ANTIHYPERLIPIDEMIC ACTIVITY OF *POLYGONUM MINUS* AND ELUCIDATION OF ITS MECHANISM OF ACTION

by

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DEDICATION

THIS THESIS IS DEDICATED

TO

THE ALMIGHTY

ALL MY TEACHERS,

MY BELOVED FATHER; VARGHESE,

MOTHER; ALEYAMMA, WIFE; GILLY, SON; JOEL,

ALL MY BELOVED FRIENDS AND RELATIVES FOR THEIR CONSTANT

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LIST OF SYMBOLS

α	Alpha
β	Beta
ºC	Celsius
Δ	Delta
γ	Gamma
g	Gram
µ	Micro
±	Plus-minus
<	Less than
>	Greater than
n	Nano

LIST OF ABBREVIATIONS

ABTS	2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt
ACAT	Acylcoenzyme A cholesterol acyltransferase
AI	Atherogenic index
ALB/GLO	Albumin to globulin ratio
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AOC	Antioxidant capacity
ARASC	Animal Research and Service Centre
AST	Aspartate aminotransferase
BAAT	Amino acid n-acyltransferase
BHA	Butylated Hydroxyanisole
BSA	Bovine serum albumin
CA	Cholic acid
CAE	Catechin equivalent
CAT	Catalase
CDCA	Chenodeoxycholic acid
CETP	Cholesterylester transfer protein
CHD	Coronary heart disease
CMC	Carboxymethylcellulose
COX	Cyclooxygenase
CUPRAC	Cupric ion reducing antioxidant capacity
CVD	Cardiovascular disease

DALY	Disability adjusted life years
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picryl-hydrazyl
DW	Dry weight
EA.hy926	Human umbilical vein cell
EC_{50}	Median effective concentration
FAs	Fatty acids
FRAP	Ferric reducing-antioxidant power
FTC	Ferric thiocyante
GAE	Gallic acid equivalent
GGT	Gamma-glutamyltransferase
GHS	Globally harmonized system
GR	Glutathione reductase
GSH	Reduced glutathione estimation
GPx	Glutathione peroxidase
GSt	Glutathione-S-transferase
HAT	Hydrogen Atom Transfer
Hb	Hemoglobin concentration
HCT116	Human colon cancer cell
HDL	High density lipoprotein
HeLa	Human cervical cancer cell
HFD	High fat diet
HMG CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography

HT29	Human colon cancer cell
IC_{50}	Median inhibitory concentration
ICP-AES	Inductively coupled plasma- atomic emission spectroscopy
ICP-FIMS	Inductively coupled plasma-flow injection mercury system
ICP-OES	Inductively coupled plasma optical emission spectrometry
IDL	Intermediate density lipoprotein
IU	International unit
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
LOX	Lipoxygenase
LPO	Lipid peroxidation
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MEM	Minimum essential medium
MIC	Minimum inhibitory concentration
MS	Mass spectroscopy
MTT	Tetrazolium salt
NAD	Nicotinamide adenine dinucleotide
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NCEs	New chemical entities
NHMS	National health and morbidity survey
OECD	Organization for Economic Co-operation and Development
ORAC	Oxygen radical absorbance capacity

PCV	Packed cell volume
QE	Quercitin equivalent
ROS	Reactive oxygen species
RBC	Red blood cells
RHD	Rheumatic heart disease
SD rats	Sprague Dawley rats
SDS	Sodium dodecyl sulfate
SEM	Standard error mean
SET	Single electron transfer
SOD	Superoxide dismutase
sPLA2	Secretary phosphlipase A2
TBA	Thiobarbituric acid
TC	Total cholesterol
TC/HDL	Total cholesterol to high density lipoprotein ratio
TEAC	Trolox equivalent antioxidant capacity
TG	Triglycerides
THF	Tetrahydrofuran
TMC	Transition metals chelation
TPTZ	2,4,6-Tri(2-pyridyl)-s-triazine
TRAP	Total radical-trapping antioxidant parameter
VLDL	Very low density lipoprotein
VSV	Vesicular stomatitis virus
WBC	White blood cells
WHO	World Health Organization

LIST OF UNITS

g/L	Gram per liter
µg/mL	Micro gram per milliliter
µm	Micrometer
µL	Microliter
mmol/L	Milli mole per liter
nmol/L	Nano mole per liter
nm	Nano meter
U/mL	Units per milliliter
U/mg	Units per milligram
W/V	Weight per volume

AKTIVITI ANTIHIPERLIPIDEMIK POLYGONUM MINUS

DAN ELUSIDASI MEKANISME TINDAKANNYA

ABSTRAK

Polygonum minus merupakan tumbuhan herba yang ditanam di negara Asia Tenggara. Ia merupakan salah satu daripada herba yang digunakan secara meluas di Malaysia sebagai pengawet makanan, ulam dan ubatan herba bagi mengubati pelbagai penyakit. Kajian ini bertujuan untuk menilai kesan hiperlipidemik ekstrak daun *P. minus* termasuklah mekanisma, aktiviti antioksida serta ketoksikan tumbuhan ini. Kesan ekstrak akueus dan metanol daun *P. minus* (1000 mg/kg) diuji pada tikus hiperlipidemik yang diaruh menggunakan polaxamer-407 bagi kajian antihiperlipidemik. Atorvastatin (60 mg/kg) digunakan sebagai rujukan piawai. Kajian ini menunjukkan kedua dua ekstrak menurunkan kolesterol total, trigliserida, LDL, indeks atherogenik dan meningkatkan paras HDL apabila dibandingkan dengan kawalan hyperlipidemia bagi ekstrak metanol dibandingkan dengan ekstrak akueus. Berdasarkan keputusan yang diperolehi, ekstrak metanol daun *P. minus* dipilih bagi kesan anti-hiperlipidemik kronik hiperlipidemia. Model tikus yang diaruh hiperlipedemia dengan diet tinggi lemak digunakan bagi menilai kesan anti-hiperlipidemik ekstrak metanol pada dos 250, 500 dan 1000 mg/kg. Kajian ini menilai kesan ekstrak metanol (1000 mg/kg) pada tikus normal dan tikus dengan diet tinggi lemak. Tiada sebarang perubahan signifikan pada lipid dan parameter lain pada tikus normal dan tikus yang diberi diet tinggi lemak manakala perubahan signifikan diperolehi bagi tikus diet tinggi lemak yang diberi dos berbeza terutamanya 500 dan 1000 mg/kg ekstrak apabila dibandingkan dengan kawalan hiperlipidemik. Efek ekstrak dapat dibandingkan dengan dadah kawalan, atorvastatin (20 mg/kg). Keputusan yang

diperolehi ini menunjukkan kesan ekstrak *P. minus* adalah bersifat anti-hiperlipidemik dan bukannya hipolipidemik kerana paras parameter lipid pada tikus normal tidak berubah. Kandungan fenolik dan flavonoid didapati lebih tinggi pada ekstrak metanol dan memberi aktiviti antioksida yang lebih tinggi berbanding ekstrak akueus. Aktiviti anti-hiperlipidemik ekstrak metanol diperolehi melalui pelbagai mekanisma termasuklah peningkatan aktiviti antioksida *in-vivo*, perencatan enzim HMG-CoA reductase, penurunan paras kolesterol di dalam hati, sintesis trigliserida, peningkatan aktiviti penyingkiran kolesterol dan hempedu melalui tinja. Kajian ketoksikan dijalankan bagi menilai aspek keselamatan ekstrak. Analisa kandungan logam berat menunjukkan ekstrak akueus dan metanol adalah bebas dari logam berat. Aktiviti sitotoksik pada sel normal (EA.hy926) dan kanser (HCT116, HT29 dan HeLa) menunjukkan kedua dua ekstrak adalah tidak sitotoksik. Aktiviti ketoksikan akut dan sub-kronik menunjukkan ekstrak metanol daun *P. minus* adalah selamat pada dos sehingga 2000 mg/kg kerana ekstrak tersebut tidak menunjukkan sebarang tanda tanda ketoksikan berdasarkan berat badan, berat organ, berat relatif organ, parameter biokimia dan hematologi serta histologi sel. Kuersetin dan miresetin dikenalpasti dalam ekstrak metanol dan kandungannya dinilai menggunakan HPLC. Kajian ini menunjukkan *P. minus* mempunyai kesan penurunan lipid dan boleh dibangunkan sebagai agen anti-hiperlipidemik.

