ANTIHYPERLIPIDAEMIC AND ANTIOXIDANT ACTIVITIES OF EXTRACTS OF DIFFERENT PARTS OF *AVERRHOA CARAMBOLA* AND ELUCIDATION OF THEIR MECHANISMS OF ACTION

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By

SULTAN AYESH MOHAMMED SAGHIR

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DEDICATION

In the name of ALLAH, The Most Gracious, The

Most Merciful

THIS THESIS IS DEDICATED

TO

MY MOTHER AND FATHER FOR DOING THEIR BEST

TO EDUCATE ME,

MY WIFE EMAN AND MY DAUGHTERS MANAL, HUDA, DUA`A AND

ALAA FOR THEIR PATIENCE, UNDERSTANDING, LOVE, AND

SINCERITY

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LIST OF SYMBOLS

α	Alpha
γ	Gamma
β	Beta
<	Less than
>	More than
µ	Micro
n	Nano
$^{\circ}$C	Celsius
g	Gram

LIST OF ABBREVIATIONS

4MUO	4-methyl umbelliferoneo
ABC	ATP-binding cassette
ABTS	2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
ACAT	Acyl-coenzyme A: cholesterol acyltransferase
AI	Atherogenic index
ARASC	Animal Research and Service Centre
BA	Bile acids
BMI	Body mass index
BSA	Bovine serum albumin
BW	Body weight
CA	Cholic acid
CAT	Catalase
CDCA	Chenodeoxy cholic acid
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CMC	Carboxymethylcellulose
CRI	Coronary risk index
CVD	Cardiovascular disease
CYP	Cytochrome P450
CYP7A1	Cytochrom P450 for cholesterol 7α-hydroxylase
CYP27A1	Cytochrom P450 sterol 27-hydroxylase
CV	Coefficient of variation

DAD	Diode array detector
DMSO	Dimethyl sulfoxide
DPPH	2, 2’-Diphenyl-1-picrylhydrazyl
DTNB	5, 5-dithio-bis-nitrobenzoic acid
FAs	Fatty acids
FFAs	Free fatty acids
FBS	Fetal bovine serum
G6PD	Glucose -6 -phosphate dehydrogenase
GAE	Gallic acid equivalent
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
GSSG	Oxidised glutathione
HAT	Hydrogen atom transfer
HDL-C	High- density lipoprotein cholesterol
HFD	High-fat diet
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A reductase
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
LCAT	Lecithin cholesterol acyl transferase
LDL-C	Low- density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptors
LPL	Lipoprotein lipase
LPO	Lipid peroxidation
MAG	Monoacylglycerol

MCAs	Muricholic acids
MDA	Malondialdehyde
mg	Milligram
µg/mL	Microgram/millilitre
µL	Microliter
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy- phenyl)-2-(4- sulfophenyl)-2H tetrazolium salt
NPC1L1	Niemann-Pick C1-like 1 protein
OECD	Organization for Economic Cooperation and Development
P-407	Poloxamer- 407
PBS	Phosphate buffer saline
PC	Phosphocholesterol
PGDH	Phospho gluconate dehydrogenase
PBMC	peripheral blood mononuclear cells
PL	Pancreatic lipase
PLs	Phospholipids
PO	Per oral
PPAR-α	Peroxisome proliferator-activated receptor
PPLA2	Pancreatic phospholipase A2
PTFE	Polytetrafluoroethylene
RPMI	Roswell Park Memorial Institute
RBCs	Red blood cells
ROS	Reactive oxygen species
RNS	Reactive nitrogen species

RSD	Relative standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error mean
SET	Single electron transfer
SOD	Superoxide dismutase
SR-B1	Scavenger receptor class B1
STZ	Streptozotocin
TC	Total cholesterol
TBARS	Thiobarbituric acid reactive substances
TE	Trolox equivalent
TFC	Total flavonoid content
TG	Triglycerides
TP	Total protein
TPC	Total phenolic content
TPTZ	2, 4, 6-Tri (2- pyridyl)-s-triazine
TWR-1339	Triton-WR- 1339
T X-100	Triton X-100
VLDL-C	Very Low- density lipoprotein cholesterol

AKTIVITI ANTIHIPERLIPIDEMIK DAN ANTIOKSIDAN EKSTRAK BAHAGIAN BERBEZA BAGI *AVERRHOA CARAMBOLA* DAN ELUSIDASI MEKANISME TINDAKANNYA

ABSTRAK

Averrhoa carambola, biasanya dikenali sebagai belimbing merupakan salah satu herba yang digunakan secara meluas dalam perubatan tradisional masyarakat Malaysia, daun dan buahnya merupakan bahagian yang paling banyak digunakan. Kajian ini bertujuan menyiasat kesan antihiperlipidemik, aktiviti anti-oksidan dan toksisiti ekstrak metanol dan akueus bahagian yang berlainan daripada *A. carambola* dengan tumpuan untuk elusidasi mekanisme tindakannya. Daripada semua ekstrak yang diuji, ekstrak metanol bahagian daun *A. carambola* menunjukkan aktiviti antihiperlipidemik terbaik dalam model tikus hiperlipidemik akut teraruh oleh poloxamer-407 berbanding kawalan hiperlipidemik yang setanding dengan aktiviti atorvastatin. Berikutan pemberian kronik sehingga lima minggu, tiada penurunan signifikan diperhatikan dalam aras parameter lipid bagi tikus normal yang dirawat dengan 1000 mg/kg ekstrak metanol daun. Sebaliknya, perbezaan yang signifikan diperhatikan dalam parameter lipid tikus hiperlipidemik teraruh diet tinggi lemak selepas dirawat dengan 500 dan 1000 mg/kg ekstrak metanol daun berbanding kawalan normal. Hasil kajian ini mencadangkan ekstrak metanol daun tersebut bertindak sebagai agen antihiperlipidemik dan bukan sebagai agen hipolipidemik. Selepas proses pemeringkatan, ujian menggunakan tikus hiperlipidemik akut teraruh oleh poloxamer-407 menunjukkan fraksi etil asetat bagi ekstrak methanol daun *A. carambola* mempamerkan kesan paling poten dalam penurunan semua parameter

lipid kecuali meningkatkan aras HDL-C. Bagi penilaian antioksidan, ekstrak metanol daun dan batang *A. carambola* menunjukkan aktiviti antioksidan paling tinggi. Kandungan fenolik dan flavonoid total bagi ekstrak *A. carambola* menunjukkan korelasi yang kuat dengan aktiviti antioksidan, tetapi tiada korelasi diperhatikan dengan kesan antihiperlipidemianya. Ekstrak metanol daun dan fraksi etil asetatnya menunjukkan kesan perencatan bergantungan dos ke atas enzim HMG-CoA reduktase pada kepekatan 5 dan 10 mg/mL, manakala kesan perencatan yang lemah dikesan pada enzim lipase pankreas *in vitro*. Tambahan lagi, ekstrak metanol daun meningkatkan aras enzim antioksidan *in vivo* secara signifikan dan menurunkan aras peroksidasi lipid dalam sampel serum dan homogenat hepar secara bergantungan dos. Selain itu, ekstrak metanol daun yang diberikan kepada tikus diet tinggi lemak pada dos 500 dan 1000 mg/kg menunjukkan keberkesanan dalam menurunkan penghasilan kolesterol dan trigliserida di dalam hepar dan meningkatkan perkumuhan kolesterol dan asid hempedu di dalam tinja. Penyiasatan menggunakan empat titisan sel kanser (K-562, HL-60, kasumi-1 dan HCT-116) mendapati kesemua ekstrak *A. carambola* tidak menunjukkan kesan sitotoksik. Kajian toksisiti akut dan sub-kronik menunjukkan ekstrak tersebut adalah selamat dan tiada perubahan signifikan diperhatikan bagi kedua-dua parameter biokimia dan hematologi dalam tikus rawatan berbanding kumpulan kawalan. Secara keseluruhannya, kajian ini mencadangkan ekstrak metanol daun *A. carambola* mempunyai kesan penurunan lipid yang boleh dibangunkan selanjutnya sebagai agen antihiperlipidemik.

