PREVENTIVE EFFECT OF FISETIN ON NEUROBEHAVIOURAL, OXIDATIVE STRESS AND HISTOPATHOLOGICAL CHANGES INDUCED BY SUBCHRONIC EXPOSURE TO CHLORPYRIFOS IN MALE ALBINO MICE

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIAIN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN VETERINARY TOXICOLOGY

DEPARTMENT OF VETERINARY PHARMACOLOGY AND TOXICOLOGY,

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ZARIA

JUNE, 2017

DECLARATION

I declare that the work in this dissertation, entitled **"PREVENTIVE EFFECT OF FISETIN ON NEUROBEHAVIOURAL, OXIDATIVE STRESS AND HISTOPATHOLOGICAL CHANGES INDUCED BY SUB-CHRONIC EXPOSURE TO CHLORPYRIFOS IN MALE ALBINO MICE**" has been carried out by me in the Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria. The information derived from the literature has been duly acknowledged in the text and in the list of references provided. No part of this Dissertation was previously presented for another degree or diploma at any other Institution.

Amaka Rosita AKPA ---------- ----------

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CERTIFICATION

The dissertation, entitled "**Preventive Effect of Fisetin on Neurobehavioural, Oxidative stress and Histopathological Changes Induced by Sub-chronic Exposure to Chlorpyrifos in Male Albino Mice" b**y Amaka Rosita AKPA meets the regulations governing the award of an MSc degree in Veterinary Toxicology and is approved for its contribution to knowledge and literary presentations.

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DEDICATION

This dissertation is dedicated to Almighty God for seeing me through this programme, my parents for their provision and assistance and to all who contributed in one way or the other to the success of this work.

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ABSTRACT

Chlorpyrifos (CPF) is one of the common neurotoxicants in Nigeria and the world. Fisetin as an antioxidant (3,3′,4′,7tetrahydroxyflavone) has been used to ameliorate deleterious effects of various neurotoxicants. The aim of the study was to evaluate the preventive effect of fisetin on neurobehavioural, histopathological and oxidative damages, induced by sub-chronic exposure of male mice to CPF. The mice were acclimatised for two weeks and grouped into six groups. Group 1 mice were exposed to distilled water, group 2 was exposed to soya oil at 2 ml/kg, group 3 was exposed to 1% dimethyl sulphoxide at 5 mg/kg, group 4 was exposed to CPF at 6.6 mg/kg, group 5 was administered fisetin at 15 mg/kg, while group 6 was administered fisetin at 15 mg/kg followed by CPF at 6.6 mg/kg 30 minutes later. The mice were administered with this regimen once daily by oral gavage for fifty days, after which they were sacrificed and the organs and tissues harvested for further studies. The neurobehavioural evaluations were carried out on day 0, weeks 3 and 6. Open-field assessment was done using the open-field apparatus, neuromuscular coordination was evaluated using inclined plane and locomotor efficiency was evaluated using ladder walk. The determination of motor strength was done using the forepaw grip time, while excitability score was assessed using the ordinal scale excitability scores. Depression was also evaluated using the forced swimming test and learning and short-term memory was assessed using the step-down inhibitory avoidance task apparatus. The brain sample from each group was used to evaluate CPF-induced histopathological and lipoperoxidative changes, through determination of brain malondialdehyde concentration. The brain was also used to assess activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), using their appropriate kits. The result showed that fisetin significantly ($P < 0.05$) prevented CPF induced impairments in neuromuscular coordination ($67.08 \pm 1.24°$) and locomotor efficiency (4.42 ± 0.64). It also significantly ($P < 0.05$)

ameliorated CPF effect on brain lipoperoxidative damages MDA (173.90 ± 18.77 nmol/mg protein), activities of antioxidant enzymes, CAT (78.55 ± 7.37 µ/mg protein), GPx (73.96 ± 4.97 mol/min/mL), SOD (143.00 ± 11.09 µ/mL) and acetylcholinesterase activity (11.60 ± 0.55 nmol/min/mL). Fisetin significantly ($P < 0.05$) mitigated the toxic effects of CPF on the serum lipid profiles of low density lipoprotein (1.14 ± 0.31 mg/gL), high density lipoprotein (0.75 ±0.13 mg/gL), total cholesterol (2.10 ± 0.34 mg/gL) and serum triglycerides (1.10 ± 0.25 mg/gL). It was concluded that fisetin prevented the sub-chronic CPF-induced toxic effects on neurobehavioural, histopathological and oxidative damages in adult male Albino mice.

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LIST OF ABBREVIATIONS

5-HT	Serotonin.
Ach	Acetylcholine.
AChE	Acetylcholinesterase.
ANOVA	Analysis of variance.
ATChI	Acetylcholine iodide.
CAT	Catalase.
CNS	Central nervous system.
COPIND	Chronic organophosphate induced neuropsychiatric disorder.
CPF	Chlorpyrifos.
D/W	Distilled water.
DDT	Dichlorodiphenyltrichloroethane.
DMSO	Dimethylsulphoxide.
DNA	Deoxyribo ucleic acid.
DTNB	Disthiobisnitrobenzoate.
E	Eosin.
EDSA	Endocrine disruption screening programme.
EJF	Environmental Justice Federation.
F	Fisetin.
F	Frequency.
FDA	Food and drug administration.
GPx	Glutathione peroxidase.
GSH	Glutathione.
H	Haematoxylin.

HDL	High Density Lipoprotein.
IMS	Intermediate Syndrome.
LD_{50}	Median Lethal Dose.
LDL	Low Density Lipoprotein.
MDA	Malondialdehyde.
OP	Organophosphate.
OPICN	Organophosphate-induced chronic neuro toxicity.
OPIDP	Organophosphate-induced delayed polyneuropathy.
ROS	Reactive oxygen species.
S	Seconds.
S/oil	Soya oil.
SEM	Standard Error of Mean.
SOD	Superoxide Dismutase.
TBA	Thiobarbituric acid.
TBARS	Thiobarbituric acid reactive substances.
USDA	United States Department of Agriculture.
USEPA	United States Environmental Protection Agency.
XO	Xanthine oxidase.

CHAPTER ONE

1.1 Background of the study

Organophosphates (OPs) constitute a heterogeneous category of chemicals, specifically designed for the control of pests, weeds or plant diseases (Yinhui *et al.,* 2015). Their application is still the most effective and accepted means for protection of plants from pests, and has contributed significantly to enhanced crop yields (Mathews *et al.,* 2011). Organophosphate (OP) pesticides are the most common and widely used pesticides in agriculture and in the household (Lovasi *et al.,* 2011). Theyhave been widely used for decades in agriculture for crop protection and pest control. Thousands of these compounds have been screened and over 100 of them have been marketed for these purposes(Subash *et al.,* 2010). Organophosphates affect the nervous system by disrupting the enzyme that regulates acetylcholine, a neurotransmitter. The OP insecticides inhibit acetylcholinesterase activity by binding to the active sites of the enzyme, preventing the break-down of acetylcholine in the nervous system of the insect or pest (Kwong, 2002; Marks *et al.,* 2010). This results in the accumulation of acetylcholine in nerve endings which causes continuous stimulation of cholinergic receptors (Timofeeva *et al.,* 2008), eventual paralysis and death (Ogueji *et al.,* 2013). The OPs were developed during the early 19th century, but their effects on insects are similar to those observed in humans. Animal studies have linked OP exposure to sensorimotor and cognitive impairments (Ambali *et al.,* 2010b). The OP compounds are most commonly associated with serious human toxicity, accounting for more than 80% of pesticide-related hospitalisations.OPs are taken in via the oral, respiratory or transdermal routes (Poojara *et al.,* 2003) is most common among farmers, and they constitute gradually-increasing cause of accidental and suicidal poisoning; with high morbidity and mortality (Poojara *etal.,* 2003).

Chlorpyrifos (CPF) is the most common used broad-spectrum chlorinated OP insecticide. It is used on fruits, grains, nuts, vegetables, livestock, ornamentals, golf courses, buildings, and for treating wood products (Tirelli*et al.,* 2007). It exerts its effects on the nervous system and its primary mode of action is by inhibiting the break-down of acetylcholine (ACh), a neurotransmitter (Smegal, 2000). When insects are exposed, CPF binds to the active site of the acetylcholinesterase (AChE) enzyme, which prevents the break-down of ACh in the synaptic cleft (Karanth and Pope, 2000). Neonates and the young are more susceptible than adults to adverse effects from exposure to CPF at levels below those causing AChE inhibition (Smegal, 2000). Thus, CPF is a developmental neurotoxicant affecting even developing foetus (Saunders *et al.,* 2012), and its low doses disrupt brain development and cognitive function (Slotkin and Seilder, 2005). Apart from effect of CPF on ACh, other neurotransmitters are also affected, including the monoamines, noradrenaline, dopamine and serotonin (Raines *et al.,* 2001). Exposure of CPF to the foetus or neonates impair brain development, synaptic function and behaviour (Dam *et al.,* 2000). Although inhibition of AChE is the primary mechanism of toxicity of CPF, studies have shown that toxicity also occurs at doses that do not inhibit acetylcholinesterase activity or long after its restoration (Haviland *et al.,* 2010; Goodman *et al.,* 2013). Otherimportant mechanism implicated in CPF toxicity, is induction of oxidative stress andexcessive production of reactive oxygen species (ROS), identified as a major mechanism of CPF toxicity (Ambali and Ayo, 2011; Lee *et al.,* 2014).

Oxidative stress is a state of increased generation of reactive oxygen species (ROS), which exceeds the scavenging capacity of the antioxidant systems in the body. The shift in the balance between oxidants and antioxidants in favor of oxidants is termed oxidative stress (El Erain *et al.,* 2015). Oxidative stress exerts toxic effects on cells (Misra *et al.,* 2009). The ROS are small, highly reactive, oxygen-containing molecules that are generated in

small amounts during the body's metabolic reactions, and can react with, and damage complex cellular molecules such as fats, proteins, or DNA. Antioxidants are agents which inhibit and scavenge ROS; Thus,protecting tissues against the adverse effects of CPF. (Beam *et al.,* 2014; Michael and Peter, 2015). Oxidative stress has been previously reported in CPF poisoning (Ambali *et al.,* 2010a, 2010b). Supplementation with exogenous antioxidants has been shown to ameliorate CPF-induced adverse health effectsin rats (Ambali *et al.,* 2012,2010).

Fisetin (3,3',4',7-tetrahydroxyflavone), is one of the naturally-occurring flavonoids, found in fruits and vegetables (Jayakumar and Subramanian, 2013). It is frequently added to nutritional supplements due to its antioxidant properties (Jing-Yuan *et al.,* 2014).Fisetin, is found in strawberry, persimmon, grape and cucumber (Adhami *et al.,* 2012; Syed *et al.,* 2016). As a hydrophobic compound, fisetin easily penetrates cell membranes, accumulating in cells to exert its antioxidative effects (Ishige *et al.,* 2001; Subramanian *et al.,* 2015). It has a wide-range of biological activities, including anti-ageing, anti-inflammatory (Khan *et al.*2013), neuroprotective (Zheng *et al.,* 2008; Prakash *et al.,* 2013) and anticancer effects (Zbarsky *et al.,* 2005; Ash *et al.,* 2015; Meng *et al.,* 2017). After oral administration, fisetin also passes the blood-brain barrier to exert neuroprotective and cognition-enhancing effects in animal model of CNS disorders (Chiruta *et al.,* 2012). Importantly, fisetin attenuates postischaemic immune cell infiltration and reduces infarct size after transient occlusion of cerebral middle artery in mice (Gelderblom *et al.,* 2012; Wang *et al.,* 2015). Recent study also revealed that orally-administered fisetin exerts neuroprotectiveeffects against reactive gliosis and behavioural deficits in aluminum chloride-induced brain pathology model (Prakash*et al.,* 2013).

1.1 Statement of Research Problem

In an effort to feed the world's rapidly-growing population, government has encouraged an increase in agricultural production, including an increase in the application of agricultural inputs such as pesticides to enhance productivity in most countries (Ramaswamy and Sanders, 1992). The use of CPF has, therefore, increased over the years, with attendant surge in agricultural productivity and domestic pest control (Poswal and Akpa, 1991). The increase has been associated with adverse health consequences as CPF is often used on non-target species including exposure to animals and man (Kumar and Singh, 2014). Chlorpyrifos is a broad spectrum, highly effective, widely used OP pesticide. Its continuous use has increased the propensity of its neurotoxic effects (El-Hossary *et al.*, 2009). Thus, concern has led to the banning of CPF from home and garden use in the United States of America and some European countries (Renner, 2004). Unfortunately, CPF remains on the shelves of agricultural shops and plazas, and is harnessed for such use, in many developing countries, including Nigeria (Asogwa and Dango, 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 a high level of CPF residues in the soils and several vegetables in some parts of northern Nigeria (Akan *et al.*, 2013).

Factors promoting exposure include eating and drinking during spraying operations, failure to use protective clothing, improper storage and disposal of insecticides, which are common among farmers and pesticide applicators in Nigeria (Sosan and Akingbohungbe, 2009). Thus, pesticide applicators, with inadequate protective gears, farmers and livestock are exposed to sub-lethal and, apparently,sub-toxic doses of CPF by aerosol and direct contacts (Cattani *et al.*, 2001; Quandt *et al.*, 2006). The CPF residues that have entered into the food web of the ecosystem in plant parts, soils and water bodies (Akan *et*

al.,2013), also constitute a source of health hazard to man and animals. At low doses, CPF induces oxidative stress, which plays an essential role in its toxicologic mechanism (Slotkin *et al.,* 2001), including its effects on a wide variety of neurotransmitter systems. The serotonin (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.,* 2011b). 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). Adverse effects of chronic exposure to CPF such as strained breathing, insomnia and loss of memory have been observed in several Nigerian farmers (Sosan and Akingbohungbe 2009).

1.2 Justification

Chlopyrifos as a pesticide is used world-wide in large quantities and in different forms in order to control pests in agriculture, industries, stores and homes. Studies have reported that these chemicals have adverse effects on health of humans and animals, especially the end-users, who are the farmers. When used at home, CPF also affects adversely the health of children, immune-compromised and poorly-nourished individuals. The CPF poisoning is a global public health problem. It has been reported to cause morbidity in three million individual's world-wide and 220000 deaths annually, with most deaths occurring in developing countries. CPF impairs motor coordination, locomotor efficiency, motor strength, learning and short-term memory (Ambali *et al.,* 2010b) and 5HT systems (Slotkin and Sielder, 2005). In developing countries, cognitive deficit, anxiety and

depression have also been reported in individuals, exposed to CPF (Lee *et al.,* 2007). The induction of oxidative stress is one of the mechanismsimplicated in CPF-induced neurotoxicity (Ambali *et al.,* 2010b). Several antioxidant vitamins and other classical antioxidants have been shown to ameliorate CPF toxicity. The lack of proper legislation and effective pesticide control policies, awareness and education of the farmers on the adverse effects of OP pesticide has led to the continual, uncontrolled use of the chemical. Therefore, the danger posed by pesticides will continue to be a major problem in many developing countries for a long time to come.

Since oxidative stress is being implicated in the adverse effects of OP, including CPF poisoning (Tuzmen *et al.,* 2008; Ambali *et al.*, 2010b), there is the possibility that pretreatment with antioxidants, including fisetin, may reduce the adverse effects of low dose exposure to CPF on neurobehavioural changes and histopathologic changes.

1.3General Aim of the Study

To evaluate the effect of fisetin on CPF induced-neurobehavioural, oxidative stress and histopathologic changes.

1.4Specific Objectives of the study

The specific objectives of this study were to evaluate in adult male Albino mice the effect of fisetin on:

1. Some neurobehavioural and cognitive changes, induced by sub-chronic CPF exposure.

2. Changes in brain oxidative stress parameters and AChE activity, induced by subchronic CPF exposure.

3. Brain histopathological changes following subchronic exposure to CPF.

1.5 Null Hypothesis

Fisetin **does** not exert any significant protective effect on neurobehavioural, oxidative stress and histopathological changes induced by subchronic CPF exposure in adult male Albino mice.

CHAPTER 2

LITERATURE REVIEW

2.1 Pesticides

The term pesticide covers a wide range of compounds, including insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides, and plant growth regulators (Ensley, 2015). Pesticides are poisonous chemicals used to control plants or animals that are detrimental to the activities of man (Patricia *et al.,* 2015). They are used to prevent, repel, destroy and mitigate any insect pests. The pests attacked by chemicals include insects, plants pathogens, weeds, molluscs, birds, mammals, fish, and nematodes. Others include microbes that compete with humans and animals for food, destroy properties, spread or act as vectors of diseases or cause nuisance. Studies have also shown that human and wildlife are also exposed to the harmful effects of pesticides. Most pesticides are not selective, therefore, they are toxic to both target and nontarget organisms (Ensley, 2015). Pesticides differ in their composition, potency, mode of action, speed of effect and the dosage required (Patricia*et al.*, 2015)

Tremendous benefits have been derived from the use of pesticides in forestry, public health, domestic sphere and in agriculture (Charles and Yipeng, 2015). Pesticides can contaminate air, soil, surface water (Kole *et al.,* 2002), ground water, and other vegetation. Insecticides are generally the most acutely toxic class of pesticides, but herbicides can also pose risks to non-target organisms (Kole *et al.,* 2002).

2.1.1 Uses of pesticides

Pesticides are legitimate and useful tools that can provide significant benefits to our society. To maximise the benefits, they are to be used in a safe and efficient manner (Beatriz *et al.,* 2015).Pesticides are extensively used throughout the world in homes, industries and in agriculture. More than 200,000 pesticide products with nearly 900 active ingredients are registered for use as insecticides, fungicides, herbicides, fumigants, wood preservatives and plant growth regulators. They are a group of heterogeneous substances that have a significant public health benefit by increasing food production and decreasing food-borne and vector borne diseases. Despite their public health use, pesticides have been reported to cause serious major economic and environmental losses as a result of their application (Maria*et al.*, 2015).

In agriculture, herbicides are used to control weeds, and insecticides are used to control invertebrate pests in order to improve the yield and quality of food and fibre crops (Curran, 2016). Chemical dips are used to protect livestock from parasites, and poison baits help to control rodents and predators such as foxes. Pesticides are also important tools in the prevention and treatment of termite infestation in buildings (Lichetenthhaler *et al.,* 2000). The control of biting insects such as mosquitoes, which can be a nuisance as well as vectors of disease, provides public health benefits and improves the quality of human lives. The use of pesticides in recreational areas such as sporting ovals, golf courses and parks has social, recreational and aesthetic benefits for the community. Pesticide use can also help control environmentally-harmful organisms, leading to the protection of native habitats and the maintenance of biodiversity.

2.2 General Classification of Pesticides

2.2.1 Fungicides

Fungicides are chemicals or biological agents that kill or inhibit the growth of fungi or fungal spores. (Burpee, 2006). Fungistats slows or prevents the growth of fungi (Pimemtel, 2005). Modern fungicides do not kill fungi, they simply inhibit growth for a period of days or weeks (Francis and Keinarth, 2010). Fungi can cause serious damage in agriculture, resulting in critical losses of yield, quality and profit. Fungicides are used both in agriculture and to fight fungal infections in animals (Latijnhouwers *et al.,* 2000).

Fungicides can either be contact, translaminar or systemic. Contact fungicides are not taken up into the plant tissue, and only protect the plant where the spray is deposited (Philips, 2001). Translaminar fungicides redistribute the fungicide from the upper, sprayed leaf surface to the lower, unsprayed surface; systemic fungicides are taken up and redistributed through the xylem vessels to the upper parts of the plant. New leaf growth is protected for a short period. Most fungicides that can be bought retail are sold in a liquid form. The most common active ingredient is sulfur (Francis and Keinarth, 2010).

