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Article

Curcumin Attenuates Lead-Induced Cerebellar Toxicity in Rats via Inhibition of Oxidative Stress and Chelating Activity

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Abstract: Lead (Pb) is a toxic environmental heavy metal that induces serious clinical defect on all organs with the nervous system being the primary target. Curcumin is the main active constituent of turmeric rhizome (*Curcuma longa*) with strong antioxidant and anti-inflammatory properties. This study is aimed at evaluating the therapeutic potentials of curcumin on Pb-induced neurotoxicity. Thirty six male Sprague Dawley rats were randomly assigned into five (5) groups with 12 rats in the control (normal saline) and 6 rats for the lead treated group (LTG) (50 mg/kg lead acetate for 4 weeks), recovery group (RC) (50 mg/kg lead acetate for 4 weeks), treatment group 1 (Cur100) (50 mg/kg lead acetate for 4 weeks, followed by 100 mg/kg curcumin for 4 weeks) and treatment group 2 (Cur200) (50 mg/kg lead acetate for 4 weeks, followed by 200 mg/kg curcumin for 4 weeks) groups each. All experimental groups received the oral treatments through orogastric-tube on alternate days. Motor function was assessed using horizontal bar method while Pb concentration in the cerebellum of the rats were evaluated using ICP-MS techniques. Pb-administered rats showed significant decrease in motor scores, SOD activity with increase MDA levels and Pb concentration in their cerebellum with marked alterations in the histological architecture of the cerebellar cortex layers. However, treatment with curcumin improved the motor score, reduced Pb concentration in the cerebellum and ameliorates the markers of oxidative stress as well as restored the histological architecture of the cerebellum. The results this in study suggested that curcumin attenuates Pb-induced neurotoxicity via inhibition of oxidative stress and chelating activity.

Keywords: Curcumin; Lead toxicity; ICP-MS; Horizontal bar; Motor coordination; Oxidative stress; Cerebellum.

1. Introduction

Lead (Pb) is a ubiquitous environmental neurotoxin that induces several physiological, behavioral and biochemical abnormalities in humans and animals [1]. Pb toxicity remain a common problem in both developing and industrialized countries due to unavoidable environmental and occupational exposure resulting to about 600,000 new cases of intellectual disabilities in children and

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account for about 143,000 deaths per year [1,2]. Although data from Adults Blood Lead Epidemiology and Surveillance (ABLES) program indicates a significant decrease in incidence of blood lead levels (BLLs) among adults' industrial workers, occupational exposure Pb remained a public health concern accounting for about 94% Pb exposures [3]. Occupational exposure to Pb is linked with several health consequences such as cognitive impairment, reproductive disorders, hypertension, motor dysfunction, cancer, hepatotoxicity, nephrotoxicity, and mortality [4,5].

The mechanism of Pb-toxicity is due to its ability to induce oxidative stress via disruption the oxidant/antioxidant balance mechanism in the cells [6]. Pb as a ubiquitous toxin is known to induce oxidative stress by increasing the generation of reactive oxygen species (ROS) such as hydroxyl radicals, lipid peroxides, superoxide radicals and hydrogen peroxide [6]. Pb-toxicity has long been linked with impaired motor function particularly deficits in visuomotor coordination among adult industrial workers and children exposed to Pb [7]. However, studies on the effect of Pb exposure on visuomotor integration among Yugoslavian and urban African children shows that blood lead levels (BLLs) contributed significantly to poorer fine motor skills as well as gross motor speed [7,8]. Animal models, when treated with Pb display increased oxidative stress, cognitive impairments, degeneration of neurons, deficits in motor coordination and mortality [9–11].

The field of phytotherapy is growing more interest in the use of phytochemical compounds such as curcumin due to its wide pharmacological safety and antioxidant properties against heavy metal induced-toxicity since the application of standard drugs such as chelators in the treatment and management of heavy metal poisoning have shown several side effects, such as brain damage, anemia, liver and kidney diseases, anaphylactic shock and others [12]. Curcumin is the main natural polyphenol in the rhizome of turmeric (*Curcuma longa*), that belong to the family of ginger (*Zingiberaceae*) which is widely used as traditional medicine and food in Asia [13]. It is lipophilic, phenolic and water insoluble compound which has antioxidant, anti-inflammatory and anticancer properties [14]. Curcumin has a strong antioxidant properties through increased production of antioxidant enzymes resulting in to the scavenging of excess ROS produced and inhibition of lipid peroxidation [15]. Curcumin as a lipophilic compound has the potency to cross the blood-brain barrier (BBB) and bind to plaques in the brain, thus inhibiting amyloid-B peptide aggregation in Alzheimer's disease [16]. Post-treatment with curcumin in subarachnoid hemorrhage (SAH) induced mice preserved the integrity of the BBB and improves brain functions via down regulation of Matrix Metallopeptidase 9 (MMP-9) and inhibition of microglia cells as well as reducing water content in the brain [17].

The neuroprotective mechanism of curcumin on neurodegenerative disorders is mainly due to its ability to bind redox-active transition metal ions such as Mn^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+} to form active and tight complexes [18]. Additionally, curcumin has a wide range of pharmacological activities and safety margin, it is identified as a natural drug against neurodegenerative disorders [18]. However, there is paucity of knowledge on the therapeutic potentials of curcumin in Pb induced cerebellar toxicity. Therefore, the present study aimed at investigating the neurotherapeutic potentials of curcumin on Pb-induced cerebellar toxicity in rats.

2. Materials and Methods

2.1. Chemicals

Lead acetate, (CH3CO2)2Pb.3H20, 99%) and animal feed were purchased from Sigma-Aldrich (St. Louis, MO USA) while MDA ELISA kit and SOD assay kit were purchased from Elabscience (Houston, TX, USA). Curcumin, sodium acetate, aniline solution and colophonium were purchased from Apical Scientific Sdn. Bhd. Malaysia. All other chemicals used were of high analytical grade.

2.2. Animals

Thirty six (36) male Sprague Dawley rats weighing 200-250g were obtained from the Animal Breeding Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). The rats were kept in plastic cages and maintained at room temperature of 25°C ± 2°C with a 12 h light- dark cycle, all rats had free access to food and water during the study period. The rats were allowed to acclimatize for one week prior to the experiment in the animal house, Faculty of Medicine and Health Science, UPM. The animal management and handling procedures was performed based on the recommended institutional animal care and use committee (IACUC) guidelines with the reference number **UPM/IACUC/AUP-R038/2018**, approved on 19th September 2018.

2.3. Experimental design

The rats were randomly divided into five (5) groups (Groups A, B, C, D, and E) with six (6) rats each in group B, C, D and E, while group A consist of twelve (12) rats. Group A (normal control group), received normal saline orally for the whole period of the experiment (8 weeks), Group B designated as the lead treated group (**LTG**), receive 50 mg/kg of Pb acetate orally for 4 weeks (induction of lead toxicity), group C designated as recovery group (**RC**) receive 50 mg/kg of Pb acetate orally for 4 weeks and left with no treatment for 4 weeks, Group D also known as the treatment group 1 (**Cur100**) received 50 mg/kg of Pb acetate for 4 weeks orally followed by 100 mg/kg of curcumin for another 4 weeks and group E designated as treatment group 2 (**Cur200**) received 50 mg/kg of Pb acetate for 4 weeks orally followed by 200 mg/kg of curcumin for another 4 weeks. At the end of 4 weeks of oral administration of Pb acetate, six rats each from group A and B were euthanatized to confirm the Pb-toxicity via histopathological examination. At the end of the experiment (at week 8) all the rats were sacrificed and tissues were harvested for biochemical and histopathological examinations. Motor activity and weight of the rats was evaluated weekly (figure 1). The dose of Pb acetate [19,20] and curcumin [21] was selected based on previous studies.

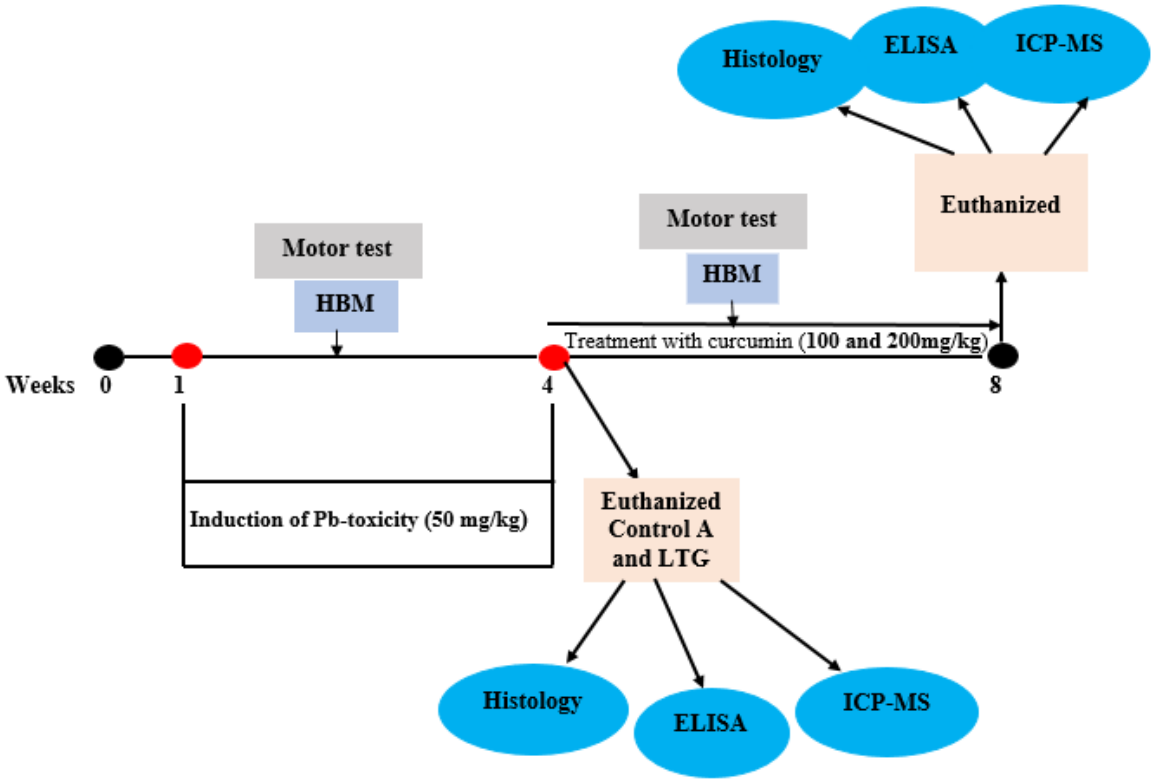

Figure 1. Schematic representation of experimental design. HBM-Horizontal bar method, LTG-lead treated group, ICP-MS-inductive coupled plasma spectrometry.

