The Effect of Morelloflavone on HMG-CoA Reductase Activity

Ku-aida Tuansulong

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biochemistry

Prince of Songkla University

2009

Thesis Title The Effect of Morelloflavone on HMG-CoA Reductase Activity

Author Miss Ku-aida Tuansulong

Major Program Biochemistry

Major Advisor :

…….....…….....

(Assoc. Prof. Dr. Nongporn Towatana)

Co-Advisor :

…….....…….....

(Dr. Rapiporn Sotthibhandhu)

Examining Committee :

……...……...……Chairperson

(Assoc. Prof. Dr. Prapaporn Utarabhand)

……...……...……Committee

(Assoc. Prof. Dr. Nongporn Towatana)

……...……...……Committee

(Dr. Rapiporn Sotthibhandhu)

……...……...……Committee

(Dr. Decha Pinkaew)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Biochemistry.

……

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

ชื่อวิทยานิพนธ์ ผลของ morelloflavone ต่อเอนไซม์ HMG-CoA reductase
ผู้เขียน นางสาวกูไอดา ต่วนสุหลง
สาขาวิชา ชีวเคมี
ปีการศึกษา 2552

บทคัดย่อ

ภาวะคอเลสเทอรอลในเลือดสูงผิดปกติเป็นบ่อเกิดสำคัญของโรคหลอดเลือดหัวใจซึ่งมีอุบัติการเพิ่มขึ้นอย่างต่อเนื่องและยังคงเป็นสาเหตุของการเสียชีวิตอันดับต้นๆ ของประชากรในหลายประเทศ การรักษาด้วยยาลดไขมันในเลือดนั้น ถึงแม้จะให้ผลดี แต่มักเกิดอาการหรือภาวะข้างเคียงบางอย่างที่เป็นอันตรายต่อสุขภาพร่วมด้วย ดังนั้น จึงมีผู้พยายามค้นหาสารออกฤทธิ์จากธรรมชาติที่มีศักยภาพในการบำบัดความผิดปกติดังกล่าวซึ่งอาจให้ผลข้างเคียงที่ไม่พึงประสงค์น้อยกว่ายาแผนปัจจุบันที่ใช้กันอยู่ในขณะนี้ จากการศึกษาที่ผ่านมาพบว่า morelloflavone ซึ่งเป็นสารไบฟลาโวนอยด์จากใบมะพูดสามารถลดระดับไขมันในเลือดของกระต่ายที่ได้รับอาหารผสมคอเลสเทอรอลในปริมาณมากติดต่อกันเป็นเวลานาน นอกจากนี้ ยังมีรายงานว่า สารฟลาโวนอยด์จากพืชบางชนิดสามารถยับยั้งการทำงานของ HMG-CoA reductase ซึ่งเป็นเอนไซม์ควบคุมวิถีการสังเคราะห์คอเลสเทอรอลในร่างกาย และยังเป็นเป้าหมายหลักในการออกฤทธิ์ของ statins ซึ่งเป็นกลุ่มยาลดไขมันในเลือดที่นิยมใช้กันอย่างแพร่หลายที่สุดในโลก ดังนั้น เพื่อให้ทราบกลไกการออกฤทธิ์ลดระดับไขมันในเลือดของ morelloflavone จึงได้ศึกษาผลของสารไบฟลาโวนอยด์ดังกล่าวต่อเอนไซม์ HMG-CoA reductase ทั้งในหลอดทดลองและในสัตว์ทดลอง

ในการทดสอบกับบริเวณเร่งของ HMG-CoA reductase ซึ่งเตรียมโดยถ่ายฝากยีน plasmid pET 17b-CD-HMG เข้าสู่แบคทีเรีย *Escherichia coli* สายพันธุ์ BL21 นั้น เอนไซม์ที่ผลิตได้สามารถเร่งปฏิกิริยาในอัตราเร็วสูงสุด (V_{max}) เท่ากับ 5 ± 0.26 μM NADPH/mg/min โดยมีค่าคงที่ของ Michaelis (K_m) สำหรับ HMG-CoA และ NADPH เป็น 74 ± 0.06 μM และ 133 ± 0.21 μM ตามลำดับ ผลการทดสอบกับ morelloflavone พบว่าสารสำคัญชนิดนี้ยับยั้งเอนไซม์แบบแข่งขันกับ HMG-CoA โดยมีค่าคงที่ของการยับยั้ง (K_i) เท่ากับ 80.87 ± 0.06 μM แต่ยับยั้งแบบไม่แข่งขันกับ NADPH โดยมีค่า K_i เป็น 103 ± 0.07 μM ในขณะที่ narigenin กับ luteolin ซึ่งเป็นฟลาโวนอยด์หน่วยย่อยของ morelloflavone ยับยั้งเอนไซม์แบบแข่งขันกับ HMG-CoA ด้วยค่า K_i ที่เท่ากัน คือ 83.58 ± 4.37 μM และ 83.59 ± 0.94 μM ตามลำดับ ซึ่งใกล้เคียงกับ morelloflavone และสารทั้งสองยังยับยั้งแบบไม่แข่งขันกับ NADPH โดยมีค่า K_i เท่ากับ 182 ± 0.67 μM และ 188 ± 0.14 μM ตามลำดับ การที่ morelloflavone และหน่วยย่อยทั้งสองชนิดสามารถยับยั้งเอนไซม์ได้ทัดเทียมกัน แสดง

ว่า แต่ละโมเลกุลของ morelloflavone อาจใช้หน่วยย่อยข้างใดข้างหนึ่งเข้าแย่งจับกับเอนไซม์ โดยคาดว่า ตำแหน่งจับที่เหมาะสมน่าจะเป็นวงแหวนฟีนอลิคเดี่ยว (ring B) ซึ่งมีลักษณะคล้ายกับของ HMG และ statin อีกทั้งเป็นโครงสร้างที่ naringenin และ luteolin มีร่วมกัน

สำหรับผลการทดสอบเบื้องต้นในสัตว์ทดลอง พบว่า หนูขาวใหญ่ที่มีภาวะคอเลสเทอรอลในเลือดสูงจากการบริโภคอาหารไขมัน ซึ่งได้รับ morelloflavone ทุกวันในขนาด 0.01% ของน้ำหนักตัว มีการเปลี่ยนแปลงของระดับไขมันในเลือดภายใน 6 สัปดาห์ไม่แตกต่างจากหนูที่ไม่ได้รับสารทดสอบ ในขณะที่หนูปกติซึ่งได้รับ morelloflavone กลับมีการเพิ่มขึ้นของปริมาณคอเลสเทอรอลใน HDL (HDL-C) ซึ่งใช้บ่งชี้ระดับไลโปโปรตีนชนิดดังกล่าวในเลือด นอกจากนี้ mRNA ของ HMG-CoA reductase และตัวรับ LDL ในตับของหนูทุกกลุ่มที่ได้รับ morelloflavone ยังมีแนวโน้มถูกกระตุ้น ทั้งนี้ อาจจะเป็นผลตอบสนองต่อกระบวนการสังเคราะห์คอเลสเทอรอลในเซลล์ที่ลดลงนั่นเอง

ผลจากการศึกษานี้แสดงให้เห็นว่า morelloflavone เป็นสารออกฤทธิ์จากมะพูดที่สามารถยับยั้งเอนไซม์ HMG-CoA reductase ได้ดี ซึ่งควรค่าแก่การค้นคว้าวิจัยเพิ่มเติมเพื่อนำไปสู่การพัฒนาเป็นยาลดระดับคอเลสเทอรอลในเลือดต่อไป

Thesis Title Effect of Morelloflavone on HMG-CoA Reductase Activity

Author Miss Ku-Aida Tuansulong

Major Program Biochemistry

Academic Year 2009

ABSTRACT

Hypercholesterolemia is a leading cause in the development of atherosclerosis-associated cardiovascular disease which still occurs increasingly and accounts for the majority of mortality worldwide. Although normalization of plasma cholesterol or LDL concentrations through drug administration is nowadays the most effective way to decrease atherogenic risk, these synthetic lipid-lowering drugs have been shown to cause serious side effects in various clinical settings. As such, there are attempts to find anti-hyperlipidemia agents from natural sources possessing lesser adverse effects. In previous studies, morelloflavone, a biflavonoid abundant in *Garcinia dulcis* leaves, has exhibited its lipid-lowering property in high-cholesterol fed rabbits. However, a mechanism underlying such beneficial effect of this compound is not known. Several plant derived flavonoids have been reported for their inhibitory activities on HMG-CoA reductase, the key enzyme in the early step of cholesterol biosynthesis pathway and the main target of statins, the most prescribed cholesterol-lowering drugs. Therefore, to investigate whether morelloflavone would reduce blood cholesterol levels primarily through the inhibition of HMG-CoA reductase, morelloflavone from *G. dulcis* was then examined for its effect on this enzyme both *in vitro* and *in vivo*.

For the kinetics study, the catalytic domain of house mouse HMG-CoA reductase prepared by transforming plasmid pET 17b-CD-HMG to *Escherichia coli* of BL21 strain, catalyzed the reaction at a maximum velocity (V_{max}) of 5 ± 0.26 μM NADPH/mg/min, with the Michaelis constant (K_m) values for HMG-CoA and NADPH calculated to be 74 ± 0.06 μM and 133 ± 0.21 μM, respectively. Morelloflavone inhibited the enzyme by competing with HMG-CoA resulting in the inhibition constant (K_i) of 80.87 ± 0.06 μM but exhibited noncompetitive inhibition towards NADPH with K_i of 103 ± 0.07 μM. In accordance with morelloflavone, both

flavonoid subunits, narigenin and luteolin, competed with HMG-CoA with similar K_i values of 83.58 ± 4.37 μM and 83.59 ± 0.94 μM, respectively. These isoflavone compounds were also noncompetitive with NADPH and gave K_i of 182 ± 0.67 μM and 188 ± 0.14 μM, respectively. Due to the finding that morelloflavone and its subunits were equally effective at inhibiting HMG-CoA reductase, it was likely that this biflavonoid would bind with the enzyme active site *via* either naringenin or luteolin subunits. The moiety favorable for such binding could be the phenolic structure (ring B), commonly existing in both flavonoids and having HMG-like feature.

In animal experiments, treatment with morelloflavone at a dose of 0.01% (w/w/day) for 6 weeks showed no effect on plasma lipids in diet-induced hypercholesterolemic rats. In normal rats receiving the compound, however, their HDL-cholesterol levels became elevated indicating the stimulation of circulating HDL particles in these animals. A tendency for enhanced hepatic mRNA levels of HMG-CoA reductase and LDL receptor in response to the suppression of cellular cholesterol synthesis was also detected upon morelloflavone administration.

The findings from this study thus indicate a potentiality of morelloflavone isolated from *G. dulcis* as an effective HMG-CoA reductase inhibitor that might be developed into a new hypocholesterolemic agent in the future.

ACKNOWLEDGEMENTS

I would like to express a sincere gratitude to my advisor, Associate Professor Dr. Nongporn Towatana, for her guidance, patience, enthusiasm, and support until completion of my study. I am also grateful to my co-advisor, Dr. Rapiporn Sotthibhandhu, for her very kind and generous support, and academic advice throughout this work.

I would like to thank Dr. Decha Pinkaew for morelloflavone, his technical advice, and criticism of my work.

My special thank is also due to Dr. Sriwan Wongwisansri at Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand for the recombinant plasmids pET 17b-CD-HMG and *E. coli* BL21 competent cells used in this study.

I feel indebt to grant-in-aids from Prince of Songkla University Graduate Research Fund, from Department of Biochemistry through Academic Strengthening Program for Excellence in Biochemistry, and from Prince of Songkla University Research Fund (Grant No. SCI5122020019S).

I am thankful to all members of Department of Biochemistry, Faculty of Science, Prince of Songkla University, for their hospitalities and helps in many ways during my study time.

My sincere thanks are due to my friends whose moral support and encouragement helped me to achieve this success.

I wish to express the deepest gratefulness to my parents and to my dear sister for their continuing support and encouragement without which it would have not been possible for me to pursue my education.

Ku-aida Tuansulong

CONTENTS

CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF ABBREVIATIONS

cDNA	=	Complementary deoxynucleic acid
DMSO	=	Dimethyl sulfoxide
DTT	=	Dithiotreitol
EDTA	=	Ethylenediamine tetraacetic acid
E. coli	=	*Escherichia coli*
g	=	Gravity force
g	=	Gram
h	=	Hour
HDL-C	=	High density lipoprotein cholesterol
HMG-CoA	=	3-Hydroxy-3-methylglutaryl coenzyme A
kDa	=	KiloDalton
K_m	=	Michalis Menten constant
LB broth	=	Luria-Bertani broth
M	=	Molar
min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
NADPH	=	β-nicotinamide adenine dinucleotide phosphate, reduced form
nm	=	Nanometer
OD	=	Optical density
pH	=	-Log hydrogen ion concentration
RT-PCR	=	Reverse transcriptase polymerase chain reaction
SDS-PAGE	=	Sodium dodecylsulfate polyacrylamide gel electrophoresis
sec	=	Second
V_{max}	=	Maximum rate of reaction
%	=	Percent
°C	=	Degree Celsius

LIST OF ABBREVIATIONS (CONTINUED)

µg	=	Microgram
µl	=	Microliter
µm	=	Micrometer
v/v	=	Volume by volume
w/w	=	Weight by weight

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Epidemiological studies have indicated that high blood cholesterol or hypercholesterolemia confers high risk for atherosclerosis and occlusive related vascular disorders (Levy and Brink, 2005). According to the world health report 2002 by the World Health Organization (WHO), hypercholesterolemia causes 18% of cerebrovascular disease and 56% of ischemic heart disease among populations all over the world (WHO, 2002). Although therapeutic life style changes including low-fat diet, weight management, and increased physical activity are important in a regulation of blood cholesterol, but in many cases, pharmaceutical lipid regulating interventions are also required. HMG-CoA reductase or 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34) catalyzes the rate-limiting step in cholesterol biosynthesis. Therefore, inhibition of this enzyme is the major target for lowering of blood cholesterol level. A class of HMG-CoA reductase inhibitors collectively called statins have been developed since the first compound was discovered by Endo and Kuroda in 1970s (Endo, 2004). They remain the drug of choice for blood cholesterol lowering in people with or at risk of cardiovascular disease until now. Although, these drugs exert health benefits, they also cause adverse effects in various clinical settings (Wagstaff *et al*., 2003). Therefore, a search for naturally occurring compounds as regulators of cholesterol metabolism has been focused. Flavonoids are a large group of natural polyphenolic compounds ubiquitous in the plant kingdom. They exert a broad range of biological and pharmacological activities (Havsteen, 2002). Flavonoids from various sources have been shown to cause significant reduction of hepatic HMG-CoA reductase in experimental animals (Lee *et al*., 1999; Choi *et al.*, 2001; Bok *et al*., 2002; Ohtsuki *et al.*, 2003) and to inhibit the activity of this enzyme *in vitro* (Sung *et al.*, 2004).

Garcinia dulcis (Roxb.) Kurz, commonly known as Maphuut in Thai, is a tropical fruit tree belonging to the Clusiaceae (Guttiferae) family that grows mainly in Southeast Asia (Yupwattanaphun *et. al*., 2002). All parts of this plant have been reported to contain a biflavonoid, morelloflavone (5, 7, 4', 5'', 7'', 3''', 4'''-heptahydroxy-[3, 8'']-flavonylflavanone) (Ansari *et al*., 1976; Deachathai *et al*., 2005; 2006; 2008). This compound possesses many remarkable biological activities such as anti-HIV (Lin *et al*., 1997), anti-inflammation (Gil *et al.*, 1997), anti-oxidant (Hutadilok-Towatana *et al.*, 2007), anti-malaria (Ngouamegne *et al.*, 2008), and anti-tumor (Pang *et al.*, 2009). In the previous study, morelloflavone from *G. dulcis* leaf has been found to lower blood cholesterol level in diet-induced hypercholesterolemic rabbits (Decha-Dier *et al*., 2008). Therefore, to elucidate the mechanism underlying that hypocholesterolemic action of morelloflavone, the current study was then undertaken both *in vitro* and *in vivo*. In Part 1, the inhibitory effect of morelloflavone on HMG-CoA reductase activity was determined. Its effect on hepatic HMG-CoA reductase expression and activity in hypercholesterolemic rats was also studied in second part.

1.2 Objectives

The general aim of this thesis is:

To investigate whether morelloflavone from *G. dulcis* could inhibit HMG-CoA reductase *in vitro* and *in vivo*.

The specific aims are:

1. To determine the effects of morelloflavone on HMG-CoA reductase kinetics in comparison with those of the enzyme inhibitors, by using HMG-CoA reductase produced from recombinant DNA as a study model.

2. To preliminarily investigate the regulatory effects of this compound on both mRNA expression and activity of hepatic HMG-CoA reductase in high-fat diet induced hypercholesterolemic rats.

1.3 Benefits Gained

Basically, *G. dulcis* or Ma-phuut will be more well-known and become more valuable. Some insights into the mechanism underlying hypolipidemic/anti-atherosclerotic action of its chemical constituent, morelloflavone, will be obtained. Taken together, all results from the recent past and present studies will substantiate the potential of this natural compound to be medicinally applicable for the management of atherosclerosis associated hypercholesterolemia in the future.

The publication arisen from this thesis is:

Tuansulong, K., Hutadilok-Towatana, N., Sotthibhandhu, R., Mahabusarakam, W., and Pinkaew, D. (2009) Morelloflavone inhibits HMG-CoA reductase activity. In: Proceedings for the 2nd Biochemistry and Molecular Biology Conference, 7-8 May 2009, Khon Kaen, Thailand, pp. 62-66.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Blood cholesterol

2.1.1 Hypercholesterolemia

Cholesterol is the most abundant steroid in our body. It serves as a membrane structural component and a precursor of adrenal and gonadal hormones, vitamin D, and bile acids. Over 70% of total cholesterol in individuals arises from endogenous biosynthesis mainly in liver and intestine, and the remainder is provided by the diet (Grundy, 1988). In blood stream, cholesterol resides within two kinds of lipoproteins: low density lipoprotein (LDL) which carries this lipid of both dietary and *de novo* synthesis origins from liver to peripheral tissues for cellular uptake and utilization, and high density lipoprotein (HDL) which transports cholesterol from tissues to liver for degradation and excretion. Since LDL has the cholesterol content about 45% whereas HDL contains only 18%, thus any changes in LDL level unquestionably impacts on the total amount of cholesterol in the circulation (Bettelheim and March, 1991). At the present time, a high LDL level together with a low HDL level is well accepted as a symptom of faulty cholesterol transport and also a warning sign for possible 'atherosclerosis' or the hardening and narrowing of the arteries, a major risk factor for cardiovascular diseases (CVD) (Myant, 1990). Therefore, the significance of any particular cholesterol level cannot be assessed without taking into account either the total cholesterol (TC)/HDL ratio or LDL/HDL ratio. According to the blood lipid guidelines shown below, an acceptable TC/HDL ratio for a low risk of CVD is 5 or less for men, and women is 4.5 or less. The ability of HDL to predict the development of coronary atherosclerosis has been estimated to be about four times greater than LDL (www.exrx.net/Testing/LDL&HDL.html).

	TC/HDL ratios	
Risk Levels	***Men***	***Women***
Very low	<3.4	<3.3
Low	4.0	3.8
Average	5.0	4.5
Moderate	9.5	7.0
High	>23	>11

	LDL/HDL ratios	
Risk Levels	***Men***	***Women***
Very low	1.0	1.5
Average	3.6	3.2
Moderate	6.3	5.0
High	8.0	6.1

Source: www.exrx.net/Testing/LDL&HDL.html.

In certain cases, however, when the blood cholesterol level rises above 7-8 mmol/L or 240 mg/dL and it is known as hypercholesterolemia (Chait *et al.,* 1993). This abnormal condition is not a disease in itself but has a good correlation with various circulatory diseases (Levy and Brink, 2005). It plays a crucial part in atherogenesis, along with accompanying high blood pressure, which may lead to heart attack or stroke. Hypercholesterolemia has been estimated to cause nearly 8% of total deaths worldwide (World Health Organization, 2002).

2.1.2 Causes of hypercholesterolemia

Both the dietary and environmental factors determine individual's circulating cholesterol concentration. Based on data from a number of survey studies, the populations in western countries have their average blood cholesterol levels higher than those in Asia (Sung *et al.,* 2002). The high blood cholesterol among western people is in connection with their lifestyles and likely to be influenced by their dietary

intakes which are rich in saturated fats (Mirmiran *et al.,* 2008). In comparison to unsaturated fat-fed animals, the conversion of cholesterol to bile acids in those fed with saturated fat is lower (Bravo *et al.,* 1997). Down-regulation of LDL receptor by saturated fatty acids has also been evident (Bucci *et al.,* 1998).

High cholesterol levels can also run in families which is called 'familial hypercholesterolemia'. Familial hypercholesterolemia is an autosomal codominant inherited disorder of lipoprotein metabolism. It is clinically characterized by dramatically elevated levels of total and LDL-cholesterol in the circulation as well as the presence of tendon xanthomas and premature atherosclerosis (Artieda *et al.,* 2005). The genetic basis of familial hypercholesterolemia resulted from the LDL receptor gene (LDLR) deficiency is well-described. A large array of mutations in LDLR with varying frequency in the population, leads to a lack of functional receptors for LDL on the cell surface and thus a reduced rate of plasma LDL clearance. As a result, the elevated LDL level causes hypercholesterolemia (Goldstein *et al.,* 2001). Similarly, mutations in the gene encoding for apolipoprotein B (APOB) also reduce LDL clearance, resulting in the familial defective apolipoprotein B, which is clinically indistinguishable from familial hypercholesterolemia (Defesche *et al.,* 1993; Boren *et al.,* 2001). Recently, a third putative familial hypercholesterolemia locus located on chromosome 1, encoding proprotein convertase subtilisin/kexin 9 (PCSK9) was identified (Abifadel *et al.,* 2003). The clinical phenotype caused by mutations in LDLR, APOB or PCSK9 , characterized by elevated levels of plasma LDL-cholesterol (LDL-C) is nowadays referred to as autosomal dominant hypercholesterolemia (Alonso *et al.,* 2009). Homozygosity for the apolipoprotein E allele which does not bind to the LDL receptor also has severe effects on familial hypercholesterolemia phenotype (Nagasaka *et al.*, 2008).

2.1.3 Hypercholesterolemia treatments

The formation of foam cells induced by oxidative modification of LDL is the hall mark of atherosclerosis (Shafi *et al.,* 2000). Accumulation of LDL particles in the circulation confers higher risk for these lipoproteins to be oxidized and taken up

by macrophages to become foam cells. For this reason, LDL-C has been recognized as an important determinant of clinical atherosclerotic diseases and thus treatment for hypercholesterolemia is usually focused on management of high LDL-C levels. In some cases, LDL-C can be lowered without medication (nonpharmacological therapy), and in other cases, medication is necessary.

2.1.3.1 Nonpharmacological therapy

Standard nonpharmacological therapy consists primarily of modifying diet and lifestyle. This therapy may modestly reduce LDL-C, but is not likely to lower the LDL-C level more than about 30 mg/dl (Sloane *et al.,* 2007).

Therapeutic lifestyle features that may lower LDL-C levels as recommended by the National Cholesterol Education Program (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001) include:

Diet change: Minimizing cholesterol and fat intake, especially saturated fat which raises cholesterol levels, together with increasing dietary fiber and plant stanols/sterols.

Weight management: Losing a modest amount of weight can reduce in LDL levels achieved through an improved diet.

Increased physical activity: Exercise can decrease LDL and increase HDL levels.

2.1.3.2 Pharmacological therapy (Medication)

Hypocholesterolemic agents are divided into five major classes.

1) Bile acid sequestrants

Bile acid sequestrants are cross-linked polymeric cationic gels (anion-exchange resins) that bind to the negatively charged bile acids in the intestine. Because these sorbents are large-sized, they are not absorbed into the body and thus the bound bile acids are excreted with the stool (Stedronsky, 1994). Consequently, the liver's pool of bile acids is depleted, leading to increased conversion of cholesterol to bile acids in hepatocytes. Decline in hepatic cholesterol content stimulates the

production of LDL receptors causing increased LDL clearance and a net decrease in blood cholesterol due to lower LDL-C level (Jain *et al.,* 2007). However, this effect is partially offset by the enhanced cholesterol synthesis caused by the up-regulation of HMG-CoA reductase (Jain *et al.,* 2007).

