ผลปกป้องของเคอร์คิวมินและแอลฟาโทโคเฟอรอลต่อไลโปโปรตีนชนิดความหนาแน่นต่ำ
ที่ถูกออกซิไดซ์โดยฮีมิน

นาง ปวีณา ยามานนท์
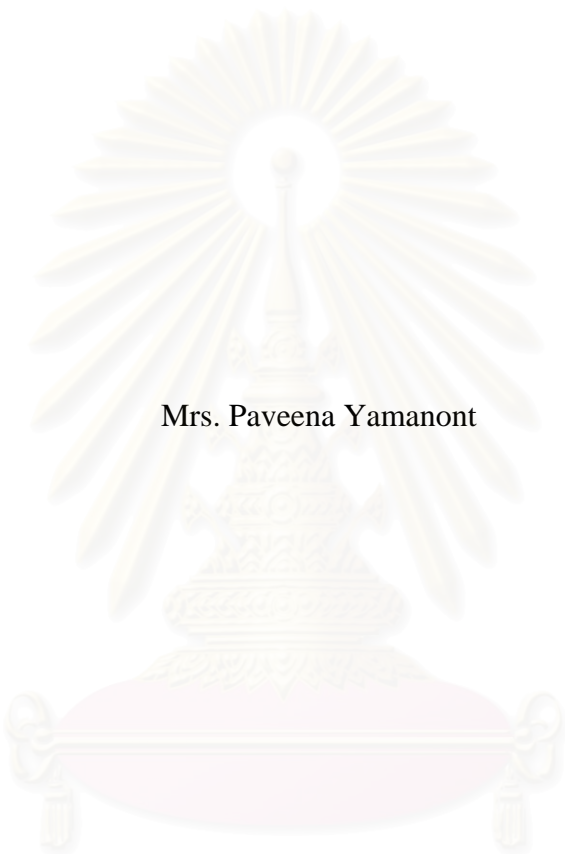
PROTECTIVE EFFECTS OF CURCUMIN AND ALPHA-TOCOPHEROL ON
HEMIN INDUCED LDL OXIDATION

Mrs. Paveena Yamanont

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Pharmacology
Interdisciplinary Program
Graduate School
Chulalongkorn University
Academic Year 2006
ISBN 974-14-2637-2

Thesis Title	PROTECTIVE EFFECTS OF CURCUMIN AND ALPHA-TOCOPHEROL ON HEMIN INDUCED LDL OXIDATION
By	Mrs. Paveena Yamanont
Field of Study	Pharmacology
Thesis Advisor	Rataya Luechapudiporn, Ph.D.
Thesis Co-advisor	Associate Professor Supeenun Unchern, Ph.D.,
	Assistant Professor Noppawan Phumala Morales, Ph.D.

Accepted by the Graduate school, Chulalongkorn University in Partial Fulfillment of the Requirements for Master's Degree

....Dean of the Graduate School

(Assistant Professor M.R. Kalaya Tingsabadh, Ph.D.)

THESIS COMMITTEE

....Chairman

(Assistant Professor Suree Jianmonkol, Ph.D.)

....Thesis Advisor

(Rataya Luechapudiporn, Ph.D.)

....Thesis Co-advisor

(Assistant Professor Noppawan Phumala Morales, Ph.D.)

....Member

(Associate Professor Pol. Lt. Col. Somsong Lawanprasert, Ph.D.)

....Member

(Associate Professor Supeecha Wittayalertpanya. M.Sc.)

ปวีณา ยามานนท์ : ผลปกป้องของเคอร์คิวมินและแอลฟาโทโคเฟอรอลต่อไลโปโปรตีนชนิดความ
หนาแน่นต่ำที่ถูกออกซิไดซ์โดยฮีมิน (PROTECTIVE EFFECTS OF CURCUMIN AND
ALPHA-TOCOPHEROL ON HEMIN INDUCED LDL OXIDAITON)
อ. ที่ปรึกษา: ดร. รัตยา ลือชาพุฒิพร, อ. ที่ปรึกษาร่วม: รศ. ดร. สุภีนันท์ อัญเชิญ,
ผศ. ดร. นพวรรณ ภู่มาลา มอราเลส 95 หน้า ISBN 974-14-3895-8

การเปลี่ยนแปลงของไลโปโปรตีนชนิดความหนาแน่นต่ำ (LDL) โดยปฏิกิริยาออกซิเดชัน มีบทบาท
สำคัญต่อพยาธิกำเนิดของการแข็งตัวของหลอดเลือด ฮีมินเป็นสารที่เกิดขึ้นจากการสลายตัวของฮีโมโกลบิน
จัดเป็นสารออกซิไดซ์ที่พบว่ามีปริมาณมากขึ้นในพยาธิสภาพของโรคบางโรค เช่น hemoglobinopathies,
sickle cell anemia และ thalassemia การศึกษานี้จึงสนใจบทบาทของฮีมินต่อการเปลี่ยนแปลงองค์ประกอบ
ของไขมันใน LDL เมื่อถูกออกซิเดชัน รวมทั้งศึกษาผลปกป้องของเคอร์คิวมินและแอลฟา-โทโคเฟอรอลต่อ
การเกิดออกซิเดชันของ LDL โดยฮีมิน

การศึกษาการเกิดออกซิเดชันของ LDL ในหลอดทดลองนี้ทดลองโดยใช้ฮีมิน (25 ไมโครโมลาร์ต่อ
350 ไมโครกรัมโปรตีน) เหนี่ยวนำให้เกิดปฏิกิริยาออกซิเดชันใน LDL เป็นเวลา 24 ชั่วโมง และการศึกษาผล
ปกป้องของสารด้านออกซิเดชันทดลองโดยเติมเคอร์คิวมินหรือแอลฟา-โทโคเฟอรอลลงใน LDL ก่อน แล้วจึง
เติมฮีมินเพื่อเหนี่ยวนำให้เกิดปฏิกิริยาออกซิเดชันเป็นเวลา 9 ชั่วโมง ผลการทดลองพบว่าฮีมินสามารถ
เหนี่ยวนำให้เกิดการออกซิเดชันของ LDL ส่งผลต่อการเปลี่ยนแปลงองค์ประกอบของไขมัน การลดลงของ
แอลฟา-โทโคเฟอรอลและการเพิ่มขึ้นของระดับ thiobarbituric acid reactive substances (TBARs) โดย
พบความสัมพันธ์แบบผกผันระหว่าง TBARs กับแอลฟา-โทโคเฟอรอล (r=-0.734, p<0.001) และพบว่า
cholesteryl arachidonate (CA) และ cholesteryl linoleate (CL) ลดลง 48.2 % และ 32.3% ตามลำดับ
ขณะที่ cholesteryl esters อื่นๆรวมทั้ง cholesteryl oleate (CO) ไม่เปลี่ยนแปลง อีกทั้งร้อยละของการลดลง
ของ CA และ CL มีความสัมพันธ์กับระดับ TBARs (r=0.837 และ 0.824 ตามลำดับ; p<0.001) นอกจากนี้
อัตราส่วน CL/CO ซึ่งเป็นตัวบ่งชี้การเกิดออกซิเดชันในไลโปโปรตีน พบว่าลดลงและมีความสัมพันธ์แบบ
ผกผันกับระดับ TBARs (r=-0.706, p<0.001) สำหรับการศึกษาผลปกป้องของเคอร์คิวมินและแอลฟา-โท
โคเฟอรอล พบว่าทั้งเคอร์คิวมินและแอลฟา-โทโคเฟอรอลสามารถปกป้องการเกิดปฏิกิริยาออกซิเดชันของ
ไขมันได้ โดยที่ความเข้มข้น 25 ไมโครโมลาร์เคอร์คิวมินมีผลยับยั้งการเพิ่มระดับ TBARs ได้มากกว่า 80%
ขณะที่แอลฟา-โทโค เฟอรอลมีผลยับยั้งได้เพียง 23% นอกจากนี้เคอร์คิวมินมีผลปกป้องการลดลงของ CA,
CL และอัตราส่วน CL/CO ได้ดีกว่าแอลฟา-โทโคเฟอรอล 2-3 เท่า

การศึกษานี้สรุปได้ว่า ฮีมินทำให้เกิดการเปลี่ยนแปลงองค์ประกอบของไขมันใน LDL โดย CA และ
CL มีความไวต่อการถูกออกซิไดซ์ด้วยฮีมิน และเคอร์คิวมินสามารถปกป้องการเกิดออกซิเดชัน ของ LDL ที่
เหนี่ยวนำโดยฮีมินได้ดีกว่าแอลฟา-โทโคเฟอรอล

สาขาวิชา เภสัชวิทยา   ลายมือชื่อนิสิต

ปีการศึกษา 2549   ลายมือชื่ออาจารย์ที่ปรึกษา

                                 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

                                 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

Oxidative modification of low-density lipoprotein (LDL) plays a major role in the pathogenesis of atherosclerosis. Hemin, a degradation product of hemoglobin, is one of oxidative mediator that found to be elevated in pathological cases like severe hemoglobinopathies, sickle cell anemia and thalassemia. This study was interested in oxidative modification of LDL induced by hemin and focusing on the change of lipid composition. The study was also investigated the protective effect of α-tocopherol and curcumin on hemin induced LDL oxidation.

This *in vitro* LDL oxidation study was performed by incubation of LDL with hemin (25 μM/350 μg protein) for 24 hours. The protective effect of antioxidants was also performed by preincubation LDL with curcumin or α-tocopherol and then incubation with hemin for 9 hours. Hemin induced LDL oxidation was shown by the alteration of lipid composition in LDL, the depletion of α-tocopherol and the increasing of thiobarbituric acid reactive substances (TBARs) level. There is an inverse correlation between TBARs level and α-tocopherol (r=-0.734, p<0.001). Cholesteryl arachidonate (CA) and cholesteryl linoleate (CL) were decreased 48.2 % and 32.3% respectively while other cholesteryl esters were not affected including cholesteryl oleate (CO). In addition, the percent decreasing of CA and CL were good correlation with TBARs level (r=0.837 and 0.824 respectively; p<0.001). The CL/CO ratio which is a direct oxidative marker in lipoprotein was also decreased. The CL/CO ratio was an inverse correlation with TBARs level (r=-0.706, p<0.001). Both curcumin and α-tocopherol showed the protective effect on lipid peroxidation. However, curcumin can inhibit TBARs level more potent than α-tocopherol at the dosing of 25 μM/350 μg protein. Curcumin was able to inhibit TBARs level more than 80% at 9 hours incubation while α-tocopherol inhibited only 23%. Curcumin also protects the decreasing of CL and CA, as well as CL/CO approximately 2-3 times more than α-tocopherol.

We concluded that lipid composition of LDL was changed by hemin. CA and CL were susceptible to oxidation in hemin induced LDL oxidation. The protective effect of curcumin against hemin induced LDL oxidation was more potent than α-tocopherol.

Field of Study: Pharmacology Student's Signature

Academic year: 2006 Advisor's Signature

Co-advisor's Signature

Co-advisor's Signature

ACKNOWLEDGEMENTS

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAPH	2,´2-azobis 2-amidinopropane hydrochloride
ADP	Adenosine 5´ diphosphate
α-tocopherol	Alpha-tocopherol
Apo	Apoprotein
BHT	butylated hydroxytoluene
β-thalassemia/hemoglobin E	β-thal/HbE
CA	Cholesteryl arachidonate
CE	Cholesteryl ester
CL	Cholesteryl linoleate
CLN	Cholesteryl linolenate
CO	Cholesteryl oleate
CP	Cholesteryl palmitate
FC	Free cholesterol
g	Gram
hr	Hour
he-oxLDL	Hemin oxidized LDL
HPLC	High performance liquid chromatography
LDL	Low-density lipoprotein
MDA	Malondialdeyde
μg	Microgram
μl	Microliter
μM	Micromolar
μmole	Micromole
min	Minute
mg	Milligram
ml	Milliter
mM	Millimolar
nm	Nanometer
nmole	Nanomole
Ox-LDL	Oxidized LDL

PBS	Phosphate buffered saline
PUFA	Polyunsaturated fatty acid
rpm	Revolution per minute
S.E.M.	Standard error of mean
TBARs	Thiobarbituric acid reactive substances
TC	Total cholesterol

CHAPTER I
INTRODUCTION

1. Background and rationale

Oxidative modification of low-density lipoprotein (LDL) was established that it plays a major role in atherosclerosis (1). Oxidative modification dramatically changes the biologic properties of low density lipoprotein (LDL), converting them into a form that is both atherogenic and pro-thrombotic (1, 2). Transition metals like iron and most notably copper were found to induce LDL oxidation. As a result, in most in vitro studies of LDL oxidative modification, copper was used as the trigger of oxidation. More recently, with the understanding that both iron and copper are tightly bound to proteins in the plasma and therefore they are unavailable in free forms in vivo. Other oxidative mediators which may endogenously reside in the vascular system were employed to study LDL oxidation.

Hemin (iron (III)-protoporphyrin IX) is a degradation product of hemoglobin that may derive from the hemoglobin in circulating erythrocytes. Hemin was found to be elevated in pathological cases like severe hemoglobinopathies, sickle cell anemia and thalassemia, which their globins tend to release hemin (3, 4). The study of hemin distribution showed that as much as 80% of total hemin bound initially to LDL and HDL, the plasma components which are the most susceptible to oxidation. Therefore, hemin may serve as an important pro-oxidant capable of promoting oxidative modification of LDL in vivo (5). Thalassemia is a pathological condition that has a high level of hemin in plasma. Normally hemin is undetectable in healthy subjects. Interestingly, Phumala et al. (6) found that the levels of hemin in plasma of β-thalassemia/hemoglobin E (β-thal/HbE) subjects were 50-280 μM. Moreover, the increasing level of hemin was readily catalyzed free radical in plasma, suggesting that hemin was a major oxidant in β-thal/HbE. Hemin is a powerful in vitro inducer of LDL oxidation. The exposure of LDL to hemin facilitates Fe^{3+} mediated oxidation of both its lipid and apolipoprotein components (7). Moreover, the LDL oxidation by hemin, producing a variety of lipid oxidation products of LDL and apo B cross linking, turns the LDL recognizable by the macrophage scavenger receptor. Consequently, formation of macrophage-derived foam cells occurs (8-10).

One of the initial events in oxidative damage to LDL is the peroxidation of lipids, particularly cholesteryl esters (CEs), phospholipids and triglycerides, which

comprise the bulk of polyunsaturated fatty acids (PUFAs) (11). In the study of Luechapudiporn (12), the level of cholesteryl linoleate (CL) and the ratio of CL to cholesteryl oleate (CO) were lowered in the β-thal/HbE. In addition, cholesteryl linoleate hydroperoxides have been found in human atherosclerotic lesions (13-15), it indicated that CEs in human LDL are the targets of oxidative damage in lipoproteins (15). Thereby, this study is interested in CEs as the targets of LDL oxidation by hemin. Beside atherogenic properties of oxidized LDL (Ox-LDL), it also interacts with platelets then stimulating their function included platelet aggregation (16). Thereby, this study also investigates effect of hemin oxidized LDL (he-oxLDL) on platelet aggregation in platelet rich plasma (PRP).

The protection of LDL oxidation by supplementation with antioxidants became an attractive therapeutic strategy to prevent and possible to treat atherosclerosis. The several studies showed protective effect of vitamin E or alpha-tocopherol (α-tocopherol) and curcumin on LDL oxidation. Vitamin E is the principal lipid-soluble antioxidant in human plasma and lipoproteins (17). It is a chain-breaking antioxidant. Raising the concentration of vitamin E in LDL increased the resistance to *in vitro* oxidation of LDL by cupric ions (18). In the study of Unchern et al. (19), thalassemic patients, who were supplemented with a daily dose of vitamin E (525 IU) for 3 months, resulted in a significant increase in plasma α-tocopherol level and reduction in plasma thiobarbituric acid reaction substances (TBARs). It indicated that vitamin E has been able to protect lipid peroxidation.

Curcumin is the major curcuminoid found in turmeric (*Curcumin longa*) which is commonly used as a spice and food colorant (20). Curcumin has been shown to exhibit a variety of biological activities including antioxidative activity (21-27). Curcumin is an effective scavenger of reactive oxygen species in the *in vitro* study (28,29). Wei-Feng Chen et al. (30) demonstrated that curcumin and some of its analogues are effective antioxidants against AAPH- and Cu^{2+}-induced LDL peroxidation, and the H-atom abstraction from the phenolic group is responsible for the activity. The previous study indicated that curcumin has protective effect on LDL oxidation however the protective effect of curcumin on LDL oxidation induced by hemin has not been investigated.

Thereby, this study has been interested in hemin induced LDL oxidation by focusing on the lipid composition and the effect of hemin oxidized LDL (he-oxLDL) on platelet aggregation. In addition, the study compares the antioxidative effect of curcumin and α-tocohoerol on hemin induced LDL oxidation.

2. Objective

2.1 To study the effect of antioxidant, α-tocopherol and curcumin, on hemin induced LDL oxidation.

2.2 To study the effect of he-oxLDL on platelet aggregation in platelet rich plasma.

3. Hypothesis

Curcumin and α-tocopherol are able to protect LDL oxidation by hemin. Hemin oxidized LDL (he-oxLDL) enhances adenosine 5´ diphosphate to induced platelet aggregation.

4. Expected Benefit and Application

The results can be used for consideration of using curcumin to protect LDL oxidation in patient who was found hemin elevated in blood circulation.

CHAPTER II
LITERATURE REVIEW

1. Low density lipoprotein

Low density lipoprotein (LDL) is one class of lipoproteins which has density range 1.006-1.063 g/ml. LDL is a spherical molecule with diameter in the range of 200-250 A°. (figure 2.1). LDL is the major carriers of cholesterol in the human circulation. Their physiological function is to carry cholesterol to the cells. LDL forms a heterogeneous group of particles varying greatly in size, composition and structure.

Figure 2.1 Structure of LDL

The core of LDL consist about 170 triglyceride (TG) and 1600 cholesteryl ester (CE) molecules. The surface monolayer comprises about 700 phospholipid molecules and a single copy of apo B-100. In addition, the particles contain about 600 molecules of free cholesterol, of which about one-third is located in the core and two-thirds in the surface. Cholesteryl esters (CEs) are most abundant in core of LDL. The structure of CE consist part of cholesterol and part of fatty acid such as arachidonic acid, linoleic

acid, oleic acid, linking with ester bound (figure 2.2). In addition to lipid, LDL also carries lipophilic antioxidants, such as α-tocopherol (about 6 molecules/LDL particle) and a minute amounts of carotenoids, oxycarotenoids and ubiquinol-10. The particles are in a dynamic state, their structure and physical properties being dependent on their lipid composition as well as on the conformation of apo B-100 (31).
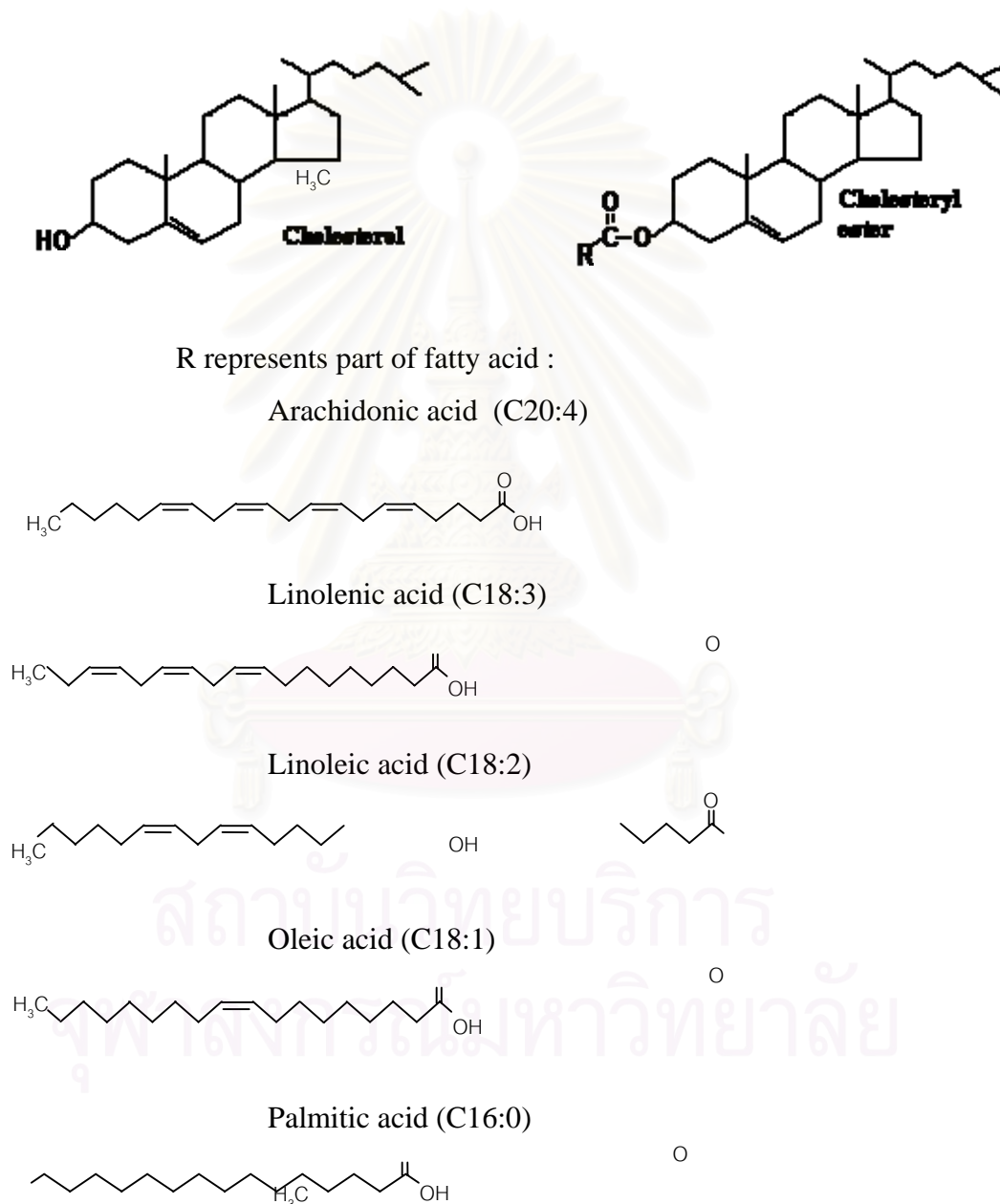
Figure 2.2 Chemical structure of cholesterol, cholesteryl ester and fatty acid

LDL contains a large proportion of the cholesterol presented in the plasma of adult humans and elevated concentrations are considered to be a major factor in the development of premature atherosclerosis. LDL is derived from VLDL. VLDL is synthesized in the liver and is converted by the action of lipoprotein lipase, situated on the blood capillary walls of adipose tissue, muscle, and other organs, to form intermediate density lipoproteins (IDL). A large part of IDL, about half in the case of humans, is normally processed further to form LDL. The conversion of VLDL into LDL results in the loss of triglyceride, phospholipids, and apolipoproteins other than apo B100 which remains as the major apolipoprotein in LDL particles. LDL is cleared from the plasma by a regulated pathway involving endocytosis following binding to apo B or apo E receptors presented in the liver and other tissues (32).

2. Oxidized low density lipoprotein

LDL is involved in the genesis of atherosclerosis. As oxidized LDL (Ox-LDL) is present in atherosclerotic areas, it is suggested that LDL oxidation may occur *in vivo* in the vascular wall (11). Ox-LDL exhibit a variety of biological properties potentially involved in atherogenesis, such as induction of adhesion protein expression and subsequent entry of mononuclear cells, formation of foam cells and fatty streak, induction of smooth muscle cell migration and proliferation, changes in the extracellular matrix, alterations of coagulation pathways and disturbance in the arterial tone regulation (11, 33). *In vitro* studies have shown that chemically modified LDL and biologically modified LDL, by incubation in the presence of endothelial cells, are taken up through a system that is insensitive to control by scavenger receptors on macrophages. This biological or chemical modification is believed to involve the oxidative breakdown of LDL cholesteryl ester, phospholipids and apo B100; since foam cells found in early atherogenic lesions. This process provides an intriguing hypothesis for the underlying cause of atherogenesis (32). The morphological changes of cultured endothelial cells associated with Ox-LDL toxicity are similar to those observed *in vivo* on endothelial cells covering atherosclerotic areas (5).

The lipid and protein component of oxidized LDL differ substantially from those of the native LDL. The characteristics of oxidized LDL include the depletion of

polyunsaturatied fatty acid and antioxidants (18), containing massive amounts of lipid peroxides and their degradation products, and numerous proteolytic fragments derived from apoB-100 (34). Substantial evidence suggested that most of the atherogenic effects of oxidized LDL be derived from the oxidized lipid components. The active lipids include both esterified and unesterified peroxidized lipid, lysophosphatidylcholine cholesterol oxidation products, aldehydes derived from breakdown of oxidized fatty acid, and perhaps proteolipids that may have peroxidized lipids bound to fragmented apoB-100 (35).

