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SCREENING OF 3-HYDROXY-3-METHYL GLUTARYL CoA REDUCTASE INHIBITORS FROM MEDICINAL PLANTS

Miss Onrapak Reamtong

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นำสมุนไพร 18 ชนิดที่ภูมิปัญญาไทยกล่าวว่าสามารถลดคอเลสเตอรอลได้นำมาคัดกรองหาสารที่มีฤทธิ์ยับยั้ง 3-ไฮดรอกซี-3-เมทิลกลูทาริลโคเอนไซม์เอรีดักเทส ได้แก่ ใบหม่อน คำฝอย แห้ม กระชายดำ หญ้าหนวดแมว โหระพา กระเจี๊ยบแดง ชะเอมเทศ แก่นขี้เหล็ก เม็ดข่อย หญ้าขัดมอญ ชะพลู ฝักส้มป่อย ชลู่ หญ้าแห้วหมู กะเม็ง ยี่หร่าและงวงตาล นำพืชเหล่านี้มาตากแห้งและบดให้ละเอียด จากนั้นนำไปสกัดด้วยเฮกเซน เอทิลอะซิเตดและเมทานอล ตามลำดับ นำส่วนสกัดของพืชที่ได้จากตัวทำละลายอินทรีย์ทั้ง 3 ชนิดไปทดสอบฤทธิ์ยับยั้ง 3-ไฮดรอกซี-3-เมทิลกลูทาริลโค-เอนไซม์เอ รีดักเทสที่ได้จากยีสต์ขนมปัง พบว่าส่วนสกัดเมทานอลความเข้มข้น 1 มิลลิกรัมต่อมิลลิลิตรที่ได้จาก ใบหม่อน คำฝอย แห้ม กระเจี๊ยบแดงและชะเอมเทศสามารถยับยั้งการทำงานของ 3-ไฮดรอกซี-3-เมทิลกลูทาริลโคเอนไซม์เอรีดักเทสได้ร้อยละ 79.1, 84.1, 63.1, 79.1 และ 73.6 ตามลำดับ ในงานวิจัยนี้จึงเลือกนำใบหม่อนมาแยกสารที่ออกฤทธิ์ยับยั้ง 3-ไฮดรอกซี-3-เมทิลกลูทาริลโคเอนไซม์เอรีดักเทสต่อไป โดยทำการแยกส่วนสกัดเมทานอลของใบหม่อนด้วยเทคนิคทางคอลัมน์โครมาโทกราฟีร่วมกับการทดสอบฤทธิ์ยับยั้ง 3-ไฮดรอกซี-3-เมทิลกลูทาริลโคเอนไซม์เอรีดักเทส พบสารที่ออกฤทธิ์ยับยั้ง 3-ไฮดรอกซี-3-เมทิลกลูทาริลโคเอนไซม์เอรีดักเทส 1 ชนิดและทำการหาสูตรโครงสร้างของสารที่แยกได้โดยอาศัยข้อมูลทางสเปกโตรสโกปี พบว่าคือ นารินจิน ซึ่งเป็นตัวยับยั้งแบบไม่แข่งขันของ 3-ไฮดรอกซี-3-เมทิลกลูทาริลโคเอนไซม์เอรีดักเทส และมีค่าความชอบในการจับกับเอนไซม์เท่ากับ 1.06 ไมโครโมลาร์

ภาควิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต .....

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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ONRAPAK REAMTONG: SCREENING OF 3-HYDROXY-3-METHYLGLUTARYL CoA REDUCTASE INHIBITOR FROM MEDICINAL PLANTS. ADVISOR: ASSOC. PROF. AMORN PETSOM, Ph. D., THESIS Co-ADVISOR : VASANA TOLIENG 89 pp. ISBN 974-17-5912-6

Eighteen Thai-traditional medicinal plants known to have hypocholestermic activity were screened for their ability to inhibit 3-hydroxy-3-methylglutaryl CoA reductase activity. They were *Morus alba* Linn., *Carthamus tinctorius* Linn., *Fibraurea recisa* Pierre., *Carum carri* Linn., *Boesenbergia pandurata* Roxb., *Orthosiphon aristatus* Miq., *Ocimum basilicum* Linn., *Hibiscus sabdariffa* Linn., *Glycyrrhiza glabra* Linn., *Senna siamea* Lam., *Streblus asper* Lour., *Sida rhombifolia* Linn., *Piper sarmentosum* Roxb., *Acacia rugata* Merr., *Pluchea indica* Linn., *Cyperus rotundus* Linn. and *Eclipta prostrata* Linn. The herbs were extracted with hexane, ethyl acetate and methanol, respectively. Each crude extract was examined for the inhibition of 3-hydroxy-3-methylglutaryl CoA reductase which obtained from baker's yeast (*Saccharomyces cerevisiae*). It was found that the crude methanol extract (1mg/ml) from *M. alba*, *C. tinctorius, F. recisa, H. sabdariffa* and *G. glabra* exhibited 79.1, 84.1, 63.1, 79.1 and 73.6 % inhibition, respectively. Using bioassay guided, chromatographic separation the crude methanol extract from *M. alba* yielded an active compound which was identified by spectroscopic method as naringin and it was a noncompetitive inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase with K_i value 1.06 μ*M*.

Department……..BIOTECHNOLOGY……..Student's signature……..…..
Field of study.....BIOTECHNOLOGY……..Advisor's signature……
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CONTENTS

Page

LIST OF FIGURES

Figure **Page**

Figure **Page**

LIST OF TABLES

LIST OF SCHEMES

Scheme **Page**

LIST OF ABBREVIATIONS AND SYMBOLS

m/z	mass to charge ratio
M	Molar
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
COSY	Correlated Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
HMBC	Heteronuclear Multiple Band Coherence
LDL	Low density lipoprotein
HMG	3-Hydroxy-3-methylglutaryl CoA
ppm	part per million
q	quartet (for NMR spectrum)
s	singlet (for NMR spectrum)
t	triplet (for NMR spectrum)
S.E.M	Standard Error of Mean
mg	milligram
ml	milliliter

CHAPTER I

INTRODUCTION

Cholesterol levels in Asian people are rising.[1] A high blood cholesterol is said to promote atherosclerosis and thus also coronary heart disease. Our body produces three to four times more cholesterol than we eat. The production of cholesterol increases when we eat little cholesterol and decreases when we eat more. This explains why the prudent diet cannot lower cholesterol more than on average a few percent. The only effective way to reduce cholesterol is with drugs. The new cholesterol-lowering drugs, the statins, do prevent cardio-vascular disease. The statins inhibit the body's production of a substance called mevalonate. 3-hydroxy-3-methylgluatryl coenzyme A reductase (HMG CoA reductase or HMGR) catalyzes the 4-electron reduction of HMG CoA into CoA and mevalonate, with oxidation of two molecules of NADPH. This is the controlled step leading to the synthesis of cholesterol, as shown in figure 1. When the production of mevalonate goes down, less cholesterol is produced by the cells and thus blood cholesterol goes down as well.[2]

In Thailand, there are many plants known by Thai traditional medics as the herbs which are able to reduce cholesterol such as white-mulberry, safflower, cumin, sweet basil leave, roselle, cassod tree, siamese rough bush, broom weed, variegatum, soap pod, nut grass, false daisy and toddy plam.[3,4,5] The goal of this research is to screen for 3-hydroxy-3-methylglutaryl CoA reductase inhibitors from medicinal plants. The baker ‘s yeast (*Saccharomyces cerevisiae*) is used to obtain 3-hydroxy-3-methylglutaryl CoA

reductase. Then, it is used to screen for inhibitory activity of crude hexane, crude ethyl acetate and crude methanol extracts from each herb.

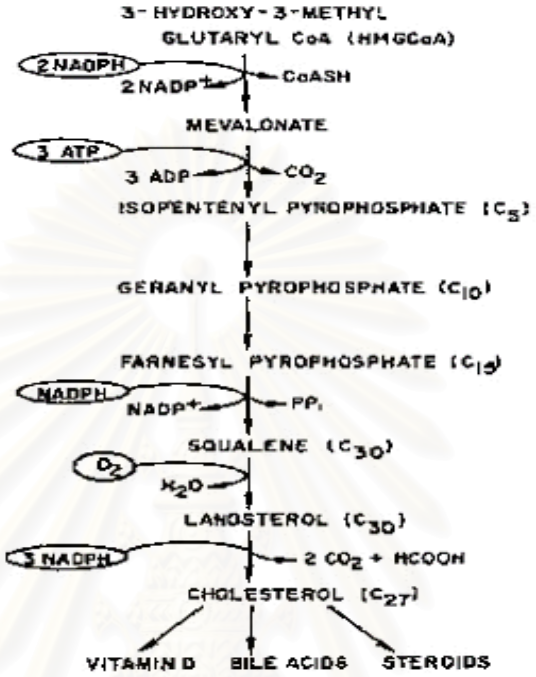


Figure 1 Cholesterol synthesis pathway[6]

Objectives of this research

1.To screen for 3-hydroxy-3-methylglutaryl CoA reductase inhibitors from crude extract of the medicinal plants which known to have hypocholestermic.

2. To isolate, purify and characterize the active compounds from selected plants.

CHAPTER II

LITERATURE REVIEW

1. CHOLESTEROL

Cholesterol is an extremely important biological molecule that has roles in membrane structure as well as being a precursor for the synthesis of the steroid hormones and bile acids. Both dietary cholesterol and that synthesized *de novo* are transported through the circulation in lipoprotein particles. The same is true of cholesteryl esters, the form in which cholesterol is stored in cells. The synthesis and utilization of cholesterol must be tightly regulated in order to prevent over-accumulation and abnormal deposition within the body. Of particular importance clinically is the abnormal deposition of cholesterol and cholesterol-rich lipoproteins in the coronary arteries. Such deposition, eventually leading to atherosclerosis, is the leading contributory factor in diseases of the coronary arteries. The structure of cholesterol is shown in figure 2.

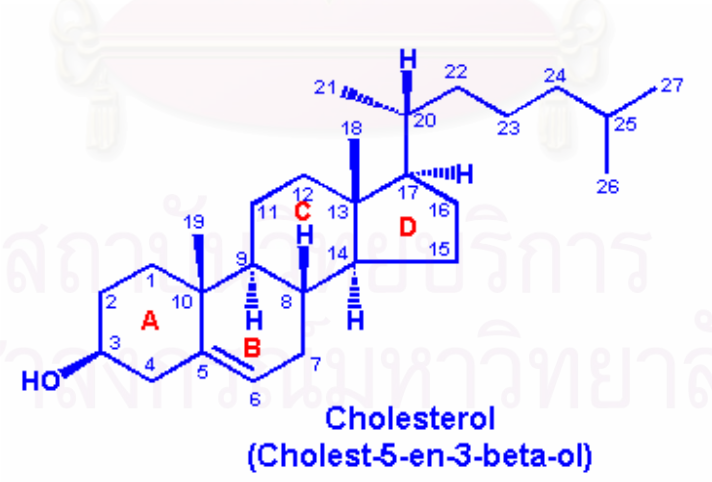


Figure 2 Structure of cholesterol (http://www.ravnskov.nu/cholesterol.htm)

Slightly than half of the cholesterol in the body derives from *de novo* biosynthesis. Biosynthesis in the liver accounts for approximately 10%, and in the intestines approximately 15%, of the amount produced each day. Cholesterol synthesis occurs in the cytoplasm and microsomes from the two-carbon acetate group of acetyl-CoA. The process has five major steps as the following:

1. Acetyl-CoAs are converted to 3-hydroxy- 3-methyl glutaryl-CoA.

2. 3-Hydroxy-3-methylglutaryl CoA is converted to mevalonate, as shown in figure 3.

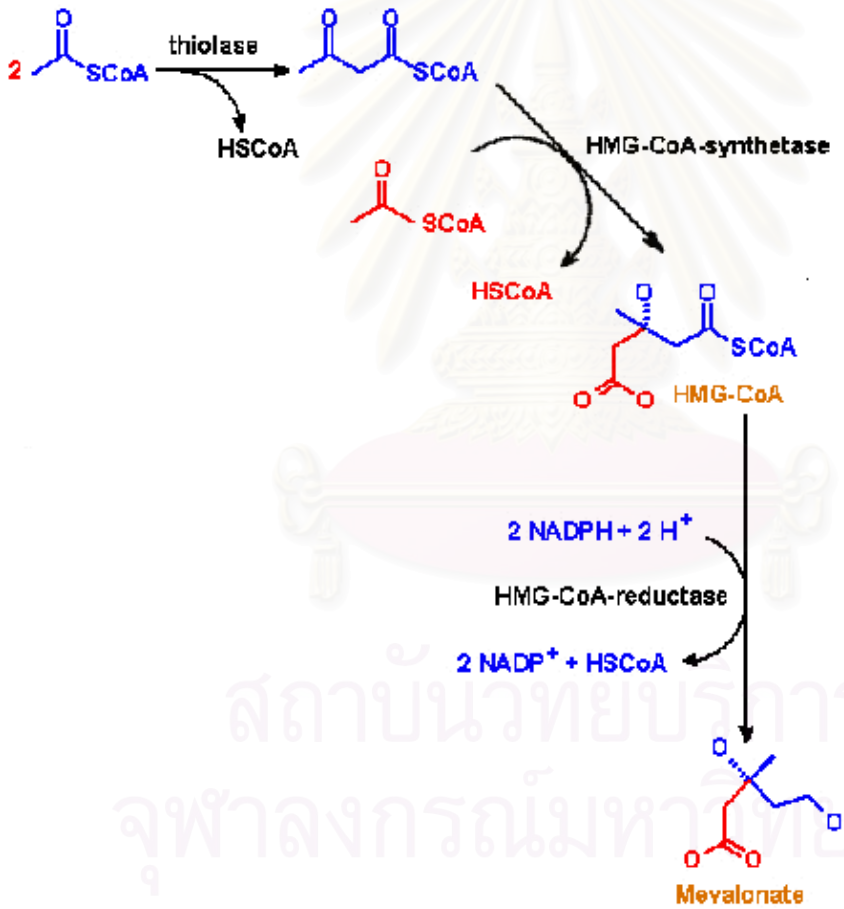


Figure 3 Pathway of the converting of acetyl-CoAs to mevalonate

3. Mevalonate is converted to the isoprene based molecule, isopentenyl pyrophosphate (IPP), with the concomitant loss of CO_2