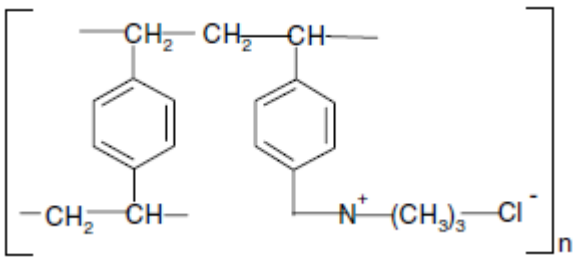


Figure 1 Structure of cholestyramine, a typical bile acid sequestrant (Jain *et al.,* 2007).

With over 30 years of clinical experience, the use of cationic polymers for sequestration of bile acids has become an established approach for treating elevated blood cholesterol levels (Zarras and Vogl, 1999). Cholestyramine, a quaternary amine, and colestipol, a mixture of tertiary and quaternary diamines, are the two widely used bile acid sequestrants (Huval *et al.,* 2001). Cholestyramine is a polymer of styrene and divinylbenzene with active sites formed from trimethylbenzylammonium groups. Colestipol is a copolymer of diethylenetriamine and chloro-2, 3-epoxypropane. These two first generation bile acid sequestrants, however, have low clinical potency, considered to be due to a competition between the polymeric sequestrant and active bile acid reuptake transporter system of the gastrointestinal tract (Huval *et al.,* 2004). Recently, a more potent bile acid sequestrant, colesevelam, has been launched (Donovan *et al*., 2000). This drug has high binding capacity, strong binding strength, and selectivity towards bile acids in the presence of competing desorbing forces of the gastrointestinal tract (Huval *et al.,* 2004). Despite the appeal of their safety profiles, bile acid sequestering agents may

contribute to calcium loss and therefore increase the risk for osteoporosis. Overtime deficiencies of vitamin A, D, E, and K may occur also (Jain *et al.,* 2007).

2) Niacin

Niacin (nicotinic acid) is a water-soluble vitamin of B type. It has been widely used clinically to regulate abnormalities in lipid/lipoprotein metabolism and in the treatment of atherosclerotic coronary heart disease (Meyers *et al.,* 2004). It inhibits the lipolysis of triglycerides by hormone-sensitive lipase, which reduces transport of free fatty acids to liver and decreases hepatic triglyceride synthesis by inhibiting both the synthesis and esterification of fatty acids that increases apolipoprotein (apo) B degradation (Aithul *et al.,* 1955 as cited by Jain *et al.,* 2007). Reduction of triglyceride synthesis reduces hepatic VLDL production, which accounts for the reduced LDL levels. Niacin enhances lipoprotein lipase activity, which promotes the clearance of chylomicrons and VLDL-triglycerides. It also raises HDL-C levels by decreasing the fractional clearance of apo A-1 in HDL, rather than by enhancing HDL synthesis (Knopp *et al.,* 1985). These unique beneficial effects of niacin on lipoproteins have been assumed to contribute to its anti-atherosclerotic properties. Clinical studies have demonstrated that niacin alone or in combination can slow or reverse the progression of atherosclerosis, and reduce cardiovascular event rates and total mortality in patients with hypercholesterolemia and established atherosclerotic cardiovascular disease (Meyers *et al.,* 2004). In recent trial studies, a combination of niacin with simvastatin has shown more protective effects than simvastatin monotherapy in patients with elevated non-HDL-C by increasing more plasma HDL-C levels as well as lowering more triglyceride levels (Sanford and Curran, 2008).

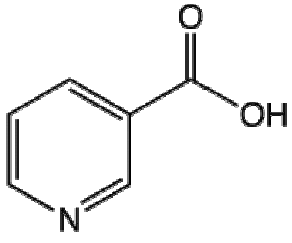


Figure 2 Structure of niacin (Jain *et al.,* 2007)

3) Fibrates (fibric acid derivatives) or lipoprotein lipase stimulants

The primary effect of fibrates is a significant reduction in triglyceride levels. Additionally, increased HDL-C and decreased LDL-C levels may also be obtained (Birjmohun *et al.,* 2005). Major anti-hypertriglyceridemic actions of fibrates are increased lipolysis, increased hepatic fatty acid uptake, and reduced hepatic triglyceride production (Steals *et al.,* 1998). Fibrates seem to reduce plasma cholesterol to a variable extent from their ability to reduce intestinal cholesterol absorption, and more importantly, to increase the biliary output of cholesterol and phospholipids while decreasing the biliary secretion of bile acids by hepatic cholesterol 7α-hydroxylase inhibition (Sirtori *et al*., 1992; Catapano *et al.*, 1992; Bertolotti *et al*., 1995). Since the discovery of clofibrate (ethyl ester of *p*-chlorophenoxyisobutyrate) as an effective anti-lipidemic agent in the mid-1960s (Gennes *et al.,* 1965), four more fibrate derivatives, gemfibrozil, fenofibrate, ciprofibrate, and benzafibrate, are now prescribed.

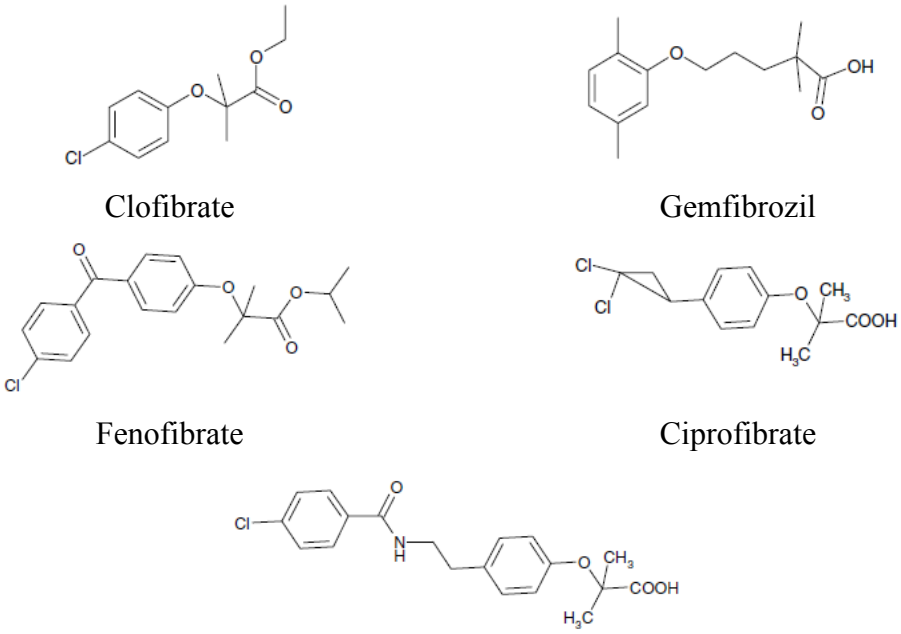


Figure 3 Structure of the five different fibrates (Jain *et al.,* 2007).

These fibrate agents are agonists of the peroxisome proliferator-activated receptor alpha (PPARα) group of nuclear receptors, which control the expression of key elements in cholesterol transport and lipid metabolism, as well as endothelial function, vascular inflammation and remodeling (Steals *et al.,* 1998; Lock *et al.,* 1989; Duval *et al.,* 2007; Zandbergen and Plutzky, 2007). Their agonistic effects on PPARα result in the peripheral lipolysis of triglyceride rich lipoproteins through the hepatic stimulation of the lipoprotein lipase gene, a reduction in apo C-III, resuting in a stimulation of the catabolism of triglyceride-rich lipoproteins, mainly VLDL and IDL (Shepherd, 1995). Fibrate-mediated PPARα stimulation of apo A-I and apo A-II expression also increases HDL-cholesterol levels (Steals *et al.,* 1998). An increased hepatic expression of sterol regulatory element binding protein-2 (SREBP-2), responsible for higher HMG-CoA reductase and phosphatidate phosphohydrolase activities in gemfibrozil administered rats, has been reported (Roglans *et al.*, 2001)

4) Ezetimibe

Ezetimibe is also known as a cholesterol absorption inhibitor. This drug localizes at the brush border of the small intestine, where it inhibits the absorption of cholesterol from the diet. Specifically, ezetimibe appears to bind to a critical mediator of cholesterol absorption, the Niemann-Pick C1-Like 1 (NPC1L1) protein on the gastrointestinal tract epithelial cells (Garcia-Calvo *et al*., 2005) as well as in hepatocytes (Temel *et al*., 2007). In addition to this direct effect, decreased cholesterol absorption leads to an up-regulation of LDL-receptors on the cell surface and thus decreases LDL levels in the blood (Repa *et al.,* 2005). In clinical practice, ezetimibe replaces bile acid sequestrants as second-line therapy for the treatment of familial hyperlipidemias and in most drug-intolerant patients. In comparison to the resins, ezetimibe shows significantly lower gastrointestinal side-effects (Sweeney *et al.,* 2007; Florentin, 2008). It is able to reduce LDL-C by 15–25% from baseline (Mikhailidis *et al.,* 2005; Capps, 2006). Besides monotherapy, ezetimibe is commonly used in combination with other agents (Toth and Davidson, 2005). Co-administration of ezetimibe and fenofibrate is more efficacious than fenofibrate alone.

It greatly reduces LDL-C, as well as significantly improves triglycerides, HDL-C total cholesterol, non-HDL-C, and apo B levels in patients with mixed hyperlipidemia (McKenney *et al.*, 2006), while the addition of ezetimibe to niacin-based regimens lowers LDL-C level by 25% but does not change the level of HDL-C (Jelesoff, 2006). Its concurrent use with simvastatin represents the most common combined therapy, due to the fact that ezetimibe can add an extra 20% reduction in LDL-cholesterol to that produced by statins alone (Wierzbicki *et al.,* 2005). The molecular mechanism for this enhanced efficacy has not been identified. Apo B-100 kinetics in pigs treated with ezetimibe plus simvastatin have revealed that such combination increases NPC1L1 expression in liver and intestine, consistent with increased SREBP2 expression, and also decreases VLDL and LDL apo B-100 concentrations through reduced VLDL production and up-regulation of LDL receptor-mediated LDL clearance (Telford *et al.*, 2007). LDL receptor protein expression as well as LDL receptor and HMG-CoA reductase gene expression upon their combination therapy have been studied in healthy men. The results demonstrate that the co-administration abrogates the ezetimibe-induced increase in cholesterol synthesis and up-regulates the LDLR gene but not protein expression, possibly through a parallel up-regulation of PCSK9 expression (Gouni-Berthold *et al.*, 2009).

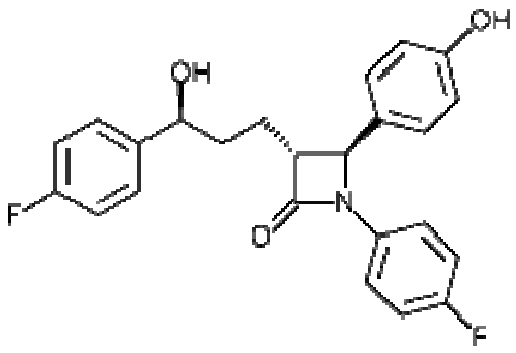


Figure 4 Structure of ezetimibe (http://www.biochemsoctrans.org/bst).

5) Statins or HMG-CoA reductase inhibitors

In the early 1970s, Dr. Akira Endo and his research team at Sankyo Co. Ltd. in Tokyo, isolated and studied various fungal metabolites from *Penicillium citrinium* for their hypocholesterolemic activities through the inhibition of HMG-CoA reductase which catalyzes the first committed step of the endogenous cholesterol biosynthetic pathway. The discovery of a potent inhibitor namely 'mevastatin' (formerly called ML-226B or compactin) in 1973 was a key step toward attaining an effective means of lowering blood cholesterol levels in humans (Endo, 2004). In 1976, lovastatin (mevinolin or monacolin K from *Monascus ruber*) produced from *Aspergillus terreus*, became the first statin to be commercially marketed. Since then, a number of statin derivatives have been developed. This class of agents, fermentation-derived and synthetic ones with varying potency, becomes the most commonly prescribed medications for blood cholesterol reduction in people with or at cardiovascular risk nowadays (Kapur *et al.*, 2008). Statins also have a wide range of 'pleiotropic effects' independent of cholesterol-lowering (Tandon *et al.*, 2005). These include improvement of endothelial dysfunction, increased nitric oxide bioavailability, antioxidant properties, inhibition of inflammatory responses, stabilization of atherosclerotic plaques, recruit endothelial progenitor cell, a putative immunosuppressive activity, and inhibition of cardiac hypertrophy (Davignon, 2009). Such beneficial effects of statins have been known to be mediated through inhibition of small GTP-binding proteins (Turner *et al.,* 2008; Rashid *et al.*, 2009).

In 1980, Drs. Michael Brown and Joseph Goldstein at University of Texas Southwestern in Dallas, USA established that statins act by competitively inhibiting HMG-CoA reductase which is the rate-limiting enzyme of the mevalonate pathway of cholesterol synthesis in mammals (Brown and Goldstein, 1980). Due to the fact that they possess a side group that is structurally similar to HMG-CoA, statins then act by competitively inhibiting HMG-CoA reductase activity in the liver which results in decreased cholesterol synthesis (Istvan, 2003). Inside the liver cell, other enzymes of the protease class sense the reduced intracellular cholesterol content. In response, they cleave a group of proteins called 'membrane-bound sterol regulatory element binding proteins', which then migrate to the nucleus and up-regulate

production of various proteins and enzymes, including LDL receptor. This results in an enhanced clearance of LDL particles from the bloodstream and eventually lower circulating cholesterol level (Ma *et al*., 1986).

Although statins are the most potent cholesterol-lowering drugs available, their treatments are associated with major side effects including increased liver enzymes and skeletal muscle pain and/or damage due to creatine kinase inhibition (Bybee *et al.,* 2004), and induced memory loss (Endres *et al.,* 1998). More serious but rare reactions include myositis and myopathy, with the potential for rhabdomyolysis leading to acute renal failure (Graham *et al.,* 2004). A common variation in the SLCO1B1 gene, which participates in the absorption of statins, has been shown to significantly increase the risk of myopathy (The research collaborative group, 2008). Among currently used statins, the risk of myopathy is lowest with pravastatin and fluvastatin probably because they are more hydrophilic and as a result have less muscle penetration (Pierno *et al.*, 1995). Lovastatin induces the expression of gene atrogin-1, which is believed to be responsible in promoting muscle fiber damage (Hanai *et al.,* 2007).

Besides inhibiting cholesterolgenesis, statin therapy also limits availability of mevalonate, the ubiquinone precursor, leading to lower levels of coenzyme Q10 (Ghirlanda *et al*., 1993). The deficiency of coenzyme Q10 which is an essential cofactor in the mitochondrial electron transport and also a lipid-soluble antioxidant, has been implicated in several clinical disorders, including heart failure, hypertension, Parkinson's disease, and malignancy (Molyneux *et al.,* 2008). Despite initial concerns that statins might increase the risk of cancer, a systematic review and meta-analysis of statin trials and observational studies have concluded later that statins have no influence on cancer risk (Browning and Martin*,* 2007). Though, a 2005 trial has revealed that patients taking statins for over 5 years reduced their risk of colorectal cancer by 50% (Poynter *et al.,* 2005). A secondary analysis of data performed in large colorectal adenoma chemoprevention trials has found no association between statin use and reduced risk of recurrence of colorectal carcinomas (Wei *et al.*, 2005). As statins inhibit the acetyl CoA pathway of mevalonate, resulting in the decreased formation of higher isoprenoids which are necessary for post-

translational processing, anti-neoplastic effects of these drugs through inhibition of protein prenylation have been demonstrated in a variety of cancer cell lines (van de Donk *et al.*, 2002; Otsuki et al., 2004; Shellman *et al.*, 2005; Glynn *et al.,* 2008; Maksimova *et al*., 2008; Nonaka *et al*., 2009).

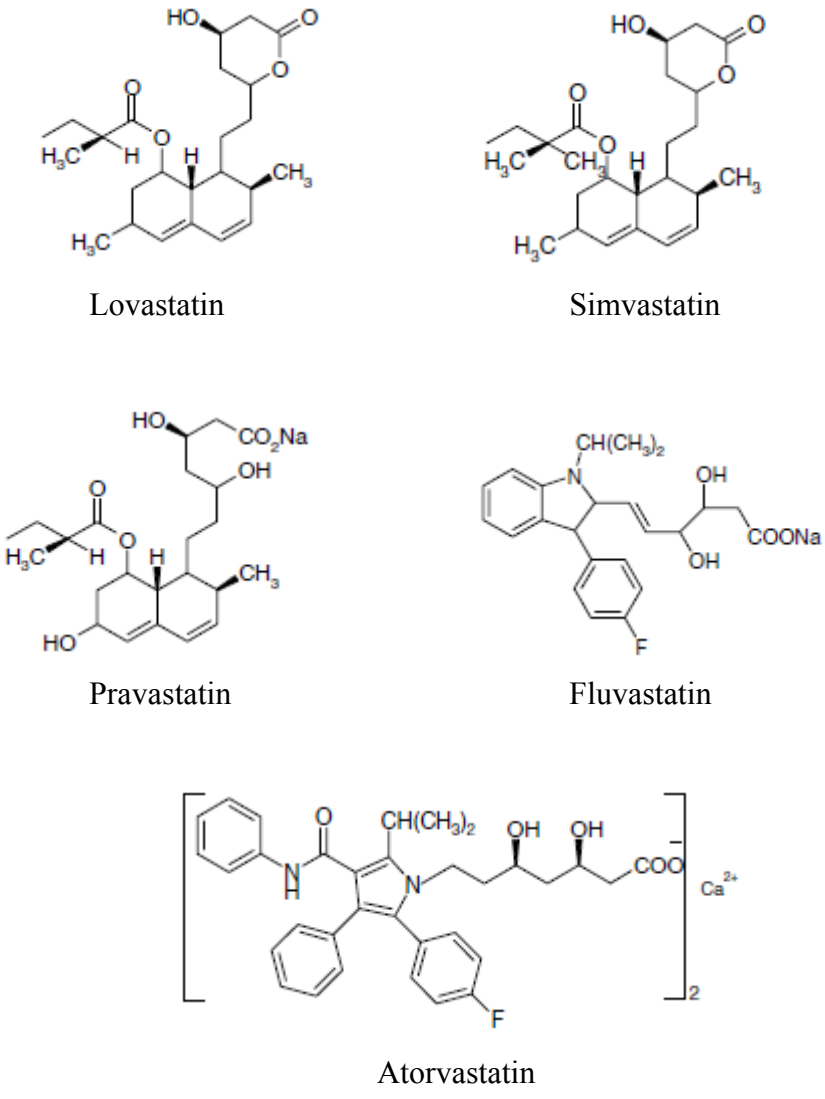


Figure 5 Structure of commonly used statins (Jain *et al.,* 2007).

2.2 HMG-CoA reductase

2.2.1 Characteristics

Basically, all body tissues containing nucleated cells are capable of synthesizing cholesterol but about two-third of endogenously produced cholesterol comes from liver alone (Williams *et al.,* 2002). The cholesterol biosynthesis pathway begins with conversion of acetyl CoA to HMG-CoA as shown in Figure 6. HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase, EC 1.1.1.88), which catalyzes the reduction of HMG-CoA to mevalonate, is the rate limiting enzyme of this pathway and provides feedback regulation of cholesterol synthesis in cells (Brown and Goldstein, 1980).

In humans, the gene for HMG-CoA reductase is located on the long arm of the fifth chromosome (5q13.3-14) (Cherng *et al.,* 2008). The enzyme has a molecular weight of about 97 kD and contains 887 amino acids in total. There are 39 out of 449 residues at the amino terminal region which act as a signal sequence for specific targeting to endoplasmic reticulum (ER) (Olender and Simoni, 1992). It is an intrinsic membrane-bound protein of the ER, containing eight transmembrane domains with the active site located in a long carboxyl terminal domain in the cytosol (Roitelman. *et al*., 1992). The enzyme normally forms a tightly associated tetramer, but it is only the r of the molecule that is crucial for catalysis (Istvan, 2003).

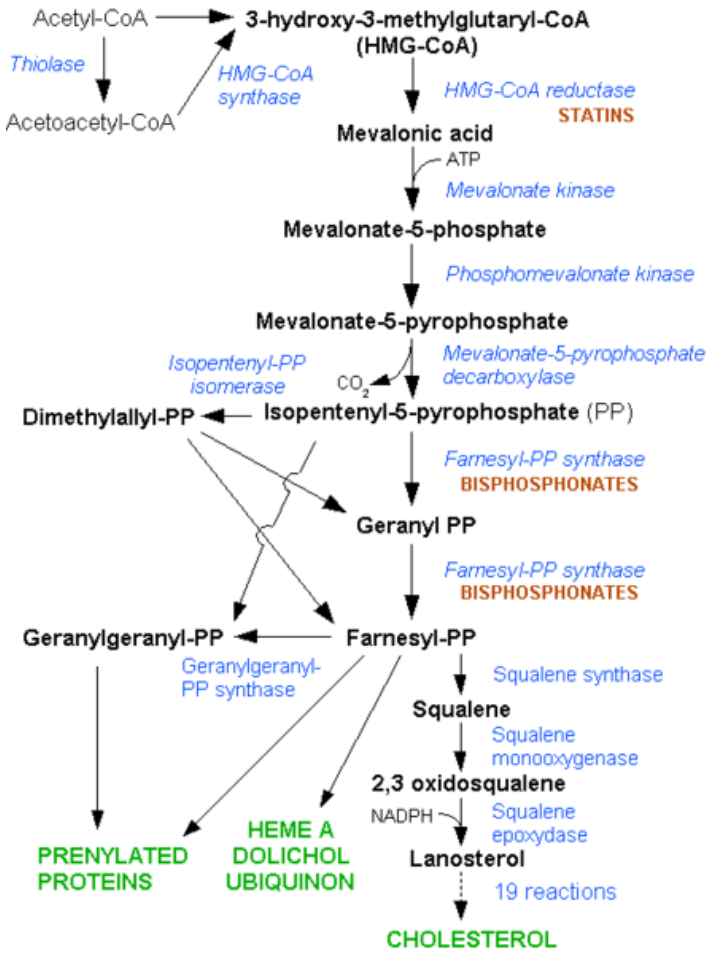


Figure 6 The mevalonate pathway of cholesterol synthesis (http://www.smbrower.com/mediawiki/images/8/8f/Cholesterol_Synthesis_Pathway.png).

