AMELIORATIVE EFFECTS OF VITAMIN C ON NEUROBEHAVIOURAL, HAEMATOLOGICAL AND BIOCHEMICAL CHANGES INDUCED BY SUBACUTE CO-ADMINISTRATION OF CHLORPYRIFOS AND CYPERMETHRIN IN WISTAR RATS.

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(D.V.M., A.B.U., 2005)

M.Sc./VET-MED/01859/2006-07

**A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL,
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA**

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN VETERINARY TOXICOLOGY

**DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY,
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31st JULY, 2011

DECLARATION

I declare that the work in this thesis, entitled "Ameliorative Effects of Vitamin C on Neurobehavioural, Haematological and Biochemical Changes Induced by Subacute Co-Administration of Chlorpyrifos and Cypermethrin in Wistar Rats" has been performed by me in the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria under the supervision of Professor J. O. Ayo, Dr S. F. Ambali and Professor J. O. Hambolu. The information derived from the literature has been duly acknowledged in the list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

_____ _____

Dayo Olufemi AKANBI Date

CERTIFICATION

This thesis, entitled "Ameliorative effects of Vitamin C on Subacute Co-administration of Chlorpyrifos and Cypermethrin in Wistar Rats" by Akanbi, Dayo Olufemi meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

"To Him who sits on the throne and unto the lamb", the Lord of Hosts, the King of kings and the Restorer of my soul, the God who leads me besides still waters, to Him only I dedicate this masterpiece.

ACKNOWLEDGEMENTS

My heartfelt and most profound expression of gratitude and encomia go to my Redeemer, the Author and Finisher of my faith. Blessed be the Lover of my soul, the God that inscribed me in the palm of His Hands that He may not forget me, the Creator of the ends of the earth, whose understanding is unsearchable. I worship the Immortal, Invisible, and the only wise God, the most Gracious and Merciful, my ever-present Help in times of trouble.

My soul showers unending blessings and praises to my dearest parents, Mr. and Mrs. E. O. Akanbi (I fondly call them Papa and Mama) for creating an enabling environment, nurturing and passionately supporting me, and also for their unfading love and relentless prayers.

I am inexplicably indebted to three rare gems - three supervisors; Professor J. O. Ayo, my major, for his fatherly guidance, excellent academic influence and detailed supervision of this project; Dr S.F. Ambali, my first minor, for being a chastising brother, a caring and persevering father and for his technical "panel-beating" of my project all the way. My erudite father, Professor J. O. Hambolu, my second minor, for his encouragement, quintessential love, fatherly counsel and guidance. They exuded the essential ingredients that made the completion of this project a reality.

My "Daddy and Mummy" Dr. and Dr. (Mrs.) V.O. Kolawole are signature to every success I have recorded so far, for their moral, financial, spiritual and emotional support, I say a huge thank you.

To my 'oga', CSP. E. J. Usifo, (Officer-in-charge), Lafiagi Prisons Farm Centre, Kwara State, I shout "all correct, sir" and give my most loyal salute and unreserved appreciation for his unwavering moral support and tireless understanding, especially for approving permissions to make progress with this project.

Dr. Olusola Oladipo is a friend indeed, we went through a lot of turbulence together- academic, physical and spiritual. Ultimately, we emerged victorious, “o se ore mi, ku itoju mi”. My heart goes out in thanks to my colleagues and senior friends who immensely contributed to the success story of this project: Drs M. Shittu, L. S. Yaqub, O. O Asala, Tijjani Abdul Fatai, D. O. Omoniwa (Scriptural brother), F. A. Olajide, T. Dzenda, S. O. Salami, M. U. Kawu, and M. S. Abubakar. The technical support offered by the entire staff of the Department of Veterinary Physiology and Pharmacology under the leadership of Chief Emmanuel Nwosu and Mr. Tony Abah is appreciated.

I acknowledge with great delight the moral support and contributions of my friends, Drs Peter Ehizibolo, Noble Igbokwe, Nde Yerima, Jibril Aliu. I render a special ‘shout’ to my colleagues and brothers with the Nigerian Prisons Service, Drs Lasisi Oluwole, Babalola Rotimi, ASP Adetutu Emmanuel and Ogunsanya Dayo.

Quintessential thanks to my spiritual mentor and father, the astute and anointed Pastor Albert Wole Soetan, whose teachings have made indelible and positive impact in my life. Also to Pastors Ken and Precious, and other brethren at the Commonwealth of Zion Assembly (COZA), I celebrate you all.

This project were made colourful by angels of destiny in Bisola Hambolu, Kikanwa Onyema, Gift Ereosun, Izeh Adava, Lynda Nwabunor, Toyin Ekundayo, Adekola Aderoju, and Sanmi Aderoju (“Pastor”) who single-handedly and painstakingly analyzed, typed data and provided me with an anointing for completion.

I appreciate Dr. and Dr. (Mrs.) G.B. Adesiji of the Benevolence unit of COZA for all the attention, love, prayers and counsels, I elevate you. Also, to Dr. Ayotunde Omotosho and members of the Social Network team, I thank you for the prayers and moral support.

The love from my siblings, Kemi, Joke and Titilayo, is a priceless asset to my existence, I declare that I love you too.

I pay a post-humous tribute to my late dear sister of blessed memory – Abiodun Jumoke Akanbi (I love and miss you) and my late dear friend – Dr Abidemi Olabode Olaniyan, you were always there for me.

Finally, when I look back and forth, I have nothing else to say but to give glory, majesty and honour to the Lord God Almighty, who was, who is and shall ever be forever more. Amen.

ABSTRACT

Experiments were performed with the aim of evaluating the effects of vitamin C on neurobehavioural, haematological and biochemical changes induced by repeated subacute co-administration of chlorpyrifos (CPF) and cypermethrin (CYP) in Wistar rats. Forty adult Wistar rats were divided into 4 groups of 10 rats (5 males and 5 females) each. The rats in group I (control) were administered with soya oil (2 ml/kg), while those in group II were given vitamin C (100 mg/kg) only. Rats in group III were co-administered with CPF (5.3 mg/kg) and CYP (14.1 mg/kg) (~ $1/16^{th}$ of their LD_{50}), while those in group IV were pretreated with vitamin C (100 mg/kg) followed by the administration of CPF (5.3 mg/kg) + CYP (14.1 mg/kg) 30 minutes later. These regimens were given orally by gavage once daily for a period of 28 days. The effects of the different treatments on body weight change, motor activity, anxiety, neuromuscular coordination, learning and short-term memory were evaluated in the rats at various times during the study period. Haematological parameters such as packed cell volume, haemoglobin concentration, total erythrocyte count, total and absolute differential leucocyte count, thrombocyte count and erythrocyte osmotic fragility were determined using standard methods. Sera obtained from another set of blood samples were evaluated for the concentrations of total protein, albumin, globulin, creatinine, urea, uric acid and glucose using standard methods. In addition, the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactic acid dehydrogenase (LDH), creatine kinase (CK) were evaluated in the sera samples. Lipid peroxidation was determined by measuring for the thiobarbituric acid reactive substance; malondialdehyde (MDA) in the brain and liver of the rats. The results demonstrated that subacute co-exposure to chlorpyrifos and cypermethrin caused an appreciable percentage decline in body weight gain although the changes were not significant (P

> 0.05). The CPF+CYP group demonstrated an increase in anxiety, sensorimotor reflex and brain malondialdehyde concentration when compared to the control and vitamin C pretreated groups. It also recorded a significant ($P < 0.05$) decrease in neuromuscular coordination, and learning and memory indices as compared to the control and vitamin C pre-treated groups. Rats in the CPF+CYP group were observed to have a relative decline in packed cell volume, haemoglobin concentration, total erythrocyte and thrombocyte counts when compared to the S/oil and vitamin C pretreated groups. Rats exposed to CPF+CYP recorded a relatively higher erythrocyte osmotic fragility and total leucocyte count than rats in the control and VC groups. Serum AST, ALP, LDH and CK values for the CPF+CYP group were relatively higher (although not significant; $P > 0.05$) than the S/oil and VC groups. The serum concentrations of creatinine and urea were increased while the serum concentrations of glucose, uric acid and albumin were decreased by CPF+CYP when compared with control and VC groups. In conclusion, the study has shown that pretreatment with vitamin C mitigates the alterations in neurobehavioural, haematological and biochemical parameters induced by co-administration of CPF and CYP.

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List of Abbreviations

2, 4-D: 2, 4-Dichlorophenoxyacetic acid
2, 4, 5-T: 2, 4, 5- Trichlorophenoxyacetic acid
2 PAM: 2-Pralidoxine aldoxime methchloride
ACh: Acetylcholine
AChE: Acetylcholinesterase
A.D.: Anno domini
A.F.R.: Ascorbate free radical
A.L.P.: Alkaline phosphatase
A.L.T.: Alanine aminotransferase
AP-1: Transcription factor
AR: Ascorbate radical
ASC: Ascorbate
ASK1: Apoptosis signal-regulating kinase
ATP: Adenine triphosphate
BuChE: Butyrylcholinesterase
cAMP: Cyclic adenylyl monophosphate
CAT: Catalase
ChE: Cholinesterase
CK: Creatine kinase
CK-B: Brain creatine kinase
CK-M: Plasma creatine kinase
CPF: Chlorpyrifos
CPF-oxon: Chlorpyrifos oxon
CPO: Chlorpyrifos oxon
CS: Choreo-athetosis
CREB: Ca^{2+}/cAMP response element binding protein
CYP: Cypermethrin
D.D.T.: Dichlorodiethyltrichoroethane
D.E.P.: Diethyl-phosphate
D.H.A: Dehydro-ascorbate
DNA: Deoxyribonucleic acid
EOF: Erythrocyte osmotic fragility
Fe^{2+}: Iron II ions
Fe^{3+}: Iron III ions
G.D.: Gestation day
GABA: γ-aminobutyric acid
IMS: Intermediate syndrome
IUPAC: International union of pure and applied chemistry
JNK: C- jun N- terminal kinases
K^{+}ATPase: Potassium adenosine triphosphatase
LD_{50}: Median lethal dose
LDH: Lactic dehydrogenase
LDL: Low density lipo-protein
LTP: Long term potentiation
MAO: Monoamine oxidase

MAPKs: Mitogen-activated protein kinases
MDA: Malondialdehyde
mRNA: Messenger ribonucleic acid
NADH: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
Na+ ATPase: Sodium adenosine triphosphatase
NMDA: N -methyl- D-aspartate
NTE: Neuropathy target esterase
OP: Organophosphate
OPICN: Organophosphate induced chronic neuropathy
OPIDPN: Organophosphate induced delayed poly-neuropathy
PCV: Packed cell volume
PNS: Peripheral nervous system
POP: Persisitent organic pollutant
PTZ: Chemoreceptor trigger zone
PUFA: Polyunsaturated fatty acid
RBC: Red blood cell
RNA: Ribonucleic acid
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
RS: Reactive species
SAPKs: Stress activated protein kinase
SOD: Superoxide dismutase
T: Tremor type
TBARS: Thiobarbituric acid reactive substance
TCPY: 3, 5, 6-trichloro-2-pyridinol
TLC: Total leucocyte count
TP: Total protein
TRX: Thioredoxin
UV: Ultra violet
VC: Vitamin C

CHAPTER 1

INTRODUCTION

1.0 BACKGROUND

Human and animal populations throughout the world are inevitably exposed on a daily basis to low and apparently non-toxic levels of environmental contaminants. The consequences of potential hazards posed by the use of these compounds to human and animal health remain unknown (Wade *et al*., 2002). Pesticides as organic contaminants are the most toxic environmental pollutants (Garg *et al*., 2004). They are primarily used in agriculture and residential settings for the control of plant pests and animal ectoparasites (WSC, 2004). Among insecticides, organophosphates (OP) remain the most widely used, accounting for about 50% of all insecticide applications worldwide (Casida and Quistad, 2004). Globally, there are approximately 3 million cases of OP poisoning reported annually, resulting in nearly 200,000 deaths (Haywood and Karalliede, 2000).

Chlorpyrifos (CPF) [O, O- diethyl-O- 3, 5, 6,-trichloro-2- pyridinyl-phosphorothioate] is a moderately toxic, broad spectrum chlorinated OP insecticide (Timchalk *et al*., 2002). It is the ingredient in commonly used OP insecticide brands such as Termicot®, Britermicot®, Dursban®, and Lorsban® (Timchalk *et al*., 2002). Like other OP insecticides, CPF inhibits acetylcholinesterase (AChE), thereby increasing the ACh level in the synaptic cleft, resulting in the stimulation of muscarinic, nicotinic (Nostrandt *et al*., 1997) and central cholinergic receptors. However, recent evidence has shown that other mechanisms such as induction of oxidative stress may be involved in the molecular mechanisms of CPF toxicosis (Slotkin *et al*., 2006; Ambali *et al*., 2007; Ambali, 2009, Ambali *et al*., 2010a-d).

Pyrethroid insecticides are widely used in agriculture, veterinary practice and gardening, among others. They are a group of chemically unique compounds with a high degree of efficacy and relatively low mammalian toxicity (Kakko, 2004). It has been estimated that they account for about 25% of the global insecticidal market in the 1990's (Williamson *et al*., 1996). Severe pyrethroid toxicosis is common in developing countries because of its widespread and frequent use in agriculture (Bateman, 2000); and the apparent poor pesticide regulation and legislation in these countries. Cypermethrin (CYP) [(*R*)-cyano-3-phenoxybenzyl (*1R*)-cis-3- (2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate] is one of the most potent pyrethroids owing to the presence of an alpha-cyano group in its chemical structure (Holloway *et al*., 1989; Tabarean and Narahashi, 1998). In Nigeria, it is marketed as Captain C®, Cymbush®, etc. CYP is known to stimulate nerves causing pronounced repetitive firing activity in peripheral nerves that may lead to frequency-dependent depression of nerve impulse. This is achieved via a mechanism of delaying the closure of sodium channels, so that a prolonged sodium tail current persists after membrane repolarization (Perger and Szadkowski, 1994). Recently, oxidative stress has also been implicated in CYP-induced toxicosis (Nasuti *et al*., 2007).

Oxidative stress is commonly observed when pro-oxidants, such as free radicals, reactive oxygen and reactive nitrogen species (i.e. superoxide anion, hydroxyl radical, hydrogen peroxide and nitric oxides) overwhelm cellular antioxidant systems and induce cellular damage. Cellular injury is primarily due to the inability of the anti-oxidants to neutralize the effects of the oxygen radicals (Saulsbury *et al*., 2009). The body is however equipped with antioxidant systems to combat the menace posed by the reactive oxygen species. These include the antioxidant enzymes and molecules. The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, etc (Chihuailaf *et al*., 2002). The antioxidant molecules include

glutathione, ceruloplasmin, albumin, vitamin C and E, and β- carotene. They act to protect the body against oxidative damage by either scavenging for the free radical and/ or repairing the damage caused by the ROS (Chihuailaf *et al*., 2002). The antioxidant vitamin C is a highly potent reducing agent (Padayatty *et al*., 2003) which is found naturally and abundantly in vegetables and fruits (Haytowitz, 1995). It is a low-molecular weight antioxidant that protects the cellular compartment against hydrophilic oxygen-nitrogen radicals; a property that makes it an efficacious antioxidant of the hydrophilic phase (Obara and Harasawa, 2008; Ogutcu *et al*., 2008).

1.1 STATEMENT OF PROBLEM

The increasing need to feed the ever-increasing human and animal population and combat public health has necessitated an increase in global insecticidal use. The exposure to pesticides in developing countries like Nigeria is rapidly increasing due to the less widely enforced regulations on pesticide importation and use, compounded by the porosity of borders, illiteracy among pesticide users and lack of awareness of the dangers of exposure (Ambali *et al*., 2010b) . Therefore, leading to over 200,000 annual global deaths due to pesticide poisoning occur in developing countries (Miranda, 2003).

The development of resistance to single insecticidal compounds has necessitated the use of a combination of pesticides in the control of agricultural pests, vectors and veterinary ecto-parasites (Alves-Branco *et al*., 2005). The different chemical groups included in each combination of pesticides often possess different mechanisms of action (Alves-Branco *et al*., 2005). In view of the complex pest situation and pervasion of resistant pest populations, the need to broaden spectrum of activity of pesticides has become very crucial. Therefore, when single insecticide failed to give adequate pest control, farmers often resort to the use of insecticide

mixtures. The most common mixtures were those of pyrethroids plus OPs (Ahmad, 2008). For example, the use of pesticide cocktails (otapiapia) is quite prevalent in Nigeria. According to Environmental Justice Foundation (2003), uncontrolled chemical cocktailing results in unpredictable consequence in biological systems. Exposures to multiple pesticides have been associated with multiple chemical sensitivity, which is a chronic pathologic condition manifesting multiple symptoms related to low levels of multiple unrelated chemical compounds (Lee *et al*., 2003).

Despite the heavy use of CPF and CYP in the Nigerian agricultural sector, the residual concentrations of these pesticides in foodstuffs are not determined for their conformity to the standards of maximum residue limits. In addition, their new biological effects have not been examined.

1.2 JUSTIFICATION OF THE STUDY

Current understanding of the toxicity of many environmental toxicants/pollutants is based primarily on toxicity studies performed on laboratory animals exposed to a single toxic agent (Brouwer *et al*., 1998; Wade *et al*., 2002). Although several toxicity studies have been conducted on some mixtures of pesticides, very little study has been done with a combination of CPF and CYP. Although numerous studies have linked single pesticide exposure to neurotoxicity, there have been very few systematic studies of mixtures/combinations of various pesticides on learning and memory (Carpenter, 2002). Khan (2006) reported the sub-acute hepatotoxic effects of a combined dose of CPF and CYP administered on alternate days to mice.

Cypermethrin is ubiquitous in Nigeria and it is one of the major pesticides found in the Nigeria pesticide market (Racke *et al*., 1997). It has also been used by the Federal Pest Control Services (FPCS), for Fadama associated pest management in Nigeria (NFDP, 2003). According

to Olorunshola (2006), CYP is one of the most available pesticides in Kaduna/Katsina agro-allied markets. Jacobsen *et al*. (2004) reported that CPF and CYP were among the five pesticides that were most frequently found as residues in food. CPF residues have been detected in exported citrus fruits in some parts of the world (Iwasaki *et al*., 2007), while CYP residues have been detected in some vegetables (USDA, 1994), and in milk from cows wearing CYP impregnated ear-tags for the control of horn flies (Braun *et al*., 1985). Similarly, these two pesticides have been consistently found as contaminants of rivers and other aquatic bodies in different parts of the world (Anderson *et al*., 2006; Hall *et al*., 2006; Syakalima *et al*., 2006). Based on local survey in Zaria, it has been found that CPF is one of the pesticides used for fish killing/harvesting by some local fishermen, through its application to streams and ponds (Personal communication). This act poses a huge exposure risk to animal and human consumers of the poisoned fish or contaminated water, since low level of the insecticidal mixture are being consumed.

Oxidative stress has been incriminated as an important mechanism of toxicosis for these two pesticides even when humans and animals are exposed to sublethal doses on a sub-chronic course (Gultekin, 2000; Etemadi-Aleagha *et al*., 2002). Many studies have indicated that pyrethroids induce oxidative stress (Giray, 2001; Vontas, 2001). Similarly, some studies have demonstrated that oxidative stress is induced by CPF in rats (Gultekin, 2000; Ambali, 2009; Ambali *et al*., 2010a-d) and humans (Banerjee *et al*., 1999; Dantoine *et al*., 2003). Therefore, the use of antioxidants such as vitamin C may ameliorate pesticide-induced neurotoxicity (Ambali *et al*., 2007; Ambali, 2009).

1.3 GENERAL AIM

The general aim of the study was to evaluate the ameliorative effects of vitamin C on neurotoxic, hemotoxic and biochemical changes induced by repeated subacute co-administration of CPF and CYP in Wistar rats.

1.4 SPECIFIC OBJECTIVES:

1. To evaluate the neurotoxic, hemotoxic and biochemical changes induced by sub-acute exposure to a combination of CPF and CYP in Wistar rats.
2. To determine the role of lipo-peroxidation on neurotoxic, haemotoxic and biochemical changes induced by sub-acute exposure to a combination of CPF and CYP in Wistar rats.
3. To evaluate the ameliorative effect of vitamin C on neurotoxic, haemotoxic and biochemical changes induced by sub-acute exposure to a combination of CPF and CYP in Wistar rats.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 WHAT ARE PESTICIDES?

According to the United States Environmental Protection Agency, pesticides are substances or mixture of substances intended for preventing, destroying, repelling, or mitigating pests; they also refer to substances intended for use as plant regulators, defoliants, or dessicants (EPA, 2005). They are synthetic chemicals or biological agents, antimicrobials, disinfectants or devices used against pests (Miller, 2004). According to Hodgson (2004), pesticides are also known as agricultural chemicals, and are defined as economic poisons, regulated by federal and state laws, that are used to control, kill or repel pests. USEPA (1996) defines pesticides as agents used to destroy pests i.e. organisms that compete for food supply, adversely affect comfort, or endanger human health. Weiss *et al*. (2004) describes them as a broad group of heterogenous chemicals that have a significant public health benefit by enhancing agricultural productivity and decreasing food-borne and vector-borne diseases.

Pesticides are deliberately released into the environment (Cox, 1998) with the aim of preventing and controlling damage to crops, human and animal health by insects, fungi, and noxious weeds (Garry, 2004).

2.1.1 Historical Perspectives

Humans have utilized pesticides to protect their crops even before 2500 B.C. The first known pesticide was elemental sulphur dusting used in Sumeria about 4,500 years ago. Toxic chemicals such as arsenic, mercury and lead were applied to crops to kill pests in the 15th century. By the 17th century, nicotine sulphate was extracted from tobacco leaves for use as an insecticide (Miller, 2002). However, in the 1800s pyrethrins; that is, compounds present in the flowers of

the *Chrysanthemum, Pyrethrum cineraefolium* were found to have insecticidal properties. In 1854, the French began using lime sulphur to eliminate downy mildew on grapes. In the United States of America, chemical pest control commenced in 1867 with the use of Paris Green (copper-acetoarsenite) for control of the Colorado potato beetle. In 1880, Bordeaux mix (hydrated lime and copper sulphate) was discovered which subsequently led to the manufacture of metal-based compounds for agricultural use. The roots of Derris plant species (*Derris elliptica* and *Lonchocarpus* species) were used by the Chinese and South American natives as fish poison. The active principle in these species, rotenone was isolated in 1895 and used for insect control (Hodgson, 2004).

By the 1900s, the pesticide manufacture had evolved leading to the emergence of newer compounds. Some of these compounds, which include petroleum oils distilled from crude mineral oils, were introduced in 1920s to control scale insects and red spider mites (Hodgson, 2004). In 1939, dichlorodiethyltrichoroethane (DDT) was discovered and lead to a huge success in preventing typhus outbreaks during World War II (making it the first war in history in which more soldiers died of battle-related wounds rather than diseases). In 1943, German scientists experimenting on nerve-gas during World War II synthesized the first organophosphate insecticide, parathion. Subsequently, the Dow Chemical Company introduced the phenoxy-herbicides; 2, 4-Dichlorophenoxyacetic acid (2,4-D) and 2,4,5-T (constituents of "agent orange") in the 1940s. DDT came into the U.S. market in 1945 after the war embargo was lifted. Pests that had plagued human history succumbed to these "miracle chemicals". DDT was promoted as a "godsent". The World Health Organization was so impressed with DDT's effect on the Anopheles mosquito that it even projected that the insecticide would eliminate malaria by 1955 (Encarta Encyclopaedia, 2009). However in the U.S.A., the use of DDT was eventually banned

in 1973 (Encarta Encyclopaedia, 2009). Triazine herbicides such as atrazine introduced in the late 1950s dominated the world herbicide market for years. Thereafter, the synthesis of pyrethroids became popular because of their relatively low mammalian toxicity, enhanced persistence, when compared to pyrethrins and low application rates (Hodgson, 2004).

The global pesticide use is increasing, especially in the third world countries (Garg *et al*., 2004). India uses approximately 85,000 tonnes of pesticides per annum (Garg *et al*., 2004). A total of at least 4.5 billion pounds of chemicals are applied annually as pesticides in the U.S. alone (Weiss *et al*., 2004). Today, several classes of compounds are used as pesticides in more than 20,000 pesticide products with nearly 900 active ingredients registered as insecticides, herbicides, rodenticides, nematocides, fungicides, fumigants, wood preservatives, and plant growth regulators (CDC, 2002).

2.1.2 Classification of Pesticides

Pesticides are classified on the basis of their main chemical constituents and mechanism of action (Weiss, *et al*., 2004). There is also a classification based on the target pests, that is, based on the organism the compound is designed to control (Hodgson, 2004; Weiss *et al*., 2004). In this study, pesticides have been classified on the basis of their target pests (CDPR, 2003).

2.1.2.1 Algicides

These are substances that are used for the control of algae in swimming pools, lakes, canals, and water used industrially or stored.

2.1.2.2 Attractants

They have the propensity to attract pests (for example, lure an insect or rodent to a trap). Pheromones are chemical sex attractants often used to confuse mating behaviour of insects.

2.1.2.3 Disinfectants and Sanitizers

These are agents that kill or inactivate disease-producing microorganisms (bacteria, viruses, etc.) on inanimate objects.

2.1.2.4 Fumigants

Refer to agents that produce gas or vapour intended to destroy insects, fungi, bacteria, or rodents, used to disinfest interiors of buildings and soil before planting.

2.1.2.5 Fungicides

Substances that reserve the capacity to kill fungi (many of which can infect and cause diseases in plants, animals and people; examples of disease-causing fungi: rusts, mildews, blights, and moulds), for example, dicloran and hexachlorobenzene.

2.1.2.6 Herbicides

Agents that are used to destroy weeds and other plants that grow where they are not wanted, for example, glyphosate and 2, 4-dichlorophenoxyacetic acid.

2.1.2.7 Insecticides

Substances used against insects in all developmental forms. They include ovicides and larvicides used against the eggs and larvae of insects, respectively. Insecticides are used in agriculture, medicine, industry and the household. Examples include organophosphates (chlorpyrifos and dichlorvos), pyrethroids (allethrin, cypermethrin, cyfluthrin, etc).

2.1.2.8 Miticides or Acaricides

They are pesticides that kill mites. Antibiotic miticides, carbamate miticides, formamidine miticides, mite growth regulators, organochlorine, permethrin and organophosphate miticides are all in this category.

2.1.2.9 Molluscicides

They are also known as snail baits and snail pellets, used against molluscs, often of value in agriculture or gardening to control slugs and snails that can damage crops by feeding on them. Iron (III) phosphate and aluminium phosphate are examples of these.

2.1.2.10 Nematicides

These kill nematodes (microscopic, wormlike organisms that feed on plant roots). Examples include, 1, 2-dibromo-3-chloro-propane, and 1, 3-dichloropropene.

2.1.2.11 Rodenticides

These are a category of pest control chemicals intended to kill rodents; for examples, include warfarin and coumatetralyl.

2.1.2.12 Defoliants

Substances that cause leaves or foliage to drop from a plant, usually to facilitate harvest. An example is agent orange.

2.1.2.13 Insect Growth Regulators.

These are products or materials that interrupt or inhibit the life cycle of a pest. They disrupt the action of insect hormones controlling moulting, maturity from pupal stage to adult, or other life processes, e.g. methoprene, hydroprene and nylar.

2.1.2.14 Plant Growth Regulators (also known as phytohormones)

These are chemicals that regulate plant growth (excluding fertilizers or other plant nutrients), they alter the expected growth, flowering, or reproduction rate of plants through hormonal rather than physical action. Auxins, cytokinins and ethylene belong to this category of substances.

2.1.3 Benefits and Bane of Pesticides

Pesticides have been of immense benefit to humanity in the control of certain communicable diseases and aversion of colossal agricultural losses that would have ensued due to ravaging effects of pests. Malaria, a disease with the greatest impact of all communicable diseases on the society living in tropical and sub-tropical areas is being controlled by pesticides (WHO, 1967).

By 1965, DDT had effectively eradicated malaria threatening about 953 million people (WHO, 1965). In addition to malaria, it controlled the outbreaks of louse-borne typhus, the plague and yellow fever, the three other most important diseases in human history (Hayes, 2001).

In the United States alone during the last 50 years, the use of synthetic organic pesticides has enabled agriculturalists to increase crop yields by as much as 50% (Ballatine, 1992). Despite the extensive use of pesticides, the U.S. Food and Drug Administration (FDA) estimated that pests destroy approximately one-third of the world's food crops annually (Ballatine, 1992). The use of pesticides also has its attendant adverse effect on human, animals and the environment. It has been estimated that three million cases of acute pesticide poisoning occur each year, with about 20, 000 accidental deaths and 200, 000 suicidal deaths (WHO, 1990; He, 2000). However, this may be a conservative estimate because not all cases of poisoning are reported (EJF, 2002). Many of these accidents arise as a result of exposure to toxic pesticides due to accidental spillages, leaking or poorly insulated knapsack sprayers, consumption of pesticide-contaminated food, drinking from a container that has been used for mixing pesticides, and accidental or

deliberate ingestion (Dinham, 1993). Pesticides have also been incriminated in the occurrence of birth defects, sterility and cancers (Dinham, 1993).

One factor that increases the risks of adverse effects of pesticides in people from developing countries is the fact that about 30 per cent of pesticides marketed in developing countries do not meet internationally accepted quality standards set by the Food and Agriculture Organization (FAO) (EJF 2003). Furthermore, EJF (2003) reported that many pesticides used in developing countries are banned or severely restricted in the industrialized world because of concerns about their safety. These concerns are generally not shared by pesticide users in the developing world, due to a widespread lack or poor awareness of the hazards of pesticide exposure both by the populace and the government.

