ANTI-HYPERLIPIDEMIC POTENTIAL OF *VITEX DONIANA*ETHANOLEXTRACTS ON POLOXAMER 407 INDUCED HYPERLIPIDEMIC AND NORMAL RATS.

BY

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BY

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NIGERIA.

SEPTEMBER, 2014

DECLARATION

I hereby declare that this thesis entitled Anti-Hyperlipidemic Potential of *VitexDoniana* Extracts on Poloxamer 407 Induced Hyperlipidemic and Normal Ratshas been carried out by me in the Department of Biochemistry,Ahmadu Bello University Zaria, under the supervision of Dr. D.B. James and Professor S.E. Atawodi, and that it was the record of my own research work. This work has not been part of any presentation for any degree or diploma. The sources of the information are specifically acknowledged by mean of references.

Victor DuniyaSheneni

_____ _____ _____

Name of student Signature Date

CERTIFICATION

This thesis entitled Anti-Hyperlipidemic Potential of *VitexDoniana* Extracts on Poloxamer 407 Induced Hyperlipidemic and Normal Ratsby Victor DuniyaShenenimeets the regulations governing the award of Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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(Dean, Postgraduate school) Signature Date

DEDICATION

This research work is dedicated to God Almighty, my family and supervisors for their support, understanding and love.

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My greatest thanksgo to God Almighty who has been the source of wisdom and understanding in my academic life that has led to the completion of this research work.

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ABSTRACT

Anti-hyperlipidemic potential of extracts (aqueous, 70% methanol, 70% ethanol and 70%, acetone) of *Vitexdoniana* leaves, stem bark and root bark on poloxamer 407 induced hyperlipidemic and normal rats was investigated. Phytochemical screening of the extracts revealed the presence of flavonoids, saponins, cardiac glycosides, alkaloids and tannins in the leaves, stem bark and root bark. The average total polyphenol contents of the leaves ethanol (36.11±3.13mg/g gallic acid) and methanol (35.75±1.72mg/g gallic acid) extracts were significantly ($p<0.05$) higher when compared with that of acetone and aqueous extracts. The $IC_{50}$of the leaves ethanol extract (0.227mg/ml) was lowerthan that ofstem bark ethanol extract (0.236mg/ml) and root ethanol extract (0.561mg/ml). Screening the extracts for the most potent anti-hyperlipidemicactivityreveals that ethanolic extracts of root bark and leaveshas the highest percentage reduction of total cholesterol (51.98%) and triacylglycerol (50.75%) respectively. The most abundant phytochemical in the most potent extract is flavonoid (4.605±0.077%) in the leaves and the least is tanins (0.035±0.008%) in the root bark extract. The LD_{50} of both leaves and stem bark was greater than 5000mg/kg body weight and that of root bark was 948.68 mg/kg body weight. Hyperlipidemic control rats significantly ($p<0.05$) increased total Cholesterol (TC), Triacylglycerol (TAG), Low density lipoprotein (LDL-c) andsignificantly ($p<0.05$) decreased High density lipoprotein (HDL-c) compared to other groups.Atherogenic risk factor of all induced treated rats shows a significant ($p<0.05$) lower levels of LDL-c/HDL-c, Log (TAG/HDL-c) and significant ($p<0.05$) higher level of HDL-c /TC ratio. There was no significant ($p>0.05$) change between normal control rats and normal treated rats in lipid profile parameters and atherogenic indices. The level of liver marker enzymes (ALT, ALP, AST) and liver function parameter (TB, IB) were significantly

($p<0.05$)higher, and lower (TB, DB) in hyperlipidemic control groups compared to all other groups. The invivo antioxidant activity shows a significantly ($p<0.05$) higher level of TBARS and a significant ($p<0.05$) lower level of SOD and CAT in hyperlipidemic groups when compared to all treated groups. In both liver and kidney, the leaves and stem bark extract significantly ($p<0.05$) lowers levels of TBARS of normal control rats compared to normal treated and all induced treated groups. All the extractsactivity in the liver and leaves extract in the kidney of normal rats show a significant higher level of CAT compared with other treated groups. The study shows that *vitexdoniana*possesses anti-hyperlipidemic potential.

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LIST OF ABBREVIATIONS

ALP	=	Alkaline Phosphatase
ALT	=	Alanine Amino Transferase
AST	=	Aspartate Amino Transferase
BW	=	Body Weight
CAT	=	Catalase
DB	=	Direct Bilirubin
DPPH	=	1, 1 diphenyl-2-picrylhydrazyl
HDL-c	=	High Density Lipoprotein Cholesterol
IB	=	Indirect Bilirubin
IC_{50}	=	Median Inhibitory Concentration
LD_{50}	=	Median Lethal Dose
LDL-c	=	Low Density Lipoprotein Cholesterol
MDA	=	Malondialdehyde
PCV	=	Packed Cell Volume
SOD	=	Superoxide Dismutase
TAG	=	Triacylglycerol
TB	=	Total Bilirubin
TBA	=	Thiobarbituric Acid

TBARS	=	Thiobarbituric Acid Reactive Substances
TC	=	Total Cholesterol
TP	=	Total Protein
V. doniana	=	*Vitexdoniana*
WHO	=	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

Polyphenols arenaturalorganic chemicals characterized by the presence of large number of phenol structural units (Quideau*et al.*, 2011). The most research-informed and chemistry-aware definition of polyphenol is termed the White–Bate-Smith–Swain–Haslam (WBSSH) definition (Haslam and Cai, 1994) which describes the polyphenol as moderately water-soluble compounds, with molecular weight of 500–4000 Dalton,having more than 12 phenolic hydroxyl groups and with 5–7 aromatic rings per 1000 Da.The number and characteristics of the phenol structures underlie the unique physical, chemical, and biological properties of a particular member of the polyphenol class(Quideau *et al.*, 2011).

Over the past 10 years, researchers and food manufacturers have become increasingly interested in polyphenols. The main reason for this interest is the recognition of the antioxidant properties of polyphenols, their great abundance in our diet, and their probable role in the prevention of various diseases associated with oxidative stress, such as cardiovascular, cancer and neurodegenerative diseases. As the major active substance found in many medicinal plants, itmodulates the activity of a wide range of enzymes and cell receptors.Polyphenols as antioxidants, helps in addressing and reversing the problems caused by oxidative stress to the walls of arteries, create a heart-healthy environment by curbing the oxidation of low density lipoprotein cholesterol which stops the potential for atherosclerosis, and they help relieve chronic pain, as seen in conditions like rheumatoid arthritis, due to their anti-inflammatory properties. In addition to having

antioxidant properties, polyphenols have several other specific biological actions that are yet to be understood(Quideau *et al*., 2011).

Plants has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine (Hostettmann *et al.,* 2000). These plants continue to play an essential role in health care, with about 90% of the world's inhabitants depending mainly on traditional medicines for their primary health care (Hostettmann *et al.,* 2000). Recently, there has been an upsurge of interest in the therapeutic potentials of medicinal plants antioxidants reducing free radical related diseases. It has been mentioned that the antioxidant activity of plants might be due to their phenolic compounds (Cook and Samman, 1996).

Vitex doniana is a deciduous tree, usually 4-8m high, with a dense rounded crown. Its bark is light grey with numerous vertical fissures. The leaves are long stalked with 5-7 leaflets. The leaflets are usually widest towards the tip, more or less hairless. The fruits are ellipsoid to oblong, green turning black on ripening. It is a savanna species in wooded grassland and can also be found along forest edges. It is extremely widespread in tropical Africa. It is commonly known as Black Plum or African olive (Glew *et al*., 1997), Dinya (Hausa),Galbihi (Fulani),Oori-nla (Yoruba), Ucha coro (Igbo), and is wide spread in the southwestern Nigeria as a perennial tree. Earlier reports have shown that aqueous root bark extract of the plant can be used for the treatment of anaemia (Abdulrahman *et al*., 2010), methanolic stem bark extract can be used for the treatment of gastroenteritis, diarrhoea, dysentery (Kilani, 2006) and aqueous leaves and stem bark extracts for the treatment of liver disorder (James *et al*., 2010). The ability of an aqueous extract of *Vitex*

doniana stem bark to protect the liver of albino rats from carbon tetrachloride-induced liver damage was reported by Ladeji and Okoye (1996).

Hyperlipidemia is an elevation of one or more of the plasma lipids, including cholesterol, cholesterol esters, triglycerides and phospholipids (Raasch, 1988). It is the most common form of dyslipidemia. It is well established that elevated blood lipid levels (hyperlipidemia)constitute the primary risk factor for atherosclerosis (Saunders, 2007). There is now overwhelming evidence that, dietary factors, nutritional habits and genetic origin influence the risk of coronary artery diseases (Van Horn, 1997). Increased levels of high-density lipoprotein cholesterol (HDL-c) are associated with a decreased cardiovascular risk (Wanner and Quaschning 2001; Kourounakis*etal.*, 2002,). Predominant cardiovascular diseases associated with hyperlipidemia arehypertension, ischemic heart diseases, stroke, coronary heart diseases and atherosclerosis.They account for at least 80% of the burden of cardiovascular disease in both developing and developed countries, which shares many of the same common risk factors (Balakumar *et al.*, 2007). Hyperlipidemia is divided into primary and secondary subtypes. Primary hyperlipidemia is usually due to genetic causes such as a mutation in a receptor protein, while secondary hyperlipidemia arises due to other underlying causes such as diabetes. (Chait and Brunzell, 1990). Traditionally, factors such as hypercholesterolemia, cigarette smoking, diabetes mellitus and sedentary life style have been implicated in the development of hyperlipidemia and atherosclerotic cardiovascular disease (Frohlich and Lear, 2002).

1.1 Statement of Research Problem

Hyperlipidemia is one of the greatest risk factors contributing to the prevalence and severity of cardiovascular disease (Grundy, 1986). It accounts for about 56% of stroke, 18% of ischemic heart disease and more than 4 million deaths per year globally (WHO 2002).In Nigeria, it accounts for about (45–73) % death per year (Ebesunum *et. al.*, 2008). Cardiovascular diseases are one of the major causes of death worldwide (Murray and Lopez, 1996).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 failure.High levels of cholesterol particularly total cholesterol, triglycerides and low density lipoprotein cholesterol is mainly responsible for the onset of CHDs (Choudhary *et al.,* 2005). About 20% reduction of blood cholesterol level can decrease about 31% of CHD incidence and 33% of its mortality rate (Marzyieh *et al.*, 2007).

Cardiovascular disease covers a wide array of disorders, including disease of the cardiac muscle and of the vascular system supplying the heart, brain, and other vital organs (Bently *et al.*, 2002). Predominant cardiovascular diseases associated with hyperlipidemia are hypertension, ischemic heart disease, stroke, coronary heart disease and atherosclerosis (Balakumar *et al.*, 2007). Hyperlipidemia is asymptomatic, characterized by elevated serum total cholesterol, low density lipoprotein, very low density lipoprotein and decreased high density lipoprotein levels.Hyperlipidemia associated with lipid disorders are considered to cause atherosclerotic cardiovascular diseases (Saravanan *et. al.*, 2003). Among these are hypercholesterolemia, hypertriglyceridemia and ischemic heart disease (Kaesancini and Krauss, 1994).

1.2 Justification

Polyphenols are natural products found in fruits and vegetables, as well as in beverages such as tea and red wine. Recent datasuggests that diet rich in these compounds is associated with a decreased risk of cardiovascular diseases such as atherosclerosis, ischemic heart disease, stroke, coronary heart disease and hypertension. Their effects are also believed to underlie part of the improved cardiovascular health ascribed to the 'French paradox'. Polyphenols are hypothesized to provide cardio-protective effects through their ability to scavenge free radicals and inhibit lipid peroxidation. As antioxidants, they help in addressing and reversing the problems caused by oxidative stress to the walls of arteries, create a heart-healthy environment by curbing the oxidation of LDL cholesterol, and they help relieve chronic pain, as seen in conditions like rheumatoid arthritis, due to their anti-inflammatory properties.

Recent works are available on the scavenging potential of the aqueous extract of *Vitex doniana* in the treatment of carbon tetrachloride induced liver damage, but there is no documentated work on the effect of itsethanol extractson hyperlipidemic rats.Hence there is a need to investigate the effect of these extracts on hyperlipidemic rats.

1.3 Aim and Objectives

The general aim of this study is to investigate theantihyperlipidemic effect of ethanol extracts from different parts (leaves, stem and root bark) of*Vitex doniana* in therats, with a view of providing a pharmacological justification and for the use of the plant in the management, control and/or treatment of hyperlipidemic relateddiseases.

1.3.1 Specific objectives

1. To carry out the plant phytochemicalscreening, extracts total polyphenol and in vitro antioxidant activity.

2.To carry out in vivoantihyperlipidemic activity of the extractsand quantitative phytochemical of most potent extract.

3. To determine the effect of the extract onlipid profile andsomebiochemicalparameters of the hyperlipidemic and normal rats.

4.To determine the effect of the extract on lipid peroxidationand endogenous antioxidant enzymesin hyperlipidemic and normal rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1*Vitex Doniana*

Vitex doniana is a deciduous tree, usually 4-8m high, occasionally up to 15m, with a heavy rounded crown. The bark is rough, pale brown or greyish-white, rather smooth with narrow vertical features. The bases of old trees have oblong scales.Leaves are opposite, glabrous, 14-34 cm long, usually with 5 leaflets on stalks 6-14 cm long. Leaflets distinctly stalked ovate, obovate, elliptic or oblong, entire, 8-22 cm long, 2-9 cm wide. Leaf tips rounded or emarginate, leaf bases cuneate, dark green above, pale greyish-green below, thickly leathery, with a few scattered stellate hairs on the upper surface, otherwise without hairs. Flower petals are white except on largest lobe, which are purple, in dense opposite and axillary cymes. Flowers small, blue or violet, 3-12 cm in diameter, only a few being open at a time. The fruit is oblong, about 3 cm long, green when young, turning purplish-black on ripening and with a starchy black pulp. Each fruit contains 1 hard, conical seed, 1.5-2 cm long, and 1-1.2 cm wide (Burkill, 2000).

2.1.1 Habitat/Distribution

Vitex doniana is the most abundant and widespread of the genus occurring in savannah regions. It is planted as an ornamental shade tree. It occurs in variety of habitats, from forest to savanna, often in wet localities and along rivers, and on termite mounds, up to 2000m altitude. It occurs in regions with a mean annual rainfall of 750-2000mm and mean annual temperature of 10-30 degree Celsius.

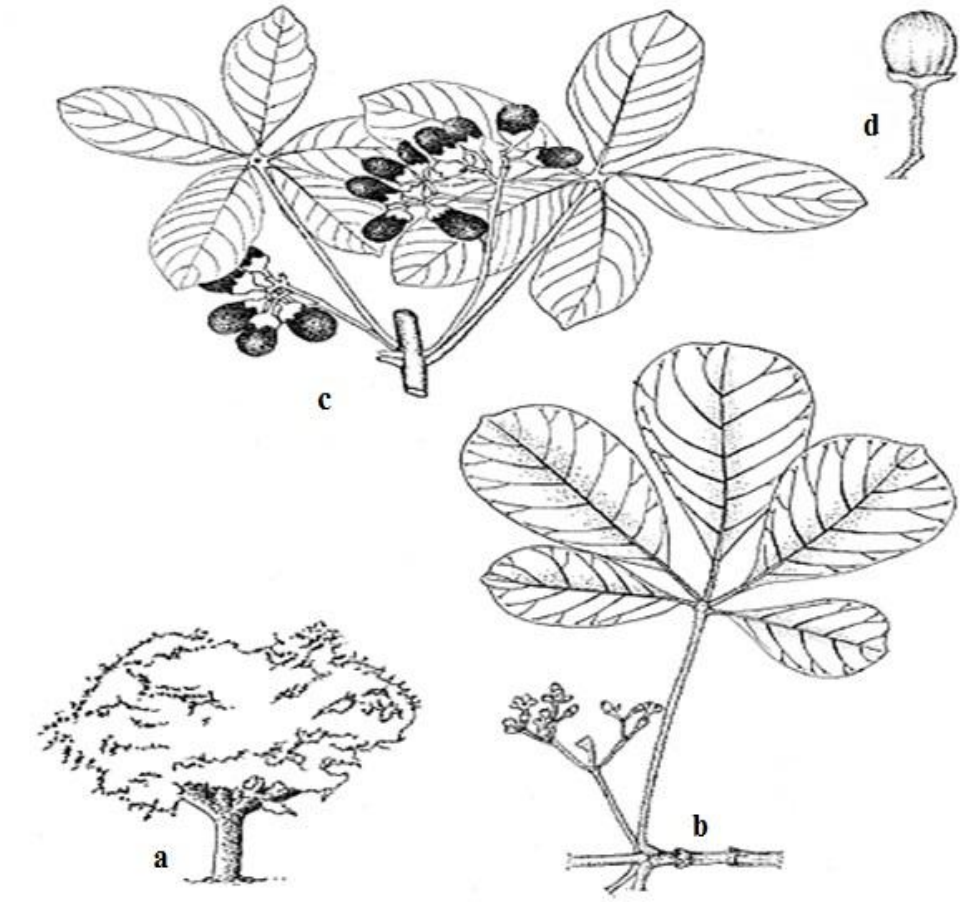


Plate 2.1 *Vitex doniana*Plant **(**Ky, 2008).

a. Full Plant

b. Part flowering branch

c. Part of fruiting branch

d. Fruit

2.1.2 Botanical classification of *vitex doniana*

The Taxonomic System of the Angiosperm Phylogeny Group (APG III) classification was used

Kingdom:Plantae

Phylum: Angiosperms

Sub-phylum: Eudicots

Class: Core eudicots

Sub-class: Asterids

Super order: Euasterids

Order: Lamiales

Family: Verbenaceae

Genus: *Vitex*

Species: *doniana*

Authority: Sweet

Name: *Vitex doniana* Sweet

2.1.3 Chemical constituents of *vitex doniana*

The mean values for the proximate composition of *Vitex doniana* (black plum) fruit are: moisture 487.7±0.5, ash 52.7±0.1, fat 30.0±0.4, fibre 67.3±0.7, protein 72.8±0.1 and carbohydrate 289.5±0.8 g kg^{-1} (Agbede and Ibitoye, 2007). Though it has lower vitamin C content when compared with other well-known fruits (LENNTECH, 2007), it is safe for consumption and could be a cheap source of raw material for juice production

(Nnajiofor, 2003).The proximate composition of *Vitex doniana* appears comparable and in some cases higher than the values reported for well-known fruits in the tropics (Agbede and Ibitoye, 2007). The macro-mineral contents of *Vitex doniana* (black plum) fruitare: calcium, magnesium, sodium, potassium and phosphorus. These macro-minerals are present at higher values than those reported for some well-known fruits (USDA, 2012) and consequently, the consumption of the fruit would help to meet the recommended levels of these minerals especially among the school children and adult weighing between 70–100 kg (LENNTECH, 2007). However, the K/Na ratio suggests the adequacy of Na in *Vitex doniana*. Fruits can be considered as rich source of crude fibre, all fall in the range of Recommended Dietary Allowance (RDA) for fibre in children, adults, pregnant and lactating mothers which are 19-25, 21-38, 28 and 29%, respectively (Ishida *et al*., 2000). Crude fibre is an important part of diet which decreases serum cholesterol levels,risk of coronary heart disease, hypertension, diabetes, colon and breast cancer (Ishida *et al*., 2000).

