ผลของผงพ่นแห้งจากน้ำคั้นผลมะขามป้อมต่อการเกิดปฏิกิริยาออกซิเดชัน ของลิโพโพรทีนจากการเหนี่ยวนำด้วยฮีมิน

นางสาวสิริรัตน์ มงคลลิขิต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาเภสัชวิทยา ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

THE EFFECT OF SPRAY-DRIED FRUIT JUICE OF *PHYLLANTHUS EMBLICA* ON HEMIN INDUCED LIPOPROTEIN OXIDATION

Miss Sirairat Mongkhollikit

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology
Department of Pharmacology and Physiology
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2008

Thesis Title	THE EFFECT OF SPRAY-DRIED FRUIT JUICE OF *PHYLLANTHUS EMBLICA* ON HEMIN INDUCED LIPOPROTEIN OXIDATION
By	Miss Sirirat Mongkhollikit
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Rataya Luechapudiporn, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

Pornpen Pramyothin .....Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pornpen Pramyothin, Ph.D.)

THESIS COMMITTEE

Pol. Lt. Col. Somsong Lawanprasert .....Chairman

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

Rataya Luechapudipon .....Thesis Advisor

(Assistant Professor Rataya Luechapudiporn, Ph.D.)

Withaya Janthasoot .....Examiner

(Assistant Professor Withaya Janthasoot)

Ubonthip Nimmannit .....External Examiner

(Associate Professor Ubonthip Nimmannit, Ph.D.)

สิริรัตน์ มงคลลิขิต : ผลของผงพ่นแห้งจากน้ำคั้นผลมะขามป้อมต่อการเกิดปฏิกิริยาออกซิเดชันของลิโพโพรทีนจากการเหนี่ยวนำด้วยฮีมิน (THE EFFECT OF SPRAY-DRIED FRUIT JUICE OF *PHYLLANTHUS EMBLICA* ON HEMIN INDUCED LIPOPROTEIN OXIDATION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. รัตยา ลือชาพุฒิพร, 124 หน้า.

การเกิดปฏิกิริยาออกซิเดชันของลิโพโพรทีนทั้งชนิดความหนาแน่นต่ำ (LDL) และชนิดความหนาแน่นสูง (HDL) มีบทบาทสำคัญต่อการเกิดภาวะหลอดเลือดแดงแข็ง ฮีมินเป็นสารที่เกิดขึ้นจากการสลายตัวของฮีโมโกลบิน โดยมีปริมาณเพิ่มขึ้นในพยาธิสภาพของโรคบางโรค เช่น hemoglobinopathies, sickle cell anemia และ thalassemia สามารถเร่งปฏิกิริยาออกซิเดชันของลิโพโพรทีนได้ แอนติออกซิแดนซ์เอนไซม์ที่พบอยู่บนผิวของลิโพโพรทีนสามารถป้องกันลิโพโพรทีนจากปฏิกิริยาออกซิเดชัน ได้แก่ เอนไซม์ Platelet-activating factor acetylhydrolase (PAF-AH) พบใน LDL และ Paraoxonase พบใน HDL โดยที่ระดับของเอนไซม์จะลดลงในภาวะที่ลิโพโพรทีนถูกออกซิเดชัน ปัจจุบันได้มีการนำสารจากธรรมชาติมาใช้ในการต้านออกซิเดชันอย่างแพร่หลาย มะขามป้อม (*Phyllanthus emblica* Linn.) จัดเป็นพืชที่ทราบกันดีว่ามีฤทธิ์ต้านออกซิเดชัน วัตถุประสงค์ของการศึกษาในหลอดทดลองครั้งนี้ เพื่อศึกษาผลของผงพ่นแห้งจากน้ำคั้นผลมะขามป้อมในการป้องกันการเกิดปฏิกิริยาออกซิเดชันของลิโพโพรทีนจากการหนี่ยวนำด้วยฮีมิน และผลต่อการทำงานของแอนติออกซิแดนซ์เอนไซม์ PAF-AH และ paraoxonase การศึกษานี้ทดลองโดยเติมสารละลายของผงพ่นแห้งจากน้ำคั้นผลมะขามป้อมที่ความเข้มข้น 0.5, 1, 2.5, 5, 10 และ 20 µg/ml ลงใน LDL และ HDL ก่อนเป็นเวลา 30 นาที โดยใช้ L-ascorbic acid เป็น positive control แล้วจึงเติมฮีมิน 5 ไมโครโมลาร์ต่อ.LDL และ HDL 300 µg protein/ml เพื่อเหนี่ยวนำให้เกิดปฏิกิริยาออกซิเดชันของ LDL และ HDL เป็นเวลา 24 ชั่วโมง ผลการทดลองพบว่าฮีมินทำให้ระดับ TBARs ใน LDL เพิ่มขึ้นประมาณ 6 เท่า จาก 5.3 nmol/mg protein ที่เวลา 0 ชั่วโมง เป็น 36.2 nmol/mg protein ที่เวลา 24 ชั่วโมง และปริมาณ α-tocopherol ลดลงอย่างรวดเร็วและหมดไปที่เวลา 4 ชั่วโมงหลังการเติมฮีมิน มะขามป้อมสามารถป้องกันการเกิดปฏิกิริยาออกซิเดชันใน LDL จากการเหนี่ยวนำด้วยฮีมินได้ในลักษณะที่ขึ้นกับความเข้มข้น โดยความเข้มข้นที่สามารถยับยั้งการเกิด TBARs ได้ 50% (IC_{50}) สำหรับมะขามป้อม และ L-ascorbic acid คือ 2.5 และ 13 µg/ml ตามลำดับ และพบว่ามะขามป้อมในขนาด 20 µg/ml ที่ 24 ชั่วโมงสามารถยับยั้งการเกิด TBARs ได้ 99.4% มีปริมาณ α-tocopherol คงเหลือ 70.1% และสามารถคงระดับการทำงานของ PAF-AH ใน LDL ได้ 93.3% เมื่อเปรียบเทียบกับ he-oxLDL มีระดับการทำงานของ PAF-AH เหลือเพียง 53.3% ($p<0.05$) นอกจากนี้ยังสามารถยับยั้งการลดลงของระดับ cholesteryl arachidonate (CA) และ cholesteryl linoleate (CL)ได้ 97.5% และ 91.5% ตามลำดับ รวมทั้งสามารถยับยั้งการลดลงของอัตราส่วน CL/CO ได้ 80.6% สำหรับใน HDL ฮีมินทำให้ระดับ TBARs เพิ่มขึ้นเล็กน้อยจาก 2.5 nmol/mg protein เป็น 4.3 nmol/mg protein ระดับ α-tocopherol ยังคงเหลือ 16.9% และเอนไซม์ paraoxonase จะลดลงอย่างรวดเร็วภายในเวลา 2 ชั่วโมง มะขามป้อมมีผลยับยั้งการเกิดปฏิกิริยาออกซิเดชันใน HDL ได้โดยสามารถยับยั้งการเกิด TBARs และป้องกันการลดลงของ α-tocopherol ได้ แต่ไม่สามารถคงระดับการทำงานของเอนไซม์ paraoxonase ได้ จากการทดลองสรุปได้ว่ามะขามป้อมมีคุณสมบัติในการต้านออกซิเดชันโดยสามารถป้องกันการเกิดปฏิกิริยาออกซิเดชันของไขมันจากการเหนี่ยวนำด้วยฮีมินได้ทั้งใน LDL และ HDL อีกทั้งยังสามารถคงระดับการทำงานของเอนไซม์ PAF-AH ใน LDL ได้

ภาควิชา....เภสัชวิทยาและสรีรวิทยา..... ลายมือชื่อนิสิต.....สิริรัตน์ มงคลลิขิต.....

สาขาวิชา.....เภสัชวิทยา..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....รัตยา ลือชาพุฒิพร.....

ปีการศึกษา..... 2551.....

4976602333: MAJOR PHARMACOLOGY
KEY WORD: LIPOPROTEIN OXIDATION / HEMIN / *PHYLLANTHUS EMBLICA* / PAF-ACETYLHYDROLASE / PARAOXONASE

SIRIRAT MONGKHOLLIKIT: THE EFFECT OF SPRAY-DRIED FRUIT JUICE OF *PHYLLANTHUS EMBLICA* ON HEMIN INDUCED LIPOPROTEIN OXIDATION. THESIS ADVISOR: ASST. PROF. RATAYA LUECHAPUDIPORN, Ph.D., 124 pp.

Oxidation of lipoprotein both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) play a major role in the pathogenesis of atherosclerosis. Hemin (Iron (III)-protoporphyrin IX), a degradation product of hemoglobin was found to be elevated in pathological cases like severe hemoglobinopathies, sickle cell anemia and thalassemia. It is a potent oxidative inducer of lipoproteins oxidation. Antioxidant enzymes located in lipoproteins can protect lipoproteins from oxidation such as platelet activating factor acetylhydrolase (PAF-AH) in LDL and paraoxonase (PON) in HDL. Enzyme activities can be depleted in oxidative stress condition. To date, natural substances are widely used for antioxidants. Ma-kham-Pom (*Phyllanthus emblica* Linn., Euphorbiaceae) is one of plants that is known to be antioxidant activity. Therefore, the aim of this *in vitro* study is to determine the protective effect of spray-dried fruit juice of *Phyllanthus emblica* on hemin induced lipoprotein oxidation and its effect on PAF-AH and paraoxonase activity. The pre-incubation of LDL and HDL with 0.5, 1, 2.5, 5, 10 and 20 μg/ml of spray-dried fruit juice of *P. emblica* were performed for 30 min; L-ascorbic acid was used as a positive control. To induce lipoprotein oxidation, hemin was added into LDL and HDL with a final concentration of 5 μM/300 μg lipoprotein, then further incubate for up to 24 hr. The results showed that TBARs levels were increased in he-oxLDL about 6 fold from 5.3 nmol/mg protein at 0 hr to 36.2 nmol/mg protein at 24 hr while α-tocopherol levels were rapidly decreased until undetectable at 4 hr of incubation. *P. emblica* was able to protect LDL from lipid peroxidation induced by hemin in a concentration dependent manner. The 50% inhibition concentration (IC_{50}) of TBARs formation was 2.5 and 13 μg/ml for *P. emblica* and L-ascorbic acid, respectively. The 20 μg/ml of *P. emblica* at 24 hr exhibited the protective effect by 99.4% inhibition of TBARs formation, and 70.1% remaining of α- tocopherol in LDL. The percent remaining of PAF-AH activity in *P. emblica* was significantly higher than that in he-oxLDL (93.3% vs. 53.3%; $p<0.05$). In addition *P. emblica* can inhibit the decrease of cholesteryl arachidonate (CA), cholesteryl linoleate (CL) and CL/CO ratio 97.5%, 91.5% and 80.6%, respectively. For he-oxHDL, TBARs levels were slightly increased from 2.5 nmol/mg protein to 4.3 nmol/mg protein while α- tocopherol level was remained 16.9%. Paraoxonase activity was rapidly decreased within 2 hr. *P. emblica* was able to protect HDL oxidation by decrease TBARs formation and inhibiting the depletion of α-tocopherol but it was not able to preserve the paraoxonase activity. We concluded that *P. emblica* possess an antioxidant activity which protected hemin-induced lipid peroxidation in both LDL and HDL including preserve the PAF-AH activity in LDL.

Department : Pharmacology and Physiology Student's signature Sririrat Mongkholllikit
Field of study : .....Pharmacology..... Advisor's signature Rataya Luechapudiporn
Academic year : .....2008.....

ACKNOWLEDGEMENTS

The success of this thesis can be attributed to the extensive support and assistance from my advisor, Assistant Professor Dr. Rataya Luechapudiporn. I would like to express my profound gratitude and deep appreciation for her excellent advice, valuable guidance and continual support during the entire duration of the work.

Special thanks to the thesis committee for their constructive suggestions and invaluable comments.

I would like to thank Associate Professor Dr. Ubonthip Nimmannit of the Pharmaceutical Technology (International) Program, Faculty of Pharmaceutical Sciences, Chulalongkorn University for her kindness supporting spray-dried *Phyllanthus emblica* powder to use in this study.

I would like to thank all blood donors, my friends and my workmates in the lab for their co-operation, assistance and encouragement.

I would like to thank staffs and students of department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for their help, informative guidance and using instrument guidance.

I also thank all staff members of the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University for facilitating instruments and work area in the study.

I am grateful to my friends for their help and encouragement. My sincere appreciation goes to Mrs. Paveena Yamanont, Miss Prapaporn Chaniad and Miss Usa Suwannasual, for their kind technical guidance, and valuable advice.

Finally, I would like to express my deepest gratitude and infinite thankfulness to my family for their endless love, understanding and encouragement that greatly support me in order to make this thesis possible.

CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

α-tocopherol	Alpha-tocopherol
Apo	Apolipoprotein
BHT	Butylated hydroxytoluene
β-thal/HbE	β-thalassemia/hemoglobin E
CA	Cholesteryl arachidonate
CE	Cholestery ester
CL	Cholesteryl linoleate
CO	Cholesteryl oleate
Conc.	Concentration
CP	Cholesteryl palmitate
Cu	copper
°C	Degree celcius
d	Density
FC	Free cholesterol
Fe	Ferric
FFA	Free fatty acid
g	gram
HDL	High Density Lipoprotein
HPLC	High perfomance liquid chromatography
hr	hour
he-oxHDL	Hemin oxidized High Density Lipoprotein
he-oxLDL	Hemin oxidized Low Density Lipoprotein
IC_{50}	Median inhibitory concentration
LDL	Low Density Lipoprotein
LPO	Lipid peroxidation
MDA	Malondialdehyde
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeter

MM-LDL	Minimally modified low density lipoprotein
µg	Microgram
µl	Microliter
µM	Micromolar
NA_2EDTA	Ethylenediamine tetraacetic acid disodium salt
nHDL	Native Low Density Lipoprotein
nLDL	Native Low Density Lipoprotein
nm	Nanometer
nmol	Nanomole
NO	Nitric oxide
OD	Optical density
ox-LDL	Oxidized Low Density Lipoprotein
PAF-AH	Platelet Activating Factor Acetylhydrolase
PBS	Phosphate buffer saline
P. emblica	*Phyllanthus emblica*
PON	Paraoxonase
PUFA	Polyunsaturated fatty acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolution per minute
RT	Retention time
S.E.M.	Standard error of mean
TBARs	Thiobarbituric acid reactive substances
TC	Total cholesterol
TG	Triglyceride
VLDL	Very Low Density Lipoprotein

CHAPTER I

INTRODUCTION

1. Background and rationale

Oxidation of lipoproteins is believed to be the key step of atherogenesis (1). In recent years increasing evidence suggests that oxidation of plasma low-density lipoproteins (LDL) is a critical factor in promoting atherosclerosis (2). LDL oxidation can be induced by transition metals, such as copper and iron, free radicals, hypochlorous acid, peroxynitrite and the activity of selected enzymes, such as myeloperoxidase and lipoxygenase (3). However, the physiological mechanisms by which LDL become oxidized is not clearly known. One postulated mechanism suggests that hemin (the complex of ferric iron (Fe^{3+}) with protoporphyrin IX) is a possible physiological iron to promote LDL oxidation (4).

Hemin is a degradation product of hemoglobin that derived from the hemoglobin in circulating erythrocytes. Hemoglobin and free hemin appear in plasma following intravascular hemolysis. Increased hemin is associated with many pathological states, such as severe hemoglobinopathies, sickle cell anemia and thalassemia (5, 6). Hemin has been detected in the serum of β-thalassemia/hemoglobin E (β-thal/Hb E) patients, by using the technique of electron spin resonance (ESR) spectroscopy (7). Hemin was present at levels ranging from 50 to 280 μM in serum of β-thal/Hb E but not detectable in serum of non-thalassemia (4). It has been established that, hemin is a powerful inducer of LDL oxidation in the *in vitro* studies. Exposure of LDL to hemin *in vitro* facilitates Fe^{3+}-mediated oxidation of both its lipid and lipoprotein components resulting in oxidatively modification of LDL that is a pro-atherogenic formation. Since hemin is available in blood plasma of β-thal/Hb E, hemin may serve as an important pro-oxidant capable of promoting oxidative (i.e. atherogenic) transformation of LDL *in vivo* (6, 8, 9).

Antioxidant enzymes present in lipoproteins exhibit an effective to prevent LDL oxidation. Once lipid peroxidation are initiated and oxidized lipid will be accumulated in LDL. The enzymes namely platelet-activating factor acetylhydrolase (PAF-AH) and paraoxonase (PON) degrade proinflammatory, oxidized phospholipids, limiting their accumulation in LDL (10).

The PAF-AH activity in human plasma is mainly associated with LDL, whereas a small proportion of the enzyme activity is found to be related to HDL (11). Normally, it catalyzes the conversion of platelet-activating factor (PAF), a potent lipid mediator involved in inflammation (12), to lyso-PAF by hydrolyzing the acetyl group at the *sn*-2 position of the glycerol backbone (13, 14). PAF-AH also degrades oxidatively fragmented phospholipids, by acting on molecules with nine carbons or less at the *sn*-2 position of phospholipids which are formed during the oxidative modification of low-density lipoprotein (LDL) and may play important roles in atherogenesis (11, 15).

PON hydrolyzes phospholipids hydroperoxides in HDL and LDL. PON has been shown to have peroxidase activity and to hydrolyze lipid peroxides in oxidized lipoproteins with a preference for cholesteryl linoleate (16). Paraoxonase activity has been found to be modified during oxidation of HDL induced by monocytic TH1 cells, copper ions, γ-radiolysis (17). PON activity has been demonstrated to be reduced in different pathologies associated with atherosclerosis such as diabetes, familial hypercholesterolemia, chronic renal failure, post-myocardial infarction and also with smoking and aging (18-22). Thus, many studies have been used paraoxonase as an indicator of antioxidant property of HDL.

The inhibition of LDL peroxidation by supplementation of antioxidants becomes an attractive therapeutic strategy to prevent and possibly to treat atherosclerosis and related diseases in human. This leads to great deal of research devoting to the prevention of lipid peroxidation in LDL by antioxidant (23).

Phyllanthus emblica Linn (also called *Emblica officinalis*) is commonly known as amla. It is a member of the family Euphorbiaceae. In Thailand, it is known as "Ma Kham Pom" (24). The fruit is the most commonly used plant part, with the fresh fruit preferred. *P. emblica* has been shown to possess several pharmacologic actions such as the anti-pyretic and analgesic activity (25), immunomodulatory effects (26), hypolipidemic effect (27), antidiabetic (28), antiulcerogenic (29), antitussive (30) and antioxidant activities (31). *P. emblica* has been used for treatment of several disorders including common cold, scurvy, cancer and heart diseases (31), diarrhea, jaundice and inflammation (25). The antioxidant activities of this plant, due to the presence of the conjugated ring structures and hydroxyl groups, most phenolic compounds have the potential function as antioxidants by scavenging the superoxide anion, singlet oxygen and lipid peroxyl radicals and stabilizing free radicals involved

in oxidative processes through hydrogenation or complexing with oxidizing species (32).

In addition, the antiatherogenic effects of this fruit have been shown in a number of studies. *P. emblica* fruit extract were effective inhibiting the progress of atherosclerosis by alleviating oxidation injury and by inhibiting ox-LDL-induced vascular smooth muscle cells (VSMC) proliferation (33). However, the protective effect of *Phyllanthus emblica* on hemin induced lipoprotein oxidation has not been determined. Therefore, in the present study, the spray-dried fruit juice of *Phyllanthus emblica* were investigated for their protective effect on hemin induced lipoprotein oxidation.

2. Objective

The objectives of this study are:

2.1 To determine the effect of spray-dried fruit juice of *Phyllanyhus emblica* on hemin induced lipoprotein oxidation.

2.2 To determine the effect of spray-dried fruit juice of *Phyllanyhus emblica* on the activities of PAF-AH in hemin induced LDL oxidation and PON in hemin induced HDL oxidation.

3. Hypothesis

Phyllanthus emblica are able to protect lipoprotein oxidation induced by hemin *in vitro*.

4. Expected Benefit and Application

Knowledge from this study leads to obtain information about the antioxidant activity of *Phyllanthus emblica* to protect lipoprotein oxidation induced by hemin.

CHAPTER II

LITERATURE REVIEW

1. Lipoproteins

Lipoproteins provide means for the transport of triacylglycerols (triglycerides) and cholesterol between organs and tissues. A lipoprotein particle contains a hydrophobic core of cholesterol esters (consist part of cholesterol and part of fatty acid such as arachidonic acid, linoleic acid, oleic acid and palmitic acid) and triglycerides (Figure 2.1). Amphipathic phospholipids and free cholesterol, together with apoproteins form an outer layer. Lipoprotein particles present in plasma form a continuum of size and density (Table 2.1). Their classification is based on their hydrated density. Thus there are chylomicrons, very-low-density lipoproteins (VLDL), remnant particles (which include intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). VLDL and remnant particles are triglyceride-rich, whereas LDL is cholesterol-rich. The density of these particles increases and the size decreases with decreasing triglyceride content, from chylomicrons (the lightest) through VLDL, remnant particles, IDL, LDL, to HDL (the heaviest). HDL contains several proteins, and some cholesterol and phospholipids, but relatively little triglyceride (34).

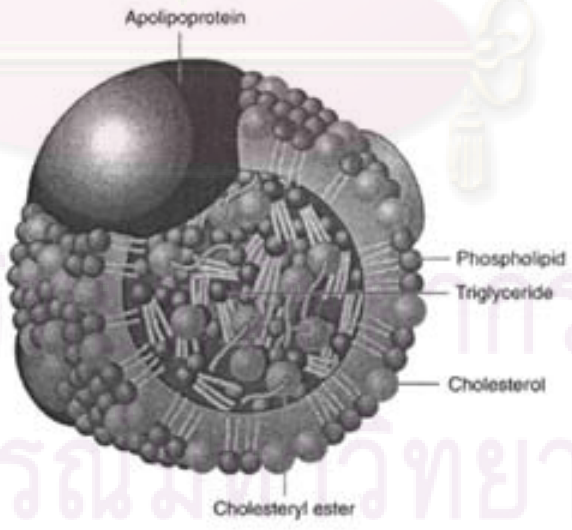


Figure 2.1 Structure of lipoprotein particle. The external monolayer of a lipoprotein particle contains free cholesterol, phospholipids, and apoproteins. Cholesterol ester and triglycerides locate in the particle core.

Table 2.1 Composition and characteristics of the lipoprotein

	Chylomicrons	VLDL	LDL	HDL
Protein (% particle mass)	2	8	22	40-55
Triglycerides (% particle mass)	86	55	6	4
Free cholesterol (% particle mass)	2	7	8	4
Cholesteryl esters (% particle mass)	3	12	42	12-20
Phospholipids (% particle mass)	7	18	22	25-30
Particle mass (daltons)	$0.4\text{-}30 \times 10^6$	$10\text{-}100 \times 10^6$	$2\text{-}3.5 \times 10^6$	$1.75\text{-}3.6 \times 10^5$
Density range (g ml^{-1})	<0.95	0.95-1.006	1.019-1.063	1.063-1.210
Diameter (nm)	80-1000	30-90	18-22	5-12
Apoproteins	AI, AII, AIV, B-48, CI, CII, CIII, E	B-100, CI, CII, CIII, E	B-100	AI, AII, AIV, CI, CII, CIII, D, E

1.1 Function of lipoproteins (34)

Chylomicrons transport dietary triglycerides from the intestine to the peripheral tissues. Their main apoprotein is apoB48, which is synthesized in the intestine. Chylomicrons also contain apoproteins A, C and E. After hydrolysis of chylomicrons by the enzyme lipoprotein lipase (LPL), their remnants are taken up by the liver. Their uptake is mediated by apoE binding to the LDL receptor and to the LDL receptor-related protein (LRP).

VLDL transport triglycerides from the liver to the periphery. The main apoprotein of VLDL is apoB100. They also contain apoC and apoE. VLDL loses triglycerides through the action of the LPL while it transform into IDL (a remnant particle), which in turn is either taken up by the liver or, by losing more triglycerides, transform further into LDL.

LDL particles are the major carriers of plasma cholesterol. They are derived from VLDL in the plasma by a series of degradative steps that remove triglycerides, resulting in a series of particles that contain a progressively lower proportion of triglycerides and are correspondingly richer in cholesterol, phospholipids and protein. The intermediate particles (IDL) are usually present at relatively low concentrations. During the transformations, the apoB component remains with the LDL particles and the apoC and E components are progressive lost. Once only apoB remains, the particle is a mature LDL particle. ApoB has an important role in the recognition of LDL by cells

since it must interact with specific cell-surface receptors before the LDL particle can be taken up and metabolized by the cell. Other receptors (for example on macrophages) recognize modified LDL and are responsible for the degradation of LDL particles that cannot be recognized by normal cell surface LDL-receptors.

There is a variety of types of particle classified as HDL. They are very numerous in plasma: it has been estimated that there are 10-20 times as many HDL particles in the circulation as all other lipoprotein particles combined. HDL transport cholesterol centripetally (from the periphery to the liver). This pathway is known as reverse cholesterol transport. The main apoproteins in HDL are apoAI and apoAII. They are synthesized in the intestine and liver. HDL also contains apoC and apoE. HDL participates in the metabolism of other particles (chylomicrons, VLDL and remnants) through component exchange, exchanging apoproteins, phospholipid, triglyceride and cholesteryl ester.

1.2 Metabolism of low- and high-density lipoproteins

Low-density lipoprotein (LDL) particles are main carriers of cholesterol in the human circulation and are thus key players in cholesterol transfer and metabolism. Their density are ranging from 1.019-1.063 g/ml and have average diameter of 22 nm. They are very rich in cholesterol and cholesteryl esters and containing apoB-100 as the major apolipoprotein. LDLs transport cholesterol to extrahepatic tissues which have specific plasma membrane receptors to recognize specifically to apoB-100. The binding of LDL receptor initiates endocytosis, which brings the LDL and its receptor in to the cell within and endosome.

High-density lipoprotein (HDL) are a heterogeneous group of small, dense lipoproteins isolated from human plasma at a density of 1.063-1.125 g/ml. Majority of HDL particles in plasma are spherical of 7 to 14 nm in diameter. The major proteins associated with HDL are apolipoprotein apoA-I (70%) and apoA-II (20%). HDLs also provide a transport vehicle for other plasma proteins that are involved in plasma lipid metabolism, including, cholesteryl ester transfer protein (CETP), lecithin:cholesterol acyltransferase (LCAT), and phospholipids transfer protein (PLTP). HDLs are formed in liver and small intestine as small, protein-rich particles that contain relatively little cholesterol and no cholesteryl esters nascent HDL. LCAT on the surface of nascent HDL particles converts the cholesterol and phosphatidylcholine (lecithin) of chylomicron and VLDL remnants to cholesteryl esters, which begin to form a core, transforming the disk-

shaped nascent HDL to mature, spherical HDL particle. This cholesterol-rich lipoprotein then returns to liver, where the cholesterol is unloaded.

2. Lipoprotein oxidation

Elevated serum cholesterol, particularly in the form of apoprotein B (apoB)-containing lipoproteins, is an important etiological factor in the pathogenesis of atherosclerosis. It is believed that modification of low-density lipoprotein (LDL) in the arterial wall, particularly by oxidation, is crucial to the cellular uptake of LDL in the first stages of atherosclerotic plaque development (35). LDL oxidation is mediated by free radicals or other oxidants, including extracellular reactive oxygen species, thiols, hypochlorous acid, metal ions, aldehyde, lipoxygenases, myeloperoxidase and reactive nitrogen species (Figure 2.2) (36).

In vitro oxidation of LDL by metal ions (e.g., Cu^{2+}, Fe^{2+}) occurs in three phases: an initial lag phase (consumption of endogenous antioxidants), a propagation phase (rapid oxidation of unsaturated fatty acids to lipid hydroperoxides), and a decomposition phase (hydroperoxides are converted to reactive aldehydes, e.g., malondialdehyde, 4-hydroxynonenol). The oxidation process induces several structural and compositional modifications in lipids of LDL (hydroperoxides, lysophosphatidylcholines, oxysterols, hydroxynonenol), and structural alterations of apoB (37). Interaction of aldehydes with positively charged ε-amino groups of lysine residues renders the LDL more negatively charged, resulting in decreased affinity for the LDL receptor and increased affinity for scavenger receptors. The metal ion-dependent enzyme lipoxygenase converts polyunsaturated fatty acids into lipid hydroperoxides and thereby oxidizes LDL. Activated macrophages secrete metal ion-independent enzyme myeloperoxidase, which generates reactive species, thereby oxidizing protein and lipid moieties of LDL. Finally, oxidized LDL is interacting with scavenger receptors present on endothelial cells, macrophages, and smooth muscle cells (Figure 2.2). Ox-LDL oxidized lipids are present in atherosclerotic plaques, and participate in their evolution toward more advanced lesions potentially associated with plaque destabilization, rapture, and thrombosis (38). Nitric oxide (NO) which is released from endothelium can inhibit copper-mediated oxidation and NO is converted under aerobic conditions to nitrite. Nitrite inhibits the myeloperoxidase-mediated oxidation of LDL.

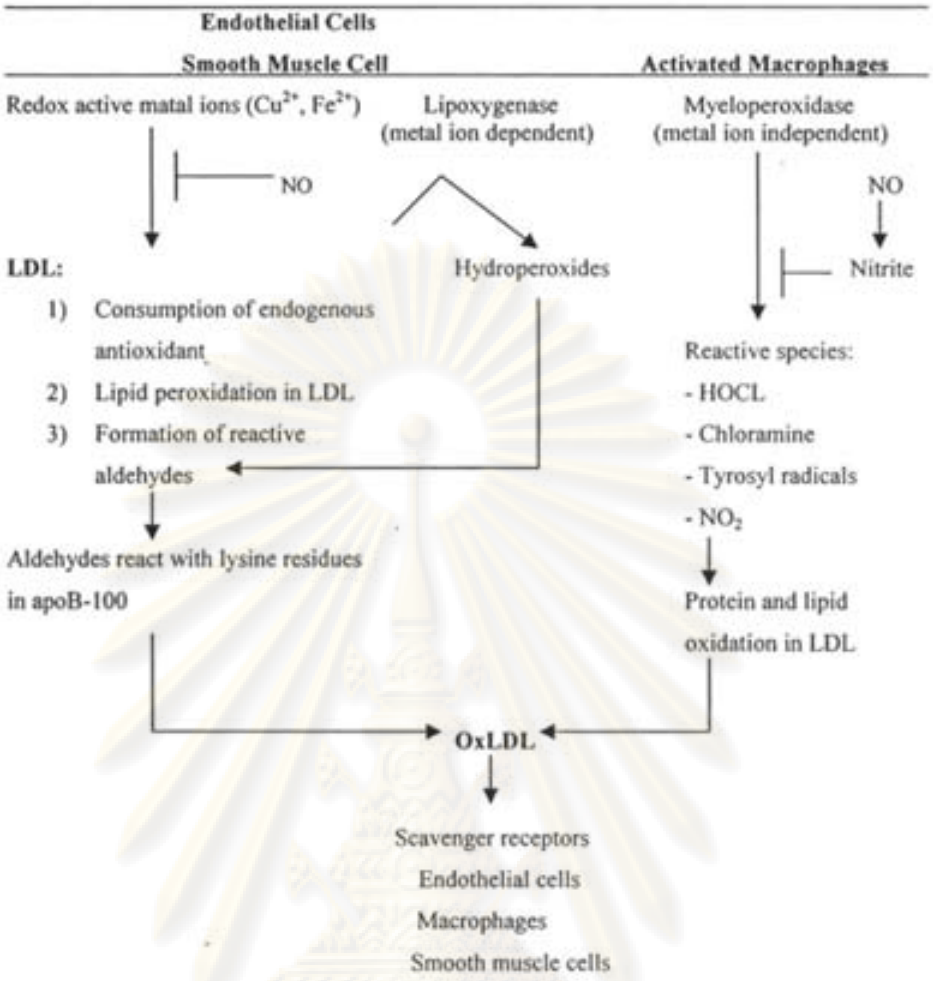


Figure 2.2 Mechanisms of LDL oxidation (39)

2.1 Lipid peroxidation

Oxidation of LDL is a free radical-driven lipid peroxidation chain reaction. Lipid peroxidation is initiated by free radical attack on double bond associated with a polyunsaturated fatty acid (PUFA). This results in the removal of a hydrogen atom from a methylene (CH_2) group, the rate of which determines the rate of initiation, a key step. Molecular rearrangement of the resulting unstable configuration, a conjugated diene. The conjugated diene reacts very quickly with molecular oxygen, and the peroxyl radical thus formed is a crucial intermediate (40) (Figure 2.3). A PUFA peroxyl radical in LDL may abstract a hydrogen atom from an adjacent PUFA to form a hydroperoxide and another lipid radical, a reaction which results in chain propagation. Removal of hydrogen atoms by the peroxyl radical from other lipids. Lipid hydroperoxides fragment to shorter-chain aldehydes, including malondialdehyde and 4-hydroxynonenol. These reactive aldehydes in turn may bind to ε-amino groups of apoB-100, giving the protein an increased net

negative charge. The classical LDL receptor recognizes a specific domain of positive chages from lysine, arginine and histidine residues on apoB. Alteration of this domain results in falure of binding by the apoB/E receptor, and an increase in negative surface charge on apoB-100 results in increased recognition by the scavenger receptor (36).

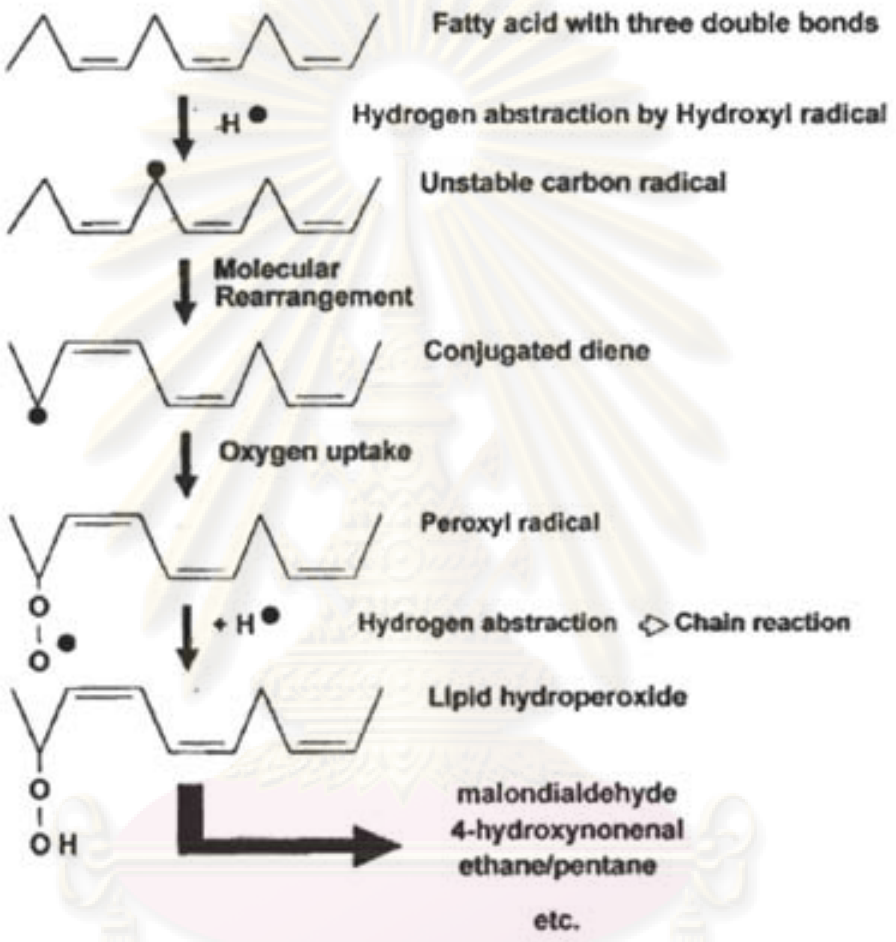


Figure 2.3 Basic reaction sequence of lipid peroxidation (36)

2.2 Biological composition of LDL and oxidized LDL

2.1.1 Biochemical composition of LDL

The central core of LDL particles contains 1600 molecules of cholesterol ester and 170 molecules of triglycerides. It is surrounded by a monolayer of 700 phospholipid molecules, and 600 molecules of cholesterol (41). Embedded in the outer later is one apoB-100 molecule. About half of the fatty acids in LDL are polyunsaturated fatty acids (PUFAs), mainly linoleic acid with minor amounts of arachidonic acid and docosahexaenoic acid. These PUFAs are protected against free radical attack and oxidation by antioxidants primary α-tocopherol (~six molecules per LDL particle) (42). The amount of PUFAs and antioxidants varies significantly within individuals, resulting in a great variation in LDL oxidation susceptibility.

