ANTI-HYPERLIPIDEMIC ACTIVITIES OF *Amauroderma rugosum* ON HUMAN HEPATOCELLULAR CARCINOMA CELLS

CHAN KAM SENG

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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CHAN KAM SENG

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ANTI-HYPERLIPIDEMIC ACTIVITIES OF *Amauroderma rugosum* ON HUMAN HEPATOCELLULAR CARCINOMA CELLS

ABSTRACT

Cardiovascular disease (CVD) is a tender killer leading to an increased mortality level in most industrially-developed countries. The fruiting bodies of the *Amauroderma rugosum* (AR) has been proclaimed to have excellent cardiovascular benefits such as high anti-oxidative capacity, anti-hyperlipidemic, anti-hypertensive, anti-inflammatory, anti-platelet aggregation and anti-thrombotic effects. In the present study, we have investigated the anti-atherosclerotic potentials by measuring the *in vitro* antioxidant capacity, anti-hyperlipidemic, lipoprotein modulating activity and its related mechanism(s) in oleate-induced HepG2 cells. The semi-polar ethyl acetate extract (EA) demonstrated the highest antioxidant capacity. Besides, the EA also exhibited the strongest inhibitory effect on Cu^{2+}-induced low-density lipoprotein (LDL) oxidation, and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity, respectively. Meanwhile, other AR extracts were non-cytotoxic (IC_{50} > 100 μg/mL) to normal human hepatic (WRL 68) and human lung fibroblast (MRC-5) cells. Assay on lipid lowering effects revealed that oleate-induced HepG2 cells treated with EA showed pronounced reductions in intracellular accumulation and secretion of total cholesterol (TC) and total triglyceride (TG). Further investigation on oleate-induced HepG2 cells treated with EA has observed a down-regulation of sterol-regulatory element binding factor-2 (*SREBF-2*) which confirmed the inhibition on mevalonate-mediated biosynthetic pathway of cholesterol. Unpredictably, our results showed an up-regulation of sterol-regulatory element binding factor-1 (*SREBF-1*) which does not correspond to the decreased endogenous synthesis of triglyceride. Further evaluation on two other key lipid regulatory transcription factors demonstrated a dose-and time dependent co-enhanced liver X receptor alpha (LXR-α) and peroxisome proliferator-activated receptor alpha (PPAR-α)

that suppressed the SREBF-1 promoting activity and *SREBF-2* gene. The activation of LXR-α was further confirmed by the over expression mRNA levels of its target genes i.e., apolipoprotein E (*APOE*), cholesteryl ester transfer protein (*CETP*) and *SREBF-1*. However, our results showed up regulation of *CETP* and *APOE* at transcription level but a down regulation at translational level leading to dose-and time-dependently inhibited secretion of both CETP and apoE which is beneficial to regress cardiovascular response. Up-regulation of LXR-α has been the hallmark of the beneficial reverse cholesterol transport (RCT) pathway which raises the "good cholesterol", the high-density lipoprotein (HDL) in the plasma. Further examination on two selected markers, i.e., apoA-1 and lecithin: cholesterol acyltransferase (LCAT) enzyme has evidenced the activation of RCT-related pathway leading to the over expression of HDL. On the contrary, down regulation of apoB-100, apoE, and CETP suppressed the production of "bad cholesterol", LDL and very low-density lipoprotein (VLDL). Finally, ten phenolic compounds were identified by Liquid Chromatography-Tandem Mass Spectrometry analysis. Among those, caffeic acid (CA), protocatechuic acid (PCA), vanillic acid (VA), and 4-hydroxybenzoic acid (4HBA) were selected as bioactive markers. The dose-dependent reduction of intracellular and secreted TC and TG demonstrated by CA, PCA, VA and 4HBA has evidenced their role in the lipid lowering effect. In summary, EA showed good anti-atherogenic potential through its high antioxidant capacity, anti-hyperlipidemic activity, and its lipoprotein modulating effect via activating the RCT-mediated pathway in raising the HDL while suppressing the LDL and VLDL productions. Therefore, EA can be used as a source of anti-atherosclerotic agent for the prevention of cardiovascular diseases.

Keywords: anti-hyperlipidemic activity, *Amauroderma rugosum*, HepG2 cells, lipoprotein metabolism, cardiovascular disease

AKTIVITI ANTI-HIPERLIPIDEMIK OLEH *Amauroderma rugosum* KEPADA SEL-SEL KARSINOMA HEPATOSELULAR MANUSIA

ABSTRAK

Penyakit kardiovaskular merupakan pembunuh senyap yang menyebabkan peningkatan kadar kematian di semua negara maju. Janabuah cendawan *Amauroderma rugosum* (AR) telah disahkan memiliki khasiat mencegah penyakit kardiovaskular seperti kapasiti antioksida yang tinggi, anti-hiperlipidemik, anti-hipertensi, anti-radang, anti-pengumpulan platelet, dan kesan anti-trombotik. Dalam kajian ini, potensi anti-atherosklerotik diuji dengan mengukur kapasiti antioksida, anti-hiperlipidemik, aktiviti modulasi lipoprotein *in vitro* dan mekanisme yang berkaitan menggunakan sel-sel HepG2-teraruh oleat. Ekstrak etil asetat (EA) dari jasad buah AR menunjukkan kapasiti antioksida yang tertinggi di kalangan semua ekstrak. Selain itu, EA juga mempamerkan kesan perencatan pengoksidaan pada penginduksian Cu^{2+} lipoprotein ketumpatan rendah (LDL) dan aktiviti 3-hydroxy-3-methylglutarul-CoA reduktase (HMG-CoA) masing-masing. Sementara itu, ekstrak pelarut-pelarut lain AR adalah tidak sitotoksik (IC_{50} > 100 µg/mL) kepada sel-sel hepatik (WRL 68) dan fibroblas paru-paru (MRC-5) manusia. Esei pada kesan penurunan lipid mendedahkan bahawa sel-sel HepG2-teraruh oleat yang dirawat dengan EA menunjukkan pengurangan ketara dalam pengumpulan intrasel dan rembesan kolesterol (TC) dan trigliserida (TG). Siasatan lanjut menggunakan sel-sel HepG2 terinduksi dengan oleat yang dirawat dengan EA memperlihatkan penurunan-regulasi pada faktor pengikat-2 elemen pengawal sterol (*SREBF-2*) di mana ia mengesahkan perencatan pada laluan biosinthesis kolesterol bermula dari mevalonat. Tanpa dijangka, keputusan kami menunjukkan peningkatan-regulasi pada faktor pengikat-1 elemen pengawalan sterol (*SREBF-1*) yang tidak sepadan dengan pengurangan sistesis dalaman trigliserida. Siasatan lanjut terhadap dua lagi faktor pengawalatur lipid transkripsi utama lipid menunjukkan peningkatan bersamaan dengan dos reseptor X alpha hati (LXR-α) dan reseptor peroksisom proliferator teraktif alpha

(PPAR-α) yang merencat aktiviti penggalak *SREBF-1* dan gen *SREBF-2*. Pengaktifan LXR-α seterusnya disahkan oleh peningkatan-ekspresi mRNA gen-gen sasaran i.e., apolipoprotein E (*APOE*), cholesteryl ester transfer protein (*CETP*) dan *SREBF-1*. Walau bagaimanapun, keputusan kami menunjukkan peningkatan-regulasi *CETP* dan *APOE* di peringkat transkripsi tetapi penurunan di peringkat translasi yang tergantung-dos dan masa menghalang rembesan kedua-dua CETP dan apoE di mana ia mendorong kemerosotan tindak balas kardiovaskular. Peningkatan-regulasi LXR-α sudah menjadi laluan kepada pengakutan kolesterol berbalik (RCT) bermanfaat yang meningkatkan "kolesterol baik", lipoprotein ketumpatan tinggi (HDL) dalam plasma. Pemeriksaan lanjut terhadap bagi dua penanda yang dipilih i.e., apoA-1 (komponen utama HDL) dan enzim lesitin:kolesterol asiltransferase (LCAT) telah membuktikan pengaktifan laluan yang berkaitan RCT membawa kepada peningkatan-ekspresi HDL. Sebaliknya, penurunan-regulasi apolipoprotein B-100, apoE dan CETP menyekat penghasilan "kolestrol jahat", i.e., LDL dan lipoprotein ketumpatan amat rendah (VLDL). Akhirnya, sepuluh sebatian fenolik telah dikenal pasti dengan kromatografi cecair seiring analisis spektrometri jisim. Di antaranya, sebatian tersebut, asid kafein (CA), asid protocatechuic (PCA), asid vanillik (VA) dan 4-hidrosibenzoik asid (4HBA) telah dipilih sebagai penanda bioaktif. Pengurangan dalam intrasel dan rembesan TG and TC yang ditunjukkan oleh CA, PCA, VA dan 4HBA telah membuktikan peranan mereka dalam kesan penurunan lipid oleh EA dalam sel-sel HepG2-teraruh oleat. Ringkasnya, EA menunjukkan potensi anti-atherogenik yang baik melalui kapasiti antioksida yang tinggi, aktiviti anti-hiperlipidemik, dan kesan lipid modulasi melalui pengaktifan RCT-laluan pengantara dalam meningkatkan HDL manakala menyekat laluan dalaman biosinthesis LDL dan VLDL. Oleh itu, EA boleh digunakan sebagai sumber agen anti-atherogenik yang bagus untuk mencegah penyakit kardiovaskular.

Kata kunci: aktiviti anti-hiperlipidemik, *Amouroderma rugosum*, sel-sel HepG2, metabolism lipoprotein, penyakit kardiovaskular

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^{o}C	Degree Celsius
µg/ml	Microgram per milliliter
µL	Microliter
µM	Micromolar
%	Percentage
x g	Time gravity
ANOVA	Analysis of variance
Apo	Apolipoprotein
AR	*Amauroderma rugosum*
BHA	Butylhydroxyanisole
BHT	Butylhydroxytoluene
BSA	Bovine serum albumin
CA	Caffeic acid
CAT	Catalase
CD	Conjugated diene
cDNA	Complementary DNA
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CM	Chylomicron
Cu^{2+}	Copper (II) ion
Cu(II)-Nc	Copper(II) Neocuproine
CUPRAC	Cupric-ion-reducing antioxidant capacity
CVD	Cardiovascular disease
DCM	Dichloromethane fraction

DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
dsDNA	Double stranded DNA
EA	Ethyl acetate fraction
EDTA	Ethylenediaminetetra acetic acid
EI	Electronic ionisation
FBS	Fetal bovine serum
FC	Free cholesterol
FFA	Free fatty acid
GAE	Gallic acid equivalent
GPx	Glutathione peroxidase
HBA	Hydroxybenzoic acid
HDL	High-density lipoprotein
Hex	Hexane fraction
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
H_2O	Water fraction
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IC_{50}	50% inhibitory concentration
ICAM-1	Intracellular adhesion molecule
IDL	Intermediate-density lipoprotein
LCAT	Lecithin-cholesterol acyltransferase
LC-MS/MS	Liquid Chromatography - Tandem Mass Spectrometry
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase

LXR	Liver X receptor
MD	Methanol-dichloromethane crude extract
MDA	Malondialdehyde
mg	Milligram
mM	Milimolar
mRNA	Messenger ribonucleic acid
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
n-LDL	Native-LDL
NO·	Nitrite oxide
PBS	Phosphate buffered saline
O_2·	Superoxide radical
OH·	Hydroxyl radical
PCA	Protocatechuic acid
PME	Pre-mixed extract
PPAR	Peroxisome proliferator-activated receptor
OD	Optical density
Ox-LDL	Oxidised-low density lipoprotein
RCT	Reverse cholesterol transport
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
R_t	Retention time
RT-PCR	Reverse transcriptase polymerase chain reaction
SOD	Superoxide dismutase
SREBP	Sterol-regulatory element-binding protein

TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
TCA	Trichloro acetic acid
TG	Total Triglyceride
UV	Ultraviolet
VA	Vanillic acid
VCAM-1	Vascular cell adhesion molecule
VLDL	Very low-density lipoprotein
WHO	World Health Organisation

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Atherosclerosis is a chronic disease of blood vessels which is referred as "hardening of the arteries". It is characterised by the accumulation of fatty streaks beneath the inner lining of endothelial layer of an artery. Major complications of atherosclerosis include angina, myocardial infarction and stroke which have been well-established as leading key factors of morbidity and mortality in Western countries. The World Health Organization (WHO) predicted that coronary heart diseases and stroke are becoming much life-threatening and projected a number of death toll reaching 24 million towards 2030 (Reinhardt, 2005). The pathogenesis of atherosclerosis is recognised primarily due to the incapability of body's physiological-defense mechanisms against a series of physiopathological responses such as hypercholesterolemia, oxidative stress, oxidation of low-density lipoprotein (LDL) and inflammation that occurred in the sub-endothelial space (Lowenstein & Matsushita, 2004).

Dyslipidemia is one of the key precursors of atherosclerotic cardiovascular disease. It is defined as elevated total triglyceride (TG), total cholesterol (TC), LDL and reduction in high-density lipoprotein (HDL) levels (Kolovou *et al.,* 2005). Current clinical management of dyslipidemia merely relies on statin, mono- or combined drug therapy (statins and fibrates), aiming to lower LDL and raising HDL levels. Despite statin and fibrate drugs having the ability to reduce cardiovascular mortality, however, long-term and intensified (high dosage) drug therapy causes patients to suffer from adverse side effects e.g., myopathy, rhabdomyolysis, hepatotoxicity, liver and renal failure. Based on this paradigm, the US National Institutes of Health (US NIH) has suggested that for those patients under drug therapies which do not achieve the LDL goal, to opt for alternative medicines, i.e., traditional complementary medicines by means of reducing atherosclerotic cardiovascular disease (ASCVD) (Allen *et al.,* 2011; NCEP ATP III,

2002; Stone *et al*., 2014). This has spurred many researchers to explore novel strategies, cost-effective, traditional-based multitarget and multicomponent lipid ameliorating agents. There are many potential molecular targets that have been identified for therapeutic intervention. Among those, modulation of plasma lipid profile, particularly the lowering of LDL and the raising of HDL remain the major goal of anti-atherosclerotic treatment.

It is strongly suggested that medicinal mushrooms are good resources to search for anti-atherosclerotic and lipid modifying agents with greatest attention. Mushroom epitomises a major and untapped source of potent new pharmaceutical products due to their great nutritional and therapeutic potentials (Selima *et al*., 2012). Mushrooms with high phenolic contents are highly correlated to their good antioxidant capacity. Research has evidenced that many phenolic compounds derived from medicinal mushrooms are able to attenuate oxidative stress and reduce blood cholesterol levels in hypercholesterolemic rats (Afonso *et al*., 2013). Hence, lipid lowering potential of medicinal mushrooms combined with their antioxidant capacity could make it a good source of anti-atherosclerotic agent.

On this basis, *Amauroderma rugosum* (Blume & T. Nees) Torrend, (a representative of black lingzhi mushroom) has been enunciated to have good antioxidative, anti-inflammatory (Chan *et al.*, 2013; Chan *et al.,* 2015), antitumor and antihyperlipidemic potentials (Babu, 2008). However, there is limited scientific proof on the felicitous uses with regards to the lipid lowering effect *in vitro*, *in vivo* and clinical trials. Nevertheless, its underlying mechanisms remain to be clarified. To validate these therapeutic benefits, more scientific data are needed to advocate either the traditional or clinical uses in treating dyslipidemia.

1.1 Objectives

1. To evaluate the antioxidant capacity, inhibitory effects on human LDL oxidation and HMG-CoA reductase activity of organic-solvent-partitioned extracts prepared from AR.
2. To examine the cytotoxicity of AR on selected normal cell lines (MRC-5 and WRL 68).
3. To investigate the lipid and apolipoprotein modulating effects on oleate-induced HepG2 cells using organic-solvent-partitioned extracts of AR.
4. To determine the underlying mechanisms of lipid, apolipoprotein and lipoprotein modulating effects on oleate-induced HepG2 cells using selected bioactive extract of AR by investigating the protein and gene expressions of selected molecular targets.
5. To characterise the chemical components present in the bioactive extract of AR and to determine their role in lipid lowering activity on oleate-induced HepG2 cells.

CHAPTER 2: LITERATURE REVIEW

2.1 Risk factors of atherosclerosis

Atherosclerosis is a chronic disease of blood vessels which is known as "hardening of the arteries". It is characterised by the accumulation of fatty streaks (the grossly visible lesion mainly consists of aggregates of lipid-engorged macrophage foam cells, inflamed T cells, and smooth muscle cells) beneath the inner lining of endothelial layer of an artery. Major complications of atherosclerosis include angina, myocardial infarction and stroke which have been well-established as leading key factors of morbidity and mortality in western countries. The WHO predicted that coronary heart diseases and stroke are becoming much life-threatening and projected a number of total death toll reaching 24 million towards 2030 (Reinhardt, 2005).

The major risk factors for atherosclerosis include elevated blood cholesterol level, high blood pressure, smoking, insulin resistance, diabetes, obesity or overweight, lack of physical activity and etc. Although ASCVD is one of the major causes of death and disability worldwide, global medical professionals have accepted that ASCVD is actually a modifiable and preventable disease which can be regressed with a healthy lifestyle.

2.1.1. Pathogenesis of atherosclerosis

Based on present studies, the pathogenesis of atherosclerosis is recognised primarily due to the incapability of the body's physiological-defense mechanisms against a series of physiopathological responses such as hypercholesterolemia, oxidative stress, oxidation of LDL and inflammation occurs in the sub-endothelial space (Lowenstein *et al.,* 2004). Henceforth, elevation of LDL, oxidised-LDL (ox-LDL) and endothelial dysfunction have been the key initiating factor of the development of atherosclerosis (Lusis, 2000).

Elevation of LDL in the plasma makes it more susceptible for oxidation. The ox-LDL particles are found to be toxic to various cells and cause the chain breaking reaction to generate even more reactive oxygen species (ROS). The attacks of those energetic and highly reactive free radicals together with the weakening of immune system eventually lead to endothelial injury or endothelial dysfunctions. Endothelial dysfunction allows the entry of lipids and inflammatory cells into the arterial walls.

Once in the artery, monocytes will undergo differentiation to form macrophages which take up the lipid and turn to foam cell macrophages. This eventually leads to the formation of lesions (or the fatty streaks) which is associated with atherosclerosis (Figure 2.1).

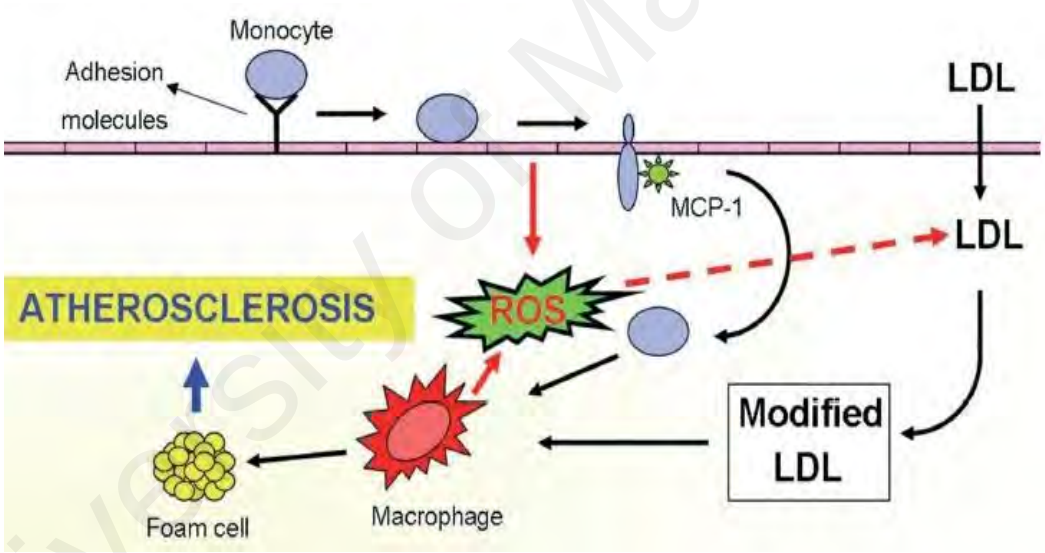

Figure 2.1: Pathogenesis of atherosclerosis (Bonomini *et al.*, 2008)

2.1.2 Role of reactive species in atherosclerosis

There are two common types of reactive species, i.e., reactive oxygen species (ROS) and reactive nitrogen species (RNS) that cause oxidative stress which can arise from the human body. They are generated by the biochemical reactions for example, superoxide radical ($O_2\cdot$) and hydrogen peroxide (H_2O_2) can be derived via direct oxidation of several biomolecules by O_2. The endogenous production of ROS and RNS

is an unavoidable consequence of these auto-oxidisable molecules present in a body during aerobic respiration. Besides, environmental exposure to low-wavelength electromagnetic radiations (gamma rays, UV light) can also split water molecules in the body to generate the vigorous reactive hydroxyl radical (OH·) (Von Sanntag, 1987). Nitric oxide (NO·**)** is an example of RNS with many useful functions such as regulation of blood pressure and platelet function. It is also produced in the body. However, higher level of NO· is toxic to various cells as well as human body compartments. Excessive production of NO· and other RNS such as nitrogen dioxide (NO_2·) have been related to chronic inflammation, stroke and septic shock (Anggard, 1994). These highly reactive free radicals would rapidly enter into oxidative cycles and attack various vital cellular components i.e., lipids, proteins, nucleic acids and carbohydrates causing cellular damage.

Over the past 2 decades, considerable evidence has been gathered in support of the hypothesis that free-radical-mediated oxidative processes do play a key role in atherosclerosis. In spite of various antioxidant defence mechanisms that occur in our body, oxidants (e.g. free radicals) are constantly generated *in vivo*, an inevitable feature of aerobic life. Researchers believed that oxidant–mediated disease occur only under circumstances in which these agents overwhelm antioxidant defences, leading to the stage notoriously known as "oxidative stress" (Sorescu *et al.,* 2002). Oxidative stress plays a key role in the pathogenesis of atherosclerosis. It permits the oxidative modification of LDL thereby producing the highly toxic ox-LDL particles which could attack the endothelial cells leading to the endothelial damages. Endothelial dysfunction thereby promotes a vascular inflammatory response, an early stage of pathogenesis of atherosclerosis.

2.1.3 Role of lipids in atherosclerosis

Lipids are mainly comprised of cholesterol, and triglyceride. They are essential components for life. Triglycerides and cholesterol are two separate classes of lipids that are present in the blood circulation. However, elevated triglyceride and cholesterol levels are the two independent factors of atherosclerosis.

Cholesterol not only serves as the main components of all cellular membranes, but also as the precursor of hormones, vitamin D and bile acids. Meanwhile, triglycerides function as a good source of energy which is normally stored and supply the calories throughout the whole body. Over 70% of cholesterol in human is derived from endogenous production in liver and intestine and the other 30% is supplied by the diet (Grundy, 2004).

Hypercholesterolemia is a condition characterised by a high level of cholesterol in the blood. The excess cholesterol in the bloodstream is deposited in the arterial walls leading to the formation of plaque which caused narrowing and hardening of artery wall. As the plaques grow bigger, they can clog the arteries and restrict the flow of blood to the heart (Joossens, 2004).

On the other hand, hypertriglyceridemia is another prevalent risk factor for cardiovascular disease (Talayero & Sacks, 2011). High triglyceride levels are the markers for atherogenic lipoproteins. The atherogenicity of triglycerides is associated with increased triglyceride-rich VLDL production and delayed VLDL clearance from circulation which in turn promotes the atherogenic inflammatory cascade (Ooi *et al.,* 2008; Zheng *et al*., 2010).

Currently, there are few pharmacological treatments available for reducing the risk of hyperlipidemia which include HMG-CoA reductase inhibitor, statins (e.g., lovastatin), bile acid sequestrants (e.g., colestipol and cholestryamie), fibrates (e.g., clofribrate, fenofibrate, ciprofibrate, and etc), and cholesterol absorption inhibitors (e.g.,

omega-3-fatty acid) (Abeysekera *et al*., 2017). According to Tucker *et al*. (2013), prostaglandins and nonsteroidal anti-inflammatory drugs (NSAIDs), also emerging as new therapeutic targets. There are not only the molecules that lead to the metabolism of lipid substances (e.g., lipid mediator), but also serve as the diagnostic indicators for diverse diseases, including atherosclerosis.

2.1.4 Role of lipoproteins in atherosclerosis

Most of the lipids are mostly insoluble in the blood and require lipoproteins for their transportation along the blood circulation. Lipoproteins are special particles made up of a core of lipid droplet {consisting mainly triglycerides, and cholesteryl ester (CE)} with a surface monolayer of phospholipids, unesterified cholesterol, and specific apolipoproteins. Apolipoproteins that are present on the surface of lipoproteins serve as cofactors for enzymes or ligands for cell-surface receptors interactions. Hence, the apolipoprotein markers on lipoproteins do play significant roles in lipoprotein metabolisms. Over production or imbalance regulation of apolipoproteins may be one of the key factors of atherogenicity.

Apolipoprotein A-1 (apoA1) is the main protein in HDL whereas apoB-100 is the key protein of all other non-HDL lipoproteins. ApoE can be present in both types of lipoproteins and serves as a ligand for the uptake and degradation of lipoproteins. The structures of lipoproteins are shown in Figure 2.2.

Lipoproteins, on the other hand, fall into five classes, i.e., chylomicron (CM), VLDL, IDL, LDL and HDL in which CM, VLDL, IDL and LDL are normally referred to as non-HDL, atherogenic lipoproteins (the “bad cholesterol”), whereas HDL is recognised as the only anti-atherogenic lipoprotein (the “good cholesterol”). Normal and healthy individuals should display an optimum lipid profile as shown in Table 2.1.

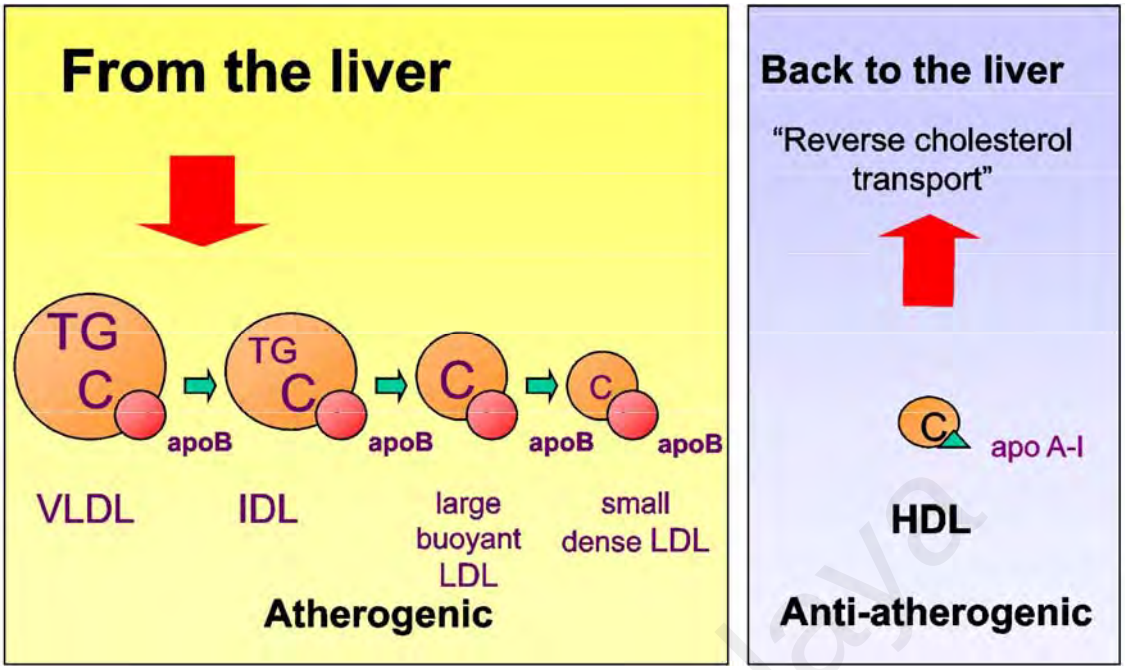

Figure 2.2: Classification of lipoproteins. The figure illustrates the structures of atherogenic apoB-containing-VLDL, IDL, LDL particles and anti-atherogenic apoA-I-containing HDL particle (Walldius & Jungner, 2006).

Table 2.1: Lipid profile: 2016 ESC/EAS Guideline for management of dyslipidemias

	Desirable (mg/dl)	Borderline (mg/dl)	High Risk (mg/dl)
Cholesterol	< 200	200-239	> 240
Triglycerides	< 150	150-199	200-499
HDL-cholesterol	60	35-45	<35
LDL-cholesterol	60-130	130-159	160-189
Cholesterol/HDL ratio	4.0	5.0	6.0

Source: Catapano *et al.*, 2016

2.1.5 Role of apolipoproteins in atherosclerosis

In addition to lipoprotein, apolipoproteins are recently recognised as other key markers for atherosclerosis. They are key protein components of lipoproteins. They play the key roles in lipoprotein metabolism and thus in atherogenesis. These multifunctional proteins serve as templates for the assembly of lipoprotein particles which bind to the

membrane receptors and regulate enzymatic activities (Karadi & Kostner, 1990). There are five major classes of apolipoproteins. The functions of apolipoproteins are summarised in Table 2.2 (Dominiczak & Caslake, 2011).