ANTIHYPERLIPIDAEMIC AND ANTIOXIDANT ACTIVITIES OF EXTRACTS OF DIFFERENT PARTS OF *AVERRHOA CARAMBOLA* AND ELUCIDATION OF THEIR MECHANISMS OF ACTION

ABSTRACT

Averrhoa carambola, commonly known as star fruit is one of the widely used herbs in the Malaysian traditional medicine, with the leaf and fruits being the most utilized parts. This study aims to investigate the antihyperlipidaemic effect, antioxidant activity and toxicity of methanolic and aqueous extracts of different parts of *A. carambola* with focus on elucidating the underlying mechanism of action. Of the tested extracts, the methanolic extract of *A. carambola* leaf showed the most potent antihyperlipidaemic activity in poloxamer-407-induced acute hyperlipidaemic rat model compared to the hyperlipidaemic control, which was comparable with that of atorvastatin. Upon chronic administration up to five weeks, no significant decrease was observed in the levels of the lipid parameters of normal rats treated with 1000 mg/kg of methanolic extract of leaf. In contrast, significant changes were observed in lipid parameters of high-fat diet induced hyperlipidemic rats after treated with 500 and 1000 mg/kg leaf methanolic extract as compared with the hyperlipidaemic control. These findings thus suggest that methanolic extract of *A. carambola* leaf works as an antihyperlipidaemic rather than a hypolipidaemic agent. Following fractionation, assessment using poloxamer-407 induced acute hyperlipidaemic rats showed that the ethyl acetate fraction of methanolic extract of *A. carambola* leaf exhibits the most potent significant effect in terms of reducing all lipid parameters except increasing high density lipoprotein cholesterol (HDL-C)

levels. For antioxidant evaluation, methanolic extract of *A. carambola* stem and leaf showed the highest antioxidant activity. The total phenolic and flavonoid contents of *A. carambola* extracts showed strong correlation with their antioxidant activities, but no correlation was found with their antihyperlipidaemic effects. Methanolic extract of leaf and its ethyl acetate fraction produced dose-dependent inhibitory effects on HMG-CoA reductase at 5 and 10 mg/mL concentrations, while weak inhibitory effect was detected on pancreatic lipase *in vitro*. In addition, methanolic extract of the leaf significantly increased the *in vivo* antioxidant enzymes levels and decreased the lipid peroxidation in liver homogenates and serum samples in a dose-dependent manner. On the other hand, methanolic extract of leaf given to high fat-diet rats at the doses of 500 and 1000 mg/kg was effective in reducing the synthesis of cholesterol and triglycerides in the liver and increasing the excretion of cholesterol and bile acids in faeces. An investigation using four cancer cell lines (K-562, HL-60, kasumi-1 and HCT-116) revealed that none of *A. carambola* extracts had cytotoxic effects. Acute and sub-chronic toxicity study of methanolic extract of *A. carambola* leaf showed that the extract was safe and no significant changes was observed in both biochemical and haematological parameters in treated rats compared with control group. Overall, this study suggests that the methanolic extract of *A. carambola* leaf has lipids lowering effect that could be further developed as an antihyperlipidaemic agent.

CHAPTER 1

INTRODUCTION

1.1 Background

Cardiovascular diseases (CVDs) are responsible for the highest burden of disease globally (Merriel et al., 2014). They are the leading causes of death, morbidity and health expenses in developed and developing countries accounting around 30 % of the annual global mortality and 10 % of worldwide health burden (Deales et al., 2013; Nair and Wang, 2013). Despite of having several therapeutic measures, focus has now been given for establishing effective preventive strategy for detecting and controlling of cardiovascular risk factors (O'Donnell and Elosua, 2008; Valdés et al., 2014).

Cardiovascular risk factors include a set of plasma lipids such as triglycerides (TG), total cholesterol (TC), very low density lipoprotein-cholesterol (VLDL-C), low density lipoprotein-cholesterol (LDL-C) and anti-atherogenic or high density lipoprotein-cholesterol (HDL-C) (Alzaid et al., 2014; Nelson, 2013). Dyslipidaemia is a highly heterogeneous class of metabolic disorders which is characterized by abnormalities in serum levels of various lipoproteins. The abnormalities of lipoproteins include elevation in TC, LDL-C and TG along with reduction in HDL-C. It is a powerful risk factor for coronary heart disease (CHD) (Cahalin et al., 2013; Pratt et al., 2014). Etiologically, dyslipidaemia relies on specific metabolic backgrounds such as insulin resistance, thyroid dysfunction and defects in the gastrointestinal absorption of cholesterol and lipids, as well as mutations in cell

surface receptors and enzymes (Yadav et al., 2014). Additionally, dyslipidaemia could occur because of suboptimal diet, obesity, inactive life style, genetic deviations and metabolism abnormalities (Xu et al., 2014).

An increase in plasma lipids concentrations (TC, TG, LDL-C, and VLDL-C) or decreased in HDL-C levels beyond certain level give rise to physiological condition known as hyperlipidaemia which is the widest form of dyslipidaemia worldwide. It has also been reported to be the most widespread marker for susceptibility to atherosclerotic heart disease (Chen et al., 2014). Oxidative modification of LDL-C, protein glycation, glucose-auto-oxidation with production of free radicals and lipid peroxidation products are the main factors responsible for ischemic heart diseases which occurs as a result of hyperlipidaemia (Yang et al., 2008).

High levels of plasma lipids, mainly cholesterol, are a common feature of atherosclerosis, a condition in which arterial damage can lead to ischemic heart disease, myocardial infarction and cerebrovascular coincidences (Prasad et al., 2012). Hypercholesterolaemia and hypertriglyceridaemia are important risk factors, either alone or together. It was found that they are extensively contributing in the acceleration of the manifestation and development of coronary heart disease as well as the progression of atherosclerosis (Cahalin et al., 2013; Merriel et al., 2014).

Accumulation of high levels of LDL-C in the extracellular sub-endothelial space of arteries is highly atherogenic and toxic to vascular cells which may lead to atherosclerosis, hypertension, obesity, diabetes and functional depression in some organs (Catapano et al., 2000; Jain et al., 2010). Several studies documented that

there is an obvious correlation between high cholesterol level in serum and cardiovascular disease (Bays et al., 2001). According to the American Heart Association report in 2004, heart disease and stroke will become the leading cause of death and disability worldwide. It is estimated that, by 2030, more than 24 million per year will suffer from the cardiovascular problems (Reinhardt, 2005). Globally, each year approximately 12 million people die due to cardiovascular diseases. Factors such as diet high in saturated fats and cholesterol, age, family history, hypertension and life style are of great significance but high level of cholesterol, particularly LDL-C is mainly responsible for the occurrence of CHD (Farias et al., 1996).

