
ORIGINAL ARTICLE**Pro-Neurogenic and Antioxidant Efficacy of *Nigella sativa* Oil Reduced Vulnerability Cholinesterase Dysfunction and Disruption in Amygdala-Dependent Behaviours in Chlorpyrifos Exposure**

Aminu Imam^{1*}, Nafeesah Abdulkareem Sulaiman¹, Aboyeji Lukuman Oyewole², Abdulbasit Amin², Sheu-Tijani Toyin Shittu³, Moyosore Salihu Ajao¹

¹Neuroscience Unit, Department of Anatomy, ²Neurophysiology Unit, Department of Physiology, College of Health Sciences, University of Ilorin, P.M.B 1515, Ilorin 240003, Nigeria, ³Endocrinology and metabolism Research Unit, Department of Physiology, College of Medicine, University of Ibadan, Ibadan, Nigeria

Abstract:

Background: Organophosphorus Pesticides (OPs) are compounds with irreversible cholinesterase activity which induce cholinergic neurotoxicity, but still remain a widely used pesticide in household and agriculture. **Aim and Objectives:** This study investigated the efficacy of a natural antioxidant *Nigella sativa* Oil (NSO) against Chlorpyrifos (CPF) induced oxidative stress and implications on emotionality behaviours. **Material and Methods:** Thirty-two adult Wistar rats were randomly divided into four groups, and exposed to (1 ml/kg b w) of normal saline, (14.9 mg/kg b w) of CPF, (14.9 mg/kg b w) of CPF plus (1 ml/kg b w) of NSO and (1 ml/kg b w) of NSO respectively for 14 consecutive days. Body weight were recorded at day 1 and 15 of the experiment, the rats were exposed to trials in both Open Field Test (OFT) and Elevated Plus Maze (EPM) to assess anxiety-like behaviours and fear related learning respectively on the 13th day. Rats were euthanized by the 15th day, the brains excised, and the amygdala area of brains were removed, homogenized to analyse for total Reactive Oxygen Species (ROS), Nitrous Oxide (NO) levels and Acetylcholinesterase (AChE) activities, while the other three were processed for histology (Nissl stain) and Proliferative marker (Ki67 immunohistochemistry). **Results:** Repeated CPF exposure caused an increase in NO and ROS levels, reduction in AChE activities and a loss in the

neurogenic cells in the amygdala. It was also a prolonged freezing period, centre squares avoidance and delayed transfer latency with CPF exposure. However, NSO prevented the overproduction of ROS and NO, and markedly reactivated AChE activities in the amygdala either with or without CPF exposure. NSO treatment was also, able to preserve neurogenic cells in the amygdala and subsequently improved amygdala-dependent behaviours in the treated rats. **Conclusion:** The antioxidant efficacy of NSO could be efficacious in CPF induced neuro-cognitive toxicity in rats.

Keywords: *Nigella sativa* Oil, Anxiety, Fear Related Learning, Chlorpyrifos, Oxidative Stress

Introduction:

Organophosphorus Pesticides (OPs) are compounds with irreversible cholinesterase activity which induce cholinergic neurotoxicity, but still remain a widely used pesticide in household and agriculture [1, 2]. They are one of the top causes of poisoning worldwide; with an annual incidence of poisoning among agricultural workers varying from 3–10% per country [3]. A single acute exposure to high doses of organophosphates can result in immediate,

devastating, and even lethal consequences [1, 3]. Chlorpyrifos (CPF) (O,O-diethyl-O-(3,5,6-trichloro-2-pyridylphosphorothionate) is a potent OP, currently used all over the world, with alarming high amounts being used in the developing world, primarily in homes and on farms. Like other OPs, it inhibits Acetylcholinesterase (AChE), leading to accumulation of ACh at the cholinergic synapses, subsequently causing hyperactivity in cholinergic pathways resulting in neurotoxicity and eventual death. It is also extensively reported that the severity in its neurotoxicity is mediated by oxidative stress and neuro-inflammation [4, 5].

