EFFECT OF Sandoricum koetjape (buah sentul) EXTRACTS ON HYPOCHOLESTEROLEMIC ACTIVITY USING 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE (HMGR) ASSAY

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by

YEONG AI LIN

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

The following abbreviations are used in the text:

$[E]_T$	Total enzyme concentration
[S]	Substrate concentration
AC	Adventitial cell
AGT	Angiotensinogen
AHA	American Heart Association
CAD	Coronary artery disease
CCR2	Chemokine (C-C motif) receptor 2
CDC42	Cell division control protein 42 homolog
CHD	Coronary heart disease
CRP	C-reactive protein
CV	Column volume
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Endothelial cell
EDN1	Endothelin-1
EDTA	Ethylenediaminetetraacetic acid

ER	Endoplasmic reticulum
E-selectin	Endothelial-selectin
FASLG	Fas ligand
GSTrapFF	Glutathione S-transferase fast flow column
H_2O_2	Hydrogen peroxide
HDL	High-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HOCl	Hypochlorous acid
IC_{50}	Half maximal inhibitory concentration
IFNγ	Interferon gamma
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
K_{cat}	Catalytic constant
K_m	Michaelis-Menten constant
LB	Luria broth
LDL	Low-density lipoprotein
LFA-1	Lymphocyte function-associated antigen 1

LPS	Lipopolysaccharide
L-selectin	Leukocyte-selectin
LTA	Lymphotoxin alpha
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony-stimulating factor
MMP-9	Matrix metalloproteinase-9
MPO	Myeloperoxidase
NaCl	Sodium chloride
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NO	Nitric oxide
OD	Optical density
ox-LDL	Oxidized low-density lipoprotein
PAD	Peripheral artery disease
PCR	Polymerase chain reaction
PECAM1	Platelet endothelial cell adhesion molecule-1
PMSF	Phenylmethanesulfonylfluoride
P-selectin	Platelet-selectin
PSGL-1	P-selectin glycoprotein ligand-1

RAC	Ras-related C3 botulinum toxin substrate
RAS	Rat sarcoma
rhHMGR	Recombinant human HMGR
RHO	Ras homolog
ROS	Reactive oxygen species
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMC	Smooth muscle cell
SOD	Superoxide dismutase
TNFα	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor
V_{max}	Maximum velocity
V_o	Initial velocity
WHO	World Health Organization

KESAN EKSTRAK *Sandoricum koetjape* (buah sentul) KE ATAS AKTIVITI HIPOKOLESTEROLEMIK MELALUI UJIAN 3-HIDROKSIL-3-METILGLUTARIL-COENZIM A REDUKTASE (HMGR)

ABSTRAK

Sandoricum koetjape, sejenis pokok buah-buahan tropika yang kaya dengan pitokimia terpenoids, telah dihipotesiskan untuk merencat sintesis kolesterol melalui perencatan ke atas aktiviti HMG-Coa reduktase (HMGR). HMGR, sejenis enzim sintesis kolesterol penghad-kadar yang mengandungi dua domain, iaitu domain sterol-sensing N-terminal (residu 1-339) dan domain katalitik C-terminal (residu 460-888). Kajian ini telah dirangka untuk mengkaji aktiviti hipokolesterolemik bagi ekstrak *S. koetjape* melalui perencatan ke atas rekombinan enzim HMGR manusia. Menerusi kajian yang dijalankan, serpihan gen yang mengkodkan domain katalitik HMGR telah diekspress dan ditulenkan sebagai protein penanda rekombinan glutathione S-transferase. Penulenan bagi subunit katalitik telah dilakukan menggunakan kolum afiniti GSTrapFF. Ujian enzim secara *in vitro* menunjukkan bahawa aktiviti katalitik adalah berdasarkan kepada pengukuran kinetik. Empat bahagian pada *S. koetjape* iaitu daun, batang, kulit dan isi buah telah diproses untuk menghasilkan ekstrak kasar metanol. Batang pokok telah dikenalpasti sebagai perencat yang paling berpotensi dengan IC_{50} < 5 µg/ml, diikuti oleh kulit buah (IC_{50} = 32.5 µg/ml), daun (IC_{50}= 37.6 µg/ml) dan isi buah (IC_{50} = 68.3 µg/ml). Kesimpulannya, keempat-empat bahagian *S. koetjape* telah menunjukkan kesan aktiviti hipokolestrolemia melalui perencatan HMGR. Hasil penemuan ini membuktikan bahawa *S. koetjape* berpotensi untuk kajian lanjutan sebagai agen hipokolestrolemia.

EFFECT OF *Sandoricum koetjape* (buah sentul) EXTRACTS ON HYPOCHOLESTEROLEMIC ACTIVITY USING 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE (HMGR) ASSAY

ABSTRACT

Sandoricum koetjape, a tropical fruit tree rich in phytochemical terpenoids, was postulated to suppress cholesterol synthesis through inhibition of HMG-CoA reductase (HMGR) activity. HMGR, a rate-limiting enzyme of cholesterol synthesis consists of two domains, N-terminal sterol-sensing domain (residues 1-339) and C-terminal catalytic domain (residues 460-888). This research was designed to study the hypocholesterolemic activity of *S. koetjape* extract through inhibition of recombinant human HMGR. In this study, the gene fragment encoding the catalytic domain of HMGR was expressed and purified as a recombinant glutathione S-transferase tagged protein. *In vitro* enzymatic assay revealed catalytic activity based on the kinetic measurements. Methanolic crude extracts of leaves, bark, fruit hull and flesh from *S. koetjape* exhibit *in vitro* inhibition activity significantly on the recombinant human HMGR. Bark was found to be the most potent inhibitor with IC_{50} <5 µg/ml, followed by fruit hull (IC_{50} = 32.5 µg/ml), leaves (IC_{50} = 37.6 µg/ml) and flesh (IC_{50} = 68.3 µg/ml). In summary, all four plant parts of *S. koetjape* exhibited hypocholesterolemic activity through HMGR inhibition. The promising finding revealed that *S. koetjape* is a good candidate for further research as a hypocholesterolemic agent.

CHAPTER 1

INTRODUCTION

1.1 Cardiovascular disease

Despite the tremendous advances in modern medicines, cardiovascular disease (CVD) remains the number one killer globally, making it the major concern in healthcare arena. In 2001, nearly one-third of global deaths were attributable to CVD where low- and middle-income countries contributed to 85% of all the CVD deaths (AHA, 2004). The number of deaths attributable to CVD continues to increase, and prevalence is increasing sharply in developing countries (Roger *et al.*, 2011). According to the World Health Statistics 2011, the relative uncertainty range for deaths from CVD is ± 12% for high-income countries and ± 25-35% for sub-Saharan Africa (WHO, 2011). CVD is greatly associated with atherosclerosis, a medical condition where atherosclerotic plaque built up slowly in the arteries and remained asymptomatic until the arteries become severely narrowed or completely blocked (Roger *et al.*, 2011). Atherosclerosis is the primary cause of cardiovascular incidents consists of stroke and heart attack. In fact, atherosclerosis is depicted by World Health Organization as the fundamental cause of cerebrovascular disease and coronary artery disease, the most common causes of morbid-mortality globally (Rader and Daugherty, 2008).