2.2.2Repellants and chemosterilants

Insect repellants are an alternative to the use of insecticides (Yap *et al.,* 2000). They may be applied to the skin to protect an individual from the bites of mosquitoes, mites, ticks and lice or, less commonly, may be used to exclude insects from an area, such as in packaging to prevent infestation of stored products (Klun *et al.,* 2001). Insect repellants act by blocking the receptors that the insects use to detect their appropriate hosts thereby preventing bites from these insects. The effectiveness of repellents varies with the active

ingredients, the concentration and the target species. Products come as lotions, pump sprays, pressurized cans, and wipes.

Chemosterilants are chemical substances that inhibits the reproduction of insets pests rather than killing them directly (Pimemtel, 2005).

2.2.3Acaricides

Acaricides are chemicals used in agriculture and medicine to control ticks and mites. (Guerrerp *et al.,* 2012).

2.2.4Rodenticides

Rodenticides, usually called ‘rat poison’, are pest control chemicals used in killing and the control of rodents. Rodenticides have also been used to control other mammals which have the same physiology as mice (Pimemtel, 2005).

2.2.5Insecticides

An insecticide is a substance used to kill insects. They include ovicides and larvicides used against insect eggs and larvae, respectively. Insecticides are used in agriculture, medicine, industry (Yao*et al*, 2012). Insecticides are claimed to be a major factor behind the increase in agricultural 20th century's productivity. Nearly all insecticides have the potential to significantly alter ecosystems, many are toxic to humans, and some concentrate along the food chains.

2.2.6Hormone mimics

These are chemicals that have properties that are similar to insect growth hormones. When they are applied to the target insects at the stage when the natural hormones is absent, they disrupt natural development (Pimemtel, 2005).

2.2.7Carbamates

Carbamates are a group of organic compounds which are of inmense importance due to their high usage in agriculture, pharmacology and chemical industries (Dariush *et al.,* 2016). Carbamate pesticides are derived from carbamic acid and kill insects in a similar fashion as organophosphate insecticides (Zhao *et al.,*, 2012). They are widely used in homes, gardens, and agriculture. Like the organophosphates, their mode of action is inhibition of cholinesterase enzymes, affecting nerve impulse transmission (Chen *et al.,* 2011).

2.2.8Organochlorines

Organochlorine pesticides are chlorinated hydrocarbons used extensively from the 1940s through the 1960s in agriculture and mosquito control (Robertson *et al.,* 2002). Organochlorine compounds include: dichlorodiphenyltrichloroethane (DDT), methoxychlor, dieldrin, chlordane, toxaphene, mirex, kepone, lindane and benzene hexachloride (Mahmoud *et al.,* 2016). As neurotoxicants, many organochlorine pesticides were banned in the United States (USEPA, 2016). Organochlorines are extremely persistent in the environment, lasting for years or even decades in soil and lake sediment and are also very long lasting in the human body (Mowry *et al.,* 2013), accumulating in fat

including breast milk (Wilson *et al.,* 2014). Organochlorines has also been associated with increased risk of cancer (Wolff *et al.,* 2000), developmental delays, and disruption of endocrine function, including thyroid hormones (Tsuda *et al.,* 2003).

2.2.9Organophosphates

Organophosphorus pesticides (OPs) are widely used for agricultural and domestic purposes (more than 50% of total pesticide consumption) due to their relatively low persistence in the environment. They are marketed for control of insects, plants, animals, soils and around the house. (Cope *et al.,* 2004). They possess high acute toxicity with many of them being extremely hazardous to human health and ecosystems. (Jeanty *et al.,* 2001).

2.1.10Botanical insecticides.

Biopesticides and botanical insecticides such as neem has been designed in the control of various organisms (Sebgottayan *et al.,* 2006). These include pyrethrum and its synthetic derivatives pyrethroids which is an extract derived from *Chrysanthemum spp* (cope *et al.,* 2004). Other botanical insecticides include nicotine, citrus oil extracted from the peels of citrus plants, rotenone derived from the roots of different plants (Cope *et al.,* 2004).

2.3 Routes of Pesticides Exposure

Pesticides enter the human body through the skin, the eyes, the lungs and the mouth. Most pesticides exposure occurs through the skin. A study shows that over 97 percent of pesticides exposure through the skin occurs during spraying operations (Fishel, 2005). A

significant amount of pesticides which are airborne gain access to the eyes and the eyes contains many blood vessels which are close to the surface of the eyes. Pesticides which are in the form of fumes, powders, aerosol or droplets suspended in the air can easily be inhaled during spraying operations (Fishel, 2005). Exposure by mouth is most common among children who ingest food or objects containing pesticides or put their contaminated fingers in their mouths (Cynthia *et al.,* 2003). Adults are exposed when pesticides splash on or into their mouth or on their lips, when food beverages or cigarettes are consumed after applying pesticides (Christos and Spyiridon, 2016).

2.3.1Occupational exposure

Farm workers and people employed in the manufacture of pesticides are especially at risk of pesticide exposure. Such risks are particularly pronounced in developing countries, where hazards are commonly less well understood, and health and safety regulations are less stringent or poorly enforced (EJF, 2003).

Exposure to pesticides has been associated with psychiatric problems among farm workers (Niece *et al.,* 2014). It has also been associated with respiratory symptoms and diseases such as asthma, bronchitis and lung cancer in farmers (Ming *et al.,* 2013). Studies have provided substantial evidence that pesticides used in agriculture by pesticide applicators are associated with cancer risk (Michael, *et al.,* 2013). It has also been associated with reproductive disorders (Toft, 2015).

2.3.2Non-occupational exposure

Non-occupational exposure can occur in a number of different ways for example by aerial drift during spraying or through contact with contaminated food, water, soil, cloth or milk (EJF, 2003). When pesticides are applied in high concentrations and frequencies, high

levels of pesticides residues remain on crops, thus at risk of exposure to consumers (Yuan *et al.,* 2014). Pesticides used in food storage may also serve as a source of exposure if the recommended interval between application and use or sales of the food product is not strictly abided to (Li *et al.,* 2014).

Children also experience different exposure risk of pesticides generally. There is the risk of pesticides affecting offspring of exposed farmers. Developing embryos can be exposed to contamination in utero. (Holland *et al.,* 2006). They can also be affected through ingestion of contaminated milk. (Ulaszewka *et al.,* 2011). Young children and toddlers that spend much time on the floor frequently come in contact with pesticides on the floor, on their toys, and other objects they come in contact with (Morgan *et al.,* 2014).

2.3.3Animal exposure

Most animal exposures occurs at homes where the animal ingests the pesticides and when ruminants graze sprayed pasture (Slotkin, 2011). Some animals are exposed dermally. Most exposures do not result in death however death is the medical outcome of exposure. Insecticide poisoning account for about 21% of animals death due to poisoning with organophosphates or carbamates (Slotkin, 2011). Pets may be affected when pesticides are used in and around the house and gardens. Pesticides are also used on pets and livestock to get rid of fleas, ticks and mosquitoes. Animals may eat mice and rats that were poisoned (Perkins *et al.,* 2016).

2.4Insecticides

Insecticides are pesticides that are formulated to kill, harm, repel or mitigate one or more species of insect. They are chemicals used to control or prevent insects from engaging in behaviors deemed undesirable or destructive. Insecticides are commonly used in agricultural, public health, and industrial applications, as well as household and commercial uses (e.g., control of cockroaches and termites) (Bouvier *et al.*, 2005). The most commonly used insecticides are the organophosphates, pyrethroids and carbamates.The USDA (2001) reported that insecticides accounted for 12% of total pesticides applied to the surveyed crops. Corn and cotton account for the largest shares of insecticide use in the United States. Insecticides are claimed to be a major factor behind the increase in agricultural 20th century's productivity (Sudakin *et al.,* 2007). They are applied and packaged in various formulations and delivery systems, including sprays, dusts, gels, slow release diffusion and baits. Due to these factors, each insecticide can pose a different level of risk to non-target insects, people, pets and the environment.

Nearly all insecticides have the potential to significantly alter ecosystems, many are toxic to humans, and some concentrate along the food chain (Boobis *et al.,* 2008). Mobilization of insecticides can occur via run-off, atmospheric deposition (primarily spray drift), or sub-surface flow. Soil erosion from high intensity agriculture, facilitates the transport of insecticides into waterbodies. Some insecticides are accumulated by aquatic organisms and transferred to their predators (Mowry *et al.*, 2013). Insecticides are designed to be lethal to insects, so they pose a particular risk to aquatic insects, but they also affect other aquatic invertebrates and fish. (Bouvier *et al.,* 2005).

Insecticides work in different ways. Some insecticides disrupt the nervous system, whereas others may damage their exoskeletons, repel them or control them by some other means

(Boobis *et al.,* 2008). Insecticides can be classified in two major groups as systemic insecticide which have residual or long term activity and contact insecticides, which have no residual activity (Mowry *et al.,* 2013).

2.4.1 Organophosphate insecticides

Organophosphate (OP) is the general name for esters of phosphoric acid. The Ops are some of the most common pesticides in use today and are similar to some of the chemical war fare agents developed during World War II, although not as toxic as nerve agents (Kwong, 2002). Organophosphate pesticides irreversibly inactivate acetylcholinesterase, which is essential to nerve function in insects, humans, and many other animals. The OP pesticides affect this enzyme in varied ways; and, thus, in their potential for poisoning.

United States Environmental Protection Agency lists OPs as very highly and acutely toxic to bees, wildlife, and humans (USEPA, 2012).Recent studies suggest a possible link to adverse effects in the neurobehavioural development of foetuses and children, even at very low levels of exposure (Saunders *et al.,* 2012).

Commonly used organophosphates have included parathion, malathion, methyl parathion, chlorpyrifos, diazinon, dichlorvos, phosmet, fenitrothion, tetrachlorvinphos, azamethiphos, and azinphos-methyl. Malathion is widely used in agriculture, residential landscaping, public recreation areas, and in public health pest control programmes such as mosquito eradication. The main source of contamination for humans are dietary ingestion and occupational exposures (Fernando *et al.,* 2016).

The OPs have been shown to induce neurological effects, malformations and micronucleus formation, birth defects and maternal toxicity (Diqui *et al.,* 2015).

The increase in accidental organophosphate poisoning as well as the rise in the number of cases of suicide attempts with organophosphate compounds is primarily due to the widespread use of these compounds in agriculture (Edibe *et al.,* 2012). Ops has also been associated with depression and high suicide rates in agricultural and other pesticide exposed workers (Freire and Kiofman, 2013).

2.4.2Toxicity of organophosphate pesticides

2.4.2.1Acute toxicity

The OPs are anti-esterase insecticides, and exert their acute effects by causing overstimulation at cholinergic nerve terminals. This process occurs in both insects and humans. Normally, acetylcholinesterase catalyzes the degradation of the neurotransmitter acetylcholine in the synapse (Fenske *et al.,* 2005). OP pesticides phosphorylate acetylcholine, thereby reducing the ability of the enzyme to break down the neurotransmitter. This produces an accumulation of acetylcholine in the central and peripheral nervous systems, resulting in an acute cholinergic syndrome via continuous neurotransmission (Leibson and Lifshitz, 2008).

Accumulation of ACh at motor nerves causes overstimulation of nicotinic expression at the neuromuscular junction. When this occurs symptoms such as muscle weakness, fatigue, muscle cramps, fasciculation, and paralysis can be seen (Jokanovic and Kosanovic, 2010). When there is an accumulation of ACh at autonomic ganglia this causes overstimulation of nicotinic expression in the sympathetic system. Symptoms associated with this are hypertension, and hypoglycemia. Overstimulation of nicotinic cholinergic receptors in the central nervous system, due to accumulation of ACh, results in anxiety, headache, convulsions, ataxia, depression of respiration and circulation, tremor, general

weakness, and potentially coma. When there is expression of muscarinic overstimulation due to excess acetylcholine at muscarinic acetylcholine receptors symptoms of visual disturbances, tightness in chest, wheezing due to bronchoconstriction, increased bronchial secretions, increased salivation, lacrimation, sweating, peristalsis, and urination can occur (Leibson and Lifshitz, 2008).

The onset and severity of symptoms, whether acute or chronic, depends upon the specific chemical, the route of exposure, the dose, and the individuals ability to degrade the compound. Reviews of case series indicate that pediatric organophosphate poisonings often manifest with mental status changes such as lethargy and coma (Searles *et al.*, 2005), as well as seizures (Reigart and Robert, 1999). The cholinergic phase usually passes off within 48-72 hours (Fenske *et al.*, 2005).

2.4.2.2Intermediate syndrome

After the acute cholinergic phase, a second stage of weakness occurs 1 - 4 days later with or without a symptom - free interval, and, if left unrecognized, can lead to fatal respiratory depression. The term intermediate syndrome (IMS) was first described by Senanayake and Karalliedde in 1987 because it arose in the interval between the end of the acute cholinergic crisis and the onset of organophosphate induced delayed neuropathy. According to that report, IMS was characterised by weakness of proximal limb muscles, neck flexors, respiratory muscles, and motor cranial nerves, and was attributed to muscle fiber necrosis following acute cholinergic crisis. Numerous studies have been published following the report of Senanayake and Karalliedde(1987) and the incidence of IMS has been reported to be as high as 80%. Although the incidence of IMS may be high and the syndrome has been considered a major contributing factor of organophosphate-related

morbidity and mortality, the pathophysiology that underlies IMS remains unclear (Chen-Chang and Jou-Fou, 2007).

Senanayake and Karalliedde (1987) in their first report of IMS suggested that the syndrome might be caused by pathologic changes in the postsynaptic end-plate region of striated muscles because such lesions were described in experimental animals (hens and rats) that developed a similar pattern of paralysis after organophosphate insecticide poisoning(John *et al.,* 2003). IMS occurs as a result of combined pre- and postsynaptic impairment of neuromuscular transmission as evidenced by electromyographic observations, and that IMS occurred after prolonged and severe acetyl- cholinesterase inhibition (Dandapani *et al.,* 2003).

Following exposure to various organophosphate insecticides, clinical manifestations of IMS typically occur within 24 to 96 hours, and affect conscious patients without fasciculation or other cholinergic signs (Senanayake and Karalliedde, 1987). Marked weakness of neck flexion and varying degree of proximal limb muscle weakness, manifesting as weak- ness of shoulder abduction and hip flexion, are the constant clinical features. Respiratory insufficiency is also common and frequently draws medical attention to the onset of the syndrome (Jayawadarne *et al.,* 2006). IMS is a major cause of morbidity and mortality in patients with acute organophosphate insecticide poisoning (Chen-Chang and Jou-Fang, 2007).

2.4.2.3 Organophosphate induced-delayed neuropathy

Also called organophosphate-induced delayed polyneuropathy (OPIDP), is a neuropathy caused by killing of neurons in the central nervous system, especially in the spinal cord, as a result of organophosphate poisoning. It is a rare, delayed neurotoxic effect, which occurs 1-5 weeks after toxicity from some cholinesterase inhibitors. However, it is not thought to

be due to the effects on acetylcholinesterase itself(Jokanovic *et al.,* 2002; Erdman 2004). The condition is associated with symmetrical sensory-motor axonal degeneration of the peripheral nerves and spinal cord (Marrs and Dewhurstm 2000).

Early symptoms include sharp cramp-like pains in the calves (Jokanovic *et al.,* 2002). Less severe cases exhibit a characteristic high-stepping gait. (Marrs and Dewhurst 2000). The initial muscle weakness gives rise to a clumsy, shuffling gait. The most disabling feature is the paralysis of the legs (Jokanovic *et al.,* 2002).

2.4.2.4 Organophosphate ester-induced chronic neurotoxicity

Organophosphorus ester-induced chronic neurotoxicity (OPICN) is also called chronic organophosphate-induced neuropsychiatric disorder (COPIND). This is a set of long-term, persistent, chronic neuropsychiatric findings that some have attributed to cholinesterase inhibitor toxicity. Some of the symptoms include: apathy, decreased visual memory, impaired vigilance, reduced abstract reasoning, anxiety, depression, increased social isolation, reduced fine motor coordination, confusion, dizziness, insomnia, decreased academic skills, emotional lability, irritability, short-term memory deficits, fatigue, problems with concentration, slowing of reaction time (Abou-Donia, 2003).

2.4.3 Mechanism of toxicity

Organophosphates cause toxicity by various mechanisms depending on the extent and duration of exposure and the type of syndrome resulting from thereof. The mechanisms of toxicity is discussed under the following sub-headings:

2.4.3.1 Acetylcholinesterase inhibition

Acetylcholinesterase is involved in the termination of impulse transmission by rapid hydrolysis of the neurotransmitter acetylcholine in numerous cholinergic pathways in the central and peripheral nervous systems (Mirjana *et al.*, 2013). Cholinesterase is a family of enzymes that catalyze the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetic acid, a reaction necessary to allow a cholinergic neurone to return to its resting state after activation

Acetylcholinesterase inactivation, induced by various inhibitors, leads to acetylcholine accumulation, hyperstimulation of nicotinic and muscarinic cholinergic receptors, and disrupted neurotransmission (Abou-Donia, 2003). Hence, acetylcholinesterase inhibitors, interacting with the enzyme as their primary target, are applied as relevant drugs and toxins.

2.4.3.2 Induction of oxidative stress

Apart from inhibition of cholinesterase and presence of cholinergic effects, oxidative stress has been reported by many authors as one of the adverse effects in poisoning by OP in both humans and animals (Anna, 2010). Oxidative stress induced by organophosphate leads to disturbances in the function of different organs and tissues. In subchronic or chronic OP exposition induction of oxidative stress has been reported as the main mechanism of its toxicity (Mates *et al.*, 1999). Both the increased production of reactive oxygen species (ROS) and attenuation of the antioxidant barrier of the organism are likely to induce oxidative stress (Milatovic *et al.*, 2006). In other words, oxidative stress can be defined as an imbalance between the production of free radicals and the body antioxidant defense system – enzymatic and non-enzymatic (Ranjbar *et al.*, 2005).

The ROS are produced during the metabolism of organophosphates by cytochrome P450s (Chambers *et al.,* 2001). ROS are unstable and highly reactive particles, as they have one or more unpaired electrons. They therefore, seek stability as radicals by attacking nearby molecules to obtain another electron, damaging the molecular structure and function. The ROS are the products of normal metabolic and signal transduction events in cells, but may also play an important role in a pathological process (Ranjbar *et al.,* 2005). All major biomolecules, such as lipids, proteins and nucleic acidsmay be attacked by free ROS, but among them lipids are probably the most susceptible to this attack.

There are several mechanisms to counteract the damage caused by ROS in the humans and animals (Luckawitz-Hassan, 2008). Many compounds have antioxidative capacity and can be categorized in two main systems. One of them is the enzymatic system which consists of such enzymes as superoxide dismutase (SOD) (manganese in the active enzymatic centre or copper and zinc), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Morgan *et al.*, 2014).

2.4.3.3 Endocrine disruptors

Chlorpyrifos was included in the 2007 draft list of initial chemicals for screening under the U.S. EPA Endocrine Disruptor Screening Programme (EDSP). The list of chemicals was generated based on exposure potential, not based on whether the pesticide is a known or likely potential cause of endocrine effects (USEPA, 2006).

The OP compounds cause endocrine impairments in both human and livestock that come in contact with these compounds; either through inhalation, ingestion and through contact with their skin (Wissem *et al.,* 2011). Research has revealed OP has anti-androgenic activities (Gunda *et al.*, 2010; Ventura *et al.*, 2016). Other hormones such as cortisol and

progesterone levels during pregnancy have also been affected by OP toxicity (Riccer*et al.,* 2006).