4. IPP is converted to geranylpyrophosphate

5. Geranylpyrophosphate is converted to farnesylpyrophosphate, as shown in figure 4.

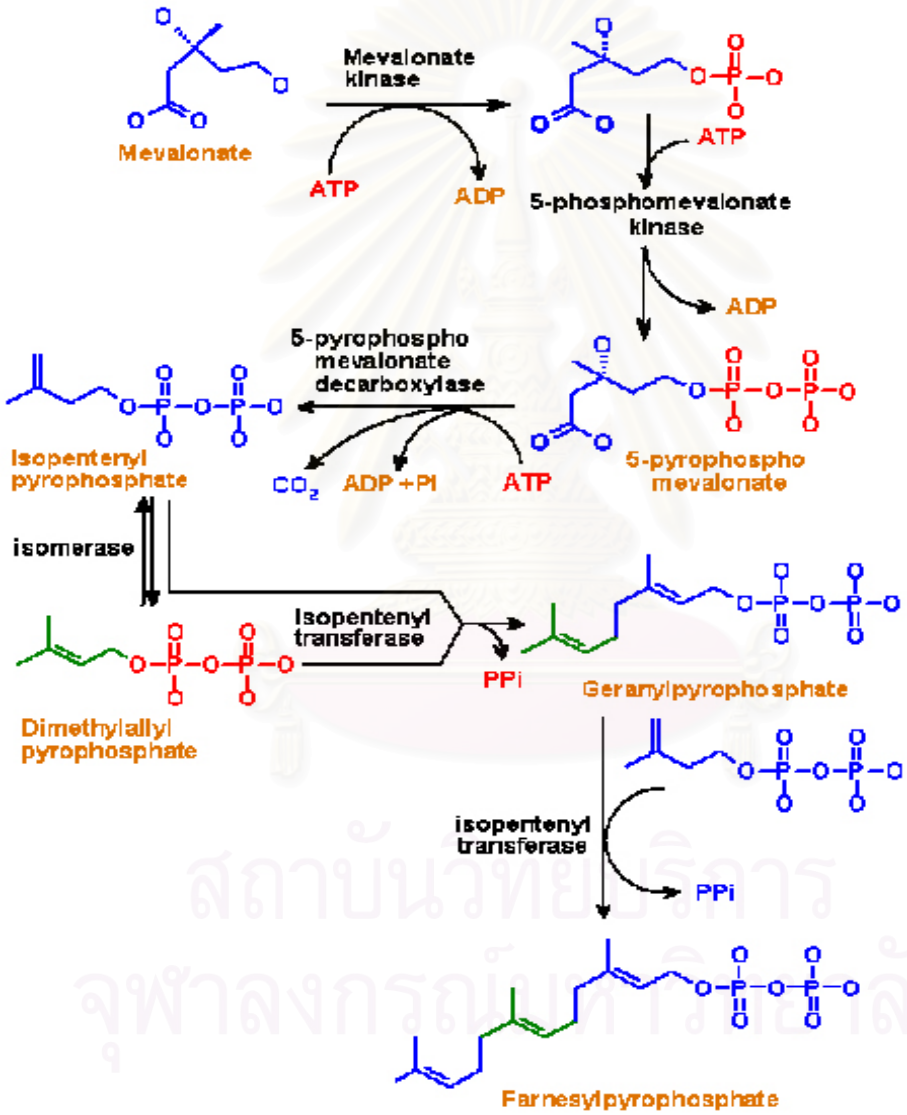


Figure 4 Pathway of the converting of mevalonate to farnesylpyrophosphate

6. Farnesylpyrophosphate is converted to squalene , as shown in figure 5.

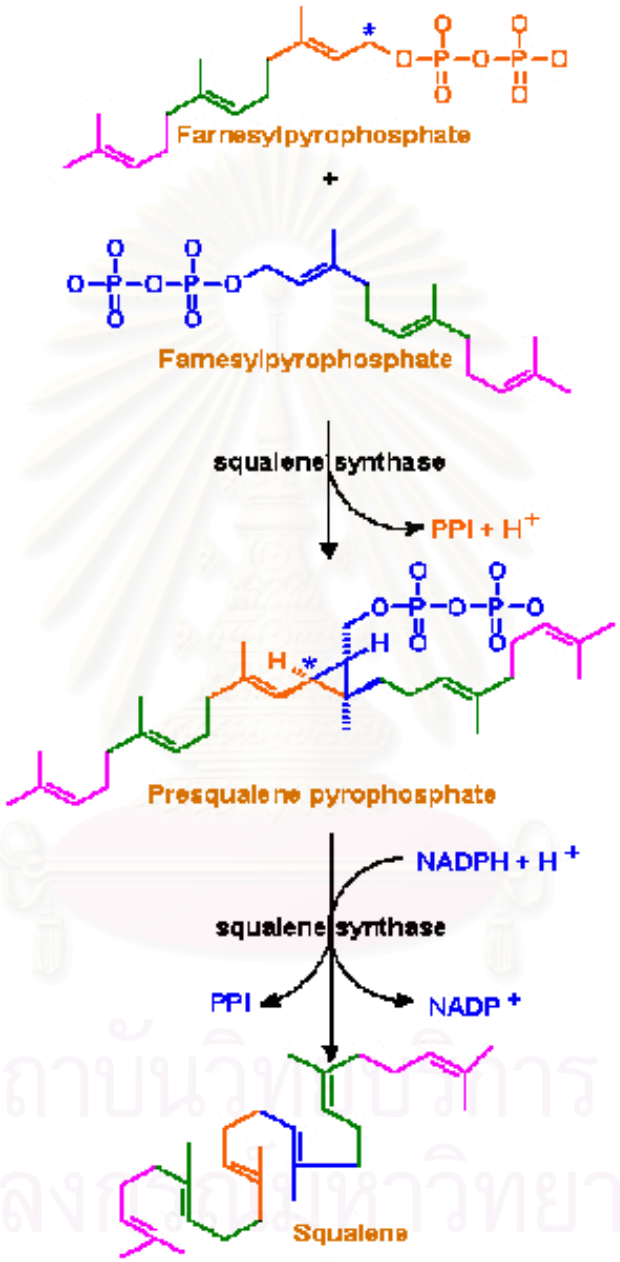


Figure 5 Pathway of the converting of farnesylpyrophosphate to squalene

7. Squalene is converted to lanosterol and to cholesterol, as shown in figure 6

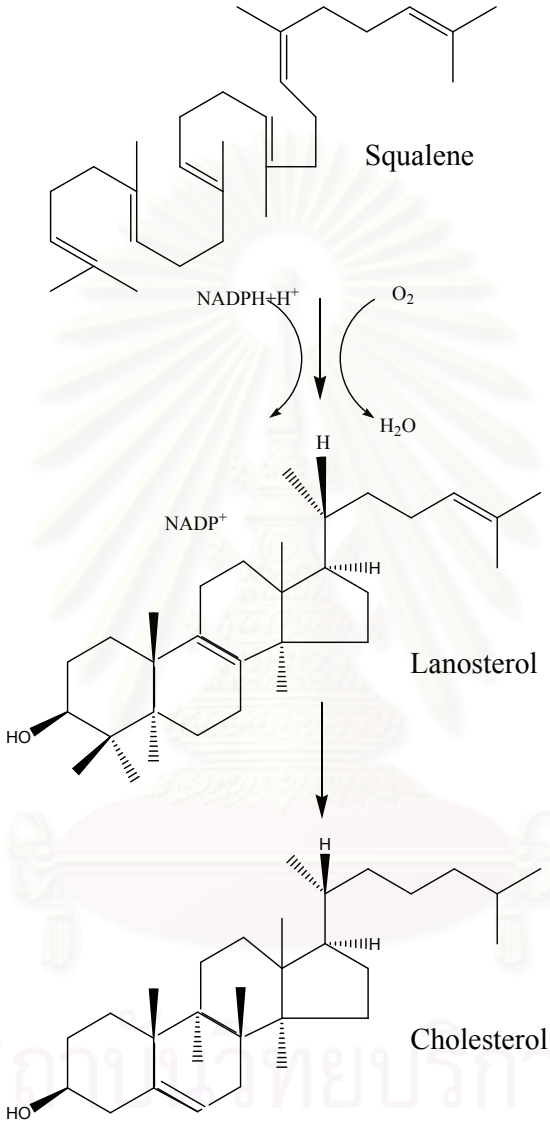


Figure 6 Pathway of the converting of squalene to cholesterol

(http://www.medscape.com/medscape/CNO/1999/ESC/ESC-01.html)

Acetyl-CoA units are converted to mevalonate by a series of reactions that begins with the formation of 3-hydroxy-3-methylglutaryl CoA. Unlike the 3-hydroxy-3-methylglutaryl CoA formed during ketone body synthesis in the mitochondria, this form is synthesized in the cytoplasm. However, the pathway and the necessary enzymes are the same as those in the mitochondria. Two moles of acetyl-CoA are condensed in a reversal of the thiolase reaction, forming acetoacetyl-CoA. Acetoacetyl-CoA and a third mole of acetyl-CoA are converted to 3-hydroxy-3-methylglutaryl CoA by the action of 3-hydroxy-3-methylglutaryl CoA synthase. 3-Hydroxy-3-methylglutaryl CoA is converted to mevalonate by 3-hydroxy-3-methylglutaryl CoA reductase (this enzyme is bound to the endoplasmic reticulum). 3-Hydroxy-3-methylglutaryl CoA reductase absolutely requires NADPH as a cofactor and two moles of NADPH are consumed during the conversion of 3-hydroxy-3-methylglutaryl CoA to mevalonate. The reaction catalyzed by 3-hydroxy-3-methylglutaryl CoA reductase is the rate limiting step of cholesterol biosynthesis, and this enzyme is subject to complex regulatory controls. Mevalonate is then activated by three successive phosphorylations, yielding 5-pyrophosphomevalonate. In addition to activating mevalonate, the phosphorylations maintain its solubility, since otherwise it is insoluble in water. After phosphorylation, an ATP-dependent decarboxylation yields isopentenyl pyrophosphate, IPP, an activated isoprenoid molecule. Isopentenyl pyrophosphate is in equilibrium with its isomer, dimethylallyl pyrophosphate, DMPP. One molecule of IPP condenses with one molecule of DMPP to generate geranyl pyrophosphate, GPP. GPP further condenses with another IPP molecule to yield farnesyl pyrophosphate, FPP. Finally, the NADPH-requiring enzyme, *squalene synthase* catalyzes the head-to-tail condensation of two molecules of

FPP, yielding squalene (squalene synthase also is tightly associated with the endoplasmic reticulum). Squalene undergoes a two step cyclization to yield lanosterol. The first reaction is catalyzed by *squalene monooxygenase*. This enzyme uses NADPH as a cofactor to introduce molecular oxygen as an epoxide at the 2,3 position of squalene. Through a series of 19 additional reactions, lanosterol is converted to cholesterol.

Normal healthy adults synthesize cholesterol at a rate of approximately 1g/day and consume approximately 0.3g/day. A relatively constant level of cholesterol in the body (150 - 200 mg/dL) is maintained primarily by controlling the level of *de novo* synthesis. The level of cholesterol synthesis is regulated in part by the dietary intake of cholesterol. Cholesterol from both diet and synthesis is utilized in the formation of membranes and in the synthesis of the steroid hormones and bile acids (see below). The greatest proportion of cholesterol is used in bile acid synthesis.

The cellular supply of cholesterol is maintained at a steady level by three distinct mechanisms:

1. Regulation of 3-hydroxy-3-methylglutaryl CoA reductase activity and levels.
2. Regulation of excess intracellular free cholesterol through the activity of acyl-CoA:cholesterolacyltransferase, ACAT.
3. Regulation of plasma cholesterol levels via LDL receptor-mediated uptake and HDL-mediated reverse transport.

Regulation of 3-hydroxy-3-methylglutaryl CoA reductase activity is the primary means for controlling the level of cholesterol biosynthesis, as shown in figure 7.

Regulation of 3-hydroxy-3-methylglutaryl CoA reductase takes place both through covalent modification and through control of the absolute level of the enzyme within cells. 3-hydroxy-3-methylglutaryl CoA reductase is a single polypeptide embedded in microsomal membranes; it is most active in its unmodified form. Phosphorylation of the enzyme decreases its activity. 3-Hydroxy-3-methylglutaryl CoA reductase is phosphorylated by reductase kinase, RK. RK itself is activated via phosphorylation. The phosphorylation of RK is catalyzed by reductase kinase kinase, RKK. There are two isoforms of RKK, one independent of cAMP and one dependent upon cAMP. The cAMP-dependent RKK is activated in the presence of cAMP. Since the intracellular level

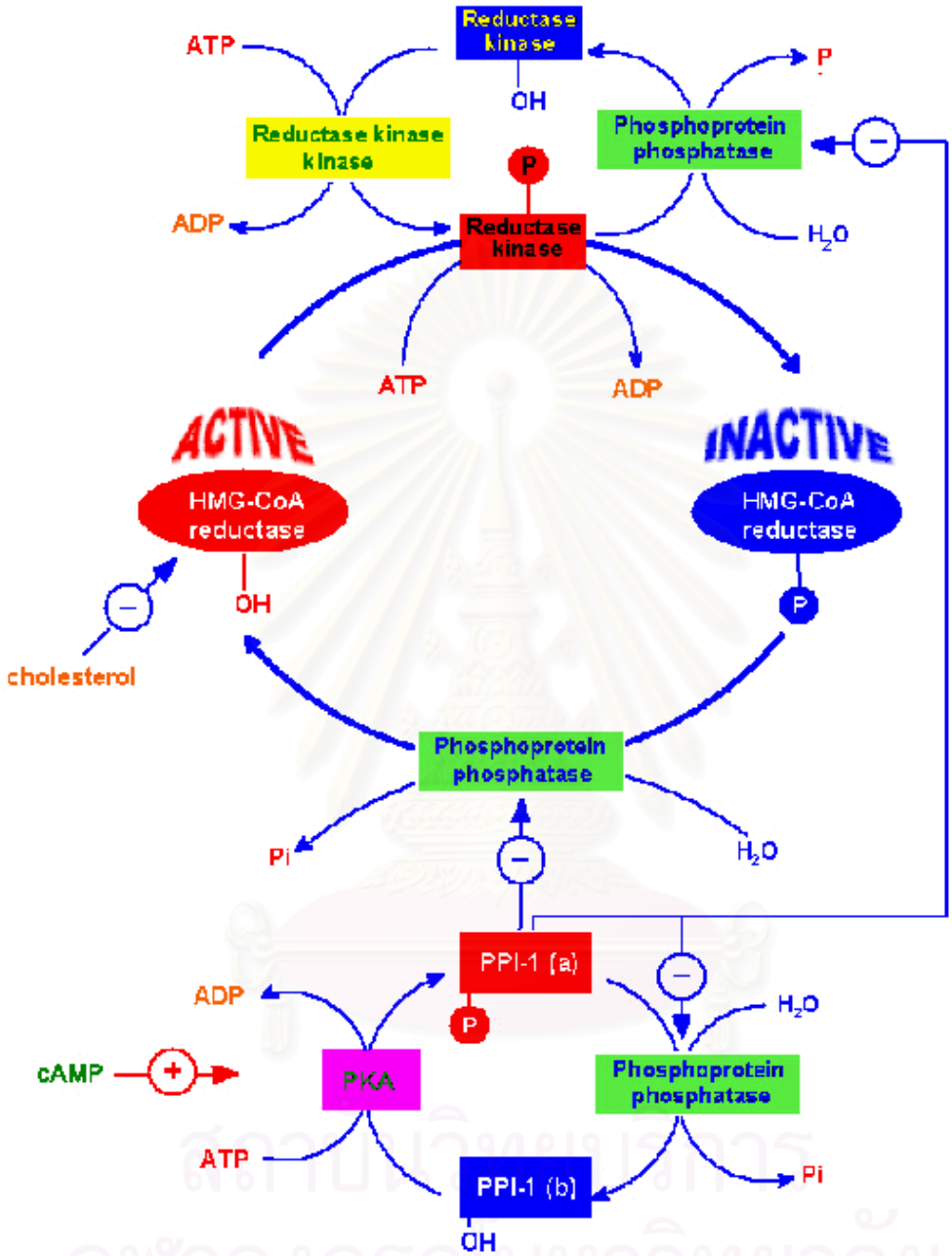


Figure 7 Regulation of 3-hydroxy-3-methylglutaryl CoA reductase activity **(**http://www.ionet.net/~jcott/homepage/drugdb/106.html**)**

of cAMP is regulated by hormonal stimuli, regulation of cholesterol biosynthesis is hormonally controlled. Insulin leads to a decrease in cAMP, which in turn activates cholesterol synthesis. Alternatively, glucagon and epinephrine which increase the level of cAMP inhibit cholesterol synthesis. The activity of 3-hydroxy-3-methylglutaryl CoA reductase is further controlled by the cAMP signaling pathway. Increases in cAMP lead to phosphorylation and activation of phosphoprotein phosphatase inhibitor-1 (PPI-1). 3-hydroxy-3-methylglutaryl CoA reductase is dephosphorylated and, thereby, activated by phosphoprotein phosphatase-1 (PP-1). At the same time, PP-1 dephosphorylates and inactivates RK. The cAMP-induced activation of PPI-1 results in a decrease in the level of active PP-1, resulting in a reduced ability of PP-1 to dephosphorylate 3-hydroxy-3-methylglutaryl CoA reductase and RK. This maintains RK in the phosphorylated and active state, and 3-hydroxy-3-methylglutaryl CoA reductase in the phosphorylated and inactive state. As the stimulus leading to increased cAMP production is removed, the level of phosphorylations decreases and that of dephosphorylations increases. The net result is a return to a higher level of 3-hydroxy-3-methylglutaryl CoA reductase activity. This regulatory cascade is additionally affected by PKA-mediated phosphorylation of PPI, which leads to reduced dephosphorylation by PP-1. The ability of insulin to stimulate, and glucagon to inhibit, 3-hydroxy-3-methylglutaryl CoA reductase activity is consistent with the effects of these hormones on other metabolic pathways. The basic function of these two hormones is to control the availability and delivery of energy to all cells of the body. Long-term control of 3-hydroxy-3-methylglutaryl CoA reductase activity is exerted primarily through control over the synthesis and degradation of the enzyme. When levels of cholesterol are high, the level of expression of the 3-hydroxy-3-

methylglutaryl CoA gene is reduced. Conversely, reduced levels of cholesterol activate expression of the gene. Insulin also brings about long-term regulation of cholesterol metabolism by increasing the level of 3-hydroxy-3-methylglutaryl CoA reductase synthesis. The rate of 3-hydroxy-3-methylglutaryl CoA turn-over is also regulated by the supply of cholesterol. When cholesterol is abundant, the rate of 3-hydroxy-3-methylglutaryl CoA reductase degradation increases.[7,8]

2. HMG CoA reductase inhibitors

3-Hydroxy-3-methylglutaryl CoA reductase inhibitors are a group of prescription drugs used to reduce cholesterol. The statins are known competitive inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, they bind enzymes at the same portion of substrate (active site). The inhibitory reaction of statin is shown in Figure 8.

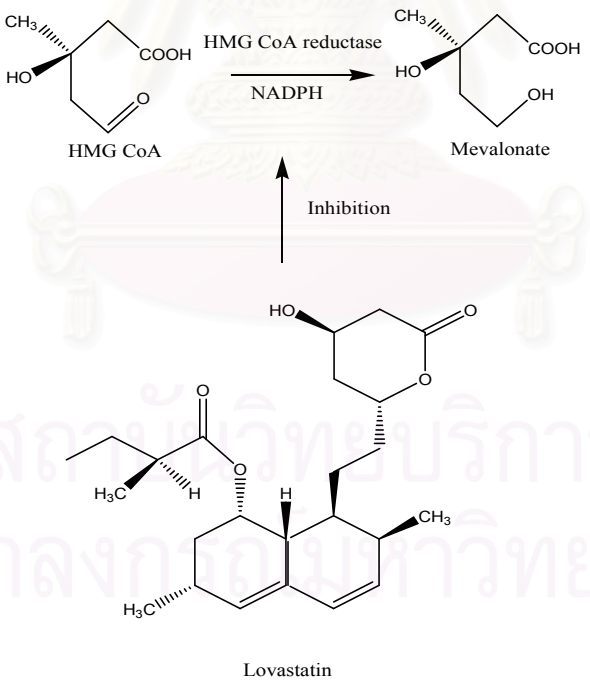


Figure 8 Inhibition of 3-hydroxy-3-methylglutaryl CoA reductase by statin (www.biochem.purdue.edu/ ~rodwell/tpuhmgr.gif**)**

Drugs in this class include atorvastatin (brand name Lipitor), fluvastatin (Lescol), lovastatin (Mevacor), pravastatin(Pravachol), simvastatin (Zocor) and cerivastatin (Baycol) as shown in figure 9

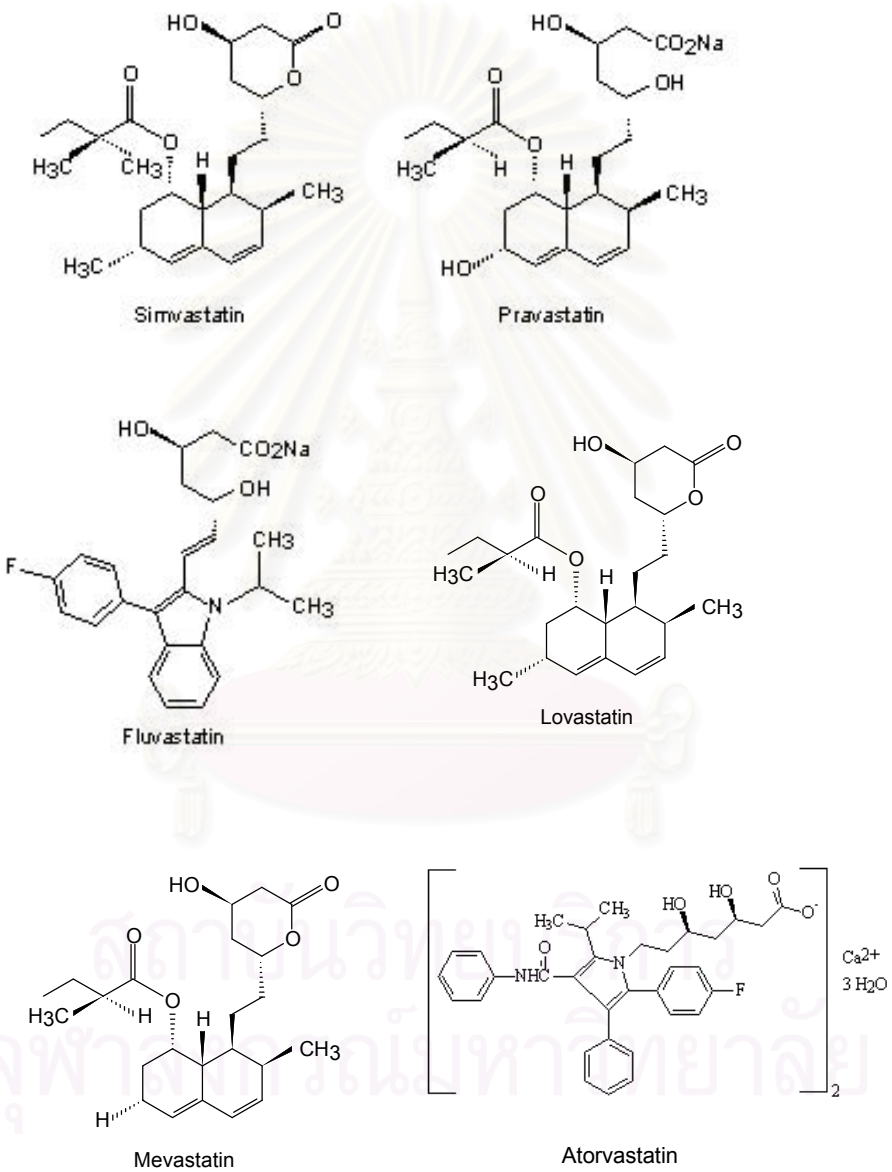


Figure 9 Structure of six 3-hydroxy-3-methyl glutaryl CoA reductase inhibitors (www.hhmi.org/news/images/ deisenhofer.gif**)**

Inhibition of 3-hydroxy-3-methylglutaryl CoA reductase reduces intracellular levels; this activates a protease, which in turn cleaves sterol regulatory element-binding proteins (SREBP's) from the endoplasmic reticulum. The SREBP's translocate to the nucleus where they upregulate expression of LDL receptor gene. Enhanced LDL receptor expression increases receptor-mediated endocytosis of LDL and thus lowers serum LDL. Inhibition of 3-hydroxy-3-methylglutaryl CoA reductase also reduces intracellular levels of isoprenoids, which are intermediates in cholesterol biosynthesis.

