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Lead Toxicity: Effect of *Launaea taraxacifolia* on the Histological and Oxidative alterations in Rat Regio III Cornu ammonis and Cerebellum

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ABSTRACT

Lead (Pb) is a widespread toxic metal found in the environment and poses potential danger to human health despite its varied daily usage. This study investigated the possible protective role of *Launaea taraxacifolia* ethanol extract (LTEE) against lead acetate-induced neurotoxicity. Thirty male adult Wistar rats were randomized into five groups of six rats each: I, Control: 0.5 mL/day of distilled water; II, 0.5 mL/rat/day of propylene glycol; III, LTEE, 400 mg/kg/day of LTEE; VI, lead acetate (PbAct) 75 mg/kg/day; V, LTEE+PbAct, 400 mg/kg/day of LTEE plus lead acetate 75 mg/kg/day. All treatments were administered for 42 days using gavage. Behavioural studies, antioxidant parameters [malondialdehyde (MDA), glutathione (GSH), and super oxide dismutase (SOD)], and histology of rat regio III cornu ammonis and cerebellum were examined in the different groups. Lead acetate caused significant ($p<0.0.05$) reduction of the behavioural parameters, and induced lipid peroxidation, reduced GSH level, increased SOD activity, and altered the microanatomy of the rat regio III cornu ammonis and cerebellum when compared with the control. The changes in MDA and GSH concentration and SOP activity and behavioural parameters were significantly ($p<0.0.05$) mitigated in rats co-treated with LTEE when compared with the lead-treated rats. Similarly, co-administration of LTEE with lead acetate inhibited lead acetate induced-neurotoxicity by reversing the altered microanatomy of the regio III cornu ammonis and cerebellum of rat. It is concluded that LTEE has beneficial effects and ameliorated lead acetate-induced neurotoxicity via its antioxidant property.

Keywords: Lead acetate, *Launaea taraxacifolia*, cornu ammonis 3, Cerebellum, Oxidative stress

INTRODUCTION

Lead is a heavy, ubiquitous, non-biodegradable, low melting metal that occurs naturally in the earth's crust, noted for its toxicity and regarded as a potent occupational toxicant (ATSDR, 2007; Sanders et al., 2009; Flora et al., 2012). It is found useful in diverse items of daily use like paints, water pipes, car batteries, leaded gasoline, ammunition, cosmetics, hair dye, airplanes, shielding for x-ray machines (ATSDR, 2007). Humans can be exposed by the ingestion

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of lead-contaminated food or drinking water containing lead leaching from old corroded pipes, and dermal contact (ATSDR, 2007). Children may be exposed to peeling or flaking lead-based paint or weathered powdered paint when engaging in activities that increase exposure (Sanders et al., 2009).

Lead toxicity is a hazard with the potential of causing health effects which may affect the nervous, hematopoietic, hepatic and renal systems, eliciting various disorders (Kalia & Flora, 2005). The mechanism of toxicity common to all toxic metals, including lead, is via oxidative stress leading to production of lipid peroxidation (LPO) secondary to generation of free radicals in the tissues (Waters et al., 2012). Reactive oxygen species (ROS) results in damage to various biomolecules like DNA, enzymes, proteins and membrane based lipids in addition to simultaneously impairing the antioxidant defense system (Flora et al., 2012). Antioxidants are known to mitigate the effects of free radical damage by mopping them up (Farombi and Owoeye, 2011; Owoeye et al., 2014). Flavonoids containing substances play important roles in detoxifying ROS activities when in excess (Van Acker et al., 1998) and one reported plant containing flavonoids is *Launaea taraxacifolia* Wild, of the Asteraceae family (Wild lettuce) known as "*Yanrin"* in south-west Nigeria. It is a common vegetable widely used in folklore medicine as treatment of restlessness, insomnia, whooping cough, bronchitis whose bioactive compounds include: phenol, ascorbic acid, flavonoids (Gbadamosi et al., 2012; Oduse et al., 2012; Arawande et al., 2013).

