Anti-hyperlipidemic activities of *Ilex latifolia* and other selected herbs

A thesis submitted in partial fulfilment of the requirements of London Metropolitan University for the degree of Doctor of Philosophy

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Abstract

Hyperlipidemia is defined by abnormally elevated levels of one or more lipids such as cholesterol or triglycerides in the bloodstream. It also involves elevated levels of lipoproteins especially LDL-cholesterol and this is the most common form of dyslipidemia. Hyperlipidemia is the result of complex interactions between environmental and genetic factors. Hyperlipidemia is the main cause of congestive heart diseases in adulthood. It is also the main cause of atherosclerosis which is the pathophysiological cause of vascular diseases such as angina pectoris, myocardial infarction, and stroke. It occurs due to disorders in lipid metabolism including elevation in cholesterol, low density lipoprotein, or triglyceride, or due to low levels of high density lipoprotein. Lifestyle is the main factor in prevention of hyperlipidemia. If lifestyle is not beneficial in the treatment or prevention of hyperlipidemia, drug therapy is required. Statins, fibrates, nicotinic acid bile acid sequestrants, and ezetimibe are approved drugs for the treatment of hyperlipidemia. Because of the tolerability problems, side effects, and low effectiveness of some of these drugs, discovery of new drugs is explored and investigated. Herbal products are not well explored as an alternative treatment for the treatment of hyperlipidemia. *Ilex latifolia* is a traditional Chinese medicine which has been used for decades for the treatment of hyperlipidemia and for weight loss. *Prunella vulgaris, Rheum palmatum*, and *Panax notoginseng* have been also used in traditional Chinese medicine. Very little work has been published about the effect of *Ilex latifolia* on hyperlipidemia. In this research project, *Ilex latifolia* hypolipidemic effect was compared to several herbs effects by assaying the effect of the extracts on pancreatic lipase. *Ilex latifolia* was the most active, and the other parts of the project focused on its effect on HMG-CoA reductase expression, and on LDL receptor expression in HepG2 and AML-12 hepatocytes. *Ilex latifolia* effect on mitochondrial metabolism, and glucose uptake was assayed in HepG2 and AML-12 hepatocytes.

Chapter I is an introduction to hyperlipidemia, its types, causes, and treatment. A review of the literature relating to *Ilex latifolia* and other herbs and herbal compounds is given.

Chapter II describes the assay of extracts from several herbs, and herbal compounds, for their inhibitory effect on pancreatic lipase, using orlistat as a positive control. It also shows that HPLC is

more sensitive and accurate than a spectrophotometric assay of lipase. *Ilex latifolia* showed the most inhibition activity on pancreatic lipase compared to other herbs.

Chapter III describes the fractionation of *Ilex latifolia* and pancreatic lipase inhibition activity of each fraction.

Chapter IV describes the effect of *Ilex latifolia* extract on HMG-CoA reductase expression in HepG2 cells and the effect of *Ilex latifolia* extract on LDL receptors expression in HepG2 and AML-12 cells.

Chapter V describes the effect of *Ilex latifolia* extracts on cell growth, mitochondrial toxicity, and glucose uptake in HepG2 and AML-12 cells. It also describes the effect of *Ilex latifolia* extract on ATP production in HepG2 cells.

This study has demonstrated for the first time that *Ilex latifolia* can play a role in the treatment of hyperlipidemia through pancreatic lipase inhibition. Effects on HMG-CoA reductase inhibition and mitochondrial inhibition were not marked, but under some circumstances glucose uptake can be significantly affected. It remains to be seen, using animal studies and other cell culture models, whether inhibition of pancreatic lipase can wholly account for the hypolipidemic activity of *Ilex latifolia*, or whether other mechanisms may be involved.

Acknowledgement

There are many people to whom I would like to express my deepest gratitude for their considerable help along this journey but first of all, I would like to thank my supervisor Dr. Kenneth White for his guidance, suggestions, discussions, and patience throughout the project. I would like to thank Professor S.W. Annie Bligh and Dr. Eileen Smith for their help in the first part of the project. I would like also to thank all London Metropolitan University technicians for their support.

Dedication

I dedicate this work to my family who supported and helped me all the time.

Declaration

I declare that whilst studying for Doctorate in Biology/Biochemistry at London Metropolitan University, I have not been registered for any other award at another university. The work undertaken for this degree had not been submitted elsewhere for any other award. The work contained within this submission is my own work and, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgement has been made in the text.

Ali Hassan Saleh

August 2016

Abbreviations and symbols

<	Less than
>	Greater than
ºC	Degrees Celsius
v/v	Volume per volume
$\lambda_{(max)}$	Maximum wavelength
µg	Microgram
µM	Micromolar
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
Asp	Aspargine
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C	Control
CHD	Congestive heart disease
CL	Control samples lysed with buffer containing leupeptin
cm	Centimeter
CMC	Critical micelle concentration
CN	Control samples lysed with buffer containing np-40
CQA	Caffeoylquinic acids
D-FCS	Dialysed foetal calf serum

DMEM	Dulbecco’s modified eagle medium
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
ECACC	The European Collection of Authenticated Cell Cultures
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
F	Fraction
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FCS	Foetal calf serum
FDA	US food and drug administration
HDL	High density lipoprotein
His	Histidine
HMBC	Heteronuclear Multiple Bond Correlation
HMGCR	3-Hydroxy-3-methylglutaryl CoA reductase
HPLC	High performance liquid chromatography
Hr	Hour

HSQC	Heteronuclear Single Quantum Coherence
I.D	Internal diameter
IL	*Ilex latifolia*
IMS	Industrial methylated spirit
ITS	Insulin, Transferrin, Sodium selenite
kDa	Kilo dalton
LCAT	Lecithin cholesterol acyl transferase
LC-MS	Liquid chromatography-mass spectroscopy
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein-cholesterol
LDLR	Low density lipoprotein receptor
mA	Milliampere
Min	Minutes
mL	Milliliter
mM	Millimolar
MTP	Microsomal triglycerides transfer protein
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5, diphenyltetrazolium bromide
MVL	Mevalonolactone
$NADP^+$	Nicotinamide adenine diphosphate oxidised form
NADPH	Nicotinamide adenine diphosphate reduced form
nm	Nanometer

NP	Nonidet-P
OPI	Oxidative phosphorylation inhibition
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffer saline
Pcsk9	Proprotein convertase subtilisin/kexin type 9
PMSF	Phenylmethylsulfonyl floride
PV	*Prunella vulgaris*
PX	*Panax notogensing*
Q	Quercetin
Ripa	Radioimmunoprecipitation assay
RP	*Rheum palmatum*
S	Simvastatin
SDS	Sodium dodecyl sulphate
Ser	Serine
SL	Cells treated with simvastatin and lysed with a buffer containing leupeptin
SN	Cells treated with simvastatin and lysed with a buffer containing np-40
TEMED	Tetramethylethylenediamine
TGF_{α}	Transforming growth factor alpha
THL	Tetrahydrolipstatin
TTB	Towbin transfer buffer
VLDL	Very low density lipoprotein

UV1	Ultraviolet

Table of Contents

List of figures

List of Tables

1 Chapter I: Introduction

1.1 Introduction

1.1.1 Hyperlipidemia

Hyperlipidemia is defined by abnormally elevated levels of one or more lipids such as cholesterol or triglycerides in the bloodstream. It also involves elevated levels of lipoproteins especially LDL-cholesterol and this is the most common forms of dyslipidemia which comprises a triad of decreased levels of high density lipoprotein (HDL), increased levels of low density lipoprotein (LDL), and elevated levels of triglycerides (Musunuru 2010). Hyperlipidemia is the result of complex interactions between environmental and genetic factors (Haffner, 1999). Hyperlipidemia is the main cause of congestive heart diseases in adulthood. It is also the main cause of atherosclerosis which is the pathophysiological cause of vascular diseases such as angina pectoris, myocardial infarction, and stroke (Klag *et al*., 1993). It occurs due to disorders in lipid metabolism including elevation in cholesterol, low density lipoprotein, or triglyceride, or due to low levels of high density lipoprotein (Laskarzewski *et al*., 1982). Hyperlipidemia is a medical condition associated with a high rate of morbidity and early mortality (Vanitallie, 1985).

Hyperlipidemia is divided into primary and secondary hyperlipidemia (Fredrickson *et al*., 1967). Primary hyperlipidemias have a genetic basis and four types have been identified, which are familial hypercholesterolemia, familial combined hyperlipidemia, familial defective apoprotein B, and familial hyper triglyceridaemia.

Familial hypercholesterolemia occurs due to mutations in the low density lipoprotein lipase receptor, and to a lesser extent, rare mutations in proprotein convertase subtilisin/kexin type 9 (PCSK9) gene and in the lipoprotein B (Apo B)(Leigh *et al*., 2008). Familial hypercholesterolemia is an autosomal co-dominant disorder characterised by elevated levels of LDL-C and a predisposition to coronary artery diseases (Soutar and Naoumova, 2007).

Familial hypercholesterolemia occurs 1 in 500 in the US, Canada, and Europe (Eisenberg *et al*., 1984).

Familial combined hyperlipidemia occurs in 1-2 % of the general adult population (Grundy *et al*., 1987) and is the most common genetic lipid disorder, accounting for at least 20% of coronary events in males under the age of 60 (Zambon *et al*., 2006). Identifying the genetic defects underlying familial combined hyperlipidemia are still a challenge despite its familial nature. Recent data (Minicocci *et al*., 2015) suggest a more complex mode of inheritance although familial combined hyperlipidemia was originally described as an autosomal dominant disorder (Goldstein *et al*., 1973). LDL could be acceptable, borderline, or high but triglycerides levels are high (Cortner *et al*., 1990).

Familial defective Apolipoprotein B occurs due to base substitution in the Apo-B gene that reduces the ability of LDL-C to bind to LDL-C receptors. Moderate to severe hypercholesterolemia occurs in familial defective Apo-B (Cefalu *et al*., 2001).

Hypertriglyceridemia can be classified as primary or secondary. Primary hypertriglyceridemia is caused by genetic defects in triglyceride synthesis and metabolism. Secondary triglyceridemia may be due to diabetes, obesity, metabolic syndrome , and medications such as anti-psychotics and estrogens (Expert Panel on Integrated Guidelines for Cardiovascular *et al*., 2011).

Familial hypertriglyceridemia is mainly expressed in adulthood and obesity is the main outcome. Triglycerides are moderately elevated (100-200mg/dL) in youths and become extremely elevated later in life.

Secondary hyperlipidemia occurs as a consequence of various conditions including obstructive liver disease, various hepatopoeitic diseases, chronic renal failure, myxedema, pancreatitis, and drugs like estrogens, corticosteroids, and retinoids (Chait and Brunzell, 1990).

1.1.2 Cholesterol

Cholesterol is a fat-like substance which is not only an essential component of cells' cytoplasmic membrane system but is also important for the synthesis of sterols, bile acids, and vitamin D.

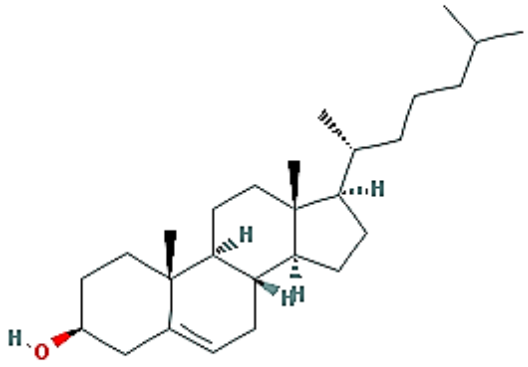

Figure 1-1: Structure of cholesterol (Song *et al*., 2014)

Lipoproteins are the carriers of cholesterol travelling in the blood. Cholesterol is synthesized via the mevalonate pathway (Figure 1-2) (Hanukoglu, 1992).

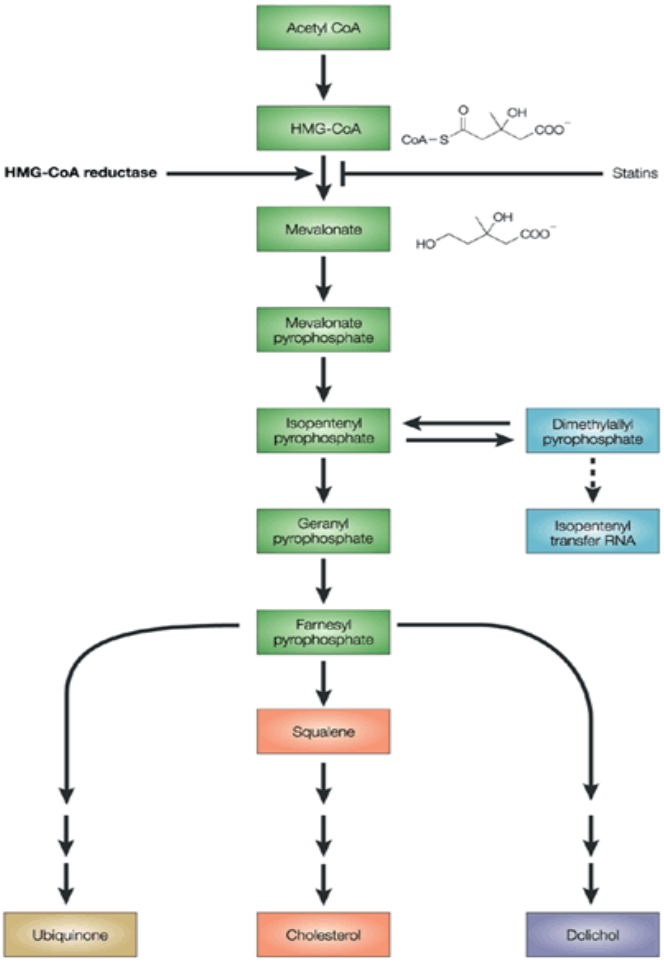

Figure 1-2: Cholesterol synthesis via the mevalonate pathway (Buhaescu and Izzedine, 2007).

1.1.3 Lipoproteins

Cholesterol, triglycerides, and phospholipids are the main components of the blood lipids. They flow through the blood vessels as mixtures or complexes known as lipoproteins. Chylomicrons, low density lipoprotein, very low density lipoprotein and high density lipoprotein are the principal plasma lipoproteins. All these complexes contain a protein component known as an apo-lipoprotein, or apo-protein.

1.1.3.1 Low density lipoprotein

Low density lipoprotein contains up to 60 -70% of the total serum cholesterol. Apoprotein B (apo-100) is the only apo-lipoprotein present in LDL. LDL is identified by the National Cholesterol Education Program of the USA (NCEP) as the primary target for cholesterol lowering therapy because it is the major atherogenic lipoprotein. The optimal LDL cholesterol

concentration is < 100 mg/dL. LDL cholesterol levels above 100mg/dL are potentially atherogenic, and are graded as follows: 100-129 mg/dL is near optimal to above optimal; 130-159 mg/dL is borderline high; 160-189 mg/dL and >190 mg/dL are high and very high respectively (Expert Panel on Detection *et al*., 2001). Clinical trials validated the focus on LDL by showing that LDL lowering therapy is efficient for reducing CHD risk (Baigent *et al*., 2005). Low levels of LDL cholesterol are not harmful (Brown and Goldstein, 1986). Animals with low levels of LDL (80mg/dL) do not have atherosclerosis. Newborn infants have an LDL cholesterol concentration of 30 mg/dL. This indicates that such low levels are safe. Similarly longevity has been documented in people because they have low levels of LDL throughout life, due to familial hypobetalipoproteinemia (Glueck *et al*., 1976).

Several large clinical trials, including the Framingham Heart Study (Wilson *et al*., 1980), the Multiple Risk Factor Intervention Trial (Stamler *et al*., 1986), and the lipid research clinics trial (Expert Panel on Detection *et al*., 2001) assessed the relationship between LDL cholesterol levels and the rate of onset of CHD, and provide compelling evidence that elevated LDL cholesterol plays a role in the development of acute coronary syndromes.

1.1.3.2 High density lipoprotein

HDL is regarded as “good” cholesterol and contains 20-30% of the total serum cholesterol. HDL contains two apo-lipoproteins Apo-AI and Apo-AII. The incidence of CHD is inversely related to HDL levels and high levels of HDL can protect against the development of atherosclerosis. Some studies indicate that low levels of HDL reflect the presence of other atherogenic factors. According to some epidemiological studies, low levels of HDL cholesterol are linked to increased CHD morbidity and mortality (Wilson *et al.*, 1998). When HDL levels are decreased by 1%, CHD risk is increased by 2-3% (Gordon *et al*., 1989). In fact, prospective studies proved HDL to be the key lipid risk factor correlated with CHD risk (Wilson *et al*., 1998). The main role that HDL plays is to collect excess cholesterol from the peripheral tissues, such as lipid-laden foam cells, and transport it to the liver for excretion in the bile (Brewer, 2004, Singh, 2007). In addition to reverse cholesterol transport, HDL shows

various activities such as antioxidant, anti-coagulation, anti-inflammatory, and vascular endothelial function improving effects. These effects may be involved in the prevention of atherosclerosis progression (Assmann and Gotto, 2004, Ansell *et al*., 2006). Researches on laboratory animals show a direct relation while researches on genetically modified animals show that high levels of HDL protect against atherogenesis (Rubin *et al*., 1991). HDL cholesterol levels could be low due to several factors including elevated triglycerides, overweight and obesity, physical inactivity, cigarette smoking, very high carbohydrate intake, type 2 diabetes, certain drugs including beta-blockers, anabolic steroids, and genetic factors (Stone, 1994).

1.1.3.3 Very low density lipoprotein

Although VLDL are triglyceride-rich lipo-proteins, VLDL contain 10-15% of the total serum cholesterol. Apo-B100, Apo-Cs(I,II,III), and Apo-E are the major Apo-lipoproteins of VLDL. VLDL are LDL precursors are produced by the liver. VLDL remnants are formed by the hydrolysis of VLDL triglycerides by lipoprotein lipase present on the surface of cells of tissues that oxidise or store free fatty acids. This allows the delivery of free fatty acids to the heart, skeletal muscles and adipose tissues (Hodis, 1999). VLDL remnants are rich in cholesterol esters and particularly appear to promote atherosclerosis similarly to LDL (Gibbons *et al*., 2004).

1.1.3.4 Chylomicrons

Chylomicrons are the fourth class of lipoproteins. They are triglyceride-rich lipoproteins formed in the intestine from dietary fat and they appear in the blood after a fat enriched meal. Apo-B48, Apo Cs (I,II,III), and Apo-E are the main apo-lipoproteins present in the chylomicrons. Like VLDL, chylomicron remnants are formed due to hydrolysis of triglycerides by lipoprotein lipase, allowing the delivery of free fatty acids to muscles and adipose tissues (Devaraj *et al*., 1998). The partially degraded chylomicrons have some atherogenic potential (Hussain, 2000).

Table 1-1: Classification of normal and abnormal levels of lipids (Expert Panel on Detection *et al*., 2001)

Lipid	Level mg/dL	Classification
Total cholesterol	< 200	Desirable
	200-239	Borderline high
	≥ 240	High
LDL-cholesterol	<100	Optimal
	100-129	Near optimal/above optimal
	130-159	Borderline high
	160-189	High
	≥ 190	Very high
HDL-cholesterol	<40	Low
	≥60	High
Triglycerides	< 150	Normal
	150-199	Borderline high
	200-249	High
	≥ 500	Very high

1.1.3.5 Transport of lipoproteins

Lipoproteins are transported in the body through endogenous and exogenous pathways, illustrated in Figure 1-3. Exogenous transportation is from ingested food to intestine and to peripheral cells and liver. Cholesterol and triglycerides which are products of fat digestion from the gut are packaged with the intestinal Apo-B48 to form nascent chylomicrons. Microsomal triglyceride transfer protein (MTP) mediates the former process (Olofsson *et al.*, 2000). Cholesterol esters, Apo-C, and Apo-E from HDL are required by nascent chylomicrons to form chylomicrons in the circulation. Lipoprotein lipase located on the luminal surface of vascular endothelium of skeletal muscles and adipose tissue breaks down the triglyceride component of chylomicrons into free fatty acids and monoglycerides to convert chylomicrons to chylomicron remnants. The remnants are rich in cholesterol and are taken up by the liver via LDL receptor-like protein receptors or Apo-E receptors (Cooper, 1997). This process ends with dietary cholesterol reaching the liver.

Endogenous transport occurs in two directions. In the first direction, endogenous transport occurs from the liver to peripheral tissues via the Apo-B100 lipoprotein system. In the liver complexes of triglycerides and Apo-B100 are formed by the action of microsomal triglyceride

transfer protein (Olofsson *et al*., 2000) to synthesize and secrete nascent VLDL. Nascent VLDL which is rich in triglycerides serve as an efficient acceptor of cholesteryl esters from HDL. Cholesteryl ester transfer protein mediates this transfer and leads to the formation of mature VLDL (Barter *et al*., 2003). Lipoprotein lipase hydrolyses VLDL triglycerides to free fatty acids and monoglycerides converting VLDL to smaller lipoproteins called intermediate density lipoproteins (IDL) and to further smaller cholesteryl esters rich LDL. As a result, LDL supplies tissues with cholesterol. Thus the liver serves as the major site for both cholesterol synthesis and LDL catabolism.

In the second direction, endogenous transport occurs from the peripheral tissues to liver via the Apo-A1 lipo-protein system. Apo-lipoprotein phospholipids complexes synthesize nascent HDL particles in the plasma, the intestine, and the liver. The peripheral tissues and the liver transfer unesterified cholesterol to nascent HDL by the membrane protein ATP-binding cassette protein A1 (Attie *et al.*, 2001). Unesterified cholesterol is esterified by lecithin-cholesterol acyl transferase which is present on the HDL to form cholesteryl esters. Triglycerides present in VLDL and chylomicrons are transferred to nascent HDL to form mature HDL which is taken by hepatocytes via the scavenger receptor class BI (Krieger, 2001). Reverse cholesterol transport is the process by which excess cholesterol is transferred from the tissues back to the liver via HDL.

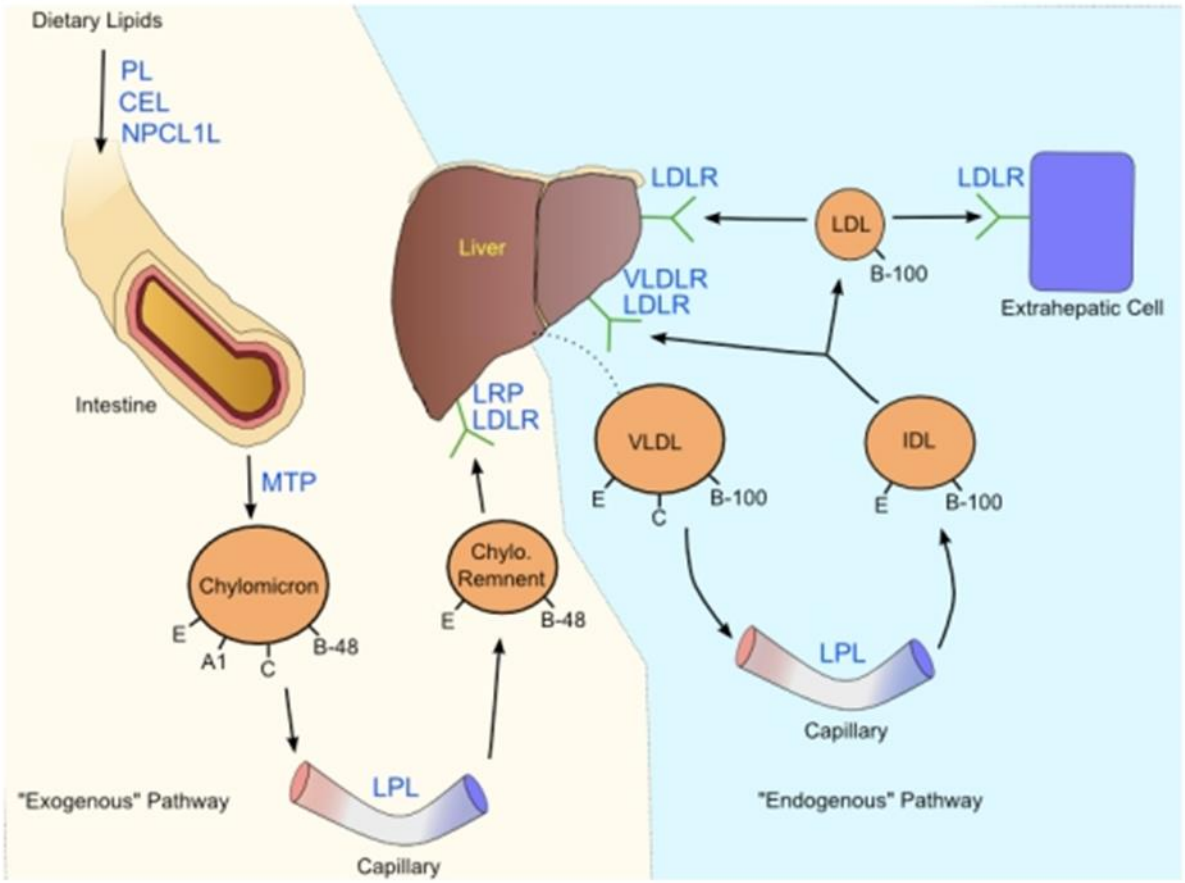

Figure 1-3: Endogenous and exogenous transport of lipoproteins (Daniels *et al*., 2009).

1.1.4 Triglycerides

Triglycerides are one of the blood lipid components and are present in the VLDL and the chylomicrons. A relationship between high levels of serum triglycerides and incidence of CHD has been reported in some epidemiological studies (Austin *et al*., 1998). Serum triglycerides are elevated due to several factors (Stone, 1994) including overweight and obesity, physical inactivity, cigarette smoking, excess alcohol intake, very high carbohydrate diets, diseases such as type 2 diabetes, chronic renal failure, or nephritic syndrome, certain drugs such as corticosteroids, protease inhibitors, beta-blockers, and estrogens, and genetic factors.

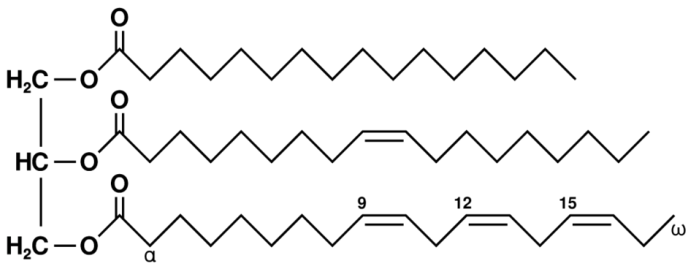

Figure 1-4: Structure of triglycerides (Lopez-Lopez *et al*., 2001)

1.1.5 Enzymes modulating lipoproteins

The metabolism of lipids ingested in the form of dietary fats is initiated in the intestine by the activity of intestinal enzymes. Around the body other key enzymes involved in the metabolism of lipoproteins and their contents, and in formation of remnants such as chylomicron remnants (Subramanian *et al*., 2003) are described below.

Lipoprotein lipase: is expressed on the surface of heart, skeletal muscle and white and brown adipose tissues. It is also located on the endothelial lining of arteries and capillaries and catalyzes the hydrolysis of lipids on lipoprotein particles such as chylomicrons and VLDL and turns them into smaller remnants that are rapidly cleared from the blood stream via the action of LDL-receptors (Olivercrona and Bengtsson-Olivecrona, 1987). The fatty acids released from the chylomicrons and VLDL are taken up by the tissues for energy metabolism or for storage.

Hepatic triglyceride lipase: is one of the hepatic endothelial cell enzymes and can hydrolyse triglycerides and probably phospholipids in VLDL remnants leading to efficient uptake of these particles and generation of LDL (Jin *et al*., 2002). It is also involved in the reverse cholesterol transport by mediating the conversion of triglycerides rich HDL to glyceride poor HDL resulting in the release of phospholipids, glycerol, and fatty acids which can be taken by the liver (Hill and McQueen, 1997).

Lecithin cholesterol acyl transferase (LCAT): is synthesized in the liver and secreted into plasma where it catalyzes, on the surface of HDLs and LDLs, the transfer of fatty acids from the C-2 position of the glycerol of phosphatidylcholine (lecithin), to the C-3 hydroxyl of cholesterol resulting in the formation of cholesteryl esters and lysolecithin. LCAT has a higher affinity for HDLs than LDLs, and the cholesterol esters move to the core of HDL making HDL particles able to acquire more cholesterol from other lipoproteins and cell membranes. LCAT is activated by Apo-A1 present on the surface of HDLs and ApoB on the surface of LDLs (Peelman *et al*., 2000).

Cholesterol ester transfer protein: transfers HDL cholesterol esters to VLDLs and LDLs. As a result, the excess cellular cholesterol is returned to the liver through the LDL receptor pathway as well as the HDL receptor pathway (Brewer, 2004).

Microsomal triglyceride protein: transfers triglycerides, cholesteryl esters, and phosphatidyl choline between membranes and lipoproteins (Olofsson *et al*., 1999).

Acyl CoA transferase: is bound to endoplasmic reticulum and catalyzes the formation of cholesteryl esters from cholesterol in a wide variety of cells. As a result, cholesteryl esters are stored as cytoplasmic storage droplets or in lipoprotein secreting cells (Chang *et al*., 1993).

Pancreatic lipase: also called triacylglycerol hydrolase, it is the principal lipolytic enzyme synthesized and secreted by the pancreas. It plays a key role in the efficient digestion of triglycerides. It catalyzes the removal of α and α' fatty acids of dietary triglycerides resulting in the formation of β-mono-glycerides and long-chain saturated and polyunsaturated fatty acids (Shi and Burn, 2004).

HMG-CoA reductase: is the rate-limiting enzyme of cholesterol biosynthesis (Brown and Goldstein, 1980), and is a 97 kDa transmembrane glycoprotein that resides in the endoplasmic reticulum of animal cells (Chin *et al*., 1982, Chin *et al*., 1984, Liscum *et al*., 1983). HMG-CoA reductase catalyzes the biosynthesis of isoprenoids, producing farnesyl

and geranylgeranyl pyrophosphates which are intermediates for the production of cholesterol and dolichol (Elson *et al*., 1999). HMG-CoA reductase catalyzes the reaction HMG-CoA + 2NADPH + $2H^+$ leading to mevalonic acid + $2NADP^+$ + CoASH. Inhibition of HMG-CoA reductase results in reduction of serum cholesterol. This is correlated with reductions in atherosclerosis and coronary heart diseases (Oates *et al*., 1988), and is a major therapeutic strategy in the treatment of heart disease.

1.1.6 Treatment of hyperlipidemia

Hyperlipidemia is a common risk factor for cardiovascular diseases which is the leading cause of mortality in the United States where 53.4% of the United States adults having abnormal cholesterol values and 32% having elevated levels of low density lipoprotein lipase cholesterol (Roger *et al*., 2011). US, UK, and Canada guidelines agree that therapeutic lifestyle changes are the main factor in hyperlipidemia management, and that LDL-cholesterol should be the primary target of therapy (Grundy *et al*., 2004, Robson, 2008, Genest *et al*., 2009).

1.1.6.1 Lifestyle and non-pharmacologic treatment

Dietary advice plays an important role in normalizing abnormal serum lipids in those who are at high risk of cardiovascular disease. Reductions in serum cholesterol levels of 3-6% are expected with diet. An early meta-analysis of 27 trials indicated that lipid levels are also improved by replacement of saturated fats with unsaturated fats (Mensink and Katan, 1992). Physical activity with associated weight loss plays an important role in controlling mixed dyslipidaemia because physical activity raises HDL-cholesterol levels and reduces very low density lipoprotein (VLDL) (Grundy *et al*., 2002, Lichtenstein *et al*., 2006). Dietary changes should include a reduction of intake of dietary cholesterol to < 200mg/day, addition of approximately 2 grams of plant sterols/stanols each day, and incorporation of 10 to 125 g/day of viscous fibres into the diet (Lipsy, 2003).

1.1.6.2 Drug treatment

If therapeutic lifestyle changes fail to achieve the goal after 3 months, it is recommended that drug therapy is added to the lifestyle modifications because lifestyle may insufficiently address LDL-C lowering which is the primary target of therapy (Grundy *et al*., 2002, Grundy *et al*., 2004). Other targets for hyperlipidemia treatment include low levels of HDL cholesterol and elevated levels of triglycerides, both of which are independent predictors of cardiovascular disease (Gordon *et al*., 1989, Bansal *et al*., 2007, Sarwar *et al*., 2007). *Statins* (Figure 1-5)**:** Development of statins, which are derived from fungi and later became purely synthetic molecules, is one of the major advances in the management of hyperlipidemia (Endo, 1992). Statins are inhibitors of HMG-CoA reductase (Table 1-2), which is the rate-limiting enzyme in cholesterol biosynthesis. Inhibition of HMG-CoA reductase leads to a decrease in intrahepatic cholesterol concentration to which the liver responds by inducing its LDL receptors leading to an increase in LDL-catabolism (Endo *et al*., 1976). Triglyceride serum levels are also reduced by statins because increased LDL receptor activity leads to removal of triglyceride rich LDL and VLDL (Table 1-3) (Expert Panel on Detection *et al*., 2001). Statins should be used for the treatment of women with a history of congestive heart diseases. Women respond to statins differently than men. Primary and secondary trials show that cholesterol lowering drugs reduce the risk of cardiovascular disease events in men. Secondary prevention trials in women with established cardiovascular disease also show a reduction in risk. Some investigators nevertheless argue that women without cardiovascular disease should not be given cholesterol lowering drugs because of insufficient evidence from primary prevention trials in women. They speculate that women with and without cardiovascular disease respond differently to the drugs; if true, women may not respond to treatment even when they have the same level of risk as men who do benefit (Grundy, 2007). Lipid lowering therapy in women can reduce the risk of coronary events in secondary but not primary prevention (Walsh and Pignone, 2004).

Statins can cause myopathy, which if not recognized and the drugs are continued, can lead to rhabdomyolysis which is defined as the breakdown of muscle tissue that leads to the release of muscle fiber contents into the blood, myoglobinuria which is defined as the presence of myoglobin in the urine, and acute renal necrosis which is an inflammatory condition that primarily affects the renal tubules of the kidneys (Pierce *et al*., 1990). Statins also have some drug-drug interactions especially involving the cytochrome p-450 drug metabolism system which is a family of isozymes responsible for the biotransformation of most foreign substances including 70 to 80 % of all drugs (Gruer *et al*., 1999).

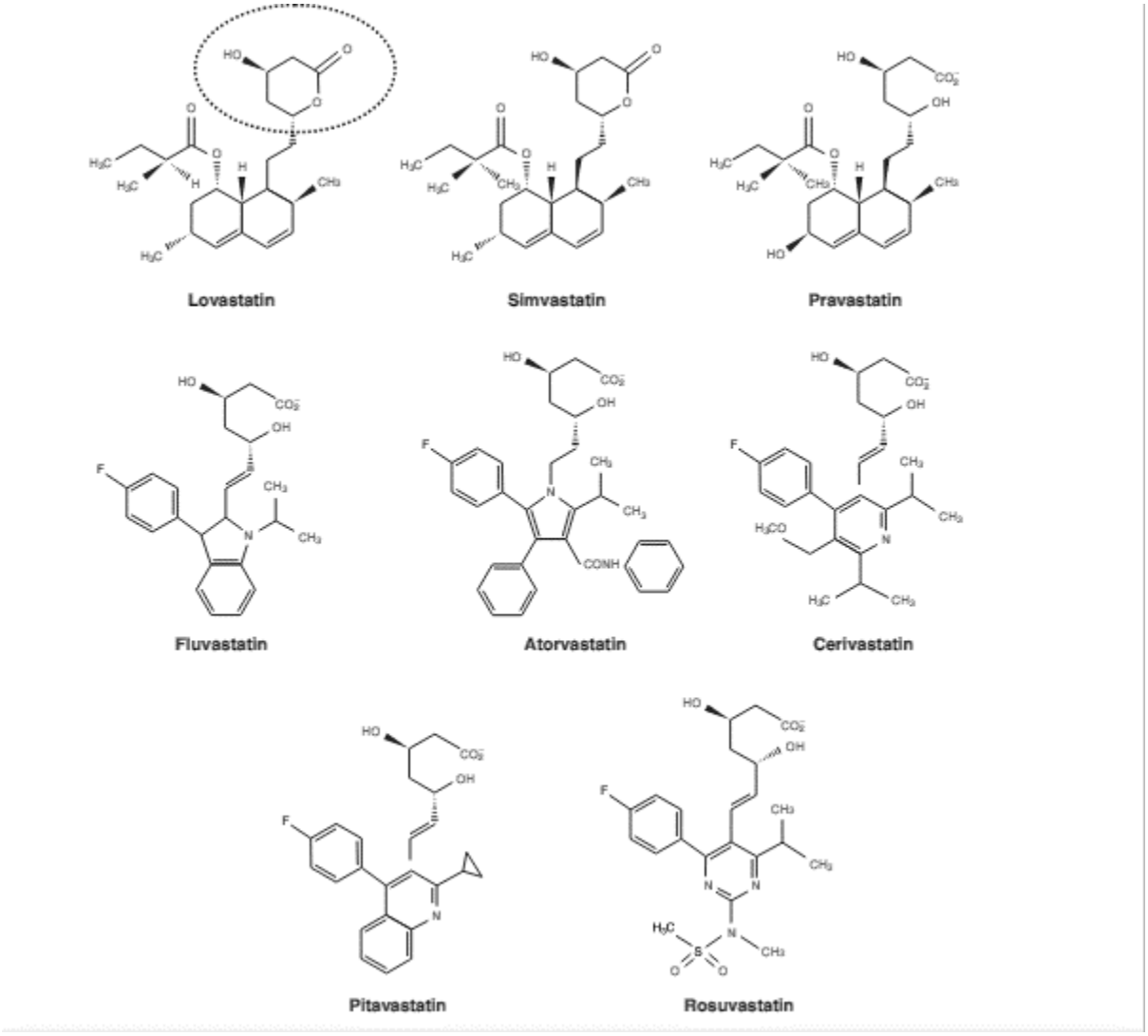

Figure 1-5: Structures of the statins where the dotted line shows the HMG-CoA like moiety which binds to the HMG-CoA-binding portion of the enzyme active domain (Schachter, 2005)

*Bile acid sequestrants***:** Bile acid sequestrants are anion exchange resins binding the bile acids in the intestinal lumen (Table 1-2). This effect prevents the reabsorption of the bile acids in the terminal ileum and their circulation back to the liver (Carlson, 2005). The reduction in bile acid levels induces the liver to produce bile acids from cholesterol which leads to depletion of intrahepatic cholesterol and increased expression of LDL receptors (Table 1-3) (Carlson, 2004). However, usage of bile acid sequestrants is hindered by inconvenient dosing and unpleasant side effects such as gastro-intestinal disorders like constipation (Guay, 2002). They also bind non-specifically with anions and interfere with the absorption of drugs such as statins, fibrates, corticosteroids, diuretics, and non-steroidal anti-inflammatory drugs (Insua *et al*., 2002).

*Nicotinic acids***:** Nicotinic acid inhibits lipolysis in adipose tissue resulting in low levels of plasma free fatty acids which are the precursors of hepatic and plasma triglycerides transported in VLDL, and as a result decreases triglyceride rich VLDL and cholesterol rich-LDL (Table 1-3) (Law *et al*., 2003). Nicotinic acid causes unpleasant side effects of flushing which hinders people from taking it (Gruer *et al*., 1999).

Fibric acid derivatives (fibrates): Fibrates activate peroxisome proliferator-activated receptors (Schoonjans *et al*., 1996), which are transcription factors that control expression of metabolic genes (Table 1-2). Fibrates reduce triglycerides by reducing expression of Apo CIII which serves as an inhibitor of lipolytic processing and receptor mediated clearance. This results in the increase of lipoprotein lipase synthesis and enhanced clearance of triglyceride rich lipoproteins. Fibrates also increase HDL by the stimulation of Apo-AI and Apo-AII expression (Eisenberg *et al*., 1984). Fibrates decrease LDL-cholesterol levels by only 10% (Table 1-3) (Law *et al*., 2003). Fibrates can lead to the formation of cholesterol gallstones (Palmer, 1987).

*Ezetimibe***:** Ezetimibe was approved by the World Health Organization (WHO) in 2002 for the treatment of primary hypercholesterolemia and homozygous sitosterolemia (Patel *et al*., 2003). Ezetimibe binds to cholesterol and blocks its dietary and biliary cholesterol absorption

from the gut. This results in reduced hepatic cholesterol levels, thus inducing LDL receptor expression (Dujovne *et al*., 2002).

Combination therapy: Some drugs can be combined with statins for the treatment of mixed hyperlipidemia. The advantage of such combinations is the usage of low doses of each drug to minimise the risk of side effects. Combining a drug with different mechanism of action than statins will reduce the levels of LDL-C by additional 10 % while doubling the statin dose with achieve only 6 % additional reduction (Lipsy, 2003).

Combinations of statin with fibrates (Vega *et al*., 2003), ezetimibe (McKenney *et al*., 2006b), or nicotinic acid (McKenney *et al*., 2007) provides an enhanced lipid control in patients with mixed dyslipidaemia where both LDL-C and triglycerides were reduced. A triple combination of ezetimibe, simvastatin, and nicotinic acid could be used to reduce LDL-C (Guyton *et al*., 2008). While combination therapy is recommended in mixed dyslipidaemia, there may be tolerability concerns (Davidson *et al*., 2007).

In case of fibrate and statin combination, there are concerns about the risk of myopathy which is a result of both drugs monotherapy (Rosenson, 2004). The most common combination used is statin plus bile acid resins. This combination showed a 50% reduction in LDL-C. This effect could be achieved with high statin dose. The gastrointestinal tract side effects and multiple daily dosage of resins limited this combination usage (Lipsy, 2003).

A statin is not always included in combined therapy. Nicotinic acids and bile acid resins have been used before statins were developed. The side effects of these drugs required to reduce the dose but the effectiveness was less compared to the higher dose (McKenney, 2000). Although not frequently used, the combination of nicotinic acid and fibrates could be useful in patients with high levels of triglycerides (Rubins *et al*., 1999).

Table 1-2: Summary of the drugs used in the treatment of hyperlipidemia (Lipsy, 2003)

Drug	Mechanism of action	Target
Statins	HMGCR inhibitors	Low LDL-C levels and low triglycerides
Bile acid sequestrants	Bind to bile acids in the intestinal lumen	Low cholesterol levels
Nicotinic acid	Inhibits lipolysis in adipose tissues	Low levels of triglycerides and LDL-cholesterol
Fibrates	Activate Peroxisome proliferator-activated receptors	Low LDL-cholesterol, high HDL cholesterol,
Ezetimibe	Binds to cholesterol	Low LDL-cholesterol

Table 1-3: Effect of each class of antihyperlipidemic drugs on lipids (Miller, 2009)

Medication	LDL-cholesterol	HDL-cholesterol	Triglycerides
Statins	↓27-50%	↑5-10 %	↓10-25%
Bile acid sequestrants	↓15-30%	↑3-5%	↓0-20%
Nicotinic acid	↓5-25%	↑10-25%	↓20-50%
Fibrates	Variable effect	↑10-25%	↓20-50%
Ezetimibe	↓18-20 %	↑3-5 %	↓ ~5%

Tetrahydrolipstatin (THL Figure 1-6): It is the hydrogenated form of lipstatin, and is a pancreatic lipase inhibitor that blocks fat absorption selectively and has antihyperlipidemic activity (Hochuli *et al*., 1987). The anti-pancreatic lipase activity of THL is due to its binding with Ser-152 which is a part of the His-Asp-Ser catalytic triad in the active site of pancreatic lipase.

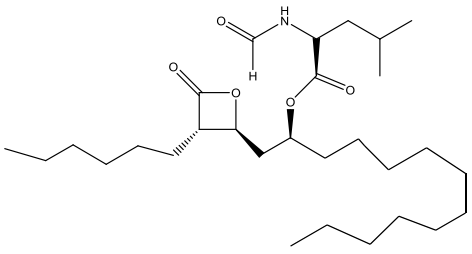

Figure 1-6: Structure of THL (Hanessian *et al*., 1993)

THL has side effects such as oily spotting, liquid stools, faecal urgency, flatulence and abdominal cramping (Chaput *et al*., 2007), decreased vitamin D absorption, and myopathy

(Harp, 1998). Some patients suffered from severe liver injuries due to use of tetrahydrolipstatin, as reported by the US food and drug administration (Douglas *et al*., 2013).

1.1.6.3 Traditional foods and herbal therapy

Despite the efficacy of statins and other lipid reducing agents, the issues of tolerability, untoward side effects, and safety remain a concern with many agents, particularly when administered at high doses or in combinations. This problem restricts use when patients cannot tolerate statins or when statin up-titration is limited due to safety concerns. In these cases, treatment is limited to agents such as bile acid sequestrants, nicotinic acid, or other less effective therapies. The natural products potential for the treatment of hyperlipidemia is not well explored and could be as an alternative strategy for the development of safe and effective drugs for the treatment of hyperlipidemia (Birari and Bhutani, 2007).

1.1.6.3.1 Polyphenols

A wealth of information indicates numerous bioactive components from nature are potentially useful in hyperlipidemia treatment. A good example of such are the polyphenols (Verger, 1997). Naturally occurring phytochemicals are an exciting opportunity for the discovery of new anti-hyperlipidaemic agents. Some of those natural products have a pancreatic lipase inhibitor effect. Many researches focused on polyphenols from teas and herbal sources (Birari and Bhutani, 2007), but there are studies showing that polyphenols from fruit sources can also have a pancreatic lipase inhibition effect (Moreno *et al*., 2003). Many polyphenols such as flavones, flavonols, tannins and chalcones are active against pancreatic lipase. Luteolin inhibited pancreatic lipase at IC_{50} 76.5 $\pm$ 7.51 µg/mL (Yamamoto *et al*., 2000). 3-Methylethergalangin and mangiferin flavonoids obtained from rhizomes of *Alpinia officinarum* (Shin *et al*., 2003) and from fruit of *Mangifera indica* (Moreno *et al*., 2003), inhibited pancreatic lipase, as did hesperidin from the peel of *Citrus unshiu* (Ono *et al*., 2006). Quercetin exhibited a pancreatic lipase inhibition effect with an IC_{50} of 40 µM (Zheng *et al*., 2010). *Emblica officinalis* is reported to have a hypolipidemic effect. Flavonoids from *Emblica officinalis* showed a significant decrease in the HMG-CoA reductase activity in the liver of

animals at a dose of 10 mg/kg body weight per day (Hofbauer, 2002). Flavonoids from *Ananas comosus* were found to inhibit HMG-CoA reductase (Xie *et al*., 2005a).

1.1.6.3.2 Prunella vulgaris

The dry spikes of a perennial herbaceous species *Prunella vulgaris* (Figure 1-7), a member of the family *Lamiacae*, which is also called a “self heal” or “heal all”, have been used. Europeans and Chinese have been using *Prunella vulgaris* as a traditional remedy for thousands of years (Pinkas *et al*., 1994). Its activity in the treatment of sore throat, fever, and wounds and its anti-inflammatory, anti-bacterial, and anti-viral activity (Chiu *et al*., 2004) increased the commercial demand for it. Recently it is reported that *P.vulgaris* extracts have anti-hyperglycemic effects (Jie *et al*., 2007). *Prunella vulgaris* is rich in triterpenoids such as oleanolic acid and ursolic acid. It is also rich in flavonoids such as quercetin, rutin, and emodin, and luteolin. In addition to the triterpenoids and flavonoids, it contains steroids, coumarins, organic acids, saccharides, and volatile oils.

Figure 1-7: Dried spikes of *Prunella vulgaris* (Psotova *et al*., 2003)

1.1.6.3.3 *Ilex latifolia*

Kudingcha is a bitter tea that has been used for long time in China. According to Chinese medicine it is known to refresh the mentalities, clear blood toxins, and disperse wind-heat. It has also been utilised to help in digestion and alleviating the adverse effects of alcohol. It has been used for the treatment of rhinitis, cold, itching eyes, conjunctional congestions, and headache (Li, 2011, Li *et al*., 2011). Many herbs which have similarities in flavour,

appearance, and traditional usage in China are named kudingcha (He *et al*., 2003). The most common plants in Chinese markets are divided into two groups. The first group is called large leaved kudingcha and it includes species *Ilex latifolia* and *Ilex kudingcha* C.J. Tseng. The other group is called the small leaved kudingcha and it contains the species *Ligustrum robustum*. The large leaved kudingcha are considered the original kudingcha species and have obvious lipid metabolism, hepatoprotective, anti-tumour, anti-inflammatory, and antioxidant activities (Wu *et al*., 2008, Nishimura *et al*., 1999a, Nishimura *et al*., 1999b, Woo *et al*., 2001). For the last few decades kudingcha has been used as a dietetic beverage and is gaining popularity with names like clearing heat tea, beauty slimming tea, and longevity tea.

1.1.6.3.3.1 Secondary metabolites of large leaved kudingcha

Phenolic acids, essential oils, flavonoids, and triterpenoids were isolated and characterized from large leaved kudingcha. Polyphenols and triterpenoids were considered the most important and abundant metabolites because they have various bioactivities (Feng, 1998).

1.1.6.3.3.1.1 Triterpenoids

Kudinlactones α, β, and δ with a lactone ring at the position of C20 and C28 are considered to be the most characteristic chemicals in kudingcha species. While β-kudinlactone was the main triterpenoid present in *Ilex latifolia*, α and δ kudinlactones were not present. *Ilex latifolia* also contains oleanane-type and lupane-type triterpenoids and their glycosides (Li *et al*., 2012).

1.1.6.3.3.1.2 Phenolic acids

Phenolic acids with anti-oxidant activities have been discovered recently. Polyphenols and their derivatives are the main types of phenolic acids found in large leaved kudingcha (Thuong *et al*., 2009, Liu *et al*., 2009).

1.1.6.3.3.1.3 Flavonoids

Quercetin, rutin, and kaempferol were the main flavones isolated from the large leaved kudingcha (Li *et al*., 2013).

1.1.6.3.3.1.4 Essential oils and other secondary metabolites

Gas chromatography coupled to mass spectroscopy (GC-MS) was used to the study the essential oils alcohol, aldehyde, ketone, ether, fatty acids, and fatty acid esters (He *et al*., 2003, Wu *et al*., 2008, Xiong B, 2003). Polysaccharides and phytosterols were also isolated and identified (Zou , 2011, Wang , 2008).

1.1.6.3.3.2 Biological activities of kudingcha

Regular usage of kudingcha as a herbal tea has a positive role in the cure and prevention of diabetes, pharyngitis, cardiovascular diseases, cerebrovascular diseases, especially the troublesome conditions related to atherosclerosis, hypertension, dizziness, insomnia, palpitations, and chest tightness caused by vascular diseases (Zhang, 1994). Lipid metabolism is the most important activity (Li *et al*., 2011).

1.1.6.3.3.2.1 Lipid metabolism activity

Acyl CoA cholesteryl acyl transferase (ACAT) catalyses the intracellular esterification of cholesterol in various tissues. Inhibitors of ACAT could be new types of medicines to treat obesity and atherosclerosis. Ilekudinol C and Ulmoidol isolated from kudingcha showed potent inhibition in an ACAT assay (Nishimura *et a*l., 1999a). Kudinoside A and ilekudinoside P had inhibitory activity on the formation of foam cells and reduced intracellular triglycerides and cholesterol content (Zheng *et al*., 2009).

1.1.6.3.3.2.2 Protection of vascular system

Many ilex species have been used in Chinese medicine for the treatment of coronary heart diseases. Their action was considered due to their coronary vasodilative effect. An aqueous extract of *Ilex latifolia* increased contractility and decreased the frequency of contraction in an isolated rat heart perfusion system. The extract inhibited Na^+/K^+ ATPase activity in rat

heart sarcolemma, as well as in the purified porcine cerebral cortex enzyme. It also inhibited Ca^{2+} dependent ATPase at a similar dose (Woo *et al*., 2001).

1.1.6.3.3.2.3 Hypoglycemic effect

Ilex latifolia extract showed inhibition of hyperglycemia in the epinephrine hyperglycemia rat model. *Ilex latifolia* has a potential as a hypoglycemic drug because the blood sugar levels of rats given a low or high dose of *Ilex latifolia* extract were significantly reduced compared to the control (Qu, 1999).

1.1.6.3.3.2.4 Antioxidant activity

Antioxidant activities of polyphenols and flavonoids of kudingcha and *Ilex latifolia* were assessed by DPPH, ABTST, and OH-free radical scavenging assays. *Ilex* kudingcha was found to contain higher polyphenol content and to have more anti-oxidant activity than *Ilex latifolia* (Zhang, 2010).

1.1.6.3.3.2.5 Anti-microbial activity

Ilex latifolia showed inhibition of the growth of *Pneumococcus*, *Acillus diphthera*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E.coli*. It also improved the survival rate of mice infected by *E.coli*, *B. dysenteriae*, *Pneumobacillus*, and *Streptococcus B* (Jiang, 2001).

1.1.6.3.3.2.6 Anti-viral activity

An aqueous extract of *Ilex latifolia* was found to have a beneficial effect on humoral and cellular immunity. *Ilex latifolia* is able to enhance and adjust the immune function of an organism as indicated by its ability to enhance phagocyte function of macrophages in the mouse abdominal cavity and increase the number of plaque-forming cells (Dong Y, 2001).

1.1.6.3.3.2.7 Antitumour activity

The anti-tumour activity of *Ilex latifolia* was assessed *in vitro* by an MTT assay. The inhibition rate against SGC-7901 human gastric cancer cells was found to be 62.4% (IC_{50} 42.86 mg/mL), while the inhibition rate against NCI-H460 human lung cancer cells was 93.33%.

These results suggested a basis for the development of new anti-tumour medicines from *Ilex latifolia* (Chen, 2007).

1.1.6.3.3.2.8 Protective activity in neuronal cells

Ilex latifolia has been used in Chinese medicine for the treatment of headache and inflammatory diseases. *Ilex latifolia* at 10-100 µg/mL inhibited elevation of intracellular calcium, glutamate-induced neuronal death, generation of reactive oxygen species, and decrease of anti-apoptotic protein, BcL-2 in cultured rat cortical neurons (Kim *et al*., 2012).

1.1.6.3.3.2.9 Toxicity

Kudingcha has been consumed as a tea with little or no acute toxicity. *Ilex latifolia* acute and long term toxicity was studied in rats using aqueous extracts. The maximum tolerable dose was 168 g/kg indicating that there is no acute toxicity (Xu, 2001).

1.1.6.3.3.2.10 Clinical usage

Studies show that drinking *Ilex latifolia* for two months was sufficient to reduce hypertension as well as, or better than, nifedipine (Mou, 2005, Huang, 1997).

Figure 1-8: *Ilex latifolia* Plant (Li *et al*., 2011)

1.1.6.3.4 Rheum palmatum

The dried rhizome and root of *Rheum palmatum* is one of the most famous and ancient herbs in China and has been used for thousands of years. The dried rhizome or root of

Rheum palmatum has many effects such as purging heat, promoting blood circulation, and removing blood stasis (Chen and Wang, 2009). *Rheum palmatum* can be used as well for the treatment of hyperlipidemia (Zhang *et al*., 2002), chorinic renal failure (Bi *et al*., 1982) and memory impairment (Tian *et al*., 1997). Anthraquinones which include rhein, emodin, chrysophanol, and physcion, are the main bioactive constituents of *Rheum palmatum* (Chai *et al*., 1998).

1.1.6.3.5 Panax notoginseng

The root of *Panax notoginseng* (Radix notoginseng) is a well known traditional Chinese medicine and it belongs to the Ginseng genus (Dong *et al*., 2003). *Panax notoginseng* has been used for the treatment of coronary heart diseases and cerebral vascular diseases (Chan *et al*., 2002). It was also effective in the promotion of blood circulation, relief of swelling, and alleviation of pain. The root of *Panax notoginseng* contains flavonoids, saponins, and polysaccharides as the main active constituents (Wu *et al*., 1995, Yang and Du, 2002).

Aims of the project

Hyperlipidemia is defined as an abnormal elevated level of lipids such as cholesterol or triglycerides in the blood. It is the main risk factor for congestive heart diseases and atherosclerosis which is the pathophysiological cause of vascular diseases such as angina pectoris, myocardial infarction, and stroke. Hyperlipidemia is associated with high rates of morbidity and early mortality. Lifestyle is the main factor in the prevention of hyperlipidemia. If lifestyle is not beneficial, drug therapy is required. Statins, fibrates, nicotinic acid bile acid sequestrants, and ezetimibe are approved drugs for the treatment of hyperlipidemia. Because of low tolerance, side effects, and low efficacy of some of these drugs, discovery of new drugs is being explored and investigated. Herbal products are not well explored as an alternative treatment for the treatment of hyperlipidemia. *Ilex latifolia* is a traditional Chinese

medicine which has been used for decades for the treatment of hyperlipidemia and for weight loss. This project describes efforts to identify new applications of Chinese herbs, and in particular *Ilex latifolia*, to treat hyperlipidemia. Two experimental approaches for testing herbs and extracts were adapted, lipid absorption and cholesterol synthesis. For lipid absorption, pancreatic lipase enzyme was the target, and for cholesterol synthesis, the target was the enzyme HMG-CoA reductase.

2 Chapter II: Pancreatic lipase assay

2.1 Introduction

Several potential therapeutic targets for hyperlipidemia have been identified due to the progress in unveiling the molecular mechanisms of lipid metabolism (Zhang and Huang, 2012). The development of molecules that inhibit nutrient digestion and absorption and reduce energy intake through gastrointestinal mechanisms, without altering central mechanisms, is one of the most important strategies in the treatment of hyperlipidemia (Birari and Bhutani, 2007). Hyperlipidemia can be prevented by inhibiting the movement of triglycerides from the intestinal lumen by inhibiting triacylglycerol lipases.

2.1.1 Pancreatic lipase

Pancreatic lipase is the principal lipolytic enzyme synthesized and released by the pancreas. It plays a key role in the efficient digestion of triglycerides. It catalyzes the removal of α and α' fatty acids of dietary triglycerides resulting in the formation of β- monoglycerides and long chain saturated and polyunsaturated fatty acids (Mukherjee, 2003, Shi and Burn, 2004, Thomson *et al*., 1997). Pancreatic lipase is responsible for the hydrolysis of 50-60% of dietary fats (Figure 2-1). Pancreatic lipase is a target for the treatment of hyperlipidemia especially mixed hyperlipidemia or secondary hyperlipidemia where the triglycerides levels are high due to obesity or diabetes mellitus type II (Christian *et al*., 2011, Shah and Wilson, 2015). Fatty acids and monoglycerides are incorporated in to bile acid micelles. These micelles deliver the fatty acids and the monoglycerides to the intestinal microvilli, where triglycerides are re-synthesized and incorporated to the chylomicrons which are secreted to the intestinal lymph. Inhibition of pancreatic lipase results in malabsorption of triglycerides and consequently inhibition of synthesis of triglycerides-rich chylomicrons synthesis (Hussain, 2000, Ros, 2000). His-263, Asp-176, and Ser-152 form a catalytic triad in the active site of human pancreatic lipase. This triad represents the lipolytic site. Chemical

modification of Ser-152 irreversibly inhibits enzymatic activity indicating that Ser-152 is essential for the catalytic activity (Winkler *et al*., 1990).