2.1.4 Pesticide Exposure

The sources and routes of exposure to pesticides in children may differ from that of adults. For adults, the most important routes include oral ingestion, inhalation and dermal. These routes may differ between chemicals in that volatile substances are more likely to be inhaled than transdermally (EJF, 2003). A simple classification of exposure to organophosphate pesticides is: (a) occupational exposure; and (b) environmental or non-occupational exposure (Muldoon and Hodgson, 1992). Unintentional, accidental, or suicidal poisoning may occur in both categories of exposures (Langely and Sumner, 2002). Homicidal poisoning includes suicide poisoning as well as intentional poisoning of an individual by another (Langely and Sumner, 2002).

2.1.4.1 Occupational Exposure

Most of the pesticide exposures in adults occur as occupational hazards, especially in farm workers, pesticide applicators and those employed in the manufacture of pesticides. Such risks are predominant in developing nations, where issues involving hazards are less commonly

understood and health and safety regulations are less stringent (EJF, 2002). According to Jaga and Dharmani (2003), exterminators who use pesticides, greenhouse workers and florists, health-care workers (nosocomial poisoning) are often exposed, office workers are also exposed occasionally, when their offices are sprayed by exterminators; veterinary employees may get exposed during application of ectoparasiticides on animals; prosectors are also at risk of exposure when performing autopsies on victims of pesticide-related death; soldiers may be exposed to nerve poisons during war; an example is the Gulf War in which soldiers of the Allied troops were attacked with nerve poisons (Jaga and Dharmani, 2003).

Accidental oral contamination can occur when farmers eat, drink, or smoke while spraying or do so immediately after spraying without first decontaminating their hands (Kishi *et al*., 1995). Although ingestion of pesticides is the most dangerous form of exposure, transdermal absorption and inhalation are probably the major causes of occupational poisoning cases among farmers in developing nations as they are often ignorant of these particular risks (Kishi *et al*., 1995).

2.1.4.2 Environmental exposure

There are a number of ways in which non-occupational pesticide exposure can occur; for example, by aerial drift during spraying or through contact with contaminated food, water, soil, clothes or mother's milk (Hong *et al*., 2002). A good example of pesticides that can cause environmental exposure is a group of chlorinated chemicals called- Persistent Organic Pollutants (POPs). Owing to their chemistry and structure, POPs persist in the environment, thereby posing risks to human and environmental health (Sturm *et al*., 2007). Furthermore, spraydrift and edge-of-field run-off are important routes of pesticide entry into streams. Pesticide contamination originating from spraydrift usually resides in the water phase, while pesticides in contaminated run-off are to a large extent associated with suspended particles (Sturm *et al*., 2007); thus,

compounding the environmental contamination. Also, studies have shown that golfers have been exposed to pesticides following their play on turf grass treated with CPF and carbaryl (Putnam *et al*., 2008).

Dietary risks- If excessive concentrations of pesticides are applied frequently to crops or when they are used close to the period of harvest, high levels of pesticide residues may remain on the crops, which leave the consumers at a high risk of exposure (ECOTOXNET, 1996). Drinking water may also be contaminated, either through direct pollution of water systems or via the use of vessels used to mix pesticides in the transport of drinking water. In many developing countries a major concern is the low intake of protein diets which increase people's susceptibility to certain pesticides (ECOTOXNET, 1996). In a survey, pesticide residues were investigated in 50 agricultural products using a multi-residue method for agricultural chemicals by gas chromatography and mass spectroscopy, the result revealed that several pesticide residues were present at levels higher than the maximum residue limits (Iwasaki *et al*., 2007). In another survey in Japan involving 490 imported cereal products on the Tokyo market, eight organophosphorus pesticides were detected (Kobayash *et al*., 2008). Dietary risks are particularly high in Cambodia, where vegetables are often sprayed with banned pesticides right up to the point of harvest (EJF, 2002).

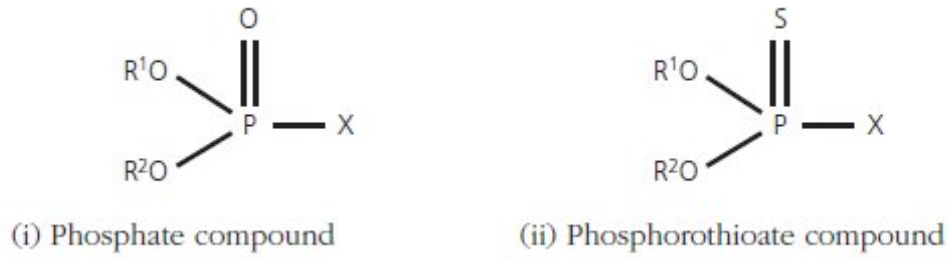
Exposure of children to pesticides- Children have multiple sources and routes of pesticide exposure that differ from adults (Cooper *et al*., 2001; Garry, 2004). They have unique behaviours and activities that place them at greater risk for heavier exposure to contaminants such as pesticides present in air, water and soil, compared with adults who live in the same environment (Weiss, 2000). The outdoor play activities of children increase their exposure to pesticides present in various elements of the environment. They are less likely than adults to wash hands

before eating or use utensils; also, their breathing zones are much closer to the ground, where most pesticide residue accumulate, increasing inhalation exposure to heavier-than-air toxicants and low-lying particulates (Bearer, 2000; Weiss, 2000). Two other characteristics further magnify their exposures to environmental toxicants: (1) their hand-to-mouth behaviour, which increases their chances of ingesting toxicants soils or dust, and (2) their playing close to the ground, which increases their exposure to environmental toxicants on floors, in carpets, soil and in the lower layers of the air (Landrigan, 2001). They can also be exposed through their pica-like behaviour of ingesting non-food items such as contaminated soil (Faustman *et al*., 2000). Furthermore, children are exposed through peculiar dietary means. Breast-feeding infants may ingest pesticides or their metabolites present in breast milk (Wickstrom *et al*., 1983); which, due to its high fat contents allow lipophilic substances to accumulate. Dichlorodiphenyldichloroethylene in breast milk, for example, may be 6-7 times higher than its concentration in blood (Al-Saleh *et al*., 2003. Compounding the situation is the report that multiple pesticides may be present at the same time in breast milk, for example, aldrin, lindane, endosulfan, methoxychlor, dieldrin and dichlorodiphenyltrichloroethane and its metabolites have been detected in milk specimen of women in Spain (Campoy, 2001). Also, a report has noted that infants experience a higher exposure to pesticide from agricultural crop residues because of their higher intake of fruits and vegetables per body weight than adults (National Research Council, 1993).

2.2 ORGANOPHOSPHATES

2.2.1 Chemical Structures and Physico-Chemical Properties

All compounds that contain both carbon and phosphorus elements practically belong to the group of organophosphorus compounds, although many of them do not inhibit the enzyme acetylcholinesterase and are not used as pesticides (COT, 1999). Meanwhile, organophosphate (OP) refers to a chemical compound in which a phosphate group, or phosphate derivative, is part of an organic (i.e. carbon-containing) molecule. In practice, it is specifically used to refer to those organophosphorus compounds which inhibit the enzyme acetylcholinesterase (COT, 1999; Jagger *et al*., 2007). They are usually esters, amides or thiol derivatives of phosphoric acids with the following general formula for (i) phosphates and (ii) phosphorothioates, respectively (Figure 2.1)



(i) Phosphate compound (ii) Phosphorothioate compound

Figure 2.1: Basic chemical structures of organophosphates (COT, 1999)

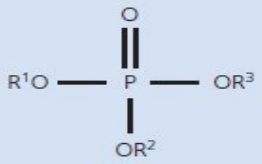
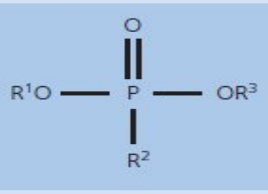
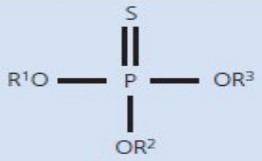
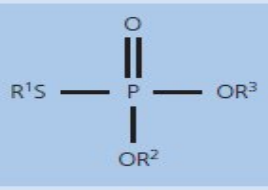
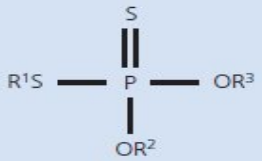
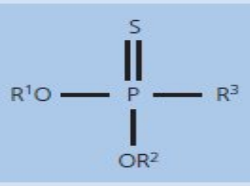
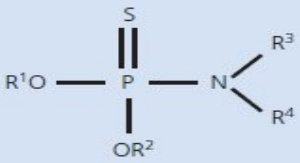
The structure of OPs determines their reactivity, biological activities, and potencies as acetylcholinesterase inhibitors; depending on the nature of R^1, R^2, and X.

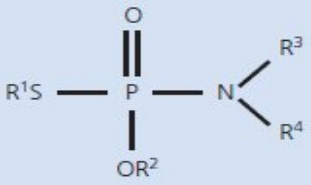
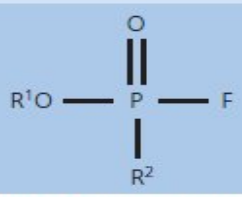
The physico-chemical properties of these compounds, including their volatility and lipid-solubility are determined by the structures of the substituents, R^1, R^2, and X. OPs, which are

nerve agent gases, such as sarin, possess low-molecular weights with simple R^1, R^2 substituent groups and are readily displaceable leaving groups (flouro- and cyano-) which are bound directly to the phosphorus without an intermediate oxygen or sulphur atom. The resultant molecules are both volatile and lipid-soluble. OPs used as insecticides have volatilities that are far less than those used as nerve agent gases. They are usually solid at room temperature and are more stable in aqueous solution. Those used in sheep dips are of low volatility and high lipid-solubility, and they easily cling onto the lanolin in the fleece (COT, 1999).

2.2.2 Classification of Organophosphates

A summary of the various classes of OPs is contained in Table 2.1; it shows the types, generic structures and examples.

Type	Structure	Examples
Phosphate		Dichlorvos Chlorfenvinphos
Phosphonate		Trichlorfon
Phosphorothioate		Bromophos Chlorpyrifos Chlorpyrifos methyl Diazinon Parathion
Phosphorothiolate (S-substituted)		Demeton-*S*-methyl Omethoate Profenofos
Phosphorodithioate		Dimethoate Disulfoton Malathion Thiometon
Phosphonothioate		EPN (this is the recognised common name of this pesticide)
Phosphorothioamidate (Phosphoramidothionate)		Isofenphos Propetamphos

Type	Structure	Examples
Phosphorothioamidate (Phosphoramidothiolate)		Methamidophos
Phosphonofluoridate		Sarin

Where R^1, R^2, R^3 and R^4 are chemical groups which may be the same or different. (The reader is referred to reference 4 for more details of the specific structures of these compounds.)

Table 2.1 (Basic chemical structures of the various groups of organophosphates, adapted from COT, 1999).

2.2.3 Historical Perspective and Uses

Organophosphorus pesticides are phosphoric acid esters or thiophosphoric acid esters and are among the most widely used pesticides for insect control (Abou-Donia, 2003). Tetraethylpyrophosphate was the first organophosphate synthesized as an AChE inhibitor in 1854. Later, dimethyl and diethyl phosphorofluoridates were synthesized (Abou-Donia, 2003).

Some OPs were developed in 1938 by Gerhard Schrader and co-workers. They were initially introduced as nerve poisons or agents of chemical warfare during World War II; and, subsequently, were developed into less potent chemicals for insecticidal uses the 1930s and 1940s (Sullivan and Blose, 1992; Hodgson, 2004). The anticholinesterase properties of the OP compounds were recognized and investigated as potential insecticides. Major research on OPs was carried out in the period preceeding the Second World War and continued in Germany during the war with the development of compounds which showed high volatility, rapid skin

penetration and potent effects on the central nervous system. Some of these agents include tabun, sarin and soman (COT, 1999).

Some OPs were developed for uses besides those based on their inhibition of acetylcholinesterase; for example, as defoliants and fire retardants, industrial fluids, lubricants, modifier of plastics while others are useful in the aviation industry where they serve as aviation fuel additives (triorthocresyl phosphate) (Sevim *et al*., 2003). During the 1950s and 1960s, OPs with selective toxicity to insects and relatively low toxicity to mammals were synthesized and their utilization soared rapidly in the 1970s (COT, 1999). Levels of use then plateaued and declined due to the introduction and increased use of pyrethroids. From the 1980s through the 1990s its use undulated (COT, 1999). Despite their undergoing increasing scrutiny and restriction these compounds still account for about 50% of global insecticides in use today (Casida and Quistad, 2004).

In the United Kingdom and many parts of the world, OPs have been used as agricultural and horticultural pesticides, in veterinary insecticidal applications (particularly in sheep dips to prevent and treat sheep scab and other ectoparasitic disorders, also in flea collars), in human medicines (malathion only – as a treatment for head lice). It is also used in various public hygiene products, both for use by professional operators (e.g. for the control of cockroaches and other insect pests in public buildings such as hospitals, schools, etc.) and for use by the general public (insecticides for home and garden use) (COT, 1999).

2.2.4 Mechanisms of Toxicity

Organophosphate insecticides are known to interfere with the metabolism or breakdown of acetylcholine (ACh) at cholinergic sites by inhibiting the activity of acetylcholinesterase (Blodgett, 2001). ACh is the neurotransmitter found between pre-and post- ganglionic neurons of

the autonomic nervous system; at the junction of post-ganglionic parasympathetic neurones in smooth muscle, cardiac muscle or exocrine glands, and at neuromuscular junctions of somatic nervous system; and in the cholinergic synapses in the CNS, It is also located on the red blood cell surface (Blodgett, 2001).

AcetylCholinesterase (AChE) or true cholinesterase (ChE) is the enzyme responsible for breaking down ACh at these sites (Blodgett, 2001). AChE has anionic and esteratic sites in which ACh is temporarily bound until hydrolysis initially releases choline from the anionic site and then acetate from the "acetylated" esteratic site (Figure 2.2). The AChE is inhibited by OP insecticides at these cholinergic sites, so that Ach keeps depolarizing the post synaptic membrane and cannot be broken down by synaptic AchE, which is inactive because it is "phosphorylated". Depolarization of postsynaptic membranes begins as a stimulatory process that may progress to paralysis (Blodgett, 2001).

The OP insecticides were designed to emulate the structure of ACh and occupy both anionic and esteratic sites of AchE. The OP moiety that fills the anionic site is quickly hydrolyzed away, leaving the phosphorus atom with multiple attached groups bound to the esteratic site. In OP insecticides, binding of the phosphorus atom with its attached groups to the esteratic site of AChE is considered "irreversible" binding, since its half-life may be for hours or days compared to the microsecond binding of ACh (Fukuto, 1990; Sultatos, 1994). At some point, the organic groups (i.e. methyl or ethyl) attached to the phosphorus may be hydrolysed away and replaced by hydrogen. This makes the bond between the phosphorus atom and the esteratic sites unbreakable. At this point, the enzyme has undergone "ageing" and will never be functional again (Blodgett, 2001). The body will have to synthesize new AchE at the rate of approximately 1% per day (Aaron and Howland, 1994). The OPs with dimethoxy groups tend to age faster than those with

diethoxy groups (Sultatos, 1994). Other esterase enzymes in the body act for most part as a cushion or prevention against clinical signs. Arylesterases or type A esterases hydrolyse OPs without being inhibited in the process while the type B esterases, consisting of AChE, pseudocholinesterase (ChE), carboxylesterase and neuropathy target esterase (NTE) are inhibited (Blodgett, 2001). Pseudocholinesterase located in serum, liver, pancreas and nervous tissues, and carboxylesterase (aliesterase) located in the serum, liver, muscle, and nervous tissue can both be inhibited without causing clinical signs (Fikes, 1997).

Death from acute OPs poisoning is associated with respiratory problems resulting from massive respiratory tract secretions, bronchiolar constriction, intercostal and diaphragm muscle paralysis, and respiratory paralysis from the CNS effects in the medulla (Blodgett, 2001).

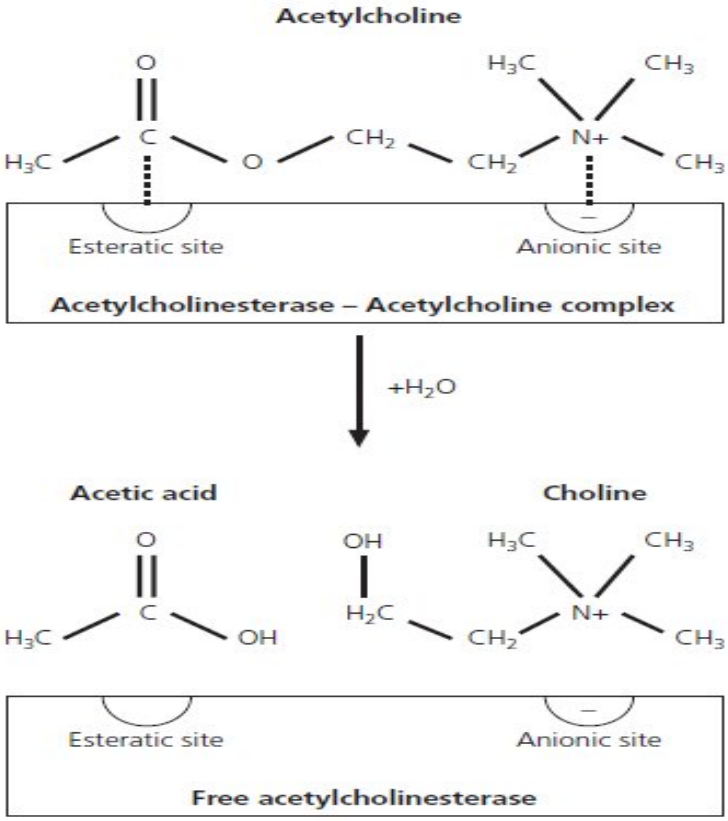


Figure 2.2: Schematic representation of the hydrolysis of acetylcholine by acetylcholinesterase (COT, 1999).

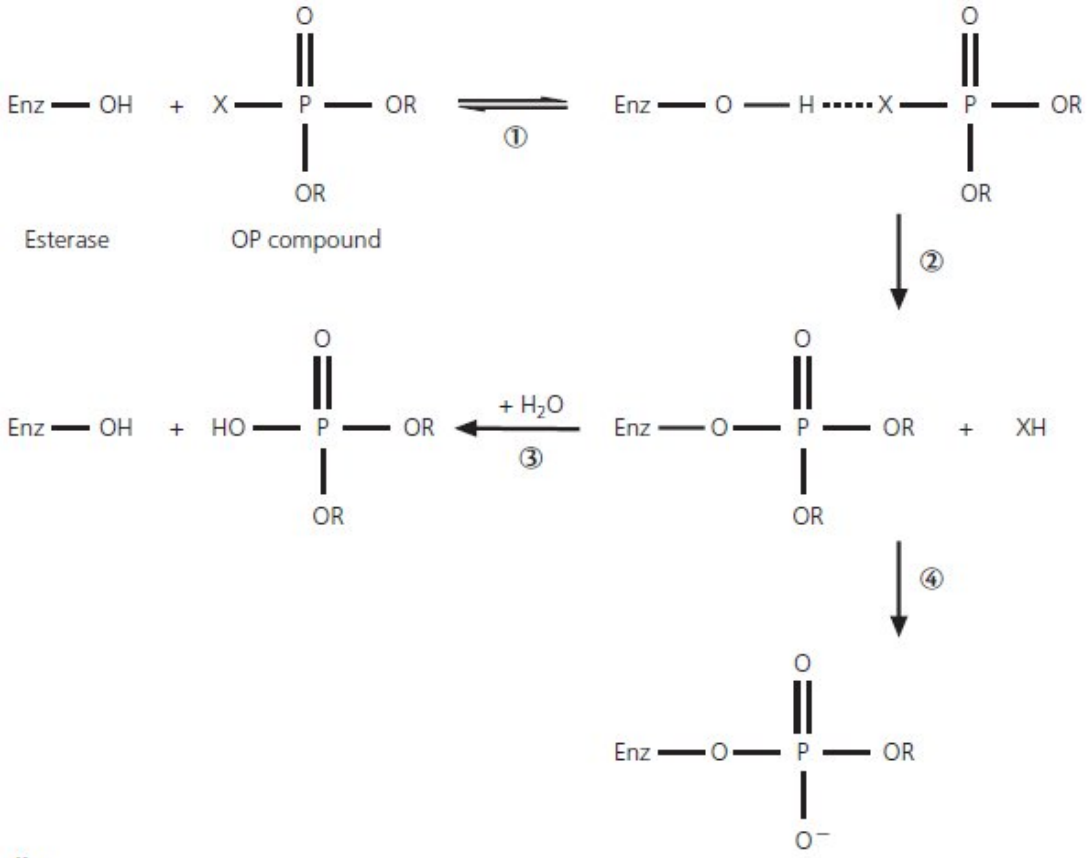


Figure 2.3: Inactivation ('ageing') of acetylcholinesterase by organophosphate compounds (COT, 1999)

2.2.5 General Health Effects

These comprise the acute cholinergic, intermediate syndrome, organophosphate-induced delayed poly-neuropathy and organophosphate-induced chronic neurotoxicity.

2.2.5.1 Acute Syndrome

Clinical signs of acute toxicity can be grouped under three broad categories: Muscarinic, nicotinic and central nervous signs (Keifer and Mahurin, 1997). These signs and symptoms of acute poisonings with OPs result from the inhibition of AChE in both the central and the peripheral nervous systems (Keifer and Mahurin, 1997).

The muscarinic signs associated with acute OP poisoning include salivation, lacrimation, increased frequency of urination (diuresis), bradycardia, abdominal pain and distress, and emesis (Blodgett, 2001). The nicotinic signs which include muscle tremors of the head, generalized muscle tetany, stiffness, weakness and paralysis, owing to inability of membranes to repolarise adequately before next action potential, tachycardia and mydriasis.

The CNS signs include anxiety, restlessness, hyperactivity, depression, clonic-tonic seizures, depressed respiration and coma (Blodgett, 2001).

2.2.5.2 Intermediate Syndrome

Prolonged dermal exposure from a single dose of insecticide or repetitive exposures to low doses of OPs may produce an intermediate syndrome (IMS) (Sultatos, 1994). The symptoms manifest 24 - 96 hours sequel to intoxication when individual is recovering from the initial cholinergic crises, and they include muscular weakness, affecting predominantly muscles of the proximal limbs and neck flexors (Karalliede *et al*., 2006; Qureshi *et al*., 2007). Unlike the delayed polyneuropathy, this syndrome carries a death risk due to respiratory depression. The clinical course may last from 5 to 18 days (Karalliede *et al*., 2006; Qureshi *et al*., 2007). This syndrome

has been associated with the production of tolerance in cholinergic receptors, resulting from prolonged bombardment with ACh (Sultatos, 1994). Prolonged exposure to ACh agonist is believed to cause down regulation of cholinergic receptors through an internalization of receptors into the cells. Muscarinic receptors are especially prone to down-regulation in tolerance situations (Nand *et al*., 2007). Other proposed mechanisms of IMS include muscle necrosis, prolonged acetylcholinesterase inhibition, inadequate oxime therapy, downregulation or desensitization of postsynaptic acetylcholine receptors, failure of postsynaptic acetylcholine release, and oxidative stress-related myopathy (Yang and Deng, 2007). The clinical manifestations of IMS typically occur within 24 to 96 hours and involve the muscles of respiration, proximal limb muscles, neck flexors, and muscles innervated by motor cranial nerves (Yang and Deng, 2007). Dermal exposure in cats tends to produce anorexia, diarrhoea, generalized weakness, muscle tremors, abnormal posturing, abnormal behaviour, cervical ventroflexion, depression and death. Severely affected animals may have clonic-tonic convulsion, with either mioisis or mydriasis (Fikes, 1992).

2.2.5.3 *Organophosphate-induced Delayed Polyneuropathy*

Organophophate-induced delayed polyneuropathy (OPIDPN) is a sensorimotor polyneuropathy, which predominantly affects the lower limbs, and also exhibiting ataxia. Symptoms and clinical signs develop over a period of 1 to 4 weeks after intoxication (Lotti, 1992; Marrs, 1993; Nand *et al*., 2007; Qureshi *et al*., 2007). OPIDPN is a neurodegenerative disorder characterized by a delayed onset of prolonged ataxia and upper motor neuron spasticity as a result of a single or repeated exposure to organophosphorus esters (Abou-Donia and Lapadula, 1990). Pathologies, such as degeneration of the distal ends of longer axons of some distal and spinal nerves are followed by myelin breakdown, Schwann cell proliferation and macrophage accumulation

(Krinke *et al*., 1997). This condition is known not to respond to anticholinergic drugs and oximes. The recovery is quite slow, often incomplete (Vasilescu and Florescu, 1980); while the CNS component never recovers (Vasilescu and Florescu, 1980).

The mechanism involved in OPIDPN was believed to be associated with the phosphorylation and ageing of an enzyme, neuropathy target esterase (NTE, formerly neurotoxic esterase) by processes similar to those described for AChE. It was thought that the generation of a negative charge at the phosphorylated site forms the basis for the ageing process and appears to be an essential step in development of OPIDN (Barrett and Oehme, 1985; Sevim *et al*., 2003; Nand *et al*., 2007). The steps from NTE inhibition to development of neuropathy remain vague but involve degeneration of long peripheral axons (Moretto and Lotti, 2006). Although, the role of NTE in the nervous system is not known for certain, its role in maintaining the integrity of peripheral motor axons is indicated by a M1012V mutation associated with a hereditary disease, characterized by progressive lower-extremity weakness and wasting of distal extremity muscles has been identified (Rainier *et al.,* 2008). According to Vose *et al*., (2008), neuropathy target esterase (NTE) plays critical roles in embryonic development and maintenance of peripheral axons. It is a secondary target of some organophosphorus toxicants, including analogues of insecticides and chemical warfare agents. However, a more convincing evidence against this hypothesis is the finding that NTE-knockout mice are sensitive to the development of OPIDN (Abou-Donia, 2003; O' Callahan, 2003), indicating that this enzyme may not be involved in the mechanism of OPIDPN (Abou-Donia, 2003). Studies have associated OPIDPN mechanism with an anomalous hyperphosphorylation of cytoskeletal elements; this neurodegenerative disorder is characterized by distally located swellings in large axons of the CNS and PNS, with subsequent axonal degeneration. Central to this protein kinase hypothesis is the observation that increased

aberrant protein kinase-mediated phosphorylation of cytoskeletal proteins could result in the destabilization of microtubules and neurofilaments, leading to their aggregation and deregulation in the axon (Abou-Donia, 1995).

In the adult hen, which is the test species of choice, this delay is 8-14 days, while in man, the delay may reach 4 weeks after acute exposure. The neuropathological lesion is a central-peripheral distal axonopathy caused by a chemical transection of the axon (known as Wallerian-type degeneration), followed by myelin degeneration of distal portions of the long and large-diameter tracts of the CNS and PNS (Cavanagh and Pantagia, 1995). Symptoms observed in this situation include cramps, weakness, tingling, and numbing of extremities, a high stepping gait, paralysis of lower limb, and in severe cases quadriplegia (Fikes, 1992).

2.2.5.4 *Organophosphate-Induced Chronic Neurotoxicity*

Organophosphate-Induced Chronic Neurotoxicity (OPICN) occurs due to exposure to large, acutely toxic- or small subclinical doses of organophosphates. The resultant clinical signs which persist for a long time ranging from weeks to years after exposure consist of neurological and neurobehavioural abnormalities (Jamal, 1997; Abou-Donia, 2003). Damage occurs in both the central and peripheral nervous systems, with the latter being more involved. Within the brain, neuropathological lesions are seen in various regions, including the cortex, hippocampal formation, and cerebellum (Jamal, 1997; Abou-Donia, 2003). The lesions are characterized by neuronal cell death resulting from early necrosis or delayed apoptosis. These neurological and neurobehavioral alterations are aggravated by concurrent exposure to stress or to other chemicals that cause neuronal cell death or oxidative stress (Jamal, 1997; Abou-Donia, 2003).

Professional pesticide applicators and farmers who had been exposed to OP pesticides manifested an elevated level of anxiety, impaired vigilance and reduced concentration (Levin *et*

al., 1976; Abou-Donia, 2003). Kaplan *et al*. (1993) reported long-term cognitive dysfunction and defects in concentration, word finding, and short-term memory in sub-clinically exposed individuals. These signs may be observed despite normal AChE activity (Srivastava *et al*., 2000).

The mechanisms of OPICN have been shown to occur by necrotic death of brain neuronal cells caused by exposure to large toxic doses of OP compounds, while small subclinical doses produce delayed apoptotic death (Abou-Donia, 2003); but it is also believed to involve increased AChE gene expression and oxidative stress (Abou-Donia, 2003).

De Groot *et al*., (2001) suggested a mechanism for OPICN-induced necrosis; in which accumulated acetylcholine, resulting from acute inhibition of AChE by OP compounds, causes the activation of glutamatergic neurons and the release of the excitatory L-glutamate amino acid neurotransmitter. This consequently increases depolarization and subsequent activation of the N-methyl- D-aspartate (NMDA) subtype of glutamate receptors—and the opening of NMDA ion channels— resulting in massive Ca^{2+} fluxes into the post-synaptic cell and causing neuronal degeneration. Thus, glutamate- induced neuronal degeneration during seizures may occur as a result of lowering of the threshold for glutamate excitation at NMDA receptor sites (Dawson *et al*., 1992). Activation of nitric oxide synthase, following stimulation of NMDA receptor sites, increases the level of nitric oxide, which functions as a signaling or cytotoxic molecule responsible for neuronal cell death (Dawson *et al*., 1992).

