HYPOLIPIDEMIC EFFECT OF BIFLAVONOID FRACTION FROM ROOT BARK, STEM BARK AND SEED OF *GARCINIA KOLA* ON POLOXAMER 407 INDUCED HYPERLIPIDEMIC RATS

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JULY, 2014

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DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA,
NIGERIA.

JULY, 2014

DECLARATION

I declare that this thesis entitled "**Hypolipidemic Effect of Biflavonoid Fraction from Root Bark, Stem Bark and Seed of *Garcinia kola* on Poloxamer 407 Induced Hyperlipidemic Rats**" is a record of my own research work under the supervision of Prof. D.A. Ameh and Dr. D.B. James.

The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this project has been presented elsewhere for the award of a degree in any other university.

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M.Sc./SCIE/00319/2010-2011	Signature	Date

CERTIFICATION

This thesis titled "HYPOLIPIDEMIC EFFECT OF BIFLAVONOID FRACTION FROM ROOT BARK, STEM BARK AND SEED OF *GARCINIA KOLA* ON POLOXAMER 407 INDUCED HYPERLIPIDEMIC RATS" meets the regulations governing the award of the degree of Masters in Science of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to God Almighty for bringing me thus far.

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ABSTRACT

Phytopreventive and phytotherapeutic effects of *Garcinia kola* (root bark, stem bark and seed) biflavonoid fractions in Poloxamer 407 (P407) induced hyperlipidemic rats were studied using standard methods. The total flavonoid content of root bark (63.99±2.94 mg quercetin equivalent/g of dry fraction) was significantly (p<0.05) lower compared to stem bark (96.54±1.70) and seed (197.29±0.64) biflavonoid fractions. The phytopreventive study showed a significant (p<0.05) reduction in serum total cholesterol (TC), triacylglycerol (TG) and low density lipoprotein cholesterol (LDL-c) concentrations of all treated groups when compared with hyperlipidemic control while the root bark and stem bark biflavonoid fractions significantly (p<0.05) increased high density lipoprotein cholesterol (HDL-c) when compared to all other groups. Atherogenic risk predictor indices showed significant (p<0.05) increase in HDL-c/TC ratio and significant (p<0.05) decrease in LDL-c/HDL-c and log (TG/HDL-c) ratios in all biflavonoid treated groups when compared to hyperlipidemic control. Aspartate aminotransferase (AST) was the only serum liver damage indicator enzyme significantly (p<0.05) reduced by root bark biflavonoid fraction when compared to hyperlipidemic control. Liver function tests showed total bilirubin (TB) level was significantly p<0.05) increased while albumin (ALB) was significantly (p<0.05) decreased in hyperlipidemic rats compared to normal rats. However all induced treated groups had significantly (p<0.05) lower TB level while ALB was not significantly (p>0.05) changed when compared to hyperlipidemic and normal control. Haematological assay showed significant (p<0.05) decrease in platelet count of all treated groups when compared to hyperlipidemic control as well as a significant (p<0.05) increase in neutrophils of all induced groups compared to normal control. All biflavonoid fractions significantly

($p<0.05$) reduced liver and spleen weights while root bark and stem bark fractions significantly ($p<0.05$) reduced heart weight compared to hyperlipidemic control but only the root bark fraction significantly ($p<0.05$) increased body weight change compared to all other groups. In the phytotherapeutic study, atorvastatin and all biflavonoid fractions also significantly ($p<0.05$) reduced TC, TG and LDL-c concentrations compared to hyperlipidemic control while only the seed biflavonoid fraction significantly ($p<0.05$) increased HDL-c compared to normal control. All treated groups had significantly ($p<0.05$) higher HDL-c/TC and significantly ($p<0.05$) lower LDL-c/HDL-c but only the seed fraction and the standard drug significantly ($p<0.05$) lowered log (TG/HDL-c) when compared to hyperlipidemic control. Analysis of liver damage indicators showed only AST was significantly ($p<0.05$) reduced by stem bark biflavonoid fraction when compared to hyperlipidemic control. Liver function test also showed TB level of treated groups was significantly ($p<0.05$) reduced and ALB level was not significantly ($p>0.05$) changed when compared to hyperlipidemic control. Haematological assay showed significant ($p<0.05$) reduction in platelet count of treated groups compared to hyperlipidemic control and significant ($p<0.05$) increase in red blood cell (RBC) count of seed biflavonoid treated group compared to normal control. Spleen weight was significantly ($p<0.05$) reduced in all treated groups compared to hyperlipidemic control while only the seed significantly ($p<0.05$) reduced body weight change compared to normal control.

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LIST OF ABBREVIATIONS

4-AAP	4-aminoantipyrine
ADP	Adenosine diphosphate
ALB	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
APG	Angiosperm Phylogeny Group
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
cAMP	Adenosine 3',5' cyclic-monophosphate
CB	Conjugated Bilirubin
CDM	Cellular Differentiation Marker
CHD	Coronary Heart Disease
CM	Chylomicron
CO	Cholesterol oxidase
CVD	Cardiovascular Disease
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCR2	Chemokine (C-X-C motif) ssreceptor 2
DB	Direct Bilirubin
DSBmT	N-N-bis(4-sulphobutyl)-*m*-toludine-disodium
FBG	Fasting Blood Glucose
g/dl	gram per decilitre
GB1	Garcinia Biflavanone one
GB2	Garcinia Biflavanone two

Hb	Haemoglobin
HCl	Hydrochloric acid
HDL	High Density Lipoprotein
HDL-c	High Density Lipoprotein Cholesterol
HMG CoA	3 hydroxy-3-methylglutaryl Coenzyme A
IDL	Intermediate Density Lipoprotein
KOH	Potassium Hydroxide
KV	Kolaviron
LD_{50}	Median Lethal Dose
LDL	Low Density Lipoprotein
LDL-c	Low Density Lipoprotein Cholesterol
LPL	Lipoprotein lipase
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
mg/dl	Milligram per decilitre
ml	Millilitre
MSG	Monosodium Glutamate
NaOH	Sodium Hydroxide
NAPQI	N-acetyl-p-benzo-quinoneimine
NEFA	Non esterified fatty acids
NITR	National Institute for Trypanosomiasis Research
P407	Poloxamer 407
PAI	Plasminogen Activator Inhibitor
PCV	Packed Cell Volume

POD	Peroxidase
RBC	Red Blood Cell
ROS	Reactive Oxygen Species
SD	Standard Deviation
TB	Total Bilirubin
TC	Total Cholesterol
TF	Tissue factor
TG	Triacylglycerol
TP	Total Protein
U/l	International Units per litre
µl	Microlitre
VLDL	Very Low Density Lipoprotein
WBC	White Blood Cell
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

Plants have been used by different countries for centuries as drug substances for relief from illnesses, as health-care products, fragrances, flavors, sweeteners and as materials for pest control (Adesina, 2005). Today, plants appear to be the major source of drugs for majority of the world's population, with substances derived from higher plants constituting about a quarter of all prescribed medicines (Kumar *et al*., 2011a). They contain diverse groups of phytochemicals such as tannins, inulins, alkaloids, flavonoids, saponins and cardiac glycosides that possess enormous medicinal potentials, as such traditional medicine in many areas of the world rely on the use of a wide variety of plant species (Edeoga *et al*., 2005; Koche *et al*., 2010).

More than 2000 plants have been listed in the Traditional (Herbal/Alternative) systems of medicine and some of these are providing comprehensive relief to the people suffering from cardiovascular diseases, like "ischemic heart disease" and its risk factor, "hyperlipidemia". The World Health Organization (WHO) reports indicate that around 80% of the global populations still rely on botanical drugs and several herbal medicines have advanced to clinical use in modern times (Mahmood *et al*., 2010).

Garcinia kola, a dicotyledonous plant in the *Clusiaceae* or *Guttiferae* family is one of such plants used in the treatment of various diseases in Sub-Saharan Africa (Penduka and Okoh, 2011). It produces seeds that are bitter and chewed like kola nuts (Keay *et al*., 1989). In Nigeria, it is found in South Western and South Eastern parts and commonly known as bitter kola due to its bitter taste, *Orogbo* (Yoruba), *Aku ilu* (Igbo) and *Namijin goro* (Hausa).

Traditionally, the seeds are served to visitors during ceremonies in the eastern part of Nigeria (Atawodi *et al*., 1995; Omode *et al*., 1995; Leakey, 2001). Unlike other kola nuts, bitter kola is believed to purge the digestive system, without side effects such as abdominal problems, even when a lot of nuts are eaten (Onochie and Stanfield, 1960). Biological activity reported for different plant part extracts include; antidiabetic activity (Iwu *et al*, 1990a), antihepatotoxic activity (Iwu *et* al., 1987; Akintonwa and Essien, 1990; Braide, 1991; Adaramoye and Adeyemi, 2006a), antimicrobial activity (Okonji and Iwu, 1988; Lutete *et al*., 1994; Sote and Wilson, 1999; Adefule-Ositelu *et al*., 2004; Akerele *et al*., 2008), anti-inflammatory and antipyretic effects (Braide, 1993), antioxidant activity (Arouma *et al*., 1990; Adaramoye *et al*., 2005a) and bronchodilatory effect (Orie and Ekon, 1993).

Hyperlipidemia is one of the greatest risk factors contributing to the prevalence and severity of cardiovascular disease (Micallef and Garg, 2009) and cardiovascular disease is regarded as a leading cause of death worldwide (Murray and Lopez, 1996; WHO 2002). Cardiovascular disease covers a wide array of disorders, including diseases of the cardiac muscle and of the vascular system supplying the heart, brain, and other vital organs (Bentley *et al*., 2002). Predominant cardiovascular diseases associated with hyperlipidemia are hypertension, ischemic heart disease**,** stroke, coronary heart disease and atherosclerosis (Balakumar *et al.*, 2007). These diseases account for at least 80% of the burden of cardiovascular disease in both developing and developed countries (Reddy, 2002), which share many of the same common risk factors.

Hyperlipidemia is characterized by elevated serum total cholesterol, triacylglycerol, low density lipoprotein, very low density lipoprotein and decreased high density lipoprotein

levels. Hyperlipidemia associated lipid disorders are considered to cause atherosclerotic cardiovascular disease (Saravanan *et al*., 2003).

1.1 Statement of Research Problem

Hyperlipidemia contributes significantly in the manifestation and development of atherosclerosis and coronary heart diseases (CHD) (Kumar *et al*., 2011b). Atherosclerosis is a major cause of mortality and morbidity worldwide (Weber and Noels, 2011). According to WHO (2005) report, cardiovascular disease accounts for 11% of total death in Nigeria. Although several factors, such as diet high in saturated fats and cholesterol, age, family history, hypertension and life style play a significant role in causing heart disease, the high levels of cholesterol particularly total cholesterol, triacylglycerol and low density lipoprotein cholesterol is mainly responsible for the onset of CHDs (Choudhary *et al*., 2005). A 20% reduction of blood cholesterol level can decrease about 31% of CHD incidence, and 33% of its mortality rate (Marzyieh *et al*., 2007).

The main aim of treatment in patients with hyperlipidemia is to reduce the risk of developing ischemic heart disease or the occurrence of further cardiovascular disease or cerebrovascular disease (Smith and Pekkanen, 1992). Currently available drugs have been associated with a number of side effects. The consumption of some synthetic drugs leads to hyperuricemic, diarrhoea, nausea, myositis, gastric irritation, flushing, dry skin and abnormal liver function (Brown, 1996; Jeyabalan and Palayan; 2009). Certainly, a plant that has documented antimicrobial, antihepatotoxic, antioxidant as well as an ability to positively affect the immune response would be of considerable clinical interest.

The potential of *Garcinia kola* seed as a hypocholesterolemic agent; a process that is largely due to the activity of its biflavonoids and other bioactive compounds has been reported (Adaramoye *et al*., 2005b) but there is paucity of information on the other parts (root bark and stem bark) as hypolipidemic agents.

1.2 Justification

Flavonoids are natural products found in fruits and vegetables, as well as in beverages such as tea and red wine. Epidemiologic evidence suggests that, consumption of a diet rich in these compounds is associated with a decreased risk of coronary artery disease, cancer, and stroke (Graf *et al*., 2005). Flavonoids are hypothesized to provide cardioprotective effects through their ability to scavenge free radicals and inhibit lipid oxidation. Although most researches on flavonoids have been based on *in vitro* experiments, growing bodies of *in vivo* studies are beginning to provide insight into their biological mechanisms and support for their health benefits (van Hoorn *et al*., 2003).

Kolaviron (KV), the predominant flavonoid in *Garcinia kola* is a biflavonoid complex that has been reported to prevent hepatotoxicity mediated by several toxins (Iwu *et al*., 1987; Adaramoye and Adeyemi, 2006a). It is known to exhibit hypoglycemic effects in normal, alloxan and streptozotocin induced diabetic animals (Iwu *et al*., 1990a; Adaramoye and Adeyemi, 2006b). Also, KV has been reported to elicit strong antioxidant activity in both *in vivo* and *in vitro* experimental models (Adaramoye *et al*., 2005a). A preliminary study by Adaramoye *et al*., (2005b) demonstrated that KV elicited hypocholesterolemic effects and reduced the relative weight of the heart in cholesterol fed animals, thus providing a background to explore the possible hypolipidemic effect of this biflavonoid complex from

the different plant parts (root bark, stem bark and seed) with a view of paving way for the future development of a relatively cheap, available chemical agent or drug with little or no adverse effects.

1.3 Aim

The aim of this study was to investigate the hypolipidemic effects of biflavonoid fractions from different parts (root bark, stem bark and seed) of *Garcinia kola* in experimental animal paradigms.

1.4 Specific Objectives

The specific objectives:

i. To qualitatively determine phytochemical constituents and total flavonoid content in root bark, stem bark and seed fractions of *G. kola*.

ii. To determine the effect of preventive and therapeutic administration of the biflavonoid fractions on lipid-profile and glucose level of the hyperlipidemic rats.

iii. To determine the effect of preventive and therapeutic administration of the biflavonoid fractions on hepatic and renal function of the hyperlipidemic rats.

iv. To determine the effect of preventive and therapeutic administration of the biflavonoid fractions on some haematological parameters of the hyperlipidemic rats.

v. To determine the effect of preventive and therapeutic administration of the biflavonoid fractions on body weight and organ weight of the hyperlipidemic rats.

1.5 Research Hypothesis

H_o: i. The root bark, stem bark and seeds of *G. kola* do not possess biflavonoids.

H_o: ii. Preventive and therapeutic administrations of the biflavonoid fractions have no effect on lipid-profile and glucose level of hyperlipidemic rats.

H_o: iii. Preventive and therapeutic administrations of the biflavonoid fractions have no effect on hepatic and renal function of hyperlipidemic rats.

H_o: iv. Preventive and therapeutic administrations of the biflavonoid fractions have no effect on some haematological parameters of hyperlipidemic rats.

H_o: v. Preventive and therapeutic administrations of the biflavonoid fractions have no effect on body weight and organ weight of hyperlipidemic rats.

CHAPTER TWO

LITERATURE REVIEW

2.1 Medicinal Plants

Throughout the ages people have turned to herbal medicine for healing. All cultures have folk medicine traditions that include the use of plants and plant products (Penduka and Okoh, 2011). Many orthodox drugs used today, originated in the herbal traditions of various cultures, such as the medication commonly used for heart failure, digitalis, which is derived from foxglove (Kumar *et al*., 2011a).

Plants are made up of various constituents that could be a reason for their medicinal purpose and hence raw materials in drug development (Oluyemi *et al*., 2007). These constituents are extracted from plants using various solvents and methods (Iwu *et al*., 1990b; Grigonisa *et al*., 2005; Durling *et al*., 2007; Spigno *et al*., 2007; Michiels *et al*., 2012). Polyphenols, tocopherols, flavonoids, alkaloids, inulin, cardiac glycosides and saponins amongst others are secondary metabolites which are the bioactive compounds contained in plants (Gordana *et al*., 2004; Lee *et al*., 2007).

Among them, flavonoids are particularly attractive as they are known to exhibit various pharmacological properties such as vasoprotection, anti-carcinogenic, anti-microbial, anti-inflammatory, anti-proliferative effects and protection against cardiovascular diseases such as hypertension of which hyperlipidemia is a risk factor (Gordana *et al*., 2004). The health benefits of flavonoids have been linked to their actions as antioxidants and free radical scavengers (Li-chem *et al*., 2006).

The biflavanones are the most dominant flavonoids in most *Garcinia* species (Waterman and Hussain, 1983). Kolaviron (KV), a biflavonoid complex is the predominant constituent in *Garcinia kola* (Iwu *et al*., 1987).

2.2 Bitter Kola (*Garcinia kola*)

Garcinia kola (Heckel), an angiospermae, belonging to the family *Guttiferae* or *Clusiaceae*, is commonly known as bitter kola. On chewing, bitter kola seed has a bitter astringent, sticky and a slight after sweetness. It improves the taste of anything eaten after it (Eisner, 1990). Bitter kola is a highly valued plant in folklore medicine because of its numerous medicinal uses (Eisner, 1990). It is ranked third among medicinal plants in Benin, Nigeria, where it is incorporated in a lot of recipes and has proved to be one of the several non timber products of high socio-economic importance (Igwe *et al.,* 2007).

2.2.1 Botanical Classification

According to the Angiosperm Phylogeny Group (APG) III system of classification, bitter kola belongs to the genus *Garcinia*, a genus with a disputed number of species ranging between 50 and 300 taxa specifically valid from various sources. *Garcinia kola* is one of the most predominant *Garcinia* in Africa.

Family: Guttiferae/*Clusiaceae*

Genus: *Garcinia*

Species: *G. kola*

Species Authority: Heckel

2.2.2 Plant Description

Garcinia kola is a well branched evergreen dicotyledonous plant found in moist rain forests and swamps, it grows as a medium sized tree up to a height of about 12m to 28m, straight bole, unbuttressed, girth up to 1.80m producing reddish yellowish or orange coloured fruits (pods) (Akerele *et al.,* 2008), each fruit containing 2 to 4 brown seeds. The seeds are oval, 3 to 4.5cm long, 1.5 to 2.5cm wide, with a fibrous coat and 5 to 8 furrows or slight ridges, brown (in the fresh state). It is cultivated through seedlings or cuttings, though grows more easily using the cuttings (Adesuyi *et al*., 2012). The flowering of the plant occurs between December and January while the fruits mature between June and August.

Plate I: Fruits and leaves of *Garcinia kola*.

2.2.3 Distribution

Garcinia kola, a wide spread ever green tree is a native of Africa though can grow anywhere especially on moist lands and tropical forest up to 1200m altitude. It is found in

West and Central African countries like; Nigeria, Ghana, Cameroon, Sierra Leone, Togo, Congo Democratic Republic, Angola, Liberia and Gambia (Adesuyi *et al*., 2012). *Garcinia kola* is largely cultivated and highly valued in West and Central Africa for its edible seeds (Hutchinson and Dalziel, 1956). In Nigeria, it is mostly found in south eastern and south western parts and known by various names such as bitter kola, male kola (English name), *orogbo* (Yoruba), *Aku ilu* (Igbo) and *Namijin goro* (Hausa). It is also known as false kola mainly due to the absence of stimulants which characterizes the kola seeds.

2.2.4 Traditional Uses

Garcinia kola is widely chewed in south eastern and south western parts of Nigeria, it is served to guest on regular visits or during ceremonies (Omode *et al*., 1995; Leakey, 2001). Unlike other kola nuts, *Garcinia kola* is believed to purge the digestive system, without side effects such as abdominal problems, even when a lot of the nuts are eaten (Onochie and Stanfield, 1960). It is also used as chewing sticks and as snake repellant when placed around the house (Nair, 1990).

In traditional medicine, the dried root soaked in local gin is taken orally for the treatments of coughs, inflammation, liver cirrhosis, tooth decay, bronchitis and gonorrhea (Adesina *et al*., 1995). The dried pod is used for the treatment of arthritis (Ebana *et al*., 1991). The pod is used externally as an antiseptic for cuts and sore throats (Iwu *et al*., 1990b). The bark of *G. kola* is taken orally for fever, cough, inflammation, respiratory tract disease and as an antihelmintic (Gill and Akinwumi, 1986). Extracts of the seeds had remarkable improvement in liver function of patients with chronic hepatitis and cholangitis after treatment for 14 days at a Nigeria herbal home (Iwu, 1982). It is also used for the treatment

of jaundice, high fever (Iwu, 1993), stomach ache and gastritis (Ajebesone and Aina, 2004). In south western Nigeria, *G. kola* is one of the constituents of traditional recipe used in the management of sickle cell disease (Egunyomi *et al*., 2009).