1.2 Atherosclerosis

Atherosclerosis came from Greek words *athero* (meaning lump of paste) and *sclerosis* (hardening), which means the nodular accumulation of a soft, flaky, yellowish materials at the centre of arteries. The atherosclerotic plaque consists of

the deposit of cholesterol and other lipids, calcium, and large number of inflammatory cells including foam cells, T lymphocytes, B lymphocytes, dendritic cells and macrophages as shown in Figure 1.1 (Galkina and Ley, 2009).

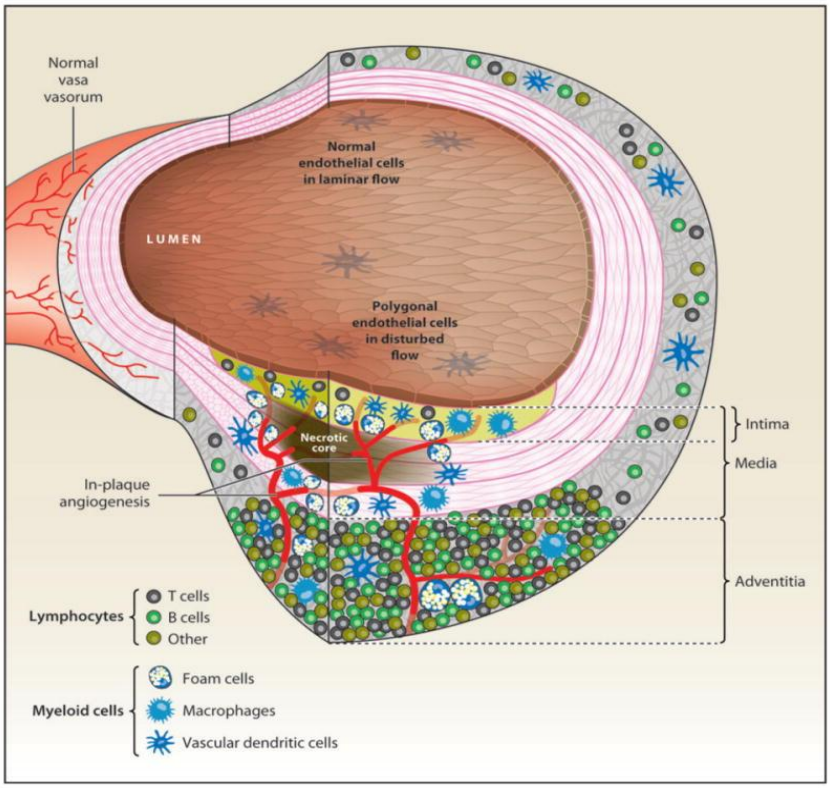

Figure 1.1: Immune cells involved in atherosclerosis (Adapted from Galkina, E. and Ley, K. 2009. Immune and inflammatory mechanisms of atherosclerosis. *Annu Rev Immunol*, 27, 165-197, Figure 1, page 33)

Two types of plaque are formed in the arterial walls, stable plaque and unstable plaque. The unique feature of stable plaque is a thick fibrous cap which comprised of smooth muscle cells. As the plaque grows, it narrows the lumen of the artery. Consequently, the supply of oxygen-rich blood to the heart, brain, and other body parts is restricted. Atherosclerosis leads to severe problems when this type of plaques causes a partial or complete obstruction of blood flow to heart and brain. The second type of plaque is unstable plaque, which is more dangerous because it has a thin cap comprised of macrophages, foam cells and less smooth muscle cells. As a result, it is more prone to plaque rupture and cause thrombus (blood clot) formation.

This sudden occlusion of the artery often leads to an acute, life-threatening heart attack.

Either type of plaques can lead to peripheral arterial disease, stroke, coronary artery disease and transient ischemic attack (Roger *et al.*, 2011). Peripheral arterial disease happens when blood flow to the arms or legs is limited. The symptoms of peripheral arterial disease are pain and numbness. If left untreated, it can result in tissue death and gangrene (AHA, 2004). Restriction of blood flow to parts of the heart can cause myocardial infarction (heart attack), angina (chest pain), weakened cardiomyocytes (heart muscles) or heart failure (Roger *et al.*, 2011). Stroke and transient ischemic attack occurs when blood supply is blocked to parts of the brain. Transient ischemic attack often has similar symptoms as stroke; the symptoms normally pass within 1 hour up to 24 hours. It serves as a warning to future attacks and stroke. It can be seen that the consequences of atherosclerosis is deleterious and detrimental. Therefore, it is necessary to understand the different stages of atherosclerosis and its pathological determinant.

1.2.1 Pathological events that lead to atherosclerosis

Atherosclerosis is a systemic coronary artery disease (CAD) with complicated pathogenesis involving a couple of highly interrelated processes, including oxidative stress, lipid disturbances, endothelial dysfunction, chronic inflammation, smooth muscle cell activation, altered matrix metabolism, platelet activation and thrombosis (Gerdes and Zirlik, 2011). Atherosclerosis arises at sites in the arterial tree where laminar flow is disrupted. It all begins with a fatty streak and develop into an intermediate lesion, subsequently into a lesion that is susceptible to

rupture. The progression of atherosclerosis may develop into an advanced obstructive lesion. Compelling evidences have indicated the role of inflammation in mediating all stages of atherosclerosis from initiation through progression (early asymptomatic vascular injury) and, ultimately the thrombotic complications (clinically manifest dysfunction).

Inflammatory signalling alters the behaviour of the vascular elements and intrinsic cells of the artery wall (endothelium and smooth muscle), further recruits immune cells that interact to promote lesion formation and complications, as shown in Figure 1.2 (Badimon *et al.*, 2009). Early injury is characterized by endothelial expression of adhesion molecules, monocyte adhesion and transmigration into the subendothelial layer, differentiate into macrophages and subsequent transformation into foam cells (Puntmann *et al.*, 2010). Parallel upregulation of hemostatic proteins induces a highly procoagulative state, in which platelets releasing their contents at the site of activated endothelium. The arterial wall remodels in response to continuous accumulation of plaque material. Initial preservation of arterial lumen cross-sectional area through means of circumferential expansion of the wall segments allows certain level of plaque burden before significant luminal narrowing, blood flow restriction, and CVD symptoms occur (Puntmann *et al.*, 2010). Ongoing release of proinflammatory cytokines, activation of matrix metalloproteinases (MMPs), and neovascularization may cause plaque instability (Puntmann *et al.*, 2010).

