ANTIHYPERLIPIDAEMIC, ANTIOXIDANT AND MOLECULAR STUDIES OF BIOACTIVE FRACTION OF ETHYL ACETATE EXTRACT FROM *Mentha piperita* LEAVES IN POLOXAMER 407-INDUCED HYPERLIPIDAEMIC RATS

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ANTIHYPERLIPIDAEMIC, ANTIOXIDANT AND MOLECULAR STUDIES OF BIOACTIVE FRACTION OF ETHYL ACETATE EXTRACT FROM PEPPERMINT LEAVES IN POLOXAMER 407-INDUCED HYPERLIPIDAEMIC RATS

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA-NIGERIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY DEGREE (Ph.D) IN BIOCHEMISTRY

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MARCH, 2021

DECLARATION

I declare that the work in this Project Thesis entitled "ANTIHYPERLIPIDAEMIC, ANTIOXIDANT AND MOLECULAR STUDIES OF BIOACTIVE FRACTION OF ETHYL ACETATE EXTRACT FROM PEPPERMINT LEAVES IN POLOXAMER 407-INDUCED HYPERLIPIDAEMIC RATS" has been carried out by me in the Department of Biochemistry, Ahmadu Bello University, Zaria, under the supervision of Prof K.M.Anigo, Prof A.B.Sallau and Prof H.C Nzelibe.The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

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CERTIFICATION

This thesis titled "ANTIHYPERLIPIDAEMIC, ANTIOXIDANT AND MOLECULAR STUDIES OF BIOACTIVE FRACTION OF ETHYL ACETATE EXTRACT FROM PEPPERMINT LEAVES IN POLOXAMER 407-INDUCED HYPERLIPIDAEMIC RATS" by Hafsat Abdullahi Mohammed, meets the regulations governing the award of the degree of Doctor of Philosophy (Ph.D) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this work to my parents Late Prof. Abdullahi Mohammed and Hajiya Amina Abdulkadir, and my husband Major General M.S. Ahmed for their untiring support and love.

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ABSTRACT

Hyperlipidaemia is a risk factor for cardiovascular diseases. The antihyperlipidaemic and antioxidant effects of bioactive fraction of ethyl acetate extract of *Mentha piperita* L. (Lamiaceae) leaves in hyperlipidaemic rats were investigated. Antihyperlipidaemic activity of hexane, ethyl acetate and methanolic extracts (100, 200 and 300 mg/kg) was evaluated using lipid profile. Twelve (12) fractions (F1-F12) were thereafter collected from the extract with the most antihyperlipidaemic activity (ethyl acetate at 100 mg/kg). The most active fractions were selected for *in vitro* antioxidant and genotoxic studies by Diphenylpicryl hydrazine (DPPH) scavenging activity and plasmid nicking assay respectively. The effects of the most antihyperlipidaemic fraction (F2) on redox sensitive biomarkers; superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and malonaldialdehyde (MDA) and lipoprotein lipase (LPL) activity at cellular and molecular levels were investigated. In addition, F2 was further subjected to Gas chromatography mass spectroscopy (GC-MS) and Liquid chromatography mass spectroscopy (LC-MS) analyses. All the fractions exhibited similar pattern of antihyperlipidaemic activity. However, fractions 2, 3, 4, 6 and 12 were significantly ($p< 0.05$) antihyperlipidaemic than other fractions, with Fraction 2 (F2) being the most potent. Half maximal inhibitory concentration (IC_{50}) values obtained from % DPPH radical inhibition of ethyl acetate extract and and its fractions (2, 3, 4, 6 and 12) were 3.0, 3.58, 3.97, 15.97, 8.13, 2.67 µg /ml respectively. Ethyl acetate extract and fractions 3, 4, 6 and 12) showed protection against Fenton's-induced genotoxicity. In addition, F2 was neither genotoxic nor geno-protective. Hyperlipidaemic animals treated with F2 showed significant ($p<0.05$) decrease in the level of MDA with concomitant enhancement of GSH, LPL activities in the liver and serum. More so, animals treated with F2 non-specifically and significantly ($p<0.05$) up regulated the expression of SOD,

CAT, and LPL in liver and blood relative to hyperlipidaemic untreated rats. GC-MS and LC-MS analyses of the most active fraction revealed oleic acid, vaccenic acid, 3, 4-dicaffeoylquinic acid, coumarin and triacontane as probable bioactive components with reported antihyperlipidaemic and antioxidant effects. Findings of this study suggest that the most active fraction (F2) from ethyl acetate extract of *M. piperita* leaves possess antihyperlipidaemic and antioxidant properties. Thus, may potentially be explored in the management of hyperlipidaemia and its related diseases.

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LIST OF ABBREVIATIONS

ACAT	Acyl CoA transferase
ANGPTL3	Angiopoietin-like 3
ANGPTL4	Angiopoietin-like 4
ANGPTL8	Angiopoietin-like 8
Apo CII	Apolipoprotein – CII
ATV	Atorvastatin
BMI	Body mass index
CAT	Catalase
CETP	Cholesteryl ester transfer protein
CVDs	Cardiovascular diseases
DEPC	Diethyl pyrocarbonate Dilution factor
DPPH	Diphenylpicrylhydrazyl
ETC	Electron transport chain
FFAs	Free fatty acids
GCMS	Gas Chromatography Mass Spectrometry
GPIHBP1	Glycosylphosphatidylinositol- anchored high lipoprotein binding density protein 1
GP_X	Glutathione Peroxidase
GR	Glutathione reductase
G6PHD	Glucose – 6- phosphate dehydrogenase
GSH	Reduced glutathione
HL	Hepatic Lipase
HDL	High-density lipoproteins
HRP	Horseradish peroxidase
IC_{50}	Inhibitory concentration at 50%
IDL	Intermediate density lipoprotein
LCAT	Lecithin cholesterol acyl transferase
LSEC	Liver sinusoidal endothelial cells

LCMS	Liquid Chromatography Mass Spectrometry
LD_{50}	Median Lethal Dose
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
MDA	Malondialdehyde
MTP	Microsomal triglyceride transfer protein
NCEP	National Cholesterol Education Programme
P407	Poloxamer 407
PPAR-α	Peroxisome proliferator activated receptor-alpha
$PPAR_S$	Peroxisome proliferator activated receptors
RF	Retention factor
SOD	Superoxide dismutase
SqS	Squalene synthase inhibitors
STZ	Streptozotocin
TC	Total cholesterol
TG	Triacylglycerol
TMB	Tetra methyl Benzidine
TRLs	Triglyceride rich lipoproteins

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Cardiovascular diseases (CVDs) are diseases that affect the heart and blood vessels and also refer to conditions that involve narrowed or blocked blood vessels which lead to heart attack, angina or stroke. CVDs have remained one of the leading causes of death globally and more people die annually from it than any other cause. According to World Health Organization (WHO, 2017), an estimated 17.9 million people died from CVDs in 2016, representing 31% of all global deaths in which 85% are due to heart attack and stroke. Risk factors for CVD, includes high blood pressure, hypercholesterolemia and high body mass index (BMI). High blood cholesterol contributes to approximately 56% of cases of cardiovascular diseases worldwide and causes about 4.4 million deaths each year (Reddy *et al.*, 2014). A review of studies carried out by Oguejiofor *et al*., (2012) showed that dyslipidemia was highly prevalent in all the geopolitical zones of Nigeria with the consistent pattern of low level of high-density lipoproteins (HDL) with high level of low density lipoprotein (LDL). The prevalence of metabolic syndrome is rising due to increased urbanization and sedentary life style (Onyenekwu *et al*., 2017).

African region has been identified with the highest burden of hypertension and non communicable diseases (Alikor and Emem - Chioma, 2018). There is high prevalence of hypertension and diabetes mellitus among the adult population in North West Nigeria (Ovosi *et al.,* 2018). Significant number of CVD risk factors (low HDL, hypertriglyceridemia, hypercholesterolemia etc.) exists in the rural and urban migrants of adult Nigerian population (Oguoma *et al.*, 2015). These factors ultimately result in disorders of lipid and lipoprotein metabolism including lipoprotein overproduction and deficiency (Karam et al., 2017). Indeed, increase in the plasma lipid levels, mainly total

cholesterol (TC), triacylglycerols (TG), and low density lipoprotein (LDL), along with a decrease in high-density lipoproteins (HDL) are known to cause a metabolic disturbance called hyperlipidaemia (Karam *et al*., 2017).

Hyperlipidaemia refers to the elevated levels of lipids and cholesterol in the blood, and is also the manifestations of different disorders of lipoprotein metabolism (Onwe *et al*., 2015). Studies have shown that hyperlipidaemia leads to lipid peroxidation and oxidative modification of proteins which may cause elevation of the level of oxidative stress (Yang et al., 2008; Csonka et al., 2016). Hyperlipidemia is also associated with the activation of NADPH oxidase and alterations in physical properties of cellular membranes which may facilitate the escape of free radicals from mitochondrial electron transport chain (Engelmann et al., 1992; Yang et al., 2008).

Consequently, endogenous and exogenous antioxidant systems are of great importance to combat this scourge. Impotantly, plants within and around our environments constitute a vital source of these antioxidants (Xu, *et al*., 2017).

Mentha piperita L. (Pepper mint) belongs to the family Lamiaceae commonly known as Peppermint which is a natural hybrid of *M. spicata* (spearmint) and *M. aquatica* (Water mint) (Barbalho, *et al*., 2017).The plant grows in the Central and Southern Europe, Asia, and Africa (Nigeria). It is widely used all over the world for flavouring, cosmetic and medicinal purposes (Dastmalchi, 2008). Pepper mint leaves are locally used in the treatment of common cold, digestive disturbances and hypertension. It is called Ruhun na'a – na'a or minti" in Hausa, "Achibo or nchiawu" in Igbo, "Awe renren or iyere" in Yoruba languages of Nigeria (Department of Biological Sciences, Herbarium section, A.B.U Zaria). *Mentha* species have also been reported as possessing antidiarrhoeal, antimicrobial, antioxidant and anti-inflammatory properties, in addition to their therapeutic potential in cardiovascular diseases (Shaikh *et al*., 1999). The aqueous

extract of *M. piperita* leaves was reported to have significant beneficial effect against fructose - induced hyperlipidaemia and strong antioxidant activity (Badal *et al*., 2011). It was reported that, cholesterol and triglycerides levels were notably reduced in streptozotocin (STZ) induced diabetic rat after treatment with aqueous extract of *M. piperita* leaf (Chandirasegaran *et al*., 2015). Equally, Peppermint juice administered to rats reduced TC, LDL body weight and atherogenic indices; and increased the levels of HDL (Barbalho *et al*., 2017). This could buttress the fact that, a strong positive correlation between hyperlipidaemia, initiation and progression of cardiovascular disorders does exist.

M. piperita leaves contain phytochemicals such as polyphenols, tannins, flavonoids and others. Phenolic compounds have been recognized as antioxidant agents (Salih *et al*., 2013). It is also rich in caffeic acid, p-cumaric, ferulic, and rosmarinic acid with highest concentration of ferulic and rosmarinic acid (Alexa *et al*., 2018). The plant has been reported to exhibit antimicrobial and antiproliferative activity (Alexa *et al*., 2018). However, its effect on LPL at cellular and molecular level vis-à-vis the link with antioxidant defence system has not been documented. It is equally important to note that, antihyperlipidaemic drugs used in the treatment of hyperlipidaemia are not fully effective, safe and free from side effect (Indumathi, *et al*., 2017). The widely used drugs for managing hyperlipidaemia are the statins which are expensive with several side effects (Kumar *et al*., 2013; Thompson *et al*., 2016; Indumathi, *et al*., 2017). Therefore, there is need to search for cheap, more effective and safe hypolipidaemic agents from foods that can be easily obtained around our microenviroment will be an advantage since it can be readily available and consumed. Hence, this study was designed to investigate the antihyperlipidaemic and antioxidant properties of bioactive fraction of ethyl acetate extract of *M. piperita* leaves in P407-induced hyperlipidaemic rats.

1.2 Statement of the Research Problem

Globally, CVDs are known to be the number one group of "killer diseases", accounting for about 17.3 million deaths annually (AHA, 2015; WHO, 2017). Survey conducted in Nigeria showed a rising trend in the prevalence of CVDs (Oguejiofor *et al*., 2012; Alikor and Emem - Chioma, 2018; Ovosi *et al*., 2018). Positive correlation exist between hyperlipidaemia and initiation as well as progression of cardiovascular disorders (Bittner, 2005).

The antihyperlipidaemic drugs used in the treatment of hyperlipidaemia are not free from side effects and statins, the widely used drugs in managing hyperlipidaemia are expensive with a lot of side effects. Some of these side effects include: kidney failure, liver failure, hypersensitivity and haematological effects et.c (Kumar *et al*., 2013; Thompson *et al*., 2016; Indumathi, *et al*., 2017).

1.3 Justification

As a result of many adverse effects associated with the use of synthetic medications, a strong interest has arisen to search for new antihyperlipidaemic substances derived from plants that are cheap, more effective and relatively safe. Accordingly, targeting hyperlipidaemia by the use of medicinal plants could be an alternative viable strategy in managing and combating cardiovascular diseases bearing in mind the issue of resistance, genotoxicity, side effects as well as cost effectiveness associated with orthodox drugs Thompson *et al*., 2016; Indumathi, *et al*., 2017). This study will look at safer antihyperlipidaemic substances to address the problem.

The plant *M. piperita* is traditionally used in Nigeria in the treatment of hypertension and other cardiovascular related diseases but has not been scientifically validated. Even though aqueous extract of the plant has been reported with antihyperlipidaemic effect, the mechanistic study is still lacking (Chandirasegaran *et al*., 2015). However, its effect

on Lipoprotein lipase at cellular and molecular level vis-à-vis the link with antioxidant defence system has not been documented. Hence, the consideration in the therapy of hyperlipidaemia and subsequent atherosclerotic effect is to reduce the elevated blood lipids. This study will ultimately highlight the mechanism of action of this plant in the prevention and treatment of cardiovascular and related diseases.

1.4 Research Hypothesis

Bioactive fractions of *Mentha piperita* leaves do not have antihyperlipidaemic and antioxidant properties.

1.5 Aim of the Study

The aim of this research was to investigate the antihyperlipidaemic, antioxidant and molecular effects of bioactive fractions of ethyl acetate from *M. piperita leaves* in Poloxamer 407- induced hyperlipidaemic rats.

1.6 Specific Objectives:

i. To establish median lethal dose (LD_{50}) of different solvents leaf extracts (hexane, ethyl acetate and methanol).

ii. To investigate the *in vivo* antihyperlipidaemic activity of the extracts in Poloxamer 407 induced hyperlipidaemic rats.

iii. To assess the *in vivo* antihyperlipidaemic activity of fractions obtained from the most active ethyl acetate extract.

iv. To evaluate the *in vitro* antioxidant and genotoxic properties of the most antihyperlipidaemic fraction(s) of ethyl acetate extract.

v. To determine the effect of the most active fraction of ethylacetate extract on the integrity of hepatocytes of hyperlipidaemic rats by histopathology.

vi. To determine the effects of the most active fraction of ethyl acetate extract on oxidative stress biomarkers and lipoprotein lipase activity in the liver and serum.

vii. To evaluate the effect of the bioactive fraction on expression of lipoprotein lipase and oxidative stress biomarkers in the liver and blood.

viii. To identify the possible components of the most active fraction of ethyl acetate extract.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Hyperlipidaemia

Hyperlipidaemia is a condition of excess fatty substances (lipids), largely cholesterol and triglycerides, in the blood. It is also called hyperlipoproteinemia because these excess lipids travel in the blood attached to proteins. Therefore, hyperlipidaemia overall is characterized by excess blood lipids which include cholesterol (CH), triglyceride (TG), low density lipoprotein (LDL) and decrease in high density lipoprotein (HDL) in blood stream. It is a modifiable risk factor for atherosclerosis, cardiovascular disease and other related conditions (Karam *et al.,* 2017).The primary cause of hyperlipidaemia is defect in lipid metabolism which is caused by the defect in lipoprotein lipase activity or the absence of the surface Apolipoprotein C-II (Harikumar *et al*., 2013). Other causes of hyperlipidaemia include various genetic abnormalities and environmental factors (Nirosha *et al.,* 2014).

The fat-protein complexes in the blood are called lipoproteins. Excess LDL-cholesterol contributes to the blockage of arteries, which eventually leads to heart attack. Population studies have clearly shown that the higher the level of LDL-cholesterol, the greater the risk of heart disease (Nirosha *et al.,* 2014). Hence, LDL-cholesterol has been labelled the "bad cholesterol". In contrast, the lower the level of HDL-cholesterol, the greater the risk of coronary heart disease. As a result, HDL-cholesterol is commonly referred to as "good cholesterol". Low HDL-cholesterol levels are typically accompanied by an increase in blood triglyceride levels (Nirosha *et al.,* 2014; Onwe *et al.,* 2015). Studies have also shown that high triglyceride levels are associated with an increased risk of coronary heart disease (Nirosha *et al.,* 2014; Karam *et al.,* 2017).

Oxidative stress is implicated in the complications of hyperlipidaemia. Elevated oxidative stress has been demonstrated in renal and liver tissues in hyperlipidaemic rats (Bhalodia *et al*., 2010; Singh *et al*., 2015) and this can increase the level of total ROS which has been shown to affect the antioxidant status of different organs (including the liver and kidney) as well as their lipoprotein levels (Singh *et al*., 2015).

2.2 Classification of Hyperlipidaemia

Hyperlipidaemia, in general, can be divided into two subcategories based on the type of lipid

i. Hypercholesterolemia, in which there is a high level of cholesterol

ii. Hypertriglyceridemia, in which there is a high level of triglycerides, the most common form of fat (Karam *et al.,* 2017).

Based on the causative factor, hyperlipidaemia may be classified as either familial (primary) which is caused by specific genetic abnormalities, or acquired (secondary) which results from another underlying disorder that leads to alterations in plasma lipid and lipoprotein metabolism. Also, hyperlipidaemia may be idiopathic, that is without known cause (Nirosha *et al.,* 2014).

Familial hyperlipidaemias are classified according to the Fredrickson classification, which is based on the pattern of lipoproteins on electrophoresis or ultracentrifugation.

Type I - Raised cholesterol with high triglyceride levels

Type II - High cholesterol with normal triglyceride levels

Type III - Raised cholesterol and triglycerides

Type IV - Raised triglycerides, atheroma, and raised uric acid

Type V - Raised triglycerides (Harikumar *et al*., 2013).

2.2.1 Hyperlipoproteinemia Type I

Type I hyperlipidaemia also known as familial hyperchylomicronemia is quite uncommon and this disorder causes high levels of chylomicrons, the lipoproteins that carry fat from the intestine to the liver. It can cause abdominal pain, pancreatitis, fat deposits in the skin and eyes, enlarged liver and spleen. Treatment involves eating a diet very low in fat. Orlistat is a medication that can also be used to improve hyperchylomicronemia by decreasing the risk of pancreatitis; it is a pancreatic lipase inhibitor given before meals (Blom *et al.*, 2018).

2.2.2 Hyperlipoproteinemia type II

Hyperlipoproteinemia Type II, by far the most common form, is further classified into Types IIa and IIb, depending mainly on whether there is elevation in the triglyceride level in addition to LDL cholesterol. Type IIa is also known as familial hypercholesterolemia and type IIb is also known as familial combined hyperlipidaemia (Karam et al., 2017). Type lIa results in high LDL or "bad cholesterol" levels. Type IIa also raises levels of LDL, as well as a similar lipoprotein, VLDL, which results in elevated fat levels in the blood. These conditions cause fat deposits under the skin and around the eyes, and are treated medically with dietary control (Harikumar *et al*., 2013; Nirosha *et al*., 2014).

2.2.3 Hyperlipoproteinemia Type III

This form is due to high chylomicrons and intermediate density lipoprotein (IDL). Also known as broad beta disease or dysbetalipoproteinemia, the most common cause of this disease is the presence of ApoE /E2 genotype. It is due to cholesterol-rich VLDL (Harikumar *et al*., 2013; Nirosha *et al*., 2014).

2.2.4 Hyperlipoproteinemia Type IV

Familial hypertriglyceridemia is an autosomal dominant condition occurring in approximately 1% of the population in which the levels of VLDL are elevated. It is also treated with medicines and proper diet (Harikumar *et al*., 2013; Nirosha *et al*., 2014).

2.2.5 Hyperlipoproteinemia Type V

Hyperlipoproteinemia Type V is s another rare type of hyperlipidaemia that is characterized by elevated chylomicrons and VLDL, very similar to Type I, but with high VLDL in addition to chylomicrons. It is also associated with glucose intolerance and hyperuricemia. Type V hyperlipoproteinemia is also known as familial mixed hyperlipidaemia (Harikumar *et al*., 2013; Nirosha *et al*., 2014).

Acquired hyperlipidaemias (also called secondary dyslipoproteinemias) may mimic primary forms of hyperlipidaemia and can have similar consequences. They may result in increased risk of premature atherosclerosis or, when associated with marked hypertriglyceridemia, may lead to pancreatitis and other complications of the chylomicronemia syndrome. The most common causes of acquired hyperlipidaemia are diabetes mellitus, use of drugs such as diuretics, beta blockers, and estrogens. Other conditions leading to acquired hyperlipidaemia include: hypothyroidism, renal failure nephrotic syndrome, alcohol, some rare endocrine disorders and metabolic disorders (Harikumar *et al*., 2013).

2.3 Signs and Symptoms of Hyperlipidaemia

Hyperlipidaemia usually has no noticeable symptoms and tends to be discovered during routine examination or evaluation for atherosclerotic cardiovascular disease such as; heart attack, chest pain, xanthoma, abdominal pain, enlarged liver, enlarged spleen (Bhatnagar *et al*., 2008).

2.4 Causes of Hyperlipidaemia

Lifestyle habits or medical conditions. Lifestyle contributors include obesity, lack of exercise, and smoking (Harikumar *et al*., 2013). Other causes include diabetes (Type 2), kidney disease, pregnancy, alcohol intake, environmental and genetic factors et.c.The risk factors for hyperlipidaemia are: high fat intake which is one of the factors that leads to hypercholesterolemia. Chronic renal failure, alcohol intake, drugs (antihypertensive agents, diuretics, Oral contraceptives et.c). Complications of hyperlipidaemia include pancreatitis, premature coronary artery disease, heart attack, stroke, atherosclerosis and myocardial infarction (Nirosha *et al.,* 2014).

2.4.1 Pathophysiology of Hyperlipidaemia

Decreased clearance of triglyceride-rich lipoproteins due to inhibition of lipoprotein lipase. Other factors such as peripheral insulin resistance, carnitine deficiency, and hyperthyroidism may contribute to lipid abnormalities (Kingsburry and Bondy, 2003; Harikumar *et al*., 2013).

2.4.2 Pathophysiology of hypercholesterolemia

LDL cholesterol normally circulates in the body for 2.5 days, and subsequently binds to the LDL receptor on the liver cells, undergoes endocytosis (Ford *et al*., 2002). LDL is removed, and synthesis of cholesterol by the liver is suppressed in the HMG-CoA reductase pathway. In familial hyperlipidaemia, LDL receptor function is reduced or absent, and LDL circulates for an average duration of 4.5 days, resulting in significantly increased level of LDL cholesterol in the blood with normal levels of other lipoproteins (Ford *et al*., 2002). Due to mutations of ApoB, reduced binding of LDL particles to the receptor causes the increased level of LDL cholesterol (Ford *et al*., 2002).

2.4.3 Pathophysiology of Hypertriglyceridemia

Hypertriglyceridemia may be as a result of two Processes: The first is overproduction of VLDL by the liver in response to an increase in free fatty acids flowing to the organ.

The other process is a defect in the lysis of VLDL triglycerides and chylomicrons by lipoprotein lipase. When lipoprotein lipase activity is deficient, triglycerides cannot be converted, hydrolyzed, or broken down, and the metabolism of chylomicron and VLDL remnants may be delayed, as in Type III hyperlipoproteinemia (Jap *et al*., 2003).

2.4.4 Prognosis

Hyperlipedemia is a condition characterized by an increased amount of fats (lipids) present in the bloodstream. The prognosis for the condition varies according to a number of different factors. The prognosis for persons is in direct proportion to their serum cholesterol levels. Persons with hypercholesterolemia are at high risk of dying from heart disease or stroke (Genest and Libby, 2011; Semenkovich, 2011).Many studies have looked at the relationship between elevated cholesterol levels, increased risk for heart attack and death. The risk of a fatal heart attack was approximately five times higher among persons having cholesterol levels of 300 mg/dL or more compared to those with cholesterol levels below 200 mg/dL (Genest and Libby, 2011; Semenkovich, 2011).

2.4.5 Management of Hyperlipidaemia

Diet changes, exercise, and medications can lower cholesterol levels for those with the milder form of this disorder, and may significantly delay a heart attack. Men and women with familial hypercholesterolemia typically are at increased risk of early heart attacks. Risk of death varies among patients with familial hypercholesterolemia (Harikumar *et al*., 2013; Nirosha *et al*., 2014).

2.5 Treatment of Hyperlipidaemia

2.5.1 HMG-COA Reductase Inhibitors (statins)

Statins are a class of drugs used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase, which plays a central role in the production of cholesterol in the

liver (Ray *et al*., 2010). Increased cholesterol levels have been associated with CVDs, and statins are therefore used in the prevention of these diseases (Ray *et al*., 2010). Statins have rare adverse effects, particularly muscle damage, and some doctors believe they are overprescribed (Ray *et al*., 2010). The approved statins are; Atorvastatin, Fluvastatin, Lovastatin, Pravastatin Rosuvastatin, Simvastatin and Pitavastatin (Ray *et al*., 2010).

Mode of Action of Statins

Statins act by competitively inhibiting HMG-CoA reductase, the first committed step of the cholesterol biosynthetic pathway. Statins are similar to HMG-CoA on a molecular level, they take the place of HMG-CoA in the enzyme and reduce the rate by which it produces mevalonate, the next molecule in the cascade that eventually produces cholesterol, as well as a number of other compounds (Nirosha *et al*., 2014).

Cholesterol biosynthetic pathway, which is blocked by statins, ultimately reduces cholesterol via several mechanisms. Statins block the production of cholesterol in the liver itself (Lewington *et al*., 2007). They lower LDL, the "bad cholesterol", and triglycerides, and have a mild effect in raising HDL, the "good cholesterol". These drugs are the first line of treatment for most people with high cholesterol (Lewington *et al*., 2007).

2.5.2 Bile acid sequestrants

The bile acid sequestrants are a group of medications used to bind certain components of bile in the gastrointestinal tract. They disrupt the enterohepatic circulation of bile acids by sequestering them and preventing their reabsorption from the gut. In general, they are classifiedas hypolipidemic agents, although they may be used for purposes other than lowering cholesterol (Harikumar *et al*., 2013; Nirosha *et al*., 2014). They are used in the treatment of chronic diarrhoea due to bile acid malabsorption. When the

statins are not sufficient to lower high cholesterol, these drugs can be added. Their use is often limited by side effects, which are primarily gastrointestinal. They can include nausea, bloating, cramping, and an increase in liver enzymes. Examples include cholestyramine, colesevelam and colestipol (Harikumar *et al*., 2013; Nirosha *et al*., 2014).

Mode of Action of Bile Acid Sequestrants

Bile acid sequestrants are polymeric compounds that serve as ion exchange resins. Bile acid sequestrants exchange anions such as chloride ions for bile acids. By doing so, they bind bile acids and sequester them from enterohepatic circulation (Wong, 2001). Since bile acid sequesterants are large polymeric structures, they are not well absorbed from the gut into the bloodstream. Thus, bile acid sequestrants, along with any bile acids bound to the drug, are excreted via the feaces after passage through the gastrointestinal tract (Wong, 2001).