Apolipoprotein A-1 is the most abundant protein in HDL. It serves as a ligand for HDL-receptor and acts as a cofactor for Lecithin-cholesterol acyltransferase (LCAT). Evidence has revealed that high levels of apoA-1 are inversely correlated to the incidence of coronary heart disease (CHD). Meanwhile, apoB-100 is the predominant protein of the VLDL and LDL particles. Elevation of apoB-100 is an indication of high levels of VLDL and LDL particles in the blood circulation, which is corresponding to the ASCVD (Kreuzer & Hodenberg, 1994).

Table 2.2: Classification and functions of apolipoproteins

Apolipoproteins	Lipoprotein Classes	Functions
A-I	Chylomicrons, HDL	Activates LCAT
A-II	Chylomicrons, HDL	Inhibits LCAT, enhances hepatic lipase activity
A-IV	Chylomicrons	Unknown function
B-100	VLDL, IDL, LDL	Necessary for binding to cell receptors, Lipoprotein lipase
B-48	Chylomicrons	Necessary for binding to cell receptors, Lipoprotein lipase (LPL)
C-1	Chylomicrons, VLDL, HDL	Cofactor for LCAT
C-II	Chylomicrons, VLDL, HDL	Activates LPLs
C-III	Chylomicrons, VLDL, HDL	Regulates LPLs
D	HDL	Essential for LCAT activity and cholesteryl ester transfer
E	All	Bind to specific cell receptors

Apolipoprotein E that is present in plasma lipoprotein is involved in the degradation of triglyceride and cholesterol-rich particles. Their exact roles in lipoprotein metabolism and atherogenesis remain unclear. There are three major apoE isoforms, E2, E3 and E4. Allele E2 is associated with lower LDL levels; however, allele E4 is related to higher LDL levels. Some researchers have demonstrated that apoE is an independent risk factor for premature development of atherosclerosis (Horejsi & Ceska, 2000).

2.1.6 Exogenous lipoprotein metabolism

Under normal circumstances, the dietary fatty acids are absorbed, and converted to triglycerides so that they can be incorporated into chylomicrons (CMs) in the intestinal epithelial cells. The CMs are the triglyceride-rich apolipoprotein (apoB) B48 containing lipoprotein which enter the plasma though the intestinal lymph.

The lipoprotein lipase (LPL) hydrolyses the triglycerides (from the CMs) to fatty acids, which are then taken up by peripheral cells for oxidation or adipocytes for storage. Then the remaining particles (the CM remnants), are cleared from the blood circulation by the liver via binding of their surface apoE to the LDL receptor and converted to the triglyceride-rich apoB-100 containing VLDL particles before they are re-circulated into the blood stream.

Similar to CMs, the VLDLs are hydrolysed by LPL to deliver the TG to peripheral cells, and the VLDL remnants or the intermediate density lipoproteins (IDLs) are taken up by the liver receptors thru apoE and then converted to LDL (Figure 2.3). The end product of this metabolism is LDL. All of those CM, VLDL and, IDL should be cleared by the liver and the LDL level should be brought to the optimal levels within 12 hours under normal physiology.

2.1.7 Endogenous lipoprotein metabolism

In the liver, the triglyceride-rich apoB-containing VLDL particles are assembled. The synthesis of apoB-containing lipoproteins is a two-step reaction that occurs in endoplasmic reticulum (ER) lumen and Golgi. At first, a partially lipidated apoB particle and VLDL that lacks of apoB are formed independently. In the following step, these two precursors are fused to form a mature VLDL particle which is transported through the Golgi apparatus and secreted from hepatic cells (Olofsson *et al.*, 1999).

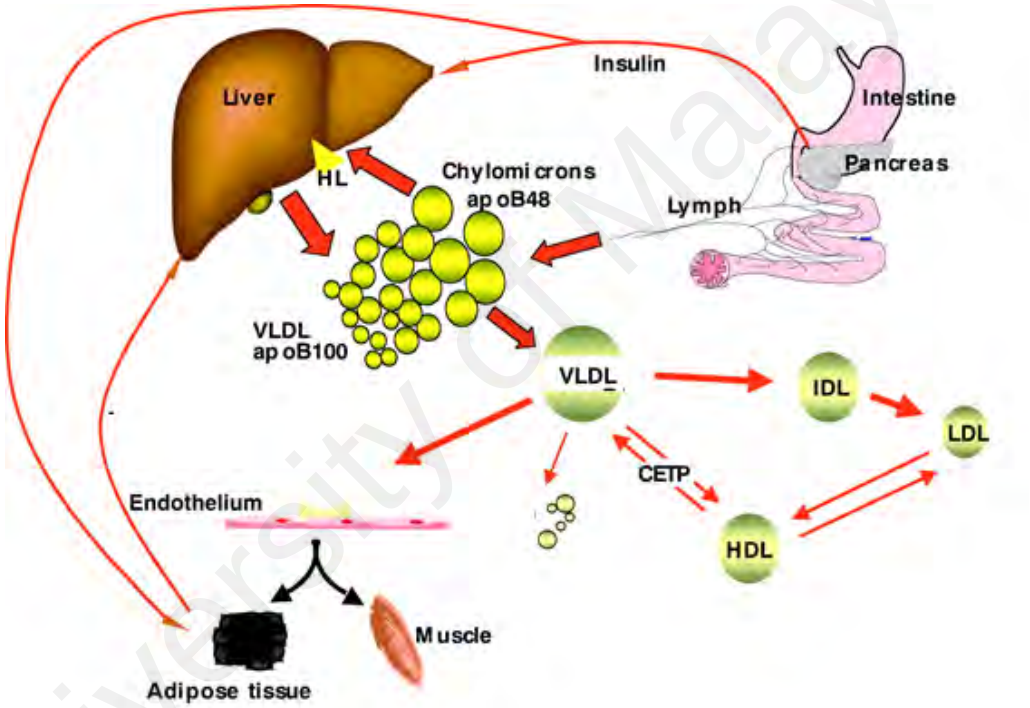

Figure 2.3: Exogenous lipid metabolism (Tushuizen *et al*., 2005).

Approximately 50% of the newly synthesized apoB is degraded intracellularly and not secreted. The degree of lipidation of apoB polypeptide chain is of great importance for stability. The secreted hepatic VLDL particles bind to peripheral VLDL receptors that is subjected to hydrolysis of triglycerides contained in the VLDL by lipoprotein lipase thereby leading to plasma clearance of triglycerides in VLDL.

The remnants of VLDL particles are converted to the intermediate-density lipoprotein (IDL) and finally the LDL particles which are taken up by the hepatic LDL receptor. Liver as the main organ involved in lipid homeostasis accounts for 60-80% for the LDL removal from the plasma (Medding *et al*., 1987). However, the disturbance of lipid metabolism may arise from the genetic factors, alcohol overuse, diabetes mellitus, hypothyroidism, chronic kidney disease and etc. The overproduction or delayed clearance of lipoproteins lead to prolonged circulation of lipoproteins. This has greatly influenced the distribution of lipoproteins, especially the atherogenic and anti-atherogenic markers, LDL/HDL ratio. The excess of VLDL, IDL and LDL (the atherogenic lipoproteins) in the circulation and low level of HDL is a defined condition of dyslipidemia.

2.1.8 Dysmetabolism and cardiovascular disease

Dyslipidemia is an integral part of the metabolic syndrome (MetS). It is the key precursor of ASCVD. Evidence has clearly shown that dyslipidemia or hypercholesterolemia does play the key initiating role in the pathogenesis of atherosclerosis. For every 1% increase in total cholesterol, there is an increase of 2-3% in CHD incidences (Law *et al*., 1994). In clinical significance, dyslipidemia is defined as elevated TGs, elevated TC, increased LDL and decreased HDL levels (Kolovou *et al*., 2005). Parini *et al*. (1999) has reported that plasma cholesterol levels increase with age, as does the incidence of CHD. The increase of the plasma cholesterol levels is attributable to (i) increase production of apoB-100 required for VLDL assembly coupled with (ii) gradual decline in fractional clearance of LDL due to reduced expression of hepatic LDL receptors (LDLRs). Besides, the capacity for body cholesterol removal through the conversion of cholesterol to bile acids is also progressively reduced with age. Undoubtedly, diseases associated with high cholesterol levels have been a significant

problem for elderly groups. Most importantly, it is now affecting younger ages than ever due to modernised lifestyle.

On the other hand, HDL is an anti-atherogenic lipoprotein. The main role of HDL is to traffic back excess cholesterol from various parts of the body (especially from arteries) to liver for clearance by converting it into the bile acid for subsequent biliary excretion. The optimum levels of LDL and HDL determine the balance of cholesterol in the circulation which is crucial in lipid homeostasis. Both the triglycerides and cholesteryl ester (CE) present in the core of lipoproteins are the determinants of lipoprotein levels and their plasma distribution. Overproduction of endogenous lipids (TG and TC) has been associated with delayed clearance of atherogenic non-HDLs. Besides, they promote the increased catabolism of anti- atherogenic HDL in liver leading to the increased LDL and decreased HDL levels. High ratio of LDL/HDL is a hallmark of atherogenic dyslipidemia as well as the central of metabolic syndrome (MetS).

Plasma cholesterol, as mentioned in the "cholesterol hypothesis" by Keizer (2012) and triglyceride are two independent causative risk markers of atherosclerosis. Elevation of the plasma TG implies the increase of the CM, VLDL, IDL and their remnants which are TG-rich lipoproteins, whereas elevation of the plasma cholesterol usually indicates that LDL is increased (Cox *et al.,* 1990). Plasma cholesterol values represent total cholesterol, which includes both unesterified cholesterol and esterified cholesterol. The primary defect due to overproduction of TGs is associated with high plasma free fatty acids (FFAs), resulted from decreased retention of FFAs by the adipocytes which could be mediated by insulin resistance. Increased flux of free fatty acids back to the liver stimulates the hepatic TG synthesis, which in turn promotes the assembly and secretion of TG containing VLDL.

Overproduction of VLDL leading to the high levels of buoyant LDL and small dense-LDL (sdLDL) as VLDL is eventually metabolised to IDL and then LDL. Besides,

the increased VLDL also leads to decreased HDL as the high TG levels delays hepatic mediated uptake and degradation of VLDL, IDL and LDL in the liver. This prolongs the retention and circulation of these atherogenic lipoproteins in the plasma and thus causes the exchange of their TG content with CE content of HDL and LDL. The TG-rich HDL is prone to hepatic uptake and degradation that leads to decreased in HDL in the blood circulation. This will defeat the cholesterol reverse pathway which is the main anti-atherogenic functions. In contrast, the TG-rich LDL particles are less susceptible to be cleared from the liver, leading to prolonged retention in the circulation and atherogenicity (Pal *et al.,* 2003).

2.2 Regulatory precursors in lipid homeostasis

Atherogenic dyslipidemia is a disruption of lipid metabolism resulting from elevated serum total cholesterol, triglyceride, LDL and lower level of HDL. Serum lipids and lipoprotein lipids are the most potent risk factors for atherosclerotic cardiovascular disease (Durrington, 2003). In human, lipid homeostasis is well-controlled by balanced mechanisms of intestinal uptake, endogenous biosynthesis and its secretion, transportation throughout the circulation by lipoprotein particles, and biliary excretion (Bei *et al*., 2012). Transcription factors such as LXRs, PPARs and SREBPs and two plasma proteins i.e., LCAT and CETP have been recognised for their roles in the pathological processes associated with atherogenesis (Shchekunova *et al*., 2013).

2.2.1 Sterol regulatory element-binding proteins (SREBPs)

Lipid homeostasis in mammalian cells is controlled by a family of membrane-bound transcription factor known as sterol regulatory element-binding proteins (SREBPs) encoded by genes *SREBF-1* and *SREBF-2*. SREBPs directly regulate the expression of genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides,

phospholipids and etc. (Brown & Goldstein, 1997; Sakakura *et al*., 2001). The major intermediates in the synthetic pathways of lipids are illustrated in Figure 2.4. In the hepatic cells, three SREBPs regulate the production and export of lipids, i.e., SREBP-1a, SREBP-1c and SREBP-2.

SREBP-1c is an activator of SREBP-responsive genes required for fatty acid synthesis while SREBP-2 preferentially activates cholesterol synthesis. Overexpression of SREBP-1c has been shown to cause hepatic steatosis (fatty liver) due to the up-regulation of fatty acid synthetic-related enzymes whereas overexpression of hepatic SREBP-2 has dramatically raised the mRNA levels of all cholesterol biosynthetic enzymes i.e., HMG-CoA reductase (*HMGR*), LDL receptor (*LDLR*) gene and etc. (Shimano *et al.,* 1997; Horton *et al,* 1998).

Inhibition of SREBP pathway can be a novel strategy to reduce lipid biosynthesis in treating hyperlipidemia, thus lower the risk of metabolic disease such as type II diabetes, insulin resistance and fatty liver.

2.2.2 HMG-CoA reductase inhibitors (statins)

Liver is the major organ that plays a critical role in endogenous cholesterol synthesis. The cholesterol biosynthetic pathway begins from the conversion of acetyl CoA to HMG-CoA as shown in Figure 2.4. The 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase that catalyses the reduction of HMG-CoA to mevalonate, is the rate determining, NADPH dependent enzyme of this pathway. It provides regulatory feedback of cholesterol synthesis in hepatic cells (Goldstein & Brown, 1990).

HMG-CoA reductase inhibitors (statins) emerged as potent lipid lowering drugs in early 1976 by inhibiting HMG-CoA reductase thereby lowering the synthesis of cholesterol in the liver (Endo, 1992). These drugs such as pravastatin, fluvastatin, atorvastatin, simvastatin and rosuvastatin have been shown to reduce mortality.

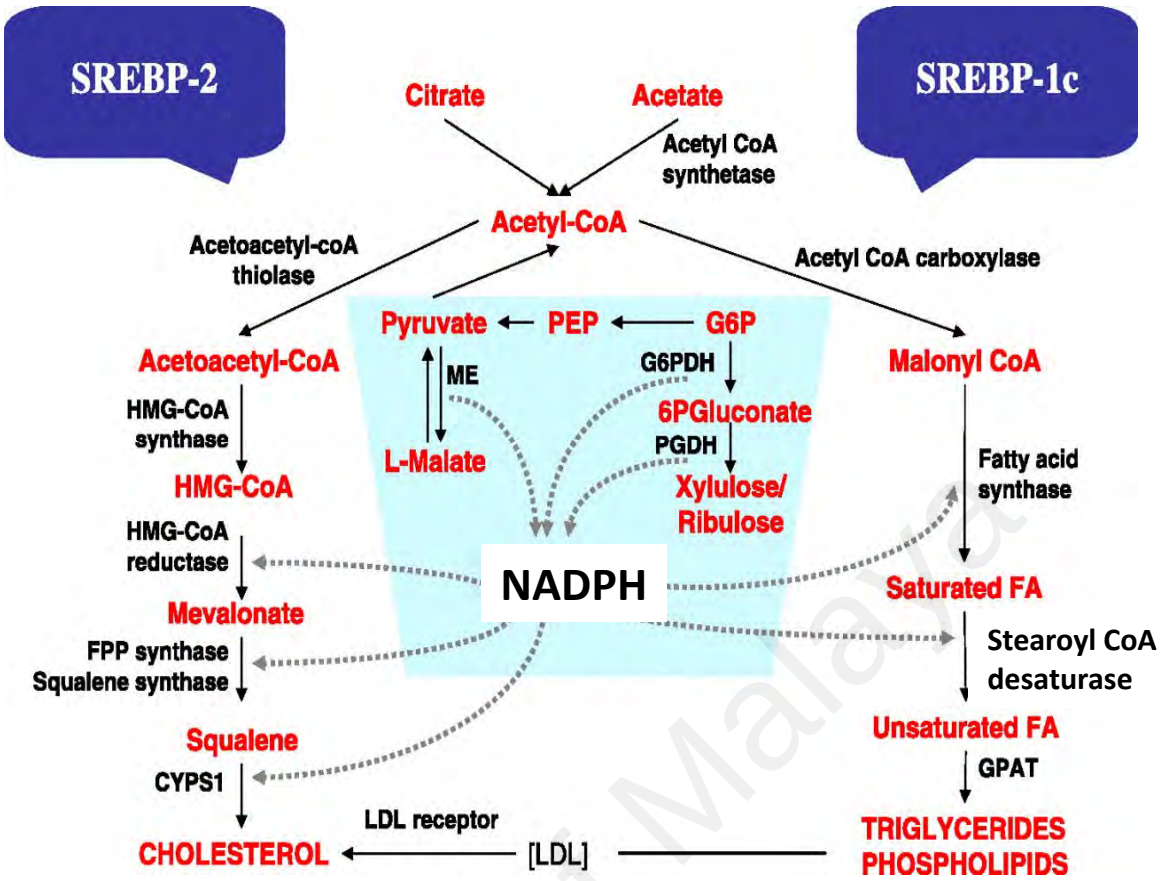

Figure 2.4: The synthetic pathways of cholesterol, fatty acid and triglycerides. (Source: Desvergne *et al*., 2006).

They are the mainstay of lipid lowering therapy for elevated LDL in preventing acute coronary heart disease strokes. However, major side effects were associated with stains, including increased liver enzymes and skeletal pain, memory loss, myopathy, acute renal failure (Bybee *et al*., 2004; Graham *et al.*, 2004). Hence, discovery of novel and safer HMG-CoA inhibitors are continuous efforts.

2.2.3 Peroxisome proliferator-activated receptors (PPARs)

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the superfamily of nuclear hormone receptors. They are three isoforms, PPAR-α, PPAR-β and PPAR-δ. The PPAR-α receptor is highly expressed in liver, kidney and skeletal muscle with high capacity of mitochondrial and peroxisomal β-oxidation (Braisant *et al*., 1996). In the liver, PPAR-α activators are well-established

for promoting genes involved in the uptake, transport and metabolism of fatty acids, i.e., fatty acid transport protein (FATP), and acyl-CoA synthetase. Conversion of fatty acids into acyl-CoA is required for further catabolism of fatty acids and triglycerides via peroxisomal, microsomal and mitochondrial fatty acid oxidation as well as ketogenesis (Rodriguez *et al*., 1994; Yu *et al*., 1998). PPARs are the major regulator of intra and extracellular lipid metabolism which could be activated by pharmacological drugs such as fibrates. They mediate the hypolipidemic action in the treatment of hypertriglycedemia. Upon activation, PPAR-α down-regulates hepatic apolipoprotein C-III and increase lipoprotein lipase gene expression which are the key players of triglyceride catabolism. Besides, PPAR-α also increases plasma HDL particles via the induction of hepatic apolipoprotein A-I expression in humans (Gervois *et al*., 2000). Hence, PPARs could be attractive targets for lipid lowering therapy.

2.2.4 Liver X receptors (LXRs)

The liver X receptors (LXRs) are ligand-activated transcription factors of the nuclear receptor superfamily. There are two LXR isoforms, i.e., LXR-α and LXR-β encoded by *NR1H3* and *NR1H2*. The liver receptors (LXRs) are key regulators of lipid homeostasis. These transcription factors govern the expressions of genes involved in the uptake, transport, efflux and excretion of cholesterol in a tissue-dependent manner (Hong & Tontonoz, 2014). LXRs were demonstrated to serve as sterol sensors protecting the cells from cholesterol overload by promoting reverse cholesterol transports and converting cholesterol into bile acids in the liver. LXR agonists have been developed as potent anti-atherogenic agent in treating dyslipidemia. However, LXR activators were also shown to induce lipogenesis via up-regulating SREBP-1c leading to hepatic steatosis (fatty liver) and hypertriglyceridemia (Baranowski, 2008). However, due to their distinct

roles, LXRs are still considered potential for use as attractive targets for the treatment of cardiovascular disease.

2.2.5 Lecithin: cholesterol acyltransferase (LCAT)

Lecithin: cholesterol acyltransferase (LCAT) is first identified by Glomset (1968). It is an enzyme that is bound to high-density lipoprotein (HDL) particles and is involved in HDL metabolism. It is the only enzyme capable of converting free cholesterol (FC) to cholesteryl ester (CE) in plasma. The esterified CE is then sequestered into the core forming the newly synthesized HDL particles from the liver to collect free cholesterol along the circulation in the plasma and forcing them back to the liver for clearance (Figure 2.5).

LCAT has long been believed to play a critical role in reverse cholesterol transport (RCT). (Alice *et al*., 2016). Animal studies have revealed a complex interaction between LCAT and atherosclerosis. LCAT may be modulated by diet or other proteins that modify lipoproteins. Nevertheless, the ability of LCAT to lower apoB in animal models has further indicated its anti-atherogenic properties. Recent studies on human subjects with LCAT deficiency have established the linkage between LCAT and cardiovascular disease (Rousset *et al*., 2009).

2.2.6 Cholesteryl ester transfer protein (CETP)

Cholesterol ester transfer protein (CETP) is a hydrophobic glycoprotein that is highly expressed from the liver. It is bound mainly to HDL and circulates in plasma. It is a plasma lipid transfer protein that facilitates the transport of cholesteryl esters and triglycerides between the lipoproteins.

The role of CETP in atherosclerosis remains unclear. Some evidence suggested that CETP decreases the concentration of HDL (Figure 2.3). Because most of the

cholesteryl esters (CEs) in plasma originate in HDL are catalysed by LCAT and most of the triglyceride enters the plasma as a component of chylomicrons (CM) and VLDL, so the overall effect of CETP is a net mass transfer of cholesteryl esters from HDL to triglyceride-rich VLDL, IDL and LDL and of triglyceride from VLDL to LDL and HDL. Hence, CETP-mediated transfer of CE from HDL is a reduction in the CE content in apoA-1 containing HDL particles (Rye *et al.,* 1999). Hence, it may decrease the anti-inflammatory impact of this lipoprotein distribution leading to pro-atherogenicity (Agellon *et al*., 1991).

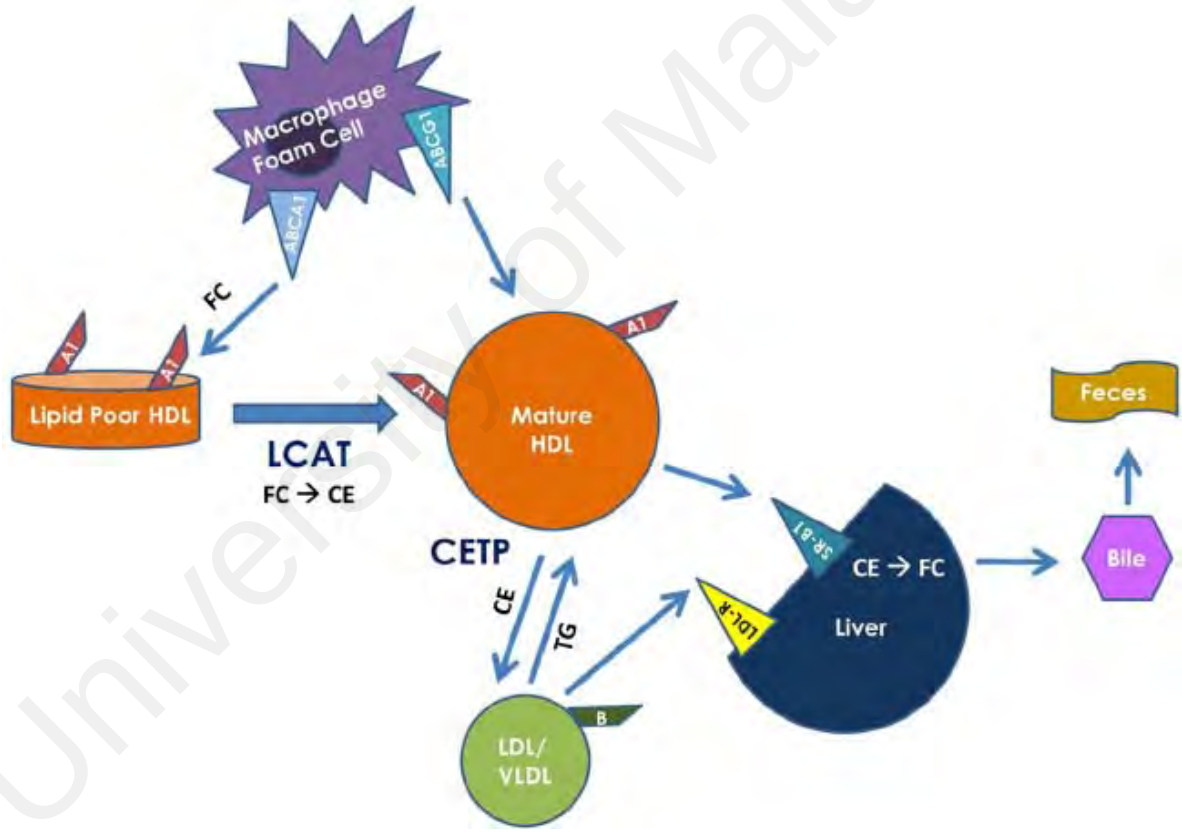

Figure 2.5: Roles of LCAT and CETP in lipid metabolism (Rohatqi, 2015).

A CETP inhibitor is a member of a class of pharmacological drugs that inhibit CETP activity. Some CETP inhibitors, i.e., Torcetrapib, Dalcetrapib and Evacetrapib are intended to reduce the risk of atherosclerosis by improving plasma lipid profile. However, these drugs generally failed in clinical trials either causing a marked increase in deaths or

intolerable side effects despite HDL increases (Drahl, 2012; Mohammadpour *et al.*, 2013).

2.3 Natural sources of anti-atherogenic agents from fungi

Mushrooms are not only well-known for their nutritional components but also their therapeutic values worldwide. The interest in mushroom research has peaked recently due to their immunity and cellular protection which are important for individuals who have critical health issues. Investigations on the medicinal and nutritional potential of mushrooms are underway throughout the world. Researchers are recently providing supportive information on the array of bioactive compounds found within the medicinal fungi (Rahi & Malik, 2016).

Since ancient times people have been consuming herbs and its derivatives as therapeutic medicine (mainly the secondary metabolites) for their primary healthcare needs (Kashani *et al.,* 2012). The pharmacological actions of natural product-based cardiovascular medicines are often dependent on the presence of phytochemicals (like alkaloids, terpenoids, phenolics, flavonoids). These bioactive constituents are not only serve as good antioxidants and lipid lowering agents, but also provide additional protection to human being from a series of pathogenic attacks. Furthermore, those bioactive compounds may promote self-healing in humans associated with cellular injuries and damages.

2.3.1 Secondary metabolites

The compounds which is synthesised from secondary metabolisms are referred to as secondary metabolites. Secondary metabolites are only produced in specific organs and the composition of secondary metabolites is varied from one species to the other.

Interestingly, the secondary metabolites provide most of the pharmacologically active natural products (Raja & Sreenivasulu, 2015).

Medicinal mushrooms have been used as an alternative therapy to promote health and longevity for the populations all over the world. Presently, there is an increasing interest in the secondary metabolites derived from higher fungi for discovering new therapeutic agents.

In the recent decades, many new secondary metabolites from higher fungi have been isolated. Furthermore, various strategies have been used for enhancing secondary metabolite production by medicinal mushroom fermentation, followed by purification of bioactive secondary metabolites for pharmaceutical application (Zhong & Xiao, 2008).

Secondary metabolites are widely distributed into many classes and play different roles in human health. Hence, in ethnopharmacognosy, herbal materials are commonly extracted by means of liquid solvents (known as the "solid-liquid solvent extraction"), followed by a sequential solvent extraction to separate the phytochemicals of interest, based on their chemical natures and polarities (Bruneton, 1999), before *in vitro* screening of bioactivity was performed (Rimando *et al*., 2001; Vaidya & Antarkar, 1994).

This bioassay-guided fractionation strategy approach is favoured by the pharmaceutical industry. For the last few decades, this approach has not been used to isolate secondary metabolites for cardiovascular incidence. Hence, searching anti-atherogenic agents from medicinal mushrooms may shed the light in reducing atherosclerotic cardiovascular disease.

2.3.2 Phenolic compounds

Mushrooms are getting popular as a foodstuff as well as in medicine due to their phenolic and antioxidant content. Polyphenols are naturally occurring secondary metabolites found largely in mushrooms (Robaszkiewicz *et al*., 2010; Yildiz *et al*., 2014).

They are divided into several classes which include phenolic acid (i.e., hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (i.e., flavonols, flavones, flavanols, flavonones, isoflavones, and proanthocyanidins), stibenes, and lignins.

Phenolic compounds are found to have multiple biological effects, including antioxidant activity. They can act as chain breakers or free radical scavengers. Polyphenols that are present abundantly in the fruiting bodies of mushrooms exhibit good antioxidant capacity in biological system.

According to Palacios *et al*. (2011), twelve common antioxidant phenolic compounds (e.g., caffeic acid, catechin, chlorigenic acid, *p*-coumaric acid, ferulic acid, gallic acid, gentistic acid, *p*-hydroxybenzoic acid, homogentistic acid, myricetin, protocatechuic acid, pyrigallol) were detected in eight types of edible mushrooms namely, *Agaricus bisporus, Boletus edulis, Calocybe gambosa, Cantharellus cibarius, Craterellus cornucopioides, Hygrophorus marzuolus, Lactarius deliciosus* and *Pleurotus ostreatus*.