ANTIHYPERLIPIDEMIC ACTIVITY

OF *POLYGONUM MINUS* AND ELUCIDATION OF ITS

MECHANISM OF ACTION

ABSTRACT

Polygonum minus is an herbaceous plant grown naturally and cultivated throughout Southeast Asian countries. It is one of the widely used herbs in Malaysia as food additive, salad leaves (Ulam) and as traditional medicine to treat different ailments. Present study aims to evaluate the antihyperlipidemic effect including possible mechanism, antioxidant capacity and toxicities of extract of *P. minus* leaves. In acute antihyperlipidemic study, the effect of aqueous and methanol extracts of *P. minus* leaves (1000 mg/kg) were tested on poloxamer-407 induced acute hyperlipidemia in rats. Atorvastatin (60 mg/kg) was used as standard drug. The study showed that both extracts reduced the total cholesterol (TC), triglycerides (TG), LDL, VLDL, atherogenic index, and increased HDL levels when compared with hyperlipidemic control and the effect was higher in methanol extract when compared to aqueous extract. Based on this result, methanol extract of *P. minus* leaves was selected for determination of antihyperlipidemic effect in chronic hyperlipidemia. High fat diet-induced hyperlipidemia rat model was employed to evaluate the antihyperlipidemic effect of *P. minus* leaves methanol extract at doses of 250, 500 and 1000 mg/kg. There were no significant changes in the lipid and other parameters observed in the normal rats treated with high dose of extract whereas significant alterations were evident in high fat diet fed rats treated with different doses especially at 500 and 1000 mg/kg extract when compared with hyperlipidemic control. The effect of the extract was comparable with standard drug atorvastatin (20 mg/kg). This finding suggests that the

effect of *P. minus* extract is antihyperlipidemic rather than hypolipidemic as the levels of lipid parameters in the normal rats was not affected. Phenolic and flavonoids content were found to be high in methanol extract leading to better antioxidant activity than aqueous extract. Antihyperlipidemic effect of methanol extract was achieved via multiple mechanisms including elevation of *in vivo* antioxidant activity, in part by inhibition of HMG-CoA reductase enzyme, reduction in hepatic cholesterol and triglyceride synthesis and increased excretion of cholesterol and bile acids through feces. Toxicity studies were conducted to assess the safety of the extract. The heavy metal content analysis suggested that the aqueous and methanol extracts are devoid of toxic heavy metals. The cytotoxicity studies on normal (EA.hy926) and cancer (HCT116, HT29 and HeLa) cells showed that both the extracts were not cytotoxic. Acute and sub-chronic toxicity studies found that the methanol extract of *P. minus* leaves was safe to administer orally up to 2000 mg/kg of body weight as the extract did not cause any signs of toxicity in body weight, relative organ weight, biochemical and hematological parameters and cell histology. Biomarkers such as quecitrin and myricetin were identified in methanol extract and the amounts were quantified using HPLC (5.5 and 0.6 µg/mg respectively). In conclusion, this study suggests that *P. minus* possess lipid-lowering effect and could be potentially developed as an antihyperlipidemic agent.

CHAPTER 1

INTRODUCTION

1.1 Background

Cardiovascular disease (CVD) is one of the four major non-communicable diseases which cause significant mortality in the world. These diseases are manifested as coronary heart disease (CHD), cardiomyopathy, strokes, rheumatic heart disease (RHD) and other heart diseases (Fakhrzadeh and Tabatabaei-Malazy, 2012; Celermajer *et al*., 2012). About one third of the global death was caused by CVD in 2020 and is expected to be one of the major health risks by 2020. The rate of mortality caused by CVD is predicted to rise dramatically in the coming decades. Dyslipidemia is one of the main metabolic comorbidities associated with excessive body fat that contribute as major factor to the development of CVD. It is projected the rate of mortality and morbidity due to CVD could be raised as the prevalence of various CVD risk factors rise as a result of adverse changes in way of life due to urbanization and industrialization. Obesity caused by lipid metabolic syndromes is one of the serious health issue faced by both industrialized and developing world. Over 115 million people in the world are suffering with obesity and its related problems (Adeboye *et al*., 2012). CVD is one of the prime causes of mortality in Malaysia in 1970 and the rate of mortality is still increasing with CHD. Many other studies also have found that the prevalence rate of CVD in Malaysia is alarming (Jeyamalar, 1991; Yunus *et al*., 2004). The treatment of CVD is based on life style modifications and drug therapy. Hyperlipidemia is the main risk factor to develop CVD. Mainly five classes of drugs are utilized to treat hyperlipidemia condition;

statins, fibtrates, derivatives of nicotinic acid, bile acid sequesterants and inhibitors of cholesterol absorption (Rohilla *et al*., 2012). Even though they are very effective and beneficial in treating hyperlipidemia, their adverse effects are limiting factors in the treatment of hyperlipidemia. The development of effective but less toxic antihyperlipidemic agents are the present focus. The current interest has extended to develop novel lipid lowering molecules from natural sources with efficacy and minimal toxicity (Saghir *et al*., 2014; Ibrahim *et al*., 2013).

Atherosclerosis is one of the common pathophysiological features to cause cardiovascular diseases. Atherosclerosis develops by the combination of several environmental and genetic factors but the pathogenesis of this condition is principally due to lipoproteins. Many studies have established the solid relationship between increased LDL level and occurrence of atherosclerosis. Reduction in the LDL level remains as the main stay in the primary target of the treatment (Kastelein *et al*., 2008; Adams, 2005). Dyslipidemia is one of the major risk factor of CVD and it is manifested by increased lipoprotein concentration in plasma. It is a highly heterogeneous class of metabolic disorder, characterized by altered serum plasma protein levels. Generally dyslipidemia includes hyperlipidemia (hypercholesterolemia) and low levels of HDL (Bersot, 2011; Fakhrzadeh and Tabatabaei-Malazy, 2012).

In epidemiological studies, LDL has been identified as atherogenic. The relationship between new onset of CHD and LDL is also an established fact. The recurrence of cardiac events in CHD patients are also related with LDL level (Wilson *et al*., 1998). The decreased level of HDL is also an important factor which increases the CHD risks. It is reported that 1% decrease in HDL may increase 2-3% risk of the CHD. An increased association between TG level and CHD risk was confirmed by

many investigations. The raised TG level significantly correlated with CHD risk and the atherogenic potential of some TGs have confirmed. Non-HDL cholesterol level is a better indicator of CHD risk than HDL level. A condition of low HDL, high TG and small dense LDL (highly atherogenic) particles but normal LDL level, atherogenic dyslipidemia, is also a risk factor for CHD (Desforges *et al*., 1989; Sarwar *et al*., 2007; Rubins *et al*., 1999; Lu *et al*., 2003).