Nigella sativa, is a medicinal plant widely used all over the world, notably recognized as one of the most famous plants whose seeds and oil have been used to promote health and fight diseases for decades [6]. Its major component, thymoquinone, has been discovered to possess cytoprotective, antioxidant, digestive, hepatoprotective, renal protective, antibacterial, anti-diabetic, and anti-inflammatory therapeutic properties for many inflammatory diseases, including edema, diabetes, neuro-inflammation and joint inflammation effects [6-7]. With respect to the antioxidant effect of this plant, many *in-vivo* and *in-vitro* studies suggest that *Nigella sativa* has neuroprotective effects in neurodegenerative disorders [10-12], and against induced toxicity in other tissues [13].

In recent years, *Nigella sativa* seeds and its extracted essential oil have been tested for their antitoxic properties against some organophosphate pesticides including dimethoate, diazinon, propoxur, pentylentetrazole and malathion. These studies have shown the ability of the plant to neutralize the most hematological,

biochemical, and immunological changes as well as reproductive toxicity effects caused by these pesticides [14-17].

Material and Methods:

Chemicals and drugs

Chlorpyrifos (PubChem Substance ID 329756699) PESTANAL[®], analytical standard was purchased from Sigma (Sigma-Aldrich) (St. Louis, MO, USA), while normal saline solution was prepared in our laboratory. *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 150 g and 170 g 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. 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

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

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

Group 2- were treated with (14.9 mg/kg b w orally) CPF daily;

Group 3- were treated with (14.9 mg/kg b w orally) CPF plus NSO (1 ml/kg b w orally) daily;

Group 4- were treated with NSO (1 ml/kg b w orally) daily;

All procedures were scheduled and carried out during the early phase of the day between 07:00 and 08:30 hours, and treatments were given for the fourteen consecutive days.

Ethical approval

This research work was approved by the University of Ilorin Ethical Review committee, following the recommendation of the College of Health Sciences Ethical Review Committee, University of Ilorin, Ilorin, Nigeria. The research was approved to be in compliance with the Institutional Animal Care and Use Committee (IACUC).

Body and brain weight evaluation

The body weights of all the rats were recorded after acclimatization at the first day of the exposures as initial weight and at the last day of exposure as the final weight. Thus, the differences between the two weights were calculated and recorded as the weight changes. The brain weights of all rats were recorded after the sacrifice, and a ratio of the brain to final body weight was calculated and recorded.

Behavioural Evaluation

The rats were subjected to behavioural evaluations on the 13th day of the treatment to assess, anxiety related behaviours and fear related learning in the Open Field Test (OFT) and the Elevated Plus Maze (EPM) paradigms.

OFT Procedure:

The animals were exposed to a trial in the OFT to evaluate exploratory and anxiety related behaviours in rats following CPF and/or NSO exposures. The rats were individually placed in the centre of the apparatus and time spent in the

centre and immobility period were recorded in a 5 minute session and all animals were monitored in a balanced design during the procedures [18]. For analysis, trial was performed in a well illuminated wooden box, divided into 4 × 4 squares. It has been researched that preference or avoidance of central squares may provide an evaluation of the anxiety level in the rats [19].

EPM Procedure:

To evaluate amygdala dependent or fear related learning, the rats were exposed to two trials in the EPM paradigm. This is consisted of 2 open arms, surrounded by a short edge to prevent falls, and two enclosed arms erected in such a way that the 2 open arms were opposite each other. The maze was raised about 35 cm above the ground with a stable stand and the arms of the maze were connected by a central platform. At each of the two trials, each rat was gently placed on an open arm, positioned to face away from the central platform and the closed arms. The time it takes the rats to recognise the treat and move to the closed arms was recorded as the transfer latency, while the first trial was for acquisition; the second was used as a measure of fear learning. The principle of this experiment is primarily based on the antipathy of rats to heights and open spaces [20].