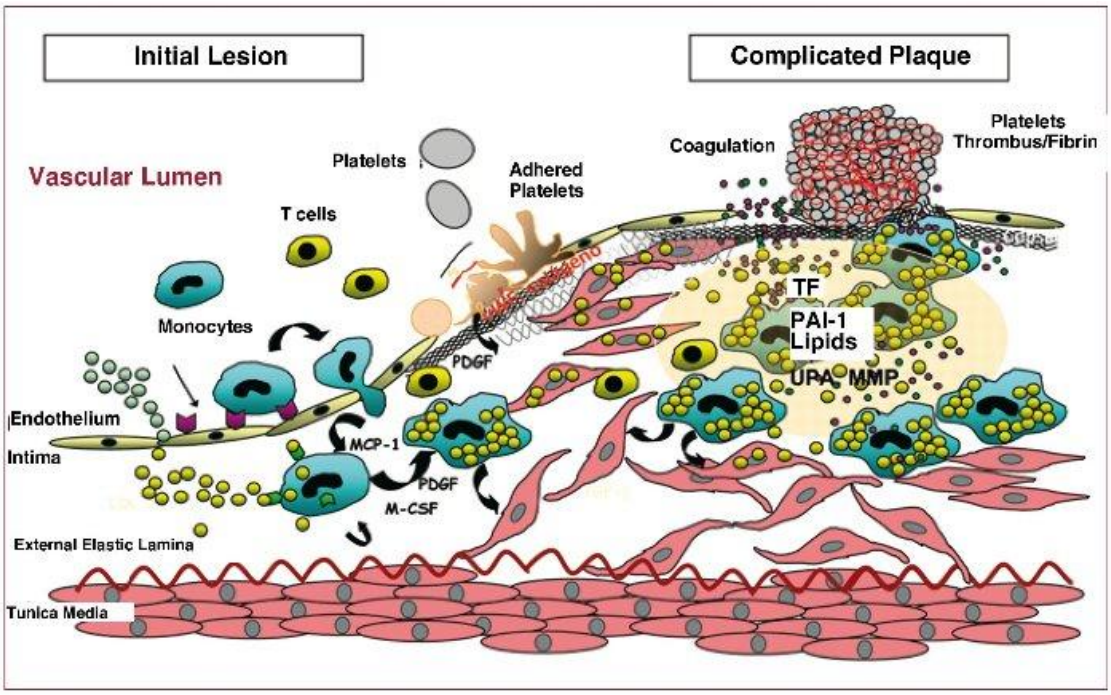

Figure 1.2: Schematic representation of the progression of atherosclerotic plaque from initial stage of endothelial dysfunction to advanced stage with the presence of complicated plaques. (Adapted from Badimon, L., Vilahur, G. and Padro, T. 2009. Lipoproteins, platelet and atherothrombosis. *Rev Esp Cardiol*, 62(10), 1161-78, Figure 3, page 1163)

1.2.1.1 Initiation of atherosclerosis

Cholesterol is insoluble organic molecules which has very low solubility in aqueous media attributable to their highly hydrophobic characteristic. Lipoproteins are carriers composed of proteins and phospholipids that facilitate the transport of cholesterol in bloodstream (Badimon *et al.*, 2009). Low-density lipoproteins (LDL) is the main cholesterol carrier that serves as an exogenous source of cholesterol and other cellular nutrients for hepatic and extrahepatic tissues, where it is taken up by receptor-mediated endocytosis (Osterud and Bjorklid, 2003).

Under physiological conditions, a multifactorial organ vascular endothelium plays an imperative role to recognize both systemic and local stimuli and thereby modify its functional status which contributes to the homeostasis of the vascular wall.

Endothelium is naturally equipped with a surface that prevents thrombosis (Badimon *et al.*, 2009). It permits the exchange of numerous substances between the blood and tissues and controls the vascular tone and trafficking of inflammatory cells towards the vascular bed.

In regions of vascular bed predisposed to atherosclerotic lesions due to increased permeability, hypercholesterolemia may cause the increased of LDL transcytosis through vascular endothelium (Badimon *et al.*, 2009). The presence of native or atherogenic LDL particles in the arterial intima led to a decrease in the bioavailability of endothelial nitric oxide (NO). The decreased in NO availability is associated with a reduction in the concentration and activation of nitric oxide synthetase (Badimon *et al.*, 2009). Parallel changes in endothelial permeability further augment the inflammatory response by promoting the entry and retention of cholesterol-containing LDL particles in arterial intima (Libby *et al.*, 2011). LDL retention in the arterial intima is a key process in the progression of atherosclerotic lesion.

LDL are subjected to a milieu conducive to various kinds of enzymatic and chemical modifications (Fan and Watanabe, 2003). Modification of LDL via oxidative processes is believed to be a prerequisite for the development of atherosclerosis. Basically, LDLs are modified by oxygen radicals (lipoxygenase, myeloperoxidase, sphingomyelinase and secretary phospholipase A2) (Rader and Daugherty, 2008). Oxidation of the particle in the arterial wall is thought to be a complex reaction involving several cell types such as endothelial cells, smooth muscle cells, granulocytes, macrophages, monocytes and lymphocytes (Osterud and Bjorklid, 2003).

On the other hand, LDL may also undergo modification processes when they interact with extracellular matrix components such as proteoglycans, collagen, elastin, hydrolytic enzymes (esterase), proteolytic enzymes (metalloproteinases, tryptase, kinase, and thrombin), lipolytic enzymes (phospholipase A2, phospholipase C and sphingomyelinase) (Badimon *et al.*, 2009). Collagen is vital for maintaining the integrity and elasticity of the vascular wall. However, it is a double-edge sword which also implicated in cell differentiation processes. The glycosylated forms of collagen are chief factor in atherogenesis as they favour LDL retention. In the nutshell, there are various chemical and structural processes that take place to generate different types of modified LDL particles.

Arterial endothelial cells, which normally resist attachment of the white blood cells streaming past them, express adhesion molecules that capture leukocytes on their surfaces when subjected to irritative stimuli such as dyslipidemia, hypertension or pro-inflammatory mediators (Libby *et al.*, 2011). Inflammation results in the generation of chemokines such as CX3, CL1 and MCP-1, which in turn signal the adhesion and transmigration of monocytes into the arterial intima (Rader and Daugherty, 2008; Libby *et al.*, 2002). Once resident in the artery wall, monocytes may differentiate into macrophages under the action of M-CSF, as shown in Figure 1.2 (Fan and Watanabe, 2003). Macrophages secrete various factors involved in propagating the atherosclerotic plaque, including factors involved in proteolysis, inflammation and lipid metabolism (Rader and Daugherty, 2008).

