EFFECTS OF *Pleurotus pulmonarius* (Fr.) QUEL ON CHOLESTEROL LEVEL, MUSCLE CONTRACTILITY, TISSUE CONDITION AND SERUM METABOLOMIC PROFILE IN HYPERCHOLESTEROLEMIC-INDUCED RATS

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EFFECTS OF *Pleurotus pulmonarius* (Fr.) QUEL ON CHOLESTEROL LEVEL, MUSCLE CONTRACTILITY, TISSUE CONDITION AND SERUM METABOLOMIC PROFILE IN HYPERCHOLESTEROLEMIC-INDUCED RATS

ABSTRACT

Cardiovascular disease is one of the leading factors of mortality worldwide and one of the risks that contributes to the disease is hypercholesterolemia. Statins have been widely prescribed as the solution to hypercholesterolemic patients after a better lifestyle practice failed to improve the total cholesterol level. However, contraindication and drug-drug interaction always jeopardize the patients to various health risks. Thus, nutraceutical or functional food received overwhelming reception from the society. *Pleurotus pulmonarius* or commonly called grey oyster mushroom is one of the best culinary mushrooms with great taste and texture. Besides, its tremendous benefits have been verified through various scientific findings. As an extended effort to explore more of its hidden benefits, this study focused on *in vivo* observations on the rats. The crude aqueous extract (CA) had been verified in a previous study as potent free radical scavenging activity *in vitro*, making it a suitable candidate to be studied furthered *in vivo* by using forty-eight Wistar-Kyoto rats. The extract was subjected to tissue integrity test; analysis on their ability to dilate a pre-constricted thoracic aortic ring. CA was able to dilate the thoracic aortic ring by 60 % and also protected the thoracic aortic tissue when concomitantly fed to hypercholesterolemic-induced rats. The higher dose of CA (2.0 g/kg body weight) fed to the rats also assisted the thoracic aortic ring to dilate when challenged with phenylephrine at the percentage of 43.11 %, followed by 34.17 % for simvastatin and finally 12.51 % for low dose CA (0.5 g/kg body weight). Besides its ability to protect thoracic aortic tissue, histopathology observations proved CA was able to contribute to the liver tissue viability in the hypercholesterolemic-induced rats. Metabolomic analysis was also performed to observe the metabolites involved in the

selected rat's sera. Metabolites levels including serotonin, L-formylkynurenine, pantothenic acid, phosphodimethylethanolamine and 5-hydroxyquinoline were altered in the sera samples. Each of the metabolite plays its own role in the pathogenesis chain by either contributing to disease progression or improvises the viability of the rats. It can be concluded that CA from *P. pulmonarius* was able to ameliorate the disease progression due to high cholesterol. This study also suggested that CA works best as preventive agent rather than curative as it not able to fully restore the structure of damaged tissues following hypercholesterolemic state.

Keywords: hypercholesterolemia, thoracic aortic ring, dilate, tissue, metabolomic

KESAN *Pleurotus pulmonarius* (Fr.) QUEL KE ATAS TAHAP KOLESTEROL, KONTRAKTILITI OTOT, KONDISI TISU DAN PROFIL METABOLOMIK SERUM PADA MODEL TIKUS TERARUH-HIPERKOLESTEROLEMIA YANG DIBERIKAN EKSTRAK AKUES *Pleurotus pulmonarius* (Fr.) QUEL.

ABSTRAK

Risiko kardiovaskular adalah salah satu faktor utama kematian di seluruh dunia dan salah satu penyumbang kepada risiko tersebut adalah hiperkolesterolemia.. Statin dipreskripsi sebagai solusi kepada pesakit hiperkolesterolemia sekiranya gaya hidup yang lebih sihat gagal membaiki tahap kandungan kolesterol. Namun, kontraindikasi dan interaksi antara ubat-ubatan selalunya menyebabkan pesakit terdedah kepada pelbagai risiko penyakit yang lain. Maka, nutraseutikal atau makanan berfungsi menerima sambutan yang tinggi daripada masyarakat. *Pleurotus pulmonarius* atau kebiasaannya dikenali sebagai cendawan tiram kelabu adalah salah satu cendawan pilihan dengan rasa dan tekstur yang enak. Pelbagai kebaikannya telah disahkan melalui pelbagai kajian saintifik yang telah dijalankan, Sebagai satu usaha untuk mengetahui kebaikan lain yang tersembunyi di dalam cendawan tersebut, kajian ini telah memfokuskan kepada pemerhatian tikus dari aspek *in vivo*. Estrak akues mentah (CA) telah dibuktikan sebagai penghalang radikal bebas secara *in vitro* dalam kajian terdahulu menjadikan ia calon yang sesuai penyelidikan lanjut secara in vivo menggunakan empat puluh lapan tikus Wistar-Kyoto. Kemudian, CA diperiksa akan kemampuan dalam ujian integriti; analisa kemampuan untuk mengembangkan gegelang aorta toraks yang dikontraksikan. CA mampu mengembangkan gegelang aorta toraks pada kadar 60 % serta berkemampuan untuk melindungi tisu aorta apabila diberikan kepada tikus secara serentak ketika pengaruhan hiperkolesterolemia. Dos tertinggi CA (2.0 g/kg berat badan) yang diberikan kepada tikus membantu mengembangkan

gegelang aorta toraks apabila dikontraksikan dengan phenylephrine pada kadar 43.11 %, diikuti 34.17 % oleh simvastatin dan akhir sekali 12.51 % untuk dos rendah CA (0.5 g/kg berat badan). Selain melindungi sel aorta toraks, CA juga mampu membantu melindungi sel hati tikus teraruh-hiperkolesterol. Analisis metabolomik juga telah dijalankan ke atas sampel serum terpilih. Paras beberapa metabolit telah diubahsuai antaranya serotonin, L-formylkynurenine, pantothenic acid, phosphodimethylethanolamine dan 5-hydroxyquinoline. Setiap metabolit memainkan peranan di dalam rantai patogenesis; sama ada sebagai penyumbang kepada penyakit atau membantu pemulihan tikus. Daripada kajian ini, dapat dirumuskan bahawa CA dari *P. pulmonarius* mampu mengurangkan perkembangan penyakit disebabkan oleh kolesterol yang tinggi. Kajian ini juga mencadangkan CA bertindak lebih baik sebagai agen pencegahan berbanding agen rawatan kerana ia tidak mampu mengembalikan struktur asal tisu yang telah rosak akibat keadaan hiperkolesterolemik.

Kata kunci: hiperkolesterolemia, gegelang aorta toraks, mengembang, tisu, metabolomik

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

NCDs	:	non-communicable diseases
CVD	:	cardiovascular diseases
CA	:	crude aqueous extract
C27H46O	:	Cholesterol
-OH	:	hydroxyl
HDL	:	high density lipoprotein
LDL	:	low density lipoprotein
NO	:	nitric oxide
CRs	:	chylomicron remnants
IDLs	:	intermediate density lipoproteins
miligram/decilltre	:	mg/dl
HMG-CoA	:	3-hydroxy-3-methylglutaryl Coenzyme A
NADPH	:	nicotinamide adenine dinucleotide phosphate
SSD	:	sterol sensing domain
SREBP	:	sterol regulatory element binding protein
SCAP	:	SCREBP cleavage activating protein
INSIG	:	insulin-induced proteins
SRE	:	sterol regulatory element
RCT	:	Reverse Cholesterol Transport
LCAT	:	lecithin: cholesterol acyltransferase

APOA1	:	apolipoprotein A1,
ABCA1	:	ATP-binding cassette transporter ABCA1
LCAT	:	lecithin cholesterol acyltransferase
PLTP	:	phopholipid transfer protein,
CETP	:	cholesterol ester transfer protein
LIPG	:	endothelial cell-derived lipase,
LIPC	:	hepatic lipase
LPL	:	lipoprotein lipase
CNS	:	central nervous system
FH	:	familial hypercholesterolemia
LDL-C	:	low-density lipoprotein cholesterol
ISH	:	isolated systolic hypertension
CoQ10	:	Coenzyme Q10
ADRs	:	adverse drug reaction
DDI	:	drug-drug interaction
ILSI	:	International Life Sciences Institute
DPPH	:	1,1-diphenyl-2-picrylhhydrazyl
SAHH	:	S-eadenosylhomocysteine hydrolase
CYP7A1	:	7α-hydroxylase
ABCG5/G8	:	G-transporters
LDLR	:	low-density lipoprotein receptor

v/v	:	volume/volume
rpm	:	rotation per minute
ml	:	mililitre
EA	:	ethyl acetate
ddH2O	:	deionized water
w/v	:	weight/volume
°C	:	degree celcius
AEU	:	Animal Experimental Unit
g	:	gram
UDP	:	Up and Down Procedure
IACUC	:	Institutional Animal Care and Use Committee
ml/kg	:	mililitre/kilogram
BW	:	body weight
L	:	litre
NaCl	:	sodium chloride
KCl	:	potassium chloride
$CaCl_2$	:	calcium chloride
$MgSO_4$	:	magnesium chloride
KH_2PO_4	:	kalium dihydrogen phosphate
$NaHCO_3$	:	sodium carbonate
mM	:	milimolar
mm	:	milimeter
PE	:	phenylephrine
Ach	:	acetylcholine

µM		micromolar
mg/ml		miligram/mililitre
TAR	:	thoracic aortic ring
cm	:	centrimeter
ACN	:	acetonitrile
eNOS	:	endothelial NO synthase
ROS	:	reactive oxygen species
SD	:	standard deviation
OCTN-1	:	organic cation transporter novel type-1
EA	:	Ellagic acid
NAD	:	nicotinamide adenine dinucleotide
L-NAME	:	N-Nitro-L-arginine methyl ester hydrochloride
TDO	:	Trp-2,3-dioxygenase
IDO	:	indoleamine-2,3- dioxygenase
TI	:	tunica intimae
TM	:	tunica media
TA	:	tunica adventitia
Da	:	delton
1H-indole-2,3-dione	:	Isatin
PA	:	pantothenic acid
NCG	:	N-carbamylglutamate
Arg	:	L-arginine
SOD	:	superoxide dismutase
GPx1	:	glutathione peroxidase 1
GR	:	glutathione reductase

MDA	:	Malondialdehyde
PPP	:	platelet-poor plasma
WB		whole blood
DPPH		1,1-diphenyl-2-picryl-hydrazyl
H_2O_2		hydrogen peroxide
kyn trp-1		kynurenine and tryptophan ratio

CHAPTER 1: INTRODUCTION

1.1 Introduction

World population is estimated to increase by the rise of approximately 20% from 6.7 billion to 8.1 billion in 2030. A major change observed in world population is the factors for death which more likely to be caused by non-communicable diseases (NCDs). One of the diseases classified as NCDs is cardiovascular diseases (CVD). Cardiovascular disease is caused by various risk factors including hypercholesterolemia, hypertension and atherosclerosis. Hypercholesterolemia for example, has shown prevalence of 44.9% among Malaysian aged between 35-70 years for the survey period of 6 years (2006-2012). The same pattern was observed in other Asia Pacific regions as well including Taiwan, Japan, Korea, China, Philippines, Thailand, Indonesia, Singapore and Australia (Jamal *et al*., 2014).

Hypercholesterolemia is a lipid dysregulation condition in the human body. This particular disease is affiliated with the sedentary lifestyle and unhealthy diet (Kumar, 2014). The disease progression is prolonged and leads to atherosclerosis. It commences with the increased of blood cholesterol. Intracellular cholesterol will achieve homeostasis if cholesterol is over produced. The exogenous cholesterol; particularly from food contribute to the elevated level of cholesterol in the blood. Cholesterol basically can be divided into high density lipoprotein (HDL) and low-density lipoprotein (LDL). Reactive oxygen species (ROS) generated by mitochondrial as by-product tends to oxidize LDL. LDL oxidation is a perpetual chain of reaction that generates countless oxidized LDL (Lusis, 2000; Mudd *et al*., 2007).

Oxidized LDL has the ability to activate T cells to produce cytokines; a stimulator to activate macrophages and for smooth muscle cells proliferation. This first phase of "response-to-injury mechanism" also attracts natural antibodies and innate effector proteins such as C-reactive protein. Oxidized LDLs are able to be recognized by a family of scavenger receptors expressed by macrophages. These scavenger receptors mediate uptake of the oxidized LDL into macrophages and the formation of foam cells. Endothelial dysfunction due to above mentioned pathogenesis will also disrupt in NO secretion and ultimately interfere with dilation in the aorta. Later, the accumulation of various cells leads to foam cells formation which later becomes plaque. The matured plaque will be covered with fibrous cap. The ruptured plaque later will cause thrombosis and block the blood flow (Amirullah *et al*., 2018; Tabas and Lichtman, 2017; Rosenbaum *et al*., 2002).

Statins are widely used to treat hypercholesterolemia. It works by binding to the active site of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which is responsible for cholesterol synthesis. It has a high affinity compared to 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA). Thus, the biosynthesis of cholesterol is interrupted. However, dolichols and Coenzyme Q10 synthesis are interrupted too. Both compounds have their respective crucial roles. For example, CoQ10 is a lipid-soluble antioxidant that protects membranes from oxidative damage. Therefore, it's low level may cause further endothelial damage thus prone to other risks including hypertension (Graveline, 2015).

Mushrooms, as one of the functional foods have been discovered long time ago for their magnificent benefits in human well-being. Countless researches have been done and still in the pipeline to discover more of their hidden advantages. Mushrooms are well-known for their capabilities as an anti-diabetes, anti-hypercholesterolemia, anti-viral, anti-oxidant and anti-cancer agents. *Lentinus edodes* (Berk.) Singer, *Agaricus bisporus* (J. E. Lange) Imbach, *Agaricus brasiliensis* Fr. and *Pleurotus* spp. are among the mushrooms discovered with potential as anti-hyperholesterolemic agent (Liu *et al*., 2010; Carrasco-Gonzalez *et al*., 2017; Yang *et al*., 2008). *Pleurotus* spp. for instance, executed the reduction of total serum cholesterol in the experimental animal model. Total serum cholesterol in rat model fed with ergosterol-rich and nicotinic acid-rich extracts from dried fruiting bodies of *Pleurotus citrinopileatus* Singer was successfully reduced in an observation by Hu *et al*. (2006). In addition, Guillamón *et al*. (2010) have summarized in their review the existence of mevinolin, the HMG-CoA reductase inhibitor in *Pleurotus ostreatus* (Jacq.) P. Kumm, *Pleurotus cornucopiae* (Paulet) Rolland and *Pleurotus eryngii* (DC.) Quél thus, interrupt the cholesterol synthesis.

Pleurotus pulmonarius (Fr.) Quél is among the widely distributed mushroom in Malaysia. It belongs to the family of basidiomycetes and enriched with tremendous benefits. Crude aqueous extract (CA) of *Pleurotus pulmonarius* was verified as an anti-antioxidant and a powerful ROS scavenger besides protecting the endothelial cells and human LDL from oxidation which was confirmed via *in vitro* observation (Abidin *et al*., 2016). This study is the extended *in vivo* observation on Wistar-Kyoto rat to further validate the anti-oxidant and cell protector activity by CA of *P. pulmonarius*. Thus, the hypothesis of this study was CA from *P. pulmonarius* is able to alleviate serum total

cholesterol and assist vasodilation in thoracic rat aorta besides contribute to the metabolomic profiling in the analyzed rat sera.

1.2 Objectives

1. To determine the anti-hypercholesterolemic effect of crude aqueous extract of *P. pulmonarius* in rat model.
2. To interpret vasomotor modulation of crude aqueous extract of *P. pulmonarius* on the thoracic aorta of Wistar-Kyoto rat.
3. To microscopically view and verify the toxicity effects of crude aqueous extract of *P. pulmonarius* on the rats' liver and aorta through histopathology observation
4. To compare the metabolites profile among the quantified rats' sera.

CHAPTER 2: LITERATURE REVIEW

2.1 Cholesterol and its Biological Functions in Human Body

2.1.1 Cholesterol Structure

Cholesterol ($C_{27}H_{46}O$) consists of four rings molecule attached to both hydroxyl (-OH) and hydrocarbon group; polar and non-polar group, respectively. The four rings are hydrocarbon rings as each corner is composed of CH_3. This particular structure belonged to all steroid hormones. The polar group is soluble in water and the presence of –OH group make the molecule as alcohol. Thus, the combination of both steroid and alcohol produces sterol (Figure 2.1). In addition, the presence of hydrocarbon tail makes the molecule less soluble in water, however very soluble in fat. Cholesterol is a sterol and mainly synthesized by liver, besides intestines, adrenal gland and reproductive organs (Ačimovič & Rozman, 2013).

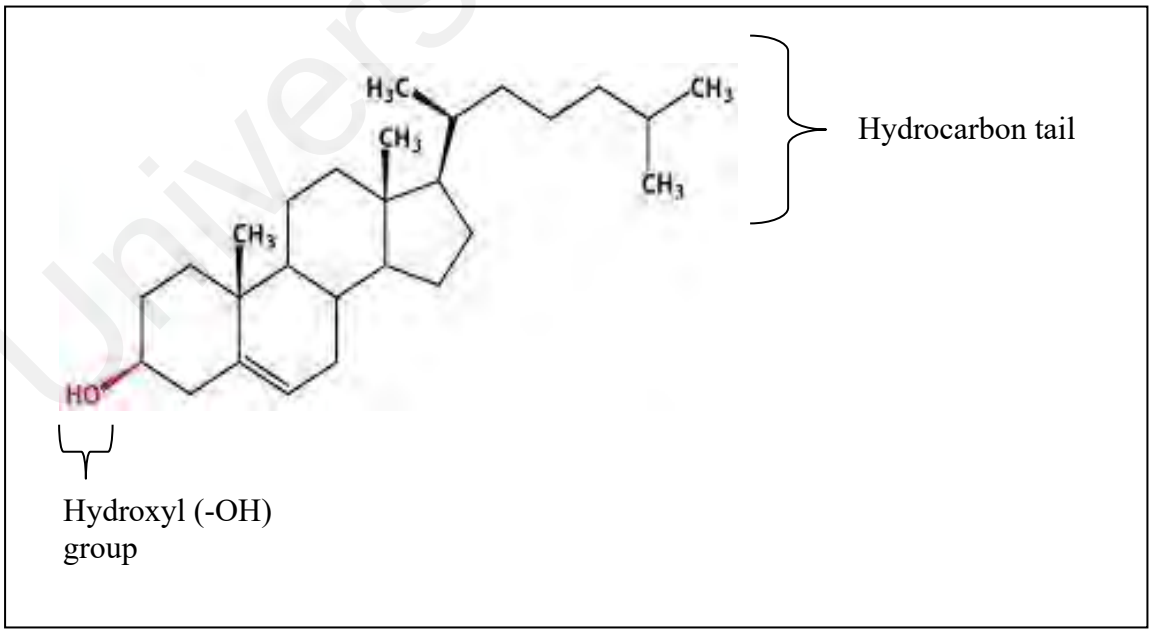


Figure 2.1: Molecular structure of cholesterol ($C_{27}H_{46}O$) which consists of four rings, hydroxyl (-OH) group and hydrocarbon tail (Ačimovič & Rozman, 2013).

Approximately, 20 to 25 % of *de novo* synthesis occurs in the liver or equal to 3000 mg in 24 hours period. Cholesterol has countless functions including as many steroid hormones' precursor and for vitamin D formation. Since cholesterol is insoluble in water, it is circulated in the body mainly as high-density lipoprotein (HDL) and low-density lipoprotein (LDL). LDL conveys the cholesterol to the tissues for their utilization; known as forward cholesterol transport. LDL also delivers cholesterol to hepatocytes, a crucial step in maintenance of plasma LDL level (Lewis, 1959).

On the other hand, HDL plays a role by collecting excessive cholesterol back to the liver to be secreted as bile acid which has termed it as 'good cholesterol'. The path used by HDL to 'return the cholesterol' is known as reverse cholesterol transport (RCT). Besides these lipoproteins, chylomicrons, chylomicron remnants (CRs) and intermediate density lipoproteins (IDLs) are also the major sub-fractions of lipoproteins (Daniels *et al*., 2009; Murray, 2013).

2.1.2 Cholesterol Functions

Cholesterol does have various functions and among these functions is for membrane plasma rigidity. Plasma membrane is constructed by many components including amphipathic lipid; consists of both hydrophilic and hydrophobic regions. Membrane lipids can be divided into three types which are phosphoglycerides, sphingolipids and cholesterol (Figure 2.2). Cholesterol is the main lipid component in animal cells' membrane. Cholesterol molecules are smaller in size compared to other lipids and also less amphipathic. Cholesterol is crucial for membrane fluidity and thickness, evenly distributed in the membrane and modulates the glycoprotein. Glycoprotein's modulation can be either direct binding to the protein, changes in membrane physiochemical properties or both. These modulations keep membrane in

shape besides restricting the movement of molecules into the plasma (Grouleff *et al*., 2015; Singer & Nicolson, 1972).

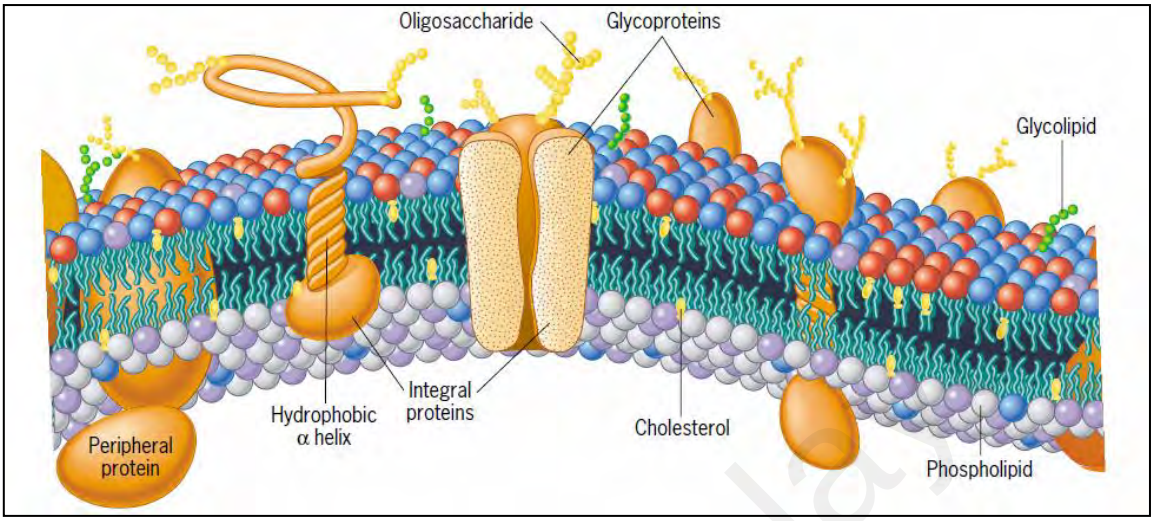


Figure 2.2: Plasma membrane structure and its components. This detailed fluid-mosaic model of plasma membrane was proposed by Singer and Nicolson (1972). The cholesterol molecules are evenly distributed in between the lipid bilayer in order to maintain the rigidity and permeability of the plasma membrane (Singer & Nicolson, 1972).

Approximately 90 % of cellular cholesterol resides in membrane plasma with almost half of the component of plasma membrane is made of cholesterol. Its prevalence is due to its prominent functions including maintaining the rigidity of the membrane and for permeability purpose. Cholesterol that structured the membrane plasma is synthesized either intracellular or acquired from circulating lipoprotein in the bloodstream (Crane & Tamm, 2004).

Cholesterol has an impact on physical properties of lipid bilayer which construct the membrane plasma. The increment of cholesterol may cause the enhancement of mechanical strength of the membrane and reduce its permeability (Kessel *et al*., 2001). Sun *et al*. (2007) cited the cholesterol role to maintain the rigidity of the fluid phase of

the membrane. The depletion of the cholesterol leads to reduction of protein mobility and decrease the diffusion of signalling across membrane, otherwise, if the cholesterol concentration increased, the membrane stiffness will also be increased. In addition, receptors, ion channel and glycoprotein are also affected by the abnormalities of cholesterol concentration (Grouleff *et al*., 2015). These observations show the importance of a balanced cholesterol level for normal cellular activity.

Increased permeability with the presence of cholesterol is also a crucial element in neuron. The neuronal transmission of the impulse in central nervous system (CNS) is mediated by neurons. Each of the neuron has a special feature of axon wrapped by myelin sheaths and separated by nodes of Ranvier. The myelin sheath is derived from oligodendrocytes and it is enriched with cholesterol. The presence of cholesterol indirectly assists in reducing membrane permeability against ions, thus the pulse transmission will occur across the axon prior to oligodendrocytes. Cholesterol is also found abundantly in synaptosomal membrane which is the main constituent in synapse formation and stability and for neurotransmitter release (Korade & Kenworthy, 2008; Orth & Bellosta, 2012).

2.1.3 Cholesterol Biosynthesis Pathway

Acetoacetyl-CoA thiolase interconverts both acetyl-CoA and acetoacetyl-CoA, which is then condensed to 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) by HMG-CoA synthase; an enzyme which is produced both in the liver and extrahepatic tissues. Next, HMG-CoA reductase catalyzes the reduction of HMG-CoA to mevalonate, utilizing two molecules of NADPH. HMG-CoA reductase is prominently regulated by cholesterol presence in the body and it is a rate-determining enzyme of the cholesterol biosynthesis pathway. Therapeutically, this enzyme is the treatment target of hypercholesterolemia.

Statins are widely used as reversible competitive inhibitors which occupy the HMG-CoA binding portion of the active site. However, complete inhibition of HMG-CoA reductase by statins will cause cells mortality due to mevalonate deprivation although exogenous cholesterol is supplied to the body. Mevalonate is metabolized by a series of enzymes localized in peroxisomes to farnesyl-diphosphate (FPP) (Liscum, 2002). The flow chart below (Figure 2.3) showed the steps involved in cholesterol formation (Liscum, 2002). Synthesized cholesterol will be esterified, attached to LDL receptor and delivered to different tissues. This process is also called as forward cholesterol transport. LDL receptor will be down regulated if cholesterol level exceeded normal rate besides of reverse cholesterol transport by HDL as discussed further below (Wüstner *et al*., 2012).

