EFFECTS OF METHANOL LEAF EXTRACT OF *MORINGA OLEIFERA* ON NEUROBEHAVIOURAL AND OXIDATIVE STRESS CHANGES INDUCED BY SUBCHRONIC EXPOSURE TO CHLORPYRIFOS IN MALE WISTAR RATS

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DEPARTMENT OF VETERINARY PHARMACOLOGY AND TOXICOLOGY, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

MAY, 2015

DECLARATION

I declare that the work reported in this thesis, entitled "**Effects of methanol leaf extract of *Moringa oleifera* on neurobehavioural and oxidative stress changes induced by subchronic exposure to chlorpyrifos in male Wistar rats**" was carried out by me in the Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, under the supervision of Professor S. F. Ambali, Professor J. O. Ayo, and Dr. A. Mohammed. The information derived from literature has been duly acknowledged in the text and in the list of references provided. No part of this thesis has been presented for another degree or diploma at any university.

Enokela Shaibu Idoga

……………………………. ………………………

Name of Student Date

CERTIFICATION

This thesis, entitled "**EFFECTS OF METHANOL LEAF EXTRACT OF *MORINGA OLEIFERA* ON NEUROBEHAVIOURAL AND OXIDATIVE STRESS CHANGES INDUCED BY SUBCHRONIC EXPOSURE TO CHLORPYRIFOS IN MALE WISTAR RATS**" carried out by Enokela Shaibu Idoga meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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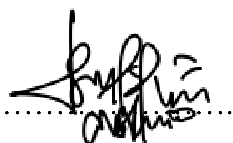
Ahmadu Bello University, Zaria  01-06-2015
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DEDICATION

I dedicate this work to the Almighty God, to my parents, and to the memory of my beloved mother.

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Firstly, my gratitude goes to the Almighty God for life, good health, inspiration and provision for this study. My ineffable gratitude also goes to members of my supervisory committee including Prof. J. O. Ayo and Dr. A. Mohammed, and the chairman of the committee Prof. S. F. Ambali, for their huge inputs, immense contribution and guidance through this study. I appreciate them for the repeated perusal, meticulous corrections and constructive criticisms, all of which have brought out the best in the thesis. I am grateful to the entire staff of the Departments of Veterinary Pharmacology and Toxicology, Veterinary Physiology, and Veterinary Anatomy, Ahmadu Bello University, Zaria. I am also grateful the staff of National Research Institute in Chemical Technology, Zaria. Their diverse contributions brought this work to fruition.

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ABSTRACT

The aim of the study was to investigate the modulatory role of methanol extract of *Moringa oleifera* leaves (MO) on neurotoxicity induced by chlorpyrifos (CPF) exposure. The methanol extract of the MO leaves were first subjected to qualitative phytochemical screening. The quantity of flavonoids was evaluated using the High performance Liquid Chromatography (HPLC) while vitamins A, C and E were also evaluated using ultraviolet-visible spectroscopy. To investigate the modulatory role of MO leaves on CPF-induced neurotoxicty, 60 male Wistar rats were divided into 6 groups of 10 animals each.Group I was administered with distilled water (2 ml/kg); Group II, with soya oil (2 ml/kg); Group III, with MO (500 mg/kg); and Group IV, with CPF (9.8 mg/kg~1/10th of the LD_{50} determined). Groups V and VI were administered with MO at 250 mg/kg and 500 mg/kg, respectively, 30 min before administration of CPF (9.8 mg/kg). The regimens were administered once daily via gavage for a period of 9 weeks. Animals were subjected to neurobehavioural tests such as open field (measuring frequency of locomotion, rearing, stretch–attends posture, defaecation and urination), beam-walk (measuring motor coordination), ladder walk, (measuring efficiency of locomotion) inclined plane (measuring neuromuscular coordination), forepaw grip time (measuring motor strength), excitability scores and forced swimming (measuring depression) on day 0, weeks 3, 6 and 9 of administration, and then step-down avoidance test (measuring learning and short-term memory) at the end of extract administration. Thereafter, the animals were sacrificed and the brain tissues harvested. The homogenates were assayed for levels of acetylcholinesterase (AChE), superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) and malondialdehyde (MDA). Brain tissue was also processed for histopathological examinations. The phytochemical screening indicated that MO extract was positive for alkaloids, flavonoids, glycosides, phenol, saponin, tannin and

terpenoids. Quantitative analysis of the antioxidant components of the plant showed that the flavonoid, vitamins A, C and E contents of the plant were 22.6% w/w, 0.3 mg/g, 6.7 mg/g and 0.22 IU/g, respectively. The result of the subchronic toxicity showed that CPF decreased ($P < 0.05$) frequency of locomotion and rearing, indicating deficiency of motor activity, and increased ($P < 0.05$) the frequency of stretch-attends posture and defaecation in the open field, demonstrating anxiogenic response. The CPF induced significant ($P < 0.05$) deficits in beam-walk score, forepaw grip time, ladder work, forced swimming time and excitability score, while it marginally increased ($P > 0.05$) the number of foot shocks, but decreased significantly ($P < 0.05$) the time spent on the platform in the step-down avoidance inhibition apparatus indicating apparent deficits in learning and significant deficit in short-term memory, respectively. The CPF group also showed an increase in brain MDA concentration and reduction in the activities of SOD, GPx but an increase in the activity of CAT, indicating oxidative stress. Histopathology revealed that CPF induced neuronal degeneration. Pretreatment with MO extract mitigated the deficit in motor activity, anxiety, motor coordination, neuromuscular coordination, motor strength, excitability scores, depression, learning and short-term memory provoked by subchronic CPF administration. MO pretreatment modulated the brain AChE activity and mitigated the neuronal degeneration provoked by CPF. MO also mitigated the CPF-induced oxidative stress in the brain by reducing MDA concentration and modulated the activities of SOD, CAT and GPx, demonstrating its antioxidant effect. In conclusion, MO pretreatment mitigated CPF-evoked neurotoxicity due to the flavonoid, vitamins C and E contents of the plant extract, which confers its antioxidant and AChE restorative properties.

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LIST OF ABBREVIATIONS

5C-SRTT	5 choice serial reaction time test
5HT	Serotonin
ACh	Acetylcholine
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
ATChI	Acetylcholine iodide
BHT	Butylated hydroxy toluene
BHA	Butylated hydroxy anisole
CAT	Catalase
CNS	Central nervous system
COPIND	Chronic organophosphate induced neuropsychiatric disorder
CPF	Chlorpyrifos
CPF- oxon	Chlorpyrifos oxon
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DTNB	Disthiobisnitrobenzoate
DW	Distilled water
g	Relative centrifugal force
GSSG	Oxidized glutathione
GSH	Reduced glutathione
GP_X	Glutathione peroxidase

HPLC	High performance liquid chromatography
IQ	Intelligence quotient
LD_{50}	Median lethal dose
MO	*Moringa oleifera*
NADPH	Nicotinamide adenine dinucleotide phosphate
NARICT	National Research Institute for Chemical Technology
NWLSS	Northwest life science specialties
OP	Organophosphate
OPICN	Organophosphate induced chronic neurotoxicity
OPIDP	Organophosphate induced delayed poly-neuropathy
PBS	Phosphate buffered saline
PNS	Peripheral nervous system
PONS1	Paraoxonase 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SO	Soya oil
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric aci-reactive substances
TCPy	3,5,6-trichloro-2-pyridinol
TOCP	Tri-o-cresyl phosphate
UK	United Kingdom

USA	United States of America
USEPA	United States Environmental Protection Agency
UV	Ultra-violet
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background

Pesticides are used in agriculture as plant protection agents for boosting food production (Abhilash and Singh, 2009) and in public health (Matthews *et al*., 2011). Organophosphate (OP) compounds are used widely as pesticides in agriculture industry (Mulchandani *et al*., 2001; Oates *et al*., 2014), and as household insecticides (Lovasi *et al*., 2011). OP insecticides inhibit actetylcholinesterase (AChE) activity by binding to the active site of the enzyme, preventing the break-down of acetylcholine in the nervous system of insects and other pests (Kwong, 2002; Marks *et al*., 2010). This leads to the accumulation acetylcholine (ACh) in nerve endings, and therefore continuous stimulation of cholinergic receptors (Timofeeva *et al*., 2008), resulting in tetany, eventually paralysis and death (Ogueji *et al*., 2013). Animal and human studies have linked OP exposure to sensorimotor and cognitive impairments (Ambali *et al*., 2010b, 2012a, b; Rauh *et al*., 2012; Lopez-Granero *et al*., 2013; Kaur *et al*., 2014). Similarly, epidemiological studies have linked acute and chronic OP exposure to affective disorders (Da Vies, 1995), resulting in depression and suicide in humans (London *et al*., 2005, 2012). The wide-spread exposure of humans and animals to OP pesticides have raised increasing concern about their toxicity (El-Hossary *et al*., 2009; Kim *et al*., 2014).

Chlorpyrifos (CPF) is an extensively used OP insecticide with many domestic and agricultural pest control applications. It is one of the most widely used organophosphorothionate pesticide (Tirelli *et al*., 2007). Neurotoxicity is the main manifestation of CPF exposure in animals and man (Sanchez-Santed *et al*., 2004), and the primary toxic mechanism is AChE inhibition (Kwong, 2002). CPF is also a

developmental neurotoxicant, affecting developing foetus (Saunders *et al*., 2012) and its low doses disrupt brain development and cognitive function (Slotkin and Seidler, 2005). CPF-induced damage extends beyond cholinergic pathways to include other neurotransmitter systems, notably the monoamines, norepinephrine, dopamine (Xu *et al*., 2012), and serotonin (5HT) (Raines *et al*., 2001). Recent studies show that 5HT systems are especially sensitive as CPF affects 5HT receptors, the presynaptic 5HT transporter, and 5HT-mediated signal transduction during a discrete critical gestational window (Aldridge *et al*., 2003). Foetal or neonatal exposure to CPF leads to abnormalities of brain cell development, synaptic function, and behaviour (Dam *et al*., 2000).

Although inhibition of AChE activity is the primary mechanism of CPF toxicity, studies have shown that toxicity also occurs at doses that do not inhibit AChE activity or long after its restoration (Haviland *et al*., 2010; Goodman *et al*., 2013). Therefore, other non-AChE mechanisms have been implicated in CPF toxicity. The generation of reactive oxygen species (ROS), and, hence, induction of oxidative stress has been identified as one of the major non-AChE mechanism of CPF toxicity (Slotkin *et al*., 2005; Tuzmen *et al*., 2007; Verma *et al*., 2007; Mansour and Mossa 2009; Ambali and Ayo 2011; Ambali *et al*., 2011 a, b, c; Lee *et al*., 2014; Ventura *et al*., 2015).

Oxidative stress is associated with increased generation of ROS, exerting toxic effects on cells (Misra *et al*., 2009). It results in increased lipoperoxidative damage to cell membranes. Under normal circumstances, the body is equipped with agents (antioxidants) that combat the menace of oxidative stress. Antioxidants are agents which play an important role in inhibiting and scavenging free radicals, thus providing protection to tissues against damaging effects of free radicals (Beam *et al*., 2014). However, oxidative stress results when the body antioxidant reserve system cannot cope with the level of

oxidants, as has been previously reported in CPF poisoning (Ambali *et al*., 2010a, b; Mansour and Mossa, 2010). In this type of situation, exogenous supplementation with antioxidants becomes imperative. Several studies in humans and animals have shown that various antioxidants including, those derived from plants (Ambali *et al*., 2012a, b) mitigated CPF-evoked adverse health effects.

Moringa oleifera Lam (MO) (Moringaceae), commonly known as Drumstick is a highly valued plant, and a small or medium sized tree of about 10 m high. *M. oleifera* is a multipurpose tree considered to have its origin in the northwest region of India, south of the Himalayan mountains (Ganesan *et al*., 2014) with high nutritional values. It is distributed in many countries of the tropics and sub-tropics (Anwar *et al*., 2007; Razis *et al*., 2014). It is cultivated for use as a vegetable for spice, cooking and cosmetic oil, and as a medicinal plant (Ajayi *et al*., 2014). Different parts of this plant contain a profile of important minerals, and are a good source of protein, vitamins, β-carotene, amino acids and various phenolics (Anwar *et al*., 2007). It contains various chemical constituents such as alkaloids, tannins, flavonoids, carbohydrates, amino acids and glycosides (Belay and Sisay 2014). Leaf extracts of *M. oleifera* have antioxidant properties, the free-radical scavenging effect of which is comparable with that of many classical antioxidants (Kumar and Pari, 2003; Siddhuraju and Becker, 2003; Sreelatha and Padma, 2009; Ratshilivha *et al*., 2014).

1.2 Statement of Research Problem

In an effort to feed the world's rapidly growing population, governments have through various means encouraged increased agricultural production. To this effect, there has been an increase in the application of agricultural inputs such as pesticides, to increase productivity in most countries (Ramaswamy and Sanders, 1992). Pesticide use has

therefore increased over the years, with attendant surge in agricultural productivity and domestic pest control (Poswal and Akpa, 1991). This has however come with its attendant adverse health consequences as they are often not target specific and cause adverse effects in exposed non-target animals and man (Kumar and Singh, 2014).

Chlorpyrifos (CPF) is a broad spectrum, highly effective OP pesticide that is widely used worldwide. Its increasing use has increased the propensity of its neurotoxic effects (El-Hossary *et al*., 2009), especially in children. This concern has led to the banning of CPF for home and garden use in the United States of America and some European countries (Renner, 2004). However, CPF remains on the shelves of agricultural shops and plazas, and is harnessed for such use in many developing countries, including Nigeria (Asogwa and Dongo, 2009). Many people are exposed to small doses of CPF when applied outdoors to fields, and when used as termiticides (Farahat *et al*., 2010). The use of CPF has been shown to leave high level of its residues in the soils and several vegetables in some parts of Northern Nigeria (Akan *et al*., 2013). Factors promoting exposure including eating and drinking during spraying operations, failure to use protective clothing, improper storage and disposal of insecticides, are common among farmers and pesticide applicators in Nigeria (Sosan and Akingbohungbe, 2009; Surajudeen *et al*., 2014). Thus, pesticide applicators with inadequate protective gears, and farmers and livestock are exposed to sub-lethal and apparently sub-toxic doses of CPF by aerosol and direct contact (Cattani *et al*., 2001; Quandt *et al*., 2006). CPF residues that have entered into the food web of the ecosystem in plant parts, soils and water bodies (Akan *et al*., 2013) therefore constitute a source of hazard to man and animals. CPF induces oxidative stress at low doses and this plays an essential role in its pathophysiology (Slotkin *et al*., 2001; Renner, 2007), including its effects on a wide variety of

neurotransmitter systems. The 5-HT system is especially affected, leading to long-lasting changes in 5-HT-related emotional behaviours such as anxiety and depression (Chen *et al*., 2011).

Recent studies suggest a link between pesticide exposure and anxiety, depression and suicide (Lee *et al*., 2007). A study in Hordaland, Norway showed that among other workers, farmers showed a significantly higher level of anxiety and depression than non-farmers. In the same study, full-time farmers showed significantly higher anxiety and depression levels than part-time farmers (Sanne *et al*., 2004). In a similar vein, studies in Sri Lanka have shown that suicide rates have more than halved since 1995, when the government placed restrictions on the import and sales of WHO Class I toxicity pesticides (Roberts *et al*., 2003). Adverse effects of chronic CPF exposure such as strained breathing, insomnia, and loss of memory have been observed in several Nigerian farmers (Sosan and Akingbohungbe, 2009; Sosan *et al*., 2010).

1.3 Justification

There has been a steep increase in the prevalence of neurodegenerative diseases in recent times, which may have been partly associated with an increase in the use of environmental chemicals, including pesticide (Zaganas *et al*., 2013). Incidences of neurodegenerative diseases and cognitive deficits, including decline in intelligent quotient (IQ) have been shown to be positively correlated with exposure to pesticides (Hayden *et al*., 2010; Parrón *et al*., 2011), including OP insecticides (Kamel and Hoppin, 2004; Wang *et al*., 2014).

Chlorpyrifos is the most commonly used OP insecticide throughout the world (De Felice *et al*., 2014) and its ability to induce neurobehavioural and cognitive deficits, and predispose to neurodegenerative diseases have been reported (Lee *et al*., 2007; Jiang *et al*., 2010). It has been shown to affect motor coordination, locomotor efficiency, motor

strength, learning and short-term memory (Ambali *et al*., 2010c) and 5-HT systems (Slotkin and Seidler, 2005) in animal models. Cognitive deficit, anxiety and depression have also been reported in individuals exposed to CPF (Lee *et al*., 2007). Induction of oxidative stress is one of the mechanisms implicated in CPF-induced neurotoxicity (Ambali *et al*., 2010a, b, c; 2012a, b, c) and several antioxidant vitamins and other classical antioxidants have been shown by several workers to ameliorate CPF toxicity (Yu *et al*., 2008; Mansour and Mossa 2009; Ambali *et al*., 2010a, b, c; 2011b, c; 2012b, c).

Moringa oleifera, a plant with high flavonoid constituents has been shown to have antioxidant properties (Siddhuraju and Becker, 2003). Although its antioxidant activity has been succinctly elucidated, the possible protective role of *M. oleifera* in CPF toxicity is yet to be proven. There is the need to add to the available potent antioxidants that have been proven to mitigate against CPF-induced neurobehavioural changes. This will be especially beneficial when the source of the antioxidant is from a natural plant that is abundant in tropical environment. Recent investigations are directed towards natural antioxidants, originated from plants due to concepts of safe therapeutics (Sreelatha and Padma, 2009).

1.4 General Aim of the Study

To evaluate the effect of methanol extract of *M. oleifera* leaves on CPF-induced neurobehavioural changes.

1.5 Objectives of the Study

The specific objectives of this study were to evaluate:

1. Qualitatively, the phytochemical constituents, and quantitatively, the flavonoids, vitamins A, C and E contents of the *M. oleifera* extract.

2. The effect of *M. oleifera* extract on some neurobehavioural and cognitive changes induced by subchronic CPF exposure in Wistar rats.

3. The effect of *M. oleifera* extract on the brain oxidative status and AChE activity of Wistar rats exposed subchronically to CPF.

4. The effect of *M. oleifera* on brain histopathological changes following subchronic exposure of Wistar rats to CPF.

5. The dose-dependency effect of *M. oleifera* in the mitigation of neurobehavioural, cognitive, oxidative and histopathological changes in Wistar rats exposed subchronically to CPF.

1.6 Research Hypothesis (H_0)

- *Moringa oleifera* extract does not have any ameliorative effect on neurobehavioural, oxidative and histopathological changes induced by subchronic CPF exposure in Wistar rats.

CHAPTER TWO

LITERATURE REVIEW

2.1 Pesticides

The term pesticide is often used to refer to chemical compounds formulated to kill or repel pests, or halt their reproduction (Gilden *et al*., 2010). Pesticides can be defined as any substance or mixture of substances, natural or synthetic, formulated to control or repel any pest that competes with humans for food, destroys property, and spreads disease (Konstantinou *et al*., 2006). They are used predominantly in agricultural production to protect crops against pests (Spiewak, 2000; Ding and Bao, 2014) and in public health to control disease vectors (de Solla *et al*., 2014). Pesticides are also used on wooden structures to prevent attack and destruction by pests, to protect farm produce from pest attack and to control pests in animal husbandry (Barrett and Jaward, 2012; El-Zaemey *et al*., 2013). Pesticides are not harmful to their target organisms alone, most of them have been found to be toxic to other organisms, including humans and animals (Raina and Hamid, 2013; Uzun and Kalender, 2013).

2.1.1 Classification of pesticides

Pesticides are classified based on different criteria. On the basis of the class of pests they target, pesticides are classified into insecticides, fungicides, herbicides and rodenticides, with insecticides being the most commonly used pesticides (Narula and Upadhyay, 2010; Mandour, 2011). Based on chemical classification, insecticides are classified into: organochlorines, organophosphates, carbamates, triazines and pyrethroids (Cortada *et al*., 2009; Afful *et al*., 2010). The organochlorines and organophosphates are most commonly used (Ogbuinya, 2013), however, the use of organochlorines have been banned in several countries because of their high toxicity and persistence in the environment (Kouzayha *et*

al., 2013). Thus organochlorines are being increasingly replaced by the more biodegradable organophosphates (Chowdhary *et al*., 2014) and pyrethroids.

2.2 Organophosphates

Organophosphates are esters of phosphoric acid and its derivatives. They are produced by the reaction of alcohols and phosphoric acid (Kwong, 2002) and are the chemical basis of many insecticides, herbicides, pesticides, and chemical warfare agents (Rusyniak and Nanagas, 2004). They are used extensively to control agricultural, household and structural pests (Bradman *et al*., 2011).

The chemical structure of organophosphates consists of a phosphate group as the basic structural framework and can largely be represented by the chemical structure in Figure 2.1 (Husain, 2013):

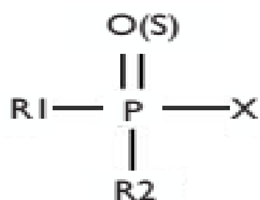


Figure 2.1. General Chemical structure of organophosphate (Gupta, 2006)

Where, $R^1$and R^2 are usually methyl or ethyl groups, the O in the OX group can be replaced with S in some compounds and the X group can take a wide diversity of forms (Prapamontol *et al*., 2013).

2.2.1 Classification of organophosphates

There are at least 13 types of OP based on their structural characteristics, these include: phosphates, phosphonates, phosphinates, and phosphorothioates (S=), phosphonothioates (S=), phosphorothioates (S substituted), phosphonothioates (S substituted),

phosphorodithioates, phosphorotrithioates and phosphoramidothioates (Gupta, 2006) (Figure 2.2)

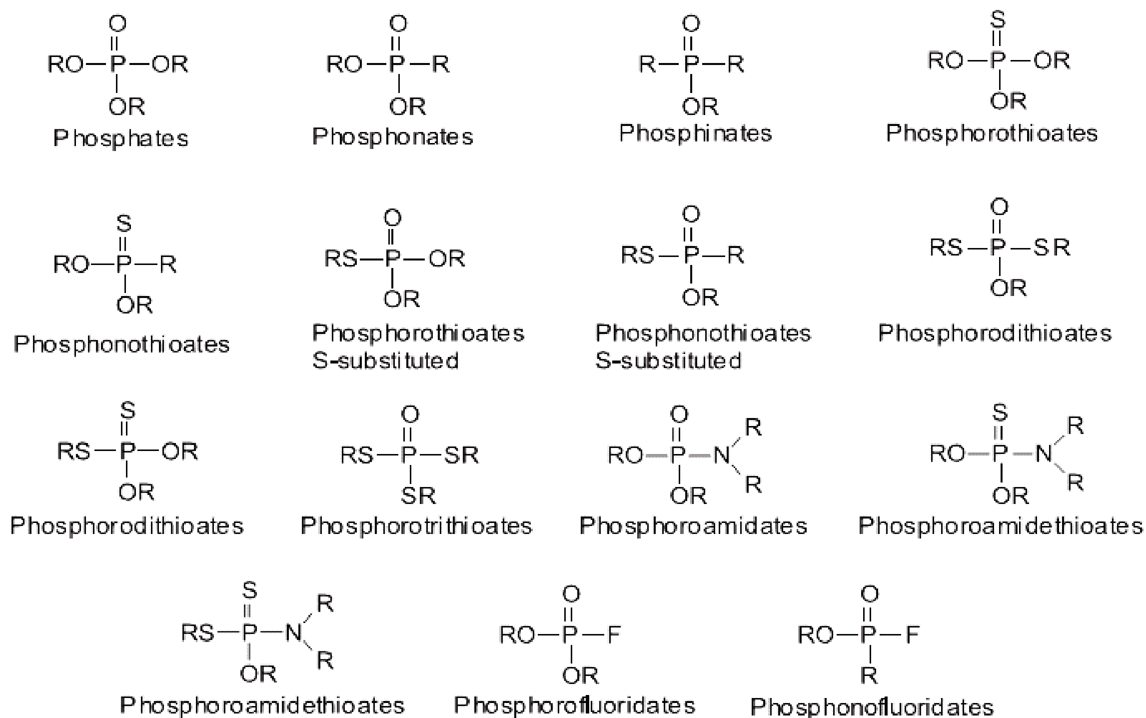


Figure 2.2: Structural classification of organophosphates (Gupta, 2006)

2.2.2 History of organophosphates

Early synthesis of organophosphates dates back to the 19th century when the compounds were first synthesized in acts of chemical curiosity (Davies *et al.*, 2000). In the 1930's, a German chemist, Gerhard Schrader, in Nazi Germany synthesized organophosphates for use as chemical warfare agents (Newmark, 2004). Schrader's work, initially aimed at formulating insecticides led to the synthesis of highly toxic ethyl-N,N-dimethylphosphor-amidocyanidate (tabun) in 1936 and isopropyl methylphosphonofluoridate (sarin) in 1937 among about 2000 OP chemicals synthesized (Szinicz, 2005). These compounds aroused the attention of the German military which anticipated their application in warfare and built plants for their industrial production in

1942. Thus, Schrader's OP research was redirected from their insecticidal properties to their potential application in warfare (Delfino *et al*., 2009).

However, the widespread use of OPs as insecticides in agriculture and public health was also hampered by the synthesis by Paul Muller (in 1939) of an inexpensive organochlorine, dichlorodiphenyltrichloroethane (DDT). This compound, which earned Paul Muller a Nobel Prize in medicine gained prominence for its efficacy as an insecticide and was soon used in the early 1940s in the control of malaria, yellow fever and many other insect-vectored diseases and as an agricultural pesticide, relegating OP pesticide popularity (Rosas and Eskenazi, 2008). In the 1960s, however, Rachael Carson's 1962 book, *Silent Spring*, alerted the public to the toxic side-effects of DDT and other organochlorines, and its propensity to accumulate in the environment and in animal tissues (bioaccumulation). This led to a public outcry and fuelled environmental movements which successfully pushed for the banning of DDT and other organochlorines from use in the USA and many other parts of the world (Heckel, 2012).

Organophosphates were found to be non-persistent in the environment; as they are hydrolyzed rapidly on exposure to sunlight, air and soil; they do not persist in animal tissues, thus the hazard of bioaccumulation posed by organochlorines is eliminated (Rastogi *et al*., 2009). The use of organophosphates, and carbamates as pesticides was therefore exploited and their use spread widely as agricultural and household pesticides (Wilson, 2013). Today, OP pesticides are among the most widely used in the world (Ding and Bao, 2014).

2.2.3 Uses of organophosphates

By their chemical structure, there are a vast number of OP chemicals applied to various uses as they remain the most widely used insecticides in various parts of the world

(Bouchard *et al*., 2010; Jafarzadeh *et al*., 2013; Ross *et al*., 2013), including Nigeria (Natala and Ochoje, 2009; Akan *et al*., 2013). OP insecticides such as malathion, parathion, methyl parathion, glyphosate, diazinon, dimethoate, chlorpyrifos, terbufos, diazinon, fenitrothion, fenthion, dichlorvos, azinphos methyl and ethion have been applied in various formulations in domestic and agriculture pest control (Van Dyk and Pletschke, 2011). Tribufos, glyphosate, glufosinate and merphos are OP chemicals used as herbicides; echothiophate and isoflurophate are OP ophthalmic agents; while trichlorfon is a common OP antihelminthic agent (Schmidt *et al*., 2010; Lopes *et al*., 2009). OP chemical warfare agents include soman, sarin, tabun and VX. Chlorpyrifos is among the most commonly used OP pesticide in the world today (Wang *et al*., 2013b).

2.2.4 Mechanism of action of organophosphates

Organophosphates act by inhibiting AChE, the enzyme responsible for hydrolysis of acetylcholine (the excitatory neurotransmitter) in insects, and vertebrates (Kumari *et al*., 2012; Ruark *et al*., 2013). Inhibition of AChE occurs after phosphorylation of hydroxyl group at serine active site of the enzyme (Figure 2.3).

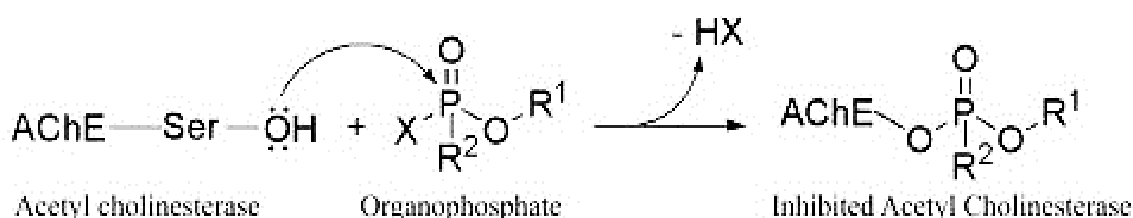


Figure 2.3: Inhibition of acetylcholinesterase by organophosphates (Guimarães *et al*., 2011).

