TITLE PAGE

RISK ASSESSMENT OF DIFFERENT ROUTES OF EXPOSURE TO ORGANOPHOSPHATE PESTICIDE (CHLORPYRIFOS 40% EC) USING ANIMAL MODEL

A THESIS SUBMITTED IN PARTIAL FULFULMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE (MSC) IN BIOCHEMISTRY (ENVIRONMENTAL AND INDUSTRIAL TOXICOLOGY), UNIVERSITY OF NIGERIA, NSUKKA.

BY
AMADI, EUSTACE UGOCHUKWU
PG/MSC/17/00448

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF BIOLOGICAL SCIENCES
UNIVERSITY OF NIGERIA, NSUKKA

SUPERVISOR: DR. UBANI, CHIBUIKE SAMUEL,

FEBRUARY, 2020

CERTIFICATION

Amadi, Eustace Ugochukwu, a postgraduate student of the Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria Nsukka, with Registration Number PG/MSc/17/00448, has satisfactorily completed the requirements for the award of the degree of Master of Science (M.Sc.) in Biochemistry (Environmental and Industrial Toxicology). The work embodied in this dissertation is original and has not been submitted in part or full for any other diploma or degree of this or any other University.

DR. C. S. UBANI
(Supervisor)

PROF. H. A. ONWUBIKO
(Head of Department)

EXTERNAL EXAMINER

DEDICATION

The work is dedicated to all young scientists striving to make an impact with limited resources.

ACKNOWLEDGEMENT

I wish to thank my Supervisor Dr. C. S. Ubani for his support, direction and constructive criticism towards the success of this work.

I am immensely grateful to my benefactor Mr. John Ogbata for his unwavering financial support and encouragement throughout this program, may God bless your Sir.

I also thank the Head, Department of Biochemistry, Prof. H. A Onwubiko, the PG Coordinator, Dr. P.E Joshua, all my distinguished Lecturers, Dr. Emeka Asogwa and my Senior Colleagues Mr. Ekene Nweze, Mr. Innocent Okagu, Mr. Collins Oguh, Mr. Ogugofor Martins, Mr. Prosper, Mr. Emeka Okwaraji, for all your supports, I am grateful.

To my friends and colleagues who supported and encouraged me during this work, Mr. Ozioko Chiedozie, Mr. Ugwu Christopher, I say thank you.

To my beloved Family, for all your support, prayers and admonitions, I love and cherish you.

Above all, to the almighty God, you are my all in all.

ABSTRACT

The loss of crops due to pest, plant disease and competition from weeds is very high. To avert or remedy these losses, farmers now increasingly deploy pesticides in their agronomic practices. This research aimed at assessing the risk assessment of the different routes (oral and inhalation) of exposure to chlorpyrifos an organophosphate pesticide and measurement marker parameters of the organs susceptible to chlorpyrifos toxicity using animal model. A total of 64 male wistar rats were used for the experiment. The animals were divided into two groups for oral (36 rats) and inhalation (28 rats) routes of exposure. The oral experimental groups consists of 20 animals, divided into 4 groups of 5 animals each and the inhalation experimental groups consists of 16 animals divided into 4 groups of 4 animals each. The four experimental groups in both routes of exposure consist of 3 treatment groups and a control group. Acute toxicity studies and the median lethal dose were carried out using a modified method. Colorimetric Method was used to determine hepatotoxicity of aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP). Standard methods were used to determine oxidative stress markers of lipid peroxidation, catalase activity and glutathione peroxidase activity. Immunoassay test kit was used to carry out the reproductive toxicity studies**.** Docking of the ligands was carried out using Autodock Vina. Standard formulae were used to estimate the risk assessment study. The results from the 24 hour acute toxicity studies revealed that oral exposure to pesticide gave a median lethal dose (LD_{50}) of 155 mg/kg b.w. while inhalation exposure gave a median lethal concentration (LC_{50}) of 1414 mg/kg b.w. for 60 minutes. The hepatotoxicity studies showed that oral exposure to Chlorview® led to a significant ($p<0.05$) increase in the activities of AST, ALT and ALP when compared to the control. Oxidative stress markers (GSH, MDA, CAT and GPx) show that the pesticide induced appreciable oxidative imbalance in the system. In oral exposure, there was a significant ($p<0.05$) increase in group 3 CAT activity when compared to control. The reproductive toxicity studies revealed that that oral exposure to pesticide led to a significant ($p<0.05$) increase in the testicular Cholesterol and a significant ($p<0.05$) decrease in testosterone and sperm count when compared to control. Molecular Docking Study shows the binding affinities of a standard ligand (Mevastatin) and chlorpyrifos docked against the binding site of β-keto acyl ACP synthase, HMG CoA synthase and the rate limiting enzyme in cholesterol synthesis pathway HMG CoA reductase revealed that chlorpyrifos potentiates the key enzymes resulting in cholesterol production. The risk assessment studies show a subchronic toxicity of chlorpyrifos at the dose of 15.5 mg/kg b.w of chlorpyrifos accumulation was recorded for oral exposure. This research concludes that exposure to pesticides can pose a reasonable risk to human health both through oral and inhalation routes of exposure which affect many biochemical processes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ach -	Acetylcholine
AChE -	Acetylcholinesterase
ACP -	Acyl Carrier Protein
ADD -	Average Daily Dose
ADI -	Acceptable Daily Intake
ALP -	Alkaline Phosphatase
ALT -	Alanine Aminotransferase
ANOVA –	Analysis of Variance
AST -	Aspartate Aminotransferase
BAF -	Bioaccumulation Factor
CAT -	Catalase
ChEs -	Cholinesterase
CPF -	Chlorpyrifos
DDTs –	Dichloro-diphenyltrichloroethane
DI -	Daily Intake
DILI -	Drug Induced Liver Injury
EC -	Emulsifiable Concentration
GGT -	γ-glutamyltransferase
GPx -	Glutathione Peroxidase
GR -	Glutathione Reductase
GSH -	Reduced Glutathione
GSSH –	Oxidized Glutathione
HCHs –	Hexachloro-cyclohexane
HMG-CoA -	ß-hydroxy-ß-methylglutaryl-Coenzyme A

HQ - Hazard Quotient

IGRs - Insect Growth Regulators

LC_{50} - Median Lethal Concentration

LD_{50} - Median Lethal Dose

LOAEL - Lowest Observable Adverse Effect Level

MDA - Malondialdehyde

MPI - Maximum Permissible Intake

NOAEL – No Observed Adverse Effect Level

NTE - Neuropathy Target Esterase

OP - Organophosphate

PCBs - Polychlorinated Biphenyls

PON 1 – Paraoxonase 1

RFD - Reference Dose

ROS - Reactive Oxygen Species

SOD - Superoxide Dismutase

TCP - 3,5,6-trichloro-2-pyridinol

CHAPTER ONE

INTRODUCTION

In Nigeria, the loss of crops due to pest, plant disease and competition from weeds is alarming. To avert these losses, farmers now increasingly deploy pesticides in their agronomic practices. Pesticides are chemical substances used in agricultural practices to aid the production and yield by repelling, preventing, and destroying pests (Oguh *et al.*, 2019a). They may also be used as insect repellants that are directly applied to the skin or clothing (United States Environmental Protection Agency, 2018). The dramatic increase in use of pesticides for agricultural, industrial, and household purposes over the past several decades has created a growing concern about this class of chemicals. Pesticide residues are now among the most ubiquitous synthetic chemicals in our environment, detectable in the tissues of humans, animals, aquatic life, and wildlife worldwide (Milatovic *et al*., 2006). Its primary purpose is the protection of agricultural products against the action of pests. The continuous application of synthetic pesticides in agriculture over the years has caused accumulation of pesticide residues such as heavy metals in the environment leading to various chronic illnesses and toxicity to non-target organisms (Oguh *et al*., 2019b). Chemicals assault or enter the body at almost every hour of the day. They may come through air, food, products use on the body, and in drinking water. Toxic buildup of these chemicals has been shown to cause several damages in the body and minimize health. Many modern pesticides (synthetic) persist in soil for years and compound the store of toxins such as heavy metals and other metabolites in the soil, air and water (Farahat *et al*., 2010; Oguh *et al.*, 2019a).

Organochlorines are a class of pesticide used in agricultural and house hold pest control. The use of organochlorines have been banned or restricted in most developed countries but are still being used in some countries (eg. dichlorodiphenyltrichloroethane (DDT) for Malaria control and benzenehexachloride (BHC) as lindane insecticide). Ninety percent of the applied synthetic pesticides like Dichlorodiphenyltrichloroethane (DDT) enter the various environmental resources as a result of run-off, exposing the farmers as well as consumers of the agricultural produce to severe health issues and sometimes take decades for some of these chemicals to break down. The accumulation of pesticides in low concentration in the body fat of mammals (through food chain) may pose health problems in the long run. Literatures have been reviewed regarding the tissue concentration of pesticide residues/metabolites in fishes. The data indicating that fish, birds, reptiles, mammals and other species inhabiting in polluted environments with number of known

and unknown synthetic compounds suffer from reproductive problems has been reported (Singh and Singh, 2008).

Potential adverse health impacts of organophosphate (OP) pesticide exposures are an ongoing public health concern for agricultural workers in low-middle income countries, particularly in regard to acute health effects. An increasing number of studies have identified neurobehavioral effects associated with occupations involving chronic or subchronic exposures to OP pesticides that did not cause systemic toxicity (Farahat *et al*., 2010). Organochlorine and organophosphate insecticides are persistent organic pollutants displaying high fish toxicity and bioaccumulation potential. These pollutants after biomagnifications in aquatic food web finally reach human beings and may cause reproductive problems. Several experiments on the exposure of organochlorine pesticide in fish have been carried out and recorded that the toxic substances affect the fish in several ways like inhibition of lipid metabolism, steroidogenesis, and reproductive failure and also as indicator of pesticide contamination (Singh and Singh 2008). Chlorpyrifos affects the nervous system by reversibly inhibiting the activity of cholinesterase (ChE), an enzyme necessary for the proper functioning of the nervous system (Smegal, 2000). This work was designed to assess the risk of exposure to pesticides.

1.1 Pesticides

The variety of pesticides available today is much greater than it was 20 years ago. It includes some made from bacteria, insect-killing fungi or viruses; products such as insecticidal soaps that kill by physical processes; and products like the clay-based deterrents that don't directly kill insects, but protect plants. Most of the insecticides in common use today are toxic to people as well as well as insects, although the degree of toxicity to people depends on the dose of the material and the mechanism of action, among other factors. Some toxicants affect the nervous system. Others affect water balance, oxygen metabolism, an insect's molting or maturation process, or other aspects of physiology. Some of the newer insecticides have toxicity mechanisms that scientists don't fully understand. New materials are being synthesized and tested constantly (Alan, 2017).

1.1.1 Classes of Pesticides

Organophosphates (OP) Chlorpyrifos and malathion are organophosphates. They interfere with the transmission of nerve impulses. Their point of action is the synapse, the tiny gap between one

nerve fiber and the next. Nerve impulses jump such gaps with the aid of chemicals called neurotransmitters. Enzymes normally destroy these chemicals immediately after the nerve impulse crosses the gap (Alan, 2017).

Carbamates Carbaryl, bendiocarb, and propoxur are examples of carbamates. Like the organophosphates, carbamates act as cholinesterase inhibitors, and insects exposed to them show similar symptoms. Unlike the OP's, the action of carbamates can be reversed (Alan, 2017).

Pyrethroids Permethrin, cypermethrin, fenvalerate and cyfluthrin are all examples of pyrethroids. The first pyrethroids were synthesized by chemists who studied the structure of insecticidal pyrethrins, chemicals found in the seeds of certain chrysanthemums. Pyrethroids last longer than their natural counterparts and seem to have a similar mode of action, disrupting the normal transmission of nerve impulses by affecting the potassium or sodium ion channel in nerve cells (Alan, 2017).

Insect Growth Regulators (IGRs) Methoprene and pyriproxifen are examples of IGRs. Insects go through a process of growth and development that necessitates molting, a process of shedding the "skin" and growing a new one. Some molts also result in a change in form — a caterpillar changes to a chrysalis, for example. Certain hormones control the entire process of molting and changing form. IGRs mimic those hormones or their actions. This results in changes that cause the insects to die, such as trying to change into an adult or pupa when the insect is not physiologically ready. IGRs only affect immature insects. If you expose adult insects to IGRs, they show no effect. Since humans don't have these insect hormones, IGRs appear to have a wide margin of safety for people (Alan, 2017).

Chlorinated Hydrocarbons Methoxychlor and dicofol are examples of this group. These chemicals interfere with the normal movement of potassium and sodium ions across nerve cell surfaces. This prevents normal nerve cell function and produces symptoms similar to those from OP and carbamate insecticides (Alan, 2017).

Microbial Insecticides These are made from microorganisms that attack insects. They are so specialized to attack insect cuticle and cells that they are not very dangerous to people. Insecticides based on viruses must be eaten by insects in order to harm them. Then the viruses take over the function of certain insect cells (those of the gut first), making many copies of themselves. As a result, the insect's cells burst and die. Elcar and Madex are examples of insecticides with insect viruses as their active ingredient. Insecticides based on the bacterium *Bacillus thuringiensis* (B.t.) also need to be eaten by insects in order to work. The microbes attack the gut lining, and then invade the insect's body and multiply. Examples are Dipel and XenTari. Most B.t. insecticides are made from strains that harm only caterpillars. A few are available that harm immature fungus gnats or mosquito and black fly larvae, and not caterpillars. Sawflies will not be harmed by these materials. Fungal insecticides attack insects from the outside. The spores land on the insect's body and grow right into it, eventually penetrating inside and growing throughout the body. They require relatively high humidity to germinate and grow into the insect's body. Examples include Met52 and Botanigard (Alan, 2017).

1.2 Chlorpyrifos

Chlorpyrifos (O,O-diethyl O-[3,5,6-trichloro-2-pyridinyl]-phosphorothioate, CPF) is a widely used organophosphorus insecticide all over the world both for agricultural and nonagricultural purposes (Quintana *et al*., 2018). Chlorpyrifos is rapidly absorbed, metabolized and excreted by experimental animals. A single dose of [36Cl]chlorpyrifos (50 mg/kg) administered to rats (strain not specified) by gavage resulted in 90.1% excretion of the administered radioactivity in 26 h, with 90% of the excreted material being in urine and 10% in feces. Excretary products identified were [36Cl]-3,5,6- trichloro-2-pyridyl phosphate (75–80%), 3,5,6-trichloro-2-pyridinol (TCP) (15–20%), and traces of chlorpyrifos, suggesting deethylation is a significant metabolic pathway in rats. However, the pyridyl phosphate is likely to be only a transitory intermediate in the hydrolysis of chlorpyrifos to the pyridinol moiety. Tissue residues of chlorpyrifos with a radioactive half-life of about 62 h were found mainly in fat (Clegg and Van Gemert, 1999).

1.2.1 Toxicity of Chlorpyrifos

Chlorpyrifos is moderately toxic following acute oral, dermal and inhalation exposures (toxicity category II). Chlorpyrifos affects the nervous system by reversibly inhibiting the activity of

cholinesterase (ChE), an enzyme necessary for the proper functioning of the nervous system. Inhibition of ChE is the most sensitive effect in all animal species evaluated and in humans, regardless of route or duration of exposure. In animals, significant inhibition of plasma and red blood cell (RBC) ChE occur at doses below those that cause brain ChE inhibition. Data from two human studies suggest that humans are similarly and possibly more sensitive than animals following acute and short-term oral exposure and acute dermal exposure based on plasma ChE inhibition and/or possible clinical signs. Females are slightly more sensitive than males based on ChE inhibition and acute toxicity (comparison of LD_{50}'s). Studies in the scientific literature suggest that neonates are more sensitive to oral chlorpyrifos exposure than adults for ChE inhibition and behavioral effects. The increased sensitivity of the young may be attributed to a reduced capacity to detoxify chlorpyrifos (Smegal, 2000).

Developmental and reproductive effects have been observed in rats, rabbits and/or mice, but only at doses that induced maternal or parental toxicity. In rats, chlorpyrifos causes delayed alterations in brain development in offspring of exposed mothers. Several studies in the peer reviewed literature and results of the guideline developmental neurotoxicity study are supportive of the possibility that chlorpyrifos exposure may affect brain development (e.g., altered synaptic development, alterations in DNA, RNA, and protein synthesis, inhibition of mitosis and mitotic figures, and disruption of the structural architecture of the brain). There are suggestive data that these effects may arise independent of cholinesterase inhibition (Smegal, 2000).

Chlorpyrifos did not induce treatment-related tumors or provide evidence of carcinogenicity in two chronic rat or two chronic mouse studies. Chlorpyrifos was not mutagenic in bacteria, or mammalian cells, but did cause slight genetic alterations in yeast and DNA damage to bacteria (Smegal, 2000).

1.2.2 Metabolism of Chlorpyrifos

Chlorpyrifos, and some other organophosphate (OP) compounds, are detoxified via a two-step pathway involving bioactivation of the parent compound to an oxon by the cytochrome P450 systems, and then hydrolysis of the resulting oxon compounds by esterases such as liver or serum paraoxonase (PON1) (located in the plasma). In the human population, serum PON1 activity is genetically determined (polymorphic) and individuals express widely different levels of this

enzyme. Therefore, it is possible that some individuals may be more sensitive to chlorpyrifos toxicity based on genetic factors that regulate serum PON1 activity resulting in a reduced capacity to detoxify chlorpyrifos-oxon (Smegal, 2000).

1.2.3 Mechanism of Toxicity

The classical mechanism of chlorpyrifos toxicity is the ability of its oxon metabolite to bind and inhibit the serine hydrolase AChE. The primary target is the nervous system because AChE hydrolyzes the neurotransmitter acetylcholine thereby terminating its synaptic action. AChE inhibition increases the availability of acetylcholine at the neural synapse leading to excessive stimulation of the cholinergic pathways in the central and peripheral nervous systems. Significant inactivation of AChE causes acute cholinergic effects, morbidity, or death. Chlorpyrifos oxon interacts with other esterases such as butyrylcholinesterase, neuropathy target esterase (NTE), carboxylesterase, and PON1. Carboxylesterases and PON1 are key enzymes involved in detoxification of chlorpyrifos. Carboxylesterases act as alternative targets to AChE for chlorpyrifos oxon thereby decreasing its concentration in blood. Gender and age differences are observed in the carboxylesterase activity in animals. PON1 is a polymorphic enzyme with allozymes differing in their ability for hydrolytic detoxification of chlorpyrifos oxon. The PON1 status is an important determinant in modulating the acute toxicity of chlorpyrifos. Butyrylcholinesterase may function as a molecular scavenger for chlorpyrifos in the blood or substitute for AChE where it is low. NTE may be involved in the OP-induced delayed neurotoxicity syndrome (Koshlukova and Reed, 2014).

Chlorpyrifos itself is a weak ChE inhibitor. In risk assessment, the co-occurrence of chlorpyrifos and chlorpyrifos oxon is addressed by applying a toxicity equivalence factor or relative potency factor of 10 to chlorpyrifos oxon. Concerns were raised regarding regulatory standards of chlorpyrifos established based on its inhibitory effects on ChE activity that may overlook potentially more sensitive non cholinergic mechanisms. Besides AChE, potential targets include neurogenesis, nervous system development, cytotoxicity, macromolecule synthesis, neurotransmitter receptors, oxidative stress, cell signaling, nuclear transcription factors, and neuronal-glial cell interactions (Koshlukova and Reed, 2014).

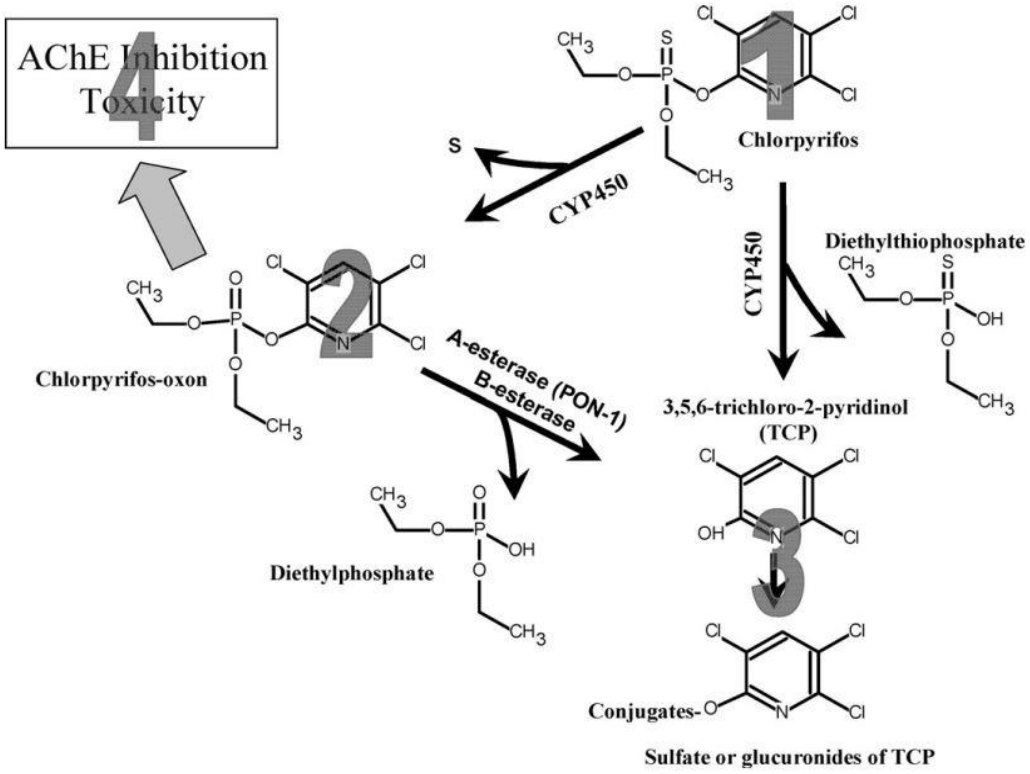

Figure 1: Metabolic scheme for (1) Chlorpyrifos (CPF), and the major metabolites (2) Chlorpyrifos-oxon (CPF-oxon), (3) Trichloropyridinol (TCP), Diethyl phosphate, and Diethylthiophosphate. (Timchalk *et al*., 2007)

1.2.4 Permissible limit of Chlorpyrifos

The acute dietary RfD of 0.005 mg/kg/day is based on a no-observed adverse effect level (NOAEL) of 0.5 mg/kg/day from an acute oral rat blood time-course study that observed 28-40% plasma cholinesterase (ChE) inhibition 3-6 hours after dosing male rats with a single dose of 1 mg/kg/day (the lowest-observable adverse effect level, LOAEL). This NOAEL is supported by statistically significant 30% RBC ChE inhibition 4 hours after a single 1.5 mg/kg/day exposure by a study in the scientific literature (Zheng et al. 2000). The chronic RfD of 0.0003 mg/kg/day is based on an oral NOAEL of 0.03 mg/kg/day for significant plasma and red blood cell (RBC) ChE inhibition at 0.22 to 0.3 mg/kg/day (LOAEL) based on a weight of the evidence consideration of 5 toxicity studies in dogs and rats. An uncertainty factor of 100 (10X for interspecies extrapolation and 10X for intraspecies variability) was applied to the NOAELs to obtain the RfDs (Smegal, 2000). The

acceptable daily intake (ADI) of chlorpyrifos is 0.003 mg/kg/day while maximum permissible limit (MPI) is 0.18 mg/kg/day (Chen *et al*., 2012).

A route-specific short-term dermal NOAEL of 5 mg/kg/day from a 21-day dermal rat study has been selected based on plasma and RBC ChE inhibition of 45% and 16%, respectively at 10 mg/kg/day (LOAEL). A dermal absorption adjustment is not necessary because a dermal study was selected. The intermediate- and long-term dermal NOAELs and long-term inhalation NOAEL are 0.03 mg/kg/day based on statistically significant plasma and RBC ChE inhibition that occurred at 0.22 to 0.3 mg/kg/day based on a weight of the evidence of 5 toxicity studies in dogs and rats. Because an oral NOAEL was selected, a 3 percent dermal absorption factor was used. Dermal absorption was estimated to be 3 percent based on the ratio of the oral LOAEL of 0.3 mg/kg/day from the rat developmental neurotoxicity study to the dermal LOAEL of 10 mg/kg/day from the 21-day rat dermal study. This absorption factor is comparable to the dermal absorption estimated from human data of 1-3% (Smegal, 2000).

The short- and intermediate-term inhalation NOAEL is 0.1 mg/kg/day from two separate 90-day rat inhalation studies that did not observe effects at the highest vapor concentration tested. HED selected a LOAEL of 0.3 mg/kg/day for 43% plasma and 41% RBC ChE inhibition from the oral developmental neurotoxicity study in rats to complete the dose-response assessment. A 100% default inhalation absorption factor (i.e., inhalation and oral absorption are equivalent) was used (Smegal, 2000).

1.2.5 Bioaccumulation and Biomagnification

Both chlorpyrifos and atrazine are bioaccumulated in L. variegatus tissues. The bioaccumulation of chlorpyrifos was clearly stronger, as expected on basis of its greater lipophilicity. The uptake clearance coefficients (ks) increased in the same order for both chemicals, those of chlorpyrifos being distinctly larger than those of atrazine. The elimination rate coefficients (ke) are of the same order of magnitude for both chemicals and largest in sediment S4, where the animals bioaccumulated the greatest amount of both compounds (Jantunen *et al*., 2008).

1.3 Oxidative Stress

In acute exposure, the main mechanism of OP toxicity is irreversible inhibition of AChE activity that results in accumulation of ACh and acute muscarinic and nicotinic effects. In subchronic or chronic OP exposition induction of oxidative stress has been reported as the main mechanism of its toxicity (Ranjbar *et al*., 2005). Both the increased production of reactive oxygen species and attenuation of the antioxidant barrier of the organism are likely to induce oxidative stress. In other words, oxidative stress can be defined as an imbalance between the production of free radicals and the body antioxidant defense system–enzymatic and non-enzymatic (Lukaszewicz-Hussain, 2010).

The reactive oxygen species (ROS) may be produced as the result of the metabolism of organophosphates by cytochrome P450s. The P450s are monooxygenases and catalyze oxidation by addition of one atom of molecular oxygen into a substrate (organophosphate) by an electron transport pathway (Lukaszewicz-Hussain, 2010). In this reaction reactive oxygen species are generated. The OP change normal antioxidant homeostasis resulting in antioxidant depletion, if the requirement of continuous antioxidants is not maintained (Possamai *et al*., 2007). The other way of ROS generation in OP toxicity is high energy consumption coupled with inhibition of oxidative phosphorylation, which has been described by Milatovic *et al*., 2006 and increased release of glucose by induction of glycogenolysis in the liver and subsequent release of ATP to meet the body's energy requirement (Rahimi and Abdollahi, 2007). High energy consumption leads to decreased ability of cells in maintenance of its energy levels. For this reason, the excessive amounts of ROS may be generated in different organs (Milatovic *et al*., 2006). Thus disturbance in the cell redox system is another mechanism implicated in the generation of reactive oxygen species in OP exposure (Lukaszewicz-Hussain, 2010). MDA may be increase due to adverse effect of chlorpyrifos on cell membrane (Uzun and Kalender, 2013).

There are several mechanisms to counteract the damage caused by ROS in the human and animal organism. Many compounds have antioxidative capacity and can be categorized in two main systems. One of them is the enzymatic system which consists of such enzymes as superoxide dismutase (SOD) (manganese in the active enzymatic centre or copper and zinc), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Morgan *et al*., 2007).