2.5.3 Fibric Acid Derivatives (fibrates)

Fibrates are cholesterol-lowering drugs that are primarily effective in lowering triglycerides and to a lesser extent in increasing HDL-cholesterol levels (Abourbih, 2009). Fibrates prescribed commonly are; bezafibrate, ciprofibrate, clofibrate, gemfibrozil and fenofibrate (Abourbih, 2009)

Mode of Action of Fibric Acid Derivatives

Fibrates are agonists of the peroxisome proliferator-activated receptors (PPAR-α) receptor in muscle, liver, and other tissues. Activation of PPAR-α signaling results in: Increased β-oxidation in the liver, decreased hepatic triglyceride secretion, increased lipoprotein lipase activity, increased VLDL clearance, Increased HDL and Increased clearance of remnant particles. Fibrates activate PPARα, a class of intracellular receptors that modulate carbohydrate and fat metabolism so also adipose tissue

differentiation (Kumar *et al.,* 2013; Nirosha *et al.,* 2014). Activating PPARs induces the transcription of a number of genes that facilitate lipid metbolism (Harikumar *et al*., 2013; Nirosha *et al.,* 2014).

2.5.4 Nicotinic Acid

Nicotinic acid reduces the production of triglycerides and VLDL which is converted to LDL in the blood. This leads to decreased LDL, increased HDL, and lowered triglycerides. Nicotinic acid raises HDL cholesterol more than other lipid-lowering medicines. The nicotinic acid form of niacin lowers cholesterol, but other forms of niacin do not lower cholesterol which includes nicotinamide and inositol nicotinate (Lee *et al.,* 2013).

There are other agents with promising antihyperlipidaemic activity which are: Acyl-CoA cholesterol acyl transferase (ACAT) inhibitors. Avasimibe and Eflucimibe act by inhibiting ACAT, decrease plasma cholesterol levels and slow the development of atherosclerosis. Some of the potent ACAT inhibitors which are in clinical development are naphthoquinone derivatives (Lee *et al.,* 2013). Microsomal triglyceride transfer protein (MTP) inhibitors also cause significant reductions in plasma triglycerides, LDL and VLDL cholesterol. A series of synthesized phosphonate esters exhibited potent *in vitro* and *in vivo* inhibition on MTP activity (Lee *et al.,* 2013).

Cholesteryl ester transfer protein (CETP) inhibitors in liver facilitate the transfer of cholesteryl esters from anti-atherogenic HDLs to apolipoprotein B containing lipoproteins, including VLDLs and LDLs (Shinkai, 2012). Dalcetrapib and anacetrapib are novel compounds in clinical trials. Dalcetrapib reduced CETP activity by 50% and elevated HDL cholesterol levels by 31% without affecting LDL cholesterol levels (Shinkai, 2012). Pharmacologists also regard Squalene synthase (SqS) inhibitors, Hydroxymethylglutaryl-CoA synthase inhibitors, ATP citrate lyase inhibitors, Squalene

epoxidase inhibitors and Lanosterol synthase inhibitors as antihyperlipidaemic compounds (Shattat, 2014).

2.6 Enzymes and Proteins involved in Lipoprotein Metabolism

2.6.1 Hepatic Lipase

Hepatic Lipase (HL) (EC 3.1.1.3) is a multifunctional protein that regulates lipoprotein metabolism. It is synthesized by hepatocytes and hydrolyzes phospholipids and triglycerides of plasma lipoproteins (Shattat, 2014).

2.6.2 Lecithin cholesterol acyl transferase

Lecithin cholesterol acyltransferase (LCAT) (EC 2.3.1.43), is a crucial enzyme in the metabolism of HDL (Shattat, 2014). It converts free cholesterol into cholesteryl esters which is then sequestered into the core of lipoprotein and finally making mature HDL (Nakhjavani *et al.,* 2013).

2.6.3 Cholesteryl ester transfer protein

Cholesteryl ester transfer protein (CETP), also called plasma lipid transfer protein, is a hydrophobic plasma glycoprotein that accelerates the transfer of esterified cholesterol esters from HDLs to chylomicrons, VLDL and LDL, in exchange for triglyceride. Deficiency of CETP is linked to increased HDL levels and decreased LDL levels (Arise *et al.,* 2012).

2.6.4 Microsomal triglyceride protein (MTP)

Microsomal triglyceride protein (MTP) is a lipid transfer protein which catalyzes the transfer of neutral lipids, triglycerides and cholesterol esters between membrane of the lumen of microsomes isolated from the liver and intestinal mucosa. It is essential for the assembly of apo B containing lipoproteins (Shattat, 2014).

2.6.5 Acyl Co-A transferase

Acyl Co-A transferase (ACAT) (EC 2.8.3.8) is membrane-bound protein that uses long-chain fatty acyl-CoA and cholesterol as substrates to produce cholesteryl esters. ACAT plays significant roles in cellular cholesterol homeostasis in various tissues and prevents the toxic accumulation of excess cholesterol in a cell (Shattat, 2014).

2.7 Lipoprotein Lipase

LPL (EC 3.1.1.34) is an extracellular enzyme essential in lipoprotein metabolism. The enzyme is a glycoprotein that is synthesized and secreted in several tissues (adipose tissue, skeletal muscle, cardiac muscle, and macrophages) (Kersten, 2014; Andrade, 2018). LPL acts at the luminal surface of the vascular endothelium where it hydrolyzes triglyceride-rich lipoproteins (e.g., chylomicrons, very low-density lipoproteins), providing FFAs and glycerol for tissue use and excess are stored in adipose tissue (Kersten, 2014).Triacylglycerols and monoacylglycerols are preferred substrates, whereas phosphatidylcholine is hydrolyzed at a slower rate (Kobayashi and Mabuchi, 2015).

Inhibition of the enzyme leads to the accumulation of triglycerides in the plasma (Momin *et al.,* 2016; Björnson *et al.,* 2017). The catalytic activity of LPL along the capillary endothelium is considered rate-limiting for the lipolytic processing of plasma triglycerides and for the subsequent uptake of fatty acids by surrounding tissues (Young and Zechner, 2013; Kersten, 2014).

Therefore, LPL plays a key metabolic role in providing substrates for lipogenesis and lipid storage, and in supplying immediate energy for different tissues. LPL activity has been reinforced by its pathophysiological relevance in chronic degenerative diseases such as dyslipidaemia, obesity, type 2 diabetes mellitus, Alzheimer's disease, and in other contexts of disordered lipid metabolism such as severe hypertriglyceridaemia,

metabolic syndrome, acute pancreatitis as well as non-alcoholic fatty liver disease (Andrade, 2018).

2.7.1 Structure of lipoprotein lipase

The structure of lipoprotein lipase is similar to the enzymes in lipase family and is composed of two distinct regions. The *N*-terminal domain has a lipolytic active site and C- terminal domain are connected by a hinge region which are attached via peptide link (Llinas *et al.* 2005; Birrane *et al.,* 2018).The LPL gene is located on chromosome 8p22, spans 30 kb and is divided into 10 exons; it has substantial sequence homology among most of the species that have been examined (Llinas *et al.* 2005; Birrane *et al.,* 2018). The cDNA codes for a 475-amino-acid protein including a 27-amino-acid signal peptide. The catalytic center is formed by three amino acids: Ser^{132}, Asp^{156}, and His^{241} (Brunzell, 1995).

2.7.2 Cofactor of Lipoprotein Lipase

Lipoprotein lipase requires apolipoprotein-CII, as a cofactor.The cofactor is carried by a chylomicron as well as by VLDL and IDL for activation (Kobayashi and Mabuchi, 2015). Lipoprotein lipase shrinks the chylomicrons by removing the fatty acids from triglycerides, and transferring them to adipocytes and skeletal muscle, while the chylomicron remnants are taken up by the liver via receptor-mediated endocytosis (Kobayashi and Mabuchi, 2015). ApoC-II is a constituent of chylomicrons, VLDL, LDL, and HDL particles. ApoC-II contains three amphipathic α-helices, an N-terminal lipid binding domain, and a C-terminal LPL binding domain (Kei *et al*., 2012).

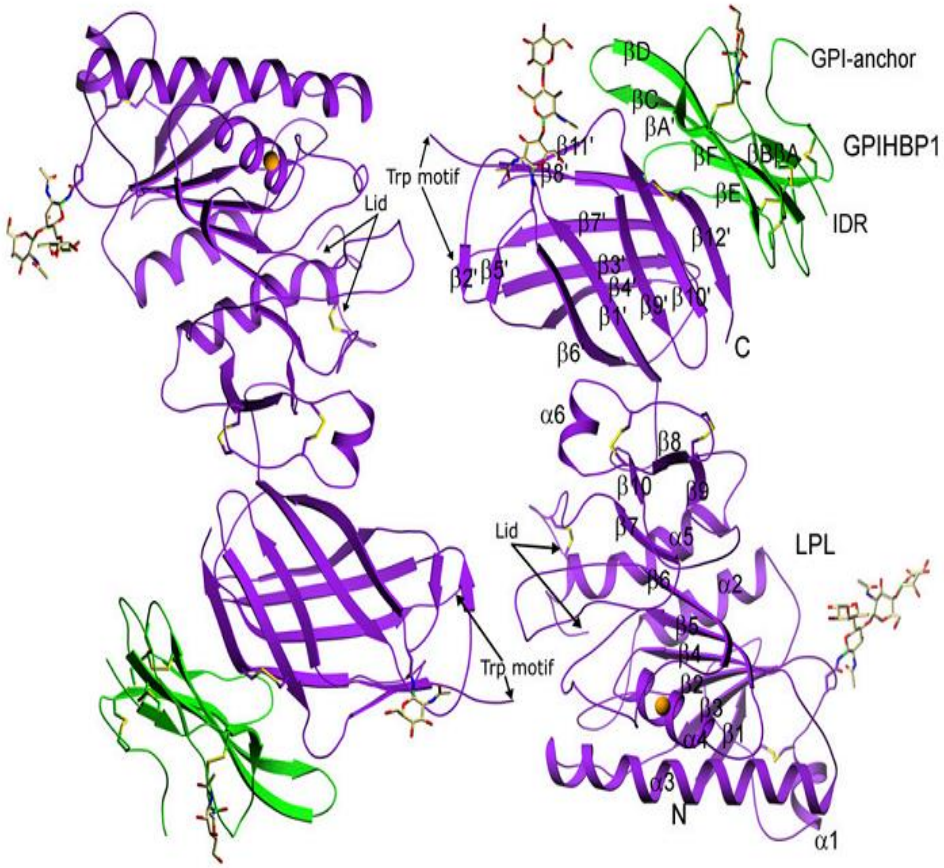


Figure 2.1: Structure of the LPL–GPIHBP1 complex, as depicted by ribbon representations of the two LPL–GPIHBP1 complexes in the asymmetric crystallographic unit (Llinas *et al.* 2005; Birrane *et al.*, 2018).

LPL (purple) has five disulfide bonds (C54–C67, C243–C266, C291–C302, C305–C310, and C445–C465); a single calcium ion (orange sphere) coordinated by A194, R197, S199, D201, and D202; and two N-linked glycans (at N70 and N386). GPIHBP1 (green) has one N-linked glycan (at N78). LPL contains an N-terminal α/β-hydrolase domain (N) containing 6α-helices and 10β-strands and a C-terminal flattenedβ-barrel domain (C) containing 12β-strands, connected by a hinge region. IDR - intrinsically disordered region.

2.7.3 Pathophysiology of lipoprotein lipase

The pathophysiology of lipoprotein lipase is evident in familial dyslipidemias (specifically type one), or hyperchylomicronemia. In both diseases there is severe LPL dysfunction; due to LPL deficiency and LPL co-factor deficiency (apolipoprotein CII) deficiency, which is necessary for activation of lipoprotein lipase (Jap *et al.,* 2003; Falko, 2018). LPL normally removes triglycerides from chylomicrons, and if this process does not function, initial triglyceride breakdown cannot take place. Therefore, triglycerides will build up in the serum and chylomicrons will grow very large as they are full of triglycerides, which are not undergoing removal (Falko, 2018). Patients with hyperchylomicronemia present with recurrent pancreatitis, enlarged liver, and Xanthomas because of lack of triglyceride removal from chylomicrons and consequent increased levels of serum triglycerides and increased chylomicrons (Falko, 2018). This build up eventually leads to triglyceride accumulation in the pancreas, the liver, and deposit on the skin causing these clinical manifestations.

Elevated triglycerides can cause acute pancreatitis; this may involve increased chylomicrons in the plasma (Fong *et al*., 2016).These chylomicrons can obstruct capillaries, and this may cause decreased blood flow and ultimately ischemia (Kroupa *et al.,* 2012; Catoire *et al.,* 2014).When vessel damage occurs, this can cause pancreatic lipases to have access to serum triglycerides. Pancreatic lipases may then break down these triglycerides, leading to the release of triglyceride breakdown products including free fatty acids. This increase in free fatty acids can injure the tissue of the pancreas leading to pancreatitis (Okopień, 2018).

Clinically, LPL plays a significant role in the progression of atherosclerosis. Furthermore, patients with advanced atherosclerosis have elevated LPL mass and

activity in their post-heparin plasma (Kroupa *et al.,* 2012; Catoire *et al.,* 2014). LPL acts on chylomicrons and VLDL to hydrolyze triglycerides from them and form VLDL remnants and chylomicron remnants (Okopień, 2018).VLDL remnants and chylomicron remnants are composed of a high concentration of cholesterol esters and then get incorporated into macrophages (Birrane et al., 2018). Free fatty acids are formed by LPL and macrophages then re-esterify them, which leads to the accumulation of cholesterol esters in macrophages, causing macrophages to transform into foam cells. LPL catalyzes the conversion of VLDL to IDL, which hepatic lipases then convert to LDL (Birrane et al., 2018). Foam cells also form when macrophages incorporate oxidized LDL via their scavenger receptor in the vascular endothelium (Okopień, 2018).

2.7.4 Regulation of Lipoprotein Lipase

LPL produced by myocytes and adipocytes is transported towards the endothelial cell surface glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1 (GPIHBP1) (Davies, 2010). GPIHBP1, a member of the LU (Ly6/uPAR) protein family, is expressed exclusively in capillary endothelial cells.Based on protein homology considerations; GPIHBP1's LU domain (75 residues in length) is predicted to adopt a three-fingered fold, stabilized by five disulfide bonds (Kristensen *et al.,* 2016). GPIHBP1 is unique within the LU protein family in having a highly acidic and intrinsically disordered N-terminal domain containing a sulfated tyrosine and numerous glutamates and aspartates (Kristensen *et al.,* 2016; Mysling *et al*., 2016a).

GPIHBP1 binds to the C-terminal domain of LPL, and both the LU domain and the acidic domain of GPIHBP1 contribute to the binding affinity (Mysling *et al*., 2016a). First, GPIHBP1 is solely responsible for capturing LPL within the interstitial spaces and shuttling it across endothelial cells to its site of action in the capillary lumen. In the absence of GPIHBP1, LPL remains stranded in the interstitial spaces.Second,

GPIHBP1-bound LPL is required for the margination of Triglyceride rich lipoproteins (TRLs) along capillaries, allowing the lipolytic processing of TRLs to proceed (Fong *et al*., 2016)). In the absence of GPIHBP1, TRLs do not stop along capillaries and simply flow in the bloodstream. Third, the binding of GPIHBP1 stabilizes the structure of LPL, thereby preserving its enzymatic activity. In the absence of GPIHBP1, LPL's hydrolase domain is highly susceptible to spontaneous unfolding, resulting in a rapid decline in catalytic activity (Mysling *et al*., 2016b). When LPL is complexed with GPIHBP1, the unfolding of LPL and the concomitant loss of activity is markedly inhibited (Mysling *et al*., 2016b). The ability of GPIHBP1 to prevent the unfolding of LPL and preserve catalytic activity depends on GPIHBP1's disordered acidic domain (Kristensen *et al.*, 2016; Mysling *et al*., 2016a).When the acidic domain is deleted, GPIHBP1 still binds to LPL, but the protective effects of GPIHBP1 on LPL structure and activity are minimal or absent (Kristensen *et al.*, 2016; Mysling *et al*., 2016b). GPIHBP1 also inhibits the unfolding of LPL catalysed by physiologic inhibitors of LPL (ANGPTL4, ANGPTL3) (Mysling *et al*., 2016b).

The central importance of both LPL and GPIHBP1 in plasma triglyceride metabolism is illustrated by the fact that missense mutations that interfere with LPL–GPIHBP1 interactions profoundly impair intravascular triglyceride processing, resulting in severe hypertriglyceridemia (chylomicronemia) (Fong *et al*., 2016).

Angiopoietin-like 3 (ANGPTL3), Angiopoietin-like 4 (ANGPTL4) and Angiopoietin-like 8 (ANGPTL8) are three members of the angiopoietin-like protein family that are involved in the post-translational regulation of LPL (Dijk *et al.*, 2015; Dijk *et al.*, 2018). ANGPTL3 and ANGPTL8 are secreted by the liver as hepatokines and cooperate to inhibit the activity of LPL in oxidative tissues such as heart and brown fat, with a primary action in the fed state (Wang *et al.*, 2013; Haller *et al.*, 2017; Chi *et al.*, 2017).

Lipoprotein lipase appears to be produced at a relatively constant rate. The activity and the distribution of the enzyme between the endothelial cell surface and other places in the tissue are determined by lipase maturation factor 1(LMF1), ANGPTL4 and GPIHBP1 and perhaps other proteins in a context dependent manner (Wang *et al.*, 2013; Haller *et al.*, 2017). The rapid daily modulations of LPL activity are mainly post-transcriptional which occurs by interplay of several factors and is important for partitioning of lipids between tissues in accordance with changes in the metabolic situation (Catoire *et al.*, 2014).

Studies have shown that ANGPTL4 plays a major role in the regulation of LPL activity during various physiological conditions such as exercise, fasting and cold exposure (Kroupa *et al.*, 2012; Catoire *et al.*, 2014; Dijk *et al.*, 2015). ANGPTL4 inactivates LPL by promoting the unfolding of the protein, leading to the conversion of the catalytically active LPL dimer into inactive monomers (Mysling *et al.*, 2016b). LPL activity is finely regulated by a multitude of factors at the transcriptional, translational, and posttranslational levels (Kersten 2014; Ioannou and Tempest, 2018).

2.7.5 Drugs affecting Lipoprotein Lipase Activity

Many compounds exert part of their beneficial effects on the disordered metabolism of lipoproteins through modulation of LPL activity. Fibrates increases expression of LPL, leading to a significant rise in LPL activity and a reduction in TG level in the bloodstream (Dhanalakshmi *et al.*, 2014; Okopien *et al.*, 2018). Nicotinic acid and its derivatives activate LPL, thereby lowering TG levels (Carlson, 2005). Some statins, such as pitavastatin, simvastatin, and atorvastatin, also increase the expression and the activity of LPL (Verd *et al.*, 1999; Schneider *et al.*, 2005; Lamon-Fava *et al.*, 2007).

2.8 *Mentha piperita* plant

The plant belongs to the Family Lamiaceae which is commonly known as peppermint. It is a hybrid of *M. spicata* L. (spearmint) and *M. aquatica* (water mint). The plant is mostly grown in Europe, Asia, Middle east (Iran) and is also found in Africa (Nigeria) but nowadays cultivated throughout in all regions of the world. The essential oil extracted from the aerial parts of the flowering plant, the dried leaves, the fresh flowering plant and the whole plant are of therapeutic properties (Singh *et al*., 2015).



Plate 1.1: The picture of *Mentha piperita* Leaves

2.8.1 Taxonomical Classification

Kingdom: Plantae

Division: Angiosperms

Class: Eudicots

Subclass: Asterids

Order: Lamiales

Family: Lamiaceae

Genus: Mentha

The plant is called “Ruhun na’a – na’a or minti” in Hausa, “Achibo or nchiawu” in Igbo, “Awe renren or iyere” in Yoruba languages of Nigeria (Department of Biological Sciences, Herbarium section, A.B.U Zaria)..

2.8.2 Chemical Composition

Menthol is the primary component of the essential oil of peppermint leaves. Other constituents of peppermint oil are limonene (1.0-5.0%), cineole (3.5-14.0%), menthone (14.0-32.0%), menthofuran (1.0 -9.0%), isomenthone (1.5-10.0%), menthyl acetate (2.8-10.0%), isopulegol (0.2%), menthol (55.0%), pulegone (4.0%) (Sujana *et al*., 2013; Balakrishnan, 2015). Other pharmacologically active ingredients include bitter substances, caffeic acid, flavonoids (12%), polymerized polyphenols (19%), carotenes, tocopherols, betaine, choline and tannins (Zheng and Wang, 2001). The chemistry of peppermint oil is very complex and highly variable. The relative concentrations vary depending on climate, cultivar, and geographic location (Singh *et al*, 2011).

Organic crude extracts (chlorofoam, hexane, and ethyl acetate) obtained from *M. piperita* leaves contain some biologically active compounds at very high concentrations. The major chemical compounds identified included eucalyptol (1.697%), 3-octanol (0.592%), borneol (0.894%), dihydrocarveol (3.512%), pulegone (3.562%), carvone (8.498%), caryophyllene (5.002%), β-cuvebene (4.658%), hexadecylene oxide

(3.102%), phytol (1.06%), α-linolenic acid (26.785%), vitamin E (5.577%), benzoic acid (1.896%), caffeic acid (31.963%), gluconic acid (9.527%), p-isopropenyl toluene (16.153%), palmitic acid (5.328%), and β-sitosterol (45.886%)etc (Hossain *et al.*,2014). The leaves extract also contains: romarinic, p-cumaric caffeic and ferullic acid (Kim et al., 2010; Alexa *et al.*, 2018).

2.8.3 Plant Description

Peppermint plant grows to about 2-3 feet in height. It produces very small purple or white flowers that bloom from July through August. The leaves are dark green and fragrant (Dorman *et al*, 2003). The leaf is the most important part from which oil is extracted. Upper epidermis composed of large, clear epidermal cells with sinuous, vertical walls and possessing few or no stomata. Few grandular trichomes are present; palisade parenchyma, comprising a layer of columnar cells rich in chloroplasts; spongy parenchyma, of 4-6 layers of irregular shaped chloroplastid-containing cells and intercellular air spaces (Dorman *et al*, 2003). Lower epidermis is composed of small epidermal cells with sinuous, vertical walls and numerous diacytic stomata; in the region of veins and midrib, exhibits non-glandular and glandular trichomes as outgrowths; non glandular trichomesuniseriate, papillose, 1-8-celled glandular trichomes, have 1-2 celled stalk and 1-8 celled glandular head containing the essential oil. Calcium oxalate crystals are absent. (Dorman *et al*, 2003)

2.8.4 Therapeutic/Medicinal Properties of Peppermint Plant

M. piperita is one of the herbs most widely used worldwide, with a long history of safe use in medicinal preparations. Its leaf is used as a remedy for common cold, inflammation of the mouth, pharynx, liver, as well as disorders in the gastro intestinal tract such as nausea, vomiting, diarrhea, cramps, flatulence and dyspepsia. It exhibits antioxidant, antimicrobial, antiviral, anti- inflammatory and anti-carcinogenic properties

(Valente *et al*., 2016; Alexa *et al.,* 2018). The essential oil of peppermint is widely used in pharmaceuticals, perfume production and as a spice (in form of tea)

2.8.4.1 Anti inflammatory effects

Peppermint compounds prevent inflammation and angiogenesis. The plant exhibits anti-inflammatory activity in carrageenan induced animal model (Mathur *et al*., 2011).

2.8.4.2 Spasmolytic Properties

Consumption of peppermint oil relaxes gastrointestinal smooth muscle which in turn lowers down the calcium inflow in the large intestine as well as jejunum (lies between ileum and duodenum) (DeSousa *et al.,* 2010: Sadraei *et al*., 2016) and thus provides relaxation in the gastrointestinal tract (Nissen and Lau 2016). Menthol and oil of peppermint obstructed the calcium deposition motion in rats, pig atrial and papillary muscles, and broilers retinal neurons (Harris 2016).

2.8.4.3 Antimicrobial effects

Peppermint oil and its extracts shows protection against the growth of various microbes and have different modes of action in bacteria and eukaryotic cells.They exhibit strong bactericidal properties, and in eukaryotic cells, they modify apoptosis and differentiation, interfere with the post translational modification of proteins and induce or inhibit certain liver detoxifying enzymes (Sharafi et al 2010). The plant also possesses maximum potency against infectious pathogens (Mathur *et al*., 2011; Pramila, *et al.,* 2012). Antibacterial activity of plants may be attributed to the presence of phenolic compounds that behave as prooxidants because they undergo high oxidation which leads to generation of superoxide and quinones (Mallick *et al.,* 2016).

2.8.4.4 Anti-fungal properties

Peppermint plant exhibits anti-fungal properties against Candida albicans**,** Aspergillus Albus, and dermatophytes fungi. Mentha spicata oils presented optimum anti-fungal property against Aspergillus fumigatus and A. niger (Bansod and Rai, 2008; Pramila, *et al.*, 2012).

2.8.4.5 Therapy for spastic bowel

Irritable bowel syndrome (IBS) is the dysfunction of bowel categorized by occurrence of diarrhoea, anxiety or pain, and having additional symptoms such as swelling, bowel urgency, and inadequate emptying of the bowel (Chey *et al*., 2015; Harris, 2016). Peppermint oil can be used as a non-toxic therapy for a spastic bowel disorder.

2.8.4.6 Antihypertensive Effects

Peppermint has traditionally been used as a rubefacient and has vasolidating properties on some animals (Hawthorn *et al*., 1999). It has a lowering effect on the heart rate and the systolic pressure. Relaxation of bronchial smooth muscles to increase ventilation is cardiovascular effect of the plant (Robbers and Tyler, 1999).

2.8.4.7 Antihyperlipidemc activity

Mentha piperita plant has significant beneficial effect against fructose induced hyperlipidaemia (Badal *et al*., 2011). Mentha piperita aqeous extract was administered to fructose fed rats and the level of glucose, cholesterol, triglycerides, VLDL, LDL, and atherogenic index were seen to be significantly reduced (Badal *et al*., 2011). In a study carried out to examine the effects of this plant on human biochemical /anthropometric profiles and blood pressure, the administration of peppermint juice twice daily for 30 days indicated that 41.5% of the subjects showed a reduction in glycemia, 66.9% in total cholesterol levels, 58.5% in triglycerides, 52.3% in low-density lipoproteins(LDL-

c), 70% in glutamic-oxaloacetic transaminase (GOT) levels, 74.5% in glutamic-pyruvic transaminase) GPT levels, and that 52% presented an increase in high-density lipoprotein cholesterol(HDL-c). Also, 52.5% showed a decrease in blood pressure and 48.7% in body mass index (BMI). The use of peppermint by humans can be considered beneficial in the prevention and treatment of risk factors of chronic degenerative diseases (Barbalho *et al*., 2011a).

The level of lipids like total cholesterol, triglycerides, LDL and VLDL were found to be high in streptozotocin (STZ) induced diabetic rats when compared with control rats. After treatment with *Mentha piperita* leaves, the total cholesterol, triglycerides, LDL, and VLDL levels were decreased in STZ induced diabetic rats. In the study, cholesterol and triglycerides levels were notably reduced after treatment of *Mentha piperita* leaf extract which indicates that the leaf extract has good ability to suppress the biosynthesis of cholesterol and triglycerides (Chandirasegaran *et al*., 2015). In another study, treatment of animals with aqeous extract of peppermint leaves showed reductions in plasma total cholesterol (44.32%), LDL-cholesterol (69.19%) and total triglycerides (55.77%) and it was found that taurine, betaine, alanine, glycine and L-leucine were responsible for the hypocholesterolemic effects of the extract (Johari *et al.*,2015).

