Abstract

Background: Aluminium Chloride ($AlCl_3$) is a heavy metal with reported damaging consequences on various tissues and organs. With the overarching aim to discern the neuropathological effects of toxic insults and explore substances that may influence the outcome of such experiences, we examined the putative impacts of dietary walnut on $AlCl_3$-induced neurotoxicity. **Results:** $AlCl_3$ treatment for 28 days induced significantly decreased catalase (CAT) activities, an overexpressed LDH level as well as a significant elevation in IL-3 levels, and nitric oxide levels markedly increased when compared with the control and walnut-treated animals ($p<0.05$) resulting in induced oxidative stress, altered glucose metabolism, and neuroinflammation; as well as increased chromatolysis and degenerative changes in the cerebellar cortex. Interestingly, walnut-enriched diet administered post and pre-treatment with $AlCl_3$ significantly improved cerebellar CAT activities, downregulated LDH levels and the activity of IL-3, and significantly reduced nitric oxide levels with a p-value <0.05 when compared with the Aluminium treated group. **Conclusion:** In this study, walnut-enriched diet diminished Aluminium-induced cerebellar perturbation in rats by modulating cellular and oxidative damage and counterbalances inflammatory cytokines and nitrosative stress proteins evoked by $AlCl_3$ neurotoxicity. These results suggest that walnut-enriched diet may play a critical role in mitigating the progression of toxic cascades witnessed in neurodegenerative disorders triggered by Aluminium neurotoxicity.

Keywords: Neurodegeneration, walnut-enriched diet, Aluminium neurotoxicity, Cerebellum, interleukin-3, Nitric oxide, oxidative stress.

Running Title: Dietary walnut abrogates $AlCl_3$ cerebellar toxicity

Type of article: Original Research Article

1.0 Introduction

Walnut is a common fruit used as a food supplement all over the world. In Nigeria, the African walnut (*Tetracarpidium conophorum*) is known as awusa or asala in Yoruba; ukpa, or oke okpokirinya in Igbo and gawudi bairi in Hausa; and okhue or okwe in Benin (Chijoke et al., 2015; Kanu et al. 2015). It is called musyabassa in Sierra Leone, and kaso or ngak in western Cameroon (Burkill 1985). All the plant parts of walnut are used as medicine in the treatment of different diseases (Rathi *et. al,* 2014). The US Department of Agriculture's food composition database states that 100 g of walnuts provide 15.2 g of protein, 65.2 g of fat, and 6.7 g of dietary fiber. Antioxidants found in walnuts, such as flavonoids, phenolic acid (ellagic acid), melatonin, folate, vitamin E, selenium, juglone, and proanthocyanidins, are abundant (3.68 mmol/oz) (Chauhan & Chauhan, 2012; Reiter et al., 2005; Fukuda et al., 2003). Walnuts, in contrast to other nuts, contain substantial levels of polyunsaturated fatty acids, particularly -linolenic acid (18:3n-3; 9.1 g) and linoleic acid (18:2n-6; 38.1 g) (Li et al., 2007) ALA, also known as linoleic acid, has strong anti-inflammatory and anti-atherogenic effects (Baum et al., 2012) and also impact cholesterol levels and other risk factors for cardiovascular disease (Ros & Mataix, 2006). Hence improved blood lipid profile is associated with walnut diet intervention without necessarily promoting body weight or adversely affecting blood pressure (Guasch-Ferre et al., 2018). In addition, iNOS, COX-2, and pro-inflammatory cytokines (IL-1, IL-6, and TNF-) are all downregulated by ALA, which prevents NO production (Reifen et al., 2015). In research on animals, nutritional supplementation with walnuts enhanced memory, learning abilities, motor development, and anxiety-related behavior in an animal model of Alzheimer's disease (Muthaiyah et al., 2014), as well as improved cognitive and motor function in aged rats (Willis et al., 2009). In addition, improved cognitive performance (Valls-Pedret et al., 2018; O'Brien et al., 2014) and a significant increase in inferential verbal reasoning (Pribis et al., 2012) were associated with walnut diet supplementation in humans.