Lipases with high affinity for their substrates work at the aqueous/oil interface (Schmid and Verger, 1998) forming an activated enzyme-substrate complex. The inhibition effect of bile salts on hydrolysis of triglycerides is due to desorption of the pancreatic lipase from its emulsified substrate (Granon and Semeriva, 1980, Gargouri *et al*., 1983, Borgstrom, 1982, Luthipeng and Winkler, 1992). Bile salts enhance lipolysis at low concentration either by stabilising the enzyme at the interface (Momsen and Brockman, 1976, Luthipeng and Winkler, 1992) or by facilitating the dissociation of the long water-insoluble fatty acid chains (Desnuelle *et al*., 1960, Luthipeng and Winkler, 1992). The effect of bile salts on pancreatic lipase depends on the concentration of the salts, substrate used, and the presence or absence of colipase which in the presence of bile salts is necessary for the binding of the enzyme to the substrate.

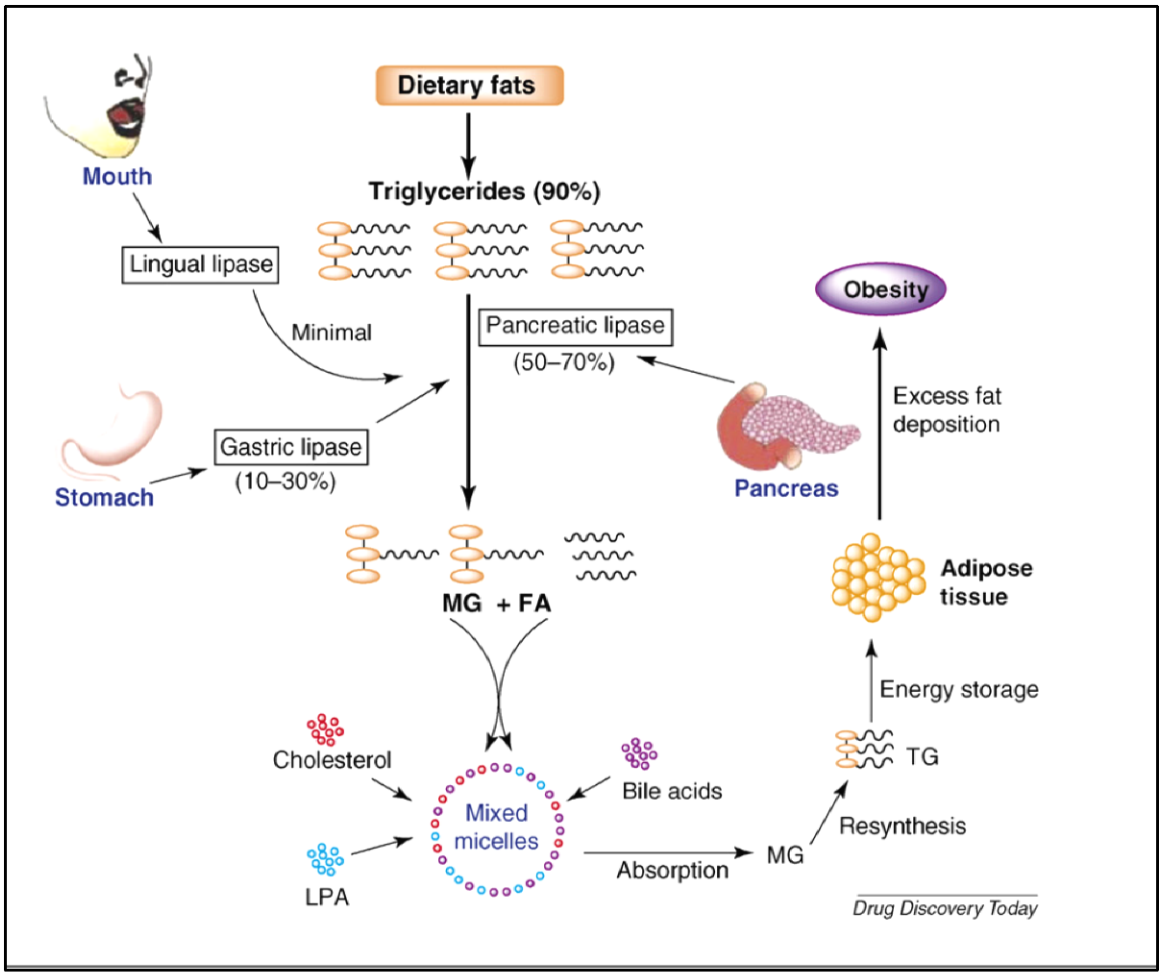

Figure 2-1: Role of pancreatic lipase in lipid absorption (Birari and Bhutani, 2007).

2.1.2 Tetrahydrolipstatin (THL)

THL, the hydrogenated product of the naturally occurring lipstatin, inhibits pancreatic lipase and two other lipolytic enzymes secreted into the gastrointestinal tract,the carboxyl esterase lipase of the pancreatic juice and the acid lipase of the human gastrointestinal tract. Esterastin, isolated from *streptomyces*, was the first lipase inhibitor to be described (Weibel *et al*., 1987; Kitahara *et al*., 1987; Umezawa *et al*., 1978; Hahn *et al*., 2013). Its structure was determined by Umezawa et.al (1978) and it was reported to exhibit a strong inhibition of pancreatic esterase. It was followed by the discovery of lipstatin (Weibel *et al*., 1987; Hochuli *et al*., 1987) and valilactone (Kitahara *et al*., 1987) (Figure 2-2).

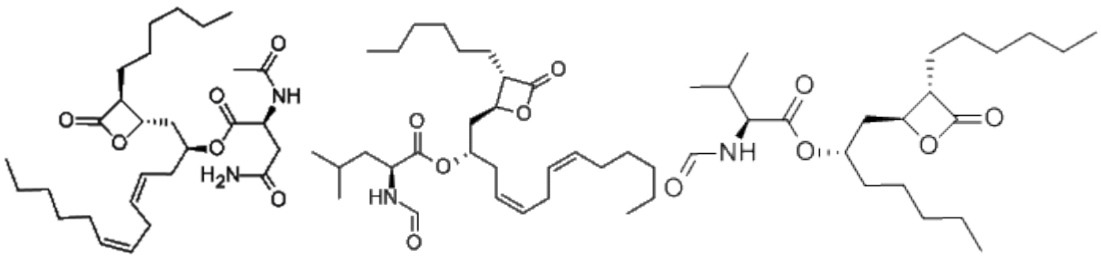

Figure 2-2: Structures of esterastin, lipstatin, and valilactone respectively

The structures of these compounds reveal that they are derived from mycolic acids which are hydroxy fatty acids possessing a 2-alkyl substitution (Borgstrom, 1988). The mycolic acid moiety of these inhibitors contains hydroxy groups in the 3- and 5- positions where the 3- hydroxy group forms a β-lactone ring and the 5-hydroxy group is esterified with N-formyl-1- leucine for lipstatin. THL (Figure 2-3) reduces intestinal fat absorption through inhibition of pancreatic lipase (Ballinger and Peikin, 2002, Drew *et al*., 2007). THL blocks fat absorption selectively and has anti-hyperlipidaemic activity (Hochuli *et al*., 1987). The anti-pancreatic lipase activity of THL is due to its binding with Ser-152 which is a part of the Asp-His-Ser triad in the pancreatic lipase structure (Ballinger and Peikin, 2002).

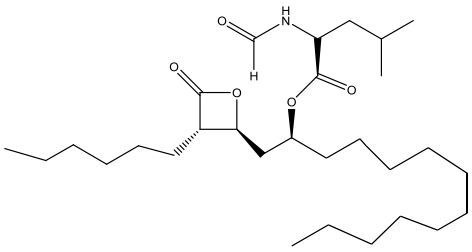

Figure 2-3: Structure of tetrahydrolipstatin (Hanessian *et al*., 1993)

THL has a low hydrophilicity and distributes in the aqueous/oil system in favour of the lipid phase. It is an insoluble non-swelling amphiphile which is surface active (Borgstrom, 1974) and forms stable monolayers at the interface. It can also form mixed micelles with bile salts and this could result in its presence at the substrate water interface of the lipase substrate. THL transfer from oil to aqueous phase increases in the presence of bile salts above their CMC (Tiss *et al*., 2002) reflecting the possibility of solubilisation of THL in the aqueous phase by forming mixed micelles with bile salts. The dependence of THL inhibitory effect of

pancreatic lipase on the presence of bile salts in the incubation medium could be explained by either the formation of THL/bile salts mixed micelles which lead to a better interfacial quality improving the lipase adsorption onto these micelles, or by conformational changes of pancreatic lipase caused by bile salts. The increased inhibition efficacy of THL in the presence of bile salts above their CMC was reported in the case of human pancreatic lipase (Luthipeng and Winkler, 1992, Tiss *et al*., 2009) human gastric lipase (Gargouri *et al*., 1991, Tiss *et al*., 2009) human lipoprotein lipase (Lookene *et al*., 1994, Tiss *et al*., 2009) as well as dog and guinea pig lipase (Cudrey *et al*., 1993, Tiss *et al*., 2009). This matches with porcine pancreatic lipase inhibition studies using diethyl p-nitrophenylphosphate (E_{600}). E_{600} in bile salts mixed micelles irreversibly inhibited porcine pancreatic lipase while aqueous solution of the organo-phosphorous compound does not (Rouard *et al*., 1978, Tiss *et al*., 2009). The same effect was reported when the compound was not emulsified with gum Arabic (Desnuelle *et al*., 1960, Tiss *et al*., 2009). It was reported that inhibition of *Chromobacterium vicosum* lipase by THL was only achieved in the presence of 50 % isopropanol (Potthoff *et al*., 1998, Tiss *et al*., 2009).

Herbal extracts of *Ilex latifolia*, *Prunella vulgaris*, *Rheum palmatum* and *Panax notogensing*, in addition to herbal compounds including quercetin, emodin, crocin, and hesperidin, will be investigated for their pancreatic lipase inhibition activity using spectrophotometric and high performance liquid chromatography assays. Orlistat, an approved pancreatic lipase inhibitor will be used as a positive control.

2.2 Experimental methods

2.2.1 Materials

p-Nitrophenyllaurate, pancreatic lipase of porcine pancreas type II, DMSO, THL, quercetin, emodin, crocin, hesperidin, sodium chloride, taurocholic acid sodium salt, sodium dihydrogen phosphate, and 2,4-dinitroaniline were obtained from Sigma-Aldrich (Gillingham, England). Hydrochloric acid and acetonitrile HPLC grade, n-butanol, and Industrial methylated spirit (IMS), were obtained from Fisher scientific UK limited (Bishop Meadow

road, Loughborough, England). *Prunella vulgaris*, *Rheum palmatum* and *Panax notogensing* were bought from Beijing Tong Ren Tang (London,UK) . *Ilex latifolia*. was a gift from Shanghai University of traditional Chinese medicine. Alli was a gift from Professor S.W. Annie Bligh (Westminster University). HPLC used was Dionex Ultimate 3000. NMR instrument used was Bruker Avance 500.

2.2.2 Methods

2.2.2.1 Extraction of orlistat from Alli

Alli contains orlistat as the active pharmaceutical ingredient. It also contains inactive ingredients such as microcrystalline cellulose, sodium starch glycolate, sodium lauryl sulphate, povidone, and talc. 0.5 g of alli powder was suspended in 200 mL deionised water. The suspension was filtered [Whatman, Whatman international Ltd, Maidstone, England] using a Buchner vacuum pump, and the filtrate was discarded. The residue was suspended in 20 mL of water and filtered, and the filtrate was discarded. The residue was suspended in 20 mL of IMS. The solution was filtered and the filtrate was dried by leaving the solution in the fume hood. The orlistat powder recovered (0.205 g) was stored at 4 ºC.

2.2.2.2 Structure elucidation of orlistat using NMR

Nuclear magnetic resonance (NMR) was used for structure elucidation. ^{1}H, ^{13}C, HMBC, and HSQC spectroscopic analysis were carried out in deuterated chloroform ($CDCl_3$). The NMR analysis was operated by Mr. John Crowder, NMR technician at London Metropolitan University. Standard orlistat purchased from Sigma Aldrich was used as a reference.

2.2.2.3 Extraction of Prunella vulgaris, Rheum palmatum, and Ilex latifolia.

Dried flower spikes of the *Prunella vulgaris* were ground to a coarse powder. Separate aliquots (20 g) were extracted in 200 mL of n-butanol using two different methods. The first method was reflux extraction at 120 ºC for 2 hours. The second method was ultrasonic extraction at room temperature for 2 hours. The extract was filtered using Buchner vacuum apparatus (Buchi vacuum pump V-700). After filtration, the solvent was evaporated at 100 ºC

using Buchner vacuum pump (Buchi vacuum pump V-700). The extracts obtained using reflux and ultrasonic extraction were weighed (1.135 and 0.7945 g respectively) and stored at 4 ºC. Separate aliquots (30 g) of course powder of *Ilex latifolia* dried leaves, separate aliquots (35 g) of *Rheum palmatum*, and separate aliquots (35 g) of *Panax notoginseng* were extracted using the same methods. The extracts were weighed and stored at 4 ºC.

2.2.2.4 Validation of pancreatic lipase inhibition assay by spectrophotometry

The assay was adapted from previous work (McDougall *et al*., 2009). Porcine pancreatic lipase type II was prepared at 10 mg/mL in ultra-pure water by pipetting up and down for a few minutes. After centrifugation at 20000 g for 5 min to remove the undissolved powder, the supernatant was used. The assay buffer was 0.1 M Tris-HCl (pH 8.2). The substrate stock solution was prepared at 0.9 mg/mL of p-nitrophenyllaurate in 0.007 M sodium acetate (pH 5.0) containing 1% Triton X-100 (17 mM) which is much higher than the critical micellar concentration of Triton X-100 (0.22-0.4 mM) to facilitate the substrate dissolution. The solution was heated in boiling water for 1 min to aid dissolution and then cooled to room temperature. Tetrahydrolipstatin (THL) solution was prepared at 0.105 mg/mL in DMSO. Five samples with a final volume of 1050 µL, containing 150 µL enzyme, 50 µL THL, and 650, 400, 200, 100, and 0 µL substrate respectively, were prepared. The volume was adjusted with 0.1 M Tris-HCl buffer (pH 8.2). Five control samples were prepared without THL. All samples were incubated at 37 °C for 2 h. They were then centrifuged at 20,000 g for 3 min and read at 405 nm using a Shimadzu UV/Vis spectrophotometer. All the samples were assayed in triplicate.

2.2.2.5 Determination of IC_{50} of THL by spectrophotometry

The method was adapted from that described in section 2.2.2.4. THL solution was prepared at 2.3 mg/mL in DMSO. Serial two-fold dilutions were done nine times to obtain a range of THL concentrations of 2.3 mg/mL to 8.9 µg/mL. Samples containing 400 µL assay buffer, 150 µL enzyme, 50 µL THL (2.3 mg/mL-8.9 µg/mL) respectively, and 450 µL of substrate were prepared. Negative controls were prepared by combining 400 µL assay buffer, 150 µL

enzyme, 50 µL DMSO respectively, and 450 µL of substrate. All the samples were incubated at 37 °C for 2 h. They were then centrifuged at 20,000 g for 3 min and read at 405 nm using a Shimadzu UV/Vis spectrophotometer. All the samples were assayed in triplicate.

2.2.2.6 Determination of IC_{50} of quercetin by spectrophotometry

The method of section 2.2.2.4 was adapted. Instead of THL, quercetin (2-0.7 mg/mL) was dissolved in DMSO and used as the inhibitor. All the samples were analysed in triplicate.

2.2.2.7 Determination of the influence of THL and quercetin on one another by spectrophotometry

The method of section 2.2.2.4 was adapted with some modifications. Briefly, to maintain the same concentrations of quercetin and THL used in the previous experiments, 25 µL of quercetin (4.5 mg/mL-17.5 µg/mL), and 25 µL of THL (42 µg/mL) were used respectively. All the samples were assayed in triplicates.

2.2.2.8 Pancreatic lipase assay using HPLC

2.2.2.8.1 Preparation of stock solutions

Substrate solution was prepared by mixing 10 mL of 0.2 M phosphate buffer (pH 7.4), 2 mL of 0.07 M sodium chloride, 2 mL of 0.03 M sodium taurocholate, 0.274 mM 4-nitrophenyllaurate solution, and 4 mL water.

2,4-Dinitroaniline was used as an internal standard. It was prepared at 0.15 and 0.2 mg/mL in ethanol. A reference standard solution was prepared following the procedure cited by Maurich *et al*. (1991) by combining 100 µL of 0.3 mg/mL p-nitrophenol solution, 1 mL of 0.15 mg/mL 2,4-dinitroaniline solution, 0.625 mL of 5 M HCl, and 5 mL of 0.2 M phosphate buffer (pH 7.4). The solution was made up to 10 mL by adding water.

2.2.2.8.2 Validation of pancreatic lipase assay using HPLC

0.0101 g of porcine pancreas lipase type II was dissolved in 5 mL of 0.1 M phosphate buffer (pH 7.4) and diluted to 25 µg/mL in 0.1 M phosphate buffer. Six samples of 700 µL final

volume were prepared. 600 µL of substrate solution and different volumes of enzyme solutions (25, 35, 50, 65, 75, and 100 µL) were added to each sample. The volume was made up to 700 µL by adding 0.1 M phosphate buffer (pH 7.4). All the samples were incubated at 37 °C for 30 min. 50 µL of 5 M HCl was added to terminate the reaction and 50 µL of 0.2 mg/mL 2,4-dinitroaniline internal standard solution were then added to the samples. Samples were centrifuged at 20,000 g for 2 min. The supernatant layer was analysed using HPLC at room temperature using a Hichrom ACE 150mm x 4.6 mm I.D. column packed with 5 µm ACE 18. The flow rate was 1mL/min. The mobile phase was 55:45 (v/v) mixture of water and acetonitrile. The peaks of 4-nitrophenol and 2,4-dinitroaniline were detected at 300 nm where the peaks areas were used to determine the 4-nitrophenol concentration in addition to the enzyme activity.

2.2.2.9 Determination of the calibration curve of p-nitrophenol and limit of detection using HPLC

0.0032 g of p-nitrophenol were dissolved in 10 mL of acetone. *p*-Nitrophenol solution was diluted 2 fold seven times using acetone. Seven samples were prepared by mixing 300 µL of 0.2 M phosphate buffer (pH 7.4), 100 µL of 0.1 M phosphate buffer (pH 7.4), 60 µL of 0.07 M sodium chloride, 60 µL of 0.03 M sodium taurocholate, 60 µL of different p-nitrophenol solutions, 50 µL of DMSO, and 120 µL of water. Ten blank samples were prepared using the same reagents used in the samples but with 60 µL of acetone instead of p-nitrophenol solution. All the samples were incubated at 37 ºC for 30 min. 50 µL of 5 M HCl and 50 µL of ethanol were added to the samples. All the samples were centrifuged at 20,000 g for 2 min. The supernatant layer was analysed using HPLC at room temperature using a Hichrom ACE 150mm x 4.6 mm I.D. column packed with 5 µm ACE 18. The flow rate was 1mL/min. The mobile phase was 55:45 (v/v) mixture of water and acetonitrile. The peaks of 4-nitrophenol were detected at 300 nm.

2.2.2.10 Determination of the IC_{50} of THL using HPLC

0.063 g of porcine pancreas lipase type II was dissolved in 2.5 mL of 0.1 M phosphate buffer (pH 7.4). THL (2.8 mg/mL) in DMSO was serially diluted two-fold in DMSO 13 times. All samples were prepared by adding 150 µL of enzyme (Final concentration 4.2 mg/mL), 50 µL of THL, and 600 µL of substrate solution. A positive control sample was prepared by adding 50 µL of DMSO instead of THL. Samples were incubated at 37 ºC for 30 min. 50 µL of 5 M HCl and 50 µL of 0.2 mg/mL 2,4-dinitroaniline solution were added to the samples. Samples were centrifuged at 20,000 g for 2 min. The supernatant layer was analysed using HPLC at room temperature using a Hichrom150mm x 4.6 mm I.D. column packed with 5 µm ACE 18. The flow rate was 1mL/min. The mobile phase was 55:45 (v/v) mixture of water and acetonitrile. The peaks of 4-nitrophenol and 2,4-dinitroaniline were detected at 312 nm. In addition, the enzyme activity was determined by calculating the concentration of 4-nitrophenol using the peaks areas.

2.2.2.11 Determination of the IC_{50} of different compounds using HPLC

The method described in section 2.2.2.10 was used. Two-fold serial dilutions in DMSO of quercetin (2.5 mg/mL), crocin (12.1 mg/mL), emodin (9.5 mg/mL), and hesperidin (12 mg/mL) were assayed. Pancreatic lipase final concentration used in this assay was 4200 µg/mL. Crocin was also tested with 383 µg/mL of pancreatic lipase.

2.2.2.12 Determination of the effect of Rheum palmatum, Prunella vulgaris, and Ilex latifolia on pancreatic lipase *using HPLC*

The method of section 2.2.2.10 was adapted with some modifications. Briefly, porcine pancreatic lipase type II was used at 2 mg/mL in 0.1 M phosphate buffer (pH7.4). All the extracts were prepared by reflux extraction in n-butanol (section 2.2.2.3). *Rheum palmatum* extract (21.1 mg/mL), *Prunella vulgaris* extract (21 mg/mL), and *Ilex latifolia* extract (60.1 mg/mL) were dissolved in DMSO and spun at 20,000 g for 2 minutes when necessary. The supernatant layer was used in the assay. *Ilex latifolia* extract was incubated with the enzyme for 10 min before adding the substrate solution.

2.2.2.13 Determination of the pancreatic lipase inhibition effect of Prunella vulgaris when combined with THL using HPLC

The method (2.2.2.8) was adapted with some modifications. *Prunella vulgaris* was prepared at 37.3 mg/mL in DMSO. This sample was diluted two-fold nine times in DMSO. THL (0.001mg/mL) was prepared. To maintain the same volume and the same concentrations, 25 µL of each *Prunella vulgaris* sample and 25 µL of THL were used in each sample.

2.2.2.14 Assay of the effect of different concentrations of pancreatic lipase on p-nitrophenyllaurate

2.2 mg of porcine pancreas lipase type II were dissolved in 1 mL of 0.1 M phosphate buffer (pH 7.4). Enzyme solution was diluted 5 times two-fold in phosphate buffer. Six samples of 900 µL final volume were prepared. Five samples were prepared by adding 150 µL enzyme, 50 µL DMSO, and 600 µL substrate. All the samples were incubated at 37 ºC for 30 min. 50 µL of 5 M HCl and 50 µL of 0.2 mg/mL 2,4-dinitroaniline solution were added to the samples. All the samples were centrifuged at 20,000 g for 2 min. The supernatant layer was analysed using HPLC at room temperature using a Hichrom 150mm x 4.6 mm I.D. column packed with 5 µm ACE 18. The flow rate was 1mL/min. The mobile phase was 55:45 (v/v) mixture of water and acetonitrile. The peaks of 4-nitrophenol and 2,4-dinitroaniline were detected at 312 nm. The 4-nitrophenol concentration and the enzyme activity were determined using the peak areas. The chart speed was 0.5 cm/min.

2.2.2.15 Determination of the IC_{50} of THL using HPLC

The method was adapted from the previous method (section 2.2.2.12) with some modification. The enzyme concentration used in this experiment was 0.2575 mg/mL. THL (0.002 mg/mL) was diluted nine times two fold in DMSO. Nine samples were prepared by adding 150 µL enzyme, 50 µL THL, and 600 µL of substrate. One positive control was prepared by adding 50 µL of DMSO instead of THL. THL was incubated with the sample for 10 minutes before the addition of the substrate. Samples were incubated at 37 ºC for 30 min. 50 µL of 5 M HCl and 50 µL of 0.2 mg/mL 2,4-dinitroaniline solution were added to the

samples. Samples were centrifuged at 20,000 g for 2 min. The supernatant layer was analysed using HPLC at room temperature using a Hichrom 150mm x 4.6 mm I.D. column packed with 5 µm ACE 18. The flow rate was 1mL/min. The mobile phase was 55:45 (v/v) mixture of water and acetonitrile. The peaks of 4-nitrophenol and 2,4-dinitroaniline were detected at 312 nm. The chart speed was 0.5 cm/min.

2.2.2.16 Determination of the effect of Rheum palmatum, Prunella vulgaris, Ilex latifolia, Panax notoginseng and quercetin using HPLC

The method of section 2.2.2.15 was adapted with some modifications. Briefly, porcine pancreatic lipase type II was used at 0.2575 mg/mL in 0.1 M phosphate buffer (pH 7.4). Extracts were prepared by reflux in n-butanol (section 2.2.2.10). *Rheum palmatum* extract (62.3 mg/mL), *Prunella vulgaris* extract (52 mg/mL), *Ilex latifolia* extract (32.1 mg/mL) *Panax notogensing* (44.5 mg/mL) and quercetin (14.4 mg/mL) dissolved in DMSO were used instead of THL. Samples were incubated with the enzyme for 10 minutes before the addition of the substrate. Samples were centrifuged at 20,000 g for 2 minutes.

2.3 Data analysis

All lipase assay data were processed and plotted using Excel, Grafit (Erithacus Software Limited) or GraphPad Prism (GraphPad Sotware Inc). The data were analysed using non-linear fitting.

2.4 Results

2.4.1 Extraction of orlistat from Alli

Alli contains orlistat as the active pharmaceutical ingredient. It also contains non-active ingredients such as microcrystalline cellulose, sodium starch glycolate, sodium lauryl sulphate, povidone, and talc. Orlistat is soluble in chloroform, methanol, ethanol, DMSO, and DMF. Microcrystalline cellulose is insoluble in water, ethanol, ether, and dilute mineral acids. Sodium starch glycolate is insoluble in water and most organic solvents, and sparingly soluble in ethanol. Sodium lauryl sulphate is soluble in water, but insoluble in ether.

Povidone is soluble in water, chloroform, alcohol, chlorinated hydrocarbons, amines and nitropariffins. Talc is insoluble in water and ethanol.

The extraction of Alli powder yielded 0.205 grams which is 85.42 % of the expected yield.

2.4.2 NMR elucidation of orlistat structure

The NMR spectrum of the extracted orlistat was compared to the standard orlistat and to the literature (Eisenreich *et al*., 2003).

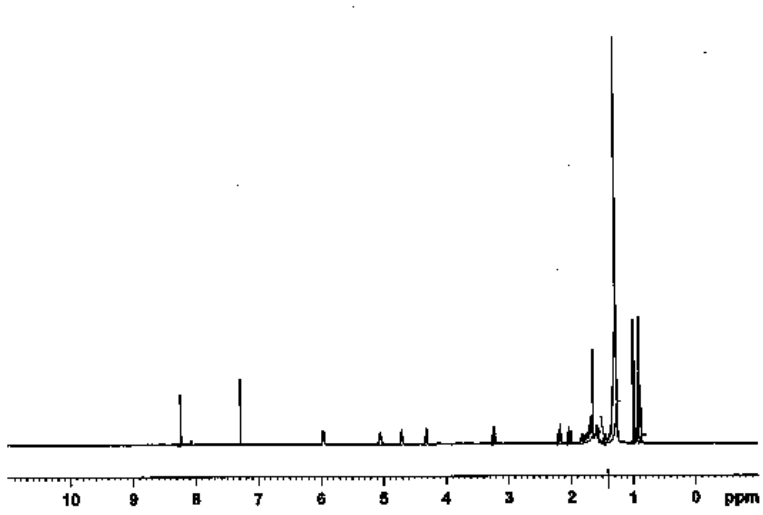

Figure 2-4: ^{1}H NMR of orlistat extracted from Alli

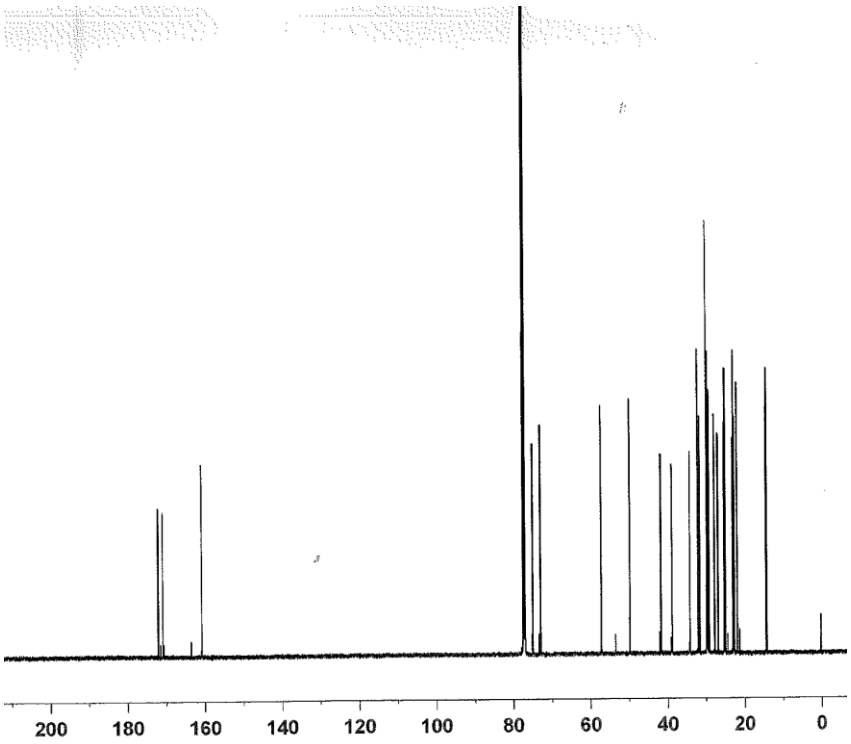

Figure 2-5: ^{13}C NMR of orlistat extracted from Alli

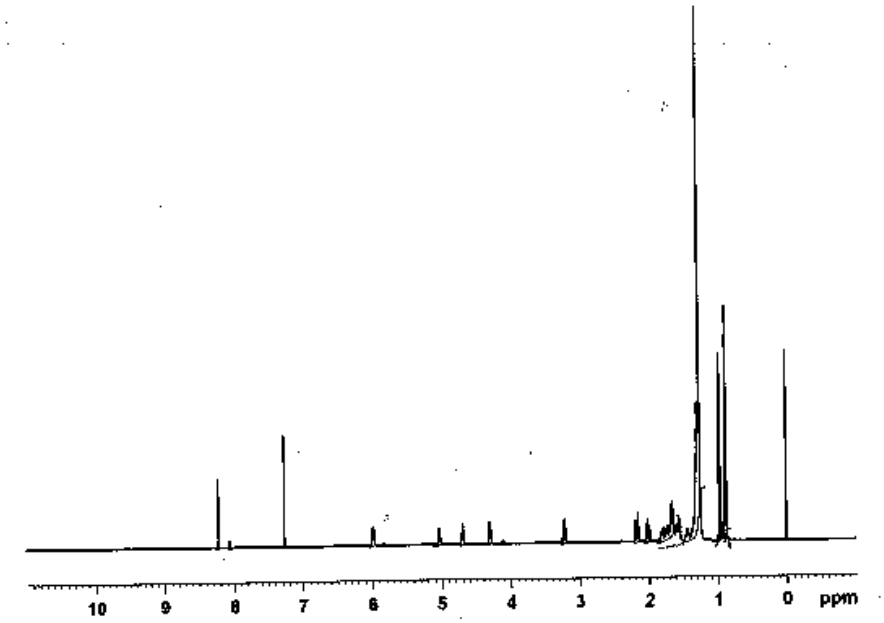

Figure 2-6: ^{1}H NMR of standard orlistat

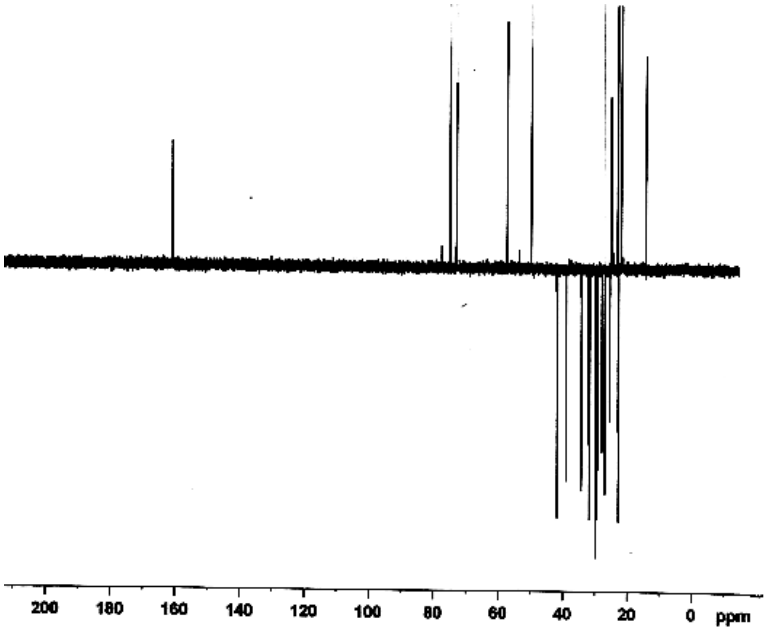

Figure 2-7: ^{13}C NMR of standard orlistat

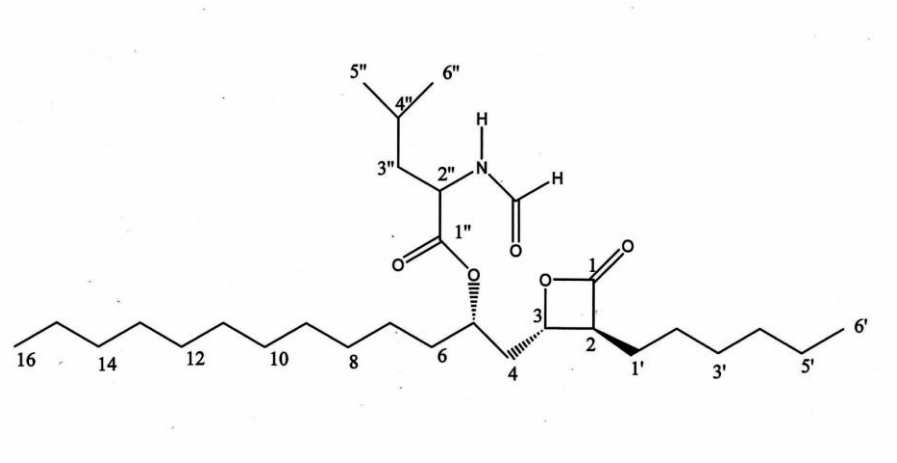

Figure 2-8: Structure of orlistat as determined by NMR.

Table 2-1: : ^{1}H and ^{13}C NMR of orlistat. Orlistat extracted from alli capsules was analysed using NMR. The spectrum was compared to the spectrum of standard orlistat purchased from Sigma Aldrich (O4139) and to literature

Extracted orlistat from Alli			Standard orlistat			literature(Eisenreich et al., 2003)		
Position	Chemical shifts ppm		Position	Chemical shifts ppm		Position	Chemical shifts ppm	
	d^{13}C	d^{1}H		d^{13}C	d^{1}H		d^{13}C	d^{1}H
1''(C)	171.96		1''(C)	171.95		1''(C)	171.85	
1(C)	170.8		1(C)	170.79		1(C)	170.76	
CHO(CH)	160.66	8.1	CHO(CH)	160.64	8.22	CHO(CH)	160.77	8.18
NH		5.97	NH		5.95	NH		5.97
3(CH)	74.77	4.29	3(CH)	74.78	4.29	3(CH)	74.71	4.26
5(CH)	72.74	5.03	5(CH)	72.76	5.03	5(CH)	72.62	5.00
2(CH)	57.02	3.2	2(CH)	57.03	3.2	2(CH)	56.91	3.19
2''(CH)	49.6	4.68	2''(CH)	49.6	4.68	2''(CH)	49.57	4.65
3''(CH2)	41.54	1.63,1.52	3''(CH2)	41.56	1.63,1.53	3''(CH2)	41.33	1.63,1.52
4(CH2)	38.7	2.14,1.99	4(CH2)	38.7	2.14,1.99	4(CH2)	38.59	2.14,1.97
4'(CH2)			4'(CH2)			4'(CH2)	38.49	1.14
6(CH2)	34.05	1.63,1.56	6(CH2)	34.05	1.63,1.56	6(CH2)	33.94	1.62,1.56
14(CH2)	31.91	1.55	14(CH2)	31.91	1.55	14(CH2)	31.81	1.22
8(CH2)	29.61	1.56	8(CH2)	29.61	1.56	8(CH2)	29.53	1.25
9(CH2)	29.53	1.55	9(CH2)	29.54	1.55	9(CH2)	29.52	1.25
10(CH2)	29.43	1.56	10(CH2)	29.43	1.56	10(CH2)	29.45	1.25
11(CH2)	29.34	1.56	11(CH2)	29.35	1.57	11(CH2)	29.34	1.25
12(CH2)	29.31	1.56	12(CH2)	29.31	1.58	12(CH2)	29.25	1.25
13(CH2)	28.97	1.57	13(CH2)	28.97	1.59	13(CH2)	29.22	1.25
5'(CH)	27.62	1.54	5'(CH)	27.62	1.53	5'(CH)	27.75	1.49
1'(CH2)		1.72	1'(CH2)			1'(CH2)	27.55	1.77,1.71
3'(CH2)	26.71	1.31	3'(CH2)	26.71	1.29	3'(CH2)	26.95	1.28
2'(CH2)	25.1	1.3	2'(CH2)	25.1	1.29	2'(CH2)	26.9	1.39,1.28
7(CH2)	25.1	1.3	7(CH2)		1.25	7(CH2)	25.00	1.27
4''(CH2)	24.88	1.65	4''(CH2)	24.88	1.65	4''(CH2)	24.78	1.65
5''(CH3)	22.88	0.94	5''(CH3)	22.88	0.96	5''(CH3)	22.79	0.94
15(CH2)	22.69	1.3	15(CH2)	22.69	1.29	15(CH2)	22.59	1.25
6'/7'(CH3)	22.53	0.87	6'/7'(CH3)	22.52	0.88	6'/7'(CH3)	22.48	0.84
6''(CH3)	21.73	0.96	6''(CH3)	21.74	0.96	6''(CH3)	21.63	0.94
16(CH_3)	14.14	0.87	16(CH_3))	14.14	0.87	16($CH_3)_3$)	14.04	0.85

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Table 2-1 represents the data obtained by NMR for orlistat extracted from alli and standard orlistat. It also includes the data from the literature. The data shows that the orlistat extracted

from alli matches with the standard and literature. This orlistat was used as a positive control in all the experiments which required orlistat as a positive control.

2.4.3 Extraction of *Prunella vulgaris*, *Rheum palmatum*, and *Ilex latifolia*, and *Panax notoginseng*

The herbs were extracted in n-butanol using reflux extraction and ultrasonic extraction. N-butanol was chosen to extract the herbs because n-butanol extraction is one of the most common methods used for the extraction of organic components that do not dissolve in water (Zhao *et al*., 2010). The yield of the extracted herbs is summarized in table 2-2.

Table 2-2: Yield of the herbal extracts using n-butanol. 20 g of herbs coarse powder were extracted in n-butanol using two different methods (section 2.2.2.3). Reflux extraction at 120 ºC yielded bigger amount of extract compared to ultrasonic extraction at room temperature.

Herb	Method	Yield (g)
Prunella vulgaris	Reflux extraction	1.135
	Ultrasonic extraction	0.7945
Ilex latifolia	Reflux extraction	1.367
	Ultrasonic extraction	0.903
Rheum palmatum	Reflux extraction	1.283
	Ultrasonic extraction	0.783
Panax notoginseng	Reflux extraction	1.421
	Ultrasonic extraction	1.05

The results in the table 2-2 show that reflux extraction of *Prunella vulgaris* at 120 ºC yielded 30% more than than the ultrasonic extraction at room temperature. Reflux extraction also yielded 34%, 39%, and 27% more than ultrasonic extraction when used to extract *Ilex latifolia, Rheum palmatum, and Panax notoginseng.* This could be due to the higher temperature used in the reflux extraction.

2.4.4 Validation of pancreatic lipase assay by spectrophotometry

p-Nitrophenyllaurate was prepared in different concentrations to validate the method for the assay of pancreatic lipase activity. A dose-response curve was obtained (Figure 2-9). It showed that the absorbance increased as the substrate concentration increased. The enzyme was active in hydrolyzing p-nitrophenyllaurate to p-nitrophenol which has a yellow

color and has a maximum absorbance at 405 nm. Control samples containing the same amount of substrate with a concentration of THL (0.1 mg/mL) showed that the absorbance increased as the substrate concentration increased. THL inhibited pancreatic lipase activity because the enzyme activity increased as the THL concentration decreased (Figure 2-9). Thus, the assay method was validated to be used for the assay of pancreatic lipase inhibition activity of herbal extracts or herbal compounds using THL as a positive control.

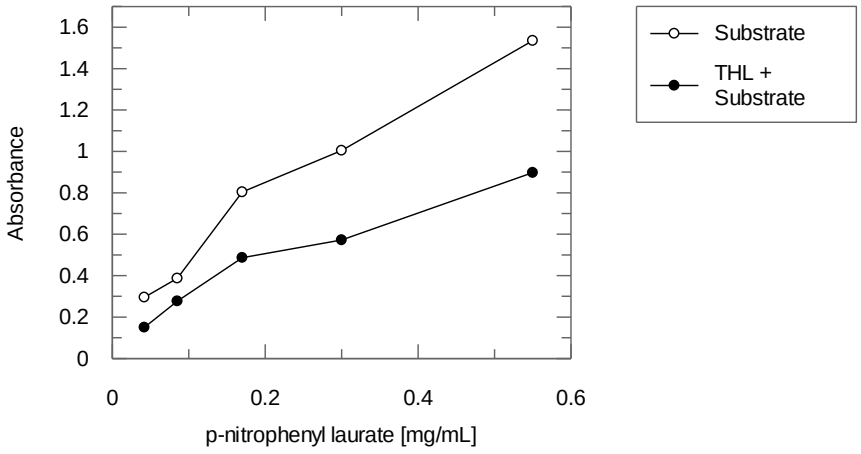


Figure 2-9: Dose-response curve of pancreatic lipase activity in the presence or absence of THL. The assay method used is described in section 2.2.2.4. The data are means of triplicates**.**

2.4.5 IC_{50} of THL assayed by spectrophotometry

THL was prepared at different concentrations and added to samples containing the same buffer, enzyme, and substrate concentrations as mentioned before. A dose-response curve (Figure 2-10) shows that THL inhibited the enzyme activity with an IC_{50} of 0.9480± 0.14 µg/mL. This was less than the IC_{50} of orlistat (5-10 µg/mL) determined by Zheng *et al*. (2010) where the inhibitory effect of herbs on porcine pancreatic lipase (1mg/mL) activity on *p*-nitrophenyl butyrate was investigated using a spectrophotometric assay . The enzyme was incubated with the tested extracts for 1 h before the addition of the substrate. But this result was much less than the IC_{50} of orlistat (0.076 µg/mL) determined by Kim (Kim *et al*., 2010) using an ELISA reader where 1.1 mg/mL of porcine pancreatic lipase were incubated with the tested compounds for 15 min at 37 ^{o}C before the addition of *p*-nitrophenyl butyrate as a

substrate. The IC_{50} of orlistat determined using titrimetric and spectrophotometric assays were 25 and 17 µg/mL (Saraphanchotiwitthaya and Sripalakit, 2014). In the titrimetric assay, the substrate triolein was incubated with the tested compounds for 30 min at 4 °C before adding the enzyme. In the spectrophotometric assay, the porcine pancreatic lipase enzyme (3.5 units) was incubated with the tested compounds for 30 min before the addition of *p*-nitrophenyl butyrate. The variation in the IC_{50} values between different assays could be due to the type of the substrate, the type of the enzyme, and the incubation time

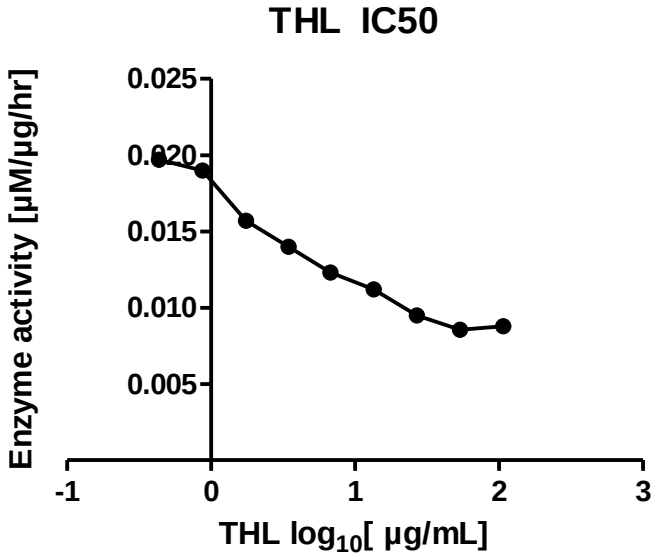


Figure 2-10: Inhibition of porcine pancreatic lipase type II by THL using a colorimetric assay The assay method is described in section 2.2.2.3. Data are the means of triplicates.

2.4.6 IC_{50} of quercetin assayed by spectrophotometry

Quercetin at different concentrations was tested for its inhibitory effect on pancreatic lipase, shown in Figure 2-11. The enzyme activity rate decreased as the quercetin concentration increased. The IC_{50} of quercetin was found to be 15.82 ± 1.19 µg/mL. In contrast quercetin inhibited the lipase by 27.4% at 25 µg/mL when assayed using the spectrophotometric assay. Quercetin was tested at 5, 10, and 25 µg/mL because at concentrations higher than that it was showing a rapid increase in the absorbance and it interfered with the results (Zheng *et al*., 2010). Quercetin showed a pancreatic lipase inhibition activity at IC_{50} of 43.9 µg/mL when assayed using a fluorometric assay method (Habtemariam, 2012).

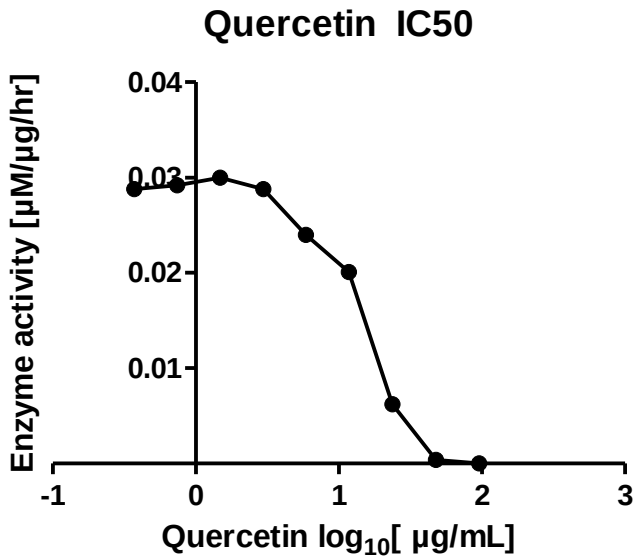


Figure 2-11: Inhibition of porcine pancreatic lipase type II by quercetin using a colorimetric assay. The assay method is described in section 2.2.2.4. Data are the means of triplicate.

2.4.7 Interaction of quercetin and THL on lipase assayed by spectrophotometry

Quercetin in the presence of THL was tested for its pancreatic lipase inhibition effect. Quercetin dissolved in DMSO was used as blank. A dose-response (Figure 2-12) curve was obtained. Quercetin had a high absorbance when the concentration was 25 µg/mL (abs 2.644) or higher where the absorbance at 95 µg/mL was 2.676. This absorbance interfered with the absorbance produced by *p*-nitrophenol. The graph below (Figure 2-12) shows that the absorbance produced by the quercetin is not proportional to the concentration and then the effect is not linear. As a result, the interference can not be cancelled by using quercetin as a blank. Thus, the method was not appropriate to assay the effect of herbal extracts on pancreatic lipase.

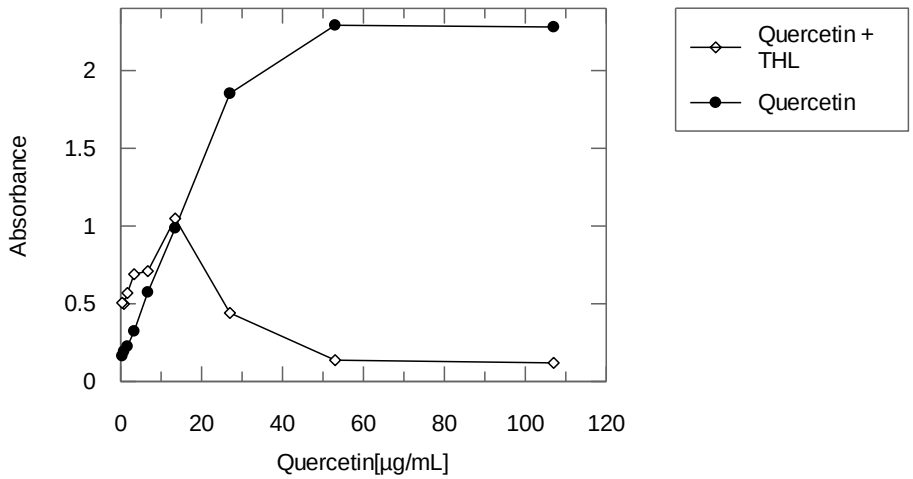


Figure 2-12: The inhibition effect of quercetin on porcine pancreatic lipase when combined with THL was assayed using colorimetric assay method. Quercetin was used as a blank and the absorbance was subtracted from the samples absorbance. Quercetin absorbance was very high at high concentrations and this resulted in non reliable results. All the samples were prepared in triplicate.

2.4.8 *P*-Nitrophenol calibration curve and the limit of detection using HPLC

The main target of this chapter is to investigate the anti-pancreatic lipase activity of herbal extracts and herbal compounds. Colorimetric assays are commonly used for this type of assay. The results obtained by using spectrophotometric assay were not accurate or reliable because there was interference from the tested samples with the results. To avoid any interference, HPLC was used because HPLC is more sensitive and specific than spectrophotometric assay methods.

p-Nitrophenol was prepared at different concentrations. All the samples were analysed using HPLC. A calibration curve (Figure 2-13) was obtained using Excel by plotting the p-nitrophenol concentration against the area under the curve. This calibration curve was used to determine the limit of detection of the method to investigate the sensitivity and the accuracy of the method. The limit of detection was 0.03 µg/mL, and the range of detection was linear up to the maximum concentration tested, 25 µg/mL. This compares favourably with the lower limit of detection previously reported for the same technique of 0.75 µg/mL (Maurich *et al*., 1991). The method is sensitive and accurate and could be used for the investigation and assay of pancreatic lipase inhibitors.

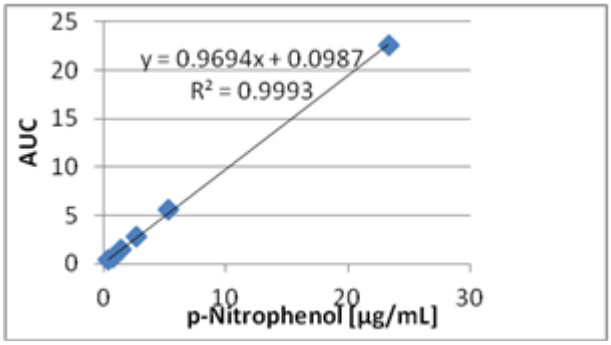


Figure 2-13: Calibration curve of p-nitrophenol using HPLC.
Samples were prepared and assayed as described in section 2.2.2.9.

2.4.9 Validation of pancreatic lipase assay using HPLC

Pancreatic lipase of porcine pancreas type II was prepared at different concentrations. Different samples containing the same substrate concentration were prepared. Enzyme solutions with different concentrations were added to each sample. The p-nitrophenol released was calculated using an internal reference standard. The p-nitrophenol released increased as the amount of the enzyme increased. Consequently, inhibition of the enzyme will result in the inhibition of the production of p-nitrophenol and this can be used to investigate the effect of herbal extracts on pancreatic lipase using orlistat as a positive control.

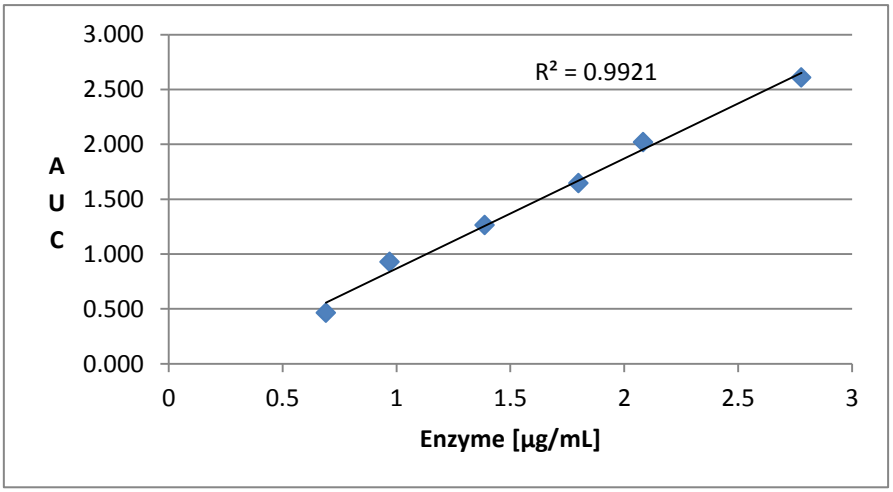


Figure 2-14: Dose-response curve of pancreatic lipase against p-nitrophenol [method from 2.2.2.8.2]

2.4.10 IC_{50} of THL assayed using HPLC

THL was added at different concentrations to the samples which contained the same substrate and the same enzyme concentration (4.2 mg/mL). A dose-response curve (Figure 2-15) was obtained. The curve shows that the enzyme activity decreases as the amount of THL increases. The IC_{50} of THL was found to be 0.8921$\pm$ 0.0415 µg/mL.

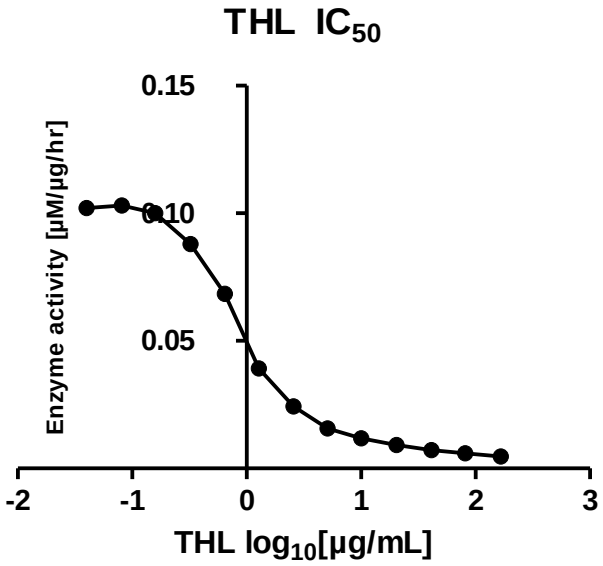


Figure 2-15: Inhibition of porcine pancreatic lipase type II by THL using HPLC assay (section 2.2.2.8). Data are the means of triplicates.

To check if the method is reproducible and reliable, 2,4-dinitroaniline was used as an internal standard. The chromatogram (Fig. 2-16) shows that the peak of p-nitrophenol (t_r =3.4 min) decreased as the concentration of THL increased, but the peak of 2,4-dinitroaniline (t_r =4.44 min) did not change. This means that the change in the p-nitrophenol is due to the inhibitory effect of THL on the pancreatic lipase and not due to sample processing or instrumental errors (Figure 2-16).

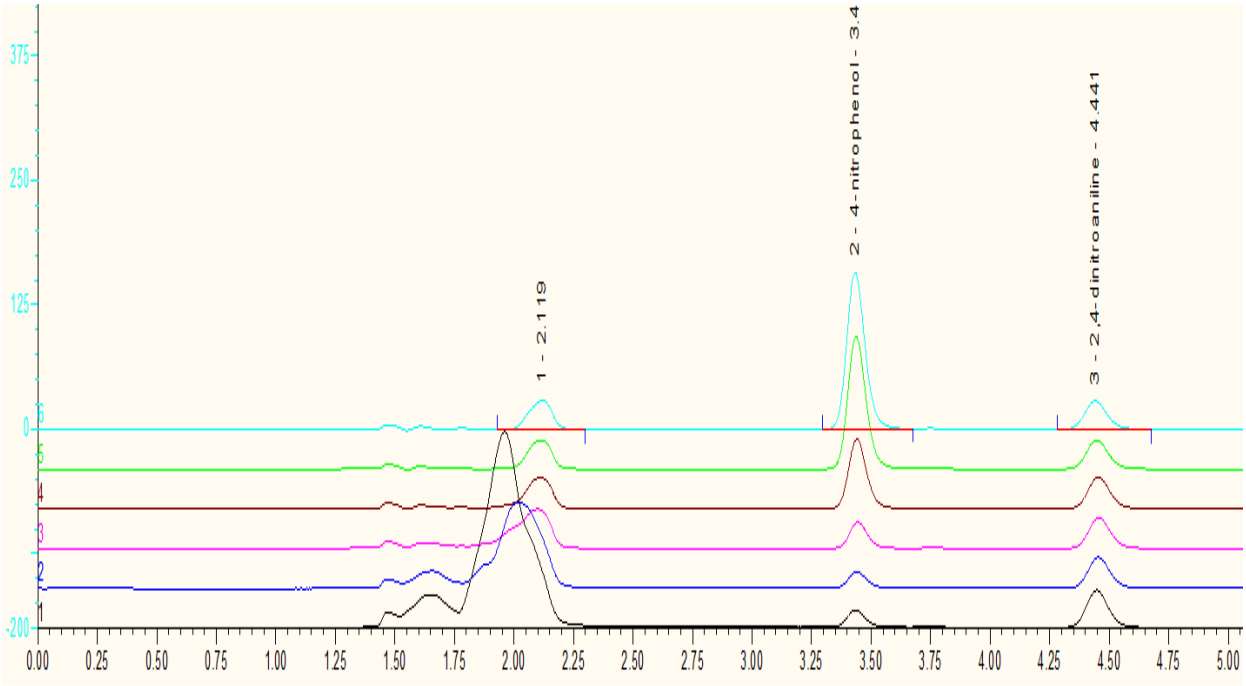


Figure 2-16: Chromatogram of 4-nitrophenol in the presence of 2,4-dinitroaniline. The peak under the area of 4-nitrophenol changed with the inhibitor concentration while the peak of 2,4-dinitroaniline, which was used as an internal standard, was constant (section2.2.2.8). The different chromatograms show that 4-nitrophenol concentration was changing while 2,4 dinitrioaniline was constant when THL concentration was changing.

2.4.11 IC_{50} of different compounds assayed using HPLC

2.4.11.1 IC_{50} of quercetin assayed using HPLC

Quercetin was added at different concentrations to samples containing constant amounts of enzyme (4200 µg/mL and substrate (0.274 mM) respectively. The enzyme activity was found to be the same in all the samples which meant that quercetin has no pancreatic lipase inhibitory activity up to a concentration of 100 µg/mL.

