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THE PROTECTIVE EFFECT OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 ON HEMIN- INDUCED LIPOPROTEIN OXIDATION

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การเกิดปฏิกิริยาออกซิเดชันของลิโพโปรตีนชนิดความหนาแน่นต่ำ (LDL) มีบทบาทสำคัญต่อการเกิดภาวะหลอดเลือดแดงแข็ง ฮีมินเป็นตัวเหนี่ยวนำให้เกิดปฏิกิริยาออกซิเดชันซึ่งพบในพลาสมาของผู้ป่วยโรคธาลัสซีเมีย บัวบก (*Centella asiatica*) เป็นพืชสมุนไพรที่มีฤทธิ์ต้านออกซิเดชันได้ วัตถุประสงค์ของการศึกษาครั้งนี้ เพื่อศึกษาผลของสารสกัดมาตรฐานบัวบก ECa233 และ asiatic acid ที่มีต่อการเกิดปฏิกิริยาออกซิเดชันจากการเหนี่ยวนำด้วยฮีมินในลิโพโปรตีน ทดลองโดยการเติม ECa233 และ asiatic acid ที่ความเข้มข้นต่างๆ ลงใน LDL ก่อนเป็นเวลา 30 นาที โดยใช้ α-tocopherol เป็นสารควบคุมบวก แล้วจึงเติมฮีมินเพื่อเหนี่ยวนำให้เกิดปฏิกิริยาออกซิเดชันที่ 37 $^{\circ}$C เป็นเวลา 24 ชั่วโมง จากนั้นจึงตรวจวัดการเกิดปฏิกิริยาออกซิเดชันของ LDL โดยการวัด thiobarbituric acid reactive substances (TBARs), α-tocopherol (α-TC) และ relative electrophoretic mobility (REM) ผลการศึกษาพบระดับของ TBARs และ REM สูงขึ้นใน oxidized LDL และพบว่า ECa233 และ asiatic acid ความเข้มข้น 20-80 μg/ml สามารถยับยั้งการเกิด TBARs ที่ 6 ชั่วโมงได้ 70% ส่วนที่ 24 ชั่วโมง ยับยั้งได้เพียง 15-20 % อย่างไรก็ตาม ECa233 และ asiatic acid ไม่สามารถลดค่า REM แสดงว่าไม่สามารถป้องกันการออกซิเดชันของโปรตีน จากผลการทดลองสรุปได้ว่า ECa233 และ asiatic acid สามารถป้องกัน lipid peroxidation ใน LDL ที่ถูกเหนี่ยวนำด้วยฮีมินได้อย่างมีนัยสำคัญเป็นระยะเวลา 6 ชั่วโมง แต่ไม่สามารถป้องกันได้ถึง 24 ชั่วโมง

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Oxidation reaction of low density lipoprotein (LDL) plays a pivotal role in atherogenesis. Hemin (protoporphyrin IX- Fe^{2+}) is oxidative mediator found in plasma of thalassemia patient. *Centella asiatica* have been shown to be antioxidants. The aim of this study was to determine the effect of standardized extract of *Centella asiatica* ECa233 and asiatic acid on hemin-induced LDL oxidation (he-oxLDL). LDL was pre-incubated with either ECa233 or asiatic acid for 30 minutes, and then oxidation was initiated by incubation with hemin for 24 hours. The degree of LDL oxidation was determined by measurement of thiobarbituric acid reactive substances (TBARs) and the relative electrophoretic mobility (REM). TBARs levels and REM values were increased in he-oxLDL. The results showed that ECa233 and asiatic acid (20-80 μg/ml) can inhibit TBARs formation at 6 hr 70% while at 24 hr they can inhibit only 15-20%. However, ECa233 and asiatic acid cannot decrease REM value, indicating that both compounds cannot protect protein oxidation. In conclusion, ECa233 and asiatic acid could protect lipid peroxidation in LDL induced by hemin significantly at 6 hr but this effect was not prolonged to 24 hr.

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LIST OF ABBREAVIATIONS

α-tocopherol	Alpha-tocopherol
Apo	Apolipoprotein
AA	Asiatic acid
BHT	Butylated hydroxytoluene
β-thal/HbE	β-thalassemia/hemoglobin E
C.asiatica	*Centella asiatica*
Conc.	Concentration
$^{\circ}$C	Degree celcius
d	Density
Fe	Ferric
g	gram
HPLC	High performance liquid chromatography
hr	hour
he-oxLDL	Hemin oxidized Low Density Lipoprotein
LDL	Low Density Lipoprotein
MDA	Malondialdehyde
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeter
μg	Microgram
μl	Microliter
μM	Micromolar
NA2EDTA	Ethylenediamine tetraacetic acid disodium salt
nLDL	Native Low Density Lipoprotein
nm	Nanometer

nmol	Nanomole
ox-LDL	Oxidized Low Density Lipoprotein
PBS	Phosphate buffer saline
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
REM	Relative Electrophoretic Mobility
rpm	Revolution per minute
S.E.M.	Standard error of mean
TBARs	Thiobarbituric acid reactive substances
VLDL	Very Low Density Lipoprotein

CHAPTER I

INTRODUCTION

1. Background & Rationale

Oxidation in low density lipoprotein (LDL) plays a pivotal role in atherogenesis. The formation of oxidized LDL occurs when the free radical process lipid peroxidation and modify apolipoprotein of LDL. Then the oxidized LDL was phagocyted by macrophage causing foam cells and plaques formation in arteries wall that leads to atherosclerosis subsequently cardiovascular complications. The LDL oxidation can be induced by transition metals such as copper and iron, free radicals and some enzymes such as myeloperoxidase and lipoxygenase (Marco, et al. 1999). In addition, some diseases like in hemoglobinopathy disorder and thalassemia patients of which the circulation was iron overload, hemin was a possible physiological iron to promote LDL oxidation.