1.2 Therapeutic challenges

A substantial proportion of deaths in developing countries are due to cardiovascular diseases. It is projected that 80-90% of the cardiovascular patients will be in under developed or developing countries by 2025. The disability adjusted life years (DALY) of premature morbidity and mortality associated with CVD is expected to double from 85 million in 1990 to 140-160 million in 2020 (Yusuf *et al*., 2002). The Malaysian scenario of coronary artery disease is a good reflection of the disease in most developing countries. According to the report of 'The National Health and Morbidity Survey, (NHMS), the prevalence rate of atherosclerosis is dangerously increasing when compared with the previous data. About 47.7% (9.6 million) Malaysian adults of 18 years and above have hypercholesterolemia, in that 9.7% have already diagnosed with hypercholesterolemia and the remaining 38.6% was undiagnosed previously (Institute of Public Health, 2015). Hyperlipidemia is associated with many other metabolic disorders such as atherosclerosis, obesity, diabetes mellitus, renal disorders, liver disorders, thyroid disorders, Cushing's syndrome, hepatic lipase deficiency and glycogen storage diseases, so the treatment of hyperlipidemia is also related with the coexisting disorders (Ibrahim *et al*., 2013). Diverse classes of drugs are available to manage hyperlipidemia. Even though they are effective, their adverse effects are considered to be serious. The side effects

include rhabdomyolysis, elevation of hepatic enzymes, myopathy, and increased risk of gall stones (Expert Panel on Detection, 2001). The escalating costs of these synthetic drugs are also a major clinical challenge. There is no single drug or class of drug is effective against all sorts of hyperlipidemia (Alsheikh-Ali *et al*., 2004). The demand for high efficacy, minimal side effect, low cost and multiple targeting in preventing and curing antihyperlipidemic agents are need of the day.

1.3 Problem statement

Prevention of hyperlipidemia using drugs with minimum adverse effects and cost effectiveness remained unresolved therapeutic challenges in the management of hyperlipidemia associated metabolic disorders. People residing in areas with rich biodiversity usually depend upon medicinal plants to cater their daily health needs. Traditional medicines have significantly contributed to the development of successful drugs for various health conditions. *Polygonum minus* is such a plant and commonly available throughout Malaysia. Traditional uses and various pharmacological activities including excellent antioxidant capacity for this plant were reported (Qader *et al*., 2012a; Narasimhulu and Mohamed, 2014). This plant is believed to have lipid lowering effect and consumed as salad leaves along with other plant leaves (Jaganath and Ng, 2000). There were no previous studies available on the lipids lowering effect of *P. minus* in any animal models other than an *in vitro* study evaluated its inhibitory effect on LDL oxidation (Saputri and Jantan, 2011). The notable scarcity of scientific studies to prove or challenge the traditional claim on lipids lowering effect created an interest to evaluate the potential antihyperlipidemic effect, investigate the probable mechanism of lipid lowering property and assesses the toxicity due to regular consumption of *P. minus*.

1.4 Objectives

The objectives of the present study are:

i. To evaluate the antihyperlipidemic effect of aqueous and methanol extracts of leaves of *P. minus* on chemical-induced acute hyperlipidemic rat model

ii. To evaluate the antihyperlipidemic effect of the most active extract of leaves of *P. minus* on diet-induced chronic hyperlipidemic rat model

iii. To evaluate the antioxidant capacity of aqueous and methanol extracts of leaves of *P. minus*

iv. To evaluate the mechanism of antihyperlipidemic effect of the most active extract of leaves of *P. minus* on

    a. Inhibition of lipid synthesizing enzymes

    b. Lipids and bile acid excretion

    c. *In vivo* antioxidant capacity and lipid peroxidation

v. To evaluate the toxicity of the most active extract of leaves of *P. minus*

vi. To standardize the most active extract of leaves of *P. minus* using selected marker compound

1.5 Flow chart of the study

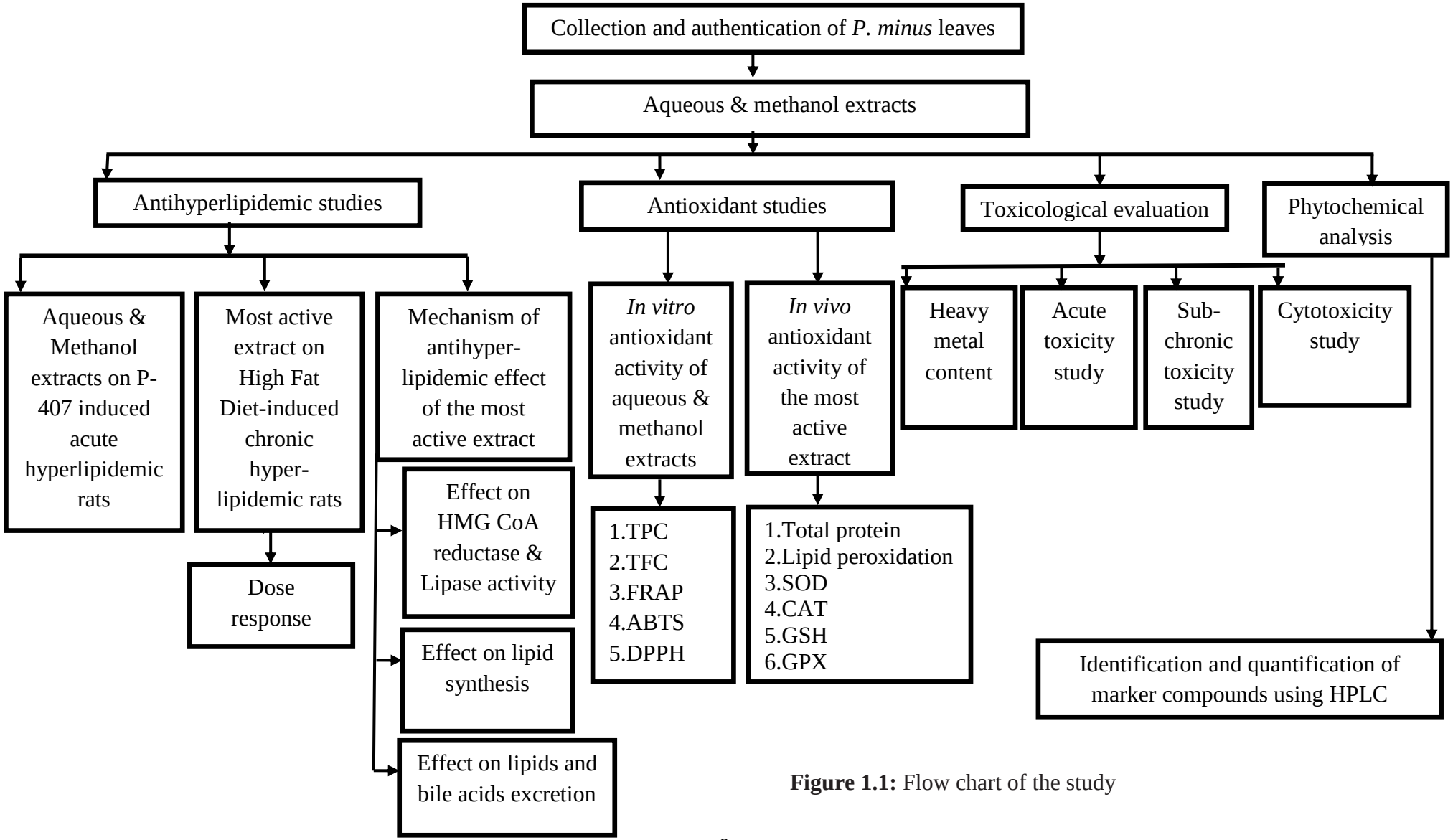


Figure 1.1: Flow chart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Lipids

The word lipid was coined by G. Bertrand in 1923 from Greek *lipos* (grease or fat) and French chemical suffix –*ide*. The exact definition for lipids does not exist. Lipids comprise wide range of natural products including terpenes, fatty acids and bile acids. They are chemically heterogeneous substances, readily soluble in non-polar solvents but insoluble in water. Lipids are classified according to their physical properties, polarity, human requirement and structure. Physically lipids are classified into liquids and solids. They are classified as essential and non-essential fatty acids according to human requirement and structurally they are divided into simple and complex lipids (Akoh and Min, 2008).