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 amygdala tissue (from Bregma -2.5 mm to -4.5 mm) were removed from the brains of five rats from each group, dipped in 30% sucrose solution, homogenized and portions centrifuged at 2500 revolutions per minute for 10 minutes, and the serum collected into tubes

containing the compounds for the Nitric Oxide (NO) and Reactive Oxygen Species (ROS) analysis. NO metabolites were measured using Griess reagent. Tissue samples were added to the Griess reagents, sulfanilamide and Naphthylethylenediamine solutions. Absorbance was measured with the aid of a microplate reader, and the level of NO metabolites was calculated from standard curve [21, 22].

Since AChE inhibition is the pathological hallmark of organophosphate poisoning, AChE levels is frequently used for the diagnosis of organophosphate exposure [23]. The remaining portions of the homogenized amygdala 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 μ L of 5 mM dithio-bisnitrobenzoic acid, also known as Ellman's reagent (DTNB), 10 μ L of 75 mM acetylthiocholine (ATCh) and 50 mM phosphate buffer (pH 8.0). Protein concentration in brain homogenates was quantified using a Bradford assay and AChE activity was calculated in micromoles of ATCh hydrolysed per hour per milligram of protein and was expressed as percentage of control activity and measured values in micromole per hour per milligram of protein.

Tissue processing and histopathology

After euthanasia and extraction the brains of three rats from each groups, the brains were fixed in 10% formalin for 24 hours, amygdala blocks (from Bregma -2.5 mm to -4.5 mm) were removed, dehydrated through ascending grades of alcohol, cleared in xylene and embedded in paraffin blocks. Every second hippocampal tissue section (5 μ m in thickness) was stained with Cresyl fast violet (CFV) for Nissl substances or immunostained to reveal Ki67 protein distributions.

Immunohistochemistry for Ki-67

The Ki-67 is a chromosome-associated protein present during division (G, S, G, and M, but absent from cells at rest, G). 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% HO 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% serum (normal goat serum) + 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. Until incubation with primary antibody, all rinses 7 in between incubations were made with TBS-Triton, afterwards with TBS alone.

Statistical Analysis

Data from the morphometry, behavior and biochemicals were analyzed using One-way Analysis of Variance (ANOVA), and subjected to post hoc Bonferroni's multiple comparison test. The results are expressed as mean \pm 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 [18].

Results:**NSO with or with CPF improves indirect metabolic function markers in exposed rats**

There was a significant loss in body and brain weight of animals exposed to CPF only ($p \leq 0.05$), with a complementary reduction in relative brain weight (-22.2%). NSO intervention or without CPF co-administration prevented the absolute and relative weight loss in the brain and the body, and markedly (-5.6%) ($p \leq 0.05$) prevented decline of these parameters when given only (Fig.1, 2 and 3).

CPF induced ROS and NO out-burst in the amygdala: antioxidant efficacy of NSO

CPF exposure caused a significant ($p \leq 0.05$) increase in ROS (13.9%) and NO (7.3%) productions in the amygdala of the exposed rats, but these out-burst of the oxidative and nitrasitic stress markers were significantly ($p \leq 0.05$) depleted following NSO intervention (ROS = -5 and NO = -6.5%) or in NSO only (ROS = -13.9% and NO = -14.6%) treated rats (Fig. 4 and 5).

Reactivation of CPF induced AChE inhibition in the Amygdala by NSO

A significant ($p \leq 0.05$) depletion in AChE activities (-25%) was observed in the amygdala tissues following CPF exposure, when compared with the saline control rats. However, a significant ($p \leq 0.05$) reactivation or promotion of amygdala AChE activities in the NSO treated rats, with (12.5%) or without (25%) CPF exposure was observed (Fig. 6).

NSO prevented amygdala related behaviours following DDVP exposures

Exposure to CPF caused a significant ($p \leq 0.05$) prolongation of the immobility time, otherwise known as the freezing period in the exposed rats,

accompanied with marked ($p \leq 0.05$) avoidance of the centre squares in the OFT paradigm. NSO, however influenced a resistance and improvements of these behaviours, by significantly ($p \leq 0.05$) enhancing centre squares visit and exploration in the treated rats. CPF exposed rats also experiences or displayed lack of acquisition with delayed ($p \leq 0.05$) transfer latency in the EPM paradigm. With consistency, NSO was able to shorten transfer latency in the CPF and/or NSO only treated rats (Fig. 7, 8 and 9).