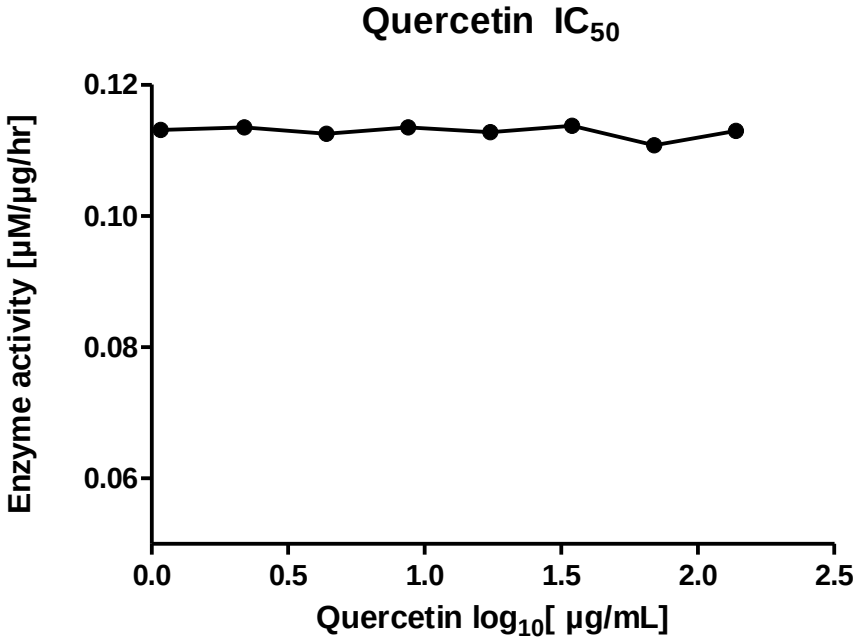


Figure 2-17: Effect of quercetin on porcine pancreatic lipase type II assayed using HPLC assay (section 2.2.2.9). Data are the means of triplicates.

2.4.11.2 IC_{50} of crocin assayed using HPLC

Crocin was added at different concentrations to samples containing the same amount of enzyme and the same amount of substrate to investigate its effect on pancreatic lipase and to determine the IC_{50}. The dose response obtained (Figure 2-18) shows that crocin doesn't have any inhibitory activity and that the enzyme activity is constant in all the samples. Crocin has been tested for pancreatic lipase inhibition activity using spectrophotometric assay method and the IC_{50} was found to be 28.6 µM (Sheng *et al*., 2006), which corresponds to 27.9 µg/mL. The difference between the two assays could be due to different conditions used. In the assay used by Sheng *et.al.,* pancreatic lipase 2.43 µg/mL were used and the substrate triolein was incubated with crocin (0.1-10000 µg/mL) for 30 min before the addition of the enzyme.

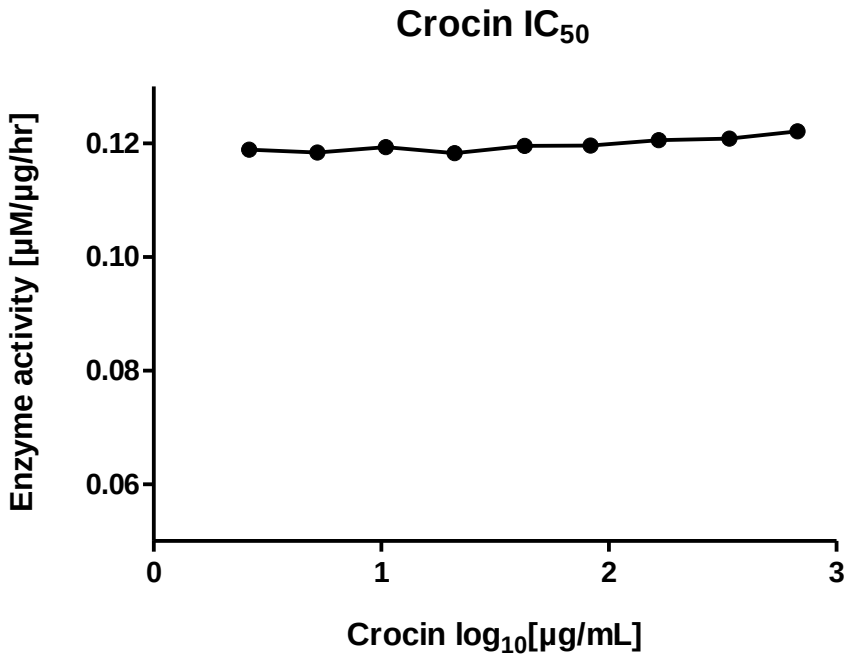


Figure 2-18: Inhibition of porcine pancreatic lipase type II by crocin using HPLC assay (section 2.2.2.9). Data are the means of triplicates.

2.4.11.3 IC_{50} of emodin assayed using HPLC

Emodin was added in different concentrations to samples containing constant substrate (0.274 mM) and enzyme (42.91 µg/mL) concentrations respectively. A dose-response curve (Figure 2-19) was obtained. It showed that emodin has no pancreatic lipase inhibition activity because the enzyme activity was constant in all the samples. In contrast others have found that emodin inhibited pancreatic lipase activity by 12 % at 25 µg/mL (Zheng *et al*., 2010). These results were concluded using a colorimetric assay where the enzyme (1 mg/mL) was incubated with the tested compounds for 1 h at 37 ^{o}C before the addition of *p*-nitrophenyl laurate substrate.

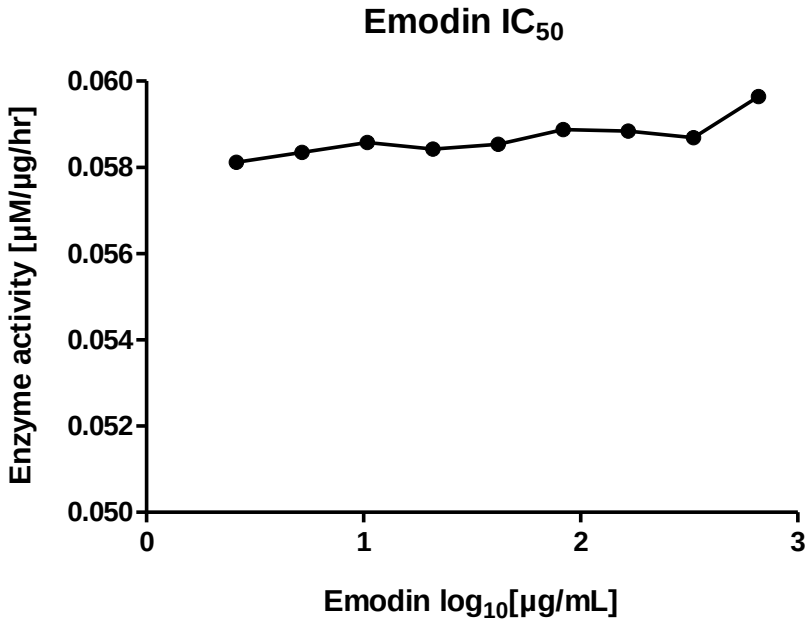


Figure 2-19: **Inhibition of porcine pancreatic lipase type II by emodin using HPLC assay (section 2.2.2.9).** Data are the means of triplicates.

2.4.11.4 IC_{50} of hesperidin assayed using HPLC

Hesperidin was added at different concentrations to the samples which contained the same enzyme and substrate concentrations respectively. The enzyme activity was the same in all the samples. A dose-response curve (Figure 2-20) shows that hesperidin does not have any pancreatic lipase inhibition activity. The IC_{50} of hesperidin is 32 µg/mL (Birari and Bhutani, 2007). The method is not mentioned but hesperidin is a polyphenol which means it's a hydrophilic compound and its binding with the pancreatic lipase enzyme is weak.

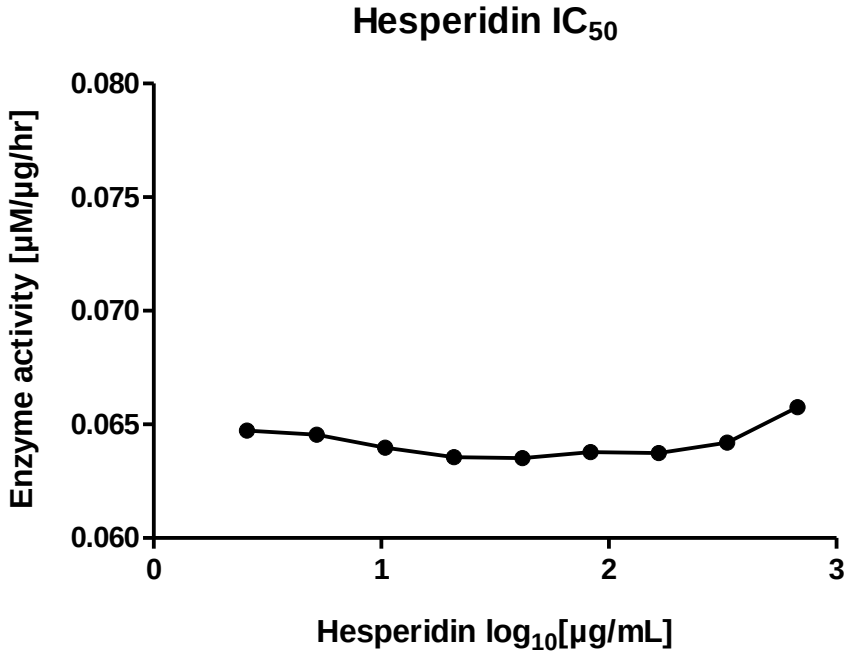


Figure 2-20: **Inhibition of porcine pancreatic lipase type II by hesperidin using HPLC assay (section 2.2.2.9).** Data are the means of triplicate.

2.4.12 Effect of *Rheum palmatum* on pancreatic lipase assayed using HPLC

Rheum palmatum extract was dissolved in DMSO. It was added at different concentrations to samples containing the same amounts of enzyme (330 µg/mL) and substrate. The enzyme activity was constant in all the samples as shown by the dose-response curve obtained (Figure 2-21).

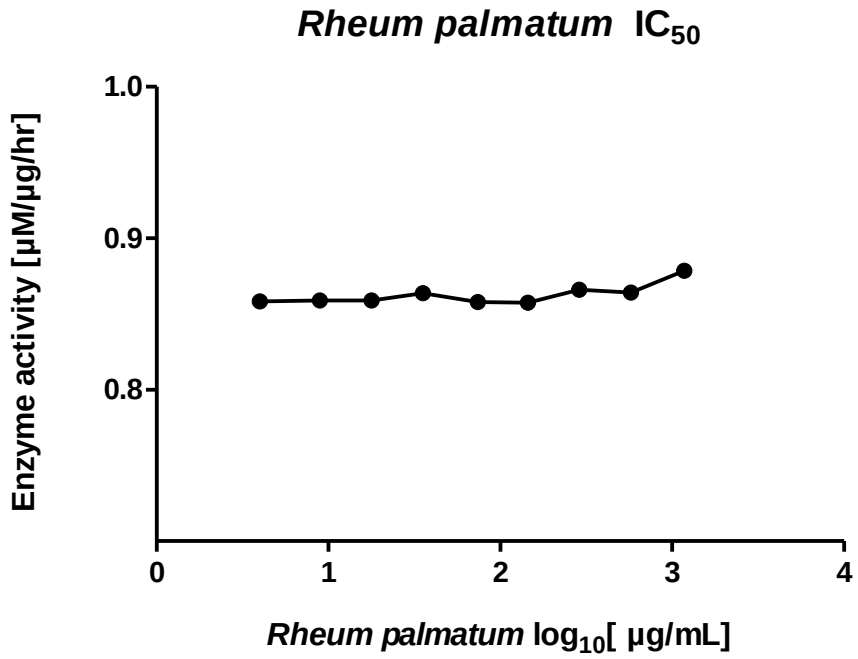


Figure 2-21: Inhibition of porcine pancreatic lipase type II by *Rheum palmatum* using HPLC assay (section 2.2.2.10). Data are the means of triplicate.

2.4.13 Effect of *Prunella vulgaris* on pancreatic lipase assayed using HPLC

Prunella vulgaris extract was dissolved in DMSO. The extract solution was added at different concentrations to samples containing the same concentrations of enzyme (300 µg/mL) and substrate (0.183 mM). A dose-response curve (Figure 2-22) was obtained. The graph shows that the enzyme activity decreased as the concentration of *Prunella vulgaris* increased, and maximal inhibition was achievable. The IC_{50} of *Prunella vulgaris* is 71.8 $\pm$ 0.51 µg/mL. *Prunella vulgaris* has a antihyperglycemic effect in diabetic rats *(Jie et al*., 2007) and showed a pancreatic lipase inhibition activity of > 50% at 25 µg/mL *(Zheng et al*., 2010). The active compounds which are pancreatic lipase inhibitors are to be explored because quercetin and other polyphenols which are present in *Prunella vulgaris* have been shown that they are not active.

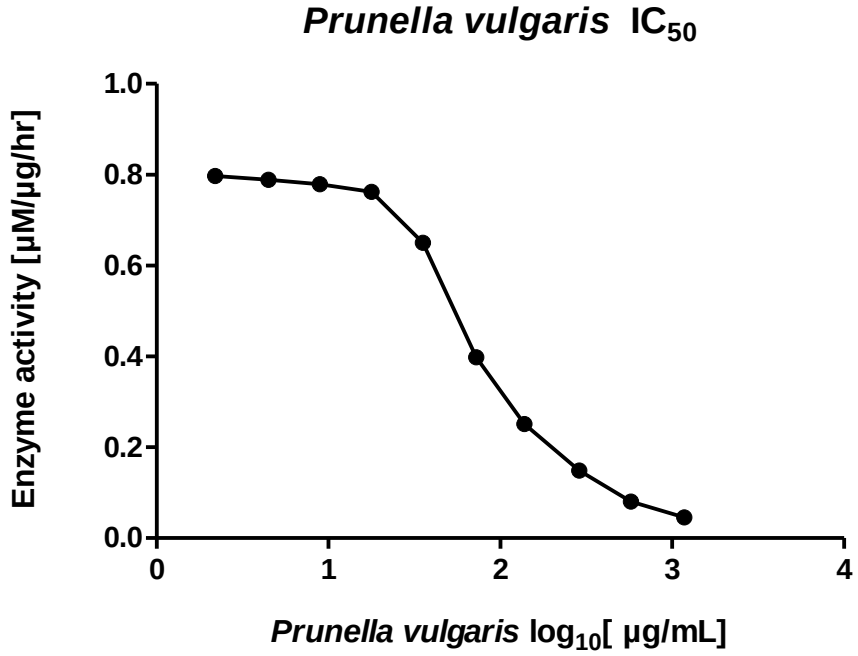


Figure 2-22: Inhibition of porcine pancreatic lipase type II by *Prunella vulgaris* using HPLC assay (section 2.2.2.10). Data are the means of triplicate.

2.4.14 Effect of *Prunella vulgaris* when combined with THL on pancreatic lipase assayed using HPLC

Prunella vulgaris solution was added at different concentrations to samples containing the same enzyme (330 µg/mL) and substrate 0.183 mM) concentrations respectively. Other samples were prepared by adding the same *Prunella vulgaris* concentrations to fixed concentration of THL (0.027 µg/mL). A control sample was prepared by adding THL but DMSO was added instead of *Prunella vulgaris*. The enzyme activity was higher in the samples containing *Prunella vulgaris* in comparison to the samples containing *Prunella vulgaris* and THL (Figure 2-23). The IC_{50} of *Prunella vulgaris* in the absence of THL was 63.7 $\pm$ 0.5 µg/mL. The IC_{50} of *Prunella vulgaris* in the presence of THL (0.027 µg/mL) was 50.2$\pm$0.6 µg/mL. THL in the control sample (0.027 µg/mL) inhibited the enzyme activity by 19.79 %. The IC_{50} of *Prunella vulgaris* was 19.75 less when it was combined with THL. The change in *Prunella vulgaris* IC_{50} was similar to the effect of THL. This means that the presence of THL did not improve the activity of *Prunella vulgaris*. This could be explained as *Prunella vulgaris* and THL could have a competitive effect on pancreatic lipase or it could be

due to another mechanism which needs further investigation because the mechanism of action of *Prunella vulgaris* is not known. This could be because they have the same or different enzyme receptors. This should be explored in the future after the active compounds are isolated.

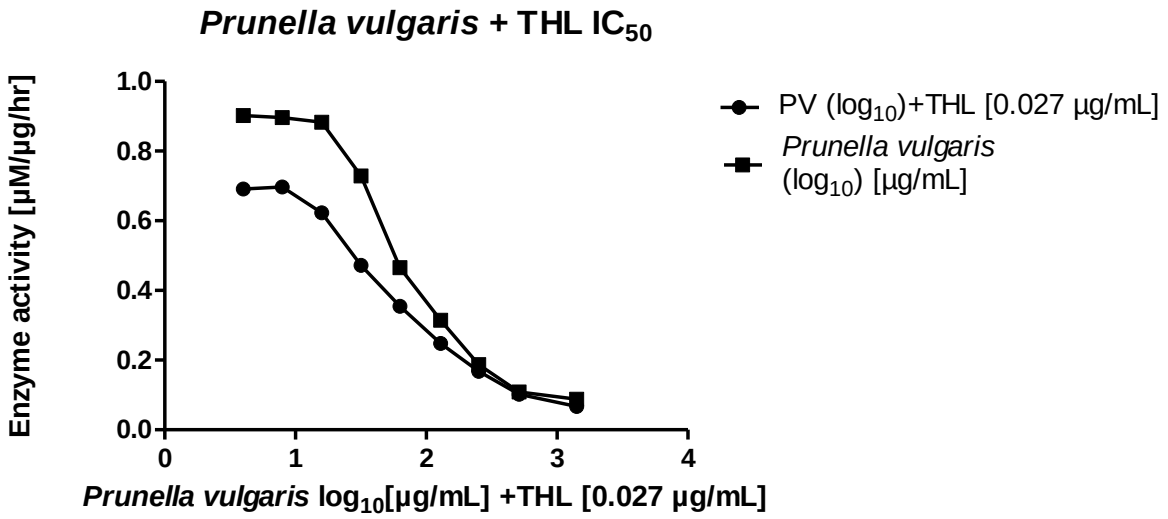


Figure 2-23: Inhibition of porcine pancreatic lipase type II by *Prunella vulgaris* (■) and in the presence of THL (●) using HPLC (section 2.2.2.11). Data are the means of triplicates**.**

2.4.15 Effect of *Ilex latifolia* on pancreatic lipase assayed using HPLC

Ilex latifolia butanol extract was dissolved in DMSO. The extract solution was added at different concentrations to samples containing constant enzyme (330 µg/mL) and substrate (0.183 mM) concentrations. The enzyme activity decreased as the concentration of *Ilex latifolia* extract increased, although maximal inhibition was not achieved. The IC_{50} of *Ilex latifolia* was calculated as 1418 $\pm$ 111 µg/mL. *Ilex latifolia* is reported to lower cholesterol and consequently used for the treatment of hyperlipidemia (Zheng *et al*., 2009).

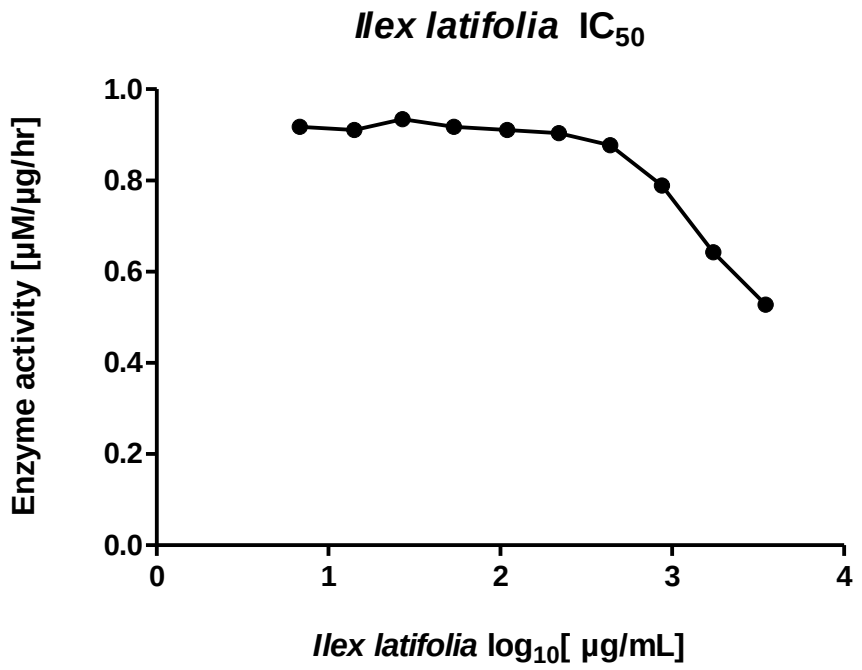


Figure 2-24: Inhibition of porcine pancreatic lipase type II by *Ilex latifolia* using HPLC assay (section 2.2.2.10). Data are the means of triplicates.

2.4.16 Effect of pancreatic lipase different concentrations on p-nitrophenyllaurate using HPLC

During the previous experiments, some of the herbal extracts were showing inhibition activity at high concentration. When the extract was diluted, the extract had been losing any inhibition activity. The analysis of the results led to two conclusions. The first conclusion was that the extracts were not active as mentioned before in the previous reports. The second conclusion was that the substrate, p-nitrophenyllaurate, was saturated with the enzyme especially that it has been used in small concentrations as mentioned in the substrate stock solution preparation. This means that the amount of enzyme remaining in the solution after the inhibition action occurs is enough to hydrolyze the substrate and give a high concentration of the p-nitrophenol. The main aim of this experiment was to dilute the enzyme and check if the amount of p-nitrophenol produced was changing with different enzyme concentrations. The graph (Figure 2-25) shows that the amount of p-nitrophenol was steady at some enzyme concentrations (366 µg/mL, 183 µg/mL, and 91.5 µg/mL, respectively). The

amount of p-nitrophenol released started to change at 45.75 µg/mL. As a result, any inhibition activity due to any compound or extract on the enzyme will result in a response difference. Consequently, all the extracts and quercetin will be assayed for their pancreatic lipase inhibition activity.

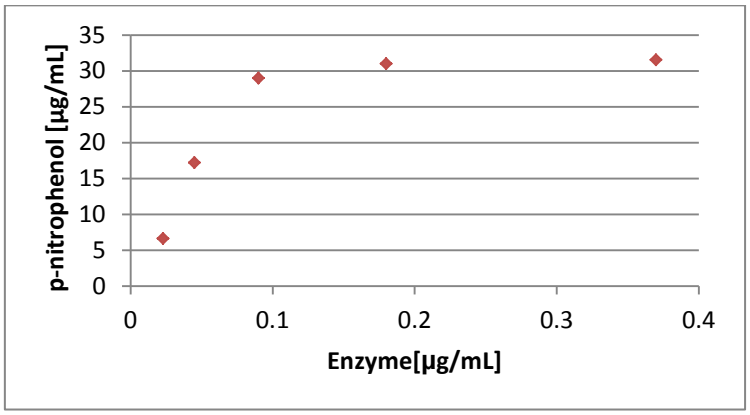


Figure 2-25: Dose-response curve of pancreatic lipase and p-nitrophenol

2.4.17 IC_{50} of THL using HPLC

THL was added at different concentrations to the samples which contained constant substrate and enzyme concentrations. A dose-response curve (Figure 2-26) was obtained. The curve shows that the enzyme activity increases as the amount of THL decreases. IC_{50} of THL was found to be 0.0017$\pm$ 0.0003 µg/mL. THL is the only clinically approved drug as a pancreatic lipase inhibitor with IC_{50} 0.75 µg/mL (Chaput *et al*., 2007). THL interacts with pancreatic lipase and inactivates it by forming of stable covalent intermediate (Bitou *et al*., 1999).

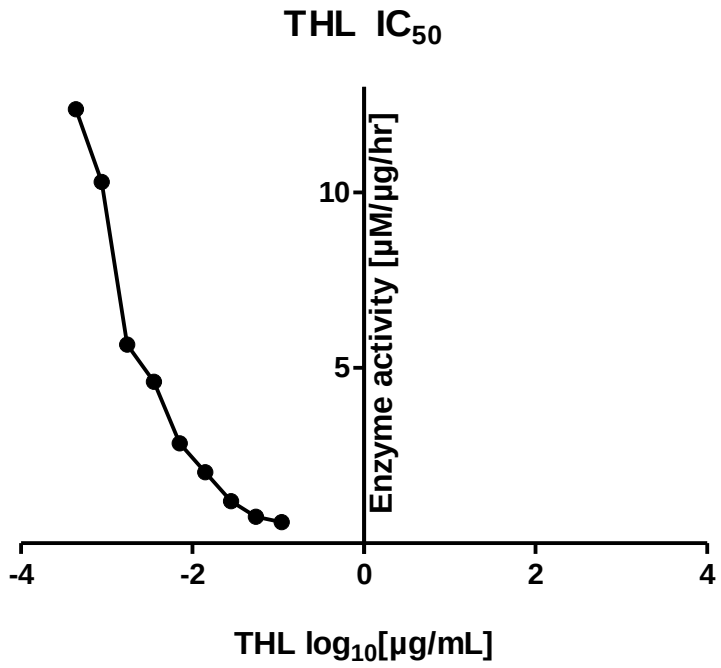


Figure 2-26: Inhibition of porcine pancreatic lipase type II by THL using HPLC assay (section 2.2.2.13). The results were analysed and plotted using graphpad prism. Data are the means of triplicates

2.4.18 IC_{50} of *Rheum palmatum* using HPLC

Rheum palmatum butanol extract different concentrations were added to samples containing constant enzyme and substrate concentration. The extract showed an inhibition activity which decreased as the concentration of the extract decreased. A dose-response curve (Figure 2-27) shows that the enzyme activity increased as the concentration of the extract decreased. IC_{50} of *Rheum palmatum* was found to be 117.1$\pm$ 12.4 µg/mL. *Rheum palmatum* at a concentration of 200 µg/mL inhibited pancreatic lipase by 53.8% (Zheng *et al*., 2010). This assay was carried out using spectrophotometry which appeared to be not suitable in previous experiments especially that all herbal extracts with high concentrations have a high absorbance in a wide range of wavelength.

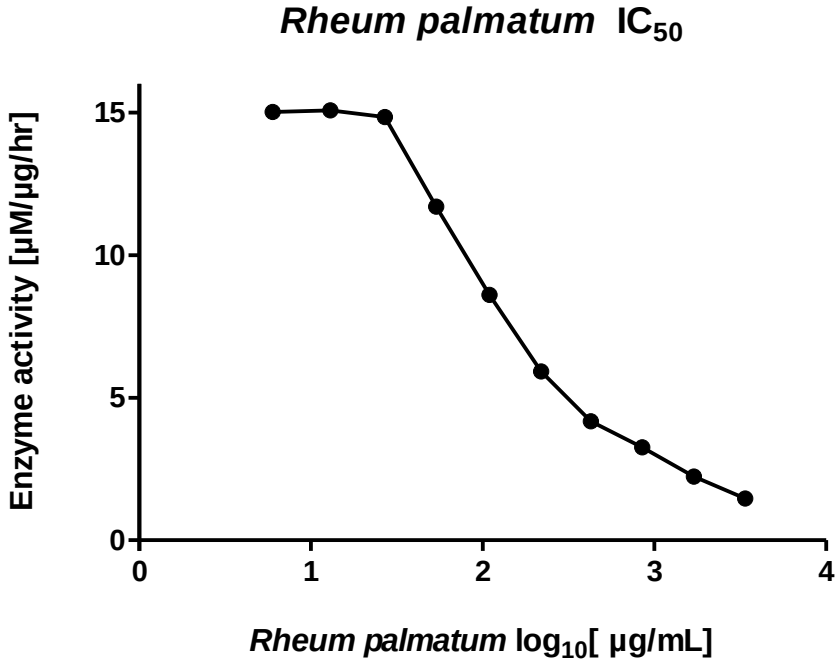


Figure 2-27: Inhibition of porcine pancreatic lipase type II by *Rheum palmatum* using HPLC assay (section 2.2.2.14).The results were analysed and plotted using graphpad prism. Data are the means of triplicate.

2.4.19 IC_{50} of *Prunella vulgaris* using HPLC

Prunella vulgaris butanol extract was dissolved in DMSO. The solution was serially diluted in two-fold resulting in different concentrations. All the *Prunella vulgaris* samples were added to samples containing constant enzyme and substrate concentrations. A dose-response curve (Figure 2-28) was obtained. It shows that the *Prunella vulgaris* extract inhibited the enzyme and the extract activity was increasing as the concentration increases. IC_{50} of *Prunella vulgaris* was found to be 250.7 ± 27.6 µg/mL. *Prunella vulgaris* with concentration of 200 µg/mL inhibited pancreatic lipase by 74.5% (Zheng *et al*., 2010). The assay of *Prunella vulgaris* extract was done using a spectrophotometer method which as mentioned before is not accurate especially that the extracts showed a high absorbance at the specified wavelength and this would interfere with the results. The assay was done using concentrations less than 50 µg/mL which affects the accuracy of the results.

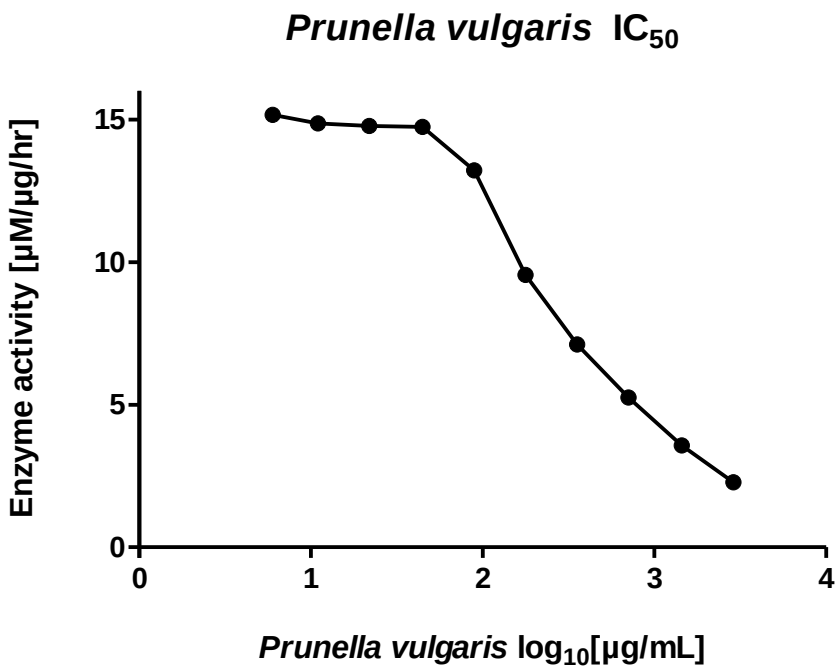


Figure 2-28: Inhibition of porcine pancreatic lipase type II by *Prunella vulgaris* using HPLC assay (section 2.2.2.14).The results were analysed and plotted using graphpad prism. Data are the means of triplicate.

2.4.20 IC_{50} of *Ilex latifolia* using HPLC

Ilex latifolia. butanol extract different concentrations were added to samples containing constant enzyme and substrate concentrations. The extract showed an inhibition activity which decreased as the concentration of the extract decreased. A dose-response curve (Figure 2-29) was obtained. It shows that the amount of p-nitrophenol released increased as the concentration of the extract decreased. The IC_{50} of *Ilex latifolia* was found to be 76.10$\pm$ 7.58 µg/mL. The pancreatic lipase activity has not been previously assayed before for the *Ilex latifolia.* The traditional use for weight loss could be due to inhibition of pancreatic lipase or other enzymes.

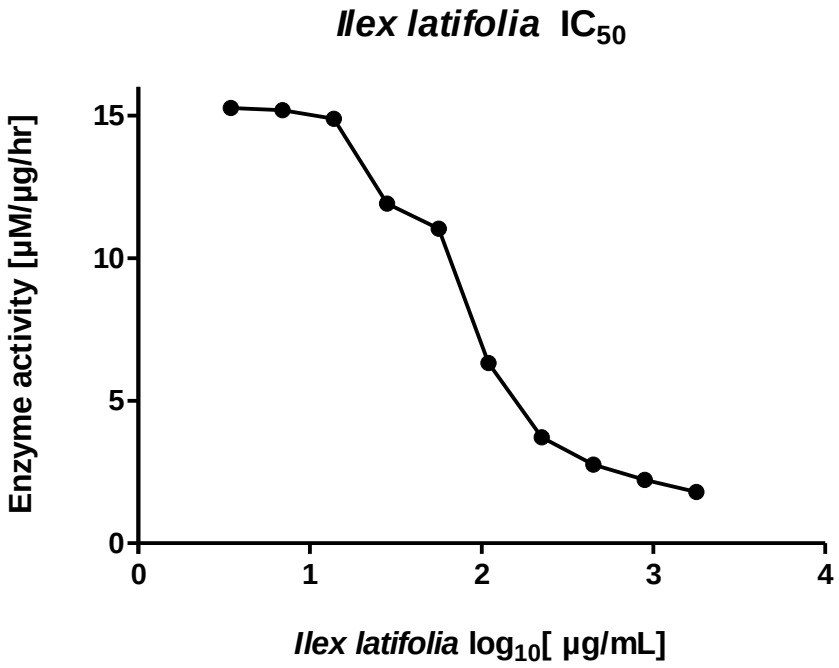


Figure 2-29 Inhibition of porcine pancreatic lipase type II by *Ilex latifolia* using HPLC assay (section 2.2.2.14).The results were analysed and plotted using graphpad prism. Data are the means of triplicate.

2.4.21 IC_{50} of *Panax notoginseng* using HPLC

Panax notoginseng bark butanol extract was added in different concentrations to samples containing constant enzyme and substrate concentrations. The dose-response curve (Figure 2-30) obtained shows that the enzyme activity increased as the extract concentration decreased. IC_{50} of *Panax notoginseng* was found to be 431.2±31 µg/mL. *Panax notogensing* reduced cholesterol at a concentration of 40 mg/kg by 30 percent compared to a positive control, pravastatin (Joo *et al*., 2010). *Panax notogensing* is a promising Chinese herb for the treatment of hyperlipidemia because in-vivo studies showed that *Panax notogensing* extracts can reduce blood cholesterol and triglycerides.

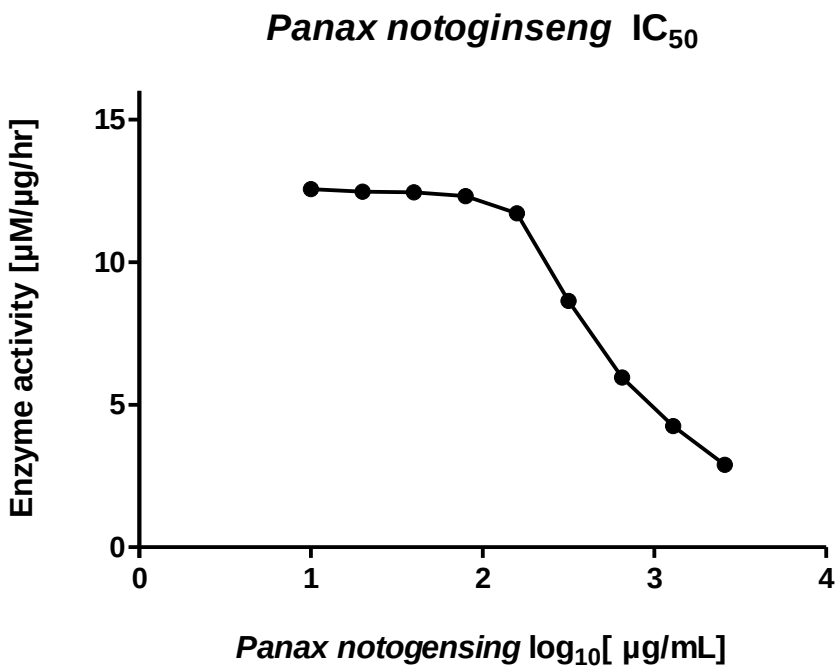


Figure 2-30: Inhibition of porcine pancreatic lipase type II by *Panax notogensing* using HPLC assay (section 2.2.2.14).The results were analysed and plotted using graphpad prism. Data are the means of triplicate.

2.4.22 IC_{50} of quercetin using HPLC

Quercetin was added at different concentrations to samples containing constant amounts of enzyme and substrate respectively. The enzyme activity was increasing as the concentration of the quercetin decreased (Figure 2-31). IC_{50} of quercetin was found to be 136.68 $\pm$ 6.96 µg/mL. Quercetin is the main compound present in *Prunella vulgaris*. It inhibited pancreatic lipase by 27.4% at a concentration of 25 µg/mL (Zheng *et al*., 2010). The difference between the results in this report and the results of other studies is due to the applied method. The results in previous studies were obtained using spectrophotometry which is not very accurate especially that quercetin was showing a high absorbance at high concentrations. Consequently, low concentrations were used. HPLC which is used in this project is more sensitive and accurate and is not affected by the absorbance of the tested compound.

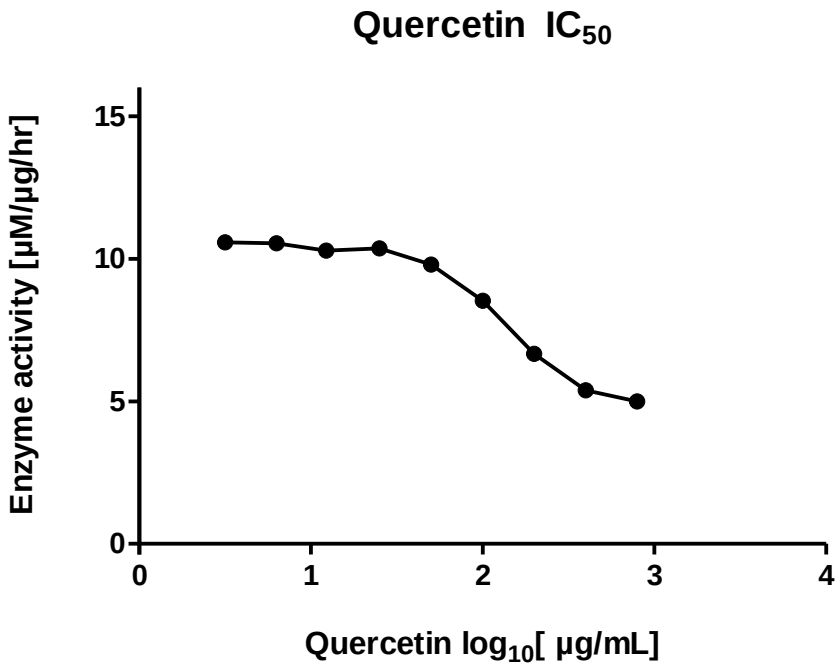


Figure 2-31:Inhibition of porcine pancreatic lipase type II by quercetin using HPLC assay (section 2.2.2.14). The results were analysed and plotted using graphpad prism. Data are the means of triplicate.

Table 2-3: Summary of the IC_{50S} of herbs and quercetin tested for their pancreatic lipase inhibition activity. THL was used as a positive control.

	Pancreatic lipase 330 µg/mL	Pancreatic lipase 42.91 µg/mL
Tested sample	IC_{50} (µg/mL)	IC_{50} (µg/mL)
Rheum palmatum	No activity	117.1 ± 12.4 (µg/mL)
Prunella vulgaris	71.8 ± 0.51 (µg/mL)	250.7 ± 27 (µg/mL)
Ilex latifolia	1418 ± 111 (µg/mL)	76.1 ± 7.5 (µg/mL)
Panax notoginseng	Not tested	431.2 ± 31 (µg/mL)
Quercetin	No activity	136.68 ± 6.96 (µg/mL)
THL (positive control)	0.8921± 0.0415 (µg/mL)	0.0017 ± 0.0003 (µg/mL)

2.5 Discussion

Dietary fat absorption and cholesterol synthesis are the main factors in the development of hyperlipidemia. Pancreatic lipase is an enzyme secreted by the intestine and is responsible for the hydrolysis of 50-70% of the dietary fats. Digestion of dietary fats leads to the hydrolysis of triglycerides into fatty acids and monoglycerides which are absorbed from the intestine in the form of micelles with bile acids. These micelles release the fatty acids and monoglycerides to form triglycerides rich chylomicrons. Inhibition of fat absorption by inhibition of pancreatic lipase is one of the main factors in the prevention of development of hyperlipidemia and consequently in the protection against heart diseases.

To investigate the pancreatic lipase inhibition activity, 4-nitrophenyl laurate was used as a substrate where pancrartic lipase hydrolyses it to produce 4-nitrophenol which has been detected using spectrophotometric method because of its reliability and because it is easy to perform.

In this project, pancreatic lipase inhibition activity was investigated using orlistat as a positive control. Orlistat decreases the absorption of dietary fats of 30 % in adults leading to an improved lipid profile (Ballinger and Peikin, 2002). Orlistat inhibited pancreatic lipase at IC_{50} 0.9480 µg/mL. This was less than the IC_{50} of orlistat (5-10 µg/mL) determined by Zheng (Zheng *et al*., 2010) using spectrophotometric assay. But this result was higher than the IC_{50} of orlistat (0.076 µg/mL) determined by Kim (Kim *et al*., 2010) using ELISA reader. The IC_{50} of orlistat using titrimetric assay was 0.025 mg/mL (Saraphanchotiwitthaya and Sripalakit, 2014). The inhibition activity of orlistat was not complete and this could be due to the substrate type and concentration, the enzyme concentration, or the retention time.

The method was used to investigate the inhibition activity of quercetin and then the inhibition activity of herbal extracts such as *Prunella vulgaris*, *Ilex latifolia*, and *Rheum palmatum*. It was also supposed for the investigation of the inhibition activity of herbal compounds such as emodin, crocin, and hesperidin.

Quercetin which showed an inhibition activity of 27 % (Zheng *et al*., 2010) and at IC_{50} 43.9 µg/mL (Habtemariam, 2012) was used to assay the efficiency of spectrophotometric method for the assay of herbal extracts and compounds pancreatic lipase inhibition activity. Quercetin showed a high absorbance at the wavelength used and consequently the spectrophotometric method was not suitable for the assay of herbal extracts.

The next step was to develop a more sensitive and accurate method where the interference from the herbal compounds can be avoided. HPLC method is more sensitive technique and was used for the determination of the suitable substrate for pancreatic lipase (Maurich *et al*., 1991). Orlistat was used as a positive control. Orlistat inhibited pancreatic lipase at IC_{50} 0.8921 µg/mL. No data about the IC_{50} of orlistat by HPLC. This IC_{50} compared to the IC_{50} mentioned before show that orlistat is a potent pancreatic lipase inhibitor and the activity was more than that obtained by spectrophotometric technique.

This method was used to assay the pancreatic lipase inhibition activity of *Prunella vulgaris*. *Prunella vulgaris* has a antihyperglycemic effect in diabetic rats (Jie *et al*., 2007) and showed a pancreatic lipase inhibition activity of > 50% at 25 µg/mL (Zheng *et al*., 2010). In this project *Prunella vulgaris* inhibited pancreatic lipase at IC_{50} of 71.8 µg/mL. This method was also used to investigate the pancreatic lipase inhibition activity of *Rheum palmatum* which has been used to treat hyperlipidemia in diabetic rats (Xie *et al*., 2005b). *Rheum palmatum* inhibited pancreatic lipase of more than 50% at 25 µg/mL (Zheng *et al*., 2010). In this project, *Rheum palmatum* did not show any pancreatic lipase inhibition activity. *Ilex latifolia* which has been used in traditional Chinese medicine because it has an inhibitory activity on the formation of foam cells and reduced intracellular triglycerides and cholesterol content (Zheng *et al*., 2009), was assayed for its pancreatic lipase inhibition activity using HPLC technique. *Ilex latifolia* showed a minimal pancreatic lipase inhibition activity at IC_{50} of 1418 $\pm$ 111 µg/mL. The same method was used to investigate the pancreatic lipase activity of selected compounds such as crocin which inhibited pancreatic lipase at 2.7 mg/mL (Lee *et al*., 2005) using titrimetric method. Crocin did not show any inhibition activity using HPLC. Hesperidin

which inhibited pancreatic lipase at IC_{50} of 32 µg/mL (Birari and Bhutani, 2007) and emodin which inhibited pancreatic lipase activity of 12 % at 25 µg/mL (Zheng *et al*., 2010) were investigated and they did not show any inhibition activity.

One of the disadvantages of this method is that the inhibition activity of the compounds was changing very quickly when going from high to lower concentration. The main concern was that the enzyme concentration is too high and even after inhibition, the remaining active enzyme is enough to hydrolyze the substrate.

The next step was to find an optimal concentration. The concentration of the enzyme was determined from the log phase because at certain concentrations, the enzyme had a static effect although the concentration was increasing. The concentration was determined as mentioned in the methods and the previously tested compounds were investigated again. Orlistat was still showing high inhibition activity at 0.0017 $\pm$ 0.0003 µg/mL. *Prunella vulgaris*, *Rheum palmatum*, *Ilex latifolia*, and *Panax notoginseng* showed inhibition activity at 250.7, 117.1, 76.10, and 431.2 µg/mL respectively. Quercetin was tested and it showed inhibition activity at 136.68 µg/mL.

In conclusion, herbal products could be an alternative treatment for hyperlipidemia resulting from high levels of triglycerides because these herbal products can inhibit pancreatic lipase which is the main enzyme in hydrolyzing dietary fats. Detection of the inhibitory activity of these herbs and their active compounds depend on the sensitivity and the accuracy of the technique used. HPLC was more efficient in the assay of pancreatic lipase inhibition activity of herbal compounds than spectrophotometric assay. Even HPLC assay is affected by different factors such as the concentration and type of the substrate, the type and strength or concentration of the enzyme, and the retention time.

3 Chapter III: Effect of *Ilex latifolia* fractions on pancreatic lipase activity

3.1 Introduction

3.1.1 *Ilex latifolia*

The dried leaves of *Ilex latifolia*, a herb known as ku-ding-cha in China, has been used as an adjuvant treatment of headache, cold, diabetes and hypertension (Benchao, 1999). It has been also used as a beverage tea to refresh the mind, improve eye sight, quench thirst, and remove phlegm for 2000 years. The most common kudingcha used in China are from two genera in different families, genus *Ligustrum* in the family Oleacceae, and the genus *Ilex* in the family Aquifoliaceae (Zhu *et al*., 2009). Phytochemical studies revealed that *Ilex latifolia* contains bioactive compounds such as triterpenes and triterpenoid saponins (Huang *et al*., 2001, Wang *et al*., 2012) which have anti-depressant, antitumor, and antiviral activities (Pemminati S., 2011, Kiplimo *et al*., 2011, Fujiwara *et al*., 2011). Triterpenoid saponins are responsible for the potential activity of *Ilex latifolia* on lipid metabolism (Zheng *et al*., 2009). It also contains caffeoylquinic acids (Negishi *et al*., 2004) which have anti-inflammatory, anti-oxidant, and antiviral activities (Deng *et al*., 2011, Li *et al*., 2005).

Ilex latifolia and *Ilex* kudingcha are the main components of the large leaved kudingcha (Feng, 1998, Zhu *et al*., 2009). The main experiments on isolation of pure and active compounds from *Ilex latifolia* and large leaved kudingcha were limited to the characterisation of flavonols such as rutin quercetin, and kaempferol, common triterpenes such as ursolic acid and oleanolic acid, and mono-and dicaffeoylquinic acids. These compounds do not represent the real activity of the herb (Fan *et al*., 2013).

Ilex latifolia was fractionated using HPLC and the collected fractions were assayed for their pancreatic lipase inhibition activity.

3.2 Experimental methods

3.2.1 Materials

p-Nitrophenyl laurate, pancreatic lipase of porcine pancreas type II, DMSO, THL, sodium chloride, taurocholic acid sodium salt, sodium dihydrogen phosphate, and 2,4-dinitroaniline were obtained from Sigma-Aldrich (Gillingham, England). Hydrochloric acid, analytical grade formic acid, acetonitrile HPLC grade, and Millipore filters (0.45 µm) were obtained from Fisher Scientific UK limited (Loughborough, England). All the experiments were done using *Ilex latifolia* butanol extract prepared as described in Chapter II, section 2.3.3. The HPLC apparatus used was a Dionex Ultimate 3000. The NMR instrument used was a Bruker Avance 500. The freeze drier used was Heto Powerdry PL 3000.

3.2.2 Methods

3.2.2.1 Separation and collection of fractions of Ilex latifolia

3.2.2.1.1 Separation into four fractions

23 mg of *Ilex latifolia* butanol extract were dissolved in 1 mL of butanol. The solution was filtered using a 0.45 µm Millipore filter. The constituents of the filtrate were separated using HPLC at room temperature using a 250mm x 8 mm I.D. column packed with 5 µm C_{18}. The flow rate was 2 mL/min. The mobile phase was 0.5 % formic acid (A) in water and acetonitrile (B). Elution was performed with 2 % B for 2 min, 2-8 % B up to 8 min, 10-30 % B up to 10 min, 30% B for 3 min, 30%-50 % B up to 27 min, 50%-70% B up to 8 min, 70%-2% B up to 2 min, and 2% B for 6 min. The peaks were detected at 260 nm. The chromatogram was divided into four fractions. Fraction 1 was collected from 0 to 22 min, fraction 2 from 22 to 31 min, fraction 3 from 31 to 40 min, and fraction 4 from 40 to 66 min. The method was repeated several times. Each Fraction contained 200 mL of the eluate. The organic solvent was diluted using Buchner vacuum pump and then the aqueous solvent was evaporated using freeze drying. The fractions were weighed and stored at 4 ºC.

3.2.2.1.2 Separation into eight fractions

The method was adapted from 3.2.2.1.1. 60 mg of *Ilex latifolia* butanol extract were dissolved in 1 mL of n-butanol. The solution was filtered using 0.45 µm Millipore filter. The flow rate was 2 mL/min. The mobile phase was 0.5 % formic acid (A) in water and acetonitrile (B). Elution was performed with 2 % B for 2 min, 2-8 % B up to 8 min, 8-35 % B up to 20 min, 35-80 % B up to 30 min, 80% B for 5 min, 80%-2% B up to 2 min, 2% B for 8 min. The detection wavelength was 260 nm. The chart speed was 0.5 cm/min. The chromatogram was divided into eight fractions. Fraction 1 (14.5-17 min), fraction 2 (17-18.8 min), fraction 3 (20.5-22.5 min), fraction 4 (22.5-23.5 min), fraction 5 (23.5-25.5 min), fraction 6 (25.5-28 min), fraction 7 (29.3-30.5 min), fraction 8 (36-39 min). The acetonitrile was evaporated using a vacuum pump (Buchi vacuum pump V-700). The aqueous solvent was removed using freeze drying. The dried materials were weighed and stored at 4 ºC. All the fractions were dissolved in 1 mL of DMSO.

3.2.2.1.3 Separation into 10 fractions

The method was adapted from 3.2.2.1.1. 65 mg *Ilex latifolia* butanol extract were dissolved in 1 mL of n-butanol. The solution was filtered using 0.45 µm Millipore filter. The flow rate was 2.5 mL/min. The mobile phase was a 0.5% formic acid (A) in water and acetonitrile (B). The mobile phase was 90 % A to 10% B for 2 min, 70 % (A) and 30 % (B) for 13 min, 40 % (A) and 60 % (B) for 10 min, 90 % A to 10% B for 2 min, and 90 % A to 10% B for 7 min to equilibrate the column. The detection wavelength was 260 nm. Ten fractions (F1-F10) were collected. Each fraction was around 45 mL. The acetonitrile was removed from the collected fractions using Buchner vacuum pump. The aqueous solvent was freeze dried. The dry material in the tubes was weighed for F5-F10 after drying. F1-F4 did not contain any material after drying.

3.2.2.2 Assay of pancreatic lipase inhibition activity of the Ilex latifolia fractions

The method (2.2.2.12) was adapted with some modifications. Briefly, pancreatic lipase of porcine pancreas type 2 was made up to 0.2575 mg/mL in 0.1 M phosphate buffer (pH 7.4).

Fraction 1 (3.75 mg/mL in 200 µL of DMSO) , fraction 2 (1.25 mg/mL in 200 µL of DMSO), fraction 3 (1.9 mg/mL in 400 µL of DMSO), and fraction 4 (5.75 mg/mL in 200 µL of DMSO) (section 3.2.2.1.1) were used instead of THL. All the fractions were serially diluted two-fold four times in DMSO. All the samples were incubated with the enzyme for 10 minutes before the addition of the substrate. The fractions collected in methods (3.2.2.1.2 and 3.2.2.1.3) were assayed for pancreatic lipase inhibition activity using the same method.

3.2.2.3 Nuclear magnetic resonance of collected fractions

Ten fractions were collected as single peaks (section 3.2.2.1.3). Fractions 5 to 10 showed pancreatic lipase inhibition activity. Nuclear magnetic resonance was used for structure elucidation. ^{1}H and ^{13}C NMR were carried out in deuterated DMSO. The NMR instrument was operated by Mr. John Crowder, NMR technician at London Metropolitan University.

3.3 Results

3.3.1 Four fractions collected from *Ilex latifolia*

In order to determine the active components, the butanol extract of *Ilex latifolia* was fractionated using preparative HPLC and the chromatogram is shown in Figure 3-1. Four fractions were collected (section 3.2.2.1.1), dried and weighed. The amounts obtained are summarized in Table 3-1.

Table 3-1: Yields of four fractions of the butanol extract obtained by HPLC (section 3.2.2.1.1)

Fraction	Elution time (min)	Yield (mg)
1	0 - 22	1.5
2	22 – 31	0.5
3	31 – 40	3.8
4	40 - 66	2.3

Each fraction contains a number of peaks, indicating that further separation is required to resolve individual peaks.

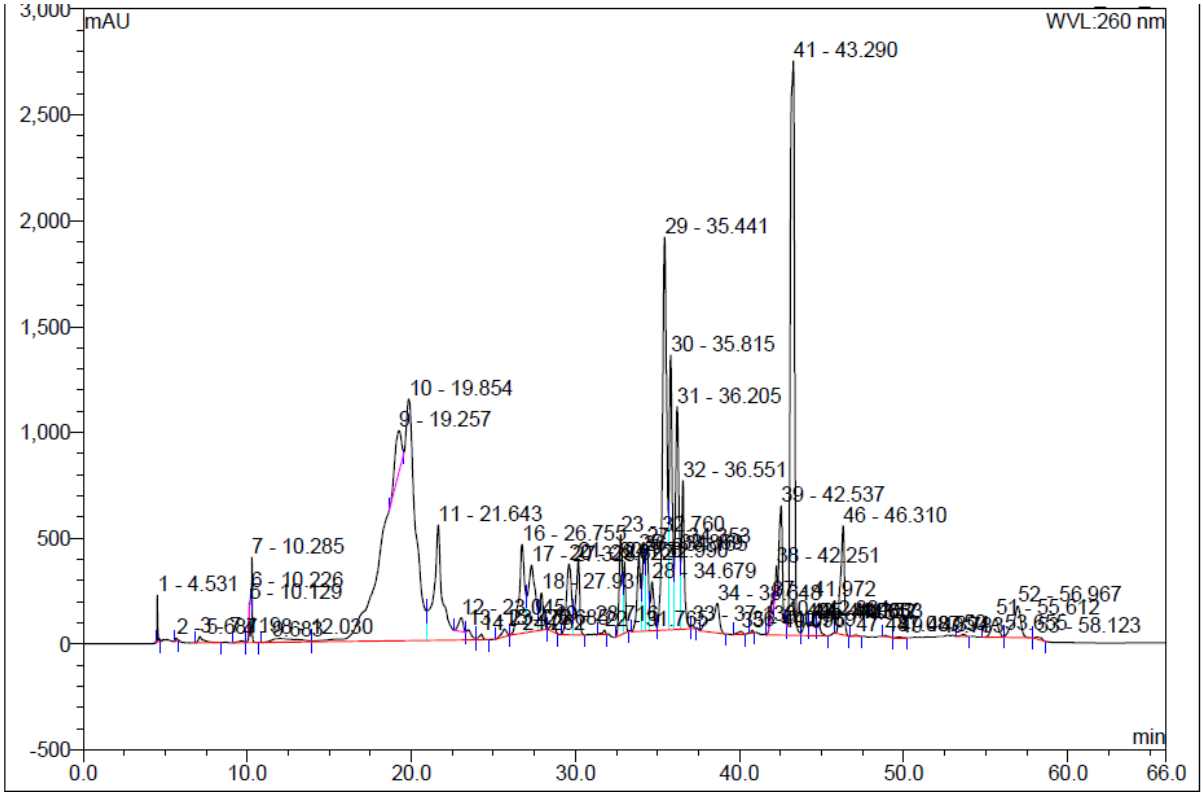


Figure 3-1: Chromatogram of separation of components of butanolic extract of *Ilex latifolia* by HPLC (section 3.2.2.1.1).

3.3.2 Effect of the four fractions on pancreatic lipase activity

The four fractions were assessed for potential inhibitory activity of pancreatic lipase, using a variant of the method described in 2.2.2.15. The results for fraction1 (3.5 mg/mL), which was serially diluted two fold in DMSO, are shown in Figure 3-2. A clear inhibition of pancreatic lipase was achieved, with IC_{50} = 6.25 $\pm$1.66 µg/mL.

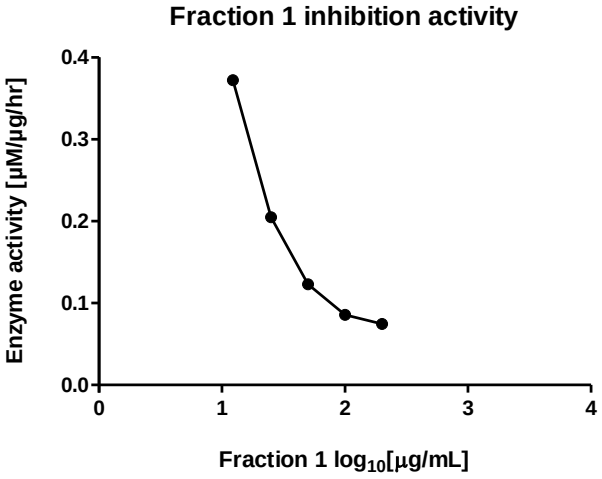


Figure 3-2: Inhibition of porcine pancreatic lipase type II by fraction 1 (section 3.2.2.1.1) assessed using HPLC assay (section 3.2.2.2). The results were analysed and plotted using GraphPad Prism. Data are the means of triplicates.

Fraction 2 (1.25 mg/mL) was serially diluted two-fold five times using DMSO. The pancreatic lipase inhibition assay showed that the enzyme activity was increasing as the concentration of the fraction decreased. The IC_{50} was found to be ~ 12.25 $\pm$2.6 µg/mL.

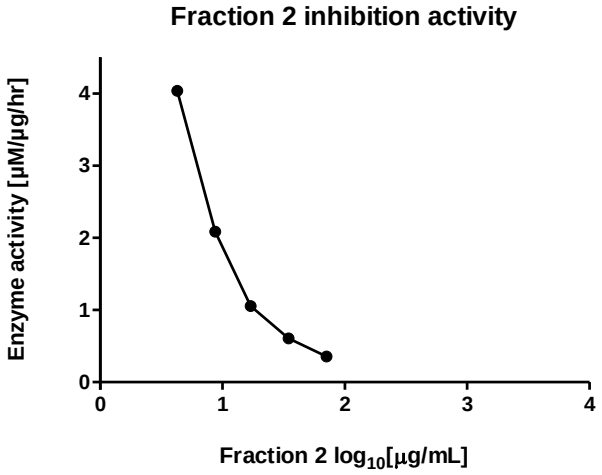


Figure 3-3: Inhibition of porcine pancreatic lipase type II by fraction 2 (section 3.2.2.1.1) using HPLC assay (section 3.2.2.2). The results were analysed and plotted using graphpad prism. Data are the means of triplicate.