Another antioxidative system is non-enzymatic and consists of a reduced form of glutathione (GSH) and such vitamins as vitamin C, vitamin E and beta-carotene. Each of these antioxidative systems has a specific activity/concentration, but they work synergistically to enhance the antioxidant capacity of the organism. In the antioxidative action, GPx uses reduced glutathione and in this reaction reduced glutathione (GSH) is converted to oxidized glutathione form (GSSG). To recycle GSSG to GSH, the cells utilize the enzyme named glutathione reductase (Lukaszewicz-Hussain, 2010). GR needs NADPH as a substrate for reduction of oxidized form of glutathione to its reduced form. The source of NADPH is the pentose phosphate pathway and glucose-6-phoshate dehydrogenase (catalyzes the initial step of this pathway)– enzyme, whose more important function is reduction of nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. Glucose 6-phosphate dehydrogenase needs glucose as a substrate for its activation (Rahimi and Abdollahi, 2007).

1.4 Hepatotoxicity

The liver is the first organ to encounter ingested nutrients, drugs and chemicals like pesticides that enter the hepatic portal vein from the digestive system, and liver function can be detrimentally altered by injury resulting from acute or chronic exposure to toxicants (Al-Attar, 2011).The most common serum biomarkers of liver damage are aspartate transaminase (AST) and alanine transaminase (ALT). There is strong evidence that both are increased by chlorpyrifos (Somayyeh *et al*., 2017).Hepatotoxicity are characterized by both intracellular and extracellular changes in hepatocytes (Kobylinska *et al.,* 2015). Some symptoms are traditionally taken as pertaining to liver lesion, such as bile ducts damage, hepatocytes lysis, transport protein degradation, T-cell cytolytic activity, apoptosis of hepatocytes, and disintegration of mitochondria (Kobylinska et al., 2015). Nevertheless, the particular mechanisms of hepatotoxicity of pharmaceuticals, including anticancer drugs, remain largely unknown. Elevated serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) are observed at liver pathologies. This is explained by their increased biosynthesis in bile ducts cells, solubilization and release in bloodstream (Panchyshyn *et al*., 2010). These phenomena are observed during intrahepatic as well as extrahepatic obstruction, when activity of the mentioned enzymes may exceed normal levels 5 to 10 times (Mohort *et al*., 2010).

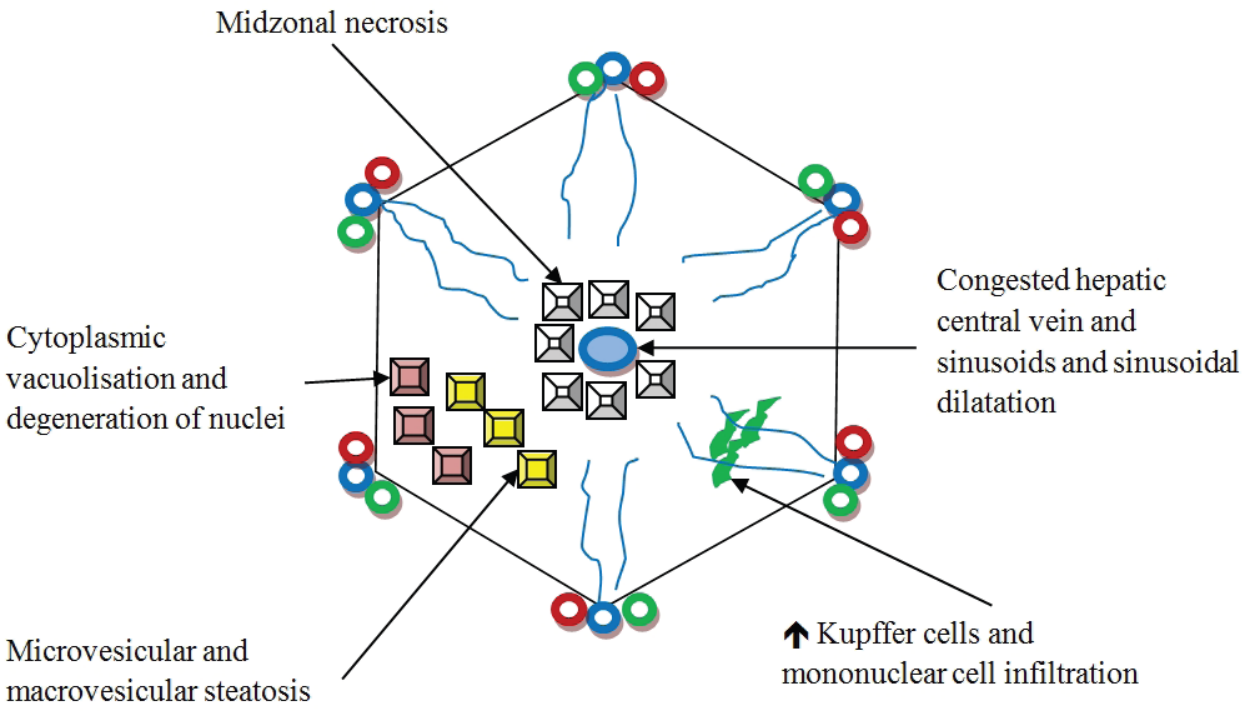

Figure 2: Histopathological disorders in the hepatic lobule after exposure to organophosphorus pesticides (Somayyeh *et al*., 2017)

In the liver, organophosphorus pesticides cause ultrastructural, biochemical, metabolic, and mitochondrial damage, evidenced by changes in hepatic biomarkers such as serum aminotransferase and direct and indirect bilirubin (Somayyeh *et al*., 2017). Many studies confirm that the liver tissue is the primary target organ of OP toxicity. Chlorpyrifos is a typical representative of the OPs, which causes detrimental effects both on liver function and that a two-day i.p. exposure to a sub-lethal dose of this insecticide resulted with mid-zonal liver necrosis, fatty deposition at the periphery, and glycogen deposition at one side of the hepatic cell and around the central vein. (Goel *et al*., 2016) studied liver histoarchitecture in chlorpyrifos treated rats and observed hepatocyte vacuolisation and necrosis, sinusoidal dilatation, and increase in binucleated cells at higher doses and longer exposure to chlorpyrifos.

Recent findings by Ezzi *et al*. 2016 suggest that chlorpyrifos had a dose-dependent effect on dilated sinusoids, central vein, and portal triad in rats. An excessive amount of liver blood and degenerative changes were found in the liver of fish exposed to chlorpyrifos through contaminated water (Topal *et al*., 2014). As for oxon OPs, studies have reported hyalinisation, vacuolisation,

nucleus necrosis, and hepatocellular oedema, and fatty degeneration in sub-acute exposure to low-dose of trichlorfon and acute exposure to omethoate (Li *et al*., 2017 and Li *et al*., 2010). These disturbances in the morphological structure of the liver could be associated with a disruption of the tissue function, which could also be related to lower antioxidative capability, change in fatty and glycogen content in the liver, and the inhibition of some enzymes contributing to lipid, protein, and carbohydrate metabolism needed to preserve the integrity of the liver tissue. Impaired bile flow and biliary excretion could also serve as indirect indicators of liver damage (Li and Chiang, 2014). Goel and Dhawan, 2001, for example, suggested that poor biliary excretion and longer half-life of 99mTc-mebrofenin in chlorpyrifos-treated rats reflected impaired hepato-biliary function. As impaired liver function affects the metabolism, consequently it also affects the concentrations of waste products. Several animal studies have pointed to the indirect effects of sub-lethal doses of phorate, fenitrothion, and dimethoate on urea and bilirubin (Jayusman *et al*., 2014 and Farghaly *et al*., 2007). In humans occupationally exposed to a wide range of OPs, blood urea nitrogen and albumin along with serum aminotransferases are frequently elevated (Malekirad *et al*., 2013 and Cecchi *et al*., 2012).

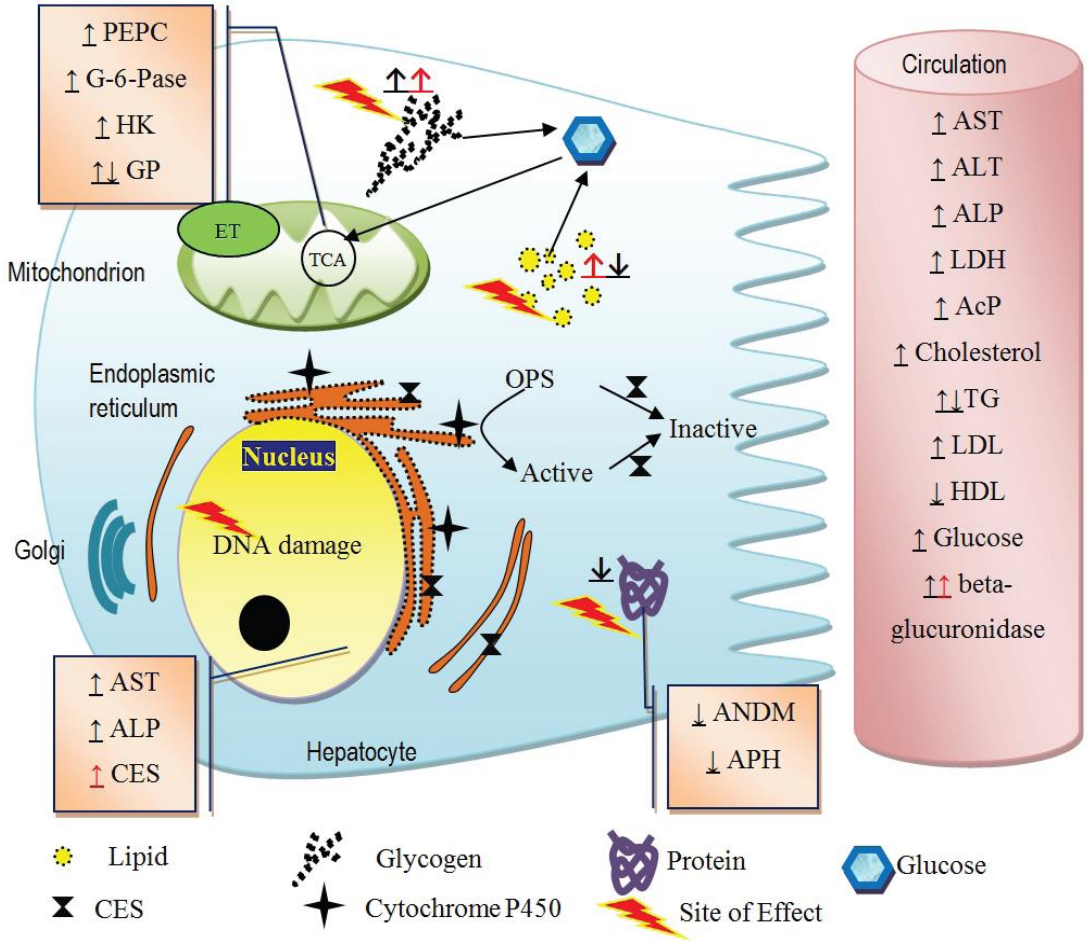

Figure 3: Possible disturbance pathways in the liver tissue after exposure to organophosphorus pesticides. Black arrows indicate long-term exposure and red ones acute poisoning (Karami-Mohajeri *et al*., 2017).

1.5 Neurotoxicity

Organophosphate (OP) and carbamate (CM) insecticides are the chemicals most commonly used in the U.S.[1]. In addition, military nerve agents such as soman, sarin, cyclosarin, tabun, and VX are also OP compounds that are chemically related to pesticides, though with greater toxicity. Recent global events have focused attention on the potential threat of international and domestic chemical terrorisms, as well as the possibility of chemical warfare proliferation. Exposure to these agents can lead to both short- and long-term neurological impairments, including: (1) acute hypercholinergic effects that occur minutes or hours following exposure, (2) a delayed intermediate syndrome affecting muscles that can occur within few days following recovery from severe acute affects, (3) a delayed peripheral polyneuropathy associated with some

anticholinesterases that usually occurs within weeks following an acute exposure, and (4) subtle, long-term neurological effects, which may last months or even years. Whereas the acute effects are manifest only after a threshold exposure is attained, it has become increasingly apparent that many Ops and CMs can act in a cumulative fashion, with the threshold for more chronic effects reached through repeated exposures at doses that are not associated with acute effects.

Pharmacologically, all these compounds are acetylcholinesterase (AChE) inhibitors, and their acute symptoms are attributed to accumulation of acetylcholine (ACh), thus exhibiting cholinergic toxicity. Phosphorylating or carbamylating the esteratic site of the enzyme[2] diminishes its capacity to catalyze its endogenous substrate ACh. Consequently, the hydrolysis of ACh is prevented, leading to accumulation of ACh in the synaptic cleft and overstimulation followed by desensitization of muscarinic and nicotinic ACh receptors. Depending on the degree of AChE inhibition, cholinergic stimulation may lead to hyperactivity of excitable tissues, causing fasciculations, seizures, convulsions, severe muscle paralysis, hypersecretion from secretory glands, respiratory failure, coma, and death. The CM and OP anticholinesterases induce neuro- and myotoxic effects that destroy neurons and muscle fibers by excitotoxic action. Additional non cholinergic mechanisms that contribute to these effects include excessive generation of free radicals and alteration in antioxidant and the scavenging system, causing lipid peroxidation. Understanding the relationships of excitotoxicity, cholinergic, and noncholinergic (oxidants/antioxidants) determinants may elucidate biochemical mechanisms that are crucial to a variety of toxicities of cholinesterase inhibitors, and relate important aspects of their toxicity to an established conceptual framework of other neurodegenerative disease (Milatovic *et al*., 2006).
In rats, chlorpyrifos causes delayed alterations in brain development in offspring of exposed mothers. Several studies in the peer reviewed literature and results of the guideline developmental neurotoxicity study are supportive of the possibility that chlorpyrifos exposure may affect brain development (e.g., altered synaptic development, alterations in DNA, RNA, and protein synthesis, inhibition of mitosis and mitotic figures, and disruption of the structural architecture of the brain) (Smegal, 2000).

1.6 Reproductive toxicity

Insecticides are widely used for insect control and are persistent in the environment. Residues of insecticides eventually reach the aquatic ecosystem and get bioaccumulated in fatty tissues of aquatic fauna from where it is concentrated in terrestrial vertebrates through the food chain. It is likely that the insecticides may be causing disturbances in the gonads at receptor levels so that estrogen functioning is disturbed thereby affecting the reproductive function of the species. Simultaneously insecticides present beyond the permissible limit must be avoided because it may cause decrease in fertility in vertebrate fauna in future (Singh and Singh 2008).

Reports have indicated that several fish species have tendency to bioaccumulate the metabolites of DDT and isomers of HCH in gonads and decreased plasma levels of sex steroid hormones during breeding phase. Earlier results have indicated that the insecticides caused inhibition of sex steroid hormone levels in the catfish, H. fossilis (Singh and Singh 2008). There were no indications that chlorpyrifos caused delayed neurotoxicity at dose levels below twice the oral LD_{50}. In another developmental neurotoxicity study, pregnant rats were given chlorpyrifos at dose levels of 0, 0.3, 1, or 5mg/kg-d without evidence of selective developmental neurotoxicity. Chlorpyrifos was not carcinogenic for male and female rats at dose levels up to 10 mg/kg-d. A teratology and reproduction study in rats indicated that oral administration of up to 3mg/kg-d of chlorpyrifos had no effect on the reproductive capacity of either the male or female, and was without teratogenic effect on the progeny. Another study revealed that oral administration of chlorpyrifos to rats at parentally toxic dose levels (3 and 15 mg/kg-d) was not embryolethal, embryo/fetotoxic, or teratogenic and did not adversely affect fertility or the function or structure of the reproductive organs. No developmental toxicity was observed at doses up to and including 10 mg/kg-d in pregnant female mice (Faraga *et al*., 2010).

In contrast, exposure to chlorpyrifos poses the greatest problem to pregnant women and small children, with evidence of neurophysiologic effects in humans. Indoor spraying of chlorpyrifos may pose a considerable risk to human health especially to children (Qiao *et al*., 2003). Therefore, the relationship between maternal chlorpyrifos exposure and birth defects has been controversial despite the importance to public health and concern over prenatal exposure (Tian., 2005). On the other hand, oral treatment with chlorpyrifos induced a significant increase in the percentage of

polychromatic erythrocytes compared to the control (Faraga *et al*., 2010). In addition, basic studies have recognized cytogenetic toxicity following exposure to chlorpyrifos in vivo and in vitro. Recently, chlorpyrifos induced significant increases in the rate of micronucleus formation, and a dose-dependent reduction in cell number, in the pre-implantation mouse embryo (Tain and Yamauchi, 2003). Chlorpyrifos can induce adverse effects on reproductive performance; showed fetotoxic and teratogenic effects at a maternal dose of 25 mg/kg-d, a dose that also produced maternal toxicity (Farag *et al*., 2003).

Chlorpyrifo, like other OP insecticides, has been shown to alter reproductive function by altering the activity of the pituitary-thyroid and pituitary-adrenal axes, giving credence to its endocrine disrupting effect. Inverse relationship between CPF exposure and sperm count has been established in humans and laboratory animals. Although the main mechanism of chlorpyrifos toxicity is inhibition of acetylcholinesterase (AChE) activity, toxicity does occur at doses that did not inhibit AChE, prompting search for other mechanisms. The induction of oxidative stress is one of the non-cholinergic mechanisms implicated in CPF toxicity (Shittu *et al*., 2013).

1.7 Enzyme Activities

Enzyme activity is a measure of the amount of active enzyme present and is thus dependent on conditions, which should be specified. The SI unit is the Katal, I katal = 1 mol s^{-1} =1 unit. It is also referred to as the rate at a particular reaction proceed in a given time. Enzymes speed up biological reactions in living organisms. These enzyme activities could function as biomarkers, such that a significant increase or decrease in a particular enzyme activity could sound off a warning bell as a direct result of a specific malfunction in cellular metabolism (Bruce, 2005).

1.7.1 Enzymes and Biomakers

Biochemical biomarkers are molecules, genes and characteristics which are prone to change depending on the physiology and metabolic rate of the organisms (Quintero and Zafra, 2016). This change could be due to a positive or a negative induction of a substance, maybe toxic material into the habitat of an organism. Since enzymes are the biocatalysts of living organisms and are at most times functional to maintain the metabolic process, an appreciable increase or decline in their respective activities may be indicative of stressors of oxidation as can be seen in most antioxidant enzymes (Obomanu *et al.,* 2009). As such enzymes as biomarkers is yet considered as one of the

most promising tool for the detection of the real changes that may have metabolic explanation (Quintero and Zafra, 2016).

1.7.2 Alanine aminotransferase (ALT)

Clinical chemistry data are routinely used for noninvasive monitoring of liver disease in preclinical species and humans, and alanine aminotransferase (ALT) is the most widely used clinical biomarker (Ozer *et al.*, 2008). ALT is responsible for the metabolism (transamination) of alanine and is found at much higher concentrations in the liver compared to other organs. When hepatocellular injury occurs, the liver-abundant enzyme ALT will leak into the extracellular space and enter the blood, wherein it shows a slow clearance rate with a half-life of approximately 42 h (Ozer *et al.*, 2008). The typical reference range is 7-35 IU/L in females and 10-40 IU/L for males (WebMD, 2013). Notably raised levels of alanine aminotransferase (ALT) in plasma can be a marker of severe liver damage with variable aetiologies and prognosis (Helgi *et al.,* 2016). It is deemed to be the clinical chemistry gold standard for DILI detection and has long been used at the FDA to facilitate regulatory decision-making (FDA, 2009). Recent studies have suggested that measuring the ALT isozymes, ALT1 and ALT2, may aid in differentiating the source of injury (Yang *et al.*, 2009). ALT1 has been noted to be localized in human hepatocytes, in renal tubular epithelial cells, and in salivary gland epithelial cells. ALT2, on the other hand, is localized to human adrenal gland cortex, neuronal cells bodies, cardiac myocytes, skeletal muscle fibers, and endocrine pancreas (Lindblom *et al.*, 2007). Compared to ALT1, ALT2 was found to contribute less to the total serum ALT activity and was probably a reflection of mitochondrial damage (Yang *et al.*, 2009).

1.7.3 Aspartate aminotransferase (AST)

Based on the same rationale as ALT, aspartate aminotransferase (AST) has also been introduced as a standard biomarker for drug induced liver injury (DILI) and is well accepted by clinicians (Shi *et al.*, 2010). AST is responsible for the metabolism (transamination) of aspartate. Even though the sensitivity of the AST test is believed to be lower than that of ALT, it is still a widely used liver biomarker. Owing to its more ubiquitous expression in extra hepatic organs, such as the heart and muscle, AST is significantly less specific than ALT in detecting DILI (Ozer *et al.*, 2008). It appears that the ratio between serum ALT and AST activity is useful in differentiating DILI from extra hepatic organ injury (Ozer *et al.*, 2008), as well as to help in diagnosing acute alcoholic

hepatitis and cirrhosis with an AST/ALT ratio at 2:1. At least two isoenzymes of AST have been found; one is cytosolic AST and another is mitochondrial AST (mAST). The relative contributions of cytosolic AST or mAST to serum AST elevation have not been critically assessed. In addition it remains unknown whether AST isoenzymes are susceptible to drug-driven induction or inhibition.

1.7.4 Alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) is an enzyme located in the liver, and its concentration in serum increases when the bile ducts are blocked (Burtis and Ashwood, 1999). ALP is another diagnostic biomarker recommended in the FDA guidance and is widely adopted by clinicians (FDA, 2009; Shi et al., 2010). The Council for International Organizations of Medical Sciences consensus criteria considered a more than two fold isolated elevation of serum ALP, or an ALT/ALP ratio of no more than 2, as a key biomarker of cholestatic DILI (Ramachandran and Kakar, 2009). It is noteworthy that conditions other than DILI, such as bone disease and pregnancy, are also associated with ALP elevation (Reust and Hall, 2001). Therefore, ALP should not be regarded as a specific biomarker of cholestatic DILI. The unique advantage of ALP is that it is at least partially predictive of biliary obstructive types of liver injury when used together with other DILI biomarkers.

1.7.5 Catalase

Catalase is a 240 kilodalton, (KDA) Tetrameric protein with four similar subunits and is encoded by ctt1 gene mapping to chromosome 11 (Ighodaro *et al.,* 2017). Each polypeptide subunit is 60KDA in weight and contains a single ferriprotoporphrin. Catalase (CAT) is a common antioxidant enzyme present in all living tissues that utilize oxygen. The enzyme uses either iron or manganese as a cofactor and catalyzes the degradation or reduction of hydrogen peroxide (H_2O_2) to water and molecular oxygen, consequently completing the detoxification process initiated by SOD (Ighodaro *et al.*, 2017). Hydrogen peroxide (H_2O_2) is one of the most stable of the reactive oxygen species (ROS). This key oxidant is chiefly generated by the reduction and protonation of the superoxide radical (O_2) or by direct reduction of Oxygen, Catalyzed by certain oxidases (Tuzet *et al.,* 2019). H_20_2 can oxidize cellular thiols, including specific protein cysteines to form sulfuric acid groups. It can become more generally reactive if transformed into the hydroxyl radical (OH),

an extremely unstable molecule that reacts very rapidly with a wide variety of biological molecules (Tuzet *et al.*, 2019). CAT is highly efficient in breaking millions of hydrogen peroxide (H_2O_2) in one second (Ighodaro *et al.,* 2017). CAT is located mainly in the peroxisomes but absent in mitochondria of mammalian cells. The only exception is in rats.

CAT also reacts efficiently with hydrogen donors such as methanol, ethanol, formic acid or phenols with peroxidase activity. The activity of CAT takes place in two steps; A molecule of hydrogen peroxide oxidizes the heme to an oxyferryl species. A porphyrin cation radical is generated when one oxidation equivalent is removed from ion and one from porphyrin ring. A second hydrogen peroxide molecule acts as a reducing agent to regenerate the resting state enzyme, producing a molecule of oxygen and water (Ighodaro *et al.,* 2017)..

Hydrogen peroxide though at low amounts tends to regulate some physiological processes such as signaling in cell proliferation, cell death, carbohydrate metabolism, mitochondria function, platelet activation and maintenance of normal thiol redox balance (Ighodaro *et al.,* 2017). However at high concentration it is very deleterious to cells. CAT is also known as a first line antioxidant defenses mechanism (Ighodaro *et al.*, 2017).

$$2H_2O_2 + \text{catalase} \longrightarrow 2H_2O + O_2$$

1.7.6 Glutathione Peroxidase

Glutatione peroxidase (GPX) is an important intracellular enzyme that breakdown hydrogen peroxides (H_2O_2) to water and lipid peroxide to their corresponding alcohols mainly in the mitochondria and sometimes in the cytosol (Ighodaro *et al.*, 2017). Most times, its activities depend on micronutrient cofactor known as selenium. For this reason, GPX is often referred to as a selenocysteine peroxidase. The enzyme plays a major role in inhibiting lipid peroxidation process and therefore protects cells from oxidative stress (Ighodaro *et al.*, 2017). There are at least eight (8) GPX enzymes in human, GPXI-GPX8 are mapped to chromosomes 3,14, 5,19,6,6,1 and 5 respectively. Among the GPXs, GPXI is the most abundant selenoperoxidase and it is present virtually in all cells. GPX2 is found much more in the gastrointestinal tract primarily in the intestine. The kidney relative to other tissues is the primary location for GPX3, though the enzyme is also present in extracellular fluids as a glycoprotein. Most forms of GPX are tetrameric in structure but GPX4 which is often regarded as phospholipid hydroperoxide is a monomer and

differ in substrate specificity. This is because GPX4 is the only GPX enzyme that breaks down phospholipid hydroperoxides. The enzyme also has a mitochondrial isoform that mediates the apoptotic response oxidative stress and has a peroxidase independent structural role in sperm maturation. GPX5 is found in both human and rodents and GPX6 differ from other glutathione peroxidases in that their activities are independent of selenium. This invariably implies that these forms of GPX may not be able to effectively scavenge H_2O_2, a feature which is a characteristic of the selenium dependent glutathione peroxidases.

1.7.7 Cholinesterase (EC 31.1.7) Activity

Cholinesterase is an esterase that lyses choline-based esters, many of which serve as neurotransmitters. Thus, it is either of the two enzymes that catalyse the hydrolysis of these cholinergic neurotrasmitters, example breaking acetylcholine into choline and acetic acid or butyrylcholine into choline and butyric acid (Richetti *et al*., 2011). Acetylcholinesterase (AChE) is found mainly in neuromuscular junctions and is the key enzyme in the central nervous system of cholinergic synapses, where its activity serves to terminate synaptic transmission. Irreversible inhibition of AChE may lead to muscle paralysis, convulsion, bronchial obstruction and death by asphyxiation (Al-Sawafi and Yan, 2013). The cholinergic system, with acetylcholine (ACh) as the neurotransmitter is involved in cognitive processes, through the activation of metabotropic muscarinic and ionotropic nicotinic cholinergic receptors (Al-Sawafi and Yan, 2013).

1.7.8 Malondialdehyde (MDA)

Malondialdehyde (MDA) is one of several byproducts of lipid peroxidation process,it is a biomarker that provides an indication of lipid peroxidation level. (Bhale *et al.,* 2015) postulates that Malondialdehyde is the most frequently used biomarker of oxidative stress in many health problems such as cancer, psychiatry, chronic obstructive pulmonary disease, asthma orcardiovascular diseases. MDA serves as a reliable biomarker for oxidative stress. MDA is mutagenic, tumorigenic and highly reactive three carbondialdehyde produced during polyunsaturated fatty acid peroxidation and arachidonic acid metabolism (Singh *et al*., 2014). The Endogenous formation of MDA during intracellular oxidative stress and its reaction with DNA forms MDA-DNA adducts which makes it an important biomarker of endogenous DNA damage.