In another study by Barbalho *et al.*, (2017), peppermint juice administered to rats reduced TC, LDL, body weight and atherogenic indices; and increased the levels of HDL. It has being reported that the total cholesterol in offspring of diabetic rats increased particularly in HDL-cholesterol levels which then helped to reduce the total cholesterol/HDL ratio and LDL/HDL ratio (Sharafi *et al*., 2010). Peppermint tea and juice administered to animals fed a hypercaloric diet reduced lipid profile and weight gain in the rats (Barbalho *et al*., 2009).

2.8.4.8 Antidiabetic Effect

The species of mint produce a significant reduction in glucose, cholesterol, LDL-c and triglycerides levels as well as significant increase in HDL-c levels in diabetic rats, contributing to the prevention of diabetes (Barbalho *et al*., 2011b).The saponins and alkaloids present in the *M. piperita* leaf exert anti-hyperglycemic and hypolipidemic effects through some mechanisms such as regulation of insulin secretion, inhibition of glucose absorption in the intestinal lumen and decreased accumulation of lipids in the liver (Figueroa-Perez *et al*., 2015).

2.8.4.9 Anti Cancer Activity

Menthol is one of the main components of the essential oil of *M. piperita* that produce anti-cancer activity inducing cell death, either by necrosis or apoptosis (in Caco-2 cell line). Methanol extract of peppermint has cytotoxic effect on L1210 cancer cells (Liu *et al.,* 2014).The cytotoxicity associated with essential oil has been attributed to various effects such as the production of reactive species, change in fluidity and membrane permeability, tubulin polymerization, imbalance in ion transport, and inhibition of protein function (Ferreira et al., 2014).

2.8.4.10 Antioxidant Activity

The plant is known for having several phytochemicals, including polyphenols that are highly effective antioxidants and are less toxic than the synthetic ones. This property makes it of great interest to the food industry, since the phenolic compounds retard the oxidative degradation of lipids improving the quality and nutritional value of food (Roblova, *et al*., 2016). Pharmacological studies have shown that *M. piperita* leaves and roots have antioxidant activity, reducing the effects of the free radicals that are related to diseases such as cancer and atherosclerosis (Mathur *et al*., 2011). Alcohol extract of peppermint was found efficient against radiation-induced morbidity and mortality.

These properties are due to the presence of bioactive compounds (Liu *et al.*,2014; Mallick *et al.,* 2016). The hydroalcoholic leaf extract of peppermint plant increased the activity of superoxide dismutase (15.3–63.5-fold) and glutathione peroxidase (34.4–73.6-fold) in lipopolysaccharide (LPS)-treated macrophages (Arruda *et al.,* 2017). The extract modulates macrophage-mediated inflammatory responses, by stimulating the antioxidant pathway in these cells (Liu *et al.,* 2014). These effects may be beneficial when the excessive activation of macrophages contributes to tissue damage during infectious disease (Arruda *et al.,* 2017).

From a study conducted by Salih *et al*., (2013) extracts of peppermint (methanolic and aqeous) contains flavonoids and phenols which has strong reducing power.

The plant is rich in total phenols and these phenolic compounds have been recognized as antioxidant agents which act as free radical oxidation terminators, the reducing properties are generally associated with the presence of protons. Several studies indicates that peppermint may be used as an antioxidant, leading to the possibility of developing natural antioxidant agent because of its constituent phytochemicals such as phenols, tannins, flavonoids and others (Salih *et al;* 2013; Dyab *et al*., 2015).

Salih *et al.,* (2013) reported that the antioxidant activity of *M, piperita* could be due to its constituent phytochemicals such as phenols, tannins, flavonoids and others; and these phenolic compounds have been recognized as antioxidant agents.

The free radical scavenging activity of different crude extracts of *M. piperita* was tested through DPPH method and the results showed gradual discolouration which indicates the scavenging capacities of the plant's crude extracts. The five different crude extracts extracted from *M. piperita* plant were able to decolourize DPPH radical (Hossain *et al.,* 2014).

The antioxidant and free radical scavenging properties of different extracts of *Mentha* species showed that *M. piperita* exhibits the strongest activity as a DPPH scavenger (Nickavar *et al;* 2008). In another study by Badal *et al.,* (2011), administration of aqueous extract of *M. piperita* leaves increased the activity of SOD and CAT in fructose-fed rats. The methanolic extract was found to contain tannins and flavanoids, with considerable free radical scavenging activity and antioxidant properties (Mairapetyan *et al.,* 2013; Daryadar, 2014). Peppermint grown in water stream hydroponics has high antioxidant property (Mairapetyan *et al.*, 2016)

2.8.4.11 Anti allergic Effects

Menthol has anti-inflammatory effects when applied topically. It could suppress antigen induced allergies and inhibits cutaneous anaphylaxis that is mediated by IgE antibody. Peppermint is a central nervous system stimulant and effective on cognitive performance (Balakrishnan, 2015). The presence of flavonoids such as eriocitrin, narirutin, hesperidin, luteolin-7-O-rutinoside, isorhoifolin, diosmin and rosmarinic acid, and 5, 7-dihydroxycromone-7- O-rutinoside exerts anti-allergic effects also (De Sousa *et al.,* 2016; Wozniak and Walasek, 2014).

2.8.4.12 Antiviral properties of peppermint

Infectious viral diseases remain an important worldwide problem, since many viruses have resisted prophylaxis or therapy longer than other microorganisms (Shalaye *et al*., 2016; Moradi *et al.,* 2016). At the moment, only few effective antiviral drugs are available for the treatment of viral diseases Peppermint extracts has significant antiviral activities, helps to improve the immune system and protect the body from viruses (Shaikh *et al*., 2014).

2.8.4.13 Effect on Hepatic Enzymes

Aqueous extract of peppermint modulates phase I and phase II drug metabolizing enzymes. The alcoholic extract of the plant ameliorated the adverse effects of CCl_4 on growth performance and liver function (Khodadust and Asadi, 2015). Treatment of mice with peppermint oil after immobility stress leads to significant decrease in alanine and aspartate transaminases, alkaline phosphatase and gamma glutamyl transferase (Marjani, *et al*., 2012).

2.8.5 Safety /Toxicity of the Plant

The aqueous extract of peppermint leaf was relatively safe at 5000mg/kg body weight (Johari *et al*., 2015). The results for safety and toxicological evaluation of the peppermint showed that there was a slight toxicity on kidney tissue of studied Wistar albino rats when peppermint tea (20g/L) was given to the rats for 30 consecutive days as drinking water. The possible toxic effect of peppermint oil was attributable to the pulegone amount in the oil, thus the toxicity could be controlled by adjusting the amount of this constituent in the oil preparations (Samojlik *et al*., 2011). Allergic reactions to peppermint have been reported. Menthol causes hepatocellular changes in rats. Chronic exposure to high concentrations of menthol vapour has shown no gross toxic effects (Robbers and Tyler, 1999). With the limitation that the concentration of pulegone should not exceed 1%, it has been concluded that the oil, extract, and water from *Mentha Piperita leaves* are safe and used in cosmetic formulations (Nair, 2001; Nickavar, 2008).

Due to the menthol constituent, topical use of peppermint oil around the facial or chest areas of infants and young children, especially around the nose, can induce apnea,

laryngeal and bronchial spasm, acute respiratory distress with cyanosis, or respiratory arrest (Nickavar, 2008).

2.9 Antihyperlipidaemic Agents of Plant Origin

Preclinical studies have demonstrated that classical antioxidant vitamins including beta carotene (Vit A precursor), folic acid (VitB9) vitamin C, and vitamin E administered alone or in combination with other drugs or in multivitamin preparations could improve serum lipid profile and myocardial oxidative/nitrative stress in hypercholesterolemia (Abbas and Sakr, 2013). In addition, multivitamin preparation supplemented with phytosterols decreased serum cholesterol level in hypercholesterolemic rats (Abbas and Sakr, 2013; Silva *et al*., 2013).

Flavonoids are a large family of natural polyphenolic compounds in the human diet and these compounds favorably affect a wide range of biological processes. Beside their antioxidant capacity, flavonoids improve lipid profile; have anti-inflammatory, antiplatelet, andAntithrombotic effects as well. Catechins are abundant polyphenols in green tea and it reduces blood cholesterol level in both animals and humans (Yousaf *et al*., 2014; Ahmad *et al*., 2015). Quercetin has been shown to reduce serum LDL and increase HDL cholesterol level in hypercholesterolemic rats and lower total cholesterol in rabbits fed a high-cholesterol and high-fat diet (Mariee *et al*., 2012; Csonka *et al*., 2016). Rutin (the glycoside between the quercetin and the disaccharide rutinose) improves cholesterol level in different hypercholesterolemic and dyslipidemic rodent models (Umarani *et al*., 2015). Naringin and hesperidin are natural flavon glycosides in citrus fruits which exhibits hypocholesterolemic effect in animal models (Csonka *et al*., 2016). Polyphenol resveratrol has beneficial effects and its sources include the skin of grapes, blueberries, raspberries, and mulberries. Grape seed and skin are rich in natural

antioxidants, so these are possible supplements to alleviate hypercholesterolemia-induced oxidative stress. In a high-fat-induced obesity model, grape seed extract and its skin was shown to improve lipid profile and cardiac lipid accumulation along with attenuated oxidative stress (Charradi *et al*., 2013; Csonka *et al*., 2016).

Plant stanols and sterols are natural compounds which are present in our daily diet. Their main dietary sources are vegetables, fruit, vegetable oils, cereals, and nuts. They are added to foods and food supplements because the intake from naturally occurring sources is too low to bring about a significant cholesterol-lowering effect. Plant stanols are added to commercially available cholesterol lowering products as plant stanol ester. This fat soluble compound is formed of plant stanols and vegetable oil based fatty acids through esterification. Adding plant stanols as plant stanol ester ensures the cholesterol-lowering efficacy of the ingredient without altering the taste or the texture of the food product (Gylling *et al*., 2014; Rosin *et al*., 2015).

Plant stanol ester works by partly inhibiting the absorption of cholesterol in the small intestine (Gylling *et al*., 2014). Plant stanol ester is first hydrolysed to plant stanols and fatty acids after which it interferes with the solubilisation of cholesterol, that is, the incorporation of cholesterol into mixed micelles which is possible because of the slight structural dissimilarities of plant stanols and cholesterol. Other mechanisms, such as activation of certain transport proteins in the enterocytes, may also be involved in the process (Rosin *et al*., 2015). Plant stanols themselves are virtually not absorbed but are rapidly excreted from the body in feces (Rosin *et al*., 2015). Consumption of plant stanol ester inhibits the absorption of both dietary cholesterol, and biliary cholesterol. The reduced cholesterol absorption achieved with plant stanol ester leads to

significantly reduced levels of serum total and LDL-cholesterol (Plat and Mensink, 2005).

Plant stanol ester is one of the most established functional food ingredients in the world. Research evidence consistently shows that a daily intake of 2 g of plant stanols (as plant stanol ester) reduces total and LDL-cholesterol concentration by 10%, with no effect on HDL-cholesterol concentration. The average reduction in LDL-cholesterol concentration can be detected only after 1 to 2 weeks daily consumption of plant stanol ester (2 g/day of plant stanols) (Gylling *et al*., 2014; Rosin *et al*., 2015).

Studies on plant stanol ester have been conducted in several population groups, including normocholesterolemic, mildly and moderately hypercholesterolemic adults and children, individuals with familial hypercholesterolemia, individuals with type 1 or type 2 diabetes, and patients with coronary heart disease (Plat *et al*., 2012). No adverse effects have been reported in connection with the use of plant stanol ester in the above-mentioned clinical studies or in regular consumption of plant stanol ester containing products (Plat *et al*., 2012; Rosin *et al*., 2015). Plant stanols are stable molecules that are not altered in food manufacturing or preparation processes. They are microbiologically inert as they are not affected by fermentation, not oxidized when heated and are not metabolized in the body but excreted (Rosin *et al*., 2015).

2.10 Bioactive Components from Plant sources

The extracts or bioactive components of many medicinal plants have been extensively explored for antioxidant activities in several cellular systems (Satish and Dilipkumar, 2015). Antioxidants are essential for maintaining optimal cellular activity, systemic health and well-being (Madhavan *et al*., 2010). The properties are attributed to a variety of constituents, including vitamins, minerals, fibers and numerous phytochemicals,

including flavonoids. Probably the most important natural phenolic compounds are flavonoids because of their broad spectrum of chemical and biological activities (Salih *et al;* 2013).

2.11 Antioxidants

Antioxidants are substances that inhibit or neutralize free radicals (Satish and Dilipkumar, 2015). An extensive range of antioxidant defenses, both the endogenous and exogenous are present to protect cellular components free radical induced-damage. Antioxidants exists both in enzymatic and non-enzymatic forms in the intracellular and extracellular environment (Satish and Dilipkumar, 2015). Enzymatic antioxidants work by breaking down and removing free radicals. The antioxidant enzymes which are Superoxide dismutase (SOD), Catalase, glutathione reductase, glutathione peroxidise convert dangerous oxidative products to hydrogen peroxide (H_2O_2) and then to water, in a multi-step process in the presence of cofactors such as copper, zinc, manganese, and iron (Madhavan *et al*., 2010). Non-enzymatic antioxidants work by interrupting free radical chain reactions. Few examples of non-enzymatic antioxidants are vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione (Satish and Dilipkumar, 2015).

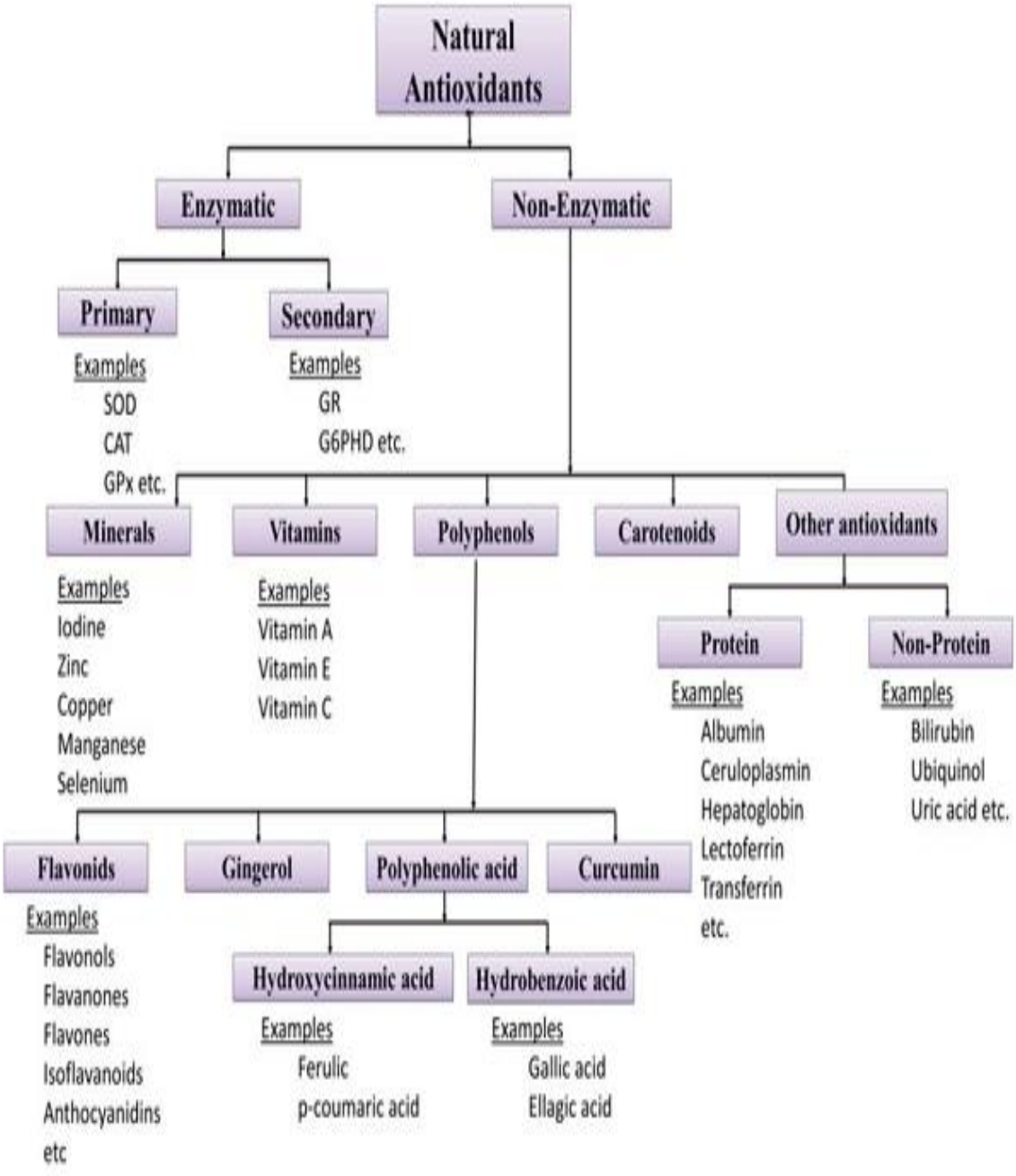


Figure 2.2: Classification and subclassification of antioxidants found in natural sources adapted from Carocho and Ferreira, (2013) and Anwar et al., (2018)

Defence mechanisms against free radical-induced oxidative stress involves: preventative mechanisms, repair mechanisms, physical defenses, and antioxidant defense (Devasagayam *et al*., 2004; Valko *et al*., 2007). Nature has endowed each cell with adequate protective mechanisms against any harmful effects of free radicals. Superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, thioredoxin, thiols and disulfide bonding are buffering systems in every cell. Different types of secondary metabolites have been identified in plants which are either nitrogen containing (alkaloids) or nitrogen deficient (terpenoids and phenolics). Nearly 20% of plant species accumulate alkaloids, which mainly include ter-penoid indole alkaloids, tropane alkaloids, and purine alkaloids.

Tetraterpenes and carotenoids have been shown to possess potent antioxidant activity within both in *vivo* and in *vitro* studies; however, some valuable carotenoids such as beta-carotene showed prooxidant effects at high concentration and oxygen pressure (Patra *et al*., 2013). Carotenoids, which are abundant in fruits and vegetables, scavenge peroxyl radicals and act pre-dominantly as antioxidants (Jomova and Valko, 2013). The antioxidant po-tential of dietary carotenoids such as beta-carotene and lycopene in biological systems is dependent on a number of factors, including the presence of other co antioxidants. Phenolic antioxidants appear to be the most important since they have shown promising antioxi-dant activity in both *in vivo* and *in vitro* investigations. Plant phenolics are mainly classified into five major groups, phenolic acids, flavonoids, lignans, stilbenes and tannins.

Ascorbic acid (vitamin C) is regarded as one of the most important dietary antioxidants that help to decrease free-radical-mediated damage (Putchala *et al*., 2013; Heo *et al*., 2013). It is also part of the normal protecting mechanism and is readily absorbed by active transport in the intestine, functions as an antioxidant by donating two of its

electrons, which prevents other compounds from being oxidized. Vitamin C has been shown to be an excellent antioxidant under both *in vitro and in vivo* study conditions. Specifically, vitamin C has been found to be capable of converting Fe^{3+}into Fe^{2+}, which subsequently reacts with oxygen or hydrogen peroxide to form superoxide and hydroxyl radicals that can subsequently damage biomolecules (Putchala *et al*., 2013; Heo *et al*., 2013).

Vitamin E is an essential nutrient which functions as a chain-breaking antioxidant which prevents the propagation of free radical reactions in all cell membranes in the human body (Sunil, 2014). Vitamin E is another useful and powerful plant antioxidant that is generally present in lipid structures of cells. This compound reacts with peroxyl radicals to inhibit the propagation of lipid peroxidation. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health (Valko *et al*., 2007).

Flavonoids are the most abundant dietary polyphenols. Flavonoids are classified into six major subclasses; flavones, flavonols, flavanones, catechins or flavanols, anthocyanidins and isoflavones. In plants, most flavonoids are attached to sugars (glycosides), although they are occasionally found as aglycones (Bunaciu et al., 2016). Occasionally, major fractions of absorbed flavonoids are metabolized to conjugates or further metabolized in the colon, where they produce a wide array of low molecular weight aromatic acids such as phenylvaleric, phenylpropionic, phenylacetic and benzoic acids as a result of their antioxidant potency being reduced.

Moreover, flavonoids and their *in vivo* metabolites have been reported to exert other potential biological activities than conventional hydrogen-donating antioxidants, such as the ability to act as signaling molecules in cells (Bunaciu *et al.,* 2016).

Most of the natural antioxidants convert lipid radicals into more stable products by breaking the chain. Antioxidants obtained from vegetables and fruits are mostly of phenolic structure, which may include vitamins, minerals, and polyphenols (Bunaciu *et al.,* 2016; Sonia *et al*., 2016)). Antioxidant minerals, such as iron, zinc, selenium, copper, and manganese, act as cofactor of many antioxidant enzymes, absence of which may certainly disturb the activity of their enzymatic scavenging activity (Sonia *et al*., 2016).

2.12 Types and Functions of Endogenous antioxidants

2.12.1 Catalase

Catalase (EC 1.11.1.6) is a tetrameric heme-containing enzyme that plays an important role in antioxidant defense (Khan *et al*., 2015). Catalase catalyzes the reduction of hydroperoxides, thereby protecting mammalian cells against oxidative damage (Durdi and Timur, 2007). In addition, catalase is active in neutralizing reactive oxygen species and so removes cellular superoxide and peroxides before they react with metal catalysts to form more reactive species (Khan *et al*., 2015). Catalase catalyses the decomposition of hydrogen peroxides to give water and molecular oxygen. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of catalase activity (Durdi and Timur, 2007). Catalyses use either an iron or manganese as cofactor. Catalyses are found in peroxisomes, in most eukaryotic cells and have hydrogen peroxide as substrate (Khan *et al*., 2015).

2.12.2 Superoxide Dismutase

Superoxide dismutases (SODs) (EC 1.15.1.1) are a class of enzymes that catalyse the breakdown of the superoxide anion into oxygen and hydrogen peroxide and belongs to a group of metalloenzymes that catalyze the dismutation of O_2•− to O_2 and H_2O. It resides in almost every subcellular compartment that generates activated oxygen (Khan *et al*., 2015). These enzymes are present in almost all aerobic cells and in extracellular fluids (Khan *et al*., 2015). SODs contain metal ion cofactors that, depending on the isozyme, can be copper, zinc, manganese or iron. For example, copper/zinc SOD is present in the cytosol of humans, while manganese SOD is present in the mitochondrion. In plants, SOD isozymes are present in the cytosol and mitochondria. There is also an iron SOD found in chloroplasts (Khan *et al*., 2015).

2.12.3 Lipid Peroxidation

Lipid Peroxidation (LP) can be described generally as a process under which oxidants such as free radicals or non radical species attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides (Ayala *et al*., 2014). Lipid peroxidation is a free radical mediated process. Initiation of a peroxidative sequence is due to the attack by any species, which can abstract a hydrogen atom from a methylene group (CH_2), leaving behind an unpaired electron on the carbon atom (•CH). The resultant carbon radical is stabilized by molecular rearrangement to produce a conjugated diene, which then can react with an oxygen molecule to give a lipid peroxyl radical (LOO•). These radicals can further abstract hydrogen atoms from other lipid molecules to form lipid hydroperoxides (LOOH) and at the same time propagate lipid peroxidation further. The peroxidation reaction can be terminated by a number of reactions. Malondialdehyde (MDA) is the

end product of lipid peroxidation. Therefore, measurement of MDA is an indirect method for accessing the extent of lipid peroxidation (Rathod *et al*., 2011).

The increased level of LP has been widely used as a marker of oxidative stress. Damage to cell membranes by malondialdehyde (MDA), one of the final products of unsaturated fatty acids and phospholipids peroxidation, is another sign of oxidative stress (Khan *et al*., 2015).

2.12.4 Reduced Glutathione

Reduced glutathione (GSH) is the most abundant thiol containing protein in mammalian cells and organs (Rathod *et al*., 2011). It is a three peptide containing glutamic acid, cystein and glycine and is found in reduced and oxidized forms in cells. High concentration of glutathione and its high reduced/oxidized potential makes GSH a powerful antioxidant and defensive against free radicals. However, glutathione is the most efficient tool for detoxification of xenobiotics. Reduced glutathione (GSH) is a major source of reducing equivalents in mammalian cells ((Rathod *et al*., 2011; Shi *et al*., 2013).

2.13 Hyperlipidaemia and Oxidative Stress

Free radicals may be either Oxygen derived (ROS) or Nitrogen derived (RNS). The most common reactive oxygen species include superoxide anion (O_2), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO) and reactive hydroxyl radicals (OH). The nitrogen derived free radicals are nitric oxide (NO), peroxy nitrite anion (ONOO), Nitrogen dioxide (NO_2) and Dinitrogen trioxide (N_2O_3). The exogenous sources of ROS include electromagnetic radiation, UV-light, cigarette smoke and low wave length electromagnetic radiations while endogenous sources are mitochondrial electron transport chain, β-oxidation of fat (Patel *et al*., 2013).

During electron transport, approximately 1–2% of the electrons slip from complex I and III of the electron transport chain (ETC), after which they react with molecular oxygen to form free radicals such as superoxide anion (O_2-●), hydroperoxyl radical (HO_2●), hydroxyl radical, hydrogen peroxide (H_2O_2), hydroperoxyl radical(●OH), peroxynitrite ($ONOO^-$) and lipid peroxyl radical ($LOO^•$). These free radicals target and damage biomolecules such as DNA, protein and lipids (Lü *et al*., 2010; Carocho and Ferreira, 2013).

Enzymatic sources for ROS formation include the mitochondrial respiratory chain, Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidases, xanthine oxidase, cyclooxygenases, uncoupled nitric oxide synthase (NOS), and peroxidases. Although there is a consensus in the literature that hypercholesterolemia is associated with increased cardiac oxidative stress but the molecular mechanism by which hypercholesterolemia induces oxidative stress in the heart are not clear (Figure 1), Evidence shows increased superoxide production in the hearts of hypercholesterolemic animals and activation of NADPH oxidase are the major sources of superoxide radicals in hyperlipidaemic rats (Varga et al., 2013; Csonka et al., 2016).