2.1 Mechanisms of LDL Oxidation (36)

Much data has been accrued supporting a proatherogenic role for Ox-LDL. Although LDL can be oxidatively modified by various mechanisms, there is no consensus on the predominant mechanism of LDL oxidation *in vivo*. LDL can be oxidatively modified in a cell-free system by transition metals, such as iron and copper, and also by all the major cells of the arterial wall, namely endothelial cells, smooth muscle cells, and monocyte-macrophages. Physiologically relevant mechanisms for in vivo oxidation of LDL are not well understood. Superoxide anion can promote oxidation of LDL lipids mediated by smooth muscle cells and phagocytes. Also, activated human neutrophils and monocytes can oxidize LDL via a pathway that is inhibited by superoxide dismutase and metal chelators. Thiols can also oxidize in the presence of metal ions, forming thiyl radicals and superoxide and thereby promoting LDL oxidation. In addition, certain cellular enzymes, such as 15-lipoxygenase (15-LO), that convert polyunsaturated fatty acids to lipid hydroperoxides can also oxidize LDL. Although some groups question the role of 15-LO in LDL oxidation, mRNA and protein of 15-LO have been found in atherosclerotic lesions. Myeloperoxidase, another enzyme, is a heme protein secreted by activated phagocytes and may also oxidize LDL by acting as a physiological catalyst. Hypochlorous acid and tyrosyl radical, products of myeloperoxidase action, are also known to promote LDL oxidation. Nitric oxide and peroxynitrite produced by the endothelial cells and macrophages are other oxidants relevant to LDL oxidation. It has been shown that peroxynitrite can result in the formation of modified LDL.

In *in vitro* systems, LDL can be oxidatively modified in the presence of transition metal ions, such as iron and copper, to a form that is physicochemically and biologically indistinguishable from LDL oxidized by a cellular system. LDL molecules can bind copper; the new molecule can promote rapid lipid peroxidation in vitro. Also, LDL can be oxidized in a metal-independent system with ˙2,2-azobis 2-amidinopropane hydrochloride (AAPH), a water-soluble azo compound that thermally decomposes producing aqueous peroxy radicals at a constant rate. The oxidizability of LDL also depends on its size. Subjects with a preponderance of small, dense LDL exhibit increased risk for coronary artery disease. Studies from numerous laboratories have shown that small, dense LDL is more susceptible to oxidation than large, more buoyant LDL.

2.2 Oxidative Modification of LDL (36)

Oxidation of LDL is a free-radical-mediated process that results in numerous structural and functional changes. The initiation of LDL oxidation occurs by the peroxidation of lipid especially the polyunsaturated fatty acids (PUFA) in LDL (figure2.4). Oxidation of LDL is initiated by reactive oxygen species that abstract an H˙ from a double bond in PUFA, followed by molecular rearrangement that leads to the formation of conjugated double bonds that are commonly referred to as conjugated dienes (CD). During this process, the rate of oxidation is dependent on endogenous antioxidants in LDL, accounting for the lag phase of oxidation. The lag phase is followed by a rapid propagation phase that occurs after depletion of endogenous antioxidants and involves abstraction of another H˙ by a PUFA-peroxyl radical (LOO˙) from another PUFA, resulting in the formation of lipid peroxides. Cholesterol in LDL can be oxidized to oxysterols, such as 7-keto cholesterol. The propagation phase is followed by a decomposition or degradation phase, in which there is cleavage of double bonds, resulting in the formation of aldehydes, such as malondialdehyde (MDA), 4-hydroxynonenal (HNE), and hexanal, those can crosslink with amino groups on apo B-100. Changes in the protein moiety of LDL also occur during oxidation. Oxidation is followed by an increase in the negative charge on the LDL, possibly due to derivatization of positively charged amino groups through the formation of schiff base with aldehydes. Also, following oxidation, the apo B-100

undergoes oxidative scission, leading to fragmentation (figure 2.3). Since MDA is a product of lipid peroxidation, MDA assay have been widely used as a measure of lipid peroxidation. A Thiobarbituric acid reactive substance (TBARs) is a technique of MDA assay. MDA form a 1:2 adduct with thiobarbituric acid and produces the end product which can be measured by fluorometry or spectrophotometry (figure 2.4).

Oxidation of apo B-100   Oxidation of unsaturated fatty acid side-shains
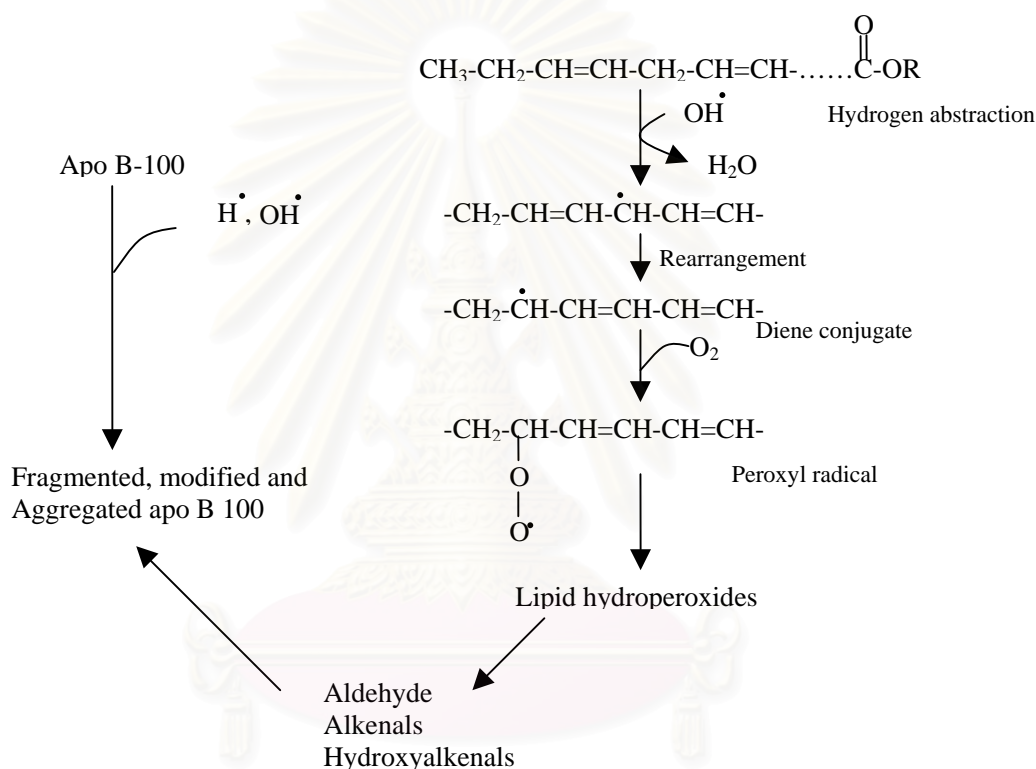
Figure 2.3 Oxidative damage to LDL affects both its lipid and apoprotein components. The dot (•) represents an unpaired electron, introduced on a reactive hydroxyl radical (OH•).
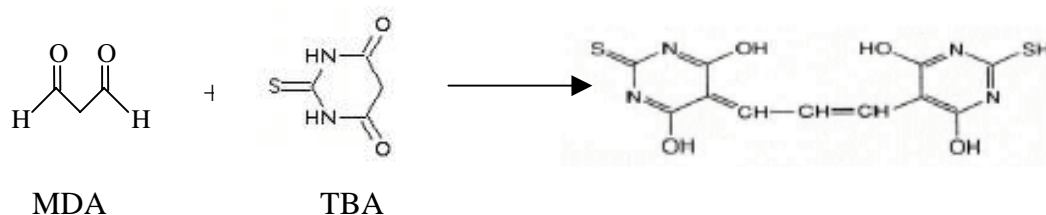
Figure 2.4 Thiobarbituric acid reactive substances

2.3 Biologic effects and in vivo existence of oxidized low-density lipoprotein (36)

Oxidized LDL exerts several biologic effects that contribute to the evolution of the atherosclerotic lesion. During oxidation minimally modified LDL (MM-LDL) is initially formed in the subendothelial space. Minimally modified LDL is typified by mild lipid peroxidation and uptake by the classical LDL receptor; it can induce monocyte-endothelial adhesion and secretion of monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF) promotes their differentiation into macrophages. Macrophages in turn oxidize MM-LDL into a more oxidized form.

Oxidized LDL is no longer recognized by the LDL receptor but is taken up by the scavenger receptor on the macrophages, resulting in foam cell formation. It is a potent chemoattractant for monocytes and inhibits macrophage motility, thereby promoting retention of macrophages in the arterial wall. Oxidized LDL is cytotoxic and promotes endothelial dysfunction and evolution of the fatty streak into a more advanced lesion; it can also promote atherogenesis by altering expression of genes in the arterial wall. Oxidized LDL can adversely affect coagulation by inducing tissue factor and plasminogen activator inhibitor-1 synthesis. Products of Ox-LDL have been shown to impair gene expression of tumor necrosis factor and platelet-derived growth factor. Oxidized LDL inhibits endothelium-dependent vasodilation by inhibiting endothelium-derived relaxation factor (EDRF). Another atherogenic property of Ox-LDL is its immunogenicity. Malondialdehyde-modified LDL has been shown to stimulate formation of autoantibodies and immune complexes of LDL aggregates that are efficiently internalized by macrophages by Fc receptors, thus promoting further cholesterol accumulation. Ox-LDL stimulates interleukin-1 (IL-1) release from macrophages. Interleukin-1 has been shown to induce smooth muscle cell proliferation and monocyte-endothelial cell adhesion. Also, IL-1b mRNA has been found to be at higher levels in atherosclerotic lesions (36). In addition, the *in vitro* Ox-LDL by hypochlorous acid produces a modified form (HOCL-LDL) that capable of stimulating platelet function. It is highly effective at inducing platelet function, causing stable aggregation and α-granule secretion. Such stimulation depended on the presence of low levels of primary agonists such as adenosine

diphosphate (ADP) and thrombin, or others like epinephrine (EPI) and macrophage-derived chemokine (MDC, CCL22) (37).

3. Hemin

Hemin, iron (III)-protoporphyrin IX, is the oxidation product of heme (iorn (II)-protoporphyrin IX) (figure 2.5). It degrades from hemoglobin during intravascular hemolysis under pathological conditions (3, 4). Hemin has cytotoxic effects to various cells (38). In the study of Miller and Shaklai (5), most of hemin in plasma binds initially to LDL and HDL and then hemin was transfer to albumin and hemopexin. The half time of the hemin-LDL complex in plasma, initially comprising 27% of total hemin, was more than 20 seconds. In addition, they suggested that the hemin-LDL complex may exist in vivo and its oxidative potential should be considered pro-atherogenic.

The largest repository of heme in the human body is in red blood cells, which have a life span of about 120 days. There is a turnover of about 6 g/day of hemoglobin which presents 2 problems. First, the porphyrin ring is hydrophobic and must be solubilized to be excreted. Second, iron must be conserved for new heme synthesis. Normally, senescent red blood cells and heme from other sources are engulfed by cells of the reticuloendothelial system. The globin is recycled or converted into amino acids, which in turn are recycled or catabolized as required.
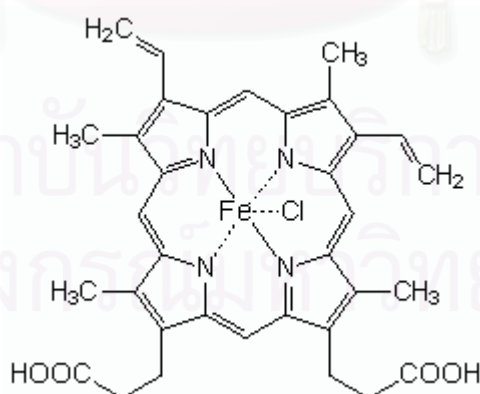
Figure 2.5 Structure of hemin

Heme is oxidized, with the heme ring being opened by the endoplasmic reticulum enzyme, heme oxygenase. The oxidation step requires heme as a substrate, and any hemin (Fe^{3+}) is reduced to heme (Fe^{2+}) prior to oxidation by heme oxygenase. The oxidation occurs on a specific carbon producing the linear tetrapyrole biliverdin,

ferric iron (Fe^{3+}), and carbon monoxide (CO). This is the only reaction in the body that is known to produce CO. Most of the CO is excreted through the lungs, with the result that the CO content of expired air is a direct measure of the activity of heme oxygenase in individual. In the next reaction a second bridging methylene (between rings III and IV) is reduced by biliverdin reductase, producing bilirubin. Peripherally arising bilirubin is transported to the liver in association with albumin, where the remaining catabolic reactions take place.

4. Platelet (39)

4.1 Morphology

Blood platelets are formed from megakaryocytes in bone marrow. The physiological platelet count in peripheral blood lies between 150,000 and 300,000 per µL blood. Platelets are the smallest corpuscular component of circulating blood and have a diameter of 2-4 µM. It do not have nucleus. The physiological lifetime of platelets in the peripheral blood stream amounts about 7 days with a daily renewal rate of about 20% of the total platelets count. Degradation of the platelet occurs in the reticulo-endothelial system of the liver and spleen. In the non-activated state platelets have a typical discoid shape with and average surface area of μm^2. Activation of the platelets by soluble agonists such as ADP or thrombin or by adhesion leads to a shape change with formation of pseudopods.

The ultrastructure of platelets can be divided into four morphological regions: the peripheral zone, the structural zone, the zone of the organelles, and the membrane system. The peripheral zone consists of the cytoplasmic membrane that is covered on its extracellular side by a thin layer composed of various glycoproteins, proteins, and mucopolysaccharides (glycocalix). The structural zone consists of microtubule located in the submucosa that represent tubulin threads and are surrounded by a network of other, widely differing structural proteins. The components of the structural zone serve to maintain the typical discoid shape of resting platelets and actively participate in the shape changes of activated platelets. The zone of the organelles is found in the cytoplasm and consists of mitochondria, glycogen stores and three different forms of storage granules: dense bodies, α-granules, and

lysosomes. The granules are characteristic for platelets and serve as storage sites for proteins and other substances essential for platelet function. The fourth morphological zone is the membrane system. It consists of the surface-connected open canalicular system (SCS) and the dense tubular system (DTS). The open canalicular system is characterized by tortuous canals reaching from their connection with the plasma membrane far inside the platelet and accessible from the extracellular space by pores. The dense tubular system is deriving form the rough endoplasmic reticulum of the megakaryocytes. It is one of the main storage sites for free calcium ions (Ca^{2+}) which play a major role in the regulation of platelet metabolism and activation.

4.2 Platelet adhesion

Adhesion of still resting platelets to the damaged vessel wall is the first step of primary hemostasis and is known as primary adhesion. Attachment of already activated platelets to structures of the subendothelium is known as secondary adhesion. Primary adhesion leads to shape change, spreading or rolling, and activation of the adhering platelets with subsequent secretion of the granule components and formation of aggregates.

The adhesion process is regulated by glycoproteins of the platelet membrane. Platelets possess membrane-bound, adhesion receptors that recognize specific structural components of the extracellular matrix in the region of the subendothelium and media. The extracellular matrix is made up of a network of various components such as collagen and elastic fibrillae that are embedded in a gel of proteoglycans, glycoproteins, and water. Fibronectin and laminin are the main components of the non-collagenic adhesion proteins occurring in the extracellular matrix. Also present are thrombospondin mainly in the proliferative tissue and von Willebrand factor (vWF) which is secreted by the endothelium, a smaller portion of this factor adheres to collagen fibrillae in the surface layers of the subendothelium while the major portion is released into the plasma compartment. The interaction of vWF with platelet receptor for von Willebrand factor (glycoprotein Ib-V-IX) is characterized by a very high affinity that makes the adhesion of the platelets to the vessel wall possible, especially in regions with high shear forces (arterial flow regions). A stabilization of the platelet adhesion proceeds via further membrane adhesion receptors (collagen,

fibro-nectin, and laminin receptors) that are assigned to the receptor family of the integrins.

The binding of the platelet collagen receptor to collagen, in particular, leads to activation and shape changes of the adherent platelets. During the shape change, pseudopods are formed which support and effective sealing of the vessel wall lesion. The final stage of adhesion is reached when the thrombocytes are complately distributed over the subendothelium and seal the endothelial lesion from the blood flow. Besides aggregation, glycoprotein IIb-IIIa plays a major role in the spreading process.

4.3 Platelet activation

Besides adhesion processes, morphological and functional changes in platelets are induced by soluble agonists such as ADP or Thrombin; this is known as activation. A primary, hemostatic clot can only form completely after activation of the platelets. The activation process is complex and regulated by changes in metabolic and biochemical mechanisms, shape changes, activation of surface receptors, and changes in the orientation of membrane phospholipids.

Many substances can activate platelets and induce them to undergo metabolic changes. The platelets can form or release a part of these activators (agonists) themselves (autocrine); the other part is formed in the surrounding tissue or plasma. Each agonist binds at its specific receptor on the platelet surface and effects the formation of signal factors (second messengers) via signal transduction pathways. These second messengers then induce a series of metabolic intracellular changes. Three enzyme complexes play a major part in the formation of second messenger: phospholipase C, phospholipase A_2, and adenylate cyclase. Each of these enzymes also participates in the regulation of free, intracellular Ca^{2+}.

When the intracellular Ca^{2+} concentration exceeds a specific threshold platelets undergo a shape change with formation of pseudopods. This shape change requires alterations in the structural zone with enrichment of microtubules in the pseudopods. At the same time, G-actin polymerizes and forms F-actin filaments that are associated with other membrane proteins such as the actin-binding protein. This stabilizes the plasma membrane of the poeudopods. In addition, F-actin binds to

myosin (actomyosin). Similar to the situation in smooth muscles, actomyosin forms a contractile apparatus that guarantees the dynamic shape change of the activated platelets. The shape change results in an increase in surface area of the platelets which then makes an increased interaction with plasmic reactions possible.

The aggregation receptors of GPIIb-IIIa are stimulated by signal transduction from the activated platelets. On the surface of resting platelets GPIIb-IIIa forms a complex that cannot bind plasmic fibrinogen. Upon activation of the platelets this receptor undergoes a shape change with formation of "fibrinogen pouches" that control the binding of fibrinogen to GPIIb-IIIa and trigger the aggregation. In the course of the structural changes of the platelets, the surface density of GPIIb-IIIa molecules is additionally increased by release of internal stores of fibrinogen receptors.

Changes in the orientation of phospholipids in the civility of the plasma membrane allow the association of coagulation factors and the formation of a catalytic prothrombinase complex on the activated surface. This leads to the increased formation of thrombin in the region of a platelet aggregate and thus to a consolidation of the hemostatic clot through fibrin cross links.

4.4 Platelet aggregation

Platelets are activated during aggregation and release granule components. This stimulates further platelets to undergo aggregation. Aggregation is defined as the process of co-adhesion between two platelets. Soluble agonists such as ADP or TxA2 bind to activation and shape change of the platelets via signal transduction pathways. Two phases of aggregation are distinguished: primary and secondary aggregation. During the primary phase the platelets are loosely linked to each other by "fibrinogen bridges." This process is reversible. Secondary aggregation sets in after a time lag and begins when the platelets have released granule components. In patients with defects in storage granules (storage pool deficiency) the secondary aggregation phase is reduced or completely absent so that there is a tendency for increased bleeding. Three conditions are decisive for a normal aggregation process: shear forces, Ca^{2+}, and fibrinogen. When one of these three components is lacking platelets cannot aggregate. Normal plasma contains sufficient Ca^{2+} and fibrinogen for aggregation. Both Ca^{2+}

and fibrinogen are additionally stored in high concentrations in platelet granules and are released into the surroundings of the growing platelet aggregate during aggregation.

The glycoprotein IIb-IIIa complex plays a central role in aggregation. In the resting state, soluble plasma fibrinogen cannot bind to the platelet surface. Binding sites for fibrinogen in the region of the glycoprotein IIb-IIIa complex only become accessible after activation of the platelets. The binding of GPIIb-IIIa is strongly dependent on Ca^{2+} and does not occur in the absence of divalent cations. A platelet microaggregate is formed through fibrinogen bridges between two adjacent platelets.

In summary, in the non-activated state platelets have a typical discoid shape. Activation of the platelets by soluble agonists such as ADP or Thrombin or by adhesion leads to a shape change with formation of pseudopods. Primary adhesion leads to shape change, spreading or rolling, and activation of the adhering platelets with subsequent secretion of the granule components and formation of aggregates. (Figure 2.6)
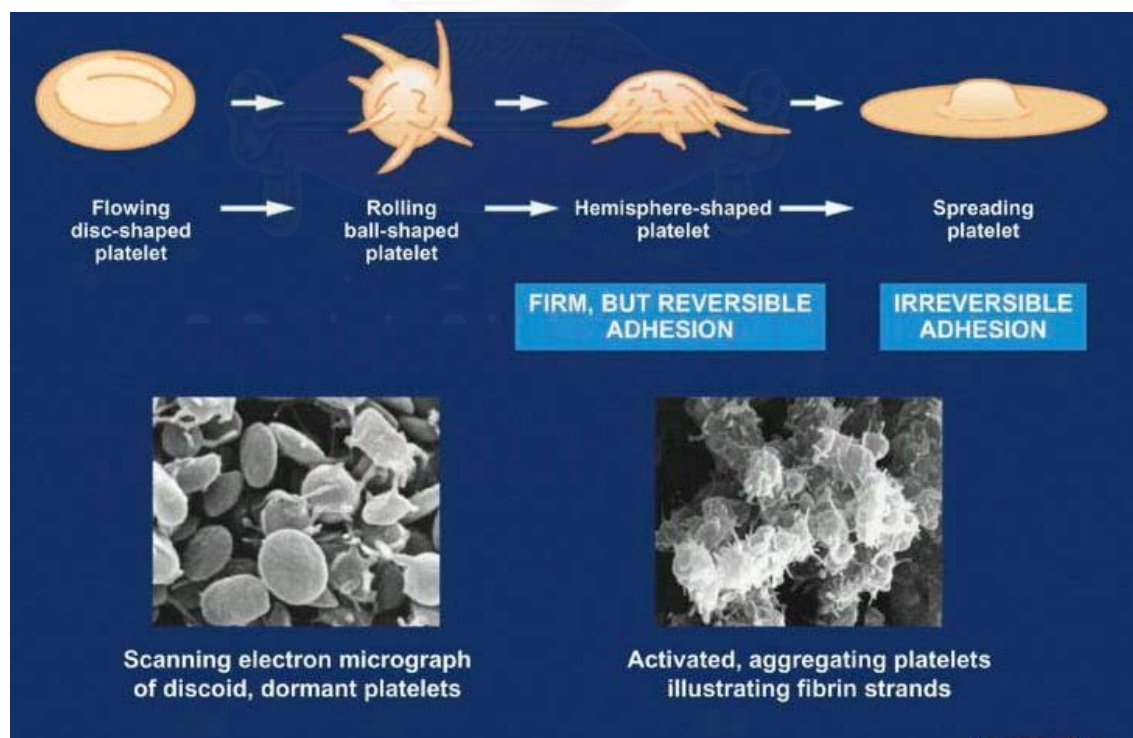
Figure 2.6 Platelet shape change on adhesion, activated and aggregation (40)

5. α-Tocopherol

α-Tocopherol is the most studied antioxidant for the lipid phase. It functions by converting chain-carrying alkylperoxyl radicals to hydroperoxides and simultaneously, being converted to tocopheroxyl radicals. The structure of α-tocopherol is shown in figure 2.7

Vitamin E (α-tocopherol)
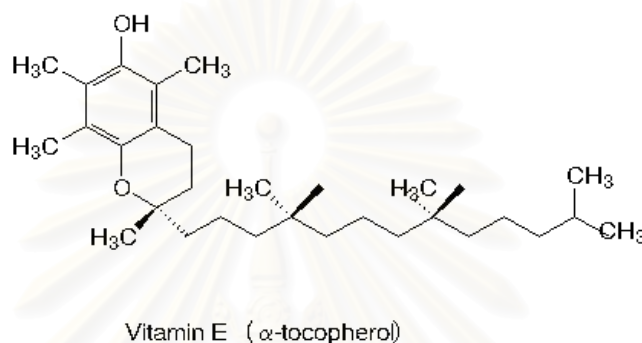
Figure 2.7 Structure of α-tocopherol

The resulting tocopheroxyl radicals are, at least in part, reduced by ascorbic acid back to the antioxidant tocopherol. The antioxidant mechanism of α-tocopherol cooperated with several antioxidant such as vitamin C and glutathione (41). A two-electron redox cycle involving α-tocopherol oxidation to 8α-substituted tocopherones followed by tocopherone reduction to α-tocopherol occurs, but would require enzymatic catalysis in vivo. Metabolism of antioxidant-inactive α-tocopheryl esters releases α-tocopherol, whereas reductive metabolism of α-tocopherylquinone, an α-tocopherol oxidation product, yields α-tocopherylhydroquinone, which also may provide antioxidant protection (42).

5.1 Absorption and transport (43)

Absorption of α-tocopherol is dependent on an individual's ability to absorb fat. Tocopherol is absorbed into the lymphatic system from the intestinal tract and enters the blood as a component of the chylomicrons. The majority of α-tocopherol in plasma is in low density lipoproteins (LDL). Alpha-tocopherol accounts for almost 87% of the total tocopherol concentration in adult human plasma. There is a high correlation between serum tocopherol and total lipid concentrations. Vitamin E levels are most frequently measured in plasma and range from 0.5 to 1.6 mg/dL in normal populations.

5.2 Sources (43)

The richest dietary sources of vitamin E are vegetable oils (primarily soybean, sunflower, and corn oils), nuts, and sunflower seeds. Whole grains and wheat germ are also major sources. Foods of animal origin are generally low in vitamin E. The amount of vitamin E (as d-α-tocopherol) supplied by the normal U.S. diet is estimated to be 7.4-9 mg/day (11.0-13.4 IU).