2.4. Motor activity

Motor activity of the rats was evaluated using the horizontal bar method.

2.4.1. Horizontal bar method

Horizontal bar method is a test that measures forelimb strength and coordination in rodents. It involves the use of 2mm diameter metal bars, 38cm long suspended horizontally above 49cm in height with support at two end by a laboratory clamp and a padded surface to ensure soft landing of the experimental animals. The rats were placed on the center of the metal bar by handling them through their tail to ensure that only the forepaws grasp the metal bar. The falling time of the rats were recorded by a stopwatch and subsequently translated into scores as described by Deacon [22].

2.5. Oxidative stress biomarkers analysis

5.5.1. Determination of protein concentration

The total protein concentrations of the cerebellar homogenates were measured using the Bicinchronic acid assay (BCA assay). Bovine serum albumin (BSA) (2 mg/mL) was used as standard with a working range between 125-2000 µg/mL

2.5.2. Antioxidant enzyme activity analysis

Superoxide dismutase (SOD) enzyme activity was determined in the rats' cerebellum homogenates using SOD Assay kit (Elabscience, E-BC-K020). The reaction mixture consist of 20µL of tissue homogenates, 20µL of enzyme working solution and 200µL of substrate application solution

fully mixed and incubated at 37^{0}C for 20minutes. The absorbance was measured at wave-length 450nm, using micro-plate reader and the results expressed as mgprot/mL.

2.5.3. Malondialdehyde (MDA) analysis

Malondialdehyde level was determined from the rats cerebellum using MDA ELISA kit (E-EL-006, Elabscience) using the principle of competitive-ELISA and the test was conducted according to manufacturer's manual (Elabscience, USA). Tissue pieces were washed, weighed and finally homogenized in PBS in a ratio of 1:9 with a glass homogenizer on ice. The homogenates were then centrifuged for 5minutes at 5000xg to get the supernatant. The obtained supernatants were used to analyze the MDA using a micro-plate reader at wave-length 450nm. The results for MDA were expressed as ng/mL.

5.6. Inductive coupled plasma mass spectrometry (ICP-MS)

2.6.1. Sample preparation

Harvested cerebellum from the rats was digested with 65% nitric acid using microwave reaction system (Anton Paar, Multiwave PRO). About 0.5 g of the samples were placed in a Teflon vessel and 4 mL of nitric acid were added, the samples were then transferred into the microwave oven for 60 minutes to obtain a free-contaminated clear digested solution, the solution was diluted with an atomic water to 25 mL in agreement with the digestion protocol as proposed by Rattanachongkiat *et al.*, (2004) and Simsek *et al.*, (2017).

2.6.2. Sample analysis with ICP-MS

Pb was determined with an Agilent 7700x inductively coupled plasma mass spectrometer (Agilent Technologies, Barcelona, Spain) equipped with a Micro Mist nebulizer (Glass Expansion, Melbourne, Australia). Table 1 shows parameters and operation conditions of Agilent 7700x ICPMS. The results were quantified using external calibration standards. Each sample was digested and analyzed in duplicate. Quality control (QC) was performed by analyzing from 20 ppb of calibration standard for every three samples.

Table 1. Work specification resume for ICP-MS Agilent 7700x and measurement parameters.

Parameters	Values	Units
RF Power	1550	W
RF Matching	1.6	V
Sample depth	9.5	mm
Torch	-0.1	mm
Torch-V	0	mm
Argon Gas Flow Rate	15	L/min
Carrier Gas Flow	1.01	L/min
Make up Gas Flow	0.15	L/min
Auxiliary Gas Flow Rate	0.58	L/min

Sample Uptake Rate	0.3	rps
Sample Uptake Rate	100	µL/ min
Sampling Depth	6 - 7.6	mm
Spray chamber temperature	2	^{0}C
Nebulizer Pump	0.1	rps
Integration Time	3	s
Internal Standard (^{103}Rh,^{208}Bi)	200	ppb

166

167 *2.7. Histopathological examination and scoring*

168 The rats' cerebellum were fixed in 10% buffered formalin and prepared for 5 days and
169 subsequently prepared for histological examination. 5µm paraffin sections of the cerebellar tissue
170 were cut with a microtome (Leica 2235 Microtome, USA) mounted and stained on glass slide with
171 hematoxylin and eosin (H&E) or toluidine blue stain [25] for histochemistry examination under a
172 light microscope (Leica DM4M, NY USA). Micrographs were captured using Moticam Pro 282A
173 5.0MP (Motic images Software Plus 2.0 TWAIN, Hong Kong).
174 The numbers of necrotic Purkinje cells in the cerebellum were quantified in all the rats groups,
175 using an image analyzer software (Motic images Software Plus 2.0 TWAIN, Hong Kong) in the
176 histopathology department, faculty of veterinary medicine, UPM. Quantification of the necrotic
177 Purkinje cells was done at x400 magnification in ten (10) non overlapping fields from six (6) different
178 sections from 3 rats of each groups. The average values of the necrotic Purkinje cells for the 10 non
179 overlapping fields were calculated for each section and analyzed with GraphPad prism.

180 *2.8. Statistical analysis*

181 The data obtained from this present study were presented as the mean ± standard error of mean
182 (SEM), using unpaired independent student t-test, one-way ANOVA and two-way ANOVA, where
183 $p < 0.05$ is considered significant using GraphPad prism (GraphPad Prism software, Inc. Version 6.01,
184 San Diego, California, USA).

185 **3. Results**

186 **Induction of Pb acetate toxicity in rats**

187 *3.1. Effect of Pb acetate on body weight of rats during Pb toxicity induction*

188 In order to evaluate the effect of Pb acetate on body weight of the experimental rats two-way
189 ANOVA was employed and the results indicates statistically significant interaction between
190 treatment effect of Pb acetate on body weight and weeks of induction of Pb-toxicity, [$F(16, 125) = 3.01$, $p = 0.0003$]. Tukey's post hoc comparison indicates a significant decrease ($p < 0.05$) in body weight
191 in rats of Group B, C, D and E in week3 and week4 respectively when compared to the body weight
192 of rats in Group A as shown in figure 2.
193

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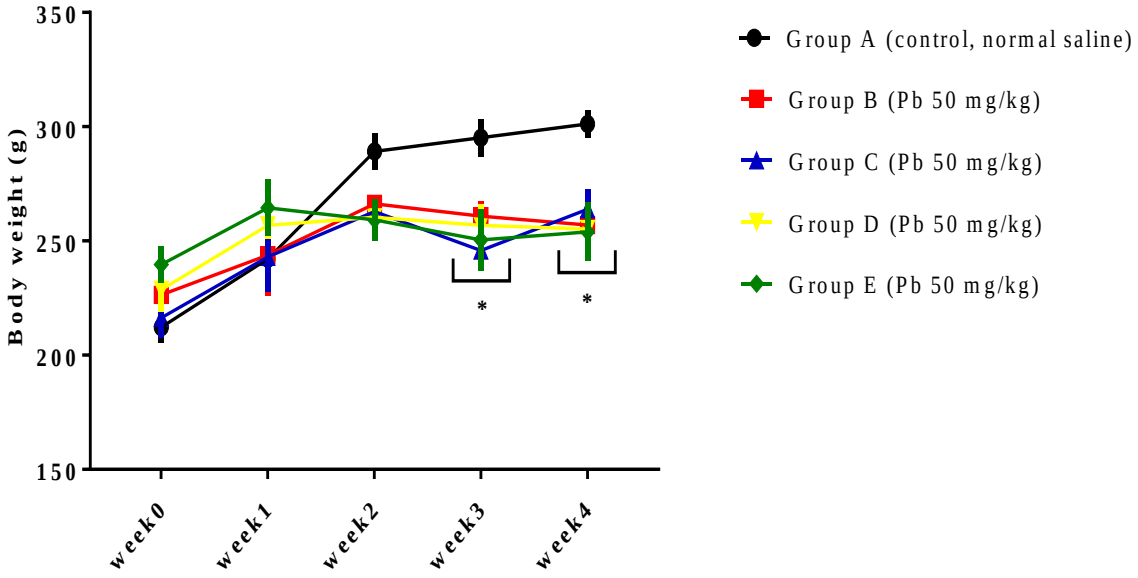

Figure 2. Effect of Pb toxicity on body weight after four (4) weeks of oral administration lead acetate during induction of Pb toxicity. Data are represented as mean ± SEM, n = 6. *p< 0.05 vs. control.

