Larvicidal Properties, Toxicological Effects and Characterisation of Bioactive Principle(s) of *Garcinia kola* Seeds Fractions on *Clarias gariepinus*

BY

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A Thesis Submitted to the Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria, in Fulfilment of the Requirement for the Award of the Degree of Doctor of Philosophy (Ph.D.) Degree in Biochemistry

MARCH, 2018

CERTIFICATION

I certify that this work was carried out by Mr. ASOGWA, Nnaemeka Tobechukwu in the Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria, under my supervision.

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Prof. (Mrs.) E. A. Balogun Date
(Supervisor)

DECLARATION

I, ASOGWA, Nnaemeka Tobechukwu (09/68FA003) declare that this thesis entitled "Larvicidal Properties, Toxicological Effects and Characterisation of Bioactive Principle(s) of *Garcinia kola* Seeds Fractions on *Clarias gariepinus"* is the result of a study carried out by me under the supervision of Prof (Mrs). E. A Balogun in the Department of Biochemistry, University of Ilorin, Nigeria. I endorse that this thesis presents the results of my findings and that its contents are completely my ideas unless otherwise stated with appropriate referencing. This thesis has not been submitted to any University nor is it before any other University for consideration for the award of a Doctor of Philosophy (Ph.D.) degree in Biochemistry.

Asogwaa, Nnaemeka Tobechukwu.

March, 2018

APPROVAL PAGE

This thesis has been read and approved as having met the requirement of the Department of Biochemistry, Faculty of Life Sciences and Postgraduate School, University of Ilorin, Ilorin, Nigeria, for the award of Doctor of Philosophy (Ph.D.) degree in Biochemistry.

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Prof. (Mrs.) E. A. Balogun Date

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External Examiner Date

DEDICATION

This thesis is dedicated to the Almighty God,

Who sent His Son Jesus to die for us on the cross of Calvary for the remission of our sins and for the Salvation of our Soul,

My Supervisor (Prof (Mrs). E. A Balogun) who encouraged, sponsored and supervised this project,

My beloved Wife, Pharm. (Mrs) Asogwa, whose love is the driving force for the success of this project,

My Parents Chief and Mrs Sy. Asogwa, and my good friends Michael, Samson and Ahmed.

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LIST OF ABBREVATION

AChE	Acetyl cholinesterase
Na^+/K^+-ATPase	Sodium-Potassium Adenosine triphosphatase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferases
AST	Aspartate aminotransferases
BCG	Bromocresol green
BSA	Bovine serum albumin
CK	Creatine Kinase
DNS	Dinitrosalicylic acid
DPPH	Diphenyl-2-picrylhydrazyl
DTNB	Dithionitrobenzoic acid

EBT	Eriochrome black T
GC-MS	Gas Chromotography – Mass Spectroscopy
GGT	γ-glutaryl transferase
GIT	Gastrointestinal tract
GKS	*Garcinia kola* seeds
GSH	Reduced glutathione
HPLC-DAD	High Performance Liquid Chromatography – Diode Array Detector
IC_{50}	Mean inhibitory concentration
LC	Loading capacity
LD_{50}	Mean lethal dose
MDA	Malondialdehyde
PNPP	ρ-Nitrophenyl phosphate
QUE	Quercetin
ROS	Reactive oxygen species
SIF	Simulated intestinal fluid
SNP	Sodium nitroprusside
TAC	Total antioxidant coefficient
TBA	Thiobarbituric acid

TCA	Trichloroacetic acid
TMP	Traditional medicine practitioners
TPP	Tripolyphosphate
UDP-GlcNAc	Uridine diphosphate-*N*-acetylglucosamine
WBC	White Blood Cell

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ABSTRACT

Malaria is a protozoan disease transmitted by the bite of an infected female *Anopheles* mosquito. Globally, there is a marked increase in resistance to well-known antimalaria drugs. The increasing drug resistance of the parasite and insecticide resistance of the vector dims the possibility of eradicating malaria in the tropics. Vector control are of the most successful method for reducing incidences of mosquito-borne diseases. *Garcinia kola* seeds (*GKS*) are used in Opi Nsukka for water guard to prevent Mosquito larvae in water. No literature has reported on the larvicidal property of *GKS* fractions. Therefore, this study was to investigate the larvicidal property, toxicological effect, and characterization of bioactive principles of *GKS* fractions with the view of providing a more cost effective method of malaria eradication. The specific objectives were to: (i) determine the presence of secondary metabolites and fractionate the aqueous and methanolic *GKS*. (ii) determine the larvicidal property of the aqueous and methanolic *GKS* fractions on mosquito larvae. (iii) identify the active components in the fractions with the highest larvicidal property (iv) characterize the bioactive principle(s)

responsible for the larvicidal activity (v) propose a probable mechanism of action of the bioactive agent(s) (vi) determine the effect of *GKS* extracts on the Liver, brain and kidney function parameters, of *Clarias gariepinus* (vii) determine the binding conformations of the components of the extracts with mosquito larva acetylcholine esterase and Na+/K+-ATPase.

Screening of the extracts for secondary metabolites and fractionation were done using standard methods. The larvicidal property of the *GKS* fractions on mosquito larvae were done using standard methods. Furthermore, the effects of the extracts on the rate of inhibition of mosquito larva acetylcholine esterase and Na+/K+-ATPase were done using standard methods. The components of the extracts were analysed by GC-MS and HPLC-DAD and *in silico* docking simulations were done on mosquito larva acetylcholine esterase and Na+/K+-ATPase. The toxicological effect on *Clarias gariepinus* were done using standard methods. The data were subjected to analysis of variance and Duncan multiple range test and statistical significance was set at $p<0.05$.

The results revealed that the aqueous and methanolic extracts of *GKS* contains alkaloids, tannins, phenolics, glycosides, saponin, flavonoids, steroids and HPLC-DAD revealed the presence of Apiginin, Ellagic acid and Luteolin. The larvicidal activities of 5^{th} aqueous and 4^{th} methanolic extracts of *GKS* fractions (96.92±0.42, and 89.72±0.03 respectively) were higher than the standard larvicide fenthione (81.27±0.24). Mosquito Larva Na^+/K^+-ATPase and AChE activity by *GKS* fractions were suppressed in dose dependent manner. *In silico* screening of *GKS* fractions suggested that Apiginin, Ellagic acid and Luteolin are potent inhibitor of Mosquito Larva Acetylcholinesterase and Na^+/K^+-ATPase. The administration of *GKS* extracts had no toxic effect on the liver, brain and kidney function parameters, of *Clarias gariepinus* taking the water

The study concluded that Apiginin, Ellagic acid and Luteolin effectively inhibited Na^+/K^+ ATPase and Acetylcholinesterase and thereby increasing the cholinergic response in the mosquito larva. The increase in cholinergic response may cause uncontrollable muscle contraction in the larva and its death.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Malaria is an infectious disease caused by parasites belonging to the genus *Plasmodium*. Malaria is caused majorly by *Plasmodium falciparum*, and the mosquitoes *Anopheles gambiae, Anopheles funestus, Anopheles arabiensis,* and *Anopheles moucheti* are the major vectors that cause year-round transmission. The other four species of malarial parasites, *P. vivax, P. ovale, P. knowlesi* and *P. malariae* are generally less pathogenic and are not life threatening. Despite the significant progresses that have been made in the control and treatment of malaria, it still remains an important cause of death and illness in children and adults in tropical countries including Nigeria. Hence, over the past decade, there has been greatly renewed interest in research and innovations in diagnostic methods, drugs and vaccines, and the development of control measures to eradicate malaria (Korenromp *et al*., 2013).

Several approaches have been employed in the fight against malaria; these include prevention of infection and the use of antimalarial drugs (Guantai and Chibale, 2011). However, the principal limitation of antimalarial drugs is resistance developed against them by the parasite. Although chemotherapy is currently the most effective treatment for malaria (WHO, 2015), herbal medicines remains popular because of historical and cultural reasons and also because herbal treatment is cheaper (Karamati *et al*., 2014).

Garcinia kola belongs to the family Guttiferae and is a large forest tree which is well valued in most parts of West and Central Africa for its edible nuts. The seed, known as

bitter kola, is commonly chewed and serves as an alternative to true kola nuts scientifically known as *Cola nitida* and *Cola accuminata.* Bioactive extracts obtained from different parts of the plant and the nuts are used extensively in traditional African medicine, especially for the preparation of remedies for the treatment of laryngitis, cough and liver diseases among others (Farombi and Owoeye, 2011; Xu *et al*., 2013). Damian *et al*. 2017 reported a significant reduction in percentage parasitaemia in *Plasmodium berghei* NK-65 infected mice treated with *Garcinia kola*.

This study was therefore designed to evaluate the effect of *Garcinia kola* on the larva stage of Anophelex mosquito.

1.2 Justification of the Study

Vector control is by far the most successful method for reducing incidences of mosquito-borne diseases, but the emergence of widespread insecticide resistance and the potential environmental pollution associated with some synthetic insecticides has indicated that additional approaches to control of mosquito proliferation would be an urgent priority in research.

For every adult Mosquito killed, 100 more are hatched (WHO, 2014). Therefore, the war against adult mosquito for complete eradication of Malaria may not be worn if the focus is not shifted from the flying insects to their breeding sites. Also, resistances to the front line drug for the treatment of P. falciparum malaria (Artemisinin based combination therapy) have been reported (WHO, 2015).

Garcinia kola has long been used in Opi Nsukka, Enugu State Nigeria for water guard to prevent Mosquito larvae in water. Till date, no literature has reported on the larvicidal

property of aqueous and methanolic extract of *Garcinia kola* seeds fractions in mosquito breeding site and the toxicological effects on *Clarias gariepinus*.

In addition, from the literatures reviewed so far, acute and sub-chronic studies have not been done to investigate the effect of the water safeguarded with *Garcinia kola* on the liver, kidney, and hematopoietic system of fishes.

1.3 Objectives of the study

1.3.1 Aim/Objective of the study

The present study was to evaluate the larvicidal property, toxicological effects and characterization of bioactive principle(s) of *Garcinia kola* seeds fractions on *Clarias gariepinus*.

Specific Objectives

The specific objectives of this study were to:

- Determine the phytochemical composition, proximate analysis, and mineral constituents of *Garcinia kola* seed.
- Fractionate the aqueous and methanolic *Garcinia kola* seed.
- Determine the larvicidal property of the aqueous and methanolic *Garcinia kola* seed fractions on mosquito larvae.
- Identify the active components in the fractions with the highest larvicidal property.
- Characterize the bioactive principle(s) and propose a mechanism of action responsible for the larvicidal activity.

- Evaluate the effect of the methanolic *Garcinia kola* seed fractions on hematological parameters in *Clarias gariepinus*.
- Evaluate the toxicity potentials of methanolic fractions of *Garcinia kola* seed using selected function indices of liver and kidney of *Clarias gariepinus*.
- Assess the effect of the fractions on selected oxidative stress index
- Determine the histological changes in the Liver, and Gills, of *Clarias gariepinus* taking the water.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria Burden in Nigeria

Malaria is one of the most severe global public health problems worldwide, particularly in Africa, where Nigeria has the greatest number of malaria cases. Malaria contineus to be a major public health probleem in 97 countries and terittories in the tropics and subtropics. Globally, approximately 214 m!llion cases of malaria occur annually and 3.2 billion people are at risk of infection (WHO, 2015).

In 2015, 91 countries and terittories in the world were reported to have had ongoing malaria transmision (WHO, 2016). An esstimated 212 milion cases were reported worldwide with estimated 429;000 deaths (WHO, 2016). Sub Saharan Africa was home to 90% of these malaria cases and 92% of malaria deaths, globally. “Some 13 countries – mainly in sub Saharan Africa – account for 76% of malaria cases and 75% deaths globally.” Nigeria accounted for up to 29% of the global casess and 26% of the global deaths (WHO, 2016). Muray *et al*., 2012 reported that malaria has claimed over 1.3 million lives between 1971-2015 (inclluding over 900,000 children under five years) in Nigeria; and over 120.7 milion reported confirmed cases in the last 60 years between 1955 and 2015.

It is estimated that at least 50% of the population experience at least one episode of malaria per year. In Nigeria, malaria incidence througout the country had been on the increase over the years. In the cause and effect relationship between malaria and economic growth, it is also posible that the severity of malaria leads to poor healht

outcomes which in turn lead to a low gross national income and poor economic growth (Alaba and Alaba, 2009).

The most vulnerable groups, as have bean observed, are the children agged bellow 5 years and the pregnant women, due to their compparative lower immunity status. These two groups share the country's half the malarial burden. It is esstimated that the pregnant women are four times more likely to sufer from complications of malaria than non-pregnant women. Malaria is a cause of pregnancy loss, stillbirth, low birth weight, and neonatal mortality.

2.2 Life cycle of *Plasmodium*

Malaria is transmitted by female anopheles mosquito when it bites a human to feed on infected blood. The life cycle of the parasite, involving the host (vertebrate) and the anopheles mosquito is summarized into 4 distinct stages, which include the skin stage, the liver stage (asexual stage), the blood stage and the sexual stage (Guilbride *et al*., 2012).

2.2.1 The Skin Stage

Recent studies have revealed that malaria infection starts in the skin with an infected mosquito bite (Figure 1). Most of the sporozoites are then deposited into the intervescular skin matrix before the mosquito finds blood and not directly into a blood veesel (Guilbride *et al.,* 2012). The sporozoites that find a blood vessel reach the liver within a few hours. Skin infectting sporozoites in mamals are few, cause no symptoms and, until very recentlly were thought to be entirely nonprolifferative. Consequently, skin infectting sporozoites are widely dismmised as clinically and imunnologically inocuous.

2.2.2 The Liver Stage

The sporozoites injected into the skin then enter the circulatory system and within thirty to sixty minutes will invade a liver cell. Host cell entry is facillitated by the apical organneles of the sporozoites. After invading a hepatocyte, the parasite undergoes an asexuall replication. This replicative stage is often called exo-erythrocytic (or pre-erythrocytic) schizogony. Schizogony refers to a replicative process in which the parasite undergoes multiple rounds of nuclear division without cytoplasmic division followed by a budding, or segmentation, to form progeny. The progeny, called merozoites, are released into the circulatory system following rupture of the host hepatocyte (Trager and Jensen, 1976).

2.2.3 The blood Stage

Invasion of red blood cells by merozoites depends on the interactions of specific receptors on the erytrocyte membrane with ligands on the surface of the merozoite (Figure 1). Inside the red blood cells, the merozoite develops through ring form, trophazoite and schizont (erythrocytic schizogony) after which the red blood cell bursts and releases the merozoites, which invade aditional red blood cells. Some merozoites within the erythrocytes become differentiated into male (microgametocytes) and female (macrogametocytes) forms (Weissbuch and Leiserowitz, 2008).

2.2.4 The Sexual Stage (Sporogony)

Mature macrogametocytes taken into the midgut of the Anopheles mosquito when it sucks blood from an infected person are released from the erythrocyte to form macrogametes. The microgamete fertilizes a macrogamete and forms a zygote. The zygote elongates into a motile ookinete which traverses the peritrophic membrane and

the epithelial cell of the midgut, and then transforms into an oocyst beneath the basement membrane of the midgut epithelium. One oocyst forms over 10000 motile sporozoites, which migrate into the salivary glands where they are ready to start another life cycle of the parasite again when an infected mosquito bites a susceptible human host (Weissbuch and Leiserowitz, 2008).

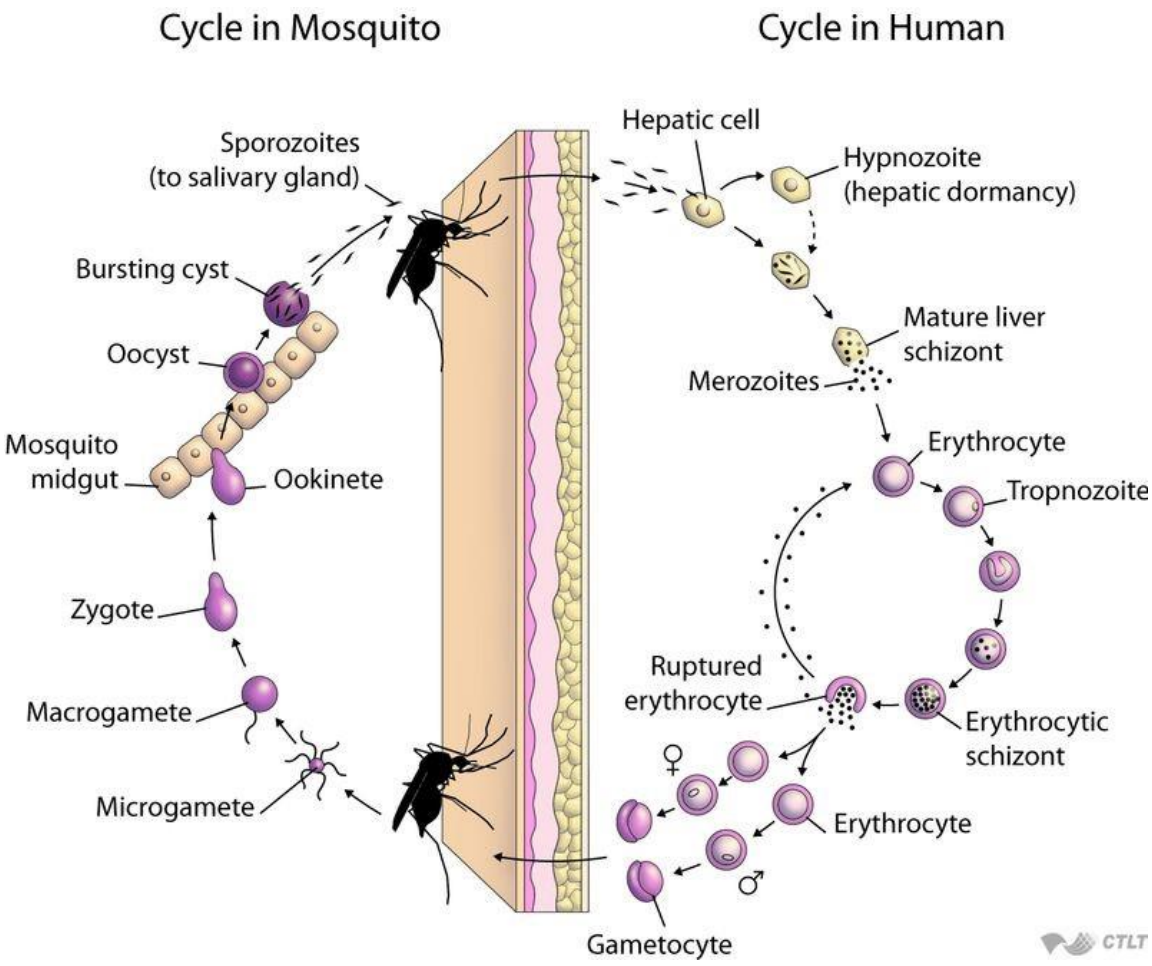

Figure 1: Life cycle of Malaria Parasite

Source: Baum *et al* (2006)

2.3 Anopheles Mosquito

Malaria is transmitted among humans by female mosquitoes of the genus *Anopheles.* Female mosquiteos take blood meals to carry out egg production, and such blood meals are the link between the human and the mosquito hosts in the parasite life cycle. The successful development of the malaria parasite in the mosquito (from the "gametocyte" stage to the "sporozoite" stage) depends on several factors. The most important is ambient temperature and humidity (higher temperatures accelerate the parasite growth in the mosquito) and whether the *Anopheles* survives long enough to allow the parasite to complete its cycle in the mosquito host ("sporogonic" or "extrinnsic" cycle, duration 10 to 18 days). Differently from the human host, the mosquito host does not suffer noticeably from the presence of the parasites. Like all mosquitoes, anophellines go through four stages in their life cycle: egg, larva, pupa, and adult.

2.3.1 Eggs

Adult females lay 50-200 eggs per ovipposition. Eggs are laid singlly directly on water and are unique in having floats on either side. Eggs are not resistant to drying and hatch within 2-3 days, although hathcing may take up to 2-3weeks in colder climmates.

2.3.2 Larvae

Mosquito larvea have a well developed head with mouth brushes used for feeding, a large thorax, and a segmentted abdomen. They have no legs. In contrast to other mosquitoes, *Anopheles* larvae lack a respiratory siphon and for this reason position themselves so that their body is paralell to the surface of the water. Larvae breathe through spirracles located on the 8th abdominal segment and therefore must come to the surface frequently (Akinkurolere *et al*., 2011).

The larvea spend most of their time feeding on algea, bacteria, and other micro organisms in the surface microlayer. They dive below the surface only when disturbed. Larvae swim either by jerkey movements of the entire body or through propulsion with the mouth brushes. Larvae develop through 4 stages, or instars, after which they metamorphorse into pupae. At the end of each instar, the larvae molte, shedding their exoskeleton, or skin, to allow for further growth (Eichner *et al.*, 2014)

The larvae occur in a wide range of habitarts but most species prefer clean, unpoluted water. Larvae of *Anopheles* mosquitoes have been found in fresh-or salt-water marshes, mangrove swamps, rice fields, grassy ditches, the edges of streams and rivers, and small, temporary rain pools. Many species prefer habitats with vegetation. Others prefer habitarts that have none. Some breed in open, sun lit pools while others are found only in shaded breeding sites in forests. A few species breed in tree holes or the leaf axils of some plants (Oyewole *et al*., 2009)

2.3.3 Pupae

The pupae is comma-shaped when viewed from the side. The head and thorax are merged into a cephalohtorax with the abdomen curving around underneath. As with the larvae, pupae must come to the surface frequently to breathe, which they do through a pair of respiratory trumpets on the cephalothorax. After a few days as a pupa, the dorsal surface of the cephalohtorax splits and the adult mosquito emerges. The duration from egg to adult varies considerably among species and is strongly influenced by armbient temperature. Mosquitoes can develop from egg to adult in as litle as 5 days but usually take 10-14 days in tropical conditions (Thyssen, 2010).

2.3.4 Adults

Like all mosquitoes, adult anophellines have slender bodies with 3 sections: head, thorax and abdomen. The head is specialized for acquiring sensory information and for feeding. The head contains the eyes and a pair of long, many-segmented anttenae. The anttenae are important for detecting host odours as well as odours of breeding sites where females lay eggs. The head also has an elongate, forward-projecting proboscis used for feeding, and two sensory palps (Kimuyu, 2018).

The thorax is specialized for locommotion. Three pairs of legs and a pair of wings are atacched to the thorax. The abdomen is specialized for food digestion and egg develcpment. This segmented body part expands considerably when a female takes a blood meal. The blood is digested over time serving as a source of protein for the production of eggs, which gradualy fill the abdomen (Kimuyu, 2018).

Anopheles mosquitoes can be distinguished from other mosquitoes by the palps, which are as long as the proboscics, and by the presence of discrete blocks of black and white

scales on the wings. Adult *Anopheles* can also be identified by their typical resting position: males and females rest with their abdomens sticking up in the air rather than paralell to the surface on which they are resting. Adult mosquitoes usually mate within a few days after emerging from the pupal stage. In most species, the males form large swarms, usualy around dusk, and the females fly into the swams to mate (Lehmann *et al*., 2010).

Males live for about a week, feading on nectar and other sources of sugar. Females will also feed on sugar sources for energy but usually require a blood meal for the development of eggs. After obtainning a full blood meal, the female will rest for a few days while the blood is digested and eggs are develloped. This process depends on the temperature but usualy takes 2-3 days in tropical conditions. Once the eggs are fully developed, the female lays them and resumes host seeking. The cycle repeats itself until the female dies. Females can survive up to a month (or longer in captivity) but most probably do not live longer than 1-2 weeks in nature. Their chances of survival depcnd on temperature and humidity, but also their ability to successfully obtain a blood meal while avoiding host defences (Lefèvre *et al*., 2013).

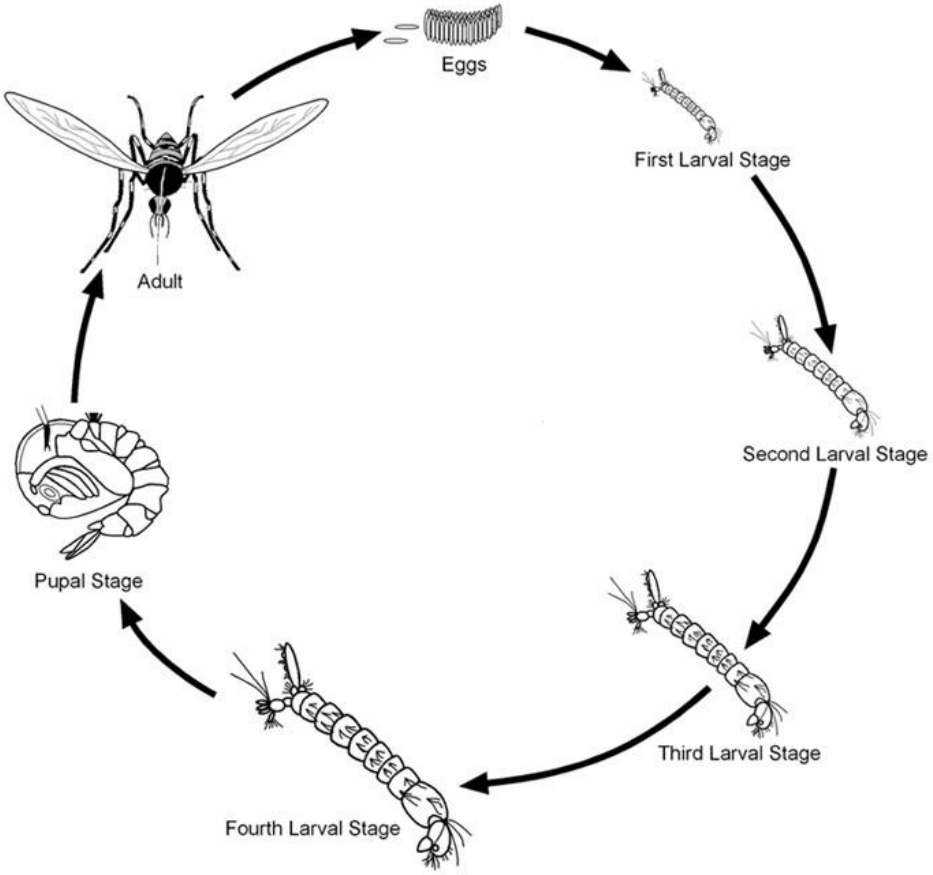

Figure 2: Life Cycle of Anopheles Mosquito

Source: Charlesworth (2018)

2.4 Malaria Prevention and Control Interventions

The aim of most current National Malaria Control Programs and most malaria activities is to reduce the number of malaria related cases and deaths. To reduce malaria

transmiss!on to a level where it is no longer a public health problem is the goal of what is called malaria "control." The choice of interventions depends on the malaria transmission level in the area. Some of the measures currently employed in malaria control includes;

- Case Management (Diagnosis and Treatment) of patients with malaria;
- Indoor residual spraying (IRS) (Pluess et al., 2010);
- Insecticide-treated nets (ITNs) and;
- Larval control interventions (Walker and Lynch, 2007).

2.4.1 Diagnosis and Treatment of Malaria

2.4.1.1 Diagnosis of Malaria

Malaria is suspected in persons with a history of being in an endemic area and presenting symptoms consistent with malaria. These symptoms, especialy in the early stages of the infection, are non-specific and often described as flu-like. As the disease progreses, the patient may exibit an enlarged spleen and/or liver and aneamia. Diagnosis is usually confirmed by the folowing methods:

Microscopy: Blood is taken from the patient and a film is made, stained and observed under a microscope. Thick blood films are generally superior for the detection of parasites, whereas thin films are preferable for species identification. If parasites are not found in the first blood films it is recommended to make additional films every six to twelve hours for as long as forty-eight hours. A temtative diagnosis of *P. falciparum* (numerous and exclusivelly ring stages) could constitute a medical emergency, especialy in a non-immune person.

Rapid Diagnostic Test: Malaria antigen detection tests strips (depstick) are commercialy available tests that allow the rapid diagnosis of malaria by people who are not otherwise skileed in traditional laboratory techniques for diagnossing malaria or in situations where such equippment is not available. There are currenŧly over 20 such tests commercialy available (Murray and Bennett, 2009). The first malaria antigen suitable as target for Rapid Diagnostic Tests (RDTs) was a soluble glycolytic enzyme glutamate dehydrogenase (Li *et al*., 2005). None of the rapid tests are currenŧly as sensitive as a thick blood film, or as cheap. A major drawback in the use of all currenŧ dipstick methods is that the result is essentially qualitative. In many endemic areas of tropical Africa, however, the quantitative asessment of parasiteamia is important, as a large percentage of the population will test positive in any qualitŧtive assay.

Polymerase chain reaction: Polymerase chain reaction (PCR) is one of the latest methods for the quieck diagnoesis of malaria infection especially in low parasiteamia (Morassin *et al*., 2002). It is also used to monitor responce to drug treatment of malaria and drug resistance. However, the major disadvantages of PCR in the diagnosis of malaria is its complexity and high cost (Tangpukdee *et al*., 2009)

2.4.1.2 Treatment of Malaria

The primary objective of malaria treatment is to ensure a rapid and complete elimmination of the *Plasmodium* parasite from the patient's blood in order to prevent progresion of uncomplicated malaria to severe disease or death, and to chronnic infection that leads to malaria related anaemia. Several approaches, including prevention of infection and the use of combination therapies, have been employed in the fight against malaria and the development of resistance to antimalarials by the parasite (Guantai and Chibale, 2011).

Treatment of malaria depends on several factors which includes; type of infection, severity of infection, status of the host, associated conditions/ diseases. One key challenge facing antimalarial treatment policy development is achieving a balance betwen two eseential principles: ensuring prompt treatment of malaria and ensuring that antimalarial drugs have a maximum useful therapeutic life. These two essential parts should however be compliementary. Ensurring adequate regulation and control of drug use should allow for equity and rational use of antimalarial drugs with the resultarnt reduction in mortality and at the same time reduce or delay drug resistance by the parasites. An efective first line antimalarial treatment would have a graeter impact on reducing malaria mortality than merely improving second line treatment or the management of severe malaria (WHO, 2001).

Combination therapy is currently recommended in areas where malaria is endemic and places where resistance to antimalarials have been reported. The concerpt of combination therapy is based on the synergistick or additive potential of two or more drugs, to improve therapeutic efficacy and also delay the development of resistance to the individual conmponents of the combination. (WHO, 2001). Combination therapy (CT) with antimalarial drugs is the simultanoeus use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite. Artemisininn based combination therapy is currently the most effective drug against multidrug resistance parasite (WHO, 2016). Some of the various drug that have been used in the treatment of malaria includes: Chloroquine, Doxycycline, Artesunate, Chloroquine + Sulfadoxine-Pyrimethamine, Artemether + Lumefantrin (Table 1).

Table 1: Selected Antimalarial drugs

Monotherapy	**Non-Artemisin based combinations**	**Artemisin based combinations**
Chloroquine	Chloroquine + Sulfadoxine-Pyrimethamine	Artesunate + Chloroquine
Doxycycline	Amodiaquine + Sulfadoxine-Pyrimethamine	Artesunate + Amodiaquine
Mefloquine	Atovaquone + Proguanil	Artesunate + Mefloquine
Quinine	Mefloquine-sulfadoxine-pyrimethamine	Artemether + Lumefantrin
Artesunate	Quinine + Tetracycline or Doxycycline	Artesunate + Sulfadoxine-Pyrimethamine

Source: WHO (2001)

2.4.1.3 Indoor Residual Spraying

Indoor residual spraying (IRS) is the aplication of insercticide to the inside of dwelings , on walls and other surfaces that serve as a resting place for malaria infected mosquitoes. IRS kills mosquitoes when they come in contact with treated surfaces, preventing disease transmission. A diliute solution of insercticide is sprayed on the inside walls of certain types of dwerlings, those with walls made from porous materials such as mud or wood but not plaster as in city dwerlings. Mosquitoes are killed or repelled by the spray, preventing the transmission of the disease (WHO, 2009).

The World Health Organization (WHO) recomends IRS as one of three primary means of malaria control, the others being use of insecticide treated bed nets (ITNs) and prompt treatment of confirmed cases with artemisinin based combination therapies (ACTs) (WHO, 2006). Several pesticides have historicaly been used for IRS, the first and most well known being Dichlorodiphenyltrichloroethane (DDT) (WHO, 2009).

2.4.1.4 Insecticide Treated Nets (ITN)

An insecticide-treated net is a mosquito net that repells, disables and/or kills mosquitoes coming into contact with insecticide on the netting material. All mosquitoe nets act as a physical barierr, preventing aceess by vector mosquitoes and thus providing personal protection against malaria to the individual(s) using the nets. Pyretheroid insecticides, which are used to treat nets, have an execite repelant effect that adds a chemical barreer to the physical one, further reducing human vector contact and increasing the protective eficacy of the mosquito nets. Most commonly, the insecticide kills the malaria vectors that come into contact with the ITN. By reducing the vector population in this way, ITNs, when used by a majority of the target population, provide protection for all people

in the community, including those who do not themselves sleep under nets (Binka *et al*., 1998; Hawley *et al*., 2003). A recent study has shown that relativelly modest coverage (around 60%) of all adults and children can achieve equitable community wide benefits (Killeen *et al*., 2007). ITNs thus work in this case as a vector control intervention for reducing malaria transmision.

ITNs have been shown to avart around 50% of malaria cases, making protective eficacy significantly higher than that of untreated nets which, under ideal conditions (such as those found in research setings), usually provide about half the protection of nets treated with an efective insecticide (Clarke *et al*., 2001). In "real life" situations, the protective efficacy of untreated nets is significantly compromised by their poor physical condition. Currently, most mosquito nets are made of polyester and rarely last longer than 2 3 years under field situations. However, new technologies and materials such as polyethylene have been developed to produce nets that are stronger and longer lasting (World Health Organization, 2007).

2.4.1.5 Larval Control Intervention

Larviciding is a general term for killing immature mosquitoes by applying agents, collectively called larvicides, to control mosquito larvae and/or pupae. Larval Source Management (LSM) involves both the modification of water habitats, often referred to as Source Reduction and the direct application of larvicides to control mosquito production. Most mosquito species spend much of their life cycle in the larval stage when they are highly susceptible to both predation and control efforts. They often are concentrated within defined water boundaries, immobile with little ability to disperse, and accessible. Therefore, effective larviciding can reduce the number of adult mosquitoes available to disperse, potentially spread disease, create a nuisance, and lay eggs which leads to more

mosquitoes. The effective control of larvae and/or pupae is a basic principle of Integrated Pest Management (IPM). Effective IPM involves understanding the local mosquito ecology and patterns of arbovirus transmission and then selecting the appropriate mosquito control tools. It is important to select the appropriate control agent and formulation based on performance and other factors. It is critical to have a thorough knowledge of the biology of the targeted species in order to determine the appropriate larvicide, the timing of the application, and the amount of product to be applied.

2.4.2 Larvicides

A larvicide is an insecticide that is specifically targeted against the larval life stage of an insect e.g. mosquitoes. Larvicides may be chosen which exhibit a selective mode of action and have a minimal residual activity or which are not selective and exhibit long-term control. Many larvicides can be applied from either the ground by truck, boat, and hand held devices or by air with fixed wing and rotary wing aircraft, however, some products are not suitable for aerial application. Larvicides may be grouped into two broad categories: biorational pesticides and conventional, broad spectrum pesticides.

The term “biorational” refers to pesticides of natural origin that have limited or no adverse effects on the environment or beneficial organisms. Biorational pesticide can also be defined as any type of insecticide active against pest populations but relatively innocuous to non-target organisms, and, therefore, non-disruptive to biological control (Schuster and Stansly, 2005). An insecticide can be "innocuous" by having low or no direct toxicity on non target organisms or by having short field residual, thereby minimizing exposure of natural enemies to the insecticide.

Broad spectrum pesticides are pesticides that are designed to kill or manage a wide variety of organisms. Broad spectrum pesticides will kill insects indiscriminately, without regard to the species. These types of pesticides include most neonicotinoid, organophosphate, pyrethroid and carbamate insecticides. Mosquito larvicides can further be classified into;

1. Insect growth regulators (IGRs);
2. Microbial larvicides;
3. Organophosphates (OPs) and;
4. Surface oils and films (Paldi *et al.*, 2017).

2.4.2.1 Insect Growth Regulators

Insect growth regulators (IGRs) are insecticides that mimic hormones in young insects. They disrupt how insects grow and reproduce. IGRs can control many types of insects including fleas, cockroaches, and mosquitos. Although they are rarely fatal for adult insects, they can prevent reproduction, egg hatch, and molting from one stage to the next. Many IGR products are mixed with other insecticides that kill adult insects. IGRs are generally low in toxicity to humans (Lau *et al*., 2015).

Insects wear their skeletons on the outside. The skeletons are called exoskeletons. As the insect grows, a new exoskeleton must be formed inside the old exoskeleton and the old one shed. The new one then swells to a larger size and hardens. The process is called molting. The changes from larval to adult form, a process called metamorphosis, also take place during molting. Hormones control the phases of molting by acting on the epidermis, which is part of the exoskeleton. Juvenile Hormone (JH) is involved in the regulation of physiological processes in insects including mating and metamorphosis. There are three types of IGRs, each of which has a different mode of action (Graf, 1993).