Vitamin E is the exception to the paradigm that synthetic and natural vitamins are equivalent because their molecular structures are identical. Natural-source vitamin E (R-R-R-alpha-tocopherol), derived from vegetable oils, is a single stereoisomer. Synthetic vitamin E (all-rac-α-tocopherol, also known as dl-α-tocopherol) is a mixture of eight stereoisomers, only one of which is d-α-tocopherol. The other seven isomers have different molecular configurations, all with lower biological activity than d-α-tocopherol.

Results of animal bioassays and human studies have demonstrated that the biological potency of natural forms of vitamin E is higher than that from synthetic sources. Research data also suggest that the lungs, red blood cells, blood plasma, and brain demonstrate preferential uptake of natural-source vitamin E compared to one of the isomers in synthetic vitamin E. Physiological difference between natural and synthetic vitamin E relate to the preferential retention of d-α-tocopherol in blood and tissues compared to other tocopherols.

5.3 Vitamin E or α-tocopherol on LDL oxidation (43)

It has been well established that cholesterol deposited in arteries originates primarily from LDL, and that elevated LDL levels are associated with an increased risk for atherosclerosis. In an early stage in development of atherosclerosis, there is accumulation in the arteries of foam cells, which are macrophages that have taken up oxidized LDL. These foam cells are a key component of the fatty streak lesion. LDL is an important target of free radicals, and LDL oxidation is considered to be an important event in development of atherosclerosis. Cell and animal data support the hypothesis that oxidative modification of LDL leads to their enhanced uptake by macrophages, leading to conversion of macrophages into foam cells, and that antioxidants may be protective against LDL oxidation.

In isolated-cell studies, cell-mediated oxidation of LDL was largely prevented over 24 hr when high quantities of vitamin E were added to the culture medium. Supplementation of plasma with increasing levels of vitamin E before LDL isolation resulted in a proportional increase in the duration of the lag phase during which there was no detectable oxidative modification of LDL. In a study of WHHL rabbits, low dose vitamin E supplementation for 6 months significantly decreased the maximal oxidation rate of LDL.

In studies of healthy, nonsmoking subjects supplemented with vitamin E, there was a significant increase in resistance of LDL isolated from plasma to induced oxidation. Resistance of LDL to oxidation also increased significantly in a group of smokers supplemented with vitamin E, and in smokers supplemented with vitamin C and E, beta-carotene, and selenium.

The minimal dose of vitamin E required to protect LDL from oxidation was evaluated in a study of healthy young adults. Resistance of LDL to oxidation increased in a dose-dependent manner. The maximum rate of LDL oxidation was significantly decreased only after daily supplementation with 400 or 800 IU vitamin E. In another study evaluating the effectiveness of varying doses of vitamin E on induced LDL oxidation in healthy men, there was no significant effect of daily supplementation with 60 or 200 IU vitamin E for 8 wk compared to baseline values. Groups that received at least 400 IU vitamin E per day showed a decreased susceptibility of LDL to oxidation and significant inverse correlations between plasma and LDL α-tocopherol levels and the oxidation rate.

5.4 Safety (43)

Research in rats has demonstrated that the acute and chronic toxicities of oral vitamin E are very low and that vitamin E is not mutagenic, teratogenic, or carcinogenic. Studies in a number of animal species have shown that oral vitamin E intakes of up to 200 mg/kg body weight were well tolerated, without evidence of side effects.

In double-blind, placebo-controlled human studies, there were very few observed side effects, and no specific side effect was consistently seen in all studies. Side effects of oral vitamin E intake were also uncommon in other clinical studies that

were not necessarily double-blind. The majority of side effects attributed to vitamin E were reported in letters to the editor as uncontrolled studies or individual case studies. Most of these reported side effects were not observed in the larger, well-controlled clinical trials. However, it should be noted that high oral intakes of vitamin E can exacerbate the blood coagulation defect of vitamin K deficiency due to malabsorption or anticoagulant therapy. Thus, high levels of vitamin E intake are contraindicated in such conditions. Blood evaluations in studies of normal adults on vitamin E supplementation did not demonstrate any blood coagulation abnormalities.

Based on a review of animal and human data on safety and tolerance of oral vitamin E, it may be concluded that vitamin E is safe at levels commonly consumed. Except for interaction with vitamin K in patients on anticoagulant therapy, there are no specific side effects associated with oral vitamin E intake.

6. Curcumin

Curcumin (figure 2.8) is the main yellow phenolic material of turmeric and widely used as food coloring additive (44). Turmeric is a spice derived from the rhizomes of Curcuma Longa, which is member of the ginger family (Zingiberaceae) (45). Rhizomes are horizontal underground stems that send out shoots as well as roots. The bright yellow color of turmeric comes mainly from polyphenolic pigments, known as curcuminoids. Curcumin is the principal curcuminoid found in turmeric, and is generally considered its most active constituent (46). Other curcuminoids found in turmeric include demethoxycurcumin and bisdemethoxycurcumin. Numerous experimental studies have demonstrated that curcumin has various biological activities, including antioxidant activity, cancer preventive activity, and antiangiogenesis activity, etc (47, 48).
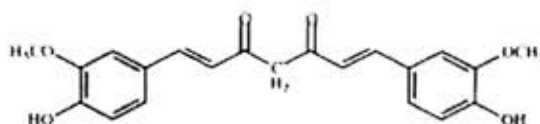
Figure 2.8 Structure of curcumin

6.1 Metabolism and bioavailability

Clinical trials in humans indicate that the systemic bioavailability of orally administered curcumin is relatively low (46). Curcumin is readily conjugated in the intestine and liver to form curcumin glucuronides and curcumin sulfates or reduced to hexahydrocurcumin (49). Curcumin metabolites may not have the same biological activity as the parent compound. In one study, conjugated or reduced metabolites of curcumin were less effective inhibitors of inflammatory enzyme expression in cultured human colon cells than curcumin itself (50). In a clinical trial conducted in Taiwan, serum curcumin concentrations peaked 1-2 hours after an oral dose, and peak serum concentration were 0.5, 0.6, and 1.8 micromoles/liter at doses of 4, 6 and 8 g/day, respectively.(51) Curcumin could not be detected in serum at lower doses than 4 g/day. More recently, a clinical trial conducted in the UK, found that plasma curcumin, curcumin sulfate and curcumin glucuronide concentrations were in the range of 10 nanomoles/liter one hour after a 3.6 g dose of oral curcumin (52). Curcumin and its metabolites could not be detected in plasma at lower doses than 3.6 g/day. Curcumin and its glucuronidated and sulfated metabolites were also measured in urine at a dose of 3.6 g/day. There is some evidence that orally administered curcumin accumulates in gastrointestinal tissues. When colorectal cancer patients took 3.6 g/d of curcumin orally for 7 days prior to surgery, curcumin was detected in malignant and normal colorectal tissue (53). In contrast, curcumin was not detected in the liver tissue of patients with liver metastases of colorectal cancer after the same dose of oral curcumin (54) suggesting that oral curcumin administration may not effectively deliver curcumin to tissures outside the gastrointestinal tract.

6.2 Antioxidant activities

Curcumin is an effective scavenger of reactive oxygen species and reactive nitrogen species *in vitro* (28, 29). However, it is not clear whether curcumin acts directly as an antioxidant *in vivo*. Due to its limited oral bioavailability in human's plasma and tissue curcumin concentrations are likely to be much lower than that of other fat-soluble antioxidants, such as α-tocopherol (vitamin E). However, the finding that 7 days of oral curcumin supplementation (3.6 g/day) decreased the number of oxidative DNA adducts in malignant colorectal tissue suggests that

curcumin taken orally may reach sufficient concentrations in the gastrointestinal tract to inhibit oxidative DNA damage (53). In addition to direct antioxidant activity, curcumin may function indirectly as an antioxidant by inhibiting the activity of inflammatory enzymes or by enhancing the synthesis of glutathione, and important intracellular antioxidant.

6.3 Antioxidant mechanism (55)

Although the antioxidant mechanism of curcumin in biological and chemical systems has been extensively investigated, there exists much controversy on this topic. Barclay et al. indicated that curcumin was a phenolic chain-breaking antioxidant and the H atom was abstracted from the phenolic group. By laser flash photolysis or pulse radiolysis, Jovanovic and co-workers suggested that the H-atom abstraction was mainly from the central active CH_2 group in the heptadienone link, and the H-abstraction from the phenolic group accounted for only ~15% of the reaction. As H-atom abstraction is mainly governed by the strength of the X-H bond (X represents O or C) now, it is clear that the O-H bond dissociation enthalpies (BDEs) for the curcumin-related compounds have been calculated using density functional theory (DFT) methods. It was found that the antioxidant mechanism of curcumin was a H-atom abstraction from the phenolic group, not from the central CH_2 group in the heptadienone link. Curcumin, methylcurcumin, and half-curcumin had similar O-H BDEs, indicating that the two phenolic groups in curcumin were independent of each other. (Figure 2.9)

The antioxidant mechanism

Hydrogen atom transfer (HAT)from the O-H group
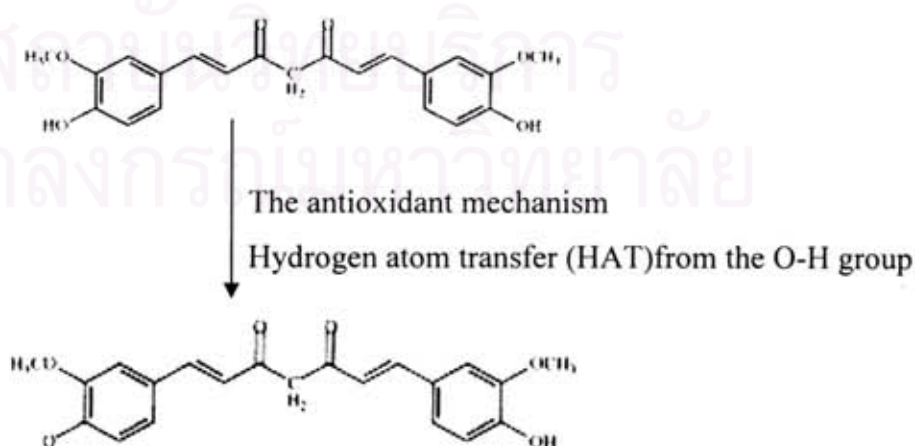
Figure 2.9 Show the antioxidant mechanism of curcumin was a H-atom abstraction from the phenolic group.

6.4 Sources

Turmeric is the dried ground rhizome of Curcuma Longa Linn. (56) It is used as a spice in Indian, Southeast Asian and Middle Eastern cuisines. Curcuminoids comprise about 2-9% of turmeric (57). Curcumin is the most abundant curcuminoid in turmeric, providing about 75% of the total curcuminoids, while demethoxycurcumin provides 10-20% and bisdemethoxycurcumin generally provides <5%. Curry pawder contains turmeric along with other spices, but the amount of curcumin in curry pawders is variable and often relatively low (58). Curcumin extracts are also used as food coloring agents (59).

6.5 Safety

In the US, turmeric is generally recognized as safe (GRAS) as a food additive by the FDA (59). Serious adverse effects have not been reported in humans taking high dose of curcumin. In a phase I trial in Taiwan, curcumin supplementation up to 8 g/day for 3 months was reported to be well-tolerated in patients with precancerous conditions or noninvasive cancer (58). In another clinical trial in the UK, curcumin supplementation ranging from 0.45-3.6 g/day for 4 months was generally well tolerated by people with advanced colorectal cancer, although two participants experienced diarrhea and another reported nausea (52). Increases in serum alkaline phosphatase and lactate dehydrogenase were also observed in several participants, but it was not clear whether these increases were related to curcumin supplementation or cancer progression (46). Curcumin supplementation of 20-40 mg has been reported to increase gall bladder contractions in healthy people (60, 61). In light of this finding, people with gallbladder disease are often advised to avoid curcumin supplements.

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Chemicals

All chemical were obtained commercially and used without further purification. The following chemicals were purchased from Sigma Chemical Co., St Louis, U.S.A.: Standard free cholesterol, cholesteryl arachidonate, cholesterol linoleate, cholesteryl linolenate, cholesteryl oleate, cholesteryl palmitate, α-tocopherol, ethylenediamine tetraacetic acid disodium salt, potassium bromide, sodium acetate, monobasic sodium phosphate anhydrous, disodium hydrogen phosphate anhydrous, tetrachloroacetic acid, 2-thiobarbituric acid, adenosine 5' phosphate (ADP), hemin and Folin & Ciocalteu's phenol reagent.

Other chemicals were obtained from commercial sources as follows : cholesterol enzyme kit from B.M. Lab Limited Partnership (Bio-medical Laboratory) Thailand, sodium dodecyl sulfate from Fiedel-deHaan Co., Ltd. Germany, methanol, n-hexane, acetonitrile and isopropanol (HPLC grade) from Lab Scan Co., Ltd. Thailand. The government pharmaceutical organization aids for curcumin.

1.2 Instruments

1) Kubota 5900 centrifuge with swing bucket rotor (RS-720 M, Japan)

2) Optima Beckman Coulter ultracentrifuge (LE 80 K) with 90 Ti Rotor

3) Perkin Elmer LS55 luminescence spectrometer

4) GBC Cintra 10 UV-visible spectrometer

5) HPLC systems (shimudsu, Japan) class LC 10

6) Aggregrometer (chrono-log) model 500CA/560CA

2. Methods

2.1 Low density lipoprotein preparation

Low density lipoprotein (LDL) fractions were separated by the sequential density gradient ultracentrifugation method, which is modified from Havel method (62). LDL (density = 1.019-1.063 g/ml) was separated from healthy volunteer plasma in salt solutions by Beckman 90 Ti rotor at 50,000 rpm 4°C. Salts were removed from LDL by using PD-10 Desalting column with PBS buffer pH 7.4. LDL were analyzed for protein content.

2.2 Preparation of hemin

Stock solution of hemin was prepared by dissolving hemin in 0.1 M NaOH and adjusted pH to 7.5 by 10 mM PBS. Hemin solution was centrifuged at 3,500 rpm for 5 min. The supernatant was collected and its concentration was determined by reading absorbance at 385 nM using molar coefficient ε=58.4 mmol l-1 cm-1. Stock solution was kept in dark at 4°C and used within a week.

2.3 Preparation of curcumin and α-tocopherol

Curcumin and α-tocopherol were dissolved in methanol. Curcumin or α-tocopherol was pipetted into the incubation tube and was dried under nitrogen gas before incubation with LDL.

2.4 Other parameters measurement

2.4.1 Thiobarbituric acid reactive substances (TBARs) (63, 64)

Lipid peroxidation of lipoproteins 1 ml was terminated by adding 50 ul of 100 mM butylated hydroxytoluene. Then 1 ml of 10% trichloroacetic acid was added and mixed for 60 sec. A 0.5 ml of 5 mM EDTA was added into the mixture. After vortexing, 0.5 ml of 8% sodium dodecyl sulfate and 1.5 ml of 1.2% thiobarbituric acid were added. The reaction mixtures was then heating at 100°C for 1 hour, the samples were cooled to room temperature and were centrifuged at 3,500 rpm at 25°C for 10 minutes. The clear supernatants were analyzed by spectrofluorometric

method, excitation and emission wavelength at 515 and 553 nm, respectively. 1, 1, 3, 3-tetraethoxypropane was used as a standard of MDA.

2.4.2 Protein determination

Protein content was determined by the Markewell modification of the Lowry protein assay (65) using bovine serum as standard. The aromatic amino acids residues (tyrosine and tryptophan), or the polar side chain of amino acid in the sample were reduced by the mixed acid chromogen in the reagent of Folin and Ciocalteu. The reaction could be measured by UV-visible spectrometer at OD 750 nm.

2.4.3 Total cholesterol

Total cholesterol was measured by enzymatic methods assays using commercially reagent (BML). All sample and standard were mixed with working solution containing enzyme mixture tubes, incubated at 37°C for 10 min and measured the absorbance at 550 nm

Principle:
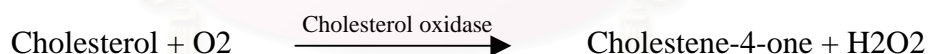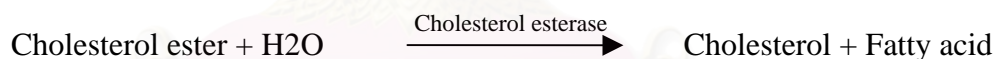
$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestene-4-one} + H_2O_2$$

$$H_2O_2 + \text{Antipyrine} + \text{Colored Substrate} \xrightarrow{\text{Peroxidase}} \text{Quinone imine}$$

2.4.4 α-Tocopherol, free cholesterol, cholesteryl esters and oxidative products

α-Tocopherol, free cholesterol, cholesteryl esters and oxidative products in LDL can be determined by reverse phase HPLC method using UV monitor at 210 nm for free cholesterol, cholesteryl esters, and 234 nm for products of oxidized lipids. α-Tocopherol can be detected by fluorescence detector at excitation 295 nm and emission 370 nm. This method modifies from Seta et al. (66) and Zaspel

and Csallany (67). Briftly, a 100 μl of LDL sample was added and mixed with 200 μl of 10 mM PBS. Then 500 μl of methanol (HPLC grade) were added and mixed on a vortex for 30s. The 2.5 ml of hexane (HPLC grade) were added into the mixture and agitated on a vortex for 1 min and centrifuged 1,700 rpm for 5 min at 4°C. The 2 ml of hexane layer was transferred into a test tube and dried under N_2 and redissolved with 200 μl of mobile phase (75%acetonitride:25%isopropanol, v/v). Preparation of standard substance is summarized in table 3.1. The standard mixture were injected 2.5, 5, 10 and 20 μl into the hypersil BDS C18 column (5 μm; 4.6 mm X 250 mm) by autosampler. The sample was injected 20 μl. The flow rate was 1.2 ml/min. The temperature of column was controlled at 50°C. The chromatogram of cholesteryl esters are shown in figure 3.1.

Table 3.1 Standard preparation of free cholesterol and cholesteryl esters

	Stock (μg/ml)	volume(μl)	amount per 20 μl injection (μg)
CHL	1000	90	3.6
CLN	1000	4.5	0.18
CA	1000	15	0.6
CL	1000	180	7.2
CO	1000	90	3.6
CP	1000	60	2.4
mobile phase		60.5	
total volume		500	

Free cholesterol (CHL), cholesteryl linolenate (CLN), cholesteryl arachidonate (CA) and cholesteryl linoleate (CL) were dissolved in isopropanal. Cholesteryl oleate and cholesteryl palmitate were dissolved in isopropanol : diethyl ether (5:2 v/v).
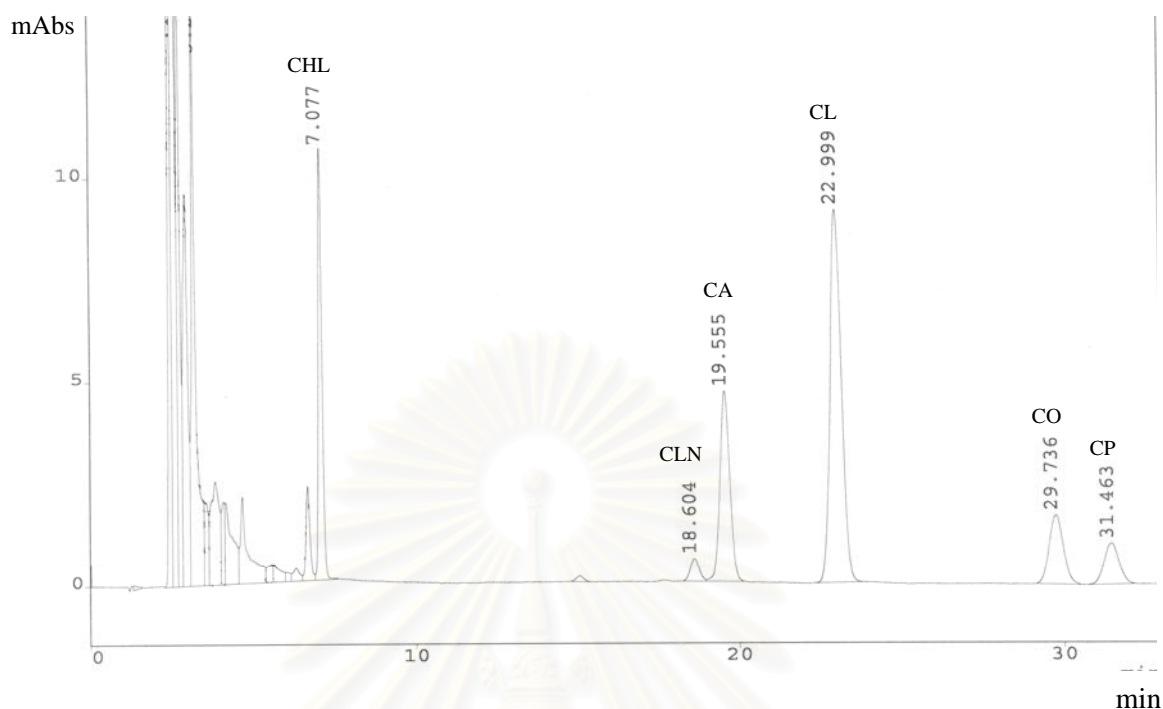
Figure 3.1 The chromatogram of individual cholesteryl esters standard

2.5 Platelet rich plasma (PRP) preparation

Blood samples were collected in 3.8% sodium citrate from same healthy volunteers who had abstained from all medication affecting platelet function for at least two weeks. The PRP was prepared by centrifugation at 800 rpm at room temperature for 10 minutes. The upper supernatant was collected as platelet-rich plasma.

2.6 Aggregation studies

Aggregation was performed on a Chronolog aggregometer linked to a computer. The change in turbidity of PRP in a aggregometer was used as an in vitro indicator for the aggregability of platelets. Following addition of an agonist into the stirring PRP, the pattern of change of light transmission was recorded with respect to time. There was performed by placing the testing PRP sample of 275 μl in a siliconized cuvette with Teflon coated stirring magnetic bar, transferred into the temperature-controlled compartment of the aggregometer equilibrating at 37°C for 3 minutes. The sample was further equilibrated to obtain the basline by stirring at 1,000

rpm for 1 minute before addition of 20 μl of Ox-LDL and 5 μl of ADP (final concentration of Ox-LDL and ADP are 23 μg/ml and 1 μM respectively)

3. Experimental procedure

3.1 Part I: The study of the effects of hemin induced LDL oxidation and effects of hemin oxidized LDL (he-oxLDL) on platelet aggregation (figure 3.2).

Low density lipoproteins (LDL) were separated by sequential ultracentrifugation from EDTA plasma of healthy volunteer and then were oxidized by hemin 25 μM per LDL 350 μg protein. He-oxLDL were collected at 0, 1, 3, 6, 9 and 24 hours and the reaction were terminated by adding 0.4 mM BHT. Then oxidation parameters (level of TBARs and α-tocopherol) and lipid composition (total cholesterol and individual cholesteryl ester) were determined. The effect of he-ox LDL on platelet aggregation was studied.

3.2 Part II: The study of protective effect of antioxidant (curcumin, α-tocopherol) on hemin induced LDL oxidation (figure 3.3)

Low density lipoproteins (LDL) were separated by sequential ultracentrifugation from EDTA plasma of healthy volunteer and then were incubated with antioxidants (α-tocopherol and curcumin) at various concentrations for 30 minutes perior to hemin oxidation (hemin 25 μM per LDL 350 μg protein). He-oxLDL were collected at 0, 6, and 9 hours and the reaction were terminated by adding 0.4 mM BHT. Oxidation parameters and lipid composition were determined.

Part I

Plasma

Sequential ultracentrifugation

LDL

Control LDL (350 μg/ml)   LDL (350 μg/ml)

+ 10 mM PBS   + 25 μM hemin in PBS

Incubate at 37°C, aliquot at 0, 1, 3, 6, 9, 24 hours
Termination of the reaction by adding 0.4 mM BHT

Determination of   Determination of
oxidation parameters   lipid composition   Study the effect on platelet
- TBARs   - Total cholesterol   - platelet aggregation
- α-tocopherol   - cholesteryl ester

Figure 3.2 study procedure of part I

Part II

Plasma

↓ Sequential ultracentrifugation

LDL

Adjust to 350 µg/ml

LDL (negative control)   LDL (control)   LDL + antioxidant

↓

Preincubate LDL with
antioxidant 30 minutes:
1) 25 µM of α-tocopherol

2) 75 µM of α-tocopherol

3) 225 µM of α-tocopherol

4) 7.5 µM of curcumin

5) 25 µM of curcumin

6) 75 µM of curcumin

+ 10 mM PBS

+ 25 µM of hemin in 10 mM

Incubate at 37°C, aliquot at 0, 6, 9 hours

Termination of the reaction by adding 0.4 mM BHT

Determination of
oxidation parameters
- TBARs
- α-tocopherol

Determination of
lipid composition
- Total cholesterol
- cholesteryl ester

Figure 3.3 study procedure of part II

Figure 3.3 study procedure of part II

3. Statistical Analysis

All data were presented as mean ± standard error of mean (S.E.M). Data between groups were compared by Mann-Whitney U Test and correlation analyses were assessed by Spearman correlation using the SPSS 12 for window software.

CHAPTER IV
RESULTS

Part I: The effects of hemin induced LDL oxidation on lipid composition

1. Lipid chemical composition and α-tocopherol of native LDL

The lipid composition was determined from low density lipoprotein (LDL) of six healthy volunteers. The baseline LDL levels of α-tocopherol, total cholesterol, free cholesterol (CHL), cholesteryl linolenate (CLN), cholesteryl arachidonate (CA), cholesteryl linoleate (CL), cholesteryl oleate (CO) and cholesteryl palmitate are shown in table 4.1. The level of α-tocopherol is approximately 22.62 μmol/g protein. The level of CL, most abundant cholesteryl esters in LDL, is approximately 1.37 mmol/g protein while minute amount of CLN. So the target of LDL oxidation induced by hemin should be CL rather than CLN.