Fraction 3 (1.9 mg/mL) was serially diluted 2 folds in DMSO. The enzyme inhibition activity was assayed and the result was that the enzyme activity increased as the concentration of the fraction decreased. The IC_{50} was found to be 50.1 $\pm$ 7.678 µg/mL.

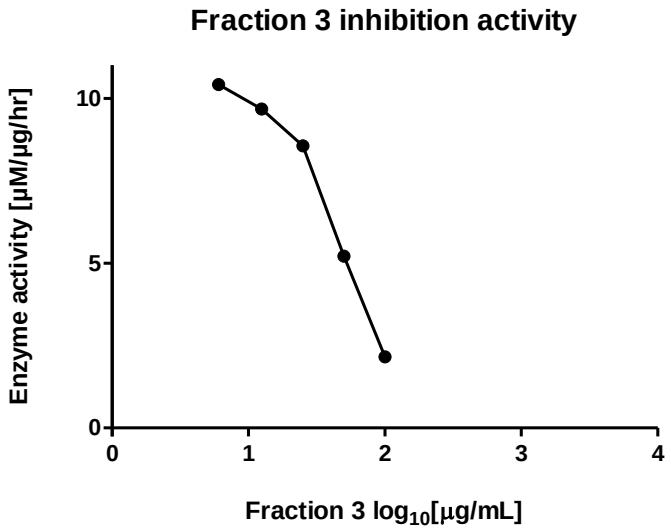


Figure 3-4: Inhibition of porcine pancreatic lipase type II by fraction 3 (section 3.2.2.1.1) using HPLC assay (section 3.2.2.2). The results were analysed and plotted using graphpad prism. Data are the means of triplicate.

Fraction 4 (5.75 mg/mL) was serially diluted 2 folds in DMSO. The enzyme inhibition activity was assayed and the result was that the enzyme activity increased as the concentration of the fraction decreased. The IC_{50} was found to be 52.34 $\pm$ 3.34 µg/mL.

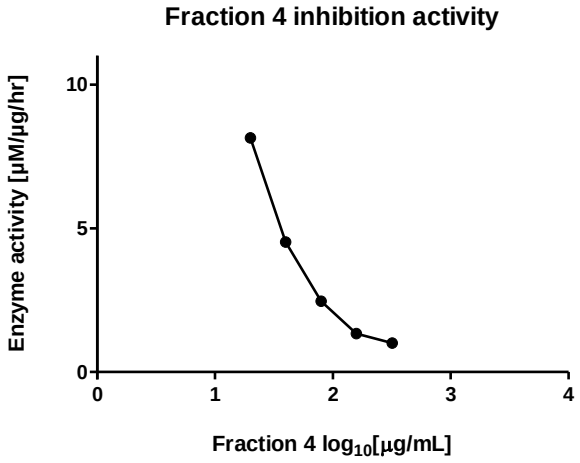


Figure 3-5: Inhibition of porcine pancreatic lipase type II by fraction 4 (section 3.2.2.1.1) using HPLC assay (section 3.2.2.2). The results were analysed and plotted using GraphPad prism. Data are the means of triplicates.

3.3.3 Eight fractions collected from *Ilex latifolia*

In an attempt to fractionate further the components of the butanol extract a second HPLC separation was attempted using a slightly higher final concentration of acetonitrile, 80%, compared with the first fractionation which used up to 70% acetonitrile. The chromatogram is shown in Figure 3-6. Eight fractions were collected, dried and weighed (Table 3-2).

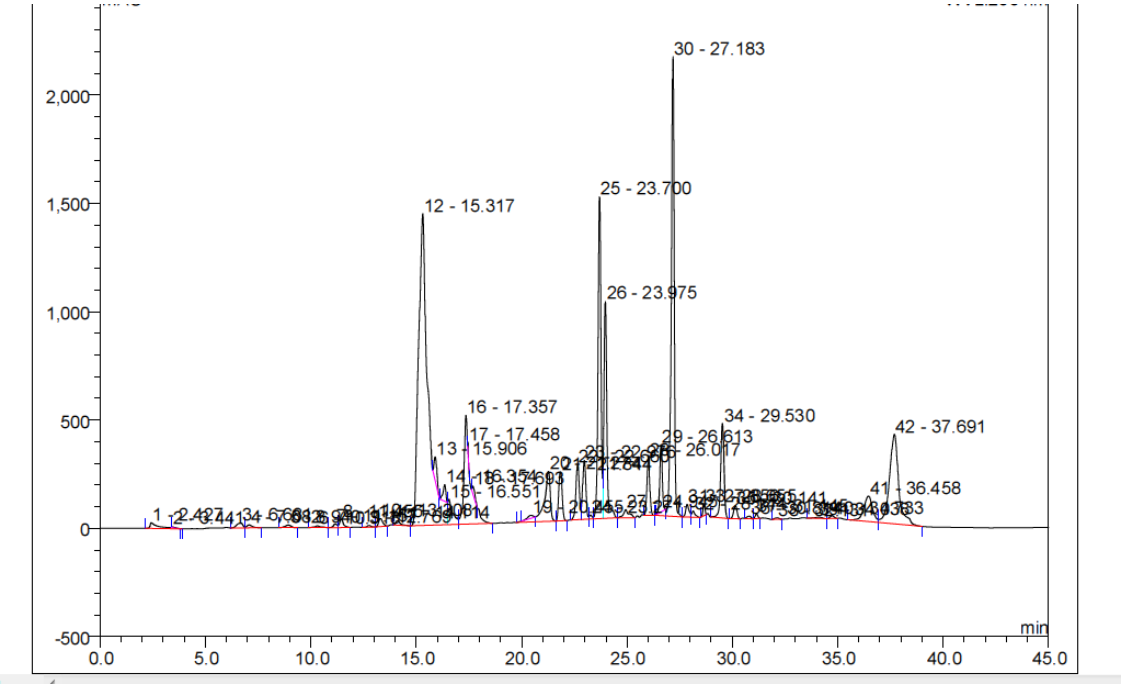


Figure 3-6: Chromatogram of separation of components of butanolic extract of *Ilex latifolia* by HPLC (section 3.2.2.1.2)

Table 3-2: A summary of the amount obtained in each fraction collected (section 3.2.2.1.2)

Fraction	Elution time (min)	Yield (mg)
1	14.5 - 17	0.14
2	17 – 18.8	0.75
3	20.5 – 22.5	1.94
4	22.5 – 23.5	No yield
5	23.5 – 25.5	0.5
6	25.5 - 28	0.97
7	29.3 – 30.5	No yield
8	36 - 39	2.3

3.3.4 Inhibitory action of the eight fractions on pancreatic lipase

The eight fractions were assessed for inhibitory activity towards pancreatic lipase. A preliminary assessment was carried out of the eight fractions at concentrations as follows: fraction 1 (0.14 mg/mL), fraction 2 (0.75 mg/mL), fraction 3 (1.94 mg/mL), and fraction 4, fraction 5 (0.5 mg/mL), fraction 6 (0.97 mg/mL), fraction 7, and fraction 8 (2.3 mg/mL) in 1 m L of DMSO. The data, shown in Table 3-3, indicate that all fractions were active, and inhibited at least about 50%, and the most active fractions were fractions 2, 6, and 8. The IC_{50} of these fractions were determined by assaying two-fold serially diluted fractions.

Table 3-3: Representative data showing inhibitory activity of *Ilex latifolia* fractions

Fraction	Conc.[mg/mL]	AUC	p-Nitrophenol [µg/mL]
F1	0.14	14.068	14.4
F2	0.75	10.635	10.86
F3	1.94	14.953	15.32
F4	_	15.869	16.27
F5	0.5	15.275	15.65
F6	0.97	10.53	10.76
F7	_	9.333	9.52
F8	2.3	7.83	8
THL	0.01	0.841	0.76
Control	DMSO	30.451	31.31

The IC_{50} of fraction 2 was measured at 187.2 ± 14.2 µg/mL (Figure 3-7), IC_{50} of fraction 6 was 86.2 ± 12.7 µg/mL (Figure 3-8) and for fraction 8 was 12.7 ± 2.6 µg/mL (Figure 3-9).

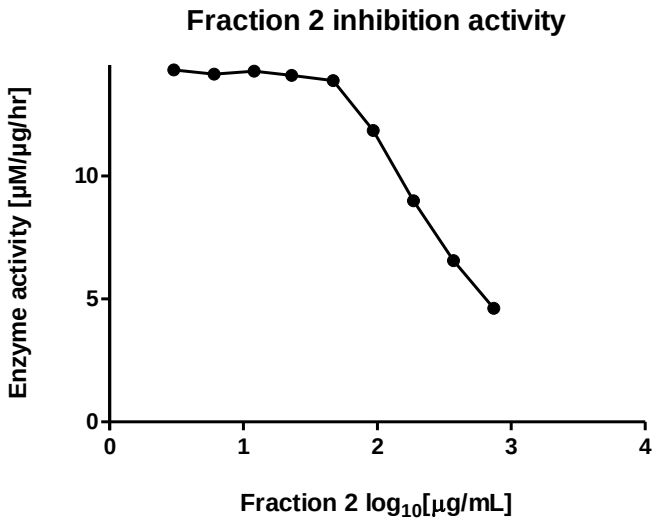


Figure 3-7: Inhibition of porcine pancreatic lipase type II by fraction 2 using HPLC assay (section 3.2.2.2). The results were analysed and plotted using GraphPad Prism. Data are the means of triplicate.

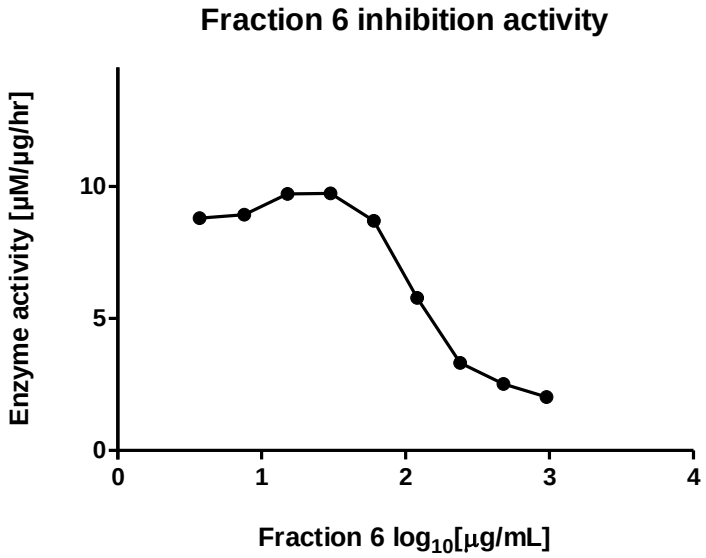


Figure 3-8: Inhibition of porcine pancreatic lipase type II by fraction 6 using HPLC assay (section 3.2.2.2). The results were analysed and plotted using GraphPad Prism. Data are the means of triplicates.

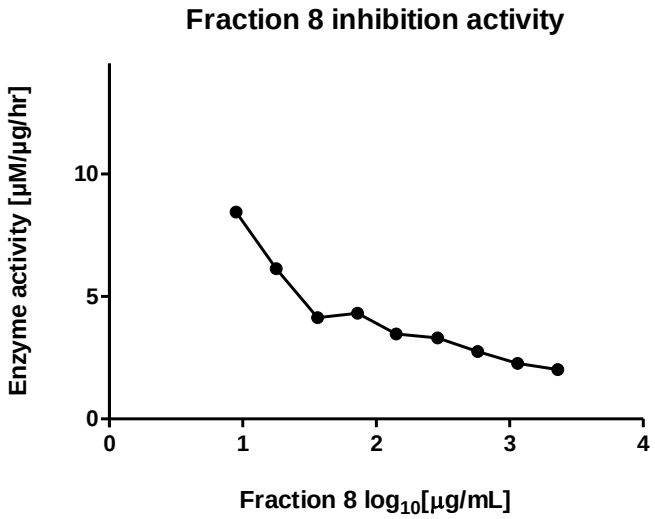


Figure 3-9: Inhibition of porcine pancreatic lipase type II by fraction 8 using HPLC assay (section 2.2.2.8). The results were analysed and plotted using GraphPad Prism. Data are the means of triplicate.

3.3.5 10 fractions collected from *Ilex latifolia*

A further attempt to separate and isolate components was carried out using a maximum proportion of acetonitrile of 60%, shown in Figure 3-10. Ten fractions were collected, dried and weighed (Table 3-4).

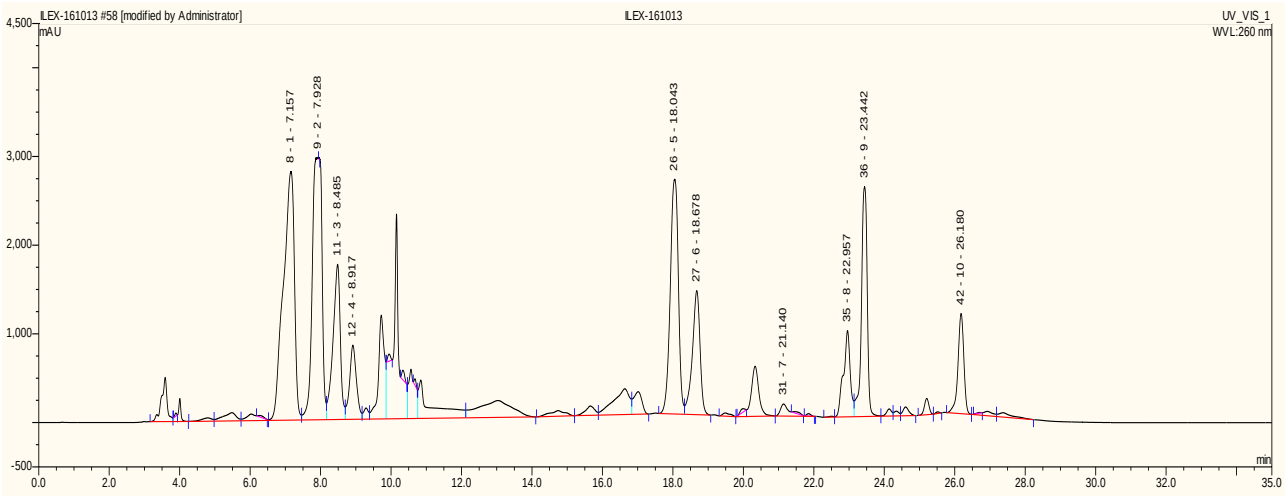


Figure 3-10: Chromatogram of *Ilex latifolia* separation using HPLC. The peaks collected are labelled by their retention time.

Table 3-4: A summary of the amount obtained in each fraction collected (section 3.2.2.1.3)

Fraction	Yield (mg)
F1	Negligible
F2	Negligible
F3	Negligible
F4	Negligible
F5	4.7
F6	20
F7	1.2
F8	4.7
F9	3.4
F10	1.1

Although more than 45 mL were collected for every fraction, fractions 1 to 4 did not yield any solid material.

3.3.6 Pancreatic lipase inhibition activity of the 10 fractions

Fractions 5 to 10 were assayed for inhibitory activity towards pancreatic lipase. A preliminary set of data are shown in Table 3-5. Fractions 5 to 7 didn't show any activity, fraction 8 showed a little inhibition activity and fractions 9 and 10 inhibited the enzyme by almost 50 %.

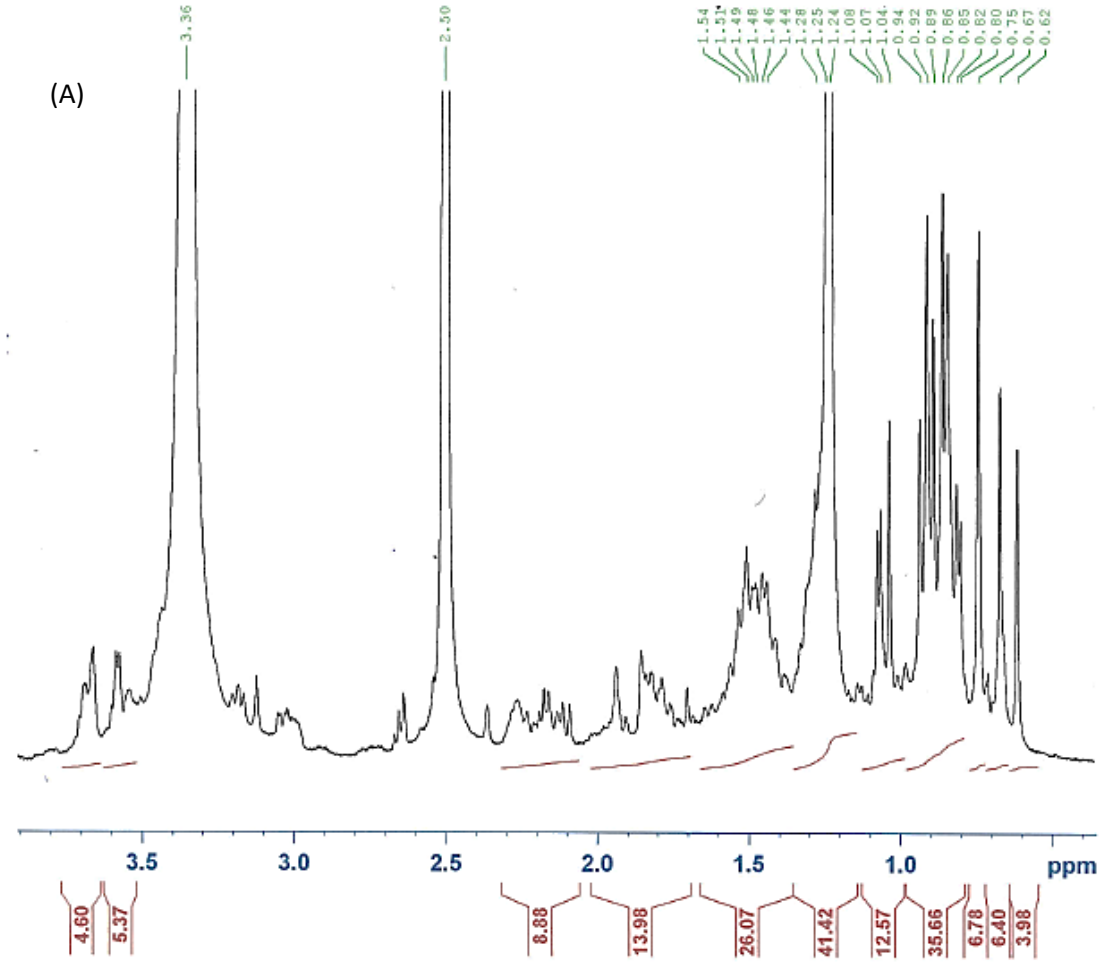
Table 3-5: A summary of the pancreatic lipase inhibition activities of the ten fractions

Tested Sample (mg/mL)	Enzyme activity [µM/µg/hr]
F5 (0.35)	12.71
F6 (1.5)	13.1
F7 (0.09)	13.24
F8 (0.35)	9.40
F9 (0.25)	6.06
F10 (0.08)	5.98
THL ($1.05*10^{-4}$)	0.11
Control	10.5

3.3.7 NMR analysis of collected fractions from *Ilex latifolia*

The pancreatic lipase inhibition activities of the isolated fractions showed that fraction 10 was the most potent with 43.04 % inhibition activity while the second potent was fraction 9 which showed inhibition activity of 42.6 % compared to the control.

The NMR spectra obtained for fractions 5 to 10 were not helpful in structure characterization of the collected compounds because the ^{13}C spectra did not show clear chemical shifts. ^{1}H NMR showed chemical shifts of the protons but without the ^{13}C data this was not enough information to deduce the expected structure of the isolated compounds.



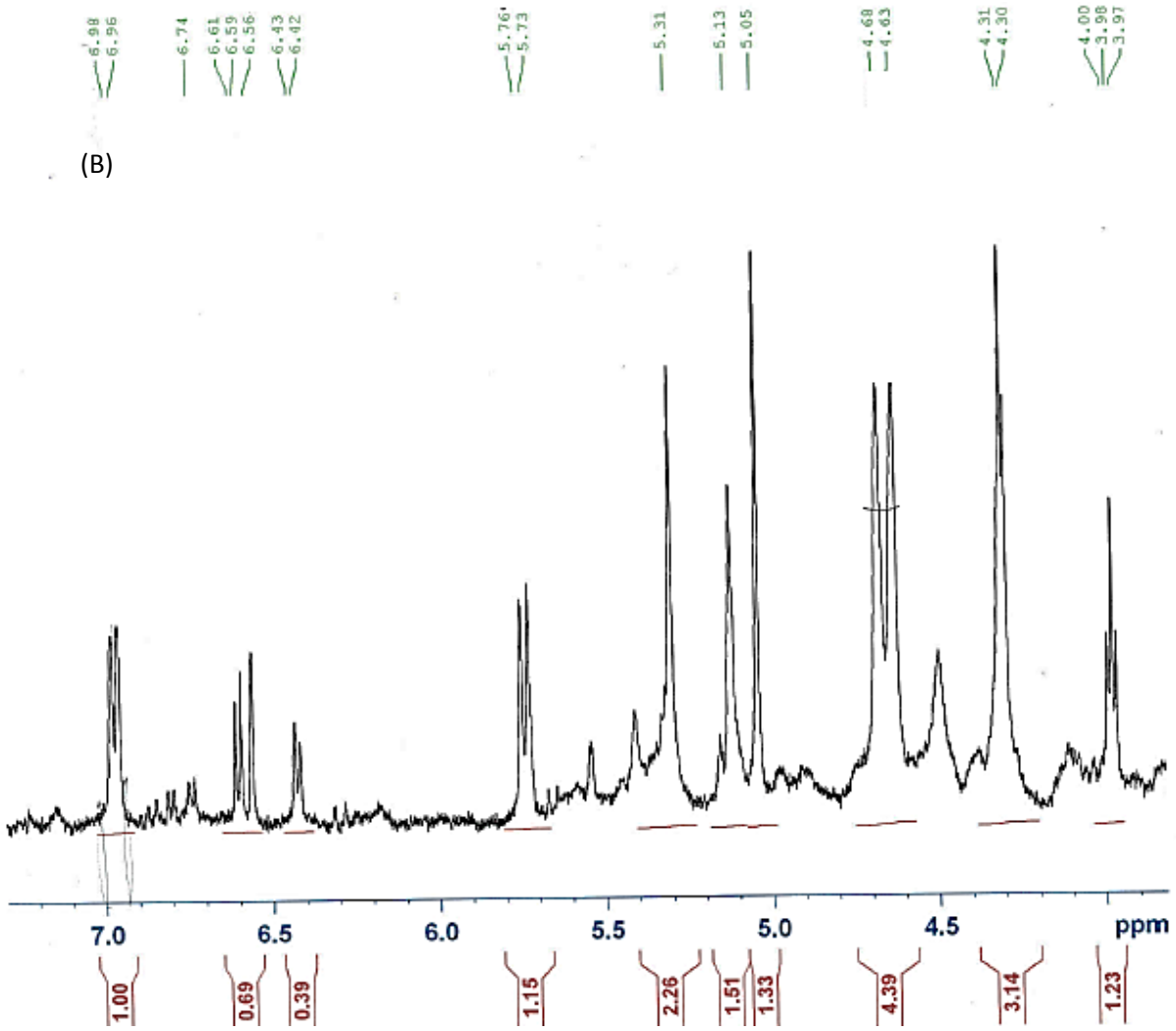


Figure 3-11: ^{1}H NMR of fraction 10. The spectrum was divided into (A) which shows the peaks detected from 0 to 4 ppm while (B) shows the spectrum from 4 to 7 ppm.

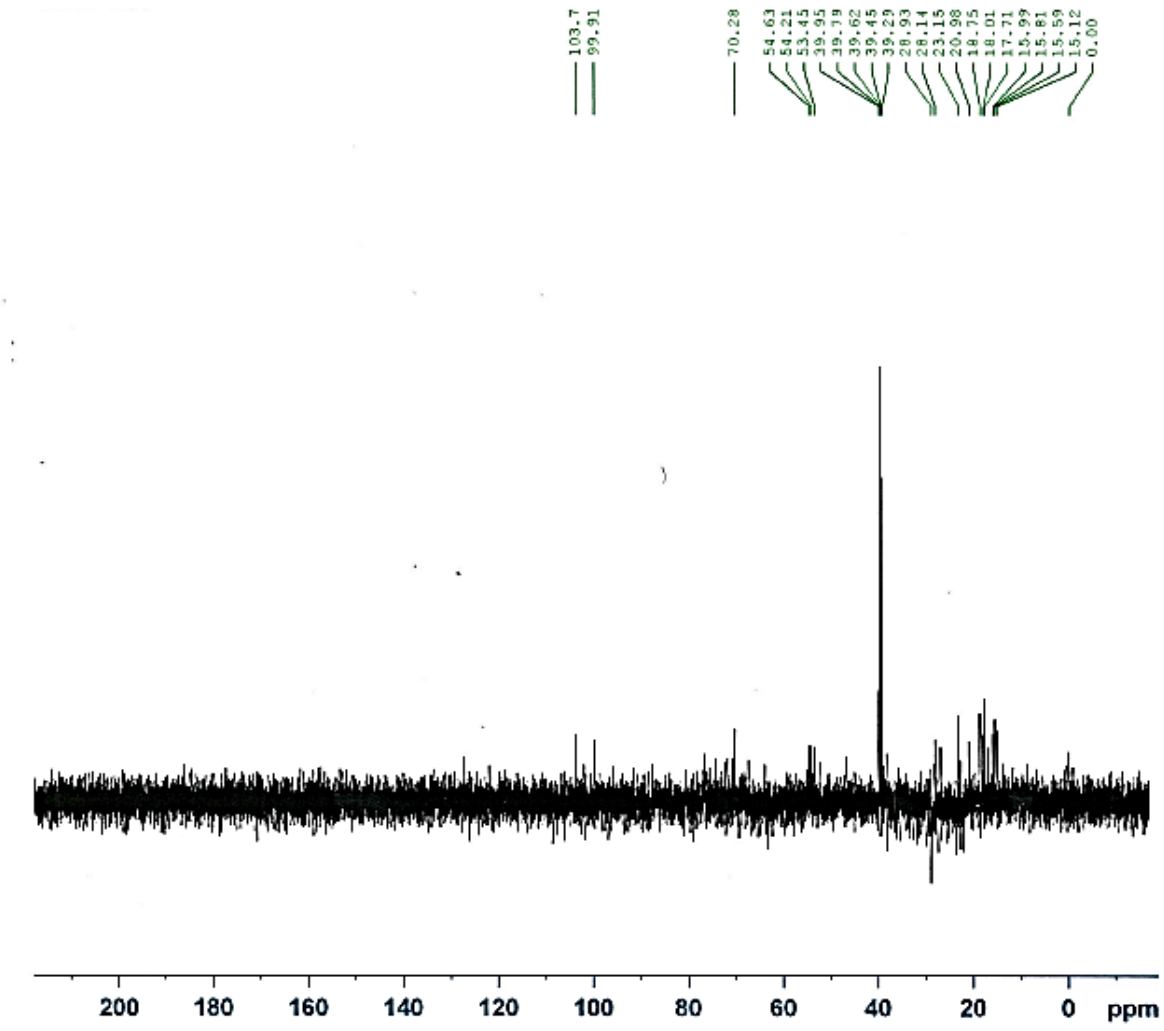


Figure 3-12: ^{13}C NMR of fraction 10

The spectra shown for fraction 10 show that the carbon NMR did not give enough information to characterize the structure *Ilex latifolia* leaves have been reported to be rich in triterpene saponins where nine ilekudinosides (k-s) were isolated and characterized . A single peak was detected at 39.29 and it could be the solvent peak where DMSO was used as a solvent. Another 2 peaks were detected at 99.91 and 103.7. The peak detected at 103.7 is similar to the anomeric carbon detected in L-rhamnose (102.0) in the terminal sugar of the triterpene saponins. ^{1}H NMR showed some signals which can help in the elucidation of the structure. The proton NMR showed five singlets for tertiary methyls at δ 0.75, 0.85, 1.04, 1.24, 1.5. It also showed two doublets at δ 4.68 (1H) and 5.76 (1H) . A singlet appeared at 3.36 and an aromatic doublet was detected at 6.98 (1H). These data are characteristic for triterpene saponins.

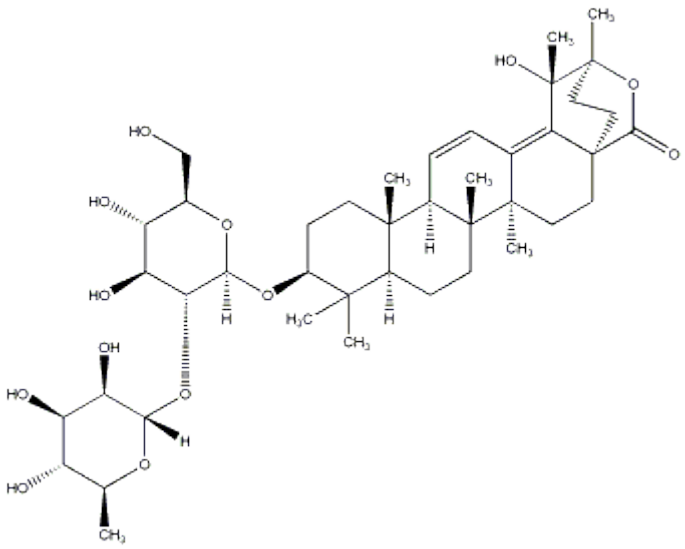
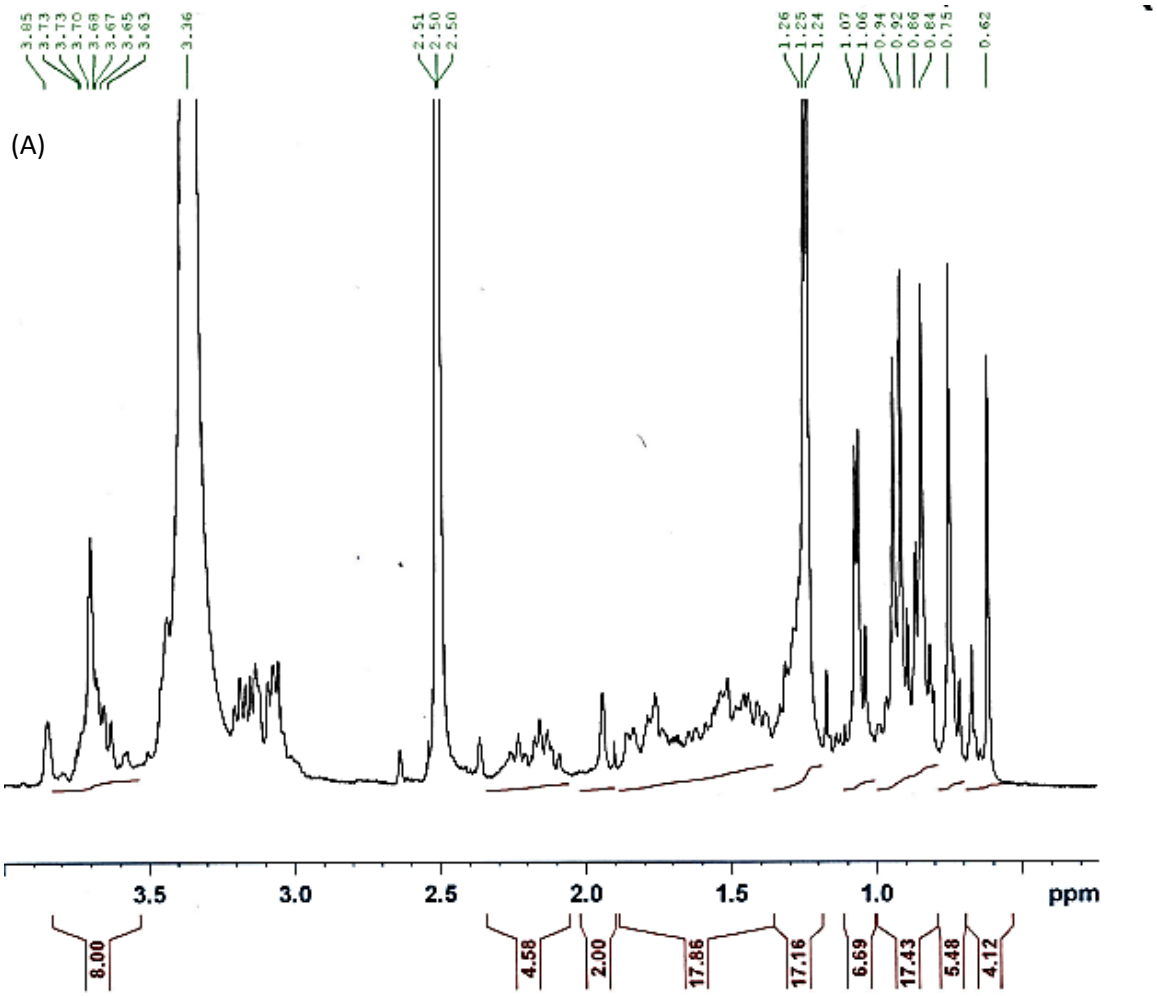


Figure 3-13: Structure of ilekudinoside k, a triterpene saponin isolated from *Ilex latifolia* (Tang *et al*., 2005). The structure shows the methyls detected as singlets.



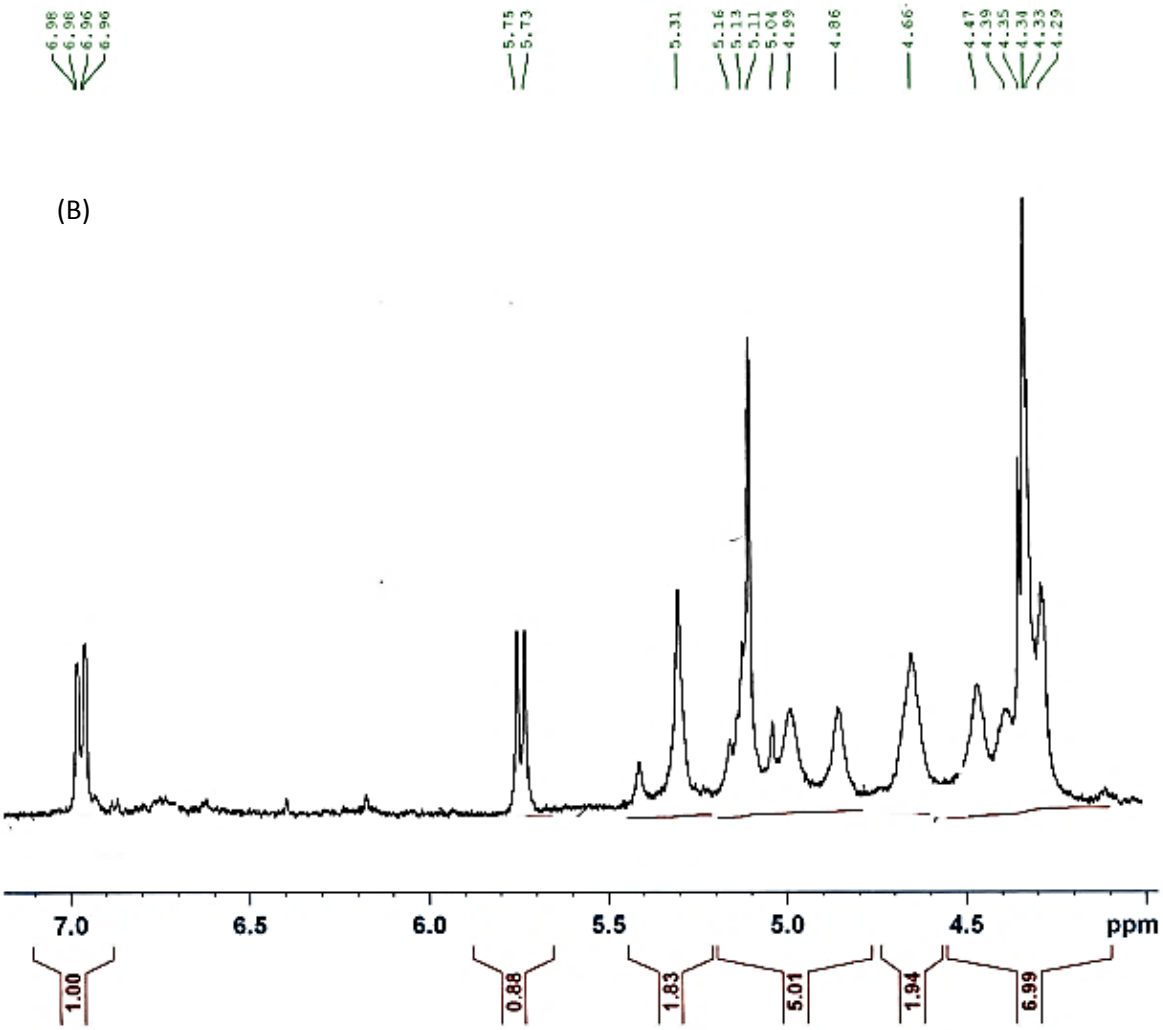


Figure 3-14: ^{1}H NMR of fraction 9. To magnify the peaks detected, the spectrum was divided into A and B. Spectrum (A) shows the peaks from 0 to 4 ppm while spectrum (B) shows the peaks from 4 to 7 ppm.

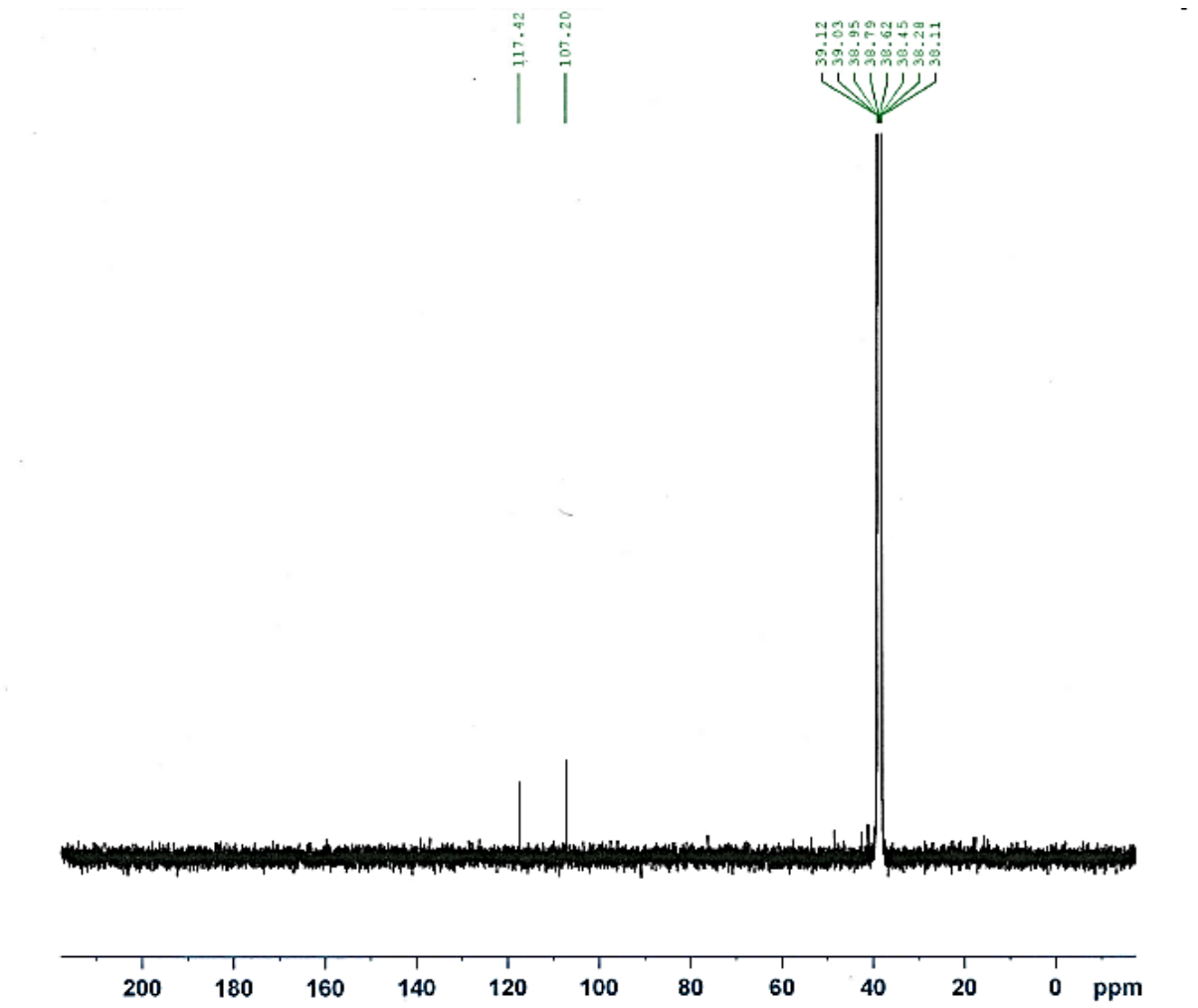


Figure 3-15: ^{13}C NMR of fraction 9

The spectra of fraction 9 are similar to that of fraction 10 with differences in the chemical shifts. ^{1}H showed some signals which can help in the elucidation of the structure. The proton NMR showed five singlets of tertiary methyls at δ 0.75, 0.84, 1.06, 1.24, and 1.26. It also showed one doublet at δ 5.73 (1H). A singlet appeared at 3.36 (1H) and an aromatic doublet was detected at 6.98 (1H). This shows that fraction 9 could be a triterpene saponin but the sugar moiety could be different between the two fractions. These signals are similar to the signals detected in fraction 10. Fraction 10 H-NMR showed two doublets at 6.42(1H) and 6.61(1H) respectively, and a singlet at 6.56 (1H). These three signals were not detected in fraction 9. A doublet detected at 4.68 in fraction 10 was detected as a singlet in fraction 9. Two carbons were detected in fraction 9 at 107.2 and 117.42. The carbon at 107.2 could be the anomeric carbon (107.4) of L-arabinose at the 3-O-sugar position or the terminal carbon

(106.4) of glucose in the triterpene saponins. If the anomeric carbon is 107.2, then the anomeric proton could be at 5.31.

Triterpene saponins inhibit Acyl CoA Cholesteryl Acyl Transferase (ACAT) which catalyzes the intracellular esterification of cholesterol in various tissues. This leads to the formation of foam-cell phenotype, a typical feature of early atherosclerotic lesions (Cignarella et al., 2005). Triterpene saponins can inhibit the formation of these foam cells through the inhibition of ACAT and may serve as a new medication for the treatment of atherosclerosis and obesity (Nishimura, 1999) .

3.4 Discussion

Fractions of the butanol extract of *Ilex latifolia* showed pancreatic lipase inhibition activity when compared to THL which is an approved pancreatic lipase inhibitor. The *Ilex latifolia* butanol extract was a crude extract which means that it contained a mixture of compounds. One or more compound could be responsible to the pancreatic lipase inhibition activity. The aim of the work described in this chapter was to try to separate components of the *Ilex latifolia* extract using HPLC and to isolate pure compounds and check their activities. The first step was to collect a group of peaks in each fraction and check the pancreatic lipase inhibition activity. Four fractions were collected and the pancreatic lipase assay showed that fractions 1,2,3, and 4 inhibited pancreatic lipase and the IC_{50} were found to be 6.25 $\pm$ 1.66, 12.25 $\pm$ 2.6, 50.1 $\pm$ 7.678, and 52.34 $\pm$ 3.4 µg/mL respectively. Fraction 1 was the most active but the other fractions showed inhibitory activity as well. Fraction 1 was 48.97 % more active than fraction 2 and around 88 % more active than fractions 3 and 4.

The next step was to do further separation of the crude extract by changing the mobile phase concentrations and the flow rate so better separation could be achieved, and more peaks could be detected and isolated. At this stage, 2 peaks were collected in each fraction and the pancreatic lipase inhibition activity was assayed. Only fractions 2, 6, and 8 showed a clear inhibition activity and the IC_{50}s were 187.2 $\pm$ 14.2, 86.2 $\pm$ 12.7, and 12.7 $\pm$ 2.6 µg/mL

respectively. Comparing these data to the effects of fractions 1 (section 3.3.2) shows that fractions 1 was 93.5, 86.4, and 50.9 % more active than fractions 2, 6, and 8 respectively (section 3.3.4). Fraction 3 (section 3.3.2) was 73 % and 41.8 %, more active than fractions 2 and 6 respectively (section 3.3.4), but it was 74.65% less active than fraction 8 (section 3.3.4). Fraction 4 (section 3.3.2) was 72.04 % and 39.28 % more active than fractions 2 and 6 respectively (section 3.3.4) but it was only 75.73% less active than fraction 8 (section 3.3.4). Comparing the inhibition activities of the fractions collected in section 3.3.3, fraction 2 was 53.95 % and 99.99 % less active than fractions 6 and 8 respectively. Fraction 6 was 85.26 % less active than fraction 8. The third step was to collect single peaks and isolate pure compounds. 10 peaks were collected and after assaying the activity on pancreatic lipase, only fractions 9 and 10 showed inhibition activity by 50% (Table 3-5). As appears from the data, the fractions were more active when they contained a mixture of compounds but when the peaks were separated and less compounds were collected, the inhibitory activity decreased. Fractions 9 and 10 (section 3.3.6) showed pancreatic lipase inhibition activity at 250 and 80 µg/mL respectively. This can lead to a conclusion that *Ilex latifolia* has a pancreatic lipase inhibition activity but the activity could be due to the effect of more than one compound. This was clear because the IC_{50} of fractions 1-4 (section 3.3.2) were much lower that the IC_{50} of the other fractions collected.

The separation was done three times and each time the solvent conditions and sometimes the flow rate were changed. Increasing the ratio of the organic solvent resulted in better separation and in reducing the separation time. Consequently, the compounds in the extract had more affinity to the stationary phase which means they needed lower mobile phase polarity to be eluted from the column and to be separated. The yield was low and the NMR detection could not be done to characterize the isolated compound. H-NMR showed that the compounds isolated could be triterpene saponins because the peaks detected are similar to the proton NMR of ilekudinosides which were isolated from *Ilex latifolia*. Large amounts of the herb should be extracted using different solvent systems in order to extract several

compounds. Solvent partitioning using two solvents with different polarities can be used to separate compounds with different polarities. This could result in better separation and isolation.

Table 3-6: A table summarizing the IC_{50} of different fractions collected from *Ilex latifolia* butanol extract (Sections 3.3.2 and 3.3.4 respectively.

Fraction	IC_{50} (µg/mL)
1 (Section 3.3.2)	6.25 ± 1.66
2 (Section 3.3.2)	12.25 ± 2.6
3 (Section 3.3.2)	50.1 ± 7.678
4 (Section 3.3.2)	52.34 ± 1.66
2 (Section 3.3.4)	187.2 ± 14.2
6 (Section 3.3.4)	86.2 ± 12.7
8 (Section 3.3.4)	12.7 ± 2.6

4 Chapter IV: Investigation of the effect of *Ilex latifolia* on cholesterol by assaying its effect on the expression of HMG-CoA reductase and LDL-receptor

4.1 Introduction

As mentioned before, the aim of this project is to investigate the efficacy of herbal products for the treatment of hyperlipidemia, focusing on two types of target. The first one is dietary fat absorption linked to the action of pancreatic lipase. The second is cholesterol biosynthesis and metabolism in the liver. From the work described in Chapter 3, *Ilex latifolia* showed the highest inhibitory effect, out of several herbs tested, on pancreatic lipase compared to orlistat. It was therefore decided to investigate the effect of *Ilex latifolia* on the expression of HMG-CoA reductase which is the rate limiting enzyme in cholesterol synthesis, and on the expression of LDL-receptors which play important role in the removal of LDL-cholesterol from the circulation by the liver. Inhibiting HMG-CoA reductase and increasing uptake of LDL-cholesterol by expressing LDL-receptors is one of the targets for treatment of hyperlipidemia.

4.1.1 HMG-CoA reductase

HMG-CoA reductase, the rate limiting enzyme of cholesterol biosynthesis (Brown and Goldstein, 1980), is a 97 kDa transmembrane glycoprotein that resides in the endoplasmic reticulum of animal cells (Chin *et al*., 1982, Chin *et al*., 1984, Liscum *et al*., 1983). HMG-CoA reductase catalyzes the biosynthesis of isoprenoids, producing farnesyl and geranylgeranyl pyrophosphates which are intermediates for the production of products such as cholesterol and dolichol (Elson *et al*., 1999). HMG-CoA reductase catalyzes the reaction HMG-CoA + 2NADPH + $2H^+$ leading to mevalonic acid + $2NADP^+$ + COASH. Inhibition of HMG-CoA reductase results in reduction of serum cholesterol. This is correlated with reductions in atherosclerosis and coronary heart diseases (Oates *et al*., 1988). An enzymatically active 62 kDa fragment can be released from endoplasmic reticulum membrane vesicles by cleavage with a Ca^{2+}-activated endogenous protease. This 62 kDa fragment is membrane bound and can be reduced to a 53 kDa fragment with a leupeptin sensitive exogenous

protease without loss of enzymatic activity (Faust *et al*., 1982). These findings suggest that the active site of the reductase is contained within a water soluble 53 kDa domain that is exposed to protease and thus must be projected onto the cytoplasm. This domain is presumably contiguous with a hydrophobic domain that fixes the reductase to the ER membrane. The less prominent bands at 62 and 52 kDa are proteolytic fragments that are produced during solubilisation of the cells. After treatment with Ca^{2+}-activated protease, 97 kDa was reduced to 53 kDa which remained in the supernatant after centrifugation.

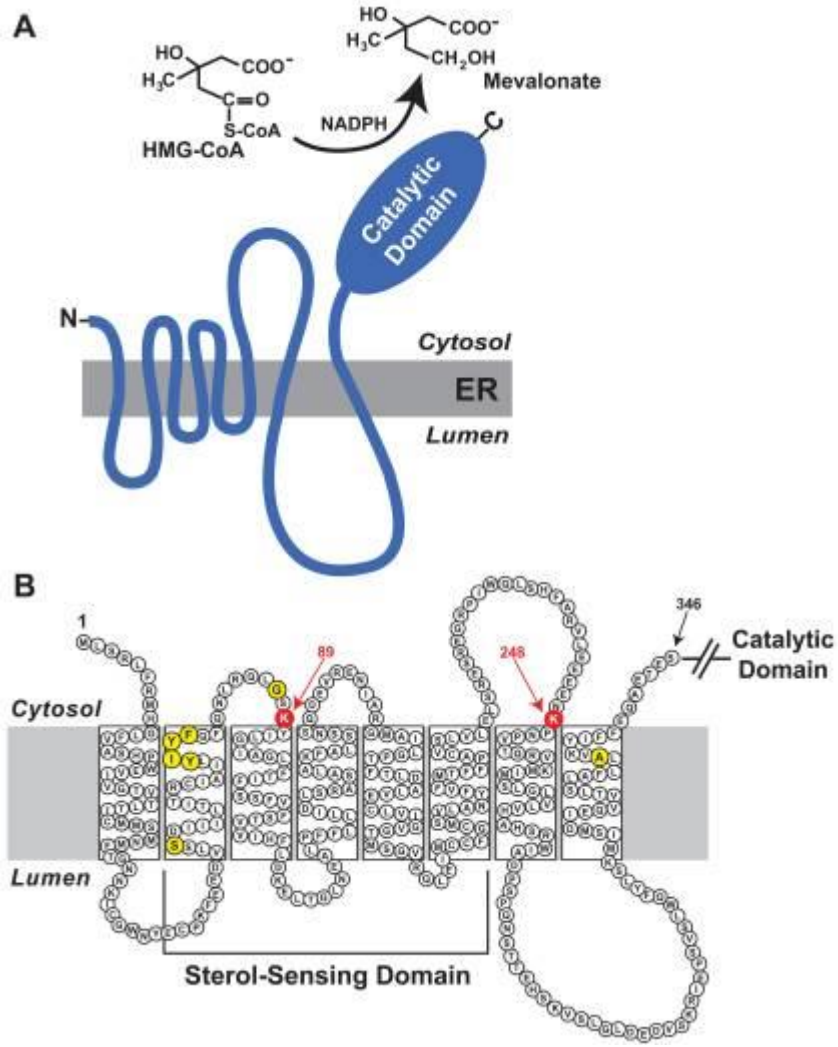


Figure 4-1: Domain structure of HMG CoA reductase. (A) HMG CoA reductase consists of two distinct domains: a hydrophobic N-terminal domain with eight membrane-spanning segments that plays a key role in sterol-accelerated degradation of the enzyme, and a hydrophilic C-terminal domain that directs enzymatic activity. (B) Amino acid sequence and topology of the membrane domain of HMG CoA reductase (Jo and DeBose-Boyd, 2010).

Development of statins, which are derived from fungi and later became purely synthetic molecules, is one of the major advances in the management of hyperlipidemia (Endo, 1992). Statins competitively inhibit HMG-CoA reductase enzyme which leads to a decrease in intrahepatic cholesterol concentration to which the liver responds by inducing expression of its LDL receptors, leading to increase in LDL uptake and catabolism (Endo *et al*., 1976). Several clinical trials have demonstrated that statins are efficient in reducing LDL-cholesterol by 24% to 60% in patients suffering from hypercholesterolemia, and in lowering triglycerides by 10% to 29% (Knopp, 1999) because the LDL and VLDL taken up by LDL receptors are rich in triglycerides (2001, Expert Panel on Detection et al., 2001). Statins are also efficient in increasing HDL-cholesterol by 12 % (Maron *et al*., 2000). Consequently, statins can reduce the incidence of congestive heart diseases including stroke, and myocardial infarction, and total mortality (Packard *et al.*, 1998). Despite the efficacy of statins in the treatment of hyperlipidemia, and protection against coronary heart diseases, some patients experienced tolerability problems and side effects. The most prevalent and important side effects associated with the statin therapy are muscle symptoms such as pain, soreness, weakness, and/or cramps or muscle signs such as creatine kinase elevation. These side effects are fortunately rare (Thompson *et al.*, 2006). Clinical trials on patients treated with statin or placebo found that myopathy occurs in 5 per 100,000 patients while rhabdomyolysis occurs in 1.6 per 100,000 patients (Law and Rudnicka, 2006). All statins roughly have the same risk of drug-related muscle toxicity which can be rarely severe, or rarely progressing to a life threatening situation (Kasiske *et al.*, 2006, Thompson *et al*., 2006). The highest frequency of rhabdomyolysis is associated with simvastatin at 80 mg or less per day. The lowest frequency is associated with fluvastatin and pravastatin which are the weakest inhibitors of HMG-CoA reductase. Muscle damage and rhabdomyolysis have been reported with pravastatin and rosuvastatin which are hydrophilic (Bays, 2006, Law and Rudnicka, 2006). Statins can also lead to asymptomatic elevations in alanine aminotransferase and aspartate aminotransferase liver enzymes > 3 times the upper limit of normal. These

elevations were seen in < 1% of patients receiving initial and intermediate doses and in 2-3% of patients receiving 80 mg or more per day (Cohen *et al*., 2006). Withdrawal of the statin or reduction of the dose results in the return of the enzyme levels to normal without any adverse consequences (McKenney *et al*., 2006a).

Statins can also cause myoglobinuria, and acute renal necrosis (Pierce *et al*., 1990). Statins also have some drug-drug interactions especially with the cytochrome P-450 drug metabolism system (Gruer *et al*., 1999).

All statins have a rigid hydrophobic group linked to an HMG moiety (Endo and Hasumi, 1989). Investigation of the binding modes of statins to HMG-CoA reductase suggest that the statin's cyclic group mimics the nicotinamide group of the NADP(H) and thus uses the binding pockets of the HMG and nicotinamide binding sites (Istvan *et al*., 2000).

4.1.2 LDL Receptor

The discovery of low density lipoprotein receptor and the understanding of its mode of action in the body and the cells resulted in an understanding of the mechanisms that control levels of low density lipoprotein (LDL), which is the main carrier of cholesterol in the human body. Human and animal cells can synthesize hormones, bile acids, and membranes, obtaining the required cholesterol through two mechanisms. The first mechanism is by activating HMG-CoA reductase. The second mechanism is by is by endocytosis of LDL, which is mediated by the LDL receptor (Goldstein and Brown, 1977). Most cells, such as fibroblasts, under the usual circumstances of tissue culture, obtain the required cholesterol by relying on LDL uptake via LDL receptors and by maintaining low levels of HMG-CoA reductase. In contrast, cells with defective LDL receptors, such as those from people with homozygous familial hypercholesterolemia, must express high amounts of HMG-CoA reductase to synthesize the required cholesterol because they have a genetic defect in the LDL receptor and consequently they cannot rely on LDL as a source of cholesterol. In cultured cells, HMG-CoA reductase and the LDL receptor levels are affected by the cholesterol concentration in the culture medium and by the cells' requirements of cholesterol. When the

cells need cholesterol, production of both HMG-CoA reductase and LDL receptors increase, but when cellular cholesterol levels rise, the synthesis of HMG-CoA reductase and LDL receptors is suppressed. The same effect has been observed in the livers of several animal species. Therefore, the body modulates the availability of cholesterol to the tissues through a complex homeostatic network which operates at both the cellular level and within the plasma compartment. Cholesterol is both synthesized in the cells and taken in with food intake. The liver is the principal site to maintain cholesterol homeostasis (Dietschy *et al*., 1993) through different mechanisms including biosynthesis via HMG-CoA reductase activity, LDL-receptors uptake, lipoproteins release in the blood stream, storage by esterification, degradation, and conversion to bile acids (Weber *et al*., 2004). If this balance is not preserved, hypercholesterolemia can result, which will eventually lead to atherosclerosis and heart disease (Goldstein and Brown, 1977). Therefore, treatment of hyperlipidemia helps to maintain this balance by retaining the liver homeostatic function.

The LDL receptor is synthesized in the endoplasmic reticulum as a precursor with an apparent molecular weight of 120 kDa, which increases to 160 kDa after lengthening due to O-linked glycosylation (Schneider *et al*., 1982). The changing in the molecular weight could affect the mobility of the protein in the SDS-gel electrophoresis and consequently affect the detection of LDL-receptors by Western Blotting.

The removal of LDL-cholesterol from the circulation increases as the expression of LDL-cholesterol receptors increase (Goldstein and Brown, 2009). LDL-receptors are another target for hyperlipidemia treatment because compounds which are capable of up-regulating LDL-receptors can be used as anti-hyperlipidemic drugs.

4.1.3 HepG2 and AML-12 cells

An adequate model of human hepatocytes is required to investigate the expression of HMG-CoA reductase and hence cholesterol biosynthesis and metabolism as well as LDL- receptor regulation.