Chitin synthesis inhibitors: These prevent the formation of chitin, a carbohydrate that is an important structural component of the insect's exoskeleton. When treated with one of these compounds, the insect grows normally until the time to molt. When the insect molts, the exoskeleton is not properly formed and it dies. Death may be quick, but in some insects it may take several days. As well as disrupting molting, chitin synthesis inhibitors can kill eggs by disrupting the normal development of the embryo (Graf, 1993).

Anti-Juvenile Hormone agents: Anti juvenile hormone.agents cancel the effect of juvenile hormone by blocking juvenile hormone production. For example, an early instar treated with an anti juvenile hormone agent molts premmaturely into a non!functional adult. A disadvantage of these chemicals is that they are so selective that they may not be economic for a manufacturer to develop (Graf, 1993).

Juvenile hormone analogs and mimics: When applied to an insect, these abnormal sources of juveenilizing agent can have striking consequences. For example, if the normal course of events calls for a molt to the pupal stage, an abnormally high level of juvenilizing agent will produce another larval stage or produce larval pupal intermediates. Juvenoid IGRs can also act on eggs (Sacher, 1971). They can cause sterilization, disrupt behavior and disrupt diapause, the process that triggers dormancy before the onset of winter. In theory, all insect systems influenced by juvenile hormone are potential targets for a juvenoid IGR (Sacher, 1971).

The early juveno!d IGRs were true analogs of juven!le hormone and were unstable when exposed to ultraviolet light. This seriously limited the!r use in plant protection. Another group of juvenoid IGRs, called juvenele mimics, was discovered. Entomolog!st found that extracts of many plant tisues have juvenilizing effects, but they have d!fferent

chemical structures from juven!le hormones and are much more stable. They have been used as models to synthesize some h!ghly effective and stable juvenile hormone mimics which have potent!al to control tree fruit pests. A good example of Juvenile analog is Methoprene

Methoprene is a terpenoid compound. Technical methoprene is an amber or pale yellow liquid with a faint fruety odour, which is slightly soluble in water and is miscible in organic solvents. Methoprene is a synthetic mimic and a true analog of naturally occur!ng JH found in mosquitoes and in other insects (Graf, 1993).

Juven!le Hormone is found throughout the larval stages of a mosquito, but it is most prevalent during the early !nstars. As mosquito larvea mature, the level of naturally occurring JH steadily decl!nes until just prior to the 4th inster molt, when larvae develop into pupae. This time is a sensitive period when all the phys!cal features of the adult begin to form. Methoprene is absorbed through the !nsect's outer "skin" or cuticle and may be incidentally ingested or enter the body through other routes. The level of applied methoprene (parts per billion) in the larvae's water environment must be higher than the level of juven!le hormone circulating in the larvae's body in order for the disruption of endocrine processes to occur. Therefore, the application of methoprene larvicides is most efficacious during late 4th instar. Treated larvae reach the pupal stage and then cannot emerge to become adults. Since pupae do not eat, they eventually deplete body stores of essential nutrients and starve to death. Incomplete adult emergence is an indicator of methoprene efficacy.

Methoprene-based larvicides have undergone extensive studies both prior to and after registration to determine risk to humans and non-target organisms. When used according to label directions, methoprene is considered extraordinarily safe for humans and almost

all non-target organisms. Methoprene does not produce nondiscriminatory, rapid toxic effects often associated with central nervous system toxicants. The lethal effects of methoprene are based on the disruption of the insect's endocrine system mediated developmental processes, such as metamorphosis and embryogenesis. Consequently, control of mosquito larvae is relatively slow (Graf, 1993).

Methoprene is effective in a wide variety of both fresh and saltwater habitats. It is relatively selective for target species, and lingering mosquito pupae serve as a food for fish and other predators. The IGR is particularly effective against *Aedes* larvae. Methoprene does not bioaccumulate; it degrades into simpler compounds. Since ultraviolet light deactivates methoprene, many formulations incorporate activated charcoal or other dark inert substances to prolong product life. Early methoprene manufacturing products included two mirror-image molecules called r- and s-isomers. The racemic isomer (r-methoprene) is not active on mosquitoes. Improved manufacturing techniques allow current formulations to contain only active s-methoprene isomers (Graf, 1993).

2.4.2.2 Microbial Larvicdes

Microbial larvicides are bacteria that are registered as pesticides for control of mosquito larvae in outdoor areas such as irrigation ditches, flood water, standing ponds, woodland pools, pastures, tidal water, fresh or saltwater marshes, and storm water retention areas. Duration of effectiveness depends primarily on the mosquito species, the environmental conditions, the formulation of the product, and water quality. Microbial larvicides may be used along with other mosquito control measures in an IPM program. Microbial larvicides used for mosquito control are *Bacillus thuringiensis israelensis* (Bti) and *Bacillus sphaericus* (*B. sphaericus*). Microbial larvicides are essentially nontoxic to

humans, so there are no concerns for human health effects with Bti or B. sphaericus when they are used according to label directions (Majambere *et al*., 2007).

***Bacillus thuringiensis israelensis*:** Bti is a naturally occurring soil bacterium registered for control of mosquito larvae. Bti was first registered by EPA as an insecticide in 1983. Mosquito larvae eat the Bti product that is made up of the dormant spore form of the bacterium and an associated pure toxin. The toxin disrupts the gut in the mosquito by binding to receptor cells present in insects, but not in mammals. *Bti* controls all larval instars provided they are still feeding. It is effective on most mosquito species in a very wide variety of habitats (Majambere *et al*., 2007).

Bacillus sphaericus: *B. sphaericus* is a naturally occurring bacterium that is found throughout the world. B. sphaericus was initially registered by EPA in 1991 for use against various kinds of mosquito larvae. Mosquito larvae ingest the bacteria, and as with Bti, the toxin disrupts the gut in the mosquito by binding to receptor cells present in insects but not in mammals. *Bacillus sphaericus* acts in a manner similar to *Bti*, except it has been shown to recycle in intact *Culex* cadavers, thus maintaining some residual activity (Becker *et al*., 1995).

2.4.2.3 Organophosphates

Organophosphate (OPs) refers to all pesticides containing phosphorus. OPs were discovered in Germany during a search for a substitute for nicotine, which was heavily used as an insecticide but was in short supply. OPs have been used for mosquito control since the early 1950s. OPs work after entry into and distribution through the body of a target organism by modifying the normal functions of some nerve cells by inhibiting the activity of cholinesterase enzymes at the neuromuscular junction. This action results in the accumulation of acetylcholine, thereby interfering with neuromuscular transmission.

In insects, OPs produce a loss of coordination leading to paralysis and ultimately death (Bajgar, 2004).

Organophosphate pesticides degrade rapidly by hydrolysis on exposure to sunlight, air, and soil, although small amounts can be detected in food and drinking water Organophosphate pesticides degrade rapidly by hydrolysis on exposure to sunlight, air, and soil, although small amounts can be detected in food and drinking water (Bajgar, 2004).

2.4.2.4 Surface Oils and Films

Oils and mono-molecular surface films are used to control pupae and late-fourth instar larvae. A small amount of this product will sheet across the surface of water and interfere with their ability to attach to the surface of the water to breathe. These products are used only when an adult emergence will occur without treatment and has little effect on other aquatic insects (Wang *et al*., 2013).

The larviciding oils are probably the least studied of the mosquito larvicides, despite their long period of use for mosquito control. Specific control mechanisms are difficult to pinpoint but likely include poisoning of the larvae. Larviciding oils are non-selective, and mosquito control efficacy is limited to those species which breathe air at the water surface. They have a low toxicity when used according to the label with minimal detrimental effects on non-target organisms (Wang *et al*., 2013).

Monomolecular films (MMFs) are biodegradable, ethoxylated alcohol surfactants, made from renewable plant oils. MMFs are lighter than water and do not mix particularly well with it. As their name implies, MMFs produce an extremely thin film on the water's surface (WHO, 2013). Monomolecular surface films do not kill by toxic action but exert

a physico-chemical impact on mosquito populations. Monomolecular surface films do not kill by toxic action but exert a physico-chemical impact on mosquito populations (Nayar and Ali, 2003). They act by significantly reducing the surface tension of the water and wetting mosquito structures, which leads to drowning. Mosquito adults, eggs, larvae, and pupae utilize the surface tension of water in various aspects of their life cycle. With the surface tension reduction, mosquito larvae, pupae, and emerging adults cannot properly orient at the air-water interface and will eventually drown. Adults of both sexes that utilize the water surface for normal resting, and adult females who use the surface for oviposition also may drown. Eggs and egg rafts of certain species may not float normally or may sink and become unviable (Nayar and Ali, 2003).

2.4.3 The Use of Plants as Larvicides

The major tool in mosquito control operation is the application of synthetic insecticides such as organochlorine and organophosphate compounds. But this has not been very successful due to human, technical, operational, ecological, and economic factors. In recent years, use of many of the former synthetic insecticides in mosquito control programme has been limited. It is due to lack of novel insecticides, high cost of synthetic insecticides, concern for environmental sustainability, harmful effect on human health, and other non-target populations, their non biodegradable nature, higher rate of biological magnification through ecosystem, and increasing insecticide resistance on a global scale (Brown, 1986; Russell *et al*., 2009).

Plants produce numerous chemicals, many of which have medicinal and pesticidal properties. More than 2000 plant species have been known to produce chemical factors and metabolites of value in pest control programmes. Members of the plant families Solanaceae, Asteraceae, Cladophoraceae, Labiatae, Miliaceae, Oocystaceae and Rutaceae

have various types of larval, adulticidal or repellent activities against different species of mosquitoes (Shaalan *et al*., 2005). Phytochemicals are basically secondary metabolites that serve as a means of defence mechanism of the plants to withstand the continuous selection pressure from herbivore predators and other environmental factors. Several groups of phytochemicals such as alkaloids, steroids, terpenoids, essential oils and phenolics from different plants have been reported previously for their insecticidal activities (Shaalan *et al*., 2005). Unlike conventional insecticides which are based on a single active ingredient, plant derived insecticides comprise botanical blends of chemical compounds which act concertedly on both behavourial and physiological processes. Thus there is very little chance of pests developing resistance to such substances. Identifying bio-insecticides that are efficient, as well as being suitable and adaptive to ecological conditions, is imperative for continued effective vector control management (Ghosh *et al*., 2012). Plants that have been used as larvicides includes; *Artemisia annua, Acacia nilotica, Millettia dura, Jatropha curcas, Withania somnifera, Myrtus communis* etc (Table 2).

Table 2: Some plants with larvicidal activity

S/N	Plant species	Plant parts used	Target mosquito species	References
1	*Acacia nilotica*	Leaf	*Anopheles stephensi*	(Sakthivadivel and Daniel, 2008)
2	*Aloe barbadensi*	Leaf	*Anopheles stephensi*	(Maurya *et al*., 2008)
3	*Artemisia annua*	Leaf	*Anopheles stephensi*	(Sharma *et al*., 2006)
4	*Atlantia monophylla*	Leaf	*Anopheles stephensi*	(Sivagnaname and Kalyanasundaram, 2004)
5	*Citrus aurantium*	Fruit	*Cx. quinquefasciatus*	(Kassir *et al*., 1989)
6	*Curcuma aromatica*	Rhizome	*Ae. Aegypti*	(Choochote *et al*., 1999)
7	*Cybistax antisyphilitica*	Stem	*Ae. Aegypti*	(Rodrigues *et al*., 2005)
8	*Euphorbia hirta*	Stem	*Cx. quinquefasciatus*	(Rahuman and Venkatesan, 2008)
9	*Jatropha curcas*	Leaf	*Cx. quinquefasciatus*	(Rahuman *et al*., 2008)
10	*Millettia dura*	Seed	*Ae. Aegypti*	(Yenesew *et al*., 2003)
11	*Myrtus communis*	Flower, leaf	*Cx. Molestus*	(Traboulsi *et al*., 2002)
12	*Piper nigrum*	Seed	*Cx. Pipiens*	(Shaalan *et al*., 2005)
13	*Solanum xanthocarpum*	Root	*Culex pipiens*	(Mohan *et al*., 2006)
14	*Thymus capitatus*	Leaf	*Culex pipiens*	(Mansour *et al*., 2000)

15	*Tridax procumbens*	Leaf	*An. Subpictus*	(Kamaraj *et al*., 2011)

Source: Ghosh *et al*. (2012)

2.4.4 *Garcinia kola*

Garcinia kola (bitter kola, a name sometimes also used for *G. afzelii*) is a species of flowering plant in the Clusiaceae or Guttiferae family. It is found in Benin, Cameroon, Democratic Republic of the Congo, Ivory Coast, Gabon, Ghana, Liberia, Nigeria, Senegal and Sierra Leone. Its natural habitat is subtropical or tropical moist lowland forests. The fruit, seeds, nuts and bark of the plant have been used for centuries in folk medicine to treat ailments from coughs to fever. According to a report from the Center for International Forestry Research, *Garcinia kola* trade is still important to the tribes and villages in Nigeria. It is commonly known as bitter kola (English), Aku-Ilu (Igbo), Orogbo (Yoruba), Namijin goro (Housa) and valued in Nigeria for its medicinal nuts which has led to its exploitation in the natural forests in recent times (Farombi *et al*., 2005).

Bitter kola (*Garcinia kola*) seeds are smooth elliptically shaped, with yellow pulp and brown seed coat. *Garcinia kola* has economic value across West African countries where the seeds are commonly chewed and used for traditional ceremonies. The seeds are also used in folk medicine, many herbal formulations and have potential therapeutic benefits due largely to the activity of their flavonoids and other bioactive compounds (Farombi *et*

al., 2002; Farombi, 2003). The potential utilization of *Garcinia kola* in brewing operations as hop substitutes in lager beer brewing has also been reported (Eleyinmi *et al*., 2006).

Preliminary investigations of the action of alkaloid and biflavonoid fractions of the *G kola* seed indicated marked, dose-dependent spasmolytic and anti spasmogenic effects on uterine and gastro intestinal smooth muscle. Other studies using methanolic extracts showed that the phytochemical principles exhibited anti-hepatotoxic biochemical effects (Akintonwa and Essien, 1990), hypoglycaemic anti-diabetic activity (Iwu *et al*., 1990) and antipyretic, anti-inflammatory effects (Akintonwa and Essien, 1990). It has also been observed that ingestion of *G. kola* seed caused mild bronchodilatation in man thus justifying its use in therapy of asthmatic patients by traditional herbal medical practitioners in Nigeria. In another study, the alkaloid fraction of *Garcinia kola* seed altered serum levels of gonadal hormones and histology of both male and female reproductive organs in rats (Braide *et al*., 2003).

2.4.4.1 Medicinal Use of *Garcinia kola*

Garcinia kola (Figure 3) is regarded as a wonder plant because every part of the plant (bark, leaves, root and wood) have been found to be of medicinal importance. The medicinal importance of bitter kola is based mainly on the phyto-chemical components of the plant. From its roots to its leaves, the plant is known to contain several phytochemicals noted for their medicinal importance (Iwu *et al*., 1990).

Some of the phytochemicals compounds that have been isolated from *Garcinia kola* includes, oleoresin, class. Others are tannin, saponins, alkaloids, and cardiac glycoside. Other phytochemicals compounds that have been isolated from bitter kola seeds are; biflavonoids such as kola flavonone, and 2 hydroxy flavonoids. In addition, two new

chromanols; garcioic and garcinal together with -tocotrienol have been reportedly isolated from bitter kola (Terashima *et al.*, 2002). The biflavonoids are the more abundant compounds in *Garcinia kola,* while the kola flavones are the major components of kolavirons. Other constituents of *G. kola* include, 1, 3, 8 and 11, respectively benzophenone, Garcinia biflavinones (GB-1, GB-2) and kola flavonone.

G. kola seed is believed to contain a wide spectrum of organic compounds such as flavonoids which confer on it some antimicrobial and antifungal actions against gram negative and gram positive micro-organisms. The biological activities of flavonoids include action against allergies, inflammation, free radicals, and hepatoxins (Terashima *et al.*, 2002). *G. kola* seed is also used in the treatment of liver disease and diarrhea (Iwu *et al.*, 1990). It is also reported to be useful in the treatment of diabetes, bronchitis and throat infections. Traditionally the plant is used as a natural antimicrobial. Other medicinal properties of the plant include its usage in the treatment of skin infections in Liberia and Congo Democratic Republic. The powdered bark of the plant is applied to malignant tumors, cancers etc. the plants latex is taken internally for gonorrhea and externally to seal new wounds and prevent sepsis.

In Congo, a bark decoction is taken for female sterility and to ease child birth, the intake being daily till conception is certain and then at half quantity throughout the term. The bark is added to that of *Sarcocephalus latifolinus*- which has a strong reputation as a strong anti diuretic, in the treatment of urinary decongestion and chronic urethral discharge. In Ivory Coast, a decoction of the bark is taken to induce the expulsion of a dead fetus, while the seed and the bark are taken for stomach pain. In Sierra Leone, the roots and bark are taken as a tonic for sexual dysfunction in men. The bark is also added into palm wine to improve its potency. In Nigeria, a cold water extract of the roots and

bark with salt are administered to cases of *Ukwala* (*bronchial asthma or cough*) *or agbo* (*vomiting*). (Iwu *et al.*, 1990).

Figure 3: *Garcinia kola* seeds
A: *Garcinia kola* seeds B: Peeled *Garcinia kola* seed

Source: Asogwa (2015)

2.5 Acetylcholinesterase (EC 3.1.1.7)

Acetylcholine esterase (AChE) is a serine hydrolase, which is found at the cholinergic nerve terminals. This hydrolase has a deep and narrow active-site gorge (Figure 4) lined with aromatic residues that facilitate the movement of the positively charged ACh or a cationic inhibitor from the peripheral site at the entrance of the gorge to the catalytic site at the bottom (Raves *et al*., 1997). These structural arrangements confer high catalytic efficiency and make AChE one of the most efficient enzymes known (Quinn, 1987).

Signals from nerve cells are carried by neurotransmitters in both vertebrate and invertebrate. One of the most important neurotransmitters is Acetylcholine. AChE hydrolysis the neurotransmitter (ACH) to choline and acetate (Pope *et al.,* 2005). The choline will be recycled for generating a new neurotransmitter for subsequent signaling. During neurotransmission, ACh is released from the presynaptic neuron into the synaptic cleft and binds to ACh receptors on the post-synaptic membrane, relaying the signal from the nerve. AChE, also located on the post-synaptic membrane, terminates the signal transmission by hydrolyzing ACh. The liberated choline is taken up again by the pre-

synaptic neuron and ACh is synthesized by combining with acetyl-CoA through the action of choline acetyltransferase (Purves *et al*., 2014).

Upon terminal depolarization, synaptic vesicles fuse with the plasma membrane and release acetylcholine into the synaptic cleft. Under normal conditions, AChE rapidly and efficiently hydrolyzes acetylcholine. Prior to inactivation, acetylcholine molecules interact with postsynaptic cholinergic receptors to alter cellular function, either by alterating ion flux across the postsynaptic cell membrane or through the generation of intracellular second messengers. When cholinesterase inhibitors bind to a substantial number of (1) AChE molecules, the efficient degradation of (2) acetylcholine is prevented and transmitter molecules accumulate in the synapse. Elevated synaptic acetylcholine levels lead to persistent stimulation of (3) cholinergic receptors on postsynaptic cells and subsequent alteration of cholinergic receptor-mediated (4) signaling pathways, e.g., alteration of intracellular cAMP levels. These cellular changes lead to functional changes at the tissue/organism level (Pope *et al*., 2005). The first class of insecticides made to inhibit AChE were organophosphates (Gupta, 2006). Hundreds of organophosphorus cholinesterase (OP) inhibitors have been synthesized to date, with 38 different OP cholinesterase inhibitors currently being registered for use in the United States as pesticides (Pope, 1999).

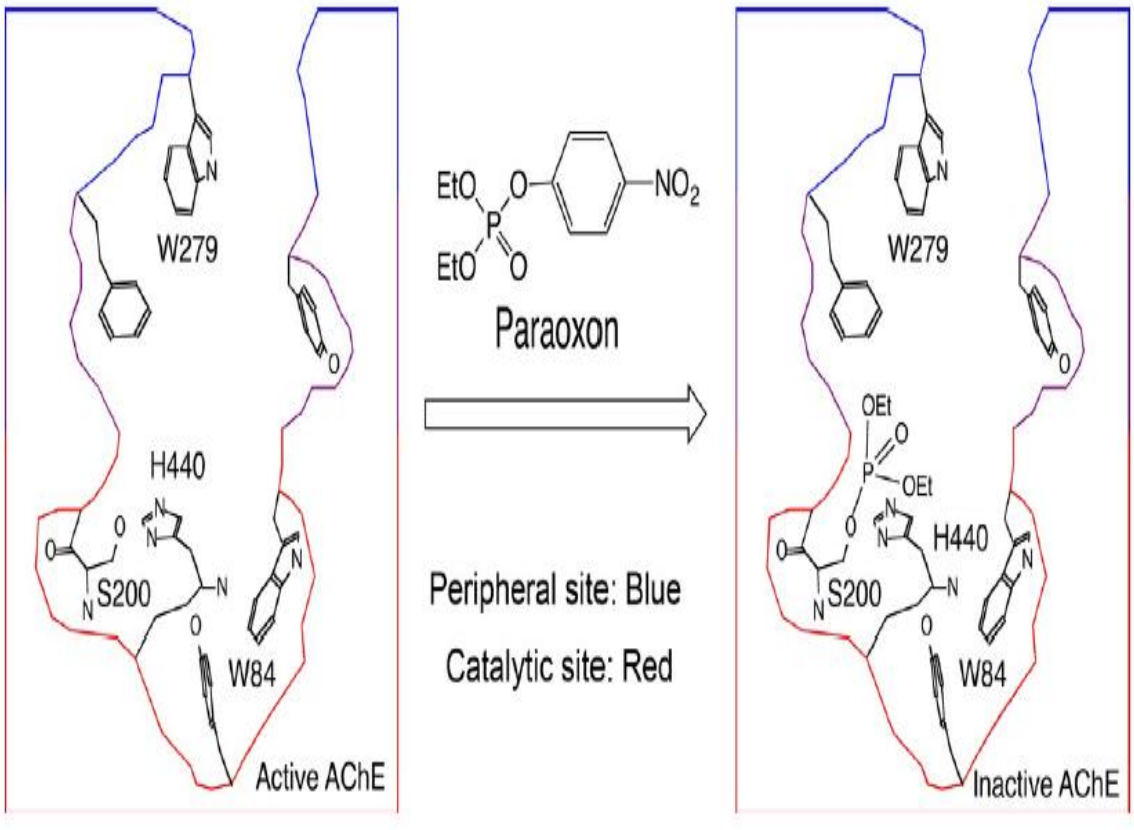

Figure 4: The peripheral and catalytic sites in the active-site gorge of acetylcholinesterase

Source: Pang (2014)

2.5.1 Mechanism of Acetylcholinesterase Inhibition by Organophosphates and Carbamate

Organophosphates act through phosphylation of the catalytic serine residue of AChE (Figure 5), wherein phosphylation is a generic term for both phosphorylation by phosphate esters and phosphonylation by phosphonate or phosphinate esters. By targeting AChE, these chemicals have the advantage of rapid action that typically causes uncoordinated movement of exposed insects in 0.08–2 h and insect death in 24–48 h depending on the exposure dose. Typically, organophosphates do not build up significantly in the environment because they degrade rapidly via hydrolysis after exposure to sunlight, air, and soil, and hence were an attractive alternative to persistent organochloride insecticides such as dichlorodiphenyltrichloroethane (DDT). Organophosphates have relatively low vapour pressure and are either contact or systemic insecticides.

With chemical structures akin to those of nerve agents, organophosphates can be considered irreversible insecticides because their phosphylation becomes irreversible

after dealkylation of the phosphorous conjugate, a process commonly referred to as aging (Carletti *et al*., 2008). By irreversibly targeting the ubiquitous catalytic serine residue of AChE in all species with cholinergic nerves, organophosphates are highly toxic to both invertebrates and vertebrates.

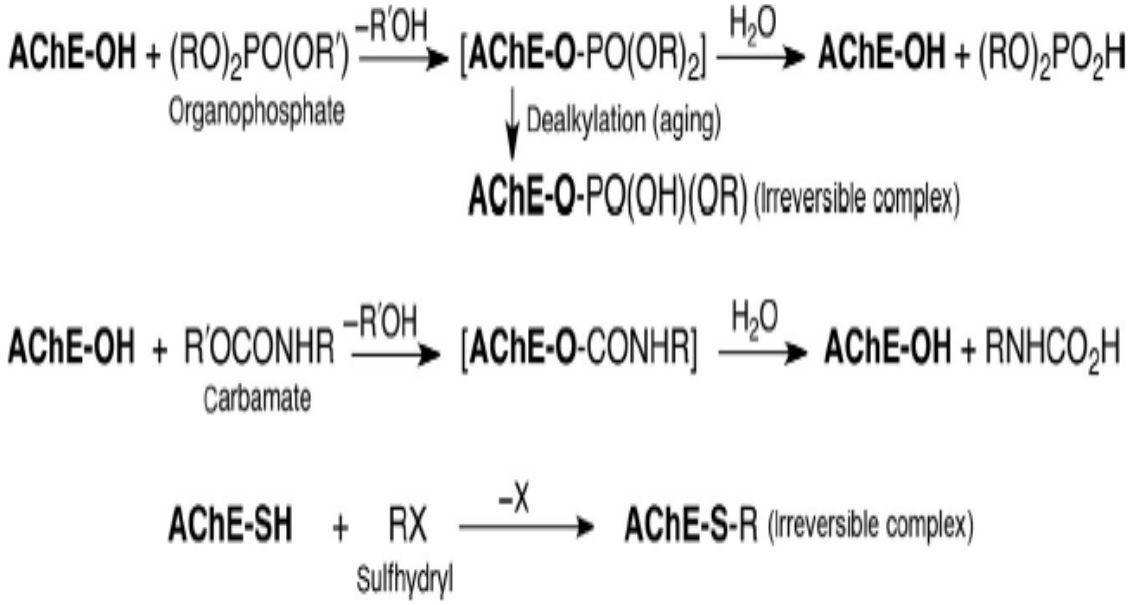

Figure 5: Mechanisms of action for inhibition of acetylcholinesterase by organophosphates, carbamates and sulfhydryl compound.

Source: Pang (2014)

2.6 Na^+ K^+ - ATPase (EC.3.6.3.9)

ATPases are enzymes that catalyse the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and phosphate (Pi) to release energy for other cellular reactions such as transport of ion across the plasma membranes, muscle contraction and removal of toxic ions from cells. Adenosine triphosphatases (ATPases) are integral parts of active transport mechanisms for cations across the cell membrane (Li *et al*., 2016). Na^+, K^+-Adenosine Triphosphatase (Na^+, K^+-ATPase) is the most well-known member of the P-type ATPase family; it pumps three Na^+ out and two K^+ into the cell hydrolyzing one ATP molecule to maintain their ionic concentration gradients across the plasma membrane.

Most insecticides are neuropoisons, but their target sites are rather limited. Organophosphate and carbamate insecticides inhibit acetylcholinesterase whereas

voltage-gated sodium channels are the major target of pyrethroids and DDT (Narahashi, 2010).

2.6.1 Sodium Channel Modulation by pyrethroids and DDT

Despite apparent differences in chemical structure, pyrethroids and DDT exert similar actions on the nervous system through modulation of the function of voltage-gated sodium channels. Pyrethroids may be divided into two groups: type I pyrethroids lack a cyano group in the alpha position, and their symptoms of poisoning are characterized by hyperexcitation, ataxia, convulsions, and paralysis; type II pyrethroids have an alpha cyano group, and cause hypersensitivity, choreoathetosis, tremors, and paralysis. At the level of nerve function, type I pyrethroids tend to produce repetitive action potentials as a result of the increase in depolarizing after-potential, whereas type II pyrethroids tend to cause membrane depolarization leading to discharges from sensory neurons. These apparent differences in nerve function alteration between the two types of pyrethroids can be ascribed to differences in modification of sodium channel kinetics. DDT has many features in common with type I pyrethroids with respect to the mechanism of action on the sodium channel (Narahashi, 2010).

2.6.2 *Clarias gariepinus*

Clarias gariepinus (Figure 6) is a species of catfish of the family Clariidae, the air breathing catfishes. Colour varies dorsally from dark to light brown and is often mottled with shades of olive and grey while the underside is a pale cream to white (Skelton, 2001). It can grow very large with a maximum reported length of 170 cm and weight of 60 kg (Robins, 1991).

Clarias gariepinus is considered to be omnivorous displaying both scavenging and predatory behaviour (Bruton, 1979). It is known to have an extremely varied diet consuming fruits and seeds, all types of aquatic invertebrates and small vertebrates, small mammals and even plankton (Skelton, 2001). Larger individuals show a specific dietary shift towards fish as they grow bigger. However, inactive foods, which it detects with its sensory barbells before securing with its array of very fine teeth prior to gulping, are generally preferred (Skelton, 2001). Alternatively, it can be an efficient predator and even hunt in 'packs' where it may herd shoals of small fish against submerged aquatic vegetation before devouring them (Merron, 1993). Solitary feeding, social hunting and coordinated pack-hunting foraging behaviours and even feeding migrations have all been observed (Merron, 1993).

Clarias gariepinus is considered to have a rapid growth rate (in length and weight), the rate of which strongly depends on ambient conditions and habitat (Britz and Pienaar, 1992). Growth has been found to be positively density dependent. Individuals have been recorded to reach 200 mm SL within a year (Skelton, 2001). In females, the growth rate decreases after 3 years resulting in the males reaching larger sizes (Skelton 2001). Individuals of this species are known to live for eight or more years.

Clarias gariepinus can endure extremely harsh conditions (Skelton 2001). It is able to tolerate very low oxygen concentrations and even survive for considerable periods out of water, via the use of a specialised suprabranchial organ (Safriel and Bruton, 1984). This organ is a large paired chamber with branches above the gill arches specifically adapted for air breathing and allows it to move over land even when not forced to do so by drought. Water temperatures between 8 and 35°C, salinities of 0 to 10‰ and a wide pH range are all tolerated (Safriel and Bruton, 1984). *C. gariepinus* exhibits high growth

rates between 25 and 33 °C, with optimum growth recorded at 30°C (Britz and Hecht, 1987). The ability of the fish to be able to tolerate these extreme conditions allows it to survive even in moist sand or in borrows with an air-water interface.

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Figure 6: ***Clarias gariepinus***

Source: Asogwa (2015)

2.7 Tissues Studied

2.7.1 The Gill

The gill is a respiratory organ found in many aquatic organisms that extracts dissolved oxygen from water and excretes carbon dioxide. Fish gills serve a variety of physiological functions including respiratory gas exchange, osmo-regulation, nitrogen excretion and control of acid-base balance (Hoar and Randall, 1984). The gills, being continuously bathed in the surrounding water are most vulnerable organs to the various aquatic pollutants. Gills usually consist of thin filaments of tissue, lamellae (Figure 7), branches, or slender, tufted processes that have a highly folded surface to increase surface area. The delicate nature of the gills is possible because the surrounding water provides support. The blood or other body fluid must be in intimate contact with the respiratory surface for ease of diffusion (Dorit *et al*., 1991).

Usually water is moved across the gills in one direction by the current, by the motion of the animal through the water, by the beating of cilia or other appendages, or by means of a pumping mechanism. In fish and some molluscs, the efficiency of the gills is greatly enhanced by a countercurrent exchange mechanism in which the water passes over the gills in the opposite direction to the flow of blood through them. This mechanism is very efficient and as much as 90% of the dissolved oxygen in the water may be recovered (Dorit *et al*., 1991).

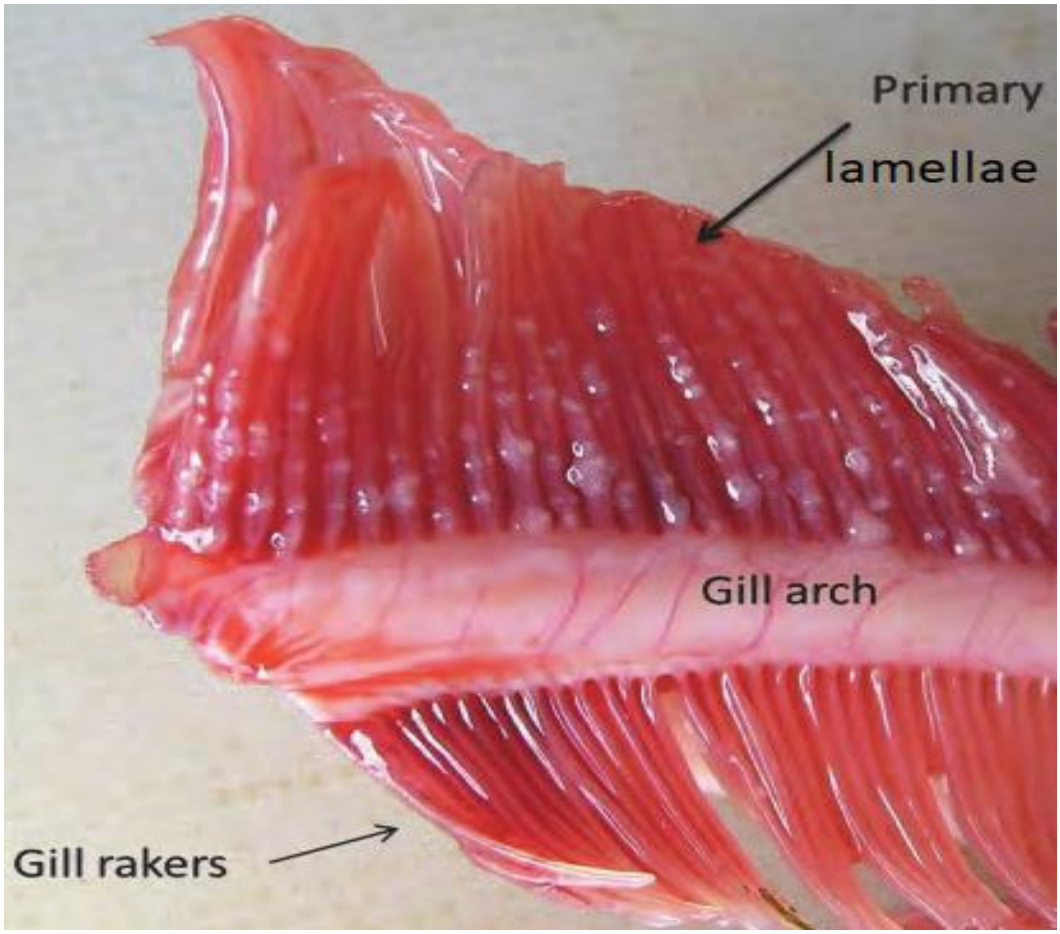

Figure 7: *Clarias gariepinus* gill

Source: Grant *et al.* (2014)

2.7.2 The Liver

The liver has many digestive and storage functions. One of these is the production of bile, a solution which emulsifies fats and may assist in changing the acidic conditions of the stomach into the neutral pH of the intestine). The liver is also responsible in some species for the storage of fats, blood sugar, and vitamins A and D. Before it was possible to synthetically create vitamins A and D, sharks were caught for their livers which have high concentrations of these vitamins.

The liver acts as a food reserve and so changes with reproductive condition particularly in viviparous species. The livers of sharks in early pregnancy are large and lightly coloured. Those of individuals that have just given birth tend to be shrunken and darker coloured. Similarly, the livers of males vary depending upon mating activity.

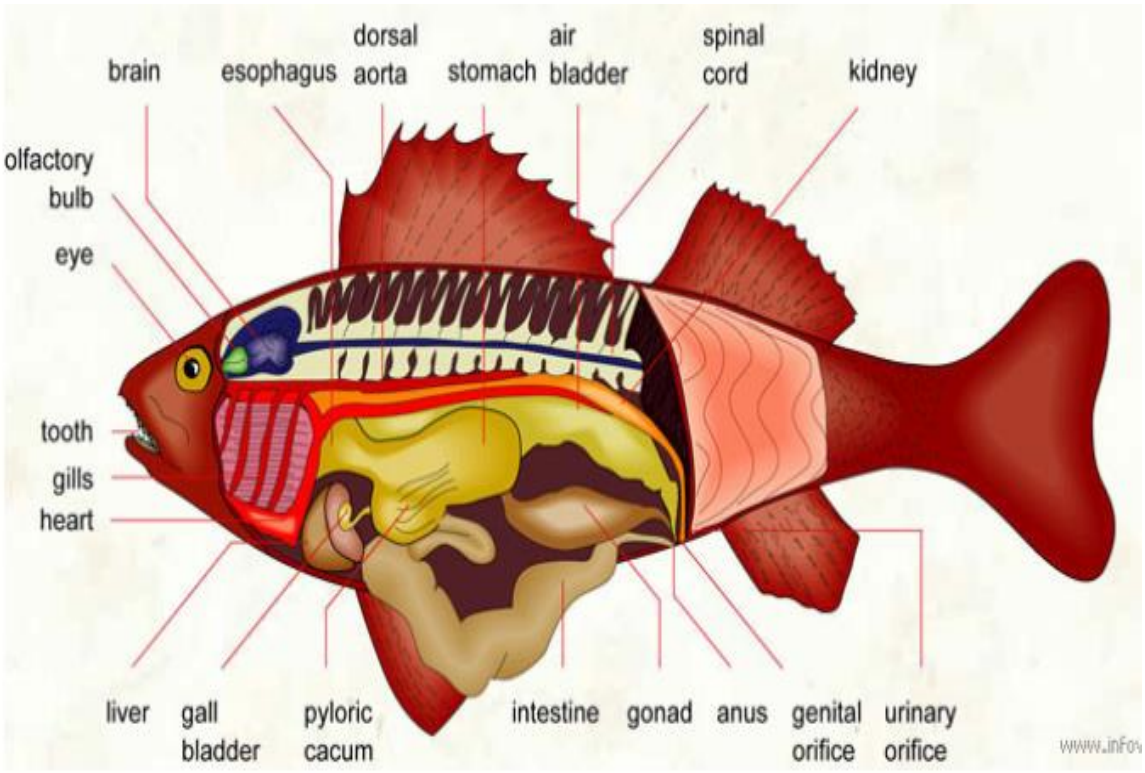

Figure 8: Anatomy of fish showing the liver, kidney and other organs

Source: Shneider and Sherman (2008)

2.7.3 Liver function indices

2.7.3.1 Total Protein

Serum protein represents a complex mixture containing a number of components which differ in properties and function. The major components of serum proteins include albumin, globulins and conjugated proteins such as glycoprotein, mucoprotein and lipoproteins. The evaluation of the protein makes up of an individual is a very important diagnostic feature because of the role of proteins in the maintenance of osmotic balance, acid-base balance, biocatalysis, hormonal action, and other important functions. The major serum proteins usually measured are albumin and globulin (Guyton and Hall, 2000).