2.2.2 Biochemical composition of oxidized LDL

From the plasma of patients with coronary artery disease, there are many characteristic of oxidized LDL. It was characterized by a 1.3-fold higher electrophoretic mobility on agarose gels compared with native LDL fractions of the linoleic acid levels. The fractions of aldehyde-substitution of lysine residues was ~30 - 40% of that in standard preparations of in vitro oxidized LDL indicating that between 60 and 90 lysine residues in the apoB-100 moiety of oxidized LDL were substituted (43, 44).

2.3 Change that occur in the LDL particle caused by oxidation (45)

LDL undergoes rapid physicochemical changes during oxidation and some of these changes have been correlated with altered biological properties.

2.3.1 Electrophoretic mobility

Alterations in electrophoretic mobility during oxidation have received considerable attention because of their potential role in the recognition by macrophages. Modifications of lysine residues of apoprotein B by-products of lipid peroxidation do not fully account for increased electrophoretic mobility. In addition to modification of lysine by aldehyde products generated from lipid peroxidation. Presence of lipid peroxides does not necessarily mean that aldehydes are the only products involved in the modification of apoprotein B. For example, a direct interaction between lysine residues of protein and peroxidized lipids that generated antigenic fluorescent adducts.

2.3.2 Chemical changes (45)

Chemical changes occurring during LDL oxidation include:

- loss of antioxidants
- loss polyunsaturated fatty acids
- generation of lipid peroxidation products including lipid peroxides, hydroxides, aldehydes, ketones, and hydrocarbons
- generation of lysophospholipids and oxidatively tailored phospholipids
- oxidative scission of apoprotein
- oxidative loss of specific amino acids of the apoprotein
- oxidative conversion of one amino acid into another
- generation of protein carbonyls
- covalent modification of the protein involving products of lipid peroxidation including intact oxidized phospholipids and oxidized cholesterol esters
- modification of the apoprotein involving cross-links by products of lipid peroxidation
- modification of the apoprotein involving cross-link by the formation of dityrosine
- modification of the protein by products derived from the oxidation of amino acids
- products formed from the interaction of oxidized lipids and proteins with nitric oxide
- modified amino lipids.

2.4 The role of oxidation in atherogenesis

There is considerable evidence that lipid oxidation within the arterial wall plays a critical role in atherogenesis (46). Typically, the initial stage of atherosclerosis is characterized by the accumulation beneath the endothelium of excessive amounts of low-density lipoprotein (LDL), the so-called "bad" cholesterol, in combination with a protein carrier. As LDL accumulates within the vessel wall, this cholesterol product becomes oxidized, primarily by oxidative wastes produced by the blood vessel cells. These wastes are free radicals, very unstable electron-deficient particles that are highly reactive. Antioxidant vitamins that prevent LDL oxidation, such as α-tocopherol, ascorbic acid, and β-carotene, have been shown to slow plaque deposition. In response to the presence of oxidized LDL and/or other irritants, the endothelial cells produce chemicals that

attract monocytes, a type of white blood cell, to the site. These immune cells trigger a local inflammatory response.

Once the oxidized LDL leave the blood and enter the vessel wall, monocytes settle down permanently, enlarge, and become large phagocytic cells called macrophages. Macrophages voraciously phagocytize the oxidized LDL until these cells become so packed with fatty droplets. Now called foam cells, these greatly engorged macrophages accumulate beneath the vessel lining and form a visible fatty streak, the earliest form of an atherosclerotic plaque. Thus the earliest stage of plaque formation is characterized by the accumulation beneath the endothelium of a cholesterol-rich deposit. The disease progresses as smooth muscle cells within the blood vessel wall migrate from the muscular layer of the blood vessel to position on top of the lipid accumulation, just beneath the endothelium. This migration is triggered by chemicals released at the inflammatory site. At their new location, the smooth muscle cells continue to devide and enlarge. Together the lipid-rich core and overlying smooth-muscle form a maturing plaque. The plaque progressively bulges into the lumen of the vessel as it continues to develop. The protruding plaque narrows the opening through which blood can flow.

Further contributing to vessel narrowing, oxidized LDL inhibits the release of nitric oxide from the endothelial cells. Nitric oxide is a local chemical messenger that causes relaxation of the underlying layer of normal smooth-muscle cells within the vessel wall. Relaxation of these smooth-muscle cells causes the vessel to dilate. Because of reduced nitric oxide release, vessels damaged by developing plaques cannot dilate as readily as normal. A thickening plaque also interferes with nutrient exchange for the cells located within the involved arterial wall, leading to degeneration of the wall in the vicinity of the plaque. The damaged area is invaded by fibroblasts (scar-forming cells), which form a connective tissue cap over the plaque. (The term sclerosis means excessive growth of fibrous connective tissue, hence the term atherosclerosis for this condition characterized by atheromas and sclerosis, along with abnormal lipid accumulation.)

In the later stages of disease, Ca^{2+} often precipitates in the plaque. A vessel so afflicted becomes hard and poorly distensible (47). Oxidative modification of LDL could result in many effects that lead to the process of atherosclerosis, as shown in table 2.2.

Table 2.2 Proposed the atherogenic properties of oxidized LDL (48, 49)

- Chemotactic for monocytes
- Enhanced uptake by macrophages, leading to cholesteryl ester enrichment and foam cell formation
- Inhibits the motility of macrophage in the artery wall
- Increased uptake by scavenger receptor
- Intimal thickening, which caused by accumulation of foam cells and by smooth muscle cell migration and proliferation
- Cytotoxic to artery wall cells
- Induced endothelium to express adhesion molecules for monocytes, intracellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule (VCAM-1)
- Induced smooth muscle cell proliferation
- Induced apoptosis in endothelial and smooth muscle cells
- Induced vasoconstriction through inhibition of NO production
- Stimulates platelet adhesion and aggregation
- Attribute to the oxidation of LDL phospholipids, which contain arachidonic acid in the *sn*-2 position

2.5 Inhibitors of LDL oxidation

The list of compounds that can inhibit LDL oxidation by virtue of their antioxidant properties is growing steadily (Table 2.3). In addition to probucol, α-tocopherol, ascorbic acid and other well-known antioxidants, and a number of natural as well synthetic chemicals have been added to the list.

Table 2.3 Inhibitors of LDL oxidation *in vitro* (45)

Simple phenol	α-Tocopherol, probucol and its analogs, BHT, estradiol, flavonoids, tocotrienol
Polyhydric phenols	Caffeic acid, nordihydroguairetic acid, polyphenols from plant extracts
Amino compounds	Diphenylphenylenediamine (DPPD), RU 486 and similar compounds, tamoxifen, aminoquanidine, nitric oxide
Metal chelators	EDTA and similar compounds, lazaroids

	(21-aminosteroids)
Antioxidant enzymes	Superoxide dismutase, catalase, paroxonase, PAF-acetyl hydrolase, GSH-peroxidase
Others	β-Carotene and other carotenoids, ascorbic acid, lipoic acid derivatives, lipoxygenase inhibitors, calcium channel blockers, nitric oxide donors, ebselen, cavedilol, captopril, dithiocarbamates
Interesting antioxidants	Oleic acid, bilirubin, boldine, uric acid, spin traps, quercetin, ubiquinol, dehydroepiandrosterone, fibrates, HMG-CoA reductase inhibitors, thiols including glutathione, garlic extract, curcumin, etya, cigarette smoke extract.

2.6 Hemin induced LDL oxidation

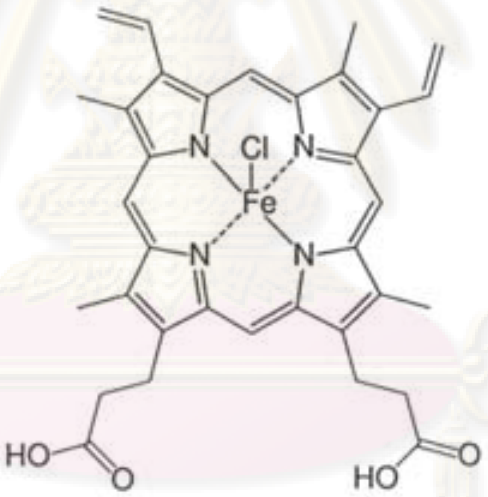


Figure 2.4 Chemical structure of hemin

The amphipatic hemin is a protoporphyrin IX with a coordinated Fe^{3+}, formed in the *in vivo* breakdown of heme (protoporphyrin IX-Fe^{2+}), that is usually bound to hemoglobin and myoglobin (8). Hemoglobin and free hemin appear in plasma following intravascular hemolysis (6). Erythrocytes lysis and hemin overload result in the accumulation of hemin in blood plasma, with further import to various organs and tissues (4). Hemin is a potent hemolytic agent (50) it was found to be elevated in pathological case like severe hemoglobinopathies, sickle cell anemia and thalassemia. Increased

hemin is associated with many pathological stages (5, 6). In the study of Miller and Shaklai (51) show that first seconds after hemin appearance in plasma, more than 80% of this powerful oxidizer binds to lipoproteins (LDL and HDL), LDL binds hemin faster than any other proteins and only the remaining 20% binds to antioxidative human serum albumin (HAS) and hemoplexin (HPX), that bind hemin most strongly. While it may be that HPX binds hemin somewhat more strongly than HAS, the large ratio of concentrations of albumin to hemoplexin (100: 1) in all species examined should make albumin the main carrier of hemin (5).

The half time of hemin-LDL complex in plasma, initially comprising 27% of total hemin, was more than 20 seconds (51).

In vivo, hemin may become available in blood plasma to participate in LDL oxidation as a result of hemoglobin oxidation and decomposition. During intravascular hemolysis, free hemoglobin released into blood plasma is rapidly oxidized to methemoglobin and, subsequently, dissociated into ferriheme (i.e. hemin) and globin (52).

Several researchers have pointed out that hemin is an effective *in vitro* inducer of LDL oxidation. Incorporation of hemin into the LDL particle results in delivery of pro-oxidant Fe^{3+} to the hydrophobic core of the lipoprotein where it initiates lipid peroxidation. The hemin oxidation, producing a variety of LDL lipid oxidation products and apoB crosslinking, resulting in transformation of the lipoprotein particle to a pro-atherogenic form that both is cytotoxic to aortic endothelial cells (4) and accumulates within monocyte-derived macrophages to generate the lipid-laden foam cell characteristic of the early atherosclerotic lesion (8).

Hemin toxic effects on erythrocytes including the induction of potassium leakage (50) the dissociation of erythrocyte membrane skeletal protein (53) and inhibition of a number of erythrocyte enzymes (54).

3. Protective effect of HDL against LDL oxidation

The enhancement of reverse cholesterol transport (RCT) with HDL, apoA-I and apoA-I mimetics is well documented (55-58). In addition, apoA-I can bind oxidized lipids ("seeding molecules") and remove them from LDL (59). This limits the oxidation of phospholipids within LDL, along with the subsequent inflammatory response of atherosclerosis (59, 60). However, HDL particles also carry enzymes that retard LDL oxidation, including paraoxonase (PON), platelet-activating factor

acetylhydrolase (PAF-AH), and lecithin-cholesterol acyltransferase (LCAT) (61, 62). These enzymes prevent LDL oxidation by degrade proinflammatory, oxidized phospholipids, limiting their accumulation in LDL.

Navab et al. (59, 60) have proposed that biologically active lipids in LDL are formed in a series of three steps.

Step 1: Seeding of LDL with products of the metabolism of linoleic acid and arachidonic acid as well as with hydroperoxides.

Step 2: Trapping LDL in the subendothelial space and the accumulation of LDL of additional reactive oxygen species derived from artery wall cells

Step 3: Nonenzymatic oxidation of LDL phospholipids that occurs when a certain threshold of specific oxidized phospholipids that induce monocyte binding, chemotaxis, and differentiation into macrophages.

Normal HDL and its major protein, apoprotein AI (apoA-I), inhibit all three steps in the formation of minimally modified LDL (MM-LDL). Pretreatment of LDL with apoA-I renders LDL resistant to oxidation and reduces the chemotactic activity of LDL. ApoA-I also renders LDL resistant to *in vivo* oxidation. It removes 13(S)-hydroxyperoxyoctadecadienoic acid [(13(S)-HPODE] and 15(S)-hydroxyperoxyeicosatetraenoic acid [(15(S)-HPETE] from LDL. [(13(S)-HPODE] and [(15(S)-HPETE] enhance the nonenzymatic oxidation of both 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine and cholesterol linoleate (65, 66). This results in the formation of some bioactive oxidized phospholipids in mmLDL. Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC), which includes three component oxidized phospholipids, containing 1-palmitoyl-2(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine (POVPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine (PGPC) and 1-palmitoyl-2-epoxyisoprostane-*sn*-glycero-3-phosphorylcholine (PEIPC).

Paraoxonase, a HDL-associated enzyme, prevents LDL oxidation by hydrolyzing lipid peroxides, cholesterol linoleate hydroperoxides, and hydrogen peroxide (63-65). Recent investigators suggest that another HDL-associated enzyme lecithin:cholesterol acyltransferase (LCAT) also prevents the accumulation of oxidized lipids in LDL (66). Once MM-LDL is present, it inhibits plasma LCAT activity and thereby impairs HDL metabolism and reverse cholesterol transport (67, 68). In addition, the degradation of oxidized phospholipids has also been attributed to

another enzyme PAF-acetylhydrolase (PAF-AH). The early inflammatory phase of atherosclerosis (69) involves the generation of PAF and oxidized phospholipids with PAF-like bioactivity in LDL (70). PAF is a potent lipid mediator that stimulates macrophages to produce superoxide anions, thus contributing to progression of atherosclerosis (71). PAF and PAF-like oxidized phospholipids are inactivated by PAF-AH, a Ca^{2+}-independent enzyme that hydrolyzes the *sn*-2 group of PAF, converting it into lyso-PAF (14). These protective effects of HDL are summarized in figure 2.5.

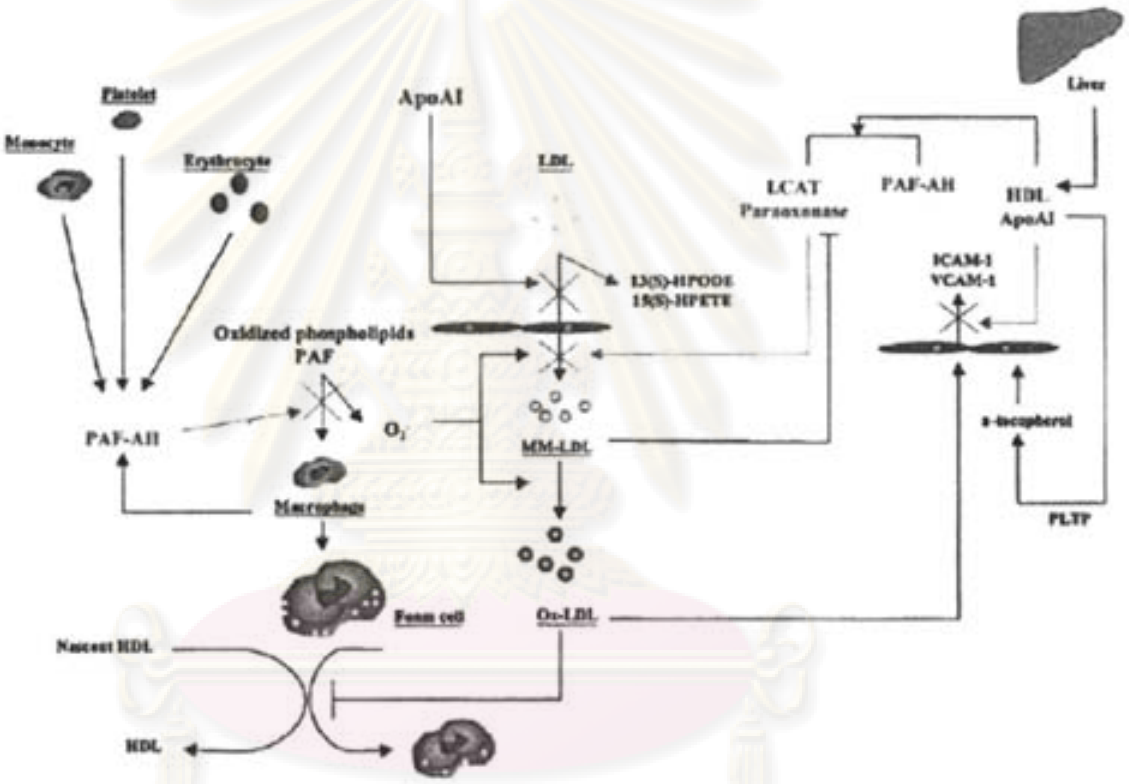


Figure 2.5 Effect of HDL and HDL-associated enzymes on LDL oxidation (49)

4. Paraoxonase

Paraoxonase (aryldialkylphosphatase) is a serum calcium-dependent esterase synthesized in the liver (72). The paraoxonase family consists of three genes, designates PON1, PON2, and PON3. The PON1 gene encodes a 354 amino acids protein with a molecular mass of 43 kDa (73, 74). PON2 and PON3 having one or two fewer amino acids.

PON1 has two qualitatively different properties, paraoxonase and arylesterase activities, because it hydrolyzes neurotoxic organophosphorus

compounds such as paraoxon (an active metabolite of insecticide called parathion, to nontoxic products, *p*-nitrophenol and diethyl phosphate). Paraoxon is toxic to human because it irreversibly inhibits acetylcholinesterase (75). The enzyme also catalyses the hydrolysis of a broad range of substrates (Figure 2.6) including aromatic esters such as arylesters, carbamates, as well as cyclic carbonates, lactone and detoxify nerve agent such as soman and sarin (76)

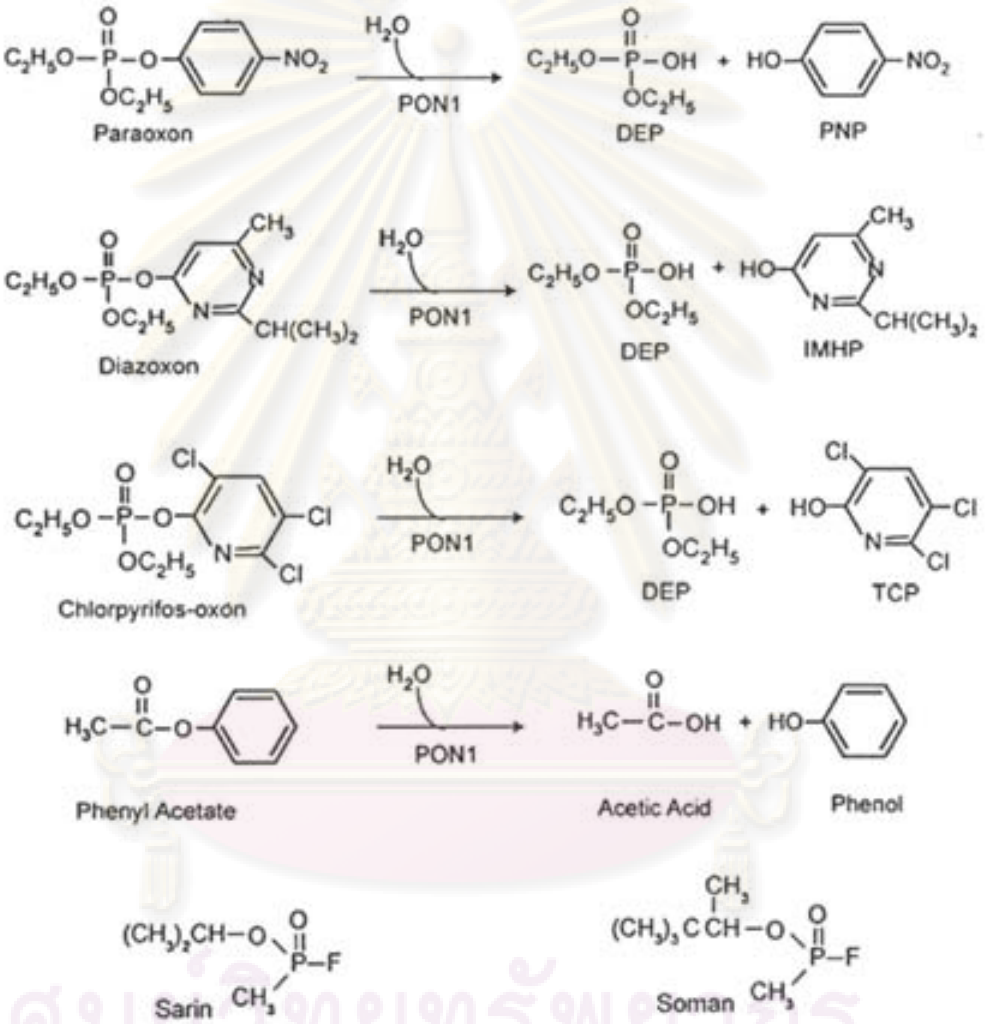


Figure 2.6 The hydrolysis of paraoxon, diazoxon, chlorpyrifos-oxon, and phenyl acetate by paraoxonase 1 (PON1), and the structures of the nerve agents sarin and soman. DEP, diethyl phosphate; PNP, *p*-nitrophenol; IMHP, isopropyl methyl pyrimidinol; TCP, trichloropyridinol (77).

However, human PON2 and PON3 lack, or have very limited on paraoxonase and arylesterase activities, but are similar to PON1 in that both

hydrolyze aromatic and long-chain aliphatic lactones, e.g. dihydrocoumarin and 5-hydroxy-6E, 8Z, 11Z, 14Z-aicosaretraenoic acid (5-HETE) lactone. Moreover, PON3 efficiently hydrolyzes aromatic lactone and 5- or 6-member ring lactones with aliphatic substituents, but not simple lactones or those with polar substituents (78).

5. Platelet-activating factor acetylhydrolase

Platelet-activating factor acetylhydrolase (PAF-AH) is a serine lipase consists of two intracellular isoforms (Ib and II), and one secreted isoform (plasma). These PAF-AHs show different biochemical characteristics and molecular structures. Among these PAF-AH family, plasma PAF-AH has been the target of many clinical studies in many disease such as atherosclerosis and inflammatory diseases because the plasma PAF-AH activity in the patients with these diseases is altered when compared with normal individuals.

The intracellular PAF-AH isoforms II is expressed in the liver and kidney which has active site serine and cysteine residues and its substrates specificity is similar to that of the extracellular plasma form. In addition, isoform II has been reported to catalyze hydrolysis of phospholipids with acyl chains containing up to five methylene groups. This suggests that one function of isoform II may be to scavenge oxidatively fragmented phospholipids (79).

Human plasma PAF-AH contains the lipase/esterase Gly-Xaa-Ser-Xaa-Gly (GXSXG) sequence commonly found in lipases and esterases. It has been investigated that several amino acid residues (Tyr-205, Trp-115 and Leu-116) were important in the binding of plasma PAF-AH to LDL particle (80). The carboxy terminus of apolipoprotein B plays a key role in the association of plasma PAF-AH with LDL.

Secreted PAF-AH is synthesized by macrophages, mast cells and also hepatocytes but the plasma pool of PAF-AH most likely originates from hematopoietic cells (81) whereas in plasma is mainly located in LDL. PAF-AHs are structurally diverse isoenzymes that catalyze the hydrolysis of the acyl group at the *sn*-2 position of glycerol in bioactive phospholipids and short residues of the PAF-like lipid (Figure 2.7). They categorized by enzymatic activity, which are group IV of phospholipases A_2 and have marked selectivity for phospholipids with short acyl chains at the *sn*-2 position; with chains longer than nine carbons there was essentially no measurable activity. Thus, the strict substrates specificity of plasma PAF-AH is

required to protect cell membranes and lipoproteins from continuous hydrolysis of the phospholipids.

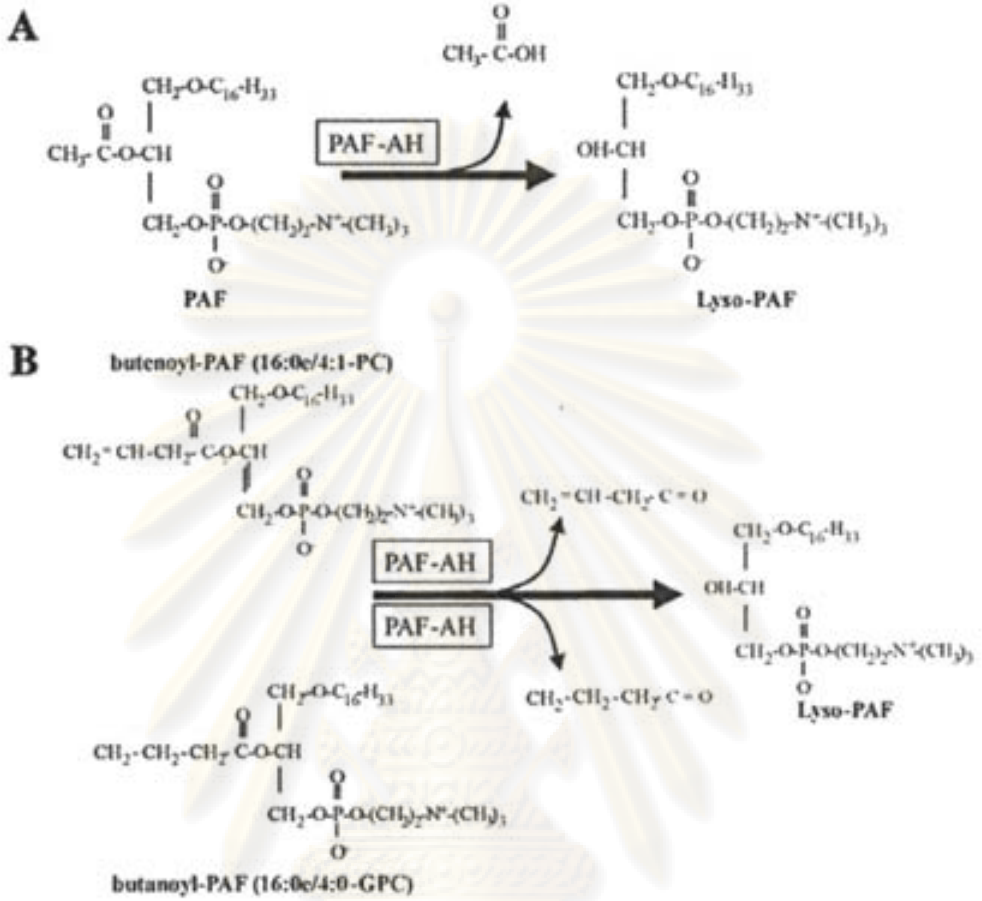


Figure 2.7 Reaction catalyzed by PAF-acetylhydrolases (80)
PAF-AH hydrolyzed PAF and oxidized PAF-like lipids to their inactive metabolite Lyso-PAF, A: hydrolysis of *sn*-2 bound acetate of PAF inactivates it to Lyso-PAF, B: hydrolysis of *sn*-2 bound oxidatively fragmented arachidonoyl residues of butanoyl or butanoyl PAF-like lipids to Lyso-PAF.

6. Effect of PON1 and PAF-AH on oxidation of lipids

PON1 circulates as a high-density lipoprotein (HDL) component in the blood of human and other vertebrates. The enzyme is a calcium-dependent esterase and tightly bound with the hydrophobic N-terminal domain to apoA-I of HDL. The presence of paraoxonase in HDL may be a major contributor to the antiatherogenicity of this lipoprotein (72). PON1 is able to hydrolyze a number of substrates, such as paraoxon and phenyl acetate, and also lipid peroxides, cholesteryl ester hydroperoxides and $H_2O_2^{2-}$ (73). PON1 has been exert the protective against

atherosclerosis development by inhibit cholesteryl influx by reducing the formation of oxidized LDL (ox-LDL), increasing the breakdown of specific oxidized lipids in ox-LDL, and decreasing monocyte adhesion to endothelial cells and macrophage chemotaxis attributable to oxidized phospholipids (82).

The early inflammatory phase of atherosclerosis (69) involves the generation of PAF and oxidized phospholipids which PAF-like bioactivity in LDL (70). PAF is a potent lipid mediator with proinflammatory properties that stimulates macrophages to produce superoxide anions, thus contributing to progression of atherosclerosis (71, 83). PAF-AH is released by monocytes and macrophages, platelets, erythrocytes, spleen and liver cells (84), and has anti-inflammatory properties (85). Human PAF-AH is associated with VLDL, LDL and HDL with approximate distribution of 6, 83 and 11%, respectively (86). This enzyme was associated with different inflammatory diseases such as asthma, sepsis, and vascular diseases (87).

The biological effects of PAF-AH differ from those exerted by PON, since PAF acts on fatty acids with nine carbons or less, while PON acts on derivatives with longer chain oxidized FA. However, recent studies suggest that PON purified from human serum is involved in HDL ability to hydrolyze PAF (88). The current and past suggest that both PAF-AH and paraoxonase can destroy active lipids in MM-LDL and that theirs effects are additive. Therefore, the protective effect of HDL may not be dependent on the absolute levels of HDL cholesterol in the blood but rather the abundance of HDL particles which contain protective enzymes relative to the concentration of oxidized LDL proximate to the artery wall cells.

7. *Phyllanthus emblica* Linn.

Family : Euphorbiaceae
Synonymn : *Emblica officinalis* Gaertn.
English name : Emblic myrobalan, Malacca tree, Indian gooseberry
Common name : Amalaka, Amla, Avla

Common names of this tree include *melaka*, *Asam melaka*, or *amlaka* in Malaya. In Thailand, it is called *ma-kham-pom*; in Laos, *mak-kham-pom*; in Combodia, *kam lam* or *kam lam ko*; in southern Vietnam, *bong ngot*; in North Vietnam, *chu me*. In the Philippines, it is called *nelli* (89).

7.1 Plant anatomy

The tree is small to medium size, reaching 8 to 18 m in height. Its fairly smooth bark is a pale grayish-brown and peels off in conchoidal flakes (89, 90). The leaves are linear-oblong blunt, small, a hundred or more on each branchlet, arranged in two ranks and thus appearing to form a pinnate leaf, 8-12 mm or more long and 2-5 mm broad, stipulate, entire, obtuse or round at the base, subcute or apiculate apex, hairless, light green outside, pale green or often pubescent beneath, almost stalk-less. Leaves fall in November-December and grow in February-March (90). Small, inconspicuous, greenish-yellow flowers are borne in compact clusters in the axils of the lower leaves, blossom in March-May, unisexual, 0.5-1.5 cm long. Usually, male flowers occur at the lower end of a growing branchlet, with the female flowers above them, but occasional trees are dioecious. The nearly stemless fruit is round or oblate, nearly spherical or globular, indented at the base, and smooth, though 6 to 8 pale lines. Normally, fruit is 12-25 mm wide and 15-20 mm long, ripen from November-February. When ripened the mesocarp is yellow and the endocarp is yellowish brown. The mesocarp is acidulous in fresh fruit and acidulous astringent in dried fruit (89, 90).

7.2 Distribution

The *P. emblica* tree is native to tropical southeastern Asia, particularly in central and southern India, Pakistan, Bangladesh, Ceylon, Malaya, southern China, the Mascarene Islands, Thailand and Combodia (89). It is commonly cultivated in home gardens throughout Indian and grown commercially in Uttar Pradesh. In India, and to a lesser extent in Malaya, the *P. emblica* is important and exteemed, raw as well as preserved, and it is prominent in folk medicine. Fruits from both wild and dooryard trees and from orchards are gathered for home use and for market. In southern Thailand, fruits from wild trees are gathered for marketing (89).

7.3 Phytochemistry

The *Phyllanthus emblica* tree contains different classes of constituents list in table 2.4

Table 2.4 The classes of chemical constituents reported in *Phyllanthus emblica* L. (24, 90, 91)

Class	Compound	Occurence
Alkaloid	Phyllantine	Leaves, fruit, and tissue culture
Benzenoid	Chebulic acid	Leaves
	Chebulinic acid	
	Chebulagic acid	
	Gallic acid	
	Ellagic acid	Leaves
	Amlaic acid	Fruit
	Corilagin	Fruit
	3-6-di-O-galloyl-glucose ethyl gallate	
	β-glucogallin	Leaves, fruit
	1,6-di-O-galloyl-β-D-glucose	Fruit
	1-di-O-galloyl-β-D-glucose	
	Putranjivain A	
	Digallic acid	
	Phyllemblic acid	Fruit
	Emblicol	
	Mucic (=galactaric) acid	
Furanolactone	Ascorbic acid	Fruit, leaves

Sesquiterpene	Phyllanemblic acid and its glycosides Phyllanemblicuns A-C	Roots
Diterpene	Gibberellin A-1 Gibberellin A-3 Gibberellin A-4 Gibberellin A-7 Gibberellin A-9	
Triterpene	Lupeol	Fruit, leaves
Flavonoid	Leucodelphinidin Kaemferol Kaemferol-3-glycoside Rutin Quercetin	Leaves
	Kaempherol-3-O-β-D-glucoside Quercetin-3-O-β-D-glucoside	Fruit
Hydrolysable tannins	Emblicanin A, B Punigluconin Pedunculagin	Fruit
Sterol	β-sitosterol	Leaves
Carbohydrate	Acidic and neutral polysaccharides Glucose	Fruit Leaves

7.4 Ethanopharmacology

P. emblica has been used for thousands of years in many of the indigenous medical preparations against a variety of disease condition and is used in traditional medicines of Ayurvedic system.

7.4.1. Anti-inflammatory, anti-pyretic and analgesic activity

P. emblica leaves and fruit have been used for fever and inflammatory treatments by rural population in its growing areas. The ethanol and aqueous extracts of *P. emblica* have shown significant anti-inflammatory activity. The phytochemical screening of the plant extract gave positive test for alkaloids, tannins, phenolic compounds, carbohydrates and amino acids, which might be in part responsible for anti-pyretic and analgesic activities (25). It was found that the water fraction of the methanol leaf extract of *P. emblica* possess marked anti-migration activity, the IC_{50} being around 10 µg/mL for both LTB_4 and FMLP-induced PMN migration (92). Leaf extracts from different solvents were tested for their inhibitory activity against human PMN function such as degranulation, migration, and leukotriene B (LTB_4) release and platelet activity and the results showed that the plant leaves contain as yet unidentified polar compound(s) with potent inhibitory activity on PMNs and a chemically different polar molecule(s) which inhibit both prostanoid and leukotriene synthesis (93).

7.4.2. Anti-microbial

Ethanolic extracts of *P. emblica* have been found to show potentially interesting activity against test bacteria using agar well diffusion method at sample concentration of 200 mg/ml, without any indication of cellular toxicity (94).

7.4.3. Anti-viral

A bioassay-guided fractionation of a methanol extract of the fruit of *P. emblica* led to the isolation of putranjivain A as a potent inhibitory substance on the effects of human immunodeficiency virus-1 reverse transcriptase in the replication of retrovirus such as HIV-1 (95).

7.4.4. Anti-atherogenic

Two compounds from *P. emblica* fruit extract, including corilagin [beta-1-O-galloyl-3,6-(R)-hexahydroxydi-phenoyl-d-glucose] and its analogue Dgg16 [1,6-di-O-

galloyl-beta-d-glucose], were effective in inhibiting the progress of atherosclerosis by alleviating oxidation injury and by inhibiting ox-LDL-induced vascular smooth muscle cells (VSMC) proliferation (33).

7.4.5. Anti-diabetic

Oral administration of 'Triphala' a traditional medicines used in human diabetes (combination of *Phyllanthus emblica, Terminalia chebula* and *Terminalia belerica*) (100 mg/kg body) reduced the blood sugar level in normal and in alloxan-induced (120 mg/kg) diabetic rats significantly within 4 h. It was also found to have a significant antioxidant activity. It may be possible that these extracts may reduce the effect of inflammatory cytokine released during diabetes which may be one of the causative agents for the tissue distraction and insulin resistance (28).

7.4.6. Anti-ulcerogenic

Methanolic *P. emblica* extract, 10-50 mg/kg administration orally, twice daily for 5 days, showed dose-dependent ulcer protective effects in rat models and significant ulcer healing effect, at the dose of 20 mg/kg, after 5 and 10 healing days treatment. The significant ulcer protective and healing effects of *P. emblica* might be due to its effect both on offensive and defensive mucosal factors (29). Pretreatment with the butanol extract of the water fraction of *P. emblica* fruits was found to enhance secretion of gastric mucous and hexosamine in the indomethacin induced ulceration of rats (96).

7.4.7. Anti-tussive

The anti-tussive activity of *P. emblica* in conscious cats was dose-dependent. The anti-tussive activity of *P. emblica* is less effective than shown by the classical narcotic antitussive drug codeine, but more effective than non-narcotic antitussive agent dropropizine. It is supposed that the antitussive activity of the dry extract of *P. emblica* is due to not only its antiphlogistic, antispasmolytic and antioxidant effects, but also to its effect on mucous secretion in the airways (30).