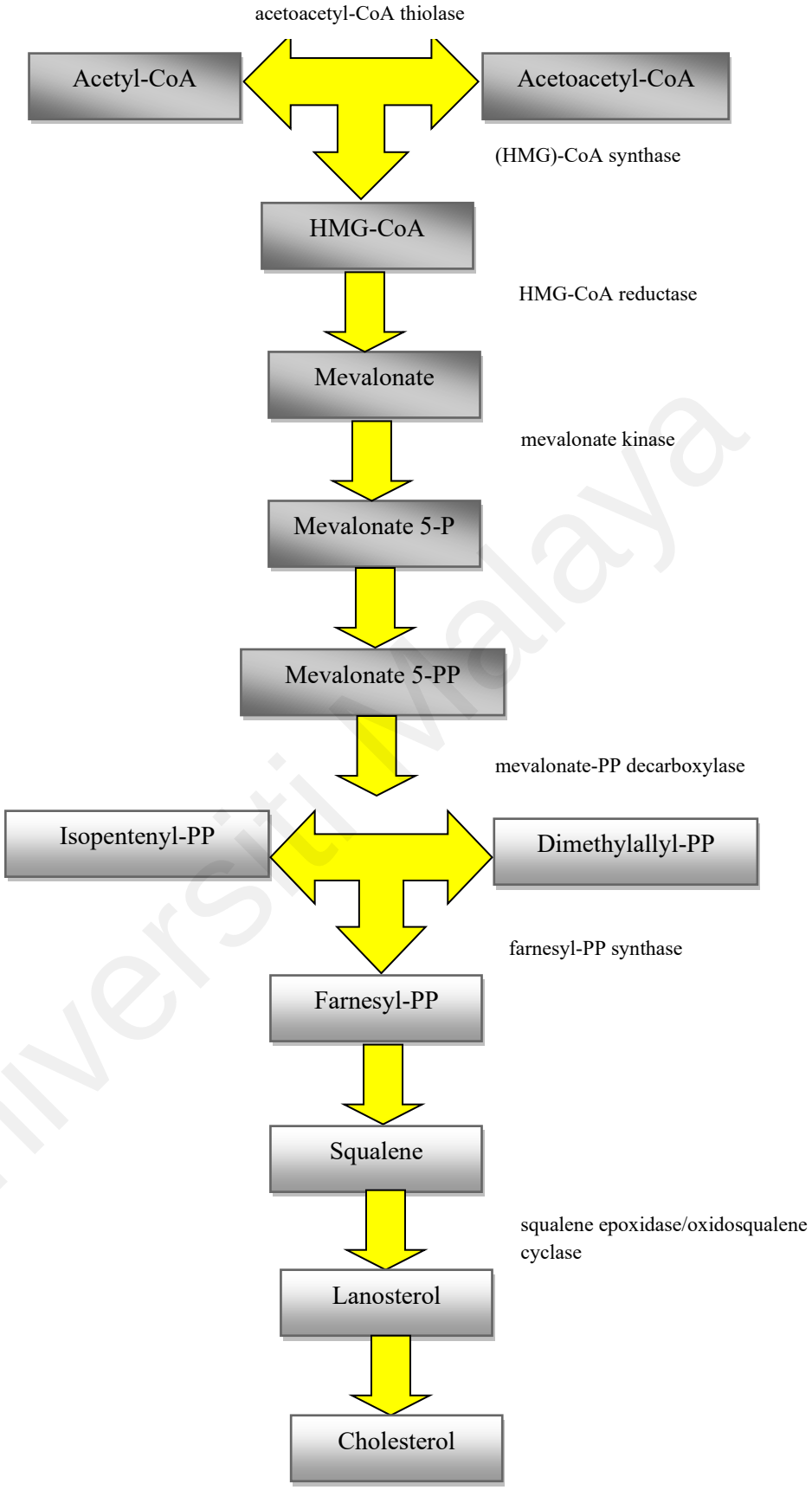


Figure 2.3: Biosynthesis pathway of cholesterol in liver (Liscum, 2002).

2.1.4 Bile Acid Synthesis

Cholesterol breakdown to bile acids in the liver is either initiated through conventional pathway by cholesterol 7a-hydroxylase (CYP7A1) or acidic pathway by mitochondrial sterol 27-hydroxylase (CYP27A1). Conventional pathway involves the alteration of the sterol nucleus and saturation of the double bond, whereas the acidic pathway involved side-chain oxidation process (Chiang, 2004). Thus, bile acids consist of two main unit; saturated bond sterol nucleus and side-chain. Bile acid synthesis a major role as the major cholesterol breakdown in human body. Approximately, 500 mg of cholesterol is converted bile acid daily (Mukhopadhyay & Maitra, 2004). The cholesterol breakdown pathway is simplified in Figure 2.4.

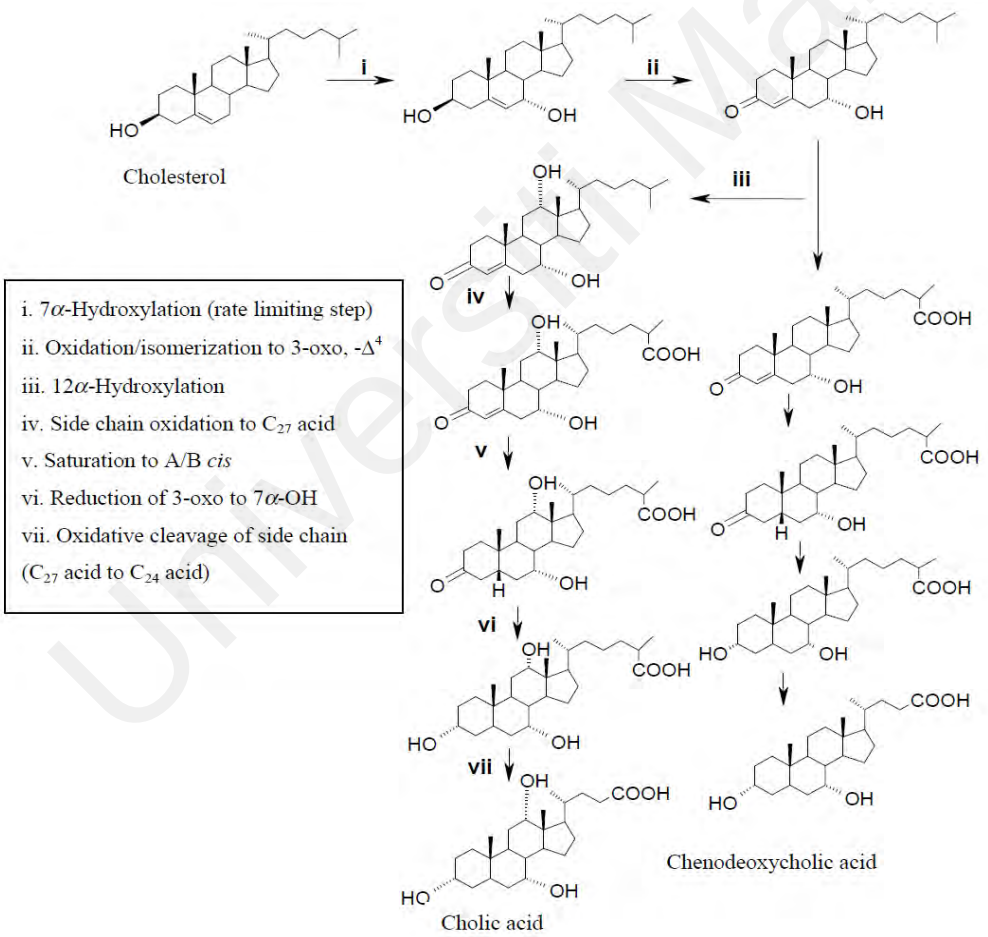


Figure 2.4: Cholesterol breakdown into bile acids (Mukhopadhyay & Maitra, 2004).

In addition, due to hypercholesterolemic conditions, the conversion of cholesterol to bile acid demands higher activity of 7 -hydroxylase. The catalysis action this enzyme requires NAPDH, oxygen and Cytochrome P-450 oxidase. Cytochrome P-450 oxidase also involves in the reticulum endoplasmic metabolism. The increased activity of Cytochrome P-450 oxidase elevates the level of ROS as the by-product. The increasing free radicals creates more oxidized LDL as they prone to interact with LDL (Wresdiyati *et al*., 2008).

2.1.5 Inhibition of Hydroxy Methyl Glutaryl Coenzyme A (HMG-CoA) Reductase

Hydroxy methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor or statin was first introduced in 1987 as the solution for high cholesterol. In addition to its benefit as lipid lowering agent, statins are also fortified with many added values. The statin family is able to reduce the production of Rho GTPases, which involves in neovascularization in cancerous tissues. Besides, statins also diminish immune system response activation by reducing the T-cell activation, major histocompatibility complex (MHC II) expression and also tissue factor or PAI-1. In addition, the statin family is capable in improving endothelial function, decreasing vascular inflammation, enhancing plaque stability and increased nitric oxide bioavailability in the endothelium as diagrammed in Figure 2.5. The nature of any drug with contraindication is reported for statins as well. Among them are acute coronary syndrome, heart failure, stroke, multiple sclerosis, chronic kidney disease and sepsis (Liao, 2002; Merx & Weber, 2008).

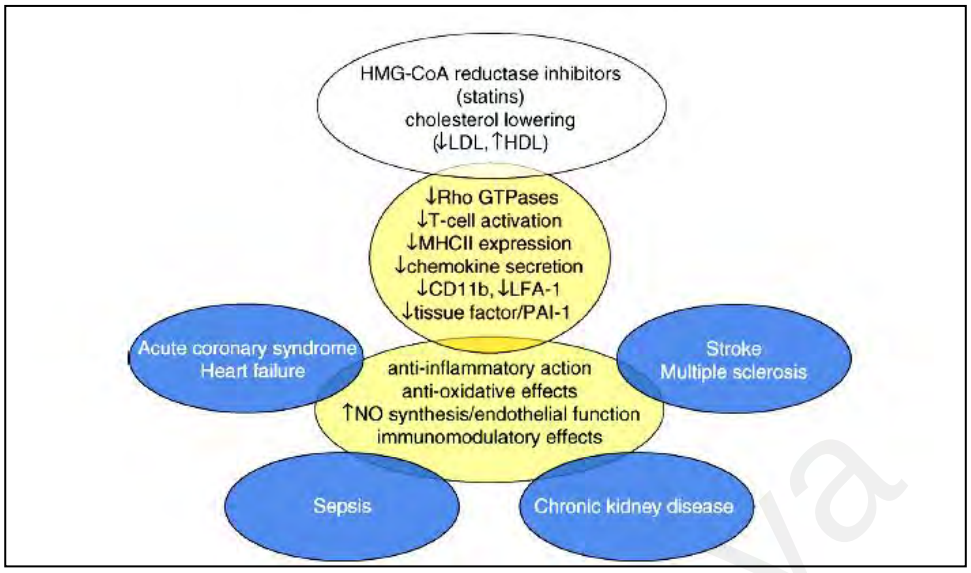


Figure 2.5: Hydroxy methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (statin) benefits (yellow) and contraindication (blue) (Merx & Weber, 2008).

There are five types of approved statins in the market viz. lovastatin, pravastatin, simvastatin, atorvastatin and fluvastatin. Lovastatin and pravastatin (mevastatin derived) are natural statins originated from fungi, while simvastatin is a semi-synthetic lovastatin derivative. Atorvastatin and fluvastatin are fully synthetic statins, derived from mevalonate and pyridine; respectively (Manzoni & Rollini, 2002).

In 2003, rosuvastatin was introduced. Each of the statin plays the same role of reducing LDL-C however, their efficiency may differ. Rosuvastatin recorded an average LDL-C reduction of 63 %, followed by 57 % for atorvastatin, 46 % for simvastatin, 34 % for pravastatin and 31 % for fluvastatin at their highest approved doses (Tiwari & Khokhar, 2014).

Statins retard the cholesterol synthesis pathway by binding to the enzyme HMG-CoA reductase as it has three times more affinity towards the enzyme compare to the

substrate, HMG-CoA. These structures basically represent their binding patterns to the enzyme (Figure 2.6). The statin actions usually will take place within hepatocytes and its concentration is the determinant of the ability to reduce cholesterol (Tobert, 2003).

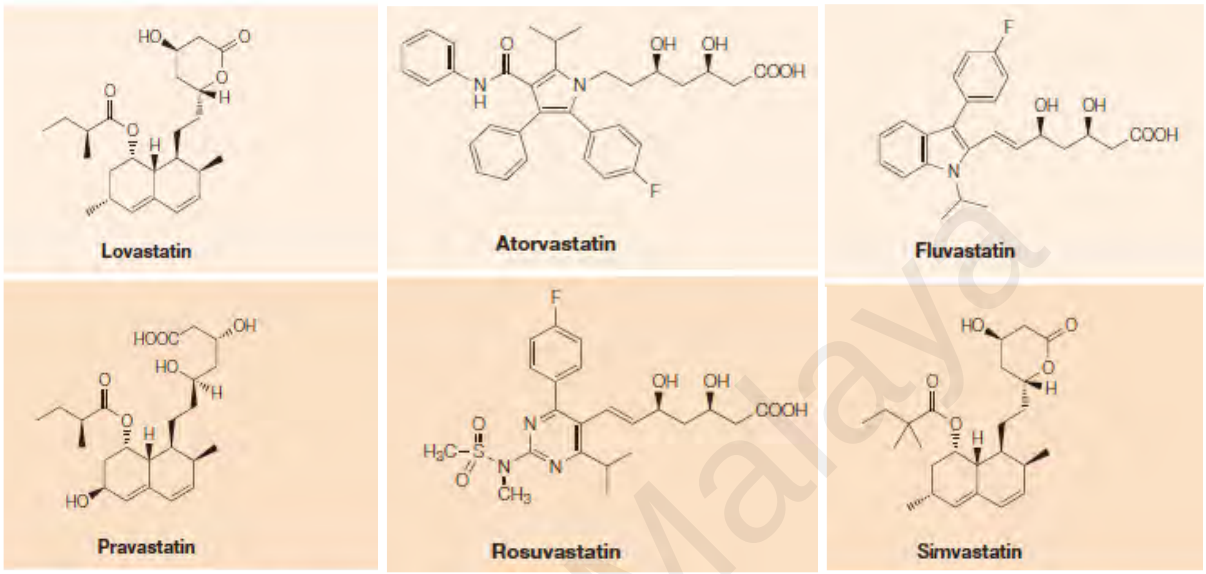


Figure 2.6: Various synthesized statins and their structures **(**Tobert, 2003).

However, statin not only inhibits cholesterol, but the formation of Coenzyme Q10 (CoQ10) and dolichols (Figure 2.7). The side effect is called as adverse drug reactions (ADRs). CoQ10 is a crucial component in human electron transport chain which usually takes place in mitochondria. Besides, CoQ10 is also a lipid-soluble antioxidant that protects membranes from oxidative damage. The reduction of CoQ10 in statin treated patients is a serious issue as it makes ones liable to further oxidative damage in cells. The finding by Marcoff and Thompson (2007) is another evident of statin side effect on the patients. It was stated in their report that CoQ10 is reduced to maximum of 54 % which represents half of the CoQ10 concentration in human body.

Another compound inhibited due to statin therapy is dolichol or in its active state in eukaryotic cell is known as dolichol phosphate. Besides its function as lipid carrier, dolichol is also involved in C- and O-mannosylation of proteins, glycosylphosphatidylinositol (GPI) anchors and N-glycosylation of protein. The defects of its de novo synthesis were reported as glycosylation disorders or termed as congenital disorders of glycosylaion (CDG). CDG can cause infant death (Denecke & Kranz, 2009; Graveline, 2015).

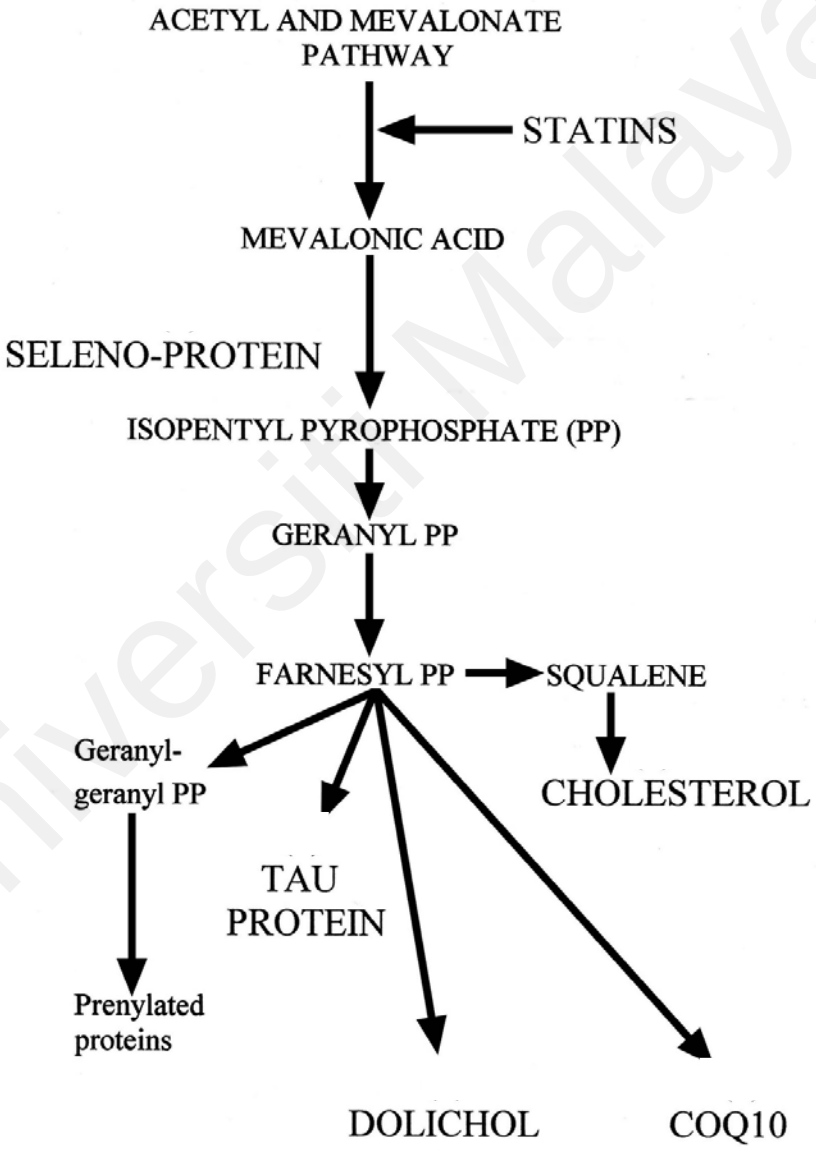


Figure 2.7: Inhibition of dolichol and CoQ10 by statin in cholesterol biosynthesis pathway (Graveline, 2015).

Besides ADRs, drug-drug interaction (DDI) is also another issue encounters by some of the elderly patients. Polypharmacy is a term used for patients under multiple drugs prescription. Each drug has its own way of healing at the targeted organ, however, the interactions among them and the endogenous environment may influence their respective performance thus create a new side effect. DDI mainly observed or reported in the patients with renal or hepatic impairment as the metabolism and excretion of the drugs will be impaired. Some of the established adverse reaction is as shown in Table 2.1 (Marengoni *et al*., 2014).

Table 2.1: Example of medication combination (warfarin and statin) and potential adverse reactions encountered by the patients (Marengoni *et al*., 2014)

Medication Combination	Potential adverse reaction
Warfarin and simvastatin	Increased anticoagulant effect
Warfarin and atorvastatin	Increased anticoagulant effect
Warfarin and rosuvastatin	Increased bleeding risk

2.2 Hypercholesterolemia

Diet, genetic, health and environment are some of the factors leading to hypercholesterolemia. Some people with other health problems are prone to develop hypercholesterolemia and consequently atherosclerosis if left untreated (Table 2.2) (Adams, 2005). Besides, unhealthy lifestyles such as smoking and excessive alcohol consumption readily increased the chances of hypercholesterolemia as well as other illnesses. In addition, genetic factor so called familial hypercholesterolemia (FH) is also one of the factors of hypercholesterolemia. Familial hypercholesterolemia (FH) is an autosomal-dominant disorder which is characterized by elevated level of plasma low-

density lipoprotein cholesterol (LDL-C) due to mutation of LDL-receptor encoding gene. Thus, genetic disorder leads to premature cardiovascular disease in FH patients (Sjouke *et al*., 2011).

Table 2.2: Secondary causes of hypercholesterolemia (Adams, 2005)

Causes	Lipid Abnormality		
Anabolic steroid use	↑ LDL		↓ HDL
Anorexia nervosa	↑ LDL		
Cigarette smoking			↓ HDL
Diabetes	↑ LDL	↑ TG	↓ HDL
Hypothyroidism		↑ TG	
Liver disease	↑ LDL		
Obesity	↑ LDL	↑ TG	↓ HDL
Transplant		↑ TG	↓ HDL

The recommended total blood cholesterol level for a human being is less than 200 mg/dl, LDL less than 130 mg/dl is desirable and HDL is recommended to be higher than 35 mg/dl as shown in Table 2.3. Total blood cholesterol to HDL and LDL: HDL ratio is referred as the cardiac risk factor ratio. Total blood cholesterol to HDL ratio should exceed 4.2 whereas LDL to HDL ratio not higher than 2.5. The risk for heart disease can be pulled down by lowering LDL but raising HDL levels. A small reduction as low as 1 in LDL level, may reduce the risk for heart attack by 2 %. Meanwhile, an increment in HDL provides a protection against heart attack (Murray, 2013).

Table 2.3: Plasma cholesterol reading and its indications

Total cholesterol level	
<200 mg/dL	Desirable
200-239 mg/dL	Borderline high
>240 mg/dL	High
LDL cholesterol level	
<100 mg/dL	Optimal
100-129 mg/dL	Near optimal/above optimal
130-159 mg/dL	Borderline high
160-189 mg/dL	High
>190 mg/dL	Very high
HDL cholesterol level	
<40 mg/dL	Increase risk of heart disease
>60 mg/dL	Lower risk of heart disease

(US Department of Health and Human Services, National Institute of Health, National Heart, Lung and Blood Institute) (Murray, 2013)

2.2.1 Hypercholesterolemia and Atherosclerosis

Reactive oxygen species mostly generated as by-product by mitochondria during electron transport and has a high affinity towards apolipoprotein-B-containing LDL. LDL oxidation is a chain of reaction which will be on-going and generates countless oxidized LDL. Oxidized LDL has the ability to activate T cells to produce cytokines; a stimulator to activate macrophages and for smooth muscle cells proliferation and various components of innate immune system including natural antibodies and innate effector proteins such as C-reactive protein. With the presence of the growth factors, these cells migrate to subendothelial space, produce collagen and take up LDL and become foam cells. The delicate layer of endothelial layer is liable to damage. Thus, the formation of form cells inhibits its function in vasodilation by interrupting the nitric oxide secretion. By time, the accumulation of foam cells will later be precipitated as plaque (Lusis, 2000; Mudd *et al*., 2007).

This first phase of "response-to-injury mechanism" also attracts natural antibodies and innate effectors proteins such as C-reactive protein. Oxidized LDLs are able to be recognized by a family of scavenger receptors expressed by macrophages. These scavenger receptors mediate uptake of the oxidized LDL into macrophages via endocytosis and carried them to lysosomes to be catalyzed. Unfortunately, oxidized LDL is less susceptible to degradation thus turned the macrophage into foam cell. Later, the repair mechanism becomes maladaptive and more aggressive. This attracts the participations of more immune cells including neutrophils and platelet-neutrophil aggregates and natural killer cells (Amirullah *et al*., 2018; Tabas & Lichtman, 2017).

The severity of inflammation and calcification will determine the state of the atherosclerotic plaques; stable or unstable. Stable plaque or the matured plaque is covered with fibrous cap and less susceptible to rupture. However, the unstable one is more prone to rupture and causes thrombosis as shown in Figure 2.8 (Wang & Butany, 2017).

The continuous inflammatory responses increase the permeability of tunica intima thus promotes the entry into tunica media. Tunica intima comprises of endothelial cells which are responsible for nitric oxide (NO) secretion. NO plays a crucial role for aorta vasodilation. Endothelial dysfunction due to above mentioned pathogenesis will disrupt NO production and ultimately interfere with dilation in aorta. This disruption will cause hypertension and the continuous pathogenesis in tunica intima and tunica media will end up with atherosclerotic risk (Tjaden *et al*., 2015; Warnholtz *et al*., 2001).

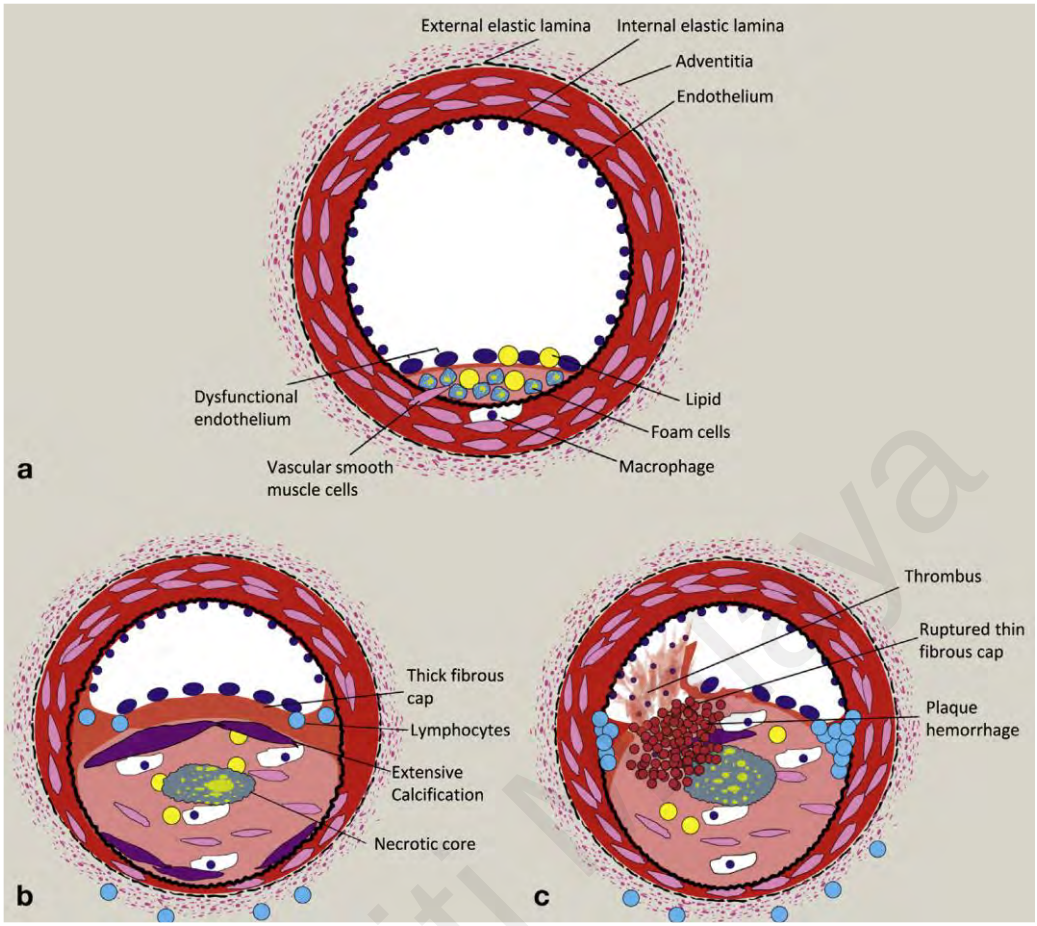


Figure 2.8: (a) Dysfunctional endothelium with foam cells (b) Stable plaque with thick fibrous cap (c) Unstable plaque has the capability to be ruptured and causes thrombosis in the blood vessels (Wang & Butany, 2017).

These conditions may contribute artery stiffness and interrupt in vasodilation of the artery. Vasodilation is a condition where the artery is in dilating condition. Due to artery stiffness, vasodilation is failed since it did not properly dilate and thus, causes prolong vasoconstriction. A prolong vasoconstriction in summary causes the blood pressure to increase and leads to hypertension. A study conducted by Ferrier *et al*. (2002) has shown the linkage between hypercholesterolemia and hypertension.

