Chlorpyrifos impaired cerebellar oxidative and cholinesterase activities in rats: Mitigating efficacy of *Nigella sativa* oil

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Date of submission: 25th January 2021 Date of acceptance: 7th May 2021 Date of publication: 1st June 2021

Abstract

Introduction: Motor dysfunctions are some of the characteristic symptoms of organophosphate (OP) poisoning and they have been associated with decreased levels of cholinesterase inhibition within motor areas of the brain. The current study aims to investigate the potential neuroprotective effects of *Nigella sativa* oil (NSO) in alleviating chlorpyrifos (CPF) induced toxicity in the cerebellar and motor cortices of the rat brains using combined behavioural, biochemical and histochemical methods.

Methods and Materials: Thirty-two rats were randomly divided into four groups (eight rats per group), exposed to 1ml/kg of normal saline, 14.9 mg/kg of CPF, 14.9 mg/kg of CPF plus 1ml/kg of NSO and 1ml/kg of NSO respectively for 14 consecutive days. The rats were each exposed to a single trial of the Open Field Test (OFT) on day 13 of the experiment. This experimental test measured locomotor activity levels (line crossing frequency (LCF)) and exploratory (rearing frequency (RF)) activities in the rats studied. The rats were euthanized on day 15 of the experiment and the brains were subsequently excised. The cerebellar cortices of five brains were removed and homogenised to analyse for total reactive oxygen species (ROS), nitric oxide (NO) levels and acetylcholinesterase (AChE) activity. The motor and cerebellar cortices from three other brains in each group were processed for histology (Nissl stain) and proliferative activity (Ki67 immunohistochemistry).

Results: Rats exposed to CPF experienced a significant increase in cerebellar NO and ROS levels, depletion in AChE activity, neurogenic cells loss and subsequent reduction in locomotor and exploratory behaviours respectively (LCF and RF). However, interventional treatment with NSO depleted markers of oxidative damage (NO and ROS), reduced AChE inhibition, preserved neurogenic (Ki67) cells distribution and motor functions.

Conclusion: These results demonstrate the potential efficacy of NSO in OP poisoning and the roles of neurogenic and oxidative functions in the pathophysiology and treatment of motor dysfunction in OP neurotoxicity.

Key words: Acetycholinesterase, Motor functions, *Nigella sativa,* Oxidative stress, Proliferation.

Access this article online
Website: https://www.nepjol.info/index.php/NJN
DOI: https://doi.org/10.3126/njn.v18i2.34525
HOW TO CITE
Aminu I, Barakat OS, Aboyeji LO, Asma'u SM,Christianah O, Rukayat JS, Fatimo A S, Adam MA, Moyosore S. Chlorpyrifos impaired cerebellar oxidative and cholinesterase activities in rats: Mitigating efficacy of Nigella sativa oil. NJNS. 2021;18(2):15-22.

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Introduction

Organophosphorus (OP) insecticides are widely used in agricultural and household activities for controlling insects and pests. The extensive use of OPs increases environmental pollution leading to potentially severe harmful effects on humans and animals alike.[1,2] The need to maximise food production in response to the constant increase in global food demand has resulted in a significant increase in the use of these compounds in

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ISSN: 1813-1948 (Print), 1813-1956 (Online)

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agriculture.[3] However, numerous studies have shown that they induce oxidative stress and are repeatedly implicated in contributing to the clinical burden of several diseases including cancer, diabetes, stroke, coronary heart disease, Alzheimer's disease, Parkinson's disease and other life threatening metabolic and neurological disorders.

Chlorpyrifos (CPF), (o,o-diethyl-o-3,5,6-trichloro-2-pyr-idyl phosphothionate), a widely used and highly effective OP,[4, 5] is an irreversible AChE inhibitor, causing accumulation of acetylcholine (ACh) at both central cholinergic axonal terminals and neuromuscular junctions.[6] Oxidative damage, inflammatory changes and neurotoxicity are cardinal characteristic features of exposure to CPF and other OPs, leading to various behavioural dysfunctions and disruption of neurochemical homeostasis.[7,8]

After decades of advancement in medicine, natural-product based therapeutics have remained a useful alternative in disease management thanks to their perceived reduced side effects.[9] One of such is *Nigella sativa*, also known as black seed and regarded in medieval medicine as "a remedy for all diseases except death", but whose pharmacological efficacies are still explored.[10] Nigella sativa is aranunculaceae plant, with more than 100 bioactive molecules, including thymoquinone (38-40%), p-cymene (7-15%), carvacrol (6-12%), 4-terpineol (2-7%), t- anethol (1-4%), sesquiterpinelongifolene (1-8%), thymohydroquinone, dithymoquinone and alpha-pinene among others.[11] Most of the beneficial activities of *Nigella sativa* are largely attributed to the presence of thymoquinone, the major bioactive and antioxidant component.[12,13]