Aluminium is one of the toxic metals that is known to be a major environmental pollutant across the world and has been reported to be associated with the onset and progression of neurodegenerative diseases. Alzheimer's dementia (AD) is thought to be influenced by aluminum, a powerful neurotoxin that induces protein misfolding and self-aggregation of highly phosphorylated cytoskeletal proteins (Kawahara et al., 1994). It is reported to be present in both senile plaques and neurofibrillary tangle (NFT)-bearing neurons within AD patients' brains (McLachlan et al., 1996). It is also a potent cholinotoxin and causes apoptotic neuronal loss, which

is a characteristic symptom of neurodegeneration associated with AD (Gulya et al., 1990). In addition, prolonged Aluminium chloride exposure induces oxidative stress and increases amyloid-beta levels in vivo treatment (Kumar *et. al.,* 2009). Due to the high permeability of Aluminium into the brain via the specific high-affinity receptors for transferrin (TfR) expressed in the blood-brain barrier (Roskams and Connor, 1990), it has been reported to induce inflammatory responses (Campbell et al., 2004), cause synaptic structural abnormalities which leads to profound memory loss, increase free radical generation, which results to lipid peroxidation, increase energy metabolism, and induce neurotoxicity in rats (Olowoyeye et. al., 2018). Therefore this study was designed to elucidate the neuroprotective and ameliorative impacts of walnut-enriched diet against $AlCl_3$-induced cerebellar neuronal damage in Wistar rats.

2.0 Materials and Methods.

2.1 Preparation of walnut diet

Dried cured African walnut (*Tetracarpidium conophorum*) were procured from a commercial market in Osogbo and then soaked in hot water to soften the nutshell. After softening, a nutcracker was used to separate the shell from the kernel. The kernel was cut into smaller sizes, air-dried for 4 weeks, pulverized with a blender into powder, and mixed with powdered standard rat chow. The mixture was pelletized into the walnut diet using a pellet machine. Table 1 shows the composition of the control and walnut diet.

Table 1: Composition of the control and walnut-enriched diet.

Ingredient	% of weight	Control Diet Amount/100g	Walnut Diet Amount/100g
Groundnut Cake	25%	25g	24.4g
Palm kernel Cake	25%	25g	24.4g
Corn starch	15%	15g	13.5g
Industrial soya	10%	10g	9.4g
Bone meal	5%	5g	5g
Wheat (Fibre)	5%	5g	4.8g
Lysine	0.2%	0.2g	0.2g
Methionine	0.3%	0.3g	0.3g
Concentrate	4.5%	4.5g	4.5g
Ground Walnut		0	11.1g
Soybean oil	10%	10g	2.63g

Total	100%	100g	100.2g

2.2 Ethical Statement:

Ethical approval was obtained from the Health Research Ethical Committee (HREC) of College of Health Sciences, Osun State University, Osogbo, Osun state (UNIOSUNHREC 2021/003C) and the study was conducted in accordance with the ARRIVE guidelines, and the ethical guidelines of the 1975 Declaration of Helsinki**.**

2.3 Animal groupings

Twenty-eight (28) male Wistar rats weighing between 150g - 170 g were housed in the Osun State University's Animal House, located in Osun state. They were acclimated to typical laboratory surroundings and fed rat meals and water at will. The rats were randomly distributed into 4 groups (A – D), each consisting of 7 rats (n = 7). Group A received distilled water and normal diet ad libitum daily for 28 days; group B received $AlCl_3$ with normal diet ad libitum daily for 28 days; group C received walnut diet ad libitum daily for the first 28 days after which $AlCl_3$ was administered with normal diet ad libitum for the last 28 days, and group D received $AlCl_3$ with normal diet for the first 28 days after which walnut diet was introduced ad libitum daily for the last 28 days. The experimental animals were housed singly in metabolic cages.

Neurotoxicity in rat models was induced by oral administration of 100mg/kg body weight of AlCl3 dissolved in distilled water. The correct volume was guaranteed by using a calibrated syringe with an oral cannula while rats were fed with walnut diet ad libitum according to the grouping.

2.4 Tissue collection and preparation

At the end of days 28 and 56 respectively, groups A-B and groups C-D were euthanized by cervical dislocation. The brain tissue in each group was carefully and quickly removed from the skull using brain forceps and fixed in 10% neutral buffered formalin (NBF) for 48 hours for histological analysis. After dehydration with ascending grades of alcohol, clearing with xylene and infiltration and embedding in paraffin wax, the paraffin block containing the tissue was sectioned by a rotary microtome at a thickness of 6µm. Histological staining was performed using Hematoxylin and Eosin as described by Bancroft & Layton (2012). Demonstration of Nissl substances using Cresyl fast violet stain was performed by the method of Pilati et al., (2008). Using a 5.0 MP Amscope

and an Olympus binocular research microscope (Olympus, New Jersey, USA), photomicrographs of the stained sections were taken. The stereological assessment of the Purkinje layer of the cerebellar sections was measured using Image J software.