Statin therapy assists in elevated isolated systolic hypertension (ISH) patient by reducing the artery stiffness although those patients were normocholesterolemic. However, for hypercholesterolemic patients, statin therapy needs a longer period in

improving artery stiffness. These observations concluded that in ISH patients, an improved condition of hypercholesterolemia, endothelial injury or smooth muscle content, will improve the arterial stiffness. Statins has the capability to improvise the nitric oxide (NO) concentration by stimulating the enzyme responsible for NO synthesis; endothelial nitric oxide synthase and also by inhibiting endothelin-1 production. The increment in NO concentration consequently will improve vasodilation (Brucket & Rosenbaum, 2011; Ferrier *et al*., 2002).

2.2.2 Hypercholesterolemia and Muscle Contractility

A close relationship between hypercholesterolemia and hypertension have been observed; specifically on vascular muscle modulation. Modulation of vascular muscle tone is mediated by many factors including endothelium derived relaxing factors (EDRFs). NO is one of the EDRFs involves in vasodilation modulation. The NO synthesis interruption, for instance, is contributed by plaque formation due to accumulation of macrophage thus deteriorate the endothelial layer. Endothelial layers are delicate and consist of tunica intima, tunica media and tunica adventitia. The oxidization of LDL by ROS activates the immune system including the actions by T-cell and macrophage. The pressure on the delicate layer of tunica intima, follows by the penetration of fibrous cells to tunica media (further explain in 2.5) (Wang & Butany, 2007; Zhu *et al*., 2007).

2.2.3 Hypercholesterolemia and Tissue Condition

Impairment of endothelial cells are microscopically observable. The spike of cholesterol esterification resulted due to cholesterol homeostasis dysregulation, yielded the plaque formation. The plaque consists of macrophages, smooth muscle cells and fibrous cells. The bulge due to the plaque formation pressurized the endothelial cells thus interrupt with its normal function. The endothelial layers are distinct in normal aortic cell. These delicate layers are prone to damage due to hypercholesterolemia (Figure 2.8). The vascular smooth cells and fibrous cells migration from tunica media to tunica intima dysregulates the endothelial function as one of the channels for EDRFs (Dianita *et al*., 2016).

Hepatic cells are involved in cholesterol homeostasis thus hypercholesterolemic conditions are observable in these cells. Microscopically, the lipid vacuoles presence was identified in the hepatic cells accompanied by cell necrosis. Lipid peroxidation due to hypercholesterolemia will generate overproduction of free radicals. As part of the homeostasis, free radicals will interact with some electrons from the bimolecular cells including lipids, proteins and carbohydrates. The consequence of it is cell impairment follow by cell necrosis (Wresdiyati *et al*., 2008).

2.3 Mushrooms and Hypercholesterolemia

Culinary mushroom is one the best options nowadays as both functional food and nutraceutical. Functional food is a term introduced by International Life Sciences Institute (ILSI) Europe which refers to the capability of a food to demonstrate a beneficial factor to mankind in order to maintain the health or provide a better conditions by improving the health status (Reis *et al*., 2017). Mushroom is the term originated from Latin word means mucus. It is a type of macro fungus belonging to the

class Basidiomycetes (fungi producing basidiospore). It has a distinctive fruiting body which is usually harvested and consumed as mushroom. It has the vegetative part which is called mycelium; a branching thread-like structure which reproduce more fruiting bodies (Guillamón, 2010; Sanchez, 2017).

Varieties of mushrooms species are enriched with polysaccharides, terpenoids, phenolic compounds, lectins, peptides, enzymes, lovastatin, glycoproteins, ergothioneine, pletirone, numerous vitamins, minerals and fibres. These mycochemical compounds are able to execute many bioactive functions including as anti-hypercholesterolemia, anti-tumor, anti-viral, anti-bacterial, hepatoprotective, anti-cancer and anti-diabetic effects (Carrasco-Gonzalez *et al*., 2017; Guillamón *et al*., 2010; Roupas *et al*., 2012).

Many mushrooms' species have been discovered as anti-hypercholesterolemic agent. *Lentinus edodes*, *Agaricus bisporus*, *Agaricus brasiliensis* and *Pleurotus* spp. are among the mushrooms discovered with potential as anti-hypercholesterolemic agents. Roncero-Romas and Delgado-Andrade (2017) has summarised that the secondary metabolite, eritadinine from *L. edodes* was found to significantly reduce the cholesterol level in rats by inhibiting S-adenosylhomocysteine hydrolase (SAHH); an enzyme involved in phospholipids metabolism. Another study on *L. edodes* discovered the capability of eritadinine to also inhibit cholesterol HMG-CoA reductase; an enzyme responsible for cholesterol metabolism (Figure 2.9) (Gil-Ramirez *et al*., 2016).

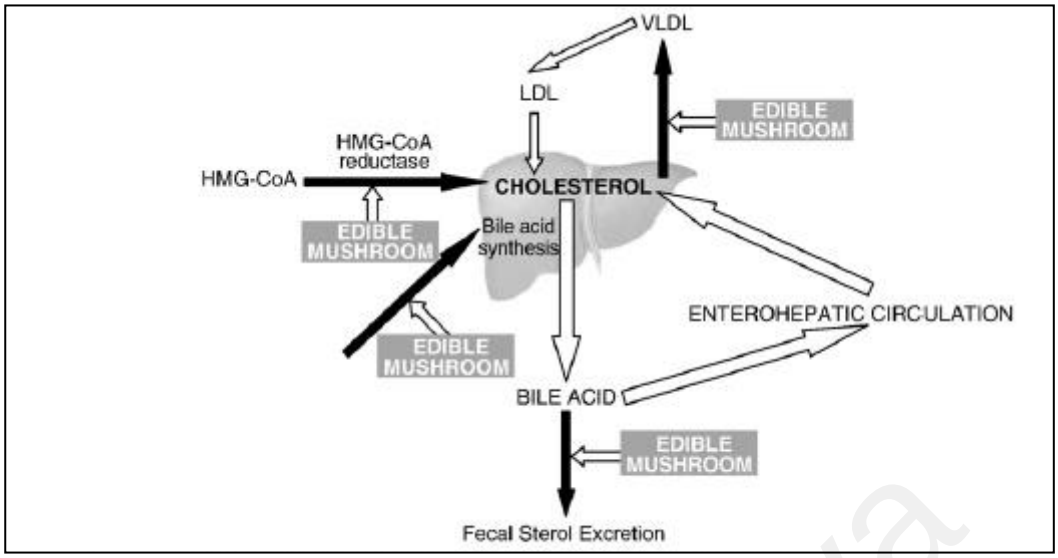


Figure 2.9: Potential pathways in reducing cholesterol level by edible mushrooms (Guillamón, 2010).

In addition, Yang *et al*. (2013) concluded that eritadinine was able to reduce serum lipid level, fat accumulation and plaque formation in the aorta of the observed mice. These reports validated *L. edodes* capability as anti-hypercholesterolemic agent. Boonsong *et al*. (2016) also summarized that *L. edodes* has a powerful antioxidant property which is chelating agent against ferrous ion; via *in vitro* observation. Antioxidant properties are also essential to halt the development of hypercholesterolemia thus antioxidant properties in mushrooms indirectly contribute to the anti-hypercholesterolemic potential.

Another mushroom species, *A. bisporus* was found to reduce total cholesterol level in rats fed with high cholesterol diet (Jeong *et al*., 2010). Another study conducted on *A. brasiliensis* revealed its advantages in reducing total cholesterol by promoting the expression of 7α-hydroxylase (CYP7A1), ATP-binding cassette subfamily G-transporters (ABCG5/G8) and low-density lipoprotein receptor (LDLR) (de Miranda *et al*., 2016).

Pleurotus spp. also had been discovered as potential anti-hypercholesterolemic agent and as powerful antioxidant in many findings. *Pleurotus* spp. is a well-known mushroom with high nutrients, wonderful taste and validated bioactivities. It has received tremendous interest worldwide since the recorded publication on *Pleurotus* spp. has spiked from 15 articles in 1985 to 788 articles in 2015 (Correa *et al*. 2016). *Pleurotus* spp. is a good source of protein need for vegetarians due to the high protein content (*P. sapidus*, 38.5 %; *P. geesteranus*, 30.3 %; *P. citrinopileatus*, 29.4 %; *P. sajor-caju*, 26.0 %; *P. ostreatus*, 23.0 %; *P. pulmonarius*, 22.9 %; *P. tuber regium* 10.8 %) (Carrasco-Gonzalez *et al*., 2017; Correa *et al*., 2016; Guillamón, 2010).

Fombang *et al*. (2016) reported their finding on *P. florida* capability in ameliorating weight gain in hypercholesterolemic-induced rats besides reducing the atherogenic index in them by secreting accumulated lipid through faecal matter. HDL-C also increased thus favoured in cholesterol homeostasis in the bloodstream. Another study by Alam *et al*. (2011) also claimed the promising feature of *P. ferulae* in reducing serum total cholesterol, triglyceride and LDL in hypercholesterolemic rats. An observation on the patients has been reported by Khatun *et al*. (2007). Their research showed that *P. ostreatus* consumption by diabetic patients significantly reduces total cholesterol and triglycerides with additional value by reducing blood pressure with no toxicity effect on liver and kidney.

Besides its tremendous benefits to reduce blood serum total cholesterol level, *Pleurotus* spp. were also able to act as antioxidant agent. Antioxidant properties are very crucial to reduce the inflammation process happens in human body due to increased oxidized LDL. Perpetual inflammation may lead to severe conditions such as atherosclerosis and thrombosis. Khatun *et al*. (2015) conducted *in vitro* observation on

antioxidant properties in three *Pleurotus* spp. and discovered their significant chelating property. Chelating ion properties are crucial to avoid further damage due to ROS and protect from ion-overload. The high ferrous ion chelating properties was found in *P. florida* which has classified it as a powerful antioxidant agent.

Jayakumar *et al*. (2011) in their review mentioned that *P. ostreatus* is a rigid scavenger against hydroxyl and superoxide radicals besides having additional benefits of inhibiting lipid peroxidation and chelating ferrous ion. Another review by Sanchez (2017) also compiled many studies which have categorized *Pleurotus* spp. as highly fortified with phenolic compounds. Among the mentioned species are *P. eryngii*, *P. ostreatus* and *P. pulmonarius*. Phenolic compounds generally are having very good antioxidant properties as they have the ability to neutralise free radicals. (Reis *et al*., 2017).

2.4 *Pleurotus pulmonarius* (Fr.) Quél.

2.4.1 Taxonomy and Structure of *P. pulmonarius*

Pleurotus pulmonarius or locally known as Grey Oyster Mushrooms is an edible fungi and mushroom of interest in this study. It is also commonly known as Indian Oyster, Phoenix Mushroom, or the Lung Oyster. Taxonomically, it is classified as below:

Kingdom: Fungi

Division: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Tricholomataceae

Genus: *Pleurotus*

Species: *pulmonarius*

Pleurotus pulmonarius is often misapplied as *Pleurotus sajor-caju*. *Pleurotus sajor-caju* has been reclassified under the Genus Lentinus [*Lentinus sajor-caju* (Fr.) Fries.] by Pegler in 1975 due to the distinct difference between both mushrooms is on the stipe structure. *Pleurotus sajor-caju* has been removed from Fungorum website, but the scientific papers continuously published their findings using the scientific name of *Pleurotus sajor-caju* (Stamets, 2000).

P. pulmonarius is feasibly cultivated in various cultivation wastes and grows well in warm weather thus suits best to be cultivated in Malaysia. Despite of its established nutritional compounds, it is among the highly produced and consumed mushrooms in Malaysia. The edible part of *P. pulmonarius* is called the fruiting body. Fruiting body encompasses of cap and stipe or stalk (Figure 2.10).

Figure 2.10: *Pleurotus pulmonarius* fruiting body (edible part).

2.4.2 Medicinal Properties of *P. pulmonarius*

Pleurotus pulmonarius is fortified with many bioactive compounds including protein, carbohydrates, ash and crude fibre which are crucial for medicinal value execution on the human or animal models. Many medicinal properties have been discovered in *P. pulmonarius* including as anticancer, antihypertensive, anti-diabetic, anti-inflammatory and anti-hyperchoelsterolemia (Carrasco-González *et al*., 2017).

A. Anticancer Properties

The polysaccharide extract from *P. pulmonarius* was found to execute anticancer activity on colon cancer cells by reducing the adherence to extracellular proteins of cancer cell matrix (Lavi *et al*., 2010). Another study reported the capability of protein extract from *P. sajor-caju* was able to reduce the viability of liver cancer cells.

Hot water extract of polysaccharide-protein complex extracted from *P. pulmonarius* was found to inhibit the cancer cell proliferation via in vitro observations. *In vivo* observations also validated its anticancer properties as oral fed and intraperitoneal infection of the extract significantly inhibited the tumour growth in the mice (Xu *et al*., 2012). The polysaccharides extract of both mycelial and fruiting body owned by *P. pulmonarius* executed antiproliferative against colon cancer cells which expressed high amounts of galectin-3. Indirectly, the tumour cell adherence is downregulated thus interrupted in cancer progression (Lavi *et al*., 2010).

B. Antihypertensive Properties

Aqueous extract of *P. sajor-caju* was found to carry angiotensin1-converting enzyme (ACE) inhibitor thus normalize blood pressure in the studied rats' model (Abdullah *et al*., 2012).

Another study on mycelial water extract of *P. pulmonarius* possess inhibitory effect against ACE activity with the IC_{50} value of 720 µg/ml. Protein purification of the extract further confirmed the strong inhibitory effect (60 times stronger) at the IC_{50} value of 12 µg/ml (Ibadallah *et al*., 2014).

The protein fraction of *P. pulmonarius* orally fed to the spontaneous hypertensive rats (SHRs) found to be able to reduce systolic blood pressure (SBP) 33.5 mm Hg in SHRs. Molecular docking analysis by Manoharan *et al*. (2018) resulted in identification of gastrointestinal (GI) enzymes, tripeptide GVR with the ability to execute as ACE inhibitor.

C. Anti-inflammatory Properties

Carrageenan and formalin –induced paw edema in rats were tested for anti-inflammatory effects by treating them with polysaccharide compound from *P. pulmonarius*. A high inhibitory percentage was recorded; 83.33% and 92.37% inhibition respectively compared to the standard drug of dichlofanac (Adebayo *et al*., 2012).

Induced mice with acetic acid for inflammatory reaction was used to demonstrate the anti-inflammatory responses by hot water extract; glucan from *P. pulmonarius*. It executed dose-dependent anti-inflammatory response on the injured cells by inhibiting

the leukocyte migration (82 ± 6%) with an ID_{50} of 1.19 (0.74–1.92) mg/kg. The pre-treated mice with glucan and later injected with acetic acid showed a significant viability of the cells which (Smiderle *et al*., 2008).

D. Anti-diabetic Properties

Another research on Swiss Albino mice found the medicinal properties as anti-diabetic by the aqueous extract of *P. pulmonarius*. The alloxan-induced mice were treated with the extract and the reduction of blood glucose was closely monitored. During the second hour of the administration of the extract, a significant reduction in serum glucose level and maximum value of reduction detected at the sixth hour. The glucose level maintained at normal value till Day 28 of the extract administration. It was concluded in the observation that dosage of 500 mg/kg showed the optimum activity (Badole *et al*., 2006).

E. Antioxidant Properties

The 1,1-diphenyl-2-picryl-hydrazy free radical scavenging activity of methanol extract (ME) from *P. pulmonarius* fruiting body was studied. 2.0 mg/mL of ME activity was comparable to the standard reference, butylated hydroxytoluene (Nyugen *et al*., 2016).

Many extracts of *P. pulmonarius* were compared in a study by Abidin *et al*. (2016). Methanol-dichloromethane extract, water fraction, hot water, aqueous extract and hexane fraction were verified to exhibit potent anti-oxidant activities in Folin-Ciocalteu, DPPH radical scavenging, metal chelating, CUPRAC and lipid peroxidation assays. The compounds responsible to execute the anti-oxidant activities were found to be flavonoids, ergosterol, ergothioneine and phenolic acid derivatives.

F. Anti-hypercholesterolemia Properties

Little is known on anti-hypercholesterolemia properties by *P. pulmonarius*. Carrasco-González *et al*. (2017) cited β-glucan extract of *P. sajor-caju* assisted in alleviating serum lipid profile in induced-obese rats. Besides, the hepatic enzymes were sustained the body weight successfully reduce. Another species of *Pleurotus*, *P. ostreatus* is well-known for higher content lovastatin thus executes anti-hypercholesterolemia effects on studied animal model (Chen *et al*., 2012).

2.5 Metabolomic Profiling for Cardiovascular-related Disorders

The holistic approach of biological system is well-explained in omic- sciences; genomic, proteomic, transcriptomic and metabolomic. Since a decade ago, the latest advance in analytical methods involves the development of metabolomic analysis. Metabolomic analysis focuses on metabolite changes in sample compares to control or normal sample.

It assists to define the disease biomarkers by analysing the altered metabolites. Metabolomic research can be divided into untargeted and targeted metabolomic. Targeted metabolites aimed at multiplexed analysis of known metabolites to assess its performance in disease pathology, diagnosis or even for post-evaluation of untargeted metabolites after a particular treatment or developed method. In contrast to untargeted metabolomics, the analysis is more specific and narrowed. Usually, it involves the known metabolites previously explored and discussed. It involves significant compounds or target treatment either to downregulate its presence due to its detrimental effects or to upregulate in order to favour cell regulation (Ribbenstedt *et al*., 2018).

Untargeted metabolomic avoids bias in analysis as the comprehensive metabolites are detected and perhaps the new biomarkers (Figure 2.11) (Ghosson *et al*., 2018).

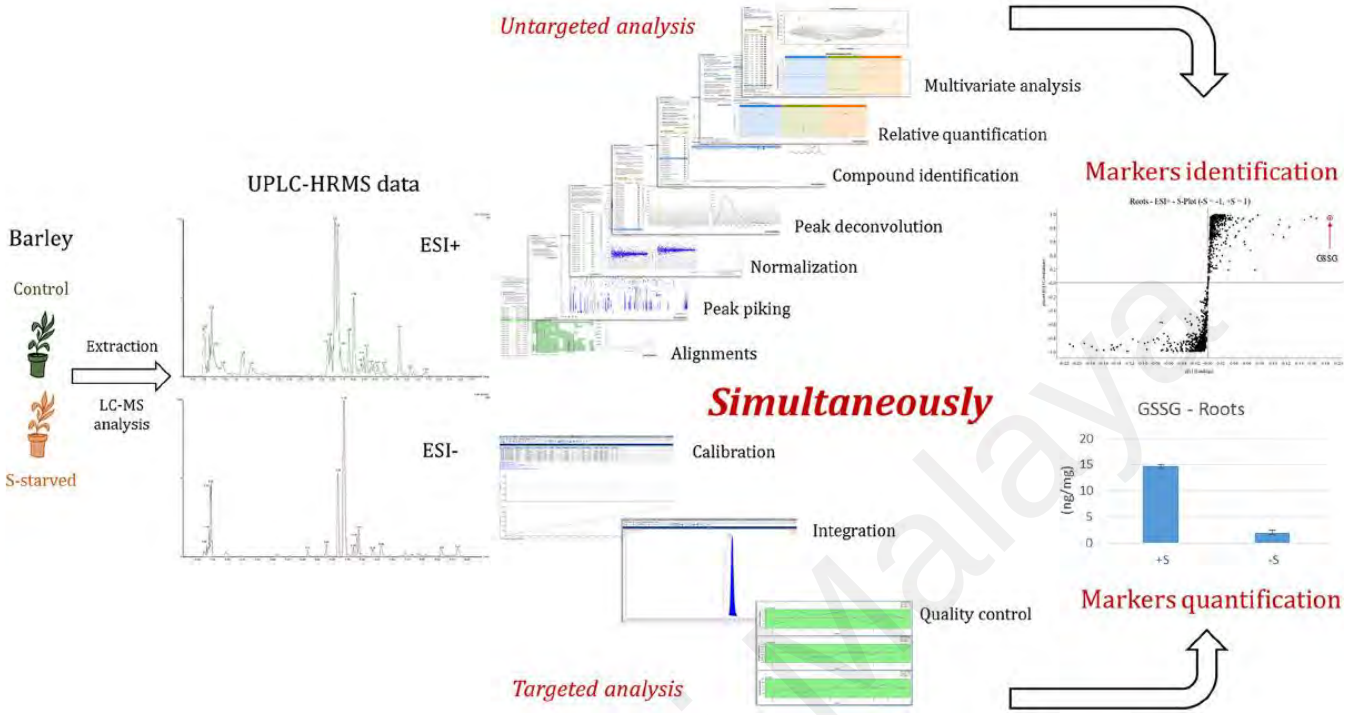


Figure 2.11: Targeted and untargeted metabolomics workflow (Ghosson *et al*., 2018).

The analysis generally involved a few steps commences from serum sample acquisition from animal model. Later, the metabolites detection follows by identification of the detected metabolites as summarized in Figure 2.12 (McGarrah *et al*., 2018).

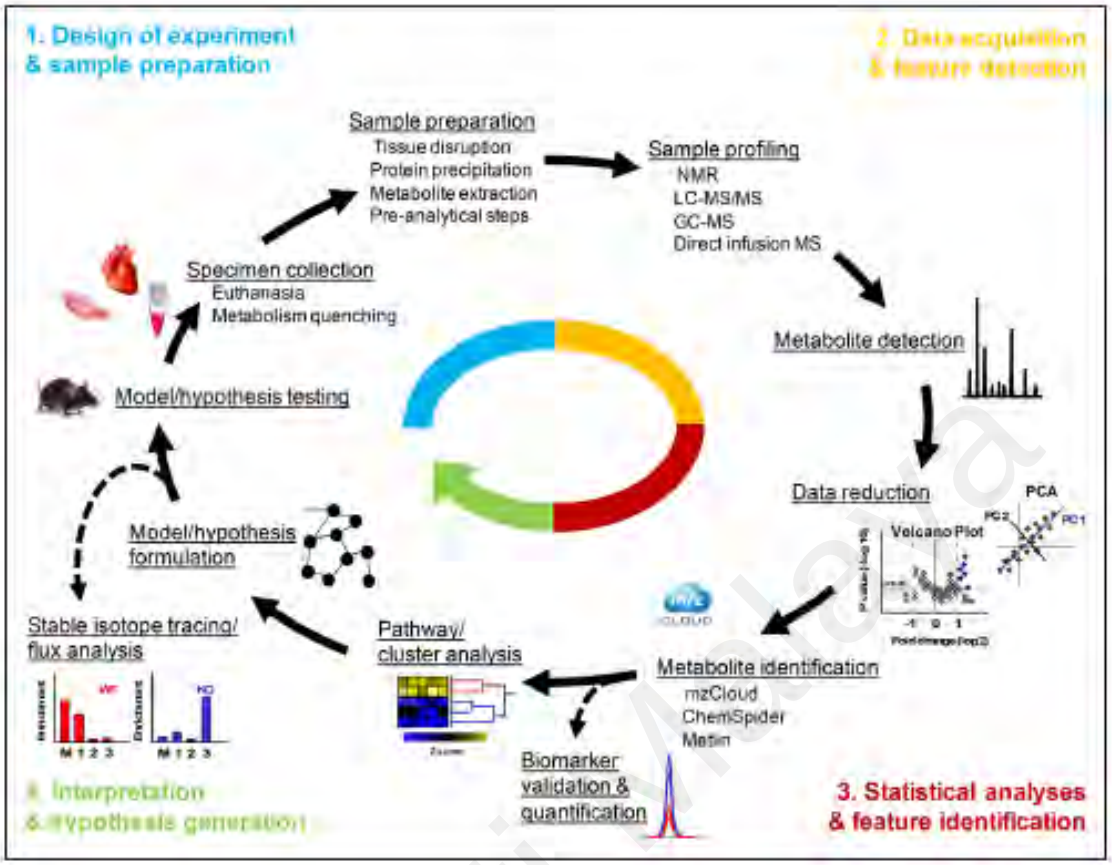


Figure 2.12: General steps involved in metabolomic analysis. It commences with sample acquisition; usually serum sample from animal model which later processed accordingly prior to metabolomic detection. A few common channels are used for identification of the detected metabolite (McGarrah *et al*., 2018).

Metabolomic has become prominent field in cardiovascular disease analytical scope. Many novel biomarkers have been identified as obligatory metabolites in contributing disease severity. Countless researches and analytical methods on metabolites profiling and identification are perpetually conducted worldwide.

In 2012, a metabolite profiling by Senn *et al*. (2012) was done on the atherosclerotic patients and its associated adverse events such as diabetes mellitus and hypercholesterolemia. Atherosclerotic and its associated risk patients are at potential risk of cardiovascular event. Dysregulation in a few metabolic pathways were found viz.

phosphatidylcholine pathway, choline metabolism and arganine pathway. Trimethylamine-N-oxide (TMAO), choline and betaine were noxious compounds discovered during metabolites identification. Another research on hypercholesterolemic rat with coronary heart risk found the significantly altered metabolites in the plasma sample; β-hydroxybutyrate, tyrosine and creatinine (Kordalewske and Markuszewki, 2015). Ming-Qian *et al*. (2012) run metabolomic analysis on mini pigs' plasma sample which previously has been induced with high-fat diet and coronary injury. Taurocholic acid was found to be the obligatory metabolite for atheroma plaque formation.

These metabolomic alteration are crucial to determine the target metabolites for further observation. Untargeted metabolomic analysed all the metabolites in compound-of-interest and assist to target all the significant metabolites (highly downregulated or highly upregulated) for further actions by using targeted metabolomic methods. Thus, untargeted metabolomic is the preliminary approach for compounds with unknown metabolites data and this study is intended to explore the compounds on interest, *Pleurotus pulmonarius* crude aqueous extract (CA) metabolites listing as it has been proven to significantly increase the cell viability through *in vitro* approach.

CHAPTER 3: MATERIALS AND METHODS

3.1 Experiments Materials

3.1.1 Chemicals

Below are the list chemicals used during the experiment:

Sodium chloride (NaCl)

Potassium chloride (KCI)

Calcium chloride (CaCl2)

Magnesium sulphate (MgSO4)

Potassium hydrogen phosphate (KH2PO4)

Sodium bicarbonate (NaHCO3)

Glucose

Ethylenediaminetetraacetic acid (EDTA)

Acetylcholine (Ach)

Phenylephrine (PE)

Formalin (HCHO)

Methanol (CH_3OH)

Ketamine

Xylazine

3.1.2 Instruments

The instruments involved in the experiments were:

A) Serum cholesterol quantification-Automatic Chemistry Analyzer: Siemens Dimension Xpand Plus

B) Aortic ring contraction - AD Instruments Pty Ltd, Bella Vista, Australia) coupled with PowerLab 4/35 (AD Instruments, Australia)

C) Histopathology - Leica EG1150 H/ Leica EG1150 H / Leica 2045 Microtome

D) Microscopic Observation - wide-field Olympus BX5 with a CCD camera

E) Metabolomic Analysis - Agilent 1290 Infinity LC System coupled to Agilent 6520 Accurate-Mass Q-TOF

3.1.3 Software

The software utilized for data analysis were:

A) Toxicity study - AOT425 Statistic Programme

B) Contractility study - LabChart software

C) Metabolomic analysis – KEGG ID/MetaboAnalyst 4.0

D) Rats' body weight/serum total cholesterol analysis - SPSS Software Package ver. 23

3.2 Sources of *P. pulmonarius* and Preparation of Crude Aqueous (CA) Extract

The locally known grey oyster mushroom or *Pleurotus pulmonarius* was purchased from Agro-tech Sdn. Bhd, Selangor, Malaysia. The species was previously identified as *P. pulmonarius* based on the morphological characteristics and via DNA online sequence data. The culture was registered as KUM61119 and maintained at the Mycological Laboratory, Mushroom Research Centre, Universiti Malaya. A voucher of the mushroom specimen was deposited in Universiti Malaya Herbarium and registered as KLU-M1234.