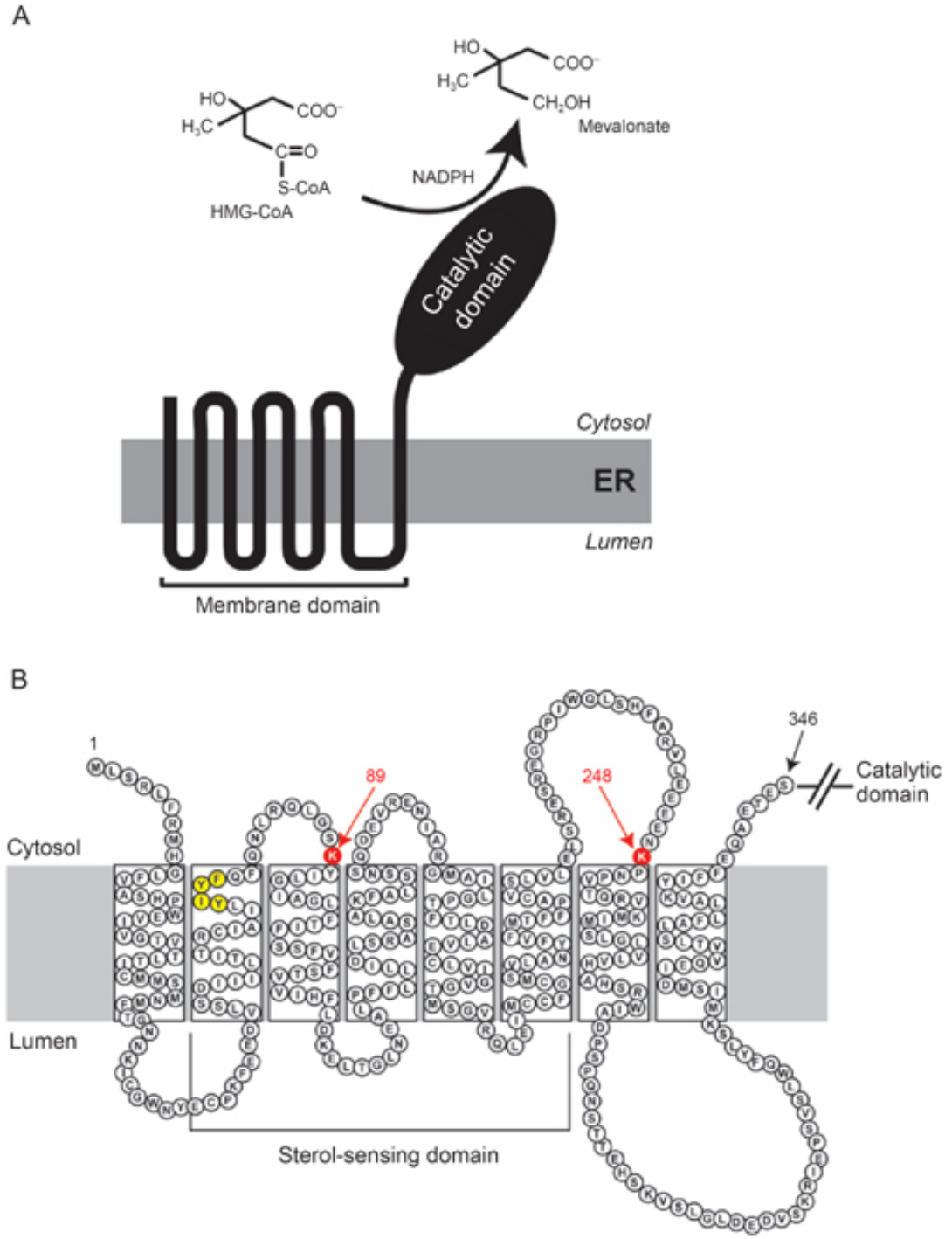
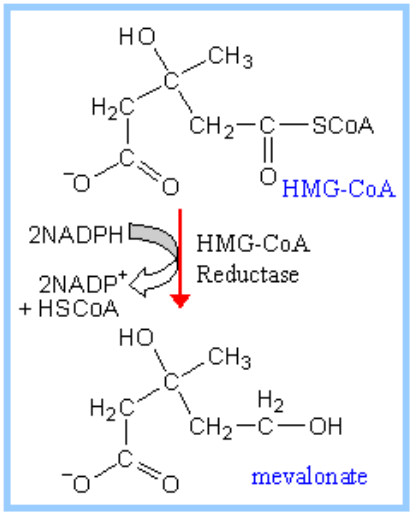


Figure 7 Structure of membrane-bound HMG-CoA reductase (Roitelman *et al.*, 1992)

HMG-CoA reductase is a NADPH dependent enzyme which catalyzes the reduction of HMG-CoA to mevalonate in two steps, as follows.



Source: www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/cholesterol.htm.

This reaction involves the transfer of 4 electrons *via* 2 molecules of NADPH cofactor to a substrate that has been activated for reaction with the sulfhydryl (-SH) containing coenzyme A (CoASH). Some of the key amino acid residues at the active site of this allosteric enzyme that bind to HMG-CoA have been identified (Jain *et al.,* 2007).

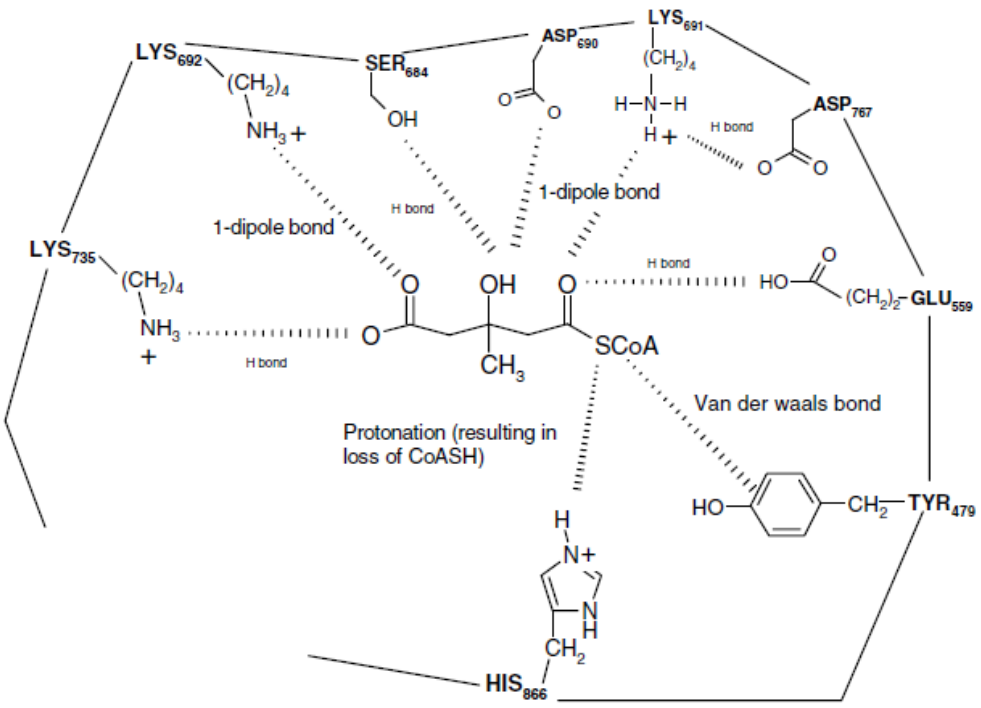


Figure 8 The substrate binding site of HMG-CoA reductase (Jain *et al.,* 2007).

According to Jain and co-workers (2007), the binding interactions of HMG-CoA reductase and its substrate include:

• Lys735 (in cationic conjugate acid form), which anchors the substrate to the enzyme through an ion-ion bond with the C5 anionic carboxylate group of HMG-CoA.

• Lys692, also in the cationic form, stabilizes the carbonyl oxygen of which group through an ion-dipole bond.

• The C3-OH group of the substrate, which is stabilized by two residues, Ser684 and Asp690. Serine acts as a H-donor to form a H-bond with the oxygen of the OH group, and anionic Asp forms an ion-dipole bond with the alcoholic hydrogen.

• Lys691 (in cationic conjugate acid form), engages in an ion-dipole bond with the carbonyl oxygen of C1. It is this carbonyl group that gets reduced to

the primary alcohol through the action of the HMGR enzyme. Lys691 is found in the region (or domain) of the receptor referred to as the *cis* loop.

• An anionic Asp767, promotes this important ion-dipole interaction by forming its own ion-ion bond with Lys residue. This interaction stabilizes the action for interaction with the HMG-CoA substrate.

• Glu559 (in unionized acid form), also forms hydrogen bonds to the same C1 carbonyl oxygen. By forming these two important bonds, this carbonyl group is held tightly to the enzyme, and is properly oriented for the reduction to occur. In addition to these interactions, Tyr479 engages through Van der Waals bond with the adenine base of the CoA portion of the substrate, forming a kind of 'hydrophobic shield' that closes down the binding pocket for effective reduction by the NADPH cofactor. During the first NADPH reduction, the doubly bonded nitrogen of His866 (in cationic acid form) acts as a hydrogen donor to the sulfur atom of the thioester (SCoA), to liberate CoASH from the substrate. This, in turn, produces the mevaldehyde intermediate.

2.2.2 Regulation of HMG-CoA reductase

The liver is the main organ responsible for controlling cholesterol homeostasis in the body. In mammalian cells, the intracellular concentration of cholesterol is regulated by the balance between endogenous synthesis and exogenous uptake). Endogenous synthesis is subjected to feedback control by hepatic HMG-CoA reductase activity, while the exogenous supply is mainly controlled by the modulation of LDL receptor (Brown and Goldstein, 1980; 1986). The feedback control of HMG-CoA reductase and LDL receptor occurs through regulation of gene transcription or by regulation of protein level through translation or degradation (Reynolds *et al.,* 1984). Regulation of HMG-CoA reductase is multi-valent as shown in Figure 7, being achieved at several levels; transcription, translation, degradation and phosphorylation (Cherng *et al.,* 2008).

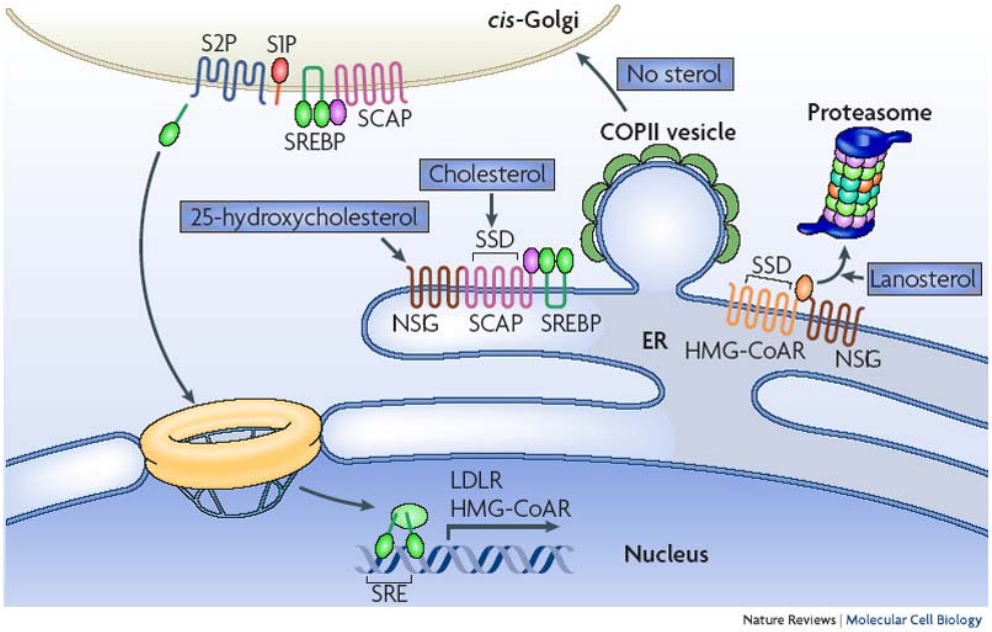


Figure 9 Regulation of intracellular cholesterol metabolism (http://www.nature.com/nrm/journal/v9/n2/fig_tab/nrm2336_F2.html).

1) Transcription of the gene

In 1993, Brown and Goldstein discovered that sterol regulatory element binding proteins (SREBPs) at ER membrane are responsible for the end-product feedback regulation of gene transcription factors controlling intracellular cholesterol and lipid metabolism (Bengoechea-Alonso and Ericsson, 2007). The SREBPs are synthesized as inactive proteins that are inserted into ER membrane through two membrane-spanning domains. In ER, the C terminus of SREBP interacts with a protein called 'SCAP' (SREBP-cleavage-activating protein), which functions as a sterol sensor. In sterol-depleted cells, SCAP escorts SREBPs as SREBP-SCAP complex from ER to Golgi body, where they are cleaved by two membrane-associated proteases, the site 1 (S1P) and site 2 (S2P) proteases. The active fragments of the SREBPs are translocated to the nucleus, where they bind to the promoters of SREBP target genes, including HMG-CoA reductase and LDL receptor genes. Under conditions of ample sterol, the ER retention protein 'INSIG' prevents entry of the SREBP-SCAP complex to Golgi *via* COPII-coated vesicles. HMG-CoA reductase is also post-transcriptionally regulated by lanosterol, the first sterol intermediate in the

cholesterol pathway, with INSIG binding of the enzyme molecule leading to its proteasomal degradation (Bengoechea-Alonso and Ericsson, 2007).

2) Translation of mRNA

It has been believed that mevalonate, the product of HMG-CoA reductase, regulates the reductase translation through its conversion to a non-sterol substance by a mechanism that is still to be defined. This proposal comes from the findings that Chinese hamster ovary cells develop high levels of both the reductase protein and activity when they are deprived of mevalonate. In the presence of sterols, the mRNA is partially suppressed whereas the level of reductase remains relatively high. When mevalonate is added, the mRNA level does not decline further but the level of the enzyme declines owing to both reduced translation and accelerated degradation of the enzyme protein (Brown and Goldstein, 1988).

3) Degradation of the reductase

Rising levels of intracellular sterols increase the susceptibility of the reductase enzyme to proteolysis. Sterol-sensing domain (SSD) at helices 2-6 of transmembrane HMG-CoA reductase senses the higher levels of lanosterol through its C4-dimethyl group, leading to ubiquitination of Lys248. This ubiquitinyl residue then serves as a signal for proteolytic degradation of the enzyme (Cherng *et al.,* 2008).

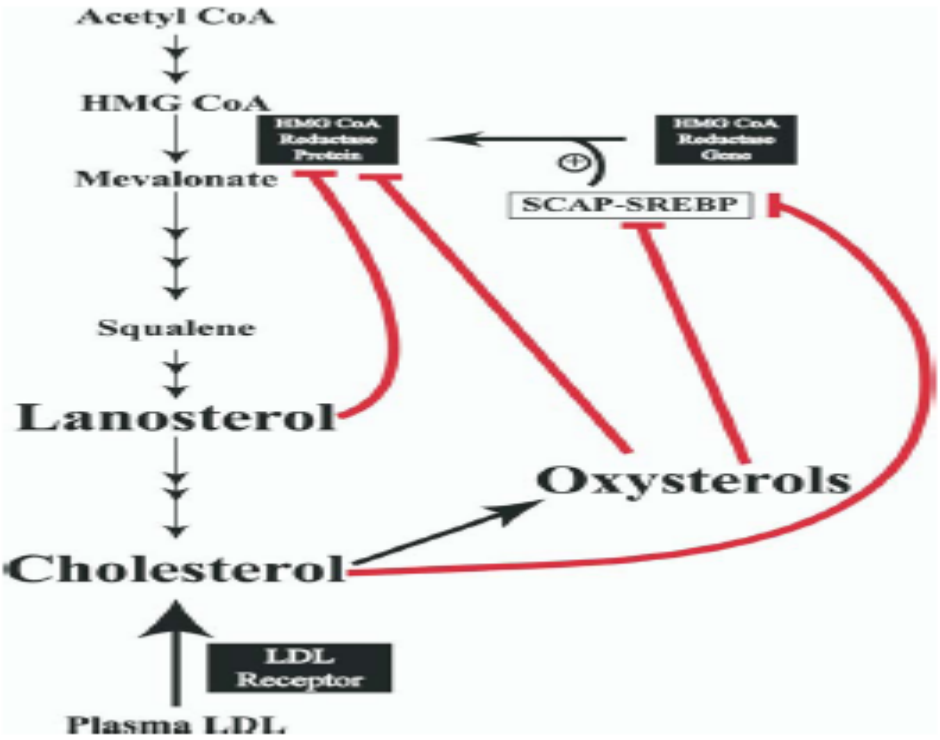


Figure 10 Model for sterol-mediated regulation of HMG CoA reductase (Song *et al.,* 2005)

Feedback control of cholesterol synthesis is also mediated in part by cholesterol and its oxy derivative (25-hydroxycholesterol) levels which induce binding of HMG CoA reductase to INSIG in ER. Such binding suppresses the activation of SREBPs, leads to ubiquitination, and accelerates proteasomal degradation of the reductase. Through these reactions, cholesterol synthesis is maintained such that important by-products of the synthetic pathway are continually provided, while at the same time avoiding the accumulation of cholesterol (Song *et al.,* 2005).

4) Phosphorylation of the reductase

Short-term regulation of the reductase is operated *via* phosphorylation and dephosphorylation processes. The principal enzymes involved

are AMP-activated kinase α (Hardie, 1992) and protein phosphatase 2A (Gaussin *et al.*, 1997). The phosphorylation site on HMG-CoA reductase has been identified at the C-terminal domain as Ser871 in rodents (Sato *et al.*, 1993) and Ser872 in humans (Istvan *et al*., 2000). The phosphorylated form of HMG-CoA reductase is inactive, while dephosphorylation activates it (Hardie, 1992).

According to a 2007 review (King, 2007), HMG-CoA reductase is phosphorylated by AMP-activated protein kinase α, (AMPKα). AMPK itself is activated *via* phosphorylation by at least 2 enzymes. The primary kinase sensitive to rising AMP levels is LKB1. The second AMPK phosphorylating enzyme is calmodulin-dependent protein kinase kinase (CaMKK). CaMKK induces phosphorylation of AMPK in response to increasing intracellular Ca^{2+}.

Additional regulation of HMG-CoA reductase occurs through the cAMP signaling pathway. An increase in cAMP leads to activation of cAMP-dependent protein kinase (PKA). PKA phosphorylates phosphoprotein phosphatase inhibitor-1 (PPI-1) leading to an increase in PPI-1 activity. PPI-1 then inhibits the activity of numerous phosphatases including protein phosphatase 2C and HMG-CoA reductase phosphatase which remove phosphates from AMPK and HMG-CoA reductase, respectively. This maintains AMPK in the phosphorylated and active state, and HMG-CoA reductase in the phosphorylated and inactive state. Conversely to glucagon and epinephrine which increase cAMP production, insulin stimulates the removal of phosphates, and thereby activates the enzyme activity.

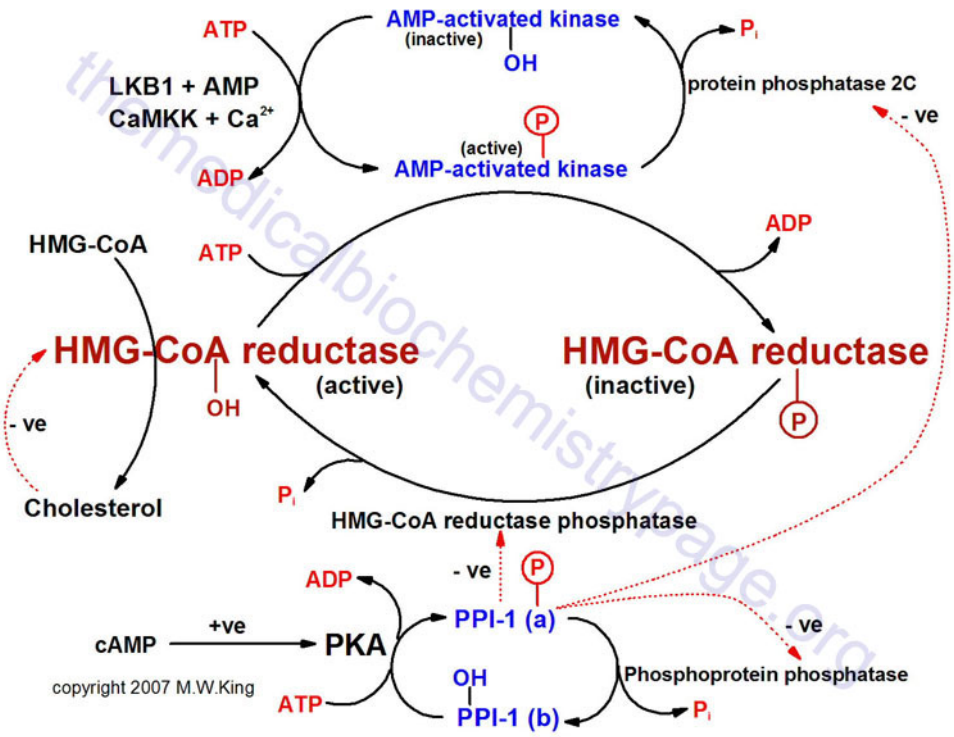


Figure 11 Regulation of HMG-CoA reductase by covalent modification as a result of phosphorylation and dephosphorylation (King, 2007).

2.2.3 Inhibitors of HMG-CoA reductase

Natural products from many sources have been reported to possess HMG-CoA reductase inhibitory properties.

In different animal studies, garlic supplemented diets decrease the hepatic cholesterol synthesis resulting from an inhibition of HMG-CoA reductase (Qureshi *et al.,* 1983a; 1983b; 1987). Organosulfur compounds present in garlic (*Allium sativum*), diallyl disulfide, allicin, allyl mercaptan, and ajoene, suppress HMG-CoA reductase in both rat hepatocytes and HepG2 cells (Gebhardt *et al.,* 1994; Gebhardt and Beck, 1996). Rai and co-workers have shown that diallyl disulfide analogs decrease the enzyme activity by reducing the mRNA level in hypercholesterolemic rat livers (Rai *et al*., 2009). Qureshi and co-workers have found that α-tocotrienol, a vitamin E analog, from barley potently inhibits HMG-CoA reductase in both rat and chick livers (Qureshi *et al.,* 1986), whereas δ- and γ-

tocotrienols show a greater effect on the enzyme inhibition than α-tocotrienol in endothelial cells (Chao *et al.*, 2002). *β*-sitosterol which is a major phytosterol present in seed oil such as black cumin seed, cashew, rice bran *etc*., decreases hepatic HMG-CoA reductase activity in rats (Gylling and Miettinen, 2005). The reduction of HMG-CoA reductase in rat livers have been detected following intake of a polyunsaturated fatty acid, docosahexaenoic acid (DHA), which is abundant in marine fish oil (Froyland *et al.*, 1996: Shirai and Suzuki, 2007). Dietary α-linolenic acid also shows the similar effect (Ihara-Watanabe *et al.*, 1999; 2000). Curcumin significantly lowers this liver enzyme in cholesterol fed rats (Murugan and Pari, 2006). Citrus flavonoids, hesperitin, hesperidin, naringenin and naringin, have been found to inhibit hepatic HMG-CoA reductase in several hypercholesterolemic animal models (Lee *et al.* 1999; Kim *et al.*, 2003; 2004). Quercetin, a bioflavonoid found in the skins of apples and red onions, significantly lowers this liver enzyme in high cholesterol fed rats (Bok *et al.*, 2002). Gugulipid from the resin of gugul tree (*Commiphora mukul*) also elicits its inhibitory effects against both liver and intestinal HMG-CoA reductase in cholesterol-fed rats (Kumari and Augusti, 2007). Resveratrol, a phytoalexin primarily found in grapes**,** significantly inhibits the hepatic HMG-CoA reductase activity in apo E-deficient mice (Do *et al.,* 2008) and attenuates the expression of this enzyme in hamsters (Cho *et al*., 2008). The hydroalcoholic extract of watercress *(Nasturtium officinale*) leaves attributes to a significantly decreased hepatic HMG CoA reductase activity in hypercholesterolemic rats (Bahramikia and Yazdanparast, 2008).

β-carotene and its precursor 'lycopene' from higher plants, inhibit this enzyme at a post-translational level, as demonstrated in rat livers (Moreno *et al.,* 1995) and in macrophages (Fuhrman *et al.,* 1997). Artichoke (*Cynara scolymus*) leaf extracts also inhibits HMG-CoA reductase activity in rat hepatocytes, and its constituents, cynaroside and luteolin, have been identified to be mainly responsible for the inhibition (Gebhardt, 1998). A flavonoid compound 'astilbin' (Chen *et al*., 2001) and tannin derivatives (Chang *et al*., 2001) isolated from different traditional Chinese herbs as well as their crude extracts (Liu *et al.*, 2002), show the inhibitory effects on HMG-CoA reductase in Vero cells. Policosanol, a defined mixture of high molecular weight aliphatic alcohols purified from sugar cane (*Saccharum officinarum*) wax,

suppresses an up-regulation of this enzyme in lipid-depleted cultured Vero fibroblasts (Menendez *et al*., 2001). FR901512, a novel compound with unique tetraline ring structure, has been isolated from an agonomycetus fungus, and strongly inhibits the enzyme activity in human hepatoma HepG2 cells with IC_{50} value of 1.0 nM (Hatori *et al.*, 2004). Soy isoflavone, genistein, and omega-3 fatty acids, eicosapentaenoic acid (EPA) and DHA, down-regulate HMG-CoA reductase activity, primarily through post-transcriptional effects in MCF-7 human breast cancer cells (Duncan *et al.,* 2005). Diosgenin, a natural furostanol saponin, decreases HMG-CoA reductase expression in human colon carcinoma cells (Raju and Bird, 2007). Green and black tea extracts also inhibit HMG-CoA reductase activity and thus decrease cholesterol synthesis in hepatoma cells (Singh *et al.,* 2008).

Extracts of medicinal plants such as *Typha augustifolia*, *Polygonum cuspidatum*, *Crataegus pinnatifida*, *Polygonum multiforum*, and *Pueraria thunbergiana,* as well as *Scutellariae radix, Coptidis rhizoma, Phellodendri cortex, Gardeniae fructus,* and *Rhei rhizoma,* contained in two oriental herbal medicines with anti-hyperlipidemic activity, 'orengedokuto' and 'daio-orengedokuto', show their high HMG-CoA reductase inhibitory activities *in vitro* (Lee and Choi., 1999; Kim *et al*. 2000; 2005). A stigmasterol from the mushroom *Pholiota adipose*, having an inhibitory activity of IC_{50} 6.8 μg, has been isolated from its fruiting body (Yu *et al.,* 2007). Vitisin A and vitisin B from *Vitis vinifera* bark exhibit a remarkable inhibitory activity against HMG-CoA reductase *in vitro* with IC_{50} values of 42.1 μM and 23.9 μM, respectively (Koo *et al.,* 2008).