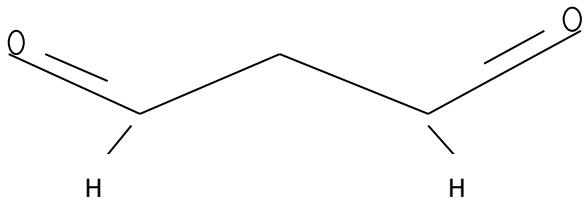

Fig 4: Malondialdehyde (Azizi *et al*., 2018)

1.7.9 Glutathione

Glutathione is a tripeptide which consists of glutamic acid, cysteine, and glycine. The sulphydryl group (SH) of cysteine is the functional group of the molecule, responsible for its biological activity (Scire *et al.*, 2018). The thiol group of glutathione is a potent reducing agent, rendering GSH (reductase) able to scavenge many dangerous endobiotic and xenobiotic electrophiles, both through direct or indirect enzymatic reactions. This Ubiquitous molecule is the main low molecular weight thiol found in living cells, also considered one of the major non-protein defenders against oxidative stress and the most important thiol-di-sulfide redox buffer of the cell (Scire *et al.*, 2018). It is present in both reduced (GSH) and oxidized (GSSG) forms. It is of great importance to maintain an optimal GSH: GSSG ratio. Under physiological conditions intracellular glutathione is mainly found as GSH form at low millimolar concentrations while the content of oxidized species does not exceed 1% of its total intracellular content (Scire *et al.*, 2018). Glutathione in its reduced form can help repair oxidant damage caused by pollution, stress, infection, radiation, aging, drugs, unhealthy diets and burns (Scire *et al*, 2018). Glutathione also contributes to the biosynthesis of iron sulfur (fe-s) clusters. These are co-factors of many proteins of crucial importance.

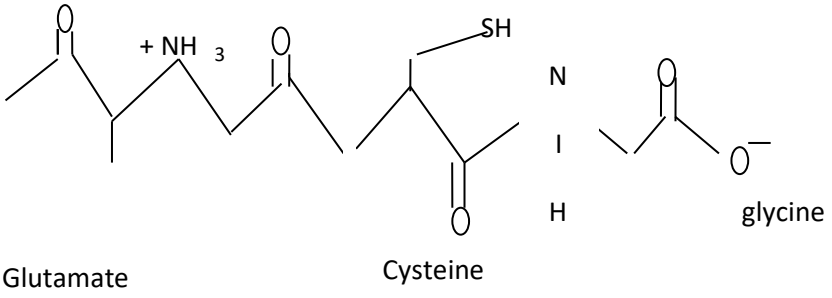

Fig 5: Glutathione (Di and Kerns 2015).

Glutathione plays a critical role in protecting redox signaling pathways. In this scenario, the electron of GSH can be utilized by several redox reactions within the cell and the formed GSSG returns to the reduced state by NADPH dependent activity of glutathione reductase (Scire *et al.*, 2018).

Synthesis of glutathione occurs exclusively in the cytosol, from where it goes through many cellular compartments such as endoplastic reticulum (ER), mitochondria, nuclear matrix and perioxisomes (Scire *et al.*, 2018). GSH is synthesized from cysteine and glutamate, linked by a typical peptide bond in a reaction catalyzed by the glutamate–cytein ligase (GCL). Then, glutathione synthetase (GS) catalyzes the condensation of Y-glutamylcysteine and glycine (Gly) to produce GSH, coupled ATP hydrolysis is required in both reactions (Scire *et al*., 2018).

1.7.10 Testosterone

Testosterone is a steroid hormone involved in many bodily processes, including reproductive physiology (e.g., spermatogenesis), morphology (e.g., development of secondary sexual characteristics), psychology (e.g. sexual desire), and behavior (e.g., aggression) each of which plays an important role in survival and reproduction. Testosterone effects clearly manifest during puberty when its levels rise significantly (Bird and Zilioli, 2018).

Through several enzymatic steps, testosterone is synthesized from cholesterol in numerous tissues, including the testes, ovaries, and adrenal glands. Testosterone secretion is ultimately governed by the hypothalamic-pituitary-gonadal (HPG) axis. In men, testosterone secretion results from a hormonal "cascade," where the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates luteinizing hormone (and follicle-stimulating hormone) from the anterior pituitary gland, which in turn stimulates testosterone pulses from the Leydig cells in the testes. In women, testosterone is primarily secreted in the ovaries, which contain cells similar to those of the testes (Bird and Zilioli, 2018).

Testosterone is the main type of circulating androgens, which is mainly synthesized by the Leydig cells. Testis also produces small amounts of other hormones such as estrogen and progesterone. Maintenance of an adequate concentration of intratesticular testosterone is essential for testicular function, especially for spermatogenesis (Sede *et al*., 2018). Once in the cell, testosterone can also

be converted to other sex steroids, such as estradiol (via enzyme aromatase) or dihydrotestosterone (via enzyme 5-a-reductase), which in turn bind to steroid hormone receptors and affect cell functioning (Bird and Zilioli, 2018)..

Testosterone is implicated in numerous processes that are critical to reproduction and survival, including the expression of secondary sexual characteristics, mate seeking, aggression, and status seeking. From an evolutionary perspective, testosterone's role in promoting reproductive fitness can be explained largely from the framework of the life history theory, which proposes that species face evolutionary trade-offs (e.g., mating versus parenting) due to the limited availability of resources (e.g., energy, time). Some of these trade-offs are mediated by testosterone (Bird and Zilioli, 2018).

1.7.11 Cholesterol

Cholesterol is essential for mammalian cell functions and integrity. It is an important structural component maintaining the permeability and fluidity of the cell membrane. Within the cells, the cholesterol homeostasis is tightly regulated to ensure normal cellular processes. Cholesterol homeostasis is strictly regulated at the cellular level. Cholesterol is also a precursor for steroid hormones synthesis such as bile acids and vitamin D. Oxidized derivatives of cholesterol, known as oxysterols, are also implicated in numerous biological processes. The main source of cholesterol is food while less than half of the cholesterol originates from de novo synthesis. The cellular efflux of cholesterol is essential to maintain the homeostasis, as cells in peripheral organs do not express genes involved in the catabolism of cholesterol. The efflux of cholesterol requires acceptors such as HDL (Sede *et al*., 2018).

Cholesterol is the precursor for bile acids (BAs) synthesis within the liver. These molecules are the main constituents of bile and ensure solubilisation and emulsification of fat to help digestion. Their synthesis and excretion are major mechanisms of cholesterol catabolism in mammals. The major role of cholesterol in the endocrine function has been well established, as cholesterol is the precursor of steroids. There are multiple sources of cholesterol that contribute to steroidogenesis such as de novo synthesized, stored cholesteryl esters, exogenous lipoprotein-supplied cholesterol as well as plasma membrane-derived cholesterol. The plasma lipoproteins are the main source of steroid synthesis. The homeostasis of the interstitial tissue, mainly in Leydig cells, involved factors

of cholesterol homeostasis such as HMG-CoA reductase, HSL, and ACAT. Sex steroid hormones share a common biosynthesis pathway, with cholesterol as unique precursor (Sede *et al*., 2018).

It is often suggested that loss of cholesterol directly affects the sperm plasma membrane lipid bilayer to make it ‘‘fusogenic.’’ This idea is consistent with the observations that the acrosomal and plasma membranes of cholesterol-inhibited sperm do not fuse upon treatment of the sperm with inducers of the acrosome reaction. There is some reason to believe that elevated sperm cholesterol content might contribute to infertility. Sperm of normospermic men who failed to fertilize eggs in vitro were found to be characterized by abnormally high cholesterol content, or by a slow loss or even an increase in cholesterol during in vitro incubation (Cross, 1998).

1.7.12 Sperm count

Spermatogenesis is the formation of the male gamete or spermatozoa. Spermatogenesis is dependent on the integrity of the architecture of the seminiferous tubules and Sertoli cells and endocrine regulation and is regulated by testosterone and FSH (Jana and Sen, 2012) A sperm concentration of 20 x10^6/ml, the ‘normal’ or ‘reference’ value cited by WHO, has been considered too low for a lower reference limit because the probability of pregnancy is essentially linear with sperm concentrations up to 40–50 x10^6/ml (Cooper *et al*., 2010)

Several recent studies have suggested that sperm concentrations and semen quality have been decreasing over the past several decades in many areas of the world. The etiology of these decreases is currently unknown. Acute events can have significant impacts on spermatogenesis and are often readily identified during the male fertility evaluation. The majority of male factor infertility, however, is idiopathic. Chronic, low-dose exposures to chemicals and nutrients are more difficult to identify, but are extremely prevalent. These exposures to chemicals have been shown to have dramatic effects on both individual and community health and interest in the cumulative and synergistic impacts of such agents on spermatogenesis have been increasing. While our understanding of these potential hazards is evolving, it is clear that they may significantly influence male reproductive potential. (Gabrielsen and Tanrikut, 2016). While many studies have reported a negative impact on sperm motility, few studies have reported significant findings between POPs and sperm counts in the general population. Pesticides are taken in via dietary consumption; however, pesticides can also be absorbed through the skin and by inhalation,

particularly in occupationally exposed men. 1,2-dibromo-3-chloropropane (DBCP) is the most well-known pesticide associated with impaired fertility (Gabrielsen and Tanrikut, 2016).

Dramatic decreases in sperm counts have also been reported for occupational exposures to other pesticides including paraquat and malathion (Hossain *et al*., 2010). Additionally, Danish greenhouse workers with high pesticide exposure were found to have a 60% decrease in total sperm count compared to those with low exposure (Gabrielsen and Tanrikut, 2016). There is also evidence to suggest that pesticides may impair spermatogenesis in those who do not have occupational-based exposures. For example, urinary concentrations of three organophosphate metabolites were found to be negatively associated with total sperm counts (Melgarejo *et al.*, 2015). Additionally, high pesticide residue fruit and vegetable intake was associated with lower sperm counts in men presenting to a fertility clinic, while total fruit and vegetable intake was not associated with changes in semen parameters (Chiu *et al*., 2015).

1.8 Risk Assessment

Occupational and residential exposures to chlorpyrifos can occur during handling, mixing, loading and application activities. Occupational post application exposure can occur for agricultural workers re-entering treated fields such as during scouting, irrigation and harvesting activities. Residential post application exposure can occur following treatment of lawns, or residences for cockroaches, carpenter ants, termites, and other insects. In addition, there is a potential for inadvertent oral exposure to children from eating chlorpyrifos-treated turf and soil or licking fingers following contact with treated areas. Post application exposure to children can occur in locations other than the home, including schools, daycare centers, playgrounds, and parks (Smegal, 2000).

1.8.1 Human Health Risk Assessment

Risk assessment is a method used to estimate people's increased risk of health problems as a result of exposure to a toxic pollutant (Boguski, 2003). In recent years, the public has become increasingly aware of the presence of harmful chemicals in our environment. Many people express concerns about pesticides and other foreign substances in food, contaminants in drinking Water, and toxic pollutants in the air. Others believe these concerns are exaggerated or unwarranted. How

can we determine which of these potential hazards really deserve attention? How do we, as a society, decide where to focus our efforts and resources to control these hazards? Health risk assessment is a scientific tool designed to help answer these questions (Winston, 2001). Human risk assessments help to determine which potential hazards are the most significant. And these orders of significance help in proffering solutions to aid in curtailing hazards. These hazards are implicated as probable causative agent or exacerbating agent in most complex diseases (Rim and Lim, 2014).

1.8.2 Steps in Human Risk Assessment

There are four steps to Human risk assessment:

Hazard identification

Exposure assessment

Dose-response assessment

Risk characterization (Winston, 2001; Boguski, 2003; USEPA, 2017)

1.8.2.1 Hazard Identification

The objective of hazard identification is to identify the types of adverse health effects that can be caused by exposure to some agent in question, and to characterize the quality and weight of evidence supporting this identification. Hazard Identification is the process of determining whether exposure to a stressor can cause an increase in the incidence of specific adverse health effects (e.g. cancer, birth defects). It is also whether the adverse health effect is likely to occur in humans. In the case of chemical stressors, the process examines the available scientific data for a given chemical (or group of chemicals) and develops a Weight of evidence to characterize the link between the negative effects and the chemical agent.

Exposure to a stressor may generate many different adverse effects in human diseases, formation of tumours, reproductive defects, death, or other effects (USEPA, 2013).

Epidemiological studies involve a statistical evaluation of human populations to examine whether there is an association between exposure to a stressor and a human health effect. The advantage of

these studies is that they involve humans while their weakness results from generally not having accurate exposure information and the difficulty of finding out the effects of multiple stressors (Winston, 2001).

When data from human studies are unavailable, data from animal studies (rats, mice, rabbits, monkeys, dogs, snails etc.) are relied on to draw inference about the potential hazard to humans. Animal studies can be designed, controlled, and conducted to address specific gaps in knowledge, but there are uncertainties associated with extrapolating results from animal subjects to humans (Rim and Lim, 2014).

Key Components of Hazard Identification

A wide variety of studies and analysis are used to support a hazard identification analysis. **Toxicokinetics** considers how the body absorbs, distributes, metabolizes, and eliminates specific chemicals. **Toxicodynamics** focus on the effects that chemicals have on the human body. Models based on these studies can describe mechanisms by which a chemical may impact human health, thus providing insights into the possible effects of a chemical (Rim and Lim, 2014).

When assessing a chemical for potential carcinogenic behaviour, current EPA practice is to focus on analysis of a mode of action. Mode of action is a sequence of key events and processes, starting with interaction of an agent and a cell, proceeding through operational and anatomical changes, and resulting in cancer formation. A given agent may work by more than one mode of action, both at different tumour sites as well as at the same site. Analysis of mode of action is based on physical, chemical, and biological information that helps to explain key events in an agent's influence on tumour development (Boguski, 2003).

A key component of hazard characterization involves evaluating the weight of evidence (WOE) category. Based on the quality and adequacy of data on carcinogenicity, EPA places a chemical in one of the following five weights of evidence categories, as specified in 51 FR 33996 (USEPA, 2017):

A. Carcinogenic to humans

B. Probable carcinogen

B1. Indicates limited human evidence;

B2. Indicates sufficient evidence in animals and inadequate or no evidence in humans;

C. Possible carcinogen;

D. Not classifiable;

E. Evidence of non-carcinogenicity regarding a chemical's potential to cause adverse human health effects (USEPA, 2017).

The weight of evidence narrative may include some standard 'descriptors' that signify certain qualitative threshold levels of evidence or confidence have been met, such as 'Carcinogenic to humans' or 'Suggestive evidence of carcinogenic potential'.

1.8.2.2 Exposure assessment

This is the process of measuring or estimating the magnitude, frequency, and duration of human exposure to an agent in the environment, it is used to calculate a numerical estimate of exposure or dose, or estimating future exposures for an agent that has not yet been released (Boguski, 2003). An exposure assessment includes some discussion of the size, nature, and types of human populations exposed to the agent, as well as discussion of the uncertainties in the above information. Exposure can be measured directly, but more commonly is estimated indirectly through consideration of measured concentrations in the environment, consideration of models of chemical transport and fate in the environment, and estimates of human intake over time (USEPA, 2017).

Different Kinds of Doses: Exposure assessment considers both the exposure pathway (the an agent takes from its source to the person(s) being contacted) as well as the exposure route (means of entry of the agent into the body). The exposure route is generally further described as intake (taken in through a body opening, e. g. as eating, drinking, or inhaling) or uptake (absorption through tissues, e.g. through the skin or eye) (Winston, 2001). EPA defines exposure as 'contact between an agent and the visible and openings into the body)'. The applied dose is the amount of agent at the absorption barrier that is available for absorption. The potential dose is the amount of agent that is ingested, inhaled, or applied to the skin. The applied dose may be less than the

potential dose if the agent is only partly bioavailable. The internal dose or absorbed dose is the amount of an agent that has been absorbed and is available for interaction with biologically significant receptors within the human body. Finally, the delivered dose is the amount of agent available for interaction with any specific organ or cell (USEPA, 2017).

Range of Exposure: For any specific agent or site, there is a range of exposures actually experienced by individuals. Some individuals may have a high degree of contact for an extended period (e.g. factory workers exposed to an agent on the job). Other individuals may have a lower degree of contact for a shorter period (e.g. individuals using a recreational site downwind of the factory). EPA policy for exposure assessment requires consideration of a range of possible exposure levels.

Two common scenarios for possible exposure are "Central Tendency" and "High End".

"Central Tendency" exposure is an estimate of the average experienced by the affected population, based on the amount of agent present in the environment and the frequency and duration of exposure. "High End" exposure is the highest dose estimated to be experienced by some individuals, commonly stated as approximately equal to the 90th percentile exposure category for individuals.

Quantifying Exposure: There are three basic approaches for quantifying exposure. Each approach is based on different data, and has different strengths and Weaknesses; using the approaches in combination can greatly strengthen the credibility of an exposure risk assessment (USEPA, 2017)

2 Point of Contact Measurement: The exposure can be measured at the point of contact (the outer boundary of the body) while it is taking place, measuring both exposure concentration and time of contact, and then integrating them.

3 Scenario Evaluation: The exposure can be estimated by separately evaluating the exposure concentration and the time of contact, then combining this information;

4 Reconstruction: The exposure can be estimated from dose, which in turn can be reconstructed through internal indicators (biomarkers, body burden, excretion levels, etc.) after the exposure has taking place (reconstruction).

1.8.2.3 Dose-response Assessment

This shows the relationship between dose and toxic effect. It describes how the likelihood and severity of adverse health effects (the responses) are related to the amount and condition of exposure to an agent (the dose provided) (Boguski, 2003). Typically, as the dose increases, the measured response also increases. At low doses there may be no response. At some level of dose, the responses begin to occur in a small fraction of the study population or at a low probability rate. Both the dose at which response begin to appear and the rate at which it creasing dose can be variable between different pollutants, individuals, increases given in exposure routes, etc. (Boguski, 2003).

The shape of the dose-response relationship depends on the agent, the kind of response (Tumour, incidence of disease, death, etc.), and the experimental subject (human, animal) in question. For example, there may be one relationship for a response such as ‘weight loss‘ and a different relationship for another response such as ‘death’. Since it is impractical to study all possible relationships for all possible responses, toxicity research typically focuses on testing for a limited number of adverse effects. Typically, as the dose increases response also increases (USEPA, 2017).Upon considering all available studies, the response (adverse effect), or a measure of response that leads to an adverse effect (known as a ‘precursor’ to the effect), that occurs at the lowest dose is selected as the critical effect for risk assessment. The underlying assumption is that if the critical effect is prevented from occurring, then no other effects of concern will occur (Rim and Lim, 2014).

As with hazard identification, there is frequently a lack of dose-response data available for human subjects. When data are available, they often cover only a portion of the possible range of the dose-response relationship, in which case some extrapolation must be done in order to extrapolate to dose levels that are lower than the range of data obtained from scientific studies. Also, as with hazard identification, animal studies are frequently done to augment the available data (USEPA, 2017). Studies using animal subjects permit the use of study design to control the number and composition (age, gender, species) of test subjects, the levels of dose tested, and the measurement of specific responses. Use of a designed study typically leads to more meaningful statistical conclusions than does an uncontrolled observational study were additional confounding factors must also be considered for their impact on the conclusions (Winston, 2001).

1.8.2.4 Risk Characterization

Summarizes and integrates information from the proceeding steps of the risk assessment to synthesize an overall conclusion about risk. Conveys the risk assessor's judgment as to the nature and presence or absence of risks, along With information about how the risk was assessed, where assumptions and uncertainties still exist, and where policy choices will need to be made. Risk characterization takes place in both human health risk assessments and ecological risk assessments (USEPA, 2017).

In practice, each component of the risk assessment (e.g. hazard assessment, dose-response assessment, exposure assessment) has an individual risk characterization written to carry forward the key findings, assumptions, limitations, and uncertainties. The set of these individual risk characterizations provide the information basis to write an integrative risk characterization analysis. The final, overall risk characterization thus consists of the individual risk characterizations plus an integrative analysis (Winston, 2001; Boguski, 2003).

1.8.3 Principles of Conducting Risk Characterizations

A good risk characterization will restate the scope of the assessment, express results clearly, articulate major assumptions and uncertainties, identify reasonable alternative interpretations, and separate scientific conclusions from policy judgments.

EPA‘s Risk Characterization Policy calls for conducting risk characterizations in a manner that is consistent with the following principles:

2 **Transparency**: The characterization should fully and explicitly disclose the risk assessment methods. Defaults assumptions, logic, rationale, extrapolations, uncertainties and the overall strength of each step in the assessment.

3 **Clarity**: The products from the risk assessment should be readily understood by readers inside and outside of the risk assessment process. Documents should be concise, free of jargon, and should use understandable tables, graphs, and equations as needed.

4 **Consistency**: The risk assessment should be conducted and presented in a manner which is consistent with EPA policy and consistent with other risk characterizations of similar scope prepared across program within the EPA

5 **Reasonableness** - The risk assessment should be based on sound judgment, with methods and assumptions consistent with the current state-of-the-science and conveyed in a manner that is complete and balanced, informative.

These four principles are referred to collectively as TCCR. In order to achieve TCCR in a risk characterization, the same principles need to have been applied in all of the prior steps in the risk assessment which lead up to the risk characterization (USEPA, 2017).

1.8.4 Ecological Risk Assessment

This is the process for evaluating the likelihood that the environment may be impacted as a result of exposure to one or more environmental stressors such as chemicals, land change, disease, invasive species and climate change (SETAC, 2004: USEPA, 2017). A key part the ecological risk assessment is the understanding of the potential effects of the stressors and managing the risk from them in order to protect the health of the natural environment and the resources that people rely on (Wang *et al*., 2013).

Ecological risk assessments are used to support many types of actions including:

Regulation of hazardous waste sites, mining sites, industrial chemicals and pesticides;

Management of watersheds

Other ecological effects by multiple chemical, physical or biological stressors.

Ecological risk assessment can be used to predict the likelihood of future effects (prospective) oe evaluate the likelihood that effects are caused by past exposures to stressors (retrospective) (SETAC. 2004).

Ecological risk assessment is divided into 23 phases

- Problem formulation
- Analysis
- Risk characterization (SETAC, 2004; USEPA, 2017).

➢ **Problem Formulation**

The objective of the problem formulation phase is to define an assessment endpoint to determine what ecological entity is important to protect. An ecological entity can be a portion of the ecosystem like a lake, species, community, specified value habitat (Wang *et al*., 2013).

- **Analysis**

The analytical phase provide the ingredient necessary for determining or predicting ecological responses to stressors under exposure conditions of interest. Analysis is the determination of what plants and animal are exposed and to what degree they are exposed and if that level of exposure is likely or not to cause harmful ecological effects. Calculations used may include: Hazard quotient. Metal pollution index, bioaccumulation factor, bioavailability and ingestion rate (Wang *et al.*, 2013; Manoj and Padhy, 2014).

- **Risk Characterization**

This phase involves the use of the results of analysis to estimate the risk posed to ecological entities. The assessor then describes the risk, indicating the overall degree of confidence in the risk estimates, summarizing uncertainties, citing evidence supporting the risk estimate and interpreting the adversity of ecological effects (SETAC, 2004; USEPA, 2017).

1.9 Justification

In Nigeria, the loss of crops due to pest, plant disease and competition from weeds is very high. To avert these losses, farmers now increasingly deploy pesticides in their agronomic practices. Pesticides produced to kill these pests in order to prevent these damages, especially those produced from synthetic materials also tend to have adverse effects on humans and environment in various ways (Yuguda *et al*., 2015). The continuous application of these pesticides in agriculture over the years have led to the accumulation of pesticide residues such as their metabolites and heavy metals, in the environment leading to various chronic illnesses and toxicity to non-target organisms (Oguh *et al*., 2019a).

The indiscriminate use of chlorpyrifos in agricultural pest control and also in household pest control has led to the increase in exposure to this pesticide. This exposure has been implicated in many health challenges, which result in sub chronic, chronic and sometimes lethal health effects.

The study into the risk of exposure to this pesticide will help to assess the effect of unguided use of chlorpyrifos.

1.10 Aim of the Study

The aim of this study is to assess the risk of the different routes (oral and inhalation) of exposure to chlorpyrifos and determine its effects on human health by analysing marker parameters of the organs susceptible to chlorpyrifos toxicity. Furthermore, to determine the risk of bioaccumulation/biomagnification of the pesticide in the tissues of mammals using a probabilistic risk assessment model.

1.11 Specific Objectives of the Study

The specific objectives of the study were:

- To determine the median lethal dose (LD_{50}) of chlorpyrifos.
- To determine the median lethal concentration (LC_{50}) of chlorpyrifos.
- To determine the activity of AST, ALT and ALP.
- To determine the activity of CAT and GPx.
- To determine the concentration of MDA and GSH.
- To determine the concentration of testicular Cholesterol, Testosterone and Sperm.
- To determine the activity of brain acetylcholinesterase.
- To estimate liver cholesterol accumulation.
- To predict alternative mechanism of chlorpyrifos toxicity.
- To determine the bioaccumulation factor (BAF) of chlorpyrifos.
- To determine the Average daily dose (ADD) and Hazard quotient (HQ).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All chemicals used in this study were of analytical grade and were obtained from Merck (Germany), May and Baker (England), Sigma Chemical Company (USA), or BDH Chemicals limited (England). Sterile distilled and deionized water were used in the preparation of the chemicals and/or reagents. The pesticide Chlorview® (Chlorpyrifos 40% E.C.) was purchased from commercial agro-chemical vendor at Ogige market in Nsukka, Enugu State, Nigeria.

2.1.2 Equipment and Instruments

The equipment used was found in the Department of Biochemistry and other laboratories in the University of Nigeria Nsukka, as well as the Central Research Laboratory and Diagnostic Laboratory, Ilorin, Kwara state, Nigeria. They include: Spectrophotometer (VIS SPEC 721) from Axion Medical United Kingdom, Microtitre plate reader, Gas chromatography-Mass Spectrophotometer (GC-MS) from Agilent USA (5975C), Omron compressor Nebulizer (NE-C28P) from Omron healthcare United Kingdom, and Sperm analyser and host of other instruments.

2.1.3 Animals

Wistar rats were purchased from animal house of the University of Nigeria Nsukka and fed with top finisher feed throughout the period of the experiment.

2.2 Methods

2.2.1 Experimental Design

A total of 64 male wistar rats were used for the experiment. The animals were divided into two groups for oral (36 rats) and inhalation (28 rats) routes of exposure. Acute toxicity studies were carried out using 16 animals for oral exposure and 12 animals for inhalation exposure. The experimental groups were composed of 20 divided into 4 groups of 5 animals each for oral route of exposure and 16 animals divided into 4 groups of 4 animals each for inhalation route of

exposure. The four experimental groups consist of 3 treatment groups and a control group. The treatment doses were obtained by dividing the value of the median lethal dose (LD50) established from the acute studies by a factor of 40, 25 and 10 to get 3 treatment doses representing groups 1, 2 and 3 respectively and group 4 (control group) for both routes of exposure.

The oral group was administered via guavage, once a day for 10days while the inhalation group was administered using an Omron nebulizer, the doses per body weight of the animals were calculated and measured into the nebulizer and then allowed to nebulize while the animals inhale for 60mins per day for 10days.

2.2.1 Acute toxicity Studies

Acute toxicity studies were carried out using a modified Lorke's (1983) method. Oral acute toxicity study was done using 16 wistar rats of weight range 140g to 200g divided into 4 groups of 4 animals each. Adjusted doses of 50, 120, 200 and 270 mg/kg b.w. of the pesticide were administered to each group representing groups 1, 2, 3 and 4 respectively. This reflects the range of LD50 values already reported in various literatures.

Inhalation acute toxicity was carried out using 12 wistar rats of weight range 133g to 223g divided into 4 groups of 3 animals each, adjusted doses of 1000, 2000, 3000 and 6000 mg/kg b.w. of the pesticide was administered to each group representing groups 1, 2, 3 and 4 respectively. This reflects the range of LD_{50} values already reported in various literatures.

Experimental animals were observed for signs of sub-acute and acute toxicity for and the median lethal dose (LD_{50}) determined after 24 hours.

2.2.2 Determination of median lethal dose (LD_{50})

The median lethal dose was determined using the following formula

$LD_{50} = \sqrt{(D_0 \times D_{100})}$

D_0 = Highest dose that gave no mortality

D_{100} = lowest dose that gave mortality

Converting Emulsifiable Concentration (E.C.) to mg/ml using the method of Lacarin and Reed (1999)

$(g/L) \div 10 = E.C.$

$\therefore (g/L) = E.C. x\ 10$

For 40% E.C.