2.4.3.4 Reproductive toxicity

Certain reproductive effects in fertility, growth, and development for males and females have been linked specifically to OP pesticide exposure (Woodruff *et al.,* 2010). Most of the research on reproductive effects has been conducted on farmers working with pesticides and insecticdes in rural areas. For those males exposed to OP pesticides, poor semen and sperm quality have been seen, including reduced seminal volume and percentage motility, as well as a decrease in sperm count per ejacuate. In females, menstrual cycle disturbances, longer pregnancies, spontaneous abortions, stillbirths, and some developmental effects in offspring have been linked to OP pesticide exposure(Peiris-John and Wickremasinghe, 2008). Prenatal exposure has been linked to impaired fetal growth and development. The effects of OP exposure on infants and children are at this time currently being researched to come to a conclusive finding (Rauh*et al.,* 2011). Evidence of OP exposure in pregnant mothers are linked to several health effects in the fetus. Some of these effects include delayed mental development (Rauh*et al.,* 2012). Pervasive developmental disorder (Eskenazi *et al.,* 1999) morphological abnormalities in the cerebral surface (Slotkin *et al.,* 2006).

2.5Chlorpyrifos

Chlorpyrifos (IUPAC name: *O*, *O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate) commonly known as CPF is a crystalline organophosphate insecticide. It was introduced in 1965 by Dow Chemical Company and is known by many trade names, including

Dursbanand Lorsban. It acts on the nervous system of insects by inhibiting acetylcholinesterase. Chlorpyrifos is moderately toxic to humans, and exposure has been linked to neurological effects, persistent developmental disorders and autoimmune disorders (Timofeeva, 2010). Exposure during pregnancy retards the mental development of children (Munoz-Quezada, *et al.,* 2013). In agriculture, it remains one of the most widely used organophosphate insecticides, according to the United States Environmental Protection Agency (USEPA, 2002).

The EPA estimated that between 1987 and 1998 about 1million metric tonnes of chlorpyrifos were annually used in the US (USEPA, 2002). In 2007, chlorpyrifos was the most commonly used organophosphate pesticide in the United States, with an estimated 360,000 to 500,000 tonnes applied (Grube *et al.,* 2011).About 18,000 tonnes of CPF has been reportedly consumed in China annually (Liu *et al.,* 2014). CPF is used against a wide variety of insects including fleas, wasps, flies, bees, cockroaches as well as other agricultural pests. It is a broad spectrum OP insecticide that has gained popularity as one of the widely used OP pesticide in domestic and industrial applications (Slotkin, 2004).

2.5.1Physical properties

Chlorpyrifos (CPF) is a white, granular, colourless solid crystals. It melts and decomposes at 41-42°C and 160°C, respectively (Mackay *et al.,* 2014). It has a mild mercaptan odour and lipid soluble in organic solvents such as methyl alcohol, iso-octane (Dow, 2011). It is available in common formulations such as dispersable powder, emulsifiable concentrate, granules, microgranules, ultra-low volume liquid, water dispensible granules and wetable powder (USEPA, 2011).

2.5.2Uses of chlorpyrifos

Chlorpyrifos (CPF) has been registered to be used both indoors and outdoors as a pesticide. It is manufactured for both home use as well as commercial use. It is used to control and eliminate fleas, insects, termites, pests and mosquitoes. It is a very broad spectrum product and has, for years, been used for so many applications. It is used in agriculture for crop protection from numerous insects that affects and destroys agricultural farm products (Lassiter and Brimijoin, 2006). Other uses are in the control of ticks in cattle (Eaton *et al.*, 2008), veterinary clinics commercial storage or processing sites and in indoor pest control (USEPA, 2002).

2.5.3 Toxicity of chlorpyrifos

Chlorpyrifos CPF exerts its toxic effects by AChE activity (Yen *et al.*, 2011). AChE depletion has been positively associated with frequency of pesticide spraying, as symptoms usually occur within hours of acute exposure and typically decline within days or weeks as new cholinesterase is synthesized (Sandhal *et al.*, 2005). Depletion of cholinesterase and significant reduction in sensory nerve conduction of both legs were some of the major findings in a study conducted on termite control operators exposed to CPF and other OP pesticides. CPF is a neuro toxin and exposure to low concentrations can affect brain development in rats (Marty *et al.*, 2012). New evidence suggests that AChE may have a direct role in nervous system differentiation, and therefore exposure to certain cholinesterase inhibitors during brain development could have serious permanent effects (Carr *et al.*, 2001). CPF has been linked to births of children with deformed heads, faces, eyes and genitals (Betancourt and Carr, 2004).

CPF, an OP insecticide, has been listed as a potential endocrine disruptor by the German Federal Environment Agency, who reported that it affects pesticide formulators, reducing blood thyroxin concentrations. Symptoms of wheezing were associated with parquets, parathion, malathion and CPF (Munk *et al.*, 2003); except for malathion, these all had dose-related trends. In a study, it was found that toxic effect of CPF is persistent in soil and on various surfaces, although not hazardous in itself, could lead to undesirable effects by accumulation, depending on the frequency of application and amount applied (US EPA, 2008).

2.5.4Health effects of chlorpyrifos

Chlorpyrifos (CPF) is an OP pesticide known for its damaging effects on the human nervous system. Like all OPs, CPF blocks an enzyme (acetylcholinesterase) that the brains need to control acetylcholine, one of the many neurotransmitters mediating communication between nerve cells (Moser *et al.*, 2006). These neurological effects pose elevated risks,especially for children as their brains and nervous systems develop (Carr *et al.*,, 2001).

Short-term symptoms of low-dose exposure may include headaches, agitation, inability to concentrate, weakness, tiredness, nausea, diarrhoea and blurred vision. Higher doses can lead to respiratory paralysis and death (Marty *et al.*, 2012). Pregnant women may also be more sensitive to CPF toxicity (Carr *et al.*, 2001).

2.5.5Chlorpyrifos and oxidative stress

Pesticides are known to increase the production of ROS, which generate oxidative stress in different tissues (Heikal *et al.*, 2012).

2.6Antioxidants

They are various substances that inhibit oxidation or reactions promoted by oxygen and peroxides and they include many vitamins held to protect the living body from the deleterious effects of free radicals (Bjelakovic *et al.,* 2013). A substance that reduces damage due to oxygen, such as that caused by free radicals. Well-known antioxidants include enzymes and other substances, such as vitamin C, vitamin E, and beta-carotene, which are capable of counteracting the damaging effects of oxidation (Crinchton *et al.,* 2013). Antioxidants are also commonly added to food products such as vegetable oils and prepared foods to prevent or delay their deterioration from the action of air. Antioxidants may possibly reduce the risks of cancer (Jiang *et al.,* 2010). Antioxidants clearly slow the progression of age-related degenerations (Rees *et al.,* 2013; Takaeda *et al.,* 2014), and inhibit the oxidation of other molecules. Oxidation is a chemical reaction that can produce free radicals, leading to chain reactions that damage cells. Antioxidants such as thiols or ascorbic acid (vitamin C) terminate these chain reactions.

To balance the oxidative state, animals maintain complex systems of overlapping antioxidants, such as glutathione and enzymes (for example, catalase and superoxide dismutase) produced internally or vitamin C, vitamin A and vitamin E obtained by ingestion.

2.6.1Mechanism of action of antioxidants

Antioxidants are compounds or systems that delay autooxidation by inhibiting formation of free radicals or by interrupting propagation of the free radicals by one of the following mechanisms; scavenging species that initiate peroxidation, chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, breaking the

autoxidative chain reaction and/ or reducing localized oxygen concentrations (Jian-Ming *et al.*, 2010).

Antioxidants are molecules that can neutralise free radicals by accepting or donating electron(s) to eliminate the unpaired condition of the radical (Zhao *et al.*,. 2008). The antioxidant molecules may directly react with the reactive radicals and destroy them, while they may become new free radicals which are less active, longer-lived and less dangerous than those radicals they have neutralised. They may be neutralised by other antioxidants or other mechanisms to terminate their radical status (Shih *et al.*, 2007).

Another important function of antioxidants is to regulate ROS-related enzymes. Antioxidants may decrease the cellular level of ROS either by inhibiting the activities or expressions of ROS-generating enzymes such as NAD(P)H oxidase and xanthine oxidase (XO), or by enhancing the activities and expressions of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Rizvi and Maurya, 2007).

2.6.2 Classification of antioxidants

According to Denisov and Afanas'ev(2005), antioxidants are classified according to their solubility into:

(1) Water-soluble antioxidants, which include: Ascorbic acid (Vitamin C), Glutathione, Lipoic acid.

(2) Fat-soluble antioxidants, including α-Tocopherol (Vitamin E), gallates, resveratrol, polyphenols (e.g. ubiquinone, or coenzyme Q10), polyene compounds (e.g. β-Carotene).

You *et al.,* (2000) also classified antioxidants into non enzymatic and enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT). Nonenzymatic antioxidants include; vitamin C, vitamin E, taurine and lipoic acid.

2.6.3Natural antioxidants

These natural antioxidant compounds include flavonoids, phenolic acids, carotenoids, and tocopherols that can inhibit Fe^{3+}/AA-induced oxidation, scavenge ROS, and act as reductants (Ozsoy *et al.,* 2009). Spices and herbs, used in foods for their flavour and in medicinal mixtures for their physiological effects, often contain high concentrations of phenolic compounds that have strong H-donating activity (Muchuweti *et al.*,2007).

Naturally occurring antioxidants are high or low molecular weight in their composition, they can be divided into the following categories:

Enzymes: such as SOD, CAT and GPx. These enzymes attenuate the generation of ROS, by removing potential oxidants or by transforming the ROS into relatively stable compounds (Ursin *et al.,* 1982).

Synthetic antioxidants: These are most effective antioxidants and are synthetic chemicals approved by the Food and Drug Administration (FDA) for addition to foods, for example, butylated hydroxyl anisole and butylated hydroxy toluene. (Ozsoyl *et al.,* 2009).

2.7 Fisetin

Fisetin, also namely 3,3′,4′,7tetrahydroxyflavone(5-deoxyquercetin) (Prasath and Subramanian, 2013), is a naturally-occurring flavonoid, abundantly found in fruits,

vegetables and Anacardiaceous plants such as strawberries, grapes and onions (Arai *et al.,* 2000). Recently, pharmacological studies and clinical practice have demonstrated fisetin to have broad spectrum of biological functions, including anticancer (Sung *et al.,* 2007; Eiman *et al.,* 2015), antiobesity (Chang *et al.,* 2013), antiangiogenic, antiviral (Pooja *et al.,* 2016) neuroprotective (Zbarsky, 2005), neurotrophic (Maher *et al.,* 2007), anti-inflammatory (Salvatore, 2016) and antiproliferative (Lee *et al.,* 2014).

2.7.1Antioxidant effect of fisetin

Fisetin was found to be a planar molecule exerting a conjugation effect. The presence of hydroxyl bond (OH) dissociation energy and dipole moment suggest that fisetin had high antioxidant capacity (Markovic *et al.,* 2009). It has been reported that fisetin strongly binds between the polar head and hydrophobic tail of the phospholipids around the interfacial region of the egg phosphatidylcholine liposomes (Sengupta *et al.,* 2004). This region is easily available to the free radicals (Haddad *et al.,* 2010) and serves as the reaction site for the antioxidant activity of the fisetin molecule and inhibits lipid peroxidation. The high antioxidant capacity was confirmed by semiempirical calculations for fisetin, with the effectiveness for extracting OH from the surrounding medium predicted to decrease.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemical Acquisition and Preparations

Commercial grade CPF 20% emulsifiable concentrate (Termifos® by Alderelm Limited, United Kingdom) was obtained from a reputable chemical store. Grand cereal Soya oil was used as vehicle to reconstitute the CPF into a 1% (w/v) working solution. All other materials used for this study such as Fisetin and Dimethylsulphoxide were of analytical grade, and were obtained from Sigma Inc., USA.

3.2 LD_{50} Determination

The median lethal dose (LD_{50}) of the working CPF solution 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, III, consisting of three animals each. mice in groups I, II, III were administered CPF at 10 mg/kg, 100 mg/kg and 1000 mg/kg body weight, respectively. Death was recorded over a period of 48 hours. The second phase, which depended on the results obtained from Phase I, was conducted using three animals divided into three groups of one animal each. The LD_{50} was determined, based on dose-response as described by Lorke (1983).

3.3 EXPERIMENTAL ANIMALS

A total of 48 adult male Albino mice were used for the experiment. The mice were obtained from the Animal house of Department of Veterinary Physiology, Ahmadu Bello University, Zaria, Nigeria. They were maintained on commercial growers'

feed(Grand CerealsVital feed), maize offal (*Dusa)* and groundnut cake (*Kuli kuli*), compounded in a ratio 4:2:1. Mice were given access to feed and water *ad libitum*. The animals were kept in metal cages at room temperature (cold dry season) throughout the study period.

3.4. Groupings/Treatments

The mice were randomly divided into six groups of 8 mice 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 0.1% dimethylsulphoxide, while Group IV was administered with CPF only, Group V was administered with Fisetin (15 mg/kg) and Group VI was administered Fisetin (15 mg/kg), followed by CPF 30 minutes later. The regimens were administered once daily for a period of seven weeks. During this period, the body weight, neurobehavioural and cognitive parameters was monitored. At the end of 7 weeks, the mice were sacrificed using jugular venesection after initial light chloroform anaesthesia and serum and tissue samples were collected for evaluations.

3.5 Evaluation of Neurobehavioral and Cognitive Changes

3.5.1 Open-field assessment

This was carried out on all experimental mice using the open field apparatus as described by Zhu *et al.,* (2001). The open-field apparatus was constructed using cardboard box with a dimension of 50 × 50 × 45 cm with a clear Plexiglas on the inner surface (base). The floor of the box was divided into 25 equal squares. The parameters analyzed were locomotor activity and anxiety, evaluated using horizontal locomotion

(number of crossings of the lines marked on the floor); frequency of rearing, locomotor activity involved placing an animal in the box and allowing it for a period of 3 minutes to walk freely and become familiarised with the environment. Thereafter and for the next 2 minutes, the number of squares crossed with all the paws was recorded. Rearing was measured by determining the frequency at which each mouse stands on its hind-limbs with the fore-paws lifted up freely or leaned against the wall of the apparatus during the next 2 minutes, after the initial 3 minutes of habitation. The frequency of grooming (protracted washing of the coat), number of faeces voided, and the number of puddles of urine voided was observed and recorded. The open-field assessment was carried out on day 0 and weeks 3 and 6 (Zhu *et al.*, 2001).

3.5.2 Assessment of neuromuscular coordination

The effect of the regime on neuromuscular coordination was assessed using the inclined plane, as described by Ambali *et al.*, (2010b). Each mouse 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 beforehand, and was obtained by propping the plank on a vertical bar with several notches. The plain was initially inclined at an angle of 35°, and there after increased stepwise by 5° until the subject can no longer hold on horizontally for 30 seconds without sliding down. The test was performed with the subjects facing left and then right across the plank. The highest angle at which the mouse can no longer stay, standing horizontally, and facing any of the directions was recorded. Two trials was performed for each testing period (Petrich, 2006). The experiment was performed on day 0 and week 3, and 6.

3.5.3 Assessment of motor coordination

The motor coordination was assessed using the beam-walk performance task as described by Ambali *et al.* (2010b). Briefly, the beam-walk performance was evaluated by allowing individual mouse 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 walks across, the width of the beam at which it steps down was recorded by one rater on each side, and this was repeated twice during each trial session. This task was performed on day 0 and weeks 3 and 6

.

3.5.4 Assessment of locomotor efficiency

This was done using the ladder walk as described by Ambali and Aliu (2012). Mice were encouraged to walk across a black wooden ladder (106× 17 cm, width 0.8-cm diameter rungs with 2.5-cm spaces between them, totaling 31 rungs). The number of times each mouse drops down through the rungs of the ladder and/or, when the subject misses a rung was counted by one rater on each side. This was performed on day 0 and weeks 3 and 6.

3.5.5 Assessment of motor strength

The fore-paw grip time was used to evaluate the motor strength of the mice hung from a 5-mm- thick diameter wood dowel, gripped with both fore-paws. The time spent by

each mouse before releasing its grip was recorded in seconds. The assessment was conducted on day 0, and weeks 3 and 6 (Ambali *et al.,* 2010a).

3.5.6 Assessment of excitability scores

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

Grade 0: Mouse showed no sign of wriggling at all.

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

Grade 2: Mouse responded through a stronger wriggling and fore-paw movement.

Grade 3: Mouse vigorously wriggled and showed strong fore and hind-limb movement.

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

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

The excitability scores was assessed on day 0 and weeks 3and 6.

3.5.7 Assessment of depression

The depression status of the rats was evaluated using the forced swimming test as described by Porsolt *et al.* (1977). All mice were acclimatized to the test room for 1 h before the beginning of the experiment. Mice were individually placed in a cylindrical

tank measuring 60 cm in height and 38 cm in width. The tank was filled with water (24 ± 1 °C) at a height of 40 cm and water was changed after each session (Dalla *et al.,* 2008). each animals was forced to swim for a 5-min swimming session (test) (Pitychoutis *et al.,* 2011). The total duration of floating (immobility), swimming, and climbing periods was manually scored by the same observer for the first 5 min of each session (test), with the use of behavioural scoring software (Kokras *et al.,* 2012)

3.5.8 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) is delivered through the floor bars. 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 mouse 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 this assessment. The number of times the mouse 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 mouse 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 mouse 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.

3.6 Preparation of Brain Samples

Freshly obtained brain sample 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 evaluation.

3.6.1 Assessment of acetylcholinesterase activity

Brain samples collected was analyzed for AChE using the assay methods 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 (break-down product of acetylcholine iodide [ATChI] and disthiobisnitrobenzoate [DTNB])

3.6.2 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). The principle was based on the measurement of the colour developed as a result of the reaction of thiobarbituric acid (TBA) with MDA. Briefly, brain samples of animals from all groups was weighed and then homogenized in a known volume of ice-cold phosphate

buffer to obtain a 10% homogenate, which was 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% TBA. The mixture was then heated in a boiling water bath for 15 min, and Butan-2-ol (2:1v/v) was added to the solution. After centrifugation (800 g, 5 min), TBARS, an index of MDA was determined from the absorbance of 532 nm using Schimadzu UV spectrophotometer (Model UV 160 Kyoto, Japan). The concentration of MDA in the brain samples was calculated by the absorbance coefficient of MDA-TBA complex 1.56 $\times 10^5$/cm/M and expressed in nmol/mg of protein for brain.

3.6.3 Assessment of brain antioxidant enzyme activities

The supernatant was immediately used to assay for activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase GPx). The specific activities of CAT, SOD and GPx was determined using the appropriate enzyme-linked immunosorbent assay kits as described by the manufacturers.

3.6.4 Histopathology

The brain was collected from at least 1 mouse from each group and fixed in Bouin solution for brain section preparation according to the method of Luna (1960). Briefly, after two weeks in Bouin solution, the tissues was dehydrated in alcohol, cleared in xylene and infiltrated with molten paraffin wax at 50°C, blocked in paraffin according to standard procedures and labelled. Section was cut at nominal thickness of 10 µm using Jung rotary microtome (Model 42339, Berlin, Germany). The sections was

mounted on glass, deparafinized, stained with haematoxylin and eosin stain. Slides prepared were viewed under light microscope at ×40 magnification.

3.7. Assessment of Serum Lipid Profile

Freshly obtained blood was collected into a test tube (without anticoagulant). The blood was allowed to coagulate and sediment, the blood was then centrifuged and the supernatant (serum) was decanted into sterile testtubes. The serum was stored in the refrigerator for use in evaluation.

The serum lipid profile was carried outusing Agappe Diagnostic Kit (Switzerland) according to the manufacturer's instructions.

3.8 Statistical Analysis

Data obtained was expressed as mean ± standard error of the mean (mean ± SEM). The value obtained for the parameters, measured repeatedly (neurobehavioural tests) were analyzed using repeated measures one way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Data obtained from the parameters measured once during the period of study (cognitive tests and biochemical assay) were analysed using ANOVA, followed by Tukeyspost-hoc test. Graph Pad Prism version 4.0 for windows (Graph Pad Software, San Diego, California, USA) was used to analyse all the data. Values of $P < 0.05$ were considered significant.