Following inhibition, AChE can be spontaneously reactivated at the rate that depends on chemical structure of the OP. Based on the ability of AChE inhibited by certain OPs to be spontaneously reactivated, OPs are classified into reversible and non-reversible AChE

inhibitors (Jokanović and Stojiljković, 2006). AChE inhibition by OPs that possess dimethyl- and diethyl phosphate structure is usually not reversible. AChE reactivation is relatively rapid with a half-life of about 1–2 h for OPs that possess dimethyl structure while reactivation after exposure to OPs with diethyl structure can be as long as 31–57 h (Buckley *et al*., 2011; Kazemi *et al*., 2012). However, for chemical warfare agents such as soman, sarin, tabun and VX, their branched radicals ensure there is no spontaneous AChE reactivation (Delfino *et al*., 2009). For certain OPs, the phosphorylated AChE may undergo a non-enzymatic time-dependent loss of one alkyl group (R) bound to the phosphorus, (breaking of the PO–C bond of the phosphylated enzyme, with the subsequent loss of an alkylic carbenium). This is termed 'ageing' and it leads to a stable non-reactivatable form of phosphorylated AChE (Jokanović and Stojiljković, 2006; Delfino *et al*., 2009; Jokanović and Prostran, 2009).

2.2.5 Organophosphate toxicity

Organophosphate pesticides are widely and extensively used globally, and in several situations, exposure is hardly controlled especially where personal protective equipments are not used (Rohlman *et al*., 2011). Despite the widely acclaimed advantage of OPs over the earlier, more popular organochlorines, OPs have been found to induce greater acute toxicity and are therefore more hazardous than the organochlorines (Leong *et al*., 2007; Sadasivam *et al*., 2010). The mechanism of toxicity of OP is mainly through their inhibition of AChE and consequent accumulation of ACh in the synaptic junction, resulting in continuous stimulation of the post-synaptic tissue (Kwong, 2002). OP toxicity is a common consequence of its widespread use since they are not target or species specific. OP toxicity therefore is an important global problem that cuts across several species of animals and man (Cemek *et al*., 2010). Suicide attempts are a common

consequence of OP exposure in humans and cases often present with classical syndromes of acute toxicity (Maruping *et al*., 2006; Hsu *et al*., 2013; Chowdhary *et al*., 2014). Acute toxicity from OP exposure can be expressed in three different forms: cholinergic syndrome, intermediate syndrome and delayed polyneuropathy (Shetye *et al*., 2014).

2.2.5.1 Cholinergic syndrome

This is caused by AChE inhibition with subsequent accumulation of ACh at synapses and neuromuscular junctions in cholinergic pathways leading to overstimulation of postsynaptic muscarinic and nicotinic receptors. The symptoms of cholinergic syndrome can therefore be predicted as an exaggeration of the physiological actions on these receptors (Jokanović and Kosanović, 2010). Being a direct consequence of OP exposure, cholinergic syndrome occurs within minutes or hours of exposure episode (Chowdhary *et al*., 2014). Symptoms of cholinergic syndrome include miosis (constriction of pupils, unreactive to light); sweating, rhinorrhea, lacrimation, and salivation; abdominal cramps and other gastro-intestinal symptoms; cough and respiratory difficulties; dyspnoea, constriction sensation in the chest, wheezing; tremors and fasciculations; bradycardia and electrocardiograph (ECG) changes, pallor, and cyanosis; anorexia, nausea, vomiting, diarrhea, and involuntary urination and defaecation (Peter *et al*., 2013). Other symptoms include dizziness, tremulousness, and confusion; ataxia; headache, fatigability, and paraesthesia, as well as seizures, convulsions, twitching, coma, and respiratory failure. Patients that survive the first day exhibit personality changes, mood swings, aggressive events, psychotic episodes (including schizoid reactions), paranoid delusions, and exacerbations of pre-existing psychiatric problems. Sleep is often interrupted by nightmares and hallucinations, while memory and attention deficits also are common findings (Balali-Mood and Saber, 2012; Jokanović and Škrbić, 2012). Although a study

by Bird *et al.* (2003) demonstrated that early death due to OP poisoning could be a centrally mediated process, it is common knowledge that death usually occurs due to respiratory failure resulting from a combination of central (depression of the brain respiratory center) and peripheral (paralysis of the respiratory muscles) effects (Eddleston *et al*., 2008; Jokanović and Škrbić, 2012).

Diagnosis of cholinergic syndrome is often based on medical history, circumstances of exposure, clinical presentation, and confirmatory diagnosis is by laboratory tests. The laboratory test is often to detect AChE inhibition in nervous tissues, indirectly by assaying erythrocyte AChE or plasma cholinesterase activity (Roberts and Aaron, 2007). Management of cholinergic syndrome involves supportive therapy to manage clinical signs such as maintenance of ventilation, by suction of respiratory and oral secretion and placing the patient on a ventilator (George and Hedge, 2013). Also, for oral exposure gastric lavage is encouraged to evacuate yet to be absorbed organophosphate from the stomach. A dose of activated charcoal can be left in the stomach at the end of the lavage (Eddleston *et al*., 2004; Jokanović and Škrbić, 2012). Specific treatment is often by intravenous administration of atropine (a competitive inhibitor of acetylcholine) and an oxime (a cholinesterase reactivator) (King and Aaron, 2015). Pyridinium oximes are mostly used, of which pralidoxime has been found to be very effective. However, depending on the nature of OP and if “ageing” has taken place, oximes might be ineffective in cholinesterase re-activation. Anti-convulsant drugs such as benzodiazepines can be useful in controlling convulsions symptoms (Balali-Mood and Saber, 2012; Jokanović and Škrbić, 2012; Basher *et al*., 2013; Husain, 2013).

2.2.5.2 Intermediate syndrome

The term Intermediate syndrome (IMS) was first described as a distinct clinical entity by Senanayake and Karalliedde (1987). It is so termed because it arises in the time interval between the acute cholinergic syndrome of fasciculations and muscle weakness and the delayed neuropathy attributed to inhibition of the neuropathy target esterase, 24–96 h after exposure (DeBleecker, 1995; Jokanović *et al.*, 2011; Abdollahi and Karami-Mohajeri, 2012), and may last 5 – 18 days in humans, but in rare cases lasts up to 21 days (Das *et al.*, 2013).

The intermediate syndrome is characterized by acute respiratory insufficiency due to paralysis of respiratory muscles. In humans, this insufficiency often draws attention to the onset of the syndrome (Jokanović *et al.*, 2011; Das *et al.*, 2013). Other symptoms include marked weakness of neck flexion and varying degree of proximal limb muscle weakness, manifesting as weakness of shoulder abduction and hip flexion (Merbl *et al.*, 2011; Abdollahi and Karami-Mohajeri, 2012; Vučinić *et al.*, 2013). Although the mechanisms underlying the intermediate syndrome are not fully known, delayed AChE inhibition, muscle necrosis, down regulation or desensitization of postsynaptic ACh receptors, failure of postsynaptic ACh release, and oxidative stress-related myopathy have been implicated (Abdollahi and Karami-Mohajeri, 2012).

Accompanied with excretion of OP metabolites in the urine and severe depression in cholinesterase levels, IMS could be explained by the reduction in number of functioning cholinergic receptors at the postjunctional membrane, or a failure of acetylcholine release. It has been suggested that a recirculation of lipid soluble OPs from body fat compartments or gastric fluids is responsible for high amounts of OP metabolites in circulation and in the urine during this syndrome (Jokanović and Škrbić, 2012). Dimethyl OPs are more

likely to produce intermediate syndrome than diethyl OPs (Indira *et al*., 2013). Management of IMS is largely by supportive therapy such as assisted ventilation. Due to poor understanding of the pathophysiology of intermediate syndrome, no specific therapy has been prescribed (Yang and Deng, 2007).

2.2.5.3 Organophosphate induced delayed polyneuropathy

Organophosphate induced delayed polyneuropathy (OPIDP) is a rare pathology that results from a single exposure to an OP insecticide, which manifests 10 to 20 days after exposure (Jokanović and Škrbić, 2012). It is essentially a sensory-motor distal axonopathy, characterized by degeneration of long axons in the central and peripheral nervous system and consequent ataxia and paralysis that appear about 2–3 weeks after exposure, or later (Emerick *et al*., 2012). OPIDP has occurred in epidemic proportions in humans leading to outbreaks of paralysis in the USA, Morocco and other locations (Lotti and Moretto, 2005). Evidence of this has also been seen in water buffalo, sheep, cats, ferrets, chickens (Kavitha *et al*., 2013), a number of other species and laboratory rodents (Husain, 2013). OPIDP has also been observed from exposure to several OPs, including tri-o-cresyl phosphate (TOCP), parathion, trichlorfon, leptophos, mipafox, metamidophos, malation, metriphonate and chlorphos (Vasconcellos *et al*., 2002).

The mechanism of OPIDP has not been fully understood, however it is believed that neuropathic organophosphates cause a transient loss of neuropathy target esterase activity in the nervous system of susceptible vertebrates, putatively disrupting membrane phospholipid homeostasis and endoplasmic reticulum functions, including axonal transport and glial–axonal interaction: the distal parts of long axons will be particularly vulnerable to loss of these support functions (Glynn, 2006; Husain, 2013). Abou-Donia *et al*. (2003) suggested that acute necrotic neuronal cell death in the brain that results from

exposure to large toxic doses of organophosphorus compounds may be responsible for OPIDP. In a study in rats, brain tissue homogenates of rats with OPIDP showed increased in malondialdehyde levels, as well as reduced activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, indicating that the development of OPIDP is mediated in part through an increased oxidative stress (Masoud and Sandhir, 2012).

2.2.5.4 Organophosphate induced chronic neurotoxicity

Organophosphate induced chronic neurotoxicity (OPICN) refers to a neurodegenerative disorder that results from large toxic or small subclinical doses of OPs (Abou-Donia, 2003). One that results from exposure to one or more large doses of OP is often a sequelae of acute toxicity, and often called Chronic Organophosphate-Induced Neuropsychiatric Disorder (COPIND) (Jamal *et al*., 2002).

Clinical signs, which continue for weeks to years, consist of neurological and neurobehavioural abnormalities. Some of the signs and symptoms include apathy, decreased visual memory, impaired vigilance, reduced abstract reasoning, anxiety, depression, increased social isolation, reduced fine motor coordination, confusion, dizziness, insomnia, reduced vibrotactile sensitivity, decreased academic skills, emotional lability, irritability, short-term memory deficits, decreased verbal attention, fatigue, problems with concentration and slowing of reaction time (Jamal, 1997; Abou-Donia, 2003).

Damage is greater in the CNS than PNS, for this reason improvement is slow and complete recovery is unlikely. Neuronal cell death is seen in various brain areas including cerebral cortex, hippocampal formation and cerebellum. Cell death arising from early necrosis or delayed apoptosis have been fingered as a possible mechanism of this

pathology. Acute necrotic neuronal cell death often results from exposure to large toxic doses of OPs, while prolonged exposure to sub-clinical doses produce apoptotic neuronal cell death and involve oxidative stress. OPICN is exacerbated by concurrent exposure to stress or other chemicals that cause neuronal cell death or oxidative stress (Abou-Donia, 2003).

2.3 Chlorpyrifos

Chlorpyrifos (0,0-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is an effective broad-spectrum diethyl organophosphate pesticide widely used to control household, as well as agricultural insect pests (Chen *et al.*, 2013). With chemical formula $C_9H_{11}Cl_3NO_3PS$, CPF is a crystalline OP with low-water solubility and high-lipid solubility (Oliver *et al.*, 2013). The chemical structure is shown in Figure 2.4:

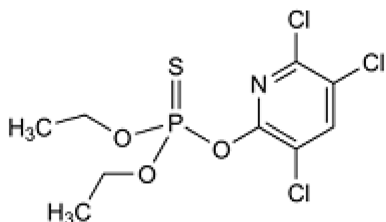


Figure 2.4: Chemical structure of chlorpyrifos (Kousba *et al.*, 2004).

First introduced, patented and marketed in 1965 by Dow Chemical Company (now Dow Elanco) Indianapolis, USA (Steenland *et al.*, 2000), CPF is used in farms, gardens and households around the world, marketed with various trade names such as Dursban®, Lorsban®, Renoban®, Termifos®, Brodan®, Detmol UA®, Dowco 179®, among others. Owing to wide reports of toxicity in humans especially children, and pets; the domestic use of CPF has been banned in the USA by USEPA since December 2001 (Banks *et al.*, 2005). CPF, however, continues to be a choice agricultural and household pesticide in many developing countries (Deb and Das, 2013).

Like other diethyl OP pesticides, CPF acts by reversibly inhibiting (by covalently binding to the active site) AChE, which is the enzyme responsible for hydrolyzing acetylcholine, an excitatory neurotransmitter in neural junctions. This results in the accumulation of acetylcholine in neural junctions, thereby causing a disruption of nerve impulses (Pope, 1999; Mullins *et al*., 2013; de Oliveira *et al*., 2014). By this mechanism, CPF exerts its toxicity on both target (insect pests) and non-target (humans and other vertebrates) organisms (Deb and Das, 2013; Shittu *et al*., 2013).

2.3.1 Pharmacodynamics of chlorpyrifos

Being highly lipophilic, CPF is readily absorbed from the respiratory and alimentary tracts, and slowly absorbed through the skin (Harishankar *et al*., 2012). Once absorbed, CPF is metabolized by cytochrome P450 enzymes to either it's oxidative desulfuration product, "superpotent" chlorpyrifos-oxon (CPF-oxon) (essentially a bioactivation process), or it's dearylation (oxidative ester cleavage) products, 3,5,6-trichloro-2-pyridinol (TCPy) and diethylthiophosphate (essentially a detoxification process) (Croom *et al*., 2010; Crane *et al*., 2012).

Chlorpyrifos-oxon is a potent inhibitor of cholinesterases, including AChE and butyrylcholinesterase (BuChE) (Smith *et al*., 2011). Chlorpyrifos-oxon is metabolized by hepatic and extrahepatic A-esterases, such as paraoxonase-1 (PON1) and tissue B-esterases (for example carboxylesterase, CaE) to form TCPy and diethylphosphate (Foxenberg *et al*., 2007). These metabolites are excreted via the kidney in urine.

2.3.2 Pharmacokinetics of chlorpyrifos

The pharmacokinetics of CPF has been shown to differ with factors such as species of animals exposed, route of exposure and the vehicle with which CPF is administered (Smith *et al*., 2009). Since CPF is mostly metabolized to its active form (CPF-oxon) or

its dearylation metabolites (TCPy and diethylthiophosphate), studies of CPF pharmacokinetics often measure blood and urinary levels of these metabolites (Poet *et al.*, 2003; Timchalk *et al.*, 2007).

The CPF is rapidly and efficiently absorbed in the gastro-intestinal tract following oral exposure. Much of this gastrointestinal absorption occurs in the intestines (Smith *et al.*, 2013) and it has been shown to exhibit a high intestinal permeability over the entire length of the small intestine (Cook and Shenoy, 2003). It is rapidly absorbed and metabolized in orally exposed rats, with peak plasma levels observed about 3 hours post dosing (Timchalk *et al.* 2002). Norlan *et al.* (1984) reported that TCP concentrations peaked at six hours after ingestion of the oral dose and at 27 hours after the 5.0 mg/kg dermal dose in human volunteers. Meuling *et al.* (2005) reported that on application of CPF to area of approximately 100 cm^2, 4.3 % of dermally applied dose of CPF was absorbed and subsequently excreted in the urine. Dermal absorption of CPF has also been reported in rats (Noaishi *et al.*, 2012), mice (Mitra *et al.*, 2008) and rabbits (Solati *et al.*, 2012).

Upon absorption, CPF is transported in the plasma and widely distributed throughout the body, also crossing the blood brain barrier (Mumtaz *et al* 2012). Being lipophilic, CPF is transported in plasma lipid compartments, thus plasma levels of CPF is directly proportional to level of plasma lipids in circulation (Lowe *et al.*, 2009). Several authors however have demonstrated the possible binding of CPF to plasma proteins, especially plasma albumin (Cui *et al.*, 2006; Jiang *et al.*, 2012; Smith *et al.*, 2012)

Almost all CPF and CPF oxon absorbed and distributed in the body of exposed individuals are excreted as TCPy and diethylthiophosphate in urine (Ellison *et al.*, 2011; Farahat *et al.*, 2011;). Griffin *et al.* (1999) reported that CPF exhibited an apparent elimination half-life (using urinary dialkylphosphates) of 15.5 hours after oral dose and

30 hours after dermal exposure in humans. Marty *et al*. (2007) reported an elimination half-life of 3 hours for neonatal rats.

2.3.3 Chlorpyrifos toxicity

Several studies in humans and animal models have shown that CPF is a potent toxicant and a hazard to human and animal populations. CPF exposure has been shown to adversely affect the liver, kidney, reproductive organs, and the nervous system (Deb and Das, 2013). Young animals have been shown to be more sensitive to the neurotoxic effects of exposure to chlorpyrifos than adult animals (Moser, 2000; Cole *et al*., 2012).

Acute exposure to high dose of CPF has been reported to produce all 3 phases of OP acute toxicity, i.e., CPF toxicity has been shown to induce cholinergic syndrome, intermediate syndrome and organophosphate induced delayed neuropathy (Eaton *et al*., 2008; Thivakaran *et al*., 2012). Signs of acute CPF toxicity, associated with cholinergic syndrome include numbness, depression, tingling sensations, incoordination, headache, dizziness, tremor, nausea, abdominal cramps, diarrhea, sweating, blurred vision, difficulty breathing or respiratory depression, and slow heartbeat. Very high doses may result in unconsciousness, incontinence, convulsions and death (Yu *et al*., 2008; Shalaby *et al*., 2013). CPF has been shown to be toxic to several body systems and organs, including the nervous and reproductive systems (Farag *et al*., 2010), liver (Ncibi, *et al*., 2008; Ambali *et al*., 2010b, d; Goel *et al*., 2005; Heikal *et al*., 2012a), kidneys (Rekha and Hamid, 2013), blood cells (Ambali *et al*., 2010a, b, d; Uzun and Kalender, 2013) and the body's immune systems (Singh *et al*., 2013a; Shahzad *et al*., 2013).

2.3.3.1 Effects of chlorpyrifos on the nervous system

Being a cholinesterase inhibitor, the nervous system is mostly affected by CPF toxicity. Several human and animal studies have shown that CPF causes behavioural and cognitive deficits.

Developmental neurotoxicity of CPF

Epidemiologic studies suggested a possible link between perinatal exposure to CPF and long-term mental delay and some behavioural alterations in humans and animals (Rauh *et al*., 2006; Braquenier *et al*., 2010). It has been shown that exposure of neonatal rats to CPF produce brain cell damage and loss, with resultant abnormalities of synaptic development (Qiao *et al*., 2002). This exposure may permanently interfere with specific key signaling proteins of the hypothalamic peptidergic system (such as oxytocin, vasopressin and prolactin), as well as central nervous system serotonergic and dopaminergic synaptic activity; and the time-, dose-, and sex-related effects remain evident at adulthood (Aldridge *et al*., 2005; Tait *et al*., 2009). Aldridge *et al*. (2005) reported that rats administered 1 mg/kg/day CPF on postnatal days 1–4 showed lasting abnormalities in behavioural tests that involve 5-HT mechanisms, resembling animal models of depression. Sherman (1996) reported teratological defects in the ventricles, *corpus callosum*, choroid plexus, and *septum pellucidum* of the brain of children exposed *in utero* to chlorpyrifos. Chanda and Pope, (1996) reported that gestational exposure of Sprague-Dawley rats to CPF-induced extensive neurochemical and neurobehavioural changes in developing rats, despite absence of overt clinical signs in the dam. Although the mechanism of CPF-induced developmental neurotoxicity is not fully understood, it has been postulated that low repeated doses of CPF target the developing brain during the critical period of cell division, causing inhibition of DNA and protein synthesis (not

secondary to generalized cell damage or suppression of cell metabolism), eventually resulting in cellular, synaptic and behavioural aberations (Whitney *et al*., 1995). In addition to this postulated mechanism, chief among other mechanisms is the induction of oxidative stress by CPF exposure, causing oxidative damage to developing neural cells (Qiao *et al*., 2005; Slotkin and Seidler, 2009). Crumpton *et al* (2000) reported that CPF, but not CPF-oxon, is responsible for much of the elicited oxidative damage in developing neural cells.

Effects of chlorpyrifos on neurobehaviour

Physiologically, there exist a dynamic relationship between the nervous system and behaviour, both influencing each other. The quality of behaviour is often dependent upon neural feedback (Sullivan *et al*., 2012). Certain behaviours therefore are very closely associated with the synaptic transmission of neurotransmitters (Zhang *et al*., 2013). The term "neurobehaviour" refers to any behaviour reflecting neuronal function. Neurobehavioural studies are important in toxicology, as neurobehavioural tests reflect sub-clinical changes of the nervous system (Ma and Xu, 2009). Examples of neurobehavioural parameters include anxiety, depression, motor coordination, motor strength, righting reflex etc. The effects of repeated low dose exposure to CPF is more prominent in young animals and man, hence the common reference to CPF as a "developmental neurotoxicant" or "neuroteratogen" (Garcia *et al*., 2002; Turgeman *et al*., 2011).

Behavioural studies of individuals exposed repeatedly over months or years to low levels of OPs reveal a relatively consistent pattern of neurobehavioural deficits (Rohlman *et al*., 2011). Steeland *et al*. (2000) found alterations in nerve conduction velocity, arm/hand tremor, vibrotactile sensitivity, vision, smell, visual/motor skills, or neurobehavioural

skills in termiticide applicators, previously exposed to CPF. The study further found alterations in some neurobehavioural parameters, including memory, emotion, fatigue and muscle strength.

Sánchez-Amate *et al*. (2001) studied the effects of two different doses (166 mg/kg and 250 mg/kg, s.c.) of CPF on anxiety using the plus-maze model and reported that both doses were anxiogenic. Exposures to CPF during perinatal period have also been shown to induce long-term alterations in mouse anxiety-like behaviours. Maternal CPF exposure in rats has been shown to cause increased anxiety in the female F1 generation (Braquenier *et al*., 2010). Conversely, Richendrfer *et al*. (2012) reported that CPF exposure in zebra fish reduces anxiety-related behaviour in zebrafish larvae as demonstrated by decreased thigmotaxis. Investigating the effects of CPF on anxiety, López-Crespo *et al*. (2009) observed that results from investigations of the effect of CPF on anxiety are quite puzzling, as both anxiolytic and anxiogenic effects have been reported in different animal models of anxiety. Inferring from results of their experiments, they concluded that CPF effects on emotional behaviour are dependent on both task contingencies and post-administration time.

Effects of chlorpyrifos on depression: Relationship between CPF exposure and incidences of depression and suicide have been reported in humans (Chen *et al*., 2014) and depression is one of the complications of repeated low level CPF exposure in man and animals (Steeland *et al*., 2000; Aldrige *et al*., 2005; Lee *et al*., 2007; Beseler and Stallones, 2008; Freire and Koifman, 2013). In animal models, Chen *et al*. (2012b) reported depression-like behaviours as manifested by increased immobility time in force swimming test in four-week-old adolescent male rats repeatedly exposed to CPF for 10 days. Reduced exposure to pesticides has been suggested as a means to reduce the risk of

depression in the farm setting (Onwuameze *et al*., 2013). The mechanism for CPF-induced depression is not fully understood, but it may not be unrelated to the effects of CPF on serotonergic and dopaminergic systems (Aldridge *et al*., 2005). It has been shown that CPF induces long-lasting changes in 5-HT receptors, presynaptic 5-HT transporter, and 5-HT-mediated signal transduction after exposure in discrete developmental windows that range from the neural tube stage through synaptogenesis (Aldridge *et al*., 2004; Aldridge *et al*., 2005; Slotkin *et al*., 2008). Moreno *et al*. (2008) reported significant depletion of monoamines, including norepinephrine, dopamine and 5-HT, and their metabolites in the striatum and *nucleus accumbens* of adult male rats 30 days after CPF intoxication.

Effects of chlorpyrifos on sensorimotor reflex: Another sequelae of repeated exposure to CPF is impairment of neuromuscular, motor coordination, and motor strength (Ambali *et al.,* 2010c). Sensorimotor deficit is also a feature of OPIDP, resulting from CPF exposure in man and animals (Masoud and Sandhir, 2012; Thivakaran *et al*., 2012). Abou-Donia *et al.* (2003, 2006) reported that *in-utero* exposure of rats to CPF induced sensorimotor deficits at adult age. Ambali and Ayo (2012) and Ambali *et al.* (2012a) reported that chronic exposure of adult rats to CPF induced deficits in locomotion efficiency, motor strength, righting reflex and excitability score. Yan *et al*. (2012) also reported that repeated exposures to low doses of CPF may reduce motor activity in the open field.

Effects of chlorpyrifos on learning and memory:The effects of CPF exposure on cognition (learning and memory) in human and animals have long been established. Prenatal CPF exposure leads to cognitive impairments in adulthood. Rauh *et al*. (2011) in a 7-year longitudinal birth cohort study reported evidence of deficits in Working Memory Index and Full-Scale intelligence quotient (IQ) as a function of prenatal CPF exposure at 7 years

of age. Prenatal exposure to CPF at 5 mg/kg/d induces selective cognitive impairments using the T-maze delayed alternation task. This may be related to morphological deficits in the dorsal hippocampus and the medial prefrontal cortex, as seen in the altered cell morphology and significantly reduced cell count in these brain sub regions (Chen *et al.*, 2012). Using the step down inhibitory avoidance task, adult rats have been shown to exhibit learning and memory deficits after repeated exposure to low doses of CPF (Ambali *et al.*, 2011c; 2012a, b, c; Ambali and Ayo, 2012; Akande *et al.*, 2014, Idris *et al.*, 2014).

Middlemore-Risher *et al* (2010), in a study using the 5-Choice Serial Reaction Time Task (5C-SRTT), an animal model of sustained attention found that repeated exposures to relatively low levels of CPF lead to protracted impairments of sustained attention and an increase in impulsive behaviours in rats. Comparing cognitive and screening tests for neurotoxicity, Bushnell *et al.* (2001) reported that CPF impaired attention and altered behaviour in the visual signal detection task (SDT) and the neurobehavioural test battery after 8 days repeated exposure of rats. In another study by Cañadas *et al.* (2005), rats injected either once or twice with CPF subcutaneously showed decreased performance in water maze, indicating spatial learning impairment and suggestive of functional central nervous system alterations. Yan *et al.* (2012) also reported that repeated exposures to low doses of CPF may lead to spatial recall impairments.

The reason for these cognitive deficits might not be unconnected to synaptic plasticity impairments that result from AChE inhibition at synapses due to CPF exposure (Ambali *et al.*, 2010c). CPF inhibits axonal growth via inhibition of the morphogenic activity of AChE (Yang *et al.*, 2008), and its disruption of basal forebrain cholinergic function may abolish cortical plasticity, thus impeding learning and memory (Conner *et al.*, 2003). CPF

has also been shown to induce alterations in mitochondrial dynamics and/or their transport in axons (Middlemore-Risher *et al*., 2011). Abnormal mitochondrial dynamics may be responsible for such symptoms as cognitive decline and memory loss seen in neurodegenerative diseases (Su *et al*., 2010), as well as cognitive impairments observed as a consequence of organophosphate exposure (Terry Jr, 2012). In addition, CPF-induced oxidative damage to cortical centres responsible for learning and memory, may be partly responsible for this impairment (Ambali *et al*., 2010c). Oxidative damage to the brain due to ageing has been shown to induce similar cognitive impairments (Fukui *et al*., 2001, 2002; Liu *et al*., 2003; Haxaire *et al*., 2012).

Effects of chlorpyrifos on brain antioxidant status

Chlorpyrifos produces oxidative stress in different human and animal cells (Uzun *et al*., 2010). Repeated exposure to low doses of CPF elicits chronic intoxication, leading to redox disturbance with irreversible brain damage and oxidative stress (Singh and Panwar, 2013). This is one of the major mechanisms by which CPF is believed to exert most of its toxic effects at low doses (Crumpton *et al*., 2000; Zama *et al*., 2007; Singh *et al*., 2013b). Excess ROS generated during oxidative stress induce a cascade of reactions that result in apoptosis (Simon *et al*., 2000; Yu *et al*., 2008; Circu and Aw, 2010; Schäfer *et al*., 2013). Several studies assaying oxidative stress biomarkers of brain tissue have shown that CPF exposure induces lipoperoxidation in brain tissues, and depletes the brain antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GP_X) (Ambali and Ayo, 2012; Ambali *et al*., 2012a, b, c; Singh *et al*., 2013b; Akande *et al*., 2014, Idris *et al*., 2014).