2.7.3.2 Albumin

Albumin is the major constituent of serum protein accounting for 55-60 % of the measured serum protein. The serum albumin concentration is a function of its rates of synthesis and degradation and its distribution between the intravascular and extravascular compartment. Albumin is involved in osmotic pressure regulation, nutrient transport and waste removal. A major function of albumin is to provide colloid osmotic pressure in the plasma, which prevents plasma loss from the capillaries and it is a marker for the synthethic ability of the liver (Guyton and Hall, 2000).

2.7.3.3 Bilirubin

Bilirubin is a tetrapyrrole obtained from the degradation of haem. The liver clears bilirubin from the blood by conjugating it with glucoronic acid, to make it water-soluble for secretion into the bile, where it is then transported to the intestine for excretion.

Elevated total bilirubin causes jaundice; this is usually caused by hemolytic anaemia and internal hemorrhage. Bilirubin, being a product of the breakdown of liver erythrocytes, is a good indicator of the liver function (Guyton and Hall, 2000).

2.7.4 The Kidney

The kidneys of fish are typically narrow (Figure 8), elongated organs, occupying a significant portion of the trunk. They are similar to the mesonephros of higher vertebrates (reptiles, birds and mammals). The kidneys contain clusters of nephrons, serviced by collecting ducts which usually drain into a mesonephric duct. However, the situation is not always so simple. In cartilaginous fish there is also a shorter duct which drains the posterior (metanephric) parts of the kidney, and joins with the mesonephric duct at the bladder or cloaca. Indeed, in many cartilaginous fish, the anterior portion of the kidney may degenerate or cease to function altogether in the adult (Romer *et al*., 1976). Hagfish and lamprey kidneys are unusually simple. They consist of a row of nephrons, each emptying directly into the mesonephric duct (Romer *et al.*, 1976).

2.7.5 Kidney Function Indices

The kidney function indices are evaluated to investigate functional capacity of the kidney. The kidney removes metabolic waste and maintains the chemical composition of the body fluids at the proper concentration. Malaria affects functional indices of various organs including the kidney, upsetting its function, such as excretion of endogenous substances like urea, creatinine and electrolytes (Das and Nanda, 1999).

2.7.5.1 Uric acid

Uric acid is a heterocyclic compound of carbon, nitrogen, oxygen, and hydrogen. Uric acid is a product of the metabolic breakdown of purine nucleotides, and it is a normal

component of urine. High blood concentrations of uric acid can lead to gout and are associated with other medical conditions including diabetes and the formation of ammonium acid urate kidney stones. Excess blood uric acid can induce gout, (Heinig and Johnson, 2006) a painful condition resulting from needle-like crystals of uric acid precipitating in joints, capillaries, skin, and other tissues (Richette and Bardin, 2010).

2.7.5.2 Urea

Urea is an end product of amino acid catabolism; deamination of amino acids either through oxidative or non-oxidative process produces ammonia, which is then converted to a less toxic compound, urea through the urea cycle. Urea formation, which takes place in the cytosol and mitochondria of hepatocytes, is the primary method of nitrogen excretion. After synthesis, urea travels through the blood and is excreted in the urine. When the kidneys are unable to excrete urea normally either due to increased amino acid catabolism in starvation or glomerular dysfunction, it accumulates in the blood and may damage the nephrons, leading to reduced glomerular filtration and uraemia (Guyton and Hall, 2000).

2.7.6 Serum Electrolytes

2.7.6.1 Serum Sodium ion

Sodium ion is the primary cation in extracellular fluids in animals and humans. These fluids, such as blood plasma and extracellular fluids in other tissues bathe cells and carry out transport functions for nutrients and wastes. Sodium ions help to maintain osmotic pressure, muscle contraction, acid-base balance and to transmit nerve impulses. Hyponatraemia in addition to other clinical findings may indicate renal tubular disease,

heart failure or diabetes mellitus; high concentrations (hypernatremia) may indicate dehydration (Guyton and Hall, 2000).

2.7.6.2 Serum Potassium ion

Potassium ion is the major intracellular cation in the blood. It helps to maintain osmotic balance and is also involved in acid-base balance along with sodium. It is needed for impulse transmission and muscle contraction. Renal tubular dysfunction may deplete potassium levels while renal glomerular dysfunction may increase its concentration (Guyton and hall, 2000).

2.7.7 The Heart

Fish have what is often described as a two-chambered heart (Jurd, 2004) consisting of one atrium to receive blood and one ventricle to pump it (Ostrander, 2000) in contrast to three chambers (two atria, one ventricle) of amphibian and most reptile hearts and four chambers (two atria, two ventricles) of mammal and bird hearts (Jurd, 2004). However, the fish heart has entry and exit compartments that may be called chambers, so it is also sometimes described as three-chambered (Ostrander, 2000) or four-chambered (Farrell, 2011), depending on what is counted as a chamber. The atrium and ventricle are sometimes considered “true chambers”, while the others are considered “accessory chambers” (Pandey and Shukla, 2005).

The four compartments are arranged sequentially:

- Sinus venosus, a thin-walled sac or reservoir with some cardiac muscle that collects deoxygenated blood through the incoming hepatic and cardinal veins (Ostrander, 2000).

- Atrium, a thicker-walled, muscular chamber that sends blood to the ventricle (Ostrander, 2000).
- Ventricle, a thick-walled, muscular chamber that pumps the blood to the fourth part, the outflow tract. The shape of the ventricle varies considerably, usually tubular in fish with elongated bodies, pyramidal with a triangular base in others, or sometimes sac-like in some marine fish (Farrell, 2011).
- The outflow tract (OFT) to the ventral aorta, consisting of the tubular conus arteriosus, bulbus arteriosus, or both. The conus arteriosus, typically found in more primitive species of fish, contracts to assist blood flow to the aorta, while the bulbus anteriosus does not (Pandey and Shukla, 2005; Icardo, 2006).

Ostial valves, consisting of flap-like connective tissues, prevent blood from flowing backward through the compartments.[36] The ostial valve between the sinus venosus and atrium is called the sino-atrial valve, which closes during ventricular contraction.[36] Between the atrium and ventricle is an ostial valve called the atrio-ventricular valve, and between the bulbus arteriosus and ventricle is an ostial valve called the bulbo-ventricular valve. The conus arteriosus has a variable number of semilunar valves (Pandey and Shukla, 2005).

The ventral aorta delivers blood to the gills where it is oxygenated and flows, through the dorsal aorta, into the rest of the body. (In tetrapods, the ventral aorta has divided in two; one half forms the ascending aorta, while the other forms the pulmonary artery) (Romer and Parsons, 1977).

The circulatory systems of all vertebrates, are closed. Fish have the simplest circulatory system, consisting of only one circuit, with the blood being pumped through the

capillaries of the gills and on to the capillaries of the body tissues. This is known as single cycle circulation.

In the adult fish, the four compartments are not arranged in a straight row but, instead form an S-shape with the latter two compartments lying above the former two. This relatively simpler pattern is found in cartilaginous fish and in the ray-finned fish. In teleosts, the conus arteriosus is very small and can more accurately be described as part of the aorta rather than of the heart proper. The conus arteriosus is not present in any amniotes, presumably having been absorbed into the ventricles over the course of evolution. Similarly, while the sinus venosus is present as a vestigial structure in some reptiles and birds, it is otherwise absorbed into the right atrium and is no longer distinguishable (Romer and Parsons, 1977).

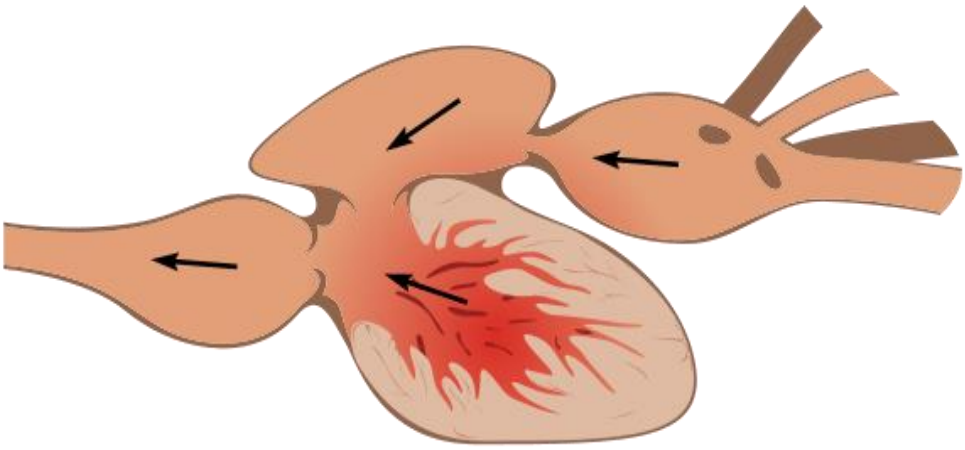

Figure 9: Blood flow through fish heart

Source: Ostrander (2000)

2.7.8 Blood

Blood is the fluid that flows in the vascular system (heart, arteries, veins and the capillaries) of humans and other vertebrates. Blood contains both cellular and liquid constituents. The liquid component obtained when blood is allowed to clot is called serum while the liquid obtained when blood is prevented from clotting by the use of anticoagulant is called plasma (Basten, 2014).

In blood, the serum is the component that is neither a blood cell nor a clotting factor. Serum includes all proteins not used in blood clotting (coagulation) and all the electrolytes, antibodies, antigens, hormones, and any exogenous substances. Serum is used in numerous diagnostic tests, as well as blood typing. Measurements of serum concentrations has proved useful in many fields including clinical trials of therapeutic and toxic response (Kaplan, 2005).

2.7.9 Cardiovascular Indices

2.7.9.1 Triglyceride

Triglyceride more popularly known as triacylglycerol, TAG or triacylglyceride is a glyceride in which the glycerol is esterified with three fatty acids which may be saturated

or not. It is the main constituent of vegetable oil and animal fats. In the human body, high levels of triglycerides in the bloodstream have been linked to atherosclerosis, besides the risk of heart disease and stroke. Reference ranges (increasing with age) for blood tests, is shown below. The American Heart Association has set guidelines for triglyceride levels (American Heart Association, 2011).

2.7.9.2 Cholesterol

Cholesterol is a waxy steroid metabolite found in the cell membranes and transported in the plasma of all animals (Leah, 2009). It is an essential structural component of mammalian cell membranes, where it is required to establish proper membrane permeability and fluidity. In addition, cholesterol is an important component for the manufacture of bile acids, steroid hormones, and several fat-soluble vitamins. Cholesterol is the principal sterol synthesized by animals, but small quantities are synthesized in other eukaryotes, such as plants and fungi. It is almost completely absent among prokaryotes, which include bacteria (Pearson *et al*., 2003).

Cholesterol is required to build and maintain membranes; it regulates membrane fluidity over the range of physiological temperatures. The hydroxyl group on cholesterol interacts with the polar head groups of the membrane phospholipids and sphingolipids, while the bulky steroid and the hydrocarbon chain are embedded in the membrane, alongside the nonpolar fatty acid chain of the other lipids. In this structural role, cholesterol reduces the permeability of the plasma membrane to protons (positive hydrogen ions) and sodium ions (Haines, 2001).

Within the cell membrane, cholesterol also functions in intracellular transport, cell signalling and nerve conduction. Recently, cholesterol has also been implicated in cell signalling processes, assisting in the formation of lipid rafts in the plasma membrane. In

many neurons, a myelin sheath, rich in cholesterol, since it is derived from compacted layers of Schwann cell membrane, provides insulation for more efficient conduction of impulses (Ross and Pawlina, 2006). Within cells, cholesterol is the precursor molecule in several biochemical pathways. In the liver, cholesterol is converted to bile, which is then stored in the gallbladder. It is an important precursor molecule for the synthesis of Vitamin D and the steroid hormones, including the adrenal gland hormones cortisol and aldosterone as well as the sex hormones progesterone, oestrogens, and testosterone, and their derivatives. Some research indicates that cholesterol may act as an antioxidant (Smith, 1991). About 20–25% of total daily cholesterol production occurs in the liver; other sites of high synthesis rates include the intestines, adrenal glands, and reproductive organs.

2.7.9.3 High Density Lipoprotein-Cholesterol (HDL-c)

High-density lipoprotein (HDL) is one of the five major groups of lipoproteins (chylomicrons, VLDL, IDL, LDL, HDL) that enable lipids like cholesterol and triglycerides to be transported within the water-based bloodstream. In healthy individuals, about thirty percent of blood cholesterol is carried by HDL. It is hypothesized that HDL can remove cholesterol from atheroma within arteries and transport it back to the liver for excretion or re-utilization, which the main reason why HDL-bound cholesterol is sometimes is called good cholesterol, or HDL-c. A high level of HDL-c seems to protect against cardiovascular diseases and low HDL cholesterol levels (less than 40 mg/dL or about 1mmol/L) increase the risk for heart disease. Cholesterol contained in HDL particles is considered beneficial for the cardiovascular health, in contrast to "bad" LDL cholesterol.

2.7.10 The Brain

Fish brains are divided into several regions. At the front are the olfactory lobes, a pair of structures that receive and process signals from the nostrils via the two olfactory nerves (Helfman *et al.*, 1997). Similar to the way humans smell chemicals in the air, fish smell chemicals in the water by tasting them. The olfactory lobes are very large in fish that hunt primarily by smell, such as hagfish, sharks, and catfish. Behind the olfactory lobes is the two-lobed telencephalon, the structural equivalent to the cerebrum in higher vertebrates. In fish the telencephalon is concerned mostly with olfaction. Together these structures form the forebrain.

The forebrain is connected to the midbrain via the diencephalon. The diencephalon performs functions associated with hormones and homeostasis. The pineal body lies just above the diencephalon. This structure detects light, maintains circadian rhythms, and controls color changes. The midbrain or mesencephalon contains the two optic lobes. These are very large in species that hunt by sight, such as rainbow trout and cichlids (Helfman *et al.*, 1997).

The hindbrain or metencephalon is particularly involved in swimming and balance. The cerebellum is a single-lobed structure that is typically the biggest part of the brain. Hagfish and lampreys have relatively small cerebellae, while the mormyrid cerebellum is massive and apparently involved in their electrical sense (Helfman *et al.*, 1997). The brain stem or myelencephalon is the brain's posterior (Helfman *et al.*, 1997). As well as controlling some muscles and body organs, in bony fish at least, the brain stem governs respiration and osmoregulation (Helfman *et al.*, 1997)

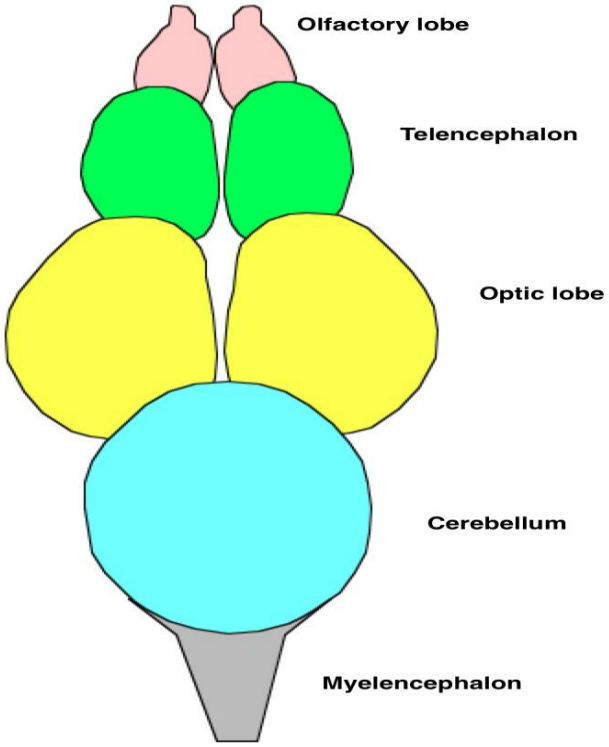

Figure 10: Dorsal view of fish brain

Source: Helfman *et al*. (1997)

The brain also produces a portion of the body's hormones that can influence organs and glands elsewhere in a body. Conversely, brains also react to hormones produced elsewhere in the body. In mammals, the hormones that regulate hormone production throughout the body are produced in the brain by the structure called the pituitary gland (Armstrong, 1983) Hormones, incoming sensory information, and cognitive processing performed by the brain determine the brain's state. Cognitive priorities are constantly shifted by a variety of factors such as hunger, fatigue, belief, unfamiliar information, or threat. The simplest dichotomy related to the processing of threats is the fight-or-flight response mediated by the amygdalia and other limbic structure (Martin, 1996).

2.8 Enzymes studied

Enzymes are remarkable molecular entities that facilitate the biotransformation in a living organism. The major clinical use of enzymes is the prognosis and diagnosis of diseases i.e. they serve as biomarkers. Generally, enzymes in circulating plasma are either plasma-specific or non-plasma-specific. Plasma-specific (plasma functional) enzymes are normally present in plasma, perform their primary function in blood, and have higher level of activity in plasma than in tissue cells. Non-plasma-specific (Non-plasma

functional) enzymes are intracellular enzymes normally present in plasma at minimal levels or at concentrations well below those in tissue cells. Their presence in plasma is normally due to turnover of tissue cells, but they are released into the body fluids in excessive concentrations as a result of cellular damage or impairment of membrane function (Murray *et al*., 2004). The enzymes studied include: Alkaline phosphatase (ALP), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT).

2.8.1 Alkaline phosphatase (E.C. 3.1.3.1)

Phosphatases are enzymes that remove a phosphate group from its substrate by hydrolysing phosphoric acid monoester into a phosphate ion and a molecule with a free hydroxyl group such as alcohol and phenol. (Jansson *et al*., 1988) classified these enzymes into two based on their sensitive pH and they are alkaline phosphatase (ALP, EC 3.1.3.1, optimum pH $\geq$ 8.0) and acid phosphatase (ACP, EC 3.1.3.2, optimum pH $\leq$ 6.0).

Alkaline phosphatase, a non-plasma specific enzyme is a metalloprotein that catalyze dephosphorylation reaction on both naturally occurring and synthetic substrates (Guyton and Hall, 2000). As the name suggests, alkaline phosphatases are most effective in an alkaline environment. It is sometimes used synonymously as basic phosphatase (Tamás *et al*., 2002). The most commonly used substrates for the study of it activity are paranitrophenyl phosphate (PNPP), phenyl phosphate and glycerol-3-phosphate. The enzyme activity increases with low level of glycine and alanine concentration as well as Mg^{2+} and Zn^{2+} as cofactor through synergistic effect (Olorunniji *et al*., 2007). However, the activity of the enzyme is inhibited by Be, Cu, Hg ions, sulphide, cyanide, adrenaline, oxalate, borate, adrenochrome and oxidizing agents (Tietz, 1995).

Alkaline phosphatase is an intracellular membrane bound enzyme (Mazorra *et al*., 2002) with various isoforms that is present in all animals as well as in bacterial cells with less molecular weight (Johnson *et al*., 1972). The most organs commonly associated with alkaline phosphatase activity are Liver, Kidney, muscle, intestines, bones and placenta in pregnant woman where it performs the following metabolic functions

i. Permeability,
ii. Growth and cell differentiation,
iii. Protein synthesis,
iv. Absorption and transport of nutrients, and
v. Gonadal maturation (Ram and Sathyanesan, 1985).

Clinical Significance: The primary clinical significance of alkaline phosphatase is in cases of suspected bone disorders and obstructive liver diseases. Raised serum levels are seen in different bone disorders and also seen in liver disease associated with extra- or intrahepatic obstruction, obstructive jaundice, diabetes (Kechrid and Kenouz, 2003) and infectious mononucleosis, biliary cirrhosis and cholestasis (Khanna and Kumar, 2002). Low serum levels are associated with protein-energy malnutrition, cardiac surgery, low dietary magnesium, hypothyroidism, pernicious anemia (Lum *et al*., 1989), hypervitaminosis D, achondroplasia in children, scurvy and estrogen replacement therapy (Lum, 1995). The increased activities of ALP observed in some tissues may be caused by increased synthesis of plasma membrane proteins. Measurement of specific isoenzymes improves the diagnostic value of the test (Murray *et al*., 2004).

2.8.2 Aspartate aminotransferase (E.C. 2.6.1.1)

Aspartate Aminotransferase also known as serum glutamate oxaloacetate transaminase (EC 2.6.1.1) is a transferase found in both the cytoplasm and mitochondrion catalyzing the reversible conversion of aspartate to oxaloacetate in which α-keto glutarate is converted to glutamate. It is also a liver function test enzyme but less sensitive than alanine aminotransferase. Elevation of it activity (normal, 0-40 IU/L) is mainly significant for muscle and heart diseases because of their cellular distribution (Satyanarayana and Chakrapani, 2006).

Clinical Significance: The measurement of activity of AST is very important in clinical diagnosis especially in the initial stage of disease as well as during recovery period. The levels of AST increase significantly over the normal in myocardial infarction, hepatic necrosis and other diseases such as active cirrhosis (Wróblewski and Ladue, 1955).

2.8.3 Alanine aminotransferase (E.C. 2.6.1.2)

Alanine aminotransferase (ALT) generally referred to as Glutamate pyruvate transaminase has its systematic name as L-alanine-2-oxoglutarate aminotransferase and catalyzes the transamination reaction between L-alanine and α-ketoglutarate, to form pyruvate and oxaloacetate. It is also a transaminase enzyme found in serum and in various bodily tissues including skeletal muscle, kidney, pancreas, spleen, lung, erythrocytes and heart but is most commonly associated with the liver (Tietz, 1987). It catalyzes the transfer of an amino group from Alanine to α-ketoglutarate, to produce pyruvate and glutamate.

Clinical significance: ALT is measured as part of liver function tests. Increase in serum ALT is considered a more sensitive indicator of hepatitis than serum AST because ALT

is found in high concentration in the liver than in heart muscle (Reitman and Frankel, 1957). An elevated level of ALT often suggests the existence of other medical problems such as alcoholic or viral hepatitis, congestive heart failure, liver damage or biliary duct problems.

2.9 Oxidative stress

Oxidative stress is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants. Oxidative stress is a pathological state that arises when free radicals (collectively known of as reactive oxygen species or ROS) chemically interact with and damage biological molecules. Oxidative stress research has largely focused on the role and effects of antioxidants in protecting these molecules from damage.

Oxygen by-products are relatively unreactive but some of these can undergo metabolism within the biological system to give rise to these highly reactive oxidants (Figure 11). Not all reactive oxygen species are harmful to the body. Some of them are useful in killing invading pathogens or microbes. However, free radicals can chemically interact with cell components such as DNA, protein or lipid and steal their electrons in order to become stabilized. This, in turn, destabilizes the cell component molecules which then seek and steal an electron from another molecule, therefore triggering a large chain of free radical reactions. Every cell that utilizes enzymes and oxygen to perform functions is exposed to oxygen free radical reactions that have the potential to cause serious damage to the cell. Antioxidants are molecules present in cells that prevent these reactions by donating an electron to the free radicals without becoming destabilized themselves. An imbalance between oxidants and antioxidants is the underlying basis of oxidative stress.

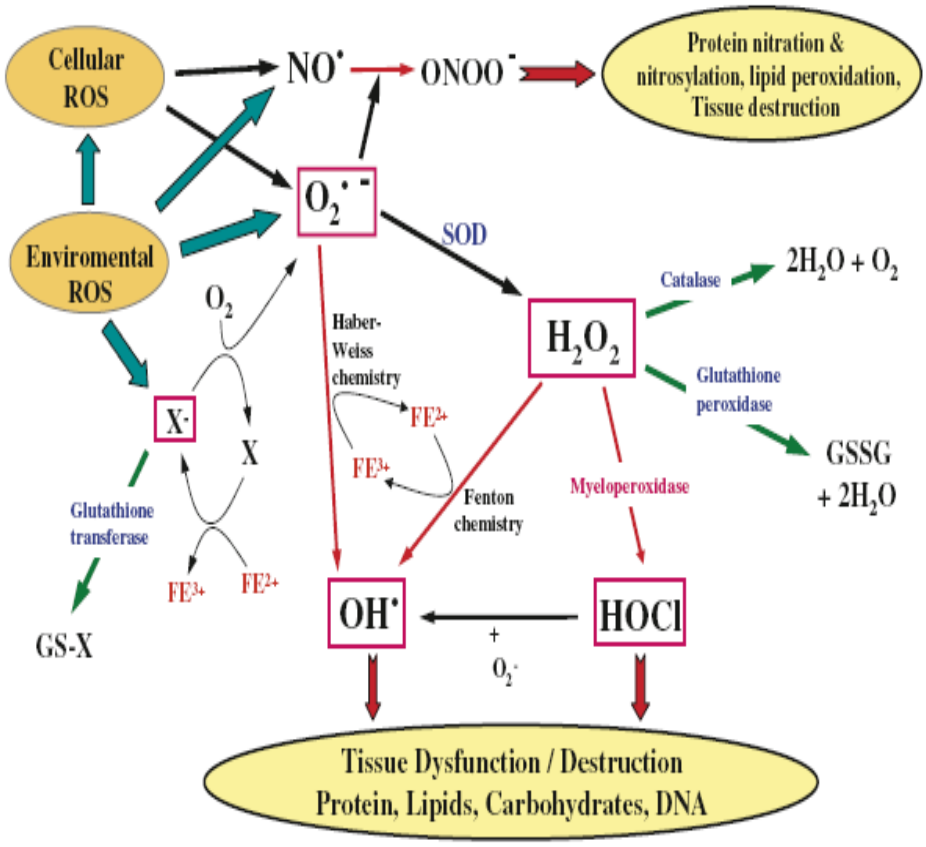

Figure 11: Molecular Consequence of oxidative stress
ROS, reactive oxygen species; RNS, reactive nitrogen species; $X^{\cdot}$, xenobiotic radical; $O_2^{\cdot-}$, superoxide anion; H_2O_2, hydrogen peroxide; $OH^{\cdot}$, hydroxide radical; HOCl, hyperchlorous acid; NO, nitric oxide; $ONOO^{-}$, peroxynitrite; GS-X, glutathione-xenobiotic conjugate; GSSG, oxidized glutathione dimers.

Source: Kirkham and Rahman (2006)

2.9.1 Antioxidant Enzymes

2.9.1.1 Superoxide Dismutase (EC 1.15.1.1)

Superoxide dismutase belongs to an important enzyme family in living cells for maintaining normal physiological conditions and for coping with stress (Otitoju *et al*., 2008). Superoxide dismutase is a prime antioxidant enzyme found in two forms. One, complexed with zinc and copper, is localized in the cytosol, while the other, bound with manganese, is found in the mitochondrial matrix. Both forms of this metalloenzyme catalyze the inactivation of destructive superoxide anion by converting it to hydrogen peroxide which is then transformed to water and oxygen by the enzyme catalase (Davis *et al*., 1997).

2.9.1.2 Catalase (EC 1.11.1.6)

Catalases are the class of enzymes which catalyze the decomposition of hydrogen peroxide to molecular oxygen and water. Catalases of many organisms contain four heme groups (Aebi, 1974). Although the predominant sub-cellular localization in mammalian cells is in peroxisomes, catalase is also found in the blood, bone marrow, mucous membranes, kidney and liver. Catalase also has functions in detoxifying other substrates, e.g., phenols and alcohols, via coupled reduction of hydrogen peroxide. One anti-oxidative role of catalase is to lower the risk of hydroxyl radical formation from H_2O_2 via the Fenton reaction catalyzed by Cu or Fe ions (Fridovich, 1999; Halliwell, 1999).

2.9.1.3 Glutathione Peroxidase (EC. 1. 11. 1. 9)

Glutathione peroxidases are an important part of the antioxidant defence system. They are present in almost every cell of animals, but the tissue distribution of the isoforms shows high variation (Mézes *et al*., 2003). There are at least four different GPx in

mammals (GPx1–4), all of them containing selenocysteine (Maiorino *et al.*, 1995). GPx1 and GPx4 (or phospholipid hydroperoxide GPx) are both cytosolic enzymes abundant in most tissues. All glutathione peroxidases catalyze the reduction of H_2O_2 using glutathione as substrate. They can also reduce other peroxides (e.g., lipid peroxides in cell membranes) to alcohols (Matés *et al.*, 1999). The catalytic mechanism proposed for reduction of hydroperoxides by GPx involves oxidation of the active site selenolate (Se^-) to selenenic acid (SeOH). Upon addition of one molecule of GSH, the selenenic acid is transformed to a selenenylsulfide adduct with glutathione (Se-SG), which can be regenerated to the active selenolate and glutathione disulfide (GSSG) by addition of a second molecule of GSH. Thus, in the reaction, two molecules of GSH are oxidized to GSSG that subsequently can be reduced by GR, the major mammalian GSSG-reducing enzyme.

2.10 Haematological Parameters

Most chemical components of blood plasma are supplied from the organs and as such may be regarded as indicators of biochemical, physiological and pathological status of the tissues and organs from which they are supplied into the blood. The levels of haemoglobin (Hb), packed cell volume (PCV), Red blood cells (RBCs), white blood cells (WBCs), Mean Corpuscular Haemoglobin, (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV), Neutrophils (NEU), lymphocytes (LYM) and platelet counts (PLT) are jointly referred to as haematological parameters and alterations in these parameters are indicators of certain pathological conditions.

2.10.1 Red blood Cell Count

The red blood cell is a non-nucleated biconcave disc produced in the bone marrow. The main function of red blood cells is to carry oxygen to the tissues and to transfer carbon dioxide to the lungs. This process is made possible by haemoglobin which combines easily with oxygen and carbon dioxide. A red blood cell count is the total number of red blood cells in the blood; alterations in the qualitative and quantitative composition as well as the biochemistry of the blood cells are warning signals and indicative of an impaired function of both bone marrow and lymphoid tissues (Guyton and Hall, 2000).

2.10.2 Haemoglobin (Hb)

Haemoglobin is the red respiratory pigment of red blood cells. It is composed of four polypeptide chains of globin, a group of amino acids that form a protein and heme which contains iron atoms and the red pigment, porphyrin. It is the main means of transport of oxygen and carbon dioxide in the blood. The tetrameric structure of haemoglobin allows for conformational changes that aid in oxygen transport. As with hematocrit, it is an important determinant of anaemia (decreased), dehydration (increased), polycythemia (increased), poor diet/nutrition, or possibly a malabsorption problem (Guyton and Hall, 2000).

2.10.3 Packed Cell Volume (PCV)

The packed cell volume is the volume of red blood cells in a litre of blood. It is reduced in anaemia, when there is increased RBC breakdown in the spleen or in over-hydration but increased in polycythemia and during dehydration (Guyton and Hall, 2000).

2.10.4 Mean Corpuscular Volume (MCV)

The Mean Corpuscular Volume reflects the size of red blood cells by expressing the volume occupied by a single red blood cell. Increased readings may indicate macrocytic anaemia or vitamin B6 or Folic acid deficiency and decreased values may indicate microcytic anaemia, possibly caused by iron deficiency (Guyton and Hall, 2000).

2.10.5 Mean Corpuscular Haemoglobin (MCH)

Mean Corpuscular Haemoglobin (MCH) gives the average weight of haemoglobin in the red blood cell in picogram. Decreased MCH is associated with microcytic anaemia and increased MCH is associated with macrocytic anaemia (Guyton and Hall, 2000)

2.10.6 Mean Corpuscular Haemoglobin Concentration (MCHC)

MCHC is the average concentration of haemoglobin in red blood cells. Low MCHC implies that a unit of packed RBCs contains less haemoglobin than normal and a high MCHC means that there is more haemoglobin in a unit of RBCs (Guyton and Hall, 2000)

2.10.7 White blood Cell

The main function of the white blood cells is to fight infection, defend the body by phagocytosis against invasion by foreign organisms and produce antibodies in the immune response. There are different types of white blood cells which include granulocytes (neutrophils, eosinophils, basophils) and agranulocytes (lymphocytes and monocytes). Low levels of white blood cells lead to an impaired immune response and increased susceptibility to infections (Guyton and Hall, 2000)

2.10.8 Platelet

Platelets or thrombocytes are the smallest formed elements of the blood. They are important in blood coagulation to prevent too much bleeding. Increased concentration implies dehydration or stimulation of the bone marrow where the cells are produced while decreased level may indicate compromised immune system, drug reactions, folic acid or vitamin B_{12} deficiency (Guyton and Hall, 2000)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

Methanol was obtained from Eagle Scientific Limited Beeston, Nothingham, UK. Sodium chloride (NaCl) was obtained from British Drug House Chemical Limited, Poole, England. Sodium citrate was obtained from Merck, Darmstadt, Germany. Disodium hydrogen phosphate and potassium dihydrogen phosphate were obtained from Kermel China. Enzyme assay kits for alkaline phosphatase (ALP), aspartate and alanine aminotransferases (AST and ALT respectively) were obtained from Randox laboratories Ltd, UK. Other reagents were of analytical grade and were prepared according to specifications.

3.1.2 Plant Material

Garcinia kola seed were obtained from the Local market in Oja Tutun, Ilorin, Kwara State, Nigeria. The identification and authentication were done at the Herbarium unit of the department of Plant Science, University of Ilorin and the Voucher No….. was given

3.1.3 Mosquito Larvae

Larvae of Anopheles and Aedes Mosquito were collected from mosquito breeding sites (open curing tank in the laboratory) in the department of Biochemistry, University of Ilorin, Kwara, Nigeria.

3.1.4 Experimental Animals

48 *Clarias gariepinus* with an average weight of 40 ± 2 g were obtained from the Department of Zoology, University of Ilorin. The fishes were allowed to acclimatize in the water for about two weeks before commencement of the experiment. They were fed with commercial fish feed.

3.1.5 Ethical Clearance

Ethical clearance for the study was obtained from the University of Ilorin Ethical Review Committee (UERC), with the UERC Approval number:

3.2 Methods

3.2.1 Preparation of Plant Extract

Aqueous and methanolic extracts were prepared by macerating 500 g of the dried powder sample in 2000 mL of distilled water and 2000 mL of methanol respectively for 48 hrs. The extracts were filtered, concentrated and refrigerated till further use.

3.2.2 Preparation of Larvae

The Mosquito Larvae were reared in a black plastic buckets containing rain water and fed with baker's yeast and fish meal till adult emergence. They were protected with net to prevent escape of the adult. The adult mosquitoes were fed on 10% glucose solution dripped on treads from the top of the net preventing the adult mosquito from escaping. A restrained albino mice were provided for the adult female mosquito to blood-feed, for egg production.

3.2.3 Larvicidal Activity Study

Standard WHO protocol with slight modification were adopted for the study (WHO, 1996). There were 18 groups of 30 larvae each, fed with baker's yeast. The fractions were concentrated and 100 mg/L administered for three days and mortality monitored as follows:

- Group 1: one litre of rain water, 30 larvae and 100 mg/L of 1st fraction of aqueous extract.
- Group 2: one litre of rain water, 30 larvae and 100 mg/L of 2nd fraction of aqueous extract.

- Group 3: one litre of rain water, 30 larvae and 100 mg/L of 3rd fraction of aqueous extract.
- Group 4: one litre of rain water, 30 larvae and 100 mg/L of 4th fraction of aqueous extract.
- Group 5: one litre of rain water, 30 larvae and 100 mg/L of 5th fraction of aqueous extract.
- Group 6: one litre of rain water, 30 larvae and 100 mg/L of 6th fraction of aqueous extract.
- Group 7: one litre of rain water, 30 larvae and 100 mg/L of 7th fraction of aqueous extract.
- Group 8: one litre of rain water, 30 larvae and 100 mg/L of residue of aqueous extract.
- Group 9: one litre of rain water, 30 larvae and 100 mg/L of 1st fraction of methanolic extract.
- Group 10: one litre of rain water, 30 larvae and 100 mg/L of 2nd fraction of methanolic extract.
- Group 11: one litre of rain water, 30 larvae and 100 mg/L of 3rd fraction of methanolic extract.
- Group 12: one litre of rain water, 30 larvae and 100 mg/L of 4th fraction of methanolic extract.