Neuronal degeneration caused by apoptosis which occur due to chronic exposure to small doses of OP compounds. It is believed to involve free radical generation (Tuzmen *et al*., 2008). OPs that cause mitochondrial damage/dysfunction, thereby depleting ATP and increased generation of ROS, resulting in oxidative stress (Tsujimoto, 1997; Murphy *et al*., 1999; Tuzmen, *et al*., 2006). ROS can cause fatal depletion of mitochondrial energy (ATP), induction of

proteolytic enzymes, and DNA fragmentation, leading to apoptotic death (Floyd, 1999; Gupta *et al*., 2001).

Calderon *et al*. (1998) believed that AChE plays a role in the pathogenesis of OPICN similar to that reported for Alzheimer's disease. Certain OPs have been shown to induce the AChE gene in the same regions of the brain that underwent neuronal degeneration (Damodaran *et al*. 2003). AChE has been shown to be neurotoxic *in vivo* and *in vitro*; it increases the rate of assembly of amyloid peptide in Alzheimer's fibrils, leading to cell death via apoptosis. Brain AChE has been shown to be toxic to neuronal (Neuro 2a) and glial-like (B12) cells (Calderon *et al*., 1998). These results suggest that OPs like sarin may provoke an endogenous cell suicide pathway cascade in susceptible neurons (for example, in the caspase-3 pathway), resulting in the release of AChE into adjacent brain tissues (Abou-Donia, 2003). The aggregation of AChE initiates more apoptotic neuronal death. Amplification of this cascade thus may result in the progressive neuronal loss that is the hallmark of sarin-induced chronic neurotoxicity (Abou-Donia, 2003). A symptom common to both OPICN and Alzheimer's disease is memory deficit, suggesting that the ageing process may be accelerated following exposure to OP compounds in OPICN (Abou-Donia, 2003).

2.2.6 Chlorpyrifos

2.2.6.1 Introduction

This is a broad spectrum OP insecticide. It belongs to the phosphorothioate (also called phosphorothionate) group, that contains the P=S sub structure. Its chemical name is *O, O*-diethyl-3,5,6 trichloro-2-pyridyl phosphorothioate based on the IUPAC nomenclature. Its molecular weight is 350.6 and the chemical formula is $C_9H_{11}Cl_3NO_3PS$ (CHID, 2008). Technical CPF is a white crystalline solid with a melting point of 41.5–42.5ºC. CPF is stable in neutral and

acidic aqueous solutions; but its stability decreases with increasing pH. CPF is practically insoluble in water, but is soluble in most organic solvents such as acetone, xylene and methylene chloride (CHID, 2008).

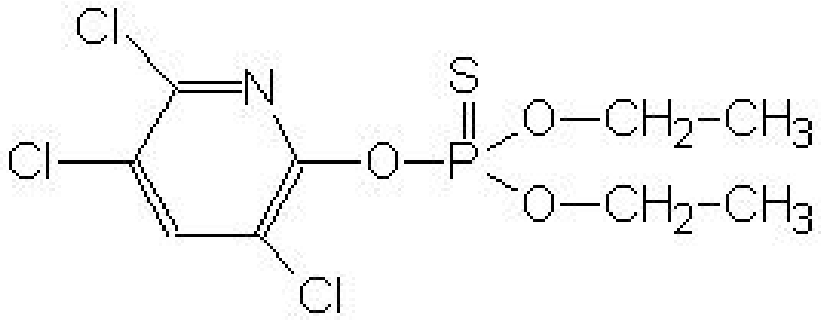


Figure 2.4: **Structure of Chlorpyrifos**

Some of the common product names of CPF include Termicot®, Britermicot®, Dursban®, Lorsban®, Pagent® F, among others (USDA, 2005). Others include Detmol U.A.®, Dowco 179®, Dursban 4E®, Dursban F®, Dursban HF®, Eradex® and Killmaster® (Kegley *et al*., 2008).

2.2.6.2 Insecticidal Uses

Chlorpyrifos was first introduced in 1965 and is one of the most commonly used OP pesticides. It is used as an acaricide and miticide to control foliage and soil-borne insect pests on a variety of food and feed crops (CHID, 2008).

Until 2000, CPF was commonly used as a residential pesticide for fire ants, cockroaches, and other household pests. The U.S. Environmental Protection Agency (EPA) cancelled most home, lawn and garden use products containing CPF in mid-2000 based on human health risks (CDPR, 2007). In addition, as of December, 2005, CPF products were no longer permitted for use in pre-construction termite control in the USA. However, CPF is still being widely used in agriculture. Data from the California Department of Pesticide Regulation's (CDPR) Pesticide Use Reports indicate a use of nearly 2 million pounds by weight in 2006 (CDPR, 2007). In forestry and

rangeland applications, CPF is used to control tent caterpillars, bagworms, leafrollers, leafcutters, borers, bark beetles, spider mites, ticks, chiggers, fire ants and other soil insects (USDA, 2005). It was registered for use in a wide variety of buildings, including residential, commercial, schools, daycare centers, restaurants, hospitals, hotels, and food manufacturing plants until restrictions were placed on its use (EPA, 2000c). CPF persists longer indoors due to diminished filtered sunlight, reduced moisture, air movement and surface area, as well as a lack of soil microorganisms (Krieger *et al*., 2001; Needham *et al*., 2005).

2.2.6.3 Toxicokinetics

Absorption- Chlorpyrifos is known to be absorbed through the respiratory tract in humans and animals but quantitative estimates were not available (ATSDR, 1997; FAO/WHO, 1999). It is readily absorbed from the gastrointestinal tract. Based on the percent of administered dose excreted in the urine, absorption of CPF in humans was about 70% in a single-dose oral study (Nolan *et al*., 1984) and in rats ranged from 84-90% in single-dose gavage studies (ATSDR, 1997, EPA, 2000b).

Absorption of CPF varies with species. In humans, about 70% was absorbed after oral exposure of volunteers (Nolan *et al.,* 1984). And for the metabolite, 3, 5, 6-trichloro-2-pyridinol (TCPY), the minimal dermal absorption was 1-3% (Mendrala and Brzak, 1998). Dermal absorption of CPF (in dipropylene glycol, methyl ether) in humans was about 1.3% of administered dose within 48 hours. Dermal absorption in animals was much higher, but results were compounded by dermal irritation and even blistering, which compromised the integrity of the skin (Nolan *et al*., 1984).

Distribution- The highest tissue concentrations were found in the liver and kidney, but CPF did not bioconcentrate in tissue (Cochran *et al*., 1992). In a time course study, when pregnant rats were dosed with CPF at 7 mg/kg-day on GD 14–18, fetal brain TCPY concentration was twice as high as the maternal brain concentration, five hours after the last dose of CPF (Hunter *et al*., 1999). It should be noted that CPF is rapidly absorbed and transported to the brain with oral dosing (Mendrala and Brzak, 1998; CHID, 2008).

Metabolism- The metabolic scheme for CPF is outlined in the figure below. CPF is bioactivated to Chlorpyrifos-oxon (CPO) in the liver through cytochrome P_{450} mediated desulfuration. CPO inhibits AChE at about 1000 times the rate of CPF; hence the rate of its detoxification is extremely important (Huff *et al*., 2001). CPO is the active metabolite of CPF, it is hydrolyzed by A- and B-esterases to diethylphosphate (DEP) and 3,5,6-trichloro-2-pyridinol (TCPY), which are major biological metabolites and environmental breakdown products of CPF (Figure 2.5) they can undergo phase II sulfuration and glucuronidation (Timchalk *et al*., 2007).

CPO mediates the toxic effects of CPF by binding irreversibly with AChE eliciting cholinergic hyperstimulation in the nervous system and in neuro-muscular junctions. A-esterase (e.g., CPF oxonase and paraoxonase) and carboxylesterase are known to play an important role in the detoxification of CPF. Paraoxonase (PON1) is a high density lipoprotein liver and plasma enzyme that hydrolyzes the active metabolites (i.e., oxons) of specific OPs, including CPF, diazinon, and parathion (Qiao *et al*., 2001). In humans, considerable variability has been seen in PON1 status across populations (Sirivarasai *et al*., 2007).

The oxon forms are much more potent inhibitors of cholinesterase than the parent compounds. Detoxification of CPF is not limited to carboxylesterase or A-esterase, bovine serum albumin has been shown to prohibit the effects of CPF on DNA synthesis *in vitro* (Huff *et al*., 2001). In a

study on human volunteers, the mean half-life ($t_{1/2}$) of CPF in the body was 26.9 hours (Nolan *et al.,* 1984).

Figure 2.5: Metabolic Scheme for Chlorpyrifos (Timchalk *et al*., 2007)

In summary, the main features of chlorpyrifos metabolism are: (ATSDR, 1997, Buratti *et al*., 2003; FAO/WHO, 1999; Tang *et al*., 2001)

(i) Activation of chlorpyrifos by cytochrome P450 oxidative desulfuration of the P=S moiety to P=O, resulting in the toxic intermediate, CPO;

(ii) Detoxification by cytochrome P450 dearylation of chlorpyrifos, resulting in TCPY and DEP;

(iii) Detoxification by A-esterases (including paraoxonases) that hydrolyse the phosphate ester bonds of CPF and CPO to form TCPY, and also diethyl thiophosphate (from chlorpyrifos), or DEP (from CPO).

(iv) Formation of glucuronide and sulphate conjugates of (TCPY).

Metabolic activation occurs predominantly in the liver. Detoxification occurs predominantly in the liver and plasma (ATSDR, 1997; FAO/WHO, 1999). Metabolism of CPF is rapid and extensive as the parent compound and oxon are not detected or are found only in trace concentrations in blood or urine. The metabolite, TCPY is the principal form found in the circulation. Chlorpyrifos metabolites are excreted primarily in the urine (ATSDR, 1997; FAO/WHO, 1999).

Excretion- Chlorpyrifos is excreted primarily through the kidneys in the urine. Approximately 70% of an oral dose was excreted in the urine of humans, while 90% was excreted in rats (Cochran *et al*., 1992). The main route of excretion in humans, rats and goats was through the urine. Less than 0.1% of the dose administered to goats was found in the milk (CHID, 2008).

2.2.6.4 Health Effects

The principal toxic effect of CPF in humans, experimental animals and insects is acetylcholinestrase inhibition (ATSDR, 2004). Acetylcholine is a neurotransmitter in the central and peripheral neurons. Inhibition of AChE, the enzyme that breaks down and terminates the action of acetylcholine by CPF, results in the accumulation of ACh at cholinergic receptors leading to continued stimulation (ATSDR, 2004). In humans and experimental animals, the accumulation of ACh results in cholinergic responses in the peripheral (muscarinic and nicotinic) and central nervous system. These cholinergic responses, seen in severe AChE inhibition include excessive glandular secretion (salivation, lacrimation, rhinitis etc.), miosis, bronchoconstriction, vasodilation, hypotension, diarrhoea, nausea, vomiting, urinary incontinence, and bradycardia associated with muscarinic receptor stimulation. Tachycardia, mydriasis, muscle fasciculations, cramping, twitching, muscle weakness, muscle paralysis, and hypertension are associated with nicotinic receptor stimulation (ATSDR, 2004).

Central nervous system toxicity includes respiratory depression, anxiety, insomnia, dizziness, loss of concentration, confusion, tremors, convulsions and coma. These effects usually appears within a few minutes to about 24 hours after exposure, depending on the extent and route of exposure (ATSDR, 2004). In non-fatal cases, the effects are usually transient with rapid and complete recovery following cessation of exposure.

Recovery from CPF poisoning results from increased availability of active AChE either from synthesis of new enzyme or the spontaneous hydrolysis of the enzyme-phosphate ester complex. In addition, treatment with atropine, a competitive antagonist of ACh at muscarinic and central nervous system receptors, with pralidoxime (2-PAM), a drug that regenerates

inhibited AChE by displacing the diethyl phosphoester bond that the CPO forms at the active site, also aid recovery from CPF poisoning (ATSDR, 1997).

Transient, delayed polyneuropathy has been reported in humans in case reports of acute – or intermediate-duration exposure to CPF; these reports did not adequately characterize exposure (ATSDR, 1997; FAO/WHO, 1999). CPF has been shown to induce delayed neurotoxicity in chickens (Richardson, 1993) and cat at a very high dose (Fikes, 1992).

Chronic toxicity due to CPF has been reported in humans, and may include weakness, anorexia and malaise (USDA, 2005), as well as neuronal degeneration and subsequent neurological, neurobehavioral and neuropsychological consequences in what has been referred to as OPICN (Abou-Donia, 2003).

2.2.6.5 Mechanism of Toxicity

The primary, but not necessarily the only, mechanism of action for the toxic effects of CPF is related to the ability of its metabolite- CPO to bind to and irreversibly inhibit AChE in target tissues, the nervous system is the primary target because AChE and/or butyrylcholinesterase (BuChE) catabolyze the neurotransmitter ACh, thereby terminating its synaptic function. The inhibition of AChE results in accumulation of the neurotransmitter acetylcholine in neural junctions causing excessive firing of the nerves resulting in cholinergic toxicity (Eaton *et al*., 2008; Croom *et al*., 2010). Specifically, AChE inhibition occurs by the phosphorylation of serine hydroxyl group in the substrate-binding domain of the enzyme, resulting in accumulation of acetylcholine at cholinergic synapses in the central and peripheral nervous systems cholinergic syndrome) and associated neurotoxicity (Oruc and Usta, 2007; Colovic *et al*., 2011).

In humans, the enzyme activity of plasma BuChE is substantially more sensitive to inhibition by CPO than erythrocyte AChE, which is widely recognized as more reflective of AChE in the brain, spinal cord, and the peripheral somatic and autonomic nervous system.

Besides the putative mechanism of action of CPF, there are alternative mechanisms proposed mainly from *in vitro* studies, these include, cytotoxicity, effects on macromolecule synthesis (DNA, RNA, proteins), interactions with neurotransmitter receptors, interactions with signal transduction pathways, effects on neuronal differentiation, interactions with various enzymes, other neurochemical effects (e.g., neurotransmitter release or uptake), and other effects (e.g. oxidative stress, effects on microtubules) (Eaton *et al*., 2008). Furthermore, there are two targets which have been identified for which CPF may cause effects at concentrations below those necessary to inhibit AChE activity. These include, serine hydrolase enzyme KIAA1363 and the CREB.

There is evidence that CPF directly targets events that are specific to the developing brain and that are not necessarily related to the inhibition of AChE (Qiao *et al.,* 2004; Qiao *et al*., 2005). Since immature animals actually recover more rapidly from AChE inhibition than do adults, measurements of AChE activity alone may not be sufficient for the assessment of adverse effects. Chlorpyrifos-induced neurochemical and neurobehavioral changes may in fact be unrelated to AChE inhibition and may be of equal concern for human health risk assessment. These include; effects on the developing brain during cell division, interference with RNA synthesis during differentiation, interruption of cell signaling (CHID, 2008), interference with important nuclear transcription factors involved in cell differentiation, impairment of cholinergic synaptic function during development, effects on the catecholamine system in the developing brain, oxidative stress in the developing brain, and interference with gliogenesis and

axonogenesis (CHID, 2008). These findings have made scientists believe that the vulnerability period for CPF may likely extend into childhood or adolescence.

CPF and CPO inhibit acetyl cholinesterase by reacting with the active site to form a stable dialkyl phosphorylated enzyme that cannot hydrolyze acetylcholine. Chlorpyrifos oxon, the active metabolic intermediate of chlorpyrifos, is much more potent than chlorpyrifos in inhibiting acetylcholinesterase (ATSDR, 1997; FAO/WHO, 1999).

Chlorpyrifos is known to inhibit erythrocyte and neural AChEs as well as plasma cholinesterase also known as pseudocholinesterase (an enzyme produced in the liver, circulates in the blood and capable of hydrolyzing acetylcholine and butyrylcholine) whose physiological function is hypothesized to be hydrolysis of esters ingested from plants (Lefkowitz *et al.,* 1990; ATSDR, 1997).

Infact rather than operating via a single defined mechanism, CPF actually disrupt brain development through several families of mechanisms, including direct interactions with ACh receptors, interference with intracellular signaling cascades that transduce receptor signals on the cell surface into intracellular events, and generation of free radicals, culminating in oxidative stress (Gupta 2004; Slotkin *et al*., 2007).

2.4 PYRETHROIDS

2.4.1 Introduction

Pyrethroids are a class of neurotoxic pesticides widely used for pest control in a wide array of indoor and outdoor applications, including medicinal, veterinary and agricultural scenario (ATSDR, 2003) and are the synthetic derivative of natural pyrethrins with greater potency and environmental stability (Shashikumar and Rajini, 2010). Synthetic pyrethroid insecticides have

been used in agriculture and home formulation for more than 30 years and account for approximately one-fourth of worldwide insecticide market (Farag *et al*., 2007).

2.4.2 Brief Historical Perspective and Uses

The history of these compounds is very old and dates back to the first century A.D. The natural pyrethrin was first used by the Chinese in the first century A.D. (Elliot, 1980; Kakko, 2004). The first written accounts of these compounds date from the 17th century literature. Pyrethroids, synthetic analogues of pyrethrin have been produced since 1940 (Elliot, 1980; Kakko, 2004). Pyrethrins are obtained from the flowers of *Chrysanthemum cinerariaefolium* (Parvez and Raisuddin, 2006; Pinpao *et al*., 2007). The first commercial synthetic pyrethroid, allethrin, was produced in 1949, followed in the 1960s by others including dimethrins, tetramethrin, and resmethrin (UKPID, 1998). They were initially not stable enough for extensive use in agriculture, thus, the light-unstable centers in the molecule had to be replaced by light-stable centers which then permitted their large scale agricultural use (Kakko, 2004). The presence of the light-unstable centres was responsible for the non-persistance of pyrethrins and lead pesticide chemists to develop compounds of similar structure having insecticidal activity but being more persistent. Therefore, pyrethroids have greater insecticidal activity and are more photostable than pyrethrins (Hodgson, 2004). Due to the increasing demand for pyrethroid compounds, newer analogues were synthesized, which had better stability in light and air, better persistence, more selectivity in target species, low mammalian toxicity, and better biodegradability (Kakko, 2004). They have a widespread use in various aspects of agriculture, veterinary practice and gardening (Kakko, 2004). They are used in the control of a variety of pests such as mosquitoes and cockroaches (Narahashi, 2000; Narendra *et al*., 2008).

In developing countries pyrethroids are extensively and intensively used for both agricultural and domestic purposes (Shafer *et al*., 2005; Narendra *et al*., 2008). Besides agriculture, they have also been widely considered ideal in public health and veterinary applications on both farm animals and pets for the prevention and control of ectoparasites (Hossain *et al*, 2001; Yilmaz *et al*., 2008). They are considered the safest among the highly potent insecticides (Bateman, 2000). Higher rates of enzymatic biotransformation, detoxification and excretion account for the lower toxicity in mammals (Kakko, 2004). Their potency in insects is five-fold greater than in mammals. This is due to the lower body temperature, slower enzymatic detoxification in insects, and, smaller body size which gives less time for the compound to be detoxified before reaching the target site (Kakko, 2004). Pyrethroid insecticides have been in use for more than four decades in view of their wide applicability; they consequently account for 25% of the global insecticidal market (Kakko *et al*., 2003; Shafer *et al*., 2005; Narendra *et al*., 2008). There has also been a dramatic rise in their use in many developing countries (Ramesh and Vijayalakshriu, 2001).

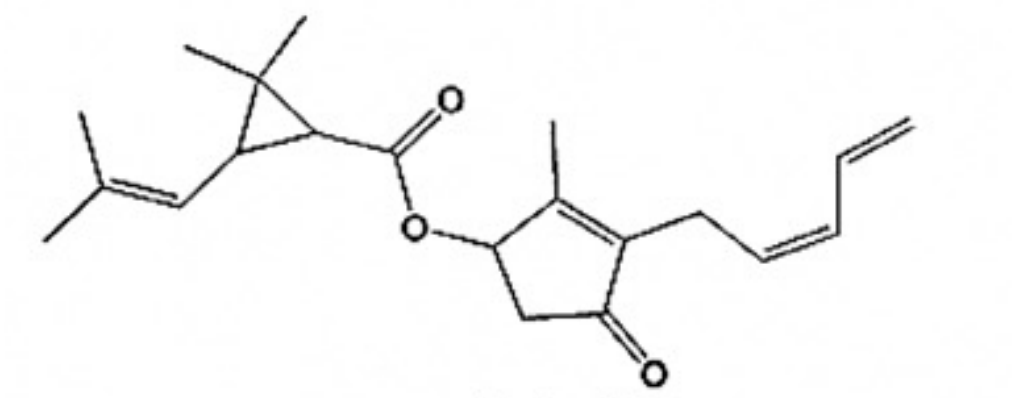


Figure 2.5: General chemical structure of a pyrethrin (non-cyano) (Eaton *et al*., 2008).

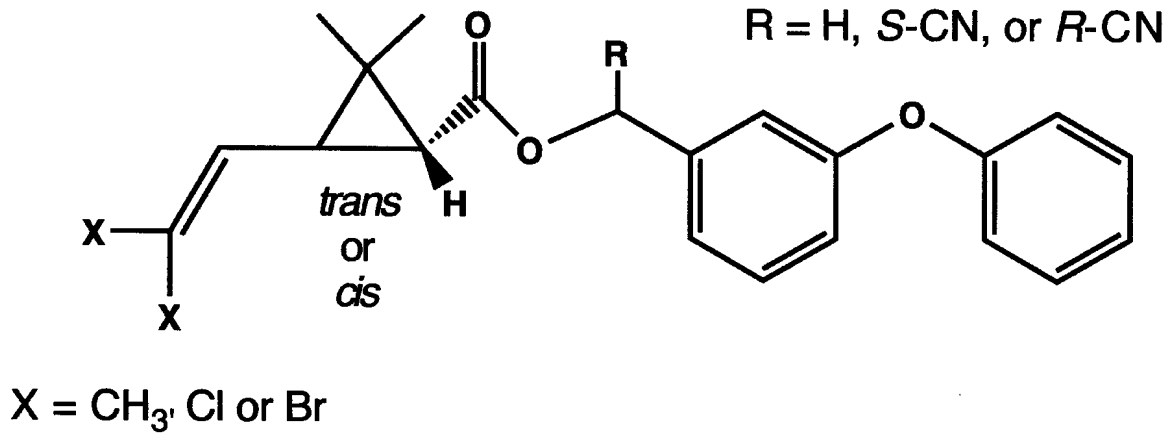


Figure 2.6: General chemical structure of a pyrethroid (Eaton *et al*., 2008).

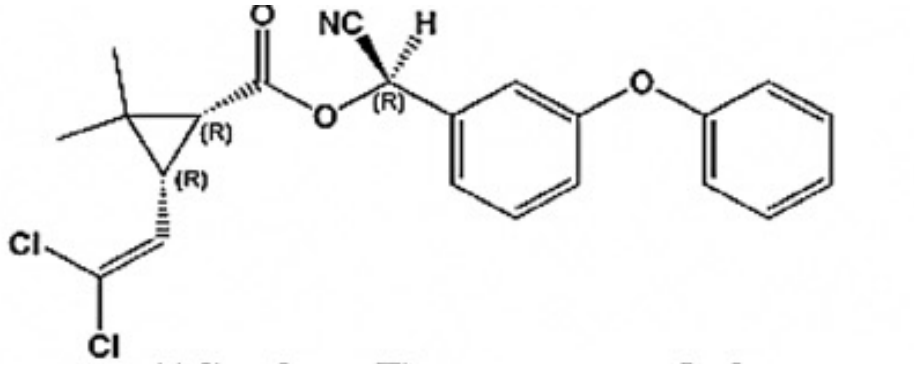


Figure 2.7: Chemical structure of α-cypermethrin (Eaton *et al*., 2008).

2.4.3 Classification of Pyrethroids

Pyrethroids may be classified in two broad groups on the basis of the presence of a cyano-group in chemical structure and the production of either a tremor (T) or choreo athetosis (CS) syndromes (Soderlund *et al*., 2002; Nieradko-Iwanicka, 2008). Type I pyrethroids (for example, allethrin, pyrethrin, permethrin etc.) lack a cyano moiety. Conversely, type II pyrethroids (for example, deltamethrin, fenvalerate, cypermethrin etc.) possess a cyano group in the α-position (Yilmaz *et al*., 2008). The two groups also have peculiar clinical signs of poisoning in mammals. Type I pyrethroids cause hyperexcitation, ataxia, convulsion, paralysis (Vijverberg and Van de Bercken, 1990; Yilmaz *et al*., 2008), and signs of repetitive nerve firing. However, type II pyrethroids' poisoning is characterized by hypersensitivity, profuse salivation, choreoathetosis, tremor and paralysis, but without signs of repetitive nerve firing in sensory nerves (Yilmaz *et al*., 2008). Although both groups show peculiarities, they also exhibit similarities in that their primary target is the sodium channel. All active pyrethroids interact with sodium current evoked by membrane depolarization (Yilmaz *et al*., 2008), thus, a prolonged tail persists after the membrane repolarization (Latuszynska *et al*., 2001). Type II pyrethroids induce a longer lasting delay in sodium channel closure than type I pyrethroids (Bloomquist *et al*., 1996).

2.4.4 Cypermethrin

Cypermethrin is a type II synthetic pyrethroid insecticide. It was first manufactured in 1974 but was not marketed until 1977 (Luty *et al*., 2000). The primary manufacturers in the U.S are Zeneca Incorporated F.M.C. Corporation., and the American Cynamide Corporation (Cox 1996). Common trade names include: Demon®, Cymbush®, Ammo®, Cynoff® etc. (Cox 1996). It is effective in the control of many pest species in agriculture, animal breeding for household use and in public health programmes (Nabioullin and Sone, 1994; Luty *et al*., 2000; Manna *et al.*, 2004; Velisek *et al.,* 2006). Over 90% of cypermethrin manufactured worldwide is used to control cotton pests; nevertheless it is used to control domestic pests of crops such as lettuce/pecan and insects such as cockroaches and termites in buildings (Cox, 1996).

2.4.4.1 Chemical Structure of Cypermethrin

Cypermethrin [(RS)-α-cyano-3-phenoxybenzyl (1RS)-cis-trans-3-(2,2-dichlorovinyl) -2,2-dimethyl cyclopropane carboxylate] is a photostablesynthetic pyrethroid with great insecticidal potency (Soderlund *et al*., 2002; Nieradko-Iwanicka and Borzecki, 2008). The technical grade cypermethrin is the racemic mixture of 8 isomers (four cis- and four trans- isomers). Two stereoisomers are termed α-isomers of cypermethrin, which is believed to be the most active isomer, and is known as α-cypermethrin (WHO, 1992).

2.4.4.2 Physico-chemical Properties

Cypermethrin has a molecular weight of 416.3, at room temperature it exists as solid crystals while its technical grade occurs as a powder (Pesticide manual, 1997: UKPID, 98).

It is normally colourless while its technical grade is white to pale (Pesticide manual, 1997: UKPID, 98). Its technical grade has a weak aromatic odour. It has a low solubility in water approximately 1×10^{-5} g/L at 25ºC and <1.11g/L at 20ºC in ethylene glycol. Its boiling point is

200 ºC at 9.3 Pa, with a density of 1.28 at 22ºC, vapour pressure of 2.3×10^{-5} Pa at 20ºC (Pesticide manual, 1997: UKPID, 98).

2.4.4.3 Toxicokinetics

Absorption- The mean dermal absorption of CYP was estimated to be 1.2 % after application of spray formulation containing 31 mg CYP to volunteers (Woollen *et al.,* 1992). When orally administered between 19 and 57 % of racemic CYP was absorbed in human studies (Woollen *et al*., 1992). According to Luty *et al.* (2000), the absorption of CYP from the digestive tract and its excretion takes a quick course (Sayim *et al*., 2005).

Metabolism- There is some stereo specificity in metabolism, with trans - isomers being hydrolyzed more rapidly than the cis-isomers for which oxidation is the more important metabolic pathway. CYP metabolism actually involves both oxidation and hydrolytic pathways (Scollon *et al*., 2008). Although the alpha-cyano group reduces the susceptibility of the molecule to hydrolytic and oxidative metabolism, the cyano group is converted to the corresponding aldehyde (with release of the cyanide ion), followed by oxidation to the carboxylic acid (UKPID, 1998).

Elimination- Dermal exposure of three mixers/loaders to CYP 0.25-5.27mg/8 hours resulted in urinary excretion of 12-23 µg cypermethrin equivalents on the day of exposure (Chester *et al*., 1987). Following oral administration of CYP to volunteers, peak excretion rates were observed between 8 and 24 hours, and 24 % of administered dose was excreted as metabolites (Woollen *et al*., 1992). Alpha-cypermethrin was shown to have 43 % of it excreted as free cyclopropane carboxylic acid in 24 hours (Eadsforth *et al.,* 1988).

2.4.5 Neurotoxic Mechanisms of Cypermethrin

Cypermethrin (CYP), like other type II pyrethroids, kill insects by disruption of the gating kinetics of sodium channels of the nervous systems (Cox, 1996). CYP thus blocks the sodium channels of nerve filaments, thereby lengthening their depolarization phase (Macdonald *et al*., 1988). Neural membrane as a target of pyrethroids membrane fluidity has been shown to be dependent on the cholesterol content and on orientation of phospholipid molecules in the membrane (Macdonald *et al*., 1988; Kakko, 2004). The lipophilicity of pyrethroids allows for rapid access into tissues, including the nervous system, and incorporation into the cell membranes of nervous tissue (Aldridge, 1990; Kakko 2004).