The hippocampal subfield, region III cornu ammonis (cornu ammonis3) is important in receiving mossy fibres from the granule neurons of dentate gyrus for onward projection to cornu ammonis1, both subfields being important in long-term memory acquisition (Snell, 2015). The Purkinje cells of the cerebellum functions as the main output from the cerebellum in its coordination of body movement, balance and posture, (Snell, 2006). Lead as a toxicant reportedly affects the central nervous system due in part to its ability to cross the blood-brain barrier (BBB) by substituting other bivalent cations like Ca2+, Mg2+, Fe2+ with Pb+ ions (Pb2+) thus concentrating it in the brain (Lidsky and Schneider, 2003; Sanders et al., 2009). Neurons have a reduced capacity to detoxify ROS making them vulnerable to increases in ROS levels (Dringen et al., 2005) which may be generated by lead intoxication thus altering the histology and neurological functions of the hippocampus and cerebellum. Since it is almost impossible to remove or reverse completely the damaging effect of lead once it enters the body, preventive measures appear to be the preferred option considering its toxicity (Guidotti and Ragain, 2007). It will therefore be beneficial to investigate and explore the potential antioxidant effect of *L. taraxacifolia ethanol* extract (LTEE) on exposure of rat brain to lead toxicity especially as literature is scanty concerning this effect.

The aim of the study was to investigate the effect of LTEE on lead acetate-induced alterations of the oxidative, behavioural and microanatomy of regio III cornu ammonis and cerebellum of Wistar rat. This is to provide an answer to the research question: 'Can the *Launaea taraxacifolia* ethanol extract protect rat brain from lead acetate-induced injury?'

MATERIAL AND METHODS

Experimental Animals

This study comprised a total number of thirty male Wistar rats, initially weighing from 80 – 140 g, obtained from Central Animal House of College of Medicine, University of Ibadan, Nigeria. They were housed in transparent plastic cages with wood shavings at a freely ventilated and illuminated Animal House of the College of Medicine, University of Ibadan. All animals were

allowed to acclimatize for a week prior to initiating the experiment, they were fed on normal rodent pellets and clean water offered *ad libitum* throughout the adaptation period. The experimental protocols were carried out according to the approval and guidelines given by the University of Ibadan Ethical Committee Number: UI-ACUREC/App/2015/022, which conformed to the acceptable guidelines on the ethical use of animals in research (Public Health Service, 1996).

Plant preparation and extraction procedures

The leaves of *Launaea taraxacifolia* were obtained in July, 2014 from a farm at Olomi area of Oluyole Local Government Ibadan, Oyo State. The leaves were identified and authenticated at the Department of Botany, University of Ibadan, Nigeria and compared with the voucher specimen with Herbarium Identification Number UIH: 22400 we had earlier deposited. The leaves were air-dried and extracted in batches each, with 2 litres of 99.1% ethyl alcohol at room temperature and the filtrate evaporated with a rotary vacuum evaporator in the University of Ibadan Central Laboratory. The term *Launaea taraxacifolia* ethanolic extract (LTEE) was given to the product we obtained. The administered dose was then calculated per kilogram body weight of the rats.

Chemicals

Lead acetate purchased from Femolak Chemicals, Yemetu, Ibadan was manufactured by May & Baker Ltd, Dagenham England, Batch number L54/18/90. Ketamine was manufactured by Rotex Medica, Trittau, Germany. Propylene glycol was manufactured by Guangdong Guanghua Science Tech. Co Ltd. (China). All other reagents were of analytical grade and were obtained from the British Drug Houses (Poole, Dorset, UK).

Preparation and administration of LTEE

One gramme of LTEE was dissolved in 10 mL of propylene glycol to form the stock solution from which the LTEE at a dose of 400 mg/kg/day was prepared and administered using oral gavage.

Preparation and administration of Propylene glycol (PrGly) and Lead acetate

Oral administration of propylene glycol was administered at a dose of 0.5 mL/rat/day using gavage. Lead acetate (PbAct) was prepared diluted in distilled water to constitute the stock solution from which the measured dose of lead acetate solution 75 mg/kg was administered orally using gavage.

Experimental Design

After one week of acclimatization to animal room conditions, the 30 rats were randomized into five groups of six animals in each group. Animal grouping was as follows:

Group I: control rats given water *ad libitum.*

Group II: PrGly, received daily dose of 0.5 mL/rat/day of propylene glycol p.o. for 42 days.

Group III: LTEE, received once daily dose of 400 mg/kg of LTEE in propylene glycol p.o. for 42 days.

Group VI: PbAct, lead acetate 75 mg/kg p.o. daily for 42 days.

Group V: LTEE+PbAct, received once daily dose of 400 mg/kg of LTEE in propylene glycol and lead acetate 75 mg/kg p.o. for 42 days. The two treatments were given separately at two hours interval.