3.2. Effect of Pb acetate on motor score and coordination in rats during Pb toxicity induction

In order to evaluate the effect of oral administration of Pb acetate on motor function of rats, horizontal bar test was performed. The results indicates statistically significant interaction between treatment effect of oral administration of Pb acetate and weeks of treatment [F (16, 125) = 2.23, p=0.0072] in motor score of among the rat groups. Tukey's post hoc comparison showed significant decreases (p<0.05) in motor score of Pb-administered rats in their ability to maintain a hand grip balance on 2mm horizontal bar at week3 and week4 in rats of Group B, C,D and E compared with the motor score of rats in Group A as seen in figure 3.

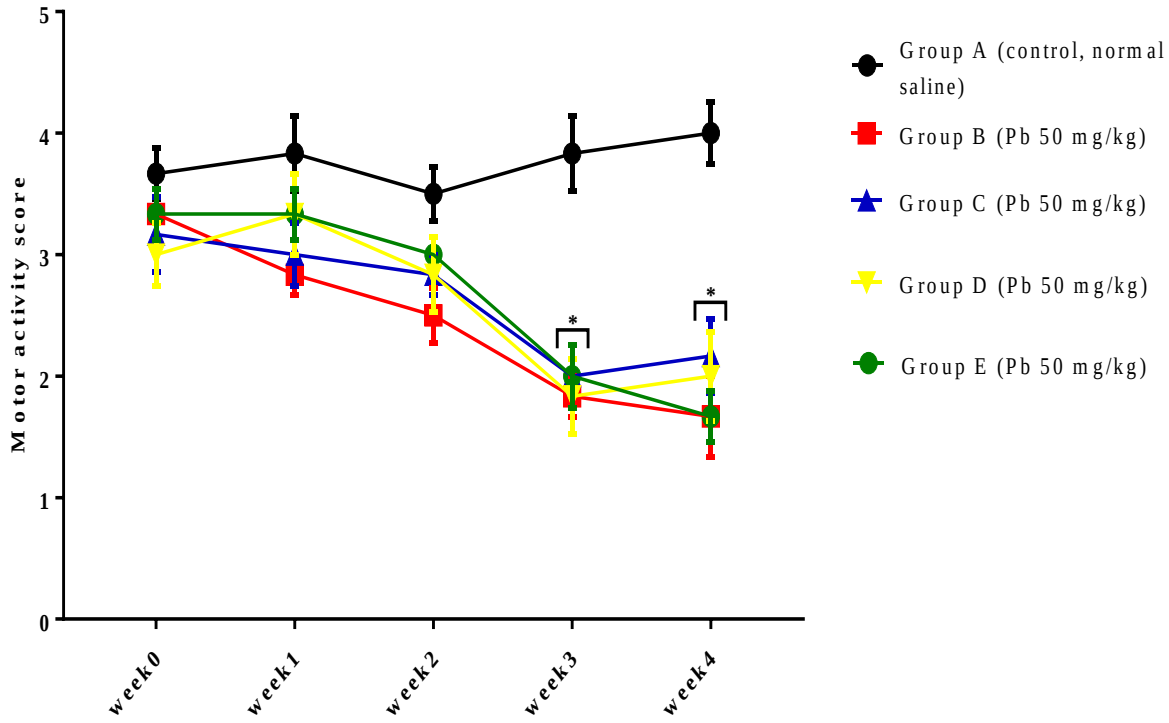

Figure 3. Effect of oral administration of Pb acetate on motor score of rats during induction of Pb toxicity. Values were presented as mean ± SEM (n = 6), * p<0.05 vs. control.

3.3. Effect of Pb acetate on oxidative stress status of cerebellum in rats of control and LTG groups during Pb toxicity induction

In order to confirm the induction of Pb toxicity in the rats, the oxidative stress status in their cerebellum were assessed through unpaired independent student t-test.

3.3.1. SOD activity

Results from the unpaired independent student t-test (figure 4**)** showed statistically significant differences of SOD activity in the cerebellum of rats in the control (M=8.4, SEM=0.4) and LTG (M=4.833, SEM=0.3283); t (4) =6.892, p =0.0023 after treatment with Pb acetate.

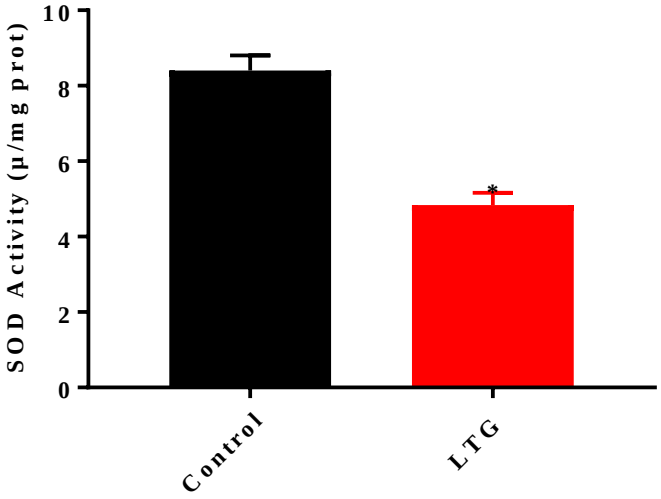

Figure 4. Effect of Pb acetate administration on SOD activity in cerebellum of rats after Pb toxicity induction. Data were express as mean ± SEM, n = 3, * p <0.005 vs. control.

3.3.2. MDA level

Unpaired independent student t-test results in figure 5 indicates statistically significant differences in MDA level in cerebellum of rats in the control (M=15.4, SEM = 2.794) and LTG (M=42.19, SEM=7.979); t (4) =3.169, p = 0.0339 after oral administration of lead acetate for four (4) weeks.

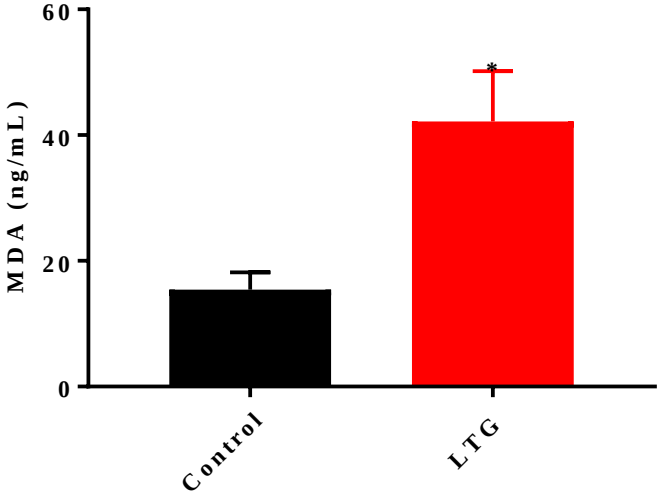

Figure 5. Effect of Pb acetate administration on MDA level in cerebellum of rats after Pb toxicity induction. Data were express as mean ± SEM, n = 3, * p <0.005 vs. control.

3.4. Determination of Pb concentration in the cerebellum of rats in control and LTG groups after Pb toxicity induction.

To determine the concentration of Pb in the rat's cerebellum, an ICP-MS techniques was used. Unpaired independent student t-test was employed to analyze the difference of Pb concentration in the cerebellum of rats in the control and LTG after oral administration of Pb acetate. The results indicates statistically significant differences in Pb concentration in the cerebellum of rats in the control (M=0.2, SEM=0.024) and LTG (M =2.58, SEM = 0.6009); t (4) = 3.981, p =0.0164 (figure 6**).**

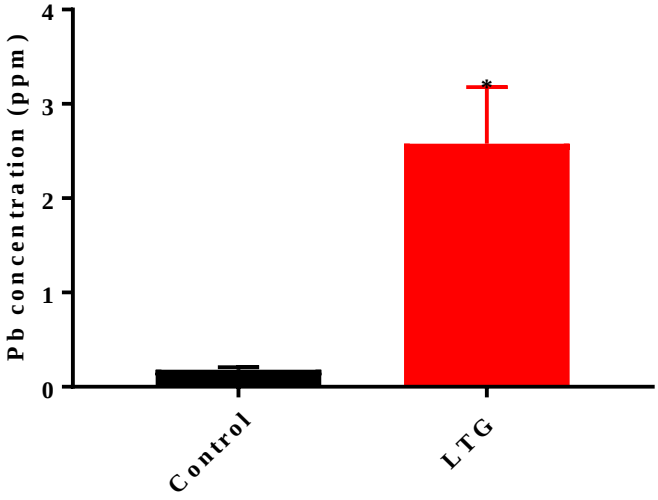

Figure 6. Concentration (ppm) of Pb in rats' cerebellum after induction of Pb toxicity for four (4) weeks. Data are represented as mean ± SEM, n = 3. *p< 0.05 vs. control.