Cell membranes possess integral proteins, some of which function as ion channels. Ion channels may be continuously open, or have gates that are opened and closed due to alterations in the membrane potential, an example of such channel is the voltage gated sodium (Na^+) channel which is the target of pyrethroids (Meachem *et al*., 2008) when carrier proteins transport substances, e.g. ions, against their chemical and electrical gradients, it involves the utilization of energy which is provided by the hydrolysis of ATP catalysed by ATPases. One carrier important to CYP toxicity is the Na^+, K^+ ATPase, which consists of α-and β-subunits, both of which are membrane spanning (Kakko, 2004).

Due to its lipophilicity CYP and other pyrethroids can incorporate into the lipid bilayer in the neural tissue. This can disrupt the phospholipids orientation and cause changes in the membrane fluidity (Kakko, 2004). It has been reported that CYP induced a significant decrease in erythrocyte membrane fluidity (Hu *et al*. 2008).

Disturbances in the Na^+, K^+, ATPase activity and changes in the membrane fluidity have been found to be the mechanisms which mediate the neurotoxicity of many lipophilic organic solvents such as CYP (Tahti *et al.,* 1999).

2.4.5.1 Effects of Cypermethrin on Ion-channels

The main target of CYP and other pyrethroids in both invertebrates and vertebrates has been shown to be sodium channels (Zlotkin, 1999; Atessahin *et al*., 2005; Meacham *et al*., 2008) and they comprise alpha beta subunits each of which have multiple isoforms that are expressed in a developmentally regulated manner (Meacham *et al*., 2008). Pyrethroids such as deltamethrin were observed to bind to and modify sodium channel function inducing tail currents (Meacham *et al*., 2008). Pyrethroids generally bind to sodium channels inducing a prolongation of their opening. Prolonged sodium current causes a depolarizing after-potential (Narahashi, 2000). When the depolarizing after-potential reaches the excitation threshold, repetitive activity can be initiated in various regions of the nervous system. An alteration of only 1 % of the total number of Na^+ channels is sufficient to induce repetitive discharges and hence the symptoms of toxicosis in animals (Narahashi, 1996; Narahashi, 2000). Type II pyrethroids such as cypermethrin are known to slow the deactivation (the tail currents upon repolarization) of sodium channels leading to a persistent depolarization of the nerve membrane which results in conduction block (Narahashi *et al*., 1998). This depolarizing action can have a dramatic effect on the sensory nervous system, which results in an increase in the number of repeated discharges (Vijverberg and Van den Bercken, 1990; Kakko, 2004). Insect sodium channels are 100-fold more sensitive to CYP than to tetrodoxin-resistant sodium channels in developing mammalian brains and dorsal root ganglia of adults (Nieradko-Iwanicka and Borzecki, 2008).

2.4.5.2 Cypermethrin and Oxidative Stress

Studies have shown that pyrethroids induce cell stress, which alters antioxidant enzymes. This alteration is indicative of the involvement of free radical intermediates in pyrethroids toxicity (Karen *et al*., 2001). The cleavage of cypermethrin and some type II pyrethroids releases cyanohydrins, which are unstable in physiological conditions decomposing into cyanides and aldehydes (Kale *et al*., 1999; Grajeda-Cota *et al*., 2004). These may generate free radicals and lead to oxidative stress (Kale *et al*., 1999; Grajeda-Cota *et al*., 2004). Cypermethrin has been shown to induce oxidative stress and alterations in antioxidant enzymes in erythrocytes (Giray *et al*., 2001). The pathogenesis of α-cypermethrin free radical induction may relate to its ability to undergo hepatic metabolism via hydrolytic ester cleavage and oxidative pathways by the cytochrome P450 microsomal enzyme system (Manna *et al*., 2004). Studies have shown that lipid peroxidation increases in plasma and some tissues in cypermethrin-induced toxicities (Atessahin *et al*., 2005).

2.4.5.3 Monoamine Oxidase Inhibition

Ullah *et al*. (2006) reported that CYP inhibits monoamine oxidase, an enzyme that is responsible for metabolizing neurotransmitters. MAO is an oxidoreductase, a particle bound flavoenzyme located in the outer mitochondrial membrane of the cell. The enzyme is distributed widely in the nervous and renal tissue, the liver and the gastrointestinal tractus (Sagin *et al*., 2004). It is responsible for the metabolism of dopamine, thus, if MAO is inhibited then dopamine metabolism is disrupted and this leads to a dysfunctioning of dopaminergic neurotransmission (Jones and Miller, 2008. This has implicated pesticides like CYP in the pathogenesis of neurodegenerative diseases such as Parkinson's (Jones and Miller, 2008).

2.4.5.4 Calcium-related mechanism

According to Clark and Symington (2008), CYP just like deltamethrin causes calcium ion influx and Ca^{2+} dependent neurotransmitter release from rat brain synaptosomes, an action that is not shared by non-alpha cyano pyrethroids.

2.4.5.5 Gamma-aminobutyric-acid Inhibition

CYP inhibits the γ-aminobutyric acid (GABA) receptor, causing excitability and convulsion (Cox, 1996; Velisek *et al*., 2006). According to Manna *et al*. (2005), α-CYP decreases GABA levels significantly both in the cerebellum and other parts of the brain. This decrease has been to related to reduced synthesis or increased catabolism of GABA resulting in the inactivation of the chloride channel, leading to excitation and convulsion (Manna *et al*. (2005). Furthermore, it has been observed that α-CYP potentiates pentobarbitone-induced sleeping time and PTZ-induced convulsion as it decreases GABA concentration in the brain (Manna *et al*. (2005).

2.5 OXIDATIVE SRESS

Oxidative stress is defined as a disruption of the prooxidant-antioxidant balance in favour of the former, leading to potential damage. It is a result of one of three factors: an increase in reactive oxygen species (ROS), an impairment of antioxidant defense system or an insufficient capacity to repair oxidative damage (Aly *et al*., 2010).

Reactive oxygen species (ROS) present a paradox in their biological function: on one hand, they help in disease prevention by assisting the immune system, mediating cell signaling and playing an essential role in apoptosis but on the other hand, they reserve the capacity to damage essential or vital macromolecules within the cells and may play a role in carcinogenesis and cardiovascular diseases (Finkel and Holbrook, 2000; Holbrook and Ikeyama, 2002; Seifried *et al*,

2007). ROS generation is a natural and continuous process within cells as a result of mitochondrial electron transfer processes or as by-products of the enzymes xanthine oxidase, lipoxygenases and cycloxygenases (Szocs, 2004; Masella *et al*., 2005). Also, they are produced as a consequence of the intracellular metabolism of xenobiotics: toxins, pesticides or drugs by cytochrome P450, monoxygenases, or due to exposure to environmental factors such as excessive iron salts or UV irradiation (Ichihashi *et al*., 2003). Other sources of ROS include macrophages and neutrophils which contain enzymes such as NADPH oxidase complex, capable of generating superoxide radicals and hydrogen peroxide (Rosen *et al*., 1995; Masella *et al*., 2005). Despite all of these productions, oxygen homeostasis- the balance between constitutive oxidants and antioxidants- is maintained through a natural series of reduction-oxidation (redox) reactions involving the transfer of electrons (oxidized) and those that gain electrons (reduced) (Sohal and Weindruch, 1996). When oxygen homeostasis is not maintained, the cellular environment becomes oxidatively stressed. Approximately 1-3% of oxygen consumed by the body is converted to ROS (Sohal and Weindruch, 1996; Seifried, 2007). Potentially damaging oxidative stress (OS) can be generated by excess ROS, which are kept in check by endogenous or exogenous cellular antioxidant mechanisms (Castro and Freeman, 2001). Transient fluctuations of ROS levels affect the activity of signal transduction pathways leading to cell proliferation, or apoptosis or necrosis, depending on the dosage and duration of such fluctuation. ROS can be mitogenic whereas medium doses will cause temporary or permanent growth arrest (replicative senescence), and high doses usually result in cell death either by apoptosis or necrosis (Finkel and Holbrook, 2000).

ROS may have adverse influence on signal transduction pathways and regulations of proliferation mediated through mitogen-activated protein kinases (MAPKs). MAPKs comprise

three subfamilies: the extracellular signal regulated kinases (ERKS), C-jun N-terminal kinases (JNKs) and the P38 kinases (Droge, 2002). It is however noteworthy that ROS has an interesting influence on two MAPK families, the JNK and P38 families, collectively known as stress activated protein kinases (SAPKs) for their role in cellular response to different stresses, including cytokines, radiations, osmotic shock, mechanical injury, heat shock and oxidative damage (Droge, 2002; Martindale and Holbrook, 2002).
ROS during oxidative stress can activate these kinases, as JNK and P38 kinases are activated by an array of stresses. For example, the redox regulated protein: thioredoxin (TRX) binds to and inhibits the activity of the apoptosis signal-regulating kinase (ASK1) under normal redox conditions. Oxidative stress causes dissociation of the Trx-ASK1 complex leading to activation of JNK and P38 by ASK1 (Droge, 2002; Martindale and Holbrook, 2002). In the same vein, binding of glutathione-s-transferase to JNK under non-stressed conditions can be disrupted by oxidative stress, resulting in activated JNK (Finkel and Holbrook, 2000; Holbrook and Ikeyama, 2002; Seifried *et al*, 2007), which leads to activation of transcription factor AP-1.Consequently, transactivation of AP-1 regulated gene ensues (Johnson and Lapadat, 2002). Other effects of oxidants (ROS) occur when they react with three general classes of biomolecules, that is: lipid, protein and DNA (Padayatty *et al*., 2003).

Membrane lipids and lipids in circulating lipoproteins such as low-density lipoprotein (LDL) can interact with ROS resulting in lipid peroxidation. Once lipid peroxides form, they can react with oxygen to form highly reactive peroxyl radicals. Continued formation of lipid hydroperoxides can result, a process termed radical propagation (Morrow, 2000; Padayatty *et al*., 2003). Proteins undergo oxidation via several mechanisms. A peptide chain can be cleaved by oxidants, or specific amino acids can be oxidized (Stadtman and Berlett, 1998). Cysteine and methionine

are the two most susceptible to oxidative attacks. As with lipids, radical propagation may occur in proteins with formation of additional radical species (RS) (Stadtman and Berlett, 1998; Padayatty *et al.*, 2003). Sugars and their oxidized products can also react with lysine moieties to form advanced glycation end-products, although other substrates contribute to these products, such as amino groups on phospholipids (Baynes, 2001; Padayatty *et al.*, 2003).

Deoxyribonucleic acid (DNA) can be directly or indirectly affected by oxidative processes (Haliwell, 2000; Haliwell, 2001). Indirect mechanisms leading to DNA damage include protein oxidation, which could alter repair enzymes and DNA polymerases. When RS interact with lipids, the resulting lipid peroxidation products might then subsequently react with DNA, inducing mutations (Lee *et al*., 2001). The most important mechanism of DNA damage, however, is believed to involve direct attack of oxidants on individual nucleotides in DNA (Haliwell, 2000). Guanine is the DNA base most prone to oxidative attack. When such attack occurs, there's formation of the nucleotide oxidation product 8-hydroxyguanine (8OHG) and its nucleotide derivative; 8-hydroxy-2-deoxyguanosine (8OHdG) (Haliwell, 2000). Reactive nitrogen species (RNS) can also cause DNA damage by reacting with DNA leading to strand breaks and point mutations (Lee *et al*., 2001; Padayatty *et al*., 2003).

2.6 ANTIOXIDANTS

Antioxidants have been studied by chemists since the late 19th and early 20th century. They are loosely defined as a group of compounds characterized by their ability to be oxidized in place of other compounds present (Seifried *et al*., 2007). Their use ranged from food storage to vulcanization of rubber, but it was later that biologists discovered the essentiality of antioxidants in health which was occasioned by the 1960s publications of vitamins and flavonoids, followed

by later research in the 1970s on ascorbic acid (vitamin C), cancer, and common cold (Cameron and Pauling, 1976; Seifried *et al*., 2007). With many well-known scientists actively researching and or discussing antioxidants as being protective (Ames, 1983; Willett and MacMahon, 1984; Seifried *et al*., 2007), elucidations for the effects of antioxidants on cancer susceptibility and overall health expanded rapidly in subsequent decade with research into mechanisms, molecular targets, and molecular interactions (AICR/WCRF, 1997; Seifried *et al.,* 2007). This in turn has increased the rate of antioxidants intake particularly in the U.S as discovered by the National Health and Nutrition Examination survey (Radimer *et al.*, 2004). Vitamin C is one of the most common antioxidant supplement (Radimer *et al.*, 2004).

2.7 VITAMIN C

2.7.1 Sources of Vitamin C

Fruits and vegetables are a rich natural source of ascorbic acid (Martin *et al*., 2002, Sanchez-Moreno *et al.,* 2004; Sanchez-Moreno *et al*., 2006). Fruits such as cantaloupe, grapefruit, kiwi, mango, orange, papaya, strawberries, tangerine, and watermelon are rich sources of ascorbic acid. Rich vegetable sources of vitamin C include asparagus, broccoli, cabbage, cauliflower, kale, mustard greens, plantains, tomato, sweet potatoes etc. (Padayatty *et al*., 2003; Sanchez-Moreno *et al*., 2006).

2.7.2 Biochemistry and Redox Metabolism of Vitamin C

Most mammalian species have the capacity to synthesize ascorbic acid (a six-carbon lactone) from glucose in their liver. However, humans, non-human primates, guinea pigs and fruit bats lack the last enzyme (gulonolactone oxidase) involved in the synthesis of vitamin C from its precursor 2-keto-1-gulonolactone, and thus, vitamin C is essential in their diet (Halliwell, 2001;

Arrigoni and De Tullio, 2002). Vitamin C has an inherent capacity to donate electrons and therefore is naturally a reducing agent. All known physiological and biochemical actions of this enzyme are related to its action as an electron donor. It donates two electrons from a double bond between the second and third carbons of the 6-carbon molecule (Duarte and Lunec, 2005). Vitamin C is called an antioxidant because it prevents the oxidation of other compounds *in vivo* by donating its electrons, even though vitamin C itself becomes oxidized in the process (Duarte and Lunec, 2005).

In addition to its antiscorbutic action, vitamin C is a potent reducing agent and scavenger of free radicals in biological systems (May, 1999; Duarte and Lunec, 2005). At physiological pH, the mono-anion form (ascorbate) is the predominant. Ascorbate undergoes two consecutive, yet reversible, one-electron oxidations to generate dehydroascorbate (DHA) and an intermediate, the ascorbate free radical (AFR) (Equation I). AFR is, however, a relatively unreactive free radical, with a reduction potential considerably low compared to the α-tocopherol radical, the glutathione radical and virtually all reactive oxygen and nitrogen species that are thought to be involved in human disease (e.g. superoxide anion, hydroxyl radical, hydroperoxyl radicals, singlet oxygen, nitrogen dioxide, nitroxide radicals and hypochlorous acid) (May *et al.*, 2001). As compared to these free radicals (species containing an unpaired electron), AFR is relatively stable with a half life of 10^{-5} seconds and is fairly unreactive (Arrigoni and De Tullio, 2002). This is why ascorbate may be a preferred antioxidant. A reactive and harmful free radical can interact with ascorbate leading to the formation of an AFR while reducing the free radical. This process is known as free radical scavenging or quenching (Arrigoni and De Tullio, 2002). This property makes ascorbate an efficient electron donor in many biological redox reactions, capable of replacing potentially damaging radicals by the poorly reactive AR.

According to equation 1 where $AscH^-$ represents ascorbate, $Asc^{\bullet -}$ represents the AFR and $X^\bullet$ represents the oxidizing species:

$AscH^- + X^\bullet \rightarrow Asc^{\bullet -} + XH$……Equation I

Furthermore, vitamin C (ascorbate) has the ability to recycle other important antioxidant molecules such as α-tocopherol and glutathione from their respective radical species. Ascorbate can also be recycled by chemical and enzymatic mechanisms. AFR is converted back to ascorbate by an NADH-dependent reductase or by dismutation of two molecules of the radical into one molecule of vitamin C and one molecule of DHA (May *et al*., 2001). DHA, due to instability is in turn reduced back to ascorbate; otherwise, it is irreversibly hydrolysed to 2, 3-diketogulonic acid (May *et al*., 2001). This compound is formed by irreversible rupture of the lactone ring structure that is a part of ascorbic acid, ascorbyl radical (AFR), and dehydroascorbic acid 2, 3-diketogulonic acid is further metabolized into xylose, xylonite, lyxonate and oxalate (Padayatty *et al*., 2003). DHA can be reduced back to ascorbate either directly by glutathione or enzymatically by a glutathione-dependent DHA reductase, glutaredoxin, or the NADPH-dependent thioredoxin reductase (Wilson, 2005).

2.7.3 Physiological Role of Ascorbic Acid (ASC)

Ascorbic acid also known as vitamin C is a water-soluble micronutrient required for numerous biological functions. Vitamin C is a co-factor for several enzymes participating in the post-translational hydroxylation of collagen, in the biosynthesis of carnitine, in the conversion of the neurotransmitter dopamine to norepinephrine, in peptide amidation and in tyrosine metabolism (Haliwell, 2001; Duarte and Lunec, 2005). Furthermore, it's being shown to be an important regulator of iron uptake. It reduces ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions, thereby promoting dietary non-haem iron absorption from gastrointestinal tract, and stabilizes iron-binding proteins

(Haliwell, 2001). Although many animals synthesize ASC from glucose, humans, other primates, guinea pigs and fruit bats lack the last enzyme involved in the synthesis of ASC, therefore, require it in diet (Haliwell, 2001; Duarte and Lunec, 2005). It has also been shown that a healthy vasculature is maintained by vitamin C (ASC) function of prostacyclin production and maintenance of nitric oxide levels (Ness *et al*., 1996; Cullum, 2003; Sanchez-Moreno *et al*., 2006).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 CHEMICALS ACQUISITION AND PREPARATION

Commercial grade chlorpyrifos marketed as Termikill® (Modern Insecticides Limited, Punjab, India) was obtained from a reputable agrochemical company in Kano, Nigeria. Termikill® is a 20 % emulsifiable concentrate of CPF. It was prepared by reconstituting in soya oil (Grand Cereals and Oil Mills Ltd., Jos, Nigeria) to 10 % stock solution.

Cypermethrin marketed as Captain-C® (Modern Insecticides Limited, Punjab, India) was obtained from a reputable agrochemical company in Kano. Captain-C® is a 10 % emulsifiable concentrate of CYP. It was reconstituted in soya oil (Grand Cereals and Oil Mills Ltd., Jos, Nigeria).

Analytical grade vitamin C (Aldrich Chemical Co. Ltd., Gillingham Dorset, England, Cat. No. 25,556-6) was obtained from a reputable scientific store in Zaria. A 5 % solution was prepared by dissolving 5 g of vitamin C in 100 ml of distilled water.

3.2 EXPERIMENTAL ANIMALS

Sixty-four adult Wistar rats of either sex were obtained from the Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria. They were housed in cages under standard environmental conditions in the Laboratory of the Department. The rats were fed on pellets made from commercial growers' mash, maize-bran, and groundnut cake at the ratio of 4:2:1 with wheat flour used as a binding agent. The rats were given access to tap water *ad libitum* and were pre-conditioned for two weeks in the laboratory before the commencement of the experiment.

3.3 DETERMINATION OF MEDIAN LETHAL DOSE FOR CHLORPYRIFOS AND CYPERMETHRIN

Twelve (12) Wistar rats were used for the determination of the median lethal dose (LD_{50}) for each of the pesticides using the method of Lorke (1983). Nine (9) rats were used for the first phase; which involved the division of the nine rats into three (3) groups of 3 rats each. Rats in each of the groups were dosed with each pesticide orally as follows: Group I: 10 mg/kg, Group II: 100 mg/kg, and Group III: 1000 mg/kg. They were subsequently observed for death over a period of 48 hours.

The dosing for the second phase was based on the result obtained from the first phase. Three (3) rats were selected at random and assigned into three groups of one rat each. The LD_{50} was calculated based on dose-response from the second phase and in accordance with the method of Lorke (1983).

3.4 SUB-ACUTE TOXICITY STUDIES

Prior to the commencement of the study, forty adult Wistar rats were divided into four groups with each group having 10 rats (5 males and 5 females). Every rat in each group was weighed and identified by means of a tail-marker. The rats in group I, which is the control group, were administered with soya oil (2 ml/kg), while those in group II were given vitamin C (100 mg/kg) only. Rats in group III were treated with a reconstituted combination of CPF (5.3 mg/kg) (~ $1/16^{th}$ of LD_{50}) + CYP (14.1 mg/kg) (~ $1/16^{th}$ of LD_{50}), while those in group IV were pretreated with vitamin C (100 mg/kg) followed by administration of CPF (5.3 mg/kg) + CYP (14.1 mg/kg), 30 minutes later. These regimens were given by gavage once daily for a period of 28 days. All the rats were observed for clinical signs within the experimental period. The body weight of each rat in all the groups was recorded using standard procedure. In addition, periodic

evaluation of some neuro-behavioural parameters was conducted during the study period as indicated below.

3.5 EVALUATION OF OPEN-FIELD ACTIVITIES

The open-field apparatus as described by Zhu *et al*. (2001) was used to evaluate open-field activities of rats exposed repeatedly to all the regimens. This was carried out on each animal in all the groups prior to the commencement of the study, and on days 7, 14, 21 and 28 of the experiment. The open-field apparatus was made of cardboard box (50 x 50 x 46 cm high) with clear plexiglass on the inner surface. The floor of the box was divided into 25 equal squares. locomotor activity was evaluated by counting the number of squares crossed over a period of 2 minutes after an intial 3 minutes of habituation. Anxiety was also determined by recording the frequency of rearing, grooming for 2 minutes after an initial 3 minutes of habituation.

3.6 EFFECTS OF TREATMENT REGIMENS ON NEUROMUSCULAR COORDINATION

The effect of the combination of CPF+CYP and the modulatory effect of vitamin C on neuromuscular coordination were assessed using inclined plane test as described by Ambali (2009). Briefly, the activity of rats on an inclined plane was evaluated by placing each rat on an apparatus made with an angled rough-wooden plank with thick foam pad at its bottom end. The plank was raised to an inclination of 35°, which was increased by 5° step-wise until the subject could no longer stay situated horizontally on the plank for 30 s without sliding down. Angles were measured and marked on the apparatus beforehand, and were obtained by propping the plank on a bar with several notches that would catch on a vertical bar. The test was performed with the rat facing left and then right, and the highest angle at which the rat could stay standing horizontally, facing each direction was recorded. Two trials were performed for each testing

session. This procedure was carried out on each animal from all the groups on week 1, 2, 3 and 4 of the study.

3.7 ASSESSMENT OF LEARNING AND MEMORY

The effect of the combination of CPF+CYP on learning task in rats and the effect of the antioxidant vitamin C was assessed 48 hours to the termination of the test using the step-down inhibitory avoidance learning task as described by Zhu *et al*. (2001). The apparatus used was a 40 x 25 x 25 cm acrylic chamber, consisting of a floor made of parallel 2-mm-caliber stainless steel bars spaced 1 cm apart through which electric shock was administered. A 2.5-cm high, 8 x 25 cm wooden platform was placed on the left extreme of the chamber. Each animal was gently placed on the platform. Upon stepping down, the animal immediately received a single 80-volt foot shock. If the animal did not return to the platform, the foot shock was repeated every 5 seconds. An animal was considered to have learned the avoidance task, if it remained on the platform for more than 2 minutes. The number of foot-shocks was recorded as an index of learning acquisition.

To assess short-term memory, each rat was placed gently on the platform 24 hours later. The time an animal remained on the platform was recorded as an index of memory retention. Staying on the platform for 2 minutes was counted as maximum memory retention (ceiling response).

3.8 DETERMINATION OF BRAIN MALONDIALDEHYDE CONCENTRATION

For the determination of lipid peroxidation in the brain, the method of Draper and Hadley (1990) as modified by Freitas *et al.* (2005) was used. A known weight of the tissue sample was homogenized in a known volume of ice-cold phosphate buffer to obtain a 10 % homogenate, which was then centrifuged at 6,000 x g for 10 minutes to obtain the supernatant. The

supernatant was used to access the level of protein and malonedialdehyde (MDA) in the sample. Briefly, 0.5 ml of the supernatant was mixed with 1 ml 10% trichloroacetic acid and 1 ml 0.67% thiobarbituric acid. The mixture was then heated in a boiling water-bath for 15 min, and butan-2-ol (2:1, v/v) was added to the solution. After centrifugation (800 x g, 5 min) thiobarbituric reactive substance (TBARS), MDA was determined from the supernatant at the absorbance of 535 nm. The concentration of MDA in the brain sample was calculated by the absorbance coefficient of MDA-TBA complex 1.56 x 105/cm/M and expressed in nmol/mg of protein. The protein content of the supernatant was determined using the method of Lowry (1951).

3.9 DETERMINATION OF HAEMATOLOGICAL PARAMETERS

3.9.1 Determination of Packed Cell Volume

Heparinized capillary tubes (John Poulten Limited, English) were inserted into the blood-containing sample bottles, leaving about 15 mm of the tubes unfilled. The open end of each capillary tube is then sealed in a flame. The tubes were then loaded onto a microhaematocrit centrifuge (Hawksley, England) and centrifuged at 1000g for 10 minutes. The PCV value for each of the tube was read using the haematocrit reader.

3.9.2 Determination of Total Erythrocytes Count

0.5 mL of blood was sucked into RBC pipette and then diluted with 100mL of RBC diluting fluid (1:200). The counting was done in an improved Neubaeur counting chamber under a light microscope at ×40 magnification.

3.9.3 Determination of Absolute and Differential Leucocyte Count

A drop of blood was placed on one end of a clean glass slide (UNISCOPE, England) and using a spreader a blood film was made and allowed to air-dry. Leishman stain was then poured to cover

the film for 2 minutes. The film was then rinsed in buffered distilled water and allowed to stand for 10 minutes. The film was later viewed under a microscope in an oil immersion at ×1000 magnification.

3.9.4 Determination of Haemoglobin Concentration

20µL of blood was pipetted into a tube and then diluted with 5 mL of modified Drabkin's fluid and allowed to stand for at least 3 minutes. The absorbance of the mixture was read using a spectrophotometer (Beckman Coulter, model BU520, Austria) at a wavelength of 540nm against reagent blank. The haemoglobin concentration was determined using the following formula:

$$Hb = \frac{T \times C \times D}{A \times 1000} \text{ (g/dL)}$$

Where T is the test absorbance, A is the standard absorbance, C is the concentration of cyanhaemoglobin standard (mg/dL), while D is the dilution factor (1: 250); 1000 converts the Hb concentration from mg/dL to g/dL.

3.10 SERUM BIOCHEMICAL EXAMINATION

Blood was collected into test tubes and allowed to stand for 60 minutes. The test tubes were then centrifuged at 1000g for 10 minutes. Thereafter, the straw coloured serum was collected from each test tube into a clean sample tube, which was subsequently used for the evaluation of the serum biochemical parameters. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactic dehydrogenase (LDH) and creatine kinase (CK) were analysed using an autoanalyzer (Bayer Clinical Chemistry Analyzer, Germany). Total serum proteins was analysed using Biuret method while serum albumin concentration was measured using the method described by Henry (1974). Serum globulin concentration was obtained by

subtracting serum albumin from total serum protein concentration. Serum glucose concentration was measured using the glucose oxidase method described by Ware and Marbach (1968).

3.11 STATISTICAL ANALYSIS

Values were expressed as mean ± standard error of the mean (mean ± SEM). Data were subjected to one-way analysis of variance (ANOVA) and Tukey's post-hoc test using Graphpad Prism version 4.0 for windows from Graphpad Software, San Diego, California, USA (www.graphpad.com) to compare the level of significance between the test groups and those in the control, treated with S/oil only. Values of $P<0.05$ were considered significant.

CHAPTER 4

4.0 RESULTS

4.1.1 Determination of Median Lethal Dose (LD_{50}) for Cypermethrin

No mortality was observed among the three rats used for the phase I of the LD_{50} determination in group I. In group II, of this phase, two of the three rats died following the administration of CYP. All the three rats in group III died. The observations above were made within 24 hours post-administration. The following signs were also observed in the rats: huddling, back-arching, spastic limb movement, ataxia, convulsion, tachypnoea, paresis, hind-quarter paralysis and death. Based on the results obtained in phase I, the sequel experiment (phase II) was conducted and the results are synopsized in Table 4.1

4.1.2 Determination of Median Lethal Dose (LD_{50}) for Chlorpyrifos

In phase I of the LD_{50} determination for CPF, the three rats in each of the three groups dosed with CPF died within 24 hours post-administration. The observed clinical signs included depression, huddling, piloerection, tremor, intermittent convulsion, respiratory depression, ataxia and death. Post-mortem gross findings observed were haemorrhagic gastromenteritis, filling of the stomach with bubbles, congestion and petechial haemorrhages of the liver, congestion of the lungs, heart, kidneys, testes and brain vessels, and flabby thigh muscle.

The results obtained in the phase II of the LD_{50} determination for CPF in the three groups are shown in Table 4.2

Table 4.1: Synopsis of results from Phase II of Cypermethrin LD_{50} evaluation

Group	Dose (mg/kg)	Number of dead rats
1	200	0/1
2	250	1/1
3	300	1/1

On the basis of the method of Lorke (1983), the LD_{50} was calculated as follows:

$$\frac{200 + 250}{2} = 225 \text{ mg/kg}$$

Table 4.2: Summary of results of the phase II of Chlorpyrifos LD_{50} evaluation

Group	Dose (mg/kg)	Number of dead rats
1	100	1/1
2	90	1/1
3	80	0/1

Based on the method of Lorke (1983), the $LD_{50} = \frac{90+80}{2} = 85$ mg/kg.