- Group 13: one litre of rain water, 30 larvae and 100 mg/L of 5th fraction of methanolic extract.
- Group 14: one litre of rain water, 30 larvae and 100 mg/L of 6th fraction of methanolic extract.
- Group 15: one litre of rain water, 30 larvae and 100 mg/L of 7th fraction of methanolic extract.
- Group 16: one litre of rain water, 30 larvae 100 mg/L of residue of methanolic extract.
- Group 17: one litre of rain water, 30 larvae and 300 mg/L of Fenthion.
- Group 18: one litre of rain water and 30 larvae (control).

The percentage mortality was corrected using Abbott's formula (Kumar *et al*., 2013).

$$CM = \frac{OMT-OMC}{OMC} \times 100$$

CM = Corrected Mortality

OMT = Observed mortality in treatment

OMC = Observed mortality in control

- The percentage mortality was calculated using the formula as state by (Kumar *et al*., 2013).

$$PM = \frac{NDL}{NLI} \times 100$$

PM = Percentage Mortality

NDL = Number of dead larvae

NLI = Number of larvae introduced

3.2.4 Phytochemical Profiling

GC-MS analysis were carried out with Agilent-6890A instrument equipped with an on-colunm authomatic injector, flame ionization detector and HP 88 capillary column (100mx0.25 um film thickness). The OVEN TEMP. were set to 180^0c, Detector temprature at 250^0c, injection temprature at 220^0c and the GC allowed to warm up. All the initial and final values were set and 1 ul of sample injected into the column A when the 'Not Ready' light turns off.

3.2.5 HPLC Analysis

High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software at the Department of Industrial Pharmacy, Federal University of Santa Maria, Build 26, room 1115, Santa Maria, CEP 97105-900, Brazil.

3.2.6 Phytochemical Screening

3.2.6.1 Qualitative Screening of Secondary Plant Metabolites.

A known volume 1.0mL of the sap of *M. paradisiaca* was screened for the presence of some secondary metabolites as described for alkaloids (Harborne, 1998), steroids, anthraquinones, cardenolides and dienolides (Trease and Evans, 1989), saponins (Wall *et al.*, 1954), phenolics and flavonoids (Awe and Sodipo, 2001), cardiac glycosides (Sofowora, 1993), tannins and triterpenes (Odebiyi and Sofowora, 1978) as follows.

Saponins: (frothing test) A known volume (2.0 mL) of the sap was boiled in 20mL of distilled water in a water bath and filtered. The filtrate (10.0 mL) was mixed with 5.0mL of distilled water and shaken vigorously for a stable persistent froth which confirms the presence of saponins.

Alkaloids: Exactly 1.0mL of the sapwas stirredwith 5.0mL of 1% v/v aqueous HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1.0mL of the filtrate was treated with two drops of Mayer's reagent (potassium mercuric iodide solution), Wagner's reagent (solution of iodine in potassium iodide), and Dragendorff's reagent (solution of potassium bismuth iodide). The formation of a creamcolour with Mayer's reagent, reddish-brown precipitate withWagner's and Dragendorff's reagents give a positive test for alkaloids.

Phenolics: Two drops of 5% w/v of $FeCl_3$ was added to 1.0mL of the plant sap. The appearance of a greenish precipitate indicated the presence of phenolics.

Cardiac Glycosides: 1.0mL of the sap was added to 2.0mL of chloroform. Thereafter, 2.0mL of H2SO4 was carefully added. A reddish-brown colour at the interface indicated the presence of aglycone portion of cardiac glycosides.

Tannins: 1.0mL of freshly prepared 10% w/v ethanolic KOH was added to 1.0mL of the sap. A white precipitate indicated the presence of tannins.

Steroids: Five drops of concentrated H_2SO_4 was added to 1.0mL of the sap. The appearance of red colour indicated the presence of steroids.

Triterpenes: A known volume (1.0 mL) of the sap was added to 5 drops of acetic acid anhydride followed by a drop of concentrated H_2SO_4. The mixture was steamed for 1 hour and neutralized with NaOH followed by the addition of chloroform. Appearance of bluish-green colour indicated the presence of triterpenes.

Anthraquinones: Exactly 3.0 mL of the sap was shaken with 10.0 mL of benzene and filtered, afterwhich 5.0 mL of 10% v/v NH4OH was added to the filtrate. The appearance of a pink colour in the ammonical (lower) phase indicated the presence of anthraquinones.

Flavonoids: Exactly 3.0mL of the filtrate was mixed with 4.0 mL of 1% potassium hydroxide in a test tube. A dark yellow colour indicated the presence of flavonoids.

Cardenolides and Dienolides: A portion (5.0 mL) of the sap was added to 2.0 mL of glacial acetic acid containing one drop of 5% w/v $FeCl_3$ solution. This was then followed by the addition of 1.0 mL of concentrated H_2SO_4. A brown ring at the interface indicated the presence of a deoxy sugar characteristic of cardenolides.

3.2.6.2 Quantitative Screening of Secondary Plant Metabolites.

3.2.6.2.1 Saponins

The Spectrophotometric method of (Brunner, 1984) was used to determine percentage saponins. For each extract, 1g was weighed into a 250 ml beaker and 100 ml of isobutyl alcohol was added. The mixture was shaken for 5 hours to ensure uniform mixing. Thereafter, the mixture was filtered through a Whatman No1 filter paper into a 100 ml beaker and 20 ml of 40% (w/v) saturated solution of maganesium carbonate was added. The mixture obtained with saturated $MgCO_3$ was again filtered through a Whatman No1 filter paper to obtain a clear colorless solution. The colorless solution (1 ml) was pipetted into 50 ml volumetric flask and 2 ml of 5% (w/v) $FeCl_3$ solution was added and made up to the mark with distilled water. It was allowed to stand for 30 min for a blood red color to develop. Standard saponin solutions (0-10 mg/ml) were prepared from saponin stock solution. The standard solutions were treated similarly with 2 ml of 5% $FeCl_3$ solution as done for each extract above. The absorbance of the extracts as well as

standard saponin solutions was read after color development in a Jenway V6300 Spectrophotometer at a wavelength of 380 nm. Percentage saponin was calculated thus:

$$\%\text{Saponin} = \frac{\text{Absorbance of each extract X gradient factor X dilution factor}}{\text{Weight of each extract X10000}}$$

3.2.6.2.2 Flavonoids

Total flavonoids were determined according to the method of (ACOA, 1979). For each extract, 0.50 g was weighed into a 100 ml beaker and 80 ml of 95% (v/v) Ethanol added and stirred with a glass rod to prevent lumping. The mixture was filtered through a Whatman No.1 filter paper into a 100-ml volumetric flask and made up to the mark with ethanol. Each extract (1ml) was pipetted into a 50-ml volumetric flask and four drops of conc. HCl was added using a dropping pipette after which 0.5 g of magnesium turnings was added to develop a magenta red coloration. Standard flavonoid solution (range 0-5 mg/ml) was prepared from 100 mg/ml stock solution and treated in a similar way with HCl and magnesium turnings like the extracts. The absorbance of magenta red coloration of sample and standard solutions were read on a digital Jenway V6300 Spectrophotometer at a wavelength of 520 nm. The percentage flavonoid was calculated using the formula:

$$\frac{\text{Absorbance of each extract X average gradient factor X dilution factor}}{\text{Weight of each extract X 10,000}}$$

3.2.6.2.3 Alkaloids

Percentage alkaloids were determined according to (ACOA, 1979) which is a distillation and titrimetric procedure. For each extract, 2 g was weighed into a 100 ml beaker and 20 ml of 80% (v/v) absolute alcohol added to give a smooth paste. The mixture was transferred to a 250-ml flask and more alcohol was added to make up to 100 ml following

which 1g magnesium oxide was added. The mixture was digested in a boiling water bath for 1.5 hrs under a reflux air condenser with occasional shaking. It was then filtered while hot through a small Buchner funnel. The residue was returned to the flask and re-digested for 30 min with 50 ml alcohol after which the alcohol was evaporated, adding hot water to replace the lost alcohol. When all the alcohol had been removed, 3 drops of 10% (v/v) HCl was added. The whole solution was later transferred into a 250 ml volumetric flask containing 5 ml of zinc acetate solution and 5 ml of potassium ferrocyanide solution was added and thoroughly mixed to give a homogenous solution. The flask was allowed to stand for a few minutes, filtered through a dry filter paper and 10 ml of the filterate was transferred into a separatory funnel and the alkaloids present were extracted vigorously by shaking with five successive portions of chloroform. The residue obtained was dissolved in 10 ml hot distilled water and transferred into a Kjeldahl tube with the addition of 0.20 g sucrose and 10 ml Conc.H_2SO_4 and 0.02 g selenium for digestion to a colorless solution to determine % nitrogen (%N) by Kjeldahl distillation method. Percentage nitrogen obtained was converted to % total alkaloid by multiplying by a factor of 3.26; i.e.

% Total alkaloid = %N X 3.26

3.2.6.2.4 Tannins

The method described by (ACOA, 1979) was used to determine the percentage tannin content of each extract. Each extract of *Clerodendrum violaceum* (0.20 g) was measured into a 50 ml beaker and 20 ml of 50% (v/v) methanol was added and covered with parafilm and placed in a water bath set at 80^oC for 1 hour. It was shaken thoroughly to ensure a uniform mixing. Each extract was filtered using a double layered Whatman No 41 filter paper into a 100 ml volumetric flask and 20 ml water was added. Following this,

2.5 ml Folin-Denis reagent and 10 ml of 17% Na_2CO_3 were added and the resulting solution was mixed properly. The mixture was made up to mark with water; thouroughly mixed and allowed to stand for 20 mins. A bluish-green color developed. Tannic acid standard (0-10 mg/ml) was treated similarly as 1ml extract above. The absorbance of the tannic acid standard solutions as well as that of each extract was read after color development on a digital Jenway V6300 spectrophotometer at a wavelength of 760 nm. Percentage tannin was calculated using the formula:

$$\% \text{ Tannins} = \frac{\text{absorbance of each extract X average gradient factor X Dilution factor}}{\text{Weight of each extract X 10,000}}$$

3.2.6.2.5 Glycosides

For each extract, 10 ml was pipetted into a 250-ml conical flask and 50 ml of chloroform was added and shaken on a vortex mixer for 1hr. The mixture was filtered into a 100 ml conical flask and 10 ml pyridine; 2 ml of 2% (w/v) sodium nitroprusside were added and shaken thoroughly for 10 minutes. 20% (w/v) NaOH (3 ml) was later added to develop a brownish yellow colour. Glycoside standards (0-5 mg/ml) were prepared from a 100 mg/ml stock glycoside standard. The series of standards 0-5 mg/ml were treated similarly like each extract above. The absorbances of each extract as well as standards (figure 38, appendix) were read on a Jenway V6300 spectrophotometer at a wavelength of 510 nm. Percentage glycoside was calculated using the formula:

$$\frac{\text{Absorbance of each extract X gradient factor X dilution factor}}{\text{Weight of each extract X10000}}$$

3.2.6.2.6 Steroids

The method of Wall *et al*. (1952) was used to determine percentage of steroids. For each extract, 0.50 g was weighed into a 100 ml beaker and 20 ml of Chloroform-Methanol

(2:1) mixture was added to dissolve the extracts upon shaking for 30minutes. The whole mixture was later filtered through a Whatman No.1filter paper into another clean, dry 100 ml conical Flask. The resultant residue was repeatedly treated with the chloroform-methanol mixture until it is free of steroids. The filtrate (1 ml) was pipetted into a 30-ml test tube and 5 ml of alcoholic KOH was added and shaken thoroughly to obtain a homogenous mixture. The mixture was later placed in a water bath set at 40^0 C for 90 minutes. It was then cooled to room temperature and 10 ml of petroleum ether was added followed by 5 ml distilled water. This was evaporated to dryness on the water bath. Liebermann Burchard reagent (6 ml) was added to the residue in a dry bottle and absorbance read at a wavelength of 620 nm on a Jenway V6300 spectrophotometer. Standard Steroids of concentration (0-4 mg/ml) were prepared from a 100 mg/ml stock steroid solution and treated similarly like each extract as above. Percentage steroid was calculated using the formula:

$$\frac{\text{Absorbance of each extract X Gradient Factor X Dilution Factor}}{\text{Weight of each extract X 10000}}$$

3.2.6.2.7 Phenolics

The method of Harborne (1973) was used to determine percentage phenolics. For each extract, 0.20 g was weighed into a 50-ml beaker and 20 ml of acetone was added and mixed properly for 1 hr to prevent lumping. The mixture was filtered through a Whatman No.1 filter paper into a 10-ml volumetric flask using acetone to rinse and made up to the mark with distilled water with thorough mixing. Each extract (1 ml) was pipetted into a 50-ml volumetric flask and 20 ml of water, and 3 ml of phosphomolybdic acid were added, followed by the addition of 5 ml of 23% (w/v) $NaCO_3$; mixed thoroughly and made up to the mark with distilled water and allowed to stand for 10 min to develop a

bluish-green colour. Standard phenolics (0-10 mg/ml) were prepared from a 100 mg/l stock phenolic solution from Sigma-Aldrich chemicals, U.S.A. The absorbances of each extract as well as that of standard concentrations of phenolics were read on a Jenway V6300 spectrophotometer at a wavelength of 510 nm. Percentage phenolic was calculated using the formula:

$$\frac{\text{Absorbance of each extract X gradient factor X dilution factor}}{\text{Weight of each extract X 10,000}}$$

3.2.7 Physicochemical Studies

Physical properties of the water such as pH, Temperature, Total Dissolved Solid, Total dissolved oxygen, Conductivity, Nitrate, Nitrite were carried out. Water for the culturing of mosquito larvae and *the G. kola seed* were tested for Arsenate, Lead, Iron, Chromium, Cadmium, Copper, Calcium, Manganese before and after the experiment using AAS.

3.2.8 Acetylcholine Esterase Inhibitory Activity (AChE)

3.2.8.1 *In-vitro* Evaluation of AChE Inhibitory activity

The method of Ellman *et al*. (1961) was employed in the determination of AChE inhibitory activity of the *Garcinia kola*.

Principle: The enzyme hydrolyzes the substrate ATCI to thiocholine and acetic acid. Thiocholine is allowed to react with DTNB, and this reaction resulted in the development of a yellow color. The color intensity of the product is measured at 405 nm, and it is proportional to the enzyme activity.

Procedure: In the 96 µl well plates, a reaction mixture of 25 µl of 15 mM ATCI in water, 125 µl of 3 mM DTNB in buffer and 25 µl of the plant extract were added and the

absorbance was measured at 405 nm. Thereafter, 25 µl of AChE solution (0.22 U/ ml) was added to the wells and the microplate was read again at the same wavelength 6 times at 1 min intervals. Galanthamine dissolved in methanol was used as standard at 1mg/ml concentrations; a blank of methanol in 50 mM Tris-HCl, (pH 8) was used.

The percentage inhibition for each test solution was then calculated using the following equation: Inhibition (%) = 1 -(Asample/Acontrol) X 100

Where Asample is the absorbance of the sample fractions and Ligands and Acontrol is the absorbance of the blank.

3.2.8.2 *In -vivo* Acetylcholinesterase activity

Principle: Acetylcholinesterase Assay is based on an improved Ellman method, in which thiocholine produced by the action of acetylcholinesterase forms a yellow color with 5,5'-dithiobis (2-nitrobenzoic acid). The intensity of the product color, measured at 412 nm, is proportionate to the enzyme activity in the sample.

Procedure: 200 µL distilled water and 200 µL calibrator was transferred separately into wells of a clear bottom 96-well Plate followed by the addition of 10 µL sample per well in separate wells. 190 µL freshly prepared Working Reagent was transferred to all sample wells and the plate taped briefly to mix. The Absorbance was read at 412nm at 2 min and at 10 min in a plate reader.

$$\text{AChE Activity (U/L\}} = \frac{OD10 - OD2 \text{ X n X } 200}{OD\ CAL - OD H_2O}$$

Estimation of IC_{50} values

The IC50 values (concentration of test compounds that inhibits the substrates by 50 %) were determined by spectrophotometric measurement of the effect of increasing concentrations of ligands and methanolic fractions on AChE activity. Each sample was assayed at five concentrations (100, 50, 25, 12.5, 6.25 mg/ml) and IC50 values were obtained from dose-effect curves by linear regression.

3.2.9 *In vivo* Na^+/K^+ ATpase activity in larva homogenate

Principle: The Na^+/K^+ ATpase activity was determined as the ouabain sensitive rate of release of inorganic phosphate (Pi) in the presence of ATP

Procedure: Each homogenate was assayed in triplicate in a control buffer lacking K^+ (NaCl 130 mmol.L-‘; MgCl, 5 mmol.L-‘; HEPES 20 mmol.L-‘; ouabain 1 mmol.L-‘; pH 7.6) to measure non-Na^+-K^+-ATPase activity and in an experimental buffer (NaCl 100 mmol.L-‘; KC1 30 mmol.L-‘; MgCl, 5 mmol.L-‘; HEPES 20 mmol.L-‘; pH 7.6) to measure total ATPase activity, the difference between the two giving the activity due to Na^+-K^+-ATPase. Fifty µl of homogenate were assayed in a volume of 1 ml and the assay started by the addition of disodium ATP to give a final concentration of 6 mM. The reaction mixture was incubated in a shaking-water bath at 25°C for 1 hour. The assay was stopped by the addition of 1 ml of ice-cold 20% (w/v) trichloroacetic acid with rapid mixing and by cooling the tubes in ice. The tubes were centrifuged at 700 g for 10 min to sediment protein and 400 µl aliquots of supernatant were taken for inorganic phosphate analysis (Phosphate analysis kit, Sigma Chemical Company). The absorbance at 660 nm was compared with the absorbance of standard solutions of sodium phosphate. Activities were expressed as µ mol of inorganic phosphate produced per mg protein per hour (µmol mg $protein^{-1}$ h^{-1}).

3.2.10 In silico studies

The 3D Structures of Acetylcholinesterase was obtained from protein databank (www.rcsb.org) with PDB 1EEA. Existing ligands and water molecules were removed and hydrogen molecules were added. SDF structures of the ligands were obtained from the open chemistry database and converted into mol2 (flexible) format using Open Babel (O'Boyle *et al*., 2011). Docking of the compounds were carried out using Autodock vina (Trott and Olson, 2010). The binding site of the protein to the ligand molecule was identified using Discovery Science 2016 visualizer.

3.2.11 *In vivo* Antioxidant Studies

3.2.11.1 Superoxide dismutase Activity (SOD)

The method described by Woolliams *et al*. (1983) was used to assay for superoxide dismutase activity.

Principle: The method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2,4 (iodophenyl)-3 (4-nitrophenol)-5 phenyltetrazolium chloride (I.N.T) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes 50% inhibition of the rate of reduction of I.N.T under assay conditions.

Procedure: From each group for each extract, 0.05 ml of lysed red blood cells/liver homogenate supernatant was diluted with 0.05 ml of 0.01 mol/L phosphate buffer (pH 7) and added to 1.7 ml of mixed substrate (0.05 mmol/L xanthine, 0.025 mmol/L I.N.T, 40 mmol/L (pH 10.2), 0.94 mmol/L EDTA) and mixed thoroughly. Then 0.25 ml of xanthine oxidase (80 U/L) was added and the initial absorbance was read after 30 secs

and 3 mins at 505 nm. Standard SOD (5.66 U/L) was used to prepare a calibration curve from which the activity of SOD was read. The % inhibition was calculated using the expression:

$$\% \text{ Inhibition} = 100 - \frac{\text{Absorbance of sample/standard/minute} \times 100}{\text{Absorbance of standard/min}}$$

3.2.11.2 Catalase Activity(CAT)

Catalase activity was measured by the method of Aebi (1974)

Principle: The method is based on the ability of catalase to reduce hydrogen peroxide (H_2O_2) to water and oxygen. One unit of catalase will decompose 1.0 µmole of H_2O_2 per minute at pH 7.0 at 25°C.

Procedure:

Hepatic supernatant or plasma (0.1 ml) was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 ml of freshly prepared 30 mM H_2O_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.

3.2.11.3 Glutathione Peroxidase (GPx)

The method described by Paglia and Valentine (1967) was used to determine the activity of glutathione peroxidase.

Principle: Glutathione peroxidase catalyzes the oxidation of reduced glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, oxidized glutathione (GSSG) is immediately reduced to GSH with concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance is then measured. One unit of

glutathione peroxidase is the activity that causes the formation of 1.0 mmol of $NADP^+$ fron NADPH per minute at pH 8.0 and 25°C in the presence of other substrates.

Procedure: From each group for each extract, 0.02 ml of the sample was added to 1 ml of reagent (4 mmol/L glutathione, 0.5 U/L glutathione reductase, 0.34 mmol/L NADPH, 0.05 mmol/L phosphate buffer. (pH 7.2), 4.3 mmol/L EDTA) and 0.04 ml of 0.18 mmo/L cumene hydroperoxide. This was mixed and the initial absorbance at 340 nm was read. Absorbance was read after 1 and 2 mins against reagent blank containing distilled water in place of sample. Glutathione peroxidase activity was calculated from the following expression:

$$\text{GPx (U/mg protein)} = \frac{\text{Absorbance/min x 8412 x dilution factor}}{\text{Protein concentration (mg/ml)}}$$

3.2.11.4 Malondialdehyde Concentration (MDA)

The rate of lipid peroxidation in tissues was assessed by quantifying malondialdehyde (MDA) levels as described by Varshney and Kale (1990).

Principle: This method is based on the measurement of thiobarbituric acid reactive substances (TBARS). In acidic medium, malondialdehyde (MDA) generated membrane fatty acid peroxidation reacts with 2-thiobarbituric acid to yield a MDA-TBA2 adduct, a pink coloured complex that absorbs maximally at 532 nm.

Procedure: 0.8 ml of Tris-KCl was added to 0.2 ml of the sample and then quenched by addition of 0.25 ml of TCA. 0.25 ml of TBA was added and the reaction mixture incubated for 45mins at 80°C and then cooled on ice. The resulting pink-coloured reaction mixture was centrifuged at 4000 x g for 15mins. The absorbance of the clear pink supernatant was then read at 532nm using distilled water as blank.

Calculations

MDA (units/mg protein) = $\frac{\text{absorbance x volume of mixture}}{E_{532} \text{ x volume of sample x mg protein}}$

Where E_{532} is molar absorbtivity at 532nm = 1.56×10^{-5}

3.2.12 Toxicological Studies

3.2.12.1 Determination of Serum Uric Acid Concentration

The concentration of Uric Acid was determined using the procedure described by Fossati *et al*. (1980)

Uric Acid + O_2 + $2H_2O$ $\xrightarrow{\text{Uricase}}$ **Allantoin + CO_2 + H_2O_2**

$2H_2O_2$ + 3, 5-Dichloro-2-hydroxybenzensulfonic acid + 4-aminophenazone $\xrightarrow{\text{peroxidase}}$ **N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzo-quinoneimine**

Procedure: 20 µL of the sample or standard (0.595 mmol/L uric acid) was mixed with 1000 µL of pre-mixed reagent 1a (50 mmol/L Hepes buffer, pH 7.0 and 4 mmol/L 3, 5-Dichloro-2-hydroxybenzensulfonic) and reagent 1b (0.25 mmol/L 4-aminophenazone, ≥1000 U/I peroxidase and ≥ 200 U/I Uricase) following manufacturer's instruction. The mixture was incubated at 25^0C for 15 min after which the absorbance of standard and samples was read at 520 nmagainst reagent blank.

Calculation of Uric Acid Concentration in (mmol/L)

Uric Acid (mg/dl) = $\frac{\text{Absorbance of the sample}}{}$ X Concentration of the Standard

Absorbance of the Standard

3.2.12.2 Determination of Serum Urea Concentration

The method described by Henry (1963) was used in the determination of serum urea concentration.

Principle: Urea in the samples was broken down to ammonia and carbon dioxide by urease. The ammonia reacts with salicylate in the presence of nitroprusside and hypochlorite to give 2,2-dicarboxyindophenol, a coloured compound; the intensity of which is measured and is proportional to the concentration of urea in the sample.

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{salicylate} \xrightarrow[\text{Hypochlorite}]{\text{Nitroprusside}} \text{2.2-dicarboxyindophenol}$$

Procedure: Three clean test tubes were labeled blank, standard and sample. To each of the test tubes, 1000 µl of the working reagent (Phosphate buffer 60 mmol/L (pH 6.9), urease 20 (U/L) was added. To the tubes labeled standard and samples, 10 µl each of the standard preparation (40 mg/dL) and sample were added. The resulting solutions were mixed and incubated for 5minutes at 37°C after which 1000 µl of colour reagent (sodium salicylate 80 mmol/L, sodium nitroprusside 4 mmol/L, sodium hypocholorite 45 mg/dL) was added to all tubes, mixed and incubated again for 5 minutes at 37° C. After the incubation, 1000 µl of distilled water was added to each tube and mixed again. The absorbance of the sample and standard against the reagent blank was read at 600 nm.

3.2.12.3 Determination of Serum Potassium Ion Concentration

Serum potassium ion concentration was determined using the method of Tietz (1995).

Principle: Potassium was estimated by a turbidimetric method. Potassium in the sample reacts with sodium-tetraphenylborate to give a turbid potassium-tetraphenylborate complex. The extent of turbidity is proportional to the potassium concentration.

Na-tetraphenylborate + K^+ ⟶ K-tetraphenylborate + Na^+

Procedure: To two test tubes standard and test, 1000 µl of the potassium reagent (sodium tetraphenylboron (TPS-Na) 0.2 mol/L) was added; 25 µl of the standard solution (5 mmol/L) was added to one tube and 25 µl of sample to the other. The contents of each tube were mixed and incubated at 37º C for 5 minutes after which the absorbance of the sample and standard were read against a distilled water blank at 578 nm. The concentration of potassium ions in the sample was calculated using the following expression:

Potassium Conc. (mg/dl) = (absorbance of sample) / (absorbance of standard) x 5

3.2.12.4 Determination of Serum Sodium Ion Concentration

The method described by Tietz (1995) was used in the determination of serum sodium ion concentration.

Principle: Sodium and proteins in the sample are precipitated together by magnesium uranyl acetate as uranyl magnesium sodium acetate salt. Excess of uranyl salt reacts with potassium ferrocyanide to produce a brownish colour, the intensity of which is inversely proportional to the sodium concentration in the sample.

Procedure: The procedure was done in 2 steps: precipitation and sodium estimation. To precipitate, 1 ml of precipitating reagent (Uranyl acetate, 19 mmol/L, magnesium acetate, 140 mmol/L) was added to serum and standard solution (150 mmol/L), shaken vigorously and incubated at room temperature for 5 minutes. The tubes were then centrifuged at

3000 rpm for 2 minutes to obtain a clear supernatant. For sodium estimation, 1ml of colour reagent (ammonium thioglycolate 550 mmol/L, ammonia 550 mmol/L) was added to all tubes and 20 µl of supernatant was added to their respective tubes and 20 µl of precipitating reagent was added to the blank to make up the volume. The tubes were mixed and allowed to stand at room temperature for 5 minutes. The absorbance for test and standard were read against reagent blank at 546 nm. The concentration of sodium in the samples was calculated from the expression:

$$\text{Sodium conc. (mmol/L)} = \frac{\text{Absorbance of blank} - \text{absorbance of test}}{\text{Absorbance of blank} - \text{absorbance of standard}} \times 150$$

3.2.12.5 Determination of Serum Total Cholesterol Concentration

The method of Friedewald *et al*. (1972) was used to determine serum total cholesterol concentration.

Principle: The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestene-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrene} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4H_2O$$

Procedure:

To three test tubes labeled blank, standard and sample, 10 µl of distilled water, standard solution and sample (serum) were added respectively followed by 1000 µl of the cholesterol reagent (4-aminoantipyrine (0.30 mmol/L), phenol (6 mmol/L), peroxidase ≥0.5 U/ml), cholesterol oxidase ≥0.1 U/ml), cholesterol estarases (≥0.1 U/ml), pipes buffer (80 mmol/L; pH 6.8) to each of the test tubes. This was thoroughly mixed and incubated for 5mins at 37°C and the absorbance of the samples and standard were read at 546 nm against the reagent blank.

Cholesterol concentration was calculated from the expression:

Conc. of cholesterol = (Absorbance of sample) / (Absorbance of standard) X 5.25 mmol/L.

3.2.12.6 Determination of Serum High Density Lipoprotein Cholesterol concentration (HDL-c)

The method of Friedewald *et al.* (1972) was used to determine the concentration of HDL-c.

Principle: Low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the HDL fraction, which remains in the supernatant, is determined.

Procedure: To 200 µl of sample and standard in separate test tubes, 500 µl of the reagent (phosphotungstic acid, 0.55 mmol/L, magnesium chloride, 25 mmol/L) was added. The contents were mixed and allowed to stand for 10mins at room temperature. The mixtures were then centrifuged at 4000 rpm for 10 mins and the clear supernatant was separated for determination of cholesterol content. The HDL-c concentration was calculated using the following expression:

HDL-C (mmol/L) = (absorbance of sample) / (absorbance of standard) X 5.25 mmol/L

3.2.12.7 Determination of Serum Triglyceride Concentration

The method of Jocobs and Van Denmark (1960) was employed in the determination of triglyceride concentration.

Principle: Enzymatic determination of triglyceride is based on following reactions:

$$TGL + H_2O \xrightarrow{LPL} Glycerol + Fatty\ acid$$

$$Glycerol + ATP \xrightarrow{GK,\ Mg^{2+}} Glycerol\text{-}3\text{-}phosphate + ADP$$

$$Glycerol\text{-}3\text{-}phosphate + O_2 \xrightarrow{GPO} Dihydroxyacetone\ phosphate + H_2O_2$$

$$2H_2O_2 + 4\text{-}Aminoantipyrine + p\text{-}chlorophenol \xrightarrow{POD} Red\ quinonemine$$

Where TGL= triglyceride, LPL= lipoprotein lipase, GK= glycerol kinase, GPO=glycerol-3-phosphate oxidase. POD= peroxidase

Procedure: Into three clean tubes labeled blank, standard and sample, 1000 µl of the working reagent (Pipes-buffer (pH 7.00) 50 mmol/L, p-chlorophenol 5,3 mmol/L, potassium ferrocynate 10 mmol/L, magnesium salt 17 mmol/L, 4-aminoantipyrine 0.9 mmol/L, ATP 3.15 mmol/L, lipoprotein lipase 1800 U/L, glycerol kinase 450 U/L, glycerol - 3- phosphate oxidase 3500 U/L, peroxidase 450 U/ L) was added; 10µl of the standard solution (200 mg/dL) and sample (serum) were added accordingly. This was mixed and incubated for 5mins at 37° C. The change in absorbance of standard and sample against the reagent blank was read at 505 nm. Triglyceride concentration was calculated from the expression:

Triglycerides Conc. (mg/dl) = (absorbance of sample) / (absorbance of standard) X 200

3.2.12.8 Determination of total protein concentration

The protein concentration was determined in serum and tissue supernatants were using the Biuret method of Gornall *et al*. (1949).

Principle: Biuret reagent detects the presence of peptide bonds in solution; when compounds containing peptide (-CO-NH-) bonds are treated with alkaline copper sulphate, it results in the formation of a purple complex, the intensity of which is a measure of the protein content in the sample.

Procedure: To 1 m1 of sample, 4 m1 of Biuret reagent was added and mixed. This was incubated for 30 mins at room temperature. The blank contained 1 ml distilled water in place of sample. The absorbance of samples was read at 540 nm and protein concentration of samples were calculated from a standard calibration curve of bovine serum albumin (BSA) having a concentration range of 1-10 mg/ml.

3.2.12.9 Determination of Albumin Concentration

Plasma albumin concentration was quantified by the method described by Doumas *et al*. (1997)

Principle: The measurement of serum albumin is based on its quantitative binding to the indicator 3, 3, 5, 5-tetrabromo-m cresol sulphonephtlhalein (bromocresol green BCG). The albumin-BCG-complex absorbs maximally at 578 nm, the absorbance being directly proportional to the concentration of the albumin in the sample.

Procedure

10 µl of plasma was added to 3000 µl of BCG concentrate solution in a test tube. Similarly, 10 µl of the standard was added to 3000 µl of BCG concentrate solution in a separate test tube while the third test tube contains a mixture of 10 µl of distilled water and 3000 µl of BCG concentrate solution. The test tubes were mixed and incubated for 20 min at 25 ^{0}C. The absorbance of the standard and test samples were read against reagent blank at 578 nm.

Albumin concentration in the test samples:

$$= \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Concentration of standard in (g/L)}$$

3.2.12.10 Determination of total bilirubin concentration

Total bilirubin concentration in the plasma was measured with slight modification as described by Winsten and Cehely (1968).

Principle: Total bilirubin is determined in the presence of dimethylsulphoxide (DMSO) by the reaction with diazotized sulphanilic acid.

Procedure: 40 µl of plasma was added to 1000 µl of diazo reagent in a test tube. Similarly, 40 µl of the standard was added to 1000 µl of diazo reagent in a separate test tube while the third test tube contains a mixture of 40 µl of distilled water and 1000 µl of diazo reagent. The test tubes were mixed and allowed to stand for 5 min at 25 ^{0}C. The absorbance of the standard and test samples were read against reagent blank at 546 nm.

Calculation:

Total bilirubin concentration in the test samples:

$$= \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Concentration of standard in (mg/dl)}$$

3.2.13 Determination of Enzyme Activities

3.2.13.1 Determination of Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was determined using the method described by Wright *et al*. (1972).

Principle: Enzyme activity was determined by monitoring the rate of formation of p-nitrophenol from the hydrolysis of p-nitrophenolphosphate. One unit of ALP is the enzyme activity which hydrolyses 1 µmole of 4-nitrophenyl phosphate in 1 minute at 37°C under assay conditions.

$$\text{p-nitrophenylphosphate} + H_2O \xrightarrow{\text{Alkaline phosphatase}} \text{phosphate} + \text{p-nitrophenol}$$

Procedure: To 0.01 ml of sample in a test tube, 0.5 ml of reagent (diethanolamine buffer 1 mmol/L, pH 9.8, $MgCl_2$, 0.5 mmol/L and p-nitrophenylphosphate, 10 mmol/L) was added, mixed and the initial absorbance read at 405 nm was read. The absorbance was read again after 1, 2, and 3 minutes against air. Specific activity of ALP was calculated as follows:

$$\text{Specific activity (U/mg protein)} = \frac{\text{Absorbance change/min x 2760 x dilution factor}}{\text{Protein concentration}}$$

3.2.13.2 Determination of Alanine Aminotransferase Activity

Alanine aminotransferase (ALT) activity was determined according to the method described by Reitman and Frankel (1957).

Principle: ALT was measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine.

α-ketoglutarate + L-alanine $\xrightarrow{\text{ALT}}$ L-glutamate + Pyruvate

Procedure: To 0.1 ml of sample 0.5 ml of reagent (phosphate buffer (100 mmol/L, pH 7.4), L-alanine 100 mmol/L, α-ketoglutarate 2 mmol/L) was added and mixed thoroughly. After incubation for 30 minutes at 37 ºC, 0.5 ml of 2,4-dinitrophenylhydrazine (2 mmol/L) was added and incubated at 37ºC for another 20 minutes after which 5 ml of 0.4 N sodium hydroxide was added and the absorbance read at 546 nm after 5 minutes. The activity of ALT was determined from a calibration curve and the specific activity was calculated using the expression:

$$\text{Specific activity (U/mg protein)} = \frac{\text{ALT activity -calibration curve (U/L) x dilution factor}}{\text{Protein concentration}}$$

3.2.13.3 Determination of Aspartate Aminotransferase Activity

The activity of Aspartate Aminotransferase (AST) was determined according to the method described by Reitman and Frankel (1957).

Principle: AST was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine. One unit of AST is the amount of enzyme that will generate 1 µmole of glutamate per minute at pH 8.0 at 37°C.

α-ketoglutarate + L-aspartate $\xrightarrow{\text{AST}}$ L-glutamate + Oxaloacetate

Procedure: To 0.1 ml of sample, 0.5 ml of reagent (phosphate buffer (100 mmol/L, pH 7.4)**,** L-aspartate 100 mmol/L**,** **α**-ketoglutarate 2 mmol/L) was added and mixed thoroughly. This was then incubated at 37ºC for 30 minutes and 0.5 ml of 2,4-dinitrophenylhydrazine (2 mmol/L) was added and incubated at 20ºC for another 20 minutes after which 5 ml of 0.4 N sodium hydroxide was added and the absorbance read at 546 nm after 5 minutes. The activity of AST was determined from a calibration curve and specific activity was calculated using the expression:

Specific activity (U/mg protein) = $\frac{\text{ALT activity (U/L) x dilution factor}}{\text{Protein concentration}}$

3.2.14 Data Analysis

Data was expressed as the mean ± SEM. Statistical Package for Social Sciences, version 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Statistical significance was set at 95% confidence interval.

CHAPTER FOUR

RESULTS

4.1.1 Phytochemical Constituents of *Garcinia kola*

Some phytochemicals present in the methanolic fraction of *Garcinia kola* extract are shown in Table 3. The results showed that the extract fraction contains alkaloids, tannins, phenolics, glycosides, saponin, phlobatanins, chalcones, cardenolics, steroids and cardiac glycosides.