Cerebellar tissue of animals across the different groups for enzymatic studies were separated from the brain, blot-dried, weighed, and homogenized with the aid of an automated homogenizer in ice-cold 0.1M phosphate buffer at 4°C. The homogenate was centrifuged at 12000g for 5 mins at 4°C. The supernatant produced was aspirated into plain labeled glass cuvette and immediately used for the different enzymatic assays.

2.5 Assay for Catalase and Lactate dehydrogenase

The activity of catalase in the tissue supernatant was determined by a dichromate method described by Sinha 1972 and modified by Hadwan 2016. For lactate dehydrogenase assay, cerebellar tissue was rinsed in phosphate-buffered saline (pH 7.4) to remove the blood and homogenized in 5 mL buffer containing 100 mM potassium phosphate (pH 7.0) and 2 mM EDTA. The homogenate was centrifuged at 10,000 g for 15 min at 4°C and the supernatant was aspirated for assay. Lactate dehydrogenase assay kit with catalog number KA1653 procured from Abnova was used for this assay. 50 µL of supernatant was transferred into 1cm cuvettes with 950µL LDH working reagent pipetted into it and mixed briefly. Absorbance was read spectrophotometrically at 565nm.

2.6 Determination of Interleukin-3 activities and Nitric oxide

Interleukin-3 (IL-3) was assessed in the supernatant using a commercially accessible enzyme-linked immunosorbent assay (ELISA) kit according to the instructions described by the manufacturer (eBIOSCIENCE, San Diego, CA, USA). The obtained values were presented in ng/ml. Absorbance was measured at 440nm. For quantification of nitric oxide activity in the tissue supernatant, the Griess reagent kit (cat # G7921 procured from Thermo Fischer Scientific, USA) was used based on the enzymatic conversion of nitrate to nitrite by the enzyme nitrate reductase (NaR), followed by quantitation of nitrite using Griess Reagent (1% sulfanilasmide, 0.1% N-(1-naphthyl) ethylenediamine hydrochloride, 2.5% H_3PO4). Absorbance was read at 540 nm using a spectrophotometer.

2.7 Statistical Analysis

GraphPad Prism® (version 8) was used to analyze quantitative data. The outcomes were plotted in One-way ANOVA followed with Turkey's multiple comparisons test. Significance was set at $p<0.05$. Using Image J software, the staining polarity of the Cresyl fast violet stain was measured.

3.0 Results

3.1 $AlCl_3$ induced oxidative stress and Lactate dehydrogenase overexpression: mediation by Walnut-enriched diet

As shown in fig. 1 and 2, we measured the neural activities of catalase (CAT) and lactate dehydrogenase (LDH). $AlCl_3$-treated rats (group B) manifested a significantly decreased level of neural CAT with a mean value of 85.00 ± 9.889 than the control with a mean value of 216.6 ± 15.47. Animals post- and pre-treated with walnut-enriched diet (groups C and D) had significantly higher levels of CAT when compared to rats exposed to $AlCl_3$ only with mean values of 201.0 ± 7.232 and 222.8 ± 11.43 respectively. Altered redox functioning is associated with reduced availability of oxygen at the subcellular level. Hence, we assessed levels of LDH in tissue lysates. LDH, an enzyme that is notable for its role in energy production, on the other hand, was overexpressed in the $AlCl_3$ treated rats with a mean value of 225.2 ± 6.468 when compared with the control group with a mean value of 136.0 ± 5.718. Treatment with walnut diet significantly modulated the profile of LDH; $AlCl_3$+Walnut diet (group C) with a mean value of 159.2 ± 3.555 and Walnut+$AlCl_3$ (group D) with a mean value of 153.2 ± 3.992; therefore presented with normalized LDH activity