CHAPTER FOUR

RESULTS

4.0Determination of LD_{50}.

All mice were administered CPF at 100 mg/kg and 1000 mg/kg during the phase I died within 24 hours of exposure. All the mice exposed 10mg/kg all survived. Each of the mice exposed to 90mg/kg, 80 mg/kg and 70mg/kg in the phase II also died. Phase III was conducted and the mouse exposed to 60mg/kg died while those exposed to 40mg/kg and 20 mg/kg survived.

The clinical signs of toxicity were restlessness, huddling together, roughened hair coat, rearing and depression.

4.1 Effect of Treatment on Neurobehavioural and Cognitive Changes

Figure 4.1 shows the effect of treatment on neuromuscular coordination. There were significant (P□ 0.05) decreases in the neuromuscular coordination in the D/W and Fisetin + CPF groups, relative to the DMSO group. Figure 4.2 shows the effect of treatment on locomotor efficiency. There was a higher (P□ 0.05) locomotor efficiency in Fisetin group than CPF group. There were no significant differences in the effect of the treatment on the motor strength of the mice across all groups. However, the group of animals exposed to CPF showed aninsignificant reduction in their forepaw grip time (Fig.4.3). Figure 4.4 shows the effect of treatment on the excitability scores of the mice. There was no significant difference across all groups, but the group exposed to fisetin alone showed an insignificant increase in their excitability scores, compared to other groups.

The effect of treatment on learning and short-term memory of the mice are shown in figures 4.5 and 4.6 respectively There were significant ($P < 0.05$) differences in learning in S/Oil, DMSO, CPF, Fisetin and Fisetin + CPF groups, relative to the control group (D/W). There were no significant differences in the short-term memory across all the groups.

4.2 Effects of Treatment on Open-Field Parameters

Table 4.1 shows the effect of treatment on open field parameters. There was no significant ($P < 0.05$) change across the groups, but a decrease in horizontal movement was recorded in the S/Oil group, when compared to other groups.

The effect of treatment on rearing was not significantly different however, there was an insignificant decrease in the rearing in the Fisetin + CPF group, when compared with the control group (D/W). The effect of treatment on grooming did not differ across the groups. The treatment significantly ($P <0.05$) increased the frequency of centre crossing, there was significant ($P <0.05$) difference in the DMSO and Fisetin groups relative to the negative control group (D/W). There was no significant ($P \square 0.05$) change across the groups in the frequency of defeacation and urination.

4.3 Effect of Treatment on Forced Swimming

Table 4.2 shows the effect of treatment on forced swimming parameters. There was a significant ($P <0.05$) increase in the swimming duration between the D/W and Fisetin groups. A significant ($P <0.05$) increase in the duration was also recorded between

Fisetin and Fisetin + CPF groups.The effect of treatment on climbing duration were insignificant across all the groups.

There was a significant ($P < 0.05$) difference in immobility duration between CPF and Fisetin + CPF groups, and between Fisetin and Fisetin + CPF groups.

4.4 Effect of Treatment on Acetylcholinesterase Activity

Figure 4.7 shows the effect of treatment on the acetylcholinesterase activity in the subjects. There was a significant ($P < 0.05$) decrease in the group exposed to CPF when compared to the groups exposed Fisetin and Fisetin + CPF.

4.5 Effect of Treatment on Brain Lipoperoxidation

Figure 4.8 shows the effect of treatment on the brain malonaldehyde concentration in the subjects. There was a significant ($P < 0.05$) decrease in the CPF group, when compared to the Fisetin and Fisetin + CPF groups.

4.6 Effects of Treatment on Activities of Brain Antioxidant Enzymes

4.6.1Catalase

Figure 4.9 shows the effect of treatment on catalase activity in the mice. There were significant ($P < 0.05$) decrease in the catalase activity of mice exposed to CPF and Fisetin,when compared to mice exposed to D/W. However, there were insignificant differences in the catalase activities of groups of mice exposed to CPF when compared to mice exposed to Fisetin + CPF.

4.6.2Glutathione peroxidase

Figure 4.10 shows the effect of the treatment on glutathione peroxidase activity. There was a significant ($P < 0.05$) decrease in the GPx activity in the CPF group, compared to the S/OIL group, and also a significant ($P < 0.05$) between the CPF and the DMSO groups. Although insignificant, there was a decrease in glutathione peroxidase activity of mice treated with CPF, when compared with the groups treated with Fisetin and Fisetin + CPF.

4.6.3Superoxide dismutase

Figure 4.11 shows the effect of treatment on the superoxide dismutase activity across groups. There was a significant ($P < 0.05$) increase in the SOD activity in the group of mice exposed Fisetin + CPF, when compared to the CPF group, but the changes between the CPF and Fisetin group were not significant.

4.7 Effect of Treatment on Serum Lipid Profiles

4.7.1Serum high-density lipoprotein

Figure 4.12 shows the effect of treatment on the serum HDL concentration in the mice. There were significant ($P \square 0.05$) differences in the serum HDL between the S/OIL group and the DMSO, Fisetin and Fisetin +CPF groups. The differences between the serum HDL between the CPF group and the Fisetin and Fisetin + CPF groups wereinsignificant ($P < 0.05$).

4.7.2Serum low-density lipoproetin

The effects of treatment on the serum LDL concentration across the groups are shown in fig.4.14. The serum LDL concentration decreased significantly ($P < 0.05$) in the D/W group, compared to that of the S/OIL group. There were no significant ($P < 0.05$) differencesin the LDL concentration between the group of mice exposed to CPF and those exposed to Fisetin + CPF.

4.7.3Serum tryglycerides

Figure 4.14 shows the effect of treatment on the serum triglyceride concentration in the mice. There was no significant difference across the groups.

4.7.4Total cholesterol

There were no significant changes in the total cholesterol concentration in the S/Oil group relative to the D/W, DMSO, CPF, Fisetin, and Fisetin + CPF groups (Fig.4.15).

4.8. Effect of treatment on histopathologic changes

Plate I, II and III shows the effect of treatment on brain histopathological changes.in the group of mice exposed to distilled water, soya oil and dimetylsulphoxide showing no apparent histopathologic changes.

There were meningeal haemorrhages and congestion of blood vessels in the brain of the group of mice exposed to CPF (Plates IV and V). Plate VI shows histopathologic changes in the group of mice exposed to Fisetin only, Plate VII represents the group

exposed to CPF + Fisetin showed meningeal haemorrhages but there was no significant histopathological observations in the brain.

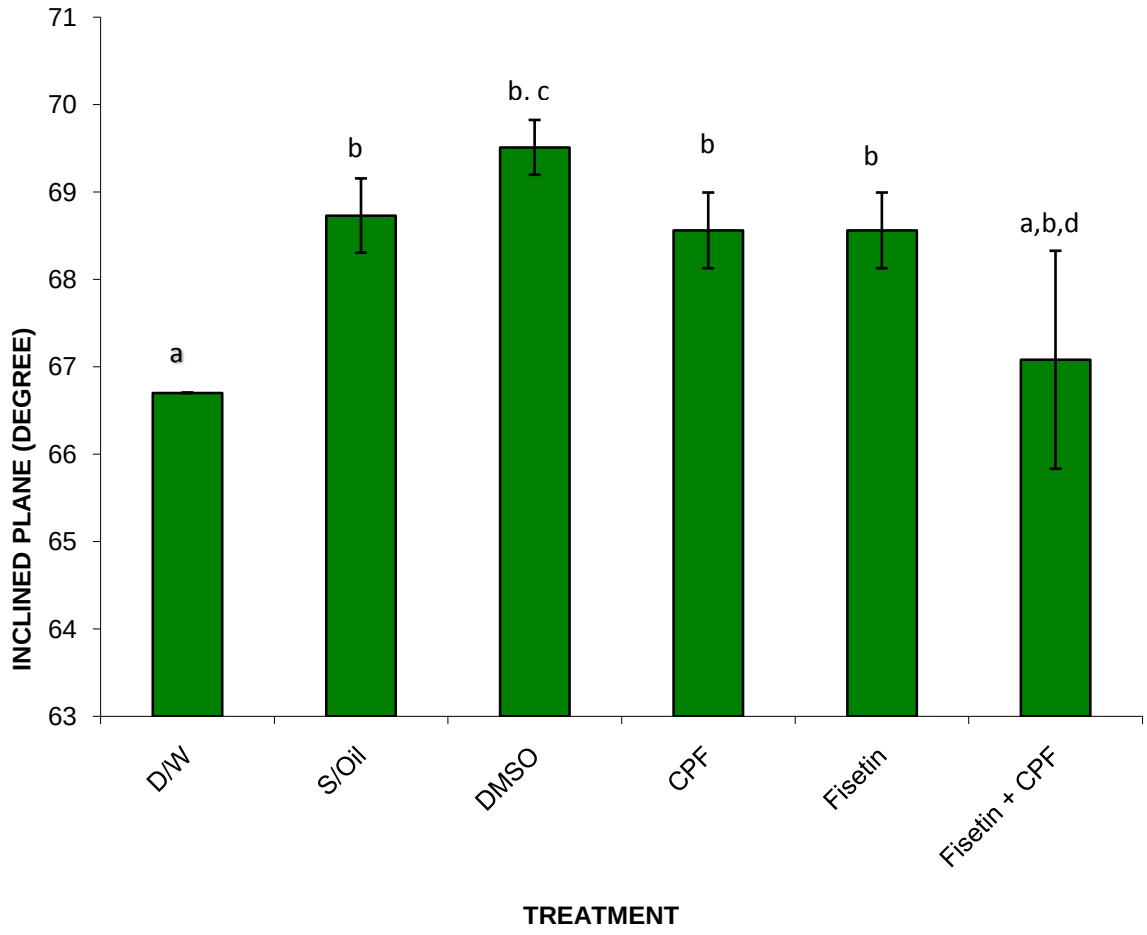


Figure 4.1. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on neuromuscular coordination in adult male albino mice.

[a, b, c, d] different superscript letters indicate significant (P □ 0.05) differences between groups

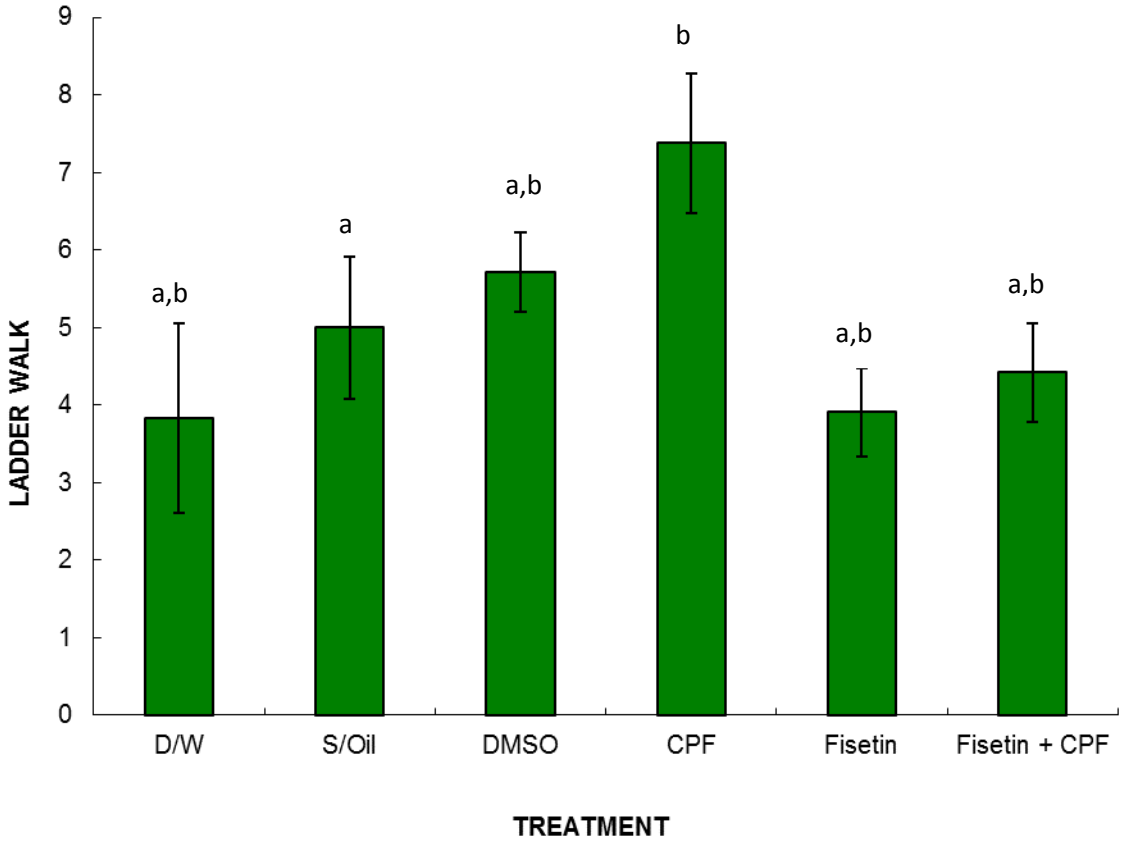


Figure 4.2. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on locomotor efficiency in adult male Albino mice.

[a, b] different superscripts indicate significant (P □ 0.05) differences between the groups

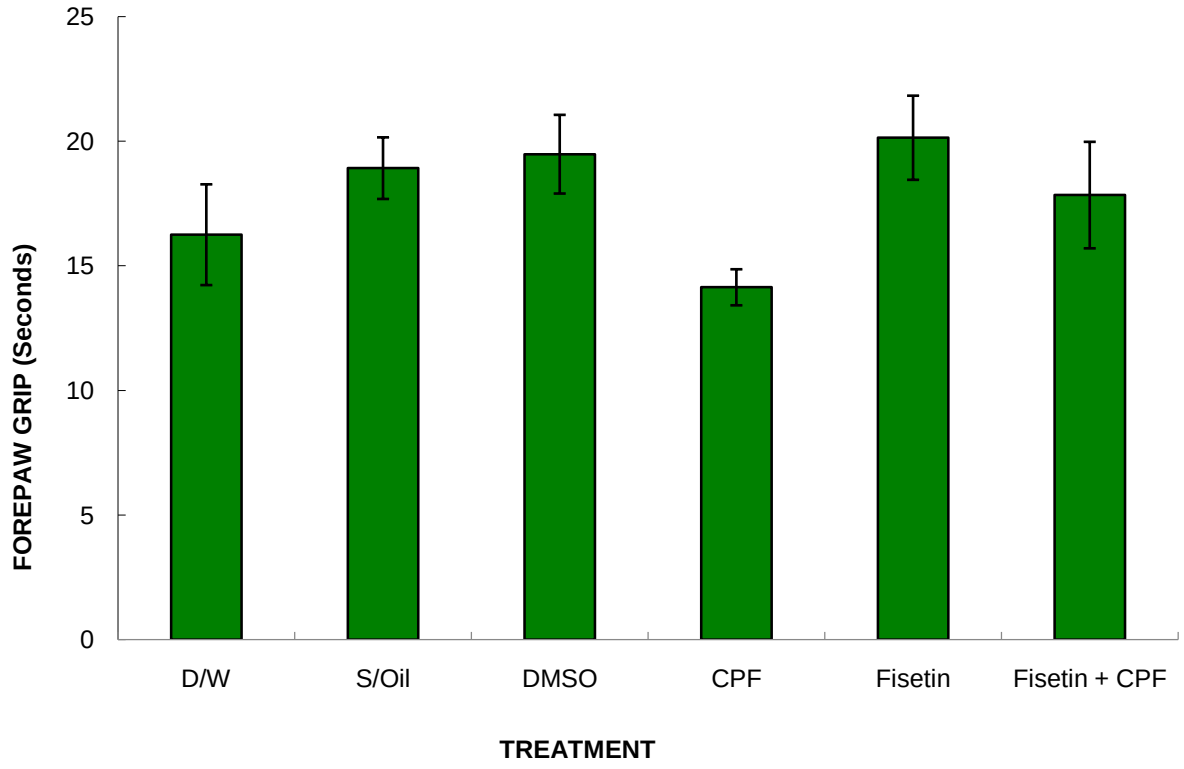


Figure 4.3. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on motor strength in adult male Albino mice.

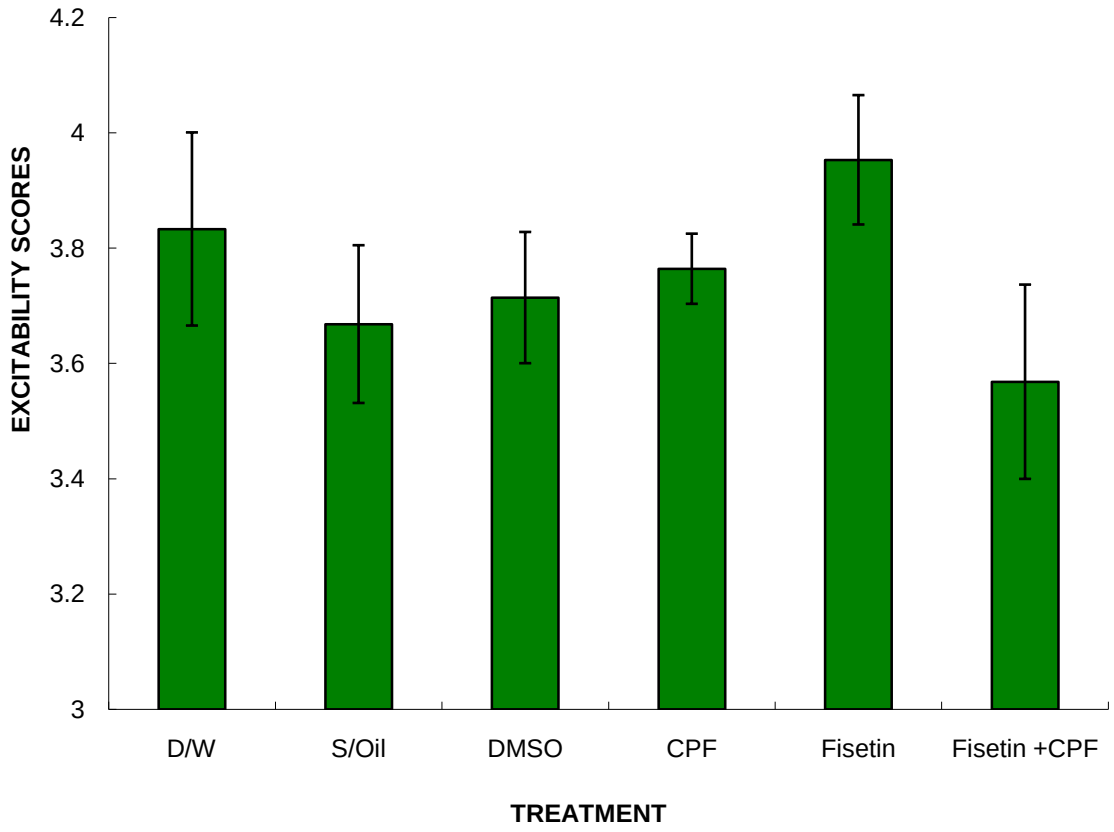


Figure 4.4. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on excitability scores in adult male Albino mice.