Results obtained from the quantitative phytochemical screening revealed that total phenolics was the most abundant in both methanolic and aqueous extract. Steroids are the least present in both methanolic and aqueous extract of *Garcinia kola* (Table 4)

4.1.2 Proximate Analysis of *Garcinia kola*

Proximate analysis of Garcina Kola reveales a 47.67% moisture content, 46.01% carbohydrate content, 2.76% crude fibre content, 0.94% ash content and 0.81% total lipids (Table 5)

4.1.3 Mineral Composition of *Garcinia kola* seeds

Results obtained from the analysis of the mineral constituents of *Garcinia kola* revealed that calcium and potassium is the most abundant (989.38 and 801.2 ppm) respectively. The least abundant of the mineral is phosphorus (0.5 ppm) (Table 6)

Table 3: Phytochemical constituent of *Garcinia kola* seeds

s/n	Phytochemical	Result
1	Alkaloid	+
2	Tannins	+
3	Phenolics	+
4	Glycosides	+
5	Saponin	+

6	Flavonoids	+
7	Steroids	+
8	Phlobatannins	+
9	Triterpenes	-
10	Chalcones	+
11	Cardenolides	+
12	Anthraquinone	-
13	Cardiac glycosides	+

+: Present -: Absent

Table 4: Quantitative Phytochemical constituent of *Garcinia kola* seeds Fractions

s/n	**Phytochemical**	**Methanolic Fraction**	**Aqueous Fraction**
1	Total Phenols mg/g (GAE)	46.2 ± 0.8^{a}	32.4 ± 0.6^{b}
2	Total Flavonoids mg/g (QE)	30.7 ± 0.5^{a}	12.05 ± 0.2^{b}
3	Ascorbic acid (AscE)	19.2 ± 0.3^{b}	23.98 ± 0.7^{a}
4	Alkaloid mg/g	26.83 ± 0.4^{b}	38.30 ± 0.3^{a}
5	Tannin mg/100g	1.03 ± 0.001^{b}	2.86 ± 0.021^{a}

6	Cyanogenic glycoside mg/100g (CNE)	0.44±0.35[c]	1.02±0.011[a]
7	Steroids mg/100g (SE)	0.07±0.001[a]	0.008±0.001[b]

Table 5: Proximate Analysis of *Garcinia kola* seeds

s/n	Parameter (%)	*Garcinia kola*
1	Moisture content	47.67±0.43[a]
2	Protein content	1.82±0.37[b]

3	Crude Fiber content	2.76±0.02[b]
4	Total Lipids	0.81±0.15[c]
5	Ash content	0.94±0.01[c]
6	Carbohydrate content	46.01±0.11[a]

Table 6: Mineral composition of *Garcinia kola* seeds

s/n	Element	(ppm)
1	Na	735.8±0.53[a]
2	K	801.2±0.14[a]
3	Zn	18.0±0.01[c]
4	Fe	6.2±0.01[d]
5	Mn	5.9±0.01[d]
6	P	0.5±0.01[e]

7	Ca	989.38±1.87[a]
8	Cu	7.2±0.01[d]
9	Mg	148±0.02[b]

4.2 Identification of compounds by GC-MS

4.2.1 Compounds identified in Aqueous fraction of *Garcinia kola*

Compounds identified by GC-MS in the Aqueous fractions of *Garcinia kola* seeds includes 2- Cyclopenten-1-one, Ethylbenzene, p-xylene, Methyl-cyclooctane, Phenoxymethyloxirane, Benzoic acid, 2-(1- methyl-2-nitro ethyl)-3,5- Hexadien-2-ol, Pentadecanoic acid, 9, 12-Octadecadienoic acid, Cis-13-Octadecenoic acid, 16-methyl-heptadecanoic acid (Table 7).

4.2.2 Compounds identified in Methanolic fraction of *Garcinia kola*

Compounds identified by GC-MS in the Aqueous fractions of *Garcinia kola* seeds includes Cyclotrisiloxane, Tetrahydro-isobutyl (2 (2-(2-methoxyethoxy) ethoxy) ethyl) caronate, 1, 3,-dimethylxylene, 1-heptyl-2-methyl-Cyclopropane, Octamethyl-Cyclotetrasiloxane, Methyl benzoic acid, Hexadecametyl-cyclooctasiloxane, Tetracocane, Octadecamethyl-Cyclononasiloxane, Heptadecane, Methylhexadecanoic acid, Eicosamethyl-octasiloxane, Octadecane, 9-methyl octadecenoic acid, 2,4-bis[(trimethylsilyl)oxy]-benzoic acid, Methyl stearate, Octadecamethyl-benzeneacetic acid, Octadecamethyl-cyclononasiloxane, Octadecamethyl-3,6-dioxo-2,4,5,7-tetrasilaoctane (Table 8)

Table 7: Compounds identified by GC-MS in the Aqueous fractions of *Garcinia kola* seeds

S/N	Compound Identified	Retention Time (RT)	% Composition
1.	2- Cyclopenten-1-one	8.103	1.321
2.	Ethylbenzene	8.810	1.130
3.	p-xylene	9.148	2.506

4.	Methyl-cyclooctane,	14.505	1.710
5.	Phenoxymethyloxirane	15.629	8.224
6.	Benzoic acid	18.826	1.812
7.	2-(1- methyl-2-nitro ethyl)-3,5-Hexadien-2-ol	30.797	4.260
8.	Pentadecanoic acid	40.043	20.502
9.	9, 12-Octadecadienoic acid	41.103	16.330
10.	Cis-13-Octadecenoic acid	41.143	32.155
11.	16-methyl-heptadecanoic acid	41.284	10.051

Table 8: Compounds identified by GC-MS in the Methanolic Fraction of *Garcinia kola* seeds

S/N	Compound Identified	Retention Time (RT)	% Composition
1.	Cyclotrisiloxane	7.475	2.864
2.	Tetrahydro-isobutyl (2 (2-(2-methoxyethoxy) ethoxy)ethyl) caronate	8.582	0.963
3.	1, 3,-dimethylxylene	9.297	2.267
4.	n-butylether	9.918	0.982

5.	1-heptyl-2-methyl-Cyclopropane	14.435	6.606
6.	Octamethyl-Cyclotetrasiloxane	14.906	2.353
7.	Methyl benzoic acid	18.787	0.869
8.	Hexadecametyl cyclooctasiloxane	37.270	0.932
9.	Tetracocane	37.820	0.724
10.	Octadecamethyl-Cyclononasiloxane	39.147	2.746
11.	Heptadecane	39.792	0.905
12.	Methylhexadecanoic acid	40.004	9.509
13.	Eicosamethyl-octasiloxane	40.279	5.019
14.	Octadecane	40.467	1.005
15.	9-methyl octadecenoic acid	41.103	23.874
16.	2,4-bis[(trimethylsilyl)oxy]-benzoic acid	41.166	8.876
17.	Methyl stearate	41.245	6.500
18.	Octadecamethyl-benzeneacetic acid	42.109	7.393
19.	Octadecamethyl-cyclononasiloxane	43.287	8.188
20.	Octadecamethyl-3,6-dioxo-2,4,5,7-tetrasilaoctane	44.898	7.424

4.3 HPLC Analysis of Aqueous and Methanolic Fraction of *Garcinia kola* seeds

Seven (7) major peaks were obtained in the HPLC spectra of the aqueous and methanolic fractions of *Garcinia kola* seeds. The peaks numbered 1, 2, 3, 4, 5, 6 and 7 were identified as catechin, caffeic acid, chlorogenic acid, ellagic acid, quercetin, luteolin and apigenin respectively (Figure 12). Luteolin had the highest concentration (6.23 mg/g)

among the compounds identified in the aqueous fraction while quercetin was the highest in concentration (8.65 mg/g) in the methanolic fraction (Table 9)

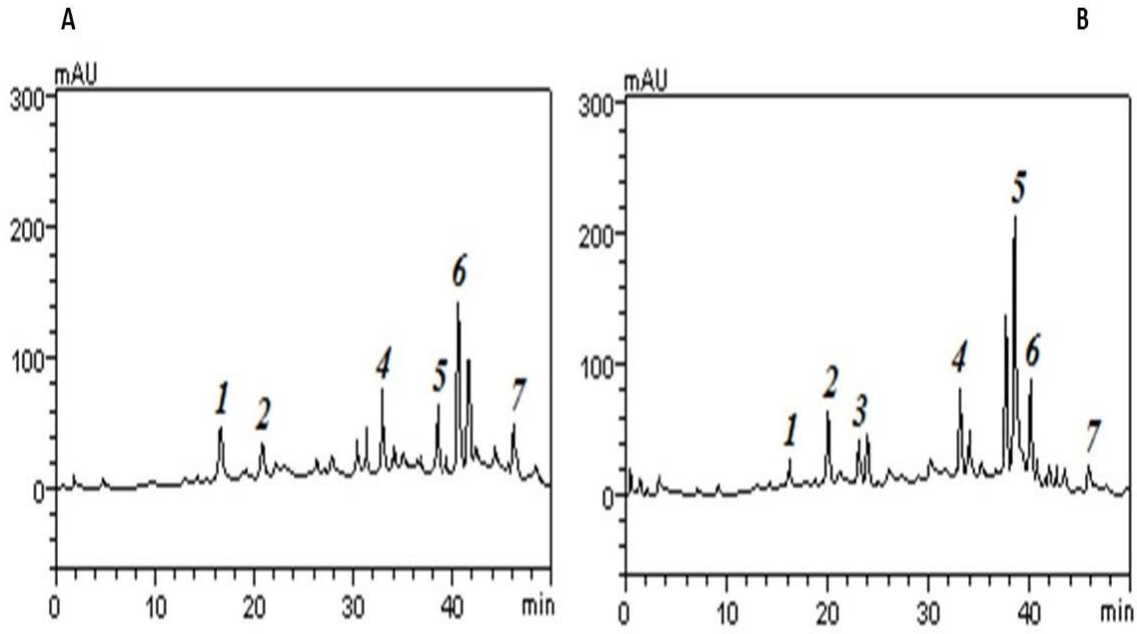

Figure 12: Reverse-phase HPLC analysis of Garcina kola (aqueous and methanolic) extract. Using standard and spectral analysis.

A – Aqueous extract B – Methanolic extract

Peaks 1, 2, 3, 4, 5, 6 and 7 were identified as catechin, caffeic acid, chlorogenic acid, ellagic acid, quercetin, luteolin and apigenin respectively.

Table 9: Composition of HPLC Results of Methanolic and Aqueous *Garcinia kola* seed fractions

Compounds	***Garcina kola***	**LOD**	**LOQ**

	Aqueous (mg/g)	*Methanolic* (mg/g)	µg/mL	µg/mL
Catechin	2.49 ± 0.02 [a]	1.09 ± 0.02 [a]	0.012	0.039
Caffeic acid	1.81± 0.01 [b]	2.67± 0.03 [b]	0.025	0.083
Chlorogenic acid	-	1.18 ± 0.01 [a]	0.009	0.030
Ellagic acid	3.07 ± 0.01 [c]	3.11 ± 0.01 [c]	0.028	0.095
Quercetin	2.98 ± 0.03 [c]	8.65 ± 0.03 [d]	0.017	0.056
Luteolin	6.23 ± 0.02 [d]	3.24 ± 0.04 [c]	0.008	0.027
Apigenin	2.45 ± 0.01 [a]	1.13 ± 0.01 [a]	0.023	0.073

Results are expressed as mean ± standard deviations (SD) of three determinations.

Averages followed by different letters differ by Tukey test at $p < 0.05$.

LOD: limit of detection and LOQ: limit of quantification.

4.4 Larvicial Activity of *Garcinia kola* seeds

4.4.1 Larvicidal Activity of Aqueous Fraction of *Garcinia kola* seeds

The highest percentage (%) mortality (96.92%) was recorded by the group treated with 100 mg/l of the 4th fraction of the aqueous extract of *Garcinia kola* followed by the group treated with 100 mg/ml of the 5th fraction of of *Garcinia kola* seed (73.91%). The lowest % mortality (11.02%) was recorded by the group treated with 100 mg/l of residue of aqueous extract of *Garcinia kola* (Table 10)

4.4.2 Larvicidal Activity of Methanolic Fraction of *Garcinia kola* seeds

Results obtained from the larvicidal studies on the methanolic fraction of *Garcinia kola* revealed that the highest mortality (89.72 %) was recorded for the froup treated with 100 mg/l of the 4^{th} fraction of the extract. The lowest % mortality was recorded for the group treated with 100 mg/l residue of the methanolic extract of *Garcinia kola* (Table 11)

Table 10: Larvicidal Activity of the aqueous fractions of *Garcinia kola* seeds

s/n	Group number	Mortality (%)
1	G1	14.0 ± 00^{e}
2	G2	23.33 ± 0.61^{c}
3	G3	22.72 ± 0.04^{c}
4	G4	96.92 ± 0.42^{a}
5	G5	73.91 ± 0.00^{b}
6	G6	60.67 ± 0.75^{c}
7	G7	54.85 ± 0.30^{c}
8	G8	11.02 ± 0.82^{e}
9	G17	81.27 ± 0.24^{a}

Table 11: Larvicidal Activity of the Methanolic fractions of *Garcinia kola* Seed

s/n	Group number	Mortality (%)
1	G9	29.30 ± 0.28^{cd}
2	G10	33.01 ± 0.00^{c}
3	G11	75.54 ± 0.80^{b}
4	G12	89.72 ± 0.03^{a}
5	G13	76.92 ± 0.65^{b}
6	G14	52.88 ± 0.02^{c}
7	G15	46.70 ± 0.51^{c}
8	G16	13.05 ± 0.37^{e}
9	G17	81.27 ± 0.24^{a}
10	G18	0.00

4.5 Na^+ K^+-ATPase Activity

The methanolic fraction of *Garcinia kola* significantly reduce ($P<0.05$) Na^+ K^+-ATPase activity (Figure 13) at higher doses when compared to the control. However, its reductive effect on ATPase activity is comparable ($P>0.05$) to that of the ethanolic fraction at all doses.

In addition, the constituents of the seed fraction (cathechin, quercetin and caffeic acid) significantly reduced Na^+ K^+-ATPase activity in a concentration dependent manner with the lowest enzyme actitivity observed at 5 mmol/l

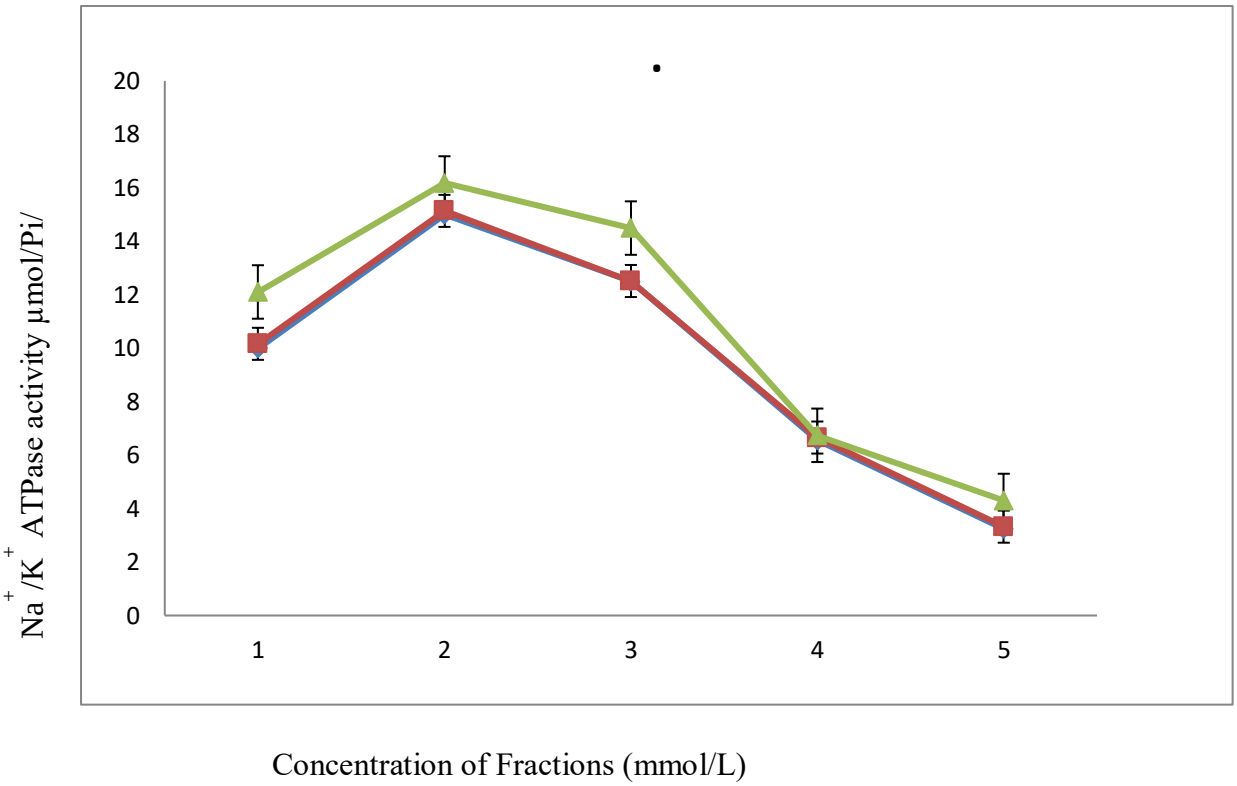

Figure 13: Suppression of Mosquito Larva Na^+/K^+-ATPase activity by *Garcinia kola* fractions

Each values represent the mean ± SD of n = 3 reading. Different superscript is significantly different (P < 0.05).

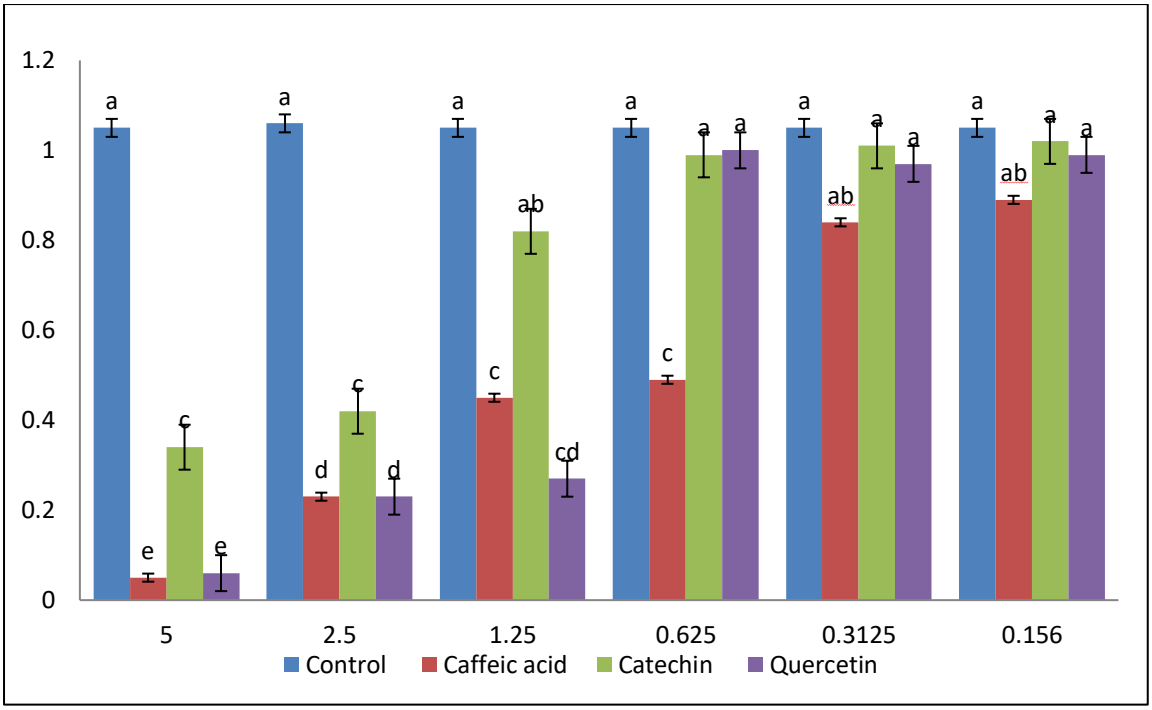

Figure 14: Effect of Cathechin, Quercetin and Caffeic Acid on Mosquito larva Na^+/K^+ ATPase activity

Each value represents the mean ± SD of n = 3 readings. Different superscript in a column is significantly different ($P < 0.05$).

4.6 Effect of *Garcinia kola* seed fractions on Acetylcholinesterase Activity

4.6.1 Effect of *Garcinia kola* seed fractions on Mosquito larva AChE

Methanolic fraction of *Garcinia kola* seed significantly decrease ($P<0.05$) the activity of Mosquito larva acetylcholinesterase when compared with the control. The methanolic fraction had a higher reduction effect on AChE when compared to the ethanolic fraction at lower doses, however, its effect was comparable to that of the ethanolic fraction at higher doses (figure 15).

4.6.2 Inhibitory effect of Apigenin, Ellagic Acid, Catechine and Methanolic fraction of *Garcinia kola* on AChE Activity

Ellagic acid had the highest % inhibitory effect on Musquito larva AChE activity when compared to apigenin, catechine and methanolic fraction of *Garcinia kola*. At higher concentrations, apigenin had a higher % inhibition when compared to catechine and methanolic fraction of *Garcinia kola* (Figure 16)

4.6.3 Inhibitory effect of Apigenin on Mosquito Larva Acetylcholinesterase Activity

Apigenin had a lower ($P<0.05$) % inhibition on acetylcholinesterase activity when compared to the standard galactamine. The inhibition of acetylcholinesterase by apigenin was non competitive (Figure 17).

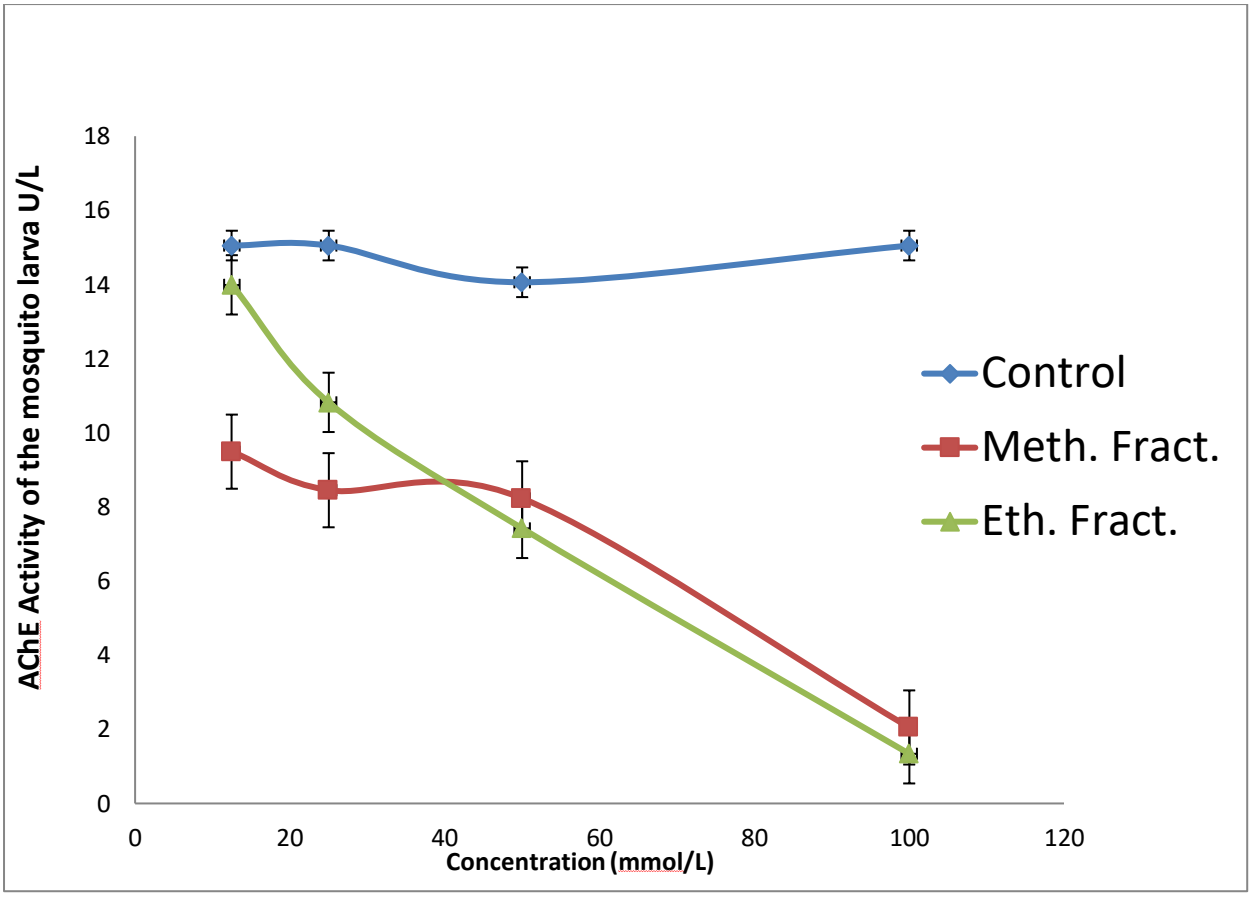

Figure 15: Inhibitory activity of *Garcinia kola* seed fractions on mosquito larva Acetylcholinesterase

Each value represents the mean ± SD of n = 3 readings. Different superscript in a column is significantly different ($P < 0.05$).

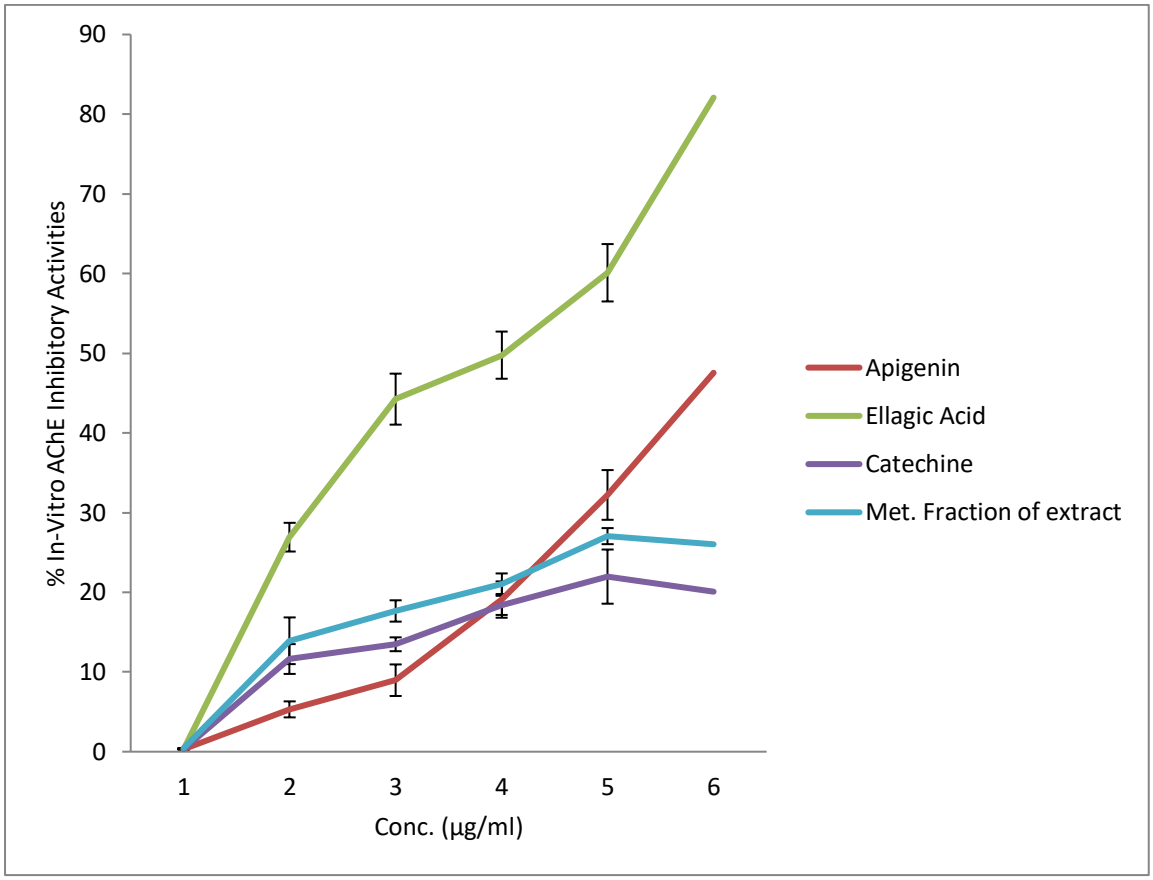

Figure 16: Inibibitory effect of apigenin, catechine, ellagic acid and methanolic fraction of *Garcinia kola* on acetylcholinesterase activity

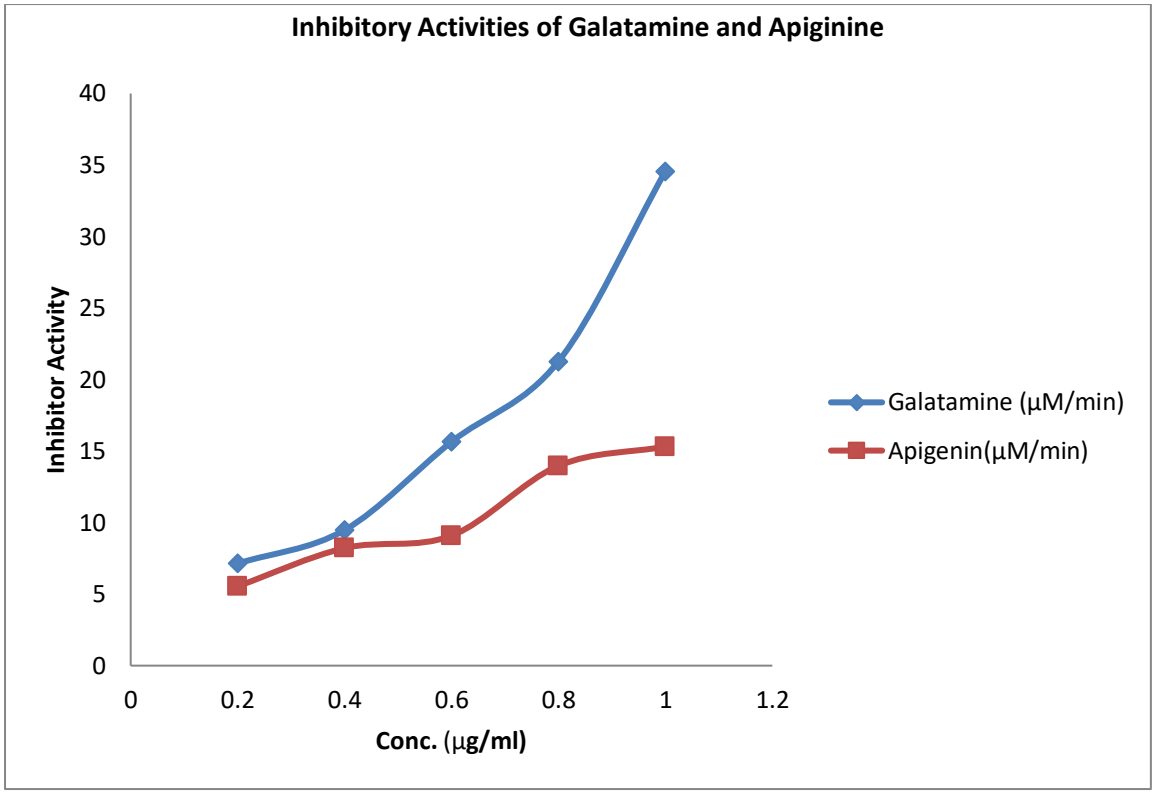

Figure 17: Inhibitory Activities of Galatamine and Apiginine

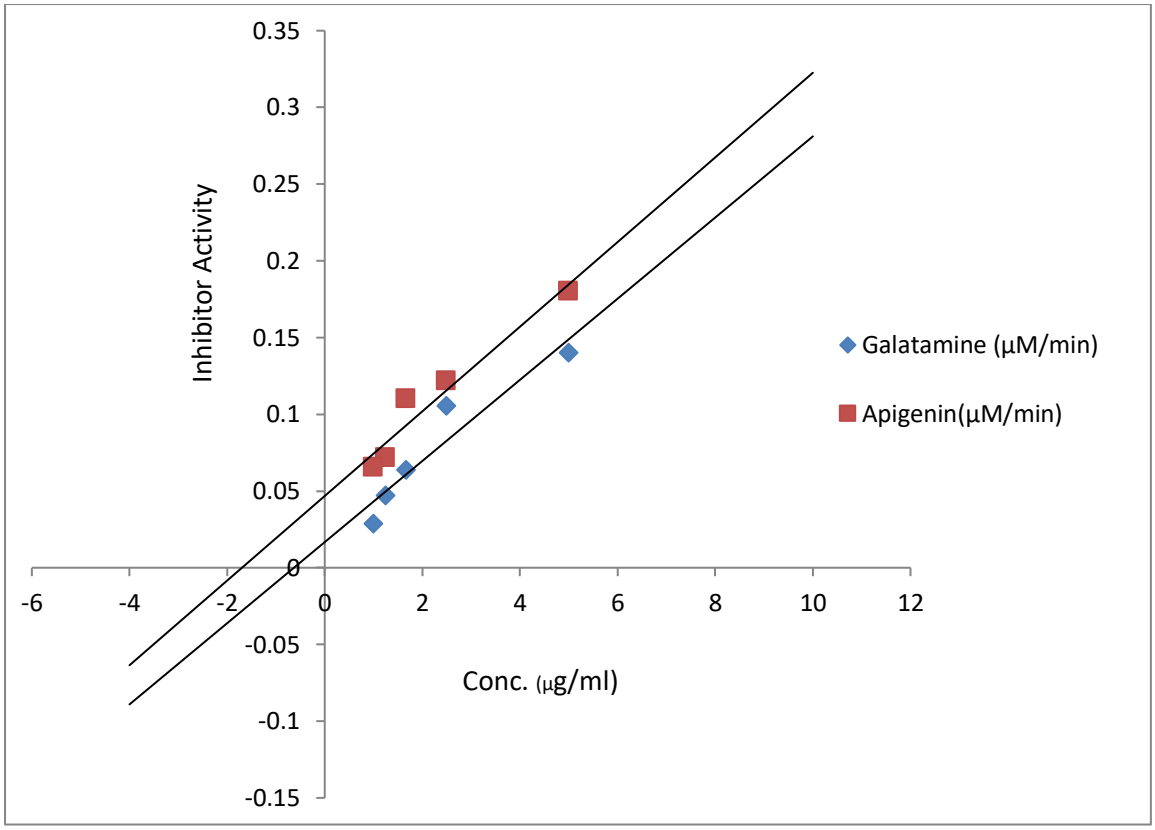

Figure 18: Non competitive Concentration dependent inhibitory activities of Apiginin on AChE

4.6.4 Inhibitory effect of Ellagic acid on Mosquito Larva Acetylcholinesterase Activity

Ellagic acid had a higher ($P<0.05$) inbitory effect on mosquito larva acetylcholinesterase activity when compared to the standard galactamine. The nature of the inhibition is competitive and concentration dependent (Figure 19)

4.6.5 Inhibitory effect of Catechine on Mosquito Larva Acetylcholinesterase Activity

The result obtained revealed that catechine significantly inhibit acetylcholinesterase activity at lower concentration compared to galactamine. However, the trend was reversed at higher concentration with galactamine significantly inhibiting AChE activity higher than catechine. The nature of inbition of AChE by catechine and galactamine was competitive (Figure 22)

4.6.6 Inhibitory effect of Methanolic fraction of *Garcinia kola* on AChE activity

Result obtained revealed that the methanolic fraction *G. kola* had a higher inhibitory effect on acetylcholinesterase compared to the standard galactamine. At 1 µg/ml, the inhibitory effect of the methanolic fraction of *Garcia kola* and galactamine was comparable. The inhibitory activity of the methanolic fraction and galactamine was competitive (Figure 24).

