognitive impairment. The morphological alterations observed are in agreement with previous neuropathological findings on lead-induced toxicity and its effects on anatomical brain structures (Augustine et al., 2021; Lazarus et al., 2018). These alterations were however considerably absent in the cerebellum, cerebrum and hippocampus of rats pre-treated with *Vernonia amygdalina*, thus indicating a protective activity. In this study, findings from the phytochemical screening of *Vernonia amygdalina* leaves revealed the presence of phenols and flavonoids which are often considered as plant antioxidant metabolites (Okechukwu et al., 2013; Omoregie et al., 2011). The protective activity of *Vernonia amygdalina* against lead-induced toxicity could be linked to the presence of phenols and flavonoids, both of which have been previously reported to have potent ROS scavenging and metal ions chelating activities. This is in agreement with previous studies indicating the potent antioxidant benefits of *Vernonia amygdalina* and its usefulness in health and diseases (Alara and Abdurahman, 2021; Oriakhi et al., 2014; Oyeyemi et al., 2018).

In conclusion, results from this study show that aqueous *Vernonia amygdalina* leaf extract protects against lead-

induced neurotoxicity in adult Wistar rats. This highlights a huge prospect for food technologists to further formulate this vegetable as a dietary composition not only for its nutritional value but also for its potential as a neuroprotective agent especially in environments with high levels of lead exposure. Molecular and mechanistic studies are recommended to further corroborate these findings.

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