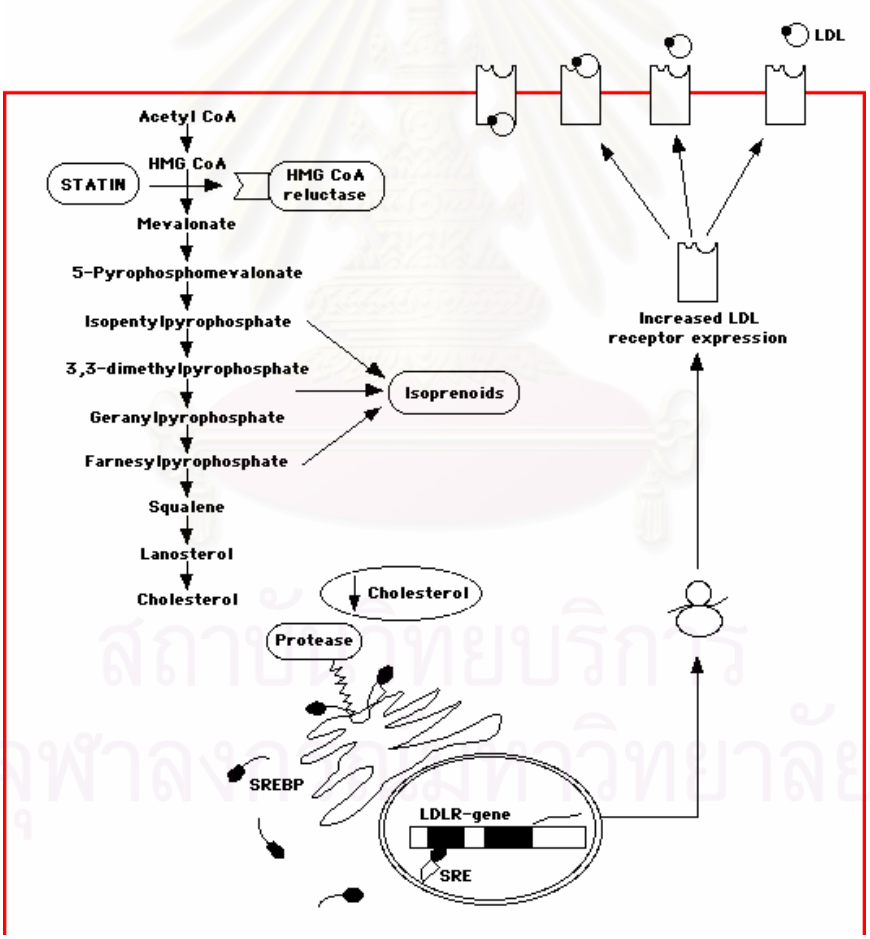


Figure 10 Reduction of serum cholesterol by statin
(www.wpu.com.cn/english/images/ 2/xuezhikang/p3.gif**)**

Statins, like all prescription drugs, have side effects. In the case of statins, the most serious and the most rare is rhabdomyolysis. This condition is more likely to occur when statins are being taken in combination with drugs called fibrates, they also have the effect of blocking the body's natural production of Coenzyme Q10. Supplementation with CoQ10 is necessary to avoid the heart disease when use statins to reduce cholesterol. In a small number of other cases, the statin drugs depletion of CoenzymeQ10 led to liver disease.

In 1967, Kirtley and co-workers[9] found that 3-hydroxy-3-methylglutaryl CoA reductase from *Saccharomyces cerevisiae* have a " Ping-Pong " mechanism. A second class of enzymatic reactions involving 2 substrates and 2 products is called the ping-pong mechanism. In this case, the first substrate binds and the first product is formed and released, leaving a modified enzyme. Then the second substrate binds to the modified enzyme, and the second product is released, restoring the enzyme. In contrast to the sequential mechanism, there is no ternary complex of EAB.

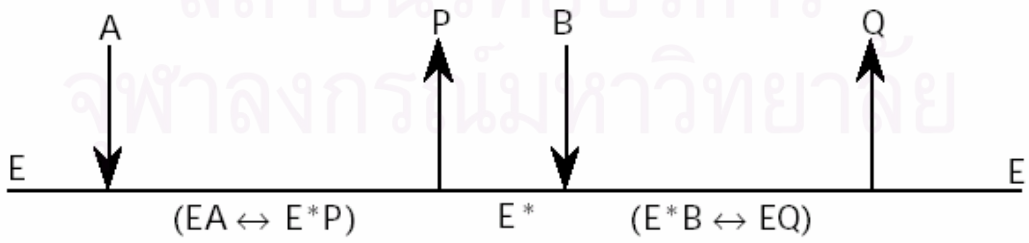


Figure 11 Ping-pong mechanism

In 1979, Endo[10] discovered that a strain of *Monascus* yeast naturally produced a substance that inhibits cholesterol synthesis, which is monacolin K known as mevinolin and lovastatin.

In 1980, Hiram and co-worker[11] found that the sulfhydryl reagent inactivate 3-hydroxy-3-methylglutaryl CoA reductase from *Saccharomyces cerevisiae* and dithiothreitol can reactivate them.

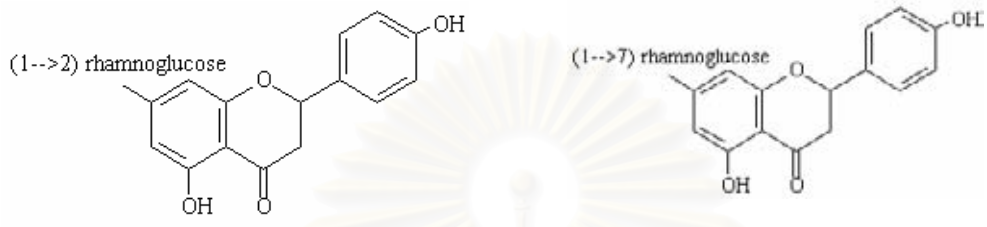
In 1988, Basson and co-workers[12] found the structural and functional similarity of 3-hydroxy-3-methylglutaryl CoA reductase, between the yeast *Saccharomyces cerevisiae* and humans. They conclude that microbial 3-hydroxy-3-methylglutaryl CoA reductase may provide a screen tool to identify new drugs that can modulate cholesterol biosynthesis.

In 1993, Gunde and co-workers[13] showed that the screening of fungi for the production of mevinolin (3-hydroxy-3-methylglutaryl CoA reductase inhibitor), the basidiomycetous genus *Pleurotus*, especially species *P. ostreatus, P. saca* and *P. sapidus* are promising source of the hypocholesterolaemic agent.

In 1998, Ignacimuthu and co-workers[14] found that the leaves extract of *Zizyphus jujuba* (Rhamnaceae) reduce serum cholesterol on diabetic rats.

In 1999, Heber and co-workers[15] discovered that the fermented product of rice on white red yeast (*Monascus purpureus*) has been grown, significantly reduces total cholesterol, LDL cholesterol and total triacylglycerol concentrations compared with placebo.

In 1999, Bok and co-workers[16] found that 3-hydroxy-3-methylglutaryl CoA reductase from rats' liver can be inhibited by mandarin peel flavonoids, whose structures are shown in figure 12.



Naringin Hesperidin

Figure 12 Structure of mandarin peel flavonoids

In 2000, Istvan and co-workers[17] discovered that the structure of 3-hydroxy-3-methylglutaryl CoA reductase is tetramers and catalysed reaction probably involves His-866 and Glu-559 as proton donating moieties.

In 2001, Lee and co-workers[18] reported that the 3-hydroxy-3-methylglutaryl CoA reductase from rats liver can be inhibited by 3,4-di(OH)-cinnamate and 3,4-di(OH)-hydrocinnamate.

In 2001, Litthilert[19] reported that the mulberry (*Morus alba* Linn.) leaves extracts reduce level of cholesterol on diabetic rats.

In 2001, Tamaki and co-workers[20] indicated that the water-soluble polysaccharides from kenaf (*Hibiscus cannabinus*) seeds significantly lower the serum cholesterol in rats fed hypercholesterolemic diet.

In 2002, Benkhalti and co-workers[21] showed that the polyphenols of virgin olive oil decrease significantly the 3-hydroxy-3-methylglutaryl CoA reductase activity in the rat liver microsome.

In 2002, Surya and co-workers[22] found that the cumin seed significantly reduce lipid in the plasma, liver, kidney, pancreas, intestine and aorta of the diabetic rats.

In 2003, Yokozawa and co-workers[23] indicated that the *Coptidis* Rhizoma extract is effective in reducing cholesterol synthesis on hypercholesterolemic rats model.

In 2003, Chang and co-workers[24] showed that a Local soft drink material and medicinal herb (*Hibiscus sabdariffa* L.) decrease the levels of triglyceride, cholesterol and low-density lipoprotein cholesterol in New Zealand white rabbits. and also significicantly reduced severe atherosclerosis in the aorta.

3. The medicinal plants.

Tropical forest plant species have been served as a source of medicines for people of the tropics for millennia. Many medical practitioners with training in pharmacology is well aware of the number of modern therapeutic agents that have been derived from tropical forest species. In fact, over 120 pharmaceutical products currently in use are plant-derived, and some 75% of these were discovered by examining the use of these plants in traditional medicine, a large portion have come from tropical forest species. Yet while many modern medicines are plant-derived, the origins of these pharmaceutical agents and their relationship to the knowledge of the indigenous people in the tropical forests is usually omitted.[25] In Thailand, there are many plants accepted by Thai traditional medics as the herbs to reduce cholesterol such as white-mulberry, safflower, cumin, sweet basil leave, roselle, cassod tree, siamese rough bush, broom weed,

variegatum, soap pod, nut grass, false daisy and toddy plam. The objective of this research is to screen for 3-hydroxy-3-methylglutaryl CoA reductase inhibitors from local medicinal plants.

4. *Morus alba* Linn.

Morus alba Linn. belongs to Moraceae family (division: Spermatophyta, class: Angiospermae, subclass: Dicotyledoneae, group: Thalamiflorae, order: Urticals)[26]

The common names of this plant are white or mulberry tree (English), silkworm mulberry, China mulberry, Russian mulberry, Beyazdut, Maulbeerbaum (German), Mon (Thai)[27,28]

Morus alba Linn. is a shrub or middle tree reaching the height of 4-5 m with straight trunk. The bark is light brown to gray and smooth, breaking into broad scales in older tree. Twigs are thin, light brown and glabrous. Buds are small, pointed and brown. Sap is milky. It has alternate simple leaves, variable in shape and degree of lobe, sometimes with 3 or 5 lobes, sometimes without on the same tree, coarsely serrate, 8-14 cm wide and 12-16 cm long, shiny green to dark green, usually smooth above and hairless below. The flowers are white or greenish white, staminate and pistillate on different trees, small, crowded in cluster 1.5-2.5 cm long in early spring. The fruits are multiple, small, crowded in cruster 1-2 cm long, white to purple when ripe in late spring. They are composed of many tiny bead of 1 seeded fruits.[29,,30,31,32,33,34]

Mulberry leaves have been consumed as a food in many countries and various parts of mulberry have been used in traditional medicine by local people which are summarized in table 1.

Table 1 Pharmacology of *Morus alba* L.

Country	Part Used	Used	Type extract /Route	Reference
Chile	-Dried leaves	-Antidiabetic	Infusion/oral	35
China	-Branches,Fruit, stembark	-Antiarthritis,Alleviate arthritic pain	-Hot H_2O extract/oral	36
	-Rootbark	-Expectorant, diuretic,antitussive	-Hot H_2O extract/oral	37
Iran	-Dried fruit	-Laxative, antitussive	-Fruit/oral	
	-Dried leaves	-Diuretic	-Decoction/oral	38
	-Dried rootbark	-Diuretic	-Decoction/oral	
Japan	-Dried bark	-Antidiabetic	-Decoction/oral	38
	-Dried fruit	-Protectant against alcohol toxicity	-Decoction/oral	39
	-Rootbark	-Antitussive, diuretic, expectorant, laxative, prevent palsy	-Hot H_2O extract/oral	
Jordan	-Leaves	-Diuretic	-Decoction/oral	39
Korea	Not specified	-Coughs, bronchitis, colds, apoplexy, cerebral, hemorrhage, cerebral anemia, hypertension, sunstroke, beri-beri	-Hot H_2O extract/oral	40
Peru	-Dried leaves and stem	-Antidiabetic	-Hot H_2O extract/oral	41
Rodrigues Islands	-Leaves	-Hypertension, hernia	-Decoction/oral	42
Spain	-Dried leaves	-Hypoglycemic, astringent	-Hot H_2O extract/oral	43
Taiwan	-Dried leaves	-Liver disease	-Hot H_2O extract/oral	44

Table 1 Pharmacology of *Morus alba* L. (cont.)

Country	Part Used	Used	Type extract /Route	Reference
Thailand	-Fruit	-Expectorant, laxative, relieve, sore throat	-Fruit, decoction/oral	45
	-Leaves	-Coughs, fever, relieve sore throat, relieve thirst, sedation	-Decoction/oral	
		-Reddening of the eyes	-Decoction/ ophthalmic	46
	-Rootbark	-Laxative	-Decoction/oral	47
Tunisia	-Dried root	-Dental pain	-Not stated/ external	48
Turkey	-Fruit	- Reddening of the eyes	-Infusion/ ophthalmic	49
Yugoslavia	-Dried leaves	-Antidiabetic	-Hot H_2O extract/oral	50

The chemical compounds present in various parts of *Morus alba* L. are shown in Table 2.

Table 2 Constituents of *Morus alba* L.

Compounds	Part Used	Reference
Alkaloid 1.adenine, iso-pentenyl	Leaves	51
2.arabinitol, D: 1-4-dideoxy-1-4-imino	Leaves	52
3.arabinitol, D: 1-4dideoxy-1-4-imino-(2-O-β-D-glucopyranosyl	Root	53
4. fagomine	Leaves, Root	53
5. fagomine, 3-epi	Root	53
6. nojirimycin, 1-deoxy	Bark, Leaves, Root	54
7. nojirimycin, 1-deoxy: 2-O-α-D-galactopyranoside	Leaves	53
8. nojirimycin, 1-deoxy: N-methyl	Leaves, Root	54
9. nojirimycin, 2-O-α-D-galactopyranosyl-1-deoxy	Root	54
10. nojirimycin, 2-O-α-D-glucopyranosyl-1-deoxy	Root	54
11. nojirimycin, 2-O-β-D-glucopyranosyl-1-deoxy	Root	54
12. nojirimycin, 3-O-α-D-galactopyranosyl-1-deoxy	Root	54
13. nojirimycin, 3-O-β-D-glucopyranosyl-1-deoxy	Root	54
14. nojirimycin, 4-O-α-D-glucopyranosyl-1-deoxy	Root	54
15. nojirimycin, 4-O-β-D-glucopyranosyl-1-deoxy	Root	54
16. nojirimycin, 6-O-α-D-galactopyranosyl-1-deoxy	Root	54
17. nojirimycin, 6-O-β-D-glucopyranosyl-1-deoxy	Root	54

Table 2 Constituents of *Morus alba* L (cont.)

Compounds	Part Used	Reference
18. ribitol, D: 1-4-dideoxy-1-4-imino	Root	54
19. zeatin	Leaves	51
20. zeatin riboside	Leaves	51
Benzenoid		
1.alboctalol	Heartwood	55
2. benzoic acid, 2-4-dihydroxy: ethyl ester	Rootbark	56
3. benzyl alcohol glucoside	Leaves	57
4. kuwanol E	Callus tissue	58
5. resorcinol	Heartwood	59
6. resorcyl aldehyde, β	Heartwood	59
Carbohydrate		
1. carbohydrate	leaves	60
2. sugar	leaves	61
Chormone		
1. chromone, 5-7-dihydroxy	Rootbark	56
Coumarin		
1.bergapten	leaves	62
2. marmesin	Shoots	63
3. scopoletin	leaves	61
4. scopolin	leaves	64

Table 2 Constituents of *Morus alba* L (cont.)

Compounds	Part Used	Reference
5. skimmin	Leaves	59
6. umbelliferone	Leaves	65,66
Flavonoid 1. albafuran C	Shoot epidermis	67,68
2. aromadendrin	Heartwood	69
3. astragalin	Leaves	70,71
4. chalcomoracin	Leaves, seed	70,71
5. chrysanthemin	Fruit	65
6. cyclomorusin	Bark, rootbark, stembark	66
7. cyclomulberrin	Bark, rootbark, stembark	65
8. kaempferol-3-O-(6”-O-acetyl)-β-D-glucopyranoside	Leaves	72
9. kaempferol-3-O-(6”-O-acetyl)-β-D-glucosice	Leaves	64
10. kaempferol-3-O-α-L-rhamnopyranosyl(1-6)-β-D-glucopyranoside	Leaves	64
11. kuwanol B	Rootbark	73
12. kuwanon A	Rootbark	64
13. kuwanon B	Rootbark	65
14. kuwanon C	Bark, rootbark, stembark, leaves	74

Table 2 Constituents of *Morus alba* L (cont.)

Compounds	Part Used	Reference
15. kuwanon D	Rootbark	75
16. kuwanon E	Rootbark	76
17. kuwanon F	Rootbark	77
18. kuwanon G	Rootbark	78
19. kuwanon H	Rootbark	79
20. kuwanon I	Rootbark, seed	79
21. kuwanon J	Callus tissue, seed	80
22. kuwanon K	Rootbark	80
23. kuwanon L	Rootbark	80
24. kuwanon Q	Callus tissue	81
25. kuwanon R	Callus tissue	68
26. kuwanon v	Callus tissue	59
27. moracenin D	Rootbark	59
28. morachalcone A	Seed	71
29. morin	Heartwood	71
30. morin, dihydro	Heartwood	71

Table 2 Constituents of *Morus alba* L (cont.)

Compounds	Part Used	Reference
31. morus flavone A	Stembark	57
32. morus flavone B	Stembark	57
33. morus flavone C	Stembark	57
34. morus prenyl-flavan 1	Leaves	82
35. morus prenyl-flavan 2	Leaves	83
36. morus prenyl-flavan 3	Leaves	84
37. morusin	Bark, Rootbark, Stembark	95
38. morusin, dihydro:oxy	Rootbark	89
39. morusin-4'-glucoside	Rootbark	58
40. morusinol	Rootbark	65
41. mulberranol	Bark	68
42. mulberrofuran T	Callus tissue	86
43. quercetin	Leaves	65
44. quercetin-3-7-di-O-β-D-glucopyranoside	Leaves	66
45. quercetin-3-7-di-O-β-D-glucoside	Leaves	65
46. quercetin-3-O-(6"-O-acetyl)-β-D-glucopyranoside	Leaves	57

Table 2 Constituents of *Morus alba* L (cont.)

Compounds	Part Used	Reference
47. quercetin-3-O-(6”-O-acetyl)-β-D-glucoside	Leaves	86
48. quercitin-3-O-β-D-glucopyranosyl((1-6)-β-D-glucopyranoside	Leaves	87
49. quercitrin	Leaves	88
50. quercitrin, iso	Leaves	89
51. rutin	Leaves	90
52. sanggenon C	Root	90
53. sanggenon D	Root	63
54. sanggenon E	Rootbark	63
55. sanggenon P	Rootbark	91
Lignan		
1. broussonin A	Shoots	91
2. broussonin B	Shoots	92
Lipid		
1.glycerol, 1-O-(cis-9-cis-12-cis-15-octatrienoyl)-2-O-(cis-9-cis-12-cis-15-octatrienoyl)-3-O-β-D-galactopyranosyl	Leaves	92
2. glycerol, 1-octadecanoyl-2-O-(cis-9-cis-12-cis-15-octatrienoyl)-3-O-β-D-galactopyranosyl	Leaves	92
Monoterpene		
1. comphor	Fruit essential oil	92
2. cineol, 1-8	Fruit essential oil	92
3. geraniol	Fruit essential oil	92

Table 2 Constituents of *Morus alba* L (cont.)