In addition to statins originated from molds, the three isoflavones obtained from soybeans, diadzein, genistein, and glycitein, also act as competitive inhibitors of this enzyme with K_i values of 27.7, 49.5, and 94.7 μM, respectively (Sung *et al.*, 2004b).

2.3 *Garcinia dulcis*

2.3.1 General description

Garcinia dulcis (Roxb.) Kurz is a sub-woody plant belonging to the Clusiaceae (Guttiferae) family that grows mainly in Southeast Asia (Yupwattanaphun *et. al*., 2002). It is called Mundu in Indonesia and Malaysia, Baniti in the Philippines, and also known as Ma-phuut in Thailand. *G. dulcis* is a medium sized tree with short trunks and brown bark with white latex. Its leaves are opposite, lanceolate-shaped, 10-30 cm long and 3-5 cm wide. They are pale green when young and become dark green and shiny on the upper surface at maturity. The lower leaves are often hairy. The midrib is prominent with numerous veinlets arranged in parallel. The thick petiole is short being only 2 cm long. Flowers are borne in the axil. They are yellowish white with a sour smell. Fruits are globose, 5-8 cm wide with slightly pointed ends, often rather compressed and crowned by the persistent stigma. The fruit is soft with a thin skin and has a light yellow color, which turns to orange when ripe. The seeds are enveloped in an edible pulp of a darker color than the skin and have a pleasant taste (Subhadrabandhu, 2001).

2.3.2 Uses

The fruits can be eaten fresh, but they are sour and can be made into an excellent jam. Its leaves and seeds have been used in traditional medicine against lymphatitis, parotitis and struma in Indonesia (Kasahara and Henmi, 1986). Its stem bark has been used in Thai folk medicine as antiseptic, the fruit juice as anti-scurvy and expectorant for the relief of cough and sore throat, as mild laxative, and as decongestant, the root extract is also used as antipyretic and antitoxin (Wuttidhammavej, 1997; Bunyapraphatsara and Chokechaichareonporn, 1999).

Accumulated data from phytochemical studies have revealed that various parts of this plant contain, in abundant, a broad range of phenolic compounds (Ansari *et al*., 1976; Harrison *et al.*, 1994; Iinuma *et al.*, 1996a, 1996b, 1996c; Ito *et al.,* 1997; Kosela *et al.*, 1999; 2000; Dechathai *et al.,* 2005; 2006; 2008). Some possess potent biological activities, for example, the five xanthones isolated from the

barks of *G. dulcis* have shown inhibitory effects on *Plasmodium falciparum* (Likhitwitayawuid *et al.,* 1998), the three prenylated paraxanthonoids from its fruits are cytotoxic against cancer cell lines (Soemiati *et al.*, 2004), and several phenolic compounds isolated from the seeds, fruits and flowers also act as anti-oxidants and anti-bacterial agents (Deachathai *et al*., 2005; 2006; 2008; Hutadilok-Towatana *et al.*, 2007). Lately, anti-atherogenesis (Decha-Dier *et al.*, 2008), anti-restenosis (Pinkaew *et al*., 2009) and anti-tumor angiogenesis (Pang *et al*., 2009) effects of a biflavoniod, morelloflavone, isolated from *G. dulcis* leaves have been demonstrated.

Figure 12 Leaves, fruit, and flowers of *Garcinia dulcis***.**

2.4 Morelloflavone

Morelloflavone (IUPAC Name: 8-[(2S,3R)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4-oxo-2, 3-dihydrochromen-3-yl]-2-(3,4-dihydroxyphenyl)-5, 7-dihydroxychromen-4-one) represents a biflavonoid consisting of a flavanone, naringenin covalently linked to a flavone, luteolin (Verbeek *et al*., 2004) (Fig. 13). This compound, also known as fukugetin (Konoshima *et al.,* 1969), is abundant in plants of *Garcinia* spp. It was firstly found in *Garcinia morella* (Karanjgaokar *et al*., 1967) and is a common constituent among *Garcinia* spp., for example, *G. volkensii* (Herbin *et al.*, 1970)*, G. talboti* (Joshi *et al.,* 1970)*, G. livingstonei* (Pelter *et al*., 1971; Mbwambo *et al.*, 2006), *G. terpnophylla and G. echinocarpa* (Bandaranayake *et al.,*

1975), *G. multiflora* (Chen *et al*., 1975; Lin *et al*., 1997), *G. dulcis* (Ansari *et al*., 1976; Deachathai *et al*., 2005; 2006; 2008), *G. indica* (Cotterill *et al.*, 1977), *G. densivenia* (Waterman and Crichton, 1980), *G. xanthochymus* (Baslas and Pradeep, 1979, 1981), *G. quadrifaria* (Waterman and Hussain, 1982), *G. spicata* (Gunatilaka *et al*., 1984), *G. nervosa* (Babu *et al.*, 1988), *G. atroviridis* (Permana *et al*., 2003), *G. scortechinii* (Sukpondma *et al*., 2005), *G. cowa* (Shen *et al.,* 2006), *G. subelliptica* (Masuda *et al.*, 2005; Terashima *et al.*, 2008), and *G. cymosa* (Elfita *et al.,* 2009). The study of this naturally occurring compound is interesting from its pharmacological point of view. Morelloflavone has been found to be an inhibitor of secretory phospholipase A_2 (Gil *et al*., 1997) and exerts potent anti-inflammatory effects in mice (Gil *et al*., 1997; Castardo *et al*., 2008). It also possesses anti-HIV activity by inhibiting both HIV-1 reverse transcriptase (HIV-1 RT) *in vitro* and HIV-1 (strain LAV-1) in human lymphocytes (Lin *et al*., 1997). In 2002, Li and co-workers demonstrated that morelloflavone inhibited fatty acid synthase, which became a potential antifungal target (Li *et al*., 2002). This compound isolated from the leaves of *Rheedia gardneriana* as fuguketin, also shows antibacterial activity (Verdi *et al.*, 2003). Its strong inhibition on tyrosinase, the major enzyme responsible for skin melanization, has been found (Masuda *et al*., 2005). Among the anti-plasmodial compounds isolated from *Endodesmia calophylloides,* morelloflavone has been found to be active against *Plasmodium falciparum* W2 strain which is resistant to chloroquine and other drugs (Ngouamegne *et al.,* 2008). Moreover, antioxidant activities of morelloflavone have been observed in various experimental systems (Sanz *et al*., 1994; Gil *et al*., 1997; Deachathai *et al*., 2005; Hutadilok-Towatana *et al*., 2007). Decha-Dier and co-workers have discovered anti-atherogenic effects of morelloflavone in hypercholesterolemic-induced rabbits (Decha-Dier *et al*., 2008). This biflavonoid also prevents restenosis by blocking injury-induced neointimal hyperplasia *via* the inhibition of vascular smooth muscle cell migration (Pinkaew *et al*., 2008). Pang and co-workers have recently shown that morelloflavone exerts anti-tumor angiogenesis by targeting the activation of Rho-GTPases and ERK signaling pathways (Pang *et al*., 2009).

Although morelloflavone reduces blood cholesterol levels and thus prevents progression of atherosclerosis in high fat-diet induced hypercholesterolemic animals (Decha-Dier *et al*., 2008), the mechanism underlying such ability of this compound is still unknown. It was postulated that morelloflavone might exert its activity *via* inhibition of cholesterol *de novo* synthesis, and therefore, the effect of morelloflavone on HMG-CoA reductase, the key regulating enzyme of cholesterol pathway in mammals, was explored in this study.

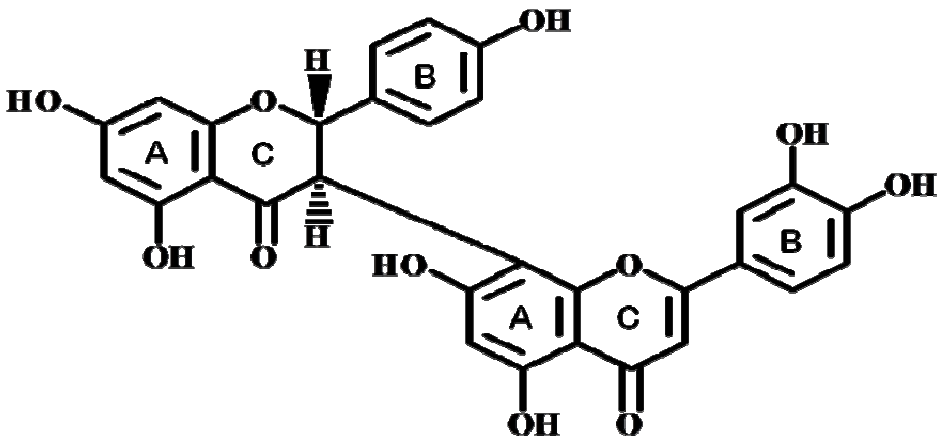


Figure 13 Structure of morelloflavone (MW = 556) consisting of naringenin (left) and luteolin (right) subunits.

CHAPTER 3

METERIALS AND METHODS

3.1 Materials

3.1.1 Morelloflavone

Morelloflavone (94% pure) was obtained from Dr. Decha Pinkaew. It was isolated from *Garcinia dulcis* leaves as previously described (Pinkaew *et al.*, 2009).

3.1.2 Recombinant plasmids and competent cells

The recombinant plasmids pET 17b-CD-HMG and *Escherichia coli* BL21 competent cells were kindly provided by Dr. Sriwan Wongwisansri of Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand.

3.1.3 Animals

Male Wistar rats at about 8 weeks of age and weighing 230-270 g, were obtained from Animal House Facility Unit, Faculty of Science, Prince of Songkla University.

3.1.4 Instruments

Name	***Model***	***Manufacturer***
Analytical balance	BP110S	Sartorius, Germany
Autoclave	HA-300M	Hirayama, Japan
Bench-top refrigerated centrifuge	5804R	Eppendorf, Germany

Name	*Model*	*Manufacturer*
Fraction collector	2110	Bio-Rad, USA
Heat block	D1100	Labnet, USA
Microtube pump	MP-3	Eyela, Japan
PCR machine	Mastercycler	Eppendorf, Germany
Polyacrylamide gel electrophoresis unit	AE-6450	ATTO, Japan
Submarine type electrophoresis unit	Mupid Ex	Takara Bio, USA
Superspeed refrigerated centrifuge	J-30I	Beckman, USA
Ultracentrifuge	XL Series	Beckman, USA
Ultrasonicator	MSE ultrasonic power unit	MSE, England
UV-visible spectrophotometer	8453	Hewlett-Packard, USA

3.1.5 Chemicals All chemicals used were of analytical grade.

Name	*Manufacturer*
Acrylamide	Merck, Germany
Agarose	Sigma-Aldrich, USA
Ammonium persulfate	Merck, Germany
Ampicillin, sodium salt	Sigma-Aldrich, USA
Blue Sepharose	Sigma-Aldrich, USA
Bovine serum albumin	Sigma-Aldrich, USA
Bromophenol blue	Fluka, Switzerland
Cholesterol, 95% pure	Sigma-Aldrich, USA
Dimethyl sulfoxide (DMSO)	Fluka, Switzerland

Name	***Manufacturer***
Dipotassium hydrogenphosphate	Fluka, Switzerland
Dithiotreitol (DTT)	Sigma-Aldrich, USA
Ethidium bromide	Sigma-Aldrich, USA
Ethylenediamine tetraacetic acid (EDTA)	Fluka, Switzerland
Folin-Ciocalteau's phenol reagent	Carlo Erba, Spain
Genistein from *Glycine max* (soybean), 98% pure	Sigma-Aldrich, USA
Glycerol	Analar, England
Glycine	Sigma-Aldrich, USA
D, L-3 hydroxy-3-methylglutarylcoenzyme A (HMG-CoA)	Sigma-Aldrich, USA
Isopropyl β-D-thiogalactopyranoside	Sigma-Aldrich, USA
LB media	Sigma-Aldrich, USA
Luteolin from synthesis, ≥98% pure	Sigma-Aldrich, USA
Naringenin from synthesis, ~95% pure	Sigma-Aldrich, USA
β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)	Sigma-Aldrich, USA
Olive oil, extra virgin (Sabroso®)	Ihoros De Ibarra, Spain
Pravastatin, sodium salt	Sigma-Aldrich, USA
Potassium chloride (KCl)	Carlo Erba, Spain
Potassium dihydrogen phosphate	Merck, Germany
SOC medium	Sigma-Aldrich, USA
Sodium chloride (NaCl)	Merck, Germany
Sucrose	Carlo Erba, Spain
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, USA
Tris (hydroxymethyl) aminomethane	Vivantis, Italy

3.2 Methods

Part 1: *In vitro* study

3.2.1 Preparation of HMG-CoA reductase

HMG-CoA reductase was expressed in *E. coli* according to Frimpong and co-workers (Frimpong *et al.*, 1993). The recombinant plasmid pET 17b-CD-HMG which contain HMG-CoA reductase gene was transformed to *E. coli* BL21 competent cell by following the on-line protocol of Sosnick Lab, University of Chicago, USA (http://sosnick.uchicago.edu/competent_cells.html). Firstly, 5 µl of the plasmid solution was added to 10 µl of the competent cells, and the resulting mixture was then put in an ice-bath for 10 min. The cell mixture was incubated at 42 ^{o}C for 90 sec, and placed back on ice for 2 min to reduce damage to the *E. coli* cells. Subsequently, 10 µl of SOC medium and 1 ml of LB broth (without antibiotic) were added, and the mixture was further incubated at 37 ^{o}C by swirling the reaction tube every 15 min for 1 h. The resulting culture (200 µl) was spread on LB agar (3 ml/plate) containing 100 µg/ml ampicillin as a selective marker and further incubated at 37 ^{o}C.

After 12-16 h of culture, a freshly transformed colony was picked and inoculated into 10 ml LB broth containing 100 µg/ml of ampicillin and incubated at 37 ^{o}C overnight. An inoculum was diluted in 200 ml LB broth containing 100 µg/ml of ampicillin and cultured until its absorbance at 600 nm reached 0.8-1.0. Expression of the house mouse (*Mus musculus*) HMG-CoA reductase catalytic domain was induced by 0.5 mM isopropyl β-D-thiogalactopyranoside. Culturing was continued for another 5.5 h. The 200 ml culture was divided into four 50 ml aliquots. Each aliquot was centrifuged (10,000×*g*, 15 min at 4 ^{o}C), and the pellet was kept at -80 ^{o}C until used. The cell pellet was resuspended in 2 ml of 20 mM phosphate buffer, pH 7.3 containing 50 mM NaCl, 10% (v/v) glycerol, 100 mM sucrose, and 10 mM DTT on ice. This suspension was then used to prepare cell-free extract for the enzyme purification.

To prepare cell-free extract, cells were lysed by sonication at 23% amplitude for 3 min (pulse on 6 sec, off 9 sec). Cell debris was separated by centrifugation (20,442×*g*, 10 min at 4 ^{o}C) and the supernatant subsequently filtered through a 0.2 μm polysulfone syringe filter (Acrodisc®, GelmanSciences, USA). The supernatant was subjected immediately to a Blue Sepharose column (1×5 cm) pre-equilibrated with 20 mM phosphate buffer, pH 7.3 containing 50 mM NaCl, 10% (v/v) glycerol, 100 mM sucrose, and 10 mM DTT. Elution was carried out using a linear gradient of 0 to 2 M NaCl in the same buffer at a flow rate of 0.25 ml/min, 4 ^{o}C. Each fraction (1 ml) was monitored for protein at OD 280 nm and HMG-CoA reductase activity at OD 340 nm. The active fractions were then analyzed by SDS-polyacrylamide gel electrophoresis.

3.2.2 Kinetic studies of HMG-CoA reductase

The HMG-CoA-dependent oxidation of NADPH was monitored at 340 nm and 37 ^{o}C, with some modifications from the procedure developed in the Bioassay Laboratory, BIOTEC**,** Thailand (S. Wongwisansri, personal communication). The standard assay mixture contained 200 μM D, L-HMG-CoA, 200 μM NADPH, 100 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 100 mM phosphate buffer, pH 6.8 and a final volume of 150 μl. The reaction mixture containing the enzyme (0.76 μg protein) and all components, except NADPH, was pre-incubated at 37 ^{o}C for 6 min. The reaction was started when NADPH was added and its initial velocity was measured as the decreasing rate of absorbance at 340 nm ($\triangle OD_{340}$/min). The rate of reaction in the unit of $\triangle OD_{340}$/min was then translated into the enzyme specific activity from following calculation:

$$OD_{340} = \varepsilon cl$$

Where ε is millimolar absorptivity or millimolar extinction coefficient of NADPH which is equal to 6.22 mM^{-1} cm^{-1}

c is the concentration of NADPH in mM

l is the optical path length in cm

Enzyme activity or reaction rate (V) was measured as the reduction of OD_{340} (ΔOD_{340}) over time investigated,

Therefore

$$V = \Delta c = OD_{340} / min / 6.22\ mM^{-1}\ cm^{-1} \times cm^{1}$$

$$V = OD_{340} / min \times 0.1608\ mM\ /min$$

For 150 µl reaction

$$V = OD_{340} / min \times 0.02412\ \mu mol/min$$

Specific activity (V_{spec}) was calculated as reaction rate per milligram protein used, therefore

$$V_{spec} = [OD_{340} / min \times 0.02412\ \mu mol/min] / \mu g\ protein\ used$$

Enzyme kinetic parameters (k_m and V_{max}), were evaluated using the non-linear regression method based on Michaelis-Menten equation and the type of inhibition was identified using Lineweaver-Burk plot.

To investigate its inhibitory effect on enzyme activity, the test compound dissolved in DMSO at various concentrations, was added into the reaction mixture. For the HMG-CoA inhibition study, the NADPH concentration was fixed at 200 µM, and, HMG-CoA was used in the range of 25-150 µM. With respect to NADPH, HMG-CoA was fixed at 200 µM, and NADPH was 50-100 µM. The type of inhibition was identified using Lineweaver-Burk plot.

Kinetic parameters were evaluated using the non-linear regression method based on Michaelis-Menten equation:

$$V_i = \frac{V_{max} \times S}{K_m(1+I/K_i) + S}$$

Where V_i is the initial velocity in the absence and presence of inhibitor; S and I, are concentrations of the substrate and the inhibitor, respectively; V_{max}, the maximum velocity; K_m, the Michaelis-Menten constant; and K_i is the inhibition constant defined as [E][I]/[EI]; [E] = enzyme concentration, [I] = inhibitor concentration, [EI] = enzyme-inhibitor complex concentration.

3.2.3 Protein assay

Samples were determined for their protein concentrations by the Lowry method (Lowry *et al*., 1951) using bovine serum albumin as a standard.

3.2.4 SDS-polyacrylamide gel electrophoresis

The samples were electrophoresed in a slab gel, composed of 3% stacking gel and 12% separating gel according to the method of Laemmli (Laemmli, 1970). Each sample and a standard protein marker solution (Sigma-Aldrich, USA) containing phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14 kDa), were mixed with a sample buffer (0.2 M Tris-HCl, pH 6.8 with 4% SDS, 40% (v/v) glycerol, 8 mM EDTA, 0.4% DTT, and 0.4% bromophenol blue as the tracking dye) in a ratio of sample: buffer = 3: 1 (v/v). They were then boiled for 5 min and loaded on the gel. Electrophoresis was carried out in the electrode buffer (25 mM Tris-HCl, 0.192 M glycine, 0.1% SDS, pH 8.3) at a constant current of 100 Volts for 1 h 45 min. After electrophoresis, protein bands were stained with Coomassie Blue. Migration distances of the samples, standard markers and bromophenol blue were measured and calculated for their relative mobility (R_f) by the following equation:

$$\text{Relative mobility } (R_f) = \frac{\text{Migration distance of protein}}{\text{Migration distance of bromophenol blue}}$$

A standard curve plotted between logarithms of the standard molecular weights and their R_f values was used to estimate the molecular weight of protein sample.

Part 2: *In vivo* study

3.2.5 Animal treatments

All procedures concerning animal treatments and experimentation in this study were reviewed and approved by the Institutional Committee for Ethical Use of Experimental Animals at Prince of Songkla University (approval no. 22/51). Male Wistar rats were randomly divided into 4 groups (n = 5). Group 1 and Group 4 were fed pelletized commercial chow (normal) diet (C.P. Mice Feed®, Charoen Phokphand, Thailand) and accessed to drinking-water *ad libitum*, whereas Group 2 and Group 3 received normal diet supplemented with 1% (w/w) cholesterol to induce hypercholesterolemia.

Each rat was housed separately in an identical stainless steel cage (28×30×40 cm) and maintained in an air-conditioned room at 22 ± 3 ^{o}C, 50-60% relative humidity, and 12 h-light/dark cycle. Blood was taken twice a month for plasma cholesterol analysis. Before blood sampling, they were overnight-fasted. Heparinized blood was obtained from ocular bed puncture under ether anesthesia. It was centrifuged at 1,700×*g* for 15 min to separate plasma.

When the rats in Group 2 and Group 3 had their plasma cholesterol levels higher than 100 mg/dl, the experiments were started. For 6 weeks of study, animals in Group 1 were given normal diet and intragastrically administered with olive oil (5 ml) daily; Group 2: normal diet plus 5 ml cholesterol suspension in olive oil (1 g% body weight/day); Group 3: normal diet plus 5 ml suspension containing cholesterol (1 g% body weight/day) and morelloflavone (0.01 g% body weight/day); Group 4: normal diet plus 5 ml morelloflavone suspension in olive oil (0.01 g% body weight/day).

All rats were observed daily for any abnormalities of activities and behaviors. They were weighed individually once a week throughout the experimental period. Food and water intakes were also measured daily. At the end of experiments, the animals were fasted overnight. Blood was collected from the abdominal aorta under ether anesthesia and then prepared for plasma. Their livers were removed immediately after cervical dislocation and stored at -80 ^{o}C for mRNA analysis and hepatic HMG-CoA reductase assays.

3.2.6 Blood lipids measurement

Plasma HDL-C, triglyceride, and total cholesterol concentrations were determined by the colorimetric methods using an autoanalyzer (Cobas® Mira, Roche, Switzerland).

3.2.7 Preparation of liver microsome

The frozen liver was excised and immediately placed in an ice-cold 50 mM potassium phosphate buffer, pH 7.0 containing 0.2 M sucrose and 2 mM DTT (2 ml/g liver). The homogenization was performed in three strokes with a motor-driven Teflon pestle in a Potter-Elvehjem type glass homogenizer. The homogenate was

centrifuged for 10 min at 15,000×*g*, 4 °C and the supernatant solution was centrifuged at 100,000×*g* for 75 min, and 4 °C. The precipitate was re-suspended in the same buffer with 50 mM EDTA and then centrifuged at 100,000×*g* for 60 min, and 4 °C to obtain microsomal pellet (Kleinsek *et al.,* 1977). It was kept frozen at -20 °C until use.

After thawing in an ice-bath, the microsomes were manually homogenized with a Teflon pestle and a glass homogenizer, in a solubilization buffer consisting of 0.1 M sucrose, 2 mM DTT, 50 mM KCl, and 30 mM EDTA in 50 mM potassium phosphate buffer, pH 7.0 (5 ml/g liver) (Heller and Gould, 1974). The homogenates were quantified for their protein contents and further used for HMG-CoA reductase assays.

3.2.8 Hepatic HMG-CoA reductase assay

The assay mixture contained 200 µM D, L-HMG-CoA, 200 µM NADPH, 100 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 100 mM phosphate buffer, pH 6.8 at a final volume of 150 µl. The homogenate of liver microsomes in 100 mM phosphate buffer, pH 6.8 containing 100 mM NaCl, 1.0 mM EDTA, and 10 mM DTT was pre-incubated at 37 °C for 2 min. The reaction was then started by addition of D, L-HMG-CoA, and followed by NADPH. The absorbance at 340 nm was continuously measured for 3 min. The initial velocity of each sample was used to calculate the specific activity of the enzyme.