2.4 Formation and Action of Reactive Oxygen Species

Oxygen is essential for life. Energy to run life processes in metazoan organisms is obtained from reactions in which oxygen is used, along with a substrate (such as glucose, glycogen, etc) via the electron transport chain in the mitochondria to produce energy (Ward *et al.*, 2013). Oxidative phosphorylation is the process by which the oxido-reduction energy of mitochondrial electron transport (via a multicomponent NADH dehydrogenase enzymatic complex) is converted to the high-energy phosphate bond of ATP, and in the process, O_2 is reduced to H_2O (Thannickal and Fanburg, 2000). The utilization of O_2 as a substrate for energy production however is not completely efficient as some O_2 molecules are not completely reduced, producing partially reduced and highly reactive metabolites of O_2 [termed reactive oxygen species (ROS) or Free Radicals] as common by-products of the reaction (Jones, 2006; Semenza, 2007). The metabolites tend to be harmful as they react rapidly with several biomolecules such as lipids, nucleic acids, and proteins, changing their structure and altering their functions and resulting in cellular dysfunction, cell injury and sometimes cell death (Ryter *et al.*, 2007; Kumar *et al.*, 2012).

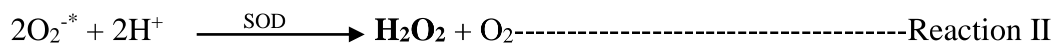
Although used mostly interchangeably, the term “free radical” differ slightly from “ROS” as not all free radicals are species of oxygen (Zhang *et al.*, 2014). Free radicals are defined as species containing one or more unpaired electrons in their atomic or molecular orbitals, which confers on them high reactivity and hence high propensity for chemical reactions (Siasos *et al.*, 2013). They can be generated from many elements, but in biological systems, ROS and reactive nitrogen species (RNS) are the most important (Burton and Jauniaux, 2011; Zhang *et al.*, 2014). ROS are therefore essentially oxygen species containing one or more unpaired electrons (Turrens, 2003). Examples of ROS include superoxide radical (O_2^{-*}); hydroxyl radical (OH^*), peroxyl radical (RO_2^*) and alkoxyl

radical (RO^*). The term is also used to refer to certain non-radicals that are either oxidizing agents and/or are easily converted into radicals, such as HOCl, ozone (O_3), peroxynitrite (ONOO−), singlet oxygen (O) and H_2O_2 (Bayr, 2005). There are several RNS, and they are mostly products of the reaction between nitric oxide (NO) and a ROS, for example superoxide react with NO to form peroxynitrite radical (Suresh and Annam, 2013; Zhang *et al.*, 2014). Examples of RNS include: nitric oxide (NO), nitrogen dioxide (NO_2), nitrite (NO_2^{-*}), nitrous acid (HNO^2) and peroxynitrite ($ONOO^{-*}$) (Suresh and Annam, 2013; Peteu *et al.*, 2014).

The superoxide radical is formed from the univalent reduction of oxygen in the electron transport chain (reaction I) (Misra and Fridovich, 1972 a, b; Liu *et al.*, 2013).

$O_2 + e^- \rightarrow \mathbf{O_2^{-*}}$--Reaction I

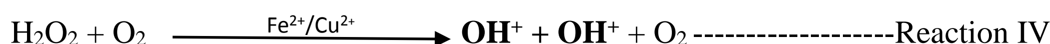
Through the process of dismutation, catalyzed by superoxide dismutase (SOD), the superoxide radical reacts with protons to form hydrogen peroxide (reaction II).



$2O_2^{-*} + 2H^+ \xrightarrow{SOD} \mathbf{H_2O_2} + O_2$------------------------------------Reaction II

Iron or copper ion may react with hydrogen peroxide to form hydroxyl radicals in what is termed the Fenton reaction (reaction III). Alternatively, transition metal ions catalyze a reaction between hydrogen peroxide and molecular oxygen to form hydroxyl radicals in a reaction termed the Haber Weiss reaction (reaction IV) (Freinbichler *et al.*, 2011).

$Fe^{2+}/Cu^{2+} + H_2O_2 \longrightarrow \mathbf{OH^+} + \mathbf{OH^+} + Fe^{2+}/Cu^{2+}$---------------Reaction III



$H_2O_2 + O_2 \xrightarrow{Fe^{2+}/Cu^{2+}} \mathbf{OH^+} + \mathbf{OH^+} + O_2$------------------Reaction IV

2.4.1 Effects of free radicals on biological systems

In a non-specific manner, ROS are extremely reactive and are not depleted in the chemical reactions they induce (Kumar *et al*., 2012). They instead form more radicals, which in turn form more radical, resulting in a radical chain reaction (Yin and Porter, 2011). Thus, one ROS molecule is capable of destroying irreversibly several biomolecules and tissues in a chain oxidation reaction (Galli *et al*., 2005; Gallego *et al*., 2013). ROS induce cellular injury by various ways, which include reactions with biomolecules such as nucleic acids, nucleotides, polysaccharides, protein, non-protein, thiols (thiol oxidation), thereby altering their structure and function. ROS also covalently bind to membrane components (proteins, lipids, enzymes, receptors, transport systems), thereby altering cellular homeostasis and initiating lipid peroxidation (Comporti, 2012).

The superoxide radical is usually the first free radical formed from the electron transport chain. It is a precursor of ROS such as singlet oxygen and hydroxyl radicals (Acharya *et al*., 2011). It is thus not only a physiological oxidant but also a pro-oxidant (Reiter and Rusnak, 2002; Cadenas and Boveris, 2011). The superoxide radical is able to oxidize biomolecules including DNA and enzymes. In fact, it is capable of directly inactivating CAT and GPx, thus exhausting the antioxidant defence system (Shah *et al*., 2013). Hydroxyl radical is extremely reactive and can oxidize almost all low-molecular weight organic compounds. It is highly unstable and is thus the most destructive ROS. It is primarily generated through the breakdown of H_2O_2 in the presence of copper or iron in the Fenton and Haber-Weiss reactions (Maezono *et al*., 2011). Peroxy radicals have been shown to mediate lipid peroxidation and its activity alters cell homeostasis as much of its deleterious effects is seen on the cell membrane (Salama *et al*., 2013). Regulation of ROS

is therefore critical for cell viability, activation, proliferation, and organ function (Birben *et al*., 2012).

Although notorious for the harm they cause on biomolecules in the cells, ROS are important physiologically. At low concentrations, they have been shown to act as intracellular second messengers involved in intracellular signaling (Finkel 2011; Kruk *et al*., 2013). They have also been shown to activate caspase 3, an important mediator of apoptosis (Ma *et al*., 2013; Wang *et al*., 2013c). Injury-induced ROS production is an important regulator of tissue regeneration as seen in Xenopus tadpole tail regeneration (Love *et al*., 2013). RNS, being products of radical chain reactions (secondary radicals), they serve several physiological roles in the living cell as signaling molecules and have been shown to be important immunological substances as they mediate inflammatory responses and possess antimicrobial effects (Fang, 2004; Koskenkorva-Frank *et al*., 2013), but, like ROS, (in nitrosative stress), they induce extensive cellular damage when in excess (Tripathi *et al*., 2013).

In-vitro studies on CPF toxicity in cell cultures, using chemiluminescence techniques have shown that exposure to CPF induces production of high amount of ROS in the cultures (Bagch *et al*., 1995; Lee *et al*., 2012; Chiapella *et al*., 2013). This has been further demonstrated *in vivo* as increased lipid peroxidation of several tissues after exposure to CPF (Ambali and Ayo, 2012; Ambali *et al*., 2012a, b, c; Singh *et al*., 2013b; Akande *et al*., 2014; Idris *et al*., 2014). Also depletion in tissue antioxidant enzymes activities has been recorded in animals exposed to CPF, indicative of CPF-induced sharp increase in ROS production in animal models and man (Singh *et al*., 2013b; Akande *et al*., 2014, Idris *et al*., 2014; Surajudeen *et al*., 2014).

2.5 Antioxidants and their Defence System

Because of the extreme threat of uncontrolled oxidation, aerobic life evolved a complex system to "quench" ROS induced oxidation chain reaction, using certain chemicals called antioxidants (Jones, 2006).

2.5.1 Antioxidants

Antioxidants are compounds which even at relatively small concentrations act as inhibitors of the process of oxidation (Naik, 2003). They are a group of substances which, when present at low concentration in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while often being oxidized themselves (Kumar, 2011). They are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu *et al*., 1998). Several types of antioxidants are known, some are synthesized endogenously while most are absorbed from food and supplements (Tewari *et al*., 2014).

2.5.1.1 Mode of action of antioxidants

Generally, antioxidants bind to and sequester ROS and transition metal ions, such as iron and copper, which contain unpaired electrons and strongly accelerate free radical formation (Cui *et al*., 2004). The mode of action of an antioxidant is via one or more of the following routes:

- Chain breaking reaction: For example, α-tocopherol act in lipid phase by donating electrons to form tocopheroxyl radical (which is relatively safe), thereby terminating the chain reaction.
- By reducing concentration of ROS, for example, glutathione (Pang and Panee, 2014).
- By scavenging initiating radicals which act in the lipid phase to trap superoxide free radicals.

- By chelating transition metal catalyst: a group of antioxidant compounds act by sequestration of transition metals that are well established proxidants. For example, transferrin, lactoferrin and ferritin function to keep iron induced oxidant stress in check while ceruloplasmin and albumin are copper sequestrants (Vaya and Aviram, 2001; Kumar, 2011).
- By repairing damages caused by ROS, antioxidants remove damaged biomolecule before they can accumulate, and their presence results in altered cell metabolism and viability (Noguchi *et al*., 2000).

2.5.1.2 Types of antioxidants

Antioxidants are classified based on two main criteria: their source and their mode of action (that is, mechanism of antioxidant defence). Based on source, antioxidants are classified into natural antioxidants and artificial antioxidants (Mishra and Bisht, 2011). Natural antioxidants occur in nature and in many cases are derived from plant sources. They are mostly phenolic compounds such as flavonoids and phenolic acid, vitamins, and volatile compounds found in different fruits, plants, herbs and spices (Ahmad *et al*., 2013). Natural antioxidants are often preferred to artificial antioxidants as they are safe and have little or no interference with the body's ability to use free radicals constructively (Wolfe *et al*., 2003). Artificial or synthetic antioxidants [such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA)] are products of chemical synthesis. They are equally effective and commonly used as preservatives in processed food, but have been found to have side effects and may be carcinogenic (Jaafar *et al*., 2013).

2.5.1.3 Classifications of antioxidant defences

Antioxidant defences refer to various mechanisms by which living cells are protected from the several deleterious effects of ROS. In principle, protection against such effects can be by prevention, interception and repair (Sies, 1993). Based on their principal mechanism, antioxidant defences are classified into:

- Primary or chain-breaking antioxidants: also called scavenger antioxidants, they neutralize free radicals by donating one of their own electrons, ending the electron "stealing" reaction, and terminating radical chain reactions (Sies, 1993; Bhalerao *et al.*, 2011).
- Secondary or preventive antioxidants: These act through various means to prevent the formation of free radicals. They act through mechanisms like sequestration of transition metal ions to prevent metal catalyzed reactions; removal of peroxides (that can react with transition metal ions to produce ROS) by catalase peroxides and glutathione peroxidase, as well as removal of ROS (Celıkyurt, 2011)
- Tertiary antioxidants defences: The repair processes, which remove damaged biomolecules before they can accumulate and before their presence, result in altered cell metabolism and viability. Examples include lipases, proteases, transferases, and DNA repair enzymes (Townsend *et al.*, 2010; Celıkyurt, 2011).

2.5.2 Antioxidant defence system

Physiologically, several cells contain certain endogenous compounds and enzymes (antioxidants) that act as scavengers of ROS, "mopping" them "up" before they cause extensive damage to biomolecules and tissues, and terminating radical chain reactions. These endogenous antioxidants make up the antioxidant defence system of the organism

and are crucial for the survival of all aerobic organisms (Birben *et al*., 2012; Sachdeva *et al*., 2012).

The antioxidant defence system is a complex mechanism involving many enzymes and substances which plays an important role in the maintenance of cellular homeostasis and in antioxidant defence, by removing ROS (Casanova *et al*., 2013; Mabrouk *et al*., 2013). The system consists two groups: the antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase; and the antioxidant molecules such as glutathione, ascorbic acid, α-tocopherol and melatonin, which are small molecular weight molecules that directly scavenge free radicals and their reactants (Rodriguez *et al*., 2004; Ognjanović *et al*., 2008; Liu *et al*., 2012; Shafaq, 2012, Reiter *et al*., 2013).

Antioxidant enzymes are the first line of defence against free radicals. They possess a transition metal at their core which takes different valences as they transfer electrons during ROS detoxification (Burton and Jauniaux, 2011). SOD catalyses the conversion of superoxide radical into H_2O_2 and O_2, (Verma, 2014). CAT on the other hand catalyzes the reduction of H_2O_2 to water and the reduction of other organic hydroperoxides (Naziroglu, 2012). Glutathione peroxidase (GPx) catalyses reduction of reduction of H_2O_2 and a variety of hydroperoxides using reduced glutathione, forming oxidised glutathione (Ferreira *et al*., 2013).

2.6 Oxidative Stress - a Mechanism of Chlorpyrifos Toxicity

In normal metazoan cells, there exist a delicate balance between the amount of free radicals and antioxidant defence molecules present in the cell for optimum functioning (Üstün *et al*., 2014). Oxidative stress is a condition in which ROS are generated extra- or intracellularly and exert toxic effects on cells (Misra *et al*., 2009). It can be defined as an imbalance between ROS production and/or impaired ROS metabolism that favours them

being present in excess of physiological levels (Chrissobolis *et al*., 2010), or an imbalance between oxidants/antioxidants because of excess oxidant and/or reduced antioxidant capacities, resulting in tissue or organ injuries (Ni *et al*., 2013; Zeng *et al*., 2013). It is simply a derangement in the balance between cellular oxidant species production and antioxidant capability, in favour of the oxidants (Elias *et al*., 2013; Liu *et al*., 2014), resulting in damage.

Oxidative stress is a consequence of either the overproduction of ROS or a decreased efficiency of the antioxidant defence system, or both (Rains and Jain, 2011; Yonar *et al*., 2012; Wei *et al*., 2013). Excess production of ROS exerts pressure on the antioxidant defence system of the organisms, thereby depleting its components. Assay of antioxidant enzymes activity have therefore been employed by many investigators as indices of oxidative stress (Ambali *et al*., 2011b, c; 2012a, b, c; Ambali and Ayo, 2012; Singh *et al*., 2013b; Akande *et al*., 2014, Idris *et al*., 2014). The term "oxidative damage" refers to cell and tissue damages that result from the excess ROS in oxidative stress. Several authors have implicated oxidative stress as one of the mechanisms by which CPF exerts its toxicity. Apart from increased ROS production seen in cell cultures exposed to CPF as demonstrated by chemiluminescence techniques (Bagch *et al*., 1995; Lee *et al*., 2012; Chiapella *et al*., 2013), cells exposed to CPF have been shown to exhibit varying degrees of oxidative damage as evidenced by increase in lipid peroxidation and increased DNA–protein crosslinks in exposed cells (Mansour and Mossa, 2010; Attia *et al*., 2012; Ma, *et al*., 2013). Several investigators (Ambali *et al*., 2011a, c; 2012 a, b, c; Ambali and Ayo, 2012; Singh *et al*., 2013b; Akande *et al*., 2014; Idris *et al*., 2014) have also linked CPF exposure to decrease in tissue antioxidant enzyme activity, indicating that oxidative stress is involved in the pathologies associated with CPF exposure.

2.7 *Moringa oleifera*

2.7.1 *Moringa oleifera*: description

Moringa oleifera (MO) (Plate I) is a tropical plant belonging to the family of Moringaceae. It is the most widely cultivated specie of the family, and is believed to be a native of the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan (Fahey *et al*., 2005). It is known by several common names including drumstick tree, moringa, horseradish tree, ben oil tree, to mention a few (Mishra *et al*., 2011). It is now cultivated across the tropical belt (Vlahov *et al*., 2002). Viable and flourishing in all ecological zones in Nigeria, all parts of the MO plant have been proven useful in nutrition, medicine, water treatment and a host of human endeavors (Okorondu *et al*., 2013; Popoola and Obembe, 2013).

Moringa oleifera grows as a shrub or small deciduous tree of 2.5–10 m in height (Vlahov *et al*., 2002). The stem is usually straight, but is occasionally poorly formed and consists of soft and white wood covered with a corky and gummy bark. The branches grow in a disorganized manner, though forming an umbrella shaped canopy. The roots have the taste of horseradish, this is probably the reason for its alternate common name "horseradish tree". The flowers are white, with yellow dots at the base, and are pleasantly fragrant. The fruits are three lobed pods which hang down from the branches and are 20-60 cm in length. When they are dry they open into 3 parts. Each pod contains between 12 and 35 seeds which are round with brownish semi-permeable seed hull. The hull has three white wings that run from top to bottom at 120-degree intervals. The leaves are usually tripinnate, 30 – 75 cm along its main axis, with leaflets 12 – 18 mm long, with yellow or white petioles (Ramachandran *et al*., 1980). The petiole are long, with 8-10 pairs of pinnae bearing two pairs of opposite, elliptic or obovate leaflets each, and one at

the apex, all 1-2 cm long; with glands at the bases of the petioles and pinnae (Foidl *et al*., 2001).

Moringa oleifera grows across Nigeria, and is known by local names that vary between ethnic groups (Popoola and Obembe, 2013). It is called Zogale, Zogale gandi and Bagaruwar makka (Hausa), Ewe igbale and Idagbo monoye (Yoruba), Ikwa oyibo (Igbo) and Kabi (Kilba) (Thilza *et al*., 2010). It is also known as Haakoobisii (Mumuye), Jeghlegede (Tiv), Gegeredi (Idoma), and Gelgedi (Igala) (Tee *et al*., 2014).

2.7.2 Nutritional properties and ethnomedical uses of *Moringa oleifera*

A host of medicinal applications have been ascribed to different parts of MO, including antihypertensive, antimicrobial, antihelminthic, antiinflammatory and antioxidant properties (Fahey *et al*., 2005; Anwar *et al*., 2007; Ferreira *et al*., 2008).

The leaves are the most widely used part of MO in Nigeria, and they are used for food and medicine (Popoola and Obembe, 2013). The leaves are highly nutritional and have been shown to contain high amounts of vitamins A, B and C, in addition to some amounts of protein, fat and fibre (Mensah *et al*., 2012). It has been reported that Moringa leaves contain more vitamin A than carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, more potassium than bananas and that its protein quality rivals that of milk and eggs (Fugie, 2000; Fahey *et al*., 2005; Nandave *et al*., 2009). The leaves have also been shown to be rich in minerals and essential amino acids (Cosme *et al*., 2014). Phyto-chemical screening results from the leaves are positive for tannin, alkaloid, glycoside, phenol and flavonoids (Mensah *et al*., 2012; Shahriar *et al*., 2012; Moyo *et al*., 2013). *Moringa oleifera* leaves have been reported to be safe for both nutritional and medicinal uses (Adedapo *et al*., 2009).

2.7.3 Antioxidant properties of *Moringa oleifera*

The presence of high amounts of vitamins A and C, phenols and flavonoid (all direct free-radical scavengers) in MO leaves hardly leaves doubt that extracts of this leaf would possess antioxidant properties (Nandave *et al*., 2009; Luqman *et al*., 2011; Vongsac *et al*., 2013). Therefore, several investigators have attempted to explore this proposed antioxidant effects to solve oxidant problems. The leaves, seeds, stem and root barks of MO have been shown to possess antioxidant activity (Anwar *et al*., 2007; Atawodi *et al*., 2010). Some authors have claimed that MO possess the highest antioxidant content among various natural food sources (Karthivashan *et al*., 2013; Pakade *et al*., 2013a) and this antioxidant property may be mediated by direct trapping of the free radicals and also through metal chelation (Verma *et al*., 2009).

Nandave *et al*. (2009) reported that leaf extract of MO prevented isoproterenol-induced myocardial damage in rats. Sinha *et al*. (2011) showed that the leaf extract of MO protected the liver against radiation-induced oxidative stress, while Eshak and Osman (2013) reported that leaf extracts of MO protected rats from biochemical and genetical alterations that result from γ-irradiation. Ouédraogo *et al*. (2013) reported that aqueous ethanolic MO leaf extract protected rabbit kidneys from gentamicin-induced oxidative stress mediated nephrotoxicity. Flavonoids such as kaempferol, quercetin, myricetin and multiflorin-B, present in MO leaf extracts have been widely reported as responsible for the antioxidant properties of MO leaves (Karthivashan *et al*., 2013; Pakade *et al*., 2013b).

Plate I: ***Moringa oleifera*** **leaves**

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant Materials

3.1.1 Plant collection, identification, extraction and preparation

Fresh leaves of *M. oleifera* were collected from Makurdi (7°44′27″ N, 8°30′43″ E), Nigeria in August 2013. They were identified at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria and a specimen was deposited there, with the voucher number 571. The identified leaves were air-dried to constant weights under shade and then pounded to powder using mortar and pestle. The powdered samples were kept in polythene bags until use.

The powdered material was extracted with methanol at a ratio of 1:5 w/v, using a cold extraction method in a glass funnel for 48 hours, with periodic shaking, to enhance the extraction process. Thereafter, the solution was filtered and the filtrate concentrated *in-vacuo,* using the rotary evaporator coupled to a thermocirculator. The residue, which was the methanol extract of *M. oleifera* leaves, was further air-dried to a constant weight and preserved in a refrigerator at 4°C until required for use. The extract yield was calculated using the formla adapted from Zhang *et al*. (2007), shown below.

$$Methanol\ yield\ (\%) = \frac{Mass\ of\ extract}{Mass\ of\ leaf\ sample} \times 100$$

3.1.2 Phytochemical screening of leaf extract

Phytochemical screening of the extract of *M. oleifera* leaves was carried out to determine the chemical constituents, using standard methods. Alkaloids were detected using Mayer’s test; carbohydrates, using Molisch’s test; glycosides, using modified Borntrager’s test; saponins, using Foam test; phenols, using ferric chloride test; tannins,

using gelatin test; diterpines, using copper acetate test; and flavonoids, using alkaline reagent test (Kaur *et al*., 2011).

3.1.3 Quantitative analysis of methanol extract of *Moringa oleifera* leaf for flavonoids and antioxidant vitamins

Quantitative analysis of the flavonoid contents of the extract of *M. oleifera* leaves was performed using high-performance liquid chromatography method with in-line connected diode-array spectrophotometer as described by Mattila *et al* (2000). The contents of vitamins A, C and E of the extract were assayed using ultraviolet–visible spectroscopy (Fadhel, 2012; Jadoon *et al*., 2013).

3.2 Chemical Acquisition and Preparation

Commercial grade CPF 20% emulsifiable concentrate (Termifos®, Alderelm Limited, United Kingdom) was obtained from a reputable chemical store. Soya oil was used as vehicle to reconstitute the CPF into a 1% (w/v) working solution.

3.3 LD_{50} Determination

The median lethal dose (LD_{50}) of reconstituted CPF was determined using a standard two-phase approach as described by Lorke (1983). The first phase involved nine animals divided into three groups – I, II and III, consisting of three rats each. Animals in groups I, II and III were administered CPF at 10 mg/kg, 100 mg/kg and 1000 mg/kg body weight, respectively. They were observed for death over a period of 48 hours. In the second phase, which depended on the results obtained from phase I, three rats were divided into three groups (groups IV, V and VI) of one animal each. Animals in groups IV, V and VI were administered with 80 mg/kg, 90 mg/kg and 100 mg/kg, respectively. They were observed for death over a period of 48 hours. The LD_{50} was determined based on dose-response as described by Lorke (1983).

The median lethal dose of the methanol extract of *M. oleifera* was determined using the same procedure. The first phase involved nine animals divided into three groups – I, II and III, consisting of three rats each. Animals in groups I, II and III were administered MO at 10 mg/kg, 100 mg/kg and 1000 mg/kg body weight, respectively. They were observed for death over a period of 48 hours. In the second phase, which depended on the results obtained from phase I, three rats were divided into three groups (groups IV, V and VI) of one animal each. Animals in groups IV, V and VI were administered with 1600 mg/kg, 2900 mg/kg and 5000 mg/kg, respectively. They were observed for death over a period of 48 hours. The LD_{50} was determined based on dose-response as described by Lorke (1983).

3.4 Experimental Animals

A total of sixty (60) adult male Wistar rats (weighing 110 – 140 g) were used for the experiment. The rats were obtained from the animal house of Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, Nigeria. They were maintained on commercial grower's feed, maize offals (*Dusa*) and groundnut cake (*Kuli kuli*), compounded at a ratio 4:2:1. Rats were given access to feed and water *ad libitum*. The animals were kept in metal cages at room temperature throughout the study.

3.5 Experimental Groupings/Treatments

The rats were randomly divided into six groups of 10 rats each. Group I was administered 2 ml/kg of distilled water only, while group II was administered soya oil (2 ml/kg). Group III was administered with methanolic extract of *M. oleifera* at 500 mg/kg (~1/5th of LD_{50}), while Group IV was administered with CPF at 9.8 mg/kg [~1/10th of the LD_{50} (Idris *et al*., 2014)]. Group V was administered with *M. oleifera* extract at 250 mg/kg (Ganguly and Guha, 2008), followed by CPF, 30 min later (Ambali *et al*., 2012c), while Group VI

was administered with *M. oleifera* extract at 500 mg/kg, followed by CPF, 30 min later. These regimens were administered once daily for a period of 9 weeks. During this period the neurobehavioural and cognitive parameters were monitored. At the end of 9 weeks, the rats were sacrificed, using jugular venesection, after light chloroform anesthesia. Blood, serum and tissue samples were collected for evaluations.

3.6 Evaluation of neurobehavioural and cognitive changes

3.6.1 Open-field assessment

This was carried out on all experimental rats using the open-field apparatus as described by Zhu *et al*. (2001). The open-field apparatus was constructed using a cardboard box with a dimension of 50 × 50 × 46 cm with a clear plexiglass on the inner surface (base). The floor of the box was divided into 25 equal squares. The parameters analysed were locomotor activity and anxiety, evaluated using horizontal locomotion (number of crossings of the lines marked on the floor); frequency of rearing, grooming, defaecation and urination as indices. In general, the procedure for assessing motor (locomotor) activity involved placing an animal in the box and allowing it for a period of 3 minutes to walk freely and become familiarized with the environment. Thereafter, the number of squares crossed with all the paws were recorded for the next 2 minutes. Rearing was measured by determining the frequency at which each rat stood on its hind-limbs with the fore-paws lifted up freely or leaned against the wall of the apparatus during the 2 minutes, after the initial 3 minutes of habitation. The frequencies of grooming (protracted washing of the coat), defaecation (number of faecal mass defaecated) and urination (the number of puddles of urine voided) were observed and recorded. The open-field assessment was carried out on day 0 and weeks 3, 6 and 9.

3.6.2 Assessment of neuromuscular coordination

The effect of the regimens on neuromuscular coordination was assessed using the incline plane method, as described by Ambali *et al*. (2010c). Briefly, each rat was placed on an apparatus made with an angled rough-wooden plank, with a thick foam pad at its base. Angles were measured and marked on the apparatus before hand, and were obtained by propping the plank on a vertical bar with several notches. The plain was initially inclined at an angle of 35º, and, thereafter, increased stepwise by 5º until the subject could no longer hold on horizontally for 30 seconds without sliding down. The test was performed with each subject facing left and then right across the plank. The highest angle at which the rat could no longer stay horizontally and facing any of the directions was recorded. Two trials were performed for each testing period. The experiment was performed on day 0 and weeks 3, 6 and 9.

3.6.3 Assessment of motor coordination

The motor coordination was assessed using the beam-walk performance task as described by Ambali *et al*. (2010c). Briefly, the beam-walk performance was evaluated by allowing individual rat from each group to walk across a wooden black beam of 106-cm length, beginning at 17.2-cm width and ending at 1.0-cm width. Periodic widths were marked on the side of the apparatus. On each side of the narrowing beam, there was a 1.8-cm step-down to a 3.0-cm area, where subjects may step, if necessary. As the subject walked across, the width of the beam at which it stepped down was recorded by one rater on each side, and this was repeated twice during each trial session. The task was performed on day 0 and weeks 3, 6 and 9.

3.6.4 Assessment of locomotor efficiency

The assessment was done using the ladder-walk as described by Ambali and Aliyu (2012). Rats were encouraged to walk across a black wooden ladder (106 × 17 cm, with 0.8-cm diameter rungs with 2.5-cm spaces between them, totaling 31 rungs). The number of times each rat dropped its forelimb down through the rungs of the ladder, when the subject missed a rung was counted by one rater on each side. This was performed on day 0 and weeks 3, 6 and 9.