The oil of *Nigella sativa* (NSO), is one of the forms in which the pharmacological potency of *Nigella sativa* has been widely examined. The beneficial qualities of NSO include antioxidant,[10,14,15] anti-inflammatory,[16,17] neuroprotective,[14,15,18] immunomodulatory,[19] efficacy against neurodegenerative disease[20] and memory-enhancing effects.[21,22]

In the present study we report the possible mitigating role of NSO in CPF-induced oxidative damage, neurogenic loss, cholinesterase dysfunction in the cerebellum and the resulting implications on motor function and neuronal integrities in the cerebellum and the primary motor cortex, using combined behavioural, biochemical and histochemical methods.

Methods and Materials

Chemicals and drugs

Chlorpyrifos (PubChem Substance ID 329756699) PESTANAL®, analytical standard was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA), while the 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.

Animals and experimental design

Thirty-two adult male Wistar rats weighing between 150g and 170g were obtained from the University of Ilorin Biological garden, Ilorin. They were housed in cages and fed with standard laboratory diet and water ad libitum, 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 hours 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 schedule

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

Group 1 (control) - were given normal saline (1 ml/kg orally) daily

Group 2- were given CPF (14.9 mg/kg orally) daily

Group 3- were given CPF (14.9 mg/kg orally) plus NSO (1 ml/kg orally) daily[14]

Group 4- were given NSO (1 ml/kg orally) daily[14,15,18,19,21,22]

The experiments were all conducted in the morning (between 07:00 and 09:00 am), and treatments with the experimental substances spanned a period of fourteen consecutive days.

Ethical approval

This research work was approved by the University of Ilorin ethical review committee (UERC) (UERC/ASN/2017/856), following the recommendation of the College of Health Sciences ethical review committee, in compliance with the Institutional Animal Care and Use Committee (IACUC).

Behavioural evaluation

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

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.[22,23]

Biochemical evaluation

At the end of the treatment period, the animals were euthanized with an overdose of ketamine (10 mg/kg ip) and the brains were quickly dissected out and weighed. Blocks of cerebellar 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). 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 cerebellar tissues were placed in a phosphate buffer with 1% Triton-X 100 and centrifuged at 5000rpm for 10 minutes. The following reagents were used; 35µL of 5mM dithio-bisnitrobenzoic acid (also known as Ellman's reagent (DTNB)), 10µL of 75mMacetylthiocholine (ATCh) and 50mM 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 a 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.Motor cortex(from Bregma –2.5 mm to –4.5 mm) and cerebellar cortex 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/ cerebellar 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 & 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

Effects of CPF and NSO on cerebellar cortices' AChE activity and Oxidative Biomarkers

CPF exposure caused significant depletion in cerebellar AChE activities when compared with the normal saline treated rats (Figure 1). However, NSO treatment prevented the depletion of AChE activities, whether given in isolation or in combination with CPF (Figure 1). A significant increase in NO levels in the cerebellum of the CPF exposed rats was observed when compared with all the other groups. However, co-administration with NSO, or exposure to NSO on its own caused significant depletion in cerebellar NO levels (Figure 1). Accordingly, ROS activities slightly increased in the cerebellum of the

CPF exposed rats compared with control, and that surge in ROS activity depleted significantly with NSO treatment (Figure 1)

Effects of CPF and NSO on locomotor and explorative activities

CPF exposure caused a significant reduction in both exploratory and locomotor indicators (line crossing frequency and rearing frequencies) in the open field paradigm. In contrast, in groups exposed to NSO on its own and NSO co-treated CPF there was a significant increase both in line crossing frequency and rearing frequency when compared with control and the CPF only treated rats (Figure 2). Therefore NSO counteracted the effect of CPF on locomotor and exploratory activity.

Effects CPF and NSO on histopathological changes in the cerebellar and motor cortices of rats

Both the cerebellar Purkinje cells and the motor pyramidal cells appeared preserved in normal saline and NSO treated rats. CPF exposed rats suffered some damage to both cerebellar Purkinje and the motor pyramidal cellular integrity, with few degenerative-like features in these cells and increased peri-cellular spaces (Figure 3). There was also a marked loss of neurogenic cells (Ki67) distribution in both the cerebellar and motor cortices of the CPF exposed rats (Figure 3), further supporting the degenerative-like reduced cellularity above (Figure 3). However, intervention with NSO or NSO-only treatment preserved the population of neurogenic cells in the two brain regions (Figure 3).