4.2 SUBACUTE TOXICOLOGICAL STUDY

4.2.1 Clinical Signs

No overt clinical signs of toxicosis were observed throughout the study period in all the treatment groups.

4.2.2 Effect of Subacute Administration of Soya oil, Combination of Chlorpyrifos and Cypermethrin, and/or Vitamin C on Body Weight Changes in Adult Wistar Rats.

The dynamics of changes in body weight in rats exposed to the various treatment regimens throughout the study period are shown on Figure 4.1. Rats in all the experimental groups showed a progression in weight gain. Those in the CPF group did not show any rapid progression in body weight gain as observed in the other groups. At week 1, there was an increase in weight gain in the rats belonging to CPF+CYP+VC group, when compared to the control and CPF+CYP groups. The rats in the control and VC groups had steady increase in body weight from day 0 to the day of termination of the experiment.

Conversely, rats in the CPF+CYP and CPF+CYP+VC groups initially showed a significant weight gain by week 1, while a decrease in weight gain was observed in week 2. Thereafter, there was an increase in weight gain of the rats belonging to the two groups. There was no significant ($P > 0.05$) difference in the values of body weight in CPF+CYP and CPF+CYP+VC groups throughout the study period.

The effect of various treatments on the mean cumulative body weight changes in adult Wistar rats is shown in Figure 4.2. The cumulative body weight change of rats in the CPF+CYP group (149.0 ± 3.5 g) over the 4-week study period was the lowest as compared to other test groups. The cumulative body weight of rats in the CPF+CYP group was significantly lower ($P < 0.05$) than that of the control group (159.0 ± 3.9g). However, the mean cumulative weight gain in the VC (156.0 ± 4.2g) and S/oil groups did not differ significantly. Although the mean cumulative

body weight of the CPF+CYP+VC group (151.0 ± 3.3g) was slightly higher than that of the CPF+CYP group (149.0 ± 3.5g), the difference in the values was not significant ($P > 0.05$).

The percentage weight gain, based on the body weight at the commencement of the study compared to the weight at termination of the experiment, for the s/oil group was 30.85 %. The VC group had 28.4 % weight gain, while the percentage weight gain of CPF+CYP and CPF+CYP+VC groups were 24.26 % and 19.14 %, respectively.

4.3 ASSESSMENT OF NEURO-BEHAVIOURAL CHANGES FOLLOWING SUB-ACUTE CO-ADMINISTRATION OF CHLORPYRIFOS AND CYPERMETHRIN AND/OR VITAMIN C IN WISTAR RATS.

4.3.1 Effect on Open - field Activities

The open-field parameters examined included motor activity and anxiety (as measured by frequency of locomotor activity and rearing).

4.3.1.1 Effect on Motor Activity

The general pattern of locomotor activity in rats from all the groups throughout the study period is shown on Figure 4.4.There were no significant changes in the dynamics of locomotor activity in S/oil, VC and VC+CPF groups throughout the study period. However, there were significant increase in the dynamics of motor activity in the CPF+CYP group at day 0 compared to days 7, 14, and 28 ($P < 0.05$), and day 21 ($P < 0.01$). There were no significant changes ($P > 0.05$) in the dynamics of locomotor activity in days 0, 7, 14, 21 and 28 in between the groups.

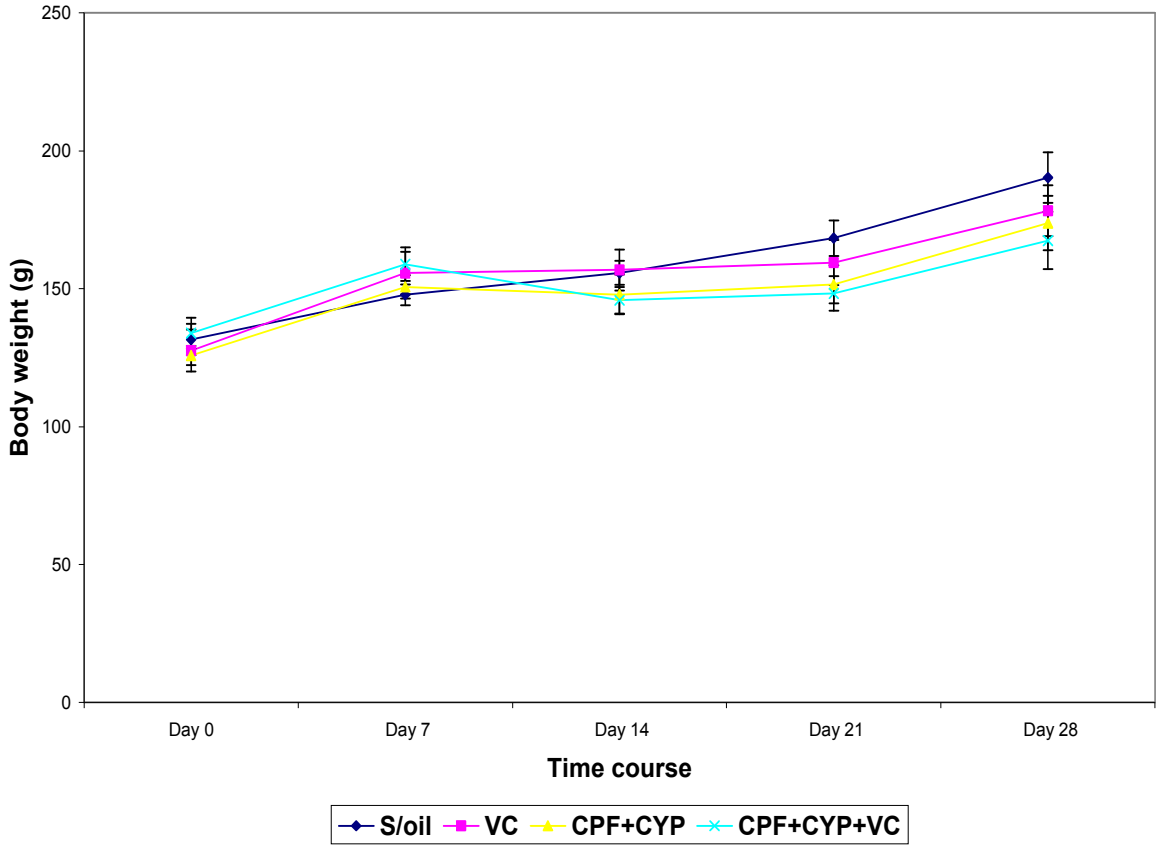


Figure 4.1: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin, and/or vitamin C on the dynamics of weekly body weight changes in Wistar rats (n = 10).

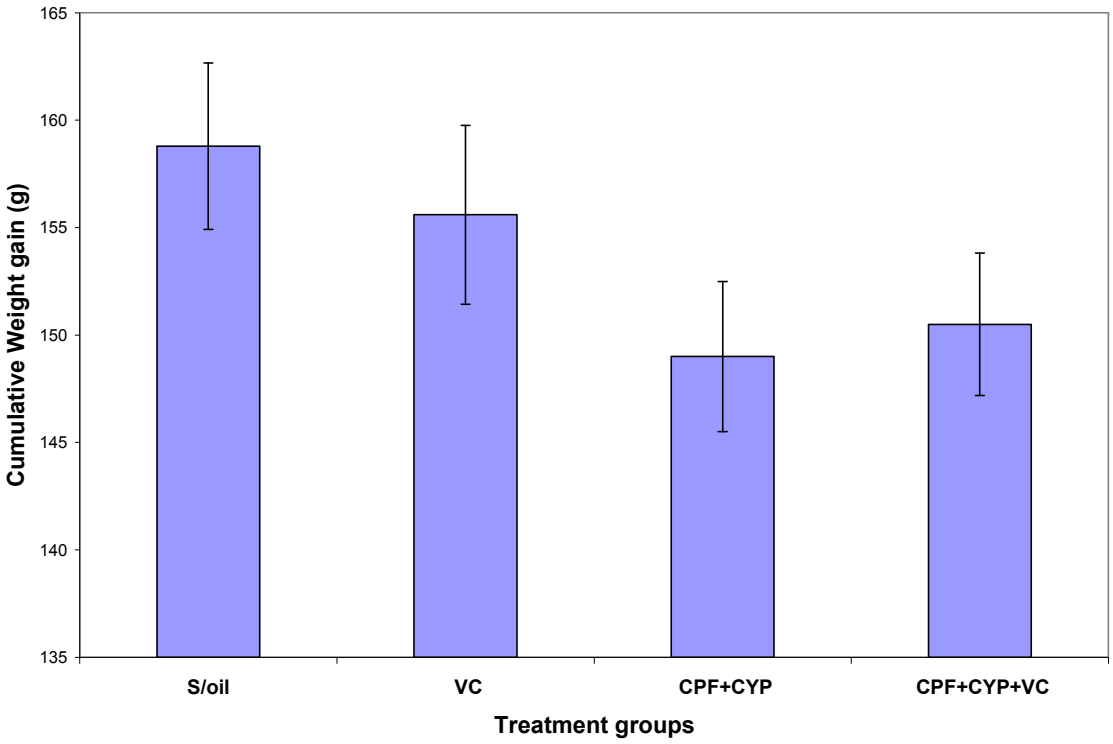


Figure 4.2: Effect of sub-acute co-administrationof chlorpyrifos (CPF) and cypermethrin (CYP), and/or vitamin C (VC) on cumulative body weights in Wistar rats (n = 10).

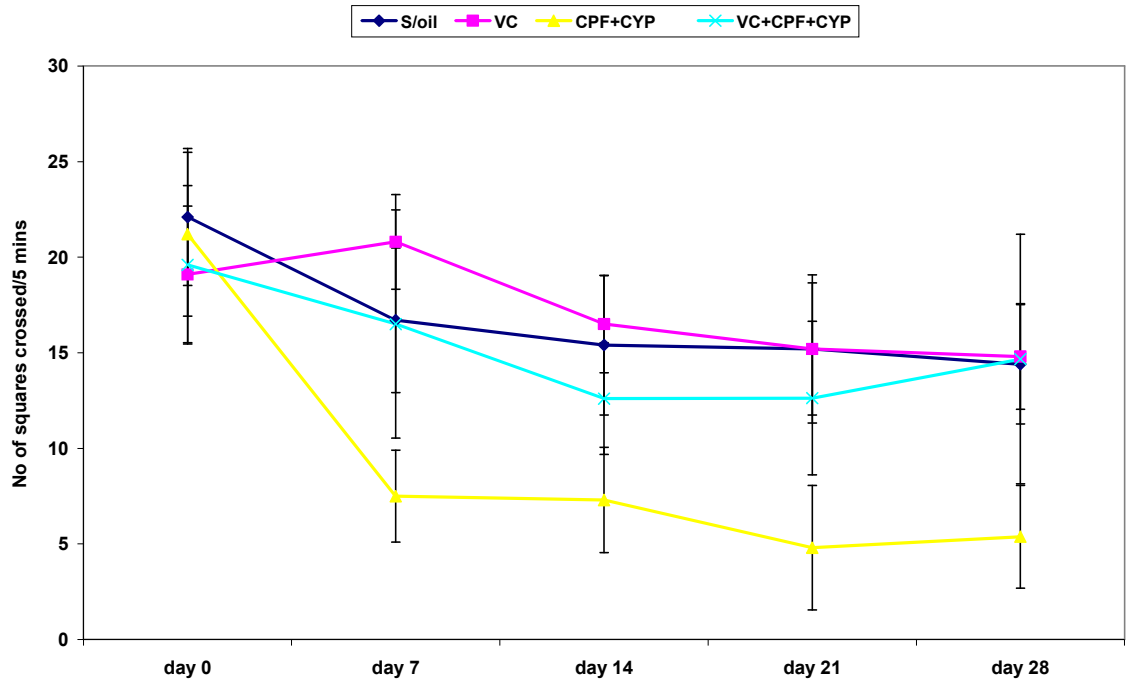


Figure 4.3: Effect of the various treatments on the time-course of locomotor activity pattern in adult Wistar rats (n= 10).

4.3.1.2 Effect on the Frequency of Rearing

The effect of repeated sub-acute administration of combination of CPF and CYP and/or VC on the pattern of frequency of rearing in Wistar rats is shown in Figure 4.4. There was no significant change ($P > 0.05$) in the frequency of rearing in between the period of evaluation in the C/oil, VC and VC+CPF+CYP groups. However, there were significant decreases in the frequency of rearing on days 14 ($P < 0.05$), 21 ($P < 0.01$) and 28 ($P < 0.05$) compared to day 0 in the CPF+CYP group.

There was no significant change ($P > 0.05$) in the frequency of rearing on days 0 and 7 in between the groups. However, there was a significant decrease in the frequency of rearing in the CPF+CYP group compared to C/oil ($P < 0.01$) or VC ($P < 0.05$) group on day 14. On day 21, there was a significant decrease ($P < 0.01$) in the frequency of rearing in the CPF+CYP group compared to C/oil, VC or VC+CPF+CYP group. There was a significant decrease ($P < 0.05$) in the frequency of rearing in the CPF+CYP group compared C/oil group.

4.4. EFFECT OF SUB-ACUTE CO-ADMINISTRATION OF CHLORPYRIFOS AND CYPERMETHRIN AND/ OR VITAMIN C ON NEURO-MUSCULAR COORDINATION

4.4.1 Effect of Treatments on Inclined Plane Performance

The effect of repeated sub-acute administration of combination of CPF and CYP and/ or VC on the inclined-plane performance of Wistar rats is shown in Figure 4.5.There was no significant change ($P > 0.05$) in the inclined plane performance on days 0 and 7 in between the groups. However, on day 14, there was a significant decrease in the inclined-plane performance of CPF+CYP when compared to VC ($P < 0.001$), S/oil ($P < 0.01$), or CPF+CYP+VC ($P < 0.001$) groups. Similarly, on days 21 and 28, there was a markedly significant decrease ($P < 0.001$) in the inclined-plane performance of the CPF+CYP group when compared to all the other groups.

There was a progressively significant increase in the inclined plane performance in the S/oil group between day 14 and day 21 ($P < 0.05$), also between day 14 and day 28 ($P < 0.01$).

The VC group had no significant change ($P > 0.05$) in the inclined plane performance in between the period of evaluation. Conversely, the CPF+CYP group had a significant decrease ($P < 0.01$) between days 7 and 14 ($P < 0.01$), also had a significant decrease ($P < 0.01$) when days 21 and 28 were compared with day 7. For the CPF+CYP+VC group, there was an initial significant increase ($P < 0.01$) between day 7 and day 14 ($P < 0.01$) followed by significant decreases ($P < 0.01$) between days 21 and 28.

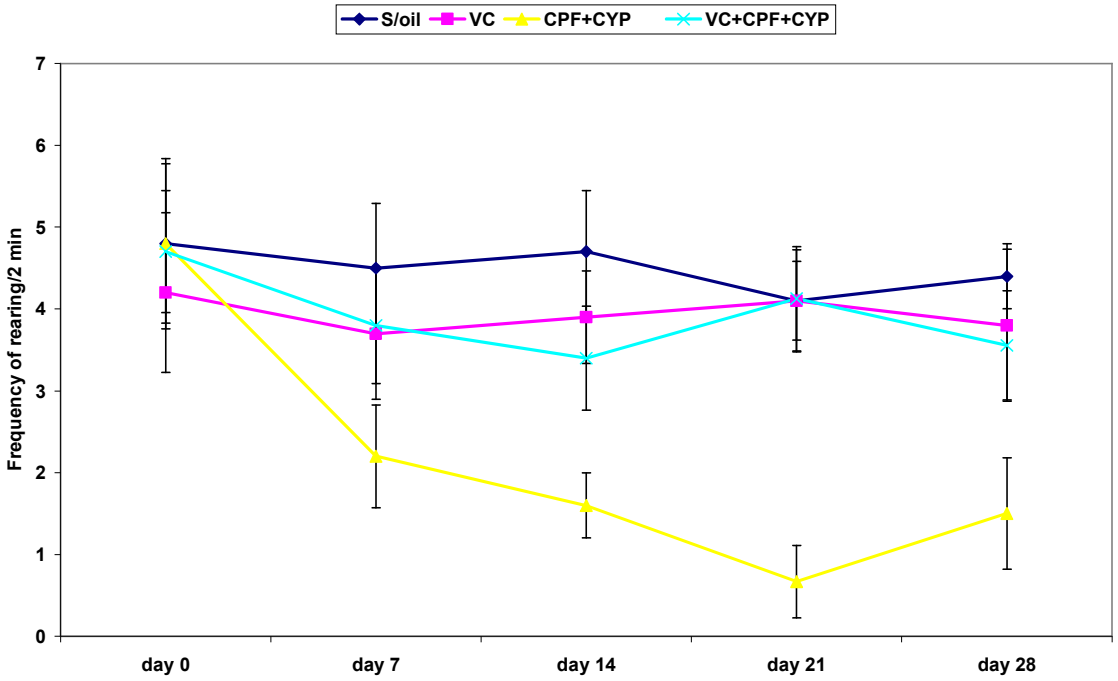


Figure 4.4: Effect of sub-acute administration of soya oil, combination of chlorpyrifos and cypermethrin and/ or vitamin C on the pattern of frequency of rearing in Wistar rats (n= 10).

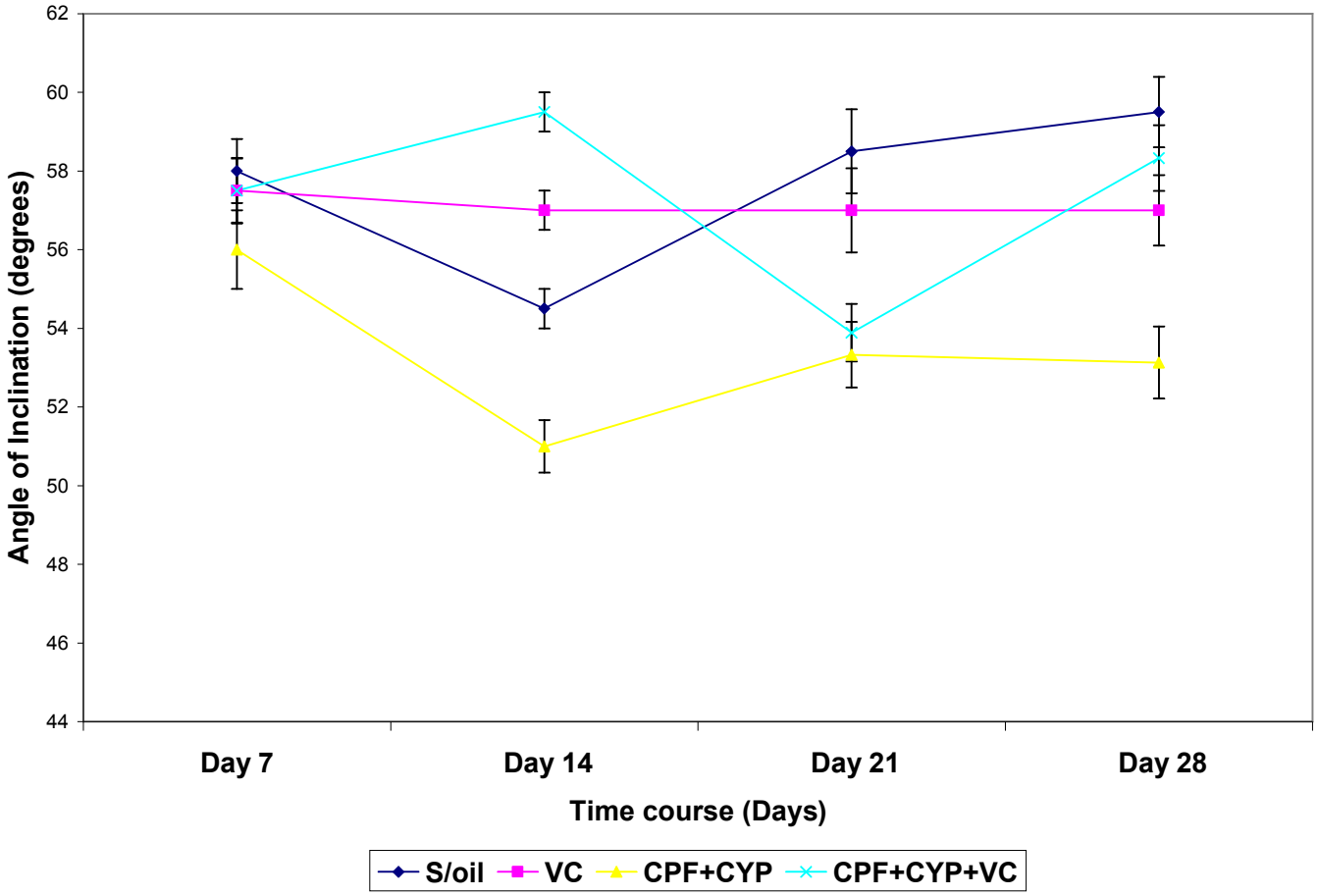


Figure 4.5 : Effect of sub-acute co-administration of chlorpyrifos (CPF) and cypermethrin (CYP) and/or vitamin C (VC) on the cumulative inclined plane performance in Wistar rats (n= 10).

4.5 EFFECT OF SUB-ACUTE CO-ADMINISTRATION OF CHLORPYRIFOS AND CYPERMETHRIN AND/ OR VITAMIN C ON COGNITION IN WISTAR RATS

4.5.1 Effect on Learning

The effect of various treatments on learning is shown in Figure 4.6. Rats in the CPF+CYP group (1.6 ± 0.3) had a significantly reduced ($P < 0.05$) ability to learn compared to those of the control group (1.2 ± 0.1). However, there was no significant difference in the values obtained on the learning ability of rats belonging to the vitamin-pretreated group (CPF+CYP+VC) and those in the VC group when compared to the control. Although there was a slightly improved learning ability in the rats of the CPF+CYP+VC group the value obtained was not significantly ($P > 0.05$) different from that of the control rats.

4.5.2 Effect of Sub-acute Co-administration of Chlorpyrifos and Cypermethrin and/ or Vitamin C on Short-term Memory

The effect of the various treatments on memory is illustrated on Figure 4.7. The rats in CPF+CYP group (80.9 ± 14.4 seconds) had a shorter ($P < 0.05$) latency period than those of the control (113 ± 6.8 seconds) group. Rats in the vitamin C pre-treated group (CPF+CYP+VC) showed a higher latency period than those in the CPF+CYP group. There was no significant difference ($P < 0.05$) in the values of the latency period obtained for the CPF+CYP and VC groups when compared to that of the control.

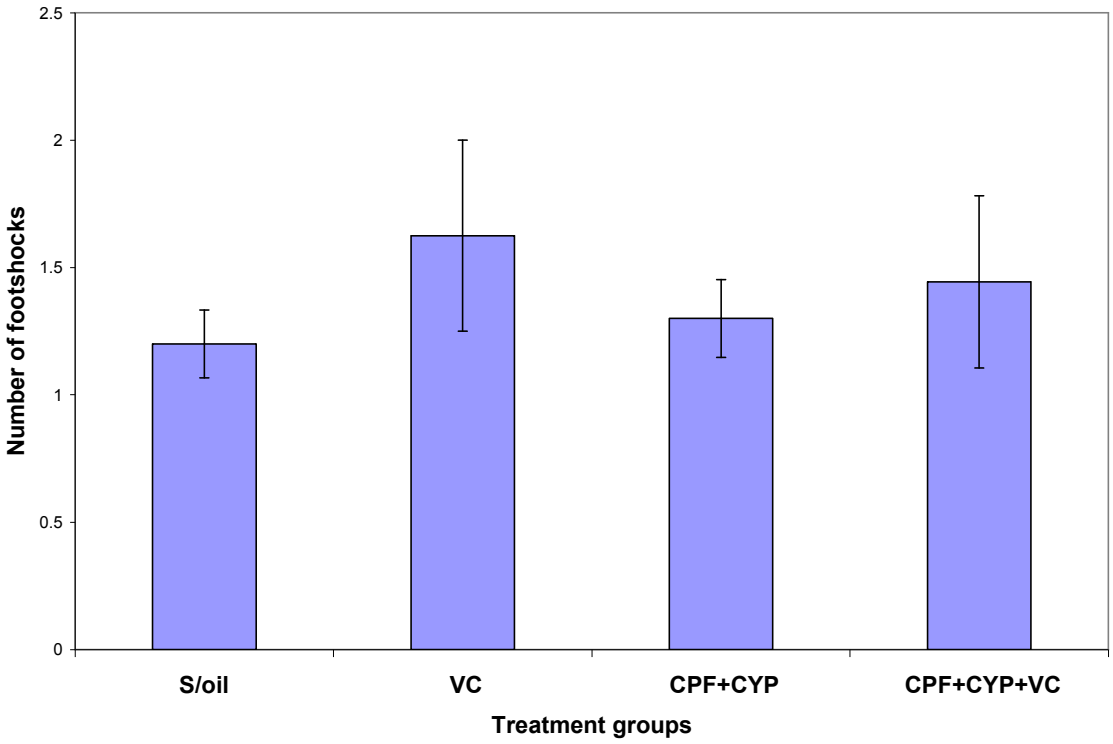


Figure 4.6: Effect of sub-acute co-administration of chlorpyrifos (CPF) and cypermethrin (CYP) and/or vitamin C (VC) on learning in Wistar rats (n= 10).

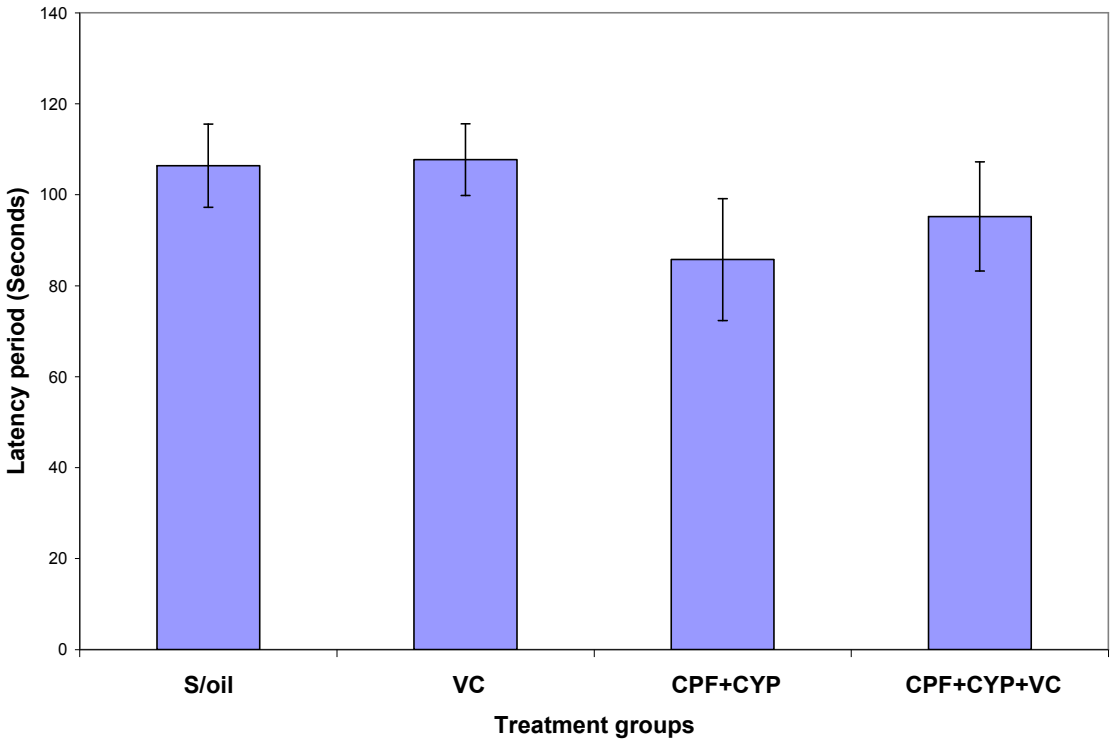


Figure 4.7: Effect of sub-acute co-administration of chlorpyrifos (CPF) and cypermethrin (CYP) and/or vitamin C (VC) on memory in Wistar rats (n= 10).

4.6 EFFECT OF SUB-ACUTE CO-ADMINISTRATION OF CHLORPYRIFOS AND CYPERMETHRIN AND/OR VITAMIN C ON HAEMATOLOGICAL PARAMETERS IN WISTAR RATS

4.6.1 Effect on Packed Cell Volume

There was no significant difference (P>0.05) in the PCV in between the groups. However, the PCV in the CPF+CYP group was comparatively lower compared to S/oil (0.3%), VC (7%) or CPF+CYP+VC (6%) group (Figure 4.8).

4.6.2 Effect of Sub-acute Co-administration of Chlorpyrifos and Cypermethrin and/or Vitamin C on Total Red Blood Cell Count

There was no significant difference (P>0.05) in the total red blood cell count in between the groups. However, the total red blood cell count in the CPF+CYP group was relatively lower compared to S/oil (0.7%) or VC (6%) groups while it was higher than the CPF+CYP+VC (0.02%) group (Figure 4.9).

4.6.3 Effect of Sub-acute Co-administration of Chlorpyrifos and Cypermethrin and/or Vitamin C on Haemoglobin Concentration

There was no significant difference (P>0.05) in the haemoglobin concentration in between the groups. However, the haemoglobin concentration of the CPF+CYP group was comparatively lower to S/oil (3%), VC (7%) or CPF+CYP+VC (3%) group (Figure 4.10).