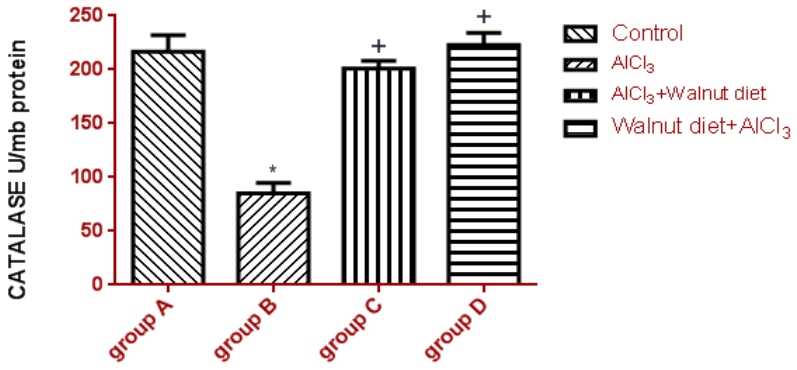

Figure 1: Activity of catalase in the cerebellum of animals. A= control, B= $AlCl_3$, C= $AlCl_3$+Walnut diet, D= Walnut diet+$AlCl_3$. The values are expressed as Mean ± SEM. $p<0.05$ is considered to be statistically significant; * indicate significant level of difference in comparison to the control group while $^+$ indicate significant level of difference in comparison to group B

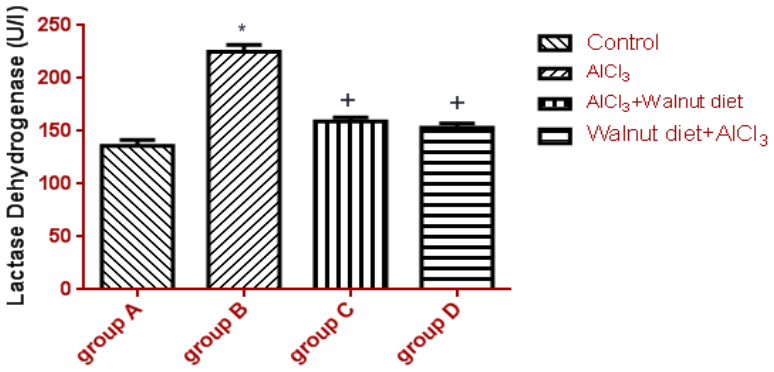

Figure 2: Activity of lactate dehydrogenase in the cerebellum of experimental animals. A= control, B= $AlCl_3$, C= $AlCl_3$+Walnut diet, D= Walnut diet+$AlCl_3$. The values are expressed as Mean ± SEM. $p<0.05$ is considered to be statistically significant; * indicate significant level of difference in comparison to the control group while $^+$ indicate significant level of difference in comparison to group B

3.2 Treatment with walnut-enriched diet counterbalances $AlCl_3$-induced activation of inflammatory cytokines and Nitrosative stress

The present study examined the effects of $AlCl_3$ on inflammatory and nitrosative stress markers as shown in figure 3 and 4 respectively. Compared to the control and walnut diet treated groups, the $AlCl_3$-treated group (B) presented a significant elevation in interleukin-3 levels with a mean value of 15.20 ± 1.393. Walnut-enriched diet significantly ($p< 0.05$) downregulated the activity of IL-3 in the cerebellum; rats post-treated with walnut diet (group C) had a mean value of 10.80 ± 1.241 while those pretreated with walnut diet (group D) had a mean value of 10.80 ± 1.200. These values were significantly different when compared to rats that received only $AlCl_3$.

Furthermore, nitric oxide levels markedly increased in the $AlCl_3$-treated group (B) with a mean value of 320.0 ± 28.20 compared with the control group with a mean value of 123.2 ± 8.680. Walnut diet pre-and post-treatment significantly counterbalanced the dysregulation in NO

metabolism induced by $AlCl_3$. Like the animal group post-treated with walnut-enriched diet after $AlCl_3$ treatment, the experimental animals pre-treated with walnut-enriched diet before exposure to $AlCl_3$ treatment presented with significantly reduced nitric oxide levels with mean values of 145.2 ± 20.17 and 140.0 ± 17.71 respectively when compared to the $AlCl_3$ group (B).