3.5. Effect of Pb-acetate on histology of cerebellum of rats in the control and LTG groups after the induction of Pb toxicity

3.5.1. Cerebellum stained with hematoxylin and eosin (H&E)

The histopathological examination of the cerebellar tissue of Pb-administered rats using H&E stain revealed histological alteration of the cerebellar cortex layers with shrinkage and degeneration of the purkinje and molecular layer cells with scattered glia cells. The purkinje cells exhibited darkly stained nuclei with eosinophilic cytoplasm and empty spaces between them indicating degeneration of the purkinje cells (figure 7B) compared with the control rats (figure 7A).

Further, in order to evaluate the effect of Pb-acetate on purkinje cells of the cerebellum, nonparametric t-test was used. Mann-Whitney test indicated that the number of degenerated purkinje cells was statistically significantly greater in the cerebellum of rats in the LTG (M = 5.5) group, when compared to the control group (M = 0.73), U = 0, p = 0.0022 (figure 7C).

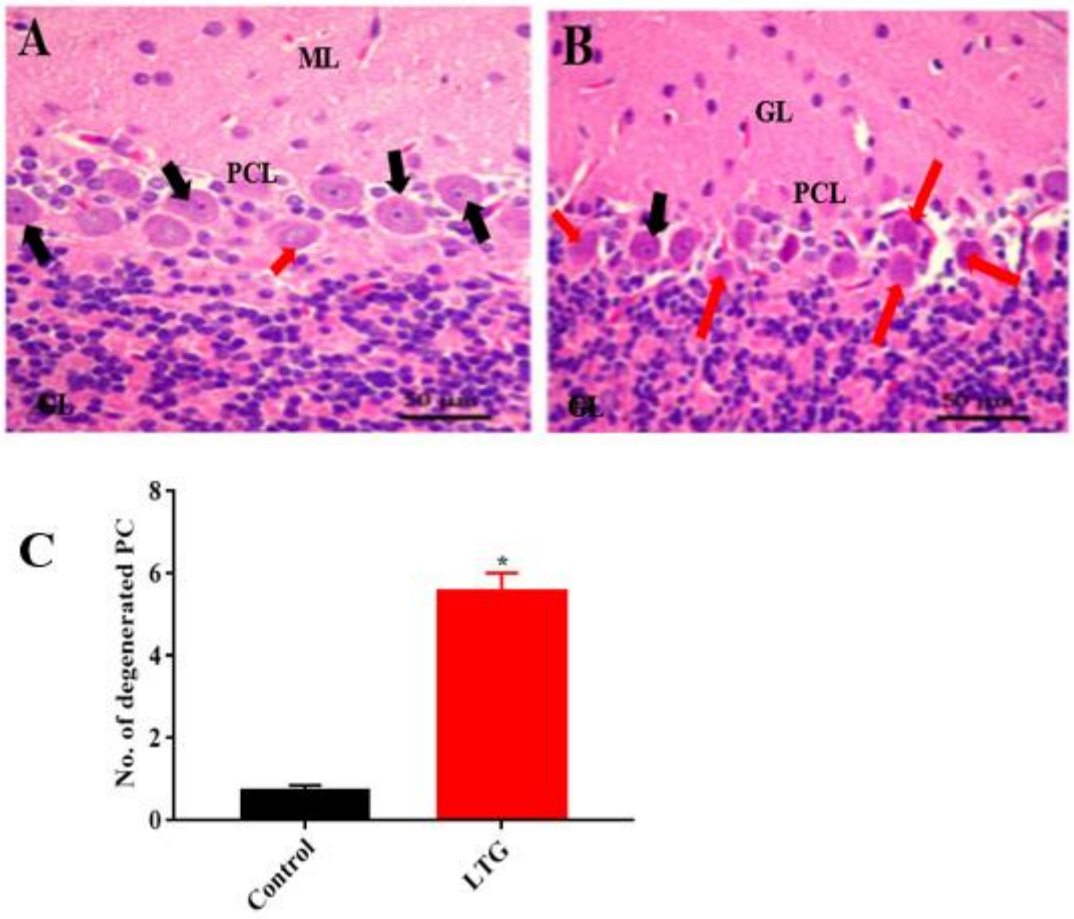

Figure 7. Photomicrograph of (A) Control Cerebellar cortex showing the layers of the cerebellum (**ML**) molecular layer, (**PCL**) middle purkinje cells layer with large pyriform shape (**black arrow**) and inner granular layer (**GL**) with aggregation of granular cells. (B) LTG group showing marked degeneration of purkinje cells (**red arrow**), (C) Semi quantitative representation of degenerated Purkinje cells of the control and LTG groups: *p < 0.05 vs. control, H&E x400, scale bar 50 μm, n = 6.

3.5.2. Cerebellum stained with toluidine blue

In order to further confirm the neurodegenerative effect of Pb-acetate induction on the cerebellum of rats, toluidine blue staining was also performed. The histological results from the cerebellum of rats in the LTG indicates degeneration of the cells of the molecular layer and distortion of the purkinje cells layer with the purkinje cells having a darkly stained cytoplasm and distorted nuclei with empty space between them indicating loss of the purkinje cells (figure 8B) compared with rats in the control (figure 8A).

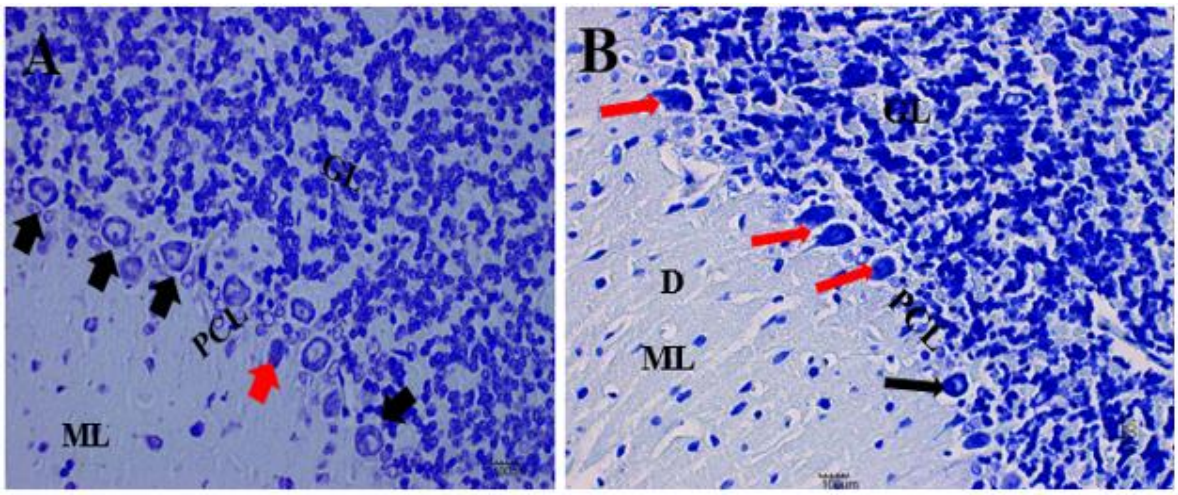

Figure 8. Photomicrograph sections of the cerebellum from rat's groups (A) Control showing molecular layer **(ML**), the granular layer (**GL**) and Purkinje cells layer (**PCL**). The Purkinje cells (**black arrow**) with prominent nuclei. (B) LTG showing molecular layer (**ML**) with degenerated area of cells (**D**), Purkinje cells with darkly stained cytoplasm and distorted nuclei leaving spaces between them (**red arrow**). The granular layer (**GL**) indicates deeply stained cells with vacuolation (Toluidine blue x200 scale bar = 100μm).

3.5.3. Effect of Pb acetate on the weight of the cerebellum of control and LTG rats groups after Pb toxicity induction

Unpaired t-test was employed to evaluate the effect of Pb acetate administration on cerebellar weight in rats of the control and LTG groups. The results showed statistically significant differences of cerebellar weight in the control group of rats (*M*= 0.5333, SEM=0.02418), when compared to LTG groups of rat (*M*=0.4033, SEM = 0.1706); t (10) =4.393, *p*=0.0013 (figure 9**)**.

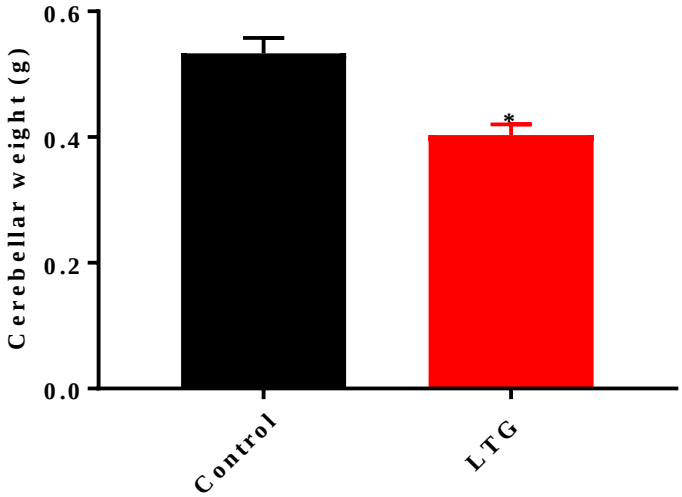

Figure 9. Effect of Pb acetate on the weight of the cerebellum of control and LTG. Data are represented as mean ± SEM, n=6, *p<0.05 vs. control

Treatments of Pb acetate induced rats with curcumin

3.6. Effect of curcumin on the body weight of Pb induced rats

Two-way ANOVA showed statistically significant interaction between treatment effect of curcumin on body weight of rats and the weeks of administration, [F (24,180) = 3.242, p=0.0001]. Tukey's post hoc test indicates a significant decrease (p < 0.05) in body weight of rats in the RC, Cur100 and Cur200 groups in week3, week4, week5, week7 and week8 respectively when compared to body weight of rats in the control as shown on figure 10.

