ORIGINAL COMMUNICATION

Anatomy Journal of Africa. 2022. Vol 11 (1): 2109-2121

NIGELLA SATIVA OIL INGESTION MITIGATES ALUMINUM CHLORIDE INDUCED CEREBELLA OXIDATIVE, NEUROGENIC DAMAGES AND IMPAIRED MOTOR FUNCTIONS IN RATS

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ABSTRACT

Varying neurological effects, and impairments to motor functions, neurochemistry and neuromorphology have been associated with Aluminium chloride ($AlCl_3$) induced neurotoxicity. This study aims to investigate the efficacy of *Nigella sativa* oil (NSO) in $AlCl_3$ induced cerebellar toxicity in rats. Thirty-two male Wistar rats were randomly divided into four groups and received: normal saline; 100 mg/kg.bw of $AlCl_3$; 100 mg/kg.bw $AlCl_3$ and 1 ml/kg.bw of NSO; and 1 ml/kg.bw, orally and daily for fourteen days. On the 13th day of the experiment, the rats were each exposed to a single trial of the Open Field Test (OFT), of which line crossing frequency, rearing frequency, and freezing period were recorded as measures of exploratory and locomotive behaviours of the animals. By day 15, the rats were euthanized, their brains were excised, the cerebellum dissected from five brains of each group, and homogenized for biochemical evaluations of nitric oxide (NO) metabolites and total reactive oxygen species (ROS) levels. The remaining three brains in each group were processed for histology and Ki67 immunohistochemistry investigations. The results of this study shows that $AlCl_3$ impaired motor related behaviours in the $AlCl_3$ exposed animals, by significantly reducing the line crossing and rearing frequencies, and increasing the freezing period. This effect was observed to be mitigated in the animal group that received NSO following $AlCl_3$ administration, as the animals showed improved motor behaviours. $AlCl_3$ also caused an increase in the cerebellar activities of NO and ROS, while it depleted Ki67 expressions and caused neurodegenerative-like effects in the cerebellar histoarchitecture of the exposed animals. Intervention with NSO depleted ROS/NO levels and protected the cerebellum from the nitrosative and oxidative stress induced by $AlCl_3$. NSO was also observed to preserve the cerebellar cortex histoarchitecture and neurogenic morphology against the neurodegenerative effect of $AlCl_3$. It can be concluded that NSO, with its high efficacy against oxidative stress and neuroinflammation, is a potent natural therapeutic agent in aluminum and heavy metal neurotoxicity.

Keywords: Aluminum chloride, *Nigella sativa,* Oxidative stress; Cerebellum; motor functions.
DOI: https://dx.doi.org/10.4314/aja.v11i1.11

INTRODUCTION

The use of a wide variety of metals in industries, other work environments, transport settings, and even homes, has been on the increase, consequently leading to an increase in environmental pollution and toxicity in living things (Bhat *et al.,* 2019; Agnihotri and Kesari, 2019; Mardare and Horhogea, 2019). Heavy metals induce their toxicity by getting attached with protein sites in cells and displacing the original metals from their natural binding sites, thereby causing malfunctioning of the cells. Continuous exposure to heavy metals have been discovered to cause progressive physical, muscular, and neurological degenerative processes that replicate diseases such as Parkinson's disease, Multiple sclerosis, Muscular dystrophy, and others (Vennam *et al.,* 2019).

Aluminum's ability to cross the blood-brain-barrier, and its relatively slow rates of elimination, contributes to its accumulation in the brain, leading to neurotoxicity (Exley, 2014; Zhang *et al.,* 2019), and exposure to aluminum is considered as a risk factor for many neurological disorders (Aljarari and Bawazir, 2019; Bhat *et al.,* 2019). Neurological disorders such as Alzheimer's disease, Parkinson's disease and multiple sclerosis show oxidative and inflammatory characteristics that suggest the involvement of metal induced toxicity (Linhart *et al*., 2020).