3.6.5 Assessment of motor strength

The fore-paw grip time was used to evaluate the motor strength of the rats as described by Abou-Donia *et al*. (2001). The procedure involved having the rats hung from a 5 mm-thick diameter wood dowel, gripped with both fore-paws. The time spent by each rat before releasing its grip was recorded in seconds. The assessment was conducted on day 0 and weeks 3, 6 and 9.

3.6.6 Assessment of excitability score

The excitability score of the rats was evaluated using the method described by Ambali and Ayo (2012). Briefly, each rat was held by the tail upside down and kept in that position for 30 seconds. Response of each rat was rated using ordinal scale of 0-5 as follows:

Grade 0: Rat did not show any sign of wriggling at all.

Grade 1: Rat wriggling was low with feeble fore-paw movement.

Grade 2: Rat responded through a stronger wriggling and forepaw movement.

Grade 3: Rat vigorously wriggled and showed a strong fore and hind-limbs movement.

Grade 4: In addition to the observations in grade 3 above, rat made unsuccessful attempt to climb on its tail.

Grade 5: In addition to the observations in grade 3 above, rat successfully climbed the tip of its tail.

The excitability score was assessed on day 0 and weeks 3, 6 and 9.

3.6.7 Assessment of depression

The depression status of the rats was evaluated using the forced swimming test as described by Porsolt *et al*. (1977). Each rat was dropped in a transparent glass (height 25 cm; diameter 24 cm) containing 10 cm height of water at 25°C and left there for 5 minutes. The rats were forced to swim and soon became immobile, floating in an upright position, making only small movements. The amount of time it took for the rat to get immobile was recorded and used as the index of the depression status of the rats. The assessment was conducted on day 0 and weeks 3, 6 and 9.

3.6.8 Evaluation of learning and short-term memory

The effect of the different regimens on learning was assessed using the step-down inhibitory avoidance task as described by Zhu *et al*. (2001). This was evaluated 48 hours to the termination of the experiment. It involved the use of an acrylic chamber 40 × 25 × 25 cm, consisting of a floor made of parallel 2 mm-calibre stainless-steel bars, spaced 1 cm apart. An electric current (80 volts) was delivered through the floor bars and a 2.5-cm-high, 8 × 25 cm wooden platform was placed on the left extreme of the chamber. Each animal was gently placed on the platform. The ability of each rat to remain on the platform, having received an initial foot shock when it stepped down from the platform the first time, was the basis of the assessment. The number of times the rat stepped down with all its limbs after receiving an electric shock was used as an index of learning. The maximum learning ability was observed, when the rat stayed on the platform for 2 minutes without stepping down.

Short-term memory was assessed 24 hours later using the same procedure. In this case, each rat was again placed gently on the platform and the period of time an animal remained on the platform without stepping down was recorded as an index of memory retention. The ceiling memory was observed when the animal remained on the platform for 2 minutes.

3.7 Preparation of Brain Samples

Freshly obtained brain sample from each rat was sonicated, suspended in phosphate-buffered saline and then vortexed for 5 – 10 seconds. The mixture was centrifuged at 1000 × g for 15 minutes and the supernatant was decanted into sterile test tubes and stored on ice for the evaluation.

3.8 Assessment of Acetylcholinesterase Activity

Brain samples collected were analysed for AChE activity using the assay method described by Ellman (1961). The principle for the AChE assay was based on spectrophotometric measurement of the yellow colour, developed following the reaction of thiocholine [breakdown product of acetylthiocholine iodide (ATChI)] and disthiobisnitrobenzoate (DTNB)

Brain homogenate was incubated with 0.01 M DTNB in 0.1 M PBS, pH 7.0. Incubations were allowed to proceed at room temperature for 10 minutes. Then, ATChI (0.075 M in 0.1 M PBS, pH 8.0) was added, and absorbance at 412 nm was measured continuously for 30 minutes using a UV spectrophotometer (T80+ UV/VIS spectrometer®, PG

Instruments Ltd., Liicestershire, UK). AChE activity expressed as IU/mg tissue protein was calculated based on the rate of color change per minute.

3.9 Assessment of Brain Lipoperoxidation

The level of lipoperoxidative changes in the brain was assessed by evaluating the concentration of thiobarbituric acid-reactive substances (TBARS), malondialdehyde (MDA), using the method described by Draper and Hadley (1990), as modified by Freitas *et al*. (2005). The principle was based on measurement of the colour developed as a result of the reaction of thiobarbituric acid (TBA) with MDA. Briefly, brain samples from rats in all the groups were weighed and then homogenized in a known volume of ice-cold phosphate buffer to obtain a 10% homogenate, which was then centrifuged at 6,000 *g* for 10 minutes to obtain a supernatant. The brain homogenate (0.5 ml) of each animal was mixed with 1 ml 10% TCA and 1 ml 0.67% thiobarbituric acid. The mixture was then heated in a boiling water bath for 15 min, and butan-2-ol (2:1, v/v) was added to the solution. After centrifugation (800 *g*, 5 min), TBARS, MDA was determined from the absorbance at 532 nm using Schimadzu UV spectrophotometer (Model UV160 Kyoto, Japan).

The concentration of MDA in the brain samples was then calculated by the absorbance coefficient of MDA-TBA complex 1.56×10^5/cm/M and expressed in nmol/mg of protein for the brain. The concentration of protein in the brain homogenate was evaluated using the method described by Lowry *et al*. (1951).

3.10 Assessment of Brain Antioxidant Enzyme Activities

The supernatant was immediately used to assay for activities of SOD, CAT and GP_X peroxidase. The activity of catalase was measured using the Northwest Life Science Specialties catalase activity assay kit (NWK-CAT01), based on the method of monitoring

the consumption of H_2O_2 substrate at 240 nm, originally described by Beers and Sizer (1952), but modified to increase robustness and convenience. The CAT enzyme activity was measured by monitoring the consumption of H_2O_2 substrate at 240 nm.

The activity of SOD was measured using the Northwest Life Science Specialties SOD kit (NWLSS™ NWK-SOD02) based on the method of monitoring the auto-oxidation rate of haematoxylin originally described by Martin *et al*. (1987) with modifications to enhance reliability. Briefly, in the presence of SOD enzyme at specific assay pH of 7.4, the rate of auto-oxidation was inhibited and the percentage of inhibition was linearly proportional to the amount of SOD present within a specific range. Sample SOD activity was determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample and expressed as McCord-Fridovich "cytochrome c" units.

Glutathione peroxidase (GPx) activity was evaluated using the Northwest Life Science Specialties (NWLSS™) glutathione peroxidase assay kits protocol NWK-GPX01, adapted from the method described by Paglia and Valentine (1967). Briefly, GPx catalyzed the reduction of hydrogen peroxide and oxidized the reduced glutathione to form oxidized glutathione (GSSG). GSSG was then reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming $NADP^+$. This resulted in decreased absorbance at 340 nm and the recycling of GSH. The decrease in absorbance at 340 nm was directly proportional to the GPx concentration.

3.11 Histopathology

The brain was collected from each rat and fixed in Bouin solution for brain section preparation according to the modified method of Luna (1960). Briefly, after two weeks in Bouin solution, the tissues were dehydrated in alcohol, cleared in xylene, infiltrated with molten paraffin wax at 50ºC and blocked in paraffin according to standard

procedures and labeled. Sections were cut at nominal thickness of 5 - 6 μm using Jung Rotary Microtone (Model 42339, Berlin, Germany). The sections were mounted on glass, deparafinized, stained with haematoxylin and eosin stain using cytoseal 60 as mounting medium. Slides prepared were viewed under light microscope at × 400 magnification.

3.12 Data Analysis

Data obtained were expressed as mean ± standard error of the mean (mean ± SEM). The values obtained for the parameters, measured repeatedly were analyzed using repeated measures analysis of variance (ANOVA), followed by Tukey's *post-hoc* test. Data obtained from the parameters measured once during the period of the study were analyzed using one-way ANOVA, followed by Tukey's *post-hoc* test. The non-parametric data of excitability scores were analysed using Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's test. Graphpad Prism version 5.0 for windows (Graphpad Software, San Diego, California, USA) was used to analyse all the data. Values of $P < 0.05$ were considered significant.

CHAPTER FOUR

RESULTS

4.0 RESULTS

4.1 Determination of Median Lethal Dose

4.1.1 Determination of median lethal dose for chlorpyrifos

All three rats in each of the groups administered CPF at 100 mg/kg and 1000 mg/kg during phase I of the LD_{50} determination died within 24 hours of administration. However, all three rats in the group administered CPF at 10 mg/kg survived. Each of the rats administered 80 mg/kg and 90 mg/kg of CPF in phase II of the LD_{50} determination survived, only the rat administered 100 mg/kg died (Table 4.1).

Based on the modified method of Lorke (1983), the $LD_{50} = \frac{90+100}{2}$ = 95 mg/kg

The clinical signs of toxicity observed prior to death included depression, huddling, piloerection, tremor, intermittent convulsion, respiratory depression and ataxia. Post-mortem gross findings, included haemorrhagic gastro-enteritis, filling of the stomach with gas bubbles, congestion and petechial haemorrhages of the liver, congestion of the lungs, heart, kidneys, testes and brain blood vessels.

Table 4.1: Summary of results from phase II median lethal dose (LD_{50}) determination for chloryrifos

Group	Dose (mg/kg)	No. dead/No. dosed
1	100	1/1
2	90	0/1
3	80	0/1

4.1.2 Determination of median lethal dose for *Moringa oleifera* leaf extract

All three rats in each of the groups administered with MO at 10 mg/kg, 100 mg/kg and 1000 mg/kg, respectively during phase I of the LD_{50} determination survived 24 hours after administration. In the same vein, all three rats administered 1600 mg/kg, 2900 mg/kg and 5000 mg/kg survived 24 hours after administration without visible clinical signs of toxicity apart from initial irritation and discomfort expressed immediately after administration of the high doses of the extract. The rat administered with 5000 mg/kg was sacrificed 48 hours after administration, and no gross lessions were noticed post-mortem. Therefore, using Lorke's method (1983), the LD_{50} of MO leaf extract was determined to be > 5000mg/kg, and the extract was considered relatively safe.

4.2 *Moringa oleifera* Leaf: Methanol Extract Characteristics

4.2.1 Methanol extract yield of *moringa oleifera* leaf

Using 300 g of grounded MO leaves initially, 1500 ml of methanol was used to extract 65 g of extract. The ethanol extract yield was calculated, thus:

$$Methanol\ yield\ (\%) = \frac{Mass\ of\ extract}{Mass\ of\ sample} \times 100$$

$$= \frac{55}{300} \times 100$$

$$= 18.33\%$$

4.2.2 Phytochemical components of methanol extracts of *Moringa oleifera* leaf

Phytochemical screening of the extract was positive for alkaloids, flavonoid, glycosides, phenols, saponin, tannin and terpenoids. The results are summarized in Table 4.2.

Table 4.2: Summary of results of the phytochemical screening of the methanol extract of *Moringa oleifera* leaf

Constituents	Status
Alkaloids	+
Flavonoids	+
Glycosides	+
Phenols	+
Saponins	+
Tannins	+
Terpenoids	+

(+) means present

4.2.3 Flavonoid and antioxidant vitamin contents of *Moringa oleifera* leaf extract

The results of the quantitative analysis of the flavonoid content of the extract showed that the extract contained 22.6% w/w of flavonoids. The results of UV visible analysis of the extract for antioxidant vitamins also showed that the extract contained vitamin A (0.3 mg/g), vitamin C (6.7 mg/g) and vitamin E (0.22 IU/g). A summary of the results is shown in Table 4.3.

Table 4.3: Summary of the concentration of antioxidant components of *Moringa oleifera* methanol leaf extract

Antioxidant Component	Concentration
Flavonoid	22.6% w/w
Vitamin A	0.3 mg/g
Vitamin C	6.7 mg/g
Vitamin E	0.22 IU/g

4.3 Subchronic Toxicity Study

4.3.1 Clinical signs

Four rats in the CPF group died in the course of the sub-chronic exposure phase of the experiment after displaying varying clinical signs prior to their death. These include weakness, fasciculations, priapism and blood-tinged ocular and nasal discharges. Animals in the CPF group showed general signs of weakness, lacrimation, huddling, relative reluctance to move and congested ocular mucous membranes. The groups pretreated with MO (250 mg/kg and 500 mg/kg) before exposure to CPF, did not show any apparent sign of toxicity. Similarly, the DW, SO and MO500 groups showed no signs of toxicity.

4.4 Effect of Treatments on Neurobehaviour

4.4.1 Effect of treatments on open-field performance

4.4.1.1 Effect of treatments on locomotion in the open-field performance test

The effect of treatments on locomotion in the open field apparatus using number of squares crossed as an index of locomotion is shown in Figure 4.1. The number of squares crossed by the rats decreased for all groups after day 0. The DW, SO and MO500 groups crossed fewer squares in weeks 3, 6 and 9, but did not differ significantly ($P > 0.05$) from each other. The CPF group crossed significantly ($P < 0.05$) fewer squares in weeks 3, 6 and 9, when compared to Day 0, while the MO250+CPF group crossed significantly ($P < 0.05$) fewer squares in week 3 and 6. When compared to day 0, the number of squares crossed by MO500+CPF group did not differ significantly ($P > 0.05$) through the course of this study.

The number of squares crossed by the CPF group was significantly ($P < 0.05$) lower when compared to the SO group and the MO500 groups, respectively in weeks 3, 6 and 9. The number of squares crossed by MO500 group differed significantly ($P < 0.05$) from the

MO250+CPF group in week 6. The MO500+CPF groups crossed 67.7% and 43.3% more squares in weeks 3 and 6, respectively, compared to the MO250+CPF group, but crossed 11.3% fewer squares in week 9.

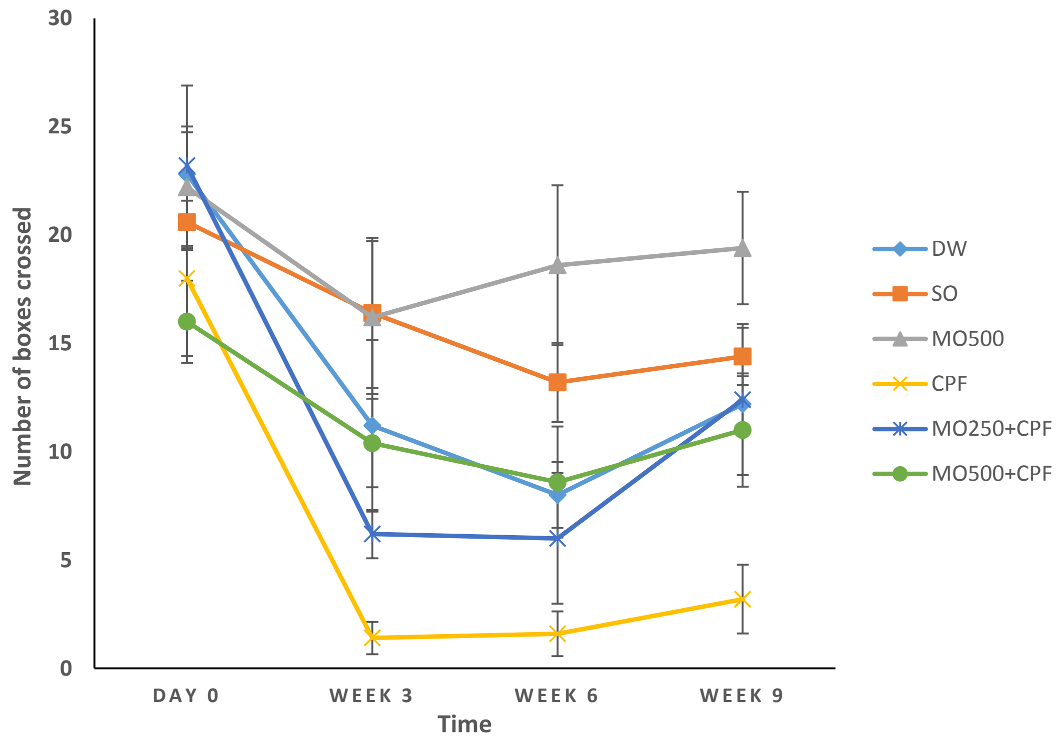


Figure 4.1: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the dynamics of locomotion of Wistar rats in the open field apparatus

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.1.2 Effect of treatments on the frequency of stretch-attends posture in the open-field performance test

The frequency of stretch-attends posture was lowest in week 3 in all the groups. In the DW, SO and MO500 groups, the frequency of the stretch-attends posture decreased significantly ($P < 0.05$) in weeks 3, 6 and 9, when compared to day 0. The CPF group, however, did not show any significant difference ($P > 0.05$) in the frequency of stretch-attends posture throughout the study period. It decreased steadily by 43.14%, 54.9% and 58.8% in weeks 3, 6 and 9, respectively, when compared to day 0. The MO250+CPF and MO500+CPF groups, showed a significant ($P < 0.05$) decrease in the frequency of stretch-attends posture in weeks 3, 6 and 9, when compared to day 0.

The frequency of stretch-attends did not differ significantly ($P > 0.05$) between groups in week 3. The CPF group had the least posture in week 3 and subsequently. In week 3, the CPF group had frequencies 69.2%, 76.1% and 67.1% lower than the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups showed stretch-attends posture frequency 17.9% and 49.6% higher, respectively, when compared to the CPF group.

In week 6, the CPF group had stretch-attends posture frequencies 48.3%, 55.0% and 70%, higher than the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups had frequencies that were 158% and 116.7% higher, respectively than the CPF group. The CPF group differed significantly ($P < 0.05$) in their frequency of stretch-attends posture from the DW group in week 6 (Figure 4.2).

In week 9, the frequency of stretch-attends posture for the CPF group was 16.7%, 19.6% and 33.9% lower than the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups had frequency of stretch-attends posture which were 70% and

45.2% higher than the CPF group, respectively. Groups, however did not differ significantly ($P > 0.05$) in this week. The MO500+CPF group showed less frequency of stretch-attends posture (10.6%, 24.7% and 5.9%) when compared to the MO250+CPF group in weeks 3, 6 and 9, respectively.

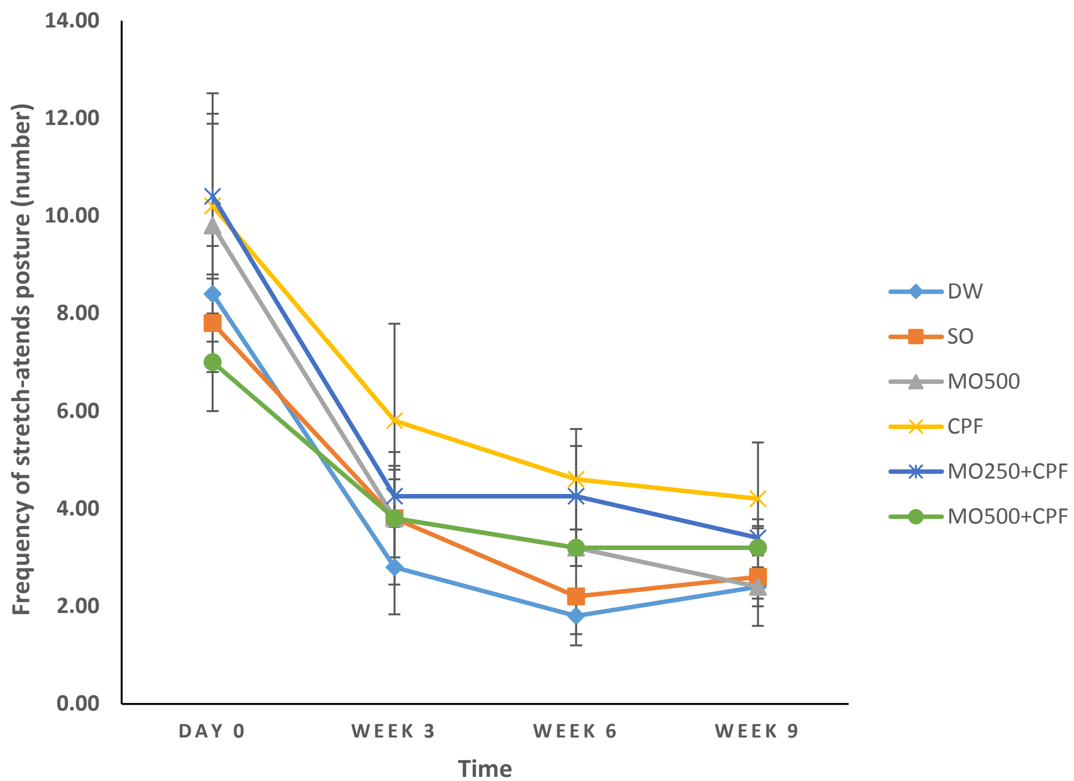


Figure 4.2: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the dynamics of the frequency of stretch-attends posture of Wistar rats in the open field apparatus

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.1.3 Effect of treatments on the frequency of rearing in the open field performance test

Frequency of rearing decreased with time in all the groups throughout the study period, but this decrease was not significant ($P > 0.05$) in the DW, SO, and MO500 groups. The frequency of rearing in the CPF group decreased significantly ($P < 0.05$) in weeks 3, 6 and 9 when compared to day 0. The MO250+CPF group also showed rearing frequency that decreased significantly ($P < 0.05$) in weeks 3, 6 and 9, when compared to day 0, but, the decrease in the MO500+CPF group was only significant ($P < 0.05$) in week 9, when compared to day 0.

Among the groups, the CPF group had the least frequency of rearing in week 3, 6 and 9, respectively (Figure 4.3). Frequency of rearing did not differ significantly ($P > 0.05$) between groups in week 3. The CPF group showed 26.7%, 52.2% and 54.2% lowered frequency of rearing, when compared to the DW, SO amd MO500 groups, respectively. The MO250+CPF and MO500+CPF groups had rearing frequencies that were 18% and significantly ($P < 0.05$) higher, respectively when compared to the CPF group.

In week 6, the CPF group showed lower frequency of rearing (33.3%, 66.7 and 72.7%) when compared the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups showed rearing frequencies 45.8% and 66.7% higher, respectively when compared to the CPF group.

The CPF group in week 9 had lower frequency of rearing (25%, 57.1% and 70%) as compared to the DW, SO and MO500 groups respectively. The MO250+CPF and MO500+CPF groups showed frequency of rearing of 33.3% and 0% higher, respectively, when compared to the CPF group.

The MO500+CPF group had higher frequency of rearing (5.3% and 14.3%) when compared to the MO250+CPF group in weeks 3 and 6, respectively, but had a lower frequency in week 9 (25%). The MO500 group had significantly ($P < 0.05$) higher frequency of rearing when compared to the CPF group in weeks 6 and 9. The MO500 group showed significantly ($P < 0.05$) higher frequency of rearing in week 9 when compared to the MO500+CPF group.

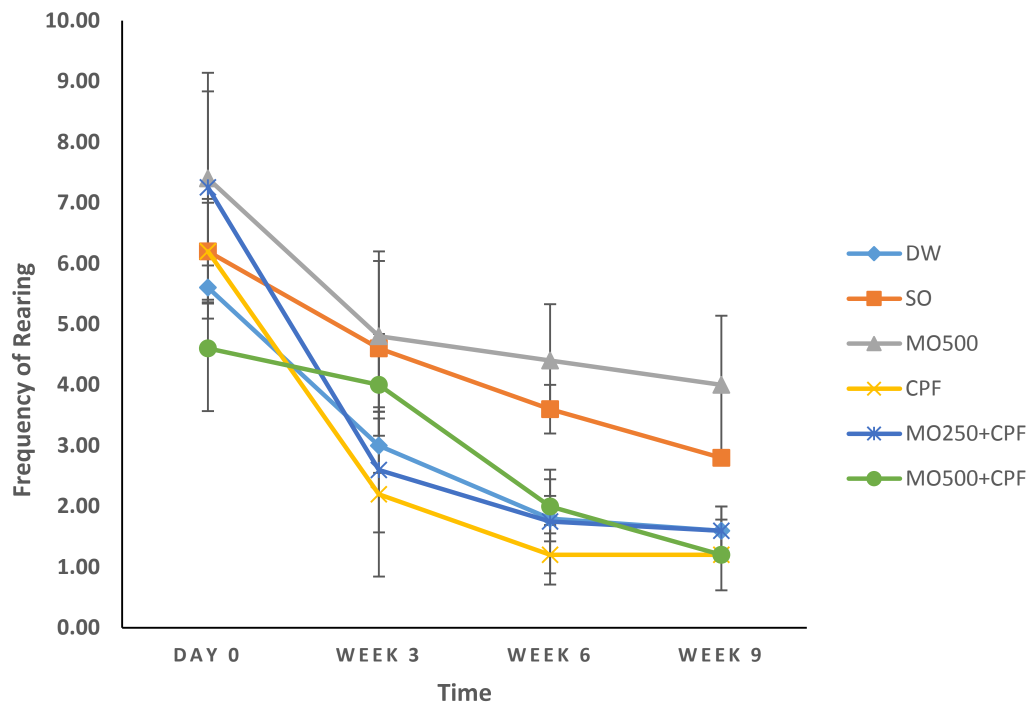


Figure 4.3: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the dynamics of rearing of Wistar rats in the open field apparatus

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF -chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.1.4 Effect of treatments on frequency of grooming in the open field performance test

The frequency of grooming seemed to undulate through the study period. The DW group showed grooming frequency values that were significantly different ($P < 0.05$) in weeks 3 and 9, when compared to that of day 0. The SO and MO250+CPF groups showed grooming frequencies that respectively differed significantly ($P < 0.05$) in weeks 3, 6 and 9, when compared to that of day 0. The MO500 and CPF groups had grooming frequencies that did not differ significantly ($P > 0.05$) throughout the study period. The MO500+CPF group showed grooming frequency values that differed significantly ($P < 0.05$) only in week 3 when compared to day 0.

Between groups, grooming frequency did not differ significantly ($P > 0.05$) through the study period. However, the CPF group showed frequency that was 14.29%, 40.0% and 36.36% higher when compared to the SO group in weeks 3, 6 and 9, respectively. The MO250+CPF group had 14.29%, 20.0% and 9.09% higher grooming frequency when compared to the SO group in weeks 3, 6 and 9, respectively; while the MO500+CPF group had 0.0%, 30.0% and 27.27% higher frequency compared the SO group in weeks 3, 6 and 9, respectively (Figure 4.4).

The MO250+CPF group had a lower frequency of grooming in weeks 3, 6 and 9 (0%, 14.3% and 20%, respectively) when compared to the CPF group. The MO500+CPF group also had a lower frequency of grooming (12.5%, 7.1% and 6.7%, respectively), when compared to the CPF group. The MO500+CPF group had grooming frequencies that were lower in week 3 (12.5%), but were higher in weeks 6 and 9 (8.3% and 16.7%, respectively) when compared to the MO250+CPF group.

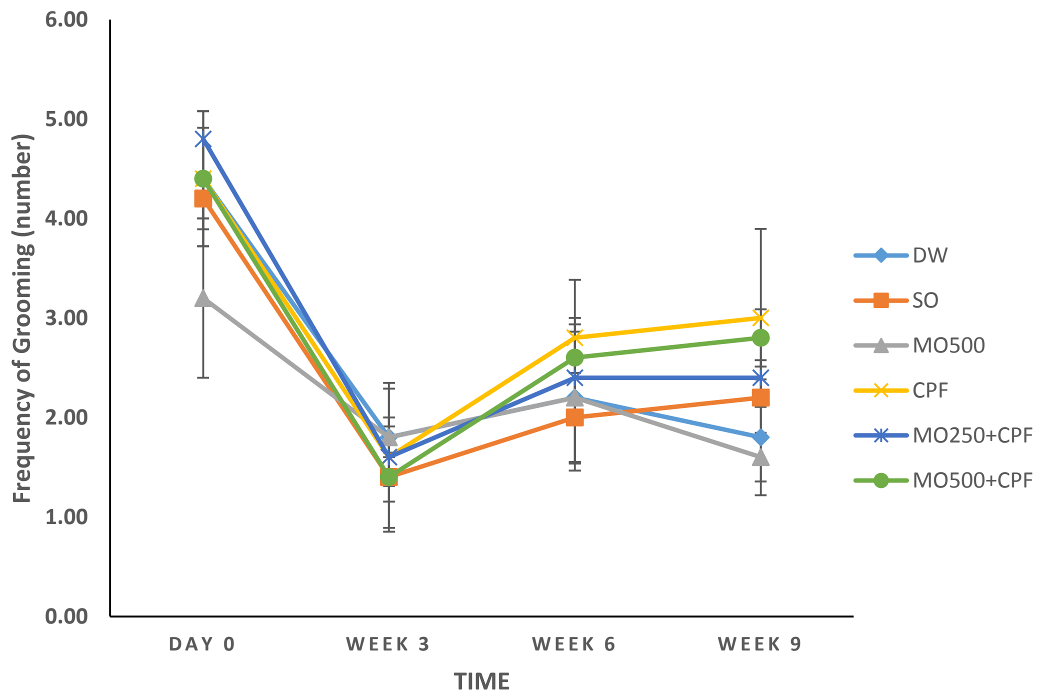


Figure 4.4: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the dynamics of grooming of Wistar rats in the open field apparatus

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.1.5 Effect of treatments on frequency of defaecation in the open field performance test

The DW and SO groups showed frequency of defaecation that did not respectively differ significantly ($P > 0.05$) in between the weeks of evaluation throughout the study period. The MO500 group however had significantly lower ($P < 0.05$) defaecation frequency in week 9 when compared to day 0. The frequency of defaecation did not differ significantly ($P > 0.05$) in the CPF group throughout the study period, but decreased by 26.6% and 20.0% in weeks 3 and 6, respectively, and increased by 40% in week 9 when compared to day 0. The frequency of defaecation in the MO250+CPF group also did not differ significantly ($P > 0.05$) during the study period, but declined to 45%, 36.4% and 45% less in weeks 3, 6 and 9, respectively, when compared to day 0. The MO500+CPF group showed a steady decline in the frequency of defaecation becoming 18.2% and 54.5% lower in weeks 3 and 6, respectively, and significantly lower ($P < 0.05$) (68.18%) in week 9, when compared to day 0.