Hemin is a product of heme (protoporphyrin IX-Fe^{2+}) oxidation and is a powerful inducer of LDL oxidation in the *in vitro* studies. Ferric component of hemin and amphipathicity property may be the cause of oxidation in lipoprotein. Hemin was found to be elevated in serum of β-thal/Hb E patients (Phumala, et al. 2003). Both plasma and lipoprotein of β-thal/Hb E patients also presented a precipitous decrease in α-tocopherol and elevation of oxidized LDL (Leuchapudiporn, et al. 2006) and lead to vascular complications. Oxidation of LDL may be protected with endogeneous antioxidants such as α-tocopherol, glutathione and ascorbic acid. So prevention of LDL oxidation by antioxidant could have a great potential for prevention of atherosclerosis or complications of vascular diseases (Esterbauer, et al., 1993).

Centella asiatica (L.) Urban has been reported to possess antioxidant, antileprotic, antitumor, antistress, wound healing, fungicidal and antibacterial properties. It is also used as tonic in Ayuvredic formulations (Thongnopnua, 2008). As a potent antioxidant, *Centella asiatica* exerted neuroprotective effect and proved efficacious in

protecting rat brain against age related oxidative damage (Subathra, et al. 2005). One study suggested potential of Centella to attenuate the age-related decline in cognition function and mood disorder in the healthy elderly (Wattanathorn, et al. 2008). These pharmacological efficacies have been proven to relate to four bioactive triterpenes from an alcoholic extract of *C. asiatica* leaves. They are asiatic acid, madecassic acid, asiaticoside and madecassoside. Asiatic acid is also an active metabolite of asiaticoside. Among the biological active triterpenoids in *Centella asiatica,* asiatic acid has been presented to be the most therapeutically active for collagen synthesis stimulation then promoting wound healing. Hence, it is interesting to study antioxidant activity of asiatic acid as well.

The standardized extract of *Centella asiatica* (ECa233) has been developed recently. ECa233 consists of not less than 80 % of triterpenoids and the ratio of madecassoside and asiaticoside is between 1.50 $\pm$ 0.5 : 1. Its stability is more than 2 years and toxicological data were shown in high level safety (Tantisira, 2011). Many studies reported a potential property of ECa233 to treat deficit in learning and memory and also wound healing property in animal models (Anchalee, et al. 2009). However, the effect of *Centella asiatica* extract and asiatic acid on hemin-induced lipoprotein oxidation has not been established. Since ECa233 have a potent antioxidant activity, we hypothesized that ECa233 might have a potential to prevent LDL oxidation induced by hemin. Thus this *in vitro* study will focus on the antioxidant effect of ECa233 and asiatic acid on hemin-induced lipoprotein oxidation.

2. Objective

1) To study the protective effect of ECa233 on hemin-induced low density lipoprotein oxidation.

2) To study the protective effect of asiatic acid on hemin-induced low density lipoprotein oxidation.

3. Hypothesis

1) ECa233 prevents hemin-induced LDL oxidation.

2) Asiatic acid prevents hemin-induced LDL oxidation.

4. Expected Benefit and Application

Information regarding antioxidant activity of ECa233 in hemin-induced low density lipoprotein oxidation *in vitro* may be useful for further study of ECa233 in the *in vivo* prevention of LDL oxidation.

CHAPTER II

LITERATURE REVIEW

Oxidation reaction of LDL plays an important role in atherogenesis and can be induced by free radicals, heavy metals such as iron, copper which cause cell damage. However these metals are not in free from in normal body fluid. It is possible that LDL oxidation may be enhanced by heme which is ferric compound and lipophilic.

Oxidation reaction which is induced by heme may involve in atherosclerosis. It was supported by the research that LDL which is oxidized by hemin is toxic to endothelial cells culture from aorta (Balla, et al., 1991). So in this study hemin was used to induce LDL oxidation.

Low density Lipoprotein

Low density Lipoprotein (LDL) is the lipoprotein that transfers cholesterol and triglycerides from liver to peripheral tissues. LDL is one of five main groups of lipoproteins (chylomicrons, VLDL, IDL, LDL, and HDL) that can move lipid and cholesterol into plasma. LDL also controls cholesterol synthesis.