Table 4.1 Levels of α-tocopherol, cholesterol and cholesteryl esters in LDL

Components	Levels
α-tocopherol (μmol/g protein)	22.62 ±2.01
Total cholesterol (mmol/g protein)	3.59±0.40
Free cholesterol (mmol/g protein)	1.22±0.06
Cholesteryl linolenate (mmol/g protein)	0.069±0.01
Cholesteryl arachidonate (mmol/g protein)	0.15±0.02
Cholesteryl linoleate (mmol/g protein)	1.27±0.12
Cholesteryl oleate (mmol/g protein)	0.53±0.07
Cholesteryl palmitate (mmol/g protein)	0.21±0.07

Data are presented as mean ± S.E.M of six individual subjects.

2. Effect of hemin on oxidation parameters

2.1 TBARs formation

TBARs is a marker to determine lipid peroxidation products. LDL incubated with hemin showed time course effect to increase TBARs levels significantly ($p < 0.05$ at 3 hours and $p < 0.01$ at 6, 9 and 24 hours of incubation) when compare to control (Figure 4.1). The result indicated that lipid peroxidation was occurred in hemin induced LDL oxidation.
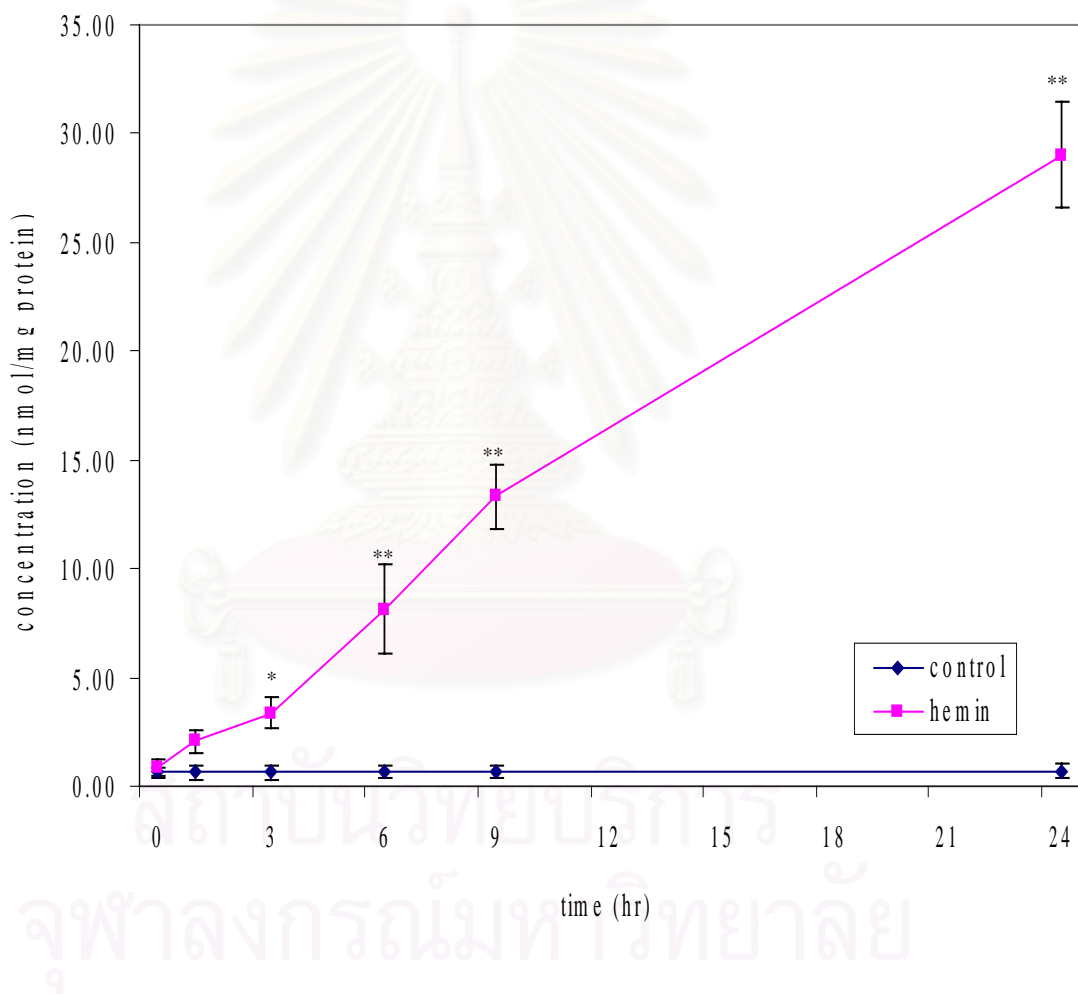
Figure 4.1 Effect of hemin 25 µM on the TBARs formation in LDL oxidation. The values are presented as mean ± S.E.M. of six individual subjects. Significant different *$p < 0.05$, **$p < 0.01$ compared to the respective control.

2.2 α-tocopherol levels

The time course effect of hemin induced LDL oxidation on levels of α-tocopherol is shown in figure 4.2. The level of α-tocopherol was rapidly decreased more than 70% at 1 hour and negligible level at 9 hours after incubation of hemin. The depletion of α-tocopherol indicated that α-tocopherol was consumed during hemin induced LDL oxidation.
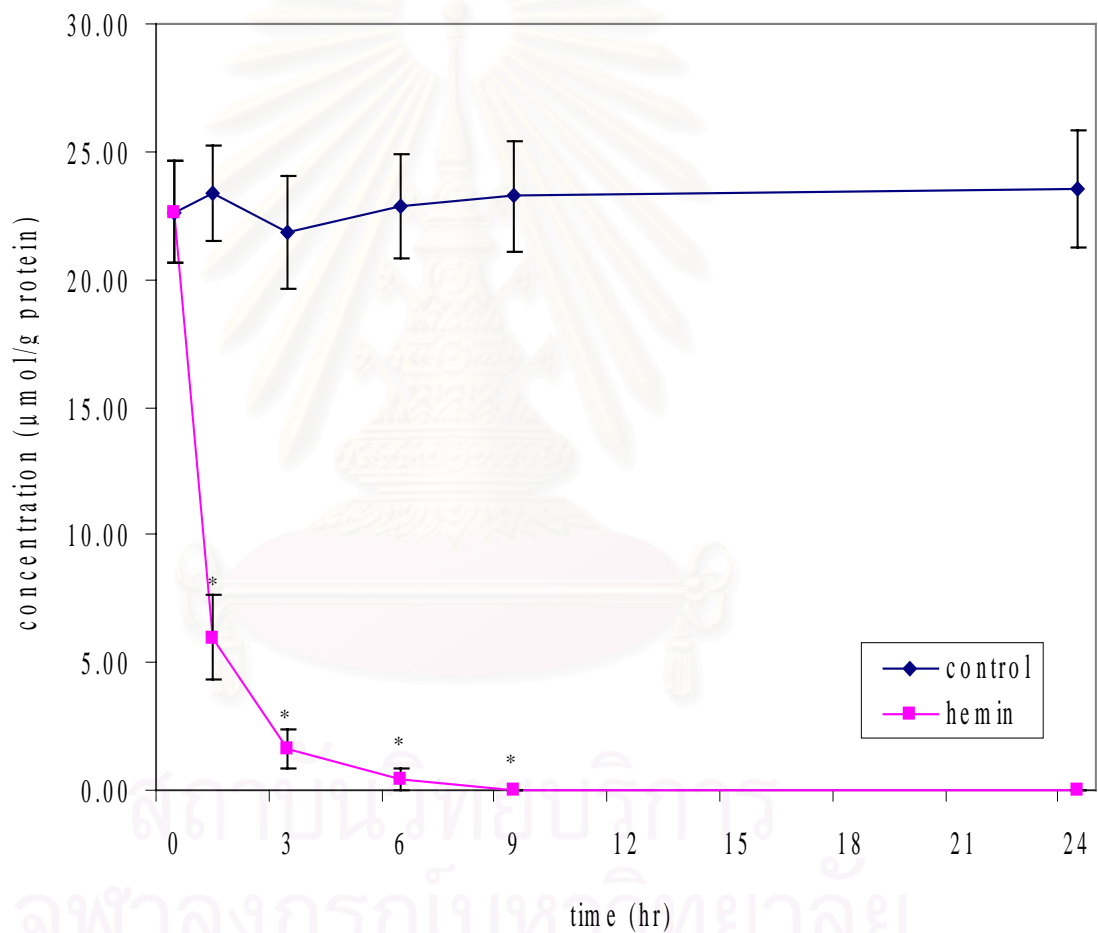
Figure 4.2 Effect of hemin 25 μM on α-tocopherol levels. The levels of α-tocopherol in LDL were presented as mean ± S.E.M. of six individual subjects. Significant different *p<0.01 compared with respective control.

2.3 The correlation between TBARs and α-tocopherol

The negative correlation between level of TBARs and α-tocopherol at 0, 1 and 3 hours of incubation was found with the r-value of -0.734 ($p < 0.001$) (figure 4.3). When the level of α-tocopherol was negligible after either 6 or 9 hours, TBARs level was dramatically increased. It indicated that hemin induced LDL oxidation can be investigated by the increasing of oxidized lipid product formation and depleting of α-tocopherol.
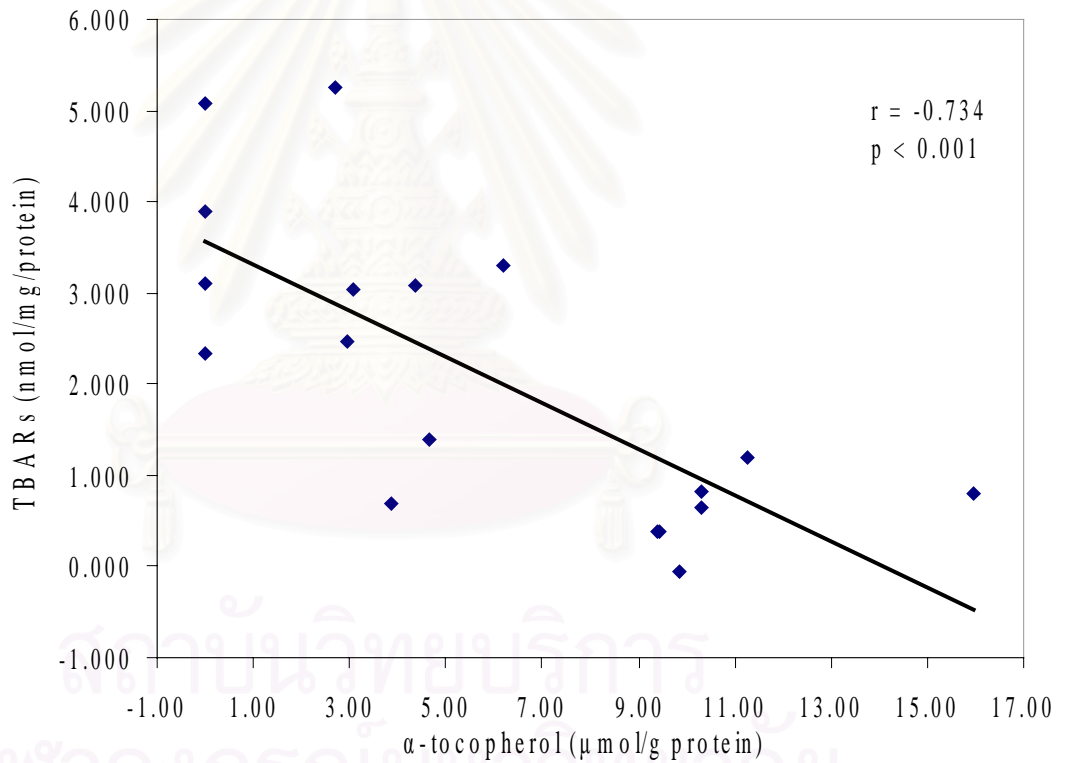
Figure 4.3 The correlation of TBARs and α-tocopherol. The data analyses   form six individual subjects on time 0, 1, 3 hours after incubation LDL 350 μg with hemin 25 μM.

3. Effect of hemin on levels of cholesterol and cholesteryl esters

The effect of hemin induced LDL oxidation on free cholesterol and cholesteryl esters are shown in figure 4.4 and figure 4.5. Levels of CA, CL and CLN were decreased while levels of free cholesterol, CO and CP were not changed after incubation with hemin.

After 24 hours incubation of hemin, CA, and CL were decreased about 48 % 32% and (figure 4.4 A and B respectively). The percent decreasing of CA and CL correlated with increasing of TBARs with the r-value of 0.837 ($p < 0.001$) and 0.824 ($p < 0.001$) respectively (figure 4.6 A and B). It indicated that CA and CL are the major targets of lipid peroxidation induced by hemin.
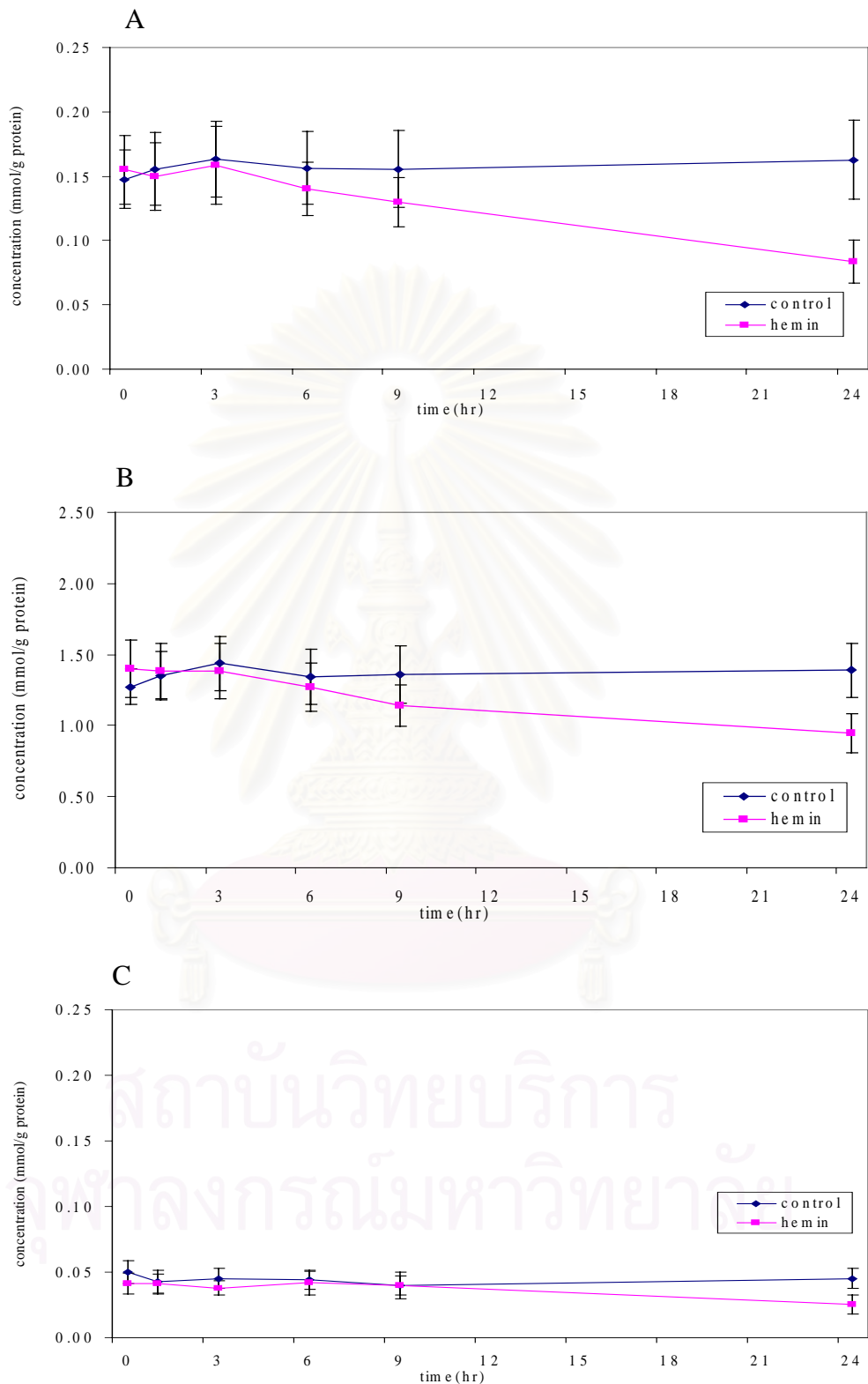
Figure 4.4 Effect of hemin 25 μM on levels of (A) cholesteryl arachidonate (CA), (B)
cholesteryl linoleate (CL) and (C) cholesteryl linolenate (CLN) in LDL.
Data were presented as mean ± S.E.M of six individual subjects.
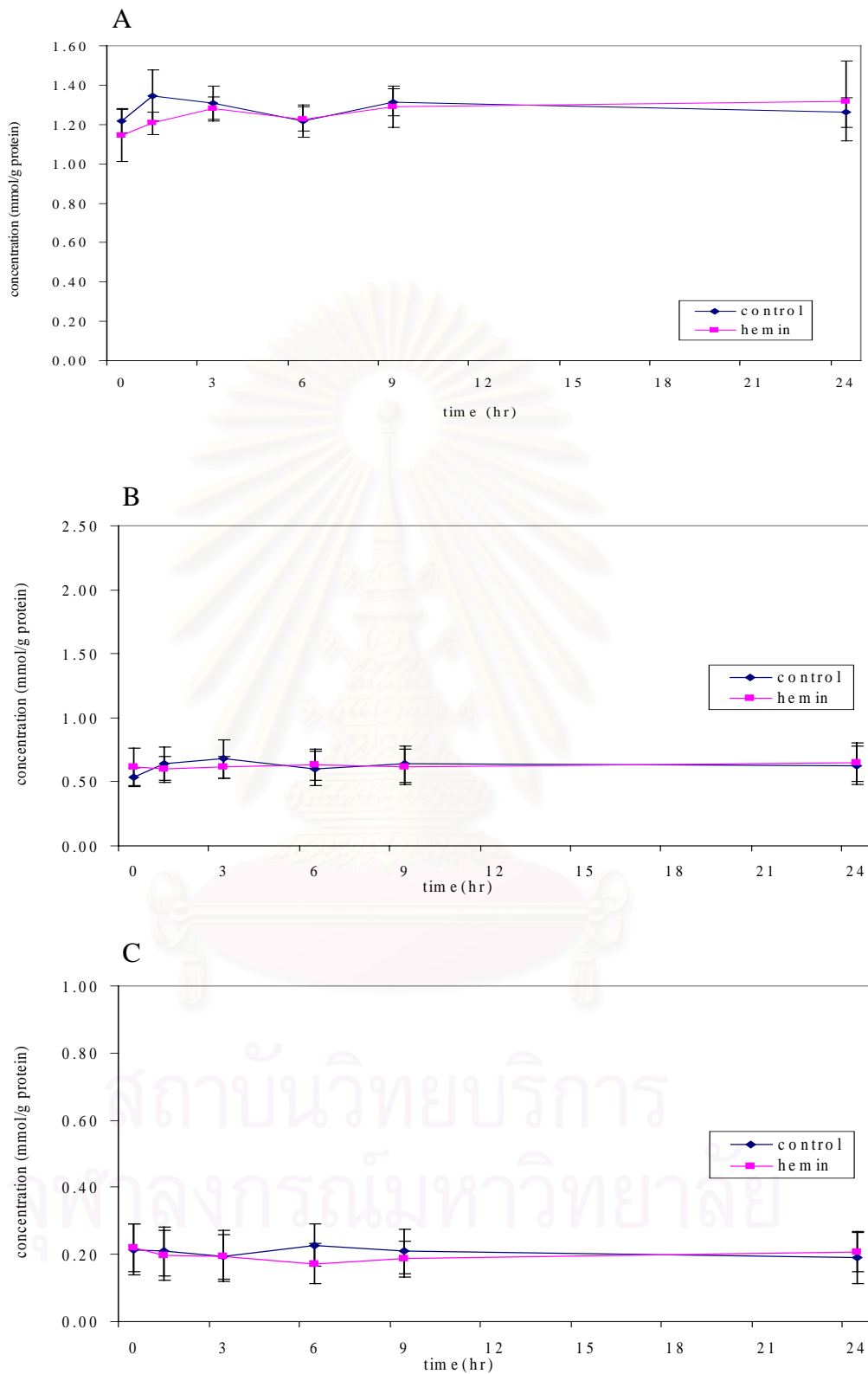
Figure 4.5 Effect of hemin 25 μM on levels of (A) free cholesterol cholesteryl, (B) cholesteryl oleate (CO) and (C) cholesteryl palmitate (CP) in LDL. Data were presented as mean ± S.E.M of six individual subjects.
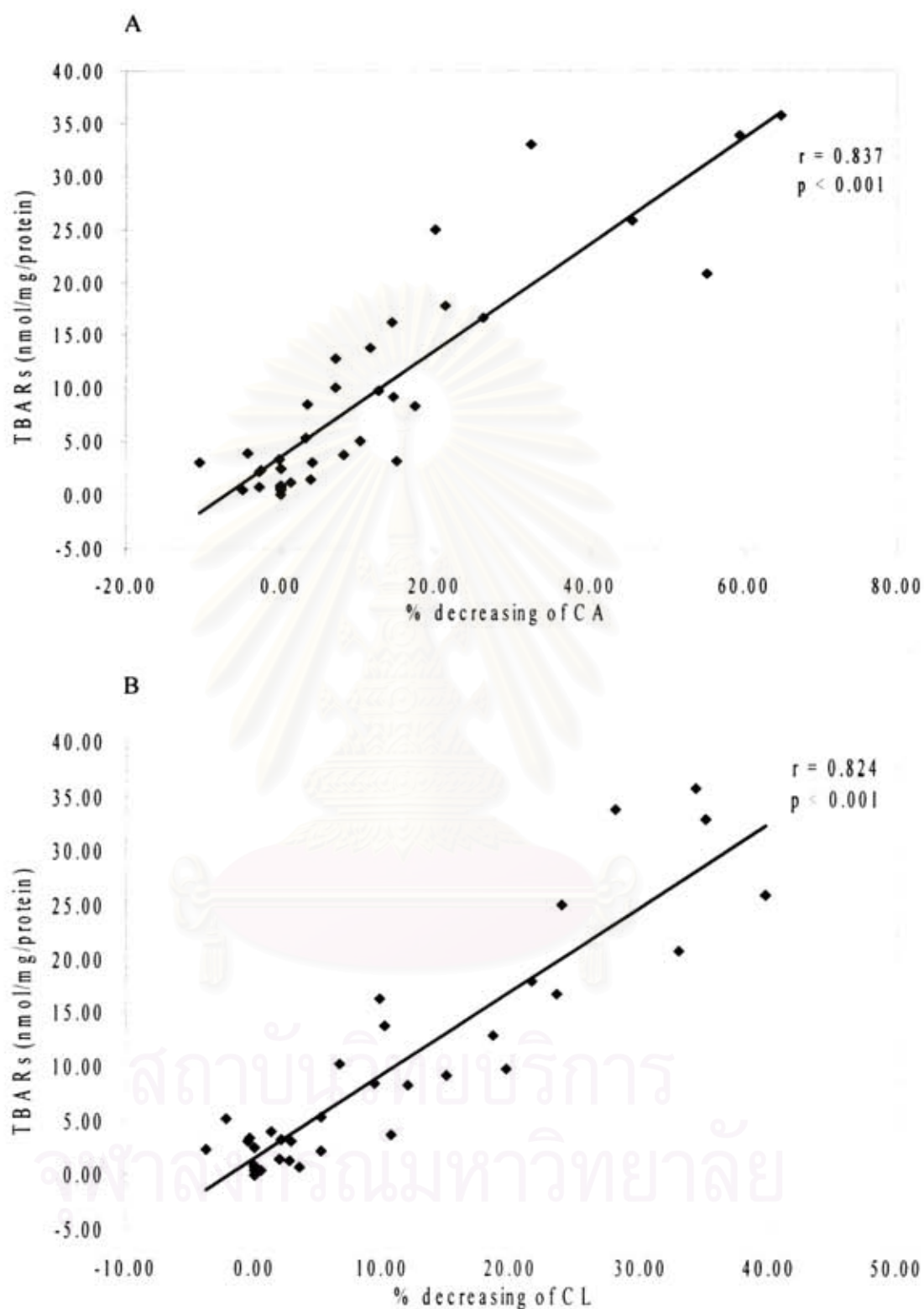
Figure 4.6 The correlation between TBARs with percent decreasing of CA (A) and CL (B). The data analyses from six individual subjects on various times after incubation LDL 350 μg with hemin 25 μM.

Figure 4.6 The correlation between TBARs with percent decreasing of CA (A) and CL (B). The data analyses from six individual subjects on various times after incubation LDL 350 μg with hemin 25 μM.