The frequency of defaecation did not differ significantly ($P > 0.05$) in between the groups on day 0, weeks 3 and 6 (Figure 4.5). In week 3, the CPF group had the least frequency of defaecation, and was 21.4%, 26.7% and 26.7% lower, when compared to the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups had defaecation frequencies of 36.4% and 63.6% higher, respectively when compared to the CPF group.

In week 6, the frequency of defaecation for the CPF group was 0% and 14.3% lower when compared to the DW and MO500 groups, respectively and was 33.3% higher, when compared to the SO group. The MO250+CPF group had defaecation frequency that was 45.8% higher, while the MO500+CPF group was 16.7% lower, when compared to the CPF group.

The frequency of defaecation for the CPF group was highest in week 9, and significantly ($P < 0.05$) higher than the MO500 and MO500+CPF groups. It was 50%, 110% and 250% higher than the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups had defaecation frequencies that were 28.6% and 66.7% lower, respectively, when compared to the CPF group. The MO500+CPF group defecated more frequently (20%) when compared to the MO250+CPF group in week 3, but less frequently in weeks 6 and 9 (42.9% and 53.3%, respectively).

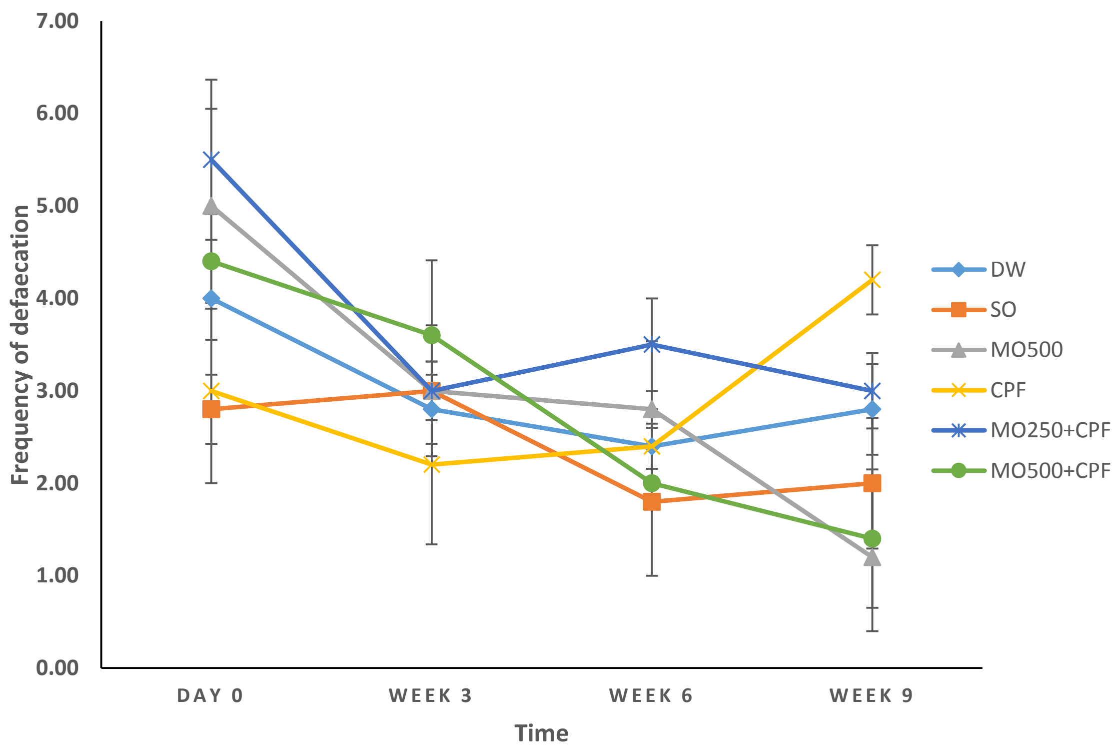


Figure 4.5: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the dynamics of defaecation of Wistar rats in the open field apparatus

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.1.6 Effect of treatments on frequency of urination in the open field performance test

Whereas the DW, SO and MO500 groups had urination frequency values that did not differ significantly ($P > 0.05$) throughout the study period, the CPF, MO250+CPF and MO500+CPF groups urinated significantly less frequently ($P < 0.05$) in weeks 3, 6 and 9, respectively, when compared to day 0 (Figure 4.6).

The frequency of urination did not differ significantly ($P > 0.05$) between groups on day 0. In week 3, the frequency of urination of the CPF, MO250+CPF and MO500+CPF groups was significantly ($P < 0.05$) lower, respectively, when compared to that of the DW or MO500 group. The SO group had a significantly ($P < 0.05$) lower frequency of urination, compared to that of DW and MO500 groups, respectively. The CPF and MO250+CPF groups showed no significant ($P > 0.05$) difference in urination frequency in week 3. The MO500+CPF group, however, had urination frequency that was 50% lower than that of the SO group.

The CPF, MO250+CPF and MO500+CPF groups had no significant difference in their frequency of urination in week 6, but the frequency in each of the groups was significantly ($P < 0.05$) lower when compared to either DW or MO500 groups. In week 9, the CPF and MO250+CPF groups had no significant ($P > 0.05$) difference in their frequency of urination, whereas the MO500+CPF group had frequency of urination that was 80%, 75% and 85.6% lower than that of the DW, SO and MO500 groups, respectively. The MO 500+CPF group had a higher frequency of urination in week 3 when compared to the MO250+CPF group, but no significant difference subsequently at weeks 6 and 9, respectively.

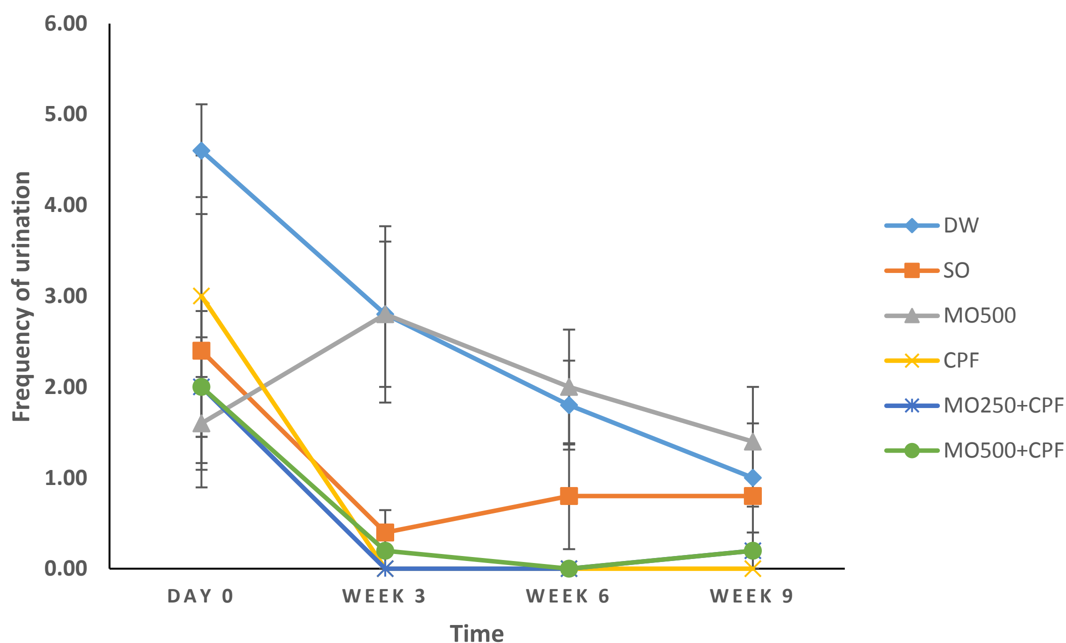


Figure 4.6: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the frequency of urination of Wistar rats in the open field apparatus

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.2 Effect of treatments on beam-walk score

The effect of the treatments on motor coordination as seen in the beam width at which the animals stepped off the beam in the beam-walk performance task is shown in Figure 4.7. The width at which the DW and MO500 groups stepped off the beam only differed significantly ($P < 0.05$) in week 9 when compared to week 3, while the SO group stepped off at significantly ($P < 0.05$) lower beam width in week 9 when compared to week 6. The CPF group stepped off at a higher beam width in weeks 6 and 9 when compared to day 0. The width at which the MO250+CPF stepped off the beam did not differ significantly ($P > 0.05$) throughout the study period, while the MO500+CPF group stepped off at a significantly ($P < 0.05$) lower beam width in week 9 when compared to week 3.

The width at which rats stepped off the beam did not differ significantly ($P > 0.05$) between groups in week 3. However, the beam width at which the CPF group stepped off was 16.7%, 35.5% and 20% higher, when compared to the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups stepped off at beam-width that were 21% and 11.9% lower, respectively when compared to the CPF group.

The width at which the CPF group stepped off the beam was significantly ($P < 0.05$) higher than all the other groups in weeks 6 and 9. The width at which MO250+CPF group stepped off the beam was 35.8% and 40.4% lower at weeks 6 and 9, respectively, when compared to the CPF group. The width at which the MO500+CPF group stepped off the beam was 37.7% and 46% lower at weeks 6 and 9, respectively, when compared to the CPF group. There was no significant difference in the width at which MO250+CPF group stepped off the beam relative to that of MO500+CPF group throughout the study period.

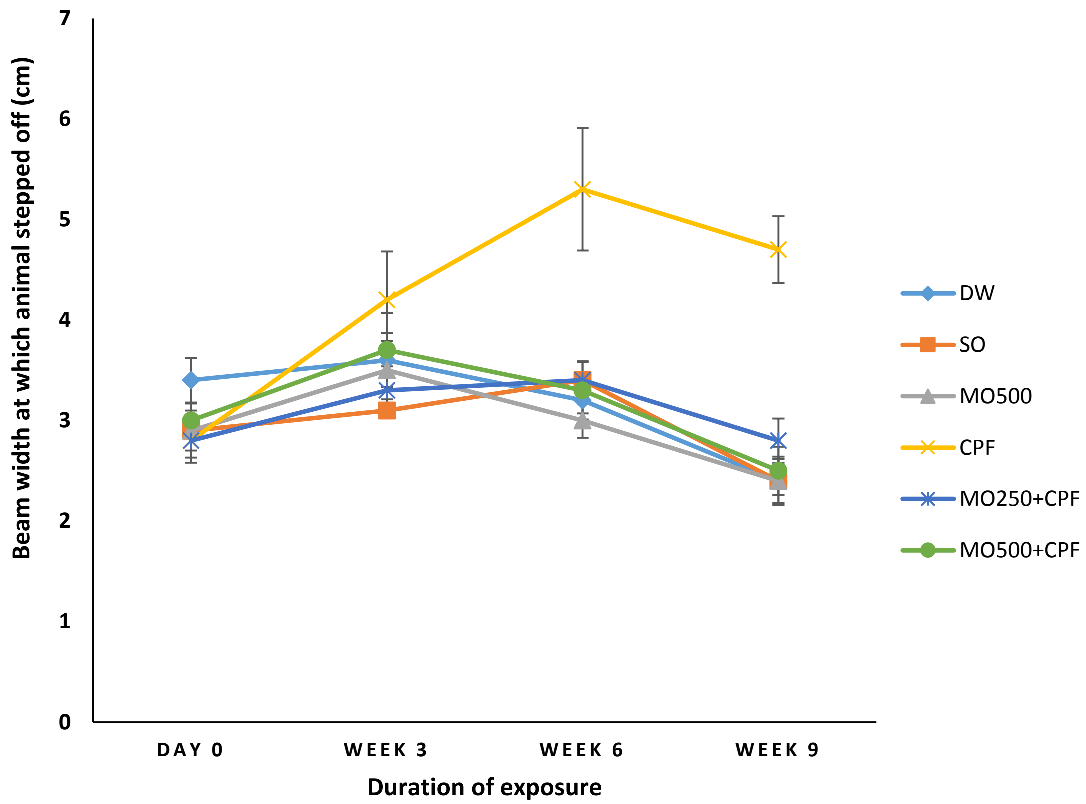


Figure 4.7: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the dynamics of motor coordination in Wistar rats using the beam-walk performance task

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.3 Effect of treatments on ladder-walk performance

The effect of treatments on efficiency of locomotion as reflected in the number of rungs missed in the ladder-walk task is shown in Figure 4.8. The number of rungs missed did not differ significantly ($P > 0.05$) for all the groups throughout the study period. The dynamics, however, showed that the number of rungs missed by the DW and SO groups increased steadily throughout the study period, while the MO500 group showed a slight decline in week 9 (3.3%) after the steady increase up to week 6. The CPF group showed a steady decline in the number of rungs missed after week 3, decreasing by 6.8% and 26.8% in weeks 6 and 9, respectively when compared to day 0. The MO250+CPF group showed a decreased number of rungs missed (5.2%) in week 3; after which it increased in weeks 6 and 9 (21.3% and 26.5% respectively), when compared to day 0. The MO500+CPF group missed fewer rungs in week 3, 6 and 9 (5.5%, 10.5% and 10.5%, respectively) when compared to that of day 0.

The number of rungs missed did not differ significantly ($P > 0.05$) between groups throughout the study period. In week 3, the CPF group missed 19.4%, 16.7% and 10.1% fewer rungs, when compared to the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups missed 10% and 20% fewer rungs, respectively, when compared to the CPF group.

The CPF group missed 31.4%, 30% and 30% fewer rungs in week 6, when compared to the DW, SO and MO500 groups, respectively. The MO250+CPF group missed more rungs (9.9%), while the MO500+CPF group missed fewer rungs (8.5%) when compared, respectively to the CPF group.

In week 9, the CPF group missed the least number of rungs. The MO250+CPF and MO500+CPF groups missed more rungs (45.9% and 16.4%, respectively), when

compared to the CPF group. The CPF group missed fewer rungs (49.2%, 50.1% and 43.2%, respectively), when compared to the DW, SO and MO500 groups. The number of rungs missed by MO500+CPF group was 12.5% higher than that of the MO250+CPF group in week 3, but subsequently, the MO500+CPF group missed fewer rungs (16.7% and 20.2%) in weeks 6 and 9, respectively.

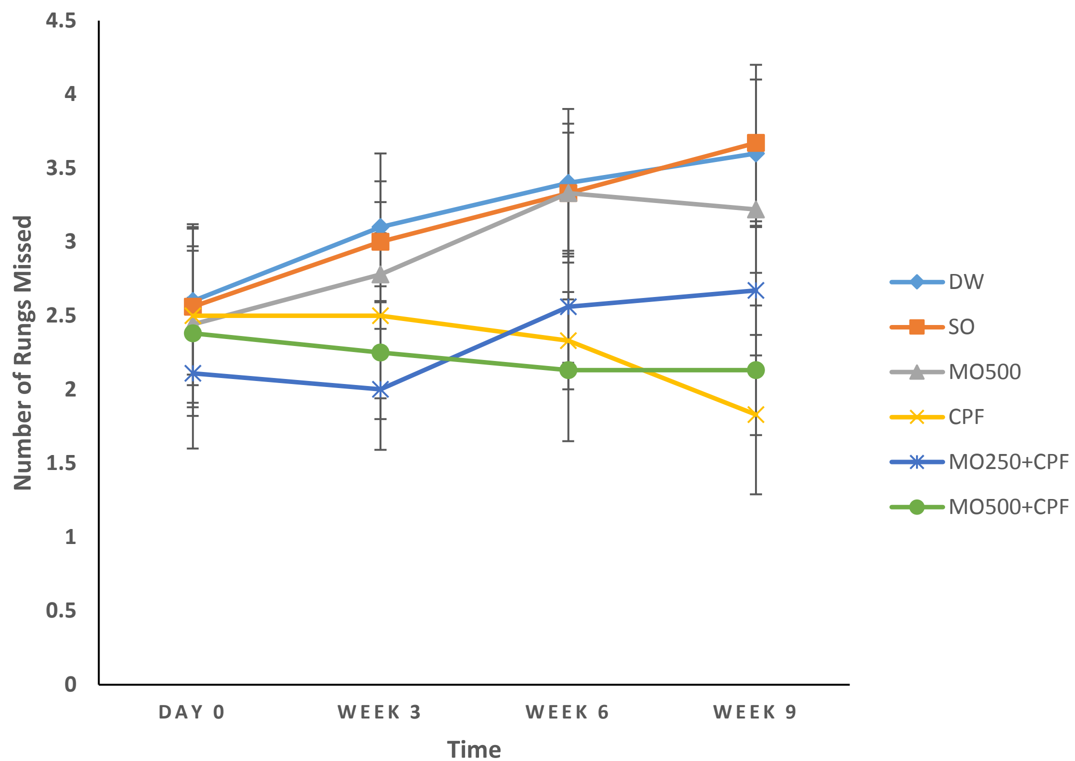


Figure 4.8: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the dynamics of locomotor efficiency of Wistar rats using the ladder walk performance task

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.4 Effect of treatments on inclined plane performance

The neuromuscular coordination of the rats was assessed using the angle at which the rats slipped on the inclined plane apparatus. Despite steady decline, the DW, SO and MO500 groups slipped at angles that did not differ significantly ($P > 0.05$) throughout the study period. The CPF, MO250+CPF and MO500+CPF groups, however, slipped at angles that were significantly ($P < 0.05$) lower in weeks 3, 6 and 9, respectively, when compared to day 0 (Figure 4.9). The dynamics showed a steady decline in the angle at which the CPF group slipped off the inclined plane.

The CPF group slipped at angles significantly ($P < 0.05$) lower than the DW, SO and MO500 groups, respectively in weeks 3, 6 and 9. The angle at which the MO250+CPF and MO500+CPF groups slipped was significantly ($P < 0.05$) lower when compared to the DW, SO and MO500 groups, respectively in week 3, but was 3%, and 5.5% higher when compared to the CPF group.

In week 6, the CPF group slipped at an angle that was significantly ($P < 0.05$) lower when compared to the DW, SO, MO500 and MO250+CPF groups, respectively. The MO500+CPF group slipped at angle that was significantly ($P < 0.05$) lower than the SO group, but was 8.4% higher when compared to the CPF group. The MO500+CPF group slipped at an angle lower than the MO250+CPF group in weeks 3 and 6 (2.3% and 2.1%, respectively), but at a slightly higher angle in week 9 (0.1%).

The CPF group slipped at angle that was significantly ($P < 0.05$) lower in week 9 when compared to all the other groups.

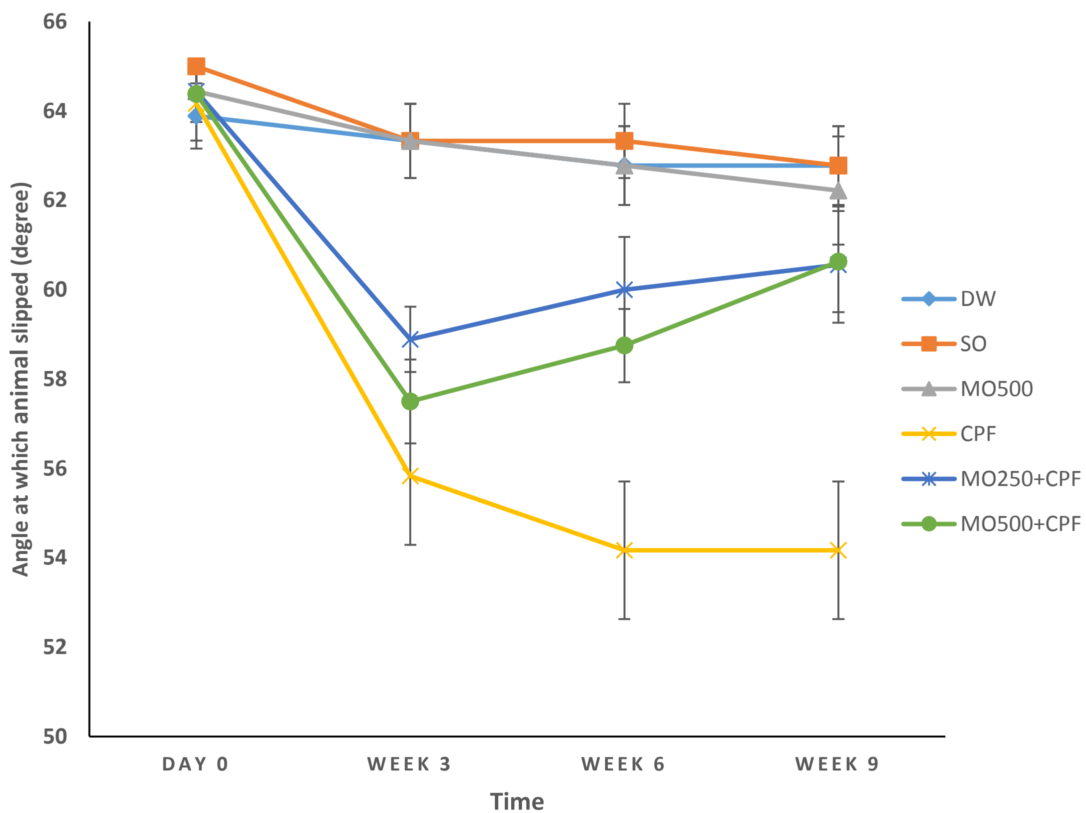


Figure 4.9: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the dynamics of neuromuscular coordination of Wistar rats using the inclined plane

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.5 Effect of treatments on forepaw grip time

The effect of the treatments on the dynamics of motor strength using the forepaw grip test is shown in Figure 4.10. The dynamics showed grip time that increased steadily in the DW, SO, MO500, MO250+CPF and MO500+CPF groups throughout the study period. The CPF group showed a steady decline (6.6%, 10.7% and 17.1% lower in weeks 3, 6 and 9, respectively when compared to day 0), but did not differ significantly ($P > 0.05$) throughout the study period. The DW and SO groups, respectively had grip time values that were significantly ($P < 0.05$) higher in week 9 when compared to day 0, while the MO500 group had significantly ($P < 0.05$) higher grip time in weeks 6 and 9 when compared to day 0. Despite steady increase, the MO250+CPF group showed no significant difference ($P > 0.05$) in forepaw grip time throughout the study period, while the MO500+CPF group had a significantly ($P < 0.05$) higher grip time in weeks 6 and 9, respectively when compared to day 0.

The grip time did not differ significantly ($P > 0.05$) between DW, SO, MO500, MO250+CPF and MO500+CPF groups throughout the study period. In week 3, the MO250+CPF and MO500+CPF groups had grip time values 21.2% and 23.5% higher, respectively when compared to the CPF group. The CPF group had 21.2%, 19.1% and 17.5% lower grip time when compared to the DW, SO and MO500 groups, respectively.

The CPF group had lower grip time when compared to the DW, SO and MO500 groups (28.4%, 25.8% and 32.9%), respectively in week 6. The MO250+CPF and MO500+CPF groups had higher grip time (32.3% and 39.7%) than the CPF group, respectively.

The grip time for the CPF group was significantly ($P < 0.05$) lower in week 9 when compared to the SO group. Although not significant, the grip time in the CPF group was 36.3% and 39.3% lower than the DW and MO500 groups, respectively. The MO250+CPF

and MO500+CPF groups had higher grip time values (49.1% and 56.9%) when compared to the CPF group, respectively.

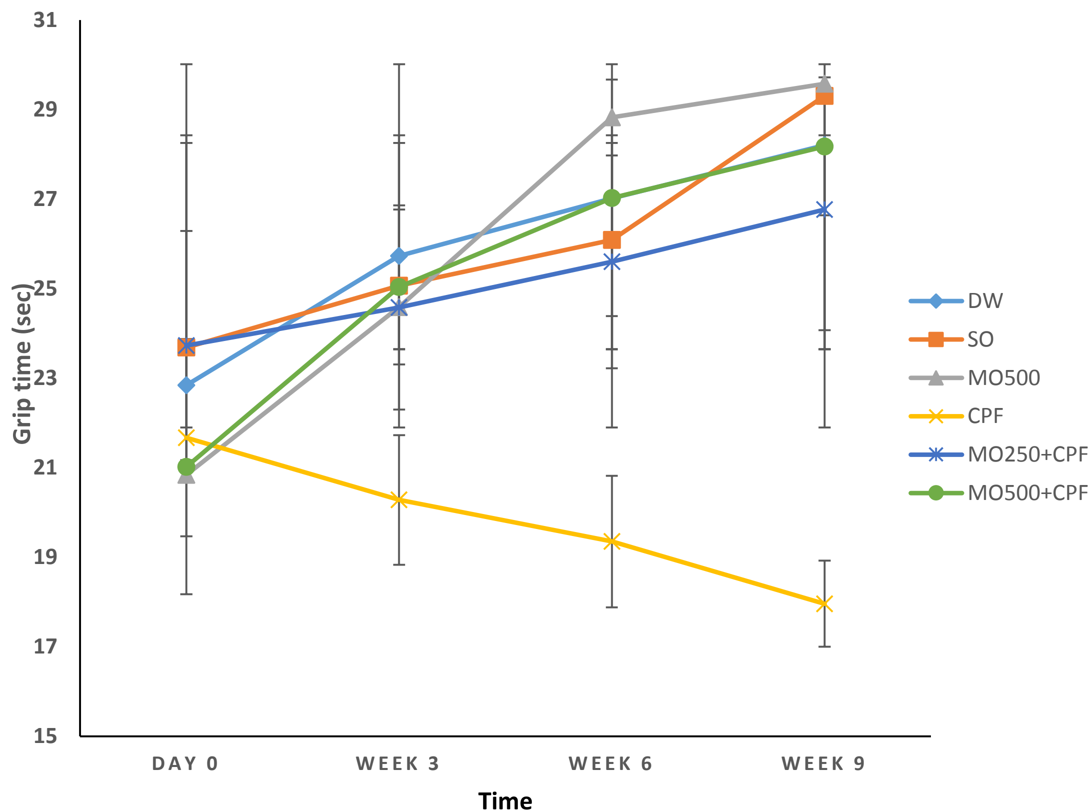


Figure 4.10: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the dynamics of the motor strength in Wistar rats

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.6 Effect of treatments on excitability scores

The dynamics of the effects of treatments on the excitability scores of rats is shown in Figure 4.11. The excitability scores of the DW and MO500 groups increased in week 3 and remained stable through weeks 6 and 9. Both groups had excitability scores that were significantly ($P < 0.05$) higher in weeks 3, 6 and 9 respectively, when compared to day 0. The SO group attained similar plateaux in week 3 through week 9, but the difference was not significant ($P > 0.05$) throughout the study period. The CPF group showed a steady [but not significant ($P > 0.05$)] decline in excitability scores throughout the study period ,being 5%, 5% and 7.5% lower in weeks 3, 6 and 9, respectively, when compared to day 0. The excitability scores for the MO250+CPF group did not differ significantly ($P > 0.05$), but the dynamics showed a steady increase (28.5% and 31.4%) in weeks 3 and 6, respectively compared to day 0, and decreased in week 9 (6.5%) when compared to week 6. The MO500+CPF group had excitability scores that increased steadily through the study period and was significantly ($P < 0.05$) higher in week 9, when compared to day 0.

The CPF group had the least excitability scores in weeks 3, 6 and 9. No significant difference ($P > 0.05$) was observed in the excitability scores between groups on day 0 and in week 3. The CPF group had excitability scores that were 15.6%, 17.4% and 22.4% higher when compared to the DW, SO and MO500 groups, respectively in week 3. The MO250+CPF and MO500+CPF groups had 13.2% and 5.2% higher excitability scores, respectively when compared to the CPF group.