1.2 Therapeutic challenges

Hyperlipidaemia has risen to the top in terms of causes of death in both developed and developing countries (Sunil et al., 2012). In Malaysia, the prevalence rate of hypercholesterolaemia accounts about 35.1 % (6.2 million) of adults (18 years and above) in which 8.4 % are known to have hypercholesterolaemia and 26.6 % are previously undiagnosed with hypercholesterolaemia (NHMS, 2011). There are various classes of synthetic lipid lowering agents used in current therapy belonging to the statins, fibrates or bile acid sequestrants groups. Although, they possess beneficial therapeutic effects, they are often associated with some serious side effects such as rhabdomyolysis, myopathy, elevation of hepatic enzyme levels and an increasing risk of gallstones (Javed et al., 2006; Laurance and Bennett, 1992; Shin et al., 2014).Thus, there is an exigent need for new lipid lowering agents with high therapeutic value and minimum tolerable side effects (Sefi et al., 2010; Shin et al., 2014).

1.3 Problem statements

Previously, a study among local plants indicated that different insoluble fibers prepared from *Averrhoa carambola* fruits have potential antihypocholesterolaemic activity (Wu et al., 2009). In addition, another study investigated the *in vivo* effect of micronized insoluble fiber and fiber-rich fraction from star fruit on lipids metabolism in a murine model (Herman-Lara et al., 2014).

However, to date there is neither detailed investigation on the lipid lowering effects of *A. carambola* nor report on the antihyperlipidaemic effect of other parts of *A. carambola*. This has created an interest to work on various parts of *A. carambola* to evaluate their antihyperlipidaemic effects and to further investigate the mechanism of action and toxicity.

1.4 Objectives

The objectives of the present study are:

i. to evaluate the antihyperlipidaemic effects of methanolic and aqueous extracts of different parts of *A. carambola* and the fractions of the most active extract in chemically-induced acute hyperlipidaemic rats model

ii. to evaluate the antihyperlipidaemic effect of the most active extract of *A. carambola* in diet-induced chronic hyperlipidaemic rats model

iii. to evaluate the antioxidant activity of methanolic and aqueous extract of different parts of *A. carambola* and the fractions of most active extract

iv. to elucidate the mechanism of antihyperlidaemic effect of the most active extract of *A. carambola* and its bioactive fraction on

   a. inhibition of enzyme involved in lipids synthesis

b. lipids and bile acids absorption and excretion

c. *in vivo* antioxidant and lipid peroxidation

v. to investigate the toxicity of the most active extract of *A. carambola*

vi. to standardize the most active extract of *A. carambola* using selected marker compound

The research scheme is presented in figure 1.1.

1.5 Flow chart of the study

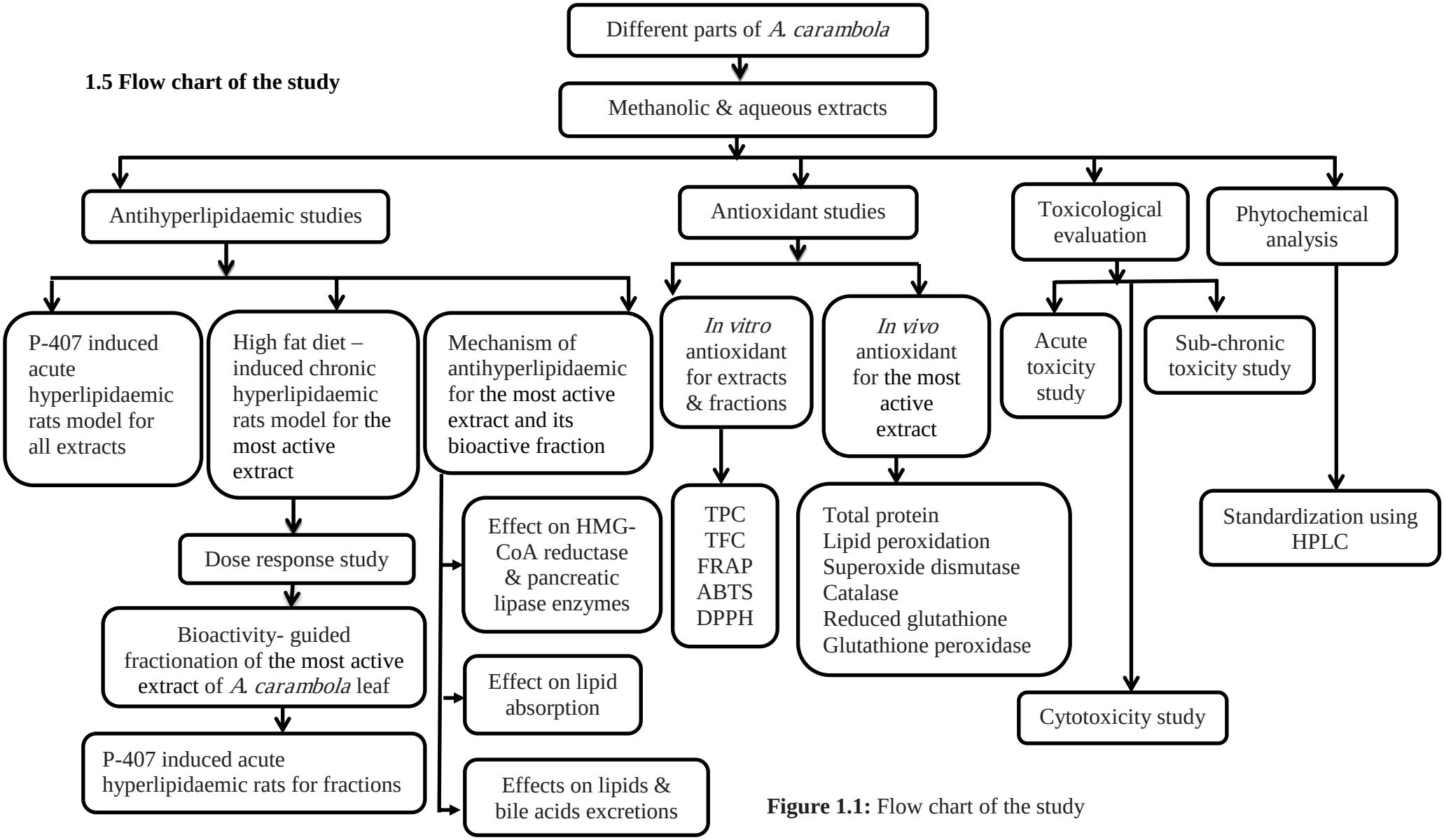


Figure 1.1: Flow chart of the study

CHAPTER 2

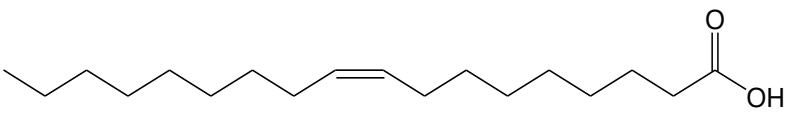
LITERATURE REVIEW

2.1 Lipids

The term ‘‘Lipid’’ is imitative from ‘‘lipos’’, which refers to animal fat or vegetable oil. Adiposity is derived from ‘‘adipo’’ that denotes to body fat (Driskell, 2009). Utmost amounts of body lipids are stored in the adipocytes and adipose tissue including triglycerides and free cholesterol (Bays et al., 2013). The term lipids also refer to an entire class of fats and fat-like substances in the blood. The most essential lipids in the body include; fatty acids (FA), cholesterol, cholesterol esters, TGs and phospholipids (PLs).