Nigella sativa (NS), also identified as black seed or black cumin, is a flowering plant belonging to the *Family* Ranunculaceae, and its phytochemical constituents include: 30% fixed oils (mainly fatty acids), 0.40- 0.45% volatile oils, vitamins, amino acids, proteins, carbohydrates, alkaloids, saponins, crude fiber, and minerals (Cascella et al., 2018). It is also rich in polyunsaturated fatty acids, phytosterols, thymoquinone (TQ), carvacrol, t-anethole, sesquiterpenelongifolene, and 4 tepinol (Cascella et al., 2018). Nigella sativa plants' flowers and seeds (and oil) are used for the traditional treatment of several ailments (Begum and Mannan, 2020).

Nigella sativa is known for its several pharmacological actions, which include antioxidant, anti-inflammatory and immunomodulator, analgesic among others (Ikhsan *et al.,* 2018; Imam *et al.,* 2018 a,b,c,d; ; Bordoni et al., 2019; Begum and Mannan, 2020), with protective efficacy against toxicity caused by heavy metals (Assi, 2019).

Nigella sativa and its major constituent thymoquinone exhibit a range of activities including neuroprotection (Alhibshi *et al.,* 2019, Imam *et al*., 2018 a,b), thus efficacious in neurotoxicity and neurological diseases (Morsy *et al.,* 2017; Abdulfadl *et al*., 2018; Butt *et al.,* 2018; Imam et al., 2018 a,b,c,d; Saleh *et al*., 2019), including motor deficits (Ajao *et al.,* 2016; Malik *et al* 2016; Folarin *et al.,* 2020).

We aimed to assess the possible mitigative efficacy of Nigella sativa oil against Aluminum Chloride (AlCl3) Induced cerebella toxicity through the evaluation of behavioural, biochemical and histological changes in wistar rats.

MATERIALS AND METHODS

Chemicals and drugs

A crystalline salt of alluminium chloride was procured from Sigma-Aldrich (Germany) while normal saline solution was prepared in our laboratory. The *Nigella sativa* oil (concentration; 100% black seed; HUSNA black seed oil, Fazhab Agency, Karachi, Pakistan) was purchased from a TIBB-medical store in Ilorin, Kwara state, Nigeria.

Antibodies for Ki67 and the NO and ROS kits was obtained from abcam.

Animals and experimental design

Thirty-two (32) adult male Wistar rats weighing about 180 ± 20 g were obtained from the University of Ilorin Biological garden, Ilorin. They were housed in cages and fed with standard laboratory diet and water adlibitum, in the animal holding unit of the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, Ilorin. The rats were exposed to a 12 h light/dark cycle at room temperature for 7 days before the commencement of the experiments. All rats were handled in accordance with the standard guide for the care and use of laboratory animals.

Treatment plan

The rats were randomly divided into four groups (n=8) as described below:

- Group 1 (control) - were given normal saline (1 ml/kg.bw orally) daily
- Group 2 – were given $AlCl_3$ (100 mg/kg.bw orally) daily
- Group 3 – were given $AlCl_3$ (100 mg/kg.bw orally) plus NSO (1ml/kg.bw orally) daily
- Group 4 – were given NSO (1 ml/kg.bw orally) daily

The experiments were all conducted in the morning (between the 07:00 and 09:00 hours), and treatments with substances lasted for the period of fourteen (14) consecutive days.

Ethical Approval

This research work was approved by the University of Ilorin ethical review committee (UERC) (UIL/UERC/11/46KA072), following the recommendation of the College of Health Sciences ethical review committee, in compliance with the Institutional Animal Care and Use Committee (IACUC).

Evaluation of Brain and Body Weight

The body weights of all the rats were recorded after acclimatization on the first day of exposure, as 'the initial weight', and on the last day of exposure, as 'the final weight'. The differences between the two weights were thus calculated and recorded as the weight fluctuated or changed. The brain weight of all the rats were recorded after the sacrifice, and a ratio of the brain to final body weight was calculated and recorded.

Behavioral evaluation

The rats were subjected to behavioural evaluations in the Open Field Test (OFT) paradigm on the 13th day of the experimental treatments to access exploratory and locomotor behaviours.

OFT Procedure:

The animals were exposed to a single trial in the OFT paradigm to evaluate exploratory and locomotor behaviours following exposure to normal saline, CPF and/or NSO. The rats were individually placed in the centre of the apparatus and left to willingly explore the paradigm (a well illuminated wooden box, divided into 4 × 4 squares) for a 5 minute session under video surveillance. After analysing the video recordings frequent line crossing (FLC) and rearing frequencies (RF) were recorded for locomotor and exploratory behaviours respectively.