The dose of LTEE was based on the method of Adejuwon et al., 2014 while that of lead acetate was based on the method of Al-Naimi et al., 2011. The rats were thereafter monitored for their daily food intake, health, and general behaviour.

Behavioural tests

The behavioural tests conducted included: (1) open field test (2) forelimb grip test (3) and negative geotaxis. The rats were weighed and then subjected to these tests on the 43rd day of the experiment so as to investigate the effects of lead toxicity.

Negative geotaxis

Negative geotaxis was tested by placing rats head-down on an inclined plane and then watch the rat orient in a head-up direction (Kreider and Blumberg, 1999). The time it takes for the rat to

orient in a head-up direction was recorded with a stopwatch. The average of two trials was obtained.

Forelimb grip test

This was conducted according to the published method of VanWijk et al. (2008). It involved the suspension of each rat with both forepaws on a horizontal steel wire (1 meter long, diameter 7 mm). While holding a rat in a vertical position, the front paws were placed in contact with the wire. Upon grasping the wire, the rat was released, and the latency to fall was recorded with a stopwatch. Rats were randomly tested and each animal was given two trials with a 30 min inter trial rest interval. The strength of the forelimb muscle was thus tested.

Open field test

The apparatus used was a slight modification of the method of Mohammad et al., (2010). It consisted of a square arena (56×56×20 cm) made of white wood and its floor divided by lines into 16 squares that allowed the definition of central and peripheral parts. At the beginning of the session, each rat was individually placed in the center of the arena and its activity was video-recorded for 5 minutes. The number of squares crossed with all paws (crossing) and standing on legs (rearing) were evaluated during the 5 minute sessions. The crossing numbers were indicators of locomotor while the rearing numbers indicated vertical and exploratory activities. Each rat was removed at the end of each session from the open field and the experimental chamber was thoroughly cleaned with a damp cloth and dried before letting in another rat.

Sample collection and histological preparation

After the completion of each session of the behavioural tests on day 43 of the experiment, all animals in all groups were weighed and then euthanized using ketamine hydrochloride (100 mg/kg) i.p. Blood samples of about 2–4 mL were collected from retro-orbital venous sinus of each rat into heparinized bottles for haematological parameters followed by cervical dislocation. Each rat was decapitated at the cervico-medullary junction for uniformity and the skulls opened after which the brains were quickly dissected out. We adopted the method of Igado et al. (2012) wherein the right hemisphere, was preserved for histology and fixed in 10% neutral buffered saline for three days before being processed by paraffin wax embedment technique. The left half of the brain preserved for biochemical assays was rapidly rinsed, mopped with filter paper, weighed and kept in freshly prepared cold phosphate buffered solution (PBS) at a pH 7.4 and then kept in the freezer till processed

Biochemical Assays

The left hemisphere of the brain samples was homogenized in 50 mM Tris–HCl buffer (pH 7.4) containing 1.15% potassium chloride, and the homogenate centrifuged at 10,000 x g for 15 minutes at 4 °C. The supernatant was collected for the estimation of the various biochemical bioassays. Lipid peroxidation was quantified as malondialdehyde (MDA) according to the method described by Farombi et al. (2000) and expressed as micromoles of MDA per milligram protein. Protein concentration was determined by the method of Lowry et al. (1951). Reduced glutathione (GSH) was determined at 412 nm using the method described by Jollow et al. (1974). The method of Misra and Fridovich (1972) was used to assay for superoxide dismutase (SOD). Biochemical experiments were performed in Prof. E.O. Farombi's Drug Metabolism and Toxicology Research Laboratories, Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria.

Histology

The cerebellum from each group was obtained and homologous sampling was assured by obtaining transverse sections of the right cerebellum from each specimen from the paravermal zone portions of the cerebella hemisphere (vermal, lateral and flocullus

portions were not utilized) for uniformity. Coronal sections of the right half of each brain were made at the level of the optic chiasma to obtain samples of the cerebral cortex and hippocampal tissue. The tissues were sectioned at 5-6 µm thickness and then stained with Haematoxylin and Eosin according to the method of Bancroft and Gamble, (2008). After staining, the slides were viewed with an Olympus CH (Japan) light microscope with 16x objective. The image capturing was performed with a Sony DSC-W610 digital camera (Japan), while photomicrograph calibration was done using Image J (Abramoff et al., 2004).