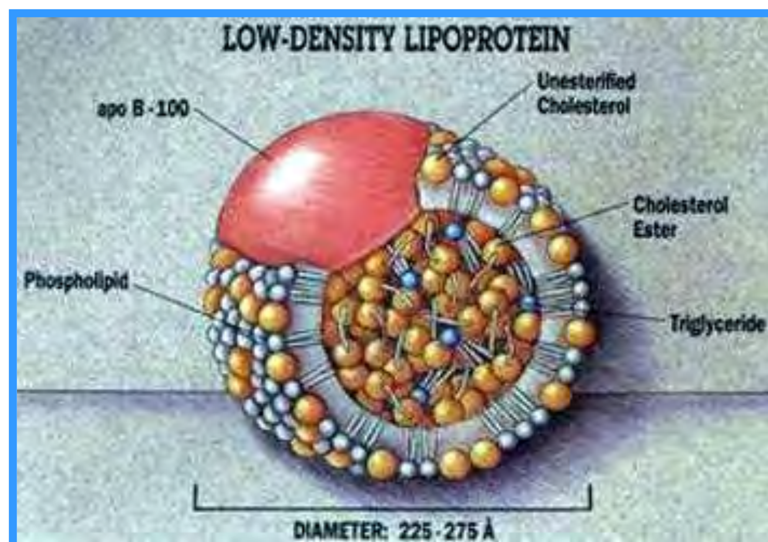


Figure 2.1 Structure of low density lipoprotein

-Structure

Native LDL particle consists of one molecule of apolipoprotein B-100 (Protein that has 4536 amino acids) and lipophilic core that consist of linoleate which is polyunsaturated fatty acid (PUFA), 200 molecules of triglycerides and 1500 molecules of cholesteryl ester. The surface layer consists of phospholipid, choleslerol and large size of B-100 protein. LDL is protected from lipid peroxidation by the presence of several antioxidants such as α-tocopherol, superoxide dismutase, catalase, peroxidase. In each LDL particle contains on average about seven molecules of α-tocopherol, the major antioxidant. Other potential antioxidants in LDL are γ-tocopherol, β-carotene, α-carotene, lycopene, cryptoxanthine, lutein, zeaxanthin, phytofluene, retinoids and ubiquinol-10. These antioxidants are present in lower amounts than α-tocopherol. The average ratio of PUFAs to total antioxidants is 150:1 but there is a large degree of variation. (Chisolm and Steinberg, 2000; Esterbauer, et al. 1993)

Lipoprotein oxidation and atherosclerosis

Lipoprotein oxidation is the important initial stage in atherosclerosis. Oxidation of LDL is a chain reaction of lipid peroxidation. LDL oxidation results in the chemical modification of certain moieties of apoB by lipid peroxidation products. The ε-amino groups of lysine residues are especially susceptible. Lipid peroxidation which is occurred from free radical attack at double bond of PUFA caused taking its atom out of methylene (CH_2) group. At initiation stage, rearrangement of unstable carbon radical initiated to conjugated diene. Conjugated diene fast reacted with oxygen caused peroxyl radical in propagation stage. Peroxyl radical in LDL may abstract H atom from adjacent PUFA causing hydroperoxide. Lipid hydroperoxide broke to short chain aldehydes, malondialdehyde and 4-hydroxynonenal. When lipids in LDL are attacked by free radicals, it will become oxidized LDL. Oxidized LDL molecule will get into artery wall and stimulate macrophage in phagocytosis. Then macrophage will change to foam cell attach and deposit to artery wall. If it collects more to be plaque may be lead to development of atherosclerosis (Chisolm and Steinberg, 2000).

Atherosclerosis is the disease that has plaque in artery. Plaque consists of fat, cholesterol, calcium and others compounds that have been found in blood. When time passes, plaque became harder and arteries became thinner. The consequent are blood vessel obstruction or plaque rupture. Then blood circulation to organs decreased, causing big problems such as coronary heart disease, stroke and can lead to death.

Lipid peroxidation

Lipid peroxidation is the process that free radicals abstract electrons from lipid in cell membrane causing cell damage and increases free radicals. Lipid peroxidation often occurred in PUFA which has many double bonds between methylene groups that contain reactive H. The reaction has 3 key steps which are initiation, propagation and termination (Young and McEneny, 2001)

-Initiation

Initiation is the step that cause fatty acid radical, mostly reactive oxygen species (ROS) such as $OH^{\bullet}$ will attach H atom to give water and fatty acid radical

-Propagation

Fatty acid radical was unstable molecule that will react with oxygen to be peroxyl-fatty acid radical which was also unstable and will react with another free fatty acid to be fatty acid radical and hydrogen peroxide or cyclic peroxide. If reacted with itself, this step will continue causing new free fatty acid radical and did the same reaction.

-Termination

Initiation step stopped when 2 radicals reacted and caused non-radical in livings. Antioxidants such as α-tocopherol or Vitamin E are the molecules that accelerated termination by attaching free radicals to protect cell membrane. Other antioxidants that are formed in body are superoxide dismutase, catalase and peroxidase.