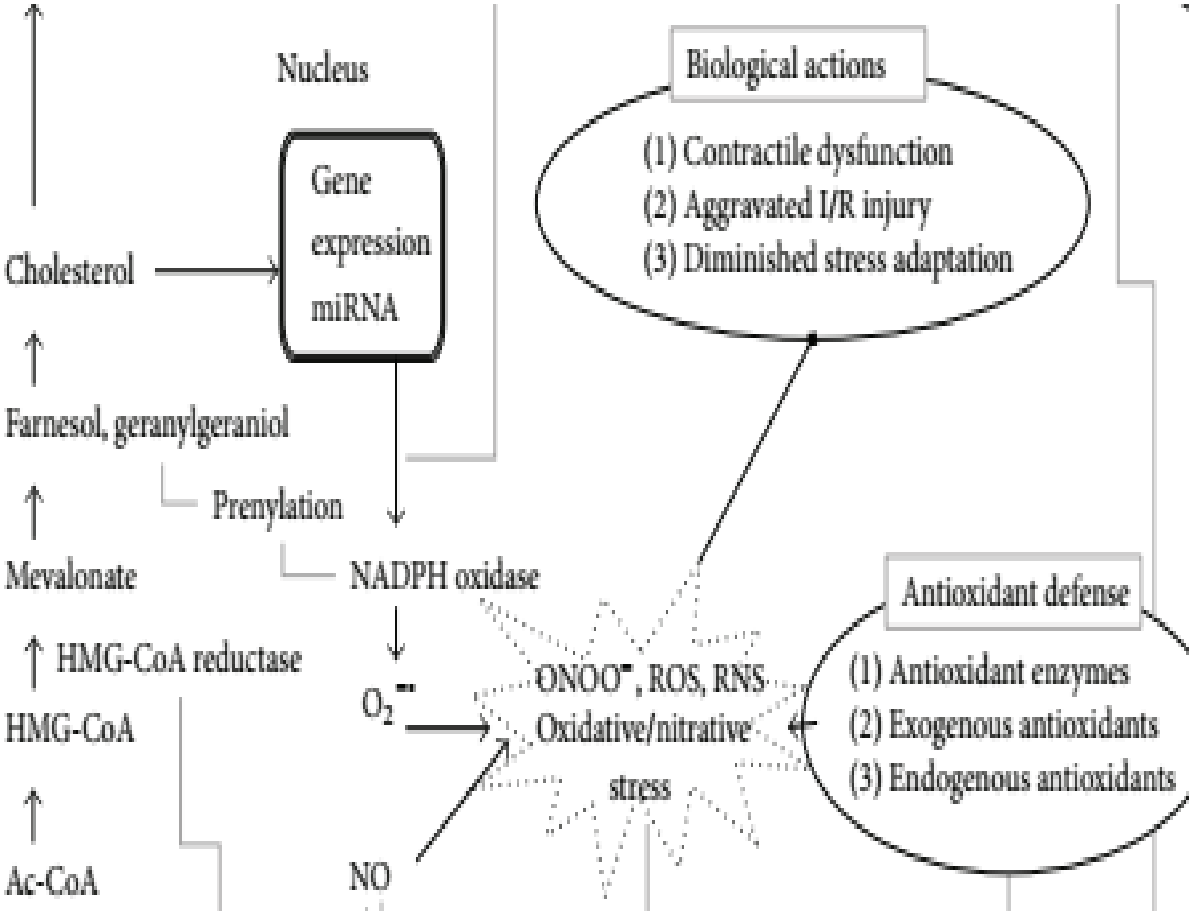


Figure 2.3: Hypercholesterolemia-induced myocardial oxidative/nitrative stress

Ac-CoA: acetyl-coenzyme A; HMG-CoA: 3-hydroxy-3-methyl glutaryl coenzyme A; eNOS: endothelial nitric oxide synthase; ONOO−: peroxynitrite; ROS: reactive oxygen species; RNS: reactive nitrogen species; miRNA: microRNA (Csonka et al., 2016).

2.14 Poloxamer 407

Poloxamer 407 (P407) is a non-ionic detergent that is used widely in pharmaceutical formulations. Structurally, P407 is a copolymer of ethylene oxide and propylene oxide with a molecular weight of 12,600 (Alessandra *et al*., 2011). As a non-ionic synthetic copolymer surfactant, it provides an attractive means of inducing hyperlipidaemia because of its rapid onset and seeming lack of toxicity; within 24 hours of its intraperitoneal (i.p) injection, a profound hyperlipidaemic state is achieved (Chaundhary and Brocks, 2013). P407 has been used to induce hyperlipidaemia and hypercholesterolemia in rats, mice and rabbits (Wout *et al*., 1992; Chaundhary and Brocks, 2013). P407 is a biocompatible, non-ionic surfactant considered safe during chronic administration for long term studies (Chaundhary and Brocks, 2013). In animals, P407 has a marked effect on the ultrastructure of liver sinusoidal endothelial cells (LSEC), which contribute to the pathogenesis of hyperlipidaemia (Megalli *et al*., 2005). Kupffer cells became massively engorged with vacuoles and took on marked honeycomb morphology (Megalli *et al*., 2005).

2.14.1 Mechanism of Action

P-407 increases serum lipoproteins via its actions at various levels in lipid metabolism, largely by inhibiting lipoprotein lipase, which facilitates the hydrolysis of triglycerides. The increase in triglycerides seen following P407 injection is mainly due to the inhibition of TG degradation, due to direct inhibitory effect of lipoprotein lipase (LPL), the enzyme responsible for TG degradation (Megalli, *et al.*,2005). P-407 also causes indirect stimulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase which is involved in cholesterol biosynthesis (Chaundry and Brocks, 2013). Another study indicated that the increase in plasma cholesterol observed after P-407

administration to rats may be due to inhibition of cholesterol 7*a* -hydroxylase, but not stimulation of (HMG CoA) reductase (Johnston *et al*., 2000; Johnston *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals/Reagents

Assay kits for lipid profile (Randox Laboratories, UK), Poloxamer 407(P407) (Lutrol F127; BASF, Ludwigshafen, Germany), Atorvastatin (Pfizer Ireland pharmaceuticals, Ireland). Accutrend GCT Meter with Cobas®test strips (Roche, Germany). Silica gel (G60-200 mesh size, China), *Diphenyl picrylhydrazine (DPPH).* Fenton's reagent (10% H_2O_2 and 1mM $FeSO_4$), Phosphate buffer (0.05M,0.1M,0.2M), gel loading dye, agarose gel (1%), biuret reagent, Bovine serum albumin (BSA), Trichloroacetic acid (TCA), Ellman's reagent, Adrenaline, hematoxylin and eosin (H& E), Rat Lipoprotein Lipase Elisa Kit (Wuhan Fine Biotech Co., Ltd. China). RNA later solution, Exiprep^{TM} Dx DNA/RNA extraction kit, cDNA synthesis kit (Bioneer, Accuprep, USA). AccuPower® 2X GreenStar qPCR Master mix, SYBR Green 2X master mix. All other reagents and chemicals were of molecular and analytical grade.

3.1.2 Equipment

Spectrophotometer (Shimadzu UV-2600 Spectrophotometer, Japan), centrifuge (Beckman, USA), vortex machine (XH-C), Shimadzu LCMS-2020 Single Quadrupole Liquid Chromatograph Mass Spectrometer (LC-MS). GCMS Model: GC– 7890B; MSD – 5977A: Agilent gas chromatograph fitted with HP-5m ultra inert 30 m × 250 mm ID × 0.25µm film thickness or equivalent column. Rotary evaporator (R-400, Gallenkamp rotary evaporator. Germany). Real-Time PCR machine (RG-3000, Version 3.5, Roche, Basel, Switzerland). Programmable thermal cycler (BIORAD PTC-100) machine. Exiprep^{TM} 16Dx machine (A-5050, Bioneer Corp, Republic of Korea), Microplate

Reader (RT-2100C, Nanodrop (2000c spectrophotometer, U.S.A), AccuPower RT PreMix tube.

3.1.3 Collection of Plant Material and Identification

The leaves of *M. piperita* were obtained from Sharada garden in Kano state, Northwest Nigeria (Latitude: 12°3'N and Longitude: 8°32'E). The plant was identified and authenticated in the Herbarium Section of the Department of Biological Sciences, Faculty of Life Sciences, Ahmadu Bello University, Zaria. The voucher specimen number 1306 was assigned and deposited for future reference.

3.1.4 Experimental Animals

One hundred and fifty (145), male healthy wistar strain albino rats (120-200g) were purchased from the Faculty of Veterinary Medicine and Department of Pharmacology, Ahmadu Bello University, Zaria.The rats were fed with water *ad libitum* throughout the experiment. Ethical clearance for the use of animals was obtained from Ethical Clearance committee, Ahmadu Bello University, Zaria (ABUCAUC/2020/39).

3.2 METHODS

3.2.1 Plant Sample Preparation and Extraction

The leaves of *M. piperita* were cleaned; air dried and pulverized using mortar and pestle. The pulverized leaves were then extracted with n-hexane. The residue was then extracted with ethyl acetate followed by methanol and filtered, and the filtrate concentrated using rotary evaporator.

3.2.2 Acute Toxicity (LD_{50}) Determination

This was carried out in two stages using oral route of administration. In stage 1, three different groups of three rats each were administered with different doses of hexane, ethylacetate and methanolic crude extracts (10, 100 and 1000 mg/kg) and observed for death or physical signs of toxicity such as weakness and rashes et.c. In stage 2, fresh

sets of rats were divided into three groups of three rats per group, and administered the crude extracts at doses of 1600, 2900 and 5000 mg/kg and were observed for another 24 hours for death or toxicity signs. The median lethal dose (LD_{50}) was calculated based on findings at this stage (Lorke, 1983).

Preparation of Poloxamer 407

Powdered P407 (10g) was dissolved in 20ml of distilled water. This makes a stock concentration of 500mg/ml. Dissolved P407 was refrigerated overnight to facilitate its dissolution. Needles and syringes to be used for administration were also cooled to prevent gelation within the syringe during injection.

3.2.3 Induction of Hyperlipidaemia

Poloxamer 407 (P407) was used as the inducing agent. P407 was administered intraperitoneally at a dose of 500mg/kg every 48 hours for 21 days according to Megalli *et al*., (2005). To confirm the induction of hyperlipidaemia, two rats were randomly selected from each induced group, sacrificed and assayed for; triacyglycerol, total cholesterol, low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL) which was compared with normal control. Lipid profile of the other rats was confirmed from the tail end using the accutrend metre.

3.2.4 Preparation of Standard Drug

Atorvastatin was purchased in a tablet form at strength of 20 mg. Two tablets (40mg) were dissolved in 2ml distilled water to make a stock concentration of 20mg/ml. The drug was administered orally to the rats by intubation.

3.2.5 Antihyperlipidaemic activity of Hexane, Ethyl acetate and Methanol Crude Extracts

A total of fifty- five (55) rats were divided into eleven (11) groups of five (5) animals per group.

3.2.5.1 Animal Grouping

Group I: Rats not induced with hyperlipidemia but given normal feed and distilled water *ad libitum* for 21 days (NC)

Group II: Rats induced with hyperlipidaemia at 500mg/kg P407 without treatment (HC).

Group III: Rats induced with hyperlipidaemia with 500mg/kg P407 and treated with Atorvastatin (ATV) at 20 mg/kg body weight/day.

Group IIIa – **Vc** : Rats induced with hyperlipidaemia at 500mg/kg P407 and orally administered crude ethyl acetate methanolic and hexane extracts respectively at different concentrations (100, 200 and 300 mg/kg).

Group VIa – VIIIc: Rats not induced with hyperlipidaemia but orally administered crude ethyl acetate, methanolic and hexane extracts at different concentrations (100, 200 and 300mg/kg).

3.2.5.2 Administration of n-hexane, ethyl acetate and methanol extracts of Mentha piperita leaves

The plant extracts were administered to the rats orally by intubation for 21 days which commenced 2hrs after induction of hyperlipidaemia.

3.2.5.3 Collection of Blood Samples for Lipid Profile Analysis

Twenty four (24) hrs after administration of the last dose and overnight fast, the rats were decapitated and blood collected for analysis, after which the blood was centrifuged at a speed of 3000 ×g for 15 minutes to obtain the serum used for lipid profile analysis.

3.2.6 Determination of Serum Lipid Profile Parameters

The serum lipid profile parameters (Total cholesterol, HDL cholesterol and Triacylglycerol) were determined by spectrophotometric methods as described in the assay kits.

3.2.6.1 Serum Total Cholesterol Assay

Principle

Cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. The intensity of the quinoneimine formed is directly proportional to the level of cholesterol present in the sample.

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

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

$$2H_2O_2 + \text{Phenol} + \text{4- Aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4H_2O$$

Procedure

Three (3) test tubes were set up and labelled as blank, standard and sample. Distilled water (10µl), cholesterol standard (10µl), and serum (10µl) were added to the blank, standard and sample tubes respectively. Cholesterol reagent (1000µl) was then added to each of the tubes and were mixed and incubated for 5 minutes at 37^0C. The absorbance was read against the reagent blank at 500 nm. The concentration of cholesterol was obtained from the relation:

$$\text{Concentration of cholesterol (mmol/l)} = \left(\frac{Absorbance\ of\ sample}{\text{Absorbance of standard}}\right) \times \text{concentration of standard}$$

Concentration of standard = 5.33mmol/l

3. 2. 6. 2 Serum High-Density Lipoproteins Assay

Principle

LDL, VLDL and chylomicrons fraction(s) are precipitated quantitatively by addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the

cholesterol concentration in the high density lipoprotein fraction(s) (HDL) which remains in the supernatant is determined.

Separation of HDL Supernatant

Sample (200µl) and HDL reagent (500µl) were mixed well, allowed to stand for 10 minutes at room temperature and was centrifuged for 10 minutes at 4000rpm.The clear supernatant was separated within two hours and HDL cholesterol concentration was determined using the cholesterol reagent.

Procedure

Three (3) test tubes were set up and labelled as blank, standard and sample. Distilled water (50µl), standard HDL (50µl), HDL supernatant (50µl), were added to the blank, standard and sample tubes respectively. Cholesterol reagent (500µl) was then added to the blank, standard and sample tubes. The reagents were mixed and incubated for 5mins at 37^0C. The absorbance of the sample and standard were measured against reagent blank at 500nm.The concentration of HDL cholesterol was obtained using the relation:

$$\text{Concentration of HDL cholesterol in supernatant (mmol/l)} = \left(\frac{Absorbance\ of\ sample}{\text{Absorbance of standard}}\right) \times \text{concentration of standard}$$

Concentration of standard = 5.33mmol/l

3. 2. 6. 3 Serum Triglycerides Assay

Principle

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. The equations are:

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LPL}} \text{glycerol} + \text{fatty acids}$$

Glycerol + ATP $\xrightarrow{GK}$ glycerol-3-phosphate + ADP

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

$2H_2O_2$ + 4-aminophenazone + 4 chlorophenol $\xrightarrow{POD}$ quinoneimine + HCl + $4H_2O$.

Where :

LPL = Lipoprotein Lipase

GK = Glycerol kinase

GPO = Glycerol -3-phosphate oxidase

POD = Peroxidase

Procedure

Distilled water, triglyceride standard and serum (10µl each) were pipetted into tubes labelled blank, standard and sample respectively. Exactly 1000µl of triglyceride enzyme reagent (containing a mixture of lipases, glycerol -kinase, ATP, 4-aminophenazone, 4-chlorophenol, peroxidise, glycerol-3-phosphate oxidase and magnesium ions) were added into each of the tubes, then mixed and incubated for 5 minutes at 37^0C. The absorbances of the sample and standard were taken against reagent blank at 500nm using a spectrophotometer.

$$\text{Triglycerides concentration (mmol/l)} = \left(\frac{Absorbance\ of\ sample}{\text{Absorbance of standard}}\right) \times \text{concentration of standard}$$

Concentration of standard = 2.20 mmol/l

3. 2. 6. 4 Serum Low Density Lipoproteins Assay

The serum level of low density lipoproteins (LDL) was measured according to the protocol of Friedewald (1972) using the equation:

$$\text{LDL Cholesterol (mmol/l)} = \left(\frac{\text{Total cholesterol} - \text{triglycerides}}{2.2}\right) - \text{HDL cholesterol}$$

3. 2. 7 Fractionation of Ethyl acetate Extract from *M. piperita* Leaves

Thin Layer Chromatography was carried out to develop a suitable solvent system. Based on the TLC profiling, combinations of n-hexane and ethyl acetate gave better separations, hence were chosen as eluting solvent systems for column chromatography.

The column (3.5cm diameter by 100cm height) was packed using the wet slurry method in which 150g silica gel (G60-200 mesh size) was mixed in 200ml of absolute hexane to make the slurry. The chromatographic column was packed with the slurry and was allowed free flow of solvent into a conical flask. The tap was locked and the column was allowed to stabilize for 24 h after which the clear solvent at the top of the silica gel was allowed to drain freely. Ethyl acetate extract was adsorbed by adding some silica gel into the extract previously dissolved in ethylacetate. The extract was allowed to dry before it was loaded into the column. A disc of cotton wool was used to make a clear distinction between the stationary phase and the mobile phase to prevent extract from mixing with the silica gel. Exactly 30g of dried adsorbed ethyl acetate extract was loaded onto the previously packed silica gel column. The fractions were eluted by gradient elution in which the chemical compounds present in the extract are separated based on the polarity of the eluting solvent System (mobile phase).

Procedure

Gradient elution with 100% hexane, followed by different combinations of hexane: ethylacetate (90:10, 80:20, 70:30, 60:40 ,50:50, 40:60, 30:70, 20:80, 10:90 and 100% ethyl acetate). The column was finally washed with 100 % methanol with a total of three hundred (300) fractions, (100 ml each) collected. The collections were pooled together based on relative Retention Factor (RF) values to a final total of 12 fractions coded F1 - F12. The percentage yield of the fractions was calculated.

3. 2. 8 Antihyperlipidaemic Activity of the Fractions

Antihyperlipidaemic activity of the fractions was determined through four (4) independent experiments.

3. 2. 8. 1 Animal Grouping for Testing Activity of Fractions

Total of seventy five (75) apparently healthy Male rats of the Wistar strain weighing between 100 to 200g were randomly divided into fifteen (15) groups comprising five (5) rats each. The rats were fed and given water *ad libitum* throughout the experiment.

The groupings are stated below:

Group I: Rats induced for hyperlipidaemia with P407 without treatment (Hyperlipidaemic Control).

Group II: Rats were induced for hyperlipidaemia with P407 and treated with Atorvastatin (ATV) at 20 mg/kg body weight/day.

Group III- XIV: Rats were induced with hyperlipidaemia and orally administered F1-F12.

Group XV: Rats were given normal feed and distilled water only for 21 days (Normal Control).

Twenty four (24) hrs after the administration of the last dose and overnight fasting, the rats were decapitated and the blood was collected for analysis. The blood was collected and centrifuged at a speed of 3000 ×g for 15 minutes and the serum obtained for lipid profile analysis using spectrophotometric methods as described in the assay kits.

3. 3 Evaluation of *In Vitro* Antioxidant Activity of the Most Active Fraction(s) of *M. piperita* Leaves Extract

3. 3. 1 Determination of DPPH Radical Scavenging Activity (Modified method of Liyana – Pathiranan and Shahidi, 2005)

Principle

Diphenyl picrylhydrazine (DPPH) is a stable nitrogen centered free radical which produces violet colour in methanol solution. Diphenyl picrylhydrazine reacts with reducing agents during which the electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up. Antioxidants donate protons to this radical, hence absorbance decreases.

Procedure

A solution of 0.0135 µM DPPH in methanol was prepared and 1000 µL of the solution was mixed with 1000 µL of the fractions/extract. The reaction mixture was vortexed thoroughly, and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The ability of the plant fractions/extract to scavenge DPPH radical was calculated by the equation:

DPPH radical scavenging activity

$$= \left(\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{\text{Absorbance of control}}\right) \times 100$$

Where Absorbance of control is the absorbance of DPPH radical + methanol;

Absorbance of sample is the absorbance of DPPH radical + fraction or extract.

The IC_{50} values of the fractions/extract were calculated from the DPPH results and were further subjected for possible genotoxic and or genoprotective property.

3. 3. 2 Genotoxicity Studies (Modified method of Lee *et al*., (2002)

Principle

The DNA damage inhibition activity of the fractions/crude extract was determined on pVax plasmid DNA (500ng/μl) using Fenton's reagent as source of free radical. Fenton radical formation is based on reaction between hydrogen peroxide and iron (II) sulphate which produces hydroxyl radical.

Procedure

A mixture containing 2.5μl of IC_{50} values of fractions 2, 3, 4, 6, 12 and ethyl acetate extract with 0.5µl of plasmid pVax (500ng) incubated for 10min at room temperature followed by the addition of 5 µl of Fenton's reagent (10% H_2O_2 and 1mM $FeSO_4$). The final volume of the mixture was made up to 10µl with 50mM Phosphate buffer (pH 7.4) and incubated for 30min at 37°C. After incubation of these compounds with DNA, 10µL of all the samples were loaded along with gel loading dye (3µl) in agarose gel (1%) for electrophoresis. The gel was scanned using the gel documentation system.

3. 4 Determination of *in vivo* antioxidant activity

3. 4. 1 Determination of total protein by biuret method (Gornall, 1949)

Principle

Cupric ions (Cu^{2+}) in the biuret reagent complex with the groups involved in the peptide bond. In the presence of alkaline media i.e. NaOH and at least two peptide bonds a violet colored chelate is formed (Gornall *et al.*, 1949).

Protein + Alkaline $CuSO_4$ ⟶ Blue-violet Coloured Complex

Procedure:

Exactly 1 ml of serum / tissue homogenate was mixed with 4 ml biuret reagent and incubated at room temperature for 30mins. The absorbance of the solution was immediately measured at 540 nm against reagent blank. Standard curve was produced by plotting absorbance of Bovine serum albumin (BSA) against its concentration. The concentration of total protein in the serum was obtained from a standard curve.

3. 4. 2 Determination of Catalase Activity (Aebi *et al.,* 1974)

Principle

The method is based on the principle that catalase catalyzes the conversion of hydrogen peroxide to water and oxygen. The difference in absorbance per minute is a measure of catalase activity.

Procedure

Exactly 100µl of sample was added to a test tube containing 2.80ml of 50mM potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1ml of freshly prepared 30mM H_2O_2 and decomposition rate of H_2O_2 was measured at 240nm for 3 minute at 1minute interval on a spectrophotometer. A molar extinction coefficient (E) of 0.041$Mm^{-1}cm^{-1}$ was used to calculate catalase activity. One unit of enzyme activity is defined as one µmole of H_2O_2 consumed/min and calculated using the following formula:

$$\text{Catalase activity} = \left(\frac{\Delta\, Absorbance\ /min}{\text{E} \times \text{Protein concentration (mg/ml)}}\right)$$

3.4.3 Lipid peroxidation (MDA) (Ohkawa *et al.*, 1979)

Principle:

Malondialdehyde concentration was determined as an index to monitor lipid peroxidation.This was determined according to the method described by Ohkawa *et al.* (1979). Malondialdehyde (MDA) is a secondary product of lipid peroxidation which is used as an indicator of tissue damage. MDA reacts with thiobarbituric acid (TBA) and produces a pink coloured product which absorbs maximally at 535 nm.

Procedure:

Exactly 150µl of serum or tissue homogenate was added to 2ml of TBA-TCA-HCL reagent (1:1:1 ratio) and placed in a water bath at 90^{o}C for 60 minutes. The mixture was cooled and centrifuged at 3000rpm for 5 minutes and absorbance of the pink supernatant (Malondialdehyde complex) was then measured at 535nm against blank. Malondialdehyde (MDA) formed was then calculated using the molar extinction coefficient of 1.56 x 10^5 $cm^{-1}M^{-1}$ as follows:

$$\text{MDA concentration} = \left(\frac{Absorbance\ of\ sample}{1.56 \text{ x } 10^5\ cm^{-1}M^{-1}}\right)$$

3.4.4 Assay of Reduced Glutathione Concentration (Ellman 1959).

Principle

It is based on the reaction of 5, 5- dithiobis nitro benzoic acid (DNTB) and reduced Glutathione (GSH).

Procedure

To 150µl of serum or tissue homogenate (in phosphate buffer pH 7.4), 1.5ml of 10% TCA were added and centrifuged at 1500g for 5 minute. Exactly 1ml of the supernatant was treated with 0.5ml of Ellman's reagent and 3ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412 nm.Standard curve was obtained by plotting absorbance of GSH concentration against its concentration.The quantity of GSH was obtained from the standard curve.

3.4.5 Determination of Superoxide Dismutase Activity (Misra and Fridovich, 1972).

Principle

The ability of superoxide dismutase (SOD) to inhibit auto oxidation of adrenaline at pH 10.2 forms the basis of this assay.

Procedure:

Exactly 0.1ml of tissue homogenate / serum was diluted with 0.9ml of distilled water to make 1:10 dilution of the sample. An aliquot (0.2ml) of the diluted sample 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 (blank) contained 2.5ml of 0.05M carbonate buffer, 0.3ml of 0.3mM Adrenaline and 0.2ml of distilled water. Increase in absorbance was measured up to 150 seconds at 480nm.

Calculations

$$\text{increase in absorbance per minute} = \left(\frac{\text{A2} - \text{A1}}{2.5}\right)$$

Where; A_2 = initial absorbance

A_1 = final absorbance

$$\%\ \text{inhibition} = 100 - \left(\frac{increase\ in\ absorbance\ of\ sample}{increase\ in\ absorbance\ of\ blank}\right) \times 100$$

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

3.4.6 Histopathological studies

Principle

The use of haematoxylin and eosin (H& E) is a widely accepted staining technique for cell morphology analysis. Haematoxylin is a dye with a deep blue-purple color and stains nucleic acids, while eosin is pink and stains proteins nonspecifically. After the staining process, nuclei are stained blue, while the cytoplasm is characterized by varying degrees of pink staining. Well-fixed and properly stained cells could provide

information on morphological changes due to the cytotoxicity of a test compound (Fischer *et al*, 2008).

Procedure

Histological studies were carried out on the liver tissues using the method described by Kiernan (2008). A portion of the liver tissues was fixed in buffered 10% neutral formol saline and processed by passing them through ascending grades of methanol from 70 to 90 and 100% methanol for a peroid of 12 hours to properly dehydrate them after which they were cleared in xylene for 2 hours, then infiltrated and embedded in liquid paraffin wax. The tissues were then sectioned using rotary microtome at 5 micron thickness, stained with hematoxylin and eosin (H and E) on a slide. The tissues were viewed microscopically and photomicrograph taken using digital microscope at a magnification of × 250.

3. 4. 7 Lipoprotein lipase (LPL) Assay Determination *in vivo*

The level of lipoprotein lipase was determined in the serum and liver tissues of hyperlipidaemic rats using Rat Lipoprotein Lipase Elisa Kit (Wuhan Fine Biotech Co., Ltd. China).

Principle

The assay is based on sandwich enzyme-linked immunoassay. The microtiter plates (96 wells) were pre-coated with an antibody specific to lipoprotein lipase. Tetra Methyl Benzidine (TMB) substrate was catalysed by Horse radish peroxidase (HRP) to produce a blue coloured product that changed into yellow after adding acidic stop solution. The density of yellow colour is proportional to the amount of lipoprotein lipase captured in the plate.

Assay Protocol

Plates were washed two (2) times before adding standard, sample and control. Standard or sample (100µL) was added to each well and incubated for 90 minutes at 37°C, then plates were washed two (2) times. Biotin labelled antibody working solution (100µL) was added to each well and incubated for 60 minutes at 37°C, then plates were washed three (3) times. Exactly 100µL Horse radish peroxidase (HRP- Streptavidin Conjugate) working solution was added to each well and incubated for 30 minutes at 37°C, then plates washed five (5) times. Tetra Methyl Benzidine (TMB) substrate solution (90µL) was added and incubated for 30 minutes at 37°C. Stop solution (50µL) was finally added to each well and concentration was read immediately at 450nm in a microplate reader.

Lipoprotein lipase activity was calculated as:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{450nm}/\text{min Test} - \Delta A_{450nm}/\text{min Blank})(\text{volume of assay in mls})(df)}{(\text{Molar Extinction Coefficient})(\text{Volume of sample/enzyme})}$$

Where;

Volume of assay (reaction mixture) = 0.24mls

Molar Extinction Coefficient = 5.9×10^4 $cm^{-1}M^{-1}$

Dilution factor (DF) = 1 for serum and 10 for liver homogenate

Volume of sample/enzyme = 0.1ml.

3. 5 Expression of Superoxide Dismutase, Catalase, and Lipoprotein Lipase Genes in Liver and Blood Tissues

3. 5.1 Collection of Samples and Preparation

Liver (0.5g) and blood (0.4mls) were collected and preserved in 1mls of RNA later solution at −20°C.

3. 5.2 Isolation of Total RNA from Liver and Blood

Total RNA was isolated from the liver and blood according to manufacturer's instructions.

Principle

RNA is extracted from samples by using a lysis buffer to disrupt the structure of the protein and binding buffer dissociates water molecules from nucleic acids and silica magnetic beads. This induces negative charge to nucleic acid and positive to silica magnetic beads. As a result, exposed RNA binds to the surface of the beads. The washing buffer rinses all impurities that may exist and the elution buffer dissolves pure RNA from the beads.