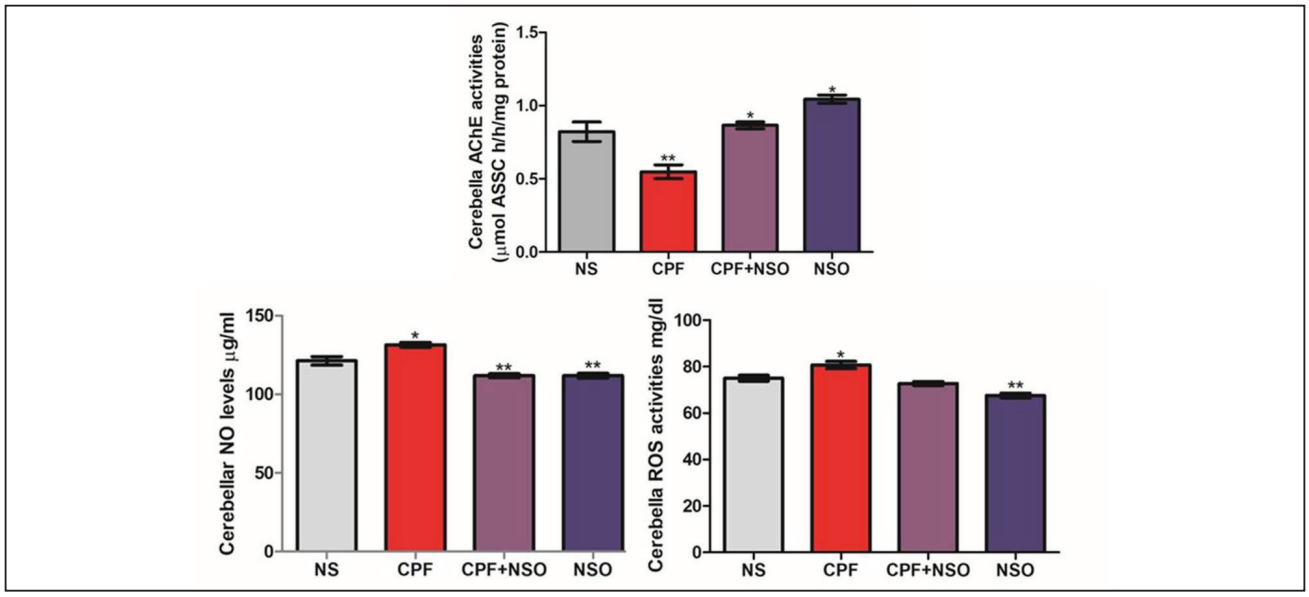


Figure 1: AChE activities and oxidative stress biomarkers (NO and ROS) in the cerebellar cortices of rats exposed to Normal saline (NS), chlopyrifos (CPF), chlopyrifos + Nigella sativa oil (CPF+NSO) and Nigella sativa oil (NSO). Double asterisk (**) indicates significant (p≤0.05) reduction, while single asterisk (*) indicates significant (p≤0.05) increase from control and/or CPF treated rats

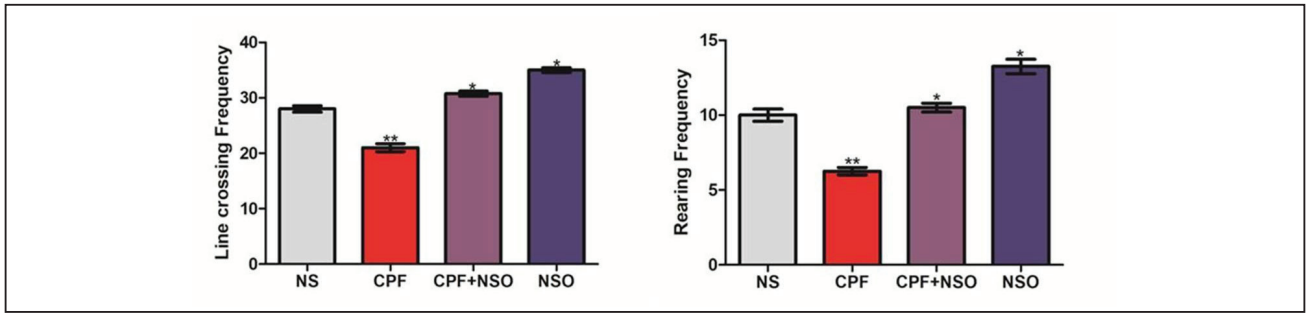


Figure 2: Line crossing frequency and Rearing frequency by rats exposed to Normal saline (NS), chlopyrifos (CPF), chlopyrifos + Nigella sativa oil (CPF+NSO) and Nigella sativa oil (NSO). Double asterisk (**) indicates significant (p≤0.05) reduction when compared with the control and other groups, while single asterisk (*) indicates significant (p≤0.05) increase from CPF and/or control rats.