HepG2, a liver cell line derived from a human hepatoblastoma, is considered a reasonable model of the human liver which is the primary location for the synthesis of cholesterol (Mensink and Katan, 1992). HepG2 cells have been used for a long time by many investigators as a model system for human hepatocytes to study various aspects of cholesterol biosynthesis and metabolism, and LDL-receptor expression (Kraft *et al*., 1992, Molowa and Cimis, 1989, Tam *et al*., 1991). Primary cultures of mammalian cells have been used for the investigation of mechanisms of cell growth, differentiation, tumorigenesis and as vectors for gene therapy. Data derived using these may be difficult to reproduce because hepatocytes are short-lived, rapidly lose their specific characters, and are unstable to replicate in culture. The cells remain non-proliferative although culture differentiation can be maintained with specialised media and substrata or by co-culture with nonparenchymal cells (Guguen-Guillouzo, 1992, Isom, 1992, Reid, 1986). Hepatocellular tumors have been used to produce hepatocyte lines which express some tissue-specific markers. Viral oncogenesis has also been used to produce well differentiated cell lines from hepatocytes. These cell lines have been useful but full transformation or transformation with repeated passaging has been the main drawback because liver cells immortalized with simian virus 40 T antigen can express xenobiotic drug-metabolising enzymes (Pfeifer *et al*., 1993). In this case, the viral antigen can form a complex with the products of the tumor suppressing genes (Decaprio *et al*., 1988). Transforming growth factor (TGFα) is a polypeptide that regulates normal growth in epithelial cells and its overproduction is correlated with malignant transformation in cells possessing epidermal growth factor receptors (Derynck, 1988, Salomon *et al*., 1990, Kaufmann *et al*., 1992). TGFα is expressed in liver and it increases in liver during liver regeneration after hepatectomy. Two hepatocyte lines (AML-12 and AML-14) which are non-tumorigenic and possess a large complement of differentiated traits were established from liver of TGFα transgenic mice. In AML-12 cells liver specific genes and TGFα have been maintained in culture. They can be up-regulated by passaging the cells in serum free medium. AML-12 cells are adherent epithelial cells. The mechanisms used by AML-12 cells

to regulate liver specific genes are unknown but are likely to involve transcriptional/post-transcriptional events responsive to intracellular contacts, growth rate, and serum compartments, as observed in other hepatocytes (Wu *et al*., 1994, Isom, 1992, Derman *et al*., 1981). AML-12 cells have been used to investigate the expression of LDL-receptors in cell cultures. They have been used to investigate the effect of fenofibrates (Huang *et al*., 2008) and pcsk9 secretion (Miranda *et al*., 2015) on LDL-receptors expression.

Ilex latifolia was the most potent inhibitor of pancreatic lipase when compared to other herbs and orlistat. The effect of Ile*x latifolia* on cholesterol biosynthesis and metabolism was investigated by checking its effect on HMG-CoA reductase enzyme activity and expression and consequently, the effect on LDL-receptor expression by using HepG2 and AML-12 cells as models for liver hepatocytes. Simvastatin, an approved HMG-CoA reductase inhibitor was used as a positive control.

4.2 Experimental methods

4.2.1 Materials

Anti-mouse IgG HRP (β-actin secondary antibody), bovine serum albumin (BSA), Dulbecco's modified eagle medium (DMEM), foetal calf serum (FCS), human HMG-CoA reductase, sodium dodecyl sulphate (SDS), phosphate buffer saline (PBS), simvastatin, protease inhibitor cocktail, tween-20, coumaric acid, luminol, hydrogen peroxide 3%, insulin, transferrin, sodium selenite, nicotinamide adenine diphosphate sodium salt, CoA, potassium dihydrogen phosphate, nicotinamide adenine diphosphate reduced salt, HMG-CoA, mevalonolactone (MVL), sodium chloride (NaCl), magnesium chloride ($MgCl_2$), manganese chloride ($MnCl_2$), EGTA, DTT, leupeptin hemisulphate, phenylmethylsulfonyl fluoride (PMSF), 2% Zwittergent 3-14, potassium chloride (KCl), Nonidet P-40 and, dimethyl sulfoxide (DMSO), and Bradford reagent were purchased from Sigma-Aldrich (Gillingham, England). *Ilex latifolia* was a gift from Shanghai University of traditional Chinese medicine. β-actin (C4) mouse monoclonal IgG (β-actin primary antibody), HMG-CoA reductase (H-300) polyclonal

rabbit IgG (HMG-CoA reductase primary antibody), goat anti-rabbit IgG (HMG-CoA reductase secondary antibody), donkey anti-rabbit IgG (LDL-R secondary antibody), RIPA buffer and Western blotting luminol reagent were bought from Santa Cruz Biotechnology, INC. (Dallas, USA). Anti-LDLR primary antibody (EP1553Y) was purchased from Novus Biologicals inc. (Abingdon, UK). Glycine, Millipore microfilter tubes (0.22 µm) were purchased from Merck Millipore (Hertfordshire, UK). HPLC grade methanol, formic acid, and HPLC grade acetonitrile were purchased from Fisher Scientific (Loughborough, UK). Precision Plus Protein-xtra dual standards marker were purchased from Bio-Rad Laboratories (Hertfordshire, UK). All the experiments were done using *Ilex latifolia* butanol extract (chapter II, section 2.3.3).

4.2.2 Methods

4.2.2.1 Assay of HMG-CoA reductase using HPLC

Determination of the calibration curve of NADP and CoA.

NADP and CoA stock solutions were serially diluted two-fold five times. Six samples were prepared by adding 910 µL activity buffer, 5 µL water, and 60 µL of each CoA samples respectively. All the samples were incubated at 37 °C for 5 min. 20 µL of different NADP samples were added. 10 µL of 5 M HCl were added. All the samples were analyzed using HPLC at room temperature using a 150mm x 4.6 mm I.D. column (Hichrom. Ltd) packed with 5 µm particle size ACE 18. The mobile phase was 55:45 100 mM potassium dihydrogen phosphate (A) and methanol (B). The elution was 10%–30% B up to 10 min, 30%-60% B for 2 min, 60% B for 2 min, 60%-10% B up to 1 min, and 10% B for 5 min at flow rate of 1 ml/min, UV/VIS detector set at 260 nm. The compartment temperature was 26 °C.

4.2.2.2 Assay of HMG-CoA reductase using LC-MS

4.2.2.2.1 HepG2 cell culture

HepG2 cells were seeded at 6 x 10^5 cells/mL in a 96 well plate. After attachment the medium was aspirated, and the cells were incubated with DMEM/ 1% BSA. For direct inhibition assay, the cells were incubated with the medium for 5 hrs and the medium was aspirated and the

cells were kept at – 80 ºC. For assaying the induction or repression of the enzyme, the cells were incubated with simvastatin at 20, 10, 5, 2.5, and 1.25 µM respectively for 18 hrs. The medium was aspirated and the cells were washed twice with 150 µL DMEM. The cells were incubated with DMEM for 15 mins at 37 ºC. The cells were then washed three times with 150 µL of PBS/1% BSA. The buffer was aspirated and the cells were frozen at -80 ºC.

4.2.2.2.2 HMG-CoA Reductase assay

In the direct inhibition assay, the cells were lysed by six freeze and thaw cycles using liquid nitrogen and a warm water bath. 25 µL buffer A (50 mM K_2HPO_4, 1 mM EDTA, and 5 mM DTT, pH 7.5) were added and the samples were incubated at for 25 min at 37 ºC. 10 µL of buffer B (125 mM K_2HPO_4, and 12.5 mM DTT, pH 7.5) were added. 5 µL of simvastatin (0.3, 0.15, 0.075, 0.0358, 0.017 mM) were added. For induction or repression assay, 5 µL of water were added. 10 µL of substrate solution (15 m M NADPH, 0.4 m M HMG-CoA) were added to both sets. The samples were incubated at 37 ºC for 70 mins with gentle shaking. 10 µL of 6 M HCl were added to stop the reaction. The plates were incubated at 37 ºC for 40 min to produce mevalonolactone (MVL). Control samples were prepared by adding DMSO instead of simvastatin. The calibration curve for MVL was prepared by adding different concentrations of mevalonolactone instead of the reaction mixture. All the samples were filtered using 0.22 µm Millipore microfilter tubes. The samples were frozen at -80 ºC for LC-MS assay. All the samples were prepared in triplicates.

4.2.2.2.3 Determination of the limit of detection of mevalonolactone

1.75 mg of MVL were dissolved in 1 mL of water and serially diluted two-fold five times. The solution was analysed using HPLC at room temperature using a 50mm x 2 mm I.D. column packed with 2 µm particle size ACE 18. The flow rate was 0.25 mL/min. The mobile phase was 0.1 % formic acid (A) in water and acetonitrile (B). Elution was performed with 2 % B for 0.5 min, 2-15 % B up to 3.5 min, 15-50 % B up to 5.7 min, 50-98 % B up to 6.0 min, 98% B for 5.1 min.

4.2.2.2.4 Determination of effect of *Ilex latifolia* on HMG-CoA Reductase expression by Western blotting

4.2.2.2.4.1 HepG2 cell culture

HepG2 cells were routinely grown in two 75 cm^2 flasks using DMEM/10 % FCS. When the flasks became fully confluent, the medium was replaced with DMEM/ 1% BSA and the cells were incubated at 37 ºC for 24 hrs. The medium was removed and the cells were treated with DMEM/1 % BSA containing 40 µM simvastatin. One flask was treated with DMEM/ 1% BSA containing DMSO as vehicle control, at 0.95 % (v/v). To assay the effect of different concentrations of simvastatin on HMG-CoA reductase expression, HepG2 cells were treated with 200, 100, 50, and 25 µM of simvastatin respectively. All the cells were incubated at 37 ºC for 24 hrs. After incubation, the medium was removed and the cells were washed with EDTA-PBS containing trypsin. After detachment, 5 mL of full medium were used to collect the cells in a 15 mL tube. The cells were spun at 800 rpm for 5 minutes at 4ºC. The cells were washed with PBS and spun as in the previous step. The cells were transferred in one mL of PBS to an eppendorf tube. The cells were spun at 12,000 rpm for 10 seconds. The supernatant layer was removed and the cells were lysed for 5 minutes in ice using 100 µL RIPA buffer (50 mM Tris, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS). The same method was repeated to check the effect of different lysing buffers by growing the cells in four flasks. Two flasks were treated with DMSO as a negative vehicle control and two flasks were treated with 100 µM simvastatin. One control (Lane CN) and one 100 µM simvastatin treated cells (Lane SN) were lysed using buffer containing 50 mM potassium di-hydrogen phosphate, 25 mM EDTA, 5 mM DTT, 0.2 M KCl, and 0.2 % NP-40. One pair of control (Lane CL) and 100 µM simvastatin treated cells (Lane SL) were lysed using buffer containing 0.15 M NaCl, 50 mM Tris, 0.5 mM $MnCl_2$, 0.5 mM $MgCl_2$, 5 mM EGTA, 10 mM DTT, 0.1 mM leupeptin hemisulphate, 0.2 mM PMSF, and 2% Zwittergent 3-14 at pH 7.4.

4.2.2.2.4.2 Determination of the protein content of cell lysates

Two 75 cm^2 flasks were seeded with HepG2 cells. The cells were grown using DMEM/10 % FCS. When the flasks became fully confluent, the medium was removed and the cells were grown using MEM/1% BSA for 24 hrs. The medium was removed and the cells in one flask were treated with 100 µM simvastatin. The other flask was treated with DMSO and used as a control. After 24 hrs, the medium was removed and the cells were lysed in 500 µL of buffer containing 50 mM potassium di-hydrogen phosphate, 25 mM EDTA, 5 mM DTT, 0.2 M KCl, and 0.2 % NP-40. The cell lysates were assayed for protein content using Bradford reagent. BSA in the range 0.1625 TO 2.6 mg/mL was used as a standard. A 96 well plate was used. 5 µL of each lysate and 5 µL of BSA standard solutions were added to the plate. 250 µL of the Bradford reagent were added to each sample. All the samples were prepared in triplicates. The plate was shaken for 10 minutes and the absorbance at 595 nm measured.

4.2.2.2.4.3 SDS-PAGE gel electrophoresis and Western blotting

For denaturing gel electrophoresis, a very clean and dry glass plate, an aluminium backing plate and two spacers (0.75 mm) were assembled into a vertical slab gel and placed into a gel caster carefully to prevent leaks. Water was added to test for leaks. The running gel solution (RGS) was prepared by combining 2.5 mL of 40% acrylamide monomer solution, 2.5 mL of 4x running gel buffer (1.5 M Tris-HCl, pH 8.8), 0.1 mL of 10% SDS, 0.2 mL of 10% (w/v) ammonium perodoxisulphate (APS) in H_2O, and 0.02 mL of TEMED. The volume was adjusted to 10 mL with H_2O. APS, and TEMED were added last. A volume of RGS was pipetted into the vertical gel caster to a level that left 1 cm to the teeth of the comb. A volume of water saturated with n-butanol solution was added on the top of the running gel to prevent the possible reaction between the gel solution and the atmospheric oxygen. The RGS polymerised within 30 min at room temperature.

After the running gel had polymerised, the n-butanol was removed by pouring from the gel onto absorbent paper. The 4% stacking gel solution (SGS) prepared by combining 0.5 mL of

40% acrylamide monomer solution, 0.625 mL of 8 x stacking gel buffer (1.0 M Tris-HCl, pH 6.8), 0.05 mL of 10% SDS, 0.1 mL of 10% APS, and 0.01 mL of TEMED. The volume was adjusted to 5 mL with H_2O. APS, and TEMED were added at last. Then a comb was inserted and the SGS was pipetted into the space at the top of the aluminium plate. The gel was left for 20 min to polymerise.

25 µL of each sample were added to 25 µL of 2 X SDS- PAGE sample buffer (125 mM Tris, 4% SDS, 20% glycerol) which was prepared by combining 12.5 mL of 8x stacking buffer, 10 mL glycerol, 10 mL 20% SDS, and 2.5 mL of 0.1% (w/v) bromophenol blue in H_2O. β-mercaptoethanol was added to 20% (v/v). The mixture was heated at 95 °C for 5 min to denature the proteins. The samples were cooled on ice before loading. The gel unit (glass and aluminium plates and spacers in the cassette) was placed into the electrophoresis tank containing running buffer. Buffer was poured into the cassette chamber until it covered the edge of the aluminium plate. The comb was slowly removed from the stacking gel. After the samples were cold, 50 µL of each sample were loaded in the gel. 30 µL (0.5 µg/µL protein) of human HMG-CoA reductase active domain (Sigma Aldrich, CS1090) was used as a positive control. 10 µL of Precision Plus Protein-xtra dual standards marker were loaded (Bio-Rad, US, 161-0377).

The stacking gel was run at 22 mA, constant current (power supply, POWER PAC 300, BioRad). When the samples migrated into the running gel the current was increased to 26mA.

The run was complete when the dye arrived at the bottom of the running gel. The run was left for 10 min after the dye arrived at the end of the gel to have better separation of the target bands. The power supply was turned off, the leads were disconnected and the safety lid was removed. The gel unit was released from the upper buffer chamber.

After electrophoresis the cassette was removed and the gel unit released. The spacers were taken out and the glass plate removed. The aluminium plate and the gel (still united) were submerged in Towbin Transfer Buffer (TTB, 3.03 g Tris-base, 14.41g glycine, 10 mL 10%

SDS, up to 800 m L with H_2O, 200 m L methanol) for 5 – 10 min. Then the stacking gel was removed and the running gel was separated from the glass plate. The proteins were transferred to a nitrocellulose membrane using wet-transfer blotting apparatus at 12 mA.

Four pieces of blotter paper (Whatman, 3MM), the same size as the sponges for the blot, were cut. One piece of nitrocellulose membrane (manufacturer) also was cut just bigger than the size of the running gel. All the papers, the sponges and the membrane were wetted with TTB. The order for the transfer cassette ("sandwich") was a grey side of the cassette, sponge, two pieces of blotter paper, membrane, running gel, two pieces of blotter paper, sponge, black side of the cassette. The transfer cassette was assembled under TTB to minimise trapping air bubbles. The cassette was closed and it was inserted into the transfer apparatus with the grey side with the positive pole (anode). The tank of the transfer apparatus was filled with TTB. The leads of the transfer apparatus were connected to the power supply and proteins transferred overnight at room temperature with a constant voltage of 12 mA. After wet transfer blotting, the membrane and the marker proteins were labelled using a marker pen. The membrane was washed soaked in TBS (100 m M Tris-HCl, 0.9% NaCl, p H 7.5) for 5 minutes to remove loose acrylamide.

The membrane was cut at 50 kDa to two pieces. Both membranes were blocked for one hour using 5% (w/v) skimmed-milk powder in washing buffer (0.2% (v/v) Tween-20 in TBS). The blocking buffer was removed and the membranes were washed with washing buffer three times for five minutes. The membrane containing proteins with molecular weights more than 50 kDa was incubated with anti-HMG-CoA Reductase antibody solution (1:200) (Santa Cruz Biotechnology, US, sc-33827) with constant shaking at 4 ºC for 24 hrs. The primary antibody was removed and the membrane was washed three times for 5 minutes. The membrane was treated with goat anti-rabbit secondary antibody solution (Santa Cruz Biotechnology, sc-2004; 1:1000 dilution) for 1 hr with constant shaking at room temperature. The second membrane was incubated with β-actin antibody solution (1:500) (Santa Cruz Biotechnology, US, sc-47778), with constant shaking at 4 ºC for 24 hr. The primary antibody

was removed and the membrane was washed three times for 5 minutes. The membrane was incubated with goat anti-mouse secondary antibody solution (1:1000 dilution; Sigma Aldrich, UK, A 4416) for 1 hour with constant shaking at room temperature. The secondary antibodies were removed and the membranes were washed three times for 5 minutes. The membranes were incubated with Western blotting luminol reagent (Santa Cruz Biotechnology, sc2048) in darkness for two minutes. Other membranes were incubated with ECL solution prepared in the laboratory by mixing equal volumes of solutions A and B. Solution A was prepared by mixing 9 mL of deionised water, 1 mL of 1 M Tris-HCl (pH 8.5), 45 µL of 90mM coumaric acid in DMSO, and 100 µL of 250 mM luminol in DMSO. Solution B was prepared by adding 9 mL of deionised water, 1 mL of 1 M Tris-HCl (pH 8.5), and 6 µL of 30% hydrogen peroxide. The treatment with chemiluminescent substrate was done two times. The signals were recorded using chemiDoc-IT imaging system.

The same Western blotting method was used to assess the effect of different concentrations of simvastatin, *Ilex latifolia*, 0.2% NP-40 lysing buffer, and buffer containing leupeptin on HMG-CoA reductase expression and detection, described below. AML-12 lysates (section 4.2.2.3.1) were probed for HMG-CoA reductase using the same method mentioned in this section.

4.2.2.2.4.4 Reprobing Western blot membranes

As an alternative to probing separate pieces of membrane independently, the membrane was stripped, after the detection of the HMG-CoA reductase protein, for re-probing. The membrane was placed in fresh stripping buffer (15 g glycine, 1 g SDS, and 10 mL Tween 20 to 1L of water, pH 2.2) for 10 min. The buffer was discarded and the membrane was washed with PBS two times for 10 min. The PBS was discarded and the membrane was washed with TBST (1% Tween-20 in TBS) two times for 5 min. After that the membrane was blocked for 1 hour using 5 % (w/v) skimmed-milk powder in washing buffer. The blocking buffer was

removed and the membrane was washed three times for 5 minutes. β-Actin was detected as mentioned before (section 4.2.2.2.4.3).

4.2.2.3 Determination of LDLR expression by Western blotting

4.2.2.3.1 AML-12 cell culture

AML-12 cells were maintained in three 75 cm^2 flasks with full DMEM-Ham F12 (1:1) medium containing 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL selenium, and 40 ng/mL dexamethasone, supplemented with 10 % FCS. Once the cells were confluent, the medium was replaced with new medium. One flask was treated with DMSO (< 1%) as a negative vehicle control. The other two flasks were treated with 100 µM simvastatin and 100 µg/mL *Ilex latifolia* butanol extract respectively.

The medium was removed, and the cells were washed with 5 mL EDTA-PBS with trypsin. The cells were incubated at 37 °C for 5 min. After the cells detached, 5 mL of full medium were added and the cells were collected in a 15 mL centrifuge tube. The tube was spun at 800 rpm at 4 °C for 5 min to collect the cells as a pellet. The medium was removed and the cells were washed with 5 mL ice-cold PBS. The cells were collected as a pellet as mentioned in the previous step. The PBS was removed and 1 mL of ice-cold PBS was added to transfer the cells into an Eppendorf tube. The cells were centrifuged for 10 sec at 5000 rpm. The PBS was removed and 100 µL of Triton-X 100 lysing buffer solution (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate) were added. The cells were kept in ice for 30 mins. The tube was spun at 12,000 rpm for 5 min at 4 °C. The supernatant layer was removed and stored at -20 °C.

4.2.2.3.2 SDS-PAGE electrophoresis and Western blotting

The method was adapted from section (4.2.2.2.4.3) with some modifications. The running gel solution (RGS 7%) was prepared by combining 1.87 mL of 40% acrylamide monomer solution, 2.5 mL of 4x running gel buffer, 0.1 mL of 10% SDS, 0.2 mL of 10% APS, and 0.02

mL of TEMED. The volume was adjusted to 10 mL with H_2O. The membrane was incubated with Anti-LDLR primary antibody (1:200) (EP1553Y, Novus Europe, Abingdon, UK) with constant shaking at 4 ºC for 24 hrs. The membrane was treated with donkey anti-rabbit IgG secondary antibody (1:500; Santa Cruz Biotechnology, US, sc-33827) for one hour. LDL-receptors were detected in lysates of HepG2 cells (section 4.2.2.2.4.1) by loading the samples in the same gel used for AML-12 cells lysates. LDL-receptors in HepG2 and AML-12 cell lysates were also assayed using LDLR (C-20) primary antibody (Santa Cruz Biotechnology sc-11824).

4.2.2.3.3 Reprobing Western blot membranes

As an alternative to probing separate pieces of membrane independently, the membrane was stripped, after the detection of the LDLR protein, for re-probing. The method was adapted from section (4.2.2.2.4.4).

4.3 Data assay

Use of imaging software in the UVI doc and of Image J for analysis of blot bands.

4.4 Results

The first experiments to assess the effect of *Ilex latifolia* on cholesterol metabolism were attempts to measure HMG-CoA reductase activity in lysates of HepG2 cells that had been treated with extracts of *Ilex latifolia*, and with simvastatin as a positive control. HPLC or MS methods were attempted to assay substrates or products of the enzyme reaction.

4.4.1 Assay of effect of *Ilex latifolia* on HMG-CoA reductase activity using HPLC

NADP and CoA are the products of the reaction of the enzyme with the substrates NADPH and HMG-CoA, respectively. Methods to measure these two products by HPLC were established, as described above, and calibration curves of CoA and NADP are shown in Figures 4-2 and 4-3 respectively.

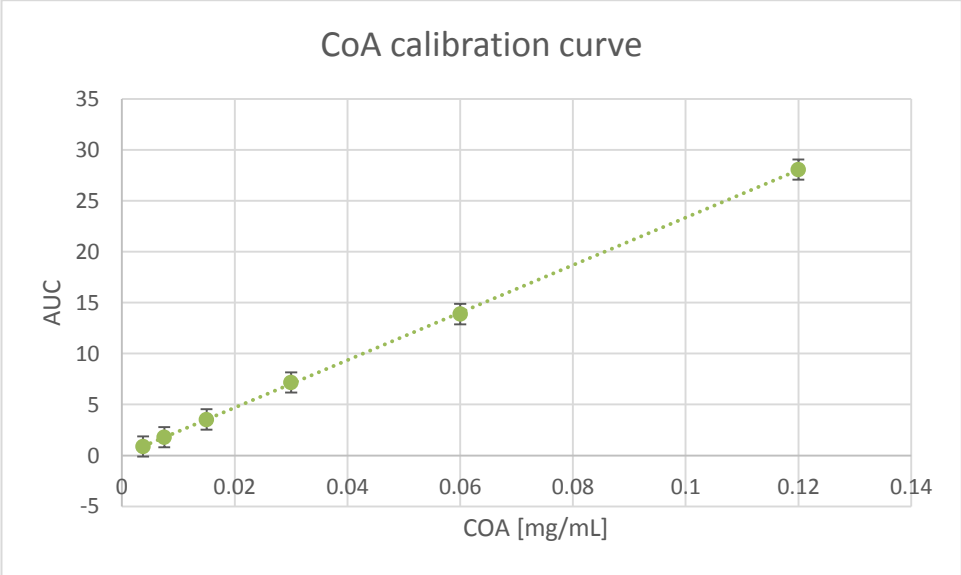


Figure 4-2: Calibration curve of CoA using HPLC (section 4.2.2.1.1). Data are the means ± SD of triplicate samples.

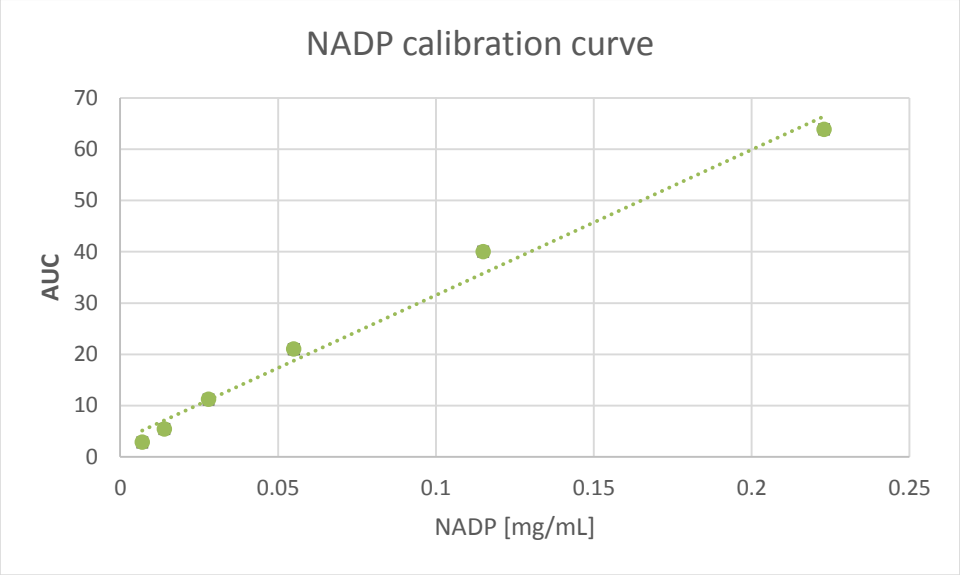


Figure 4-3: Calibration curve of NADP using HPLC (section 4.2.2.1.1). Data are the means ± SD of triplicate samples.

4.4.2 Assay of the effect of *Ilex latifolia* on HMG-CoA reductase activity using LC-MS

LC-MS is an accurate and sensitive analytical method. This technique can detect nano-grams of the analyte and was chosen to assay HMG-CoA reductase activity through detection of mevalonolactone which is the product of the reaction of the enzyme with the substrates, NADPH and HMG-CoA (as described in the methods (section 4.2.2.2.2). The enzyme is present in the HepG2 lysates. To ensure that LC-MS can detect mevalonolactone, HPLC was used to determine the minimum detectable amount of mevalonolactone because HPLC can detect micrograms but the work done here was on cells and the amount of mevalonolactone produced was expected to be in nanograms or less.

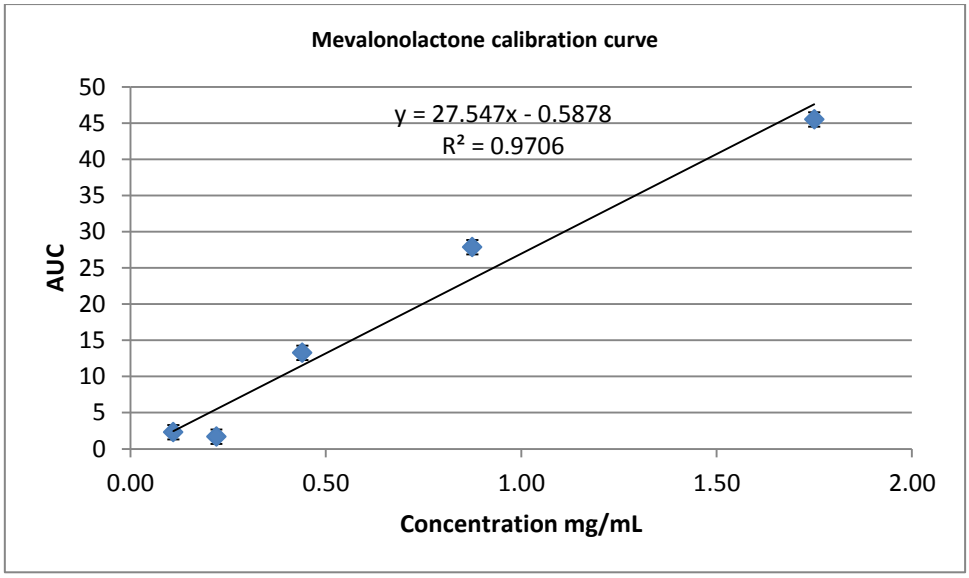


Figure 4-4: Calibration curve of mevalonolactone detected using HPLC (section 4.2.2.2.3). The results are the means ±SD of triplicate samples. The AUC was almost zero at concentrations less than 0.25 mg/mL and this means that the limit of detection is very high and the method is not reliable.

The results of HPLC showed that liquid chromatography has limitations as a method to detect mevalonolactone. One of these is the lack of precision when estimating peak areas, due to the interference from the absorbance of the mobile phase at the wavelength used, 200nm. The λ_{max} of mevalonolactone is 200 nm and this resulted in interference from the mobile phase, which contained formic acid, in the form of trough just prior to the peak of mevalonolactone. An example is shown in Figure 4-5. Although the peak is clear, it is not easy to discern where the peak starts, and hence estimates of the AUC will not be as precise as when the peak is on a level background of absorbance.

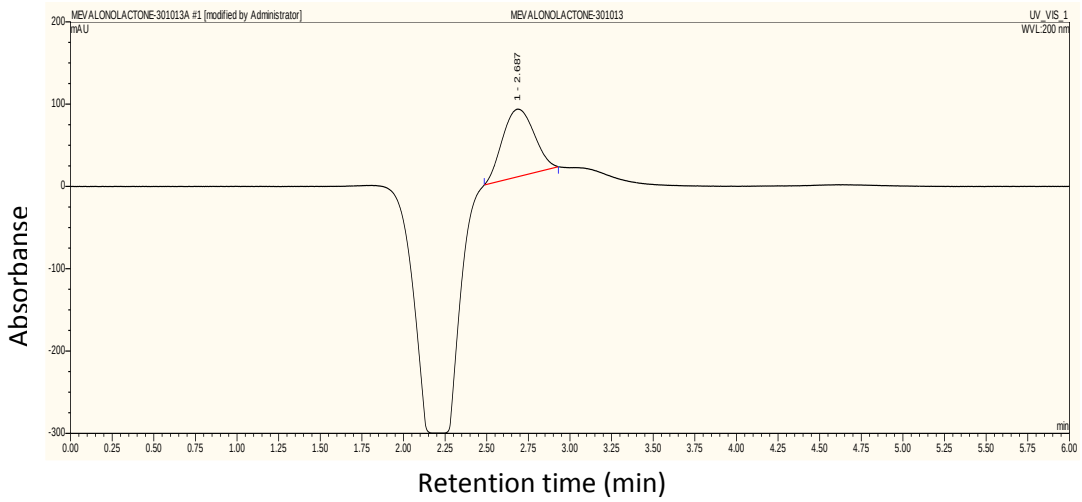


Figure 4-5: Chromatogram of mevalonolactone using HPLC (section 4.2.2.2.3). Mevalonolactone was detected at 2.687. The negative peak at 2.25 could be due to formic acid and consequently the solvent peak.

The other conclusion is that the liquid chromatography detected mevalonolactone at microgram amounts (Fig. 4-4) which means that if mevalonolactone is present only in nanograms in the samples which are cells lysates, mevalonolactone cannot be detected by HPLC quantitatively. To address this issue an attempt was made to use LC-MS to detect mevalonolactone. Samples were prepared as described in the methods (section 4.2.2.2.2), and the assay using LC-MS was planned but the main problem was that the technique was not available because the LC-MS machine had not been working. The only possibility had been to assay the samples using MS manually but this does not yield the necessary quantitative data since manual MS can yield qualitative results only.

4.4.3 Expression of HMG-CoA reductase by Western blotting

Detection of the effect of *Ilex latifolia* on the HMG-CoA reductase activity by detecting NADP, CoA, and mevalonolactone which are the products of the reaction of HMG-CoA reductase with the substrates, HMG-CoA and NADPH, was not possible for the reasons mentioned before (sections 4.4.1 and 4.4.2). The third type of assay method was to detect the effect of *Ilex latifolia* on HMG-CoA reductase protein expression, especially because hepatocytes counteract HMG-CoA reductase inhibition by overexpression of HMG-CoA reductase (Gerber *et al*., 2004), in a feedback control loop.

4.4.3.1 Protein content of cell lysates

In order to normalise measurements as much as possible the protein content of HepG2 cell lysates was assayed so that equal amounts of protein could be compared. HepG2 cells were treated with 100 µM simvastatin. Cells treated with DMSO (<1 %) and were used as vehicle control. The cells were lysed in 500 µL of buffer containing 50 mM potassium dihydrogen phosphate, 25 mM EDTA, 5 mM DTT, 0.2 M KCl, and 0.2 % NP-40. The protein content was assayed using the Bradford reagent. BSA (3.25 to 50 µg/mL) was used as a standard. PBS was used as blank. Lysing buffer was assayed for any interference in the protein assay (section 4.2.2.2.4.2). Protein contents of the lysates were determined using the BSA standard curve (Figure 4-6).

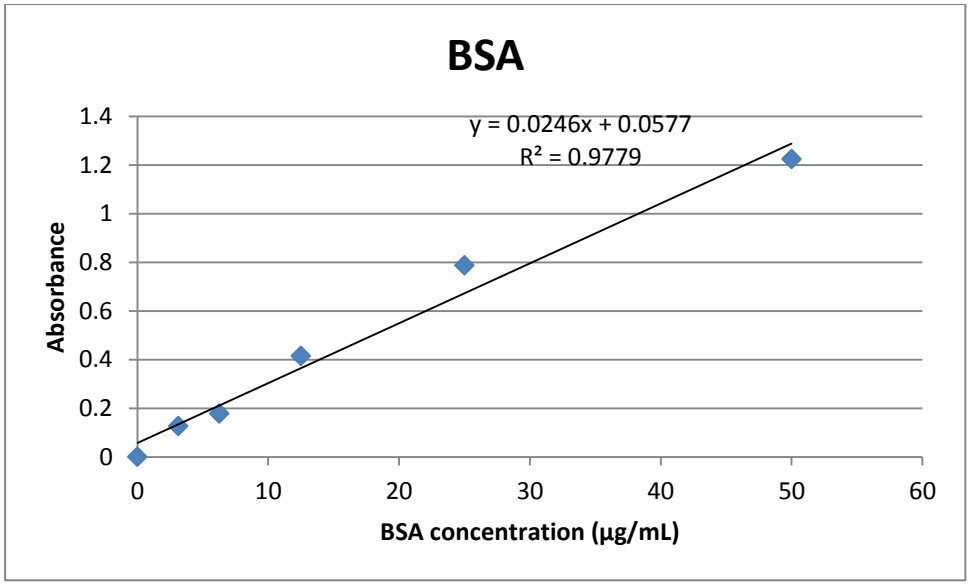


Figure 4-6: Standard graph of BSA using Bradford reagent assay (section 4.2.2.2.4.2). The results are the means of triplicate samples.

The standard graph (Figure 4-6) was used to measure the protein content of the cells lysates by using the absorbance measured using the Bradford reagent assay.

Table 4-1: Protein concentrations of cell lysates using BSA standard graph

Sample	Concentration (µg/mL)	Absorbance
Cells treated with simvastatin	49.1	1
Control	19.8	0.3

The concentrations (Table 4-1) show that the protein content in the cells treated with simvastatin is 2.5 times the protein content of the control cells. Sample volumes for Western blotting were adjusted to achieve equal loading of protein.

4.4.3.2 Validation of the primary antibody

Western blotting was used to detect HMG-CoA reductase expression in HepG2 cells and to detect the effect of *Ilex latifolia* on HMG-CoA reductase expression compared to simvastatin. To make sure that the method was reliable and the results were not affected by the sensitivity or the specificity of the primary antibody (Santa Cruz Biotechnology, US, sc

33827), human HMG-CoA reductase active domain (Sigma Aldrich, CS-1090) was used as a positive control.

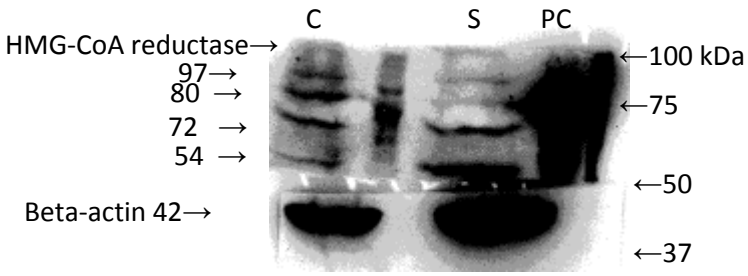


Figure 4-7: Immunoblot to detect HMG-CoA reductase in HepG2 cells. Lysates of HepG2 cells were analysed which had been treated with 40 µM simvastatin (lane S) or DMSO vehicle control (Lane C). HMG-CoA reductase active domain (Sigma-Aldrich) (lane PC) was used as a positive control to confirm the specificity of the primary anti-body activity. HMG-CoA reductase and beta-actin proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was cut to two pieces at 50 kDa and HMG-CoA reductase and β-actin were probed in parallel before detection using a chemiluminescent substrate. PrecisionPlus protein dual xtra standards marker (BioRad) was used as a reference (section 4.2.2.2.4.2). Four bands at 97, 80, 72, and 54 kDa were detected in HepG2 lysates.

HepG2 cells were treated with simvastatin (40 µM) (Figure 4-7, lane S) to detect the effect of HMG-CoA reductase inhibition on protein expression. Other cells were treated with DMSO (< 1 %) and used as control (Figure 4-7, lane C). Human HMG-CoA reductase active domain was used as a positive control (Figure 4-7, lane PC) to insure that the primary antibody used was suitable for the assay. After SDS-PAGE gel electrophoresis, the proteins were transferred to nitrocellulose membrane. HMG-CoA reductase proteins were probed using anti-HMG-CoA reductase primary antibody H-300 (Santa Cruz Biotechnology, US, sc 33827), which is a rabbit polyclonal IgG that reacts with an epitope corresponding to amino acids 589-888 mapping at the C-terminus of HMG-CoA reductase of human origin. It is recommended for detection of HMGCR of mouse, rat and human origin by Western blotting, immunoprecipitation, immunofluorescence and ELISA. It is also reactive with additional species, including equine, canine, bovine and avian. H-300 was used for the detection of HMG-CoA reductase in mouse (Scheving et al., 2014).

Loading control proteins are commonly used as internal standards to accurately measure protein levels in a sample. These controls are usually derived from ubiquitously expressed housekeeping genes and they have been widely used due to their consistent level of

expression across a wide range of samples. The most frequently used loading controls in biochemical research are actin and tubulin (Suzuki et al., 2011, Wishart et al., 2008, Mutsaers et al., 2011). Actins are an essential component of the cytoskeleton, with critical roles in cellular processes including gene expression, cell division, and cell migration. There are six isoforms of actins in vertebrates (Rubenstein, 1990) where four of these isoforms are expressed in smooth (α_{sm} and $\gamma_{sm)}$ and in striated (α_{sk} and $\alpha_{ca)}$ muscle cells. The two cytoplasmic β-actin and γ-actin isoforms are ubiquitously expressed (Bunnell et al., 2011). Beta-actin, at 42kDa, was used as a loading and blotting control in this study (Fig. 4-7).

The image was analysed using Image J. Lysate of control HepG2 cells (Fig. 4-7 Lane C) showed four HMG-CoA reductase bands which were estimated to have molecular weights 97, 80, 72 and 54 kDa respectively. The positive control lane showed bands which means that the primary anti-body used was active. Because the positive control was used at 0.5 µg/mL, The bands are appearing thick and very strong.

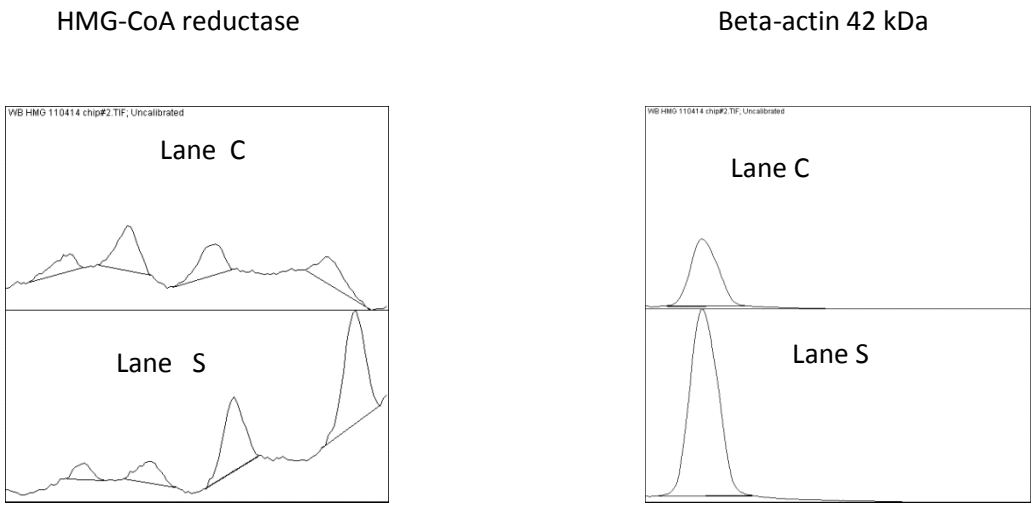


Figure 4-8: Estimation of HMG-CoA reductase protein expression by transforming the bands from Fig. 4-7 into peaks using image J. Lane C is the control (DMSO <1%). Lane S corresponds to the cells treated with simvastatin (40 µM). The intensities of the bands of HMGCR will be estimated by measuring the peak areas and by comparing these intensities to the beta-actin intensities.

Table 4-2: Estimation of the intensities of the detected bands (Figures 4-7,4-8) for HMG-CoA reductase proteins in lanes S and C. The intensities of the bands in lane C were normalized to 1.0. The intensities of the bands in lane S were calculated as a ratio to the bands in C. The normalized intensities were calculated as a ratio to the beta-actin normalized intensity in lane S.

		Lane	
		C	**S**
HMG-CoA reductase 97 kDa	Intensity	877	566
	Normalized intensity	1.0	0.64
HMG-CoA reductase 80 kDa	Intensity	1966	1033
	Normalized intensity	1.0	0.53
HMG-CoA reductase 72 kDa	Intensity	1589	3095
	Normalized intensity	1.0	1.95
HMG-CoA reductase 54 kDa	Intensity	1409	4855
	Normalized intensity	1.0	3.45
Beta-actin 42 kDa	Intensity	6055	17365
	Normalized intensity	1.0	2.87
Ratio of HMG-CoA reductase 97 kDa / beta-actin		1.0	0.223
Ratio of HMG-CoA reductase 80 kDa / beta-actin		1.0	0.185
Ratio of HMG-CoA reductase 72 kDa / beta-actin		1.0	0.68
Ratio of HMG-CoA reductase 54 kDa /beta-actin		1.0	1.2

The results and the image show that HMG-CoA reductase can be detected using HMG-CoA reductase (H-300) polyclonal rabbit IgG. It also shows that simvastatin can be used as a positive control as it stimulated the expression of HMG-CoA reductase. The bands (Figure 4-7) were transformed into peaks where the intensity of each peak was determined using ImageJ (Table 4-2). The intensity of each peak was normalized to the intensity of the beta-actin intensity. Table 4-2 shows that the intensities of the bands detected at 97, 80, 72, kDa were higher than those detected in the simvastatin sample. The intensity of the band detected at 54 kDa was 20 % higher than the intensity of the band detected in the control sample. If the 54 kDa fragment is the soluble fragment which represents the whole enzyme protein, this means that simvastatin stimulates the HMG-CoA reductase expression.

As an alternative to separate probing of two pieces of membrane independently, after detection of HMGCR, the membrane was stripped using a stripping buffer and β-actin was detected (section 4.2.2.2.4.4)

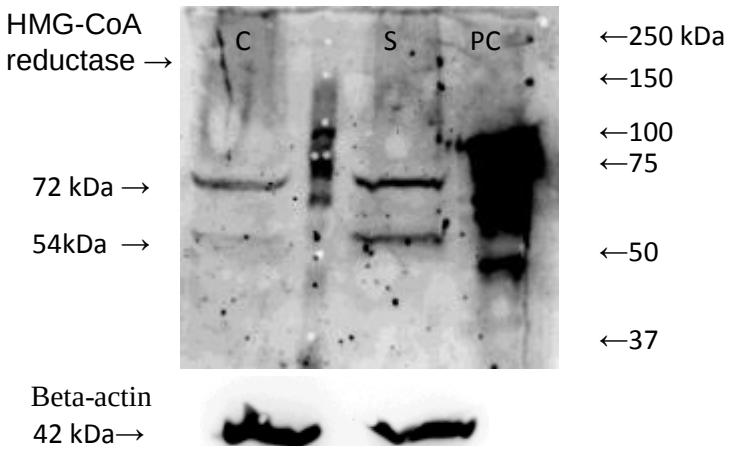


Figure 4-9: Immunoblot of proteins from lysates of HepG2 cells which had been treated with 40 μM simvastatin (lane S) or DMSO vehicle control (Lane C). HMG-CoA reductase active domain (Sigma-Aldrich) (lane PC) was used as a positive control to confirm the specificity of the primary anti-body activity. HMG-CoA reductase and beta-actin proteins were analysed by SDS-PAGE and transferred to nitrocellulose membrane before detection using a chemiluminescent substrate. Precision plus protein dual xtra standards marker (BioRad) was used as a reference (section 4.2.2.2.4.4). Two bands at 72 and 54 kDa were detected.

Lanes C and S showed two HMGCR bands which were estimated to have molecular weights 72 and 54 kDa respectively. The beta-actin was detected at 42 kDa (Figure 4-9).

HMG-CoA reductase

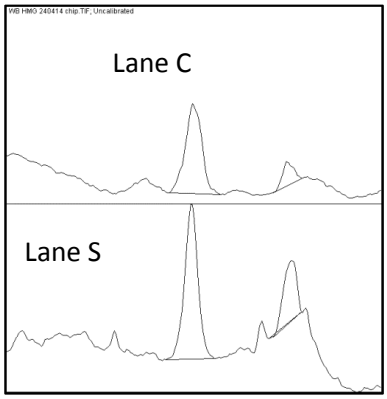


Figure 4-10: Estimation of HMG-CoA reductase protein expression by transforming the bands into peaks using image J. Lane C is the control (DMSO <1%). Lane S corresponds to the cells treated with simvastatin (40 µM).

Beta-actin 42

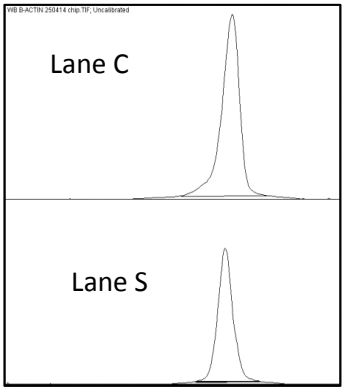


Figure 4-11: Estimation of the expression of beta-actin by transforming the bands into peaks using image J. Lane C is the control (DMSO <1%). Lane S corresponds to the cells treated with simvastatin (40 µM).

Table 4-3: The intensities of HMG-CoA reductase 54 and 72 kDa bands (Figure 4-10) as calculated by Image J. Lane C was used as a reference for lanes S.

		Lane	
		C	**S**
HMG-CoA reductase 72 kDa	Intensity	5202	7263
	Normalized intensity	1.0	1.4
HMG-CoA reductase 54 kDa	Intensity	938	2720
	Normalized intensity	1.0	2.9
Beta-actin 42 kDa	Intensity	12946	8023
	Normalized intensity	1.0	0.62
Ratio of HMG-CoA reductase 72 kDa / beta-actin		1.0	2.26
Ratio of HMG-CoA reductase 54 kDa /beta-actin		1.0	4.7

Table 4-4: The intensities of the peaks from figure 4-10 as calculated by Image J. The intensities of HMG-CoA reductase 54 and 72 kDa bands were combined. Lane C is used as a reference for lanes S.

		Lane	
		C	**S**
HMG-CoA reductase 72 kDa	Intensity	5202	7263
HMG-CoA reductase 54 kDa	Intensity	938	2720
Combined HMG-CoA reductase 54 and 72 kDa	Intensity	6140	9983
	Normalized intensity	1.0	1.62
Beta-actin 42 kDa	Intensity	12946	8023
	Normalized intensity	1.0	0.62
Ratio of HMG-CoA reductase / beta-actin		1.0	2.61

The intensities of the HMGCR and beta-actin bands were estimated by using Image J software to analyse a cross-section of the bands of the blot. Plots of the cross-sections are shown in figures 4-10 and 4-11. The areas under the peaks were estimated and are given in Table 4-3. The intensities of the bands from the control sample in lane C were normalised to 1. The intensities of the bands in lane S were normalised as a ratio to the corresponding bands in lane C. HMG-CoA reductase bands intensities were calculated as a ratio to the beta-actin to correct for any variation in the protein content of the cells lysates. The data show that simvastatin stimulated the expression of HMGCR in HepG2 cells 2.6 to 4.7 times compared to levels of HMG-CoA reductase in control cells. These data indicate that the HepG2 cells can be used to assess inhibitors of HMG-CoA reductase through modulation of the regulatory loop that controls level of enzyme protein. The Western blotting technique detected truncated forms of HMG-CoA reductase, but, under the conditions used for cell lysis, the full length form of HMG-CoA reductase, which has a molecular weight of 97 kDa, was not visible. Despite this, the response of the HepG2 cells to simvastatin was as expected, namely an up-regulation of HMG-CoA reductase expression and it was decided to

continue to use HepG2 cells to assess effects of extracts of *Ilex latifolia* in comparison to simvastatin.

4.4.3.3 Effect of different concentrations of simvastatin and Ilex latifolia on HMG-CoA reductase expression

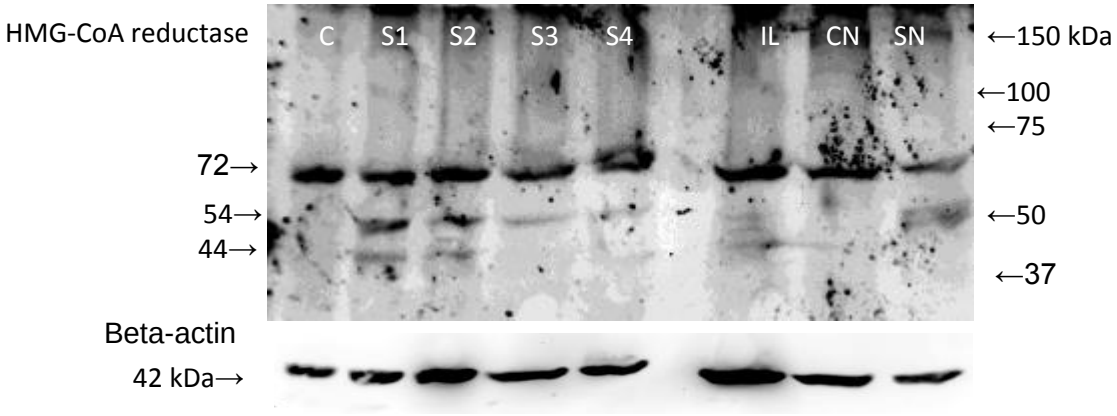


Figure 4-11: Immunoblot of proteins from lysates of HepG2 cells. Lane C is the control sample. Lanes S1, S2, S3, and S4 are the lysates of cells treated with 200, 100, 50, and 25 µM simvastatin respectively. Lane IL is the lysate of cells treated with 100 µg/m L of *Ilex latifolia*. Lanes CN and SN are the lysates of the control and cells treated with 100 µM simvastatin respectively. The cells were lysed using 0.2 % NP—40 lysing buffer.

To further confirm the effect of simvastatin on HMG-CoA reductase expression, the cells were treated with different concentrations of simvastatin. HepG2 cells were grown in six 75 cm^2 flasks. One flask was treated with DMSO (<1% v/v) as a negative vehicle control. The other flasks were treated with 200, 100, 50, 25 µM simvastatin, and 100 µg/mL *ilex latifolia* extract respectively. All the flasks were lysed using RIPA buffer containing protease inhibitor cocktail (Sigma Aldrich, P8340). RIPA buffer dissolves all the cellular membranes and release all the cellular fluids including the nuclear DNA which rendered the lysates very viscous and this resulted in difficulties in measuring the protein contents of the cell lysates and in loading of the denatured samples onto the running gel. To avoid the setbacks of RIPA buffer, one control (Lane CN) and one 100 µM simvastatin treated cells (Lane SN) were lysed using buffer containing 50 mM potassium dihydrogen phosphate, 25 mM EDTA, 5 mM DTT, 0.2 M KCl, and 0.2 % NP-40 with protease inhibitor cocktail (Sigma Aldrich, P8340)

(Figure 4-12). The SDS-PAGE electrophoresis and the Western blotting were done as mentioned before (section 4.2.2.2.4.3). All the lanes showed the two HMG-CoA reductase bands detected previously, 72 and 54 kDa respectively, and samples in lanes S1 and S2 showed an additional band at 44 kDa. The beta-actin was detected at 42 kDa (Figure 4-12).

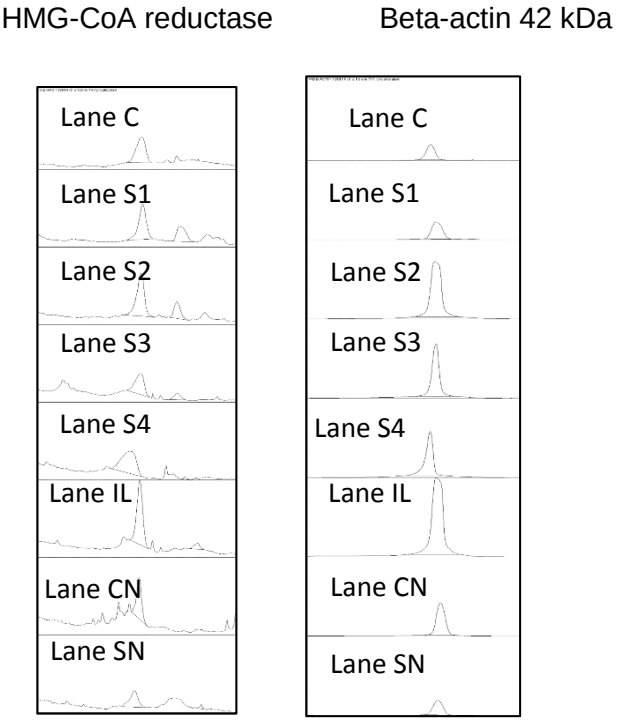


Figure 4-12: Estimation of HMG-CoA reductase protein expression by transforming the bands from Fig. 4-11 into peaks using image J. Lane C is the control (DMSO <1%). Lanes S1, S2, S3 and S4 correspond to the cells treated with 200, 100, 50, and 25 µM simvastatin respectively. Lane IL corresponds to the cells treated with 100 µg/mL *Ilex latifolia.* Lanes CN and SN correspond to cells lysed with buffer containing 0.2% NP-40.

Table 4-5: The intensities of the peaks (Figure 4-12) as calculated by Image J. Lane S1 is used as a reference for lanes C, S2, S3, S4 , and IL. Lane CN is used as a reference for lane SN.

		Lane							
		C	**S1**	**S2**	**S3**	**S4**	**IL**	**CN**	**SN**
HMG-CoA reductase 72 kDa	Intensity	3906	4310	5353	3050	5231	6188	3324	2157
	Normalized intensity	0.90	1.0	1.24	0.7	1.21	1.44	1	0.65
HMG-CoA reductase 54 kDa	Intensity	0	2249	1707	706	98.31	568	0	200
	Normalized intensity	0	1.0	0.76	0.31	0.043	0. 25	0	1.0
Beta-actin 42 kDa	Intensity	1963	2630	8932	5657	4846	13008	4111	2107
	Normalized intensity	0.75	1.0	3.4	2.15	1.84	4.94	1.0	0.51
Ratio of HMG-CoA reductase 72 kDa / beta-actin		1.20	1.0	0.36	0.32	0.66	0.29	1.0	1.27
Ratio of HMG-CoA reductase 54 kDa /beta-actin		0	1.0	0.22	0.14	0.02	0.05	0	1.0

Table 4-6: The intensities of the peaks (Figure 4-12) as calculated by Image J. The intensities of HMG-CoA reductase 54 and 72 kDa were combined. Lane C is used as a reference for lanes S1, S2, S3, S4, and IL. Lane CN is used as a reference for lane SN.

		Lane							
		C	**S1**	**S2**	**S3**	**S4**	**IL**	**CN**	**SN**
HMG-CoA reductase 72 kDa	Intensity	3906	4310	5353	3050	5231	6188	3324	2157
HMG-CoA reductase 54 kDa	Intensity	0	2249	1707	706	98.31	568	0	200
Combined HMG-CoA reductase 54 and 72 kDa	Intensity	3906	6569	7060	3756	5329	6757	3324	2357
	Normalized intensity	1.0	1.68	1.8	0.96	1.36	1.73	1.0	0.70
Beta-actin 42 kDa	Intensity	1963	2630	8932	5657	4846	13008	4111	2107
	Normalized intensity	1.0	1.34	4.55	2.88	2.47	6.63	1.0	0.51
Ratio of HMG-CoA reductase / beta-actin		1.0	1.25	0.4	0.33	0.55	0.26	1.0	1.4

Simvastatin, at the highest concentration used, 200 µM, stimulated the expression of HMG-CoA reductase in HepG2 cells by 25 % (Figure 4-12, Lane S1) compared with the control cells (Figure 4-12, Lane C) but it did not show any stimulation at lower concentrations (Figure 4-12, Lanes S2 to S4), instead having an inhibitory effect of 45% to 65%. Similarly the extract of *Ilex* also inhibited HMG-CoA reductase expression, by about 70% (Figure 4-12, Lane IL). In a separate pair of samples, cells were extracted with a milder lysis buffer compared with RIPA buffer. Simvastatin used at 100 µM was found to stimulate the expression of the HMG-CoA reductase (Figure 4-12, Lane SN), 40 % compared with the control (Figure 4-12, Lane CN).

Taken together these data indicate that higher concentrations can induce measurable, but variable increases in HMGCR expression, as assessed by detection of partially degraded forms of HMGCR. *Ilex latifolia* did not stimulate HMG-CoA reductase expression but on the contrary it inhibited HMG-CoA reductase expression. The mechanism by which this happened could be explored in future work by using different cell lines such as UT-1 cells, a line of Chinese hamster ovary cells that have been used for the determination of HMG-CoA reductase structure (Goldstein and Brown, 1984) or JJN-3 human myeloma cell lines (Roelofs et al., 2007) and by investigating the effect of *Ilex latifolia* on the gene which expresses HMG-CoA reductase protein. The effect of *Ilex latifolia* on HMG-CoA reductase expression could be assayed in-vivo by assaying HMG-CoA reductase levels in liver homogenates such as rats livers (Bergstrom et al., 1997).