Total weight of 500 g *P. pulmonarius* fruiting bodies were shredded into small pieces prior to freeze-drying. After a week, the sample was ground into a fine powder (~50 g) and soaked in deionized water (ddH_2O) in a ratio of 1:10 (w/v) overnight at 4°C. Later, it was sieved to collect the liquid and the residue was discarded. The collected liquid was centrifuged at 4000 rpm for 20 minutes and the clear supernatant was

transferred into a new collection tube and sent for freeze-drying. After a few days, the freeze-dried sample was stored at 4°C in air-tight bottle until further use (Abidin *et al*., 2016).

3.3 Acute Oral Toxicity of CA

Five Wistar-Kyoto male rats were purchased from Animal Experimental Unit (AEU), University of Malaya aged six weeks (~200 g). The procedure below has received approval from University of Malaya Institutional Animal Care and Use Committee (UM IACUC) (UM IACUC No: 2015-181006/IBS/R/NFMY). All the animals were housed in AEU under 12:12 h light-dark cycle conditioned at 24°C (±1°C). Food and water were *ad libitum*.

Up and Down Procedure (UDP) was chosen as the procedure is more humane and less animals involved. By using AOT425 Statistic Programme, the default starting dosage for CA for this test was 175 mg/kg. Only one rat for each dosage of each extract and 24 hours interval observation was done. The observation includes physical changes such weight and fur losses and behavioural changes such as aggressiveness and eating habit. Five rats were labelled as R1, R2, R3, R4 and R5 for CA toxicity study. Since R1 survived, next orally feed dosage for R2 was 550 mg/kg. Subsequently, 2000 mg/kg was feed to R3 which was the highest limit dose set for the study. The limit dose repeated for three consecutive days prior to LD_{50} calculation. Since R3, R4 and R5 survived (no physical or behavioural change), CA was concluded as safe for the Wistar-Kyoto rats (Rispin *et al*., 2002).

3.4 Effects of CA and Simvastatin on the Rats Aortic Rings*: ex vivo* and *in vivo*

3.4.1 Induction of Hypercholesterolemia in Rats

Forty-eight Wistar-Kyoto male rats were purchased from Animal Experimental Unit (AEU), University of Malaya aged six weeks (~200 g). Male rats were preferred to minimize the hormone interference in measured parameters. The procedure below has received approval from University of Malaya Institutional Animal Care and Use Committee (UM IACUC) (UM IACUC No: 2015-181006/IBS/R/NFMY). All the animals were housed in AEU under 12:12 h light-dark cycle conditioned at 24°C (±1°C). Food and drink were maintained *ad libitum* for all the rats. The cholesterol diet was prepared by dissolving the purchased cholesterol powder (Cholesterol powder 95% stabilized; Brand: Acros Organics) in palm oil (10 ml/kg BW). Crude aqueous (CA) was prepared by dissolving the freeze-dried CA powder in distilled water; according to the dosage for the rats which were 0.5 g/kg BW and 2.0 g/kg BW.

The rats were divided into 8 groups as summarized in Table 3.1. The comparison studies were among the normal, hypercholesterolemia, treatment and prevention groups. Two dosages of CA (0.5 g/kg BW and 2.0 g/kg BW) were given to the rats and compared with commercial drug, simvastatin. 10 mg. Simvastatin 10 mg is a commercial drug with active compound of statin (weight of 10 mg) in each tablet. The dosage is according to the prescription by the medical practitioner or pharmacist to the hypercholesterolemic patients. Body weight of each rat was taken on Day 1, Day 15, Day 30 and Day 45. The rats' body weight of rats for treatment group was also taken on Day 75.

Table 3.1: Diet formulation for all the rats' group

No	Group	Category	Description
1	G1	Normal	Healthy rats
2	G2	Hypercholesterolemic	Rats fed with cholesterol powder* (200 mg/kg BW) dissolved in palm oil (10 ml/kg BW) (Day 1-45)
3	G3	Treatment 1	Rats fed with cholesterol powder* (200 mg/kg BW) dissolved in palm oil (10 ml/kg BW) (Day 1-45) + CA (0.5g/kg BW) (Day 46-75)
4	G4	Treatment 2	Rats fed with cholesterol powder* (200 mg/kg BW) dissolved in palm oil (10 ml/kg BW) (Day 1-45) + CA (2.0 g/kg BW) (Day 46-75)
5	G5	Treatment 3	Rats fed with cholesterol powder* (200 mg/kg BW) dissolved in palm oil (10 ml/kg BW) (Day 1-45) + simvastatin 10 mg/kg BW (Day 46-75)
6	G6	Prevention 1	Rats fed with cholesterol powder* (200 mg/kg BW) dissolved in palm oil (10 ml/kg BW) + CA (0.5g/kg BW) (simultaneously) (Day 1-45)
7	G7	Prevention 2	Rats fed with cholesterol powder* (200 mg/kg BW) dissolved in palm oil (10 ml/kg BW) + CA (2.0 g/kg BW) (simultaneously) (Day 1-45)
8	G8	Prevention 3	Rats fed with cholesterol powder* (200 mg/kg BW) dissolved in palm oil (10 ml/kg BW) + simvastatin 10 mg/kg BW (simultaneously) (Day 1-45)

*Cholesterol powder 95% stabilized; Brand: Acros Organics

Blood was taken on Day 1 for all the groups utilising lateral tail veins (hypodermic needle 26 G) after anaesthesia [ketamine:xylazine; 80:10 (mg/kg)]. Approximately, 0.5 ml blood was withdrawn each time. Whole blood was collected through intracardiac puncture (3 ml; 23 G) on Day 45 for normal, hypercholesterolemia and prevention groups whereas for treatment group, intracardiac puncture (3 ml; 23 G) was done on Day 75. Oral gavage (18g curved stainless needle) was carried out for 45 days for G1, G2, G6, G7 and G8 groups whereas G3, G4 and G5 groups were lasted till Day 75 (Dikshit *et al*., 2016).

3.4.2 Aortic Rings Contractility Observations

TAR for contractility study were preserved in Krebs-Heinseleit buffer solution (mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 11.1 Glucose; 0.25 EDTA) for approximately 10 min. TAR was then mounted in the organ bath chamber filled with 30 ml of Krebs-Heinseleit buffer solution. The chamber was continuously aerated with carbogen (95% oxygen: 5% carbon dioxide) and maintained at 37°C. A baseline tension of 1.50 g was applied to the TAR and variations in the basal tension were recognised with a 50 g force transducer (ADInstruments Pty Ltd, Bella Vista, Australia) coupled with PowerLab 4/35 (ADInstruments, Australia) and let to equilibrate for 45 min. The solution was changed every 15 min as a precaution against any interfering metabolite (Chen *et al*., 2014; Qu *et al*., 2014).

Later, a high concentration of potassium (KCl) (80 mM) was added to the chamber for tissue priming purpose. Once it reached the plateau, TAR was flushed till it achieved the baseline. Later, integrity test was assembled on TAR by challenging it with phenylephrine (PE) (10 µM) and acetylcholine (Ach) was subsequently administrated to TAR (cumulatively added to the solution in the chamber from 1 µM to 20 µM) and

dilation responses towards Ach were transmitted to LabChart software (Koon *et al*., 2014; Niazmand *et al*., 2014). The raw data was later entered and analysed using Excel for graph patterns and SPSS for data analysis.

Integrity test is the pre-requisite to confirm the vitality of the aortic rings used in this study. Once the integrity test succeeded, TAR of the GI and G2 were challenged again with PE and CA was then administrated cumulatively (0.1- 6.0 mg/ml) to the solution in the chamber (*ex vivo*). The whole procedures were repeated using another tissue segment and replacing CA with simvastatin. For G3 to G8 groups, TAR was used for integrity test only in order to observe the TAR responses towards Ach after the rats were orally fed with CA/simvastatin prior to organ bath analysis.

3.5 Quantification of Serum Total Cholesterol in Rats

Whole blood was collected through intracardiac puncture (3 ml; 23 G) centrifuged at 2200 rpm for 6 min and the serum finally sent to Histopathology Department, Faculty of Veterinary, Universiti Putra Malaysia for serum cholesterol quantification.

3.6 Histopathology of Rats' Aorta and Liver

Histopathology viewing of the tissues was intended to observe the conditions of isolated liver and thoracic aorta to observe any significant changes in tissues. The methods explained below were extracted from Slaoui and Fiette (2011) with some modifications.

3.6.1 Fixation

Liver was harvested from each rat once it was sacrificed for organ bath analysis. The liver was cut into 1 cm x 1cm block and swiftly transferred to 10 % formalin in Bijour bottle. Cut organs were left immersed overnight in the bottles to ensure the formalin are fully absorbed into the tissues so called as fixation process. This particular process is very much crucial to halt autolysis process in order to preserve the tissues for observations. The following steps (dehydration till staining) were outsourced to Histopathology Department, Faculty Veterinary, Universiti Putra Malaysia.

3.6.2 Dehydration

Later, dehydration process was taken place. The tissues were dehydrated by soaking them in a series of ethanol with different concentration. The step commenced with soaking the tissues in lowest concentration of ethanol; 80 % for 2 hours followed by 95 % for 2 hours and ended up with 100 % for 3 hours.

3.6.3 Embedding

Embedding is a crucial process to infiltrate tissue samples with paraffin in order to replace the water content in the tissue samples. The paraffin was heated till it reached its melting point around 57°C and poured into Leica EG1150 H heated paraffin dispensing module. The tissue was positioned properly to make sure the paraffin fully covered the tissue once solidified. Later, the mould was transferred onto Leica EG1150 C cold plate. The procedure was repeated for the tissue samples. Once solidified, the paraffin provided an affirm medium to keep intact of the tissue before sectioning.

3.6.4 Pre-sectioning

Paraffin-embedded tissues blocks were chilled by placing them on ice as cold wax allows thinner section to be obtained by providing support for harder elements within the tissue specimen. The moisture derived from the melting ice also made the tissues easier to be sectioned.

3.6.5 Sectioning

The blade is positioned in the holder of Leica 2045 Microtome. The paraffin block was inserted and properly orientated for accurate angle of sectioning. Prior to sectioning, trimming process took place at thickness of 10 – 30 µm. Later, sectioning was done at the thickness of 4-5 µm. The first few sections were discarded as they contained holes caused by trimming. The ribbons (comprised of a few sections) were floated in the water bath and separated into individual section using a tweezer. Later, each section is taken out from the water bath using microscopic slides and stored at upright position in a slide rack and kept in the oven overnight at 37°C.

3.6.6 Staining

The slides were deparaffinised by submerging in xylene for 5 minutes. Later, these slides were dehydrated by submerging them in 100% alcohol and continued with another 5 minutes in 70% alcohol. The slides were then rinsed and submerged in Haematoxylin for 5 minutes prior to re-rinsed for 3 to 5 times. Next, the slides were dipped in 1% acid alcohol for 3 seconds and put under the running tap water for 5 minutes. Later, the slides were submerged in Eosin for 1 minute followed by spraying them with 95% alcohol. The slides again rinsed under running tap water for 5 to 10 seconds. The slides were sprayed with 95% alcohol, cleaned and left to dry. Finally, the

slides were mounted with DPX prior to microscopic viewing using wide-field Olympus BX51(Olympus Optical Co. Ltd., Tokyo, Japan) with a CCD camera to capture all images. Image viewing and interpretation were done in-house following the guide and explanation in 'Histopathology of Animal Model' course.

3.7 Metabolomic Analysis of Rats' Sera

3.7.1 Rats' Sera Sample Preparation

Serum sample from four groups were selected; G1, G2, G4 and G7. The selections were based on the organ bath data recorded by the aortic rings belonged to these groups. The serum samples were thawed at room temperature. 200 µl of serum were pipetted and transferred to Appendoff tube and followed by 400 µl methanol in order to precipitate protein. The mixtures were vortexed and centrifuged at 12,500 g for 15 minutes. The supernatant was collected and re-centrifuged, dried and stored at -80°C (Yang *et al*., 2013).

3.7.2 Liquid Chromatography and Mass Spectrometry

The serum samples were analyzed with Agilent 1290 Infinity LC System coupled to Agilent 6520 Accurate-Mass Q-TOF. The HPLC column used for the analysis was ZORBAX Eclipse Plus C18 column (100 MM x 2.1 mm x 1.8 µm, Agilent Technologies, SA, USA). Two mobile phases were used: 0.1% formic acid in Mili Q water (A): 0.1% formic acid in acetonitrile (ACN) (B), based on an established protocol developed in the laboratory (Yang *et al*., 2013; Manoharan *et al*., 2018). The gradients of both mobile phases were as follow:

Time (min)	A%	B%	Flow (ml/min)
0	95	5	0.25
35	5	95	0.25
41	5	95	0.25
41.1	95	5	0.25
48	95	5	0.25

Dual Agilent Jet Stream ESI was used to perform ElectroSpray Ionization (ESI) under positive mode with following parameters:

a) Capillary voltage: 4000 V
b) Nozzle voltage: 750 V
c) Fragmentor voltage: 175 V
d) Nebulizer pressure: 30 psi
e) Gas temperature:325°C
f) Gas flow: 10 L/min
g) Reference masses: 121.050873, 922.009798
h) MS data range: 100 – 1000 *m/z*

Data was processed with Agilent Mass Hunter Qualitative Analysis B.05.00. Later, the data were exported to Mass Profiler Professional software to run statistical analysis and metabolites identification via ID Browser and METLIN metabolite PCDL database (Manoharan *et al*., 2018).

At least three runs of one treatment is pre-requisite to detect the frequency of occurrence of each compound prior to filter and data normalization. Significant analysis

via one-way ANOVA ($p < 0.01$) and fold change analysis ($FC \geq 2$) was conducted using Mass Profiler Professional. Bejamini-Hochberg Procedure was applied for multiple testing corrections to comply to false discovery rate (FDR) ≤ 1%. KEGG ID was used to confirm the presence of the compound in *Rattus norvegicus* via KEGG Pathway Database (https://www.genome.jp/kegg/pathway). Once confirmed, Metabo Analyst software (http://www.metaboanalyst.ca/) platform used to run pathway analysis. Both sites are free online platforms specifically for metabolomic analysis (Yang *et al*., 2013; Manoharan *et al*., 2018).

3.8 Statistical Analysis

Overall, SPSS Software Package ver. 23 was used to interpret the significant difference for all the recorded data. The rats' body weight was analyzed using One Way ANOVA ($p < 0.05$). Total serum cholesterol was analyzed using Paired Sample-Test. The contractility values of prevention group used Two Way ANOVA ($p < 0.05$).

CHAPTER 4: RESULTS AND DISCUSSION

Endothelium is a delicate layer in the blood vessel. It has a prominent role in secreting various compounds favourable for blood vessel vasodilation including nitric oxide (NO). NO is very crucial as a blood vasodilator and its deterioration may create a pressure and causes more detrimental effects to the blood vessel. Deterioration of blood vessel could be due to many factors including hypercholesterolemia.

Hypercholesterolemia is linked to the plaque formation in the blood vessel after years of cholesterol accumulation. It begins with the build-up of cholesterol in the intima tunica and after a long process of smooth muscle cell migration underneath the endothelium. The endothelial layer will be disrupted and damaged due to the presence of these cells. These interruptions may contribute to the inadequate of NO secretion by the endothelial layer which later impairs the blood vessel to dilate. The bulge will continue to grow thus narrowing the blood vessel creating pressure to the blood flow (Kim *et al*., 2012).

Above mentioned disease progression leads to severe health conditions including stroke and cardiac attack. The increasing number of patients succumbed to cardiac risks is the main concern by most of the researchers worldwide. The development of a new class of drugs and discovery of functional food with countless benefits are very crucial. As part of the contribution towards a better life, this study is constructed to reveal the hidden benefits of well-known grey oyster mushroom or scientifically known as *P. pulmonarius* against hypercholesterolemic risks. The hypercholesterolemic rats' model was developed to mimic hypercholesterolemic

conditions and observed the responses towards *P. pulmonarius*. Organ bath analysis, histopathology examination on liver and aortic tissues together with metabolomic analysis are the elements of this study to serve its purpose to find a potential solution to lower the hypercholesterolemic risk factors.

4.1 Toxicity of CA on Rats

Toxicity study to confirm the CA extract is safe for the rat is crucial. Up and Down Procedure (UPD) applied in this study is an improved version of conventional method practiced in acute oral toxicity study besides significantly reduces the number of animals sacrificed. In addition, UPD conforms to OECD guideline which was adopted on 17th December 2001. UPD applies a simple yet efficient AOT 425 Software program developed by Rispin *et al*. (2002). The dose of CA started at 175 mg/kg body weight and ended at 2000 mg/kg body weight. It was a 24 hours observation of each dose for short term outcome. Since the first rat survived, the dosage was increased to 550 mg/kg BW for the second rat on Day 2. The last dosage of 2000 mg/kg body weight was given to the third rat and repeated to the fourth and fifth rat.

All the rats were observed for another 14 days if any physical or behavioural changes. Overall results are summarized in Table 4.1. In summary, CA was found to be safe for the rats even at the highest dosage of 2000 mg/kg body weight recommended for the rats. The finding was in accordance with the report by Blanche *et al*. (2019). The authors found that the aqueous extract of *P. pulmonarius* did not show any sign of toxicity on the rats during acute toxicity observation at the concentration of 2000 mg/kg body weight. In addition, an observation by Abidin *et al*. (2016) also found that IC_{50} value for CA was more than 200 µg/ml with endothelial cell viability rate exceeded 80 %. Thus, the toxicity recorded in this study was in accordance with previous findings.

Table 4.1: Acute oral toxicity of CA on the observed rats

No	Rat ID	Dosage (mg/kg body weight)	Short–term outcome (1 day)	Long-term outcome (2 weeks)
1	R1	175	o	o
2	R2	550	o	o
3	R3	2000	o	o
4	R4	2000	o	o
5	R5	2000	o	o

Legend
o - represents rat still alive

Once CA was found to be safe for the rats, newly purchased 48 rats were grouped into eight experimental groups; normal, hypercholesterolemia, treatment (three subgroups) and prevention (three subgroups). The compound-of-interest in this study was CA versus commercial drug, simvastatin 10 mg. The rats were fed orally with these compounds either concomitantly with dissolved cholesterol or fed after the serum total cholesterol was successfully elevated.

4.2 Body Weight Pattern and Serum Total Cholesterol Quantification

Table 4.2 summarized the increment of the body weight (BW) throughout the experiment. The body weight increment percentage for all the rat groups was in accordance with the age increment. Percentage of 30.1% in body weight increment in G1 and 40.0% in G2 were recorded. In the treatment groups (G3-G5), 45.7%, 31.9% and 48.6% increments were recorded for these groups accordingly. In addition, prevention groups (G6-G8), the increment percentage of 30.4%, 32.2% and 31.5%; were observed respectively. The body alleviation was consistent for all the groups except for G3 and G5, the percentage exceeded 40 %. The spike could be due to the

different body responses towards the treatment given to the rats. The induced hypercholesterolemic rats are basically in average range for their serum total cholesterol, however, there are many other uncontrolled factors due to hypercholesterolemia especially liver activity to eliminate the excessive cholesterol. None of them showed a reduction in the body weight as the treatment did not involve induction of severe infection or disease.

Table 4.2: Changes of body weight (g) in rats (A) Normal and hypercholesterolemic groups (B) Treatment groups and (C) Prevention groups.

A

Group	Normal (g)	Hypercholesterol (g)
	G1	G2
Day 1	199.3 ± 2.50	197.9 ± 2.64^{w}
Day 15	218.0 ± 1.67	227. 5 ± 3.39^{a,w}
Day 30	239.7 ± 1.37	247.8 ± 2.14^{b,w}
Day 45	259.3 ± 1.21	276.2 ± 3.6^{c,w}

B

Group	Treatment (g)		
	G3	G4	G5
Day 1	199.0 ± 2.37	200.2 ± 2.40^{w}	198.8 ± 1.47
Day 15	228 ± 2.37	227.7 ± 2.23^{a,w}	225.8 ± 1.33
Day 30	246.5 ± 1.87	245.6 ± 1.03^{b,w}	245.5 ± 1.05
Day 45	271.5 ± 2.35	271.3 ± 2.07^{c,w}	272.2 ± 1.72
Day 75	290.0 ± 2.28	294.0 ± 1.55^{d,w}	295.5 ± 1.38

Table 4.2, continued.

C

Age \ Group	Prevention (g)		
	G6	G7	G8
Day 1	200.2 ± 2.14	196.7 ± 1.37^{w}	199.8 ± 2.04
Day 15	219.8 ± 1.47	219.3 ± 1.03^{a,w}	221 ± 2.28
Day 30	238.7 ± 0.82	241.8 ± 1.72^{b,w}	241.5 ± 1.87
Day 45	261.0 ± 0.89	260.0 ± 1.89^{c,w}	262.8 ± 1.72

Letter a, b, c and d constitute significant differences ($p < 0.05$) within the groups while w represents significant difference between the groups ($p < 0.05$; analyzed with One Way ANOVA). The values are expressed as mean ± SD (SD= Standard Deviation)

Table 4.3 shows the serum total cholesterol values of rats in all the groups. The initial total cholesterol ranges of the groups were 1.80 – 2.00 mmol/L. The serum total cholesterol values increased in accordance with age increment for G1 group (2.53 mmol/L) (Table 4.3A). The average value recorded for the hypercholesterolemic-induced rats (G2 – G5) was > 3.30 mmol/L. In addition, treated groups (G3-G5) managed to reduce its cholesterol level following the treatment (Table 4.3B) whereas for the prevention groups (G6-G8), serum total cholesterol maintained within the normal range as shown in the below table (Table 4.3C).

Serum total cholesterol for treatment groups (G3-G5) were significantly reduced on Day 75 after successfully elevated to be hypercholesterolemic on Day 45. The percentage of 17.4, 23.6 and 28.3 reduction were recorded all these groups (G3-G5); respectively. The serum total cholesterol achieved normal rate of < 3.3 mmol/L on Day 75. Serum total cholesterol for the prevention groups (G6 – G8) was within the normal range (2.68 – 3.00 mmol/L), thus verified on CA and simvastatin potent effect on cholesterol homeostasis in the bloodstream. Above finding on cholesterol alleviation in

rats' blood, is as claimed in previous findings. Previous researchers who worked on *Pleurotus* spp. have discovered anti-hypercholesterolemic properties in this particular mushroom species (Valverde *et al*., 2015). Guillamón *et al*. (2010) for instance, has summarized many edible mushrooms with various benefits including *Pleurotus* spp. capability to reduce total cholesterol level.

Table 4.3: Serum total cholesterol quantification (mmol/L) in (A) G1 – G2 (B) G3-G4 (C) G5-G8.

A

	mmol/L	
	Day 1	Day 45
G1 (Normal)	1.90 ± 0.02	2.53 ± 0.67[a]
G2 (Hypercholesterol)	1.89 ± 0.6	3.83 ± 1.03[b]

B

		mmol/L		
		Day 1	Day 45	Day 75
Treatment Groups	G3	1.86 ± 0.06	3.85 ± 0.12[c,d]	3.18 ± 0.11
	G4	1.89 ± 0.67	3.86 ± 0.97[e,f]	2.95 ± 0.09
	G5	1.90 ± 0.70	3.92 ± 0.10[g,h]	2.81 ± 0.98

Table 4.3, continued.

C

		mmol/L	
		Day 1	Day 45
Prevention Groups	G6	1.91 ± 0.03	3.00 ± 0.11[i]
	G7	1.89 ± 0.07	2.78 ± 0.97[j]
	G8	1.90 ± 0.07	2.68 ± 0.06[k]

Letter a-k represented within group comparison [a-b and i-k represented significant differences ($p < 0.05$) between Day 0 and Day 45; c-h represents significant differences ($p < 0.05$) between Day 0 and Day 45/Day 45 and Day 75 when analysed using Paired Sample T-test. The values are expressed in mean ± SD; SD: Standard Deviation.

4.3 Aortic Rings Contractility Study

Organ bath technique is one of the oldest methods used to observe aortic ring contractility towards compound of interest. In this study, CA was observed in its integrity to dilate the contracted thoracic aortic rings (TAR).

4.3.1 Integrity Test on G1 and G2 Groups

TAR achieved almost 60 to 70 % vasodilation (Figure 4.1). Although previous studies reported that the TAR should achieve 70 to 90 % vasodilation, however when the rats age is taken into account, the vasodilation values vary. Since it took almost 2 months to induce hypercholesterolemia in rats, thus normal group rats have to be at the same age for comparable results in this study. The rats' age during contractility response study has achieved almost 15 weeks which has categorized them into adult rats. As reviewed by Matz *et al*. (2000), the maximal effect of Ach is reduced from 100

% in young rats (4-6 weeks) to 50 % in adult rats (3-6 months) and reduced to 25 % in old rats (12-25 months).

However, TAR of hypercholesterolemic group G2 showed no response to Ach as the value recorded via the LabChart software was zero vasodilation. Based on this observation, the endothelium of hypercholesterolemic aortic ring is probably damaged as no response was recorded.

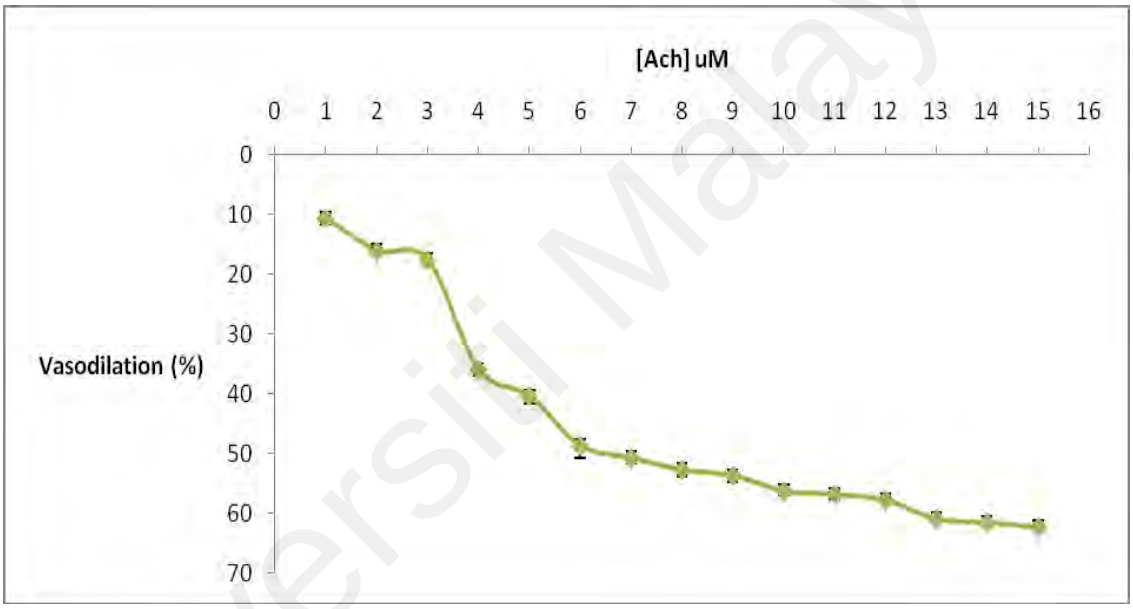


Figure 4.1: Integrity test on TAR of G1 group; Ach successfully caused vasodilation (Average mean of six replicates; Mean ± SD; SD = Standard Deviation).