4.6.4 Effect of Sub-acute Co-administration of Chlorpyrifos and Cypermethrin and/or Vitamin C on Platelet Count

There was no significant difference (P>0.05) in the platelet count in between the groups. However, the platelet count in the CPF+CYP group was comparatively lower than the S/oil (7%), VC (5%) or CPF+CYP+VC (2.5%) groups (Figure 4.11).

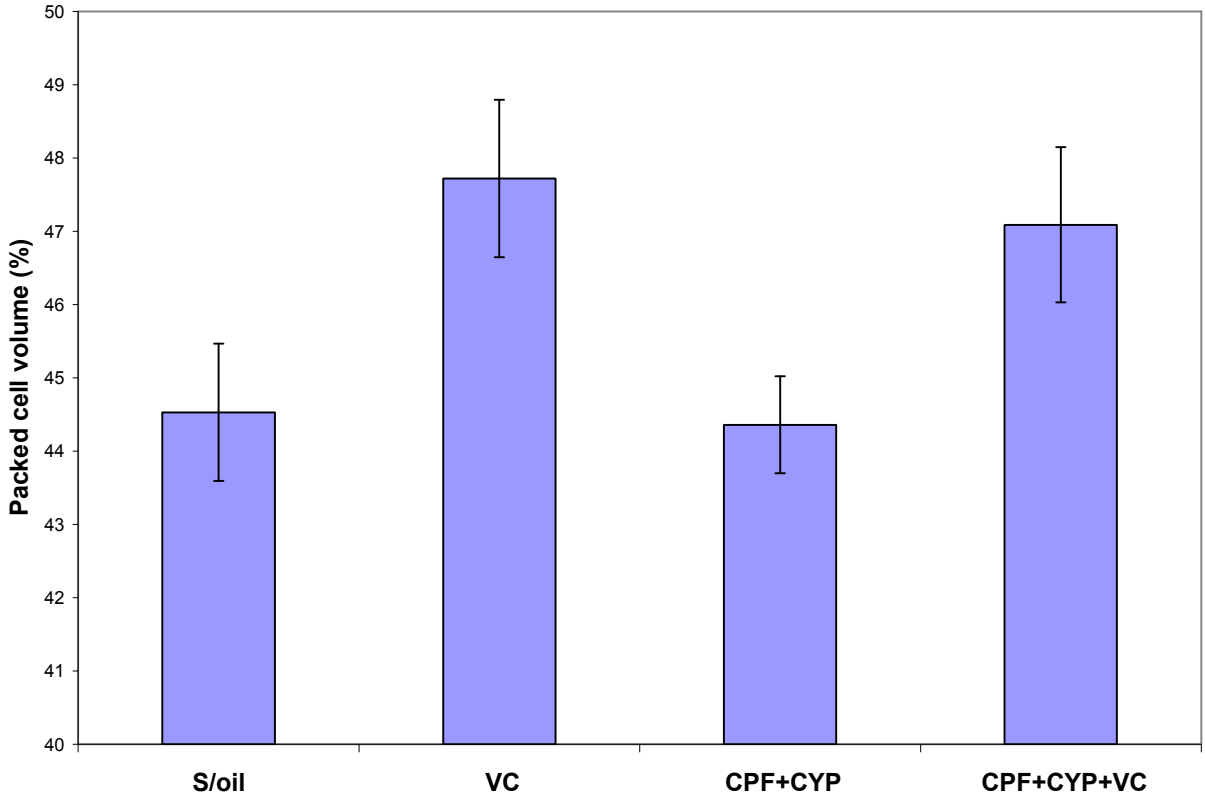


Figure 4.8: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on packed cell volume in Wistar rats (n= 10).

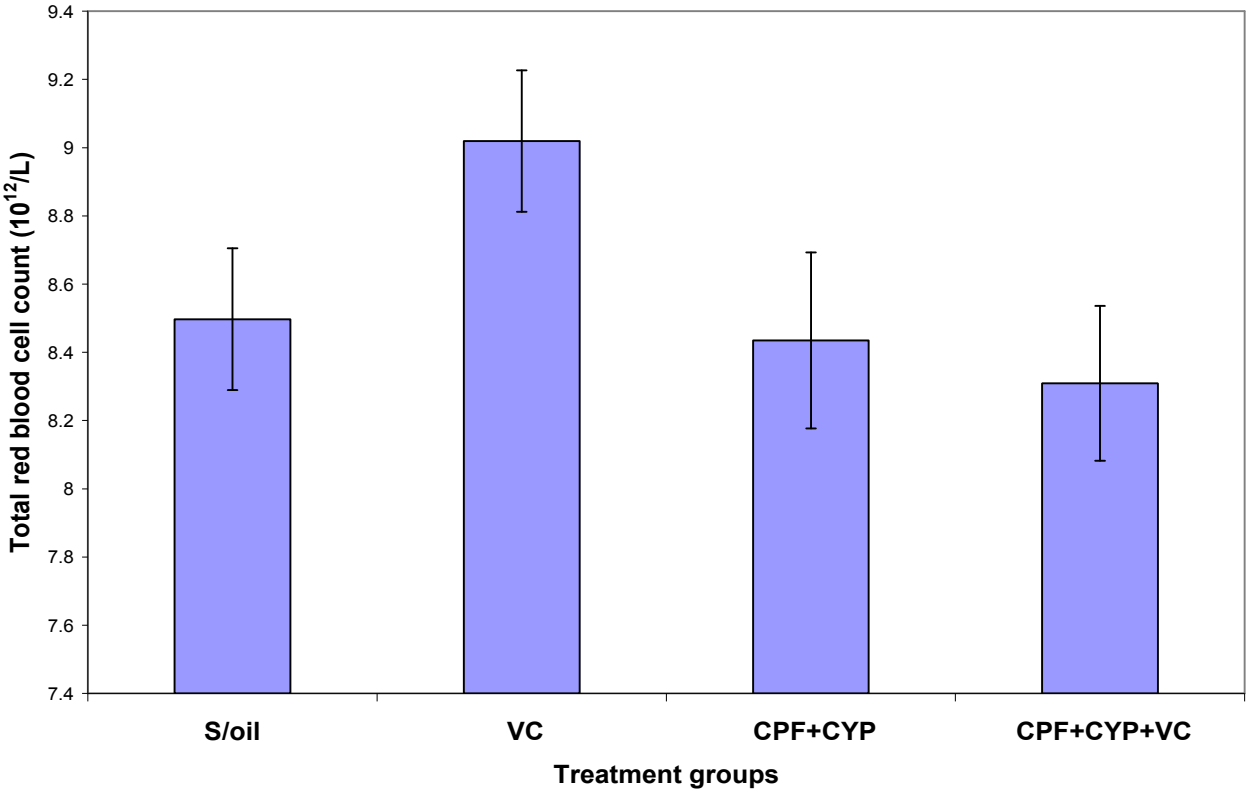


Figure 4.9: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on total erythrocyte count in Wistar rats (n= 10).

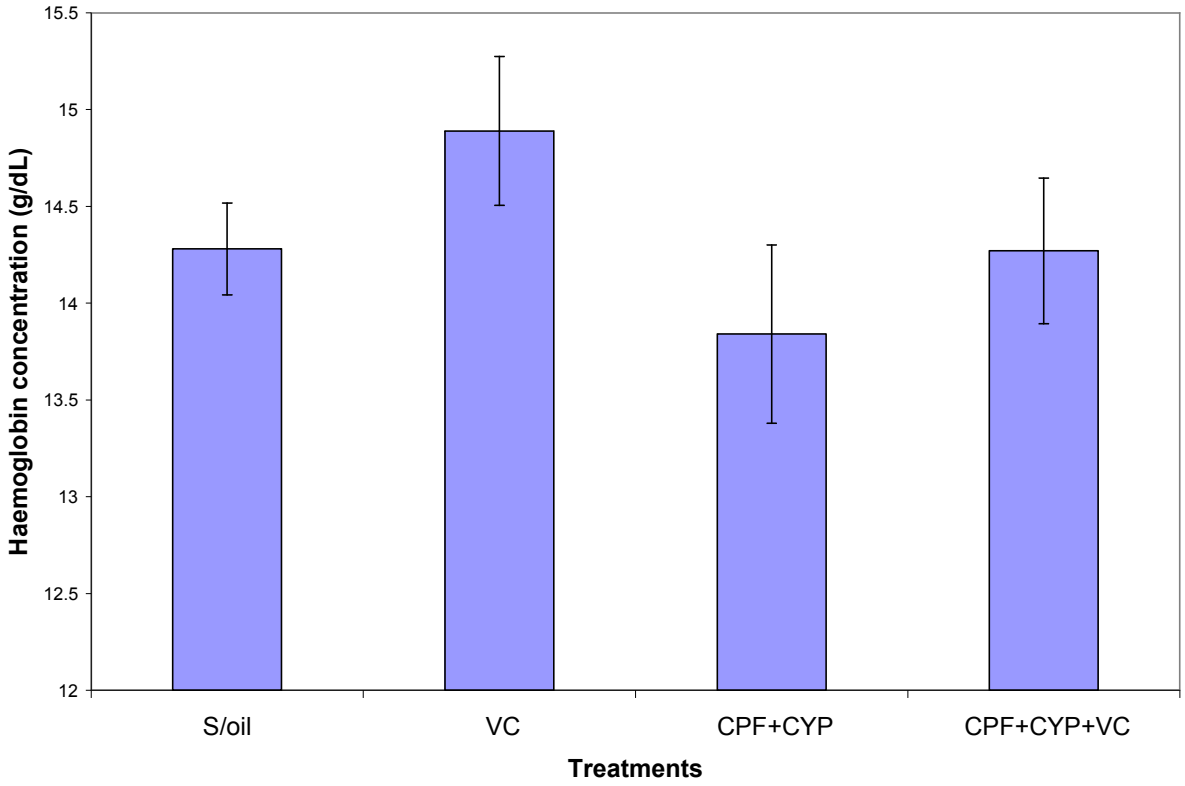


Figure 4.10: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on haemoglobin concentration in Wistar rats (n= 10).

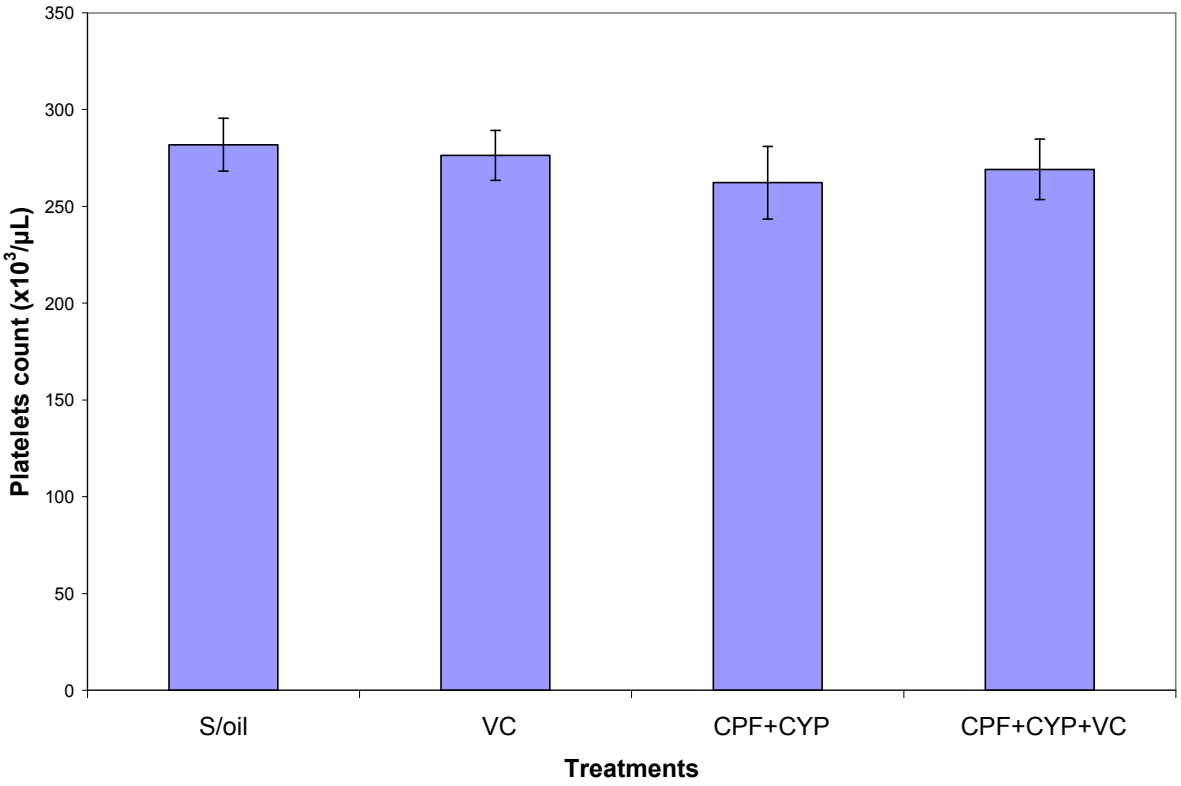


Figure 4.11: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on platelet count in Wistar rats (n= 10).

4.6.5 Effect on Total White Blood Cell Counts

There was no significant difference ($P > 0.05$) in the white blood cell count in between the groups. However, the white blood cell count in the CPF+CYP group was comparatively higher than the S/oil (5.75%), VC (7.7%) or CPF+CYP+VC (7.7%) groups (Figure 4.12).

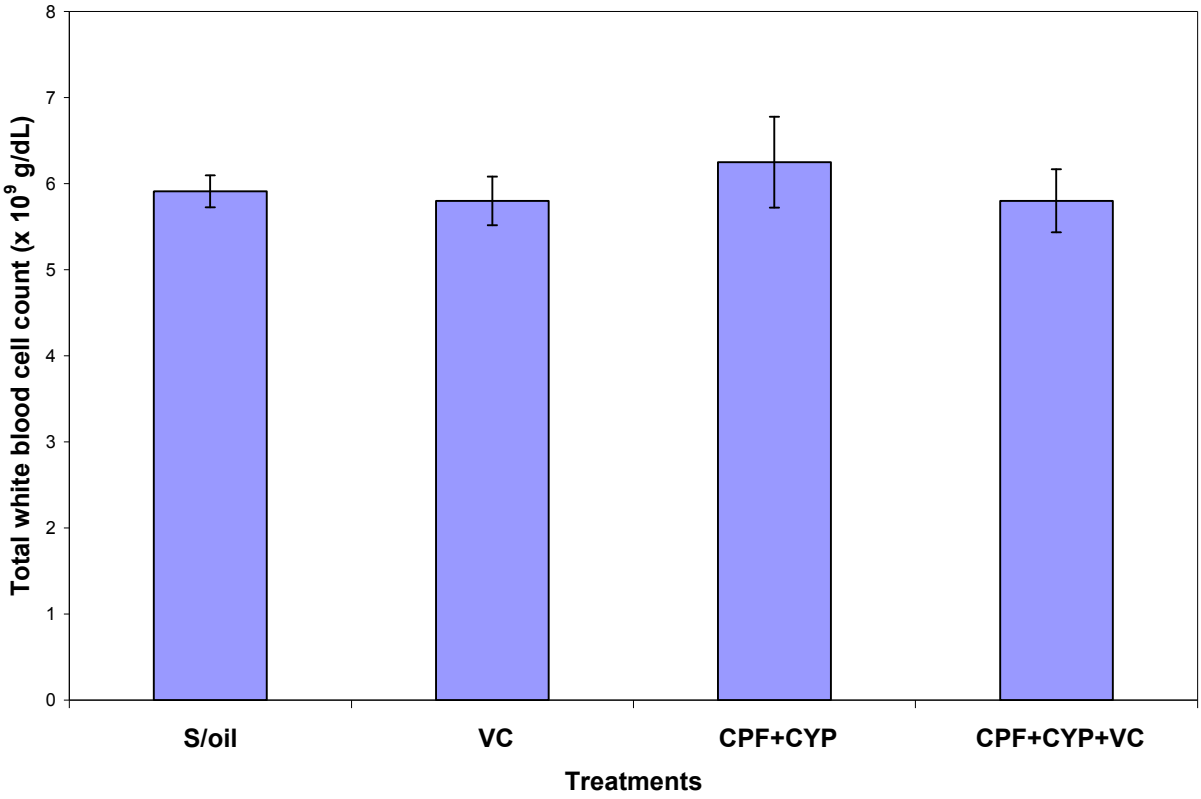


Figure 4.12: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on total leucocyte count in Wistar rats (n= 10).

Groups	WBC Absolute ($x10^6$ g/dL)	Neutrophil ($x10^6$ g/dL)	Lymphocytes ($x10^6$ g/dL)	Monocytes ($x10^6$ g/dL)	Eosinophils ($x10^6$ g/dL)	Basophils ($x10^6$ g/dL)
Soya oil	5.91±0.19	1.31±0.15	4.39±0.21	0.06±0.01	0.1±0.02	0.02±0.01
Vitamin C only	5.80±0.28	1.56±0.22	3.97±0.17	0.06±0.02	0.16±0.03	0.03±0.01
CPF+CYP	6.25±0.53	1.73±0.24	4.45±0.35	0.01±0.01	0.05±0.02	0.0±0.0
CPF+CYP +VC	5.80±0.37	1.28±0.18	4.26±0.35	0.07±0.03	0.18±0.04	0.01±0.01

Table 4.3 Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on absolute total and differential white blood cell counts in Wistar rats (Mean±SEM, n=10).

4.6.6 Effect of Sub-acute Co-administration of Chlorpyrifos and Cypermethrin and/or Vitamin C on *In vitro* Erythrocyte Osmotic Fragility

The effect of various treatments on percent haemolysis is illustrated in Figure 4.13. The control solvent (distilled water) had complete haemolysis (100%). Generally, no significant difference was recorded in percentage haemolysis of rats in the test groups when compared respectively to the control at each concentration of the NaCl solution.

At 0.5 % NaCl concentration, there was no significant difference ($P > 0.05$) in between the groups. However, the percent haemolysis was highest in CPF+CYP group it was comparatively higher than S/oil (9%), VC (10%) or CPF+CYP+VC (30%) groups.

4.7 EFFECT OF SUB-ACUTE CO-ADMINISTRATION OF CHLORPYRIFOS AND CYPERMETHRIN AND/OR VITAMIN C ON BIOCHEMICAL PARAMETERS

4.7.1 Effect of Sub-acute Co-administration of Chlorpyrifos and Cypermethrin and/or Vitamin C on Serum Glucose levels

There was no significant difference ($P>0.05$) in the serum glucose levels in between the groups. However, the serum glucose level in the CPF+CYP group was comparatively lower than the S/oil (11.2%) or VC (3.2%) groups while it was higher than the CPF+CYP+VC (2.9%) group (Figure 4.14).

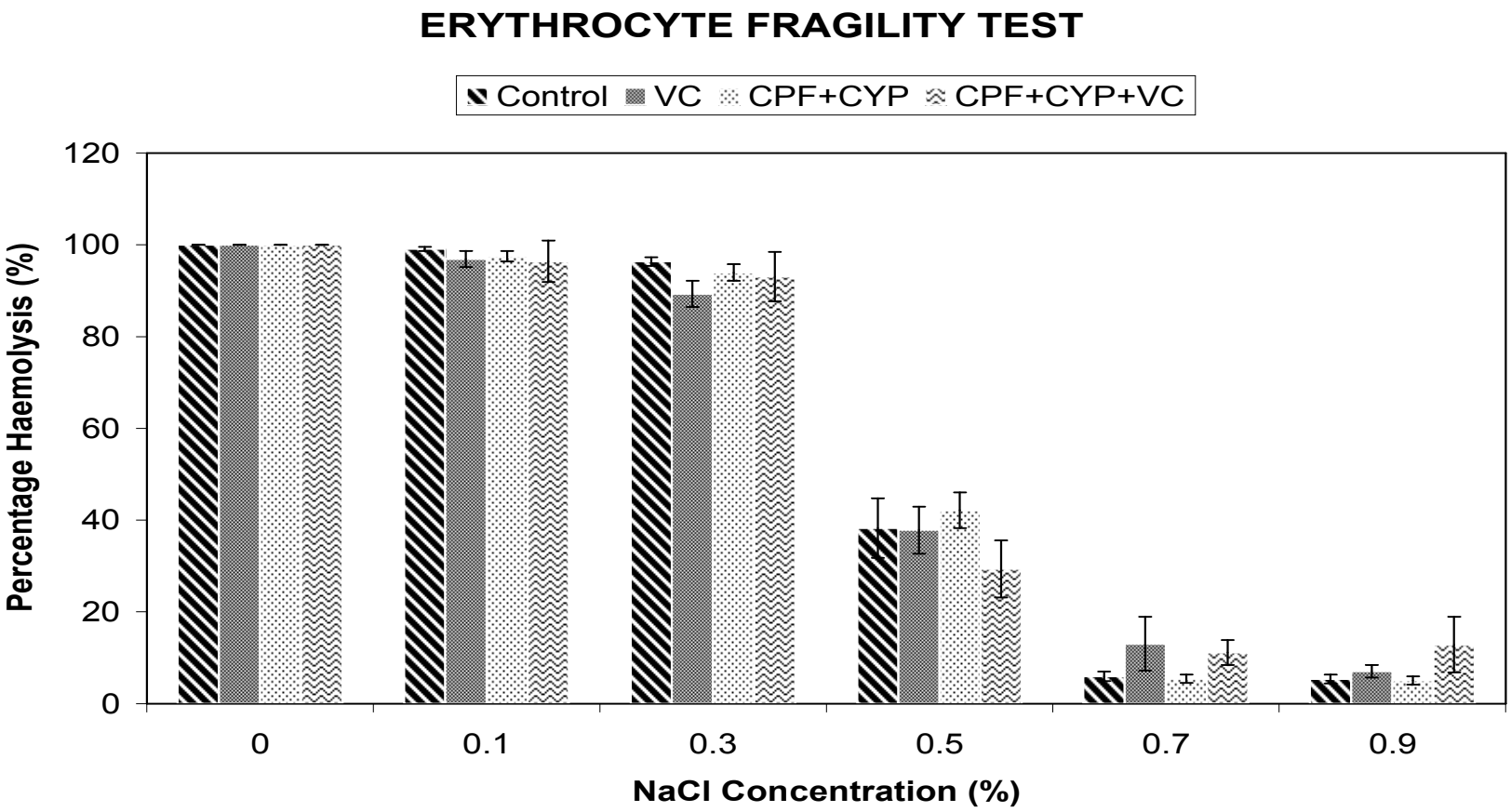


Figure 4.13: Effect of sub-acute administration of soya oil, combination of chlorpyrifos and cypermethrin and/ or vitamin C on *in vitro* erythrocyte osmotic fragility of Wistar rats (n= 10).

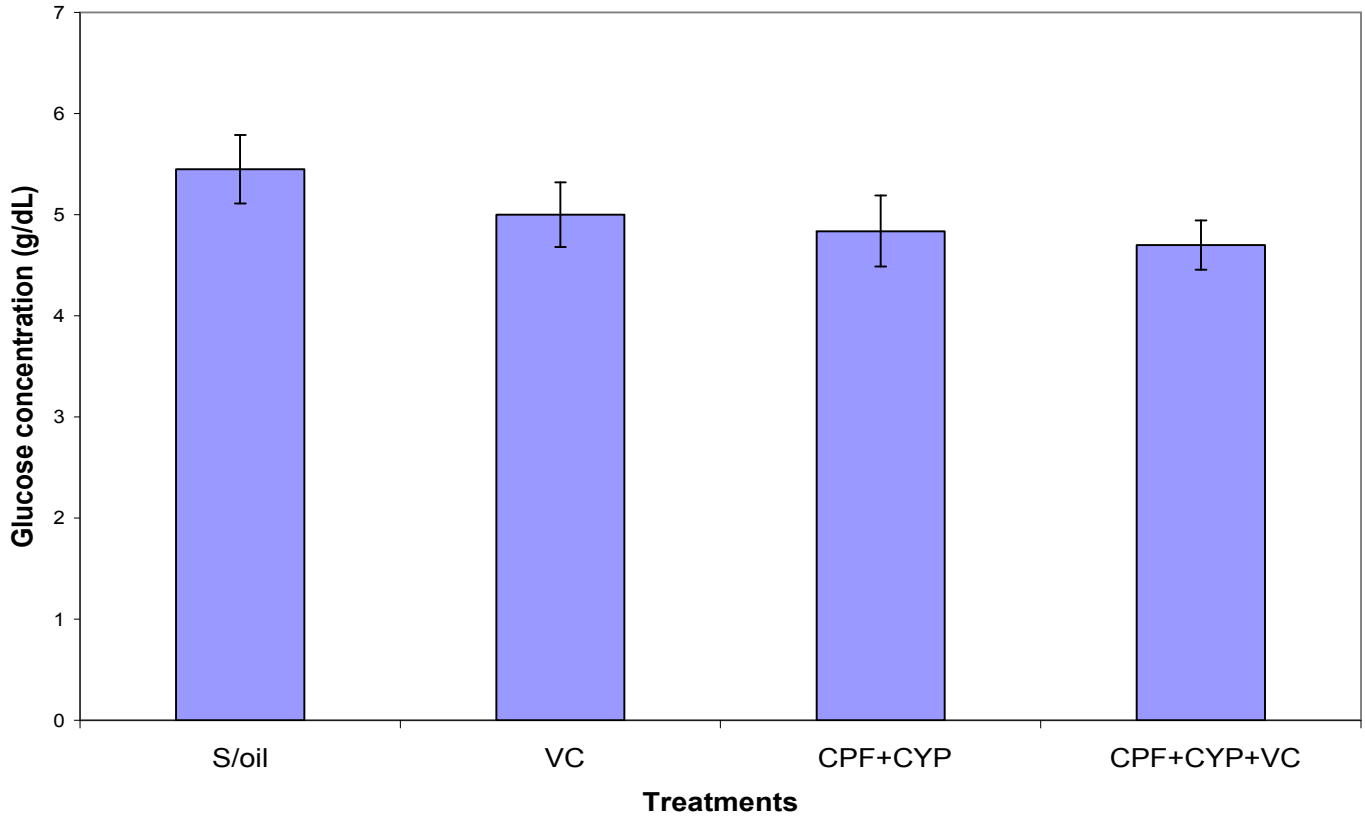


Figure 4.14: Effect of sub-acute administration of soya oil, combination of chlorpyrifos and cypermethrin and/or vitamin C on serum glucose level of Wistar rats (n= 10).

4.7.2 Effect of Sub-acute Co-administration of Chlorpyrifos and Cypermethrin and/or Vitamin C on Serum Electrolytes Concentration.

The effect of the various treatments on some serum electrolyte concentration is shown in Figure 4.15. There was no significant difference ($P > 0.05$) in the serum concentrations of K^+ and Na^+ in between the groups. Nevertheless, the Na^+ level in the CPF+CYP group was comparatively higher than the S/oil (4.3%) group while it was comparatively lower than the VC (0.7%) or CPF+CYP+VC (0.2%) groups. The K^+ level in the CPF+CYP group was comparatively higher than the S/oil (0.3%), VC (12.7%) or CPF+CYP+VC (18.7%) groups.

4.7.3 Effect of Sub-acute Co-administration of Chlorpyrifos and Cypermethrin and/or Vitamin C on Serum Proteins

The effect of various treatments on serum levels of total protein, albumin and globulin concentrations in all experimental groups is shown in Figure 4.16. There was no significant difference ($P > 0.05$) in the serum protein concentrations in between the groups. However, the serum total protein concentration in the VC group was comparatively higher than the S/oil (4.9%), CPF+CYP (3.8%) or CPF+CYP+VC (4.7%) groups. Similarly, the serum globulin concentration for the VC group was comparatively higher than the S/oil (9.5%), CPF+CYP (3.9%) or CPF+CYP+VC (6.0%) groups. However, the serum albumin concentration in the CPF+CYP group was comparatively lower than the S/oil (2.3%) or VC (3.7%) group but comparatively higher than the CPF+CYP+VC (0.34%) group.

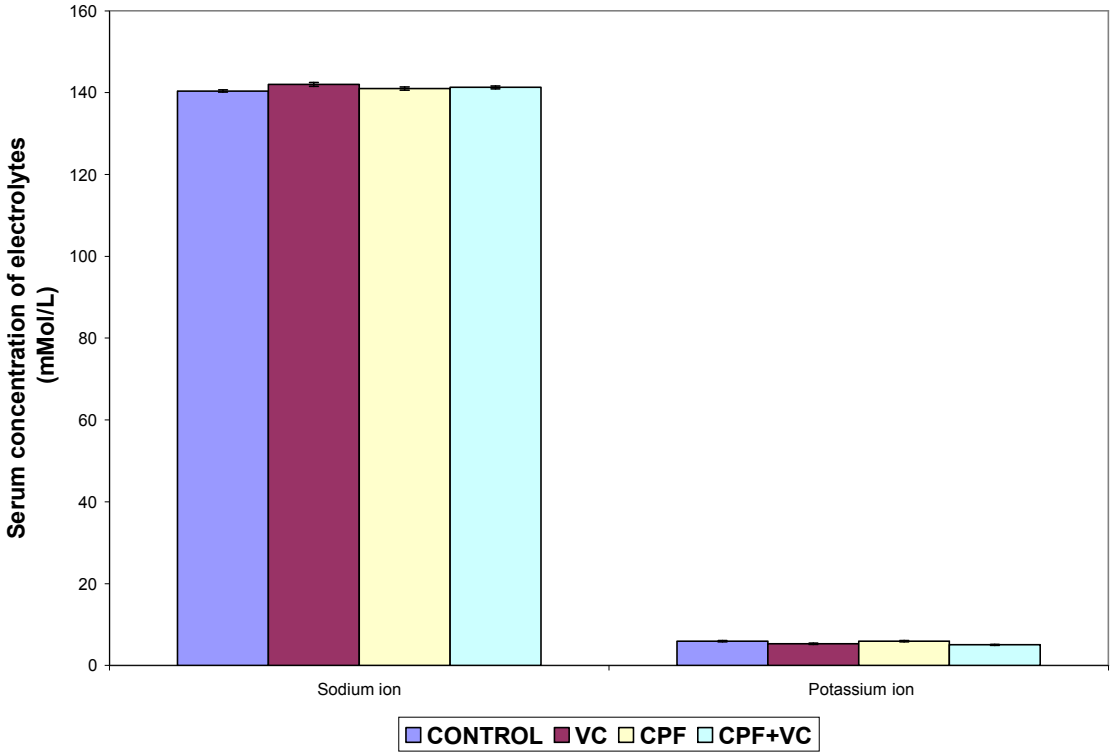


Figure 4.15: Effect of sub-acute administration of soya oil, combination of chlorpyrifos and cypermethrin and/ or vitamin C on serum electrolytes level of Wistar rats (n= 10).