1.2.1.2 Progression of atherosclerosis

A key feature in the development of atherosclerotic lesions is the infiltration of circulating monocytes into the subendothelial space. Under the condition of normal blood flow, selectin-mediated interactions are not sufficient to arrest rolling leukocytes. However, endothelial cells are susceptible and sensitive to the shear stresses provided by blood flow, which can change their morphology and trigger many signalling cascade, resulting LDL accumulation at sites of arterial branching or curvature, where flow is disturbed (Mestas and Ley, 2008).

The first step of atherosclerotic lesion development is leukocyte recruitment to the arterial wall. Leukocyte homing in this event proceeds through a well-defined dynamic adhesion cascade, including tethering, rolling, slow rolling, firm adhesion, and transmigration (Huo and Xia, 2009). Capture or tethering is the first step of leukocyte adhesion, which functions to decelerate fast-flowing leukocytes from the central blood stream and enables them to interact closely with the activated endothelium (Huo and Xia, 2009). Rolling leukocytes transduce signals from adhesion receptors, which activate downstream adhesion molecules that mediate slow rolling and firm adhesion to the endothelium, permitting the adherent leukocytes to infiltrate into the arterial wall (McNeill *et al.*, 2010). Each of these steps is mediated by the coordinated actions of different adhesion molecules and chemokines.

It was found that the interactions of leukocyte integrins with members of the immunoglobulin family mediate firm adhesion and migration (Huo and Xia, 2009). Integrins are heterodimeric cell surface receptors that support both rolling and adhesion of leukocytes (Mestas and Ley, 2008). Upon activation, integrins undergo a series of conformational changes that result in increased binding affinity to their

respective ligands (Mestas and Ley, 2008). VLA-4 and the β2 integrin LFA-1 constitutively, express E-selectin, L-selectin and P-selectin ligands (Figure 1.3) (Mestas and Ley, 2008). These selectins and their ligand (PSGL-1) also mediate the initial tethering and rolling events of leukocyte on the endothelium. Interactions between selectins and PSGL-1 serve as a braking system to decelerate fast-flowing leukocytes from the central blood stream, enabling them to adhere to and transmigrate underneath the activated endothelium (Huo and Xia, 2009; Mestas and Ley, 2008; McNeill *et al.*, 2010).

Chemokines and chemokine receptors also play an imperative role in leukocyte adhesion and migration (Huo and Xia, 2009). For instance, MCP-1 plays a key role by means of the CCR2 receptors (Huo and Xia, 2009). Integrin activation is typically mediated by signals induced by chemokine receptor engagement that triggers arrest and firm adhesion (Mestas and Ley, 2008). Some chemokines can bind to the surface of endothelial cells, immobilize, and mediate arrest of rolling leukocytes. These chemokines can also bind to heparin sulphate, a glycoaminoglycan present on the surface of endothelial cells (Mestas and Ley, 2008). The association of chemokines with heparin sulphate immobilizes chemokines on the vessel wall, providing strong and localized signals for integrin activation (Mestas and Ley, 2008).

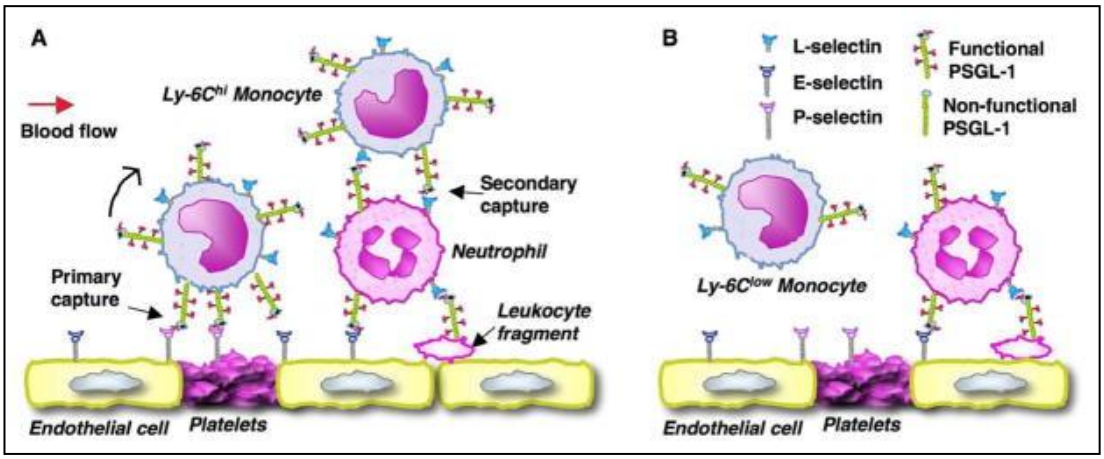

Figure 1.3: PSGL-1 mediated selective homing of monocytes. This picture gives a closer view to the process of monocyte tethering and rolling on the endothelium through classic adhesion cascade, involving adhesion molecules and their ligands. (Adapted from Huo, Y. and Xia, L. 2009. P-selectin glycoprotein ligand-1 plays a crucial role in the selective recruitment of leukocytes into the atherosclerotic arterial wall. *Trends Cardiovasc Med*, 19(4), 140-5, Figure 2, page 143)

Macrophages are the predominant cellular components in atherosclerotic lesions. Macrophages found in lesions are differentiated from circulating blood monocytes, and these macrophages behave very differently (Huo and Xia, 2009). In the nascent atheroma, macrophages may take up deposited atherogenic LDL through scavenger receptors, engulf the lipoprotein particles and transformed into foam cells (lipid-laden macrophages) (Fan and Watanabe, 2003; Libby *et al.*, 2002; Rader and Daugherty, 2008; Libby *et al.*, 2011). M-CSF contributes to the differentiation of the blood monocyte into macrophage foam cell (Libby *et al.*, 2002). The accumulation of foam cells in the atherosclerotic plaque leads to the formation of fatty streaks (Rader and Daugherty, 2008).

Atheroma formation also involves the recruitment of SMCs in the tunica media into the tunica intima (Libby *et al.*, 2011). SMCs undergo phenotypic changes from differentiation to the acquisition of a synthetic phenotype under the effects of atherogenic stimuli (Badimon *et al.*, 2009). Thus, SMCs with a nonproliferative

contractile phenotype, typically in healthy arteries, transform into actively proliferative cells (Badimon *et al.*, 2009). During atherogenesis, SMCs migrate from the media into the intima when they are attracted by chemotactic agents, and proliferate within the intima in response to mediators like PDGF (Figure 1.2) (Libby *et al.*, 2011). Vascular SMCs contributes to development of atherosclerotic lesion by secreting large amounts of extracellular-matrix molecules, including interstitial collagen and elastin, and form a fibrous cap that covers the plaque (Rader and Daugherty, 2008; Libby *et al.*, 2011; Badimon *et al.*, 2009). The presence of extracellular matrix increases the retention and aggregation of atherogenic lipoproteins (Rader and Daugherty, 2008).