7.4.8. Hepatoprotective

P. emblica extract significantly inhibited hepatocarcinogenesis induced by *N*-nitrosodiethylamine (NDEA) in a dose dependent manner. Male Wistar rat treated

with NDEA alone showed 100% tumor incidence and significantly elevated tissue levels of drug metabolizing enzymes such as glutathione S-transferase (GST) and aniline hydroxylase (AH). Treatment with *P. emblica* significantly reduced these levels. Serum levels of lipid peroxidase (LPO), alkaline phosphatase (ALP) and glutamate pyruvate transamunase (OPT), which are markers of liver injury, were also significantly reduced in the treated group. Morphology of liver tissue and levels of marker enzymes indicated that *P. emblica* extract offered protection against chemical carcinogenesis. It may be due to the scavenging of the reactive oxygen radicals from the system, as well as inhibition of the enzymes responsible for the activation of NDEA (97).

Another study revealed the aqueous fruit extract of *P. emblica* counteracted the increased lipid peroxide levels induced by acute CCl_4 treatment and offered partial protection against increase in glutamate-pyruvate transaminase (GPT) and alkaline phosphatase (ALP) levels. Moreover, *P. emblica* extract could inhibit the induction of fibrosis due to chronic CCl_4 administration in rats, indicating that *P. emblica* have hepatoprotective activity (98).

7.4.9. Hypolipidemic

Feeding of *P. emblica* fresh juice at a dose of 5 ml/kg body weight per cholesterol-fed rabbit per day for 60 days leading to reduce serum cholesterol, TG, phospholipids and LDL by 82%, 66%, 77% and 90%, respectively, and the tissue lipid levels showed a significant reduction following P. *emblica* juice administration. Therefore, *P. emblica* fresh is an effective hypolipidemic agent can be used as pharmaceutical tool in hyperlipidaemic subjects (27).

Flavonoids from *P. emblica* effectively reduce lipid levels in serum and tissues of rats with induced hyperlipidemia. The mechanism of hypolipidemic action is by the concerted action of inhibition of synthesis and enhancement of degradation (99).

7.4.10. Anti-Tumor and Anti-Proliferative

Aqueous extract of *P. emblica* could inhibit the growth of L929 cells significantly in culture and inhibit cell cycle regulating enzymes cdc 25 phosphatase in a dose dependent manner. Concentration needed for 50% inhibition was found to be 16.5 and 5 μg/ml, respectively, and that needed for inhibition of cdc2 kinase was > 100 μg/ml. P. emblica extract significantly reduced solid tumors induced cell lines,

Dalton's lymphoma ascites (DLA) cells while having only a moderate effect on ascites tumor. The results suggest that antitumor activity of *P. emblica* extract may particularly be due to its interaction with cell cycle regulation (100).

Pyrogallol, a compound present in dried fruit extract of *P. emblica*, was an active inhibitor of *in vitro* tumor cell growth of K562 human tumor cell lines. *P. emblica* extracts (5-500 ng/ml) were able to fully suppress K562 cell growth. Antiproliferative effects of pyrogallol were therefore determined on human tumor cell lines, thus identifying pyrogallol as the active component (101).

7.4.11. Immunomodulating

Administered orally, *P. emblica* enhanced two natural defense mechanisms including natural killer (NK) cell activity and antibody dependent cellular cytotoxicity (ADCC) in vivo through the induction of interferon and, thus conferred, protection on Dalton's lymphoma ascites (DLA) tumor (a transplantable murine T cell lymphoma) bearing mice by boosting the host natural immune response. The antitumor activity of *P. emblica* is mediated primarily through the ability of the drug to augment natural cell mediated cytotoxicity (26).

The immunomodulatory property of *P. emblica* extract was evaluated in adjuvant induced arthritis (AIA) rat model using Complete Freunf's Adjuvant (CFA) induced inflammation. The result showed that this fruit extract caused immunosuppression in AIA rats, indicating that it may provide an alternative approach to the treatment of arthritis.

7.4.12. Antioxidant

The two emblicanin A and B have been found to preserve erythrocytes against oxidative stress induced by asbestos, a generator of superoxide radical. Emblicanin A oxidates when put in contact with asbestos, becoming emblicanin B, together they have a stronger protective action to erythrocytes than vitamin C (24).

In another study, an emblicanin-A and –B enriched fraction of fresh fruit juice of *P. emblica* was investigated for antioxidant activity against oxidative stress in rat heart with vitamin E as the standard antioxidant agent. The results clearly showed that the administration of *P. emblica* extract given orally twice daily for 14 days prior to the sacrifice of the animals provided a significant protection against the stressor agent induced decrease in the activities of cardiac antioxidant enzymes such as superoxide

dismutase, catalase, and glutathione peroxidase, leading to aconsequent decrease in lipid peroxidation. The study thus confirmed the antioxidant effect of *P. emblica* and indicated that the fruits of the plant may have a cardioprotective effect (102). The ethanolic extract of *P. emblica* (100 μg/ml) showed the highest cardioprotective effect in patients receiving doxorubicin compared with other medicinal plants tested (103).

Methanolic extract of *P. emblica* on antioxidant activity against free radical-induced lipid peroxidation using bovine brain phospholipids liposomes as model membranes. The *P. emblica* fruit extract inhibited lipid peroxidation with IC50 value of 13 μg/ml (104).

The radio protective effect of the fruit pulp of *P. emblica* in adult Swiss albino mice can change the antioxidant enzyme levels in the body, leading to induce DNA strand breaks and mutation and induced peroxidative changes to lipids and proteins. *P. emblica* extract has been shown to have significant antioxidant activity, which enhanced the activity of the various antioxidant enzymes and GST as well as glutathione system in the blood (105). Treatment with *P. emblica* also lowered the elevated levels of lipid peroxides in the serum. The data clearly indicated that the extract significantly reduced the bioeffects of radiation. *P. emblica* extract may be useful in reducing the side effects produced during therapeutic radiation. *P. emblica* extract for its ability to inhibit γ-radiation-induced lipid peroxidation (LPO) in rat liver microsomes and inhibit the damage to antioxidant enzyme SOD (31).

The protective effect of *P. emblica* on oxidative stress and toxicity in rats challenged with dimethyl hydrazine (DMH). Administration of *P. emblica* at 5% and 10% levels increased the hepatic GSH and reduced the conjugated dienes. 10% *P. emblica* enhanced the catalase, glutathione peroxidase (GSH-Px) and superoxide dismutase activities in the liver and increase the hepatic ascorbic acid and glutathione (GSH) in rat treated with DMH. The activity of γ-glutamyl transpeptidase, which was increased significantly in kidney upon DMH injection, was reduced by 50% on feeding of *P. emblica*. The results showed that P. emblica has the ability to detoxify DMH partly by enhancing the multicompartment antioxidant system in the rat (106).

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Chemicals

All chemicals were obtained commercially and used without further purification. The following chemicals were perchased from Sigma Chemical Co., St Louis, U.S.A.: Standard free cholesterol, cholesteryl arachidonate, cholesteryl linoleate, cholestryl oleate, cholesteryl palmitate, α-tocopherol, L-ascorbic acid, Trizma hydrochloride, 5,5'-dinitro-bis-(2-nitrobenzoic acid) (DTNB), copper sulfate, ethylenediamine tetraacetic acid disodium salt, monobasic sodium phosphate anhydrous, disodium hydrogen phosphate anhydrous, sodium carbonate, sodium bicarbonate, sodium potassium tartrate tetrahydrate, bovine serum albumin, 2-thiobarbituric acid, sodium dodecyl sulfate, hemin and Folin & Ciocalteu's phenol reagent.

Other chemicals were purchased from commercial sources as follow: cholesterol enzyme kit from Biotechmedical Laboratory Thailand, 2-thio PAF from Cayman chemical company Michica America, sodium hydroxide from Ajax Finechem Australia, heparin from Leo Phamaceutical Products Denmark, potassium bromide, sodium chloride, trichloroacetic acid from Merck Germany, methanol, ethanol, n-hexane, butanol, acetronitrile, isopropanol and diethylether (HPLC grade) from Lab Scan Co., Ltd Thailand.

Spray-dried fruit juice of *Phyllanthus emblica* was supported by Assoc. Prof. Ubonthip Nimmannit; Pharmaceutical Technology (International Program), Faculty of Pharmaceutical Sciences, Chulalongkorn University.

1.2 Instruments

1) Refrigerated centrifuge (Beckman coulter model Allegra X-12R)
2) Optima Beckman Coulter ultracentrifuge (LE 80 K) with 90 Ti roter
3) Spectrophotometer (Jasco model UVDEC 650)
4) Spectrofluorometer (Jasco model FP-777)

5) Microplate reader, VICTOR®, multilabel coulter, Perkin Elmer Ltd., U.S.A.
6) HPLC systems (Shimadzu, Japan) class LC 10

2. Methods

2.1 Plasma preparation

Blood was obtained from overnight fasting healthy volunteers and collected in heparin. All volunteers signed informed consent, and the study was approved by the Ethics Committee of The Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Plasma was separated by centrifugation at 3,250 rpm for 15 min at 4 °C then pooled plasma were stored at -80 °C until used for separation of lipoproteins (LDL and HDL).

2.2 The separation of LDL and HDL

LDL and HDL were separated from plasma by sequential density gradient ultracentrifugation (107) in salt solution using a Beckman 90 Ti rotor, speed 50,000 rpm at 4 °C. Thick-wall polycarbonate tubes were used. LDL (density = 1.019-1.063 g/ml) and HDL (density = 1.210-1.063 g/ml) were separated after 19 hr and 39 hr of ultracentrifugation (Figure 3.1). Salts were removed from LDL and HDL by dialysis against 10 mM of PBS buffer pH 7.4. LDL and HDL were then analyzed for protein content.

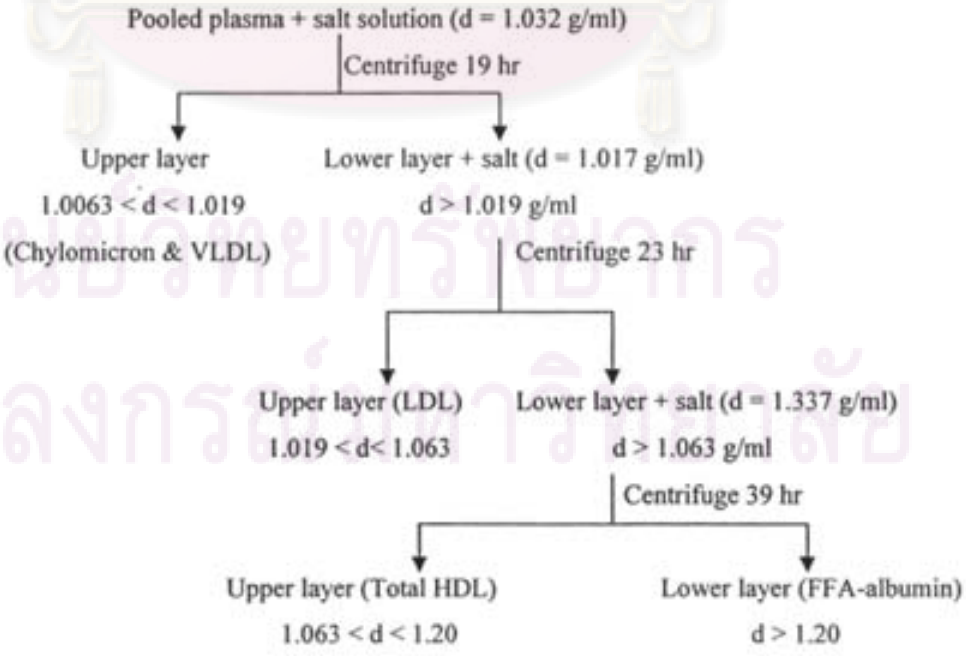


Figure 3.1 Lipoproteins separation

2.3 Determination of protein content

Protein content was determined by the Markewell modification of the Lowry protein assay (108) using bovine serum albumin 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 spectrophotometer at OD 660 nm.

2.4 Preparation of hemin

Stock solution of hemin was prepared by dissolving hemin in 500 µl of 0.1 M NaOH and adjusted pH to 7.8 by 4.5 ml of 10 mM PBS. Hemin suspension 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.

2.5 Parameters measurement

2.5.1 Oxidative Markers

- Thiobarbituric acid reactive substances (TBARs) (109).

Lipid peroxidation of lipoproteins was terminated by adding 25 µl of 100 mM butylated hydroxytoluene. Then 0.5 ml of 10% trichloroacetic acid was added and mixed for 1 min. A 0.25 ml of 5 mM EDTA was added into the mixture. After vortexing, 0.25 ml of 8% sodium dodecyl sulfate and 0.75 ml of 0.6% thiobarbituric acid were added and vortex, respectively. The reaction mixtures was then heating at 100 °C for 1 hr, then samples were cooled to room temperature and measured by spectrofluorometer, excitation and emission wavelength at 515 and 553 nm, respectively. 1,1,3,3-tetraethoxypropane was used as a standard. The results are presented as nanomoles thiobarbituric acid reactive substances (TBARs) per milligram protein.

2.5.2 Enzyme Activity assays

- Platelet activating factor acetylhydrolase (PAF-AH) Activity

PAF-AH activity was measured in LDL by using 200 µM 2-thio PAF substrate (110) in 0.1 M Tris-HCl buffer (pH 7.2) containing 1 mM EDTA and 1

mM DTNB in a total volume of 225 µL. Twenty microlitre of LDL samples was added to start the reaction. Upon hydrolysis of the acetyl thioester bond at the sn-2 position by PAF-AH, free thiols are detected using 5,5'-dinitro-*bis*-(2-nitrobenzoic acid) (DTNB; Ellman's reagent), the increase in absorbance at 405 nm was record continuously for 10 min. The amount of 5-thio-2-nitrobenzoic acid formed was calculated from the molar extinction coefficient of 10,000 $M^{-1}cm^{-1}$. The blank contained substrate without LDL. One unit of enzyme hydrolyzes one µmol of 2-thio PAF per minute at 25 °C.

- Paraoxonase (PON) activity

PON activity was measured in HDL samples. PON activity was measured with a 1.0 mmol/l paraoxon substrate in 50 mmol/l Tris-HCl buffer (pH 7.4) containing 1.0 mmol/l $CaCl_2$ in a total volume of 1000 µl (111). One hundred microlitre of HDL was added to start the reaction, and the increase of 4-nitrophenol formed was calculated from the molar extinction coefficient of 10,000 $M^{-1}cm^{-1}$. The blank contained substrate without HDL. One unit of PON activity is defined as 1 nmol of 4-nitrophenol formed per minute under the above assay conditions.

2.5.3 Chemical Composition

- Total cholesterol

Total cholesterol was measured by enzymatic methods assays using commercially reagent. All sample and standard were mixed with working solution containing enzyme mixture tubes, stand for 10 min and measured the absorbance at 500 nm.

Principle:

$$\text{Cholesteryl ester} + H_2O \xrightarrow{\text{Cholesteryl esterase}} \text{Cholesteryl} + \text{Fatty acid}$$

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

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

- α- tocopherol, free cholesterol, cholesteryl esters and its oxidative products

α- tocopherol, free cholesterol, cholesteryl esters and oxidized lipid products in lipoproteins can be determined by reverse phase HPLC method using UV monitor at 210 nm for free cholesterol and cholesteryl esters, 234 nm for oxidized lipid products and 292 nm for α- tocopherol. This method modifies from Seta et al, Zaspel and Casllany (112, 113).

Briefly, 100 µl of lipoproteins was added and mixed with 100 µl of 10 mM PBS. Then 500 µl of ice-cold methanol were added and mixed on a vortex for 30 s. The 2.5 ml of hexane were added into the mixture and vortexed vigorously for 1 min and then centrifuged at 1,700 rpm at 4 °C for 5 min. The 2 ml of hexane layer was transferred into test tube and dried under nitrogen and redissolved with 200 µl of mobile phase (75% acetronitrile : 25% isopropanol, v/v). preparation of standard substance is summarized in table 3.1. The standard mixture of α-tocopherol (5, 10, 20 and 40 µl) and standard mixture of free cholesterol and cholesteryl esters (5, 10, 25, 50 µl) were injected into the hypersil BDS C18 column (5µm: 4.6 mm × 250 mm) by autosampler. The sample was injected 50 µl. The flow rate was 1.2 ml/min and the temperature of column was controlled at 50 °C.

Table 3.1 Standard preparation of free cholesterol and cholesteryl esters

Standard substance	Stock concentration (µg/ml)	Final concentration (µg/ml)
Free cholesterol (FC)	500	60
Cholesteryl arachidonate (CA)	1000	10
Cholesteryl linoleate (CL)	500	120
Cholesteryl oleate (CO)	500	60
Cholesteryl palmitate (CP)	500	40

Free cholesterol, cholesteryl arachidonate and cholesteryl linoleate were dissolved in isopropanol. Cholesteryl oleate and Cholesteryl palmitate were dissolved in isopropanol : diethylether (5:2 v/v).

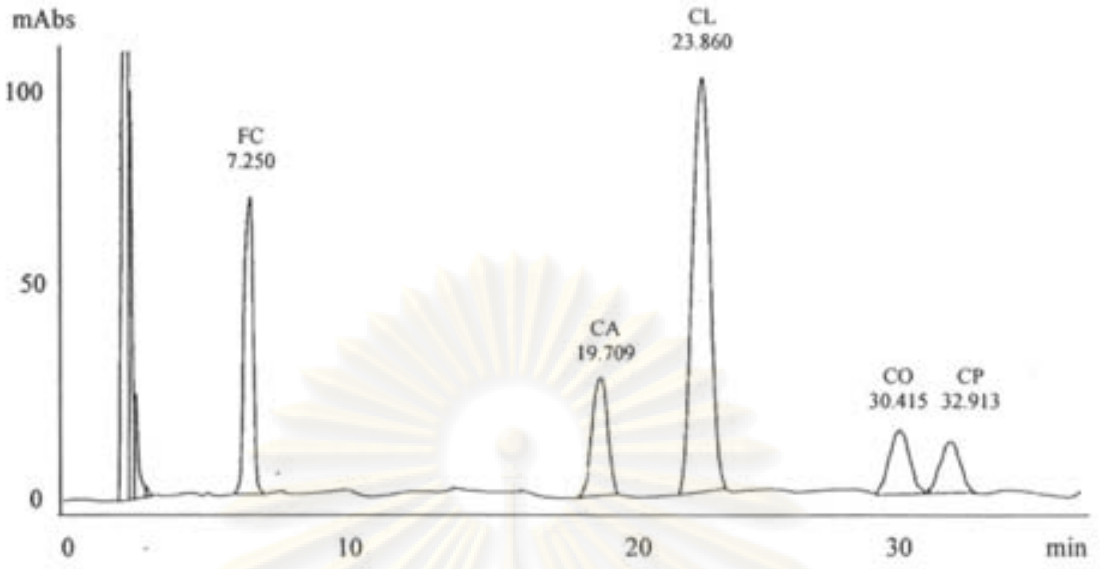


Figure 3.2 Typical HPLC chromatogram of free cholesterol (FC) and a series of cholesteryl esters standards: cholesteryl arachidonate (CA), cholestery llinoleate (CL), cholesteryl oleate (CO) and cholesteryl palmitate (CP).

3. Experimental procedure

Part I: Study the concentration dependent effects of *Phyllanthus emblica* on hemin induced LDL oxidation (he-oxLDL).

LDL were preincubated with spray-dried fruit juice of *P .emblica* at concentrations 0.5, 1, 2.5, 5, 10 and 20 μg/ml for 30 min, L-ascorbic acid use as a positive control and then were incubated with hemin 5 μM per 300 μg LDL protein at 37 °C for 24 hr. During the oxidation, aliquots were removed at various time points (0, 6, 9 and 24 hr). Oxidation reaction were terminated by adding 50 μM BHT. Then enzyme activity (PAF-AH), oxidative markers (TBARs and α-tocopherol) and lipid composition (total cholesterol, free cholesterol, cholesteryl esters and oxidized lipid products) were determined (Figure 3.1).

Part II: Study the time dependent effects of *Phyllanthus emblica* on hemin induced LDL oxidation (he-oxLDL).

LDL were pre-incubated with spray-dried fruit juice of *P .emblica* at concentration 2.5 μg/ml for 30 min, L-ascorbic acid at concentration 13 μg/ml was used as a positive control and then were incubated with hemin 5 μM per 300 μg LDL protein at 37 °C for 24 hr. During the oxidation, aliquots were removed at various time points. Oxiadtion reaction were terminated by adding 50 μM BHT. Then enzyme activity (PAF-AH), oxidative markers (TBARs and α-tocopherol) and lipid composition (free cholesterol, cholesteryl esters and oxidized lipid products) were determined (Figure 3.2).

All samples were collected at various time points for each parameter:

PAF-AH activity:	0, 1, 2, 4, 6, 9, 12, 16, 20 and 24 hr.
TBARs level:	0, 1, 2, 4, 6, 9, 12, 16, 20 and 24 hr.
α-Tocopherol level:	0, 1, 2, 4, 6 and 9 hr.
Lipid composition :	0, 6, 9, 12, 16, 20 and 24 hr.

Part III: Study the concentration dependent effects of hemin induced HDL oxidation (he-oxHDL).

HDL was incubated with hemin 2.5, 5, 10 and 20 μM per 300 μg HDL protein at 37 °C for 24 hr. The oxidation reaction was terminated by adding 50 μM BHT. Then the TBARs level and α-tocopherol level were determined (Figure 3.3).

Part IV: Study the concentration dependent effects of *Phyllanthus emblica* on hemin induced HDL oxidation (he-oxHDL).

HDL were pre-incubated with spray-dried fruit juice of *P .emblica* at concentrations 0.5, 1, 2.5, 5, 10 and 20 μg/ml for 30 min, L-ascorbic acid was used as a positive control at same concentrations and then were incubated with hemin 5 μM per 300 μg HDL protein at 37 °C for 24 hr. During the oxidation, aliquots were removed at various time points (0, 6, 9 and 24 hr). Oxidation reaction were terminated by adding 50 μM BHT. Then oxidative markers (TBARs and α-tocopherol) were determined (Figure 3.4).

Part V: Study the time dependent effects of *Phyllanthus emblica* on hemin induced HDL oxidation (he-oxHDL)

HDL was pre-incubated with spray-dried fruit juice of *P. emblica* at concentration 8 μg/ml for 30 min and then was incubated with hemin 5 μM per 300 μg HDL protein at 37 °C for 24 hr. During the oxidation, aliquots were removed at various time points. Oxidation reaction were terminated by adding 50 μM BHT. Then enzyme activity (paraoxonase) and oxidative markers (TBARs and α-tocopherol) were determined (Figure 3.5).

All samples were collected at various time points for each parameter:

TBARs level:	0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 hr.
α-Tocopherol level:	0, 1, 2, 4, 6, 8, 10 and 24 hr.
PON activity:	0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 hr.

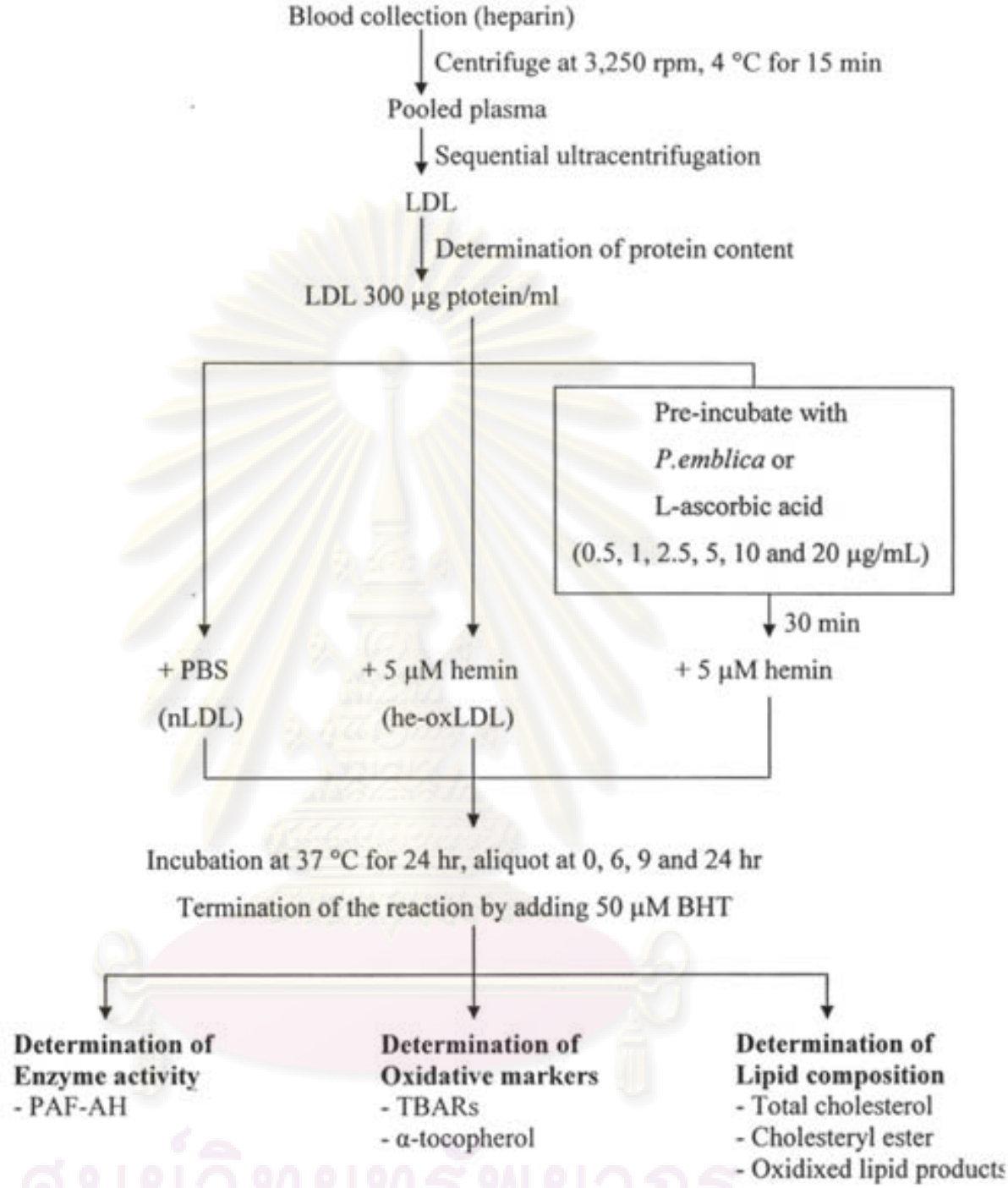


Figure 3.3 Study the concentration dependent effects of *Phyllanthus emblica* on hemin induced LDL oxidation (he-oxLDL).

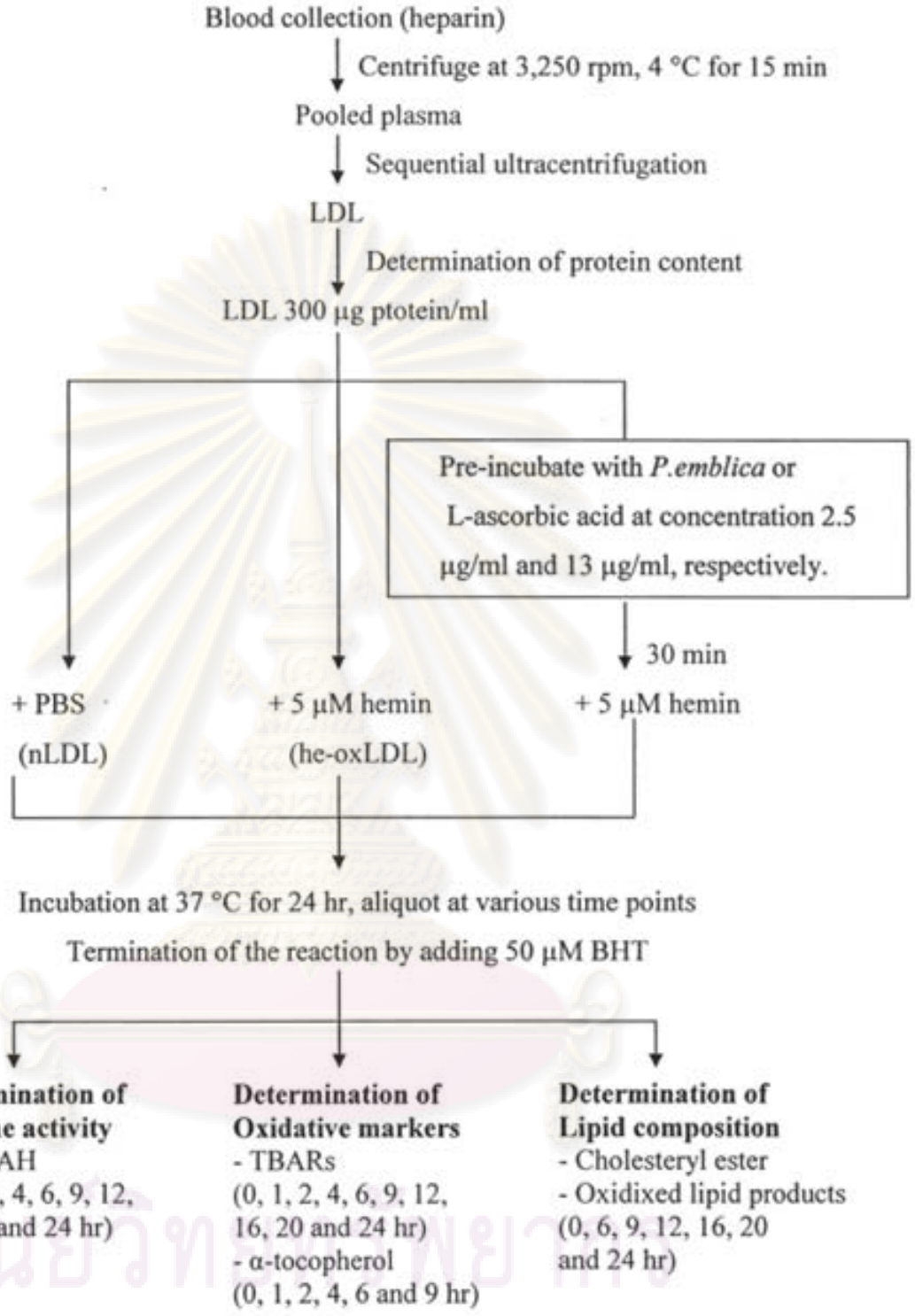


Figure 3.4 Study the time dependent effects of *Phyllanthus emblica* on hemin induced LDL oxidation (he-oxLDL).

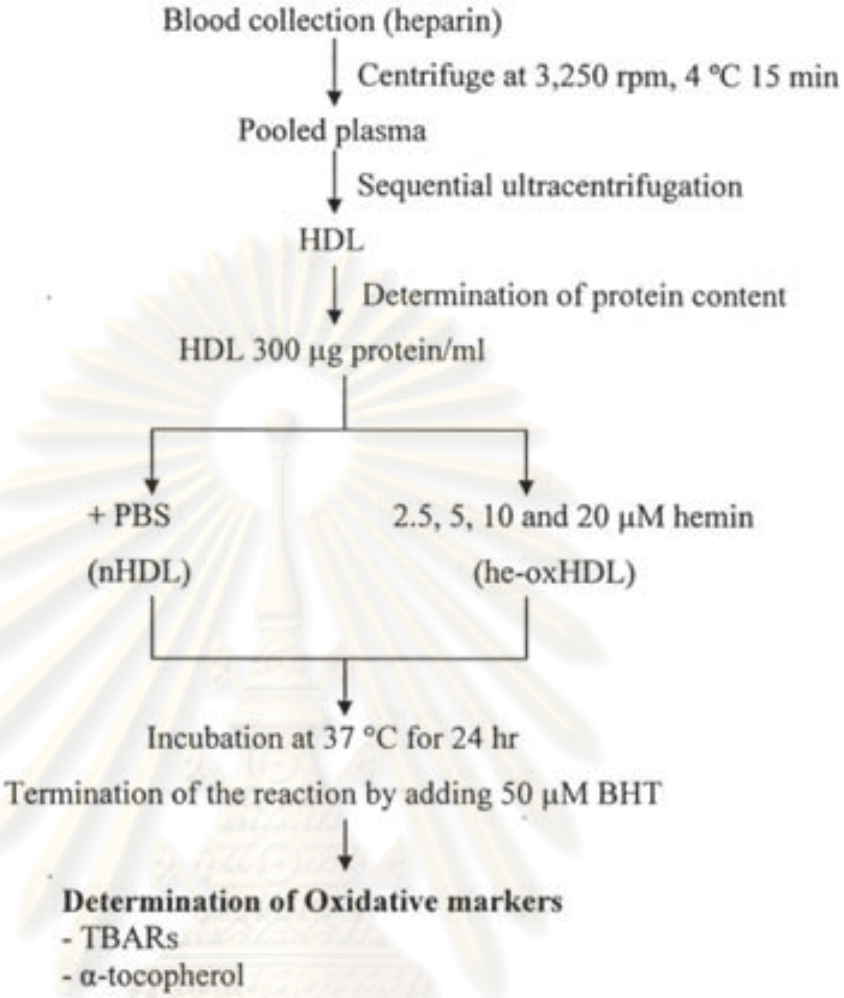


Figure 3.5 Study the concentration dependent effects of hemin induced HDL oxidation (he-oxHDL).

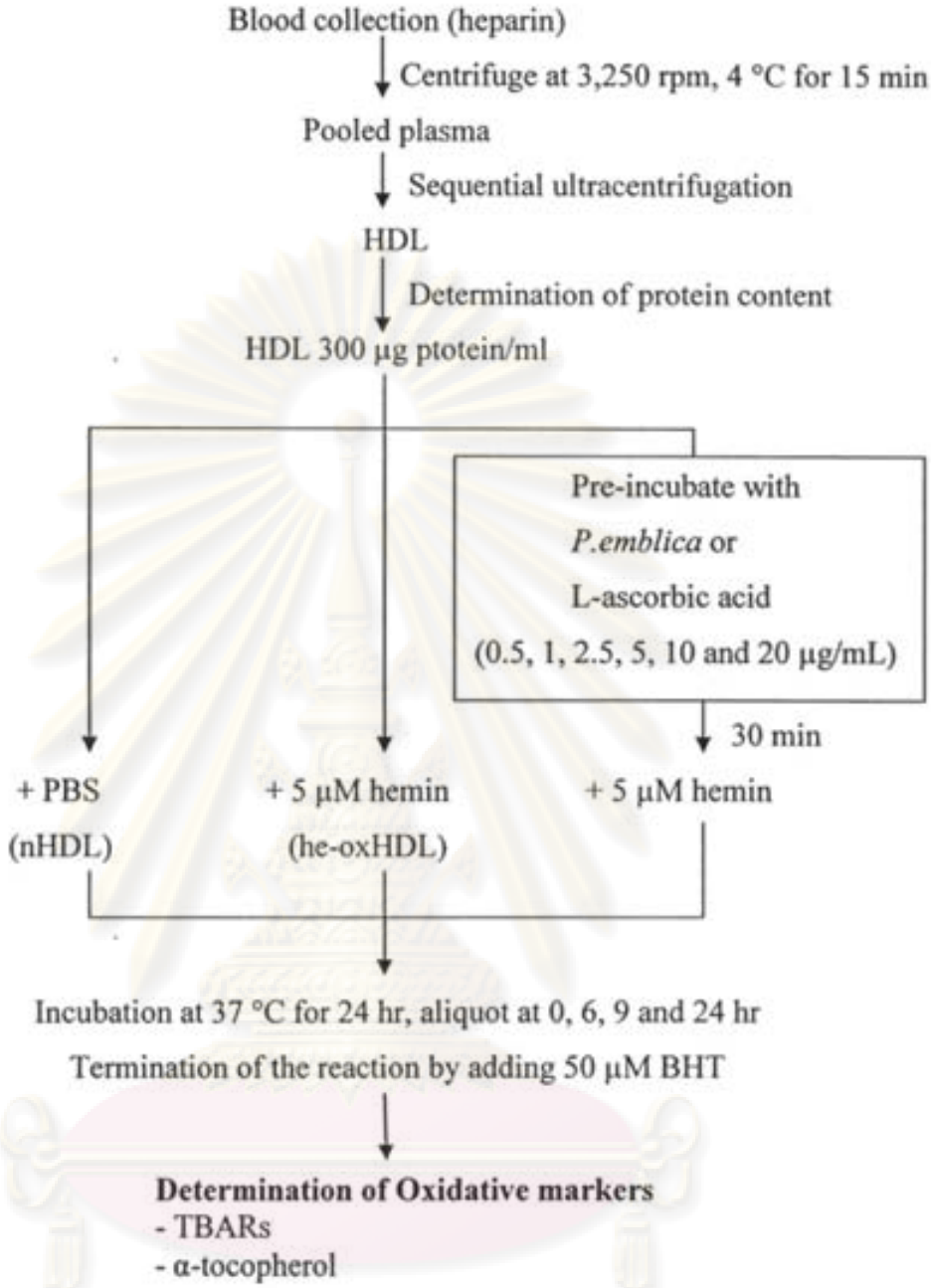


Figure 3.6 Study the concentration dependent effects of *Phyllanthus emblica* on hemin induced HDL oxidation (he-oxHDL).