2.1.1 Fatty acids

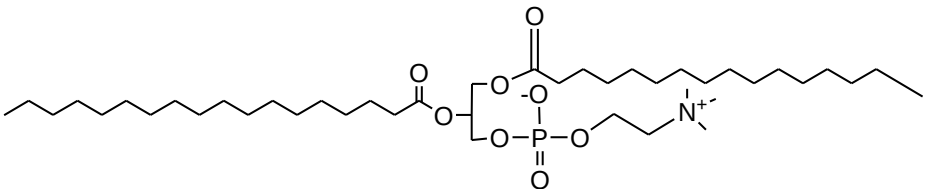
Fats are defined mainly as carboxylic acids (esters) with long hydrocarbon chains which are either saturated or unsaturated. Mostly, they are derived from triglycerides or phospholipids. They are named "free" fatty acids because of not attached to the other molecules. They represent an important source of energy because they yield large quantities of ATP when metabolized (Ibrahim et al., 2013).



1 Free fatty acid

2.1.2 Phospholipids

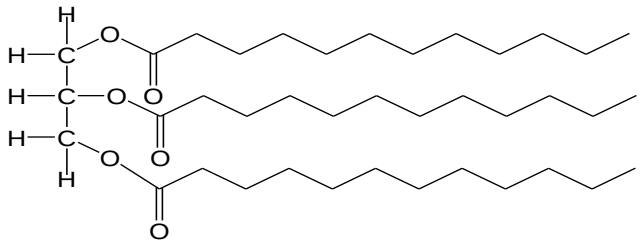
Phospholipids (PLs) resemble the TG with small different in which one fatty acid in TG is replaced by phosphate and a nitrogenous base (Ibrahim et al., 2013).



2 Phospholipids

2.1.3 Triglycerides

Triglycerides (TG) are esters consisting of a glycerol molecule attached to three fatty acid residues. It could be found in dietary fats and can be synthesized in the liver and adipose tissue (Phan and Tso, 2001). It offers a source of stored energy when it is required, especially in case of starvation. It is found in all plasma lipoproteins and are the major component of lipoproteins with density less than 1.019 kg/L (Rosenson et al., 2002). The ideal or normal value of TG is less than 150 mg/dL (1.69 mmol/L) and values between 150 to 199 mg/dL is considered at the borderline high, while a values from 200 to 499 mg/dL are high and above that considered very high (Ducharme and Radhamma, 2008, Raza et al., 2004). They are atherogenic because they are rich in apo C-III, which delays the lipolysis of VLDL and inhibits its uptake and clearance from plasma (Poirier et al., 2006).



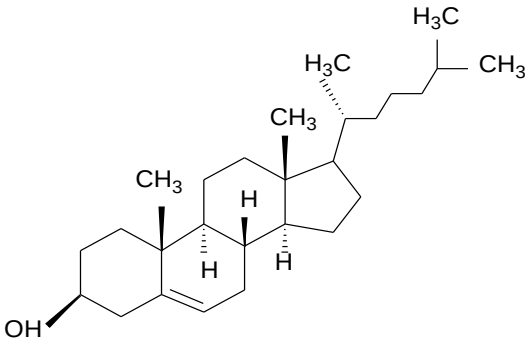
3 Triglycerides

2.1.4 Cholesterol and cholesterol esters

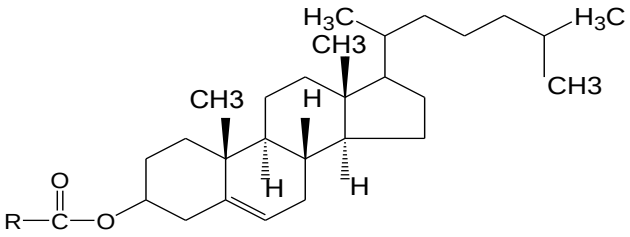
Cholesterol and cholesterol esters are important elements found in all human cell membranes. Cholesterol is an essential constituent of steroid hormones and bile acids. They could be synthesized in liver and many tissues as well as may be acquired from dietary fat. Their main functions are to build, repair cells and produce hormones such as oestrogen and testosterone (Rudel et al., 2005). In addition, they modulate cell membrane fluidity and work as a precursor of bile acids, which play an important role in the digestion of fats (Ahmed et al., 2009).

Cholesterol molecule is an amphipathic lipid, due to the presence of hydrophilic group (3β-hydroxyl group) attached to the hydrophobic part of the molecule. In addition to polarity, the 3β-hydroxyl reduces cholesterol ability to form esters (Pikuleva and Curcio, 2014). The desired value of TC is less than 200 mg/dL (5.17 mmol/L) and value between 200 to 239 mg/dL (5.17-6.18 mmol/L) is considered at the borderline high, while a value of 240 mg/dL(6.21 mmol/L) or more is high (Ducharme and Radhamma, 2008).

Cholesterol is stored in the cells in the form of cholesteryl esters (one cholesterol molecule bound to one fatty acid by an ester bond). Esterification is carried out by Acyl-CoA: cholesterol acyltransferase (ACAT) 1 and 2. ACAT 1 is universally expressed, while ACAT 2 is expressed only in enterocytes and hepatocytes. Esterification of cholesterol will produce a different shape molecule, which is greater in size and hydrophobicity (Lemaire-Ewing et al., 2012; Rudel et al., 2005).



4 Cholesterol



5 Cholesterol ester

Free cholesterol could be eliminated from the liver into the bile via the ATP-binding cassette (ABC) G5/G8 heterodimer. The cholesterol ring structure formed is highly stable and not easily metabolized (Parini et al., 2004). Cholesterol and other types of fats cannot dissolve in the blood. Thus, they have to be transported by attachment to specific molecules called lipoproteins in order to form macromolecular complexes (Abrass, 2004).

2.2 Lipoproteins

Lipoproteins are macromolecule complexes, which consist of spherical particles containing a hundreds of lipids and protein molecules. The main functions of lipoproteins is carrying and transporting the plasma lipids (Kanakavalli et al., 2014). There are five major lipoproteins; each one has its own function: chylomicrons, VLDLs, intermediate-density lipoproteins (IDLs), LDL-C and HDL-C (Kanakavalli

et al., 2014, Von Zychlinski et al., 2014). Table 2.1 demonstrates the physical properties of lipoproteins and their contents of apolipoproteins (Babin and Gibbons, 2009; Crook, 2012; Von Zychlinski et al., 2014). Apolipoproteins are known as protein components of the lipoproteins or apoproteins. They assist as cofactors for enzymes and ligands for receptors. Disturbances in lipid handling will occur if there is any defect happened in apolipoprotein metabolism (Ducharme and Radhamma, 2008).