Close interactions among infiltrated macrophages, foam cells, T lymphocytes and smooth muscle cells together with diverse cytokines and other biological effects decide the fate of the progression of atherosclerotic lesions (Fan and Watanabe, 2003).

1.2.1.3 Plaque rupture and thrombosis

Composition and stability of the plaque is an important determinant of atherosclerosis complications as plaques with high lipid content and excess macrophages in the cap is more susceptible to rupture (Rader and Daugherty, 2008). As the plaque grows, compensatory remodelling takes place, the size of the lumen is preserved while its overall diameter increases (Rader and Daugherty, 2008). Paradoxically, thrombotic complications do not always occur at the sites of the most severe arterial narrowing by plaques. Instead, thrombi often arise after fracture of the fibrous cap or erosion of endothelium, resulting in the exposure of plasma

thrombogenic materials including tissue factor, triggering thrombosis (Libby *et al.*, 2011).

In atherosclerotic plaque, macrophages degrade extracellular matrix by phagocytosis or by secreting proteolytic enzymes, in particular MMP9. MMP9 is a proteolytic enzyme of different extracellular matrix related proteins such as type IV collagen, laminin, and elastin, which may weaken the fibrous cap, predisposing its rupture (Badimon *et al.*, 2009). In addition to macrophages, SMCs also release MMPs; further augment the progression and rupture of atherosclerotic plaques. Therefore, regulation of MMPs is crucial to reduce cardiovascular complications due to its inextricable role in the degradation of extracellular matrix in both physiological and pathological processes of plaque rupture (Bellosta *et al.*, 1998). Emphasis also placed on another class of proteases, cathepsins, which have also been suggested to contribute to lesion disruption (Rader and Daugherty, 2008).

Plaque rupture with superimposed thrombosis is the major cause for acute coronary syndrome of unstable angina, heart attack, and sudden death (Rader and Daugherty, 2008). The underlying causes of these cardiac events are artery occlusion by large thrombus or relentless lesion growth. Large thrombus formation during plaque rupture can block artery and accountable for acute coronary syndrome or heart attack. If the plaque does not rupture and the lesion continues to grow, the lesion can encroach on the lumen and result in clinically obstructive disease (Rader and Daugherty, 2008).

1.2.2 Therapeutic options and clinical perspectives in the management of atherosclerosis

The therapeutic options proven to be promising in the management of atherosclerosis are limited to HDL modulators, anti-restenotic agents, anti-coagulant agents and HMG-CoA reductase (HMGR) inhibitors (Charo and Taub, 2011). Other therapies such as LDL modulators, anti-inflammatory agents, anti-oxidative agents and therapeutic cure targeting at angiogenesis are still under clinical trials investigation to prove their efficacy (Charo and Taub, 2011).

One of the established therapeutic targets in atherosclerosis is HMGR. HMGR inhibitor, generally referred to as statins is the leading therapeutic regimen for treating hypercholesterolemia (Greenwood *et al.,* 2006). Currently, statins has become so prevalent in clinical use that they are now prescribed to more than 25 million people worldwide, with the number expected to rise rapidly. Statins intercede biological effect through HMGR inhibition, an upstream rate-limiting enzyme in the cholesterol synthesis pathway (Greenwood *et al.*, 2006).

Although statins were developed to specifically reduce cholesterol synthesis, clinical trials have indicated their beneficial effects extend beyond lipid lowering efficacy (Charo and Taub, 2011). The anti-inflammatory actions of statins described by previous evidences are decreased in expression of VCAM-1 by endothelial cell, attenuation of the growth of macrophages and their MMPs activity, and inhibition of interferon-γ production (Charo and Taub, 2011). The aforementioned activities had contributed to decrease in monocyte recruitment, T cell activation and thereby improve plaque stability (Charo and Taub, 2011).

Owing to the tremendous benefits attributed by HMGR inhibitors and their clinically proven effectiveness in lowering cholesterol levels and reducing cardiovascular incidence, HMGR has been targeted for research. This research study has been initiated to search novel molecules that can reduce cholesterol level through HMGR inhibition.

1.3 HMG-CoA reductase (HMGR)

HMG-CoA reductase (protein accession number: P04035) is involved in the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) into mevalonate, which is the precursor of cholesterol and other isoprenoids (Carbonell and Freire, 2005).

1.3.1 Structure and binding properties

Human HMGR consists of a single polypeptide chain of 888 amino acids. The amino-terminal 1-339 residues are membrane bound and reside in the endoplasmic reticulum membrane, while soluble C-terminal portion (residues 460-888), the catalytic domain of HMGR resides in its cytoplasm (Istvan and Deisenhofer, 2000). A linker region (residues 340-459) connects the two portions of the protein (Istvan and Deisenhofer, 2000).

1.3.1.1 The architecture of HMGR

HMGR is anchored to membranes of the ER through its hydrophobic NH_2-terminal domain, which consists of eight membrane-spanning regions separated by short loops, as shown in Figure 1.4 (Roitelman *et al.*, 1992). The membrane domain of HMGR precedes a large COOH-terminal domain that projects into the cytosol and exerts all of the catalytic activity of the enzyme (Gil *et al.*, 1985; Liscum *et al.*, 1985).

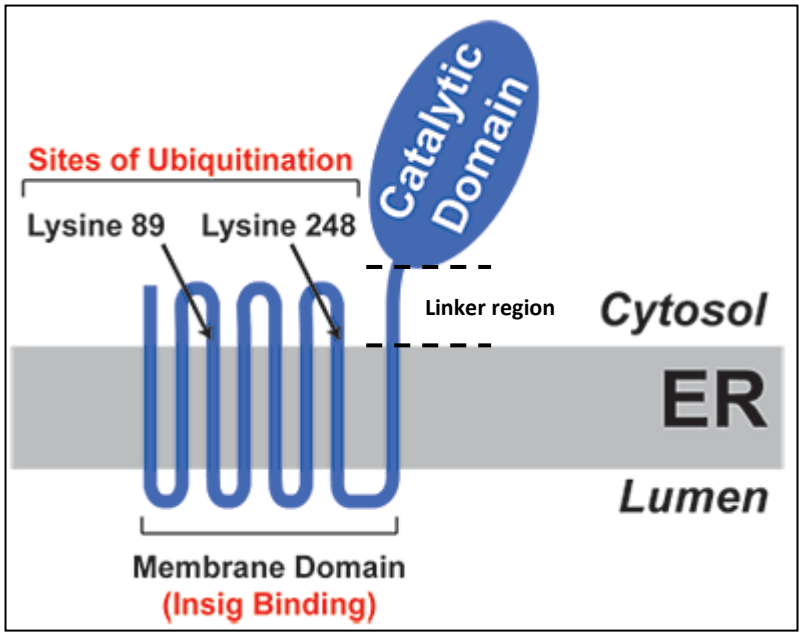

Figure 1.4: Schematic diagram of HMGR. (Adapted from Debose-Boyd, R. A. 2008. Feedback regulation of cholesterol synthesis: sterol-accelerated ubiquitination and degradation of HMG CoA reductase. *Cell Res*, 18(6), 609-21, Figure 2)