Neurodegenerative like activities of CPF and Protective efficacy of NSO

CPF caused severe histopathological changes in the amygdala of the exposed rats, evidenced by numerous necrotic-like pyknotic and reduced cellularity observed with the neurons. There was also a marked loss in the potent proliferating cells. NSO is observed to prevent the severity of the degenerative like activities of the CPF and preserved the density of neurogenic cells in the treated rats (Fig. 10 and 11).

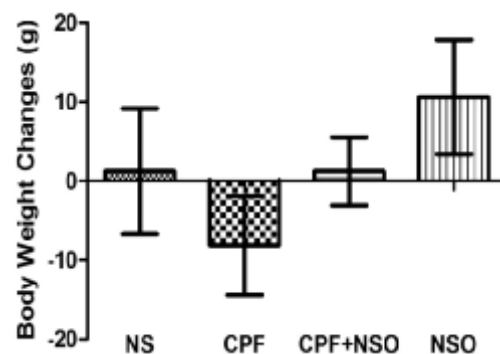


Fig. 1: Average Body Weights of Rats Exposed to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* Oil (CPF+NSO) and *Nigella sativa* Oil Only (NSO).

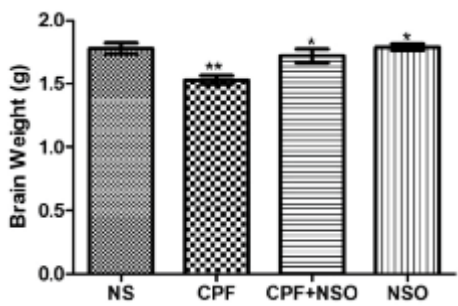


Fig. 2: Average Body Weights of Rats Exposed to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* Oil (CPF+NSO) and *Nigella sativa* Oil Only (NSO). Double Asterisks (**) Indicates Significant ($p \leq 0.05$) Reduction When Compared All Other Treatments, While Single Asterisk (*) Indicates Significant ($p \leq 0.05$) Increase When Compared With the CPF Exposed Rats

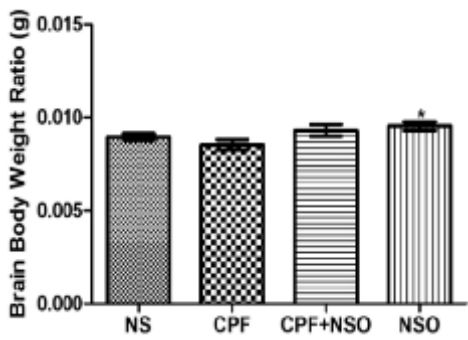


Fig. 3: Average Body Weights of Rats Exposed to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* Oil (CPF+NSO) and *Nigella sativa* Oil Only (NSO). Asterisks (*) Indicates Significant ($p \leq 0.05$) Increase When Compared with the DDVP Treated Rats

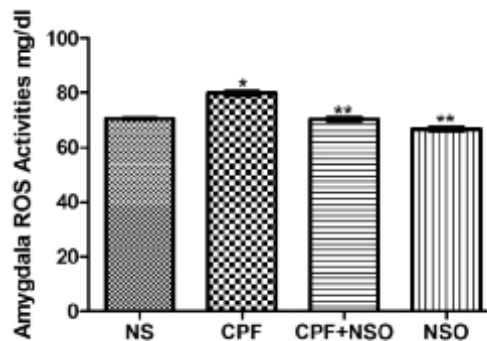


Fig. 4: Amygdala ROS Activities Following Exposures to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* Oil (CPF+NSO) and *Nigella sativa* Oil Only (NSO). Double Asterisks (**) Indicates Significant ($p \leq 0.05$) Reduction When Compared with CPF Exposed Rats, While Single Asterisk (*) Indicates Significant ($p \leq 0.05$) Increase from Control and Other Groups