Table 2.1: Physical properties of plasma lipoproteins

	Chylomicron	**VLDL-C**	**IDL-C**	**LDL-C**	**HDL-C**
Source	Gut	Liver	VLDL-C	VLDL-C via ILD-C	Gut /liver
Density g/mL	<0.95	0.95-1.006	1.006-1.019	1.063-1.09	1.063-1.21
Diameter nm	800-5000	300-800	250-350	180-280	50-120
Desired values (mg/dL)	Undetectable	<30 mg/dL	Undetectable	<130	>40
Borderline-high (mg/dL)	Undetectable	Undetectable	Undetectable	130-159	40-59
High (mg/dL)	Undetectable	Undetectable	Undetectable	>160	≥ 60
Functions	Transport exogenous TG & cholesterol from intestine to all cells and tissues	Transport endogenous TG & cholesterol from intestine to all cells and tissues	Formed during the conversion of VLDL-C to LDL-C	Formed from VLDLs, they carry cholesterol from liver to the cells and tissues	Transport endogenous cholesterol from the cells and tissues back to the liver (scavenger)
Apolipoproteins	A1,A4,B48, C1,C2,C3,E	B100,C1,C2, C3,E	B100,C1,C2, C3,E	Abo B100	A1,A2,A4, C1, C2,C3,E
Total lipid (%)	98 – 99	90 – 94	89	79	45 - 55
Protein	1	8	10	20	50
Cholesterol	4	25	32	55	20
TGs	90	55	25	5	5
PL	5	12	25	20	25

(Babin and Gibbons, 2009; Crook, 2012; Ducharme and Radhamma, 2008; Von Zychlinski et al., 2014)

2.3 Bile acids

Bile acids (BA) are functional compounds that simplify emulsification, absorption, and transportation of fats and sterols in the liver and intestine through formation of soluble mixed micelles with lipids (Ye et al., 2013). Daily, in the liver of the adult human about 500 mg of cholesterol is transformed into BA (Staels et al., 2010).

They are the cornerstones, which play vital role in maintenance of mammalian cholesterol homeostasis. The liver represents the unique source and site of BA formations (Cherrington et al., 2013).

Besides their role in lipid digestion, bile acids also denature dietary proteins, enhancing their rate of cleavage by pancreatic proteolytic enzymes. Bile acids also possess antimicrobial activity which remain poorly understood (Liu et al., 2013b). Synthesis of bile acids requires a group of enzymes belonging to the cytochrome P450 (CYP450) superfamily (Alrefai and Gill, 2007). BA biosynthesis involves modification of the ring structure of cholesterol, oxidation and shortening of the side chain and lastly conjugation with an amino acid (Zwicker and Agellon, 2013). These modifications are paramount to increase the polarity and consequently the solubility of these molecules (Hofmann et al., 2010).

Figure 2.1 illustrates the two main pathways of bile acids synthesis; the standard (classic) and the alternative pathway. The standard pathway is controlled by an enzyme encoded by cholesterol 7α-hydroxylase (CYP7A1) gene which is the rate limiting enzyme in bile acid synthesis. It catalyses and initiates the major pathway in cholesterol catabolism and bile acid synthesis (Alnouti et al., 2008, Ogawa et al., 2013, Pols et al., 2011).

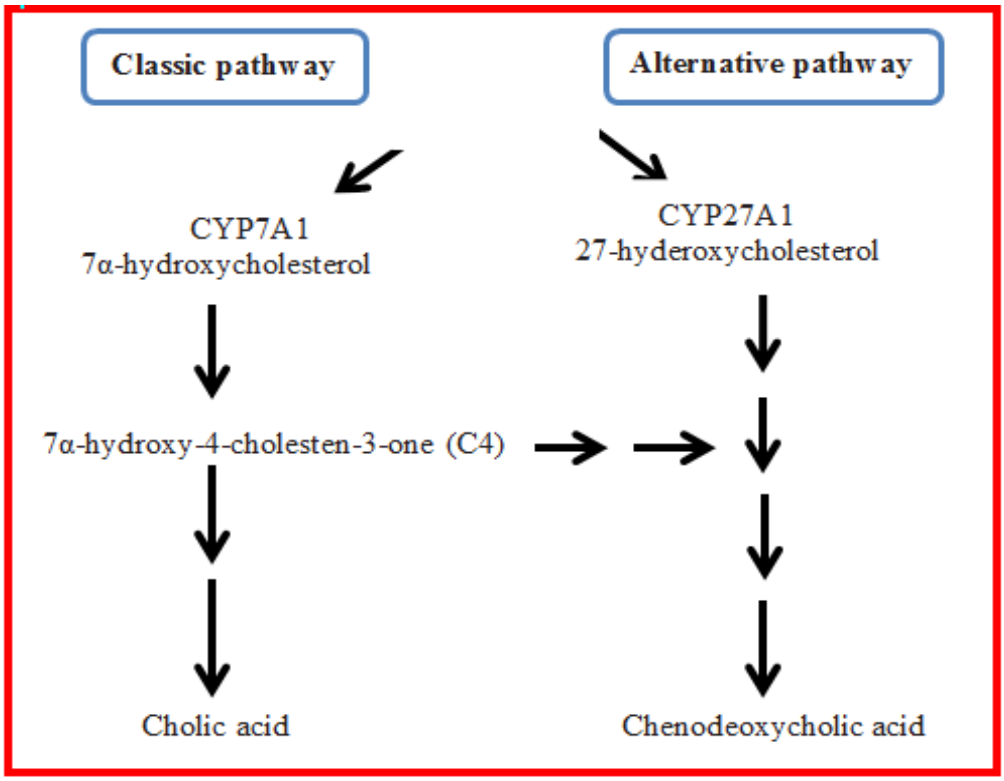


Figure 2.1: Simple outlines of the classic and alternative pathways in bile acids synthesis. (CYP7A1, cholesterol 7alpha-hydroxylase; CYP27A1, sterol 27-hydroxycholesterol) (Adapted from Thomas et al., 2008a).

The standard pathway initiates with hydroxylation of the 7α position in the cholesterol ring structure, which will end with the formation of either cholic acid (CA) or chenodeoxycholic acid (CDCA) (Hofmann, 2009). CA and CDCA constitute the two primary bile acids in humans. CA and CDCA share a common precursor; 7α-hydroxy-4-cholestene-3-one (C4), which can be hydroxylated in the C12 position by sterol 12α-hydroxylase (CYP8B1) to form CA. Otherwise, without 12α-hydroxylation, it is converted to CDCA (Lake et al., 2013). The alternate pathway is commenced by sterol 27-hydroxylase (CYP27A1) and results in formation of CDCA. Small changes appear in the bile acid synthesis in mice compared with human. In mice, most of the synthesized CDCA is converted into muricholic acids (MCAs) (Alnouti et al., 2008).

2.4 Cholesterol biosynthesis

Cholesterol is an essential constituent in the plasma membranes of the eukaryotic organisms and it acts as a precursor for the biosynthesis of some vitamins, steroid hormones and bile acids (Faust and Kovacs, 2014). The biosynthesis of cholesterol is initiated by the reaction of acetate with citrate to produce acetyl-coenzyme A (acetyl-CoA). Then, two molecules of acetyl-CoA under the action of thiolase forms acetoacetyl-CoA. Acetoacetyl-CoA is transformed to hydroxyl methylglutaryl-CoA (HMG-CoA) upon the effect of HMG-CoA synthase. The subsequent reduction of the thioester in HMG-CoA in the presence of HMG-CoA reductase produces mevalonate. HMG-CoA reductase enzyme is called the rate limiting enzyme and this step is called the rate-limiting step in cholesterol biosynthesis (Groen et al., 2014).