In year 2000, the structure of the catalytic domain of human HMGR (residues 460-888) has been published and discussed (Protein Database codes: 1DQ8, 1DQ9, 1DQA). The HMGR monomer reveals a unique structure comprised of three domains (Figure 1.5): an N-terminal helical domain, N-domain (residues 460-527), a large domain, L-domain (residues 528-590 and 694-872) and a small domain, S-domain (residues 592-682) (Istvan *et al.*, 2000). Substrate HMG-CoA binds to the L-domain, while NADP(H), its co-substrate binds predominantly to the S-domain.

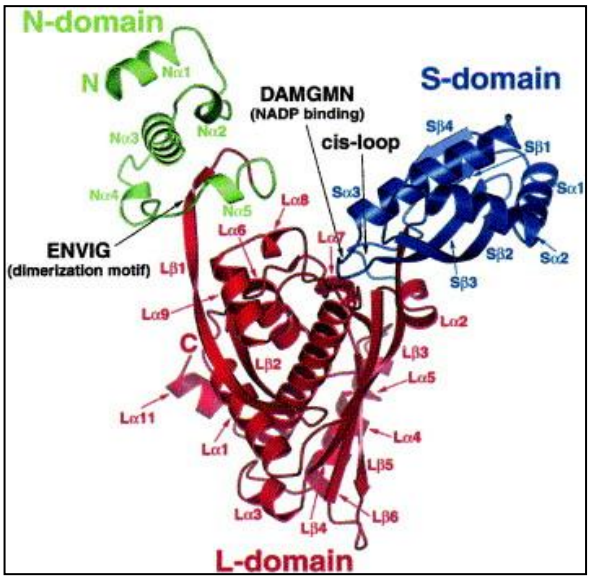

Figure 1.5: Ribbon diagram of the human HMGR monomer. The monomer consists of three domains: (1) the small, helical amino-terminal N-domain (green), (2) the large, central L-domain which contains the dimerization motif ENVIG (red), and (3) the small, ferredoxin-like S-domain which is inserted into the L-domain (blue). (Adapted from Istvan, E. S. and Deisenhofer, J. 2000. The structure of the catalytic portion of human HMG-CoA reductase. *Biochimica et Biophysica Acta*, 1529, 9-18, Figure 3, page 11)

The enzyme forms tetramers, shown in Figure 1.6. Four monomers of the catalytic portions of human HMGR are arranged in two dimers and combine to form a tetramer. The observation of large contact areas and residues at the interfaces which are typical for protein-protein interactions suggest the tetrameric structure of the catalytic portion of human HMGR in solution (Istvan *et al.*, 2000). This is supported by results of analytical sedimentation and velocity ultracentrifugation experiments (Istvan *et al.*, 2000).

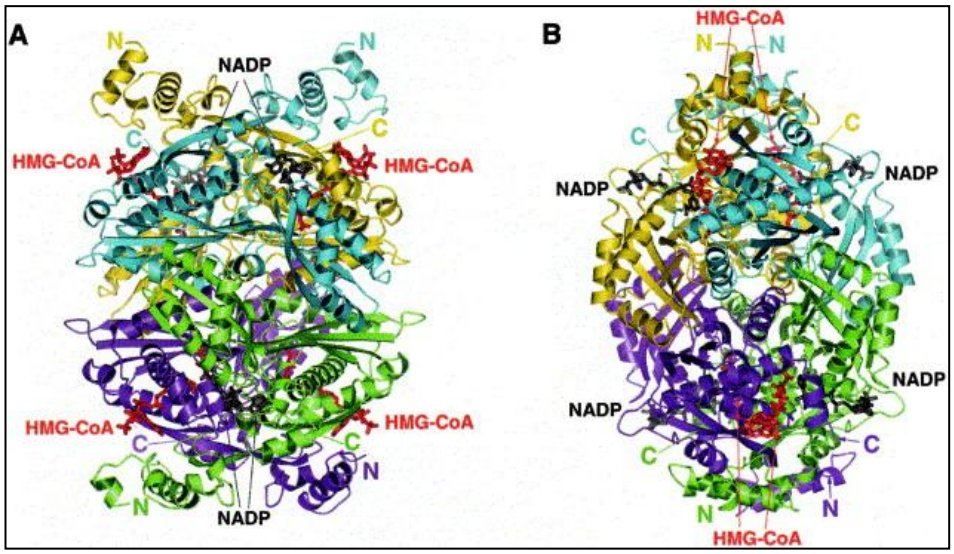

Figure 1.6: Ribbon diagram of the human HMGR tetramer. The four different subunits are colored in yellow, blue, green, and purple. The N- and C-terminal of the four monomers are indicated. Each monomer contains one HMG-CoA molecule and one NADP molecule. (A) Front view of the human HMGR tetramer. (B) Side view of the human HMGR tetramer. (Adapted from Istvan, E. S. and Deisenhofer, J. 2000. The structure of the catalytic portion of human HMG-CoA reductase. *Biochimica et Biophysica Acta*, 1529, 9-18, Figure 2, page 10)

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1.3.1.2 Sterol-sensing domain

The membrane-bound domains of mammalian HMGRs contain a 167 residue segment, termed as sterol sensing domain, shares approximately 25% sequence identity with membrane regions of other proteins that are also influenced by cholesterol (Istvan and Deisenhofer, 2000). This domain is responsible for the enhanced degradation of HMGR in response to increased concentrations of oxysterols (Istvan and Deisenhofer, 2000). The degradation of HMGR appears to be initiated when a membrane-bound cysteine protease cleaves within the 8 transmembrane-span region of HMGR (Istvan and Deisenhofer, 2000; Moriyama *et al.*, 1998).

ER protein insig-1 appears to play an essential role in the sterol-mediated degradation of HMGR (Sever *et al.*, 2003). Part of the underlying mechanism is associated with sterol-activated binding of the sterol-sensing domain of HMGR to

insig-1 as determined by coimmunoprecipitation (Sever *et al.*, 2003). The N-terminal of sterol-sensing domain, which is the most conserved region mediates accelerate degradation of HMGR in the presence of sterols (Luskey and Stevens, 1985). This sterol-regulated degradation is an important mechanism for feedback suppression of HMGR activity and regulation of cholesterol metabolism in humans, one of several strategies animal cells use to limit production of cholesterol (Luskey and Stevens, 1985; Sever *et al.*, 2003; Song *et al.*, 2005).