According to Nnamani *et al* (2009), the proximate analysis of leaves of *Vitex doniana* showed moisture contents of 10.8%, protein contents of 8.74% and carbohydrate value of 58.94%. The Micro nutrients showed high level of Ca (72.08mg/100g).Phytochemical reports on *Vitex* species indicate that they are rich sources of ketosteroids, iridiods, terpenoids, saponins and flavonoid glycosides (Ono *et al*., 2000). The investigation of some *Vitex* species have resulted in the isolation of iridoid glycosides named agnuside, eurostoside, negundoside (2α-p-hydroxybenzolymussaenosidic acid), 6α-phydroxybenzoylmussaenosidic acid, nishindaside and isonishindaside from the leaves.The phytochemical analysis of aqueous leaves extract of *V. doniana* revealed the

presence of alkaloids, terpenoids, flavonoids, saponins, tannins, phenols and absence of resins, steroids and glycosides (Ukwuani *et al*., 2012).

Agnuside and peduncullariside have been found to be present in the stem barks of*Vitex* species (Kuruuzum *et al*., 2003).Phytochemical analysis of ethanol stem bark extract revealed the presence of flavonoids, tannins, steroids, saponins, carbohydrates, terpenes and cardio-active glycosides (Abdulrahman*et al.*, 2012).

Phyto**chemical analysis** of the various parts (root bark, stem bark and leaves) of the plant extract revealed the presence of saponin, tannins, phenols, cardiac glycosides, flavonoids, sterols and triterpenes as well as high concentration of sodium, potassium, calcium, iron, phosphorus and sulphur (Abdulrahman and Onyeyili, 2006).

2.1.4Uses of the plant

1. *Functional uses*

The fruit is sweet and tastes like prunes; it is occasionally sold and eaten as food. It contains vitamins A and B and can be made into a jam (Katende, 1995). The leaves are often used as a herb and for cooking. The leaves, pods and seeds are used as fodders and a favourite tree for hanging bark beehives. The Wood is used for firewood, charcoal and timber. The tree produces a stalk-like termite-resistant timber which is quite hard and suitable for light building material, furniture, carvings and boats (Katende, 1995). The fruit can be made into wine, and pounded leaves can also be added to warm filtered grain beer and then drunk. The bark yields a dye that can be used for cloth (Katende, 1995).

2. *Medicinal use*

Vitex doniana is widely used in traditional system of medicine. The plant has been used as medication for liver disease, anodyne, stiffness, leprosy, backache, hemiplegia, conjunctivity, rash, measles, rachitis, febrifuge, as tonic galactagogue to aid milk production in lactating mothers, sedative, digestive regulator and treatment of eye troubles, kidney troubles and as supplement for lack of vitamin A and B (Burkill, 2000). It has also been recommended for treatment of disease conditions such as infertility, anemia, jaundice, dysentery, gonorrhea, headaches, diabetes, chickenpox, rash and fever (Beentje, 1994). Several authors have evaluated the medicinal application of the *V.doniana*. Sofowora, (1993) reported that various parts of the plant are used for treatment of several disorders which include rheumatism, hypertension, cancer and inflammatory diseases. It has been reported to have significant analgesic and anti-inflammatory activities mediated through sequential inhibition of the enzymes responsible for prostaglandin synthesis from arachidonic acid (Iwueke *et al.*, 2006). The essential oil extracted from the plant has been shown *in vitro* to have antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Klebsiella pneumoniae, Candida albicans, Streptococcus pyogens, Shigella dysenteriae, Listeria monocytogenes* and *Bacillus cereus* (Egharevba *et al.*, 2010). Research report by James *et al.*, (2010) on the antihepatotoxic effects of aqueous leaves and stem extracts of *V. doniana* showed that it was effective against carbon tetrachloride induced liver injury in rats. The anti-hypertensive effect of extract of stem bark of *V. doniana* has been reported by Olusola *et al*, (1997), and shows that the extract exhibited a marked dose-related hypotensive effect in both normotensive and hypertensive rats. Extracts of stem bark of *V. doniana* have also demonstrated some

level of *in vitro* trypanocidal activity against Trypanosoma *brucei brucei* (Atawodi, 2005).

2.2 Polyphenols

2.2.1 Classes

Polyphenols are natural organic chemicals characterized by the presence of large number of phenol structural units (Quideau *et al*., 2011). They are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8,000 polyphenols structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins.Theyare classified into the following groups:phenolic acids, flavonoidsand tannins (Jin Dai and Russell, 2010).

Phenolic acids can be divided into two classes: derivatives of benzoic acid such as gallic acid, and derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid. Caffeic acid is the most abundant phenolic acid in many fruits and vegetables, most often esterified with quinic acid as in chlorogenic acid, which is the major phenolic compound in coffee. Another common phenolic acid is ferulic acid, which is present in cereals and is esterified to hemicelluloses in the cell wall (D'Archivio*et. al.*, 2007).

Flavonoids are the most abundant polyphenols in our diets. The basic flavonoid structure is flavan nucleus, containing 15 carbon atoms arranged in three rings (C6-C3-C6). Flavonoids are themselves divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins, according to the oxidation state of the central carbon ring. Their structural variation in each subgroup is partly due to the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation. Some of the most

common flavonoids include quercetin, a flavonol abundant in onion, broccoli, and apple; catechin, a flavanol found in tea and several fruits; naringenin, the main flavanone in grapefruit; cyanidin-glycoside, an anthocyanin abundant in berry fruits (black currant, raspberry, blackberry, *etc.*); and daidzein, genistein and glycitein, the main isoflavones in soybean (D'Archivio *et. al.*, 2007).

Tannins are another major group of polyphenols in our diets and usually subdivided into two groups: hydrolysable tannins and condensed tannins. Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins. The great variety in the structure of these compounds is due to the many possibilities in forming oxidative linkage. Intermolecular oxidation reactions give rise to many oligomeric compounds having a molecular weight between 2,000 and 5,000 Daltons (Khanbabaee *et. al.*,1997). Condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond. They are also referred to as proanthocyanidins because they are decomposed to anthocyanidins through acid-catalyzed oxidation reaction upon heating in acidic alcohol solutions.

2.2.2 Extraction

The extraction of bioactive compounds from plant materials is the first step in the utilization of phytochemicals in the preparation of drugs. Phenolics can be extracted from fresh, frozen or dried plant samples. Usually before extraction plant samples are treated by milling, grinding and homogenization, which may be preceded by air-drying or freeze-drying. Generally, freeze-drying retains higher levels of phenolic content in plant samples than air-drying (Abascal*et.al.* 2005).Solvent extractions are the most commonly used

procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability. It is generally known that the yield of chemical extraction depends on the type of solvents with varying polarities, extraction time andtemperature, sample-to-solvent ratio as well as on the chemical composition and physical characteristics of the samples. The solubility of phenolics is governed by the chemical nature of the plant sample, as well as the polarity of the solvents used. Plant materials may contain phenolics varying from simple (e.g., phenolic acids, anthocyanins) to highly polymerized substances (e.g. tannins) in different quantities. Moreover, phenolics may also be associated with other plant components such as carbohydrates and proteins. Therefore, there is no universal extraction procedure suitable for extraction of all plant phenolics.

Depending on the solvent system used during extraction, a mixture of phenolics soluble in the solvent will be extracted from plant materials. It may also contain some non-phenolic substances such as sugar, organic acids and fats. As a result, additional steps may be required to remove those unwanted components. Solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water. Selecting the right solvent affects the amount and rate of polyphenols extracted (Xu and Chang, 2007). In particular, methanol hasbeen generally found to be more efficient in extraction of lower molecular weight polyphenols whilethe higher molecular weight flavanols are better extracted with aqueous acetone (Metivier *et.al.,* 1980, Labarbe *et.al.* 1999).Ethanol is another good solvent for polyphenol extraction and is safe for human consumption (Shi *et.al.,* 2005).

2.2.3 Pharmacological action/effect

Polyphenols are a vast family of natural compounds exhibiting a variety of activities relevant for health-care endeavors such as protection of the cardiovascular system, improving performance impaired by old age or neurodegeneration, and prevention and therapy of cancer. The mechanisms of action of polyphenols are by no means limited to their reactivity as anti- or pro-oxidants. Members of the family are known to modulate signal-transducing proteins ranging from channels to cyclooxygenases. Several biochemical pathways underlying these effects have been identified. Effects on gene expression are important, and in fact much of the antioxidant activity of polyphenols seems to be actually mediated by redox-sensitive transcription factors and enzymes. The notoriously low bioavailability of these compounds is a major obstacle for their pharmacological exploitation.Once absorbed, polyphenols are subjected to 3 main types of conjugation: methylation, sulfation, and glucuronidation. Since they are ready-made Phase II metabolism substrates, they are rapidly converted by sulfo- and glucuronosyl-transferases in enterocytes into conjugates which are to a large extent re-exported to the intestinal lumen. Liver enzymes then intervene on the molecules which have entered the circulation, administering other rounds of Phase II metabolism. The metabolic fate in the liver of the conjugates that are produced in the intestine is not yet clear. After penetration into cells, they undergo 2 types of metabolism: methylation and deglucuronidation followed by sulfation (O'Leary *et. al.*, 2003). However, in the same conditions, conjugates are not metabolized. This could result from a lower rate of penetration into the cells or a lower affinity of the metabolizing enzymes for this substrate. A complex set of conjugating enzymes and carrier systems is probably involved in the regulation of uptake

and the production and release of the various polyphenol metabolites by the hepatocytes (O'Leary *et. al.*, 2003). The activity of these enzymes and carrier systems may depend on the nature of the polyphenol and may be influenced by genetic polymorphisms that lead to important inter-individual differences in the capacity to metabolize polyphenols.

2.3 Hyperlipidemia

2.3.1 Definition

Hyperlipidemia is the abnormal elevation of lipids in the blood, largely cholesterol and triglycerides. It is also known as hyperlipoproteinemia due to abnormal elevations of lipoproteins that transports lipids in the blood (Saunders, 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, triglycerides, phospholipids and free acids (Satyanarayana, 2002). 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 lipoprotein (LDL), and high-density liproteins (HDL). Most triglyceride is transported in chylomicrons or VLDL, and most cholesterol is 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 (CVDs) (Rerkasan *et al.,* 2008) and pancreatisis. 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).

2.3.2 Classification

Hyperlipidemias resulting from either elevations of cholesterol or triglyceride are called hypercholesterolemia or hypertriglyceridemia respectively and are both regarded as sub-categoreis 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).

1. *Primary hyperlipidemia*

This is as a result of a stimulation or deficiency in one or more enzymes such as lipoprotein lipase deficiency etc responsible in cholesterol and triglyceride metabolism could result in hyperlipidemia. It 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: elevations of chylomicrons (CM) resulting in elevations of triacylglycerol (TAG).
* Type II A: elevations of low density lipoprotein cholesterol (LDL-c) only
  Type II B: elevations of both LDL-c and TAG
* Type III: develops as result of defect in VLDL remnant clearance
* Type IV: characterized by hyper TAG

* Type V: characterized by elevated levels of CM and VLDL

2. *Secondary/Acquired hyperlipidemia*

This results from an underlying disease or treatment of other diseases, therefore called acquired hyperlipidemia. 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 factors associated with secondary hyperlipidemia are as follows:

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

2.3.3 Etiology of hyperlipidemia

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

Type I: is due to a deficiency of lipoprotein lipase (LPL) or altered apolipoprotein C2,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 (James *et. al.,* 2006). It is rare and usually present in childhood with eruptive xanthomata and abdominal colic. Complications include retinal vein occlusion, acute pancreatitis, steatosis and organomegaly, and lipaemia retinalis.

Type II A: may be sporadic (due to dietary factors), polygenic, or truly familiar as a result of a mutation either in the LDL receptor gene or the ApB gene (Rader *et.al.,* 2003). This form is also called familial hypercholesterolemia; it is very common characterized by tendon xanthoma, xanthelasma and premature cardiovascular disease (Tsouli *et. al*., 2005).

Type II B: have very high VLDL levels due to overproduction of substrates, including triglycerides, acetyl CoA, and an increase in B-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 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 (Fung *et. al*., 2011).

Type IV: also known as familial hypertriglyceridemia is an autosomal dominant condition. It is also a common type and can also result from metabolic/endocrine diseases, renal disease, liver disease, 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.

2.3.4 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), TAG, HDL directly while VLDL cholesterol levels are calculated by dividing the triglyceride value by 5 and

LDL cholesterol is calculated by subtracting HDL cholesterol and VLDL cholesterol from total cholesterol. When triglycerides are about 400mg/dl, LDL calculation is inaccurate, and specialized laboratory tests measuring direct LDL should be conducted. 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 (2002), the desirable levels of TC, TAG, HDL-c and LDL-c in mg/dl is <200, <150, >50 and <130 respectively and individual with levels above the borderline level of 200-239 for TC, 150-199 for TAG and 130-159 for LDL-c are considered hyperlipidemic.

2.3.5 Treatment of hyperlipidemia.

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

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 a eliminate alcohol intake
* Consumption of light fiber foods such as fruits, vegetables, whole grains and beans.

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.

3.*Medication*

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

*Statins: They act by inhibiting 3-hydroxy-3-methylglutary 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.

2.3.6 Experimental models of hyperlipidemia

Poloxamer Model:Poloxamer 407(P407) is a ubiquitous surfactant and non-ionic detergent. It is a tri-block copolymer composed of a central hydrophobic chain of polyoxypropylene flanked by two hydrophilic chains of polyozyethylene. P407 has unusual thermoreversible properties. It is a 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.P407 has also been found to induce dose dependent hyperlipidemia observed in experimental

animals after parenteral administration (Johnston and Palmer 1997; Palmer*et. al.*, 1998;Johnston, 2004). This is done by inhibition of lipoprotein lipase, which is an endothelial enzyme that is responsible for converting chylomicrons to chylomicrons remnants (Johnston and Palmer 1993;Johnston 2004) and indirectly inhibiting HMG CoA reductase, a rate limiting enzyme in cholesterol synthesis, hence hypertriglyceridemia and hypercholesterolemia respectively. The increase in TC and TG is evident 2 hours after a single intraperitoneal injection of 0.5-1g/kg of P407 (Palmer *et. al*., 1997). Repeated administration of P407 is associated with the development of atherosclerotic lesions in rodents and this model has been developed by Johnston and colleagues for the study of the pathogenesis and treatment of atherosclerosis (Palmer *et. al.*, 1998). It is non-toxic and safe for chronic administration and long term studies (Megallis *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).
* Isoproterenol: decreases lipid peroxidation generating free radicals that potentiate the raise of blood cholesterol (Sheela and Shymala, 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 earns excessive production of these two types of lipoproteins (Gehard, 2001).

2.3.7 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. Hyperlipidemia is one of the disease conditions that are 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 triglycerides 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 tratohepatitis, fibrosis and necrosis (Day and James, 1998).

2.3.8 Hyperlipidemia and kidney

Kidney helps in maintaining homeostasis of the body by reabsorbing important material and excreting waste products (James *et. al*., 2010). Hyperlipidemia is a major risk for cardiovascular diseae like atherosclerosis, which may result to End Stage Renal Disease (ESRD) (Kumari *et. al*., 2003). Hyperlipidemia specifically hypercholesterolemia can

induce or exacerbate glomerular injury in mammals (Grone *et. al*., 1994; Tolins *et. al*., 1992) 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 and Mackensen-Haen *et. al*., 1987). Hypercholesterolemia also results in interstitial fibrosis and tubular atrophy (Grone *et. al.*, 1996; Eddy, 1998). Hyperlipidemia can lead to ischemic nephropathy (Anja *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).

2.3.9 Hyperlipidemia and hematological 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 1999; 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 obviously 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.,* 2009). 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 lessons has been seen in human atherosclerosis specimens and murine models of atherosclerosis (Van Leeuwen *et. al*., 2008; Yvan-Charvet *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, phagocrytic capacity, and cytokine release (Soehnlein *et. al.*, 2009; Soehnlein and Lindbom, 2010). There is a positive correlation between plasma triglycerides 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 contains less myeloperoxidase, whereas plasma myeloperoxidase levels of 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 3

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

The *Vitex doniana* plants were harvested from its natural habitat at the Institute of Agricultural Research (IAR), Ahmadu Bello University (ABU), Zaria, Kaduna State in the month of April 2012, and authenticated at the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria with a voucher number 1162.

3.1.2Chemicals and reagents

All assays kits were from Randox laboratories Ltd. Ardmore, Co. Antrm UK. Chemicals and reagents used were of analytical grade unless otherwise stated. Folin Ciocalteu Phenol Reagent, Gallic Acid, Hydrogen Peroxide, 2,2-Diphenyl-2-picrylhydrazyl (Sigma-Aldrich), Sodium chloride, Sodium hydroxide, Sodium carbonate, Sodium bicarbonate, Acetic acid, Potassium dichromate, Potassium phosphate, Dipotassium phosphate,Trichoroacetic acid, Adrenalin, Thiobarbituric acid (Sigma-Aldrich) were all obtained from Okey Medicals Kaduna, Kaduna State.

3.1.3 Equipment

Sherwood Colorimeter 257, Grant JB Series Water Bath, Heraeus Labofuge 300 Centrifuge, Gallenkamp Incubator, MicropH 2000, RS-232C Weighing Balance, Thomas-Wile Laboratory mill.

3.1.4 Experimental animals

A total of one hundred and eight (108) apparently healthy rats of both sexesweighing between100 to 150 g were obtained from the Department of Physiology and Pharmacology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The rats were were kept in a well aerated cages andallowed to acclimatize for two weeks before the commencement of experiment. They were maintained on a commercial mash (PLS feed, Zaria, Kaduna state) and water *ad libitum* throughout the experiment.

3.2 Methods

3.2.1 Extraction

The collected plant sample was rinsed in clean water and dried at room temperature for two weeks. The dried plant sample was ground into powder using a mortar and pestle, the powder obtained was used to prepare the extracts. 10 g of each of the grounded sample (leave, stem and root bark) was suspended in 100 ml of different extractants(aqueous, 70% methanol, 70% ethanol and 70% acetone) for 48 hours at room temperature and filteredoff using a Whatman filter paper (Cat no 1001 125) of pore size 125mm.. The filtrate was concentrated by drying in a water bath maintained at a temperature of 45°C until a brownish black residue for the leaves, stem bark and root was obtained. This was kept in a sealed container and refrigerated at (2-4) °C until further required.