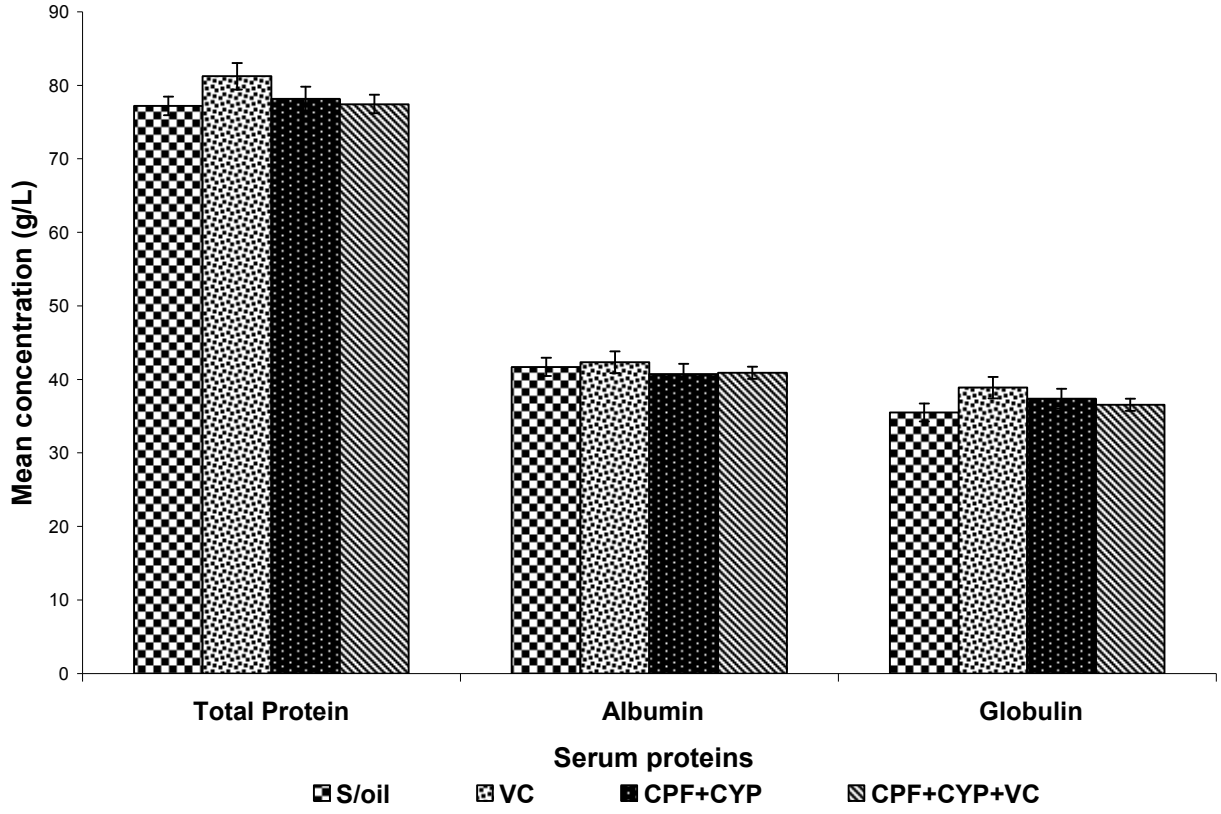


Figure 4.16: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on serum protein levels in Wistar rats (n= 10).

4.7.4 Effect of Treatments on Serum Uric acid, Urea and Creatinine levels of Wistar Rats

The serum concentrations of uric acid in between the groups were not significantly different ($P > 0.05$). However, the serum uric acid concentration in CPF+CYP group was comparatively lower than S/oil (2.8%) or CPF+CYP+VC (14.2%) group but higher than that recorded in VC group (11.0%) (Table 4.4).

The serum urea concentrations in between the groups were not significantly different ($P > 0.05$). However, the serum concentration of urea in CPF+CYP group was comparatively higher than the S/oil (11.0%), VC (24.0%) or CPF+CYP+VC (22.0%) group (Table 4.4).

The serum creatinine concentration in between the test groups did not differ significantly ($P > 0.05$) from those of the control. However, the serum concentration of creatinine in CPF+CYP group was comparatively higher than the S/oil (2.5%), VC (2.5 %) or CPF+CYP+VC (3.8%) groups (Table 4.4).

Table 4.4: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on serum uric acid, urea and creatinine levels in Wistar rats (Mean±SEM, n= 10).

Parameter	Soya oil (mMol/L)	Vitamin C (mMol/L)	CPF+CYP (mMol/L)	CPF+CYP+VC (mMol/L)
Uric acid	89.8 ± 5.1	78.6 ± 3.7	87.3 ± 5.9	101.8 ± 9.1
Urea	5.9 ± 0.3	5.9 ± 0.3	7.3 ± 0.2	6.0 ± 0.6
Creatinine	79 ± 3.3	79 ± 3.6	81 ± 5.2	78 ± 3.6

4.7.5 Effect of Sub-acute Co-administration of Chlorpyrifos and Cypermethrin and/or Vitamin C on Serum Creatine Kinase Activity

The effect of the various treatments on creatine kinase (CK) concentration is shown on Figure 4.17. The serum CK activities in between the groups were not significantly different ($P > 0.05$). However, the serum CK activity in CPF+CYP group was comparatively higher compared to the S/oil (2.0%), VC (12.5%) or CPF+CYP+VC (8.9%).

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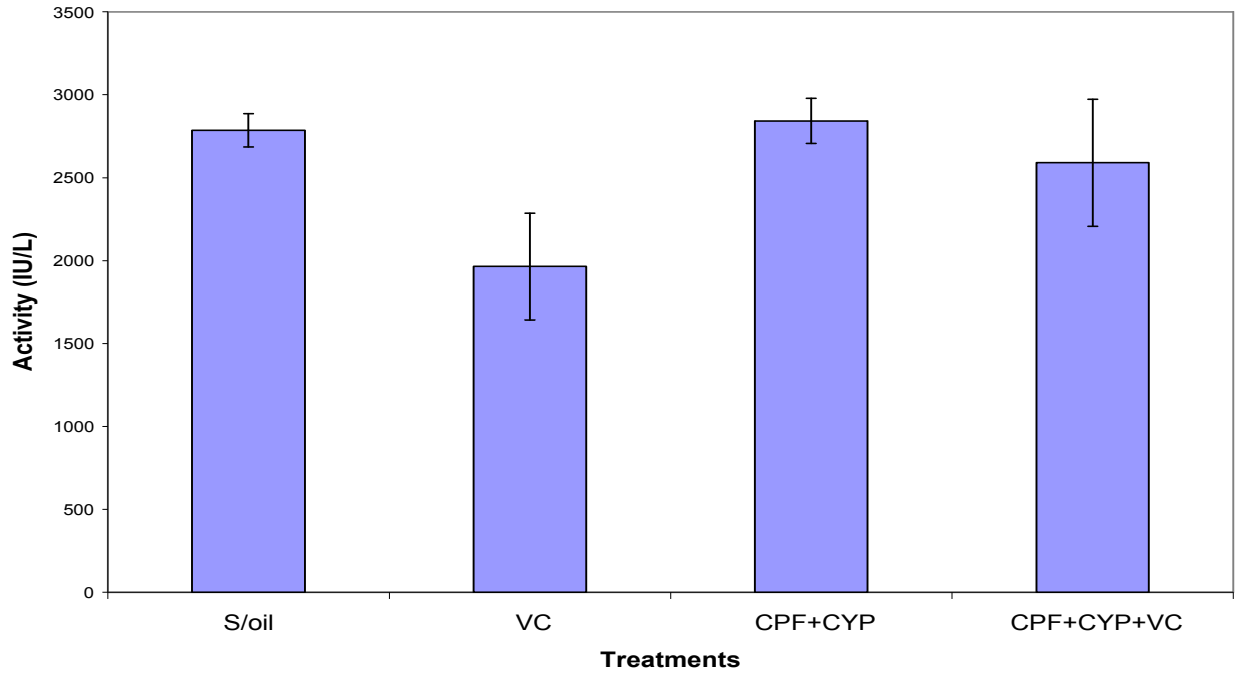


Figure 4.17: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on serum creatine kinase activity in Wistar rats (n= 10).

4.7.6 Effect of Sub-acute Co-administration of Chlorpyrifos and Cypermethrin and/or Vitamin C on Serum Liver enzymes

The serum AST activity in the all the groups was not significantly different ($P > 0.05$). However, the serum AST activity in CPF+CYP group was comparatively higher compared to the other groups: S/oil (2.1%), VC (9.4%) or CPF+CYP+VC (7.5%) (Figure 4.19).

The serum ALT activity in the all the groups was not significantly different ($P > 0.05$). However, the serum ALT activity in CPF+CYP group was comparatively lower compared to the other groups: S/oil (4.6%), VC (2.5%) or CPF+CYP+VC (2.2%) (Figure 4.19).

The serum ALP activity in the all the groups was not significantly different ($P > 0.05$). However, the serum ALP activity in CPF+CYP group was comparatively higher compared to the other groups: S/oil (20.2%), VC (7.4%) or CPF+CYP+VC (20%) (Figure 4.19).

The serum LDH activity in the all the groups was not significantly different ($P > 0.05$). However, the serum LDH activity in CPF+CYP group was comparatively higher compared to the other groups: S/oil (2.4%), VC (12.5%) or CPF+CYP+VC (0.6%) (Figure 4.19).

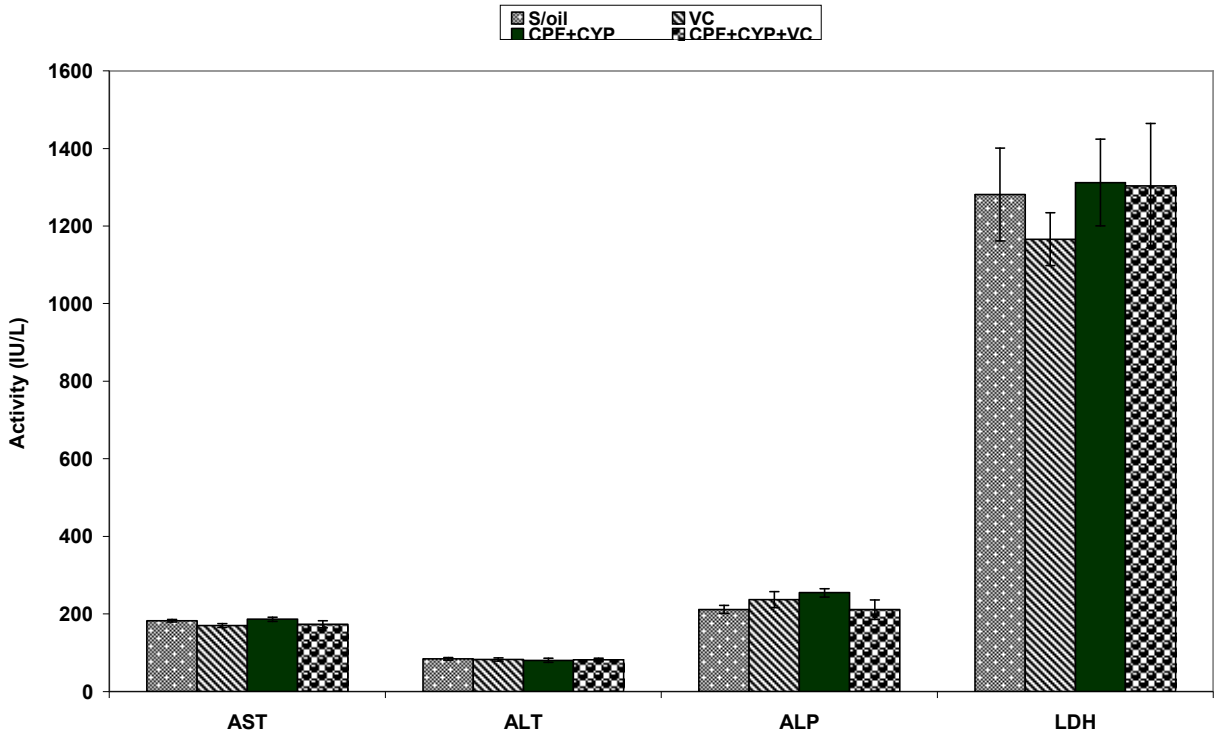


Figure 4.18: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on serum liver enzymes of Wistar rats (n= 10).

4.8 EFFECT OF SUB-ACUTE CO-ADMINISTRATION OF SOYA OIL, COMBINATION OF CHLORPYRIFOS AND CYPERMETHRIN AND/ OR VITAMIN C ON LIPID PEROXIDATION

4.8.1 Effect on Malondialdehyde Concentrations of the Liver

The effect of various treatments on lipid peroxidation in the liver is as shown in Figure 4.19. A significantly ($P < 0.05$) higher MDA concentration was observed for the CPF+CYP group (when compared with the S/oil group. However, the VC and CPF+CYP groups showed MDA concentrations that did not significantly ($P < 0.01$) differ from that of the S/oil group.

4.8.2 Effect of Treatments on Brain Malondialdehyde Concentrations in Wistar Rats

The rats in the CPF+CYP (0.032 ± 0.002 mMol/mg) group had an MDA concentration which was significantly ($P < 0.05$) higher than that of rats in the control (0.023 ± 0.005 mMol/mg) and the VC (0.022 ± 0.0022 mMol/mg) groups. Although, the concentration recorded by rats in the CPF+CYP+VC (0.025 ± 0.005) group was slightly lower than that of the CPF+CYP group they did not differ significantly (Figure 4.20).

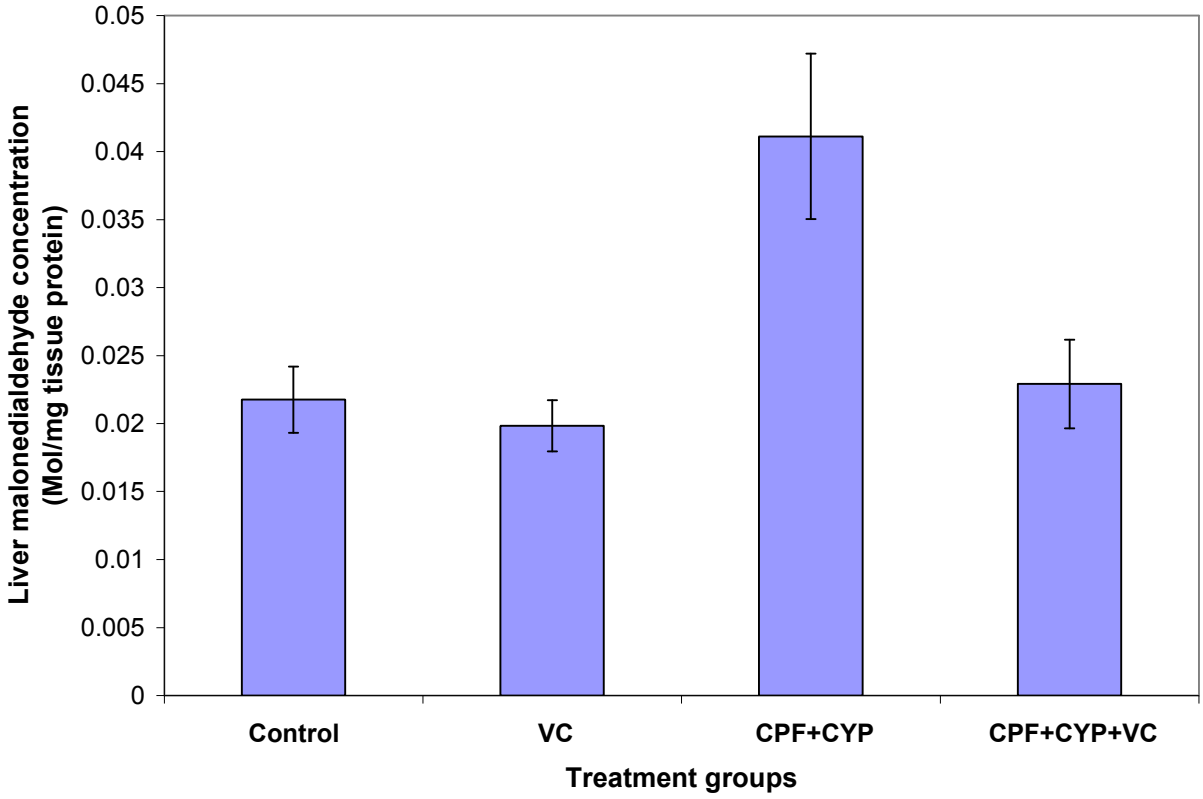


Figure 4.19: Effect of sub-acute co-administration of chlorpyrifos and cypermethrin and/or vitamin C on malondialdehyde concentration in the liver of Wistar rats (n= 10).

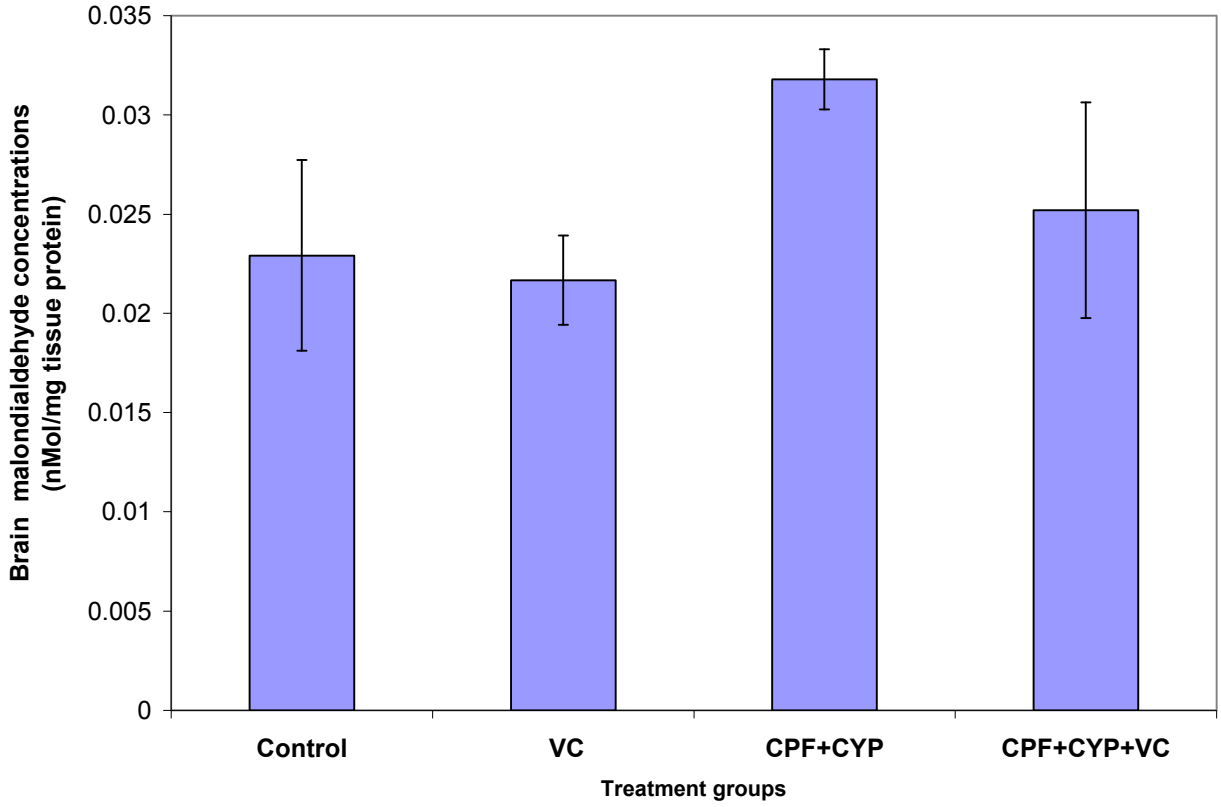


Figure 4.20: Effect of sub-acute co-administration of chlorpyrifos (CPF) and cypermethrin (CYP) and/or vitamin C (VC) on malondialdehyde concentration in the brain of Wistar rats (n= 10).

CHAPTER 5

5.0 DISCUSSION

The LD_{50} value of 225 mg/kg body weight obtained in this present study following oral administration of CYP fell within the range of 100 – 500 mg/kg body weight which was obtained following single epidermal administration in mice as reported by Luty *et al*. (1998); it was also within the range of 160 – 300 mg/kg for cis-isomer of cypermethrin (Das and Parajuli, 2005). The clinical signs observed during the LD_{50} study were typical of type II pyrethroid acute poisoning in which CYP interacts with the sodium channels, disrupting its gating kinetics thus producing long delays in sodium channel inactivation leading to a persistent depolarization of the nerve membrane without repetitive discharge, consequently, a hyperexcitable state occurs (Yousef *et al*., 2006; Nieradko-Iwanicka and Norzecki, 2008).

A median lethal dose value of 85 mg/kg body weight was obtained which actually fell within the range of 82 – 270 mg/kg as documented by Berg, (1986). According to Eaton *et al*. (2008) oral LD_{50} can range from 60 mg/kg in mice to 163 mg/kg in male Dow-Wistar rats. The clinical signs observed during the LD_{50} study demonstrate that CPF caused muscarinic, nicotinic and central cholinergic effect peculiar to organophosphate poisoning. OPs are known to interfere with the metabolism or breakdown of acetylcholine (ACh) at cholinergic sites by inhibiting the activity of acetylcholinesterase (Blodgett, 2001), found between pre-and post- ganglionic neurons of the autonomic nervous system; at the junction of post-ganglionic parasympathetic neurones in smooth muscle, cardiac muscle or exocrine glands, and at neuromuscular junctions of somatic nervous system; and in the cholinergic synapses in the CNS (Blodgett, 2001).

The generally progressive pattern in body weight gain with no significant differences in the weight gain of rats in the various groups even in those rats exposed to CPF+CYP demonstrates

that the dose administered was very well subclinical. However, the mean cumulative weight changes revealed that rats in the control and VC groups had the highest cumulative body weights while those administered with CPF+CYP recorded the lowest mean cumulative weight. This finding concurs with that of Ambali (2009), in which rats chronically exposed to CPF alone had a relatively lower body weight gain compared to the rats belonging to the control group. Similarly, some studies reported a dose-dependent change in body weight following repeated exposure to CPF, and a decline in weight gain was recorded in rats exposed to high doses of CPF alone (Terry *et al*., 2003; Ambali, 2009; Ambali *et al*., 2011a). However, a 28- day study in rats repeatedly exposed to cypermethrin alone recorded no significant differences in weight gain when compared to rats belonging to the control group (Yavasoglu *et al*., 2006). The mean cumulative weight of rats in the CPF+CYP+VC group was relatively higher than that those in the CPF+CYP group, this improvement in the vitamin C pre-treated group suggests that oxidative stress may play a role in the CPF+CYP induced changes in weight gain. Chlorpyrifos is known to be potentiated in the presence of pyrethroids, as pyrethrum has been shown to potentiate CPF by 2-7 times when they are combined (Axelrad *et al*., 2002). Thus, synergism is most likely to be involved in the relatively lower body weight recorded in the CPF+CYP group. In addition, the induction of lipid peroxidation by the two pesticides may have caused depletion of body tissue mass. Furthermore, CPF-oxon has been demonstrated to inhibit cholesterol ester hydroxylase which eliminates the normal reaction to stress (Civen *et al.*, 1977; Ambali, 2009). Therefore, the adverse effect of CPF+CYP on body weight gain observed in the present study may be due to oxidative stress and adrenal-mediated stress (Pung *et al.,* 2006). The relative improvement in the mean weight of the rats in the CPF+CYP+VC group over those in the CPF+CYP group suggests that oxidative stress plays a role in the weight changes seen in the latter group.

The fact that the dynamics of locomotor activity in the CPF+CYP group significantly decreased at day 21 compared to day 0 showed that the insecticide cocktails impaired locomotor activity. The fact that the CPF+CYP group had the lowest further showed that the insecticide combination impaired locomotor activity. Ambali (2009) had shown that chronic CPF exposure impairs locomotion activity. Farag *et al.* (2007) have shown that CYP impairs locomotion activity in the F1 progeny rats. Furthermore, CYP has also been shown to cause dyskinesia (Wolansky and Harril, 2008). The impaired locomotion activity may be related partly to the ability of CPF to evoke lipoperoxidation and decrease AChE activity in the bicep brachii (Ambali and Ayo, 2011b) leading to its structural and functional impairments of the muscle, hence impaired locomotion activity.

The dynamics of locomotion activity did not differ significantly in rats pretreated with vitamin C at day 0 compared to the other evaluation days. This clearly demonstrated the protective effect of the antioxidant vitamin. This observation partly agreed with the findings of Ambali (2009) that showed that pretreatment with vitamin C ameliorated chronic CPF-induced locomotion deficits. This protective effect of vitamin C may be due to its antioxidant and AChE restoration effects (Ambali *et al*., 2010c; Ambali and Ayo, 2011a and b).

According to Boucwknecht and Paylor (2002), rearing reflects adaptive ability of animals to explore their environment. It also reflects responses to and stress levels in rodents (Sadile, 1996). Rearing can be used to evaluate xenobiotic-induced dopaminergic interferences (Nasuti *et al*., 2007); anxiety in animals, with high frequency indicating low anxiety level (Brown *et al*., 2007; Ambali, 2009). It also measures habituation abilities in animals, cholinergic activity in the fore-brain and hippocampus (Thiel *et al*., 1998). Since dopamine is the major neurotransmitter involved in the control of the motor system (Nasuti *et al*., 2007), thus, the slight decrease in

cumulative frequency of rearing manifested by the rats in CPF+CYP group may reveal a dopaminergic impairment. Therefore, the decrease in the frequency of rearing observed in the group co-administered CPF and CYP compared to the other groups on days 14, 21 and 28 and a decrease in the frequency of rearing on days 14, 21 and 28 compared to day 0 is an indication of decreased motor response, increased anxiety and lowered habituation learning in this group. However, in the vitamin C pretreated group (CPF+CYP+VC) there was a remarkable increase in the frequency of rearing which suggests that oxidative stress has a role in the anxiogenesis induced by subacute co-administration of CPF and CYP. Similarly, vitamin C is a cofactor in the synthesis of dopamine (Naidu, 2003; Padayatty *et al.,* 2003) hence may have modulated the level of dopamine following the suspected dopaminergic impairment by either or combined effect of the insecticides.

This present study has shown a poor incline plane performance of Wistar rats repeatedly exposed to low dose of a combination of CPF and CYP. This demonstrates the ability of CPF and CYP to cause impairment to neuromuscular coordination and sensorimotor performance. Some pyrethroids (Wolansky *et al*., 2006; Wolansky and Harill, 2008) and CPF (Ambali *et al*., 2010c; Ambali and Aliyu, 2012) have been shown to impair neuromuscular coordination. This finding Dopamine is the main neurotransmitter involved in the control of the motor system (Nasuti *et al*., 2007). Pyrethroids have been shown to decrease the dopamine content in striatum by selectively inhibiting dopamine synthesis; this occurs via the inhibition of mRNA activity and protein expressions of tyrosine hydroxylase (Liu and Shi, 2006). This may be the reason for the impairment to the sensorimotor performance. Also, CYP has been reported to depress neuromuscular function (Wolansky and Harill, 2008). Abou-Donia *et al*. (2002) demonstrated poor inclined plane performance following the chronic exposure of rats to CPF. The rats in the

vitamin C pretreated group showed an improved inclined plane performance indicating that oxidative stress/lipid peroxidation played an important role in the poor inclined plane performance exhibited by co-administration of the two insecticides.

The present study has demonstrated that low doses of CPF+CYP caused profound impairment to learning and memory. The result obtained here concurs with several earlier studies involving exposure to CPF only (Prendergast *et al*., 1998; Moser *et al*., 2005; Prendergast *et al*., 2007; Ambali *et al*., 2010c; Ambali and Aliyu, 2012). Furthermore, studies have revealed persistent cognitive deficits in farmers and termiticide applicators repeatedly exposed to OPs but symptoms free (Dick *et al*., 2001; Samsam *et al*., 2005). Individually, CPF and CYP have been widely reported to cause cognitive deficits (Schuh *et al*., 2002; Carvajal *et al*., 2005; Samsam *et al*., 2005; Hossain *et al*., 2008).

The molecular basis for the neurotoxicity of pyrethroids is primarily their action on voltage-sensitive sodium channels, receptor regulated ion channels, and on subsequent neurotransmitter release (Motomura and Narahashi, 2001). Many studies have implicated other target sites such as calcium and chloride channels (Hossain *et al*., 2006). Hippocampal long-term potentiation (LTP) has been reported to be the neurochemical basis for learning and memory (Kullman *et al*., 2000; Hossain *et al*., 2008).