Simple lipids are esters of unsaturated fats with various alcohols, mainly fats and waxes. Compound lipids are simple lipids containing other groups along with an alcohol and a fatty acid. They are mainly phospholipids, glycolipids and other complex lipids. Lipids have additional functions in the body such as fat-soluble vitamins, coenzymes and steroid hormones. The deficiencies or imbalance of lipid metabolism leads to major clinical problems.

2.1.1 Fatty acids

Fatty acids (FAs) are carboxylic acids containing long chain hydrocarbon side groups. Fatty acids occur in the body as esters, but it is found to be free fatty acids in the plasma. Fatty acids are stored as triacylglycerols. They functioned as the

fuels for body by providing energy. Most fatty acids possess even number of carbon atoms since they are synthesized by C_2 units' concatenation. Alterations in the fatty acid metabolism are associated with obesity and diabetes. The role fatty acids in biological systems involve different mechanism of action. The roles and mechanisms of are outlined in Figure 2.1

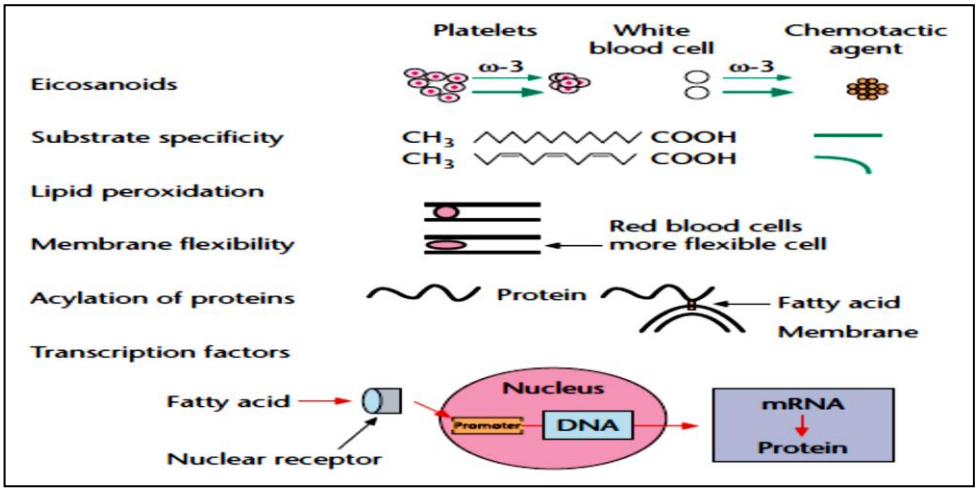


Figure 2.1: Scheme of mechanisms of action for fatty acids- adapted from Rustan and Drevon, (2005).

2.1.2. Phospholipids

Phospholipids made up of two fatty acids, a glycerol unit, a phosphate group and a water soluble molecule. They frequently have nitrogen-containing bases and substituent. They are regarded as the derivatives of phosphatidic acid. The phosphate group and water soluble head region of the molecule is hydrophillic while the fatty acid tail is hydrophobic. When placed in water, phospholipids will situate themselves into a bilayer in which the non-polar region faces the inner area. Phospholipids are the predominant cell membrane lipids. Membrane phospholipids functions as a storage depot for intracellular messengers where as non-membrane phospholipids are functions as lung surfactant and essential components of bile.

2.1.3. Triglycerides

Neutral fats are also called as triglyceride (TG) or triacylglycerol. It is classified under simple lipids. These comprise esters of, glycerol, trihydric alcohol with fatty acids. The old names like monoglyceride, diglyceride and triglyceride were corrected to monoacyl glycerol, diacyl glycerol and triacyl glycerol by International Union of Biochemistry. But the old popular names are still in use. Triacylglycerols are hydrophobic and insoluble in water. They include oils and fats. Triacylglycerols are stored in the form of lipids in adipose tissues. Triglycerides are hydrolysed by lipases to di and monoacylglycerol (Vasudevan and Sreekumari, 2006).

Elevated levels of triglycerides are considered as a cardiovascular risk factor. The mild to moderately elevated concentration (2–10 mmol/L) of triglyceride lipoproteins can be a reason for atherosclerosis. It is reported that the raised level of fasting and non-fasting triglycerides (hypertriglyceridemia) were directly associated with the coronary heart diseases like myocardial infarction, ischemic heart disease, ischemic stroke and mortality caused by all of these diseases (Nordestgaard and Varbo, 2014). The suggested role of increased triglyceride level in the development of atherosclerosis is depicted in Figure 2.2.

Triglyceridemia levels are classified as normal (less than 150 mg/dL; less than 1.70 mmol/L), borderline high (150 to 199 mg/dL; 1.71-2.25 mmol/L), high (200 to 499 mg/dL; 2.26-5.65 mmol/L), very high (greater than 500 mg/dL; greater than 5.66 mmol/L). The coagulability and viscosity of the blood is increased by hypertriglyceridemia and leads to atherothrombosis. Triglycerides have atherogenicity due to the rich presence of a polipoprotein C-III. These Apo C-III causes delay the breakdown of VLDL and block its plasma clearance.

Hypertriglyceridemia is usually connected with low HDL levels (Ducharme and Radhamma, 2008; Raza *et al*., 2004; de Graaf *et al*., 1991).

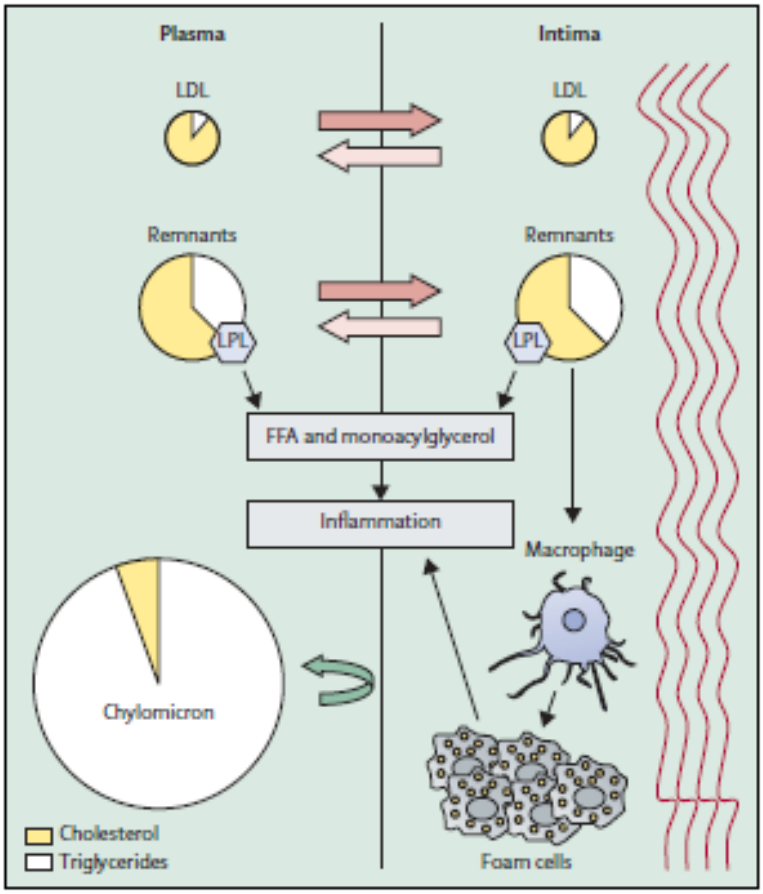
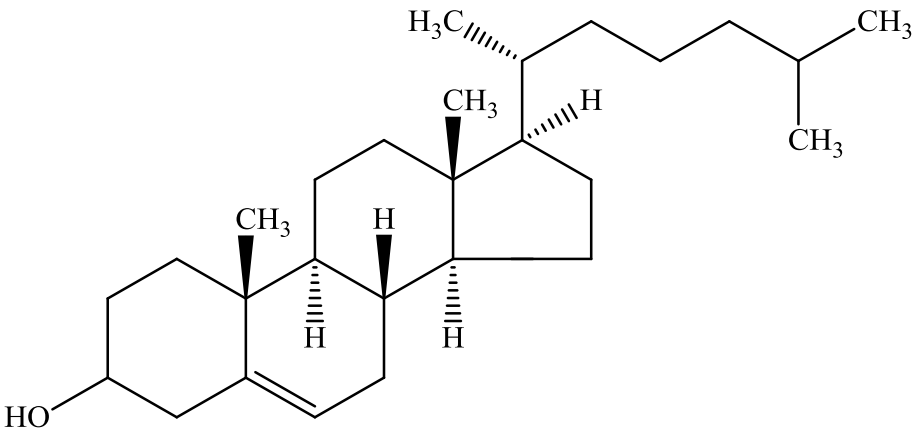