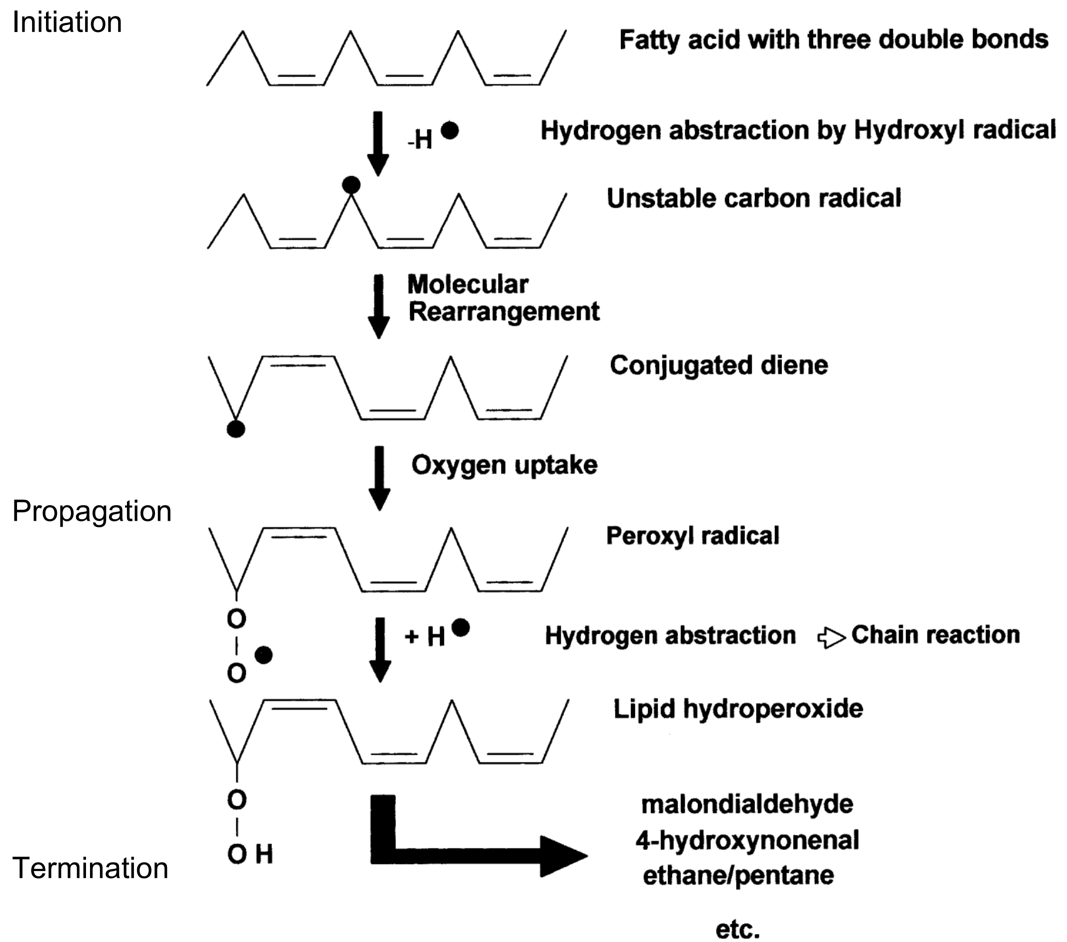


Figure 2.2 Lipid peroxidation process

Hemin

Hemin is porphyrin that contains iron. It is chloride crystal of heme with red brown color. The structure formula is $C_{34}H_{32}N_4O_4$ FeCl. When hemoglobin reacts with glacial acetic acid and sodium chloride, hemin is occurred.

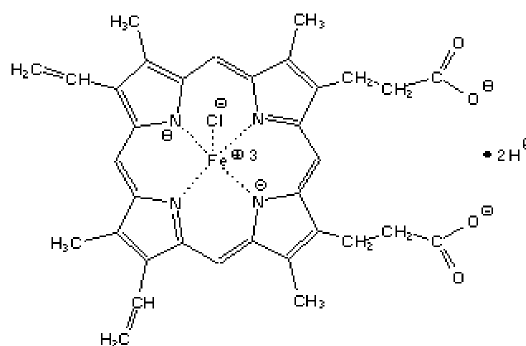


Figure 2.3 Structure of hemin

Hemin is the product of hemoglobin degradation. It is ferric oxidation product of heme. It is an effective inducer in LDL oxidation in the *in vitro*. At low concentration, hemin serves as oxidant, but at high concentration it becomes antioxidant.

The analysis of hemin in the circulation found that 80% of total hemin bound with LDL and HDL initially. Then some of hemin will bind with antioxidants, albumin and hemopexin. Half life of hemin-LDL complex in plasma (initially 27% of total hemin) is more than 20 seconds. So the researcher proposed that hemin-LDL complex may be exist *in vivo* and their oxidation lead to atherosclerosis (pro-atherogenic) (Miller, 1999)