2.2.5 Biological and Pharmacological Activities

Garcinia kola (different parts and extracts) exhibits a wide range of reported biological and pharmacological activities. *G. kola* possess remarkable antimicrobial (Akerele *et al*., 2008) activity; antilisterial (Penduka and Okoh, 2012) and biocidal activity (Akinpelu *et al*., 2008) as well as broad spectrum antibacterial (gram negative and positive organisms) activity (Adegboye *et al*., 2008). It also has both trypanocidal and trypanostatic effect (Johnson *et al*., 2011; Ogbadoyi *et al*., 2011) which results from synergistic interactions of all or some of its active components interfering with cell cycle progression in the parasite, possibly causing cell cycle arrest, thereby halting cell proliferation and/or the modulation of the animal's immune system consequently enabling the animal withstand the ravaging parasites for a long time.

G. kola inhibits cytochrome P450 which converts paracetamol to N-acetyl-p-benzo-quinoneimine (NAPQI), an intermediate toxic to the liver, hence hepatoprotective (Akintonwa and Essein, 1990) and an ability to reduce kidney dysfunction (Okoko and Awhin, 2007) attributed to its inhibition of lipid peroxidation. Its antimalarial activity is due to the presence of quinone, an antimalarial agent (Kayembe *et al*., 2010).

The contraceptive and fertility control property of *G. kola* (Akpantah *et al*., 2005) are linked to its anti inflammatory activity (Braide, 1993; Madubunyi, 1995) attributed to the presence of flavonoids which inhibits cyclo-oxygenase, thereby causing abnormal follicle

rupture (Gaytan *et al*., 2002), a mechanism similar to that induced by indomethacin, a potent cyclo-oxygenase enzyme inhibitor (Epsey, 1983). Its antisickling property has also been attributed to inherent phytochemicals (Adejumo *et al*., 2011).

G. kola contains a complex mixture of phenolic compounds such as biflavonoids, xanthones and benzophenone known to cause a reduction in glucose level and reactive oxygen species (ROS), hence its antidiabetic (Chandrika *et al*. 2006; Omage *et al*., 2010; Duze *et al*., 2012; Nwangwa, 2012) and antioxidant activity (Farombi *et al*., 2002; Terashima *et al*., 2002; Okoko, 2009); a property crucial to its effectiveness as an anti cancer (tumor) agent (Adaramoye, 2010).

2.3 Biflavonoids

Flavonoids are low molecular weight (Heim *et al*., 2002; Fernandez *et al*., 2006) bioactive polyphenols (Hollman and Katan, 1999) which play a vital role in photosynthesizing cells (Cushnie and Lamb, 2005) but are not essential for the survival of such cells (Buer *et al*., 2010). There are more than 9000 structural variants (Williams and Grayer, 2004) of flavonoids, many of which are responsible for the attractive colors of flowers, fruit, and leaves (de Groot and Rauen, 1998). Flavonoids are present in foods like berries, raspberries, red wine, straw berries, tea, broccoli, lettuce, grapes, olives, kale, onion, citrus fruits, fruit peels and legumes (Middleton, 1998) and are known for their health benefits before successful isolation as effective compounds (Nijveldt *et al*., 2001).

Flavonoids are secondary metabolites characterized by flavan (2-phenyl-benzo-γ-pyrane) nucleus (Heim *et al*., 2002) consisting of two benzene rings (A and B) linked through a heterocyclic pyran ring (C) as shown in Fig 2.2 (Cushnie and Lamb, 2005). They differ in

their arrangement of hydroxyl, methoxy and glycosidic side groups and in the conjunction between A and B rings (Heim *et al*., 2002). A variation in C ring provides division of subclasses (Tsuchiya, 2010). These varying structure, size, physical and biochemical properties allow them to interact with targets in different sub cellular locations influencing biological activity in plants, animals, and microbes (Taylor and Grotewold, 2005; Peer and Murphy, 2007). According to their molecular structure, they are divided into eight classes namely; Flavonol, Flavone, Flavonone, Isoflavone, Anthocyanin, Catechins, Chalcone and Dihydroflavonol (Tsuchiya, 2010).

Biflavonoids are a type of flavonoids characterized by flavonoid-flavonoid dimer with different chemical structures (Chen *et al*., 2006) (Fig 2.3). Many varied combinations of flavonoid dimer are possible; for example, flavanone-flavone, flavone-flavone, flavone-flavonol are among possible structures. There are also two general types of bond connections between the flavonoids: C-C bond or C-O-C bond, this connecting bond may have diverse positions: 3-4´´´, 4´-4´´´, etc. Many hydroxyl/methoxyl groups are substituted at different positions in biflavonoids of natural sources. Therefore, theoretically, numerous biflavonoids may exist (Chen *et al*., 2006).

However, plants that contain biflavonoids as major constituents are not widely distributed. Only a few plant species have biflavonoids; *Ginkgo biloba*, *Selaginella* species (Chen *et al*., 2006; Kim *et al*., 2008b) and *Garcinia* (Iwu, 1986). More than 100 biflavonoids have been identified from plants and a variety of biological activities of biflavonoids have been published, including anti-inflammatory, antimicrobial, antioxidant activities (Blazso *et al*., 1997; Lin *et al*., 2001; Ursini *et al*, 2001). In Nigeria, Kolaviron (KV), a biflavonoid

complex from *Garcinia kola* is the most reported (Iwu *et al*, 1987; Farombi *et al*., 2002; Adaramoye *et al*., 2005a).

2.3.1 Kolaviron

Kolaviron (KV), a biflavonoid complex from *Garcinia kola*, contains Garcinia biflavanone one (GB1), Garcinia biflavanone two (GB2) and kolaflavanone in an approximate ratio of 2: 2: 1 (Farombi *et al*., 2005) (Fig 2.4)

Most of the biological and pharmacological activities of *G. kola* have been attributed to kolaviron. KV modulates the hepatotoxicity of carbon tetrachloride, galactosamine, amanita toxin, paracetamol, thioacetamide and 2-acetylaminofluorene in various experimental animal models (Iwu, 1985; Iwu *et al*., 1987; Akintonwa and Essien, 1990; Iwu *et al*., 1990b; Farombi *et al*., 2000; Adaramoye and Adeyemi, 2006a). It prevents accumulation of lipid peroxidation products and protects biomembranes against oxidative damage by acting as *in vivo* antioxidant (Farombi, 2000) as well as a scavenger of reactive oxygen species *in vitro* (Farombi *et al*., 2002; Adaramoye *et al,* 2005a); though a weak analgesic a very strong anti inflammatory agent (Olaleye *et al*., 2000). It has also shown neuroprotective potential and can provide some protection to the hippocampus (Ijomone *et al*., 2011).

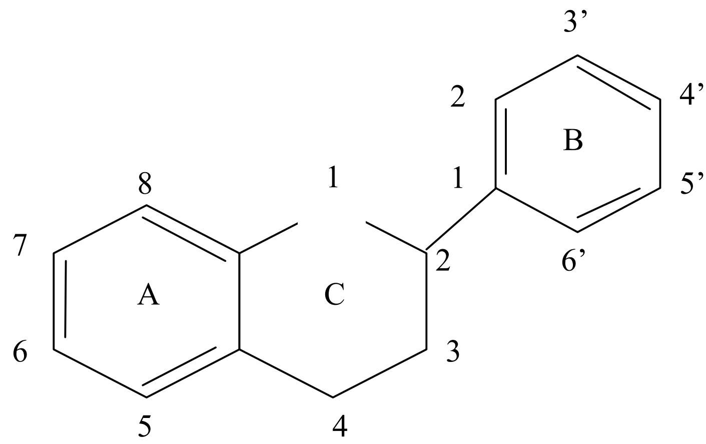


Figure 2.1: Structure of Flavan Nucleus (Cushnie and Lamb, 2005)

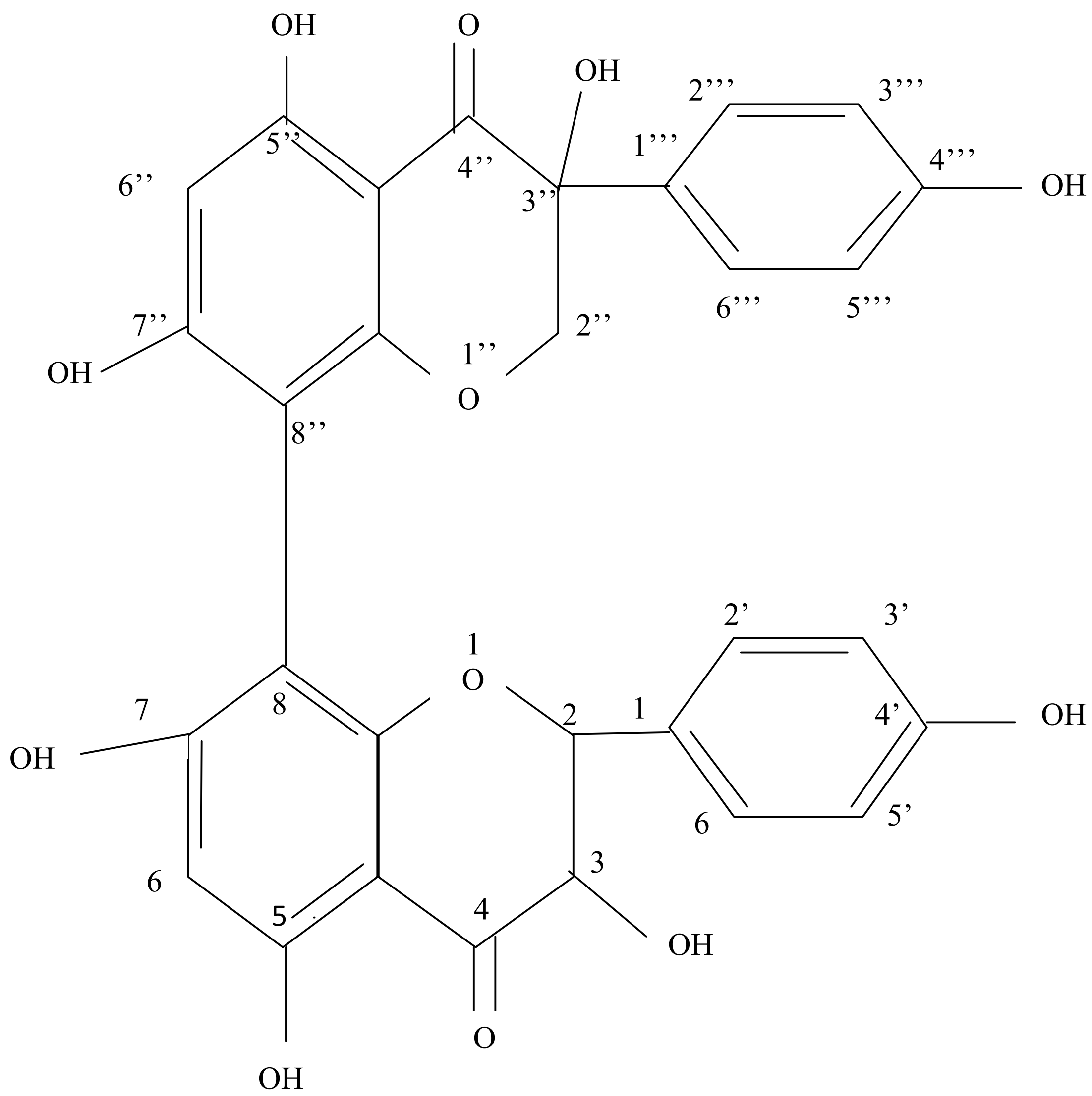
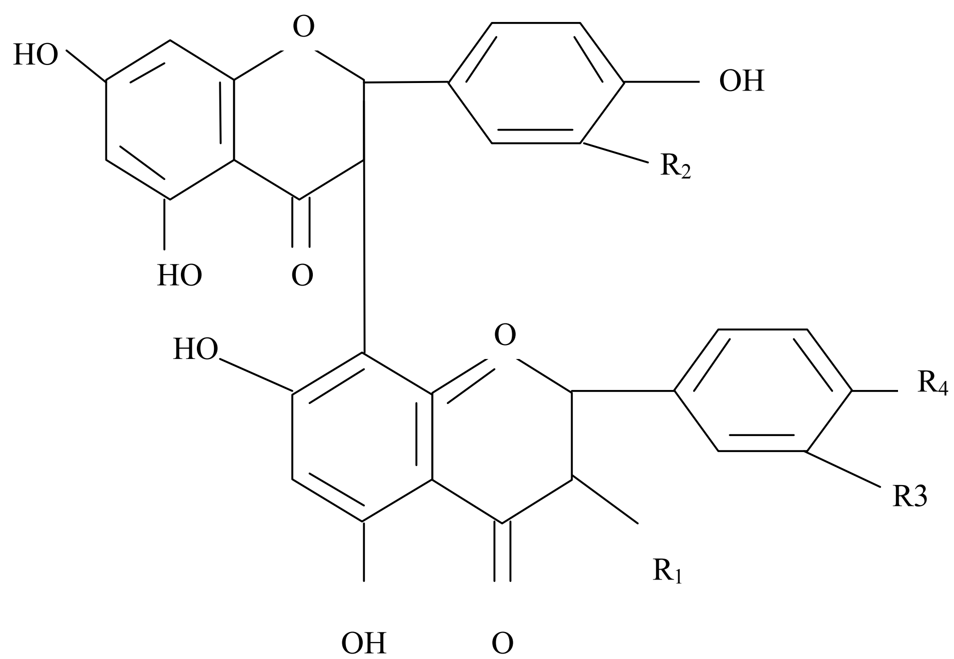


Figure 2.2: Structure of Biflavonoid (Flavonoid-Flavonoid Dimer) (Chen *et al*., 2006).



	R_1	R_2	R_3	R_4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OMe	OH

Figure 2.3: Structure of Kolaviron (Farombi *et al*., 2005).

2.4 Hyperlipidemia

2.4.1 Definition

Hyperlipidemia is the abnormal elevation of lipids in the blood, largely cholesterol and triacylglycerol. It is also known as hyperlipoproteinemia due to abnormal elevations of lipoproteins that transport lipids in the blood (Dorland, 2007).

Lipids are biological molecules soluble in organic solvents but insoluble in aqueous solutions. The major lipids found in the blood stream include cholesterol, cholesterol esters, triacylglycerols, phospholipids and free fatty acids (Dowhan *et al*., 2008). Lipids are transported in a protein capsule called lipoprotein. The size of this capsule, or lipoprotein, determines its density. The lipoprotein density and type of apolipoproteins it contains determines the fate of the particle and its influence on metabolism (Raja *et al*., 1996). Lipoproteins are divided into five major classes, based on density: chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Most triacylglycerols are transported in chylomicrons or VLDL, and most cholesterols are carried in LDL and HDL (Biggerstaff and Wooten, 2004).

Hyperlipidemia is the most common form of dyslipidemia (which also includes any decreased lipid levels) and one of the most important risk factors in the development and progression of atherosclerosis leading to cardiovascular diseases (CVDs) (Rerkasen *et al*., 2008). The close relationship between CVDs and lipid abnormalities poses a major problem to many societies as well as health professionals (Ramachandran *et al*., 2003; Matos *et al*., 2005).

2.4.2 Classification

Hyperlipidemias resulting from either elevations of cholesterol or triacylglycerol are called hypercholesterolemia or hypertriacylglycerolemia respectively and are both regarded as sub-categories of hyperlipidemia being the form in which hyperlipidemia manifests; these two forms could occur together as mixed hyperlipidemia (Baron, 2005). However, hyperlipidemia depending on its etiology is divided in two major classes, primary and secondary or acquired hyperlipidemia (American Heart Association, 2005).

2.4.2.1 Primary Hyperlipidemia

This is as a result of deficiency in enzymes such as lipoprotein lipase or altered apolipoprotein C II responsible in triacylglycerol metabolism. It also results from high intake of foods rich in saturated fat and cholesterol but it is most often genetic, hence also called familial hyperlipidemia (American Heart Association, 2005). It may or may not be ameliorated with the use of antilipidemic drugs. There are 5 types of primary hyperlipidemia (Kumar *et al*., 2002).

- **Type I:** severe elevations of chylomicrons (CM) resulting in elevations of triacylglycerol (TG)
- **Type II A:** elevations of low density lipoprotein cholesterol (LDL-c) only

  Type II B: elevations of both LDL-c and TG
- **Type III:** develops as result of defect in VLDL remnant clearance
- **Type IV:** characterized by hyper TG
- **Type V:** characterized by elevated levels of CM and VLDL

2.4.2.2 Secondary/Acquired Hyperlipidemia

This results from an underlying disease or treatment of other diseases, therefore called acquired hyperlipidemia (Chait and Brunzell, 1990). It does not require treatment of its own but treatment of the underlying disease. According to Coughlan and Sorrentino (2000), American Heart Association (2005) and Castilla-Guerra *et al*. (2009), some of the risk factors associated with secondary hyperlipidemia are as follows:

- Metabolic influences: diabetes, obesity, hyperuricemia
- Hormonal influences: insulin, glucagon, thyroxine
- Nutritional influences: alcohol, high carbohydrate intake
- Disease state: renal disease, renal failure, nephritic syndrome
- Drugs: diuretics, beta blockers, glucocorticoids, estrogen replacement therapy.

2.4.3 Etiology of Hyperlipidemia

Lifestyle choices such as high intake of carbohydrate, alcohol, obesity and lack of exercise are contributing factors to the progression of hyperlipidemia. However hyperlipidemia is mainly caused by either genetic or secondary factors.

- **Type I:** is due to a deficiency of lipoprotein lipase (LPL) or altered apolipoprotein C II, resulting in elevated chylomicrons, the particles that transfer fatty acids from the digestive tract to the liver. It can also result from lack of LPL activator and circulating inhibition of LPL. It is rare and usually present in children with eruptive xanthomata and abdominal colic. Complications include retinal vein occlusion,

acute pancreatitis, steatosis and organomegaly, and lipaemia retinalis (James *et al*., 2006).

- **Type II A:** may be sporadic (due to dietary factors), polygenic, or truly familial as a result of a mutation either in the LDL receptor gene or the ApoB gene (Rader *et al*., 2003). This form is also called familial hypercholesterolemia; it is very commonly characterized by tendon xanthoma, xanthelasma and premature cardiovascular disease (Tsouli *et al*., 2005).
- **Type II B:** has a very high VLDL level due to overproduction of substrates, including triacylglycerol, acetyl CoA, and an increase in ApoB-100 synthesis. They may also be caused by the decreased clearance of LDL (Dallari *et al*., 2003).
- **Type III:** is due to high CM and IDL level also known as broad beta disease or dysbetalipoproteinemia. The most common cause of this form is the presence of ApoE E2/E2 genotype which is due to cholesterol-rich VLDL (β-VLDL). Its prevalence has been estimated to be approximately 1 in 10,000 worldwide (Fung *et al*., 2011).
- **Type IV:** also known as familial hypertriacylglycerolemia is an autosomal dominant condition. It is a common type and can also result from metabolic/endocrine diseases, renal disease, liver diseases, ethanol use/abuse, pregnancy and drug use (Boman *et al*., 1975).
- **Type V:** is very similar to type IV and a rare type, but with high VLDL in addition to chylomicrons. It is also associated with glucose intolerance and hyperuricemia.

2.4.4 Diagnosis

Hyperlipidemia may be asymptomatic; therefore the medical and lifestyle history of an individual must be taken into account during diagnosis and laboratory tests for assessing the lipid profile. The laboratory test measures total cholesterol (TC), TG, HDL-c directly while VLDL-c level is calculated by dividing the triacylglycerol value by 5 and LDL-c is calculated by subtracting VLDL-c and HDL-c from TC. The individual must fast for 12 hours before the laboratory test, because chylomicron clearance can take up to 10 hours). According to National Cholesterol Education Program Lipid Assessments Guideline (2001), individuals with TC, TG, HDL-c and LDL-c above the borderline shown in Table 2.1 below would be considered hyperlipidemic.