Biochemical evaluation

At the end of the treatment period, the animals were euthanised with an overdose of ketamine (10 mg/kg ip) and the brains were quickly dissected out and weighed. Blocks of cerebella tissue (from Bregma –10

mm to –15 mm) were removed from the brains of five rats in each of the four groups, dipped in 30% sucrose solution, homogenized and portions centrifuged at 2500 revolutions per minute for 10 minutes. The supernatant was then collected in tubes containing the compounds fornitric oxide (NO) metabolites and reactive oxygen species (ROS) analysis. ROS activity was measured by monitoring the increasing fluorescence of dichloro-dihydro-fluorescein diacetate (DCFH-DA) using flow cytometry technology (Partec, Deutschland) equipped with a 488 nm argon ion laser and supplied with the Flomax software. The signals were obtained using a 530 nm band pass filter (FL-1 channel), and each determination was based on the mean fluorescence intensity of 10,000 counts.

The remaining tissue homogenate was added to the Griess reagents, sulfanilamide and naphthyl ethylene diamine solutions to measure nitrate/nitrite production (NO metabolites). Absorbance was measured with the aid of a microplate reader and the levels of NO metabolites were calculated from a standard curve. The remaining portions of the homogenized motor cortex and cerebella tissues were placed in phosphate buffer with 1% Triton-X 100 and centrifuged at 5000 rpm for 10 minutes. The following reagents were used; 35 µL of 5 mM dithio-bisnitrobenzoic acid (also known as Ellman's reagent [DTNB]), 10 µL of 75 mM acetylthiocholine (ATCh) and 50 mM phosphate buffer (pH 8.0). Protein concentration in brain homogenates was quantified using a Bradford assay. AChE activity was calculated in ⋅ M of ATCh, hydrolysed per hour per mg of protein and was expressed as percentage of control activity.

Tissue processing and histopathology

After euthanasia and brain extraction from 3 rats in each experimental group,the brains were fixed in 10% formalin for 24 hours. Cerebella cortices blocks (from Bregma –10 mm to –15 mm) were removed, dehydrated through ascending grades of alcohol, cleared in xylene and embedded in paraffin blocks. Every second motor cortex/cerebella tissue section (5µm thickness) was stained with Cresyl fast violet (CFV) for Nissl substance or immuno-stained to reveal Ki67 protein containing nuclei in the tissues. The sections were finally examined under an AmScope 40X-2500X LED Lab Compound Microscope and photographed using the AmScope 5.0 MP USB Still Photo and Live Video Microscope Imager Digital Camera 5MP, manufactured by iSCOPE corp., USA.

Immunohistochemistry for Ki-67

Ki-67 is a chromosome-associated protein that is expressed during division (G_1, S, G_2, and M phases) but absent from cells at rest (G_0). Paraffin embedded sections were incubated for epitope retrieval in citrate buffer, pH 6.0, at 90˚C for 40 minutes, followed by incubation in endogenous peroxidase blocking reagent, 0.6% H_2O_2 in tris-buffered saline (TBS)-Triton (0.05% Triton X-100 in TBS, pH 7.4) for 30 minutes at room temperature. Thereafter, sections were pre-incubated in 2% normal goat serum (NGS) + 0.1% bovine serum albumin (BSA) + 0.25% Triton in TBS for 60 minutes at room temperature. Afterwards, sections were incubated with polyclonal rabbit-anti-lyophilized-Ki-67p antibody (Novocastra, Newcastle, UK; 1:5,000 in preincubation solution) overnight at 4˚C. Incubation with biotinylated goat anti-rabbit IgG (1:1,000 + 2% normal goat serum + 0.1% BSA in TBS; Vector lab, CA, USA;1:250) was performed for 2 hours at room temperature followed by incubation with streptavidin-biotin complex (Vectastain Elite ABC kit) and

stained with 3,3′-diaminobenzidine (DAB) as chromogen. All rinses until incubation in primary antibody were made with TBS-Triton and afterwards with TBS alone.