3.2.2 Phytochemical screening of the plant

1. *Flavonoids*

Shinoda Test: About 0.5g of extract was dissolved in 1-2ml of 50% methanol and heated. Metallic magnesium and four to five drops of concentrated HCI were added. A red or orange colour indicates the presence of flavonoid aglycones.

Sodium Hydroxide Test: Few drops of aqueous NaOH were added to 5ml of extract, a yellow colouration shows the presence of flavonoid (Trease and Evans, 1983).

2. *Saponins*

Frothing test: 0.5g of the extract was dissolved in 10ml of distilled water. This was then shaken vigorously for 30 seconds and was 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):Equal volume of acetic anhydride was added to the extract. 1ml of concentrated sulphuric acid was added down side the tube. The colour change was observed immediately and later. Red, pink or purple colour indicates the presence of triterpenes while blue or blue-green indicate steroids (Trease and Evans, 1983).

3. *Cardiac glycosides*

Kella Killiani Test: One gram (1g)of the extract was dissolved in glacial acetic acid containing traces of ferric chloride. The test tube was held at an angle of 45°C, 1ml of concentrated sulphuric acid was added down the side purple ring colour at the interface indicate cardiac glycosides (Trease and Evans 1983).

Kadde Test*:* One millilitre of 2% 3, 5-dinitrobenzole acid in 95% alcohol was added to extract, the solution was made alkaline with 5% sodium hydroxide. Appearance of purple-blue colour indicates the presence of cardenolides in the ring.

4. *Alkaloids*

Meyers Test:Two drops of Meyers reagent were added to sample of the extract in a test tube Green precipitate indicates alkaloids.

Dragendoffs Test: Two drops of Dragendoffs reagent were added to the extract. Rose-red precipitate indicates the presence of alkaloids.

Wagners Test: Two drops of Wagnersreagent were added to a small amount of the extract. Whitish precipitate indicates alkaloids.

Picric Acid Test: Two drops of 1% picric acid solution were added to the extract. Yellow coloured solution indicates alkaloids.

Tannic Acid Test: Two drops of tannic acid were added to a sample of the extract (1g). Black precipitate indicates the presence of alkaloids (Sofowora, 1982).

5. *Tannins*

Lead Sub-Acetate Test: Lead sub-acetate solution (3 drops) was added to a solution of 1g of the extract. A red precipitate indicates tannins.

Ferric Chloride Test:About 0.5ml of extract was dissolved in 10ml of distilled water, and then filtered. Few drops of ferric chloride solution were added to the filterate. Formation

of a blue-black precipitate indicates hydrolysable tannins and green precipitates indicate the presence of condensed tannin.

3.2.3In vitro screening of the extracts

A preliminary study was carried out using four different extracts (aqueous, 70% methanol, 70% ethanol and 70% acetone) from which the percentage yield, total polyphenol and $IC_{50}$was determined.

1. *Percentage yield*

The percentage yield of theextracts were determined as percentage of the weight of the extracts to the original weight of the dried sample used, using the formula below;

$$\text{Percentage yield} = \frac{\text{weight of extract}}{\text{weight of sample}} \times 100$$

2. *Total phenolic*

The total phenolic content was determined using the Folin-Ciocalteu method as modified by Amin *et al*., 2004

Principle:The reaction is based on the reduction of phosphor-wolframate-phosphomolybdate complex by phenolics to a blue reaction product with a maximum absorptionat 765nm which can be measured spectrophotometrically (Amin *et al*., 2004).

Procedure:The Folin-Ciocalteu reagent was diluted 10 times (2.5 ml) and mixed with 2 ml of saturated sodium carbonate (75 g/litre) and 6050 µl of sample (supernatant) and homogenized for 10 seconds and heated for 30 minutes at 45°C. The absorbance was measured at 765 nm after cooling at room temperature. The data obtained was calculated

by comparison between a standard curve (µg Gallic acid/ml) and the absorbance of each sample. The data obtained were expressed as mg Gallic acid equivalents per gram of dry matter.

3. *DPPH free radical scavenging activity*

The DPPH radical scavenging activity of the extracts was assayed by the DPPH radical scavenging method of Chan *et al.,* (2007).

Principle:1, 1-Diphenyl-2-picrylhydrazyl (DPPH) contains an odd electron in its structure. Its purple colour is reduced to yellow-coloured diphenylpicryhydrazine when it reacts with an antioxidant, which can donate a hydrogen atom or an electron to it. The change in colour was measured spectrophotometrically at 520 nm using a UV/Visible light spectrophotometer.

Procedure: DPPH solution was prepared by dissolving 6 mg of DPPH in 100 ml of methanol. To 1ml of various concentrations of the extracts (0.020, 0.040, 0.060, 0.080, 0.100 mg/ml), 2 ml of DPPH solution (0.1mM) was added. An equal amount of methanol and DPPH served as control. The mixture was shaken vigorously and left to stand in the dark for 30min. The absorbance of the resulting solution was measured spectrophotometrically at 520 nm. The experiments were performed in triplicate and the percentage scavenging activity of each extract on DPPH radical was calculated using the following formula:

Scavenging activity (%) = {(1- Absorbance of the sample) / (Absorbance of the control)} × 100

DPPH radical scavenging activities of the extracts were expressed as IC_{50} values. IC_{50}, the effective concentration of the extracts required for 50% scavenging of DPPH radical was calculated from the graph of scavenging activity plotted against sample concentration using Microsoft Excel software.

3.2.4 In vivobiological activity of the extracts

Four different extracts (aqueous, 70% methanol, 70% ethanol and 70% acetone) were used for invivo study to ascertain the extract with the highest antihyperlipidemic activity. A total of fourteen (14) apparently healthy rats were used for this study. Treatment with the extracts lasted for 4 days. On the 5^{th} day, the animals were sacrificed and blood sample were collected for total cholesterol and triacylglycerol analysis.

3.2.5 Quantitative phytochemical analysisof ethanol extract of *vitex doniana*

1. *Flavonoids*

Flavonoid determination was done using the method of Boham and Kocipal-Abyazan (1974). Ten grams of the extract were extracted with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper no 42 (125mm), the filtrate was later transferred into a crucible and was evaporated to dryness in a water bath and weighed as flavonoids.

Calculation

% Flavonoids = weight of flavonoids/ weight of sample x 100/1

2. *Saponin*

A gravimetric method of AOAC (1984) employing the use of a soxhlet extractor and two different organic solvents was used. The first solvent (Acetone) extracts lipids and interfering pigments while the second solvent (methanol) extracts saponin. Two grams each of the extract were weighed into a thimble and put in a soxhlet extractor with a condenser fitted on top. Extraction was done with acetone in a 250ml round bottom flask for 3hrs, after which the other weighed 250ml round bottom flask containing methanol was fitted to the same extractor continued for another 3hrs. At the end of second extraction, the methanol was recovered by distillation and the flask oven-dried to remove the remaining solvent in the flask. The flask was allowed to cool in a dessicator and weighed.

Calculation:

% **S**aponin = A- B / W × 100

Where A= weight of flask and extract (saponin), B = weight of empty flask

W = weight of sample.

3. *Cardiac glycosides*

The procedure of AOAC (1984) was used in the determination of cardiac Glycosides. Five grams of extract was placed in a 200ml conical flask; 50ml of distilled water was added. The content of the flask was stirred and allowed to stand for 4hrs. The filtrate was steam distilled into 20ml of 2.5% NaOH. About 75ml of the distillate was collected. This was titrated with 0.02N $AgNO_3$ (Silver nitrate) after the addition of 8ml of 6N Ammonium hydroxide and 2ml of 2% potassium iodide. Permanent turbidity indicated

end-point. The cardiac Glycosides was calculated by multiplying the volume of 0.02N $AgNO_3$ used (titre value) by 1.08mg, using the equation below;

1ml 0.02N $AgN0_3$= 1.08mg HCNTherefore c cardiac Glycosides content in the sample = titre value x 1.08mg

4. *Tannin*

Concentration of tannin was determined using the standard method described by AOAC (1980). Two grams of the extract was boiled with 300ml of distilled water. This was diluted in a standard volumetric flask and filtered through a non-absorbent cotton wool. Twenty five millilitre(25ml) of the infusion was measured into a 2 litre porcelain dish and titrated with 0.1N potassium permanganate (0.1N potassium permanganate was standardized against 0.1N Oxalic acid) until the blue solution changed to green, Then 0.5ml of 0.1N potassium permanganate was added at a time until the solution turned golden yellow.

The tannin content in the sample was calculated by multiplying the volume of 0.1N potassium permanganate used (titre value) by 0.0066235g. Using the equation below;

1ml of 0.1N potassium permanganate (titre value) = 0.0066235g Tannins

Therefore Tannins content in the sample = titre value x 0.0066235g

3.2.6Acute toxicity study

The median lethal dose (LD_{50}) of the plant extracts(leaves, stem bark and root bark) were conducted in order to determine a suitable dose for the evaluation of effects of the extracts.This was done using the method described by Lorke (1983). In the initial phase, rats were divided into 3 groups of 3 rats each and were treated with 10mg, 100mg and

1000mg of the extract per kg body weight orally. 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 with the extracts based on the findings in the first phase. For the leaves and stem bark, the rats were treated with 1200mg, 1600mg, 2900mg and 5000mg of the extracts per kg body weight orally whereas for the root bark, the rats were treated with 200mg, 350mg, 550mg and 900mg of the extracts per kg body weight orally. 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.

3.2.7 Induction of hyperlipidemia

Hyperlipidemia was induced with the method described by Megalli *et.al.*,(2005). 1000mg/kg dose of Poloxamer 407 was introduced intraperitoneally. All syringes were placed on ice prior to Poloxamer 407 administration to maintain the polymer in a mobile viscous state during the injection. To confirm the induction of hyperlipidemia, 24 hours after induction blood sample was collected from the rat tail in each group and assayed for; triacylglycerol (TAG) and total cholesterol (TC). TC and TAG levels above borderline of (200-239) mg/dl and (150-199) mg/dl respectively were considered hyperlipidemic.

3.2.8Animal grouping

A total of 54 rats were randomly divided into 9 groups of 6 rats each.

Group 1: Normal Rats control given feed and water only. This served as the normal control group (NC)

Group 2: Hyperlipidemic Rats control given feed and water only.This served as the hyperlipidemic control group (HC)

Group 3:Hyperlipidemic Rats treated with 20 mg/kg b.wt Atorvastatin according to Kumar *et al.,* 2010. (H+SD)

Group 4: Hyperlipidemic Rats treated with 100mg/kg b.wt ethanolic extract of leaves of *Vitex doniana* (H+LE)

Group 5: Hyperlipidemic Rats treated with 100mg/kg b.wt ethanolic extract of stem bark of *Vitex doniana* (H+SE)

Group 6: Hyperlipidemic Rats treated with 30mg/kg b.wt ethanolic extract of root bark of *Vitex doniana*(H+RE)

Group 7:Normal rats treated with 100 mg/kg b.wt ethanolic extract of leaves of *Vitex doniana*(N+LE)

Group 8:Normal rats treated with 100 mg/kg b.wt ethanolic extract of stem bark of *Vitex doniana*(N+SE)

Group 9:Normal rats treated with 30 mg/kg b.wt ethanolic extract of root bark of *Vitex doniana*(N+RE)

3.2.9Collection and preparation of samples

At the end of 21 days of treatment, the rats were sacrificed by decapitation after using chloroform anesthesia and blood samples were collected from the head wound in a plain bottles(for biochemical parameters) and in Ethylene diamine tetraacetic acid (EDTA) coated bottles (haematological parameters). Blood samples collected in plain tubes were allowed to clot and the serum separated by centrifugation using Labofuge 300 centrifuge

(Heraeus) at 3000 rpm for 10 minutes and the supernatant (serum) collected was subjected to biochemical screening.

1. *Kidney and Liver*

Immediately after the blood was collected, the liver and kidneys were quickly excised, trimmed of connective tissues, rinsed with saline to eliminate blood contamination, dried by blotting with filter paper and weighed (so as to calculate the relative weight) and were kept in the freezer until required. The liver and kidney were crushed using mortar and pestle. 50mM potassium phosphate buffer (PH 7.4) was used for homogenization. One gram(1g) of the organ was taken and 10ml of the buffer was added and then homogenized. It was then centrifuged at 4000rpm for 15 minutes. Then the supernatant was collected using Pasteur pipette.

3.2.10Determination of serum lipid profile

1. *Serum total cholesterol (TC)*

The serum level of total cholesterol was quantified by methods described by Stein (1987), using assay kits (Randox Laboratories Ltd). The value of TC present in the serum was expressed in mg/dl.

Principle: A large portion of the cholesterol in blood is in the form of cholesteryl esters, which is hydrolysed by cholesterol esterase into cholesterol. 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.

Cholesterol

Cholesterol ester + H_2O ⟶ Cholesterol + Fatty acids

Esterase

Cholesterol

Cholesterol ester + O_2 ⟶ Cholestene-3-one + H_2O_2

Oxidase

Peroxidase

2 H_2O_2 + Phenol + 4-Aminoantipyrine ⟶ Quinoneimines + 4 H_2O

Procedure: Exactly, 1000µl of the cholesterol reagent which is made up of 4-Aminoantipyrine, phenol, peroxidase, cholesterol esterase, cholesterol oxidase and buffer was added into three clean test tubes labelled as test, standard and reagent blank containing 10µl serum, 10µl standard reagent and 10µl distilled water respectively. The content in each of the test tube was mixed and incubated for 5 minutes at 37^0C. The absorbance was read against the reagent blank at 500nm within 60 minutes.

Calculation; Using the standard, the concentration of cholesterol in the sample is given as:

$$\text{Conc. Of cholesterol (mg/dl)} = \frac{\Delta A\text{sample} \times \text{Conc. Of Standard}}{\Delta A\text{standard}}$$

ΔAsample = Change in absorbance of sample

ΔAstandard = Change in absorbance of standard.

2. *Serum triacylglycerol (TAG)*

The serum triacylglycerol level was determined by enzymatic method described by Stein (1987), using assay kits (Randox Laboratories Ltd). The values of TAG were expressed in the unit of mg/dl.

Principle: The triglycerides 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 (Tietz, 1990).

Lipases

$$\text{Triglycerides} + H_2O \longrightarrow \text{Glycerol} + \text{Fatty acids}$$

Glycerol Kinase

$$\text{Glycerol} + \text{ATP} \longrightarrow \text{Glycerol-3-phosphate} + \text{ADP}$$

GPO

$$\text{Glycerol-3-phosphate} + O_2 \longrightarrow \text{Dihydroxyacetone} + \text{Phosphate} + H_2O_2$$

POD

$$H_2O_2 + \text{4-Aminophanazone} + \text{4-Chlorophenol} \longrightarrow \text{Quinoneimine} + HCl + 4H_2O$$

Reagent Composition:Rla. Buffer: Pipes Buffer (40mmol/l, pH7.6), 4-chloro-pheno (5.5mmol/l), Magnesium-ions (17.5mmol/l). Rlb. Enzyme Reagent: 4-aminophenazo

(0.5mmol/l), ATP (1.0mmol/l), Lipases (≥ 150U/ml), Glycerol-kinase (≥ 0.4U/ml), Peroxidase (≥ 0.5U/ml). CAL. Standard: (196mg/dl).

Reagent Preparation:One vial of Enzyme reagent R l b was reconstituted with 15 mlof Buffer Rl a.

Procedure: Exactly, 1000µl of the tricylglycerols reagent which is made up of buffer and enzyme reagent was added into clean test tubes containing 10µl of serum, 10µl of standard, 1000µl of the tricylglycerols reagent was also added to empty test tube (serving as reagent blank). The content in each of the test tubes was mixed and incubated for 5 minutes at 37^{O}C. The absorbance was read against the reagent blank at 500nm.

Calculation; Conc. of triacylglycerol (mg/dl) = $\frac{\Delta A\text{sample} \times \text{Conc. Of Standard}}{\Delta A\text{standard}}$

GPO = Glycerol-3-phosphate oxidase

POD = Peroxidase

ΔAsample = Change in absorbance of sample

ΔAstandard = Change in absorbance of standard

3. *Serum high density lipoprotein-cholesterol (HDL-c)*

The serum levels of HDL-c were measured by the method of Wacnic and Albers (1978), using assay kits (Randox Laboratories Ltd). The values were expressed in the unit of mg/dl.

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

Reagent Composition:R1: Phosphotungstic Acid (0.55mmol/l), Magnesium Chloride (25mmol/l), and Concentration of standard (210mg/dl).

Reagent Preparation:The precipitating reagent was prediluted in the ratio 4 + 1 with redistilled water (the contents of80 mlbottles was diluted with 20 ml redistilled water**).**

Procedure: Exactly, 0.5ml of reagent1 (R1) which is made up of phosphotungstic acid and magnesium chloride was added into two clean test tubes; one containing 0.2ml of serum and the other containing 0.2ml of standard, the content in each test tubes was mixed thoroughly and allowed to stand for 10minutes at room temperature, centrifuged at 4000 rpm for 10 minutes and the supernatant was collected. 1.0ml of reagent 2 (cholesterol reagent) made up of phosphate, cholesterol esterase, cholesterol oxidase, peroxidase, 4-Aminoantipyrine, sodium cholate and dichlorophenolsulfonate was added into clean test tubes containing 0.05ml of sample supernatant, 0.05ml standard supernatant and an empty test tube (reagent blank), mixed thoroughly and incubated for 30 minutes at room temperature. The absorbance was read against the reagent blank at 500nm within 60 minutes.

Calculation; HDL-c Concentration (mg/dl) $= \frac{\Delta A\text{sample} \times \text{Conc. Of Standard}}{\Delta A\text{standard}}$

ΔAsample = Change in absorbance of sample

ΔAstandard = Change in absorbance of standard

4. *Serum low density lipoprotein-cholesterol (LDL-c)*

The serum levels of (LDL-c)were calculated in mg/dl according to the protocol of Friedewald *et al*., (1972) using the equation below;

LDL-c = (TC - TAG/5) – HDL-c

3.2.11Determination of biochemical parameters

Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)were assayed for by the method of Reitman and Frankel 1957 using Randox assay kits.

1. *Alanine aminotransferase (ALT)*

Principle

$$\alpha\text{-oxoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{L-glutamate} + \text{pyruvate}$$

Alanine Aminotrasferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine at 540 nm and 37^{o}C.

Reagent Composition:R1. Buffer: Phosphate buffer (100mmol/l, pH 7.4), L-alanine (200mmol/l), α-oxoglutarate (2mmol/l). R2. 2, 4-dinitrophenylhydrazine: (2mmol/l).