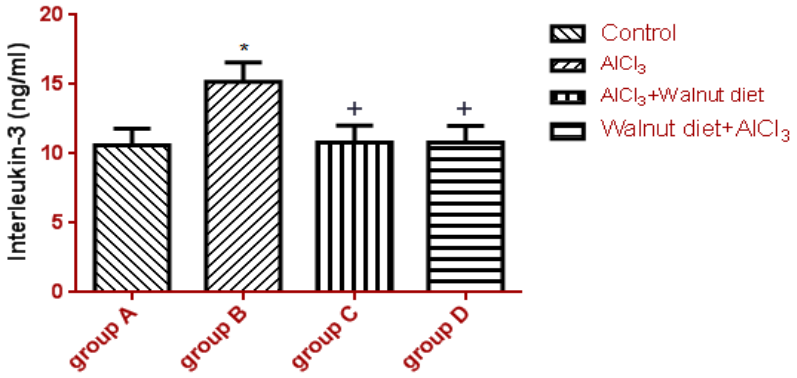

Figure 3: Activity of interleukin-3 in the cerebellum of experimental animals. A= control, B= $AlCl_3$, C= $AlCl_3$+Walnut diet, D= Walnut diet+$AlCl_3$. The values are expressed as Mean ± SEM. $p<0.05$ is considered to be statistically significant; * indicates significant level of difference in comparison to the control group while $^+$ indicates significant level of difference in comparison to group B.

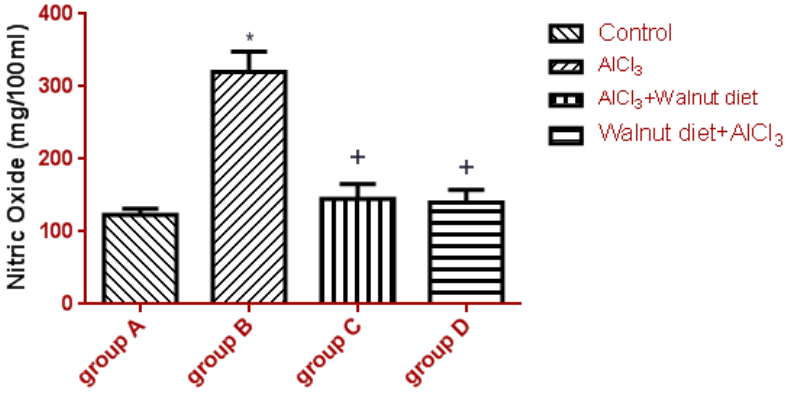

Figure 4: Activity of Nitric oxide in the cerebellum of experimental animals. A= control, B= $AlCl_3$, C= $AlCl_3$+Walnut diet, D= Walnut diet+$AlCl_3$. The values are expressed as Mean ± SEM. $p<0.05$ is considered to be statistically significant; * indicate significant level of difference in comparison to the control group while $^+$ indicate significant level of difference in comparison to group B

3.3 Cerebellar histomorphology following treatment with $AlCl_3$ and walnut-enriched diet

Figure 5 showed the morphological changes in the cerebellum following $AlCl_3$ and walnut-enriched diet treatments. The histology of the cerebellar cortex across the control and walnut-enriched diet groups (A, C, and D) appeared unperturbed. The distinct Purkinje cells present in groups A, C, and D groups appeared conspicuous (black arrow) with large soma and apparent projections into the molecular layer. However, group B rats showed $AlCl_3$ induced degenerative changes in the cerebellar cortex characterized by necrotic Purkinje cell layer and cytoplasmic vacuolations (yellow circle and arrow respectively).