Statistical Analysis

Data from the behavioural and biochemical assays were analysed using one-way analysis of variance (ANOVA) and subjected to post hoc Bonferroni's multiple comparison test. The results were expressed as mean±SEM. Statistical analyses were performed using Graphpad Prism software (version 5.0, La Jolla, CA). Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

NSO Protected the Cerebellum from Nitrosative and Oxidative Stress Induced by $AlCl_3$

The NO concentration of the NSO and $AlCl_3$+NSO group were significantly lower ($p \leq 0.05$) than that of $AlCl_3$ group (Fig. 1). $AlCl_3$ was found to be a nitrosative stressor as the mean NO level of $AlCl_3$ exposed group was significantly higher than that of the normal control group (Fig. 1). Oxidative stress is one of the mechanisms of action of $AlCl_3$, which was also observed in this study that the cerebella ROS activity was significantly higher in the $AlCl_3$ administered group when compared to the control group, while the cerebella ROS activity of NSO administered group was found to be significantly lower ($p \leq 0.05$) when compared to $AlCl_3$ group (Fig. 2).

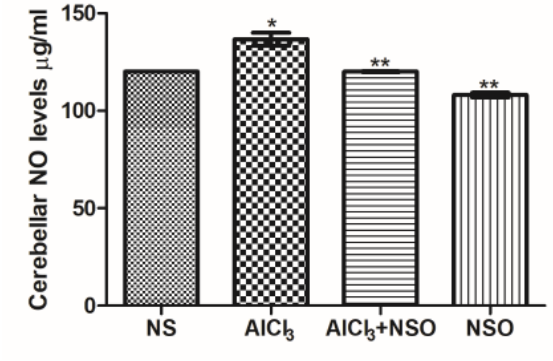

Figure 1: Cerebella NO levels of rats exposed to normal saline (NS), aluminum chloride(AlCl3), aluminum chloride+ Nigella sativa Oil(AlCl3+NSO) and nigella sativa Oil Only(NSO). Doubleasterisks (**) indicates significant ($p \leq 0.05$) difference when compared with AlCl3 exposed rats, while single asterisk (*) indicates significant ($p \leq 0.05$) difference from the normal control group.

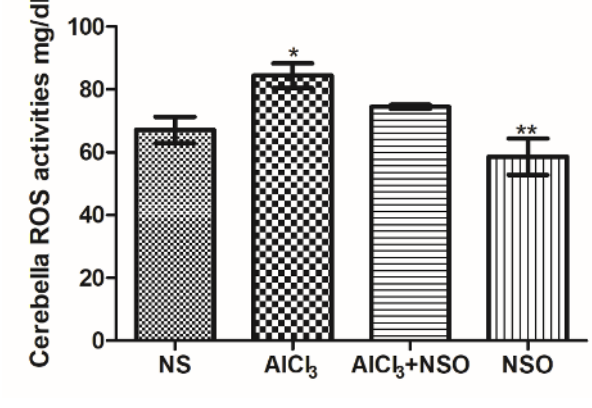

Figure 2: Cerebella ROS activities of rats exposedto normal saline (NS), aluminum chloride(AlCl3), aluminum chloride+ nigella sativa Oil(AlCl3+NSO) and nigella sativa oil only(NSO). Doubleasterisks (**) Indicates Significant ($p \leq 0.05$) difference when compared with AlCl3 exposed rats, while single asterisk (*) indicates significant ($p \leq 0.05$) difference from control group.

NSO retained the motor related behaviours following $AlCl_3$ administration

Administration of $AlCl_3$ caused a significant ($p \leq 0.05$) lower line crossing frequency (Fig 3) of the experimental animals. In the same vein, the group in which $AlCl_3$ and NSO were co-administered had a lower line crossing frequency compared to the normal control and NSO (only) group, but higher than $AlCl_3$ group (Fig 3). This was equally supported by the finding from other motor behavior parameters measured from the OFT procedure. The period of time spent in the centre was found to be higher in the $AlCl_3$+NSO administered group compared to the group in which only $AlCl_3$ was administered (Fig 4). This was also found in rearing frequency as well as the freezing period data observed from the OFT. As the rearing frequency of the both groups that are exposed to NSO were higher than that of $AlCl_3$ group which is indicative of higher motor related behavior exhibition by these group of experimental rats (Fig 5 and 6).