Procedure: Exactly, 0.5ml of reagent 1 which is made up of phosphate buffer, L-alanine and α-Oxoglutarate was added into two clean test tubes, one containing 0.1 ml of serum and the other containing 0.1ml distilled water (blank). The content in each test tube was

mixed, incubated for exactly 30 minutes at 37^{O}C. 0.5 ml of reagent 2 which is made up of 2, 4-dinitrophenylhydrazine was added to each of the test tubes, mixed and allowed to stand for exactly 20 minutes at room temperature (20-25^{O}C). Then 0.5ml of sodium hydroxide solution was added to each of the test tubes, the content in each of test tubes was mixed and absorbance was read against the blank at 540nm after 5 minutes. The ALT concentration (U/I) was determined from the standard calibration table provided in the manual of Randox Lab. Ltd, UK Reagent Kit.

2. *Aspartate aminotransferase (AST)*

Principle

$$\alpha\text{-oxoglutarate} + \text{L-aspartate} \xrightarrow{\text{AST}} \text{L-glutamate} + \text{Oxaloacetate}$$

AST was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine at 540 nm and 37^{o}C.

Reagent Composition:R1. Buffer: Phosphate buffer (100mmol/l, pH 7.4), L-aspartate (100mmol/l), α-oxoglutarate (2mmol/l). R2. 2, 4-dinitrophenylhydrazine (2mmol/l).

Procedure: Exactly, 0.5ml of reagent 1 which is made up of phosphate buffer, L-aspartate and α-Oxoglutarate was added into two clean test tubes labelled as test sample and reagent blank, containing 0.1 ml of serum and 0.1 ml of distilled water, the content in each test tubes was mixed and incubated for exactly 30 minutes at 37^{O}C. 0.5 ml of reagent 2 which is made up of 2, 4-dinitrophenylhydrazine was added to each of the test tubes, the content of each of the test tubes was mixed and allowed to stand for exactly 20 minutes at room

temperature(20-25^{O}C). To each of the test tubes, 0.5ml of sodium hydroxide solution was added, mixed and absorbance was read against the blank at 540nm after 5 minutes. The AST concentration (U/l) was determined from the standard calibration table provided in the manual of Randox Lab. Ltd, UK Reagent Kit.

3. *Alkaline phosphatase (ALP)*

The serum level of alkaline phosphatase was quantified by optimized standard method described by Haussament (1977) using Randox assay kits.

Principle

P-nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ phosphate + p-nitrophenol

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

Reagent Composition: Rla: Buffer. Diethanolamine buffer (1mol/l, pH 9.8), MgCl2(0.5mmol/l), Rlb: Substrate. p-nitrophenylphosphate (10 mmol/l).

Reagent Preparation: One vial of substrate Rlb was reconstituted with the 20ml of Buffer Rla.

Procedure: Exactly 1ml of reagent 1 containing Diethanolamine buffer, magnesium chloride and substrate (P-nitrophenylphosphate) was added into a clean test tube containing 0.02ml of serum. This was mixed, initial absorbance was read and timer was started simultaneously, absorbance was read again after 1, 2 and 3 min at 405nm.

Calculation

The ALP activity was calculated using the following formulae:

U/l = 2760 x ΔA 405 nm/ min.

ΔA 405 nm/ min= change in absorbance at 405nm per min

4. *Total protein (TP)*

Total protein was determined colorimetrically according to the method described by Fine (1935) using Randox assay kits.

Principle: The protein assay (using the Randox laboratories assay kit) is based on the reaction of protein with an alkaline copper tartarate solution and Folin reagent. Two steps lead 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.

Reagent Composition:R1: Biuret reagent. Sodium hydroxide (100mmol/l), Na-K-tartrate: (16mmol/l), Potassium io dide (15mmol/l), Cupric Sulphate (6mmol/l). R2: Blank Reagent. Sodium hydroxide (100mmol/l), Na-K-tartrate: (16mmol/l). CAL. Standard: Protein (58.48g/l).

Reagent Preparation:R:Biuret reagent.The contents of bottle R1 was diluted with 400 ml of double distilled water, rinsing the bottle thoroughly. *R2:* Blank reagent.The contents of bottle R2 was diluted with 400 ml of double distilled water, rinsing the bottle thoroughly.

Procedure:Exactly 1ml of biuret reagent was added to three clean test tubes labeled as sample, standard and blank containing 0.02ml of serum, 0.02ml of standard and 0.02ml of distilled water respectively, the content in each of the test tube was mixed, incubated for 10 minutes under room temperature. The absorbance was read at 540nm. The total protein concentration was calculated using the formula below

$$\text{Total Prot. Conc.} = \frac{\Delta\mathbf{A} \text{ sample}}{\Delta\mathbf{A} \text{ standard}} \times \text{Standard Conc.}$$

ΔA sample = Change in absorbance for sample

ΔA standard = Change in absorbance for standard.

5. *Serum total bilirubin (TB)*

The serum TB concentration was determination using the Randox Kit (Randox laboratories limited U.K) based on the method described by Sherlock (1951).

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

Procedure:Exactly, 200ml of reagent 1 (sulphanillic acid) was dispensed each into two different test tubes labeled 'sample blank' and 'sample' followed by the addition of 1 drop (50µl) of reagent 2 (nitrite) and the 1000 µl of reagent 3 (caffeine). 200 µl of the test serum was the dispensed into each of the test tubes and the mixtures incubated in a water bath for 10 minutes at 25 ^{o}C. This was followed by the addition of 100 µl of reagent 4 (tartrate) and the mixture incubated again at 25 ^{o}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 then calculated using the formula provided by the manufacturers viz:

Total bilirubin (mg/dl) = $10.8 \times A_{TB}$ (578nm)

6. *Serum direct and indirect bilirubin*

The serum indirect bilirubin concentration was determined using the Randox Kit (Randox laboratories limited UK) based on the method described by Jendrassik and Grof (1938) and Sherlock (1951).

Principle:DB/IBreacts with diazotized sulphanillic acid in alkaline medium to form a blue coloured complex**.**

Procedure:Exactly, 200ml of reagent 1 (sulphanillic acid) was dispensed each into two different test tubes labeled 'sample blank' and 'sample' followed by the addition of 1 drop (50µl) of reagent 2 (nitrite) and the 2000 µl of 0.9 % NaCl. 200 µl of the test serum was the dispensed into each of the test tubes and the mixtures incubated in a water bath for 10 minutes at 25 ^{o}C. The absorbance of the sample (A_{TB}) was then read against the sample blank using a colorimeter at 546 nm wavelength. The direct and bilirubin concentration was then calculated using the formula provided by the manufacturers viz:

Direct bilirubin (mg/dl)=$14.4 \times A_{DB}$ (546nm).

Indirect bilirubin (mg/dl) =Total Bilirubin – Direct Bilirubin.

7. *Serum creatinine*

The colourimetric method was used to determine serum creatinine concentration according to Bartels and Bohmer (1973) using Randox assay kits.

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.

Reagent Composition:CAL. Standard (2.06 mg/dl), Rla: Picric Acid (35 mmol/l), Rlb: Sodium Hydroxide (0.32 mol/l).

Reagent Preparation:An equal volume of solutions Rla + Rlb was mixed.

Procedure:Exactly1ml of working reagent containing picric acid and sodium hydroxide was added into two clean test tubes labelled sample test and standard, containing 0.1ml of sample and 0.1ml of standard solution. The content in each test tube was mixed and after 30 seconds, the absorbance A_1 of the standard and sample was read. Exactly 2 minutes later, absorbance A_2 of the standard and sample was read at 490nm.

Calculation

The Concentration of creatinine in serum was calculated using the formular below;

$$\frac{\Delta \mathbf{A} \text{ sample}}{\Delta \mathbf{A} \text{ standard}} \times \text{Standard conc. (mg/dl)} = \text{mg/dl}$$

$A_2 - A_1 = \Delta \mathbf{A}$ sample or $\Delta \mathbf{A}$ standard

8. *Serum urea*

Principle:The serum urea concentration was determined using urea Randox assay kit. Urea in serum is hydrolysed to ammonia in the presence of urease. The ammonia formed is then measured photometrically by Berthelot's reaction (Fawcett and Scout, 1960).

. Urease

Urea + H_2O ⟶ $2NH_3$ + CO_2

NH_3 + hypochlorite + phenol ⟶ indophenols (blue compound).

Reagent Composition: R1: EDTA (116mmol/l), Sodium nitroprusside (6mmol/l), Urease (1mmol/l). R2: Phenol (diluted) (120mmol/l). R3: Sodium hypochlorite (diluted) (27mmol/l), NaOH (0.14 N). CAL. Standard: (80.65 mg/dl).

Reagent Preparation: R1. Sodiumnitroprusside and urease (Solution Rl). The contents of vial Rla was transferred into bottle Rlb and mixed gently. R2(Phenol) content of bottle R2 was diluted with 660 ml of distilled water. R3 (Sodium hypochlorite) content of bottle R3 was diluted with 750 ml of distilled water.

Procedure: Exactly 100 µl of reagent 1 containing sodium nitropusside and urease was added into three clean test tubes labelled as test sample, standard and reagent blank containing 10 µl sample, 10 µl standard reagent and 10 µl distilled water respectively. The content in each of the test tube was mixed and incubated at 37^{o}C for 10 minutes, 2.5ml of the reagent 2 containing diluted phenol and reagent 3 containing diluted sodium hypochlorite and sodium hydroxide were added to each of the three test tubes, the content in each of the test tube was mixed immediately and incubated for 15 minutes. The absorbance of the test sample (A sample) and standard (A standard) were read against blank at 500nm.

Calculation

The serum urea concentration was calculated using the formular below;

Urea Concentration $= \frac{\Delta \mathbf{A} \text{ sample}}{\Delta \mathbf{A} \text{ standard}} \times$ Standard Conc. (mg/dl)

Δ**A** sample = Change in absorbance for sample

Δ**A** standard= Change in absorbance for standard

9. *Packed cell volume (PCV)*

The 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 (Cheesbrough, 2000).

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 centrifuge, they settle and packed 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 EDTA bottle was allowed by capillary action to flow through the capillary tube and one end of the tube was sealed by flaming. It was then centrifuged at a speed of 3000rpm for 10 minutes. The PCV was estimated using a microheamatocrit reader and expressed as percentage erythrocytes the blood contain.

3.2.12Determination of in vivo antioxidant activity

1. *Thiobarbituric acid reactive substances (TBARS)*

Lipid peroxidation of LDL was assessed by Thiobarbituric acid reactive substances (TBARS) formation (Ohkawa *et. al.*, 1979).

Principle: Malondialdehyde (MDA) is a product of lipid peroxidation and is used as an indicator of tissue damage. The MDA form a 1:2 adduct with thiobarbituric acid (TBA) and produces a pink coloured product which has an absorption maximum of 532nm.

Reagents:Thiobarbituric acid (100mg of TBA in 30ml distilled water and 30ml of acetic acid)

Trichloroacetic (TCA) 10% 10g of TCA was dissolved in distilled water and made up to 100ml with distilled water. Normal saline solution (0.9%): 0.9g of NaCl was dissolved in distilled water and made up to 100ml with distilled water.

Procedure: 0.2ml of normal saline was pipetted into test tubes labelled as; sample test and sample blank, 0.2ml of tissue homogenate was pipetted into the sample test tube only, 0.5ml of TCA solution was added to each of the sample test and sample blank tubes followed by addition of 0.1ml of TBA solution, 0.2ml of distilled water was added into sample blank tube in place of tissue homogenate. The mixture in each test tube was heated for 60 minutes in a water bath at 95^{o}C. After cooling to room temperature on ice bath, 3ml of n-butanol was added to the content in each test tube and then mixed vigorously, the butanol phase was mixed by centrifugation at 1000 X g for 5 minutes and the absorbance of the sample test was read against the absorbance of the sample blank at 532nm

Calculations

The concentration of TBARS is expressed in terms of Malondialdehyde (MDA) in mol/ml.

Molar extinction of MDA 1.56 x $10^5 cm^{-1}m^{-1}$

MDA concentration = Absorbance / 1.56 x $10^5 cm^{-1}m^{-1}$

TBARS = (Volume of homogenate x MDA Concentrate) / wt of tissue

2. *Superoxide dismutase (SOD)*

Superoxide dismutase (SOD) was determined by a method described by Fridovich (1989).

Principle: The ability of superoxide dismutase (SOD) to inhibit auto oxidation of adrenanline at pH10.2 forms the basis of this assay.

Reagents: Carbonate buffer (0.05M): 14.3g of Na_2CO_3 and 4.2g of $NaHCO_3$ was dissolved in distilled water and made up of 100ml mark in a volumetric flask. The buffer was adjusted to pH 10.2. Adrenaline (0.3mM): 0.01g of adrenaline was dissolved in 17ml of distilled water. The solution was prepared fresh.

Procedure**:** Tissue homogenate of 0.1ml was diluted in 0.9ml of distilled water to make 1:10 dilution of microsome. An aliquant mixture of 0.20ml of the diluted microsome was added to 2.5ml of 0.05M Carbonate buffer. The reaction was started with the addition of 0.3ml of 0.3mM Adrenaline. The reference mixture contains 2.5ml of 0.05M Carbonate buffer, 0.3ml of 0.3mM Adrenaline and 0.20ml of distilled water. Absorbance was measured over 30s up to 150s at 480nm.

Calculations

Increase in absorbance per minute $= (A_5 - A_1)/ 2.5$

$$\% \text{ inhibition} = 100 - \frac{\text{Increase in absorbance for substrate} \times 100}{\text{Increase in absorbance of blank}}$$

1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adenochrome in 1 minute.

SOD activity = % inhibition / (50 x weight of tissue).

3. *Catalase(CAT)*

Catalase activity was determined using the method described by Sinha (1972).

Principle: The method is based on the reduction of Dichromate in acetic acid to chromic acetate, when heated in the presence of hydrogen peroxide with the formation perchromic acid as unstable intermediate. Chromic acetate so produced is measured colorimetrically using spectrophotometer at 570nm. This is because dichromate has no absorbance at 570nm and does not interfere with the determination of catalase.

Reagents:0.01M Phosphate buffer (pH 7.0): 5% potassium heptaoxochromate (V1), $K_2Cr_2O_7$: 5g of $K_2Cr_2O_7$ was dissolved in little quantity of distilled water and made up to 100ml.0.2M H_2O_2: 0.6ml was dissolved in little quantity of distilled water and made up to 100ml. It was stored at 4^oC.Dichromate / Acetic acid solution: 5% potassium heptaoxochromate (V1), $K_2Cr_2O_7$, was mixed with gacial acetic acid in the ratio 1:3, and was stored in brown bottle at room temperature.

Procedure: Exactly 1ml of phosphate buffer, 0.2ml of tissue homogenate and 0.4ml of H_2O_2 were added into two separate test tubes each, 1ml of potassium dichromate/glacial acetic acid was added immediately to one of the test tubes immediately after the addition

of H_2O_2 (T0), while 1ml of potassium dichromate was also added to the second test tube 10 minutes after the addition of H_2O_2 (T1). The mixture in the test tubes was heated in water bath for 10 minutes at 80^0C. Absorbance was read at 570nm.

Calculations

The quantity of H_2O_2 was calculated using

$C_T/C_S = A_T/A_S$

C_T = Concentration of the test

C_S = Standard concentration = 0.2M

AT = Absorbance of test (T0 – T1)

A_S = Absorbance of blank (T0)

$$\text{Catalase activity} = \frac{C_T X \text{ total volume of homogenate}}{10 \text{ minutes X volume of homogenate used X weight of tissue}}$$

Catalase activity was expressed in moles of H_2O_2/min/g tissue.

3.2.13Statistical analysis

The data was analyzed by the analysis of variance (ANOVA) using SPSS program (version 16.0 SPSS Inc., Chicago, IL, USA). The difference between the various extracts and animal groups were compared using the Duncan Multiple Range Test. The results were expressed as mean ± standard deviation (SD). P value less than 0.05 was considered as significant ($P< 0.05$).

CHAPTER 4

4.0 RESULTS

4.1 Phytochemical Constituent of*Vitex Doniana* Plant.

The result of the phytochemical screening of the plant is presented in Table 4.1. The result shows the presence of flavonoids, Terpenoids, Cardiac glycosides, alkaloids, saponins, and tanins in the leaves, stem bark and root bark of*Vitex doniana.* Terpenoids is present only in stem bark, Anthraquinone in leaves and root bark, whereas Steroids is present in the leaves and stem bark.

4.2 Percentage Yield, Total Polyphenol and $IC_{50}$of *Vitex Doniana* Extracts.

The result of the percentage yield, total polyphenol and IC_{50} is presented in Table 4.2. The result shows that in all the parts,aqueous extract had the highest percentage yield while ethanolic extract had the highest total polyphenol content and least IC_{50} value when compared with the other extracts.

4.3 InVivoBiological Activity of *Vitex Doniana* Extracts

The result of the invivo biological activities of the extracts is presented in Table 4.3. The result shows that ethanolic extract had the highest reduction effect on serum total cholesterol and triacylglycerol when compared to the other extracts.

4.4 Quantitative Phytochemical Analysis of Ethanolic Extract of *Vitex Doniana*

The quantitative phytochemicals constituent of the ethanolic extract of *vitex doniana* is presented in Table 4.4. The results show that flavonoids and saponins was significantly

(P< 0.05) higher in leaves while Cardiac glycoside was highest in both leaves and stem bark. Alkaloids and tanins was significantly (P< 0.05) high in the root bark.

Table 4.1:Phytochemicals Present in theLeaves, Stem Bark and Root Bark of*Vitex Doniana*.

Phytochemicals	Leaves Extract	Stem bark Extract	Root bark Extract
Flavonoids	Positive	Positive	Positive
Terpenoids	Negative	Positive	Negative
Anthraquinone	Positive	Negative	Positive
Cardiac glycosides	Positive	Positive	Positive
Alkaloids	Positive	Positive	Positive
Saponins	Positive	Positive	Positive
Steroids	Positive	Positive	Negative
Tanins	Positive	Positive	Positive

Table 4.2: Percentage Yield, Total Polyphenol and IC_{50} of Leaves, Stem Bark and Root Bark Extracts of *Vitex Doniana*.

Extraction Source	Solvent	Yield of Crude extract (%)	Total Polyphenol (mg Gallic acid/g extract)	50% Inhibitory Concentration
Leaves	Aqueous	12.90	30.56 ± 1.22[a]	0.342
	70%Methanol	10.30	35.75 ± 1.72[b]	0.322
	70% Ethanol	10.10	36.11 ± 3.13[b]	0.228
	70% Acetone	8.90	31.03 ± 4.49[a]	0.302
Stem bark	Aqueous	15.90	23.82 ± 1.42[a]	0.443
	70%Methanol	9.90	27.73 ± 4.39[b]	0.347
	70% Ethanol	9.20	28.05 ± 1.22[b]	0.236
	70% Acetone	7.50	23.49 ± 1.33[a]	0.409
Root bark	Aqueous	17.30	20.42 ± 1.94[a]	0.436
	70%Methanol	13.00	23.72 ± 2.60[b]	0.313
	70% Ethanol	12.80	24.43 ± 2.04[b]	0.261
	70% Acetone	9.20	20.66 ± 2.44[a]	0.401

Total polyphenols expressed as mean ± SD of triplicate determination. Values in the same columnwith different Lettersuperscripts are significantly different $p<0.05$

4.3:InVivo Biological Activity of Leaves, Stem bark and Root bark Extracts of *Vitex Doniana*.