In addition, polyphenols have been documented to suppress the progression of cancer, anti-ageing, anti-inflammatory and protect against cardiovascular diseases (Robaszkiewicz *et al*., 2010). Epidemiological studies have suggested that long term consumption of polyphenol-rich diets could protect against the development of cancer, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Pandey & Razvi, 2009).

2.3.3 Mushrooms with antioxidant capacity

Apart from the endogenous antioxidant defence mechanism of the human body, its dietary intake of antioxidants may contribute to oxidative homeostasis. Antioxidant-containing mushrooms may be useful to help humans to reduce oxidative damage. The growing interest for healthy mushroom food is influenced by the growth of the global antioxidant market. One of the significant findings from Xu *et al*. (2017) has

demonstrated that the purified fraction of polysaccharide obtained from the mycelia of *P. erynii* var. tuoliensis showed marked increase of antioxidant enzymes (SOD, GSH-Px and CAT) in high-fat induced hyperlipidemic mice. It supports the use of mushrooms as functional foods and natural drugs in preventing the hyperlipidemia and non-alcoholic fatty acid.

Synthetic phenolic antioxidants i.e., butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT) effectively inhibited cellular oxidation. However, some synthetic antioxidants may lead to adverse side effects under certain conditions (Ferreira *et al*., 2009). In recent years, edible and medicinal mushrooms have gained the greatest attention as a commercial source of antioxidants. They might directly enhance the antioxidant defence system through dietary supplementation to reduce the oxidative stress. Edible and medicinal mushrooms that are either harvested in wild or cultivated domestically are now available in the market. The true nutritive potential of mushrooms has been rapidly explored and recognised not only by mushroom researchers but also consumers. Some researchers claim that mushrooms could be a good source in the prevention of atherogenic cardiovascular disease due to their antioxidant capacity (Kozarski *et al*., 2015).

Recently, there is a wealth of scientific data coming from both *in vitro* and *in vivo* studies models to support the oxidation hypothesis of atherosclerosis. A corollary of this hypothesis is that antioxidants prevent LDL oxidation and therefore, protect against the development of atherosclerosis (Chisolm & Steinberg, 2008). Many of antioxidant compounds derived from higher fungi showed its activity in *in vitro* model, but its mechanism on anti-atherosclerotic effects remains unclear. Sanchez *et al*. (2017), has shown that extracts obtained from mushrooms contain antioxidant components (e.g., phenolics, polysaccharides, tocopherols, flavonoids, carotenoids, glycosides, ergothioneine and ascorbic acid) that demonstrated good anti-oxidant capacity in DPPH

and ABTS assays, superoxide anion radical scavenging activity assay, and etc. Hence, mushrooms are good natural source of food and antioxidants. The antioxidant potential in mushrooms is higher than that in most vegetables and fruits.

Besides antioxidant property, mushrooms have gained considerable attention for other medicinal properties which include antitumor, antiviral, anticoagulant, anti-diabetic, hypolipidemic, hepatoprotective, immunological and immunostimulant potentials making them suitable as foods or supplements.

2.3.4 Mushrooms with lipid lowering capacity

It has been suggested that mushrooms have beneficial lipid modulating effects. From 380 strains of fungi tested, those especially from the basidiomycetous genus *Pleurotus*, e.g, *P. ostreatus, P. saca* and *P. sapidus* have been found to contain statin (the inhibitor of HMG-CoA reductase). They could be promising source of the anti-hypercholesteromic agent (Gunde-Cimerman *et al*., 1993). Besides, Zhao *et al*. (2015) has also reported that a purified extract from the fermentation of marine fungus *(Fusarium soloni*) demonstrated good anti-atherosclerotic effect by directly inhibited HMG-CoA reductase activity.

The cholesterol lowering effect of the mushroom *Pleurotus ostreatus* (oyster mushroom) has been rapidly and extensively studied. Recently, research had shown that the *P. ostreatus* demonstrated hypolipidemic activities in hypercholesterolemic rats. Feeding a diet containing 5% of *P. ostreatus* fruiting bodies to hypercholesteromic rats reduced plasma triglyceride, cholesterol, low-density lipoproteins (LDL) and LDL/HDL ratio. This finding has suggested the health benefits of mushroom on improving lipid profile in hypercholesterolemic rats (Alam *et al*., 2011). Similarly, research conducted by Bobek *et al*. (1993 & 1994) using Wistar rat fed with diet containing 0.3% cholesterol as well as 4% dried oyster mushroom has led to increased catabolism of LDL and promote

excretion of bile acid via increasing the cholesterol 7α-hydroxylase (a rate-determining enzyme of cholesterol catabolism) and the LCAT activity. According to Xu *et al*. (2017), high-fat induced hyperlipidemic mice treated with purified polysaccharide fractions obtained from the mycelia of *P. eryngii* var. tuoliensis showed significant marked decrease in levels of LDL-C, VLDL-C, TC, TG and marked increase of HDL-C in serum lipid. This may suggest the use of mushrooms as functional foods or natural drugs in the prevention of hypercholesterolemia and cardiovascular diseases.

One of the most popular hypolipidemic agents, lovastatin is a naturally occurring compound derived from oyster mushroom. However, there are many other lipid modulating agents awaiting to be explored from other mushrooms and its bioactive secondary metabolites responsible for lipid ameliorating effects are warranted for future investigation.

From the literature, polyphenols present in the mushrooms have also been reported to play significant roles in improving lipid profile via regulating cholesterol absorption, lowering triglyceride production, and lowering plasma oxidised low-density lipoproteins (Dai *et al*., 2015). However, its exact mechanisms remain to be clarified.

There is increasing evidence suggesting that polyphenols may play important roles in reducing cardiovascular disease (CVD) risk via modulating vascular function and inflammation. Hence, searching for the new source of secondary phytochemicals from medicinal fungi and their roles in anti-atherogenic potentials will provide new information in the cardiovascular disease herbal database (CVDHD) (Gu *et al*., 2013).

2.3.5 Standardisation of herbal medicine

There is increasing interest and public acceptability towards the consumption of natural resources of therapeutics in medical practice. Over 80% of world population currently relies on herbal medicines and supplements for healthy living. (Bodeker *et al*.,

2005). The rise in the use of naturally-derived herbal medicines has also caused increase in various forms of misuse and abuse leading to disappointment and in some instances fatal consequences.

Generally, all herbal medicines should fulfill the basic requirements of being effective and safe. Herbal medicines denote plants that have been prepared into phytopharmaceuticals by means of processes involving harvesting, drying, storage, and extraction. Hence, there are several factors that could lead to variation. The variability could be due to geographical location, differences in growth and time of harvesting.

Standardisation of herbal medicines is the procedure of prescribing a list of standards or constant parameters, definitive qualitative and quantitative values that provide an assurance of quality with regards to efficacy, safety and reproducibility. One of the standardisation methods is chromatographic examination where the major bioactive constituents of the herbal medicines were identified and their amounts were estimated. This has provided information on quality assessment and quality control for better use of herbal medicines (Folashade *et al*., 2012).

Ginkgo biloba standardised extract, for example, is one of the most characterised US FDA approved herbal medicines which is used for the prevention of Alzheimer's disease in elderly groups. This extract was generally standardised to 24% flavonoids and 6% terpenoids (Bent, 2008).

2.4 Lingzhi mushrooms

Historically for over 2000 years lingzhi mushrooms have been well-known as the most respected medicinal mushrooms in Asia; known as the "King of Medicines" as well as "mushroom of immortality" (Arora, 1986). Up to present, lingzhi mushroom is still one of the greatest tonic herbs on earth and has been used as tonic and strengthening medicine for thousands of years. Its Chinese name "Lingzhi", means "spiritual potency"

(or literally mean "Supernatural Fungus"), has been demonstrating life extending properties.

According to the Chinese Pharmacopeia "The Shen Nong's Medica", these miraculous lingzhi mushrooms were appraised and filed as superior medicinal natural herb or unparalleled natural healing herb among all others. They are classified into six types based on its shape, color (green, red, white, yellow, black and purple) (Babu, 2008) and medicinal properties (Yang, 1998).

Although there are more than 2000 known species of lingzhi, only black and red lingzhi have demonstrated the most significant health-enhancing effects, scientifically proven to be safe, and free from side effects (Komoda *et al*., 1989). For the last few decades, the red lingzhi (*G. lucidum*), (the most popular lingzhi mushroom) has been used as medicinal remedy in China, Korea, and Japan for its health promoting properties which include antioxidant, antitumor, antifungal, anti-hypertensive, anti-hyperlipidemic, antiviral, anti-inflammatory agents to treat various diseases, including allergies, arthritis, bronchitis, gastric ulcer, hyperglyceridemia, hypertension, hypercholesterolemia, chronic hepatitis, hepatic disease, insomnia, nephritis, scleroderma, inflammation and cancer (Yang, 1998).

2.4.1. *Amauroderma* species

Ganoderma and Amauroderma are two genera of the *Ganodermataceae* family. An extensive work has been conducted on genus *Ganoderma*. Due to the medicinal properties, two of its species, *G. lucidum* and *G. sinense* are recorded in the Chinese Pharmacopoeia (Zhang *et al.,* 2013). However, there is only little information with regards to the genus *Amauroderma. Amauroderma* species is widespread in tropical areas and contains about 30 species.

Among them, *Amauroderma rude* is being newly described and gaining interest to explore its biological properties. Research conducted by Jiao *et al.* (2013) has demonstrated anticancer potential of *A. rude* which is equally potent as *G. lucidum*. Recently, chemical investigation of the fruiting body of *A. subresinosum* has led to identification of two new bioactive compounds, namely amaurosubresin, and erythro (23, 24)-5α, 6α-epoxyergosta-8-ene-7-one-3β,23-diol which both exhibited inhibitory effect against acetylcholinesterase (AChE) activity (Wang *et al*., 2016).

Similarly, three other bioactive compounds which include a newly identified amauroamoienien and two other known compounds derived from *A. amoiensis* demonstrating inhibitory effect against AChE activity (Zhang *et al*., 2013). The chemical and biological activities of many other *Amauroderma* species are still awaiting to be determined.

2.4.2 *Amauroderma rugosum*

Based on the literature, the black lingzhi, *A. rugosum* (Blume & T. Nees) Torrend (Figure 2.6) (synonym: *Polyporus rugosum*, *G. rugosum*), belongs to the family *Ganodermataceae*, which are commonly known as "black chi" or "false *G. lucidum*". The species is widespread in tropical areas, particularly in China provinces that include Fujian, Guangzhou, Hainan, Guangxi, Guizhao, Yunnan, and other countries including Japan, Malaysia and Philippine. *A. rugosum* (AR) is tough, woody, wild mushroom with black stipes (4 to 15cm) and its fruiting bodies that are typically 3-13 cm diameter (Figure 2.6 a). The cap is kidney-shaped, underside are small white pale grey pores. The polypore fungus grows on the ground, for a year to reach its maturation (Figure 2.6 b)

As recorded in the Compendium of Materia Medica, the black lingzhi, possesses excellent cardiovascular preventive effects, anti-atherosclerotic potential that takes into account the antioxidant capacity, anti-hyperlipidemic, anti-hypertensive, anti-

inflammatory, anti-platelet aggregation and anti-thrombotic effects (Yang, 1998). However, there are only limited reports documents with regards to its cardiovascular benefits.

Studies performed by Chan *et al*. (2013 & 2015) have demonstrated good antioxidant capacity and anti-inflammatory activity of *A. rugosum* on LPS-stimulated RAW264.7 cells. However, there is no scientific proof with regards to its anti-atherogenic mechanisms. Hence, in the present study, the antioxidant capacity, anti-LDL-oxidative property, lipid and lipoprotein modulating potentials and mediated pathway of AR are further investigated. Hereafter, our findings on this investigation definitely will provide new insights on the development of AR as a good source of anti-atherosclerotic agents in the prevention of cardiovascular disease.

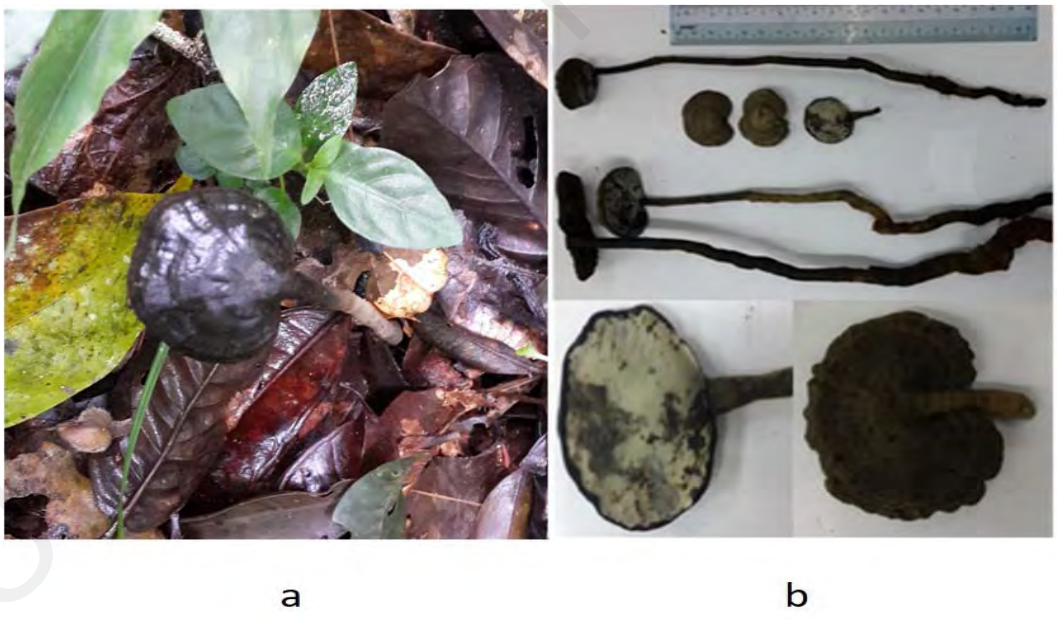

Figure 2.6: The fruiting bodies of *Amaurodema rugosum* (Blume & T. Nees) Torrend

CHAPTER 3: MATERIALS AND METHODS

3.1 **Mushroom material**

The fruiting bodies of the *A. rugosum* used in this study were collected from Brinchang Tropical Forest, Cameron Highland, Malaysia. The sample was identified and authenticated via DNA sequencing method by experts and molecular sequencing by the Mushroom Research Centre, University of Malaya and voucher specimen was deposited in the University of Malaya herbarium (KLU-M1234).

3.2 Chemicals and reagents

Caffeic acid (Catalog no. 205546; purity ≥ 95% by HPLC), vanillic acid (Catalog no. 841025; purity ≥ 98%); protocatechuic acid (Catalog no. 841533; purity ≥ 96%), and 4-hydroxybenzoic acid (Catalog no. 821814; purity ≥ 98%) were purchased from Merck Millipore, USA. Lovastatin was purchased from Sigma-Aldrich, USA and sodium oleate was purchased from Nacalai Tesque, Japan.

3.3 Preparation of mushroom extracts

The preparation of mushroom extract and solvent-partitioned fractions was performed according to the method of Rahman *et al*. (2014) with some modifications. The method was summarised in (Figure 3.1). The mushroom fruiting bodies were sliced, oven-dried, and ground to powder. The mushroom powder (120g) was extracted with (2400 mL) of methanol: dichloromethane (2:1) in shaking flasks (200rpm) at room temperature for 3 days, followed by filtration through Whatman number 1 filter paper.

The extraction was repeated thrice and the total organic solution collected was pooled and evaporated using rotary evaporator (Büchi Rotavapor R-114, Switzerland) that yielded the methanol: dichloromethane (MD) crude extract. The dried, crude extract

was dissolved in 90% aqueous-methanol and partitioned with hexane (1:1). The upper hexane layer was separated using a separating funnel and rota-evaporated to obtain hexane fraction (Hex).

The partition process was repeated until the color of organic solvent become colorless. The bottom aqueous methanolic layer was rota-evaporated which yield a semisolid fraction, followed by re-dissolving it in distilled water (100 mL). Similarly, the successive partitioning process was performed, using other solvents with increasing polarity (e.g. dichloromethane and ethyl acetate) to obtain dichloromethane fraction (DCM) and ethyl acetate fraction (EA), respectively.

Finally, the remaining aqueous layer was freeze-dried to attain the water-soluble fraction (H_2O). A total of five mushroom extracts including the MD crude extract, and the other four extracts (Hex, DCM, EA and H_2O) were kept at -4^{o}C prior to use.

3.4 Evaluation of antioxidant capacity

The antioxidant capacity of mushroom extracts was investigated based on the standard methods. Standard chemicals including quercetin dihydrate, 6-hydroxy-2,5,7,8-tetramethylchlomane-2-carboxylic acid (Trolox), $FeSO_4 \cdot 7H_2O$, sodium ethylenediamine tetraacetate (Na_2EDTA) and gallic acid were obtained from Sigma-Aldrich (St. Louis, USA). Other chemicals and solvents used were of analytical grade. All extracts were dissolved in 50% (v/v) Dimethyl sulfoxide (DMSO) in water to produce stock solution of 20 mg/ml and diluted to desired concentration for the following assays:

3.4.1 Folin-Ciocalteu assay

The total phenolic content of the mushroom extracts was estimated using Folin-Ciocalteu reagent according to the method of Abdullah *et al*. (2012) (Appendix A: Figure

A.1). Briefly, equal volume (250 µL) of mushroom extract (100 µg/mL) was added into 10% (v/v) Folin-Ciocalteu reagent and incubated for 2 minutes at room conditions.

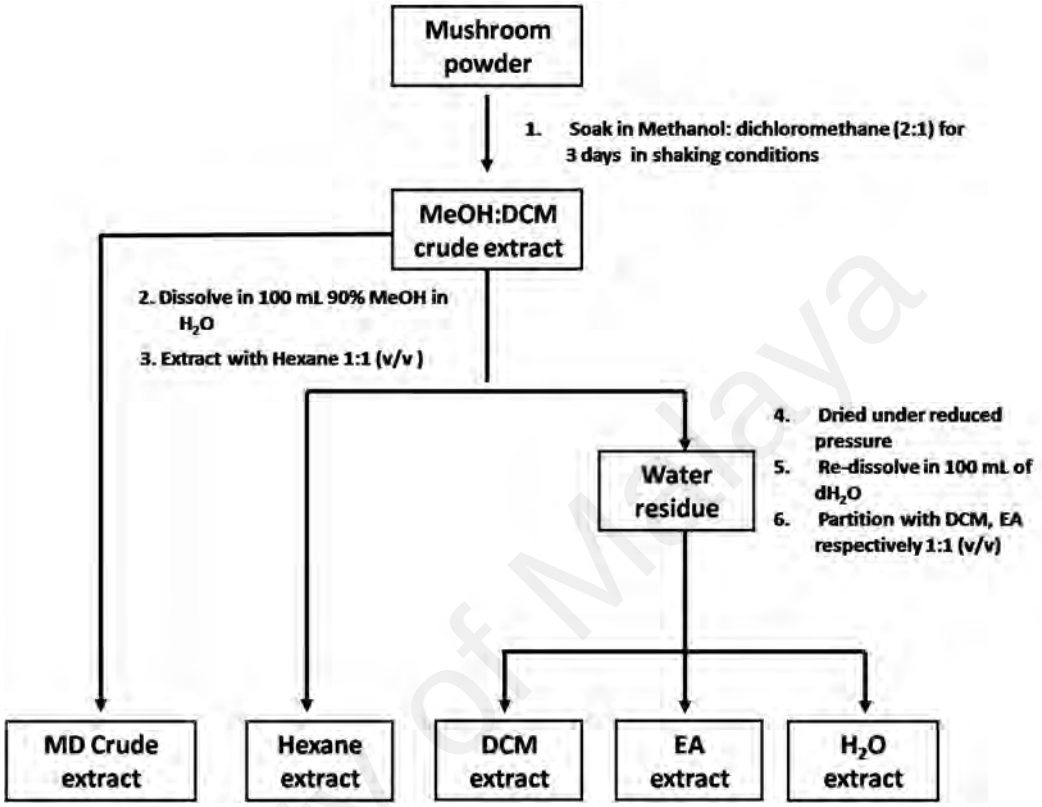

Figure 3.1: The preparation of mushroom extracts by organic solvent partition method.

Next, 500 µL of 10% (w/v) of aqueous sodium carbonate was added and the mixture was kept for another 1 hour in dark conditions before the absorbance at 750 nm was measured using UV-Vis Spectrophotometer (Shimadzu, Japan). A calibration curve was established using standard, gallic acid (2-10 µg/mL). The results were expressed as mg gallic acid equivalents (GAEs)/g of dried extract based on the standard curve in Appendix B: Tables B.1, Table B.2, and Figure B.1.

3.4.2 1,1-Diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity

The DPPH free radical scavenging activity of the extracts was measured according to method Abdullah *et al*. (2012) (Appendix A: Figure A.2). Briefly, 5 µL of

mushroom extract (100 µg/mL) was added to 195 µL of 1 mM DPPH solution in 96-well plate. The mixture was incubated at room conditions for 30 minutes in dark before absorbance at 515 nm was measured using ELISA plate reader (Sunrise, Tecan). The DPPH free radical scavenging activity was expressed as IC_{50} value (the concentration of extract required to produce 50% inhibition). Quercetin dihydrate was used as positive control while methanol is used as blank in this assay. The percentage of DPPH radical-scavenging activity was calculated using the formula as below:

$$\text{DPPH radical scavenging activity} = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 is the absorbance of the black and A_1 is absorbance of sample

3.4.3 Metal-chelating activity

The metal chelating effect was determined according to the method of Lau *et al.* (2014) (Appendix A: Figure A.2). Briefly, 1 mL of the mushroom extract (100 µg/mL) was mixed with 100 µL of 2 mM $FeCl_2$ and the mixture was incubated for 1 minute. Then, 200 µL of 5 mM ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate) and water were added to reach a final volume (5 mL). The absorbance was measured at 562 nm using UV-Vis Spectrophotometer (Shimadzu) after 20 minutes of incubation in dark at room conditions. The metal chelating activity was expressed as IC_{50} value. Na_2EDTA was used as positive control while distilled water was used as blank in this experiment.

3.4.4 Cupric ion-reducing antioxidant capacity (CUPRAC)

CUPRAC assay was performed according to the method of Abdullah *et al.* (2012) (Appendix A: Figure A.1). Briefly, 1 mL of the mushroom extract at 100 µg/mL was added to the reaction mixture containing 1 mL of Neocuproine (7.5 mM), 1 mL of copper

(10 mM) and 1 mL of ammonium acetate (1M) at pH 7.0. The mixture was incubated at room conditions for 30 minutes before the absorbance at 450 nm were measured using UV-Vis Spectrophotometer (Shimadzu). A calibration curve was established using Trolox (1-100 µg/mL) as shown in Appendix B: (Figure B.2 and Table B.5), and the result was expressed as mg Trolox equivalent/g of dried extract.

3.4.5 Inhibition of lipid peroxidation

The inhibitory effect of the extract against lipid peroxidation was determined based on a method that measure thiobarbituric-acid-reactive substances (TBARS) in $FeSO_4$-induced lipid peroxidation in egg yolk homogenates (Lau *et al*., 2014) (Appendix A: Figure A.3). Briefly, 1 mL of fowl egg yolk was added to 0.1 M phosphate buffer at pH 7.4 to prepare a buffered egg yolk mixture (25g/L) before 100 µL of Fe^{2+} solution (1 mM) was added. Then the mushroom extract (100 µg/mL) was added to the mixture and subjected to 1 hour incubation at 37ºC. After this, 0.5 mL of 15% trichloroacetic acid (TCA) and 1.0 mL of 1% thiobarbituric acid (TBA) was added to the reaction mixture and allowed to incubate in boiling water for another 15 minutes. The mixture was then cooled to room condition and subjected to centrifugation at 3500 x g for 10 min. The supernatant was collected and measured at 532 nm using ELISA microplate reader (Tecan) for the formation of thiobarbituric acid reactive substances (TBARS).The α-tocopherol was used as positive control while the buffered egg solution with Fe^{2+} only served as control. The percentage inhibition was calculated based on the following equation and the inhibitory activity (%) was expressed as IC_{50} value.

$$\text{Inhibition (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of blank and A_1 is the absorbance of the sample

3.5 Inhibitory effect on Cu^{2+}-induced LDL oxidation

The native low-density lipoprotein (nLDL) from human plasma (formulated in 150 mM NaCl, 0.01% EDTA, pH 7.4) used in this assay was purchased from EMD Millipore (Billerica, MA, USA). The human LDL (density 1.02-1.063g/mL), consists of 78-81% lipids and 19-22% LDL protein. To measure the effect of fractions on the inhibition of the oxidation of human LDL, the method of Rahman *et al*. (2014) (Appendix A: Figure A.3) was performed with some modifications by measuring the formation of conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS), respectively. Quercetin dihydrate was used as positive control.

To measure the effect of extracts on conjugated diene (CD) formation, the LDL was adjusted to a final concentration of 150 µg/mL in the reaction mixture containing 193 µL 0.1M phosphate buffer, pH 7.4 (to simulate human physiological and blood pH), 4 µL LDL and 1 µL extract (1µg/mL) in 96-well plate . The reaction was initiated by adding 2µL of 1mM $CuSO_4$ solution and incubated at 37ºC. The kinetics of LDL oxidation was monitored by measuring absorbance at 234nm every 20-minutes intervals for a period of 5 h using spectrophotometer (Synergy™ H1, Bio Tek). The mixture without $CuSO_4$ inducer was used as blank.

To determine the formation of thiobarbituric acid reactive substances (TBARS), the assay was performed according to the method of Ahmadvand *et al.* (2011) with some modifications. Briefly, 191 µL 20 mM $CuSO_4$ solution was added into a reaction mixture that contained 9 µL human LDL (adjusted to 150 µg/mL in phosphate buffered saline, pH 7.4) and 100 µL extract (1 mg/mL). The reaction mixture was then incubated for 3 h before the reaction was terminated by adding 50 µL of 100 mM Na_2EDTA solution. To the mixture, 240 µL of 15% trichloroacetic acid (TCA) and 400 µL of 1% thiobarbituric acid (TBA), both freshly prepared, were added and incubated in boiling water for 30 min. Upon cooling, 200 µL of aliquot was transferred into 96-well plate and absorbance at 532

nm was recorded. LDL without $CuSO_4$ served as negative control, and the LDL with $CuSO_4$ inducer was used as blank. A calibration curve was established using malondialdehyde (MDA) standard (Merck, Darmstadt, Germany) (0.625-100 μM), assayed under similar conditions, and the result was expressed as nmol malondialdehyde (MDA) equivalent/ mg LDL protein.

3.6 Inhibitory effect of HMG-CoA reductase activity

The HMG-CoA reductase assay kit from Sigma-Aldrich (St. Louis, MO, USA) with the catalytic domain of human enzyme was used, under conditions recommended by the manufacturer. The concentration of the purified human enzyme stock solution was 0.50-0.70 mg protein/mL. The reference drug, pravastatin was used as positive control. The mushroom extract was hydrolysed according to the method reported by Yang *et al*. (2006) (Appendix A: Figure A.4). Briefly, 25 μL of 0.1 N NaOH solution was added to mushroom extract (10 mg/mL) and incubated at 50°C for 2 h, followed by neutralisation with 25 μL of 0.1 N HCl. The hydrolysed sample was kept in 4°C before use.

To characterise HMG-CoA reductase inhibition, under defined assay conditions, reaction containing 4 μL of NADPH (to obtain a final concentration of 400 μM) and 12 μL of HMG-CoA substrate (to obtain a final concentration of 400 μM) in a final volume of 0.2 mL of 100 mM potassium phosphate buffer, pH 7.4, containing 120 mM KCl, 1 mM EDTA and 5 mM DTT, were initiated (time 0) by addition of 2 μL of the catalytic domain of human HMG-CoA reductase and incubated in 96-well plate at 37°C in the presence and absence (control) of 1 μL aliquots of extracts (5mg/mL), pre-treated with 0.1 M HCl and 0.1 M NaOH for the activation of compounds, if any, to bind with the HMG-CoA reductase. The pravastatin, transformed into active open dihydroxy carboxylic acid form, was used as positive control.

The rate of NADPH consumed was monitored every 20 sec for up to 20 min by scanning spectrophotometrically the decrease in absorbance at 340 nm, (represents the oxidation of NADPH in the presence of the substrate HMG-CoA). The activity of the extracts was calculated according to the equation provided by the manufacturer. Results were expressed as specific activity of the enzyme (μmol of NADPH oxidised/min/mg HMG-CoA reductase protein).

3.7 Chromatographic and mass-spectrometric analyses

Chemical analysis on the bioactive extract was conducted based on the method of Lau *et al*. (2014) using Liquid Chromatography –Tandem Mass Spectrometry (LC-MS/MS) (UHPLC, PerkinElmer, Inc., Massachesetts, USA) coupled with an AB SCIEX 3200 QTrap hybrid linear ion trap triple-quadruple mass spectrometer equipped with a turbo ion spray source. Chromatographic separation was performed on a Phenomenex Aqua C18 (5 μm, 50 mm×2 mm) column. Mobile phase A (composed of water with 0.1% (v/v) formic acid and 5 mM ammonium formate), whereas the mobile phase B (consisted of acetonitrile containing 0.1% (v/v) formic acid and 5 mM ammonium formate) were prepared.