$(g/L) = 40\ x\ 10 = 400g/L$

- To convert g/L to mg/m

1g = 1000mg

1L = 1000ml

$400\text{g/L} = (400 \text{ x } 1000)\ /1000 = 400\text{mg/ml}$

∴ there is 400mg of chlorpyrifos in 1ml of the pesticide.

2.2.3 Assay for Aspartate Aminotransferase Activity

PRINCIPLE: AST present in the sample catalyzes the transfer of the amino group from L-aspartate to 2-oxoglutarate, in the presence of pyridoxal-5'- phosphate, forming oxaloacetate and L-glutamate. Oxaloacetate in the presence of NADH and malate dehydrogenase (MDH) is reduced to L-malate.

PROCEDURE: Aspartate Aminotransferase Activity using Reitman and Frankel (1957) method. Standard procedures of RANDOX test kit was used for the assay.

2.2.4 Assay for Alanine Aminotransferase Activity

PRINCIPLE: Alanine aminotransferase (ALT/GPT) catalyzes the transfer of the amino group from alanine to oxoglutarate with the formation of glutmate and pyruvate. The latter is reduced to lactate by lactate dehydrogenase (LDH) in the presence of reduced nicotinamide adenine dinucleotide (NADH). The reaction is monitored kinetically at 340 nm by the rate of decrease in absorbance resulting from the oxidation of NADH to NAD+, proportional to the activity of ALT present in the sample.

PROCEDURE: Alanine Aminotransferase Activity using Reitman and Frankel (1957) method. Standard procedures of RANDOX test kit was used for the assay.

2.2.5 Assay for Alkaline phosphatase Activity

PRINCIPLE: Alkaline phosphatases catalyze the hydrolysis of a wide variety of physiologic and non-physiologic phosphoric acid esters in alkaline medium. The natural substrates for these enzymes have not yet been identified. Thus, a variety of synthetic substrates have been used in assay methods for ALP, the selection of which has been largely a matter of convenience. This method requires measuring the rate of phosphate liberation against the background level of endogenous phosphate.

PROCEDURE: Colorimetric Method using RX Monza

Standard procedures of RX Monza test kit was used for the assay.

Calculation:

To calculate the ALP activity, the following formula was used

U/L = $2760 \times \Delta A\ 405 nm/min$ MICRO

2.2.6 Determination of Reduced Glutathione Concentration

The method proposed by Moron *et al*. (1979) was used for the estimation of reduced glutathione. A 20% serum was obtained by homogenizing 0.5g of sample in 2.5 ml of 5% TCA. To precipitate the protein 125 µl of 25% TCA was added to 0.5 ml of serum. The precipitated protein was centrifuged at 1000rpm for 10 mins. The serum was cooled on ice and 0.1 ml of the supernatant was taken for the estimation. The supernatant was made up to 1 ml with 0.2M sodium phosphate buffer (pH 8.0). 2.0 ml of freshly prepared DTNB solution was added to the tubes and the intensity of the yellow colour formed was read at 412 nm in a spectrophotometer after 10 mins. A standard curve of GSH was prepared using concentrations ranging from 2-10 nmoles of GSH in an electronic calculator set to the linear regression mode and the values of the samples were read off it. The values are expressed as nmoles of GSH /g serum.

2.2.7 Determination of Malondialdehyde Concentration

The level of Lipid peroxides was estimated by Thiobarbituric acid reaction method described by Ohkawa *et al*., (1979). To 0.2 ml of test sample, 0.2 ml of SDS, 1.5ml of acetic acid and 15 ml of TBA were added. The mixture was made up to 4 ml with water and then heated in a water bath 95^{o} C for 60 minutes. After cooling, 1ml of water and 5 ml of n-butanol/pyridine mixture were added and shaken vigourously. After centrifugation at 4000 rpm for 10 minutes, the organic layer was taken and its absorbance was read at 532 nm. The level of lipid peroxides was expressed an nmoles of MDA released/g wet tissue.

Calculation

$$\text{The concentration of MDA} = \frac{\text{Absorbance at 532 nm x D}}{\text{L x } \varepsilon}$$

Where,

L= light path (1cm).

ε = extinction coefficient 1.56 x 105 M-1.Cm-1.

D: dilution factor = Total Vol (10ml)/ Vol of the sample (0.2ml)

2.2.8 Assay for Catalase Activity

Catalase activity was measured by the method of Aebi.1983. 0.1 ml of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate butter (pH 7.0). Reaction was started by the addition of l.0ml of freshly prepared 50 mM H_20_2. The rate of decomposition of H_2O_2 Was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as units/mg protein. A unit is defined as the velocity constant per second. The reaction occurs immediately after the addition of H_20_2. Solutions are mixed well and the first absorbance (A1) is read after 15 seconds (t1) and the second absorbance (A2) after 30 seconds
(t2). The absorbance is read at wave length 240 nm.

Calculation

$$K = \frac{Vt}{Vs} x \frac{2.3}{t} \text{ x Log} \frac{A1}{A2} \text{ x } 60$$

Where,
K= Rate constant of the reaction.
t= (t2- t1) = 15 seconds.
Al= absorbance after 15 seconds.
A2 = absorbance after 30 seconds.
Vt = total volume (3 ml).
Vs=volume of the sample (0.1ml).

2.2.9 Assay for Glutathione Peroxidase activity

About 3-ml cuvette containing 2.0 ml of phosphate buffer(75mmol/L, PH 7.0) , 50ul of (60mmol/L) glutathione reductase solution, 50ul of (0.12mol/L) NaN_3, 0.I ml of (0.15mmol/L) Na^{2+} EDTA , 100uL of (3.0 mmol/L) NADPH, and 100ul of tissue supernatant was added. Water was added to make a total volume of 2.9ml. The reaction was started by the addition of 100uL of (7.5 mmol/L) H_20_2, and the Conversion of NADPH to NADP was monitored by a continuous recording of the change of absorbance at 340 nm at 1-minutes interval for 5 min. Enzyme activity of GPx was expressed in terms of mg of proteins (Ellman, 1959).

Calculations

$$\text{Enzyme activity (M/min/ml)} = \text{A340/min x } \frac{Vt}{e} \text{ x d x Vs}$$

Where,
e= 6.22 x 10^6 $M^{-1}cm^{-1}$
d = 1cm
Vt = Total volume (3.0)
Vs = Sample volume (0.1 ml)

2.2.10 Determination of Testosterone Concentration

Testosterone level concentration was determined using the method of Ekins, (1990). The desired number of coated wells was secured in a holder, 25µl of the standard, specimen and control was dispensed into appropriate well. 50µl of rabbit anti-testosterone reagent was dispensed to each well

and mixed thoroughly for 30 minutes. A 100µl of testosterone-HRP conjugate reagent was dispensed into each well and incubated at 37°C for 60 minutes. The microwells were rinsed and flicked 5 times with washing buffer (1x). 100µl of TMB substrate was dispensed to each well and mixed gently for 10 seconds and then incubated at room temperature (18-22°C) for 20 minutes. The reaction was stopped by adding 100µl of stop solution to each well and gently mixed for 30 seconds for complete color change. The absorbance was read at 450 nm with a microtiter well reader within 15 minutes.

2.2.11 Determination of Cholesterol Concentration

Cholesterol level was determined using the method of Allian *et al*., (1974). Reagents were prepared according to instructions on the vial label, Test tubes were labeled: blank, standard, control, sample. 1.0ml of the reagent was added to all tubes and pre warmed at 37°C for at least two minutes. 0.01 ml of sample was added to respective tubes, mix and returned to 37°C. All tubes were incubated at 37°C for ten minutes. The reagent blank was used to zero the spectrophotometer at 520 nm and then absorbance read and recorded for all tubes.

2.2.12 Sperm Analysis

Sperm Analysis carried out using the method of Ochei and Kolhatkar (2000). The Neubauer chamber having a grid containing 1-5 large squares was used. The central square is subdivided into 25 smaller squares of which the 4 corner squares are designated 5a, 5b, 5c, 5d and the central small square as 5e. The depth of the chamber in 0.1 mm, so the volume of fluid held between cover slip and chamber is 0.1cu.mm, and the volume in 5a, 5b, 5c, 5d and 5e is 0.02 cu.mm

To calculate the number of spermatozoa per ml counted in the chamber, a multiplication factor is used. The multiplication factor for square 5 is 10,000, for all large squares 1-5, the factor is 2000: for the smaller squares 5a, 5b, 5c, 5d and 5e, the multiplication factor is 50,000.

Calculation

No of sperm cells counted in 5a, 5b, 5c, 5d and 5e = n
Multiplication factor = 50,000
Dilution factor = 20
Sperm count per ml = n x 50,000 x 20

$= n \times 10^6$

Total sperm count $= n \times 10^6 \times$ volume of semen

2.2.13 Assay for Acetylcholinesterase Activity

Acetylcholinesterase activity was determined using the method of Doctor *et al*. (1987).

1. Standard wells, testing sample wells were set and 50µl of standard was added to standard well.
2. Blank wells were set separately (for blank comparison wells, do not add sample and HRP-conjugate reagent, other each step operation is the same). 40µl of sample dilution was added to testing sample well, then 10µl testing sample was added (sample final dilution is 5-fold), and gently mixed.
3. The enzyme, HRP-conjugate reagent 100µl was added to each well, except blank well.
4. Mixture was incubated by closing plate with closure membrane for 60mins at 37°C.
5. Configurate liquid containing 20-fold wash solution diluted 20-fold with distilled water was prepared and reserved.
6. Plate was washed after uncovering closure plate membrane, liquid discarded, dry by swinging and washing buffer added to every well. Still for 30 secs then drained, repeated 5 times, and dried by pat.
7. Chromogen solution A 50µl and Chromogen solution B was added to each well to produce colour, and preserved in the dark for 15 min at 37°C
8. The reaction was stopped by adding 50µl of stop solution to each well, (the blue color change to yellow color).
9. Blank well was read as zero, and absorbance read at 450nm using microtiter plate reader within 15 minutes.

2.2.14 Identification of volatile compounds

A gas chromatography from Agilent USA hypherated to a mass spectrophotometer (5975C) with triple axis detector equipped with an auto injector (10µl syringe) was used, Helium gas was used as a carrier gas.

All chromatographic separation was performed on a capillary column having specification, length; 30m, internal diameter; 0.2µm, thickness; 250µm, treated with phenyl methyl silox. Other GC-MS conditions are ion source temperature (EI), 250°C, interface temperature; 300°C, pressure; 16.2 psia, out time; 1.8mm, 1µl injector in split mode with split ratio 1.50 with injector temperature of 300°C. The column temperature started at 35°C for 5mins and changed to 150°C at the rate of 4°C/min. the temperature was raised to 250°C at the rate of 20°C/min and held for 5mins. The total elution was 47.5mins; MS solution software provided by supplier was used to control the system and to acquire the data. Identification of compounds was carried out by comparing the mass spectra obtained with those of the standard mass spectra from NIST library (NISTII). Machine Model: 7890A GC system, 5675C Inert MSD with triple-Axis detector.

2.2.15 Molecular Docking Study on Chorview® (Chlorpyrifos 40% E. C.)

3D Structures of HMG CoA reductase, HMG CoA synthase synthase and β ketoacyl ACP synthase with PDB IDs: 3BGL, 1TVZ and 2IWZ respectively were obtained from protein databank (www.rcsb.org). The existing ligands and water molecules were removed and Hydrogen molecules were added. SDF structures of Chlorpyrifos and standard inhibitor i.e. Mevastatin was obtained from the PubChem database. The molecules were converted to mol2 chemical format using Open babel (O'Boyle et al., 2011). The protein and ligand molecules were further converted to the dockable pdbqt format using Autodock tools. Docking of the ligands to various protein targets and determination of binding affinities was carried out using Autodock Vina (Trott & Olson, 2010). Also the proteins were docked to standard inhibitor and the binding affinities were determined. Molecular interactions between proteins and ligands were viewed with Discovery Studio 2016.

2.2.16 Determination of bioaccumulation factor (BAF)

The transfer coefficient was calculated by dividing the concentration of chlorpyrifos in animal tissue by the total chlorpyrifos concentration applied. (Olowoyo *et al*., 2010).

$$\text{BAF} = \frac{\text{C liver}}{\text{C admin}}$$

Where; BAF represent the transfer factor of liver

C_{liver} = pesticide concentration in liver tissue,

C_{admin} = pesticide concentration in soil,

BAF > 1 indicates that the liver accumulated pesticide residues (Bio-accumulation)

BAF < 1 means that the liver excluded the pesticide residues from the body.

2.2.17 Determination of Average daily dose (ADD)

The Daily intake of metal was calculated using the following formula used by (Olowoyo and Lion, 2013).

$ADD = DI \times MF_{liver}/WB$

Where; ADD = represents the average daily dose (mg/kg/d) of the pesticide.

DI = is the daily intake of chlorpyrifos (for adults & for children).

MF_{LIVER} = is the trace pesticide concentration in the tissues (mg/kg)

WB = represent the body weight of investigated animal

.

2.2.18 Determination of Hazard quotient (HQ)

The Hazard Quotient (HQ) was used to calculate the possible human health risks associated with the exposure of chlorpyrifos to farmers, applicators and household. The following equation (Nabulo *et al.,* 2010) for calculating human health risk of exposure of chlorpyrifos used to calculate the Hazard Quotient of animal tissue.

HQ is the ratio between exposure and the reference oral dose (RFD)

If the ratio is lower than one 1, there will be no obvious risk.

HQ = ADD/RFD

Where; ADD = represents the average daily dose (mg,kg/d) of the chlorpyrifos.

RFDM = is the reference dose (mg,kg/d)

RFDM = is define as the maximum tolerable daily intake of chorpyrifos with no adverse effect.

2.2.19 Statistical Analysis

IBM SPSS software version 23 was used to carry out the statistical analysis. A one way analysis of variance (ANOVA) was carried out at $\alpha = 0.05$, and Duncan's multiple range test was used to show the source of the observed differences.

CHAPTER THREE

RESULTS

3.1 Acute Toxicity

3.1.1 Oral Acute Toxicity

A 24 hour Oral acute toxicity study was carried out in order to establish a median lethal dose for Chorview® (Chlorpyrifos 40% E. C.). Table 1 result shows that group 1 to group 4 with dose of 50, 120, 200, and 270 mg/kg respectively were observe and there were mortality rate at group 3 and 4 with 1 and 2 death respectively. No sign of toxicity was observed in group 1 with 50 mg/kg dose and group 2 were not fully active as usual which indicate a sign of toxicity also. The acute toxicity test shows that the higher the concentration of Chloropyrifos the more the toxicity.

Table 1: 24 hour oral acute toxicity study

Groups	Dose in mg/kg body weight	Observation	Mortality
Group 1	50	No sign of toxicity	0/4
Group 2	120	Signs of toxicity	0/4
Group 3	200	Signs of toxicity	1/4
Group 4	270	Signs of toxicity	2/4

Median Lethal Dose (LD_{50}) = 155 mg/kg b.w.

3.1.2 Inhalation Acute Toxicity

A 24 hour inhalation toxicity studies was carried out in order to establish a median lethal concentration for Chorview® (Chlorpyrifos 40% E. C.). Table 2 result shows that group 1 to group 4 with dose of 1000, 2000, 3000, and 6000 mg/kg respectively were observed for signs of toxicity/death and there were mortality for group 2, 3 and 4 with 33%, 100% and 100% deaths respectively. No toxicity sign was observed in group 1 with dose of 1000 mg/kg b.w. The acute toxicity test shows that the higher the concentration of Chloropyrifos the more the toxicity as shown in table 2.

Table 2: 24 hour inhalation acute toxicity study

Groups	Dose in mg/kg body weight	Observation	Mortality
Group 1	1000	No sign of toxicity	0/3
Group 2	2000	Signs of toxicity	1/3
Group 3	3000	Signs of toxicity	3/3
Group 4	6000	Signs of toxicity	3/3

Median Lethal Concentration (LC_{50}) = 1414 mg/kg b.w.

3.2 Hepatotoxicity

3.2.1 Oral Hepatotoxicity

Table 3 shows the effects of oral exposure to Chorview® (Chlorpyrifos 40% E. C.) on the Liver marker enzymes of wistar rats. The activities of Aspartate amino transferase (AST), Alanine amino transferase (ALT) and Alkaline phosphatase (ALP) in group 1(3.8mg/kg b.w) and group 2 (6.2mg/kg b.w) increased non-significantly ($p>0.05$) when compared with the Control group.

However there was a significant ($p<0.05$) increase in the activities of Aspartate amino transferase (AST), Alanine amino transferase (ALT) and Alkaline phosphatase (ALP) in group 3 (15.5mg/kg b.w) when compared to group 4 (Control).

Table 3: The effect of oral exposure to Chorview® (Chlorpyrifos 40% E. C.) on Liver marker enzymes

GROUPS	TREATMENT	AST (U/L)	ALT (U/L)	ALP (U/L)
Group 1	**3.8mg/kg CPF**	27.00±1.73[a]	5.00±1.00[a,b]	30.63±1.12[a,b]
Group 2	**6.2mg/kg CPF**	29.33±3.79[a]	7.33±1.53[b]	31.86±2.07[a,b]
Group 3	**15.5mg/kg CPF**	46.00±4.00[b]	11.67±2.08[c]	32.92±1.89[b]
Group 4	**Control**	25.67±3.21[a]	4.33±0.58[a]	29.53±0.42[a]

Means with the same superscript across the groups are non-significantly (p>0.05) different. n = 3

AST: Aspartate amino transferase,

ALT: Alanine amino transferase,

ALP: Alkaline phosphatase,

Group 1: 3.8mg/kg b.w of chlorpyrifos,

Group 2: 6.2mg/kg b.w of chlorpyrifos,

Group 3: 15.5mg/kg b.w of chlorpyrifos,

Group 4: control.

3.2.2 Inhalation Hepatotoxicity

Table 4 shows the effects of Inhalation exposure to Chorview® (Chlorpyrifos 40% E. C.) on the Liver marker enzymes of wistar rats. There was a significant ($p<0.05$) increase in the activities of Aspartate amino transferase (AST), Alanine amino transferase (ALT) and Alkaline phosphatase (ALP) in group 2 (56.57 mg/kg b.w) and group 3 (141.421 mg/kg b.w) when compared to group 4 (Control). However there was a non-significant ($p>0.05$) increase in the activities of Aspartate amino transferase (AST) and Alkaline phosphatase (ALP) of group 1 when compared to group 4 (Control).

Table 4: The effect of inhalation exposure to Chorview® (Chlorpyrifos 40% E. C.) on Liver marker enzymes

GROUPS	TREATMENT	AST (U/L)	ALT (U/L)	ALP (U/L)
Group 1	**35.36 mg/kg CPF**	53.12±2.09^{a,b}	41.33±8.73^{b}	238.74±26.67^{a,b}
Group 2	**56.57 mg/kg CPF**	69.62±9.79^{b}	60.80±5.73^{c}	235.47±3.84^{a,b}
Group 3	**141.421 mg/kg CPF**	94.47±6.87^{c}	64.50±0.71^{c}	269.56±57.25^{b}
Group 4	**Control**	49.81±2.99^{a}	21.04±1.96^{a}	170.27±9.00^{a}

Means with the same superscript across the groups are non-significantly (p>0.05) different. n = 2

AST: Aspartate amino transferase,

ALT: Alanine amino transferase,

ALP: Alkaline phosphatase

Group 1: 35.36 mg/kg b.w of chlorpyrifos,

Group 2: 56.57 mg/kg b.w of chlorpyrifos,

Group 3: 141.421 mg/kg b.w of chlorpyrifos,

Group 4: control

3.3 Oxidative Stress

3.3.1 Oral Oxidative Stress

Table 5 shows the effects of oral exposure to Chorview® (Chlorpyrifos 40% E. C.) on the oxidative stress markers and antioxidant enzymes of wistar rats. There was a significant ($p<0.05$) increase in the Catalase (CAT) activity of group 3 (15.5mg/kg b.w) when compared to group 4 (Control). However, there was a non-significant ($p>0.05$) increase in the Catalase activity of group 1 (3.8mg/kg b.w) and group 2 (6.2mg/kg b.w) when compared to group 4 (Control).

Furthermore, there was a non-significant ($p>0.05$) decrease in the activities of Glutathione peroxidase (GPx) across group 1(3.8mg/kg b.w), group 2 (6.2mg/kg b.w) and group 3 (15.5mg/kg b.w) when compared to group 4 (Control).

The concentration of Glutathione (GSH) also showed a non-significant ($p>0.05$) decrease across group 1(3.8mg/kg b.w), group 2 (6.2mg/kg b.w) and group 3 (15.5mg/kg b.w) when compared to group 4 (Control).

In addition, there was a significant ($p<0.05$) increase in the concentration of Malondialdehyde (MDA) of group 3 (15.5mg/kg b.w) when compared to group 4 (Control). However, there was a non-significant ($p>0.05$) increase in the concentration of Malondialdehyde (MDA) of group 1 (3.8mg/kg b.w) and group 2 (6.2mg/kg b.w) when compared to group 4 (Control).

Table 5: The effect of Oral exposure to Chorview® (Chlorpyrifos 40% E. C.) on antioxidant biomarkers

GROUPS	TREATMENT	GSH (mg/dL)	MDA (mg/dL)	CAT (U/mg)	GPx (U/mg)
Group 1	**3.8mg/kg CPF**	2.96±0.79^{y}	1.22±0.14^{y}	5.29±0.50^{y}	29.97±2.05^{y}
Group 2	**6.2mg/kg CPF**	2.94±0.41^{y}	1.26±0.04^{y}	5.50±0.80^{y}	28.93±0.57^{y}
Group 3	**15.5mg/kg CPF**	2.71±0.57^{y}	1.59±0.43^{z}	6.82±1.10^{z}	28.58±1.16^{y}
Group 4	**Control**	3.09±0.19^{y}	1.12±0.10^{y}	4.68±0.71^{y}	30.48±3.61^{y}

Means with the same superscript across the groups are non-significantly (p>0.05) different.

n = 3

GPx: Glutathione peroxidase,

GSH: Glutathione,

MDA: Malondialdehyde,

CAT: Catalase,

Group 1: 3.8mg/kg b.w of chlorpyrifos,

Group 2: 6.2mg/kg b.w of chlorpyrifos,

Group 3: 15.5mg/kg b.w of chlorpyrifos,

Group 4: control

3.3.2 Inhalation Oxidative Stress

Table 6 shows the effects of Inhalation exposure to Chorview® (Chlorpyrifos 40% E. C.) on the oxidative stress markers and antioxidant enzymes of wistar rats. There was a significant ($p<0.05$) increase in the concentration of Malondialdehyde (MDA) and Catalase activity of group 3 (15.5mg/kg b.w) when compared to group 4 (Control). However, there was a non-significant ($p>0.05$) increase in the concentration of MDA and Catalase activity of group 1 (3.8mg/kg b.w) and group 2 (6.2mg/kg b.w) when compared to group 4 (Control).

Furthermore, there was a significant ($p<0.05$) decrease in the concentration of Glutathione (GSH) in group 1 (35.36 mg/kg b.w.), group 2 (56.57 mg/kg b.w) and group 3 (141.421 mg/kg b.w) when compared to group 4 (Control). Although there was a non-significant ($p>0.05$) decrease between group 2 (56.57 mg/kg b.w)and group 3.

In addition, there was a significant ($p<0.05$) increase in Glutathione peroxidase (GPx) activity in group 2 (56.57 mg/kg b.w) and group 3 (141.421 mg/kg b.w) when compared to group 4 (Control). Although there was a non-significant ($p>0.05$) decrease between group 2(56.57 mg/kg b.w) and group 3 (141.421 mg/kg b.w).

Table 6: The effect of Inhalation exposure to Chorview® (Chlorpyrifos 40% E. C.) on antioxidant biomarkers

GROUPS	TREATMENT	GSH (mg/dL)	MDA (mg/dL)	CAT (U/mg Protein)	GPx (U/mg Protein)
Group 1	**35.36 mg/kg CPF**	146.39 ± 0.50^{y}	0.64 ± 0.001^{x}	138.81 ± 19.15^{x}	315.39 ± 6.99^{x}
Group 2	**56.57 mg/kg CPF**	142.14 ± 0.50^{z}	0.67 ± 0.010^{x}	155.13 ± 3.39^{x}	151.56 ± 1.25^{y}
Group 3	**141.421 mg/kg CPF**	140.02 ± 1.50^{z}	1.33 ± 0.110^{y}	195.73 ± 47.09^{y}	130.61 ± 2.39^{y}
Group 4	**Control**	149.94 ± 1.50^{x}	0.61 ± 0.83^{x}	112.04 ± 9.57^{x}	334.52 ± 16.14^{x}

Means with the same superscript across the same groups are non-significantly ($p>0.05$) different. n = 2

GPx: Glutathione peroxidase,

GSH: Glutathione,

MDA: Malondialdehyde,

CAT: Catalase

Group 1: 35.36 mg/kg b.w of chlorpyrifos,

Group 2: 56.57 mg/kg b.w of chlorpyrifos,

Group 3: 141.421 mg/kg b.w of chlorpyrifos,

Group 4: control

3.4 Reproductive Toxicity

3.4.1 Oral Reproductive Toxicity

Table 7 shows the effects of oral exposure to Chorview® (Chlorpyrifos 40% E. C.) on the reproductive toxicity markers of wistar rats. There was a significant ($p<0.05$) increase in the Cholesterol concentration of group 3 (15.5mg/kg b.w) when compared to group 4 (Control). Whereas there was a non-significant ($p>0.05$) increase in group 1 (3.8mg/kg b.w) and group 2 (6.2mg/kg b.w) when compared to group 4 (Control).

However, there was a significant ($p<0.05$) decrease in the Testosterone concentration of group 3 (15.5mg/kg b.w) when compared to group 4 (Control). Although there was a non-significant ($p>0.05$) decrease in Testosterone concentration of group 1 (3.8mg/kg) and group 2 (6.2mg/kg b.w) when compared to group 4 (Control).

In addition**,** there was a significant ($p<0.05$) decrease in the Sperm count of group 1 (3.8mg/kg b.w), group 2 (6.2mg/kg b.w) and group 3 (15.5mg/kg b.w) when compared to group 4 (Control).

Table 7: The effect of Oral exposure to Chorview® (Chlorpyrifos 40% E. C.) on reproductive toxicity markers

GROUPS	TREATMENT	CHOL (mg/dl)	TESTOS (ng/ml)	SPERM ($X10^6$/mL)
Group 1	**3.8mg/kg CPF**	165.87 ± 17.78^{p}	2.87 ± 0.29^{p}	70.00 ± 2.00^{q}
Group 2	**6.2mg/kg CPF**	185.59 ± 13.92^{p}	2.78 ± 0.27^{p}	$66.00\pm1.00^{p,q}$
Group 3	**15.5mg/kg CPF**	224.65 ± 6.57^{q}	1.30 ± 0.12^{q}	60.67 ± 5.77^{p}
Group 4	**Control**	151.96 ± 32.01^{p}	3.76 ± 1.09^{p}	82.00 ± 6.00^{r}

Means with the same superscript across the groups are non-significantly (p>0.05) different. n = 3

CHOL: Cholesterol,

TESTOS: Testosterone,

SPERM: Sperm count

Group 1: 3.8mg/kg b.w of chlorpyrifos,

Group 2: 6.2mg/kg b.w of chlorpyrifos,

Group 3: 15.5mg/kg b.w of chlorpyrifos,

Group 4: control

3.4.2 Inhalation Reproductive Toxicity

Table 8 shows the effects of inhalation exposure to Chorview® (Chlorpyrifos 40% E. C.) on the reproductive toxicity markers of wistar rats. There was a significant ($p<0.05$) increase in the Cholesterol concentration of group 1 (35.36 mg/kg b.w), group 2 (56.57 mg/kg b.w) and group 3 (141.421 mg/kg b.w) when compared to group 4 (Control).