Group	Total Cholesterol		Triacylglycerol	
	Concentration (mg/dl)	Percentage Reduction (%)	Concentration (mg/dl)	Percentage Reduction(%)
NC	202.35±0.01[a]	59.84	126.72±0.10[a]	84.10
HC	503.97±7.54[f]	0.00	797.36±6.30[e]	0.00
H+Laq	346.63±22.63[d]	31.22	734.99±72.60[de]	7.82
H+Lme	305.30±22.61[c]	39.42	632.54±0.01[c]	43.49
H+Let	261.32±22.60[b]	48.14	574.63±31.49[b]	50.75
H+Lac	431.96±7.54[e]	14.28	659.27±37.80[d]	17.31
H+Saq	335.87±32.77[d]	33.35	775.08±25.19[d]	2.79
H+Sme	250.64±7.54[b]	50.26	552.36±0.35[b]	30.72
H+Set	314.64±7.13[c]	37.56	645.90.18.90[c]	18.99
H+Sac	398.64±0.01[e]	20.90	786.68±25.19[e]	1.33
H+Raq	495.96±22.62[e]	1.58	664.18±12.60[d]	16.69
H+Rme	277.81±0.45[c]	44.86	577.08±50.40[c]	27.62
H+Ret	241.98±7.40[b]	51.98	468.18±4.67[b]	44.28
H+Rac	383.97±15.08[d]	23.80	677.54±18.89[d]	15.02

Values are means ± SD of triplicate determination. Values in the same column with different letter superscripts are Significantly different $p<0.05$.

NC: Normal Control rat. HC: Hyperlipdemic control rats. H: Hyperlipdemic rats. L: Leaves. S: Stem Bark. R: Root Bark. aq: Aqueous. me: Methanol.et:Ethanol. ac: Acetone.

Table4.4:Quantitative Phytochemical of Leaves, Stem Bark and Root Bark of Ethanol Extract of *Vitex Doniana*.

Parameter	Leaves	Stem BarkBark	Root
Flavonoids(%)	4.605±0.077[b]	2.035±0.035[a]	1.975±0.021[a]
Saponins(%)	3.945±0.091[b]	2.080±0.014[a]	1.940±0.070[a]
Cardiac glycosides (%)	0.185±0.007[b]	0.175±0.007[b]	0.040±0.041[a]
Alkaloids (%)	0.016±0.007[a]	0.040±0.014[a]	0.085±0.009[b]
Tannins (%)	0.015±0.007[a]	0.030±0.001[a]	0.035±0.008[b]

Values are mean ± SD for triplicate determinations with different superscript down the column are significantly different ($p<0.05$).

4.5 Lethal Dosage (LD_{50}) of Ethanol Extract of *Vitex Doniana*

The Lethal Dosage (LD_{50}) of ethanolic extracts of *Vitex doniana* is represented in Table 4.5.The result of phase one shows that there was no mortality for the leaves and stem bark extract within 48 hours oral administration at doses of 10, 100 and 1000 mg/kg body weight whereas there was mortality at a dose of 1000 mg/kg body weight for root bark extracts. The phase two result shows that there is no mortality even at a dose of 5000 mg/kg body weight for leaves and stem bark extract. Also, there was no mortality for the root bark extract at doses lower than 1000 mg/kg body weight.The $LD_{50}$for leaves and stem bark extracts was > 5000 mg/kg body weight, whereas that of the root extract was calculated as shown below.

LD_{50} = √ Highest non-lethal dose × Lowest lethal dose,

Highest non-lethal dose = 900 mg/kg body weight

Lowest lethal dose = 1000 mg/kg body weight

LD_{50} = √ 900 × 1000

LD_{50} = √ 900000

LD_{50} = 948.68 mg/kg body weight.

TABLE 4.5:Lethal Dosage (LD_{50}) of Leaves, Stem Bark and Root Bark of Ethanol Extract of *Vitex Doniana*.

Phase	Plant part	Dose (mg/Kg body weight)			
1		10	100	1000	-
	Leaves	No death	No death	No death	
	Stem	No death	No death	No death	
	Root	No death	No death	Death	
2a		1200	1600	2900	5000
	Leaves	No death	No death	No death	No death
	Stem	No death	No death	No death	No death
2b		200	350	550	900
	Root	No death	No death	No death	No death

$LD_{50} = \sqrt{}$ (highest non-lethal dose) * (lowest lethal dose)

4.6Effect of EthanolExtract of *Vitex Doniana* on Lipid Profile and Atherogenic RiskPredictor Indices of P407 InducedHyperlipidemic and Normal Rats.

The effect of *Vitex doniana* (leaves, stem bark and root bark) ethanolic extract on lipid profile is shown in Table 4.6.The result reveals that hyperlipidemic rats shows a significant ($p<0.05$) increase in TC, TAG and LDL-c and decrease in HDL-c when compared with rats in other groups. Hyperlipidemic treated rats shows that the leaves extract significantly ($p<0.05$) decreased the TC, TAG and LDL-c when compared to other hyperlipidemic treated groups. Hyperlipidemic rats treated with leaves, stem bark and standard drug shows a significant ($p<0.05$) increase in HDL-c when compared with the root treated group. All the normal rats treated with the extract shows no significant ($P>0.05$) difference in TC, TAG, LDL-c and HDL-c when compared with animals in the normal control groups.

The effects of *Vitex doniana* (leaves stem and root bark) ethanolic extracts on serum atherogenic risk predictor indices of P407 induced hyperlipidemic rats is presented in Table 4.7. The results reveal that hyperlipidemic rats shows a significant ($P<0.05$) reduction in HDL-c/TC ratio, increase in LDL-c/HDL-c and Log (TAG/HDL-c) ratio when compared with all other groups. Hyperlipidemic treated rats shows that the leaves, stem bark and standard drug significantly ($P<0.05$) increased the HDL-c/TC ratio and decrease LDL-c/HDL-c ratio when compared to the induced root treated group. Also, hyperlipidemic leaves treated ratssignificantly ($p<0.05$) decreased the LOG (TAG/HDL-c) ratio when compared to other induced treated groups.All the animals treated with the extract without induction shows no significant ($P>0.05$) difference HDL-c/TC, LDL-

c/HDL-c and LOG (TAG/HDL-c) ratio when compared with animals in the normal control group.

Table 4.6: Effects of *Vitex Doniana* Ethanolic Extract on Lipid Profiles ofP407 Induced Hyperlipidemic and Normal Rats.

Group (n=6)	Serum TC (mg/dl)	Serum TAG (mg/dl)	Serum HDL –c (mg/dl)	Serum LDL-c (mg/dl)
NC	143.73 ±4.75^{a}	126.77 ±13.30^{a}	63.37 ±0.66^{d}	41.75±5.34^{a}
HC	269.60 ±3.40^{e}	437. 99±9.23^{e}	49.16 ±1.09^{a}	91.17±4.62^{d}
H+SD	194.66 ±2.196^{c}	170.06 ±9.48^{c}	61.65 ±1.32^{c}	72.31 ±2.93^{b}
H+LE	186.90 ±2.19^{b}	162.16 ±19.69^{b}	61.23 ±1.07^{c}	75.70 ±6.04^{b}
H+SE	195.88 ±1.45^{c}	199.15 ± 7.87^{c}	61.39±0.61^{c}	77.43 ±2.25^{bc}
H+RE	207.76 ±1.50^{d}	224.88 ±13.18^{d}	59.72 ±0.33^{b}	82.51 ±3.76^{c}
N+LE	142.27± 5.01^{a}	123.56 ±15.46^{a}	63.48 ±0.35^{d}	41.04 ±6.36^{a}
N+SE	143.24 ±5.34^{a}	126.77 ±14.63^{a}	63.58 ± 0.33^{d}	41.48 ±6.44^{a}
N+RE	143.75 ±3.52^{a}	125.17±13.18^{a}	63.37 ±0.35^{d}	42.07 ± 5.70^{a}

Values are mean ± SD. Values with different superscripts down the column are significantly different ($p<0.05$).

NC: Normal Control rat. HC: Lipid control rats. H+SD: Hyperlipidemic rats + 20mg/kg Atorvastatin. H+LE: Hyperlipidemic rats + 100mg/kg ethanolic leaf extract. H+SE: Hyperlipidemic rats + 100mg/kg ethanolic stem bark extract. H+RE: Hyperlipidemic rats + 30mg/kg ethanolic root bark extract. N+LE: Normal rats +100mg/kg ethanolic leave extract. N+SE: Normal rats + 100mg/kg ethanolic stem extract. N+RE: Normal rats + 30mg/kg ethanolic root bark extract.

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

Table 4.7: Effects of *Vitex Doniana* Ethanolic Extracts on Atherogenic Predictor Indices of P407 Induced Hyperlipidemic and Normal Rats

Group (n=6)	HDL-c /TC	LDL-c/HDL-c	LOG (TAG /HDL-c)
NC	0.441 ± 0.012^{d}	0.658 ± 0.081^{a}	0.299 ± 0.044^{a}
HC	0.182 ± 0.004^{a}	1.854 ± 0.082^{d}	0.949 ± 0.014^{e}
H+SD	0.316 ± 0.009^{c}	1.173 ± 0.053^{b}	0.552 ± 0.020^{c}
H+LE	0.327 ± 0.003^{c}	1.235 ± 0.086^{b}	0.420 ± 0.054^{b}
H+SE	0.313 ± 0.002^{c}	1.261 ± 0.034^{b}	0.510 ± 0.015^{c}
H+RE	0.287 ± 0.001^{b}	1.381 ± 0.056^{c}	0.575 ± 0.027^{d}
N+LE	0.446 ± 0.017^{d}	0.646 ± 0.100^{a}	0.286 ± 0.053^{a}
N+SE	0.444 ± 0.019^{d}	0.652 ± 0.102^{a}	0.297 ± 0.049^{a}
N+RE	0.441 ± 0.012^{d}	0.664 ± 0.091^{a}	0.293 ± 0.044^{a}

Values are mean ± SD. Values with different superscripts down the column are significantly different ($p<0.05$).

NC: Normal Control rat. HC: Lipid control rats. H+SD: Hyperlipidemic rats + 20mg/kg Atorvastatin. H+LE: Hyperlipidemic rats + 100mg/kg ethanolic leaf extract. H+SE: Hyperlipidemic rats + 100mg/kg ethanolic stem bark extract. H+RE: Hyperlipidemic rats + 30mg/kg ethanolic root bark extract. N+LE: Normal rats +100mg/kg ethanolic leave extract. N+SE: Normal rats + 100mg/kg ethanolic stem extract. N+RE: Normal rats + 30mg/kg ethanolic root bark extract.

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

4.7Effects of Ethanol Extracts of *Vitex Doniana* on Serum LiverMarker Enzymesand Function Parameters of P407 Induced Hyperlipidemic and Normal Rats.

The effect of *Vitex doniana ethanolic* extract on liver marker enzymes ispresented in Table 4.8. The result shows that hyperlipidemia significantly (p<0.05) increased the serum level of AST, ALT and ALP of hyperlipidemic rats when compared to normal rats. All the hyperlipidemic treated rats significantly (p<0.05) lowered the serum levels of AST, ALT and ALP when compared with the hyperlipidemic control rats. The AST, ALT and ALP levels of all treated normal rats were notsignificantly (P>0.05) changed when compared to normal control rats.

The serum concentrations of total protein (TP), total bilirubin (TB), indirect bilirubin (IB) and direct bilirubin (DB) of hyperlipidemic and normal rats is presented in Table 4.9. The result shows that there was a significant (p<0.05) decrease in TP, DB and increase in TB, IB of hyperlipidemic rats when compared with normal rats. All the treatment of hyperlipidemic groups show a significant (p<0.05) increase in TP, DB and decreasein TB, IB when compared with hyperlipidemic control rats. The TP, DB, TB and IB levels of all normal treated rats were not significantly (p>0.05) changed when compared to normal control rats.

4.8:Effects of Ethanol Extracts of *Vitex Doniana* on Liver Marker Enzymesof P407 Induced Hyperlipidemic and Normal Rats.

Group (n=6)	ALT (U/I)	AST(U/I)	ALP(U/I)
NC	53.01 ± 2.09[a]	13.33 ± 2.58[a]	36.80 ± 14.25[a]
HC	90.50 ± 1.01[e]	41.25 ± 2.50[d]	158.70 ± 13.80[c]
H+SD	57.66 ± 1.50[b]	24.16 ± 3.76[c]	96.60 ± 15.11[b]
H+LE	57.33 ± 1.63[b]	19.16 ± 2.04[b]	78.20 ± 11.26 [b]
H+SE	63.50 ± 1.02[c]	26.25 ± 2.50[c]	92.00 ± 15.93[b]
H+RE	70.03 ± 0.90[d]	26.66 ± 2.58[c]	94.62 ± 14.75[b]
N+LE	52.66 ± 1.03[a]	12.89 ± 2.25[a]	32.20 ± 11.26[a]
N+SE	54.40 ± 2.01[a]	13.00 ± 2.44[a]	36.80 ± 14.25[a]
N+RE	54.33 ± 1.50[a]	13.16 ± 2.13[a]	36.82 ± 14.27[a]

Values are mean ± SD. Values with different superscripts down the column are significantly different ($p<0.05$).

NC: Normal Control rat. HC: Lipid control rats. H+SD: Hyperlipidemic rats + 20mg/kg Atorvastatin. H+LE: Hyperlipidemicrats+100mg/kg ethanolic leaf extract. H+SE: Hyperlipidemic rats + 100mg/kg ethanolic stem bark extract. H+RE: Hyperlipidemic rats+30mg/kg ethanolic root bark extract. N+LE: Normal rats +100mg/kg ethanolic leave extract. N+SE: Normal rats+100mg/kg ethanolic stem extract. N+RE: Normal rats + 30mg/kg ethanolic root bark extract.

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, TP: Total protein.

4.9:Effects of Ethanol Extracts of *Vitex Doniana* on Liver Function Parameters of P407 Induced Hyperlipidemic and Normal Rats.

Group (n=6)	Total protein (g/dl)	Total bilirubin (mg/dl)	Indirect bilirubin (mg/dl)	Direct bilirubin (mg/dl)
NC	5.95 ± 0.10^{f}	2.44 ± 0.05^{a}	2.41 ± 0.05^{a}	0.03 ± 0.01^{d}
HC	2.95 ± 0.13^{a}	5.75 ± 0.16^{e}	5.73 ± 0.16^{d}	0.01 ± 0.03^{a}
H+SD	5.05 ± 0.11^{c}	4.01 ± 0.08^{d}	3.98 ± 0.08^{c}	0.02 ± 0.01^{b}
H+LE	5.56 ± 0.08^{e}	3.42 ± 0.11^{b}	3.39 ± 0.11^{b}	0.02 ± 0.01^{b}
H+SE	5.21 ± 0.24^{d}	4.02 ± 0.10^{d}	3.99 ± 0.10^{c}	0.02 ± 0.02^{b}
H+RE	4.82 ± 0.14^{b}	3.87 ± 0.08^{c}	3.63 ± 0.57^{b}	0.02 ± 0.01^{b}
N+LE	5.84 ± 0.12^{f}	2.37 ± 0.06^{a}	2.36 ± 0.08^{a}	0.03 ± 0.02^{d}
N+SE	5.88 ± 0.11^{f}	3.39 ± 0.08^{a}	2.34 ± 0.68^{a}	0.03 ± 0.01^{d}
N+RE	5.34 ± 0.82^{f}	2.39 ± 0.08^{a}	2.36 ± 0.90^{a}	0.03 ± 0.01^{d}

Values are mean ± SD. Values with different superscripts down the column are significantly different ($p<0.05$).

NC: Normal Control rat. HC: Lipid control rats. H+SD: Hyperlipidemic rats + 20mg/kg Atorvastatin. H+LE: Hyperlipidemicrats + 100mg/kg ethanolic leaf extract. H+SE: Hyperlipidemic rats + 100mg/kg ethanolic stem bark extract. H+RE: Hyperlipidemic rats + 30mg/kg ethanolic root bark extract. N+LE: Normal rats +100mg/kg ethanolic leave extract. N+SE: Normal rats+100mg/kg ethanolic stem extract. N+RE: Normal rats + 30mg/kg ethanolic root bark extract.

4.8Effects of Ethanol Extracts of *Vitex Doniana*on Kidney Function Parameters and Packed Cell Volume of P407 Induced Hyperlipidemic and Normal Rats.

The Creatinine, urea and packed cell volume of poloxamer (407) induced hyperlipidemic and normal rats are presented in Table 4.10. The result shows that hyperlipidemic control group significantly ($p<0.05$) increased the serum levels of creatinine and urea when compared with all other groups. All the treatment of hyperlipidemic groups show that the leaves extract significantly ($p<0.05$) decreased the creatinine and urea concentration when compared to other treatment of hyperlipidemic groups.All the treatment of normal groups showno significant ($P>0.05$) difference in creatinine and urea concentration when compared with animals in the normal control groups.Also,hyperlipidemic control group showed a significant ($p<0.05$) decrease in packed cell volume when compared with rats in all other groups. All the induced and normal treated rats show a significant ($p<0.05$) increase in packed cell volume when compared with the hyperlipidemic control rats.

4.10:Effects of Ethanol Extracts of *Vitex Doniana* on Kidney Function Parameters and Packed Cell Volume of P407 Induced Hyperlipidemic and Normal Rats.

Group (n=6)	Creatinine (mg/dl)	Urea (mg/dl)	Packed Cell Volume (%)
NC	2.72 ± 1.05^{a}	81.55 ± 1.72^{a}	44.83 ± 3.12^{bcd}
HC	$11\text{-}73 \pm 1.02^{d}$	150.36 ± 2.96^{g}	40.25 ± 0.50^{a}
H+SD	6.80 ± 1.05^{c}	117.08 ± 1.45^{f}	42.83 ± 3.43^{abc}
H+LE	5.10 ± 1.11^{b}	97.56 ± 1.25^{c}	42.00 ± 2.52^{ab}
H+SE	6.63 ± 1.02^{c}	109.04 ± 1.32^{d}	43.50 ± 3.00^{abc}
H+RE	6.68 ± 1.05^{c}	112.87 ± 1.72^{e}	41.66 ± 2.65^{ab}
N+LE	2.38 ± 0.83^{a}	80.73 ± 0.93^{a}	46.16 ± 2.31^{cd}
N+SE	2.06 ± 1.01^{a}	80.73 ± 1.04^{a}	44.83 ± 2.92^{bcd}
N+RE	2.38 ± 0.83^{a}	80.35 ± 1.01^{a}	47.16 ± 0.98^{d}

Values are mean ± SD. Values with different superscripts down the column are significantly different ($p<0.05$).