Elution was programmed following a linear gradient from 10−90% B (0−8 min) held for 3 min, decreased back to 10% B in 0.1 min, and then re-equilibrated for 4 min before the next injection. Ionisation was done via electrospray ionisation on the AB Sciex Turbo V source with an ionisation temperature of 500°C and purified nitrogen gas (99%). Full scan with MS/MS data collection analyses was conducted in negative mode. Data analysis, processing, and interpretation were performed using the AB SCIEX Analyst 1.5 and Advanced Chemistry Development, Inc., (ACD/Labs, Ontario, Canada) MS Processor software. The MarkerView Software (AB SCIEX, Massachusetts, USA) was employed for principal component analysis (PCA). Peak identification was achieved by

comparing the molecular weights and the fragmentation patterns of the molecular ions with those available in the natural products library and the published data in the literature review.

3.7.1 Quantification of selected bioactive compounds

The concentrations of selected bioactive compounds in the EA extract was determined by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) method in HPLC system (Perkin Elmer FX15), equipped with AB Sciex 3200 Trap MS/MS. The separation was performed on Zorbax C-18 column (150 mm x 4.6 mm, 5 µm). Two solvents were used for the mobile phase: (A) water with 0.1% formic acid and 5mM ammonium formate, (B) acetonitrile with 0.1% formic acid and 5mM ammonium formate. Elution was carried out at a linear gradient from 5% B to 95%B (0-5 min), held for 2 min, and return to 5%B in 0.1 min and re-equilibrated for 3 min. The bioactive extract and standard pure compounds were dissolved in methanol and injected at 10 uL. The concentrations of each bioactive compounds in the bioactive extract was measured based on the calibration curves established, using standard compounds, respectively.

3.8. Cell culture

The human hepatocellular liver carcinoma cells (HepG2, ATCC) and the human hepatic cells (WRL-68, ATCC) were supplied by Medical Biotechnological Laboratory, UM, whereas the human lung fibroblast (MRC-5) was purchased from American Type Culture collection (ATCC). Except for MRC-5 cells (grown in MEM medium), the cells were grown in T75 flasks and maintained in a monolayer culture at 37^{o}C and 5% CO_2 in DMEM (Dulbecco's Modified Eagles's Medium) (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10 % fetal bovine serum (FBS), 10U/mL penicillin-streptomycin, and 0.25 µg/mL amphotericin B. Medium was replaced with fresh medium every 3-4 days.

3.8.1 Cytotoxicity study

The cytotoxicity of mushroom extracts on HepG2, WRL 68 and MRC-5 cells was evaluated using 3-(4,5-dimethythiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the method of Lau *et al*. (2004), with slight modifications. Viable cells (3-5 x 10^3 cells/well) were seeded into 96-well plate and incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. Next, the cells were treated with the mushroom extracts (6.25 – 400 µg/mL) (final concentration < 1% DMSO). The untreated cell (< 0.1 % dimethyl sulfoxide (DMSO) in the medium served as control. After 72 h of incubation, the supernatant from the well was replaced with fresh culture medium before 20 µL of MTT reagent (5mg/mL) was added and the mixture was incubated for another 4 h at 37°C. Thereafter, medium was replaced with 100 µL DMSO to dissolve the formazan salt crystals. The amount of formazan product was measured at 570 nm using microplate reader and the percentage of cell viability was calculated relative to the control (untreated cells). Results were expressed as mean of IC_{50} value $\pm$ standard deviation.

3.8.2 Lipid and apolipoprotein assays

Prior to each experiment, cell density at 1 x 10^6 cells/dish were seeded into 6-well plates and allowed to attach for 24 h. Pre-treatment period was initiated by incubating cells with serum-free DMEM containing 1% of lypophilised fraction V Bovine serum albumin (1% BSA) by mass for 24 h before cells were treated with serum-free DMEM containing 1%BSA complexed with 0.8 mM sodium oleate with the presence of the mushroom extracts or lovastatin (positive control). HepG2 cells treated with 0.8 mM sodium oleate only served as control).

After the completion of the treatment, the culture medium and cell sample were collected. Due to different nature of cellular lipids and apolipoproteins, the cellular contents were collected in different methods (Appendix A: Figure A.5). Briefly, for lipid

assay, the cell was washed with cold phosphate-buffered saline (PBS), detached by scrapper and harvested without lysing the cells. The cell suspension was then transferred into 15 mL centrifuge tube and centrifuged at 1800 rpm at 4ºC. The suspension was discarded and the cell pellet was collected.

The cellular and media lipids were extracted immediately according to the manufacturer's instruction. However, as for apolipoprotein assay, cells were washed, detached and lysed with RIPA lysis buffer (25mM Tris.HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1%SDS)(Merck Millipore, USA) pre-mixed with protease and phosphate inhibitor cocktail. The suspension was transferred into 2 mL Eppendorf tube and centrifuged at 1800 rpm at 4ºC. The supernatant (cell lysate) was collected. The medium and cell sample were kept in -80ºC prior to assay. Cell protein was quantified with Coomassie Plus™ (Bradford) Assay Kit (Thermo Scientific, USA) using BSA as a standard.

3.8.2.1 Extraction of cholesterol and triglyceride

The intracellular and secreted lipids were extracted using freshly prepared organic solvent mixture [hexane: isopropanol: NP-40 (7:11:0.1)] according to the manufacturer's protocol (Appendix A: Figure A.5). Briefly, (2.0-5.0) mL of the organic solvent was added to the medium and cell pellet, respectively. The suspension was vortexed periodically for 2 hours before organic phase was transferred into 2.0 mL Eppendorf tube followed by air-dried at 50ºC in oven. The dried lipid extract was kept in -20ºC prior to assay.

3.8.2.2 Determination of TC, FC and CE

The intracellular and media cholesterol was determined by using commercial enzymatic assay kit (Cat. No: K603-100) (BioVision, Inc., USA) based on the Abell-

Kendall (A-K) method (Appendix A: A.6). Briefly, the dried lipid extract was re-dissolved in 50 µL cholesterol assay buffer, and the assay was initiated by adding 50 µL of freshly mixed enzymatic solution (containing 44 uL of Cholesterol Assay Buffer, 2 µL Cholesterol Probe, 2 µL Cholesterol Enzyme Mix, and 2 µL Cholesterol Esterase). The reading at 570 nm was measured after 1h incubation at 37^{o}C and the amount of total cholesterol was calculated based on the calibration curve established using cholesterol standard (1-5) µg/mL. Similarly, total free cholesterol was determined by adding freshly mixed enzymatic solution (with the absence of Cholesterol Esterase) to the extract, while the amount of cholesteryl ester (CE) was determined by subtracting the value of free cholesterol (FC) from the value of total cholesterol (TC). The amount of TC, FC and CE was normalised to cellular protein content and the results were expressed as µg of total cholesterol/mg cell protein $\pm$ standard deviation.

3.8.2.3 Determination of TG

The cellular and media TG was determined by using commercial enzymatic assay kit (Cat. No: 10010303) (Cayman Chemical Company, USA) (Appendix A: Figure A.7). Briefly, the dried lipid extract was re-dissolved in 50 µL diluted standard diluent assay reagent. The assay was initiated by adding 150 µL of freshly mixed enzymatic buffer solution (consisting of 10 µL Triglyceride Enzyme Mixture and 140 µL of 50 mM sodium phosphate buffer, pH 7.2) to 10 µL of re-dissolved lipid extract. The reading at 540 nm was measured spectrophotometrically after 15 min incubation at room conditions. The amount of triglyceride determined based on the TG standard calibration curve (0-200 mg/dL) was normalised to cellular protein content. The result was expressed as µg triglyceride/mg of cell protein $\pm$ standard deviation.

3.8.2.4 Determination of apoA1, apoB-100 and apoE

The intracellular and secreted apoA1, apoB-100 and apoE levels were measured by using respective sandwich enzyme-linked immunosorbent assay (ELISA) apoA1 (cat. No.: KA0460), apoB (human) (Cat. No: KA1028) and apoE (Cat. No: KA1031) kits (Abnova, Taiwan). The experiment was performed separately according to the manufacturer's instructions at room conditions. Otherwise stated, the same protocol applied to all three independent assays (Appendix A: Figures A.8, A.9, and A.10). Briefly, for apoA1 measurement, 25 µL of biotinylated apoA1 primary antibody and 25 µL of diluted sample were added together (in triplicate) to commercial microplate pre-coated with goal polyclonal anti-human apolipoprotein and then incubated for 2 hours.

Unlike apoA1 measurement, 25 µL of the diluted sample containing apoB-100 or apoE was added to the pre-coated microplate. The primary antibody was added to the washed plates after 2 hour incubation. Then, plate (containing either apoA1, apoB-100 or apoE-bound protein) was washed, followed by addition of secondary antibody diluted 1: 100 in MIX Diluent to each sample and incubated for another 30 min. After incubation, the plate was washed before 50 µL of chromogen substrate was added to the well containing apoAI, apoB-100 or apoE-bound target for 10-15 minutes incubation. A Stop Solution was added and the colour intensity of the solution was read at 450 nm using an ELISA plate reader. The level of apoA1, apoB-100 or apoE determined from standard curve plotted with known concentration of apoA1, apoB-100 and apoE standards, was normalised to total cellular protein. The result was expressed as µg apolipoprotein (apoA1, apoB-100 or apoE)/mg cell protein.

3.9 Transcription factor binding assay

After the treatment, HepG2 cells were harvested with Nuclear Extraction kit (Cat. No: 10009277) (Cayman Chemical, USA) according to the manufacturer's instruction

(Appendix A: Figure A.11). The extent of DNA binding of PPAR-α, SREBP-1 and SREBP-2 transcription factors was determined by an immunosorbent assay (ELISA) using PPAR-α (Cat. No: KA1356), SREBP-1 (Cat. No: KA 1377) and SREBP-2 (Cat. No: KA1379) transcription factor assay kits (Abnova, Taipei City, Taiwan) respectively. Assays were performed in triplicate in three separate experiments independently (Appendix A: Figure A.12).

Briefly, a specific double stranded DNA sequence containing transcription factors were immobilized onto the bottom of the wells of the 96-well plates. Aliquots of nuclear extract containing the target transcription factor bind specifically to their response elements. The amount of transcription factors present were detected by addition of specific primary antibody directed against PPAR-α, SREBP-1 and SREBP-2 transcription factors respectively.

A common secondary antibody conjugated to Transcription Factor Goat anti-rabbit HRP was added to the well and the mixture was read spectrophotometrically at 450 nm. The positive control of the transcription factor (an activated lysate) supplied by the manufacturer was diluted in 2x-serial dilution and used to generate a calibration curve.

The results of transcription factor-dsDNA binding activity was measured based on the calibration curves established. The OD absorbance measured was normalised to cellular nuclear protein. The result of binding activity was expressed as fold change of transcription binding activity $\pm$ standard deviation, relative to untreated cells (control), respectively.

3.10 Determination of LXR-α, LCAT and CETP

The LXR-α, and LCAT protein levels were determined by using Human Liver X Receptor α (LXRα) (Cat. No.: CSB-E-14346h) and human Lecithin Cholesterol Acyltransferase (LCAT) (Cat. No.: CSB-E-13469h) ELISA kits, respectively purchased

from Cusabio, USA while CETP protein level was determined by using human cholesterol ester transfer protein (CETP) (Cat. No.: STA-614) ELISA Kit (Cell Biolabs, INC., San Diego, USA). The primary antibodies used were biotin-conjugated antibody specific for LXR-α and LCAT, anti-CETP antibody, respectively. The common secondary antibody used was avidin conjugate Horseradish Peroxide (HRP).

The assay was conducted in a standard procedure as in ELISA Kits. After washing steps upon addition of primary and secondary antibody, a TMS substrate and Stop Solution were added subsequently to produce the color intensity before the readout was measured at 450 nm. The protein levels of LXR-α, LCAT and CETP were quantified based on the standard curves established, respectively. The results were expressed as mean of fold changes $\pm$ standard deviation, relative to untreated HepG2 cells (control).

3.11 Quantitative real time polymerase chain reaction (RT-PCR)

Total RNA was extracted from HepG2 cells using total RNA mini kit (FavorPrep^{TM}, Taiwan) (Appendix A: Figure A.13). The purity and concentrations of total RNA were determined by spectrophotometry at 260/ 280 nm and 260/230 nm (NanoDrop 2000, Thermo Scientific). The RNA sample was then reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad) to produce cDNA (final concentration 2000 ng/ml) (Appendix A: Table A.1).

Real time PCR was conducted on cDNA samples using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Genes i.e., *APOA1* (Accession number: NM_000039), *APOB* (NM_000384), *APOE* (NM_000041), *PPAR-α* (NM_001001928), *SREBF-1* (NM-_001005291), *SREBF-2* (NM_004599), *NR1H3* (NM_001130101), *LCAT* (NM_000229), *CETP* (NM_000078) and *GADPH* (NM_002046) primers purchased from Genecopoeia, USA. *GADPH* was used as a housekeeping reference gene.

The sample mixtures were incubated at 95ºC for 10 min for pre-denaturation, followed by 40 cycles of denaturation at 95ºC for 10s, then combined annealing and extension at 72º C for 15s. The amplification of specific transcripts was confirmed by melting curves analysis at the end of each run. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA expression levels.

3.12 Statistical analysis

All the results were the mean (± standard deviation) of triplicates and subjected to one-way analysis of variance (ANOVA). The significance difference was determined by Duncan test at 95% ($P<0.05$) using STATGRAPHICS plus for Windows 3.0. Correlation and regression analysis were done by performing Microsoft EXCEL 2010 statistical analysis.

CHAPTER 4: RESULTS

4.1 Antioxidant and lipid lowering potential of AR extracts

Validation of AR as anti-atherogenic agent was initially carried out by searching for antioxidant capacity exerted by the mushroom extracts in an *in vitro* cell free system. The inhibition on LDL oxidation and HMG-CoA reductase activity was further investigated using enzymatic assays. These two assays are important to confirm the lipid lowering and anti-LDL oxidation potentials of AR by means of preventing atherogenesis. Then the mechanism of lipid ameliorating effect was postulated based on the protein and gene expression studiesusing ELISA and qRT-PCR methods.

4.1.1 Antioxidant capacity

There are several methods available for the determination of antioxidantcapacity. In the present study, screening for exogenous antioxidants was selected based on different defense mechanisms, i.e., hydrogen atom transfer (HAT) based methods which include lipid peroxidation inhibition capacity (LPIC) and transition metal ion chelating assays while electron transfer-based methods which include Folin-Ciocalteu method used for the determination of total phenolic content, DPPH radical-scavenging assay and CUPRAC reducing capacity assay. The estimation of total phenolic content, radical-scavenging activities, metal-chelating activities, reducing ability and inhibitory effects on lipid peroxidationevaluated onfive different extracts, i.e., Methanol: dichloromethane crude extract (MD), hexane (Hex), dichloromethane (DCM), ethyl acetate (EA), and water (H_2O) fractions. The results are summarised in Table 4.1(Appendix B: TablesB.1, B.2, B.3, B.4, B.5, B.6 and B.7; FiguresB.1, B.2 and B.3).

Mushroomsare well-known for its antioxidant property and for that reason; the phenolic content was measured using Folin-Ciocalteu method. The EA ulteriorly was

found to contain significantly ($p< 0.05$) higher total phenolic content (255.9±0.02 GAEs/g of extract), as compared to other factions e.g.,Hex, DCM, H_2O and MDon average 10-fold lesser in a range of 25.2-30.2 GAEs/g of extract.

DPPH assay was employed to test the free radical scavenging ability of AR. The lower the IC_{50} value indicated the better radical scavenging activity. Consistent with its high phenolic content, the EA showed the strongest scavenging effects among the five extracts tested (($p< 0.05$). Moreover, it was found that the radical scavenging ability of EA (IC_{50}: 0.34±0.06 mg/mL) was comparable to the positive control, quercetin dihydrate (IC_{50}: 0.24±0.05 mg/mL).

According to the "antioxidant hypothesis", some species, in spite of directly attacking living system, by which they can indirectly induce the generation of reactive radical species, are considered oxidants. In this connection, the transition metal ions (Cu^{2+} and Fe^{2+}) potentially stimulate lipid peroxidation through Fenton reactions, to produce highly reactive hydroxyl (OH·) radicals (Maxwell*et al.,* 1997). Both the EA (IC_{50}:1.37±0.15 mg/mL) and DCM fractions (IC_{50}:2.83±0.75 mg/mL), exhibited good ferrous ion (Fe^{2+}) chelating ability($p< 0.05$) compared to other extracts (IC_{50}: 10.6-17.2 mg/mL). The chelating activity of DCM fraction was not significantly different compared to that of EA and the positive control, Na_2EDTA (IC_{50}:0.09±0.01 mg/mL).

The CUPRAC method is based on the absorbance measurement of Cu(I)-neocuproine (Nc) chelate formed as a result of the redox reaction (via electron transfer) of chain-breaking antioxidants with the CUPRAC reagent.The EA showed the most potent reducing power ($p< 0.05$) among the five extracts exhibiting1492.9±54.5mg Trolox equivalent/g of extract.

Table 4.1: Antioxidant capacity of ARextracts.

Extracts	[i]Total phenolic content (mg of GAEs/g of extract)	DPPH (IC_{50}, mg/ml)	Metal chelating (IC_{50} , mg/ml)	[ii]CUPRAC (mg Trolox equiv./g extract	Inhibition of lipid peroxidation (IC_{50}, mg/ml)
Hex	30.2 ± 0.02^{a}	29.6 ± 1.46^{d}	13.5 ± 0.71^{c}	66.1 ± 0.65^{a}	10.5 ± 0.68^{f}
DCM	25.2 ± 0.01^{a}	7.06 ± 0.72^{b}	2.83 ± 0.75^{b}	270.5 ± 0.43^{c}	3.15 ± 0.11^{b}
EA	255.9 ± 0.02^{b}	0.34 ± 0.06^{a}	1.37 ± 0.15^{b}	1492.9 ± 54.5^{d}	2.83 ± 0.07^{c}
H_2O	29.8 ± 0.04^{a}	15.0 ± 1.20^{c}	17.2 ± 0.75^{d}	135.2 ± 10.8^{b}	8.08 ± 0.18^{e}
MD	26.8 ± 0.01^{a}	6.91 ± 1.02^{b}	10.6 ± 0.47^{c}	243.2 ± 5.56^{c}	6.05 ± 0.18^{d}
Positive control					
Quercetin dihydrate	465 ± 0.014^{c}	0.24 ± 0.05^{a}	-	1580.9 ± 180.9^{d}	0.29 ± 0.02^{a}
Na_2EDTA	-	-	0.09 ± 0.01^{a}	-	-
α-tocopherol	-	-	-	-	0.87 ± 0.05^{b}

The extracts were dissolved in 50% (v/v) DMSO in water for the antioxidant assays. Results were expressed as mean ±standard deviation of triplicate measurements (n=3). The different letters (a-f) within a column represent means with significant difference ($p<0.05$). [i]The concentration of extracts used in total phenolic content estimation was 100 µg/mL, except for the positive control, quercetin dihydrate (10 µg/mL). [ii]The concentration of extracts in CURPAC assay was 1mg/mL, and Trolox was used as the standard. Quercetin dihydrate was used as positive control for total phenolic content estimation, DPPH, CUPRAC and lipid peroxidation assays, Na_2EDTA was used as positive control for Metal chelating test, whereas α-tocopherol was used as positive control for lipid peroxidation test. Hex = hexane; DCM = dichloromethane; EA = ethyl acetate; H_2O = water, MD = methanol-dichloromethane.

It was shown to haveequal strength as the quercetin dihydrate (1580.9±180.9 mg Trolox equivalent/g of extract) in this experiment. However, other extracts showed significantly ($p< 0.05$) low reducing power in a range of 66-270 mg Trolox equivalent/g of extract.

Our results showed that EA and DCM have similar inhibitory effect with IC_{50} values 2.83±0.07 mg/mL and 3.15±0.11 mg/mL, respectively. Their inhibitions on lipid peroxidation were significantly ($p< 0.05$) higher than those of MD, H_2O and Hex with IC_{50} values 6.05±0.18, 8.08±0.18 and 10.5±0.68 mg/mL, respectively. However, inhibitory activity of both the EA and DCM were significantly lower ($p< 0.05$) than the positive controls, quercetin dihydrate (0.29±0.02 mg/mL) and α-tocopherol (0.87±0.05 mg/mL), respectively.

4.1.2 Inhibitory effect on LDL oxidation

To explore the defense mechanism of the early stage of atherosclerosis, the inhibitory activity of AR extracts on n-LDL was studied and the results arepresented in Figure 4.1 (Appendix B: TablesB.8 and B.9; Figure B.3).

In this context, two different methods, the formation of conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS) were employed to assess the oxidation of human n-LDL. The CD formation is one of the most widely used methods for monitoring LDL oxidation *in vitro* in a cell-free system.

Oxidation of polyunsaturated fatty acid (PUFA) side chains of LDL is accompanied by the formation of dienes that absorb ultraviolet light at 234 nm. Since ox-LDL, and its oxidised, acidic by-products remains fully soluble in the alkaline buffer (pH 7.4), the increase of 234 nm diene absorption can be measured directly in solution.

In the present study, it was found that all AR extracts showeda gradual increase in the absorbance at 234 nm (Figure 4.1A).

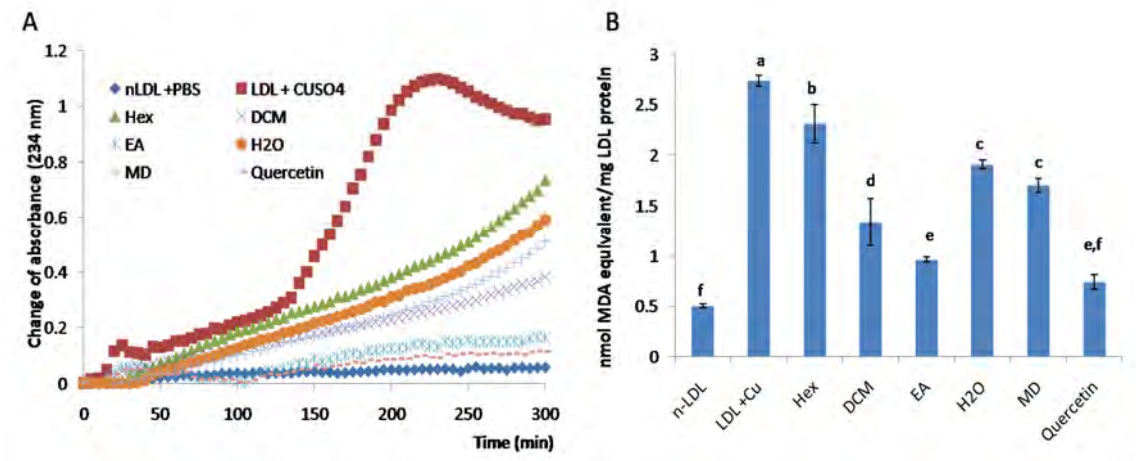

Figure 4.1: Inhibitory effect on LDL oxidation by AR extracts through monitoring of conjugated diene (CD) formation and TBARS.

Results were expressed as mean±standard deviation of triplicate measurements (n=3) in three independent experiments. The different letters (a-f) within a column represent means with significant different (p<0.05). In the assay of CD formation (panel A), the concentration of mushroom extracts was (1µg.mL) and quercetin dihydrate was used as positive control. LDL, preserved in PBS (pH 7.4) without Cu^{2+}inducer was used as blank whereas LDL and $CuSO_4$ solution was used as control. For the TBARS assay (panel B), all extracts including quercetin dihydrate (1mg/mL) were tested. LDL and Cu^{2+} inducer served as control, and the test mixture contained LDL only was used as blank. Hex = hexane; DCM = dichloromethane; EA = ethyl acetate; H_2O = water, MD = methanol-dichloromethane.

This indicated that the oxidation of LDL had taken place, and the lag phase was detected at 130 min for control (induced by transition metal, Cu^{2+}). However, the typical lag and log phases were not observed for the AR extracts, probably due to delayed LDL oxidative action or unknown protective mechanism. Hence, the lag phase was not clearly shown.

However, the slope pattern(the change of the CD absorbance over time) can be used to compare the extent of LDL oxidation. Absorbance increment as an indication of LDL oxidation showed the strength of the inhibitory effect was in the following descending order EA > DCM > MD > H_2O > Hex. To confirm the inhibitory effect, the TBARS method was performed to estimate the MDA product formed. Under acidic condition, the MDA-TBA adducts formed can be directly measured at 532 nm and the higher the absorbance reading implies a higher concentration of MDA-TBA adduct, that could imply faster rate of LDL oxidation.

The results were expressed as nmol MDA equivalent/mg LDL protein based on the MDA standard curve established ($y=0.0481x+0.0626$; $r^2=0.9764$). The inhibitory pattern was observed to be similar as the CD formation (Figure 4.1B). As compared to the control, it was found that the EA (64.9%, IC_{50} 0.77 mg/mL) showed the strongest inhibitory effect against LDL oxidation compared to DCM (51.2%, IC_{50} 0.92 mg/mL), MD (37.8%), H_2O (30.0%) and Hex (15.6%), respectively. The inhibitory effect of EA was found to be comparable to the positive control used, quercetin dihydrate (73.0%, IC_{50} 0.58 mg/mL).

4.1.3 Inhibitory effect of HMG-CoA reductase activity

The previous two hypotheses have been addressed; we now come to the third mechanismto investigate whether ARpossesses the cholesterol lowering property, and hereinafter reduce the cardiovascular disease risk. To achieve this, a rapid method, the HMG-CoA reductase assay kit was employed. The result was obtained by measuring the decrease of NADPH absorbance in the test mixture, at 340 nm.

The slower change of the absorbance indicates the slower rate of NADPH oxidation and thus suggesting that better inhibitory effect towards the enzymatic activity catalysed by HMGR. In the present study, the positive control, pravastatin demonstrated the strongest inhibitory activity. By measuring the initial negative slope (Figure 4.2A) of the absorbance change at 340 nm, the inhibitory activity of mushroom extracts can be compared. The results were expressed in μmol of NADPH oxidised/min/mg protein enzyme (Figure 4.2B) using the slope, calculated based on the mathematical formula according to the manufacturer's instruction (Appendix B: Table B.10).

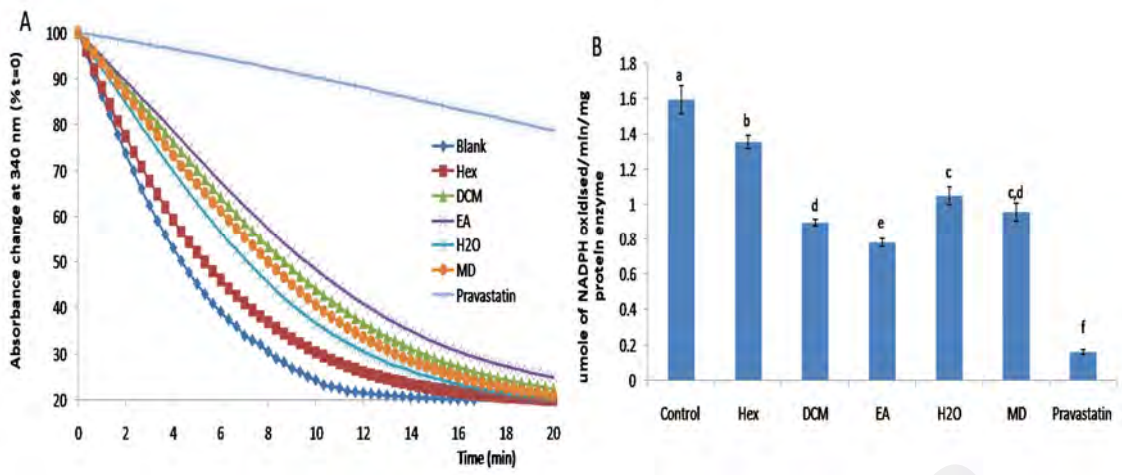

Figure 4.2: Inhibitory effect on HMG-CoA reductase activity by AR extracts (5mg/mL). Results were presented as the change of absorbance at 340 nm over time, and (µmol of NADPH oxidised/min/mg protein enzyme)as mean ±standard deviation of triplicate measurements (n=3) in three independent experiments. The different letters (a-f) represent means with significant different (p<0.05). Pravastatin, from the kit (Sigma) was used as positive control. Hex = hexane; DCM = dichloromethane; EA = ethyl acetate; H_2O = water, MD = methanol-dichloromethane.

In this regard, consistent with the previous antioxidant and LDL oxidation tests, the EA fraction again showed significantly higher and the most potential inhibitory effect on HMGR activity (50.8%), followed by DCM fraction (45.2%), MD crude extract (36.7%), H_2O fraction (36.2%) and Hex fraction (17.5%), relative to control (100%), respectively.

4.2 Cytotoxicity studies

Three cell lines i.e., WRL 68, MRC-5 and the HepG2 were employed and the cytotoxicity study was performed using MTT assay and the results are presented in Table 4.2 (Appendix C: FiguresC.1, C.2, C.3 and C.4).