Procedure

Exactly 200 mg of liver tissues chopped using disposable pestle, and transferred to a tube containing 400µl lysis buffer and then vortexed vigorously for 3 minutes. The mixture was incubated for 90 minutes at 60°C, and vortexed at an interval of 10 minutes. The solution was centrifuged at 6,000rpm for 5 seconds, and supernatant (200µl) transferred into sample tubes containing 200µl PBS 1x. RNA was isolated and purified using Exiprep^{TM} 16Dx machine.While for blood samples, 400µl PBS 1x was added to 200µl of blood samples, votexed and centrifuged. Supernatant (400µl) was transferred into sample tubes. RNA was isolated and purified using Exiprep^{TM} 16Dx machine.

3. 5. 3 Assessment of RNA Concentration and Purity

The isolated RNA (2µl) from liver and blood samples were quantified using nanodrop spectrophotometer where elution buffer (2µl) served as blank. Nanodrop displays

absorbance at 260, 280, and A260/A280 nm with also concentration of RNA in ng/µl. RNA purity was assessed using A260/A280.

3. 5. 4 Synthesis of Complementary DNA (cDNA)

Principle

Using the polymerase chain reaction, template RNA is transcribed into a more stable complementary DNA (cDNA) which requires the enzyme reverse transcriptase.

Procedure

The isolated RNA was reverse transcribed into complementary DNA using the cDNA synthesis kit according to the manufacturer's instructions. Reverse transcription was performed in total reaction volume of 20µl. Eluted RNA (0.5 µg) and primers (0.5µg) were mixed and incubated at 70°C for 5min and placed on ice. The incubated mixture was transferred to an AccuPower RT PreMix tube, and reaction volume was filled up with DEPC - distilled water. The blue lyophilized pellet was dissolved by vortexing, and briefly spun down and mounted on a programmable thermal cycler. The reaction condition was as follows: primer annealing at 30˚C for 5 minutes, cDNA synthesis at 42^{o}C for 60 minutes and RTase inactivation at 94^{o}C for 5 minutes.

3. 5. 5 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Principle

Real-time PCR is carried out in a thermocycler with the capacity to illuminate beam of light and detect fluorescence. This involves the use of an intercalating dye whose fluorescence increases as more copies of gene are generated through cycles of polymerase chain reaction until the fluorescence is strong enough to be detected (threshold frequency).

Procedure

Quantitative Real Time Polymerase Chain Reaction (Q-PCR) was performed using the AccuPower® 2X GreenStar qPCR Master mix. The reaction mixture consisted of 5µl cDNA, 12µl of SYBR Green 2X master mix, 1.0µl of each of the primer (forward and reverse) (Table 3.1), and final volume was made up to 20µl with DEPC-distilled water. For efficacy, q-PCR master mix containing SYBR Green 2X master mix, primer (forward and reverse), DEPC-distilled water except cDNA was prepared for simultaneous analysis of all samples (negative control). The reaction mixture was mixed properly by vigorous vortexing and loaded onto the real-time PCR machine to run the q-PCR programme. Each sample was tested in duplicate and β- actin was used as an internal control (housekeeping gene). Thermocycler conditions consisted of 1 cycle of pre-denaturation at 95°C for 15 minutes. Forty cycles of denaturation at 95°C for 20 seconds, annealing at 59.36°C for 30 seconds and extension at 72°C for 30 seconds. Melting curve was obtained for each quantitative PCR run. Fluorescence was measured once every cycle after the extension step using filters for SYBR Green.

3. 5. 6 Analysis of Relative Target Gene Expression (Livak and Schmittgen, 2001)

Generation of quantitative data by real-time PCR is based on the number of cycles required for optimal amplification generated fluorescence to reach a specific threshold of detection (the quantification cycle). The quantitative PCR data were analysed as follows:

$$\Delta C_T \text{ calibrator} = C_T \text{ calibrator} - C_T \text{ housekeeping gene}$$

$$\Delta C_T \text{ gene of interest} = C_T \text{ gene of interest} - C_T \text{ housekeeping gene}$$

$$\Delta\Delta C_T = \Delta C_T \text{ gene of interest} - \Delta C_T \text{ calibrator}$$

Fold change = $2^{-\Delta\Delta C}T$

Where;

C_T – Threshold cycle

ΔC_T – Difference between Threshold cycles

$2^{-\Delta\Delta C}T$ = Fold difference in mRNA abundance

Table 3.1: List and base composition of Primers used for qRT-PCR

Gene	Accession number	Forward Primer	Reverse Primer	Product Size (bp)
β – actin	V01217	5′-CCTGCTTGCTGATCCACA-3′	5′-CTGACCGAGCGTGGCTAC-3′	505
SOD	NM000237	5′-GCAGAAGGCAAGCGGTGAAC-3′	5′-TAGCAGGACAGCAGATGAGT-3′	387
CAT	X05634	5′-CGAATGGAGAGGCAGTGTAC-3′	5′-GAGTGACGTTGTCTTCATTAGCACTG-3′	670
LPL	AH004967	5′-ACTGGTGGGACAGGATGTGG-3′	5′-CCGTTCTGCATACTCAAAGTTAGG-3′	180

3.6 Gas Chromatography-Mass Spectrometric (GC-MS) Analysis of Fraction 2

Principle

Gas chromatography works on the principle that a mixture will separate into individual substances when heated. The heated components are carried through a column with an inert gas such as Helium, which flows into a mass spectrometer. Mass spectrometry identifies compounds by the mass to charge ratio of the analyte when compared to a library of spectra (Gohlke and McLafferty, 1993).

Procedure

One microliter (1µl) of fraction 2 (2mg) dissolved in hexane (1 ml) was injected into HP5- MS column (30 m X 250 mm ID X 0.25µm film thickness) of GC-MS. Helium was used as carrier gas at a flow rate of 2.5 ml/min. Temperature programming initially was at 50°C for 1min, temperature was maintained from 100°C to 200°C, and then increased to 300°C, at the rate of 3°C per minute for 15 min. The injector and ion source temperature was 250°C with detector temperature of 260°C. Exactly 1µL volume of fraction 2 was injected, mass spectra was taken at 70eV; solvent delay 3.0 min, program run time: 99 minutes, scan range 30-350 amu and EV voltage is 3000 volts. Fragments obtained were compared with National Institute of Science and Technology (NIST) library (2014).

3.6.1 Liquid Chromatography-Mass Spectrometric (LC-MS) Analysis

Principle:

Liquid chromatography mass spectrometry (LCMS) combines two approaches to separate and identify compounds present in a sample. High performance liquid chromatography (HPLC), which separates the components of a mixture, and mass

spectrometry (MS) which identifies them. In the first phase (HPLC), a liquid mixture is passed through a column which separates components based on hydrophobic interactions or ion exchange. Retention time, which is the time taken for a solute to pass through the column is characteristic of each component of the mixture but could be similar between different components. The separated components are converted to an ionized state. MS uses mass to charge ratio which is a more specific characterizing factor to identify components (Pitt, 2009).

Procedure

One microliter (1µl) of fraction 2 (2mg) dissolved in hexane (1 ml) was injected into via a loop into a RTX-7 column of LC-MS. The operating parameters were as follows: Pump: LC-2030 Pump, Flow: 0.2000 mL/min, A: 5%,B Conc.: 95.0%; C Conc.: 0.0%; D Conc.: 0.0%; Mobile Phase A: Water; Mobile Phase B: Methanol; Start Wavelength: 190 nm; End Wavelength: 800 nm; Cell Temp.: 40^{o}C; Start Time: 0.00 min; End Time: 4.00 min; Acquisition Mode: Scan Polarity: Positive; Event Time: 1.00 sec; Detector Voltage: +1.00 kV; Threshold: 0; Start m/z: 50.00; End m/z: 1700.00; Scan Speed: 1667 u/sec. Compounds were identified by direct comparison of mass spectral data with those in the NIST library (2014).

3.7 Statistical analysis

To address the biological variability and stability of the samples, each and every experiment was repeated at least three times and results expressed as Mean ± Standard deviation where appropriate. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by post hoc test of Turkey-Krammer's multiple and Dunnett's comparison test using Graphpad instat software version 3.05. P-values < 0.05 were considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Acute Toxicity (LD_{50}) Studies of *M. piperita* Leaves Extracts

Result of acute oral toxicity study of extracts in the experimental rats (Table 4.1) shows that the three extracts (hexane, ethylacetate and methanolic) were relatively safe for consumption since LD_{50} of extracts of *M. piperita* leaves was above 5000 mg/kg. The experimental rats tolerated the extracts with no symptoms of acute toxicity even at higher doses.

Table 4.1: Acute Oral Toxicity Studies of Hexane, Ethyl acetate and Methanol Extracts of *M. Piperita* Leaves

EXTRACTS	LD_{50} (mg/kg)
Hexane	> 5000
Ethyl acetate	> 5000
Methanol	> 5000

4.2 Antihyperlipidaemic Effects of *M. piperita* Leaves Extracts in Experimental Rats

The hyperlipidaemic untreated rats showed high serum levels of total cholesterol, LDL-cholesterol, and triglycerides but oral administration of ethylacetate, methanol and hexane extracts of *M. piperita* leaves at variable concentrations significantly ($p<0.05$) reduced the lipid levels in the experimental rats (Table 4.2).There was also reduction in HDL level in hyperlipidaemic untreated rats while the animals treated with the extracts showed higher HDL levels. Ethyl acetate extract at a dose of 100mg/kg produced the highest reduction in lipid profiles compared to the other extracts which qualified it as a candidate for further experiments.

Table 4.2: Antihyperlipidaemic Effect of *M. piperita* Leaves Extracts in Experimental Rats

GROUPS	TREATMENT	TG(mmol/L)	CHOL(mmolL	HDL(mmol/L)	LDL(mmol/L)
I	Normal, untreated	0.82 ± 0.24^{a}	2.38 ± 0.50^{a}	1.37 ± 0.57	0.63 ± 0.46^{a}
II	P407 ,untreated	2.45 ± 0.41^{b}	6.60 ± 0.85^{b}	0.93 ± 0.39^{b}	4.51 ± 0.33^{b}
III	P407 + artovastatin	1.05 ± 0.35^{a}	4.55 ± 1.80^{b}	1.80 ± 0.49	2.27 ± 1.69
IIIa	P407 + 100mg/kg ethyl acetate	1.13 ± 0.26^{a}	5.1 ± 0.48	2.63 ± 0.80	1.84 ± 0.54^{a}
IIIb	P407 + 200 mg/kg ethyl acetate	1.32 ± 0.51	5.45 ± 0.17	2.77 ± 0.79^{a}	2.07 ± 0.54
IIIc	P407 + 300 mg/kg ethyl acetate	1.39 ± 0.69	7.21 ± 1.46^{b}	3.45 ± 1.19^{ab}	3.13 ± 0.89
IVa	P407 + 100 mg/kg methanol	1.74 ± 0.88	6.15 ± 2.14^{b}	1.80 ± 0.65	3.56 ± 1.49^{b}
IVb	P407 + 200 mg/kg methanol	1.63 ± 0.93	5.79 ± 2.29^{b}	1.94 ± 0.39^{a}	2.74 ± 1.99^{a}
IVc	P407 + 300 mg/kg methanol	4.77 ± 0.59^{ab}	7.48 ± 0.16^{b}	1.48 ± 0.33	3.83 ± 0.30^{b}
Va	P407 + 100 mg/kg hexane	2.15 ± 0.69^{b}	5.37 ± 1.41	1.63 ± 0.39	2.77 ± 0.76
Vb	P407 + 200 mg/kg hexane	2.07 ± 0.58	5.61 ± 1.88^{b}	3.63 ± 1.85^{ab}	1.04 ± 0.45^{a}
Vc	P407 + 300 mg/kg hexane	1.47 ± 0.97	5.40 ± 1.56	2.33 ± 0.75	2.58 ± 1.34
VIa	Normal +100mg/kg ethyl acetate	0.3 ± 0.08^{a}	2.0 ± 0.55^{a}	1.38 ± 0.48	0.48 ± 0.08^{a}
VIb	Normal+200mg/kg ethyl acetate	0.49 ± 0.25^{a}	1.83 ± 0.84^{a}	1.17 ± 0.67	0.44 ± 0.16^{a}
VIc	Normal +300mg/kg ethyl acetate	0.62 ± 0.37^{a}	1.78 ± 0.69^{a}	0.94 ± 0.33	0.26 ± 0.29^{a}
VIIa	Normal +100 mg/kg methanol	0.67 ± 0.22^{a}	1.65 ± 0.61^{a}	0.94 ± 0.12	0.60 ± 0.22^{a}
VIIb	Normal +200 mg/kg methanol	0.60 ± 0.34^{a}	1.65 ± 0.40^{a}	1.11 ± 0.44	0.46 ± 0.37^{a}
VIIIc	Normal +300 mg/kg methanol	0.84 ± 0.17^{a}	1.61 ± 0.41^{a}	1.07 ± 0.38	0.16 ± 0.18^{a}
VIIIa	Normal +100 mg/kg hexane	0.98 ± 0.11^{a}	1.90 ± 0.21^{a}	1.15 ± 0.33	0.66 ± 0.06^{a}
VIIIb	Normal +200 mg/kg hexane	0.72 ± 0.35^{a}	1.77 ± 0.88^{a}	1.05 ± 0.79	0.39 ± 0.17^{a}
VIIIc	Normal +300 mg/kg hexane	0.91 ± 0.09^{a}	1.75 ± 0.60^{a}	0.95 ± 0.13	0.55 ± 0.05^{a}

Values are Mean ± S.D (n = 5). Values with [a] are significantly different ($p < 0.05$) when compared with the hyperlipidaemic control (grp 1), values with [b] are significantly different ($p < 0.05$) when compared with the normal control grp (4), Values with [ab] are significantly different ($p < 0.05$) when compared with both the hyperlipidaemic and normal control .Values with neither [a] nor [b] are not significantly different (($p < 0.05$) when compared with both hyperlipidaemic and normal control.

P407: Poloxamer 407, TG: triglycerides, CHOL: cholesterol, HDL: High density lipoprotein, LDL: Low density lipoprotein

4.3 Antihyperlipidaemic Effect of Fractions from Ethyl Acetate Extract in Experimental Rats

Table 4.3 presents the twelve fractions obtained after fractionation of ethyl acetate by different solvent system of similar RF values while Table 4.4 indicates the respective antihyperlipidaemic effects of the fractions. There was significant ($p<0.05$) reduction in triglycerides (TG), total cholesterol (CH) and LDL cholesterol (LDL) in the serum of treated rats (groups I – XV) with a significant ($p<0.05$) increase in HDL cholesterol in some of the treated rats (groups IV, X and XV) compared to the hyperlipidaemic untreated rats. Also, rats treated with fraction 2 (F2) exhibited lowest values of TG, CH and LDL. F2 was found to be most antihyperlipidaemic, with an LD_{50} beyond 5000mg/kg.

Table 4.3: Ethyl acetate Fractions of *Mentha piperita* Leaf Extracts

Fractions	Solvents system (%Hex : %EA)	% yield
F1	100 % Hexane	1.57
F2	90 : 10	21.83
F3	80 : 20	4.40
F4	70 : 30	4.87
F5	60 : 40	5.43
F6	50 : 50	2.83
F7	40 : 60	3.93
F8	30: 70	1.97
F9	20: 80	4.40
F10	10: 90	1.53
F11	100 % Ethyl acetate	1.00
F12	100 % methanol	20.27

KEY: Hex = Hexane, EA = Ethyl acetate

Table 4.4: Antihyperlipidaemic Effect of the Ethyl acetate Fractions in Experimental rats

GROUPS	TREATMENT	TG(mmol/L)	CHOL(mmol/L	HDL(mmol/L)	LDL (mmol/L
I	normal control	1.04±0.29 [a]	2.11± 0.27 [a]	0.91 ± 0.23 [a]	0.81 ± 0.35[a]
II	P407 ,untreated	5.12±1.31 [b]	9.07 ± 1.64 [b]	1.76 ± 0.38[b]	4.46 ± 1.19[b]
III	P407+artovastatin	1.96±0.36[ab]	4.10 ±0.41[ab]	1.99 ±0.48[b]	1.02 ± 0.49 [a]
IV	P407+F1	2.92±0.42[ab]	5.64 ± 0.89[ab]	2.53 ± 0.20[b]	2.2 ± 0.47 [a]
V	P407+F2	1.10±0.14 [a]	1.93 ± 0.19[a]	1.51 ± 0.53	0.28 ± 0.08 [a]
VI	P407+F3	1.89±0.48[a]	4.58± 0.52[ab]	1.04 ±0.24	3.14 ± 0.57[b]
VII	P407+F4	2.69±0.49[ab]	5.49 ± 0.81[ab]	1.44 ± 0.27	2.81 ± 0.54 [ab]
VIII	P407+F5	2.06±0.47 [a]	3.77 ± 0.60[a]	1.25 ± 0.13	1.76 ± 0.30 [ab]
IX	P407+F6	2.31± 0.53 [a]	3.61 ± 0.69[a]	1.44± 0.51	1.20 ± 0.40 [ab]
X	P407+F7	3.41±0.72[ab]	6.24± 0.99[ab]	1.74 ± 0.13[b]	3.17 ± 0.75 [a]
XI	P407+F8	3.23±0.44[ab]	3.86 ± 0.49[a]	1.39 ± 0.20	1.99 ± 0.63 [a]
XII	P407+F9	3.05±0.32[ab]	4.78 ± 0.40[ab]	1.46 ± 0.38	1.88 ± 0.39 [a]
XIII	P407+F10	3.14±0.29[ab]	5.93 ± 0.86[ab]	1.45 ± 0.27	3.19 ± 0.60[b]
XIV	P407+F11	2.91±0.50[ab]	4.88 ±0.76[ab]	1.21 ± 0.21	2.47 ± 0.39[ab]
XV	P407+F12	1.18±0.35 [a]	4.84 ±0.68[ab]	2.46 ± 0.24[b]	1.43 ± 0.62[a]

Values are Mean ± S.D (n = 5).Values with [a] are significantly different ($p < 0.05$) when compared with the hyperlipidaemic control (grp 1), values with [b] are significantly different ($p < 0.05$) when compared with the normal control (grp I), Values with [ab] are significantly different ($p < 0.05$) when compared with both hyperlipidaemic and normal control .Values with neither [a] nor [b] are not significantly different ($p < 0.05$) when compared with both hyperlipidaemic and normal control.

P407: Poloxamer 407, TG: triglycerides, CHOL: cholesterol, HDL: High density lipoprotein, LDL: Low density lipoprotein

4.4 Evaluation of *in vitro* Antioxidant Properties of Most Active Fraction(s) of the extract

Table 4.5 shows the *in vitro* antioxidant (IC_{50}) properties of the most active fraction(s) of the extract. Fractions 2, 3, 4, 6 and 12 were chosen for further in *vitro* studies because they showed the best antihyperlipidaemic effects. The *in vitro* antioxidant activity of the fractions indicates increased reduction of DPPH which is related to the high scavenging activity of the extract and fraction. The fractions exhibited significant antioxidant capacity by their ability to cause reduction in DPPH radical.

Table 4.5: *In Vitro* Antioxidant Activity (IC_{50} Values) Of Ethyl acetate Extract and Antihyperlipidaemic Fractions of *M.Piperita* Leaves

EXTRACT/ FRACTIONS	HEXANE:ETHYL ACETATE IN %	IC_{50} (µg/ml)
Ethyl acetate	100% ethyl acetate	2.96 ± 0.14^{b}
Fraction 2	90 :10	3.63 ± 0.54^{b}
Fraction 3	80 :20	4.00 ± 0.40^{a}
Fraction 4	70 :30	5.32 ± 0.87^{a}
Fraction 6	50 :50	5.98 ± 0.37^{a}
Fraction 12	100% methanol	2.68 ± 0.14^{b}
Ascorbic acid		2.49 ± 0.30

Values are Mean ± SD.Values with a are statistically different when compared with standard drug (ascorbic acid). Values with b are not statistically different when compared with the standard drug.

IC_{50} – Inhibitory concentration at 50%

4.5 Genotoxic Characteristics of Ethylacetate Extract and the Most Active Fraction(S) of the Extract

Genotoxic properties of ethylacetate extract and most active fractions of the extract of *M.piperita* leaves on pVax plasmid is presented in Plate 4.1. The gel electrophoregram shows that all the most active fractions (fractions 2, 3, 4, 6, 12) and the extract were not genotoxic as observed in lanes 2, 4, 6, 8, 10 and 12 to the plasmid DNA in the absence of Fenton's reagent. The results also indicates that all the fractions have genoprotective activity as observed in lanes 3, 5, 7, and 9 in the in the presence of fenton's reagent except fraction 2 (lane 1**).** However, fraction 2 is not genotoxic as evident from the band (lane 2).

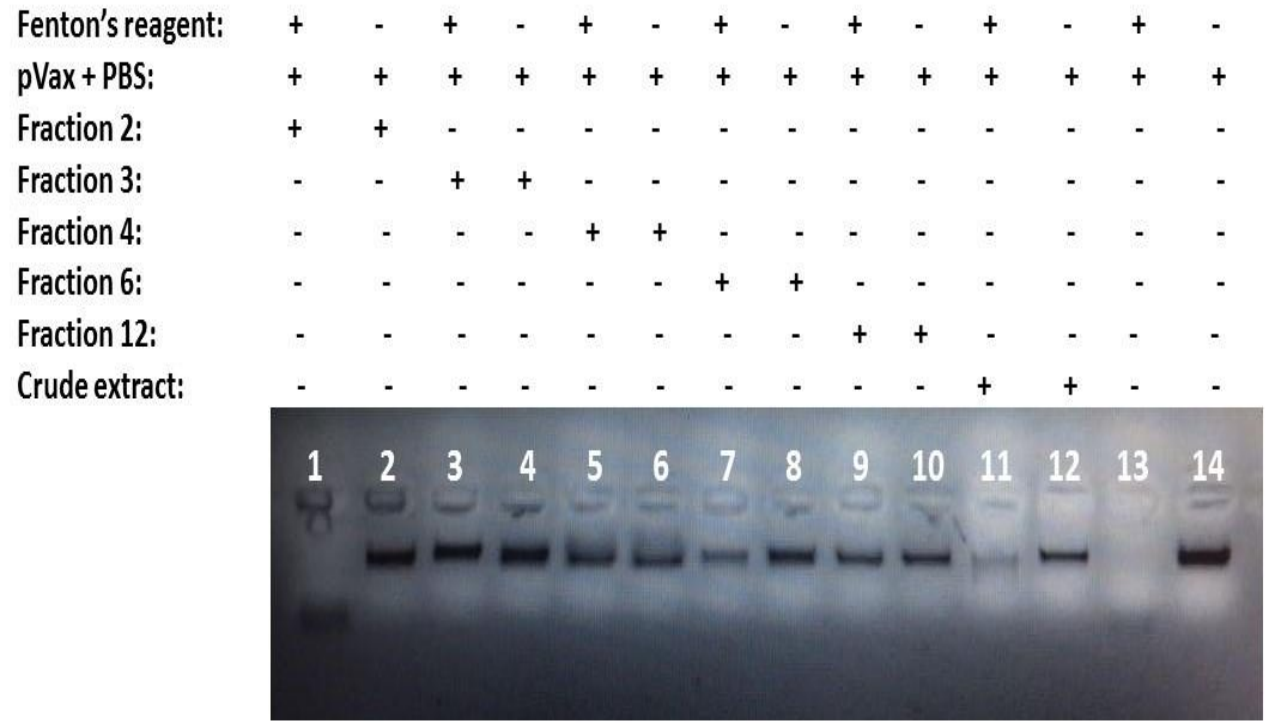


Plate 4.1: Electrophoregram Showing Non Genotoxic Property of Mentha piperita IC50 (Fractions and Ehyl acetate Crude Extract) on pVax Plasmid (3000bp) in Vitro.

Key : lanes 1and 2- f2, lanes 3 and 4-f3, lanes 5and 6 –f4,lanes 7 and 8 – f6, lanes 9 and 10 – f12, lanes 11 and 12- ethylacetate extract, lane 13- positive control, lane 14 – negative control.

4. 6 Effect of Most Active Fraction of the Extract on Integrity of Hepatocytes in Experimental Rats

Fraction 2 (of ethyl acetate extract of *M. piperita* leaves) show hepatoprotective effect based on histological analysis (Plate 4.2) in hyperlipidaemic rats. Normal features of hepatocytes were observed in liver tissues of normal control rats (Plate A) but in hyperlipidaemic untreated rats, kupffer cell hyperplasia was observed with slight hepatocellular necrosis (Plate B). The hepatocytes of animals treated with fraction 2 show slight vacuolation and necrosis (Plate C), while that for atorvastatin treated rats deposits slight foci necrosis with infiltration of lymphocytes (Plate D).

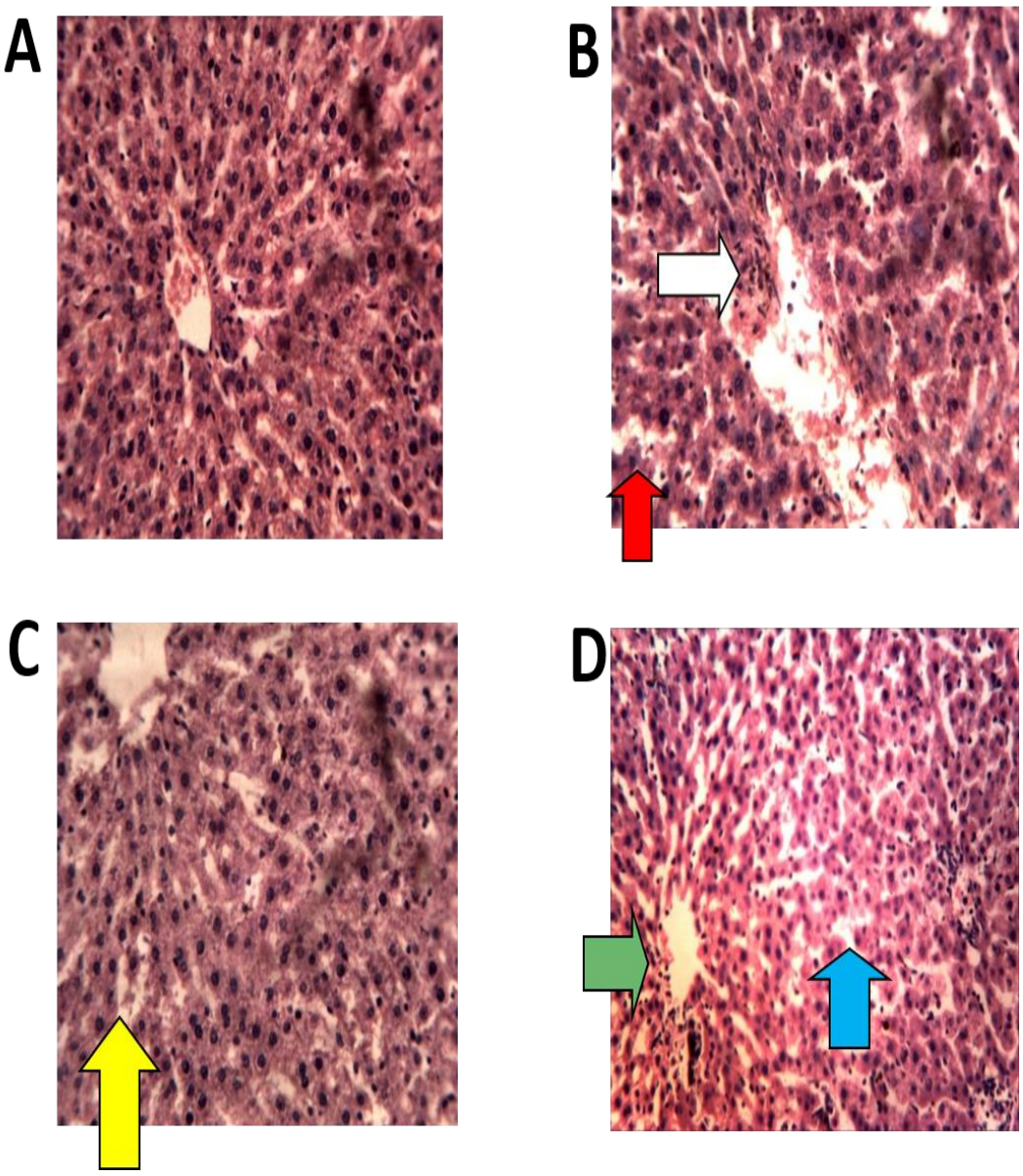


Plate 4.2: Effects of Fraction 2 (from Ethyl acetate Extract of *M. piperita* Leaves) in Liver Tissues of P407 Induced Hyperlipidaemic Rats. Magnification × 250, H & E Stain

Key: A: Normal control (Normal features), B: P407 untreated (white arrow indicates kupffer cell hyperplasia while red arrow indicates hepatocellular necrosis), C: P407 +F2 (yellow arrow indicates slight vacuolation with necrosis), D: P407 + Artovastatin (blue arrow indicates slight foci necrosis while green arrow indicates infiltration of lymphocytes).