Table 2.1: National Cholesterol Education Program Lipid Assessments

Parameters (mg/dl)	Desirable level	Borderline level	Undesirable level
TC	< 200	200-239	> 240
TG	< 150	150-199	> 200
HDL-c	> 40	Limitless	< 40
LDL-c	< 130	130-159	> 200

National Cholesterol Education Program Lipid Assessments Guideline (2001)

2.4.5 Treatment of Hyperlipidemia

Diet and lifestyle changes can help treat hyperlipidemia (Kelly, 2010). In some cases a combination of diet, lifestyle and medication may be required (Safeer and Lacivita, 2000).

2.4.5.1 Diet Changes

- Limit sugar and carbohydrate in diet, that is, diabetic diet.
- Eat a diet low in total fat, saturated fat and cholesterol.
- Reduce or eliminate alcohol intake.
- Consume high fiber foods such as fruits, vegetables, whole grains and beans.

2.4.5.2 Lifestyle Changes

- Obtain and maintain optimal body weight.
- Smoking cessation.
- Regular exercise. Patients should confirm from the doctor first because hyperlipidemic patients may have hardening of the arteries and other heart diseases which increases the risk of heart attack and death while exercising.

2.4.5.3 Medication

This is used if diet and lifestyle change cannot manage the condition. Different drugs with different mechanisms of action are used in the treatment of hyperlipidemia (Safeer and Lacivita, 2000). They include;

- **Statins:** act by inhibiting 3-hydroxy-3-methylglutaryl Coenzyme A (HMG CoA) reductase, a rate limiting enzyme in cholesterol synthesis. Examples are Atorvastatin, Pravastatin and Simvastatin.
- **Bile acid binding resins:** form insoluble complex with bile acids and prevent bile acids reabsorption into enterohepatic circulation. Examples include Cholestyramine, Colestipol.
- **Fibrates:** increase hydrolysis of TG in CM and VLDL particles by stimulating lipoprotein lipase activity. Examples are Clofibrate, Gemfibrozil and Bezafibrate.

2.4.6 Experimental Models of Hyperlipidemia

- **Poloxamer Model:** Poloxamer 407 (P407) is a ubiquitous man made surfactant and non ionic detergent. It is a triblock copolymer composed of a central hydrophobic chain of polyoxypropylene flanked by two hydrophilic chains of polyoxyethylene. P407 has unusual thermoreversible properties. It is liquid at cold temperature, while at body temperature it self-assembles into micelles that then aggregate into a gel. These temperature-dependent micellization and gelation properties have led to the widespread use of P407 in personal care products such as mouthwashes, deodorants, and skin care products and also as an excipient in a variety of pharmaceutical preparations (Dumortier *et al*. 2006).

  P407 has also been found to induce dose dependant hyperlipidemia observed in experimental animals after parenteral administration (Johnston and Palmer 1993; Palmer, *et al*., 1998; Johnston, 2004). This it does by inhibition of lipoprotein lipase, which is an endothelial enzyme that is responsible for converting

chylomicrons to chylomicron remnants (Johnston and Palmer 1993; Johnston 2004) and indirectly stimulating HMG CoA reductase activity, a rate limiting enzyme in cholesterol synthesis, hence hypertriacylglycerolemia and hypercholesterolemia respectively. The increase in TC and TG is evident 2 hours after a single intraperitoneal injection of 0.5 to 1g/kg of P407 (Palmer *et al*., 1997). It is non toxic and safe for chronic administration and long term studies (Megalli *et al*., 2005).

Other experimental models and their mechanisms of action are;

- **Diet (Fat):** increases availability of acetyl CoA thereby stimulating the rate of cholesterogenesis (Zulet *et al*., 1999).
- **Triton:** increases hepatic cholesterol synthesis or by decreasing cholesterol excretion (Gehard, 2001).
- **Genetic Models**

    Monosodium glutamate (MSG): produces destructive lesions on the arcuate nucleus of the hypothalamus followed by hypoplastic-hypotrophic obesity despite normophagia (Kaur and Kulakarni, 2001).

    Hereditary Hypercholesterolemia in rats or rabbits: decreased rate of catabolism of chylomicrons and LDL. But more specifically causes excessive productions of these two types of lipoproteins (Gehard, 2001).

2.4.7 Hyperlipidemia and Liver

The liver is a vital organ present in all vertebrates, it plays a major role in metabolism and has a number of functions in the body, including glycogen storage, erythropoietic function, plasma protein synthesis, hormone production, and detoxification (Maton *et al*., 1993).

Liver regulates total body and plasma cholesterol level, by decreasing biliary cholesterol synthesis and absorption efficiency whereby excess cholesterol is converted to bile acid and eventually excreted through faeces (Sumbull and Ahmed, 2012). Hyperlipidemia is one of the risk factors of coronary heart disease injurious to the liver. It sometimes results in fatty infiltration of the liver leading to a condition known as non alcoholic fatty liver (Assy *et al*., 2000).

Fatty liver is the accumulation of triacylglycerol and other fats in the liver cells. The amount of fatty acid in the liver depends on the balance between the processes of delivery and removal (Reid, 2001); though reversible, if not treated leads to inflammation of the liver. It is characterized by varying degree of liver injury from steatosis to fibrosis and necrosis (Day and James, 1998).

2.4.8 Hyperlipidemia and Kidney

Kidney helps in maintaining homeostasis of the body by reabsorbing important material and excreting waste products (James *et al*., 2010). Hyperlipidemia, a major risk factor for cardiovascular disease like atherosclerosis, may result to End Stage Renal Disease (ESRD) (Kumai *et al*., 2003). Hyperlipidemia specifically hypercholesterolemia can induce or exacerbate glomerular injury in mammals (Tolins *et al*., 1992; Grone *et al*., 1994) as well as tubulointerstitium injury in other animals (Grone *et al*., 1996); tubules and interstitium are major determinants of renal excretory function (Risdon *et al*., 1968; Mackensen-Haen *et al*., 1981). Hypercholesterolemia also results in interstitial fibrosis and tubular atrophy (Grone *et al*., 1996; Eddy, 1998). Hyperlipidemia can lead to ischemic nephropathy (Mühlfeld *et al*., 2004). The pathogenetic mechanisms, leading to all these conditions have

been associated with oxidative stress accompanied with hyperlipidemia (Malle *et al*., 2000; Bhalodia *et al*., 2010).

2.4.9 Hyperlipidemia and Haematological Parameters

Hyperlipidemia is closely related to thrombus formation, hence contributing to atherosclerosis (Middleton *et al*., 2000). It is accompanied by platelet hyperactivity, hypercoagulability with increased factor VII, and hypofibrinolysis with increased plasminogen activator inhibitor (PAI-1) (Juhan-Vague and Vague 1990; Middleton *et al*., 2000). The influx of non esterified fatty acids (NEFAs) from the adipocytes increases tissue factor (TF) and PAI-1 levels and enhances platelet aggregation; all of these promote the development of thrombosis (Eckel *et al.* 2002).

Hyperlipidemia also activates both endothelial cells and cells of the myeloid lineage, thereby representing a major risk factor for atherosclerosis (Hansson and Libby, 2006; Soehnlein *et al*., 2009a). Hypercholesterolemia increases circulating inflammatory monocytes counts and renders these cells more prone for emigration into atherosclerotic lesions (Swirski *et al*., 2007). The presence of neutrophils and neutrophil-derived mediators in atherosclerotic lesions has been seen in human atherosclerosis specimens and murine models of atherosclerosis (Van Leeuwen *et al*., 2008; Yvan-Charvet *et al*., 2008; Zernecke *et al*., 2008). For instance, neutrophil granule proteins such as defensins, azurocidin, and LL-37 have been detected within plaques (Soehnlein and Weber, 2009). In particular, the function of monocytes, macrophages, and dendritic cells is regulated by neutrophil-derived mediators in terms of recruitment, phagocytic capacity, and cytokine release (Soehnlein *et al*., 2009b; Soehnlein and Lindbom, 2010).

There is a positive correlation between plasma triacylglycerol and low-density lipoprotein and neutrophilic reactive oxygen species formation (Araujo *et al*., 1995). An increased rate of superoxide release and CD11b surface expression positively correlates with the severity of hyperlipidemia (Mazor *et al*., 2008). In addition, circulating neutrophils contain less myeloperoxidase, whereas plasma myeloperoxidase levels elevates (Mazor *et al*., 2008), indicating granule discharge from neutrophils in patients with hyperlipidemia.

Clinical studies correlates systemic neutrophil counts with severity of atherosclerosis in humans supporting an association of neutrophils with disease progression (Giugliano *et al*., 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Samples

Root bark, stem bark, and seeds of *Garcinia kola* were collected from their natural habitat, Abak, Akwa Ibom state in the month of August, 2012. The plant was identified and authenticated at the herbarium unit of Biological Sciences Department, Ahmadu Bello University, Zaria, where a voucher specimen number 1783 was given and deposited for future reference.

3.1.2 Chemicals/Reagents

Potassium Acetate, Quercetin, Sodium chloride, Sodium hydroxide, Potassium hydroxide, Hydrochloric acid, Sulphuric acid, Petroleum ether (Sigma-Aldrich), Acetone (Sigma-Aldrich), ethylacetate (Sigma-Aldrich), Methanol (Sigma-Aldrich) and ethanol (Sigma-Aldrich) .

3.1.3 Equipments

Sherwood Colorimeter 257, Grant JB Series Water Bath, Heraeus Labofuge 300 Centrifuge, RS-232C Weighing Balance, Sysmex Automated Haematology System (XE-5000) and Haematocrit reader.

3.1.4 Animals

A total of sixty (60) apparently healthy Wistar albino rats of both sexes weighing between 150-200g were obtained from National Institute for Trypanosomiasis Research (NITR), Kaduna, and kept according to sexes in well aerated laboratory cages in the Animal house, Department of Pharmacology, Ahmadu Bello University, Zaria. The animals were allowed to acclimatize to the laboratory environment for a period of two weeks before the commencement of the experiment. They were fed with water and grower mash from Vital Feeds Company *ad libitum.*

3.2 Methods

3.2.1 Extraction and Fractionation of Biflavonoid Fraction (Iwu *et al.* 1990c)

All the plant parts collected were washed with clean water. The seeds were peeled, sliced and pulverized with a hand grater and air-dried in the laboratory (25-28°C) alongside the root bark and stem bark which were coarsely ground after drying. The plant parts were not finely ground to avoid diffusion of fine powder from the thimble into the solvent in the soxhlet extractor. The coarsely ground root bark, stem bark and seeds (1kg) each were defatted for about 6hrs with 2.5L of light petroleum ether (bp 40-60°C) in a soxhlet extractor respectively. The defatted, dried marc of each part was separately repacked and then re-extracted with 2L of acetone (bp 55.5-56.5°C) in a soxhlet extractor. The soxhlet extraction process continued until the solvent became colourless. The acetone extract of each part was concentrated by evaporation to dryness in a water bath at 50°C for 8hrs.

The concentrated extract of each plant part was fractionated using liquid–liquid partitioning. Fifty grams of each concentrated extract was suspended in 500ml of distilled water and then transferred to a separating funnel and 500ml of ethylacetate was added, shaken and left standing for 1hr for clear separation. The two immiscible liquid phase layers were separated into ethylacetate-soluble (EAS) and water soluble (WAS) fractions respectively based on density. The two fractions were then carefully run out of the separating funnel into separate conical flasks. The water soluble fraction was poured back into the separating funnel and another 500ml of ethylacetate was added for further partitioning. The partitioning process was carried out 3 times for each plant part extract respectively; collecting all EAS fractions of each plant part into one conical flask. The ethylacetate fractions of the different plant parts were then concentrated by evaporating to complete dryness in a water bath at 50°C. The concentrated ethylacetate fractions of the plant parts (root bark, stem bark and seed) had a golden colour and were considered biflavonoid rich. These fractions obtained from the three plant parts were then stored for further analysis. On each day of the experiment the biflavonoid fractions were further dissolved in 2-3 drops of tween-80 to give a water-soluble fraction and diluted to desired concentrations with distilled water.

The percentage yields of biflavonoid fractions from root bark, stem bark and seeds were determined as a percentage of the weight of the fraction obtained to the original weight of the ground sample used:

$$\text{Percentage yield} = \frac{\text{Weight of obtained fraction}}{\text{Weight of ground sample used}} \times 100$$

3.2.2 Preliminary Phytochemical Screening (Trease and Evans, 1983)

3.2.2.1 Test for Carbohydrates

- **Molisch's Test:** Each sample (0.5g) was dissolved in 10ml of distilled water and heated for 5mins, cooled and then filtered. Two to three drops of Molisch reagent (alcoholic α-naphtol solution) was added to the filtrate (2ml) of each sample in a test tube and 2ml concentrated sulphuric acid was allowed to run down the side of the test tube. The mixture was allowed to stand for 2 to 3 mins; formation of purple to violet colour at the interface of the two layers indicates the presence of carbohydrate.

3.2.2.2 Test for Glycosides

Fehlings Solution Test: Five milliliter of concentrated sulphuric acid was added to 0.5g of each sample and boiled for 15 minutes. This was then cooled and neutralized with 20% KOH. Two to three drops of Fehlings solutions A and B were added and boiled for few minutes, red precipitate indicates the glycone portion as a result of hydrolysis.

3.2.2.3 Test for Anthraquinones

- **Test for Free Anthraquinones (Bornirager's Test):** Each sample (0.5g) was mixed with 10ml of benzene and filtered. Five millilitres of 10% ammonia solution was added to the filtrate and stirred. The production of a pink-red or violet colour indicates the presence of free anthraquinones.

- **Test for Combined Anthracene (Modified Bornirager's Test):** Each Sample (0.5g) was boiled with 5ml of 10% hydrochloric acid for 3 minutes. This hydrolyzes the glycosides to yield aglycones which are soluble in hot water only. The solution was filtered hot; the filtrate was cooled and extracted with 5ml of benzene. The benzene layer was filtered off and shaken gently with half its volume of 10% ammonia solution. A rose-pink or cherry red colour indicates combined anthracene.

3.2.2.4. Test for Cardiac Glycosides

- **Kella Killiani Test:** Each sample (0.5g) was dissolved in 2ml of glacial acetic acid containing 1 drop of ferric chloride. The test tube was held at an angle of 45 degree and 1ml of concentrated sulphuric acid was added down the side. A brown ring colour at the interface indicates the presence of deoxysugar characteristic of cardenolides. A violet or purple ring may appear below the ring while in the acetic acid layer, a greenish ring may be formed.

3.2.2.5 Test for Saponins

- **Frothing test:** Each sample (0.5g) was dissolved in 10ml of distilled water and heated for 5mins, cooled and then filtered. The filtrate was then shaken vigorously for 30 seconds and allowed to stand for 30 minutes. A honey comb formed for more than 30 minutes indicates saponin.

- **Test for Steroid and Triterpenes (Liberman-Burchards Test):** One milliliter of acetic anhydride was added to 0.5g of each sample. Concentrated sulphuric acid (1ml) was then added down the side of each tube. The colour change was observed

immediately and 5mins later. Red, pink or purple colour indicates the presence of triterpenes while blue or blue-green indicates steroids.

3.2.2.6 Test for Flavonoids

- **Shinoda Test:** Each sample (0.5g) was dissolved in 1-2ml of 50% methanol in the heat. Metallic magnesium and 4 to 5 drops of concentrated HCl were added. A red or orange colour indicates the presence of flavonoid aglycones.

3.2.2.7 Test for Tannins

- **Ferric Chloride Test:** Each sample (0.5g) was dissolved in 10ml of distilled water, heated for 5 mins, cooled and then filtered. Three drops of ferric chloride solution were added to the filtrate. Formation of a blue-black precipitate indicates hydrolysable tannins and green precipitate indicates the presence of condensed tannin.

3.2.2.8 Test for Alkaloids

- **Dragendoff's Test:** Exactly 0.5 g of each plant sample was dissolved in 5 ml of 1% HCl on steam bath. A millilitre of the filtrate was treated with 2 drops of Dragendorff's reagent. Rose red precipitation indicates the presence of alkaloids.

3.2.3 Determination of Total Flavonoid Content (Chang *et al.* 2002)

Total flavonoid content of each biflavonoid fraction was determined by the aluminum chloride colorimetric method.

***Principle of Reaction*:** Aluminium chloride forms stable acid complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids (Fig 2.2). The complex formed has maximum absorbance at 415nm.

Assay Procedure:

A 100mg of each dried fraction was dissolved in 10ml of 80% ethanol and filtered; 1ml from each sample filtrate (separately) was mixed with 3ml of 95% ethanol (v/v), 0.2ml of 10% aluminium chloride, 0.2ml of 1mol/litre potassium acetate and 5.6ml of distilled water to make final volume up to 10ml, and incubated for 30 minutes, the absorbance of the reaction mixture was measured at 415nm. A volume of test sample and aluminium chloride was substituted by the same volume of distilled water and taken as blank solution.
Stock solution of Quercetin of 1000µg/ml was prepared by dissolving 100mg of quercetin in 100ml of 80% ethanol (v/v); further, various dilutions were made to prepare 6.25, 12.5, 25.0, 50.0, 80.0 and 100µg/ml solution of quercetin; absorbance was recorded at 415nm; standard curve (Appendix 1) was plotted for various concentrations versus absorbance. Sample concentrations were extrapolated from the standard curve.

3.2.4 Acute Toxicity Study (Lorke, 1983)

The median lethal dose (LD_{50}) of *Garcinia kola* biflavonoid fractions was conducted in order to select a suitable dose for the evaluation of effects of the biflavonoid fractions. In the initial phase, rats were divided into 3 groups of 3 rats each and were orally treated with 10mg, 100mg and 1000mg of the each fraction per kg body weight respectively. They were observed for 24 hours for signs of toxicity, including death. In the final phase, 3 rats were

divided into 3 groups of one rat each, and were treated orally with 1600mg, 2900mg and 5000mg of each fraction per kg body weight respectively. The rats were also observed for 24 hours for signs of toxicity, including death same as the first phase. The LD_{50} was calculated from the results of the final phase as the square root of the product of the lowest lethal dose and the highest non-lethal dose, i.e. the geometric mean of the consecutive doses which 0 and 100% survival rates were recorded.

3.2.5 Experimental Design

3.2.5.1 Animal grouping

The rats were randomly divided into 2 major groups (Phytopreventive and Phytotherapeutic); with a total of 10 sub-groups comprising of 6 rats each. Sub-groups 3 to 6 were given atorvastatin, root bark, stem bark and seed biflavonoid fractions respectively for 19 days and on the 19th day, injected with Poloxamer 407 (P407) (500mg/kg b. wt) and sacrificed 48 hrs after (Phytopreventive study). Sub-groups 7 to 10 were administered P407 (500mg/kg b. wt) at 48h interval for 21 days; treatment commenced with respective biflavonoid fraction 2 hours after induction (Phytotherapeutic Study).

Group 1: Rats were fed normal chow and water *ad libitum* and served as normal control (NC).

Group 2: Rats were given Poloxamer 407 (500mg/kg b.wt) and served as hyperlipidemic control (P407).

Group 3: Rats were given atorvastatin (10mg/kg b.wt) + Poloxamer 407 (500mg/kg b.wt). This served as standard control for the phytopreventive study (ATV+P407).

Group 4: Rats were given root bark biflavonoid fraction (200mg/kg b.wt) + Poloxamer 407 (500mg/kg b.wt) (RBBF+P407).

Group 5: Rats were given stem bark biflavonoid fraction (200mg/kg b.wt) + Poloxamer 407 (500mg/kg b.wt) (SBBF+P407).

Group 6: Rats were given seed biflavonoid fraction (200mg/kg b.wt) + Poloxamer 407 (500mg/kg b.wt) (SBF+P407).

Group 7: Rats were given Poloxamer 407 (500mg/kg b. wt) + atorvastatin (10mg/kg b.wt). This served as a standard control for phytotherapeutic study (P407+ATV).

Group 8: Rats were given Poloxamer 407 (500mg/kg b. wt) + root bark biflavonoid fraction (200mg/kg b.wt) (P407+RBBF).

Group 9: Rats were given Poloxamer 407 (500mg/kg b. wt) + stem bark biflavonoid fraction (200mg/kg b.wt) (P407+SBBF).