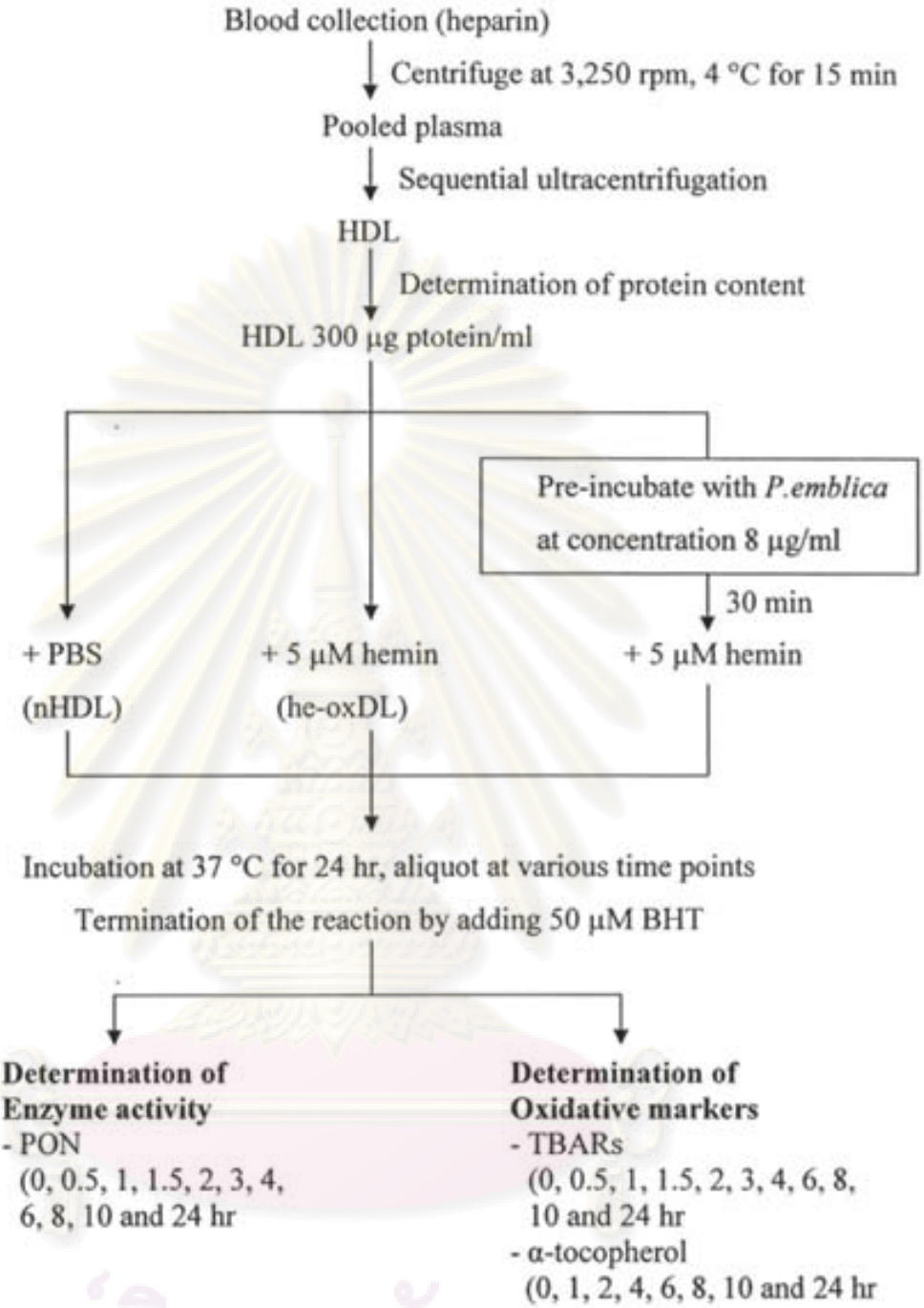


Figure 3.7 Study the time dependent effects of *Phyllanthus emblica* on hemin induced HDL oxidation (he-oxHDL).

4. Statistical Analysis

All data were presented as mean ± standard deviation (S.D.). The comparisons between groups were performed by Student's t test and correlation analyses were assessed by Pearson correlation using the SPSS 13 for window software. P-value less than or equal to 0.05 was accept as statistically significant.

CHAPTER IV

RESULTS

Part I: Study the concentration dependent effects of *Phyllanthus emblica* on hemin induced LDL oxidation (he-oxLDL).

1. PAF-acetylhydrolase activity, α-Tocopherol and lipid composition in native LDL.

The native LDL (nLDL) was separated from heparinized plasma by sequential ultracentrifugation method. The baseline levels of PAF-acetylhydrolase activity, α-tocopherol, total cholesterol, free cholesterol (FC), cholesteryl arachidonate (CA), cholesteryl linoleate (CL), cholesteryl oleate (CO), and cholesteryl palmitate (CP) in nLDLare shown in table 4.1.

Table 4.1 Levels of PAF-aceylhydrolase activity, α-tocopherol and lipid composition in native LDL.

Composition	Levels
PAF-acetylhydrolase activity (nmol/min/mg protein)	25.81 ± 3.76
α-Tocopherol (nmol/mg protein)	19.11 ± 4.26
Total cholesterol (μmol/mg protein)	4.62 ± 0.54
Free cholesterol (μmol/mg protein)	0.972 ± 0.17
Cholesteryl arachodonate (μmol/mg protein)	0.297 ± 0.05
Cholesteryl linoleate (μmol/mg protein)	1.305 ± 0.24
Cholesteryl oleate (μmol/mg protein)	0.435 ± 0.09
Cholesteryl palmitate (μmol/mg protein)	0.284 ± 0.07

Data were presented as mean ± S.D. of five independent experiments.

2. The effect of hemin on the PAF-acetylhydrolase activity

As shown in figure 4.1, hemin induced decreased in PAF-acetylhydrolase activity. PAF-AH activity was significantly decreased at 6 hr of incubation compared with nLDL (21.00 ± 1.99 *vs.* 27.06 ± 1.84 nmol/mg protein). PAF-AH activity was remained about 60% after 24 hr of incubation.

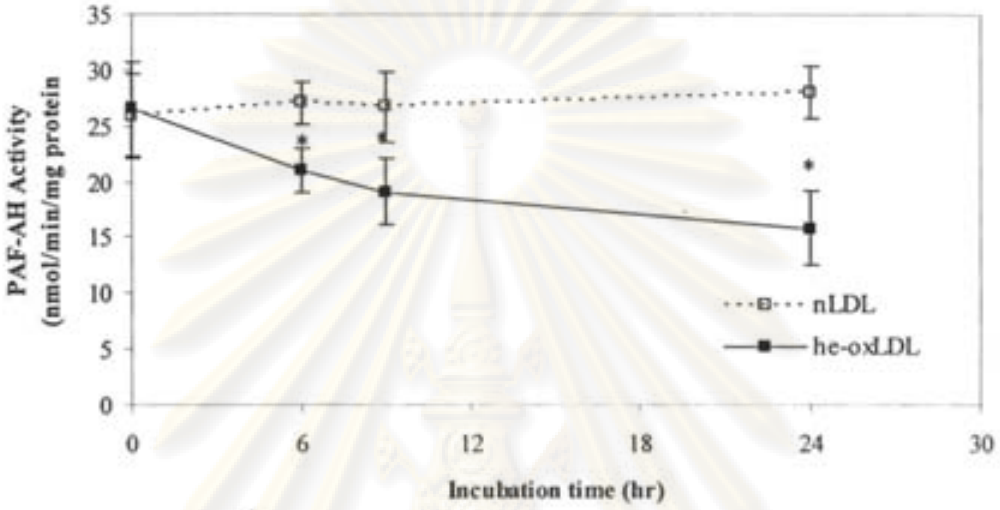


Figure 4.1 Effect of 5 μM hemin on PAF-acetylhydrolase activity in LDL oxidation. Data were presented as mean ± S.D. of five independent experiments. *Significant different at $p<0.01$ compared to respective native LDL control.

3. The effect of hemin on the oxidative markers; TBARs formation and α-tocopherol levels.

TBARs is a marker to determine lipid peroxidation products. The results showed that the TBARs levels in nLDL were not significantly changed when incubation at 37 ºC for 24 hr (5.48 ± 3.36 *vs.* 5.97 ± 3.07 nmol/mg protein). However, when incubation LDL with 5 μM hemin, TBARs levels were significantly increased from 5.34 ± 3.32 nmol/mg protein to maximal level of 36.24 ± 8.36 nmol/mg protein (Figure 4.2). The results indicated that hemin can induce lipid peroxidation inLDL.

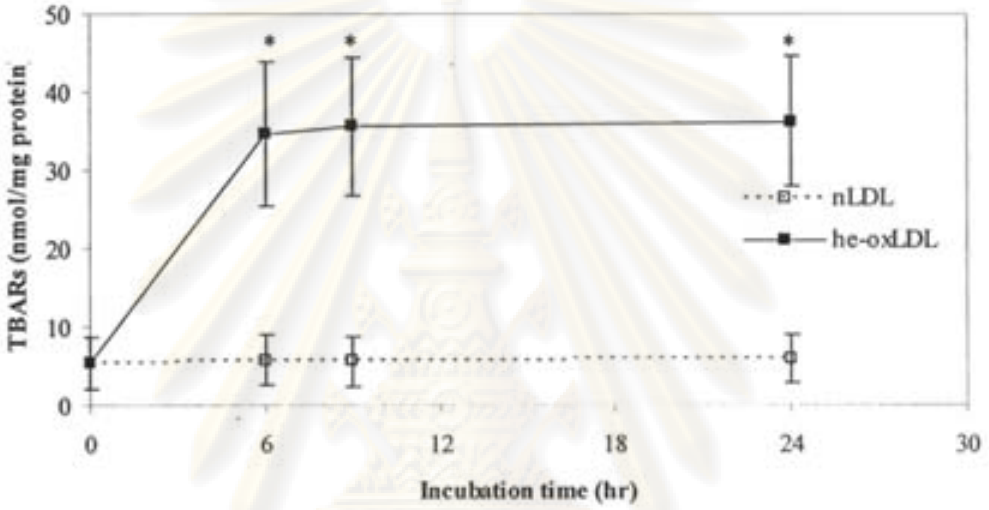


Figure 4.2 Effect of 5 μM hemin on TBARs formation in LDL oxidation.
Data were presented as mean ± S.D. of five independent experiments.
*Significant different $p<0.01$ compared to the respective nLDL control.

α-Tocopherol is the major lipid soluble endogenous antioxidant in LDL. The effect of hemin induced LDL oxidation on levels of α-tocopherol was shown in figure 4.3. The α-tocopherol levels in he-oxLDL were rapidly decreased and undetectable at 6 hr of incubation time. The depletion of α-tocopherol levels indicated that α-tocopherol was consumed during hemin induced LDL oxidation.

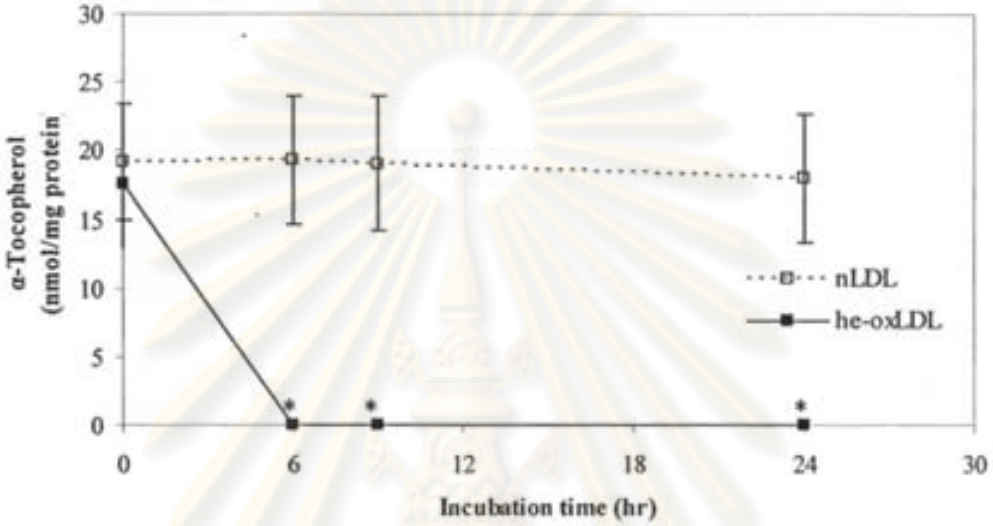


Figure 4.3 Effect of 5 μM hemin on α-tocopherol levels in LDL oxidation.
Data were presented as mean ± S.D. of five independent experiments.
*Significant different at $p<0.01$ compared to respective nLDL control.

4. The effect of hemin on the level of free cholesterol and cholesteryl esters.

The effect of hemin on the level of free cholesterol and cholesteryl esters in nLDL and he-oxLDL are shown in table 4.2 A and B respectively. Levels of CA and CL were decreased while levels of FC, CO and CP were not changed after incubation with hemin. After 6 hr of incubation, there were significantly decreased in the level of cholesteryl arachidonate (CA) ($p<0.05$) in he-oxLDL but not changed in native LDL. At 24 hr incubation of hemin, the levels of CA and CL were significantly decreased 67.4% and 50.8% ($p<0.001$), respectively.

Table 4.2 Free cholesterol and cholesteryl ester in native LDL and he-oxLDL.

A. native LDL

Time (hr)	FC (μmol/mg prot)	CA (μmol/mg prot)	CL (μmol/mg prot)	CO (μmol/mg prot)	CP (μmol/mg prot)
0	0.972 ± 0.18	0.297 ± 0.04	1.305 ± 0.25	0.435 ± 0.09	0.284 ± 0.07
6	1.046 ± 0.09	0.286 ± 0.09	1.331 ± 0.29	0.478 ± 0.11	0.288 ± 0.04
24	1.012 ± 0.16	0.287 ± 0.04	1.251 ± 0.25	0.425 ± 0.11	0.262 ± 0.07

B. He-oxLDL

Time (hr)	FC (μmol/mg prot)	CA (μmol/mg prot)	CL (μmol/mg prot)	CO (μmol/mg prot)	CP (μmol/mg prot)
0	1.061 ± 0.29	0.316 ± 0.07	1.279 ± 0.11	0.427 ± 0.11	0.298 ± 0.04
6	0.919 ± 0.16	0.194 ± 0.07*	1.010 ± 0.31	0.387 ± 0.09	0.239 ± 0.07
24	0.866 ± 0.11	0.103 ± 0.02**	0.629 ± 0.13**	0.286 ± 0.09	0.221 ± 0.07

FC: Free cholesterol, CA: cholesteryl arachidonate, CL: cholesteryl linoleate ,CO cholesteryl oleate ,CP: cholesteryl palmitate.

Data were presented as mean ± S.D. of five independent experiments. *$p<0.05$, ** $p<0.001$ comparing with the respective control at 0 hr.

5. Relationship between PAF-acetylhydrolase activity, oxidative markers and lipid composition.

The correlation between each parameter in he-oxLDL is shown in table 4.3. PAF-acetylhydrolase activity was significant inversely correlated with the level of TBARs ($r = -0.618$, $p<0.01$). The correlation between PAF-AH activity with α-tocopherol level ($r = 0.741$, $p<0.001$), cholesteryl arachidonate ($r = 0.772$, $p<0.01$) and cholesteryl linoleate ($r = 0.738$, $p<0.01$) were found in this study. Whereas TBARs formation had a strong and significant inversely correlated with the level of α-tocopherol ($r = -0.817$, $p < 0.001$), cholesteryl arachidonate ($r = -0.714$, $p<0.01$), cholesteryl linoleate ($r=-0.565$, $p<0.05$) and CL/CO ratio ($r = -0.557$, $p<0.05$) suggesting that hemin induced decreasing in PAF-acetylhydrolase activity, α-tocopherol, cholesteryl arachidonate and cholesteryl linoleate in LDL may result in the generation of oxidative products; TBARs under oxidation process.

Table 4.3 Correlation coefficients (*r*-values) between each parameter.

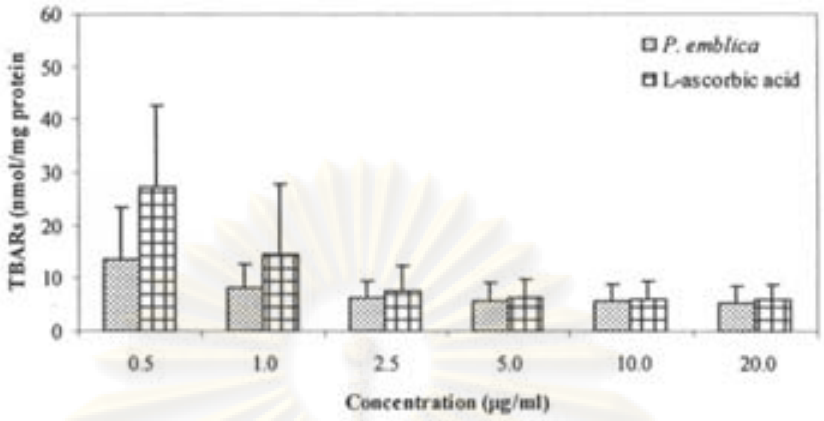
	vs. parameters	Correlation (r)	*p*-value
PAF-acetylhydrolase activity	α-tocopherol	0.741	<0.001
	TBARs	-0.618	<0.01
	Cholesteryl arachidonate	0.772	<0.01
	Cholesteryl linoleate	0.738	<0.01
TBARs	α-tocopherol	-0.817	<0.001
	Cholesteryl arachidonate	-0.714	<0.01
	Cholesteryl linoleate	-0.565	<0.05
	CL/CO ratio	-0.557	<0.05

6. The effect of *Phyllanthus emblica* and L-ascorbic acid on TBARs formation and α-tocopherol levels.

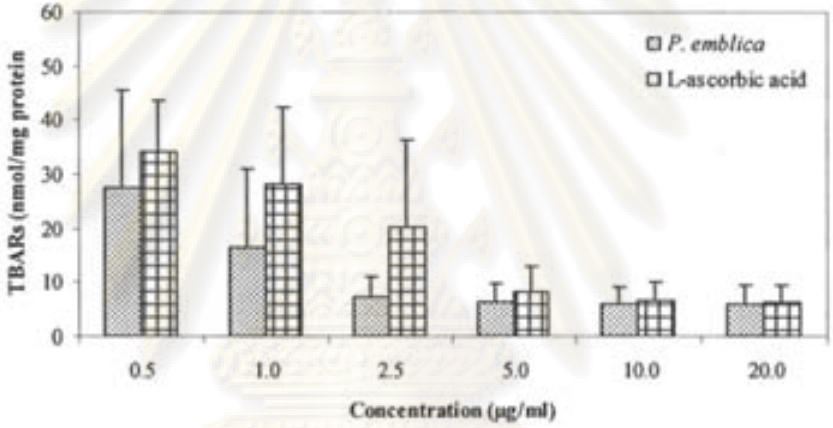
Phyllanthus emblica and L-ascorbic acid were able to inhibit TBARs formation. The level of TBARs formation in he-oxLDL incubated with *P. emblica* and L-ascorbic acid at 6, 9 and 24 hr of incubation time were shown in figure 4.4A-C. Both compounds possess the inhibitory effect on lipid peroxidation in concentration dependent manner. The percentage of inhibition of TBARs formation was shown in figure 4.5. The 50% inhibition concentration (IC_{50}) was obtained from the plot between % inhibition of TBARs formation and concentration of tested compounds. The IC_{50} was 2.5 and 13 μg/ml in *P. emblica* and L-ascorbic acid, respectively. The result indicated that *P. emblica* had more potency of protective effect than L-ascorbic acid, a positive control ($p<0.05$).

The level of α-tocopherol in he-oxLDL was decreased until disappear however α-tocopherol was preserved by *P. emblica* resulting in approximately 40-75% remaining in LDL at 24 hr of incubation. The concentration of *P. emblica* to protect the decreasing of α-tocopherol was 10-20 μg/ml. but L-ascorbic acid can not protect at all test concentrations (Figure 4.6A-C). The result indicated that *P. emblica* had more effective than L-ascorbic acid to protect the decreasing of α-tocopherol.

A) 6 hr



B) 9 hr



C) 24 hr

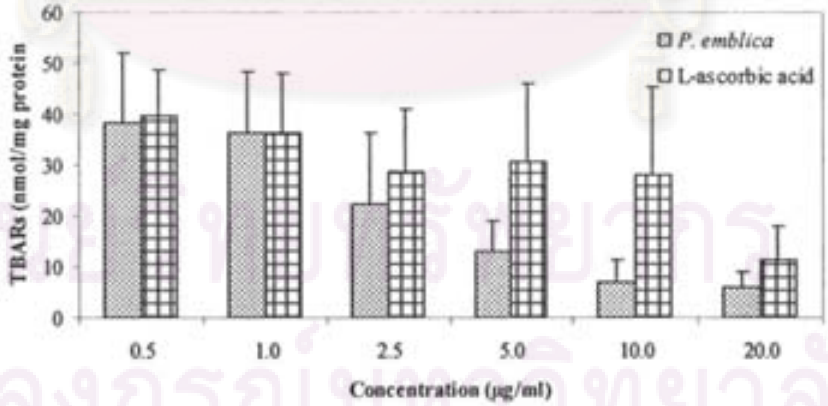


Figure 4.4 The level of TBARs formation in he-oxLDL incubated with *Phyllanthus emblica* and L-ascorbic acid at 6 hr (A), 9 hr (B) and 24 hr (C) of incubation time. Data were presented as mean ± S.D. of five independent experiments.

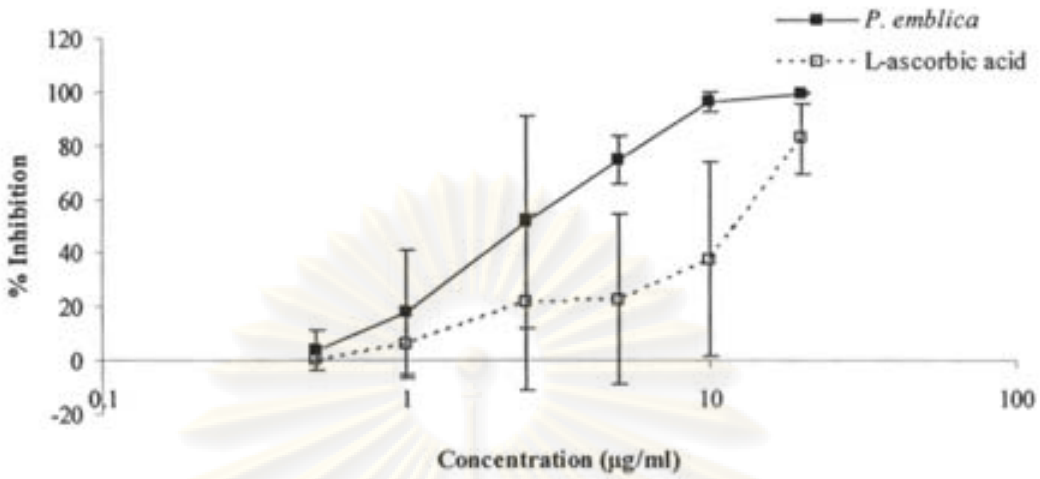
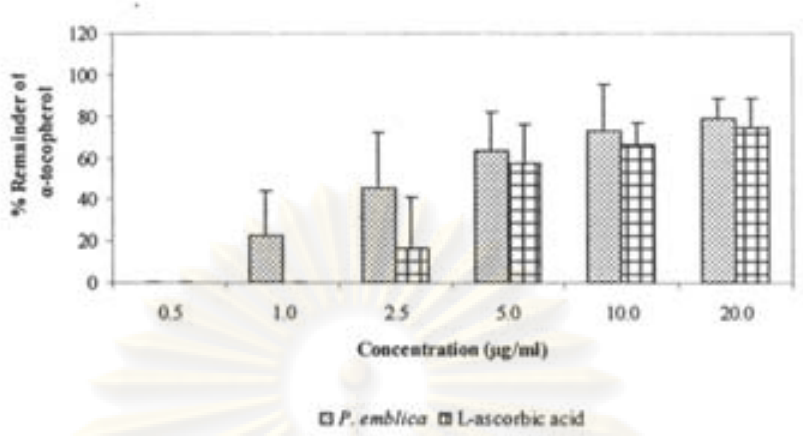
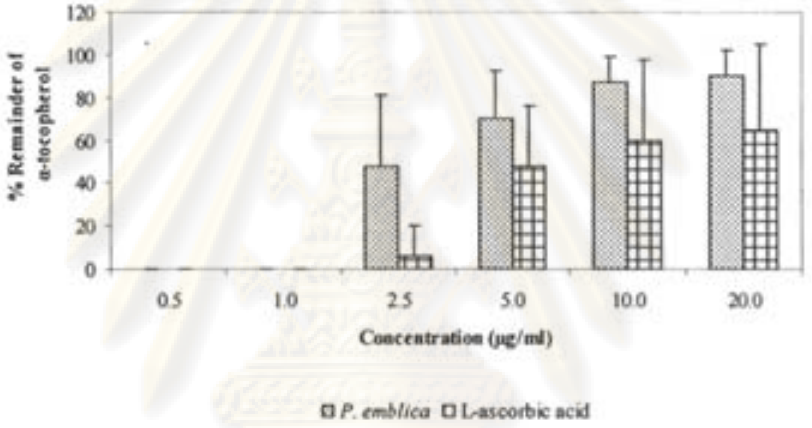


Figure 4.5 The percent inhibition of *Phyllanthus emblica* and L-ascorbic acid on TBARs formation at 24 hr of incubation LDL with hemin. The lines were acquired by plotting the concentrations of tested compounds (0.5, 1, 2.5, 5, 10 and 20 µg/ml) against the percent inhibition of TBARs formation. Data were presented as mean ± S.D. of five independent experiments.

A) 6 hr



B) 9 hr



C) 24 hr

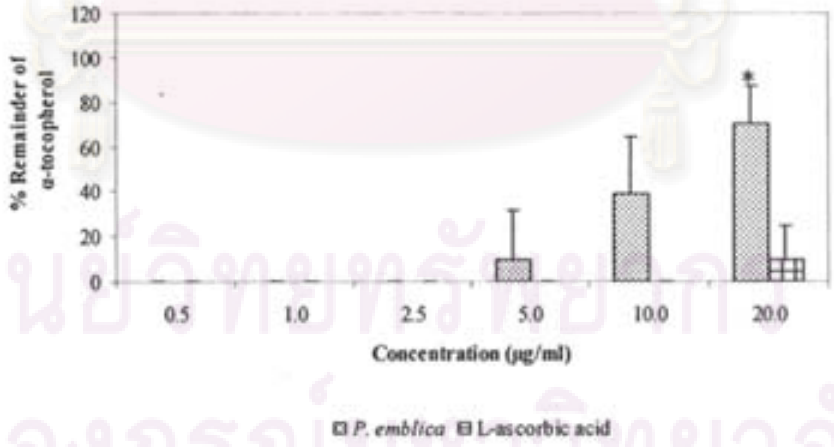
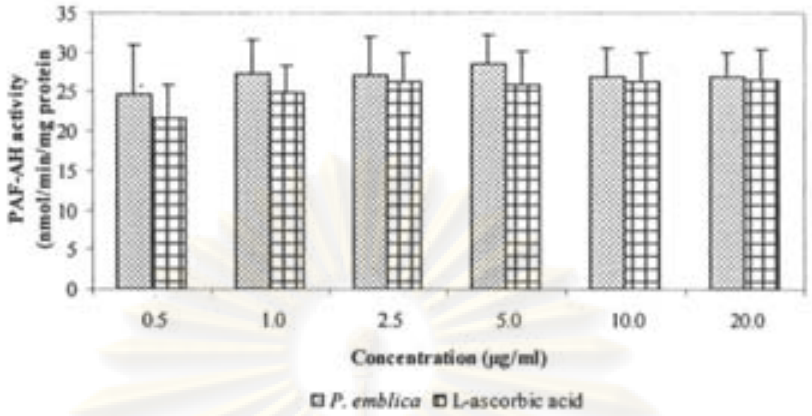


Figure 4.6 The % remainding of α-tocopherol in he-oxLDL incubated with *Phyllanthus emblica* and L-ascorbic acid at 6 hr (A), 9 hr (B) and 24 hr (C) of incubation time. Data were presented as mean ± S.D. of five independent experiments. *Significant different at $p<0.05$ compare to L-ascorbic acid.

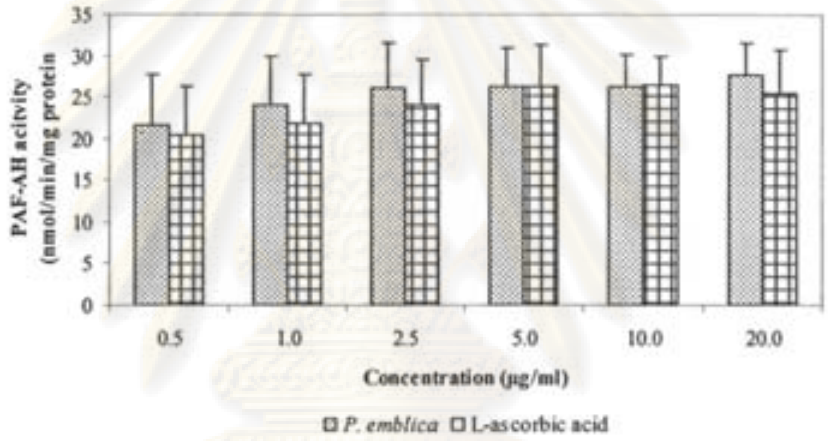
7. The effect of *Phyllanthus emblica* and L-ascorbic acid on PAF-acetylhydrolase activity.

The PAF-AH activity in he-oxLDL was depleted as shown in figure 4.1. *P. emblica* and L-ascorbic acid can preserve the PAF-AH activity at 24 hr of incubation depend on the concentration of each compound. The maximum percentage inhibition of decreasing of PAF-AH activity was 90% and 84% at a concentration of 20 µg/ml for *P. emblica* and L-ascorbic acid, respectively (Figure 4.7A-C).

A) 6 hr



b) 9 hr



c) 24 hr

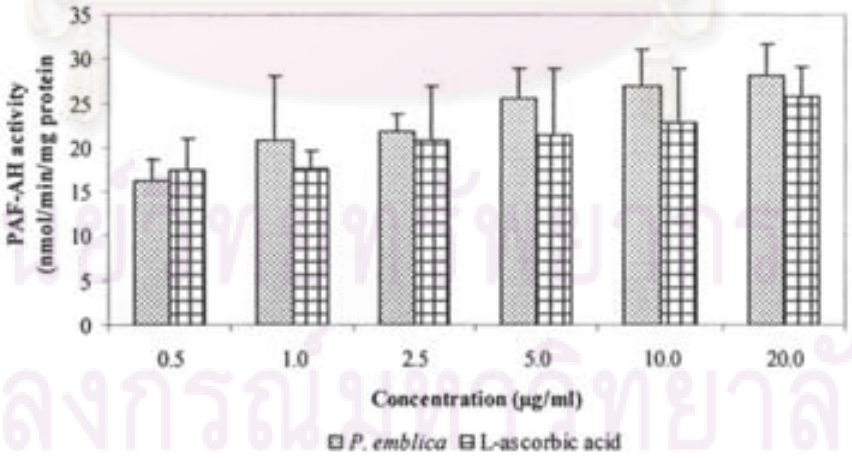


Figure 4.7 The PAF-acetylhydrolase activity in he-oxLDL incubated with *Phyllanthus emblica* and L-ascorbic acid at 6 hr (A), 9 hr (B) and 24 hr (C) of incubation time. Data were presented as mean ± S.D of five independent experiments.

8. The effect of *Phyllanthus emblica* and L-ascorbic acid on cholesteryl esters.

The level of free cholesterol and cholesteryl ester in he-oxLDL incubated with *P. emblica* and L-ascorbic acid at 24 hr of incubation are shown in table 4.4. *P. emblica* was able to protect the decrease of CA and CL in he-oxLDL. The percent inhibition of decreasing of CA was approximately 60-95% for 2.5-20 μg/ml *P. emblica* while only 20 μg/ml L-ascorbic acid can be protect decreasing of CA, approximately 70%. *P. emblica* can inhibit decreasing of CL approximately 70-90% at the concentration 10-20 μg/ml but L-ascorbic acid can not protect the decreasing of CL at all concentrations (Figure 4.8A-B). The results indicated that *P. emblica* had more effective than L-ascorbic acid to protect the decreasing of CA and CO levels.

In addition, each *P. emblica* and L-ascorbic acid was able to protect the decrease of CL/CO ratio in he-oxLDL. The % maximum inhibition of decreasing of CL/CO ratio was approximately 80% for *P. emblica* and L-ascorbic acid (Figure 4.8C).

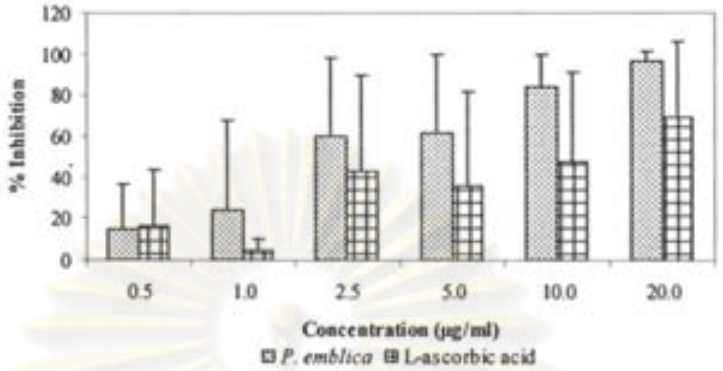
Table 4.4 The effect of *Phyllanthus emblica* and L-ascorbic acid on free cholesterol and cholesteryl esters.

		Cholesteryl esters (μmol/mg protein)				
Condition	Conc. (μg/.ml)	FC	CA	CL	CO	CP
nLDL	-	1.012 ± 0.16	0.287 ± 0.04**	1.251 ± 0.25**	0.425 ± 0.11	0.262 ± 0.07
He-oxLDL	-	0.866 ± 0.11	0.103 ± 0.02	0.629 ± 0.13	0.286 ± 0.09	0.221 ± 0.07
P. emblica	0.5	0.823 ± 0.20	0.113 ± 0.07	0.730 ± 0.22	0.352 ± 0.13	0.222 ± 0.04
	1.0	0.857 ± 0.22	0.134 ± 0.09	0.786 ± 0.25	0.357 ± 0.11	0.256 ± 0.04
	2.5	1.034 ± 0.17	0.214 ± 0.09*	0.972 ± 0.38	0.348 ± 0.13	0.235 ± 0.04
	5.0	1.035 ± 0.09	0.229 ± 0.09*	1.050 ± 0.41	0.384 ± 0.16	0.251 ± 0.09
	10.0	0.928 ± 0.20	0.259 ± 0.04**	1.082 ± 0.25*	0.354 ± 0.11	0.235 ± 0.09
	20.0	0.982 ± 0.20	0.314 ± 0.07**	1.256 ± 0.27*	0.428 ± 0.11	0.246 ± 0.07
L-ascorbic acid	0.5	0.854 ± 0.18	0.122 ± 0.07	0.735 ± 0.20	0.330 ± 0.09	0.227 ± 0.04
	1.0	0.835 ± 0.20	0.101 ± 0.04	0.707 ± 0.16	0.317 ± 0.09	0.232 ± 0.04
	2.5	0.921 ± 0.22	0.175 ± 0.11	0.974 ± 0.45	0.389 ± 0.16	0.260 ± 0.09
	5.0	0.972 ± 0.20	0.167 ± 0.11	0.870 ± 0.40	0.313 ± 0.11	0.209 ± 0.04
	10.0	0.975 ± 0.22	0.186 ± 0.09	0.855 ± 0.31	0.335 ± 0.13	0.235 ± 0.09
	20.0	0.880 ± 0.29	0.229 ± 0.09*	0.984 ± 0.40	0.338 ± 0.17	0.246 ± 0.07

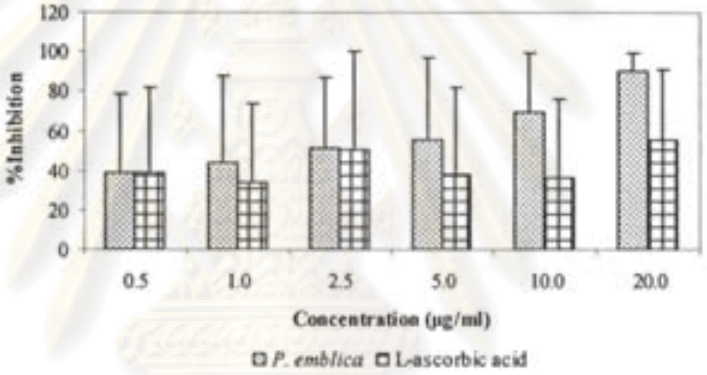
Free cholesterol (FC), cholesteryl arachidonate (CA), cholesteryl linoleate (CL), cholesteryl oleate (CO) and cholesteryl palmitate (CP)

Data were presented as mean ± S.D. of five independent experiments. *$p<0.05$, **$p<0.001$ comparing with the he-oxLDL.