Statistical analysis

Data are expressed as mean ± deviation (SD). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to compare the means of the different treatment groups. Differences between the treatment groups with a p- value < 0.05 were considered significant. Data were analysed with GraphPad Prism Version 5.0 for Windows (GraphPad® Software), San Diego, CA, USA.

RESULTS

Antioxidant parameters

Figure 1 demonstrated that lead acetate treatment elicited a significant ($p<0.05$) elevation of the MDA level (46%), and an increase in the activity of SOD (79%) in the brain of rats, whereas it reduced the level of GSH by 41% when compared with the control. In contrast, co-treatment of lead acetate with LTEE caused a significant ($p<0.05$) reduction of the elevated MDA level by 30%, and a reduction in the activity of SOD (29%). In addition, co-treatment of lead acetate with LTEE caused a significant 79% elevation of the GSH level when compared with the lead treated group.

Behavioural parameters

Behavioural tests results are presented in Figure 2. Lead acetate significantly reduced the number of lines crossed by the rats, the rearing number and the forelimb grip strength when compared with the control ($p<0.05$). However, co-treatment with lead acetate with LTEE significantly alleviated this effect by increasing the values ($p<0.05$) when compared with lead acetate. There was however, no differences in the effect of either treatment on geotaxis.

Regio III cornu ammonis (CA3)

Control rats CA3 showed typical normal histological features, namely: the alveus, stratum oriens, stratum pyramidalis, stratum radiatum, and stratum lacunosum moleculare (Figure 3A, 3B, and 3C). In Figure 3D, lead acetate toxicity on the pyramidal neurons demonstrated by neuronal degeneration (arrow and arrows-heads). Figure 3E shows the effect of co-treatment of Lead acetate with LTEE, the pyramidal neurons are relatively less damaged when compared with the lead acetate treated brain in Figure 3D as shown by increased viable of the neurons.

Cerebellum

The three typical histological layers of an adult rat cerebellum namely: granular, molecular, and Purkinje are observed in Figures 4A, 4B, and 4C. In Figure 4D lead acetate effect was observed to cause complete eosinophilia of the Purkinje cell bodies when compared with the control and the other groups. However, Figure 4E shows the reversal of this effect by LTEE co-treatment shown by a normal basophilic staining of the Purkinje cells as compared with the group of Figure 4D.

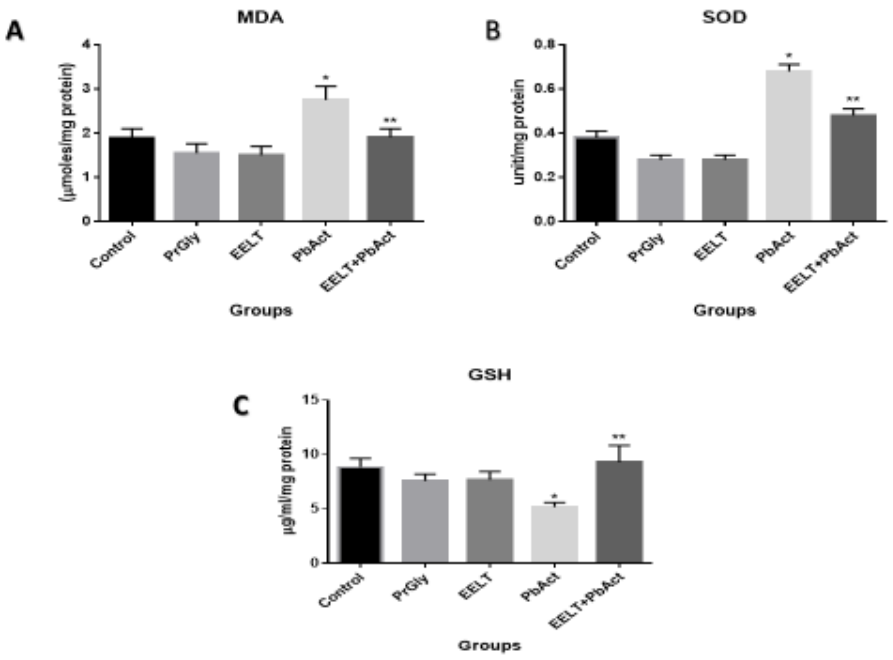

Figure 1: **Effect of Lead acetate and *Launaea taraxacifolia* ethanolic extract on oxidative parameters in male Wistar rats.** Values are presented as Mean ± Standard deviation for six rats per group after 42 consecutive days of oral treatment period. MDA-Malondialdehyde, GSH- reduced glutathione, SOD-superoxide dismutase, PrGly- Propylene glycol, LTEE-*Launaea taraxacifolia* Ethanolic Extract, PbAct- Lead acetate. * P< 0.05 versus Control group, ** P< 0.05 versus PbAct group.