All extracts were non-cytotoxic (IC_{50}> 200 µg/mL; except for DCM fraction, IC_{50}= 195±4.18 µg/ml) to the three respective cell lines incubated in DMEM (WRL-68 and HepG2) or MEM (MRC-5) supplemented with 10% FBS up to 72 hrs.

Table 4.2: The cytotoxicity study of the AR extracts of on WRL-68, MRC-5 and HepG2 cells.

Cell lines/ Extracts	WRL-68	MRC-5	HepG2	*HepG2
Hex	> 400	> 400	361 ±5.68	> 400
DCM	289 ± 7.81	268 ± 8.23	195 ± 4.18	278 ± 8.79
EA	335 ± 6.62	307 ± 5.68	252 ± 6.75	348 ± 6.45
H_2O	> 400	> 400	> 400	> 400
MD	348 ± 5.95	381 ± 7.33	322 ± 3.93	389 ± 9.51

The mushroom extracts were dissolved in 50% DMSO in water (v/v) to prepare the stock solution. WRL-68 and HepG2 cells were treated with AR mushroom extracts (final concentration <1% DMSO) in DMEM (except for MRC-5 in MEM), supplemented with 10% FBS up to 72 h incubation. *Experiment was performed using serum free-DMEM containing 1% BSA enriched with 0.8 mM sodium oleate for 24 h incubation. Results were expressed as mean of IC_{50} value ± standard deviation of three independent experiments (n = 3) in triplicate.Hex = hexane; DCM = dichloromethane; EA = ethyl acetate; H_2O = water, MD = methanol-dichloromethane.

Besides, all AR extracts, at 200 µg/mL showed no significant reduction in cell viability (IC_{50}> 200 µg/mL) as compared to the oleate-induced cells (as control) incubated in 1% BSA, serum-free, oleate-enriched DMEM medium for 24 hrs. Therefore, the concentration at 200 µg/mL of mushroom extract was used as the therapeutic range to ensure its efficacy and safety in all the subsequent experiments.

4.3 Effect of AR on lipid and apolipoprotein level

Lipids and apolipoproteins are the two classes of markers involved in the development of atherosclerosis. The neutral lipids such as triglycerides and cholesteryl ester are form the core of the lipoproteins whereas the apolipoproteins act as ligands of lipoproteins that are involved in lipid metabolism. To search for new and novel lipid or lipoprotein modulating agents, screening on both the lipids and apolipoproteins using AR extracts provides relevant information for further development.

4.3.1 Effect of AR on TG, FC and CE levels

To determine whether the AR extracts modulate TG, FC and CE levels in hepatocytes, HepG2 cells were initially fasted with serum free media and 1% BSA for 24 hours before the treatment was performed in oleate-enriched medium together with AR mushroom extracts (200 µg/mL) or 0.25 µM lovastatin (positive control). HepG2 cells cultured in oleate-enriched media only served as control. The intracellular (Figure 4.3A) and secreted (Figure 4.3B) TG, FC and CE were measured using enzymatic assay kits. The total intracellular and secreted levels of TG, FC and CE detected in oleate-induced HepG2 cells (control) were 278.26 ± 41.5; 23.5±1.18; 8.39± 2.79 and 30.36±4.53; 3.81±0.23; 2.40±0.29µg of (TG, FC or CE)/mg cell protein, respectively. (Appendix D: TablesD.1, D.2, D.3, D.4, D.5, D.6 and D.7; FiguresD.1, D.2 and D.3)

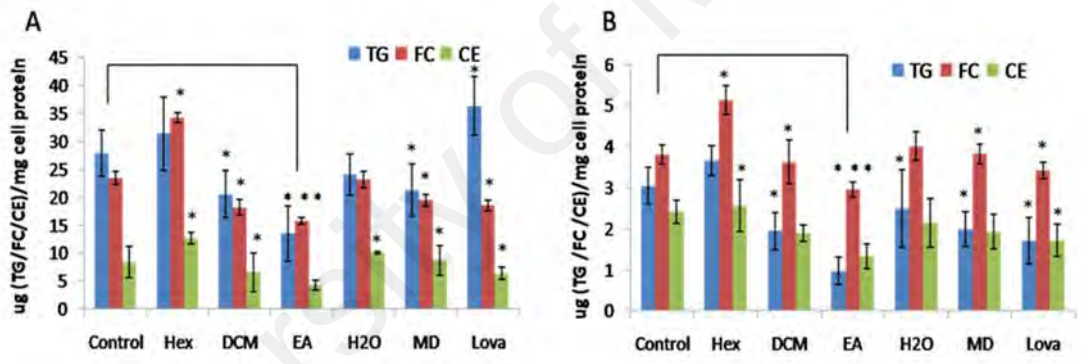

Figure 4.3: The effect AR mushroom extracts (200 µg/mL) on intracellular (A) and secreted (B)TG, FC and CE was presented.
HepG2 cells were incubated for 24 h without (control) or with AR mushroom extracts (200 µg/mL) or 0.25 µM of lovastatin (as positive control) in serum free-DMEM medium containing 1% BSA with 0.8 mM sodium oleate. Amount of TG, FC and CE measured using respective commercial enzymatic kits were normalised to intracellular protein. Results were presented as mean of µg (TG x10, FCorCE)/ mg cell protein ± standard deviation of triplicate measurements (n=3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The differences between means were significant at * ($p< 0.05$), relative to oleate enriched culture (control) (0.1% < DMSO). Hex = hexane; DCM = dichloromethane; EA = ethyl acetate; H_2O = water, MD = methanol-dichloromethane; Lova =lovastatin.

As shown in Figure 4.3, oleate-induced HepG2 cells treated with EA showed the strongest effect amongst the five extracts tested and showed a marked decrease ($p< 0.05$) in total triglyceride in intracellular content (-51.5%) and medium (-68.0%) compared to the control (100%), respectively.

Oleate-induced HepG2 cells treated with lovastatin showed no significant difference on TG production, but a significant decrease in secreted TGby (-43.9%) as compared to control, respectively. Cells treated with other AR extracts showed a moderate reduction in TG production and secretion in the descending order as follow: DCM (-26.2%, -36.0%) > MD (-23.5%, -34.5%) >H_2O (-13.7%, -18.1%), respectively. However, effect of hexane fraction on TG production and secretion was non-significantly(p> 0.05) increased.

On the other hand, FC and CE contributed to the pool of TC as FC can be converted to CE under normal physiological condition that is required for the assembly of atherogenic lipoproteins. As shown in Figure 4.3, EA again showed the most significant reduction (p< 0.05) amongst all extracts tested, on intracellular and secreted TC in oleate-induced HepG2 cells by (-37.2%) and (-31.1%) relative to control, respectively. Nonetheless, this reduction was even significantly lower than that of cells treated with lovastatin (-22.0% and -17.5%). A detailed investigation showed that the FC and CE levels in HepG2 cells treated with EA were found to be significantly lower in both intracellular (-32.9%, -49.1%) and secretion (-22.4%, -44.9%) as compared to control, respectively. However, the DCM, MD and H_2O showed no significant difference in the FC and CE production and secretion in oleate-induced HepG2, except for Hex fraction, showed a marked increase (p< 0.05) in intracellular and secreted FC by (+45.6% and +34.6%), respectively, above the control levels. Similarly, a significant increase by (+51.6%) was observed in the intracellular CE production (p< 0.05) but not for its secretion.

4.3.2Effect of AR on apolipoprotein level

To determine whether the AR mushroom extracts modulate apoA1, apoB-100 and apoE levels in hepatocytes, HepG2 cells were cultured in oleate-enriched medium with

or without various mushroom extracts (200 µg/mL) or 0.25 µM lovastatin as the positive control. The results obtained with regard to the intracellular (Figure 4.4A) and secreted (Figure 4.4B) apolipoproteins are shown.

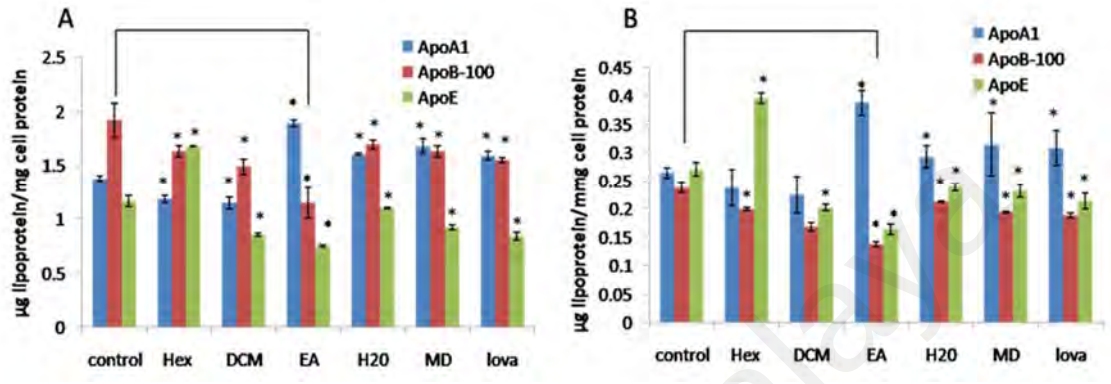

Figure 4.4:Effect of AR extracts on apoA1, apoB-100 and apoE in intracellular (A) and its secretion (B).
HepG2 cells were incubated for 24 h without (control) or with AR extracts (200 µg/mL) or 0.25 µM of lovastatin (positive control) in serum free-DMEM, 0.8 mM oleate-enriched medium containing 1% BSA. Amount of apoAI, apoB-100 and apoE measured by respective ELISA kits was normalised to intracellular protein. Results were presented as mean of µg (apoA1 x 5, apoB-100 x 10 and apoE)/ mg cell protein ± standard deviation of triplicate measurements (n=3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The differences between means were significant at * (p<0.05), relative to 1% BSA, serum-free, oleate-enriched medium (control) (0.1% < DMSO). Hex = hexane; DCM = dichloromethane; EA = ethyl acetate; H_2O = water, MD = methanol-dichloromethane; Lova = lovastatin.

The total intracellular and secreted levels ofapoA1, apoB-100 and apoE detected in oleate-induced HepG2 cells (control) were 6.88 ± 0.25; 19.2±1.57; 1.16± 0.05 and 1.32±0.05; 2.38±0.09; 0.27±0.01µg of (apoA1, apoB-100 and apoE)/mg cell protein, respectively(Appendix D: TablesD.8, D.9, D.10, D.11, D.12 andD.13; FiguresD.4, D.5 and D.6).

Except for Hex and DCM, cells incubated with various mushroom extracts or lovastatin at 24 h showed increments in both intracellular and secreted apoA1 in oleate-enriched medium.

Amongst the five extracts tested, cells treated with EA fraction showed highest significant increase in accumulated (+37.4%) and secreted (+47.0%) apoA1 relative to

the control. Nonetheless, the increase of apoA1 is significantly higher than that of lovastatin treated cells at 0.25 μM in both the intracellular (+16.8%) and secreted (+15.6%) levels.

Cells treated with H_2O and MD showed equal potency as lovastatin and marked an incremental of intracellular (+16.5% and +21.9%) and secreted (+10.8% and +19.1%) apoA1. However, opposite trends were observed in apoB-100 and apoE production and secretion. Cells treated with EA demonstrated a marked decrease ($p< 0.05$) in the intracellular and secreted apoB-100 by -32.7% and -42.3%, respectively, followed by DCM > MD > H_2O > Hex in a descending order, all of them are significantly lower than that of the untreated HepG2 cells (control) (100%).

Similarly, HepG2 cells treated with lovastatin showed a marked reduction in the intracellular and secreted apoB-100 accounting for -20.8% and -19.8%, respectively. Following this, HepG2 cells treated with EA marked a significant decrease in the intracellular (-39.41%) and secreted (-37.13%) apoE levels relative to control, respectively. The EA showed greater reduction ($p < 0.05$) compared to that of HepG2 cells treated with lovastatin (0.25 μM)on intracellular and secreted apoE (-27.6% and -22.2%).

A moderate reduction in the intracellular and media apoE was also observed in HepG2 cells treated with other mushroom extracts in a descending order as follow: DCM > MD > H_2O. However, an unexpected significant increase in the intracellular (+41.2%) and media (+46.3%) apoE from the HepG2 cells treated with hexane fraction was observed.

4.4 Chemical constituents in EA

The LC-MS/MS chromatogram of EA constituents is shown in Figure 4.5, and the interpreted result is presented in Table 4.3. The identification of compounds was literally made by comparing with the published data in the literature review.

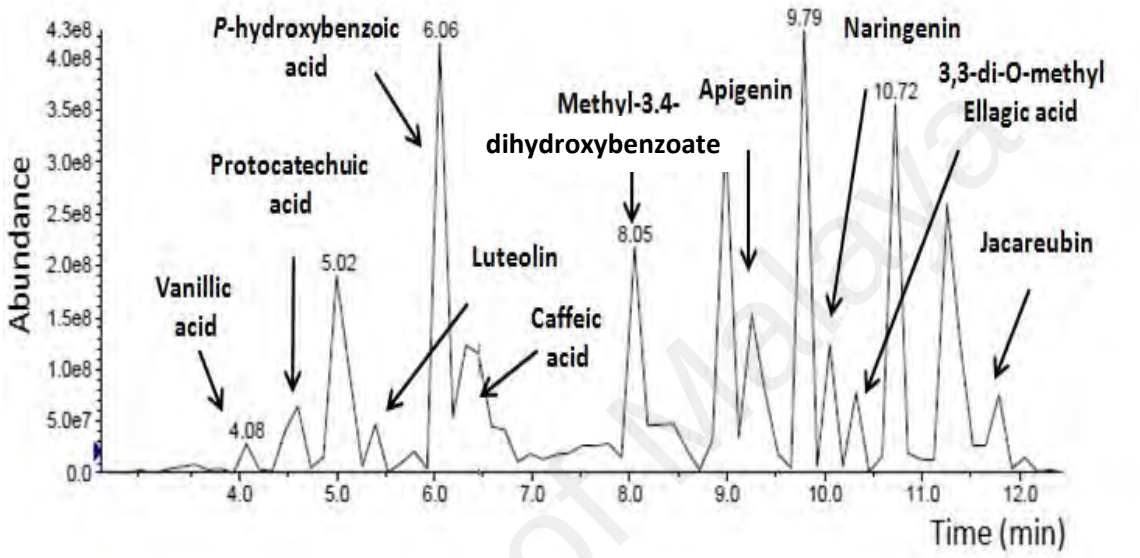

Figure 4.5: The UHPLC-ESI-MS/MS-TLC (negative mode) of the chemical components present in EA

Ten phenolic compounds were identified which include four benzoic acid derivatives (vanillic acid, *p*-hydroxybenzoic acid, protocatechuic acid, and methyl-3,4-dihydroxybenzoate), three flavonoids (luteolin, apigenin and naringenin), cinnamic acid (caffeic acid), dilactone of hexahydroxydiphenic acid (3,3'-di-O-methyl ellagic acid), and xanthone derivative (jacareubin). (Appendix E: Figures E.1, E.2, E.3, E.4, E.5 and E.6) The chemical structures are shown in Figure 4.6.

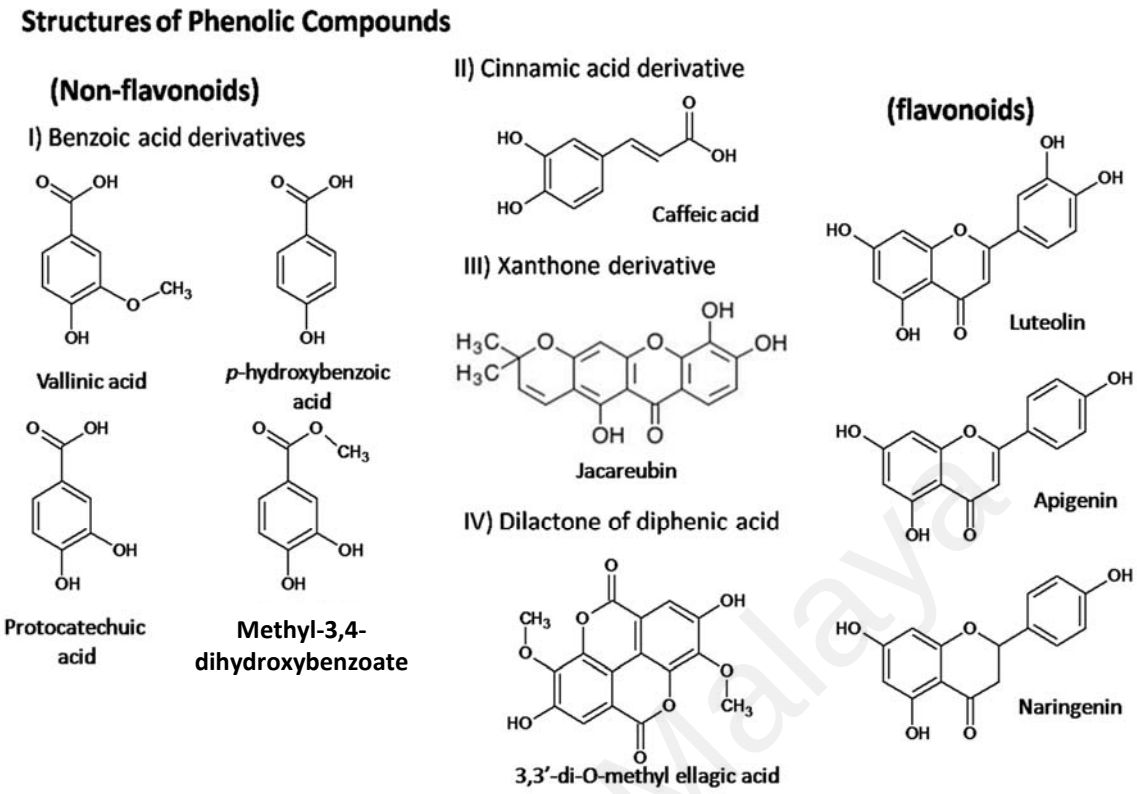

Figure 4.6: The chemical structures of phenolic compounds in EA

4.4.1Quantification of bioactive compounds in EA

A sensitive and specific method combining liquid chromatography with electrospray ionisation tandem mass spectrometry has been optimised to simultaneously quantify protocatechuic acid (PCA), caffeic acid (CA), vanillic acid (VA) and 4-hydroxybenzoic acid (4HBA) in EA.

Multiple reaction monitoring (MRM) was employed for quantification with switching electrospray ion source polarity between positive and negative modes in a single run. The mass spectra of the PCA, CA, 4HBA and VA are shown in Figure 4.5 and their mass transitions of were m/z 152.9 →108.9, 178..8 →134.9, 136.9 →92.8, 166.9 →122.9, respectively.

Table 4.3: Chemical constituents in EA based on LC-MS analysis

R_T (min)	Molecular mass	Molecular formula	$[M-H]^-$ Mass fragments, MS/MS	Suggested identification	Reference(s)
4.071	168.0	$C_8H_7O_4$	167(100), 149 (10), 137(25), 123(80), 109(30), 93(20), 65(30)	Vanillic acid	Hossain*et al*. (2010) Sudawadee *et al*. (2009)
4.468	154.0	$C_7H_5O_4$	153(32), 109(100), 108(43), 91(10)	Protocatechuic acid	Hossain*et al*. (2010) Rabaneda*et al.* (2003)
5.790	285.1	$C_{15}H_9O_6$	284(25), 283(100), 257(10), 163(57)	Luteolin	Hossain*et al*., (2010)
6.056	138.0	$C_7H_5O_3$	137(100), 136(38), 108(18), 92(10)	*p*-hydroxybenzoic acid	Hossain*et al*. (2010) Chen *et al*.(2012)
6.454	180.0	$C_9H_7O_4$	179(15), 135(100), 137(27), 89(10)	Caffeic acid	Hossain*et al*.(2010) Sudawadee *et al*. (2009) Rabaneda *et al*. (2003)
8.050	168.0	$C_8H_8O_4$	167(100), 135(21), 124(34), 123(18), 111(21), 91(25)	Methyl-3,4-dihydroxybenzoate	Su *et al*.(2013)
9.253	271.0	$C_{15}H_{10}O_5$	269(100), 197(38), 167(71), 141(61), 113(15)	Apigenin	Rabaneda *et al*. (2003) Cui *et al*. (2014)
10.06	272.1	$C_{15}H_{12}O_5$	271(100), 255(63), 239(18), 145(18), 109(46	Naringenin	Rabaneda *et al*. (2003)
10.32	331.2	$C_{16}H_{10}O_8$	330(75), 329(100), 229(18), 211(29), 171(79), 139(18)	3,3'-di-O-methyl ellagic acid	Braunberger *et al.* (2013)
11.79	327.2	$C_{18}H_{14}O_6$	236(16), 325(21), 183(100)	Jacareubin	Wei *et al*. (2011)

For chromatographic separation, the analytical method was optimised to achieve good sensitivity and peak shape as well as a relative short run. The representative mass spectral (Figure 4.7) and chromatograms (Figure 4.8) of each targeted compound while the chromatogram of EA that contains the selected compounds are shown in Figure 4.9. The concentration of PCA, CA, 4HBA and VA in EA was measured using HPLC-MS/MS method and the results summarised in Table 4.4.

It is clearly shown that EA contains high phenolic contents particularly PCA (5.74 μM), followed by CA (5.06 μM), VA (1.11 μM) and 4HBA (0.32 μM), respectively in 200 μg/mL of EA. Hence, the concentration of these four bioactive compounds were prepared individually (0.1-10 μM) and PM was prepared with corresponding concentration as present in EA to further examine their roles in anti-atherosclerotic potential.

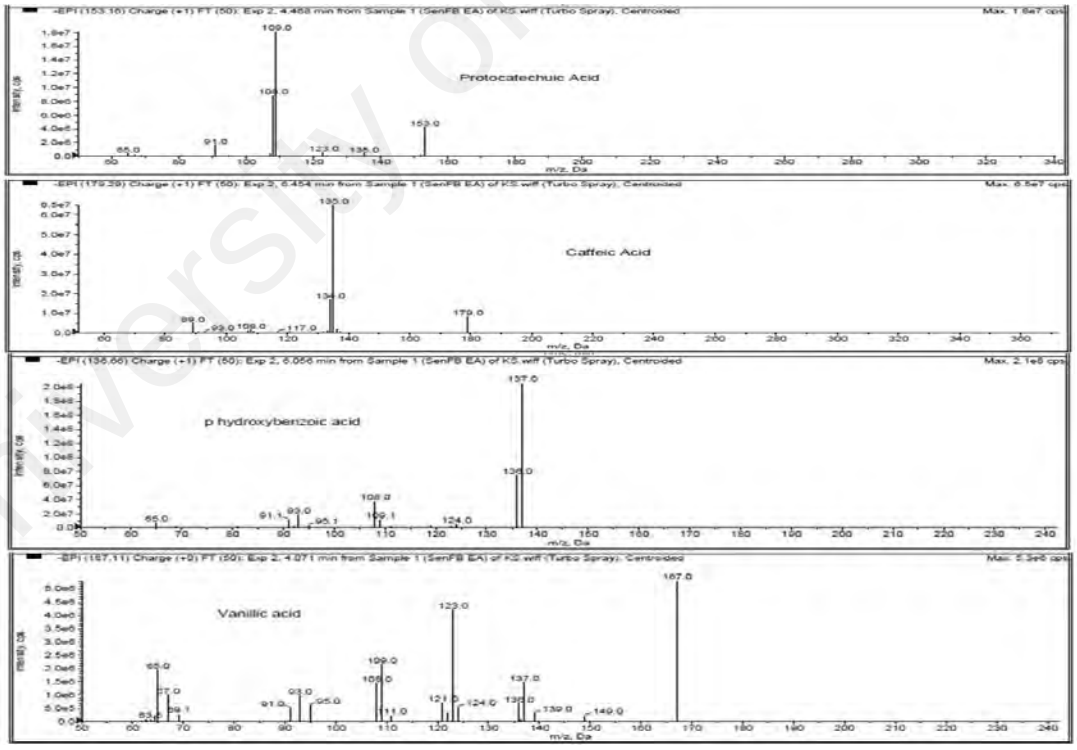

Figure 4.7: The mass spectra of PCA, CA, 4-HBA and VA in EA

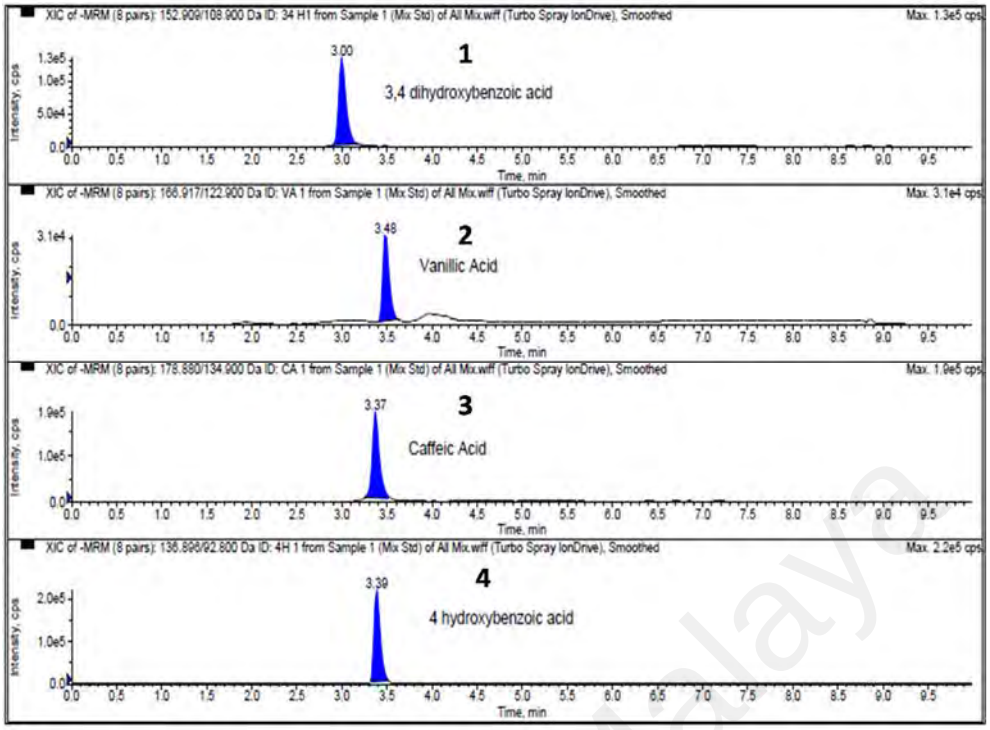

Figure 4.8: UPLC-MS/MS chromatogram of (1) PCA, (ii) VA, (iii) CA and (iv) 4HBA of pure compounds commercially purchased.

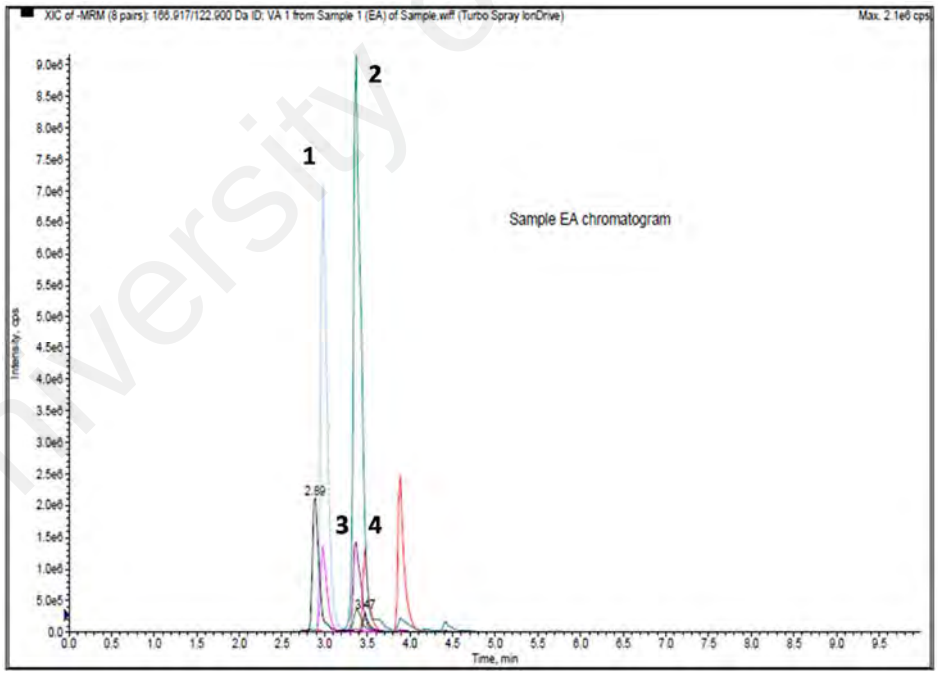

Figure 4.9: The chromatogram of EA with extractable target compounds, (1) PCA, (2) VA, (3) CA and (4) 4HBA.