4. Effect of hemin on CL/CO ratio

According to the previous study of Luechapudiporn (12) suggested that the CL/CO ratio is a marker for oxidative damage and can determine the severity of lipoprotein damage The effect of hemin induced LDL oxidation on CL/CO ratio is showed in figure 4.7. The CL/CO ratio in LDL control was 2.4 ± 0.2 and was not changed through out 24 hours incubation at 37 ^{o}C. The CL/CO ratio in LDL oxidation was significantly decreased to 1.5 ± 0.1. ($p < 0.01$) at time 24 hr after incubation with hemin (figure 4.7) and found negative correlation with TBARs ($r = -0.706$, $p < 0.001$) (figure 4.8).
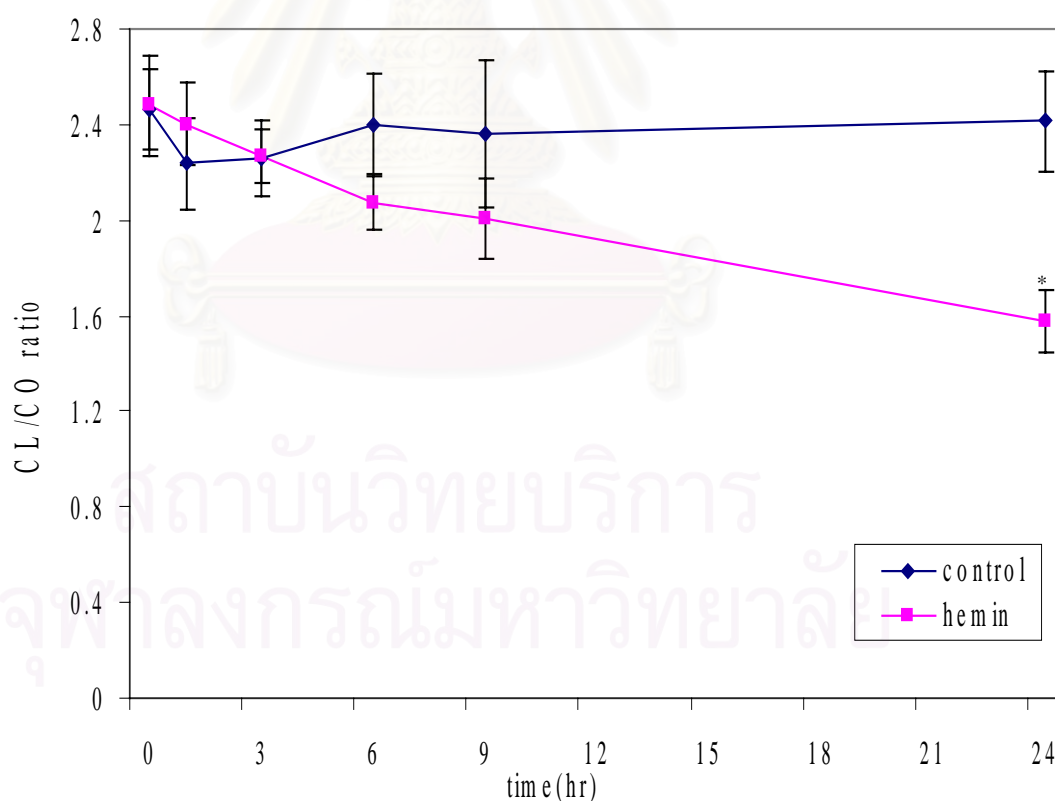
Figure 4.7 Effect of hemin 25 μM on CL/CO ratio. Levels of CL/CO ratio in LDL were presented as mean $\pm$ S.E.M. of six individual subjects. *$p < 0.01$ compared with respective control.
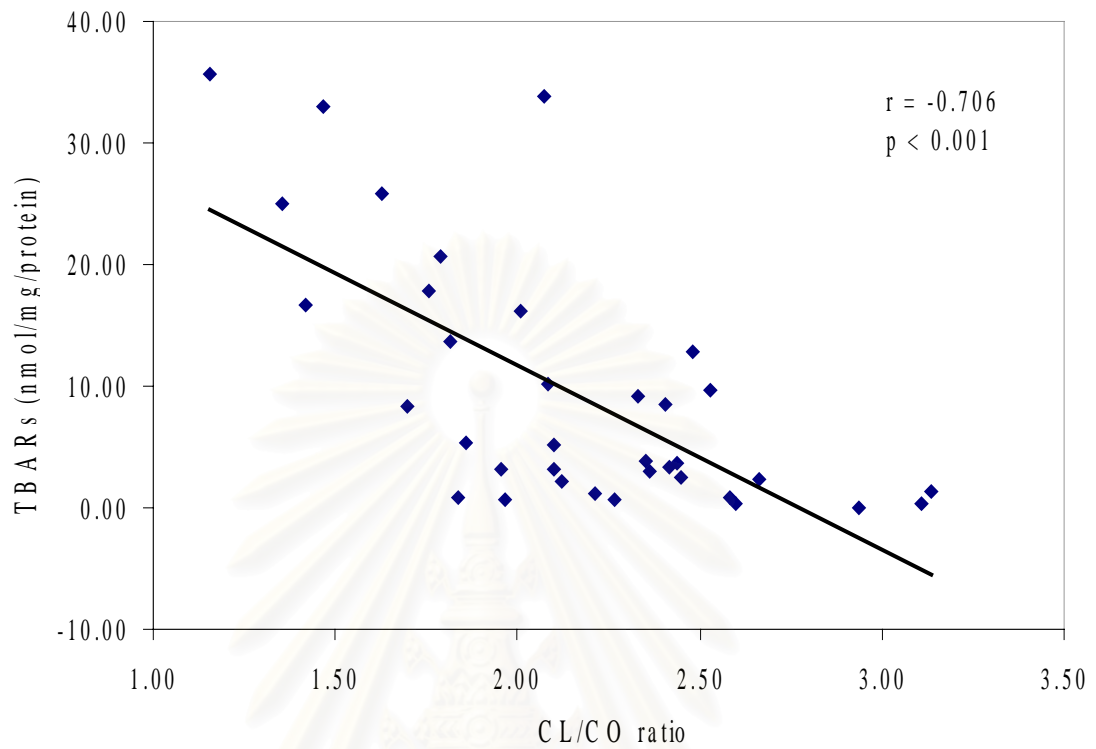
Figure 4.8 The correlation between TBARs and CL/CO ratio. The data analyses from six individual subjects on various times after incubate LDL with hemin 25 μM.

5. Effect of hemin on oxidized lipid products formation

The oxidized lipid products of hemin induced LDL oxidation were determined by HPLC-UV-detector 234 nm. Peaks at retention time of 9.5, 10.2, 11.7, and 12.2 min were detected during oxidation (figure 4.9 A, B, C, D, E and F). Peaks at retention time of 9.5 and 12.2 min were appeared at 1 hour incubation while peaks at retention time of 10.2 and 11.7 min were detected after incubation with hemin at least 9 hours. The increase of these 4 oxidized lipid products formation showed time course effect in hemin induced LDL oxidation (figure 4.10).

A) LDL 24 hr (control)
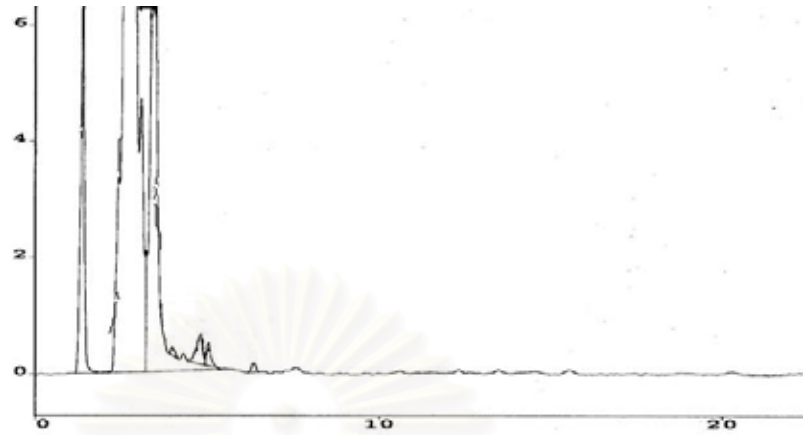
B) LDL+hemin 1 hr
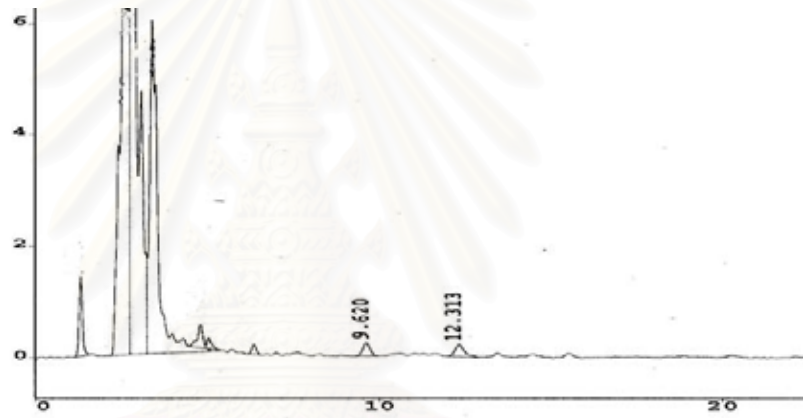
C) LDL+hemin 3 hr
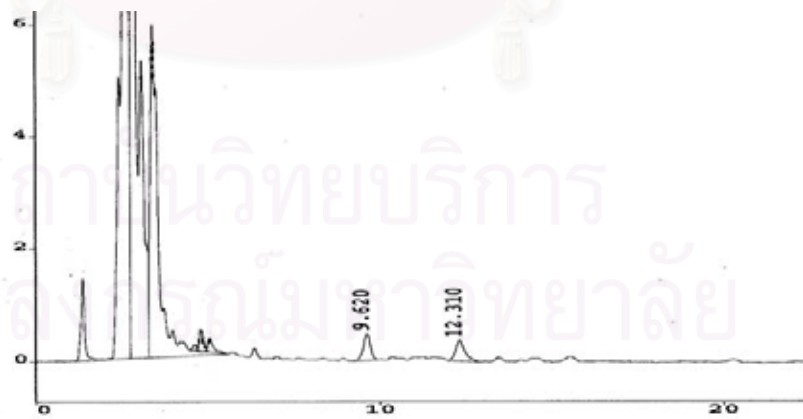
Figure 4.9 cont.

D) LDL+hemin 6 hr
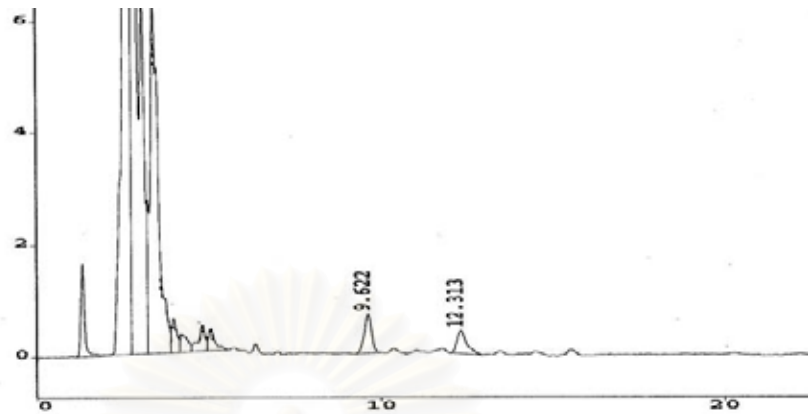
E) LDL+hemin 9 hr
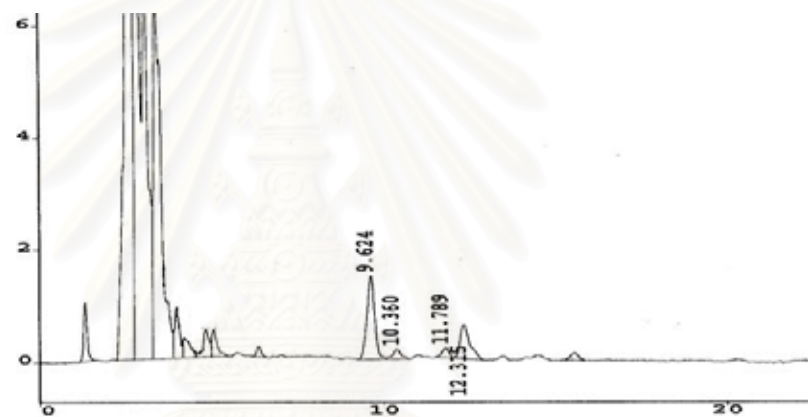
F) LDL+hemin 24 hr
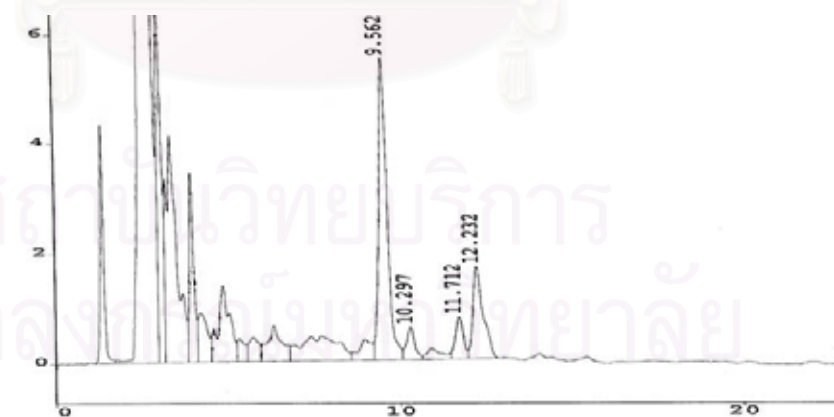
Figure 4.9 Effect of hemin on oxidized lipid products formation. The chromatograms
show peak of oxidized lipid products at retention time of 9.5, 10.2, 11.7,
and 12.2 min. A represented chromatogram of LDL control 24 hours, B, C,
D, E and F represented chromatograms of oxidized lipid products after
incubation with hemin 1, 3, 6, 9 and 24 hours respectively.
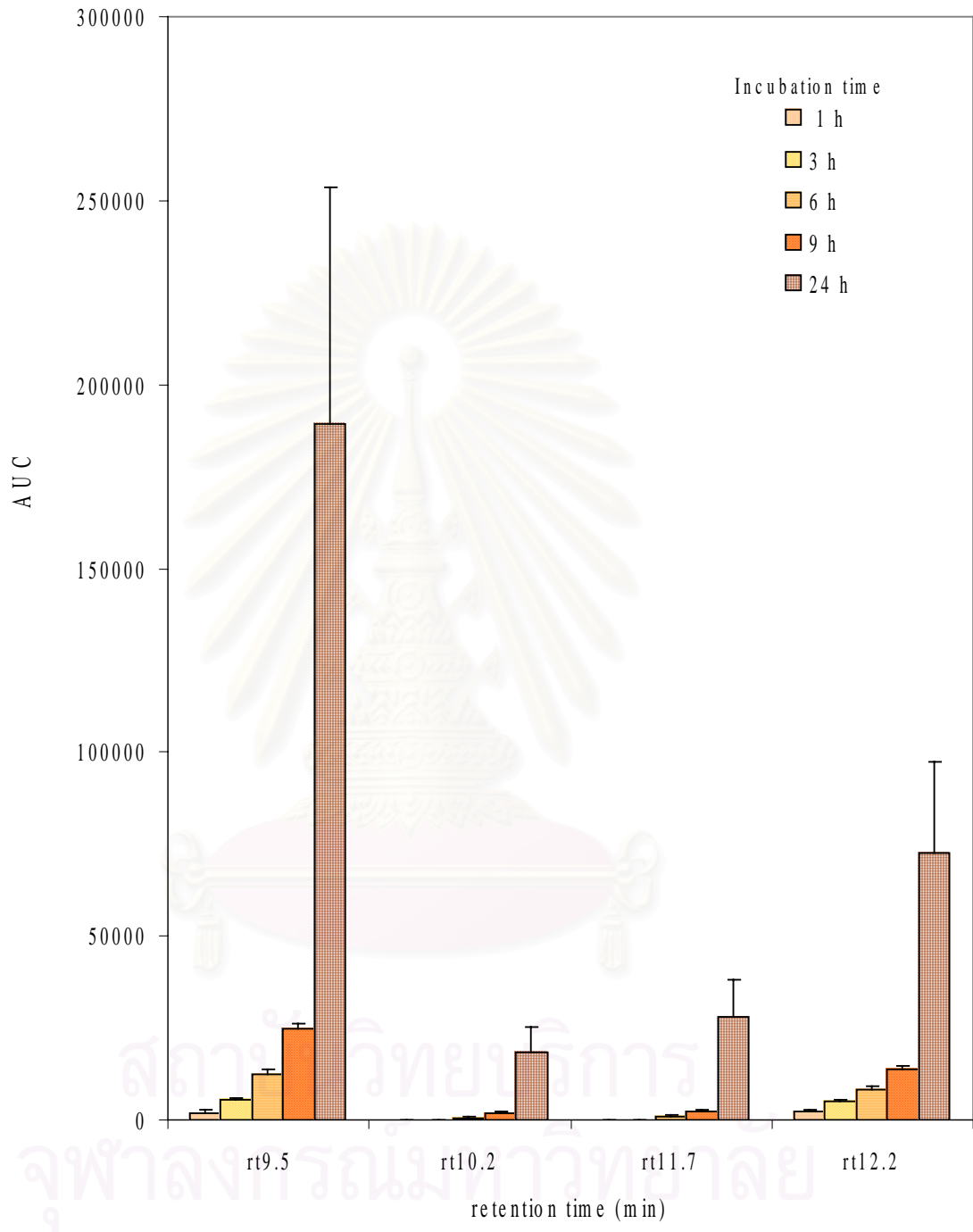
Figure 4.10 Effect of hemin on oxidized lipid products formation. The results were presented as mean ± S.E.M. of six individual subjects. Significant different *p<0.05, **p<0.01 compared to the respective control.

6. The effects of hemin oxidized LDL (he-oxLDL) on platelet aggregation

The results show that he-oxLDL can enhance ADP to stimulate platelet aggregation. But only two from six individual subjects whose platelets response to he-oxLDL (figure 4.11 A and B). The low dose of ADP (1 μM) usually initiated minimal aggregation, which was completely reversed within 1 minute. He-oxLDL alone did not activate platelets, but when combined with ADP (1 μM) caused vigorous, irreversible aggregation. The effects of he-oxLDL on enhance ADP stimulated platelet aggregation was increased according to the degree of oxidative modification of he-oxLDL. The combination with he-oxLDL from 3 and 6 hours of incubation were not completely reversed platelet aggregation. The irreversible platelet aggregation was occurred with the maximum aggregation about 50% when induced by the combination with he-oxLDL from 9 hours of incubation.
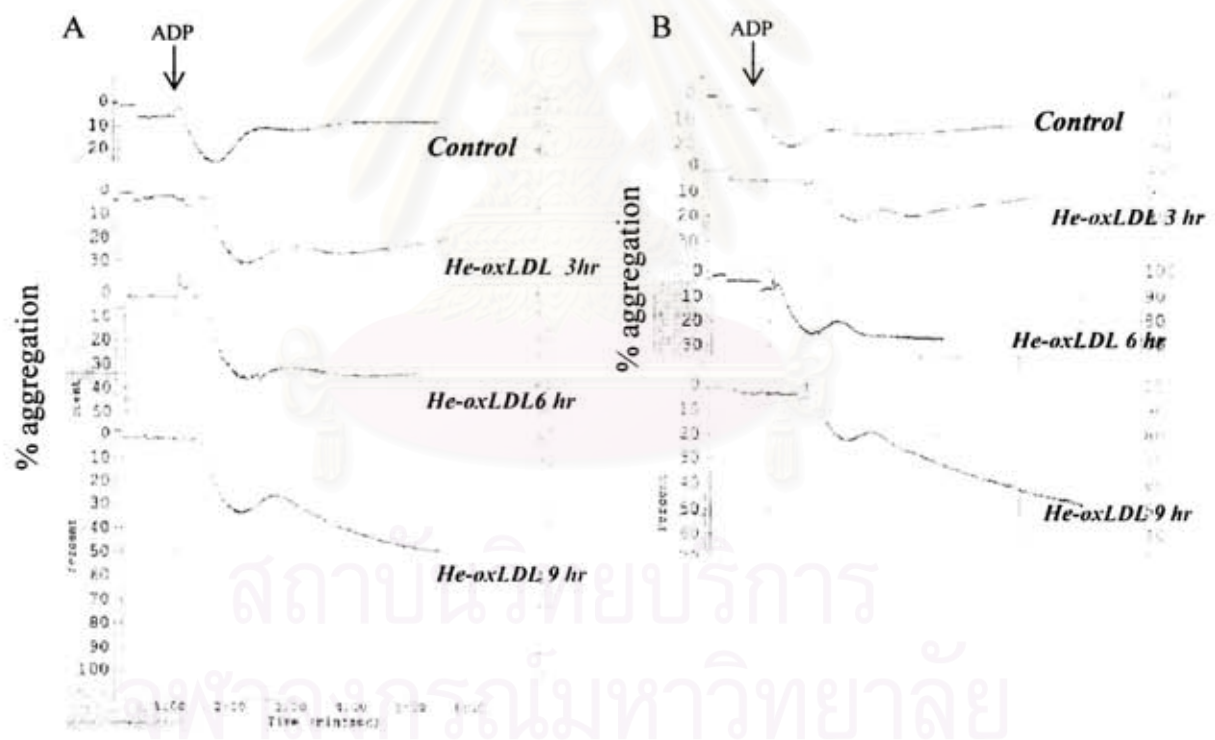
Figure 4.11 The platelet aggregation tracing induced by he-OxLDL (23 ug/ml) combined with ADP (1 μM). Control represents platelet aggregation tracing induced by ADP alone. Panel A and B represents tracing of two subjects. The letters: he-oxLDL 3 hr, he-oxLDL 6 hr and he-oxLDL 9 hr represent platelet aggregation tracing induced by he-oxLDL which are collected from the *in vitro* LDL oxidation induced by hemin at 3, 6 and 9 hours respectively.

collected from the *in vitro* LDL oxidation induced by hemin at 3, 6 and 9 hours respectively.

PART II: The study of protective effect of antioxidant (curcumin, α-tocopherol) on hemin induced LDL oxidation

1. The effect of curcumin and α-tocopherol on TBARs formation.

The curcumin and α-tocopherol were able to protect LDL oxidation induced by hemin. Both antioxidants had protective effect in dose dependent manner (figure 4.12). Curcumin was able to protect the LDL oxidation along 9 hours and reached the maximum inhibition of TBARs formation (81.84 ± 2.3%) at dose of 25 μM. In addition, at dose of 25 μM curcumin showed to inhibit TBARs formation more than α-tocopherol approximately 3 times at 9 hours incubation (table 4.2) The result indicated that curcumin has more effective than α-tocopherol to protect LDL oxidation induced by hemin.
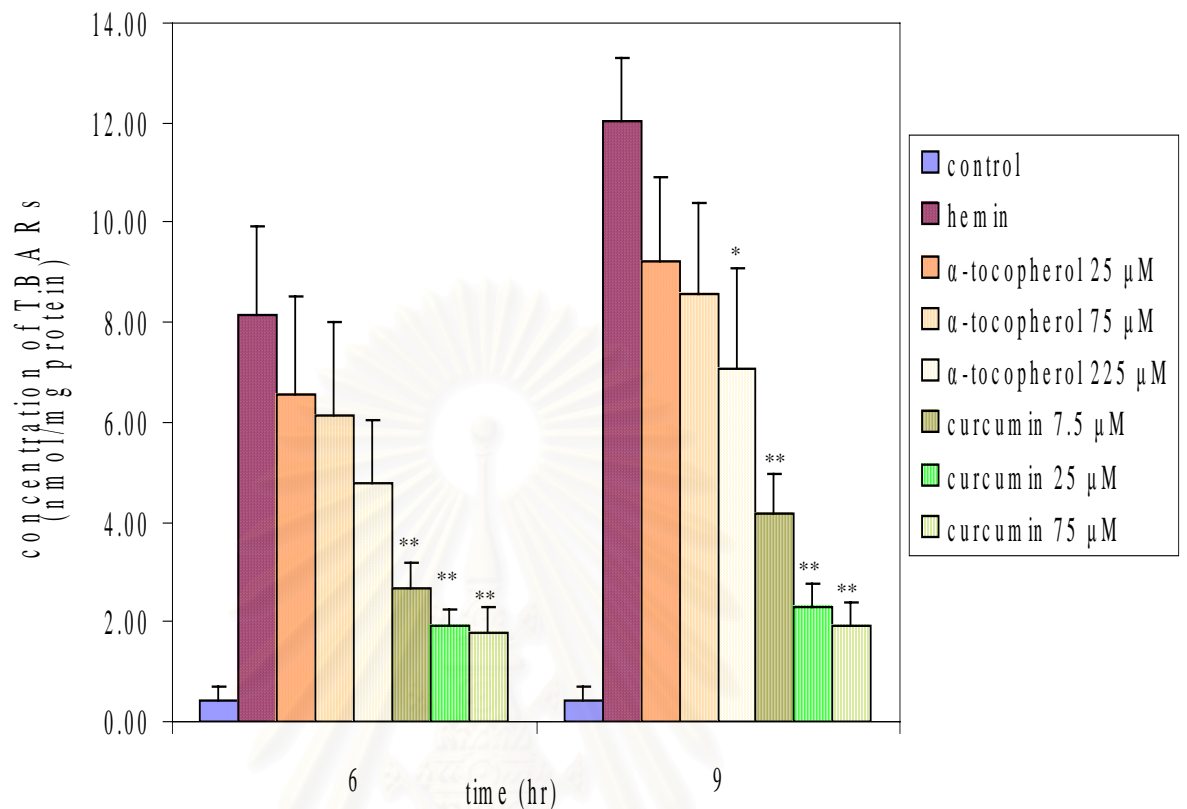
Figure 4.12 The inhibition effect of curcumin and α-tocopherol on TBARs formation. The data were presented by means of six individual subjects. Significant different * p<0.05, ** p<0.01 compared to hemin.