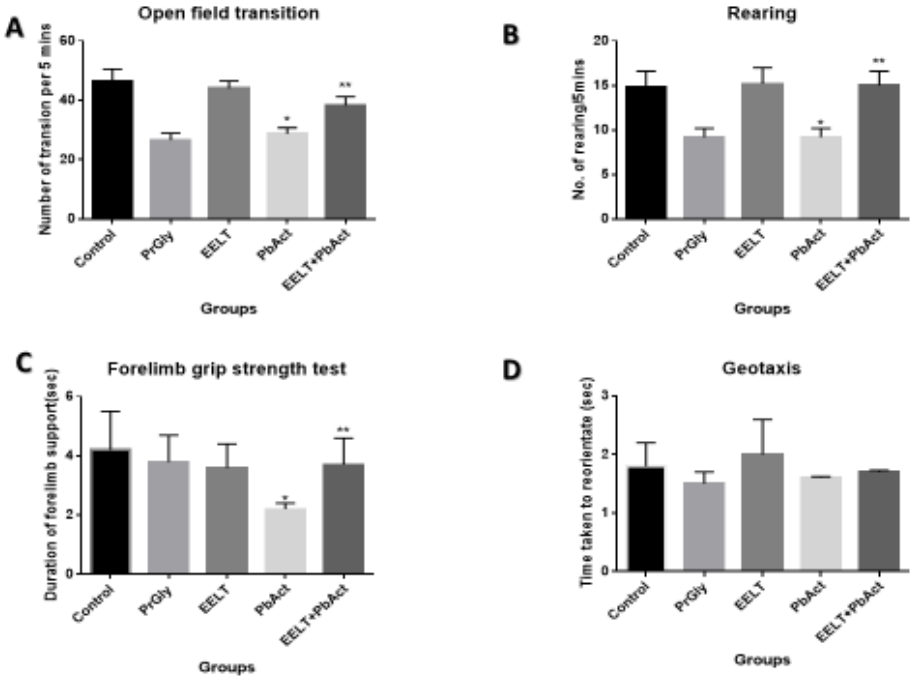

Figure 2: Behavioural tests. Histogram of behavioural tests in the control and treated groups. A: horizontal movements measured as number of open field transitions, B: Vertical movements measured as number of open field rearings, C: forelimb muscular strength measured as the length of time rats remain suspended on the metal wire, D: negative geotaxis, measured

as the time taken for the rats to re-orient in a head-up direction. Transitions, rearing and forelimb grip strength were significantly reduced by PbAct treatment. Reduction in forelimb grip strength indicated weakness of muscle strength while reduction in rearing behaviour suggested reduction of motor and exploratory skills. Pre-treatment with EELT ameliorated the reduced parameters significantly ($p<0.05$). Values are presented as Mean± Standard deviation for six rats per group after 42 consecutive days of oral treatment period. PrGly- Propylene glycol, LTEE- *Launaea taraxacifolia* Ethanolic Extract, PbAct- Lead acetate. * P< 0.05 versus Control group, ** P< 0.05 versus PbAct group.