4.7 Effects of Most Active Fraction of Ethyl acetate extract on Oxidative Stress Biomarkers and Lipoprotein Lipase Activity

A significant increase in MDA with a decrease in GSH levels were observed in liver and serum of hyperlipidaemic untreated rats (Figure 4.1 and 4.2).The animals treated with F2 show a significant ($p<0.05$) decrease in the MDA level with a concomitant increase in the GSH level when compared with hyperlipidaemic untreated rats. Treatment with F2 and atorvastatin significantly ($p<0.05$) reduced CAT and SOD activities in the liver and serum when compared with the control groups.

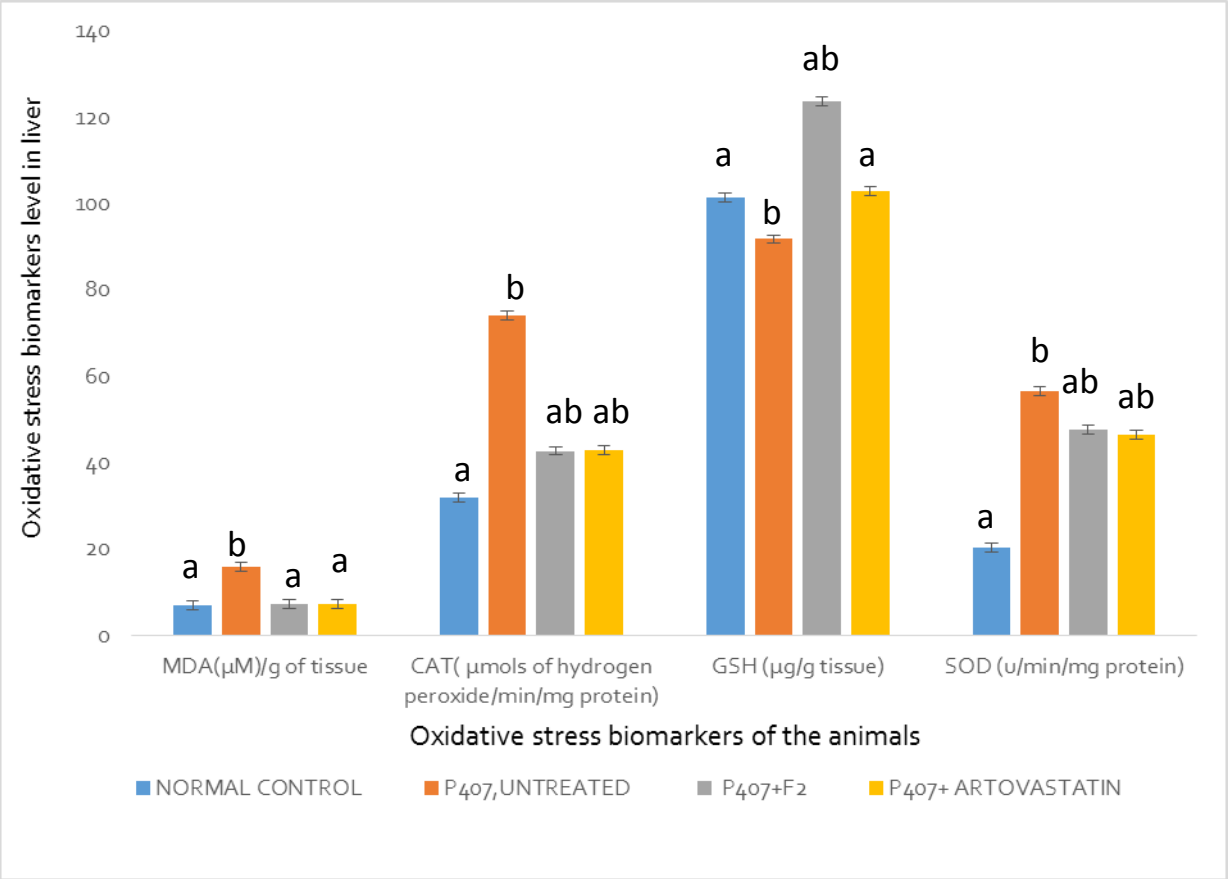


Figure 4.1: Effect of Fraction 2 (from Ethyl acetate Extract of *M. Piperita* Leaves) on Antioxidant Activity of Liver Tissues of Hyperlipidaemic Rats.

Values are Mean ± S.D (n=5) .Values with [a] are significantly different when compared with the hyperlipidaemic control while values with [b] are significantly different when compared with the normal control group. Values with [ab] are significantly different when compared with both the hyperlipidaemic and normal control values at $p < 0.05$.

MDA: Malonaldialdehyde, CAT: Catalase, GSH: Reduced glutathione, SOD : Superoxide dismutase, P407: Poloxamer 407.

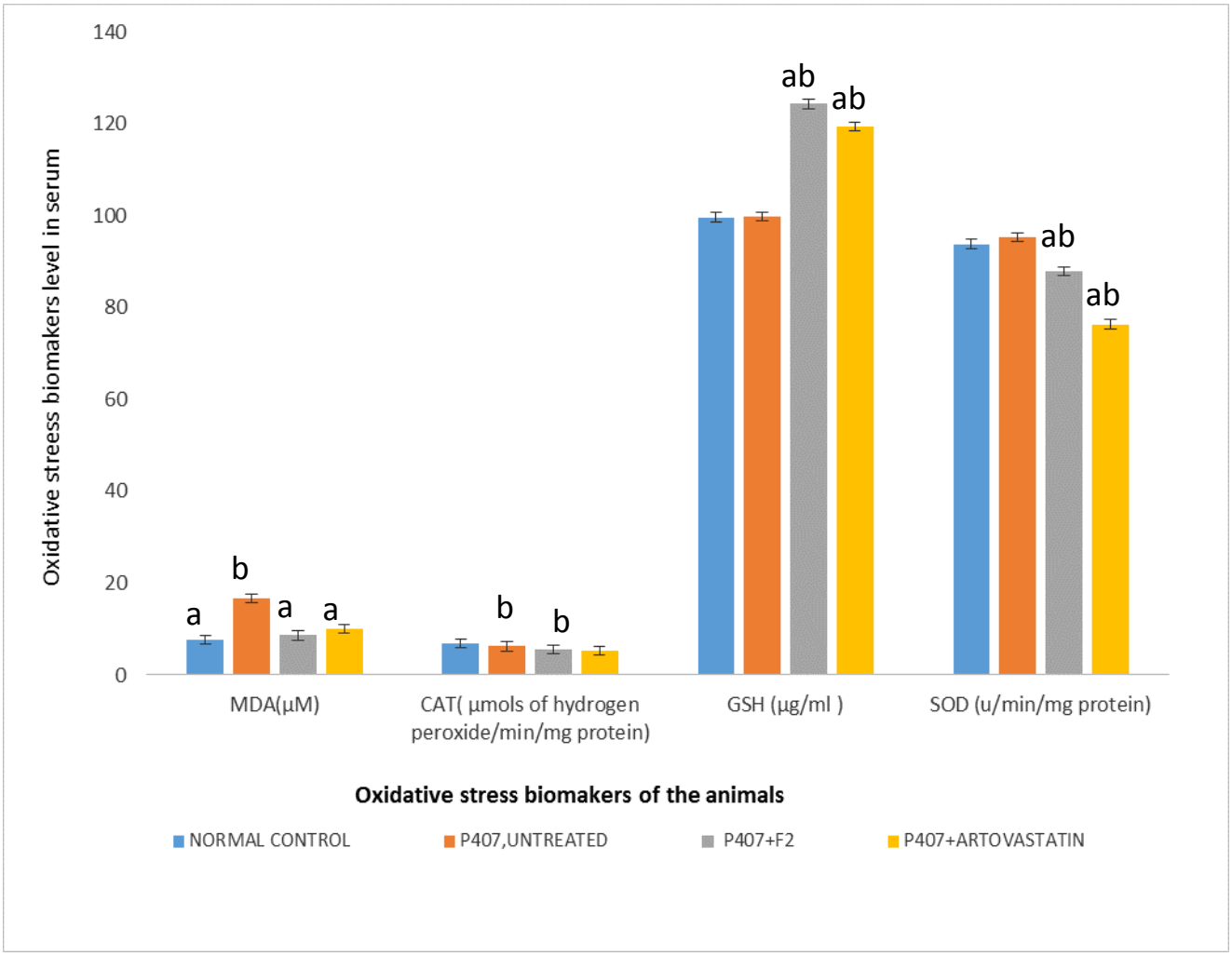


Figure 4.2: Antioxidant Activity of Serum of Hyperlipidaemic Rats.

Values are Mean ± S.D (n=5). Values with [a] are significantly different when compared with the hyperlipidaemic group while values with [b] are significantly different when compared with the normal control group. Values with [ab] are significantly different when compared with both the hyperlipidaemic and normal control .Values with neither [a] nor [b] are not statistically significant when compared with both hyperlipidaemic and normal control at $p < 0.05$.

MDA: Malonaldialdehyde, CAT: Catalase, GSH : Reduced glutathione, SOD : Superoxide dismutase, P407: Poloxamer 407.

Figures 4.3 and 4.4 shows that induction of hyperlipidaemia significantly ($p<0.05$) enhances LPL activity in the liver (8.41 U/min/g tissue) and serum (0.52 U/min/ml) when compared with the hyperlipidemic untreated rats (3.08 U/min/g tissue), in the liver and 0.28 U/min/ml), for the serum.

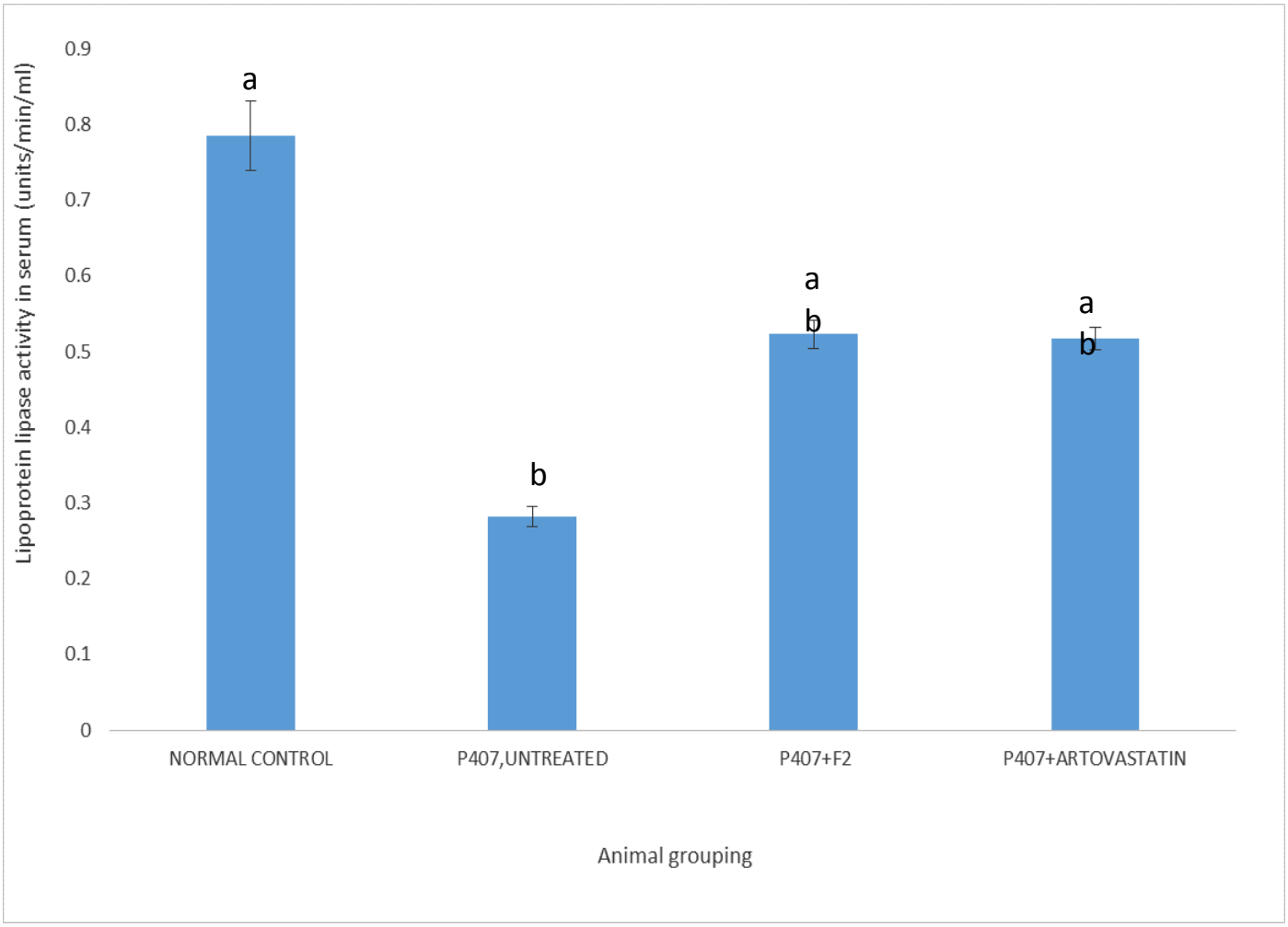


Figure 4.3: Effect of Fraction 2 (from Ethyl acetate Extract of *M. Piperita* Leaves) on Lipoprotein Lipase Activity in Serum of Hyperlipidaemic Rats.

Values are Mean ± S.D (n=5). Values with [a] are significantly different when compared with the hyperlipidaemic group while values with [b] are significantly different when compared with the normal control group. Values with [ab] are significantly different when compared with both the hyperlipidaemic and normal control at $p < 0.05$. P407: Poloxamer 407, F2: Fraction 2.

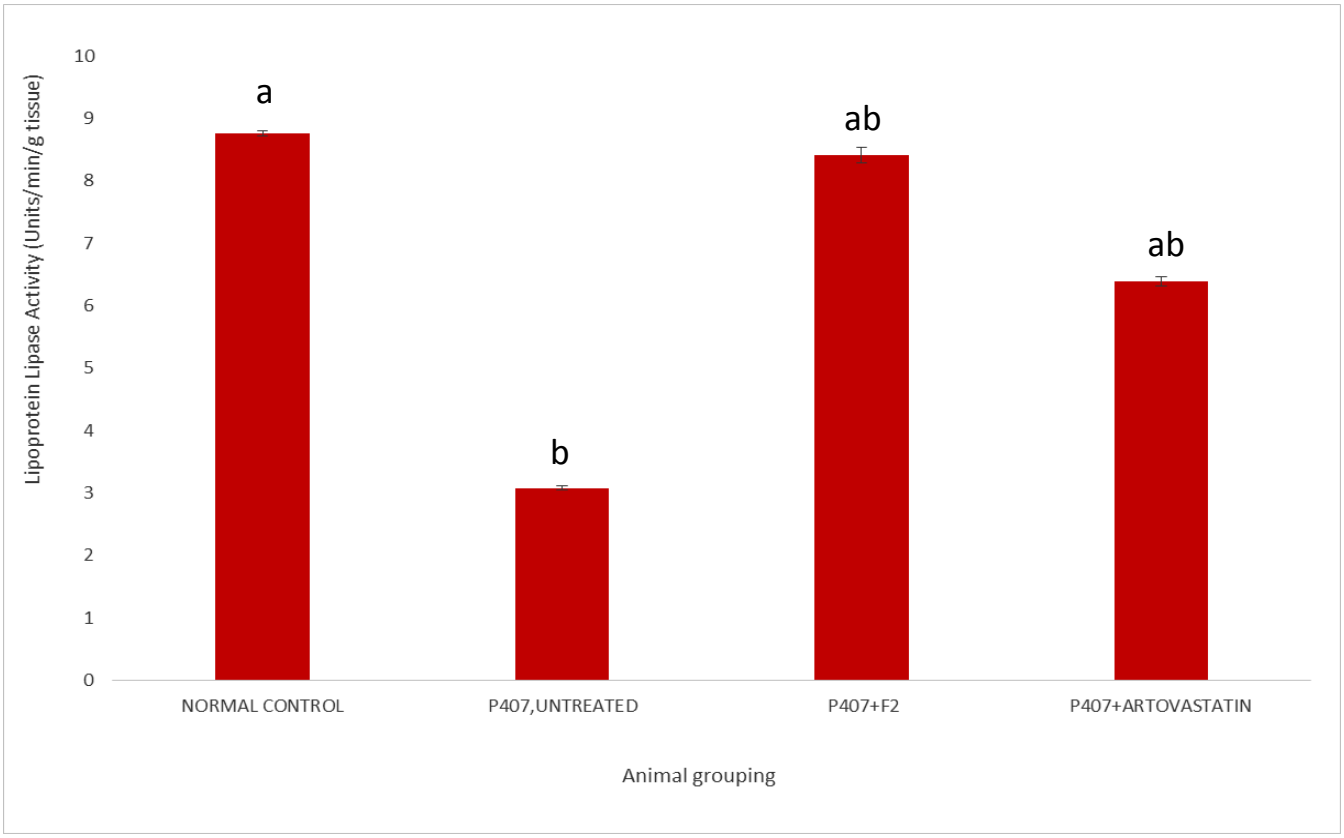


Figure 4.4: Effect of Fraction 2 (from Ethyl acetate Extract of M. Piperita Leaves) on Lipoprotein Lipase Activity in Liver Tissues of Hyperlipidaemic Rats.

Values are Mean ± S.D (n=5). Values with [a] are significantly different when compared with the hyperlipidaemic group while values with [b] are significantly different when compared with the normal control group. Values with [ab] are significantly different when compared with both the hyperlipidaemic and normal control at $p < 0.05$.

P407: Poloxamer 407, F2: Fraction 2.

4.8 Effects of Most Active Fraction of the Extract on Expression of Lipoprotein Lipase, Oxidative Stress Biomarkers (Catalase and Superoxide Dismutase) *in Vivo*

Figures 4.5, 4.6 and 4.7 shows effects of fraction 2 from ethyl acetate extract of *M. piperita* leaves on expression of catalase, superoxide dismutase and lipoprotein lipase in the liver tissues and blood of experimental rats. Down regulation of *CAT*, *SOD* and *LPL* genes were observed in the liver and blood of hyperlipidaemic untreated rats. Treatment of rats with F2 significantly ($p<0.05$) upregulated the expression the *CAT*, *SOD* and *LPL* relative to hyperlipidaemic untreated rats.

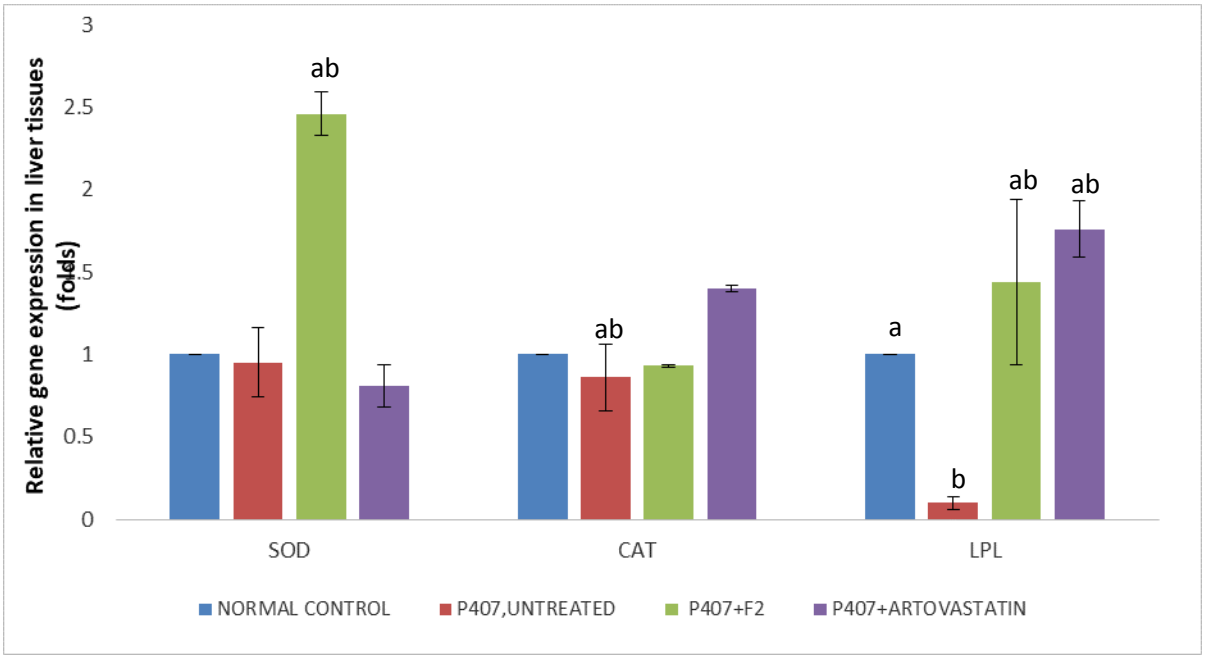


Figure 4.5: Effect of Fraction 2 (from Ethylacetate Extract of *M. Piperita Leaves*) on Relative Gene Expression of SOD, CAT and LPL in Liver Tissues of Hyperlipidaemic Rats.

Values are Mean ± S.D (n=5). Values with [a] are significantly different when compared with the hyperlipidaemic group while values with [b] are significantly different when compared with the normal control group. Values with [ab] are significantly different when compared with both the hyperlipidaemic and normal control .Values with neither [a] nor [b] are not statistically significant when compared with both hyperlipidaemic and normal control at $p < 0.05$.

SOD: Superoxide dismutase, CAT: Catalase, LPL : Lipoprotein Lipase, P407: Poloxamer 407, F2: Fraction 2.

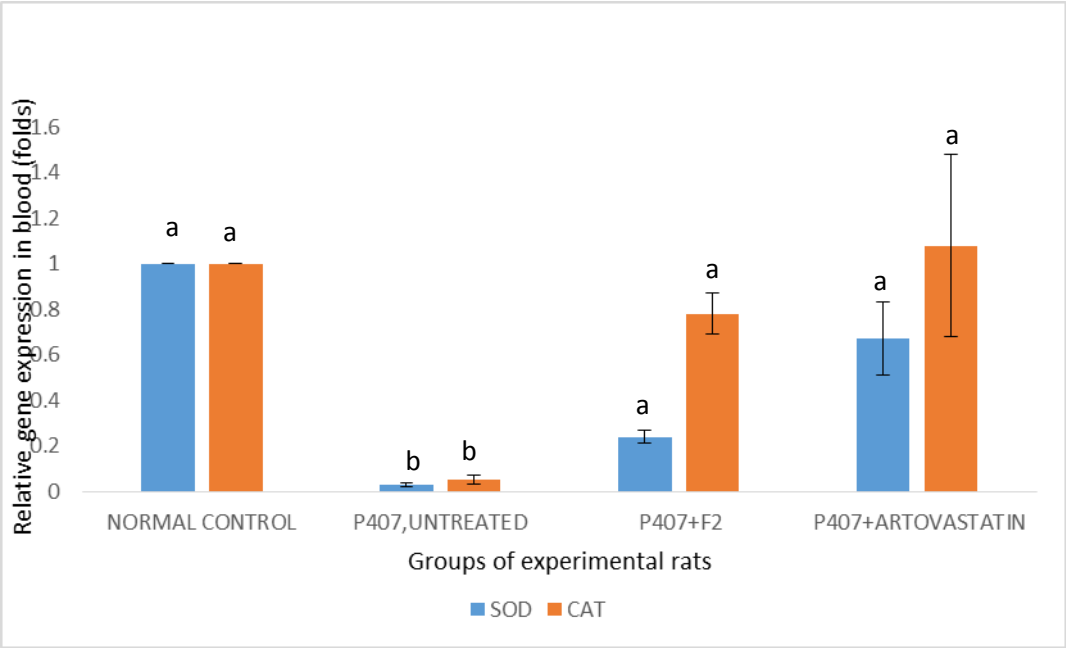


Figure 4.6: Effect of Fraction 2 (from Ethyl acetate Extract of *M. Piperita* Leaves) on Relative Gene Expression of SOD and CAT in Blood of Hyperlipidaemic Rats.

Values are mean ± S.D (n=5). Values with [a] are significantly different when compared with the hyperlipidaemic group while values with [b] are significantly different when compared with the normal control group. Values with [ab] are significantly different when compared with both the hyperlipidaemic and normal control .Values with neither [a] nor [b] are not statistically significant when compared with both hyperlipidaemic and normal control at $p < 0.05$.

SOD: Superoxide dismutase, CAT: Catalase, P407: Poloxamer 407, F2: Fraction 2.

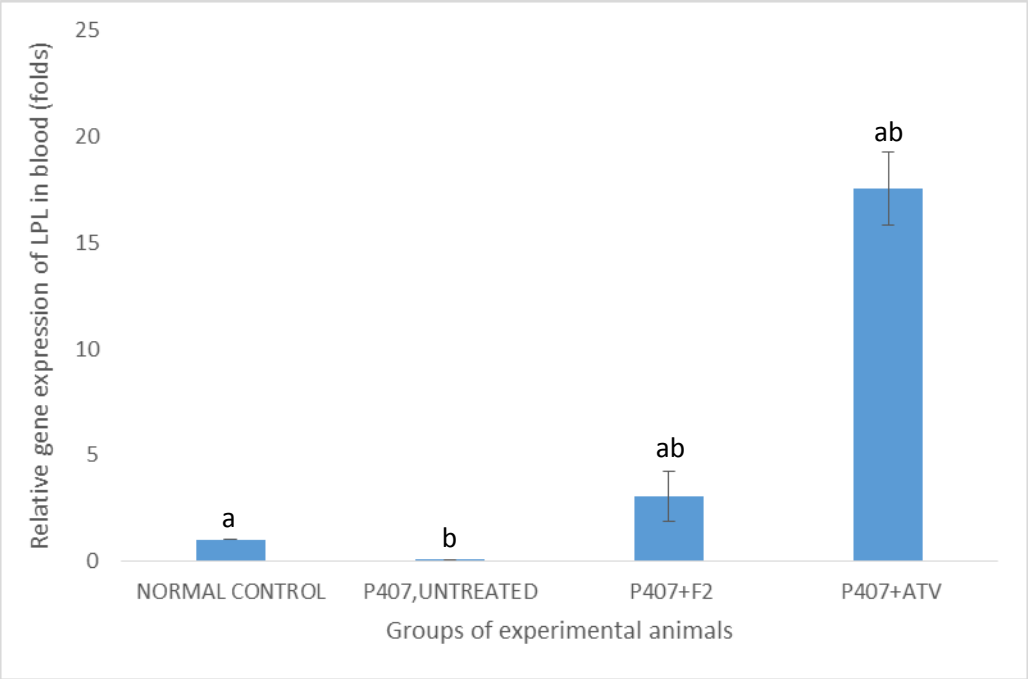


Figure 4.7: Effect of Fraction 2 (from Ethyl acetate Extract of *M. Piperita* Leaves) on Relative Gene Expression of LPL in Blood of Hyperlipidaemic Rats.