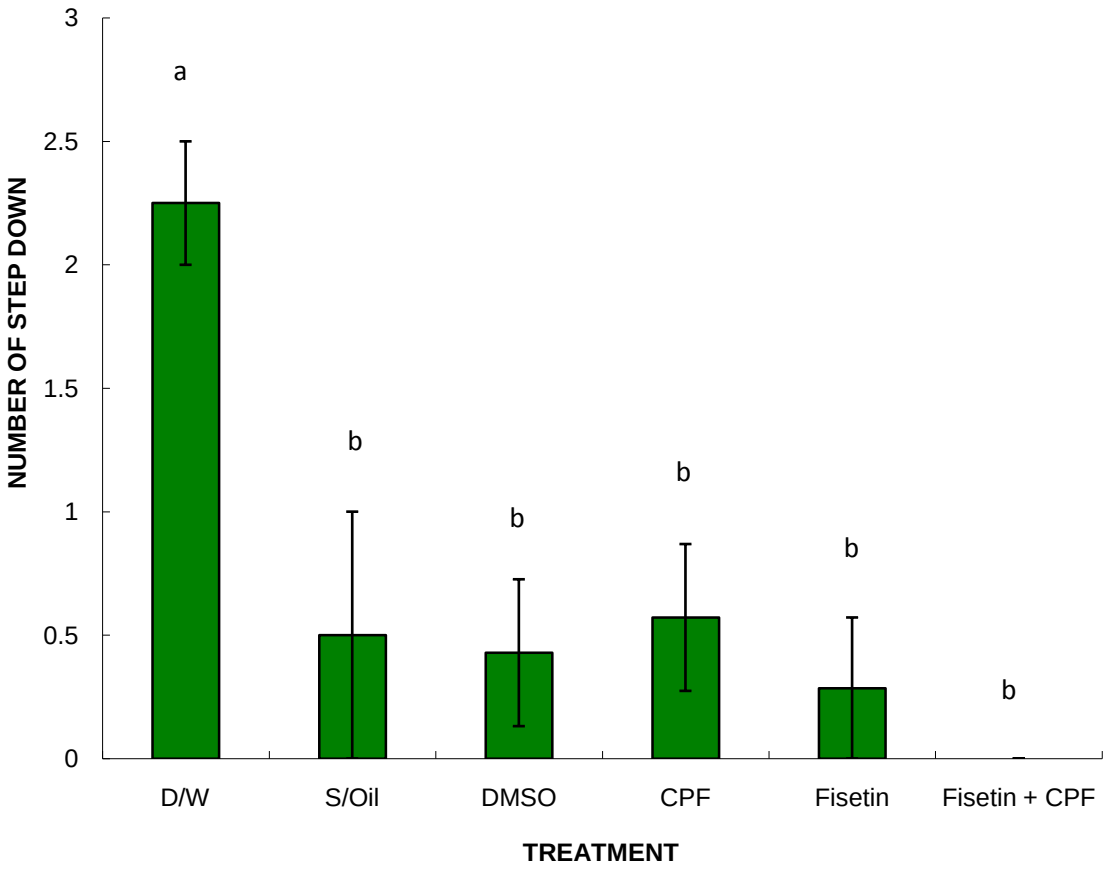


Figure 4.5. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on learning in adult male Albino mice.

[a, b] Different superscript letters indicate significant (P □ 0.05) differences between groups.

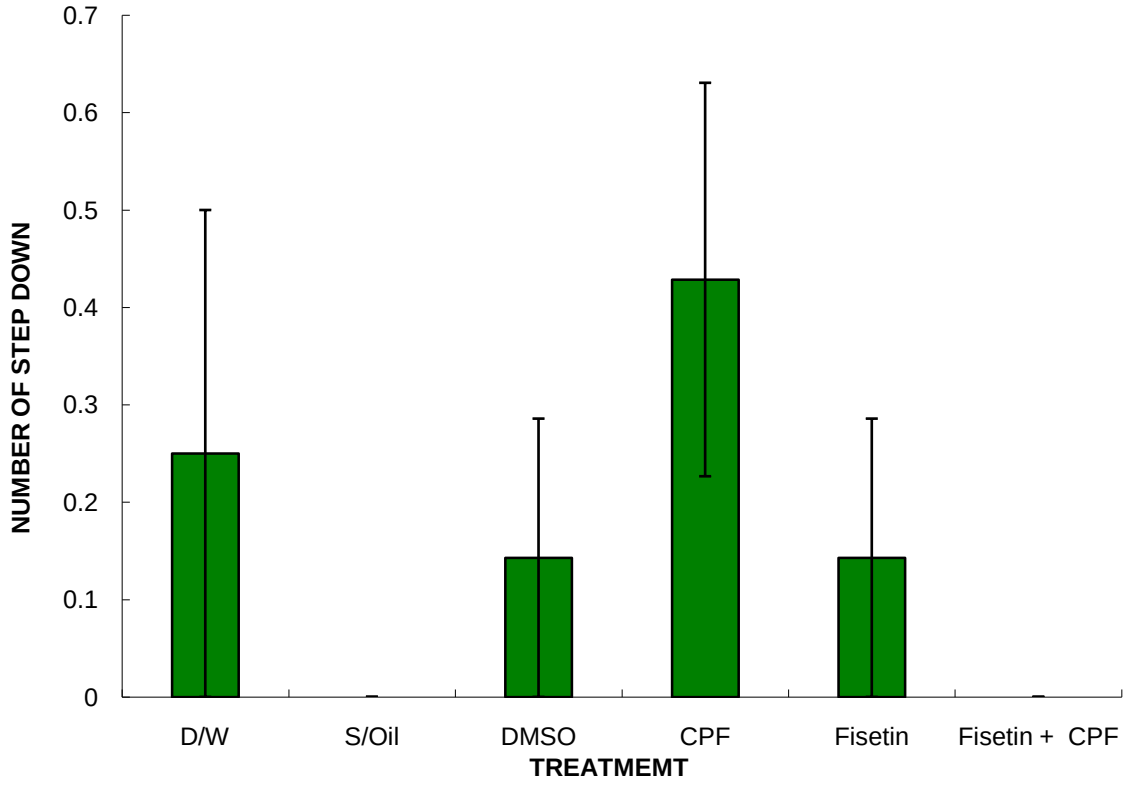


Figure 4.6. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on short term memory in adult male Albino mice.

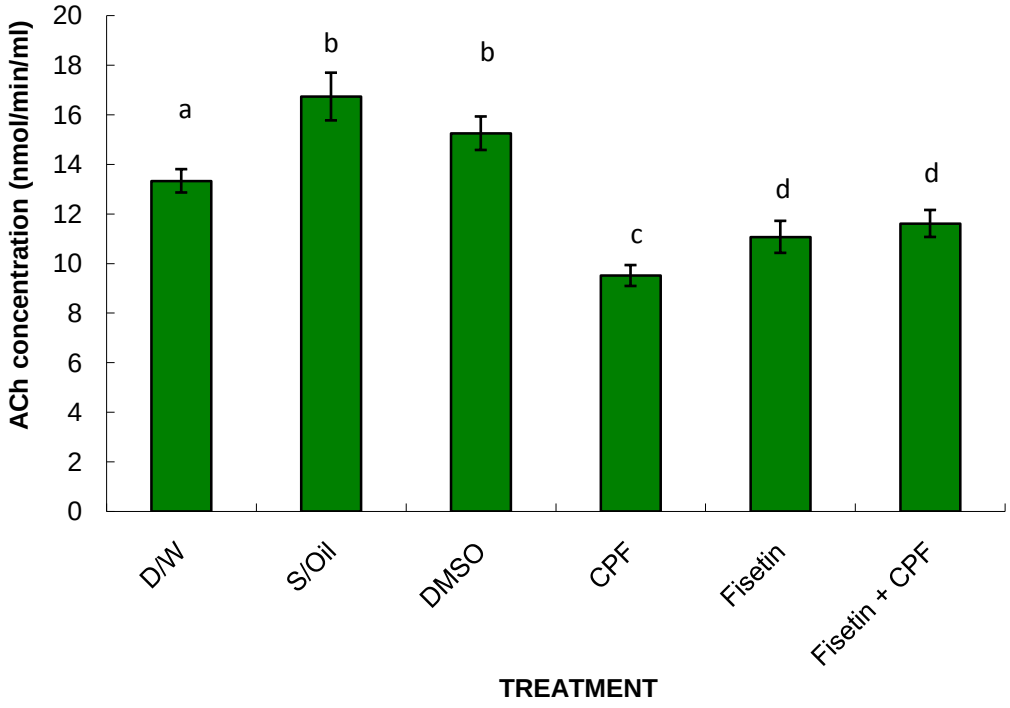


Figure 4.7. Effects of subchronic exposure to chlopyrifos (CPF) and fisetin on brain acethlcholinesterase activity in adult male Albino Mice.

a, b, c, d Different superscript letters indicate significant (P □ 0.05) differences between groups

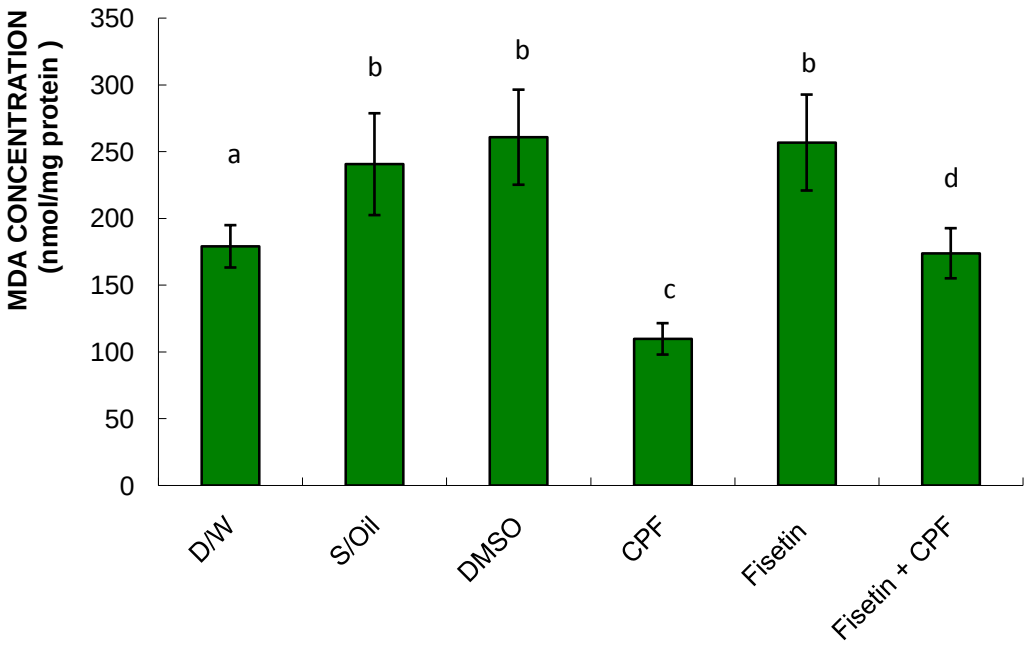


Figure 4.8. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on brain lipoperoxidation in adult male Albino mice. .

a, b, c, d, Different superscripts indicate significant (P □ 0.05) differences between groups.

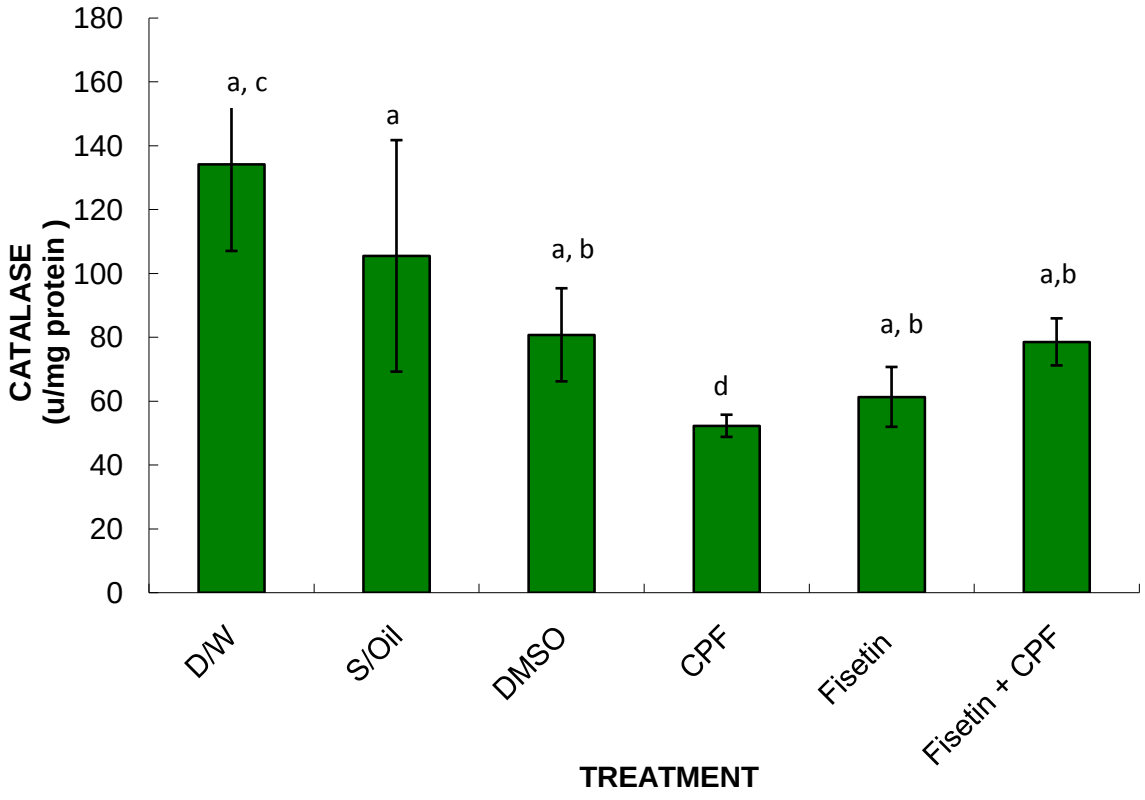


Figure 4.9. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on brain catalase activity in adult male Albino mice.

$^{a, b, c, d}$ different superscript indicates significant ($P < 0.05$) differences between groups.

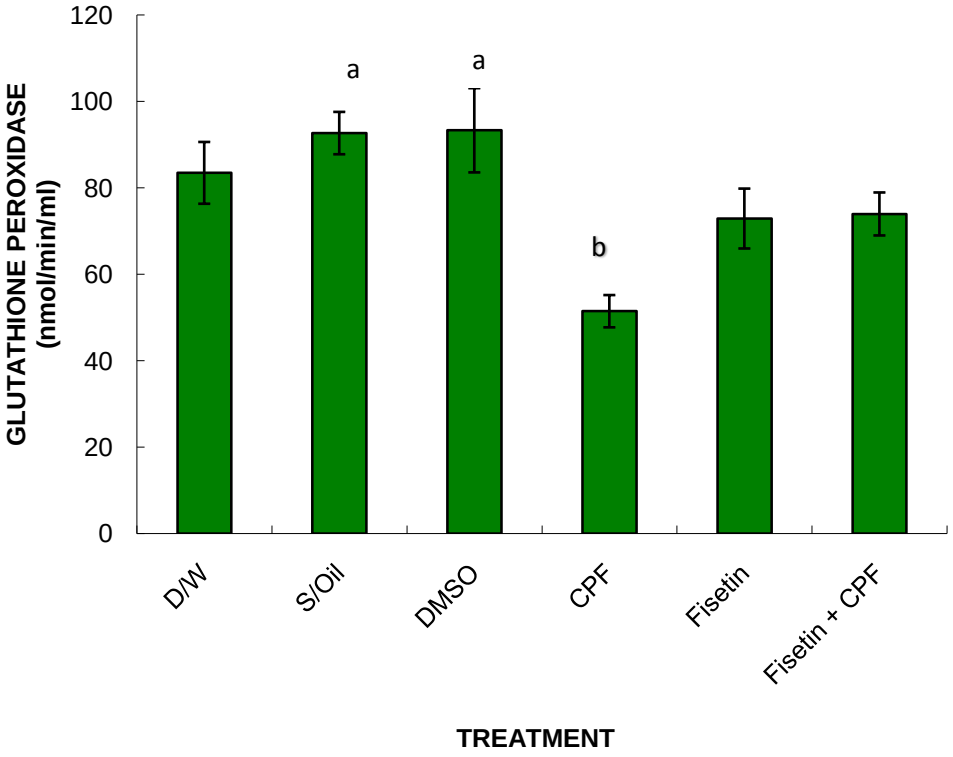


Figure 4.10. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on brain glutathione peroxidase activity in adult male Albino mice. .

[a, b,] Different superscript indicate significant ($P < 0.05$)differences between groups.

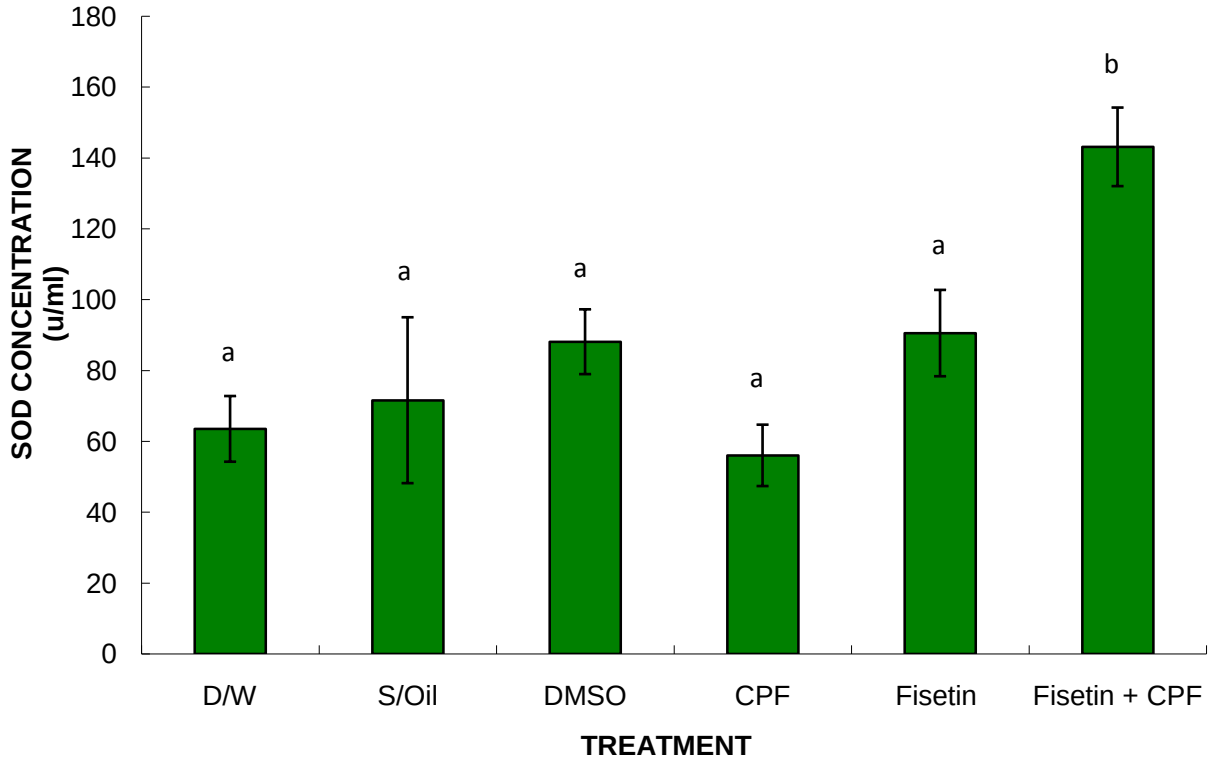


Figure 4.11. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on brain superoxide dismutase activity in adult male Albino mice.