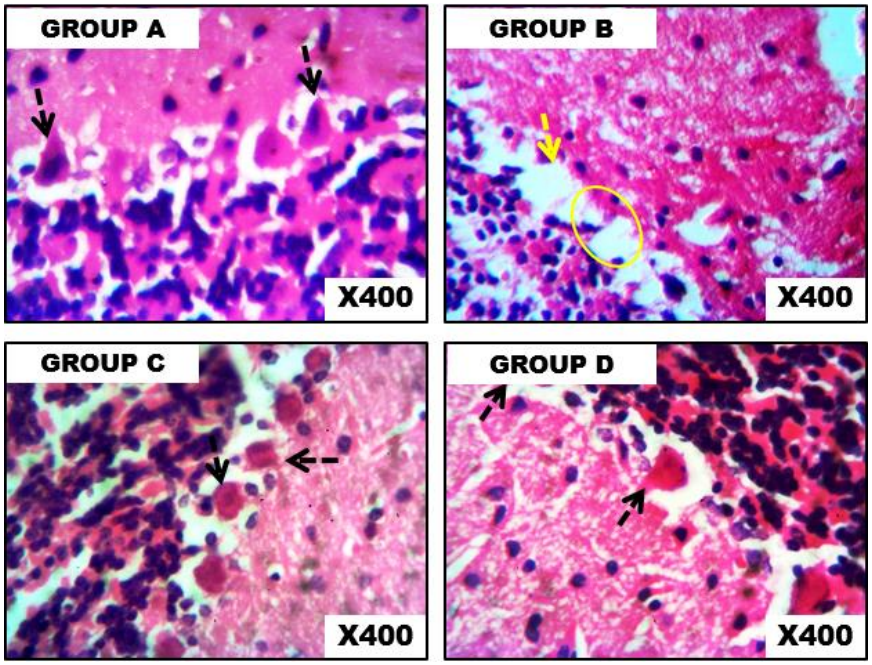

Figure 5: General morphological presentations of cerebellar cortex across the various study groups. Hematoxylin and Eosin stain (x400). A= control, B= $AlCl_3$, C= $AlCl_3$+Walnut diet, D= Walnut diet+$AlCl_3$. Necrotic Purkinje cells and vacuolations (yellow circle and arrows) observed in B.

3.4 $AlCl_3$ altered the Cerebellar Nissl Protein Profile

Nissl profile demonstration by Cresyl fast violet stain as shown in figure 6, across cerebellar sections within the study groups showed normal Nissl staining intensity in control (group A) and dietary walnut treatments (groups C and D), characterized with normal and densely populated Nissl proteins, with well stained and outlined neurons (black arrows). However, chromatolytic changes were observed in group B rats; the Purkinje and granule cell layers with reduced cytoplasmic Nissl proteins (red arrows). The stereological analysis showed a significant reduction in the relative number of Purkinje cells in $AlCl_3$-treated rats when compared with animals exposed to dietary walnut (groups C and D).

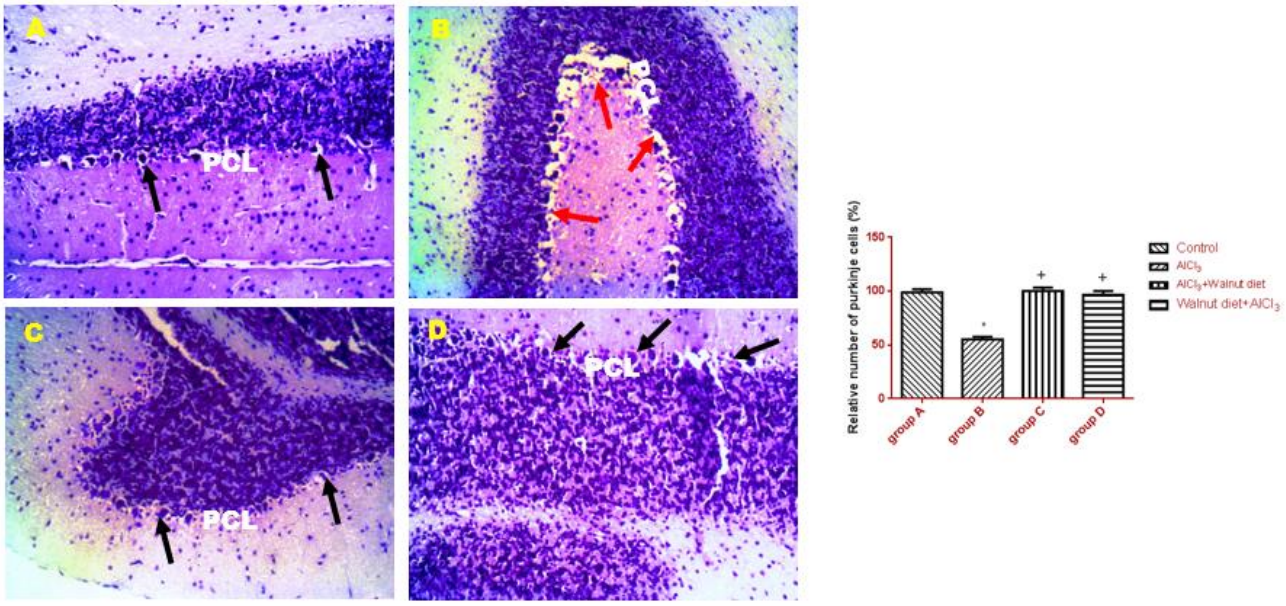

Figure 6: Expression of Nissl substance in Wistar rats across the various study groups. Cresyl fast violet stain (x100). A= control, B= $AlCl_3$, C= $AlCl_3$+Walnut diet, D= Walnut diet+$AlCl_3$. PCL=Purkinje cell layer