Decarboxylation, dehydration and double phosphorylation of mevalonate provide isopentenyl pyrophosphate. Condensation of six isoprenyl moieties of isopentenyl pyrophosphate results in the triterpene squalene. In cholesterol biosynthesis process, NADPH is used as a cofactor in all reduction reactions. Epoxidation of squalene to squalene oxide and cyclization afford the steroid lanosterol (Burg and Espenshade, 2011). Conversion of lanosterol to cholesterol required 19 steps including threefold demethylation and double-bond isomerization (Figure 2.2).

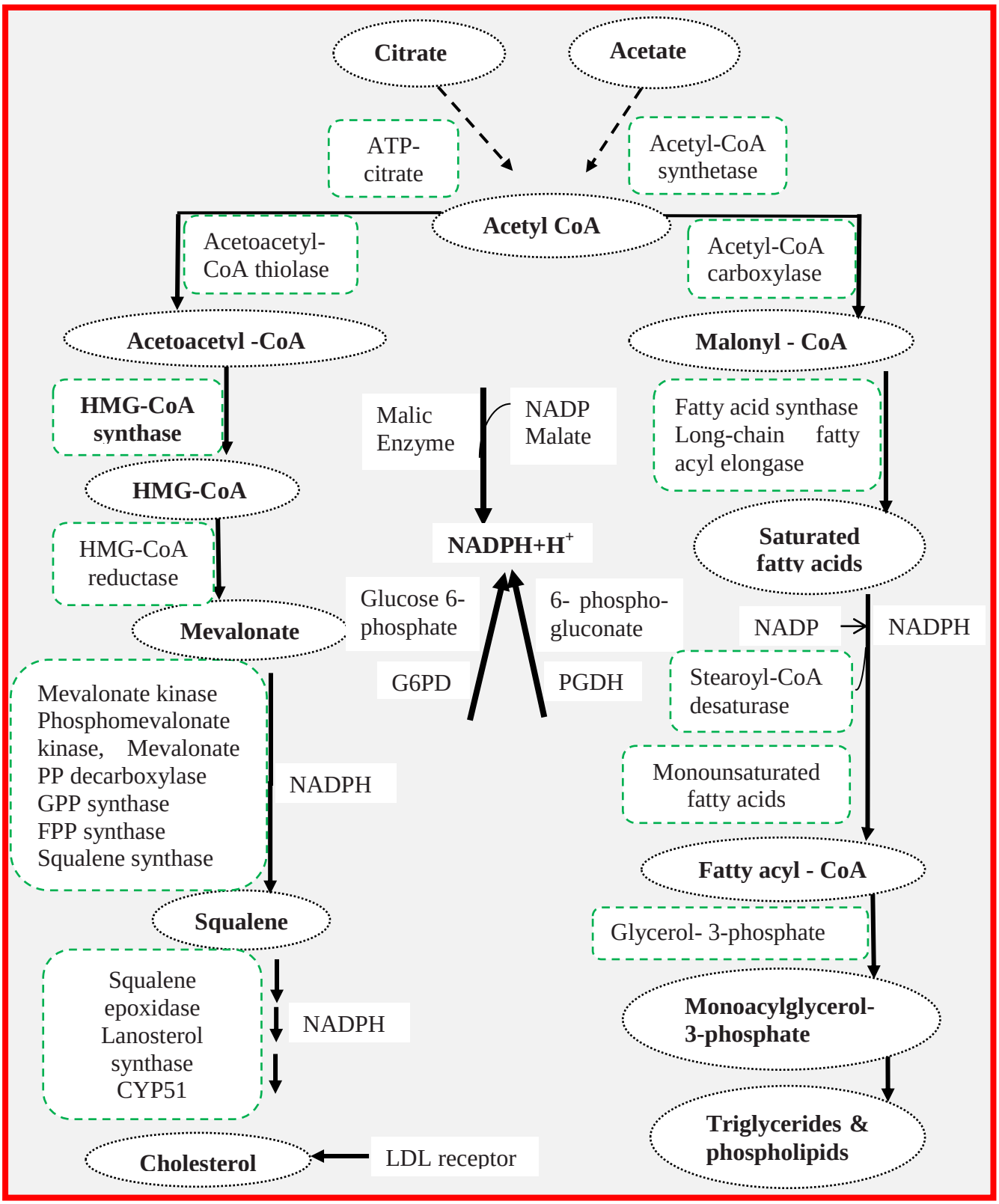


Figure 2.2: Biosynthesis of cholesterol, triglycerides and phospholipids.

(HMG-Co A, hydroxymethylglutrylco A reductase; LDL, low density lipoprotein; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced from of NADP) (Adpated from Groen et al., 2014).

2.5 Digestion and absorption of lipids

Lipids digestion is initiated in the stomach and catalysed by lingual lipases, which are secreted by tongue glands. In the stomach, lingual and gastric lipases continue digestion and emulsification of dietary fat and fat-soluble vitamins. Emulsified lipids enter the duodenum and subsequently mix with bile and pancreatic juice to go through many chemical and physical changes and the emulsification continues beside hydrolysis to be prepared for the absorption throughout the intestinal wall (Phan and Tso, 2001).

Pancreatic lipase, colipase and bile salts are working together to enhance the competence of lipid digestion and absorption. Decreased rate of lipid absorption in humans reflects the importance of bile. It was found that, the activity of pancreatic lipase could be inhibited through elevated concentrations of bile salts in the duodenum. Nevertheless, colipase plays a critical role in dietary lipids digestion and it could be restored the pancreatic lipase activity *in vitro* (Lowe, 2002).

2.5.1 Digestion and absorption of cholesterol

In the body, two types of cholesterol exist; endogenous, which is being produced in the liver and peripheral tissues and exogenous (dietary), which is absorbed from the intestine. Free sterol composed the most dietary cholesterol, while cholesterol esters represent only 10-15%. Cholesterol esters will be hydrolysed by cholesterol esterase to release free cholesterol for absorption (Nair and Wang, 2013, Van Heek et al., 2000). About 400 mg of cholesterol being provided via human diet daily and around 1g cholesterol is secreted by the liver (Siddiqi, 2008). Almost 50 % of the cholesterol is absorbed in the intestine and the residue is excreted in faeces (Clearfield, 2003).

Only non-esterified cholesterol can be combined into bile acid micelles and absorbed by enterocytes. Cholesterol enters bile salt micelles, and then it will be transported to the brush border of the enterocyte to be absorbed (Voshol et al., 2001). Cholesterol absorption depends on the presence of bile acids in the intestinal lumen and it related directly with the total bile acid pool (Voshol et al., 2001). Subsequently, cholesterol will be transferred to the unstirred water layer under the help of bile salt micelles (Moreau et al., 2002).