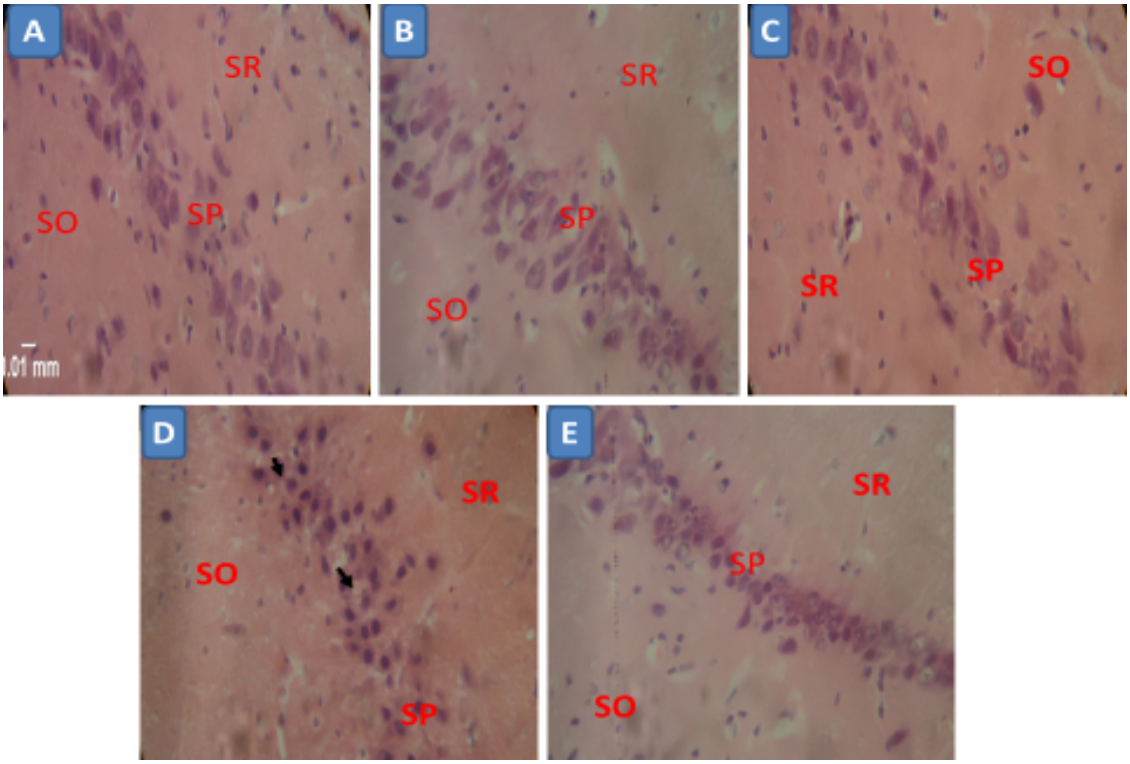

Figure 3: Representative stained sections of Cornu Ammonis3 of rats: (A) Control (B) PrGly-treated (C) LTEE-treated (D) PbAct-treated rats show degenerating pyknotic pyramidal neurons (*arrows*), and (E) LTEE+ PbAct-treated rats show normal pyramidal neurons with few scaterred pyknotic. PrGly-Propylene glycol, LTEE, *Launaea taraxacifolia* ethanolic extract; PbAct-Lead acetate. SO, stratum oriens; SP, stratum pyramidalis; SR, stratum radiatum. H&E. Calibration bar = 0.01mm (10 µm) for all figures.