[a, b] Different superscripts indicate significant (P □0.05)differences between groups

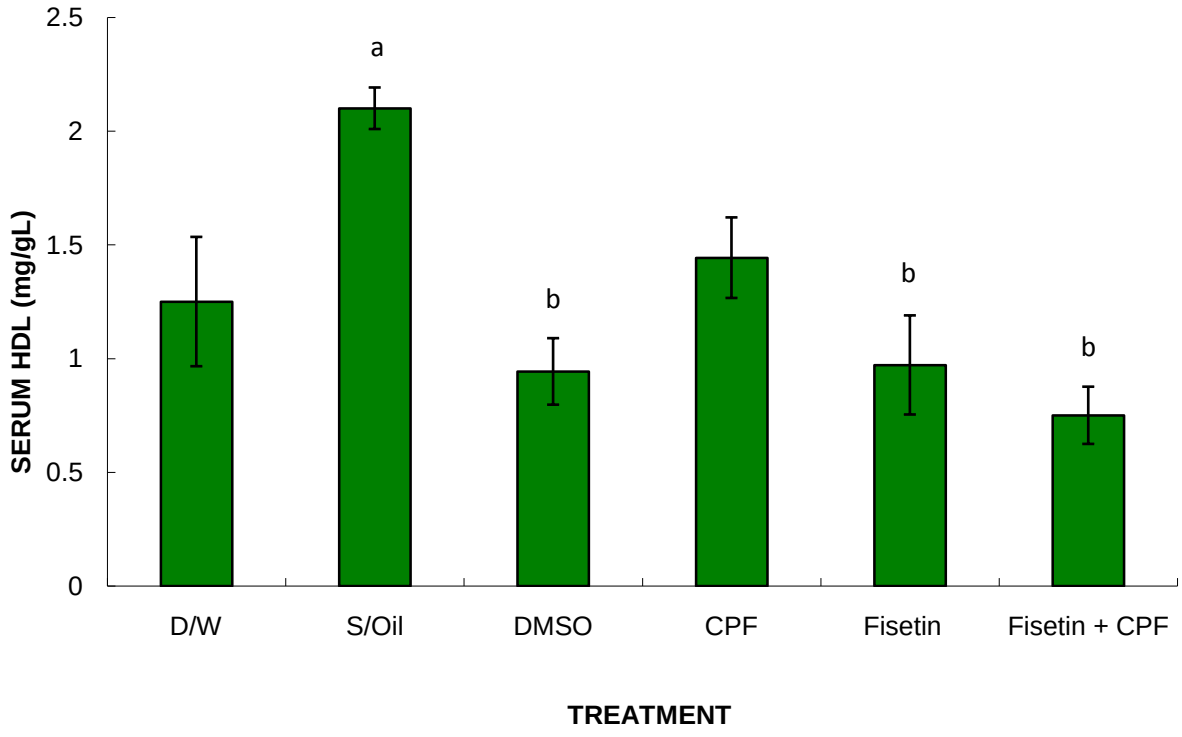


Figure 4.12. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on the serum high density lipoprotein (HDL) concentration in adult male Albino mice.

[a, b] different superscripts indicate significant (P □ 0.05) differences between groups

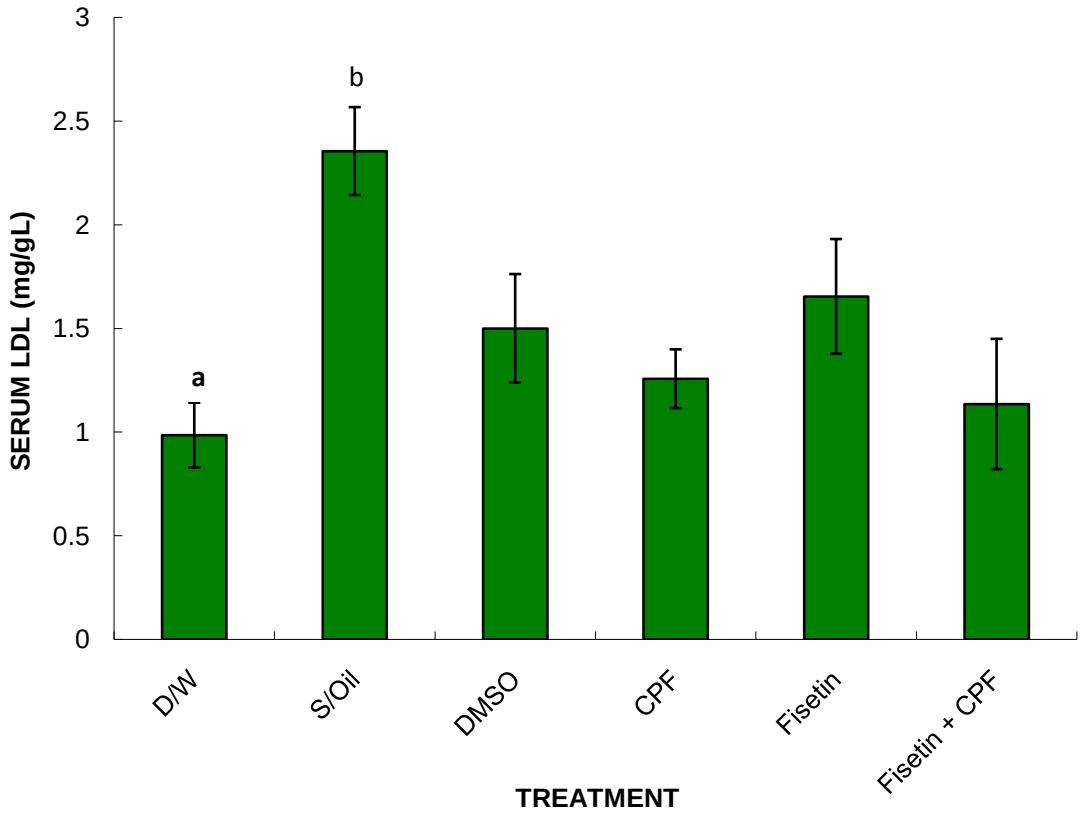


Figure 4.13. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on serum low density lipoprotein (LDL) concentration in adult male Albino mice.

[a, b] different superscripts indicate significant (P □ 0.05) differences between groups.

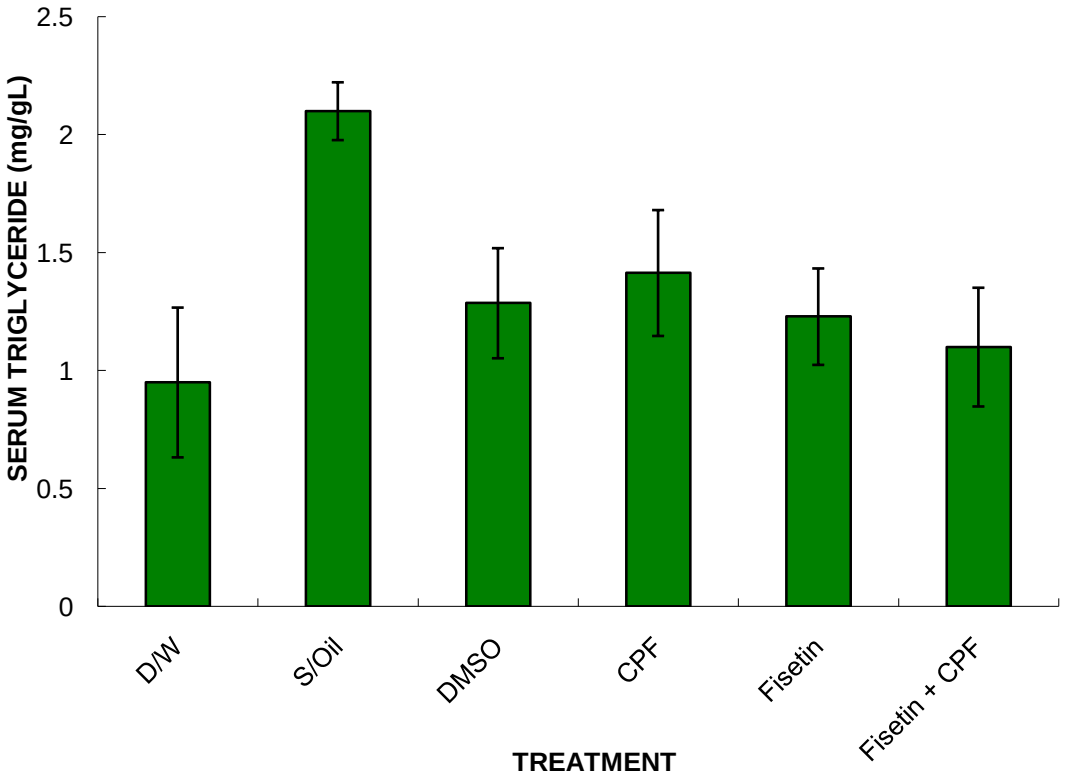


Figure 4.14. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on serum triglyceride concentration in adult male Albino mice.

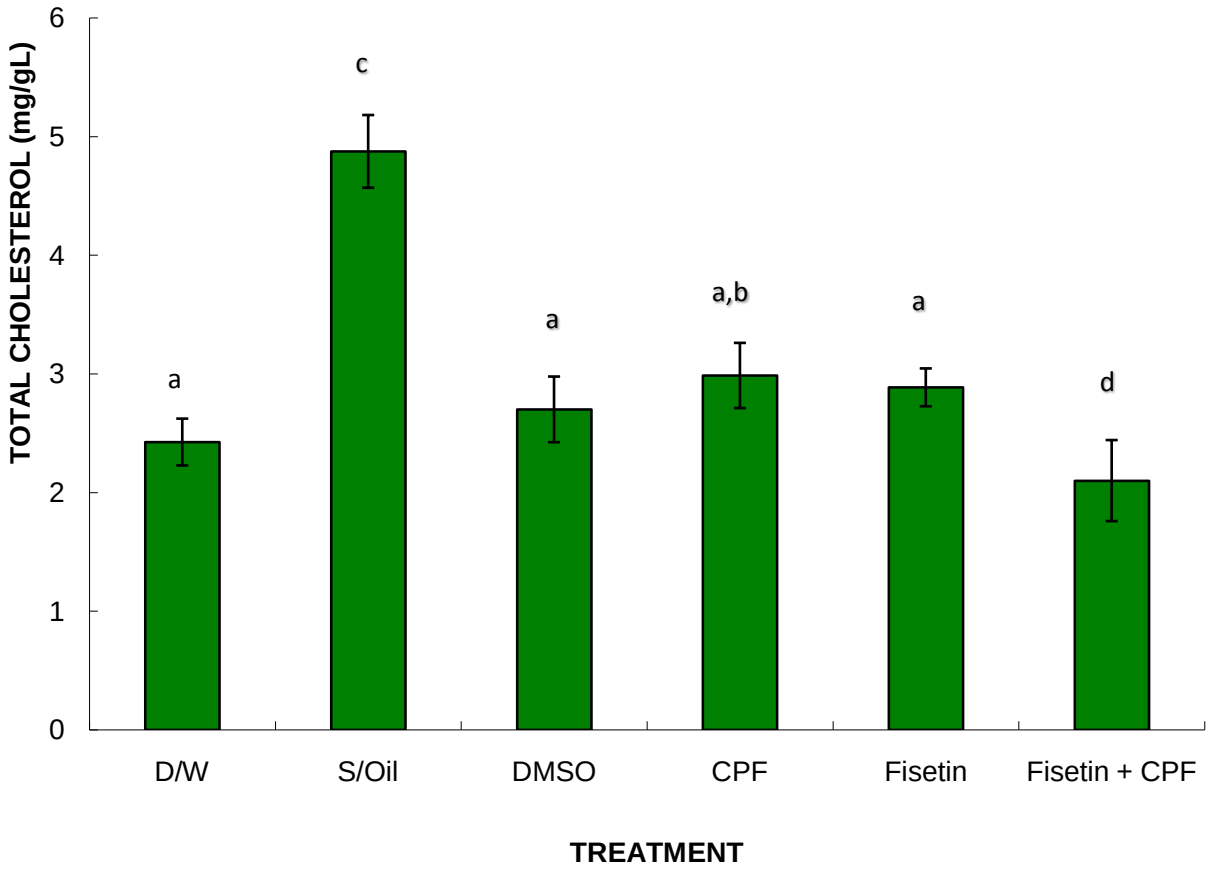


Figure 4.15. Effect of subchronic exposure to chlopyrifos (CPF) and fisetin on serum total cholesterol concentration in adult male Albino mice.

$^{a, b, c, d}$ Different superscripts indicate significant($P \square 0.05$) differences between groups.

Table 4.1: Effect of subchonic exposure to chlopyrifos and fisetin on open field parameters in adult male albino mice (Mean ± SEM)

Open field parameters (f)	GRP1(D/W)	GRP2(S/O)	GRP3(DMSO)	GRP4(CPF)	GRP5(F)	GRP6(F+CPF)
Horizontal movement	24.17 ± 3.64	7.08 ± 2.99	14.19 ± 2.82	21.86 ± 5.13	17.14 ± 2.65	13.42 ± 4.30
Rearing	11.08 ± 1.42	7.25 ± 1.42	7.81 ± 1.47	8.05 ± 1.76	6.80 ± 1.30	3.42 ± 1.08
Grooming	11.00 ± 3.60	10.71 ± 2.71	12.67 ± 1.16	16.77 ± 3.91	10.48 ± 1.12	11.50 ± 4.26
Center crossing	2.00 ± 0.59[a]	0.33 ± 0.24	0.38 ± 0.18[b]	1.10 ± 0.26	0.29 ± 0.23[b]	0.92 ± 0.70
Defaecation	2.08 ± 0.37	1.17 ± 0.42	2.10 ± 0.40	1.62 ± 0.25	2.43 ± 0.35	1.42 ± 0.37
Urination	0.42 ± 0.21	0.33 ± 0.14	0.14 ± 0.10	0.00 ± 0.00	0.48 ± 0.05	0.08 ± 0.08

GRP = group, f = frequency, D/W = distilled water, DMSO = dimethylsulphoxide, CPF = chlopyrifos, F = fisetin

[a, b,] Different superscripts indicate significant (P □ 0.05) differences between groups

Table 4.2: Effect of subchonic exposure to chlopyrifos and fisetin onforced-swimming parameters in adult male Albino mice (Mean ± SEM)

Forced swimming parameters(S)	GRP1(D/W)	GRP2(S/O)	GRP3(DMSO)	GRP4(CPF)	GRP5(F)	GRP6(F+CPF)
Swimming duration	158.80±22.29[b]	150.00±9.44	151.40±15.47[b]	118.90±8.01	98.29±9.49[a]	176.80±11.48[b]
Climbing duration	17.50±4.27	2.25±1.32	38.86±14.16	41.57±18.79	5.71±3.24	51.25±13.34
Immobility duration	123.80±21.29[b]	147.80±8.46	122.60±15.58[b]	147.40±20.04[c]	203.60±15.36[a,b]	72.00±15.35[d]

GRP = group, S = seconds, D/W = distilled water, DMSO = dimethylsulphoxide, CPF = chlopyrifos, F = fisetin

[a, b, c] Different superscripts indicate significant(P □ 0.05) differences between groups

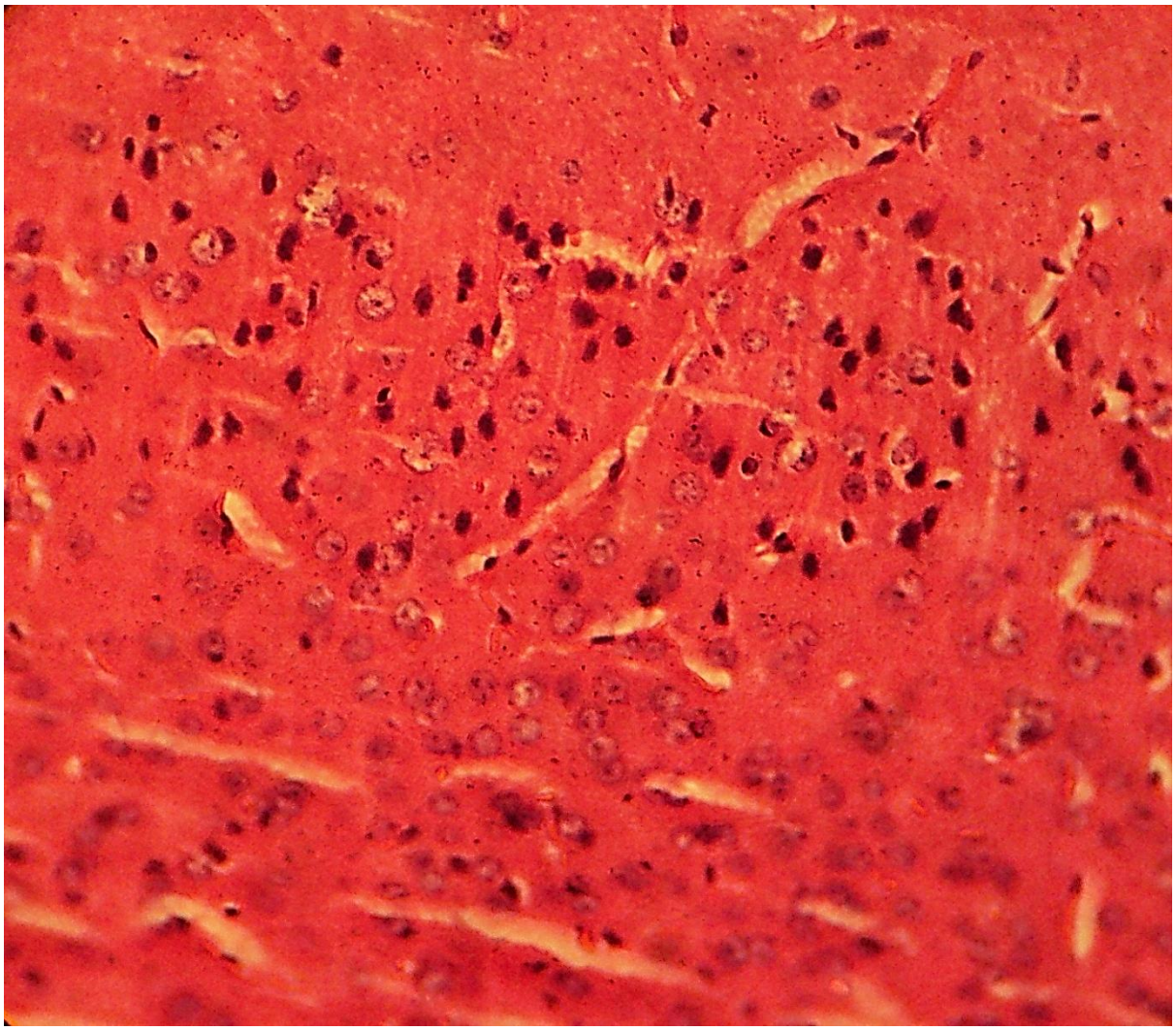


Plate I: Photomicrograph of a section of the brain of mouse pretreated with distilled water showing no apparent histopathologic lesions (H and E × 400).