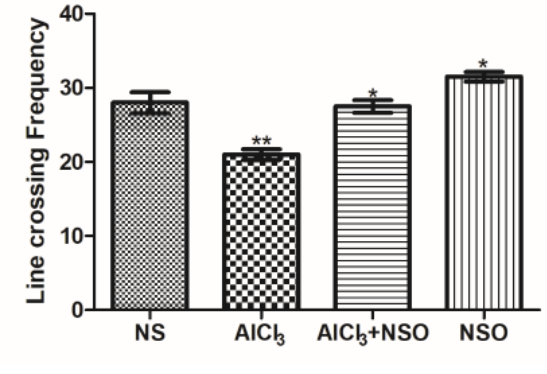

Figure 3: Line crossing frequency of the rats following the administration of normal saline (NS), aluminum chloride(AlCl3), aluminum chloride+ nigella sativa Oil(AlCl3+NSO) and nigella sativa oil only(NSO). singleasterisk (*) indicates significant ($p \leq 0.05$) difference when compared with AlCl3 exposed rats, while double asterisks (**) indicates significant ($p \leq 0.05$) difference from control group.

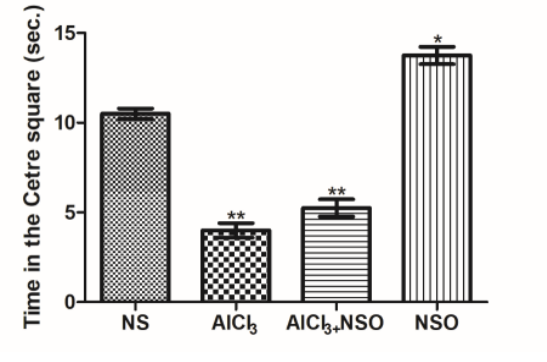

Figure 4: Time spent in centre square by rats following exposures to normal saline (NS), aluminum chloride (AlCl3), aluminum chloride+ nigella sativa oil (AlCl3+NSO) and nigella sativa oil only (NSO). Doubleasterisk (**) indicates significant ($p \leq 0.05$) avoidance time when compared with all other treatment groups, whilesingle asterisk (*) indicates significant($p \leq 0.05$) exploration when compared with the AlCl3 Exposed and/or Control Rats.

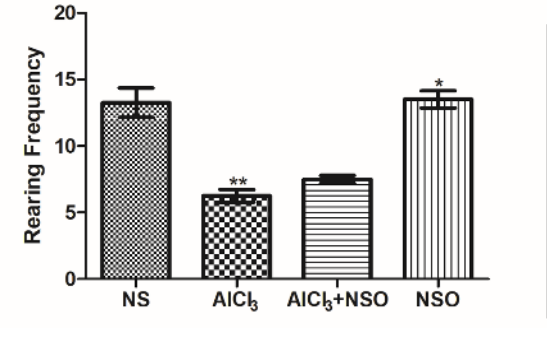

Figure 5: The rearing frequency of rats followingexposures to normal saline (NS), aluminum chloride (AlCl3), aluminum chloride+ nigella sativa Oil (AlCl3+NSO) and nigella sativa oil only (NSO). Double asterisk (**) indicates statistically significant ($p \leq 0.05$) less rearing when compared with all other group treatments while single asterisk (*) indicates statistically significant ($p \leq 0.05$) higher rearing frequency when compared to the AlCl3 group.

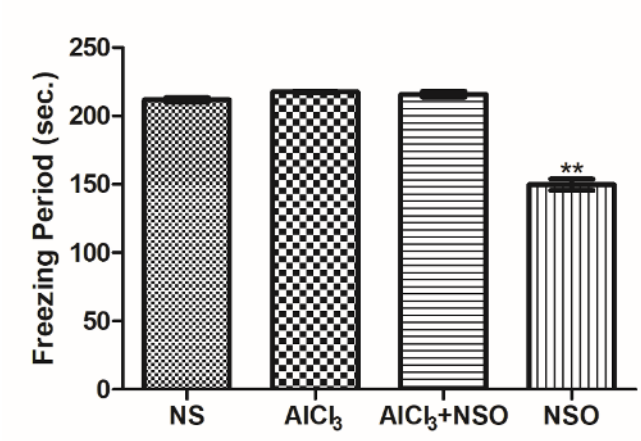

Figure 6: The Freezing period in rats following exposures to normal saline (NS), aluminum chloride (AlCl3), aluminum chloride+ nigella sativa oil (AlCl3+NSO) and nigella sativa oil only(NSO). Doubleasterisk (**) indicates significant ($p \leq 0.05$) less freezing when compared with all other group treatments.