The excitability scores for the CPF group was significantly ($P < 0.05$) lower in week 6, when compared to the MO500 group. The CPF group had lower excitability scores (15.6%, and 17.4%) when compared to the DW and SO groups, respectively. The

MO250+CPF and MO500+CPF groups had higher excitability scores (21.6% and 13.2%, respectively), when compared to the CPF group.

In week 9, the excitability scores was significantly lower ($P < 0.05$) in the CPF group when compared to the MO500, and was 17.8% and 19.6% lower than the DW and SO groups, respectively. The MO250+CPF and MO500+CPF groups had higher excitability scores (16.2% and 18.9%) than the CPF group, respectively. The MO500+CPF had lower excitability scores when compared to the MO250+CPF group in weeks 3 and 6 (6.9% and 6.5%, respectively), but had slightly higher excitability scores (2.3%) in week 9.

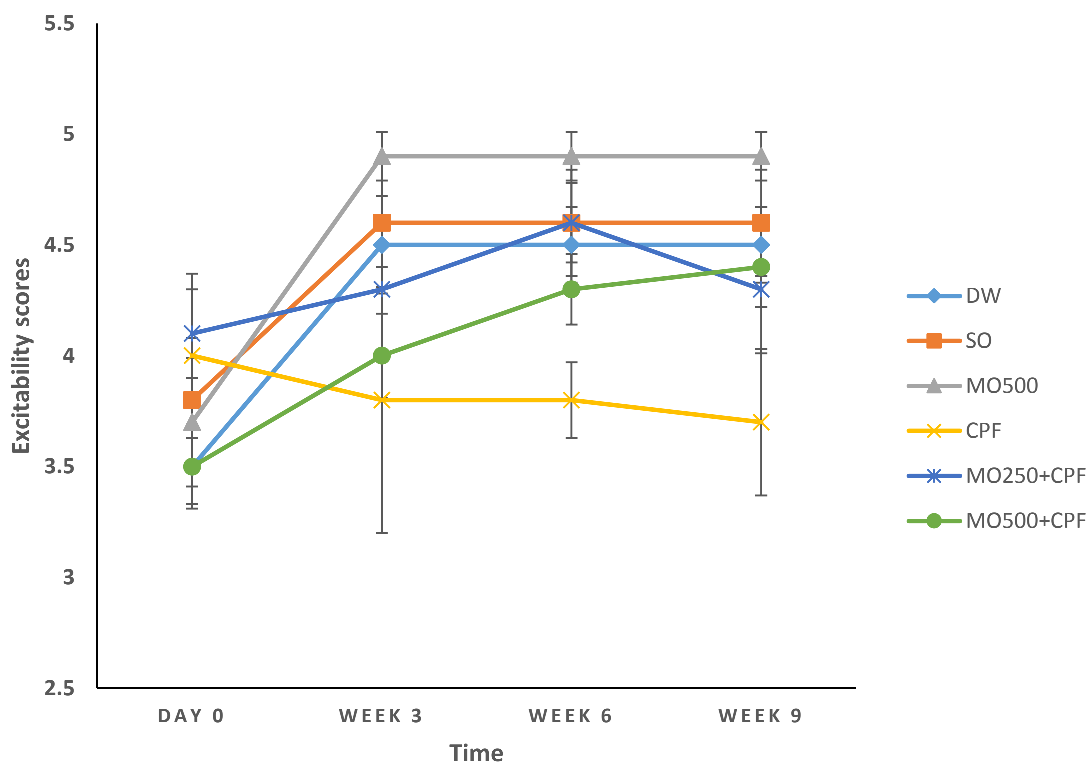


Figure 4.11: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on excitability scores of Wistar rats

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.7 Effect of treatments on forced swimming test

The DW, SO and MO500, MO250+CPF and MO500+CPF groups showed forced swimming time that did not differ significantly ($P > 0.05$) throughout the study period. The CPF group, however, showed swimming time values that decreased steadily throughout the study period and were significantly ($P < 0.05$) lower in week 9 when compared to week 0 (Figure 4.12). The swimming time did not differ significantly ($P > 0.05$) between groups on day 0 and in week 3.

The CPF group had lower forced swimming time (1.2%, 7.3% and 5.9% lower) when compared to the DW, SO and MO500 groups, respectively in week 3. The MO250+CPF group had lower (2.9%), while the MO500+CPF group had higher (8.9%) forced swimming time when compared to the CPF group.

In week 6, the forced swimming time was relatively lowrer in MO250+CPF (4.4%) and MO250+CPF (19.9%) when compared to the CPF group, respectively. The CPF group had lower forced swimming time (8.2%, 12% and 14.6%) when compared to the DW, SO and MO500 groups. The MO500 and MO500+CPF groups showed significantly ($P < 0.05$) higher forced swimming time than the CPF group.

The CPF group had lower forced swimming time in week 9 (8.4%, 13.3% and 17.1%) when compared to the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups showed forced swimming time 3.4% and 17.1% higher than the CPF group. The SO, MO500 and MO500+CPF groups had significantly ($P < 0.05$) higher forced swimming time than the CPF group, respectively. The MO500 group had significantly ($P < 0.05$) higher forced swimming time when compared to the MO250+CPF group.

The MO500+CPF group showed higher forced swimming time (4.4%, 12.1%, 14.9% and 13.2% higher) when compared to the MO250+CPF group on day 0 and week 3, 6 and 9, respectively. The MO500+CPF group showed significantly ($P < 0.05$) higher forced swimming time than the MO250+CPF group in week 6 and 9, respectively.

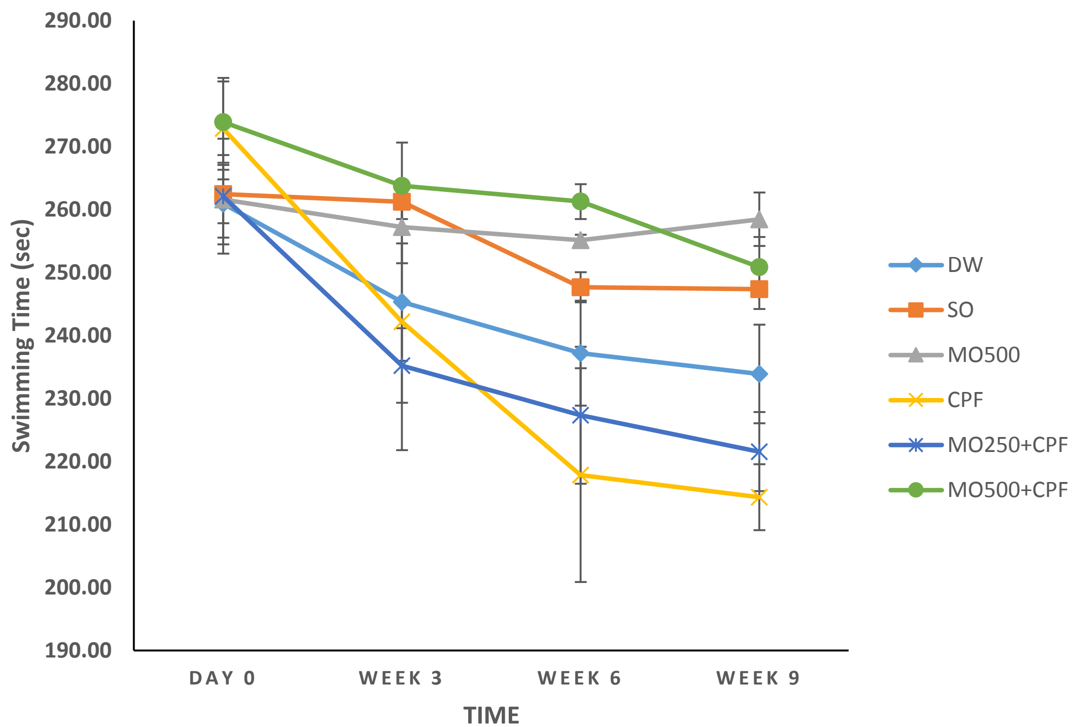


Figure 4.12: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on swimming time of Wistar rats in the forced swimming test

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.4.8 Effect of treatments on learning acqusition

The number of foot shocks did not differ significantly ($P>0.005$) between the groups in this study (Figure 4.13). However, the CPF group showed a higher number of foot shocks, which was 30%, 31.5% and 31.5% higher when compared to the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups showed number of foot shocks that were 14.5% and 29% less, respectively, when compared to the CPF group. The MO500+CPF group had 12% fewer foot shocks than the MO250+CPF group.

4.4.9 Effect of treatments on short-term memory

The CPF group spent significantly ($P < 0.05$) less time on the platform when compared to all the other groups. The CPF group spent 10.7%, 11.3% and 11.4% more time on the platform when compared to the DW, SO and MO500 groups, respectively. The MO250+CPF and MO500+CPF groups spent 12.8% and 12.1% more time, respectively on the platform when compared to the CPF group (Figure 4.14). The MO500+CPF group spent slightly less time on the platform (0.6%) when compared to the MO250+CPF group.

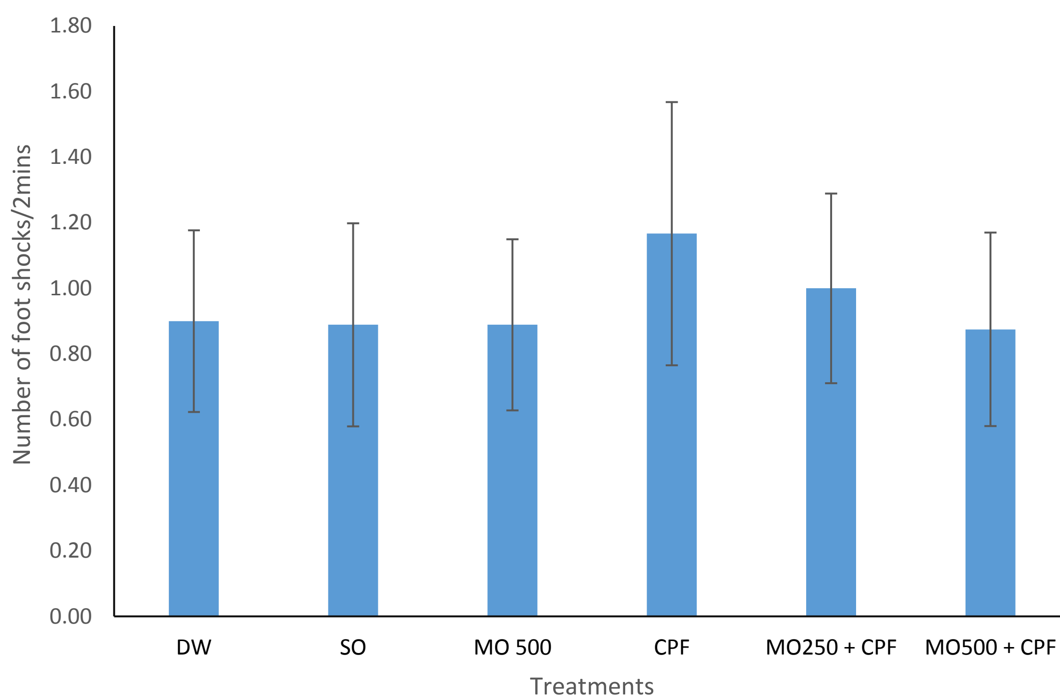


Figure 4.13: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the number of foot shocks received by Wistar rats in the step down inhibition apparatus

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

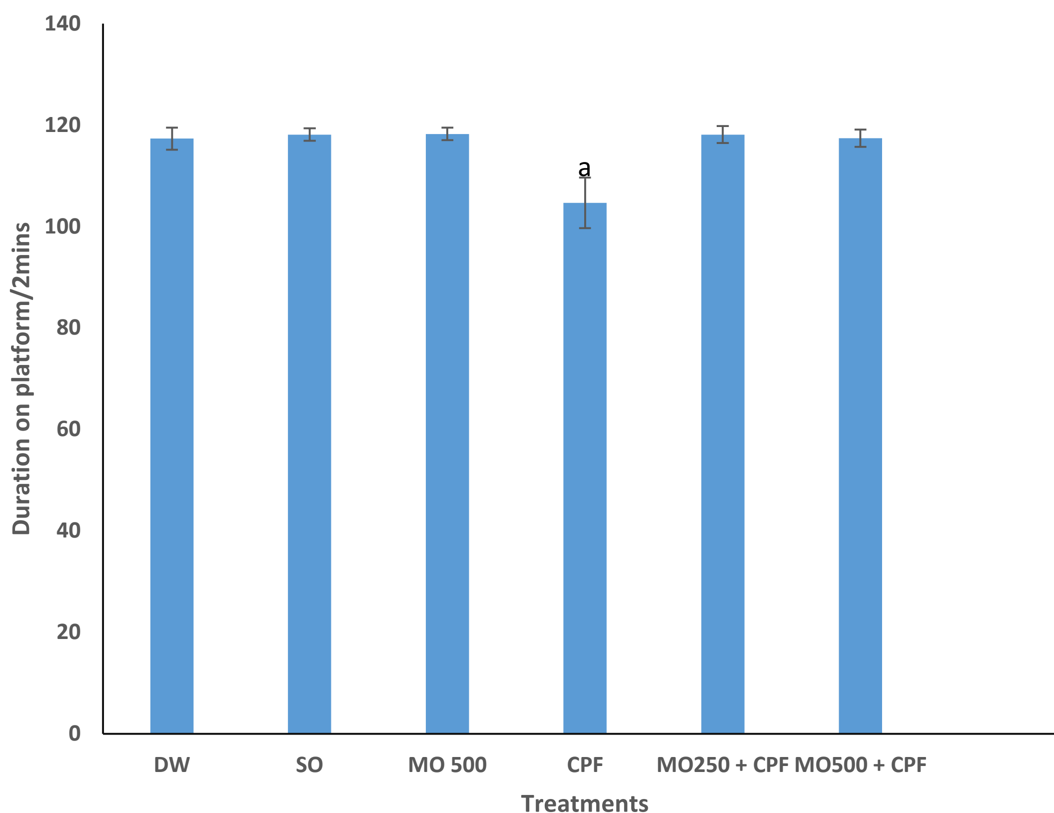


Figure 4.14: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the duration of stay of Wistar rats on the platform of a step down inhibition apparatus

[a]P < 0.05 versus DW, SO, MO500, MO250+CPF and MO500+CPF, respectively

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.5 Effect of Treatments on Brain Acetylcholinesterase Activity

The brain AChE activity was significantly ($P < 0.05$) lower in the CPF group when compared to the SO, DW and MO250+CPF groups. Although 32.31% higher, the MO500+CPF group AChE activity did not differ significantly from the CPF group (Figure 4.15). The MO500+CPF group had 11.3% lower AChE activity, when compared to the MO250+CPF group.

4.6 Effect of Treatments on Brain Malondialdehyde Concentration

The CPF group had significantly ($P < 0.05$) higher MDA concentration when compared to the DW, SO MO500 and MO250+CPF groups, respectively. The brain MDA concentration was 9.52% lower in the MO500+CPF group, when compared to the CPF group (Figure 4.16). The MO500+CPF group had higher MDA concentration (11.7%) when compared to the MO250+CPF group.

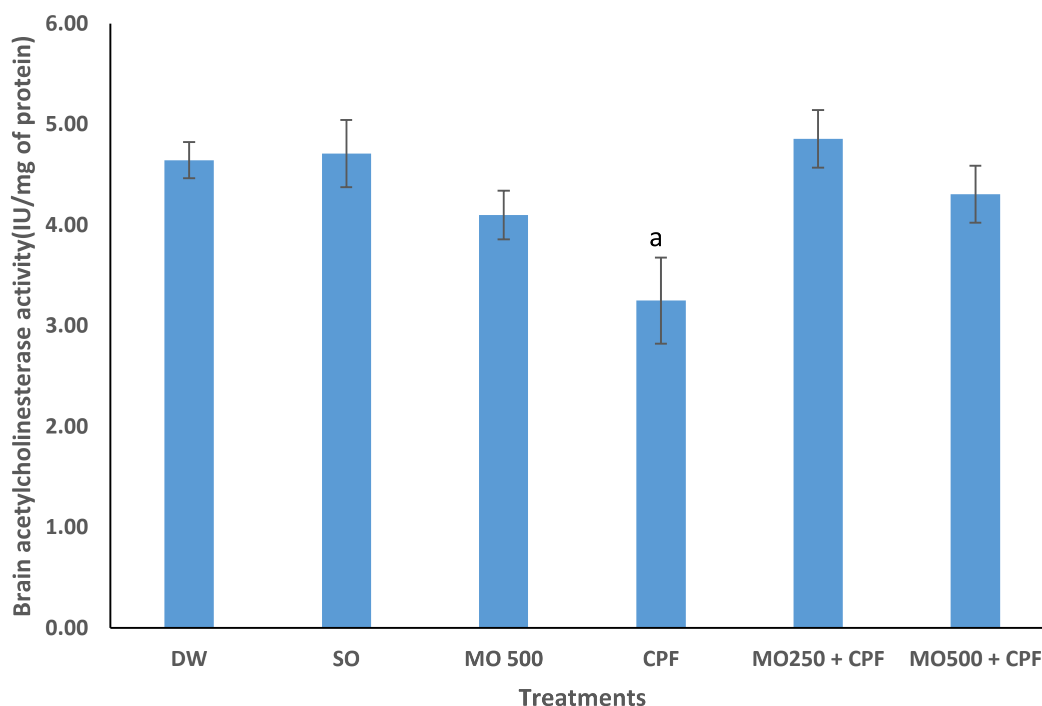


Figure 4.15: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on brain cholinesterase activity of Wistar rats. [a]P < 0.05 versus DW, SO, and MO250+CPF, respectively

[a]P < 0.05 versus DW, SO, and MO250+CPF, respectively

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

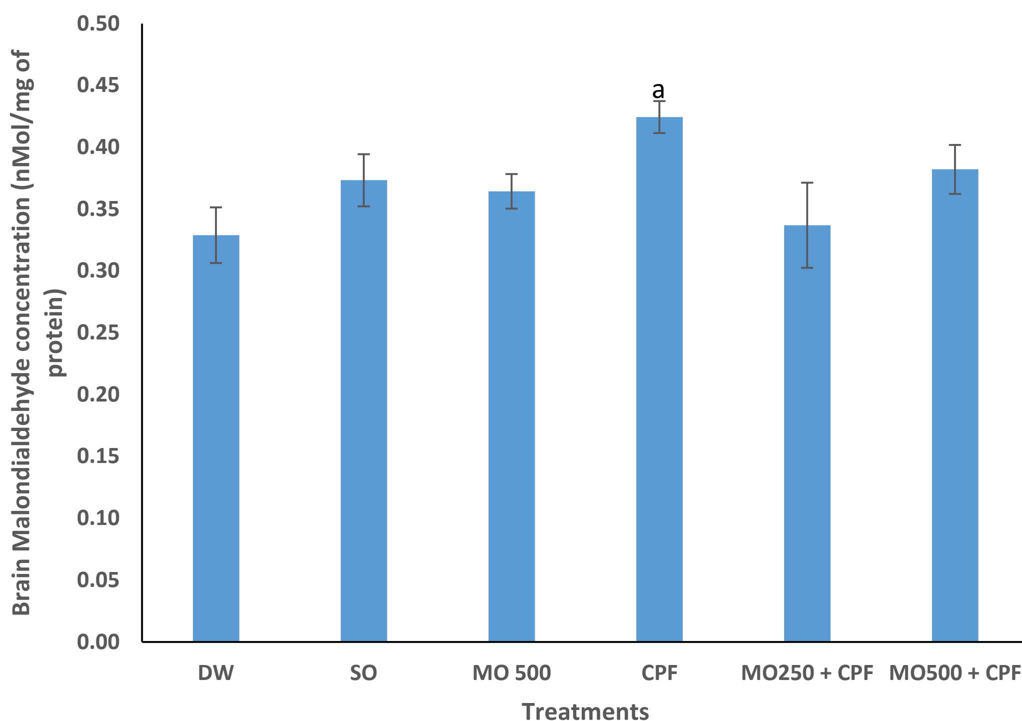


Figure 4.16: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the brain malondialdehyde concentration of Wistar rats

[a]P < 0.05 versus DW, SO, MO500 and MO250+CPF, respectively

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.7 Effect of Treatments on Brain Antioxidant Enzymes

4.7.1 Effect of treatments on brain superoxide dismutase activity

The CPF group showed the least SOD activity, which was significantly ($P < 0.05$) lower when compared to the DW, SO and MO500 groups, respectively. The SOD activity for the MO250+CPF and MO500+CPF groups was significantly ($P < 0.05$) lower, when compared to the SO and MO500 groups, respectively. Compared to the CPF group, the MO250+CPF and MO500+CPF groups were 17.2% and 10.3% higher, respectively (Figure 4.17). The MO500+CPF group had 5.9% less SOD activity when compared to the MO250+CPF group.

4.7.2 Effect of treatments on brain glutathione peroxidase activity

The GP_X activity was significantly ($P < 0.05$) lower in the CPF group, when compared to the SO and the DW groups, respectively, and was 18.1% lower when compared to the MO500 group. The MO500+CPF group had GP_X activity that was significantly ($P < 0.05$) lower than the SO group. The MO250+CPF and MO500+CPF groups showed GP_X activity that was 6.1% and 11.4% higher, respectively, when compared to the CPF group (Figure 4.18). The MO500+CPF group had 5% higher GP_X activity, when compared to the MO250+CPF group.

4.7.3 Effect of treatments on brain catalase activity

The CAT activity for the CPF group was significantly ($P < 0.05$) lower, when compared to the SO and DW groups, respectively, but was 3.5% higher compared to the MO500 group. The MO250+CPF and MO500+CPF groups showed CAT activities that were significantly ($P < 0.05$) lower when compared, respectively to the DW group. The MO250+CPF and MO500+CPF groups had CAT activities that were 10.86% and 8.63%

respectively lower than the CPF group (Figure 4.19). The CAT activity of the MO500+CPF group was 2.5% higher when compared to that of the MO250+CPF group.

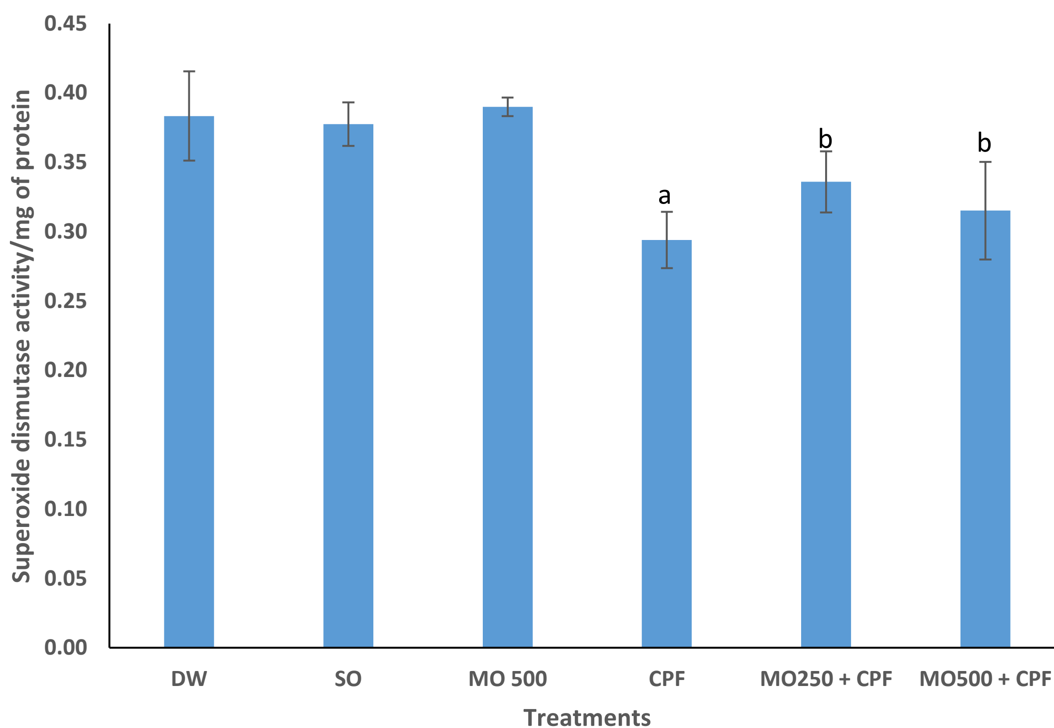


Figure 4.17: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the brain SOD specific activity of Wistar rats

[a]P < 0.05 versus DW, SO and MO500, respectively. [b]P < 0.05 versus SO and MO500, respectively

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

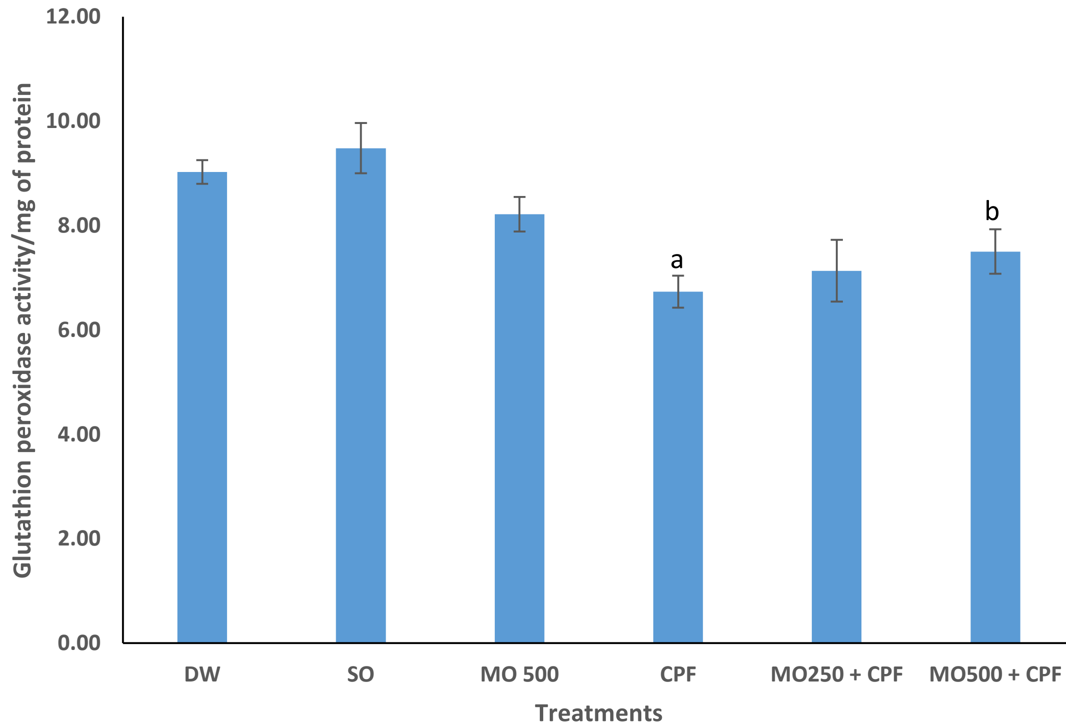


Figure 4.18: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on brain GPx specific activity of Wistar rats

[a]P < 0.05 versus DW and SO, respectively. [b]P < 0.05 versus SO

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

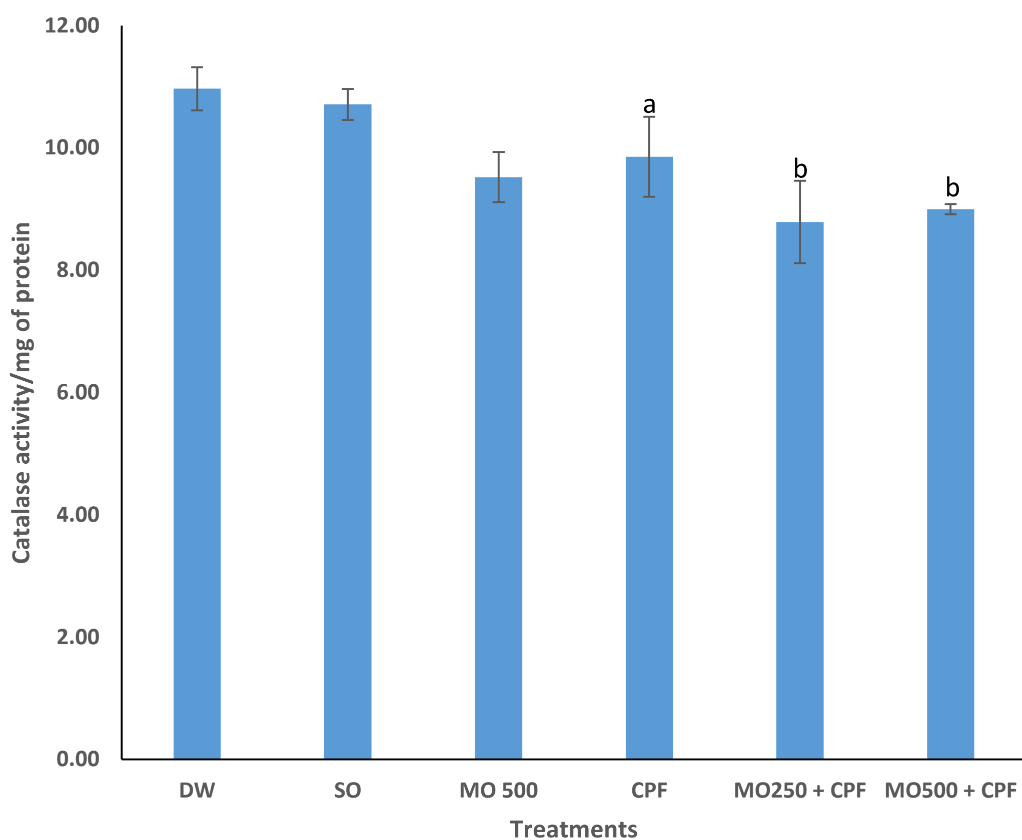


Figure 4.19: Effects of sub-chronic oral administration of methanol leaf extract of *Moringa oleifera* and chlorpyrifos on the brain CAT activity of Wistar rats

P < 0.05 versus DW and SO, respectively. [b]P < 0.05 versus DW.