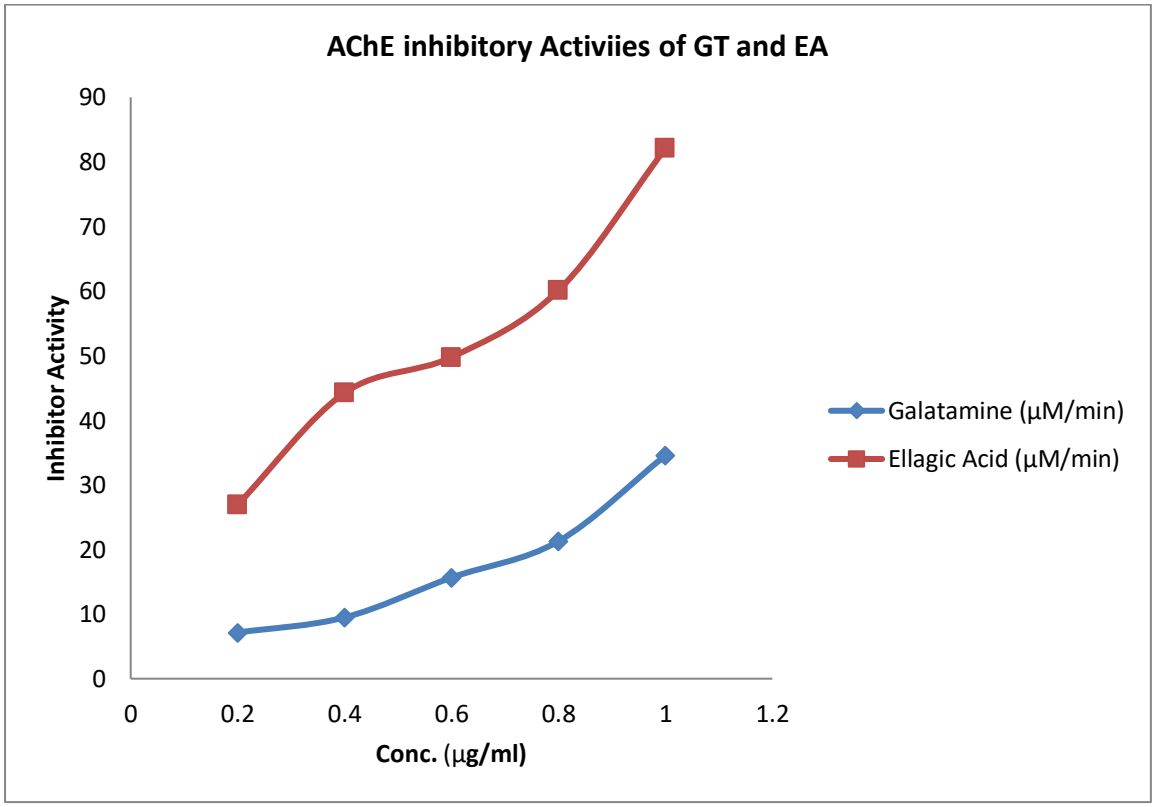

Figure 19: Concentration dependent inhibitory activities of Ellagic Acid on AChE

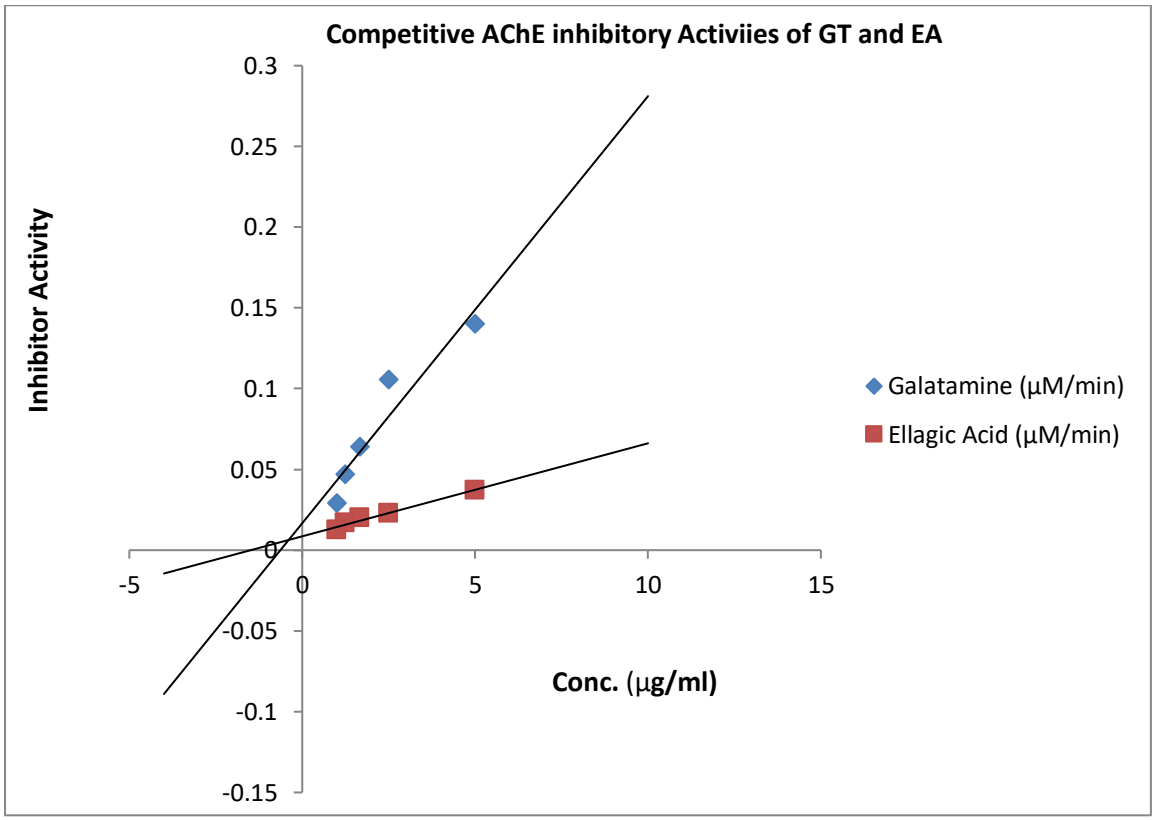

Figure 20: Concentration dependent competitive inhibitory activities of Ellagic Acid on AChE

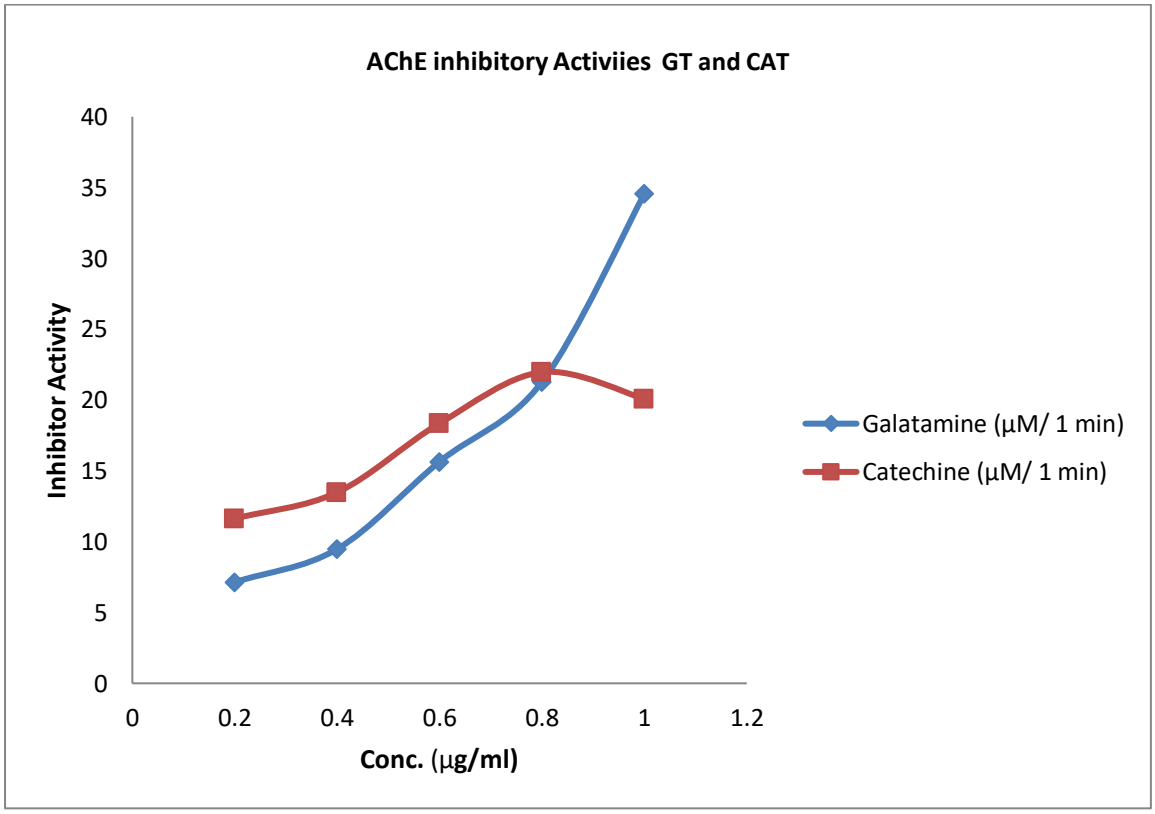

Figure 21: Inhibitory effect of Catechin on AChE activity

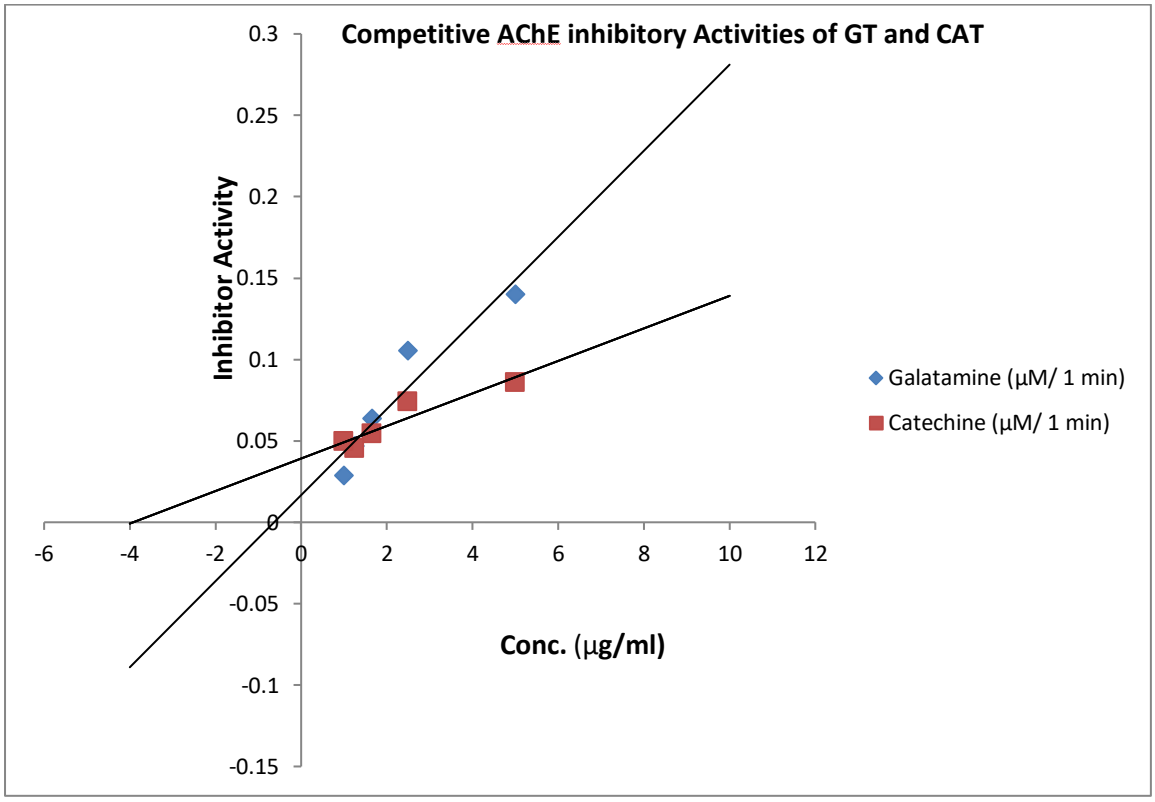

Figure 22: Inhibitory activities of Catechin on AChE

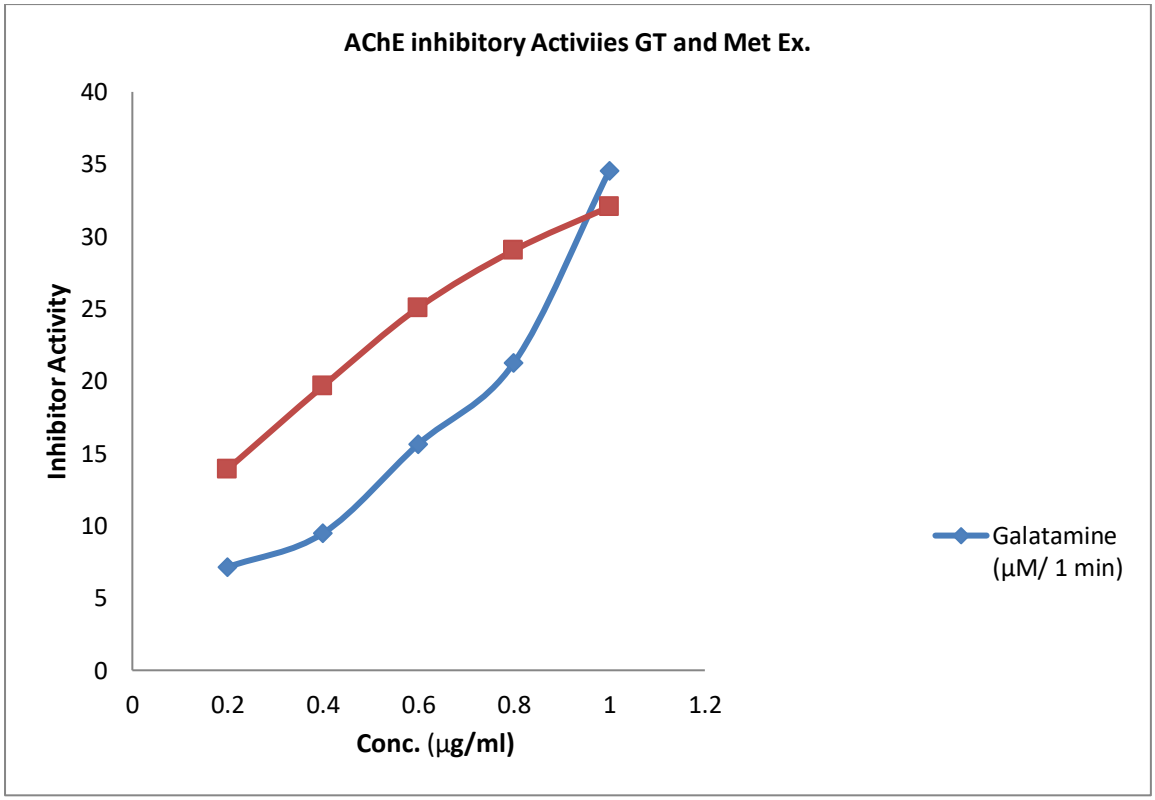

Figure 23: Inhibitory effect of Methanolic Fraction of Extract on AChE activity

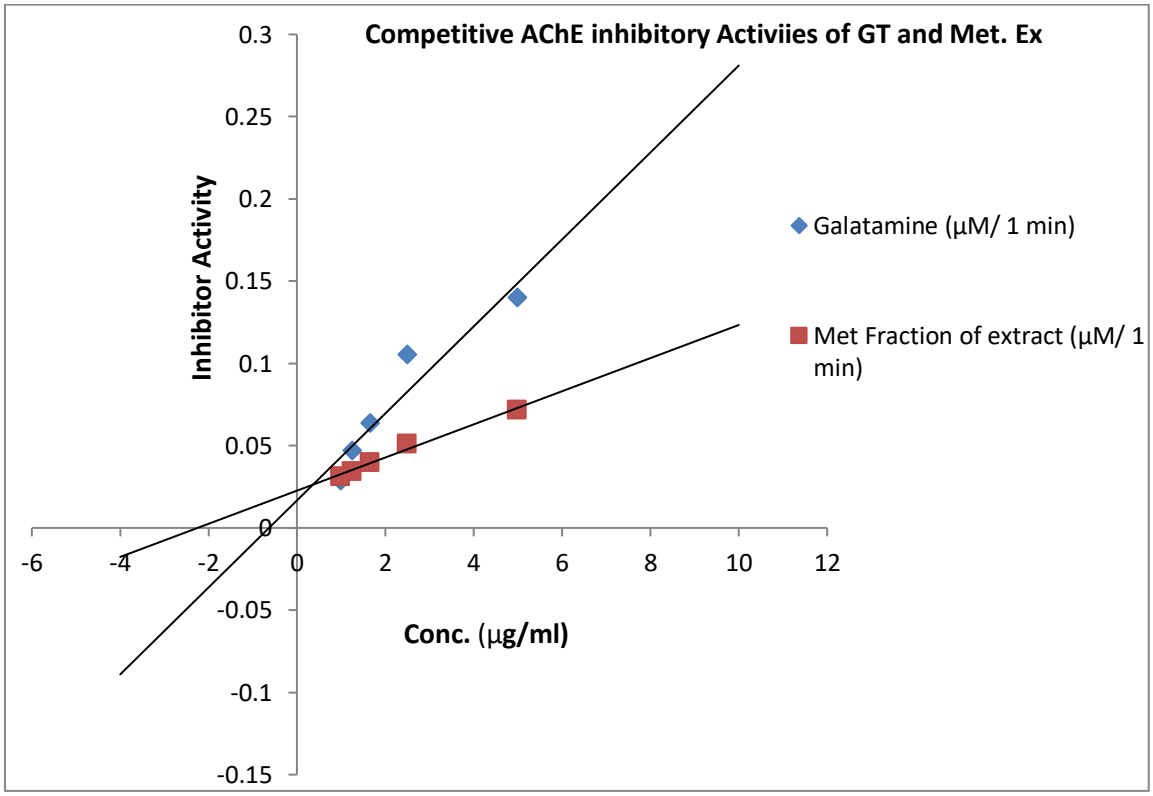

Figure 24: Inhibitory activities of Methanolic Fraction of Extract on AChE

4.7 *In vivo* antioxidant studies

4.7.1 Superoxide dismutase activity

The group treated with 300 mg/l of methanolic fraction of *Garcinia kola* significantly increased ($P<0.05$) superoxide dismutase (SOD) activity when compared to the control and standard acetylcholinesterase activity ellagic acid and quercetin. However, the group treated with standard larvicide Fenthion recorded a higher SOD activity when compared to other treatment (Table 12).

4.7.2 Catalase activity

Results obtained revealed that the group treated with 300 mg/l of methanolic fraction of *Garcinia kola* recorded a higher catalase activity when compared to the control. Fenthion, a standard larvicide significantly reduced catalase activity compared to other treatments. At all concentrations administered, the activity of the extract treated group was significantly lower than standard acetylcholinesterase inhibitor (Table 12).

4.7.3 Glutathione peroxidase activity

Fenthion significantly increased glutathione peroxidase activity when compared to other treatment group, however, the methanolic fraction of *Garcinia kola* significantly reduced glutathione peroxidase activity at 300 mg/l treatment when compared to the untreated control (Table 12).

4.7.4 Malondialdehyde concentration

The methanolic fraction of *Garcinia kola* significantly reduced malondialdehyde concentration at 100 and 300 mg/l treatment when compared to the control. The standard larvicide fenthion, significantly increased malondialdehyde concentration when compared to the untreated control (Table 12).

Table 12: Effect of methanolic fraction of *Garcinia kola* on selected antioxidant parameters in *C. garipinus*

	SOD (U/L)	CAT (U/L)	GPx (U/L)	MDA (U/L)
Group 1	433.89±2.50	636.68±11.20	27.08±1.04	14.8894±0.84
Group 2	487.06±3.14	532.28±6.18	25.9±3.76	12.259±0.57
Group 3	871.47±3.58	780.57±5.93	9.27±2.11	8.004±0.63
Group 4	640.23±2.01	657.48±7.56	29.79±3.72	8.764±0.21
Group 5	296.42±7.02	921.028±1.16	22.6±2.67	11.353±0.98
Group 6	1208.96±4.11	1103.97±10.42	93.95±3.23	21.058±0.47

Values are mean ± SEM of 5 determinations.

Means in each column with different superscripts are significantly different ($p<0.05$).

4.8 *In Vivo* Animal Studies

4.8.1 Haematoligical parameters

After 60 days of administration, the methanolic fraction of *Garcinia kola* and standard ligands caused no significant alteration ($p>0.05$) in RBC, Hb, PCV, MCV, MCH and MCHC at all doses when compared to the control (Table 19). Also, the hybrid molecule caused no significant changes ($p>0.05$) in WBC, lymphocyte, neutrophil count when compared to the control (Table 20). However, the methanolic fraction at 100 mg/l treatment significantly increased ($p<0.05$) platelet count (Table 13)

4.8.2 Lipid profile

Administration of the methanolic fraction of *Garcinia kola* and standard ligands significantly increased ($p<0.05$) total cholesterol and HDL-cholesterol concentration when compared to the control. However, the methanolic fraction of the extract and standard ligands caused no significant ($p>0.05$) difference in triglyceride concentration (Figure 25)

4.8.3 Bilirubin, Albumin and Total Protein Concentration

Total bilirubin and albumin concentration was not significantly altered after 60 days of administration of methanolic fraction of *Garcinia kola* and standard ligands. However, there was a significant increase in total protein concentration in all treatment groups when compared to control (Figure 26).

Table 13: Effect of pure ligands and methanolic fraction of *Garcinia kola* on haematological parameters in *C. garipinus* after 60 days of administration

	CTRL	MET	MET 3	ELA	QA	FT
WBC						
X103/µL	250.2	246.7	244.1	236.07	241.8	242.9
RBC						
$X10^6$/µL	2.48	2.62	2.51	1.97	2.06	2.08
HGB g/dl	8.6	8.9	9	9.23	8.09	8.39
HCT %	29.5	30.3	30.9	30.51	30.08	29.8
MCV Fl	119	115.6	123.1	113.5	112.8	121.8
MCH pg	34.7	34	35.9	33.9	33.1	35.9
MCHC g/dl	29.2	29	29.1	20.9	26.9	28.8
PLT 103/µL	3	12	4	2	3	3
PCV %	25.8	26.7	27	27.69	24.27	25.17

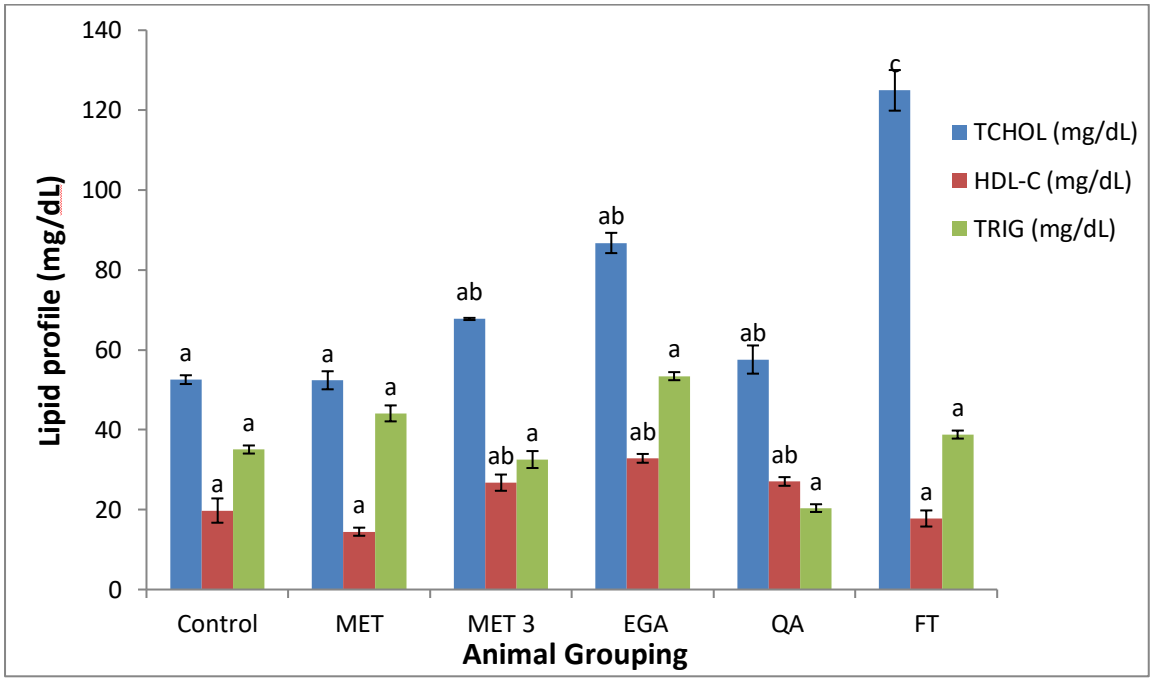

Figure 25: Effects of pure ligands and methanolic fraction of *Garcinia kola* seeds lipid profile in the serum of *C. garipinus* after 60 days of administration

Values are means ± SD of 4 determinations. Columns with different alphabets are significantly different ($p<0.05$)

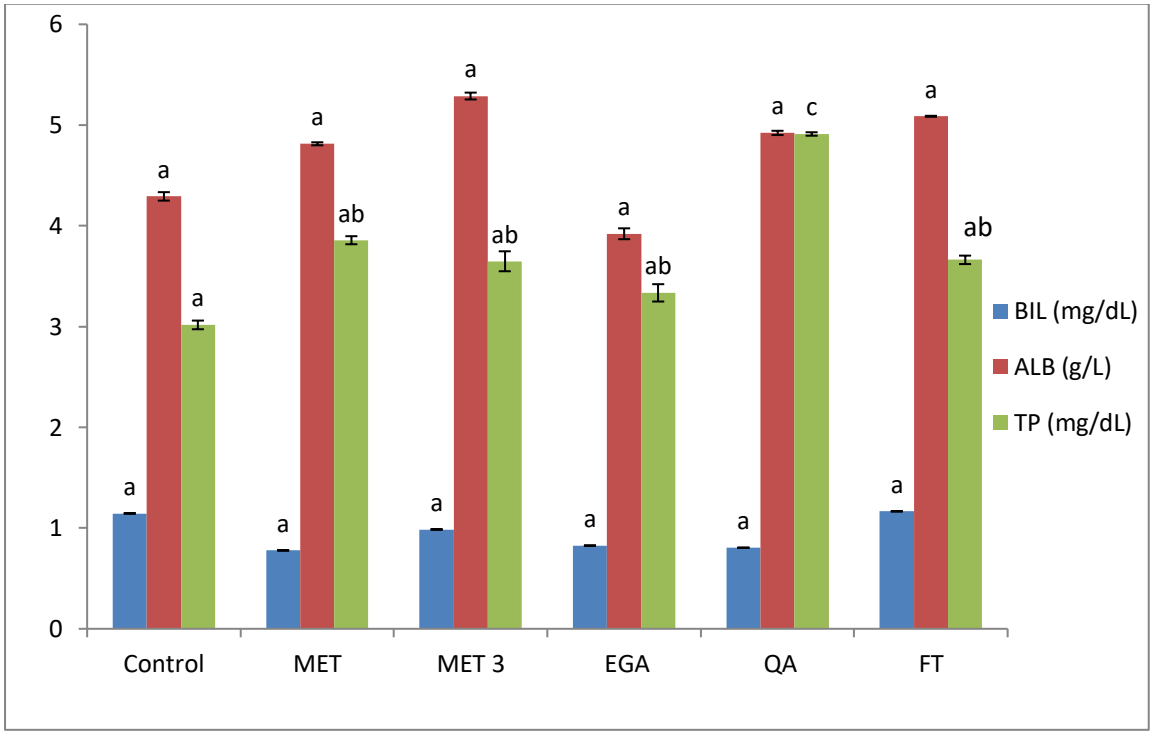

Figure 26: Effects of pure ligands and methanolic fraction of *Garcinia kola* seeds on the concentration of Bilirubin, Albumin and Total Protein in the serum of *C. garipinus* after 60 days of administration

Values are means ± SD of 4 determinations. Columns with different alphabets are significantly different ($p<0.05$)

4.8.4 Kidney function indices

The result obtained after 60 days of administration revealed that the methanolic fraction of *Garcinia kola*, acetylcholinesterase inhibitors and standard larvicides revealed an increase in serum uric acid concentration in all the treatment groups when compared to the control. Also a significant increase was recorded in the concentration of Na^+ after treatment with 100 and 300 mg/l of methanolic fraction of *Garcinia kola* seed (Figure 27).

4.8.5 ALP, AST and ALT activity

No significant increase was recorded in the activity of ALP upon 60 days' treatment with methanolic fraction of *Garcinia kola* seed when compared to the untreated control. However, administration of the methanolic fraction of the plant extract significantly decreased AST and ALT activities when compared to the control (Figure 28).

4.8.6 *In vivo* acetylcholinesterase activity

After 60 days of administration, the methanolic fraction of *Garcinia kola* seed at 200 mg/l caused a significant decrease ($p<0.05$) in acetylcholinesterase activity compared to the control. However, administration of the standard larvicide did not significantly alter ($p>0.05$) acetylcholinesterase activity (Figure 29).

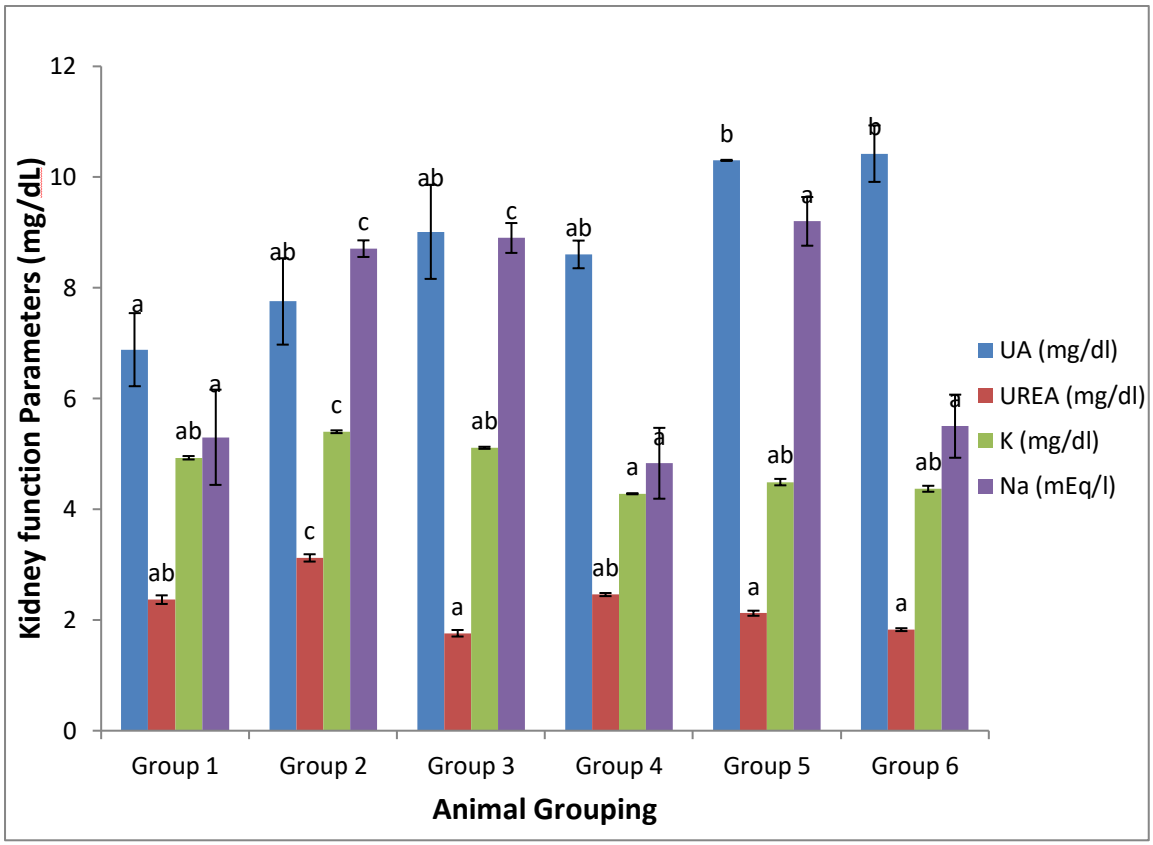

Figure 27: Effects of methanolic fraction of *Garcinia kola* seeds on selected kidney function indices in *C. garipinus* after 60 days of administration

Values are means ± SD of 4 determinations. Columns with different alphabets are significantly different ($p<0.05$)

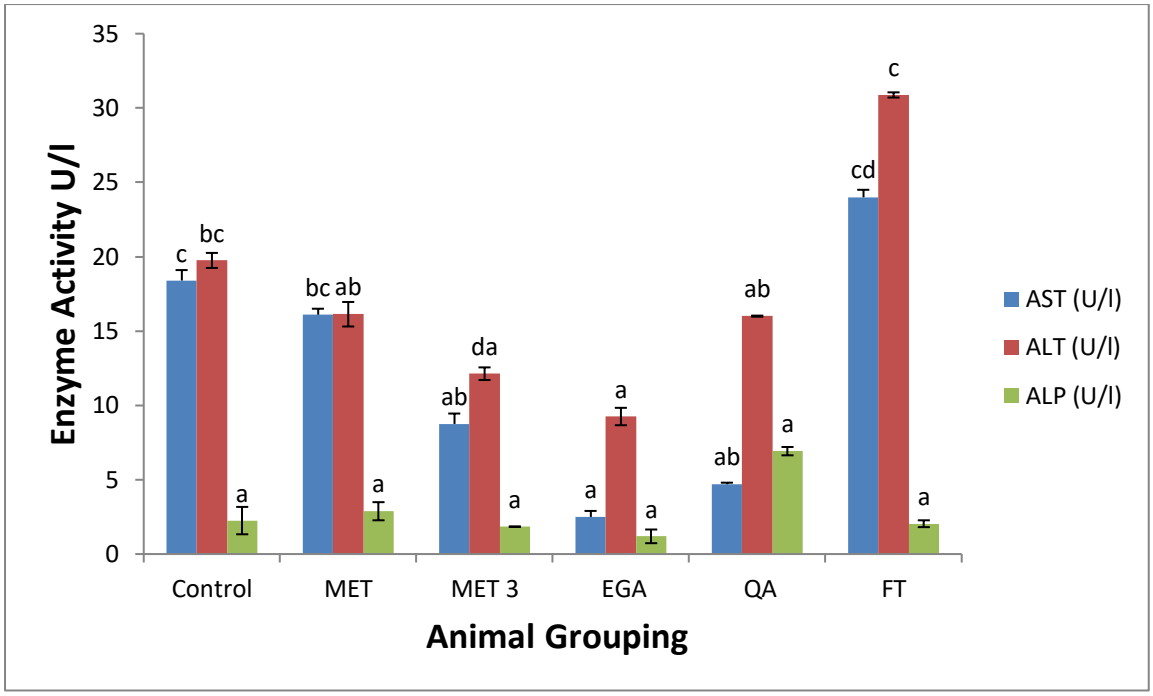

Figure 28: Effects of pure ligands and methanolic fraction of *Garcinia kola* seeds on aspartate aminotransferase and alaninie aminotransferase activity in the serum of *C. garipinus* after 60 days of administration

Values are means ± SD of 4 determinations. Columns with different alphabets are significantly different ($p<0.05$)

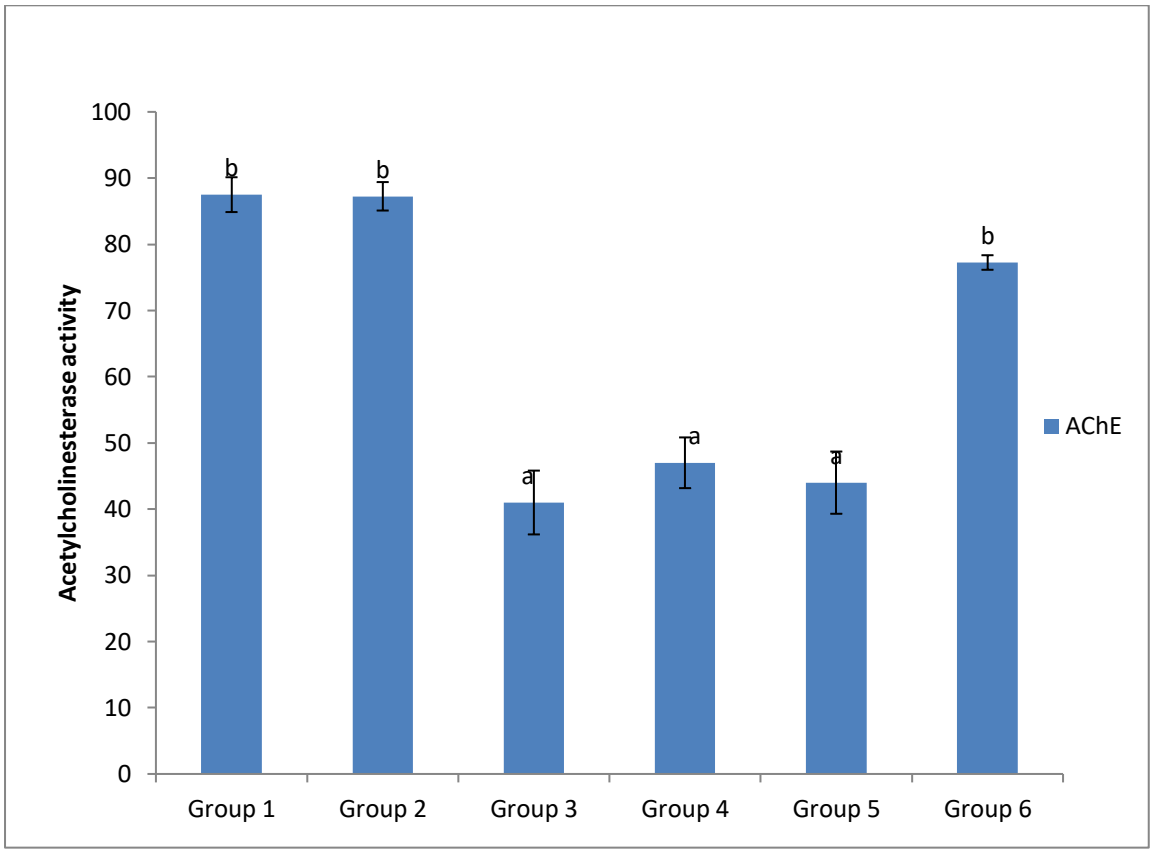

Figure 29: Effects of methanolic fraction of *Garcinia kola* seeds on acetylcholinesterase activity in *C. garipinus* after 60 days of administration

Values are means ± SD of 4 determinations. Columns with different alphabets are significantly different ($p<0.05$)

4.9 Histological studies

Histological evaluation of *C. gariepinus* gill revealed that the control group shows no perturbation of the gill arch, epithellial cells and gill filament (Plate 1). Also, the group treated with 100, 200 and 300 mg of the methanolic fraction of *G. kola* showed no Perturbation of the gill Arch, secondary lamellae and gill raker (Plate 1). A mild adipose deposit in the afferent pseudobranchial artery containing red blood cells was observed in the group treated with 400 m/kg of the methanolic fraction *G. kola*. The group treated 500 mg of the methanolic fraction Severe depletion of the secondary lamellae, severe lesion of the afferent brachial artery and severe degradation of afferent pseudobranchial artery containing red blood cells.