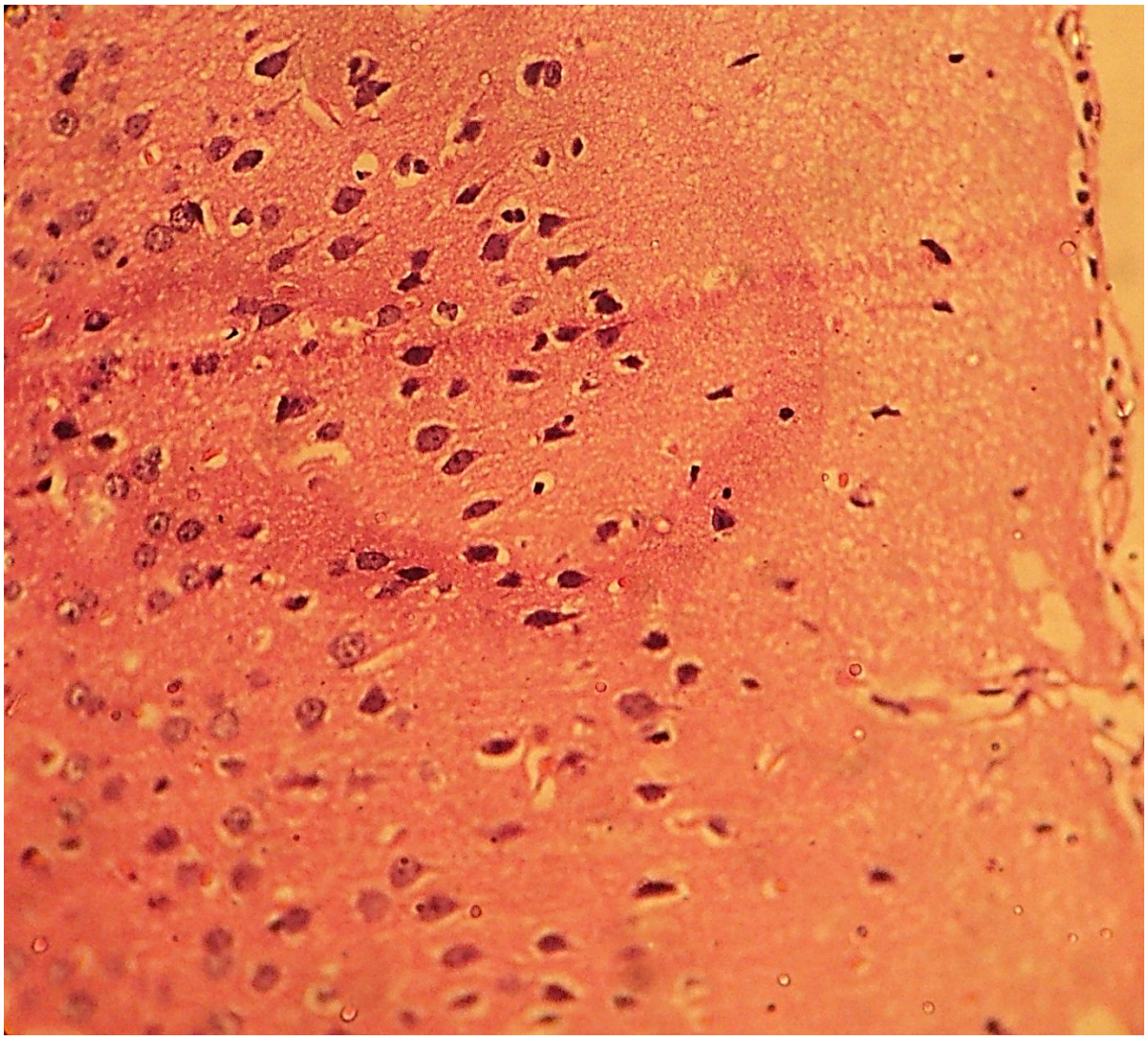


Plate II: Photomicrograph of a section of the brain of mouse exposed to soya oil (2ml/kg) showing no significant histopathological findings (H and E × 400)

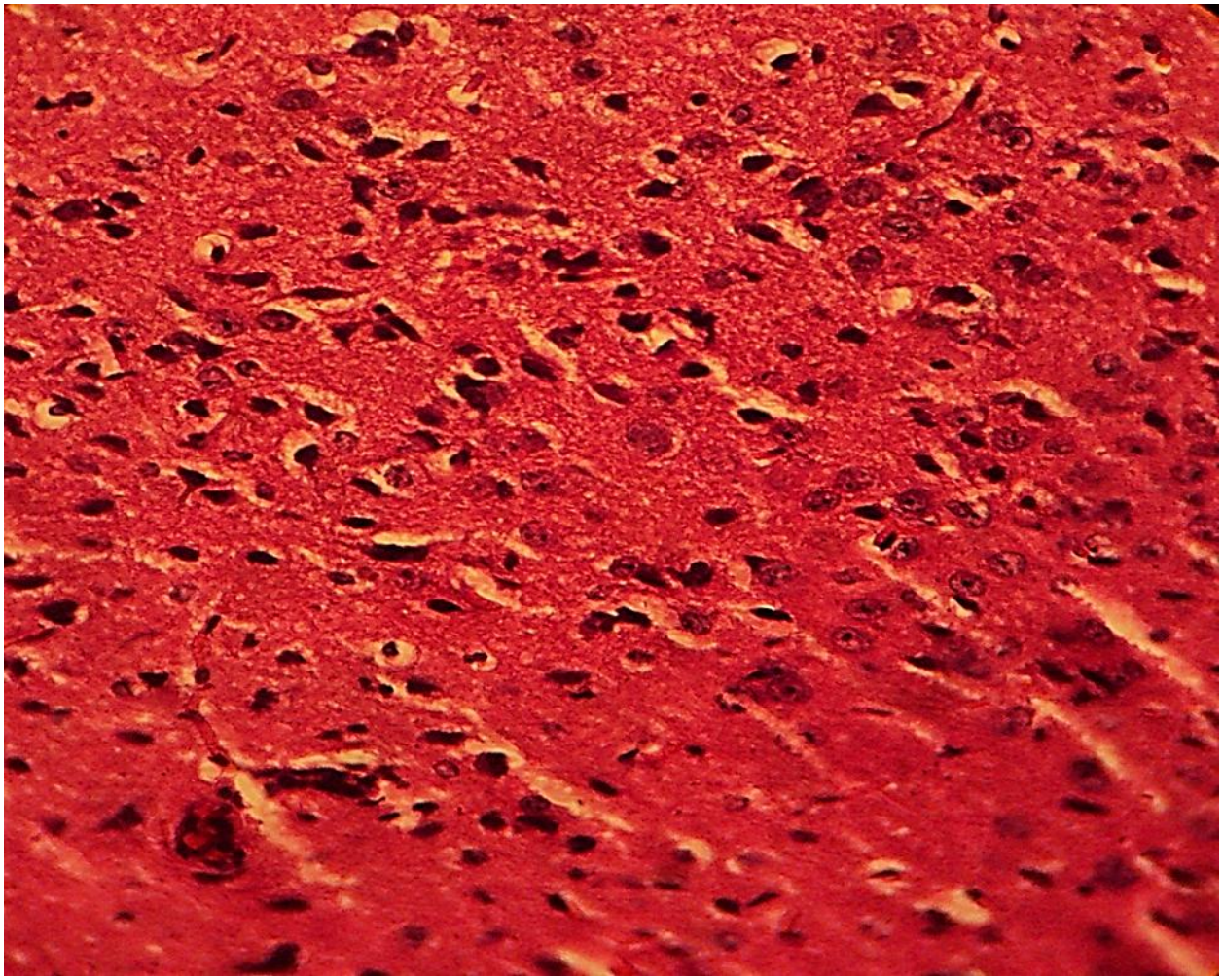


Plate III: Photomicrograph of a section of the brain of mouse exposed to dimethylsulfoxide showing no histopathologic leisions. (H and E × 400).

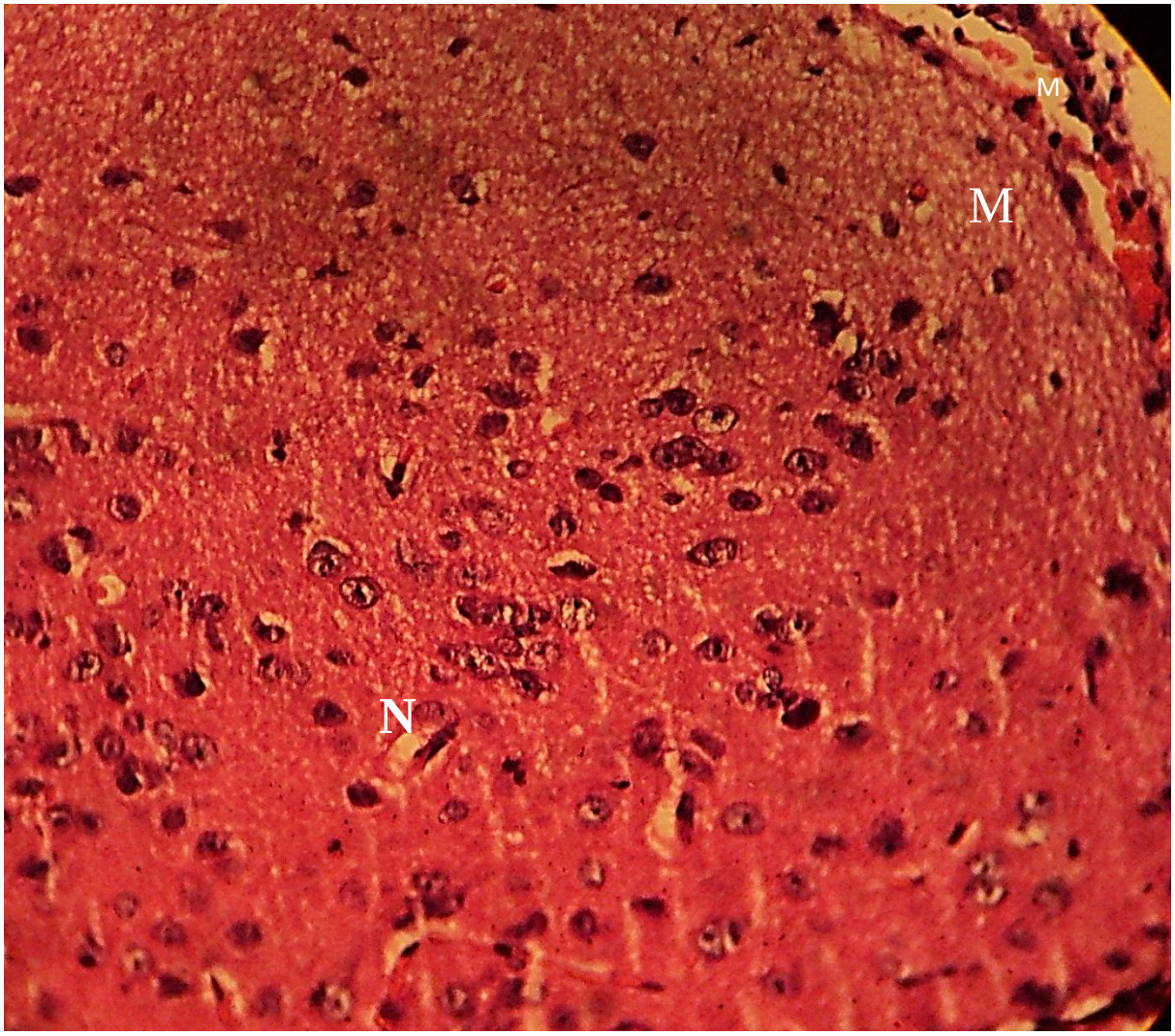


Plate IV: Photomicrograph of a section of the brain of a mouse exposed to CPF showing neuronal degeneration (N), meningeal haemorrhages (M) (H and E × 400).

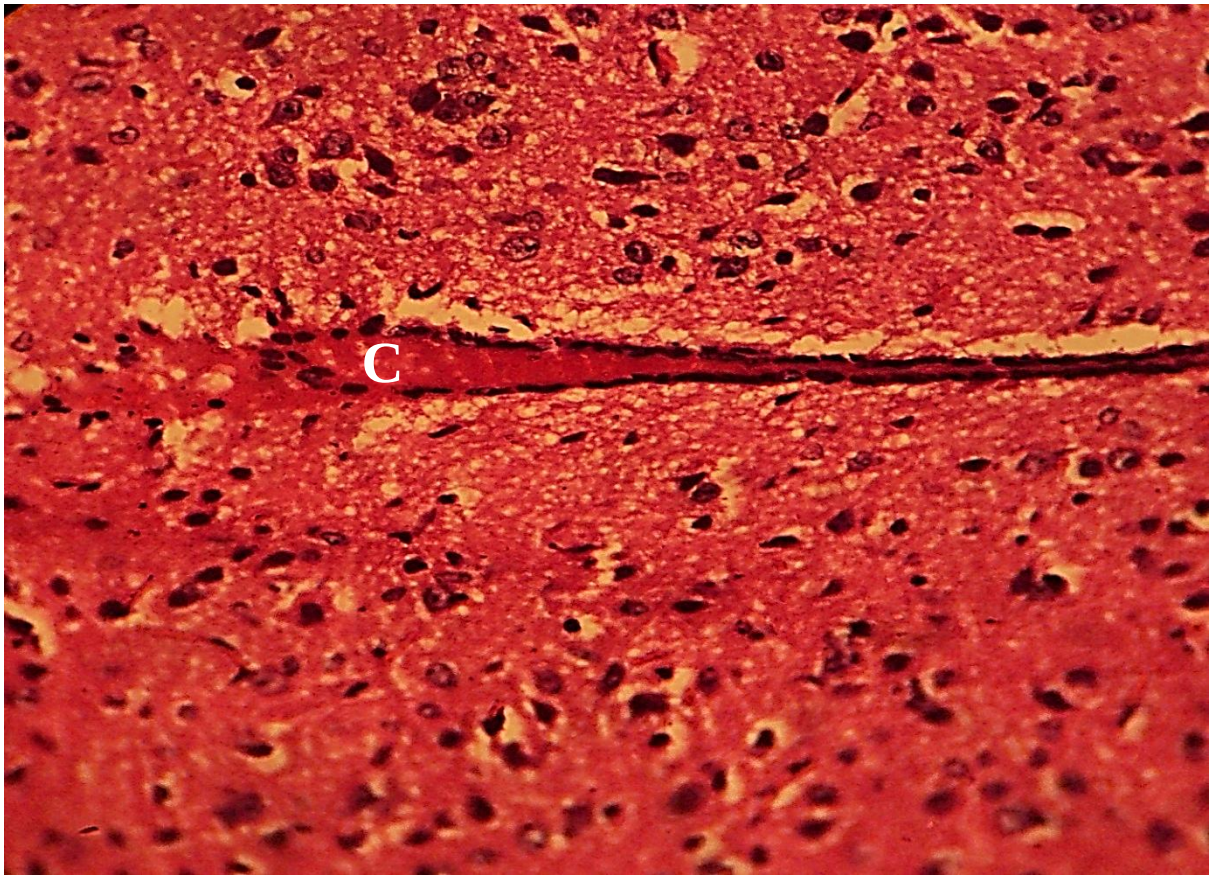


Plate V: Photomicrograph of a section of the brain of a mouse exposed to chlopyrifos (6.6mg/kg) showing congestion of blood vessel (C) (H and E × 400).

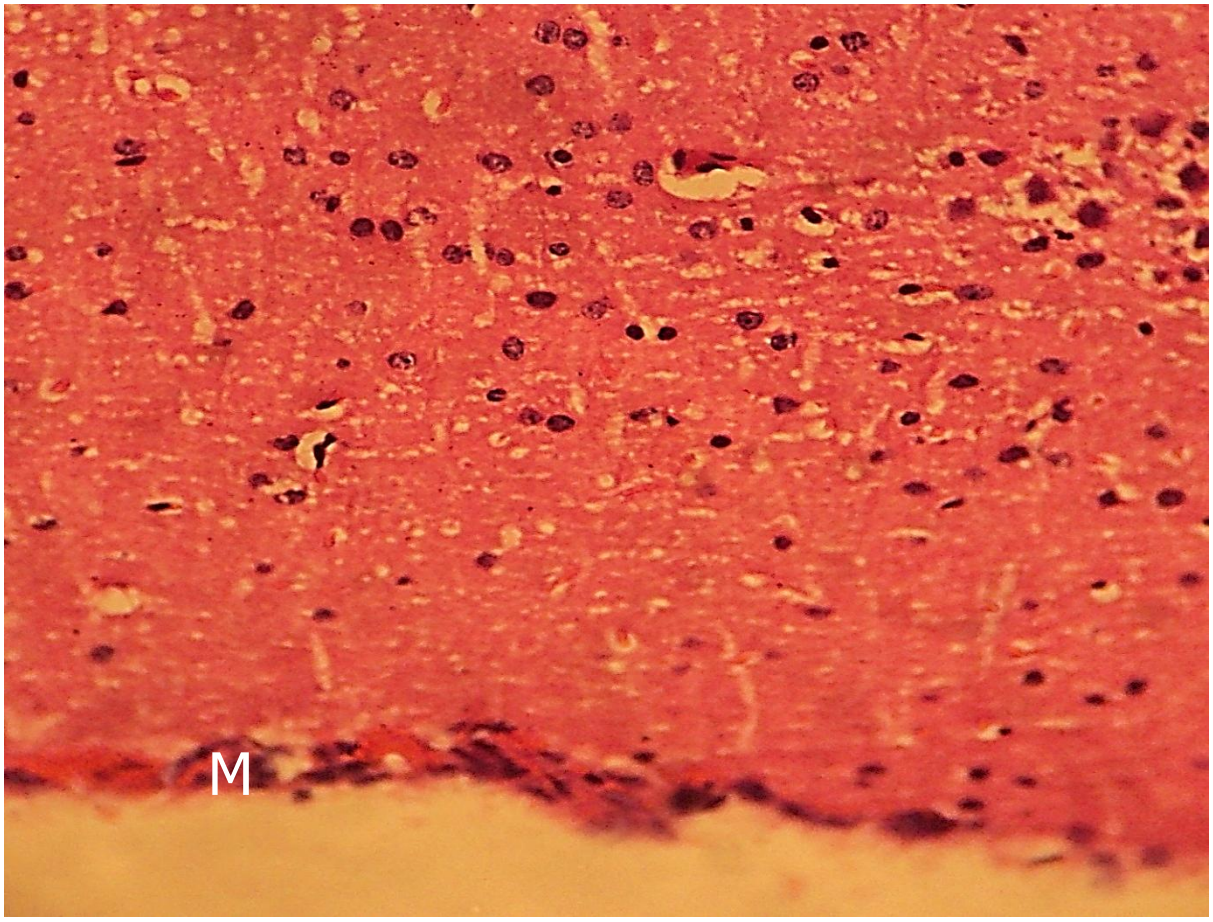


Plate VI: Photomicrograph of a section of the brain of a mouse exposed to fisetin (15mg/kg) showing meningeal haemorrhages (M) and neuronal degeneration (N). (H and E × 400).

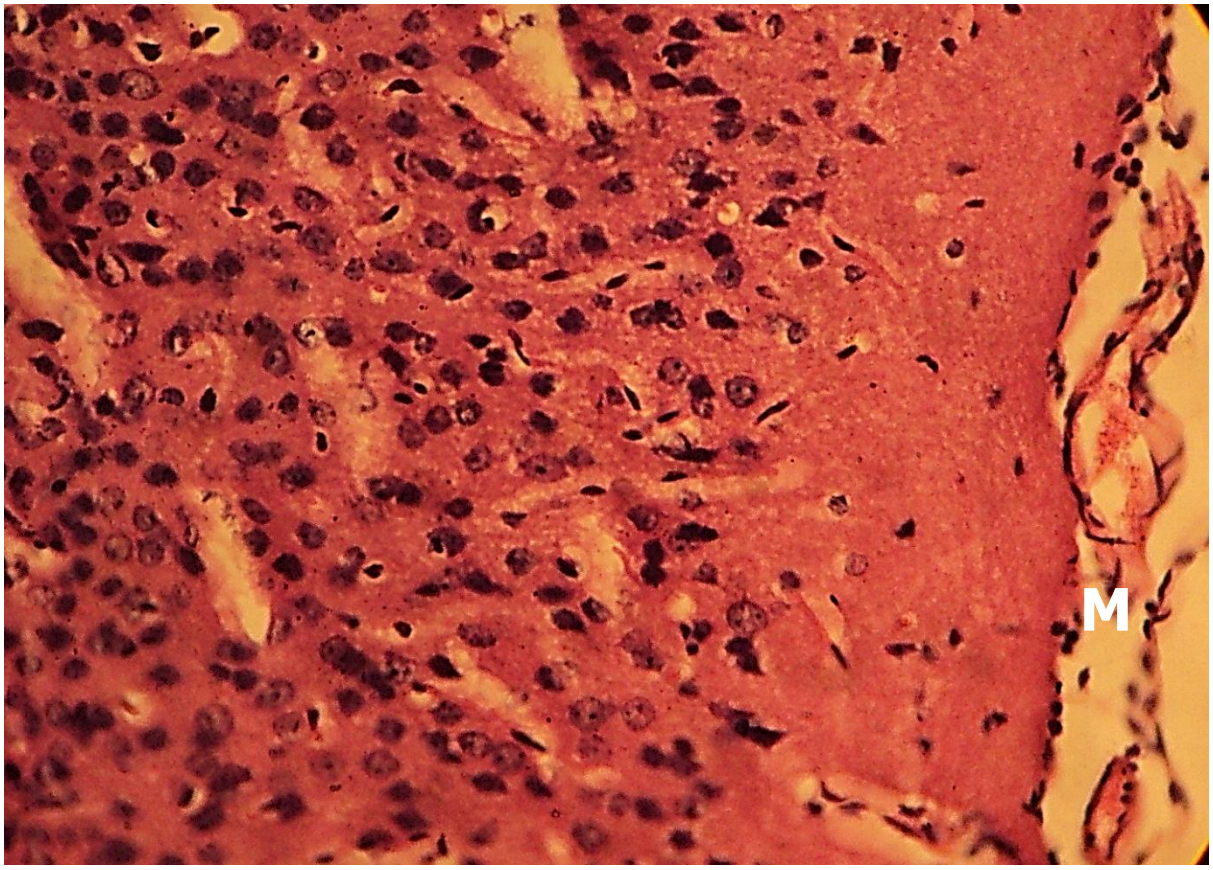


Plate VII: Photomicrograph of the brain of a mouse pretreated with Fisetin (15mg/kg) and chlopyrifos (6.6mg/kg), showing no significant histological findings in the brain and meningeal haemorrhages (M) (H and E × 400).