Group 10: Rats were given Poloxamer 407 (500mg/kg b. wt) + seed biflavonoid fraction (200mg/kg b.wt) (P407+SBF).

Groups 1-6- Phytopreventive Grouping

Groups 1, 2 & 7-10- Phytotherapeutic Grouping

All animals in groups 2 to 10 were also fed normal chow and water *ad libitum*. Weekly determination of parameters such as body weight, packed cell volume (PCV) and fasting blood glucose (FBG) were carried out during the period of the experiment.

3.2.5.2 Induction of Hyperlipidemia (Johnston and Palmer, 1993)

Poloxamer 407 (Lutrol F127; BASF, Ludwigshafen, Germany) was used as the inducing agent. Prior to the administration, Poloxamer 407 (P407) was dissolved in distilled water and refrigerated overnight to facilitate its dissolution. Needles and syringes used for administration were also cooled prior to administration to prevent gelation within the syringe. Administration of P407 (500mg/kg) was intra peritoneal.

To confirm the induction of hyperlipidemia, blood samples were collected via retro orbital puncture of the induced animals after 2 hrs of administration; total cholesterol and triacylglycerol concentrations of the blood samples were determined using a standard diagnostic kit. Rats with total cholesterol and triacylglycerol levels above 200mg/dl and 160mg/dl respectively were considered hyperlipidemic (Delvin, 2006).

3.2.5.3 Preparation of Standard Drug

Atorvastatin (Pfizer Ireland Pharmaceuticals, Ireland) was purchased in a tablet form at a strength of 20mg. Tablets were dissolved in distilled water to desired concentrations and administered orally. This preparation was done daily at time of administration.

3.2.6 Collection and Preparation of Blood and Organ Samples

On the 21st day of the experiment, chloroform-inhalation anesthesia was performed on all experimental animals. The anesthetized animals were bled by decapitation and blood samples were collected into plain bottles (for biochemical analysis) and vacutainers containing Ethylene diamine tetraacetic acid (EDTA) (for haematological analysis). The blood samples collected in plain sample bottles were centrifuged at a speed of 1400 x *g* for

10 minutes and serum collected for biochemical analysis. Organs (heart, liver, kidney and spleen) were also harvested and weighed.

The percentage organ weight of each animal was calculated as follows:

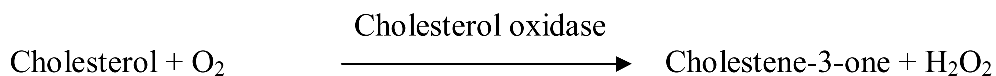
$$\% \text{ change in weight} = \frac{\text{Organ weight}}{\text{Animal weight}} \times 100$$

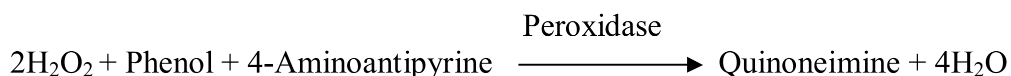
3.2.7 Determination of Biochemical Parameters

3.2.7.1 Determination of serum total cholesterol (TC) concentration

The serum level of TC was quantified by spectrophotometric method described by Stein (1987) using Randox® kit (Randox Laboratories Limited UK).

Principle: A large portion of the cholesterol in blood is in the form of cholesteryl esters, which is hydrolysed by cholesterol esterase into cholesterol and fatty acids. Cholesterol is then oxidized by cholesterol oxidase to yield H_2O_2. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase which can be detected spectrophotometrically.





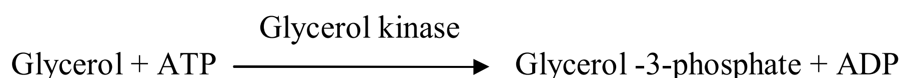
Procedure: Distilled water (10µl), cholesterol standard (10µl) and test serum (10µl) were dispensed into the test tubes labelled reagent blank, standard and sample respectively. One millilitre of the cholesterol reagent was then added to each of the tubes and mixed. The mixture was incubated at 37°C for 5 minutes in a water bath. The absorbance of the sample (A_{sample}) and that of the standard ($A_{standard}$) were measured against the reagent blank at 500nm wavelength using a colorimeter. The values obtained were used to calculate the total cholesterol concentration using the formula:

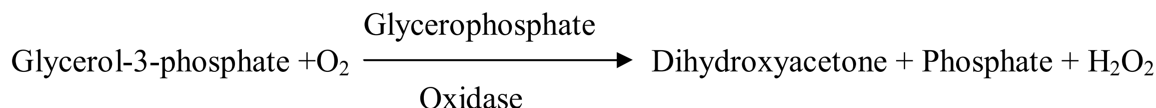
$$\text{Total cholesterol (mg/dl)} = \frac{A_{sample}}{A_{standard}} \times \text{conc. of standard}$$

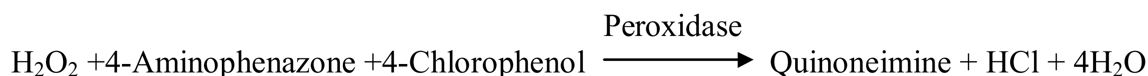
3.2.7.2 Determination of serum triacylglycerol (TG) concentration

The serum level of TG was determined by enzymatic method described by Stein (1987) using Randox® kit (Randox Laboratories Limited UK).

Principle: The triacylglycerol are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.







$$H_2O_2 + \text{4-Aminophenazone} + \text{4-Chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + HCl + 4H_2O$$

Procedure: One millilitre of the randox TG enzyme reagent (containing a mixture of lipases, glycerol-kinase, ATP, 4-aminophenazone, 4-chlorophenol, peroxidase, glycerol-3-phosphate oxidase and magnesium ions) was dispensed into three separately labelled test tubes; standard, sample and reagent blank. The standard, contained 10µl standard triacylglycerol, the tube labelled sample contained 10µl test serum while that labelled reagent blank contained 10µl of distilled water. The mixture was then incubated for 5 minutes at 37°C in a water bath. After the period of incubation the absorbance of the sample (A_{sample}) and that of the standard ($A_{standard}$) was then read against the reagent blank within 60 minutes using a colorimeter at 500nm wavelength. The triacylglycerol concentration was calculated using the formula:

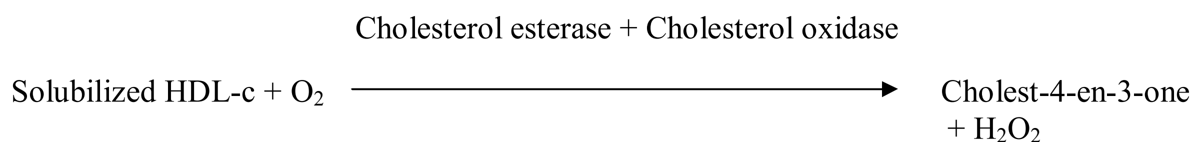
$$\text{Triacylglycerol concentration (mg/dl)} = \frac{A_{sample}}{A_{standard}} \times \text{conc. of standard}$$

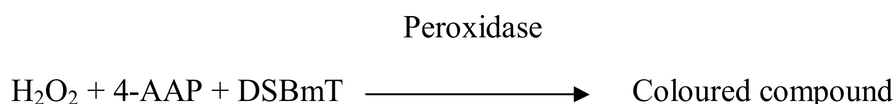
3.2.7.3 Determination of serum high density lipoprotein cholesterol (HDL-c) concentration

HDL-c level was determined using ELITech® kit (ELITech Clinical Systems, France) based on the method described by Naito (2003).

Principle: When a sample is mixed with reagent R1 containing a selective accelerator, cholesterol of non HDL is subject to enzymatic reactions to be eliminated.

When R2 is added, HDL is solubilized by a specific detergent and HDL-c is measured by enzymatic reactions.





Procedure: Exactly 240µl of reagent 1 (cholesterol oxidase, peroxidise, ascorbate oxidase and N-N-bis (4-sulphobutyl)-*m*-toludine-disodium) was dispensed into three separate test tubes labelled "blank"(containing 2.4µl of distilled water), "standard" (containing 2.4µl of HDL-c calibrator) and "sample" (containing 2.4µl of test serum). The mixture was incubated at 37°C for 4 minutes and 40 seconds in a water bath and the absorbance (A_1) measured using a colorimeter at 578nm wavelength against the blank. Then, 80µl of Reagent R_2 (4-Aminoantipyrine, cholesterol esterase) was then added to each of the tubes and the resultant mixture incubated at 37°C for 4 minutes in a water bath and absorbance (A_2) measured using a colorimeter at 578nm wavelength against the blank. The concentration of HDL-c was calculated using the formula:

$$\text{HDL-c (mg/dl)} = \frac{A_2 - A_{1\,(sample)}}{A_2 - A_{1\,(standard)}} \times \text{conc. of standard}$$

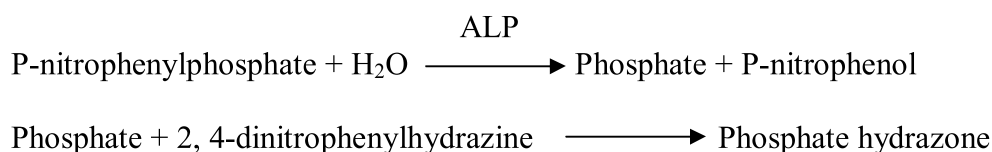
3.2.7.4 Determination of serum low density lipoprotein- cholesterol (LDL-c) concentration

The serum level of LDL-c was calculated according to the protocol of Friedewald (1972) using the equation: LDL-c (mg/dl) = TC – (TG/5 + HDL-c)

3.2.7.5 Determination of serum alkaline phosphatase (ALP) activity

The ALP activity was determined by a standard method described by Haussament (1977) using Randox® kit (Randox Laboratories Limited UK).

Principle: Alkaline phosphatase is measured by monitoring the concentration of phosphate hydrazone formed with 2, 4-dinitrophenylhydrazine at 405nm and 37^0C.



Procedure: Exactly, 0.01ml of test serum was dispensed into a 1ml cuvette followed by the addition of 0.05ml of the reagent (diethanolamine buffer, $MgCl_3$, p-nitrophenylphosphate). The absorbance of the mixture was immediately taken using a colorimeter at a wavelength of 405nm, and the timer started simultaneously as the initial absorbance was been recorded. The absorbance was read again at 1, 2, and 3 minutes respectively at 405nm. The initial absorbance reading was subtracted from the absorbance readings at 1, 2 and 3 minutes (i.e.

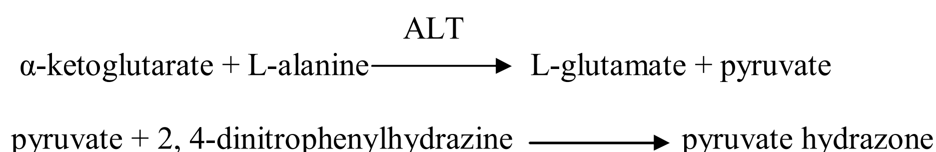
$A_{1/2/3} - A_{initial}$). The average of the three values obtained was then computed and used to calculate the ALP activity using the formula shown below:

ALP (U/I) = 2760 x ΔA at 405nm/min

3.2.7.6 Determination of serum alanine aminotransferase (ALT) activity

The ALT activity was determined using Randox® kit (Randox Laboratories Limited UK) based on the method described by Reitman and Frankel (1957) and expressed in U/l.

Principle: Alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine at 540nm and 37°C.

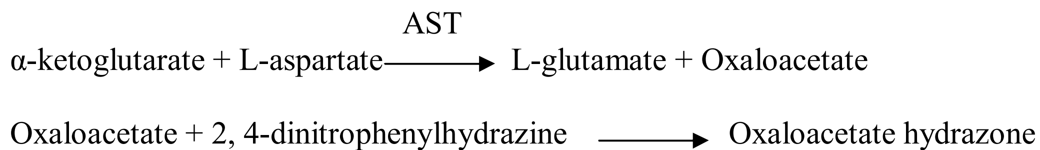


Procedure: Exactly, 0.5ml of reagent 1 (phosphate buffer, α-ketoglutarate and L-alanine) was dispensed into labelled test tubes: one containing 0.1ml of distilled water (Reagent blank) and the other 0.1ml of the test serum (sample). The mixture was incubated at 37°C for 30 minutes in a water bath. This was followed by the addition of 0.5ml of reagent 2 (2,4-dintrophenylhydrazine) to each of the test tubes and the mixture allowed to stand in a water bath at 25°C for 20 minutes. At the end of the 20 minutes, 5ml of sodium hydroxide (0.4mol/l) was then dispensed into each of the tubes and the absorbance of the sample (A_{sample}) was read against the reagent blank using a colorimeter at 546nm. The activity of ALT in the serum was obtained by extrapolating the corresponding absorbance from the plot of the standard calibration curve for the enzymes provided in the manual of the kit.

3.2.7.7 Determination of serum aspartate aminotransferase (AST) activity

The AST activity was determined using Randox® kit (Randox Laboratories Limited UK) based on the method described by Reitman and Frankel (1957) and expressed in U/l.

Principle: AST was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine at 540nm and 37°C.



Procedure: Exactly, 0.5ml of reagent 1 (phosphate buffer, α-ketoglutarate and L-aspartate) was dispensed into labelled test tubes: one containing 0.1ml of distilled water (Reagent blank) and the other 0.1ml of the test serum (sample). The mixture was incubated at 37°C for 30 minutes in a water bath. This was followed by the addition of 0.5ml of reagent 2 (2,4-dintrophenylhydrazine) to each of the test tubes and the mixture allowed to stand in a water bath at 25°C for 20 minutes. At the end of the 20 minutes, 5ml of sodium hydroxide (0.4mol/l) was then dispensed into each of the tubes and the absorbance of the sample (A_{sample}) was read against the reagent blank using a colorimeter at 546nm. The activity of AST in the serum was obtained by extrapolating the corresponding absorbance from the plot of the standard calibration curve for the enzymes provided in the manual of the kit.

3.2.7.8 Determination of serum total protein (TP) concentration

The serum TP concentration was determined using Randox® kit (Randox Laboratories Limited UK) based on the method described by Fine (1935).

Principle: The protein assay is based on a two step reaction of protein with an alkaline copper tartarate solution and Folin reagent leading to colour development. First is the reaction between protein and copper at alkaline pH, and subsequently, the reduction of Folin reagent by the copper-treated protein. Colour formation is due to amino acids that reduce the Folin reagent, yielding reduced species that imparts a characteristic blue colour. Bovine serum albumin is used as standard.

Procedure: Exactly, 0.02ml of distilled water, 0.02ml of standard and 0.02ml of test serum were dispensed into the test tubes labelled reagent blank, standard and sample respectively and 1ml of the Biuret reagent was then added to each of the test tubes and the mixture incubated at 25°C for 30 minutes in a water bath. After the period of incubation, the absorbance of the sample (A_{sample}) and that of the standard ($A_{standard}$) were then read against the reagent blank using a colorimeter at a wavelength of 546nm and the values recorded. The total protein concentration was then calculated using the formula:

$$\text{Total protein concentration (g/dl)} = \frac{A_{sample}}{A_{standard}} \times \text{conc. of standard}$$

3.2.7.9 Determination of serum albumin (ALB) concentration

The serum ALB concentration was determined using Randox® kit (Randox Laboratories Limited UK) based on the method described by Doumas *et al*., (1971).

Principle: The measurement of serum albumin is based on its quantitative binding to the indicator 3, 3', 5, 5'-tetrabromo-m cresol sulphonephthalein (bromocresol green, BCG). The absorbance of the blue green colour of albumin-BCG-complex at 630nm is directly proportional to the concentration of albumin in the sample.

Procedure: Three milliliter of bromocresol green (BCG) was dispensed into each of the tubes labelled reagent blank, standard and sample containing 0.01ml distilled water, 0.01ml standard albumin and 0.01ml test serum respectively. The mixture was then incubated for 5 minutes at 25°C in a water bath. After the incubation, the absorbance of the sample (A_{sample}) and that of the standard ($A_{standard}$) against the reagent blank were read using a colorimeter at a wavelength of 578nm. The values obtained were used to calculate the albumin concentration using the formula:

$$\text{Albumin concentration (g/dl)} = \frac{A_{sample}}{A_{standard}} \times \text{conc. of standard}$$

3.2.7.10 Determination of serum bilirubin concentrations

3.2.7.10.1 Determination of serum total bilirubin (TB) concentration

The serum TB concentration was determined using Randox® kit (Randox Laboratories Limited UK) based on the method described by Jendrassik and Grof (1938) and Sherlock (1951).

Principle: Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotised sulphanilic acid.

Procedure: Exactly, 200µl of reagent 1 (sulphanilic acid) was dispensed into two different test tubes labelled “sample blank” and “sample” followed by the addition of 1 drop (50µl) of reagent 2 (nitrite) into ‘‘sample’’ test tube only and 1000µl of reagent 3 (caffeine) into both test tubes. Test serum (200µl) was then dispensed into each of the tubes and the mixture incubated in a water bath for 10 minutes at 25°C. This was followed by the addition of 1000µl of reagent 4 (tartrate) and the mixture incubated again at 25°C for 10

minutes. The absorbance of the sample (A_{TB}) was then read against the sample blank using a colorimeter at 578nm wavelength. The total bilirubin concentration was calculated using the formula:

Total bilirubin (mg/dl) = 10.8 x A_{TB} (578nm)

3.2.7.10.2 Determination of serum direct/conjugated bilirubin (DB/CB) concentration

The serum CB concentration was determined using Randox® kit (Randox Laboratories Limited UK) based on the method described by Jendrassik and Grof (1938) and Sherlock (1951).

Principle: Direct/Conjugated bilirubin reacts with diazotised sulphanilic acid in alkaline medium to form a blue coloured complex.

Procedure: Exactly, 200µl of reagent 1 (sulphanilic acid) was dispensed into two different test tubes labelled “sample blank” and “sample” followed by the addition of 1 drop (50µl) of reagent 2 (nitrite) into the “sample” only and 2000µl of 0.9% sodium chloride into both test tubes. Two hundred microlitre of the test serum was then dispensed into each of the tubes and the mixture incubated in a water bath for 10 minutes at 25°C. The absorbance of the mixture (A_{DB}) was read against the sample blank using a colorimeter at 546nm wavelength. The direct bilirubin concentration was then calculated using the formula:

Conjugated bilirubin (mg/dl) = 14.4 x A_{DB} (546nm)

3.2.7.11 Determination of serum creatinine concentration

The serum creatinine concentration was determined using Randox® kit (Randox Laboratories Limited UK) based on the method described by Bartels and Bohmer (1973).

Principle: Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is proportional to the creatinine concentration.

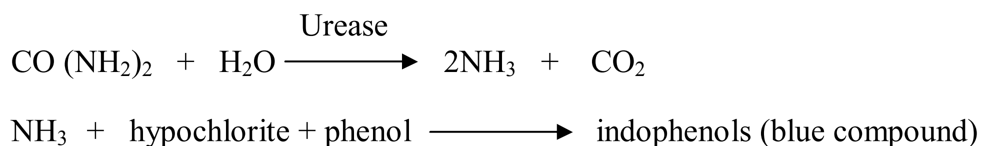
Procedure: Exactly, 0.1ml of creatinine standard solution and 0.1ml of test serum were dispensed into separate cuvettes labelled standard and sample respectively. This was followed by addition of 1.0ml of the working reagent (picric acid + NaOH). The absorbances of the mixtures were then taken after 30 seconds (A_1) and at exactly 2 minutes later (A_2) using a colorimeter at 492nm wavelength. The ΔA of the sample or standard was calculated by subtracting A_2 from A_1 i.e. $A_2 - A_1 = \Delta A$ of sample or standard. The values obtained were then used for calculating the serum creatinine concentration with the aid of the formula:

$$\text{Creatinine concentration (mg/dl)} = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times \text{conc. of standard}$$

3.2.7.12 Determination of serum urea concentration

The serum urea concentration was determined using Randox® kit (Randox Laboratories Limited UK) based on the method of Berthelot's reaction (Fawcett and Scout, 1960).