Figure 2.2 The suggested role of increased TG level in the development of atherosclerosis- adapted from Nordestgaard and Varbo, (2014).

FFA=free fatty acids, LPL=lipoprotein lipase. Triglycerides and remnant cholesterol could act through triglyceride hydrolysis and cholesterol accumulation in arterial wall foam cells leading to development of atherosclerosis.

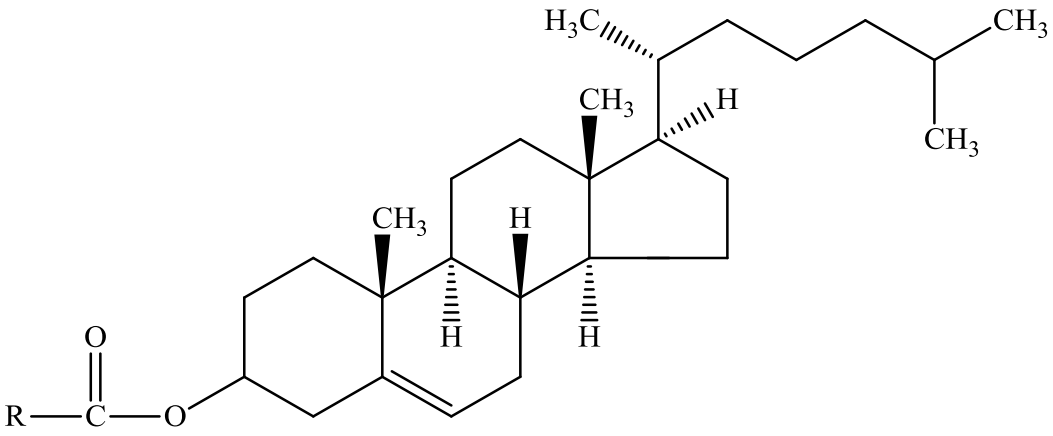
2.1.4 Cholesterol and cholesterol esters

The word cholesterol is originated from Greek words, chole-bile; steros-solid; ol-alcohol. Cholesterol is widely distributed and performs many essential functions in the body. It is the primary steroid from which other steroids are formed. The functions of the cholesterol are many and they include insulation of nerve fiber, component of membranes which maintain the fluidity, bile acids and salts are derived from cholesterol, source of steroid hormones and vitamin D etc. The imbalance between influx and efflux of cholesterol leads to its deposition in the tissue. The deposition on the lining of blood vessel leads to potentially life-threatening atherosclerosis (Harvey and Ferrier, 2011).



[1] Cholesterol

Cholesterol esters have long-chain fatty acids connected to the hydroxyl group. They are less polar when compared with free cholesterol. They are the transportation forms of cholesterol. Plasma cholesterols usually have high content of proteins due to the difference in synthesis. During membrane and lipoprotein formation, the cholesterol esters hydrolyzed to liberate cholesterol (Sebedio and Christie, 1998).



[2] Cholesterol ester

Increased total cholesterol level in plasma has a direct correlation with increased risk of CHD. The maintenance of cholesterol level in the desirable level is important. The desirable total cholesterol level is less than 5 mmol/L. A total cholesterol level of 5 to 6 mmol/L is borderline high, whereas a value of 6 mmol/L or more is high. The therapeutic decisions are mainly made based on the LDL and HDL level rather than TC level (Ducharme and Radhamma, 2008).

2.2 Lipoproteins

Cholesterol is mainly synthesized in the liver and it must be transported to the cells where it is needed. Cholesterol is a lipid and insoluble in blood plasma. Cholesterol requires a transport carrier which can shield the aqueous nature of blood plasma. Lipoproteins, complex of various proteins and lipids achieve the vascular cholesterol transport. They are different in size, shape, composition and function. The lipoproteins are made up of a core of water insoluble lipids surrounded by phosphatidylglycerols and proteins. The lipoproteins are classified according to their density, the relative amount of lipids to protein in the complex. The density of the lipoproteins increases as the protein content increases. Chylomicrons (lowest

density), VLDLs (Very low density lipoproteins), IDLs (Intermediate density lipoproteins), LDLs (Low density lipoproteins) and HDLs (High density lipoproteins) are the major lipoproteins help in the transport of cholesterol (Daniels *et al*., 2009).

Apoproteins (Figure 2.3) are the protein component present on the outer surface of lipoproteins. They function as receptors at cell surface and regulation of enzymes. Apoproteins usually dissociate from lipoproteins and bind with another except apoprotein B. Metabolism of apoproteins are important as any defect may disturb the lipid handling (Brunton *et al*., 2011).

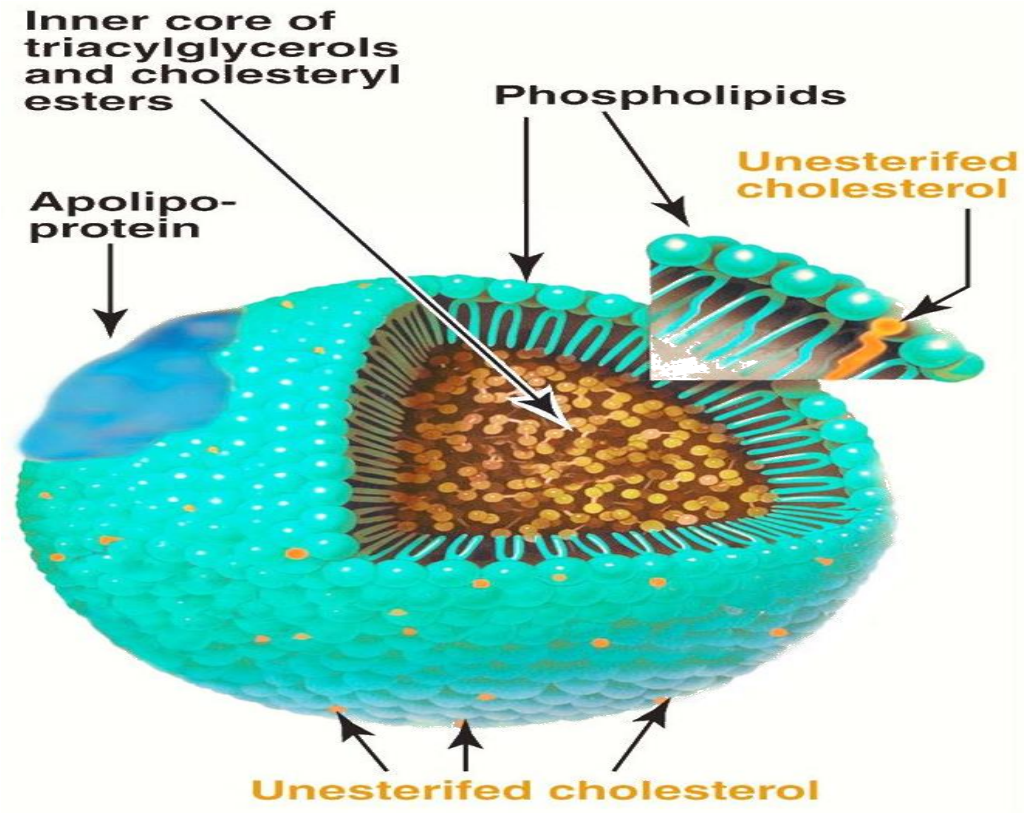


Figure 2.3: Typical structure of a lipoprotein- adapted from Harvey and Ferrier, (2011).