A.



B.



C.

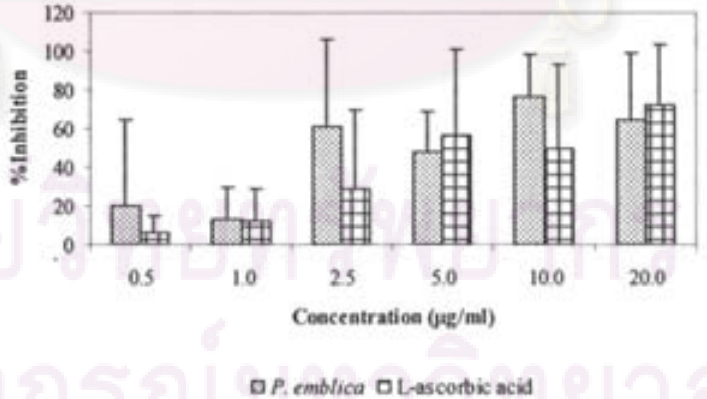
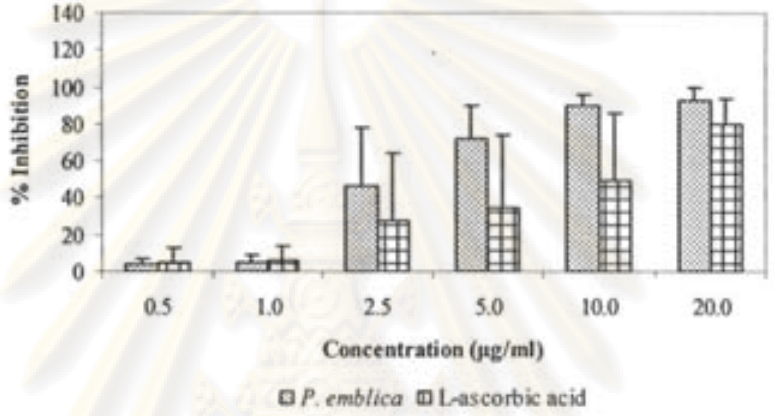


Figure 4.8 The percent inhibition of *Phyllanthus emblica* and L-ascorbic acid on decreasing of CA (A), CL (B) and CL/CO (C) ratio at 24 hr of incubation LDL with hemin. Data were presented as mean ± S.D of five independent experiments.

9. The effect of *Phyllanthus emblica* and L-ascorbic acid on oxidized lipid products.

At 24 hr of incubation LDL with hemin, *P. emblica* and L-ascorbic acid were able to inhibit oxidized lipid products formation in concentration dependent manner at RT 9.8 and 12.6 min (Figure 4.9A and D). While at RT 10.6 and 11.2 min, *P. emblica* at 5, 10 and 20 µg/ml completely inhibited oxidized lipid products formation (Figure 4.9B and C).

A) RT 9.8 min



B) RT 10.6 min

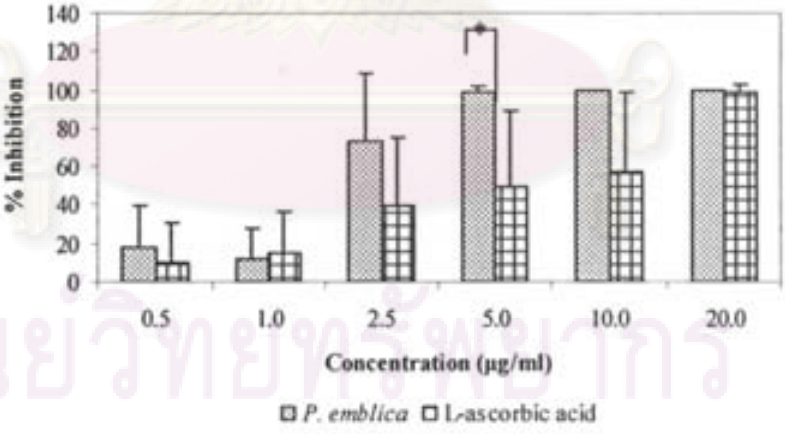
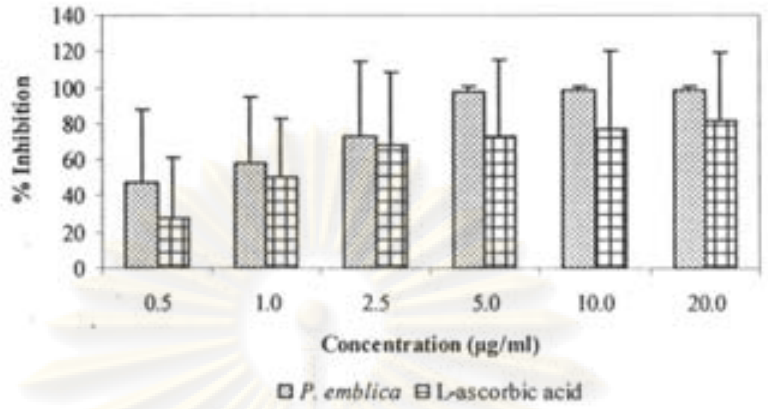


Figure 4.9 The effect of *Phyllanthus emblica* and L-ascorbic acid on % inhibition of oxidized lipid products formation at 24 of incubation LDL with hemin. At retention time (RT) 9.8 min (A), 10.6 min (B), 11.2 min (C) and 12.6 min (D). *Significant different at $p<0.05$. Data were presented as mean ± S.D of five independent experiments.

C) RT 11.2 min



D) RT 12.6 min

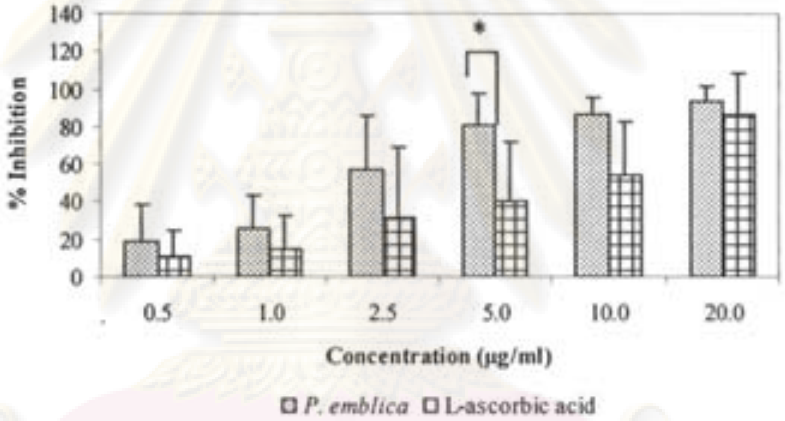


Figure 4.9 The effect of *Phyllanthus emblica* and L-ascorbic acid on % inhibition of oxidized lipid products formation at 24 of incubation LDL with hemin. At retention time (RT) 9.8 min (A), 10.6 min (B), 11.2 min (C) and 12.6 min (D). *Significant different at $p<0.05$. Data were presented as mean ± S.D of five independent experiments (Cont.).

Part II: Study the time dependent effects of *Phyllanthus emblica* on hemin induced LDL oxidation (he-oxLDL).

1. The effect of *Phyllanthus emblica* and L-ascorbic acid on TBARs formation and α-tocopherol levels.

Our results in part I showed that the 50% inhibition concentration on TBARs formation were 2.5 and 13 μg/ml for *P. emblica* and L-ascorbic acid, respectively. The IC_{50} of *P. emblica* and L-ascorbic acid were used for study the time dependent effects in each test compounds.

The TBARs formation was shown in figure 4.10. The results showed that TBARs formation in he-oxLDL have 3 phases, first phase was in 0-4 hr, second phase was in 4-6 hr and third phase was 9-24 hr. The TBARs formation in he-oxLDL incubated with *P. emblica* and L-ascorbic acid was not different. Maximum inhibitory effect of *P. emblica* and L-ascorbic acid on TBARs formation can inhibit until 9 hr of incubation, was not different from 0 hr of incubation. At 12-16 hr of incubation TBARs levels were significantly increased from 0 hr of incubation ($p<0.05$) and more significantly increased at 20-24 hr of incubation ($p<0.001$).

The levels of α-tocopherol in he-oxLDL were decreased until undetectable at 4 hr of incubation. *P. emblica* was able to prolong the time to maintain α-tocopherol levels throughout 9 hr of incubation while L-ascorbic acid can maintain only 4 hr of incubation (Figure 4.11)

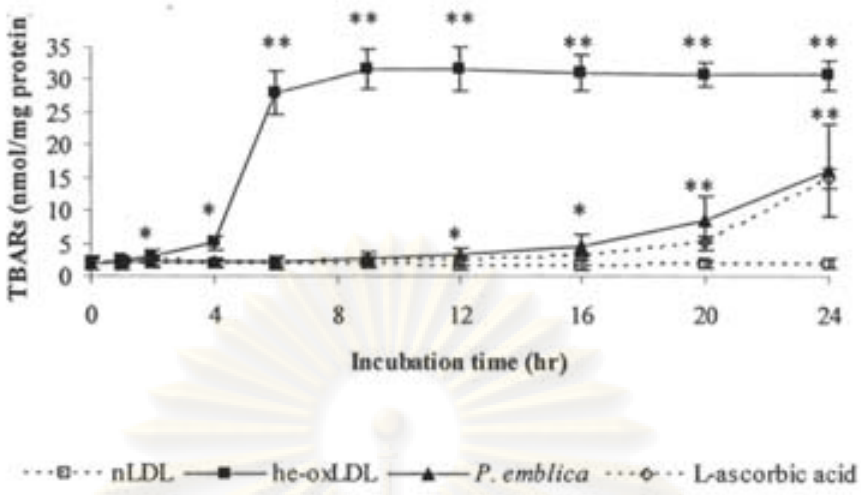


Figure 4.10 The time course effect of 2.5 µg/ml *Phyllanthus emblica* and 13 µg/ml L-ascorbic acid on levels of TBARs formation in he-oxLDL. *$p<0.05$, **$p<0.001$ (comparing with the respective control at 0 hr). Data were presented as mean ± S.D of six independent experiments.

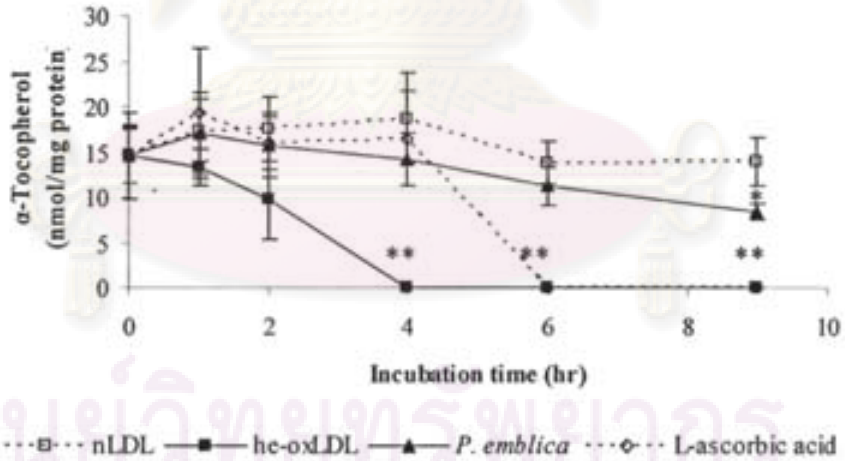


Figure 4.11 The time course effect of 2.5 µg/ml *Phyllanthus emblica* and 13 µg/ml L-ascorbic acid on levels of α-tocopherol in he-oxLDL. *$p<0.05$, **$p<0.001$ comparing with the respective control at 0 hr. Data were presented as mean ± S.D of six independent experiments.

2. The effect of *Phyllanthus emblica* and L-ascorbic acid on PAF-acetylhydrolase activity.

As shown in figure 4.12, hemin induced time dependent decreased in PAF-AH activity. PAF-AH activity was completely preserved for 4 hr (the lag phase). After 9 hr of incubation PAF-acetylhydrolase activity was significantly decreased compared with 0 hr control ($p<0.001$). *P. emblica* and L-ascorbic acid were able to maintain the PAF-AH activity completely until 20 hr and maintain approximately 50% at 24 hr.

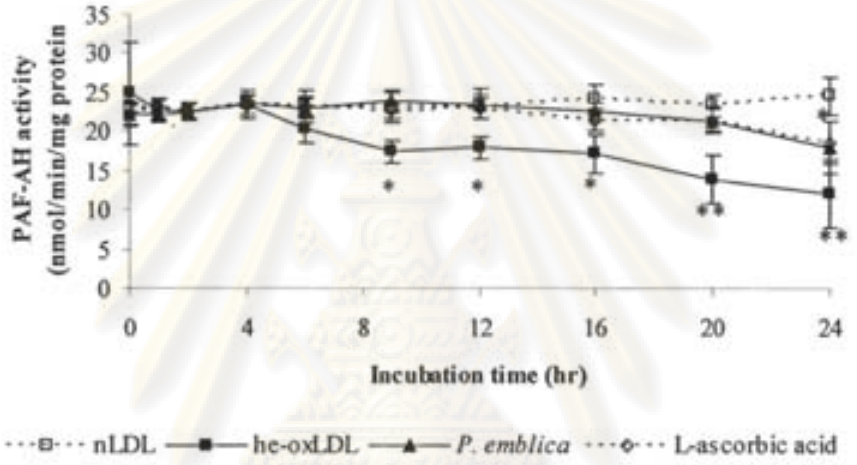


Figure 4.12 The time course effect of 2.5 µg/ml *Phyllanthus emblica* and 13 µg/ml L-ascorbic acid on PAF-acetylhydrolase in he-oxLDL. *$p<0.05$, **$p<0.001$ comparing with the respective control at 0 hr. Data were presented as mean ± S.D of six independent experiments.

3. The effect of *Phyllanthus emblica* and L-ascorbic acid on cholesteryl esters.

P. emblica and L-ascorbic acid were able to protect the damaged of lipid in he-oxLDL along 24 hr of incubation. The results of this study show the time course effect of *P. emblica* and L-ascorbic acid on cholesteryl ester as shown in table 4.5.

In addition, *P. emblica* and L-ascorbic acid were able to inhibit the decreased of CL/CO ratio in he-oxLDL along 24 hr of incubation (Figure 4.13). The results showed that *P. emblica* and L-ascorbic acid were significantly inhibited decreasing of CL/CO ratio ($p<0.05$) and these ratio have a comparable levels with 0 hr of incubation.

Table 4.5 The time course effect of 2.5 μg/ml *Phyllanthus emblica* and 13 μg/ml L-ascorbic acid on cholesteryl esters in he-oxLDL.

		Cholesteryl esters (μmol/mg protein)				
Time (hr)	Condition	FC	CA	CL	CO	CP
0	nLDL	0.852 ± 0.12	0.219 ± 0.02	1.004 ± 0.07	0.396 ± 0.07	0.190 ± 0.05
	He-oxLDL	0.927 ± 0.12	0.239 ± 0.05	1.129 ± 0.17	0.430 ± 0.10	0.204 ± 0.05
	P. emblica	0.909 ± 0.10	0.220 ± 0.02	0.967 ± 0.12	0.353 ± 0.07	0.173 ± 0.07
	L-ascorbic acid	0.872 ± 0.15	0.217 ± 0.02	0.965 ± 0.10	0.357 ± 0.05	0.191 ± 0.05
6	nLDL	0.901 ± 0.07	0.221 ± 0.02	1.013 ± 0.17	0.414 ± 0.05	0.231 ± 0.05
	He-oxLDL	0.869 ± 0.17	0.145 ± 0.02*	0.938 ± 0.07*	0.428 ± 0.07	0.207 ± 0.05
	P. emblica	0.898 ± 0.12	0.237 ± 0.02	0.944 ± 0.42	0.443 ± 0.05	0.223 ± 0.05
	L-ascorbic acid	0.925 ± 0.15	0.224 ± 0.02	0.995 ± 0.12	0.381 ± 0.07	0.215± 0.02
9	nLDL	0.864 ± 0.10	0.239 ± 0.02	1.060 ± 0.12	0.412 ± 0.07	0.189 ± 0.05
	He-oxLDL	0.877 ± 0.15	0.133 ± 0.05*	0.876 ± 0.17	0.407 ± 0.07	0.185 ± 0.05
	P. emblica	0.945 ± 0.20	0.232 ± 0.02	1.043 ± 0.07	0.407 ± 0.07	0.169 ± 0.05
	L-ascorbic acid	0.864 ± 0.10	0.215 ± 0.02	0.969 ± 0.12	0.385 ± 0.07	0.191 ± 0.05
12	nLDL	0.979 ± 0.10	0.271 ± 0.02	1.265 ± 0.20	0.445 ± 0.07	0.211 ± 0.05
	He-oxLDL	0.914 ± 0.12	0.127 ± 0.02*	0.900 ± 0.17*	0.463 ± 0.07	0.173 ± 0.07
	P. emblica	0.984 ± 0.07	0.288 ± 0.05	1.311 ± 0.12	0.487 ± 0.10	0.256 ± 0.02
	L-ascorbic acid	1.051 ± 0.15	0.279 ± 0.02	1.295 ± 0.12	0.480 ± 0.10	0.257 ± 0.05
16	nLDL	0.998 ± 0.10	0.273 ± 0.02	1.009 ± 0.42	0.456 ± 0.07	0.203 ± 0.05
	He-oxLDL	0.925 ± 0.07	0.107 ± 0.02*	0.775 ± 0.15*	0.416 ± 0.07	0.205 ± 0.05
	P. emblica	0.944 ± 0.10	0.276 ± 0.05	1.278 ± 0.25	0.448 ± 0.05	0.250 ± 0.02
	L-ascorbic acid	0.998 ± 0.15	0.303 ± 0.05	1.375 ± 0.27	0.496 ± 0.10	0.259 ± 0.05
20	nLDL	1.032 ± 0.20	0.283 ± 0.02	1.263 ± 0.15	0.462 ± 0.10	0.245 ± 0.05
	He-oxLDL	0.960 ± 0.12	0.116 ± 0.02*	0.781 ± 0.17*	0.428 ± 0.10	0.213 ± 0.05
	P. emblica	0.991 ± 0.07	0.248 ± 0.02	1.132 ± 0.17	0.470 ± 0.05	0.218 ± 0.07
	L-ascorbic acid	0.991 ± 0.07	0.261 ± 0.02	1.213 ± 0.12	0.496 ± 0.05	0.241 ± 0.05
24	nLDL	0.970 ± 0.12	0.249 ± 0.02	1.093 ± 0.17	0.420 ± 0.02	0.199 ± 0.05
	He-oxLDL	0.949 ± 0.17	0.120 ± 0.02*	0.908 ± 0.15*	0.460 ± 0.07	0.234 ± 0.07
	P. emblica	0.950 ± 0.12	0.219 ± 0.02	1.027 ± 0.15	0.403 ± 0.02	0.227 ± 0.07
	L-ascorbic acid	0.955 ± 0.10	0.247 ± 0.02	1.208 ± 0.12	0.463 ± 0.02	0.242 ± 0.05

Data were presented as mean ± S.D. of six independent experiments.

*$p<0.05$ comparing with the respective control at 0 hr.

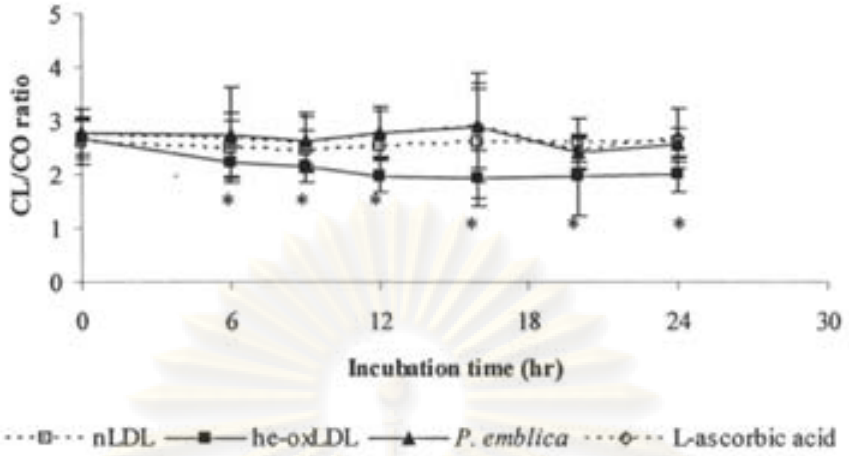
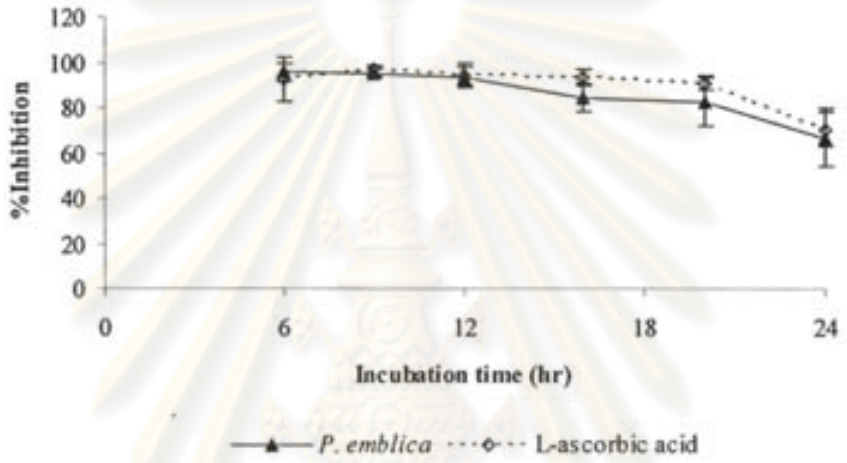


Figure 4.13 The time course effect of 2.5 µg/ml *Phyllanthus emblica* and 13 µg/ml L-ascorbic acid on CL/CO ratio in he-oxLDL. Data were presented as mean ± S.D. of six independent experiments. *$p<0.05$ comparing with the respective control at 0 hr.

4. The effect of *Phyllanthus emblica* and L-ascorbic acid on oxidized lipid products.

P. emblica and L-ascorbic acid were able to inhibit the oxidized lipid products formation in he-oxLDL all retention time along 20 hr of incubation (Figure 4.14A-D). However, no difference was found in he-oxLDL incubated with *P. emblica* and L-ascorbic acid on the formation of oxidized lipid products.

A) RT 9.8 min



B) RT 10.6 min

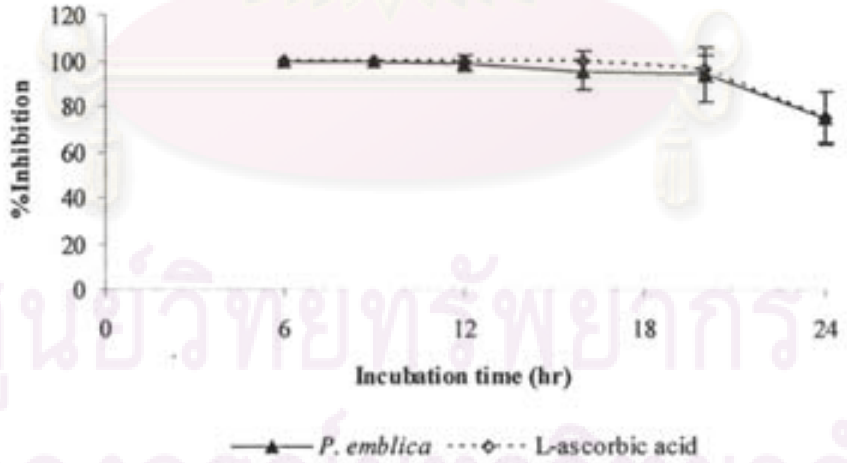
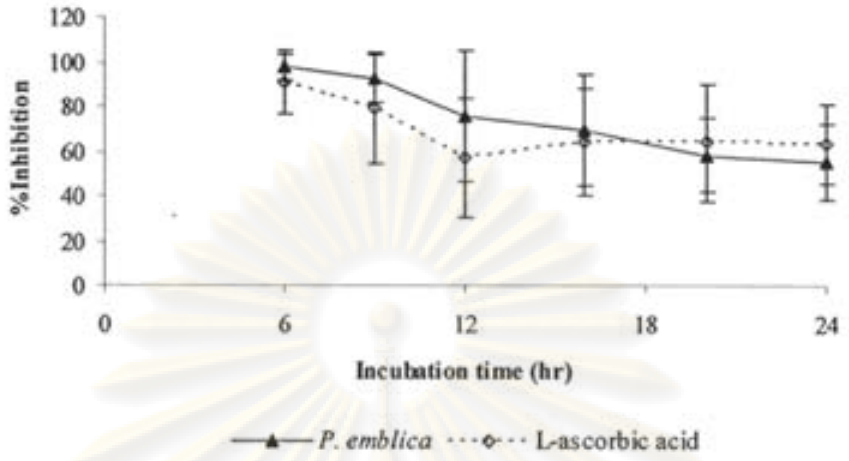


Figure 4.14 The time course effect of *Phyllanythus emblica* and L-ascorbic acid on % inhibition of oxidized lipid products formation in he-oxLDL at retention time (RT) 9.8 min (A), 10.6 min (B), 11.2 min (C) and 12.6 min (D). Data were presented as mean ± S.D. of six independent experiments.

C) RT 11.2 min



D) RT 12.6 min

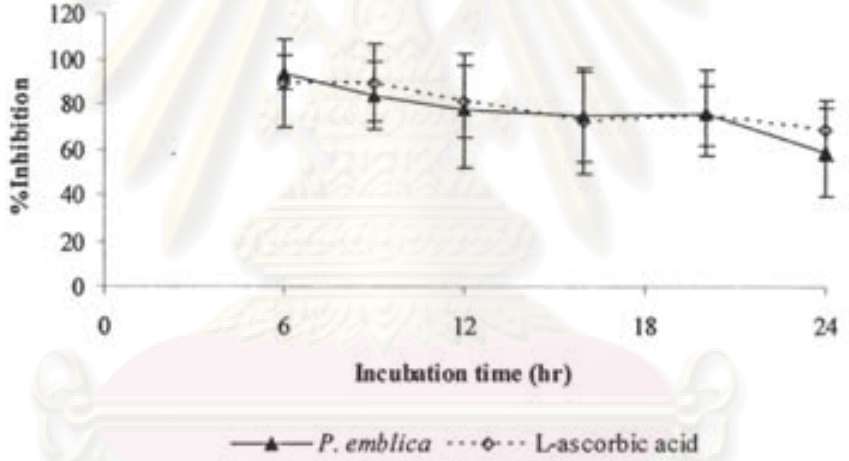


Figure 4.14 The time course effect of *Phyllanythus emblica* and L-ascorbic acid on % inhibition of oxidized lipid products formation in he-oxLDL at retention time (RT) 9.8 min (A), 10.6 min (B), 11.2 min (C) and 12.6 min (D). Data were presented as mean ± S.D. of six independent experiments (Cont.).

Part III: Study the concentration dependent effects of hemin induced HDL oxidation (he-oxHDL).

1. The effect of hemin on the formation of lipid peroxidation products; TBARs.

Figure 4.15 shows that, no difference was found in any incubation time of control and all concentrations of hemin incubated with HDL in the TBARs formation. These observations may indicate that HDL was less susceptible to oxidation induced by hemin than LDL.

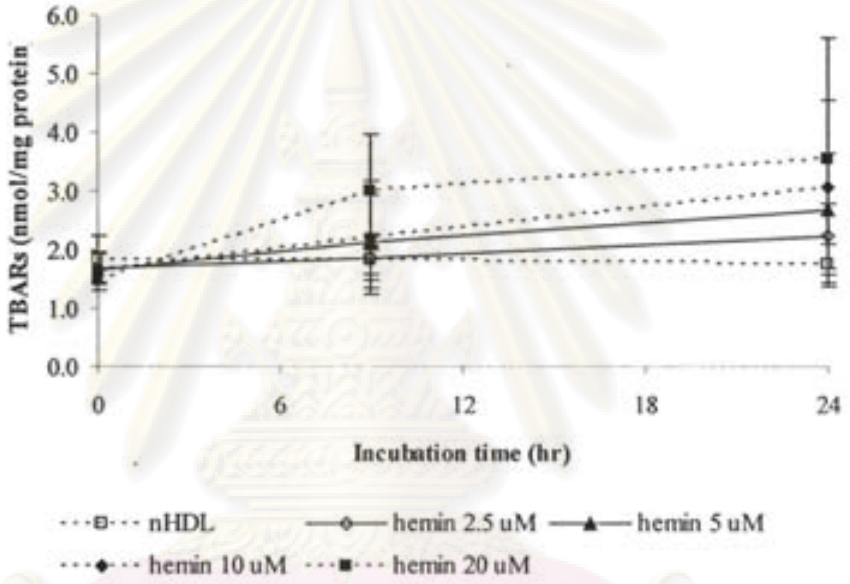


Figure 4.15 TBARs formation in HDL after oxidation with various concentrations (2.5, 5, 10 and 20 μM) of hemin. Data were presented mean ± S.D. of two independent experiments.

2. The effect of hemin on the α-tocopherol levels.

During the oxidation of HDL with a various concentration of hemin, there was shown a significant reduction in α-tocopherol (Figure 4.17).

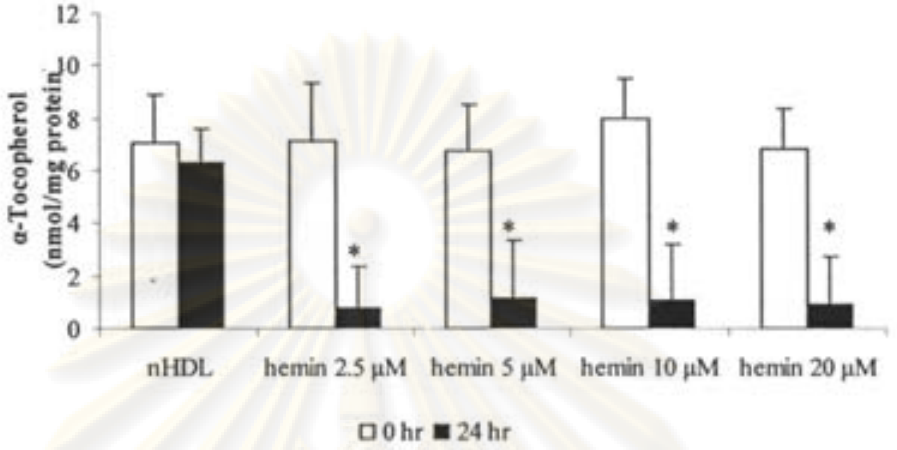


Figure 4.16 α-Tocopherol levels in HDL after oxidation with various concentrations (2.5, 5, 10 and 20 μM) of hemin.

Data were presented mean ± S.D. of four independent experiments.

*p<0.01 (comparing with the respective control at 0 hr).

Part IV: Study the concentration dependent effects of *Phyllanthus emblica* on hemin induced HDL oxidation (he-oxHDL).

1. The effect of *Phyllanthus emblica* and L-ascorbic acid on TBARs formation.

TBARs formation was significantly increased in HDL after 24 hr of incubation with hemin. TBARs formation in he-oxHDL was significantly increased compared with nHDL (2.90 ± 0.36 *vs.* 4.34 ± 0.45 nmol/mg protein, respectively). The results showed that, no difference was found in he-oxHDL incubated with each concentration of *P. emblica* and native HDL in the TBARs formation. While TBARs formation was significantly increased in he-oxHDL incubated with 0.5 and 1.0 μg/ml for L-ascorbic acid compared with native HDL (3.71 ± 0.31, 3.77 ± 0.22 and 2.90 ± 0.36 nmol/mg protein, respectively) (Figure 4.17).

The percentage of inhibition of TBARs formation was shown in figure 4.18. All concentrations of *P. emblica* were able to protect TBARs formation approximately 75-95%. The results showed that each concentration of *P. emblica* had much more protective effect on TBARs formation in he-oxHDL than L-ascorbic acid, a positive control.

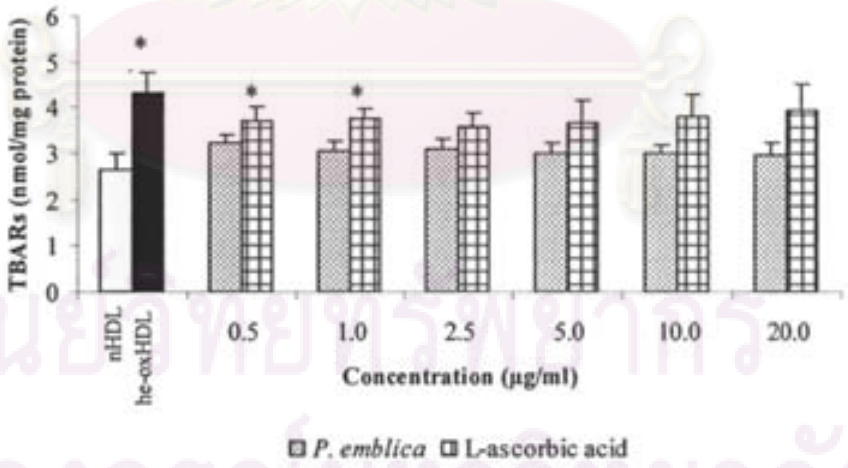


Figure 4.17 The level of TBARs formation in he-oxHDL incubated with *Phyllanthus emblica* and L-ascorbic acid at 24 of incubation.
Data were presented as mean ± S.D. of three independent experiments. *Significant different at $p<0.05$ compared to respective native HDL control.

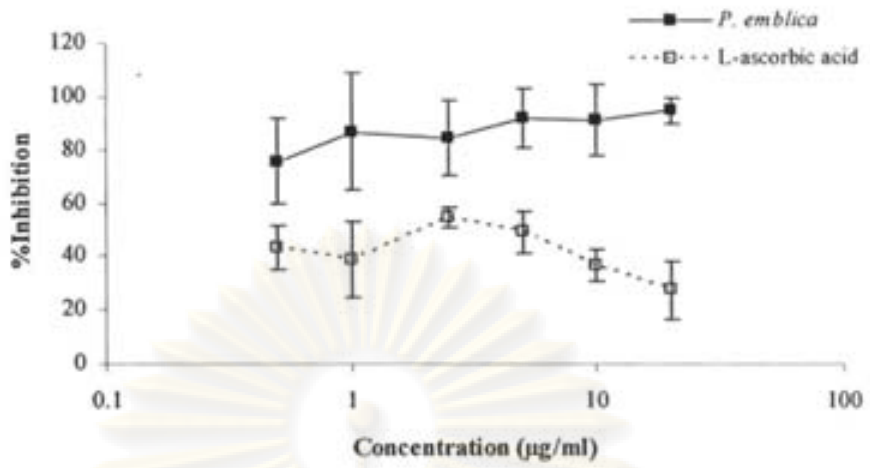


Figure 4.18 The percent inhibition of *Phyllanthus emblica* and L-ascorbic acid on TBARs formation at 24 hr of incubation HDL with hemin. The lines were acquired by plotting the concentrations of tested compounds (0.5, 1, 2.5, 5, 10 and 20 µg/ml) against the percent inhibition of TBARs formation. Data were presented as mean ± S.D. of three independent experiments.