3.2.9 Determination of hepatic mRNA expression of HMG-CoA reductase and LDL receptor

Total mRNA was isolated from rat liver tissues based on the protocol of Genlute™ Direct mRNA Miniprep kit (Sigma-Aldrich, USA). Reverse transcription was carried out by using oneStep RT-PCR kit (Quigen, USA) and operated by a thermal cycler PCR. The appropriate primers of the target gene were designed corresponding to the positions of 104-123 and 2327-2346 of Wistar rat HMG-CoA reductase cDNA (Ihara-Watanabe *et al.*, 1999) as follows:

sense primer: 5′-ACAATGTTGTCAAGACTTTT-3′

antisense primer: 5′-CCTCCTATGCTACCAGCCCAT-3′

The primers for LDL receptor gene were constructed according to Chen and Cheng (2005):

sense primer: 5′-CAGCTCTGTGTGAACCT-3′

antisense primer: 5′-TTCTTCAGGTTGGGGATCA-3′

The program of the thermal cycler was set up as follows:

Step	***Duration***	***Temperature***
Reverse transcription	30 min	50 ^{o}C
Initial PCR activation	15 min	95 ^{o}C
Three step cycling		
- Denaturation	1 min	94 ^{o}C
- Annealing	1 min	68 ^{o}C
- Extension	1 min	72 ^{o}C
Number of cycler	40	
Final extension	10 min	72 ^{o}C

The PCR products were electrophoresed on a 2% agarose gel at 100 Volts for 45 min. The gel was stained by ethidium bromide. DNA bands were detected under UV light illumination and their densities were quantified by a Gel Document System using Labworks 4.6 Image Acquisition and Analysis Software (Bio Imagine System, UVP, USA).

3.2.10 Statistical analysis

All data were expressed as mean ± SEM. They were analyzed for statistical significance by one-way analysis of variance using SPSS 11.5 (SPSS Institute, USA) and individual comparisons were obtained by LSD's multiple-range test. Significance was judged at $p < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

Part I: *In vitro* study

4.1 Preparation of the catalytic portion of mouse HMG-CoA reductase

The DNA portion harboring a catalytic domain of mouse HMG-CoA reductase was successfully introduced into *E. coli* cells *via* the plasmid vector. After its expression was induced, the harvested protein was applied to Blue Sepharose affinity column. Upon eluting with 0-2.0 M NaCl gradient, enzyme-containing fractions were obtained at 0.40-0.64 M NaCl as in Figure 14. They were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the results are shown in Figure 15. Each fraction appeared as one major protein band on SDS-PAGE with an estimated molecular weight of 50 kDa, the same as reported previously (Wongwisansri and Kitikara, 2004). These enzyme fractions were then pooled, concentrated, and assayed for protein and the enzyme activity. Each preparation batch showed not less than 26% of protein yield and was purified about 4.5 folds from the starting material.

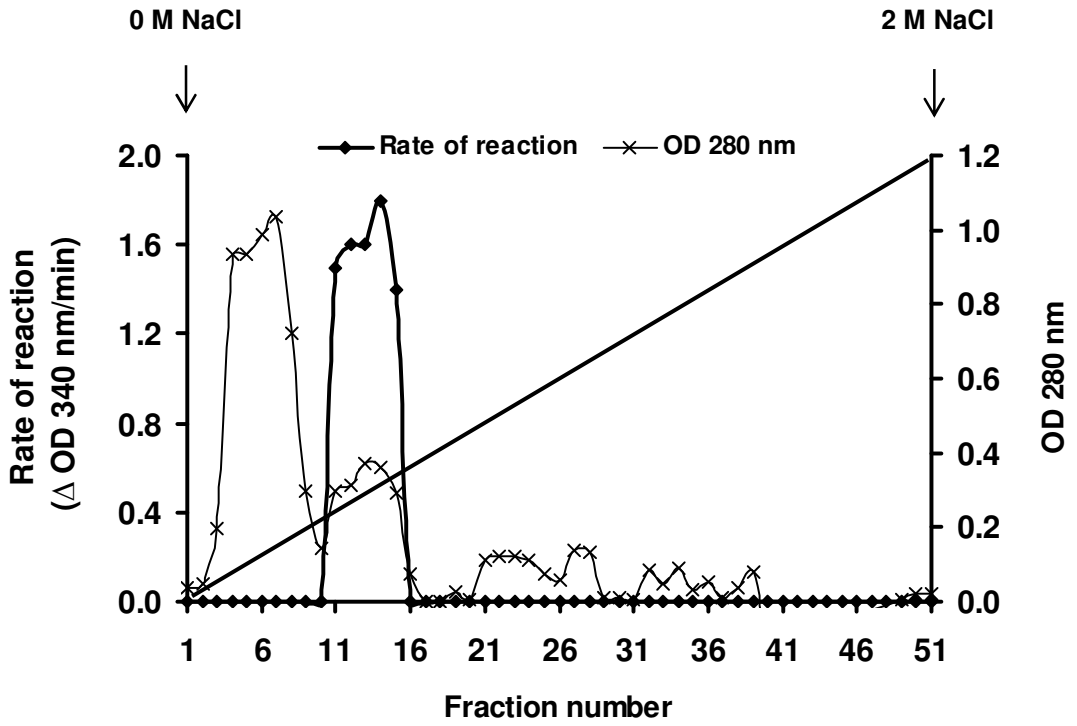


Figure 14 Chromatogram of Blue Sepharose column (1×5 cm). The bound proteins were eluted with a linear gradient of 0-2.0 M NaCl in 20 mM phosphate buffer, pH 7.3 containing 50 mM NaCl, 10% (v/v) glycerol, 100 mM sucrose, and 10 mM DTT at a flow rate of 0.25 ml/min. Each fraction (1 ml) was collected and measured for protein and the enzyme activity as described in Chapter 3.

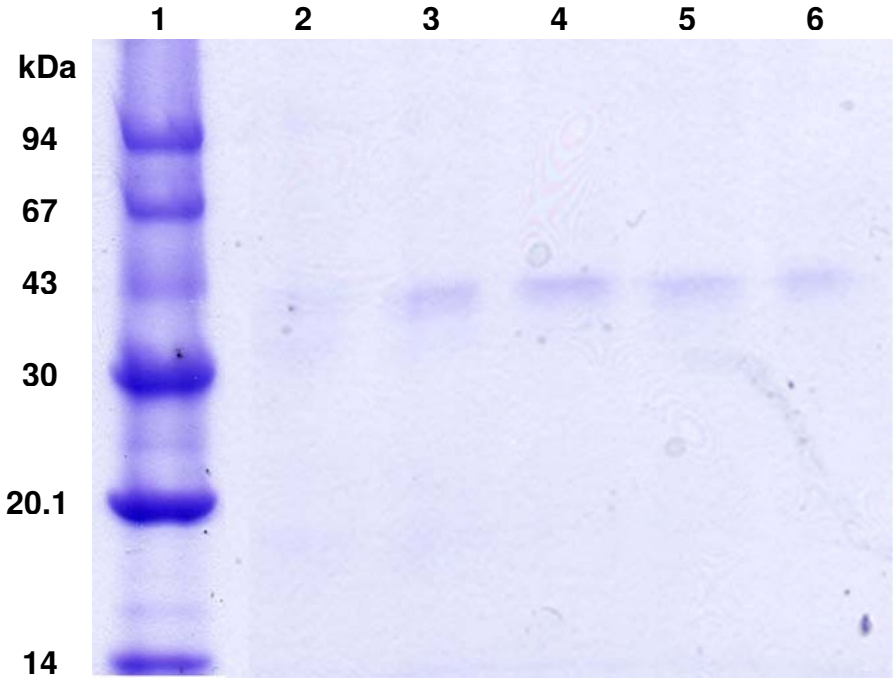


Figure 15 SDS-PAGE of HMG-CoA reductase fractions on 12 % polyacrylamide slab gel. The protein bands were stained with Coomassie Blue. Lane 1; low molecular weight markers: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14 kDa). Lane 2-6; HMG-CoA reductase fractions eluted from Blue Sepharose column containing protein of 21, 25, 32, 24, and 25 μg, respectively.

4.2 Enzyme kinetics and characterization

The HMG-CoA reductase preparation was tested for its activity. The enzyme was catalytically active yielding a high rate of oxidation and HMG-CoA concentration dependent as shown in Figure 16.

Its kinetic parameters, Michaelis-Menten constant (K_m) and maximum rate of reaction (V_{max}), were evaluated from the Lineweaver-Burk plots (Figure 16) using activity assay conditions as described in Chapter 3.

When the standard enzyme reaction was performed, V_{max} value was calculated as 5 ± 0.26 µmole NADPH/min/mg protein. Under the same conditions, K_m values for the two substrates, D, L-HMG-CoA and NADPH, were 74 ± 0.06 and 133 ± 0.21 µM, respectively, similar to those previously obtained (S. Wongwisansri, personnel communication).

The recombinant HMG-CoA reductase was also assayed in the presence of test substance at various concentrations. The Lineweaver-Burk plots (Figure 17 and Figure 18) were employed to predict the type of inhibition. Morelloflavone (A) competed with HMG-CoA, whereas it was noncompetitive towards NADPH, the second substrate of the enzyme. The inhibition constants (K_i) of this compound with regard to HMG-CoA and NADPH were 80.87 ± 0.06 µM and 103 ± 0.07 µM, respectively. From these results, it was concluded that morelloflavone occupied a portion of the binding site of HMG-CoA, thus blocking access of this substrate to the active site, whereas the nicotinamide binding pocket of NADPH was unoccupied by the inhibitor molecule.

In order to investigate which of morelloflavone subunits would participate in such binding, naringenin (B) and luteolin (C) were examined for their enzyme inhibitory activity. As evident from the results in Figure 17, both aglycone isoflavones were inhibitory to HMG-CoA reductase in the same manner as morelloflavone. They competed with HMG-CoA at the K_i values of 83.59 ± 4.37 µM and 83.58 ± 0.94 µM, respectively, comparable to morelloflavone but rather weaker than genistein (D), a HMG-CoA reductase inhibitor from soy (Sung *et al.,* 2004) (K_i = 49 ± 1.28 µM). The more hydrophobic characteristics may allow genistein to bind

more strongly to the enzyme than naringenin and luteolin (Breton *et al.,* 1975). These isoflavones were also noncompetitive with respect to NADPH (Figure 18). Their K_i values were: narigenin 182 ± 67 µM, luteolin 188 ± 14 µM, and genistein 104 ± 0.64 µM. These findings thus suggest that morelloflavone molecule would function by binding with a hydrophobic pocket of the enzyme active site *via* either subunit. In this regard, a common structural feature of isoflavone binding to the enzyme is most likely "ring B". This cyclic structure is favorable for the enzyme binding based on the fact that it resembles a pyran ring of both HMG and statin as shown in Figure 19. It has been reported, however, that in addition to bonds formed by the HMG-like moiety, compactin and simvastatin exhibit binding *via* a decalin ring structure (Istvan, 2003), and this might the same case for "ring A and ring C" of isoflavones.

In this study, inhibition constant values of pravastatin (E) for HMG-CoA and NADPH were 0.6 ± 0.02 µM. and 23.43 ± 0.93 µM, respectively (Figure 17 and Figure 18). Although morelloflavone had relatively high K_i values compared with statins which usually inhibit the enzyme in the nanomolar range (Istvan, 2002), this compound appeared to be a novel naturally occurring HMG-CoA reductase inhibitor. However, structural mechanism for morelloflavone inhibition of HMG-CoA reductase needed to be further elucidated. Through x-ray crystallographic studies, statins have been found to basically use their common HMG-like moiety to occupy the active site of the enzyme, with the hydrophobic groups of the statins being positioned in a shallow groove formed by rearrangement of the C-terminal residues of the enzyme catalytic domain and thus sterically preventing the substrate from binding (Istvan, 2003).

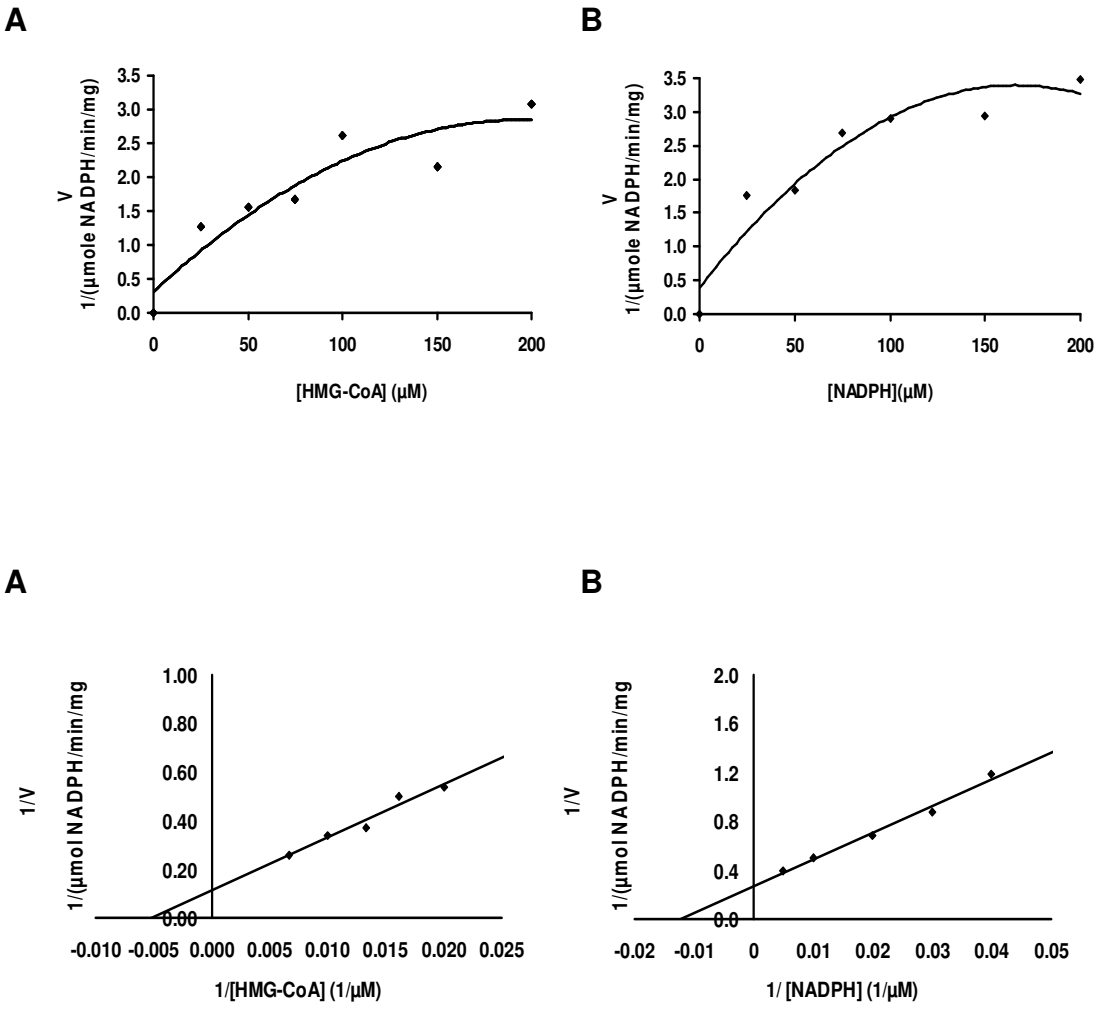


Figure 16 Michaelis-Menten plots (upper panel) and Lineweaver-Burk plots (lower panel) of HMG-CoA reductase for the two substrates, HMG-CoA (A), and NADPH (B). All analyses were conducted in six replicates.

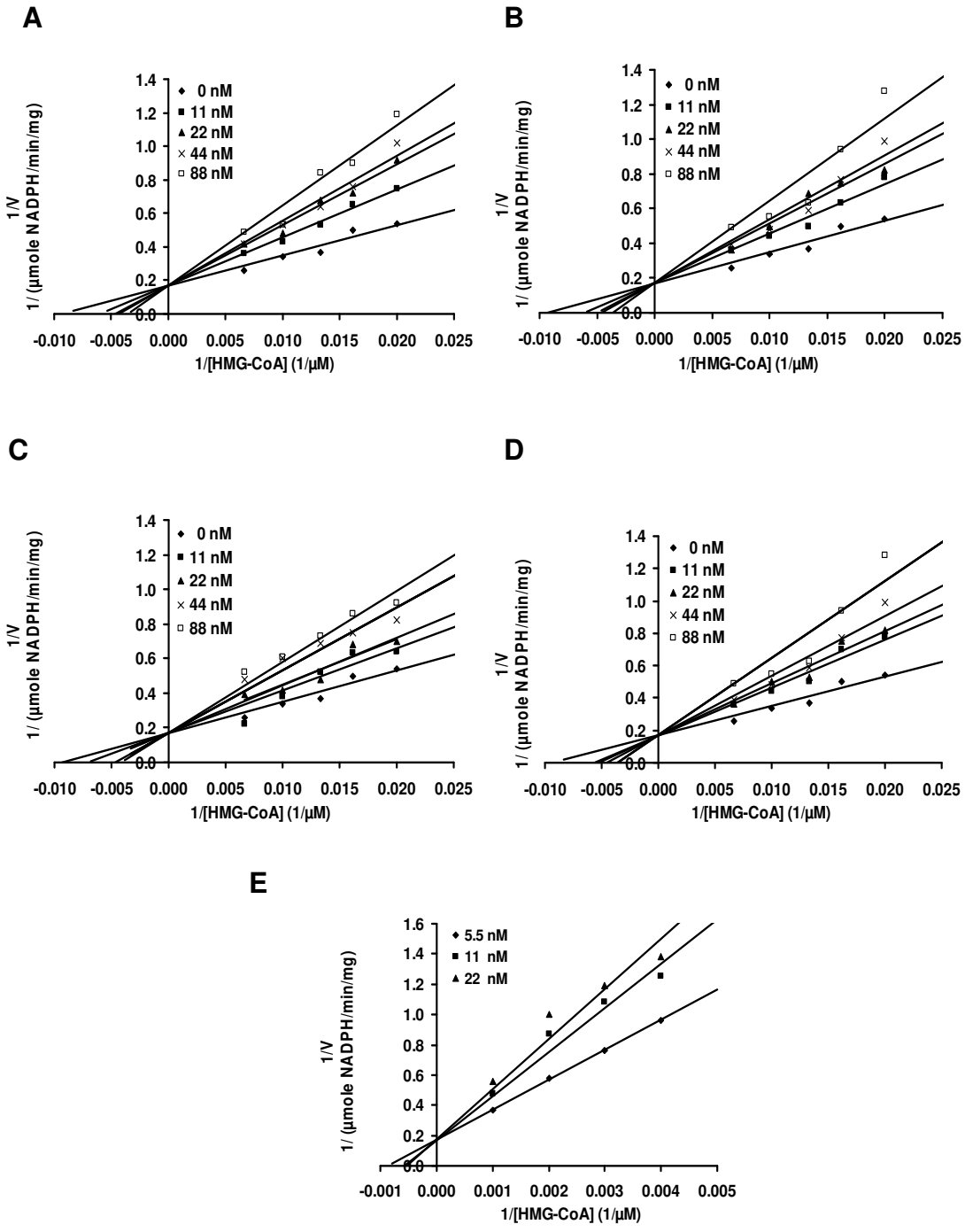


Figure 17 Lineweaver-Burk plots for the inhibition of HMG-CoA reductase by morelloflavone (A), naringenin (B), luteolin (C), genistein (D), and pravastatin (E), with respect to HMG-CoA. All analyses were conducted in six replicates.

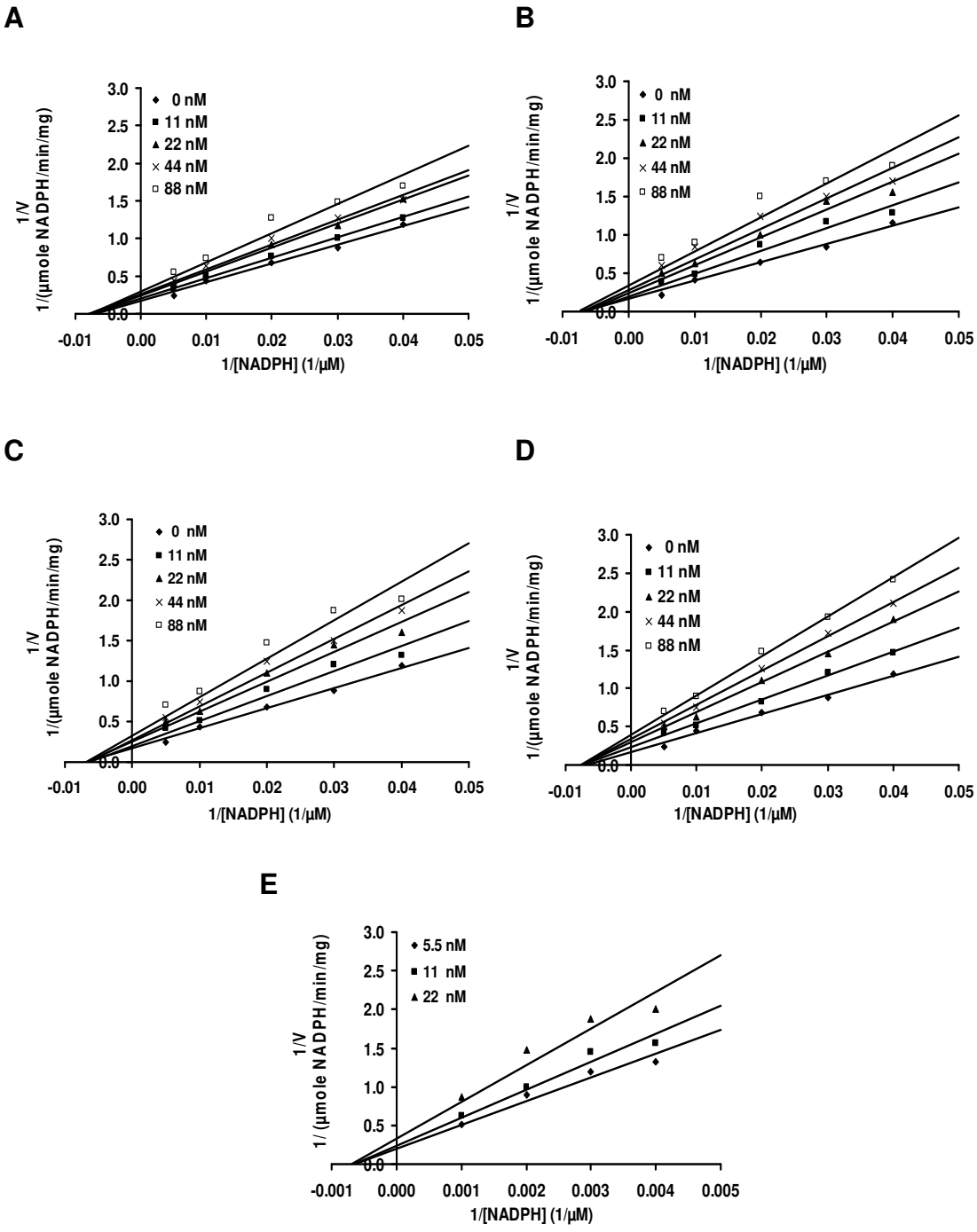


Figure 18 Lineweaver-Burk plots for the inhibition of HMG-CoA reductase by morelloflavone (A), naringenin (B), luteolin (C), genistein (D), and pravastatin (E), with respect to NADPH. All analyses were conducted in six replicates.

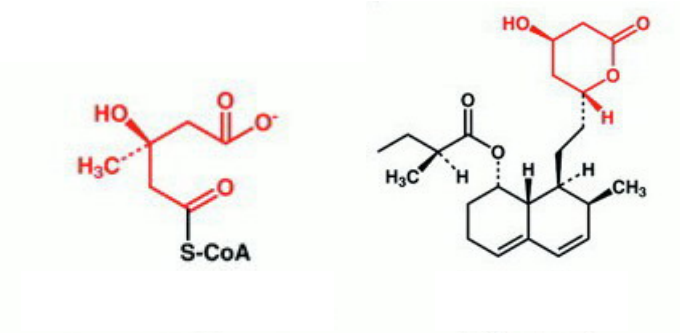


Figure 19 Cyclic structures of HMG-CoA (left) and statin drug (right) with their pyran rings as designated in red (www.nature.com/statins).

Part II: *In vivo* study

4.3 Effects on animal growth and food intake

Throughout the experimental period, daily food intake among the control rats (Group 1) and those receiving 0.01% (w/w) morelloflavone (Group 4) were not altered whereas both untreated (Group 2) and morelloflavone-treated hypercholesterolemic animals (Group 3) consumed lesser amounts of food until the end of study (Appendix I). This apparent effect has been reported previously in high-fat diet rats (Bahramikia and Yazdanparast, 2008). Surprisingly, their body weight levels were unchanged throughout the entire period (Appendix II). As a result, average food intake per body weight ratios of Group 2 and Group 3 were significantly lower than the rest (Figure 20).