4.4.3.4 Effect of leupeptin on the pattern of HMG-CoA reductase forms

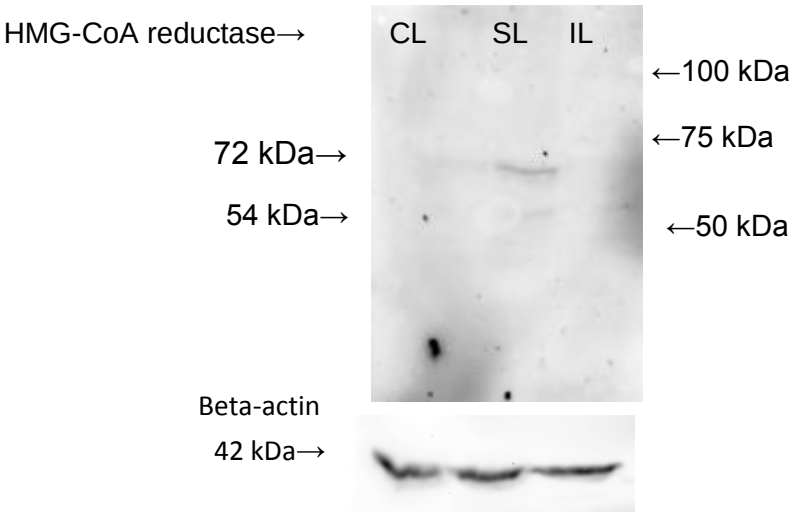


Figure 4-13: Immunoblot to detect HMG-CoA reductase in lysates of HepG2 cells prepared with leupeptin. Cells had been treated with 100 □M simvastatin (Lane SL), 100 µg/mL *Ilex latifolia,* or DMSO which was used as vehicle control (Lane CL) . Cells were lysed using with 0.15 M NaCl, 50 mM Tris-base, 0.5 mM $MnCl_2$, 0.5 mM $MgCl_2$, 5 mM EGTA, 10 mM DTT, 0.1 mM leupeptin hemisulphate, 0.2 mM PMSF, and 2% Zwittergent 3-14 at p H 7.4. HMG-CoA reductase and beta-actin proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane before detection using chemiluminescent. Precision plus protein dual xtra standards marker (BioRad) was used as a reference.

The molecular weight of HMG-CoA reductase is 97 kDa according to the amino acid sequence as deduced from the sequence of a full length of cDNA (Liscum et al., 1983). A doublet of 94-97 kDa was observed in autoradiographs of Western blots of HepG2 cell microsomal membranes probed with polyclonal anti-HMG-CoA reductase IgG (Parker et al., 1993). A Ca^{2+}-dependent protease in cell extracts is known to cleave the 97 kDa reductase to an active 62 kDa form that remains membrane bound (Chin et al., 1982). Further cleavage by a leupeptin-sensitive protease releases a soluble 50 to 55 kDa fragment that also retains full enzymatic activity. Because it was difficult, or impossible, to detect the full length HMGCR by Western blotting of cell lysates prepared using commonly used lysis buffers, the leupeptin-sensitive protease may be active and may cleave the full length form of HMGCR. To detect if the leupeptin-sensitive protease has an effect on the detection of HMG-CoA reductase using Western blotting, one sample of control cells and one sample of cells treated with 100 µM simvastatin were lysed using buffer containing 0.15 M NaCl, 50 mM Tris, 0.5 mM $MnCl_2$, 0.5 mM $MgCl_2$, 5 mM EGTA, 10 mM DTT, 0.1 mM leupeptin hemisulphate,

0.2 mM PMSF, and 2% Zwittergent 3-14 at pH 7.4. The presence of EGTA in the buffer would chelate free Ca^{2+} and leupeptin would inhibit the leupeptin-sensitive protease. SDS-PAGE electrophoresis and Western blotting were done as mentioned before. Lanes CL and SL in Figure 4-14 show two HMG-CoA reductase bands of 72 and 54 kDa respectively, as detected in previous extracts, but no band of full length HMGCR. The beta-actin was detected at 42 kDa (Figure 4-15).

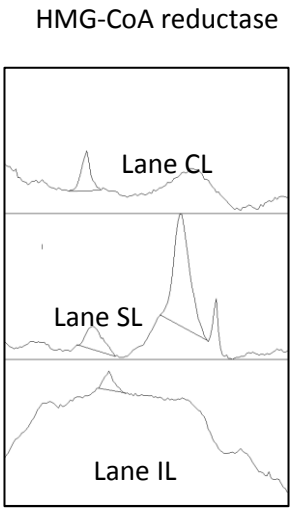


Figure 4-14: Estimation of HMG-CoA reductase protein expression by transforming the bands (Figure 4-13) into peaks using image J. Lane CL is the control (DMSO <1%). Lane SL corresponds to the cells treated with simvastatin.

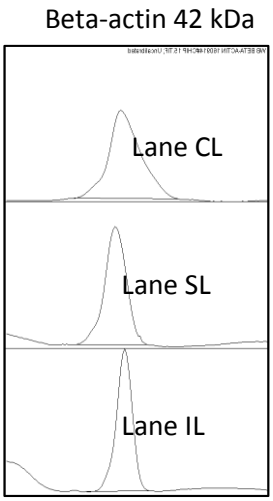


Figure 4-15: Estimation of Beta-actin protein expression by transforming the bands into peaks using image J. Lane CL is the control (DMSO <1%). Lane SL corresponds to the cells treated with simvastatin.

Table 4-7: The intensities of the bands (Figures 4-14, 4-15) as calculated by Image J. Lane SL is used as a reference for CL and IL.

		Lane		
		CL	**SL**	**IL**
HMG-CoA reductase 72 kDa	Intensity	1655	8333	829
	Normalized intensity	0.171	1.0	0.099
HMG-CoA reductase 54 kDa	Intensity	0	1797	0
	Normalized intensity	0	1.0	0
Beta-actin 42 kDa	Intensity	12580	11593	10240
	Normalized intensity	1.05	1.0	0.883
Ratio of HMG-CoA reductase 72 kDa/ beta-actin		0.163	1	0.112
Ratio of HMG-CoA reductase 54 kDa/beta-actin		0	1	0

Table 4-8: The intensities of the bands (figures 4-14,4-15) as calculated by Image J. The intensities of HMG-CoA reductase 54 and 72 kDa were combined. Lane CL is used as a reference.

		Lane		
		CL	**SL**	**IL**
HMG-CoA reductase 72 kDa	Intensity	1655	8333	829
HMG-CoA reductase 54 kDa	Intensity	0	1797	0
Combined HMG-CoA reductase 54 and 72 kDa	Intensity	1655	10130	829
	Normalized intensity	1.0	6.12	0.5
Beta-actin 42 kDa	Intensity	12580	11593	10240
	Normalized intensity	1.0	0.92	0.814
Ratio of HMG-CoA reductase / beta-actin		1.0	6.65	0.614

Analysis of the intensities of the HMG-CoA reductase and beta-actin bands revealed a marked 7.5 fold stimulation of expression of HMG-CoA reductase by simvastatin, with both 74 kDa and 54 kDa bands up-regulated (Figures 4-15, 4-16). In this experiment, simvastatin still stimulated the expression of the HMGCR but the 74 kDa was the main band while the intensity of the 54 kDa band was very low in both CL and SL. This could be due to the inhibition of the leupeptin-sensitve enzyme and consequently the inhibition of the cleavage of the enzyme from 74 kDa to 54 kDa.

Using RIPA buffer, the membranes were solubilised and this resulted in the release of both bands in to the supernatant layer after centrifugation. Because both fragments contain the active site, the primary antibody used reacted with both bands. If the soluble 54 kDa band represents the intact 97 kDa band, this means that the simvastatin stimulated the HMGCR expression but the *Ilex latifolia* inhibited HMG-CoA reductase expression compared to the control cells (Table 4-8). If the 72 kDa is released due to the effect of the endogenous Ca^{+2} activated protease on the intact protein or due to thawing and freezing (Ness *et al*., 1981) and the 54 kDa released due to the effect of the leupeptin sensitive protease, this means that the two fragments together represent the intact protein. The intensities of both fragments can be combined to model the effect of simvastatin and *Ilex latifolia* on HMGCR expression. Simvastatin stimulated the HMGCR expression but the *Ilex latifolia* extract inhibited the HMGCR expression compared to the control cells treated with DMSO. This hypothesis was checked by using leupeptin in the lysing buffer to inhibit the leupeptin sensitive protease. The ratio of 54/72 kDa forms was 0.22 while the ratio of 54/72 kDa was 0.4 when the cells were lysed using RIPA buffer with protease inhibitor cocktail.

The same method applied to detect HMG-CoA reductase expression, was used to detect the effect of *Ilex latifolia* extracts on HMG-CoA reductase expression in AML-12 cells.

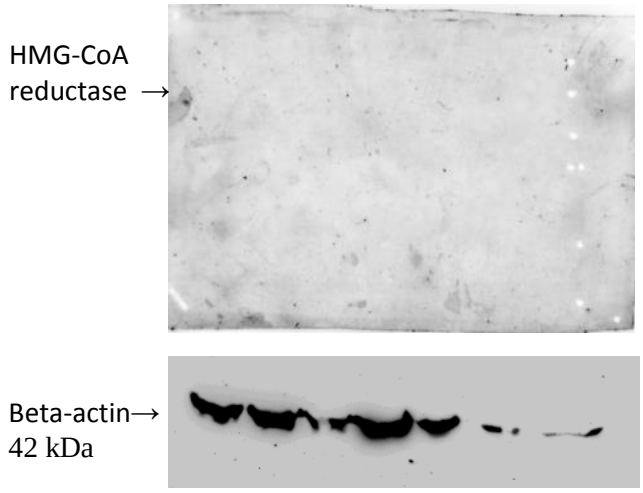


Figure 4-16: Assay of HMG-CoA reductase expression in AML-12 cells.

Although beta-actin was detected in AML-12 cells lysates, HMG-CoA reductase was not detected at all. The method used was the same method used for the detection of HMG-CoA reductase in HepG2 cells where the bands were detected at 54 and 72 kDa (section 4.4.3.2). The antibody detects mouse forms of HMG-CoA reductase and should have worked for AML-12 cells. The results suggest that expression of HMG-CoA reductase is very low in AML-12 cells, or the protein is very unstable.

4.4.3.5 LDLR expression in AML-12 cells using Western blotting

HepG2 cells were initially used to detect the expression of LDL-receptors but no bands were detected (data not shown). Since AML-12 cells have been used for the detection of LDL-receptors (Miranda et al., 2015), they were used here to detect the expression of LDLR and to assay the effect of *Ilex latifolia* on LDLR expression.

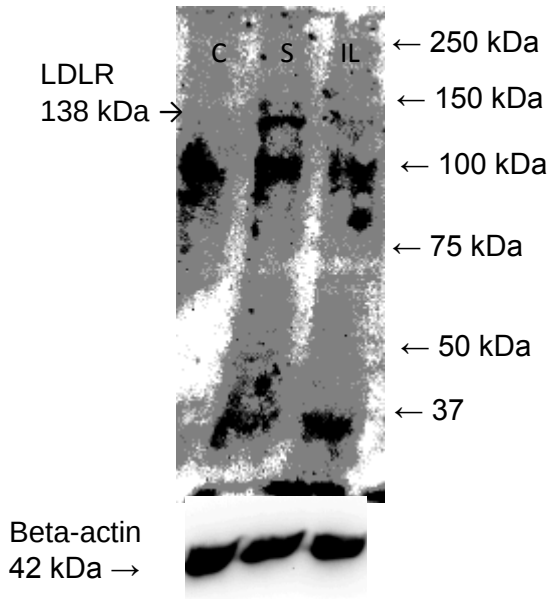


Figure 4-18: Immunoblot of proteins from lysate of AML-12 cells which had been treated with 100 µM simvastatin (Lane S), 100µg/mL *Ilex latifolia* extract (Lane IL), or DMSO which was used as a simvastatin vehicle (Lane C). Cells were lysed using Triton-X-100 buffer. LDLR and beta-actin proteins were separated by SDS-PAGE followed by transfer to nitrocellulose membrane and detection using chemiluminescence. Precision plus protein dual xtra standards marker (BioRad) was used as a reference .

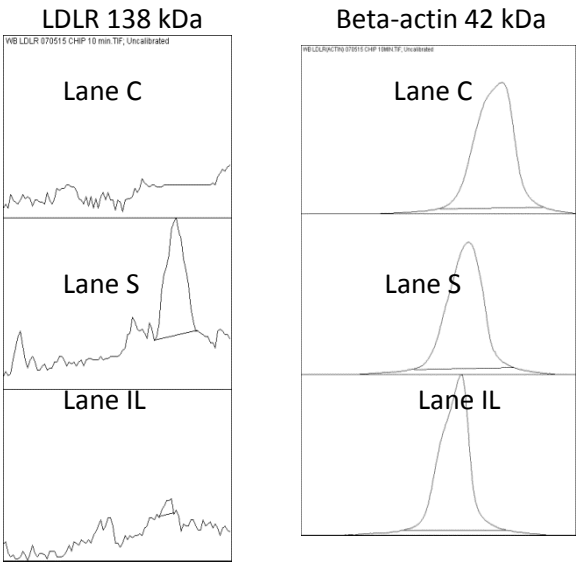


Figure 4-17: Plots of LDLR and beta-actin band intensities (Figure 4-18) using Image J. Lane CL is the control (DMSO <1%). Lane SL corresponds to the cells treated with simvastatin while lane IL corresponds to the cells treated with *Ilex latifolia.*

AML-12 cells were maintained in three 75 cm^2 flasks with full medium supplemented with 10 % FBS. Once the cells were confluent, the medium was replaced with new medium. One flask was treated with DMSO (< 1%) as a negative vehicle control (Lane C). The other two flasks were treated with 100 µM simvastatin (Lane S) and 100 µg/mL *Ilex latifolia* butanol extract (Lane IL) respectively. The cells were lysed using triton x-100 buffer as mentioned in section 4.2.2.3. After SDS-PAGE gel electrophoresis, the proteins were transferred to nitrocellulose membrane. LDLR proteins were probed using LDLR primary antibody . Beta-actin, which is a house-keeping protein, was detected using beta-actin primary antibody. Lanes S and IL showed LDLR bands at 138 kDa, but no LDLR was detected in the control cell extract. The beta-actin was detected at 42 kDa (Figure 4-19) at approximately equal intensity in all three

extracts. At 100 kDa there appear strong bands. These bands could be LDLR protein or they could be artefacts especially that they appeared after the adjustment of the image brightness. The 38 kDa band appeared without any adjustment but after adjustment of the brightness, it was more clear.

Table 4-9: The intensities of the bands (Figure 4-17) as determined using Image J. Lane S is used as a reference for lanes C, and IL.

		Lane		
		C	**S**	**IL**
LDLR 138 kDa	Intensity	0	6065	325
	Normalized intensity	0	1.000	0.054
Beta-actin 42 kDa	Intensity	26800	24214	24226
	Normalized intensity	1.107	1.000	1.005
Ratio of LDLR/beta-actin		0	1.000	0.054

Due to the lack of LDLR signal it was not possible to calculate changes in LDLR expression relative to the control. Instead the intensities of the LDLR and beta-actin were quantified using Image J. (Figure 4-18) and the band of LDLR from the cells treated with simvastatin (Lane S) was used as a reference for lanes C and IL. The intensities of the bands in lane S were normalized to 1. The intensities of the bands in lanes C and IL were calculated as a ratio to the bands in lane S. (Table 4-9) LDLR bands intensities were calculated as a ratio to the beta-actin bands to cancel any variation in the results due to the amount of protein loaded in the gel. The data show that LDLR expression was considerably enhanced, by about 20 times, in cells treated with simvastatin compared with cells treated with *Ilex latifolia*.

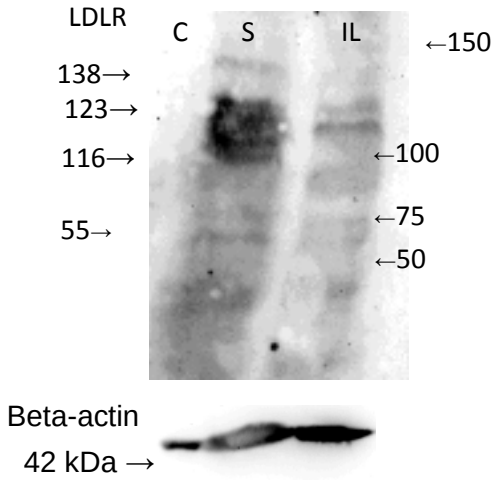


Figure 4-19: Immunoblot to detect LDLR in lysates of AML-12 cells which had been treated with 100 µM simvastatin (Lane S), 100 µg/mL *ilex latifolia* extract (Lane IL), or DMSO which was used as a simvastatin vehicle (Lane C) . All the flasks were lysed using triton-x100 buffer. LDLR and beta-actin proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane before detection using chemiluminescence. Precision plus protein dual xtra standards marker (BioRad) was used as a reference.

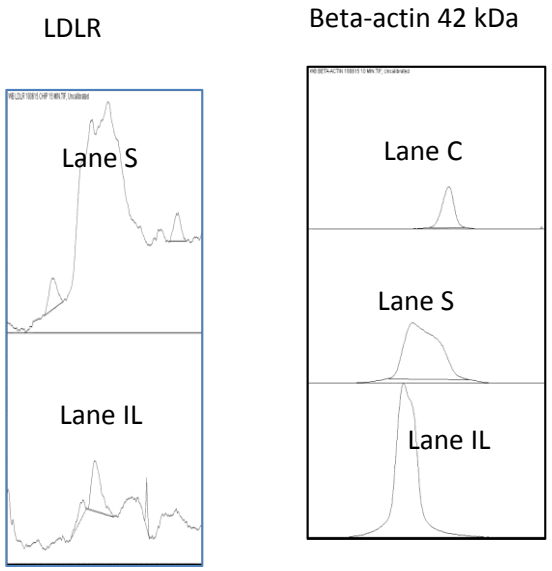


Figure 4-20: Plots of LDLR and beta-actin bands (Figure 4-19) using image j. Lane CL is the control (DMSO <1%). Lane SL corresponds to the cells treated with simvastatin).

Table 4-10: The intensities of the bands (Figures 4-19, 4-20) as calculated by Image J. Lane S is used as a reference for lanes C, and IL for bands with 138, 116, and 55 kDa molecular weight . Lane IL is used as a reference for lane S with 116 kDa molecular weight.

		Lane		
		C	**S**	**IL**
LDLR 138 kDa	Intensity	0	497	0
	Normalized intensity	0	1.0	0
LDLR 123 kDa	Intensity	0	0	440
	Normalized intensity	0	0	1.0
LDLR 116 kDa	Intensity	0	830	409
	Normalized intensity	0	1.0	0.49
LDLR 55 kDa	Intensity	0	196.7	0
	Normalized intensity	0	1.0	0.0
Beta-actin 42 kDa	Intensity	3259	13070	22626
	Normalized intensity	0.25	1.0	1.73
Ratio LDLR 138 kDa/ Beta-actin		0	1.0	0
Ratio LDLR 123 kDa/ Beta-actin		0	0	1.0
Ratio LDLR 116 kDa/ Beta-actin		0	1.0	0.28
Ratio LDLR 55 kDa/ Beta-actin		0	1.0	0

LDLR were also detected in HepG2 and AML-12 cells using LDLR (C-20) antibody (Santa Cruz Biotechnology sc-11824). No bands were detected for LDLR in HepG2 or AML-12 cells. This antibody was used to detect the LDLR before the antibody purchased from Novus Biological was used. It did not detect the bands but because LDLR was detected in AML-12 cells, the search for another antibody was the second step.

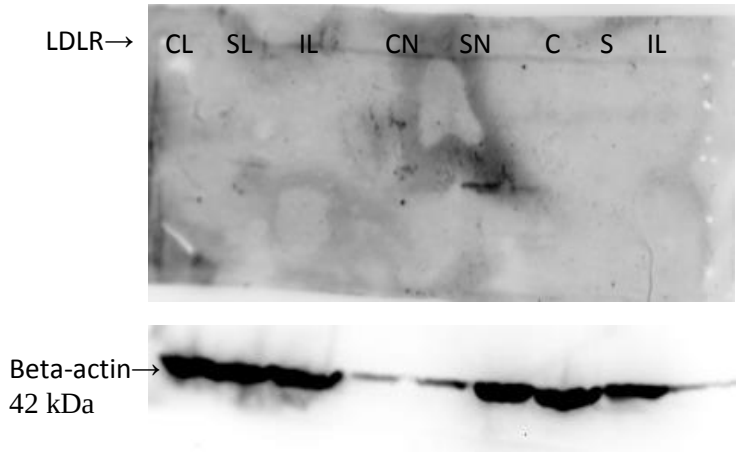


Figure 4-21: Immunoblot to detect LDLR in lysates of AML-12 and HepG2 cells using Santa Cruz primary antibody. LDLR and beta-actin proteins were analysed by SDS-PAGE and transferred to nitrocellulose membrane before detection using chemiluminescence. Precision plus protein dual xtra standards marker (BioRad) was used as a reference.

The blot, shown in Figure 4-21, shows that although beta-actin was detected in most of the loaded samples, no bands were detected for LDLR. This indicates that the primary antibody used in the previous experiments (EP1553Y from Novus Biologicals, Abingdon, UK) is more sensitive for detection of LDLR in AML-12 cells.

4.5 Conclusion

A major effort was made to try to detect effects of *Ilex latifolia* extract on cholesterol metabolism in hepatocyte model cell lines. The first phase was to focus on enzymic assay of HMG-CoA reductase, as the rate-limiting enzyme in cholesterol synthesis. HMG-CoA reductase catalyzes the reaction HMG-CoA + 2NADPH + 2H+ leading to mevalonic acid + 2NADP+ + COASH. $NADP^+$ has a yellow colour and consequently cannot be measured using colorimetric methods. The enzyme is unstable for prolonged periods at assay temperatures and the colour of the product is not stable as well, so that the readings should be done within seconds to 5 minutes as mentioned by the supplier (Sigma Aldrich, SC 1090). This method is complicated and not reliable. The second method was to detect the reaction products CoA and NADP using HPLC. However, HPLC was found not to be suitable method to assay HMGCR activity because the assay was done on a very small scale and HPLC is not sensitive to small changes in concentrations which could be in nanogram amounts.

The third method was to detect mevalonolactone using LC-MS which is more sensitive than HPLC. LC was required to separate the peak of mevalonolactone, and MS was to be used as a detector. For this reason, the minimum detected concentration of mevalonolactone was determined using HPLC but this method was not reliable because mevalonolactone was detected in microgram amounts only and the wavelength used was 200 nm and this resulted in interference from the mobile phase which contained formic acid.

Considering all the disadvantages of these methods, the samples were prepared for assay by LC-MS but unfortunately the assay could not be done because the instrument became unavailable.

Consequently, in the second phase, Western blotting was used to detect expression of HMG-CoA reductase. The inhibitory effects of *Ilex latifolia* and simvastatin, which is an approved HMG-CoA reductase inhibitor, would be manifested as an increase in HMG-CoA reductase protein expression. HepG2 cells were initially used to do the assay. HMG-CoA reductase as a full length protein has a 97 kDa molecular weight. After gel electrophoresis and Western blotting, the enzyme was detected at 72 and 54 kDa, but no full length bands were ever detected. Nevertheless it was possible in two experiments (Figs. 4-9 and 4-13) to show that simvastatin stimulated the expression of the enzyme to varying amounts, depending on how the extracts were prepared, but *Ilex latifolia* did not show any effect compared to the control. However it was difficult to reproduce the results in a consistent way, and other experiments showed a down-regulation of HMG-CoA reductase bands after treatment with simvastatin (Figs. 4-17, 4-11, 4-13). The levels of expression of HMG-CoA reductase in HepG2 cells are not very high and it was hoped that AML-12 mouse liver cells would have higher levels of detectable protein. This turned out not to be the case (Fig. 4-12) and no HMG-CoA reductase could be detected. The variable patterns could be due to the poor stability of the enzyme. The enzyme is sensitive to the endogenous Ca^{2+} activated enzymes which break it to a 62 kDa fragment which remains membrane bound. This 62 kDa fragment is sensitive to leupeptin sensitive enzyme which breaks it down to 52-56 kDa soluble fragment. The enzyme is not stable under freezing and thawing conditions which could have the same effect as the enzymes mentioned before. The 72 kDa fragment detected in HepG2 cells could be similar the 62 kDa fragment detected in other cells. To inhibit the leupetin sensitive protease, RIPA buffer was replaced by a lysing buffer containing leupeptin. The result was that the 72 kDa band was more intense than the previous assays but there was no indication that *Ilex latifolia* induced HMGCR expression compared to simvastatin (Figure 4-14). In order to make the assay reliable it is necessary to establish conditions in which consistent results are obtained. Although attempts were made to optimise conditions e.g by using different lysis buffers and protease inhibitors, the process of optimisation was very time consuming and not productive. It seems likely that the enzyme is

turned over especially quickly in the liver and not highly expressed (discussed in Liscum *et al*. 1983), which means a hepatocyte model is difficult to use. The early studies on HMG-CoA reductase by Brown and Goldstein were based on a variety of non-liver cell lines e.g. Chin *et al.* (1982) used Chinese hamster ovary cells, in which levels of full-length HMG-CoA reductase were easily detectable. Although the liver is a key organ for control of cholesterol in the body, other tissues employ the same homeostatic mechanisms (Brown and Goldstein 1980). Future assessment of modulators of HMG-CoA reductase could be tried using a more robust cell culture model such as Chinese hamster ovary cells.

Inhibition of HMG-CoA reductase induces expression of LDL receptors, and in the third phase of the investigation LDLR expression was assayed using AML-12 cells, which are known to express measurable levels of LDLR. The effect of *Ilex latifolia* extract was compared to simvastatin. Simvastatin stimulated LDLR expression as shown by detection of a band at 138 kDa (Fig. 4-19) but *Ilex latifolia* did not have any effect on the expression of LDLR band at 138 kDa, although small amounts of other bands were detected. Taken together, and although the data are fragmentary, the results indicate that the butanol extract of *Ilex latifolia* has little effect on pathways of cellular cholesterol metabolism.

5 Chapter V: Assessment of *Ilex latifolia* extracts on hepatocyte glucose metabolism

5.1 Introduction

5.1.1 Glucose uptake

Accelerated atherosclerosis and congestive heart diseases are usually associated with diabetes (Steiner, 1985). The majority of people with type 2 diabetes die of atherosclerosis and congestive heart disease because diabetic people are at greater risk of developing cardiovascular disease (Rudermann NB, 1990), although the mechanism relating diabetes to atherosclerosis is not well understood. Stimulation of glucose uptake and metabolism could help in reducing the risk of hyperlipidemia. Another concern is that if oxidative phosphorylation is affected, there may be potential for toxicity. This chapter describes an assessment of the effect of *Ilex latifolia* extract on glucose uptake and metabolism in AML-12 and HepG2 cells, as models of hepatocytes, and investigates the possibility that oxidative phosphorylation could be mildly inhibited.

5.1.2 Mitochondrial toxicity

In most mammalian cells, mitochondria produce >90% of the cell's energy in the form of adenosine triphosphate (ATP), required for survival. Agents that inhibit or undermine mitochondrial function will result in less cell viability, and according to the severity, result in tissue or organ injury and toxicity (Masubuchi *et al*., 2006). Liver, cardiovascular system, skeletal muscles, nervous system, and kidneys could be injured by different drugs in this way. Due to safety concerns, 38 of the new drugs approved by the US Food and Drug administration (FDA) were withdrawn from the market because they have had hepato- and cardiotoxicities (Shah, 2006). Failure to detect the adverse reactions of these drugs, although it only represents a small percentage (2-3 %) of the total, results in human suffering, reduces trust in the pharmaceutical industry and leads to financial losses (Shah, 2006). Toxicity is missed and could not be revealed by even a large phase III trial because it rarely occurs. If 0.1 % of patients experience side effects of a drug, this means more than 10,000

patients should be exposed before the probability of the event occurring becomes realistic. The relation between mitochondrial impairment due to drug exposure and etiology of various organ toxicities has been acknowledged recently. Some drugs such as troglitazone, cerivastatin, and tolcapone have been withdrawn from the market because they caused organ toxicities related to mitochondrial dysfunction (Ong *et a*l., 2007). Tumour derived immortalized cell lines are mainly used in the cell-based assays used in drug discovery and development because they can be grown in a reproducible and controlled way. However, unlike normal cells they can metabolically grow under hypoxic and acidic conditions, and they produce all the required energy from glycolysis rather than depending on mitochondrial oxidative phosphorylation (OXPHOS) (Rodriguez-Enriquez *et al*., 2001). HepG2 cells are not affected by mitochondrial toxicants due to the high glycolytic capacity, which is typical of cancer cells. When HepG2 cells are grown in galactose medium instead of glucose medium, they will increase the respiration rates to maintain ATP levels because galactose has to be converted to glucose inside the cell, which has an energy cost, before entering glycolysis (Warburg *et al*., 1967). Using galactose as a metabolic fuel means that cells can no longer generate most of the ATP requirement through glycolysis. Oxidation of galactose to pyruvate via glycolysis will force the HepG2 cells to use the mitochondrial OXPHOS to generate sufficient ATP for survival because the galactose oxidation to pyruvate yields no net ATP (Rossignol *et al*., 2004). The effect of different concentrations of *Ilex latifolia* extract on growth of HepG2 cells in glucose compared with galactose was investigated by using the MTT assay. Further work was done to investigate the effect of *Ilex latifolia* extract on the ATP levels in HepG2 cells. The toxicity of *Ilex latifolia* was assessed by replacing galactose for glucose in the medium to investigate its effect on the mitochondrial function of HepG2 cells. AML-12 cells are non-tumorigenic cells. They are a hepatocyte cell line derived from transforming growth factor-alpha (TGF-α) transgenic mice (Wu *et al*., 1994), and do not have the tumour phenotype of energy metabolism. AML-12 cells are more sensitive than HepG2 cells to mitogenic toxins. For example when AML-12 cells were treated with the mitogenic toxin pentachlorophenol (PCP), the LD_{50} was 16.2 $\pm$ 2.0 µg/mL compared with an LD_{50} of

23.4 ± 9.7 µg/mL in HepG2 cells (Dorsey *et al*., 2004). The effect of *Ilex latifolia* extract on AML-12 cell growth was investigated using the MTT assay. The effect of *Ilex latifolia* on the mitochondrial function of AML-12 was assayed when the cells were grown in medium containing glucose and when galactose was replaced for glucose. FCCP, an approved mitochondrial toxicant, was used as a positive control. The effect of *Ilex latifolia* on glucose uptake in HepG2 and AML-12 cells was investigated and the glucose consumption was normalized to the cell growth. In the case of AML-12 cells, the effect of the presence of insulin in the medium was also investigated.

5.2 Experimental methods

5.2.1 Materials

HepG2 cells were purchased from ECACC (Salisbury, UK). Dulbecco's modified Eagle's medium (DMEM), DMEM glucose deprived medium, glucose, galactose, 3-(4,5 dimethylthiazol-2-yl)-2,5, diphenyltetrazolium bromide (MTT), carbonyl cyanide4-(trifluoromethoxy) phenylhydrazone (FCCP), L-glutamine and DMSO were purchased from Sigma-Aldrich (Gillingham, UK). Sterile 24 well plates were purchased from Fisher Scientific (Loughborough, UK). Trypsin was purchased from Gibco (Paisley, UK). Dialysed foetal calf serum (FCS) was purchased from First Link UK limited (Wolverhampton, UK). All the experiments were done using *Ilex latifolia* butanol extract (Chapter II, section 2.3.3).

5.2.2 Assessment of *Ilex latifolia* on HepG2 cell growth

The HepG2 cells were seeded at 1.3 x 10^4 cells per ml of DMEM/ 10 % FCS in a 24 well plate. After the cells were almost fully confluent, the medium was changed and the cells were treated with DMSO (<1%) as a negative, vehicle, control, and with *Ilex latifolia* butanol extract. *Ilex latifolia* extract (17 mg/mL) was diluted two-fold five times in DMSO. The assay was done in triplicate. Cell growth and cell toxicity was assessed using an MTT assay. After overnight treatment of cells, 300 µL of the medium was removed. 50 µL of MTT solution (5 mg/ml in PBS) was added to each well. The solutions in the wells were mixed by gentle swirling and the cells were incubated at 37 ºC for 3.5 hours. After incubation the medium

mixed with MTT was removed and 500 µL of DMSO were added to lyse the cells and dissolve the purple crystals that develop by reduction of the MTT to formazan, by pipetting up and down several times. The plates were covered with aluminium foil to protect the solution from light followed by shaking on an orbital shaker for 15 mins. After 15 minutes of shaking, the absorbance was read at 590 nm corrected for light scattering at 620 nm using a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK).

5.2.3 Assay of mitochondrial toxicity of *Ilex latifolia* on HepG2 cell

HepG2 cells were grown at 1.3 x 10^5 cells/mL in two 24 well plates. After full confluency, the medium was changed to 10 mM glucose and 10 mM galactose medium in each plate respectively. The first columns (wells A1-A4) were treated with 5 µL of 93 mg/mL *Ilex latifolia* solution. The second columns (wells B1-B4) were treated with 5µL 46.5 mg/mL of *Ilex latifolia* solution. The third columns (wells C1-C4) were treated with 5 µL 32.25 mg/mL of *Ilex latifolia* solution. The fourth columns (wells D1-D4) were treated with 5 µL of 16.125 mg/mL of *Ilex latifolia*. The fifth columns (wells E1-E4) were treated with 5 µL of DMSO (<1%) as a vehicle, negative control. The sixth columns (wells F1-F4) were treated with 5 µL of 10 mM FCCP as a positive control. The treatment was applied to cells grown in glucose and galactose respectively. All the treated cells were incubated overnight at 37 ºC with 5 % CO_2 supply. The following day cell survival was assessed by an MTT assay as described in section 5.2.2.

5.2.4 Assay of ATP levels in HepG2 cells

5.2.4.1 Treatment of cells

HepG2 cells were seeded in a 6 well plate at 2.3 x10^4 cells per mL. After growing to full confluence, the cells were treated overnight with *Ilex latifolia* butanol extract at 920, 460, 230, and 115 µg/mL respectively. DMSO was used as vehicle, negative control. One well was left without any treatment to investigate if DMSO will have any effect on cell viability and consequently on ATP levels. After incubation overnight, the cells were lysed with boiling water by pipetting up and down several times (Yang *et al*., 2002). The lysed cells were

transferred to an Eppendorf tube and centrifuged at 17226 g for 5 minutes. The supernatant layer was separated.

5.2.4.2 Determination of the protein content of cell lysates using Bradford reagent

Bovine serum albumin was prepared at 3.5 mg/mL in PBS. BSA was diluted two-fold five times. To a 96 well plate, 5 µL of the BSA standard solutions, or 5 µL of the cells lysates, were added. Boiled water was used as a blank. 250 µL of the Bradford reagent were added to each well. The plate was shaken on an orbital shaker for 15 seconds and left for 15 mins. After shaking the absorbance was measured 595 nm using a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK).

5.2.4.3 Assay of ATP levels in the cell lysates

ATP levels in the cells lysates were assayed using an ATP assay kit purchased from Abcam (Cambridge,UK, cat. No ab83355). ATP standards were prepared as a 10 mM stock solution, which was further diluted to 1 mM and lower concentrations as indicated in Table 5-1.

Table 5-1: Serial dilutions of 1mM standard ATP

Final [ATP] in well	ATP 1mM Standard (µL)	Assay Buffer (µL)	Total Volume (µL)
0mM = 0 nmol/well	0	150	150
0.04mM = 2 nmol/well	6	144	150
0.08mM = 4 nmol/well	12	138	150
0.12mM = 6 nmol/well	18	132	150
0.16mM = 8 nmol/well	24	126	150
0.2mM = 10 nmol/well	30	120	150

ATP assay reaction mixture was prepared by adding 1100 µL of ATP assay buffer and 50 µL of ATP probe, ATP converter, and ATP developer mix respectively. These solutions were provided in the assay kit and did not have contents listed. To the wells, 50 µL of reaction mixture were added. 50 µL of each standard solution or cell lysate were added and incubated at room temperature for 30 mins protected from light. After incubation, the absorbance was measured 570 nm corrected for light scattering at 620 nm using a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK).

5.2.5 Effect of *Ilex latifolia* extract on glucose uptake by HepG2 cells

In preliminary experiments, a time course of glucose consumption was established with a view to selecting an appropriate time point for assessing the effect of treatments. HepG2 cells were seeded in a 24 well plate and 5 µL of *Ilex latifolia* butanol extract at 10, 3, 1, 0.3, and, 0.1 mg/mL were added respectively. The medium glucose was checked with a glucometer (Codefree blood glucose monitoring apparatus made by SD Biosensor Inc, Korea, purchased from Home Health UK, Watford, Herts) and found to be 10.8 mM glucose. The glucose content of the wells was measured for 3 days using the glucometer. The assay was done in quadruple, and the experiment was repeated. To examine the effect of treatments a 24-hour period was chosen. HepG2 cells were grown till confluency. The medium was changed to DMEM containing 11 mM glucose. 5 µL of 1.0, 0.3, 0.1, 0.03 and 0.01 mg/mL *Ilex latifolia* extract were added to each set of cells. They were incubated for 24 hrs and the glucose in the medium was measured using the glucometer. DMSO was used as vehicle negative control. An MTT assay was done to determine the cell quantity.

5.2.6 The effect of *Ilex latifolia* extracts on glucose metabolism on mouse liver AML-12 cells

5.2.6.1 AML-12 cell treatment

AML-12 cells were grown in DMEM-Ham F12 (1:1) medium containing 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL selenium, and 40 ng/mL dexamethasone, supplemented with 10 % FCS at 1.3 x 10^5 cells/mL in four 24 well plate. After full confluency, the medium in two plates was changed to 10 mM glucose with and without insulin respectively. The medium in the other two plates was changed to 10 mM galactose with and without insulin respectively. The first columns (wells A1-A4) were treated with 5 µL of 100 mg/mL *Ilex latifolia* extract. The second columns (wells B1-B4) were treated with 5µL 50 mg/mL of *Ilex latifolia* extract. The third columns (wells C1-C4) were treated with 5 µL 25 mg/mL of *Ilex latifolia* extract. The fourth columns (wells D1-D4) were treated with 5 µL 12.5 mg/mL of *Ilex latifolia* extract. The fifth columns (wells E1-E4) were treated with 5 µL of DMSO (<1%) as a vehicle negative control. The sixth columns (wells F1-F4) were treated with 5 µL of 10 mM FCCP as a positive control. The treatment was done to cells grown in glucose and galactose respectively. All the treated cells were incubated overnight at 37 ºC with 5 % CO_2 supply. An MTT assay was performed as described in section 5.2.2.

5.2.6.2 Glucose uptake

AML-12 cells were grown in a 24 well plate until confluent. The medium was changed to DMEM/Ham F-12 (1:1) containing 11.3 mM glucose, ITS, and dexamethasone. 5 µL of 10, 3, 1, 0.3, and, 0.1 mg/mL *Ilex latifolia* extract were added to each set of cells. DMSO was used as vehicle negative control. They were incubated for 24 hrs. Glucose was measured for 4 consecutive days using the glucometer. The same method was repeated by treating the cells with 5 µL of 1, 0.3, 0.1, 0.03 and 0.01 mg/mL *Ilex latifolia* extract using insulin and insulin free medium. Glucose was measured using the glucometer for three consecutive days and an MTT assay was done to determine the cell quantity, on the third day. The assay was done in quadruple.

5.2.7 Data analysis

GraphPad Prism was used to model the data. One-way and two-way ANOVA were used to compare the means of controls with treatments.

5.3 Results

5.3.1 Investigation of the mitochondrial toxicity of *Ilex latifolia* on HepG2 cells

HepG2 cells were seeded about $1.3x10^5$ cells/ mL in normal full DMEM and left to grow overnight. The medium was changed next day to lab-prepared DMEM / dialysed FCS / 1 mM pyruvate / 4 mM glutamine and 10 mM glucose or galactose. The cells were treated with 10 mM FCCP as a positive control and with DMSO as a negative vehicle control or *I. latifolia* extract in two-fold serial dilutions. All the treatments were done in quadruplicate. Cells were treated overnight and an MTT assay was done. The aim of the assay was to compare the effect of the treatments in cells grown in glucose with cells grown in galactose, shown in Figure 5-1.

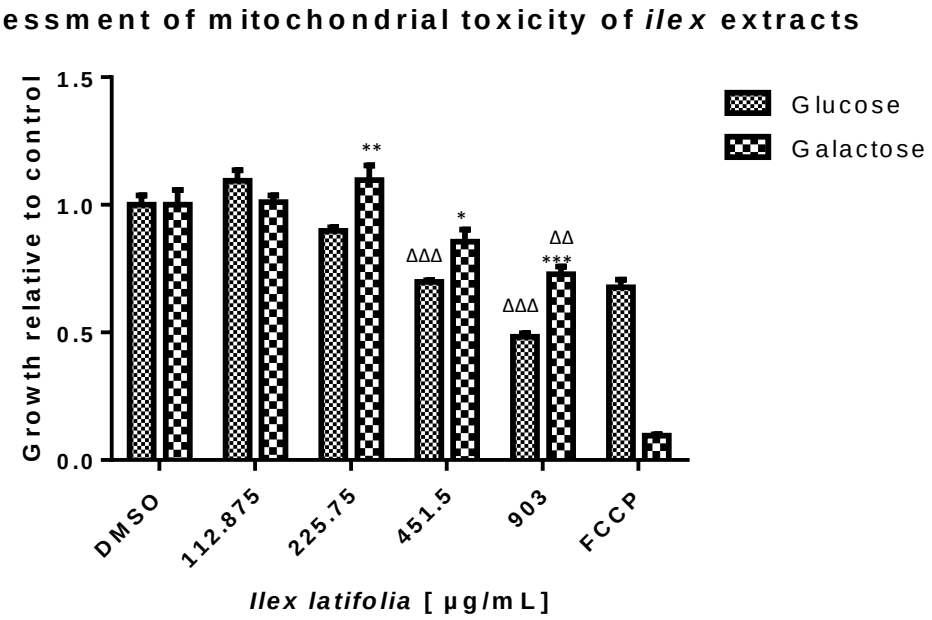


Figure 5-1: Assessment of effect of *Ilex* on HepG2 mitochondrial function. Data are the mean + SD pooled from four independent MTT assays of HepG2 cells grown in glucose , or galactose . Cells were treated with extract of *Ilex latifolia* at the concentrations indicated or with the mitochondrial inhibitor FCCP. $^{*}p< 0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ compared to glucose containing medium. $^{\Delta\Delta}p<0.01$ and $^{\Delta\Delta\Delta}$ p<0.001 compared to control untreated cells as determined by 2-way ANOVA followed by Bonferroni posttests. *Ilex latifolia* has a greater inhibitory effect on HepG2 growth when the cells are grown in glucose compared with galactose, although the cells in galactose are aerobically poised, as shown by the inhibition of growth by FCCP.

The graph above (Figure 5-1) shows the effects of *Ilex latifolia* extract compared to FCCP and DMSO on cells grown in DMEM-glucose or galactose medium. The data are normalized to controls to make comparison easier, but it is important to note that cells grow twice as much in glucose compared with galactose (data not shown). The difference in rates may be because the rate of ATP production is lower with galactose. In order to grow, cancer cells need a good supply of ATP from glycolysis, so the rate of glucose utilization in the glycolytic pathway is higher than normal. If galactose is the only fuel, it must first be converted to glucose, via the Leloir pathway, and the rate through this pathway may not be as high as the capacity of glycolysis. The FCCP is an approved mitochondrial toxicant and was used to confirm that the mitochondria of HepG2 cells are metabolically active in galactose, but less so in glucose. The effect of FCCP on the cells grown in galactose medium is very high (90.4 % growth inhibition) compared to the effect on the cells grown in the glucose medium (33 %

growth inhibition), and confirms that the HepG2 cells grown in galactose are aerobically poised. *Ilex latifolia* inhibited the growth of cells grown in both media, but the effect of the *Ilex latifolia* extracts on the cells grown in glucose medium was higher than the effect on the cells grown in galactose medium. *Ilex latifolia* at 112.875 µg/mL had the same effect on HepG2 cells growth in glucose and galactose containing media. At 225.75 µg/mL *Ilex latifolia* inhibited the growth of HepG2 cells by 11 % ($P < 0.001$) when the cells were grown in glucose containing medium. When galactose was replaced for glucose, *Ilex latifolia* did not show any effect. At 451.5 µg/mL, *Ilex latifolia* inhibited HepG2 cells growth by 30.8 % in glucose ($P<0.001$) compared to the control, but it only inhibited HepG2 cells growth by 14.4 % in galactose ($P>0.05$). *Ilex latifolia* inhibited HepG2 cells growth in glucose 16% more than in galactose ($P<0.05$). *Ilex latifolia* at 903 µg/mL inhibited HepG2 cells growth by 52% ($P<0.001$) in glucose while in galactose the inhibition effect was 27.2% ($P<0.01$) at 903 µg/mL compared to the control. *Ilex latifolia* inhibition effect in glucose was 24.8 % more than in galactose ($P<0.001$). Cells growing in glucose are more sensitive to *Ilex latifolia* than cells growing in galactose, but the glucose cells grow at twice the rate. It is possible that *Ilex latifolia* can affect the rate of glycolysis. This effect, compared to the effect of FCCP, shows that the *Ilex latifolia* toxicity on HepG2 cells is not due to mitochondrial toxicity but it could be due to another reason which needs to be investigated in the future.

5.3.2 Assessment of the effect of *Ilex* extract on cellular ATP

Lysates from cells treated with *Ilex latifolia* at different concentrations, with DMSO as vehicle negative control, or without any treatment, were assayed for their protein content, to normalise ATP measurements with respect to cell mass. Bovine serum albumin (BSA) was used as a standard. The absorbance was measured at 595 nm. A standard calibration curve for BSA was obtained, shown in Figure 5-2. Using the calibration curve (Figure 5-2), the protein content of different cell lysates was calculated. All the protein contents are summarized in Table 5-2.

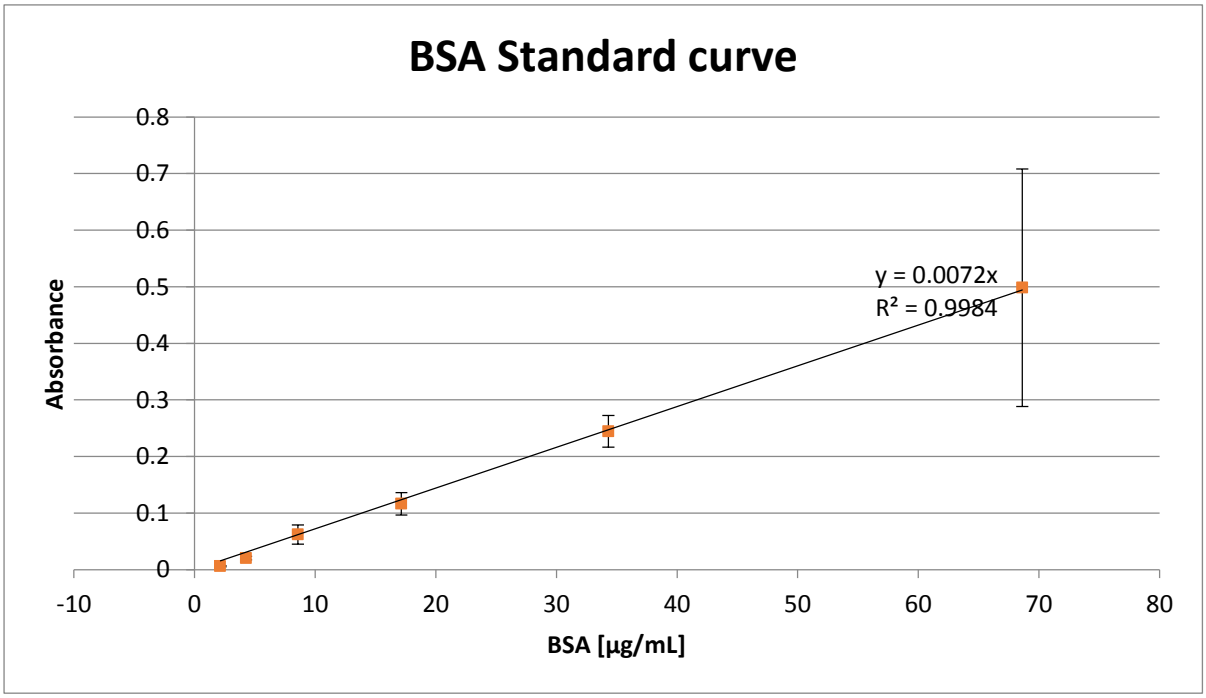


Figure 5-2: Standard curve of BSA for the Bradford protein assay. The assay was done in triplicate.

Table 5-2: Protein content of cells lysates using Bradford assay

Sample	*Ilex latifolia* [µg/mL]	Absorbance	Protein [µg/mL]
S1	920	0.136	18.88
S2	460	0.165	22.92
S3	230	0.197	27.36
S4	115	0.206	28.61
S5	DMSO<1%	0.224	31.11
S6	Control (no treatment)	0.204	28.33

The results in Table 5-2 reflect the data obtained from the MTT assays described in section 5.3.1, with reduced protein content, compared with controls, in cells treated with the higher concentrations of extract.

The ATP content of the cell extracts was determined using the calibration curve of standards shown in Figure 5-3.

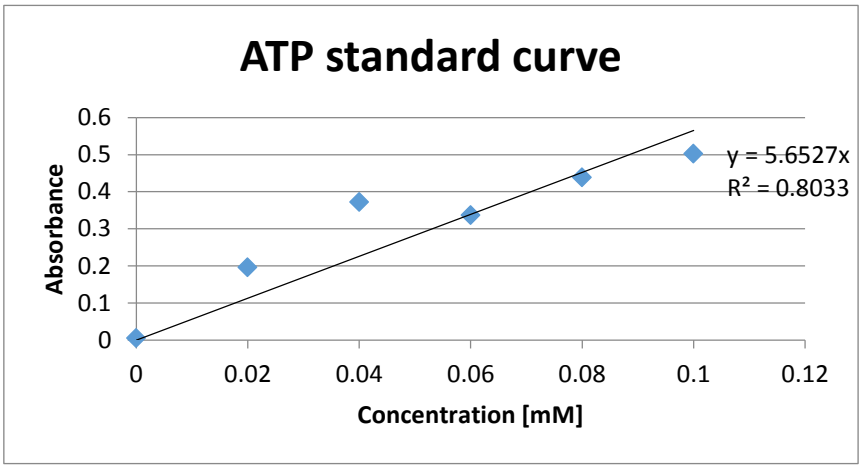


Figure 5-3: Standard curve of ATP. Data are means of duplicates.

The ATP content of lysates from cells treated with *Ilex latifolia* or DMSO, and lysates of cells without any treatment are given in Table 5-3. The standard calibration curve was used to determine the ATP level in the cells lysates. The ratio of ATP to the total protein content was determined, and is shown in Table 5-3.

Table 5-3: ATP content of lysates of cells treated with different concentrations of *Ilex latifolia*

Sample	*Ilex latifolia* [µg/mL]	Absorbance	Protein [µg/mL]	ATP(µM)	ATP [µg/mL]	ATP / protein(%)
S1	920	0.136	18.88	7.32	3.71	19.7
S2	460	0.165	22.92	16.52	8.38	36.6
S3	230	0.197	27.36	21.86	11.00	40.2
S4	115	0.206	28.61	19.18	9.73	34.0
S5	DMSO<1%	0.224	31.11	18.80	9.53	30.6
S6	Control (no treatment)	0.204	28.33	23.25	11.8	41.6

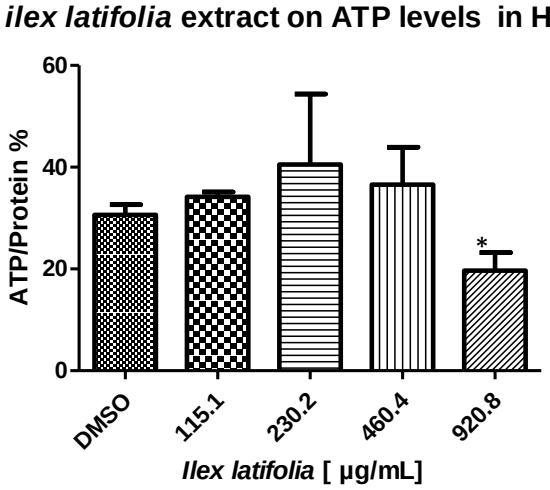


Figure 5-4: Effect of *Ilex latifolia* on ATP levels in HepG2 cells grown in medium containing glucose. Data are the mean + SD of duplicate assays of ATP in HepG2 cells treated with *Ilex latifolia* at different concentrations. $^{*}P<0.05$ compared to 115.1 µg/mL as determined by one-way ANOVA followed by Bonferroni post-hoc tests.

The graph (Figure 5-4) shows the effect of different concentration of *Ilex latifolia* on ATP levels in HepG2 cells compared to control untreated cells. The results are the means of duplicates. To avoid the effect of the herb on the cells' viability, ATP levels were calculated as a ratio to the total protein of each cell lysate. *Ilex latifolia* at 920 µg/mL inhibited the production of ATP by 35% compared with control ($P>0.05$). This effect is not significant as calculated by one-way ANOVA. On the other hand, *Ilex* at 920 µg/mL inhibited ATP production by 42% when compared to the effect at 115.1 µg/mL ($P<0.05$). It was interesting to investigate the effect of *Ilex* on ATP production in HepG2 cell when galactose is replaced for glucose especially that HepG2 cells were more sensitive to *Ilex* at 903 µg/mL in glucose than galactose.

5.3.3 Effect of *Ilex latifolia* glucose consumption by HepG2 cells

The effect of *Ilex latifolia* on glucose consumption was investigated by growing the cells in 24 well plates in full medium till confluency. After 90% confluency, the medium was changed to a glucose free medium to which glucose was added at 11.3 mM. The cells were incubated overnight and the glucose was measured using a Codefree blood glucose monitoring apparatus for three consecutive days. The results (Figure 5-5) show that the *Ilex latifolia* had little stimulation effect on glucose consumption at low concentrations (3 and 1 µg/mL), but it did not have any effect at higher concentrations compared to DMSO which was used as vehicle negative control. The main drawback of this method is that the glucose uptake is measured without considering the cells' densities in the wells, which will affect the glucose uptake. It was not expected that HepG2 cell growth would be much affected by the concentrations of *Ilex latifolia* extract used in these experiments compared with earlier experiments, shown in Figure 5-1, which used a range of 112 to 903 µg/mL.

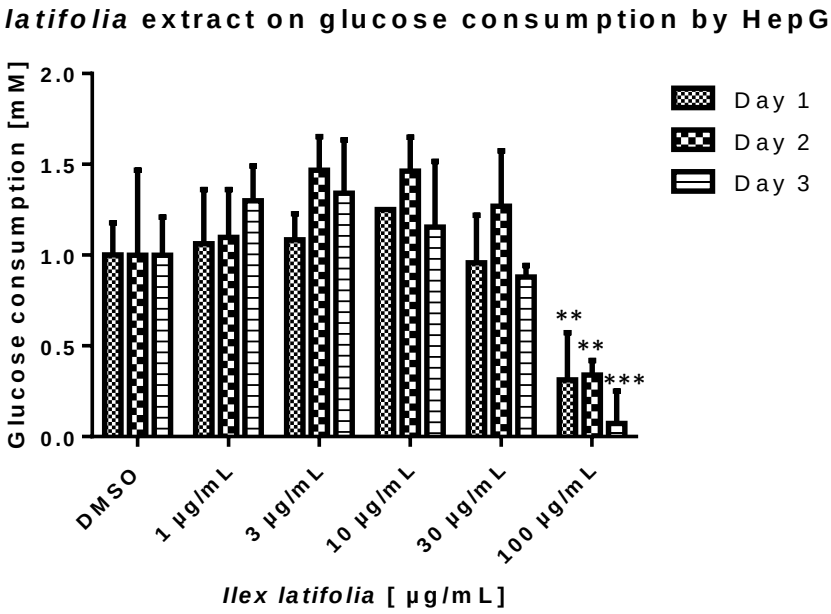


Figure 5-5: Effect of *Ilex latifolia* on glucose consumption by HepG2 cells. Data are the mean +SD of 4 assays of glucose consumption in HepG2 cells treated with *Ilex latifolia* and compared to control untreated cells. The assay was done for three consecutive days by measuring the glucose taken by the cells per day. **P<0.01 and ***P<0.001 compared to the control untreated cells in each day as determined by two-way ANOVA followed by Bonferroni post-hoc tests.

Ilex latifolia at 100 µg/mL inhibited glucose consumption in the three day assays (P<0.01 for days 1 and 2 and P<0.001 for day 3). The other concentrations (Figure 5-5) showed a little bit of stimulation of the glucose consumption especially 1, 3, and 10 µg/mL but the effect was not significant (P >0.05). To take into account the effect of the cell number on glucose consumption, an MTT assay was carried out at the end of the treatment period and the results were normalised by calculating the glucose uptake relative to cell amount.

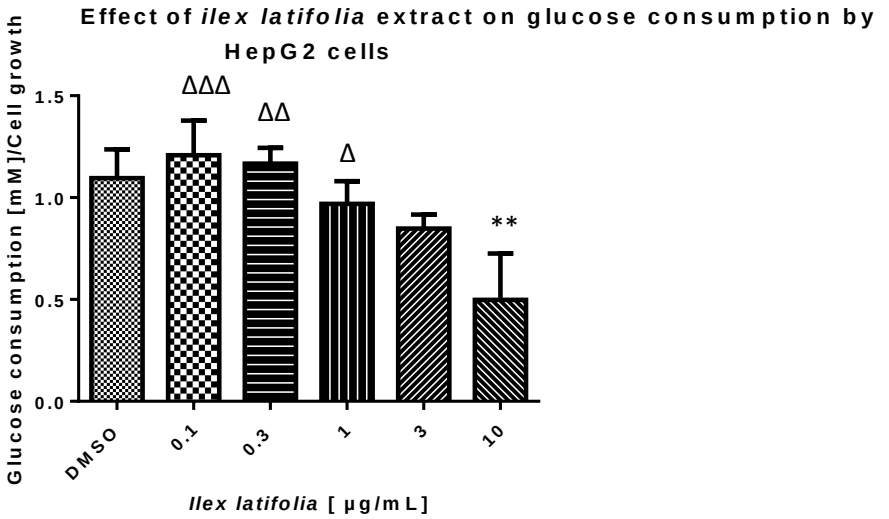


Figure 5-6: Effect of *Ilex latifolia* on glucose consumption by HepG2 cells. Data are the mean +SD of 4 assays of glucose consumption normalized to cell growth in HepG2 cells treated with *Ilex latifolia* at indicated concentrations and compared to control untreated cells. **P<0.01 compared to the control untreated cells, and $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01, and $^{\Delta\Delta\Delta}$P<0.001 compared to 10 µg/mL as determined by one-way ANOVA followed by Bonferroni post-hoc tests.

The assay of the effect of low concentrations of *Ilex latifolia* (Figure 5-6) on glucose consumption normalized to cell growth in HepG2 cells showed that *Ilex latifolia* at 0.1, and 0.3 µg/mL stimulated glucose consumption but the effect was not significant (P>0.05) as determined using one-way ANOVA. 1, 3, and 10 µg/mL inhibited glucose consumption in HepG2 cells. While the effect in the 1 and 3 µg/mL was not significant (P>0.05), the effect of 10 µg/mL was significant (P<0.01). The mechanism of action of *Ilex latifolia* in stimulating or inhibiting glucose consumption needs to be explored.