2. The effect of *Phyllanthus emblica* and L-ascorbic acid on α-tocopherol levels.

During the oxidation of HDL with hemin, there was a significant reduction in α-tocopherol compared with nHDL (9.21 ± 1.89 *vs.* 1.45 ± 2.51 nmol/mg protein, respectively). The results showed that at the concentration 10 and 20 μg/ml for *P. emblica* was able to preserve α-tocopherol. α-Tocopherol in he-oxHDL incubated with *P. emblica* 20 μg/ml was remained almost 100% (Figure 4.19).

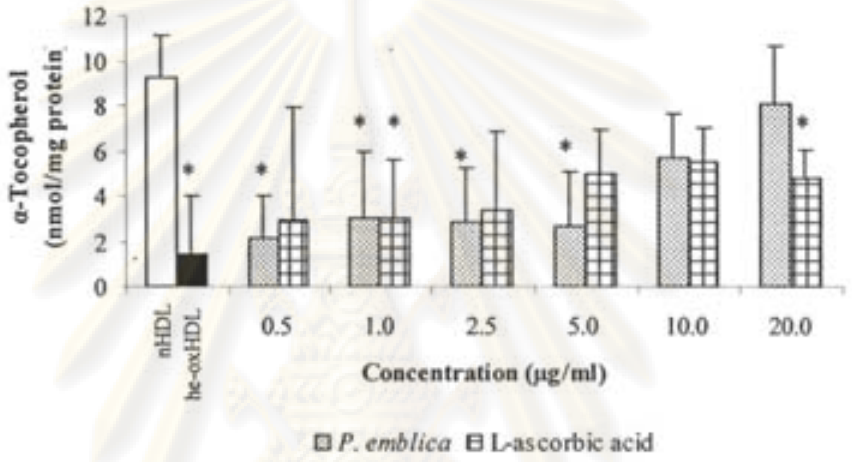


Figure 4.19 The level α-tocopherol in he-oxHDL incubated with *Phyllanthus emblica* and L-ascorbic acid at 24 of incubation.

Data were presented as mean ± S.D. of three independent experiments. *Significant different at $p<0.05$ compared to respective native HDL control.

Part V: Study the time dependent effects of *Phyllanthus emblica* on hemin induced HDL oxidation (he-oxHDL).

1. The effect of *Phyllanthus emblica* on TBARs formation and α-tocopherol levels.

Figure 4.20 shows that TBARs formation in he-oxHDL incubated with *P. emblica* was decreased compared with he-oxHDL (2.55 ± 1.21 *vs.* 1.52 ± 0.69 nmol/mg protein, respectively). However, no significantly difference was found in any incubation time of nHDL, he-oxHDL and he-oxHDL incubated with *P. emblica.*

The time course effect of *P. emblica* on levels of α-tocopherol was shown in figure 4.21. Normally α-tocopherol in he-oxHDL was rapidly decreased within 1 hr of incubation. α-Tocopherol remain in he-oxHDL incubated with *P. emblica* at 24 hr of incubation approximately 40%.

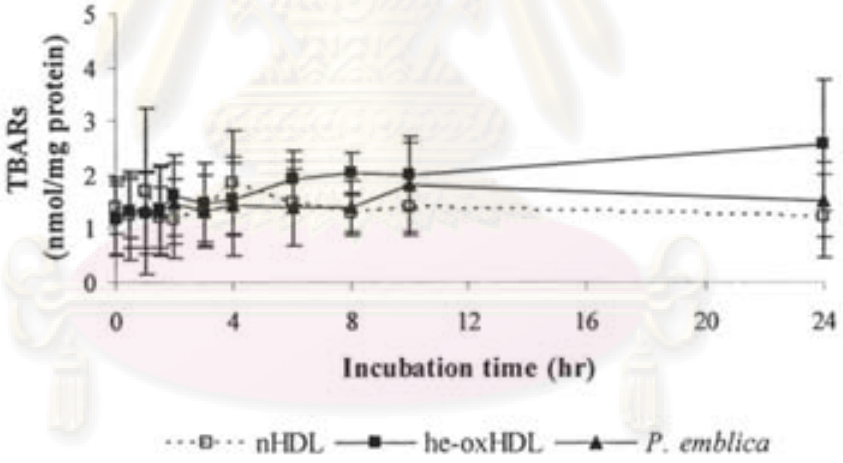


Figure 4.20 The time course effect of 8 μg/ml *Phyllanthus emblica* on TBARs formation in he-oxHDL.

Data were presented as mean ± S.D. of two independent experiments.

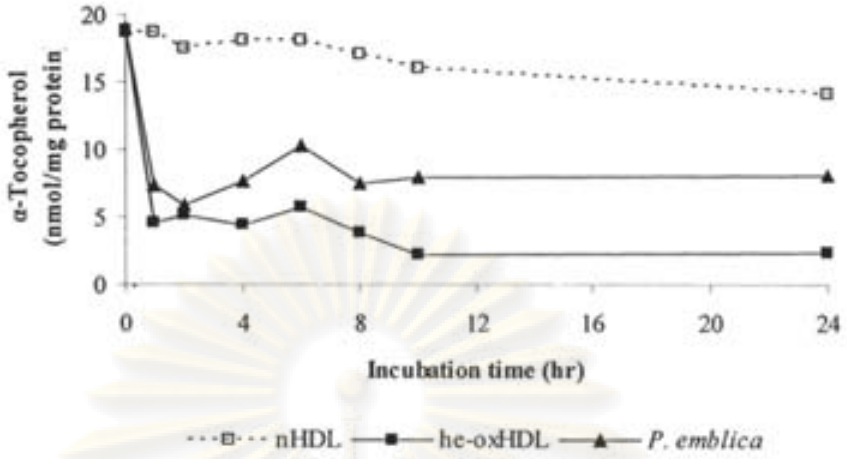


Figure 4.21 The time course effect of *Phyllanthus emblica* on levels of α-tocopherol in he-oxLDL.

2. The effect of *Phyllanthus emblica* on paraoxonase activity.

As shown in figure 4.23, no difference found in any incubation time of nHDL, he-oxHDL and he-oxHDL incubated with *P. emblica* in the measure of paraoxonase activity. Our results show that decreasing in paraoxonase activity for all conditions.

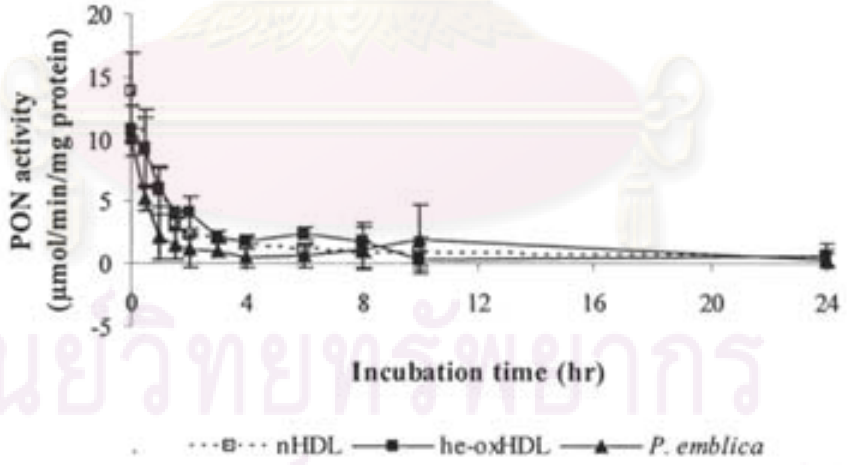


Figure 4.22 The time course effect of 8 μg/ml *Phyllanthus emblica* on paraoxonase activity. Data were presented as mean ± S.D. of two independent experiments.

CHAPTER V

DISCUSSION AND CONCLUSION

Our results clearly demonstrated that hemin is able to induce lipid peroxidation in LDL. Hemin was observed at levels ranging from 50 to 280 μM in serum of β-thal/Hb E but not detectable in serum of non-thalassemia (7). A significant oxidative effect was observed at physiologically relevant hemin concentration in thalassemia patients. Oxidative stress is an important mechanism for evidence of clinical complications in thalassemia. Miller et al., showed that hemin binds initially to LDL and HDL (51). Incorporation of hemin into LDL particles results in release of iron (Fe^{3+}) to the hydrophobic core of the lipoprotein. Iron exerts its toxicity through a series of reactions with reactive oxygen species called the Fenton reaction, generating the highly toxic hydroxyl radical (84). The hydroxyl radical can react with the unsaturated fatty acid containing of cholesteryl ester in hydrophobic core lipoprotein to initiate lipid peroxidation, resulting in the lipid peroxidation product (4). The result of this study also demonstrated that TBARs levels were increased (Figure 4.2) while α-tocopherol levels were rapidly decreased in LDL oxidized by hemin (Figure 4.3). α-Tocopherol show the inverse correlation with the TBARs formation ($r = -0.817$, $p<0.001$). This confirms that the consumption of endogenous α-tocopherol in LDL is occurred during LDL oxidation (114). In thalassemia patients, there is evidence as LDL is exposed to oxidative conditions, it becomes increase of MDA production, the final product of lipid peroxidation and a marked deplete of plasma lipid antioxidant, α-tocopherol (as known chain-breaking antioxidant activity) (115). In addition, hemin induced LDL oxidation was characterized by a decrease in the lipid component; cholesteryl arachidonate (CA) and cholesteryl linoleate (CL). The content of CA and CL show an inverse correlation with TBARs formation ($r = -0.714$, $p<0.01$ and $r = -0.565$, $p<0.05$, respectively). The decreasing of CA and CL suggests that both CA and CL are the target for lipid peroxidation induced by hemin and results in cholesteryl ester hydroperoxide formation (Figure 4.9 A-D). The CL/CO ratio of less than 2 has been suggested to be used as a clinical marker to determine the degree of clinical severity in β-thal/HbE patients (127). This study also found that CL/CO ratio was decreased of less than 2 in hemin induced LDL oxidation and was inverse correlated with TBARs formation ($r = -0.557$, $p<0.05$), indicating that CL/CO ratio can be used

as a marker of lipid damage during oxidation of LDL by hemin. These findings agree with previous study that there were high TBARs levels and corresponding low α-tocopherol levels as well as the low levels of CEs and CL/CO ratio in both plasma and lipoprotein of β-thal/HbE patients, especially in moderately and severely affected patients (116). This *in vitro* study of LDL oxidation induced by hemin mimicked the characteristics of LDL in iron overloaded thalassemia patients.

It was known that PAF-AH in LDL degrades oxidized lipid products. During induction oxidation of LDL by hemin, there was also a loss in the activity of PAF-AH (Figure 4.1). The PAF-AH activity show the inverse correlation with TBARs formation ($r = -0.618$, $p<0.01$), indicating that PAF-AH was used to hydrolyze oxidized lipid products generated from lipid peroxidation reaction induced by hemin. The depletion of PAF-AH hydrolytic activity in LDL may be attributable to an increase in the amount of biologically active phospholipids that are toxic to the cell and induce inflammatory responses in endothelial cells which lead to monocyte recruitment, adhesion, and differentiation into macrophages (117).

The present study showed that spray-dried fruit juice of *P. emblica* can protect LDL against lipid peroxidation induced by hemin. *P. emblica* exhibits the inhibition of lipid peroxidation products; TBARs and oxidized lipid products, the preservation of α-tocopherol and PAF-AH activity as well as the protection of cholesteryl esters damage (CA and CL) in a dose dependent manner (Figure 4.4 - 4.9). The two possible mechanisms of action based on its antioxidant activity may be due to firstly, its free-radical scavenger. It has been hypothesized that *P. emblica* may scavenge the hydroxyl radicals which generates by Fenton's reaction. So the hydroxyl radicals can not be reacting with unsaturated bond at cholesteryl ester. Finally, the lipid peroxidation is terminated. The previous study examined the antioxidant activity of *P. emblica* for its ability to scavenge the superoxide anion, generated by γ-radiation and protect the damage of antioxidant enzyme superoxide dismutase (SOD) in rat liver mitochondria (31). Secondly, the mechanism may be based on transition metal chelating ability. In the presence of Fe^{3+} in hemin induced LDL oxidation, *P. emblica* chelate Fe^{3+} in the constituent of hemin. Resulting that, hemin can not incorporate into LDL particle. Then the lipid peroxidation was not occurred. This hypothesis is supported by the experimental on skin exposure to sunlight in the presence of iron (or copper) and H_2O_2 will generate superoxide anion and increases the presence of superoxide dismutase, which converts the superoxide anion to hydrogen peroxide.

Fe^{2+} will react with the hydrogen peroxide to generate hydroxyl radical and Fe^{3+}. *P. emblica* antioxidant is a chelator for Fe^{3+} and Cu^{2+}, thereby eliminating the generation of hydroxyl radical and its detrimental effects on the skin. As an antioxidant, it quenches free radicals that happen to form on the skin (118). *P. emblica* is proved to be very safe as the single-dose acute oral LD50 is > 5,000 mg/kg BW in male and female rats (118). From these proposed mechanism of antioxidative effect of *P. emblica*, it may be a good reason to further study the use of this plant as medicinal herb in iron overloaded-thalassemia patients.

It is believed that the major constituent of *P. emblica* responsible for antioxidant activity is L-ascorbic acid. This present study showed that *P. emblica* had five times more potent than L-ascorbic acid. So we suggest that the inhibition of LDL oxidation of *P. emblica* may not be attributed to L-ascorbic acid alone but the overall effect is due to other polyphenols (31). For the time course effect of *P.emblica* and L-ascorbic acid at 50% inhibition concentration on hemin induced LDL oxidation found that have 3 phase. First phase was in 0-4 hr of incubation, called lag phase, TBARs levels were slightly increased while α-tocopherol was rapidly decreased until undetectable. Indicating that α-tocopherol was consumed to protected LDL oxidation. There was not changed in PAF-AH activity in this phase. Second phase was in 4-6 hr of incubation when completely loss of α-tocopherol, TBARs formation was dramatically increased (log phase) and increased up to reach the maximum levels in the third phase during 9-24 hr of incubation. Furthermore, when α-tocopherol was disappeared; the PAF-AH activity was decresed. Under oxidation of LDL by hemin that the accumulation of lipid peroxidation products reflect initially the loss of antioxidant vitamin (α-tocopherol) which then leading to lack of antioxidant enzyme (PAF-AH), altered in the chemical compositions (CA, CL) in LDL, including that the increasing of oxidized lipid products. In this study found that the pattern of the protection effect of *P. emblica* and L-ascorbic acid at 50% inhibition concentration was very similar. *P. emblica* and L-ascorbic acid can inhibit TBARs formation that prolonged the lag phase of lipid peroxidation from 4 hr to 20 hr of incubation. Inhibition oxidized lipid products as well as preserve PAF-AH activity and lipid composition completely for 20 hr. Our results suggested that the other constituent in *P. emblica* effectively act as antioxidants with the same mechanism as L-ascorbic acid but more potent.

In case of HDL oxidation, hemin at the concentration of 5 μM induced the TBARs formation less than in the LDL oxidation. The reason for this effect may be due to the antioxidant enzymes which present in HDL which protect lipoprotein from oxidation. And the major component in HDL is not lipid so it is less susceptible to oxidation than LDL. The biological effects of free radical-mediated oxidation of HDL are much less characterized than those of LDL (8). Several enzymes including paraoxonase, lecithin cholesterol acyltransferase, PAF-acetylhydrolase are associated with HDL (119). α-Tocopherol was remained at 24 hr. We observed that paraoxonase activity was rapidly decreased both in he-oxHDL and native HDL. The effect of *P. emblica* on hemin induced HDL oxidation, we found that *P. emblica* can inhibit TBARs formation and preserve α-tocopherol.

In conclusion, spray-dried fruit juice of *Phyllanthus emblica* possesses antioxidant activities and has protective effect against hemin-induced lipoproteins oxidation. *Phyllanthus emblica* was able to preserve PAF-AH activity, α-tocopherol and inhibit TBARs formation in hemin induced LDL oxidation. *P. emblica* has more potent than L-ascorbic acid. The study provides useful information regarding the antioxidant effect of *P. emblica* in hemin induced lipoprotein oxidation. The further *in vivo* study on the protective effect of *P. emblica* on lipoprotein oxidation and the prevention of complication related to thalassemia patients should be investigated.

REFERENCES

(1) Steinberg D. 1997. Low density lipoprotein oxidation and its pathological significance. Journal of Biological Chemistry. 272: 20963-20966.

(2) Berliner J.A. and Heinecke J.W. 1996. The role of oxidized lipoproteins in atherosclerosis. J Biol Chem. 20: 707-727.

(3) Marco B., et al. 1999. When and why a water-soluble antioxidant becomes pro-oxidant during copper-induced low-density lipoprotein oxidation: a study using uric acid. Biochem J. 340: 143-152.

(4) Bella G., Jacob HS., Eaton JW., Belcher JD. And Vercellotti GM. 1991 Hemin: a possible physiological mediator of low density lipoprotein oxidation and endothelial injury. Atheroscler Thromb. 11: 1700-1711.

(5) Shaklai N., Shviro Y., Rabizadeh E. and Kirschner Z.I. 1985. Accumulation and drainage of hemin in the red cell membrane. Biochim Biophys Acta. 821: 355-366.

(6) Miller Y.I., Felikman Y. and Shaklai N. 1995. The involvement of low-density lipoprotein in hemin transport potentiates peroxidative damage. Biochim Biophys Acta. 1272: 119-127.

(7) Pumala N., et al. 2003. Hemin: a possible cause of oxidative stress in blood circulation of β-thalassemia/Hemoglobin E disease. Free Radic Res. 37(2): 129-135.

(8) Camejo G., et al. 1998. Hemin binding and oxidation of lipoproteins in serum: mechanism and effect of the interaction of LDL with human macrophages. J Lip Res. 39: 755-766.

(9) Everse J., Hsia N. 1997. The toxicities of native and modified hemoglobins. Free Radic Biol Med. 22: 1075-1099.

(10) Benjamin J., Mohamad N., Karol E., Gregg C. and Alan M. 2004 Anti-inflammatory properties of HDL. Review in Endocrine & Metabolic Disorder 5: 351-358.

(11) Tselepis AD., Chapman MJ. 2002. Inflammation, bioactive lipids and atherosclerosis; potential roles of a lipoprotein-associated phospholipase A2, platelet activating factor-acetylhydrolase. Atherosclerosis. 3: 57-68.

(12) Lee C., Segrado T., et al. 1999. All apo B-containing lipoproteins induce monocyte chemotaxis and adhesion when minimally modified. Modulation of lipoprotein bioactivity by platelet-activating factor acetylhydrolase. Arterioscler Thromb Vasc Biol. 19: 1437-1446.

(13) Stafforini DM., Prescott SM. and McIntyre TM. 1987. Human plasma platelet-activating factor acetylhydrolase. Purification and properties. J Biol Chem. 262: 4223-4230.

(14) Stafforini DM., McIntyre TM., Carter ME., Prescott SM. 1987. Human plasma platelet-activating factor acetylhydrolase. Association with lipoprotein particles and role in the degradation of platelet-activating factor. J Biol Chem. 262: 4215-1222.

(15) Karasawa K., Harada A., Satoh N., Inoue K., Setaka M. 2003. Plasma platelet activating factor-acetylhydrolase (PAF-AH). Prog Lipid Res. 42: 93-114.

(16) Avirum M., Ronsenblat M., Bisgaier CL., et al. 1998. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its function. A possible role for paraoxonase. J Clin Invest. 101: 1581-1590.

(17) Jaouad L., Milochevitch C., Khalil A, 2003. PON1 paraoxonase activity is reduced during HDL oxidation and is an indicator of HDL antioxidant capacity. Free Radic Res. 37(1): 77-83.

(18) McElveen J., Mackness MI., Colley CM., Peard T., Warner S., Walker CH. 1986. Distribution of paraoxon hydrolytic activity in the serum of patients after myocardial infarction. Clin Chem. 32: 671-673.

(19) Hasselwander O., McMaster D., Fogarty DG., Maxwell AP., Nicholls DP., Young IS. 1998. Serum paraoxonase and platelet-activating factor acetylhydrolase in chronic renal failure. Clin Chem. 44: 179-181.

(20) James RW., Leviev I., Righetti A. 2000. Smoking is associated with reduced serum paraoxonase activity and concentration in patients with coronary artery disease. Circulation. 101: 2252-2257.

(21) Mackness B., Durrington PN., Boulton AJ., Hiene D., Mackness MI. 2002. Serum paraoxonase activity in patients with type 1 diabetes compared to healthy control. Eur J Clin Invest. 32: 259-264.

(22) Milochevitch C., Knalil A. 2001. Study of the paraoxonase and platelet-activating factor acetylhydrolase activities with aging. Prostaglandins Leukot Essent Fatty Acids. 65: 241-246.

(23) Abbey M., Nestel P.J., Baghurst P.A., 1993. Antioxidant vitamins and low-density lipoprotein oxidation. Am J Clin Nutr. 58: 525-532.

(24) Ghosal S. 1996. Active constituents of *Emblica officinalis*: Part 1- The chemistry and antioxidative effects of two new hydrolysable tannins, Emblicanin A and B. Indian J Chem. 35B: 941-948.

(25) Perianayagam J.B., Sharma S.K., Joseph A., and Christina A.J.M. 2004. Evaluation of anti-pyretic and analgesic activity of *Emblica officinalis* Gaertn. J Ethanopharmacol. 65: 83-85.

(26) Sureh K. and Vasudevan D.M. 1994. Augmentation of murine natural killer cell and antibody dependent cellular cytotoxicity activities by *Phyllanthus emblica*, a new immunomodulator. J Ethanopharmacol. 4(1): 55-60.

(27) Mathur R., Sharma A., Dixit VP. And Varma M. 1996. Hypolipidaemic effect of fruit juice of *Emblica officinalis* in cholesterol-fed rabbits. J Ethanopharmacol. 50: 61-68.

(28) Sabu MC. And Kuttan R. 2002. Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. J Ethanopharmacol. 81: 155-160.

(29) Sairam K., Rao Ch. V., Babu MD., Kumar V., Agrawal VK. And Goel RK. 2002. Antiulcerogenic effect of methanolic extract of *Emblica officinalis*: an experimental study. J Ethanopharmacol. 8: 1-9.

(30) Nosal GO., Mokry J. and Hassan K.M.T. 2003. Antitussive activity of the fruit extract of *Emblica officinalis* Gaertn. (Euphorbiaceae). Phytomedicine. 10: 583-589.

(31) Khopde SM., Priyadarsini KI., Mohan H., et al. 2001. Characterizing the antioxidant activity of amla (*Phyllanthus emblica*) extract. Current Sci. 81(2): 185-190.

(32) Anila L. and Vijayalakshmi NR. 2003. Antioxidant action of flavonoids from *Mangifera indica* and *Emblica officinalis* in hypercholesterolemic rats. Food Chem. 83: 569-574.

(33) Duan W., Ya Y. and Zhang L. 2005. Antiatherogenic effects of *Phyllanthus emblic*a associated with corilagin and its analogue. Yakugaku Zasshi. 125(7): 587-591.

(34) John WB., Marek HD. 2005. Medical Biochemistry. 2nd ed. Harcourt Brace and Company Limited.

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

(36) Steinbrecher UP., Zhang HF., Lougheed M. 1990. Role of oxidatively modified LDL in atherosclerosis. Free Radic Biol Med. 9: 155-168.

(37) Chisolm GM., Steinberg D.2000. The oxidative modification hypothesis of atherogenesis: an overview. Free Radic Biol Med. 28: 1815-1826.

(38) Napoli C., de Nigris F., Palinski W. 2001. Multiple role of reactive oxygen species in the arterial wall. J Cell Biochem. 82: 674-682.

(39) Heinecke JW. 1997. Mechanisms of oxidative damage of low density lipoprotein in human atherosclerosis. Curr Opin Lipidol. 8: 268-274.

(40) I.S. Young and J. McEneny. 2001. Lipoprotein oxidation and atherosclerosis. Biochemical Society Transactions. 29(2): 358-362.

(41) Gotto AM., Jr., Pownall HJ. And Havel RJ. 1986. Introduction to the plasma lipoproteins. Methods Enzymol. 128: 3-41.

(42) Ramos P., Gieseg SP., Schuster B. amd Esterbauer H. 1995. Effect of temperature and phase transition on oxidation resistance of low density lipoprotein. J Lipid Res. 36: 2113-2128.

(43) Holvoet P., Stassen JM., Van Cleemput J., Collen D. and Vanhaecke J. 1998. Oxidized low density lipoproteins in patients with transplant-associated coronary artery disease. Arterioscler Thromb Vasc Biol. 18: 100-107.

(44) Holvoet P., Vanhaecke J., Janssens S., Van de Werf F. and Collen D. 1998. Oxidized LDL in patients with acute coronary syndromes and stable coronary artery disease. Circulation. 98: 1487-1494.

(45) Parthasarathy S., Santanam N. and Auge N. 1999a. Antioxidants and low density lipoprotein oxidation. In A.M. Papas (ed.). Antioxidant status, diet, nutrition, and health. (ed.). Florida: CRC Press USA.

(46) Witztum JL., Steinberg D. 2001. The oxidative modification hypothesis of atherosclerosis: Does it hold for human? Trends Cardiovasc Med. 11(3/4): 93-102.

(47) Lauralee S. 2004. Human physiology from cells to systems. 5th edition. West Virginia University. 333-335.

(48) Parthasarathy S., Rankin SM. 1992. Role of oxidized low density lipoprotein in atherogenesis. Prog Lipid Res. 31: 127-143.

(49) Mertens A., Holvoet P. 2001. Oxidized LDL and HDL: antagonists in atherothrombosis. FESEB J. 15(12): 2073-2084.

(50) Fitch CD., et al. 1983. Intracellular ferriprotoporphyrin IX is a potent lytic agent. Blood 62: 1165-1168.

(51) Miller Y.I. and Shaklai N. 1999. Kinetics of hemin distribution in plasma reveals its role in lipoprotein oxidation. Biochim Biophys Acta. 1454: 153-164.

(52) Shviro Y., Shaklai N. 1987. Glutathione as a scavenger of free hemin. Biochem Pharma. 36: 3801-3807.

(53) Liu SC., Zhai S., Lawler J. and Palek J. 1985. Hemin-mediated dissociation of erythrocyte membrane skeletal proteins. J Biol Chem. 260: 12234-12239.

(54) Harvey JW. and Beutler E. 1982. Binding of heme by glutathione S-transferase: a possible role of the erythrocyte enzyme. Blood 60: 1227-1230.

(55) Shah PK., Yano J., Reyes O., Chyu KY., Kaul S., Bisgaier CL., Drake S., Cercek B. 2001. High-dose recombinant apolipoprotein A-I (milano) mobilizes tissue cholesterol and rapidly reduces plaque lipid and macrophage content in apolipoprotein e-deficient mice. Potential implications for acute plaque stabilization. Circulation. 103(25): 3047-3050.

(56) Zhang Y., Zanotti I., Reilly MP., Glick JM., Rothblat GH., Rader DJ. 2003. Overexpression of apolipoproteins A-I promotes reverse transport of cholesterol from macrophages to feces *in vivo*. Circulation. 108(6): 661-663.

(57) Remaley AT., Thomas F., Stonik JA., Demosky SJ., et al. 2003. Synthetic amphipathic helical peptides promote lipid efflux from cells by an

ABCA 1-dependent and an ABCA1-independent pathway. J Lipid Res. 44(4): 828-836.

(58) Martinez LO., Agerholm-Larsen B., Wang N., Chen W., Tall AR. 2003. Phosphorylation of a pest sequence in ABCA1 promotes calpain degradation and is reversed by ApoA-I. J Biol Chem. 278(39): 37368-37374.

(59) Navab M., Hama SY., Anantharamaiah GM., et al. 2000. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: Step 2 and 3. J Lip Res. 41(9): 1495-1508.

(60) Navab M., Hama SY., Anantharamaiah GM., et al. 2000. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: Step 1. J Lip Res. 41(9): 1481-1494.

(61) Van Lenten BJ., Navab M., Shih D., Fogelman AM., Lusis AJ. 2001. The role of high-density lipoproteins in oxidation and inflammation. Trends Cardiovasc Med. 11(3/4): 155-161.

(62) Durrington PN., Mackness B., Mackness MI. Paraoxonase and atherosclerosis. 2001. Arterioscler Thromb Vasc Biol. 21(4): 473-480.

(63) Watson AD., Berliner JA., Hama SY., La Du BN., et al. 1995. Protective effect of high density lipoprotein associated paraoxonase. J Clin Invest. 96: 2882-2891.

(64) Mackness MI., Arrol S., Abbott C., Durrington PN. 1993. Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein asscociated paraoxonase. Atherosclerosis. 104: 129-135.

(65) Avirum M., Ronsenblat M., Bisgaier CL., et al. 1998. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its function. A possible peroxidative role for paraoxonase. J Clin Invest. 101: 1581-1590.

(66) Vohl MC., Neville TA., Kumarathasan R., et al. 1999. A novel lecithin-cholesterol acyltransferase antioxidant activity prevents the formation of oxidized lipids during lipoprotein oxidation. Biochemistry. 38: 5976-5981.

(67) Bielicki JK. And Forte TM. 1999. Evidence that lipid hydroperoxides inhibit plasma lecithin:cholesterol acyltransferase activity. J Lipid Res. 40: 948-954.

(68) Holvoet P., Danloy S., Derider E., et al. 1998. Substitution of carboxyl-terminal domain of apo AI with apo AII sequences restores the potential of HDL to reduce the progression of atherosclerosis in apo E knockout mice. J Clin Invest. 102: 379-385.

(69) Ross R. 1993. The pathogenesis of atherosclerosis: a prospective for the 1990s. Nature. 362: 801-809.

(70) Subbanagounder G., Leitinger N., Shih PT., Faull KF., Berliner JA. 1999. Evidence that phospholipids oxidation products and/ or platelet-activating factor play an important role in early artherogenesis: in vitro and in vivo inhibition by WEB 2086. Circ Res. 85: 311-318.

(71) Imaizumi TA., Stafforini DM., Yamada T., et al. 1995. Platelet-activating factor: a mediator for clinicians. J Intern Med. 238: 5-20.

(72) Dubravka J., Milena T., Branka R., et al. 2001. Serum paraoxonase activities in hemodialyzed uremic patients : Cohort study. Clinical Sciences. 42(2): 146-150.

(73) Primo-Parma SL., Sorenson RC., Teiber J., La Du BN. 1996. The human serum paraoxonase/arylesterasegene (PON1) is one member of a multigene family. Genomics. 33: 498-509.

(74) Mackness MI., Mackness B., Durington PN., et al. 1996. Paraoxonase: biochemistry, genetics and relationship to plasma lipoproteins. Curr Opin Lipidol. 7: 69-76.

(75) Aldridge WN. A-esterases and B-esterases in perspective. In: Reiner E., Aldridge WN., Hoskin FCG, editors. 1989. Enzymes hydrolyzing organophosphorous compounds. Chichester (UK): Elis Horwood Limited. 1-14.

(76) Costa LG., Li WF., Richer RJ., Shih DM., et al. 1999. The role of paraoxonase (PON1) in the detoxification of organophosphates and its human polymorphism. Chem Biol Interact. 119-120: 429-438.

(77) Janice E. Chamber. 2008. PON1 multitasks to protect health. PNAS. 105(35): 12639-12640.

(78) Dragonov DI., Stetson PL., Watson CE., Billecke SS., La Du BN. 2000. Rabbit serum paraoxonase 3 (PON3) is a high density lipoprotein-associated lactonase and protects low density lipoprotein against oxidation. J Biol Chem. 275(43): 33435-33442.

(79) Hattori K., Hattori M., Adachi H., et al. 1995. Purification and characterization of platelet-activating factor acetylhydrolase II from bobine liver cytosol. J Biol Chem. 270 (38): 22308-22313.

(80) Stafforini DM., McIntyre TM., Zimmerman GA., Precott SM. 1997. Platelet-activating factor acetylhydrolases. J Biol Chem. 272(29): 17895-17898.

(81) Asano K., Okamoto S., Fukunaga K., Shiomi T., et al. 1999. Cellular source(s) of platelet activating-factor acetylhydrolase activity in plasma. Biochem Biophys Res Commun. 261(2): 511-514.

(82) Ahmed Z., Babaei S., Maguire GF., Draganov D., et al. 2003. Paraoxonase and coronary heart disease. Curr Opin Lipidol. 9: 319-324.

(83) Evangelou AM. 1994. Platelet-activating factor (PAF): implications for coronary heart and vascular diseases. Prostaglandins Leukot Fatty Acids. 50: 1-28.

(84) Halliwell B., Gutteridge JMC. 1999. Free radicals in Biology and Medicine, 3 rd edition. Oxford, Oxford University Press. p 53.

(85) Moore RE., Navab M., Millar JS., et al. 2005. Increased atherosclerosis in mice lacking apolipoprotein A-I attributable to both impaired reverse cholesterol transport. Annu Rev Nutr. 18: 297-330.

(86) Caslake MJ., Packard CJ., Suckling KE., Holmes SD., Chamberlain P., Macphee CH. 2000. Lipoprotein-associated phospholipase A2, platelet-activating factor acetylhydrolase: a potential new risk factor for coronary artery disease. Atherosclerosis. 150: 413-419.

(87) Karasawa K., Harada A., Satoh N., Inoue K., Setaka M. 2003. Plasma platelet activating factor acetylhydrolase (PAF-AH). Prog Lipid Res. 42: 93-114.

(88) Rodrigo L., Mackness B., Durrington PN., Hernandez A., Mackness MI. 2001. Hydrolysis of platelet-activating factor by human serum paraoxonase. Biochem J. 15: 1-7.

(89) Morton J. 1987. Emblic. In: Fruit of warm climates [Online]. 213-217. http://www.hort.purdue.edu/newcrop/morton/emblic.html.

(90) Scartezzini P. and Speroni E. 2000. Review on some plants of Indian traditional medicine with antioxidant activity. J Ethanolpharmacol. 71: 23-43.

(91) Zhang YJ., Nagao T., Tanaka T., Yang CR., Okabe H. and Kouno T. 2004. Antiproliferative activity of the main constituents from *Phyllanthus emblica*. Biol Pharm Bull. 27(2): 251-255.

(92) Asmawi MZ., Kankaanranta H., Moilanen E. and Vapaato H. 1993. Anti-inflammatory activities of *Emblica officinalis* Gaertn leaf extracts. J Pharm Pharmacol. 45: 581-584.

(93) Ihantola-Vormisto A., Summanen J., Kankaanranta H., et al. 1997. Anti-inflammatory activity of extracts from leaves of *Phyllanthus emblica*. Planta Medica. 63: 518-524.

(94) Ahmad I., Mehmood Z. and Mohammad F. 1998. Screening of some Indian medical plants for their antimicrobial properties. J Ethanopharmacol. 62: 183-193.

(95) El-Mekkawy S., Meselhy MR., Kusumoto IT., et al. 1995. Inhibitory effects of Egyptian folk medicines on human immunodeficiency virus (HIV) reverse transcriotase. Chem Pharm Bull. 43(4): 641-648.

(96) Bandyopadhyay SK., Pakrashi SC. And Pakrashi A. 2000. The role of antioxidant activity of *Phyllanthus emblica* fruits on prevention from indomethacin induced gastric ulcer. J Ethanopharmacol. 70: 171-176.

(97) Jeena KJ., Joy KL. And Kuttan R. 1999. Effect of *Emblica officinalis*, *Phyllanthus amarus* and *Picrorrhiza kurroa* on N-nitrosodiethylamine induced hepatocarcinogenesis. Cancer Lett. 136: 11-16.

(98) Jeena KJ. and Kuttan R. 2000. Hepatoprotective activity of *Emblica officinalis*. J Ethanopharmacol. 72: 135-140.

(99) Anila L. and Vijayalakshmi NR. 2002. Flavonoids from *Emblica officinalis* and *Mangifera indica*-effectiveness for dyslipidemia. J Ethanopharmacol. 79: 81-87.

(100) Jeena KJ., Kuttan G. and Kuttan R. 2001. Antitumor activity of *Emblica officinalis*. J Ethanopharmacol. 75: 65-69.

(101) Khan MTH., Lampronti I., Martello D., et al. 2002. Identification of pyrogallol as an antiproliferative compound present in extracts from the medicinal plant *Emblica officinalis*: Effects on in vitro cell growth of human tumor cell lines. Int J Oncol. 20: 187-192.

(102) Bhattacharya SK., Bhattacharya A., Sairam K. and Ghosal S. 2002. Effect of bioactive tannoid principles of *Emblica officinalis* on ischemia-reperfusion- induced oxidative stress in rat heart. Phytomedicine. 9: 171-174.

(103) Wattanapitayakul SK., Cholarojmontri L., Herunsalee A., Charuchongkolwongse S., Niumsakul S. and Bauer JA. 2005. Screening of antioxidants from medicinal plants for cardioprotective effect against doxorubicin toxicity. Basic Clin Pharmacol Toxicol. 96: 80-87.