However, there was a significant ($p<0.05$) decrease in the testosterone concentration of group 1 (35.36 mg/kg b.w), group 2 (56.57 mg/kg b.w) and group 3 (141.421 mg/kg b.w) when compared to group 4 (Control).

In addition, there was a significant ($p<0.05$) decrease in the Sperm count of group 1 (35.36 mg/kg b.w), group 2 (56.57 mg/kg b.w) and group 3 (141.421 mg/kg b.w) when compared to group 4 (Control).

Table 8: The effect of Inhalation exposure to Chorview® (Chlorpyrifos 40% E. C.) on reproductive toxicity markers

GROUPS	TREATMENT	CHOL (mg/dl)	TESTOS (ng/mL)	SPERM ($X10^6$/mL)
Group 1	35.36 mg/kg $_{CPF}$	196.10±21.80^{b}	10.18±0.23^{b}	71.50±2.12^{c}
Group 2	56.57 mg/kg $_{CPF}$	237.07±22.90^{b}	9.73±0.36^{a,b}	63.50±0.71^{b}
Group 3	141.421 mg/kg $_{CPF}$	300.29±0.28^{c}	9.20±0.17^{a}	53.00±1.41^{a}
Group 4	Control	86.28±5.68^{a}	10.96±0.06^{c}	86.50±0.71^{d}

Means with the same superscript across the groups are non-significantly (p>0.05) different. n = 2

CHOL: Cholesterol,

TESTOS: Testosterone,

SPERM: Sperm count

Group 1: 35.36 mg/kg b.w of chlorpyrifos,

Group 2: 56.57 mg/kg b.w of chlorpyrifos,

Group 3: 141.421 mg/kg b.w of chlorpyrifos,

Group 4: control

3.5 Neurotoxicity

3.5.1 Oral neurotoxicity

Table 9 shows the effects of oral exposure to Chorview® (Chlorpyrifos 40% E. C.) on the Brain Acetylcholinesterase activity of wistar rats. There was a significant ($p<0.05$) decrease in the Acetylcholinesterase activity of group 3 (15.5mg/kg b.w) when compared to group 4 (Control). Whereas there was a non-significant ($p>0.05$) increase in Acetylcholinesterase activity of group 1 (3.8mg/kg b.w) and group 2 (6.2mg/kg b.w) when compared to group 4 (Control).

Table 9: The effect of Oral exposure to Chorview® (Chlorpyrifos 40% E. C.) on Acetylcholinesterase activity

GROUPS	TREATMENT	AChE (U/mg)
Group 1	**3.8mg/kg CPF**	0.66 ± 0.03^{a}
Group 2	**6.2mg/kg CPF**	0.62 ± 0.04^{a}
Group 3	**15.5mg/kg CPF**	0.52 ± 0.03^{b}
Group 4	**Control**	0.66 ± 0.03^{a}

Means with the same superscript across the groups are non-significantly (p>0.05) different. n = 3

AChE: Acetylcholinesterase

Group 1: 3.8mg/kg b.w of chlorpyrifos,

Group 2: 6.2mg/kg b.w of chlorpyrifos,

Group 3: 15.5mg/kg b.w of chlorpyrifos,

Group 4: control

3.5.2 Inhalation neurotoxicity

Table 10 shows the effects of inhalation exposure to Chorview® (Chlorpyrifos 40% E. C.) on the Brain Acetylcholinesterase activity of wistar rats. There was a significant ($p<0.05$) decrease in the Acetylcholinesterase activity of group 1 (35.36 mg/kg b.w), group 2 (56.57 mg/kg b.w) and group 3 (141.421 mg/kg b.w) when compared to group 4 (Control).

Table 10: The effect of Inhalation exposure to Chorview® (Chlorpyrifos 40% E. C.) on Acetylcholinesterase activity

GROUPS	TREATMENT	AChE (U/l)
Group 1	**35.36 mg/kg CPF**	2.60 ± 0.04^{c}
Group 2	**56.57 mg/kg CPF**	2.34 ± 0.09^{b}
Group 3	**141.421 mg/kg CPF**	1.60 ± 0.14^{a}
Group 4	**Control**	2.89 ± 0.07^{d}

Means with the same superscript across the groups are non-significantly (p>0.05) different. n = 2

AChE: Acetylcholinesterase

Group 1: 35.36 mg/kg b.w of chlorpyrifos,

Group 2: 56.57 mg/kg b.w of chlorpyrifos,

Group 3: 141.421 mg/kg b.w of chlorpyrifos,

Group 4: control

3.6 Liver Cholesterol Accumulation

Table 11 shows the accumulation of the Cholesterol in the Liver of wistar rats exposed to the pesticide via oral and inhalation route. Cholesterol was detected in group 2 of both oral and inhalation route of exposure and also in group 3 of inhalation exposure in extremely high concentration.

However, cholesterol was not detected in group 1 and group 4 of both oral and inhalation route of exposure and also in group 3 of oral route of exposure to chlorpyrifos.

Table 11: Liver Cholesterol Accumulation in the liver of wistar rats exposed to Chorview® (Chlorpyrifos 40% E. C.)

GROUPS	Cholestrol concentration (mg/dl)	
	Oral	Inhalation
Group 1	ND	ND
Group 2	53782.4	53783.3
Group 3	ND	99820.7
Group 4	ND	ND

ND – Not Detected

3.7 Molecular Docking Study on Chorview® (Chlorpyrifos 40% E. C.)

3.7.1 Molecular Docking Study on HMG CoA reductase

Table 12 shows the binding affinities of a standard ligand (Mevastatin) and chlorpyrifos docked against the binding site of the rate limiting enzyme in cholesterol synthesis pathway, HMG-CoA reductase.

Higher binding affinity of the sample (chlorpyrifos) compared to the standard ligand (Mevastatin) indicates activation/potentiation of the enzyme whereas lower binding affinity of the sample (chlorpyrifos) compared to the standard ligand (Mevastatin) indicates an inhibition of the enzyme HMG-CoA reductase.

Table 12: Binding affinity of mevastatin and chlorpyrifos to HMG-CoA reductase

S/N	Compounds	Enzyme	Binding affinity (Kcal/mol)
1	Mevastatin (standard ligand)	HMG-CoA reductase	-9.0
2	Chlorpyrifos	HMG-CoA reductase	-6.7

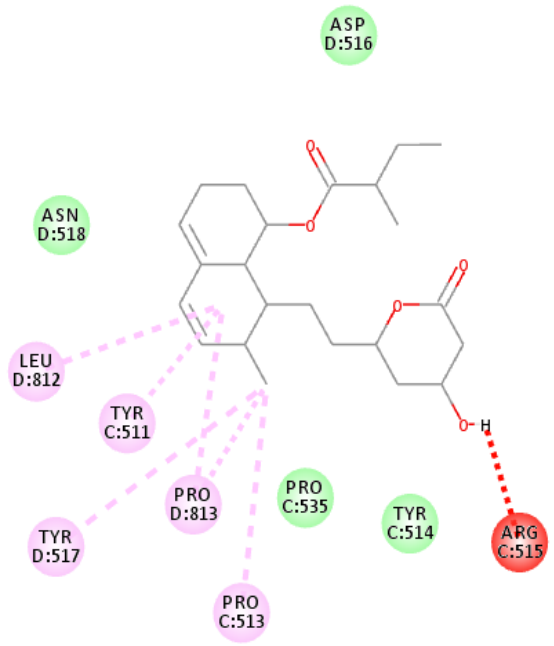

Figure 6: Interaction between amino acid in the binding site of HMGCoA reductase and mevastatin.

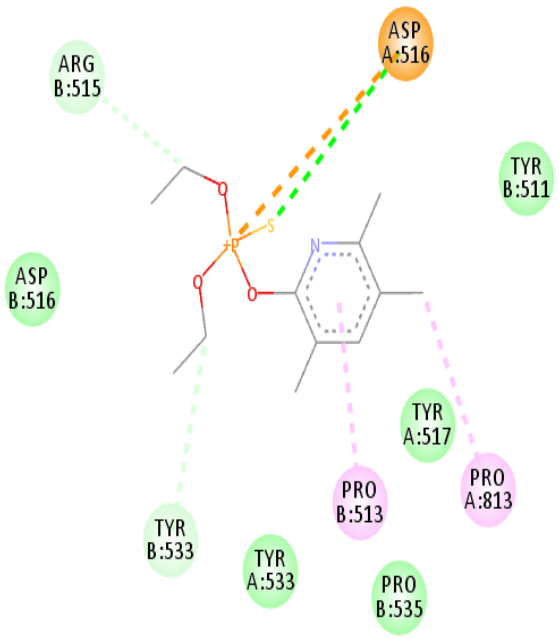

Fig 7: Interaction between amino acid in the binding site of HMGCoA reductase and chlorpyrifos.

COLOUR:

Green dotted line – Conventional hydrogen bond

Purple dotted line – π-sigma bond (hydrophobic interaction)

Light pink dotted line – π-alkyl bond (hydrophobic interaction)

Dark pink dotted line – π-π stacking (hydrophobic interaction)

Yellow dotted line – electrostatic force of attraction (π-cation/anion interaction)

Red dotted line - Unfavorable donor interaction.

3.7.2 Molecular Docking Study on HMG-CoA synthase

Table 13 shows the binding affinities of a standard ligand (Mevastatin) and chlorpyrifos docked against the binding site of HMG-CoA synthase

Higher binding affinity of the sample (chlorpyrifos) compared to the standard ligand (Mevastatin) indicates activation/potentiation of the enzyme whereas lower binding affinity of the sample (chlorpyrifos) compared to the standard ligand (Mevastatin) indicates an inhibition of the enzyme HMG-CoA synthase.

Table 13: Binding affinity of mevastatin and chlorpyrifos to HMG-CoA synthase

S/N	Compounds	Enzyme	Binding affinity (Kcal/mol)
1	Mevastatin (standard ligand)	HMG-CoA synthase	-9.5
2	Chlorpyrifos	HMG-CoA synthase	-6.1

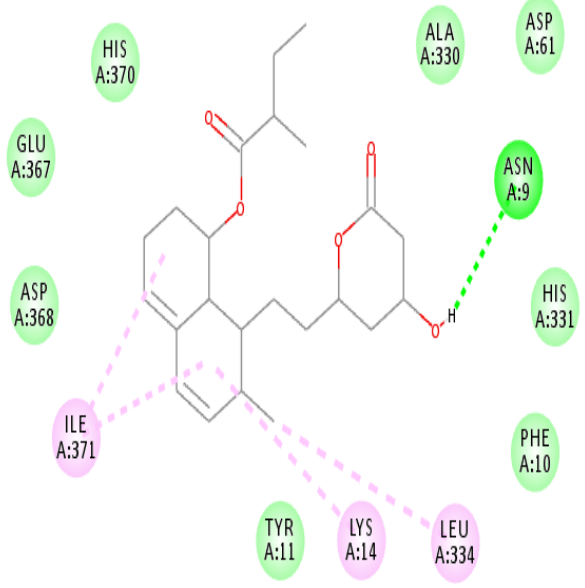

Fig 8: Interaction between amino acid in the binding site of HMG CoA synthase and mevastatin.

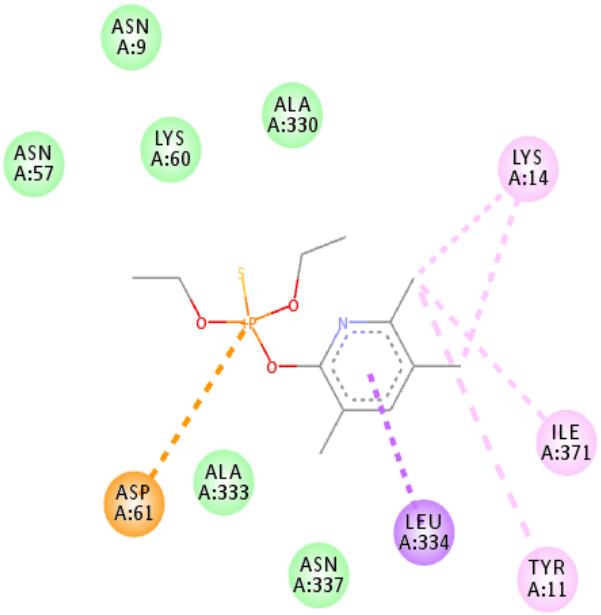

Figure 9: Interaction between amino acid in the binding site of HMG CoA synthase and chlorpyrifos.

COLOUR:

Green dotted line – Conventional hydrogen bond

Purple dotted line – π-sigma bond (hydrophobic interaction)

Light pink dotted line – π-alkyl bond (hydrophobic interaction)

Dark pink dotted line – π-π stacking (hydrophobic interaction)

Yellow dotted line – electrostatic force of attraction (π-cation/anion interaction)

Red dotted line - Unfavorable donor interaction.

3.7.3 Molecular Docking Study on β-keto acyl ACP synthase

Table 14 shows the binding affinities of a standard ligand (Mevastatin) and chlorpyrifos docked against the binding site of β-keto acyl ACP synthase.

Higher binding affinity of the sample (chlorpyrifos) compared to the standard ligand (Mevastatin) indicates activation/potentiation of the enzyme whereas lower binding affinity of the sample (chlorpyrifos) compared to the standard ligand (Mevastatin) indicates an inhibition of the enzyme β-keto acyl ACP synthase

Table 14: Binding affinity of mevastatin and chlorpyrifos to β-keto acyl ACP synthase

S/N	Compounds	Enzyme	Binding affinity (Kcal/mol)
1	Mevastatin (standard ligand)	β-keto acyl ACP synthase	-8.7
2	Chlorpyrifos	β-keto acyl ACP synthase	-6.0

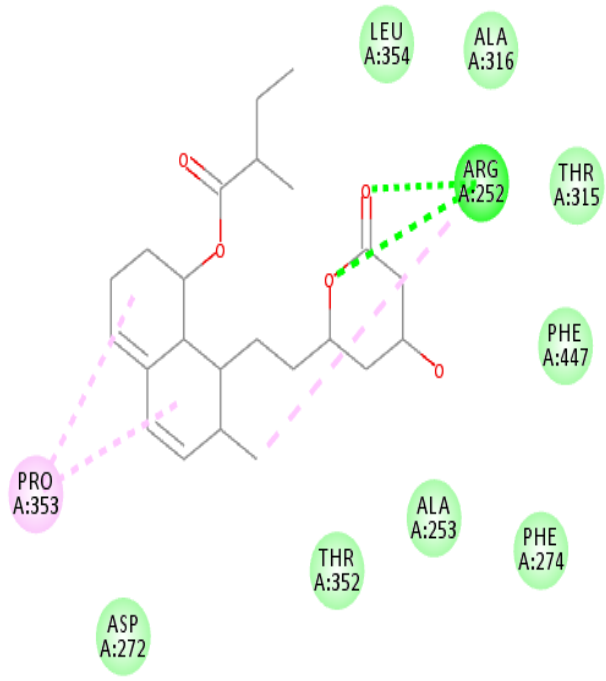

Figure 10: Interaction between amino acid in the binding site of β-keto acyl ACP synthase and mevastatin

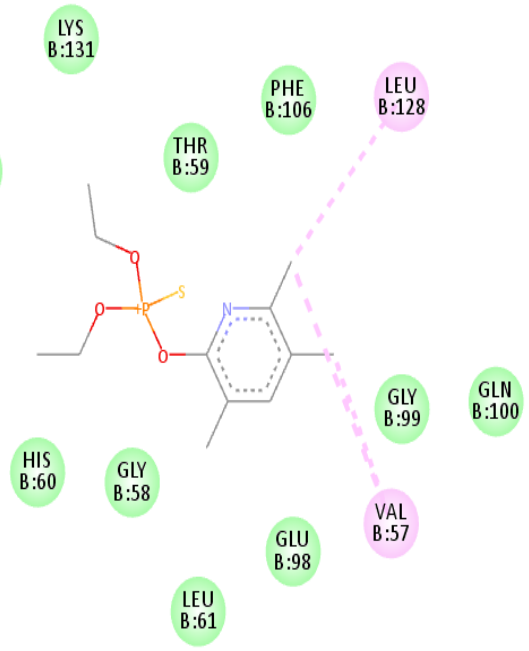

Figure 11: Interaction between amino acid in the binding site of β-keto acyl ACP synthase and chlorpyrifos.

COLOUR:

Green dotted line – Conventional hydrogen bond

Purple dotted line – π-sigma bond (hydrophobic interaction)

Light pink dotted line – π-alkyl bond (hydrophobic interaction)

Dark pink dotted line – π-π stacking (hydrophobic interaction)

Yellow dotted line – electrostatic force of attraction (π-cation/anion interaction)

Red dotted line - Unfavorable donor interaction.

3.8 Risk studies

3.8.1 Oral bioaccumulation studies

Table 15 shows the bioaccumulation of the total biotransformed derivatives of chlorpyrifos (3,5,6-trichloro-2-pyridinol (TCP) and diethylphosphate) in the Liver of wistar rats exposed to the pesticide via oral route. TCP was detected in group 2 and group 3 whereas group 1 and group 4 (control) showed total absence of TCP.

Bioaccumulation factor greater than one (BAF>1) is estimated to be significant. However, Bioaccumulation factor less than one (BAF<1) is estimated to be non-significant.

Table 15: Bioaccumulation of derivatives chlorpyrifos in wistar rat exposed to Chorview® (Chlorpyrifos 40% E. C.) via oral route.

Groups	Treatment	Dose administered (mg/kg b.w.)	Concentration administered (g/l)	Concentration in Liver (g/l)	Bioaccumulation factor (BAF)
Group 1	3.8mg/kg $_{CPF}$	3.8	400	ND	_
Group 2	6.2mg/kg $_{CPF}$	6.2	400	79.43	0.19
Group 3	15.5mg/kg $_{CPF}$	15.5	400	998.21	2.50
Group 4	Control	_	_	ND	_

ND – Not detected

Group 1: 3.8mg/kg b.w of chlorpyrifos,

Group 2: 6.2mg/kg b.w of chlorpyrifos,

Group 3: 15.5mg/kg b.w of chlorpyrifos,

Group 4: control

```
File       :C:\msdchem\1\Essential Oils\ KABIRU L\UGOCHUKWU.D
Operator   : TIJANI I A
Acquired   : 24 Sep 2019  15:21     using AcqMethod ESSENTIAL OILS_SCAN.M
Instrument :   UNILORIN MSD
Sample Name: 1
Misc Info  :
Vial Number: 2
```

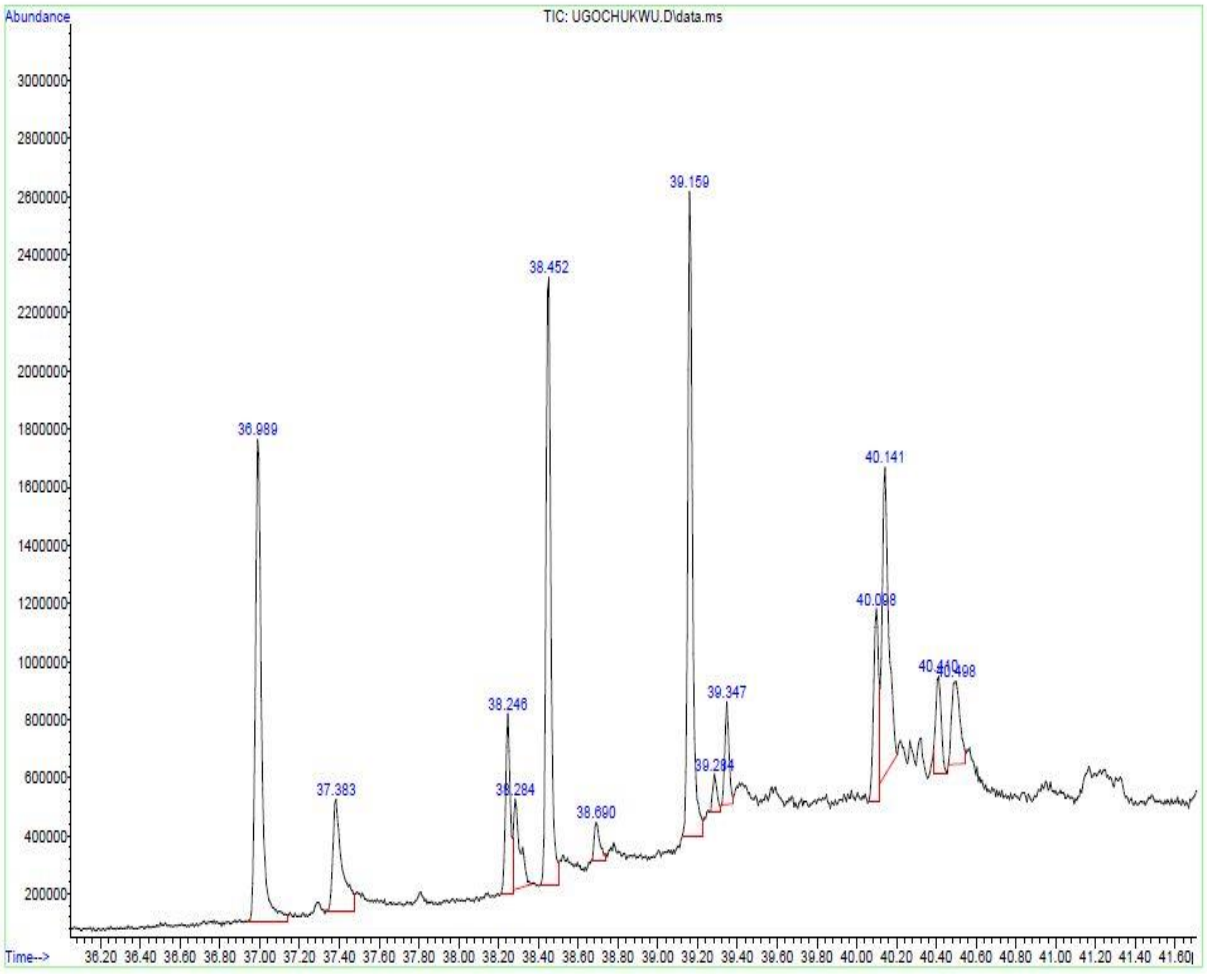

Fig 12: GC-MS Chromatograph of the Liver for group 1 Oral

```
File       :C:\msdchem\1\Essential Oils\ KABIRU L\UGOCHUKWU2.D
Operator   : TIJANI I A
Acquired   : 24 Sep 2019  16:13     using AcqMethod ESSENTIAL OILS_SCAN.M
Instrument :   UNILORIN MSD
Sample Name: 2
Misc Info  :
Vial Number: 3
```

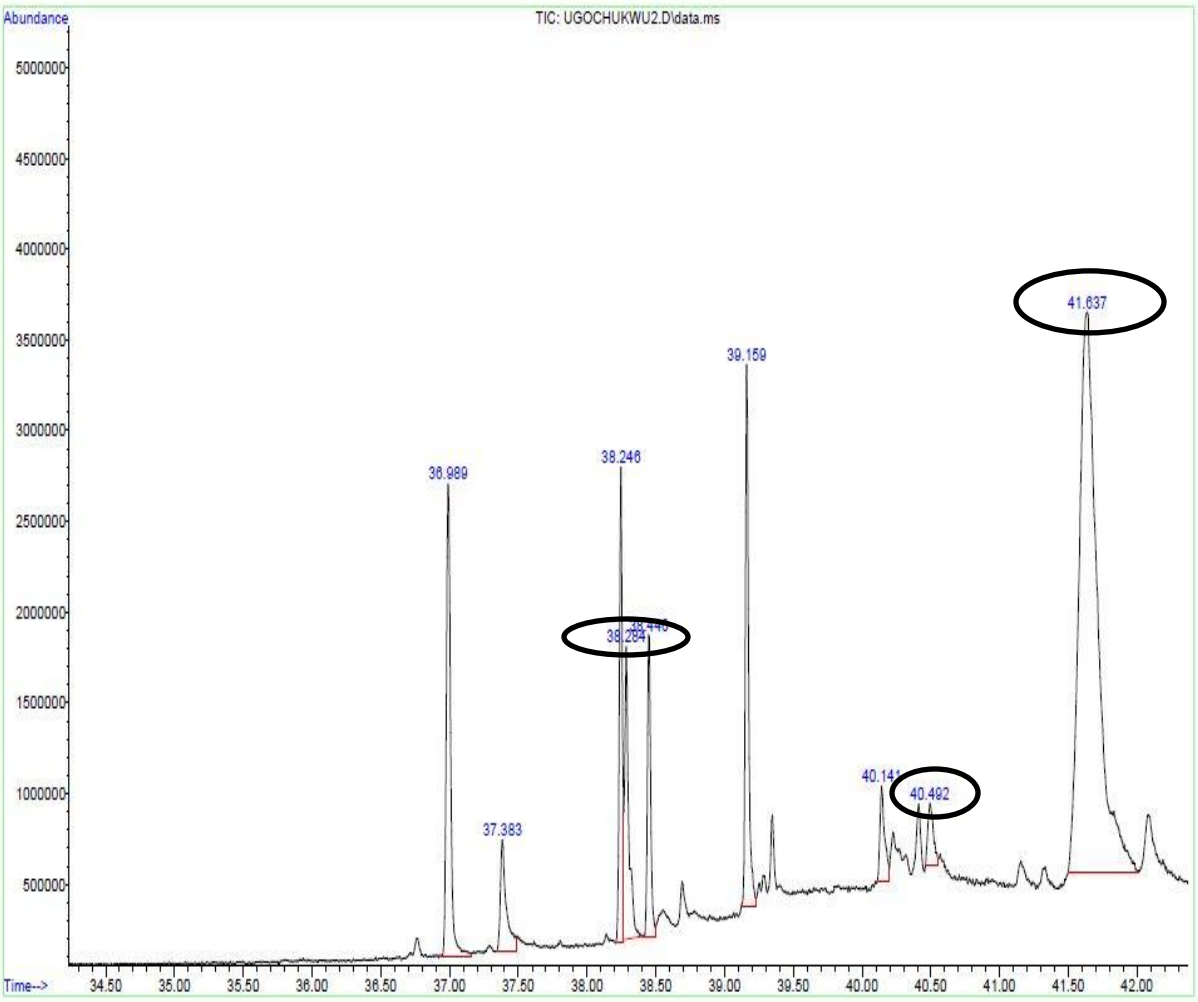

Fig 13: GC-MS Chromatograph of the Liver for group 2 Oral

```
File      :C:\msdchem\1\Essential Oils\ KABIRU L\UGOCHUKWU3.D
Operator  : TIJANI I A
Acquired  : 24 Sep 2019  17:05     using AcqMethod ESSENTIAL OILS_SCAN.M
Instrument :   UNILORIN MSD
Sample Name: 3
Misc Info  :
Vial Number: 4
```