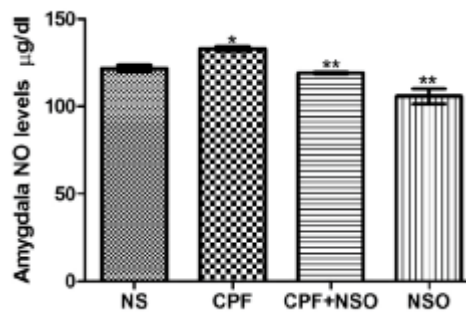


Fig. 5: Amygdala NO Levels Following Exposures to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* Oil (CPF+NSO) and *Nigella sativa* Oil Only (NSO). Double asterisks (**) Indicates Significant ($p \leq 0.05$) Reduction When Compared with CPF Exposed Rats, While Single Asterisk (*) Indicates Significant ($p \leq 0.05$) Increase from Control and Other Groups

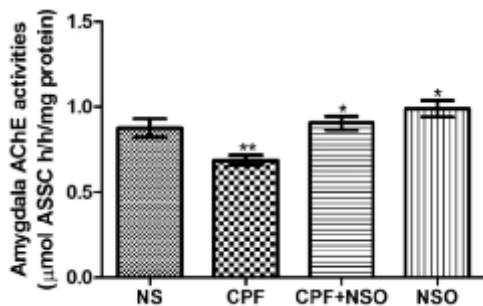


Fig. 6: Amygdala NO Levels Following Exposures to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* Oil (CPF+NSO) and *Nigella sativa* Oil Only (NSO). Double Asterisk (**) Indicates Significant ($p \leq 0.05$) Reduction When Compared with All Other Group Treatments, while Single Asterisk (*) Indicates Significant ($p \leq 0.05$) Increase When Compared with the CPF Exposed Rats.

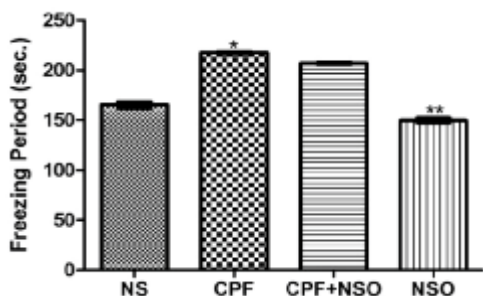


Fig. 7: The Freezing Period in rats following Exposures to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* oil (CPF+NSO) and *Nigella sativa* oil only (NSO). Double Asterisk (**) Indicates Significant ($p \leq 0.05$) Prolongation When Compared with all Other Group Treatments, while Single Asterisk (*) Indicates Significant ($p \leq 0.05$) Exploration When Compared with the Control and the NSO Treated Rats

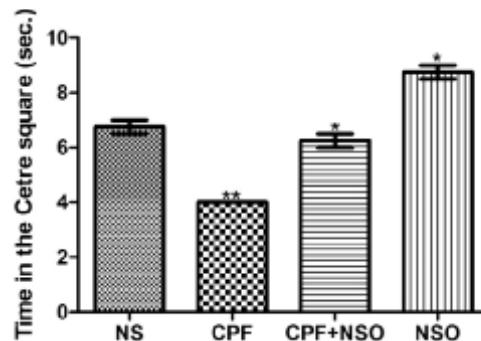


Fig. 8: Time Spent in Centre Square by Rats following Exposures to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* Oil (CPF+NSO) and *Nigella sativa* Oil Only (NSO). Double Asterisk (**) Indicates Significant ($p \leq 0.05$) Avoidance Time When Compared with all other Treatment Groups, while Single Asterisk (*) Indicates Significant ($p \leq 0.05$) Exploration When Compared with the CPF Exposed and/or Control Rats.