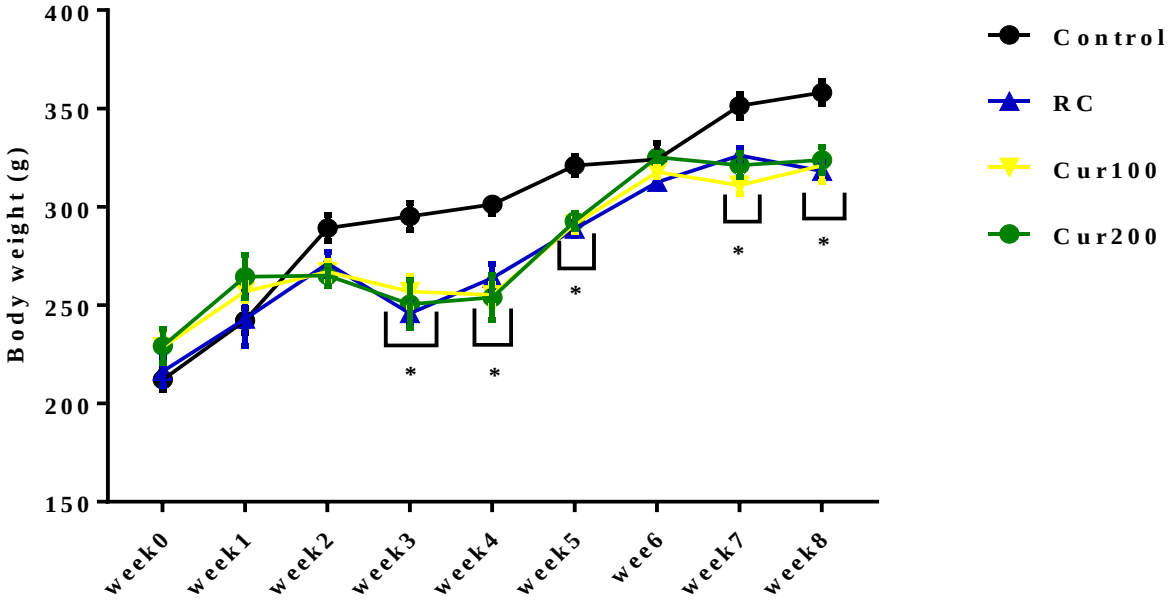

Figure 10. Effect of oral administration of curcumin on body weight of Pb acetate induced rats. Data are represented as mean ± SEM, n = 6. *p< 0.05 vs. control.

3.7. Curcumin ameliorates Pb-induced alteration of motor coordination of rats in horizontal bar test.

Two-way ANOVA showed statistically significant interaction between treatment effect of oral administration of curcumin and weeks of treatment, [F (24, 180) = 2.448, p =0.0004] in the motor score of Pb induced rats treated with curcumin (figure 11). Tukey's post hoc comparison indicates statistically significant decrease (p <0.05) in motor score of rats in the RC, Cur100 and Cur200 in the ability to maintain a hand grip balance on the 2mm horizontal bar in week3, week4, week5 and week6 when compared the control group of rats. Additionally, Tukey's post hoc test further reveal a significant increase (p <0.05 in the motor score of rats in the control group in week7 and week8, when compared to motor score of rats in the RC group rats.

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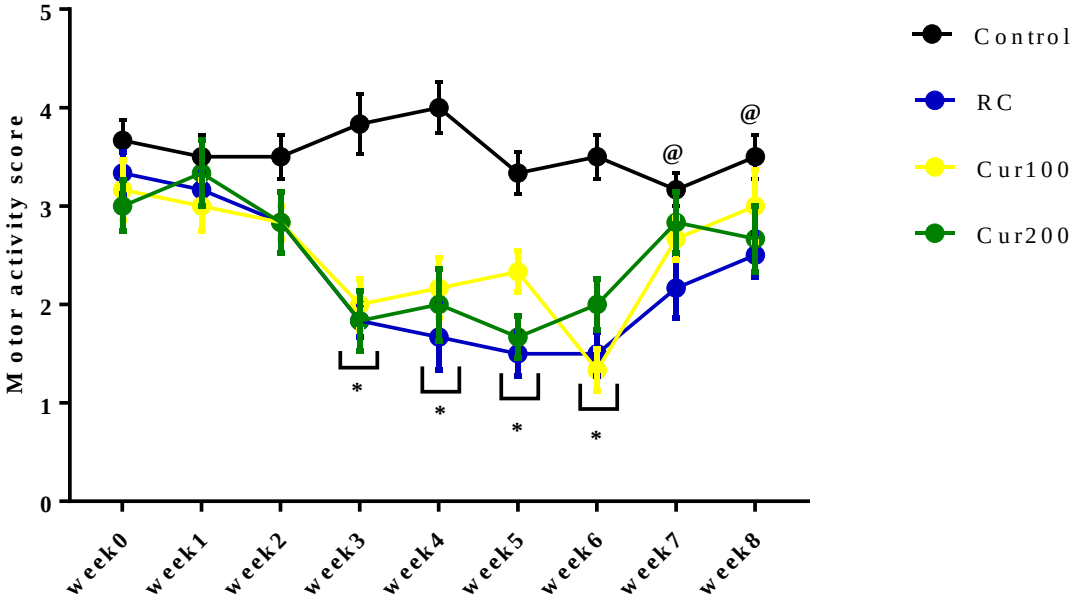

Figure 11. Effect of oral admiration of curcumin on Pb induced rats in horizontal bar test. Values were presented as mean ± SEM (n = 6), * p<0.05 vs. control, @ p<0.05 vs. RC.

3.8. Curcumin reverse Pb induced oxidative stress in rats' cerebellum.

In order to assess the antioxidant properties of curcumin on Pb-induced oxidative stress in rats, the cerebellar homogenates of the rats were analyzed for SOD activity and MDA levels.

3.8.1. SOD activity

As shown in figure 12, one-way ANOVA revealed statistically significant differences in SOD activity in the cerebellum of different rats groups [F (3, 8) =3.768, p = 0.0493]. Tukey's post hoc test showed statistically significant decrease in SOD activity in cerebellum of the RC (5.167 ± 0.133, p = 0.0443) when compared with the control group of rats (8.4 ± 0.4).

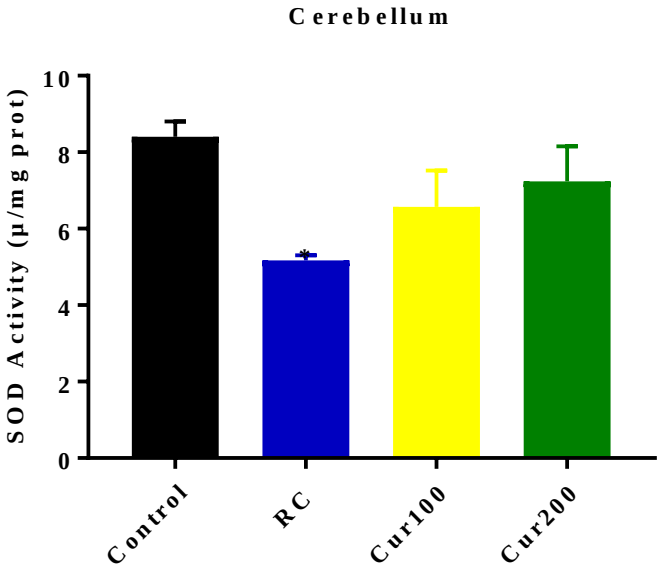

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Figure 12. Effect of curcumin on SOD activity in the cerebellum of Pb induced rats. Data were express as mean ± SEM, n = 3, * p <0.05 vs. control.

3.8.2. MDA level

One-way ANOVA showed statistically significant differences of MDA level in the cerebellum of all the rats groups, [F (3, 8) =5.844, p = 0.0205]. Tukey's comparison test revealed significant increase in MDA level in the cerebellum of rats from RC group (22.78 ± 1.579, p =0.0153), when compared to the control group of rats (15.4 ± 1.062) as shown in figure 13.

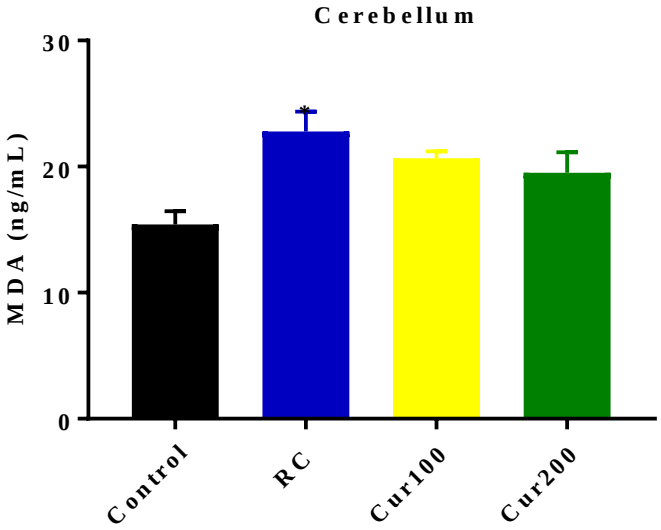

Figure 13. Effect of curcumin on MDA level in the cerebellum of Pb induced rats. Data were express as mean ± SEM, n = 3, * p <0.05 vs. control.