Table 4.4: The concentration of bioactive compounds in EA

Compounds	Retention time min (R_t)	Molecular mass	Molecular formula	Concentrations (μM)
Protocatechuic acid (PCA)	3.00	154.0	$C_7H_5O_4$	5.74 ± 0.85
Caffeic acid (CA)	3.37	180.0	$C_9H_7O_4$	5.06 ± 0.29
4-Hydroxybenzoic acid (4HBA)	3.39	138.0	$C_7H_5O_3$	0.32 ± 0.05
Vanillic acid (VA)	3.48	168.0	$C_8H_7O_4$	1.11 ± 0.12

The commercially purchased standard compounds (PCA, CA, VA and 4HBA) were dissolved in methanol. The samples were injected into the HPLC column, Zorbax C_{18} with running mobile phase A (consists of water with 0.1% formic acid and 5mM ammonium formate) and mobile phase B (consists of acetonitrile with 0.1% formic acid and 5mM ammonium formate). The four selected compounds present in the EA (200 μg/mL) were confirmed with based on their molecular masses (and their fragmentation) as well as retention times as compared with the standard compounds while the concentrations were determined based on the calibration curves established, respectively. The results were expressed as mean ± standard deviation of triplicate measurement (n=3).

4.5 Effect of EA on lipid lowering activity

Based on the previous results, EA at 200 μg/mL, has demonstrated marked reductions in intracellular and secreted total cholesterol, triglycerides in oleate-induced HepG2 cells, relative to control, respectively. Hence, the EA was selected for further evaluations.

4.5.1 Effect of EA on TG, TC, FC and CE levels

High levels of TG and CE have been attributed to high risk of cardiovascular incidence. Both lipids are essential components of the lipoproteins which carry the cholesterol in the blood circulation.In order to understand how EA influences the lipid production and secretion, HepG2 cells were fasted with serum free media and 1% BSA for 24 hours, and then the HepG2 cells were treated with different concentrations of EAin oleate-enriched medium for another 24 hours. The amount of intracellular and secreted lipids were measured independently with commercial enzymatic assay kits, respectively.

The results of lipid production (Figure 4.10A) and secretion (Figure 4.10B) are presented. In the present study, oleate (0.8 mM)(added together with mushroom extracts) was used as a lipogenic factor and it showed a significantincreased (p<0.05) in intracellular productions and secretion of TG, TC, FC and CE respectively.

Notably, oleate-induced HepG2 cells treated with a low dose lovastatin (0.25 µM), positive control showed a significant increase of TG production (21%), but markedly decrease in its secretion (-44%). However, significant decrease (p<0.05) in the production and secretion on other lipids such as TC (-31%, -18%), FC (-29%, -17%) and CE (-39%, -20%) were observed in the similar treatment using lovastatin.

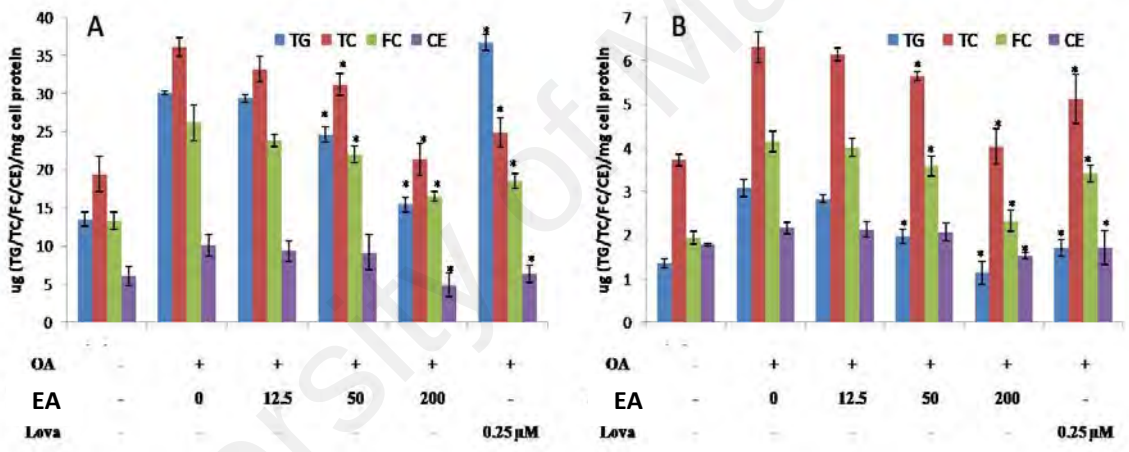

Figure 4.10: The effect of EA (200 µg/mL) on intracellular (A) and secreted (B) TG, TC, FC and CE.
HepG2 cells induced with 0.8 mM sodium oleate (in the form of sodium salt) were incubated for 24 h without (control) or with EA (0-200 µg/mL). Lovastatin (0.25 µM) was used as a positive control. Cells incubated without oleate served as negative control. Amount of TG, TC, FC and CE measured using respective commercial enzymatic assay kits were normalised to intracellular protein. All results were presented as mean µg of (TG x 10^1; TC, FC, or CE)/mg cell protein ± standard deviation of triplicate measurements (n = 3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The differences between means were significant at * (p< 0.05), relative to oleate-induced HepG2 cells (control). EA = ethyl acetate; Lova = lovastatin; OA = oleic acid.

From the results, it is shown that oleate-induced HepG2 cells treated with increasing dose (0-200 µg/mL) of EA marked a significant decrease on the intracellular productions and secretion of lipids. In particular, a marked decrease of intracellular TG, TC, FC and CE (-18%, -13%, -16% and -14%) as well as its secretion (-36%, -10%, -

14% and -4%), relative to control (100%) was detected when HepG2 cells were treated with 50 µg/mL of EA. Further reductions were observed when the cells were treated with 200 µg/mL of EA. Interestingly, a greater reduction of intracellular TG, TC, FC and CE (-48%, -40%, -37% and -54%) and secreted TG, TC, FC and CE (-63%, -36%, -44% and -30%), relative to control were observed, respectively. (Appendix F: TablesF.1, F.2, F.3, F.4, F.5, F.6 and F.7)

4.5.2 Effect of selected bioactive compounds in TG and TC levels

The lipid lowering activity of EA in oleate-induced HepG2 cells could be associated to its bioactive constituents present. To confirm this hypothesis, the extracts of the four selected phenolic compounds, PCA, CA, 4HBA and VA (purchased from Merck Millipore, USA) were dissolved in 50% DMSO-water to make the stock solution. Later, treatment was performed under similar conditions as using the AR mushroom extracts in oleate-enriched HepG2 cells using various concentrations (0.1-10 µM) independently and PM (5.74 µM PCA, 5.06 µM CA, 0.32 µM 4HBA and 1.11 µM VA) was prepared with the concentrations of those constituents as they present in the EA (all the extracts contain < 1% DMSO at final concentration). Their roles in intracellular production and secretion of TG and TC were examined.

The results are presented in Table 4.5. It showed that there were significant decreases ($p< 0.05$) in both intracellular and secreted TG and TC in dose-dependent fashion. However, CA (10.0 µM) showed the best lipid lowering activity among the four bioactive compounds exhibit a significant decrease of intracellular and secreted TG (-26.%, -35%) and TC (-23%, -25%), followed by PCA (-21%,-29% and -18%,-20%), VA (-18%,-24% and -11%,-15%) and 4HBA (-11%, -16% and -9%, -10%), respectively.

Table 4.5: Effect of selected bioactive compounds on TG and TC levels

Compounds	Total Triglyceride (µg TG/mg cell protein)		Total Cholesterol (µg TC/mg cell protein)	
	Intracellular	Secreted	Intracellular	Secreted
Control	300.1±2.38[a]	30.7±1.90[a]	36.1±1.23[a]	6.31±0.34[a]
Caffeic acid (CA)				
0.1 µM	269.7±7.72[c]	26.5±0.72[d,e]	33.7±0.91[c,d,e]	5.83±0.10[d]
1.0 µM	251.1±2.30[d,e]	23.7±0.97[f,g]	30.7±1.08[g]	5.29±0.17[f]
10.0 µM	222.4±6.41[g]	20.0±0.49[h]	27.9±0.59[h]	4.72±0.13[h]
Protocatechuic acid (PCA)				
0.1 µM	282.4±5.89[b]	27.6±1.42[b,c,d]	34.4±0.50[b,c,d]	5.90±0.10[c,d,e]
1.0 µM	259.4±8.31[c,d]	26.0±0.71[d,e]	31.2±1.01[f,g]	5.67±0.07[e]
10.0 µM	238.5±9.03[f]	21.7±1.19[g,h]	29.6±0.66[g]	5.05±0.08[g]
Vanillic acid (VA)				
0.1 µM	286.9±8.05[b]	28.7±1.51[a,b,c]	34.6±0.47[a,b,c]	6.02±0.14[b,c,d]
1.0 µM	268.3±6.17[c]	26.6±1.56[c,d,e]	33.3±0.80[c,d,e]	5.82±0.12[d,e]
10.0 µM	246.0±11.4[e,f]	23.4±0.56[f,g]	32.3±0.68[e,f]	5.37±0.11[f]
4-Hydroxybenzoic acid (4HBA)				
0.1 µM	294.5±4.17[a,b]	29.5±0.51[a,b]	35.5±0.38[ab]	6.24±0.14[a,b]
1.0 µM	282.3±5.13[b]	27.7±0.86[b,c,d]	34.2±1.07[b,c,d]	6.12±0.10[a,b,c]
10.0 µM	265.7±5.02[c]	25.5±1.30[e,f]	32.8±0.64[d,e]	5.67±0.14[e]

Table 4.5: Continued

Compounds	Total Triglyceride (µg TG/mg cell protein)		Total Cholesterol (µg TC/mg cell protein)	
*Pre-mixed mixture (PM)	192.0±6.41^{h}	15.8±0.96^{i}	22.5±1.59^{i}	4.48±0.09^{h}

The effect of bioactive compounds on intracellular and secreted TG and TC was presented. HepG2 cells induced with 0.8 mM sodium oleate were incubated for 24 h without (control) or with selected bioactive compounds (0.1-10 µM). Amount of TG and TC measured using respective commercial enzymatic assay kits were normalised to intracellular protein. All results were presented as mean µg of (TG or TC)/mg cell protein ± standard deviation of triplicate measurements (n = 3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. Means with different letters (a-i) within a column are significantly different ($p< 0.05$), relative to oleate-induced HepG2 cells (control).
*PM contains 5.74 µM PCA, 5.06 µM CA, 0.32 µM 4HBA and 1.11 µM VA.

Surprisingly, PM showed greater reductions in intracellular and secreted TG (-36%, -49%) and TC (-37%, -29%), which are comparable to lipid lowering effect observed in the treatment with 200 μg/mL of EA. (Appendix F: TablesF.8 and F.9)

4.5.3. Effect of EA on transcription factor-dsDNA binding activity

To explore the underlying mechanism of EA on lipid lowering activity in HepG2 cells, three transcription factors, i.e. PPAR-α, SREBP-1 and SREBP-2 were selected to further examine thebinding activity of their nuclear extracts to the specific double stranded DNA (dsDNA) sequence pre-coated in the 96-well ELISA plate. The experiments were performed using EA at various concentrations and at different time intervals. The transcription factors (PPAR-α, SREBP-1 and SREBP-2) contained in the nuclear extract will bind specifically to the respective response element.

Transcriptional binding activity was detected using a primary antibody directed against PPAR-α, SREBP-1 and SREBP-2 independently. Then, secondary antibody conjugated to HRP was added to provide a sensitive colorimetric readout at 450 nm. The transcriptional binding activity of the three transcription factors was determined independently based on the calibration curves plotted using positive control (2x serial dilution), respectively. (Appendix G: Figures G.1, G.2 and G.3). The results of binding activity were expressed in fold changes relative to untreated HepG2 cells (control) as presented in Figure 4.11.

From the experiments, HepG2 cells treated with EA showed significant increases of PPARα, and SREBP-1 in dose and time-dependent manner while opposite trend was observed for SREBP-2. In the similar experiment where oleate-induced HepG2 were treated with 200 μg/mL, the binding activity of PPAR-α, SREBP-1 and SREBP-2 was observed to be 1.68-fold, 1.43-fold and 0.36-fold change, relative to untreated cells, respectively (Appendix G: Table G.1, G.2, G.3, G.4, G.5, G.6, G.7 and G.8).

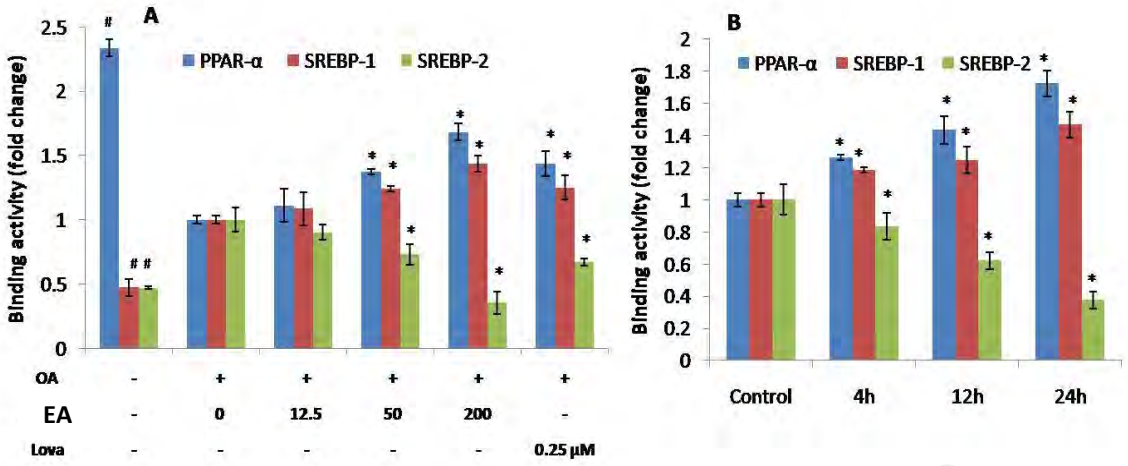

Figure 4.11: Effect of EA on transcription factor-dsDNA binding activity of PPARα, SREBP-1 and SREBP-2 in oleate-induced HpeG2 cells.
HepG2 cells induced with 0.8 mM sodium oleate were incubated for 24 h without (control) or with EA (0-200 µg/mL). Lovastatin (0.25 µM) was used as a positive control. Cells incubated without oleate served as negative control. The binding activity was measured using respective commercial ELISA assay kits were normalised to intracellular protein. All results were presented as % of control ± standard deviation of triplicate measurements (n = 3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The differences between means were significant at * (p< 0.05), relative to oleate-induced HepG2 cells (control). EA = ethyl acetate; Lova = lovastatin; OA = oleic acid.

However, in the present research, the protein levels of the corresponding transcription factors were not determined. Hence, the correlation between the protein level and transcriptional binding activity remain uncertain.Further studies need to be done to confirm the mechanisms that lead to the significant changes of the binding activity of PPAR-α, SREBP-1 and SREBP-2.

From the previous section, the results have shown that there are enhanced binding activities on PPAR-α and SREBP-1 to their corresponding dsDNA whereas decreased binding activity of SREBP-2 to its corresponding dsDNA was pronounced. Further investigation on the expression of mRNA levels of the corresponding gene coding for the synthesis of the proteins is required to explain whether the significant changes observed on the binding activities to their corresponding dsDNAs is due to the activation or deactivation of the transcription factors by their ligands present in the EA or the overexpressed of the protein themselves.

The results of mRNA expression levels of PPAR-α, SREBP-1 and SREBP-2 gene expression are presented in Figure 4.12. (Appendix G: Tables G.9, G.10, G.11, G.12, G.13, G.14, G.15, G.16, G.17, G.18, G.19, G.20, G.21 andG.22).

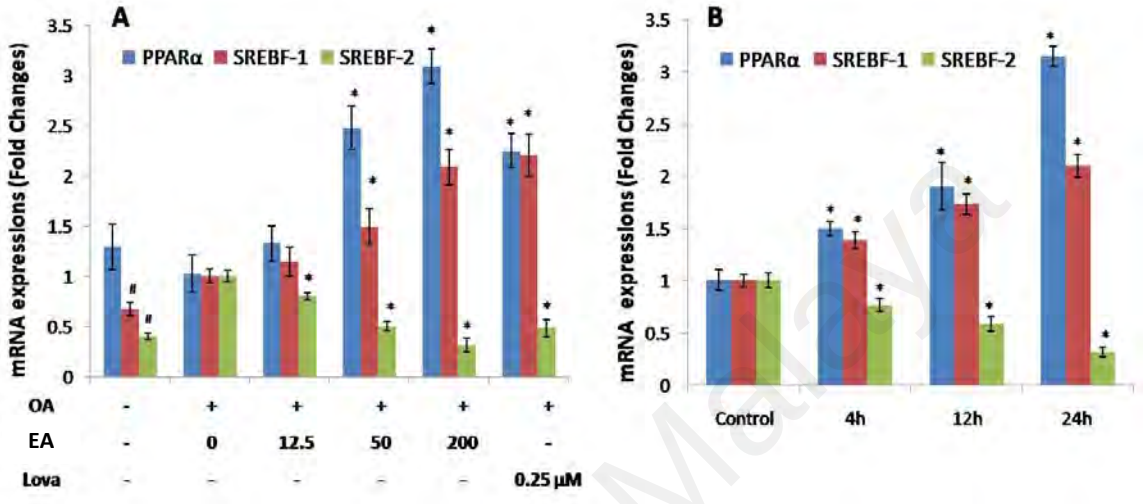

Figure4.12: The effect of EA on PPAR-α, SREBP-1 and SREBP-2 mRNA expression in oleate-induced HepG2 cells.

HepG2 cells induced with 0.8 mM sodium oleate were incubated for 24 h without (control) or with EA (0-200 µg/mL) and at different time intervals (at 200 µg/mL). Lovastatin (0.25 µM) was used as a positive control. Cells incubated without oleate served as negative control. Expression levels of genes were assessed with qRT-PCR method and the result was calculated using $2^{-\Delta\Delta Ct}$ method normalised to GADPH mRNA level. All results were presented as mean ± standard error (SEM) of triplicate measurements (n = 3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The differences between means were significant * ($p< 0.05$), relative to oleate-induced HepG2 cells (control). EA = ethyl acetate; Lova = lovastatin; OA = oleic acid.

HepG2 cells treated with EA (50 µg/mL and 200 µg/mL) marked a significant increase($p< 0.05$) of PPAR-α (2.48-fold and 3.09 fold change) and SREBP-1 (1.49-fold and 2.08 fold change) while significant decrease of SREBP-2 (0.50 fold and 0.32 fold change), relative to controlin dose-and time dependent response. At time point basis, significant changes observed were begun at 4 h for all the genes under studied, relative to control.

4.6Effect of EA on lipoprotein level

Based on the previous results, oleate-induced HepG2 cells treated with EA showed remarkable decrease in intracellular and secreted apoB-100 and apoE while increase of apoA1. Down-regulation of apoB-100 and apoE correspondedto lower production of VLDL and LDL whereas up-regulation of apoA1 is correlated to overexpression of HDL. To confirm these observation, further investigations on lipoprotein regulators i.e., LXR-α, LCAT and CETP were conducted.

4.6.1 Effect of EA on apolipoprotein level

To investigate whether EA modifies lipid metabolism in oleate-induced HepG2 cells were treated with EA at various concentrations, and different time point (at 200 µg/mL), the intracellular and secreted apoAI, apoB-100 and apoE were quantified using ELISA Kits, respectively and the results are shown in Figure 4.13.

The results showed that oleate-induced HepG2 cells treated with EA at doses 50 µg/mL and 200 µg/mL exhibited marked significant changes ($p< 0.05$) on intracellular and secreted apoAI, apoB-100 and apoE protein levels. Apparently, significant increase was detected for both intracellular and secreted apoA1 in dose and time dependent manner. At doses 50 µg/mL and 200 µg/mL, the intracellular (+12.2% and +39.2%) and secreted (+22.3% and +49.5%) apoA1 were significantly higher relative to the control, respectively. Incremental trend was observed for both intracellular and secreted apoA1 at 4h, 12h and 24h (Appendix H: TablesH.1, H.2, H.3 andH.4).

Unlike apoA1, the protein levels of apoB-100 and apoE were detected significantly lower in dose-and time dependent response compared to the untreated cells (control), respectively. At 50 µg/mL, marked decrease of intracellular and secreted apoB-100 were -23.5%,-28.5%, whereas at 200 µg/mL the intracellular and secreted apoB-100

were -33.5%, -42.8% compared to untreated cells (Appendix H: Tables H.5, H.6, H.7 and H.8).

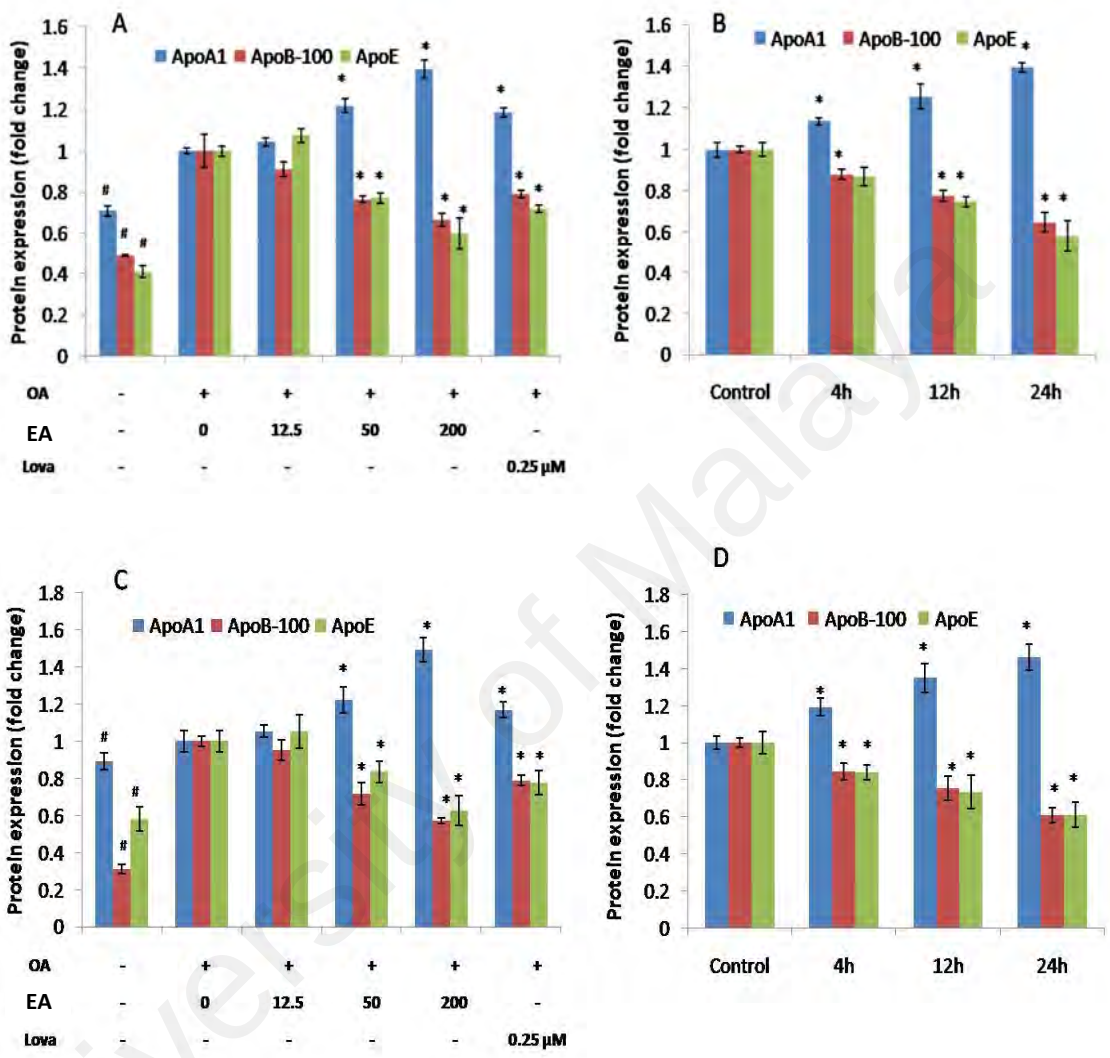

Figure 4.13: The effect of EA on intracellular (A and B) and secreted (C and D) apolipoprotein apoA1, apoB-100 and apoE at different concentration and time.
HepG2 cells induced with 0.8 mM sodium oleate were incubated for 24 h without (control) or with EA (0-200 µg/mL). Lovastatin (0.25 µM) was used as a positive control. Cells incubated without oleate served as negative control. Amount of apoA1, apoB-100 and apoE measured using respective commercial ELISA kits were normalised to intracellular protein. All results were presented as mean µg of (apoA1, apoB-100, or $1x10^{-1}$ apoE)/mg cell protein ± standard deviation of triplicate measurements (n = 3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The differences between means were significantat # or * ($p< 0.05$), relative to oleate-induced HepG2 cells (control). EA = ethyl acetate; Lova = lovastatin; OA = oleic acid.

Similarly, significant reduction in intracellular and secreted apoE were also detected at 50 µg/mL (-22.9%,-16.5%) and 200 µg/mL(-39.4%,-37.1%),relative to control, respectively (Appendix H: Tables H.9, H.10, H.11 and H.12).

Unlike apoAI and apoB-100, significant change ($p< 0.05$) of apoE only was observed after 12h in cellular accumulation. However, significant changes of the secretion for all three apolipoproteins were observed at 4h onwards.

4.6.2Effect of EA on LXR-α, LCAT and CETP levels

To examine whether the lipoprotein metabolism is governed by the regulatory protein, LXR-α, the nuclear protein level was determined. In addition, the expression of two other enzymes i.e., CETP and LCAT protein levels were also quantified. The results are presented in Figure 4.14. (Appendix I: TablesI.1, I.2, I.3, I.4, I.5, I.6, I.7, I.8, I.9 and I.10; FiguresI.1, I.2 and I.3).

The results showed that there is marked increase in the nuclear LXR-α in dose and time dependent response. Significant increase ($p<0.05$) was detected at 50 µg/mL (1.26-fold) and 200 µg/mL (1.77-fold), respectively. Meanwhile, significant change was detected at 4h, 12h and 24 h upon treatment with 200 µg/mL of EA. Similarly, the results also revealed that oleate-induced HepG2 cells treated with EA markedly increase ($p<0.05$) cellular accumulation of LCAT and CETP in dose-and time dependent manner. At (50 µg/mL and 200 µg/mL) the LCAT and CETP protein levels detected intracellularly were 1.37-fold, 1.93-fold and 1.44-fold, 1.60-fold, respectively.

Interestingly, further examination on LCAT and CETP secretions has shown an uptrend of LCAT but a downtrend of CETP protein levels in dose- and time-dependent response. Significant changes (($p<0.05$) of LCAT and CETP protein levels were observed at 50 µg/mL (1.51 fold, 0.66 fold) and 200 µg/mL (1.76 fold, 0.51 fold) relative to control, respectively. Significant changes were detected starting at 4h onwards for all three LXR-α, LCAT and CETP in oleate-induced HepG2 cells treated with EA at 200 µg/mL, respectively.

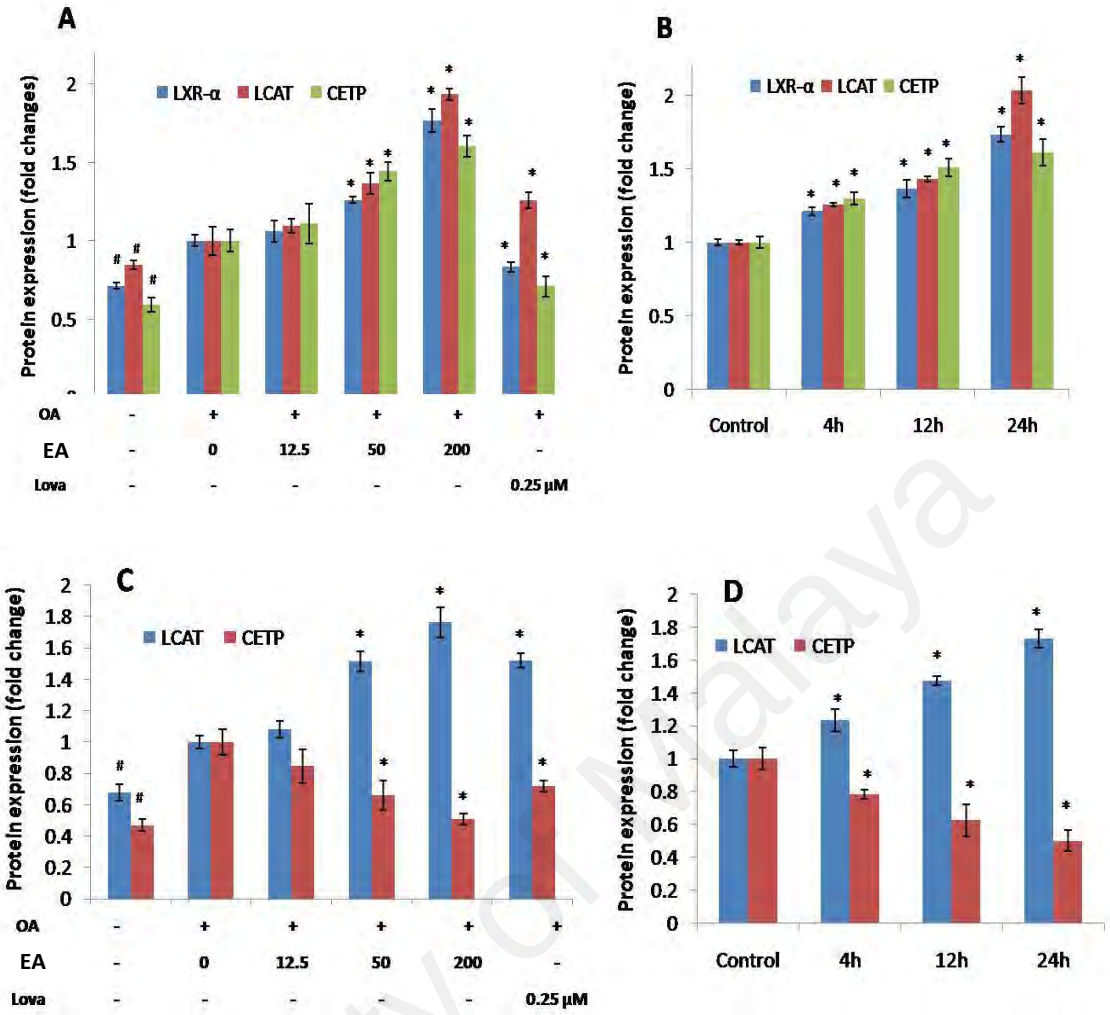

Figure 4.14: The effect of EA on intracellular (A and B) and secreted (C and D) LXR-α, LCAT and CETP at different concentrations and times.