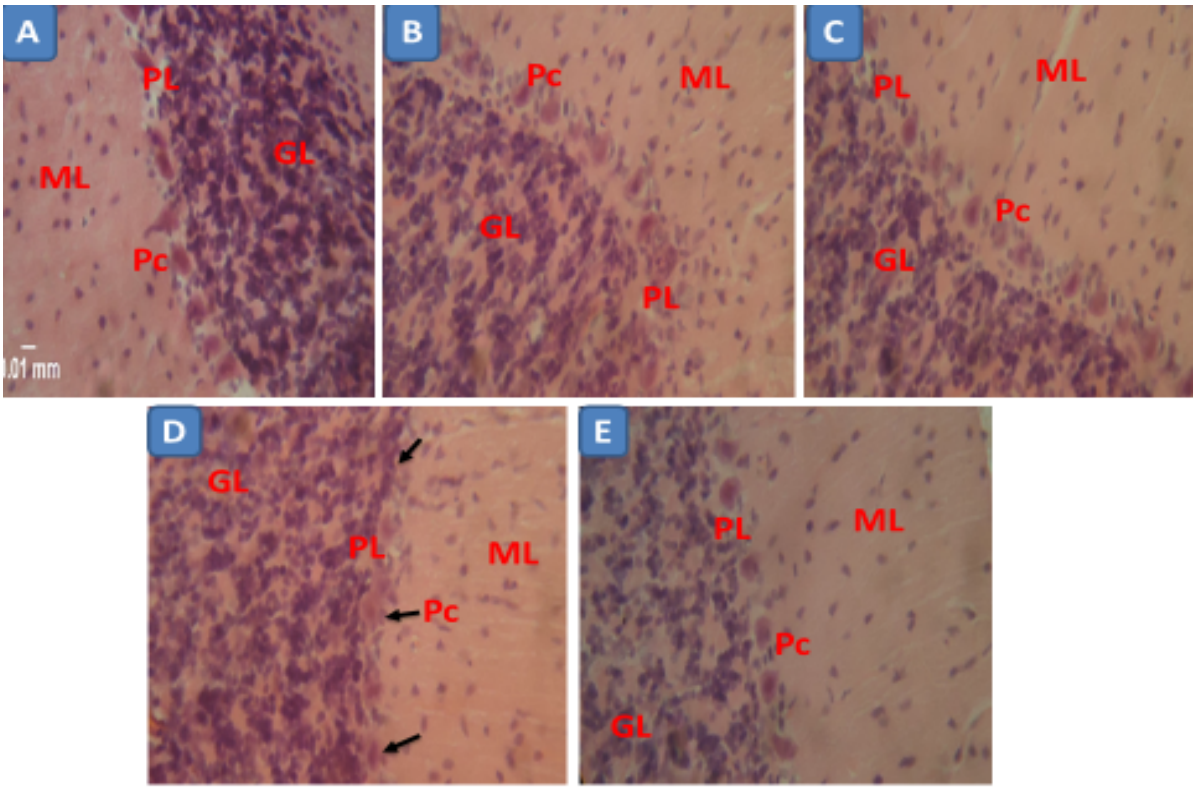

Figure 4: Representative stained sections of cerebellum of rats: (A) Control (B) PrGly-treated (C) LTEE-treated (D) PbAct-treated cerebellum with degenerating Purkinje neurons without basophilia *(arrows)*, and (E) LTEE+ PbAct-treated. Purkinje cells exhibit normal basophilic staining nuclei in all other groups. PrGly-Propylene glycol, LTEE, *Launaea taraxacifolia* ethanolic extract; PbAct- Lead acetate. ML - molecular layer; GL - granular layer; PL, Purkinje layer; Pc - Purkinje cells. H&E. Calibration bar = 0.01mm (10 µm) for all figures.

DISCUSSION

The present investigation demonstrated that lead acetate treatment of Wistar rats for 42 days consecutively, altered the antioxidant and behavioural parameters and the microanatomy of the regio III cornu ammonis (cornu ammonis3) subfield of the hippocampus, as well as the cerebellum of male Wistar rats when compared with the control. These alterations were however, ameliorated by co-treatment with the *Launaea taraxacifolia* ethanol extract.

The state of oxidative stress generated in the brain of lead acetate-treated rats was shown by the significant increase in the MDA level which was accompanied by a concomitant significant decrease in the level of glutathione (GSH) in the present study. The raised MDA level agrees with published reports (Hassan and Jassim, 2010; Attia et al., 2013; Abdel-Wahab and Metwally, 2014), who reported a similar observation of rats' response to lead treatment. This increased levels of MDA as evidenced in this study suggests an excessive generation of free radicals by the lead acetate treatment (Waters et al., 2012), which may affect both the morphology and function of the brain. Similarly, the reduction of the GSH level agrees with previous report on the response of rats to lead toxicity (Hassan and Jassim, 2010; Attia et al., 2013; Abdel-Wahab and Metwally, 2014). Apart from the generation

of reactive oxygen species (ROS), lead toxicity is also known to deplete antioxidant reserves. Lead exposure affects glutathione, an important endogenous antioxidant by the binding of its sulfhydryl complex directly to lead thus inactivating glutathione molecule and making it unavailable as an antioxidant (Sanders et al., 2009; Flora *et al.,* 2002). Glutathione's level was enhanced by LTEE co-treatment, thus enhancing GSH ability to perform its main protective roles against oxidative stress namely, as a cofactor of several detoxifying enzymes, participating in amino acid transport through the plasma membrane and scavenging hydroxyl radical and singlet oxygen directly (Valko et al., 2006). The significant increase in SOD activity in the brain of lead acetate treated rats is in agreement with the report of Attia et al. (2013) who reported similar finding after lead treatment. This response is an adaptive response of SOD to mitigate the event of increased free radical generation by lead acetate as indicated by the increased MDA level. The increase in SOD concentration by LTEE co-treatment thus enhances the capacity of SOD as an antioxidant enzyme to catalyse the dismutation of O_2 •– to O_2 and to the less-reactive species H_2O_2 (Valko et al., 2006). Overall, LTEE was able to reduce the extent of ROS generation which might have affected the stability of macromolecules like DNA, proteins and lipids which uncontrolled generation by lead acetate caused.