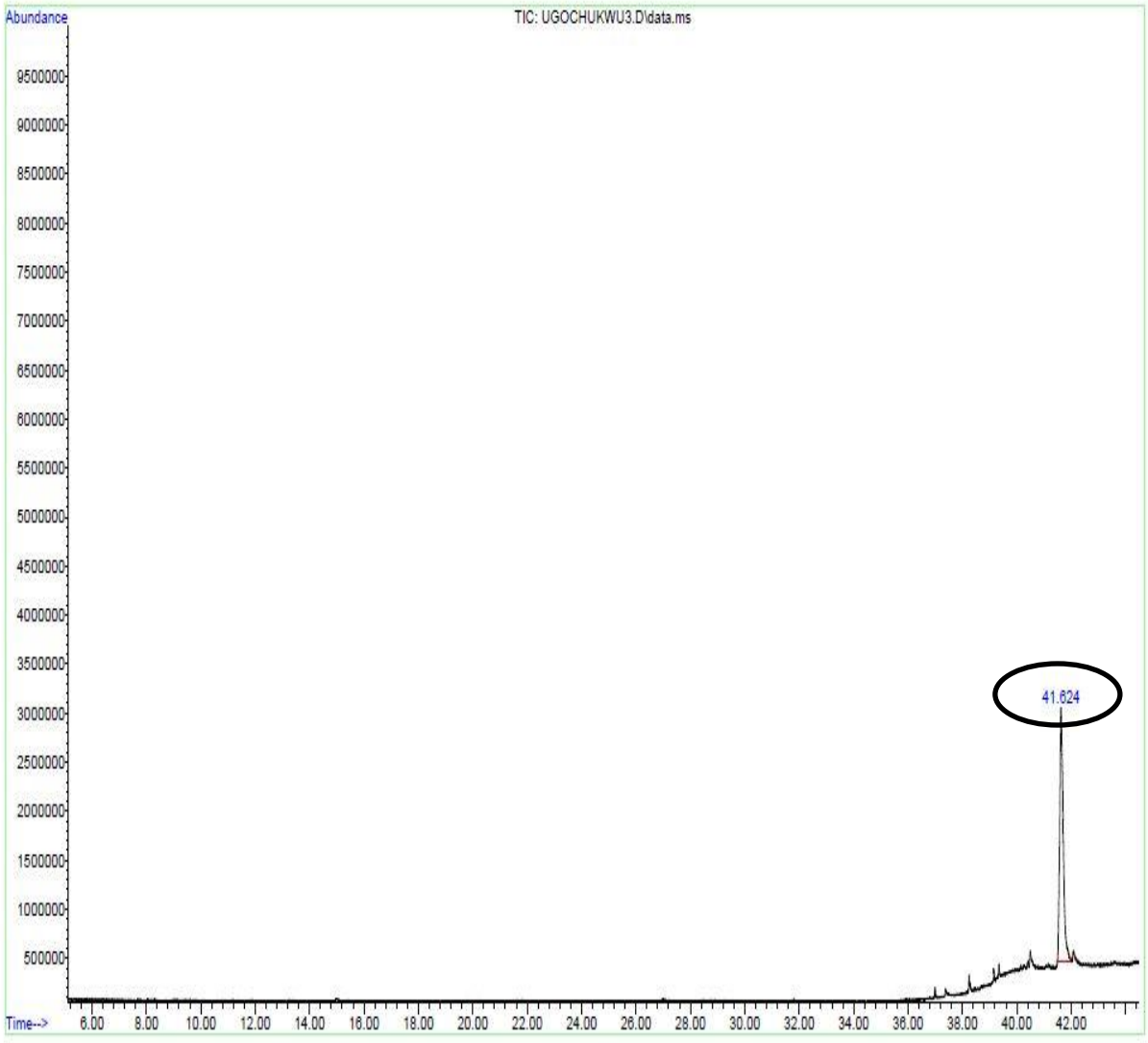

Fig 14: GC-MS Chromatograph of the Liver for group 3 Oral

```
File       :C:\msdchem\1\Essential Oils\ KABIRU L\UGOCHUKWU4.D
Operator   : TIJANI I A
Acquired   : 24 Sep 2019  17:57     using AcqMethod ESSENTIAL OILS_SCAN.M
Instrument :   UNILORIN MSD
Sample Name: 4
Misc Info  :
Vial Number: 5
```

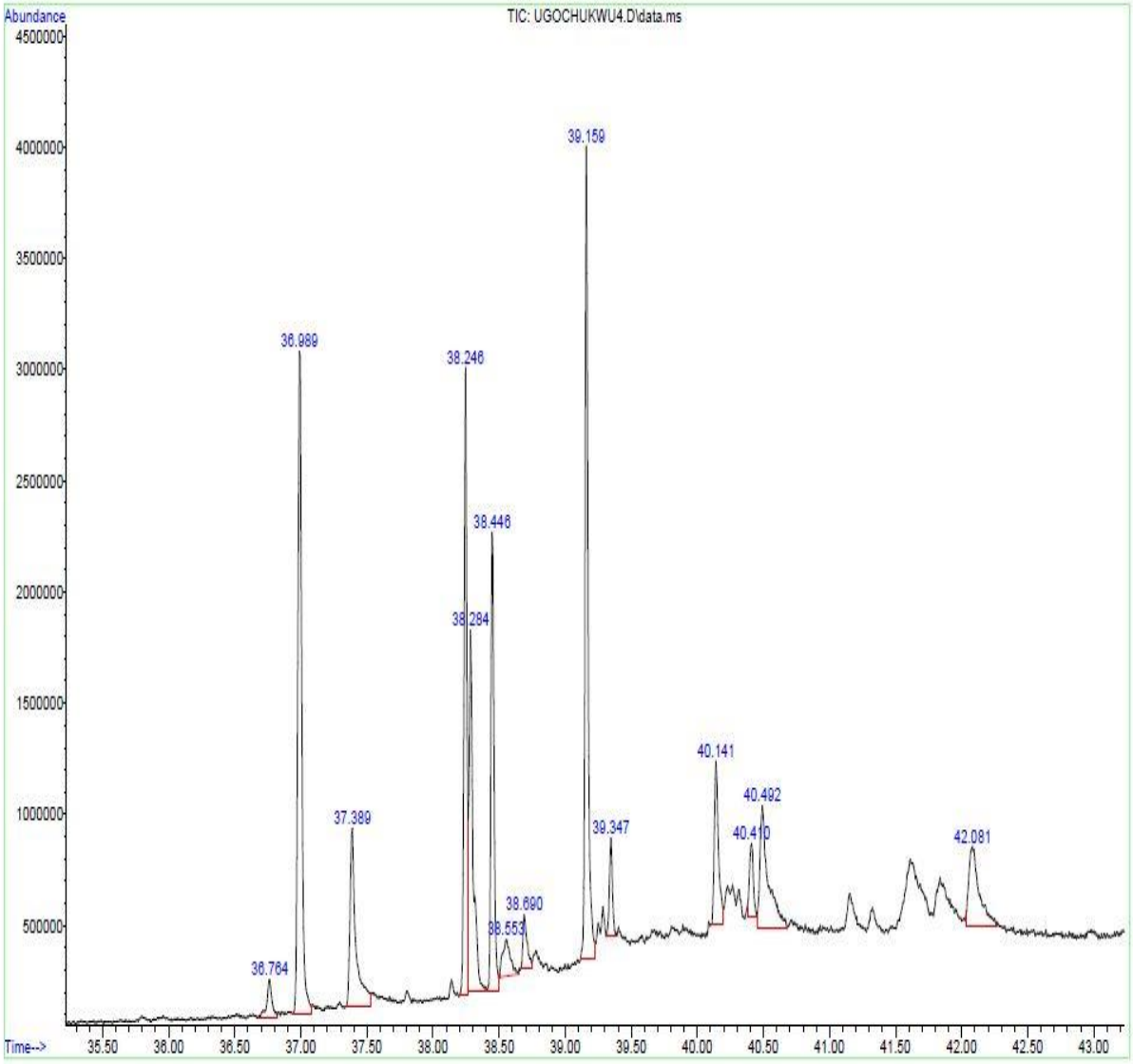

Fig 15: GC-MS Chromatograph of the Liver for group 4 Oral

3.8.2 Inhalation bioaccumulation studies.

Table 16 shows the bioaccumulation of the biotransformed derivative of chlorpyrifos, 3,5,6-trichloro-2-pyridinol (TCP) in the Liver of wistar rats exposed to the pesticide via inhalation route. TCP was not detected in group 1, group 2, group 3 and group 4 (control).

Bioaccmulation factor greater than one (BAF>1) is estimated to be significant. However, Bioaccmulation factor less than one (BAF<1) is estimated to be non-significant.

Table 16: Bioaccumulation of derivatives chlorpyrifos in wistar rat exposed to Chorview® (Chlorpyrifos 40% E. C.) via Inhalation.

Groups	Treatment	Dose administered (mg/kg b.w.)	Concentration administered (mg/l)	Concentration in Liver (mg/l)	Bioaccumulation factor (BAF)
Group 1	**35.36 mg/kg CPF**	35.36	400	ND	ND
Group 2	**56.57 mg/kg CPF**	56.57	400	ND	ND
Group 3	**141.421 mg/kg CPF**	141.421	400	ND	ND
Group 4	**Control**	_	_	ND	ND

ND – Not detected

Group 1: 35.36 mg/kg b.w of chlorpyrifos,

Group 2: 56.57 mg/kg b.w of chlorpyrifos,

Group 3: 141.421 mg/kg b.w of chlorpyrifos,

Group 4: control

```
File       :C:\msdchem\1\Essential Oils\ KABIRU L\UGOCHUKWU.D
Operator   : Dr Emeka
Acquired   : 24 Sep 2019  15:21     using AcqMethod Total Scan.M
Instrument :   CRL ILORIN MSD
Sample Name: 1
Misc Info  :
Vial Number: 2
```

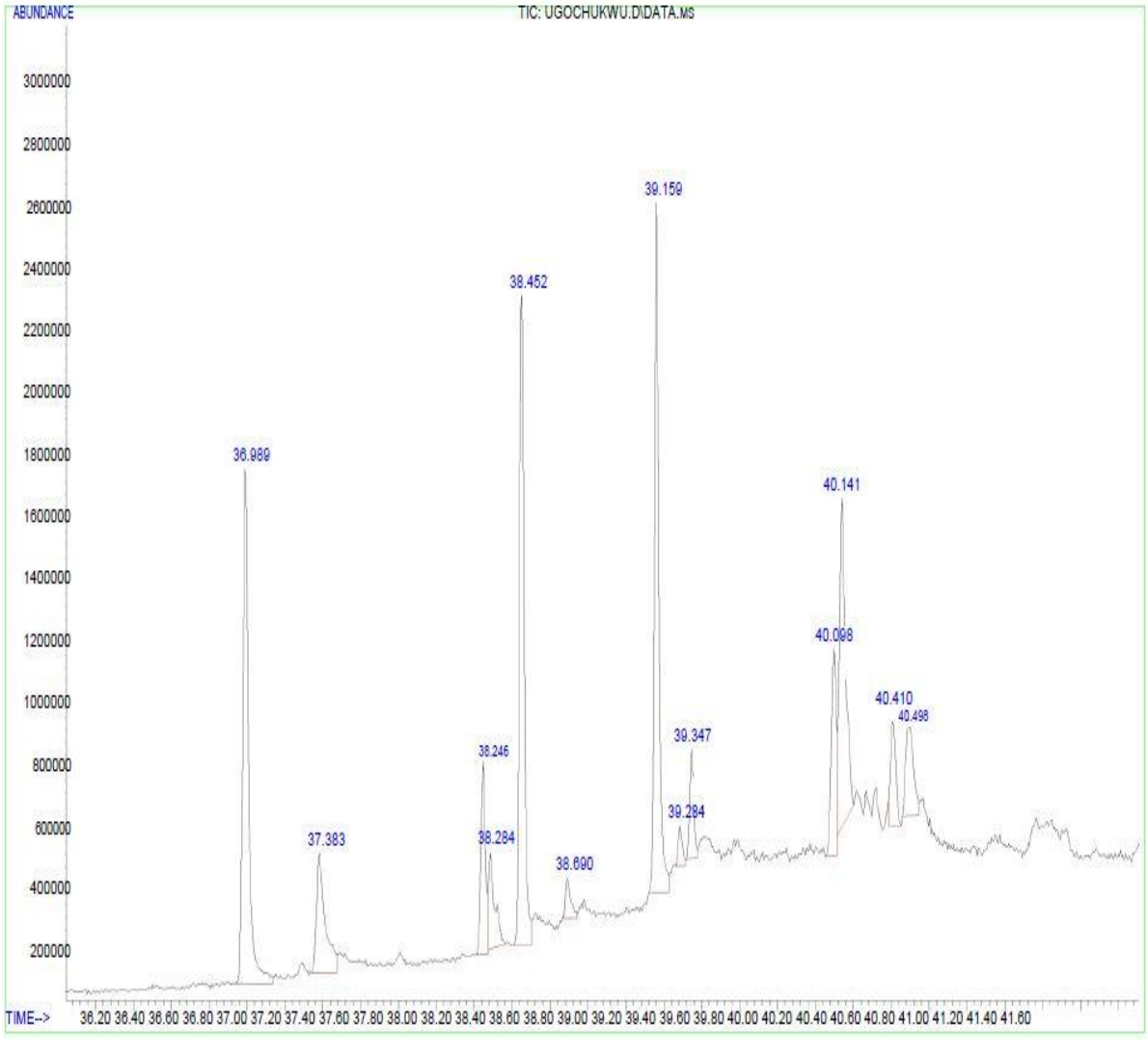

Fig 16: GC-MS Chromatograph of the Liver for group 1 Inhalation

```
File       :C:\msdchem\1\Essential Oils\ KABIRU L\UGOCHUKWU2.D
Operator   : Dr Emeka
Acquired   : 24 Sep 2019  16:13     using AcqMethod Pesticide Residue SCAN.M
Instrument :  ILORIN MSD
Sample Name: 2
Misc Info  :
Vial Number: 3
```

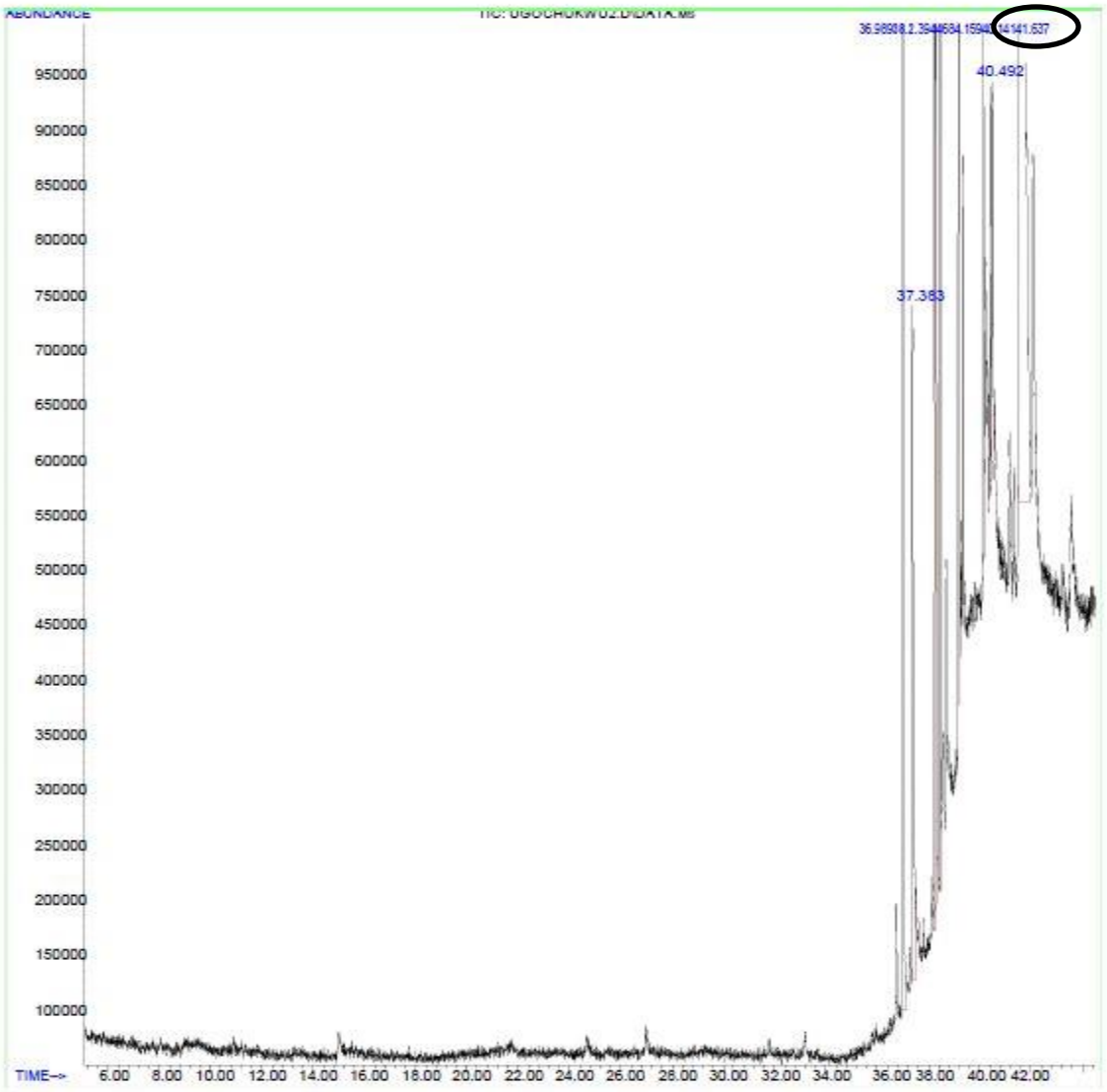

Fig 17: GC-MS Chromatograph of the Liver for group 2 Inhalation

```
File       :C:\msdchem\1\Essential Oils\ KABIRU L\UGOCHUKWU3.D
Operator   : Dr Emeka
Acquired   : 24 Sep 2019  17:05     using AcqMethod Pesticide Residue SCAN.M
Instrument : ILORIN MSD
Sample Name: 3
Misc Info  :
Vial Number: 4
```

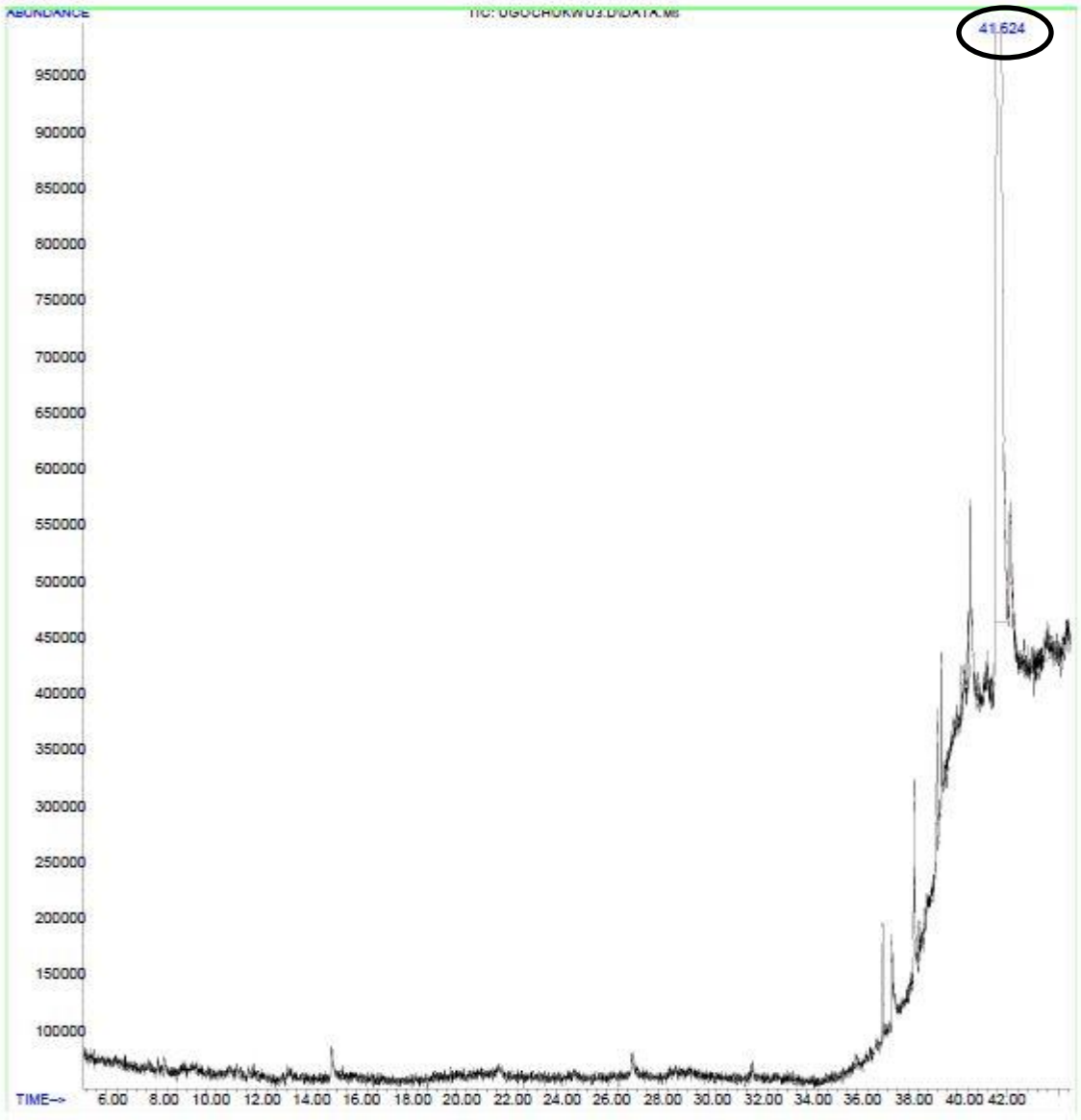

Fig 18: GC-MS Chromatograph of the Liver for group 3 inhalation

```
File       :C:\msdchem\1\Essential Oils\ KABIRU L\UGOCHUKWU4.D
Operator   : Dr Emeka
Acquired   : 24 Sep 2019  17:57     using AcqMethod Pesticide Residue SCAN.M
Instrument : ILORIN MSD
Sample Name: 4
Misc Info  :
Vial Number: 5
```

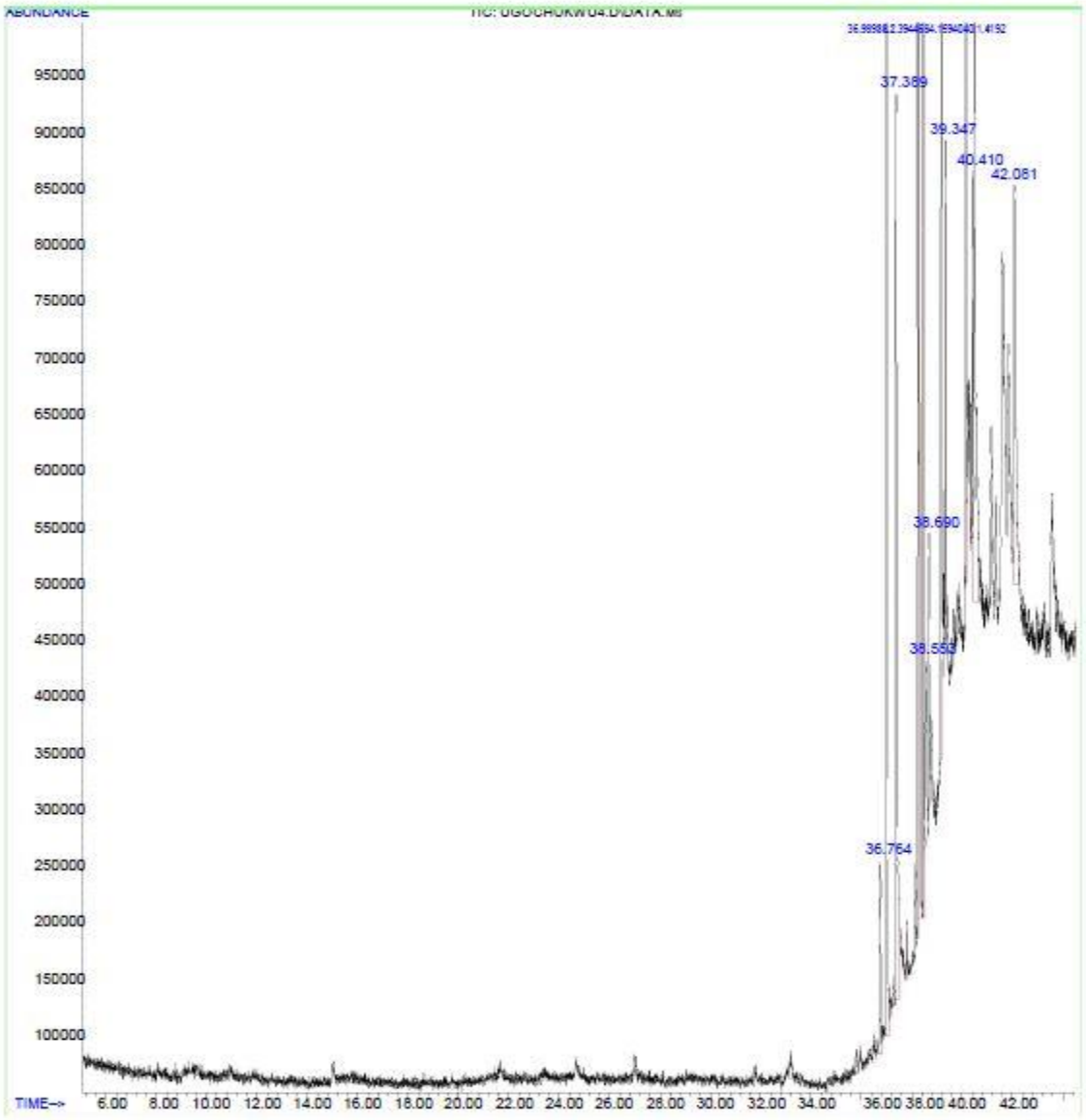

Fig 19: GC-MS Chromatograph of the Liver for group 4 inhalation

3.8.3 Oral average Daily Dose (ADD) and Hazard Quotient (HQ)

Table 17 shows the average daily dose and hazard quotient of wistar rats exposed to Chorview® (Chlorpyrifos 40% E. C.) via oral route. ADD and HQ were calculated for group 2 and group 3, who recorded accumulation of the pesticide in the liver of the wistar rats.

Table 17: Oral average Daily Dose and Hazard Quotient

Groups	Treatment	Dose administered (mg/kg b.w.)	Concentration in Liver (g/l)	Average daily dose (ADD) (mg/kg b.w.)	Hazard quotient (HQ)
Group 1	**3.8mg/kg CPF**	3.8	ND	_	_
Group 2	**6.2mg/kg CPF**	6.2	79.43	0.0012	0.24
Group 3	**15.5mg/kg CPF**	15.5	998.21	0.017	3.4
Group 4	**Control**	_	ND	_	_

ND – Not detected

Group 1: 3.8 mg/kg b.w of chlorpyrifos,

Group 2: 6.2 mg/kg b.w of chlorpyrifos,

Group 3: 15.5 mg/kg b.w of chlorpyrifos,

Group 4: control

3.8.4 Inhalation Average Daily Dose (ADD) and Hazard Quotient (HQ)

Table 18 shows the average daily dose and hazard quotient of wistar rats exposed to Chorview® (Chlorpyrifos 40% E. C.) via inhalation route. ADD and HQ were not calculated for group 1, group 2 and group 3, who recorded no accumulation of the pesticide in the liver of the wistar rats.

Table 18: Inhalation Average Daily Dose and Hazard Quotient

Groups	Treatment	Dose administered (mg/kg b.w.)	Concentration in Liver (g/l)	Average daily dose (ADD)	Hazard Quotient (HQ)
Group 1	**35.36 mg/kg $_{CPF}$**	35.36	ND	_	_
Group 2	**56.57 mg/kg $_{CPF}$**	56.57	ND	_	_
Group 3	**141.421 mg/kg $_{CPF}$**	141.421	ND	_	_
Group 4	**Control**	_	ND	_	_

ND – Not detected

Group 1: 35.36 mg/kg b.w of chlorpyrifos,

Group 2: 56.57 mg/kg b.w of chlorpyrifos,

Group 3: 141.421 mg/kg b.w of chlorpyrifos,

Group 4: control

CHAPTER FOUR

DISCUSSION

The present study majorly investigates the risk posed by both oral and inhalation exposure to organophosphate (Chlorpyrifos) pesticide. Humans are exposed to this pesticide mainly through agricultural products, direct contact, and/or household pest control. Potential adverse health impacts of organophosphorus (OP) pesticide exposures are an ongoing public health concern (Farahat *et al*., 2010). Chlorview® (Chlorpyrifos 40% EC) is a widely used brand of pesticide in the south east region of Nigeria which necessitated the study into the risk of exposure to it, via oral and inhalation routes.