DW - distilled water; SO - soya oil; MO500 - *Moringa oleifera* (500 mg/kg), CPF - chlorpyrifos, MO250+CPF - *Moringa oleifera* (250 mg/kg) and chlorpyrifos; MO500+CPF - *Moringa oleifera* (500 mg/kg) and chlorpyrifos

4.8 Effect of Treatments on Brain Histo-architecture

No histopathological changes were observed in the brains of rats administered with DW, SO and MO500 (Plates II, III and IV respectively). The CPF group had several neurones at different stages of cellular degeneration and apparent apoptosis (Plate V). The MO250+CPF group showed few degenerating neurones, and more viable cells (Plate VI). The MO500+CPF group also showed few degenerating neuronal cells and more viable cells (Plate VII).

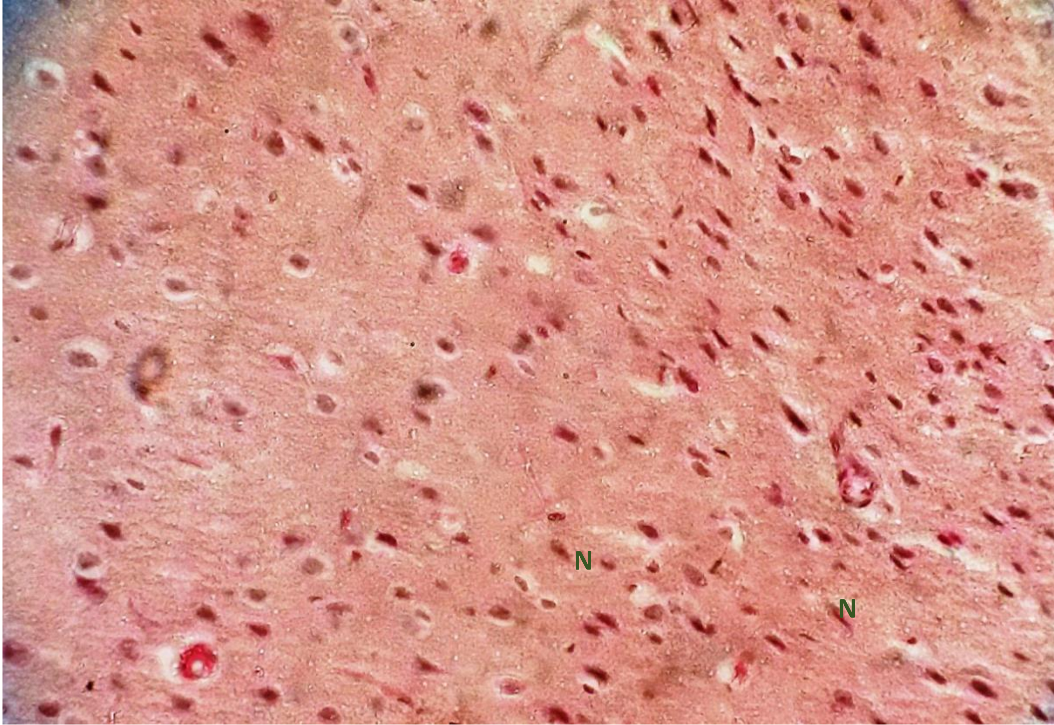


Plate II: Photomicrograph of a section of the brain of rat exposed to distilled water showing normal neurons (N), with no apparent histopathological lesions (H and E × 400)

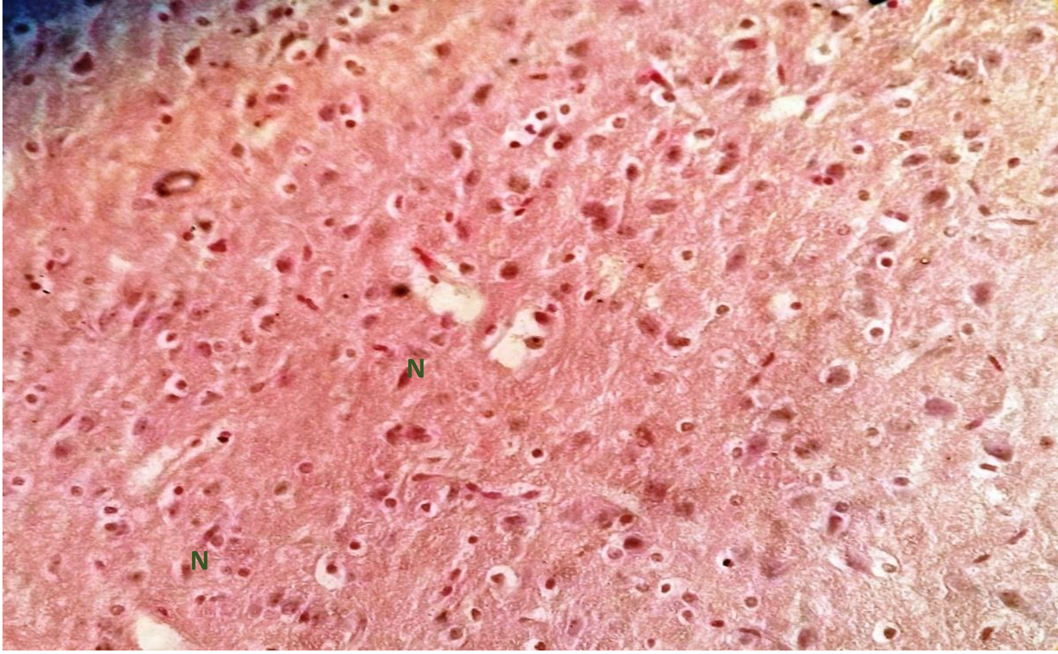


Plate III. Photomicrograph of a section of the brain of rat exposed to Soya oil, showing normal neurons (N) with no apparent histopathological lesions (H and E × 400)

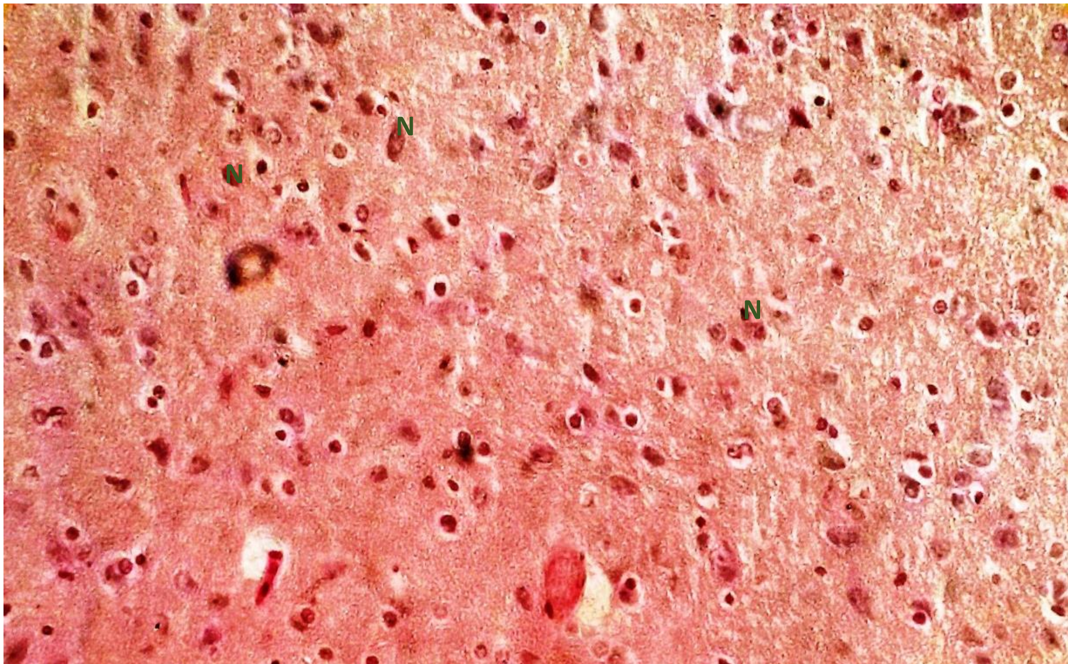


Plate IV: Photomicrograph of a section of the brain of rat exposed to *Moringa oleifera* (500 mgkg) leaf extract showing normal neurones (N), with no apparent histopathological lesions (H and E × 400)

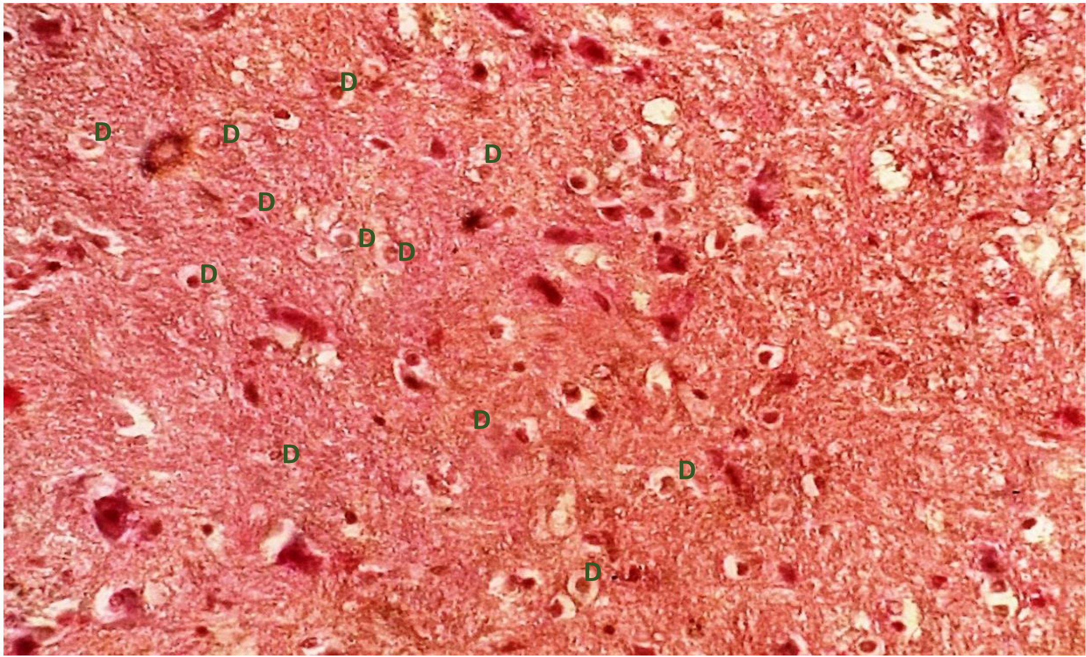


Plate V: Photomicrograph of a section of the brain of rat exposed to Chlorpyrifos showing widespread neuronal degeneration (D) (H and E × 400)

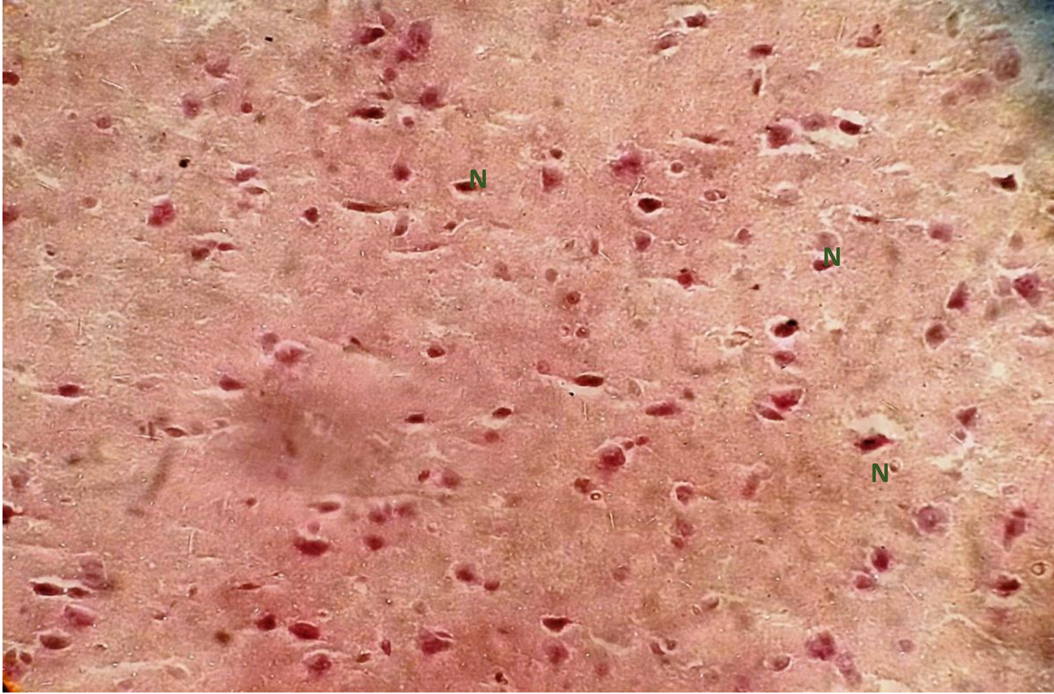


Plate VI: Photomicrograph of a section of the brain of rat pretreated with *Moringa oleifera* (250 mgkg) leaf extract before exposure to Chlorpyrifos, showing apparently normal neurones (N) (H and E × 400)

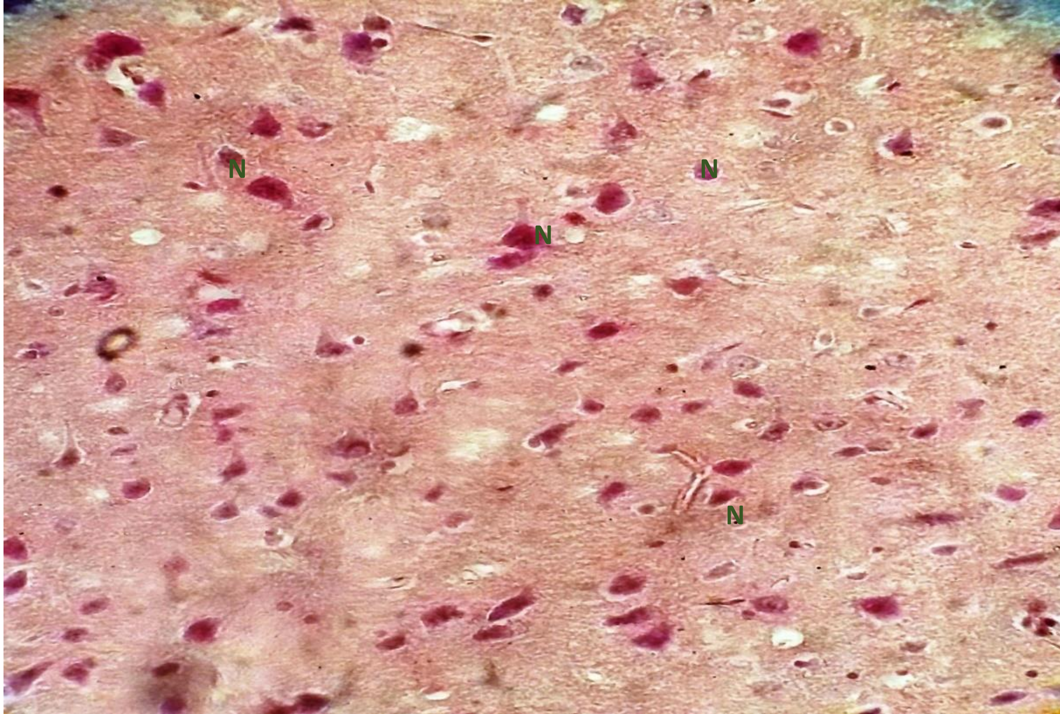


Plate VII: Photomicrograph of a section of the brain of rat pretreated with *Moringa oleifera* (500 mgkg) leaf extract before exposure to Chlorpyrifos showing apparently normal histoarchitecture of the neurones (N) (H and E × 400)

CHAPTER FIVE

DISCUSSION

The median lethal dose (LD_{50}) of 95 mg/kg obtained for CPF is within the range of 95 – 270 mg/kg reported by Ambali (2009) and Parmar *et al*. (2014) for rats. The clinical signs of piloerection, tremor, intermittent convulsions, and ocular and nasal discharges observed prior to death in acute toxicity were essentially exaggerated cholinergic activities. They were attributable to an overstimulation of cholinergic receptors due to its anticholinesterase effects, characteristic of organophophate toxicity (Kamanyire and Karalliedde, 2004). Huddling as observed in this acute toxicosis can be attributed to CPF induction of hypothermia as reported by Mack and Gordon (2007). Depression has also been reported in CPF toxicity and is attributable to alterations in serotonergic mechanisms (Slotkin and Seidler, 2005; Lee *et al*., 2007). Chen *et al*. (2014) reported that CPF exposure can be related to mood disorders, often manifested as despair behaviour.

With the median lethal dose (LD_{50}) of the methanol extract of MO leaves determined to be higher than 5000 mg/kg, the MO leaves are considered to be relatively safe. This finding is consistent with the reports of several authors (Rathi *et al*., 2006; Awodele *et al*., 2012) that MO leaf extracts have LD_{50} higher than 5000 mg/kg and are relatively safe. Akunna *et al*. (2012) reported an LD_{50} of >3000 mg/kg for orally administered MO leaf extract in rats. Kasolo *et al*. (2012) reported an LD_{50} of 39600 mg/kg for ethanol extract, and 16100 mg/kg for aqueous extract of MO leaves, following intragastric administration in mice. This apparent non-toxicity probably explains the widespread use of MO as food and medicine without adverse effects (Anwar *et al*., 2007).

The methanol yield of 18.33% for MO leaves is slightly higher than that reported by Pal *et al*. (1995) (8%), Lampronti *et al*. (2008) (14.54%), Patel *et al*. (2011) (6.36%). Hannan *et al*. (2014) reported an ethanol extract yield of 12.77% w/w. This wide variation in methanol yield reported for MO leaves may be related to variations in the climate in which the plant was cultivated, and the season of the year in which the leaves were harvested, among other factors (Ju *et al*., 2014).

The positive results obtained for the test for presence of alkaloids, flavonoids, glycosides, phenols, saponins, tannins and terpenoids were consistent with the reports of several authors (Patel *et al*., 2011; Shahriar *et al*., 2012; Okorondu *et al*., 2013; Misra *et al*., 2014). However, Koruthu *et al*. (2011) reported absence of alkaloids, saponins and glycosides in methanol leaf extracts of MO. This variation again may be related to the climate in which the plant was cultivated, season of harvest, stage of maturity, variety and soil conditions (Mukinzi *et al*., 2011).

The total flavonoid content of the MO leaf extract of 22.6% w/w is considered very high and by far above that reported by Sreelatha and Padma (2009) (2.7 % w/w) in India, but lower than that reported by Moyo *et al*. (2012) (29.5 w/w quercetin equivalent) in South Africa. The variation in the quantity of flavonoids may be dependent of the soil of cultivation, as flavonoid contents of plants have been shown to vary depending on many factors, including cultivar, growing location, climate, agricultural practices, processing techniques, preparation methods, and analytical variability. Flavonoids are secondary metabolites produced by plants in response to various environmental stresses such as climate and ultraviolet radiation (Haytowitz *et al*., 2013).

The extract was low in vitamin A (0.3 mg/g), when compared to 1.93mg/g total carotene reported by Nambiar and Seshadri (2001), and differs from that reported by Yang *et al*.

(2006) (15 mg/100g fresh weight of MO leaves). The value of 6.7 mg/g of vitamin C observed was close to that obtained by Sreelatha and Padma (2009), who obtained a mean value of 6.6 mg of vitamin C per gram of aqueous extract of matured MO leaves; but lower than the value reported by Nambiar and Seshadri (2001) who obtained 6.6 mg per gram of dry MO leaves. Also, 0.22 IU/g [147µg/g (d-alpha-tocopherol) or 198 µg/g (dl-alpha-tocopherol)] of vitamin E in extract, or 0.04 IU/g [30 µg/g (d-alpha-tocopherol) or 36 µg/g (dl-alphatocopherol)] of vitamin E in dry leaves, as obtained in this study may be considered high and beyond the range of 5.7 µg/g (adult leaves) to 27.8 µg/g (6 month-old plants) of dry leaves as reported by Ferriera *et al*. (2008).

The clinical signs of congested ocular mucous membrane, weakness, fasciculations, and lacrimation seen in CPF exposed rats in this study have also been reported previously by Ambali *et al*. (2010c). Priapism was reported by Moser (2000) as a sign of CPF toxicity. The signs may be attributed to over-stimulation of cholinergic receptors, a consequence of AChE inhibition (Ambali *et al*., 2010c). Huddling can be attributed to CPF induction of hypothermia as reported by Mack and Gordon (2007). Noaishi *et al*. (2013) reported bloody nasal discharges as one of the signs of OP intoxication. Pretreatment with MO ameliorated these signs, consistent with previous reports (Verma *et al*., 2007; Ambali *et al*., 2010c; Demir *et al.,* 2011) that antioxidants mitigate CPF toxicity. This amelioration may be due to the ability of flavonoids to protect paraoxonase I (PON1), an enzyme chiefly involved in CPF metabolism, from oxidative damage, thus enhancing the detoxification of CPF (Fuhrman and Aviram, 2002; Hamidia *et al*., 2014). In addition, flavonoids have been shown to up-regulate PON1 in the serum (Jaiswal and Rizvi, 2014).

The results demonstrated that CPF increased anxiety and decreased motor activity in exposed rats. This was evidenced from the reduced locomotion and exploration observed,

as fewer numbers of squares crossed by the CPF group. CPF treatment has been reported to cause a reduction in locomotor activity by several authors (Pope *et al*., 1992; Carr *et al*., 2001; Al-Badrany and Mohammad, 2007). This deficit may have resulted from oxidative damage to the brain. Decreases in locomotion and exploration of an open field have been reported in rats with induced hippocampal oxidative stress (de Oliveira *et al*., 2007; Masood *et al*., 2008). Oxidative damage to the muscles may also be implicated, resulting in reluctance to move (Ambali *et al*., 2012a). Such damage has been reported in rats following OP exposure (Abdou and El Mazoudy, 2010; Jafari *et al*., 2012).

The antioxidant-rich MO extract appeared to mitigate the anxiogenic and impaired motor effects of CPF, as can be inferred from the improved locomotion and exploration in the rats pretreated with MO leaf extract. This finding established that oxidative stress is involved in the locomotor deficit. Antioxidant compounds such as vitamins C and E (Ambali, 2009), acetyl-l-carnitine (Lasisi, 2011), melatonin (Idris *et al*., 2014) have demonstrated restoration of anxiogenic and locomotion deficits engendered by CPF in Wistar rats. Similarly, methanol extract of *Phyllanthus niruri* have been shown to mitigate locomotion deficit and anxiogenesis caused by CPF in Wistar rats (Ambali *et al*., 2012c)

The relatively higher frequency of stretch-attends posture in the CPF group relative to other groups throughout the period of evaluation is indicative of the anxiogenic properties of CPF, coherent with the report of Venerosi *et al*. (2006) and Ambali (2009). The stretch-attends posture is a risk assessment behaviour which aims to maintain a constant environment feedback that allows the animals to prevent and avoid dangerous situation in a strange environment (Sáenz *et al*., 2006). The result agree with the findings of Bolivar *et al*. (2000) in rats, who reported that normal rats show a higher frequency of stretch-attends posture in the first open field test, which decreases subsequently due to increased

familiarity with the open-field arena, and anxiogenic agents induce relatively stable or even increased stretch-attends posture frequency.

This anxiety-like behaviour induced by CPF in the present study was attenuated by pretreatment with methanol extract of MO leaves, as the frequency of stretch-attends posture decreased significantly in pretreated groups. The mechanism underlying the attenuation may be protection of the limbic system from oxidative damage, since anxiety disorders have been related to oxidative damage to the limbic system (Masood *et al.*, 2008; Ding *et al.*, 2014). Several antioxidants have been shown to be useful neuro-protective agents, exerting anxiolytic effects when they protect the limbic system from oxidative damage (Xu *et al.*, 2014). Antioxidants such as vitamin C and/or E (Ambali, 2009), acetyl-l-carnitine (Lasisi, 2011) and melatonin (Idris *et al.*, 2014) have been shown to mitigate CPF-evoked anxiety-like behaviour, using the frequency of stretch-attends posture in an open-field assessment in Wistar rats. Thus, oxidative damage may have been partly involved in anxiety-like behaviour caused by CPF exposure.

Rearing, also termed vertical movement, like locomotion is a measure of exploration and is inversely proportional to the anxiety status of the animal (Krishna and Ramachandran, 2009). The frequency of rearing decreased significantly in the CPF group, demonstrating the anxiogenic and impaired motor activity effects of CPF. The result agreed with the findings of Nostrandt *et al*. (1997), Karen *et al*. (2001), Terry *et al*. (2003), Ambali (2009) and Idris *et al*. (2014), who reported anxiety-like behaviour and impaired motor activity, characterized by decreased rearing frequency in an open-field arena following exposure to CPF in rats. On the contrary, Chen *et al*. (2014) reported no difference in rearing frequency in rats repeatedly exposed to CPF when compared to the control animals. This

contrary finding may be related to the short duration of exposure (10 days), relative to the duration in the present study.

Although pretreatment with 250 mg/kg of MO leaf extract did not ameliorate this apparent abnormality significantly, the higher dose (500 mg/kg) of the extract did (Figure 4.3). The possible role of oxidative stress in this behavioural aberration may have been validated by the improvement in the frequency of rearing in the MO500+CPF group. This may be due to the antioxidant properties of MO leaves (Siddhuraju and Becker, 2003; Sreelatha and Padma, 2009). This result shows that oxidative stress may be involved in the anxiogenicity (Ambali, 2009; Xu *et al*., 2014) and impaired motor activity (Ambali, 2009) induced by CPF.

Grooming behaviour has been shown to be highly sensitive to a number of experimental factors (Kalueff *et al*., 2007), and this is probably responsible for the undulations observed across the groups throughout the study period. The CPF group had relatively higher frequency of grooming throughout the study period, demonstrating anxiogenesis induced by CPF (Ambali, 2009; Lasisi, 2011). The relatively lower frequency of grooming in the extract pretreated groups when compared to that of CPF group is a demonstration that oxidative stress is partly involved in the CPF-evoked anxiogenic response. Previous studies have shown that antioxidants mitigate increase in frequency of grooming evoked by CPF (Ambali, 2009; Lasisi, 2011)

CPF exerted anxiogenic effects on the animals exposed to it alone, since they showed a relatively steady, but rising defaecation frequency after week 3. This agreed with results obtained in CPF exposed rats in the open-field apparatus (Ambali, 2009; Lasisi, 2011) and elevated plus maze (Malhotra *et al*., 2011). Salazar *et al*. (2011) and Yan *et al*. (2012) reported increased frequency of defaecation following OP toxicity. Thus, the increase

may have resulted from overstimulation of muscarinic receptors (Bhattarai *et al*., 2006), or anxiety due to oxidative damage to the brain (Rammal *et al*., 2008; Bouayed *et al*., 2009; Ambali and Ayo, 2011). The reduced defaecation observed in the MO pretreated rats suggests that MO leaf extract may be anxiolytic in CPF toxicosis, either by protecting the brain cells from oxidative damage, or by attenuating the anticholinesterase effects of CPF, thereby depleting the cholinergic signs.