The pyknotic and karyolytic pyramidal cell neurons observed evidenced the alteration of the normal microanatomy of the region III cornu ammonis (CA3) subfield of the hippocampus of rats by lead acetate. This finding of the neurotoxic effect of lead is in agreement with the report of Abdel-Wahab and Metwally. (2014) who demonstrated lead acetate-induced hippocampal neuronal degeneration microscopically using Fluoro-Jade B. The authors went on to confirm neuron degeneration by Tunnel staining of hippocampal slices that showed increased percentage of positively stained cells in lead acetate-exposed rats. This is to support the report that lead, not only induces neurotoxicity and increases oxidative stress, but also induces neuronal DNA damage (Abdel-Wahab and Metwally, (2014). This neural damage could result in the interruption of the nervous pathway between the granule cell of the granule cell layer of the dentate gyrus (DG) via the Mossy fibres of the hippocampus to the CA3 (Viva & Praag, 2013). Regio III cornu ammonis propagates neural information via the Schaffers fibres to the subiculum and entorhinal cortex. The effect of damage to pyramidal neurons will be disruption in the functions of the CA3 which includes memory acquisition and storage. Decision making is linked with the memory and this may also be affected thus affecting the behaviour of the rats as indicated in the reduction of the number of lines crossed and rearing in the treated rats.

The alteration of the microanatomy of the cerebellum of rats by lead acetate shown by the loss of the basophilic staining of the Purkinje cell nuclei is in agreement with the report of Al-Naimi et al. (2011) who reported a similar finding. The eosinophilia observed in the Purkinje neurons in this experiment is an evidence of karyolysis (Stevens and Lowe, 2000). Cerebellar Purkinje cells are sensitive to oxidative stress hence their succumbing to free radical generated by lead acetate (Kern and Jones, 2006). Neuronal cell death may lead to poor control and processing of new neuronal protein synthesis, necessary for axonal flow and the maintenance of the integrity of the Purkinje neuron. It is known that the Purkinje cells are the predominant output regulator of the cerebellar cortex and that afferent fibres to the paravermal zone of the cerebellum from which the specimen was obtained come from the spinal cord, brain stem and cerebral cortex. The paravermal zone modulates the velocity, force and pattern of muscular movements (Afifi and Bergman, 2005). Cellular degeneration of the Purkinje neurons from the paravermal zone might lead to disorder of movements in the affected rats. This was reflected in the reduction of muscle strength reflected by reduction of latency of the forelimb grip strength and the reduced number of line

crossing, which is related to locomotor activity (Reckziegela et al., 2011). However, the alterations in both CA3 and Purkinje cells were normalized upon LTEE supplementation. The micro-anatomic alteration caused by neural degeneration induced by lead acetate was paralleled by behavioral deficits as observed in the open field activity tests namely, line crossing, rearing and forelimb grip strength all of which were reduced in contrast to the report of Hassan and Jassim. (2010) who reported an increase of all the three parameters. The difference in our results may be due to the age of the rats used because while our animals were adults, they used young rats.

Although lead's ability to damage the brain have been attributed to its ability to penetrate the blood-brain barrier (BBB) by substituting for calcium ions (Sanders et al., 2009), the vulnerability of the brain to oxidative stress which is due to the high rate of ROS generation without commensurate levels of antioxidant defenses and the fact that neurons have a reduced capacity to detoxify ROS (Villeda-Hernandez et al., 2001; Dringen et al., 2005) is equally of importance. Our observation that the alterations were normalized upon LTEE supplementation underscores the essential role played by LTEE in maintaining the integrity of regio III cornu ammonis and cerebellum of rats initially marred by lead acetate administration which agrees with previous reports concerning the antioxidant activity of *Launaea taraxacifolia* (Gbadamosi et al., 2012; Oduse et al., 2012; Arawande et al., 2013).

In conclusion, the present study demonstrated that lead acetate elicited significant oxidative, behavioural and histological alterations in male rats which 400 mg/kg of LTEE co-treatment for 42 days ameliorated. These alterations appear to be mediated through oxidative stress induced by lead which the antioxidant capacity of *Launaea taraxacifolia* ethanolic extract treatment mitigated. This suggests that *Launaea taraxacifolia,* could be further investigated for a possible protective role in management of lead toxicity and raises the suggestion of this vegetable being considered as one of the component of the regular diet for people who may be exposed to lead occupationally or environmentally.

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