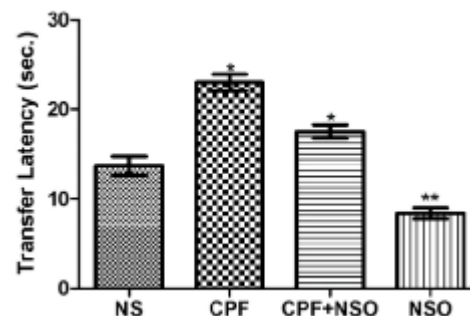


Fig. 9: Transfer Latency in Rats in the EPM Following Exposures to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* Oil (CPF+NSO) and *Nigella sativa* Oil Only (NSO). Single Asterisk (*) Indicates Significant ($p \leq 0.05$) Delay When Compared with the NSO and/or the Control, while Double Asterisk (**) Indicates Significant ($p \leq 0.05$) Shortened Latency When Compared with all Treatment Groups.

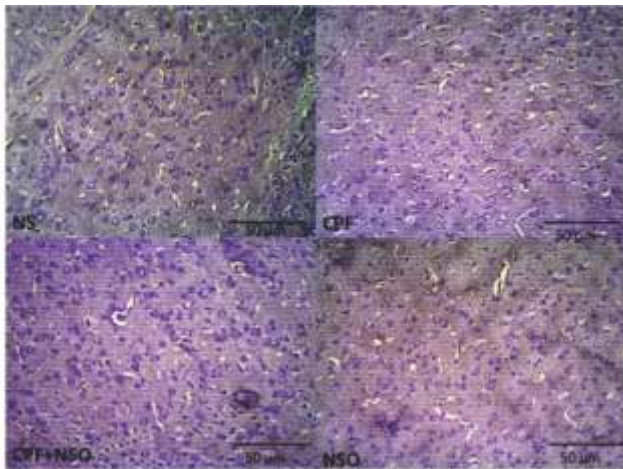


Fig. 10: Representative Photomicrographs of Amygdala of Rats Exposed to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* Oil (CPF+NSO) and *Nigella sativa* Oil Only (NSO). Pycnotic and Reduced Cellularity of the Amygdala Neurons CFV 100X

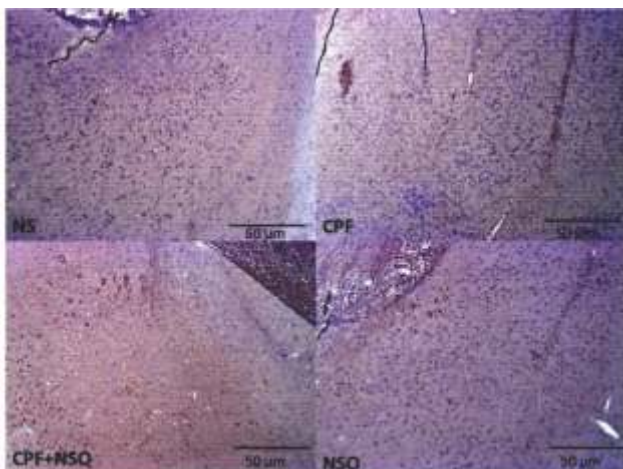


Fig. 11: Representative Photomicrographs of the Distribution of Ki67 Immunoreactive Cells in the Amygdala of Rats Exposed to Normal Saline (NS), Chlorpyrifos (CPF), Chlorpyrifos + *Nigella sativa* Oil (CPF+NSO) and *Nigella sativa* Oil Only (NSO). Ki67-ir Cells. Ki67 IHC 100X

Discussion:

Accidental or occupational exposures to OPs are prevalent globally, and toxicity to the nervous system has been cited as one of the primary points of concern and health burden from the exposures [24]. Clinical evidences show some of the burdens of the neurotoxicity from OPs exposure to include higher percentage of anxiety, depression [25], and suicide [26]. Vulnerability of the nervous system to poisoning with short term oral, acute inhalation or dermal exposure to CPF was reported to be more susceptible in humans than the primary targets (insects or pests) [27], with cases of paresthesia, headedness, seizure-like motor dysfunction, coma and subsequently, death [28]. Cholinergic dysfunction mediated by the deactivation of AChE activities remain the primary mechanism of OPs induced neurotoxicity, including CPF [29-31], and a measure of AChE activity has been confirmed to be a standard biomarker of organophosphate poisoning.