Values are mean ± S.D (n=5). Values with [a] are significantly different when compared with the hyperlipidaemic group while values with [b] are significantly different when compared with the normal control group. Values with [ab] are significantly different when compared with both the hyperlipidaemic and normal control .Values with neither [a] nor [b] are not statistically significant when compared with both hyperlipidaemic and normal control at $p < 0.05$.

LPL: Lipoprotein Lipase, P407: Poloxamer 407, F2: Fraction 2.

4.9 Chromatogram of Potent Fraction (F2) of Ethylacetate Extract from *M. Piperita* Leaves

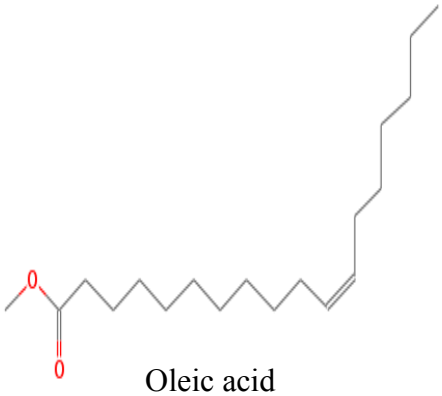
The possible identified compounds from GCMS analysis with a similarity index of 70 and above were 2-octyl-cyclopropaneoctanal, 2-methyl-3, 13-octadecadienol, cis-vaccenic acid, oleic acid, 9-Oxabicyclo [6.1.0] nonane and 9-octadecenal (Table 4.6). Two of these compunds, cis-vaccenic acid and oleic acid were reported to have antihyperlipidaemic and antioxidant activity (Figure 4.7) whereas compounds identified from LCMS (Figure 4.8, Table 4.7) which include coumarin, isoeugenitin, isoferreirin, 3, 4-dicaffeoylquinic acid, dihydromorelloflavone and triacontane were reported to have antihyperlipidaemic and antioxidant activity.

Table 4.6: Proposed Compounds Present in Fraction 2 Identified by GCMS

COMPOUND	SIMILARITY INDEX
2-octyl-Cyclopropaneoctanal	89
2-Methyl-3,13-octadecadienol	70
cis-Vaccenic acid	80
Oleic Acid	95
9-Oxabicyclo[6.1.0]nonane	86
9-Octadecenal	70



Cis-vaccenic acid



Oleic acid

Figure 4.7: Possible Compounds with Reported Activity Present in Fraction 2 Identified by GCMS

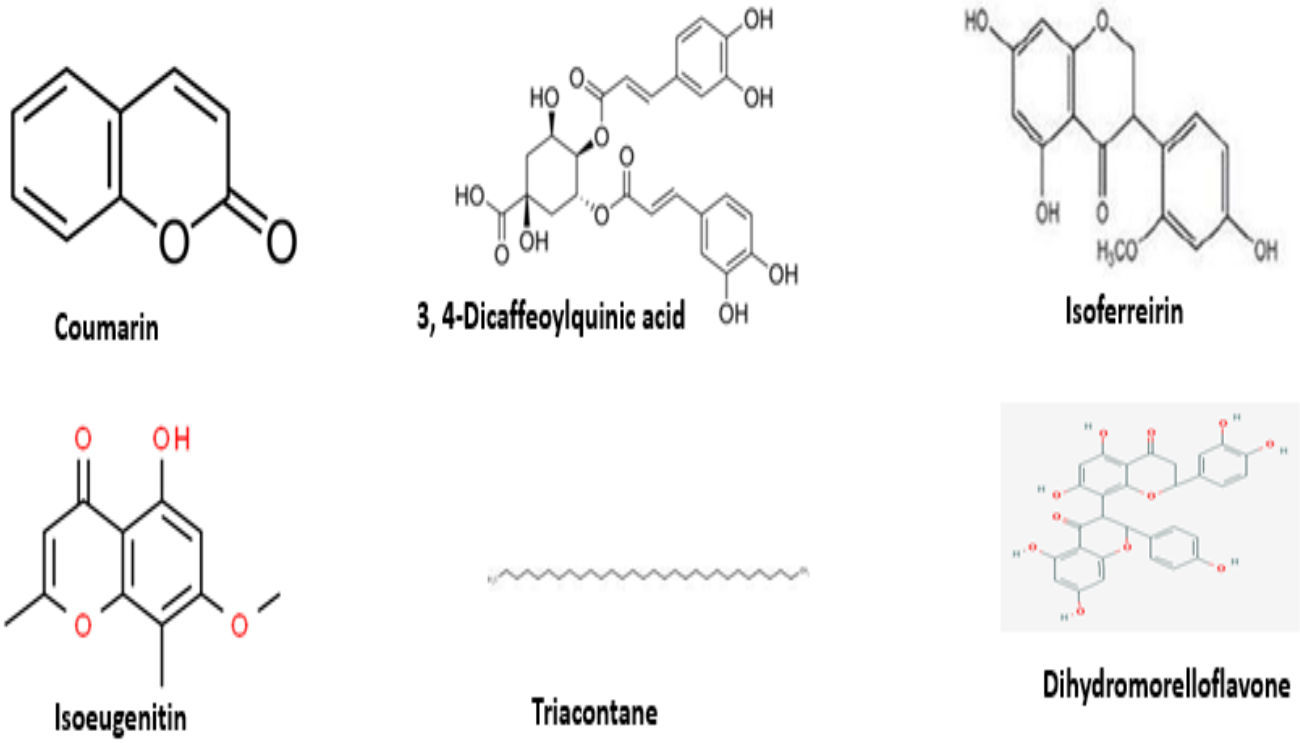


Figure 4.8: Possible Compounds Present In Fraction 2 Identified By LCMS

Table 4.7: Relative abundance of compounds present in fraction 2 identified by LCMS

Mass	Compound name	Relative abundance (%)
208	Coumarin	18.69%
297	Isoeugenitin	3.74%
379	Isoferreirin	37.38%
593	3,4-Dicaffeoylquinic acid	9.35%
635	Dihydromorelloflavone	2.8%
873	N-Triacontane	28.03%

CHAPTER FIVE

5.0 DISCUSSION

The three extracts (hexane, ethylacetate and methanol) and most active fraction of *M. piperita* leaves studied were tolerated by the experimental rats and relatively safe for consumption since the LD_{50} of the extracts of were above 5000 mg/kg. This is because experimental rats tolerated the extracts without symptoms of acute toxicity even at larger doses. Lower LD_{50} implies a more toxic extract while LD_{50} value beyond 5000 mg/kg is of no toxicological significance (Lorke, 1983). High LD_{50} exhibited by the extracts prompted the continuation with antihyperlipidaemic assessment.

The reduction of total cholesterol, LDL-cholesterol, and triglycerides and elevation of HDL-cholesterol in hyperlipidaemic rats after treatment with the crude extracts of *M. piperita* leaves implies that ethyl acetate extract has potential use in reducing the risk of coronary artery disease since it was effective in lowering plasma lipids. *Mentha piperita* leaf extract may help to suppress the biosynthesis of cholesterol and triglycerides (Chandirasegaran *et al.*,2015). The result of this work is also in agreement with the study reported by Badal *et al.*,(2011) and Chandirasegaran *et al.*, (2015) in which aqueous extract of *M. piperita* leaves reduced the level of cholesterol, triglycerides, VLDL, LDL, in streptozotocin (STZ) and fructose-induced diabetic rats respectively. Treatment of animals with aqueous extract of peppermint leaves reduced serum TC (44.32%), LDL (69.19%) and TG (55.77%) (Johari *et al.,* 2015). In another study Peppermint juice administered to rats reduced TC, LDL, body weight and atherogenic indices with concomitant increase in the levels of HDL (Barbalho *et al.*,2017). These findings have invariably corroborated our experimental outcome.

The antihyperlipidaemic property of the fractions of ethyl acetate extract from *M. piperita* leaves shows that they are effective lipid lowering agents and may protect

against cardiovascular diseases resulting from hyperlipidaemia, with fraction 2 being the most active.

In *vitro* antioxidant activity demonstrated by the most active fractions shows that reduction of DPPH is related to the high scavenging activity given by the fractions. Our findings from *in vitro* antioxidant activity study indicate fraction 12 as the most active radical scavenger compared to other fractions. The DPPH radical scavenging potentials was expressed based on the IC_{50} values. The lower the IC_{50} the higher the scavenging potentials.

M. piperita is rich in total phenols which have been recognized as antioxidant agents which act in neutralizing free radical. Therefore, it is likely that the phenolic constituents present in the *Mentha* spp may be responsible for the antioxidant and free radical scavenging activities (Nickavar *et al.,* 2008). Salih *et al.,*(2013) reported that antioxidant and free radical scavenging activity of *Mentha piperita* plant may be due to its constituent phytochemicals such as phenols, tannins, flavonoids and others; and the fact that these phenolic compounds have been recognized as antioxidant agents which act as free radical oxidation terminators. This study is consistent with the study carried out by Hossain *et al.,* (2014) in which different crude extracts of *M.piperita* scavenged dpph free radical. Also, this study correlates with the work by Nickavar *et al.,* (2008) in which ethanol extract of five *Mentha* spp showed strong antioxidant and free radical scavenging activities.

The fractions and ethylacetate extract were not genotoxic to the plasmid DNA with most fractions being genoprotective which might be due to the prevention of reaction between Fe_3^+ and H_2O_2 in the fentons reagent by components present in the extract or fraction. Indeed, natural polyphenols can influence the free radicals mediated oxidation

of DNA through simple mechanism including quenching of reactive oxygen species by donating hydrogen atom or electron (Lee *et al.*, 2002; Wei *et al*., 2006). This type of action might diminish the reduction potential of Fe_3^+ leading to the inhibition of Fenton-like reaction, and also directly scavenge $OH^{\cdot}$ and therefore, protecting the plasmid DNA from $OH^{\cdot}$- dependent strand breakage (Adejo and Atawodi, 2014).

In a related experiment, Singh *et al*., (2009) showed that incubation of plasmid DNA with Fenton's reagent resulted in the cleavage of the DNA indicating that $OH^{\cdot}$ generated from iron-mediated decomposition of H_2O_2 produced DNA breakage. Gutteridge (1984) also reported that H_2O_2 and $^{\cdot}O_2$- are potentially cytotoxic; most of the oxidative damage in biological systems is caused by $OH^{\cdot}$, which is generated by the reaction between H_2O_2 and O_2 in the presence of redox-active metals. This type of oxidative damage can be ameliorated in the presence of plants rich in antioxidants, by converting H_2O_2 into water and oxygen.

Also non-genotoxic characteristics of the extracts and fractions is consistent with the findings of Singh *et al.*, (2009) in which the aqueous fruit and seed extracts of *Moringa oleifera* exhibited partial and complete protection of plasmid DNA, mitigated the oxidative stress produced by Fenton-like reactions. Redox-active metals in solution could bind to phenolic antioxidant compounds and form complexes, and consequently prevent the reduction of redox-active metal ions with H_2O_2 (Wei and Shiow, 2001).

Due to non-genotoxic/non-protective properties of fraction 2 as seen from the results with its strong antihyperlipidaemic activity, it was therefore, selected for further assays/studies.

The slight vacuolation and necrosis seen in the liver of rats treated with fraction 2 (F2) suggest that tissue repair occured upon treatment with the fraction even though it was not completely restored to the normal features within the period of the experiment.

Thus, F2 may be capable of ameliorating tissue damage such as hepatocytes injury that may occur in hyperlipidaemic condition. This finding is in agreement with the study carried out by Helal *et al.*,(2012) in which lymphocytic infiltration, slight vacuolation; increase in number of kupffer cells were observed in the liver tissues of hyperlipidaemic rats. Necrosis observed in the liver tissues of hyperlipidaemic rats also agrees with a previous study (Korolenko *et al.,* 2010; Korolenko *et al.,* 2016). Slight foci necrosis showed by the liver of atorvastatin treated groups is in conformity with findings of Zuberu *et al*., (2017) in P407 - induced hyperlipidaemic rats.

In this research, the significant ($p<0.05$) decrease in MDA level and increase in GSH in the liver and serum demonstrated by F2 indicates it has protective/antioxidant effect on hyperlipidaemic-induced oxidative stress. Pharmacological studies have shown that *M. piperita* plant has antioxidant activity, reducing the effects of the free radicals that are related to diseases such as cancer and atherosclerosis which may be due to the presence of bioactive compounds (Liu *et al*., 2014; Mallick *et al*., 2016). Similar results was observed in a study by Rathod *et al*., (2011) which showed decrease in the level of MDA and increased GSH level in P-407 induced hyperlipidaemic rats. This study also agrees with the antioxidant studies carried out by Singh *et al.*, (2015).

Reduction in the levels of MDA by fraction 2 of *Mentha piperita* leaves is also in accordance with other studies by Ramesh *et al.,* (2011) which suggests that *Mentha* spp contain the natural antioxidants necessary for the protection against free radical damage. Previous study by Megalli *et al.,* (2005) implicated P407 in causing cellular damage through mechanisms involving lipid peroxidation, oxidative modification of proteins, and disruption of antioxidant enzyme activities invariably support the current findings of this study.

Lipoprotein lipase (LPL) is vital in the metabolism of triglycerides and is implicated in several pathological disorders, including atherosclerosis and obesity (Megalli *et al*., 2005). Low activity of LPL in liver and serum of hyperlipidaemic untreated rats may be due to the inhibitory effect of P407 on the enzyme. Enhancement of LPL activity by agents in fraction 2 of ethylacetate extract of *M. piperita* leaves may be one of the mechanisms by which the plant reduces lipid levels in the rat model. Likely, the bioactive fraction may have activated apolipoprotein CII which is a cofactor of LPL thereby increasing lipolysis as clearly depicted from this study. The enzyme functions by hydrolyzing triglycerides to form free fatty acids and alcohol. Inhibition of LPL by P407 leads to accumulation of triglycerides which leads to hyperlipidaemia paving way for production of free radicals (Megalli *et al*., 2005). This may ultimately bring about oxidative stress thereby negatively affecting enzymatic and non-enzymatic antioxidant systems. Interestingly, fraction 2 of ethylacetate extract of *M. piperita* leaves was able to reverse this metabolic scourge.

Downregulation of *SOD* and *CAT* genes observed in the liver and blood of hyperlipidaemic untreated rats may be due to oxidative stress caused by hyperlipidaemic condition. Up regulation of *SOD* and *CAT* genes in the rats treated with F2 may contribute to the cleavage of free radicals generated in HPL rats thereby mitigating oxidative stress. Similarly, up regulation of LPL gene observed in the treated rats may enhance the activity of the enzyme which may trigger lipolysis thereby leading to the amelioration of hyperlipidaemic condition. These findings are similar with other studies carried out by Sadi and Konat, (2015) and Lim *et al*., (2009)**.** These effects elicited by F2 may also be due to its polar and non-polar constituents, as most of these compounds have been reported with strong biological activities (Jayakumar, 2014; Umarani *et al*., 2015; Ramalingayya, *et al*., 2017).

The compounds revealed from chromatogram of F2 of ethyl acetate extract from *M. piperita* leaves with antihyperlipidaemic and antioxidant activities include oleic acid, cis-vaccenic acid, coumarins, 3,4-di-caffeoylquinic acid and triacontane.

Oleic acid has been proven to cause significant reduction in low density lipoprotein, triacylglycerol with an increase in HDL in rats and also exhibits antioxidant activity *in vivo* (Emekli-Alturfan, *et al*., 2010; Fattore *et al*., 2014; Kassem *et al*., 2014; Liu, *et al*., 2014).Oleic acid was also found to scavenge DPPH radical (Elagbar, *et al*., 2016; Belyagoubi-Benhammou, *et al*., 2018) and also upregulates LPL gene in primary bovine satellite cells isolated from bovine muscles (Belal *et al*., 2018). It modulates the effects of angiopoietin-like protein 4 on LPL by binding tightly to the N-terminal domain of the protein, thereby reducing inhibition of LPL by angiopoietin - like 4 protein (Angptl4) (Robal *et al*., 2012).

Cis vaccenic acid has been favourably implicated in reducing plasma TG and LDL-cholesterol to the level of the normolipidemic lean rats (Pintus et al., 2013). It also has protective effect on structure of CAT and SOD in the hyperglycaemic environment (Mirmiranpour *et al*., 2018). Coumarin analogues and coumarin-3-carboxamides were reported with *in vitro* antioxidant activity (Tasdemir *et al*., 2016). Pre-treatment with coumarin and its derivatives was found to prevent CCl_4 induced hyperlipidaemia in the rats (Tasdemir *et al*., 2016). Coumarin–indole hybrids have been reported to inhibit the biosynthesis of cholesterol and enhanced the activity of lipoprotein lipase **(**Sandhu *et al*., 2014). The Phenolic coumarin (scopoletin) was also found to greatly increase LPL activity in 3T3-L1 adipocytes at the transcriptional level and also showed significant reduction in blood glucose level and lipid level in streptozotocin induced diabetic rats (Yang *et al*., 2007;Verma *et al*., 2013).

Naturally derived coumarins such as esculetin and synthetic coumarins are good antihyperlipidaemic compounds (Sandhu *et al*., 2014). Hybridization of coumarin with an indole moiety, which is present in various synthetic statins yielded coumarin–indole hybrids which significantly decreases the plasma triglycerides (TG), total cholesterol (TC), and increases HDL-C. The synthesized coumarin-chalcone fibrates reverse the triton-induced increased plasma levels of lipid (Sandhu *et al*., 2014). These hybrids inhibited the biosynthesis of cholesterol and enhanced the activity of lipolytic enzyme, lipoprotein lipase, to facilitate early clearance of lipids from circulation in triton-induced hyperlipidaemia (Sandhu *et al*., 2014).

Treatment with coumarin derivatives isolated from *Ionidium suffruticosum* decreased total cholesterol in rats fed with high fat diet (Arumugam and Muthu, 2016). In another study, coumarin isolated from ethanolic extract of *Micromelum minutum seeds* decreased the cholesterol, triglycerides and LDL levels and increased the HDL level in diabetic rats (Koriem *et al*., 2013). Coumarins (xanthotoxin and umbelliferone) isolated from *Salvadora indica* exhibit antihyperlipidaemic activity (Iyer and Patil, 2014).

Caffeoylquinic acid derivatives, isolated from leaves of *L. japonica* showed strong antioxidant activities (Wang *et al*., 2017). Studies have shown that 3, 4 -di-O-caffeoylquinic acid were found to be good scavengers of free radicals (Li *et al*., 2018). Fruits of *P.tectorius* plant contain dicaffeolyquinic acid accounting for the anti-hyperlipidaemic effect of the plant (Liu *et al*., 2013). *Ligularia fischeri* and its major chemical constituent 3, 4-dicaffeoylquinic acid inhibits lipid accumulation by altering lipid metabolism towards fatty acid oxidation through activation of the AMP-activated protein kinase (AMPK) (Randy *et al*., 2016). 3,4-dicaffeoylquinic are also effective antioxidants that protect against oxidation reactions *in vivo* by up-regulating redox-related nuclear transcription factors involved in the expression of antioxidant enzymes

(Liang and Kitts, 2015). *Ilex paraguariensis* which is also composed of 3,4-dicaffeoylquinic acid increased gene expression of *SOD* and *CAT* in the hippocampus and liver tissues of high-fat diet fed rats (Silva *et al*., 2018).

Terpenoids including triacontane also isolated from *Scutellaria* (Lamiaceae) possess antioxidant activities (Xiong *et al.,* 2011; Sripathi and Ravi, 2017). The extracts of Cyperus rotundus rhizomes are rich in triacontane which has shown a potent antioxidant and free radical scavenging activity (Nidugala *et al*., 2015). Triacontane was also found in the extract of *T. bentzone* leaves which exhibited both antioxidant and antihyperlipidaemic properties (El-Rafie *et al.,* 2016). The leaves of *Breynia vitis-idaea* also contains triacontane and has shown antihyperlipidaemic activity (Nagar and Chauhan, 2016).

Isoferrerin (isoflavone) also is one of the chemical constituents of methanolic extract of *Dolichos biflorus* which is known to have antioxidant (Ahmad *et al*., 2014) and antihyperlipidaemic activities (Muthu *et al*., 2005).

Based on the aforementioned reports on these biologically important proposed compounds, it is indeed possible that the compounds may be responsible for the antihyperlipidaemic and antioxidant effects of *M. piperita* leaves observed in this study. It is against this background that it is proposed that hyperlipidaemic condition and redox imbalance caused by administration of P407 led to decrease in LPL, GSH and HDL with increased MDA, CAT, LDL, TG, TC and SOD levels. Contrariwise, bioactive fraction of *M. piperita* leaves extract stimulates LPL, GSH and HDL with simultaneous reduction in MDA, CAT, LDL, TG, TC and SOD levels. At the molecular level, bioactive fraction of *M. piperita* leaves extract stimulated the transcription of *LPL, CAT* and *SOD* genes. Thus, antihyperlipidaemic and antioxidant effect of *M.*

piperita leaves extract could hypothetically be due to synergistic and/or addictive effects of the proposed bioactive compounds in the plant (Figure 4.9).

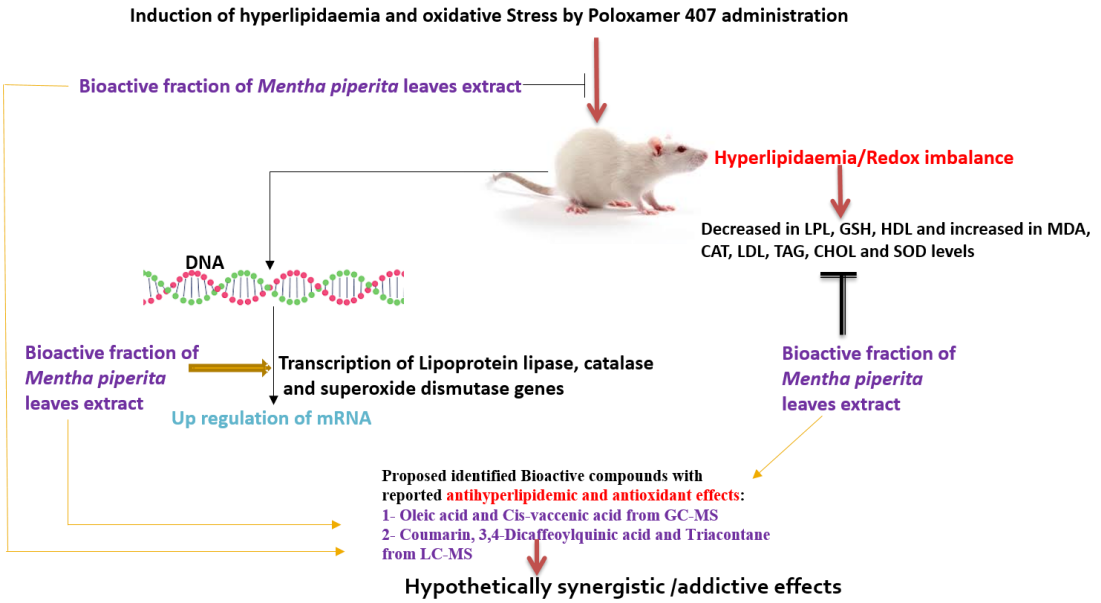


Figure 4.9: Proposed mechanism of action of bioactive fraction of *M. piperita* leaves extract on Poloxamer 407 induced hyperlipidaemia and oxidative Stress

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The outcome of this study can be summarized as follows:

i. Twelve fractions were obtained from most active ethyl acetate extract of *M.piperita* leaves by gradient elution and fraction (F2) determined as the most antihyperlipidaemic fraction.

ii. The extracts of *M.piperita* leaves and the most active fraction (F2) were relatively safe for consumption with an LD_{50} greater than 5000mg/kg body weight.

iii. The extracts of *M.piperita* leaves and F2 were found to be antihyperlipidaemic.

iv. The most active ethylacetate extract and its fractions (2, 3, 4, 6 and 12) were non-genotoxic and exhibited strong antioxidant activities with IC_{50} of ; ethyl acetate (3.0µg/ml), F2 (3.58µg/ml), F4 (15.97µg/ml), F6 (8.13µg/ml) and F12 (2.67 µg/ml).

v. The most potent fraction (F2) ameliorated liver tissue damage induced by P407 *in vivo*.

vi. Administration of fraction 2 reduced level of MDA by 45 and 51% with simultaneous increase in GSH level by 35 and 25% in the liver tissues and serum respectively compared to hyperlipidaemic untreated rats.vii. Fraction 2 significantly ($p<0.05$) enhanced LPL activity in the liver (8.41 U/min/g of tissue) and serum (0.52U/ml) compared with the hyperlipidaemic untreated rats (3.08 U/min/g of tissue, in the liver and 0.28 U/ml for the serum).

viii. Fraction 2 upregulated the gene expression of *LPL*, SOD and CAT in liver and blood relative to hyperlipidaemic untreated rats respectively.

ix. GCMS and LCMS analysis indicates that fraction 2 is rich in polar and non-polar compounds which are oleic acid, cis - vaccenic acid, Coumarin, 3, 4 dicaffeoylquinic acid and Coumarin and these might be responsible for the antihyperlipidaemic and antioxidant effects of *M. piperita* leaves extract observed in this study.

6.2 Conclusion

Bioactive fraction of *Mentha piperita* leaves from ethyl acetate was able to reverse hyperlipidaemia induced by P407 in hyperlipidaemic rats with partial restoration of the integrity of hepatocytes and exhibited very good antioxidant activity.

Mechanistically, this study demonstrates the potentials of most active fraction of *Mentha piperita* leaves in stimulating lipoprotein lipase via augmentation of antioxidant defence system in hyperlipidaemic rats at cellular and molecular levels while underscoring its richness in bioactive polar and non-polar compounds. The proposed bioactive compounds may be responsible for the antihyperlipidaemic, antioxidant and associated characteristics observed after treatment with *M.piperita* leaves in hyperlipidaemic rats.

Overall, ethyl acetate extract of *M. piperita* leaves and its fractions may be explored in the management of hyperlipidaemia and its related diseases.

6.3 Recommendations

i. *M. piperita* leaves are relatively safe to consume as an antihyperlipidaemic agent.

ii. Further studies should be carried out on the antihyperlipidaemic and antioxidant effect of the proposed bioactive components individually, addictively/synergistic action on P407-induced hyperlipidaemic rats.

iii. Effect of fraction 2 on lipoprotein lipase activity in adipose, pancreas and heart tissues of P407-induced hyperlipidaemic rats should also be determined.

CONTRIBUTIONS TO KNOWLEDGE

1. The extracts of *M.piperita* leaves and the most active fraction (F2) were relatively safe for consumption and were found to be antihyperlipidaemic.

2. The most active ethylacetate extract and its fractions (2, 3, 4, 6 and 12) were non-genotoxic with strong antioxidant activity.

3. The most potent fraction (F2) was able to ameliorate liver tissue damage induced by P407 *in vivo*.

4. Administration of fraction 2 reduced level of MDA with simultaneous increase in GSH level in the liver tissues and serum compared to hyperlipidaemic untreated rats.