CHAPTER FIVE

DISCUSSION

5.1 Effect of Treatment on Neurobehavioural and Cognitive Changes

5.1.0 Effect of treatment on neuromuscular coordination

The present study showed a significant ($P < 0.05$)decrease in the neuromuscular coordination in the fisetin +CPF group. This agrees with the workdone by Mayte *et al.* (2014), who showed that sirtuin activation was responsible for the decrease in neuromuscular coordination observed during the acute phase of administration of polyphenolic compounds. Fisetin and other polyphenolic compounds have been shown to inhibit neuromuscular coordination at the acute stage of administration as a result of sirtuin activation (Mayte *et al.*, 2014; Gan *et al.*, 2017). Sirtuin, which is a protein that influences energy metabolism, induces starvation in cells (Guarente, 2007). Previous work by Yoonsu *et al.* (2013) and Xu, (2015) has shown that fisetin reduces circulating glucose. In the current study energy level was reduced in mice exposed to fisetin.

5.1.1 Effect of locomotor efficiency

The significant decrease in the locomotor efficiency in the CPF group relative to the fisetin and fisetin + CPF groups agrees with the finding in previous studies by Ambali *et al.* (2011) and Ambali *et al.*(2012) that CPF causes a deficit in locomotor efficiency as a result of oxidative stress, induced by CPF (Alavanja *et al.*, 2004), and reduction in muscle AChE activity (Ambali and Ayo, 2012). Fisetin is a polyphenolic compound that has been proven to have strong antioxidant potential (Naghma *et al.*, 2013; Seo and Jeong, 2015). It also has a

neuroprotective effect, hence it ameliorated the deficit in the locomotor efficiency recorded in mice exposed toCPF (Zbarsky *et al.*, 2005; Maher *et al.*, 2011).

5.1.2 Effect of treatment on motor strength

The present study showed no significant difference across the groups. However, there was an insignificant decrease in the forepaw grip time in the group exposed to CPF when compared to the group exposed to fisetin + CPF. This finding indicates that fisetin ameliorated the toxic effect of CPF on fore-paw grip time. Previous work done by Ambali and Ayo (2012) has shown that CPF causes a deficit in the fore-paw grip time (motor strength), as a result of decreased muscle Acetylcholinesterase activity and lipoperoxidation (Terry *et al.*, 2003). Fisetin ameliorated these effects through its antioxidant activity, by its ability to reduce ROS production (Subramanian *et al.*, 2015).

5.1.3 Effect of treatment on excitability scores

In the current study, the group exposed to fisetin alone showed an insignificant increase in excitability scores, whereas the group exposed to fisetin+CPF showed a decrease in the excitability of the mice compared to the CPF group. This result is contrary to the finding of Ambali and Ayo (2012), who showed that CPF decrease the excitability score in rats. The decrease in the excitability in the group of mice exposed to fisetin + CPF may have been due to the pro-oxidant effect of fisetin (Watjen *et al.*, 2005).

5.1.4 Effect of treatment on open-field parameters

There were no significant differences in the open-field parameters, except in the frequency of centre crossings; where the group of mice exposed to fisetin showed a significant increase, when compared to the control group D/W. However, there were increases in the horizontal movement, frequency of grooming, frequency of centre crossings, and urination, when compared to the Fisetin+CPF group. There was also a non-significant decrease in the frequency of grooming and number of defaecation.

The ability of fisetin to decrease (compared to the CPF alone group) the horizontal movement, frequency of grooming, number of centre crossings and the number of urination indicates that fisetin ameliorated the toxic effects of CPF. Historically, open-field test has been used to assess the fearfulness or emotional reactivity of rodents (Weiss and Greenberg, 1998). An increase in the horizontal movement, grooming, and urination is an index of fearfulness and anxiety (Diaz-Moran *et al.,* 2014). Increase in the frequency of the centre crossings shows a high level of exploratory behaviour and low anxiety (Gould *et al.,* 2009). Frequency of rearing shows the exploratory behaviour of mice. (Gould *et al.,* 2009). Thus, the ability of fisetin to ameliorate these effects of CPF on the mice was due mainly to the antioxidant capacity of fisetin (Subramanian *et al.,* 2015). Fisetin has the ability to penetrate the blood-brain barrier to exert its neuroprotective effect (Chiruta *et al.,* 2012) and to reduce ROS production, thereby reducing oxidative stress in the tissues (Prakash *et al.,* 2013; Koneru *et al.,* 2016).

5.1.5 Effect of treatment on learning

This study showed that there were significant differences between the D/W and S/OIL groups, D/W and DMSO, D/W and CPF, D/W and fisetin and D/W and Fisetin+CPF. The

group exposed to CPF had a decrease in learning, when compared to the Fisetin+CPF group. It has been shown that CPF impairs cognitive performance (Levin *et al.,* 2002), especially at prenatal exposure. The cognitive deficit recorded in this study could be as a result of neuronal damage induced by oxidative stress (Idris *et al.,* 2014). The reduced number of foot-shocks in the Fisetin+CPF group agrees with the work done on mice by Prakash *et al.*, (2013) and shows that fisetin prevented cognitive deficits induced by CPF exposure. This agrees with the work done by Currias *et al.*, (2014) and Maher (2009) who showed that fisetin corrected cognitive deficit in mice through its neuroprotective property.

5.1.6 Effect of treatment on short-term memory

The higher number of step down in mice exposed to CPF indicates that CPF causes a deficit in the memory (Ambali *et al.,* 2010a). This decline in the cognitive performance could be as a result of oxidative damage to the brain tissue especially in the hippocampus, which plays a vital role in learning and memory (Liu *et al.*, 2011).

The improvement in memory following pretreatment with fisetin indicates that fisetin mitigated the oxidative stress, produced by CPF, and this finding agrees with the work done by Rendiero *et al.* (2013) and Maher (2009) which demonstrated that fisetin improve spatial memory in mice (Maurya and Trigun, 2015).

5.1.7 Effect of treatment on forced swimming parameters

Previous studies have shown that CPF causes immobility and reduces swimming duration (Chen *et al.,* 2011b). Antidepressant agents like sertraline increases swimming duration and decreases immobility duration (Mikail *et al.,* 2012). In the present study, fisetin decreased

swimming duration and increased immobility duration contrary to the work done by Lee *et al.*, (2014) who reported fisetin to have antidepressant activity following acute administration. The depressant like effect seen in the current study may have been as a result of the prooxidant effect of fisetin caused by subchronic administration (Constantin *et al.,* 2011).

5.2 Effect of Treatment on AChE Activity

The significant difference between CPF and the D/W group was due to the established finding that CPF inhibits the AChE activity in mice (Ambali *et al.,* 2011; Nguyen *et al.,* 2016), although it reduced significantly AChE activity. (Ambali *et al.,* 2011). The lipophilic nature of CPF maybe responsible for the clinical and behavioural toxicity observed in the group exposed to CPF.

The antioxidant, fisetin ameliorated the effect of CPF on the brain AChE activity (Khan *et al.,* 2013). Fisetin exhibits its antioxidant effect through ROS scavenging, thereby reducing the lipoperoxidative damages, inflicted on the tissues by CPF (Naghma *et al.,* 2013).

5.3 Effect of Treatment on Brain Lipoperoxidation

The study showed a significant difference between the CPF group and the DMSO group. CPF exposure has been shown to cause lipoperoxidative damages exerting adverse effects on the brain lipids, thereby further increasing the generation of ROS (Singh and Panwar, 2014). CPF elicits chronic intoxication, leading to redox disturbances and irreversible brain damage via oxidative stress mechanism (Kaur*et al.,* 2014).

The effect of oxidative stress has been shown by an increase in the concentration in the MDA being a lipid peroxidation marker, indicating increased levels of ROS in the tissues (Abdollahi*et al.,* 2004)

The result of the present study has shown that there was a reduction in the MDA concentration in mice treated with Fisetin + CPF, due to the antioxidant effect of fisetin and its ability to combat the production of ROS, thereby reducing oxidative stress in the brain tissues (Brajesh and Surendra, 2016).

5.4 Effect of Treatment on Brain Antioxidant Enzymes Activity

5.4.1Effect of treatment on brain catalase activity

The present study revealed that there were significant differences in the CPF and fisetin groups, when compared to the negative control group (D/W). There was a decrease in the catalase activity in the group exposed to the CPF, and the decrease was ameliorated in the group exposed to fisetin and CPF. The decrease in the catalase activity induced by CPF exposure is as a result of oxidative damages to the brain tissue (Monsour and Mossa, 2009), which in turn resulted in a decreased synthesis of the enzyme or oxidation inactivation of the enzyme protein (Heikal *et al.,* 2012b; Koneru *et al.,* 2016).

5.4.2Effect of treatment on brain superoxide dismutase activity

The CPF treatment significantly decreased SOD activities of the groups exposed to fisetin + CPF, when compared to the group exposed to CPF alone. This result shows that fisetin has the ability to ameliorate the effects of CPF on the brain SOD activities.

Previous studies revealed that CPF decreases significantly the SOD activity in rats (Ronika *et al.,* 2011). Treatment with fisetin restored the SOD activity to its normal state, through its potent antioxidant activity (Maurya and Trigum, 2015; Brajesh and Surendra, 2016).

5.4.3Effect of treatment on glutathione peroxidase activity

The present study showed a significant decrease in mice exposed to CPF, when compared with the controls.Previous studies have shown that CPF causes a decrease in the brain glutathione activity (Naseer *et al.,* 2013) and fisetin has been demonstrated to modulate activities of antioxidant enzymes, including glutathione peroxidase (Maurya and Trigum, 2015; Brajesh and Surendra 2016).

5.5 Effect of Treatment on Serum Lipid Profiles

The present study showed an increase in the serum HDL, triglycerides and total cholesterol in the group exposed to CPF, when compared to the control group (D/W). There was also a decrease in the levels of the lipid profiles in the group exposed to Fisetin+CPF. The study also showed a non-significant decrease in the LDL, compared to the control group and there was a slight increase in the LDL level in the group of mice exposed to fisetin +CPF.

Previous studies has showed that CPF has the effect of increasing the serum lipid profiles (Uchendu *et al.,* 2013). The decreased levels of serum lipid profiles in the group exposed to fisetin+CPF shows that fisetin ameliorated the effect of CPF on serum lipids (Shin *et al.,* 2013; Jung *et al.,* 2013).

5.6 Effect of Treatment on Brain Histopathology

The present study showed histopathological changes in the meninges and the brain, which agree with the result obtained by El-Hossary *et al.* (2009), that CPF causes brain tissue damages, meningeal haemorrhages and congestion (Zama *et al.,* 2007).

The group exposed to CPF + Fisetin showed milder histopathologic changes in the meninges, while there was no significant observable changes in the brain,indicating that fisetin may ameliorate the toxic effects of CPF in the brain and meninges. This amelioration may be due to the ability of fisetin to penetrate the blood-brain barrier, exert its antioxidant effects on the (Chiruta *et al.,* 2012), or its ability to reduce the production of ROS (Subramanian *et al.,* 2015).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

It can be concluded that:

1) Subchronic exposure to CPF affected neurobehavioural activities in Mice
2) Subchronic exposure to CPF caused oxidative stress and adversely affected the Histo-architecture of the mice
3) Fisetin administration provided protection against the CPF induced adverse effects on the neurobehaviour of mice
4) Fisetin provided protection against oxidative damages induced by sub-chronic exposure to CPFin mice
5) Fisetin protected the mice from brain histopathological damages induced by sub-chronic exposure to CPF.

6.2 Recommendations

It is recommended that:

1) Fisetin containing fruits and vegetables such as berries, cucumber and onions should be consumed regularly to protect individuals against harmful environmental pollutants.
2) Farmers and other CPF users should take fisetin supplements prior to the use, in order to prevent adverse effects of CPF

3) Further studies should be carried out in order to ascertain the ideal dose of fisetin to be administered for the prevention of CPF induced toxic effects.
4) Studies should be conducted to elucidate the mechanism by which fisetin administration could have ameliorated neurobehavioural, oxidative stress and histopathological changes induced by subchronic CPF exposure.

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APPENDIX

Appendix 1: Preventive Effect of Fisetin on Neurobehavioural and Cognitive Changes Induced by Sub-chronic Exposure to Chlorpyrifos in Adult Male Albino Mice (MEAN ± SEM)

PARAMETERS	GRP 1 (D/W)	GRP2 (S/OIL)	GRP3 (DMSO)	GRP4 (CPF)	GRP5 (FISETIN)	GRP6 (CPF+FISETIN)
Neuromuscular coordination (Degrees)	66.70 ± 0.00	68.73± 0.43	69.51 ± 0.31	68.56 ± 0.43	68.56 ± 0.43	67.08 ± 1.25
Locomotor efficiency	3.83 ± 1.22	4.99 ± 0.91	5.72 ± 0.52	7.38 ± 0.91	3.91 ± 0.57	4.42 ± 0.64
Motor strength (Seconds)	16.25 ± 2.02	18.92 ± 1.24	19.48 ± 1.58	14.14 ± 0.72	20.14 ± 1.69	17.84 ± 2.14
Excitability scores	3.83 ± 0.17	3.67 ± 0.14	3.71 ± 0.11	3.76 ± 0.6	3.95 ± 0.11	3.57 ± 0.17
Learning (No. of stepdown)	2.25 ± 0.25	0.50 ± 0.50	0.43 ± 0.30	0.57 ± 0.50	0.29 ± 0.29	0.00 ± 0.00
Memory (No. of stepdown)	0.25 ± 0.25	0.00 ± 0.00	0.14 ± 0.14	0.43 ± 0.20	0.14 ± 0.14	0.00 ± 0.00

Appendix 2: Preventive Effect of Fisetin on Open-Field Parameters Changes Induced by Sub-chronic Exposure to Chlorpyrifos in Adult Male Albino Mice (MEAN ± SEM)

Parameters (Frequency)	GRP1 (D/W)	GRP2 (S/OIL)	GRP3 (DMSO)	GRP4 (CPF)	GRP5 (FISETIN)	GRP6 (FISETIN + CPF)
Horizontal Movement	24.17 ± 3.64	7.08 ± 2.99	14.19 ± 2.82	21.86 ± 5.13	17.14 ± 2.65	13.42 ± 4.30
Rearing	11.08 ± 1.42	7.25 ± 1.42	7.81 ± 1.47	8.05 ± 1.76	6.80 ± 1.30	3.42 ± 1.08
Grooming	11.00 ± 3.60	10.71 ± 2.71	12.67 ± 1.16	16.77 ± 3.91	10.48 ± 1.12	11.50 ± 4.26
Centre crossing	2.00 ± 0.59	0.33 ± 0.24	0.38 ± 0.18	1.10 ± 0.26	0.29 ± 0.23	0.92 ± 0.70
Defaecation	2.08 ± 0.37	1.17 ± 0.42	2.10 ± 0.40	1.62 ± 0.25	2.43 ± 0.35	1.42 ± 0.37
Urination	0.42 ± 0.21	0.33 ± 0.14	0.14 ± 0.10	0.00 ± 0.00	0.48 ± 0.05	0.08 ± 0.08

Appendix 3: Preventive Effect of Fisetin on Forced Swimming Parameters Induced by Sub-chronic Exposure to Chlorpyrifos in Adult Male Albino Mice (MEAN ± SEM)

Parameters (Seconds)	GRP1 (D/W)	GRP2 (S/OIL)	GRP 3 (DMSO)	GRP 4 (CPF)	GRP 5 (FISETIN)	GRP 6 (FISETIN + CPF)
Swimming Duration	158.80 ± 22.29	150.00 ± 9.4 4	151.40 ± 15.47	118.90 ± 8.01	98.29 ± 9.49	176.80 ± 11.48
Climbing Duration	17.50 ± 4.27	2.25 ± 1.32	38.86 ± 14.16	41.57 ± 18.79	5.71 ± 3.24	51.25 ± 13.34
Immobility Duration	123.80 ± 21.29	147.80 ± 8.46	122.60 ± 15.58	147.40 ± 20.04	203.60 ± 15.36	72.00 ± 15.35

Appendix 4: Preventive Effect of Fisetin on Oxidative Stress Biomarkers Induced by Sub-chronic Exposure to Chlorpyrifos in Adult Male Albino Mice (MEAN ± SEM)

Parameters	GRP 1 (D/W)	GRP 2 (S/oil)	GRP 3 (DMSO)	GRP 4 (CPF)	GRP 5 (FISETIN)	GRP 6 (FISETIN + CPF)
ACh concentration (nmol/min/ml).	13.33 ± 0.47	16.73 ± 0.96	15.25 ± 0.68	9.51 ± 0.42	11.07 ± 0.65	11.61 ± 0.54
MDA (nmol/mg protein)	179.1 ± 15.85	240.60 ± 38.12	260.80 ± 35.60	109.80 ± 11.75	256.80 ± 35.92	173.9 ± 18.77
CAT (µ/mg protein)	134.20 ± 27.16	105.50 ± 36.26	80.77 ± 14.58	52.26 ± 3.47	61.32 ± 9.37	78.55 ± 7.37
GPx (nmol/min/ml)	83.47 ± 7.15	92.67 ± 4.90	93.32 ± 9.75	51.46 ± 8.73	72.90 ± 6.93	73.96 ± 4.97
SOD (µ/ml)	63.49 ± 9.27	71.58 ± 23.41	88.09 ± 9.14	56.01 ± 8.67	90.53 ± 12.19	143.10 ± 11.09

Appendix 5: Preventive Effect of Fisetin on Serum Lipid Profiles Induced by Sub-chronic Exposure of Chlorpyrifos in Adult Male Albino Mice (MEAN ± SEM)

Parameters	GRP 1 (D/W)	GRP 2 (S/OIL)	GRP 3 (DMSO)	GRP 4 (CPF)	GRP 5 (FISETIN)	GRP 6 (CPF +FISETIN)
Serum HDL (mg/gL)	1.25 ± 0.28	2.10 ± 0.09	0.94 ± 0.15	1.44 ± 0.17	0.97 ± 0.22	0.75 ± 0.13
Serum LDL (mg/gL)	0.99 ± 0.16	2.36 ± 0.21	1.50 ± 0.26	1.26 ± 0.14	1.65 ± 0.28	1.14 ± 0.31
Triglycerides (mg/gL)	0.95 ± 0.32	2.10 ± 0.12	1.29 ± 0.23	1.41 ± 0.27	1.23 ± 0.20	1.10 ± 0.25
Total Cholesterol (mg/gL)	2.43 ± 0.20	4.86 ± 0.31	2.70 ± 0.28	2.99 ± 0.27	2.89 ± 0.16	2.10 ± 0.34