(104) Kumar KC. And Muller K. 1999. Medicinal plants from Nepal; II. Evaluation as inhibitors of lipid peroxidation in biological membranes. J Ethanopharmacol. 64: 135-139.

(105) Kumar HKB., Sabu MC., Lima PS. And Kuttan R. 2004. Modulation of haematopoetic system and antioxidant enzymes by *Emblica officinalis* Gaertn and its protective role against γ-radiation induced damages in mice. J Radiat Res. 45: 549-555.

(106) Anilakumar KR., Nagaraj NS. And Santhanam K. 2004. Protective effects of amla on oxidative stress and toxicity it rats challenged with dimethyl hydrazine. Nutr Res. 24: 313-319.

(107) Havel RJ., Eder H., Bragdon JH. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest. 34(9): 1345-1353.

(108) Markwell MAK M., Hass SM., Bieber LL. And Tolbert NE. 1978. A modification of the Lowery procedure to simplify protein determination in membrane and lipoprotein samples. Analytical Biochemistry 87: 206-210.

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

(110) Aarsman AJ., Neys FW., Van den Bosch H. 1991. Catabolism of platelet-activating factor and its acyl analog. Differentiation of the activities of lysophospholipase and platelet-activating-factor acetylhydrolase. Eur J Biochem. 200: 187-193.

(111) Gan KN., Smolen A., Eckerson HW., La Du BN. 1991. Purification of human serum paraoxonase/arylesterase : evidence for one esterase catalyzing both activities. Drug Metab Dispos. 19: 100-106.

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

(113) Zaspel B. and Csallany A. 1983. Determination of alpha-tocopherol in tissues and plasma high performance liquid chromatography. Analytical Biochemistry 130: 146-150.

(114) Jessup W., Rankin SM., De Whalley CV, Hoult JR., Scott J., Leake DS. 1990. Alpha-tocopherol consumption during low-density-lipoprotein oxidation. Biochem J. 265(2): 399-405.

(115) Livrea MA., Tesoriere L., Maggio A., et al. 1998. Oxidative modification of low-density lipoprotein and atherogenetic risk in β-thalassemia. Blood. 92(10): 3936-3942.

(116) Luechapudiporn R., Morales NP. Fucharoen S. and Chantharaksri U. 2006. The reduction of cholesteryl linoleate in lipoproteins: an index of clinical severity in beta-thalassemai/Hb E. Clin Chem Lab Med. 44(5): 574-581.

(117) Watson AD., Navab M., Hama SY., Sevanian A., et al. 1995. Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. J Clin Invest. 95: 774-782.

(118) Ratan K. Chaudhuri. 2002. Emblica cascading antioxidant: A novel natural skin care ingredient. Skin Pharmacol Appy skin Physiol. 15: 374-380.

(119) Mackness MI., Durrington PN. 1995. HDL, its enzymes and its potential to influence lipid peroxidation. Atherosclerosis. 115: 243-253.

APPENDICES

APPENDIX A

Study the concentration dependent effects of *Phyllanthus emblica* on hemin induced LDL oxidation (he-oxLDL).

1. The effect *Phyllanthus emblica* and L-ascorbic acid on TBARs formation at 24 hr of incubation time.

Condition	Conc. (µg/ml)	TBARs (nmol/mg protein)					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	3.67	6.51	11.06	3.74	4.86	5.97	3.07
he-oxLDL	-	44.64	35.01	42.28	36.10	23.19	36.24	8.35
P. emblica	0.5	57.30	35.12	43.25	36.84	19.75	38.45	13.62
	1.0	47.19	36.11	44.99	36.96	16.57	36.36	12.08
	2.5	9.48	29.88	42.96	18.62	10.17	22.22	14.22
	5.0	9.30	17.34	21.15	9.37	8.41	13.11	5.77
	10.0	4.36	7.72	14.22	4.40	3.91	6.92	4.36
	20.0	4.08	6.58	11.27	4.17	4.23	6.07	3.09
L-ascorbic acid	0.5	48.63	39.80	46.32	37.20	25.69	39.53	9.03
	1.0	49.28	36.37	41.27	36.73	17.31	36.19	11.77
	2.5	14.76	38.66	39.14	34.68	16.47	28.74	12.12
	5.0	10.84	36.92	45.90	41.36	18.91	30.79	15.14
	10.0	6.09	38.94	42.71	39.98	11.59	27.86	17.53
	20.0	4.78	15.08	20.38	11.18	5.24	11.33	6.63

2. The effect *Phyllanthus emblica* and L-ascorbic acid on % inhibition of TBARs at 24 hr of incubation time.

Tested compounds	Conc. (µg/ml)	% Inhibition of TBARs formation					Mean	S.D.
		N1	N2	N3	N4	N5		
P. emblica	0.5	0.00	0.00	0.00	0.00	18.82	3.76	7.71
	1.0	0.00	0.00	0.00	54.00	36.13	18.03	23.30
	2.5	85.82	18.00	0.00	82.60	71.05	51.49	39.69
	5.0	86.26	62.00	67.68	76.72	80.62	74.66	8.96
	10.0	98.32	95.75	89.88	97.95	100.00	96.38	3.61
	20.0	99.00	99.75	99.33	98.68	100.00	99.35	0.49
L-ascorbic acid	0.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	32.11	6.42	13.13
	2.5	72.93	0.00	0.00	0.00	36.69	21.92	32.64
	5.0	82.50	0.00	3.24	4.36	23.40	22.70	31.70
	10.0	94.09	0.00	10.06	20.66	63.30	37.62	36.31
	20.0	97.29	69.93	70.15	76.99	97.92	82.46	12.90

3. The effect *Phyllanthus emblica* and L-ascorbic acid on α-tocopherol levels at 24 hr of incubation time.

Condition	Conc. (μg/ml)	α-Tocopherol (nmol/mg protein)					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	14.57	19.27	25.08	13.10	13.60	18.01	4.64
he-oxLDL	-	0.00	0.00	0.00	0.00	0.00	0.00	0.00
P. emblica	0.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	2.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	5.0	0.00	0.00	0.00	0.00	6.81	0.00	2.78
	10.0	0.00	6.01	13.44	5.70	9.18	6.29	4.52
	20.0	8.64	20.17	16.16	7.49	9.65	13.11	5.02
L-ascorbic acid	0.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	2.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	5.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	10.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	20.0	5.43	0.00	0.00	0.00	10.80	1.36	4.41

4. The effect *Phyllanthus emblica* and L-ascorbic acid on % remainder α-tocopherol levels at 24 hr of incubation time.

Tested compounds	Conc. (μg/ml)	% Remainder of α-tocopherol					Mean	S.D.
		N1	N2	N3	N4	N5		
P. emblica	0.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	2.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	5.0	0.00	0.00	0.00	0.00	48.14	9.63	21.53
	10.0	0.00	31.17	53.59	43.52	66.27	38.91	25.30
	20.0	59.31	100.00	64.43	57.14	69.84	70.14	17.40
L-ascorbic acid	0.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	2.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	5.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	10.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	20.0	37.27	0.00	0.00	0.00	17.56	9.97	14.60

5. The effect *Phyllanthus emblica* and L-ascorbic acid on PAF-AH activity at 24 hr of incubation time.

Condition	Conc. (µg/ml)	PAF-AH activity (nmol/min/mg protein)					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	31.90	26.30	27.40	26.30	28.10	28.00	2.31
he-oxLDL	-	17.80	17.80	16.90	9.90	16.90	15.86	3.36
P. emblica	0.5	17.90	12.60	18.40	15.20	17.30	16.28	2.40
	1.0	32.80	14.30	15.80	18.80	21.80	20.70	7.35
	2.5	22.50	18.40	21.80	22.10	24.00	21.76	2.06
	5.0	30.90	22.10	26.10	23.40	25.10	25.52	3.38
	10.0	32.80	22.90	29.40	23.80	25.10	26.80	4.19
	20.0	33.80	28.10	28.50	24.30	25.50	28.04	3.67
L-ascorbic acid	0.5	22.50	15.00	16.90	13.70	18.90	17.40	3.47
	1.0	20.60	17.80	15.90	15.20	18.20	17.54	2.13
	2.5	30.00	15.90	18.80	15.40	23.60	20.74	6.12
	5.0	32.80	13.50	19.70	15.90	24.40	21.26	7.66
	10.0	30.00	16.90	21.60	17.30	28.10	22.78	6.05
	20.0	30.00	23.60	28.10	21.60	25.10	25.68	3.38

6. The effect *Phyllanthus emblica* and L-ascorbic acid on cholesteryl esters at 24 hr of incubation time.

Free cholesterol (FC)

Condition	Conc. (µg/ml)	FC (µmol/ mg protein)					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	0.989	0.754	0.984	1.236	1.096	1.012	0.16
he-oxLDL	-	0.969	0.765	0.817	0.779	1.000	0.866	0.10
P. emblica	0.5	0.812	0.472	0.870	0.965	0.997	0.823	0.19
	1.0	0.817	0.609	0.667	1.024	1.168	0.857	0.22
	2.5	0.980	0.818	0.923	1.168	1.283	1.034	0.19
	5.0	1.033	1.079	0.984	0.907	1.171	1.035	0.09
	10.0	1.166	0.657	0.793	0.868	1.155	0.928	0.21
	20.0	1.013	0.631	1.104	0.987	1.174	0.982	0.19
L-ascorbic acid	0.5	0.869	0.671	0.629	0.987	1.113	0.854	0.19
	1.0	0.864	0.652	0.745	0.725	1.191	0.835	0.19
	2.5	1.026	0.778	0.681	0.876	1.244	0.921	0.22
	5.0	1.209	0.700	0.994	0.824	1.132	0.972	0.19
	10.0	1.113	0.709	1.118	0.715	1.220	0.975	0.22
	20.0	1.133	0.537	0.955	0.537	1.237	0.880	0.30

Cholesteryl arachidonate (CA)

Condition	Conc. (µg/ml)	CA (µmol/ mg protein)					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	0.301	0.222	0.317	0.313	0.284	0.287	0.04
he-oxLDL	-	0.097	0.098	0.109	0.062	0.151	0.103	0.03
P. emblica	0.5	0.059	0.056	0.118	0.111	0.219	0.113	0.06
	1.0	0.089	0.084	0.092	0.119	0.286	0.134	0.08
	2.5	0.290	0.061	0.207	0.244	0.266	0.214	0.09
	5.0	0.333	0.119	0.320	0.145	0.228	0.229	0.09
	10.0	0.321	0.228	0.250	0.239	0.259	0.259	0.03
	20.0	0.318	0.214	0.428	0.291	0.317	0.314	0.07
L-ascorbic acid	0.5	0.091	0.086	0.089	0.108	0.235	0.122	0.06
	1.0	0.113	0.085	0.068	0.070	0.167	0.101	0.04
	2.5	0.300	0.068	0.111	0.133	0.264	0.175	0.10
	5.0	0.331	0.090	0.083	0.088	0.244	0.167	0.11
	10.0	0.278	0.121	0.159	0.075	0.297	0.186	0.09
	20.0	0.320	0.163	0.287	0.095	0.278	0.229	0.09

Cholesteryl linoleate (CL)

Condition	Conc. (µg/ml)	CL (µmol/ mg protein)					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	1.352	0.820	1.299	1.504	1.281	1.251	0.24
he-oxLDL	-	0.722	0.534	0.751	0.402	0.734	0.629	0.14
P. emblica	0.5	0.665	0.350	0.798	0.850	0.988	0.730	0.22
	1.0	0.781	0.468	0.619	0.944	1.119	0.786	0.24
	2.5	1.405	0.364	0.997	1.112	0.983	0.972	0.38
	5.0	1.647	0.591	1.347	0.813	0.854	1.050	0.40
	10.0	1.541	0.837	0.922	1.075	1.037	1.082	0.25
	20.0	1.531	0.781	1.479	1.319	1.169	1.256	0.28
L-ascorbic acid	0.5	0.663	0.533	0.629	0.780	1.071	0.735	0.19
	1.0	0.785	0.574	0.639	0.586	0.949	0.707	0.15
	2.5	1.508	0.413	0.672	0.981	1.297	0.974	0.45
	5.0	1.521	0.440	0.613	0.689	1.087	0.870	0.40
	10.0	1.371	0.548	0.841	0.512	1.005	0.855	0.32
	20.0	1.605	0.656	1.186	0.485	0.987	0.984	0.41

Cholesteryl oleate (CO)

Condition	Conc. (µg/ml)	CO (µmol/ mg protein)					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	0.453	0.237	0.421	0.439	0.574	0.425	0.11
he-oxLDL	-	0.335	0.247	0.398	0.145	0.306	0.286	0.09
P. emblica	0.5	0.384	0.173	0.451	0.239	0.511	0.352	0.13
	1.0	0.396	0.193	0.293	0.441	0.462	0.357	0.10
	2.5	0.452	0.135	0.497	0.310	0.348	0.348	0.14
	5.0	0.622	0.205	0.482	0.270	0.342	0.384	0.15
	10.0	0.533	0.215	0.356	0.307	0.359	0.354	0.11
	20.0	0.483	0.242	0.571	0.344	0.501	0.428	0.12
L-ascorbic acid	0.5	0.330	0.236	0.323	0.285	0.478	0.330	0.08
	1.0	0.432	0.196	0.332	0.229	0.397	0.317	0.09
	2.5	0.472	0.185	0.364	0.337	0.587	0.389	0.15
	5.0	0.471	0.180	0.302	0.200	0.412	0.313	0.12
	10.0	0.475	0.200	0.442	0.143	0.413	0.335	0.14
	20.0	0.554	0.231	0.428	0.128	0.350	0.338	0.15

Cholesteryl palmitate (CP)

Condition	Conc. (µg/ml)	CP (µmol/ mg protein)					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	0.287	0.209	0.384	0.225	0.205	0.262	0.07
he-oxLDL	-	0.240	0.238	0.317	0.139	0.170	0.221	0.06
P. emblica	0.5	0.216	0.213	0.307	0.140	0.235	0.222	0.05
	1.0	0.312	0.201	0.226	0.276	0.266	0.256	0.04
	2.5	0.268	0.168	0.295	0.235	0.209	0.235	0.05
	5.0	0.338	0.195	0.383	0.183	0.155	0.251	0.09
	10.0	0.346	0.203	0.330	0.139	0.155	0.235	0.09
	20.0	0.276	0.205	0.365	0.193	0.191	0.246	0.07
L-ascorbic acid	0.5	0.254	0.209	0.240	0.169	0.261	0.227	0.03
	1.0	0.299	0.199	0.241	0.197	0.222	0.232	0.04
	2.5	0.377	0.163	0.271	0.220	0.271	0.260	0.08
	5.0	0.295	0.169	0.233	0.181	0.168	0.209	0.05
	10.0	0.255	0.146	0.254	0.122	0.168	0.189	0.06
	20.0	0.391	0.214	0.299	0.071	0.233	0.242	0.11

7. The effect *Phyllanthus emblica* and L-ascorbic acid on CL/CO ratio at 24 hr of incubation time.

Condition	Conc. (µg/ml)	CL/CO ratio					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	2.985	3.460	3.086	3.426	2.232	3.038	0.50
he-oxLDL	-	2.155	2.162	1.887	2.772	2.401	2.275	0.33
P. emblica	0.5	1.732	2.023	1.769	3.556	1.933	2.146	0.64
	1.0	1.972	2.425	2.113	2.141	2.423	2.164	0.20
	2.5	3.108	2.696	2.006	3.587	2.827	2.845	0.58
	5.0	2.648	2.883	2.795	3.011	2.495	2.838	0.23
	10.0	2.891	3.893	2.590	3.502	2.891	3.150	0.43
	20.0	3.170	3.227	2.590	3.834	2.335	2.993	0.49
L-ascorbic acid	0.5	2.009	2.258	1.947	2.737	2.239	2.238	0.28
	1.0	1.817	2.929	1.925	2.559	2.392	2.324	0.42
	2.5	3.195	2.232	1.846	2.911	2.209	2.142	0.88
	5.0	3.229	2.444	2.030	3.445	2.637	2.757	0.53
	10.0	2.886	2.740	1.903	3.580	2.435	2.709	0.56
	20.0	2.897	2.840	2.771	3.789	2.821	3.024	0.39

8. The effect *Phyllanthus emblica* and L-ascorbic acid on total cholesterol at 24 hr of incubation time.

Condition	Conc. (µg/ml)	Total cholesterol (µmol/ mg protein)					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	5.775	4.153	3.760	4.413	3.008	4.222	0.93
he-oxLDL	-	6.034	3.442	3.049	5.081	4.668	4.455	1.11
P. emblica	0.5	5.969	3.571	3.998	4.672	4.517	4.545	0.83
	1.0	5.926	3.743	3.933	5.103	3.612	4.463	0.92
	2.5	5.948	4.325	4.515	6.137	4.754	5.136	0.84
	5.0	5.797	4.131	4.687	4.844	5.314	4.955	0.58
	10.0	6.099	3.722	4.321	4.974	4.840	4.791	0.81
	20.0	5.560	3.635	3.998	5.189	4.969	4.670	0.75
L-ascorbic acid	0.5	4.956	3.765	3.200	3.767	4.021	3.942	0.59
	1.0	5.581	4.260	3.782	4.457	4.215	4.459	0.62
	2.5	5.474	3.916	3.998	4.564	4.969	4.584	0.66
	5.0	6.357	3.571	3.631	4.521	5.379	4.692	1.09
	10.0	6.077	4.045	3.976	4.672	7.060	5.166	1.24
	20.0	6.056	4.239	4.493	4.500	4.538	4.765	0.67

APPENDIX B

Study the time dependent effects of *Phyllanthus emblica* on hemin induced LDL oxidation (he-oxLDL).

1. The effect of *Phyllanthus emblica* and L-ascorbic acid on TBARs formation.

Incubation time (hr)		TBARs (nmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
0	N1	0.34	0.29	0.46	0.37
	N2	2.68	2.36	2.47	3.56
	N3	2.26	2.19	2.17	2.14
	N4	2.14	2.01	2.08	2.05
	N5	2.00	1.88	1.92	1.77
	N6	1.45	1.34	1.42	1.41
	Mean	1.81	1.68	1.75	1.88
	S.D.	0.83	0.76	0.71	1.05
1	N1	0.30	1.64	1.23	1.07
	N2	2.66	3.14	2.96	3.20
	N3	2.10	2.64	2.32	2.35
	N4	2.25	2.58	2.41	2.32
	N5	2.03	2.35	2.04	2.30
	N6	1.22	2.33	1.44	1.64
	Mean	1.76	2.45	2.07	2.15
	S.D.	0.86	0.49	0.64	0.74
2	N1	0.52	1.80	0.71	0.72
	N2	2.47	4.01	2.72	2.95
	N3	2.08	3.27	2.47	2.30
	N4	1.99	3.63	2.15	2.34
	N5	1.93	2.96	3.38	1.98
	N6	1.56	2.71	2.27	1.81
	Mean	1.76	3.06	2.28	2.02
	S.D.	0.66	0.78	0.88	0.74
4	N1	0.59	3.25	0.80	1.20
	N2	2.56	6.63	2.82	2.91
	N3	2.27	4.76	2.32	2.17
	N4	1.87	5.20	2.25	2.37
	N5	2.18	5.39	2.18	2.03
	N6	1.46	5.03	2.20	2.13
	Mean	1.82	5.04	2.10	2.14
	S.D.	0.71	1.10	0.69	0.56
6	N1	0.39	21.84	0.98	0.95
	N2	2.97	27.01	3.33	3.30
	N3	1.82	28.42	2.20	2.30
	N4	2.11	31.36	2.70	2.63
	N5	1.84	28.27	2.15	2.41
	N6	1.43	30.31	2.05	2.10
	Mean	1.76	27.87	2.24	2.28
	S.D.	0.86	3.33	0.78	0.78

Incubation time (hr)		nLDL	he-oxLDL	*P. emblica*	L-ascorbic acid
9	N1	0.57	27.11	0.98	1.39
	N2	3.02	30.49	3.02	3.27
	N3	1.71	32.82	2.33	2.76
	N4	2.31	35.75	2.52	3.64
	N5	2.20	30.69	2.34	2.79
	N6	1.61	33.03	2.53	2.86
	Mean	1.90	31.65	2.29	2.79
	S.D.	0.82	2.93	0.69	0.76
12	N1	0.26	25.93	0.98	1.74
	N2	2.41	32.99	2.88	3.32
	N3	1.88	32.44	2.56	3.96
	N4	2.04	36.23	2.92	4.37
	N5	1.92	31.28	2.58	3.73
	N6	1.11	31.14	2.06	3.42
	Mean	1.60	31.67	2.33	3.42
	S.D.	0.78	3.36	0.73	0.91
16	N1	0.25	27.13	2.33	1.71
	N2	2.57	31.22	3.33	3.59
	N3	1.71	31.56	2.97	6.28
	N4	2.01	35.07	3.14	6.52
	N5	1.95	29.07	3.04	4.76
	N6	1.33	32.18	2.58	5.38
	Mean	1.64	31.04	2.90	4.71
	S.D.	0.79	2.72	0.37	1.81
20	N1	0.60	27.87	3.16	3.88
	N2	2.83	31.72	5.90	5.91
	N3	1.86	31.37	5.02	12.89
	N4	2.20	32.86	4.90	11.98
	N5	1.99	28.89	4.96	7.65
	N6	1.45	31.52	6.39	9.00
	Mean	1.82	30.71	5.06	8.55
	S.D.	0.75	1.90	1.11	3.48
24	N1	0.74	27.58	13.31	8.07
	N2	2.87	32.00	14.53	8.77
	N3	1.93	30.42	16.29	23.15
	N4	2.40	33.86	16.91	24.86
	N5	2.15	28.91	13.01	15.19
	N6	1.44	30.94	14.82	17.31
	Mean	1.92	30.62	14.81	16.23
	S.D.	0.75	2.22	1.56	7.03

2. The effect of *Phyllanthus emblica* and L-ascorbic acid on α-tocopherol.

Incubation time (hr)		α -Tocopherol (nmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
0	N1	10.99	10.51	8.95	10.42
	N2	11.50	10.68	11.45	11.92
	N3	14.06	16.97	14.61	17.63
	N4	17.82	16.01	14.78	14.21
	N5	18.48	16.30	23.11	17.98
	N6	15.78	17.34	14.32	15.24
	Mean	14.77	14.64	14.54	14.57
	S.D.	3.15	3.17	4.78	3.03
1	N1	12.94	12.02	11.00	12.19
	N2	14.25	10.79	14.34	13.22
	N3	20.59	11.80	21.23	13.96
	N4	17.90	15.77	32.14	21.77
	N5	21.38	13.93	18.67	18.93
	N6	17.59	15.43	18.38	22.49
	Mean	17.44	13.29	19.29	17.09
	S.D.	3.35	2.06	7.26	4.54
2	N1	12.77	4.86	11.18	12.70
	N2	14.22	6.49	14.12	12.76
	N3	21.25	11.03	18.60	12.56
	N4	18.71	16.94	18.00	17.05
	N5	17.38	7.95	16.57	21.17
	N6	21.22	11.37	17.82	17.99
	Mean	17.59	9.77	16.05	15.71
	S.D.	3.53	4.33	2.87	3.59
4	N1	11.78	0.00	9.82	11.47
	N2	13.83	0.00	11.10	11.16
	N3	19.95	0.00	22.57	18.49
	N4	25.62	0.00	21.65	14.76
	N5	20.86	0.00	17.54	16.50
	N6	20.46	0.00	16.64	12.80
	Mean	18.75	0.00	16.55	14.20
	S.D.	5.08	0.00	5.26	2.92
6	N1	9.92	0.00	0.00	8.75
	N2	12.14	0.00	0.00	9.98
	N3	15.47	0.00	0.00	14.96
	N4	15.49	0.00	0.00	11.53
	N5	16.26	0.00	0.00	10.93
	N6	14.01	0.00	0.00	11.68
	Mean	13.88	0.00	0.00	11.31
	S.D.	2.43	0.00	0.00	2.10
9	N1	9.53	0.00	0.00	7.61
	N2	12.47	0.00	0.00	6.99
	N3	16.82	0.00	0.00	9.01
	N4	14.60	0.00	0.00	9.27
	N5	15.15	0.00	0.00	9.05
	N6	15.47	0.00	0.00	9.02
	Mean	14.01	0.00	0.00	8.49
	S.D.	2.61	0.00	0.00	0.95

3. The effect of *Phyllanthus emblica* and L-ascorbic acid on PAF-AH activity.

Incubation time (hr)		PAF-AH activity (nmol/min/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
0	N1	23.00	21.00	21.00	19.00
	N2	23.00	38.00	24.00	23.00
	N3	24.40	24.40	20.60	23.30
	N4	22.90	22.50	22.90	22.50
	N5	22.50	21.40	22.90	22.50
	N6	22.90	22.10	21.80	22.50
	Mean	23.12	24.90	22.20	22.13
	S.D.	0.66	6.53	1.30	1.57
1	N1	21.00	21.00	22.00	21.00
	N2	22.00	23.00	24.00	23.00
	N3	22.50	21.60	21.60	21.00
	N4	23.60	23.80	23.60	21.90
	N5	22.50	23.40	23.30	22.70
	N6	25.10	23.60	23.80	23.60
	Mean	22.78	22.73	23.05	22.20
	S.D.	1.41	1.16	1.00	1.08
2	N1	23.00	23.00	23.00	23.00
	N2	23.00	23.00	23.00	23.00
	N3	21.40	20.60	20.30	21.00
	N4	22.50	22.90	22.50	22.90
	N5	21.00	22.50	22.90	21.00
	N6	23.60	24.00	22.30	23.80
	Mean	22.42	22.67	22.33	22.45
	S.D.	1.01	1.13	1.04	1.17
4	N1	23.40	23.40	22.50	24.40
	N2	24.40	24.60	23.40	24.40
	N3	22.10	21.20	21.80	23.10
	N4	23.80	22.50	22.50	24.40
	N5	24.20	22.50	23.80	23.10
	N6	24.40	26.30	24.40	23.40
	Mean	23.72	23.42	23.07	23.80
	S.D.	0.88	1.81	0.97	0.67
6	N1	22.50	20.80	22.10	22.50
	N2	24.40	21.90	26.30	24.40
	N3	21.00	18.80	20.60	20.60
	N4	22.90	18.60	22.50	22.90
	N5	24.00	18.90	23.10	23.10
	N6	24.40	22.50	24.90	23.40
	Mean	23.20	20.25	23.25	22.82
	S.D.	1.34	1.72	2.05	1.26
9	N1	21.90	18.20	21.80	25.30
	N2	26.30	18.80	24.40	25.70
	N3	20.60	15.00	21.40	21.90
	N4	22.50	18.80	22.10	22.50
	N5	23.40	16.90	23.40	24.20
	N6	24.00	16.90	22.70	23.40
	Mean	23.12	17.43	22.63	23.83
	S.D.	1.96	1.47	1.11	1.51

Incubation time (hr)		nLDL	he-oxLDL	*P. emblica*	L-ascorbic acid
12	N1	20.60	16.50	21.40	20.60
	N2	24.80	18.80	23.30	24.90
	N3	22.50	16.90	22.50	21.90
	N4	22.50	17.30	22.30	23.40
	N5	23.40	17.80	22.50	24.40
	N6	24.60	20.40	25.10	25.90
	Mean	23.07	17.95	22.85	23.52
	S.D.	1.56	1.44	1.26	1.98
16	N1	21.40	14.10	19.30	21.40
	N2	25.70	14.80	22.30	24.40
	N3	23.30	16.50	21.20	21.80
	N4	23.80	17.60	22.10	21.40
	N5	24.90	20.40	20.60	22.70
	N6	25.90	19.30	23.40	23.60
	Mean	24.17	17.12	21.48	22.55
	S.D.	1.70	2.48	1.44	1.25
20	N1	21.90	11.40	20.60	20.60
	N2	24.40	9.40	22.50	23.10
	N3	21.20	13.90	19.30	18.90
	N4	23.40	15.60	21.20	21.20
	N5	23.60	15.90	21.60	21.60
	N6	25.30	17.40	23.40	23.40
	Mean	23.30	13.93	21.43	21.47
	S.D.	1.53	3.02	1.44	1.66
24	N1	21.68	6.00	16.10	19.10
	N2	28.10	7.50	18.80	20.80
	N3	23.30	13.50	16.30	13.10
	N4	23.40	14.30	16.70	14.60
	N5	24.00	14.10	20.80	20.10
	N6	27.20	17.10	22.70	20.40
	Mean	24.61	12.08	18.57	18.02
	S.D.	2.49	4.34	2.71	3.31

3. The effect of *Phyllanthus emblica* and L-ascorbic acid on cholesteryl esters.

Free cholesterol (FC)

Incubation time (hr)		FC (μmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
0	N1	0.939	0.868	0.765	0.916
	N2	0.845	0.970	0.925	0.906
	N3	0.920	1.030	1.032	1.025
	N4	0.980	1.063	1.068	0.970
	N5	0.729	0.837	0.741	0.762
	N6	0.700	0.792	0.702	0.874
	Mean	0.852	0.927	0.872	0.909
	S.D.	0.116	0.110	0.158	0.090
6	N1	0.903	0.791	0.786	0.769
	N2	0.988	1.143	0.946	1.052
	N3	0.878	0.884	0.969	0.946
	N4	0.932	0.966	1.147	0.941
	N5	0.728	0.705	0.771	0.739
	N6	0.974	0.724	0.930	0.940
	Mean	0.901	0.869	0.925	0.898
	S.D.	0.094	0.167	0.138	0.120
9	N1	0.802	0.773	0.811	0.965
	N2	0.968	1.028	0.902	0.958
	N3	0.966	0.922	0.968	1.239
	N4	0.922	1.055	0.987	1.025
	N5	0.706	0.757	0.773	0.717
	N6	0.817	0.724	0.742	0.763
	Mean	0.864	0.877	0.864	0.945
	S.D.	0.105	0.145	0.103	0.189
12	N1	0.916	0.929	0.967	0.916
	N2	0.980	0.872	0.961	1.022
	N3	1.080	1.106	1.260	1.035
	N4	1.048	0.998	1.187	1.100
	N5	0.850	0.800	0.897	0.944
	N6	0.999	0.776	1.032	0.884
	Mean	0.979	0.914	1.051	0.984
	S.D.	0.085	0.125	0.142	0.082
16	N1	1.012	1.016	0.925	0.919
	N2	1.119	0.997	1.112	1.040
	N3	1.077	0.925	1.073	1.074
	N4	1.040	0.946	1.203	0.920
	N5	0.903	0.834	0.796	0.835
	N6	0.838	0.831	0.878	0.878
	Mean	0.998	0.925	0.998	0.944
	S.D.	0.107	0.079	0.156	0.093
20	N1	0.977	1.096	0.865	0.905
	N2	0.981	0.869	1.103	1.026
	N3	1.300	0.992	1.088	1.037
	N4	1.217	1.078	1.142	1.063
	N5	0.805	0.762	0.854	0.894
	N6	0.909	0.963	0.900	1.020
	Mean	1.032	0.960	0.992	0.991
	S.D.	0.189	0.127	0.132	0.072

Incubation time (hr)		FC (μmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
24	N1	0.987	0.831	0.999	0.880
	N2	1.017	0.937	0.920	1.031
	N3	1.099	0.909	1.000	0.941
	N4	1.053	1.252	0.984	1.164
	N5	0.838	0.804	0.773	0.825
	N6	0.828	0.959	1.055	0.861
	Mean	0.970	0.949	0.955	0.950
	S.D.	0.113	0.160	0.099	0.127

Cholesteryl arachidonate (CA)

Incubation time (hr)		CA (μmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
0	N1	0.267	0.223	0.182	0.197
	N2	0.223	0.289	0.243	0.257
	N3	0.203	0.186	0.226	0.206
	N4	0.236	0.280	0.241	0.235
	N5	0.201	0.251	0.225	0.211
	N6	0.186	0.206	0.184	0.214
	Mean	0.219	0.239	0.217	0.220
	S.D.	0.029	0.041	0.027	0.022
6	N1	0.204	0.174	0.216	0.249
	N2	0.225	0.182	0.247	0.250
	N3	0.224	0.095	0.212	0.208
	N4	0.207	0.163	0.216	0.226
	N5	0.229	0.119	0.240	0.231
	N6	0.236	0.134	0.213	0.258
	Mean	0.221	0.145	0.224	0.237
	S.D.	0.013	0.034	0.015	0.019
9	N1	0.253	0.105	0.224	0.234
	N2	0.248	0.221	0.198	0.250
	N3	0.237	0.098	0.217	0.248
	N4	0.227	0.159	0.236	0.238
	N5	0.195	0.097	0.242	0.217
	N6	0.276	0.119	0.170	0.206
	Mean	0.239	0.133	0.215	0.232
	S.D.	0.027	0.049	0.027	0.017
12	N1	0.255	0.091	0.253	0.264
	N2	0.283	0.171	0.264	0.368
	N3	0.258	0.130	0.290	0.269
	N4	0.271	0.147	0.280	0.309
	N5	0.268	0.118	0.298	0.277
	N6	0.293	0.105	0.288	0.242
	Mean	0.271	0.127	0.279	0.288
	S.D.	0.015	0.029	0.017	0.045
16	N1	0.247	0.134	0.283	0.252
	N2	0.329	0.090	0.415	0.391
	N3	0.262	0.101	0.247	0.257
	N4	0.269	0.152	0.311	0.246
	N5	0.254	0.062	0.272	0.244
	N6	0.274	0.101	0.291	0.266
	Mean	0.273	0.107	0.303	0.276
	S.D.	0.029	0.032	0.059	0.057
20	N1	0.279	0.165	0.227	0.226
	N2	0.281	0.086	0.303	0.214
	N3	0.317	0.078	0.229	0.254
	N4	0.310	0.123	0.291	0.276
	N5	0.222	0.123	0.268	0.260
	N6	0.288	0.122	0.246	0.260
	Mean	0.283	0.116	0.261	0.248
	S.D.	0.034	0.031	0.032	0.023

Incubation time (hr)		CA (μmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
24	N1	0.291	0.119	0.287	0.227
	N2	0.235	0.128	0.241	0.249
	N3	0.268	0.110	0.252	0.184
	N4	0.232	0.135	0.238	0.220
	N5	0.257	0.104	0.201	0.211
	N6	0.210	0.126	0.263	0.224
	Mean	0.249	0.120	0.247	0.219
	S.D.	0.029	0.012	0.029	0.021

Cholesteryl linoleate (CL)

Incubation time (hr)		CL (μmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
0	N1	1.072	0.993	0.794	0.875
	N2	0.950	1.191	0.977	1.055
	N3	0.945	0.922	1.062	0.965
	N4	1.066	1.259	0.987	1.018
	N5	1.055	1.363	1.100	1.055
	N6	0.937	1.046	0.880	0.819
	Mean	1.004	1.129	0.967	0.965
	S.D.	0.066	0.170	0.114	0.098
6	N1	0.814	0.922	0.901	0.981
	N2	1.035	1.079	1.060	1.130
	N3	0.907	0.833	0.920	0.900
	N4	0.882	0.979	0.857	1.052
	N5	1.214	0.922	1.209	1.138
	N6	1.227	0.891	1.025	1.300
	Mean	1.013	0.938	0.995	1.084
	S.D.	0.176	0.084	0.130	0.413
9	N1	1.045	0.762	0.901	0.969
	N2	1.118	1.225	0.933	1.144
	N3	0.940	0.738	0.908	1.096
	N4	0.985	0.839	1.037	1.003
	N5	0.974	0.828	1.171	1.039
	N6	1.295	0.866	0.862	1.008
	Mean	1.060	0.876	0.969	1.043
	S.D.	0.131	0.178	0.115	0.065
12	N1	1.153	0.603	1.156	1.186
	N2	1.286	0.985	1.259	1.483
	N3	1.172	0.979	1.305	1.177
	N4	1.123	1.023	1.263	1.411
	N5	1.223	0.983	1.506	1.385
	N6	1.631	0.825	1.282	1.224
	Mean	1.265	0.900	1.295	1.311
	S.D.	0.188	0.161	0.115	0.131
16	N1	0.953	0.673	1.162	1.068
	N2	1.385	0.587	1.872	1.703
	N3	1.035	0.696	1.081	1.166
	N4	1.202	0.931	1.326	1.064
	N5	1.202	0.873	1.343	1.224
	N6	1.278	0.891	1.465	1.440
	Mean	1.176	0.775	1.375	1.278
	S.D.	0.426	0. 141	0.279	0.250
20	N1	1.070	0.612	1.037	0.978
	N2	1.224	0.574	1.227	0.917
	N3	1.392	0.758	1.084	1.179
	N4	1.308	0.853	1.229	1.078
	N5	1.155	0.876	1.370	1.416
	N6	1.428	1.014	1.330	1.221
	Mean	1.263	0.781	1.213	1.132
	S.D.	0.139	0.168	0.131	0.181