NC: Normal Control rat. HC: Lipid control rats. H+SD: Hyperlipidemic rats + 20mg/kg Atorvastatin. H+LE: Hyperlipidemicrats + 100mg/kg ethanolic leaf extract. H+SE: Hyperlipidemic rats + 100mg/kg ethanolic stem bark extract. H+RE: Hyperlipidemic rats + 30mg/kg ethanolic root bark extract. N+LE: Normal rats +100mg/kg ethanolic leave extract. N+SE: Normal rats+100mg/kg ethanolic stem extract. N+RE: Normal rats + 30mg/kg ethanolic root bark extract.

4.9Effect of EthanolExtract of *Vitex Doniana* on Body Weight of P407 Induced Hyperlipidemic and Normal Rats.

The effect of administration of ethanolic leaves, stem bark and root bark extracts of *Vitex doniana* on body weight of poloxamer (407) induced hyperlipidemic and normal rats is shown in Table 4.11. The result reveals that hyperlipidemic control rats shows a significant ($p<0.05$) decrease in body weight when compared to all other groups. The treated hyperlipidemic groups shows that allthe extracts including standard drug significantly ($p<0.05$) increased body weight when compared to hyperlipidemic control group.All the treated normal groups showed no significant ($P>0.05$) difference in body weight when compared with rats in the normal control group.

Table 4.11Effect of EthanolExtract of *Vitex Doniana* on Body Weight ofP407 Induced Hyperlipidemic and Normal Rats.

Group (n=6)	Initial BW(g)	Final BW(g)	Difference BW(g)	% Difference BW (%)
NC	123.33	133.00	9.67	7.84 ± 1.67^{d}
HC	125.25	108.00	-17.25	-13.77 ± 5.25^{a}
H+SD	116.33	105.16	-11.17	-9.60 ± 2.48^{b}
H+LE	126.33	117.00	-9.33	-7.38 ± 8.47^{c}
H+SE	122.00	113.50	-8.50	-6.96 ± 1.29^{c}
H+RE	139.83	131.50	-8.33	-5.95 ± 1.21^{c}
N+LE	117.66	125.33	7.67	6.51 ± 1.36^{d}
N+SE	138.50	145.66	7.16	5.16 ± 1.60^{d}
N+RE	116.83	123.00	6.17	5.28 ± 3.06^{d}

Values are mean ± SD. Values with different superscripts down the column are significantly different ($p<0.05$).

NC: Normal Control rat. HC: Lipid control rats. H+SD: Hyperlipidemic rats + 20mg/kg Atorvastatin. H+LE: Hyperlipidemicrats + 100mg/kg ethanolic leaf extract. H+SE: Hyperlipidemic rats + 100mg/kg ethanolic stem bark extract. H+RE: Hyperlipidemic rats + 30mg/kg ethanolic root bark extract. N+LE: Normal rats +100mg/kg ethanolic leave extract. N+SE: Normal rats+100mg/kg ethanolic stem extract. N+RE: Normal rats + 30mg/kg ethanolic root bark extract.

BW: Body weight.

4.10Effect of EthanolExtract of *Vitex Doniana*on Lipid Peroxidation and In Vivo Antioxidant Enzymes of P407 Induced Hyperlipidemic and Normal Rats.

4.9.1 Lipid Peroxidation (TBARS) in Liver and Kidney

The result of the study on the liver and kidneyTBARS is presented in Table 4.12. The table shows that hyperlipidemia significantly ($p<0.05$) increase the level of liver and kidney TBARS when compared with rats in normal control and hyperlipidemic treated groups.All the extracts activity in the liver and in the kidney (leaves and stem bark) significantly ($p<0.05$) lower TBARS of normal treatedrats when compared to normal control and induced treated (with extracts and standard drug) group. In Hyperlipidemia, rats treated with standard drug shows a significant ($p<0.05$) increase in lipid peroxidation when compared with rats treaded with the extracts.

4.9.2 Liver and Kidney Catalase and SOD

Poloxamer 407 induced hyperlipidemia significantly ($p<0.05$) decrease the level of liver and kidney CAT when compared with rats in normal control and induced treated groups (Table 4.12).All the extracts activity in the liver and only leaves extract in the kidney significantly($p<0.05$) increase in CAT level of normal treated rats when compared with the normal control and hyperlipidemic treated (with extracts and standard drug) groups. In hyperlipidemia group,standard drug significantly ($P<0.05$) reduced the level of catalase in the liver and show no significant ($P>0.05$) change in the kidney of rats treated with root extract.All other extracts were significantly higher. The level of kidney and liver SOD was also significantly ($P<0.05$) reduced in hyperlipidemia rats compared to control and treated group. Normal rats treated with extracts in both liver and kidney

shows no significant ($P<0.05$) difference in SOD compared with normal control, but were significantly ($P<0.05$) higher when compared with hyperlipidemia treated group. Hyperlipidemia rats treated with standard drug shows no significant change compared with extract treated group.

Table 4.12:Effect of Ethanol Extract of *Vitex Doniana* on Lipid Peroxidation and Endogenous Antioxidant Enzymes of P407 Induced Hyperlipidemic and Normal Rats.

Group(n=6)	Liver			Kidney		
	TBARS (mmol/g of tissue)	SOD (mmol/min/g of tissue)	CAT (moles of H_2O_2/min/g of tissue)	TBARS (mmol/g of tissue)	SOD (mmol/min/g of tissue)	CAT (moles of H_2O_2/min/g of tissue)
NC	5.12 ± 0.05^{b}	2.05 ± 0.03^{d}	1.20 ± 0.00^{f}	5.64 ± 0.15^{c}	2.04 ± 0.03^{d}	1.05 ± 0.07^{e}
HC	8.75 ± 0.06^{f}	1.85 ± 0.03^{a}	0.12 ± 0.02^{a}	8.27 ± 0.07^{f}	1.87 ± 0.01^{a}	0.22 ± 0.02^{a}
H+SD	6.41 ± 0.01^{e}	1.97 ± 0.02^{bc}	0.58 ± 0.02^{c}	6.51 ± 0.10^{e}	1.96 ± 0.03^{bc}	0.66 ± 0.04^{bc}
H+LE	5.17 ± 0.00^{c}	1.98 ± 0.00^{c}	1.00 ± 0.01^{e}	5.38 ± 0.00^{b}	1.97 ± 0.01^{c}	0.98 ± 0.01^{d}
H+SE	5.13 ± 0.01^{b}	1.95 ± 0.02^{b}	0.61 ± 0.02^{d}	5.45 ± 0.05^{b}	1.94 ± 0.02^{b}	0.71 ± 0.01^{c}
H+RE	5.67 ± 0.02^{d}	1.96 ± 0.00^{bc}	0.51 ± 0.01^{b}	5.76 ± 0.01^{d}	1.95 ± 0.02^{bc}	0.64 ± 0.02^{b}
N+LE	5.02 ± 0.01^{a}	2.01 ± 0.01^{d}	1.39 ± 0.01^{h}	5.15 ± 0.02^{a}	2.05 ± 0.01^{d}	1.13 ± 0.01^{f}
N+SE	5.04 ± 0.01^{a}	2.04 ± 0.00^{d}	1.23 ± 0.04^{g}	5.43 ± 0.01^{b}	2.04 ± 0.02^{d}	1.10 ± 0.04^{ef}
N+RE	5.05 ± 0.02^{a}	2.03 ± 0.01^{d}	1.24 ± 0.02^{g}	5.68 ± 0.01^{b}	2.04 ± 0.02^{d}	1.09 ± 0.04^{ef}

Values are mean ± SD. Values with different superscripts down the column are significantly different ($p<0.05$).

NC: Normal Control rat. HC: Lipid control rats. H+SD: Hyperlipidemic rats + 20mg/kg Atorvastatin. H+LE: Hyperlipidemic rats + 100mg/kg ethanolic leaf extract. H+SE: Hyperlipidemic rats + 100mg/kg ethanolic stem bark extract. H+RE: Hyperlipidemic rats + 30mg/kg ethanolic root bark extract. N+LE:Normal rats +100mg/kg ethanolic leave extract. N+SE: Normal rats + 100mg/kg ethanolic stem extract. N+RE: Normal rats + 30mg/kg ethanolic root bark extract.

TBARS: Thiobarbituric Acid Reactive Substances. SOD: Superoxide Dismutase. CAT: Catalase.

CHAPTER FIVE

5.0 DISCUSSION

Polyphenols are the major group of antioxidants that have generated a great interest among the scientists for the development of natural cure for cardiovascular diseases(Pietta, 2000). Antioxidant activity of a compound has been ascribed to the mechanism exerted by polyphenols (Anoosh and Fatemeh 2010). Recently, polyphenols have been considered powerful antioxidants *in vitro* and proven to be more potent antioxidants than Vitamin C, E and carotenoids (Rice-Evans, 1995; Rice-Evans, 1996). The inverse relationship between fruit and vegetable intake and the risk of oxidative stress associated diseases such as cardiovascular diseases has been partially ascribed to polyphenols (Hollman, 1999). Therefore, it was reasonable to determine the total polyphenol content of extracts from different part of the plant. The result shows that the ethanolic extracts had the highest polyphenol content when compared to the other extracts.Another remarkable observation is the high percentage yield shown by aqueous extracts. Aqueous extracts though gave a better yield of crude extract, but not good for polyphenol extraction. It extracted only the water-soluble bioactive compounds, moreover much other residual substances and impurities are present in the aqueous extract. It has been proposed that the antioxidant properties of polyphenols can be mediated by the following mechanisms; scavenging radical species such as ROS/RNS, suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production and upregulatingor protecting antioxidant defense (Cotelle, 2001).

1, 1-diphenyl 2-picryl hydrazyl (DPPH) radical Scavenging activity is considered to be primary antioxidant activity (Norshazila *et. al.,* 2010), because it is one of the most effective methods for evaluating the concentration of radical-scavenging materials actively by a chain-breaking mechanism (Maisuthisakul *et. al*., 2005) and is a model for a lipophilic radical. DPPH is anunstable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et. al.,* 1997). The reduction capability of DPPH was determined by the decrease in its absorbance at 520 nm, which is induced by antioxidants. The result is expressed as IC_{50}, which is defined as the amount of antioxidant required to inhibit 50% of DPPH free radicals under the experimental conditions (Antolovich *et al*., 2002; Nanasombat and Teckchuen, 2009). A higher DPPH radical-scavenging activity is associated with a lower IC_{50} value (Pattanayak *et. al.*, 2012). The result shows that the ethanolic leaves extract had the lowest IC_{50} value followed by ethanolic stem bark extract while the ethanolic root bark extract had the highest. Preliminary studies of possible hypolipidemic effect of the extracts invivo shows that the ethanolic extract has a better reduction effect on serum levels of total cholesterol and triacylglycerol both for leaves extracts and root bark extracts while methanol for stem bark extracts. Due to the high toxicity of methanolic extracts, ethanolic extracts of all the parts was used for these studies.

A medicinal plant is a plant which one or more of its organs contains substances that may be used for therapeutic purposes or as precursors for the synthesis of useful drugs (WHO 1998). It is well known that these plants generally contain secondary metabolites, which have been reported to be responsible for protecting them (Smith and Eyzaguine, 2007; Kumar *et. al*., 2009) and some associated with numerous physiological activities in

mammalian cells in various studies (Mishra *et. al*., 2009).The presence of these secondary metabolites in plants probably explains the various uses of plants for traditional medicine.The pharmacological and other beneficial effects of these secondary metabolites in plants have been reviewed by Soetan (2008). The result of the phytochemical constituent of ethanolic extract shows the presence of flavonoids, saponins,cardiac glycosides, alkaloids and tannins.

Flavonoids are group of phytochemicals whichhave been found in varying amounts in foods and medicinal plants. It has been shown to exert potent anti-oxidant activity against the superoxide radical (Hertog *et. al.,* 1993). Its consumption has been proven not to be associated with mortality due to coronary heart disease. This may be as a result of its antioxidant activity and subsequent inhibition of low density lipoproteins (LDL) oxidation known to have been attributed to the dietary and supplemental intake of flavonoids and other micronutrients. Epidemiologic studies indicate an inverse relationship between intake of dietary flavonoids and coronary atherosclerotic disease (Knekt *et. al.,* 1996).

Saponins are glycosides of both triterpenes and steroids having hypotensive and cardiac depressant properties (Olaleye, 2007). They bind to cholesterol to form insoluble complexes. Dietary saponins in the gut combine with endogenous cholesterol and thus excreted via the bile. This prevents cholesterol reabsorption and thus results in a reduction of serum cholesterol (Cheeke, 1971). Saponins have been found to be potentially useful for the treatment of hypercholesterolaemia which suggests that saponins might be acting by interfering with intestinal absorption of cholesterol (Malinow *et. al.,* 1977a, b).

Cardiac glycosides are cardioactive compounds belonging to triterpenoids class of compounds (Brian *et. al.,* 1985). They possess inherent activity which resides in the aglycone portions of their sugar attachment. Their clinical effects in cases of congestive heart failure are due to increase in the force of myocardiac contraction (Brian *et. al.*, 1985). They exert their hypotensive effect by inhibiting Na+-K+ ATPase. They also act directly on the smooth muscle of the vascular system. They exert a number of effects on neural tissue and thus indirectly influence the mechanical and electrical activities of the heart and modify vascular resistance and capacitance (Olaleye, 2007).

Alkaloids are basic natural products occurring primarily in plants interfering with cell division. They are generally found in the form of salts with organic acids and they are haemolytically active and are also toxic (Cheeke, 1989). Alkaloids, comprising a large group of nitrogenous compounds are widely used as therapeutic agents in the management of cancer (Horwitz, 1998; Noble, 1990). Alkaloids also interfere with cell division. Chewonarin et al. (1999) isolated an alkaloid from *Hibiscus sabdariffa* and demonstrated its ability to prevent mutagenesis.

Tannins are complex phenolic polymers which can bind to proteins and carbohydrates resulting in reduction in digestibility of these macromolecules and thus inhibition of growth (Nwogu *et al.,* 2008; Bulter, 1989).Tannins from the bark, roots and other parts of many plants especially Euphorbiaceae are used to treat cells that have gone neoplastic (Duke and Wain, 1981). Tanninsare reported to have astringent properties on mucous membranes (Egunyomi *et. al.,* 2009).

Poloxamer 407, a non-ionic surfactant is well known to induce dose dependent hyperlipidemia (Johnston 2004) by inhibiting capillary (heparin releasable) lipoprotein lipase (LPL), the major enzyme responsible for the hydrolysis of plasma lipoprotein triglycerides (TAG) and indirectly stimulating the activity of 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase, the rate limiting enzyme in cholesterol synthesis, thereby leading to hypertriglyceridemia and hypercholesterolemia respectively.Lipids are class of organic compounds that are fatty acids or their derivatives. They are insoluble in water but soluble in organic solvents. Lipids are known to perform a number of functions in the body which includes; chemical messengers, storage and provision of energy, maintenance of temperature and membrane lipid layer formation (Akoh 2005). However, abnormal elevations of lipids such as total cholesterol (TC), triglyceride (TG) and low density lipoprotein cholesterol (LDL-c) results in a condition known as "Hyperlipidemia".

Hyperlipidemia is responsible for the onset and progression of atherosclerosis (Poss *et. al.,* 20011), a major risk factor in the development of coronary heart diseases (CHDs) such as ischemic heart disease, myocardial infarction and stroke (Vaziri and Morris 2011).In clinical practice, effective and intensive lipid-lowering is important in order to reduce (Murphy *et. al.* 2007, Nissen *et. al.*, 2004) and prevent CHDs (Abdulazeez 2011). *Vitex doniana* (leaves, stem and root bark) ethanolic extract significantly ($P<0.05$) reduced TC, TG and LDL-c concentrations. These reductions in TC, TG and LDL levels suggest the ameliorative potential of *Vitex doniana* extracts inhyperlipidemia.

The elevation of TC concentration in this study was achieved by the indirect stimulation of HMG CoA reductase following induction of hyperlipidemia (Johnston 2004). Hence the possible TC lowering effects of *Vitex doniana* (leaves, stem and root bark) extracts

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. It could also be due to the presence of saponins, a phytochemical which forms insoluble complexes with cholesterol or their bile salt precursor, thus making them unavailable for absorption (Messina 1999). Besides, the standard drug (Atorvastatin) used in this study inhibits HMG CoA reductase, a rate limiting enzyme in the biosynthesis of cholesterol. The results obtained in this work conform to earlier report by (Beckmann *et. al.*, 2009) that polyphenols possesses antilipidemic activity.

The increase in TAG concentration following induction of hyperlipidemia results primarily from an inhibition of TAG degradation, P407 directly inhibits capillary lipoprotein lipase (LPL) responsible for plasma TAG hydrolysis (Johnston 2004). Although the standard drug might not decrease TAG concentrations by activating lipoprotein lipase, the ethanolic extract from *Vitex doniana* could have reduced TAG levels by either activating endothelium bound lipoprotein lipase which hydrolyses the triglyceride into fatty acid hence decreasing triglyceride levels as seen in a report by Sikarwar and Patil (2011) or by inhibiting lipolysis so that fatty acids do not get converted to triglyceride.

Low density lipoprotein (LDL) 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 sub-endothelial space, as well as to undergo oxidation. The oxidized LDL is ingested by the scavengers of macrophages and the fat-laden macrophage is left with the lipid core filled with cholesterol after necrosis

and then arteriosclerosis is initiated (Beckmann *et. al.,* 2009). It was reported that some isoflavones (a type of flavonoid) increase resistance to LDL-c oxidation, like soybean isoflavones and genistein derivatives. This work also shows significant ($P<0.05$) reduction in LDL c levels by all *Vitex doniana* ethanolic extracts. This result is in accordance with the work of Baum *et. al,* (1998), who reported that phenolics may work by increasing LDL-c receptors densities in the liver binding to apolipoprotein B thereby making liver cells more efficient to remove 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 (Kim *et. al.,* 2008, Karmarkar 2008) therefore known as the protective cholesterol. The present studies shows significant ($P<0.05$) increase in HDL-c by the standard drug, leaves and stem bark extracts. This could possibly be due to increasing activity of lecithin-cholesterol acyl transferase (LCAT), an enzyme responsible for incorporating free cholesterol into HDL-c as suggested by 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 (Nicholls *et. al.,* 2007, 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 (Dobiavosa *et. al.,* 2005). It has been suggested that log (TG/HDL-c) values of -0.3 to 0.1, 0.1 to 0.24 and above 0.24 are associated with low, medium and high cardiovascular risk disease (Dobiasova 2006). The study showed that the leaves, stem bark and root bark extract significantly ($P<0.05$) increased HDL-c/TC ratio, and lowered LDL-c/HDL-c and log (TG/HDL-c) ratio when compared with the induced not treated group. The results suggest the anti-atherogenic potential of *Vitex doniana* (leaves, stem and root bark) ethanolic extracts and hence, reducing the development of coronary atherosclerosis as suggested by Dobiasova and Frohlich (Dobiasova and Frohlich 2001).