Compounds	Part Used	Reference
4. limonene	Fruit essential oil	92
5.linalool	Fruit essential oil	93
6. linalool acetate	Fruit essential oil	93
7. pinene, α	Fruit essential oil	94
Oxygen heterocycle 1. albafuran A	Shoot epidermis	94
2. albafuran B	Shoot epidermis	73
3. albanol A	Bark	84
4. albanol B	Bark, Rootbark	68
5. albanol G	Rootbark	95
6. benzofuran, 3'-5'-6-trihydroxy-2-phenyl	Seed	96
7. dimoracin	Shoot epidermis	96
8. moracin A	Stembark	97,98
9. moracin B	Stembark	97
10. moracin C	Leaves, Seed, Shoot	97
11. moracin N	Leaves	99
12. moracin N, ω-hydroxy	Leaves	84

Table 2 Constituents of *Morus alba* L (cont.)

Compounds	Part Used	Reference
13. mulberrofuran B	Rootbark	80
14. mulberrofuran D	Rootbark	73
15. mulberrofuran E	Callus tissue	84
16. mulberrofuran F	Rootbark	72
17. mulberrofuran K	Rootbark	72
18. mulberrofuran M	Rootbark	100
19. mulberrofuran P	Rootbark	101
20. mulberrofuran Q	Rootbark	101
Protein		
1. moran 20-K	Rootbark	60
2. moran A	Rootbark	102
3. protein	Leaves	103
4. transferase, asp-sulfo	Leaves	57
Polyprenoid		
1. moraprenol II	Leaves	104
Sesquiterpene	Leaves	104
1. roseoside II		
Steroid	Fruit	73
1. campesterol		
2. cholesterol	Fruit	104

Table 2 Constituents of *Morus alba* L (cont.)

Compounds	Part Used	Reference
3. daucosterol	Leaves	104
4. sitosterol, β	Bark, Leaves, Seed, Seed oil	104
5. stigmast-5-en-3-β-ol-7-one	Seed	104
6. stigmast-7-en-3-ol-acetate	Leaves	104
7. stigmasterol	Fruit, Leaves Seed oil	68
Stilbene		
1. kuwanon Y	Rootbark	73
2. kuwanon Z	Rootbark	104
3. mulberrofuran A	Rootbark	104
4. mulberroside A	Bark, Root	104
5. mulberroside A, cis	Root	95
6. resveratrol	Heartwood	95
7. resveratrol, dihydro: oxy	Heartwood	96
8. resveratrol, dihydroxy	Heartwood	97,98
9. resveratrol, oxy	Heartwood, Root	98
10. resveratrol, oxy:3'-O-β-D-glucopyranoside	Root	55
Triterpene		
1. amyrin, β	Bark, Leaves	55

Table 2 Constituents of *Morus alba* L (cont.)

Compounds	Part Used	Reference
2. amyrin, β: acetate	Bark	59
3. betulinic acid	Bark	59
4. friedelin	Leaves	94
5. hederagenin	Leaves	55
6. lupeol acetate	Leaves	55
7. ursolic acid	Leaves	55
Tropane alkaloid		
1. calytegin B-2	Root	73
2. calytegin C-1	Root	91
3. calytegin B-2	Leaves	61
Vitamin		
1. vitamin B	Leaves	54
2. vitamin C	Leaves	104

The pharmacological effects of *Morus alba* L. have been studied as follows:

In 1952, Winter and co-workers[105] indicated that the water extract of leaves was active against *S. aureus, B. subtilis* and *E. coli*

In 1964, Sharaf and co-workers[106] showed that the water and ethanol extraction of leaves had uterine stimulant effect in female mice and rats.

In 1967, Ray and co-workers[107] indicated that the methanolic extraction of leaves had estrogenic effect in female mice.

In 1995, Chen and co-workers[108] reported that the hot water extraction of leaves in a dose of 80 mg/kg and 200 mg/kg showed hypoglycemic activity in streptozotocin induced diabetic mice and ethanol insoluble fraction had strongest activity. Moreover, the hot water extraction of leaves in dosage of 100 and 200 mg/kg had potentiating effect on pilocarpine-induced saliva flow in streptozotocin induced diabetic mice

In 1995, Huang and co-workers[109] found that the fresh leaves extract at a concentration of 1% had antioxidant activity and radical scavenging effect for hydroxyl radical generated by Fenton system.

In 1997, Gaoat and co-workers[110] showed that the leaves extract had antipyretic action at a dosage of 1.25 g/kg in *Candida albicans* induced fever. Significant antipyretic effects were observed for both the extract and paracetamol between the second and sixth hours after the administration.

In 1997, Alkofahi and co-workers[111] showed that the 100% ethanol extract of dried leaves had mutagenic activity for *Salmonella* typhimurium, with an ED_{50} 38 μg/ml.

In 2001, Singh and co-workers[112] showed that the water extraction of dried leaves in concentration of 1 g/ml had antifungal activity against *Fusarium oxysporum*.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. PLANT MATERIALS

The plants materials were leaves of white mulberry (*Mora alba* Linn.), flowers of safflower (*Carthamus tinctorius* Linn.), lianas of Heam (*Coscinium fenestratum* Colebr.), seeds of cumin (*Carum carri* Linn.), rhizomes of *Boesenbergia pandurata* Roxb., whole part of cat's whisker tem plant (*Orthosiphon aristatus* Miq.), leaves of sweet basil leaves (*Ocimum basilicum* Linn.), flowers of roselle (*Hibiscus sabdariffa* Linn.), roots of *Glycyrrhiza glabra* Linn., cores of cossod tree (*Senna siamea* Lam.), seeds of siamese rough bush (*Streblus asper* Lour.), whole part of broom weed (*Sida rhombifolia* Linn.), variegatum (*Piper sarmentosum* Roxb.), pods of soap pod (*Acacia rugata* Merr.), leaves of *Pluchea indica* Linn., whole part of nut grass (*Cyperus rotundus* Linn.), leaves of false daisy (*Eclipta prostrata* Linn.) and pistils of toddy palm (*Borassus flabellifer*).

2. CHEMICAL REAGENTS

2.1 For chemical isolation

1.Hexane, chloroform, ethyl acetate and methanol used in this research are all commercial grade solvents.

2. Merck's TLC aluminum sheets, silica gel, 20x20 cm^2, layer thickness 0.2 mm were used for TLC analysis.

3. Merck's silica gel Art. 1.07734.1000

4. Merck's silica gel Art. 1.07736.1000

2.2 For extraction and assay of 3-hydroxy-3-methylglutaryl CoA reductase activity from baker yeast

1. Baker yeast (*Saccharomyces cerevisiae*), Fermipan^{R} instant dry yeast
2. Merck's Potassium phosphate buffer, 1*M*, pH 7.0
3. Sigma's 1,4-Dithio-DL-threitol, 0.2 *M*
4. Sigma's DL-3-Hydroxy-3-methylglutaryl Coenzyme A disodium salt trihydrate, 18 m*M*
5. Sigma's *p*-Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt, 1.6 m*M*
6. Merck's Bovine serum albumin (BSA, special quality for molecular biology)
7. Sigma's 3*S*,4*S*-4-Amino-3-hydroxy-methylheptanoic acid
8. Sigma's Ethylene diamine tetraacetic acid
9. Merck's Dimethyl sulfoxide

3. INSTRUMENTS AND EQUIPMENTS

1. Centrifuge, KUBOTA KR-20000T
2. Water bath shaker, Aquathem^{R}
3. Sunrise microplate reader, TECAN
4. Spectrophotometer, SHIMADZU UV-3100
5. Rotary evaporator, YAMATO RE 52
6. High performance liquid chromatography, CHROMATOPAC C-R6A
7. Electronic balance, ER-1804, FX-3000

8. Aspirator, YAMATO BP-51
9. pH Meter, HORIBA F-13
10. Nuclear magnetic resonance spectrometer, VARIAN MERCURY 400

METHODS

1. EXTRACTION OF 3-HYDROXY-3-METHYLGLUTARYL CoA REDUCTASE FROM BAKER'S YEAST

3-Hydroxy-3-methylglutaryl CoA reductase is solubilized from Fermipan^{R} instant dry yeast by a modification of the autolysis procedure of Qureshi and co-workers.[113] The 500 g of dry yeast are suspended in 1 liter of 0.3*M* dibasic phosphate, containing 1 m*M* dithiothreitol and 1 m*M* EDTA and then stirred for 12 hr at 4^{o}C. This suspension is centrifuged for 15 min at 20,000 *g*. The supernatant is discarded, and the gummy precipitate is resuspended in 800 ml of the same buffer and stirred for another 48 hr at 4^{o}C. The suspension is centrifuged as before, and the precipitate is resuspended in 800 ml in the same buffer and stirred for 40 hr. The supernatant solution obtained after centrifugation is retained. This contains the major portion of the solubilized enzyme: total volume is 625 ml (15 ml in each microcentrifuge tube was stored at –20^{0}C).[114,115]

2. DETERMINATION OF 3-HYDROXY-3-METHYLGLUTARYL CoA REDUCTASE ACTIVITIES

The 3-hydroxy-3-methylglutaryl CoA reductase activity can be conveniently determined by a modification of the spectrophotometric method of Qureshi N. and co-workers[113] which measures the oxidation of NADPH at 340 nm. The complete buffer, pH 7.0, 5μ*M* of dithiothreitol, 300 n*M* of 3-hydroxy-3-methylglutaryl CoA, 0.16

μ*M* of NADPH, and 3-hydroxy-3-methylglutaryl CoA reductase, in a total volume of 500 μl. Following a 10 min preliminary incubation at 30°C to establish a base line rate of absorbance change at 340 nm, the reaction is initiated by the addition of 3-hydroxy-3-methylglutaryl CoA. The rate of the reaction is monitored by following the oxidation of NADPH at 340 nm ($\varepsilon^{1\ cm}_{340\ nm}$ = 6.22 x 10^3). Protein assays are carried out by the method of Lowry *et al.* [116]after the protein solution is dialyzed against water to remove dithiothreitol (from 1 ml to 7.5 ml of crude extract). The concentration of protein crude extract is 50.1 mg/ml.

One nano-unit of 3-hydroxy-3-methylglutaryl CoA reductase is defined as the amount of enzyme that catalyzes the oxidation of 1 nmol of NADPH per minute per milliliter of incubation mixture. Specific activity is expressed as units per milligram of protein.

3. DETERMINATION OF THE KINETIC PROPERTIES OF 3-HYDROXY-3 METHYLGLUTARYL CoA REDUCTASE FROM BAKER'S YEAST

The 3-hydroxy-3-methylglutaryl CoA reductase was assayed using 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 20.0 μ*M* of 3-hydroxy-3-methylglutaryl CoA. The Lineweaver-Burk plot was created to analyze for K_m and V_{max} values using the following equation.

$$\frac{1}{v_0} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S_0]} + \frac{1}{V_{\max}}$$

4. SCREENING OF MEDICINAL PLANTS FOR INHIBITION 3-HYDROXY-METHYLGLUTARYL CoA REDUCTASE

4.1 Extraction of dried plant materials with hexane, ethyl acetate and methanol

Each plant material (500 g) was extracted with hexane, ethyl acetate and methanol at room temperature and the organic solvent was removed by rotary evaporator, as shown in Scheme 1.

Scheme 1 Isolation procedures

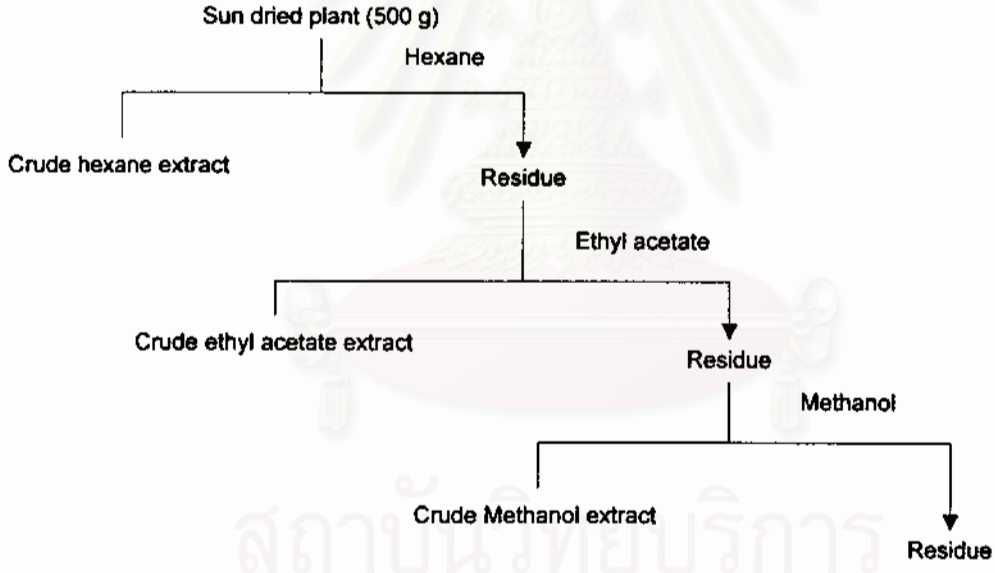


Table 3 Extraction of dried plant materials with hexane, ethyl acetate and methanol

Plant	Extracted solvent	Remark	Weight (g)
M. alba	Hexane	Dark green sticky oil	0.22
M. alba	Ethyl acetate	Dark green sticky oil	0.18
M. alba	Methanol	Dark green sticky oil	0.21
C. tinctorius	Hexane	Dark yellow oil	0.42
C. tinctorius	Ethyl acetate	Dark yellow sticky oil	0.21
C. tinctorius	Methanol	Brown sticky oil	0.07
F. resica	Hexane	Dark yellow sticky oil	0.15
F. resica	Ethyl acetate	Dark yellow sticky oil	0.17
F. resica	Methanol	Brown sticky oil	0.23
B. pandurata	Hexane	Dark brown sticky oil	0.34
B. pandurata	Ethyl acetate	Dark brown sticky oil	0.12
B. pandurata	Methanol	Dark brown sticky oil	0.24
O. aristatus	Hexane	Dark brown sticky oil	0.23
O. aristatus	Ethyl acetate	Dark brown sticky oil	0.47
O. aristatus	Methanol	Dark brown sticky oil	0.35
O. basilicum	Hexane	Dark brown sticky oil	0.15
O. basilicum	Ethyl acetate	Dark brown sticky oil	0.07
O. basilicum	Methanol	Dark brown sticky oil	0.09
H. sabdariffa	Hexane	Brown red stick oil	0.23

Plant	Extracted solvent	Remark	Weight (g)
H. sabdariffa	Ethyl acetate	Brown red stick oil	0.29
H. sabdariffa	Methanol	Brown red stick oil	0.25
G. glabra	Hexane	Brown oil	0.36
G. glabra	Ethyl acetate	Brown sticky oil	0.35
G. glabra	Methanol	Brown sticky oil	0.22
S. siamea	Hexane	Dark brown sticky oil	0.18
S. siamea	Ethyl acetate	Dark brown sticky oil	0.29
S. siamea	Methanol	Dark brown sticky oil	0.34
S. asper	Hexane	Dark brown sticky oil	0.11
S. asper	Ethyl acetate	Dark brown sticky oil	0.08
S. asper	Methanol	Brown sticky oil	0.37
S. rhambifalia	Hexane	Dark green oil	0.06
S. rhambifalia	Ethyl acetate	Dark green sticky oil	0.59
S. rhambifalia	Methanol	Dark green sticky oil	0.21
P. sarmentosum	Hexane	Dark brown sticky oil	0.37
P. sarmentosum	Ethyl acetate	Dark brown sticky oil	0.24
P. sarmentosum	Methanol	Dark brown sticky oil	0.56
A. Rugata	Hexane	Dark brown sticky oil	0.21
A. Rugata	Ethyl acetate	Dark brown sticky oil	0.16
A. Rugata	Methanol	Dark brown sticky oil	0.32
P. indica	Hexane	Dark brown sticky oil	0.15

Plant	Extracted solvent	Remark	Weight (g)
P. indica	Ethyl acetate	Dark brown sticky oil	0.16
P. indica	Methanol	Dark brown sticky oil	0.24
C. rotundus	Hexane	Dark brown sticky oil	0.28
C. rotundus	Ethyl acetate	Dark brown sticky oil	0.23
C. rotundus	Methanol	Dark brown residue	0.11
E. prostrate	Hexane	Dark green sticky oil	0.18
E. prostrate	Ethyl acetate	Dark green sticky oil	0.25
E. prostrate	Methanol	Dark brown residue	0.17
C. carri	Hexane	Dark brown oil	0.09
C. carri	Ethyl acetate	Brown sticky oil	0.15
C. carri	Methanol	Brown sticky oil	0.14
B. flabellifer	Hexane	Dark brown sticky oil	0.18
B. flabellifer	Ethyl acetate	Dark brown sticky oil	0.13
B. flabellifer	Methanol	Dark yellow sticky oil	0.54

4.2 Screening of the crude extracts for inhibition of 3-hydroxy-3-methylglutaryl CoA reductase

Dissolved 1 milligram of each crude extract in 1 milliliter of dimethyl-sulfoxide, then assay for 3-hydroxy-3-methylglutaryl CoA reductase inhibitory activity and compare with positive control (mevastatin) according to the procedure of Qureshi and coworks[113].