Although endothelium is just a simple monolayer, the healthy endothelium plays a prominent role by optimally responding to physical and chemical signals via secretion of a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation and vessel wall inflammation. The importance of the endothelium was first recognized by its effect on vascular tone. This

vasomotion plays a direct role in the balance of tissue oxygen supply and metabolic demand by regulation of vessel tone and diameter (Deanfield *et al*., 2007).

The pioneering experiment of Furchgott and Zawadzki (1980) first demonstrated NO as an endothelium-derived relaxing factor. NO is generated from L-arginine by the action of endothelial NO synthase (eNOS) in the presence of cofactors such as tetrahydrobiopterin. This gas diffuses to the vascular smooth muscle cells and activates guanylate cyclase, which leads to cGMP-mediated vasodilation. Shear stress is a key activator of eNOS in normal physiology. In addition, some other vasodilators synthesized by endothelial cells are prostacyclin, hydrogen peroxide, carbon monoxide and epoxyeicosatrienoic acids as well as vasoconstrictors, like thromboxane A_2, prostaglandin H_2 and endothelin-1 (Furchgott & Zawadzki, 1980; Deanfield *et al*., 2007; Wang *et al*., 2010).

4.3.2 Effects of CA and Simvastatin towards TAR Dilation of G1 and G2 Groups

Both TAR isolated from normal group G1 and hypercholesterolemic group G2 were further exposed to CA and simvastatin. Figure 4.2 showed a consistent increase in vasodilation of TAR of G1 as the CA concentration increased. The final 100% vasodilation was achieved at CA concentration of 3.0 mg/ml. The same pattern was observed following treatment with simvastatin, whereby at the concentration of 2.4 mg/ml, the vasodilation of 100% has been successfully achieved.

Although TAR of hypercholesterolemic group G2 failed to respond to Ach during integrity test, treatment with CA successfully dilated the TAR. Vasodilation increased parallel with cumulative CA concentration and finally achieved almost 73.0%

at the CA concentration of 6.0 mg/ml. In comparison, at simvastatin concentration of 4.8 mg/ml, the vasodilation achieved almost 76.8% (Figure 4.2). Basically, NO is the main vasodilator and endothelial-derived. Major modulation in vasodilation is endothelial-dependent thus endothelial damage interfere with NO secretion. Vasomotor modulation by compound-of-interest can be either endothelium-dependent or endothelium-independent. CA is claimed to be endothelial-independent as it can assist the aortic ring to dilate (Chen *et al*., 2014).

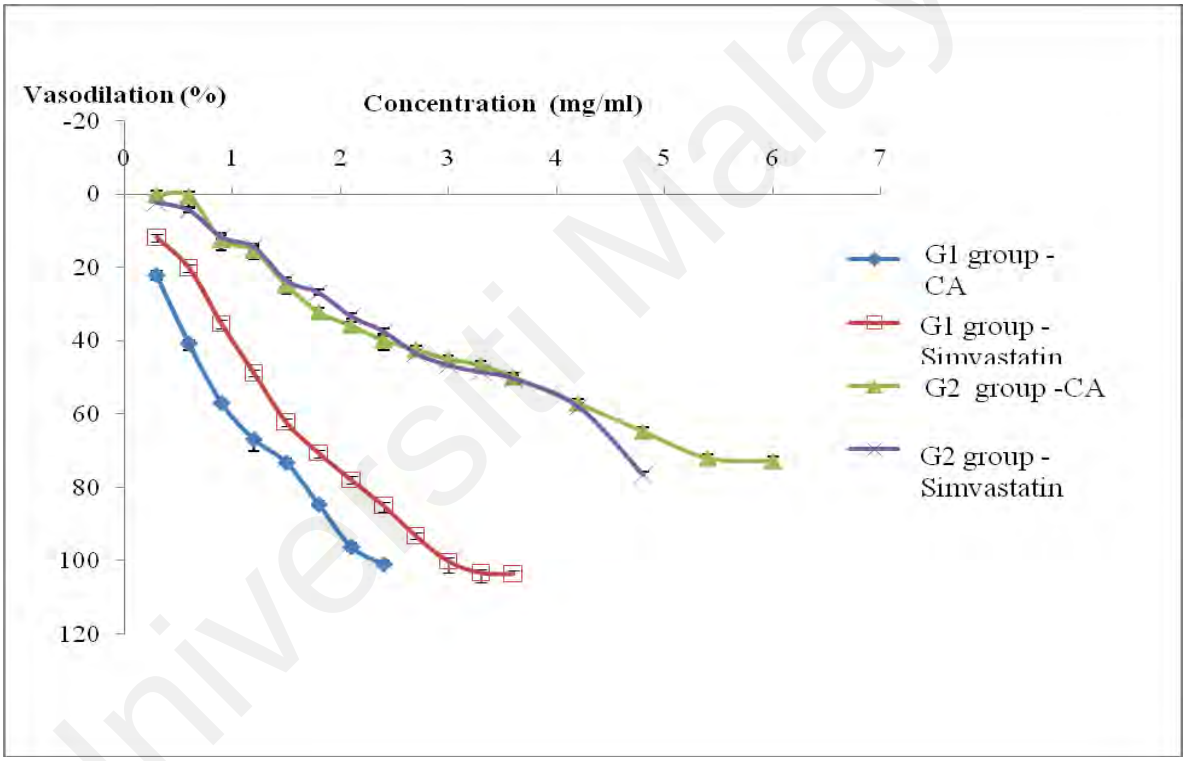


Figure 4.2: Vasodilation effects of CA and simvastatin on TAR belonged to G1 and G2 groups (Average mean of six replicates; Mean ±SD; $p < 0.05$).

4.3.3 Integrity Test on TAR of G3 to G8 Groups

None of the TAR of treatment groups; G3, G4 and G5 dilated when challenged with Ach after pre-constricted with PE during integrity test although their serum total cholesterol reduced when treated with CA/simvastatin. It perhaps due to damage and inability of the blood vessel to dilate although the serum total cholesterol had successfully decreased.

However, TAR of prevention groups; G6, G7 and G8 have transmitted its contraction and were observed in the myograph. Figure 4.3 showed the comparison of CA- and simvastatin-treated groups where a higher vasodilation percentage was achieved by TAR of G7 with 43.11 %, followed by TAR of G8 (34.17 %) and finally TAR of G6 with 12.51 %. From this result, it showed that 2.0 g/kg body weight CA performed better compared to commercial drug. The roles of each compound detected in CA are discussed below.

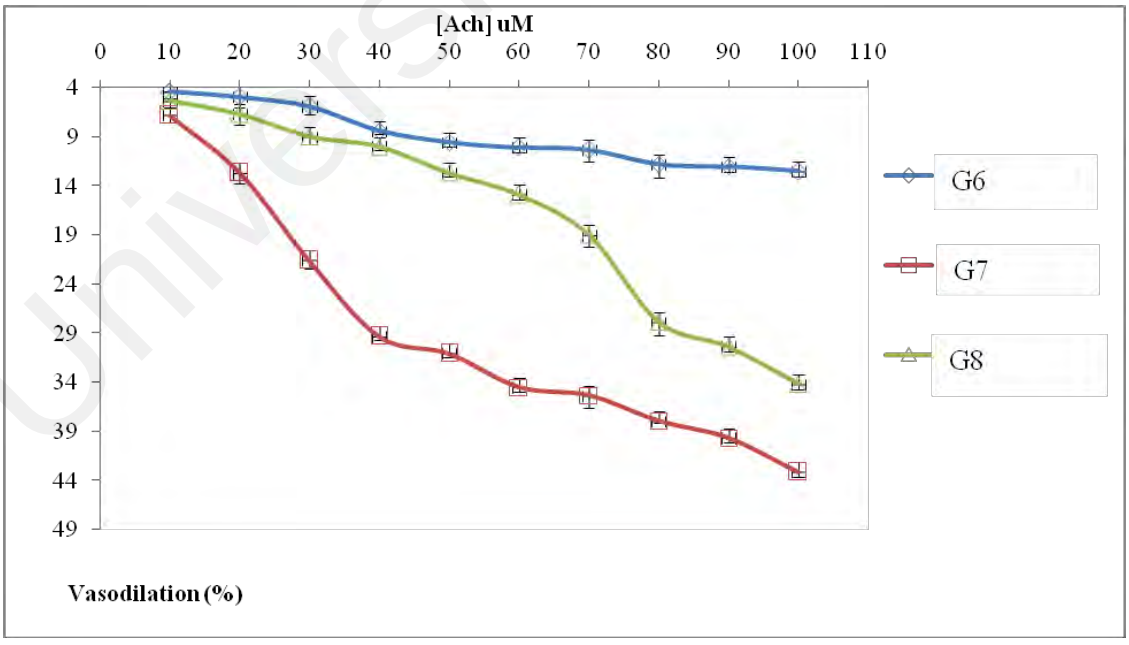


Figure 4.3: TAR responses towards Ach in G6, 7 and G8 groups (Average mean of six replicates; Mean ±SD; $p < 0.05$); *: there is a significant different among the three group with Two Way ANOVA analysis).

The compounds in CA were previously identified by Abidin *et al*. (2016) thus the repetitive work on identification was not done in this research. Some of the potent compounds identified in CA were ergothioniene, tryptophan and methyl ellagic acid (Abidin *et al*, 2016). Ergothioniene is one of the powerful scavengers of reactive oxygen species and has the ability to prevent further damage by those ROS besides preserving NO. Ergothioniene exists as a trace element in many foods however; it is present abundantly in some of the mushroom's species such as *Lentinus edodes* and *Pleurotus ostreatus*. It was proven as an antioxidant in its first cell-free study where it inhibited the formation of hydroxyl radicals and lipid peroxidation. This particular compound needs a specific carrier protein organic cation transporter novel type-1 (OCTN-1) to cross the cell membrane which was interfered if the expression of OCTN-1 was silenced (Deiana *et al*., 2004; Li *et al*., 2014). Above mentioned findings were in accordance with this study since successful vasodilation was achieved in TAR of hypercholesterolemic group (G2) in this study.

Ellagic acid has many potential properties as anti-oxidant, anti-inflammatory, anti-hypercholesterolemia and anti-carcinogenic (Devipriya *et al*., 2007; Berkban *et al*., 2015). It has the capability to be an endothelium-dependent and endothelium-independent vasodilator. Previous research reported that the endothelial-denuded aortic ring was still able to reach vasodilation value of 46.20 %. Although the percentage of vasodilation in endothelium denuded aortic ring was slightly reduced compared to endothelial intact aortic ring with vasodilation value of 75.20 %, but the vasodilation value could still be recorded (Yilmaz & Usta, 2013). In another study, they revealed that ellagic acid has the capability in normalizing the systolic blood pressure of hypertensive rats which was previously induced via N-Nitro-L-arginine methyl ester hydrochloride

(L-NAME). Orally feed L-NAME had caused decreased in both plasma NO and eNOs expression in hypertensive rats. Ellagic acid has proven to play a prominent role by restoring NO and prevents oxidative stress (Berkban *et al*., 2015).

Tryptophan is another valuable compound detected in CA (Abidin *et al*., 2016). It is mainly catalyzed by two types of enzymes; Trp-2,3-dioxygenase or Trp pyrrolase (TDO) and indole amine-2,3- dioxygenase (IDO). TDO is mainly expressed in liver and brain whereas IDO is secreted in peripheral tissues, immune system cells and central of nervous system. Kynurenine is catalysed from tryptophan by IDO during inflammation. It is usually up-regulated in response to inflammation as a novel marker of immune activation including in atherosclerosis (Sakakibara *et al*., 2015). The plasma ratio of kynurenine/tryptophan increased during systemic inflammation and contributed to vessel relaxation via the activation of adenylate and soluble guanylate cyclase pathways. However, hypotension was totally inhibited when the selective IDO inhibitor 1-methyl-D-tryptophan (1-Me-Trp) is administered. This observation proved the presence of IDO during systemic inflammation. This study by Wang *et al*. (2010) concluded that kynurenine is important for blood pressure homeostasis.

The potent effects of CA were previously verified *in vitro* on the endothelial cells. The authors cited the cell protective activity of CA on the endothelial cells (Abidin *et al*., 2016). Thus, *in vivo* antioxidant activity in G6-G8 perhaps improved the TAR condition thus vasodilation was observable during organ bath experiment. The compound found in CA executed the activity as ROS scavenger to minimize cell damage due to high cholesterol. Antioxidant properties are crucial to determine the well-being of the cell from further damage. LDL-oxidation will cause inflammation in

the cell. Since integrity test was conducted on the rats orally fed with CA/simvastatin, thus antioxidant contribution in cell viability during organ bath experiment is crucial.

TAR of treatment groups G3, G4 and G5 groups failed to show any responses towards Ach at any concentration. These observations actually tally with the previous findings. Among the most cited paper on vasomotion response of hypercholesterolemic aortic ring towards Ach was the research done by Anderson *et al*. (1995). They emphasized that the attenuation instead of full recovery of endothelium may be observed in the patients with minor hypercholesterolemia. In 1999, Kinlay and Plutzky summarized that the vasomotor function can be improved with statins therapy for at least six months to one year for endothelium-dependent vasodilation of acetylcholine to be observed. A research work by Vita *el al*. (2000) demonstrated no significant vasodilation responses towards Ach by coronary artery after the hypercholesterolemic patients were treated with cholesterol-lowering therapy for six months. They concluded that a more prolonged duration of the therapy and patients with minor elevation of cholesterol level should be taken into account for endothelium recovery observations.

Another work by Iraculis *et al.* (2001) also claimed that a positive response of left anterior descending coronary artery towards Ach was observed when the lipid level reduced which has taken almost 2 years of observations on the hypercholesterolemic patients. Besides, a 2 years therapy with atorvastatin on atherosclerotic patients by Yonemura *et al*. (2009) concluded an attenuation of vessel area reduction which also involved a very long duration of observation. These few previous researches strongly suggest the need of long-term treatment to correct or rectify cholesterol-related diseases.

4.4 Histopathology Studies

4.4.1 Aorta

Microscopic observation was conducted on the prepared slides using Leica microscope system. The morphology of aorta and liver were observed and differences were highlighted. Figure 4.4a showed the cross section of aortic tissue segment belonged to G1 group. The aorta layers; tunica intimae (TI), tunica media (TM) and tunica adventitia (TA) were observable and structured well. No abnormalities were observed and it was used as comparison for other groups (G2-G8). Figure 4.4 (b-c) are TAR of G2 group. TM thickening with fatty streak and fat deposits were found to be visible along the tunica intima. The findings were as reported by researchers. Ahmadi *et al.* (2017) reported the observations of foam cells with irregular TI in the histopathological image of rats' aorta. Besides, the damaged endothelial layer also strengthens the previous observations in organ bath study as hypercholesterolemic aortas failed to respond to Ach.

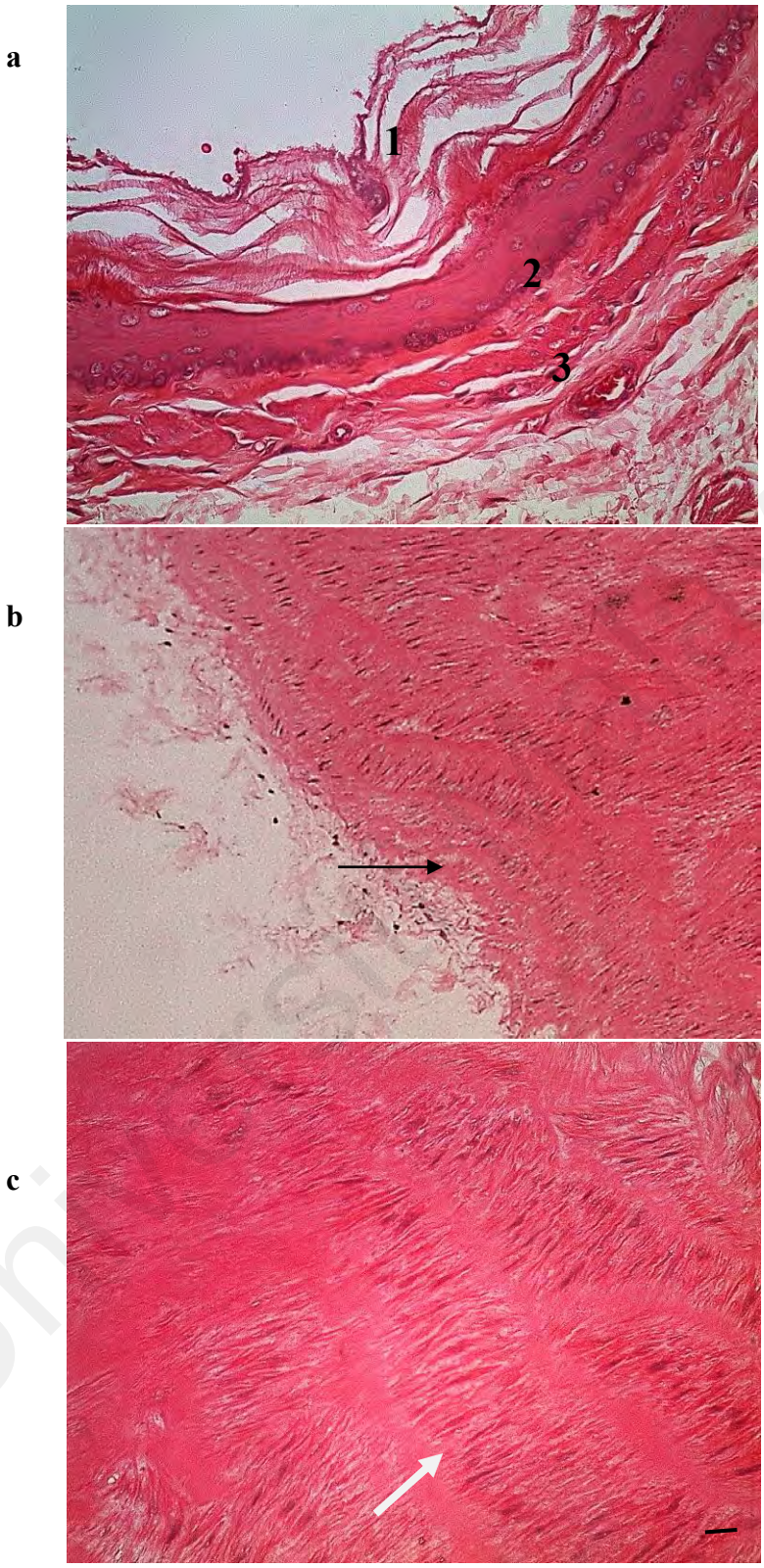


Figure 4.4: Cross section of aorta (a) G1 group (1- endothelium (tunica intima); 2- tunica media; 3- tunica adventitia); (b and c) G2 group (black arrow: tunica intimae with foam cells; white arrow: fibrous tissues migration from tunica media to tunica intima (magnification: 40x) (scale bar: 200 µm).

TM in TAR of treatment groups G3 to G5 were thicken with fatty streak observed on TI (Figure 4.5a-c). Previous reports by Dianita *et al*. (2016) and Ahmadi *et al*. (2017) have described the impact of hypercholesterolemia and followed-up treatment on the treated rats. Thickening of tunica media was reported in their studies. Dianita *et al*. (2016) has also indicated the changes observed including thickening of TM as early stage of atheroma lesion. The widening of TM due to smooth muscle cells migration from TA to TM also contributed to TM widening (Figure 4.5a-c). The short-duration treatment has shown a minor improvement in terms of the aortic structure compared to TAR of hypercholesterolemia group G2. However, the minor improvement observed did not result in endothelium recovery as the TAR failed to respond when challenged with Ach in organ bath study.

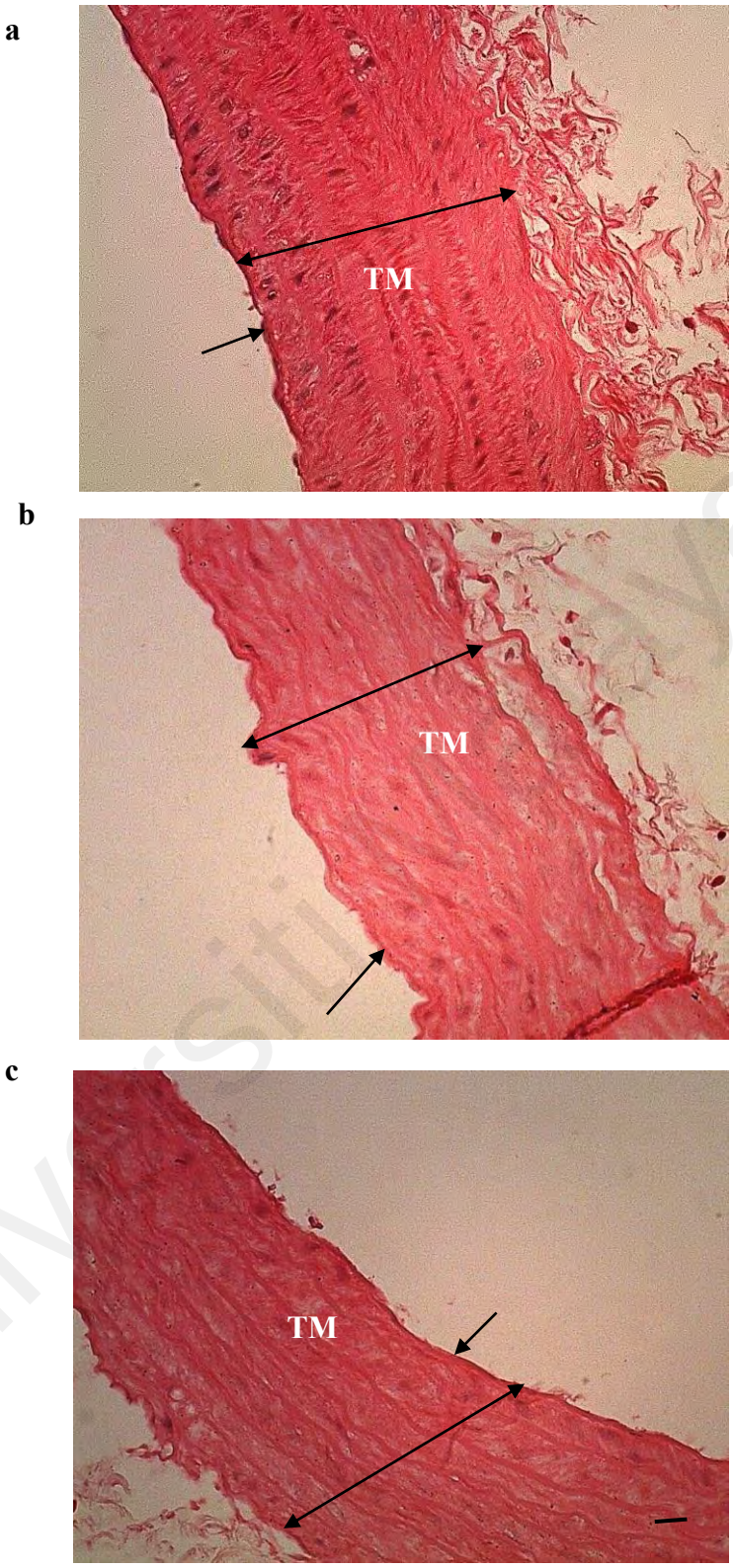


Figure 4.5: Cross section of aorta (a) G3 black arrows in (a) G3 (b) G4 (c) G5 showed TM thickening of TM (⟷) Widening of TM (Magnification: 40x) (scale bar: 200 µm).

The TAR of prevention groups G6 to G8 also showed improvement in their structures but the thickening of TM is still observable compared to Figure 4.4a. However, the diameter of the TM was smaller compared to Figure 4.5a-c. Rats in the prevention group had the aorta partially protected as both cholesterol and mushroom/simvastatin were fed concomitantly. The observations via organ bath study also revealed TAR viability by responding to Ach when cumulatively added to the organ bath chamber. Kamesh and Sumathi (2012) also reported the similar observation when the induced rats' aorta shown a thickening and the aorta condition improved when the rat was treated with selected extract. The aorta has shown a mild thickening and lesser foam cells count. Thus, the observations tally with previous finding and this suggest that CA was able to protect from further deterioration of the cell conditions.