Table 4.2 the percentage inhibition of TBARs formation by curcumin and α-tocopherol.

Dose of antioxidant	Percent inhibition of TBARs (%)	
	Curcumin	α-tocopherol
25 μM	81.84 ± 2.3	23.29 ± 7.0
75 μM	84.89 ± 1.9	28.77 ± 7.7

Data are presented as mean of percent inhibition ± S.E.M of six individual subjects at 9 hours incubation.

2. The effect of curcumin and α-tocopherol on cholesteryl esters.

The curcumin and α-tocopherol was able to protect the decreasing of CA and CL in LDL oxidation induced by hemin. Both antioxidants have protective effect in dose dependent manner (figure 4.13 A and B). However curcumin has more protective effect than α-tocopherol. Curcumin was able to protect the decreasing of CL along 9 hours and reached to approximately 80% inhibition. When comparison between curcumin and α-tocopherol at 25 μM after incubation with hemin 9 hours indicated that curcumin protect the decreasing of CA and CL more than α-tocopherol approximately 2 times (table 4.3)
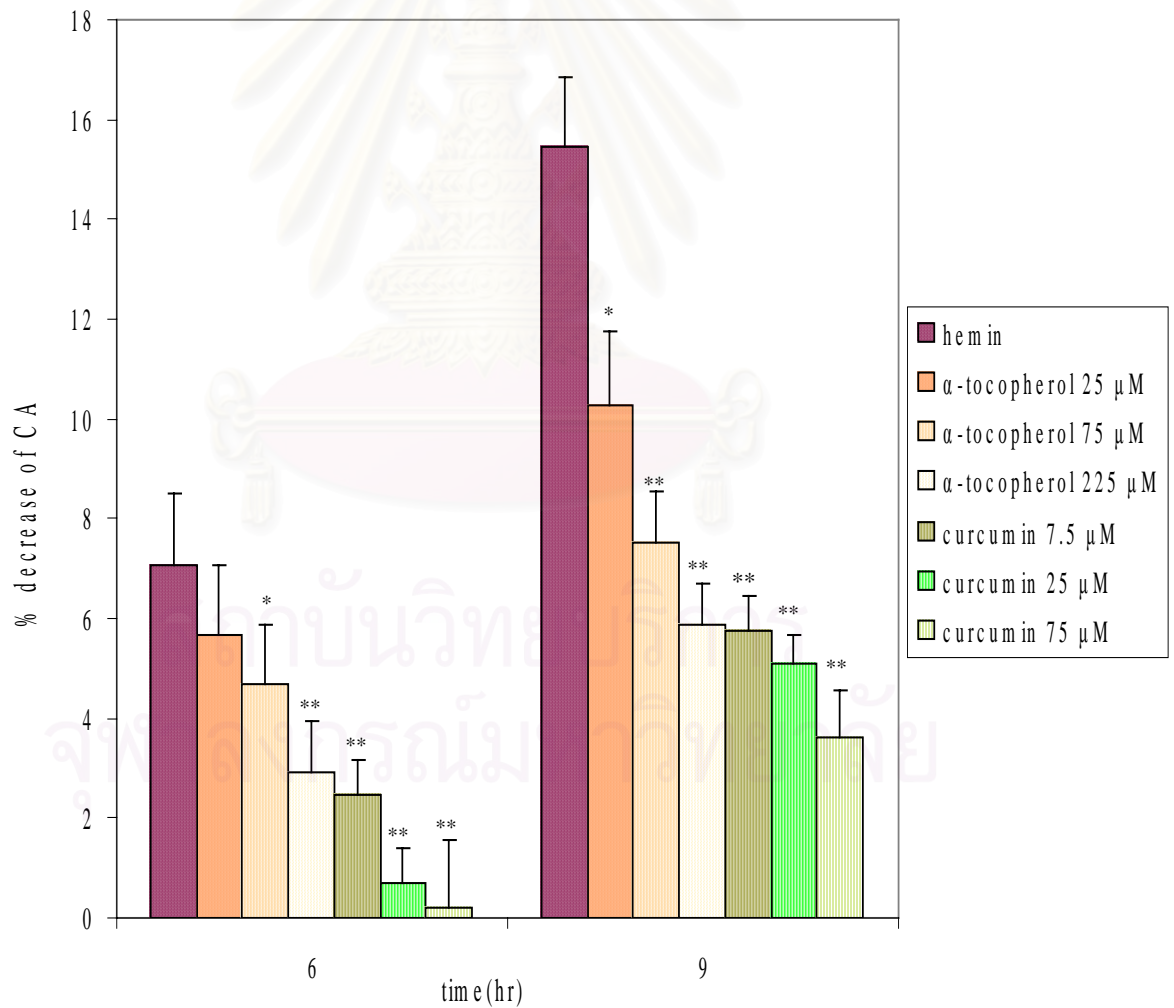
Figure 4.13 cont.
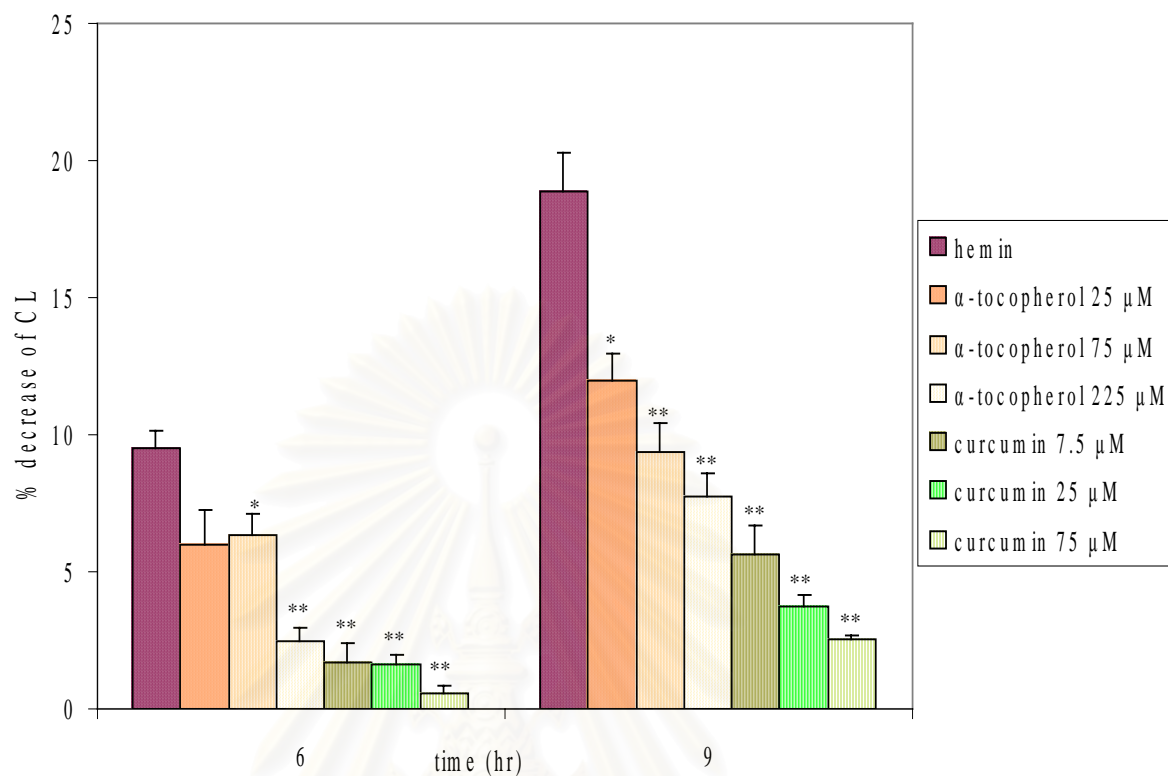
Figure 4.13 The effect of curcumin and α-tocopherol on % decrease of CA (A) and CL (B). The data were presented by mean of six individual subjects. Significant different * p<0.05, ** p<0.01 compared to hemin.

Table 4.3 the percentage inhibition of % decreasing of CA and CL by curcumin and α-tocopherol.

Dose of antioxidant	Percent inhibition of decreasing of CA (%)		Percent inhibition of decreasing of CL (%)	
	Curcumin	α-tocopherol	Curcumin	α-tocopherol
25 μM	65.35 ± 3.45	32.92 ± 7.09	79.90 ± 2.41	35.17 ± 5.95
75 μM	75.23 ± 7.79	50.82 ± 5.49	86.27 ± 1.65	48.23 ±7.52

Data are presented as mean of percent inhibition ± S.E.M of six individual subjects at 9 hours incubation.

3. The effect of curcumin and α-tocopherol on CL/CO ratio

The effect of hemin induced LDL oxidation on CL/CO ratio is showed in figure 4.7. The curcumin and α-tocopherol was able to protect decreasing of CL/CO ratio. However α-tocopherol showed significantly protection decreasing of CL/CO ratio on time 9 hours (figure 4.14). The comparison between curcumin and α-tocopherol at 25 μM after incubation with hemin 9 hours indicated that curcumin protect decreasing of CL/CO ratio more than α-tocopherol about 3 times (table 4.4).
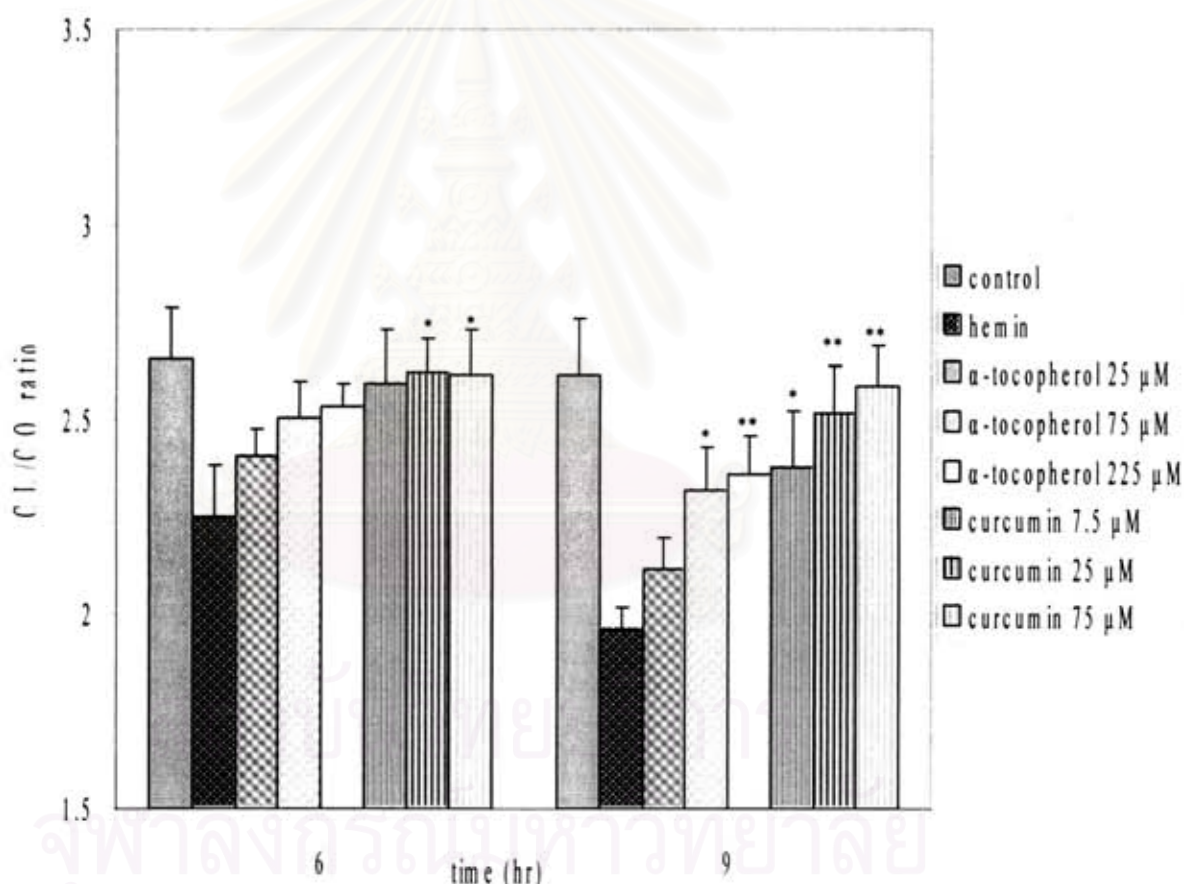
Figure 4.14 the effect of curcumin and α-tocopherol on CL/CO ratio. The data were presented by mean of six individual subjects. * $p < 0.05$, **$p < 0.01$ compared with hemin.

Table 4.4 the percentage inhibition of decreasing of CL/CO ratio by curcumin and
α-tocopherol.

Dose of antioxidant	Percent inhibition of decreasing of CL/CO (%)	
	Curcumin	α-tocopherol
25 μM	73.58 ± 6.10	23.85 ± 7.7
75 μM	82.37 ± 2.88	45.50 ± 5.5

Data are presented as mean of percent inhibition of six individual subjects at 9 hours
incubation.

4. The effect of curcumin and α-tocopherol on oxidized lipid products

The studies on part I showed that oxidized lipid products were occurred in hemin induce LDL oxidation. The study on part II (figure 4.15 A, B, C, D) found that α-tocopherol can not inhibit oxidized lipid products formation while curcumin significantly inhibited and completely inhibited oxidized lipid product formation at retention time of 10.2 and 11.7 min. Table 4.5 shows the percent inhibition of oxidized lipid products formation at 9 hours after incubation with hemin.

A

oxidized lipid product at RT 9.6 min

Fig 4.15 cont.

oxidized lipid product at RT 10.2 min

C

oxidized lipid product at RT 11.7 min
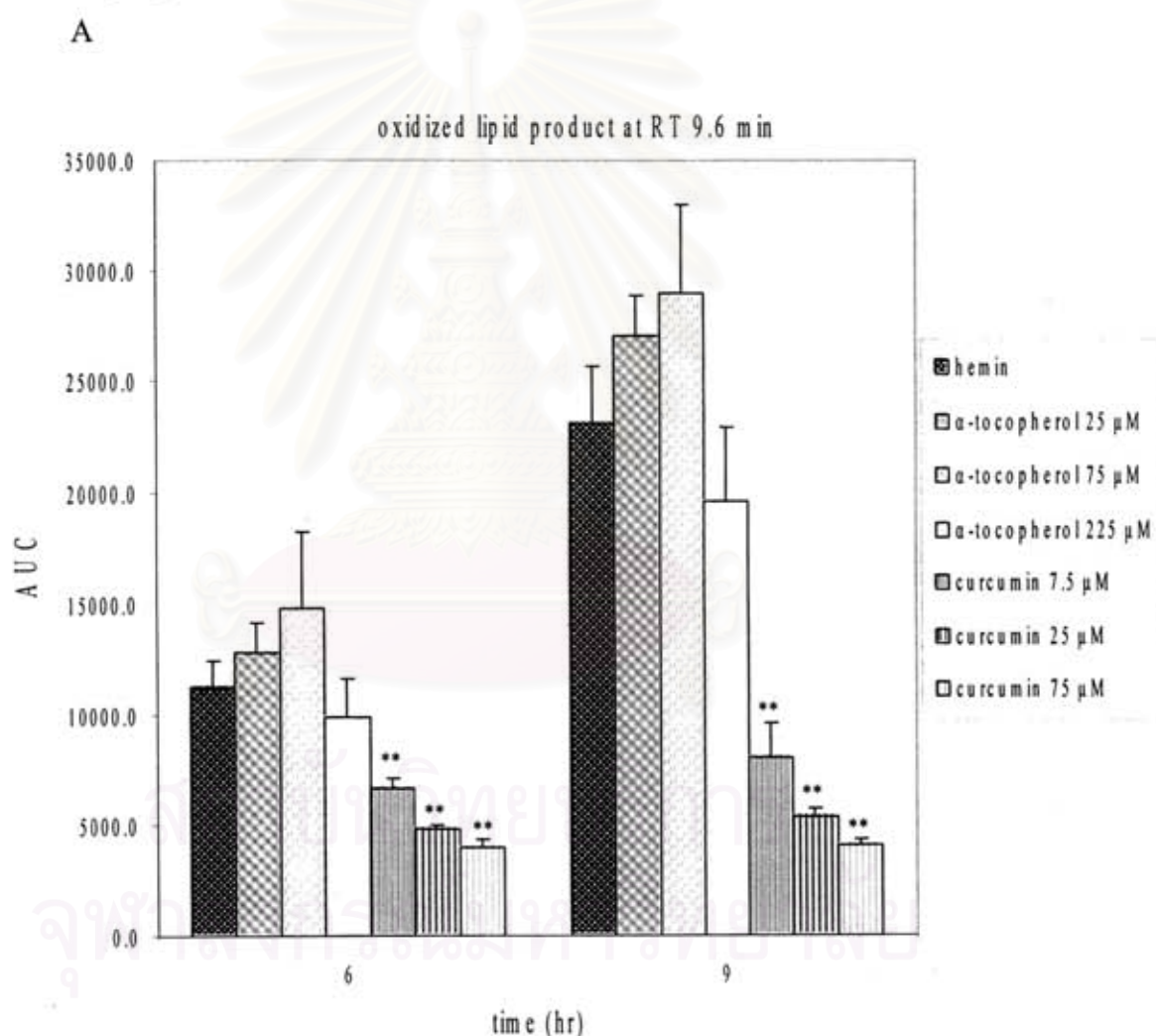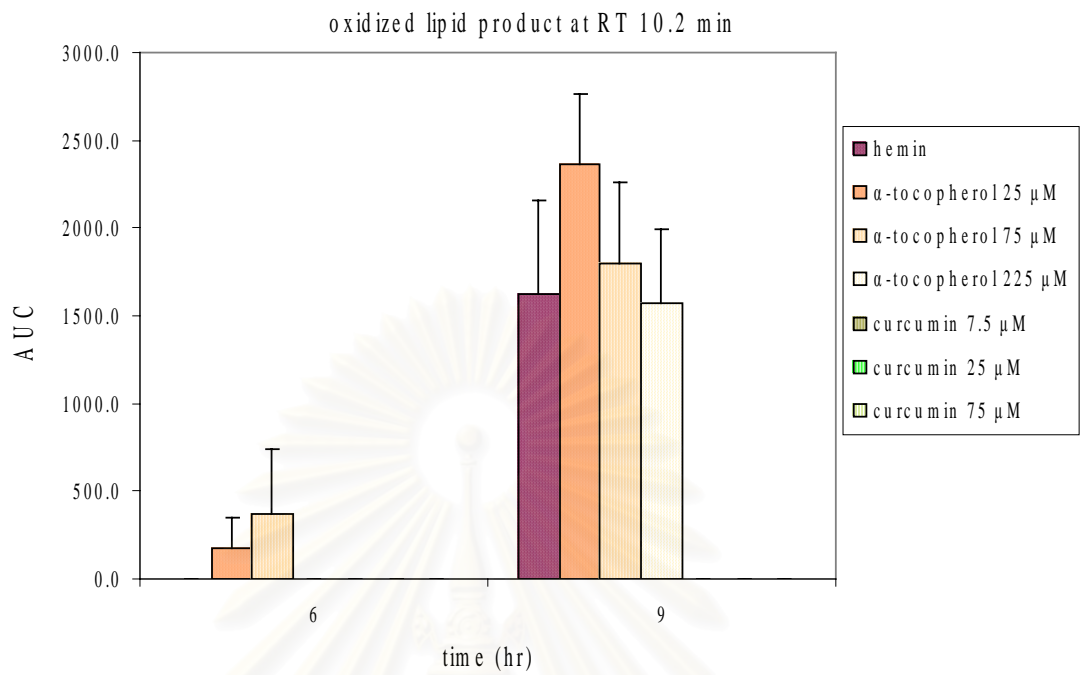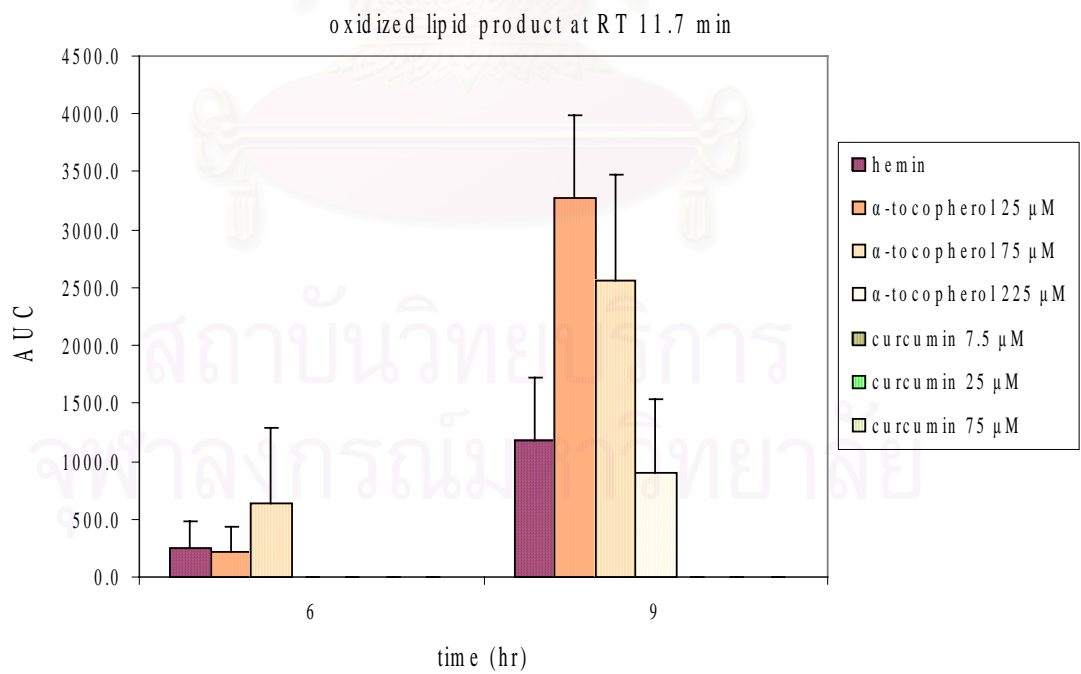
Fig 4.15 cont.

D
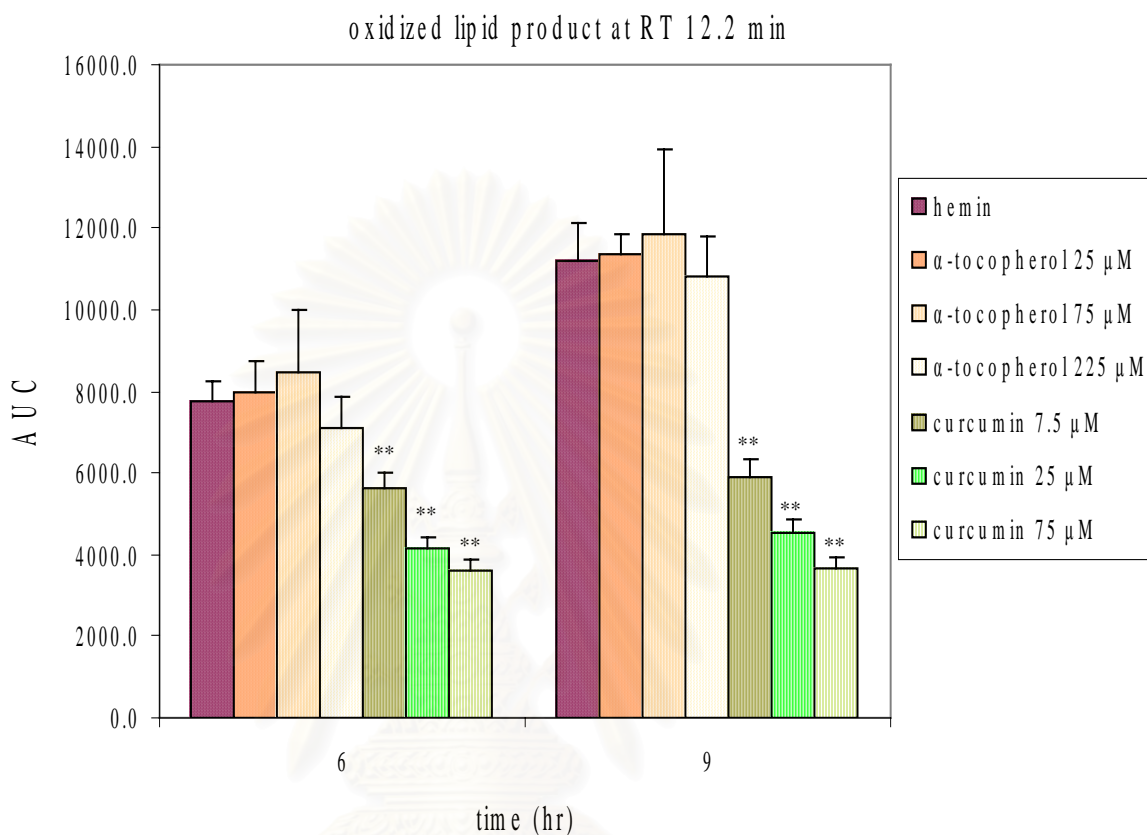
Figure 4.15 The effect of curcumin and α-tocopherol on oxidized lipid products. Figure showed the oxidized lipid products after pre incubate LDL 350 μg with PBS, α-tocopherol and curcumin which followed study design and then incubate with hemin 25 μM. The results were presented as mean ± S.E.M. of six individual subjects. Significant different *P<0.05 , **P <0.01 compared with hemin.