Physical properties and roles of major classes of lipoproteins (Voet and Voet, 2011) are summarized in Table 2.1

Table 2.1: Physical properties and roles of major classes of lipoproteins

	Chylomicrons	**VLDL**	**IDL**	**LDL**	**HDL**
Source	Synthesized from the fatty acids of dietary TGs and cholesterol by intestinal epithelial cells	Produced in the liver when TG production is stimulated by an increased flux or de novo synthesis of fatty acids	Formed during the transition of VLDL to LDL	Arise from the catabolism of IDL	Multiple mechanisms by which HDL particles are formed in blood, liver and intestine.
Density (g/cm^3)	<0.95	<1.006	1.006-1.019	1.019-1.063	1.063-1.210
Particle diameter (Å)	750-12000	300-800	250-350	180-250	50-120
Particle mass (kD)	400000	10000-80000	5000-10000	2300	175-360
Surface components					
%Protein	1.5-2.5	5-10	15-20	20-25	40-55
%Phospholipids	7-9	15-20	22	15-20	20-25
%Free cholesterol	1-3	5-10	8	7-10	3-4
Core lipids					
Triacylglycerols	84-89	50-65	22	7-10	3-5
Cholesterol esters	3-5	10-15	30	35-40	12
Major apolipoproteins	A-I, A-II, B-48, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100	A-I, A-II, C-I, C-II, C-III, D, E
Role	Transport exogenous TGs and TC from the intestine to tissues	A group of related particles. They transport endogenous TGs and TC from liver to tissues.			Transport endogenous TC from tissues to liver

2.3 Bile acids

Bile acids (BAs) contain 24 carbon atoms. They are synthesized in the liver from cholesterol. All of them have α hydroxyl and β methyl group so that they exhibit both polar and non-polar nature and act as emulsifying agents. In the intestine it helps the lipid degradation by pancreatic enzymes. Different types of bile acids were identified (Chenodeoxycholic acid, ursodeoxycholic acid, deoxycholic acid, hyocholic acid,β-muricholic acid and cholic acid) from bile of different mammalian groups. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are common to many species including human but some bile acid like ursodeoxycholic acid is specific to bear. The hamster bile acids are almost comparable with human (Agellon, 2002). Cholic acid and chenodeoxycholic acid are free bile acids while many others are conjugated bile acids. Bile acid acts as signaling molecules. They enter in the nucleolus and interact with nuclear receptors to stimulate or inhibit its own and its precursor, cholesterol production. Bile acids play important role in the cholesterol homeostasis (Stamp and Jenkins, 2008).

Apart from the emulsification and absorption of lipids, BAs have many roles. Bile acid-dependent bile flow, stimulation of bilary lipid secretion, emulsion formation of lipid soluble vitamins in the gut and their absorption, facilitate intestinal calcium absorption, modulation of pancreatic enzymes and cholecystokinin and prevention of small intestinal bacterial over growth are the important physiological roles of BAs (Monte *et al*., 2009).

Liver is the major site of BA synthesis. About 500 mg of cholesterol in an adult human is converted to bile acids every day. There are two main synthetic pathways, classical and alternative pathways accounted for the formation of BAs. The other minor pathways are relevant in some species. The classical pathway is also

called as neutral pathway due to the formation of neutral sterol intermediate metabolites. This route synthesizes two types of bile acids in human, CA and CDCA. The series of reactions in this route are catalyzed by enzymes in mitochondria, cytosol, peroxisomes and microsomes (Russell, 2003).

In the neutral pathway, about 12 enzymatic reactions are carried out for the conversion of water insoluble cholesterol to water soluble BAs. The cholesterol is first converted to 7-alpha-hydroxy cholesterol, followed by cascades of enzymatic reactions. The cytochrome P-450 enzyme, cholesterol 7 alpha hydroxylase (CYP 7A1), expressed in the liver is a rate-limiting enzyme of this conversion. The final step in BA synthesis is the amino acids glycine or taurine conjugation with terminal-side chain carboxylic acid. This step is mediated by bile acid CoA: amino acid N-acyltransferase (BAAT). The elaborated classical pathway is depicted in Figure 2.4.

In the alternative pathway, the intermediates formed are acidic in nature so the pathway is regarded as acidic pathway. The first step is the oxidation of cholesterol to 27-hydroxycholesterol by sterol 27-hydroxylase (CYP27A1), then it is converts into 7α, 27-dihydroxycholesterol by microsomal oxysterol 7α-hydroxylase (CYP7B1). The oxidized sterols must be transported to the liver to be converted to bile acids because the enzyme for that is available only in the liver. CDCA is the main bile acid formed in this pathway.

Alternative pathway may turn into the major bile acid biosynthetic pathway in patients with liver disorders (Agellon, 2002). The amount of bile acid in the body is closely regulated in liver and intestine to inhibit the accumulation thereby cytotoxicity (Hofmann, 1999).

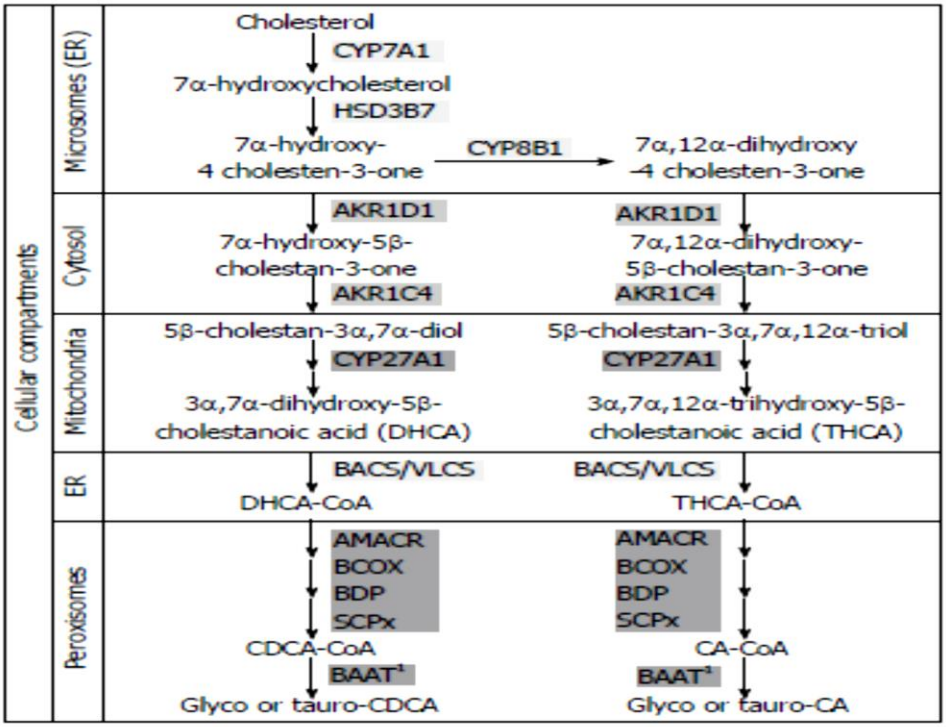


Figure 2.4: Scheme of classical pathway of bile acid synthesis- adapted from Monte *et al*., (2009).