Principle: Urea in serum is hydrolysed to ammonia in the presence of urease. The ammonia formed is then measured photometrically in the presence of hypochlorite and phenol



$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{hypochlorite} + \text{phenol} \longrightarrow \text{indophenols (blue compound)}$$

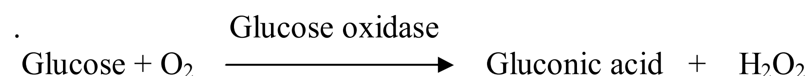
Procedure: Exactly, 100µl of reagent 1 (sodium nitroprusside [6mmol/l] + urease [1g/l]) was dispensed into three separately labelled test tubes: reagent blank, standard and sample containing 10µl of distilled water, 10µl of urea standard and 10µl of test serum respectively. The mixture was then incubated at 37°C for 10 minutes in a water bath. After the period of incubation, 2.5ml of reagent 2 (phenol [120mmol/l]) was added to the contents of each test tube followed by 2.5ml of reagent 3 (Sodium hypochlorite [27mmol/l]). The mixture was incubated for 15 minutes at 37°C in a water bath. The absorbance of the sample (A_{sample}) and that of the standard ($A_{standard}$) were read against the reagent blank using a colorimeter at wavelength of 546nm and the readings obtained were used for the calculation of the serum urea concentration with the aid of the formula:

$$\text{Urea concentration (mg/dl)} = \frac{A_{sample}}{A_{standard}} \times \text{conc. of standard}$$

3.2.7.13 Determination of fasting blood glucose concentration

The fasting blood glucose concentration was determined using the Reckon diagnostics kit based on the method of Barham and Trinder (1972).

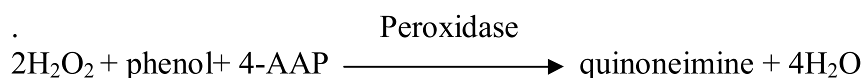
Principle: Glucose oxidase oxidizes the specific substrate β-D-Glucose, to gluconic acid and hydrogen peroxide is generated.



$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

Hydrogen peroxide thus produced is acted upon by peroxidase and oxygen is liberated. The liberated oxygen is transferred to chromogen system consisting of 4-aminoantipyrine and

phenolic compound to produce red quinoneimine dye. The intensity of colour is directly proportional to the concentration of glucose and is measured photometrically at 505nm.



$$2H_2O_2 + \text{phenol} + \text{4-AAP} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + 4H_2O$$

Procedure: The animals were fasted for 12hrs, after which blood samples were collected via retro orbital puncture into plain sample bottles, centrifuged at 1400 x *g* for 10mins and serum collected. One millilitre of glucose working reagent containing glucose oxidase, peroxidase and 4-aminoantipyrine as chromogen was pippetted into test tubes labelled reagent blank, standard and sample containing 10µl of distilled water, 10µl of standard reagent and 10µl of test serum respectively. The mixtures were all incubated at room temperature for 30 minutes and absorbance of standard ($A_{standard}$) and sample (A_{sample}) were read against the blank using a colorimeter at a wavelength of 505nm. The readings obtained were used to calculate the fasting blood glucose concentration with the aid of the formula:

$$\text{Glucose concentration (mg/dl)} = \frac{A_{sample}}{A_{standard}} \times \text{conc. of standard}$$

3.2.8. Haematological Assay

3.2.8.1 Determination of packed cell volume (PCV) (Cheesbrough, 2000)

PCV is the volume of red blood cells (RBC) expressed as a fraction of the total volume of the blood. The microhaematocrit method was used.

Principle: The red blood cells are heavier than plasma with specific gravity of 1090 and 1030 respectively. When blood is placed in a capillary tube and centrifuged, they settle and

pack together because of the centrifugal force acting on them. The volume occupied by the cells is measured and its ratio with that of the volume of the whole blood is calculated.

Procedure: Blood from the rat's tail flows into the capillary tube via capillary action, one end of the tube was then sealed by flaming and centrifuged at a speed of 650 x *g* for 10 minutes. The PCV was estimated using a microhaematocrit reader and expressed as percentage erythrocytes contained in the blood.

3.2.8.2 Determination of Other Haematological Parameters

Platelet, neutrophils, lymphocytes, Haemoglobin (Hb), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV), Red Blood Cell (RBC) Count and White Blood Cell (WBC) Count were determined using the Sysmex Automated Haematology System (XE-5000).

Principle: The Sysmex Automated Haematology System (XE-5000) combines the fluorescent flow cytometry and hydrodynamic technologies to analyze blood samples. The fluorescent flow cytometry utilizes the ability of polymethin dye to stain the different population cells differently. The machine has a diode laser bench which detects the fluorescence of nucleic acid, nucleus to plasma ratio of the stained cells and the volume of the cell as they pass through the aperture to discriminate between cells.

The hydrodynamic technology utilizes direct current to count cells as they pass through a dedicated aperture of known dimension. As the cells pass through this aperture, they create an electrical resistance which is detected as electronic pulse. The intensity of the electronic pulse from each cell is proportional to the cell volume.

The system, then, uses complex algorithm to automatically discriminate and separate the different cell population from the data.

3.3 Statistical Analysis

The results were expressed as mean ± standard deviation (SD) and data was analyzed by one way analysis of variance (ANOVA). The difference between the various biflavonoid fractions and animal groups were compared using the Duncan Multiple Range Post Hoc Test. *P* value less than 0.05 was considered significant ($p < 0.05$).

CHAPTER FOUR

RESULTS

4.1 Percentage Yield and Phytochemical Constituents of *Garcinia kola* Biflavonoid Fractions

4.1.1 Percentage Yield of *Garcinia kola* Biflavonoid Fractions

The percentage yield of *Garcinia kola* biflavonoid fractions is presented in Table 4.1. There was a significant (p<0.05) difference in the yield between the root bark, stem bark and seed with the root bark having the highest yield (31.93%) and the seed, the lowest yield (5.92%).

4.1.2 Qualitative Screening of Phytochemicals in *Garcinia kola* Biflavonoid Fractions

Phytochemical screening of the root bark, stem bark and seed biflavonoid fractions of *Garcinia kola* shows the presence of alkaloids, carbohydrates, cardiac glycosides, flavonoids, glycosides, saponins, tannins and unsaturated steroids and triterpenes. Combined anthracene as well as free anthracene derivatives were absent in all the fractions (Table 4.2).

4.1.3 Total Flavonoid Content of *Garcinia kola* Biflavonoid Fractions

The total flavonoid content of the root bark, stem bark and seed biflavonoid fractions of *Garcinia kola* is presented in Table 4.3. The result showed the root bark fraction had the least total flavonoid content (63.99± 2.94mg quercetin equivalent/g of dry fraction) followed by stem bark fraction (96.54± 1.70) while the seed (197.29± 0.64) had significantly (p<0.05) the highest total flavonoid content.

Table 4.1: Percentage Yield of *Garcinia kola* Biflavonoid Fractions

Biflavonoid Fractions	Percentage yield (%)
Seed	5.92 ± 0.08^{a}
Stem bark	7.56 ± 0.42^{b}
Root bark	31.93 ± 0.60^{c}

Values are means ± SD of three (3) determinations
Values with different superscripts down the column differ significantly ($p<0.05$)

Table 4.2: Phytochemical Constituents of *Garcinia kola* Biflavonoid Fractions

Phytochemicals	*RBBF	*SBBF	*SBF
Alkaloids	+	+	+
Carbohydrates	+	+	+
Cardiac glycosides	+	+	+
Combined anthracene	–	–	–
Flavonoids	+	+	+
Free anthracene derivatives	–	–	–
Saponins	+	+	+
Saponin glycosides	+	+	–
Tannins	+	+	+
Unsaturated sterols	–	+	+
Unsat. steroids and triterpenes	+	+	+

*RBBF: Root Bark Biflavonoid Fraction, *SBBF: Stem Bark Biflavonoid Fraction, *SBF: Seed Biflavonoid Fraction

Table 4.3: Total Flavonoid Content of *Garcinia kola* Biflavonoid Fractions

Biflavonoid Fraction	Total Flavonoid Content (mg quercetin equivalent/g of dry fraction)
Root bark	63.99 ± 2.94^{a}
Stem bark	96.54 ± 1.70^{b}
Seed	197.29 ± 0.64^{c}

Values are means ± SD of three (3) determinations
Values with different superscripts down the column differ significantly ($p<0.05$)

4.2 Lethal Dose (LD_{50}) Determination for *Garcinia Kola* Biflavonoid Fractions

The Lethal dose (LD_{50}) determination for root bark, stem bark and seed biflavonoid fractions of *Garcinia kola* is shown in Table 4.4. No death was recorded up to 5000mg per kg body weight.

Table 4.4: Lethal Dose (LD_{50}) Determination for *Garcinia kola* Biflavonoid Fractions

Dose of Biflavonoid Fraction (mg/kg)	*RBBF	*SBBF	*SBF
10	No death	No death	No death
100	No death	No death	No death
1000	No death	No death	No death
1600	No death	No death	No death
2900	No death	No death	No death
5000	No death	No death	No death

*RBBF: Root Bark Biflavonoid Fraction, *SBBF: Stem Bark Biflavonoid Fraction, *SBF: Seed Biflavonoid Fraction

$LD_{50} = \sqrt{\text{(lowest lethal dose)} \times \text{(highest non lethal dose)}}$

4.3 Biochemical Parameters

4.3.1 Effects of *Garcinia kola* Biflavonoid Fractions on Lipid Profile of P407 Induced Hyperlipidemic Albino Rats

The 19-day oral administration of *Garcinia kola* (root bark, stem bark and seed) biflavonoid fractions on lipid profile of P407 induced hyperlipidemic rats is presented in Figure 4.1. There was a significant ($p<0.05$) reduction in total cholesterol (TC), triacylglycerol (TG) and low density lipoprotein cholesterol (LDL-c) concentrations in all treated groups when compared to P407 induced hyperlipidemic control. The seed had the most significant ($p<0.05$) reduction in TC (79%) and TG (67%) levels when compared to other biflavonoid fractions. A significant ($p<0.05$) increase in high density lipoprotein cholesterol (HDL-c) concentration was seen in the groups administered root bark and stem bark biflavonoid fractions compared to all other groups.

The phytotherapeutic effect of *Garcinia kola* biflavonoid fractions on lipid profile is shown in Figure 4.2. Atorvastatin and *Garcinia kola* biflavonoid fractions also significantly ($p<0.05$) reduced TC, TG and LDL-c concentrations when compared with hyperlipidemic control with the root bark and stem bark having the most reduction in TC (77%, 78%) when compared to all treated groups. However, only the seed fraction significantly ($p<0.05$) increased HDL-c concentration compared to other groups.

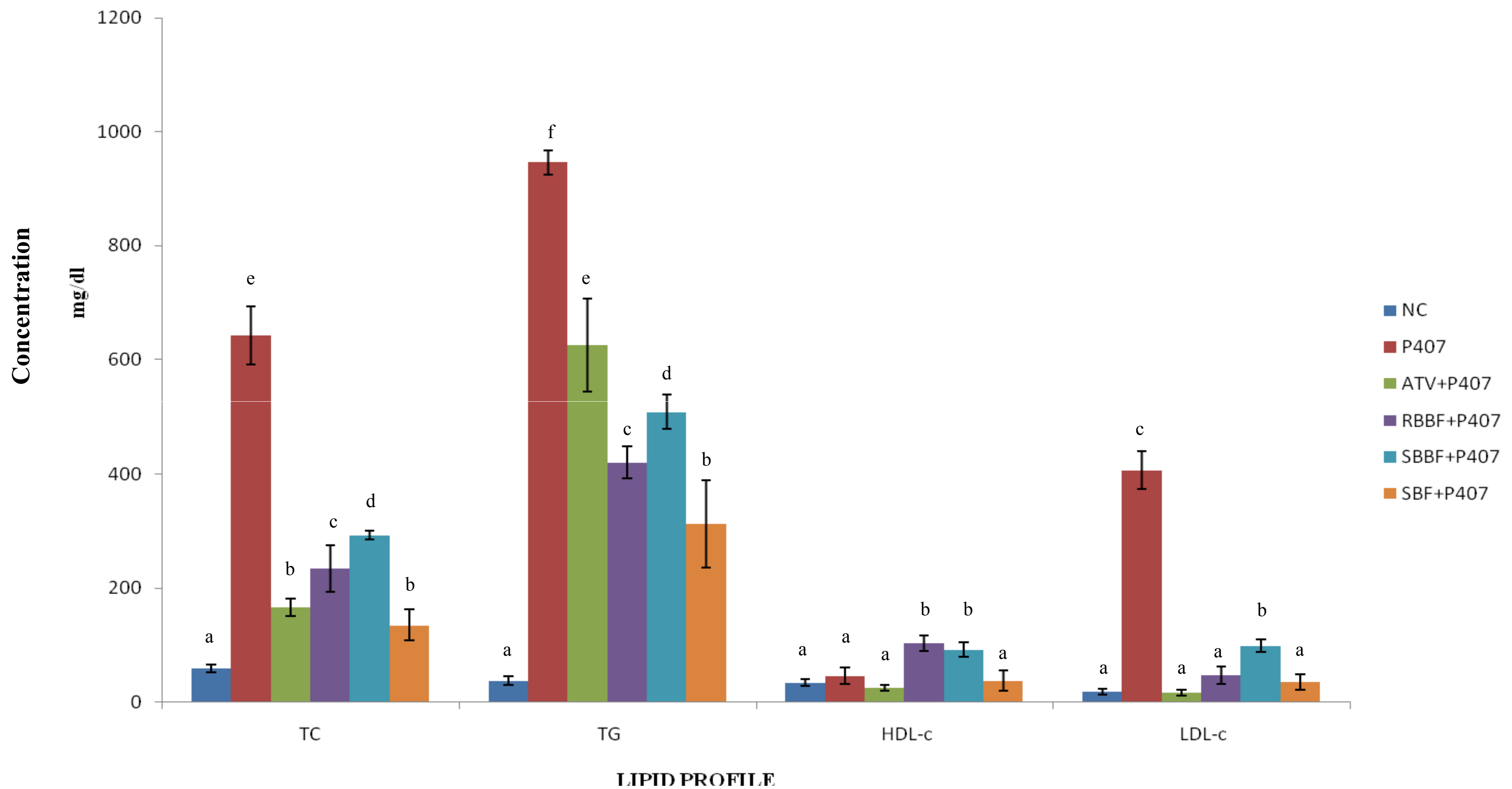


Figure 4.1: Phytopreventive Effect of *Garcinia kola* Biflavonoid Fractions on Lipid Profile of P407 Induced Hyperlipidemic Albino Rats.

TC: Total cholesterol, TG: Triacylglycerol, HDL-c: High Density Lipoprotein Cholesterol, LDL-c: Low Density Lipoprotein Cholesterol
Bars bearing different alphabets in each bar cluster, differ significantly (p<0.05)

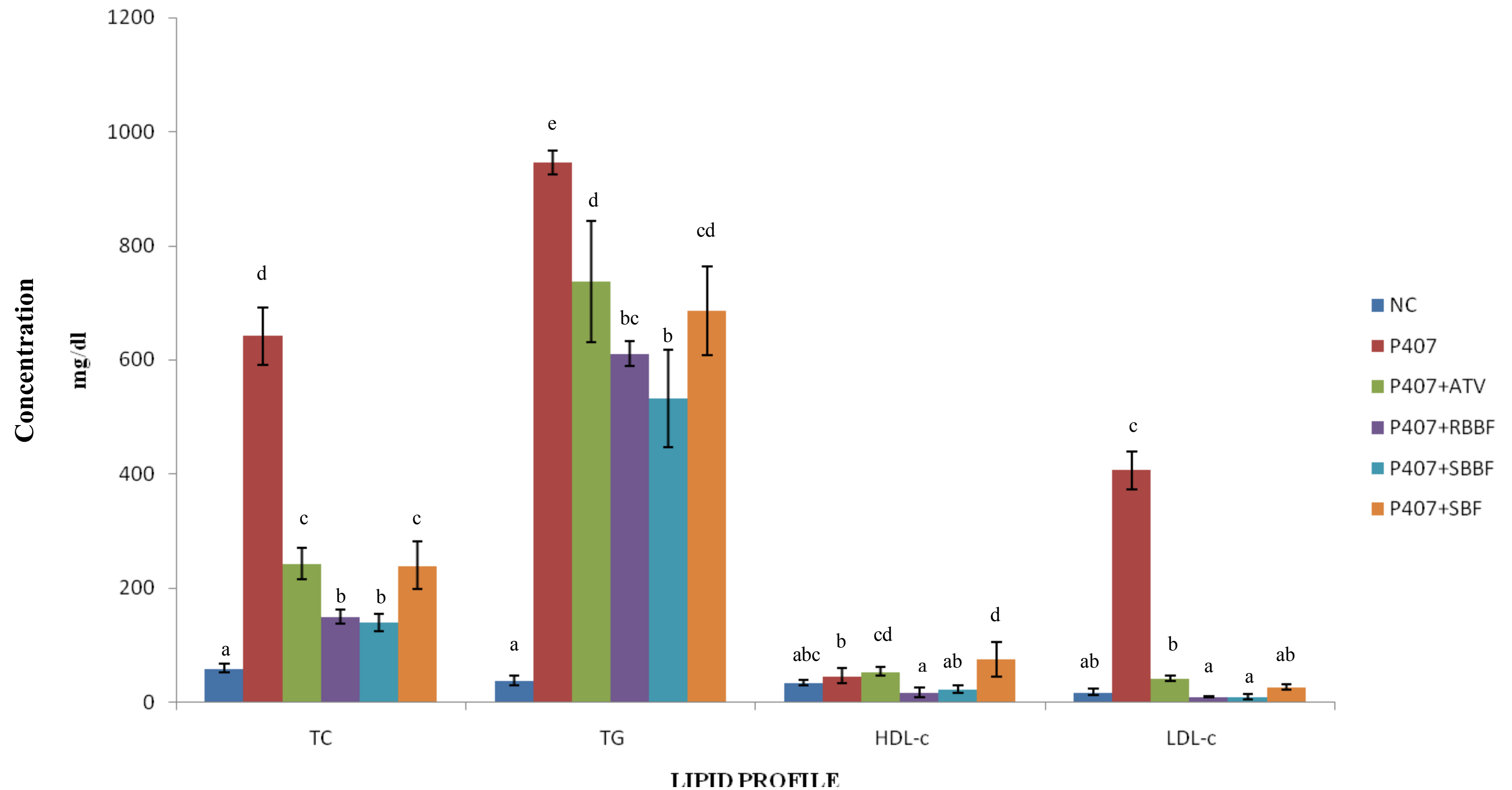


Figure 4.2: Phytotherapeutic Effect of *Garcinia kola* Biflavonoid Fractions on Lipid Profile of P407 Induced Hyperlipidemic Albino Rats.

TC: Total cholesterol, TG: Triacylglycerol, HDL-c: High Density Lipoprotein Cholesterol, LDL-c: Low Density Lipoprotein Cholesterol
Bars bearing different alphabets in each bar cluster, differ significantly ($p<0.05$)

4.3.2 Effects of *Garcinia kola* Biflavonoid Fractions on Serum Atherogenic Risk Predictor Indices of P407 Induced Hyperlipidemic Albino Rats

The effect of oral administration of *Garcinia kola* biflavonoid fractions before induction (phytopreventive) of hyperlipidemia with P407 on serum atherogenic risk predictor indices is shown in Table 4.5. The result showed significantly ($p<0.05$) higher HDL-c/TC ratio in all biflavonoid treated groups when compared to hyperlipidemic control and atorvastatin treated group with root bark and stem bark treated groups having HDL-c/TC ratio greater than 0.30. All induced treated groups had significantly ($p<0.05$) lower LDL-c/HDL-c ratio compared to hyperlipidemic control with their value less than 2.3 while only biflavonoid fractions significantly ($p<0.05$) reduced log (TG/HDL-c) compared to hyperlipidemic control.

Table 4.6 presents the phytotherapeutic effect of *Garcinia kola* biflavonoid fractions on serum atherogenic risk predictor indices of P407 induced hyperlipidemic rats. All treated groups had significantly ($p<0.05$) higher HDL-c/TC ratio except root biflavonoid treated group when compared to hyperlipidemic control but only the seed biflavonoid fraction treated group had HDL-c/TC ratio greater than 0.30. The LDL-c/HDL-c ratio of all treated groups was significantly ($p<0.05$) lower than that of hyperlipidemic control with their LDL-c/HDL-c ratio less than 2.3. However, only the seed fraction and atorvastatin significantly ($p<0.05$) reduced log (TG/HDL-c) compared to other induced treated groups.