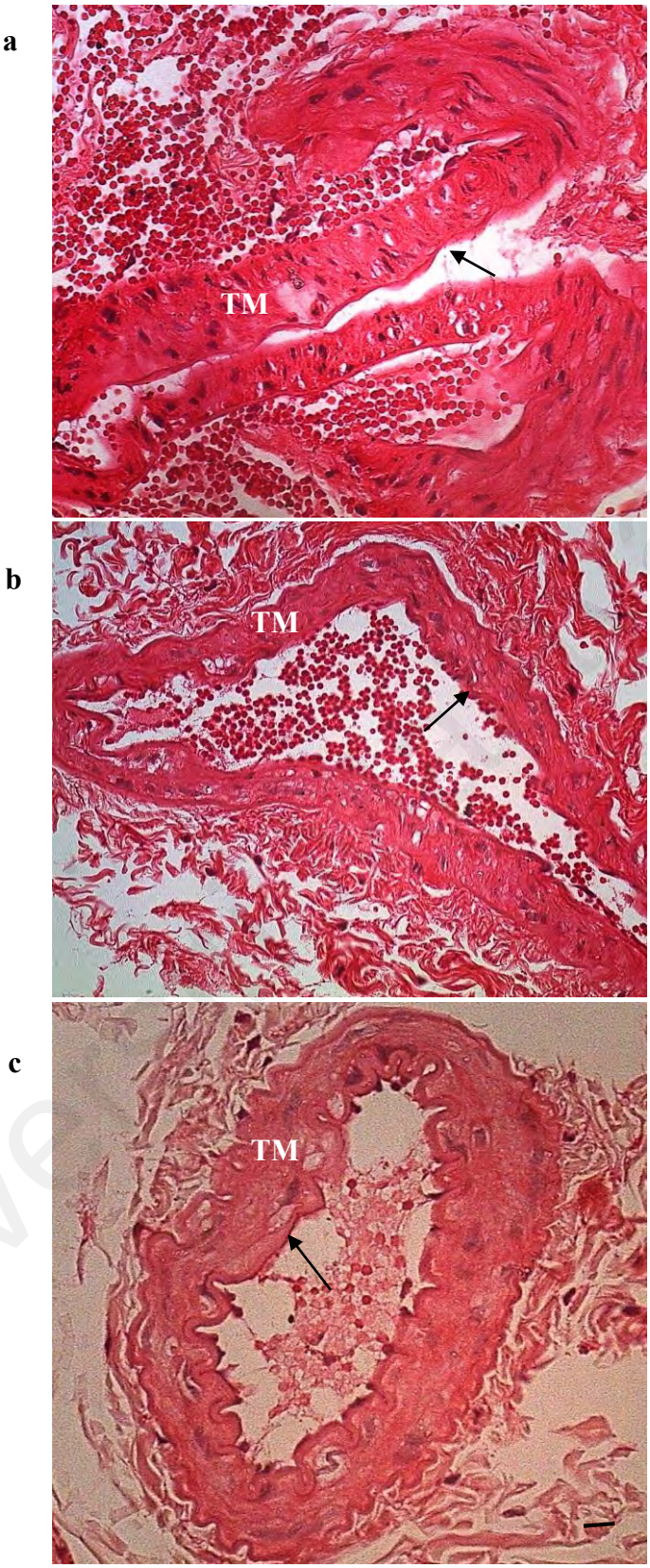
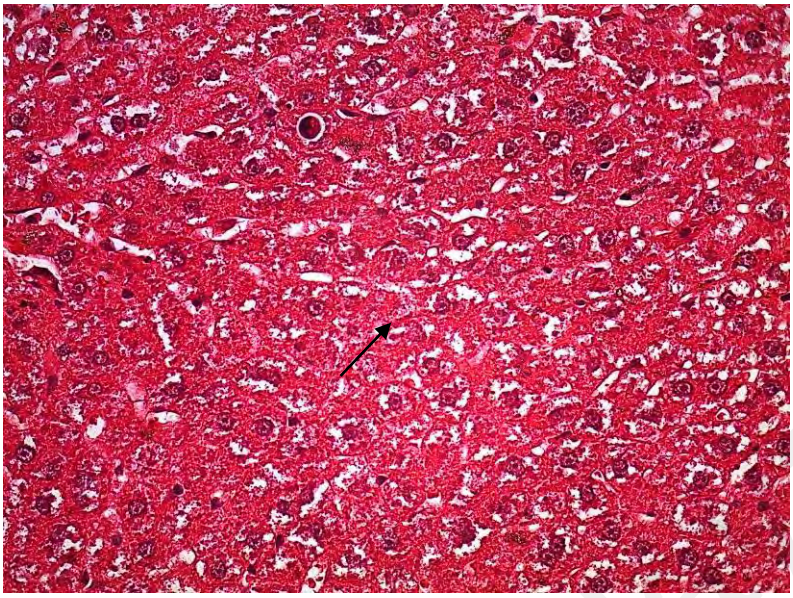


Figure 4.6: Cross section of aorta (a: G6 group, b: G7 group, c: G8 group). Tunica media (TM) shows an enlargement in these 3 groups compared to normal (G1). The black arrows in all the images show the foam cell accumulation (magnification: 40x) (scale bar: 200 µm).

4.4.2 Liver

The cross-section of liver tissues segment showed the differences between both normal group G1 and hypercholesterolemia group G2. Liver tissue in Figure 4.7a is normal with tangible nucleus, intact with cells and no inflammation observed whereas Figure 4.8b showed the presence of numerous lipid vacuoles which confirmed the condition of hypercholesterolemia in G2 group. Makni *et al*. (2008) and Alam *et al*. (2011) also reported their observations of lipid vacuoles in hypercholesterolemic liver tissues due to cholesterol accumulation in cytoplasm. Excessive ingested cholesterol had caused imbalance between free radicals' activation and antioxidant defence system. Oxidized LDL had triggered the expression of scavenger receptor on the macrophage thus causes atheroma or the formation of lipid vacuoles in hepatic cells. Besides, imbalance in cholesterol homeostasis contributed to lipid vacuoles formation in the hepatic cells (Gluchowski *et al*., 2017). In addition, Beighs *et al*. (2013) also reported the inflamed hepatic cells due to LDL oxidation is linked with lipid trapping in lysosomes.

a



b

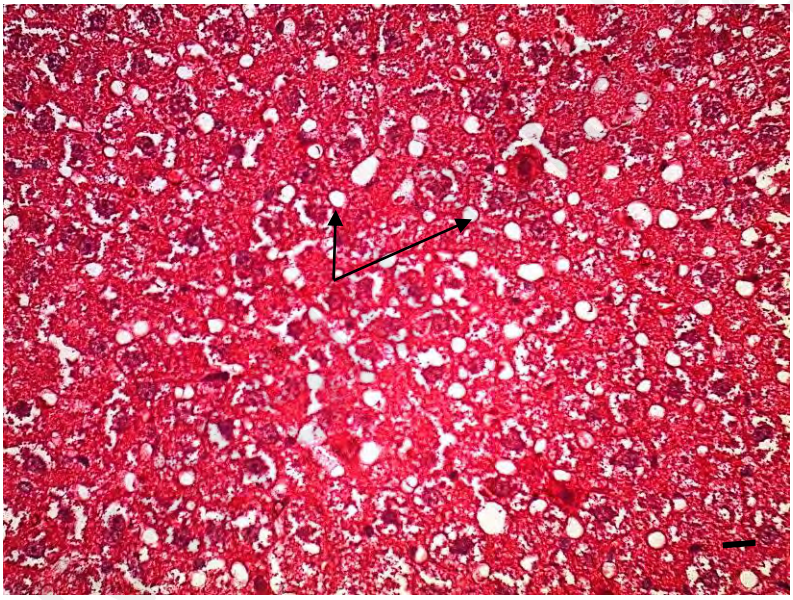


Figure 4.7: Cross section of the liver (a) G1 group (black arrow shows the normal nucleus shape) (b) G2 group (black arrows show the lipid vacuoles existed in the liver tissues) (magnification: 40x) (scale bar: 200 µm).

The liver conditions for treatment groups G3, G4 and G5 groups were as shown in Figure 4.8 (a-c). The hepatic cells were likely hypercholesterolemic hepatic cells (G2 group). Lipid vacuoles were observable with necrotic hepatocytes were seen in these figures.

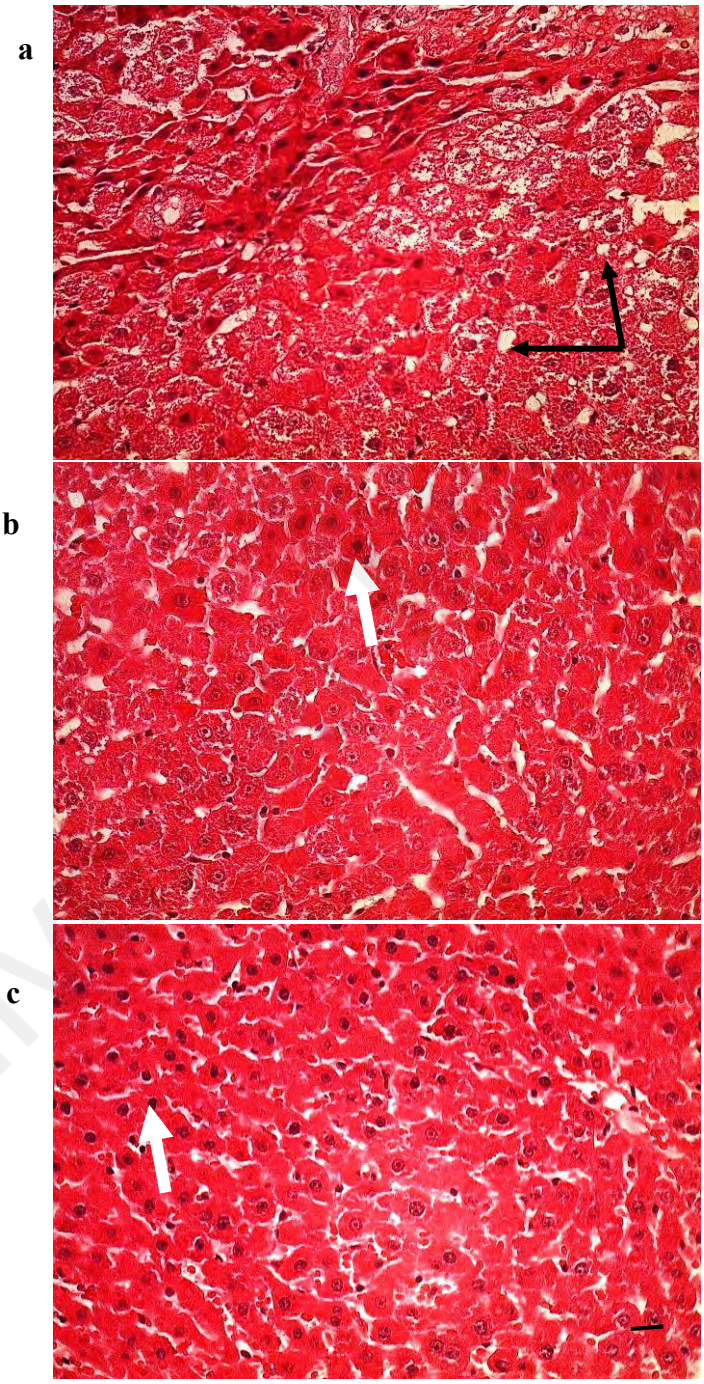


Figure 4.8: Liver cross section (a: G3 group, b: G4 group, c: G5 group). The white arrows in all the images are hepatocellular necrosis and black arrows are pointing at lipid vacuoles (magnification: 40x) (scale bar: 200 µm).

However, the conditions of the hepatic cells improved in Figure 4.9 (a-c). Alam *et al.* (2011) also claimed *Pleurotus eringii* increased the cells viability when it was simultaneously fed with cholesterol to the rats. Besides lowering the serum total cholesterol in rats, *P. eryngii* was also found to protect the hepatic cells from further damage when compared with hepatic cells belonged to hypercholesterolemic rats. The same observation was published previously by Wresdiyati *et al*. (2008). They found lipid vacuoles were vastly distributed in hypercholesterolemic rats' liver tissue and less lipid vacuoles were found in liver cells in rats fed with cholesterol and 5 % seaweed powder. The liver cells almost resembled to normal liver cells in rats fed with cholesterol and 10 % of seaweed powder. In our study, Figure 4.9b and c showed better cells viability compared to Figure 4.9a. CA 2.0 g/kg body weight and simvastatin had the ability to protect the cells compared to CA .0.5 g/kg body weight.

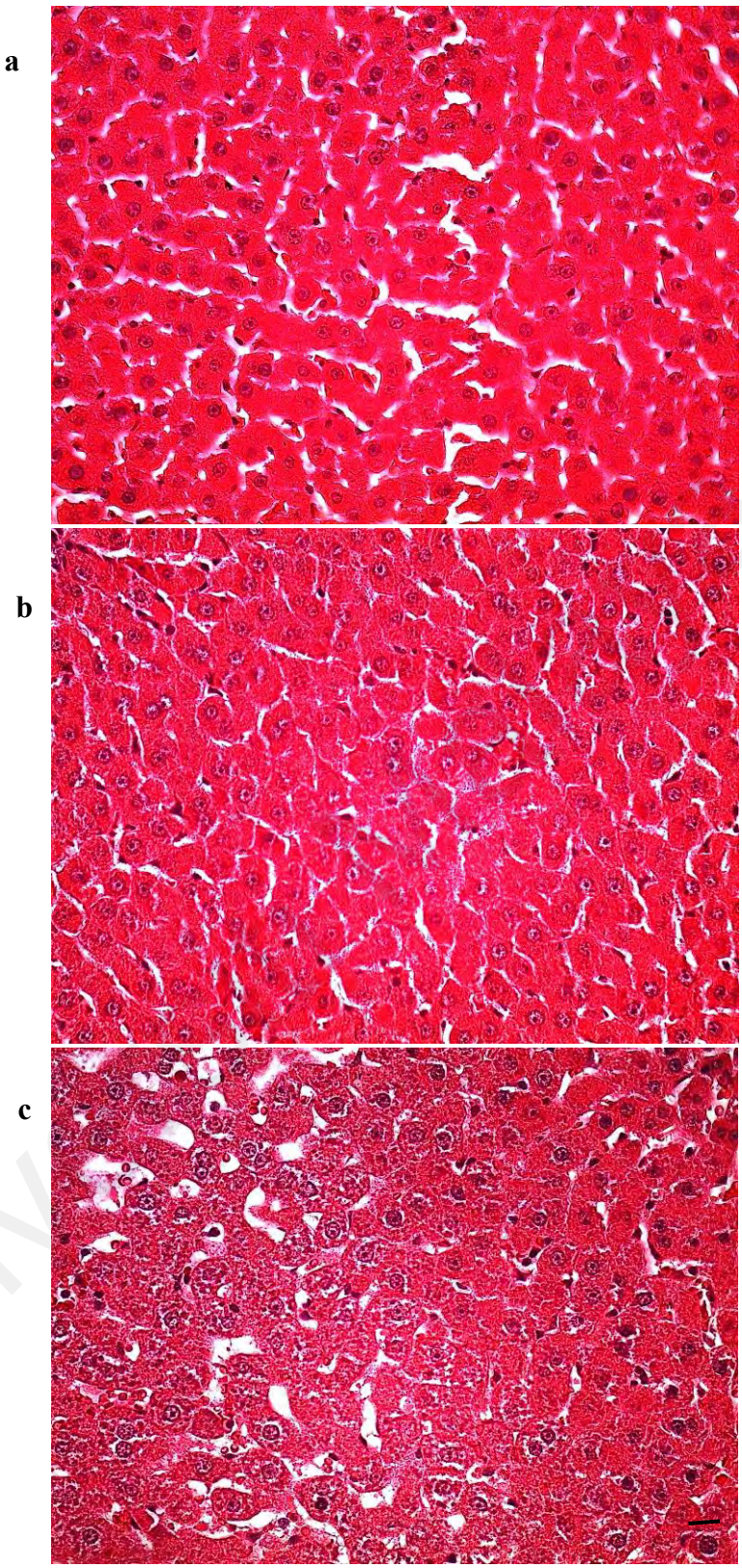


Figure 4.9: Liver cross section (a: G6 group; b: G7 group, c: G8 group). (a) Hepatocytes cells were necrotic (b-c) The nuclei of cells were intact and no major inflammations were observed (magnification: 40x) (scale bar: 200 µm).

All the published studies on hypercholesterolemic liver histopathology focused on the extract activity when simultaneously fed with cholesterol to the rats. To this date, no comparison works between treatment and prevention have been reported using CA extracted from *P. pulmonarius* on hypercholesterolemic-induced rats. Thus, this finding is believed to be novel. Furthermore, the findings in liver histopathology were in accordance with previous results of TAR performance in organ bath and aorta histopathology in this study.

4.5 Metabolomic Analysis

Metabolomic is one of the –omic science that also includes genomics, transcriptomics, and proteomics. These disciplines provide a holistic approach of molecular field commences from a single cell till the complex organisms. Metabolomic studies the metabolites secreted via metabolic biochemical processes in one's biological system. While other disciplines have been explored further, metabolomic is recognized as the current state of the studied model. The low molecular weight molecules (<1500 Da) are of interest in metabolomic observations (Kordalewska & Markuszewski, 2015).

This evolved technology has also facilitated cardiovascular disease research in many ways including to identify the pathways involved in the disease progression. Targeted and untargeted methods are two channels applied in metabolites identification. However, untargeted method is preferred compared to targeted method to avoid bias in metabolites profiling. Untargeted method assists in identifying the complete set of metabolites in the sample of interest. The increased value of certain metabolites in a sample compared to control probably involves in disease progression. Thus, that

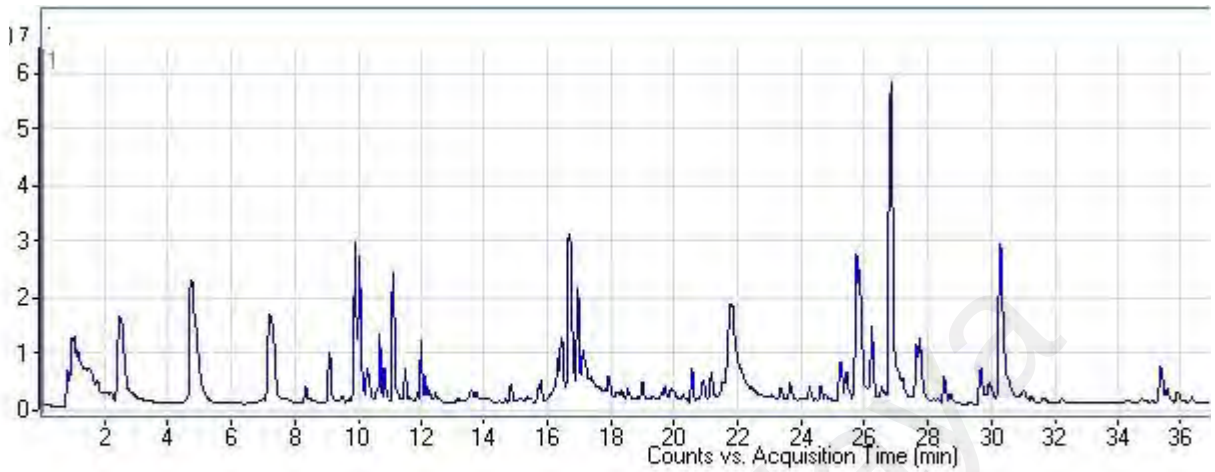
particular metabolite could be the target for treatment to reduce the disease risk and pathogenesis (Rhee & Gerszten, 2012).

Kordalewska and Markuszewski (2015) have published a compiled data on metabolites altered in almost all disorders underlying CVD risks. Atherosclerosis was found to alter many metabolites including purine, pyrimidine and ceramides. The method applied was untargeted on analyzed plaque tissue sample. Another disorder captured in the journal was hyperlipidemia from coronary artery disease with altered tyrosine and creatinine in rats' plasma.

The terms used in this study for altered metabolites were either up-regulated or down-regulated for the purpose to specify and link the altered metabolite with their value compared to control. One of the subtle advantages is to identify the target metabolites for further treatment or analysis as summarized in Table 4.4. Prior to the summary, the analysis of the selected serum samples captured the complete metabolite profiles as shown in Figure 4.10 (a-b).

The sera for metabolomic analysis were selected base on the result obtained from aortic rings contractility and histopathology observation. A total of 938 metabolites were identified. Based on the generated KEGG code and pathways, 15 metabolites were selected as they were closely related to hypercholesterolemia. The pathways were identified by using KEGG Database platform (www.genome.jp/kegg/). In addition, MetaboAnalyst 4.0 platform (www.metaboanalyst.ca/) favoured in order to analyze the pathways impact through bubble map generated by the system (Figure 4.11).

A



B

Figure 4.10a: Chromatogram of 938 metabolites in rat sera identified through LC/TOF-MS (positive ion mode). A: normal group G1; B: hypercholesterolemic group G2. *Y*-axis represents counts; X-axis represents acquisition time (min).

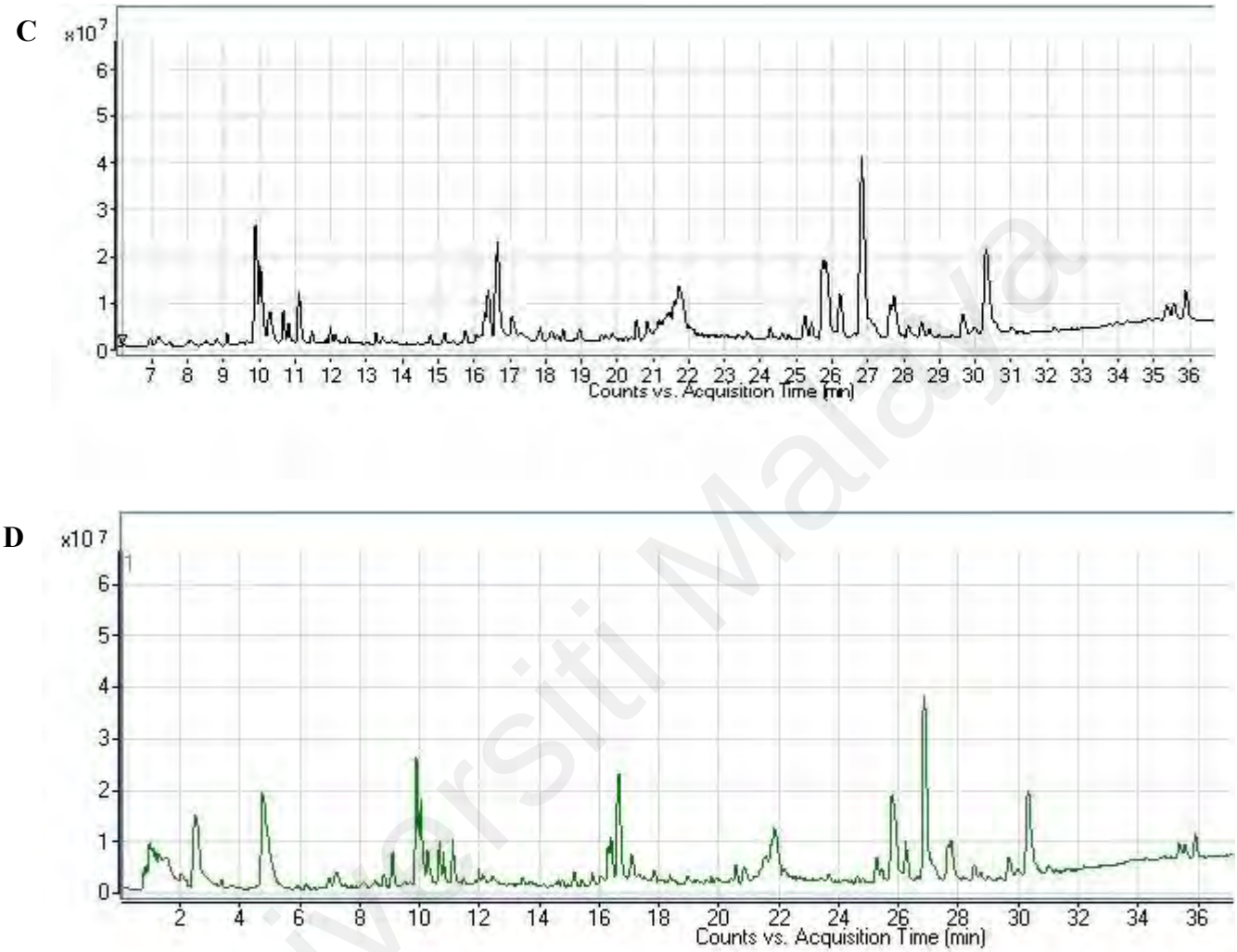
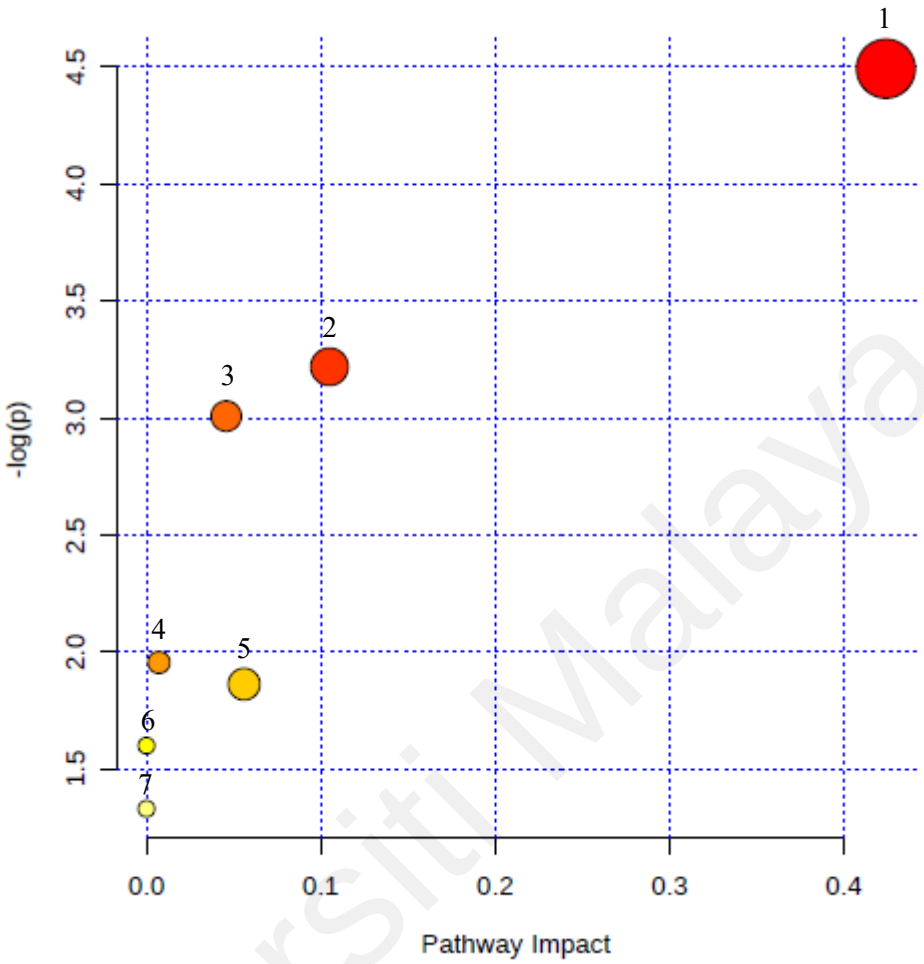


Figure 4.10b: Chromatogram of 938 metabolites in rat sera identified through LC/TOF-MS (positive ion mode). C: treatment group G4; D: prevention group G7. *Y*-axis represents counts; X-axis represents acquisition time (min).



4.11: Summary of pathways impact analysis relates to hypercholesterolemia (1) Sphingolipid metabolism (2) Tryptophan metabolism (3) Primary bile acid synthesis (4) Pantothenate and CoA biosynthesis (5) Beta-alanine metabolism (6) Glutathione metabolism (7) Arginine and proline metabolism (colour indication: light yellow – red: represents the severity of the hypercholesterolemia impact on the involved pathways in the analyzed rats' sera).

Table 4.4: Upregulation and downregulation of selected metabolites in three comparative groups; hypercholesterolemia group, prevention group (G7) and treatment group (G4).

Metabolites	KEGG ID	Log FC (cholesterol fed)	Log FC (prevention)	Log FC (treatment)	Retention time	p-value	Pathway
Pantothenic acid	C00864	-4.619034	6.4275074	-14.784496	2.7104	6.87E-09	beta-Alanine metabolism; Pantothenate and CoA biosynthesis
Ouabain	C01443	3.5537	0.208642	3.101871	10.9129	4.05E-07	Bile secretion
Glycocholic acid	C01921	0.053475887	0.20864195	16.48431	15.2556	9.15E-06	Bile secretion, Primary bile acid biosynthesis, Cholesterol metabolism
Taurochenodeoxycholic acid	C05465	7.477525	14.799491	14.9162655	11.8906	2.42E-04	Bile secretion, Primary bile acid biosynthesis, Cholesterol metabolism
Phosphodimethylethanolamine	C13482	-17.138626	0.30876923	-0.5167084	14.1146	4.96E-09	Glycerophospholipid metabolism
N-Carbamylglutamate	C05829	0.053475887	15.594496	0.08586981	11.2980	3.81E-39	Histidine metabolism
Chloranil	C18933	0.053475887	15.172274	3.6469731	0.7804	3.31E-07	Metabolomic pathway

Table 4.4, continued.