%Inhibition = [(A-B)/A] X100

Where “A” was the means of specific activity of enzyme in control (only DMSO at the same volumn being used for tested samples)

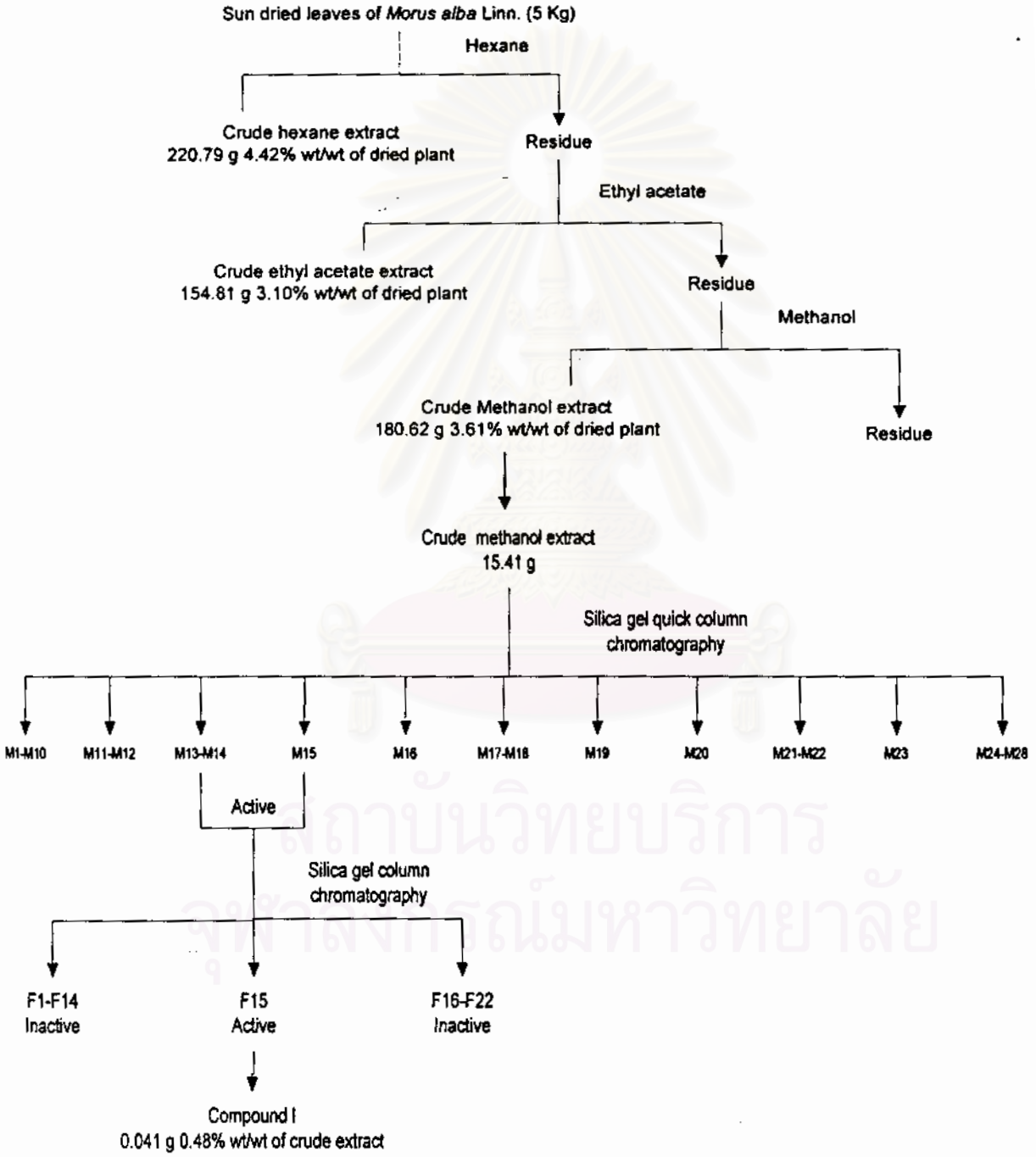
“B” was the means of specific activity of enzyme (with inhibitor)

5. ISOLATION, PURIFICATION AND STRUCTURE ELUCIDATION OF ACTIVE COMPOUND(S) FROM WHITE MULBERRY.

Five kilogram of white mulberry dried leaves (*Morus alba* Linn.) was extracted with hexane (15 *l*), ethyt acetate (15 *l*) and methanol (15 *l*) respectively. The crude methanol extract was obtained as a dark-green gummy residue (180.62 g, 3.61% wt./wt. of dried plant). For further purification, the methanol crude extract (15.41 g) was mixed with silica gel (12.68 g) and applied on top of silica gel column (142.25 g) and the column was eluted with 100% dichloromethane, dichloromethane-methanol gradient in a stepwise fashion (99:1, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 50:50, 30:70) and finally by 100% methanol. There were 28 fractions (each fraction contained 100 ml). The similar fractions that can inhibit 3-hydroxy-3-methylglutaryl CoA reductase were combined and removed the solvent by rotary evaporator. Then, the M13-M15 (0.35 g) were fractionated again by open column chromatography using Merck’s silica gel Art. 1.07734.1000 as an adsorbent. The column was eluted with dichloromethane-methanol gradient in a stepwise fashion. There were 22 fractions (each fraction contained 50 ml). The similar fractions that can inhibit 3-hydroxy-3-methylglutaryl CoA reductase were combined and solvent was removed by rotary evaporator to give compound 1 (0.041 g, 0.48% yield of

methanol crude extract). The isolation and purification of compound 1 from *Morus alba* Linn. is shown in Scheme 2.

Scheme 2 Isolation and purification *Morus alba* Linn



Inactive = 0-70 %inhibition Active = 71-100 %inhibition

Compound **1** was obtained from the elution of silica gel column chromatography with 10% methanol and was purified by re-crystallization with methanol to obtain a slightly-yellow solid crystal.

The structure of the active compound was identified by the ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectra recorded at 400 MHz on Varian Mercury +400 NMR Spectrometer. Chemical shifts are expressed in parts per million (ppm) using residual protonated solvents as reference. COSY, NOESY, HSQC and HMBC experiments were performed on the Varian Mercury + 400 NMR Spectrometer. The mass spectra were recorded on a Bruker Model Biflex TOF+MS.

6. DETERMINATION OF THE IC_{50} VALUE OF COMPOUND 1

The IC_{50} value of compound **1** was determined by dissolved 15, 30, 60 and 120 μ*M* of compound **1** in 1 milliliter of dimethyl-sulfoxide, then assay for 3-hydroxy-3-methylglutaryl CoA reductase inhibitory activity compared with mevastatin (positive control) according to the procedure of Qureshi and coworkers[113].

7. INVESTIGATION OF INHIBITORY PROPERTIES OF COMPOUND 1

The K_i values of the enzyme for mevastatin and the active compound were determined by using 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 20.0 μ*M* of 3-hydroxy-3-methylglutaryl CoA as a substrate and the inhibitor concentrations of 15, 30, 60 and 120 μ*M* for the active compound and 1.1, 5.7, 28.5 and 57.1 n*M* for mevastatin were used**.** The K_i values were determined from the Lineweaver-Burk plots.

8. STATISTICAL ANALYSIS.

All data were presented as the mean ± SEM. Significant differences among the groups were determined by one-way *t-test* at *P*=0.05.

CHAPTER IV

RESULTS AND DISCUSSION

1. EXTRACTION OF 3-HYDROXY-3-METHYLGLUTARYL CoA REDUCTASE FROM BAKER'S YEAST

The total volume of 3-hydroxy-3-methylglutaryl CoA reductase extracted from baker's yeast is 625 ml and the protein concentrations was 50.1 mg/ml.

2. DETERMINATION OF THE Km VALUE FOR 3-HYDROXY-3-METHYLGLUTARYL CoA OF KINETIC PROPERTIES OF 3-HYDROXY-3 METHYLGLUTARYL CoA REDUCTASE FROM BAKER'S YEAST

Kinetic properties of 3-hydroxy-3-methylglutaryl CoA reductase of baker's yeast are shown in Figures 13 and 14. When concentrations of 3-hydroxy-3-methylglutaryl CoA are increased, the initial velocities were increased. However, at excess concentration of 3-hydroxy-3-methylglutaryl CoA (12.5 μ*M*), the initial velocities were constant. The K_m and V_{max} values for the substrate of this enzyme were 2.0 μ*M* and 4.22 nmol/min/mg protein, respectively. The results were comparable to those reported by Qureshi and co-workers[113] who reported the K_m values for the substrate of this enzyme at 2.4 μ*M* and V_{max} of this reaction at 4.00 nmol/min/mg protein[113]. The concentration of substrate for enzyme activities assay was selected according to the K_m. The specificity of enzymes from baker's yeast with substrate was similarly to that of the enzyme from hamster liver, with K_m values at 3 μ*M*[117]. To prevent substrate inhibition, 20 μ*M* of substrate was used in subsequent experiments.

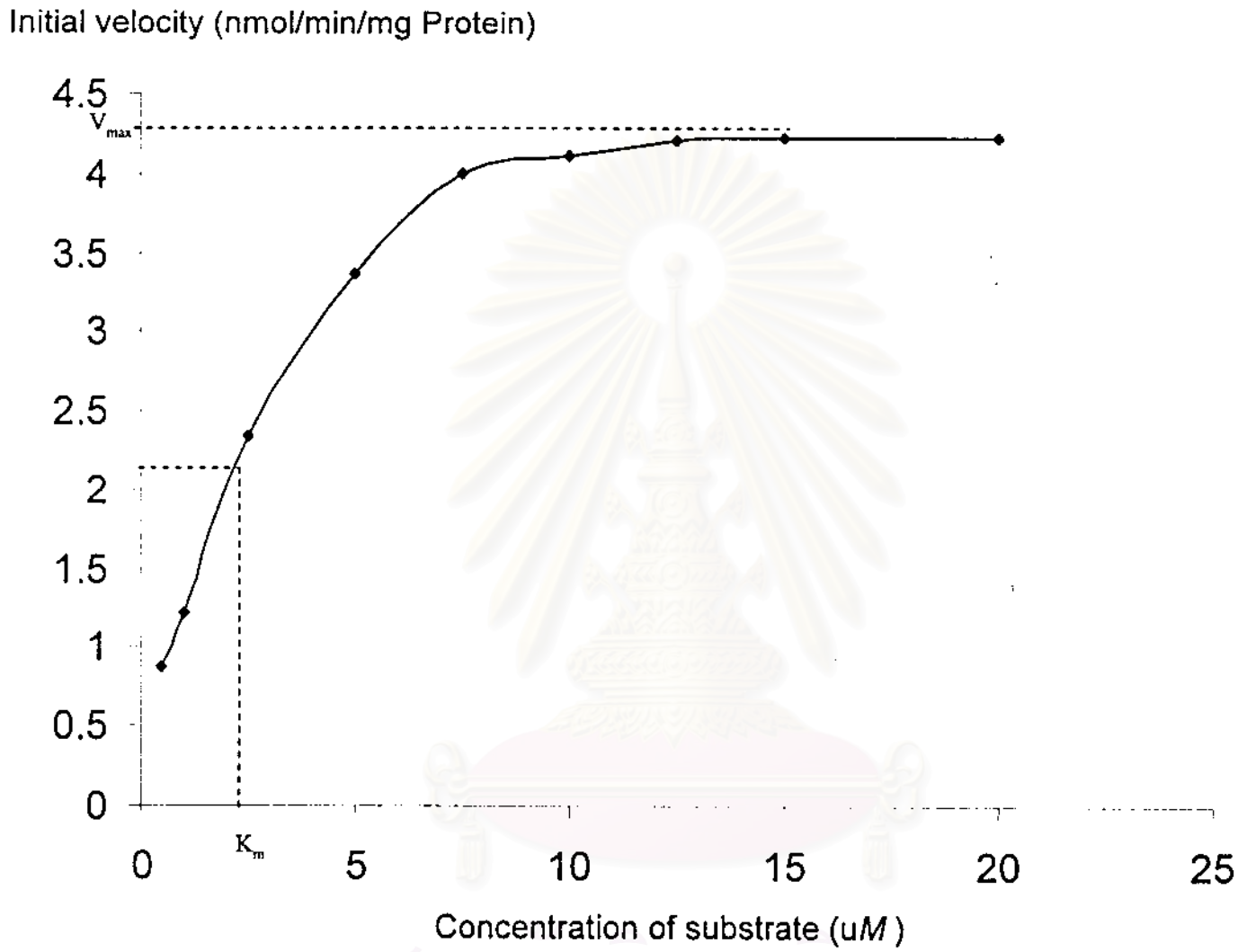


Figure 13 Relation of substrate concentration and initial velocity of 3-hydroxy-3-methylglutaryl CoA reductase from baker's yeast

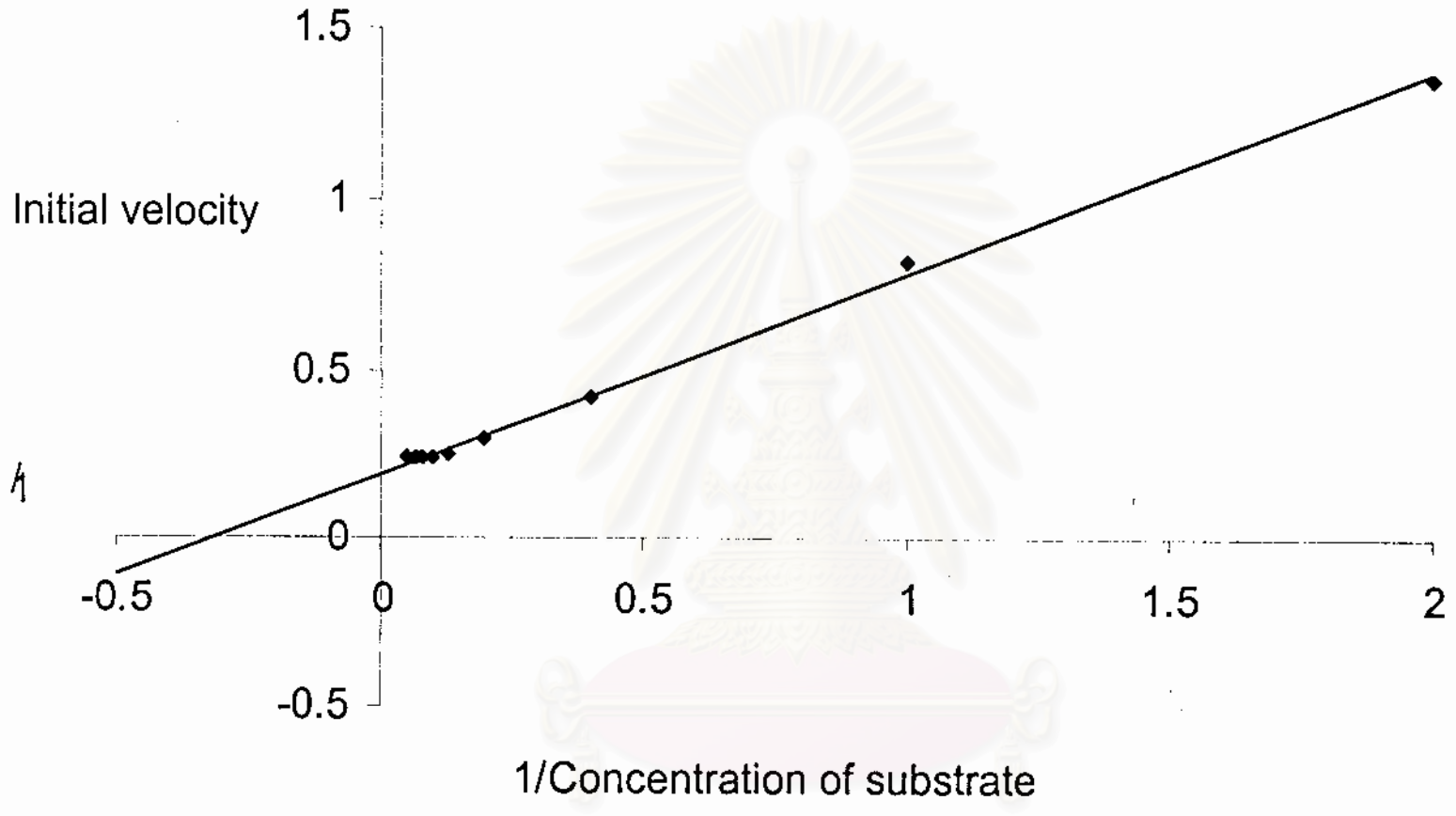


Figure 14 Lineweaver-Burk plot analysis of the kinetic of 3-hydroxy-3-methylglutaryl CoA reductase. The X-interception is $1/K_m$ and Y-interception is $1/V_{max}$

3. SCREENING OF MEDICINAL PLANTS FOR INHIBITOR OF 3-HYDROXY- METHYLGLUTARYL CoA REDUCTASE

Results from the screening of crude extract from medicinal plants for inhibition of 3-hydroxy-3-methylglutaryl CoA reductase are shown in Table 4. There were no significant differences among crude hexane extracts and the control. Crude ethyl acetate extract from *B. flabellifer*, crude methanol extracts from *M. alba, C. tinctorius, F. recisa, H. sabdariffa* and *G. glabra* gave significant %inhibition of 52.9±0.14, 79.1±0.06, 84.1±0, 63.4±0.04, 79.1±0.14 and 73.6±0.1, respectively (*P<0.05)*.

Table 4 Screening for inhibition of 3-hydroxy-3-methylglutaryl CoA reductase of crude extracts from medicinal plants

No	Crude plant extracts		Concentration (mg/ml DMSO)	Specific Activity nUnits/mg protein	% inhibition
	Plants	Solvent			
Control	-	-	0	4.22	-
1	*M. alba*	Hexane	0.1	4.19	0.55
2	*M. alba*	Hexane	1	3.95	3.95
3	*M. alba*	Hexane	10	3.79	10.19
4	*M. alba*	Ethyl acetate	0.1	4.21	0.16
5	*M. alba*	Ethyl acetate	1	4.21	0.24
6	*M. alba*	Ethyl acetate	10	2.91	13.1
7	*M. alba*	Methanol	0.1	1.32	68.64
8	*M. alba*	Methanol	1	0.88	79.07
9	*M. alba*	Methanol	10	0.45	89.26

No	Crude plant extracts		Concentration (mg/ml DMSO)	Specific Activity nUnits/mg protein	% inhibition
	Plants	Solvent			
10	*C. tinctorius*	Hexane	0.1	4.21	0.16
11	*C. tinctorius*	Hexane	1	4.17	0.95
12	*C. tinctorius*	Hexane	10	4.21	0.13
13	*C. tinctorius*	Ethyl acetate	0.1	4.21	0.08
14	*C. tinctorius*	Ethyl acetate	1	4.17	1.11
15	*C. tinctorius*	Ethyl acetate	10	3.5	17.1
16	*C. tinctorius*	Methanol	0.1	1.11	73.7
17	*C. tinctorius*	Methanol	1	0.67	84.1
18	*C. tinctorius*	Methanol	10	0.44	89.5
19	*F. resica*	Hexane	0.1	4.21	0.24
20	*F. resica*	Hexane	1	4.21	0.32
21	*F. resica*	Hexane	10	4.21	0.32
22	*F. resica*	Ethyl acetate	0.1	4.21	0.32
23	*F. resica*	Ethyl acetate	1	4.06	3.87
24	*F. resica*	Ethyl acetate	10	4.21	0.32
25	*F. resica*	Methanol	0.1	1.54	63.43
26	*F. resica*	Methanol	1	0.67	84.2
27	*F. resica*	Methanol	10	0.54	87.2
28	*B. pandurata*	Hexane	0.1	4.21	0.32
29	*B. pandurata*	Hexane	1	4.21	0.32
30	*B. pandurata*	Hexane	10	4.18	1.03
31	*B. pandurata*	Ethyl acetate	0.1	4.19	0.71
32	*B. pandurata*	Ethyl acetate	1	4.12	2.45

No	Crude plant extracts		Concentration (mg/ml DMSO)	Specific Activity nUnits/mg protein	% inhibition
	Plants	Solvent			
33	*B. pandurata*	Ethyl acetate	10	3.19	24.4
34	*B. pandurata*	Methanol	0.1	4.21	0.16
35	*B. pandurata*	Methanol	1	3.89	7.82
36	*B. pandurata*	Methanol	10	2.21	47.71
37	*O. aristatus*	Hexane	0.1	4.2	0.39
38	*O. aristatus*	Hexane	1	4.21	0.24
39	*O. aristatus*	Hexane	10	4.21	0.32
40	*O. aristatus*	Ethyl acetate	0.1	4.21	0.24
41	*O. aristatus*	Ethyl acetate	1	4.17	1.27
42	*O. aristatus*	Ethyl acetate	10	3.05	27.7
43	*O. aristatus*	Methanol	0.1	4.19	0.71
44	*O. aristatus*	Methanol	1	3.16	25.2
45	*O. aristatus*	Methanol	10	2.44	42.1
46	*O. basilicum*	Hexane	0.1	4.19	0.63
47	*O. basilicum*	Hexane	1	4.1	2.76
48	*O. basilicum*	Hexane	10	3.04	27.9
49	*O. basilicum*	Ethyl acetate	0.1	4.21	0.24
50	*O. basilicum*	Ethyl acetate	1	4.21	0.24
51	*O. basilicum*	Ethyl acetate	10	4.21	0.16
52	*O. basilicum*	Methanol	0.1	4.21	0.16
53	*O. basilicum*	Methanol	1	3.34	20.93
54	*O. basilicum*	Methanol	10	2.44	42.1
55	*H. sabdariffa*	Hexane	0.1	4.2	0.47