Table 4.5: Phytopreventive Effect of *Garcinia kola* Biflavonoid Fractions on Serum Atherogenic Risk Predictor Indices of P407 Induced Hyperlipidemic Albino Rats

Group (n=6)	HDL-c/TC	LDL-c/HDL-c	Log(TG/HDL-c)
NC	0.56±0.06[d]	0.53±0.18[a]	-0.32±0.16[a]
P407	0.07±0.02[a]	9.16±1.89[b]	1.00±0.07[d]
ATV+P407	0.15±0.02[a]	0.68±0.23[a]	0.98±0.09[d]
RBBF+P407	0.44±0.02[c]	0.45±0.10[a]	0.25±0.03[b]
SBBF+P407	0.31±0.04[b]	1.10±0.29[a]	0.37±0.04[b]
SBF+P407	0.27±0.10[b]	1.19±0.88[a]	0.59±0.15[c]

Values are means ± Standard deviations

Values with different superscripts down the column differ significantly ($p<0.05$)

Values of HDL-c/TC ratio >0.30 and values of LDL-c/HDL-c ratio <2.3 indicate reduced risk of peripheral arterial disease (Ojiakor and Nwanjo, 2005)

NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), ATV+P407: Atorvastatin (10mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), RBBF+P407: Root bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBBF+P407: Stem bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBF+P407: Seed Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg)

TC: Total cholesterol, TG: Triacylglycerol, HDL-c: High density lipoprotein cholesterol, LDL-c: Low density lipoprotein cholesterol

Table 4.6: Phytotherapeutic Effect of *Garcinia kola* Biflavonoid Fractions on Serum Atherogenic Risk Predictor Indices of P407 Induced Hyperlipidemic Albino Rats

Group (n=6)	HDL-c/TC	LDL-c/HDL-c	Log(TG/HDL-c)
NC	0.56 ± 0.06^{e}	0.53 ± 0.18^{a}	-0.32 ± 0.16^{a}
P407	0.07 ± 0.02^{a}	9.16 ± 1.89^{b}	1.00 ± 0.07^{d}
P407+ATV	0.22 ± 0.01^{cd}	0.79 ± 0.16^{a}	0.78 ± 0.02^{c}
P407+RBBF	0.12 ± 0.05^{ab}	0.66 ± 0.30^{a}	0.99 ± 0.02^{d}
P407+SBBF	0.17 ± 0.07^{bc}	0.50 ± 0.42^{a}	0.95 ± 0.12^{d}
P407+SBF	0.31 ± 0.07^{d}	0.41 ± 0.19^{a}	0.56 ± 0.08^{b}

Values are means ± Standard deviations
Values with different superscripts down the column differ significantly ($p<0.05$)
Values of HDL-c/TC ratio >0.30 and values of LDL-c/HDL-c ratio <2.3 indicate reduced risk of peripheral arterial disease (Ojiakor and Nwanjo, 2005)
NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), P407+ATV: P407 Induced Hyperlipidemic Rats (500mg/kg)+Atorvastatin (10mg/kg), P407+RBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Root bark Biflavonoid Fraction (200mg/kg), P407+SBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Stem bark Biflavonoid Fraction (200mg/kg), P407+RBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Seed Biflavonoid Fraction (200mg/kg)
TC: Total cholesterol, TG: Triacylglycerol, HDL-c: High density lipoprotein cholesterol, LDL-c: Low density lipoprotein cholesterol

4.3.3 Effects of *Garcinia kola* Biflavonoid Fractions on Serum Liver Damage Indicators, Liver Function Parameters and Fasting Blood Glucose of P407 Induced Hyperlipidemic Albino Rats

4.3.3.1 Serum liver damage indicators

The phytopreventive and phytotherapeutic effects of *Garcinia kola* biflavonoid fractions on serum liver damage indicators of P407 induced hyperlipidemic rats presented in Figures 4.3 and 4.4 showed that P407 significantly ($p<0.05$) increased serum aspartate aminotransferase (AST) but had no significant ($p>0.05$) effect on alkaline phosphatase (ALP) and alanine aminotransferase (ALT) levels of hyperlipidemic rats when compared to normal rats.

In the phytopreventive study, serum AST and ALP levels were significantly ($p<0.05$) reduced by the root biflavonoid fraction and atorvastatin respectively when compared to the hyperlipidemic control (Fig. 4.3).

The phytotherapeutic group showed that the root bark and stem bark fractions significantly ($p<0.05$) reduced serum ALP and AST levels respectively when compared to hyperlipidemic control (Fig 4.4). However, serum ALT level of all groups in both studies were not significantly ($p>0.05$) different (Figs 4.3 and 4.4).

4.3.3.2 Liver function parameters and fasting blood glucose

Serum concentrations of total protein (TP), albumin (ALB), total bilirubin (TB), conjugated bilirubin (CB) and fasting blood glucose (FBG) of the phytopreventive study is shown in Table 4.7. There was no significant ($p>0.05$) difference in the concentrations of serum TP

and CB of all the groups. Both atorvastatin and biflavonoid fractions significantly ($p<0.05$) reduced TB concentration when compared to hyperlipidemic control. Although, a significant ($p<0.05$) decrease in the serum ALB concentration of hyperlipidemic control compared to the normal control was observed, treatments (atorvastatin and biflavonoid fractions) had no significant ($p>0.05$) effect when compared to hyperlipidemic control.

Table 4.8 shows serum TP, ALB, TB, CB and FBG concentrations of phytotherapeutic study. Treatments (atorvastatin and *Garcinia kola* biflavonoid fractions) had no significant ($p>0.05$) effect on serum TB concentration when compared to hyperlipidemic and normal control. Serum ALB concentration of all induced groups was significantly ($p<0.05$) lower than normal control while no significant ($p>0.05$) change was observed in TP and CB concentrations of all the groups.

The FBG level of all the groups in both studies did not significantly ($p>0.05$) change as well (Tables 4.7 and 4.8).

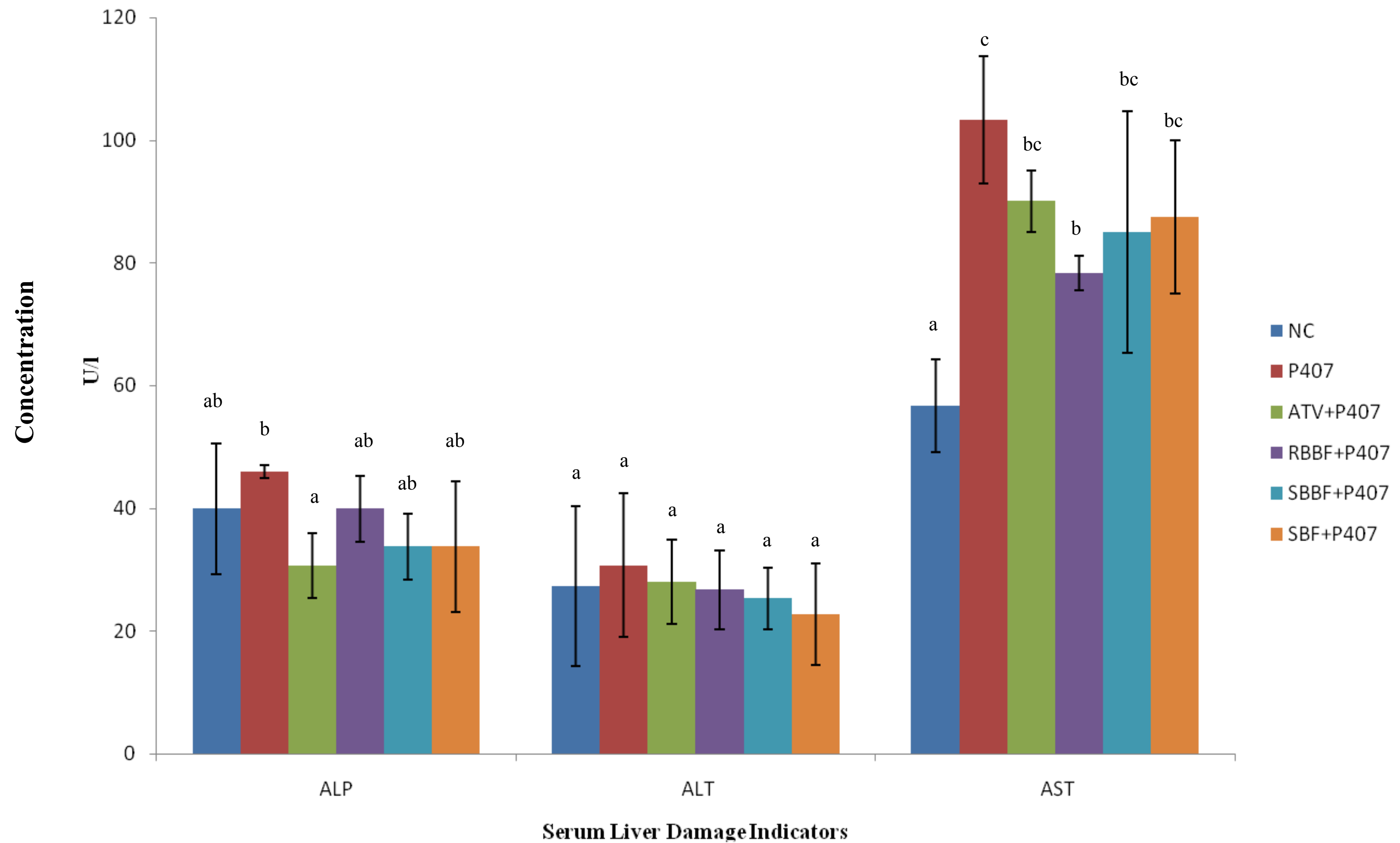


Figure 4.3: Phytopreventive Effect of *Garcinia kola* Biflavonoid Fractions on serum liver damage indicators of P407 Induced Hyperlipidemic Albino Rats.

ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, AST: Aspartate transaminase
Bars bearing different alphabets on each bar cluster, differ significantly ($p<0.05$)

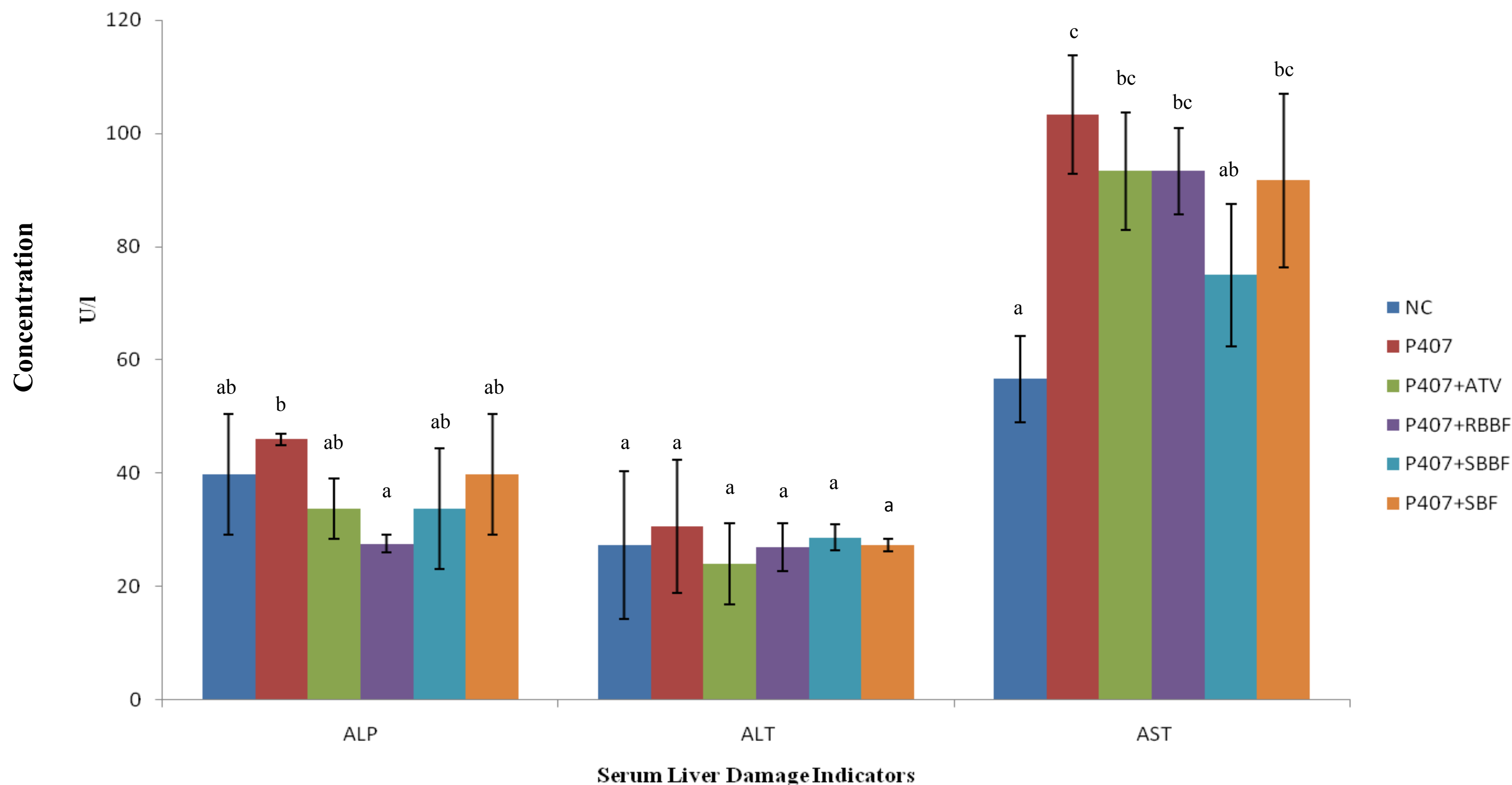


Figure 4.4: Phytotherapeutic Effect of *Garcinia kola* Biflavonoid Fractions on serum liver damage indicators of P407 Induced Hyperlipidemic Albino Rats.

ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, AST: Aspartate transaminase
Bars bearing different alphabets on each bar cluster, differ significantly (p<0.05)

Table 4.7: Phytopreventive Effect of *Garcinia kola* Biflavonoid Fractions on Liver Function Parameters of P407 Induced Hyperlipidemic Albino Rats

Group (n=6)	TP (g/dl)	ALB (g/dl)	TB (mg/dl)	CB (mg/dl)	FBG (mg/dl)
NC	$8.69{\pm}0.72^{a}$	$4.16{\pm}1.00^{b}$	$0.24{\pm}0.07^{a}$	$0.14{\pm}0.03^{a}$	$115.20{\pm}4.76^{a}$
P407	$9.13{\pm}0.85^{a}$	$2.99{\pm}0.86^{a}$	$0.86{\pm}0.07^{b}$	$0.20{\pm}0.05^{a}$	$116.40{\pm}5.55^{a}$
ATV+P407	$8.95{\pm}1.63^{a}$	$3.71{\pm}0.71^{ab}$	$0.29{\pm}0.04^{a}$	$0.15{\pm}0.07^{a}$	$120.00{\pm}2.08^{a}$
RBBF+P407	$8.55{\pm}0.45^{a}$	$3.58{\pm}0.47^{ab}$	$0.30{\pm}0.12^{a}$	$0.16{\pm}0.09^{a}$	$116.40{\pm}3.74^{a}$
SBBF+P407	$8.69{\pm}1.37^{a}$	$3.46{\pm}0.26^{ab}$	$0.27{\pm}0.07^{a}$	$0.17{\pm}0.09^{a}$	$120.00{\pm}2.87^{a}$
SBF+P407	$8.78{\pm}1.80^{a}$	$3.67{\pm}0.57^{ab}$	$0.32{\pm}0.03^{a}$	$0.18{\pm}0.05^{a}$	$115.80{\pm}6.43^{a}$

Values are means ± Standard deviations
Values with different superscripts down the column differ significantly ($p<0.05$)
NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), ATV+P407: Atorvastatin (10mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), RBBF+P407: Root bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBBF+P407: Stem bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBF+P407: Seed Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg)
TP: Total protein, ALB: Albumin, TB: Total bilirubin, CB: Conjugated bilirubin, FBG: Fasting blood glucose

Table 4.8: Phytotherapeutic Effect of *Garcinia kola* Biflavonoid Fractions on Liver Function Parameters of P407 Induced Hyperlipidemic Albino Rats

Group (n=6)	TP (g/dl)	ALB (g/dl)	TB (mg/dl)	CB (mg/dl)	FBG (mg/dl)
NC	8.69±0.72[a]	4.16±1.00[b]	0.24±0.07[a]	0.14±0.03[a]	115.20±4.76[a]
P407	9.13±0.85[a]	2.99±0.86[a]	0.86±0.07[b]	0.20±0.05[a]	116.40±5.55[a]
P407+ATV	8.60±0.85[a]	3.39±0.20[a]	0.49±0.05[ab]	0.23±0.02[a]	111.60±6.49[a]
P407+RBBF	8.51±0.37[a]	3.23±0.20[a]	0.55±0.15[ab]	0.18±0.07[a]	106.80±4.16[a]
P407+SBBF	8.54±1.14[a]	3.18±0.21[a]	0.43±0.18[ab]	0.20±0.09[a]	108.40±3.12[a]
P407+SBF	8.25±0.44[a]	3.39±0.15[a]	0.55±0.20[ab]	0.17±0.02[a]	105.80±7.90[a]

Values are means ± Standard deviations
Values with different superscripts down the column differ significantly ($p<0.05$)
NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), P407+ATV: P407 Induced Hyperlipidemic Rats (500mg/kg)+Atorvastatin (10mg/kg), P407+RBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Root bark Biflavonoid Fraction (200mg/kg), P407+SBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Stem bark Biflavonoid Fraction (200mg/kg), P407+SBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Seed Biflavonoid Fraction (200mg/kg)
TP: Total protein, ALB: Albumin, TB: Total bilirubin, CB: Conjugated bilirubin, FBG: Fasting blood glucose

4.3.4 Effects of *Garcinia kola* Biflavonoid fractions on Kidney Function Parameters of P407 Induced Hyperlipidemic Albino Rats

Serum creatinine and urea concentrations after preventive and therapeutic administration of root bark, stem bark and seed biflavonoid fractions of *Garcinia kola* are shown in Table 4.9 and 4.10 respectively. The results showed no significant (p>0.05) difference in creatinine and urea concentrations of all the groups in both studies (Tables 4.9 and 4.10)

Table 4.9: Phytopreventive Effect of *Garcinia kola* Biflavonoid Fractions on Kidney Function Parameters of P407 Induced Hyperlipidemic Albino Rats

Group (n=6)	Creatinine (mg/dl)	Urea (mg/dl)
NC	0.54±0.17[a]	31.86±7.20[a]
P407	0.59±0.19[a]	33.75±5.15[a]
ATV+P407	0.51±0.20[a]	35.90±2.53[a]
RBBF+P407	0.51±0.34[a]	44.18±7.47[a]
SBBF+P407	0.50±0.17[a]	36.72±4.79[a]
SBF+P407	0.56±0.17[a]	34.51±4.17[a]

Values are means ± Standard deviations
Values with different superscripts down the column differ significantly ($p<0.05$)
NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), ATV+P407: Atorvastatin (10mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), RBBF+P407: Root bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBBF+P407: Stem bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBF+P407: Seed Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg)

Table 4.10: Phytotherapeutic Effect of *Garcinia kola* Biflavonoid Fractions on Kidney Function Parameters of P407 Induced Hyperlipidemic Albino Rats

Group (n=6)	Creatinine (mg/dl)	Urea (mg/dl)
NC	0.54±0.17[a]	31.86±7.20 [a]
P407	0.59±0.19[a]	33.75±5.15 [a]
P407+ATV	0.51±0.34[a]	33.67±7.52[a]
P407+RBBF	0.43±0.17[a]	36.26±2.32[a]
P407+SBBF	0.57±0.39[a]	35.62±3.31 [a]
P407+SBF	0.48±0.19[a]	36.88±1.88 [a]

Values are means ± Standard deviation
Values with different superscripts down the column differ significantly (p<0.05)
NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), P407+ATV: P407 Induced Hyperlipidemic Rats (500mg/kg)+Atorvastatin (10mg/kg), P407+RBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Root bark Biflavonoid Fraction (200mg/kg), P407+SBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Stem bark Biflavonoid Fraction (200mg/kg), P407+SBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Seed Biflavonoid Fraction (200mg/kg)

4.4 Changes in Haematological Parameters, Body and Organ Weights of the Experimental Rats

4.4.1 Effects of *Garcinia kola* Biflavonoid Fractions on Some Haematological Parameters of P407 Induced Hyperlipidemic Rats

Tables 4.11 and 4.12 show the phytopreventive and phytotherapeutic effects of oral administration of *Garcinia kola* biflavonoid fractions on some haematological parameters of P407 induced hyperlipidemic rats respectively. The results showed no significant ($p>0.05$) difference in the levels of haemoglobin (Hb), lymphocytes, Mean corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV), Packed Cell Volume (PCV) and White Blood Cell (WBC) count in all the groups. Hyperlipidemia significantly ($p<0.05$) increased platelet count compared to control and treated group (Tables 4.11 and 4.12). There is insignificant ($p>0.05$) decrease in the level of neutrophils of all treated groups compared to hyperlipidemic group (Tables 4.11 and 4.12). However, therapeutic administration of the seed biflavonoid fraction significantly ($p<0.05$) increased RBC when compared to all the groups except stem bark biflavonoid fraction treated group (Table 4.12) while preventive administration of *G. kola* biflavonoid fractions had no significant effect on RBC of all the groups (Table 4.11).