The relationship between hemin *in vivo* and pro-oxidant activity was shown in β-thalassemia/hemoglobin E which is the type that often found in Thai. There was a study about typing and amount of high spin ferric heme or hemin (iron (III)-protoporphyrin IX) by electron spin resonance spectroscopy technique at low temperature in serum of β-thalassemia/hemoglobin E. The level of hemin found between 50 to 280 μm, while hemin was absent in normal subject. Higher level of hemin was probably the cause of

oxidative stress in these patients. Hemin in serum immediately accelerates reaction of free radical, and may accelerate important oxidation reaction in blood of β-thalassemia/hemoglobin E, including lipoprotein oxidation. Because of molecular movement of lipid in lipoprotein was an important factor in metabolism and reactions of lipoprotein, the measurement in molecular movement of lipid in lipoprotein that isolated from plasma with hemin was determined. The reduction of molecular movement of lipid both at the outer surface and at the core of lipoprotein was revealed. The results accorded to reduction of alpha-tocopherol. Thus hemin was suggested to promote atherosclerosis in β-thalassemia/hemoglobin E by altering molecular movement of lipid in lipoprotein (Phumala, et al., 2003).

Centella

Figure 2.4 *Centella asiatica*

The Scientific name of Centella is *Centella asiatica* (Linn.) Urban or *Hydrocotyle asiatica* and is in Umbelliferae Family. English name is Asiatic pennywort. There are many Thai local names including Bua-bok, Pak-wan, Pak-Hnok, and Pa-hna.

Centella asiatica has been found and easily harvested in almost all over the world Centella creeps on the ground with thin stem. The leaves are green and round and the flowers are pink to red. Centella fully grows in three months and well grows in tropic and flood area. It was found in Thai, India, Srilanka, Madacasscar, Africa, Australia, China and South America. Centella has been served as food and beverages for long time in many countries.

Using of Centella in medicine (Ayuravej therapy) has been firstly found in India and then distributed to all Asia and Europe. An Indian system of medicine has used Centella in brain disease, skin disease and digestive system. In addition, many studies reported the used of *C. asiatica* in various diseases including bronchitis asthma, kidney disease, urinary tract infection. It is weakly brain tonics, antianxiety, stimulate blood circulation and diuretic (Piyawan, et al., 2005).

Centella posseses many kinds of biological activities and studies about activities of Centella are the following :

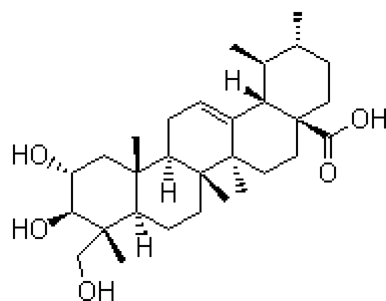
1. Antioxidative activity

It has been reported *that C. asiatica* improved memory in lack of intelligence child. Subathra (2005) found with regarding to its potent antioxidant that Centella protects neurons and brain from oxidation reaction. Centella has also anti-lipid peroxidative and free radical scavenging activities.

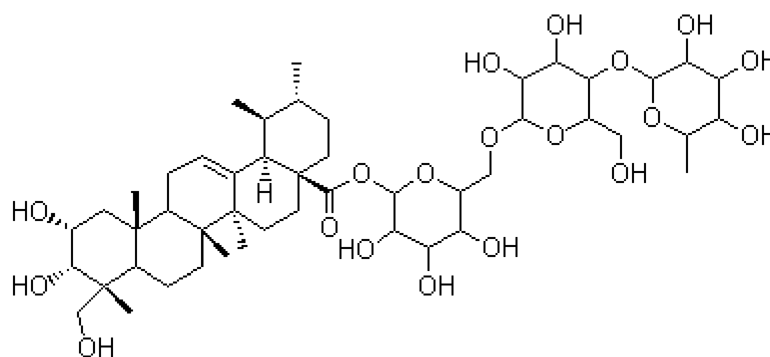
Active compounds found in Centella are polyphenols and triterpenes (Zainol, et al. 2003). Triterpenes such asiatic acid, asiaticoside are active compounds in Centella. Compounds that were found in many parts of Centella are phenolic. (Gnanapragasm, et al. 2004.) The study about antioxidative activity of Centella extracts using the ferric thiocyanate (FTC) method and thiobarbituric acid (TBA) test has been done. Total phenolic compounds were determined according to the Folin-Ciocalteu method. The results found that both leaf and roots of Centella had high antioxidant activity which was as good as that of α-tocopherol. Its antioxidative activity depends on ability to give hydrogen atoms to free radical (Zainol 2002).

In leaves, roots and petioles of Centella from different places had different antioxidative activity. Antioxidative activity from many parts of Centella was good as α-tocopherol (Zainol, 2003.)