There has been conflicting reports on the effect of poloxamer 407 induced hyperlipidemia on biochemical parameters related to hepatic functions (ALP, AST and ALT). Report on the effects of poloxamer 407 induced hyperlipidemiaon serum levels of the above enzymes showed that hyperlipidemia elevated serum levels of ALT and AST (Hyeung, *et. al.,* 2006).Ameh *et. al.,* 2013 found no effect on ALT except on AST, while Johnston et al., 1999 reported that P407 does not cause hepatic injury or damage. The discrepancy in the serum levels of the enzymes could be attributed to the levels and duration of hyperlipidemia (Lu *et. al.,* 2007).

Hyperlipidemia is one of the disease conditions that are injurious to the liver; it sometimes results into fatty infiltration of the liver leading to a condition known as non-alcoholic fatty liver (Assy*et. al.*, 2000). Fatty liver is an accumulation of triglycerides and other fats in the liver cells, if not treated leads to inflammation of the liver. It is characterized by varying degree of liver injury from steatosis to hepatitis, fibrosis and

necrosis (Day and James, 1998). In these studies, the elevated levels of AST, ALT and ALP observed in the serum of induced not treated group may be due to injuries inflicted to the liver due to the accumulation of triglycerides and other fats in the liver cells and these conform to the work of Hyeung *et al.*, 2006. There was a significant ($P<0.05$) restoration of these liver marker enzyme levels in all the induced treated groups on administration of the extracts and atorvastatin. The reversal of this liver marker enzymes towards normalcy by the extracts observed in this study may be due to the prevention of the leakage of intracellular enzymes by the presence of polyphenol in the extracts and their membrane stabilizing activity (Muthu*et. al.*, 2008; Chavan*et. al*., 2012). This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Chavan*et. al*., 2012). It is therefore, a clear manifestation of hepatopreventiveeffects of the extracts especially the leaves and stem bark extracts.

Serum levels of total protein (TP), total bilirubin (TB) and indirect bilirubin (IB) and direct bilirubin (DB) are indices used to assess liver function as well as disease progression (Uthandi and Ramasamy 2011; Saad*et. al.*,2006). The levels of these liver function parameters were affected as a result of fatty liver injuries inflicted by hyperlipidemia. Total protein and bilirubin are indices for liver function; bilirubin is excreted by the liver and as such interference with the normal liver function which in turn affects its rate of conjugation or excretion. Thus a high level of total protein and bilirubin is used as indices for liver function and bile excretion status (Usha*et. al.*, 2008). The present studies showed a significant ($p<0.05$) decrease in the levels of total protein and direct bilirubin and increase in the levels of total bilirubin and indirect bilirubin in

hyperlipidemic control when compared to normal control. These changes could be as a result of fatty liver injuries inflicted by hyperlipidemia. Although, the levels are however restored towards normalcy by treatment with the plant extracts, with the leaves extract having the highest effect. Thus, suggesting the enhancement of liver functions by the extracts. All the animals treated with the extracts without induction showed no significant ($P>0.05$) difference in the total protein, total bilirubin, direct bilirubin and indirect bilirubin when compared with animals in the normal control group, thus showing the safe nature of the extracts in normal animals.

Creatinine and urea are indices for assessment of kidney function.Creatinine is a waste product formed in muscle by creatine metabolism. It is synthesized in the liver, passes into the circulation and is taken up almost entirely by skeletal muscle. Its retention in the blood is an evidence of kidney impairment. Urea is the main end product of protein catabolism. Amino acid deamination takes place in the liver where ammonia is converted into urea and excreted through urine. Urea varies directly with protein intake and inversely with the rate of excretion. Renal diseases which diminish glomerular filtration leads to urea retention. Studies have shown that poloxamer (407) is non-toxic, safe for chronic administration (Megallis *et. al.,* 2005) and capable of inducing hyperlipidemia in experimental animals (Johnson, 2004; Johnston and Palmer 1994; Palmer, *et. al.,* 1998).

Hyperlipidemia is one of the disease conditions that are injurious to the kidney. It has been found to be capable of inducing glomerular injury (Grone *et. al*., 1994; Tolins *et. al*., 1992). The elevated levels of creatinine and urea observed in the serum of induced not treated group animals may be as a result of injuries or damage of the nephron structural integrity due accumulation of triglycerides and other fats in the kidney (Khan

and Siddique, 2012). However, there was a significant ($P<0.05$) restoration of the kidney serum levels of creatinine and urea in all the induced treated groups on administration of the extracts and atorvastatin.All the animals treated with the extracts without induction showed no significant ($P>0.05$) difference in creatinine and urea when compared with animals in the normal control group.

Packed cell volume (PCV) provides information on the general pathophysiology of the blood and reticuloendothelial system (Baker and Silverton, 1985). It increases circulating inflammatory monocytes counts and renders the cells more prone for emigration into atherosclerotic lesions (Swirski *et. al.*, 2007).In these studies, the reduction in the levels of PCV observed in induced not treated groups may be due to damage to the entire reticuloendothelial system by accumulation of triglycerides and other fats (Baker and Silverton, 1985).However, there was a restoration of the levels ofPCV and Hb in all the induced treated groups on administration of the extracts and atorvastatin. The animals treated with the extract without induction shows that only the root treated group significant ($P<0.05$) increased the PCVwhen compared to the animals in the normal control group.

The decreasein body weight observed in hyperlipidemic ratscould be as a result of damages inflicted to the liver and kidney by high level of triacylglyceride and other fats, thus impairing the functions of the organs. This probably may lead to reduction in the food intake and degradation of structural proteins.However, hyperlipidemic treated groups shows a significant ($P<0.05$) increase in body weight. The increase in body weightmay be due to increasing in glucose uptake in peripheral tissues or inhibition of catabolism of fat and structural protein. All the normal treated rats shows no significant

(P>0.05) difference in body weight when compared with normal control animals, thus showing the safe nature of the extracts.

Hyperlipidemia is associated with increased oxidative stress and overproduction of oxygen free radicals (Zalba *et. al.,* 2001). The measurement of thiobarbituric acid is commonly used to monitor lipid peroxidation and indirectly, oxidative stress *in vitro* and *in vivo* (Beltowski *et. al.,* 2000). Lipid peroxidation is initiated by free radical attack on membrane polyunsaturated fatty acids leading to their transformation and fragmentation to alkanes and reactive aldehyde compounds. The observed increase in lipid peroxidation (TBARS) in hyperlipidemic control rats is consistent with experimental studies which have shown that poloxamer induced hyperlipidemia leads to increased lipid peroxidation (Dutta and Bishayi 2009).Treatment with the extracts of *Vitex doniana* significantly ($p<0.05$) reduced the TBARS concentration. The ability of the extracts to inhibit the process of lipid peroxidation in hyperlipidemia *rats* may be due to the presence of steroids, flavonoids phenols and tannins found in the preliminary phytochemical screening as previously been reported that steroids (Ghule *et. al.,* 2009), and flavonoids (Patel *et. al.,*2009) possess antihyperlipidemic activity. According to previous reports (Hideo *et al.,* 1993; Marja *et al.* 1999; Ock *et. al.,* 2003), a highly positive relationship between phenolic compounds and antioxidant activity appears to be the trend in many plant species. This activity is believed to be mainly due to their redox properties (Zheng and Wang 2001),

The body also has an effective defence mechanism to prevent and neutralize free radicals-induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione

S-transferase. Under normal conditions, antioxidant enzymes such as superoxide dismutase catalyse the conversion of superoxide radicals (O_2-) into hydrogen peroxide (H_2O_2) and O2 (Oyedemi, 2010) and catalase further detoxifies H_2O_2 into H_2O and O2 (Fridovich, 1986) while glutathione peroxidase also functions in detoxifying H_2O_2 like catalase and GST plays an essential role in the liver by eliminating toxic compounds by conjugating them with GSH. However, imbalance between the formation of reactive oxygen species and their elimination occasioned by hypercholesterolemia has been implicated in oxidative-induced diseases.

In this study, the liver and kidney antioxidant enzymes (SOD, CAT) activities significantly decreased in rats induced with hyperlipidemia compared to those in normal control group.The decrease in the activities of these enzymes could be attributed to the excessive utilization of these enzymes in inactivating the free radicals generated due to the induced hyperlipidemia (Ma *et. al.,* 2011) or insufficient availability of GSH. Our results are in agreement with Cui, 2011 who studied the effect of high fat diet on liver (Cui, 2011) antioxidant enzyme systems.Our results indicate that *V. doniana*had a free radical scavenging activity which probably provides organ protection from hypercholesterolemia. The reduction of liver and kidney TBARS, and increased liver and kidney antioxidant enzyme activities in rats treated with *V. doniana* may be related to lipid-lowering ability because its activity compared favorably with antrovastin, a lipid lowering drug (Ozansoy, 2001). The different effect of these extracts show that the extracts might have different effects on different antioxidant enzymes in liver and kidney, respectively, or the rat organs might absorb extracts differently and the metabolic enzymes in organs were different as well. Therefore, the activities of antioxidant enzymes

may vary in different organs. We hypothesize that the change of enzyme activities is related to the components or metabolites of extracts, which could affect enzymatic activities or enzyme contents. Further studies are needed to confirm this hypothesis. It can also be hypothesized that the anti-oxidant enzyme in liver and kidney may be up-regulated by administration of *V. doniana* in response to hypercholesterolemia-enhanced free radical production.

CHAPTER 6

6.0 SUMMARY, CONCLUSIONS AND RECOMMENATIONS

6.1 Summary

- The phytochemical screening of *Vitex doniana* plant shows the presence of flavonoids, Cardiac glycosides, alkaloids, saponins and tanins in the leaves, stem bark and root bark.
- The percentage yield, total polyphenol and $IC_{50}$of the extracts show that in all the parts, aqueous extract had a higher percentage yield while ethanolic extract had the highest total polyphenol and least IC_{5O} value when compared with the other extracts.
- The in vivo biological activity of the extracts shows that ethanolic extract has a higher percentage reduction of total cholesterol and triacylglycerol when compared to the other extracts.
- The quantitative phytochemical content of ethanolic extract shows that flavonoids and saponins were significantly ($P<0.05$) higher in leaves while Cardiac glycoside was highest in both leaves and stem bark. Alkaloids and tanins was significantly ($P< 0.05$) high in the root bark.
- All the parts have lowering effect on total cholesterol, triacylglycerol and low density lipoprotein-cholesterol with a concomitant increase in high density lipoprotein-cholesterol for hyperlipidemic ratsand no-significant ($P>0.05$) effects on normal rats.
- All the parts showed hepatopreventive effect as evident in the reduction of serum liver enzymes, total bilirubin, indirect bilirubin and increase in total protein and

direct bilirubin for hyperlipidemic rats, and no-significant ($P>0.05$) effects on normal rats.

- All the parts enhanced kidney function parameters (creatinine and urea) as evident in the reduction of serum creatinine and urea for hyperlipidemic rats, and no-significant ($P>0.05$) effects on normal rats.
- All the parts increased packed cell volume of hyperlipidemic and normal rats.
- All the parts increased the body weight of hyperlipidemic treated rats and had no significant ($P>0.05$) effect on normal rats.
- All the parts possessed significant ($P<0.05$) antioxidant activity by reducing lipid peroxidation and significantly ($P<0.05$) increased the endogenous antioxidant enzymes in the liver and kidney of hyperlipidemic animals.

6.2 Conclusion

The result demonstrates that all the extracts (aqueous, 70% methanol, 70% ethanol and 70% acetone) of *Vitex doniana* (leaves, stem bark and root bark) possess antihyperlipidemic activity. The ethanolic leaves extract significantly ($p<0.05$) lowered TC, TAG, LDL-c and Log (TAG/HDL-c) which is the most accurate atherogenic risk predictor seems to have the highest antiatherogenic index. All the ethanolic extracts (leaves, stem bark and root bark) significantly ($p<0.05$) increased the SOD and CAT in the liver and kidney. The ethanolic stem bark significantly ($p<0.05$) reduced lipid peroxidation in both liver and kidney.This work thus provides the phytotherapeutic potential of leaves, stem bark and root bark of *Vitex doniana* that may be needed by scientists and researchers in nutraceutical industry.

6.3 Recommendations

From these studies we recommend that the extracts (aqueous, 70% methanol, 70% ethanol and 70% acetone) of *vitex doniana* posseses high anti-hyperlipidemic potential,thus the use in traditional medicineare validated.

More work should be done on the ethanol leaves extract to ascertain the mechanism of action the extract.

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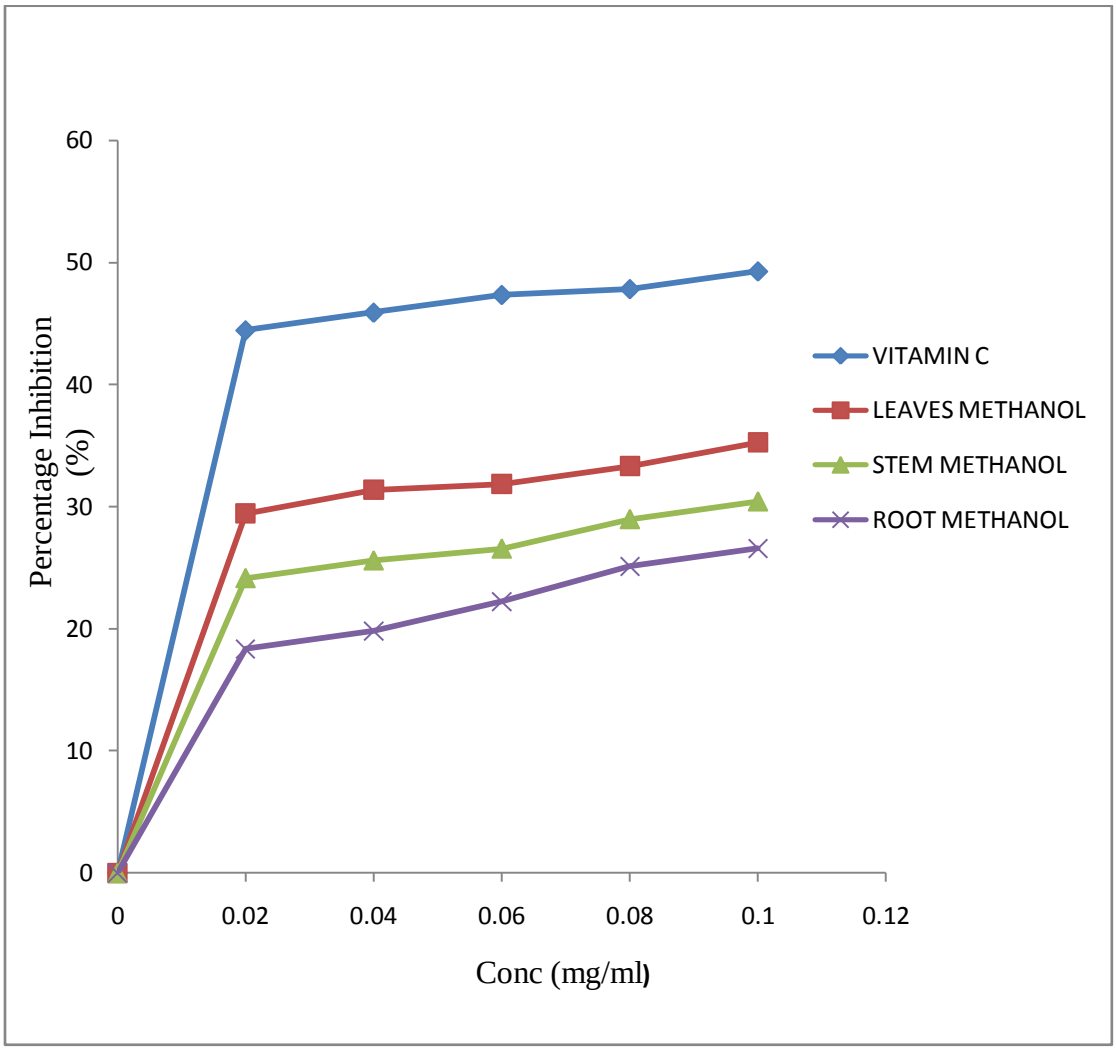
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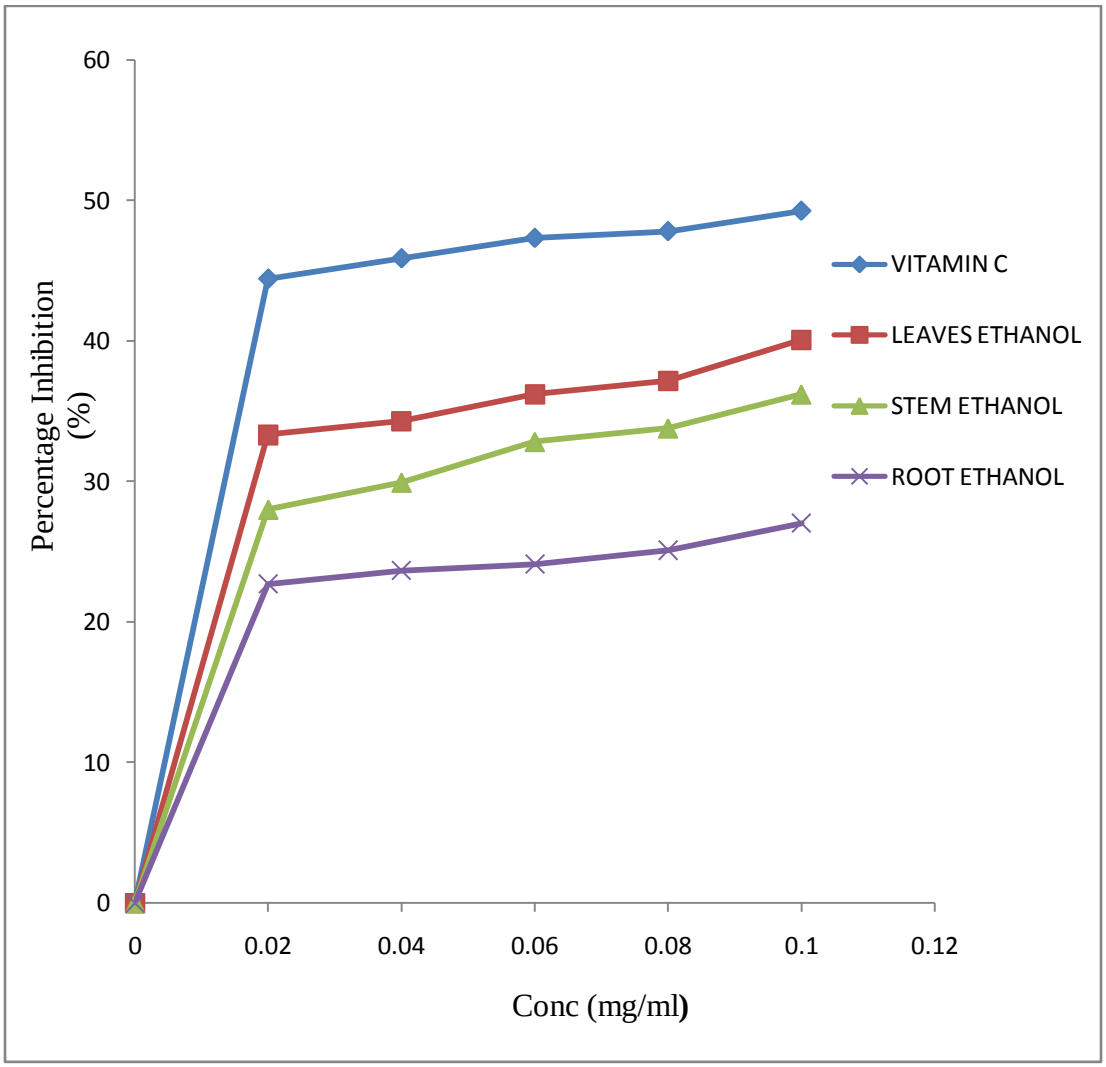
APPENDICES