3.9. Chelating potentials of curcumin onPb induced toxicity in rats

The results obtained from ICP-MS analysis was subjected to one-way ANOVA to evaluate the mean Pb concentration in the cerebellum of rats in the control, RC, Cur100 and Cur200. Results obtained showed statistically significant differences in Pb concentration in the cerebellum of different rats groups, [F (3, 8) = 27.55, p = 0.0001]. Tukey's post hoc comparison indicated significant decreases in Pb concentration in the cerebellum of rats in the control (0.1828 ± 0.02414, p = 0.0001), Cur100 (0.6319 ± 0.1545, p = 0.0014) and Cur200 (0.4848 ± 0.08147, p = 0.006), when compared to rats in the RC group (figure 14).

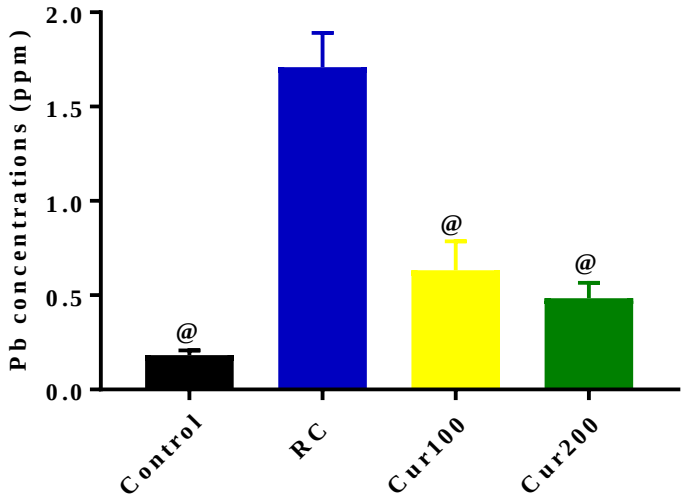

Figure 14. Concentration of Pb in rats' cerebellum after oral administration of curcumin and withdrawal of Pb acetate for four (4) weeks. Data are represented as mean ± SEM, n=3. @ p< 0.05 vs. RC.

3.10. Curcumin attenuates Pb induced cerebellar damage in rats cerebellum

2.10.1. H&E staining

The histology results from the cerebellum of rats in the RC groups of rats stained with H&E after withdrawal of Pb-acetate indicated no recovery when compared to rats in control group (figure 15A). The RC group cerebellum revealed eosinophilic purkinje cells with dark and irregular nuclei with the molecular layer appeared to have scattered glia cells with perineural spaces, thus the granular layer appeared to have normal histological appearance (figure 15B).

Oral administration of curcumin for four (4) weeks attenuated the pathological changes in the cerebellum of rats in the Cur100 (figure 15C) and Cur200 (figure 15D)

In addition, semi quantitative analysis of the purkinje cells of the cerebellum revealed statistically significant differences in degenerated purkinje cells of the experimental rats, [H (3) = 19.75, p = 0.0002], with a mean rank of 3.583 for control, 21.5 for RC, 13.83 for Cur100 and 11.08 for Cur200. Dunn's multiple comparison test further showed statistically significant increases (4.333 ± 0.2246, p=0.0001) in degenerated purkinje cells in the cerebellum of the RC group of rats, when compared to the rats in the control group (figure 15E).

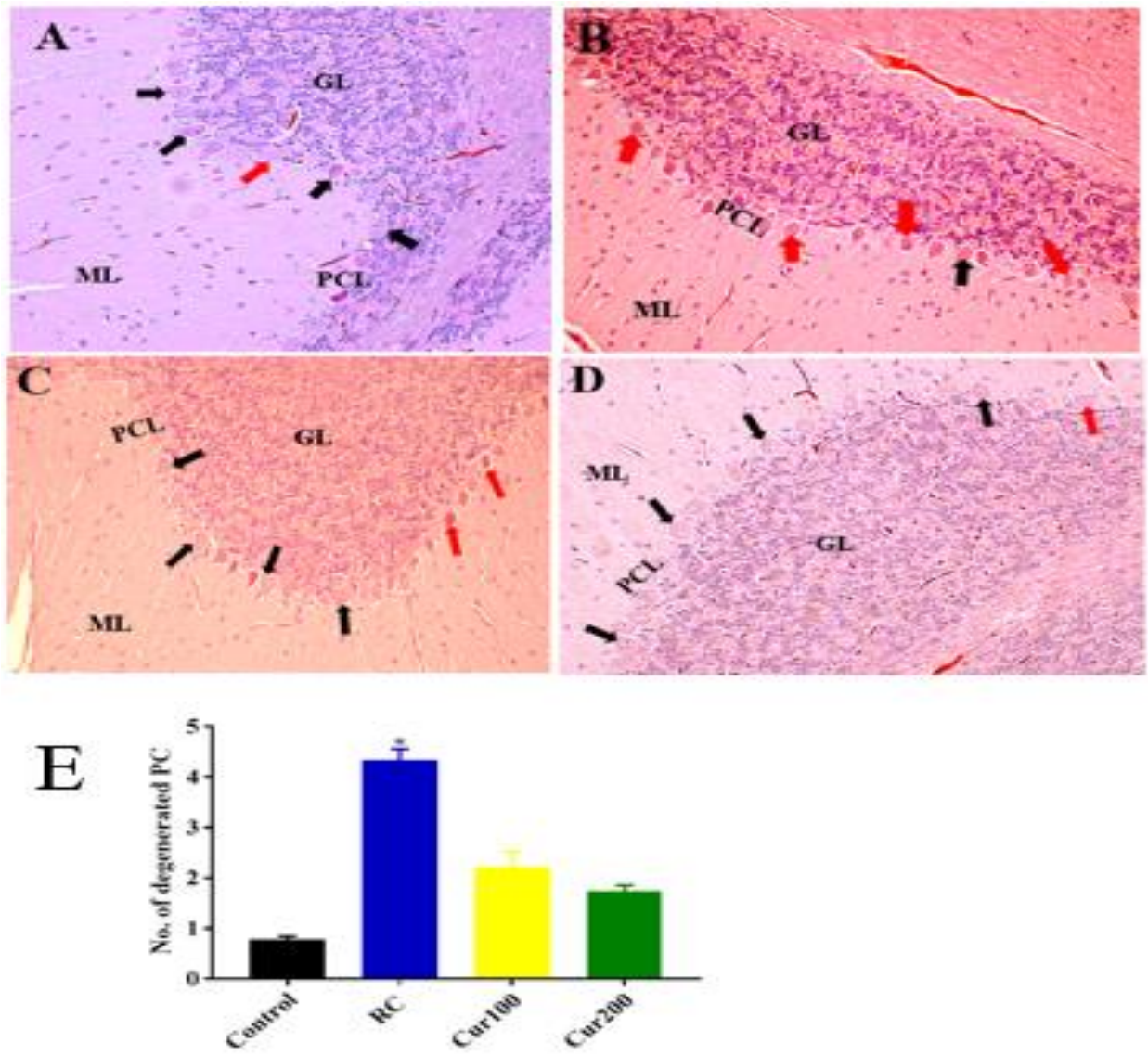

Figure 15. Photomicrograph sections of the cerebellum of rat's groups (A) control indicating layers of the cerebellar cortex, the molecular layer (**ML**) with glia cells (**red arrow**), middle Purkinje cells layer (**PCL**) with Purkinje cell having a large pyriform shape (**black arrow**) and inner granular layer with aggregation of granular cells (**GL**). (B) RC showing eosinophilic Purkinje cells with irregular dark cytoplasm (**red arrow**) and scattered glia cells in the molecular layer (**ML**). (C) Cur100 showing the Purkinje cells with prominent nuclei and regular shape (**black arrow**) and the molecular and granular layer appear normal. (D) Cur200 showing normal pyriform shaped purkinje cells with prominent nuclei (**black arrow**) with normal granular and molecular layer. (E) Semi quantitative representation of degenerated Purkinje cells: *p< 0.005 vs. control, H&E x200, scale bar 100 μm.

3.10.2. Toluidine blue staining

The photomicrograph section from the cerebellum of the control group of rats indicates normal histological structure of the cerebellum with the three layers of the cerebellar cortex. Purkinje cells appeared to have a regular prominent central nucleus with an apical dentrites. Granular and molecular layer showed normal cells with dark stained nuclei (figure 16A).

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Photomicrograph section from the cerebellum of RC group of rats revealed alteration in the purkinje cells layer with the purkinje cells appeared to have darkly stained cytoplasm with irregular nuclei. The molecular layer showed scattered glial cells, although the granular layer appeared to be normal with granular cells (figure 16B).

The photomicrograph section from the Cur100 and Cur200 showed restoration of the purkinje cells layer with healthy purkinje cells. The molecular and granular layers appeared to be normal with normal cells (figure 16C and figure 16D).