Table 4.5 the percentage inhibition of oxidized products formation at 9 hours.

Dose of curcumin	Percent inhibition of oxidized products formation (%)			
	RT 9.6	RT 10.2	RT 11.7	RT 12.2
7.5 μM	62.85 ± 7.9	100	100	45.18 ± 6.30
25 μM	74.95 ± 3.22	100	100	58.54 ± 4.10
75 μM	80.56 ± 3.45	100	100	65.34 ± 4.76

CHAPTER V
DISCUSSION AND CONCLUSION

DISCUSSION

This *in vitro* LDL oxidation demonstrated that hemin was powerful oxidant. During hemin induced LDL oxidation, α-tocopherol was rapidly decreased and shows correlation with TBARs (r=0.734, p<0.001). The depletion of α-tocopherol was indicated that α-tocopherol was consumed to protect the LDL oxidation. Hemin could trigger lipid peroxidation via free radical generated by the Fenton reaction (7, 8, 9). Hemin is taken up into the lipid core then degradation on the heme ring by LDL causes the release of free Fe^{3+} into the LDL particle. When introduced in the form of hemin, releasing Fe^{3+} is highly effective in oxidizing LDL core components and promoting oxidative changes in LDL constituents (68). In this study showed that hemin can decrease cholesteryl esters in the core of LDL, especially CA and CL. Moreover the percent decreasing of CA and CL were good correlation with TBARs (r=0.837 and 0.824 respectively; p<0.001), indicating that CA and CL were damaged by lipid peroxidation induced by hemin. Since CA and CL are polyunsaturated fatty acid which susceptible to oxidation more than monounsaturated and saturated fatty acid (36). CL is the most abundant of cholesteryl esters in LDL so it is a major target of lipid peroxidation. Also it has been previously found that cholesteryl linoleate hydroperoxides, intermediate products of lipid peroxidation, were accumulated in atherosclerotic lesions (13, 14). In addition, the severity degree of lipoprotein damage in β-thal/HbE patients can be determined by the CL/CO ratio of less than 2 (12). Our *in vitro* study showed that CL/CO ratio was also decreased less than 2 and was an inverse correlation with TBARs formation (r=-0.706, p<0.001), indicating that hemin cause highly oxidative damage of LDL similar as LDL of severe β-thal/HbE patients. This result confirmed that hemin might be an oxidant to induce oxidative modification in LDL of patient who was found hemin elevated in blood circulation.

This study found that curcumin and α- tocopherol were able to protect LDL oxidation by hemin. However curcumin inhibited TBARs formation more effective than α-tocopherol about 3 times (table 4.2). In addition, curcumin protected percent decreasing of CA and CL more than α-tocopherol approximately 2-3 times and also

protected decreasing of CL/CO ratio more than α-tocopherol approximately 3 times. Wei-Feng Chen et al. (30) demonstrated that curcumin and its analogues are potent inhibitors against AAPH- and Cu^{2+}-induced LDL peroxidation and suggested that the H-atom abstraction from the phenolic group is responsible for the antioxidant activity. The structure of curcumin have phenolic group more than α-tocopherol. In addition, H atom of curcumin was also abstracted from the central active CH_2 group in the heptadienone link (69). For the study of the effect of curcumin and α-tocopherol to protect the oxidized lipid products formation found that α-tocopherol can not protect oxidized lipid products formation while curcumin significantly inhibited and completely inhibited oxidized lipid product formation at retention time of 10.2 and 11.7 min. These results revealed that curcumin protected lipid peroxidation more than 80% so the oxidized lipid products formation was occurred in low levels. Thereby, curcumin has a potential to protect against oxidation more than α-tocopherol. Our result also demonstrated that curcumin have much potential to be use to protect LDL oxidation induced by hemin.

LDL modification by lipid composition change in hemin oxidized LDL (he-oxLDL) cause alteration of lipid fluidity (70) and may result in biological alteration. We study the effect of he-oxLDL on platelet aggregation in platelet rich plasma (PRP) and found that he-oxLDL enhanced ADP induced platelet aggregation. However two from six subjects were response to he-oxLDL. Several study showed the effect of oxidized LDL to platelet differently (37, 71, 72). Copper oxidized LDL, despite being able to augment ADP induced platelet aggregation, were not capable of independently triggering an aggregation response (71). In contrast, hypochlorite oxidized LDL modified to a comparable extent was revealed to be potent promoters of platelet aggregation (71). These different effects may be involved variation of apo B modification. LDL modification by hypochlorite resulted in almost exclusive modification of the apo B moiety without inducing the formation of lipid peroxides (72). It seem that the effect of oxidized LDL on platelet function arise from modification of apo B. In present study, apo B modification was not investigated, so the variation in apo B modification may result in non consistent effect on platelet aggregation. In addition, the non consistent effect of he-oxLDL on platelet

aggregation in PRP may be result from variations of components in PRP and LDL of each subject such as antioxidant level, antiplatelet aggregation mediator and lipid profile. These variations varied even in blood of the same donor from different time collecting (16). Therefore, further study of apo B modification in he-oxLDL and the effect on platelet aggregation should be investigated in washed platelet.

CONCLUSION

This study was concluded that during hemin induced LDL oxidation, lipid composition in LDL was changed especially CA and CL. Both curcumin and α-tocopherol have the protective effect on hemin induced LDL oxidation. However curcumin was more potent than α-tocopherol to protect against hemin induced LDL oxidation. The results can be used for consideration of using curcumin to protect LDL oxidation in patient who was found hemin elevated in blood circulation.

The effect of he-oxLDL on platelet aggregation in PRP is still not clear. So, further study to clarify this effect should be investigated.
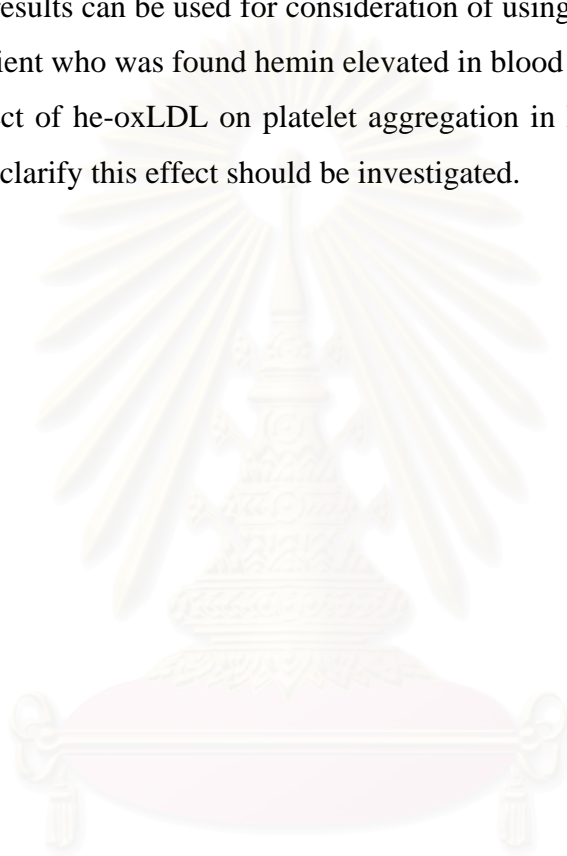
REFERENCES

(1)   Witztum J.L., Steinberg D. 1991. Role of oxidized low density lipoprotein in atherogenesis. <u>J. Clin. Invest.</u> 88: 1785-1792

(2)   Berliner J.A., Navab M., Fogelman Am., Frank J.S., Demer L.L., Edwards P.A., Watson A.D. Lusis A.J. 1995. Atherosclerosis: Basic mechanisms: Oxidation, inflammation, and genetics. <u>Circulation</u>. 91: 2488-2496.

(3)   Shaklai N., Shviro Y., Rabizadeh E., Kirschner-Zilber I. 1985. Accumulation and drainage of hemin in the red cell membrane <u>Biochim, Biophys. Acta.</u> 821: 355-366.

(4)   Hebbel R.P., Eaton JW. 1989. Pathobiology of heme interaction with the erythrocyte membrane. <u>Semin, Hematol.</u> 26: 136-149.

(5)   Miller Y., Shaklai N. 1999 Kinetic of hemin distribution in plasma reveals its role in lipoprotein oxidation, <u>Biochim Biophys Acta</u>. 1454: 153-164.

(6)   Phumala N., Porasuphatan, S., Unchern S., Pootraku P., Fucharoen S. Chantharaksri U. 2003. Hemin: a possible cause of oxidative stress in blood circulation of β-thalassemia/Hemoglobin E disease. <u>Free Radic Res</u>. 37: 129-135.

(7)   Balla G., Jacob H.S., Eaton J.W., Belcher J.D., Vercellotti G.M. 1991. Hemin : a possible physiological mediator of low density lipoprotein oxidation and endothelial injury. <u>Arterioscler. Thromb</u>. 11: 1700-1711.

(8)   Miller Y.I., Felikman Y., Shanklai N. 1995. The involvement of low-density lipoprotein in hemin transport potentiates peroxidative damage. <u>Biochim Biophys Acta</u>. 1272: 119-27.

(9)   Camajio G., Halberg C., Maschik-Lundin A., Hurt-Camejo E., et al. 1998. Hemin binding and oxidation of lipoproteins in serum: mechanism and effect of the interaction of LDL with human macrophages. <u>J Lipid Res</u> 39: 755-56.

(10) Miller Y.I., Felikman Y., Shanklai N., 1996. Hemoglobin induced apolipoprotein B crosslinking in low-density lipoprotein peroxidation. <u>Biochim Biophys</u>.326(2):252-260.

(11) Steinberg, D., 1997. Oxidative modification of LDL and atherogenesis. Circulation. 95:1062-1071.

(12) Luechapudiporn R., Phumala M.N., Jucharoen, S., Chantharaksri, U. 2006. The reduction of cholesteryl linoleate in lipoproteins: an index of clinical severity in β-thalassemia/Hb E.

(13) Chisolm G., Ma G., Irwin K., Martin L., Gunderson K., Linberg L. 1994. 7β-hydroperoxycholest-5-en-3β-ol, a component of human atherosclerotic lesions, is the primary cytotoxin of oxidized low density lipoprotein. Proc Natl Acad Sci USA. 91: 11452-6.

(14) Brook C.J., Harland W.A., Steel G. 1970. Lipids of human atheroma: isolation of hydroxyoctadecadienoic acids from advanced aortal lesions. Biochim Biophys Acta. 202:563-6.

(15) Belkner J., Stender H., Kuhn H. 1998. The rabbit 15-lipoxygenase preferentially oxygenates LDL cholesterol esters, and this reaction does not require vitamin E. J Biol Chem. 273:23225-32.

(16) Volf I., Moeslinger T., Cooper J., Schmid W., Koller E., 1999. Human platelets exclusively bind oxidized low density lipoprotein showing no specificity for acetylated low density lipoprotein. FEBS Lett. 449 : 141-145.

(17) Esterbauer H., Dieber-Rothenneder M., Striegl G., Wang G. 1991. Role of vitamin E in preventing the oxidation of low-density lipoprotein. Am. J. Clin. Nutri. 53: 314s-321s.

(18) Esterbauer H., Gebicki J., Puhl H., Jurgens G. 1992. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Rad Biol Med. 13:341-90.

(19) Unchern S., Laoharuangpanya N., Phumala N., Sipankapracha P., Pootrakul, P., Fucharoen S., Wanachivanawin W., and Chantharaksri U. 2003. The effects of vitamin E on platelet activity in β-thalassaemia patients. British Journal of Haematology. 123, 738-744.

(20) Buescher R., Yang L. 2000. Turmeric, in natural food colorants. In: Lauro, G. J., Francis, F.J., eds. New York: Dekker; 205-226.

(21) Rukkumani R., Balasubashini M.S., Menon V.P. 2003. Protective effects of curcumin and photo-irradiated curcumin on circulatory lipids and lipid peroxidation products in alcohol and polyunsaturated fatty acid-induced toxicity. Phytother. Res. 17, 925-929.

(22) Naidu K.A., Thippeswamy N.B. 2002 Inhibition of human low density lipoprotein oxidation by active principle from spices. Mol. Cell. Biochem. 229, 19-23.

(23) Venkatesan P., Rao M.N.A. 2000. Structure-activity relationships for the inhibition of lipid peroxidation and the scavenging of free radicals by synthetic symmetrical curcumin analogues. J. Pharm. Pharmacol. 52, 1123-1128.

(24) Daniel S., Limson J.L., Dairam A., Watkins G.M., Daya S. 2004. Throuh metal binding, curcumin protects against lead-and cadmium induced lipid peroxidation in rat brain homogenates and against lead induced tissue damage in rat brain. J. Inorg. Biochem. 98, 266-275.

(25) Priyadarsini K.I., Maity D.K., Naik G.H., Kumar M.S., Unnikrishnan M.K., Satav J.G., Mohan H. 2003. Role of phenolic O-H and methylene hydrogen on the free radical reaction and antioxidant activity of curcumin. Free Radic. Biol. Med. 35, 475-484.

(26) Wright J.S. 2002. Predicting the antioxidant activity of curcumin and curcuminoids. J. Mol. Struct. (Theochem) 591, 207-217.

(27) Patro B.S., Rele S., Chintalwar G.J., Chattopadhyay S., Adhikari S., Mukherfee T. 2002. Protective activities of some phenolic 1,3-diketones against lipid peroxidation: possible involvement of the 1,3-diketone moiety. ChemBioChem. 3,364-370.

(28) Sreejayan N. Rao M.N. 1997. Nitric oxide scavenging by curcuminoids. J Pharm Pharmacol. 49(1) : 105-107.

(29) Sreejanya N., Rao M.N. 1996. Free radical scavenging activity of curcuminoids. Arzneimittelforschung. 46(2) : 169-171.

(30) Wei-Feng C., Sui-Ling Deng., Bo Z., Li Y., Zhong-Li L. 2006. Curcumin and its analogues as potent inhibitors of low density lipoprotein oxidation: H-atom abstraction from the phenolic groups and possible

involvement of the 4-hydroxy-3-methoxyphenyl groups. <u>Free Radical Biology & Medicine.</u> 40: 526-535.

(31) Hevonoja T., Pentikainen M.O., Hyvonen M.T., Kovanen P.T., Ala-Karpela M., 2000. Structure of low density lipoprotein (LDL) particles: Basis for understanding molecule changes in modified LDL. <u>Biochim Biophys Acta. </u>1488:189-210.

(32) Converses C.A., Skinner E.R. 1992. <u>Lipoprotein Analysis A Practical Approach</u>. Cambrian Typesetters, Frimley, surrey : Information Press Ltd, Eynsham, Oxford.

(33) Berliner J.A., Henecke J.W. 1996. The role of oxidized lipoproteins in atherogenesis. <u>Free Radic. Biol. Med.</u> 20 : 707-727.

(34) Parthasarathy S., Santanam N., Ramachandran S., Meilhac O. 1999. Oxidants and antioxidants in atherogenesis: an appraisal. <u>J. Lipid. Res</u>. 40: 2143-2157.

(35) Steinbrecher U., Parthasrathy S., Leake D.S., Witztum J.L., Steinberg D., 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. <u>Proc. Natl. Acad. Sci.</u> 81: 3883-3887.

(36) Okezie L.A., Susan L.C. 1997. <u>Antioxidant Methodology In vivo and in vitro Concepts. </u>AOCS Press. United States.

(37) Leon G., Coleman Jr., Renata K., Ploanowska-Grabowska, Marek Marcinkiewicz, Adrian R.L. Gear. 2004. LDL oxidized by hypochlorous acid causes irreversible platelet aggregation when combined with low levels of ADP, Thrombin, epinephrine, or macrophage-derived chemokine (CCL22). <u>Blood</u>. 104 : 2, 380-389.

(38) Evers J., Hsia N. 1997. Toxicities of native and modified hemoglobins. Free Radical Biol Med. 22: 1075-99.

(39) Gawaz M. 2001. <u>Blood platelets: physiology, pathophysiology, membrane receptors, antiplatelet principles, and therapy for atherothrombotic diseases</u>. Georg Thieme Verlag. Germany.

(40) Kuwahara M., Sugimoto M., Tsuji S., Matsue H., Mizuno T., Miyata S., Yoshioka A. 2002. Platelet shape changes and adhesion under high shear flow. Arterioscler. Throm. Vasc. Biol. 22: 329-34.

(41) Packer L. 1993. The vitamin E antioxidant cycle in health and disease. NATO ASI Ser., Ser. A, 10: 197-204.

(42) Liebler D.C. 1993. The role of metabolism in the antioxidant function of vitamin E. Crit. Rev. Toxidol., 23 : 147-169.

(43) Steven I., Baskin, Harry Salem. 1997. Oxidants, Antioxidants, and Free Radicals. Taylor & Francis : United States of America.

(44) Govindarajan V.S. 1980. Turmeric-Chemistry, technology and quality. CRC Crit. Rev. Food Sci. Nutr. 12 : 199-246.

(45) Aggarwal B.B., Kumar A., Aggarwal M.S., Shishodia S. 2005. Curcumin derived from tumeric (Curcuma longa) : a spice for all seasons. In: preuss H, ed. Phytopharmaceuticals in Cancer Chemoprevention. Boca Raton: CRC Press. 349-387.

(46) Sharma R.A., Gescher A.J., Steward W.P. 2005. Curcumin: The story so far. Eur J Cancer. 41 (13) : 1955-1968.

(47) Ruby A.J., Kuttan G., Babu K.D. 1995. Anti-tumour and antioxidant activity of natural curcuminoids. Cancer Lett. 94 : 79-83.

(48) Sreejayan R.M.N., 1994. Curcuminoids as potent inhibitors of lipid peroxidation. J Pharm. Pharmacol. 46 : 1013-1016.

(49) Ireson C.R., Jones D.J., Orr S., et al. 2002. Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. Cancer Epidemiol Biomarkers Prev. 11(1) : 105-111.

(50) Ireson C.R., Orr S., Jones D.J., et al. 2001. Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production. Cancer Res. 61(3) : 1058-1064.

(51) Cheng A.L., Hsu Ch., Lin J.K., et al. 2001. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res. 21(4B) : 2895-2900.

(52) Sharma R.A., Euden S.A., Platton S.L., et al. 2004. Phase I clinical trial of oral curcumin : biomarkers of systemic activity and compliance. <u>Clin Cancer Res</u>. 10(20) : 6847-6854.

(53) Garcea G., Berry D.P., Jones D.J., et al. 2005. Consumption of the putative chemopreventive agent curcumin by cancer patients : assessment of curcumin levels in the colorectum and their pharmacodynamic consequences. <u>Cancer Epidemiol Biomarkers Prev.</u> 14(1) : 120-125.

(54) Garcea G., Jones D.J., Singh R., et al. Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration. <u>Br J Cancer.</u> 90(5) : 1011-1015.

(55) You-Min Sun, Hong-Yu Zhang, De-Zhan Chen, Cheng-Bu Liu. 2002. Theoretical elucidation on the antioxidant mechanism of curcumin : A DFT study. <u>Organic Lett.</u> 4(17) : 2909-2911.

(56) Joe B., Vijaykumar M., Lokesh B.R. 2004. Biological properties of curcumin-cellular and molecular mechanism of action. <u>Crit Rev Food Sci Nutr.</u> 44(2) : 97-111.

(57) Lechtenberg M., Quandt B., Nahrstedt A. 2004. Quantitative determination of curcuminoids in Curcuma rhizomes and rapid differentiation of *Curcuma domestica* Val. And *Curcuma xanthorrhiza* Roxb. By capillary electrophoresis. <u>Phytochem Anal.</u> 15(3):152-158.

(58) Heath D.D., Khwaja F., Rock C.L. 2004. Curcumin content of turmeric and curry powders. <u>FASEB J</u>. 18(4) : A125.

(59) U.S. 2005. <u>Food and Drug Administration</u>. Food Additive Status List. Available at : http://www.cfsan.fda.gov/~dms/opa-appa.html.

(60) Rasyid A., Lelo A. 1999. The effect of curcumin and placebo on human gall-bladder function: an ultrasound study. <u>Aliment Pharmacol Ther.</u> 13(2) : 245-249.

(61) Rasyid A., Rahman A.R., Jaalam K., Lelo A. 2002. Effect of different curcumin dosages on human gall bladder. <u>Asia Pac J Clin Nutr.</u> 11(4) : 314-318.

(62) Harvel R.J., Eder H.A., Bragdon J.H. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest. 34 : 1345-1353.

(63) Uchiyama U., Mihara M. 1978. Determination of malonaldehyde precursor in tissue by thiobarbituric acid test. Analytical Biochem. 86 : 271-278.

(64) Asakawa T., Matsushita S., 1980. Coloring condition of thiobarbituric acid test for detecting lipid hydroperoxide. Lipids. 15 : 137-140.

(65) Markwell MAK, M., Haas SM., Bieber, LL., Tolbert, NE. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem. 87 : 206-210.

(66) Seta K., Nakamura H., Okuyama T. 1990. Determination of α-tocopherol, free cholesterol, esterified cholesterol and triacylglycerols in human lipoproteins by high performance liquid chromatography. J Chromatogr. 515 : 585-589.

(67) Zaspel B. Csallany A. 1983. Determination of alpha-tocopherol in tissues and plasma high performance liquie chromatography. Anal Biochem. 130 : 146-150.

(68) Tribble DL, Chu BM., Levine GA., Krauss RM., Gong EL. 1996. Selective resistance of LDL core lipid to iron-mediated oxidation: implications for the biological properties of iron-oxidized LDL. Arterioscler. Thromb. Vasc. Biol. 16: 1580-1587.

(69) Jovanovie S.V., Steenken S., Boone C.W., Simic M.G. 1999. H-atom transfer is a preferred antioxidant mechanism of curcumin. J. Am. Chem. Soc. 121: 9677-9681.

(70) Chalermkhwan Ch. 2004. Studies of hemin and Fe-NTA induced lipid fluidity change in low- and high- density lipoprotein. The degree of master of science (pharmacology), Faculty of graduate studies, Mahidol university.

(71) Volf I., Roth A., Cooper J., Moeslinger T., Koller E. 2000. Hypochlorite modified LDL are a stronger agonist for platelets than copper oxidized LDL.FEBS Letters. 483, 155-159.

(72) Hazell L.J., Stocker R. 1993. Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages. Biochem. J. 290: 165-172.