NSO Preserved the Cerebella Cortex Histoarchitecture and Neurogenic Morphology Following the Exposure to Aluminum

Aluminum chloride caused a Neurodegenerative like effect on the cerebella cortex which was protected by NSO. $AlCl_3$ lead to chromatolytic like changes in the Purkinje cells of the cerebellum exposed to it (Fig. 7), which was not as severe in the group who took $AlCl_3$ and NSO concurrently, in which there are both chromatolytic cells as well as viable neurons. There are also numerous necrotic-like pyknotic and loss of Purkinje cells treated rats (Fig. 7 and 8).

observed with the neurons of the $AlCl_3$, there was also a marked loss in the potent proliferating cells (Fig. 8). This neurodegenerative like changes in the cerebella cortex was not limited to the Purkinje cell layer, it was also apparent in the molecular and the granular cell layer as there were numerous perineural spaces and reduction of the neuronal density in these regions indicative of shrinkage of the cells. NSO was observed to protect the cerebellum against the severity of the degenerative like activities of the $AlCl_3$ and preserved the density of neurogenic cells in the

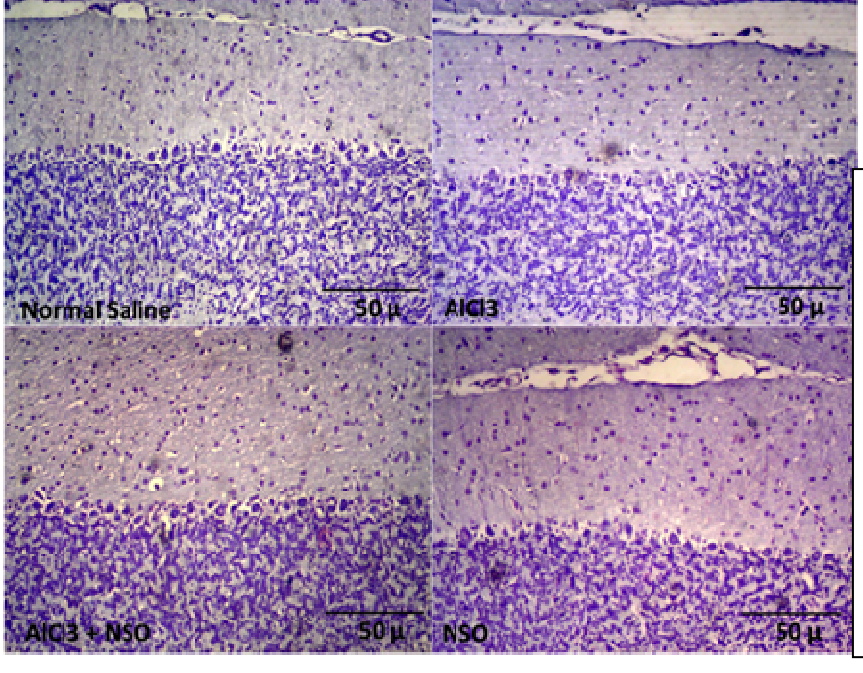

Figure 7: Representative photomicrographs of the cerebella cortex of rats exposed to normal saline (NS), aluminum chloride(AlCl3), aluminum chloride+ nigella sativa Oil(AlCl3+NSO) and nigella sativa oil only (NSO).chromatolytic and loss of Purkinje neurons. This photomicrograph indicates that the protein synthesizing machinery of the cerebella cortical neurons were preserved by NSO.CFV, X100, 50µ.