To examine whether the treatments had any effects on the vital organ responsible for detoxification, all rat livers were excised, weighed and gross-examined at the termination. Their relative liver weight to final body weight ratios are also shown in Figure 21. A high-cholesterol containing diet has been reported to induce an over-weight liver from hepatic lipids accumulation (Seifalian *et al.*, 1999; Jayasooriya *et al.,* 2000). The same evidence was also obtained in this study (Appendix III). Therefore, those significantly greater ratios of liver weight to body weight in hypercholesterolemic groups as compared to the normal controls were

attributed to increased liver weights among these animals. Due to hepatic enlargement, both cholesterol groups did not lose weight despite lower food intake.

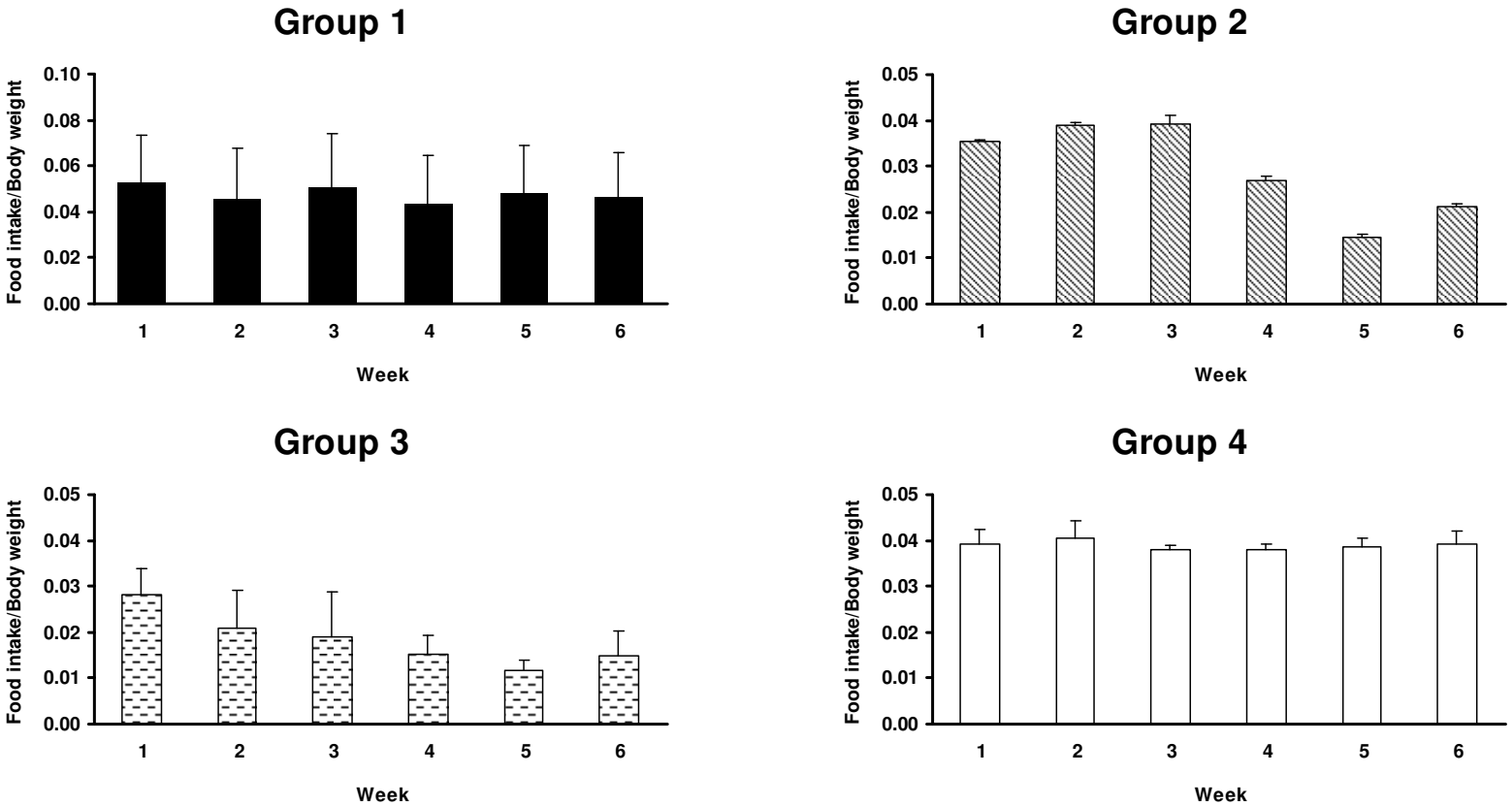


Figure 20 Average food intake/body weight ratios of the four experimental groups during six weeks. Data are given as mean ± SEM (n = 4). Group 1 = normal, Group 2 = hypercholesterolemic, Group 3 = hypercholesterolemic + morelloflavone, Group 4 = normal + morelloflavone.

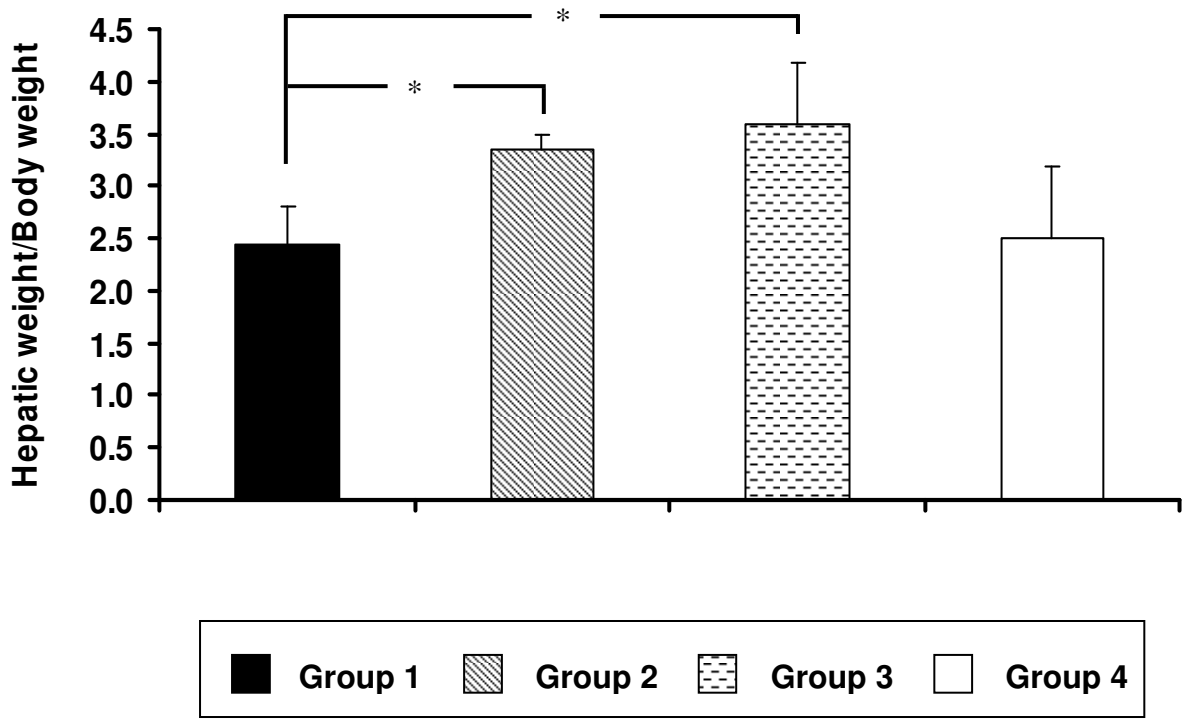


Figure 21 Rat hepatic weight/body weight ratios at the final week. Data are given as mean ± SEM (n = 4), * $p < 0.05$. Group 1 = normal, Group 2 = hypercholesterolemic, Group 3 = hypercholesterolemic + morelloflavone, Group 4 = normal + morelloflavone.

4.4 Effects on blood lipid profiles

Oral administration of 0.01% (w/w) morelloflavone for 4 months has previously shown its anti-hyperlipidemic effects in the rabbits receiving high-cholesterol diet (Decha-Dier *et al*., 2008). In the present study, however, the serum total cholesterol levels in hypercholesterolemic rats were not significantly improved after receiving the same dose of this compound continuously for 6 weeks as shown in Figure 22A. These controversial results are still unknown, yet most likely reflect differences in lipoprotein metabolism among animal species (Tall, 1993). High-cholesterol fed rats seem to respond to the HMG-CoA reductase inhibitors variably. Statins do not contribute to the plasma cholesterol-lowering action in this animal model (Endo *et al*., 1979; Lee *et al*., 2001) as they do in hamsters and rabbits (Ma *et al*., 1986), whereas anti-HMG-CoA reductase flavonoids such as hesperitin (Lee *et al*., 1999), quercetin (Bok *et al*., 1999), naringenin (Shin *et al*., 1999), and eriocitrin (Miyake *et al*., 2006), have shown their hypocholesterolemic effects in these rats. In normocholesterolemic rats (Group 4), however, morelloflavone caused some but not significantly increased total cholesterol levels as compared to those of Group 1 (Figure 22A). Meanwhile, this compound seemed to stimulate the good cholesterol "HDL-C" in these animals. In contrast to the other groups, their plasma HDL-C levels did not decline with time but became slightly increased (Figure 22B). The reduction of this plasma lipoprotein fraction normally occurs in aging (Walter, 2009) and is speculated to be another risk factor for developing atherosclerosis (Nofer *et al.*, 2002). From the above results, it was therefore postulated that such alteration of circulating HDL-C in Group 4-rats would partially account for their increased plasma total cholesterol levels, although the true reason is still unknown. It should be noted, however, that flavonoids have been demonstrated to increase HDL-C in various hypercholesterolemic subjects including rats (Kim *et al*., 2003). This compound produced no influence on blood triglyceride level in any rat group as shown in Figure 22C.

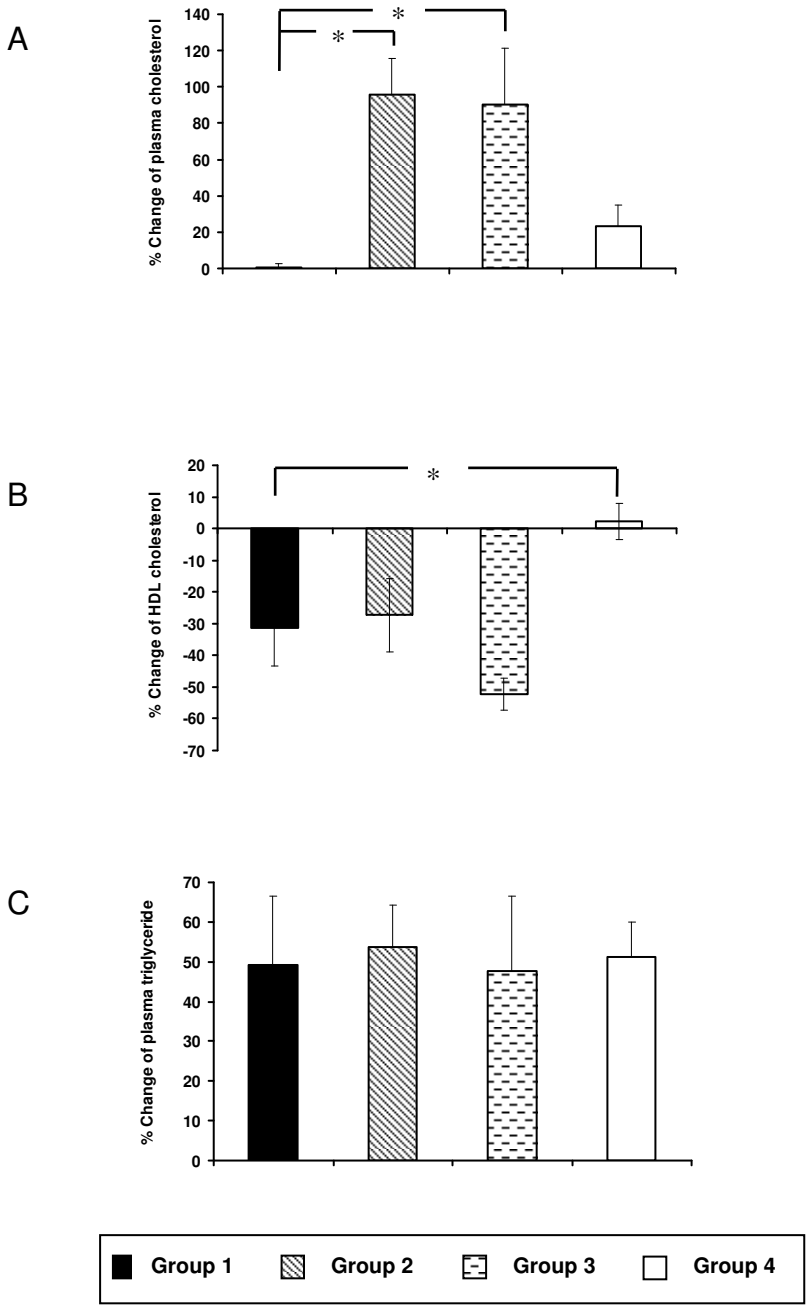


Figure 22 Relative changes of plasma cholesterol (A), HDL-C (B), and triglyceride (C) among the experimental rats. Means were normalized with 0 week value in each group. Data are given as mean ± SEM (n = 4), * $p < 0.05$. Group 1 = normal, Group 2 = hypercholesterolemic, Group 3 = hypercholesterolemic + morelloflavone, Group 4 = normal + morelloflavone.

4.5 Effects on HMG-CoA reductase and LDL receptor mRNA expressions

To investigate whether morelloflavone would affect HMG Co-A reductase in the experimental rats, both the activity and the mRNA expression of this enzyme were measured in their livers. Among the four treatment groups, levels of hepatic HMG-CoA reductase mRNA (A) and its activity (B) correlated well (Figure 23). As anticipated, dietary cholesterol in rat livers of group 2 and 3 significantly inhibited the enzyme (Figure 23A). In mammalian cells, the intracellular concentration of cholesterol is maintained by a balance between endogenous synthesis and exogenous supply. This balance is achieved through feedback regulation on the biosynthesis pathway, and also on the LDL receptor synthesis (Brown and Goldstein, 1990). Intake of high-cholesterol diet increases the intracellular sterol pool, which alternatively suppresses the expression of HMG-CoA reductase mRNA *via* sterol regulatory elements (SREs) (White *et al.,* 1997). In the presence of HMG-CoA reductase inhibitor, however, the *de novo* cholesterol biosynthetic pathway is depressed resulting in a decreasing pool. As a compensatory mechanism, HMG-CoA reductase gene is up-regulated leading to an elevation of the mRNA expression (Bergstrom *et al.,* 1998). The phenomena whereby HMG-CoA reductase inhibitors trigger an over-expression of HMG-CoA reductase gene transcription are well-documented (Bergstrom *et al.*, 1998; Pitman *et al.,* 1998). In the current study, such *in vivo* effect of morelloflavone was also evidenced (Figure 23 B). It was probable that either the compound itself or its metabolite would limit the availability of newly synthesized cholesterol, thereby promoting a hepatic expression of HMG-CoA reductase mRNA with a simultaneous increase in the enzyme activity in groups 3 and 4 (Figure 23A).

LDL receptor which plays an important role in blood cholesterol clearance is also modulated by cholesterol mainly from exogenous source (Brown and Goldstein, 1990). Its gene is down-regulated by high-cholesterol diet through the regulatory process mediated by negative effectors of transcription (SREBPs) (White *et al*., 1997). Such LDL lowering effect of dietary cholesterol also occurred in this study (Figure 23C). When cellular cholesterol content becomes lower, SREBPs undergo proteolytic cleavage and the amino-terminal domain then activates LDL receptor gene expression (Horton *et al.*, 2002). Although, there is a suggestion that

HMG-CoA reductase and LDL receptor may be regulated independently in the liver (Spady *et al.*, 1985), a coordinate regulation of the expression of the genes coding for both proteins was demonstrated in this study (Figure 23). In accompany with HMG-CoA reductase gene (A), LDL receptor gene (B) was enhanced in the rat livers after morelloflavone treatment (Figure 23C). Accordingly, an increased cellular uptake of this lipoprotein *via* the receptor might explain those higher HDL levels in Figure 22.

Based on the above results, morelloflavone supplementation seemed to improve the lipid metabolism in animal fed high-cholesterol diet through hepatic responses to HMG-CoA reductase inhibition. However, more extensive investigation in an appropriate animal model to confirm the effects of this compound on the plasma and hepatic lipids level, apolipoproteins level, hepatic cholesterol-regulating enzymes and sterol excretion is required to establish its mechanism of action.

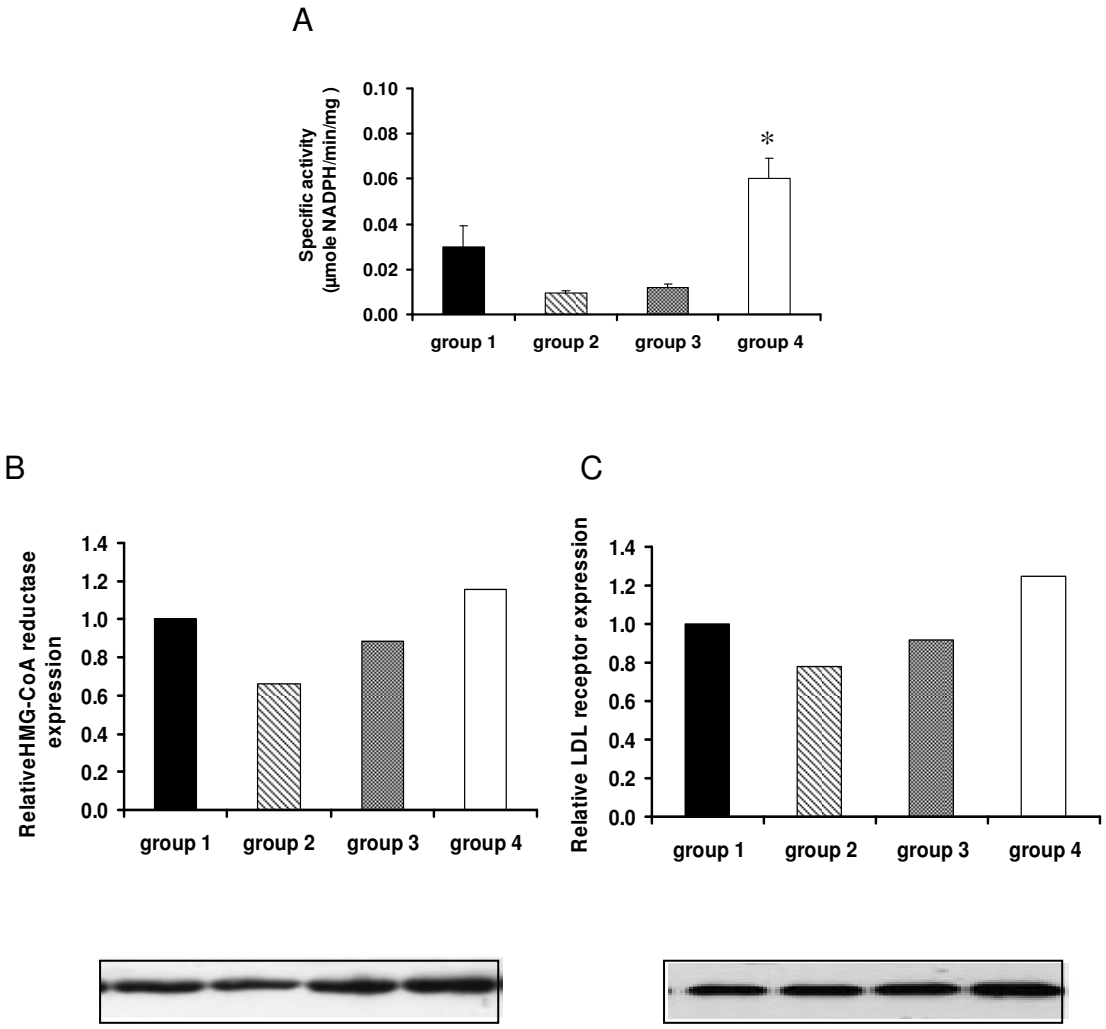


Figure 23 Hepatic HMG-CoA reductase activity (A), mRNA expressions of HMG-CoA reductase (B), and LDL receptor (C) of the experimental rats. The values of the enzyme activity are given as mean ± SEM, (n = 3), * $p < 0.05$. Representative results of liver HMG-CoA reductase and LDL receptor mRNA levels are shown in each column. Group 1 = normal, Group 2 = hypercholesterolemic, Group 3 = hypercholesterolemic + morelloflavone, Group 4 = normal + morelloflavone.

CHAPTER 5

CONCLUSIONS

House mouse catalytic portion of HMG-CoA reductase was successfully produced from *E. coli* strain BL21 by using the recombinant plasmid pET 17b-CD-HMG. The partially purified enzyme containing a major protein of 50 kDa was obtained. Morelloflavone isolated from *Garcinia dulcis* leaves showed its inhibitory effects on this enzyme *in vitro* by competing with HMG-CoA, whereas it was noncompetitive towards NADPH. The same compound and both isoflavone subunits, naringenin and luteolin, were found equally effective at the enzyme inhibition. Based on these results, it was postulated that morelloflavone could exert the HMG-CoA reductase inhibitory activity through binding of the enzyme active site with either of its subunits.

The animal study further revealed that morelloflavone caused a significant increase of circulating HDL particles in normal rats, and also up-regulated hepatic HMG-CoA reductase and LDL receptor mRNA expressions in both normal and hypercholesterolemic animals in response to the enzyme inhibition.

The findings from this study thus suggested that morelloflavone derived from *G. dulcis* could be considered as an effective HMG-CoA reductase inhibitor that might be developed into a new hypocholesterolemic agent in the future. However, further studies are necessary to confirm the above effects of this compound and to establish its mechanism of action.

REFERENCES

http://www.biochemsoctrans.org/bsl (accessed 27 October 2008)

http://exrx.net/Testing/LDL&HDL.html (accessed 13 December 2008)

http://www.nature.com/nrm/journal/V9/nz/fig_tab/nrm2336-F2.html (accessed 15 May 2009)

http://www.nature.com/statins (accessed 26 July 2007)

http://www.smbrower.com/mediawiki/images/8/8f/Cholesterol_Synthesis_Pathway.png (accessed 15 July 2009)

http://www.sosnick.uchicago.edu/competent_cells.html (accessed 15 May 2008)

http://www.rpi.edu/dept/bcbp/mulbiochem/MBWeb/mb2/part1/cholesterpl.html (accessed 2 May 2009)

Abifadel, M., Varret, M., Rabes, J.P., Allard, D., Ouguerram, K., Devillers, M., Cruaud, C., Benjanet, S., Wickham, L., Erlich, D., Derré, A., Villéger, l., Farnier, M., Beucler, I., Bruckert, E., Chambaz, J., Chanu, B., Lecerf, J.M., Luc, G., Moulin, P., Weissenbach, J., Part, A., Krempf, M., Junien, C., Seidah, N.G., Buileau, C. 2003. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nature Genetic* 34(2): 154-156.

Alonso, R., Defesche, J.C., Tejedor, D., Castillo, S., Stef, M., Mata, N., Gomez-Enteria, P., Martinez-Faedo, C., Forga, L., Mata, P. 2009. Genetic diagnosis of familial hypercholesterolemia using a DNA-array based platform. *Clinical Biochemistry* 42: 899-903.

Ansari, W.H., Rahman, W., Barraclough, D., Maynard, R., Scheinmann, F. 1976. Biflavonoids and a flavone-chromone from the leaves of *Garcinia dulcis*. *Journal of the Chemical Society-Perkin Transactions 1*: 1458-1463.

Artieda, M., Cenarro, A., Junquera, C., Lasierra, P., Martı´nez-Lorenzo, M.J., Pocovı´, M., Civeira, F. 2005. Tendon xanthomas in familial hypercholesterolemia are associated with a differential inflammatory response of macrophages to oxidized LDL. *FEBS Letters* 579: 4503–4512.

Babu, V., Ali, S.M., Sultana, S., Ilyas, M. 1988. A biflavonoid from *Garcinia nervosa.* *Phytochemistry* 27(10): 3332-3335.

Bahramikia, S. and Yazdanparast, R. 2008. Effect of hydroalcoholic extracts of *Nasturtium officinale* leaves on lipid profile in high-fat diet rats. *Journal of Ethnopharmacology* 115(1): 116-121.

Bandaranayake, W.M., Gunasekera, S.D., Karunanayake, S., Sotheeswaran, S., Sultanbawa, M.U.S. 1975. Chemical investigations of Ceylonese plants. 13 terpenes of *Dipterocarpus* and *Doona* species. *Phytochemistry* 14: 2043-2045.