The sterol-accelerated degradation of HMGR is depicted in Figure 1.7. After binding to insig-1, HMGR is degraded within the ER, by a process mediated by ubiquitination and proteasomal degradation (Sever *et al.*, 2003). Figure 1.7 shows that gp78, a membrane bound E3, is an insig-1-associated protein. It was found that insig-1 binds to the membrane domain of gp78 in the absence and presence of sterols. Upon the addition of sterols, HMGR is recruited to the complex (Song *et al.*, 2005). Binding of HMGR to insig-1, along with its associated proteins gp78 and VCP, triggers the polyubiquitination of HMGR in a reaction mediated by gp78 and its cognate E2 (Song *et al.*, 2005). Gp78 is the ubiquitin ligase that initiates sterol-dependent degradation of HMGR, and insig-1 is the bridge between gp78 / VCP and the HMGR (Song *et al.*, 2005). Ubiquitination of an enzyme is an oblidate reaction of recognition and degradation of enzyme and VCP is an ATPase that participates in post-ubiquitination steps of ER-accelerated degradation (Song *et al.*, 2005). In other words, VCP is required for HMGR degradation. Ubiquitination of HMGR marks the enzyme for recognition by VCP and its cofactors, which act to extract ubiquitinated HMGR molecules from the ER membrane and present them to cytosolic 26S proteasomes for degradation, as shown in Figure 1.7 (Song *et al.*, 2005).

In conclusion, the increase in the rate of HMGR degradation occurs when sterol concentrations are high. Sterol-mediated degradation does not influence the degradation of the catalytic portion in cytoplasmic region but the sterol-sensing domain resides in the transmembrane region.

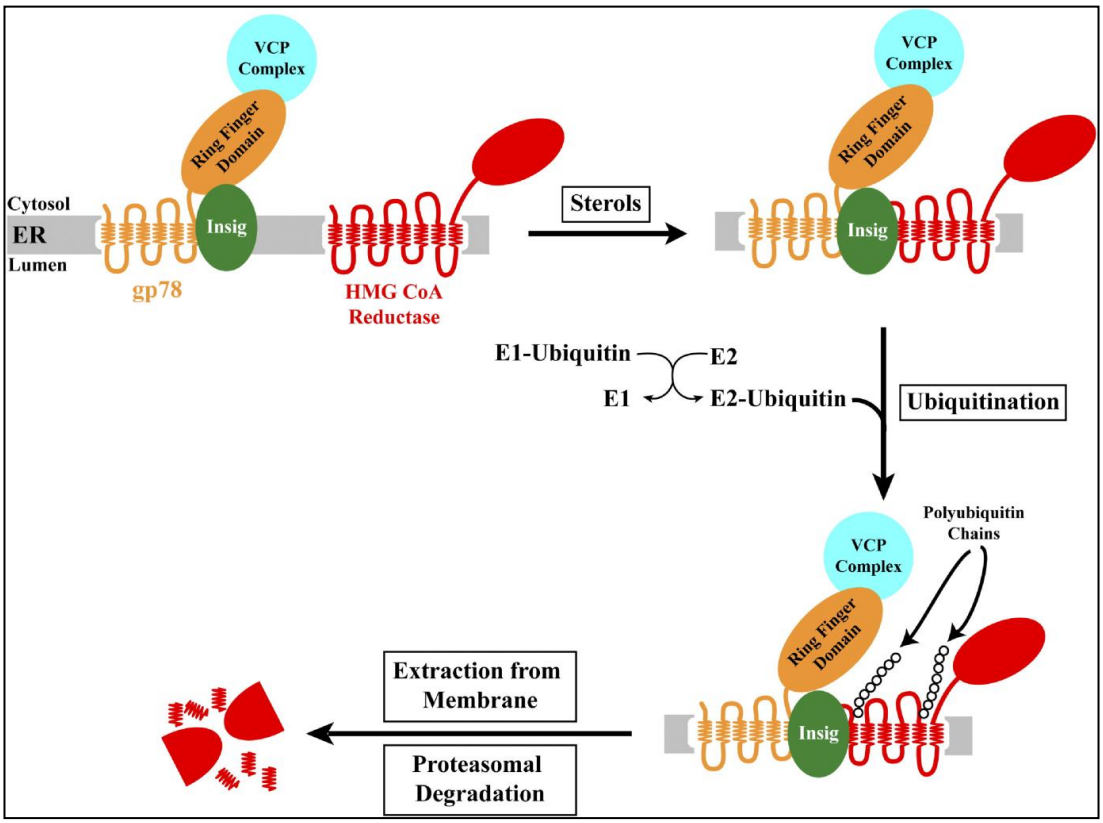

Figure 1.7: Schematic representation for the pathway of sterol-accelerated degradation of HMGR. (Adapted from Song, B. L., Sever, N. and Debose-Boyd, R. A. 2005. Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG-CoA reductase. *Molecular Cell*, 19, 829-840. Figure 6, page 837)

1.3.1.3 Substrate binding and catalysis

The extended substrates HMG-CoA and NADPH molecules make numerous contacts with L- and S-domains of the protein to form the four active sites in the tetramer (Istvan *et al.*, 2000). At each active site, the HMG moiety of one HMG-CoA molecule, which is predominantly bound to a single monomer, comes into proximity of the nicotinamide ring of an NADP(H) molecule, whose binding pocket is located

in the neighboring monomer (Istvan *et al.*, 2000). Thus, the active site is located at the interface of the two monomers of a dimer. The binding of NADP(H) causes a conformational change in the C-terminus of the enzyme that results in the ordering of helix Lα11 and the complete closure of the active site. (Istvan and Deisenhofer, 2000)

(a.) Coenzyme A binding

Coenzyme A binds in an extended conformation: the ADP moiety of CoA is located in a positively charged pocket near the enzyme surface. Multiple interactions between HMGR and CoA are formed by the L-domain of one monomer (Figure 1.5) (Istvan and Deisenhofer, 2000).

(b.) NADP(H) binding

The NADP(H)–binding site is formed primarily by the S-domain. Among the residues with specific interactions with NADP(H), the loop region between helix Sα2 and strand Sβ2 participates in binding of the NADP(H) ADP moiety. The diphosphate is stabilized primarily by main chain interactions with residues located in the highly conserved sequence element DAMGXN and by the dipole of helix Sα3 (Istvan *et al.*, 2000). The binding of NADP(H) causes a conformational change in the C-terminus of the enzyme, resulting in complete closure of the active site (Istvan *et al.*, 2000).

(c.) HMG binding

The site of catalysis and active site in HMGR is the 3-hydroxy-3-methylglutaryl (HMG) binding pocket. It is located between the L- and S-domain. The residues from neighboring monomers contribute to the binding of HMG and the most important

structural element in the binding of HMG is the '*cis*-loop' that bends over the top of HMG (Istvan and Deisenhofer, 2000).