No	Crude plant extracts		Concentration (mg/ml DMSO)	Specific Activity nUnits/mg protein	% inhibition
	Plants	Solvent			
56	*H. sabdariffa*	Hexane	1	4.21	0.24
57	*H. sabdariffa*	Hexane	10	4.21	0.24
58	*H. sabdariffa*	Ethyl acetate	0.1	4.21	0.24
59	*H. sabdariffa*	Ethyl acetate	1	4.21	0.16
60	*H. sabdariffa*	Ethyl acetate	10	3.76	10.9
61	*H. sabdariffa*	Methanol	0.1	1.07	74.56
62	*H. sabdariffa*	Methanol	1	0.88	79.15
63	*H. sabdariffa*	Methanol	10	0.44	89.65
64	*G. glabra*	Hexane	0.1	4.21	0.24
65	*G. glabra*	Hexane	1	4.05	3.95
66	*G. glabra*	Hexane	10	3.11	26.2
67	*G. glabra*	Ethyl acetate	0.1	4.2	0.39
68	*G. glabra*	Ethyl acetate	1	4.21	0.24
69	*G. glabra*	Ethyl acetate	10	4.19	0.79
70	*G. glabra*	Methanol	0.1	1.77	58.14
71	*G. glabra*	Methanol	1	1.11	73.77
72	*G. glabra*	Methanol	10	0.88	79.15
73	*S. siamea*	Hexane	0.1	4.2	0.39
74	*S. siamea*	Hexane	1	4.21	0.24
75	*S. siamea*	Hexane	10	4.2	0.39
76	*S. siamea*	Ethyl acetate	0.1	4.19	0.63
77	*S. siamea*	Ethyl acetate	1	4.12	2.29
78	*S. siamea*	Ethyl acetate	10	3.77	10.7

No	Crude plant extracts		Concentration (mg/ml DMSO)	Specific Activity nUnits/mg protein	% inhibition
	Plants	Solvent			
79	*S. siamea*	Methanol	0.1	4.21	0.16
80	*S. siamea*	Methanol	1	4.21	0.16
81	*S. siamea*	Methanol	10	3.11	26.38
82	*S. asper*	Hexane	0.1	4.19	0.79
83	*S. asper*	Hexane	1	4.16	1.34
84	*S. asper*	Hexane	10	3.56	15.6
85	*S. asper*	Ethyl acetate	0.1	4.20	0.39
86	*S. asper*	Ethyl acetate	1	4.21	0.32
87	*S. asper*	Ethyl acetate	10	4.20	0.39
88	*S. asper*	Methanol	0.1	4.21	0.15
89	*S. asper*	Methanol	1	4.20	0.39
90	*S. asper*	Methanol	10	3.76	10.82
91	*S. rhambifalia*	Hexane	0.1	4.21	0.32
92	*S. rhambifalia*	Hexane	1	4.21	0.24
93	*S. rhambifalia*	Hexane	10	4.21	0.24
94	*S. rhambifalia*	Ethyl acetate	0.1	4.21	0.32
95	*S. rhambifalia*	Ethyl acetate	1	4.20	0.39
96	*S. rhambifalia*	Ethyl acetate	10	4.21	0.24
97	*S. rhambifalia*	Methanol	0.1	4.20	0.47
98	*S. rhambifalia*	Methanol	1	4.10	2.76
99	*S. rhambifalia*	Methanol	10	3.77	10.74
100	*P. sarmentosum*	Hexane	0.1	4.21	0.24
101	*P. sarmentosum*	Hexane	1	4.21	0.16

No	Crude plant extracts		Concentration (mg/ml DMSO)	Specific Activity nUnits/mg protein	% inhibition
	Plants	Solvent			
102	*P. sarmentosum*	Hexane	10	4.21	0.32
103	*P. sarmentosum*	Ethyl acetate	0.1	4.21	0.32
104	*P. sarmentosum*	Ethyl acetate	1	4.21	0.24
105	*P. sarmentosum*	Ethyl acetate	10	4.21	0.16
106	*P. sarmentosum*	Methanol	0.1	4.20	0.39
107	*P. sarmentosum*	Methanol	1	4.21	0.24
108	*P. sarmentosum*	Methanol	10	3.77	10.66
109	*A. Rugata*	Hexane	0.1	4.19	0.79
110	*A. Rugata*	Hexane	1	4.02	4.8
111	*A. Rugata*	Hexane	10	3.67	13.1
112	*A. Rugata*	Ethyl acetate	0.1	4.21	0.16
113	*A. Rugata*	Ethyl acetate	1	4.21	0.16
114	*A. Rugata*	Ethyl acetate	10	4.21	0.24
115	*A. Rugata*	Methanol	0.1	3.24	23.3
116	*A. Rugata*	Methanol	1	2.52	40.2
117	*A. Rugata*	Methanol	10	1.11	73.78
118	*P. indica*	Hexane	0.1	4.20	0.39
119	*P. indica*	Hexane	1	4.21	0.24
120	*P. indica*	Hexane	10	4.21	0.32
121	*P. indica*	Ethyl acetate	0.1	4.20	0.39
122	*P. indica*	Ethyl acetate	1	4.21	0.24
123	*P. indica*	Ethyl acetate	10	4.21	0.32
124	*P. indica*	Methanol	0.1	4.20	0.39

No	Crude plant extracts		Concentration (mg/ml DMSO)	Specific Activity nUnits/mg protein	% inhibition
	Plants	Solvent			
125	*P. indica*	Methanol	1	4.21	0.24
126	*P. indica*	Methanol	10	4.21	0.16
127	*C. rotundus*	Hexane	0.1	4.21	0.32
128	*C. rotundus*	Hexane	1	4.21	0.24
129	*C. rotundus*	Hexane	10	4.21	0.24
130	*C. rotundus*	Ethyl acetate	0.1	4.21	0.32
131	*C. rotundus*	Ethyl acetate	1	4.20	0.39
132	*C. rotundus*	Ethyl acetate	10	4.21	0.11
133	*C. rotundus*	Methanol	0.1	4.21	0.24
134	*C. rotundus*	Methanol	1	4.19	0.79
135	*C. rotundus*	Methanol	10	3.10	26.46
136	*E. prostrate*	Hexane	0.1	4.21	0.24
137	*E. prostrate*	Hexane	1	4.20	.39
138	*E. prostrate*	Hexane	10	4.20	0.39
139	*E. prostrate*	Ethyl acetate	0.1	4.21	0.16
140	*E. prostrate*	Ethyl acetate	1	4.21	0.16
141	*E. prostrate*	Ethyl acetate	10	4.21	0.24
142	*E. prostrate*	Methanol	0.1	4.20	0.3
143	*E. prostrate*	Methanol	1	4.20	0.3
144	*E. prostrate*	Methanol	10	4.20	0.3
145	*C. carri*	Hexane	0.1	4.21	0.34
146	*C. carri*	Hexane	1	4.20	0.39
147	*C. carri*	Hexane	10	4.20	0.47

No	Crude plant extracts		Concentration (mg/ml DMSO)	Specific Activity nUnits/mg protein	% inhibition
	Plants	Solvent			
148	*C. carri*	Ethyl acetate	0.1	4.21	0.16
149	*C. carri*	Ethyl acetate	1	4.21	0.32
150	*C. carri*	Ethyl acetate	10	4.20	0.47
151	*C. carri*	Methanol	0.1	4.21	0.23
152	*C. carri*	Methanol	1	4.19	0.71
153	*C. carri*	Methanol	10	3.32	21.72
154	*B. flabellifer*	Hexane	0.1	4.21	0.16
155	*B. flabellifer*	Hexane	1	4.21	0.25
156	*B. flabellifer*	Hexane	10	2.78	34
157	*B. flabellifer*	Ethyl acetate	0.1	3.33	21.1
158	*B. flabellifer*	Ethyl acetate	1	1.99	52.9
159	*B. flabellifer*	Ethyl acetate	10	1.09	74.2
160	*B. flabellifer*	Methanol	0.1	4.17	1.24
161	*B. flabellifer*	Methanol	1	4.09	3.07
162	*B. flabellifer*	Methanol	10	3.25	23.05

5. ISOLATION, PURIFICATION AND STRUCTURE ELUCIDATION OF ACTIVE COMPOUND(S) FROM WHITE MULBERRY.

Using bioassay guided, the crude methanol extract of *M. alba* was chosen for further study by chromatographic separation and spectroscopic identification of its components. The crude methanol extract of *M. alba* was fractionated by silica gel quick column chromatography to give 28 fractions. Fraction no.13th, 14th and 15th had 76.2±0.14, 78.5±0.08 and 73.4±0.08 %inhibition respectively, as shown in Table 5.

Table 5 Screening for inhibition of 3-hydroxy-3-methylglutaryl CoA reductase of each fraction from crude methanol extract of *M. alba* after silica gel quick column separation

Fractions (1mg/ml DMSO)	Eluent	Remark	Weight (g)	Specific Activity nUnits/mg protein	% inhibition
Control	-	-	-	4.21	0
M1	100% Dichloromethane	Yellow oil	0.07	4.21	0
M2	100% Dichloromethane	Yellow oil	0.09	4.21	0
M3	100% Dichloromethane	Yellow oil	1.02	4.21	0
M4	100% Dichloromethane	Yellow oil	0.05	4.21	0
M5	100% Dichloromethane	Dark yellow oil	1.00	4.20	0.10
M6	100% Dichloromethane	Dark yellow oil	0.04	4.21	0
M7	100% Dichloromethane	Dark brown oil	1.03	4.20	0.11
M8	100% Dichloromethane	Brown oil	0.07	4.20	0.08
M9	100% Dichloromethane	Brown oil	0.09	4.21	0
M10	100% Dichloromethane	Brown oil	2.04	4.20	0.11
M11	1% MeOH-Dichloro	Brown oil	0.08	4.19	0.37
M12	1% MeOH-Dichloro	Dark green oil	1.53	3.99	5.10

Fractions (1mg/ml DMSO)	Eluent	Remark	Weight (g)	Specific Activity nUnits/mg protein	% inhibition
M13	5% MeOH-Dichloro	Dark green oil	0.22	0.90	78.5
M14	5% MeOH-Dichloro	Dark green oil	0.09	1.12	73.4
M15	10% MeOH-Dichloro	Dark green oil	0.04	1.00	76.2
M16	15% MeOH-Dichloro	Green oil	1.34	4.20	0.11
M17	20% MeOH-Dichloro	Green oil	0.09	4.20	0.13
M18	20% MeOH-Dichloro	Brown oil	0.02	4.21	0
M19	25% MeOH-Dichloro	Brown oil	0.05	4.21	0
M20	30% MeOH-Dichloro	Brown oil	0.08	4.21	0
M21	50% MeOH-Dichloro	Dark brown tar	1.02	4.21	0
M22	50% MeOH-Dichloro	Dark brown tar	0.03	4.20	0.10
M23	70% MeOH-Dichloro	Dark brown tar	1.67	4.20	0.08
M24	100% MeOH	Dark brown tar	0.04	4.21	0
M25	100% MeOH	Dark brown tar	1.13	4.21	0
M26	100% MeOH	Dark brown tar	1.20	4.20	0.08
M27	100% MeOH	Dark brown tar	1.10	4.21	0
M28	100% MeOH	Dark brown tar	0.05	4.21	0

Fractions no.13th, 14th and 15th were combined and subjected to open column chromatography. There were 22 fractions and fraction no.15th gave compound **1** with 83±0.14 %inhibition, as shown in Table 6.

Table 6 Screening for inhibition of 3-hydroxy-3-methylglutaryl CoA reductase of each fraction from combined M13-M15, after silica gel quick column separation

Fractions (1mg/ml DMSO)	Eluent	Remark	Weight (g)	Specific Activity nUnits/mg protein	% inhibition
Control	-	-	-	4.22	0
F1	100% Dichloromethane	Yellow oil	0.011	4.22	0
F2	100% Dichloromethane	Yellow oil	0.017	4.22	0
F3	1% MeOH-Dichloro	Brown oil	0.013	4.22	0
F4	1% MeOH-Dichloro	Yellow soilid in brown oil	0.002	4.21	0.08
F5	1% MeOH-Dichloro	Brown oil	0.006	4.21	0.10
F6	2% MeOH-Dichloro	Brown oil	0.009	4.22	0
F7	2% MeOH-Dichloro	green oil	0.021	4.22	0
F8	2% MeOH-Dichloro	Dark green oil	0.013	4.22	0
F9	3% MeOH-Dichloro	Dark green oil	0.017	4.21	0.13
F10	3% MeOH-Dichloro	Dark green oil	0.002	4.22	0
F11	3% MeOH-Dichloro	White solid in Green oil	0.024	4.22	0
F12	5% MeOH-Dichloro	White solid in Green oil	0.015	4.22	0
F13	7% MeOH-Dichloro	Brown oil	0.013	4.21	0.11
F14	10% MeOH-Dichloro	Semi solid in yellow oil	0.003	4.12	2.31
F15	10% MeOH-Dichloro	White crystal In yellow oil	0.092	0.72	83.0
F16	15% MeOH-Dichloro	Yellow oil	0.035	3.70	12.4

Fractions (1mg/ml DMSO)	Eluent	Remark	Weight (g)	Specific Activity nUnits/mg protein	% inhibition
F17	15% MeOH-Dichloro	Dark brown oil	0.006	4.21	0.11
F18	15% MeOH-Dichloro	Brown oil	0.004	4.21	0.05
F19	20% MeOH-Dichloro	Brown oil	0.009	4.22	0
F20	20% MeOH-Dichloro	Yellow crystal in brown oil	0.007	4.22	0
F21	30% MeOH-Dichloro	Yellow crystal in brown oil	0.002	4.22	0
F22	30% MeOH-Dichloro	Yellow crystal in brown oil	0.023	4.21	0.10

Compound **1** was obtained as slightly yellow crystal (0.041 g, 0.0096 %wt/wt of dried plant). The IR spectrum of compound **1** showed the presence of hydroxyl group and phenolic hydroxyl group according to the broad and strong absorption band at 3,317 cm^{-1}, the strong absorption band at 1649 cm^{-1} due to α, β-unsaturated carbonyl moiety and the strong absorption band at 1519 cm^{-1} due to aromatic moiety. The molecular formula of compound **1** was assigned to be $C_{27}H_{32}O_{14}$ from TOF+[M+](m/z) = 580.The ^{1}H-NMR and ^{13}C-NMR spectra of Compound **1** were shown in Table 7 and 8. The ^{1}H-NMR spectrum of compound **1** the signals at δ_H 1.20 ppm which correspondihg to methyl protons (table 7). A proton of hydroxyl group showed the signal at δ_H 3.40-3.90 ppm and the signal at δ_H 5.20 ppm was assigned to be methane proton. The The ^{13}C-NMR spectrum showed 27 signals comprising twelve olefinic carbon signals at δ_c 165.0, 163.5,

163.0, 158.0, 129.5, 128.0, 128.0, 115.0, 115.0, 104.0, 96.4 and 95.3 ppm, the carbonyl group of α, β-unsaturated ketone at δ_c 71.0 and 101.5 ppm, respectively (Table 8). Based on the spectral data discussed above the structure of compound **1** was assigned to be naringin and the structure is shown in Figure 15 [118].

Table 7 The ^1H spectral data (CD_3 OD) of Compound I and Naringin (Ficarra R. and co-workers[118])

No.	Chemical Shift of Compound **1** (ppm)	Chemical Shift of Naringin (ppm)	No.	Chemical Shift of Compound **1** (ppm)	Chemical Shift of Naringin (ppm)
2	5.40 (*J*=13)	5.34	6'	7.30(*J*=9)	7.30
3	2.80(*J*=17,3)-3.20(*J*=17,13,6)	2.73-3.13	1"	5.20(*J*=8)	5.24
4	-	-	2"	3.85(*J*=8,7)	3.85
5	-	-	3"	3.50(*J*=10,7)	3.58
6	6.20(*J*=2)	5.24	4"	3.30(*J*=10,9)	3.13
7	-	-	5"	3.90(*J*=10,6,2)	3.93
8	6.20(*J*=2)	5.24	6"	3.60-3.80(*J*=11,2)	3.63-3.88
9	-	-	1"'	5.28(*J*=2)	5.24
10	-	-	2"'	3.40(*J*=3,2)	3.38
1'	-	-	3"'	3.50(*J*=10,3)	3.58
2'	7.30(*J*=9)	7.30	4"'	3.30(*J*=10,10)	3.13
3'	6.80(*J*=9)	6.80	5"'	3.45(*J*=6)	3.42
4'	-	-	6"'	1.20d	1.28
5'	6.80(J=9)	6.80			

Table 8 The ^{13}C NMR spectral data (CD_3OD) of Compound **1** and Naringin (Ficarra R. and co-workers[118])

No.	Chemical Shift of Compound **1** (ppm)	Chemical Shift of Naringin (ppm)	No.	Chemical Shift of Compound **1** (ppm)	Chemical Shift of Naringin (ppm)
2	79.5d	80.7	6'	128.0d	129.1
3	43.0t	44.1	1"	98.0	99.4
4	197.0s	198.5	2"	71.0d	72.1
5	163.0s	164.5	3"	78.0d	79.0
6	95.3	97.8	4"	73.0d	73.9
7	165.0	166.5	5"	68.5d	70.0
8	96.4	96.7	6"	61.0d	62.2
9	163.5s	164.6	1"'	101.5d	102.5
10	104.0s	104.9	2"'	77.0d	78.9
1'	129.5s	130.8	3"'	71.0d	72.1
2'	128.0d	129.1	4"'	70.0d	71.2
3'	115.0d	116.3	5"'	78.0d	78.1
4'	158.0s	159.0	6"'	17.0t	18.2
5'	115.0d	116.3			

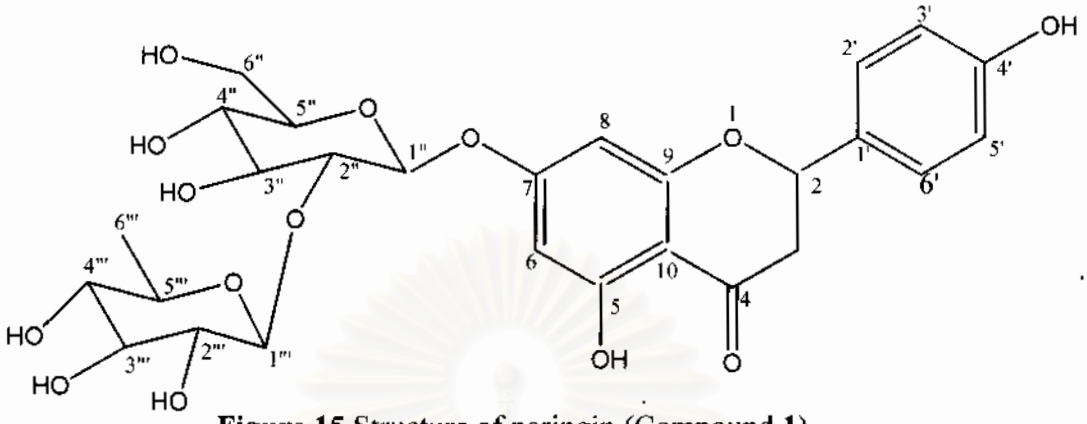


Figure 15 Structure-of naringin (Compound **1**)

6. DETERMINATION OF THE IC_{50} VALUE OF NARINGIN

%Inhibition at 15, 30, 60 and 120 μ*M* naringin and 1.1, 5.7, 28.5, 57.1 n*M* mevastatin were shown in table 9. IC_{50} is the concentration of the inhibitor required to inhibit 50 %inhibition. The IC_{50} value of mevastatin and naringin were 5.7 n*M* and 30 μ*M*, respectively.