Incubation time (hr)		CL (μmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
24	N1	1.140	0.782	1.302	0.934
	N2	0.963	0.829	1.185	0.944
	N3	1.163	0.770	1.098	0.854
	N4	0.984	1.028	1.110	1.079
	N5	1.369	0.925	1.149	1.073
	N6	0.938	1.111	1.404	1.275
	Mean	1.093	0.908	1.208	1.027
	S.D.	0.165	0.139	0.121	0.150

Cholesteryl oleate (CO)

Incubation time (hr)		CO (μmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
0	N1	0.454	0.417	0.336	0.358
	N2	0.377	0.502	0.352	0.404
	N3	0.423	0.336	0.401	0.399
	N4	0.462	0.536	0.423	0.396
	N5	0.376	0.464	0.348	0.312
	N6	0.285	0.326	0.283	0.250
	Mean	0.396	0.430	0.357	0.353
	S.D.	0.066	0.086	0.050	0.061
6	N1	0.458	0.381	0.458	0.427
	N2	0.494	0.555	0.430	0.523
	N3	0.351	0.431	0.364	0.381
	N4	0.384	0.447	0.260	0.430
	N5	0.389	0.427	0.439	0.415
	N6	0.410	0.327	0.335	0.483
	Mean	0.414	0.428	0.381	0.443
	S.D.	0.053	0.076	0.076	0.051
9	N1	0.453	0.367	0.484	0.358
	N2	0.455	0.529	0.325	0.494
	N3	0.436	0.442	0.420	0.463
	N4	0.333	0.389	0.438	0.433
	N5	0.313	0.382	0.346	0.408
	N6	0.483	0.335	0.299	0.288
	Mean	0.412	0.407	0.385	0.407
	S.D.	0.071	0.069	0.073	0.075
12	N1	0.390	0.402	0.403	0.470
	N2	0.493	0.485	0.436	0.534
	N3	0.462	0.500	0.511	0.454
	N4	0.379	0.581	0.601	0.667
	N5	0.388	0.461	0.547	0.446
	N6	0.558	0.349	0.380	0.348
	Mean	0.445	0.463	0.480	0.487
	S.D.	0.072	0.081	0.087	0.107
16	N1	0.428	0.480	0.528	0.412
	N2	0.555	0.459	0.380	0.386
	N3	0.402	0.346	0.411	0.508
	N4	0.534	0.499	0.604	0.480
	N5	0.372	0.328	0.504	0.413
	N6	0.443	0.381	0.551	0.491
	Mean	0.456	0.416	0.496	0.448
	S.D.	0.073	0.073	0.085	0.051
20	N1	0.399	0.533	0.435	0.429
	N2	0.424	0.543	0.518	0.442
	N3	0.540	0.264	0.425	0.495
	N4	0.621	0.433	0.576	0.488
	N5	0.378	0.406	0.488	0.556
	N6	0.407	0.386	0.536	0.408
	Mean	0.462	0.428	0.496	0.470
	S.D.	0.097	0.103	0.059	0.054

Incubation time (hr)		CO (μmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
24	N1	0.415	0.394	0.552	0.371
	N2	0.397	0.410	0.489	0.410
	N3	0.441	0.458	0.473	0.408
	N4	0.371	0.614	0.509	0.420
	N5	0.467	0.449	0.334	0.374
	N6	0.428	0.437	0.422	0.436
	Mean	0.420	0.460	0.463	0.403
	S.D.	0.034	0.079	0.076	0.026

Cholesteryl palmitate (CP)

Incubation time (hr)		CP (μmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
0	N1	0.222	0.209	0.157	0.104
	N2	0.235	0.235	0.231	0.228
	N3	0.234	0.216	0.248	0.224
	N4	0.208	0.233	0.223	0.222
	N5	0.104	0.197	0.166	0.101
	N6	0.137	0.134	0.123	0.156
	Mean	0.190	0.204	0.191	0.173
	S.D.	0.056	0.037	0.050	0.060
6	N1	0.214	0.166	0.148	0.214
	N2	0.212	0.223	0.237	0.208
	N3	0.238	0.197	0.230	0.292
	N4	0.175	0.239	0.226	0.221
	N5	0.249	0.267	0.208	0.233
	N6	0.295	0.148	0.240	0.169
	Mean	0.231	0.207	0.215	0.223
	S.D.	0.041	0.045	0.035	0.040
9	N1	0.232	0.142	0.179	0.162
	N2	0.212	0.186	0.235	0.141
	N3	0.208	0.259	0.143	0.212
	N4	0.204	0.220	0.223	0.218
	N5	0.111	0.185	0.225	0.121
	N6	0.169	0.120	0.143	0.161
	Mean	0.189	0.185	0.191	0.169
	S.D.	0.043	0.051	0.042	0.039
12	N1	0.191	0.15	0.281	0.302
	N2	0.265	0.15	0.292	0.228
	N3	0.205	0.214	0.294	0.258
	N4	0.229	0.276	0.28	0.215
	N5	0.142	0.091	0.238	0.279
	N6	0.234	0.155	0.155	0.252
	Mean	0.211	0.173	0.257	0.256
	S.D.	0.042	0.064	0.054	0.032
16	N1	0.179	0.142	0.269	0.250
	N2	0.219	0.170	0.285	0.233
	N3	0.286	0.227	0.235	0.216
	N4	0.205	0.172	0.240	0.247
	N5	0.178	0.211	0.326	0.289
	N6	0.151	0.305	0.196	0.262
	Mean	0.203	0.205	0.259	0.250
	S.D.	0.047	0.058	0.045	0.025
20	N1	0.188	0.158	0.141	0.209
	N2	0.272	0.187	0.295	0.180
	N3	0.293	0.195	0.227	0.292
	N4	0.268	0.181	0.268	0.299
	N5	0.214	0.255	0.234	0.161
	N6	0.237	0.301	0.281	0.169
	Mean	0.245	0.213	0.241	0.218
	S.D.	0.040	0.054	0.056	0.06

Incubation time (hr)		CP (μmol/mg protein)			
		nLDL	he-oxLDL	L-ascorbic acid	*P. emblica*
24	N1	0.187	0.162	0.159	0.166
	N2	0.206	0.221	0.292	0.222
	N3	0.270	0.238	0.267	0.268
	N4	0.184	0.201	0.225	0.318
	N5	0.192	0.227	0.209	0.155
	N6	0.156	0.357	0.299	0.231
	Mean	0.199	0.234	0.242	0.227
	S.D.	0.038	0.066	0.054	0.061

APPENDIX C

Study the concentration dependent effects of hemin in HDL oxidation (he-oxHDL).

1. The effect of hemin on the formation of lipid peroxidation products; TBARs.

Incubation time (hr)	Conc. (μM)	TBARs (nmol/mg protein)		Mean	S.D.
		N1	N2		
0	nHDL	2.11	1.56	1.84	0.38
	2.5	1.88	1.51	1.69	0.27
	5.0	1.86	1.49	1.67	0.27
	10.0	1.64	1.66	1.65	0.01
	20.0	1.62	1.37	1.49	0.16
9	nHDL	2.01	1.65	1.83	0.25
	2.5	2.14	1.58	1.86	0.40
	5.0	2.69	1.57	2.13	0.79
	10.0	2.88	1.51	2.20	0.98
	20.0	3.69	2.29	2.99	0.99
24	nHDL	2.00	1.49	1.74	0.37
	2.5	2.62	1.85	2.24	0.55
	5.0	3.37	1.99	2.68	0.98
	10.0	4.11	2.02	3.07	1.48
	20.0	5.02	2.06	3.54	2.09

2. The effect of hemin on α-tocopherol levels.

Incubation time (hr)	Conc. (μM)	α-tocopherol (nmol/mg protein)				Mean	S.D.
		N1	N2	N3	N4		
0	nHDL	9.73	6.89	6.10	5.55	7.07	1.86
	2.5	10.11	6.89	6.73	4.98	7.17	2.14
	5	9.11	6.65	6.39	4.96	6.78	1.72
	10	9.02	7.43	5.95	9.39	7.95	1.58
	20	8.97	6.63	6.31	5.57	6.87	1.46
24	nHDL	8.18	5.92	5.15	5.91	6.29	1.30
	2.5	3.11	0.00	0.00	0.00	0.78	1.56
	5	4.46	0.00	0.00	0.00	1.12	2.24
	10	4.21	0.00	0.00	0.00	1.05	2.10
	20	3.64	0.00	0.00	0.00	0.91	1.82

APPENDIX D

Study the concentration dependent effects of *Phyllanthus emblica* on hemin induced HDL oxidation (he-oxHDL).

1. The effect of *Phyllanthus emblica* and L-ascorbic acid on TBARs formation.

Incubation time (hr)	Condition	Conc. (μg/ml)	TBARs (nmol/mg protein)			Mean	S.D.
			N1	N2	N3		
0	nHDL	-	2.27	2.20	2.92	2.46	0.40
	he-oxHDL	-	2.29	2.29	2.79	2.46	0.29
	P. emblica	0.5	2.33	2.21	2.89	2.48	0.36
		1.0	2.30	2.22	2.93	2.48	0.38
		2.5	2.31	1.87	2.87	2.35	0.50
		5.0	2.28	2.31	2.86	2.48	0.33
		10.0	2.17	1.97	2.87	2.34	0.47
		20.0	2.31	2.22	2.84	2.46	0.33
	L-ascorbic acid	0.5	2.00	2.16	2.91	2.36	0.48
		1.0	2.25	2.17	2.98	2.47	0.45
		2.5	2.21	2.27	2.97	2.48	0.42
		5.0	2.19	2.26	2.92	2.46	0.40
		10.0	2.34	2.02	2.95	2.44	0.47
		20.0	2.30	2.26	3.00	2.52	0.42
6	nHDL	-	2.36	2.34	2.78	2.49	0.24
	he-oxHDL	-	3.18	3.07	3.52	3.26	0.24
	P. emblica	0.5	2.93	2.59	3.08	2.87	0.24
		1.0	2.79	2.43	3.04	2.75	0.31
		2.5	2.86	2.52	3.18	2.85	0.33
		5.0	2.66	2.46	3.23	2.78	0.40
		10.0	2.62	2.62	3.24	2.83	0.36
		20.0	2.59	2.59	3.23	2.80	0.36
	L-ascorbic acid	0.5	2.84	3.19	3.34	3.12	0.26
		1.0	2.72	2.74	3.38	2.95	0.38
		2.5	2.86	2.36	3.37	2.86	0.50
		5.0	2.87	2.86	3.47	3.07	0.35
		10.0	2.99	3.02	3.57	3.19	0.33
		20.0	3.13	2.86	3.98	3.32	0.59
9	nHDL	-	2.44	2.60	3.22	2.91	0.43
	he-oxHDL	-	3.36	3.79	4.12	3.95	0.23
	P. emblica	0.5	2.75	2.83	3.15	2.99	0.23
		1.0	3.03	2.99	3.15	3.07	0.12
		2.5	2.82	2.88	3.21	2.97	0.21
		5.0	2.76	2.88	3.05	2.97	0.12
		10.0	2.59	2.93	3.29	3.11	0.26
		20.0	2.59	3.11	3.00	3.06	0.07
	L-ascorbic acid	0.5	3.12	3.60	3.69	3.65	0.07
		1.0	3.13	3.29	3.74	3.52	0.31
		2.5	2.78	3.14	3.64	3.19	0.43
		5.0	3.05	3.30	3.72	3.51	0.29
		10.0	3.03	3.31	3.97	3.64	0.47
		20.0	3.30	3.58	4.42	4.00	0.59

Incubation time (hr)	Condition	Conc (µg/ml)	N1	N2	N3	Mean	S.D.
24	nHDL	-	2.64	2.73	3.31	2.89	0.36
	he-oxHDL	-	3.90	4.33	4.79	4.34	0.45
	P. emblica	0.5	3.16	3.11	3.44	3.24	0.17
		1.0	3.13	2.75	3.22	3.03	0.24
		2.5	3.01	3.00	3.32	3.11	0.19
		5.0	2.90	2.79	3.26	2.98	0.24
		10.0	2.95	2.78	3.21	2.98	0.23
		20.0	2.76	2.85	3.28	2.96	0.28
	L-ascorbic acid	0.5	3.47	3.59	4.07	3.71	0.31
		1.0	3.58	3.72	4.01	3.77	0.23
		2.5	3.23	3.52	3.92	3.56	0.35
		5.0	3.18	3.56	4.18	3.64	0.50
		10.0	3.40	3.68	4.35	3.81	0.48
		20.0	3.48	3.78	4.58	3.95	0.57

2. The effect of *Phyllanthus emblica* and L-ascorbic acid hemin on α-tocopherol levels.

Incubation time (hr)	Condition	Conc. (μg/ml)	α-Tocopherol (nmol/mg protein)			Mean	S.D.
			N1	N2	N3		
0	nHDL	-	10.56	8.21	7.87	8.88	1.47
	he-oxHDL	-	10.91	8.12	6.79	8.61	2.10
	P. emblica	0.5	11.06	8.53	7.84	9.14	1.70
		1.0	10.56	5.63	7.75	7.98	2.48
		2.5	9.94	6.17	7.42	7.84	1.92
		5.0	11.29	5.47	7.04	7.93	3.01
		10.0	9.81	8.86	7.09	8.59	1.39
		20.0	10.46	7.53	7.33	8.44	1.75
	L-ascorbic acid	0.5	10.19	9.05	8.51	9.25	0.87
		1.0	12.60	6.67	9.03	9.43	2.98
		2.5	11.67	5.95	9.33	8.98	2.88
		5.0	9.88	7.46	7.60	8.31	1.35
		10.0	11.61	7.83	7.76	9.07	2.20
		20.0	9.95	6.53	7.06	7.85	1.84
6	nHDL	-	11.24	7.95	7.20	8.80	2.15
	he-oxHDL	-	5.64	0.00	0.00	1.88	3.26
	P. emblica	0.5	12.48	7.61	5.04	8.38	3.78
		1.0	7.27	7.34	5.33	6.65	1.14
		2.5	6.30	9.30	5.21	6.94	2.11
		5.0	8.09	8.01	5.21	7.10	1.65
		10.0	12.04	8.06	4.83	8.31	3.62
		20.0	8.27	7.92	5.01	7.07	1.78
	L-ascorbic acid	0.5	12.42	0.00	4.49	5.64	6.29
		1.0	10.7	0.00	3.59	4.76	5.44
		2.5	12.72	0.00	5.25	5.99	6.39
		5.0	8.49	7.67	5.37	7.18	1.61
		10.0	7.42	6.65	4.83	6.30	1.33
		20.0	6.61	7.40	3.55	5.85	2.03
9	nHDL	-	10.47	9.83	6.83	8.33	2.11
	he-oxHDL	-	5.86	0.00	0.00	1.95	3.38
	P. emblica	0.5	3.16	0.00	4.90	2.45	3.46
		1.0	8.56	0.00	6.00	3.00	4.24
		2.5	4.79	0.00	4.00	2.93	2.56
		5.0	9.10	0.00	4.10	2.05	2.89
		10.0	14.66	0.00	4.30	2.15	3.05
		20.0	3.45	0.00	3.82	1.91	2.70
	L-ascorbic acid	0.5	11.99	0.00	4.71	2.36	3.33
		1.0	7.64	0.00	3.36	1.68	2.37
		2.5	10.40	0.00	3.02	4.47	5.35
		5.0	11.07	0.00	3.66	1.83	2.58
		10.0	3.23	0.00	4.32	2.16	3.05
		20.0	5.24	0.00	3.70	1.85	2.62

Incubation time (hr)	Condition	Conc (µg/ml)	N1	N2	N3	Mean	S.D.
24	nHDL	-	10.81	9.71	7.12	9.21	1.89
	he-oxHDL	-	4.35	0.00	0.00	1.45	2.51
	P. emblica	0.5	3.22	0.00	3.28	2.17	1.87
		1.0	5.96	0.00	3.08	3.01	2.98
		2.5	4.33	0.00	4.17	2.83	2.46
		5.0	4.46	0.00	3.57	2.68	2.36
		10.0	7.28	6.27	3.49	5.68	1.96
		20.0	10.80	7.87	5.60	8.09	2.62
	L-ascorbic acid	0.5	8.70	0.00	0.00	2.90	5.02
		1.0	4.75	0.00	4.20	2.98	2.60
		2.5	6.91	0.00	3.11	3.34	3.46
		5.0	4.97	6.98	3.09	5.01	1.94
		10.0	6.14	6.63	3.72	5.50	1.56
		20.0	4.54	6.19	3.72	4.82	1.26

APPENDIX D

Study the time dependent effects of *Phyllanthus emblica* on hemin induced HDL oxidation (he-oxHDL).

1. The effect of *Phyllanthus emblica* on TBARs formation.

Incubation time (hr)	Condition	TBARs (nmol/mg protein)		Mean	S.D.
		N1	N2		
0	nHDL	1.03	1.72	1.38	0.49
	he-oxHDL	0.70	1.61	1.16	0.65
	P. emblica 8 µg/ml	0.72	1.75	1.24	0.74
0.5	nHDL	0.66	1.84	1.25	0.83
	he-oxHDL	0.83	1.78	1.31	0.68
	P. emblica 8 µg/ml	0.98	1.76	1.37	0.55
1	nHDL	0.60	2.78	1.69	1.54
	he-oxHDL	0.75	1.80	1.28	0.75
	P. emblica 8 µg/ml	0.86	1.85	1.36	0.71
1.5	nHDL	0.81	1.61	1.21	0.57
	he-oxHDL	0.77	1.93	1.35	0.82
	P. emblica 8 µg/ml	0.72	1.91	1.32	0.85
2	nHDL	0.65	1.70	1.18	0.75
	he-oxHDL	1.08	2.15	1.62	0.76
	P. emblica 8 µg/ml	0.94	2.01	1.48	0.76
3	nHDL	0.87	1.80	1.34	0.66
	he-oxHDL	0.97	1.99	1.48	0.72
	P. emblica 8 µg/ml	0.84	1.80	1.32	0.68
4	nHDL	1.15	2.54	1.85	0.99
	he-oxHDL	1.08	2.02	1.55	0.66
	P. emblica 8 µg/ml	0.76	2.07	1.42	0.93
6	nHDL	0.91	2.02	1.47	0.79
	he-oxHDL	1.58	2.28	1.93	0.49
	P. emblica 8 µg/ml	0.87	1.88	1.38	0.72
8	nHDL	1.05	1.54	1.30	0.35
	he-oxHDL	1.73	2.30	2.02	0.41
	P. emblica 8 µg/ml	1.01	1.74	1.38	0.52
10	nHDL	1.08	1.69	1.39	0.44
	he-oxHDL	1.55	2.41	1.98	0.61
	P. emblica 8 µg/ml	1.14	2.44	1.79	0.92
24	nHDL	0.68	1.76	1.22	0.76
	he-oxHDL	1.69	3.41	2.55	1.22
	P. emblica 8 µg/ml	1.03	2.00	1.52	0.69

2. The effect of *Phyllanthus emblica* on α-tocopherol levels.

Incubation time (hr)	α-tocopherol (nmol/mg protein)		
	nHDL	he-oxHDL	*P. embica* 8 μg/ml
0	18.62	18.79	18.95
1	18.72	4.50	7.34
2	17.47	5.13	5.90
4	18.16	4.36	7.70
6	18.11	5.70	10.30
8	17.07	3.89	7.46
10	16.10	2.15	7.95
24	14.16	2.37	8.12

3. The effect of *Phyllanthus emblica* on paraoxonase activity.

Incubation time (hr)	Condition	PON activity (μmol/mg protein)		Mean	S.D.
		N1	N2		
0	nHDL	11.72	16.02	13.87	3.04
	he-oxHDL	12.10	9.25	10.67	2.02
	P. emblica 8 μg/ml	10.17	10.03	10.10	0.10
0.5	nHDL	7.03	11.46	9.25	3.14
	he-oxHDL	7.17	10.94	9.06	2.67
	P. emblica 8 μg/ml	4.57	5.86	5.22	0.92
1	nHDL	4.55	7.29	5.92	1.94
	he-oxHDL	5.07	7.16	6.11	1.48
	P. emblica 8 μg/ml	3.38	0.91	2.15	1.74
1.5	nHDL	3.52	2.98	3.25	0.38
	he-oxHDL	3.90	4.17	4.03	0.18
	P. emblica 8 μg/ml	2.33	0.65	1.49	0.19
2	nHDL	2.22	2.47	2.34	0.18
	he-oxHDL	3.25	5.08	4.16	1.29
	P. emblica 8 μg/ml	2.10	0.13	1.12	1.40
3	nHDL	1.97	2.47	2.22	0.35
	he-oxHDL	2.22	2.21	2.21	0.00
	P. emblica 8 μg/ml	1.44	0.65	1.04	0.55
4	nHDL	2.08	0.78	1.43	0.92
	he-oxHDL	1.56	2.08	1.82	0.37
	P. emblica 8 μg/ml	1.03	0.00	0.52	0.74
6	nHDL	1.55	0.78	1.17	0.55
	he-oxHDL	2.08	2.87	2.47	0.55
	P. emblica 8 μg/ml	1.30	0.00	0.65	0.92
8	nHDL	1.56	0.00	0.78	1.10
	he-oxHDL	2.87	0.91	1.89	1.39
	P. emblica 8 μg/ml	2.47	0.00	1.23	1.74
10	nHDL	1.83	0.00	0.92	1.30
	he-oxHDL	0.78	0.00	0.39	0.55
	P. emblica 8 μg/ml	3.90	0.00	1.95	2.76
24	nHDL	1.03	0.00	0.52	0.74
	he-oxHDL	1.44	0.00	0.72	1.02
	P. emblica 8 μg/ml	0.65	0.00	0.33	0.47

Study Protocol Approval

The Ethics Committee of The Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol dated and/ or amended as follows:

Study Title: **The effects of *Phyllanthus emblica* on hemin induced lipoprotein oxidation**

Study Code: -

Centre: CHULALONGKORN UNIVERSITY

Principal Investigator : Miss Sirirat Mongkhollikit

Protocol Date : **February 22, 2008**

A list of the Ethics Committee members and positions present at the Ethics Committee meeting on the date of approval of this study has been attached.

This Study Protocol Approval Form will be forwarded to the Principal Investigator.

Chairman of Ethics Committee: .....

(Rungpetch Sakulbumrungsil, Ph.D.)

Secretary of Ethics Committee: .....

(Suyanee Pongthananikorn, Ph.D.)

Date of Approval: **March 18, 2008**

Thai Journal of Pharmacology

hartherst.or

Official Publication of
Pharmacological and Therapeutic Society of Thailand

Proceedings of 31st Pharmacological and Therapeutic Society of Thailand Meeting

18-20 March 2009

Vol.31, No.1, 2009 ISSN 0125-3832

Original article P26

The Effect of *Phyllanthus emblica* on Hemin Induced Lipoprotein Oxidation

Sirirat Mongkhollikit [1*], Ubonthip Nimmannit [2], Rataya Luechapudiporn [1]

[1]*Department of Pharmacology, Faculty of Pharmaceutical Sciences,* [2]*Pharmaceutical Technology (International) Program, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand*

E-mail: m_sirirat@yahoo.com

Abstract

Lipoprotein oxidation is a key early stage in the development of atherosclerosis. Hemin (Iron (III)-protoporphyrin IX) can be detected in plasma of thalassemia patients. It is a potent oxidative inducer of lipoprotein oxidation. Dietary antioxidants can be used to protect lipoprotein oxidation. Ma-kham-pom (*Phyllanthus emblica* Linn) has been reported to have antioxidant activities. The aim of this study was to determine the protective effect of *P. emblica* on hemin induced lipoprotein oxidation. The pre-incubation of low-density lipoproteins (LDL) with various concentration of *P.emblica* (0.5, 1, 2.5, 5, 10 and 20 µg/mL) were performed; and then oxidized by hemin 5 µM/300 µg LDL protein (he-oxLDL) for 24 hr. L-ascorbic acid was used as a positive control. Lipoprotein oxidation was determined by measuring thiobarbituric acid reactive substances (TBARs) formation, a marker of lipid peroxidation, and platelet-activating factor acetylhydrolase (PAF-AH) activity, a lipid hydroperoxides scavenging enzyme. The results showed that *P.emblica* 20 µg/mL inhibited TBARs formation almost 100%. The 50% inhibition concentration (IC_{50}) of TBARs were 2.5 and 13 µg/mL for *P.emblica* and L-ascorbic acid, respectively. The PAF-AH activity in he-oxLDL incubated with 2.5-20 µg/mL of *P.emblica* were significant higher than he-oxLDL ($p<0.01$). We concluded that *P.emblica* can inhibit lipid peroxidation in he-oxLDL and preserved PAF-AH activity in a dose dependent manner.

Keywords: Lipoprotein oxidation, Hemin, *Phyllanthus emblica*

Introduction

Lipoprotein oxidation plays a role in the pathogenesis of atherosclerosis. There is increasing evidence that oxidation of plasma low density lipoprotein (LDL) is a critical factor in promoting atherosclerosis (1). Hemin (Iron (III)-protoporphyrin IX) is a degradation product of hemoglobin. Hemin was found to be elevated in pathological cases like severe hemoglobinopathies, sickle cell anemia and thalassemia (2). Several *in vitro* studies have revealed that hemin is an effective inducer of LDL oxidation. Therefore, the inhibition of LDL oxidation by supplementation of antioxidants becomes an attractive therapeutic strategy to prevent and possibly to treat atherosclerosis. *Phyllanthus emblica* Linn is commonly known as amla and names "Ma Kham Pom" in Thailand. It is a member of the family Euphorbiaceae. *P.emblica* has been shown to possess several pharmacologic actions and antioxidants. However, its effects on lipoprotein oxidation induced by hemin have not been established. Therefore, the aim of study was to determine the effect of *Phyllanthus emblica* on hemin induced lipoprotein oxidation by monitoring thiobarbituric acid reactive substances

(TBARs) formation, a marker of lipid peroxidation, and platelet-activating factor acetylhydrolase (PAF-AH) activity, a lipid hydroperoxides scavenging enzyme.

Methods

1. Oxidation of Lipoprotein The separation of LDL was performed by the sequential density gradient ultracentrifugation method. The powder of spray-dried from fruit juice of *P.emblica* was used in this study. LDL was pre-incubated with *P.emblica* at concentrations 0.5, 1, 2.5, 5, 10, 20 µg/mL for 30 minutes. Then hemin was added into LDL to induce lipoprotein oxidation (final concentration 5 µM hemin/300 µg LDL protein). L-ascorbic acid was used as a positive control. After incubation at 37 °C for 24 hr, the oxidation reaction was terminated by adding 50 µM of BHT. Then TBARs formation and PAF-AH activity were determined.

2. Thiobarbituric acid reactive substances The lipid peroxidation products were determined by spectrofluorometric method, with excitation and emission wavelength at 515 and 553 nm, respectively (3). 1,1,3,3-Tetraethoxypropane was used as a standard.

3. Platelet activating factor acetylhydrolase activity assay PAF-AH activity was measured by using 2-thio PAF substrate in 0.1 M Tris-HCl buffer (pH 7.2) containing 1 mM EDTA and 1 mM DTNB. Upon hydrolysis of the acetyl thio-ester bond at *sn*-2 position by PAF-AH, free thiols are detected using 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), the increase in absorbance at 405 nm was recorded continuously for 10 minutes (4).

4. Statistical analysis Data were expressed as mean±S.E.M. Differences between groups were tested with one-way ANOVA and correlation analyses were assessed by pearson correlation using the SPSS version 13.0 for window software. Statistically significant differences were accepted at $P<0.05$.

Results

P.emblica inhibited the TBARs formation in a dose dependent manner (Fig 1A). The maximum percent inhibitions of TBARs were 99.35% and 82.45% for *P.emblica* and L-ascorbic acid (20 µg/mL), respectively (Fig 2A.). The 50 percent inhibition concentration (IC_{50}) of *P.emblica* and L-ascorbic acid on TBARs formation was obtained from the plot between the concentrations against the percent inhibition of TBARs formation (Fig. 2A). The IC_{50} were 2.5 and 13 µg/mL for *P.emblica* and L- ascorbic acid, respectively. The PAF-AH activities in he-oxLDL incubated with *P.emblica* at 5, 10 and 20 µg/mL and with L-ascorbic acid at 20 µg/mL were significantly higher than he-oxLDL control (25.50±1.50, 26.8±1.90, 28.0±1.6 and 25.7±1.5 vs. 15.9±1.5 nmol/min/mg LDL protein, respectively, $p<0.001$) (Fig 1C and 1D). These results indicated that *P.emblica* was more effective than L-ascorbic acid to inhibit TBARs formation and to preserve PAF-AH activity. Significant inverse relationships were found between TBARs formation and PAF-AH activity with the r-value of -0.70 ($p<0.001$) as shown in the Figure 2B. While PAF-AH activity was depleted, the TBARs levels were increased.

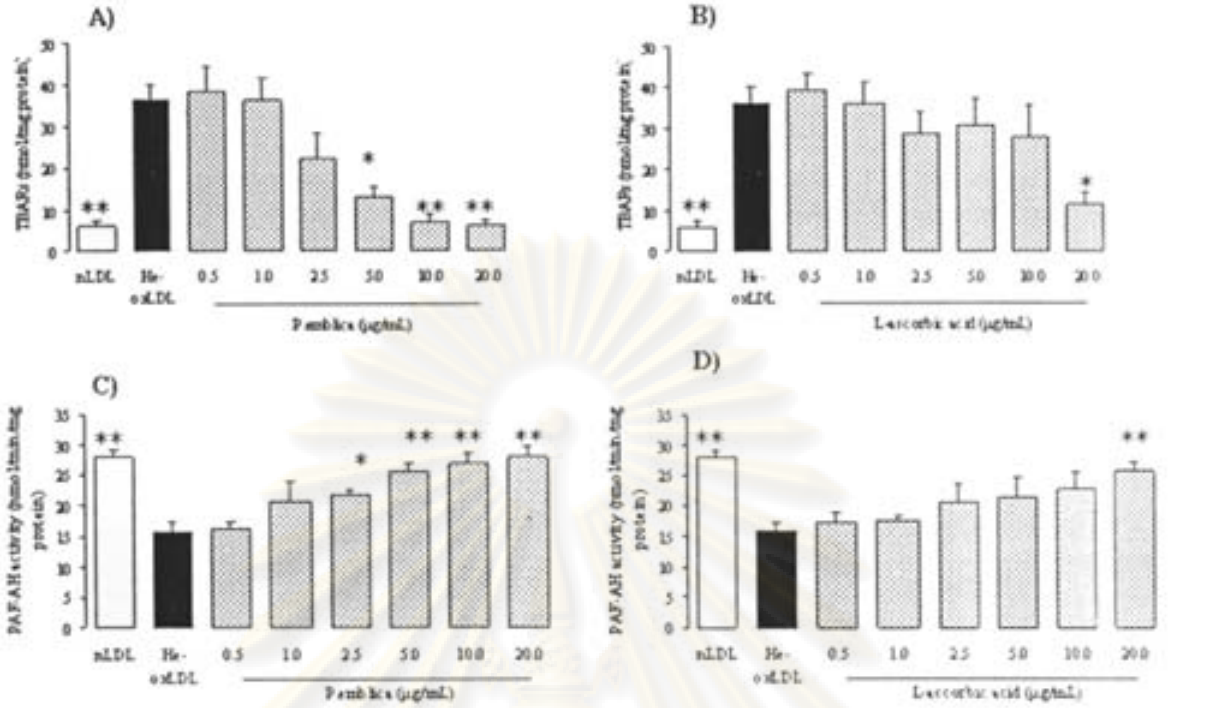


Figure 1 Effects of *P.emblica* and L-ascorbic acid on TBARs formation (A-B) and PAF-AH activity (C-D). Data are shown as mean±S.E.M. obtained from five independent experiments. * $p \leq 0.01$ compared with He-oxLDL control, ** $p<0.001$ compare with He-oxLDL control

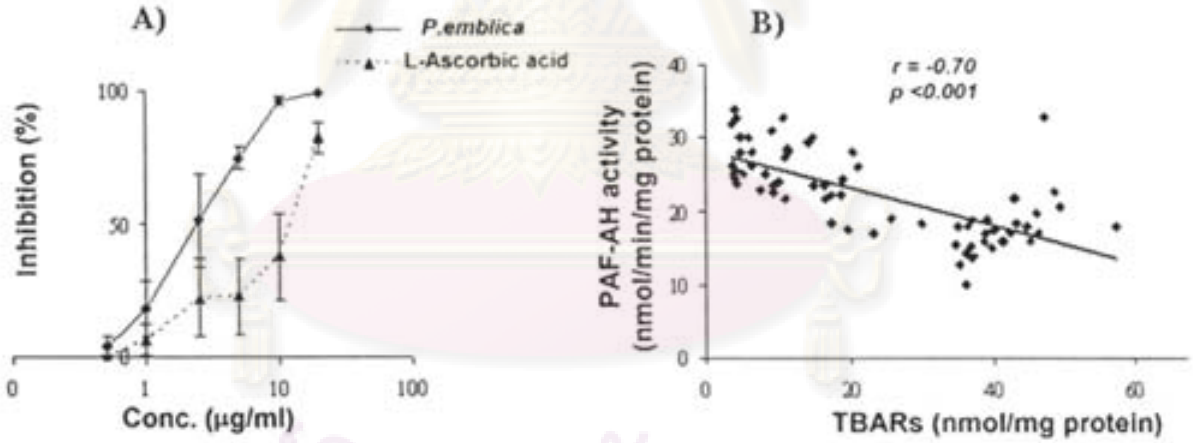


Figure 2 The percent inhibition of *P.emblica* and L-ascorbic acid on TBARs formation. The lines were acquired by plotting the concentrations of tested compounds (0.5, 1, 2.5, 5. 10 and 20 μg/mL) against the percent inhibition of TBARs formation (A). The correlation between TBARs with PAF-AH activity (B). The data were analysed from five independent experiments.

Discussion & Conclusion

P.emblica was successfully protected TBARs formation and preserved the PAF-AH activity. These effects possess the antioxidant activities. Since *P.emblica* contain with ascorbic acid and many phenolic compounds which have the potential to function as antioxidants. *P.emblica* has also been reported as a very effective free-radical scavenger (5). Our finding indicated that *P.emblica* was more potent to protect he-oxLDL than L-ascorbic acid. So we suggested that the inhibition of LDL oxidation of *P.emblica* may not be attributed to ascorbic acid alone but the overall effect is due to other polyphenols such as

ellagic acid, gallic acid, tannin, etc (6). In addition, the two new hydrolysable tannins, called emblicanin A and B are active constituents of *P.emblica* discovered, and have been reported to be a very strong antioxidant (7). Further study is needed to clarify the active substances and comparison of the potency of each compound in *P.emblica* extracts on the effect of hemin induced lipoprotein oxidation.

Acknowledgements

We thank the National Research Council of Thailand for a grant.

References

1. Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherosclerosis. Free Rad Biol Med 1996;20:707-727.
2. Shaklai N, Shviro Y, Rabizadeh E, Kirschner-Zilber I. Accumulation and drainage of hemin in the red cell membrane. Biochim Piophys Acta 1985;821:355-66.
3. Asakawa T, Matsushita S. Coloring condition of thiobarbituric acid test for detecting lipid hydroperoxide. Lipids 1980;15: 137-140.
4. Aarsman AJ, Ney FW, Van den Bosch H. Catabolism of platelet-activating factor and its analog. Differentiation of the activities of lysophospholipase and platelet-activating-factor acetylhydrolase. Eur J Biochem 1991;200:187-193.
5. Bhattacharya A, Chatterjee A, Ghosal S, Bhattacharya SK. Antioxidant activity of active tannoid principles of Emblica. Indian J Exp Biol 1999;37:676-680.
6. Khopde S.M, Indira Priyadarsini K. Characterizing the antioxidant activity of amla (Phyllanthus emblica) extract, Current Science 2001;81:185-190.
7. Ghosal S, Triethi VK, Chauhan S. Active constituents of Emblica officinalis: Part 1.-The chemistry and antioxidative effects of two new hydrolysable tannins, Emblicanin A and B. Indian Journal of Chemistry 1996;35B:941-948.

BIOGRAPHY

NAME	Miss Sirirat Mongkhollikit
DATE OF BIRTH	25 June 1982
PLACE OF BIRTH	Chonburi
INSTITUTIONS ATTENDED	Huachiew Chalermprakiet University, 2005: Bachelor of Pharmacy
HOME ADDRESS	6/13 Moo.8 Bueng, Sriracha, Chonburi, Thailand 20230