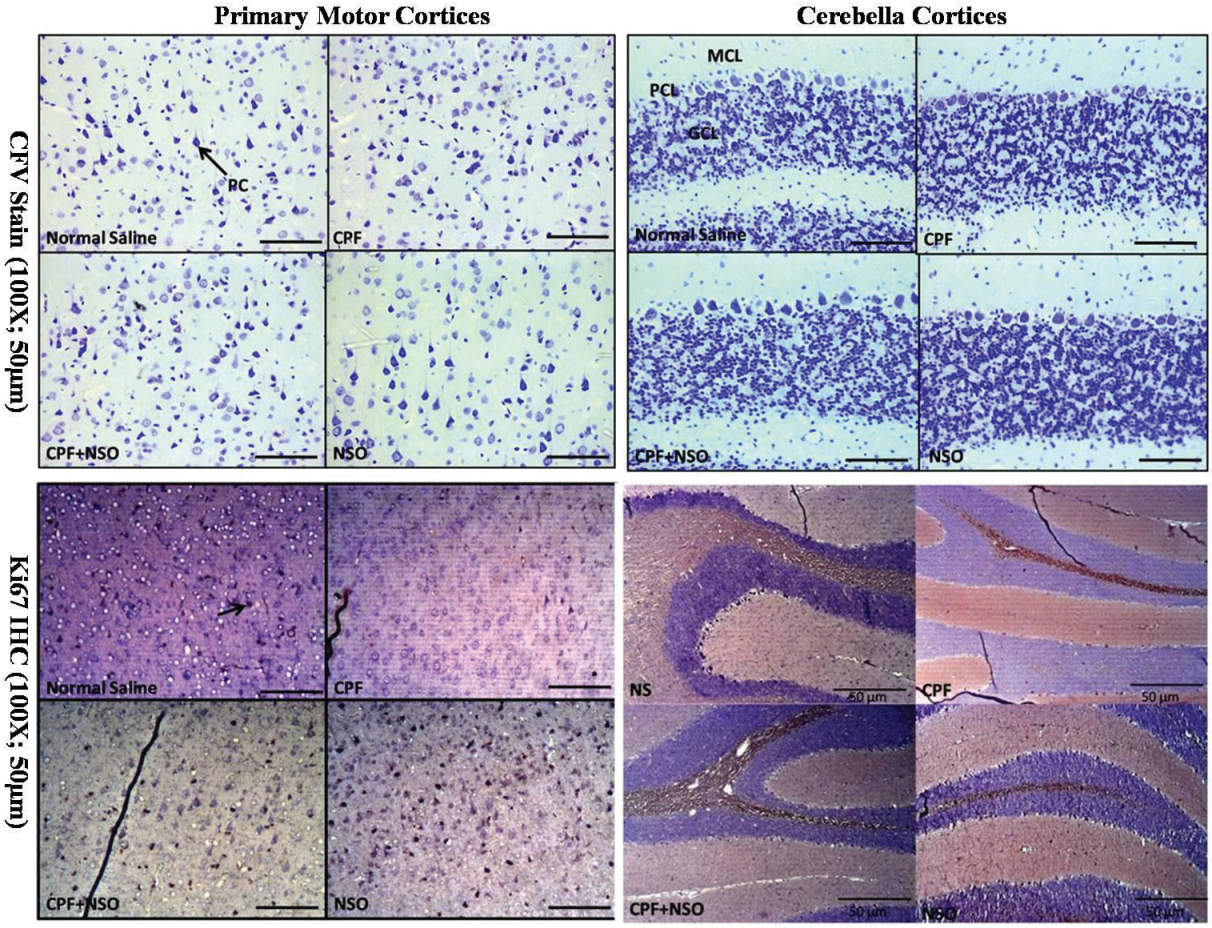


Figure 3: The pyramidal and Purkinje cells architecture (CFV) and the distribution of Ki67 immunoreactive cells in the layer 3 of the motor cortices and cerebellar cortices of rats exposed to Normal saline (NS), chlorpyrifos (CPF), chlorpyrifos + Nigella sativa oil (CPF+NSO) and Nigella sativa oil (NSO) 100X; 50µm

Discussion

The extensive use of insecticidal agents both agriculturally and domestically for pest and insect control has led to acute and chronic toxicity, that has resulted in several neurological disturbances. In this study we demonstrated that chlorpyrifos induces oxidative stress, locomotor impairment, diminished exploratory activity and cerebellar and neocortical damage in adult male Wistar rats.