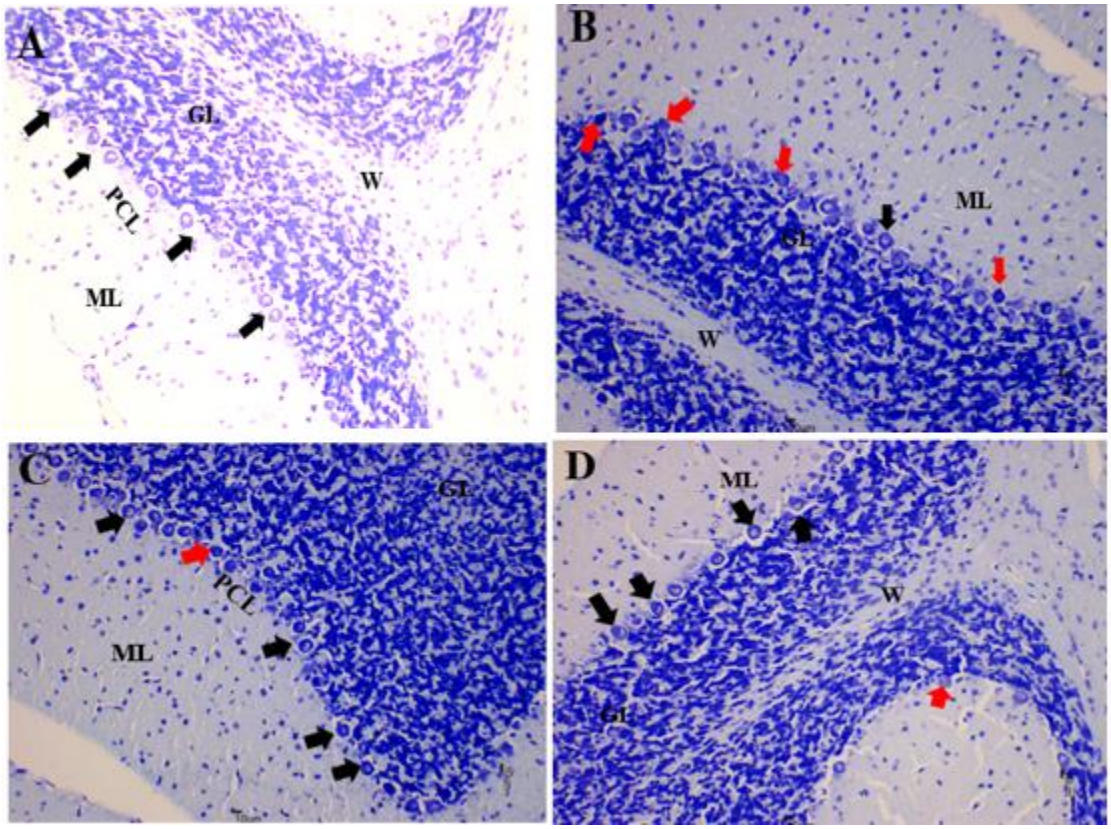

Figure 16. Photomicrograph sections of the cerebellum from rat's groups (A) Control showing the white matter (W) and the three layers of the cerebellar cortex, molecular layer (**ML**), the granular layer (**GL**), the purkinje cells layer (**PCL**) with the Purkinje cells (**black arrow**) with prominent nuclei. (B) RC showing the purkinje cells having deeply stained cytoplasm with distorted shape (**red arrow**) and the molecular layer showing scattered glia cells (**ML**). (C) Cur100 and (D) Cur100 showing the restoration of the molecular (**ML**) and granular layer (**GL**), the Purkinje cells layer (**PCL**) appeared to be arranged in a linear pattern with the Purkinje cells (**black arrow**) having prominent nuclei and regular shape. Toluidine blue, x200 scale bar = 100μm.

3.10.3. Effects of curcumin administration on cerebellar weight

One-way ANOVA indicates statistically significant differences in weight of the cerebellum among the groups of rats [F (3. 20) =3.195, p=0.457].Tukey's post hoc comparison revealed statistically significant decreases in the cerebellum weight of RC group of rats (0.4617 ± 0.01778, p = 0.0486), when compared to control group as shown in figure 17.

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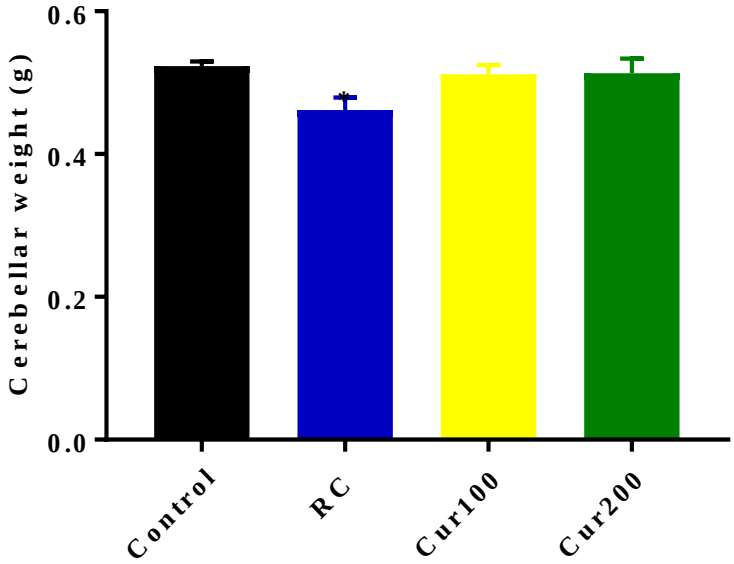

Figure 17. Effect of curcumin administration on cerebellar weight of Pb induced rats. Data are represented as mean ± SEM, n=6. *p<0.05 vs. control.

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4. Discussion

Preceding studies have documented the toxic effects of Pb on biological systems resulting into several pathologies and clinical implications with morbidity on almost all the organs with the brain, kidney and liver serving as primary targets [12,26,27]. These pathological alterations may include increase oxidative stress [9], neurological deficit [28], decrease motor coordination and cognitive deficit [29,30]. Alteration in astrocyte maturation, degeneration of neural cells [28,30], renal dysfunction, degenerative changes in tubular epithelium and hepatotoxicity [2,31] were also reported in Pb poisoning both in humans and different rodents species. In the present study, rat model of Pb toxicity was used to investigate the therapeutic potentials of curcumin on fundamental mechanism of motor dysfunctions and neurodegeneration caused by Pb toxicity. The Pb induced rats developed a remarkable deficiency in cerebellum dependent horizontal bar test for motor functions, increased oxidative stress, besides marked degeneration of cells in molecular and purkinje cell layers of the cerebellum and high concentration of Pb in their cerebellum. However, treatment with curcumin irrespective of the dose given attenuated the above aforementioned alterations and aberrations caused by Pb acetate induced toxicity in the rats.

Worthy to note in this study, is the choice of male Sprague Dawley rats for all the experimentations. As female sex hormones such as estrogen, prolactin and progesterone may influence cognitive function, emotion and motor behavior in rats, because their cyclical hormonal changes usually comes with mood swing. Hence, female rats were less preferred by the authors for pharmacological testing of phytochemical substance like curcumin in order not to be a confounding factor [32–34]. Therefore, healthy male Sprague Dawley rats were used in this present study to evaluate the neurotherapeutic effects of oral administration of curcumin on Pb-induced toxicity of motor function and coordination.

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Coordination of motor behavior and cognitive function depend on the integrity of the nervous system and core psychological functions of the animals [22,35]. The cerebellum is a delicate structure that is vulnerable to intoxication and poisoning with the cerebellar circuit especially the purkinje cells susceptible to injury after exposure to environmental toxins such as Pb [36]. Further, the cerebellum plays a vital role in the unification of motor and sensory functions, any lesion on the cerebellum may resulted into deformation of motor coordination and balance [37]. In this study decrease in motor score on horizontal bar method test were observed in Pb induced rats which is in agreement with the previous works documented by Nehru and Sidhu [38], Barkur and Bairy [39], and Sabbar *et al*. [40] who also reported decrease in cognitive and motor function in rat models of Pb toxicity. Mason *et al*. [7] also reported that Pb exposure affect motor function such as deficits in visuomotor coordination in adult Pb workers and children exposed to Pb. The mechanism in which Pb execute this decrease motor function could be due to its ability to induce oxidative stress in rats exposed to Pb [6]. However, the present study observed increase in motor score of rats treated with curcumin regardless of the dose given. Moore *et al*. [41], also observed a similar findings and attributed it to the antioxidant and anti-inflammatory properties of curcumin on motor function. Chongtham and Agrawal [42] also reported that curcumin ameliorates disease symptoms in Drosophila model of Huntington disease (HD). However, supplementation of diet with curcumin for 12 weeks in healthy aging population does not influenced their motor performance [43].

Alterations of oxidative status either by overproduction of oxidant or deficit in antioxidant activity could be one of the direct consequences of Pb-toxicity and poisoning in living organisms [6]. Preceding studies indicates that oxidative stress is linked to the pathogenesis of Pb-toxicity resulting in lipid peroxidation [6], neurodegeneration [44], oxidation of hemoglobin [45] and impairment of fundamental biological cellular process such as cell adhesion, intra and inter cellular signaling, ionic transportation, enzyme regulation, neurotransmitters release and apoptosis [12]. The fundamental oxidant that plays vital role in redox reaction includes hydroxyl radical (OH), hydroxyl anion (OH^-), hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite ($ONOO^-$) [46]. MDA or thiobarbituric acid-reactive species (TBARS) is the end product of lipid peroxidation that plays a vital role in lipid membrane damage in cells due to increase reactive oxygen species (ROS) [6]. The present study revealed an increased level of MDA and decreased SOD activity in the cerebellum of Pb-induced rats. However, these alterations were ameliorated by treatment with curcumin irrespective of the dose given. These findings were in agreement with the previous studies that have reported the antioxidant properties of curcumin [47–49].