They also found when lymphoma bearing mice were fed with crude methanol extract of *Centella asiatica* at dose 50 mg/kg/day for 14 days , there is significantly increase in antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx) and significant increasing of antioxidant such glutathione (GSH) and ascorbic acid both in liver and kidney. (It was known that reactive oxygen species (ROS) were the cause of lymphoma which affected to antioxidant enzymes and antioxidants). Reduce activity of SOD, catalase and GSHPx in liver, kidney may involve in inactivating these enzymes or reduce aerobic metabolism in cell lymphoma. Low level of antioxidants GSH and ascorbic acid may involve cell damage. Giving methanol extract of *C. asiatica* make antioxidant system to normal express anti-oxidant property to cell line of lymphoma bearing mice (Jayashree, et al., 2003)



(a) Asiatic acid ($C_{30}H_{48}O_{5}$; M.W.= 488.70)



(b) Asiaticoside ($C_{48}H_{78}O_{19}$; M.W.= 959.12)

Figure 2.5 Chemical structure of asiatic acid (a) and asiaticoside (b)

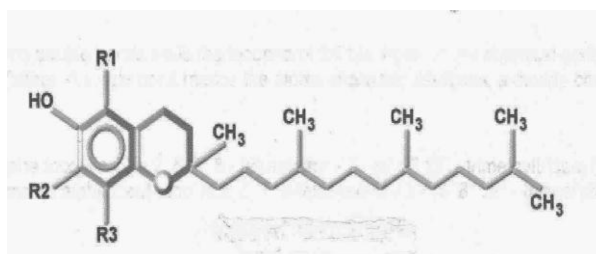


Figure 2.6 Chemical structure of α-tocopherol (R1=R2=R3=CH_3, M.W.=430.71)

There is one study about protective cardio effect of *Centella asiatica* on myocardial markers enzymes and antioxidant enzymes in adriamycin induced cardiomyopathy in rats. The rats administered with adriamycin (2.5 mg/kg i.p.) to cause myocardial change was observed with the increase of serum marker enzymes –lactate dehydrogenase (LDH), creatine phosphokinase (CPK), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and significant changes in antioxidant enzymes -SOD, CAT, GPx, glutathione S-transferase(GST). Pre-co-treated with *Centella asiatica* extract (200 mg/kg, oral) significantly protected these alterations and maintained enzyme activities. (Gnanapragasam, et al., 2004)

2. Anti-inflammatory

Triterpenes found in Centella such asiaticoside, madecassic acid, asiatic acid has anti-inflammatory activity. (Vogel, 1990) When mice were induced gastric ulcer by acetic acid and were fed with aqueous extract of whole plant at dose 0.1 and 0.25 gram per kilogram and asiaticoside at dose 5 and 10 milligram per kilogram. It reduced inflammation by inhibit inducible nitric oxide synthase (NOS) (Guo, et al., 2004). Saponin from Centella can reduce inflammation and edema in rat which was induced ear edema by croton oil (Bombardelli, et al.,1992). Madecassoside in Centella can reduce skin inflammation from abnormal increase. (Lepetit J-C., 2005).

3. Skin diseases

Centella was shown to have wound healing property by increasing cellular proliferation and collagen synthesis at the wound site (Suguna et al, 1996). Some reports found when mice were fed with Centella extract at dose 100 milligram per kilogram per day, it can effect wound healing by accelerate outer skin layer formation and reduce wound size (Poizot and Daniele, 1978). Asiaticoside has wound repairing activity and fastened wound healing in mice (Rosen, et al., 1967; Shim, et al., 1996; Velasco and Romero, 1976.), rat (Boiteau and Batsimamanga, 1956; Kim, et al., 1998) and human (Theirs, et al., 1957)

In addition, Centella can be used for treatment of leprosy combine with steroid (Subathra 2005) and had weakly antibacterial and antiviral property (Piyawan, et al., 2005). It has been used to treat burn and wounds in France since 1880's. Drug formulary from hydrophilic Centella extract that contains 60% of asiaticoside was used to treat wound, excision, burn, scar, thicken skin and reduce abnormal tissue formation of liver and kidney (Wang, et al., 2005).

4. Stop bleeding

Aqueous extract of *Centella asiatica* can stop bleeding by reduced activated partial thromboplastin time and prothrombin time (Songsriphiphat, et al., 1968; Ravivongse, et al., 1988)

5. Gastric ulcer

Aqueous extract from whole plant (Cheng and Koo, 2000) and leaves at dose 250 mg/kg can treat gastric ulcer in mice which gastric ulcer was induced by stress and hydrochloric acid in ethanol.

When whole plant extract at dose 0.05, 0.1 and 0.25 g/kg and asiaticoside at dose 1, 5 and 10 mg/kg were given to mice which had acetic acid induced gastric ulcer, they found that Centella can treat gastric ulcer by reducing wound size, increasing amount of arteries in tissue, increasing numbers and distribution of cell at wound. It also increased expression of basic fibroblast growth factor and suppressed myeloperoxidase activity (Cheng, et al., 2004)

When mice were given Centella juice at dose 200 and 600 mg/kg two times a day for 5 days, it can treat gastric ulcer which induced by ethanol, aspirin, coldness and fundoplication. At dose 600 mg/kg of juice can increase release of gastric juice mucin and increase mucosal cell glycoproteins (Sairam, et al., 2001)

In vivo experiment also found that Centella extract (Madecassol) can treat gastric ulcer and duodenal ulcer. (Shin, et al., 1982; Cho, et al., 1981)

6. Vascular disease

When total triterpenic fraction of *Centella asiatica* (TTFCA) were given to venous hypertension patient, it can improve leg edema, pain, cramps and weakness. TTFCA can effect microcirculation of veins and permeable of capillary (capillary filtration rate ; CFR) The more effective dose is 180 mg/day (TTFCA 60 mg three times a day) for four weeks (De Sanctis, et al., 2001)