The results from acute toxicity studies revealed that oral exposure to Chlorview® (Chlorpyrifos 40% EC) gave a median lethal dose (LD_{50}) of 155 mg/kg b.w. while inhalation exposure gave a median lethal concentration (LC_{50}) of 1414 mg/kg b.w. for 60 minutes. This result agrees with the findings of Clegg and Van Gemert (2010) who reported an oral LD_{50} of 118-245 mg/kg b.w. The result varies with the report of Smegal (2000), who reported an oral LD_{50} of 223 mg/kg and an inhalation LC_{50} of 200mg/m^3. This might be due to the difference in the formulation of the pesticide used for the experiment. Some components of the formulation may have added to the toxicity of the pesticide as opposed to the used of pure chlorpyrifos.

The results from hepatotoxicity studies showed that oral exposure to Chlorview® (Chlorpyrifos 40% EC) led to a significant ($p<0.05$) increase in the activity of Aspartate amino transferase (AST) in group 3 (46.00±4.00) when compared to group 4 (25.67±3.21 U/L). A significant ($p<0.05$) increase in Alanine amino transferase (ALT) activity was recorded in group 3 (11.67±2.08) when compared to group 4 (4.33±0.58 U/L). Alkaline phosphatase (ALP) activity in group 3 (32.92±1.89 U/L) significantly ($p<0.05$) increased when compared to group 4 (29.53±0.42).However, the activities of Aspartate amino transferase (AST), Alanine amino transferase (ALT) and Alkaline phosphatase (ALP) in group 1(3.8mg/kg b.w) and group 2 (6.2mg/kg b.w) increased non-significantly ($p>0.05$) when compared to the group 4 (Control). This implies that oral exposure to Chlorview® (Chlorpyrifos 40% EC) caused a significant liver damage on wistar rats at 15.5mg/kg b.w. This result is *in tandem* with the report of Uzun and Kalender (2013), who report a statistically significant increase in ALP, ALT and AST activities (50%, 41%, 38% respectively) on exposure to chlorpyrifos.

Hepatotoxicity studies on wistar rats exposed to Chlorview® (Chlorpyrifos 40% EC) via inhalation follow the same trend as those exposed via oral route. There was a significant ($p<0.05$) increase in the activity of Aspartate amino transferase (AST) in group 3 (94.47±6.87 U/L) when compared to group 4 (49.81±2.99 U/L). A significant ($p<0.05$) increase in Alanine amino transferase (ALT) activity was recorded in group 3 (64.50±0.71) when compared to group 4 (21.04±1.96 U/L). Alkaline phosphatase (ALP) activity in group 3 (269.56±57.25 U/L) significantly ($p<0.05$) increased when compared to group 4 (170.27±9.00 U/L). This may be due to death of the hepatocytes and subsequent release of these marker enzymes caused by administration of higher doses.

However, the activities of Aspartate amino transferase (AST) and Alkaline phosphatase (ALP) in group 1(35.36 mg/kg b.w) increased non-significantly ($p>0.05$) when compared to the group 4 (Control). There was also a non-significant ($p>0.05$) difference between ALT and ALP activities of group 2 and group 3. Across treatment groups in both oral and inhalation studies, there is a significant increase in the activities of liver marker enzymes (AST, ALT and ALP) of group 1 (least dose) when compared to group 3 (highest dose). This implies that the toxicity of chlorpyrifos is dose dependent and increases with increase in dose. This finding corresponds with the report of Karami-Mohajeri1 *et al*. (2017), who reported a dose-dependent liver damage in rats exposed to sub-lethal doses of organophosphate pesticide.

Results from the study of oxidative stress markers of wistar rats exposed to Chlorview® (Chlorpyrifos 40% EC) suggest that the pesticide induced appreciable oxidative imbalance in the system. In oral exposure, There was a significant ($p<0.05$) increase in the activity of Catalase of group 3 (6.82±1.10 U/mg) when compared to group 4 (4.68±0.71 U/mg), however, there is a non-significant ($p>0.05$) increase in group 1 and group 2 when compared to group 4. Glutathione peroxidase activity showed a non-significant ($p>0.05$) decrease across the treatment groups when compared to group 4 (control). There was a significant ($p<0.05$) increase in the concentration of Malondialdehyde of group 3 (1.59±0.43 mg/dL) when compared to group 4 (1.12±0.10 mg/dL), however, there is a non-significant ($p>0.05$) increase in group 1 and group 2 when compared to group 4. Also the concentration of Glutathione showed a non-significant ($p>0.05$) decrease across the treatment groups when compared to group 4 (control). These findings are supported by the

report of Uzun and Kalender (2013) who reported a decrease of GPx activity in rats intoxicated with chlorpyrifos. MDA may be increase due to adverse effect of chlorpyrifos on cell membrane. Increased MDA content is an important indicator of oxidative membrane damage. These toxic effects probably occur through the generation of ROS causing damage to various membranous components of the cell (Uzun and Kalender 2013).

The inhalation exposure showed similar trend in the oxidative stress markers in wistar rats exposed to Chlorview® (Chlorpyrifos 40% EC). There was a There was a significant ($p<0.05$) increase in the activity of Catalase of group 3 (195.73±47.09 U/mg) when compared to group 4 (112.04±9.57 U/mg), however, there is a non-significant ($p>0.05$) increase in group 1 and group 2 when compared to group 4. Glutathione peroxidase activity of group 2 (151.56±1.25 U/mg) and group 3 (130.61±2.39 U/mg) significantly ($p<0.05$) decreased when compared to group 4 (334.52±16.14 U/mg), however, there is a non-significant ($p>0.05$) increase in group 1 when compared to group 4. There was a significant ($p<0.05$) increase in the concentration of Malondialdehyde of group 3 (1.33±0.110 mg/dL) when compared to group 4 (0.61±0.83 mg/dL), however, there is a non-significant ($p>0.05$) increase in group 1 and group 2 when compared to group 4. Also the concentration of Glutathione showed a significant ($p<0.05$) decrease across the group 1 (146.39±0.50 mg/dL), group 2 (142.14±0.50 mg/dL) and group 3(140.02±1.50 mg/dL) when compared to group 4 (149.94±1.50 mg/dL).

GSH level may be decreased due to its participation in the conjugation reactions or decreased cell ability to regenerate GSH. GPx uses reduced glutathione and in this reaction reduced glutathione (GSH) is converted to oxidized glutathione form (GSSG) (Lukaszewicz-Hussain, 2010). Lipid peroxidation is one of the main processes of oxidative damage, which plays a critical role in the toxicity of many xenobiotics (Ongjanovic´ *et al*., 2010). Increased MDA content is an important indicator of oxidative membrane damage. (Lukaszewicz-Hussain, 2010; Uzun and Kalender 2013). The reactive oxygen species (ROS) may be produced as the result of the metabolism of organophosphates by cytochrome P450s. The P450s are monooxygenases and catalyze oxidation by addition of one atom of molecular oxygen into a substrate (organophosphate) by an electron transport pathway. Organophosohates change normal antioxidant homeostasis resulting in antioxidant depletion, if the requirement of continuous antioxidants is not maintained (Lukaszewicz-Hussain, 2010).

Results from the reproductive toxicity studies revealed that that oral exposure to Chlorview® (Chlorpyrifos 40% EC) led to a significant ($p<0.05$) increase in the Cholesterol concentration of group 3 (224.65±6.57 mg/dl) when compared to group 4 (151.96±32.01 mg/dl), however, there was a non-significant ($p>0.05$) increase in group 1 and group 2 when compared to group 4(control). The testosterone concentration of group 3 (1.30±0.12 ng/ml) significantly ($p<0.05$) decreased when compared to group 4 (3.76±1.09 ng/mg). Although there was a non-significant ($p>0.05$) decrease in Testosterone concentration of group 1 (3.8mg/kg b.w.) and group 2 (6.2mg/kg b.w.) when compared to group 4 (Control). There was a significant ($p<0.05$) decrease in the Sperm count of group 1 (70.00±2.00 $X10^6$/mL), group 2 (66.00±1.00 $X10^6$/mL) and group 3 (60.67±5.77 $X10^6$/mL) when compared to group 4 (82.00±6.00 $X10^6$/mL). This is consistent with the published report that administration of chlorpyrifos to male mice by oral gavage resulted in significant adverse effects that included cholinergic signs, decreased acetylcholinesterase and testosterone levels, and histological changes in testis and epididymis in the 15 and 25 mg/kg-d treated groups. Testicular spermatid and epididymal sperm counts indicated that spermatogenesis was partially arrested at the middle and high dose groups (15 and 25 mg/kg-d) (Farag *et al*., 2010).

Inhalation exposure to Chlorview® (Chlorpyrifos 40% EC) was found in this study to result in disruption of vital markers of reproductive toxicity. There was a significant ($p<0.05$) increase in the Cholesterol concentration of group 1 (196.10±21.80 mg/dl), group 2 (237.07±22.90 mg/dl) and group 3 (300.29±0.28 mg/dl) when compared to group 4 (86.28±5.68 mg/dl). Testosterone concentration showed a significant ($p<0.05$) decrease in group 1 (10.18±0.23 ng/mL), group 2 (9.73±0.36 ng/mL) and group 3 (9.20±0.17 ng/mL) when compared to group 4 (10.96±0.06 ng/mL). The Sperm count of group 1 (71.50±2.12 $X10^6$/mL), group 2 (63.50±0.71 $X10^6$/mL) and group 3 (53.00±1.41 $X10^6$/mL) was significantly ($p<0.05$) decreased when compared to group 4 (86.50±0.71 $X10^6$/mL). Across the above parameters, there is an observable trend of increasing toxicity with increasing dose of the pesticide as also reported by Li *et al*., 2019. Chlorpyrifos significantly decreased total sperm count, serum testosterone and gonadotropin levels and the activity of enzymes involved in spermatogenesis, as well as lead to oxidative damage in the testis (Li *et al*., 2019). When chronically exposed to a low dose of CPF, there was a disturbance in the secretion of endocrine hormones (Ventura *et al*., 2016) that led to an obese (Lassiter and Brimijoin, 2008) or diabetic (Peris-Sampedro *et al*., 2015) phenotype. This is explained by the excess fat deposition on the treatment groups, observed in the present study. Chronic exposure to chlorpyrifos

resulted in a significant decrease in sperm count and motility and an increase in the ratio of immotile and morphologically abnormal sperm in male rats (ElMazoudy *et al*., 2011; Dutta and Sahu, 2013; Alaa-Eldin *et al*., 2017; Peiris and Dhanushka, 2017). The mechanism of CPF-induced reproductive toxicity was mainly attributed to the decreased content of testosterone (Joshi *et al*., 2007; Sai *et al*., 2014; Usmani *et al*., 2004; Mandal and Das, 2012), or oxidative damage caused by CPF to Leydig cells (Chen *et al*., 2018; Joshi *et al*., 2007) where testosterone was synthesized. The metabolites of CPF, such as the active sulfur atom, inhibit the activity of cytochrome P450 3A4 (CYP3A4) (Buratti *et al*., 2003), an enzyme involved in testosterone metabolism (Usmani *et al*., 2004), which leads to the decrease in testosterone. The values the sperm count in the present study is are still within the limit of normal sperm count reported to be about 40–50 x10^6/ml (Cooper *et al*., 2010), although there was a significant decrease, the maybe due to short duration of the present study. The peak effect of the exposure may be seen more in the next cycle of spermatogenesis.

The classical mechanism of chlorpyrifos toxicity is the inhibition of acetylcholinesterase at the neural synapse of the nervous system (Koshlukova and Reed, 2014). Result of the neurotoxicity studies showed that oral exposure to Chlorview® (Chlorpyrifos 40% EC) led to a significant ($p < 0.05$) decrease in the Acetylcholinesterase activity of group 3 (0.52±0.03 U/mg) when compared to group 4 (0.66±0.03 U/mg), however there was a non-significant ($p>0.05$) increase in Acetylcholinesterase activity of group 1 (3.8mg/kg b.w) and group 2 (6.2mg/kg b.w) when compared to group 4 (Control). Inhalation exposure to Chlorview® (Chlorpyrifos 40% EC) resulted in a significant ($p < 0.05$) decrease in the Acetylcholinesterase activity of group 1 (2.60±0.04 U/l), group 2 (2.34±0.09 U/l) and group 3 (1.60±0.14 U/l) when compared to group 4 (2.89±0.07 U/l). This result is consistent with the findings of Farag *et al*., 2010. Muscle AChE activity was more sensitive to inhibition by chloropyrifos than brain AChE activity. Brain and muscle AChE activities were significantly decreased in the 15 mg/kg-d (84.98 and 78.16% of the control value for brain and muscle AChE activities, respectively) and 25 mg/kg-d (72.78 and 68.21% of the control value for brain and muscle AChE activities, respectively) treated groups (Farag *et al*., 2010). Early studies mainly focused on acute toxicity at high doses, and found CPF irreversibly inhibited the activity of cholinesterase, especially acetylcholinesterase (AchE) (Yen *et al*., 2011), and led to oxidative damage in organs such as the liver (Suke *et al*., 2018), kidney (Saoudi *et al*.,

2018). From the result of this present study, it can be inferred that inhalation route of exposure to chlorpyrifos has more significant inhibition of acetylcholinesterase than oral route of exposure. This may be due to a more active detoxification and biotransformation action of the liver that reduces the toxicity of the orally treated groups whereas the inhalation route get more chlorpyrifos to brain. Inhibition of the brain acetylcholinesterase activity is a better marker of neurotoxicity. This explains the physical sign of toxicity such as tremor, lack of coordination, salivation, lacrimation, observed in groups 2 and 3.

The high cholesterol accumulation macroscopically observed on liver sample of the treatment groups and also confirmed by GC-MS analysis of the liver homogenate suggest a disrupting action of the chlorpyrifos and/or its derivatives on the pathway of cholesterol synthesis. Cholesterol was detected in group 2 of both oral (53782.4 mg/dl) and inhalation (53783.3 mg/dl) route of exposure and also in group 3 (99820.7 mg/dl) of inhalation exposure in extremely high concentration. However, cholesterol was not detected in group 1 and group 4 of both oral and inhalation route of exposure and also in group 3 of oral route of exposure to chlorpyrifos. This observation is way higher than the normal liver cholesterol concentration and may be implicated in diverse health effects. The mechanism of cholesterol synthesis induction by chlorpyrifos was studied to proffer explanation to the high accumulation of cholesterol in the liver of treatment groups.

Results from the molecular Docking Study shows the binding affinities of a standard ligand (Mevastatin) and chlorpyrifos docked against the binding site of β-keto acyl ACP synthase, HMG CoA synthase and the rate limiting enzyme in cholesterol synthesis pathway, HMG-CoA reductase. Mevastatin gave binding affinities of -8.7, -9.5 and -9.0 Kcal/mol while Chlorpyrifos gave binding affinities of -6.0, -6.1 and -6.7 Kcal/mol for the three enzymes respectively. Higher binding affinity of the sample (chlorpyrifos) compared to the standard ligand (Mevastatin) indicates an inhibition of the enzymes whereas lower binding affinity of the sample (chlorpyrifos) compared to the standard ligand (Mevastatin) indicates a activation/potentiation of the enzymes. The polar interaction images (Figures 6, 7, 8, 9, 10 and 11) shows the interaction of the two ligands with specific amino acids in the binding sites of the enzyme which resulted in the binding affinity scores. The most dominant interaction is the Hydrophobic interaction (purple and pink dotted lines) which suggest the stability of the ligand in the binding site of the enzymes. In HMG-CoA reductase, mevastatin gave and unfavourable interaction (red dotted line) with Arginine 515

suggesting that the enzyme may be oriented in a conformation less liable to bind its physiological substrate whereas the electrostatic interaction (yellow dotted lines) and hydrogen bonding (green dotted lines) of chlorpyrifos with Aspartate 516 suggests that the enzyme may be able to bind its physiological substrate. This trend is also consistent in β-keto acyl ACP synthase and HMG CoA synthase, hence promoting the synthesis of cholesterol.

The risk studies for the exposure to Chlorview® (Chlorpyrifos 40% EC) shows the bioaccumulation of the total biotransformed derivatives of chlorpyrifos (3,5,6-trichloro-2-pyridinol (TCP) and diethylphosphate) in the Liver of wistar rats. In oral route of exposure, GS-MS analysis of the liver homogenate detected TCP and diethylphosphate in group 2 (79.43 g/l) and only TCP group 3 (998.21 g/l) whereas group 1 and group 4 (control) showed total absence of chlorpyrifos derivatives. This is supported by the report of Clegg and Van Gemert (2010), who stated that TCP is the major metabolite of chlorpyrifos following oral dosing.

Bioaccumulation factor (BAF) value of group 2 (0.19) and group 3 (2.50) shows that the pesticide accumulated more in group 3 treated with 15.5mg/kg b.w of chlorpyrifos and in group 2 treated with 6.2 mg/kg b.w of chlorpyrifos. This may be due to the sequestration of the pesticide derivatives in the Liver. This result is supported by the report of Tanvir *et al*. (2015) who reported accumulation of chlorpyrifos in the liver. In contrast, Tanvir *et al*. (2016) reported that nearly 90% of the administered dose was absorbed and deposition of chlorpyrifos was detected only in adipose tissue. Almost 80% CPF was absorbed with dose dependent effects seen in rat's plasma and brain Timchalk *et al*. (2002). Chlorpyrifos bioaccumulates over time and exerts toxic effects on animals (Tanvir *et al*., 2016). Bioaccmulation factor greater than one (BAF>1) is estimated to be significant while bioaccmulation factor less than one (BAF<1) is estimated to be non-significant. Hence, groups 3 (BAF = 2.50) showed a significant bioaccumulation, this implies a high risk of subchronic toxicity at the dose of 15.5mg/kg b.w of chlorpyrifos. Accumulation of chlorpyrifos residues and derivatives were not detected in groups exposed via inhalation route, however there was macroscopic (high) deposition of fats in the liver of the wistar rats with majorly was identified as cholesterol. The calculated Average daily dose (ADD) for groups exposed via oral route was given as group 2 (0.0012 mg/kg b.w.) and group 3 (0.017 mg/kg b.w.), while the Hazard quotient (HQ) was given as group 2 (0.24) and group 3 (3.4).

4.2 CONCLUSION

This study suggests that exposure to Chlorview® (Chlorpyrifos 40% EC) poses a reasonable risk to human health both through oral and inhalation routes of exposure affecting many biochemical processes. Chlorpyrifos accumulates in the liver at high oral doses of exposure (≥ 6.2 mg/kg bw). The observations suggests that oral exposure affects risk assessment indices significantly whereas inhalation exposure affects biochemical indices significantly. The mechanism of toxicity of chlorpyrifos at low doses involves pathways other than the classical acetylcholinesterase inhibition. The liver, the testis and the brain represent a highly susceptible organ for the subchronic toxicity of chlorpyrifos at low dose.

4.3 Recommendation

Based on the findings the present study the following recommendations are forwarded.

- Household pest control using chlorpyrifos should be highly regulated.
- There is a need to create awareness among consumer and farmers on appropriate withdrawal and re-entry period following chlorpyrifos application.
- Pesticide applicators should be trained and provided with protective gadgets for working.
- Further studies into the mechanism of toxicity of chlorpyrifos derivatives should be investigated.

REFERENCES

Aebi, H. E. (1983). Catalase ed HV. *Method of Enzymatic Analysis*, **3**: 273-286.

Alaa-Eldin, E. A., El-Shafei, D. A., and Abouhashem, N. S. (2017). Individual and combined effect of chlorpyrifos and cypermethrin on reproductive system of adult male albino rats. *Environmental Science and Pollution Research*, **24**(2): 1532-1543.

Alan T. E. (2017). How Insecticides Work. Retrieved from http://extension.unh.edu.

Al-Attar, A. M. (2011). Vitamin E attenuates liver injury induced by exposure to lead, mercury, cadmium and copper in albino mice. *Saudi journal of biological sciences*, *18*(4), 395-401.

Allian, C. C. (1974). Enzymatic determination of total serum cholesterol. *Clinical Chemistry*, **20**: 470-475.

Al-Sawafi, A. G. A., and Yan, Y. (2013). Alterations of acetylcholinesterase activity and antioxidant capacity of zebrafish brain and muscle exposed to sublethal level of cadmium. *International Journal of Environmental Science and Development*, **4**(3): 327.

Azizi, S., Soleymani, J., Khoubnasabjafari, M., Samadi, A., and Jouyban, A. (2018). Liquid Chromatographic Determination of Malondialdehyde in Plasma Samples After Liquid–Liquid Microextraction. *Current Analytical Chemistry*, **14**(4): 416-422.

Bhale, D. V., Patil, D. S., and Mahat, R. K. (2014). Study of malondialdehyde (MDA) as a marker of oxidative stress in obese male individuals. *International Journal of Recent Trends in Science and Technology*, **10**: 51-52.

Boguski, T. K. (2003). Human health risk assessment, environmental science and technology brief for citizens, Hazard Substance Research Centre; Retrieved from https://www.engg.ksu.edu/CHSR/outreach/resources/docs/healthrisk.pdf

Bruce A. C. (2005). Enzyme activities as a component of soil biodiversity: A review. *Pedobiologia*, **49**: 637 – 644.

Buratti, F. M., Volpe, M. T., Meneguz, A., Vittozzi, L., and Testai, E. (2003). CYP-specific bioactivation of four organophosphorothioate pesticides by human liver microsomes. *Toxicology and applied pharmacology*, **186**(3): 143-154.

Burtis, C. A., and Ashwood, E. R. (1999). Tietz textbook of clinical chemistry 3rd ed. *Philadelphia, London Pub. Saunders WB & CO*.

Cecchi, A., Rovedatti, M. G., Sabino, G., and Magnarelli, G. G. (2012). Environmental exposure to organophosphate pesticides: assessment of endocrine disruption and hepatotoxicity in pregnant women. *Ecotoxicology and environmental safety*, **80**: 280-287.

Chen, C., Qian, Y., Liu, X., Tao, C., Liang, Y., & Li, Y. (2012). Risk assessment of chlorpyrifos on rice and cabbage in China. *Regulatory toxicology and pharmacology*, **62**(1): 125-130.

Chen, R., Cui, Y., Zhang, X., Zhang, Y., Chen, M., Zhou, T., Lan, X., Dong, W. and Pan, C., (2018). Chlorpyrifos induction of testicular-cell apoptosis through generation of reactive oxygen species and phosphorylation of AMPK. *Journal of agricultural and food chemistry*, ***66***(47): 12455-12470.

Chiu, Y.H., Afeiche, M.C., Gaskins, A.J., Williams, P.L., Petrozza, J.C., Tanrikut, C., Hauser, R. and Chavarro, J.E., (2015). Fruit and vegetable intake and their pesticide residues in relation to semen quality among men from a fertility clinic. *Human Reproduction*, **30**(6): 1342-1351.

Clegg, D. J., and Van Gemert, M. (1999). Determination of the reference dose for chlorpyrifos: proceedings of an expert panel. *Journal of Toxicology and Environmental Health Part B: Critical Reviews*, **2**(3: 211-255.

Cooper, T.G., Noonan, E., Von Eckardstein, S., Auger, J., Baker, H.W., Behre, H.M., Haugen, T.B., Kruger, T., Wang, C., Mbizvo, M.T. and Vogelsong, K.M., (2010). World Health Organization reference values for human semen characteristics. *Human reproduction update*, **16**(3): 231-245.

Cross, N. L. (1998). Role of cholesterol in sperm capacitation. *Biology of reproduction*, **59**(1): 7-11.

Di, L., and Kerns, E. H. (2015). *Drug-like properties: concepts, structure design and methods from ADME to toxicity optimization*. Academic press.

Doctor, B. P., Toker, L., Roth, E., and Silman, I. (1987). Microtiter assay for acetylcholinesterase. *Analytical biochemistry*, **166(**2): 399-403.

Dutta, A. L., and Sahu, C. R. (2013). Emblica officinalis Garten fruits extract ameliorates reproductive injury and oxidative testicular toxicity induced by chlorpyrifos in male rats. *SpringerPlus*, *2*(1), 541.

Ekins, R. (1990). Hirsuitism: Free and Bound Testosterone. *Annals of clinical biochemistry*, **27**(1): 91-93.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, **82**(1): 70-77.

ElMazoudy, R. H., Attia, A. A., and El-Shenawy, N. S. (2011). Protective role of propolis against reproductive toxicity of chlorpyrifos in male rats. *Pesticide biochemistry and physiology*, **101**(3): 175-181.

El-Sayed El-Agamyd, and El-Khaliek El-Sebaea (2010). Chlorpyrifos induced reproductive toxicity in male mice. *Reproductive Toxicology* **29:** *80–85*

Ezzi, L., Salah, I. B., Haouas, Z., Sakly, A., Grissa, I., Chakroun, S., ... & Cheikh, H. B. (2016). Histopathological and genotoxic effects of chlorpyrifos in rats. *Environmental Science and Pollution Research*, **23**(5): 4859-4867.

Farag A. T., El-Okazy A. M., El-Aswed A. F. (2003) Developmental toxicity study of chlorpyrifos in rats. *Reproductive Toxicology*; **17**: 203–208.

Farag, A. T., Radwan, A. H., Sorour, F., El Okazy, A., El-Agamy, E. S., and El-Sebae, A. E. K. (2010). Chlorpyrifos induced reproductive toxicity in male mice. *Reproductive Toxicology*, **29**(1): 80-85.

Farahat, F. M., Fenske, R. A., Olson, J. R., Galvin, K., Bonner, M. R., Rohlman, D. S., Farahat, T. M., Lein, P. J. and Anger, W. K., (2010). Chlorpyrifos exposures in Egyptian cotton field workers. *Neurotoxicology*, **31**(3): 297-304.

Farghaly, M., Mahdy, F., Taha, H., and Fathy, U. (2007). Behavior of the organophosphorus insecticide fenitrothion in stored faba beans and its biological effects towards experimental animals. *Journal of Environmental Science and Health, Part B*, **42**(6): 655-662.

FDA, U. (2009). Guidance for industry drug-induced liver injury: premarketing clinical evaluation. *US FDA, Silver Spring, MD, USA*.

Gabrielsen, J. S., and Tanrikut, C. (2016). Chronic exposures and male fertility: the impacts of environment, diet, and drug use on spermatogenesis. *Andrology*, **4**(4): 648-661.

Goel, A. and Dhawan, D. K. (2001). Zinc supplementation prevents liver injury in chlorpyrifos-treated rats. *Biological trace element research*, **82**(1-3): 185.

Goel, A., Dani, V., and Dhawan, D. K. (2005). Protective effects of zinc on lipid peroxidation, antioxidant enzymes and hepatic histoarchitecture in chlorpyrifos-induced toxicity. *Chemico-biological interactions*, **156**(2-3): 131-140.

Helgi, K. B., Sigurdur, O., Ottar, M. B. and Einar, S. B. (2016). A prospective study on the causes of notably raised alanine aminotransferase (ALT). *Scandinavian Journal of Gastroenterology*, **51**: 594-600.

Hossain, F., Ali, O., D'souza, U. J. and Naing, D. K. S. (2010). Effects of pesticide use on semen quality among farmers in rural areas of Sabah, Malaysia. *Journal of occupational health*, 1009270143-1009270143.

Ighodaro, O. M., and Akinloye, O. A. (2018). First line defense antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, **54**(4), 287-293.

Jana, K., and Sen, P. C. (2012). Environmental toxicants induced male reproductive disorders: identification and mechanism of action. In *Toxicity and Drug Testing*. In Tech Rijeka, Croatia.

Jantunen, A. P. K., Tuikka, A., Akkanen, J., and Kukkonen, J. V. K. (2008). Bioaccumulation of atrazine and chlorpyrifos to Lumbriculus variegatus from lake sediments. *Ecotoxicology and Environmental Safety*, **71**(3), 860-868.