5.3.4 Assessment of mitochondrial toxicity of *Ilex latifolia* extracts on AML-12 cells

Cells are metabolically active in the presence of galactose because they use the oxidative phosphorylation pathway in mitochondria to produce ATP. Cancer cells often have an abnormally large capacity to produce ATP by glycolysis and are less reliant on mitochondrial

respiration to produce ATP. AML-12 cells are mouse liver cells that are not derived from a cancer, unlike HepG2, and should have a more limited capacity to produce ATP by glycolysis only. In addition AML-12 cells should be more sensitive to treatments that affect mitochondrial activity than HepG2 cells. AML-12 cells grown in the presence of glucose or galactose were treated with extracts of *Ilex latifolia* or with FCCP and DMSO and assayed for cell growth. Insulin is normally added to the growth medium for AML-12 cells and cells grown in AML-12-glucose or galactose medium in the presence or absence of insulin were compared.

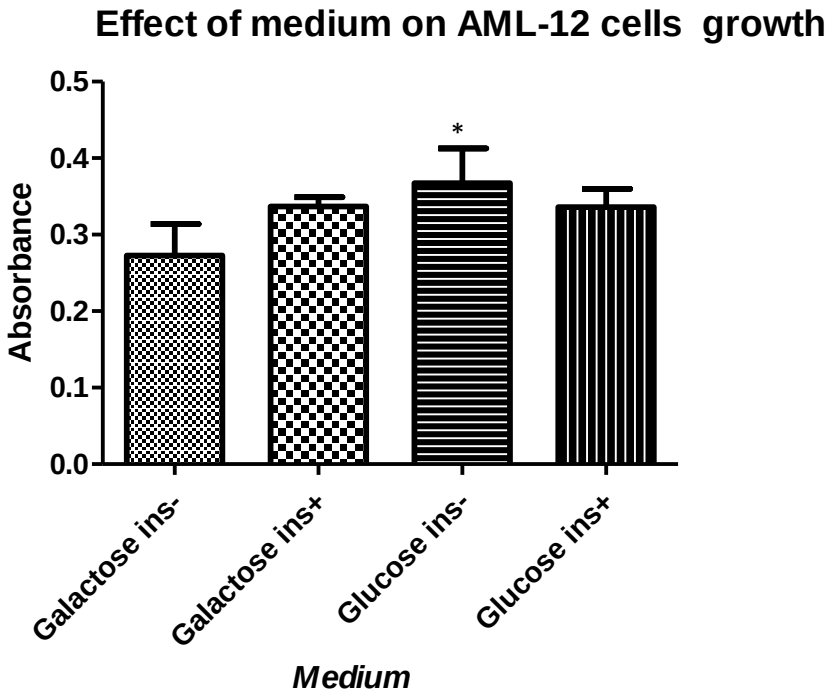


Figure 5-7: Effect of medium on AML-12 cell growth. The data are from MTT assays of cell growth and are the mean+SD of 4 different assays of the effect of glucose or galactose in the presence or absence of insulin. All the columns are compared to each other using one-way ANOVA followed by Bonferroni post-hoc tests. $^{*}P<0.05$ compared to galactose ins-.

Before the assay of the effect of *Ilex latifolia* on AML-12 cell growth, it was important to investigate if replacing glucose with galactose in the presence or absence of insulin has any effect on the cell growth. Figure 5-7 shows that there was a significant difference was between cells grown in glucose without insulin and galactose without insulin ($P<0.05$), with a

slower growth in galactose by about 20%. In the presence of insulin there was no difference between glucose and galactose, and insulin added to the medium made no difference.

The first experiment was to check the effect of *Ilex latifolia* on AML-12 cells growth when glucose was replaced for galactose in the presence of insulin.

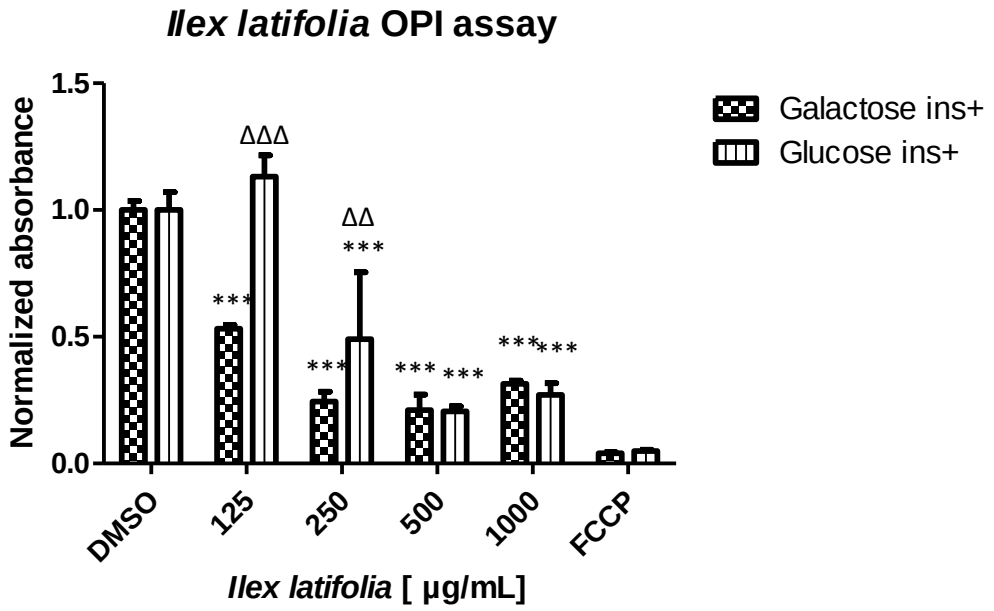


Figure 5-8: Effect of *Ilex latifolia* on AML-12 cells mitochondrial function. Data are the mean +SD from four independent MTT assays of AML-12 grown in glucose or galactose in the presence of insulin. Cells were treated with *Ilex latifolia* at the indicated concentrations or with the mitochondrial inhibitor FCCP. ***P<0.001 compared to the control untreated cells in each medium respectively, and $^{\Delta\Delta}$P< 0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to the effect of the same concentration when glucose is replaced to galactose as determined by two-way ANOVA followed by Bonferroni post-hoc tests.

The first analysis was to check the effect of *Ilex latifolia* on the cell growth when the cells are grown in galactose with insulin compared to the control untreated cells. *Ilex latifolia* at 125, 250, 500, and 1000 µg/mL inhibited AML-12 cell growth by 47 % (P< 0.001), 77.5% (P< 0.001), 82% (P<0.001), and 68.7% (P< 0.001) respectively. The second analysis was to check the effect of *Ilex latifolia* when the cells are grown in a medium containing glucose and insulin. While the 125 µg/mL did not show any inhibition effect, 250, 500, and 1000 µg/mL

inhibited AML-12 cells growth by 51% (P<0.001), 79.5% (P< 0.001), and 73% (P< 0.001) respectively. The third step was to compare the effect of *Ilex latifolia* on the cells when grown in galactose to the effect of *Ilex* on the cells when grown in glucose. The only significant effects were at 125 µg/mL where *Ilex latifolia* inhibited the cell growth in galactose by 60% (P<0.001) compared to the *Ilex latifolia* inhibition effect on cells growth in glucose, at 250 µg/mL where the inhibition effect of *Ilex latifolia* was 26.5% (P<0.01) more in the galactose medium compared to the glucose medium. These effects can be compared to the effect of FCCP which inhibited cell growth by 95% and 96% in glucose and galactose respectively. This shows that AML-12 cells are aerobically poised when grown in glucose or in galactose, and the different effects of *Ilex latifolia* on cells grown in glucose and galactose must be due to a mechanism which is not mitochondrial toxicity.

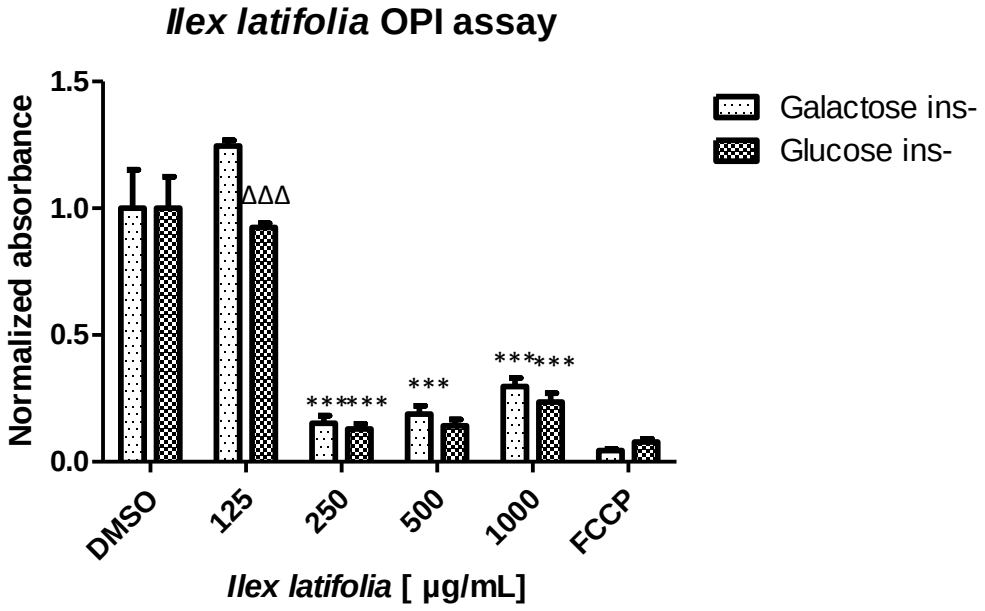


Figure 5-9: Effect of *Ilex latifolia* on AML-12 cells' mitochondrial function. Data are the mean +SD from four independent MTT assays of AML-12 growth in glucose or galactose in the absence of insulin. Cells were treated with *Ilex latifolia* at the indicated concentrations or with the mitochondrial inhibitor FCCP. ***P<0.001 compared to the control untreated cells in each medium respectively, and $^{\Delta\Delta\Delta}$P< 0.001 compared to the effect of the same concentration when glucose is replaced to galactose as determined by two-way ANOVA followed by Bonferroni post-hoc tests.

The next experiment was to check if changing glucose to galactose in the insulin depleted medium can result in any changes in the cells' growth and in the effect of *Ilex latifolia*. *Ilex*

latifolia at 125 µg/mL inhibited AML-12 cells growth by 7.7 % (P>0.05) in the medium containing glucose but did not show any effect in galactose containing medium. *Ilex latifolia* at 250, 500, and 1000 µg/mL inhibited the growth of AML-12 cells by 83.3% (P<0.001), 80% (P<0.001), and 68% (P<0.001) respectively when the cells were grown in galactose. Similarly *Ilex latifolia* at 250, 500, and 1000 µg/mL inhibited AML-12 cell growth by 87% (P<0.001), 86% (P< 0.001), and 76% (P<0.001) respectively when the cells were grown in glucose. By comparing the effect of *Ilex latifolia* on the cells when grown in galactose to the effect of *Ilex latifolia* on the cells when grown in glucose, the only significant effect was at 125 µg/mL where *Ilex latifolia* inhibited the cell growth in glucose by 32% (P<0.001) compared to the *Ilex latifolia* inhibition effect on cells growth in galactose. In the absence of insulin, AML-12 cells behaved similarly in glucose or galactose containing medium and this can be confirmed by the inhibition effect of FCCP which was 95.3% and 92.2% in galactose and glucose respectively.

To confirm that the change in the cells' behavior is due to the replacing of glucose with galactose and not because of the insulin, the effect of *Ilex latifolia* was checked in the cells grown in glucose containing medium in the presence or absence of insulin.

.FCCP is an approved mitochondrial toxicant and was used to assess how much the mitochondria of AML-12 cells are metabolically active in galactose, compared with glucose. The effect of FCCP on AML-12 cells (Figure 5-9) grown in galactose containing medium (94.3% growth inhibition) is slightly higher than the effect on the cells grown in the medium containing glucose (87% growth inhibition), but this pattern is different compared with HepG2 cells (Figure 5-1) which were inhibited 90% in galactose but only 33% when grown in glucose. These data show that AML-12 cells are aerobically poised when growing in glucose or in galactose, whereas HepG2 cells are only aerobically poised when grown in galactose.

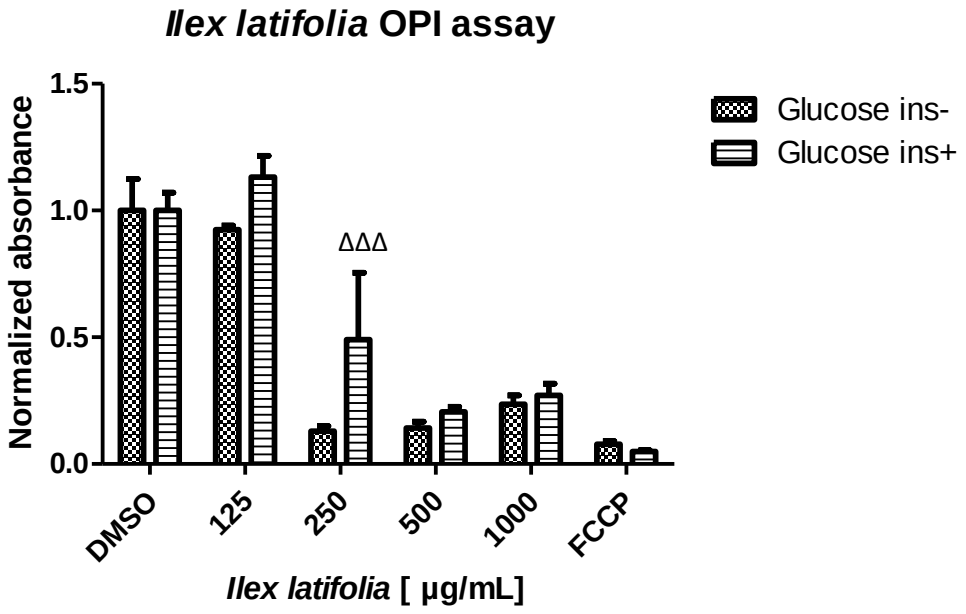


Figure 5-10: Effect of *Ilex latifolia* on AML-12 cells mitochondrial function. Data are the mean +SD from four independent MTT assay of AML-12 grown in glucose in the presence or absence of insulin. Cells were treated with *Ilex latifolia* at the indicated concentrations or with the mitochondrial inhibitor FCCP. $P^{\Delta\Delta\Delta} < 0.001$ compared to the effect of the same concentration when insulin was removed from the medium as determined by two-way ANOVA followed by Bonferroni post-hoc tests.

By comparing the effect of *Ilex latifolia* on the cells when grown in glucose in the presence or absence of insulin, the only significant effect was at 250 µg/mL where *Ilex latifolia* inhibited the cell growth in glucose without insulin by 36% (P<0.001) compared to the *Ilex* inhibition effect on cells growth in glucose with insulin (Figure 5-10). In the presence of glucose, AML-12 cells behaved similarly with or without insulin and this can be confirmed by the inhibition effect of FCCP which was 95% and 92.2% in galactose and glucose respectively.

A similar analysis for cells grown in galactose is shown in Figure 5-11. *Ilex latifolia* at 125 µg/mL inhibited the cell growth in galactose with insulin by 57% (P<0.001) compared to its effect on cell growth in galactose without insulin. At other concentrations of *Ilex latifolia,* in the presence of galactose, AML-12 cells behaved similarly with or without insulin. The inhibition

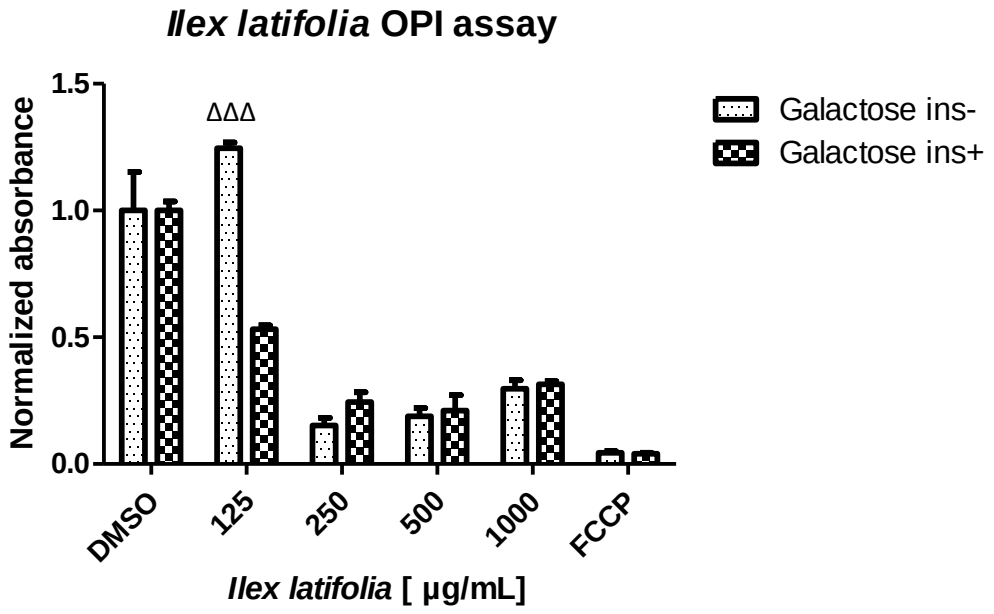


Figure 5-11: Effect of *Ilex latifolia* on AML-12 cells mitochondrial function. Data are the mean +SD from four independent MTT assay of AML-12 grown in galactose in the presence or absence of insulin. Cells were treated with *Ilex latifolia* at the indicated concentrations or with the mitochondrial inhibitor FCCP. $^{\Delta\Delta\Delta}$P< 0.001 compared to the effect of the same concentration when insulin was removed from the medium as determined by two-way ANOVA followed by Bonferroni post-hoc tests.

effect of FCCP was 95% and 92.2% with and without insulin respectively. Comparing the data in Figures 5-10 and 5-11 insulin seems to have a small, but opposing effect in which it opposes inhibition of growth by *Ilex latifolia* when cells are grown in glucose but enhances the inhibitory effect *Ilex latifolia* when cells are grown in galactose. Cells are equally aerobically poised when grown in glucose or galactose, suggesting that the effect be linked to the Leloir pathway of galactose metabolism that feeds into the glycolytic pathway.

5.3.5 Glucose uptake effect of *Ilex latifolia* on AML-12 cells

Ilex latifolia inhibits AML-12 growth at high concentrations. The effect of *Ilex latifolia* on glucose uptake was investigated by growing the cells in 24 wells plate in full medium till confluency. AML-12 cells were grown in insulin depleted medium to investigate the effect of insulin on cells response and glucose uptake. After 90% confluency, the medium was changed to a glucose depleted medium and glucose was added at 11.8 mM. The cells were

incubated overnight and the glucose was measured using a Codefree blood glucose monitoring apparatus for 4 consecutive days.

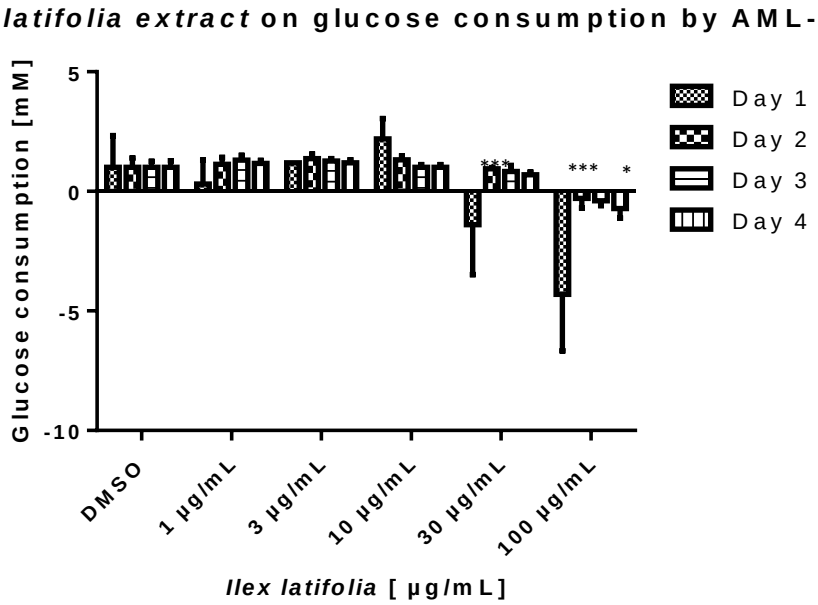


Figure 5-12: Effect of *Ilex latifolia* on glucose consumption by AML-12 cells. Data are the mean +SD of 4 assays of glucose consumption in AML-12 cells treated with *Ilex latifolia* at the indicated concentrations and compared to control untreated cells. The assay was done for 4 consecutive days by measuring the glucose taken by the cells per day. **P<0.01 and ***P<0.001 compared to the control untreated cells in each day as determined by two-way ANOVA followed by Bonferroni post-hoc tests.

The results (Figure 5-12) show that the *Ilex latifolia* did not stimulate glucose uptake at low concentrations (10, 3 and 1 µg/mL) compared to DMSO (P >0.05) which was used as negative control vehicle. At higher concentrations glucose consumption was inhibited or was apparently negative, suggesting stimulation of gluconeogenesis. These changes were significantly different from controls - at 30 µg/mL *Ilex* induced an apparent negative glucose consumption by 140% (P<0.001) at day one, and at 100 µg/mL by 543% (P<0.001) at day one and by 171% (***P<0.001) at day four compared to the control untreated cells. These measurements indicate that glucose could have been released by the cells, possibly through gluconeogenesis at these higher concentrations of *Ilex latifolia*. The main drawback of this

method is that the glucose uptake is measured without considering the cells densities in the wells and then the effect on glucose uptake.

To take into account the effect of the cell density on the results, an MTT assay was done and the results were normalised by calculating the glucose uptake relative to cell growth.

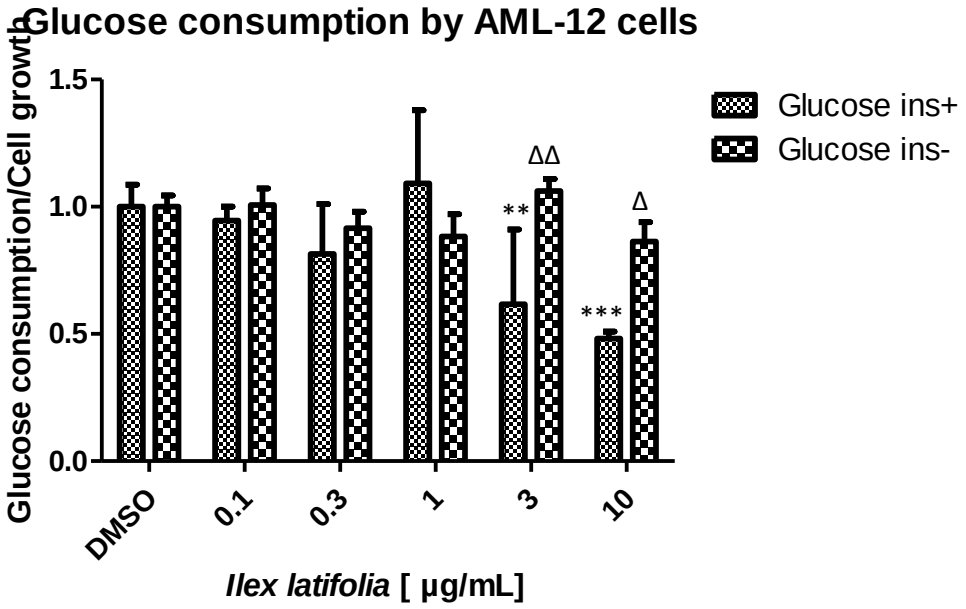


Figure 5-13: Effect of *Ilex latifolia* on glucose consumption by AML-12 cells. Data are the mean +SD of 4 assays of glucose consumption normalized to cell growth in AML-12 cells grown in glucose containing medium in the presence and absence of insulin and treated with *Ilex latifolia* for 2 days at the indicated concentrations and compared to control untreated cells. $^{**}P<0.01$ and $^{***}P<0.001$ compared to the control untreated cells, and $^{\Delta}P<0.05$ and $^{\Delta\Delta}P<0.01$ compared to the effect of the same concentration after changing the medium as determined by two-way ANOVA followed by Bonferroni post-hoc tests.

Ilex latifolia inhibited glucose consumption in AML-12 cells grown in insulin containing medium at 3 µg/mL ($P<0.01$) by 38.3% and at 10 µg/mL ($P<0.001$) by 51.8% respectively when compared to the untreated control cells. It did not have any significant effect when the cells were grown in insulin depleted medium ($P >0.05$). The effect of insulin on *Ilex latifolia* induced changes in glucose consumption can be seen at 3 µg/mL of *Ilex latifolia* when

glucose consumption was 41.9% (P<0.01) less in the cells grown in the presence of insulin compared to glucose consumption in cells grown without insulin. At 10 µg/mL, glucose consumption was 44.2% (P<0.05) less in cells grown with insulin compared to cells grown without insulin. From the data mentioned above, *Ilex latifolia* inhibits glucose consumption in AML-12 cells in a manner that is dependent on insulin.

A one-way ANOVA was done to check the significance of the effects of *Ilex latifolia* followed by Bonferroni post-hoc tests to compare each pair of results. The statistical analysis was done for the assays done in the presence or absence of insulin.

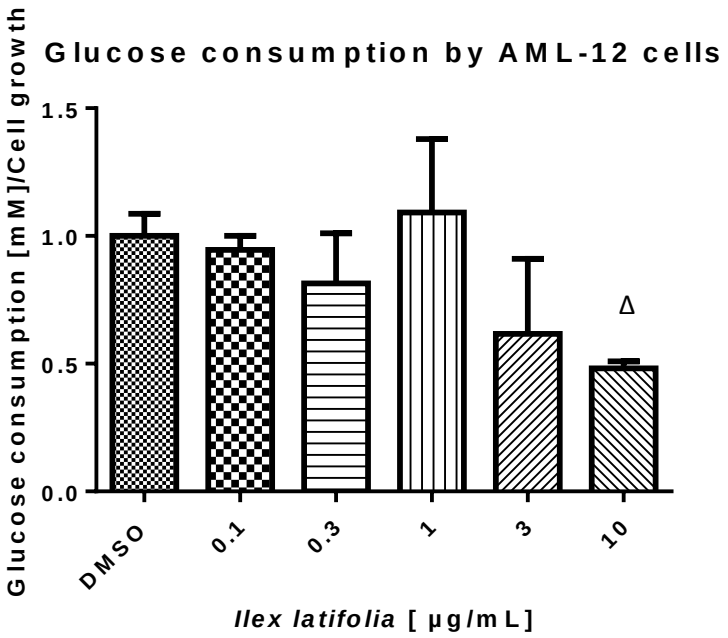


Figure 5-14: Effect of *Ilex latifolia* on glucose consumption by AML-12 cells. Data are the mean +SD of 4 assays of glucose consumption normalized to cell growth in AML-12 cells grown in glucose containing medium in the presence of insulin and treated with *Ilex latifolia* at the indicated concentrations and compared to control untreated cells. $^{\Delta}$P<0.05 compared to the effect of 1 µg/mL as determined by one-way ANOVA followed by Bonferroni post-hoc tests.

Ilex latifolia at 10 µg/mL inhibited glucose consumption in AML-12 cells in the presence of insulin compared to all the other concentrations. The only significant effect was that *Ilex latifolia* at 10 µg/mL inhibited glucose consumption by 55.9% (P<0.05) compared to the 1 µg/mL as determined by one-way ANOVA followed by Bonferroni post-hoc tests.

Figure 5-15: Effect of *Ilex latifolia* on glucose consumption by AML-12 cells. Data are the mean +SD of 4 assays of glucose consumption normalized to cell growth in AML-12 cells grown in glucose containing medium in the absence of insulin and treated with *Ilex latifolia* at the indicated concentrations and compared to control untreated cells. $^{\Delta}$P<0.05 compared to the effect of 3 µg/mL as determined by one-way ANOVA followed by Bonferroni post-hoc tests.

Ilex latifolia at 10 µg/mL again inhibited glucose consumption in AML-12 cells in the absence of insulin, but by a small amount, 15%, compared to the control, 0.1 and 0.3 µg/mL. The only significant effect was that *Ilex latifolia* at 10 µg/mL inhibited glucose consumption by 18.7% (P< 0.05) compared to the 3 µg/mL as determined by one-way ANOVA followed by Bonferroni post-hoc tests.

5.4 Discussion

The majority of diabetic people with type 2 diabetes are at high risk of dying from heart diseases because diabetes is associated with accelerated atherosclerosis and congestive heart diseases (Steiner, 1985, Rudermann NB, 1990). Agents which can stimulate glucose consumption and metabolism can help in the treatment of hyperlipidemia but potential toxicity due to inhibition of oxidative phosphorylation is a concern. Mammalian cells

produce >90% of the required energy in the form of ATP. Agents which inhibit mitochondrial function can result in organ toxicity and failure (Masubuchi *et al*., 2006). Carbonylcyanide *p*-triflouromethoxyphenylhydrazine (FCCP), is an approved mitochondrial toxicant and has been widely used in experimental research (Park *et al*., 2002, To *et al*., 2010). HepG2 cells like most cancer cells depend mainly on glycolysis rather than mitochondrial oxidative phosphorylation (OXPHOS) to produce energy.

To investigate if *Ilex latifolia* has any toxic effect on hepatocytes, HepG2 cell viability was tested in glucose and galactose because in galactose containing medium, cells depend mainly on OXPHOS as a source of energy. FCCP was used as a positive control. All the assay results were analyzed using two-way ANOVA and only significant results are mentioned. Cell viability was tested using MTT assay. FCCP inhibited HepG2 growth by 33% in glucose but it inhibited the growth by 90.4% in galactose. This confirms that the cells in the galactose depend mainly on mitochondria OXPHOS for generation of ATP while in glucose they depend mainly on glycolysis. When the cells were treated with *Ilex latifolia* , high concentrations at 451.5 and 903 µg/mL inhibited the growth of HepG2 cells in glucose by 30.8 % ($P<0.001$) and 52% ($P<0.001$) respectively while in galactose the inhibition effect of the same concentrations was 14.4% ($P>0.05$) and 27.2% ($P<0.01$) respectively. This means that the inhibitory effect of *Ilex latifolia* was higher in glucose, and this means that *Ilex latifolia* could interfere with the glycolysis process of HepG2 cells. The lower inhibitory effect of galactose may be linked to the slower rate of growth of HepG2 cells in galactose compared with glucose, about 50% slower. The glycolytic pathway is active in the cells grown in galactose, but not as much as the cells grown in glucose. The *Ilex latifolia* could also be affecting glycolysis in the galactose cells.

To check if *Ilex latifolia* has an effect on the mitochondrial function and consequently on ATP production, the ATP production was measured and to take into account that *Ilex latifolia* inhibited cells growth at high concentrations, the amount of ATP was normalized to the protein content of the cell lysates. *Ilex latifolia* at 920 µg/mL inhibited ATP production in

HepG2 cells by 42% ($P<0.05$) compared to 115.1 µg/mL and it also showed a reduction in ATP production compared to the control but the effect was not significant as determined by one-way ANOVA followed by Bonferroni post-hoc tests. Inhibition of the growth in the glucose containing medium raised the possibility that *Ilex latifolia* could interfere with the glucose consumption by the cells. When the glucose consumption was analyzed by treating the cells with *Ilex latifolia*, glucose consumption was inhibited at 100 µg/mL ($P<0.001$) but at lower concentrations, *Ilex latifolia* stimulated glucose consumption but the effect was not significant ($P>0.05$). To minimize the effect of *Ilex latifolia* on cells growth and consequently on the results, glucose consumption was normalized to cell growth after treating the cells with 0.1, 0.3, 1, 3, and 10 µg/mL respectively. *Ilex latifolia* at 10 µg/mL inhibited glucose consumption ($P<0.01$) compared to the control. It also inhibited glucose consumption compared to 0.1 µg/mL ($P<0.001$), 0.3 µg/mL ($P<0.01$), and 1 µg/mL ($P<0.05$).

HepG2 cells are tumorigenic cells which depend mainly on glycolysis for ATP production, and it was of interest to use another cell line AML-12, which is non-tumorigenic and depends mainly on mitochondrial function as a source of energy, to assay the effect of *Ilex latifolia* on the mitochondrial function.

Insulin is included in the culture medium for AML-12 cells which suggests it is an essential factor for AML-12 cell growth. It was interesting to assay the effect of insulin on *Ilex latifolia* activity on the cells. FCCP was used to assess the aerobic state of AML-12 cells, and strongly inhibited cell growth, by more than 90%, regardless of the presence or absence of insulin or whether the medium contained glucose or galactose. This confirms that AML-12 cells depend mainly on OXPHOS for ATP generation. AML-12 cells were more sensitive to inhibition by *Ilex latifolia* than HepG2 cells, with almost complete inhibition of growth at 250 µg/mL or higher, in the absence of insulin, in either glucose or galactose. In the presence of insulin *Ilex latifolia* at 125 µg/mL inhibited cell growth in galactose by 60% ($P<0.001$) compared to the *Ilex latifolia* inhibition effect on cells growth in glucose, and at 250 µg/mL

where the inhibition effect of *Ilex latifolia* was 26.5% (P<0.01) more in the galactose medium compared to the glucose medium.

To further investigate the effect of insulin on cell growth and sensitivity to *Ilex*, the cells were grown in glucose but the only variable was insulin. The only significant effect was at 250 µg/mL where *Ilex latifolia* inhibited the cell growth in glucose without insulin by 36% (P<0.001) compared to the *Ilex latifolia* inhibition effect on cell growth in glucose with insulin. This means that the cells were more resistant to the *Ilex latifolia* treatment in the presence of insulin. In contrast galactose cells were more sensitive to *Ilex latifolia* in the presence of insulin. AML-12 cells were sensitive to125 µg/mL of *Ilex latifolia* which inhibited the cell growth in galactose with insulin by 57% (P<0.001) compared to its effect on cells growth in galactose without insulin.

The next step was to check if *Ilex latifolia* has an effect on the glucose uptake by AML-12 cells. A glucose consumption assay was done by treating the cells with *Ilex latifolia* for 4 consecutive days and the glucose consumption was measured. *Ilex latifolia* 30 µg/mL inhibited glucose consumption by 140% (P<0.001) in day one. 100 µg/mL of *Ilex altifolia* inhibited glucose consumption by 543% (P< 0.001) in day one and by 171% (P<0.001) in day four compared to the control untreated cells. To avoid the effect of *Ilex latifolia* on cells growth and consequently on glucose consumption data, and to check the effect of insulin on glucose consumption by AML-12 cells, glucose consumption was measured in the presence or absence of insulin and was normalized to the cells growth by using MTT assay. *Ilex latifolia* inhibited glucose consumption in AML-12 cells grown in insulin containing medium at 3 µg/mL by 38.28% (P<0.01) and at 10 µg/mL by 51.8% (P<0.001) respectively when compared to the untreated control cells. It did not have any significant effect when the cells were grown in glucose depleted medium (P>0.05). By comparing the effect of *Ilex latifolia* on glucose consumption by the cells when insulin was removed from the medium, at 3 µg/mL of *Ilex latifolia,* glucose consumption was 41.9% (P<0.01) less in the cells grown in the presence of insulin compared to glucose consumption in cells grown without insulin. At 10

µg/mL, glucose consumption was 44.2% ($P<0.05$) less in cells grown with insulin compared to cells grown without insulin. *Ilex latifolia* inhibits glucose consumption in AML-12 cells but the mechanism of action and the role of insulin in this effect should be explored.

Overall the data show that at higher concentrations, greater than 100 µg/mL, *Ilex latifolia* inhibits cell growth, but in a manner that is not linked to mitochondrial toxicity or a partial inhibition or uncoupling of oxidative phosphorylation. At lower concentrations, up to 100 µg/mL, *Ilex latifolia* can inhibit glucose consumption. Both responses are affected by the presence of insulin. The significance of these responses to lipid metabolism requires further investigation, especially looking at processes such as gluconeogenesis and fatty acid metabolism, both affected by insulin.

6 Chapter VI: Discussion

Dietary fat absorption and cholesterol synthesis are the main factors in the development of hyperlipidemia. Pancreatic lipase is an enzyme secreted by the intestine and is responsible for the hydrolysis of 50-70% of the dietary fats. Digestion of dietary fats leads to the hydrolysis of triglycerides into fatty acids and monoglycerides which are absorbed from the intestine in the form of micelles with bile acids. These micelles release the fatty acids and monoglycerides to form triglyceride rich chylomicrons. Inhibition of fat absorption by inhibition of pancreatic lipase is one of the main factors in the prevention of development of hyperlipidemia and consequently in the protection against heart diseases.

HMG-CoA reductase is the rate-limiting enzyme in cholesterol synthesis. HMG-CoA reductase catalyses the reaction HMG-CoA + 2NADPH + 2H+ leading to mevalonic acid + 2NADP+ + COASH. Inhibition of HMGCR stimulates the cells to produce more enzyme in order to produce the required cholesterol.

The majority of diabetic people with type 2 diabetes are at high risk of dying from heart diseases because diabetes is associated with accelerated atherosclerosis and congestive heart diseases (Steiner, 1985, Rudermann NB, 1990). Agents which can stimulate glucose consumption and metabolism can help in the treatment of hyperlipidemia but potential toxicity due to oxidative phosphorylation inhibition is a concern. Mammalian cells produce >90 % of the required energy in the form of ATP. Agents which inhibit mitochondrial function can result in organ toxicity and failure (Masubuchi *et al*., 2006). Herbs such as *Prunella vulgaris*, *Ilex latifolia*, *Rheum palmatum*, and *Panax notoginseng* have been used in the Chinese traditional medicine for the treatment of hyperlidaemia. Some herbal compounds such as quercetin, crocin, emodin, and hesperidin showed pancreatic lipase inhibition activities.

In this project, the aim was to identify hypolipidemic components of these herbs, and in the first phase to check if these compounds have inhibitory activities to pancreatic lipase, comparing with the activity of orlistat, the only pancreatic lipase inhibitor in clinical use. A

colorimetric assay of pancreatic lipase was used initially and indicated that orlistat inhibited pancreatic lipase with an IC_{50} of 2.6 µg/mL. The data obtained are summarised as follows:

- The IC_{50} of Orlistat was 0.9480 µg/mL.
- The herbal extracts produced a high absorbance which overlapped the absorbance produced due to *p*-nitrophenol.
- Consequently, colorimetric methods were not suitable for this assay and another assay method was required.

The next assay method tried was using HPLC. The HPLC method is a more sensitive technique and has been used to determine a suitable substrate for pancreatic lipase (Maurich *et al*., 1991). Throughout the work described in the thesis Orlistat was used as a positive control for inhibition of pancreatic lipase and the data from the work in this thesis showed that:

- Orlistat inhibited pancreatic lipase with an IC_{50} of 0.8921 µg/mL (Chapter 2, section 2.3.1). This value is in the middle of the range of published values.
- *Prunella vulgaris* showed pancreatic lipase inhibition activity with an IC_{50} of 71.8 µg/mL.
- *Rheum palmatum,* which has been used to treat hyperlipidemia in diabetic rats (Xie *et al*., 2005b), did not show any pancreatic lipase inhibition activity when assayed by HPLC. In contrast an apparent inhibition of pancreatic lipase by more than 50% at 25 µg/mL was measured using the spectrophotometric assay, confirming an observation by Zheng *et al.* (Zheng *et al*., 2010).
- *Ilex latifolia* showed a pancreatic lipase inhibition activity at IC_{50} of 1418 µg/mL.
- Crocin, hesperidin, and emodin did not inhibit pancreatic lipase when assayed by HPLC. In contrast crocin has been reported to inhibit pancreatic lipase at 2.7 mg/mL (Lee *et al*., 2005) assayed using a titrimetric method, hesperidin inhibited pancreatic lipase at IC_{50} of 32 µg/mL as mentioned by Birari (Birari and Bhutani, 2007) and

emodin inhibited pancreatic lipase by 12 % at 25 µg/mL as mentioned by Zheng (Zheng *et al*., 2010) respectively.

The variability in the published values of IC_{50} could reflect the different amounts of enzyme used in different labs in the assay. One concern in the present work was that the enzyme concentration was too high so that even in the presence of inhibitor there is enough active enzyme to hydrolyze the substrate.

The next step was to find an optimal concentration of the enzyme. The concentration was determined as mentioned in the methods (section 2.3.16) and the previously tested compounds were investigated again.

- Orlistat was still showing high inhibition activity at 0.0017 µg/mL (Chapter II, section 2.3.17).
- *Ilex latifolia* had the highest inhibition activity at IC_{50} 76.10 µg/mL.
- *Prunella vulgaris, Rheum palmatum, and Panax notogensing* showed pancreatic lipase inhibition activity at IC_{50} 250.7, 117.1, and 431.2 µg/mL respectively.
- Quercetin showed inhibition activity at 136.68 µg/mL.

The difference in activities between orlistat and the herbal extracts could result from the fact that the herbal activities were tested using crude extracts which contain lots of compounds and pigments and this means that the active compounds are actually present in small concentrations. Another reason for low activity is the quality of the herbs which could be affected by different factors such as storage, and shipment.

The last set of data mentioned showed that *Ilex latifolia* had the highest pancreatic lipase inhibition activity. *Ilex latifolia* showed pancreatic lipase inhibition activity when compared to THL which is an approved pancreatic lipase inhibitor. The *Ilex latifolia* butanol extract was a crude extract which means that it contained a mixture of compounds. One or more compounds could be responsible for the pancreatic lipase inhibition activity.

The aim of the work described in Chapter III was to try to fractionate the *Ilex latifolia* extract using HPLC and to isolate pure compounds and check their activities.

In one separation procedure 10 peaks were collected and after assaying the activity on pancreatic lipase, only two fractions showed inhibition activity, of about 50% (Chapter III, section 3.3.6). One major constraint was that not enough material of each fraction was collected due to the limited amount of the herb that was available.

The next part of the work was to assess the effect of *Ilex latifolia* on other metabolic pathways that affect lipid metabolism, and HMG-CoA reductase was chosen as a target in view of the success of statins in controlling blood lipids, through inhibition of HMG-CoA reductase.

The first approach was to use an HMG-CoA reductase assay kit (Sigma-Aldrich CS1090) to assess inhibitory activity of *Ilex latifolia* extracts in comparison with simvastatin. The principle of the assay is to measure oxidation of NADPH to $NADP^+$ at 340 nm using a spectrophotometric assay method. The oxidation of NADPH to $NADP^+$ was not clear and could not be detected. The enzyme activity should be measured within 5 minutes and this proved to be unreliable because the assay was done at a large scale of samples. The third concern was that *Ilex latifolia* extract could interfere with the absorbance produced by $NADP^+$ especially that spectrophotometric assay method proved to be unsuitable when used for the detection of the inhibition effect of herbal extracts on pancreatic lipase as mentioned in chapter II.

The second method attempted was to detect CoA and NADP using HPLC. The result was that HPLC is not a suitable method to assay HMG-CoA reductase activity because the assay was done on a very small scale and HPLC is not sensitive to small changes in concentrations which could be in nanogram amounts.

The third method tried was to detect mevalonolactone using LC-MS which is more sensitive and accurate than HPLC. LC would be required because the assay needs to be quantitative

and the MS was used as a detector. The samples were prepared for assay using LC-MS but unfortunately the assay could not be done because the instrument was not available.

As an alternative to enzyme assays, an HMG-CoA reductase expression assay by Western blotting was chosen to investigate the inhibition effect of *Ilex latifolia* extract on HMG-CoA reductase using simvastatin, which is an approved HMG-CoA reductase inhibitor, as a positive control. HepG2 cells were used for the assay. HMG-CoA reductase has a molecular weight of 97 kDa but after gel electrophoresis and Western blotting of HepG2 extracts, the enzyme could only be detected as 72 and 54 kDa forms (Chapter IV, section 4.4.3.2). The results were as follows:

- In some experiments simvastatin stimulated the expression of the enzyme, detected as 72 kDa and 54 kDa fragments; in others simvastatin had the opposite effect.
- *Ilex latifolia* mainly showed a very small, or no, effects compared to the control (Chapter IV, section 4.4.3.3), but in one experiment *Iex latifolia inhibited the expression of the enzyme in HepG2 cells (Table 4-6).*

To avoid the effect of leupetin sensitive enzyme, RIPA buffer was replaced by a lysing buffer containing leupeptin. The result was that the 72 kDa band was more intense than the previous assays but the *Ilex latifolia* was still not inhibiting the HMGCR and consequently not stimulating the HMG-CoA reductase expression compared to simvastatin (Chapter IV, section 4.4.3.4).

These variable and inconsistent findings could be due to the instability of the enzyme. The enzyme is sensitive to the endogenous Ca^{2+}-activated enzymes which break it to a 62 kDa fragment which remains membrane bound. This 62 kDa fragment is sensitive to a leupeptin sensitive protease which breaks it down to a 52-56 kDa soluble fragment (Parker *et al*., 1993). Even though steps were taken to control proteolytic activity, it was never possible to detect the full length protein. The HepG2 cells do not express very high levels of HMG-CoA reductase and the protein is especially unstable in this cell line.

Future work in this area may be more successful if another cell line, such as Chinese hamster ovary cells, is used.

The next target was expression of LDLR, using AML-12 cells, which have been used for the detection of LDLR expression. *Ilex latifolia* extract effect was compared to simvastatin. Simvastatin stimulated LDLR expression as shown by detection of a band at 138 kDa but *Ilex latifolia* did not have any effect on the expression of LDLR (Chapter IV, section 4.4.3.5).

In another approach it was investigated if *Ilex latifolia* has any mildly toxic effect on hepatocytes when they were oxidatively poised by culturing in galactose instead of glucose. HepG2 cell viability was tested in glucose compared with galactose because in galactose containing medium, cells depend mainly on OXPHOS as a source of energy. FCCP was used as a positive control, to show that cells growing in galactose were more sensitive to FCCP and therefore were using OXPHOS more to generate ATP, compared with cells growing in glucose, which generate most of their ATP by glycolysis.

All the assay results were analyzed using two-way ANOVA and only significant results are mentioned. Cell viability was tested using the MTT assay.

- *Ilex latifolia* at relatively high concentrations of 451.5 and 903 µg/mL growth of HepG2 cells in glucose was inhibited by 30.8% ($P<0.001$) and 52% ($P<0.001$) respectively while in galactose the inhibition effect of the same concentrations was 14.4% ($P>0.05$) and 27.2% ($P<0.01$) respectively. There was no inhibition of growth at lower concentrations of *Ilex latifolia*.
- FCCP inhibited HepG2 growth by 33% in glucose but it inhibited the growth by 90.4% in galactose (Chapter V, section 5.3.1).

This confirms that the cells in the galactose medium depend mainly on mitochondria OXPHOS while in glucose they depend mainly on glycolysis. This means that although *Ilex latifolia* inhibited HepG2 cells growth in galactose, it was very modest and is unlikely to be

due to mitochondrial toxicity. Interestingly the inhibition effect in glucose was higher and this means that *Ilex latifolia* could interfere with the glycolysis process of HepG2 cells.

To investigate further the metabolic effects of *Ilex latifolia,* ATP production was measured.

- *Ilex latifolia* at 920 µg/mL there was a reduction in ATP production in HepG2 cells compared to the control but the effect was not significant.
- In the cells treated with 920 µg/mL *Ilex latifolia* ATP production was lower by 42% ($P< 0.05$) compared to ATP levels in cells treated with *Ilex latifolia* at 115.1 µg/mL as determined by one-way ANOVA followed by Bonferroni post-hoc tests (Chapter V, section 5.3.2).

Inhibition of the growth in the glucose containing medium raised the possibility that *Ilex latifolia* could interfere with the glucose consumption by the cells. When the glucose consumption was analyzed by treating the cells with *Ilex latifolia*, glucose consumption was normalized to cell growth after treating the cells with 0.1, 0.3, 1, 3, and 10 µg/mL respectively.

- *Ilex latifolia* at 10 µg/mL inhibited glucose consumption by 50.5% ($P<0.01$) compared to the control.
- It also inhibited glucose consumption by 58.7% compared to 0.1 µg/mL ($P<0.001$), 57.3% compared to 0.3 µg/mL ($P<0.01$), and by 48.6% 1 µg/mL ($P<0.05$) (Chapter V, section 5.3.3).

HepG2 cells are tumorigenic cells which depend mainly on glycolysis for production of ATP. Another liver cell line AML-12, which is non-tumorigenic and depends mainly on mitochondrial function as a source of energy was used to assay the effect of *Ilex latifolia* on the mitochondrial function.

Insulin is an essential factor in the AML-12 cells growth medium, and it was interesting to assay the effect of insulin on *Ilex latifolia* activity on the cells.

Ilex latifolia toxicity was assayed using the MTT assay and FCCP was used as a positive control. The first step was to check if AML-12 cells behave differently when galactose was replaced for glucose in the presence of insulin.

- FCCP inhibited AML-12 cell growth by 95% and 96% in glucose and galactose respectively. This confirms that AML-12 cells depend mainly on OXPHOS reaction to generate ATP and that is why they were sensitive to FCCP in both media.
- The only significant effects of *Ilex latifolia* were at 125 µg/mL where *Ilex latifolia* inhibited the cells growth in galactose by 60% ($P<0.001$) compared to the *Ilex latifolia* inhibition effect on cells growth in glucose, and at 250 µg/mL where the inhibition effect of *Ilex latifolia* was 26.5% ($P<0.01$) more in the galactose medium compared to the glucose medium.

The FCCP data show that AML-12 cells mainly utilize mitochondria for ATP generation regardless of whether glucose or galactose is used as metabolic fuel. The greater inhibition of cells by *Ilex latifolia* when grown in galactose cannot be explained by a mitochondrial effect and may relate to an effect on galactose entry into glycolysis, involving enzymes of the Leloir pathway. Inhibition when glucose was the fuel could suggest an effect on flux through the glycolytic pathway, and this links to the inhibition of HepG2 cell growth, albeit at much higher concentrations of *Ilex latifolia*, when cells are grown in glucose, but not when in galactose.

The data discussed so far are when the cells were grown in glucose and galactose but in the absence of insulin. *Ilex latifolia* had the same effect on the cell viability in both media where FCCP inhibited the cells growth by 95.3% in galactose and by 92% in glucose respectively.

To further investigate the effect of insulin on cells growth and sensitivity to *Ilex latifolia*, the cells were grown in glucose but the only variable was insulin.

- The only significant effect was at 250 µg/mL where *Ilex latifolia* inhibited the cells growth in glucose without insulin by 36% ($P<0.001$) compared to the *Ilex latifolia* inhibition effect on cells growth in glucose with insulin.
- While in the case of galactose without insulin, AML-12 cells were sensitive to125 µg/mL of *Ilex latifolia* which inhibited the cells growth in galactose with insulin by 57% ($P<0.001$) compared to its effect on cells growth in galactose without insulin (Chapter V, section 5.3.4).

Although the effect here is opposite to the one where glucose without insulin was used but this confirms that insulin is an essential factor in the growth medium of AML-12 cells but the mechanism of action needs to be explored.

The next step was to check if *Ilex latifolia* has an effect on the glucose uptake by AML-12 cells.

To take into account the possible effect of *Ilex latifolia* on cells growth and consequently on glucose consumption data, and to check the effect of insulin on glucose consumption by AML-12 cells, glucose consumption was measured in the presence or absence of insulin and was normalized to the cell growth by using MTT assay.

- *Ilex latifolia* inhibited glucose consumption in AML-12 cells grown in insulin containing medium at 3 µg/mL by 38.3% ($P<0.01$) and at 10 µg/mL by 51.8% ($P<0.001$) respectively when compared to the untreated control cells. It did not have any significant effect when the cells were grown in insulin depleted medium ($P>0.05$).
- By comparing the effect of *Ilex latifolia* on glucose consumption by the cells when insulin was removed from the medium, at 3 µg/mL of *Ilex latifolia,* glucose consumption was 41.9 % ($P<0.01$) less in the cells grown in the presence of insulin compared to glucose consumption in cells grown without insulin.
- At 10 µg/mL, glucose consumption was 44.2% ($P<0.05$) less in cells grown with insulin compared to cells grown without insulin (Chapter V, section 5.3.5).

- *Ilex latifolia* inhibits glucose consumption in AML-12 cells at relatively low concentrations, and this could potentially be linked to anti-lipid activity. More direct assessments of energy metabolism are needed to explore the association.

In this project, the search for new agents for the treatment of hyperlipidemia was the main target. This aim was done through targeting key steps in lipid metabolism. The first approach was to find inhibitors of the absorption of the dietary fats by inhibiting pancreatic lipase. *Ilex latifolia* had the most inhibitory activity out of the herbs tried, compared to orlistat which is an approved pancreatic lipase inhibitor. The next approach was to check if *Ilex latifolia* has an inhibition effect on HMGCR which is the rate limiting enzyme in cholesterol synthesis in the liver, and consequently on the expression of LDL-receptors which take the LDL-cholesterol from the circulation. *Ilex latifolia* did not show any clear effect on HMGCR or LDLR expression. Other enzymes that play important roles in lipoproteins metabolism are lipoprotein lipase (Ylaherttuala *et al*., 1991), hepatic lipase (Jin *et al*., 2002), lecithin cholesteryl acyl transferase (Barter *et al*., 2003) and cholesteryl ester transfer protein (Drayna *et al*., 1987), and these could the focus of further work to find the mechanism of action of *Ilex latifolia*.

In addition the work in this project has identified subtle effects of *Ilex latifolia* on glucose metabolism, perhaps involving modulation of glycolytic flux, that also suggest a path for further work

7 References

Ansell, B. J., Fonarow, G. C. & Fogelman, A. M. 2006. High-density lipoprotein: is it always atheroprotective? *Current Atherosclerosis Reports,* 8**,** 405-11.

Assmann, G. & Gotto, A. M., Jr. 2004. HDL cholesterol and protective factors in atherosclerosis. *CIrculation,* 109**,** III8-14.

Attie, A. D., Kastelein, J. P. & Hayden, M. R. 2001. Pivotal role of abca1 in reverse cholesterol transport influencing HDL levels and susceptibility to atherosclerosis. *Journal of lipid research,* 42**,** 1717-1726.

Austin, M. A., Hokanson, J. E. & Edwards, K. L. 1998. Hypertriglyceridemia as a cardiovascular risk factor. *American journal of cardiology,* 81**,** 7b-12b.

Baigent, C., Keech, A., Kearney, P. M., Blackwell, L., Buck, G., Pollicino, C., Kirby, A., Sourjina, T., Peto, R., Collins, R., Simes, J. & Collaborators, C. T. T. 2005. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet,* 366**,** 1267-1278.

Ballinger, A. & Peikin, S. R. 2002. Orlistat: its current status as an anti-obesity drug. *European Journal of Pharmacology,* 440**,** 109-117.

Bansal, S., Buring, J. E., Rifai, N., Mora, S., Sacks, F. M. & Ridker, P. M. 2007. Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. *Jama-Journal of the american medical association,* 298**,** 309-316.

Barter, P. J., Brewer, H. B., Chapman, M. J., Hennekens, C. H., Rader, D. J. & Tall, A. R. 2003. Cholesteryl ester transfer protein - a novel target for raising HDL and inhibiting atherosclerosis. *Arteriosclerosis Thrombosis And Vascular Biology,* 23**,** 160-167.

Bays, H. 2006. Statin safety: an overview and assessment of the data-2005. *American Journal of cardiology,* 97**,** 6c-26c.

Benchao, Z. 1999. Chinese materia medica, editorial committee of chinese materia medica, Shanghai Science And Technology, Shanghai.

Bergstrom, J. D., Bostedor, R. G., Rew, D. J., Giessler, W. M., Wright, S. D. & Chao, Y. S. 1997. Hepatic responses to inhibition of HMG-CoA reductase: A comparison of atorvastatin and simvastatin. *Atherosclerosis,* 130**,** 27-27.

Bi, Z., Zheng, F. & Kang, Z. 1982. Treatment of chronic renal failure by retention enema with rhizoma rhei compound decoction. *Journal of traditional Chinese medicine,* 2**,** 211-214.

Birari, R. B. & Bhutani, K. K. 2007. Pancreatic lipase inhibitors from natural sources: unexplored potential. *Drug Discovery Today,* 12**,** 879-889.

Bitou, N., Ninomiya, M., Tsujita, T. & Okuda, H. 1999. Screening of lipase inhibitors from marine algae. *Lipids,* 34**,** 441-445.

Borgstrom, B. 1974. Fat digestion and absorption. *Biomembranes,* 4b**,** 555-620.

Borgstrom, B. 1982. The temperature-dependent interfacial inactivation of porcine pancreatic lipase - effect of colipase and bile-salts. *Biochimica Et Biophysica Acta,* 712**,** 490-497.

Borgstrom, B. 1988. Mode of action of tetrahydrolipstatin - a derivative of the naturally-occurring lipase inhibitor lipstatin. *Biochimica Et Biophysica Acta,* 962**,** 308-316.

Brewer, H. B. 2004. High-density lipoproteins: a new potential therapeutic target for the prevention of cardiovascular disease. *Arteriosclerosis Thrombosis And Vascular Biology,* 24**,** 387-391.

Brown, M. S. & Goldstein, J. L. 1980. Multivalent feedback-regulation of HMG-CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell-growth. *Journal Of Lipid Research,* 21**,** 505-517.

Brown, M. S. & Goldstein, J. L. 1986. A receptor-mediated pathway for cholesterol homeostasis (nobel lecture). *Angewandte Chemie-International Edition in English,* 25**,** 583-602.

Buhaescu, I. & Izzedine, H. 2007. Mevalonate pathway: a review of clinical and therapeutical implications. *Clinical Biochemistry,* 40**,** 575-584.

Bunnell, T. M., Burbach, B. J., Shimizu, Y. & Ervasti, J. M. 2011. Beta-actin specifically controls cell growth, migration, and the g-actin pool. *Molecular Biology of the cell,* 22**,** 4047-4058.

Carlson, L. A. 2004. Niaspan, the prolonged release preparation of nicotinic acid (niacin), the broad-spectrum lipid drug. *International Journal of Clinical Practice,* 58**,** 706-713.

Carlson, L. A. 2005. Nicotinic acid: the broad-spectrum lipid drug. a 50th anniversary review. *Journal of Internal Medicine,* 258**,** 94-114.

Cefalu, A. B., Barbagallo, C. M., Sesti, E., Caldarella, R., Polizzi, F., Marino, G., Noto, D., Rolleri, M., Travali, S., Scalisi, G., Notarbartolo, A., Corsini, A., Bertolini, S. & Averna, M. R. 2001. Italian familial defective apolipoprotein b patients share a unique haplotype with other caucasian patients. *Clinical and Experimental Medicine,* 1**,** 151-154.

Chai, Y. F., Ji, S. G., Wu, Y. T., Liang, D. S. & Xu, Z. M. 1998. The separation of anthraquinone derivatives of rhubarb by miceller electrokinetic capillary chromatography. *Biomedical Chromatography,* 12**,** 193-195.

Chait, A. & Brunzell, J. D. 1990. Acquired hyperlipidemia (secondary dyslipoproteinemias). *Endocrinology and Metabolism Clinics of North America,* 19**,** 259-278.