5. Fraction 2 enhanced LPL activity in the liver and serum when compared with the hyperlipidaemic untreated rats

6. Fraction 2 upregulated the expression of *LPL, CAT* and SOD in liver and blood relative to hyperlipidaemic untreated rats.

7. Fraction 2 is rich in polar and non-polar compounds as indicated by GCMS and LCMS analysis which may be responsible for the antihyperlipidaemic and antioxidant effects of *M. piperita* leaves extract observed in this study.

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APPENDICES

Appendix I: Tables showing rat mortality in phases I and II lethal dose test of hexane, ethylacetate and methanolic Extracts

TABLE 1.1: Rat Mortality in Phase I Lethal Dose Test of N – Hexane Extract

Groups	Number of Rats	Dose (mg/kg)	Mortality
1	3	10	0
2	3	100	0
3	3	1000	0

TABLE 1.2: Rat Mortality in Phase I Lethal Dose Test of Ethylacetate Extract

Groups	Number of Rats	Dose (mg/kg)	Mortality
1	3	10	0
2	3	100	0
3	3	1000	0

TABLE 1.3: Rat Mortality in Phase I Lethal Dose Test of Methanolic Extract

Groups	Number of Rats	Dose (mg/kg)	Mortality
1	3	10	0
2	3	100	0
3	3	1000	0

Tables 1.1,1.2 and 1.3 showed that no death was recorded in the phase I of the acute toxicity study after twenty four hours of administration of 10mg/kg, 100mg/kg and 1000mg/kg of **hexane, ethylacetate and methanolic extracts** of *Mentha piperita leaves* to groups 1, 2 and 3, respectively.

TABLE 1.4: Rat Mortality in Phase II Lethal Dose Test of N- Hexane Extract

Groups	Number of Rats	Dose (mg/kg)	Mortality
1	3	1600	0
2	3	2900	0
3	3	5000	0

TABLE 1.5: Rat Mortality in Phase II Lethal Dose Test of Ethylacetate Extract

Groups	Number of Rats	Dose (mg/kg)	Mortality
1	3	1600	0
2	3	2900	0
3	3	5000	0

TABLE 1.6: Rat Mortality in Phase II Lethal Dose Test of Methanolic Extract

Groups	Number of Rats	Dose (mg/kg)	Mortality
1	3	1600	0
2	3	2900	0
3	3	5000	0

Tables 1.4, 1.5 and 1.6 showed no death recorded in phase II experiment which was carried out in view of the results of phase I. Therefore, hexane, ethylacetate, and methanolic extract of Mentha *piperita* leaves were relatively safe even at 5000 mg/kg body weight of the rats. By implication, the LD_{50} will be > 5000 mg/kg.

Appendix II: Agarose Gel-electrophorensis of Ethylacetate Extract and Antihyperlipidaemic Fractions

Stock solution of 100µg/ml was prepared by dissolving 1g of each fractions /crude extract in 10mls distilled water, and then the IC50 values of the fractions 2,3, 4,6,12,and ethyl acetate (3.58µg/ml, 3.97µg/ml, 15.97µg/ml, 8.13µg/ml, 2.67µg/ml, 3.0µg/ml) respectively that were calculated from the DPPH results was prepared from stock solution (100µg/ml) by serial dilution. The set up was done as shown in the table below

Sample/tube	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Plasmid DNA (µl)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Fraction 2 (µl)	2.5	-	-	-	-	-	-	-	2.5	-	-	-	-	-
Fraction 3 (µl)	-	2.5	-	-	-	-	-	-	-	2.5	-	-	-	-
Fraction 4 (µl)	-	-	2.5	-	-	-	-	-	-	-	2.5	-	-	-
Fraction 6 (µl)	-	-	-	2.5	-	-	-	-	-	-	-	2.5	-	-
Fraction 12 (µl)	-	-	-	-	2.5	-	-	-	-	-	-	-	2.5	-
Ethyl acetate extract (µl)	-	-	-	-	-	2.5	-	-	-	-	-	-	-	2.5
Fentons (µl)	-	-	-	-	-	-	5	-	5	5	5	5	5	5
PBS (µl)	7.0	7.0	7.0	7.0	7.0	7.0	4.5	9.5	2.0	2	2	2	2	2

During the set up addition of the fractions /extract to the plasmid was incubated for 10min at room Temperature (plasmid + fraction/extract). After which Fenton's reagent and phosphate buffer solution (PBS) was added and incubated at 37^{o}C for 30minutes. During the set up the agarose gel was prepared and set for loading samples as follows

The casting tray was rinsed, dried and site in a level surface and the comb was inserted, 1% of the agarose was prepared by dissolving 1g of agarose in 100ml of 1x TAE and melted by storing in 68^{o}C water bath.It was gently swirl to make sure they were no particles left, the melted agarose was cooled down to 55^{o}C and poured inside the casting tray with the inserted comb and the gel was allowed to solidify (for about 10 minute). The comb was then removed and, the casting tray was than inserted into the electrophoresis chamber. Tris-acetate-EDTA (1x TAE) (electrophoresis buffer) was then pour into the chamber until the buffer covers the gel

After the incubation, the sample was removed from the incubation chamber and prepared for loading into the gel by adding the loading dye (3µl) to each sample. The sample was loaded into the electrophoresis and voltage was applied to run the electrophoresis.

The electrophoresis was run until the bromophenol blue has migrated to within ¾ of the positive electrode which took about 30 minutes. The power was shut off to end the electrophoresis.The casting tray was removed from the chamber for photographing were the gel was captured for analysis

Appendix III: Tables showing procedure for determination of lipid profile, protein and standard curves

TABLE 1.7: Procedure for Determination of Cholesterol

The procedure involved pipetting the following into different test tubes

Parameters	Reagent blank (µl)	Standard (µl)	Sample (µl)
Distilled H_2O	10	---	---
Standard	--	10	--
Sample	--	--	10
Reagent	1000	1000	1000

TABLE 1.8: Procedure for HDL Cholesterol Determination

Parameters	Reagent blank (µl)	Standard (µl)	Sample (µl)
Distilled H_2O	50		
HDL supernatant			50
Standard(HDL)		50	
Cholesterol reagent	500	500	500

TABLE 1.9: Procedure for Triglycerides Determination

The procedure involves pipetting the following into different test tubes

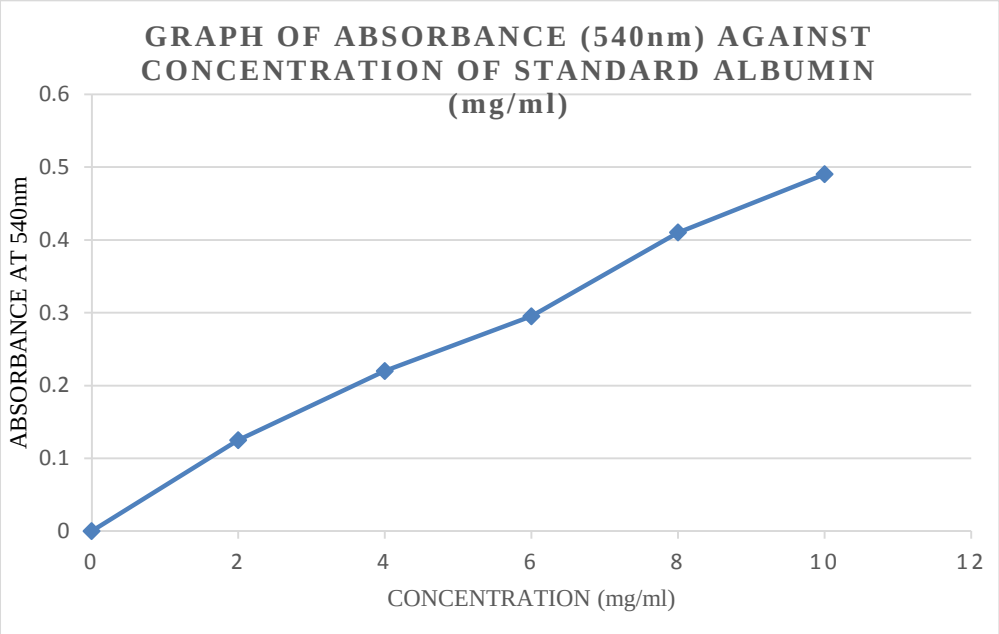
Parameters	Reagent blank (µl)	Standard (µl)	Sample(µl)
Distilled water	10	--	
Standard (CAL)	---	10	---
Reagent (R1)	1000	1000	1000
Sample			10

TABLE 2.0: Procedure for Determination of Protein

Pipette into test tubes:				
Tube Number	Standard Albumin (mg/ml)	Sample (ml)	Distilled Water (ml)	Biuret Reagent (ml)
Blank	0.00	---	1.00	4.00
Tube 1	2	---	0.80	4.00
Tube 2	4	---	0.60	4.00
Tube 3	6	---	0.40	4.00
Tube 4	8	---	0.20	4.00
Tube 5	10	---	0.00	4.00
Tube 6	---	1		4.00

The above solutions were mixed gently by swirling and incubated at room temperature for 30mins. The absorbance of the developed solutions was immediately measured at 540nm against the reagent blank.

CALCULATION: The concentration of total protein in the serum was obtained from a standard curve.



Pipette into test tubes:				
Tube Number	**Standard Albumin (mg/ml)**	**Sample (ml)**	**Distilled Water (ml)**	**Biuret Reagent (ml)**
Blank	0.00	---	1.00	4.00
Tube 1	2	---	0.80	4.00
Tube 2	4	---	0.60	4.00
Tube 3	6	---	0.40	4.00
Tube 4	8	---	0.20	4.00
Tube 5	10	---	0.00	4.00
Tube 6	---	1		4.00

Preparation of GSH Standard Curve

Absorbance (412nm)	GSH Conc. (µg/ml)
0.040	8
0.101	20
0.194	40
0.380	80
0.572	120
0.749	160

Appendix IV: Figures Showing the Effect of Fraction 2 on Lipoprotein Lipase Concentration in the Liver and Serum of Hyperlipidemic Rats

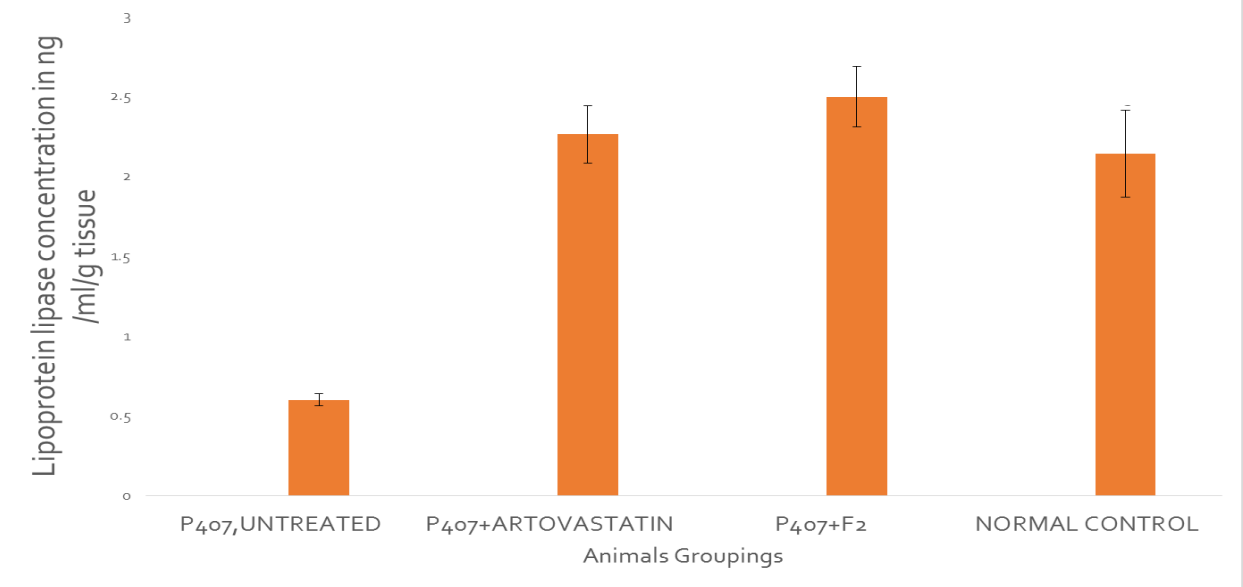


Figure 1: Effect of fraction 2 from Ethyl acetate extract of M. Piperita leaves on lipoprotein lipase concentration of the liver tissues in hyperlipidaemic rats.

Values are expressed as mean value ± S.D (n=5). Values with [a] are statistically significant when compared with the hyperlipidaemic group (group 1) while values with [b] are statistically significant when compared with the normal control group Values with [ab] are statistically significant when compared with both hyperlipidaemic and normal control at $p < 0.05$

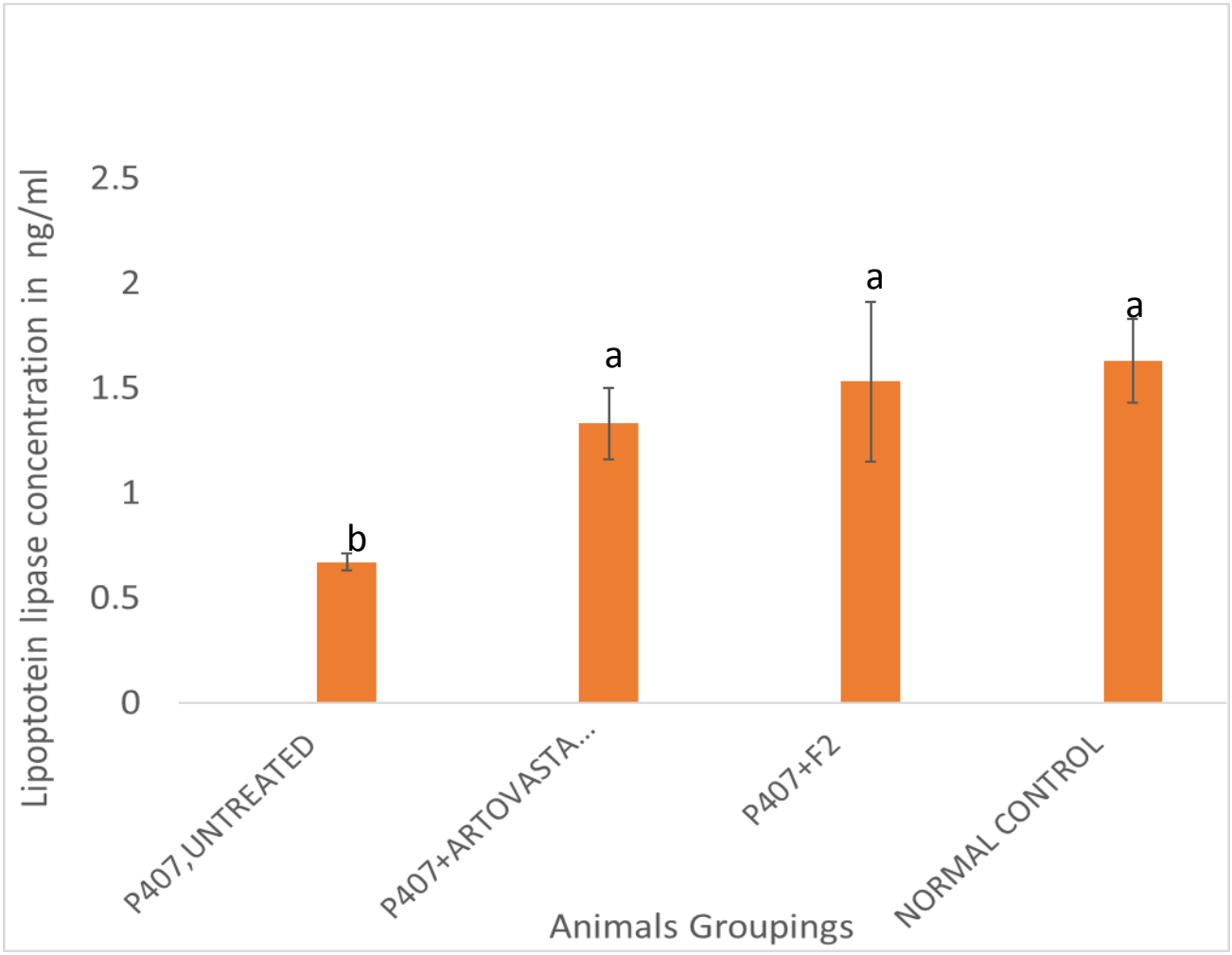


Figure 2: Effect of fraction 2 from Ethyl acetate extract of *M. Piperita leaves* on lipoprotein lipase concentration of the serum in hyperlipidaemic rats

Values are expressed as mean value ± S.D (n=5). Values with [a] are statistically significant when compared with the hyperlipidaemic group (group 1) while values with [b] are statistically significant when compared with the normal control group at $p < 0.05$.

Appendix V: Reagents for Determination of Lipid Profile and Antioxidant Enzymes

The reagents for cholesterol determination included the following;

Calibrator	**Standard**	119.5 mg/dl
R1 reagent	4-Aminoantipyrine	0.30 mmol / l
	Phenol	6 mmol/l
	Peroxidase	0.5 U/ml
	Cholesterol oxidase	$\geq$ 0.1 U/ml
	Pipes Buffer	80 mmol/l: pH 6.8

The reagents for HDL determination include

Reagent	**Concentration**
R1 Phosphotungsticc acid	0.55 mmol/l
Magnessium Chloride	25 mmol/l

Reagents for triglycerides assay

R1a. Buffer	**Concentrations**
Pipes buffer	40 mmol/l; pH 7.6
4-chloro-phenol	5.5 mmol/l
Magnessium ions	17.5 mmol/l
R1b. Enzyme reagent	
4 aminophenazone	0.5 mmol/l
ATP	1.0 mmol/l
Lipases	$\geq$ 150 U/ml

Glycerol-kinase	≥ 0.4 U/ml
Glycerol-3-phosphate oxidase	≥ 1.5 U/ml
Peroxidase	≥ 0.5 U/ml

Reagents for measuring catalase activity

- Potassium phosphate buffer (50 mM, Ph 7.0)
  Solution A: 8.90g of $Na_2HPO_4.2H_2O$ dissolved in distilled water and volume made to 1 litre.
  Solution B: 6.81g of K_2HPO_4 dissolved in distilled water and volume made to one litre. To solution A solution B was added until pH reached to 7.0.
- H_2O_2 (30mM): 340µl of 30 % H_2O_2 was diluted to 100 ml with potassium phosphate buffer. The solution was made fresh before use.

Reagents for Lipid peroxidation determination (MDA)

- 5% Trichloro acetic acid (TCA)
  5.0 g of TCA was dissolved in 100 ml of distilled water.
- Hydrochloric acid (HCl: 0.25N)
  1.08ml of 35.4% HCl was diluted with 49ml of double distilled water.

- Thiobarbutaric acid (TBA: 0.375%)
  0.375 g of TBA was dissolved in 100 ml hot distilled water.
  All three reagents were mixed in 1:1:1 ratio.

Reagents for determining reduced Glutathione (GSH).

0.2M phosphate buffer: 8.40g of NaH_2PO4 and 9.94g of Na_2HPO4 were dissolved in distilled water and made up to 1000ml in a volumetric flask. The buffer was adjusted to pH 8.0.

10% Trichloroacetic acid (TCA): 10g of TCA was dissolved in distilled water and make up to 100ml mark in a volumetric flask.

Ellman's reagent: 19.8mg of 5, 5-dithiobis nitro benzoic acid (DTNB) in 100ml of 0.1% sodium nitrate.

Reagents for determination of Superoxide Dismutase Activity

0.05M carbonate buffer: 114.3g of Na_2CO3 and 4.2g of $NaHCO_3$ was dissolved in distilled water and was made up to 1000ml in a volumetric flask. The buffer was adjusted to pH 10.2.

0.3mM Adrenaline: 0.01g of adrenaline was dissolved in 17ml of distilled water, the solution was prepared fresh.

Appendix VI: Possible compounds present in fraction 2 identified by GCMS and LCMS with fragmentation patterns

Table 4: Proposed Compounds Present in Fraction 2 Identified by GCMS

S.NO	COMPOUND NAMES	SIMILARITY INDEX
1	Undec-10-ynoic acid, dodecyl ester	50
2	1,6-Cyclodecadiene	60
3	1,5-Cyclodecadiene,	53
4	9-Octadecenal	52
5	14-Methyl-8-hexadecen- 1-ol	52
6	13-Octadecenal,	53
7	Undec-10-ynoic acid, undecyl ester	83
8	12-Methyl-2,13-octadecadien-1-ol	58
9	Cyclopropaneoctanal, 2-octyl	89
10	2-Methyl-3,13-octadecadienol	70
11	9,12-Octadecadienal	50
12	Oleic Acid	89
13	5-Eicosene	53
14	3-Eicosene	53
15	Palmitoleic acid	53
16	cis-13-Octadecenoic acid	51
17	cis-Vaccenic acid	80
18	2-Methyl-3,13-octadecadien-1-ol	70
19	Oleic Acid	95
20	9-Oxabicyclo[6.1.0]nonane	86
21	2-Methyl-3,13-octadecadienol	70
22	Oleic Acid	64
24	9-Eicosenoic acid	55
25	9-Octadecenal	70
27	2-Octadecen-1-ol	64

Table 5: Proposed compounds Present in Fraction 2 Identified by LCMS

Mass	Compound name	Relative abundance (%)
208	Coumarin	18.69%
297	Isoeugenitin	3.74%
379	Isoferreirin	37.38%
593	3,4-Dicaffeoylquinic acid	9.35%
635	Dihydromorelloflavone	2.8%
873	N-Triacontane	28.03%

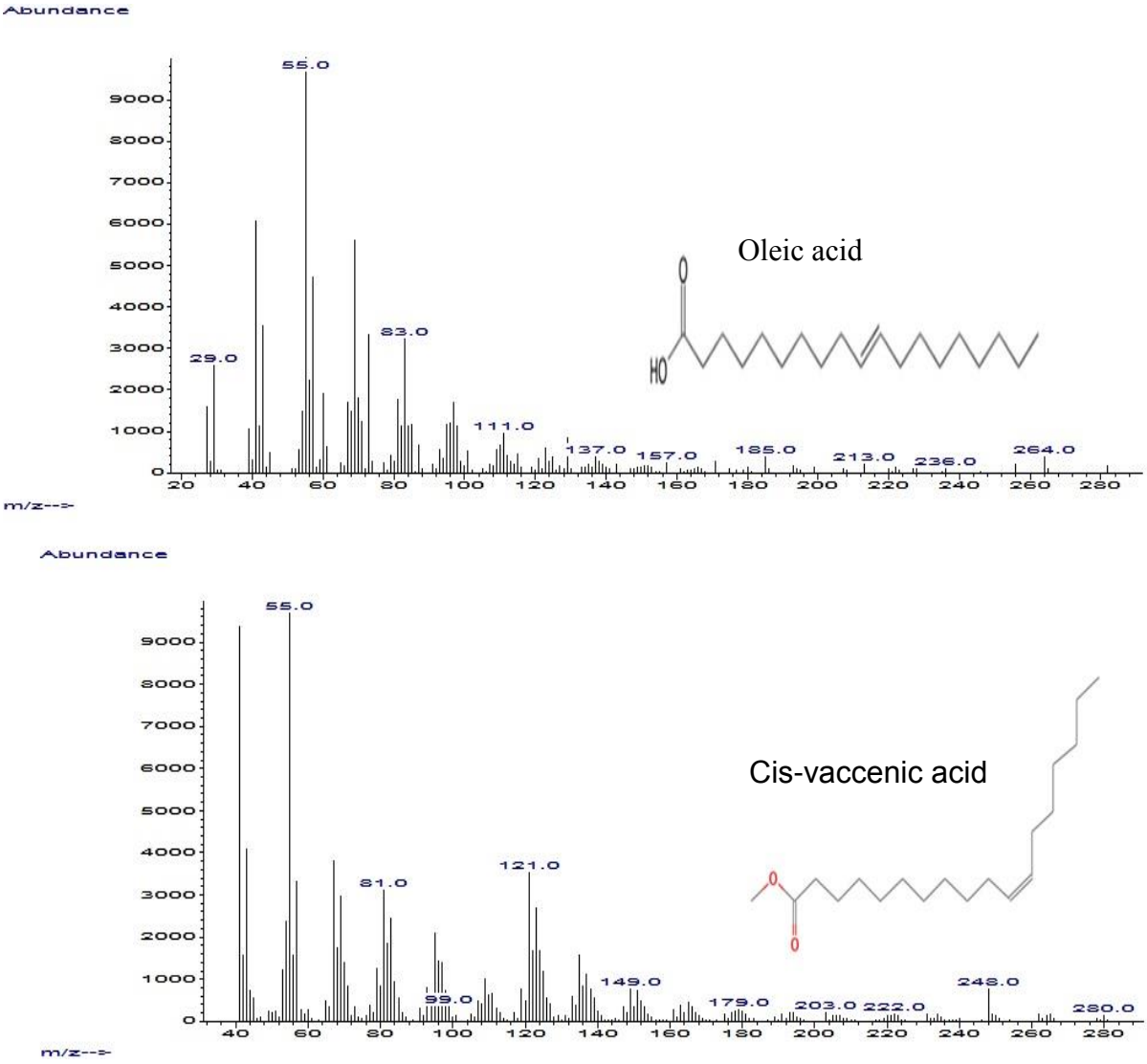


Figure 3: Fragmentation Pattern of Oleic and Cis Vaccenic acid by GCMS

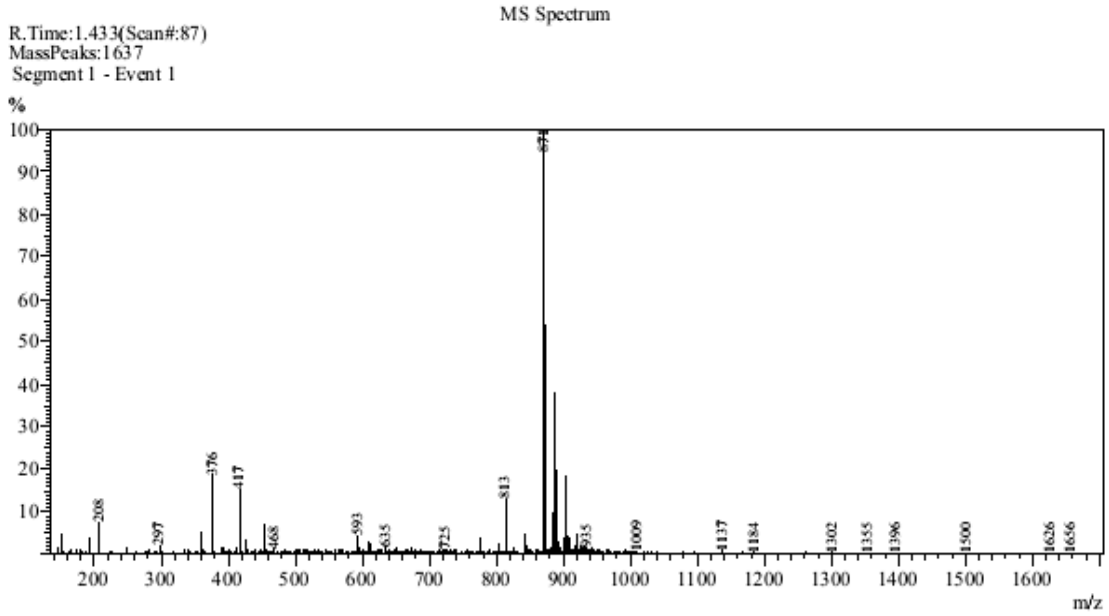


Figure 4: Fragmentation Pattern of Fraction 2 by LCMS