APPENDICES

APPENDIX A

Experiment 1

1. TBARs levels in oxidized LDL after incubate with hemin

				TBARs (nmol/mg)			
Time of incubation (hr)	0	1	3	6	9	24
N						
N1	-0.05	0.39	0.70	2.12	9.16	20.75
N2	0.64	3.09	5.25	10.10	13.73	24.96
N3	0.80	1.19	3.03	8.26	16.69	35.72
N4	2.47	3.10	5.09	16.22	17.81	32.93
N5	0.83	3.31	3.89	8.43	12.77	25.88
N6	0.39	1.40	2.33	3.67	9.70	33.79
Mean	**0.85**	**2.08**	**3.38**	**8.14**	**13.31**	**29.01**
S.E.	0.35	0.51	0.71	2.05	1.44	2.43

2. α-tochopherol levels in oxidized LDL after incubate with hemin

			α-tochopherol (μmol/g protein)				
Time of incubation (hr)	0	1	3	6	9	24
N						
N1	21.71	9.41	3.86	0.00	0.00	0.00
N2	13.12	4.35	2.68	2.61	0.00	0.00
N3	26.33	11.27	3.08	0.00	0.00	0.00
N4	23.94	0.00	0.00	0.00	0.00	0.00
N5	25.58	6.17	0.00	0.00	0.00	0.00
N6	25.06	4.64	0.00	0.00	0.00	0.00
Mean	**22.62**	**5.98**	**1.60**	**0.43**	**0.00**	**0.00**
S.E.	2.01	2.67	0.54	0.19	0.00	0.00

3. Total cholesterol levels in oxidized LDL after incubate with hemin

Time of incubation (hr) N	Total cholesterol (mmol/g protein)					
	0	1	3	6	9	24
N1	2.04	2.50	2.73	2.78	3.08	2.53
N2	2.40	2.43	2.41	2.40	2.50	2.88
N3	4.96	4.62	4.67	4.69	4.74	5.36
N4	3.14	2.76	3.02	3.65	3.64	3.88
N5	3.68	3.80	4.08	3.88	4.46	4.38
N6	3.63	5.06	4.25	4.69	4.92	4.58
Mean	**3.31**	**3.53**	**3.53**	**3.68**	**3.89**	**3.94**
S.E.	0.39	0.42	0.34	0.36	0.36	0.40

4. Free cholesterol levels in oxidized LDL after incubate with hemin

Time of incubation (hr) N	Free cholesterol (mmol/g protein)					
	0	1	3	6	9	24
N1	0.546	1.021	1.127	1.000	0.950	0.526
N2	1.062	1.073	1.140	1.144	1.136	1.178
N3	1.456	1.278	1.489	1.364	1.709	1.807
N4	1.129	1.183	1.198	1.164	1.244	1.235
N5	1.348	1.338	1.324	1.395	1.344	1.270
N6	1.330	1.352	1.398	1.299	1.357	1.896
Mean	**1.145**	**1.207**	**1.279**	**1.228**	**1.290**	**1.319**
S.E.	0.134	0.057	0.060	0.062	0.104	0.202

5. Cholesteryl linolenate in oxidized LDL after incubate with hemin

Time of incubation (hr)	Cholesteryl linolenate (mmol/g protein)					
N	0	1	3	6	9	24
N1	0.014	0.017	0.021	0.012	0.011	0.005
N2	0.043	0.042	0.036	0.041	0.035	0.032
N3	0.072	0.061	0.039	0.075	0.086	0.050
N4	0.053	0.062	0.062	0.059	0.045	0.041
N5	0.034	0.033	0.035	0.033	0.032	0.013
N6	0.033	0.032	0.034	0.032	0.031	0.012
Mean	**0.041**	**0.041**	**0.038**	**0.042**	**0.040**	**0.026**
S.E.	0.008	0.007	0.005	0.009	0.010	0.007

6. Cholesteryl arachidonate levels in oxidized LDL after incubate with hemin

Time of incubation (hr)	Cholesteryl arachidonate (mmol/g protein)					
N	0	1	3	6	9	24
N1	0.067	0.070	0.069	0.069	0.057	0.030
N2	0.185	0.178	0.179	0.172	0.164	0.148
N3	0.265	0.261	0.293	0.219	0.195	0.093
N4	0.140	0.122	0.128	0.123	0.115	0.101
N5	0.137	0.138	0.143	0.133	0.128	0.075
N6	0.136	0.130	0.139	0.125	0.119	0.055
Mean	**0.155**	**0.150**	**0.158**	**0.140**	**0.130**	**0.084**
S.E.	0.027	0.026	0.031	0.021	0.019	0.017

7. Cholesteryl linoleate levels in oxidized LDL after incubate with hemin

Time of incubation (hr) N	Cholesteryl linoleate (mmol/g protein)					
	0	1	3	6	9	24
N1	0.92	0.92	0.89	0.87	0.78	0.62
N2	1.40	1.40	1.32	1.30	1.25	1.06
N3	2.36	2.29	2.29	2.07	1.80	1.55
N4	1.25	1.22	1.28	1.13	0.98	0.81
N5	1.18	1.18	1.16	1.07	0.96	0.71
N6	1.30	1.27	1.35	1.16	1.05	0.93
Mean	**1.40**	**1.38**	**1.38**	**1.27**	**1.14**	**0.95**
S.E.	0.20	0.19	0.19	0.17	0.15	0.14

8. Cholesteryl oleate levels in oxidized LDL after incubate with hemin

Time of incubation (hr) N	Cholesteryl oleate (mmol/g protein)					
	0	1	3	6	9	24
N1	0.314	0.353	0.393	0.412	0.337	0.346
N2	0.711	0.718	0.712	0.626	0.692	0.784
N3	1.283	1.037	0.969	1.222	1.268	1.341
N4	0.510	0.582	0.608	0.610	0.558	0.555
N5	0.456	0.489	0.494	0.473	0.442	0.436
N6	0.418	0.406	0.506	0.476	0.413	0.451
Mean	**0.615**	**0.598**	**0.614**	**0.637**	**0.618**	**0.652**
S.E.	0.144	0.103	0.084	0.122	0.139	0.151

9. Cholesteryl palmitate levels in oxidized LDL after incubate with hemin

Time of incubation (hr)	Cholesteryl palmitate (mmol/g protein)					
N	0	1	3	6	9	24
N1	0.193	0.193	0.200	0.073	0.051	0.036
N2	0.558	0.543	0.490	0.448	0.431	0.342
N3	0.053	0.013	0.009	0.037	0.207	0.424
N4	0.202	0.097	0.193	0.176	0.136	0.148
N5	0.207	0.228	0.182	0.186	0.184	0.177
N6	0.110	0.111	0.082	0.116	0.116	0.120
Mean	**0.220**	**0.198**	**0.193**	**0.173**	**0.188**	**0.208**
S.E.	0.176	0.185	0.164	0.147	0.131	0.146

10. Total cholesterol levels in oxidized LDL after incubate with hemin

Time of incubation (hr)	TBARs (nmol/mg)					
N	0	1	3	6	9	24
N1	0.546	1.021	1.127	1.000	0.950	0.526
N2	1.062	1.073	1.140	1.144	1.136	1.178
N3	1.456	1.278	1.489	1.364	1.709	1.807
N4	1.129	1.183	1.198	1.164	1.244	1.235
N5	1.348	1.338	1.324	1.395	1.344	1.270
N6	1.330	1.352	1.398	1.299	1.357	1.896
Mean	**1.145**	**1.207**	**1.279**	**1.228**	**1.290**	**1.319**
S.E.	0.134	0.057	0.060	0.062	0.104	0.202

Appendix B

Experiment 2

11. Effect of α-tochopherol and curcumin on TBARs levels in hemin oxidized LDL
(he-oxLDL)

Time of incubation (hr)	N	TBARs (nmol/mg)						
		control	Dose of α-tochopherol (μM)			Dose of curcumin (μM)		
			25	75	225	7.5	25	75
0	N1	0.28	0.21	0.09	0.17	0.23	0.37	0.36
	N2	0.43	0.47	0.73	0.43	0.50	0.55	0.45
	N3	2.47	3.17	3.76	3.16	2.63	2.62	2.75
	N4	0.25	0.18	0.05	0.14	0.20	0.34	0.33
	N5	0.83	1.13	1.33	1.20	0.93	1.12	1.36
	N6	0.39	0.64	0.55	0.48	0.33	0.44	0.57
	Mean	**0.77**	**0.97**	**1.08**	**0.93**	**0.80**	**0.91**	**0.97**
	S.E.	0.35	0.46	0.57	0.47	0.38	0.36	0.39
6	N1	6.83	4.02	3.57	3.18	2.08	1.27	1.31
	N2	8.44	7.93	7.43	5.14	2.74	2.00	1.37
	N3	16.22	15.78	14.80	10.76	4.83	3.31	4.34
	N4	5.18	4.26	3.35	2.10	1.48	1.29	0.96
	N5	8.43	4.87	4.12	4.34	3.32	2.48	1.79
	N6	3.67	2.42	3.63	3.20	1.61	1.18	0.81
	Mean	**8.13**	**6.55**	**6.15**	**4.79**	**2.67**	**1.92**	**1.76**
	S.E.	1.79	1.99	1.84	1.27	0.52	0.35	0.53
9	N1	9.23	7.74	4.31	3.70	2.80	1.54	1.38
	N2	11.90	10.40	10.27	6.74	4.74	2.76	1.86
	N3	17.81	16.45	16.31	16.59	4.98	4.13	4.18
	N4	10.75	6.22	6.28	3.82	7.25	1.02	1.39
	N5	12.77	9.72	9.31	7.68	3.44	2.83	1.87
	N6	9.70	4.83	4.92	3.96	1.90	1.37	0.88
	Mean	**12.03**	**9.23**	**8.57**	**7.08**	**4.19**	**2.28**	**1.93**
	S.E.	1.28	1.68	1.83	2.02	0.78	0.48	0.48

12. Effect of α-tochopherol and curcumin on α-tochopherol levels in he-oxLDL

Time of incubation (hr)	N	α-tochopherol (μmol/g protein)						
		control	Dose of α-tochopherol (μM)			Dose of curcumin (μM)		
			25	75	225	7.5	25	75
0	N1	17.19	30.74	31.71	33.45	11.67	14.50	14.45
	N2	10.29	11.22	13.54	12.70	4.49	5.79	6.00
	N3	2.97	3.71	4.17	4.42	5.57	3.37	3.06
	N4	6.04	6.09	5.64	4.98	2.59	4.44	4.60
	N5	10.33	11.75	15.12	20.33	8.01	9.22	9.06
	N6	9.37	10.95	14.46	17.78	7.44	8.05	7.82
	Mean	**9.37**	**12.41**	**14.11**	**15.61**	**6.63**	**7.56**	**7.50**
	S.E.	1.96	3.90	4.01	4.44	1.29	1.65	1.65
6	N1	2.36	5.82	8.22	6.28	6.10	8.39	7.57
	N2	2.61	2.77	5.05	6.23	2.77	3.95	5.30
	N3	0.00	2.08	3.40	2.69	0.00	2.81	3.34
	N4	0.00	3.78	6.48	4.61	2.42	4.42	3.21
	N5	6.17	4.24	4.87	5.72	4.55	4.96	6.70
	N6	4.64	3.23	3.68	4.35	3.50	4.02	5.70
	Mean	**2.63**	**3.65**	**5.28**	**4.98**	**3.22**	**4.76**	**5.30**
	S.E.	1.01	0.53	0.74	0.56	0.84	0.78	0.72
9	N1	0.00	2.36	2.52	5.58	2.40	6.25	7.32
	N2	1.90	2.62	2.60	2.65	2.64	3.25	5.08
	N3	0.00	2.08	3.36	3.02	0.00	2.75	3.29
	N4	0.00	3.36	2.45	3.40	0.00	2.83	2.89
	N5	0.00	4.05	4.82	5.62	4.25	4.79	6.49
	N6	0.00	3.46	3.80	4.58	3.31	3.68	5.89
	Mean	**0.32**	**2.99**	**3.26**	**4.14**	**2.10**	**3.93**	**5.16**
	S.E.	0.32	0.31	0.38	0.53	0.71	0.55	0.72

13. Effect of α-tochopherol and curcumin on Total cholesterol levels in he-oxLDL

Time of incubation (hr)	N	Total cholesterol (mmol/g protein)						
		control	Dose of α-tochopherol (μM)			Dose of curcumin (μM)		
			25	75	225	7.5	25	75
0	N1	2.97	3.49	3.29	3.28	3.35	3.61	2.98
	N2	2.50	3.07	2.77	2.41	2.82	2.77	2.76
	N3	3.14	3.11	3.52	3.46	3.62	3.26	3.69
	N4	5.92	5.92	5.49	5.83	6.18	6.14	6.79
	N5	3.68	4.70	3.91	4.39	3.69	4.31	4.37
	N6	3.63	3.73	4.60	4.68	4.07	4.63	4.46
	Mean	**3.64**	**4.00**	**3.93**	**4.01**	**3.96**	**4.12**	**4.17**
	S.E.	0.45	0.41	0.37	0.45	0.43	0.45	0.54
6	N1	3.68	3.28	2.90	3.03	3.47	3.23	3.43
	N2	2.82	2.67	2.70	2.87	2.82	2.68	2.55
	N3	3.65	2.99	3.26	3.59	4.05	3.60	3.75
	N4	6.58	6.14	5.92	6.49	6.75	6.36	6.36
	N5	3.88	3.74	4.30	4.46	4.07	3.94	4.06
	N6	4.69	4.85	4.58	4.75	4.18	4.65	4.52
	Mean	**4.22**	**3.94**	**3.94**	**4.20**	**4.22**	**4.08**	**4.11**
	S.E.	0.48	0.49	0.46	0.50	0.50	0.48	0.48
9	N1	3.39	3.05	3.11	3.16	3.00	3.27	3.26
	N2	2.89	2.73	2.60	2.68	2.64	2.83	2.91
	N3	3.64	3.27	3.94	3.60	3.25	3.67	3.48
	N4	6.36	6.27	6.31	6.53	6.58	7.23	6.49
	N5	4.46	4.17	3.73	3.91	4.56	4.27	4.25
	N6	4.92	4.53	4.73	4.35	4.41	4.70	4.06
	Mean	**4.28**	**4.00**	**4.07**	**4.04**	**4.07**	**4.33**	**4.08**
	S.E.	0.47	0.49	0.49	0.50	0.54	0.59	0.48

14. Effect of α-tochopherol and curcumin on free cholesterol in he-oxLDL

Time of incubation (hr)	N	Free cholesterol (mmol/g protein)						
		control	Dose of α-tochopherol (μM)			Dose of curcumin (μM)		
			25	75	225	7.5	25	75
0	N1	1.037	1.036	0.996	1.238	1.031	0.999	1.141
	N2	0.897	0.890	0.890	0.951	0.907	0.914	0.850
	N3	1.129	1.104	1.156	1.175	1.268	1.179	1.166
	N4	0.948	0.925	0.900	1.022	0.974	0.996	1.072
	N5	1.348	1.397	1.305	1.334	1.397	1.397	1.255
	N6	1.330	1.332	1.333	1.266	1.297	1.304	1.548
	Mean	**1.115**	**1.114**	**1.097**	**1.164**	**1.146**	**1.131**	**1.172**
	S.E.	0.071	0.078	0.073	0.055	0.075	0.072	0.085
6	N1	1.283	0.967	1.247	1.093	1.280	0.993	1.051
	N2	0.987	0.912	0.894	0.882	0.858	0.889	0.907
	N3	1.164	1.214	1.219	1.527	1.169	1.096	1.222
	N4	1.342	1.296	1.334	1.355	1.335	1.138	1.167
	N5	1.395	1.368	1.423	1.265	1.326	1.321	1.512
	N6	1.299	1.405	1.421	1.535	1.613	1.358	1.508
	Mean	**1.245**	**1.194**	**1.256**	**1.276**	**1.264**	**1.133**	**1.228**
	S.E.	0.055	0.078	0.073	0.095	0.092	0.068	0.091
9	N1	1.293	1.109	1.168	1.141	1.032	1.028	1.087
	N2	0.867	0.756	0.837	0.863	0.870	0.886	0.882
	N3	1.244	1.260	1.089	1.186	1.219	1.136	1.207
	N4	1.308	1.138	0.882	1.247	1.119	1.130	1.000
	N5	1.344	1.368	1.447	1.400	1.582	1.340	1.373
	N6	1.357	1.426	1.529	1.386	1.486	1.348	1.420
	Mean	**1.236**	**1.176**	**1.159**	**1.204**	**1.218**	**1.145**	**1.161**
	S.E.	0.069	0.090	0.106	0.073	0.101	0.067	0.079

15. Effect of α-tochopherol and curcumin on cholesteryl linolenate in he-oxLDL

Time of incubation (hr)	N	control	Dose of α-tochopherol (μM)			Dose of curcumin (μM)		
			25	75	225	7.5	25	75
0	N1	0.023	0.017	0.021	0.019	0.019	0.019	0.018
	N2	0.032	0.028	0.029	0.029	0.027	0.026	0.029
	N3	0.053	0.046	0.057	0.058	0.051	0.062	0.047
	N4	0.020	0.012	0.010	0.019	0.016	0.012	0.014
	N5	0.034	0.030	0.039	0.028	0.031	0.036	0.039
	N6	0.033	0.029	0.038	0.027	0.029	0.035	0.038
	Mean	**0.032**	**0.027**	**0.032**	**0.030**	**0.029**	**0.032**	**0.031**
	S.E.	0.004	0.004	0.006	0.005	0.005	0.006	0.005
6	N1	0.017	0.021	0.018	0.020	0.017	0.022	0.019
	N2	0.026	0.030	0.027	0.030	0.029	0.028	0.029
	N3	0.059	0.048	0.055	0.055	0.059	0.051	0.059
	N4	0.015	0.021	0.022	0.021	0.019	0.016	0.013
	N5	0.033	0.036	0.034	0.033	0.049	0.030	0.045
	N6	0.032	0.035	0.033	0.032	0.048	0.029	0.045
	Mean	**0.030**	**0.032**	**0.031**	**0.032**	**0.037**	**0.029**	**0.035**
	S.E.	0.006	0.004	0.005	0.005	0.007	0.004	0.007
9	N1	0.021	0.018	0.020	0.017	0.020	0.020	0.019
	N2	0.026	0.027	0.027	0.026	0.027	0.029	0.029
	N3	0.045	0.022	0.050	0.056	0.051	0.052	0.055
	N4	0.028	0.015	0.017	0.016	0.015	0.019	0.016
	N5	0.032	0.031	0.038	0.031	0.036	0.036	0.035
	N6	0.031	0.030	0.037	0.030	0.035	0.035	0.034
	Mean	**0.030**	**0.024**	**0.032**	**0.029**	**0.031**	**0.032**	**0.031**
	S.E.	0.003	0.002	0.005	0.005	0.005	0.005	0.005

16. Effect of α-tochopherol and curcumin on cholesteryl arachidonate in he-oxLDL

Time of incubation (hr)	N	Cholesteryl arachidonate (mmol/g protein)						
		control	Dose of α-tochopherol (μM)			Dose of curcumin (μM)		
			25	75	225	7.5	25	75
0	N1	0.177	0.177	0.177	0.150	0.170	0.176	0.163
	N2	0.148	0.162	0.165	0.157	0.164	0.155	0.156
	N3	0.140	0.133	0.133	0.132	0.124	0.130	0.132
	N4	0.186	0.149	0.181	0.181	0.174	0.169	0.165
	N5	0.137	0.143	0.145	0.142	0.141	0.137	0.136
	N6	0.136	0.130	0.139	0.133	0.139	0.135	0.149
	Mean	**0.154**	**0.149**	**0.157**	**0.149**	**0.152**	**0.150**	**0.150**
	S.E.	0.009	0.007	0.008	0.008	0.008	0.008	0.006
6	N1	0.171	0.164	0.166	0.149	0.163	0.169	0.162
	N2	0.138	0.154	0.164	0.154	0.161	0.154	0.155
	N3	0.123	0.122	0.125	0.124	0.118	0.129	0.131
	N4	0.170	0.134	0.165	0.172	0.173	0.170	0.175
	N5	0.133	0.141	0.141	0.137	0.137	0.139	0.134
	N6	0.125	0.127	0.134	0.133	0.138	0.134	0.143
	Mean	**0.143**	**0.140**	**0.149**	**0.145**	**0.148**	**0.149**	**0.150**
	S.E.	0.009	0.007	0.007	0.007	0.008	0.007	0.007
9	N1	0.158	0.161	0.164	0.146	0.160	0.169	0.160
	N2	0.122	0.149	0.150	0.147	0.158	0.148	0.150
	N3	0.115	0.117	0.124	0.124	0.116	0.123	0.129
	N4	0.151	0.123	0.160	0.165	0.164	0.156	0.159
	N5	0.117	0.132	0.138	0.134	0.135	0.132	0.133
	N6	0.119	0.119	0.132	0.126	0.127	0.127	0.137
	Mean	**0.130**	**0.134**	**0.145**	**0.140**	**0.143**	**0.143**	**0.145**
	S.E.	0.008	0.007	0.007	0.006	0.008	0.007	0.006

17. Effect of α-tochopherol and curcumin on cholesteryl linoleate in he-oxLDL

Time of incubation (hr)	N	Cholesteryl linoleate (mmol/g protein)						
		control	Dose of α-tochopherol (μM)			Dose of curcumin (μM)		
			25	75	225	7.5	25	75
0	N1	1.392	1.384	1.511	1.391	1.408	1.383	1.453
	N2	1.259	1.280	1.384	1.273	1.322	1.349	1.354
	N3	1.249	1.267	1.268	1.251	1.217	1.234	1.179
	N4	1.350	1.414	1.409	1.412	1.352	1.339	1.382
	N5	1.178	1.177	1.168	1.168	1.173	1.174	1.175
	N6	1.299	1.231	1.369	1.322	1.315	1.361	1.386
	Mean	**1.288**	**1.292**	**1.352**	**1.303**	**1.298**	**1.307**	**1.321**
	S.E.	0.031	0.037	0.049	0.037	0.036	0.034	0.048
6	N1	1.254	1.328	1.423	1.353	1.339	1.347	1.429
	N2	1.120	1.246	1.259	1.233	1.328	1.317	1.338
	N3	1.128	1.169	1.163	1.198	1.208	1.226	1.179
	N4	1.261	1.337	1.341	1.392	1.330	1.319	1.375
	N5	1.068	1.121	1.117	1.142	1.155	1.152	1.175
	N6	1.160	1.091	1.293	1.308	1.290	1.350	1.382
	Mean	**1.165**	**1.215**	**1.266**	**1.271**	**1.275**	**1.285**	**1.313**
	S.E.	0.032	0.043	0.046	0.039	0.031	0.032	0.045
9	N1	1.110	1.242	1.387	1.324	1.311	1.314	1.415
	N2	0.991	1.088	1.202	1.187	1.269	1.311	1.318
	N3	0.980	1.148	1.190	1.156	1.115	1.182	1.144
	N4	1.182	1.270	1.271	1.314	1.238	1.295	1.345
	N5	0.960	1.024	1.036	1.048	1.141	1.147	1.149
	N6	1.046	1.057	1.262	1.188	1.268	1.297	1.359
	Mean	**1.045**	**1.138**	**1.225**	**1.203**	**1.224**	**1.258**	**1.288**
	S.E.	0.035	0.041	0.047	0.042	0.032	0.030	0.047

18. Effect of α-tochopherol and curcumin on cholesteryl oleate in he-oxLDL

Time of incubation (hr)	N	Cholesteryl oleate (mmol/g protein)						
		control	Dose of α-tochopherol (μM)			Dose of curcumin (μM)		
			25	75	225	7.5	25	75
0	N1	0.521	0.522	0.535	0.541	0.521	0.533	0.555
	N2	0.450	0.463	0.491	0.476	0.465	0.481	0.486
	N3	0.510	0.519	0.531	0.563	0.471	0.492	0.494
	N4	0.503	0.529	0.529	0.539	0.515	0.512	0.503
	N5	0.456	0.467	0.467	0.449	0.454	0.471	0.469
	N6	0.418	0.394	0.431	0.412	0.428	0.432	0.424
	Mean	**0.476**	**0.482**	**0.497**	**0.497**	**0.476**	**0.487**	**0.488**
	S.E.	0.017	0.021	0.017	0.024	0.015	0.014	0.017
6	N1	0.581	0.539	0.529	0.536	0.540	0.536	0.574
	N2	0.479	0.480	0.494	0.483	0.480	0.489	0.496
	N3	0.610	0.529	0.543	0.471	0.563	0.515	0.503
	N4	0.597	0.589	0.554	0.589	0.533	0.517	0.543
	N5	0.479	0.490	0.456	0.469	0.461	0.447	0.479
	N6	0.413	0.421	0.466	0.470	0.410	0.450	0.439
	Mean	**0.527**	**0.508**	**0.507**	**0.503**	**0.498**	**0.492**	**0.506**
	S.E.	0.033	0.024	0.017	0.020	0.024	0.015	0.020
9	N1	0.573	0.542	0.553	0.573	0.566	0.544	0.563
	N2	0.501	0.480	0.501	0.479	0.491	0.493	0.488
	N3	0.558	0.643	0.604	0.566	0.566	0.551	0.502
	N4	0.611	0.611	0.611	0.590	0.622	0.538	0.527
	N5	0.486	0.498	0.461	0.447	0.453	0.476	0.474
	N6	0.476	0.474	0.470	0.432	0.442	0.427	0.449
	Mean	**0.534**	**0.541**	**0.533**	**0.515**	**0.523**	**0.505**	**0.500**
	S.E.	0.022	0.029	0.027	0.028	0.029	0.020	0.016

19. Effect of α-tochopherol and curcumin on cholesteryl palmitate in he-oxLDL

Time of incubation (hr)	N	Cholesteryl arachidonate (mmol/g protein)						
		control	Dose of α-tochopherol (μM)			Dose of curcumin (μM)		
			25	75	225	7.5	25	75
0	N1	0.202	0.310	0.397	0.222	0.290	0.273	0.261
	N2	0.642	0.345	0.487	0.461	0.423	0.508	0.440
	N3	0.207	0.231	0.201	0.199	0.184	0.187	0.218
	N4	0.110	0.122	0.154	0.154	0.174	0.096	0.089
	N5	0.441	0.369	0.412	0.441	0.378	0.398	0.424
	N6	0.440	0.435	0.425	0.433	0.437	0.430	0.442
	Mean	**0.340**	**0.302**	**0.346**	**0.318**	**0.314**	**0.315**	**0.312**
	S.E.	0.075	0.041	0.050	0.052	0.044	0.059	0.054
6	N1	0.176	0.288	0.294	0.236	0.291	0.227	0.271
	N2	0.504	0.750	0.579	0.638	0.588	0.604	0.541
	N3	0.186	0.220	0.179	0.187	0.239	0.194	0.186
	N4	0.116	0.241	0.165	0.151	0.142	0.106	0.157
	N5	0.410	0.418	0.407	0.410	0.404	0.387	0.367
	N6	0.445	0.421	0.443	0.428	0.429	0.429	0.431
	Mean	**0.306**	**0.390**	**0.345**	**0.342**	**0.349**	**0.324**	**0.326**
	S.E.	0.062	0.073	0.060	0.069	0.059	0.068	0.055
9	N1	0.136	0.437	0.264	0.257	0.290	0.268	0.297
	N2	0.558	0.595	0.433	0.598	0.609	0.495	0.445
	N3	0.184	0.184	0.184	0.190	0.201	0.185	0.240
	N4	0.116	0.234	0.202	0.214	0.139	0.080	0.104
	N5	0.446	0.391	0.382	0.401	0.398	0.388	0.386
	N6	0.431	0.428	0.437	0.433	0.434	0.441	0.428
	Mean	**0.312**	**0.378**	**0.317**	**0.349**	**0.345**	**0.309**	**0.317**
	S.E.	0.070	0.056	0.043	0.059	0.064	0.060	0.049

BIOGRAPHY

NAME   Mrs. Paveena Yamanont

DATE OF BIRTH   04 October 1973

PLACE OF BIRTH   Prachuapkhirikhan

INSTITUTIONS ATTENED   Chulalongkorn University, 1992:
                           Bachelor of Sciences (marine science)

RESEACH GRANT   This work was supported by Graduate school

POSITION&OFFICE   Position: Scientist
                           Department of Pharmacology,
                           Faculty of sciences,
                           Mahidol University,
                           Bangkok, Thailand

HOME ADDRESS   104/247 Talingchan-Supanburi road,
                           Bangrukputtana, Bangbouthong,
                           Nonthaburi, Thailand