The brain of *C. gariepinus* treated with 100 mg of quercetin shows normal arrays of pyramidal neurons within the the hippocampal cells while the group treated with fenthion shows profound histological alterations in the cellular structure (Plate 2).

The liver of *C. gariepinus* treated with 100 mg of the methanolic fraction *G. kola* showed no perturbation of hepatic cells and clear central vein. However, the group treated with fenthion showed duct inflammation and mild cholestasis (Plate 3 and 4).

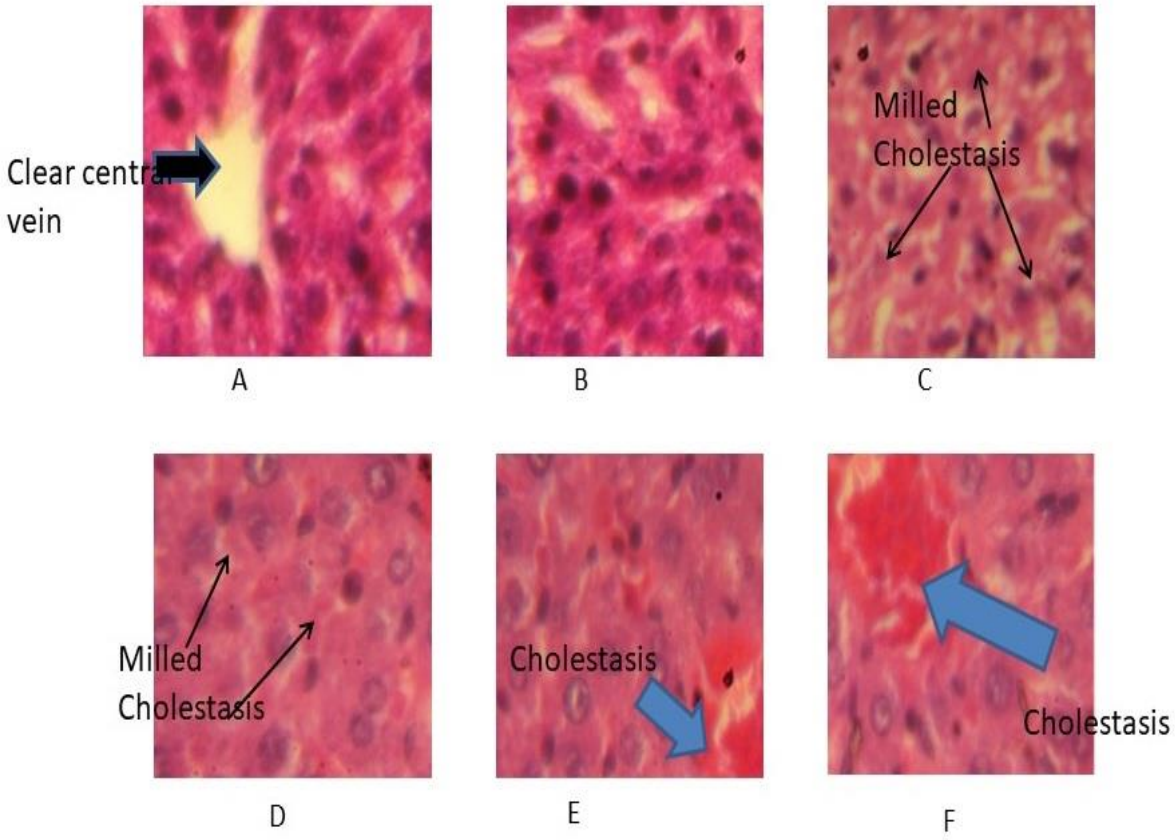

Plate 1: Cross section of *C. gariepinus* liver after treatment with methanolic fraction of *Garcinia kola* and various ligands (x 400; HandE)
A: Control, B: 100 mg of methanolic fraction, C: 300 mg of methanolic Fraction D: 100 mg of Ellaggic acid, E: 100 mg of Quercetin

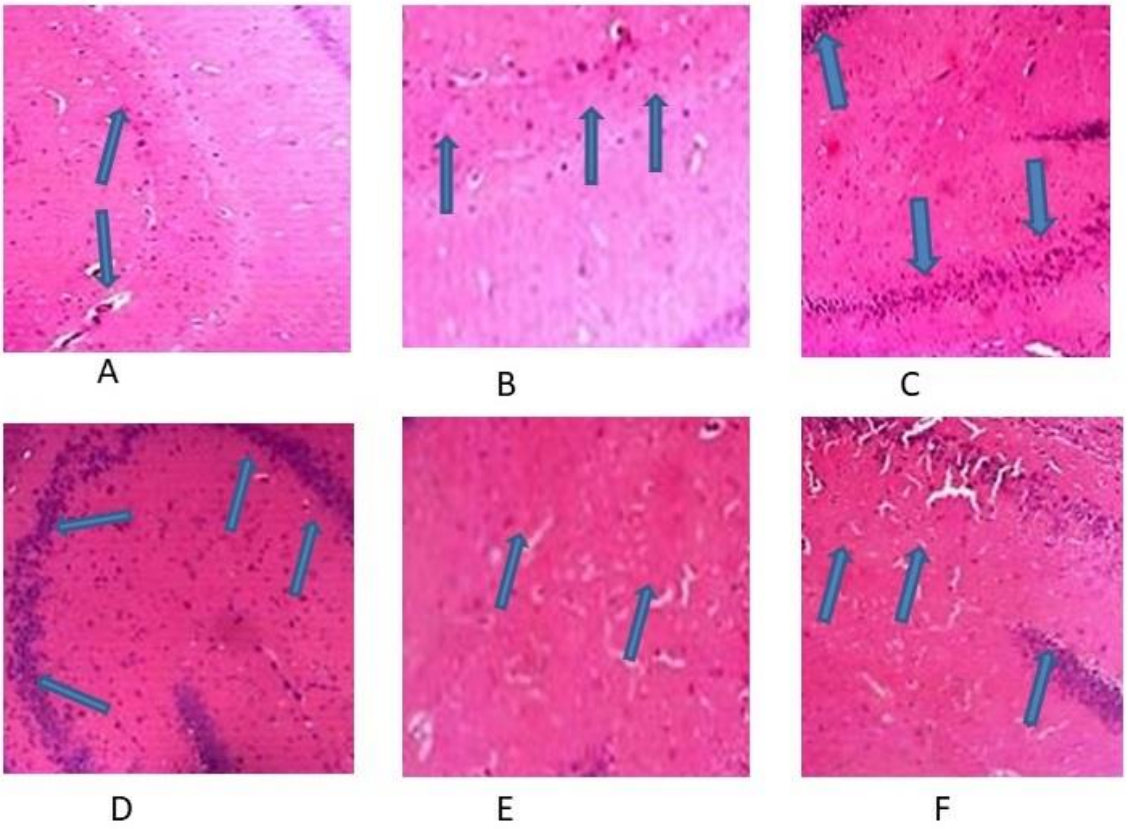

Plate 2: Cross section of *C. gariepinus* brain after treatment with methanolic fraction of *Garcinia kola* and various ligands (x 400; HandE)
A: Control, B: 100 mg of methanolic fraction, C: 300 mg of methanolic Fraction D: 100 mg of Ellaggic acid, E: 100 mg of Quercetin

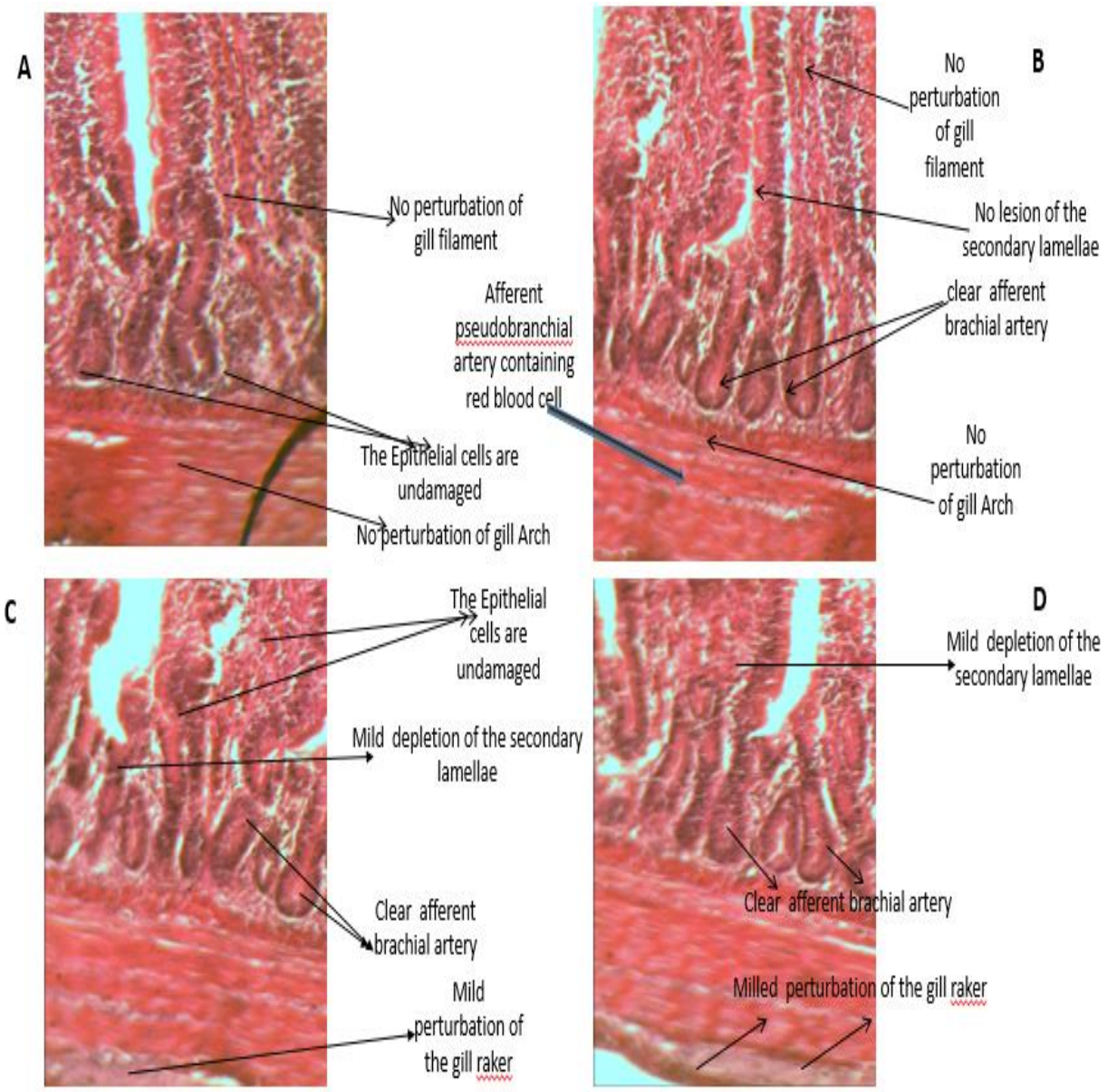

Plate 3: Cross section of *C. gariepinus* gill after treatment with methanolic fraction of *Garcinia kola* and ellagic acid (x 400; HandE)

A: Control group B: 100 mg/l of methanolic fraction C: 200 mg/l methanolic fraction D: 300 mg/l 200 mg/l methanolic fraction

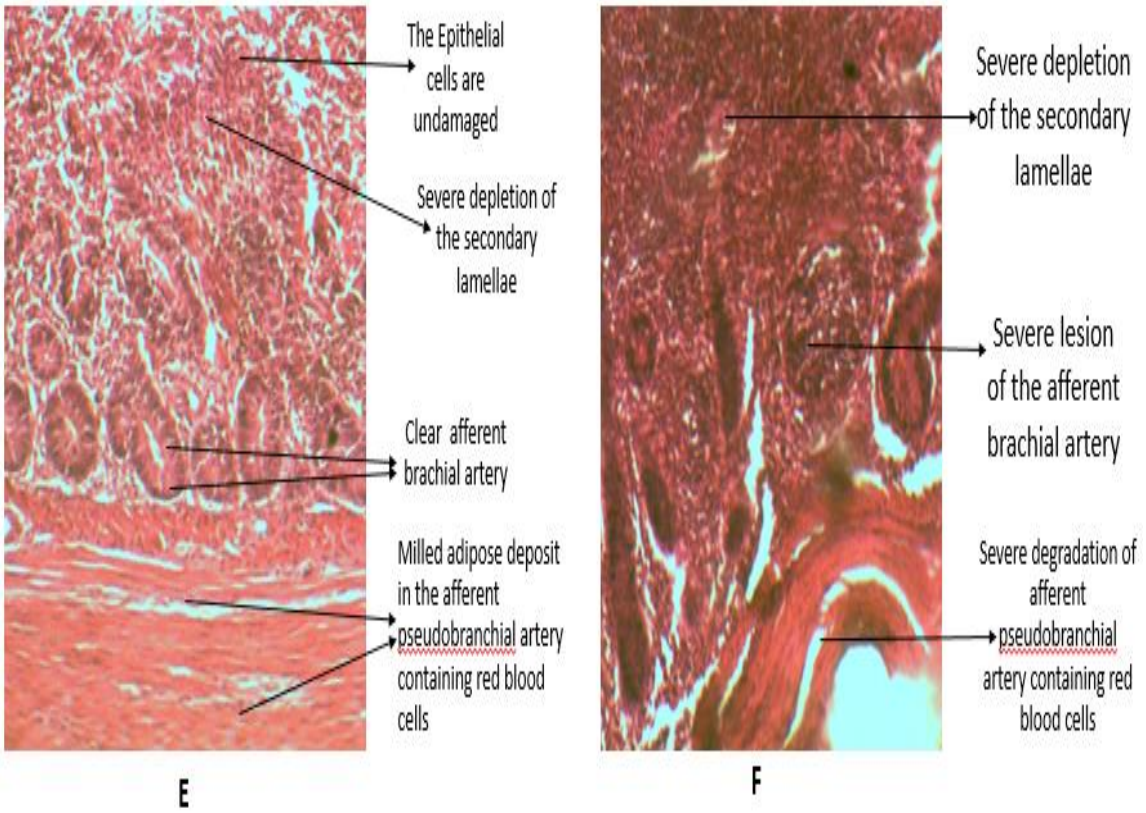

Plate 4: : Cross section of *C. gariepinus* gill after treatment with 400 and 500 mg/l methanolic fraction of *Garcinia kola*(x 400; HandE)
E: 400 mg/l methanolic fraction F: 500 mg/l methanolic fraction

4.10 *In silico* studies

4.10.1 Molecular docking

Molecular docking studies revealed that chlorogenic acid and ellagic acid had a higher binding affinity for acetylcholinesterase (-14.0 and -13.0 respectively) when compared to the standard inhibitor i.e. galantamine (-12.4) (Table 14). Cathechin, quarcetin and luteolin (-12.4, -12.3 and -12.0 respectively) were comparable to galantamine in their binding affinities. 2, 4 dibromophenol (-6.6), apigenin (-11.7), caffeic (-8.6), tacrine (-9.5) and fenthion (-8.8) all recorded a lower binding affinity when compared to standard acetylcholinesterase inhibitor galantamine (Figure 30-41). Hydrogen bonding with SER200, GLU327 was observed in the bonding of all but apigenin to acetylcholineesterase (Table 15) (Figure 42-43).

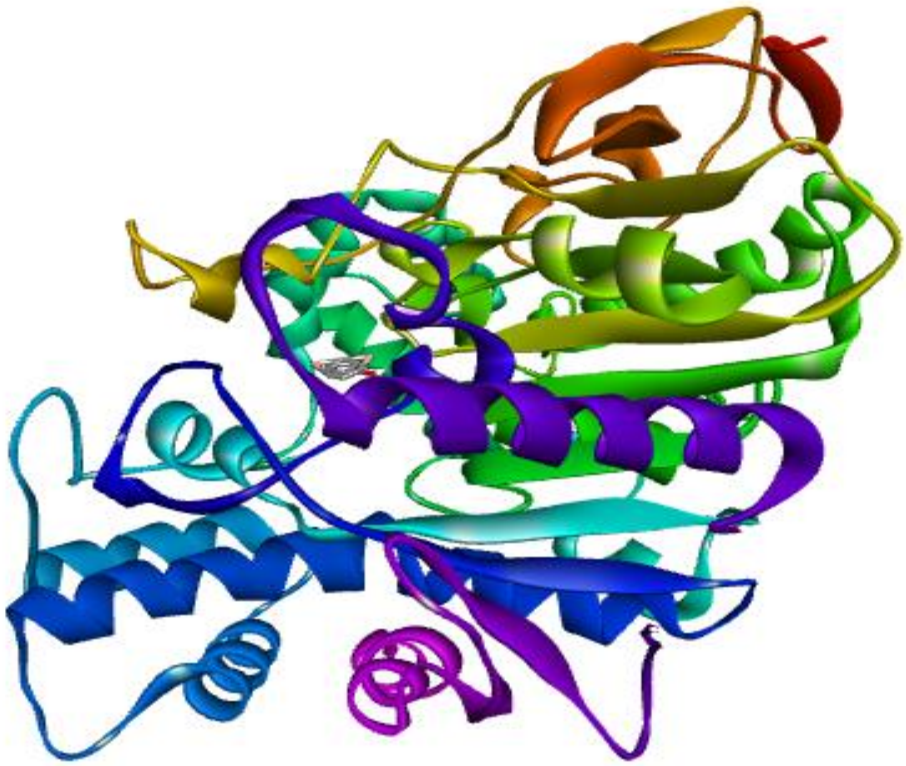

Figure 30: 3D view of the binding of 2, 4 dibromophenol to the active site of acetylcholinesterase

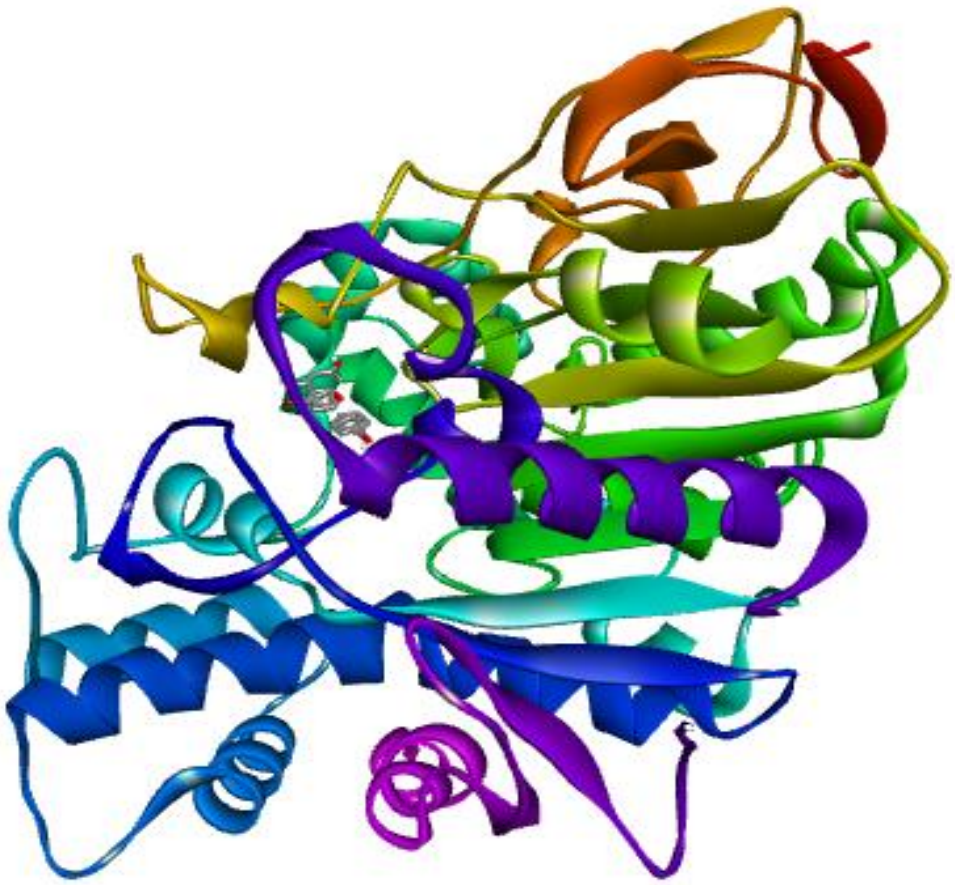

Figure 31: 3D view of the binding of apigenin to the allosteric site of acetylcholinesterase

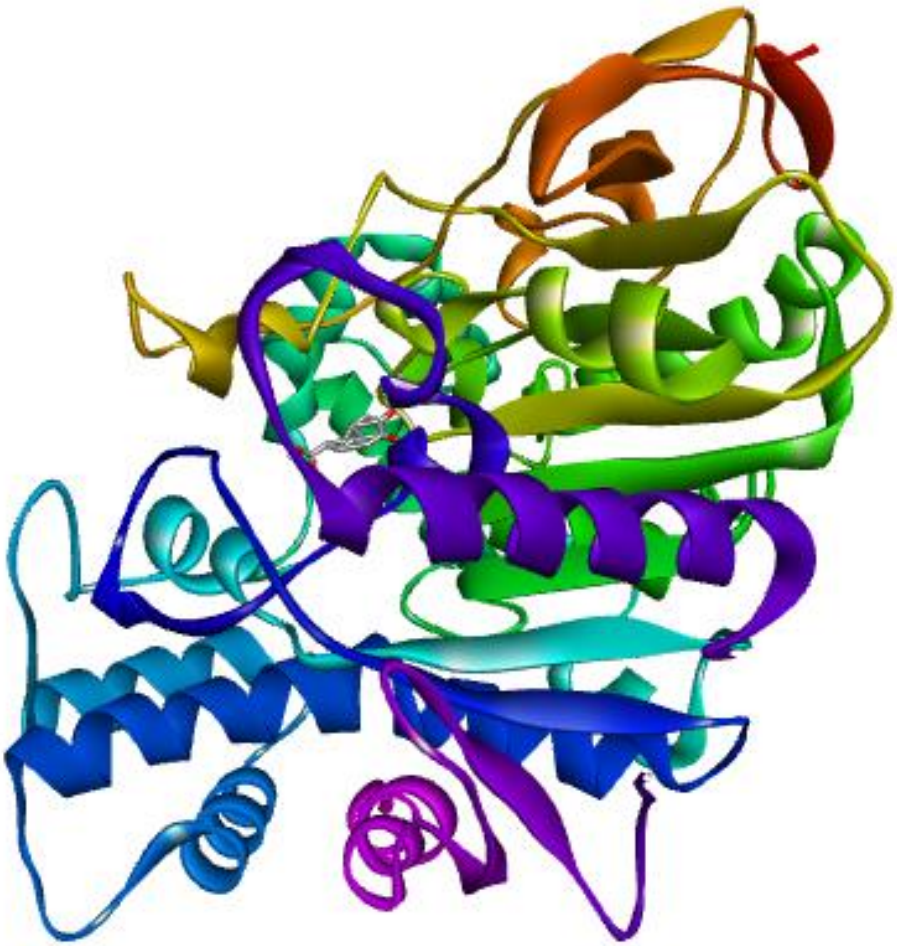

Figure 32: 3D view of the binding of caffeic acid to the active site of acetylcholinesterase

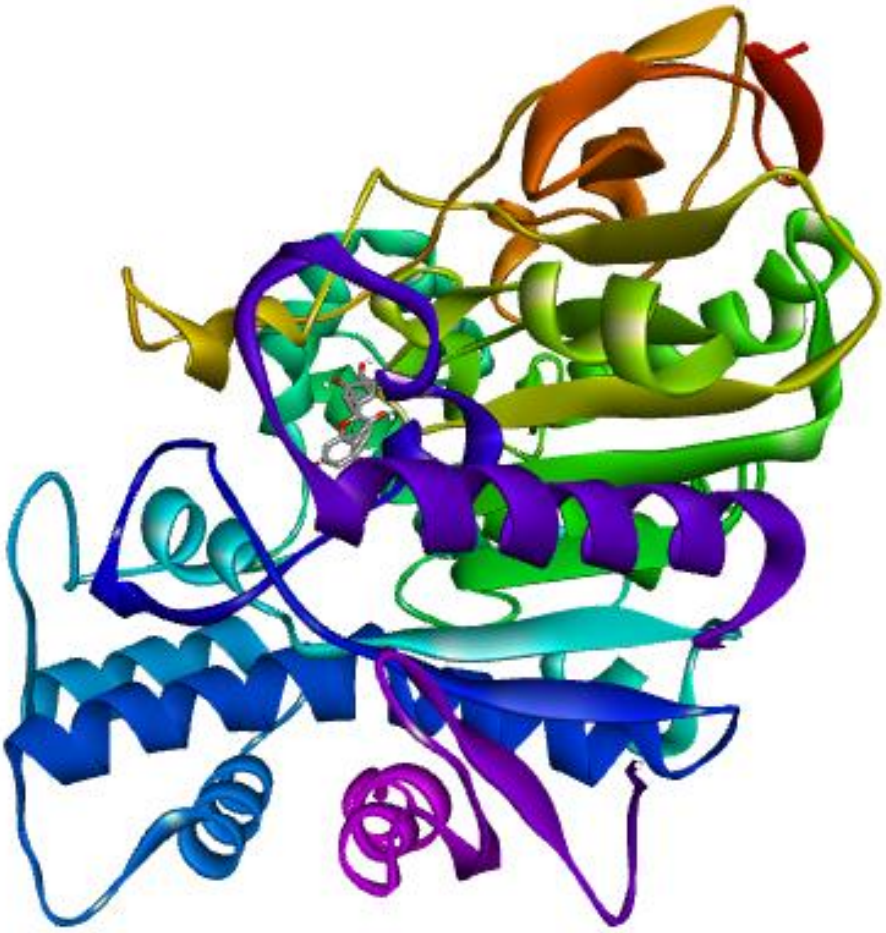

Figure 33: 3D view of the binding of catechin to the active site of acetylcholinesterase

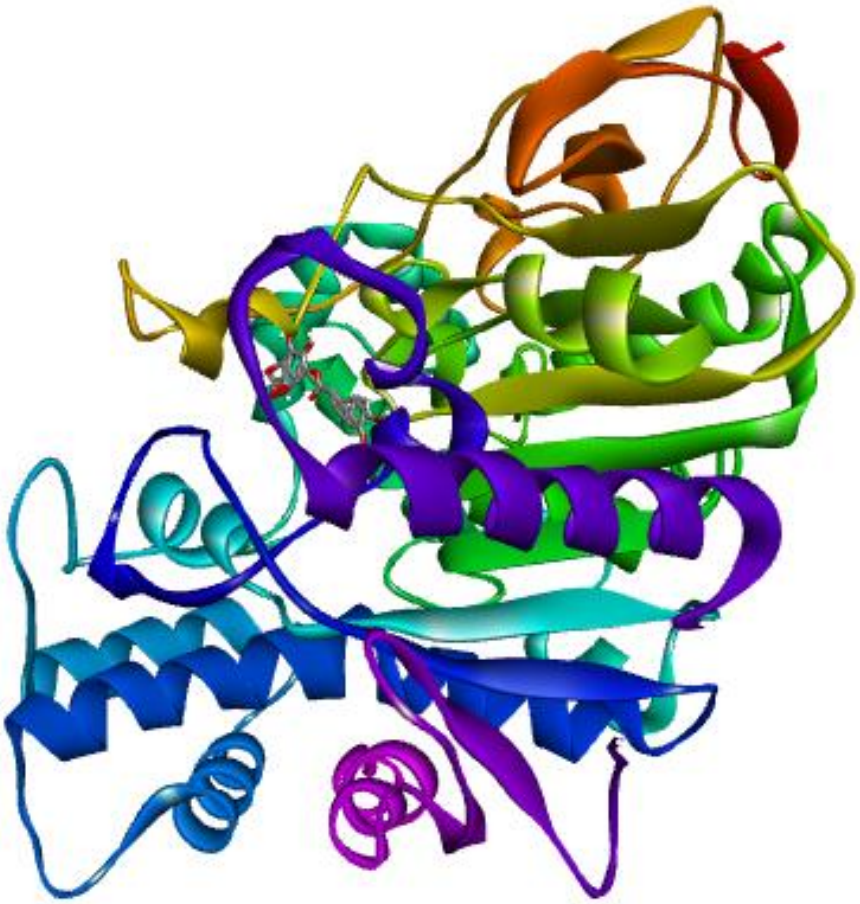

Figure 34: 3D view of the binding of chlorogenic acid to the active site of acetylcholinesterase

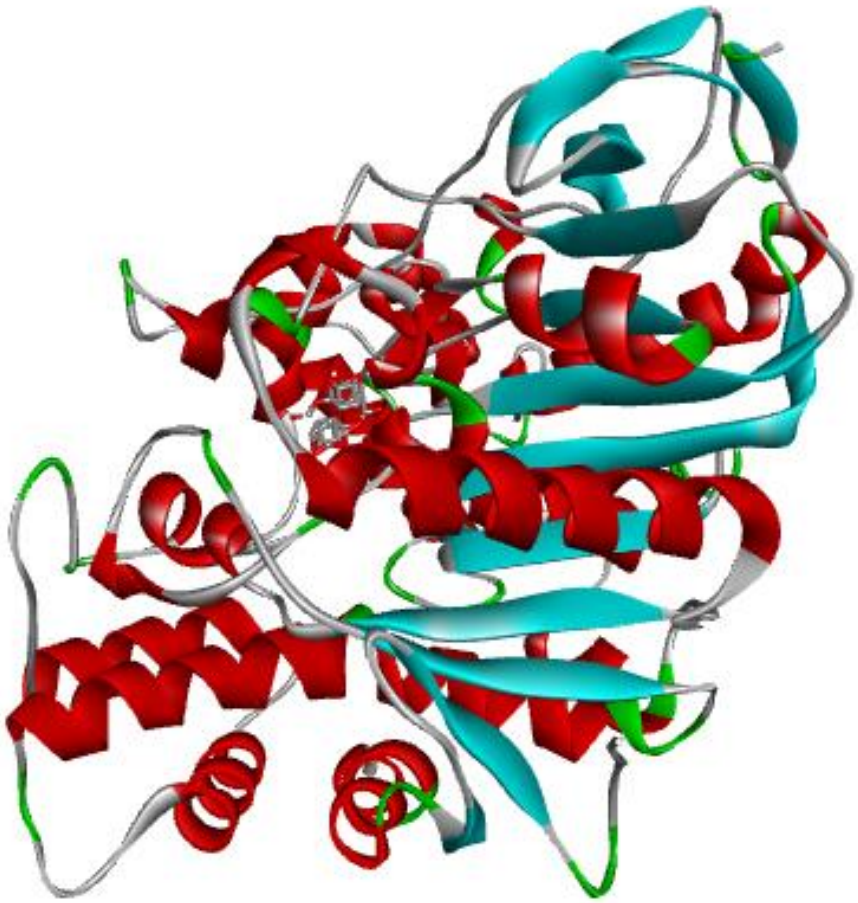

Figure 35: 3D view of the binding of ellagic acid to the active site of acetylcholinesterase

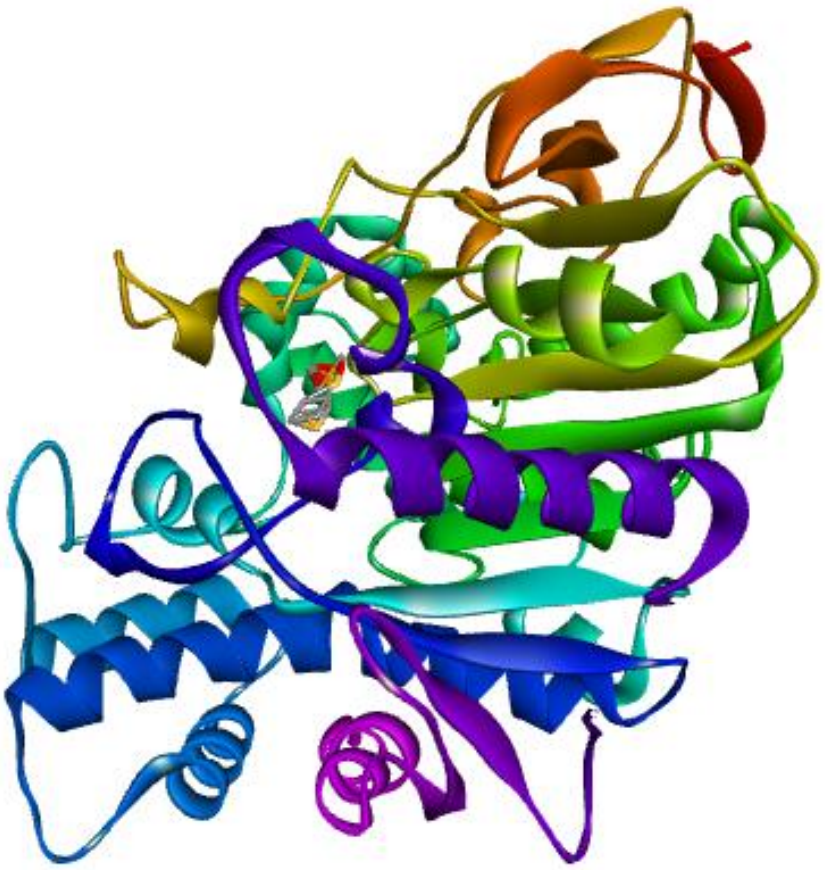

Figure 36: 3D view of the binding of fenthion to the active site of acetylcholinesterase

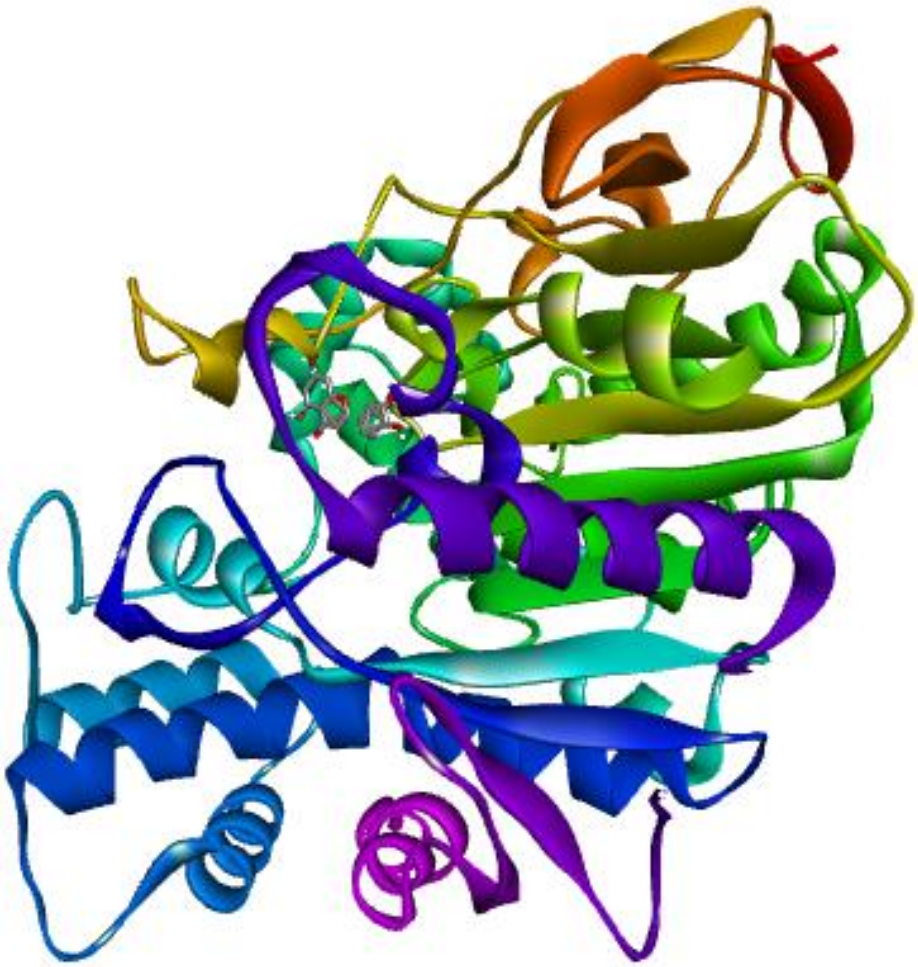

Figure 37: 3D view of the binding of luteolin to the active site of acetylcholinesterase