2.5.1.1 Cholesterol and bile acid cross-talk

There is an association between cholesterol and BA metabolism and control of various important processes. The literature confirmed the existing opinion that BA excretion represents the central route for eliminating cholesterol from the body (Briones et al., 1986, Groen et al., 2014, Kesaniemi et al., 1981, Zwicker and Agellon, 2013). Thus, it was found that loss of BA significantly contributes to cholesterol turnover. Nevertheless, around 95 % of the BA present in the intestinal lumen is reabsorbed and the BA pool remains effective in the enterohepatic circulation. Consequently, it is concluded that removal of excess cholesterol is definitely not the main role of BA synthesis in whole body cholesterol metabolism (Dietschy, 1968, Zwicker and Agellon, 2013). In human, a cycling frequency of BA is about 4–5 cycles per day and in every cycle about 5 % of the BA pool is lost. As the faecal BA loss is adequately recompensed for by hepatic synthesis to maintain BA pool size, de novo synthesis of BA (0.5 g/day in human) is a quantitatively significant pathway to maintain cholesterol homeostasis (Lefebvre et al., 2009).

2.5.2 Cholesterol excretion

Approximately, every day 1g of cholesterol is removed from the body which is approximately equal to the amount of absorbed and synthesized cholesterol. Almost, half of cholesterol is excreted in the faeces after conversion to bile acids in liver, and the remainder is excreted as free cholesterol. BAs serve to remove undesired cholesterol from the body and to aid in lipid digestion in the intestine (Nair and Wang, 2013). 7α-hydroxylase enzyme is the rate limiting enzyme of bile acid biosynthesis which converts cholesterol into 7-hydroxycholesterol. Then, 7-hydroxycholesterol is converted to one of the two primary bile acids, cholic acid and chenodeoxycholic acid. Bile acids are then delivered to the intestines where they aid in the absorption of lipids. In the intestine, intestinal bacteria act to modify some of bile acids to form secondary bile acids such as lithocholic acid and deoxycholic acid. However, the majority of bile acids delivered to intestine are recycled by reabsorption in the ileum and returned to the liver by enterohepatic circulation. In liver, glyco- and tauro-conjugate bile acids are formed and stored in gall bladder, from where they are released into the intestinal lumen for aid in the digestion and absorption process of fats or lipids (Nair and Wang, 2013).

2.5.3 Digestion and absorption of triglycerides

Pancreatic lipase is the enzyme responsible for digestion of TG, which starts in the upper part of the jejunum. The action of this enzyme breaks down the triglycerides in the micelles at positions 1 and 3 leaving two free fatty acids, glycerol and a 2-monoglycol (2-MAG) (Soutar and Naoumova, 2007). The predominant form in which MAG is absorbed from the small intestine is the 2-MAG. The uptake of 2-MAG from the small intestine is faster compared with the formation of 2-MAG

and 1-MAG through isomerization in an aqueous medium. Cholesterol esterase can also hydrolyse the acyl group at the *sn*-2 position to form glycerol and FFAs. FFAs are taken up from the intestinal lumen into the enterocytes and used for the biosynthesis of neutral fats (Ibrahim et al., 2013; Marks et al., 2003; Soutar and Naoumova, 2007;).

2.5.4 Digestion and absorption of phospholipids

In the lumen of the small intestine, phosphocholesterol (PC) is the major PL, which is found in mixed micelles that contain cholesterol and bile salts. Pancreatic phospholipase A2 (PLA2) with lipases secreted by the pancreas are responsible for the primary digestion process of PLs in response to food intake. These lipases interact with PLs at the *sn*-2 position to yield FFAs and lysophosphatidylcholine (Huggins et al., 2002). These products of lipolysis are removed from the water-oil interface when they are incorporated into the mixed micelles that form spontaneously when they interact with bile salts. PLA2 deficiency has a greater effect on the digestion of TG than that of PL hydrolysis (Huggins et al., 2002). It does not affect PL hydrolysis and absorption, possibly because its activity is compensated by other PLA2 enzymes (Richmond et al., 2001).

2.6 Lipid metabolic pathways

Generation and transport of lipids within the body are controlled through three main pathways which include; exogenous, endogenous and reverse cholesterol transport pathway (Ducharme and Radhamma, 2008).

2.6.1 Exogenous pathway

The free fatty acids will be combined with glycerol to form triglycerides once the digestion and absorption of dietary fat is completed. Then, cholesterol is esterified by acyl-coenzyme A: cholesterol acyltransferase (ACAT) to form cholesterol esters (Ibrahim et al., 2013). Triglycerides and cholesterol are present in the intracellular as chylomicrons. In the blood circulation, at the capillaries of the adipose tissue and muscle cells, chylomicrons interact to release the triglycerides into the adipose tissue to be stored and made available according to the body's energy needs (Crook, 2012).

Pancreatic lipase enzyme secreted from the pancreas is a key enzyme responsible for absorption and hydrolysis of triglyceride in the small intestine into glycerol and fatty acids (Sugiyama et al., 2007). Hence, the inhibition of lipase activity can lead to suppression of triglyceride absorption in the small intestine which could prevent obesity (Sugiyama et al., 2007). The enzyme lipoprotein lipase (LPL) hydrolyzes the triglycerides and releases the free-fatty acids. Some of the components of the chylomicrons are ‘‘repackaged’’ into other lipoproteins; for example, some apolipoproteins are transferred to HDL and the remaining chylomicrons particles are removed from the plasma by way of chylomicrons remnant receptors found on the liver (Ducharme and Radhamma, 2008) (Figure 2.3).