Inductive coupled plasma mass spectrometry (ICP-MS) is a robust technique for molecular analysis of element and physiochemical compound with separation techniques that makes it suitable for detecting elements in pharmaceutical research and for specific investigation of element present in molecule [50]. ICP-MS is a multi-element system techniques in the analysis of biological fluids with greater sensitivity and selectivity when compared to inductive coupled plasma-optical emission spectrometry (ICP-OES) and graphite furnace atomic absorption spectrometry (GF-AAS) [24]. Concentration of trace elements beyond physiological limits in organs can be toxic in both animals and humans. Likewise, concentration of heavy metals such as Pb in the biological system are known to be toxic thus, affecting biochemical reactions [24]. Further, bones remain a vital site for Pb accumulation after exposure, although circulating Pb in the blood can be distributed to various vital organs such as the brain, kidney and liver [51,52]. This present study decided to use ICP-MS for its

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467 superiority over other techniques and found significantly high concentration of Pb in the cerebellum
468 of Pb-induced rats when compared to their control counterparts. The results is in agreement with the
469 previous works of Sousa *et al.* [52] and Simsek *et al*. [24] that reported increased concentration of Pb
470 in the brains of Pb-induced rats. Noteworthy, is administration of curcumin to Pb induced rats
471 decreased the concentration of Pb in their cerebellum. These findings were in accordance with the
472 previous studies of Mary *et al*. [18] and Shen *et al.* [53], who reported the chelating properties of
473 curcumin.

474 In the present study, multiple pathological lesions were observed in the cerebellum of Pb
475 induced rats. These changes includes neuronal damage and alteration of the histological architecture
476 of the cerebellar cortex. The purkinje cells exhibited eosinophilic cytoplasm with darkly stained and
477 irregular nuclei leaving empty spaces between them. Semi quantitative analysis of purkinje cells
478 showed significant increase ($P<0.05$) in the number of necrotic purkinje cells. Further, Pb-induced rats
479 showed decreased ($P<0.05$) cerebellar weight, which could be attributed to the marked degeneration
480 of the cerebellar cells observed. These results are in accordance with several preceding studies
481 [9,26,37,54,55] that reported similar findings of neuronal degeneration in Pb and other heavy metals
482 toxicity on the cerebellum with more consequences on the purkinje cells due to sensitivity of the
483 purkinje cells layer. However, treatment with curcumin reverses the above mentioned Pb-induced
484 morphological aberrations in the rats. This could be due to the antioxidant properties of curcumin as
485 seen in the present study or combined antioxidant and anti-inflammatory role of curcumin in
486 neurotoxicity and neurodegenerative diseases as documented by previous studies [16,56,57].

487 The most important step in dealing with Pb-toxicity is to avoid exposure to the sources of Pb
488 contamination which might not always be feasible. Therefore, there exists standard drugs chelators
489 in the markets that are being used in terms of heavy metal poisoning, however, they are expensive
490 and exhibits a lot of adverse effects [12]. This study revealed that withdrawal of Pb acetate alone was
491 not enough to restore the damages inflicted by Pb-toxicity in rats of the RC group. The persistent
492 changes observed after the Pb withdrawal includes, morphological aberrations of the cerebellum,
493 decrease in motor score on horizontal bar test, oxidative stress as well as high concentration of Pb the
494 cerebellum on ICP-MS analysis when compared to the curcumin treated groups. These could be due
495 to specific kinetics, where portion of Pb absorbed is accumulated in the bone [11,58,59], thus
496 gradually released to soft tissues such as the brain, kidney and liver [58–60]. Similar findings were
497 reported by Omobowale [61]; Nehru and Sidhu [38]; and Khalaf *et al*. [62] after withdrawal of Pb
498 exposure in rats model of Pb-toxicity.

499 **5. Conclusions**

500 In consideration to the results obtained from this study, Pb-toxicity resulted into decreased motor
501 functions, increased oxidative stress, aberrations in the histological structure of the cerebellum as
502 well as accumulation of Pb in the cerebellum of affected rats. Treatment with curcumin regardless to
503 the dose attenuates the abnormalities caused by Pb-toxicity, which could be due to the antioxidant
504 and chelating properties of curcumin (figure 18). It is noteworthy, to conclude that curcumin could
505 be develop as natural drug for treatment of Pb-toxicity due to its therapeutic potentials and wide
506 pharmacological safety margin. Finally, the authors recommend future research on longer treatment
507 periods with to assess the effectiveness of its long term preclinical applications in Pb-induced toxicity
508 and neurodegeneration.

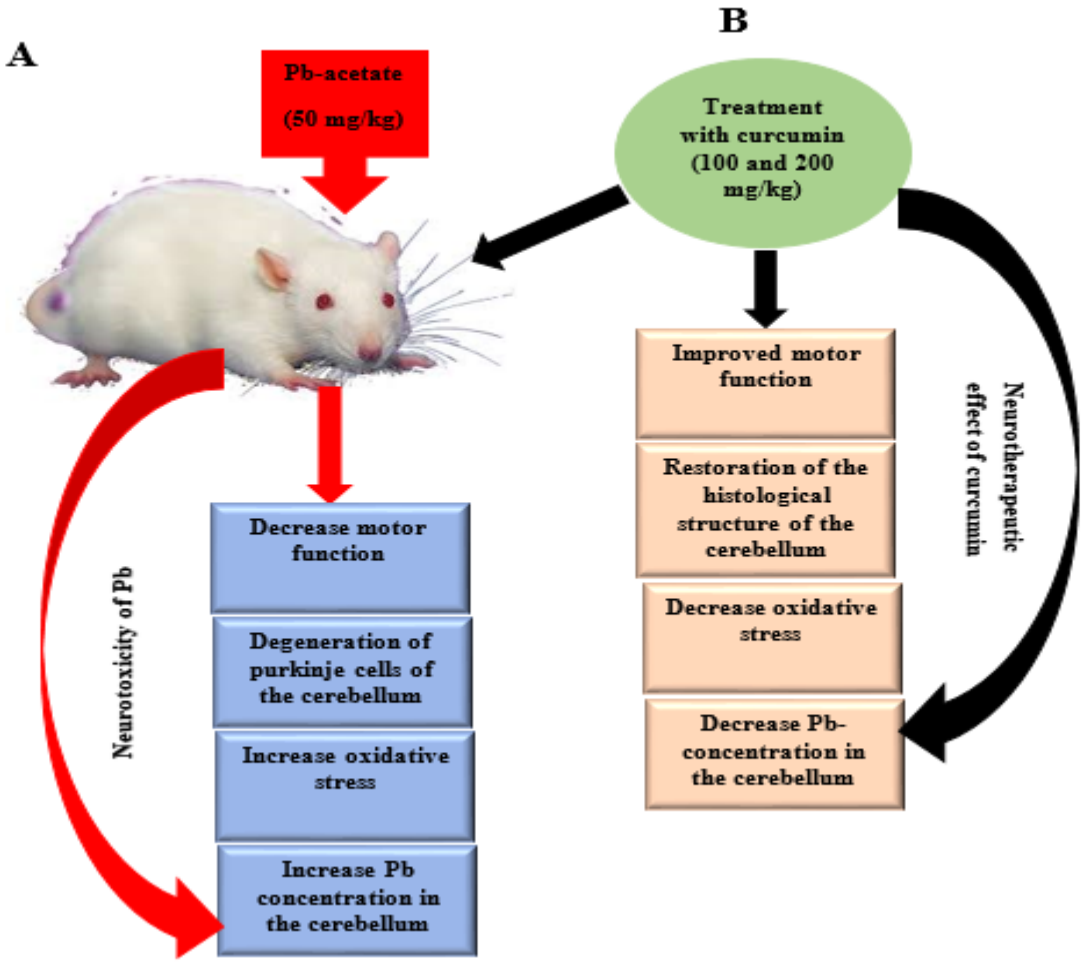

Figure 18. Proposed mechanism of Pb-induced cerebellar toxicity and the ameliorative effects of curcumin.

Abbreviations

ABLES: adults blood lead epidemiology and surveillance; BLLs: blood lead levels; Pb: lead; Pb-acetate: lead acetate; ROS: reactive oxygen species; BBB: blood brain barrier; SAH: subarachnoid hemorrhage; MDA: malondialdehyde; MMP-9: matrix Metallopeptidase 9; Mn^{2+}: manganese ion; Fe^{2+}: iron ion; Cu^{2+}: copper ion, Zn^{2+}: zinc ion, HBM: horizontal bar method, BCA: Bicinchronic acid assay, BSA: bovine serum albumin; SOD: superoxide dismutase; OD: optical density; ICP-MS: inductive coupled plasma spectrometry; QC: quality control; ANOVA: analysis of variance; H&E: hematoxylin and eosin; HD: Huntington disease.

Author Contributions: "conceptualization, K.A.; E.A.R and Md.Z.A.; methodology K.A.; A.D.; E.A.R and Md.Z.A, validation, S.M.C; A.D.; and Md.Z.A.; formal analysis K.A.; A.D. and M.M.M. investigation, K.A.; M.M.M.; A.D.; E.A.R, and Md.Z.A.; data curation, K.A; M.M.M.; S.M.C.; and; A.D; writing—original draft preparation, K.A.; writing—review and editing, K.A.; M.M.M.; A.D. and S.M.C.; supervision, E.A.R..; Md. Z.A.

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