HepG2 cells induced with 0.8 mM sodium oleate were incubated for 24 h without (control) or with EA (0-200 µg/mL). Lovastatin (0.25 µM) was used as a positive control. Cells incubated without oleate served as negative control. Amount of LXR-α, LCAT and CETP measured using respective commercial ELISA kits were normalised to intracellular protein. All results were presented as mean of fold change ± standard deviation of triplicate measurements (n = 3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The differences between means were significantat * ($p< 0.05$), relative to oleate-induced HepG2 cells (control). EA = ethyl acetate; Lova = lovastatin; OA = oleic acid.

To further evaluate whether the intracellular assembly of apoA1, apoB-100, apoE, LXR-α, LCAT and CETP proteins was initiated at their transcriptional levels, the mRNA levels of the abovesaid targets were then purified and quantified using qRT-PCR technique and the mRNA expression calculated were normalised to GADPH reference gene. The results with regards to dose effect (A) and time course (B) were presented in Figure 4.15.

Overall, the results marked significant changes with increasing dose of treatment and on time basis. The mRNA level of apoB-100 was detected to be significant ($p < 0.05$) in oleate-induced HepG2 cells treated with a lower dose at 12.5 µg/mL of EA. However, significant changes of mRNA expression of all targets were observed at 50 µg/mL and 200 µg/mL, respectively. Particularly, oleate-induced HepG2 cells treated with (50 µg/mL and 200 µg/mL) showed significant changes on mRNA levels of apoA1 (1.76 fold, 2.24 fold), apoB-100 (0.65 fold, 0.36 fold), apoE (1.74 fold, 2.08 fold), NR1H3 (2.03 fold, 3.02 fold), LCAT (2.19 fold, 3.83 fold) and CETP (2.53 fold, 2.88 fold), relative to untreated cells (control) respectively. Similarly, oleate-induced HepG2 cells treated with 0.25 µM lovastatin showed marked changes on all molecular targets except for LXR-α (Appendix H: TablesH.13, H.14, H.15, H.16, H.17 and H.18; Appendix I: TablesI.11, I.12, I.13, I.14, I.15 and I.16).

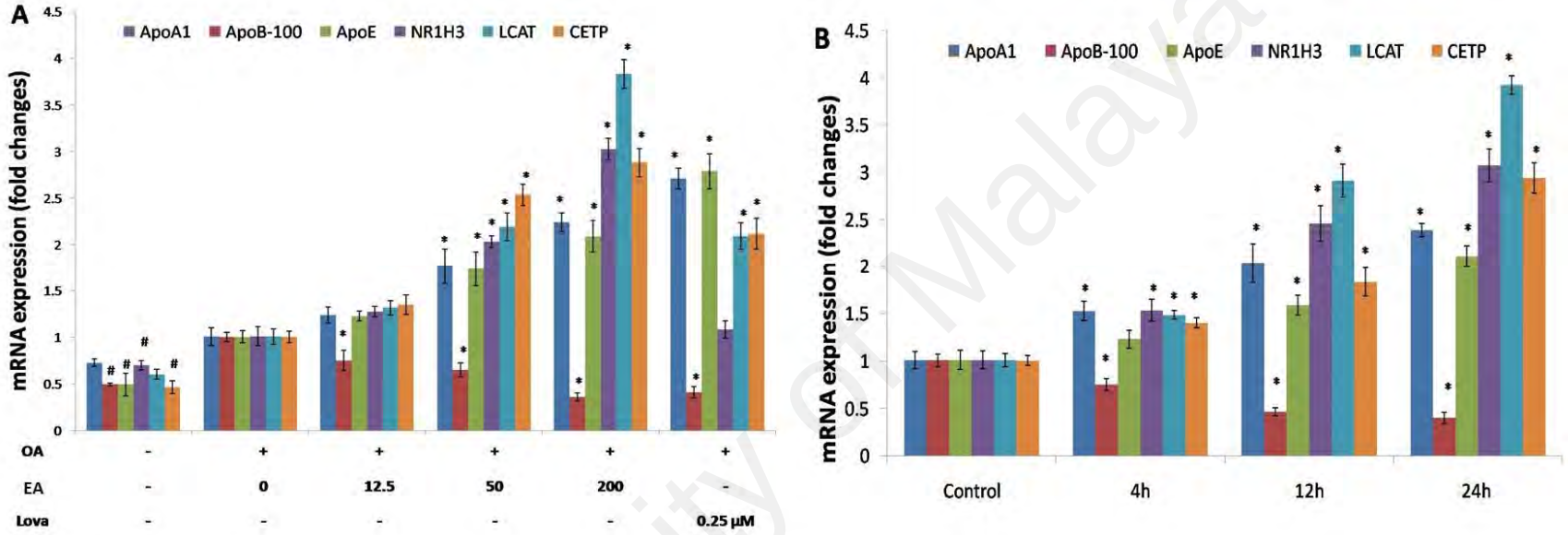

Figure 4.15: The effect of EA on mRNA expression levels of apoA1, apoB-100, apoE, NR1H3, LCAT, CETP at different concentration (A) and time (B).

HepG2 cells induced with 0.8 mM sodium oleate were incubated for 24 h without (control) or with EA (0-200 µg/mL). Lovastatin (0.25 µM) was used as a positive control. Cells incubated without oleate served as negative control. The mRNA expression levels ApoA1, ApoB-100, ApoE, LXR-α (NR1H3), LCAT and CETP normalised to GADPH were calculated by using $2^{-\Delta\Delta Ct}$ method. All results were presented as mean of fold change ± standard error (SEM) of triplicate measurements (n = 3) in three independent experiments. Data were analysed by one-way ANOVA followed by Duncan test. The differences between means were significantat # or * ($p < 0.05$), relative to oleate-induced HepG2 cells (control). EA = ethyl acetate; Lova = lovastatin; OA = oleic acid.

CHAPTER 5: DISCUSSION

5.1 Antioxidant and lipid lowering potential of AR

Over the last decade, the research on medicinal mushrooms has gained the greatest attentions. For a long time, medicinal mushrooms have demonstrated its beneficial properties in human health. Hence, they become attractive source for the development of anti-atherogenic agents. Until recent, the *in vitro* antioxidative potential, inhibitory effect of LDL oxidation and cholesterol lowering via inhibition on HMG-CoA reductase activity using secondary metabolites derived from the fruiting bodies of AR is yet to be elucidated. The organic solvent extraction, followed by liquid-liquid partition served three purposes, (i) to separate low-molecular weight compounds based on their chemical nature/structure and polarity, (ii) to concentrate bioactive compounds of interest, and (iii) to eliminate undesirable or toxic compounds that could mask the potential use of the fraction tested, thereafter, facilitating further bioassay screenings.

5.1.1 Antioxidant capacity

Oxidants are the products of normal aerobic metabolism and inflammatory response. They play critical role in the pathogenesis of atherosclerotic cardiovascular diseases. The black lingzhi is believed to exert polypharmocological potencies to alleviate various pathological conditions due to oxidative stress and its related cardiovascular disease.

Based on the antioxidant results, EA has demonstrated the highest concentration of the semi-polar phenolics (e.g., flavonoids, and non-flavonoids), which have been plausibly to strive for its protective effect against cardiovascular disease (Firuzi *et al.,* 2011). In fact, most of the treacherous free radicals in human are derived from oxygen. The reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot}$), hydroxyl radical

(OH·) are probably responsible for much of the biological damage *in vivo* and hence, a rich array of low-molecular weight molecules that preferentially scavenge free radicals is crucial to maintain the cardiovascular health status (Maxwell *et al.,*1997).

DPPH assay is good test to demonstrate the free radical scavenging ability exerted by AR extracts. Consistent with its high phenolic content, the EA showed the strongest scavenging effects among the five extracts tested. Therefore, EA is considered as a good source of primary antioxidants (Panda, 2012) and acts as excellent radical scavenger to neutralise the highly energetic radicals. This mechanism is very important in preventing free radicals from continuously attacking the cellular components that cause chain breaks and damages. Amongst all the extracts tested, both the EA and DCM exhibited good ferrous ion (Fe^{2+}) chelating ability. This suggested that they are good source of metal chelators that have the ability to remove the toxic heavy metals and preventing them from attacking the body system. This mechanism is only shown by secondary preventative antioxidants which remove the toxic particles that could possibly generate free radicals. Next, EA also showed the most potent reducing power among five extracts tested through CUPRAC assay. This suggested that EA is a good source of antioxidants via electron-transfer-based redox mechanism, possibly owing to its high phenolic content.

Cardiovascular disease could be the deleterious actions of oxygen-derived radicals on cellular lipid peroxidation. Modification of membrane lipids has been proposed to play a major role in endothelial dysfunction making it much susceptible for the pathogenesis of atherosclerosis (Rikans *et al.,* 1997). It is acceptable that upon aging, there is an increased rate of cellular lipid peroxidation that occurs in the body system. Weakening of immune system combined with low endogenous antioxidant levels in the body has been related to various chronic diseases. Hence, mushroom-derived natural antioxidants could be a good source in addressing this issue. Furthermore, the roles of

such novel mushroom-derived antioxidants and their preventive mechanisms should be given much attention.

In this study, the inhibitory effect of AR extracts on lipid peroxidation was assayed using buffered egg yolk as the source of unsaturated fatty acids (oleic, linoleic, palmitolic and linolenic acids). These unsaturated fatty acids help to simulate the polyunsaturated fatty acid components as per biological membranes as they shared similar lipid peroxidation mechanistic attacks (Maxwell *et al.,* 1997). Our results showed that the EA and DCM exhibited good inhibitory effect on lipid peroxidation suggesting that these two extracts contain the bioactive molecules which can protect cellular damages induced by oxidative stress.

Response-to-injury hypothesis of atherosclerosis stated that, endothelial denudation represents the first step in atherosclerosis, probably caused by free radical attacks, hypercholesterolemia and elevation of ox-LDL in plasma (Ross, 1999). Under normal homeostasis, our body is well protected by diverse groups of antioxidants especially, the first line of antioxidants (those naturally-occurring cellular enzymatic defence antioxidants) including superoxide dismutase (SOD), glutathione peroxidse (GPx) and catalase (CAT), which directly involve in the neutralisation of ROS/RNS, and keep in check their level and repair oxidative cellular damage. However, the ROS are produced continuously and at high rate as by-products of aerobic metabolism as demonstrated by Sohal *et al.* (1994), together with the decrease in antioxidant enzymes, as a consequence of aging (Rikans *et al.,*1997), the first line of oxidative defence comes to failure, and the second line antioxidants (radical scavengers, preventative and chain-breaking antioxidants), appeared to be playing a crucial role to maintain the antioxidant status by means of any defence mechanisms.

Hence, the antioxidant effects of EA may attribute to the primary mechanism that mediates the cardio-protective effects against cardiovascular dysfunctions. The

antioxidant agents may be effective for universal target. Granting all this, much effort is needed to underline its mechanism; firstly, preventing cellular oxidative stress, LDL oxidation comes in second, and successively other critical developmental states due to elevated ox-LDL-induced toxicity.

5.1.2 Inhibitory effects on LDL oxidation

Although a high level of plasma LDL is considered to trigger atherosclerosis, the oxidation of LDL seems to be a necessary step. There is a wealth of scientific data coming from *in vitro* and *in vivo* studies that supports validity of the oxidation hypothesis of atherosclerosis, i.e. that the oxidative modification of lipoprotein is a vital event in the evolution of atherosclerotic plaques (Steinberg *et al.*, 2002). Therefore, antioxidant therapy is supposed to be effective in the early stages of atherosclerosis by preventing LDL oxidation and oxidative lesion occurring in sub-endothelial space.

LDL oxidation is believed to occur in the intima of the artery. LDL can be oxidatively modified by copper and iron (transition metals) in a cell-free system. Many studies have revealed that the free radical such as superoxide anion, O_2^-, promotes oxidation of LDL lipids. Besides, thiols autooxidise in the presence of metal ions (Cu^{2+} and Fe^{2+}) producing thinly radicals and superoxide, which also promote LDL oxidation. Nevertheless, the cellular enzyme (e.g., 15-lipoxygenase) also has been recognised to convert polyunsaturated fatty acids (PUFAs) into lipid hydroperoxides which may oxidise LDL (Sparrow *et al*., 1988; Heinecke *et al*., 1993; Jialal *et al*., 1996). The EA extract from the *A. rugosum*, provides a wide range of water soluble antioxidants (mostly phenolic compounds) to prevent oxidation of LDL by chelating the transition metal ions, or act as free-radical scavengers to remove free radicals generated during the experiment. However, the complete mechanisms in preventing LDL oxidation by EA is yet to be clarified.

In principle, the n-LDL particles are not taken up by macrophages rapidly enough to generate foam cells; thus it was proposed that LDLs might somehow be modified in the vessel walls. It has subsequently been shown that trapped LDLs do indeed undergo modification, including oxidation, lipolysis, proteolysis and aggregation, and those modifications lead to the change on the conformation of LDL particle surface, initially serve as receptor for LDL-receptor (to be metabolised by liver for clearance), are now switched to the new receptor for macrophage scavenger receptor, contribute to inflammation as well as to foam-cell formation.

LDL modifications initially give rise to "minimally oxidised" LDL species which may not be recognised by macrophage scavenger receptors, as still susceptible for antioxidant repair mechanism, however, heavily oxidised LDLs may represent a terminal by-product (Cherubini *et al.,* 2005). Thus, this receptor can bring cholesterol-rich particles into already cholesterol-saturated macrophages. As the oxidatively-modified LDL is taken up by macrophages, the cell become cholesterol-laden, sluggish, and trapped in the sub-endothelial space, undergo series of inflammation, eventually forming atherosclerotic lesion (Harris, 1992).

A corollary of this hypothesis is that antioxidants should prevent LDL oxidation and therefore protect against the development of atherosclerosis. Undoubtedly, the EA over again showed its protective effect on Cu^{2+}-induced LDL oxidation, serving as a good source for the early prevention of atherosclerosis.

5.1.3 Inhibitory effect of HMG-CoA reductase activity

Hyperlipidemia resulted from lipid metabolic changes is a major cause of atherosclerosis. Hypercholesterolemia or more specifically elevated plasma LDL is an important risk factor for the development and progression of atherosclerosis. The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), is an enzyme that catalyses the four-

electron reduction of HMG-CoA to coenzyme A (CoA) and mevolonate, which is the rate-limiting step in sterol synthesis.

Inhibiting the HMGR enzymatic activity is considered as an important tool for the basic research of cholesterol and other related metabolic pathways. Since AR mushroom extracts were obtained via organic solvent, liquid-liquid partition, the compounds present may be primarily preserved in an inactive form, if any, unlike statin-like compounds, present in aqueous (in an active open-ring hydroxy carboxylic acid form). The ionised form is crucial for the binding of the compound with the active site of HMGR to inhibit its enzymatic activity. Therefore, the hydrolysis of AR extracts was done prior to this assay to give a better measurement.

In this regards, consistent with the previous antioxidant and LDL oxidation findings, the EA again showed significantly higher and the most potential inhibitory effect on HMGR activity and the hypothesis can be drawn that the EA contains the bioactive compounds to make it possible to interfere with cholesterol biosynthetic pathway by inhibiting the conversion of HMG-CoA to mavalonate.

5.2 Cytotoxicity study

Mushrooms represent one of the world's greatest untapped resources of food and therapeutic agents of the future. They have been documented to be effective against cancer, cholesterol accumulation, heart disease, insomnia, obesity and diabetes (Wani *et al*., 2010). However, due to cultural background, the content of the mushrooms greatly varies from one country to another. Wild mushrooms may gain bad reputation for causing intoxications due to consumption of misidentified mushrooms. There is an important need to investigate the toxicity of mushroom species with good medicinal value for the development of new drugs or therapeutic agents. The toxicity of mushrooms can be determined via *in vitro* studies using cell lines (Parasuraman, 2011).

The cytotoxicity study revealed that the AR extracts are non-cytotoxic to normal human MRC-5 and WRL 68 cell lines (IC_{50} value >100 μg/mL) and hence merits consideration for further development as anti-atherogenic agents. However, more specific investigation should be conducted to further examine long term consumption and particular safety-related issues with regards to black lingzhi mushroom. Since the AR extracts showed no significant reduction in HepG2 cell viability at 200 μg/mL, thus, this concentration was used for the investigation in lipid and apolipoprotein assays.

5.3 Effect of AR on lipid and apolipoprotein level

Preliminary screening on the lipid lowering effects is an initial step for developing anti-hyperlipidemic agents. The studies were conducted by examining how the AR extracts alter the expression of lipids, i.e., total cholesterol, total triglycerides and apolipoproteins, i.e., apoA1, apoB-100 and apoE. According to Javitt *et al*. (1990), HepG2 cell is considerably suitable research tool as it resembles many typical functions of the normal hepatocytes, particularly those metabolisms involved lipids, apolipoproteins, lipoproteins, bile acids and many other lipid regulating specific proteins mainly responsible for maintenance of blood cholesterol homeostasis.

5.3.1 Effect of AR on TG, FC and CE levels

The effect of AR extracts on accumulation and secretion of TGs, TC, FC and CE in hepatic cells was investigated. Sodium oleate (0.8 mM), the lipogenic factor, significantly stimulated cholesterol and TG productions, and lovastatin (an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, HMG-CoA reductase) was used as the positive control.

Our results clearly demonstrated that oleate-induced HepG2 cells treated with EA showed a marked reduction (p<0.05) in TG accumulation and secretion relative to

control, and this suggests that the EA interferes with TG metabolism possibly by inhibiting TG biosynthesis and thus its secretion. Unlike the HepG2 cells treated with lovastatin, no significant difference in the cellular accumulation of TG was observed; however, the secretion of TG in the medium was significantly much lower than that of the control.

EA and lovastatin showed similar reduction in TG secretion via different mechanisms, in which EA reduced the TG production and secretion, whereas lovastatin only retained the TG from secretion without interfering with its production. This observation was in agreement with Gienberg (1998) and Scharnagl *et al*. (2001). According to Scharnagl *et al.* (2001), some statins (e.g., atorvastatin, lovastatin and simvastatin), are the potent HMG-CoA reductase inhibitor in reducing cholesterol biosynthetic pathway. However, HepG2 cells treated with those corresponding statins showed marked increase in expression of *FAS* gene, and its *promoter genes* (e.g. ACC), which are essential for fatty acid synthesis, and thus triglyceride production. Therefore, statins, appear to affect the biosynthetic pathways of cholesterol and triglyceride in an opposite manner.

However based on our current data, the exact mechanism in TG lowering needs further verification.

Besides, oleate-induced HepG2 cells treated with EA showed a marked reduction of intracellular and secreted TC, FC, and CE. These observations led to the hypothesis that EA intervened with cholesterol metabolism in oleate-induced HepG2 cells. Besides, a reduction in the conversion of FC to CE was also observed.

Lowering in the bioavailability of TG and CE are the main concern of cellular production, assembly and secretion of TRL particles such as VLDL and IDL, and CE-rich end product LDL as TG and CE are not only the major lipid core but also one of the determinants of the distribution of circulating lipoproteins (VLDL, IDL, LDL and HDL).

Therefore, detailed investigation should be conducted to confirm the mechanisms that underline the TG and TC lowering activity by AR.

5.3.2 Effect of AR on apolipoprotein level

In addition to TG and CE in the core, plasma lipoprotein particles also contain specific apolipoproteins, which determine their density and size, and their biological roles in the body system (Getz *et al.,* 2009; Lu *et al*., 2011; Ooi *et al*., 2012). Our results demonstrated the oleate-induced HepG2 cells with the EA (200 µg/mL) showed marked reduction in intracellular and secreted apoB-100 implying that EA down-regulated its assembly at the cellular level and thus its secretion.

Besides, similar marked reduction was also observed on the intracellular and secreted apoE. Both apoB-100 and apoE are apolipoproteins present in all atherogenic lipoproteins (VLDL, IDL and LDL), hence, it can be hypothesised that EA inhibited the atherogenic lipoprotein assembly and secretion on oleate-induced HepG2 by intervention of apoB-100 and apoE cellular synthesis and secretion pathway. The reduction of the secreted atherogenic lipoproteins could be due to the reduction of lipid (TG and CE) availability, or down-regulation of apoB-100 and apoE production.

Interestingly, oleate-induced HepG2 cells treated with EA (200 µg/mL), showed a greater reduction in lipids (TG, FC, CE) and apolipoproteins (ApoB-100 and ApoE) compared to low-dose lovastatin (0.25 µM). High dose lovastatin therapy has been reported with regards to its toxicity. Although lovastatin is a specific inhibitor of the rate-determining enzyme in cholesterol synthesis (HMG-CoA reductase), however during the preclinical assessment using dogs, rates and rabbits, researcher has found that lovastatin induced hepatotoxicity, liver and kidney damage, hepatic necrosis and etc (Kornbrust *et al*., 1989; Mullen *et al*., 2011; Zhang *et al*., 2013).

The benefits that EA has brought the benefits eventually lead to lowering of LDL-C and non-HDL, i.e., TG-rich VLDL, IDL and their remnants as recommended by NCEP ATP III. Thus, the fruiting bodies of AR showed the cholesterol lowering potential in oleate-induced HepG2 cells, and the bioactive components are most likely present in the EA hinting the future development using AR as one of the lipid lowering agents.

In addition, oleate-induced HepG2 cells with the EA showed a marked increase in cellular and secreted apoAI, suggesting that EA up-regulated the production of apoAI and thus HDL. The HDL has been recognised to play significant role in the reverse cholesterol transport (RCT) to remove excessive cholesterol deposited on the artery wall as well as other anti-atherogenic benefits, i.e., anti-oxidative, anti-LDL oxidation, anti-inflammatory, anti-thrombotic effects. Hence, raising apoAI by any means are new potential targets for therapies designed to prevent atherosclerosis.

5.4 Chemical constituents in EA

Based on the previous results, EA demonstrated the potential anti-atherosclerotic effect. EA was then analysed using LC-MS/MS to correlate its chemical constituents to its potential/possible functions.

However, from the chemical analysis, lovastatin or any other statin-like compounds were not detected in the EA extract. Hence, the mechanisms that underlined lipid lowering activity of EA may be different from that of lovastatin. The lipid lowering activity of EA in oleate-induced HepG2 cells could be via the action of different molecular targets (LXR-α, PPAR-α, SREBPs, etc) by its phenolic content instead of directly inhibited the HMG-CoA reductase by the statin drugs.

Epidemiological evidence indicates that consumption of polyphenol-rich antioxidant diets has remarkably reduced cardiovascular risk. The water-soluble phenolics act as extrinsic antioxidants that quench reactive oxygen species and hence

potentially modify pathogenic mechanisms and be protective against cardiovascular disease (Renaud *et al.,* 1993). Furthermore, there are reports claiming that the polyphenols, especially flavonoids, act as potent free-radical scavengers *in vitro*, and may prevent the oxidation of LDL thereby delaying atheroma formation. (Viana *et al.,* 1996).

Consistent with other findings, the polyphenols are also effective on other independent mechanisms including anti-platelet, anti-inflammatory, increasing HDL plasma level, and improving endothelial function, thus making it a possible protective compound against cardiovascular diseases (Pandey *et al.,* 2009). To correlate our chemical finding to atherosclerotic cardiovascular disease, it was found that the hydroxy phenolic acids (protocatechuic acid, caffeic acid, vanillic acid, *p*-hydroxybenzoic acid and methyl-3,4-dihydroxybenzoate) present in the EA had been reported as potent antioxidants, radical scavengers, and inhibitors of lipid peroxidation in cellular component.

Caffeic acid has been found to exert good antioxidant capacity *in vitro* and *in vivo*. It is an active antioxidant via DPPH, ABTS and superoxide anion scavenging capacity. In addition, it also acts as metal chelator on ferrous ions. Its inhibitory effect on lipid peroxidation and LDL oxidation via lowering TBARS levels were observed in rats (Gulcin *et al*., 2006; Nardine *et al*., 1995; Yeh *et al*., 2009). Nevertheless, many studies have revealed that caffeic acid plays important roles in lipid metabolisms by lowering the hepatic HMG-CoA reductase activity in rats (Liao *et al*., 2013) and enhancing LDL receptor binding activity (Tabbon *et al*., 2016). Besides, it also lowers the liver FAS activity, free fatty acid, TG and TC in rats (Bezerra *et al*., 2012; Liao *et al*., 2014). Studies also found that caffeic acid raised the serum HDL by upregulating apoA1 expression (Kempf *et al*., 2010).

On the other hand, the anti-atherogenic effects of protocatechuic acid have been well-established. Protocatechuic acid is not only a good DPPH and superoxide anion

scavenger but also a strong metal chelator (Li *et al*., 2011). As a good antioxidant, it was demonstrated to inhibit lipid peroxidation and hence supresses LDL formation (Semaming *et al*., 2015; Yeh *et al*., 2009). Investigation on lipid effect revealed that protocatechuic acid decreased serum VLDL, IDL, and LDL (Akiko *et al*., 2004; Borate *et al*., 2011; Li *et al.,* 2011). The decrease of such lipoproteins may be associated with decrease hepatic apoB-100 and apoE expression in rats (Akiko *et al*., 2004). In contrast, protocatechuic acid was shown to increase HDL and LCAT (Borate *et al*., 2012) in promoting reverse cholesterol transport as well as enhancing hepatic LDL receptor binding activity (Atre *et al.,* 2012).

Some evidence also documented that vanillic acid is a good antioxidant through its free radical scavenging activity (Palafox-Carlos *et al*., 2012), inhibition on lysosomal membrane damage in rats (IIamathi *et al*., 2015) and decrease lipid peroxidation (Dianat *et al*., 2014). Its mechanism on lipid modulating effect was demonstrated via decrease of serum TGs, free fatty acids in high-fat diet rats (Chang *et al*., 2015), serum TC, LDL and VLDL while increasing HDL (Mol *et al*., 2010).

Ellagic acids, on the other hand, have been shown to act as potent antioxidants inhibiting LDL oxidation *ex vivo* and/or increasing plasma antioxidant capacity (Baharun *et al.,* 2006). Nevertheless, it was found to inhibit oxidised LDL-mediated LOX-1 (lectin-like receptor) expression, ROS generation and inflammation in human endothelial cells. Expression of LOX-1 involved in the pathobiological effect of ox-LDL in endothelial cells, including reactive oxygen species (ROS) generation, suppression of endothelial nitric oxide synthase (eNOS) activity, and leukocytic adhesion. Hence, ellagic acid, may have a protective effect against the development of atherosclerosis via the antioxidant capacity at the endothelial level.

The polyphenolic flavonoids, besides its antioxidant, and cholesterol lowering properties, have been shown to interact with LOX-1 expression. The flavones (luteolin

and apigenin) were found to inhibit THP-1 cell adhesion onto oxidised LDL-activated human umbilical vein endothelial cells (HUVEC). This finding demonstrated that they are effective in the different initial steps of atherosclerosis process by inhibiting oxidised-induced endothelial monocyte adhesion and/or oxidised LDL uptake. Therefore, certain flavonoids bear the quality as anti-atherogenic agents in LDL systems, which may have implication for strategies attenuating endothelial dysfunction-related atherosclerosis (Jeong *et al.*, 2007).

Naringenin has been widely studied in issues related to atherosclerosis. One of its metabolites, 4-hydroxybenzoic acid, has been shown to be beneficial by lowering plasma total cholesterol, triglyceride, and attenuated atherogenic index in high-cholesterol-fed rats. It was demonstrated to raise the HDL concentration and on the other hand reduced the HMG-CoA activity. Further investigation showed that the 4-hydroxybenzoic acid was effective in improving the cholesterol and antioxidant metabolism (Jeon *et al*., 2007).

In comparison with statin drugs, EA could be a safer alternative in treating atherosclerotic cardiovascular disease as it is not only proven to lower the lipid production, but also serve as excellent antioxidants to protect human from oxidative stress, which is the key initial step of various chronic diseases. High dose statin therapy has been associated with adverse side effects (e.g., myalgia and rhabdomyolysis) whereas low dose therapy may not achieve the lipid lowering goal (Ramkumar *et al*., 2016). In fact, there are many herbs have been accepted with their hypolipidemic effects. For example, basil, blueberry, celery, dandelion, dill, eugenol, evening primrose oil, funugreek, ginger, ginseng, grape, green tea, nigella and psyllium have been proven to play an important role in lipid lowering effects. The mechanisms of action of those herbs have been extensively studied. In this regard, there is increasing number of foods containing herbs has raised the concern of the food and drug administration (FDA).