Appendix 1



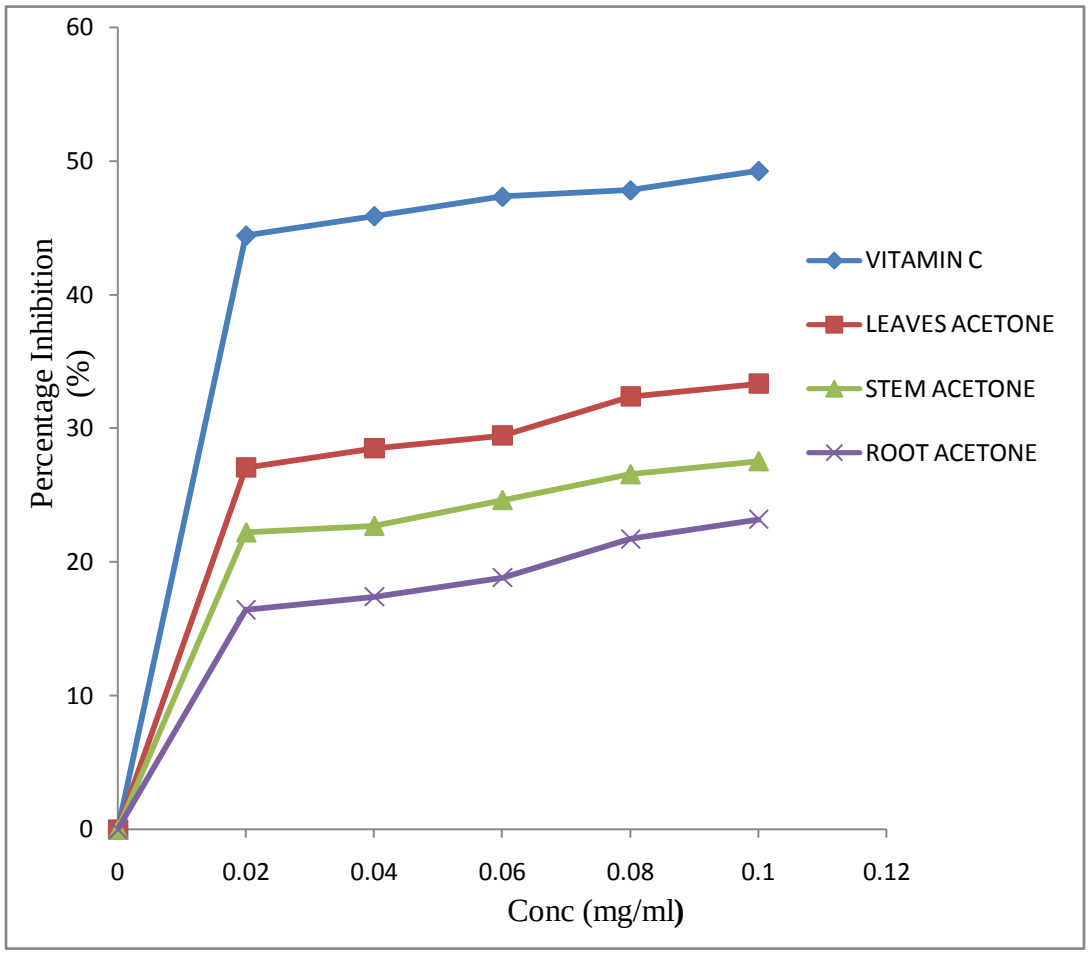
DPPH Radical Scavenging Activity of 70% of Methanol Extracts of Leaves, Stem Bark and Root Bark of *Vitex doniana*.

Appendix 2



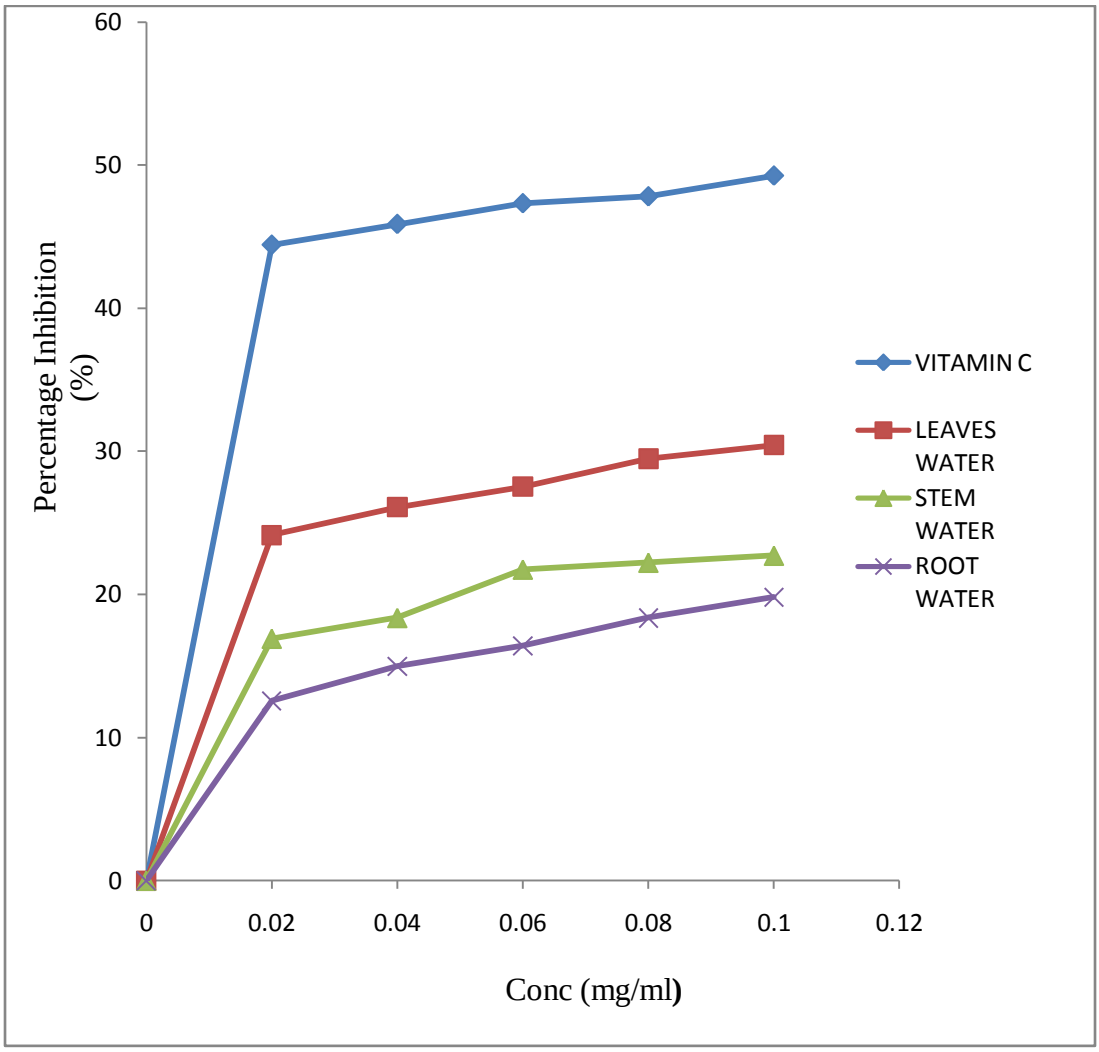
DPPH Radical Scavenging Activity of 70% of Ethanol Extracts of Leaves, Stem Bark and RootBarkof*Vitex doniana*.

Appendix 3



DPPH Radical Scavenging Activity of 70% of Acetone Extracts of Leaves, Stem Barkand RootBarkof*Vitex doniana*.

Appendix 4



DPPH Radical Scavenging Activityof Aqueous Extracts of Leaves, Stem Bark and Root Bark of*Vitex doniana.*

Appendix 5

Percentage Inhibition of DPPH at 100 µg/ml and IC_{50} Values of the Extracts of *Vitex doniana* Leaves, Stem Bark and Root Bark Compared to Vitamin C.

Extract Source		Percentage Inhibition at 100µg/ml	IC50	Equation
Vitamin C		48.792±0.008[h]	0.113	Y=57.97x + 43.47
Aqueous	Leaves	29.952±0.008[d]	0.342	Y=79.71x + 22.75
	Stem	22.705±0.008[b]	0.443	Y=77.29x + 15.74
	Root	19.807±0.008[a]	0.436	Y=89.37x + 11.06
70% Methanol	Leaves	35.265±0.008[f]	0.322	Y=67.63x + 28.21
	Stem	30.435±0.014[d]	0.347	Y=79.71x + 22.36
	Root	26.570±0.008[c]	0.313	Y=108.7x + 15.89
70% Ethanol	Leaves	40.097±0.017[g]	0.228	Y=82.12x + 31.30
	Stem	35.748±0.008[f]	0.236	Y=101.4x + 26.08
	Root	27.053±0.008[c]	0.561	Y=50.72x + 21.49
70% Acetone	Leaves	33.333±0.014[b]	0.302	Y=82.12x + 25.21
	Stem	27.053±0.008[c]	0.409	Y=72.46x + 20.38
	Root	22.705±0.008[b]	0.401	Y=89.37x + 14.15

Appendix 6

Calculations and Conversions Used In Some Analyses

(A) Calculations and Conversions Required For Analysis of Total Polyphenol Content of Extracts.

(1) Folin-ciocaltau reagent was diluted 10 times to prepare a 15ml solution by mixing 1.5 ml of Folin-ciocaltau reagent with 13.5 ml of distilled water.

(2) 0.75g of Na_2CO_3 was dissolved in distilled water and volume made up to 10ml solution to produce 75g/l solution of Na_2CO_3.

(3) To determine the mass of sample to be dissolved in distilled water producing stock from which 6050µl of sample solution was pipetted and used, the following procedure was followed.

- The percentage yield of the extract was obtained. Let the percentage yield of the extract be y
- This implies out of 100g of sample, y gram of extract was obtained. Therefore out of 10g of sample, y/10g of extract is obtained.
- y/10 g of extract obtained from 10g of sample is dissolved in 100ml of distilled water

Therefore, Xg is dissolved in 6050µl (6.05ml)

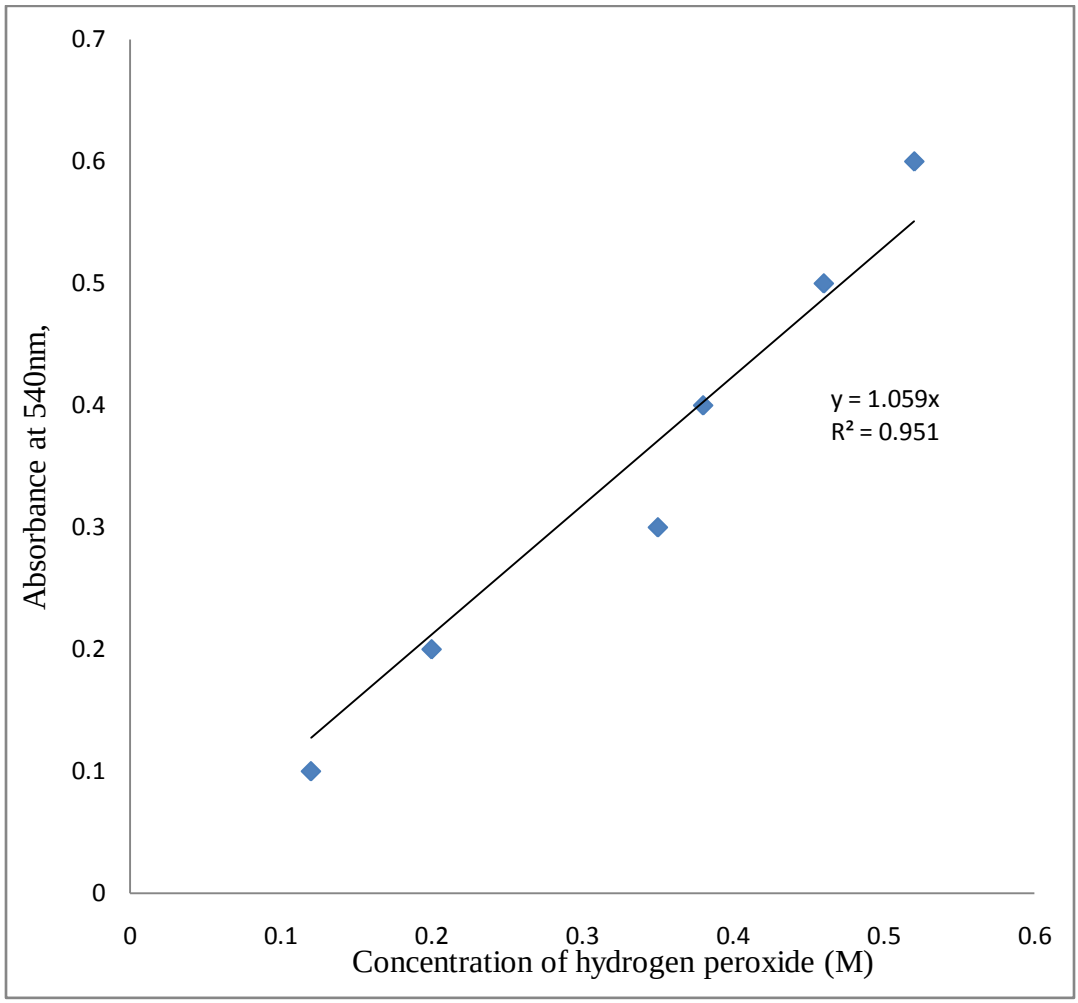
X=[y/10] x 6.05/100

(B) Conversions and calculations required in the Preparation of Varying Concentration of Extracts for DPPH Radical Scavenging Activity of the Extracts

An electronic balance was used to weigh 100µg (0.1mg) of extract. The extract was dissolved in distilled water to produce 10ml stock solution of concentration 0.1mg/ml. Stock solution was further diluted in distilled water to obtain desired concentrations of extract as demonstrated in the table below. Each dilution process was done in triplicate.

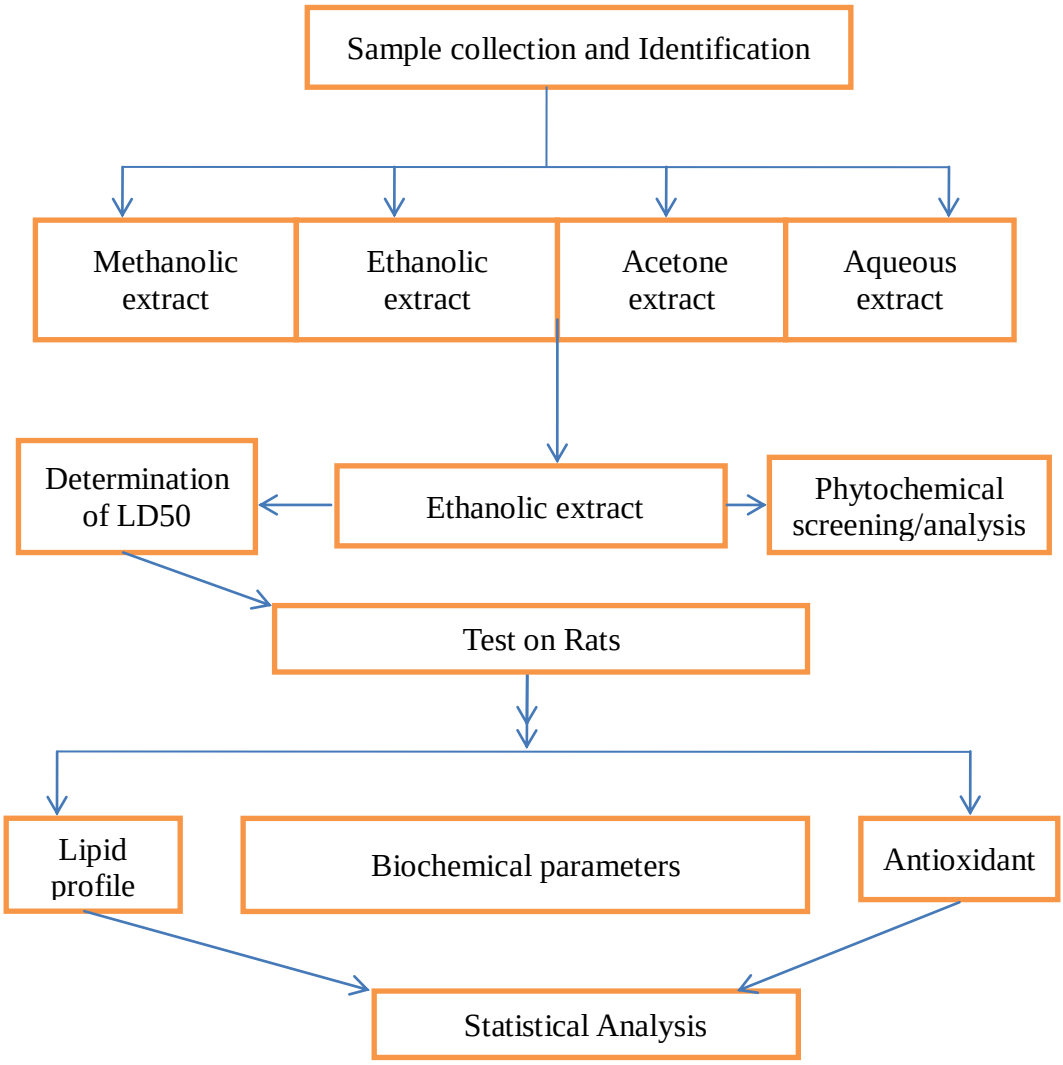
Volume of extract solution (ml)	0.2	0.4	0.6	0.8	1.0
Volume of distilled water (ml)	0.8	0.6	0.4	0.2	0.0
Final concentration of solution (mg/ml)	0.02	0.04	0.06	0.08	0.1

Appendix 7



Standard Curve for Hydrogen Peroxide

Appendix 8



The Experimental Design