AKR1C4: 3α-hydroxysteroid dehydrogenase; AKR1D1: Δ4–3-oxosteroid-5β-reductase; AMACR: Alpha methylacyl-CoA racemase; BAAT: Bile acid; CoA: Amino acid N-acyltransferase ([1]A minor cytosolic fraction does also exist); BACS: Bile acid CoA synthetase; BCOX: Branchedchain acyl CoA oxidase; BDP: D-bifunctional protein hydratase; CYP27A1: Sterol 27-hydroxylase; CYP7A1: Cholesterol 7α-hydroxylase; CYP8B1: Sterol 12α-hydroxylase; HSD3B7: 3β-hydroxy-Δ5-C27-steroid dehydrogenase/ isomerase; SCPx: Sterol carrier protein X; VLCS: Very long-chain acyl CoA synthetase; ER: Endoplasmic reticulum.

2.4 Biosynthesis of cholesterol

All carbon atoms of cholesterol are derived from acetyl CoA. Liver, adrenal cortex, testis, ovaries and intestine are the major sites of cholesterol synthesis. All tissues in human body synthesize cholesterol. The biosynthetic pathway was depicted by Sir John Cornforth and Vladimir Prelog and they were awarded with Nobel prizes in 1975. All nucleated cells can synthesize cholesterol and the enzymes involved in these reactions are located in endoplasmic reticulum as well as in cytoplasm.

The reaction starts with the condensation of two molecules of acetyl CoA mediated by cytoplasmic acetoacetyl CoA synthase to form acetoacetyl CoA. One more acetyl CoA molecule condenses to the acetoacetyl CoA and form HMG CoA by HMG CoA synthase. HMG CoA is present in the cytosol and mitochondria of the liver. The cytosolic HMG CoA pool is utilized to synthesis cholesterol. HMG CoA reductase reduces the HMG CoA to mevalonate, this step is unique to cholesterol synthesis where- as the two previous steps are common to ketogenic pathway. The reduction of HMG CoA to mevelonate is a rate limiting step. Mevelonate is phosphorylated and finally converted to 3-phospho 5-pyrophospho-mevelonate (mevelonate 5-PP). Decarboxylation of mevelonate 5-PP is to produce isopentyl pyrophosphate. All these steps are considered as the primary phase of cholesterol synthesis. The 5-carbon unit formed in the first phase is then condensed to give a 30 carbon compound called squalene. These condensation reactions are catalyzed by many enzymes. Then squalene undergoes oxidation by epoxidase to form squalene epoxide and then it is converted to lanosterol by cyclase enzyme. The additional methyl groups are removed to form zymosterol. Double bond rearrangement leads to the formation of desmosterol. Finally, the double bond in the side chain is reduced by NADPH and the cholesterol is formed (Vasudevan and Sreekumari, 2006; Liscum, 2002). The reaction summary of the cholesterol biosynthesis is given in Figure 2.5.

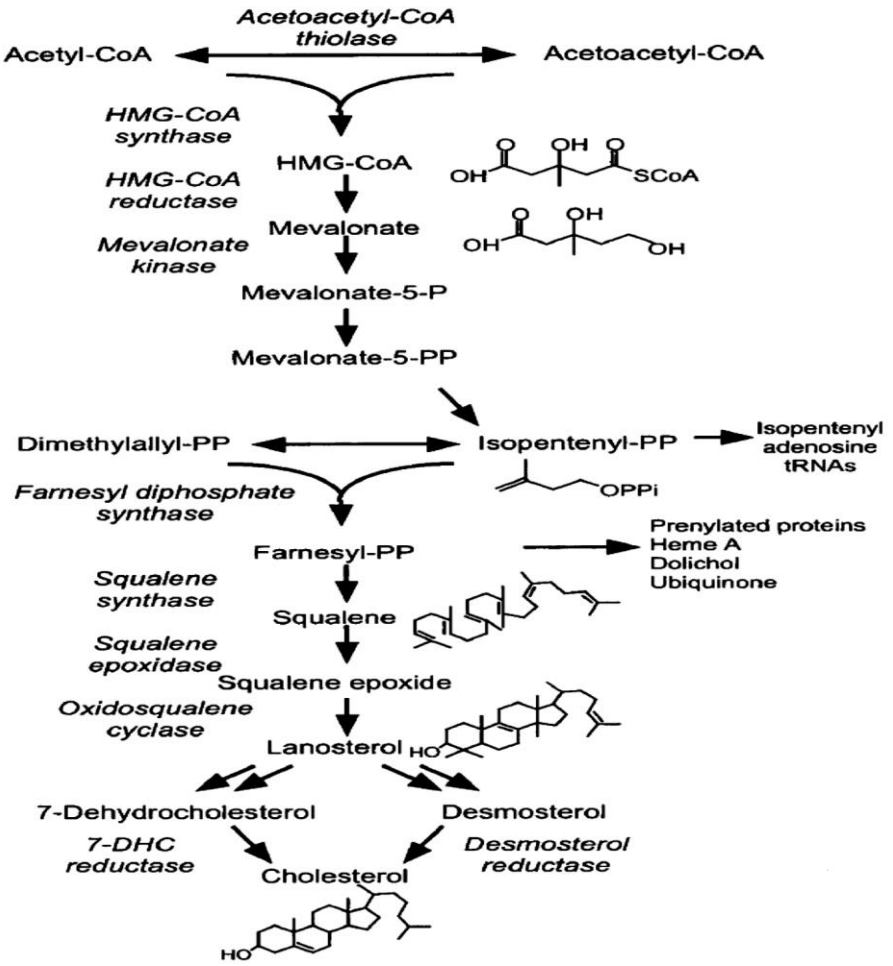


Figure 2.5: The cholesterol biosynthetic pathway, major intermediates and end-products are indicated-adapted from Olivier and Krisans, (2000).

HMG; 3-hydroxy-3-methylglutaryl, DHC; dehydrocholesterol

2.5 Digestion and absorption of lipids and cholesterol

Natural fats, especially triglycerides are the major source of dietary lipids. Diet also contains minute quantities of cholesterol, phospholipids and cholesterol esters. The absorption of triglycerides is started in the stomach by lingual lipase, an enzyme secreted by Ebner's gland on the dorsal surface of the tongue. This metabolism is insignificant. The lingual lipase and gastric lipase along with the gastric motility and retropulsion the gastric content become a crude emulsion called as chime (Wilson and Rudel, 1994). Chyme is delivered to duodenum in small

quantities. Most of the fats are started to get digest in the duodenum. The pancreatic juice contains hydrolytic enzyme and bile contains bile salts. Together they contribute to the fat digestion. Pancreatic lipase is the most important enzyme along with phospholipase A_2 and cholesterol esterase, involved in the duodenal fat digestion. Colipase is a pancreatic enzyme which was activated by trypsin and helps in the restoration of pancreatic lipase. The insoluble fat molecules are restricted to pass through the luminal membrane. They are well emulsified by the surfactant action of bile acids, lecithin and monoglycerides. Lipids and bile salts interact to form micelles. Formation of micelles facilitates the transport of fats across membrane. Then the lipids diffuse out of the micelles to the blood stream (Senior, 1964). The presence of bile, fatty acids and pancreatic juice helps in the ready absorption of cholesterol from small intestine. The absorbed cholesterol is incorporated with chylomicrons and enters in to the lymphatics.