Table 4.11: Phytopreventive Effect of *Garcinia kola* Biflavonoid Fractions on Some Haematological Parameters

Group (n=6)	Hb (mg/dl)	Lym (%)	Neut (%)	MCH (pg)	MCHC (g/dl)	MCV (fl)	PCV (%)	PLT ($\times10^3$/ul)	RBC ($\times10^6$/ul)	WBC ($\times10^3$/ul)
NC	13.53±1.22[a]	81.80±6.31[a]	5.13±0.46[a]	20.43±1.15[a]	33.23±1.02[a]	63.13±3.46[a]	42.17±2.31[a]	601.43±64.90[a]	6.46±0.29[a]	17.97±2.08[a]
P407	13.23±1.04[a]	77.47±0.60[a]	7.43±0.98[b]	20.33±1.23[a]	33.03±1.27[a]	61.53±2.97[a]	40.10±1.97[a]	1154.00±25.36[d]	6.11±0.27[a]	19.60±1.11[a]
P407+ ATV	13.13±0.51[a]	82.07±9.55[a]	6.59±0.70[ab]	19. 37±1.31[a]	32.67±0.06[a]	59.33±4.06[a]	40.20±1.54[a]	772.00±80.52[bc]	6.81±0.70[a]	18.17±1.45[a]
P407+RBBF	13.07±0.19[a]	72.50±0.26[a]	5.24±0.80[ab]	19.20±0.78[a]	32.63±1.12[a]	58.80±0.96[a]	40.10±1.81[a]	792.00±19.05[c]	6.82±0.83[a]	17.73±3.64[a]
P407+SBBF	12.27±0.29[a]	82.83±6.31[a]	5.45±1.10[ab]	18.53±0.65[a]	32.27±0.72[a]	57.40±1.12[a]	38.67±1.16[a]	651.33±99.00[ab]	6.62±0.12[a]	18.17±2.48[a]
P407+SBF	13.23±0.55[a]	81.93±2.80[a]	5.67±1.50[ab]	19.57±1.86[a]	32.20±0.35[a]	60.75±5.06[a]	41.10±1.34[a]	744.33±93.09[ab]	6.80±0.56[a]	17.60±1.65[a]

Values are means ± Standard deviations

Values with different superscripts down the column differ significantly ($p<0.05$)

NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), ATV+P407: Atorvastatin (10mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), RBBF+P407: Root bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBBF+P407: Stem bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBF+P407: Seed Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg)

Hb: Haemoglobin, Lym: Lymphocytes, Neut: Neutrophils, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration, MCV: Mean corpuscular volume, PCV: Packed Cell Volume, PLT: Platelet, RBC: Red Blood Cell, WBC: White Blood Cell

Table 4.12: Phytotherapeutic Effect of *Garcinia kola* Biflavonoid Fractions on Some Haematological Parameters

Group (n=6)	Hb (mg/dl)	Lym (%)	Neut (%)	MCH (pg)	MCHC (g/dl)	MCV (fl)	PCV (%)	PLT ($\times10^3$/ul)	RBC ($\times10^6$/ul)	WBC ($\times10^3$/ul)
NC	13.53 ± 1.22^a	81.80 ± 6.31^a	5.13 ± 0.46^a	20.43 ± 1.15^a	33.23 ± 1.02^a	63.13 ± 3.46^a	42.17 ± 2.31^a	601.43 ± 64.90^a	6.46 ± 0.29^{ab}	17.97 ± 2.08^a
P407	13.23 ± 1.04^a	77.47 ± 0.60^a	7.43 ± 0.98^b	20.33 ± 1.23^a	33.03 ± 1.27^a	61.53 ± 2.97^a	40.10 ± 1.97^a	1154.00 ± 25.36^c	6.11 ± 0.27^a	19.60 ± 1.11^a
P407+ ATV	11.40 ± 2.69^a	75.27 ± 12.08^a	7.10 ± 1.41^{ab}	20.23 ± 0.64^a	32.33 ± 1.00^a	62.35 ± 2.20^a	37.20 ± 6.32^a	815.33 ± 56.58^b	6.16 ± 0.14^a	16.87 ± 1.37^a
P407+RBBF	13.17 ± 0.15^a	81.37 ± 8.23^a	6.40 ± 1.21^{ab}	20.33 ± 0.15^a	32.37 ± 1.14^a	61.90 ± 1.87^a	40.70 ± 1.98^a	723.00 ± 45.03^b	6.44 ± 0.05^{ab}	16.40 ± 4.37^a
P407+SBBF	11.40 ± 3.05^a	76.13 ± 7.92^a	7.10 ± 0.28^{ab}	19.80 ± 2.00^a	31.03 ± 0.97^a	63.77 ± 4.49^a	39.13 ± 3.78^a	612.00 ± 68.46^a	6.90 ± 0.22^{bc}	16.30 ± 1.52^a
P407+SBF	13.33 ± 0.46^a	82.83 ± 6.90^a	6.40 ± 1.21^{ab}	18.73 ± 0.95^a	31.03 ± 1.18^a	60.47 ± 0.87^a	41.07 ± 3.09^a	786.67 ± 23.96^b	7.12 ± 0.59^c	17.83 ± 2.04^a

Values are means ± Standard deviations
Values with different superscripts down the column differ significantly ($p<0.05$)
NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), P407+ATV: P407 Induced Hyperlipidemic Rats (500mg/kg)+Atorvastatin (10mg/kg), P407+RBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Root bark Biflavonoid Fraction (200mg/kg), P407+SBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Stem bark Biflavonoid Fraction (200mg/kg), P407+SBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Seed Biflavonoid Fraction (200mg/kg)
Hb: Haemoglobin, Lym: Lymphocytes, Neut: Neutrophils, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration, MCV: Mean corpuscular volume, PCV: Packed Cell Volume, PLT: Platelet, RBC: Red Blood Cell, WBC: White Blood Cell

4.4.2 Effects of *Garcinia kola* Biflavonoid Fractions on Body Weight of P407 Induced Hyperlipidemic Albino Rats

The change in body weights of the phytopreventive study is shown in Table 4.13. The result showed a significant ($p<0.05$) increase in the body weight change of root bark biflavonoid treated group when compared to all other groups.

Table 4.14 presents the change in body weights of rats in the phytotherapeutic study. The seed biflavonoid fraction significantly ($p<0.05$) reduced change in body weight when compared to all the groups except stem bark fraction.

There was no significant ($p>0.05$) difference in the feed intake between the groups during the period of study (Tables 4.13 and 4.14)

4.4.3 Effects of *Garcinia kola* Biflavonoid Fractions on Percentage Organ Weight of P407 Induced Hyperlipidemic Albino Rats

Table 4.15 shows the percentage heart, kidney, liver and spleen weights of the phytopreventive study of orally administered *Garcinia kola* biflavonoid fractions. Liver and spleen weights were significantly ($p<0.05$) reduced by atorvastatin and all biflavonoid fractions while only root bark and stem bark fractions significantly ($p<0.05$) reduced heart weight when compared to hyperlipidemic control. However, there was no significant ($p>0.05$) change in kidney weight of all the groups.

In the therapeutic study, the percentage organ weights of P407 induced hyperlipidemic rats treated with *Garcinia kola* biflavonoid fractions presented in Table 4.16 showed no significant ($p>0.05$) change in heart and kidney weights of all the groups. All treated groups

had significantly ($p<0.05$) reduced spleen weights when compared to hyperlipidemic control. Despite the significant ($p<0.05$) increase in liver weights, biflavonoid fractions and atorvastatin had no significant ($p>0.05$) effect when compared to hyperlipidemic control.

Table 4.13: Phytopreventive Effect of *Garcinia kola* Biflavonoid Fractions on Body Weight of P407 Induced Hyperlipidemic Albino Rats

Group	Mean Daily Feed Intake (g/100g/day)			Mean Initial Body weight (g)	Mean Final Body Weight (g)	Change in Body Weight (g)
	Week 1	Week 2	Week 3			
NC	70.56±2.40[a]	68.80±1.44[a]	69.32±3.06[a]	207.50	235.75	28.50±14.52[a]
P407	66.92±3.50[a]	68.68±1.40[a]	68.12±0.61[a]	164.00	193.33	29.33±13.87[a]
ATV+P407	70.80±0.80[a]	68.00±2.00[a]	70.00±2.00[a]	189.75	216.00	26.25±9.21[a]
RBBF+P407	69.20±1.20[a]	67.60±2.12[a]	68.92±0.61[a]	181.75	229.75	48.00±9.02[b]
SBBF+P407	67.32±1.20[a]	66.68±3.06[a]	67.72±2.05[a]	184.00	213.50	29.50±11.70[a]
SBF+P407	65.88±1.70[a]	67.48±1.67[a]	68.40±1.44[a]	193.80	221.60	27.80±10.66[a]

Values are means ± Standard deviations
Values with different superscripts down the column differ significantly (p<0.05)
NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), ATV+P407: Atorvastatin (10mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), RBBF+P407: Root bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBBF+P407: Stem bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBF+P407: Seed Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg)

Table 4.14: Phytotherapeutic Effect of *Garcinia kola* Biflavonoid Fractions on Body Weight of P407 Induced Hyperlipidemic Albino Rats

Group	Mean Daily Feed Intake (g/100g/day)			Mean Initial Body weight (g)	Mean Final Body Weight (g)	Change in Body Weight (g)
	Week 1	Week 2	Week 3			
NC	70.56±2.40 ^{a}	68.80±1.44 ^{a}	69.32±3.06 ^{a}	207.50	235.75	28.50±14.52 ^{b}
P407	66.92±3.50 ^{a}	68.68±1.40 ^{a}	68.12±0.61 ^{a}	164.00	193.33	29.33±13.87 ^{b}
ATV+P407	68.12±2.34 ^{a}	68.12±2.28 ^{a}	68.52±1.29 ^{a}	191.33	221.00	29.67± 10.01 ^{b}
RBBF+P407	68.40±1.44 ^{a}	67.32±2.36 ^{a}	67.72±2.05 ^{a}	189.50	215.00	25.50±12.23 ^{b}
SBBF+P407	67.72±2.41 ^{a}	66.92±1.66 ^{a}	69.60±1.44 ^{a}	197.00	216.25	16.75±5.12 ^{ab}
SBF+P407	66.67±1.40 ^{a}	67.02±1.66 ^{a}	67.48±2.20 ^{a}	194.67	200.67	6.00±2.64 ^{a}

Values are means ± Standard deviations
Values with different superscripts down the column differ significantly (p<0.05)
NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), P407+ATV: P407 Induced Hyperlipidemic Rats (500mg/kg)+Atorvastatin (10mg/kg), P407+RBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Root bark Biflavonoid Fraction (200mg/kg), P407+SBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Stem bark Biflavonoid Fraction (200mg/kg), P407+SBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Seed Biflavonoid Fraction (200mg/kg)

Table 4.15: Phytopreventive Effect of *Garcinia kola* Biflavonoid Fractions on Percentage Organ Weight of P407 Induced Hyperlipidemic Albino Rats

Group (n=6)	Heart (g)	Kidney (g)	Liver (g)	Spleen (g)
NC	$0.29{\pm}0.03^{ab}$	$0.55{\pm}0.04^{a}$	$3.25{\pm}0.44^{ab}$	$0.42{\pm}0.09^{a}$
P407	$0.31{\pm}0.04^{b}$	$0.61{\pm}0.08^{a}$	$4.66{\pm}0.88^{c}$	$0.87{\pm}0.17^{b}$
ATV+P407	$0.29{\pm}0.02^{ab}$	$0.55{\pm}0.04^{a}$	$3.57{\pm}0.39^{b}$	$0.44{\pm}0.97^{a}$
RBBF+P407	$0.26{\pm}0.02^{a}$	$0.51{\pm}0.08^{a}$	$2.94{\pm}0.12^{a}$	$0.38{\pm}0.08^{a}$
SBBF+P407	$0.26{\pm}0.01^{a}$	$0.54{\pm}0.03^{a}$	$3.25{\pm}0.28^{ab}$	$0.45{\pm}0.06^{a}$
SBF+P407	$0.28{\pm}0.01^{ab}$	$0.52{\pm}0.05^{a}$	$3.13{\pm}0.24^{ab}$	$0.38{\pm}0.05^{a}$

Values are means ± Standard deviations
Values with different superscripts down the column differ significantly ($p<0.05$)
NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), ATV+P407: Atorvastatin (10mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), RBBF+P407: Root bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBBF+P407: Stem bark Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg), SBF+P407: Seed Biflavonoid Fraction (200mg/kg)+P407 Induced Hyperlipidemic Rats (500mg/kg)

Table 4.16: Phytotherapeutic Effect of *Garcinia kola* Biflavonoid Fractions on Percentage Organ Weight of P407 Induced Hyperlipidemic Albino Rats

Group (n=6)	Heart (g)	Kidney (g)	Liver (g)	Spleen (g)
NC	$0.29{\pm}0.03^{a}$	$0.55{\pm}0.04^{a}$	$3.25{\pm}0.44^{a}$	$0.42{\pm}0.09^{a}$
P407	$0.31{\pm}0.04^{a}$	$0.61{\pm}0.08^{a}$	$4.66{\pm}0.88^{b}$	$0.87{\pm}0.17^{c}$
P407+ATV	$0.29{\pm}0.03^{a}$	$0.59{\pm}0.03^{a}$	$4.37{\pm}1.23^{ab}$	$0.63{\pm}0.07^{b}$
P407+RBBF	$0.28{\pm}0.03^{a}$	$0.56{\pm}0.02^{a}$	$4.07{\pm}0.78^{ab}$	$0.52{\pm}0.05^{ab}$
P407+SBBF	$0.27{\pm}0.04^{a}$	$0.56{\pm}0.03^{a}$	$4.19{\pm}0.86^{ab}$	$0.63{\pm}0.11^{b}$
P407+SBF	$0.30{\pm}0.02^{a}$	$0.57{\pm}0.05^{a}$	$4.33{\pm}0.84^{ab}$	$0.72{\pm}0.10^{b}$

Values are means ± Standard deviations
Values with different superscripts down the column differ significantly ($p<0.05$)
NC: Normal Control Rats, P407: Poloxamer 407 Induced Rats (500mg/kg), P407+ATV: P407 Induced Hyperlipidemic Rats (500mg/kg)+Atorvastatin (10mg/kg), P407+RBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Root bark Biflavonoid Fraction (200mg/kg), P407+SBBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Stem bark Biflavonoid Fraction (200mg/kg), P407+SBF: P407 Induced Hyperlipidemic Rats (500mg/kg)+Seed Biflavonoid Fraction (200mg/kg)

CHAPTER FIVE

DISCUSSION

Plants are being used in virtually all cultures as a source of medicine especially in Africa and Asia (Girach *et al.,* 2003). The preliminary screening of the root bark, stem bark and seed fractions in the present study reveals the presence of alkaloids, flavonoids, saponins, tannins, glycosides, cardiac glycosides, unsaturated steroids and triterpenes (Table 4.2). The natural substances contained in plants are the reason for their therapeutic use or as precursors for the synthesis of useful drugs (Abolaji *et al*., 2007). This could be responsible for the wide use of *Garcinia kola* in ethnomedicine. These natural substances from plants also termed "secondary metabolites" have been reported to be the potent source for their excellent biological activities; anticancer, antioxidant activities, hepatoprotective and hypolipidemic effects (Adaramoye *et al*., 2005b; Adaramoye and Adeyemi, 2006a, b; Adebayo *et al*., 2010).

The determination of total flavonoid content in the different plant part fractions after following an established method of extracting kolaviron (Iwu *et al*., 1990c) was to give an idea of the quantity of flavonoid present in each fraction. Kolaviron (KV), the predominant biflavonoid complex present in *Garcinia kola* consist of kolaflavanone, Garcinia Biflavanone 1 (GB1) and Garcinia Biflavanone 2 (GB2); all of which possess orthodihydroxyl groups in their structures, therefore the choice of aluminium chloride method described by Chang *et al*., (2002). Aluminium chloride forms stable acid complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with the

orthodihydroxyl groups in the A- or B-ring of flavonoids (Mabry *et al*., 1970) which has maximum absorbance at 415nm. This result showed the root bark which had the highest yield contained the least total flavonoid, followed by the stem bark and the seed with the highest total flavonoid content. This could explain one of the reasons; the seed despite its low yield of 5.92% in accordance with literature, is the most used part for extracting kolaviron (Iwu *et al*., 1987; Iwu *et al*., 1990b; Braide, 1991; Farombi, 2000; Olaleye *et al*., 2000; Adaramoye *et al*., 2005b; Farombi *et al*., 2005; Adaramoye and Adeyemi, 2006b; Adegboye *et al*., 2008; Adaramoye, 2010; Ijomone *et al*., 2011).

Hyperlipidemia is responsible for the onset and progression of atherosclerosis (Poss *et al*, 2011), a major risk factor in the development of coronary heart diseases (CHDs) such as ischemic heart disease, myocardial infarction and stroke (Vaziri and Norris, 2011). CHDs are responsible for about 17 million deaths in the world (Boutayeb, 2006).

In this research, Poloxamer 407 (P407) was the hyperlipidemic inducing agent (Johnston, 2004) used and the results showed significant (p<0.05) increase in total cholesterol (TC) (25 fold), TG (11 fold) and LDL-c (23 fold) in hyperlipidemic models administered 500mg/kg body weight of P407 intraperitoneally (Joo *et al*, 2010), indicating successful induction of hyperlipidemia.

In clinical practice, effective and intensive lipid-lowering is important in order to reduce (Nissen *et al*., 2004; Murphy *et al*., 2007) and prevent (Abdulazeez, 2011) CHDs. *Garcinia kola* (root bark, stem bark and seed) biflavonoid fractions significantly (p<0.05) reduced TC, TG and LDL-c concentrations in both studies. These reductions in TC, TG and LDL

levels suggest the ameliorative capabilities of *Garcinia kola* fractions in hyperlipidemia (Figures 4.1 and 4.2).

The elevation of TC concentration in this study was achieved by the indirect stimulation of HMG CoA reductase activity, a rate limiting step in cholesterol biosynthesis, following an intraperitoneal (i.p) injection of P407 (Johnston, 2004) . Hence the possible TC lowering effects of *Garcinia kola* (root bark, stem bark and seed) biflavonoid fractions could be attributed to decreased activity of hepatic HMG CoA reductase and/or stimulation of cholesterol-7-alpha-hydroxylase, which converts cholesterol into bile acids. Besides, the standard drug (Atorvastatin) used in this study inhibits HMG CoA reductase. The results obtained in this work conform to earlier report by Patel *et al*. (2009) that flavonoids possess antilipidemic activity.