Baslas, R.K. and Pradeep, K. 1979. Chemical examination of the fruits of *Garcinia xanthochymus*. *Current Science* 48(18): 810-815.

Baslas, R.K. and Pradeep, K. 1981. Isolation and characterization of biflavanone and xanthones in fruits of *Garcinia xanthochymus.* *Acta Ciencia Indica, Chemistry* 7(1-4): 31-34.

Bettelheim, F.A. and March, J. 1991. *Introduction to general, organic & Biochemistry.* P.158. Saunders: New York.

Benguechea-Alonso, M.T. and Ericson, J. 2007. SREBP in signal transduction: cholesterol metabolism and beyond. *Current Opinion in Cell Biology* 19(2): 215-222.

Bergstrom, J.D., Bostedor, R.G., Rew, D.J., Geissler, W.M., Wright, S.D., Chao, Y.S. 1998. Hepatic responses to inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase: a comparision of atorvastatin and simvastatin. *Biochemica et Biophysica Acta* 1389: 213-221.

Bertolotti, M., Concari, M.P., Loria, P., Abate, N., Pinetti, A., Guicciardi, M.E., Carulli, N. 1995. Effect of different phenotypes of hypercholesterolemia and treatment with fibric and derivatives on the rates of cholesterol 7 α-hydroxylation in human. *Arteriosclerosis Thrombosis and Vascular Biology* 1064-1069.

Birjmohun, R.S., Hutten, B.A., Kastelein, J.J.P., Strues, E.S. 2005. Efficacy and safety of high-density lipoprotein cholesterol increasing compounds: a meta-analysis of randomized trials. *Journal of the American College of Cardiology* 45: 185-197.

Bok, S.H., Park, S.Y., Park, Y.B., Lee, M.K., Jeon, S.M., Jeong, T.S., Choi, M.S. 2002. Quercetin dehydrate and gallete supplements lower plasma and hepatic lipids and change activities of hepatic antioxidant enzyme in high cholesterol-fed rats. *International Journal of Vitamin and Nutrition Research* 72(3): 161-169.

Boren, J., Elstrom, U., Agren, B., Nilsson-Elle, P., Innerality, T.L. 2001. The molecular mechanism for the genetic disorder familial defective apolipoprotein B100. *Journal of Biological Chemistry* 276: 9214-9218.

Bravo, E., Flora, L., Cantafora, A., Luca, V.D., Tripodi, M., Avella, M., Botham, K.M. 1997. The influence of dietary saturated and unsaturated fat on hepatic cholesterol metabolism and the biliary excretion of chylomicron cholesterol in the rat. *Biochimica et Biophysica Acta* 1390: 134–148.

Breton, M., Precigoux, G., Courseille, C., Hospital, M. 1975. Genistéin. *Acta Crystalographica* B31: 921-923.

Brown, M.S., and Goldstein, J.L. 1980. Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *Journal of Lipid Research* 21: 505–517.

Brown, M.S., and Goldstein, J.L. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science* 232: 34–47.

Browning, D.R.L., Martin, R.M. 2007. Statins and risk of cancer: a systematic review and meta-analysis. *International Journal of Cancer* 120: 833-843.

Bucci, C., Seru, R., Annella, T., Vitelli, R., Lattero, D., Bifulco, M., Mondola, P., Santillo, M. 1998. Free fatty acids modulate LDL receptor activity in BHK-21 cells. *Atherosclerosis* 137: 329–340.

Bunyapraphatsara, N. and Chokechaichareonporn, O. 1999. *Thai Traditional Herbs 3,* pp. 639-640 Bangkok: Prachachon (In Thai)

Bybee, K.A., Prasad, A., Barsness, G.W., Lerman, A., Jaffe, A.S., Murphy, J.G., Wright, R.S., Rihal, C.S. 2004. Clinical characteristics and thrombolysis in myocardial infarction frame counts in women with transient left ventricular apical ballooning syndrome. *Journal of the American College of Cardiology* 94: 343–346.

Capps N. 2006. Total and low-density lipoprotein cholesterol responses to ezetimibe in clinical practice. *International Journal of Clinical Practice* 60: 867–869.

Castado, J.C., Prudente, A.S., Ferreira, J., Guimaraes, C.L., Monache, F.D., Filho, V.C., Chuki, M.F., Cabrini, D.A. 2008. Anti-inflamatory effect of hydroalcoholic extract and two biflavonoids from *Garcinia gardneriana* leaves in mouse paw oedema. *Journal of Ethnopharmacology* 118: 405-411.

Catapano, A.L. 1992. Mode of action of fibrates. *Phamacological Research* 26: 331-340.

Chang, J.J., Chen, T.H., Chan, P., Chen, Y.J., Hsu, F.L., Lo, M.Y., Lin, J.Y. 2001. The *in vitro* inhibitory effect of tannin derivatives on 3-hydroxy-3-methylglutaryl-coenzyme A reductase on Vero cells. *Pharmacology* 62(4): 224-228.

Chait, A., Brazg, R.L., Tribble, D.L., Krauss, R.M. 1993. Succeptibility of small low dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phonotype, pattern B. *the American Journal of Medical Association* 94: 350-356.

Chen, F.C., Lin, Y.M., Hung, J.C. 1975. Phenolic compounds from the heartwood of *Garcinia multiflora.* *Phytochemistry* 14(1): 300-303.

Chen, T.H., Liu, J.C., Chang, J.J., Tsal, M.F., Hsreh, M.H., Chan, P. 2001. The *In vitro* inhibitory effect of flavonoids Astilbin on 3-hydroxy-3-methylglutaryl coenzyme A reductase on Vero cells. *Chinese Medical Journal* 64: 382-387.

Chen, C.W. and Cheng, H.H. 2005. A rice bran oil diet increase LDL-receptor and HMG-CoA reductase mRNA expressions and insulin sensitivity rats with streptozotocin/nicotinamide-induced type 2 diabetes. *Journal of Nutrition* 136: 1472-1476.

Cherng, S., Young, J., Ma, H. 2008. HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase) (HMGR). *Journal of American Science* 4(3): 62-64.

Cho, I.J., Ann, J.Y., Kim, S., Choi, M.S., Ha, T.Y. 2008. Resveratrol attenuates the expression of HMG-CoA reductase mRNA in hamsters. *Biochemical and Biophysical Research Communications* 367: 190-194.

Choi, M.S., Do, K.M., Park, Y.S., Jeon, S.M., Jeong, T.S., Lee, Y.K., Lee, M.K., Bok, S.H. 2001. Effect of naringin supplementation on cholesterol metabolism with different levels of vitamin E. *Annals of Nutrition and Metabolism* 45(5): 193-201.

Cotterill, P.J., Scheinmann, F., Puranik, G.S. 1977. Phenolic compounds from the heartwood of *Garcinia indica.* *Phytochemistry* 16(1): 148-149.

Davignon, J. 2009. Beneficial cardiovascular pleiotropic effects of statins. *Circulation* 109: III39-III43.

Deachathai, S., Mahabusarakam, W., Phongpaichit, S., Taylor, W.C. 2005. Phenolic compounds from the fruit of *Garcinia dulcis.* *Phytochemistry* 66(19): 2368-2375.

Deachathai, S., Mahabusarakam, W., Phongpaichit, S., Taylor, W.C., Zhang, Y.-J., Yang, C.R. 2006. Phenolic compounds from the flowers of *Garcinia dulcis.* *Phytochemistry* 67(5): 464-469.

Deachathai, S., Phongpaichit, S., Mahabusarakam, W. 2008. Phenolic compounds from the seeds of *Garcinia dulcis*. *Natural Product Research* 22(15): 1327-1332.

Decha-Dier, U., Hutadilok-Towatana, N., Mahabusarakam, W., Sawangjaroen, K., and Pinkaew, D. 2008. Anti-atherogenic effects of morelloflavone from *Garcinia dulcis* leaf in hypercholesterolemic rabbits. *Journal of Natural Remedies* 8(2): 151-159.

Defesche, J.C., van Dierman, D.E., Lansberg, P.J., Lumping, R.J., Reymer, P.W.A., Hayder, M.R., Kastolein, J.J.P. 1993. South Africa founder mutations in the low-density lipoprotein gene causing familial hypercholesterolemia in the Dutch population. *Human Genetic* 92: 567-570.

Do, G.M., Kwon, E.Y., Kim, H.J., Jeon, S.M., Ha, T.Y., Park, T., Choi, M.S. 2008. Long-term effects of resveratrol supplementation on suppression of atherogenic lesion formation and cholesterol synthesis in apo E-deficient mice. *Biochemical and Biophysical Research Communications* 374(1): 55-59.

Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pralt, S.J., Moynihan, J., Paw, B.H., Drejer, A., Barut, B., Zapata, A., Law, T.C., Brugnara, C., Lux, S.E., Pinkus, G.S., Pinkus J.L., Kingsley, P.D., Palis, J., Fleming, M.D., Andrews, N.C., Zon, L.I. 2000. Positional cloning of zebiafish ferroportin 1 identifies a conserved vertebrate iron exporter. *Nature* 403(6771): 776-781.

Du, C., Sato, A., Watanabe, S., Wu, C.Z., Ikeemoto, A., Ando, K., Kikugawa, K., Fujii, Y., Okuyama, H. 2003. Cholesterol synthesis in mice is suppressed but lipofuscin formation is not affected by long-term feeding of n-3 fatty acid-enriched oils compared with lard and n-6 fatty acid-enriched oils. *Biological & Pharmaceutical Bulletin* 26: 766-770.

Duncan, R.E., Ei-Sohemya, A., Archer M.C. 2005. Regulation of HMG-CoA reductase in MCF-7 cells by genistein, EPA, and DHA, alone and in combination with mevastatin. *Cancer Letters* 224: 221–228.

Duval, C., Muller, M., Kersten, S. 2007. PPARα and dyslipidemia. *Biochemica et Biophysica Acta* 1997: 961-971.

Endo, A., Tsujita, Y., Kuroda, M., Tanzawa, K. 1979. The discovery of statins. *Biochemica et Biophysica Acta* 575: 266–276.

Endo, A. 2004. The discovery and development of HMG-CoA reductase inhibitors. *Atherosclerosis Supplements* 5: 67-80.

Endres, M., Laufs, U., Huang, Z., Nakamura, T., Huang, P., Moskowitz, M.A., Liao, J.K. 1998. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proceedings of National Academy of Sciences U.S.A.* 95: 8880-8885.

Elfita, E., Muharni, M., Latief, M., Darwati, D., Widiyantoro, A., Supriyatna, S., Bahti, H.H., Dachriyanus, D., Cos, P., Maes, L., Kenne, F., Sandra, A., Luc, P. 2009. Antiplasmodial and other constituents from four Indonisian *Garcinia* spp. *Phytochemistry* 70(7): 907-912.

Florentin, M., Liberopoulos, E.N., Elisaf, M.S. 2008. Ezetimibe-associated adverse effect: what the clinician needs to know. *International Journal of Clinical Practice* 62(1):88-96.

Frimpong, K., Darnay, B.G., Rodwell, V.W. 1993. Syrian hamster 3-hydroxy-3-methylglutaryl-coenzyme A reductase expressed expressed in *Escherichia coli*: production of homogeneous protein. *Protein Expression Purification* 4: 337-344.

Froyland, L., Vaagenes, H., Asiedu, D.K., Garras, A., Lie, O., Berge, R.K. 1996. Chronic administration of eicosapentaenoic acid and docosahexaenoic acid as ethyl ester reduced plasma cholesterol and changes the fatty acid composition in rat blood organ. *Lipids* 31: 169-178.

Garcia-Calvo, M., Lisnock, J., Bull, H.G., Hawes, B.E., Burnett, D.A., Braun, M.P. 2005. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proceedings of National Academy of Sciences U.S.A* 102(23): 8132-7.

Gaussin, V., Skarlas, P., Ching, Y.P., Hardie, G., Hue, L. 1997. Distinct type-2A protein phosphatases activate HMG-CoA reductase and acetyl-CoA carboxylase in liver. *FEBS Letters* 413: 445–448.

Gebhardt, R., Beck, H., Wagner, K.G. 1994. Inhibition of cholesterol biosynthesis by allicin and ajoene in rat hepatocytes and HepG2 cells. *Biochimica et Biophysica Acta 1213*: 57-62.

Gebhardt, R. and Beck, H. 1996. Differential inhibitory effects of garlic-derived organosulfur compounds on cholesterol biosynthesis in primary rat hepatocyte culture. *Lipids* 31: 1269-1276.

Gebhardt, R. 1998. Inhibition of cholesterol biosynthesis in primary cultured rat hepatocytes by artichoke (*Cynara Scolymus L.*) extracts. *Journal of Pharmacology and Experimental Therapeutics* 286(3): 1122-1128.

Gennes, J.L., Maunand, B., Salman, D., Laudat, P., Truffet, I. 1965. Results of atromid or CPIB treatment with and without andro sterone in hyperlipidamia (110 therapeutric trial in 2 years). *Bulletin of Rajendra Memorial Research Institute of Medical Sciences Paris* 116: 759-784.

Ghirlanda, G., Oradei, A., Manto, A., Lippa, S., Uccicli, L., Capato, S., Greco, A., Littarru, G.P. 1993. Evidence of plasma CoQ-10 lowering effect by HMG-CoA reductase inhibitors: a double-blind, placebo controlled study. *Journal Clinical Phamacology* 33(3): 226-229.

Gil, B., Sanz, M.J., Terencio, M.C., Gunasegaran, R., Paya, M., Alcaraz, M.J. 1997. Morelloflavone, a novel biflavonoid inhibitor of human secretory phospholipase A2 with anti-inflammatory activity. *Biochemical Pharmacology* 53(5): 733-740.

Glynn, N.C., Comstock, J.C., Sood, S., Dang, P., Chaparro, J.X. 2008. Isolation of NBS-LRR resistance gene analogues and kinase analogues from sugar cane (*Saccharum* spp.). *Pest Management Science* 64: 48–56

Goldstein, J.L. and Brown, M.S. 1990. Regulation of mevalonate pathway. *Nature* 343: 425-430.

Goldstein, D.J., Mallinckodt, C., Lu, Y. 2001. Efficacy and safety of duloxetine in the treatment of major depression. Presented at the Annual Meeting of the American Psychiatric Association (APA). May 2001, New Orleans, USA.

Gouni-Berthold, I., Berthold, H.K., Gylling. H., Hallikainen, M., Glannakidou, E., Stier, S., Ko, Y., Patel, D., Soutar, A.K., Seedorf, U., Mantzoros, C.S., Plat, J., Krone, W. 2009. Effect of ezetimibe and/or simvastatin on LDL receptor protein expression and on LDL receptor and HMG-CoA reductase gene expression: a randomized trial in healthy men. *Atherosclerosis* 198(1): 198-207.

Graham, D.J., Staffa, J.A., Shatin, D. 2004. Incidence of hospitalized rhabdomyolysis in patients treated with lipid lowering drug. *Journal of the American Medical Association* 292(21): 2585-2590.

Grundy, S.M. and Vegn, G.L. 1988. Plasma cholesterol responsiveness to saturated fatty acid. *American Journal of Clinical Nutrition* 47: 822-824.

Gylling, H. and Miettinen, T.A. 2005. The effect of plant stanol- and sterol-enriched foods on lipid metabolism, serum lipids and coronary heart disease. *Annals of Clinical Biochemistry* 42: 254–263.

Hanai, J., Cao, P., Tanksale, P., Imamura, S., Koshimizu, E., Zhao, J., Kishi, S., Yamashita, M., Phillips, P.S., Sukhatme, V.P., Lecker, S.H. 2007. The muscle-specific ubiquitin ligase atrogin-1/MAFbx mediates statin-induced muscle toxicity. *Journal of Clinical Investigation* 117(12): 3490-3951.

Hardie, D.G. 1992. Regulation of fatty acid and cholesterol metabolism by the AMP-activated protein kinase. *Biochimica et Biophysica Acta* 1123: 231-238.

Harrison, L.J., Leong, L.S., Leong, Y.W., Sia, G.L., Sim, K.Y., Tan, H.T.W. 1984. Xanthone and flavonoida constituents of *Garcinia dulcis* (Guttiferae). *Natural Product Letter* 5(2): 111-116.

Havsteen, B.H. 2002. The biochemistry and medical significance of flavonoids. *Pharmacology & Therapeutic* 96: 67-202.

Herbin, R.K., Jackson, B., Locksley, H.D., Scheinmann, F., Wolstenholme, W.A. 1970. Extractives from Guttiferae. XV Biflavonoids of *Garcinia volkensii*. *Phytochemistry* 9(1): 221-226.

Horton, J.D., Goldstein, J.L. , Brown, M.S. 2002. SREBPs: activators of the compete program of cholesterol and fatty acid synthesis in the liver. *Journal of Clinical Investigation* 190: 1125-1131.

Hutadilok-Towatana, N., Kongkachuay, S., Mahabusarakam, W. 2007. Inhibition of human lipoprotein oxidation by morelloflavone and camboginol from *Garcinia dulcis*. *Natural Product Research* 21(7): 655-662.

Huval, C.C., Holmes-Farley, S.R., Mandeville, W.H., Sacchiero, R., Dhal, P.K. 2004. Syntheses of hydrophobically modified cationic hydrogels by copolymerization of alkyl substituted diallylamine monomers and their use as bile acid sequestrants. *European Polymer Journal* 40: 693–701.

Iinuma, M., Ito, T., Tosa, H., Tanaka, T., Riswan, S. 1996a. Fives new xanthones from *Garcinia dulcis*. *Journal of Natural Product* 59(5): 472-475.

Iinuma, M., Ito, T., Tosa, H., Tanaka, T., Riswan, S. 1996b. A xanthone from *Garcinia cambogia*. *Heterocycle* 43(3): 535-538.

Iinuma, M., Ito, T., Tosa, H., Tanaka, T., Riswan, S. 1996c. Three new bensophenone-xanthones dimers from the root of *Garcinia dulcis*. *Chemical & Phamaceutical Bulletin* 44(9): 1744-1747.

Ihara-Watanabe, M., Umekama, H., Takahashi, T., Furuichi, Y. 1999. Effect of dietary alpha-or gamma-linolenic acid on level and fatty acid compositions of serum and hepatic lipids, and activity and mRNA abundance of 3-hydroxy-3-methylglutarly CoA reductase in rat. *Comparative Biochemistry and Physiology Part A* 122: 213-220.

Ihara-Watanabe, M., Umekama, H., Takahashi, T., Furuichi, Y. 2000. Comparative effect of safflower oil and perilla oil on serum and hepatic lipid levels, fatty acid compositions of serum and hepatic phospholipids, and hepatic mRNA expressions of 3-hydroxy-3-methylglutarly CoA reductase, LDL receptor, and cholesterol 7 alpha-hydroxylase in young and adult rats. *Food Research International* 33(10): 893-900.

Istvan, E.S., Deisenhofer, J. 2000. The structural of catalytic portion of human HMG-CoA reductase. *Biochemica et Biophysica Acta* 1529: 9-18.

Istvan, E.S., Deisenhofer, J. 2001. Strutural mechanism for statin inhibition of HMG-CoA reductase. *Science* 292: 1160-1164.

Istvan, E.S. 2003. Statins inhibition of HMG-CoA reductase; a 3-dimensional view. *Atherosclerosis Supplements* 4: 3-8.

Ito, C., Miyamoto, Y., Nakayama, M., Kawai, Y., Rao, K.S., Furukawa, H. 1997. A novel depsidone and some new xanthones from *Garcinia* species. *Chemical & Phamaceutical Bulletin* 45(9): 1403-1413.

Jain, K.S., Kathiravan, M.K., Somani, R.S., Shishoo, C.J. 2007. Review of the biology and chemistry of hyperlipidemia. *Bioorganic & Medical Chemistry* 15: 4674-4699.

Jayasooriya, A.P., Sakono, M., Yukisaki, C., Kawano, M., Yamamoto, K., Fukuda, N. 2000. Effect of *Momordica charantia* powder on serum glucose level and various lipid parameters in rats fed with cholesterol-free and cholesterol-enriched diets. *Journal of Ethnophamacology* 72: 331-336.

Jelesoff, N.E., Ballantyne, C.M., Xydakis, A.M., Chiou, P., Jones, P.H., Guyton, J.R. 2006. Effectiveness and tolerability of adding ezetimibe to niacin-based regimens for treatment of primary hyperlipidemia. *Endocrine Practice* 12: 159–64.

Joshi, B.S., Kamet, V.N., Viswanathan, N. 1970. Isolation and structure of two biflavones from *Garcinia talboti*. *Phytochemistry* 9(4): 881-888.

Kapur, N.K., Ashen, D., Blumenthal, R.S. 2008. High density lipoprotein cholesterol: an evolving target of therapy in the management of cardiovascular disease. *Journal of Vascular Health and Risk Management* 4: 39–57.

Karanjgaokar, C.G., Radhakrihnan, P.V., Venkataraman, K. 1967. Morelloflavone, a 3-(8-) flavonylflavanone, from the heartwood of *Garcinia morella. Tetrahedron Letter* 8(33): 3195-3198.

Khoo, K.L., Tan, H., Liew, Y.M., Deslypere, J.P., Jenus, E. 2003. Lipid and coronary heart disease in Asia. *Atherosclerosis* 169: 1-10.

Kim, K.H., Jeong, T.S., Lee, M.K., Park, Y.B., Choi, M.S. 2003. Lipid-lowering efficacy of hesperetin metabolites in high-cholesterol fed rats. *Clinica Chimica Acta* 327: 129-137.

Kim, H.J., Oh, G.T., Park, Y.B., Lee, M.K., Seo, H.J., Choi, M.S. 2004. Naringin alters the cholesterol biosynthesis and antioxidant enzyme activities in LDL receptor-knockout mice under cholesterol fed condition. *Life Science* 74: 1621-1634.

Kim, H.J., Lee, D.H., Hwang, Y.Y., Lee, K.S., Lee, J.S. 2005. Characterization of β-hydroxy-β-methylglutaryl coenzyme A reductase inhibitor from *Pueraria thunbergiana. Journal of Agricultural and Food Chemistry* 53: 5882-5888.

Kleinsek, D.A., Ranganathan, S., Popter, J.W. 1977. Purification of 3-hydroxy-3-methylglutaryl-coenzyme A reductase from rat liver. *Proceedings of National Academy of Sciences U.S.A.* 74: 1431-1435.

Knopp, R.H., Ginsberg, J., Albert, J.J., Hoff, C., Ogilvie, J.T., Warnick, G.R., Burrows, E., Retzlaff, B., Poole, M. 1985. Contrasting effects of unmodified and time-release forms of niacin on lipoproteins in hyperlipidemic subjects: clues to mechanism of action of niacin. *Metabolism* 34(7): 642-650.

Konoshima, M., Ikeshiro, Y., Nishinaga, A., Matsuura, T., Kubota, T., Sakamoto, H. 1969. The constitution of flavonoids from *Garcinia spicata* Hook. f. *Tetrahedron Letter* 10(2): 121-124.

Koo, M., Kim, S.H., Lee, N., Yoo, M.Y., Ryu, S.Y., Kwan, D.Y., Kim, Y.S. 2008. 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitory effect of *Vitis vinifera*. *Fitotherapia* **79**: 204–206

Kosela, S., Hu, L.H., Yip, S.H., Rachmatia, T., Sukri, T., Daulay, T.S., Tan, G.K., Vittal, J.J., Sim, K. 1999. Dulxanthone E: a pyranoxanthone from the leaves of *Garcinia dulcis*. *Phytochemistry* 52(7): 1375-1377.

Kosela, S., Hu, L.H., Yip, S.H., Rachmatia, T., Hanafi, M., Sim, K.Y. 2000. Dulxanthones F-H, three new pyranoxanthone from the of *Garcinia dulcis*. *Journal of Natural Product* 63(3): 406-407.

Kumari, K. and Augusti, K.T. 2007. Lipid lowering effect of S-methyl cystein sulfoxide from *Allium cepa* Linn in high cholesterol diet fed rats. *Journal of Ethnopharmacology* 109: 367-371.

Leammli, U.K. 1970. Clevage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 15:680-685.

Lee, H.J. and Choi, M.S. 1999. Measurement of inhibitory activities on 3-hydroxy-3-methylglutaryl CoA reductase and acyl-CoA: cholesterol acyltransferase by various plant extracts *in vitro.* *Journal of Korean Society Food Science and Nutrition* 28: 958-962.

Levy, D. and Brink, S. 2005. *A Change of Heart: How the people of Framingham, Massachusetts, helped unravel the mysteries of cardiovascular disease*. p.268. New York: Alfred A. Knopf.