Jayusman, P. A., Budin, S. B., Ghazali, A. R., Taib, I. S., and Louis, S. R. (2014). Effects of palm oil tocotrienol-rich fraction on biochemical and morphological alterations of liver in fenitrothion-treated rats. *Pakistan journal of pharmaceutical sciences*, **27**(6): 1873-1880.

Joshi, S. C., Mathur, R., and Gulati, N. (2007). Testicular toxicity of chlorpyrifos (an organophosphate pesticide) in albino rat. *Toxicology and Industrial Health*, **23**(7): 439-444.

Karami-Mohajeri, S., Ahmadipour, A., Rahimi, H. R., and Abdollahi, M. (2017). Adverse effects of organophosphorus pesticides on the liver: a brief summary of four decades of research. *Arhiv za higijenu rada i toksikologiju*, **68**(4): 261-275.

Klein, B., Read, P. A., and Babson, A. L. (1960). Rapid method for the quantitative determination of serum alkaline phosphatase. *Clinical chemistry*, **6**(3): 269-275.

Koshlukova S. E. and Reed N. R. (2014). Chlorpyrifos, Environmental Protection Agency, Sacramento, CA, USA.

Kobylinska, L.I., Havrylyuk, D.Y., Ryabtseva, A.O., Mitina, N.E., Zaichenko, O.S., Lesyk, R.B., Zimenkovsky, B.S. and Stoika, R.S., (2015). Biochemical indicators of hepatotoxicity in blood serum of rats under the effect of novel 4-thiazolidinone derivatives and doxorubicin and their complexes with polyethyleneglycol-containing nanoscale polymeric carrier. *The Ukrainian Biochemical Journal*, **87**(2): 122-132.

Lacarin, C and Reed, B. (1999). Emergency vector control using chemicals. In *Emergency vector control using chemicals*. United Kingdom. Loughborough Unioversity. Water, Engineering and Development Centre

La ssiter, T. L. and Brimijoin, S. (2008). Rats gain excess weight after developmental exposure to the organophosphorothionate pesticide, chlorpyrifos. *Neurotoxicology and teratology*, **30(**2), 125-130.

Li, B., Ma, Y., and Zhang, Y. H. (2017). Oxidative stress and hepatotoxicity in the frog, Rana chensinensis, when exposed to low doses of trichlorfon. *Journal of Environmental Science and Health, Part B*, **52**(7): 476-482.

Li, J., Pang, G., Ren, F., and Fang, B. (2019). Chlorpyrifos-induced reproductive toxicity in rats could be partly relieved under high-fat diet. *Chemosphere*, **229:** 94-102.

Li, T., and Chiang, J. Y. (2014). Bile acid signaling in metabolic disease and drug therapy. *Pharmacological reviews*, **66**(4): 948-983.

Lindblom, P., Rafter, I., Copley, C., Andersson, U., Hedberg, J.J., Berg, A.L., Samuelsson, A., Hellmold, H., Cotgreave, I. and Glinghammar, B. (2007). Isoforms of alanine aminotransferases in human tissues and serum—differential tissue expression using novel antibodies. *Archives of biochemistry and biophysics*, **466**(1): 66-77.

Lorke, D. (1983). A new approach to practical acute toxicity testing. *Archives of Toxicology*, **54**(4): 275-287.

Lu, L., Wang, X., Lang, L., and Fu, F. (2010). Protective effect of reduced glutathione on the liver injury induced by acute omethoate poisoning. *Environmental toxicology and pharmacology*, **30(**3): 279-283.

Lukaszewicz-Hussain, A. (2010). Role of oxidative stress in organophosphate insecticide toxicity–Short review. *Pesticide biochemistry and physiology*, **98**(2): 145-150.

Malekirad, A. A., Faghih, M., Mirabdollahi, M., Kiani, M., Fathi, A., and Abdollahi, M. (2013). Neurocognitive, mental health, and glucose disorders in farmers exposed to organophosphorus pesticides. *Archives of industrial hygiene and toxicology*, **64**(1): 1-8.

Mandal, T. K. and Das, N. S. (2012). Testicular gametogenic and steroidogenic activities in chlorpyrifos insecticide–treated rats: a correlation study with testicular oxidative stress and role of antioxidant enzyme defence systems in Sprague-Dawley rats. *Andrologia*, **44**(2): 102-115.

Manoj, K. and Padhy, P. K. (2014). Distribution, enrichment and ecological risk assessment of six elements in bed sediments of a tropical river, Chottanagpur Plateau: a spatial and temporal appraisal. *Journal of Environmental Protection*, **5**(14): 1419.

Melgarejo, M., Mendiola, J., Koch, H. M., Moñino-García, M., Noguera-Velasco, J. A., and Torres-Cantero, A. M. (2015). Associations between urinary organophosphate pesticide metabolite levels and reproductive parameters in men from an infertility clinic. *Environmental research*, **137:** 292-298.

Milatovic, D., Gupta, R. C. and Aschner, M. (2006). Anticholinesterase toxicity and oxidative stress. *The Scientific World Journal*, ***6*:** 295-310.

Mohort, M. A., Seredinska, M. N., and Kyrychok, L. M. (2010). Cardiotoxic effects of doxorubicin and expedience of their pharmacological correction by the antagonists of calcium of dihydropyrydin type and activators of the АТР-sensitive potassium channels of guanidine type. *Pharmacol. Drug Toxicol*, **4**(7): 35-44.

Morgan, M. J., Kim, Y. S., and Liu, Z. (2007). Lipid rafts and oxidative stress–induced cell death. *Antioxidants & redox signaling*, **9**(9): 1471-1484.

Moron, M. S., Depierre, J. W., and Mannervik, B. (1979). Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochimica et Biophysica Acta (BBA)-general subjects*, **582**(1): 67-78.

Nabulo, G., Young, S. D., and Black, C. R. (2010). Assessing risk to human health from tropical leafy vegetables grown on contaminated urban soils. *Science of the Total Environment*, **408**(22): 5338-5351.

O'Boyle, N. M., Banck, M., James, C. A., Morley, C., Vandermeersch, T., and Hutchison, G. R. (2011). Open Babel: An Open chemical toolbox. *Journal of Cheminformatics*, **3**(10): 33.

Obomanu, F. G., Gabriel, U. U., Edori, O. S., and Emetonjor, J. N. (2009). Biomarker enzymes in muscle tissue and organs of Clarias gariepinus after intramuscular injection with aqueous extracts of Lepidagathis alopecuroides leaves. *Journal of Medicinal Plants Research, 3*(12), 995-1001.

Ochei J. and Kolhatkar A. (2000). Medical Laboratory Science: Theory and Practice. Tata McGraw Hill Publishing Company, New Delhi. pp 239 - 240.

Oguh C. E, Okpaka C. O, Ubani C. S, Okekeaji U, Joseph P. S, and Amadi E. U. (2019b). Natural Pesticides (Biopesticides), and uses in Pest management- a critical review. *Asian Journal of Biotechnology and Genetic Engineering*, 2(3): 1-18.

Oguh C. E., Musa A. D., Orum T. G., Iyaji R. O. and Musa A. (2019a). Risk Assessment of Heavy Metals Level in soil and Jute Leaves (*Corchorus olitorius*) Treated with Azadirachtin Neem seed Solution and Organochlorine. *International Journal of Environment, Agriculture and Biotechnology (IJEAB)*, **4**(3): 256-266.

Ohkawa, H., Ohishi, N., and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, **95(**2): 351-358.

Olowoyo, J. O, Lion G. N. (2013). Population health risk due to dietary intake of toxic heavy metals from *Spinacia oleracea* harvested from soils collected in and around Tshwane, South Africa. *South African Journal of Botany,* **88**(11): 178–182.

Olowoyo, J. O., Van Heerden E., Fischer J. L. and Baker C. (2010). Trace metals in soil and leaves of *Jacaranda mimosifolia* in Tshwane area, South Africa. *Atmospheric Environment*, **44**(20): 1826–1830.

Ongjanovic, L. B. I., and Markovic, L. (2010). Dor. d-evic SD, Trbojevic NZ, Tajn ISA, Saicic LZS. Cadmium-induced lipid peroxidation and changes in antioxidant defense system in the rat testes: Protective role of coenzyme Q10 and Vitamin E. *Reproductive. Toxicology*, **29**: 191-197.

Ozer, J., Ratner, M., Shaw, M., Bailey, W., and Schomaker, S. (2008). The current state of serum biomarkers of hepatotoxicity. *Toxicology*, **245**(3): 194-205.

Peiris, D. C. and Dhanushka, T. (2017). Low doses of chlorpyrifos interfere with spermatogenesis of rats through reduction of sex hormones. *Environmental Science and Pollution Research*, **24**(26): 20859-20867..

Peris-Sampedro, F., Cabré, M., Basaure, P., Reverte, I., Domingo, J. L. and Colomina, M. T. (2015). Adulthood dietary exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice. *Environmental research*, **142:** 169-176.

Possamai, F. P., Fortunato, J. J., Feier, G., Agostinho, F. R., Quevedo, J., Wilhelm Filho, D. and Dal-Pizzol, F. (2007). Oxidative stress after acute and sub-chronic malathion intoxication in Wistar rats. *Environmental toxicology and pharmacology*, **23**(2): 198-204.

Qiao, D., Seidler, F. J., Tate, C. A., Cousins, M. M., and Slotkin, T. A. (2003). Fetal chlorpyrifos exposure: adverse effects on brain cell development and cholinergic biomarkers emerge postnatally and continue into adolescence and adulthood. *Environmental health perspectives*, **111**(4): 536-544.

Quintana, M. M., Osimani, V. R., Magnarelli, G., Rovedatti, M. G., and Guiñazú, N. (2018). The insecticides chlorpyrifos and acetamiprid induce redox imbalance in umbilical cord blood erythrocytes in vitro. *Pesticide biochemistry and physiology*, **148**: 87-92.

Quintero, W. and Zafra, G. (2016). Use of molecular biomarkers in studies of aquatic environmental impact. *Journal of Industrial Pollution Control*.

Rahimi, R., and Abdollahi, M. (2007). A review on the mechanisms involved in hyperglycemia induced by organophosphorus pesticides. *Pesticide biochemistry and physiology*, **88**(2): 115-121.

Ramachandran, R., and Kakar, S. (2009). Histological patterns in drug-induced liver disease. *Journal of clinical pathology*, **62**(6): 481-492.

Ranjbar, A., Solhi, H., Mashayekhi, F. J., Susanabdi, A., Rezaie, A., and Abdollahi, M. (2005). Oxidative stress in acute human poisoning with organophosphorus insecticides; a case control study. *Environmental toxicology and pharmacology*, **20**(1): 88-91.

Reitman, S., and Frankel, S. (1957). Colorimetric methods for aspartate and alanine aminotransferase. *American. Journal of Clinical Pathology*, **28:** 55-60.

Reust, C. E., and Hall, L. (2001). What is the differential diagnosis of an elevated alkaline phosphatase (AP) level in an otherwise asymptomatic patient?. *Journal of Family Practice*, **50**(6): 496-496.

Richetti, S. K., Rosemberg, D. B., Ventura-Lima, J., Monserrat, J. M., Bogo, M. R., and Bonan, C. D. (2011). Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. *Neurotoxicology*, **32**(1): 116-122.

Rim, K. and Lim, C. (2014). Biological hazardous agents at work and efforts to protect worker's health: A review of recent reports. *Safety and Health at Work*, **5**(2): 43 – 52.

Sai, L., Li, X., Liu, Y., Guo, Q., Xie, L., Yu, G., Bo, C., Zhang, Z. and Li, L., (2014). Effects of chlorpyrifos on reproductive toxicology of male rats. *Environmental toxicology*, **29**(9):1083-1088.

Saoudi, M., Hmida, I. B., Kammoun, W., Rebah, F. B., Jamoussi, K. and Feki, A. E. (2018). Protective effects of oil of Sardinella pilchardis against subacute chlorpyrifos-induced oxidative stress in female rats. *Archives of environmental & occupational health*, **73**(2): 128-135.

Scirè, A., Cianfruglia, L., Minnelli, C., Bartolini, D., Torquato, P., Principato, G., Galli, F. and Armeni, T. (2019). Glutathione compartmentalization and its role in glutathionylation and other regulatory processes of cellular pathways. *Biofactors*, **45**(2): 152-168.

Sèdes, L., Thirouard, L., Maqdasy, S., Garcia, M., Caira, F., Lobaccaro, J.M.A., Beaudoin, C. and Volle, D.H., (2018). Cholesterol: A gatekeeper of male fertility?. *Frontiers in endocrinology*, **9:** 369.

Shi, Q., Hong, H., Senior, J., and Tong, W. (2010). Biomarkers for drug-induced liver injury. *Expert review of gastroenterology & hepatology*, **4**(2): 225-234.

Shittu, M., Ambali, S. F., Ayo, J. O., Fatihu, M. Y., Sulaiman, M. M. and Yaqub, L. S. (2013). Evaluation of chronic chlorpyrifos-induced reproductive toxicity in male wistar rat: protective effects of vitamin C. *Journal of Experimental & Integrative Medicine*, **3**(1).

Singh, P. B., and Singh, V. (2008). Pesticide bioaccumulation and plasma sex steroids in fishes during breeding phase from north India. *Environmental toxicology and Pharmacology*, **25**(3): 342-350.

Singh, P. B., Singh, V., and Nayak, P. K. (2008). Pesticide residues and reproductive dysfunction in different vertebrates from north India. *Food and chemical toxicology*, **46**(7): 2533-2539.

Smegal, D. C. (2000). Human health risk assessment chlorpyrifos. *US Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Office of Pesticide Programs, Health Effects Division, US Government Printing Office: Washington, DC*, 1-131.

Society of Environmental Toxicology and Chemistry (SETAC) (2004). Ecological Risk Assessment (ERA). Technical Issue Paper retrieved from https://c.ymcdn.com/sites/www.setac.org/resource/resmgr/publications_and_resources/setac_tip_era.pdf.

Suke, S. G., Sherekar, P., Kahale, V., Patil, S., Mundhada, D. and Nanoti, V. M. (2018). Ameliorative effect of nanoencapsulated flavonoid against chlorpyrifos-induced hepatic oxidative damage and immunotoxicity in Wistar rats. *Journal of biochemical and molecular toxicology*, *32*(5), e22050.

Tanvir, E. M., Afroz, R., Chowdhury, M. A. Z., Gan, S. H., Karim, N., Islam, M. N., and Khalil, M. I. (2016). A model of chlorpyrifos distribution and its biochemical effects on the liver and kidneys of rats. *Human and experimental toxicology*, **35**(9): 991-1004.

Tanvir, E. M., Afroz, R., Chowdhury, M. A.., Khalil, M. I., Hossain, M.S., Rahman, M.A., Rashid, M. H. and Gan, S. H. (2015). Honey has a protective effect against chlorpyrifos-induced toxicity on lipid peroxidation, diagnostic markers and hepatic histoarchitecture. *European Journal of Integrative Medicine*, **7**(5): 525-533.

Tian, Y. and Yamauchi, T. (2003). Micronucleus formation in 3-day mouse embryos associated with maternal exposure to chlorpyrifos during the early preimplantation period. *Reproductive Toxicology*, **17**(4): 401-405.

Tian, Y., Ishikawa, H., Yamaguchi, T., Yamauchi, T. and Yokoyama, K., 2005. Teratogenicity and developmental toxicity of chlorpyrifos: Maternal exposure during organogenesis in mice. *Reproductive Toxicology*, **20(**2): 267-270.

Timchalk, C., Campbell, J. A., Liu, G., Lin, Y. and Kousba, A. A. (2007). Development of a non-invasive biomonitoring approach to determine exposure to the organophosphorus insecticide chlorpyrifos in rat saliva. *Toxicology and applied pharmacology*, **219**(2-3): 217-225.

Timchalk, C., Nolan, R. J., Mendrala, A. L., Dittenber, D. A., Brzak, K. A., and Mattsson, J. L. (2002). A physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the organophosphate insecticide chlorpyrifos in rats and humans. *Toxicological Sciences*, **66**(1): 34-53.

Topal, A., Atamanalp, M., Oruç, E., Demir, Y., Beydemir, Ş., and Işık, A. (2014). In vivo changes in carbonic anhydrase activity and histopathology of gill and liver tissues after acute exposure to chlorpyrifos in rainbow trout. *Archives of Industrial Hygiene and Toxicology*, **65**(4): 377-385.

Trott, O. and Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, **31(**2): 455–461.

Tuzet, A., Rahantaniaina, M. S., and Noctor, G. (2019). Analyzing the function of catalase and the ascorbate–glutathione pathway in H2O2 processing: insights from an experimentally constrained kinetic model. *Antioxidants & redox signaling*, **30**(9): 1238-1268.

United State Environmental Protection Agency (USEPA).(2017). Human Health Risk Assessment Model Ecological Risk Assessment; Retrieved from https://www.epa.gov/risk/human-health-risk-assessment#self.

United States Environmental Protection Agency, 2018. Risk Assessment and Pesticide Decisions. [WWW Document].

Usmani, K. A., Hodgson, E., and Rose, R. L. (2004). In vitro metabolism of carbofuran by human, mouse, and rat cytochrome P450 and interactions with chlorpyrifos, testosterone, and estradiol. *Chemico-biological interactions*, **150**(3): 221-232.

Uzun F. G. and Kalender Y. (2013). Chlorpyrifos induced hepatotoxic and hematologic changes in rats: The role of quercetin and catechin. *Food and Chemical Toxicology* **55**: 549–556

Ventura, C., Nieto, M. R., Bourguignon, N., Lux-Lantos, V., Rodriguez, H., Cao, G., Randi, A., Cocca, C. and Núñez, M., (2016). Pesticide chlorpyrifos acts as an endocrine disruptor in adult rats causing changes in mammary gland and hormonal balance. *The Journal of steroid biochemistry and molecular biology*, **156**: 1-9.

Wang, J., Lui, W., Yang. R., Zhang, L. and Ma, J. (2013). Assessment of the potential ecological risk of heavy metals in reclaimed soils at an opencast coal mine, *Disaster Advances*, **6** (3): 366-377.

WebMD, L. (2013). Lutein. *WebMd Editorial Team*.

Winston, H. H., Davis, G. and Denton, J. (2001). A guide to health risk assessment, California Environmental Protection Agency; Retrieved from https://oehha.ca.gov/media/downloads/risk-assessment/document/hrsguide2001.pdf.

Yang, R. Z., Park, S., Reagan, W. J., Goldstein, R., Zhong, S., Lawton, M., Rajamohan, F., Qian, K., Liu, L. and Gong, D. W. (2009). Alanine aminotransferase isoenzymes: molecular cloning and quantitative analysis of tissue expression in rats and serum elevation in liver toxicity. *Hepatology*, **49**(2): 598-607.

Yen, J., Donerly, S., Levin, E. D., and Linney, E. A. (2011). Differential acetylcholinesterase inhibition of chlorpyrifos, diazinon and parathion in larval zebrafish. *Neurotoxicology and teratology*, **33**(6): 735-741.

Yuguda A. U., Abubakar Z. A., Jibo A. U., AbdulHameed A. and Nayaya A. J. (2015). Assessment of Toxicity of Some Agricultural Pesticides on Earthworm (Lumbricus Terrestris). *American- Eurasian Journal of Sustanaible. Agriculture*, **9**(4): 49-59.

APPENDICES

Appendix I - Oral

Homogeneous Subsets

AST (U/L)

	Experimental Groups	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Group 4 (Control)	3	25.6667	
	Group 1 (3.875 mg/kg b.w. CPF)	3	27.0000	
	Group 2 (6.2 mg/kg b.w. CPF)	3	29.3333	
	Group 3 (15.5 mg/kg b.w. CPF)	3		46.0000
	Sig.		.229	1.000

ALT (U/L)

	Experimental Groups	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Group 4 (Control)	3	4.3333		
	Group 1 (3.875 mg/kg b.w. CPF)	3	5.0000	5.0000	
	Group 2 (6.2 mg/kg b.w. CPF)	3		7.3333	
	Group 3 (15.5 mg/kg b.w. CPF)	3			11.6667
	Sig.		.580	.078	1.000

ALP (U/L)

	Experimental Groups	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Group 4 (Control)	3	29.5367	
	Group 1 (3.875 mg/kg b.w. CPF)	3	30.6333	30.6333
	Group 2 (6.2 mg/kg b.w. CPF)	3	31.8633	31.8633
	Group 3 (15.5 mg/kg b.w. CPF)	3		32.9167
	Sig.		.111	.116

GSH (mg/dL)

	Experimental Groups	N	Subset for alpha = 0.05

			1
Duncan[a]	Group 3 (15.5 mg/kg b.w. CPF)	3	2.7100
	Group 2 (6.2 mg/kg b.w. CPF)	3	2.9467
	Group 1 (3.875 mg/kg b.w. CPF)	3	2.9600
	Group 4 (Control)	3	3.0933
	Sig.		.434

MDA (mg/mL)

			Subset for alpha = 0.05	
	Experimental Groups	N	1	2
Duncan[a]	Group 4 (Control)	3	1.1200	
	Group 1 (3.875 mg/kg b.w. CPF)	3	1.2233	1.2233
	Group 2 (6.2 mg/kg b.w. CPF)	3	1.2600	1.2600
	Group 3 (15.5 mg/kg b.w. CPF)	3		1.5900
	Sig.		.493	.097

CAT (U/mg)

			Subset for alpha = 0.05	
	Experimental Groups	N	1	2
Duncan[a]	Group 4 (Control)	3	4.6800	
	Group 1 (3.875 mg/kg b.w. CPF)	3	5.2933	5.2933
	Group 2 (6.2 mg/kg b.w. CPF)	3	5.5033	5.5033
	Group 3 (15.5 mg/kg b.w. CPF)	3		6.8200
	Sig.		.265	.057

GPx (U/mg)

			Subset for alpha = 0.05
	Experimental Groups	N	1
Duncan[a]	Group 3 (15.5 mg/kg b.w. CPF)	3	28.5833
	Group 2 (6.2 mg/kg b.w. CPF)	3	28.9267
	Group 1 (3.875 mg/kg b.w. CPF)	3	29.9633
	Group 4 (Control)	3	30.4800
	Sig.		.344

CHOL (mmol/L)

	Experimental Groups	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Group 4 (Control)	3	3.9333	
	Group 1 (3.875 mg/kg b.w. CPF)	3	4.2900	
	Group 2 (6.2 mg/kg b.w. CPF)	3	4.8000	
	Group 3 (15.5 mg/kg b.w. CPF)	3		5.8100
	Sig.		.083	1.000

TESTOS (ng/mL)

	Experimental Groups	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Group 3 (15.5 mg/kg b.w. CPF)	3	1.2967	
	Group 2 (6.2 mg/kg b.w. CPF)	3		2.7833
	Group 1 (3.875 mg/kg b.w. CPF)	3		2.8700
	Group 4 (Control)	3		3.7600
	Sig.		1.000	.085

SPERM (X106/mL)

	Experimental Groups	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Group 3 (15.5 mg/kg b.w. CPF)	3	60.6667		
	Group 2 (6.2 mg/kg b.w. CPF)	3	66.0000	66.0000	
	Group 1 (3.875 mg/kg b.w. CPF)	3		70.0000	
	Group 4 (Control)	3			82.0000
	Sig.		.168	.289	1.000

ACHE (mmol/min/g)

	Experimental Groups	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Group 3 (15.5 mg/kg b.w. CPF)	3	.5267	
	Group 2 (6.2 mg/kg b.w. CPF)	3		.6200
	Group 1 (3.875 mg/kg b.w. CPF)	3		.6600
	Group 4 (Control)	3		.6600
	Sig.		1.000	.209

Appendix II – Inhalation

Homogeneous Subsets

AST (U/L)

	Experimental Groups	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Group 4 (Control)	2	49.8077		
	Group 1 (35.36 mg/kg b.w)	2	53.1148	53.1148	
	Group 2 (56.57 mg/kg b.w.)	2		69.6154	
	Group 3 (141.42 mg/kg b.w.)	2			94.4744
	Sig.		.625	.058	1.000

ALT (U/L)

	Experimental Groups	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Group 4 (Control)	2	21.0365		
	Group 1 (35.36 mg/kg b.w)	2		41.3250	
	Group 2 (56.57 mg/kg b.w.)	2			60.8000
	Group 3 (141.42 mg/kg b.w.)	2			64.5000
	Sig.		1.000	1.000	.525

ALP (U/L)

	Experimental Groups	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Group 4 (Control)	2	170.2733	
	Group 2 (56.57 mg/kg b.w.)	2	235.4733	235.4733
	Group 1 (35.36 mg/kg b.w)	2	238.7400	238.7400
	Group 3 (141.42 mg/kg b.w.)	2		269.5600
	Sig.		.103	.352

GSH (U/mg protein)

	Experimental Groups	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Group 3 (141.42 mg/kg b.w.)	2	140.0186		
	Group 2 (56.57 mg/kg b.w.)	2	142.1455		
	Group 1 (35.36 mg/kg b.w)	2		146.3993	
	Group 4 (Control)	2			149.9441
	Sig.		.131	1.000	1.000

MDA (U/mg protein)

	Experimental Groups	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Group 4 (Control)	2	.6144	
	Group 1 (35.36 mg/kg b.w)	2	.6411	
	Group 2 (56.57 mg/kg b.w.)	2	.6704	
	Group 3 (141.42 mg/kg b.w.)	2		1.3259
	Sig.		.471	1.000

CAT (U/mg protein)

	Experimental Groups	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Group 4 (Control)	2	112.0378	
	Group 1 (35.36 mg/kg b.w)	2	138.8051	138.8051
	Group 2 (56.57 mg/kg b.w.)	2	155.1343	155.1343
	Group 3 (141.42 mg/kg b.w.)	2		195.7300
	Sig.		.178	.098

GPx (U/mg protein)

	Experimental Groups	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Group 3 (141.42 mg/kg b.w.)	2	130.6069	
	Group 2 (56.57 mg/kg b.w.)	2	151.5570	
	Group 1 (35.36 mg/kg b.w)	2		315.3890
	Group 4 (Control)	2		334.5190
	Sig.		.078	.098

Total Protein (mg/dl)

	Experimental Groups	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Group 3 (141.42 mg/kg b.w.)	2	11.2280		
	Group 2 (56.57 mg/kg b.w.)	2		15.3225	
	Group 1 (35.36 mg/kg b.w)	2		16.4960	16.4960
	Group 4 (Control)	2			17.7065
	Sig.		1.000	.076	.070

Cholesterol (mg/dl)

	Experimental Groups	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Group 4 (Control)	2	86.2765		
	Group 1 (35.36 mg/kg b.w)	2		196.0976	
	Group 2 (56.57 mg/kg b.w.)	2		237.0732	
	Group 3 (141.42 mg/kg b.w.)	2			300.2927
	Sig.		1.000	.063	1.000

Testosterone (ng/ml)

	Experimental Groups	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Group 3 (141.42 mg/kg b.w.)	2	9.1988		
	Group 2 (56.57 mg/kg b.w.)	2	9.7249	9.7249	
	Group 1 (35.36 mg/kg b.w)	2		10.1769	
	Group 4 (Control)	2			10.9641
	Sig.		.083	.120	1.000

Sperm count (X106/mL)

			Subset for alpha = 0.05			
	Experimental Groups	N	1	2	3	4
Duncan[a]	Group 3 (141.42 mg/kg b.w.)	2	23.0000			
	Group 2 (56.57 mg/kg b.w.)	2		31.0000		
	Group 1 (35.36 mg/kg b.w)	2			45.5000	
	Group 4 (Control)	2				62.5000
	Sig.		1.000	1.000	1.000	1.000

Acetylcholinesterase (nmol/mL)

			Subset for alpha = 0.05			
	Experimental Groups	N	1	2	3	4
Duncan[a]	Group 3 (141.42 mg/kg b.w.)	2	98.2611			
	Group 2 (56.57 mg/kg b.w.)	2		140.4586		
	Group 1 (35.36 mg/kg b.w)	2			155.8089	
	Group 4 (Control)	2				173.5159
	Sig.		1.000	1.000	1.000	1.000