Similarly low-level CPF exposure has been reported to cause long term cognitive impairments (Samsam *et al*., 2005; Ambali, 2009). According to Terry *et al*., (2007), CPF-induced cognitive deficits may result from functional changes in brain cholinergic pathways resulting from alteration in bi-directional fast axonal transport. Overstimulation followed by depression of cholinergic receptors, downregulation of muscarinic receptors (Chaudhuri *et al*., 1993) or decreased white matter of the brain (which has been observed in methylparathion poisoning)

(Nagymajtenyi *et al*., 1988) following repeated exposure may be involved in memory impairment encountered following CPF exposure.

Chlorpyrifos has been reported to cause down-regulation of muscarinic and nicotinic acetylcholine receptors (Ecobichon, 2001), modulate the glutamatergic system by increasing extracellular glutamate concentrations and glutamate-mediated neurotransmission (Pavlovsky *et al*., 2003; Pung *et al*., 2006) and alter concentrations of monoamines (Pung *et* al., 2006). These include changes in dopamine uptake and turnover reported following exposures to CPF (Pung *et* al., 2006). Glutamatergic, cholinergic and monoaminergic pathways are closely connected to the hippocampus and cerebral cortex which are the seats of cognition and emotion. The hippocampus is particularly prone to reversible damage due to stress-induced release of excitatory amino acids (glutamate) (Pung *et al.*, 2006). Another possible mechanism responsible for the cognitive deficit is the effect of low doses of CPF altering the Ca^{2+}/cAMP response element binding protein (CREB), a molecule critical to brain development and cognitive function (Schuh *et al*., 2002). An elevation in the hippocampal level of CREB could have been partly responsible for the cognitive deficits observed in the CYP+CPF group in this study.

Vitamin C was able to mitigate the cognitive impairment induced by co-administration of CPF and CYP. Ambali *et al.* (2010c) and Ambali and Aliyu (2012) have shown that vitamins C and E mitigated the cognitive impairment induced by CPF due to their antioxidant and AChE restoration properties. These may have also been involved in the mitigation of cognitive impairments recorded in the present study.

The increased brain MDA concentration obtained in the CYP+CPF group in the present study, demonstrates the role of brain lipoperoxidation in the sensorimotor and cognitive impairments recorded in the insecticide treated group. CPF has been shown induce brain lipoeproxidation and

oxidative stress (Aly *et al*., 2010; Ambali *et al*., 2010c; Ambali and Ayo, 2011a; Ambali and Aliyu, 2012). This is consistent with several studies conducted using CYP or CPF only (Giray *et al*., 2001; Nasuti *et al*., 2007; Zama *et al*., 2007; Verma *et al*., 2009). The vulnerability of the brain tissues to lipid peroxidation is due to its high rate of oxidative metabolic activity, intense production of reactive oxidative metabolites, relatively low antioxidant capacity (antioxidant enzymes and glutathione) of the brain, high iron concentration and catecholamines (Evans, 1993), non-replicatory nature of neuronal cells and the high molecular surface to cytoplasm ratio (Shohami *et al*., 1997; Qureshi *et al*., 2004). In addition, the brain contains vast amounts of polyunsaturated fatty acids which is a "predilection site" for free radicals and very prone to lipid peroxidative reactions.

Lipid peroxidation occurs due to the actions of free radicals generated when xenobiotics such as CYP and CPF are been oxidatively metabolized (Giray *et al*., 2001; Verma *et al*., 2009). Free radicals (reactive oxygen and nitrosating species) are released during biodegradation and biotransformation of xenobiotics and phagocyte activation (Verma and Srivastava, 2003). Lipid peroxidation can alter deleteriously affect the membrane lipids, membrane receptors and membrane-bound enzymes, thus, altering their functions, structure and membrane fluidity and may result in altered ion flux (Halliwell and Chirico, 1993).

The slight decrease in value of MDA for the vitamin C pre-treated group (CPF+CYP+VC) shows that some level of amelioration by vitamin C occurred, that is, vitamin C reduced the level of brain lipid peroxidation. This can be explained by the scavenging actions of vitamin C at the molecular level. Vitamin C is a potent reducing agent and scavenger of free radicals in biological systems (May, 1999; Duarte and Lunec, 2005). Ascorbate undergoes two consecutive, yet reversible, one-electron oxidations to generate dehydroascorbate (DHA) and an intermediate, the

ascorbate free radical (AFR). AFR is relatively stable with a half life of 10^{-5} seconds and is fairly unreactive (Arrigoni and De Tullio, 2002).

The comparative decrease in the PCV, Hb and RBC count in the CPF+CYP group was a demonstration of the ability of the chemical cocktail to cause some level of anemia. Previous studies have shown the ability of CPF to cause anemia (Goel *et al*., 2006; Ambali *et al*., 2010a; 2011b). Similar result has been observed following CYP exposure (Manna *et al*., 2004; Sayim *et al*., 2005). Although the cause of anemia by the chemical cocktail may be speculative at this stage, studies have shown the ability of CPF to decrease iron concentration (Goel *et al*., 2006) and increases erythrocyte fragility resulting from increased lipoperoxidation of the erythrocyte membranes (Ambali *et al*. 2010a,b,c). Similarly, CYP's ability to decrease haematological parameters has been attributed to interruption with erythropoiesis or increased rate of RBC destruction (Sayim *et al*., 2005). The improvement in these hematological parameters in the VC+CPF+CYP group reflects the ability of this vitamin to ameliorate the apparent anaemia caused by this cocktail. This may be due to the ability of the vitamin to decrease CPF-induced lipoperoxidation of the erythrocyte membrane (Ambali *et al*., 2010a; 2011b) and increase iron absorption from the gut in its reduced form (Wardlaw *et al*., 1999; Iqbal *et al*., 2004).

The increase in TLC that occurred in CPF+CYP group in this study was due to neutrophilia, similar to the finding of Yousef *et al.* (2003) in which CYP was administered to rabbits for 12 weeks. These findings agree with those of Ambali (2010a), where neutrophilia was reported following CPF exposure. On the contrary, Goel *et al*. (2006) and Ambali *et al*. (2010b) reported leucopaenia due to neutropaenia following CPF exposure. This increase in leucocyte count in the present study may be an indication of an acute phase of inflammatory response in which there is an activation of defence mechanism and immune system, specifically, neutrophils are generally mobilized to all the affected tissues (Yousef *et al*., 2003), lipoperoxidation causes a generalized

undermining of cellular and organellar membranes resulting in the seepage of proteolytic enzymes from organelles such as the mitochondria, this in turn causes genralised cytotoxicity (Ambali, 2009).

The administration of VC modulated the increase in TLC induced by CPF suggesting indicating that lipoperoxidation/oxidative stress are implicated in leukocytosis evoked by CPF+CYP. Vitamin C has been shown to be actively transported into leucocytes (Heinecke, 2004), which enables neutrophils to accumulate the vitamin exceeding the plasma levels by 25 folds (Levine *et al*., 1996; Food and Nutrition Board, 2000). This therefore aid in mitigating the cellular damage to the plasma membranes of some of the organelles, especially the mitochondria preventing it from releasing the cytotoxic proteolytic enzymes that may trigger inflammatory responses.

The decrease in platelets count of the rats in the CPF+CYP group although was not significant shows that sub-acute exposure of Wistar rats to CPF+CYP caused an appreciable level of thrombocytopaenia. This finding is consistent with that of Ambali *et al.* (2011b). However, the result contradicted thrombocytosis reported following CPF exposure (Ambali *et al*., 2010a). The thrombocytopaenia may be related to CPF+CYP-induced oxidative damage to the platelet membranes. The relative improvement in the number of thrombocytes observed in the group pre-treated with VC further supports the role of oxidative stress in CPF+CYP induced thrombocytopaenia.

The higher the level of AST activity observed in rats sub-acutely exposed to CPF+CYP compared to the control revealed the ability of these pesticides to cause damage to the liver and/or muscle. However, serum AST activity is considered a less specific biomarker of liver

function compared to ALT activity. AST is released from damaged myocytes as well as hepatocytes (Ozer *et al.,* 2008). An increased AST activity in the serum is a sensitive marker of liver damage, even if the damage is of a sub-clinical nature (Meyer *et al*., 1998).

Similarly, the ALP activity which was also observed to be higher in the CPF+CYP group is said to be associated with cell membranes in multiple tissues, particularly hepatocytes (Ozer *et al*., 2008), it is primarily a marker of hepato-biliary effects and cholestasis (Ozer *et al*., 2008). The elevation of the serum ALP activity of the rats in the CPF+CYP group higher than rats of the other groups reflect the degree of damage to organs, such as liver, muscles and intestinal tissues that produce this enzyme. ALP is associated with cell membranes in mutiple tissues, particularly hepatocytes. It hydrolyzes monophosphatases at an alkaline pH. Several ALP iso-enzymes (e.g liver, bone, intestinal, kidney, and placental forms) occur in humans and preclinical species. Either some or all the organs producing ALP may have experienced various degrees of pathological changes as a result of exposure to CPF+CYP.

The low ALT activity recorded in CPF+CYP group agrees with the findings of Ambali *et al*. (2007; 2011a) in which subjects were exposed subchronically to CPF alone. Low ALT (Ambali *et al*., 2007) activity has no known toxicological implications. ALT and AST are involved in amino acids metabolism and synthesis of proteins. In dying or damaged cells, these enzymes leak into the bloodstream (Yoshida *et al*., 1985). Serum AST activity is associated with liver toxicity, but may also be elevated in association with the heart and skeletal muscle injury (Nathwani *et al*., 2005; Ozer *et al*., 2008). The high activities of AST and ALP in this study concur with the findings of Mansour and Mossa (2010), who observed increased levels of AST and ALP following low dose of CPF exposure in lactating rats. The present study shows that pretreatment with vitamin C resulted in an alleviation of AST and ALP activities. This further demonstrates

the protective effect of vitamin C on CPF+CYP induced liver and/or muscle damage.

Serum lactic dehydrogenase (LDH) activity is very valuable in the verification of skeletal muscle injury especially when correlated with serum creatinine kinase and/or aldolase activities. In this study, there was increased LDH activity for the CPF+CYP group, which may indicate the presence of skeletal muscle injury. This result was similar to those of Mansour and Mossa (2010) where LDH activity was elevated following CPF exposure. Also Manna, (2004) reported increased LDH activity following administration of alpha-cypermethrin in rats. The elevation in LDH activity induced by the pesticides cocktails was only slightly reduced by pretreatment with vitamin C.

The comparatively elevated CK activity in the CPF+CYP group agrees with the observation that repeated inhalation exposure of cats to CPF resulted in elevated CK (Jaggy and Oliver, 1990). Furthermore, a suicidal attempt with CPF equally resulted in an increased CK activity (Aiuto *et al*., 1983). However, our finding contradicts that of Ambali (2009) where a lowering of CK activity was observed. The relationship between the CK isozyme in the brain (CK-B) and those in the serum or plasma (CK-M) is not exactly known, but a study has shown that the plasma CK-M level paralleled CK activity (Wang *et al*., 2002).

CK is present in neurons, astrocytes, and oligodendrocytes (Manos *et al*., 1991) and plays a critical role in the continuous replenishment of ATP from phosphocreatine in these cells (Wyss *et al*., 1992). CK has isoenzymes such as CK-B and CK-M in the central nervous system and plasma respectively. Neurotoxic agents such as ethylene oxide and acrylamide suppress the activity of CK in the brain and blood of rats (Wang *et al*., 2002).

The apparent reduction in CK activity in VC pretreated group shows the ability of the vitamin to decrease skeletal muscle injury induced by the pesticides cocktails, partly due to its antioxidant effect.

The present study has revealed that exposure to a combination of CPF and CYP on a subacute course culminates in an increase in erythrocyte osmotic fragility (EOF) in Wistar rats, apparently due to oxidative modifications of the erythrocyte membrane (Wagner *et al*., 1988). Erythrocytes are highly susceptible to oxidative damage due to their constant exposure to high oxygen tension, high concentration of peroxidizable polyunsaturated fatty acids (PUFAs) (Kollanjiappan, 2002), furthermore, this is complicated by the innate presence of transition elements (Cu^{2+} and Fe^{2+}) and haeme proteins (cytochromes and haemoglobin) which behave as pro-oxidants. These increase the susceptibility of erythrocytes to oxidative stress by exacerbating free radical attack on their membranes resulting in their destruction (Urano, 1993; Surai, 2001; Ambali *et al*., 2010a). The consequences of the oxidative damage of the erythrocyte membrane PUFAs are the alteration of their cohesion, membrane fluidity, permeability and metabolic functioning (Chilualaif *et al.,* 2002). Due to damage Fe^{2+} ions are liberated which further propels oxidative stress in the system (Ambali *et al*., 2010a). According to Chihualaif *et al*., (2002), EOF is a good indirect index of oxidative stress. CYP and CPF have been implicated in the alteration of erythrocyte antioxidant system and increase of lipid peroxidation in erythrocytes (Atessahin *et al.,* 2005); CYP induces a significant decrease in erythrocyte membrane fluidity (Cinzia *et al*., 2003; Hu *et al*., 2008). This is particularly significant because it directly correlates to lower erythrocyte flexibility or an increased rigidity which increases the chances of haemolysis. CYP and CPF have been implicated individually in the alterations of erythrocyte antioxidant systems and increase lipid

peroxidation (Atessahin *et al*., 2005; Mansour and Mossa, 2008; Ambali *et al*., 2010a and b; Chargui *et al*., 2010).

According to Axelrad *et al*. (2002), CPF is 2-8 times potentiated in the presence of pyrethrum (pyrethroids), therefore, it is anticipated that the combination (CPF+CYP) although administered at low doses was nevertheless deleterious to erythrocytes, thus, decreasing their osmotic resistance. The remarkable improvement of the erythrocyte osmotic fragility by antioxidant vitamin C at 0.5% concentration further confirms the role of oxidative stress in the toxico-dynamics of the combination in osmotic erythrocyte damage. The ameliorative effect of vitamin C was manifested at 0.5% NaCl concentration. This agrees with the findings of Ambali (2009) and Ambali *et al*. (2010a) where supplementation resulted in a decrease of EOF following repeated CPF exposure in Wistar rats. Also, Kraus *et al*. (1997) showed that supplementation of vitamins C, E, β+-carotene decreased EOF and oxidative damage in zinc-deficient rats.

The study revealed an insignificantly lower level of uric acid in the serum of rats sub-acutely exposed to CPF+CYP compared to the control. Elevation of uric acid has been associated with obstruction of kidney since uric acid is an end product of the catabolism of tissue nucleic acid, i.e. pyrimidine and purine metabolism, and the increase in uric acid level may be as a result of overproduction or inability of its excretion by the kidney (Ambali, 2009; Mansour and Mossa, 2010). Pretreatment with vitamin C did not significantly alter the serum uric acid concentration.

It has been shown in the present study that sub-acute low-dose CPF+CYP exposure in rats marginally increased the concentration of serum urea. This finding is in agreement with the result obtained by Ambali *et al*. (2007; 2009) where renal tubular degeneration was reported following exposure to CPF. The marginal increase in serum urea concentration in rats exposed to CPF+CYP indicates kidney damage. Yousef *et al*. (2003) who administered CYP alone to rats

also reported that an increased serum urea concentration is associated with renal dysfunction. The low urea concentration observed in the serum of rats pretreated with antioxidant vitamin C showed the ability of these vitamins to ameliorate kidney damage induced by subacute CPF+CYP exposure. This further emphasized the role of oxidative stress in CPF+CYP-induced kidney damage and the ameliorative effect of antioxidant vitamin C.

Serum creatinine was significantly elevated in rats exposed to subacute CPF+CYP compared to the control. The elevation of plasma levels of creatinine are considered as significant markers of renal dysfunction (Yousef *et al*., 2003). This observation agreed with the finding of Ambali *et al*. (2007) and Ambali (2009). The elevation of creatinine may be as a result of pathological lesions in the muscle (Quintanilla, 1982) and kidneys, since elevated creatinine has been shown to be associated with renal insufficiency and renal obstruction (Ambali *et al*., 2007). However, pretreatment with vitamin C caused lowered level of creatinine and may reflect its protective effect on oxidative damage to the muscle and/or kidneys caused by the insecticides, apparently due to its antioxidant effect.

In the present study, the serum glucose obtained was lower than the control. This contradicted the finding of Ambali, (2009). According to Rahimi and Abdollahi (2007), many OP insecticides have been shown to provoke hyperglycemia. However, Rezg *et al*. (2006) reported hypoglycaemia induced by OPs. Rodrigues *et al*. (1986) reported that the hyperglycaemic effect of some OPs is temporary as the gradual increase may be followed by a decrease in blood glucose that can even reach hypoglycaemia. A study has shown that sub-chronic effect of Ops has effect on insulin expression and secretion in favour of increased insulin levels (Pournourmohammadi *et al*., 2005), which may be responsible for the apparent decrease in blood

glucose concentration observed in the CPF+CYP group. Pretreatment using VC however did not ameliorate the apparent hypoglycaemia.

The present study showed insignificant differences in the levels of total serum protein present in rats subacutely exposed to low-dose of CPF+CYP when compared to other groups. This finding contradicted the results obtained by Ambali *et al*. (2007) in mice who observed high level of serum proteins following exposure to CPF attributed to diarrhea. The present study also revealed a higher level of serum total protein in VC than all other treatment groups; this reflects the ability of VC to synthesize proteins through hydroxylation of proline and lysine (Ganong, 1999). This high level of total protein was also obtained by Yousef *et al*. (2003) who experimented with isoflavones, thus, it boosts the total protein level.

In the present study, the serum globulin level was highest for the VC group; this reveals the immune-boosting capacity of VC, also, the relatively high globulin level observed for the CPF+CYP group was due to the antigenic nature of the combination which tends to elicit immunologic response *in vivo* and thus induces increased production of gamma-globulins which tend to increase the circulating globulin level, this particular observation agrees with the result obtained by Mansour and Mossa, (2010) in which CPF alone elicited the production of a significantly high globulin level in rats .

The present study revealed a marginally lower level of serum albumin in the CPF+CYP group as compared to the control. The major binding protein found in the rat plasma has been found to be albumin (Tarhoni *et al*., 2007). This result contradicts the findings of Ambali *et al*. (2007) in mice who observed high level of serum proteins following exposure to CPF attributed to diarrhoea. Both CPF and CYP have been implicated in the lowering of serum proteins particularly albumin (Patil *et al*., 2003; Yousef *et al*., 2003; Mansour and Mossa, 2010). Albumin

has been shown to bind and, subsequently, hydrolyse CPF-oxon and parathion (Ortigoza-Ferado *et al.,* 1984). The ability of albumin to not only bind OP but also scavenge for OP molecules so that they are less available for AChE inhibition makes them a suitable biomarker for OP poisoning (Peeples *et al*., 2005). The marginal decrease in the serum albumin level observed in this present study may partly be attributable to the binding of CPF and/ or CYP to albumin and thus reducing the availability of its freely circulating forms. Also, due to the potentiation of CYP by CPF via the inhibition of esterases that are responsible for metabolizing CYP, there is a synergy which easily would result in liver damage and therefore negatively affect free amino acid metabolism and their synthesis in the liver (Rahman *et al*., 1990; Yousef *et al*., 2003). In addition, nephrosis, increase in proteolytic activity or degradation may be responsible for the reduced albumin level (Yousef *et al*., 2003).

Despite the pretreatment with VC, the serum albumin level was not alleviated. The reason for this has not been fully comprehended.

CHAPTER 6

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

The present study has shown that sub-acute co-exposure of Wistar rats to CPF and CYP caused:

1. Poor weight gain compared to the soya oil-treated control.
2. Impaired motor activity (locomotion and rearing).
3. deficits in sensorimotor performance and neuromuscular coordination.
4. impairment in cognition (learning and memory).
5. reduction in haematological parameters such as PCV, Hb and total erythrocyte count. It also caused marked leucopaenia due to neutrophilia.
6. a decline in the platelets count (thrombocytopaenia).
7. increased erythrocyte osmotic fragility which made the RBC susceptible to lysis.
8. decrease in the level of serum protein- albumin, and while serum globulin level was relatively unaffected.
9. slight increase in serum urea and creatinine concentration resulting from an early muscle and renal damage.
10. increased activity of some serum enzymes such as AST, ALP and LDH which reflected some level of hepatic, skeletal and/ or intestinal tissue damage.

The study has also shown that pretreatment with antioxidant vitamin C improved the body weight changes, and ameliorated deficits in neurobehavioural and cognitive changes induced by sub-acute co-exposure to CPF and CYP. Furthermore, the antioxidant vitamin C to a varying degree mitigated CPF+CYP-induced deficits in PCV, Hb, RBC and WBC. This study has for the first time revealed the deleterious effects of combining CPF with CYP even at clinically sub-

lethal doses. The combination had a far reaching effect on various organ-systems even though the co-exposure period was subacute (28 days) in the Wistar rats. Also, the study also for the first time revealed the ability of vitamin C to protect the integrity of RBC membrane as observed in amelioration of the CPF+CYP-induced increased erythrocyte osmotic fragility. Similarly, pretreatment with vitamin C protect against CPF+CYP-induced hypoalbuminaemia. The study has also for the first time shown the ability of vitamin C to ameliorate the toxicity due to CPF+CYP. The antioxidant vitamin C was also observed to ameliorate CPF+CYP-induced increased activity of serum AST, ALP and LDH levels. This indirectly indicates that one of the principal mechanisms of toxicosis of this combination is via invocation of lipid peroxidation/ oxidative stress in various tissues in Wistar rats.

6.2 CONCLUSIONS

In conclusion, the study has shown that:

i. Sub-acute co-exposure of Wistar rats to CPF and CYP caused varying percentages of pathological alterations in neuro-behavioural, clinical, haematological and biochemical parameters in Wistar rats.

ii. Besides the typical modes of action of these pesticides, lipid peroxidation seem to be an important molecular mechanism involved in the neurobehavioural, cognitive, haematological and biochemical pathologies observed due to sub-acute co-exposure to low-doses of CPF+CYP in rats.

iii. Pretreatment with antioxidant vitamin C mitigated CPF+CYP-induced clinical, neurotoxicological, haematologic and biochemical pathological changes in rats.

6.3 RECOMMENDATIONS

6.3.1 Specific Recommendations

i. Further research should be done specifically on the chemo-interactive dynamics of CPF+CYP which will monitor these compounds in the rats' organ-systems.

ii. A more detailed and thorough study is required on the antioxidant status of the animals exposed to this combination; that is, endogenous antioxidant enzymes, vitamins and other antioxidant molecules should be assessed for.

iii. Sub-chronic and chronic toxicity studies of CPF+CYP should be carried out, because this will most likely reflect the reality of exposure of pesticide applicators and it will definitely confirm some uncertainties with respect to mild pathological alterations caused by the sub-acute study.

iv. Further research should be done to identify specific biomarkers involved in the toxicology of this combination. The effect of the combination on the plasma AChE activity should be compared to the effect that each of the pesticides will have on the enzyme; this will further consolidate the theory of synergism of the combination.

v. The effect of CPF+CYP on more organ systems should be investigated and supported with histopathology.

vi. More antioxidant vitamins should be experimented with in the search for an excellent therapeutic or mitigating agent.

6.3.2 General Recommendations

i. The use of a plethora of pesticide protection should be encouraged by the stake holders such as farmers, pesticide applicators, veterinary attendants, laboratory attendants, veterinarians. Direct contact with pyrethroid and OP compounds should be strictly avoided.

ii. Rural extension workers should be used to educate illiterate heavy pesticide users and farmers in the rural areas and suburbs on the dangers of irresponsible and careless pesticide use.

iii. Government and non-governmental organizations and individuals should organize awareness campaign and sensitization of the general populace on the danger of indiscriminate use of pesticides.

iv. Livestock and arable crop farmers should be sensitized on the need to observe withdrawal periods stated by the manufacturers before turning pesticide-treated products into the market for human and animal consumption.

v. Government should have a political will and put policy in place measures that will check importation of dangerous and banned pesticides into the country through effective border policing and control. In addition, pesticides that have instructions on their labeling in foreign languages other than English language should not be allowed into the country.

vi. Periodic evaluation of haematological and biochemical parameters of individuals or animals who are frequently exposed to low-dose CPF+CYP should be put in place, to enable professional counselling of the individual as it relates to exposure.

vii. Individuals who are at risk of coming in contact with CPF and CYP continuously should be pretreated with the cheap and readily available antioxidant vitamin C to reduce damages caused by frequent exposure to low-dose exposure.

viii. The Nigerian government should put in place effective regulatory and legal framework through the enactment of pesticide control Act that will check the toxicological and environmental menace caused by pesticides.

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APPENDIX 1

Effects of treatments on Locomotion

Time	Soya oil (no. of squares crossed)	VC (no. of squares crossed)	CPF+CYP (no of squares crossed)	CPF+CYP+VC (no of squares crossed)
Day 0	22.10 ± 3.58	19.10 ± 3.58	21.20 ± 4.28	19.60 ± 4.15
Day 7	16.70 ± 3.78	20.80 ± 2.47	7.50 ± 2.41	16.50 ± 5.97
Day 14	15.40±3.65	16.50±2.54	7.30 ± 2.75	12.60 ± 2.92
Day 21	15.20 ± 3.89	15.20 ± 3.45	4.80 ± 3.26	12.63 ± 4.01
Day 28	16.89 ± 3.67	5.33 ± 3.59	14.29 ± 4.22	14.67 ± 6.53

APPENDIX 2
Effect of treatments on the frequency of Rearing

Time	Soya oil	VC	CPF+CYP	CPF+CYP+VC
Day 0	4.8 ± 1.04	4.2 ± 0.98	4.8 ± 0.98	4.7 ± 0.75
Day 7	4.5 ± 0.79	3.7 ± 0.80	2.2 ± 0.63	3.8 ± 0.71
Day 14	4.7 ± 0.75	3.9 ± 0.57	1.60 ± 0.40	3.4 ± 0.64
Day 21	4.1 ± 0.48	4.1 ± 0.62	0.67 ± 0.44	4.125 ± 0.64
Day 28	4.4 ± 0.40	3.8 ± 0.93	1.5 ± 0.68	3.556 ± 0.69

APPENDIX 3
Effects of treatments on cognition

Parameters	Soya oil	VC	CPF+CYP	CPF+CYP+VC
Learning (number of foot shocks)	1.20 ± 0.13	1.20 ± 0.13	2.13 ± 0.44	1.22 ± 0.15
Memory (Latency on platform in seconds)	106.40 ± 9.13	107.7 ± 7.89	85.75 ± 13.39	103.4 ± 7.368

APPENDIX 4
Effect of treatments on haematological and biochemical changes in Wistar rats

Parameters	Soya oil	VC	CPF+CYP	CPF+CYP+VC
PCV (%)	44.53 ± 0.94	47.72 ± 1.07	44.36 ± 0.66	47.09 ± 1.06
RBC (x10^{12}g/dL)	8.49 ± 0.21	9.02 ± 0.21	8.44 ± 0.26	8.31 ± 0.23
Hb (g/dL)	14.28 ± 0.24	14.89 ± 0.39	13.84 ± 0.46	14.27 ± 0.38
WBC (x 10^6 g/Dl)	5.91 ± 0.19	5.80 ± 0.28	6.25 ± 0.53	5.80 ± 0.37
Glucose (g/dL)	5.45 ± 0.34	5.00 ± 0.32	4.84 ± 0.35	4.70 ± 0.24
Na^+ (mMol/L)	140.4 ± 0.27	142.0 ± 0.47	141.0 ± 0.38	141.3 ± 0.33
K^+ (mMol/L)	5.93 ± 0.16	5.28 ± 0.14	5.95 ± 0.18	5.01 ± 0.10
Total protein (g/dL)	77.20 ± 1.28	81.22 ± 1.67	78.13 ± 1.80	77.44 ± 1.26
Albumin (g/dL)	41.70 ± 1.22	42.33 ± 1.34	40.75 ± 1.46	40.89 ± 0.82
Globulin (g/dL)	35.5 ± 1.22	38.89 ± 1.34	37.38 ± 1.46	36.55 ± 0.82
Urea (mMol/L)	6.60 ± 0.19	5.94 ± 0.29	7.25 ± 0.16	6.00 ± 0.55
Creatinine (mMol/L)	79.20 ± 3.34	77.56 ± 2.79	83.25 ± 5.47	78.33 ± 3.61
Uric acid (mMol/L)	89.80 ± 5.19	78.56 ± 3.71	98.75 ± 5.94	88.67 ± 7.17
AST (IU/L)	182.40 ± 3.48	170.30 ± 5.08	186.30 ± 5.58	173.30 ± 8.72
ALT (IU/L)	84.10 ± 2.98	82.25 ± 4.23	80.22 ± 5.23	82.00 ± 3.39
ALP (IU/L)	211.8±10.62	237.0±20.57	254.5±10.72	211.3±25.19
LDH(IU/L)	1281 ±119.8	1166 ± 68.30	1312 ± 111.8	1304 ± 160.7
CK (IU/L)	2785 ± 100.7	1964 ± 322.0	2842 ± 136.0	2590 ± 382.5
Brain MDA (nmol/mg tissue protein)	0.023 ± 0.005	0.032 ± 0.002	0.022 ± 0.002	0.025 ± 0.005

APPENDIX 5
Open-field assessment apparatus



APPENDIX 6

Incline plane apparatus



APPENDIX 7
Step-down inhibitory avoidance learning instrument



APPENDIX 8
Step-down inhibitory avoidance learning instrument with a voltage regulator