Therefore, herbal remedies, could be the natural alternatives of statins in treating dyslipidemia (Rouhi-Boroujeni *et al*., 2015).

Taken together, AR has been reported to contain various chemical substances, phenolic antioxidants, flavonoids, triterpenes which play multifunctional roles in maintaining human cardiovascular health (Fakoya *et al.,* 2013). This may be due to the presence of the bioactive compounds identified in EA.

5.4.1 Quantification of bioactive compounds in EA

Although there are many other potential bioactive compounds present in EA, however, only those compounds that exist as major components and of known identity and potential antioxidative and lipid lowering activities were selected for quantitation. The chemical compounds such as protocatechuic acid, caffeic acid, vanillic acid and 4-hydroxybenzoic acid present in EA were identified from LC/MS/MS method and confirmed by comparing their molecular weights and fragmentation pattern of ions, with the natural product library and other published date from the literature review. Based on the literature in section 5.4, the *in vitro* and *in vivo* antioxidant and lipid modulating effect of protocatechuic acid, caffeic acid, vanillic acid and 4-hydroxybenzoic acid have been well-studied by other researchers (Akiko *et al*., 2004; Atre *et al*., 2012; Bezerra *et al*., 2012; Borate *et al*., 2012; Chang *et al*., 2015; Guicin *et al.,* 2006; IIamathi *et al*, 2015; Kempf *et al*., 2010; Li *et al*., 2011; Liao *et al*., 2013 & 2014; Mol *et al*., 2010; Nardine *et al.,* 1995; Palafox-Carlos *et al*., 2012; Semaming *et al*., 2015; Yeh *et al*., 2009).

Hence, the concentration of those compounds was quantified for EA extract standardisation. The presence of these compounds contributes to the high antioxidant capacity in EA as well as a good source of lipid ameliorating agent. Further investigation using the individual compounds and mixed compounds on lipid lowering effect in oleate-

induced HepG2 cells was conducted and the results were in agreement with the hypothesis.

5.5 Effects of EA on lipid and apolipoprotein level

There are two independent pathways to demonstrate how would the EA improves the lipid metabolism in oleate-induced HepG2 cells. It could be related to the effect of EA on both the lipids (TG and TC), as well as apolipoprotein (apoA1, apoB-100 and apoE) level, respectively.

5.5.1 Effects of EA on lipid level

In our preliminary research, the EA showed lipid lowering potential in oleate-induced HepG2 cells. Further investigation has revealed that EA reduced TG, TC, FC and CE in oleate-induced HepG2 cells in dose-dependent manner.

Previous chromatographic chemical analysis on EA performed using HPLC-MS/MS has qualitatively identified ten of the phenolic compounds. Among those, four bioactive compounds (PCA, CA, VA and 4HBA) have been documented and hence they were selected to further examine their roles in lipid lowering effect in oleate-induced HepG2 cells. Accordingly, PCA, CA and VA have been reported for their antioxidant capacity, anti-atherosclerotic and anti-hyperlipidemic activities (Kaklar *et al*., 2014; Prince *et al.*, 2011; Yeh *et al*., 2009). Besides, Borate *et al.* (2011) has also reported that PCA possesses anti-hyperlipidemic in hyperlipidemic rats by decreasing TG, TC and LDL levels, while increasing HDL level via up-regulating LDL-receptor pathway.

5.5.2 Effects of selected bioactive compounds on TG and TC level

Our results showed that HepG2 cells treated with EA marked a significant decrease in all lipid (TG, TC, FC and CE) accumulations and secretions in dose dependent

manner. Although EA has shown its efficacy in lipid lowering activity in the present study, variation of results could be obtained using the same source of mushroom due to extraction method, geographical factors, genetic issue and time of harvest and etc (Kunle *et al.,* 2012). Hence, quantification of four selected bioactive compounds, PCA, CA, VA and 4-HBA present in EA using HPLC-MS/MS method was performed in order to standardise EA as it is conceived that the lipid modulating effect could be affiliated with its phenolic bioactive compounds present.

Further evaluation on lipid effect was carried out using the selected four bioactive compounds on oleate-induced HepG2 cells and results were compared with EA. Our results showed that HepG2 cells treated with PCA, CA, VA and 4HBA alone demonstrated marked significant decrease in both intracellular and secreted lipids (TG and TC) in dose-dependent manner but with lesser extent when compared to EA. When similar experiment was performed using a PM, a further reduction in both intracellular and secreted lipids (TG and TC) comparable with EA was observed. Therefore, the conclusion can be drawn is that the lipid lowering activity of EA could partially be due to the phenolic constituents present.

Further reduction on lipid production and secretion using PM in oleate-induced HepG2 cells was due to synergistic effect between the multiple bioactive components and the molecular targets involved in lipid metabolisms (Zhao *et al.,* 2016).

5.5.3 Effects of EA on apolipoprotein level

Our hypotheses were whether the EA alters apolipoprotein expressions on oleate-induced HepG2 cells and what was the underlying mechanism that supports the modifying activity. Hence, in the present study, the research focused on the apolipoprotein modifying effect in oleate-induced HepG2 cells treated at various concentrations and different time points by EA.

Plasma lipoprotein metabolism is well-regulated by the specific apolipoproteins. The major apolipoproteins include apoA, apoB, apoC and apoE. Specific apolipoproteins function in the regulation of lipoprotein metabolism through their involvement in the transport and redistribution of lipids among various cells and tissues. (Mahley *et al*., 1984).

To investigate whether EA exhibits anti-atherogenic potential in oleate induced HepG2 cells, the mRNA and protein levels of apoA1, apoB-100 and apoE were measured. Our results demonstrated that oleate-induced HepG2 cells treated with EA up-regulated the apoA1 endogenous synthesis at both transcriptional and translational levels in dose and time dependent event. ApoA1 is the major apolipoprotein of HDL. It has been reported to have anti-atherogenic effect through several mechanisms, including the ability to coordinate the reverse cholesterol transport (Larach *et al.*, 2013). In contrast, through our experiment, a down-regulation was observed at the intracellular and secreted apoB-100 due to the suppression at transcriptional levels. Reductions in the apoB-100 secretion in hyperlipidemic model can be beneficial to the prevention of atherosclerosis.

Accordingly, apoB-100 is the primary apolipoprotein of VLDL, LDL which are responsible for transporting lipids around the body. Study performed by Choy *et al.* (2004) evidenced that high level of apoB-100 secretion in hepatic cells is strongly associated to the development of atherosclerosis. In addition, elevated apoB-100 or its related triglyceride-rich lipoproteins (VLDL or LDL) repressed the high density lipoproteins. These favourable biochemical alterations required further understanding to strengthen the hypothesis that EA significantly enhanced endogenous HDL assembly concomitantly suppressed VLDL and LDL production.

Interestingly, up-regulation of apoE observed only at gene transcriptional level but not at protein translational levels. A down trend was detected at intracellular and secreted apoE levels suggesting that newly synthesised apoE is incompletely secreted and

partially degraded by HepG2 cells. ApoE is a component of several classes of lipoproteins, i.e., HDL, LDL, VLD and CM. The decrease in secreted hepatic apoE could be due to the suppression of apoB-100 associated VLDL and LDL synthesis simply because apoE serves as a ligand on the surface of VLDL and LDL particles to bind with LDL receptor family which promotes remnant lipoprotein clearance by the liver.

Although overexpression of apoE observed in other research using lipid-loaded macrophages (and other non-hepatic tissues) was beneficial to cardiovascular protection as it promotes reverse cholesterol transport (efflux) by stimulating VLDL secretion. Some researchers even suggested that apoE may be anti-atherogenic via its modulation of anti-oxidative, anti-coagulant, anti-proliferation, and anti-inflammatory properties with optimum level in the blood circulation. However, the exact relationship between apoE and CVD risk is not well established (Ooi *et al.,* 2012).

Favourably, apoE secreted from other non-hepatic cells depends on its plasma concentration. On the contrary, in our research using hepatic cells, a modifiable RCT pathway seemed to block apoE production and minimise its secretion (triglyceride rich, apoE-containing VLDL and LDL particles). The main roles of hepatic cells in RCT is to stimulate the conversion of cholesterol into bile acids (not promoting the efflux of lipid-loaded apoE-containing VLDL or LDL) in facilitating biliary clearance and faecal excretion (Getz *et al.,* 2009).

In contrast, apoE-containing HDL is highly susceptible for liver uptake and degradation (Schmitt et al., 1999). Moreover, Huang *et al.* (1998) has found that overexpression of apoE causes hypertriglyceridemia attributed to high level of VLDL and decrease level of HDL in transgenic mice. Hence, our results co-ordinately demonstrated that EA enhanced HDL production while suppressed LDL and VLDL secretions in hepatic HepG2 cells. However, its mechanism behind this requires further work to be investigated.

5.6 Effects of EA on lipoprotein metabolism

To demonstrate how EA modulate the lipid metabolism in oleate-induced HepG2 cells, six molecular targets i.e., PPAR-α, LXR-α, SREBP-1, SREBP-2, LCAT and CETP were selected for mechanistic study.

5.6.1. Effects of EA on transcription factor-dsDNA binding activity

Sterol regulatory element binding proteins (SREBPs) are a family of transcription factors that regulate lipid homeostasis by governing the expression of a range of enzymes engaged in the endogenous lipid synthesis. This family has three isoforms that play different roles. SREBP-1 has been implicated as a major factor that up-regulates lipogenic genes involved in fatty acid uptake, activation, oxidation, synthesis and assembly of VLDL. Thus, overexpression of SREBP-1 has resulted in lipid synthesis and hence TG accumulation (Li *et al*., 2014). SREBP-2 is distinctive for cholesterol synthesis and uptake. Overexpression of SREBP-2 up-regulates its responsive genes involved in the cholesterol biosynthetic pathway i.e. HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase and squalene synthase (Horton *et al*., 2002).

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that belong to the superfamily of nuclear hormone receptors. PPARs regulate the expression of several genes linked to metabolic processes which is critical for hyperlipidemia (Contreras *et al*., 2013). PPAR-α is a ligand-activated transcription factor which is expressed predominantly in liver. It has been reported to regulate the expression of genes critical for lipid and lipoprotein metabolism (Yoon, 2009).

Activation of PPAR-α has evidenced of the high rates of beta-oxidation in the liver, suppressing hepatic apolipoprotein C-III and up-regulating lipase gene expression which play a crucial role in triglyceride metabolism. PPAR-α also plays a role in intracellular lipid metabolism by up-regulating the expression of enzymes involved in

mitochondrial fatty acid catabolism, which suppresses the activated SREBP-1 gene involved in fatty acid synthesis (Gervois *et al.,* 2000).

Besides, study conducted by Konig *et al.* (2007) also suggested that PPAR-activation led to a decrease of transcriptionally active nuclear SREBP-2, as well as its target genes such as HMG-CoA reductase and LDL receptor, hence reducing cholesterol synthesis in rat liver and Fao cells.

Specific binding of transcription factors to their dsDNA determines in a great part the connectivity of gene regulatory networks as well as the quantitative level of gene expression (Geertz *et al*., 2010). Our results undoubtedly showed that oleate-induced HepG2 cells treated with EA at various concentrations significantly co-enhanced the PPAR-α and SREBP-1 transcription factors-dsDNA binding activity in dose dependent and time course.

Activation of PPAR-α by EA due to the presence of caffeic acid explained the reduction in TG level intracellular production and secretion but activation of SREBP-1 should promote fatty acid synthesis and stimulate TG accumulation via increasing TG synthesis. In consistent to other research finding, caffeic acid (one of the main ingredients of chicory (*Cirhorium intybus* L) seed extract had been proven to up-regulate PPAR-α gene in oleate-induced HepG2 cells and thus significantly inhibited the accumulation of triglyceride in the liver (Ziamajidi *et al.,* 2013).

Few studies have reported that activation of PPAR-α causes the down-regulation of SREBP-1 (Konig *et al.,* 2009). Furthermore, overexpression of SREBP-1c has been linked to fatty liver (Li *et al*., 2014). Our results showed that activation of PPAR-α failed to suppressed the SREBP-1 in both mRNA and protein levels. This discrepancy could be explained by the activation of liver X receptor (LXR) transcription factor. Our observation was in agreement with research performed by Yoshikawa *et al.* (2003) which reported that activation of LXR-α induced SREBP-1 mRNA and protein levels.

Furthermore, overexpression of PPAR-α dose dependently inhibited SREBP-1c promoter activity. Hence, the inhibition of TG synthesis was confirmed by activation of PPAR-α which will supress SREBP-1 by blocking its promoter genes but not *SREBF-1* gene itself. On the other hand, the binding of SREBP-2 transcription factor to dsDNA was inhibited in dose and time dependent manner after the oleate-incuded HepG2 cells were treated with EA. This has suggested that EA directly suppressed the binding activity of SREBP-2 to its dsDNA and thus lead to inhibition of cholesterol synthesis.

There is no correlation between the transcription factors protein levels and its binding activity to their dsDNA responsive elements since the quantity of protein levels was not determined. Hence, further investigation on their gene expressions was performed. To validate the results obtained from the previous section, the mRNA levels of *PPAR-α, SREBF-1* and *SREBF-2* were determined. Our results showed that oleate-induced HepG2 cells treated with EA at various concentrations caused a marked increase of mRNA levels of *PPAR-α* and *SREBF-1* but a marked decrease of mRNA level of *SREBF-2*.This has proposed that EA contains the bioactive constituents that could be acting not only at the transcriptional level, but also the post-transcriptional, translational and post-translational levels.

Our results are in agreement with Cho *et al.* (2010) and Kim *et al*. (2014) where caffeic acid significantly inhibited fatty acid synthase, increased fatty acid beta oxidation activity and PPAR-α expression in the liver in high fat fed mice leading to lowering of TG level. Besides, research done by Ou *et al.* (2011), using Mulberry extract containing protocatechuic acid and caffeic acid has shown suppression on cholesterol biosynthesis by inhibiting SREBP-2 protein expression at post-translational level using oleate-induced HepG2 cells. Hence, we believe that the lipid lowering activity by EA in oleate-induced HepG2 cells via activating PPAR-α pathway by promoting mitochondrial beta-oxidation

and suppressing SREBP-2, the cholesterol biosynthetic pathway due to the presence of such phenolic compounds in EA.

5.6.2 Effect of EA on LXR-α, LCAT and CETP level

Up-regulation of hepatic ApoA1 has been an indication of putative reverse cholesterol transport (RCT) pathway. The newly synthesised HDL by the liver is secreted as "free or lipid poor", disc-shaped nascent particles whereby the lipidation should occur along the blood circulation and return to liver for its clearance. To further evaluate whether the RCT pathway is activated, three molecular targets were selected i.e., LXR-α, LCAT and CETP were used to further confirm this hypothesis.

The liver X receptors (LXRs) that belong to nuclear receptor superfamily of ligand-activated transcription factors has been recognised to play a vital role in lipid homeostasis. It is highly expressed in liver, small intestine, kidney, macrophages and adipose tissues. LXR-α activation regulates RCT, bile acid synthesis and excretion in the liver (Zhao *et al.,* 2010). Many studies with regards to RCT were performed using macrophages as researchers believed that accumulated cholesterol from peripheral tissue should be transported back to liver followed by biliary secretion or disposal via the faeces. Hence, the synthetic LXR-α agonists, T0901317 and GW3965 were used to stimulate cholesterol efflux from peripheral tissues to liver via RCT pathway.

However, little research was conducted to investigate how liver, as the main organ of lipid metabolisms to coordinate the RCT. Being non-selective, LXR-α agonists not only activated LXR-α in the macrophages, but also hepatic cells. Activation of LXR-α in the liver cells co-stimulate the overexpression of other lipogenic genes i.e., *SREBF-1*, *FAS* and *ACC1* that increase the plasma triglyceride synthesis, overproduction of VLDL and eventually, leading to hepatic steatosis (Grefhorst *et al*., 2002). This has raised the

challenge where activated LXR-α promotes RCT in non-hepatic tissues but triggers hypertriglyceride in hepatocytes.

Does it mean hepatic LXR activity is undesirable from a therapeutic perspective? Research carried out by Zhang *et al.* (2012), has documented that liver LXR-α expression is crucial for the whole body cholesterol homeostasis and RCT in consideration of the development of LXR-α agonists. In particular, liver-specific deletion of LXR-α substantially decreased RCT, cholesterol catabolism, and cholesterol excretion in mice.

In agreement with this, our results clearly demonstrated that oleate-induced HepG2 cells treated with EA significantly up-regulated the LXR-α in dose and time-dependent manner at post-transcriptional and post-translational levels suggesting that EA plays a vital role in coordinating RCT pathway. Research conducted by Kondo *et al*. (2010), has clearly shown that caffeic acid, a metabolite of chlorogenic acid present in coffee enhance reverse cholesterol transport (RCT). It was shown to up-regulate the HDL in THP-1 macrophages. Besides, Xia *et al*. (2005) also reported that phenolics-rich coffee consumption enhances cholesterol efflux from macrophages by up-modulating ABCA1 expression via PPAR-γ and LXR-α pathway. Hence, we believed that the up-regulation of LXR-α was due to the presence of caffeic acid in EA.

In our previous work, oleate-induced HepG2 cells treated with EA marked significant increase in the production of *SREBF-1* mRNA and protein levels which is believed to be upregulated by LXR-α. Surprisingly, lower level of intracellular and secreted TG was detected. Such discrepancy can be explained by the overexpression of PPAR-α dose dependently inhibited SREBP-1c promoter activity according to Yoshikawa *et al.* (2003).

Furthermore, activation of PPAR-α has evidenced the high rates of beta-oxidation in the liver which play a crucial role in triglyceride metabolism and mitochondrial fatty acid catabolism (Gervois *et al.,* 2000). The multifactorial therapeutic approaches using

herbal extracts were common in traditional medicine. Our observation was strengthened by other research performed. In 2015, Wu *et al.,* has reported that *Zanthoxylum bungeanum* extract attenuated reversed lipid accumulation, decreased apoB and enhanced apoA1 secretion in HepG2 *in vitro* model by regulating the expression of genes involved in RCT, such as *CYP27A1*, *NR1H3*, *ABCG1*. Nevertheless, reduction of serum TC and TG levels were observed in apoE-KO mice.

APOE and *CETP* were two target genes of LXR-α involved in cholesterol and lipid metabolism. Undoubtedly, activation of LXR-α up-regulates ApoE and CETP especially in non-hepatic tissues such as macrophages promotes RCT by cholesterol efflux via secretion of apoE-containing VLDL, and LDL particles (but not in hepatic tissues). Meanwhile, in the liver, the cholesterol efflux as described in the RCT should be modified (prevent excessive efflux) to allow bile acid conversion to occur before the remnants of lipoproteins (VLDL, LDL or HDL) are re-circulated into the plasma. This cholesterol homeostasis should be well balanced.

Besides, CETP has been reported to be atherogenic although it participates in RCT as it mediates the exchange of CE in HDL for TG in VLDL. This action causes the lowering of anti-atherogenic HDL and increasing pro-atherogenic VLDL and LDL particles (Okamoto *et al.,* 2000). Our results has evidenced that activation of LXR-α in oleate-induced HepG2 cells treated with EA only showed marked increase of ApoE and CETP expression at transcriptional levels in dose and time course but marked decrease at post-translational levels. The newly synthesised apoE and CETP were believed to be partially degraded and suppressed for their secretions. The exact mechanism underlying this modified RCT remains unclear but this has to be beneficial in retarding atherosclerosis at least *in vitro* in HepG2 cells.

Unlike CETP, Lecithin-cholesterol acyltransferase (LCAT) is another beneficial transfer protein that plays a central role in the RCT process. It is a key enzyme that

catalyses the conversion of free cholesterol in plasma lipoproteins to cholesterol ester (CE) and then sequestered into the core of HDL (Kunnen *et al.*, 2012). With this, excessive cholesterol deposited in the macrophages can be removed (lipid-laden macrophages is the main factor of atherosclerosis). Consistent with our results, overexpression of *LCAT* mRNA and protein levels in oleate-induced HepG2 cells in dose and time-dependent fashion suggested that EA up-regulated the *LCAT* target gene and protein.

However, LCAT is neither regulated by LXR-α nor apoA1. The regulation on its biosynthesis and secretion required further investigation. However, being the major compartments of HDL, overexpression of both apoAI and LCAT targets has further confirmed the activation of the RCT pathway in promoting cholesterol clearance in the plasma (Cheema, 2006). The mechanism of action of EA on oleate-induced HepG2 cells is summarised in Figure 5.1.

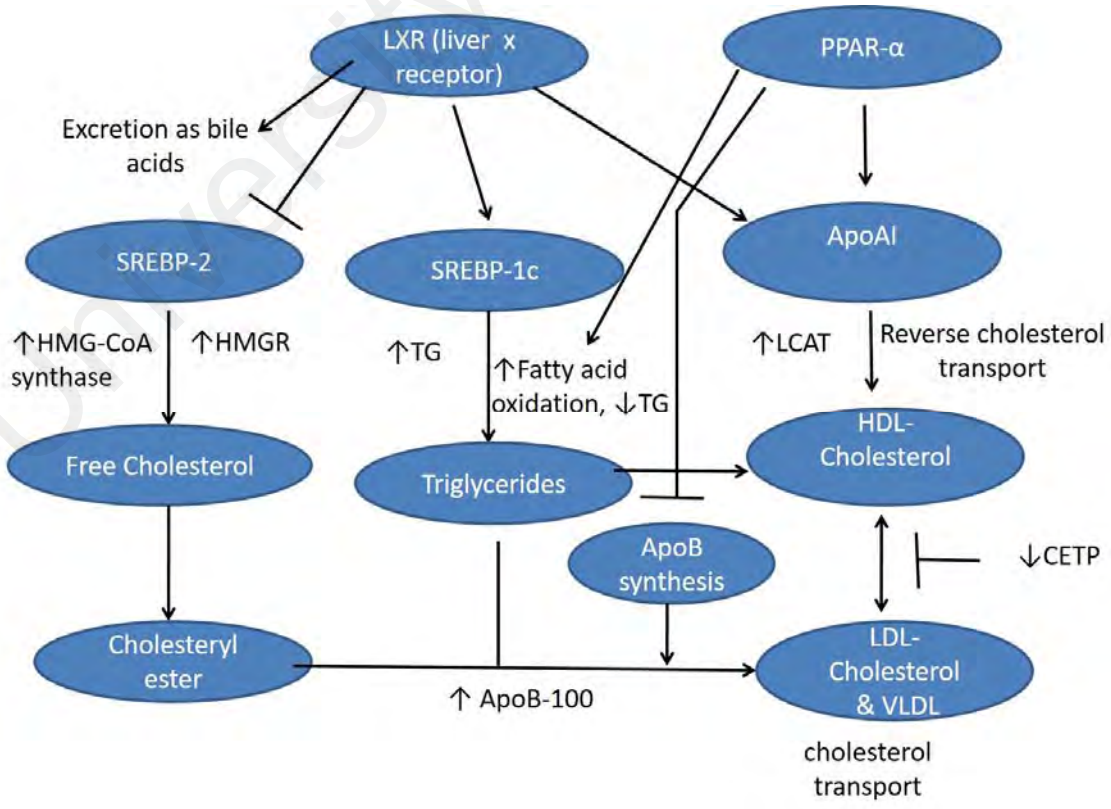

Figure 5.1: Mechanism of actions of EA on oleate-induced HepG2 cells.

CHAPTER 6: CONCLUSION AND FUTURE WORK

In the present study, we have investigated the anti-atherosclerotic potentials by measuring the *in vitro* antioxidant capacity, anti-hyperlipidemic, lipoprotein modulating activity and its related mechanism(s) in oleate-induced HepG2 cells treated with organic-solvent-partitioned extracts prepared from the fruiting bodies of AR. The semi-polar ethyl acetate extract (EA) demonstrated the highest antioxidant capacity amongst all other extracts i.e., hexane, dichloromethane, water, and methanol-dichloromethane. Besides, the EA also exhibited the strongest inhibitory effect on Cu^{2+}-induced low-density lipoprotein (LDL) oxidation (via conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS) formations), and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity, respectively. Meanwhile, all five extracts were non-cytotoxic (IC_{50} > 100 µg/mL) to normal human hepatic (WRL 68) and human lung fibroblast (MRC-5) cells. Assay on lipid lowering effects revealed that oleate-induced HepG2 cells treated with EA showed pronounced reductions in intracellular accumulation and secretion of total cholesterol (TC) and total triglyceride (TG). Further investigation at transcriptional level using oleate-induced HepG2 cells treated with EA has observed a down-regulation of sterol-regulatory element binding factor-2 (*SREBF-2*) which confirmed the inhibition on mevalonate-mediated biosynthetic pathway of cholesterol. Unpredictably, our results showed an up-regulation of sterol-regulatory element binding factor-1 (*SREBF-1*) (the main regulatory factor of fatty acid synthase-mediated pathway), which does not correspond to the decreased endogenous synthesis of triglyceride. Further evaluation on two other key lipid regulatory transcription factors demonstrated a dose-and time dependent co-enhanced liver X receptor alpha (*NR1H3*) (which up-regulated the *SREBF-1*), and peroxisome proliferator-activated receptor alpha (*PPAR-α*) that suppressed the *SREBF-1* promoting activity (instead of *SREBF-1* itself) and *SREBF-2* gene. The

activation of *NR1H3* was further confirmed by the over expression of mRNA levels of its target genes i.e., apolipoprotein E (*APOE*), cholesteryl ester transfer protein (*CETP*) and *SREBF-1*. However, our results showed down-regulation of CETP and apoE at translational levels leading to dose-and time-dependently inhibited secretion of both CETP and apoE which is beneficial to regress cardiovascular response. Up-regulation of LXR-α has been the hallmark of the beneficial reverse cholesterol transport (RCT) pathway which raises the "good cholesterol", high-density lipoprotein (HDL) in the plasma. Further examination on mRNA and protein levels of two selected markers, i.e., apolipoprotein A-1 (apoA-1) (main component of HDL) and the lecithin: cholesterol acyltransferase (LCAT) enzyme has evidenced the activation of RCT-related pathway leading to the overexpression of HDL. On the contrary, down regulation of apoB-100, apoE, and cholesteryl ester transfer protein (CETP) suppressed the production of bad cholesterol, i.e., low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) dose-dependently. Finally, ten phenolic compounds (four benzoic acid derivatives, three flavonoids, one cinnamic acid, one dilactone of hexahydroxydiphenic acid and one xanthone derivative) were identified by Liquid Chromatography-Tandem Mass Spectrometry analysis. Among those, four bioactive compounds, i.e., caffeic acid (CA), protocatechuic acid (PCA), vanillic acid (VA), and 4-hydroxybenzoic acid (4HBA) were identified as bioactive components. The dose-dependent reduction of intracellular and secreted TC and TG demonstrated by CA, PCA, VA and 4HBA has evidenced their role in the lipid lowering effect by EA in oleate-induced HepG2 cells. In summary, EA showed good anti-atherogenic potential through its high antioxidant capacity, anti-hyperlipidemic activity, and its lipoprotein modulating effect via activating the RCT-mediated pathway in raising the HDL while suppressing the endogenous biosynthetic pathways of LDL and VLDL particles. Therefore, EA merits a future development

making it as a potential source of anti-atherosclerotic agent for the prevention of cardiovascular diseases.

Since this study was performed using the fruiting bodies of *Amauroderma rugosum* collected from the wild, the supply of the mushrooms may be limited. More importantly, the chemical composition and its biological activities of the mushroom may be varied due to different factors (e.g., quality of the soil, humidity, season of harvesting, and other environmental issues). Hence, it is recommended that a systematic cultivation technique should be developed and optimised in order to produce consistent supply of this mushroom under controlled conditions. Perhaps, similar assays should also be conducted on the domesticated basidiocarps of *Amouroderma rugosum* in order to compare their bioactivity.

On the other hands, our research was performed using HepG2 (an immortalised cell line), which could be different between human hepatocytes. So far, there is no similar research was performed using animal models. To validate our *in vitro* findings, further investigation to evaluate *in vivo* antioxidant capacity, inhibitory effects of LDL oxidation and HMG-CoA reductase activity, lipid modulation effects of EA using animal models, like mice, rabbits, or clinical trials are warranted to corroborate the safety and efficacy of AR in treating dyslipidemia.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications

1. **Chan, K. S**., Abdullah, N., & Aminudin, N. (2017). Antioxidative and inhibitory effects of the fruiting body of black lingzhi mushroom, *Amauroderma rugosum* (Agaricomycetes), on LDL oxidation and HMG-CoA reductase activity. *International Journal of Medicinal Mushrooms, 19*(9), 797-807.

2. **Chan, K. S**., Abdullah, N., & Aminudin, N. (2017). Lipid-modulating effect of black lingzhi medicinal mushroom, *Amauroderma rugosum* (Agaricomycetes), on oleate-induced human hepatocellular liver carcinoma cells. *International Journal of Medicinal Mushrooms, 19*(12), 1101-1111.