Li, A.C. and Glass, C.K. 2002. The macrophage foam cell as a target for therapeutic intervention. *Nature Medicine* 8: 1235-1242.

Li, X.C., Joshi, A.S., Elsohly, H.N., Khan, S.I., Jacob, M.R., Zhang, Z., Khan, I.A., Ferreira, D., Walker, L.A., Broedel, Jr. S.E., Raulli, R.E., Cihlar, R.L. 2002. Fatty acid synthase inhibitors from plants: isolation, structure elucidation and SAR studies. *Journal of Natural Product* 65: 1909-1914.

Likhitwitayawuid, K., Chanmahasathien, W., Ruangrungsi, N., Krungkrai, J. 1998. Xanthones with antimalarial activity from *Garcinia dulcis*. *Planta Medica* 64(3): 281-282.

Lin, Y.M., Anderson, H., Flavin, M.T., Pai, Y.H.S. 1997. *In vitro* anti-HIV of biflavonoids isolated from *Rhus succedanea* and *Garcinia multiflora.* *Journal of Natural Product* 60: 884-888.

Lock, E.A., Mitchell, A.M., Elcombe, C.R. 1989. Biochemical mechanisms of induction of hepatic peroxisome proliferation. *Annual Review of Pharmacology and Toxicology* 29: 145–63.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193: 265-275.

Ma, P.T.S., Gil, G., Sudhog, T.C., Biheimer, D.W., Goldstein, J.L., Brown, M.S. 1986. Mevinolin and inhibitor of cholesterol synthesis, induces mRNA for low density lipoprotein receptor in livers of hamster and rabbits. *Proceedings of National Academy of Sciences of U.S.A* :8370-8376.

Maksimova, E., Yie, T.A., Rom, W.N. 2008. *In vitro* mechanisms of lovastatin on lung cancer cell lines as a potential chemopreventive agent. *Lung* 186 (1): 45–54.

Masuda, T., Yamashita, D., Takeda, Y., Yonemori, S. 2005. Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent inhibitors from *Garcinia subelliptica*. *Bioscience, Biotechnology and Biochemistry* 69(1): 197-201.

Mbwambo, Z.H., Kapingu, M.C., Moshi, M.J., Machumi, F., Apers, S., Cos, P., Fereira, P., Marais, J.P., Vanden-Berghe, D., Maes, L., Vlietinck, A., Pieters, L. 2006. Antiparasitic activity of some xanthones and biflavonoids from the root bark of *Garcinia livingstonei*. *Journal of Natural Product* 69(3): 369-372.

McKenney, J.M., Jones, P.H., Bays, H.E., Knopp, R.H., Kashyap, M.L., Ruoff, G., Stanek, E.J., McGovern, M.E. 2006. Comparative lipid effects of combination therapy with a statin 300 and extended-release niacin versus statin plus ezetimibe versus a statin alone. *Atherosclerosis Supplements* 7:174.

Menendez, R., Amor, A.M., Rodiero, I., Gonzalez, R.M., Gonzalez, P.C., Alfonso, J.L., Mas, R. 2001. Policosanol modulates HMG-CoA reductase activity in culture fibroblast. *Archives of Medical Research* 32: 8-12.

Meyers, C.D., Kamanna, V.S., Kashyap, M.L. 2004. Niacin therapy in atherosclerosis. *Current Opinion Lipidology* 15: 659-665.

Mirmiran, P., Ramezankhani, A., Azizi, F. 2008. Combined effects of saturated fat and cholesterol intakes on serum lipids: tehran lipid and glucose study. *Nutrition* 25: 526–531.

Miyake, Y., Suzuki, E., Ohya, S., Fukumoto, S., Hiramitsu, M., Sakaida, K., Osawa, T., Furuichi, Y. 2006. Lipid-lowering effect of eriocitrin, the main flavonoid in lemon fruit, in rats on a high-fat and high-cholesterol diet. *Journal of Food Science* 71: S633–S637.

Molyneux, S.L., Young, J.M., Florkowski, C.M., Lever, M., George, P.M. 2008. Coenzyme Q 10: is there a clinical role and a case for measurement? *Clinical Biochemistry* 29(2): 71-82.

Moreno, F.S., Rossiello, M.R., Manjeshwan, S., Nath, R., Rao, F.M., Rajalakshmi, S., Sarma, D.S.R. 1995. Effect of β-carotene on the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase in rat liver. *Cancer Letters* 96: 201-208.

Myant, N.B., 1990. Current approaches to the genetic of coronary heart disease (CHD) including an account of work clone at Hammersmith hospital. *Bollettino Della Società Italiana di Biologia Sperimentale* 66: 1015-1041.

Nagasaka,Y., Fernandez, B., Garcia-Saura, M.F., Petersen, B., Ichinose, F., Bloch, K., Feelisch, M., Zapol, W. 2008. Brief periods of nitric oxide inhalation protect against myocardial ischemia-reperfusion injury. *Anesthesiology* 109: 675-682.

Ngouamegne, E.T., Fongang, R.S., Ngovela, S., Boyom, F.F., Rohmer, M., Tsamo, E., Gut, J., Rosenthal, P.J. 2008. Endodesmiadiol, a friedeland triterpenoid, and other antiplasmodial compounds from *Endodesmia calophylloides*. *Chemical & Pharmaceutical Bulletin* 56(3): 374-377.

Nofer, J.R., Kehrel, B., Fobker, M. 2002. HDL and arteriosclerosis: beyond cholesterol transport. *Atherosclerosis* 161: 1-16.

Nonaka, T., Kametani, F., Arai, T., Akiyama, H., Hasegawa, M. 2009. Truncation and pathogenic mutations facilitate the formation of intracellular aggregates of TDP-43. *Human Molecular Genetic* 18(18): 3353-3364.

Ohtsuki, K., Abe, A., Mitzusumi, H., Kendo, M., Vemura, K., Iwasaki, Y., Kendo, Y. 2003. Glucosyl hesperidin improves serum cholesterol composition and inhibits hypertrophy in vasculature. *Journal of Nutrition Science and Vitaminology* 49(6): 447-450.

Olender, E.H. and Simoni, R.D. 1992. The intra cellular targeting and membrane topology of 3-hydroxy-3-methylglutaryl Co A reductase. *Journal of Biological Chemistry* 267: 4223-4235.

Otsuki, T., Sakaguchi, H., Hatayama, T., Fujii, T., Tsujioka,T., Sugihare, T., Takata, A., Hyodon, F., Eto, M. 2004. Effects of an HMG-CoA reductase inhibitors, simvastatin, on human myeloma cells. *Oncology Report* 11(5): 1053-1058.

Pang, X., Yi, T., Yi, Z., Cho, S.G., Gu, W., Pinkaew, D., Fujise, K., Liu, M. 2009. Morelloflavone, a biflavonoids inhibits tumor angiogenesis by targeting Rho GTPases and extracellular signal regulated kinase signaling pathway. *Cancer Research* 69(2): 518-525.

Pelter, A., Warren, R., Chexel, K.K., Handa, B.K., Rahman, W. 1971. Biflavonyls from Guttiferae*, Garcinia livingstonei. Tetrahedron* 27(8): 1625-1634.

Permanaa, D., Lajis, N.H., Shaari, K., Ali, A.M., Mackeen, M.M., Kitajima, M., Takayama, H., Aimi, N. 2003. A new prenylated hydroquinone from roots of *Garcinia atroviridis* Griff ex T. Anders (Guttiferae). *Zeitschrift für Naturforschung* 58bb: 332-335.

Pierno, S., De Luca, A., Tricarico, D., Roselli, A., Natuzzi, F., Ferannini, E., Laico, M., Camerino, D.C. 1995. Potential risk of myopathy by HMG-CoA reductase inhibitor: a comparison of pravastatin and simvastatin effects on membrane electrical properties of rat skeletal muscle fibers. *Journal of the American Society for Pharmacology and Experimental Therapeutics* 275(3): 1490-1496.

Pinkaew, D., Cho, S.G., Hui, D.Y., Wiktorowicz, J.E., Hutadilok-Towatana, N., Mahabusarakam, W., Tonganunt, M., Stafford, L.J., Phongdara, A., Liu, M. Fujise, K. 2009. Morelloflavone block injury-induced neointimal formation by inhibiting vascular smooth muscle cell migration. *Biochemica et Biophysica Acta* 1790(1): 31-39.

Poynter, J.N., Gruber, S.B., Higgins, P., Almog, R., Bonner, J.D., Rennert, H.S., Low, M., Greenson, J.K., Rennert, G. 2005. Statins and the risk of colorectal cancer. *The New England Journal of Medicine* 352(21): 2184-2192.

Qureshi, A. A., Abuirmeileh, N., Din, Z. Z., Elson, C. E., Burger, W. C. 1983a. Inhibition of cholesterol and fatty acid biosynthesis in liver enzymes and chicken hepatocytes by polar fractions of garlic. *Lipids* 18: 343-348.

Qureshi, A. A., Din, Z. Z., Abuirmeileh, N., Burger, W. C., Ahmad, Y., Elson, C. E. 1983b. Suppression of avian hepatic lipid metabolism by solvent extracts of garlic: impact on serum lipids. *Journal of Nutrition* 113: 1746-1755.

Qureshi, B.A., Burger, W.C., Peterson, D.M., Elson, C.E. 1986. The structure of an inhibitor of cholesterol biosynthesis isolated from barley. *Journal of Biological Chemistry* 261: 10544–10550.

Qureshi, A. A., Crenshaw, T. D., Abuirmeileh, N., Peterson, D. M., Elson, C. E. 1987. Influence of minor plant constituents on porcine hepatic lipid metabolism: impact on serum lipid. *Atherosclerosis* 64: 109-115.

Rai, S.K., Sharma, M., Tiwari, M. 2009. Inhibitory effect of novel diallydisulfide analog on HMG-CoA reductase expression in hypercholesterolemic rats: CREB as a potential upstream target. *Life Science* 85: 211-219.

Raju, J. and Bird, R.P. 2007. Diosgenin, a naturally occurring furostanol saponin suppresses 3-hydroxy-3-methylglutaryl coenzyme A reductase expression and induces apotosis in HCT-116 human colon carcinoma cells. *Cancer Letter* 255: 194-204.

Rashid, M., Butzner, J.D., Warren, R., Molloy, M., Case, S., Zarkadas, M., Burrows, V., Switzer,C. 2009. Home blood testing for celiac disease: recommendations for management. *Canadian Family Physician* 55: 151-153.

Repa, J.J., Turley, S.D., Quan, G., Dietschy, J.M. 2005. Delienation of molecular changes in intrahepatic cholesterol metabolism resulting from diminished cholesterol absorption. *Journal of Lipid Research* 46: 779–789.

Reynolds, G.A., Basu, S.K., Osburne, T.F., Chin, D.J., Gil, G., Brown, J.L., Goldstein, J.L., Lusky, K.L. 1984. HMG CoA reductase: a negatively regulated gene with unusual promoter and 5′ untranslated regions. *Cell* 38: 275–285.

Roglans, N., Peris, C., Verd, J.C., Alegret, M., Vázquez,M., Sánchez, R.M., Laguna, J.C. 2001. Increase in hepatic expression of SREBP-2 gemfibrozil administration to rats. *Biochemical Pharmacology* 62: 803-809.

Roitelman, J., Olender, E.H., Bar-Nun, S., Dunn, W.A., Simoni, R.D. 1992. Immunological evidence for 8 spans in the membrane domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase: implications for enzyme degradation in the endoplasmic reticulum. *Journal of Cell Biology* 117: 959-973.

Sanford, M. and Curran, M.P. 2008. Niacin extended-release/simvastatin. *Drug* 68(16): 2373-2386.

Sanz, M.J., Ferrandiz, M.L., Cejudo, M., Terencio, M.C., Gil, B., Bustos, G., Ubeda, A., Gunasegaran, R., Alcaraz, M.J. 1994. Influence of a series of natural flavonoids on free radical generating systems and oxidative stress. *Xenobiotica* 24(7): 689-699.

Seifalian, A.M., Giudiceandrea, A., Schmitz-Rixen, T., Hamilton, G. 1999. Noncompliance: the silent acceptance of a villan. In *Tissue Engineering of Prosthetic Grafts* Vol. 2, pp. 45-56: P. Zilla and H.P. Greisler (Eds), Austin: R.G. Landes

Shafi, S., Stepanova, I.P., Fitzsimmons, C., Bowyer, D.E., Welzel, D., Born, G.V.R. 2000. Effects of reserpine on expression of LDL receptor in the liver and on plasma and tissue lipids, low density lipoprotein and fibrinogen in rabbits *in vivo*. *Atherosclerosis* 149: 267-275.

Shellman, Y.G., Ribble, D., Miller, L., Gendall, J., Van Buskirk, K., Kelly, D., Norris, D.A., Dellavalle, R.P. 2005. Lovastatin-induced apoptosis in human melanoma cell lines. *Melanoma Research* 15(2): 83-89.

Shen, J. and Yang, J. 2006. Chemical constituents from fruit of *Garcinia cowa. Zhongguo Yaoxue Zazhi* 41(9): 660-661.

Shepherd, J. and Schonfeld, G. 1995. Introduction. *American Journal of Cardiology.* 76: 1A-2A.

Shirai, N. and Suzuki, H. 2007. Effect of hexaenoic acid on brain 3-hydroxy-3-methylglutarly CoA reductase activity in male ICR mice. *Journal of Nutritional Biochemistry* 18: 488-494.

Singh, D.K., Banajee, S., Porter, T.D. 2008. Green and black tea extracts inhibit HMG-CoA reductase and activate AMP kinase to decrease cholesterol synthesis in hepatoma cell. *Journal of Nutritional Biochemistry* 20 (10): 816-822.

Sirtori, C.R., Calabresi, L., Werba, J.P., Francesshini, G., 1992. Tolerability of fibric acids. Comparative data and biochemical bases. *Pharmacological Research* 26: 243-260.

Soemiati, A., Kosela, S., Hanafi, M., Harrison, L.J. 2004. Elucidation and cytotoxic L 1210 evaluation of prenylated pyranoxanthonoids from the Indonesian *Garcinia dulcis* fruit (Guttiferae) n-hexane extract. *ITE Letter on Batteries, New Technology & Medicine* 5(4): 389-393.

Song, B.L., Javitt, N.B., DeBose-Boyd, R.A. 2005. Insig-mediated degradation of HMG CoA reductase stimulated by lanosterol, an intermediate in the synthesis of cholesterol. *Cell Metabolism* 1: 179-189.

Spady, D.K., Turley, S.D., Dietshy, J.M. 1985. Rates of low density lipoprotein uptake and cholesterol synthesis are regulated independently in the liver. *Journal of Lipid Research* 26: 465-472.

Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E., Fruchart, J. C. 1998. Mechanism of action of fibrates on lipid and lipoprotein metabolism. *Circulation* 98: 2088–2093.

Stedronsky, E.R. 1994. Interaction of bile acids and cholesterol with non-systemic agents having hypocholesterolemic properties. *Biochimica et Biophysica Acta* 1210: 255-287.

Subhadrabandhu, S. 2001. *Under-utilized Tropical Fruits of Thailand*, RAP publication: 2001/26. December 2001, 75 p. Bangkok: Food and Agriculture Organization of the United Nations Regional Office for Asia and the Pacific.

Sukpondma, Y., Rukachaisirikul, V., Phongpaichit, S. 2005. Xanthone and sesquiterpene derivatives from the fruits of *Garcinia scortechinii*. *Journal of Natural Products* 68(7): 1010-1017.

Sung, J.H., Choi, S.J., Lee, S.W., Park, K.H., Moon, T.M. 2004a. Isoflavones inhibit 3-methylglutaryl coenzyme A reductase in vitro. *Biosciences Biotechnology, and Biochemistry* 68(5): 428-432.

Sung, J.H., Choi, S.J., Lee, S.W., Park, K.H., Moon, T.M. 2004b. Isoflavones found in Korean soybean paste as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Biosciences Biotechnology, and Biochemistry* 68(5): 1051-1058.

Sweeney, M.R., Mcpartlin, J., Scott, J. 2007. Folic acid fortification and public health: report on threshold dosed above, which unmetabolised folic acid appear in serum. *BMC Public Health* 7: 41-47.

Tall, A.R. 1993. Review. Plasma cholesteryl ester transfer protein. *Journal of Lipid Research* 34: 1255-1274.

Tandon, V., Bano, G., Khajurta, V., Parihar, A., Gupta, S. 2005. Pleiotropic effects of ststins. *Indian Journal of Pharmacology* 37(2): 77-85.

Telford, D.E., Sutherland, B.G., Edwards J.D., Barrett, P.H.R., Hutt, M.W. 2007. The molecular mechanisms underlying the reductase of LDL Apo B-100 by ezetimibe plus simvastatin. *Journal of Lipid Research* 48(13): 699-708.

Temel, R.E., Tang, W., Ma, Y., Rudel, L.L., Willingham, M.C., Ioannou, Y.A., Davies, J.P., Nilsson, L.M., Yu, L. 2007. Hepatic Niemann-Pick C1-like 1 regulates biliary cholesterol concentration and is a target of ezetimibe. *Journal of Clinical Investigation* 117(7): 1968-1978 (2007).

Terashima, K., Ishida, T., Furugawa, T., Takaya, Y., Niwa, M. 2008. Constituents of green and ripened fruit of *Garcinia subelliptica.* *Heterocycle* 75(2): 407-413.

The research collaborative group. 2008. SLC1B1 variants and statin-induced myopathy a genomewise study. *New England Journal of Medicine* 359(8): 789-799.

Toth, P.P., Davidson, M.H. 2005. Simvastatin plus ezetimibe: combination therapy for the management of dyslipidaemia. *Expert Opinion on Pharmacotherapy* 6: 131–139.

Turner, S.J., Zhuang, S., Zhang, T., Buss, G.R., Pilz, R.B. 2008. Effects of lovastatin on Rho isoform expression, activity, and association with guanine nucleotide dissociation inhibitors. *Biochemical Pharmacology* 75(2) 405-413.

van de Donk, N.W., Kamphuis, M,M., Lokhorst, H.M., Bloem, A.C. 2002. The cholesterol lowering drug lovastatin induces cell death in myeloma plasma cells. *Leukemia* 16: 1362--1371.

Verbeek, R., Plomp, A.C., van Tol, E.A., van Noort, J.M. 2004. The flavones luteolin and apigenin inhibit *in vitro* antigen-specific proliferation and interferon-gamma production by murine and human autoimmune T cells. *Biochemical Pharmacology* 68, 621-629.

Verdi, L.G., Pizzolatti, M.G., Montanher, A.B.S., Brighente, I.M.C., Smania, J.A., Smania, E.F.A., Simionatto, E.L., Delle, M.F. 2004. Antibacterial and brine shrimp lethality tests of biflavonoids and derivatives of *Rheedia gardneriana*. *Fitoterapia* 75(3-4): 360-363.

Walter, M. 2009. Interrelationships among HDL metabolism, aging, and atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 10 doi: 10.1161/ATVBAHA.108.181438

Wagstaff, L.R., Mitton, M.W., Arvik, B.M., 2003. Statin-associated memory loss: analysis of 60 case reports and review of the literature. *Pharmacotherapy.* 23**:** 871–880.

Waterman, P.G. and Hussain, R.A. 1982. Major xanthones from *Garcinia quedrifaria* and *G. staudtii* stem barks. *Phytochemistry* 21(8): 2099-2101.

Wei, E. K., Giovannucci, E., Fuchs, C.S., Willett, W.C., Mantzoros, C.S. 2005. Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. *Journal of the National Cancer Institute* 97: 1688–1694.

White, D.A., Bennett, A.J., Billet, M.A., Salter, A.M. 1997. Genetic determinants of plasma lipoprotein levels and their dietary response. *Prostaglandins and Essential Fatty Acids* 57(4&5): 455-462.

Wierzbicki, A.S., Nishtar, S., Lumb, P.J., Lambert-Hammill, M., Turner, C.N., Crook, M.A., Marber, M.S., Gill, J. 2005. Metabolic syndrome and risk of coronary heart disease in a Pakistani cohort. *Heart* 91: 1003–1007.

William, D.A., Foye, W.O., Lemki, T.L. 2000. *Foy's Principle of Medical Chemistry,* 583 p. Philadephia: Lippincott Williams & Wilkins.

Wongwisansri, S. and Kitikara, K. 2004. Development of a rapid screening assay to determine inhibitory effects on 3-hydroxy-3-methylglutaryl co-enzyme A reductase (HMG-CoA reductase): the rate limiting enzyme in the early step of cholesterol biosynthesis pathway. Presented at the 30th Congress on Sciences and Technology of Thailand. October 2004,19-21, Bangkok, Thailand.

World Health Organization. 2002. *The World Health Report 2002-Reduce risk, Promoting health life*, Geneva, World Health Organization. p.248. (http://www.who.int/whr/2002/en).

Wuttidhammavej, V. 1997. Thai Traditional Medicine, second edition, Bankok: Odien Store. (In Thai)

Yupwattanaphun, C., Subhadrabandhu, S., Sugiura, A., Yonemori, K., Utsunomiya, N. (2002) *Utilization of Some Garcinia Species in Thailand*. In: R. Drew (ed.) Proceedings of International Symposium on Tropical and Subtropical Fruits, 26 November-1 December 2000, Cairne, Australia, Acta Horticulturae 575, ISHS 2002, pp. 563-570.

Zandbergen, F. and Plutzky, J. 2007. PPARα in atherosclerosis and inflammation. *Biochimica et Biophysica Acta.* 1771: 972–982.

Zarras, P. and Vogl, O. 1999. Polycationic salts as bile acid sequestering agents. *Progress in Polymer Science* 24: 485-516.

APPENDIX

Appendix I: Average daily food intake (g) of the four experimental groups during 6-week period

	Group 1	**Group 2**	**Group 3**	**Group 4**
Week 1	27.86±11.14	17.00±0.42	13.06±2.02	18.03±0.59
Week 2	24.54±12.09	18.18±0.35	10.38±3.07	19.29±1.23
Week 3	26.75±12.47	17.96±0.81	9.03±2.89	17.77±0.92
Week 4	23.18±11.05	12.04±0.52	7.03±1.13*	17.34±0.49
Week 5	25.32±11.31	6.57±0.30*	5.09±1.15*	17.57±1.37
Week 6	24.32±10.73	9.32±0.49*	6.80±2.21*	18.00±1.39

Data are given as mean ± SEM (n = 4), * $p < 0.05$. Group 1 = normal, Group 2 = hypercholesterolemic, Group 3 = hypercholesterolemic + morelloflavone, Group 4 = normal + morelloflavone.

Appendix II: Average body weight (g) of the four experimental groups during 6-week period

	Group 1	**Group 2**	**Group 3**	**Group 4**
Week 1	524.75±12.49	480.25±10.59	448.80±30.18	454.80±20.56
Week 2	504.50±27.40	465.25±7.42	439.60±32.49	461.40±20.65
Week 3	496.50±27.24	456.75±10.09	433.80±31.72	458.60±21.53
Week 4	495.50±28.69	445.50±12.22	443.80±30.14	450.80±20.65
Week 5	497.75±26.68	448.75±10.57	439.80±35.70	465.80±22.02
Week 6	498.75±25.77	440.50±10.05	429.00±44.18	477.80±29.83

Data are given as mean ± SEM (n = 4), Group 1 = normal, Group 2 = hypercholesterolemic, Group 3 = hypercholesterolemic + morelloflavone, Group 4 = normal + morelloflavone.

Appendix III: Average hepatic weight (g) of the four experimental groups at the end of study period

Group	Weight (g)
Group 1	12.29±1.51
Group 2	14.92±1.52*
Group 3	14.72±0.28*
Group 4	11.30±1.03

Data are given as mean ± SEM (n = 4), * $p < 0.05$. Group 1 = normal, Group 2 = hypercholesterolemic, Group 3 = hypercholesterolemic + morelloflavone, Group 4 = normal + morelloflavone.

VITAE

Name Miss Ku-aida Tuansulong

Student ID 5010220013

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biotechnology)	Prince of Songkla University	2006

Scholarship Awards during Enrolment

PSU's Acadamic Excellence Strengthening Program in Biochemistry from Department of Biochemistry, Faculty of Science Prince of Songkla University

List of Publications and Proceedings

Proceeding

Tuansulong, K., Hutadilok-Towatana, N., Sotthibhandhu, R., Mahabusarakam, W., Pinkaew, D. 2009. Morelloflavone inhibits HMG-CoA reductase activity. The 2nd Biochemistry and Molecular Biology Conference, 7-8 May 2008, Khon Kaen, Thailand, pp. 62-66.