4.0 Discussion

This study evaluated the redox, inflammatory activities, bioenergetics, and histomorphology of the cells in the cerebellum following exposure to $AlCl_3$ and treatment with walnut-enriched diet.

For neural redox activity, the study highlighted the effect of $AlCl_3$ on catalase (CAT). CAT is an enzyme in the antioxidant spectrum that catalyzes the breakdown of hydrogen peroxide to water

and oxygen, thereby preventing neural oxidative damage by reactive oxygen species (ROS). The biochemical level for CAT was seen to decrease in the cerebellum of the $AlCl_3$ treated animals which agree with the work of Prema et al., (2017) who pointed out that the effect of $AlCl_3$ may be due to the inhibition of key free-radical scavenging enzymes and intracellular thiols resulting in reduced antioxidant activity. Bains and Shaw, (1997) also suggested that catalase depletion may be a common early event leading to neuron death. Our findings suggest that $AlCl_3$ could possibly result to a worsening of the CAT cerebellar levels, thereby significantly perturbing the activities of the antioxidant status of the cerebellum.

However, this study's findings demonstrate that walnut-enriched diet maintained the integrity of the antioxidant profile of the cerebellum. T*etracaarpidium conophorium* has been shown to contain antioxidant-rich phytochemicals and phytonutrients like terpenoids, tannins, phlobatanins, flavonoids, polyphenols, vitamin E, and a variety of minerals. The presence of these phytonutrients may provide a direct augmentation of the antioxidant capacity in the neural cells that may possibly result in efficient scavenging and accumulation of reactive oxygen species.

Dysregulation in glucose bioenergetics has been fingered as a major mediator in the onset and progression of several neurodegenerative diseases. In this study, $AlCl_3$ exacerbated the levels of Lactate dehydrogenase (LDH) in the cerebellum. $AlCl_3$ may have caused the up-regulation of LDH by eliciting a state of cellular hypoxia that may have resulted in increased production of LDH since LDH typically catalyzes the conversion of pyruvate to lactate when there is a shortage of oxygen supply to drive oxidative phosphorylation. Invariably, the observed increase in LDH activities as induced by $AlCl_3$ in this study suggests the role $AlCl_3$ may play in causing cellular hypoxia and driving accumulation of lactate, which has the propensity to activate the apoptotic cascade. LDH is an enzyme in the energy-producing glycolytic pathway which is affected by oxidative stress and may contribute to the alteration in glucose metabolism documented in neurodegenerative diseases (Di Domenico et al., 2017). Studies have also been documented that show that exacerbated free radical production leads to impairment in neuronal bioenergetics and alteration in mitochondrial functions (Olajide et al., 2017; Abayomi et al., 2021a). Furthermore, several works suggested that increase in the level of LDH may be due to the reaction between free radicals and the polynoids of the cell membrane, which brings about leakages of the enzymes and the dissociation of glutamate, and the release of ammonia, which subsequently causes brain injury and production of LDH (Onyema et al., 2006; Tawfik and Al-Badr, 2012; Sadek et al., 2016).

This study suggests that walnut-enriched diet enhanced energy derivation by providing antioxidant support which promotes the proper scavenging of ROS, thereby, regulating the activity of LDH. Findings from this study agree with the works of Poulose et al., (2014) and Gorji et al., (2018) where the role of walnut in glucose metabolism was elucidated.