Table 9 %Inhibition at varied concentration of naringin and mevastatin

Concentration of mevastatin (n*M*)	%Inhibition	Concentration of naringin (μ*M*)	%Inhibition
No inhibitor	-	No inhibitor	-
1.1	18.3	15	38.8
5.7	51.1	30	49.1
28.5	91.4	60	71.2
57.1	100	120	82.9

7. INVESTIGATION OF INHIBITORY PROPERTIES OF THE ACTIVE COMPOUND (S)

The Lineweaver-Burk plot of mevastatin (positive control) indicated that mevastatin was a competitive inhibitor because every concentration of mevastatin have the same Y-intercepts in Lineweaver-Burk plot and had very potent inhibitory activity, as shown in Figure 16. The K_i values of mevaststin were determined from the slope of each graph in Lineweaver-Burk plot. The equation for calculation of K_i value for the competitive inhibitor is

$$Slope = \frac{k_m}{v_{max}}\left[1 + \frac{[I_0]}{K_i}\right]$$

The K_i value of mevastatin was obtained at 1.71 n*M* which was comparable to that using the enzyme from microsomes of hamster liver, which gave the K_i value at 1.4 n*M*[117]. The K_i value of mevastatin are shown in Table 10.

Table 10 The K_i values of mevastatin

Concentration of mevastatin (n*M*)	K_i (n*M*)
No inhibitor	-
1.1	1.71
5.7	1.67
28.5	1.73
57.1	1.75

The Lineweaver-Burk plot of naringin indicated that naringin was a noncompetitive inhibitor because every concentration of naringin had the same X-intercepts in Lineweaver-Burk plot and had potent inhibitory activity, as shown in Figure 17. The kinetic of naringin for inhibition of 3-hydroxy-3-methylglutaryl CoA reductase have never been reported except the one using naringin from mandarin peel for the reduction of serum and liver cholesterol in rats and rabbits.[16] Thus, this report explain why naringin could lower the cholesterol in vivo. The K_i values of naringin were determined from the slope of each graph in Lineweaver-Burk plot. The equation for calculation K_i value of noncompetitive inhibitor is

$$Slope = \frac{k_m}{v_{max}}\left[1+\frac{[I_0]}{K_i}\right]$$

The K_i value of naringin is 1.06 μM, as shown in Table 11

Table 11 The K_i values of naringin

Concentration of naringin (μM)	K_i (μM)
No inhibitor	-
15	0.89
30	1.12
60	0.948
120	1.30

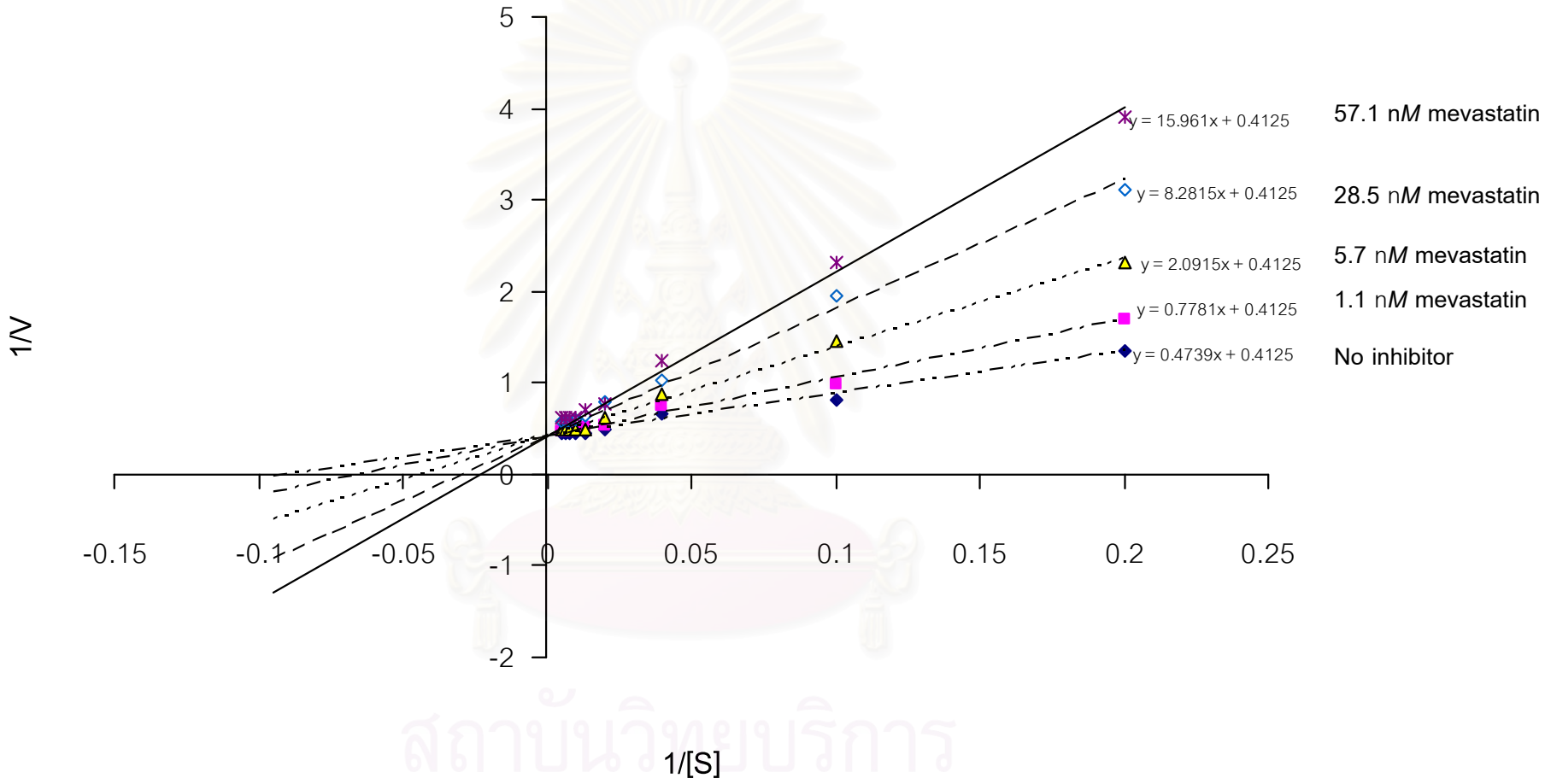


Figure 16 Lineweaver-Burk plot analysis of the inhibition kinetics of 3-hydroxy-3-methylglutaryl CoA reductase by mevastatin.

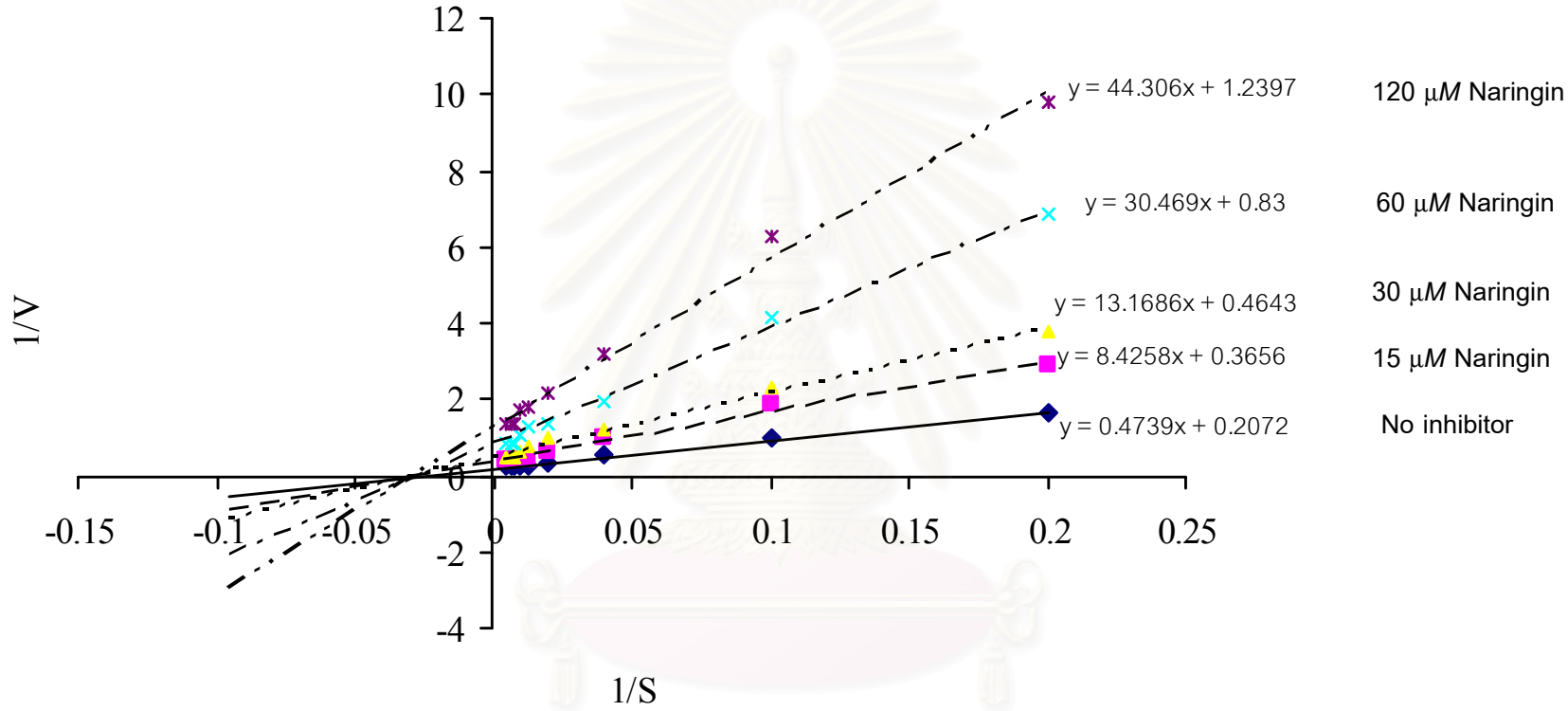


Figure 17. Lineweaver-Burk plot analysis of the inhibition kinetics of 3-hydroxy-3-methylglutaryl CoA reductase by naringin

CHAPTER V

CONCLUSION

In this research, the 3-hydroxy-3-methylglutaryl CoA reductase was extracted from baker's yeast (*Saccharomyces cerevisiae*) and eighteen Thai-traditional medicinal plants which known to have hypercholestermic activity were screened for their ability to inhibit 3-hydroxy-3-methylglutaryl CoA reductase activity. It was found that crude ethyl acetate from *B. flabellifer*, crude methanol extract from *M. alba, C. tinctorius, F. recisa, H. sabdariffa* and *G. glabra* gave significant %inhibition (52.9±0.14, 79.1±0.06, 84.1±0, 63.4±0.04, 79.1±0.14 and 73.6±0.1 %inhibition, respectively, $P<0.05$). Using bioassay guided chromatographic separation, the crude methanol extract (180.62 g) from *M. alba* was separated. The active fractions (M13-M15) that inhibited 3-hydroxy-3-methylglutaryl CoA reductase was purified and it's structure was identified by spectroscopic method as naringin (0.041g, 0.048%wt./wt. of dried plant). The IC_{50} value of mevastatin and naringin were 5.7 n*M* and 30 μ*M*, respectively. From this results showed, mevastatin was more effective than naringin 5000 folds. However, Naringin was a noncompetitive inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase. From the study of Bok and co-workers[16], the peel of mandarin containing naringin could also inhibit 3-hydroxy-3-methylglutaryl CoA reductase *in vivo*. Therefore, the results of this thesis agree well with the in *vivo* experiment carried out by Bok and coworkers. Naringin is a noncompetitive inhibitor of 3-hydroxy-3-methylglutayl CoA reductase with, the K_i value of 1.06 μ*M*. Under the same condition the K_i value of mevastatin, a competitive inhibitor was 1.4 n*M*.