One study which evaluates TTFCA efficiency to control collagen synthesis in one year found increase of echogenicity of plaque at femur bone because plaque with low echogenicity found with cerebrovascular disease. This plaque has very low collagen. The active compound is deposit lipid or occult blood. In conclusion group that receive TTFCA oral tablet dose 60 milligram three times a day. There is the increase of echogenicity and homogenicity of plaque. (Incandela, et al., 2001)

7. Brain disease

In the past ten years, there were increasing of studies about effect of *Centella asiatica* to the brain *in vitro* and *in vivo*. (Pharmacy Newsletter, Sakonnakorn Hospital, 2007)

1) Nootropic effect, neuronal dendritic growth stimulating property

In the year 2005 (Rao, et al., 2005) and 2006 (Mohandas, et al. 2006), studies about nootropic effect of aqueous extract of *Centella asiatica* in 3 month old male Swiss albino mice and in rats reported the increase of acetylcholine esterase activity in hippocampus and increase in neuronal dendrites branching. The results showed that Centella had effect to neuronal growth in postnatal period and enhanced higher brain function in adolescent rats

2) Neuroprotective effect, prevent cognitive deficits and oxidative stress

There was the study to screen protective effect of asiatic acid derivatives in cultured cortical neurons from glutamate-induced excitotoxicity.(Lee, et al., 2000) They observed that three derivatives could reduce glutamate toxicity by slowly reduced

glutathione and glutathione peroxidase. Asiatic acid derivatives also significantly reduced excessive nitric oxide production induced by glutamate. Thus, these compounds had neuroprotective effect by increasing cellular mechanism to defense oxidation.

3) Nerve regeneration acceleration

In the year 2005 (Soumyanath, et al., 2005), one experiment found that administration of ethanol extract of *Centella asiatica* 100 μg/ml in human SH-SY5Y cells with nerve growth factor (NGF) caused neurite elongation but it was not presented with aqueous extract. They thought that it may be the effect of asiatic acid. Administration of ethanol extract of *Centella asiatica* 300-330 mg/kg/day in Sprague-Dawley rats could increase new axon production.

4) Anxiolytic properties

One study in the year 2006 (Wijeweera, et al., 2006) reported asiaticoside in Centella which extracted with hexane, ethyl acetate and methanol had anxiolytic property without side effect in rodents.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Chemicals

ECa233 was supported by Assoc. Prof. Ekarin Saifah and co-workers; Faculty of Pharmaceutical Sciences, Chulalongkorn University.

The chemicals were purchased from Sigma Chemical Co., U.S.A. as follow : α-tocopherol, bovine serum albumin (BSA), butylated hydroxytoluene (BHT), copper sulfate ($CuSO_4$), ethylenediaminetetraacetic acid-Na salt (Na_2EDTA), folin-Ciocalteu's phenol reagent, hemin, sodium bicarbonate, sodium carbonate, sodium dihydrogen phosphate, sodium dodecyl sulfate, sodium hydrogen phosphate, tetraethoxypropane (TEP), thiobarbituric acid (TBA).

Potassium bromide (KBr), sodium chloride (NaCl), sodium hydroxide (NaOH), tricholoroacetic acid (TCA) were purchased from Merck Germany. Acetonitrile, butanol, ethanol, glacial acetic acid, hexane, isopropanol, methanol were purchased from Lab Scan Co., Ltd Thailand. Agarose and coomassie blue were purchased from Bio-Rad Laboratories, Inc., Spain.

1.2 Instruments

Ultracentrifugation (Beckman, German), Centrifuge (model H-103N, Kokusan, Japan), UV-VIS spectrophotometer (model 160A, Shimadzu, Japan), Spectrofluorometer (model FP-777, Jasco, Japan), HPLC class LC 10 (Shimadzu, Japan), D_2 Lamp (Shimadzu, Japan), Gel electrophoresis power supplied (model AE8130, ATTO, Japan), pH meter (Model CG842, Schott, German), Water bath Shaker (model MM-10, Taitec, Japan).

2. Methods

2.1 Preparation of plasma

Blood was collected from healthy volunteer age between 20-40 years. Additionally, each volunteer was free of any herbal or prescribed medication prior to participation for at least 2 weeks. The protocol of this *in vitro* study has been approved by The Ethics Committee of The Faculty of Pharmaceutical Sciences, Chulalongkorn University. All subjects gave written informed consent. Blood was collected from overnight fasting subject for 30 ml in EDTA (1mg/ml). Plasma was separated by centrifugation at 3,250 rpm, 4ºC for 15 minutes, then pooled and stored at -80ºC until used.

2.2 Preparation of salt for lipoprotein separation

LDL was separated from plasma using the sequential density ultracentrifugation modified from Havel method in fixed angle rotor (Beckman 90Ti) at 50,000 rpm, 4ºC (Havel, et al.,1955). The plasma was centrifuged sequentially at densities of 1.019 and 1.063 to separate lipoproteins. The first fraction (d<1.019) contained chylomicrons and VLDL, the second fraction (1.019<d<1.063) contained LDL. The plasma was adjusted to the desired density by the addition of concentrated salt solution –Mock solution and Stock solution. The initial preparation of two stock solutions from which the other solutions may be prepared.