Serotonin	C00780	18.515701	0.56415176	0.65921974	2.0665	7.23E-07	Tryptophan metabolism
Spinganine	C00836	1.3905106	-17.831316	-13.973977	22.4375	1.993-05	Sphingolipid metabolism
Ceramide	C00195	11.493731	0.20864195	0.08586981	10.2018	2.53E-09	Sphingolipid signalling pathway
5-Hydroxyquinoline	C05639	-7.770853	8.027875	-7.738459	3.7964	3.10E-04	Tryptophan metabolism
N-Acetylisatin	C02172	0.053475887	8.633935	0.08586981	6.3745	5.53E-06	Tryptophan metabolism
L-Formylkynurenine	C02700	17.905008	0.20864195	0.08586981	8.8158	0	Tryptophan metabolism
L-Arginine phosphate	C05945	0.20864195	3.9286823	0.08586981	3.7658	5.83E-04	Arginine and proline metabolism
Spermine	C00750	0.20864195	15.424756	0.08586981	0.8090	3.53E-04	Arginine and proline metabolism

Fifteen metabolites were selected based on the two platforms; KEGG ID & MetaboAnalyst 4.0

A few pathways were found to be involved in cholesterol regulation when comparison was done on the metabolites profiling of the observed rat groups (G2, G4 and G7). Fifteen identified metabolites were involved in these pathways and the sphingolipid metabolism pathway found to be highly impacted in term of severity due to cholesterol dysregulation. Spinganine is closely linked to cholesterol dysregulation thus the pathway was highly interrupted. Other pathways involved were tryptophan metabolism, primary bile acid synthesis, pantothenate/CoA biosynthesis, beta-alanine metabolism, glutathione metabolism and arginine/proline metabolism. The pathways involved were summarized in the bubble map as shown in Figure 4.11. The colour coding of the bubbles referring to the severity impact on the pathways due to cholesterol dysregulation.

In this study, pantothenic acid (PA) involved in beta-alanine metabolism and it was highly secreted in the prevention group (G7) compared to hypercholesterolemic group G2 and treatment groups G4 (Table 4.4). Pantothenic acid may preserve the cells viability from further damage thus it was highly secreted in prevention group G7 compared to treatment group G4. Pantothenic acid (PA) is one of the building blocks of coenzyme A and actively involves in CoA biosynthesis (Figure 4.11). It belongs to vitamin B group. PA and its derivatives have been found to beneficially contribute in many ways especially their protective effects against ROS injury (Wojtczok *et al*., 2003).

In addition, Wojtczok *et al*. (2003) run experimental observations using Ehrlich ascites tumour cells. The cells were exposed to lipid peroxidation by ROS. Later, the cells were incubated with pantothenic acid and concentration-dependent protection

against further damage to the plasma was observed. Its protective effects were also observed in pantothenic derivatives such as pantothenol and pantethine (a thiol-containing derivative of pantothenic acid). This partial protection had indicated that PA is not a ROS scavenger, otherwise as a partial protector from further detrimental impact on the cells. Previously, in 1985, Bon *et al*. has shown that one of the pantothenic acid derivatives, pantethine was able to execute its role as antioxidant by inhibiting LDL peroxidation *in vitro*.

Ouabain is involved in bile secretion pathway (Figure 4.11) and found to be up-regulated in all the analyzed rats' sera. However, the up-regulation was higher in hypercholesterolemic group G2 and treatment group G4 (Table 4.4) as the cholesterol level in these sera sample exceeded normal value range. Besides, ouabain is a type of endogenous hormone which plays its role by halting the Na+ pump thus contributes to intracellular increased of Na+ concentration. This inhibition leads to interrupted $Na+/Ca^{2+}$ exchange in plasma membrane which later elevates Ca^{2+} in vascular smooth muscle cell, vasomotor neurons and endothelial cells. Due to its high concentration in these cells, the cells become highly sensitive and responsive to cytoplasm transient. Thus, the cells easily contract and increase the tension especially in blood vessels (Hamlyn and Blaustein, 2016). Previous researches reported that administration of ouabain to normal rat was found to be able to induce hypertension and another observation also revealed that ouabain level was increased in hypertensive human adults (Manunta *et al*., 1994; Rossi *et al*., 1995).

Besides, taurochenodeoxycholic acid was another compound found to be part of the three pathways; bile secretion, primary bile secretion and cholesterol metabolism

(Figure 4.11). It is highly up-regulated in all the analyzed rats' sera. As explained above for ouabain, taurochenodeoxycholic acid is also another type of bile acid; a conjugated primary bile acid of chenodeoxycholic acid with taurine during enterohepatic circulation of bile acid. Chenodeoxycholic acid has more binding affinity towards taurine instead of glycine. Homeostasis will automatically take place when cholesterol level increased above normal as the cholesterol is the precursor for bile acid synthesis (Murakami *et al*., 2002; Feher, 2012). Thus, taurochenodeoxycholic acid contributes in increased bile acid flow and lipid solubility (Table 4.4).

Although chenodeoxycholic acid has more preference towards taurine, it is unequivocal that cholic acid; another primary bile acid has its preference towards glycine to form glycocholic acid. Glycocholic acid is also formed during enterohepatic bile acid circulation which plays a role to solubilise fat (Park *et al*., 1999; Feher, 2012). Although glycocholic acid is up-regulated in all the analysed sera, it was highly detected in G4. It probably due to the increment of serum total cholesterol and followed by the treatment with either CA or simvastatin. The homeostasis perhaps highly took place to break down cholesterol into bile acid thus spike its level in treatment group G4 (Table 4.4).

Phosphodimethylethanolamine is another metabolite which was down-regulated in hypercholesterolemic group G2 and treatment group G4 compared to control rats' sera (Table 4.4). It is a type of extensive phospholipids in mammal heart tissues. It has lipotropic properties; a beneficial compound to catalyse fat to generate energy for body use. Phosphodimethylethanolamine is synthesized *de novo* through CDP-ethanolamine as it is a major component in heart tissue (Ansell & Spanner, 1982). Another study also

detected the presence of Phosphodimethylethanolamine via observation of radioactivity. Thus, it was upregulated in the serum of prevention group (P1) probably as it was actively involved in fat catalysis to form energy (McMaster *et al*., 1992).

N-carbamylglutamate (NCG) and L-arginine (Arg) were to be part of the histidine metabolism and arginine proline metabolism, respectively. Both metabolites were up-regulated in all the analysed sera. However, the up-regulation in prevention group G7 sera sample is significantly higher compared to others (Table 4.5). Previous findings have reported the ability of these metabolites to play the role as anti-oxidant and ROS scavenger. Perhaps these roles were highly executed in prevention group G7 as part of the cell repair and survival. Real–time PCR analysis on total RNA in rats' spleen revealed that NCG and Arg were able to upregulate the expression of some of the antioxidant genes including superoxide dismutase (SOD), glutathione peroxidase 1 (GPx1), glutathione reductase (GR) and catalase activities. These enzymatic antioxidants are ROS scavengers, alleviate the free radicals' activities and subsequently mitigate the cells damage (Mo *et al*., 2018).

A previous study by Xiao *et al*. (2016) also found the ability of both NCG and Arg in promoting antioxidant activity. Malondialdehyde (MDA), a marker of oxidative stress was evaluated and it was found that supplementation with 0.1 % NCG and 1% Arg, 0.1% NCG, managed to mitigate the MDA content in rat jejunum. The catalase activity was upregulated by both NCG and Arg thus assisted in increased antioxidant activity. Hydroxyl radicals are converted into oxygen and water as part of the antioxidant defence mechanism. In accordance with above mentioned previous researches, both metabolites were found to be up-regulated in prevention group G7. It

shows that these metabolites are highly excreted in the prevention group thus probably increased the rats' overall viability.

In addition, Arg is also known as the precursor for nitric oxide (NO) synthesis. As explained previously, NO is crucial for vasodilation of the aorta. Besides of its important role in vessel relaxation, Arg is famed as angiotensin-converting enzyme inhibitor thus modulates blood pressure. In this study, the metabolomic analysis observed the up-regulation of Arg in hypercholesterolemic group as well. Tousoulis *et al*. (2002) reported that Arg concentration will increase as well in order to reduce the oxidative stress by interacting with H_2O_2. The concentration of H_2O_2 is usually increased due to cytokinine activation which usually occurs when atheroma plaque is present as part of the auto-recovery process.

Chloranil is a type of quinine which is also known as tetrachloro-1,4-benzoquinone. They are basically found in animals and plants. It poses antioxidant activity besides anti-inflammatory activities too as reported by Dandawate *et al.* (2010) in their review. It may execute the same activities in the studied metabolomic in serum as it was up-regulated in the serum of prevention group G7 and involved in general metabolomic pathway. Basically, the main focus for this particular compound in previous researches was on its function and structure chemically. Thus, more observations are needed for chloranil biological functions.

Serotonin was detected as part of the tryptophan metabolism and up-regulated in hypercholesterolemia group G2 (Table 4.4 and Table 4.5). Serotonin is basically stored in platelets and its stimulation due to vascular lesion and during vasoconstriction phase,

stimulates the secretion of serotonin from the platelets. The increment of serotonin level in the blood is one of the biomarkers associated with cardiovascular risk. It contributes to development and progression of atherosclerotic plaques and also linked to endothelial damage in blood vessels. Ratio platelet-poor plasma (PPP) and whole blood (WB) represents the amount of serotonin released in the bloodstream as PPP values increases proportionate with atherosclerotic stage and age. In this study, it was found that serotonin was up-regulated in hypercholesterolemia group G2 compared to other groups. The escalated increment of serotonin in this particular group is in accordance with previous findings which have indicated that in the endothelial dysfunction, serotonin exerts thrombus formation and causes vasoconstriction; subsequently increases the risk of atherosclerosis (Figueras *et al*., 2005; Hara *et al*., 2004; Sugiura *et al*., 2016).

Spingolipids and its metabolism pathway are vital for cell protection and cellular signals transmission however; it also contributes to pathogenesis of major cardiovascular risk including hypercholesterolemia. An observation on spinganine; one of the compounds in the pathway, found the hamsters fed induced with cholesterol showed an increment in hepatic spinganine level. In accordance with this finding, spinganine was detected to be up-regulated in hypercholesterolemia group G2 but down-regulated in both treatment group G4 (Table 4.4) and prevention group G7 (Table 4.5) (Dekker *et al*., 2013).

Another metabolite namely ceramide is involved in sphingolipid pathway and found to be up-regulated in all the analysed sera. The value was found to be highest in hypercholesterolemia group G2. Ceramide belongs to sphingolipid family of lipids. It is

crucial as cholesterol especially for cell regulation. However, it accrued presence in atherosclerotic plaque does increase the risk of myocardial infarction. In a study on coronary arterial disease patients, revealed the high concentration of ceramide in their bloodstream. The detection of serum ceramide in a healthy population pool also affiliated with future hidden cardiac event as ceramide can triggers lipoprotein accumulation in blood vessel. Thus, probably due to its contribution in increasing the risk, it was highly up-regulated in hypercholesterolemia group G2. Perhaps, the increment in treatment group G4 (Table 4.4) and prevention group G7 (Table 4.5) were much lower compared to hypercholesterolemia group G2 as these groups were introduced with either CA or simvastatin during the observations period. In addition, some participants established a serum ceramide with insulin resistance. Insulin resistance is modulated by ceramide thus increased the cardiac pathogenesis (Siskind *et al*., 2010; Summers, 2018).

Isatin (1*H*-indole-2,3-dione) which is also part of tryptophan metabolism was highly up-regulated in prevention group G7. Isatin is synthesized endogenously via tryptophan pathway. Isatin is widely known as small and potent metabolites with tremendous benefits pharmacologically and its derivatives are synthesized for various medical applications such as antioxidant, antitumor, antimicrobial, anti-inflammatory, analgesic, anti-mycobacterial, anticonvulsant, antiviral, anthelmintic, anti-HIV and CNS depressant activities. N-acetyl isatin which was found in rats' sera in this study mainly used as substrate in synthesizing isatin derivatives (Silva, 2013; Grewal, 2014; Souza & Chattree, 2015). N-acetyl Isatin was up-regulated in serum of all the groups (G2, G4 and G7) however the highest up-regulation was found for prevention group G7 as the rats were simultaneously fed with CA extract and induced with hypercholesterolemia.

Isatin and its derivatives possess chelating activity when evaluate using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and hydrogen peroxide (H_2O_2) (Grewal, 2014).

Kynurenine is highly up-regulated in hypercholesterolemia group G2 compared to others and it is involved in tryptophan pathway. Further breakdown of tryptophan produces kynurenine; by the action of enzymes tryptophan dioxygenase and indoleamine 2,3-dioxygenase. In a study by Wirleitner *et al*. (2003) discovered the increased kynurenine and tryptophan ratio (kyn trp^{-1}) in the patients with coronary heart diseases. Besides, Wang *et al*. (2016) also reported L-3-Hydroxy- kynurenine can cause oxidative injury to the cells as it is known as ROS generator. Another compound under the same pathway was 5-hydroxyquinoline which was down-regulated in hypercholesterolemic group G2. Little is known about this particular compound especially it was mentioned as lipophilic chelator (Deslauriers *et al*., 1986). Its role is more common in chemistry reactions. It was discussed to be crucial for cytoprotection thus, 5-hydroxyquinoline may execute the same activity as 8-hydroxyquinoline as metal chelator and protect the cells from further damage due to ROS. Perhaps it was highly upregulated in prevention group G7 as ROS scavenger (Kanizsai *et al*., 2018). The cytoprotection properties perhaps unable to be executed in G4 due to insufficient period for recovery during the experiment.

Polyamines are biologically crucial for cell cycle, scavenger of ROS and also for anti-inflammation. Spermine as one of the polyamines, has the capability to reduce oxidative stress in cells by scavenging ROS and was found to be up-regulated in G7 group. It might be one of the defence systems activated to maintain the cell from further damage. Treatment group G4 was also upregulated however the value was so close to

hypercholesterolemia group G2. Spontaneous activation of the cell defence system might take place against the ROS activity. Arginine and proline metabolism was the pathway involved by this particular metabolite. Lipid peroxidation thus can be reduced and alleviate MDA besides protecting the cells from further damage. MDA is one of the biomarkers of oxidative stress (Zhang *et al*., 2017). In addition, spermine was found to augment catalases activity in liver and spleen in piglet as reported by Wu *et al*. (2017). Catalase is a type of enzyme responsible to neutralise hydrogen peroxide. Overall, antioxidant activity will be enhanced with the presence of spermine.

From these observations, many metabolites are up-regulated for the cholesterol breakdown purpose in these three groups of hypercholesterolemia (G2), treatment (G4) and prevention group (G7). However, the metabolites that involve in cells viability and survival are highly up-regulated in prevention group G7 only such as pantothenic acid, N-carbamylglutamate, chloranil and spermine. Although N-carbamylglutamate, chloranil and spermine were also up-regulated in hypercholesterolemia group G2 and treatment group G4, perhaps as part of the self-recovery system, the upregulation is not as significant as prevention group G7. Thus, it can be concluded prevention was found to be better compared to treatment based on the observed metabolomic result.

CHAPTER 5: CONCLUSION

5.1 Conclusion

Nutraceutical has become a popular theme in food science which refers to the functional food execution on its ability for cell recovery and viability due to disease pathogenicity. The concern towards 'green life' has become more crucial and emphasized worldwide. As one of the functional food, mushroom has received overwhelming receptions among the world population.

Previous research has found ergothioniene, tryptophan and ellagic acid in CA extract from *Pleurotus pulmonarius*. These compounds have been proven to play a crucial role in endothelial cells viability through *in vitro* observations (Abidin *et al*. 2016). This research is an extended effort from previous research to further observe the attributes of the above-mentioned compounds on rat models. Acute toxicity of CA at the concentration up to 2000 mg/kg body weight on Wistar-Kyoto rat was also found to be safe for the rat consumption.

The observations through organ bath analysis, histopathology of hypercholesterolemic-induced rats and metabolomic analysis were found to be crucial to relate to the pathogenicity of hypercholesterolemia. In organ bath analysis, although hypercholesterolemic thoracic aortic ring failed to respond towards acetylcholine (Ach) after being challenged with phenylephrine (PE) during integrity test in organ bath analysis, CA and simvastatin managed to dilate the thoracic aortic rings when added to the Krebs-Heinseleit buffer solution in organ bath chamber. Besides, hypercholesterolemic rats fed with either CA (0.5 g/kg body weight and 2.0 g/kg body

weight) or simvastatin showed a reduction in serum total cholesterol level (< 3.3 mmol/L). However, the thoracic aortic rings isolated from these rats failed to response to Ach after being challenged with PE. The cross section of the aorta found a swollen tunica media and presence of fatty streak along the tunica intima. Virtually, the tissue has improved in its structure compared to hypercholesterolemic aortic cells which had fibrous cells migration from tunica adventitia to tunica media. However, the recovery did not increase its viability to response to Ach.

The rats fed with either CA and cholesterol or simvastatin and cholesterol simultaneously revealed a different pattern of reactions. The thoracic aortic ring responded to Ach by dilating after contracted with PE. CA performance was at par with commercial drug; simvastatin. Histopathology examination also showed a better structure of tunica intima, tunica media and tunica adventitia. Fatty streak was still observable but with a lesser enlargement of tunica media.

Metabolomic analysis of rats' sera was in accordance with the previous findings in this study. Many metabolites have been detected including pantothenic acid, ouabain, glycocholic acid, taurochenodeoxycholic acid, phosphodimethylethanolamine, N-carbamylglutamate, chloranil, serotonin, spinganine, eramide, 5-hydroxyquinoline, N-acetylisatin, L-formylkynurenine, L-arganine phosphate and spermine. The metabolites were involved in several pathways including bile secretion, primary bile acid biosynthesis, cholesterol metabolism and glycerophospholipid metabolism. A few metabolites were up-regulated in hypercholesterolemic rat sera such as serotonin, spinganine, ceramide and L-formylkynurenine whereas, pantothenic acid, phosphodimethylethanolamine and 5-hydroxyquinoline were down-regulated compared to control rat sera. Each of the metabolite plays its own role in pathogenesis chain;

either contribute in disease progression or improvise the viability of the rats' tissues. It can be concluded that CA is a potent extract with countless health benefits specifically to reduce cardiovascular risks and as shown in this study; hypercholesterolemia and hypertension.

Previous researches on organ bath focused solely the normal thoracic aortic rings responses towards extract of interest. None of the previous findings used a damaged thoracic ring as sample and run the integrity test on it. Thus, this study was the first to record the response of the damaged thoracic aortic ring towards Ach after being challenged with PE. Besides, in this study, the linked observation between tissue responses and histopathology was also found to be very crucial. Future work is highly needed to prolong the CA fed to rat in order to see the capability of the extract to repair the damaged thoracic aortic ring following hypercholesterolemic episode.

5.2 Limitation of the Study

The analysis of the metabolomics only till detection and the function of each metabolite was explained in this research base on the previous studies published in the journals and not observed in this study.

5.3 Future Perspective

Nitric Oxide excretion in all the groups can be analysed to further confirm the aortic ring damage in the rats. A further verification work on metabolites detected in the sera is highly recommended for future research.

REFERENCES

Abdullah, N., Ismail, S. M., Aminudin, N., Shuib, A. S. & Lau, B. F. (2012). Evaluation of selected culinary0medicinal mushrooms for antioxidant and ACE inhibitory activities. *Evidence-Based Complementary and Alternative Medicine*, *2012*, 1-12.

Abidin, M. H. Z, Abdullah, N., & Abidin, N. Z. (2016). Protective effect of antioxidant extracts from grey oyster mushroom, *Pleurotus pulmonarius* (Agaricomycetes) against human low-density lipoprotein oxidation and aortic endothelial cell damage. *International Journal of Medicinal Mushrooms*, *18(2),* 109-121.

Ačimovič, J., & Rozman, D. (2013). Steroidal triterpenes of cholesterol synthesis. *Molecules*, *18*, 4002-4017.

Adams, L. B. (2005). Chapter 10: Hyperlipidemia. In**:** Stang, J. & Story, M. (eds.). Guidelines for adolescent nutrition services. Minneapolis, MN: Center for Leadership, Education and Training in Maternal and Child Nutrition, Division of Epidemiology and Community Health, School of Public Health, University of Minnesota.

Abebayo, E. A., Oloke, J. K., Majolagbe, O. N., Ajani, R. A. & Bora, T. C. (2012). Antinicrobial and anti-inflammatory potential of polysaccharide from *Pleurotus pulmonarius* LAU 09. *African Journal of Microbiology Research*, *6(13)*, 3315-3323.

Ahmadi, K. G. S., Wulansari, A, Subroto, Y, & Estiasih, T. (2017). Protective effect of food products enriched with unsaponifiable matter from palm fatty acid distillate on the aorta of hypercholesterolemic rats. *Journal of Applied Pharmaceutical Science*, *7*, 90-96.

Alam, N, Yoon, K. N., Lee, J. S., Cho, H. J., Shim, M. J. & Lee, T. S. (2011). Dietary effect of *Pleurotus eryngii* on biochemical function and histology in hypercholesterolemic rats. *Saudi Journal of Biological Sciences, 18*, 403-409.

Amirullah, N. A., Abidin, N. Z. & Abdullah, N. (2018). The potential applications of mushrooms against some facet of atherosclerosis: A review. *Food Research International*, *105*, 517-536.

Anderson, T. J., Meredith, I.T., Yeung, A.C., Frei, B., Selwyn, A.P. & Ganz P. (1995). The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion. *The New England Journal of Medicine*, *332*, 488-493.

Ansell, G. B. & Spanner, S. (1982). Phosphotidylserine, phosphatidylethanolamine and phosphatidylcholine. In: Hawthorne, Ansell GB (Eds.). *Phospholipids*. Vol 4. 1st edition. Elsevier Inc.

Badole, S. L., Shah, S. N., Patel, N. M., Thakurdesai, P. A., & Badhankar, S. L. (2006). Hypoglycemic activity of aqueous extract of *Pleurotus pulmonarius* in alloxan-induced diabetic mice. *Pharmaceutical Biology*, *44*, 421-425.

Berkban, T., Boonpram, P., Bunbupha, S., Welbat, J., Kukongviriyapan, U., Kukongviriyapan, V., Pakdeechote, P. & Prachaney, P. (2015). Ellagic acid prevents L-NAME-induced hypertension via restoration of eNOS and $p47^{phax}$ expression in rats. *Nutrients*, *7*, 5265-5280.

Bieghs, V., Walenbergh, S. M. A., Hnedrikx, T., van Gorp, P. J., Verheyen, F., Damink, S. W. O., … Shiri-Sverdlov, R. (2013). Trapping of oxidized LDL in lysosomes of Kuffer cells I a trigger for hepatic inflammation. *Liver International*, *33(7)*, 1056-1061.

Blanche, E. O. C., Valĕre, K. T. C., Judith, M. M. A. & Anatole, P. C. (2019). Study of acute toxicity and the effect of the aqueous extract of a formulation of three edibles mushrooms on oxidative stress induced in rats. *World Journal of Food Science and Technology*, *3(1)*, 6-13.

Bon, G. B., Cazzalato, G., Zago, S. & Avogaro, P. (1985). Effects of patethine on in-vitro peroxidation of low density liporpoteins. *Atherosclerosis*, *57*, 99-106.

Boonsong, S., Wanwimol, K. W. & Pongtep, W. P. (2016). Antioxidant activities of extracts from five edible mushrooms using different extractants. *Agriculture and Natural Resources, 50*, 89-97.

Bruckert, E. & Rosenbaum, D. (2011). Lowering LDL-cholesterol through diet: potential role in the statin era. *Current Opinion in Lipidology, 22*, 43–48.

Carrasco-Gonzalez, J. A, Serna-Saldivar, S.O. & Gutiérrez-Uribe, J.A. (2017). Nutritional composition and nutraceutical properties of the Pleurotus fruiting bodies: Potential use as food ingredient. *Journal of Food Composition and Analysis, 58*, 69-81.

Chen, H., Li, S., Wang, P., Yan, S., Hu, L., Pan, X., Yang, C. & Leung, G. P. (2014). Endothelium-dependent and –independent relaxation of rat aorta induced by extract of *Schizophyllum commune*. *Phytomedicine*, *21*, 1230-1236.

Chen, S-Y., Ho, K-J., Hsieh, Y-J., Wang L-T. & Mau, J-L. (2012). Contents of lovastatin, γ-aminobutyric acid and ergothioneine in mushroom fruiting bodies and mycelia. *LWT-Food Science and Technology*, *47(2)*, 274-278.

Chiang, J. Y. L. (2004). Regulation of bile acid synthesis: pathways, nuclear receptors and mechanisms. *Journal of Hepatology*, *40*, 539-551.

Crane, J. M. & Tamm, L. K. (2004). Role of Cholesterol in the Formation and Nature of Lipid Rafts in Planar and Spherical Model Membranes. *Biophysical Journal, 86*, 2965–2979.

Correa, R. C. G., Brugnari, T., Bracht, A., Peralta, R.M. & Ferreira I. C. F. R. (2016). Biotechnological, nutritional and therapeutic uses of *Pleurotus* spp. (oyster mushroom) related with its chemical composition: A review on the past decade findings. *Trends in Food Science & Technology, 50*, 103-117.

Dandawate, P. R., Vyas, A. C., Padhye, S. B., Singh, M. W. & Baruah, J. B. (2010). *Mini-Reviews in Medicinal Chemistry*, *10*, 436-454.

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

de Miranda, A. M., Rossoni Jr, J. V., Souza, S. E. L., Dos Santos, R. C., Silva, M.E. & Pedrosa, M. L. (2016). *Agaricus brasiliensis* (sun mushroom) affects the expression of genes related to cholesterol homeostasis. *European Journal of Nutrition*, *56(4)*, 12 pages.

Deanfield, J.E., Halcox, J. P. & Rabelink, T. J. (2007). Endothelial function and dysfunction: testing and clinical relevance. *Circulation*, *115*, 1285-1295.

Deiana, M., Rosa, A., Casu, V. Piga, R. Assunta, D.M. & Aruoma, O. I. (2004). L-ergothioneine oxidative damage in the kidney and liver of rats in vivo: studies upon the profile of polyunsaturated fatty acids. *Clinical Nutrition*, *23*, 183-193.

Dekker, M. J., Baker, C., Naples, M., Samsoondar, J., Zhang, R., Qiu, W., Sacco, J. & Adeli, K. (2015). Inhibition of sphingolipid synthesis imporves dyslipidemia in the diet-induced hamster model of insulin resistance: evidence for the role of spingosine and sphinganine in hepatic VLDL-apoB100 overproduction. *Atherosclerosis*, *228*, 98-109.