Exposures to CPF in this study greatly inhibited AChE activities in the amygdala of the exposed rats, an observation that is strengthened with reports of such effects [32, 33]. This cholinergic effects with CPF exposure was further complicated with the over production of ROS and NO in the amygdala, suggestive of a possible severity in the lipoidal matrices in cells, leading to oxidative damages and possibly cell death as previously reported with other OPs [32].

The amygdala is a vital brain structure that is critically involved in the processing and expression of anxiety and fear learning [34], establishing the neural basis or link between anxiety related behaviours and fear learning, sometimes called “Pavlovian learning” or fear or threat conditioning [35-38]. Centre square

exploration and immobility periods from the OFT, and the latency to find enclosure in the in the EPM, where used in this study as measures of anxiety-like and fear learning behaviours respectively. CPF exposure severed both anxiety and fear leaning behaviours, by causing relative avoidance of centre square visits, prolonged immobility and delayed transfer latency in the exposed rats, and this is supported by recent submissions in the literature [39, 40].

There were also evidences of necrotic-like damages in the amygdala of the CPF rats, with accompanying loss of neurogenic cells, which we suggest may have contributed to the behavioural outcomes discussed above. Consequently, the neurodegenerative-like effects may be associated with the combined severity of the oxidative damage and the cholinergic dysfunction, as induced over production of ROS and RNS have been implicated to trigger cellular damages and subsequent impairments in neuronal function [21, 41, 42].

These effects are also observed in relative or absolute weights which have been used extensively as indirect markers of metabolic disturbances. In this study CPF exposure caused a marked loss in both body weight and relative brain and body weight, an effect has previously been attributed to the possibility of reduced food consumption in the exposed rats [43, 44]. Also, cholinergic dysfunctions, emotional behaviours like anxiety, fear and amygdala related cognitive dysfunctions associated with the gut-brain connections, can be implicated to mediate the weight loss, as have been implicated in metabolic dysfunctions [45-49].

Natural antioxidants, such NS have been

extensively reported to be efficacious against models of neurotoxicity, including OPs. Co-administration of NSO in this study revealed its antioxidant efficacy at large; by preventing the overproduction of both ROS and NO in the amygdala of CPF exposed rats, supported by its previously reported antioxidant capacity [50].

NSO was also able to minimise AChE depletion following CPF exposure, and improved AChE activities in the amygdala of rats treated with only NSO, an interaction that can be associated with its efficacy against neuro-cognitive, neuropsychiatry and neurodegenerative impairments [51]. The therapeutic efficacy of the combined antioxidant and pro-cholinesterase activities of NSO reported above are evidenced in the improved amygdala related behaviours recorded in this study. And such effects are strengthened by the efficacies of this essential oil in models of neurodegenerative disorders and neurotoxicity [18, 21, 50-52].

Complementing its neurocognitive activities, NSO treatment preserved the integrities of hippocampal subfield pyramidal and dentate gyrus granularcells, and such activities are evidenced in its efficacies against degenerative exposures to neurotoxic models [12, 51]. NSO also enhanced the survival of neurogenic cells in the hippocampus, which may have contributed to its neurocognitive effects, none the less, NSO and/or its constituent thymoquinone are extensively reported to restore neuronal integrity and functions by increasing neuronal density, decreasing apoptosis and preventing inflammatory processes. All of these effects have been related to its antioxidant capacities which prevent oxidative damage which initiates pathological processes in the brain tissues [21].

Conclusion:

The antioxidant efficacy of NSO could be efficacious in CPF induced neuro-cognitive toxicity in rats.

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***Author for Correspondence:** Aminu Imam, Neuroscience Unit, Department of Anatomy, College of Health Sciences, University of Ilorin, P.M.B 1515, Ilorin 240003, Nigeria
E-mail:imam.a@unilorin.edu.ng Cell:+2348165663947