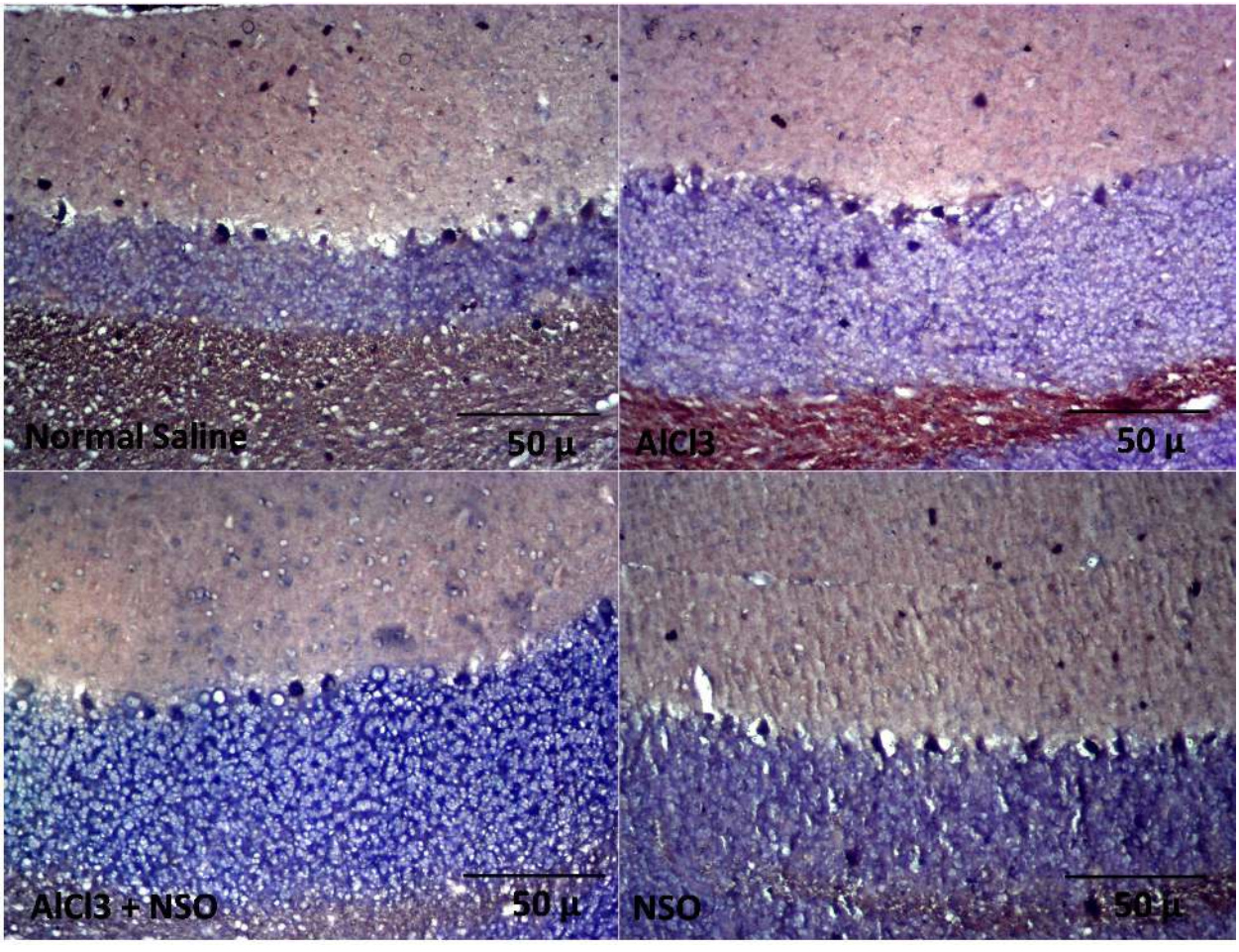

Figure 8: Representative photomicrographs of the distribution of Ki67 immunoreactive cells in the cerebellum of rats exposed to normal saline (NS), aluminum chloride (AlCl3), Aluminum chloride + Nigella sativa Oil (AlCl3+NSO) and Nigella sativa Oil Only (NSO). Ki67-ir Cells. Ki67 IHC, X100, 50µ.