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APPENDICES

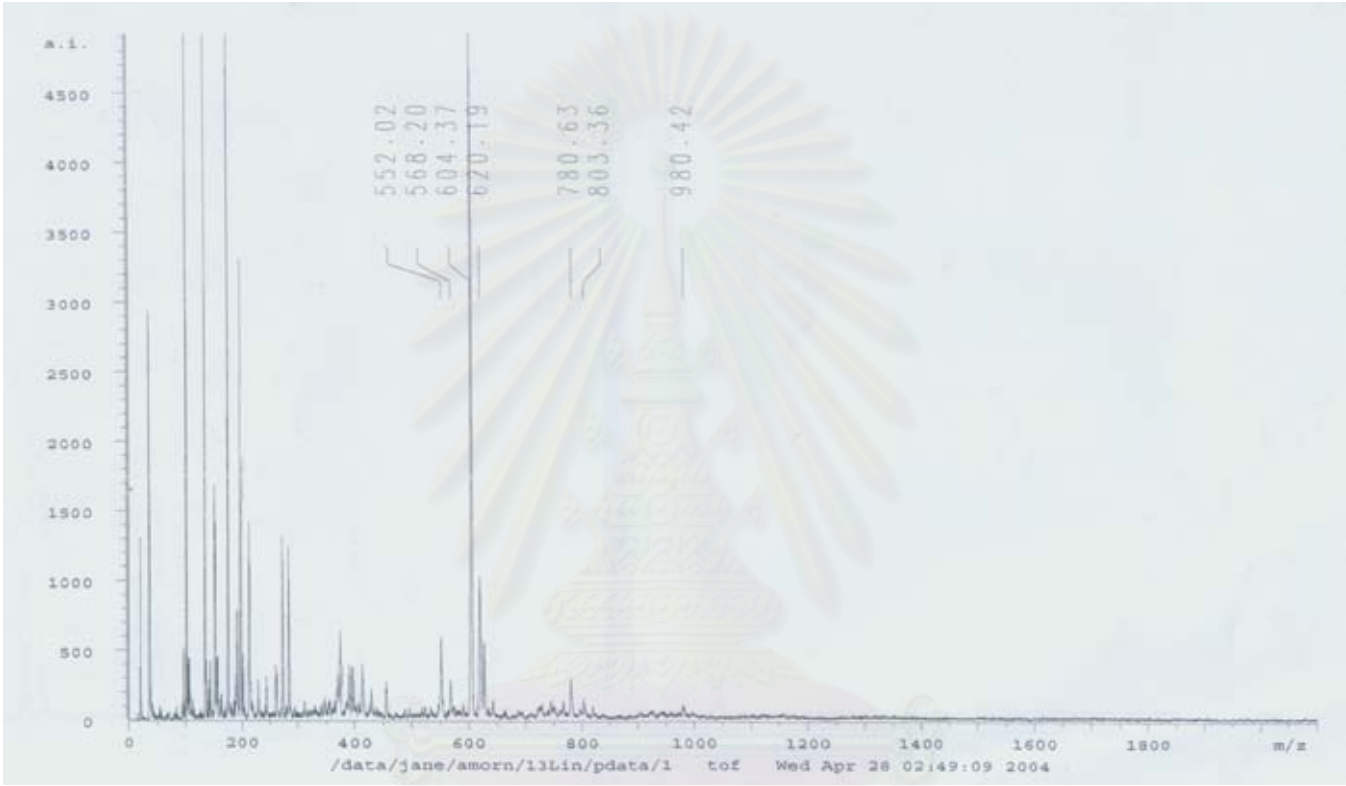

Figure 18. The MS spectrum of Compound I

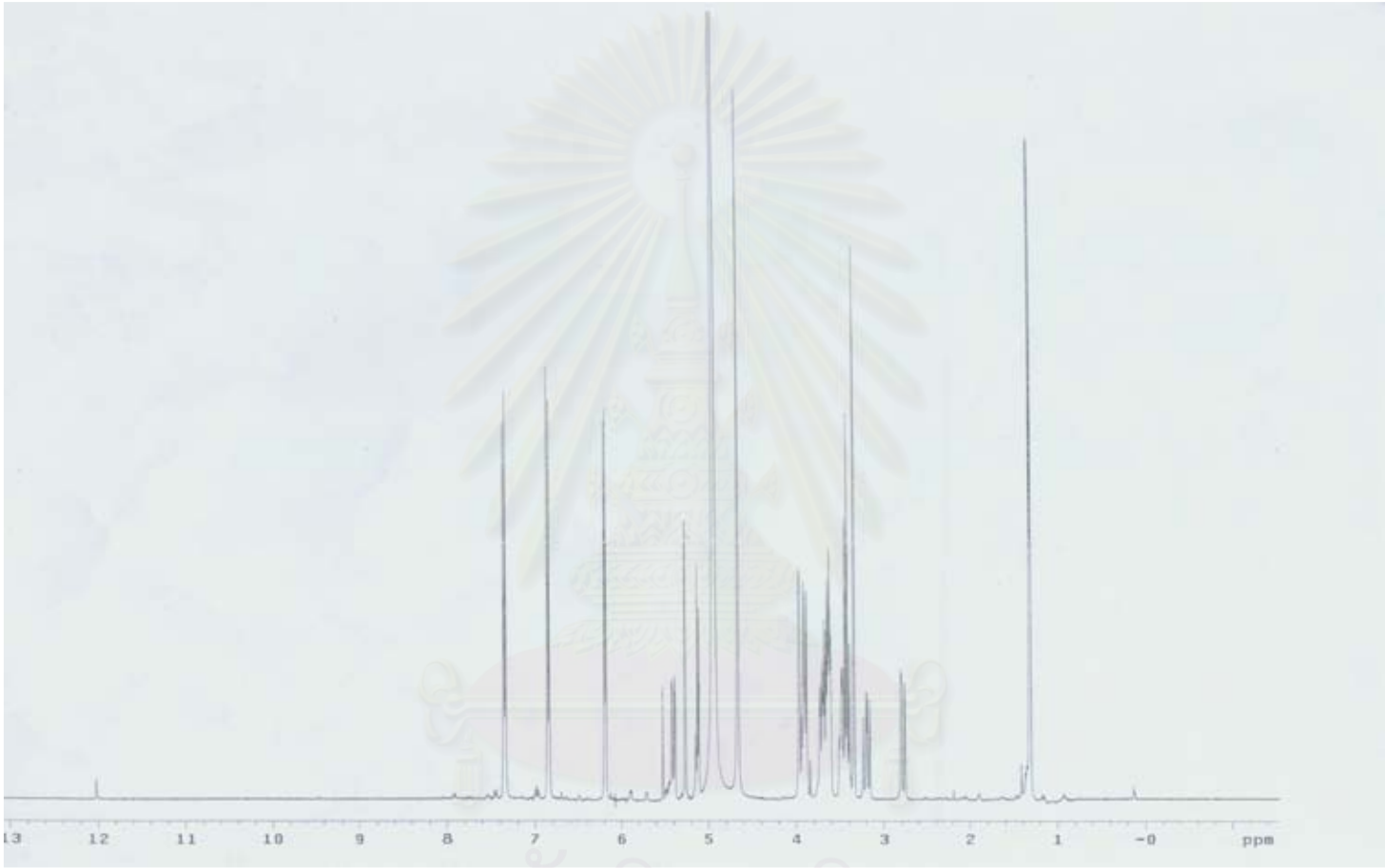

Figure 19. The ^{1}H-NMR spectrum of Compound **1**

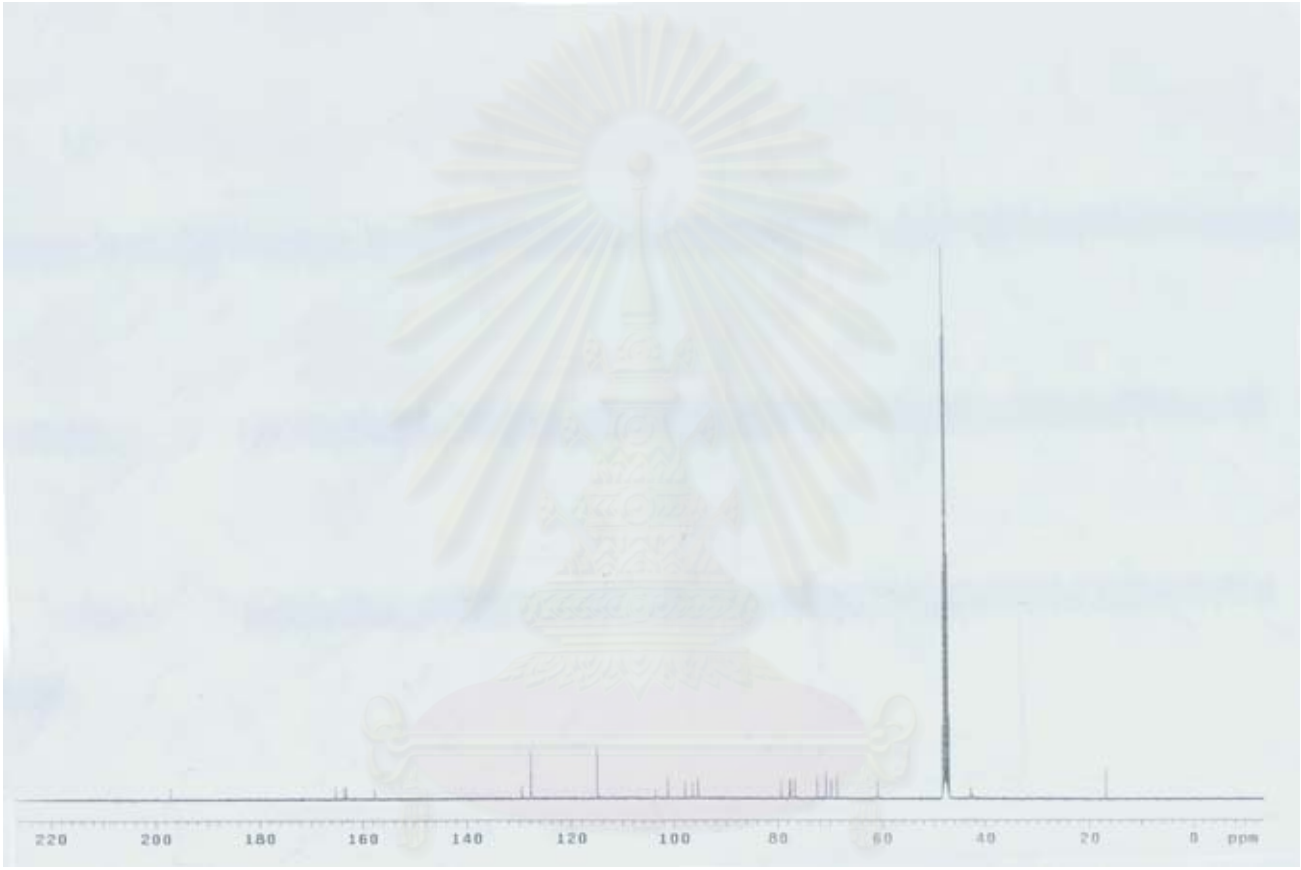

Figure 20. The ^{13}C-NMR spectrum of Compound **1**

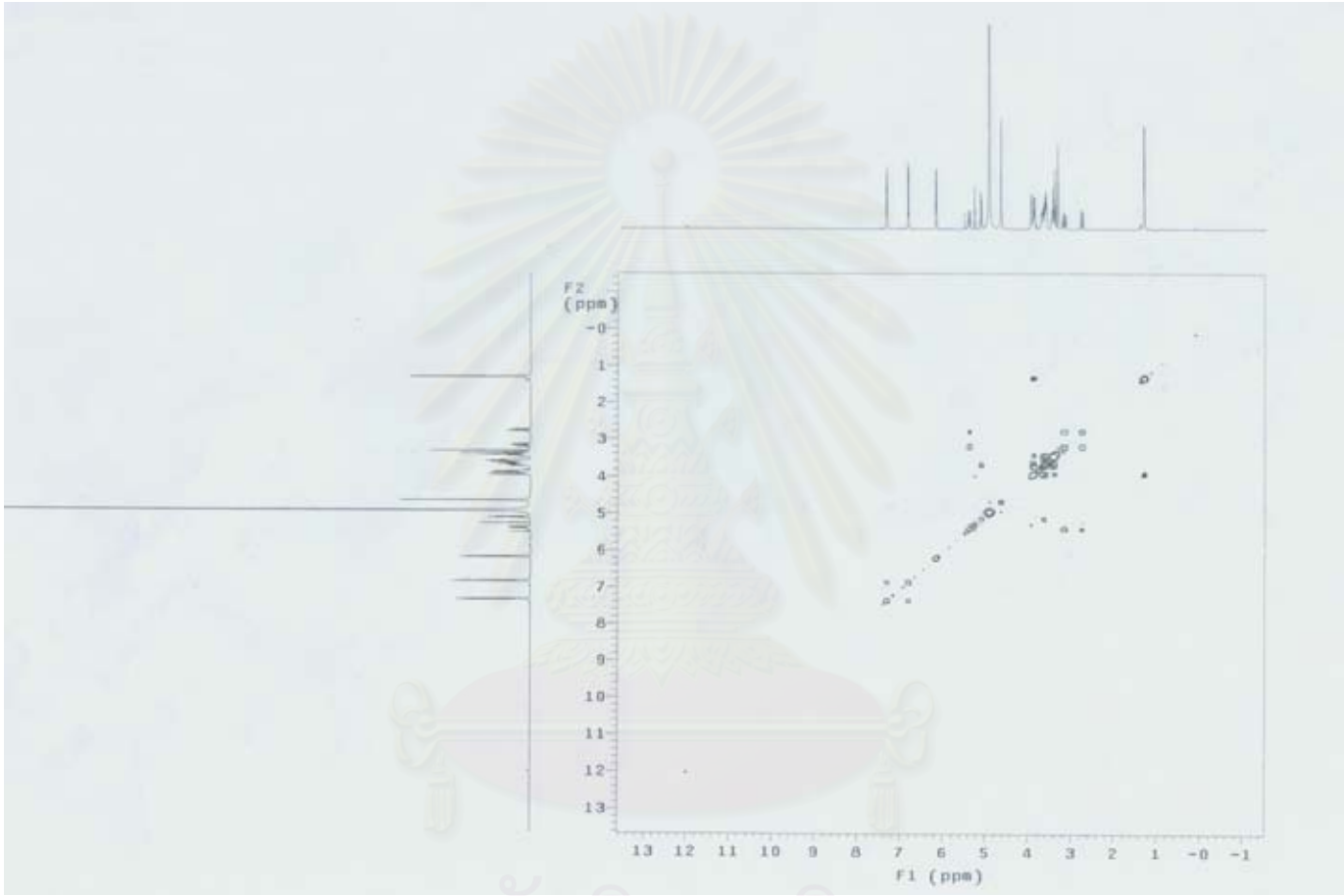

Figure 21. The COSY-NMR spectrum of Compound **1**

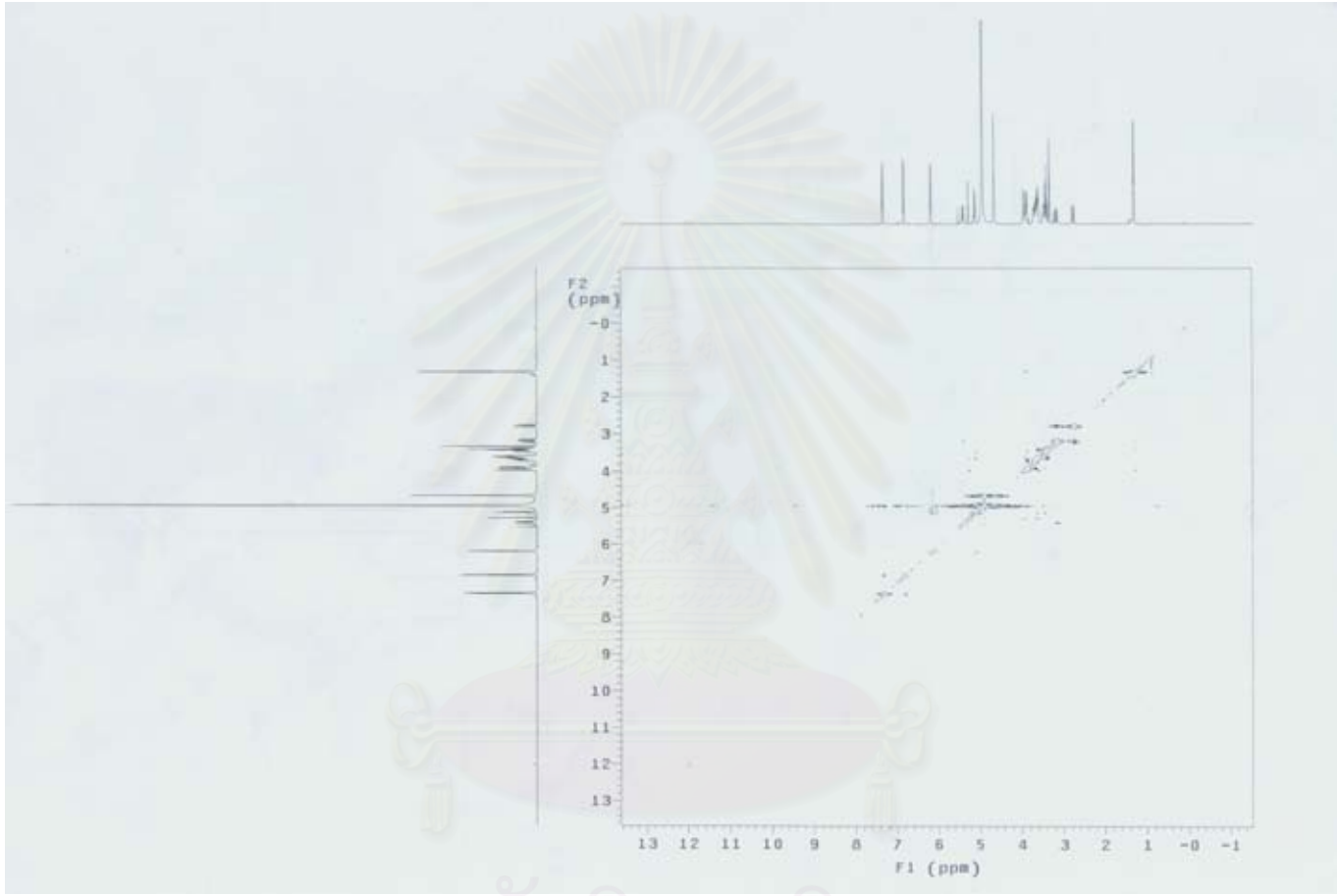

Figure 22. The NOESY-NMR spectrum of Compound I

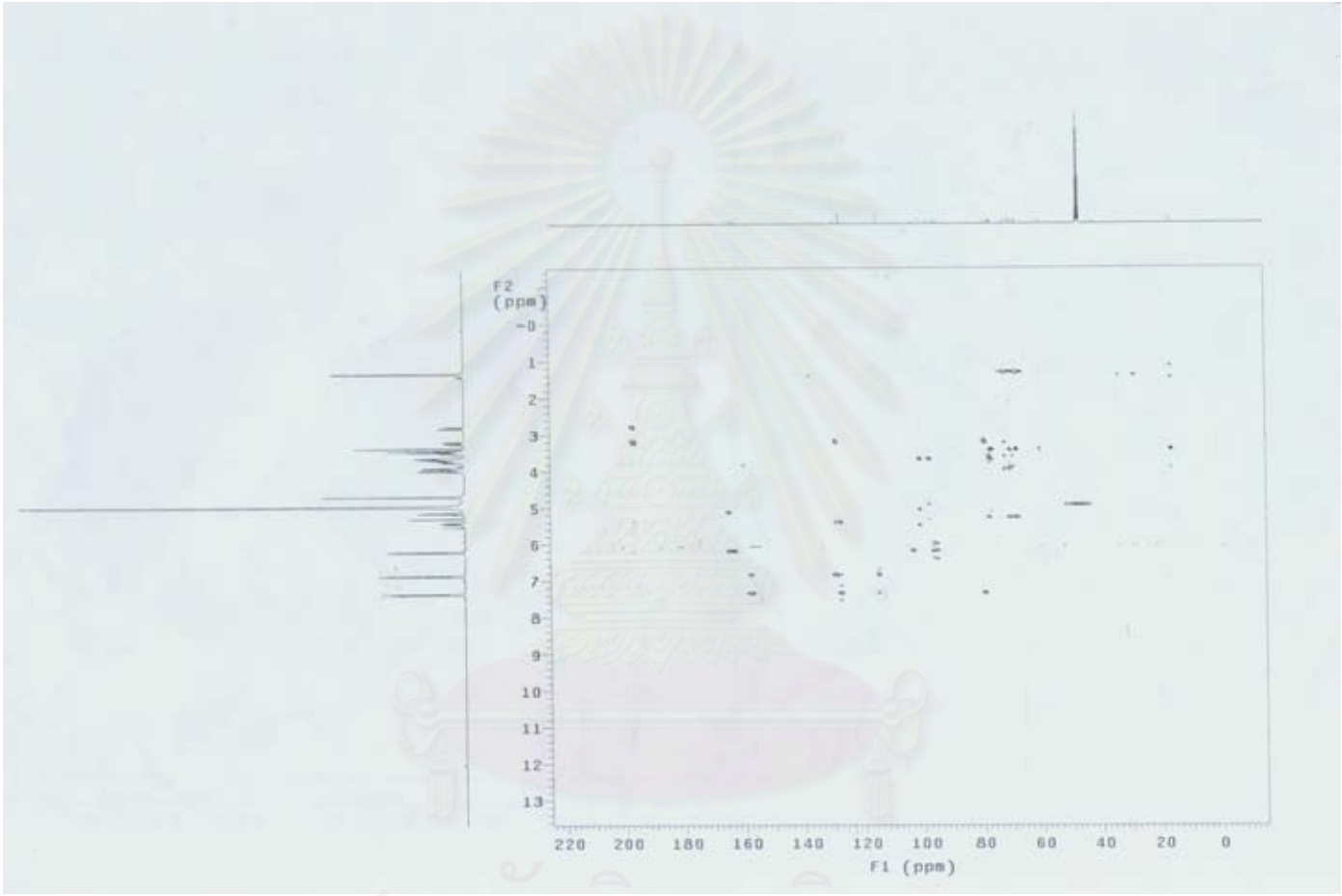

Figure 23. The HMBC-NMR spectrum of Compound **1**

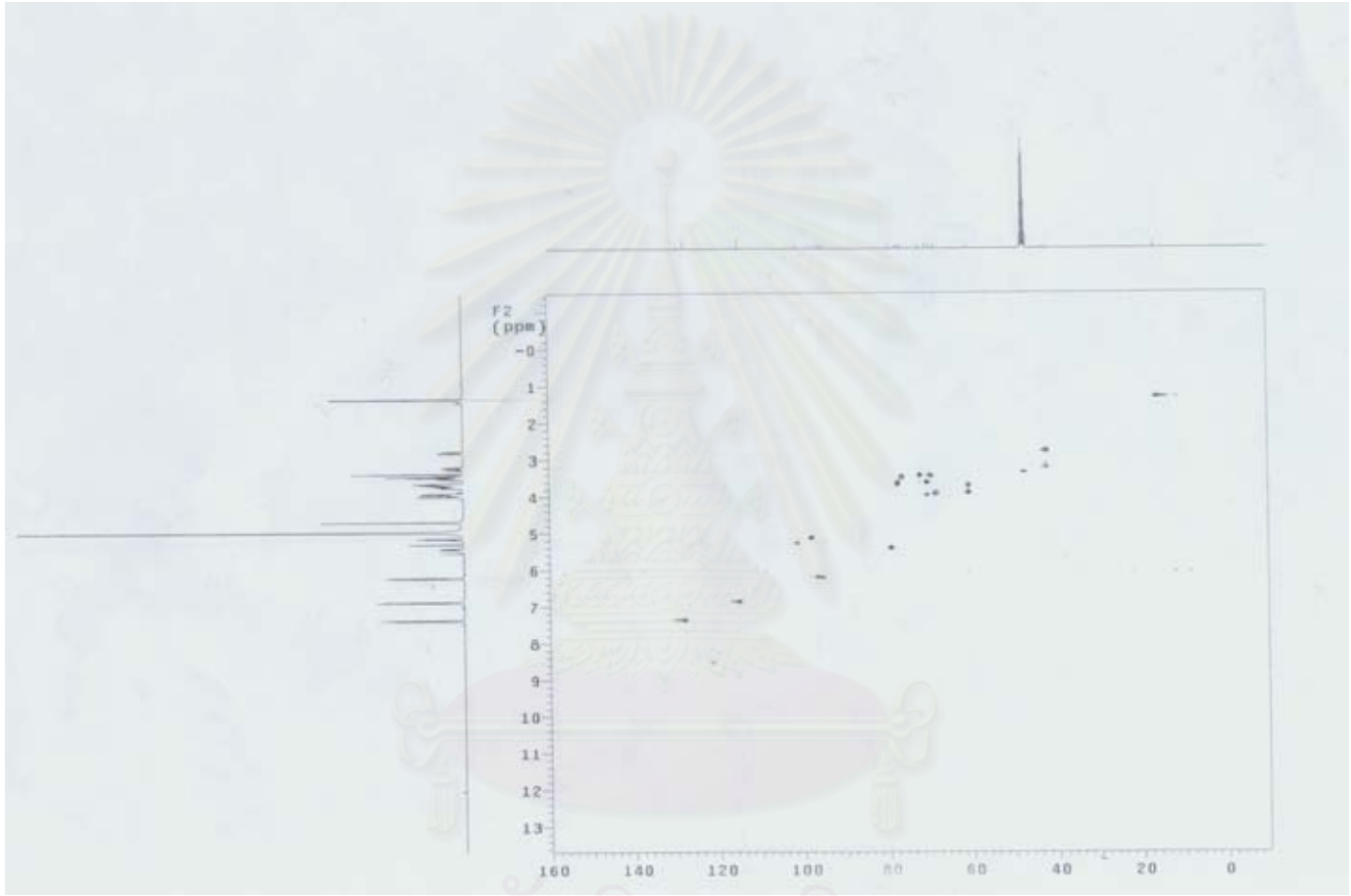

Figure 24. The HMQC-NMR spectrum of Compound **1**

VITA

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