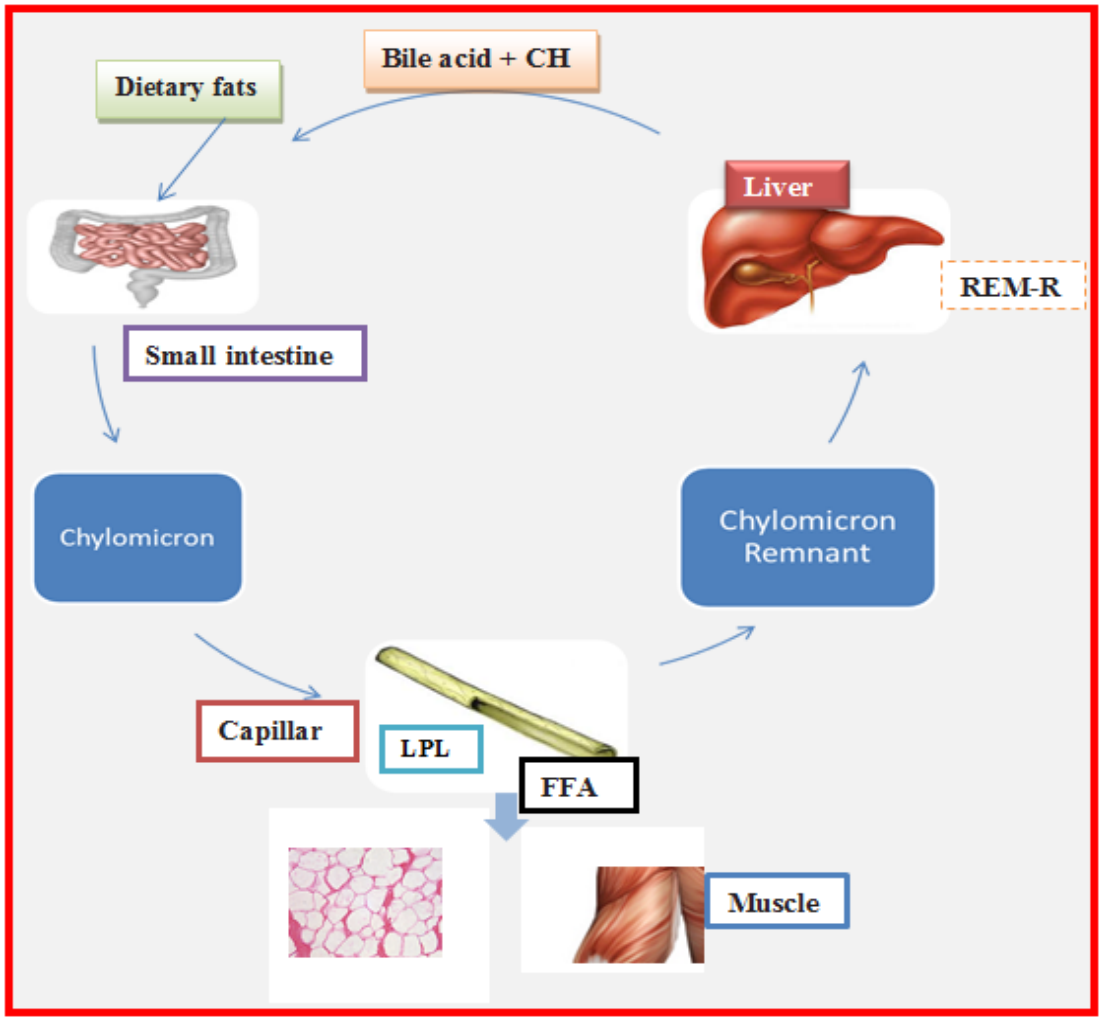


Figure 2.3: Exogenous pathway of lipid metabolism.(CM, chylomicron; FFA, free fatty acids; LPL, lipoprotein Lipase; CM-REM, chylomicron remnant; REM-R, remnant receptor; CH, cholesterol) (Adapted from Ibrahim et al., 2013).

2.6.2 Endogenous pathway

The endogenous pathway involves the liver-synthesizing lipoproteins. Triglycerides and cholesterol esters are packaged into VLDL particles and released into the blood circulation. Subsequently, fatty acids and glycerol will be released due to the hydrolysis of VLDL by tissue LPL (Groen et al., 2014). Once processed by LPL, the VLDL-C becomes a VLDL-C remnant. Most VLDL-C remnants are taken up by the liver by way of the LDL-C receptor and the remaining remnant particles become

IDL-C which is a smaller and denser lipoprotein than VLDL-C. Some IDL-C particles will be reabsorbed by the liver through the LDL-C receptor, whereas others are hydrolysed in the liver by hepatic triglyceride lipase to form LDL-C, which is smaller and denser particle than IDL-C (Rezen et al., 2011).

LDL-C is considered the main carrier of circulating cholesterol within the body. It is used by extrahepatic cells for cell membrane and steroid hormone synthesis. Most LDL-C particles are taken up by LDL-C receptors in the liver and the remaining particles are removed by way of scavenger pathways at the cellular level (Nair and Wang, 2013). Thus, this process will lead to suppression of the synthesis of new LDL-C receptors in the cells and activates the enzyme ACAT, which is responsible for the esterification of free cholesterol into cholesterol ester and store cholesterol in the cell (Ducharme and Radhamma, 2008).

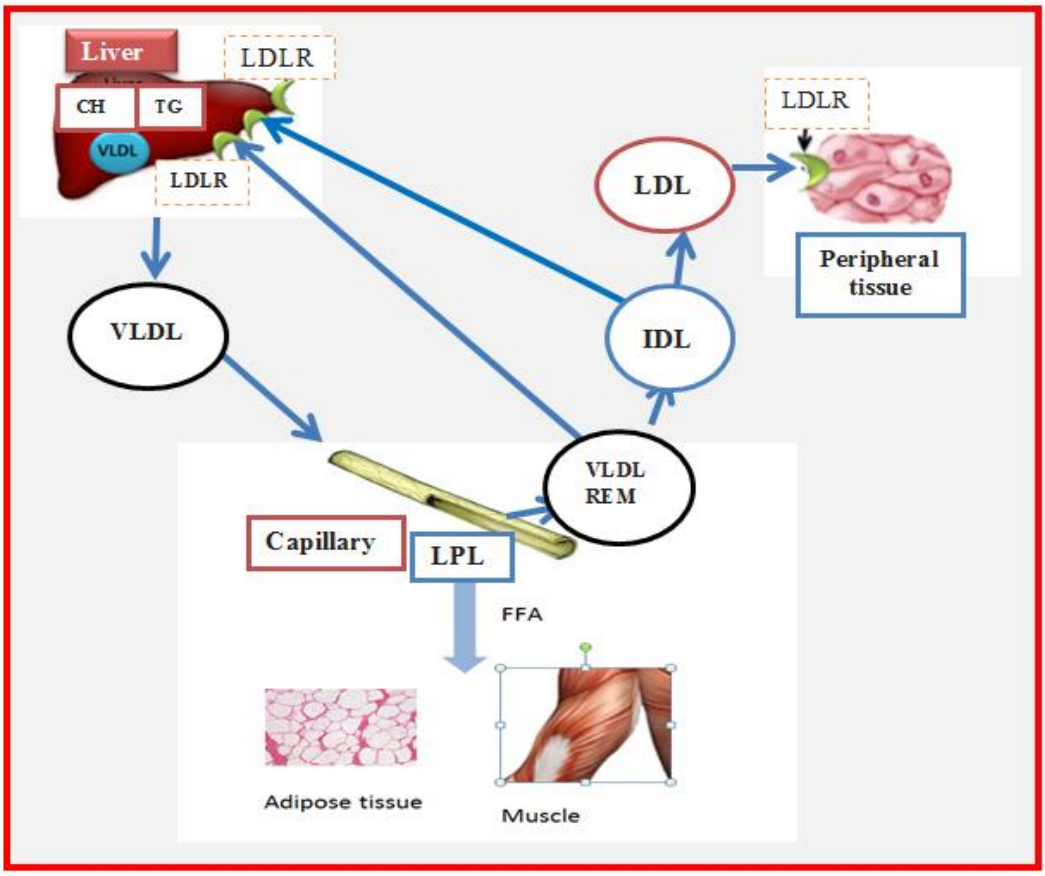


Figure 2.4: Endogenous pathway of lipid metabolism. (LPL, lipoprotein lipase; FFA, free fatty acids; VLDL, very low density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low density lipoproteins; LDLR, low density lipoproteins receptor; CH, cholesterol; TGs, triglycerides) (Adapted from Ibrahim et al., 2013).

2.6.3 Reverse cholesterol transport pathway

In this process, cholesterol is removed from the tissues and returned back to the liver. HDL-C plays the main role in the process of reverse cholesterol transport and in transferring of cholesteryl esters between lipoproteins (Groen et al., 2014). HDL-C, which is considered the smallest and the most dense lipoprotein particle is being formed through a maturation process in which the precursor particles (nascent HDL) secreted by the liver and intestine carry on through a series of conversions known as the HDL-C cycle to attract cholesterol from cell membranes and free cholesterol to the HDL particle core (Groen et al., 2014).