Increase in TG concentration following P407 i.p. injection results primarily from an inhibition of TG degradation, P407 directly inhibits capillary lipoprotein lipase (LPL) responsible for plasma TG hydrolysis (Johnston, 2004). Although the standard drug might not decrease TG concentrations by activating lipoprotein lipase, the biflavonoid fractions from *Garcinia kola* could have reduced TG levels by activating endothelium bound lipoprotein lipase which hydrolyses the triacylglycerol into fatty acid hence decreasing triacylglycerol levels as reported by Sikarwar and Patil (2011).

LDL (low density lipoprotein) is responsible for transporting cholesterol to the body cells. It transports about 60-70% of total cholesterol. Therefore, an increase in TC level consequently increases LDL-c. The increased LDL-c which was not removed in the process of lipid metabolism is likely to flow into the subendothelial space, as well as to undergo

oxidation. The oxidized LDL is phagocytized by the scavengers of macrophages and the fat-laden macrophage is left with the lipid core filled with cholesterol after necrocytosis and then arteriosclerosis is initiated (Beckmann *et al*., 2009). This work shows significant (p<0.05) reduction in LDL-c levels by all *Garcinia kola* biflavonoid fractions (Figs 4.1 and 4.2). This result is in accordance with the work of Baum *et al*. (1998), who reported that flavonoids may work by increasing LDL-c receptors densities in the liver binding to apolipoprotein B thereby making liver cells more efficient in removing LDL-c from blood.

HDL-c act as cholesterol scavengers, they pick up excess cholesterol and cholesterol esters from the blood and peripheral tissues to the liver where it is broken down to bile acids. It plays an important role in reducing blood and peripheral cholesterol concentrations and inhibits formation of atherosclerotic plaque in the aorta (Karmarkar, 2008; Kim *et al*, 2008a), therefore known as the protective cholesterol. The result of the present studies showed significant (p<0.05) increase in HDL-c by root bark and stem bark biflavonoid fractions (Fig 4.1) and significant (p<0.05) increase in HDL-c by seed biflavonoid fraction (Fig 4.2). This could possibly be due to increasing activity of lecithin-cholesterol acyl transferase (LCAT), an enzyme responsible for incorporating free cholesterol into HDL-c (Geetha *et al*., 2011), there by promoting reverse cholesterol transport and competitively inhibiting the uptake of LDL-c by endothelial cells and preventing the generation of oxidized LDL-c (Yokozawa *et al*., 2006).

Atherogenic risk predictor indices (HDL-c/TC, LDL-c/HDL-c and log (TG/HDL-c)) are mathematical relationships between TC, TG, LDL-c and HDL-c that have been successfully used as markers of assessing atherosclerosis development (Kastelein *et al*., 2008) and extent of CHDs. HDL-c/TC ratio greater than 0.3 and LDL-c/HDL-c ratio less

than 2.3 indicate a reduced risk of peripheral arterial disease (Ojiakor and Nwanjo, 2005). However, log (TG/HDL-c) has been considered the most accurate in determining the extent of atherosclerosis and the risk of myocardial infarction (Dobiasova *et al*., 2005). It has been suggested that log (TG/HDL-c) values of -0.3 to 0.1 are associated with low, 0.1 to 0.24 with medium and above 0.24 with high cardiovascular disease risk (Dobiasova, 2006).

According to these ranges provided by Ojiakor and Nwanjo (2005) for HDL-c/TC and LDL-c/HDL-c ratios and Dobiasova (2006) for log (TG/HDL-c), the most important atherogenic risk predictor index, all induced animals in both studies were at high cardiovascular disease risk after intra peritoneal administration of 500mg/kg body weight of P407 with log (TG/HDL-c) > 0.24 but the biflavonoid fractions did significantly ($p<0.05$) reduce this risk (Tables 4.5 and 4.6) suggesting anti-atherogenic abilities of *Garcinia kola* (root bark, stem bark and seeds) biflavonoid fractions, hence reduction in development of cardiovascular disease.

The HDL-c/LDL-c ratio also affects other balances in the body; according to Ginsberg and Goldberg (2001) as HDL-c/LDL-c ratio increases, platelet aggregation decreases. The results of this research agrees to these reports as seen in Tables 4.5 and 4.6, where LDL-c/HDL-c ratio which is the inverse of HDL-c/LDL-c ratio was significantly ($p<0.05$) decreased by *Garcinia kola* biflavonoid fractions, the platelet count of the hyperlipidemic rats were also significantly ($p<0.05$) decreased (Tables 4.11 and 4.12).

Alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are enzymes localized in the liver (Mohan *et al*., 2007) with ALT being the most specific liver injury marker (Sherlock and Dooley, 2002) and a more

selective liver parenchymal enzyme (Afzal *et al*., 2013). An injury or damage to liver is assessed by increased serum concentrations of these hepatic enzymes (ALP, ALT and AST) (Huo *et al*., 2011) associated with massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver (Shankar *et al*., 2008) conforming to the degree of injury (Venukumar and Latha, 2004).

The result of this work showed induction with Poloxamer 407 (P407) did not cause any damage to the rats' liver as only AST level of hyperlipidemic control was significantly ($p<0.05$) higher than normal (Figs 4.3 and 4.4) and AST is not specific to the liver. It is also found in skeletal and cardiac muscles (Kachmar, 1976; Varley *et al*., 1984) as well as brain, kidney, pancreas, lungs, red and white blood cells (Giboney, 2005), therefore damage to these tissues can also result in an elevated serum AST level. The result of this work conforms to earlier report that P407 does not cause hepatic injury or damage (Johnston *et al*., 1999). The rise in AST level may therefore have resulted from the effect of hyperlipidemia on other tissues other than the liver. However, this effect was reduced in the groups administered the root bark and stem bark biflavonoid fractions (Figs 4.3 and 4.4). Though, this study could not ascertain the particular tissue which was damaged, the ability of the fractions to decrease the increased AST level indicates preventive and therapeutic (Figs 4.3 and 4.4) efficacy of these fractions on some damaging effects of hyperlipidemia.

Serum levels of total protein (TP), albumin (ALB), total bilirubin (TB) and conjugated bilirubin (CB) are indices used to assess liver function as well as disease progression (Saad *et al*., 2006; Uthandi and Ramasamy, 2011). Albumin is an important component in plasma antioxidant activity as it primarily binds free fatty acids, divalent cations and hydrogen oxychloride (HOCl) (Akiniibu *et al*., 2008) and also transports bilirubin to the liver for

conjugation. The present study revealed significantly ($p<0.05$) lower levels of albumin in hyperlipidemic control when compared to normal control (Tables 4.7 and 4.8). The decreased levels of albumin may be due to reduction in protein uptake/absorption from the intestine as a result of a high levels of lipid in the blood (Rolls, 2000), an indication of reduced production by the liver due to its reduced function or due to increased mobilization from the circulation to enhance tissue repair (Lu *et al*., 2007).

The results of this work also showed a significant ($p<0.05$) increase in the levels of TB after induction (Tables 4.7 and 4.8). This could be attributed to the significant ($p<0.05$) decrease in ALB which is required to transport bilirubin, a metabolic end product of erythrocyte lysis to the liver for conjugation with glucuronic acid in the liver to increase its water solubility (Sreepriya *et al*., 2001; Ravi *et al*., 2005; Rajib *et al*., 2009). Although, *Garcinia kola* (root bark, stem bark and seed) biflavonoid fractions did not significantly ($p<0.05$) increase ALB levels, they significantly ($p<0.05$) reduced TB levels when compared to hyperlipidemic control in the phytopreventive studies (Table 4.7). The ability of the biflavonoid fractions to protect against further reduction in albumin resulting from hyperlipidemia and reducing TB levels may be attributed to their free radical scavenging properties (Farombi *et al*., 2002) thereby improving the function of the liver.

Glucose is an essential nutrient for the human body; it is one of the clinically important carbohydrates. It is the major energy source for many cells, which depend on the bloodstream for a steady supply. Blood glucose levels, therefore, are carefully maintained. The liver plays a central role in this process by balancing the uptake and storage of glucose via glycogenesis and the release of glucose via glycogenolysis and gluconeogenesis (Nordlie *et al*., 1999). The results of this research showed no significant ($p>0.05$) increase

in fasting blood glucose levels in all the groups (Tables 4.7 and 4.8). This further suggests that the gluconeogenic function of the liver is not impaired and sensitivity of beta cells to glucose is normal. Although, previous studies have reported that P407 inductions in neonatal rats resulted in hyperglycemia, an impaired response to the glucose tolerance test (Portha *et al*., 1979) and loss of β-cell sensitivity to glucose (Giroix *et al*., 1983), making it a good experimental model for testing anti diabetic agents (Szkudelski, 2001; Yamagishi *et al*., 2001, Bharti *et al*., 2012). The difference observed in this present study and the earlier reported works could be attributed to the dose of P407 administered, the frequency of administration (Bharti *et al*., 2012), the fact that the experimental animals used in this study were not neonates and the possibility of strain difference.

Kidney helps in maintaining homeostasis of the body by reabsorbing important material and excreting waste products (James *et al*., 2010). Its function is usually assessed by the levels of urea and creatinine in the blood; creatinine being the most specific. Urea is the main end product of protein catabolism; it varies directly with protein intake and inversely with the rate of excretion. Renal diseases which diminish the glomerular filtration lead to urea retention and decrease in urea is seen in severe liver disease with destruction of cells leadings to impairment of the urea cycle (Ranjna, 1999). Creatinine is a waste product formed in muscle by creatine metabolism. Creatine is synthesized in the liver, passes into the circulation and is taken up almost entirely by skeletal muscle. Its retention in the blood is evidence of kidney impairment. There was no significant ($p>0.05$) change in urea and creatinine levels of all the groups in both studies (Tables 4.9 and 4.10) indicating no impairment of kidney function by the administration of P407 and *Garcinia kola* biflavonoid fractions did not also significantly ($p>0.05$) exert any effect.

Assessment of haematological parameters can be used not only to determine the extent of deleterious effect of a disease on the blood of an animal, but also to explain blood relating functions of a plant part or its extract (Yakubu *et al*., 2007). Haematological evaluation in this study showed a significant (p<0.05) increase in platelet count of hyperlipidemic control compared to normal control (Tables 4.11 and 4.12). The high platelet count could be attributed to increased platelet aggregation, resulting in influx of platelet from the bone marrow due to increased thromboxane A_2 biosynthesis (Vaughn *et al*., 2002) which is in agreement with other studies (Juhan-Vague and Vague, 1990; Eckel *et al*., 2002). However, the *Garcinia kola* (root bark, stem bark and seed bark) biflavonoid fractions significantly (p<0.05) reduced the platelet count compared to the hyperlipidemic control. The *Garcinia kola* biflavonoid fractions could have done this by lowering intracellular Ca^{2+} levels; altering the metabolism of adenosine 3',5' cyclic-monophosphate (cAMP) thereby inhibiting thromboxane A_2 formation which was stimulated by high levels of cholesterol as suggested by Roy *et al*. (1999), Kang *et al*. (2001) and Sandhar *et al*. (2011). Thus preventing or reducing thrombosis. This is an additional benefit in the use of *Garcinia kola* in managing cardiovascular diseases and in agreement with studies that have shown flavonoids prevent platelet activity-related thrombosis (Harnafi and Amrani, 2007).

The seed biflavonoid fraction significantly (p<0.05) increased red blood cell (RBC) count compared to all the groups except the stem bark fraction (Table 4.12). This could be that the stem bark and seed biflavonoid fractions stimulate the cytokine erythropoietin which increases RBC lines as reported by Ahumibe and Braide (2009) which is based on the antioxidant property of *Garcinia kola* (Wang *et al*., 2000) thereby elevating the total antioxidant capacity of the blood. This implies that the stem bark and seed biflavonoid

fractions have erythropoietic, protective and stimulating effect validating the use of the seed extracts in antisickling studies (Adejumo *et al*., 2011).

This research also showed significant ($p<0.05$) increase in neutrophils level of the hyperlipidemic control group (Tables 4.11 and 4.12) compared to normal control. Hypercholesterolemia enhances serum chemokine (C-X-C motif) ligand 1 (CXCL1) levels, which promotes neutrophil mobilization via chemokine (C-X-C motif) receptor 2 (CXCR2). Hence, hyperlipidemia disturbs the tightly regulated cytokine system controlling neutrophil homeostasis at various levels, ultimately increasing peripheral neutrophil counts (Drechsler *et al*., 2010). This also buttresses the successful induction of hyperlipidemia in this study. Although, *Garcinia kola* fractions insignificantly ($p>0.05$) decreased neutrophil levels, an increase in concentration or duration of treatment could be of advantage in preventing or reducing the risk of atherosclerosis which is associated with high level of neutrophils (Giugliano *et al*., 2010). There was no deleterious effect on lymphocytes (Lym), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV), Packed Cell Volume (PCV) and White Blood Cell (WBC) by administration of P407 and biflavonoid fractions in both studies (Tables 4.11 and 4.12).

This work showed no significant ($p>0.05$) difference in feed intake and body weight change of the hyperlipidemic control compared to the normal control (Tables 4.13 and 4.14). A result agreeing with an earlier report by Johnston *et al*., (1999) that P407 does not significantly ($p>0.05$) affect body weight of animals. However, the root bark biflavonoid fraction was seen to significantly ($p<0.05$) increase body weight change (Table 4.13) while the seed fraction significantly ($p<0.05$) reduced body weight compared to all other groups

(Table 4. 14). The significant ($p<0.05$) increase in weight despite the insignificant ($p>0.05$) difference in feed intake (Table 4.13) by the root biflavonoid fraction implies that it has the ability to produce healthy, lean meat in animals because of its lipid lowering abilities seen in Figures 4.1 and 4.2 as suggested by Iwuji and Herbert (2012). The significant ($p<0.05$) weight loss by seed fraction (Table 4.14) is in accordance with earlier reports which attributed the weight loss to the anti-atherogenic effect of *Garcinia kola* seed biflavonoid fraction (Adaramoye *et al*., 2005b) and its anti-adipogenic effect, which inhibits the accumulation of lipid droplets in fat cells (Noboru, 2001). However, weight loss without decreased feed intake has been documented (Weight *et al*., 1987; Esonu *et al*., 2003) as observed in this study (Table 4.14).

The significantly ($p<0.05$) increased liver and spleen weights in hyperlipidemic control (Tables 4.15 and 4.16) could be as a result of fatty infiltration of the liver and increased blood cells in the spleen as suggested by Sheyla *et al.* (2005) and Amr and Abeer (2011) respectively. However, all biflavonoid fractions reduced the spleen (Table 4.16) and liver weights to normal but only the root bark and stem bark fractions reduced heart weight (Table 4.15) which was not significantly ($p>0.05$) increased by P407. These reductions in organ weights show the protective or restoring potentials of the plant biflavonoid fractions on the organs (heart, liver, spleen).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The following are the major findings of this study

- The phytochemical screening of the root bark, stem bark and seed biflavonoid fractions from *Garcinia kola* showed the presence of carbohydrates, flavonoids, cardiac glycosides, alkaloids, saponins and tannins.

- The seed biflavonoid fraction had the highest total flavonoid content (197.29± 0.64mg quercetin equivalent/g of dry fraction) compared to root bark (63.99± 2.94) and stem bark (96.54± 1.70).

- The seed biflavonoid fraction had significantly ($p<0.05$) higher total cholesterol (79%) and triacylglycerol (67%) reducing activity in the phytopreventive study while root bark and stem bark biflavonoid fractions had the highest TC (77%, 78%) reducing activity in the phytotherapeutic study.

- Preventive administration of root bark and stem bark fractions significantly ($p<0.05$) increased high density lipoprotein cholesterol (122%, 98%) whereas therapeutic administration of seed biflavonoid fraction increased HDL-c (62%) significantly ($p<0.05$).

- The root bark and stem bark fractions ameliorated the undesirable effects of Poloxamer 407 (P407) induced hyperlipidemia as indicated by the significant ($p<0.05$) reduction in aspartate aminotransferase (AST) and total bilirubin levels.
- *G. kola* biflavonoid fractions significantly ($p<0.05$) reduced platelet count in both studies and red blood cell count was increased in the phytotherapeutic study by the stem bark and seed fractions
- Preventive administration of *G. kola* fractions reduced liver and spleen weights.

6.2 Conclusion

The results of this research have shown that the root bark, stem bark and seed biflavonoid fractions of *Garcinia kola* possess both preventive and therapeutic effects in the management of hyperlipidemia and favourably compare with the standard drug (atorvastatin). However, the seed biflavonoid fraction had the highest phytopreventive effect; the root bark and stem bark, the most phytotherapeutic effect. The preventive and therapeutic administration of the biflavonoid fractions from *G. kola* (root bark, stem bark and seeds) had no deleterious effect on liver function, fasting blood glucose, kidney function, haematological parameters and body and organ weights. The phytopreventive and phytotherapeutic effect/potential of the different biflavonoid fractions of *G. kola* revealed in this study justifies the wide use in ethnomedicine.

6.3 Recommendations

1. The *G. kola* biflavonoid fractions should be subjected to further purification and subsequent characterization.

2. Specific bioactive components contained in this plant parts should also be employed in an *in vivo* study of transgenic hyperlipidemic knockout mice.

3. Kolaviron, the most predominant biflavonoid complex in *Garcinia kola* seeds is made up of Garcinia Biflavanone 1 (GB1), Garcinia Biflavanone 2 (GB2) and kolaflavanone. Therefore, further studies should be directed towards elucidating which of these components has the highest lipid lowering effect and also elucidate if the effect is synergistic as well as the mechanism of action as hypolipidemic agents.

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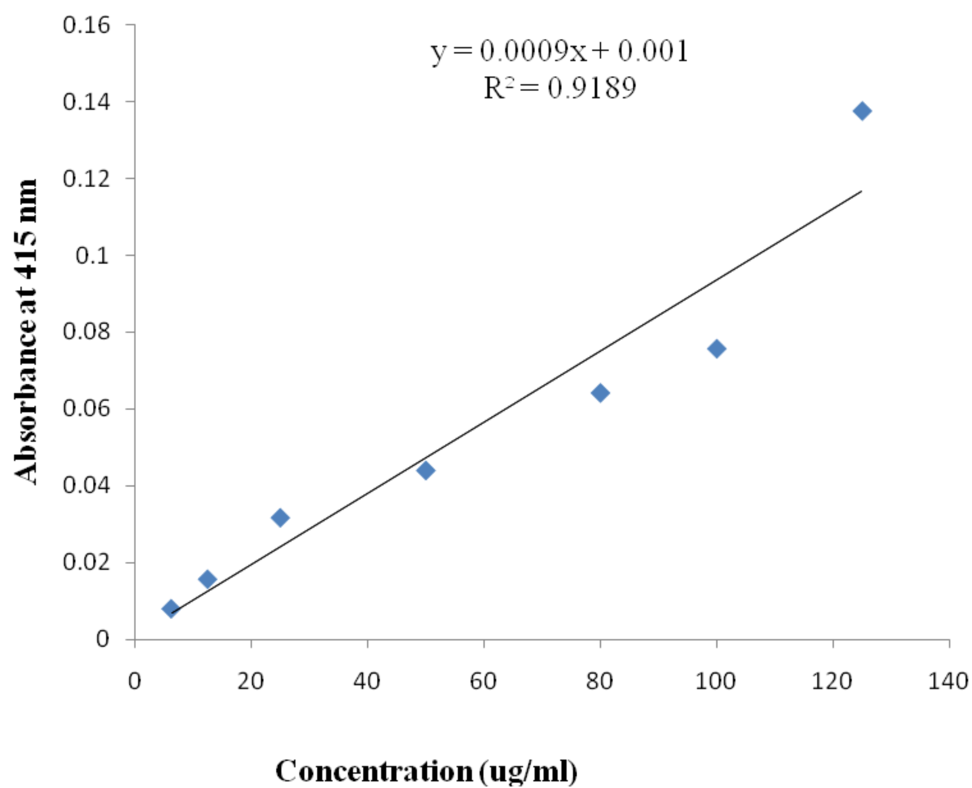
APPENDICES

APPENDIX 1.0



Quercetin Standard Curve

APPENDIX 2.0

Seeds of *Garcinia kola*

APPENDIX 3.0

Seed pods of *Garcinia kola*

APPENDIX 4.0

Stem bark of *Garcinia kola*

APPENDIX 5.0

Root of *Garcinia kola*