Various studies have shown links between behavioral and memory changes caused by pesticide poisoning and cellular damage resulting from oxidative stress. Neurological symptoms typically appear in such instances due to environmental pollutants such as organophosphates, causing imbalance in the cellular oxidative metabolic pathways.[25]

Locomotor activities which are central to social behaviours were also evaluated in this study and they were impaired by acute exposure to CPF. The level of locomotor activity was remarkably lower in the CPF group compared to either the CPF+NSO group or NSO group. The decrease in the exploratory activity where a reduced line crossing and exploratory activities were reported may be linked to a possible inhibition of transmission of neural signals through the basal ganglia and cerebellum.[22]

The study showed that exposure to CPF caused a slight increase in the levels of oxidative stress biomarkers, reactive oxygen species and Nitric Oxide which is confirmed in the literature based on previous studies.[7,26,27] The body's primary defence against organophosphate poisoning can be attributed to antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) and these have been shown to be significantly affected by CPF administration.[28] Oxidative stress is known to be a key factor in several diseases, and recent findings indicate that the toxic manifestations induced by CPF may be associated with the enhanced ROS production, which causes damage to cell membrane components.[29,30] Co-treatment with *Nigella sativa* in this study decreased elevated levels of NO and ROS. We suggest that this is due to the antioxidant properties of NSO, which resulted

in scavenging of ROS and neutralizing the effects of the oxidant before they could cause damage to the cerebellum or other areas associated with motor function.

Inhibition of the AChE activity in OP exposure is a cardinal sign of OP poisoning mechanism, because these compounds generally elicit their effects by inhibiting AChE activities irreversibly. The present study revealed that exposure to CPF caused a reduction in tissue AChE activities in the exposed rats, similar to what was reported by Chidiebere and colleagues in rats exposed to a combination of deltamethrin and chlorpyrifos [31]. However, co-treatment with NSO led to an increase in AChE activity, even above baseline levels, showing restorative effects of NSO.Histopathological studies have been widely used to study biomarkers for toxicological investigations, including those of pesticides. The cerebellum plays a key role in motor coordination and also in cognitive and emotional behaviours.[32] Oral ingestion of CPF caused several histopathological changes in the cerebellum, and these can be supported by other studies conducted on CPF and other pesticides in other regions of the brain.[15] Goel et al, administered 13.5 mg/kg chlorpyrifos to rats and it caused biochemical and histopathological changes such as necrosis, vacuolization and dilatation of sinusoids, and these they suggested can be associated with the surged generation of ROS in the exposed rats.[33] In this study, treatment with CPF showed widened spaces, altered distribution of cell masses and dilatation of sinusoids. Co-treatment with NSO ameliorated the effects of CPF on the architecture of the cerebellum.

Ki-67 is a chromosome associated nuclear antigen[34] present in cells throughout the G_1, G_2, S and M phases of the cell cycle.[35] Ki-67 distribution in brain regions allowed a clear distinction between the groups affected by OP poisoning, and suggest their pathological responses. Qualitative assessment of stained nuclei distribution allowed for the determination of the extent of the effects of the chlorpyrifos poisoning. Fewer Ki-67 protein positive nuclei were seen in the CPF groups, suggesting a loss of neurogenic properties.

We conclude here that chlorpyrifos induces mild cerebellar toxicities through AChE activities suppression, oxidative and proliferative functions disruptions resulting in impaired motor functions. All of these are however prevented by the introduction of *Nigella sativa oil* suggesting its efficacy and the efficacy of strengthening antioxidant systems as antidote against CPF poisoning induced cerebellar toxicity.

Acknowledgments

The Ilorin Neuroscience Group (ING) whose chairman is Prof. B.V. Owoyele of the department of physiology, University of Ilorin, is appreciated for affording some of the authors which are her members the opportunity to use her behavioral paradigms. The Neuroscience research group is also appreciated for allowing free use of some consumables during the experiment.

Conflict of Interest: None
Source(s) of support: None

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