DISCUSSION

Exposure to heavy metals such as aluminum, cadmium, lead, arsenic, mercury and others have increased with industrialization (Mardare and Horhogea, 2019). These metals when exposed to humans could induce toxicity by mechanisms of oxidative stress, mitochondrial dysfunction expressed as movement disorders or cognitive deficits (Exley 2016; Agnihotri and Kesari, 2019; Vennam *et al.,* 2019). In the present study, repeated ingestions of $AlCl_3$ caused over productions of the total reactive oxygen species (ROS) in the cerebella tissue of the exposed rats, leading to oxidative stress. Nonetheless, the mitigative efficacy of the Nigella sativa against alluminium induced oxidative events in this study can be strengthened by our previous reports on its antioxidant effect against environmental toxin (pesticide – chlorpyrifos), where it mitigated against the rise of ROS levels in the brain (Imam *et al.,* 2018 b,c).

The observed outburst in nitrite/nitrate metabolites (NO) and the ROS in the cerebellum caused by $AlCl_3$ are however not a surprise. This is in agreement with previous studies, where exposure to $AlCl_3$ was reported to cause marked reduction in antioxidant enzymes, DNA fragmentation were recorded (Liaquat *et al*., 2018), and induced oxidative damage in rats, affecting their cognitive function (Zahedi *et al.,* 2018). In the work of Zahedi and colleagues, the levels of antioxidants like Glutathione S-transferase and catalase, which protect the body cells against free radicals, were greatly depleted, while lipid peroxidation was increased following exposure to $AlCl_3$ (Zahedi *et al.,* 2018). The effects of $AlCl_3$ on the antioxidants defense was also reported evidenced in markedly induced oxidative stress and pathological lesson in the hippocampus (Zhang *et al.,* 2019). These further support the affective productions of NO and ROS in the present study which are oxidative and neuroinflammatory response biomarkers following $AlCl_3$ exposures.

Oxidative stress and neuroinflammation are extensively associated to the pathogenesis of neurological dysfunctions, including those caused by environmental toxins like alluminium (Muhammad *et al.,* 2019; Linhart *et al*., 2020). The reported oxidative damages and neuroinflammatory events following alluminium ingestions above was observed to contributed significantly to exploratory and locomotor activities in the exposed rats. While some studies reported that Aluminium chloride induced neurotoxicity does not have significant impact on locomotion, it has been shown to impair cognitive functions, movement and balance, and worsen motor disorders (Buraimoh *et al*., 2014; Sathiya *et al.,* 2016; Chiroma *et al*., 2019). More evidences supporting the impaired motor functions in this study following alluminium exposure, are its previously reported trigger of locomotive and cognitive deterioration in exposed animals (Auti and Kulkarni, 2019; Chaudary *et al.,* 2019). Progressively, intervention with Nigella sativa was observed to rescue the impaired motor related behaviours, and this is somewhat related to reports of improved motor coordination (parallel bars and static rods tests) by Nigella sativa in phenol-induced essential tremor in mice (Folarin *et al*., 2020), and improve exploratory and locomotor behaviours in cannabis overdose (Imam et al., 2016).

As aluminium is very biologically reactive, there is a high risk of it accumulating over time and crossing the blood brain barrier (Exley, 2014), and can cause oxidative damages and inflammation as observed in the increased ROS and NO levels, consequently leading to neuronal cell loss/damage (Agnihotri and Kesari, 2019). Repeated ingestions of alluminium in this study led to some degenerative features in the general cytoarchitecture of the cerebella cortices of the exposed rats, and induced apoptotic responces observed with the expressed Ki67 immunoreactive cells. Previously established in the literature, the mechanisms and out-turns of aluminium induced toxicity include oxidative stress, inflammation, apoptosis, tissue necrosis and protein denaturation/transformation (Buraimoh *et al.,* 2014; Igbokwe, 2019; Mardare and Horhogea, 2019; Mesole *et al.,* 2020; Verma *et* al., 2020), as are observed in this study. While Nigella sativa was also observed to have neuroprotective effects against the induced neurodegenerative like features following alluminium exposure, just like what is reported in other brain regions after exposing rats to different environmental toxicants (Imam et al., 2016; 2018 a,b,c,d).

CONCLUSION

In conclusion, the antioxidant and anti-inflammatory efficacy of Nigella sative mitigated against the alluminium chloride induced oxidative stress, apoptotic responses and cerebella degenerative activities in Wistar rats. Thus, Nigella sativa may be a potent natural agent in alluminium and heavy metal neuro-toxicity.

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