2.6 Role of bile acids on cholesterol metabolism

Regulation of cholesterol homeostasis and facilitation of small intestinal lipid digestion and uptake are the well-known functions of bile acids. Bile acids also regulate the synthesis and enterohepatic circulation of triglyceride, cholesterol and glucose. Cholesterol catabolism is mainly mediated by bile acids and they account about 50% of daily cholesterol turnover. The extent of bile acid excretion is believed to be the direct indication of cholesterol excretion but this hypothesis was discredited as 95% BA is reabsorbed in the ileum and through portal circulation transported back to liver. The remaining 5% BA is excreted and that deficiency is replenished by *de novo* synthesis in the liver. Excess cholesterol removal is not the main function of BA production in the body (Insull Jr, 2006; Lefebvre *et al*., 2009). Many

physiological functions of BA associated with cholesterol have been identified. Elimination of cholesterol is one of the most important among them. BAs convert cholesterol to bile acids, they increase the solubilization of cholesterol in bile and transport to ileum from liver cell, and then it is excreted through fecal route. Next main function is the transport of cholesterol from intestine as micelles. They diffused through the unstirred layer of mucosa, so the absorption of cholesterol is increased. The other function is the negative feedback regulation of cholesterol biosynthesis. The concentration of bile acid is a signal, when then concentration is low its hepatic synthesis will be increased. Cholesterol synthesis is also increased parallel to this (Vlahcevic *et al*., 1991).

2.7 Excretion of cholesterol

The source of cholesterol in the body is from diet and synthesized *de novo*. About 300 mg of cholesterol is obtained from the diet normally whereas 700 mg is synthesized in the body itself. About 500 mg of cholesterol is excreted through bile but partly reabsorbed from the intestines. The unabsorbed portion interacts with intestinal flora and form cholestanol and coprostanol (fecal sterols). The remaining 500 mg of cholesterol is converted to bile acids and then excreted as bile salts (Vasudevan and Sreekumari, 2006).

2.8 Cholesterol metabolism

The exogenous, endogenous pathways and reverse cholesterol transport are responsible for the generation and movement of cholesterol in human body.

2.8.1 Exogenous pathway

The dietary fats are digested to form cholesterol and fatty acids along with bile. This mixture is absorbed to intestinal mucosa and they are re-esterified to form cholesterol esters and triglycerides. These combined with phospholipids and apoA and apoB and released in to lymph as chylomicrons. When the chylomicron enters in to the circulation, apolipoprotein C and apoE are combine with it in lymph and plasma. The lipoprotein lipase located on the capillary walls hydrolyzes triglycerides to fatty acids and glycerol. Some components of chylomicrons are reattached with lipoproteins where as other remnants are cleared by chylomicron remnant receptors present in the liver. In the liver cells the cholesterol is utilized and apolipoproteins are catabolized. Ultimately the exogenous pathway delivers triglycerides to adipose tissue and muscle while cholesterol to liver (Crook, 2012; Ducharme and Radhamma, 2008).

2.8.2 Endogenous pathway

Liver is the major source of endogenous lipids in the body. The endogenous pathway of lipid metabolism involves the synthesis of lipids in the liver and they are transported by lipoproteins. Triglycerides are synthesized from fatty acids, glycerol from glucose and cholesterol from chylomicron remnants via exogenous pathway or synthesized locally. From the liver, these lipids are transported by VLDL.

VLDL is a large triglyceride with apoproteins. During circulation it adds apoC from HDL. Lipoprotein lipase hydrolyzes VLDL in the peripheral tissues. This leads to the formation of IDL or hepatic uptake, later converted to LDL by the action of hepatic liapse. LDL is rich in cholesterol and containing only apoB. 70% of the total plasma concentration is represented by LDL. In the cell, LDL is broken down

and release cholesterol by lysosomes. This cholesterol is utilized to synthesis steroid. Almost all the plasma LDL is removed by LDL receptors. If the LDL concentration is excessive then it can infiltrate tissues and cause damage. Removal of reticuloendothelial system is an alternative method to remove oxidized LDL, called as scavenger cell pathway. Reduced synthesis of LDL receptors in the cells activates the enzyme acylcoenzymeA cholesterol acyltransferase (ACAT), so the free cholesterol is esterified into cholesterol ester, storage form of cholesterol in the cell (Crook, 2012; Ducharme and Radhamma, 2008; Daniels *et al*., 2009).

The dietary intake of saturated fat rich food (egg yolk, red meat and dairy products) influences the endogenous pathway. High saturated fat can suppress the LDL receptors there by alter the cholesterol metabolism. Transport of exogenous cholesterol and endogenous pathway of cholesterol synthesis is illustrated in Figure 2.6.

2.8.3 Reverse cholesterol transport

Reverse cholesterol transport is the process of transport of non-hepatic cell cholesterol to liver involving HDL. HDL has an important role in the reverse transport of cholesterol. The HDL is synthesized from liver as well as intestine. They secrete HDL, which is rich in free cholesterol, phospholipids, apoA and apoE. HDL also carries apoC when the VLDL or chylomicron levels are low.

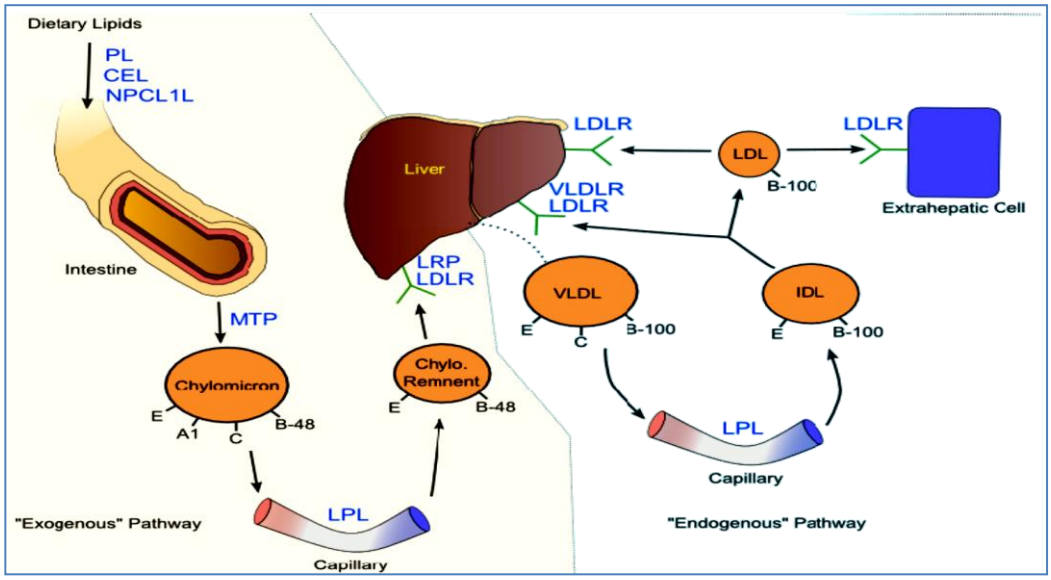


Figure 2.6: Transport of exogenous cholesterol and endogenous pathway of cholesterol synthesis- adapted from Daniels *et al*., (2009).

HDL also can be formed from the surface caot of VLDL and chylomicrons. The esterification of free cholesterol is catalyzed by an enzyme lecithin-cholesterol acyltransferase (LCAT) present on HDL. LCAT is activated by apoA_1. Most of these esterified cholesterol is transferred to LDL, VLDL and chylomicron remnants there by reaches the liver. Some cholesterol may be stored in core HDL particle and take directly to the liver. Apart from the removal cholesterol from cells, HDL also has other functions. HDL contains enzymes such as paroxanase, which may possess antioxidant capacity. Increased atherosclerotic plaque stability, protection of LDL from oxidation and maintaining the vascular integrity properties are exhibited by HDL (Crook, 2012; Ducharme and Radhamma, 2008; Groen *et al*., 2014). The reverse cholesterol pathway is depicted in Figure 2.7.