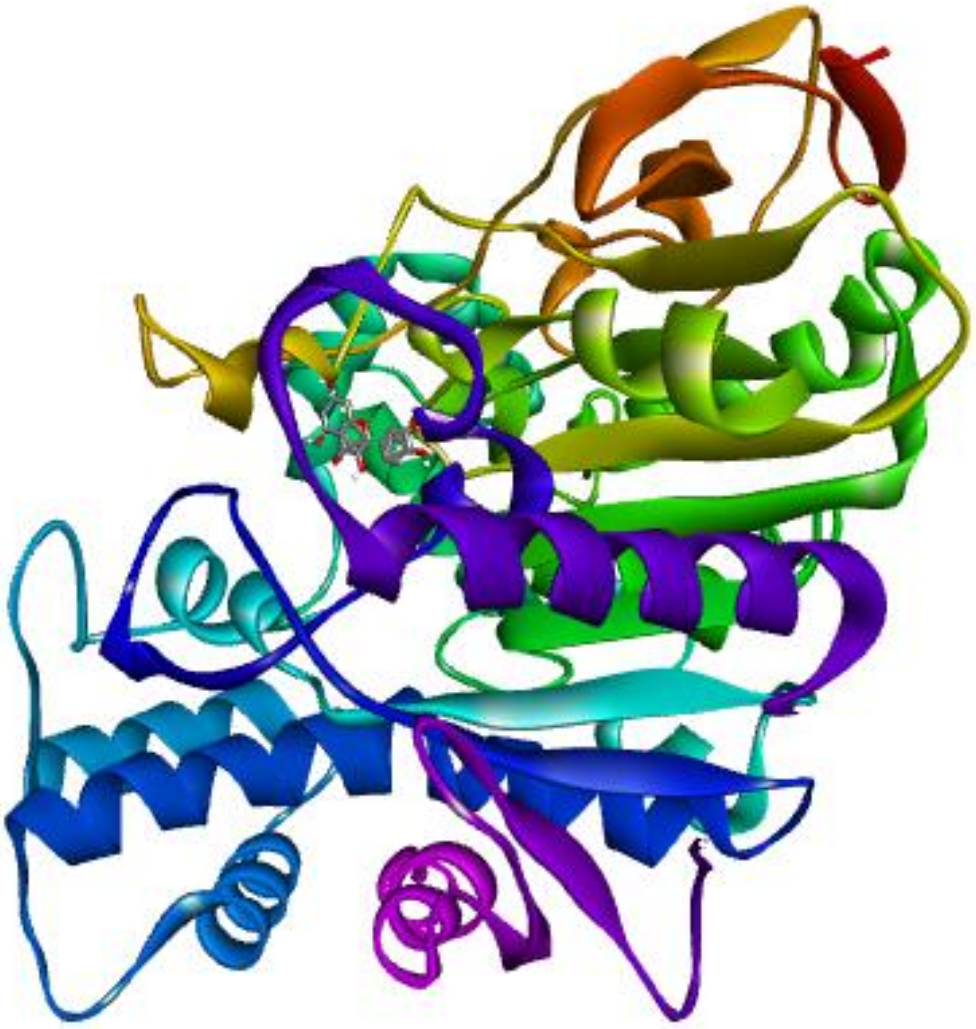

Figure 38: 3D view of the binding of quarcetin to the active site of acetylcholinesterase

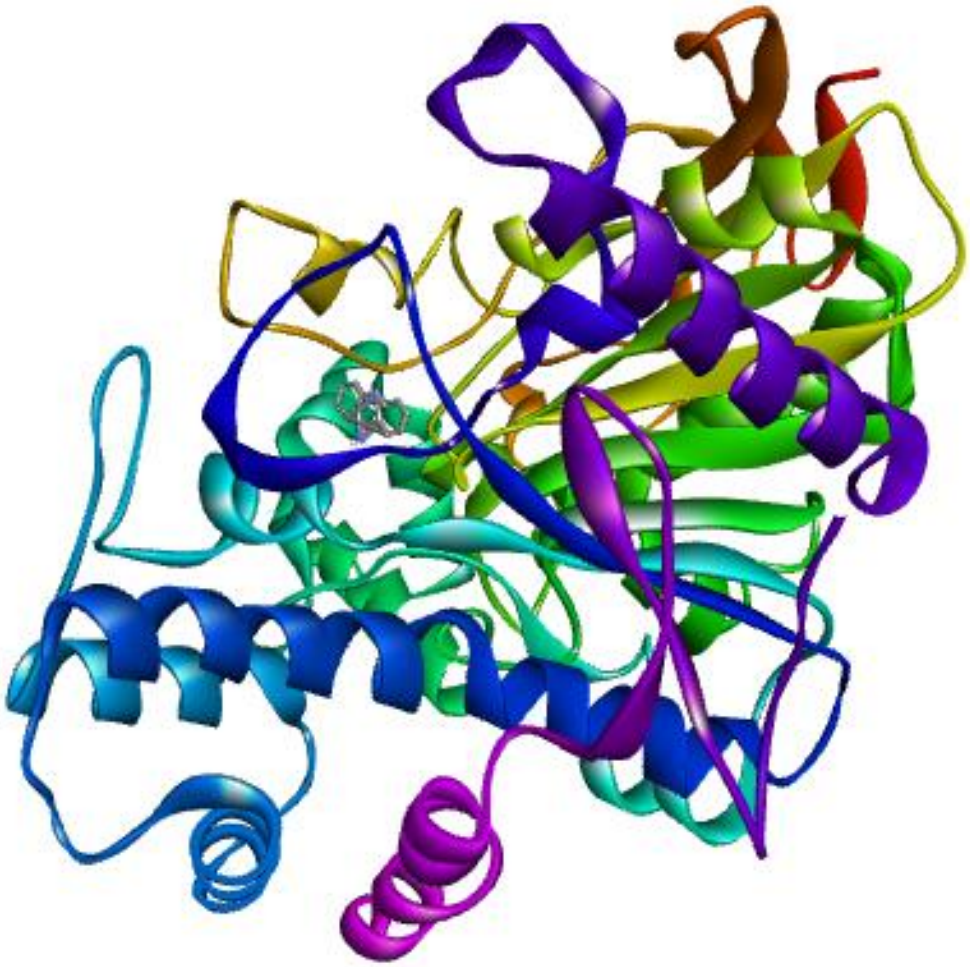

Figure 39: 3D view of the binding of tacrine to the active site of acetylcholinesterase

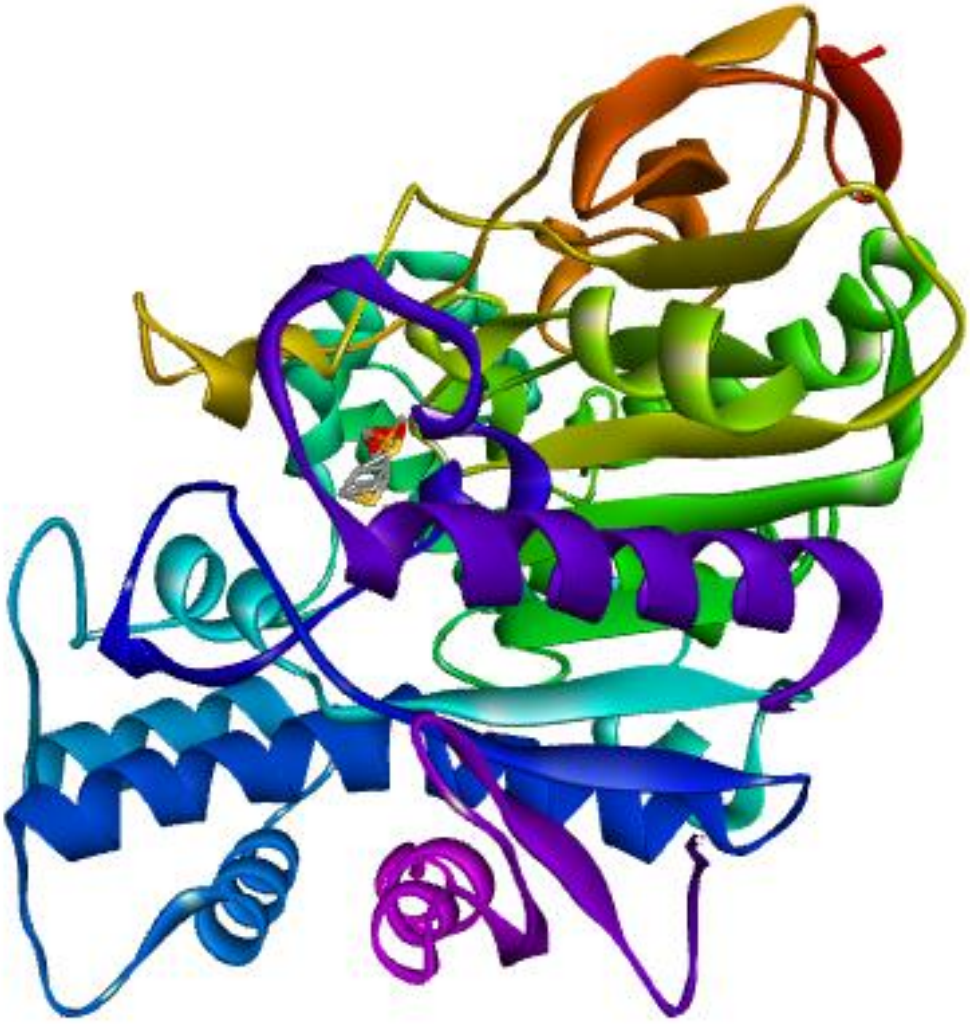

Figure 40: 3D view of the binding of fenthion to the active site of acetylcholinesterase

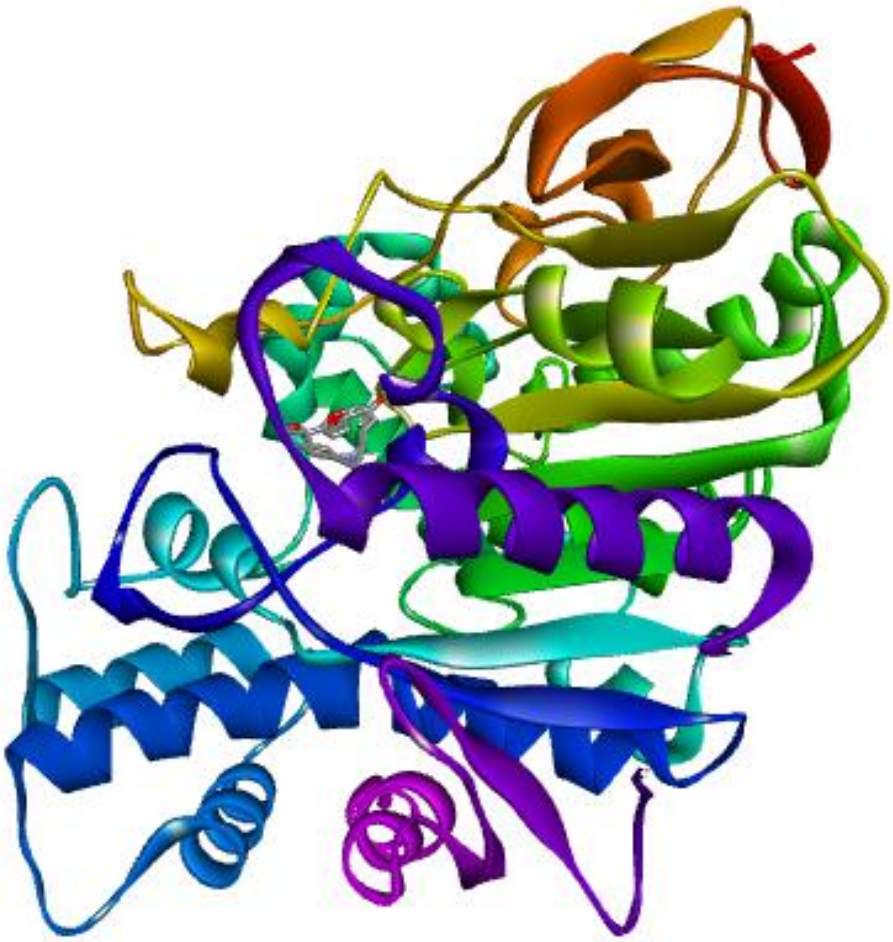

Figure 41: 3D view of the binding of galantamine to the active site of acetylcholinesterase

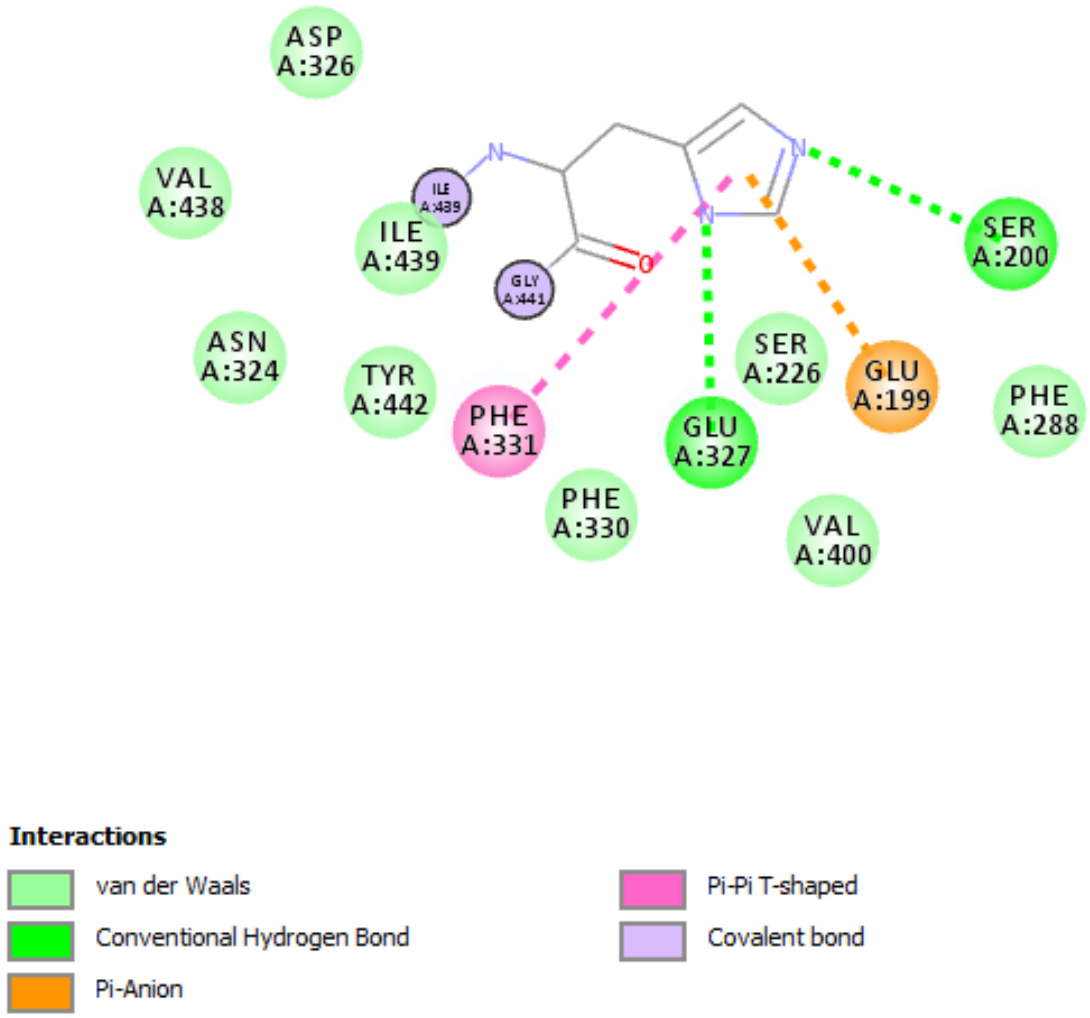

Figure 42: 2D view of the interaction of catechin, chlorogenic acid, ellagic acid, luteolin, quarcetin and galantamine to amino acids in the active loop of acetylcholinesterase

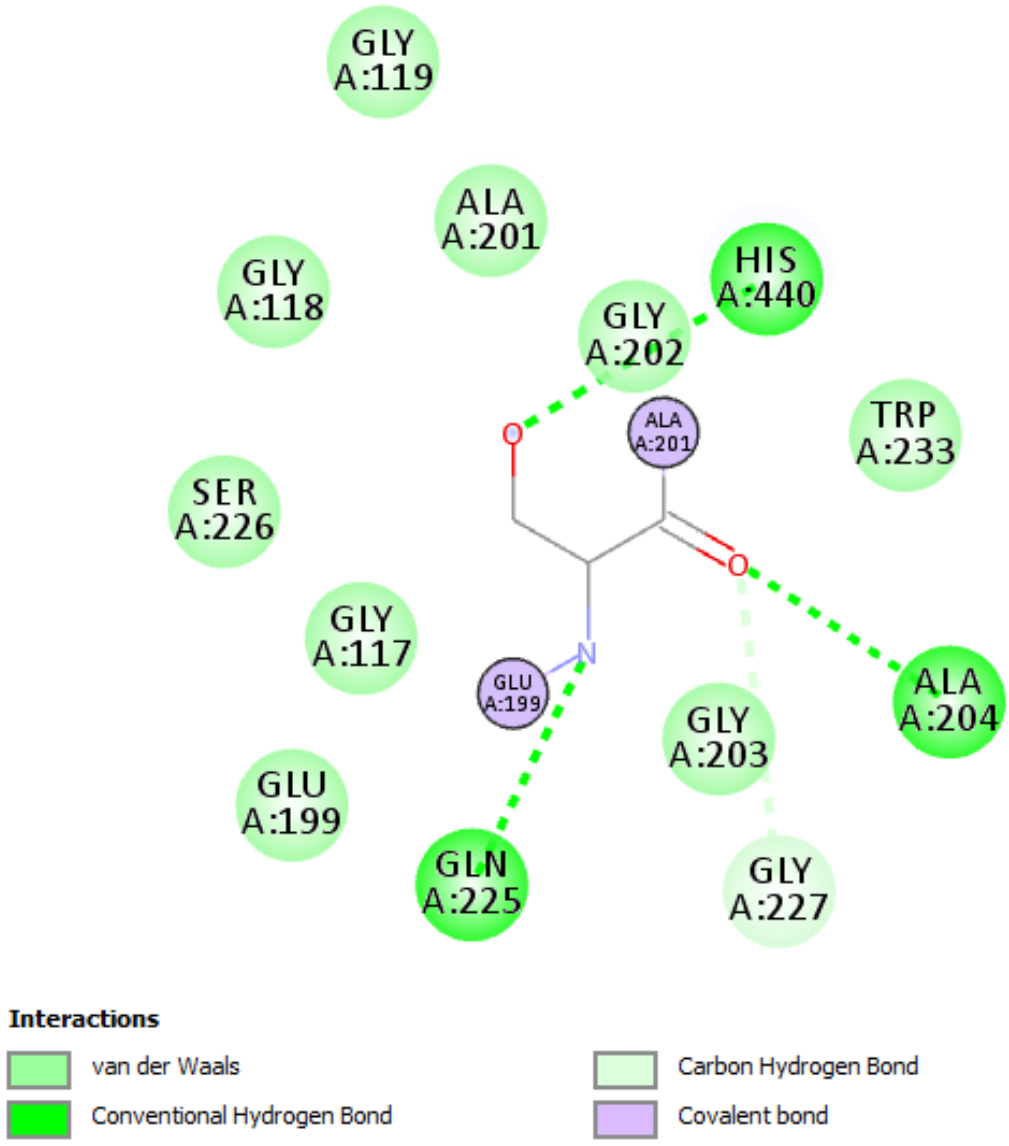

Figure 43: 2D view of the interaction between apigenin and amino acid residues in the allosteric site of acetylcholinesterase

Table 14: Binding Affinities of ligands to acetylcholinesterase

		Binding Affinity (Kcal/mol)
s/n	**Ligands**	**Acetylcholinesterase**
1	2,4-dibromophenol	-6.6
2	Apigenin	-11.7
3	Caffeic acid	-8.6
4	Catechin	-12.4
5	Chlorogenic acid	-14.0
6	Ellagic acid	-13.0
7	Luteolin	-12.0
8	Quercetin	-12.3
9	Tacrine	-9.5
10	Fenthion	-8.8
11	Galantamine	-12.4

Table 15: Amino acid residue interaction with ligands

s/n	**Ligands**	**Amino acid Interaction Acetylcholinesterase**
1	Apigenin	HIS440, ALA204, GLN225, GLY227
2	Catechin	**SER200, GLU327, GLU199, PHE331**
3	Chlorogenic acid	**SER200, GLU327, GLU199, PHE331**
4	Ellagic acid	**SER200, GLU327, GLU199, PHE331**
5	Luteolin	**SER200, GLU327, GLU199, PHE331**
6	Quercetin	**SER200, GLU327, GLU199, PHE331**
7	Tacrine	**SER200, GLU327, GLU199, PHE331**
8	Galantamine	**SER200, GLU327, GLU199, PHE331**

CHAPTER FIVE

DISCUSSION

5.1 Phytochemical constituents of *Garcinia kola*

The qualitative study carried out on the aqueous extract of all the three plants revealed the presence of medicinally active constituents such as alkaloids, phenolics, tannins, saponins, glycosides, saponins, steroids, phlobatanins, cardiac glycosides, cardenolides and chalcones. It has been reported that secondary metabolites produced by plants are responsible for their chemical defense and toxicity to other animals. Several secondary metabolites such as steroids (Bykhovets *et al*., 2002; Chowdhury *et al*., 2008; Ghosh *et al*., 2008; Rahuman *et al*., 2008), phenolics (Tripathi and Rathore, 2001), essential oils (Carvalho *et al*., 2003; Amer and Mehlhorn, 2006; (Rafael *et al*., 2008), alkaloids (François *et al*., 1996; Lee, 2000), etc. have been isolated from plant extracts having good larvicidal properties.

Mosquito larvae of different species display different susceptibilities to the same phytochemicals. In general, *Aedes* larvae are more robust and less susceptible to insecticides and botanical extracts than *Culex* larvae (Shaalan *et al*., 2005). Saponin extracted from the fruit of *Balanites aegyptiaca* showed 100% larvicidal activity against *A. aegypti* mosquito larvae (Chapagain and Wiesman, 2005). It has been suggested that saponin molecules interact with the cuticle membrane of the larvae, ultimately disarranging the membrane could be the most probable reason for the larval death. The deficiency of dissolved oxygen and active presence of the antioxidant saponin molecule might be the reason for larval death (Morrissey and Osbourn, 1999). A piperidine alkaloid

from *Piper longum* fruit was found to be active against mosquito larvae of *C. pipiens* (Lee, 2000). Similarly, an alkaloid derived from the tropical vine *Triphyophyllum peltatum* (Dioncophyllaceae), was found to have larvicidal activity against the malaria vector *Anopheles stephensi* (Francois *et al*., 1996).

5.1.1 Bioassay guided fractionation and isolation

Bioassay guided fractionation of *Garcinia kola* seed extracts revealed the presence of catechin, caffeic acid, chlorogenic acid, ellagic acid, quercetin, luteolin and apigenin. Previous studies have identified plant flavonoids as potent larvicides (Perumalsamy *et al*., 2015)

5.2 Proximate content of *Garcinia kola* seeds

Results obtained showed clearly that *Garcinia kola* seed has a high moisture and carbohydrate content. Protein, ash, lipids and crude fibre were also present in subtle amount. Animals require a number of complex organic compounds as added caloric requirements to meet the need for their muscular activities. Carbohydrates, fats and proteins form the major portion of the diet, while minerals and vitamins form comparatively a smaller part. The carbohydrates are main source and store of energy. They are the starting substances for biological synthesis of many compounds. The trace elements, together with other essential nutrients, are necessary for growth, normal physiological functioning and maintenance of life. They must be supplied in the food, since the body cannot synthesize them. Fibre is the portion that provides structural strength and form. Food fibres have been reported to aid absorption of trace elements in the gut and reduce absorption of cholesterol (Vinayaka *et al*., 2009). Dietary fibre plays an important role in decreasing the risks of many disorders such as constipation, diabetes,

cardiovascular diseases, obesity. Although crude fibre enhances digestibility in animals, the presence of high fibre levels in diet can cause intestinal irritation, lower digestibility and overall decreased utilization (Vinayaka *et al*., 2009).

The moisture content influences the rate of food absorption and digestion (Adeagbo *et al*., 2013). Also high moisture content of the fruits indicates high water retention capacity and could therefore easily be spoiled by fungi if not consumed on time

5.3 Mineral composition of *Garcinia kola* seed

Result obtained from this study reveals that *Garcinia kola* is rich in various minerals with calcium (Ca) being the most abundant followed by potassium (k) and sodium (Na). Magnessium (Mg), zinc (Zn), copper (Cu), Iron (Fe) and manganese (Mn) were also present in considerable amounts. Mineral elements also play important roles in health and disease states of humans (Ugbaja *et al*., 2017). Both Ca and Mg are chiefly found in the skeleton. In addition to its structural role, Mg also activates enzymatic processes. Calcium and phosphorous containing substances are required by children, pregnant and lactating woman for bones and teeth development (Sodamade *et al*., 2013). Na and K are required to maintain osmotic balance of body fluid, the pH of the body, regulation of muscle and nerve irritability, control glucose absorption and enhance normal retention of protein during growth. Fe is an essential component in the transfer of oxygen (a Component of cytochromes) and is the element most closely associated with anaemia. Mn activates enzymes involved in the transfer of phosphate and hydroxyl groups as well as some dehydrogenation reactions. Manganese is required for building immune system; regulation of cellular growth and acts as a co enzyme for carbohydrates, protein and nucleic acids

metabolism; regulation of blood sugar level and production of energy (Sodamade *et al.*, 2013).

5.4 Larvicidal activity of *Garcinia kola* seeds

Prevention of mosquito population is very important because they transmit a variety of diseases by acting as primary vectors for the pathogens that cause severe infections (Rajesh and Shamsudin, 2017). In this study aqueous and methanolic fractions of *Garcinia kola* seeds was found to be effective for mosquito larva control as indicated by the rate of mortalities recorded. The 4th fraction of the aqueous and the 5th fraction of the methanolic extract caused a 96.92 and 89.72% mortality rate respectively. Also, the group treated with the 3rd and 5th fraction of the methanolic extract, the 6th fraction of the aqueous extract were toxic to the mosquito larva. The observed larvicidal activities may be due to the secondary metabolites as phytochemical analysis of the plant extracts reveals the presence of several bioactive secondary metabolites that singly or in combinations may be responsible for the larval toxicity. Several groups of phytochemicals such as alkaloids, steroids, terpenoids, essential oils and phenolics from different plants have been reported previously for their insecticidal activities (Shaalan *et al*., 2005). The high mortality recorded for the larvae of the insect could also be related to the active feeding of the larvae. During feeding, the larvae must have ingested some active compounds in the oil extract of the plants, thereby leading to stomach poisoning (Ileke *et al*., 2014). The result from this study are in accordance with the findings of (Raji and Akinkurolere, 2010; Akinkurolere *et al*., 2011; Ghosh *et al*., 2012); Ileke *et al*., 2014; (Dinesh *et al*., 2015); Yousaf and Zuharah, 2015); who reported the toxicity of some indigenous plants extracts on developmental stages of mosquitoes.

5.5 Acetylcholinesterase Inhibitory effect of *Garcinia kola* seed fractions

Disruption of synaptic transmission is mainly brought about by the inhibition of acetylcholinesterase, a major mode of action for most insecticides especially organophosphates and carbamates (Alout *et al.*, 2012). The methanolic fraction of *Garcinia kola* seeds effectively inhibited acetylcholinesterase although not at the same rate with the compounds isolated from the fraction. Acetylcholinesterase inhibitory activity of the methanolic fractions may be due to alkanols and phytosterol in the extract which interfere with the normal functioning of mosquito cholinergic neuronal transmission by inhibiting AChE. Both the compounds were significantly effective against larval stages of mosquitoes than the adult stages, which is advantageous considering the fact that it is easier to target larvae (Gade *et al*., 2017).

The modes of insecticidal action of naturally occurring compounds are mainly due to AChE inhibition and interference with the octopaminergic system (Isman, 2006). In this study, ellagic acid was identified as the most potent inhibitor of AChE. Studies on intrahippocampal amyloid beta-microinjected rats by Kiasalari *et al*. (2017) reported the inhibitory effect of ellagic acid on acetylcholinesterase activity.

5.6 *In vivo* antioxidant studies

5.6.1 Superoxide dismutase Activity (SOD)

SOD deals with the superoxide radical by alternately adding or removing an electron from the superoxide molecules it encounters, thus changing the O_2^- into one of two less damaging species: either molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) (Zelko *et al*., 2002). The significant increase in SOD concentration in the group treated with

methanolic fraction of *Garcinia kola* when compared to the control may be due to the presence of polyphenols in the extract. Joshua *et al*. (2017) reported that administration of *G. kola* reduced the tissue damage induced by H_2O_2 in a dose-dependent manner and hence, significantly ($p < 0.05$) increased the activities of CAT and SOD.

5.6.2 Catalase (CAT)

Catalases are enzymes present in the peroxisomes of nearly all aerobic cells protecting the cell from the toxic effects of hydrogen peroxide by catalyzing its decomposition into molecular oxygen and water without the production of free radicals (Chelikani *et al*., 2004). The increase in catalase activity recorded in the methanolic fraction of *Garcinia kola* might be as a result of the stress imposed on *C. gariepinus* due to prolonged administration, it could also be due to the presence of flavonoids in the fraction. CAT collaborates with the removal of H_2O_2 formed during the reaction catalyzed by SOD (Liu *et al*., 2010). It has been reported that the increased activity ofendogenous antioxidant enzymes served as protective responsesto eliminate reactive free radicals (Celik and Suzek, 2009). The increase in catalase activity recorded in the ellagic acid and quercetin treated group may be due to the presence of hydroxyl group in their respective structures as flavonoids can prevent oxidative damage as a result of their ability to scavenge ROS, such as hydroxyl radical and superoxide anion (Galati *et al*., 2002).

5.6.3 Glutathione peroxidase

Glutathione peroxidase are known to directly reduce and eliminate intracellularly occurring hydrogen peroxide and lipid peroxides and to play the very important role in the protection against oxidative stress (Nagata *et al*., 1999). The significant reduction in

glutathione peroxidase activity after treatment with the methanolic fraction of *Garcinia kola* could be as a result of utilization of the enzyme in combating the oxidative stress imposed due to prolonged administration of the methanolic fraction. This could also be the reason why the activity of glutathione peroxidase is reduced in quercetin treated group when compared to the control.

5.6.4 Malondialdehyde Concentration (MDA)

Malondialdehyde is a product lipid peroxidation of polyunsaturated fatty acids (Davey *et al*., 2005). Increased level of MDA may reflect the degrees of lipid peroxidation injury in tissues (Jia *et al.*, 2014; Sinhorin *et al*., 2014). The significant reduction in MDA concentration in the ellagic acid treated group may be as a result of the antioxidant properties of ellagic acid. Also the significant reduction in MDA concentration in the group treated with methanolic fraction of *G. kola* when compared to the control may be due to the synergestic effect of the numerous polyphenols present in the extract. Insecticides may induce oxidative stress, leading to generation of free radicals and alterations in antioxidants or reactive oxygen species (ROS) scavenging enzymes (Bagchi *et al*., 1995; Ahmed *et al*., 2000; Gultekin *et al*., 2000) . Studies suggest that LPO is one of the molecular mechanisms involved in OPIs-induced toxicity (Baghci *et al.*, 1995). The significant increase in MDA concentration after 60-day treatment with fenthion may be as a result of fenthion induced oxidative stress.

5.7 Toxicological studies

5.7.1 Haematological Parameters

Evaluation of haematological parameters can be used in determining the effect of extracts/drugs on the blood; any change observed can be used to explain their effects on the functions of blood and its various components. The extract fraction did not significantly alter WBC count which suggests that the methanolic fraction of *G. kola* possess no immune modulatory effect (Balogun *et al*., 2014). Also, no significant difference was observed in the RBC, HCT, MCH, MCHC and HGB. This suggest that the extract fraction may not induce haemolysis. However, the significant increase in platelet count recorded in the after treatment with 100 mg/kg bw of methanolic fraction of *G. kola* suggests that the extract fraxtion may stimulate thrombosis.

5.7.2 Lipid profile

Alteration in the concentration of these lipids can give useful information to the predisposition of subjects to atherosclerosis and coronary heart disease (Abolaji *et al*., 2007). The significant increase in HDL-c in methanolic fraction treated group may be advantageous since HDL-c is responsible fo the transportation of cholesterol from the pheripheral tissues to the liver for metabolism. HDL-c inhibits oxidation of LDL by transition metal ions and also prevents 12-lipoxygenase-mediated formation of lipid hydroperoxides (Nofer *et al*., 2002).

5.7.3 Liver Function Indices

Albumin is the most abundant serum protein is produced in the liver. Albumin in conjunction with other plasma proteins cannot diffuse through the thin capillary wall membranes because they are large colloidal molecules. They are thus entrapped in the vascular system and exert a colloidal osmotic pressure which serves to maintain a normal blood volume. No significant alteration was observed upon treatment with the methanolic fraction of *Garcinia kola* which suggests that the extract may not alter the secretory ability and functional capacity of the liver.

Bilirubin is the main bile pigment that is formed from the breakdown of heme in red blood cells. The serum bilirubin concentration is considered a true test of liver function as it reflects the liver's ability to take up, process and secrete bilirubin. The non significant difference in bilirubin concentration suggests that methanolic fraction of *Garcinia kola* may not induce haemolysis.

5.7.4 Kidney Function Indices

Renal function indices are used to assess the normal functioning capacity of the different parts of the nephron (Guyton and Hall, 2000). In this study, the administration of the methanolic fraction of *G. kola* significantly increased uric acid concentration. This suggests that the extract may not adversely affect the may enhance nucleotide catabolism at higher concentrations. Moderate increase in uric acid has been reported to contribute to the antioxidant defence system in the blood (Ou *et al*., 2017). Increase in urea concentration suggests that the extract may stimulate the excretory function of the kidney. The significant increase in potassium ion concentration after prolonged administration of

the extract fraction suggest that the extract may induce hyperkalamia. Also the significant increase in sodium ion concentration suggest that the extract fraction may predispose subjects to hypernatramia after prolonged administration. Electrolyte imbalance may also be due to the ability of the extract fraction to inhibit Na^+/K^+ ATPase

5.7.5 Enzyme activities

Determination of enzyme activities in tissues and body fluids plays a significant role in disease investigation and diagnosis because it provides information on the effect and nature of pathological damage to tissues.

Alkaline phosphatase (ALP), is a membrane-bound enzyme that catalyze the hydrolysis of phosphate esters. It is most significantly used as a marker enzyme for plasma membrane integrity. The result obtained from this study revealed that treatment with the methanolic fraction of *G. kola* and various ligands did not significantly alter ALP activities in the serum. This suggests that the integrity of the plasma membrane is not compromised upon treatment with various compounds.

AST and ALT are two closely related enzymes of clinical significance in assessment of liver function. They are normally localized within the cells of the liver, heart, kidney, gills, muscles and others. They are considered to be sensitive indicators of hepatocellular damage and within limit can provide a quantitative evaluation of the degree of damage to the liver. The significant reduction in AST and ALT activities after treatment with methanolic fraction of *G. kola* may be as a result of the inhibition of the enzyme in situ since the integrity of the plasma membrane is not compromised.

5.7.6 Molecular docking

The high binding affinity to acetylcholinesterase recorded by chlorogenic acid, ellagic acid, catechin, quercetin and luteolin when compared to galantamine (standard acetylcholinesterase inhibitor) may be due to hydrogen bonding to amino acid residues in the active site of acetylcholinesterase (SER200 and GLU327). Pang (2014) reported that the structural arrangements of the catalytic site of acetylcholinesterase which consist of three adjacent serine, histidine, and glutamate residues (via a catalytic triad) with a hydrogen bond network that increases the nucleophilicity of the serine hydroxy group for hydrolysis of the substrate ACh confer high catalytic efficiency and make AChE one of the most efficient enzymes. Current anticholinesterase insecticides target the catalytic site, although inhibition kinetics studies have suggested that the mechanism of action for some anticholinesterases might involve binding at the peripheral site (Dou *et al.*, 2013).

SUMMARY OF THE FINDINGS

The results obtained from this study suggests that *Garcinia kola* seed fractions:

- Possesses secondary metabolites such as alkaloids, tannins, phenolics, glycosides, saponin, phlobatanins, chalcones, cardenolics, steroids and cardiac glycosides
- Possesses larvicidal activites
- May have the in hibition of acetylcholinesterase as its principal mechanism of larvicidal activities.

- *Garcinia kola* seed fractions inhibited the activities of Na^+/K^+ ATPase and Acetylcholinesterae Activities.
- *Garcinia kola* seed fractions did not significantly alter serum albumin and bilirubin concentration.
- *In silico* screening suggests that chlorogenic acid, ellagic acid and catechin are potent inhibitors of mosquito larve acetylcholinesterase
- The administration of *GKS* extracts had no toxic effect on the Liver, brain and kidney function parameters, of *Clarias gariepinus* taking the water

CONCLUSION

The study concluded that methanolic fraction of *Garcinia kola* seed possesses larvicidal activity and could effectively inhibit acetylcholinesterase as its principal mechanism of action. The increase in cholinergic response may cause uncontrollable muscle contraction in the larva and its death.

RECOMMENDATIONS

Synergistic approaches such as application of mosquito predators with botannical blends and microbial larvicides will provide a better effect in reducing the vector population and the magnitude of epidemiology. Therefore, further studies should be conducted on combination of various fraction to see if there is a synergistic effect on larvicidal activities.

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APPENDIX I