Following the numerous signs attributed to an anxiogenic effect of CPF observed in other neurobehavioural parameters in the open field apparatus, one would expect that CPF would also increase the frequency of urination (being an index of anxiety). However, that was not the case in this study. Instead the CPF exposed animals showed minimal urination in the open field. The reason for this is not clear, but may be partly due to the nephrotoxic effect of CPF, as less urine is voided by compromised kidneys. Chlorpyrifos has been shown to induce apoptosis in the nephron and tubular cells of the kidneys (Ahmed *et al*., 2010). Pretreatment with MO caused a decrease in the frequency of urination compared to the DW, SO and MO500 groups. The reason for this is not clear and deserves further study.

The beam width at which the CPF-treated rats stepped off the beam increased progressively through the study period. This is consistent with earlier reports (Abou-Donia *et al*, 2003; Ambali *et al*. 2010c; 2012a, b; Ambali and Aliyu, 2012; Krishnan *et al.*, 2012). Abou-Donia *et al*. (2006) also reported beam-walk deficits in adult offspring mice following *in-utero* exposure to CPF. The beam-walk performance deficit recorded in the CPF group of the present study may be indicative of cortical and cerebellar injury (Potts *et al*., 2009), which may have resulted from oxidative stress (Masoud and Sandhir, 2012). Also, cortical and cerebellar neuronal apoptosis may have been triggered by the

excessive ROS generated due to CPF exposure (Caughlan *et al*., 2004; Yu *et al*., 2008; Gupta *et al*., 2010; Lee *et al*., 2012). This is verifiable from the histopathological lesions of widespread cellular degeneration and apparent apoptosis observed in the brain sections of CPF-exposed rats.

Pretreatment with methanol leaf extract of MO (250 mg/kg and 500 mg/kg) before exposure to CPF, however, ameliorated this beam-walk performance deficit. This may be attributed to oxidative damage to the brain. Brain histopathology in the MO pretreated group showed fewer degenerated neurones compared to CPF, indicating less brain oxidative damage. This may be attributed to the high flavonoid content, and high antioxidant capacity of the extract (Ambali *et al*., 2010c). Flavonoids have been shown to have potent antioxidant and free radical scavenging abilities (Heim *et al*., 2002). Ishige *et al*. (2001) reported that flavonoids protect neuronal cells from oxidative stress by increasing intracellular glutathione, directly lowering levels of ROS, and preventing the influx of Ca^{2+}, despite high levels of ROS. Apart from flavonoids, other non-flavonoid phenols that are part of the total phenolic content of the extract and its vitamin C and E contents may have protected the neuronal cells from extensive oxidative damage and apoptosis, hence averted the CPF-induced beam-walk deficits.

The present study, similar to previous studies (Ambali and Ayo, 2012; Ambali and Aliyu, 2012) showed that animals exposed to CPF missed fewer number of rungs when compared to the control, which worsened with the duration of exposure. Similarly, Richendrfer *et al*, (2012) reported that CPF interfered with the speed of locomotion of zebra fish larvae. This is indicative of poor efficiency of locomotion. The reluctant, slow movement of the CPF-exposed animals is a plausible explanation for their missing fewer rungs than the control animals. According to Ambali and Ayo (2012), the legs of the rats

were held stationary above the rungs for a relatively longer period, hence they missed fewer number of rungs on the ladder. The locomotion deficit can be related to deficits associated with CPF exposure as observed in the open field apparatus in chicks (Al-Badrany and Mohammad, 2007), and demonstrated in the present study. These deficits could be partly due to either oxidative damage to the muscles (Ambali and Aliyu, 2012) or impairment of neuronal transmission due to down-regulation of cholinergic receptors following prolonged acetylcholinesterase inhibition (Ray, 1998). This down-regulation of cholinergic receptors following prolonged AChE inhibition could be responsible for the decline in ladder walk score observed with increasing duration of exposure to CPF. However, pretreatment with flavonoid rich MO leaf extract improved the ladder walk score, validating the role of oxidative stress in this motor dysfunction. This result is similar to those reported by Ambali and Aliyu (2012) and Ambali and Ayo (2012), in which vitamin E and C, respectively attenuated ladder walk score deficits induced by CPF exposure.

The inclined plane test was used to test muscle performance capacity, and thus neuromuscular coordination of the rats (Chang *et al*., 2008). The results of this study indicate that exposure to CPF induced deficits in neuromuscular coordination as seen in the steady decrease of the slip-angle with time in the CPF exposed animals. This is consistent with the findings from previous studies (Ambali *et al*., 2010c; 2012a, b; Ambali and Aliyu, 2012; Krishnan *et al*., 2012) in which CPF exposure in rats induced deficits in neuromuscular coordination. The possible reason for this observed deficit is oxidative damage of cells in the motor centres of the cerebral cortex, as well as ROS-evoked apoptosis of neuronal cells (Ambali and Aliyu, 2012; Ambali *et al*., 2012a, b). This again brings to the fore the lesions of widespread neuronal degeneration and apparent apoptosis observed at histopathology in the CPF exposed group. Giordano *et al*. (2007) reported

that CPF and its metabolites (CPF-oxon and TCP) exerted varying degrees of neurotoxicity, which involves generation of ROS and may involve disturbances in intracellular homeostasis of calcium. Similarly, Yu *et al*. (2008) reported that CPF exposure alters calcium homeostasis in mouse retina and evoked oxidative stress induced apoptosis of the retina cells.

The result of the present study showed that pretreatment with MO attenuated CPF-induced deficits in neuromuscular coordination. This is evident, as pre-treated rats showed lower inclined plane performance deficits when compared to the animals exposed to CPF only, and it agrees with the findings of Giordano *et al*. (2007), Yu *et al*. (2008) and Ambali *et al*. (2012a). The high phenolic content of MO leaf extract, and specifically its high flavonoid content, as well as the high vitamins C and E contents of the leaves could be responsible for this attenuating effect on CPF-evoked deficit in neuromuscular coordination observed in the current study. Ambali *et al*. (2012a) reported that rats pre-treated with methanol extract of *Phyllanthus niruri*, a flavonoid rich plant before exposure to CPF showed improved neuromuscular coordination, on the inclined plane. Pre-treatment with antioxidants, including, intracellular glutathione (Giordano *et al*., 2007), and vitamin C and vitamin E (Yu *et al*., 2008) have been reported to attenuate CPF-induced neurotoxicities.

The decrease in fore-limb grip time in relation to the duration of exposure observed in the CPF group is consistent with the reports of Ambali *et al*. (2010c) and Ambali and Ayo (2012), and indicative of deficit in motor strength following exposure to the insecticide. This result could also be related to paralysis observed at sites of dermal exposure to CPF in a 61-year-old carpenter as reported by Meggs (2003). The decline in motor strength following CPF exposure may be due to oxidative damage to the muscle tissues, reducing

the ability of the muscles to carry weight (Ambali *et al*., 2012a). It may also result from the down-regulation of cholinergic receptors. Following prolonged acetylcholinesterase inhibition, reduced expression of cholinergic receptors in the neuromuscular junction may result, thus, reduced muscle stimulation and consequently, reduced muscle strength is observed (Biebuyck *et al*., 1992; Abdollahi and Karami-Mohajeri, 2012; Moshiri *et al*., 2012).

In addition to the afore-mentioned factors, the effect of CPF on axonal transport could be implicated in this apparent pathology. Terry *et al*. (2003, 2007) reported that repeated exposures to sub-threshold doses of CPF may lead to behavioural abnormalities, and muscle weakness, which could be attributed to CPF inhibition of fast anterograde and retrograde axonal transport. Steeland *et al*. (2000) reported that CPF exposed termiticide applicators had significantly lower nerve conduction velocity, when compared to non-exposed control subjects. Although not fully understood, the mechanism of this altered axonal transport may involve organophosphorylation of tubulin and disruption of microtubule structures, thereby altering the neuronal cytoskeleton (Jiang *et al*., 2010). The number, structure and axonal transport of the neuronal mitochondria is also altered in CPF toxicity, even at apparently sub-toxic doses (Middlemore-Risher *et al*., 2011).

Pre-treatment with MO leaf extract (250 mg/kg and 500 mg/kg) before exposure to CPF eliminated this motor strength deficits. This again may have resulted from the mitigation of oxidative stress by flavonoids and other antioxidants contained in the extract, thereby retaining (relatively) neuronal cytoskeleton and mitochondria structure and function; and maintaining fast anterograde and retrograde axonal transport. Similar results were obtained by other investigators (Ambali *et al*., 2010c; 2012c; Ambali and Aliyu, 2012; Ambali and Ayo, 2012) who demonstrated that vitamin C, methanol extract of

Phyllanthus niruri, vitamins C + E, respectively, attenuated the adverse effects of CPF on motor strength.

This study demonstrated that exposure to CPF caused a steady decline in the physical and mental alertness of the rats as observed in the lower excitability scores of the CPF group. This result is consistent with the report of Ambali and Ayo (2012), and is indicative of poor sensorimotor reflex and neuromuscular coordination. Ambali and Ayo (2012) showed that the deficit was due to the impairment of AChE activity, altering neuronal activities in the nervous tissue.

The results obtained show that treatment with MO before CPF exposure improved the excitability of the central nervous system of rats. This could have resulted from a possible antagonism of the anticholinesterase effect of CPF by MO extract. Several antioxidants and antioxidant-containing extracts that have been shown to attenuate AChE inhibition in CPF toxicity include methanol extract of *Phyllanthus niruri* (Ambali *et al*., 2012a), vitamin E (Ambali and Ayo, 2012) and vitamin C (Ambali *et al*., 2010c).

The steady decrease in swimming time through the period of the experiment in the CPF exposed rats is indicative of increasing despair and depression as daily exposure progressed. This validates earlier reports by Chen *et al*. (2011, 2012a) and Lee *et al*. (2007) who reported a direct relationship between CPF exposure and incidence of depression and suicide in Iowa and North Carolina, USA. CPF exposure leads to despair behaviour (Chen *et al*., 2014) which may be due to alterations in serotonergic systems, probably caused by oxidative stress. Several authors have reported the involvement of serotonergic mechanisms in the developmental toxicity of CPF and have linked defects of this system to behavioural deficits in adulthood (Raines *et al*., 2001; Aldridge *et al*., 2004; Aldridge *et al*., 2005; Slotkin and Seidler, 2005). Pre-treatment with MO decreased

the depression behaviour as the animals swam for a longer period, and were apparently less depressed than the CPF-only group. This effect may be due to the antioxidant effect of the leaf extract, protecting serotonergic systems and other associated tissues in the brain from oxidative damage.

The high number of foot shocks received by rats in the CPF group is indicative of impairment in learning acquisition induced by CPF exposure. Similar results of CPF-induced learning impairment were reported by Cohn and Macphail (1997), Ambali *et al*. (2010c, 2012a, b, c), Ruiz-Muñoz *et al*. (2011), Ambali and Ayo (2012) and Ambali and Aliyu (2012). Jett *et al*. (2001), Terry *et al*. (2003) and López-Granero *et al*. (2013) showed that repeated CPF exposure at sub-threshold doses in rats caused deficits in spatial learning. The underlying mechanism of this deficit in cognition may involve alterations in mitochondrial dynamics and/or their transport in axons (Middlemore-Risher *et al*., 2011), and oxidative damage to relevant brain portions (cerebral cortex and hippocampus) responsible for learning acquisition (Ambali *et al*., 2010c) as observed in the brain histoarchitecture of CPF exposed rats in this study. Several investigators (Ghadrdoost *et al*., 2011; Alzoubi *et al*., 2012; Patki *et al*., 2013) have demonstrated that oxidative stress induces impairment of cognitive function. Cognitive impairment in ageing has also been related to the oxidative damage to synapses in the cerebral cortex and hippocampus (Fukui *et al*., 2001, 2002; Liu *et al*., 2003; Haxaire *et al*., 2012). In addition to oxidative stress, AChE inhibition at synapses that results from CPF exposure may inhibit synaptic plasticity, and thus impair learning acquisition (Ambali *et al*., 2010c). CPF has been reported to inhibit axonal growth via inpairment of the morphogenic activity of AChE (Yang *et al*., 2008). Conner *et al*. (2003) reported that disrupting basal forebrain cholinergic function may abolish cortical plasticity and impede learning.

In this study, MO leaf extract attenuated the learning acquisition deficit induced by CPF exposure, probably through the antioxidant and AChE restorative activity of its flavonoid and antioxidant vitamins' components. Antioxidants have been shown to mitigate oxidative stress-induced deficits in learning acquisition (Fukui *et al*., 2002; Liu *et al*., 2003; Ghadrdoost *et al*., 2011; Ambali and Ayo, 2012; Ambali and Aliyu 2012). Furthermore, flavonoids exert the same mitigation effects on CPF-induced learning deficit (Ambali *et al*., 2010c; 2012a). Rendeiro *et al*. (2012) reported that flavonoids and their metabolites interact with, and modulate critical signaling pathways, transcription factors and gene and/or protein expression, which control memory and learning processes in the hippocampus; the brain structure where spatial learning occurs.

The relatively low amount of time spent on the platform in the step-down apparatus by the CPF group was indicative of short-term memory deficit induced by CPF exposure. This is consistent with the findings in previous studies (Bushnell *et al*., 1993, Clegg and Van Gemert, 1999; Ambali *et al*., 2010c; 2012a; Ruiz-Muñoz *et al*., 2011; Ambali and Ayo, 2012; Ambali and Aliyu, 2012). Pre-pubertal guinea pigs exposed to a single sub-lethal dose of CPF showed significant memory retention impairment (Mullins *et al*., 2013). Steenland *et al*. (2000) reported that termiticide applicators exposed to CPF reported more symptoms of memory loss than the non-exposed control subjects. Rauh *et al*. (2011) reported deficits in working memory index and full-scale IQ as a function of prenatal CPF exposure at seven years of age. However, the results of this study contradicts the report of Maurissen *et al*. (2000), who reported that adult Long-Evans rats did not show short-term memory deficits after four weeks of daily exposure to CPF and this may be attributed to the shorter duration of exposure, as compared to the present study. Although cautiously excluding CPF applicators and workers with low educational levels from the inferences of their research, Berent *et al*, (2014) reported that CPF

manufacturing workers did not exhibit any evidence for impaired neurobehavioural domains, including verbal memory domain score.

The reason for the short-term memory deficits recorded in the CPF exposed animals may be related to alterations in mitochondrial dynamics and/or their transport in axons (Middlemore-Risher *et al*., 2011), as well as oxidative damage to brain portions responsible for memory (Ambali *et al*., 2010c), including the lobes of the cerebral cortex, specifically the parietal and temporal lobes for short term memory (Jonides *et al*., 2008). The widespread neuronal cellular degeneration in the CPF group, apparently due to oxidative stress and subsequent apoptotic damage, may have contributed to the memory deficit. Pretreatment with methanol extract of MO (250 mg/kg and 500 mg/kg) attenuated the short-term memory deficit, and the altered histoarchitecture. This may have resulted from the antioxidant neuro-protective action of antioxidants contained in the extract. In addition, the flavonoid components, their metabolites and the antioxidant vitamins component of the MO extract may have interacted with and modulated critical signalling pathways, transcription factors and gene and/or protein expression that control memory (Rendeiro *et al*., 2012).

Brain acetylcholinesterase activity was lowest in the rats exposed to CPF only. This is a direct consequence of CPF exposure, since cholinesterase inhibition is the mechanism by which OP pesticides exert toxic effects on target and non-target species (Lee *et al*., 2011). Similar results were reported by previous workers (Ambali *et al*, 2010c; 2012a, b, c; Marty *et al*, 2012; Ambali and Ayo, 2012; Ambali and Aliyu, 2012; Newairy and Abdou, 2013; Mohammad *et al*., 2014).

But for accessibility and convenience of ante-mortem collection of brain tissue samples, the AChE status of the brain should be the bio-marker for OP toxicity and/or exposure.

Brain AChE status is, therefore, replaced by erythrocyte AChE or plasma cholinesterase status as biomarkers of OP (and other AChE-inhibitors) toxicity (Jokanović and Kosanović, 2010). It is, therefore, not surprising that brain AChE activity, using brain tissue homogenate has been serially reported as low for animals exposed to apparently sub-toxic doses of CPF (Ambali and Ayo, 2012; Ambali *et al*., 2011c; 2012a, b; Akande *et al*., 2014, Idris *et al*., 2014). Farahat *et al*. (2011) reported an inverse correlation between urinary TCPy (a CPF biomarker) and blood butyrylcholinesterase (BuChE) and AChE activities in Egyptian cotton field workers. Chlorpyrifos, like other OP pesticides inhibits AChE directly through occupation of the esteritic site of the enzyme, and indirectly through oxidative stress (Ambali *et al*., 2012a), resulting in ACh build-up at synapses, and leading to over-stimulation of post-synaptic tissues. In the present study, treatment with MO leaf extract only has been shown to also inhibit brain AChE activity, but to a lesser extent. This may be linked to the AChE inhibition effect of flavonoids. Flavonoids have been widely investigated and found to be inhibitors of AChE, and may have partly formed the basis for the exploration of their potential use as drugs for the symptomatic treatment of Alzheimer's disease (Uriarte-Pueyo and Calvo, 2011). Akkol *et al*. (2012) reported that certain flavonoids isolated from *Cistus laurifolius* leaves inhibited AChE activity, and some flavonoids and their derivatives have been known to bind simultaneously to the peripheral and catalytic sites of the enzyme (Shen *et al*., 2009). Apart from this, the redox status of some flavonoids, and the vitamins C and E constituents of the MO leaf may have caused pro-oxidation, which may affect the activity of membrane–bound enzymes, including AChE (Ambali *et al.*, 2012c).

Pretreatment with MO methanol leaf extract (250 mg/kg and 500 mg/kg) attenuated CPF-induced AChE inhibition. Several antioxidants and antioxidant sources including vitamin C (Ambali *et al*., 2010c; Ambali and Ayo, 2012), vitamin E (Ambali *et al*., 2012b),

Phyllanthus niruri (Ambali *et al*., 2012c), propolis (Newairy and Abdou, 2013), grape seed extract (Singh *et al*., 2013) and *Hibiscus sabdariffa* extract (Shittu *et al*., 2014) have been demonstrated to have AChE restorative property. This may have resulted from the antioxidant protection of the cell membranes and AChE (being a membrane-bound enzyme) from oxidative damage (Ambali *et al*., 2012c). The protection of AChE from oxidative damage by other antioxidant components of the extract such as vitamins A, C and E may have contributed to this attenuation.

Since MDA concentration is indicative of the level of lipid peroxidative changes in a tissue, increased oxidative damage can be inferred from the elevated brain MDA concentration in the rats exposed to CPF in this study. The increased lipoperoxidative changes in the brain may have been partly responsible for the widespread cellular degeneration and apparent apoptosis observed histologically in the brains of CPF exposed rats. This is consistent with the reports of previous workers (Yu *et al*., 2008; Ambali *et al*, 2012a, b; Ambali and Ayo, 2012; Ambali and Aliyu, 2012; Kalender *et al*., 2012; Singh *et al*., 2013b; Singh and Bhatia, 2013; Wang *et al*., 2014) that showed that CPF-exposure increased lipid peroxidation in brain tissues. The brain is particularly prone to oxidative stress because of its high use of energy (oxygen and glucose), high peroxidizable fatty acid content, and paucity of antioxidant defences (Floyd, 1999). Also attributable to oxidative stress, CPF has been shown to induce significant changes in nerve cell morphology, inhibit anti-inflammatory cytokines and influenced important protein signalling pathways which are involved in regulation of apoptosis in the CNS (Schäfer *et al*., 2013). These changes are often only observed in histological sections of the brain and parts of the nervous system. Several investigators have reported depletion in the number of viable neurones and glial cells in the brain (Roy *et al*., 2004; Farag *et al*., 2010; Wang *et al*., 2013a). Often, there is also loss of dendrite and spine processes of

neurones in brain regions (Ruiz-Muñoz *et al*., 2011). Singh and Panwar (2013) reported histological sections that showed damage to brain morphology with increased protein levels of caspase-3 (a mediator of apoptosis) in CPF-treated animals.

Pretreatment with MO leaf extract (250 mg/kg and 500 mg/kg) attenuated the elevated lipid peroxidation induced by CPF exposure as seen in the lower MDA levels, as well as fewer degenerative neuronal cells observed histologically in the brain of pretreated rats. This again validates the reported antioxidant properties of MO leaves (Sreelatha and Padma, 2009; Verma *et al*., 2009; Atawodi *et al*., 2010; Mansour *et al*., 2014), apparently due to the high contents of flavonoids and antioxidant vitamins such as vitamins C and E contained in it. Excessive ROS produced in cells as a consequence of CPF exposure are capable of overwhelming the antioxidant defence system (Bai *et al*., 2014; Li *et al*., 2014). It is therefore only logical that exogenous antioxidant supplementation would not only restore the cell oxidant status, but also prevent oxidative damage and its features in exposed cells (Karthivashan *et al*., 2013). This logic has been investigated and proven true by several authors, using various antioxidants (Ambali *et al*., 2010b; Aly *et al*., 2010; Demir *et al*., 2011; Elsharkawy *et al*., 2012; Shittu *et al*., 2012a, b; Uchendu *et al*. 2013).

Apart from pure antioxidant chemicals, antioxidants in crude extracts have been explored in these investigations, and interesting results have been obtained. For example, Singh *et al*. (2013b) reported that grape seed extract significantly ameliorated CPF-exposure sequelae, such as increased lipid peroxidation levels and inhibition in brain AChE, SOD and CAT activity, and decreased reduced glutathione concentration in the brain. Ambali *et al*. (2012c) reported that pretreatment with methanol extract of *Phyllanthus niruri* ameliorated sensorimotor cognitive and lipid peroxidation changes induced by subacute chlorpyrifos exposure, and the flavonoid content of this extract was reported as

responsible for this response. Green tea extract has also been shown to attenuate CPF-induced renal toxicity, hepatotoxicity, lipid peroxidation and tissue antioxidant status in rats (Heikal *et al*., 2012b, 2013). Whereas the antioxidant vitamins act directly by scavenging ROS (Bjelakovic *et al*., 2013), the mechanisms by which flavonoids exert antioxidant effects include suppressing ROS formation, either by inhibition of enzymes or chelating trace elements involved in free radical production, scavenging ROS formed and up-regulating or protecting antioxidant defences (Pieta, 2000). Wang *et al*. (2014) demonstrated that hydrogen molecules protected rats from oxidative stress-induced neuronal damage.

A decline in brain SOD activity was observed in the rats exposed to CPF only. This is indicative of oxidative stress and can be attributed to increased generation of ROS due to CPF exposure as reported by Bagchi *et al*. (1995) and Lee *et al*. (2012). CPF has been shown to decrease brain SOD activity by several investigators (Ambali *et al*., 2010c; 2012a, b, c; Kalender *et al*., 2012; Demir *et al*., 2011; Umosen *et al*., 2012). Demir *et al*, (2011) attributed this decrease to the saturation of SOD during the process of dismutation of excessive superoxide radical produced by CPF exposure. SOD is the only eukaryotic enzyme capable of detoxifying superoxide radical, an initiator of radical chain reaction (Van Raamsdonk and Hekimi, 2012). It catalyzes the conversion of superoxide radical to hydrogen peroxide, which may be subsequently converted to water by catalase or peroxiredoxin (Barbosa *et al*., 2014). The fact that all aerobic organisms, and even some anaerobic ones, express SOD underscores the importance of this enzyme (Kabel, 2014).

Pre-treatment with MO mitigated the CPF-induced SOD activity decline. This may have confirmed the antioxidant properties of MO leaves, and agrees with the report of earlier workers (Zama *et al*., 2007; Newairy and Abdou, 2013; Singh and Panwar, 2013) who

observed protective effects of several antioxidants and antioxidant sources against CPF-induced depletion of SOD specific activity. Uzun and Kalender (2013) reported that catechin and quercetin, two common flavonoids exhibited protective effects on CPF induced SOD depletion in Wistar rats.

Exposure to CPF only induced a decline in brain GP_X activity. Similar results were obtained by other authors (Verma and Srivastava, 2003; Goel *et al.*, 2005; Aly *et al.*, 2010; Kalender *et al.*, 2012). This decline could be attributed to oxidative damage of the enzyme by ROS, especially the superoxide radical. The superoxide radical may directly inactivate several enzymes like GP_X, if not scavenged effectively (Shah *et al.*, 2013). GP_X is the major protective enzyme against H_2O_2 toxicity. It reduces H_2O_2 to water using glutathione as a substrate, thereby hindering the formation of hydroxyl radicals (the most noxious of the ROS) via iron-catalyzed Fenton-type reactions (Barlow-Walden *et al.*, 1995).

Pre-treatment with MO leaf extract, however, ameliorated this GP_X decline, probably by scavenging much of the excessive superoxide radicals, generated by the CPF exposure. The antioxidants present in the extract would be responsible for these effects which agree with the report of Goel *et al* (2005) and Aly *et al* (2010), using zinc and vitamin C as the antioxidants, respectively.

Unlike other antioxidant enzymes in this study, exposure to CPF did deplete the brain CAT activity, but not as much as exposure to MO only or as a pretreatment to CPF exposure. The decrease in catalase activity due to CPF exposure may have resulted from oxidative damage to the enzyme as a result of reactions with superoxide radical. Like GP_X, superoxide radical is capable of inactivating CAT. Thus if not scavenged effectively, a depletion of CAT is expected (Shah *et al.*, 2013).

The depletion of CAT specific activity in animals exposed to MO leaf extract only is attributable to a possible binding of the enzymes to flavonoid components of the extract. Zhu *et al*. (2007) reported that flavonoids, quercetin and myricetin bind to CAT when mixed and they alter the conformation of the enzyme. Krych and Gebicka (2013) reported that flavonoids inhibit CAT. In their words "the inhibition is, at least partially, due to the formation of hydrogen bonds between catalase and flavonoids", and they reported detecting an unreactive catalase compound, formed in the presence of flavonoids. It, therefore, can be implied that the lower level of CAT activity recorded for the MO pre-treated animals (when compared to the animals exposed to CPF only or MO only) may be partly attributed to the binding with flavonoids to form unreactive compounds.

On the whole, the ameliorative effects of the methanol extract of MO in this study is not dose-dependent. In most cases, the MO250+CPF group performed better than the MO500+CPF group. This may indicate that MO extracts may have pro-oxidant effect, when administered at higher doses, perhaps due to redox status. Vitamin C, a component of the MO extract, has been shown to have pro-oxidant effect (Podmore *et al*., 1998; Putchala *et al*., 2013; Grosso *et al*., 2013; Chakraborthy *et al*., 2014), therefore, the selection of appropriate doses is critical in using MO to mitigate oxidative damage induced by pesticides. This requires further investigation and the elucidation of active constituents of the leaf extract via bioassay guided methods.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, this study has shown that:

1. Methanol leaf extract of MO ameliorated the CPF-induced neurobehavioural and cognitive changes in Wistar rats.
2. Methanol leaf extract of MO mitigated the CPF-evoked brain oxidative status as evidenced by reduced brain MDAconcentration and increased activities of antioxidant enzymes (SOD and GP_X), in addition to improving brain AChE activities.
3. Pretreatment with MO leaf extract protected nerve tissues from oxidative stress by mitigating neuronal degeneration, evoked by subchronic CPF exposure.
4. The ameliorative effect of pretreatment with MO was not dose-dependent, but the MO250+CPF group showed better amelioration in some of the CPF-evoked clinical, neurobehavioural and oxidative changes than the MO500+CPF group. This shows that MO leaves may have a pro-oxidant effect when given at higher doses.

6.2. Recommendations

6.2.1 Specific recommendations

1. Individuals that are likely to be occupationally and environmentally exposed to CPF and other organophosphate insecticides should use protective devices to reduce the risk of exposure.
2. Individuals that are likely to be exposed to CPF and other organophate insecticides should be encouraged to prophylactically take flavonoid-containing substances

such as MO, within the safety limits, to assist in mitigating CPF-induced pathologic changes.

3. Further research should be carried out to identify the exact constituent(s) of this MO leaf extract that is most effective in the protection of neurones from oxidative damage, and they should be harnessed for this purpose in pesticide exposed populations.
4. Further studies on the other associated mechanisms of action of MO in mitigating the neurobehavioural and pathologic changes induced by CPF should be conducted.

6.2.2 General recommendations

1. The government should enact laws that restrict the domestic use of CPF and control its use in agriculture.
2. The government should enact laws that promote the use of protective gears by pesticide applicators and other risk populations, and ensure strict compliance to these laws.
3. The general public should be sensitized on the dangers of indiscriminate use of pesticides.
4. Periodic evaluation of health status of individuals and animals who are frequently exposed to low-dose CPF should be carried out to enable them seek for professional help and counselling.

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