Chan, P., Thomas, G. N. & Tomlinson, B. 2002. Protective effects of trilinolein extracted from panax notoginseng against cardiovascular disease. *Acta Pharmacologica Sinica,* 23**,** 1157-1162.

Chang, C. C. Y., Huh, H. Y., Cadigan, K. M. & Chang, T. Y. 1993. Molecular-cloning and functional expression of human acyl-coenzyme-a cholesterol acyltransferase cdna in mutant chinese-hamster ovary cells. *Journal of Biological Chemistry,* 268**,** 20747-20755.

Chaput, J. P., St-Pierre, S. & Tremblay, A. 2007. Currently available drugs for the treatment of obesity: sibutramine and orlistat. *Mini-Reviews in Medicinal Chemistry,* 7**,** 3-10.

Chen, D.-C. & Wang, L. 2009. Mechanisms of therapeutic effects of rhubarb on gut origin sepsis. *Chinese Journal of traumatology = Zhonghua Chuang Shang Za Zhi / Chinese Medical Association,* 12**,** 365-9.

Chen MF, L. X., Yang SH, Feng YJ, Jiang JH 2007. The antitumor activities of the leaves of *Ilex latifolia* Thunb. *China for Sci Technol,* 21**,** 30-31.

Chin, D. J., Gil, G., Russell, D. W., Liscum, L., Luskey, K. L., Basu, S. K., Okayama, H., Berg, P., Goldstein, J. L. & Brown, M. S. 1984. Nucleotide-sequence of 3-hydroxy-3-methyl-glutaryl coenzyme-a reductase, a glycoprotein of endoplasmic-reticulum. *Nature,* 308**,** 613-617.

Chin, D. J., Luskey, K. L., Anderson, R. G. W., Faust, J. R., Goldstein, J. L. & Brown, M. S. 1982. Appearance of crystalloid endoplasmic-reticulum in compactin-resistant chinese-hamster cells with a 500-fold increase in 3-hydroxy-3-methylglutaryl-coenzyme-a reductase. *Proceedings of the national academy of sciences of the United States Of America-biological sciences,* 79**,** 1185-1189.

Chiu, L. C. M., Zhu, W. & Ooi, V. E. C. 2004. A polysaccharide fraction from medicinal herb prunella vulgaris downregulates the expression of herpes simplex virus antigen in vero cells. *Journal of Ethnopharmacology,* 93**,** 63-68.

Christian, J. B., Juneja, M. X., Meadowcroft, A. M., Borden, S. & Lowe, K. A. 2011. Prevalence, characteristics, and risk factors of elevated triglyceride levels in us children. *Clinical Pediatrics,* 50**,** 1103-1109.

Cignarella, A., Engel, T., Von Eckardstein, A., Kratz, M., Lorkowski, S., Lueken, A., Assmann, G. & Cullen, P. 2005. Pharmacological regulation of cholesterol efflux in human monocyte-derived macrophages in the absence of exogenous cholesterol acceptors. *Atherosclerosis,* 179**,** 229-236.

Cohen, D. E., Anania, F. A. & Chalasani, N. 2006. An assessment of statin safety by hepatologists. *American Journal of Cardiology,* 97**,** 77c-81c.

Cooper, A. D. 1997. Hepatic uptake of chylomicron remnants. *Journal of Lipid Research,* 38**,** 2173-2192.

Cortner, J. A., Coates, P. M. & Gallagher, P. R. 1990. Prevalence and expression of familial combined hyperlipidemia in childhood. *Journal of Pediatrics,* 116**,** 514-519.

Cudrey, C., Vantilbeurgh, H., Gargouri, Y. & Verger, R. 1993. Inactivation of pancreatic lipases by amphiphilic reagents 5-(dodecyldithio)-2-nitrobenzoic acid and tetrahydrolipstatin - dependence upon partitioning between micellar and oil phases. *Biochemistry,* 32**,** 13800-13808.

Daniels, T. F., Killinger, K. M., Michal, J. J., Wright, R. W., Jr. & Jiang, Z. 2009. Lipoproteins, cholesterol homeostasis and cardiac health. *International Journal of Biological Sciences,* 5**,** 474-488.

Davidson, M. H., Armani, A., Mckenney, J. M. & Jacobson, T. A. 2007. Safety considerations with fibrate therapy. *American Journal of Cardiology,* 99**,** 3c-18c.

Decaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E. & Livingston, D. M. 1988. Sv40 large tumor-antigen forms a

specific complex with the product of the retinoblastoma susceptibility gene. *Cell,* 54**,** 275-283.

Deng, J., Fan, C. & Yang, Y. 2011. Identification and determination of the major constituents in deng's herbal tea granules by rapid resolution liquid chromatography coupled with mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis,* 56**,** 928-936.

Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M. & Darnell, J. E. 1981. Transcriptional control in the production of liver-specific messenger-rnas. *Cell,* 23**,** 731-739.

Derynck, R. 1988. Transforming growth factor-alpha. *Cell,* 54**,** 593-595.

Desnuelle, P., Sarda, L. & Ailhaud, G. 1960. Inhibition of pancreatic lipase by diethyl-p-nitrophenyl phosphate in emulation. *Biochimica et Biophysica Acta,* 37**,** 570-1.

Devaraj, S., Vega, G., Lange, R., Grundy, S. M. & Jialal, I. 1998. Remnant-like particle cholesterol levels in patients with dysbetalipoproteinemia or coronary artery disease. *American Journal of Medicine,* 104**,** 445-450.

Dietschy, J. M., Turley, S. D. & Spady, D. K. 1993. Role of liver in the maintenance of cholesterol and low-density-lipoprotein homeostasis in different animal species, including humans. *Journal of Lipid Research,* 34**,** 1637-1659.

Dong, T. T. X., Cui, X. M., Song, Z. H., Zhao, K. J., Ji, Z. N., Lo, C. K. & Tsim, K. W. K. 2003. Chemical assessment of roots of panax notoginseng in china: regional and seasonal variations in its active constituents. *Journal of agricultural and food chemistry,* 51**,** 4617-4623.

Dong Y, B. X., Shi XK, Song BH, Zhang Xl, Liu YW 2001. The effect of kudingcha on immune function in mice. *J Mudanjiang Univ,* 30**,** 14-16.

Dorsey, W. C., Tchounwou, P. B. & Sutton, D. 2004. Mitogenic and cytotoxic effects of pentachlorophenol to aml 12 mouse hepatocytes. *International Journal of environmental research and public health,* 1**,** 100-5.

Douglas, I. J., Langham, J., Bhaskaran, K., Brauer, R. & Smeeth, L. 2013. Orlistat and the risk of acute liver injury: self controlled case series study in uk clinical practice research datalink. *Bmj-British Medical Journal,* 346.

Drayna, D., Jarnagin, A. S., Mclean, J., Henzel, W., Kohr, W., Fielding, C. & Lawn, R. 1987. Cloning and sequencing of human cholesteryl ester transfer protein cdna. *Nature,* 327**,** 632-634.

Drew, B. S., Dixon, A. F. & Dixon, J. B. 2007. Obesity management: update on orlistat. *Vascular Health and Risk Management,* 3**,** 817-21.

Dujovne, C. A., Ettinger, M. P., Mcneer, J. F., Lipka, L. J., Lebeaut, A. P., Suresh, R., Yang, B. & Veltri, E. P. 2002. Efficacy and safety of a potent new selective cholesterol absorption inhibitor, ezetimibe, in patients with primary hypercholesterolemia. *American Journal of cardiology,* 90**,** 1092-1097.

Eisenberg, S., Gavish, D., Oschry, Y., Fainaru, M. & Deckelbaum, R. J. 1984. Abnormalities in very low, low, and high-density lipoproteins in hypertriglyceridemia - reversal toward normal with bezafibrate treatment. *Journal of clinical investigation,* 74**,** 470-482.

Eisenreich, W., Kupfer, E., Stohler, P., Weber, W. & Bacher, A. 2003. Biosynthetic origin of a branched chain analogue of the lipase inhibitor, lipstatin. *Journal of medicinal chemistry,* 46**,** 4209-4212.

Elson, C. E., Peffley, D. M., Hentosh, P. & Mo, H. B. 1999. Isoprenoid-mediated inhibition of mevalonate synthesis: potential application to cancer. *Proceedings of the society for experimental biology and medicine,* 221**,** 294-311.

Endo, A. 1992. The discovery and development of HMG-CoA reductase inhibitors. *Journal of lipid research,* 33**,** 1569-1582.

Endo, A. & Hasumi, K. 1989. Biochemical aspect of HMG-CoA reductase inhibitors. *Advances in enzyme regulation,* 28**,** 53-64.

Endo, A., Kuroda, M. & Tanzawa, K. 1976. Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme a reductase by ml-236a and ml-236b fungal metabolites, having hypocholesterolemic activity. *Febs Letters,* 72**,** 323-326.

Expert panel on detection, e., treatment of high, b. & cholesterol in, a. 2001. Executive summary of the third report of the national cholesterol education program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). *Jama,* 285**,** 2486-97.

Expert panel on integrated guidelines for cardiovascular, h., risk, reduction in, c., adolescents, national heart, l. & blood, i. 2011. Expert panel on integrated guidelines for cardiovascular health and risk reduction in children and adolescents: Summary report. *Pediatrics,* 128 suppl 5**,** s213-56.

Fan, C., Deng, J., Yang, Y., Liu, J., Wang, Y., Zhang, X., Fai, K., Zhang, Q. & Ye, W. 2013. Multi-ingredients determination and fingerprint analysis of leaves from *Ilex latifolia* using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. *Journal of pharmaceutical and biomedical analysis,* 84**,** 20-29.

Faust, J. R., Luskey, K. L., Chin, D. J., Goldstein, J. L. & Brown, M. S. 1982. Regulation of synthesis and degradation of 3-hydroxy-3-methylglutaryl-coenzyme-a reductase by low-density lipoprotein and 25-hydroxycholesterol in UT-1 cells. *Proceedings of the national academy of sciences of the United States of America-biological sciences,* 79**,** 5205-5209.

Feng Yx., C. S., Zhao Rf., 1998. On the identity of kudingcha in chinese holly(*Ilex* l.). *Acta Phytotaxon Sin,* 36**,** 353-358.

Fredrickson, D. S., Levy, R. I. & Lees, R. S. 1967. Fat transport in lipoproteins--an integrated approach to mechanisms and disorders. *The new England Journal of medicine,* 276**,** 273-81.

Fujiwara, Y., Komohara, Y., Kudo, R., Tsurushima, K., Ohnishi, K., Ikeda, T. & Takeya, M. 2011. Oleanolic acid inhibits macrophage differentiation into the M2 phenotype and glioblastoma cell proliferation by suppressing the activation of stat3. *Oncology reports,* 26**,** 1533-1537.

Gargouri, Y., Chahinian, H., Moreau, H., Ransac, S. & Verger, R. 1991. Inactivation of pancreatic and gastric lipases by THL and C-12-0-TNB - a kinetic-study with emulsified tributyrin. *Biochimica et Biophysica Acta,* 1085**,** 322-328.

Gargouri, Y., Julien, R., Bois, A. G., Verger, R. & Sarda, L. 1983. Studies on the detergent inhibition of pancreatic lipase activity. *Journal of lipid research,* 24**,** 1336-1342.

Genest, J., Mcpherson, R., Frohlich, J., Anderson, T., Campbell, N., Carpentier, A., Couture, P., Dufour, R., Fodor, G., Francis, G. A., Grover, S., Gupta, M., Hegele, R. A., Lau, D. C., Leiter, L., Lewis, G. F., Lonn, E., Mancini, G. B. J., Ng, D., Pearson, G. J., Sniderman, A., Stone, J. A. & Ur, E. 2009. Canadian cardiovascular society/canadian guidelines for the diagnosis and treatment of dyslipidemia and prevention of cardiovascular disease in the adult-2009 recommendations. *Canadian Journal of Cardiology,* 25**,** 567-579.

Gerber, R., Ryan, J. D. & Clark, D. S. 2004. Cell-based screen of hmg-coa reductase inhibitors and expression regulators using LC-MS. *Analytical Biochemistry,* 329**,** 28-34.

Gibbons, G. F., Wiggins, D., Brown, A. M. & Hebbachi, A. M. 2004. Synthesis and function of hepatic very-low-density lipoprotein. *Biochemical society transactions,* 32**,** 59-64.

Glueck, C. J., Gartside, P., Fallat, R. W., Sielski, J. & Steiner, P. M. 1976. Longevity syndromes - familial hypobeta and familial hyperalpha lipoproteinemia. *Journal of laboratory and clinical medicine,* 88**,** 941-957.

Goldstein .Jl, Schrott, H. G., Hazzard, W. R., Birman, E. L. & Motulsky, A. G. 1973. Hyperlipidemia in coronary heart-disease .2. Genetic analysis of lipid-levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. *Journal of clinical investigation,* 52**,** 1544-1568.

Goldstein, J. L. & Brown, M. S. 1977. Low-density lipoprotein pathway and its relation to atherosclerosis. *Annual review of biochemistry,* 46**,** 897-930.

Goldstein, J. L. & Brown, M. S. 1984. Progress in understanding the ldl receptor and HMG-CoA reductase, 2 membrane-proteins that regulate the plasma-cholesterol. *Journal of Lipid Research,* 25**,** 1450-1461.

Goldstein, J. L. & Brown, M. S. 2009. The LDL receptor. *Arteriosclerosis thrombosis and vascular biology,* 29**,** 431-438.

Gordon, D. J., Probstfield, J. L., Garrison, R. J., Neaton, J. D., Castelli, W. P., Knoke, J. D., Jacobs, D. R., Bangdiwala, S. & Tyroler, H. A. 1989. High-density lipoprotein cholesterol and cardiovascular-disease - 4 prospective american-studies. *Circulation,* 79**,** 8-15.

Granon, S. & Semeriva, M. 1980. Effect of taurodeoxycholate, colipase and temperature on the interfacial inactivation of porcine pancreatic lipase. *European Journal of Biochemistry,* 111**,** 117-124.

Gruer, P. J. K., Vega, J. M., Mercuri, M. F., Dobrinska, M. R. & Tobert, J. A. 1999. Concomitant use of cytochrome p450 3a4 inhibitors and simvastatin. *American Journal of cardiology,* 84**,** 811-815.

Grundy, S. M. 2007. Should women be offered cholesterol lowering drugs to prevent cardiovascular disease? *British Medical Journal,* 334**,** 982-983.

Grundy, S. M., Becker, D., Clark, L. T., Cooper, R. S., Denke, M. A., Howard, W. J., Hunninghake, D. B., Illingworth, R., Luepker, R. V., Mcbride, P., Mckenney, J. M., Pasternak, R. C., Stone, N. J., Van Horn, L., Brewer, H. B., Cleeman, J. I., Ernst, N. D., Gordon, D., Levy, D., Rifkind, B., Rossouw, J. E., Savage, P., Haffner, S. M., Orloff, D. G., Proschan, M. A., Schwartz, J. S., Sempos, C. T., Shero, S. T., Murray, E. Z., Keller, S. A., Jehle, A. J. & Natl Cholesterol Educ Program, E. 2002. Third report of the national cholesterol education program (ncep) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel iii) final report. *Circulation,* 106**,** 3143-3421.

Grundy, S. M., Chait, A. & Brunzell, J. D. 1987. Familial combined hyperlipidemia workshop. *Arteriosclerosis,* 7**,** 203-207.

Grundy, S. M., Cleeman, J. I., Merz, C. N. B., Brewer, H. B., Clark, L. T., Hunninghake, D. B., Pasternak, R. C., Smith, S. C., Stone, N. J. & Coordinating Comm Natl, C. 2004. Implications of recent clinical trials for the national cholesterol education program adult treatment panel iii guidelines. *Circulation,* 110**,** 227-239.

Guay, D. R. P. 2002. Update on fenofibrate. *Cardiovascular drug reviews,* 20**,** 281-302.

Guguen-Guillouzo, C. (1992) Isolation and culture of animal and human hepatocytes, in Culture of epithelial cells (Freshney, R. I., ed.) pp. 197-223.

Guyton, J. R., Brown, B. G., Fazio, S., Polis, A., Tomassini, J. E. & Tershakovec, A. M. 2008. Lipid-altering efficacy and safety of ezetimibe/simvastatin coadministered with extended-release niacin in patients with type iia or type iib hyperlipidemia. *Journal of the American college of cardiology,* 51**,** 1564-1572.

Habtemariam, S. 2012. Identification of the antidiabetic and antihyperlipidemic principles of *Cassia auriculata*. *Planta Medica,* 78**,** 1084-1084.

Haffner, S. M. 1999. Diabetes, hyperlipidemia, and coronary artery disease. *American Journal of cardiology,* 83**,** 17f-21f.

Hahn, J.; Seeber, F.; Kolodziej, H.; Ignatius, R.; Laue, M.; Aebischer, T.; & Klotz, C. 2013. High sensitivity of giardia duodenalis to tetrahydrolipstatin (orlistat) in vitro. PLoS One 8, No. e71597.

Hanessian, S., Tehim, A. & Chen, P. 1993. Total synthesis of (-)-tetrahydrolipstatin. *Journal of organic chemistry,* 58**,** 7768-7781.

Hanukoglu, I. 1992. Steroidogenic enzymes - structure, function, and role in regulation of steroid-hormone biosynthesis. *Journal of steroid biochemistry and molecular biology,* 43**,** 779-804.

Harp, J. B. 1998. An assessment of the efficacy and safety of orlistat for the long-term management of obesity. *Journal of nutritional biochemistry,* 9**,** 516-521.

He, Z. D., Lau, K. M., But, P. P. H., Jiang, R. W., Dong, H., Ma, S. C., Fung, K. P., Ye, W. C. & Sun, H. D. 2003. Antioxidative glycosides from the leaves of ligustrum robustum. *Journal of natural products,* 66**,** 851-854.

He, Z. D., Ueda, S., Akaji, M., Fujita, T., Inoue, K. & Yang, C. R. 1994. Monoterpenoid and phenylethanoid glycosides from ligustrum pedunculare. *Phytochemistry,* 36**,** 709-716.

Hill, S. A. & Mcqueen, J. 1997. Reverse cholesterol transport - a review of the process and its clinical implications. *Clinical Biochemistry,* 30**,** 517-525.

Hochuli, E., Kupfer, E., Maurer, R., Meister, W., Mercadal, Y. & Schmidt, K. 1987. Lipstatin, an inhibitor of pancreatic lipase, produced by streptomyces-toxytricini .2. Chemistry and structure elucidation. *Journal of antibiotics,* 40**,** 1086-1091.

Hodis, H. N. 1999. Triglyceride-rich lipoprotein remnant particles and risk of atherosclerosis. *Circulation,* 99**,** 2852-2854.

Hofbauer, K. 2002. Molecular pathways to obesity. *International Journal of obesity,* 26**,** s18-s27.

Huang, J., Wang, X., Ogihara, Y., Shimizu, N., Takeda, T. & Akiyama, T. 2001. Latifolosides i and j, two new triterpenoid saponins from the bark of *Ilex latifolia*. *Chemical & Pharmaceutical bulletin,* 49**,** 239-241.

Huang Z. 1997. Kudingcha treatment for hypertension - 35 case reports. Chin J Inform Trad Chin Med 4: 25.

Huang, Z., Zhou, X., Nicholson, A. C., Gotto, A. M., Jr., Hajjar, D. P. & Han, J. 2008. Activation of peroxisome proliferator-activated receptor-alpha in mice induces expression of the hepatic low-density lipoprotein receptor. *British Journal of pharmacology,* 155**,** 596-605.

Hussain, M. M. 2000. A proposed model for the assembly of chylomicrons. *Atherosclerosis,* 148**,** 1-15.

Insua, A., Massari, F., Rodriguez Moncalvo, J. J., Ruben Zanchetta, J. & Insua, A. M. 2002. Fenofibrate of gemfibrozil for treatment of types IIA and IIB primary hyperlipoproteinemia: A randomized, double-blind, crossover study. *Endocrine practice : Official Journal of the American college of endocrinology and the American association of clinical endocrinologists,* 8**,** 96-101.

Isom, H. C., & Strom, S.C. 1992. The role of cell types in hepatocarcinogenesis. CRC Press, Boca Raton, 266-298.

Istvan, E. S., Palnitkar, M., Buchanan, S. K. & Deisenhofer, J. 2000. Crystal structure of the catalytic portion of human hmg-coa reductase: insights into regulation of activity and catalysis. *Embo Journal,* 19**,** 819-830.

Jiang JM, W. B., Xu SB, & Jiang RX 2001. Research on the antibacterial acivity of kudingcha . *Pharm Clin Chin Mat Met* 17**,** 18-19.

Jie, Z., Jiguo, H., Baoping, J., Ye, L. & Xiaofeng, Z. 2007. Antihyperglycemic activity of prunella vulgaris l. in streptozotocin-induced diabetic mice. *Asia Pacific Journal of clinical nutrition,* 16**,** 427-431.

Jin, W. J., Marchadier, D. & Rader, D. J. 2002. Lipases and HDL metabolism. *Trends in endocrinology and metabolism,* 13**,** 174-178.

Jo, Y. & Debose-Boyd, R. A. 2010. Control of cholesterol synthesis through regulated er-associated degradation of hmg coa reductase. *Critical reviews in biochemistry and molecular biology,* 45**,** 185-198.

Joo, I. W., Ryu, J. H. & Oh, H. J. 2010. The influence of sam-chil-geun (panax notoginseng) on the serum lipid levels and inflammations of rats with hyperlipidemia induced by poloxamer-407. *Yonsei Medical Journal,* 51**,** 504-510.

Kasiske, B. L., Wanner, C. & O'neill, W. C. 2006. An assessment of statin safety by nephrologists. *American journal of cardiology,* 97**,** 82c-85c.

Kaufmann, W. K., Zhang, Y. C. & Kaufman, D. G. 1992. Association between expression of transforming growth factor-alpha and progression of hepatocellular foci to neoplasms. *Carcinogenesis,* 13**,** 1481-1483.

Kim, J. Y., Lee, H. K., Hwang, B. Y., Kim, S., Yoo, J. K. & Seong, Y. H. 2012. Neuroprotection of *Ilex latifolia* and caffeoylquinic acid derivatives against excitotoxic and hypoxic damage of cultured rat cortical neurons. *Archives of Pharmacal research,* 35**,** 1115-1122.

Kim, Y. S., Lee, Y. M., Kim, H., Kim, J., Jang, D. S., Kim, J. H. & Kim, J. S. 2010. Anti-obesity effect of morus bombycis root extract: anti-lipase activity and lipolytic effect. *Journal of Ethnopharmacology,* 130**,** 621-624.

Kiplimo, J. J., Koorbanally, N. A. & Chenia, H. 2011. Triterpenoids from vernonia auriculifera hiern exhibit antimicrobial activity. *African Journal of pharmacy and pharmacology,* 5**,** 1150-1156.

Kitahara, M., Asano, M., Naganawa, H., Maeda, K., Hamada, M., Aoyagi, T., Umezawa, H., Iitaka, Y. & Nakamura, H. 1987. Valilactone, an inhibitor of esterase, produced by actinomycetes. *Journal of antibiotics,* 40**,** 1647-1650.

Klag, M. J., Mead, L. A., Ford, D. E., Coresh, J. & Levine, D. M. 1993. Serum-cholesterol in young men and risk of subsequent injuries. *Circulation,* 88**,** 124-124.

Knopp, R. H. 1999. Drug treatment of lipid disorders. *New England Journal of medicine,* 341**,** 498-511.

Kraft, H. G., Demosky, S. J., Schumacher, K., Brewer, H. B. & Hoeg, J. M. 1992. Regulation of LDL receptor, Apo-B, and Apoe protein and messenger-RNA in hepG2 cells. *DNA and Cell biology,* 11**,** 291-300.

Krieger, M. 2001. Scavenger receptor class b type i is a multiligand HDL receptor that influences diverse physiologic systems. *Journal of clinical investigation,* 108**,** 793-797.

Laskarzewski, P. M., Khoury, P., Morrison, J. A., Kelly, K., Mellies, M. J. & Glueck, C. J. 1982. Prevalence of familial hyperlipoproteinemias and hypolipoproteinemias - the princeton school-district family study. *Metabolism-clinical and experimental,* 31**,** 558-577.

Law, M. & Rudnicka, A. R. 2006. Statin safety: a systematic review. *American Journal of cardiology,* 97**,** 52c-60c.

Law, M. R., Wald, N. J. & Rudnicka, A. R. 2003. Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. *British Medical Journal,* 326**,** 1423-1427.

Lee, I. A., Lee, J. H., Baek, N. I. & Kim, D. H. 2005. Antihyperlipidemic effect of crocin isolated from the fructus of gardenia jasminoides and its metabolite crocetin. *Biological & Pharmaceutical Bulletin,* 28**,** 2106-2110.

Leigh, S. E. A., Foster, A. H., Whittall, R. A., Hubbart, C. S. & Humphries, S. E. 2008. Update and analysis of the university college london low density lipoprotein receptor familial hypercholesterolemia database. *Annals of Human Genetics,* 72**,** 485-498.

Li, L., Peng, Y., Ma, G., He, C., Feng, Y., Lei, Q. & Xiao, P. 2012. Quantitative analysis of five kudinosides in the large-leaved kudingcha and related species from the genus *Ilex* by uplc-elsd. *Phytochemical analysis,* 23**,** 677-683.

Li, L., X. L., Peng, Y., & He, ZD. 2011. Simultaneous determination of five phenylethanoid glycosides in small-leaved kudingcha from ligustrum genus by UPLC/PDA. *Food Chem,* 131**,** 1583-1588.

Li, L., Xu, L., Peng, Y., Shi, R. & Xiao, P. 2011. Comparison of green tea and four other kind of teas. *China Journal Of Chinese Materia Medica,* 36**,** 5-10.

Li, L., Xu, L. J., Ma, G. Z., Dong, Y. M., Peng, Y. & Xiao, P. G. 2013. The large-leaved kudingcha (*Ilex latifolia* thunb and *Ilex* kudingcha CJ Tseng): A traditional chinese tea with plentiful secondary metabolites and potential biological activities. *Journal of natural medicines,* 67**,** 425-437.

Li, Y. L., But, P. P. H. & Ooi, V. E. C. 2005. Antiviral activity and mode of action of caffeoylquinic acids from Schefflera heptaphylla (l.) frodin. *Antiviral Research,* 68**,** 1-9.

Lichtenstein, A. H., Appel, L. J., Brands, M., Carnethon, M., Daniels, S., Franch, H. A., Franklin, B., Kris-Etherton, P., Harris, W. S., Howard, B., Karanja, N., Lefevre, M., Rudel, L., Sacks, F., Van Horn, L., Winston, M., Wylie-Rosett, J. & American Heart Association Nutrition, C. 2006. Diet and lifestyle recommendations revision 2006: A scientific statement from the american heart association nutrition committee. *Circulation,* 114**,** 82-96.

Lipsy, R. J. 2003. The national cholesterol education program adult treatment panel III guidelines. *Journal of managed care pharmacy,* 9**,** 2-5.

Liscum, L., Cummings, R. D., Anderson, R. G. W., Demartino, G. N., Goldstein, J. L. & Brown, M. S. 1983. 3-Hydroxy-3-methylglutaryl-coa reductase - a transmembrane glycoprotein of the endoplasmic-reticulum with n-linked high-mannose oligosaccharides. *Proceedings of the national academy of sciences of the United States of America-biological sciences,* 80**,** 7165-7169.

Liu, L., Sun, Y., Laura, T., Liang, X., Ye, H. & Zeng, X. 2009. Determination of polyphenolic content and antioxidant activity of kudingcha made from *Ilex* kudingcha C.J. Tseng. *Food chemistry,* 112**,** 35-41.

Lookene, A., Skottova, N. & Olivecrona, G. 1994. Interactions of lipoprotein-lipase with the active-site inhibitor tetrahydrolipstatin (orlistat)(R). *European Journal Of Biochemistry,* 222**,** 395-403.

Lopez-Lopez, A., Castellote-Bargallo, A. I., Campoy-Folgoso, C., Rivero-Urgell, M., Tormo-Carnice, R., Infante-Pina, D. & Lopez-Sabater, M. C. 2001. The influence of dietary palmitic acid triacylglyceride position on the fatty acid, calcium and magnesium contents of at term newborn faeces. *Early human development,* 65**,** s83-s94.

Luthipeng, Q. & Winkler, F. K. 1992. Large spectral changes accompany the conformational transition of human pancreatic lipase induced by acylation with the inhibitor tetrahydrolipstatin. *European Journal of biochemistry,* 205**,** 383-390.

Maron, D. J., Fazio, S. & Linton, M. F. 2000. Current perspectives on statins. *Circulation,* 101**,** 207-213.

Masubuchi, Y., Kano, S. & Horie, T. 2006. Mitochondrial permeability transition as a potential determinant of hepatotoxicity of antidiabetic thiazolidinediones. *Toxicology,* 222**,** 233-239.

Maurich, V., Moneghini, M., Zacchigna, M., Pitotti, A. & Lencioni, E. 1991. High-performance liquid-chromatographic assay of pancreatic lipase activity. *Journal of pharmaceutical and biomedical analysis,* 9**,** 427-431.

Mcdougall, G. J., Kulkarni, N. N. & Stewart, D. 2009. Berry polyphenols inhibit pancreatic lipase activity in vitro. *Food chemistry,* 115**,** 193-199.

Mckenney, J. M. 2000. Improving cholesterol control in managed care populations. *American Journal of managed care,* 6**,** s997-s1007.

Mckenney, J. M., Davidson, M. H., Jacobson, T. A. & Guyton, J. R. 2006a. Final conclusions and recommendations of the national lipid association statin safety assessment task force. *American Journal of cardiology,* 97**,** 89c-94c.

Mckenney, J. M., Farnier, M., Lo, K. W., Bays, H. E., Perevozkaya, I., Carlson, G., Davies, M. J., Mitchel, Y. B. & Gumbiner, B. 2006b. Safety and efficacy of long-term co-administration of fenofibrate and ezetimibe in patients with mixed hyperlipidemia. *Journal of the American college of cardiology,* 47**,** 1584-1587.

Mckenney, J. M., Jones, P. H., Bays, H. E., Knopp, R. H., Kashyap, M. L., Ruoff, G. E. & Mcgovern, M. E. 2007. Comparative effects on lipid levels of combination therapy with a statin and extended-release niacin or ezetimibe versus a statin alone (the compell study). *Atherosclerosis,* 192**,** 432-437.

Mensink, R. P. & Katan, M. B. 1992. Effect of dietary fatty-acids on serum-lipids and lipoproteins - a metaanalysis of 27 trials. *Arteriosclerosis and thrombosis,* 12**,** 911-919.

Miller, M. 2009. Dyslipidemia and cardiovascular risk: The importance of early prevention. *QJM-An international Journal of medicine,* 102**,** 657-667.

Minicocci, I., Prisco, C., Montali, A., Di Costanzo, A., Ceci, F., Pigna, G. & Arca, M. 2015. Contribution of mutations in low density lipoprotein receptor (LDLR) and lipoprotein lipase (LPL) genes to familial combined hyperlipidemia (FCHL): A reappraisal by using a resequencing approach. *Atherosclerosis,* 242**,** 618-24.

Miranda, M. X., Van Tits, L. J., Lohmann, C., Arsiwala, T., Winnik, S., Tailleux, A., Stein, S., Gomes, A. P., Suri, V., Ellis, J. L., Lutz, T. A., Hottiger, M. O., Sinclair, D. A., Auwerx, J., Schoonjans, K., Staels, B., Luescher, T. F. & Matter, C. M. 2015. The sirt1 activator Srt3025 provides atheroprotection in Apoe(-/-) mice by reducing hepatic pcsk9 secretion and enhancing LDLR expression. *European Heart Journal,* 36**,** 51-59.

Molowa, D. T. & Cimis, G. M. 1989. Coordinate regulation of low-density-lipoprotein receptor and 3-hydroxy-3-methylglutaryl-CoA reductase and synthase gene-expression in HepG2 cells. *Biochemical Journal,* 260**,** 731-736.

Momsen, W. E. & Brockman, H. L. 1976. Inhibition of pancreatic lipase-b activity by taurodeoxycholate and its reversal by colipase - mechanism of action. *Journal of biological chemistry,* 251**,** 384-388.

Moreno, D. A., Ilic, N., Poulev, A., Brasaemle, D. L., Fried, S. K. & Raskin, I. 2003. Inhibitory effects of grape seed extract on lipases. *Nutrition,* 19**,** 876-879.

Mou, L. 2005. Contrast observation on clinical efficacy of *Ilex latifolia* kudingcha from wuyuan jiangxi on hypertension. *Mod Diag Treat,* 16**,** 223.

Mukherjee, M. 2003. Human digestive and metabolic lipases - a brief review. *Journal of molecular catalysis B-enzymatic,* 22**,** 369-376.

Mutsaers, C. A., Wishart, T. M., Lamont, D. J., Riessland, M., Schreml, J., Comley, L. H., Murray, L. M., Parson, S. H., Lochmueller, H., Wirth, B., Talbot, K. & Gillingwater, T. H. 2011. Reversible molecular pathology of skeletal muscle in spinal muscular atrophy. *Human molecular genetics,* 20**,** 4334-4344.

Negishi, O., Negishi, Y., Yamaguchi, F. & Sugahara, T. 2004. Deodorization with ku-ding-cha containing a large amount of caffeoyl quinic acid derivatives. *Journal of agricultural and food chemistry,* 52**,** 5513-5518.

Ness, G. C., Way, S. C. & Wickham, P. S. 1981. Proteinase involvement in the solubilization of 3-hydroxy-3-methylglutaryl coenzyme-a reductase. *Biochemical and biophysical research communications,* 102**,** 81-85.

Nishimura, K., Fukuda, T., Miyase, T., Noguchi, H. & Chen, X. M. 1999a. Activity-guided isolation of triterpenoid acyl CoA cholesteryl acyl transferase (acat) inhibitors from *Ilex* kudincha. *Journal of Natural Products,* 62**,** 1061-1064.

Nishimura, K., Miyase, T. & Noguchi, H. 1999b. Triterpenoid saponins from *Ilex* kudincha. *Journal of Natural Products,* 62**,** 1128-1133.

Oates, J. A., Wood, A. J. J., Donowitz, G. R. & Mandell, G. L. 1988. Drug therapy beta lactam antibiotics. *New England Journal of medicine,* 318**,** 419-426.

Olivecrona T, & Bengtsson-Olivecrona G. 1987. Lipoprotein lipase from milk-the model enzyme in lipoprotein lipase research. In: Borensztajn J, editor. Lipoprotein Lipase. Evener; Chicago: 15–58.

Olofsson, S. O., Asp, L. & Boren, J. 1999. The assembly and secretion of apolipoprotein b-containing lipoproteins. *Current opinion in lipidology,* 10**,** 341-346.

Olofsson, S. O., Stillemark-Billton, P. & Asp, L. 2000. Intracellular assembly of VLDL - two major steps in separate cell compartments. *Trends in cardiovascular medicine,* 10**,** 338-345.

Ong, M. M. K., Latchoumycandane, C. & Boelsterli, U. A. 2007. Troglitazone-induced hepatic necrosis in an animal model of silent genetic mitochondrial abnormalities. *Toxicological sciences,* 97**,** 205-213.

Ono, Y., Hattori, E., Fukaya, Y., Imai, S. & Ohizumi, Y. 2006. Anti-obesity effect of nelumbo nucifera leaves extract in mice and rats. *Journal of ethnopharmacology,* 106**,** 238-244.

Packard, C. J., Shepherd, J., Cobbe, S. M., Ford, I., Isles, C. G., Mckillop, J. H., Macfarlane, P. W., Lorimer, A. R., Norrie, J. & Grp, W. S. C. P. S. 1998. Influence of pravastatin and plasma lipids on clinical events in the west of scotland coronary prevention study (WOSCOPS). *Circulation,* 97**,** 1440-1445.

Palmer, R. H. 1987. Effects of fibric acid-derivatives on biliary lipid-composition. *American Journal of medicine,* 83**,** 37-43.

Park, K. S., Jo, I., Pak, Y. K., Bae, S. W., Rhim, H., Suh, S. H., Park, S. J., Zhu, M. H., So, I. & Kim, K. W. 2002. FCCP depolarizes plasma membrane potential by

activating proton and na+ currents in bovine aortic endothelial cells. *Pflugers Archiv-European Journal of physiology,* 443**,** 344-352.

Parker, R. A., Pearce, B. C., Clark, R. W., Gordon, D. A. & Wright, J. J. K. 1993. Tocotrienols regulate cholesterol production in mammalian-cells by posttranscriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme-a reductase. *Journal of biological chemistry,* 268**,** 11230-11238.

Patel, J., Sheehan, V. & Gurk-Turner, C. 2003. Ezetimibe (zetia): A new type of lipid-lowering agent. *Proceedings (Baylor University. medical center),* 16**,** 354-8.

Peelman, F., Vandekerckhove, J. & Rosseneu, M. 2000. Structure and function of lecithin cholesterol acyl transferase: New insights from structural predictions and animal models. *Current opinion in lipidology,* 11**,** 155-160.

Pemminati S.,Venkatesh V., Rai S., 2011. Anxiolytic effect of acute administration of ursolic acid in rats. *Res. J. Pharm. Biol. Chem.Sci. 2***,** 431-437.

Pfeifer, A. M. A., Cole, K. E., Smoot, D. T., Weston, A., Groopman, J. D., Shields, P. G., Vignaud, J. M., Juillerat, M., Lipsky, M. M., Trump, B. F., Lechner, J. F. & Harris, C. C. 1993. Simian virus-40 large tumor antigen-immortalized normal human liver epithelial-cells express hepatocyte characteristics and metabolize chemical carcinogens. *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 5123-5127.

Pierce, L. R., Wysowski, D. K. & Gross, T. P. 1990. Myopathy and rhabdomyolysis associated with lovastatin-gemfibrozil combination therapy. *Journal of the American medical association,* 264**,** 71-75.

Pinkas, M., Trotin, F., Peng, W. & Torck, M. 1994. Use, chemistry and pharmacology of ten chinese medicinal plants. *Fitoterapia,* 65**,** 343-354.

Potthoff, A. P., Haalck, L. & Spener, F. 1998. Inhibition of lipases from chromobacterium viscosum and rhizopus oryzae by tetrahydrolipstatin. *Biochimica et Biophysica Acta-lipids and lipid metabolism,* 1389**,** 123-131.

Psotova, J., Kolar, M., Sousek, J., Svagera, Z., Vicar, J. & Ulrichova, J. 2003. Biological activities of *Prunella* vulgaris extract. *Phytotherapy research,* 17**,** 1082-1087.

Qu Lz, , Lu Pj, Xi Jy 1999. The effect of kudingcha on epinephrine hyperglycemia rats. *Tradit Chin Drug Res Clin Pharmacol,* 10**,** 279-280.

Reid L.M., Narita M., Fujita M., Murray Z., Liverpool C. &, Rosenberg L.: Matrix and hormonal regulation of differentiation in liver cultures. In A. Guillouzo and C. GuguenGuillouzo (Eds.): Research in Isolated and Cultured Hepatocytes, John Libbey Eurotext Ltd., Montrouge 1986, pp. 225–258.

Robson, J. 2008. Lipid modification: cardiovascular risk assessment and the modification of blood lipids for the primary and secondary prevention of cardiovascular disease. *Heart,* 94**,** 1331-1332.

Rodriguez-Enriquez, S., Juarez, O., Rodriguez-Zavala, J. S. & Moreno-Sanchez, R. 2001. Multisite control of the crabtree effect in ascites hepatoma cells. *European Journal of biochemistry,* 268**,** 2512-2519.

Roelofs, A. J., Edwards, C. M., Graham, R., Russell, G., Ebetino, F. H., Rogers, M. J. & Hulley, P. A. 2007. Apomine enhances the antitumor effects of lovastatin on

myeloma cells by down-regulating 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Journal of Pharmacology and Experimental Therapeutics,* 322**,** 228-235.

Roger, V. L., Go, A. S., Lloyd-Jones, D. M., Adams, R. J., Berry, J. D., Brown, T. M., Camethon, M. R., Dai, S., De Simone, G., Ford, E. S., Fox, C. S., Fullerton, H. J., Gillespie, C., Greenlund, K. J., Hailpem, S. M., Heit, J. A., Ho, P. M., Howard, V. J., Kissela, B. M., Kittner, S. J., Lackland, D. T., Lichtman, J. H., Lisabeth, L. D., Makuc, D. M., Marcus, G. M., Marelli, A., Matchar, D. B., Mcdermott, M. M., Meigs, J. B., Moy, C. S., Mozaffarian, D., Mussolino, M. E., Nichol, G., Paynter, N. P., Rosamond, W. D., Sorlie, P. D., Stafford, R. S., Turan, T. N., Turner, M. B., Wong, N. D., Wylie-Rosett, J., American Heart Assoc Stat, C. & Stroke Stat, S. 2011. Heart Disease And Stroke Statistics-2011 Update a report from the american heart association. *Circulation,* 123**,** e18-e209.

Ros, E. 2000. Intestinal absorption of triglyceride and cholesterol. dietary and pharmacological inhibition to reduce cardiovascular risk. *Atherosclerosis,* 151**,** 357-379.

Rosenson, R. S. 2004. Current overview of statin-induced myopathy. *American Journal of medicine,* 116**,** 408-416.

Rossignol, R., Gilkerson, R., Aggeler, R., Yamagata, K., Remington, S. J. & Capaldi, R. A. 2004. Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. *Cancer Research,* 64**,** 985-993.

Rouard, M., Sari, H., Nurit, S., Entressangles, B. & Desnuelle, P. 1978. Inhibition of pancreatic lipase by mixed micelles of diethyl para-nitrophenyl phosphate and bile-salts. *Biochimica et Biophysica Acta,* 530**,** 227-235.

Rubenstein, P. A. 1990. The functional importance of multiple actin isoforms. *Bioessays,* 12**,** 309-315.

Rubin, E. M., Krauss, R. M., Spangler, E. A., Verstuyft, J. G. & Clift, S. M. 1991. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein-A-I. *Nature,* 353**,** 265-267.

Rubins, H. B., Robins, S. J., Collins, D., Fye, C. L., Anderson, J. W., Elam, M. B., Faas, F. H., Linares, E., Schaefer, E. J., Schectman, G., Wilt, T. J., Wittes, J. & Vet Affairs High-Density Lipoprotein, C. 1999. Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. *New England Journal of medicine,* 341**,** 410-418.

Rudermann Nb, G. S., Sussman I, 1990. Hyperglycemia, diabetes, and vascular disease: an overview. in hyperglycemia, diabetes, and cardiovascular disease. Rudmann N, Williamson J, Brownlee M, Eds. New York, Oxford University press,. 3-20.

Salomon, D. S., Kim, N., Saeki, T. & Ciardiello, F. 1990. Transforming growth factor-alpha - an oncodevelopmental growth-factor. *Cancer cells-a monthly review,* 2**,** 389-397.

Saraphanchotiwitthaya, A. & Sripalakit, P. 2014. Effect of morinda citrifolia linn. Leaf extracts on in vitro lipase activity. *Chiang Mai Journal Of Science,* 41**,** 1182-1193.

Sarwar, N., Danesh, J., Eiriksdottir, G., Sigurdsson, G., Wareham, N., Bingham, S., Boekholdt, S. M., Khaw, K.-T. & Gudnason, V. 2007. Triglycerides and the risk of coronary heart disease - 10 158 incident cases among 262 525 participants in 29 western prospective studies. *Circulation,* 115**,** 450-458.

Schachter, M. 2005. Chemical, pharmacokinetic and pharmacodynamic properties of statins: An update. *Fundamental & clinical pharmacology,* 19**,** 117-125.

Scheving, L. A., Zhang, X., Garcia, O. A., Wang, R. F., Stevenson, M. C., Threadgill, D. W. & Russell, W. E. 2014. Epidermal growth factor receptor plays a role in the regulation of liver and plasma lipid levels in adult male mice. *American Journal of physiology-gastrointestinal and liver physiology,* 306**,** g370-g381.

Schmid, R. D. & Verger, R. 1998. Lipases: interfacial enzymes with attractive applications. *Angewandte Chemie-International Edition,* 37**,** 1609-1633.

Schneider, W. J., Beisiegel, U., Goldstein, J. L. & Brown, M. S. 1982. Purification of the low-density lipoprotein receptor, an acidic glycoprotein of 164,000 molecular-weight. *Journal of biological chemistry,* 257**,** 2664-2673.

Schoonjans, K., Staels, B. & Auwerx, J. 1996. Role of the peroxisome proliferator-activated receptor (Ppar) in mediating the effects of fibrates and fatty acids on gene expression. *Journal of lipid research,* 37**,** 907-925.

Shah, A. S. & Wilson, D. P. 2015. Primary hypertriglyceridemia in children and adolescents. *Journal of clinical lipidology,* 9**,** s20-s28.

Shah, R. R. 2006. Can pharmacogenetics help rescue drugs withdrawn from the market? *Pharmacogenomics,* 7**,** 889-908.

Sheng, L., Qian, Z., Zheng, S. & Xi, L. 2006. Mechanism of hypolipidemic effect of crocin in rats: Crocin inhibits pancreatic lipase. *European Journal of pharmacology,* 543**,** 116-122.

Shi, Y. G. & Burn, P. 2004. Lipid metabolic enzymes: emerging drug targets for the treatment of obesity. *Nature reviews drug discovery,* 3**,** 695-710.

Shin, J. E., Han, N. J. & Kim, D. H. 2003. 3-Methylethergalangin isolated from *Alpinia officinarum* inhibits pancreatic lipase. *Biological & pharmaceutical bulletin,* 26**,** 854-857.

Singh, I. M. 2007. High-density lipoprotein as a therapeutic target: a systematic review (vol 298, pg 786, 2007). *Journal of the American medical association,* 298**,** 1516.

Song, Y., Kenworthy, A. K. & Sanders, C. R. 2014. Cholesterol as a co-solvent and a ligand for membrane proteins. *Protein science,* 23**,** 1-22.

Soutar, A. K. & Naoumova, R. P. 2007. Mechanisms of disease: Genetic causes of familial hypercholesterolemia. *Nature clinical practice cardiovascular medicine,* 4**,** 214-225.

Stamler, J., Wentworth, D. & Neaton, J. D. 1986. Is relationship between serum-cholesterol and risk of premature death from coronary heart-disease continuous and graded - findings in 356 222 primary screenees of the multiple risk factor intervention trial (MRFIT). *Journal of the American medical association,* 256**,** 2823-2828.

Steiner, G. 1985. Atherosclerosis, the major complications of diabetes. *Adv Exp Med Biol* 189**,** 277-297.

Stone, N. J. 1994. Secondary causes of hyperlipidemia. *Medical Clinics of North America,* 78**,** 117-141.

Subramanian, R., Ramaswamy, M. & Wasan, K. M. 2003. Role of lipid and lipoprotein metabolizing enzymes in the development of atherosclerosis. *Indian Journal of experimental biology,* 41**,** 14-25.

Suzuki, O., Koura, M., Noguchi, Y., Uchio-Yamada, K. & Matsuda, J. 2011. Use of sample mixtures for standard curve creation in quantitative western blots. *Experimental animals,* 60**,** 193-196.

Tam, S. P., Brissette, L., Ramharack, R. & Deeley, R. G. 1991. Differences between the regulation of 3-hydroxy-3-methylglutaryl-coenzyme-a reductase and low-density-lipoprotein receptor in human hepatoma-cells and fibroblasts reside primarily at the translational and posttranslational levels. *Journal of biological chemistry,* 266**,** 16764-16773.

Tanaka, R. D., Edwards, P. A., Lan, S. F. H., Knoppel, E. M. & Fogelman, A. M. 1982. Purification of 3-hydroxy-3-methylglutaryl co-enzyme a reductase from human-liver. *Journal of lipid research,* 23**,** 523-530.

Tang, L., et al., Triterpene saponins from the leaves of Ilex kudingcha. *Journal of Natural Products*, 2005. 68(8): p. 1169-1174.

Thomson, A. B. R., Depover, A., Keelan, M., Jarockacyrta, E. & Clandinin, M. T. 1997. Inhibition of lipid absorption as an approach to the treatment of obesity. *in:* Rubin, B. & Dennis, E. A. *Lipases, Pt B: Enzyme characterization and utilization. B. Rubin and E.A. Dennis, Editors. 1997. p. 3-44.*

Thompson, P. D., Clarkson, P. M. & Rosenson, R. S. 2006. An assessment of statin safety by muscle experts. *American Journal of cardiology,* 97**,** 69c-76c.

Thuong, P. T., Su, N. D., Ngoc, T. M., Hung, T. M., Dang, N. H., Thuan, N. D., Bae, K. & Oh, W. K. 2009. Antioxidant activity and principles of vietnam bitter tea *Ilex* kudingcha. *Food chemistry,* 113**,** 139-145.

Tian, J., Du, H., Yang, H., Liu, X. & Li, Z. 1997. A clinical study on compound da Huang (Radix et Rhizoma Rhei) preparations for improvement of senile persons' memory ability. *Journal of Traditional Chinese Medicine,* 17**,** 168-73.

Tiss, A., Lengsfeld, H., Carriere, F. & Verger, R. 2009. Inhibition of human pancreatic lipase by tetrahydrolipstatin: further kinetic studies showing its reversibility. *Journal of molecular catalysis B-enzymatic,* 58**,** 41-47.

Tiss, A., Lengsfeld, H., Hadvary, P., Cagna, A. & Verger, R. 2002. Transfer of orlistat through oil-water interfaces. *Chemistry and physics of lipids,* 119**,** 41-49.

To, M.-S., Aromataris, E. C., Castro, J., Roberts, M. L., Barritt, G. J. & Rychkov, G. Y. 2010. Mitochondrial uncoupler fccp activates proton conductance but does not block store-operated Ca2+ current in liver cells. *Archives of biochemistry and biophysics,* 495**,** 152-158.

Umezawa, H., Aoyagi, T., Hazato, T., Uotani, K., Kojima, F., Hamada, M. & Takeuchi, T. 1978. Esterastin, an inhibitor of esterase, produced by actinomycetes. *Journal of antibiotics,* 31**,** 639-641.

Vanitallie, T. B. 1985. Health implications of overweight and obesity in the united-states. *Annals of internal medicine,* 103**,** 983-988.

Vega, G. L., Ma, P. T. S., Cater, N. B., Filipchuk, N., Meguro, S., Garcia-Garcia, A. B. & Grundy, S. M. 2003. Effects of adding fenofibrate (200 mg/day) to simvastatin (10 mg/day) in patients with combined hyperlipidemia and metabolic syndrome. *American Journal of cardiology,* 91**,** 956-960.

Verger, R. 1997. 'Interfacial activation' of lipases: facts and artifacts. *Trends in biotechnology,* 15**,** 32-38.

Walsh, J. M. E. & Pignone, M. 2004. Drug treatment of hyperlipidemia in women. *Journal of the American medical association,* 291**,** 2243-2252.

Wang, C.-Q., Wang, L., Fan, C.-L., Zhang, D.-M., Huang, X.-J., Jiang, R.-W., Bai, L.-L., Shi, J.-M., Wang, Y. & Ye, W.-C. 2012. Ilelic acids a and b, two unusual triterpenes with a seven-membered ring from *Ilex latifolia*. *Organic Letters,* 14**,** 4102-4105.

Wang X, H. L.,& Liu B 2008. Study on extraction, purification and scavenging activity to hydroxyl radicals of polysaccharides from leaves of *Ilex* kudingcha ,*Food Sci* 29**,** 37-40.

Warburg, O., Geissler, A. W. & Lorenz, S. 1967. On growth of cancer cells in media in which glucose is replaced by galactose. *Hoppe-Seyler's Zeitschrift Fur Physiologische Chemie,* 348**,** 1686-7.

Weber, L. W., Boll, M. & Stampfl, A. 2004. Maintaining cholesterol homeostasis: Sterol regulatory element-binding proteins. *World Journal of gastroenterology,* 10**,** 3081-3087.

Weibel, E. K., Hadvary, P., Hochuli, E., Kupfer, E. & Lengsfeld, H. 1987. Lipstatin, an inhibitor of pancreatic lipase, produced by streptomyces-toxytricini .1. Producing organism, fermentation, isolation and biological-activity. *Journal of antibiotics,* 40**,** 1081-1085.

Wilson, P. W. F., Abbott, R. D., D'agostino, R. B., Silbershatz, H., Rodriquez, B. L. & Curb, J. D. 1998. Prediction of coronary heart disease in japanese-american men. *Circulation,* 98**,** 323-323.

Wilson, P. W., Garrison, R. J., Castelli, W. P., Feinleib, M., Mcnamara, P. M. & Kannel, W. B. 1980. Prevalence of coronary heart-disease in the framingham offspring study - role of lipoprotein cholesterols. *American Journal of cardiology,* 46**,** 649-654.

Winkler, F. K., Darcy, A. & Hunziker, W. 1990. Structure of human pancreatic lipase. *Nature,* 343**,** 771-774.

Wishart, T. M., Pemberton, H. M., James, S. R., Mccabe, C. J. & Gillingwater, T. H. 2008. Modified cell cycle status in a mouse model of altered neuronal vulnerability (Wallerian Degeneration Slow; WLDS). *Genome biology,* 9.

Woo, A. Y. H., Jiang, J. M., Chau, C. F., Waye, M. M. Y., Cheung, W. T., Kwan, H. S. & Cheng, C. H. K. 2001. Inotropic and chronotropic actions of *Ilex latifolia* - inhibition of adenosine-5 '-triphosphatases as a possible mechanism. *Life Sciences,* 68**,** 1259-1270.

Wu, J. C., Merlino, G. & Fausto, N. 1994. Establishment and characterization of differentiated, nontransformed hepatocyte cell-lines derived from mice transgenic for transforming growth-factor-alpha. *Proceedings of the national academy of sciences of the United States of America,* 91**,** 674-678.

Wu, W., Zhang, X. M., Liu, P. M., Li, J. M. & Wang, J. F. 1995. Effects of panax-notoginseng saponin RG(1) on cardiac electrophysiological properties and ventricular-fibrillation threshold in dogs. *Acta Pharmacologica Sinica,* 16**,** 459-463.

Wu, Z., Raven, P. H. & Hong, D. 2008. *Flora of china illustrations hippocastanaceae through theaceae*.

Xie, W. D., Xing, D. M., Sun, H., Wang, W., Ding, Y. & Du, L. J. 2005a. The effects of ananas comosus l. leaves on diabetic-dyslipidemic rats induced by alloxan and a high-fat/high-cholesterol diet. *American Journal of Chinese medicine,* 33**,** 95-105.

Xie, W. D., Xing, D. M., Zhao, Y. N., Su, H., Meng, Z., Chen, Y. Y. & Du, L. J. 2005b. A new tactic to treat postprandial hyperlipidemia in diabetic rats with gastroparesis by improving gastrointestinal transit. *European Journal of pharmacology,* 510**,** 113-120.

Xiong B, L. H., Liu L, Xie Xm, Deng Qy 2003. GC-MS determination of essential oil from *Ilex* kudingcha C.J. Tseng. *j instrum anal,* 22**,** 67-69.

Xu Sb, Xu Dh, Jiang Rx, Kong Yc, Hu Xy 2001. The toxicological studies of the water extraction from *Ilex latifolia* Thunb. *Acta Sci Nat Univ,* 3**,** 92-94.

Yamamoto, M., Shimura, S., Itoh, Y., Ohsaka, T., Egawa, M. & Inoue, S. 2000. Anti-obesity effects of lipase inhibitor CT-II, an extract from edible herbs, nomame herba, on rats fed a high-fat diet. *International Journal of obesity,* 24**,** 758-764.

Yang, F. & Du, Y. G. 2002. Synthesis of oligosaccharide derivatives related to those from Sanqi, a Chinese herbal medicine from *Panax notoginseng*. *Carbohydrate Research,* 337**,** 485-491.

Yang, N. C., Ho, W. M., Chen, Y. H. & Hu, M. L. 2002. A convenient one-step extraction of cellular ATP using boiling water for the luciferin-luciferase assay of atp. *Analytical Biochemistry,* 306**,** 323-327.

Ylaherttuala, S., Lipton, B. A., Rosenfeld, M. E., Goldberg, I. J., Steinberg, D. & Witztum, J. L. 1991. Macrophages and smooth-muscle cells express lipoprotein-lipase in human and rabbit atherosclerotic lesions. *Proceedings of the national academy of sciences of the United States of America,* 88**,** 10143-10147.

Yosioka, I., Sugawara, T., Ohsuka, A. & Kitagawa, I. 1971. Soil bacterial hydrolysis leading to genuine aglycone .3. Structures of glycosides and genuine aglycone of sanguisorbae radix. *Chemical & pharmaceutical bulletin,* 19**,** 1700.

Zambon, A., Brown, B. G., Hokanson, J. E., Motulsky, A. G. & Brunzell, J. D. 2006. Genetically determined Apo-B levels and peak LDL density predict angiographic response to intensive lipid-lowering therapy. *Journal of internal medicine,* 259**,** 401-409.

Zhang, C. 1994. Survey of kudingcha original plant and comodity. *Chin Herbal Med,* 26**,** 595-598.

Zhang, C. B., Teng, L., Shi, Y., Jin, J. H., Xue, Y. F., Shang, K. G. & Gu, J. 2002. Effect of emodin on proliferation and differentiation of 3t3-l1 preadipocyte and fas activity. *Chinese Medical Journal,* 115**,** 1035-1038.

Zhang WQ, X. W., Sun Y, Ye H, Zeng XX 2010. Comparative evaluation of antioxidant activity in vitro of aqueous extracts from *Ilex* kudingcha C.J. Tseng and *Ilex latifolia* Thunb leaf teas. *Food Sci,* 31**,** 22-26.

Zhang, Y. & Huang, C. 2012. Targeting adipocyte apoptosis: A novel strategy for obesity therapy. *Biochemical and biophysical research communications,* 417**,** 1-4.

Zhao, L., Tao, J.-Y., Zhang, S.-L., Jin, F., Pang, R. & Dong, J.-H. 2010. n-Butanol extract from melilotus suaveolens ledeb affects pro- and anti-inflammatory cytokines and mediators. *Evidence-based complementary and alternative medicine,* 7**,** 97-106.

Zheng, C.-D., Duan, Y.-Q., Gao, J.-M. & Ruan, Z.-G. 2010. Screening for anti-lipase properties of 37 traditional chinese medicinal herbs. *Journal of the Chinese medical association,* 73**,** 319-324.

Zheng, J., Tang, L., Xian, X.-D., Zhou, S.-X., Shi, H.-M., Jiang, Y., Gu, Y.-Q., Liu, G. & Tu, P.-F. 2009. Inhibitory effect of triterpenoid saponins from the leaves of *Ilex* kudingcha on aggregated LDL-induced lipid deposition in macrophages. *Planta Medica,* 75**,** 1410-1414.

Zhu, F., Cai, Y.-Z., Sun, M., Ke, J., Lu, D. & Corke, H. 2009. Comparison of major phenolic constituents and in vitro antioxidant activity of diverse kudingcha genotypes from *Ilex* kudingcha, *Ilex* cornuta, and *Ligustrum* robustum. *Journal of agricultural and food chemistry,* 57**,** 6082-6089.

Zou WJ, Li XD, Wang ZH, Dai HF 2011. Chemical constituents of *Ilex* kudingcha leaves. *Chin Tradit Herbal,* 2**,** 18-20.