1.3.2 Modulation of HMGR activity by phosphorylation

The activity of HMGR is controlled through synthesis, degradation and phosphorylation to maintain the concentrations of mevalonate-derived products (Istvan and Deisenhofer, 2000). *In vitro*, HMGR may be phosphorylated by several protein kinases: AMP-activated protein kinase, protein kinase C and a calmodulin-dependent protein kinase (Istvan and Deisenhofer, 2000). The AMP-activated protein kinase appears to be the major HMGR kinase in liver, which is the site of cholesterol synthesis (Istvan and Deisenhofer, 2000). All three kinases phosphorylate serine 872, a residue close to the C-terminus of HMGR, and this may reduce the activity of the protein (Istvan and Deisenhofer, 2000).

1.3.3 HMGR, mevalonate pathway and cholesterol synthesis

Cholesterol, an essential component of all animal cell membranes, is derived from the uptake of plasma LDL via receptor-mediated endocytosis and de novo cellular synthesis (Goldstein and Brown, 1990). By maintaining a balance between these external and internal cholesterol sources, the excessive accumulation of cholesterol in mammalian cells can be avoided. This balance is achieved through feedback regulation on the cholesterol biosynthetic pathway and LDL receptor synthesis (Fuhrman *et al.*, 1997).

Several mechanisms for feedback regulation of LDL receptors and of two imperative enzymes involved in cholesterol synthetic pathway ensure the production

of sufficient mevalonate for end-products cholesterol. Besides cholesterol synthesis, it is essential to regulate mevalonate pathway wisely as it produces non-sterol isoprenoids such as dolichols, ubiquinones, steroid hormones, vitamin D, bile acids, lipoproteins that are vital for diverse cellular functions, ranging from cell cycle progression to cell growth to cell survival (Goldstein and Brown, 1990). In order to ensure a constant production of the multiple isoprenoid compounds at all stages of growth, cells must precisely regulate mevalonate synthesis to avoid over-accumulation of potentially toxic products such as cholesterol (Goldstein and Brown, 1990). Mevalonate homeostasis is achieved through sterol-mediated feedback repression of genes for HMGR, HMG-CoA synthase, and LDL receptor as well as the post-transcriptional regulation of HMGR by nonsterol isoprenoids (Goldstein and Brown, 1990).

In mammalian systems, mevalonate is formed from acetyl-CoA and acetoacetyl-CoA via the intermediate HMG-CoA (Istvan and Deisenhofer, 2000). The four-electron reduction of HMG-CoA to mevalonate, which utilizes two molecules of NADPH, is the committed step in the biosynthesis of cholesterol (Istvan and Deisenhofer, 2000). This reaction is catalyzed by HMGR. The activity of HMGR in mammalian cells is sensitive to accelerate degradation by both sterols and non-sterol products of mevalonate pathway (Fuhrman *et al.*, 1997; Goldstein and Brown, 1990). HMGR is among the most highly regulated enzymes known (Espenshade and Hughes, 2007). Transcription and translation of the HMGR gene are low when products of the mevalonate pathway are abundant. Regulation of HMGR is also achieved through the decrease in catalytic efficiency of the enzyme by phosphorylation of serine 872 by AMP-activated protein kinase (mentioned in

Section 1.2.2) and concentration of active protein is regulated by sterol-mediated degradation (Espenshade and Hughes, 2007).

Therefore, this reiterates that regulation of HMGR activity is indeed a promising approach in treating hypercholesterolemia individuals due to its vital role in cholesterol synthesis.

1.3.4 Effect of natural products on HMGR

Some natural products had been discovered to reduce the activity of HMGR, like berberine. Berberine is an isoquinoline alkaloid found in barberry and goldenseal plants, Oregon grapes, and the traditional Chinese herb golden thread (Wu *et al.*, 2011). It was found that berberine could interact with the common active sites on HMGR. The longer the aliphatic chain on berberine, the stronger its inhibition to HMGR activity (Ye *et al.*, 2011). Research showed that hyperhomocysteinemic rats treated with berberine for 5 days inhibited HMGR activity and reduced hepatic cholesterol content. Such an inhibitory effect was intervened by increased phosphorylation of HMGR (Wu *et al.*, 2011). In other words, berberine could inhibit hepatic cholesterol biosynthesis via increased phosphorylation of HMGR (Wu *et al.*, 2011).

Some inhibitors, flavonoid conjugates brutieridin and melitidin quantified in the bergamot fruit extracts were identified to be structural analogues to simvastatins, an HMGR inhibitor (Leopoldini *et al.*, 2010). The geometrical and electronic features affecting the binding of these flavonoids to the active site of HMGR and their respective inhibition process was researched using computational tool (Leopoldini *et al.*, 2010). Besides, four compounds from hawthorn fruit (*Crataegus*

pinnatifida Bge.) like quercetin, hyperoside, rutin and chlorogenic acid were also shown to inhibit HMGR, their inhibition to HMGR was enhanced by their weak hydrophilic ability (Ye *et al.*, 2010).

Phytochemical tocotrienols are farnesylated benzopyran natural products that exhibit hypocholesterolemic activity *in vitro* and *in vivo* (Pearce *et al.*, 1992). It was unveiled that the mechanism of their hypolipidemic action involves post-transcriptional suppression of HMGR by a process distinct from other known inhibitors of cholesterol biosynthesis. γ-Tocotrienol exhibits a 30-fold greater activity toward cholesterol biosynthesis inhibition compared to α-tocotrienol in HepG2 cells *in vitro* (Pearce *et al.*, 1992). On the other hand, the synthetic (racemic) and natural (chiral) tocotrienols exhibit virtually identical cholesterol biosynthesis inhibition as natural tocotrienols and HMGR suppression activities as demonstrated *in vitro* and *in vivo* (Pearce *et al.*, 1992).

Phenolic compound resveratrol was significantly shown to downregulate HMGR mRNA expression by real-time PCR analysis (Cho *et al.*, 2008). These results indicate that dietary resveratrol reduces serum cholesterol by down-regulating hepatic HMGR mRNA expression in hamsters fed a high fat diet (Cho *et al.*, 2008). Besides, many extracts or single compounds from *Salvia miltiorrhiza, Curcuma longa, Coptis chinensis, Rheum undulatum* and *Panax notoginseng* were shown to regulate multiple key targets involved in the initiation and propagation of atherosclerosis, possibly through the inhibition of HMGR (Zeng *et al.*, 2012).

Evidence clearly revealed that certain secondary metabolites derived from natural products are potent HMGR inhibitors. Among the tropical plants available in Malaysia, the tropical fruit plant that was found fascinating is *S. koetjape*. Owing to