In the CNS, NO functions as a neurotransmitter molecule, but when it is produced in excess, it becomes neurotoxic. $AlCl_3$ injection in the present investigation markedly increased the nitrosative stress in the cerebellar tissues, as shown by an increase in NO levels. Again, this may be as a result of excessive production of ROS, which is associated with increased synthesis of NO. Induction of oxidative-nitrosative stress in brain tissues in response to exposure to $AlCl_3$ has been confirmed in previous works carried out by many authors (Kasbe et al., 2015; Justin et al., 2017; Rather et al., 2018). High concentrations of NO, peroxynitrite, and other reactive nitrogen species have been found to correlate with greater severity of neurodegenerative disorders. Previous investigations supported the association between dysregulated Nrf2 signaling and rats' brain injuries brought on by AlCl3 (Mustafa et al., 2014). In the present study, walnut-enriched diet significantly attenuated and prevented $AlCl_3$-induced nitrosative damage. *Tetracarpidium conophorum* is well known for having a potent antioxidant activity. Additionally, because of its capacity to scavenge free radicals, it can restore the redox balance, preventing the peroxidation of membrane lipids. Previous research have shown that walnuts have a neuroprotective effect against oxidative-nitrosative stress in the rat brain, which is relevant to our findings (Banel and Hu, 2009; McKay et al., 2010). *Tetracarpidium conophorum's* remarkable antioxidant potential may be explained by its capacity to stimulate Nrf2 signaling and induce the production of antioxidant enzymes (Zhao et al., 2021).

Inflammation is a critical factor in the pathogenesis of neurodegenerative diseases. Microglia, the immune cells of the central nervous system, are activated early in neural pathogenesis and can both trigger and propagate early disease processes via innate and adaptive immune mechanisms such as upregulated immune cells and antibody-mediated inflammation (Lee et al., 2021). The proinflammatory cytokines IL-3 play a role in organ injury, tissue inflammation, and immunological dysfunction. This research suggests that $AlCl_3$ primarily impacts macrophage activities and promotes the development of brain damage since interleukin-3 levels were noticeably elevated in the $AlCl_3$-treated rats. Findings from this current study show that walnut-enriched diet modulated the activities of the inflammatory cytokine. The inhibition of proinflammatory cytokine expression in the cerebellum may be dependent upon the presence of

omega-3 PUFAs in *Tetracarpidium conophorum*. These phytonutrients have been shown to possess anti-inflammatory properties which ensure the efficient working of the immune system and provide cellular defense against chronic inflammation (Delpech et al., 2015).

Our biochemical findings were confirmed by the histological and histochemical studies, where $AlCl_3$ administration in rats induced severe cerebellar damage, particularly in the Purkinje cell layer. The current findings showed pronounced necrosis, pyknotic changes, and cytoplasmic vacuolations in the cerebellar cortex of rats treated with $AlCl_3$. These alterations in the cerebellar cortex are consistent with previous reports where $AlCl_3$ has been shown to cause neuronal damage in rats (Auer and Sutherland, 2002; Aboelwafa et al., 2020; Abayomi et al., 2021b). However, walnut enriched diet in our study was seen to reverse as well as prevent the deteriorating impact of $AlCl_3$ treatment on the cerebellar cortex especially on the Purkinje layer, thereby mitigating neuronal damage triggered by Aluminium toxicity.

Neural chromatolysis is defined by the loss of Nissl bodies, which are responsible for protein synthesis in the neurons. The present study revealed that $AlCl_3$ induced neural chromatolysis in cerebellar neurons, causing inflammatory changes in the cerebellum. The effect of walnut-enriched diet, however, was seen in this study to improve the harmful impact of $AlCl_3$ as mentioned above which were seen to be evident due to its cascade of detoxification through its antioxidant property; and anti-inflammatory pathways.

4.1 Conclusion

Walnuts contain large amounts of chemicals that can boost brain health such as α-linoleic acid (ALA) and linoleic acid (LA). Walnut-enriched diet was shown to modulate neuroinflammatory, bioenergetics, and neural redox balance in $AlCl_3$ neurotoxicity. These results suggest that walnut-enriched diet may play a critical role in mitigating against the progression of toxic cascades witnessed in neurodegenerative disorders.

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Conflict of interests: The authors declare that they have no competing interests

Authors' contributions:

Taiwo Abayomi & Olorunfemi Tokunbo contributed to the study conception and design. Material preparation, data collection and analysis were performed by **Anne Aina, Olawale Abayomi, Babatunde Dare, Opeyemi Osuntokun** & **Olawale Obembe**. The first draft of the manuscript was written by **Taiwo Abayomi** & **Olorunfemi Tokunbo**. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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