Denecke, J. & Kranz, C. (2009). Hypoglycosylation due to dolichol metabolism defects. *Biochimica et Biophysica Acta, 1792*, 888-895.

Deslauriers, R., Moffatt, D. J. & Smith, I. C. (1986). Oxygen consumption *in Plasmodium berghei*-infected murine red cells; a direct spectrophotometric assay in intact erythrocytes. *Biochimica et Biophysica Acta*, *886*(3), 319-326.

Devipriya, N., Sudhher, A. R. & Menon, V. P. (2007). Dose–response effect of ellagic acid on circulatory antioxidants and lipids during alcohol-induced toxicity in experimental rats. *Fundamental in Clinical Pharmacology*, *21*, 621–630.

Dianita, R., Jantan, I., Jalil, J. & Amran, A. Z. (2016). Effects of *Labisia pumila* var *alata* extracts on the lipid profile, serum antioxidant status and abdominal aorta of high-cholesterol diet rats. *Phytomedicine*, *23*, 810-817.

Dikshit, P., Tyagi, M. K., Shukla, K., Gambhir, J. K. & Shukla, R. (2016). Antihypercholesterolemic and antioxidant effect of sterol rich methanol extract of stem of *Musa sapientum* (banana) in cholesterol fed Wistar rats. *Journal in Food Science & Technology*, *53(3)*, 1690-1697.

Feher, J. (2012). Chapter 8.4: Pancreatic and biliary secretion. In: Feher, J. (Ed.). *Quantitative Human Physiology: An Introduction*. 1st edition. Elsevier Inc. page 721-730.

Ferrier, K.E., Muhlmann, M. H., Baquet, J. P., Cameron, J.D. & Jennings, G. L. (2002). Intensive cholesterol reduction lowers blood pressure and large artery stiffness in isolated systolic hypertension. *Journal of the American College of Cardiology*, *39*, 1020-1025.

Figueras, J., Damingo, E., Cortadellas, J., Padilla, F., Darado, D. G., Segura, R., Galard, R. & Soler, J. S. (2005). Comparison of plasma serotonin level in patients with variant angina pectoris versus healed myocardial infarction. *American Journal of Cardiology, 96* (2), 204-207.

Fombang, E. N., Lobe, E. E. & Mbodunf, M. F. (2016). *Pleurotus florida* aqueous extracts and powder influence lipid profile and suppress weight gain in rat fed high cholesterol diet. *Journal of Nutrition and Food Science*, *6(2)*, 1-7.

Furchgott, R. F. & Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, *288*, 373-376.

Ghosson, H., Schwarzenberg, A. & Jamois, F. (2018). Simultaneous untargeted and targeted metabolomics profiling of underivatized primary metabolites in sulfur-deficient barley by ultra-high performance liquid chromatography-quadrupole/time-of-flight mass spectrometry. *Plant Methods*, *14(62)*, 1-17.

Gil-Ramirez, A., Caz, V., Smiderle, F. R., Martin-Hernandez, R., Largo, C., Tabernero, M., Marı´n, F. R., Iacomini, M., Reglero, G. & Soler-Rivas, C. (2016). Water-soluble compounds from *Lentinula edodes* influencing the HMG-CoA reductase activity and the expression of genes involved in the cholesterol metabolism. *Journal of Agricultural Food Chemistry, 6*, 1910-1920.

Gluchowski, N. L., Becuwe, M., Walther, T. C. & Farese Jr, R. V. (2017). Lipid droplets and liver disease: from basic biology to clinical implications. *Nature Gastroenterology & Hepatology*, *14(6)*, 343-355.

Graveline, D. (2015). Adverse effects of statin drugs: a physician patient's perspective. *Journal of American Physicians and Surgeons*, *20(1)*, 7-11.

Grewal, A. S. (2014). Isatin derivatives with several biological activities. *International Journal of Pharmaceutical Research*, *6*, 1-7.

Grouleff, J., Irudayam, S. J., Skeby, K. K. & Schiøtt, B. (2015). The influence of cholesterol on membrane protein structure, function, and dynamics studied by molecular dynamics simulations. *Biochimica et Biophysica Acta*, *1848*, 1783–1795.

Guillamón, E., García-Lafuente, A., Lozano, M., D´Arrigo, M., Rostagno, M. A.,Villares, A. & Martínez, J. A. (2010). Edible mushrooms: Role in the prevention of cardiovascular diseases. *Fitoterapia, 81*, 715–723.

Hamlyn, J. M. & Blaustein, M. P. (2016). Endogenous ouabain. *Hypertension*, *68*, 526-532.

Hara, K., Hirowatari, Y., Yoshika, M., Kamiyama, Y., Tsuka, Y. & Takahashi, H. (2004). The ratio of plasma to whole-blood serotonin may be a novel marker of atherosclerotic cardiovascular disease. *Journal Lab Clinical Medicine*, *144*, 31-37.

Hu, S. H., Liang, Z.C., Chia, Y. C., Lien, J. L., Chen, K. S., Lee, M. Y. & Wang, J. C. (2006). Antihyperlipidemic and antioxidant effects of extracts from *Pleurotus citrinopileatus*. *Journal of Agricultural and Food Chemistry*, *54(6)*, 2103-2110.

Ibadallah, B. X., Abdullah, N. & Shuib, A. S. (2015). Identification of angiotensin-converting enzyme inhibitory proteins from mycelium of *Pleurotus pulmonarius* (oyster mushroom). *Planta Medica*, *81*, 123-129.

Iraculis, E., Cequier, A., Sabate, M., Pinto, X., Antoni, G., Hospital, J, … Espluqas, E. (2001). Improvement of endothelial function in patients with hypercholesterolemia and normal coronary arteries with lipid-lowering therapy. *Revista Espanola de Cardiologia*, *54*, 685-692.

Jamal, R., Zakaria, S. Z. S., Kamaruddin, M. A., Jalal, N. A., Ismail, N., Kamil, N. M., … Mahadi, N. M. (2014). Cohort profile: The Malaysian cohort (TMC) project: aperspective study of non-communicable diseases in a multi-ethnic population. *International Journal of Epidemiology*, *44(2)*, 423-431.

Jayakumar, T., Thomas, P.A., Sheu, J. R. & Geraldine, P. (2011). *In-vitro* and *in-vivo* antioxidant effects of the oyster mushroom *Pleurotus ostreatus*. *Food Research International, 44*, 851–861

Jeong, S. C., Jeong, Y. T., Yang, B. K., Islam, R., Koyyalamudia, S. R., Panga, G., Choa, K. Y. & Song, C. H. (2010). White button mushroom (*Agaricus bisporus*) lowers blood glucose and cholesterol levels in diabetic and hypercholesterolemic rats. *Nutrition Research, 30*, 49–56.

Kamesh, V. & Sumathi, T. (2012). Antihypercholesterolemic effect of *Bacopa monniera* linn. on high cholesterol diet induced hypercholesterolemia in rats. *Asian Pacific Journal of Tropical Medicine, 5(12)*, 949-955.

Kanizsai I., Madácsi, R., Hackler Jr, L., Gyuris, M., Szebeni, G.J., Huzián, O. & Puskás, L. G. (2018). Synthesis and cytoprotective characterization of 8-Hydroxyquinoline Betti products. *Molecules*, *23*, 1934-1959.

Kessel, A., Ben-Tal, N. & May, S. (2001). Interactions of cholesterol with lipid bilayers: The preferred configuration and fluctuations. *Biophysical Journal*, *81*, 643–658.

Khatun, K., Mahtab, H., Khanam, P. A., Sayeed, M. A. & Khan, K. A. (2007). Oyster mushroom reduced blood glucose and cholesterol in diabetic subjects. *Mymensigh Medical Journal*, *16(1)*, 94-99.

Khatun, S., Aminul Islam, A., Cakilciogluc, U., Gulerd, P. & Chatterjee, N. C. (2015). Nutritional qualities and antioxidant activity of three edible oyster mushrooms (*Pleurotus* spp.). *NJAS - Wageningen Journal of Life Sciences*, *72–73*, 1–5.

Kim, J-a., Montagnani, M., Chandrasekran, S. & Quon, M. J. (2012). Role of lipotoxicity in endothelial dysfunction. *Heart Failure Clinic, 8(4)*, 589-607.

Kinlay, S., Plutzky, J. (1999). Effect of lipid–lowering therapy on vasomotion and endothelial function. *Current Cardiology Reports*, *1*, 238-243.

Koon, C. M., Fong, S., Wat, E., Wang, Y. P., Cheung, W. S. D., Lau, B. S. C., Leung, O. C., Sun, H. D., Zhao, Q. S., Fung, K. P. (2014). Mechanisms of the dilator action of the *Erigerontis herba* on rat aorta. *Journal of Ethnopharmology*, *155*, 1561-1567.

Korade, Z. & Kenworthy, A. K. (2008). Lipid rafts, cholesterol, and the brain. *Neuropharmacology, 5*, 1265–1273

Kordalewska, M. & Markuszewski, M. J. (2015). Metabolomics in cardiovascular diseases. *Journal of Pharmaceutical and Biomedical Analysis*, *113*, 121-136.

Kumar, A. (2014). Changing trends of cardiovascular risk factors among Indians: a review of emerging risks. *Asian Pacific Journal of Tropical Biomedicine*, *4(12)*, 1001-1008.

Lavi, F., Levinson, D., Peri, I., Tekoah, Y., Hadar, Y. & Schwartz, B. (2010). Chemical characterization, antiproliferative and antiadhesive properties extracted from *Pleurotus pulmonarius* mycelium and fruiting bodies. *Applied Microbiology and Biotechnology*, *85*, 1977-1990.

Lewis, B. (1959). The metabolism of cholesterol. *Post Graduate Medical Journal*, 208-215.

Li, R. W. S., Yang, C., Sit, A. S. M., Kwan, Y. W., Lee, S. M. Y., Hoi, M. P. M, Chan, S.W., Hausman, M., Vanhoutte, P. M. & Leung, G. P. H. (2014). Uptake and protective effects of ergothioneine in human endothelial cells. *The Journal of Pharmacology & Experimental Therapeutics*, *350*, 691-700.

Liao, J. K. (2002). Beyond lipid lowering: the role of statins in vascular protection. *International Journal of Cardiology, 86*, 5–18.

Liscum, L. (2002). Chapter 15: Cholesterol biosynthesis. In: Vance, DE. & Vance, JE (Eds.) *Biochemistry of lipid, lipoprotein and membrane* .4th edition. Elsevier Science B.V.

Liu, X., Zhou, B., Lin, R., Jia, L., Deng, P., Fan, K., Wang, G., Wang, L.& Zhang, J. (2010). Extraction and antioxidant activities of intracellular polysaccharide from *Pleurotus* sp. mycelium. International *Journal of Biological Macromolecules, 47*, 116-119.

Lusis, A. J. (2000). Atherosclerosis. *Nature*, *407(6801)*, 233-241.

Makni, M., Fetoui, H., Gargouri, N. K., Garoui, E. M., Jaber, H., Makni, J., Boudawara, T. & Zeghal, N. (2008). Hypolipidemic and hepatoprotective effect of flax and pumpkin seed mixture rich in ω-3 and ω-6 fatty acids in hypercholesterolemic rats. *Food and Chemical Toxicology*, *46*, 3714-3720.

Manoharan, S., Shuib, A.S., Abdullah, N., Ashrafzadeh, A. & Kabir, N. (2018). Gly-Val-Arg, an angiotensin-1-converting enzyme inhibitory tripeptide ameliorates hypertension on spontaneously hypertensive rats. *Process Biochemistry*, *69*, 224-232.

Manunta, P., Rogowski, A. C., Hamilton, B. P. & Hamlyn, J.M. (1994). Ouabain induced hypertension in the rat: Relationships among plasma and tissue ouabain and blood pressure. *Journal of Hypertension*, *12*, 549-560.

Manzoni, M. & Rollini, M. (2002). Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. *Applied Microbiology Biotechnology, 58*, 555–564.

Marengoni, A., Pasina, L., Concoreggi, C., Martini, G., Brognoli, F., Nobili, A., Onder, G. & Bettoni, D. (2014). Understanding adverse drug reactions in older adults through drug–drug interactions. *European Journal of Internal Medicine, 25 (9)*, 843–846.

Marcoff, T. & Thompson, P. D. (2007). The role of coenzyme Q10 in statin-associated myopathy: a systematic review. *Journal of American College of Cardiology*, *49*, 2231-2237.

Matz, R. L., Schott, C., Stoclet, J. C. & Andriansitohaina, R. (2000). Age-related endothelium dysfunction with respect to nitric oxide, endothelium-derived hyperpolarizing factor and cyclooxygenase products. *Physiological Research*, *49*, 11-18.

McGarrah, R. W., Crown, S.B., Zhang, G-H., Shah, S. H. & Newgard, C. B. (2018). Cardiovascular metabolomics. *Circulation Research*, *122*, 1238-1258.

McMaster, C. R., Tardi, P. G. & Choy, P. C. (1992). Modulation of phosphatidylethanolamine biosynthesis by exogenous ethanolamine and analogues in the hamster heart. *Molecular Cell Biochemistry*, *11*, 69-73.

Merx, W. M. &, Weber, C. (2008). Benefits of statins beyond lipid lowering. *Drug Discovery Today: Disease Mechanisms, 5*, 3–4.

Ming-Qian, S., Jian-Xun, L., Lan, M., Jin, C., Cheng-Ren, L., Lei, L. & Jianxun, R. (2012). LC-coupled with TOFMS for metabonomics study of mini-pigs with atherosclerosis. *Chromatographia*, *75*, 491–497.

Mo, W., Wu, X., Jia, G., Zhao, H., Chen, X., Tang, J., Wu, C., Cai, J., Tian, G., Wang, J. & Liu, G. (2018). Role of dietary supplementation with arganine or N-carbamylglutamate in modulating the inflammation, antioxidant property, and mRNA expression of antioxidant-relative signalling molecules in the spleen of rats under oxidative stress. *Animal Nutrition*, *4*, 322-328.

Mudd, J. O., Barlaug, B. A., Johnston, P. V., Kral, B. G., Rouf, R., Blumenthal, R. S. & Kwiterovich, P. O. (2007). Beyond low-density lipoprotein cholesterol: Defining the role of low-density lipoprotein heterogeneity in coronary artery disease. *Journal of the American College of Cardiology*, *50(18)*, 1735-1741.

Mukhopadhyay, S. & Maitra, U. (2004). Chemistry and biology of bile acids. *Current Science*, *87(12)*, 1666-1683.

Murakami, S., Kordo, Y., Toda, Y., Kitajima, H., Kameo, K., Sakono, M. & Fukuda, N. (2002). Effect of taurine on cholesterol metabolism in hamsters: Up-regulation of low-density lipoprotein (LDL) receptor by taurine. *Life Science*, *70(20)*, 2355-2366.

Murray, M. T. (2013). Cholesterol and heart disease what the drug companies won't tell you and your doctor doesn't know. Mind Publishing Inc.

Niazmand, S., Elahe, F., Maryam, M.& Seyed, M. M. (2014). Endothelium-independent vasorelaxant effects of hydroalcoholic extract from *Nigella sativa* seed in rat aorta: the role of CA^{2+} and K^{+} channels. *Biomed Research International*, *2014*: Article ID 247054, 7 pages.

Nguyen, T. K., Im, K. H., Choi, J., Shin, P. G. & Lee, T. S. (2016). Evaluation of antioxidant, anti-cholinesterase and anti-inflammatory effects of culinary mushroom *Pleurotus pulmonarius*. *Mycobiology*, *44(4)*, 291-301.

Orth, M. & Bellosta, S. (2012). Cholesterol: Its Regulation and Role in Central Nervous System Disorders. *Cholesterol*, *2012*, Article#292598.

Parthasarathy, S., Raghavamenon, A., Garelnabi, M. O. & Santanam, N. (2010). Oxidized low-density lipoprotein. *Methods in Molecular Biology, 610*, 403-417.

Park, T., Oh, J. & Lee, K. (1999). Dietary taurine or glycine supplementation reduces plasma and liver cholesterol and triglyceride concentrations in rats fed a cholesterol-free diet. *Nutrition Research*, *19*, 1777-1789.

Qu, Z., Zhang, J., Gao, W., Chen, H., Guo, H., Wang, T., Li, H. & Liu, C. (2014). Vasorelaxant effects of Cerebralcare Granule® are mediated by NO/cGMP pathway, potassium channel opening and calcium channel blockade in isolated rat thoracic aorta. *Journal of Ethnopharmacology*, *155*, 572-579.

Reis, F. S., Martins, A., Vasconcelos, M. H., Morales, P., Isabel C. F. R. & Ferreira, I. C. F. R. (2017). Functional foods based on extracts or compounds derived from mushrooms. *Trends in Food Science & Technology, 66*, 48-62.

Ribbenstedt, A., Ziarrusta, H. & Benskin, J. P. (2018). Development, characterization and comparisons of targeted and non-targeted metabolomics methods. *PLoS ONE*, *13 (11)*, 1-18.

Rispin, A., Farrar, D., Margosches, E., Gupta, K., Stitzel, K., Carr, G., Michael G., Meyer, W. & McCall, D. (2002). Alternative methods for the median lethal dose (LD (50) test: The up-and-down procedure for acute oral toxicity. *ILAR Journal*, *43*, 233-243.

Roncero-Ramos, I. & Delgado-Andrade, C. (2017). The beneficial role of edible mushrooms in human. *Current Opinion in Food Science, 14*, 122–128.

Rosenbaum, M. A., Miyazaki, K., Graham, L. M. (2002). Hypercholesterolemia and oxidative stress inhibit endothelial cell healing after arterial injury. *Journal of Vascular Surgery*, *55*, 489-496.

Rossi, G., Manunta, P., Hamlyn, J. M., Pavan, E., Detoni, R., Semplicini, A., Pessina, A. C. (1995). Endogenous ouabain in primary aldosteronism and essential hypertension: Relationship with plasma renin, aldosterone and blood pressure levels. *Journal of Hypertension 13*, 1181—1191.

Roupas, P., Jennifer Keogh,J., Noakes, M., Margettsa, C. & Taylor, P. (2012). The role of edible mushrooms in health: Evaluation of the evidence. *Journal of Functional Foods, 4*, 687-709.

Sakakibara, K., Feng, G. G., Li, J., Akahori, T., Yasuda, Y., Nakamura, E., Hatakeyama, N., Fujiwara, Y., Kinoshita, H. (2015). Kynurenine causes vasodilation and hypotension induced by activation of KCNQ-encoded voltage-dependent K^+ channels. *Journal of Pharmacological Sciences*, *129*, 31-37.

Sanchez, C. (2017). Reactive oxygen species and antioxidant properties from mushrooms. *Synthetic and Systems Biotechnology, 2*, 13-22.

Senn, T., Hazen, S. L. & Tang, W. H. W. (2012). Translating metabolomics to cardiovascular biomarkers. *Progress in Cardiovascular Diseases*, *55*, 70-76.

Singer, S. J. & Nicolson, G. L. (1972). *Science* 175:720. American Association for The Advancement of Science.

Siskind LJ, Mullen TD, Obeld LM. 2010. Chapter 148: The role of ceramide in cell regulation. Handbook of Cell Signaling.: 2nd ed. Elsevier Inc.

Silva, B.V. 2013. Isatin, a versatile molecule: studies in Brazil. *Journal of Brazilian Chemical Society*, *24*, 707-720.

Slaoui, M. & Fiette, L. 2011. Chap 4: Histopathology procedures: from tissue to histopathological evaluation. In: Gautier, J. C. (Ed.). Drug Safety Evaluation: *Methods and Protocols, Methods in Molecular Biology*, Vol. 691: Page: 69-82, Springer Science+Business Media, LLC.

Sjouke, B., Kusters, D. M., Kastelein, J. J. P. & Hovingh, G. K. (2011). Familial Hypercholesterolemia: Present and Future Management. *Current Cardiology Reports, 13*, 527–536.

Smiderle, F. R., Olsen, L. M., Carbonera, E. R., Bagio, C. H., Freitas, C. S., Marcon, R., Santos, A. R. S., Gorin, P. A. Y. & Iacomini, M. (2008). Anti-inflammatory and analgesic properties in a rodent model of a (1→3),(1→6)-linked β-glucan isolated from *Pleurotus pulmonarius*. *European Journal of Pharmacology*, *597*, 89-91.

Souza, R. D. & Chattree, A. (2015). Design, synthesis and biological activities of Isatin derivatives. *Chemical Science Transactions*, *4*, 208-212.

Stamets, P. (2000). Growth parameters for gourmet and medicinal mushroom species.*Growing gourmet and medicinal*. Ten Speed Press.

Sugiura, T., Dohi, Y., Yamashita, S., Hirowatari, Y., Fujii, S. & Ohte, N. (2016). Serotonin in peripheral blood reflects oxidative stress and plays a crucial role in atherosclerosis: novel insights towards holistic anti-atherothrombotic strategy. *Atherosclerosis*, *246*, 157-160.

Summers, S. A. (2017). Could ceramides become the new cholesterol? *Cell Metabolism*, *27*, 276-280.

Sun, M., Northup, N., Marga, F., Tamas Huber, T., Byfield, F. J., Irena Levitan, I. & Forgacs, G. (2007). The effect of cellular cholesterol on membrane cytoskeleton adhesion**.** *Journal of Cell Science, 120*, 2223-2231.

Tabas, I. & Lichtman, A. (2017). Monocyte-macrophages and T cells in atherosclerosis. *Immunity*, *47(4)*, 621-634.

Tiwari, V. & Khokhar, M. (2014). Mechanism of action of anti-hypercholesterolemia drugs and their resistance. European *Journal of Pharmacology, 741*, 156–170.

Tjaden K., Pardali, E. & Waltenberger, J. (2015). Hypercholesterolemia induces vascular cell dysfunction: molecular basis for atherosclerosis. *Austin journal of Vascular Medicine*, *2(1)*, 1011-1020.

Tobert, J. A. (2003). Lovastatin and beyond the history of the HMG-CoA reductase inhibitors. *Nature Reviews: Drug Discovery*, 2, 517.

Tousoulis, D., Antoniades, C., Tentolouris, C., Goumas, G., Stefanad, C. & Pavlos, T. (2002). L-arganine in cardiovascular disease: dream or reality? *Vascular Medicine*, *7*, 203-211.

Vita, J.A., Yeung, A.C., Winnford, M., Hodgson, J.M., Treasure, C.B., Klein, J.L., Werns, S., Kern, M., Plotkin, D., Shin, J., Mitchel, Y. & Ganz, P. (2000). Effect of cholesterol-lowering therapy on coronary endothelial vasomotor function in patients with coronary artery disease. *Circulation*, *102*, 846-851.

Wang, T. & Butany, J. (2017). Pathogenesis of atherosclerosis. Diagnostic Histopathology, *23(11)*, 473-478.

Wang, Y., Liu, H., McKenzie, G., Witting, P. K., Stasch, J. P., Hahn, M., … Stacker, R. (2010). Kynurenine is a novel endothelium-derived relaxing factor produced during inflammation. *Nature Medicine*, *16(3)*, 279-285.

Warnholtz, A., Mollnou, H., Oelze, M., Wendt, M. & Munzel, T. (2001). Antioxidants and endothelial dysfunction in hyperclipidemia. *Current Hypertension Reports*, *3*, 53-60.

Wirleitner, B., Neurauter, G., Schrocksnadel, K.., Frick, B. & Fuchs, D. (2003). Interferon-γ-Induced conversion of tryptophan: immunologic and neuropsychiatric aspects. *Current Medical Chemistry*, *10*,1581-1591.

Wojtczak, L. & Slyshenkov, V. S. (2003). Protection by pantothenic acid against apoptosis and celldamage by oxygen free redicals- the role of glutathione. *BioFactors*, *17*, 61-73.

Wresdiyati, T., Hartanta, A. B., & Astawan, M. (2008). The effect of seaweed *Eucheuma cottonii* on superoxide dismutase (SOD) liver of hypercholesterolemic rats. *HAYATI Journal of Biosciences*, *15*, 105-110.

Wu, X., Cao, W., Jia, G., Zhao, H., Chen, X., Wu, C., Tang, J., Wong, J. & Liu, G. (2017). New insights into the role of spermine in enhancing the antioxidant capacity of rat spleen and liver under oxidative stress. *Animal Nutrition*, *3*, 85-90.

Wüstner, D., Solanko, L. M. & Lund, F. W. (2012). Chapter 1**:** Cholesterol trafficking and distribution between cellular membranes. In: Irena Levitan, I., Barrantes, F. J. (Eds). *Cholesterol Regulation of Ion Channels and Receptors*, 1st Edition. John Wiley & Sons, Inc. Published 2012 by John Wiley & Sons, Inc.

Xiao, L., Cao, W., Liu, G., Fang, T., Wu, X., Jia, G., Chen, X., Zhao, H., Wang, J., Wu, C. & Cai, J. (2016). Arganine, N-carbamylglutamate and glutamine exert protective effects against oxidative stress in rat intestine. *Animal Nutrition*, *2*, 242-248.

Xu, W., Huang, JJ-h. & Cheung, P. C. K. (2012). Extract of *Pleurotus pulmonarius* suppresses liver cancer development and progression through inhibition of VEGF-induced Pl3K/AKT signalling pathway. *PLoS ONE*, *7(3)*, 1-13.

Yang, H., Hwang, I., Kim, S., Hong, E. & Jeung, E. (2013). *Lentinus edodes* promotes fat removal in hypercholesterolemic mice. *Experimental and. Therapeutic Medicine*, *6*, 1409-1413.

Yang, R.L., Shi, Y.H., Hao, G., Li, W. & Le, G. W. (2008). Increasing oxidative stress with progressive hyperlipidemia in human: relation between malondialdehyde and atherogenic index. *Journal of Biochemistry and Nutrition*, *43*, 154-158.

Yang, Y., Cruickshank, C., Armstrong, M., Mahaffey, S., Reisdorph, R. & Reisdorph, N. (2013). New sample preparation approach for mass spectrometry-based profiling of plasma results in improved coverage of metabolome. *Journal of Chromatography A*, *1300*, 217-226.

Yilmaz, B. & Usta, C. (2013). Ellagic acid-induced endothelium-dependent and endothelium-independent vasorelaxation in rat thoracic aortic rings and the underlying mechanism. *Phytotherapy Research*, *27*, 285-289.

Yonemura, A., Momiyama, Y., Fayad, Z. A., Ayaori, M., Ohmori, R., Kihara, T., … Ohsuzu, F. (2009). Effect of lipid-lowering therapy with atorvastatin on atherosclerotic aortic plaques: a 2 years follow-up by non-invasive MRI. *European Journal of Cardiovascular Prevention & Rehabilitation*, *2*, 222-228.

Zhang, H., Wang, J., Li, L., Chai, N., Chen, Y., Wu, F., Zhang, W., Wang, L., Shi, S., Zhang, L., Bian, S., Xu, C., Tian, Y. & Zhao Y. (2017). Spermine and spermidine reversed age-related cardiac deterioration in rats. *Oncotarget*, *8*, 64793-64808.

Zhu, X-M., Fang, L-H., Li, Y-J. & Du, G-H. (2007). Endothelium-dependent and – independent relaxation induced by pinocembrin in rat aortic rings. *Vascular Pharmacology*, *46*, 160-165.