1) Mock solution with the same background salt density as plasma (containing 1.142 g of NaCl, and 10 mg Na_2EDTA per 100 ml; density 1.0063 g/ml)

2) Concentrated stock salt solution (containing 35.4 g of KBr, 15.3 g of NaCl, and 10 mg of Na_2EDTA; density 1.346 g/ml)

From both solutions the required salt solutions will be prepared according to a formula:

$$A*Y + B*Z = (A+B) X$$

Where X is the desired density of the mixture, A and B were the volume of mock and stock solution, Y and Z are their densities, respectively.

LDL can keep at -80 ºC for one month. Before use in the experiment, LDL in dialysis membrane was dialyzed against 10 mM PBS (Phosphate buffer saline) pH 7.4 at 4 ºC overnight to remove salts.

2.3 Preparation of hemin

The stock solution of hemin was prepared by dissolving hemin in 0.1 M NaOH, then adjusted pH to 8 with 10 mM PBS and protect from light. Hemin solution was centrifuged at 3,500 rpm for 10 minutes. Supernatant was collected and detected for UV absorbance at wavelength 385 nm. Then hemin concentration was calculated by using molar coefficient (ε) = 58.4 mmol $L^{-1}cm^{-1}$ The solution can be kept at 4ºC for one week.

2.4 Determination of Protein content (Markwell et al, 1978)

Protein concentration was determined by modified Lowry method using BSA as a standard. BSA Standard was prepared at various concentrations of 50, 100, 200, 400 and 800 µg/ml. Working solution contain 100 parts of Reagent A (2 g of Na_2CO3, 1 g of SDS, 0.16 g of sodium potassium tartrate in 0.4% NaOH) and 1 part of 4% $CuSO_{4.}$ Working solution 1.5 ml was added to 100 µl of LDL samples, standard albumin or blank. The solutions were left for ten minutes, before 50% Folin & Ciocalteu 's phenol reagent 150 µl was added. After 45 minutes, tested solutions were measured the absorbance at wavelength 660 nm with UV-visible spectrophotometer.

2.5 Preparation of ECa233, asiatic acid and α-tocopherol

ECa233 (2 mg/ml), asiatic acid (2mg/ml) and α-tocopherol (10mg/ml) was dissolved in methanol and kept at -20ºC. The calculated volume of each test compound was pipette into incubation-tube in the final concentrations of 40 and 80 µg/ml for ECa233, 40 µg/ml (81.8 µM) and 80 µg/ml (163.6 µM) for asiatic acid, and 80 µg/ml (186 µM) for α-tocopherol (a positive control). Each test compound was dried under nitrogen gas to remove methanol before adding LDL.

2.6 Oxidation reaction of hemin-induced Low density lipoprotein

The *in vitro* LDL oxidation induced by hemin was performed in this study. First, ECa233 or asiatic acid (20, 40, 80 µg/ml) was dried under nitrogen gas, The pre-incubation of LDL 300 µg protein/ml with tested compound was performed at 37ºC for 30 minutes. α-Tocopherol 80 µg/ml (186 µM) was used as a positive control. Then, LDL samples were aliquoted (time 0) before adding 5 µM of hemin. LDL were furthered incubated at 37ºC for 24 hours and then were stopped reaction with 5µM EDTA and 50 µM BHT. Lipid peroxidation products (TBARS), α-tocopherol and REM were measured at time 0, 6 and 24 hours of incubation.

2.7 Lipid peroxidation measurement with TBARS (Asakawa & Matsushita, 1980)

Standard TEP in various concentrations of 0.1, 0.2, 0.4, 0.8, 1.2, 1.6 nmol/ml were prepared and purified water was used as a blank. 25 µl of 100 mM BHT was added into LDL samples, standard and blank 0.5 ml. Then 10% TCA 0.5 ml, 5mM EDTA 0.25 ml, 8%.SDS 0.25 ml, 0.6% TBA 0.75 ml were added in order. The solutions were boiled in water bath at 100ºC for one hour. When the solutions were cooled to room temperature, butanol 2 ml was added and vortexed vigorously before centrifuged at 3,000 rpm for 15 min. Malondialdehyde levels in butanol layer were measured by spectrofluorometer at the excitation wavelength of 515 nm and the emission wavelength of 553 nm.

2.8 Determination of α-tocopherol (Zaspel and Csallany, 1983)

LDL samples 100 µl was added with 100 µl of 10 mM PBS, 500 µl of methanol (cool), and then α-tocopherol was extracted into 2.5 ml of hexane. Samples were centrifuged at 1,700 rpm, 4ºC for 5 min and hexane layer were dried under nitrogen gas. After that, they were redissolved with 200 µl of mobile phase (Acetonitrile: Isopropanol = 75:25). α-tocopherol was determined by reverse phase HPLC (hypersil BDS C18 column with the flow rate of mobile phase 1.2 ml/min, at 50ºC and absorbance were detected with UV detector at 292 nm).

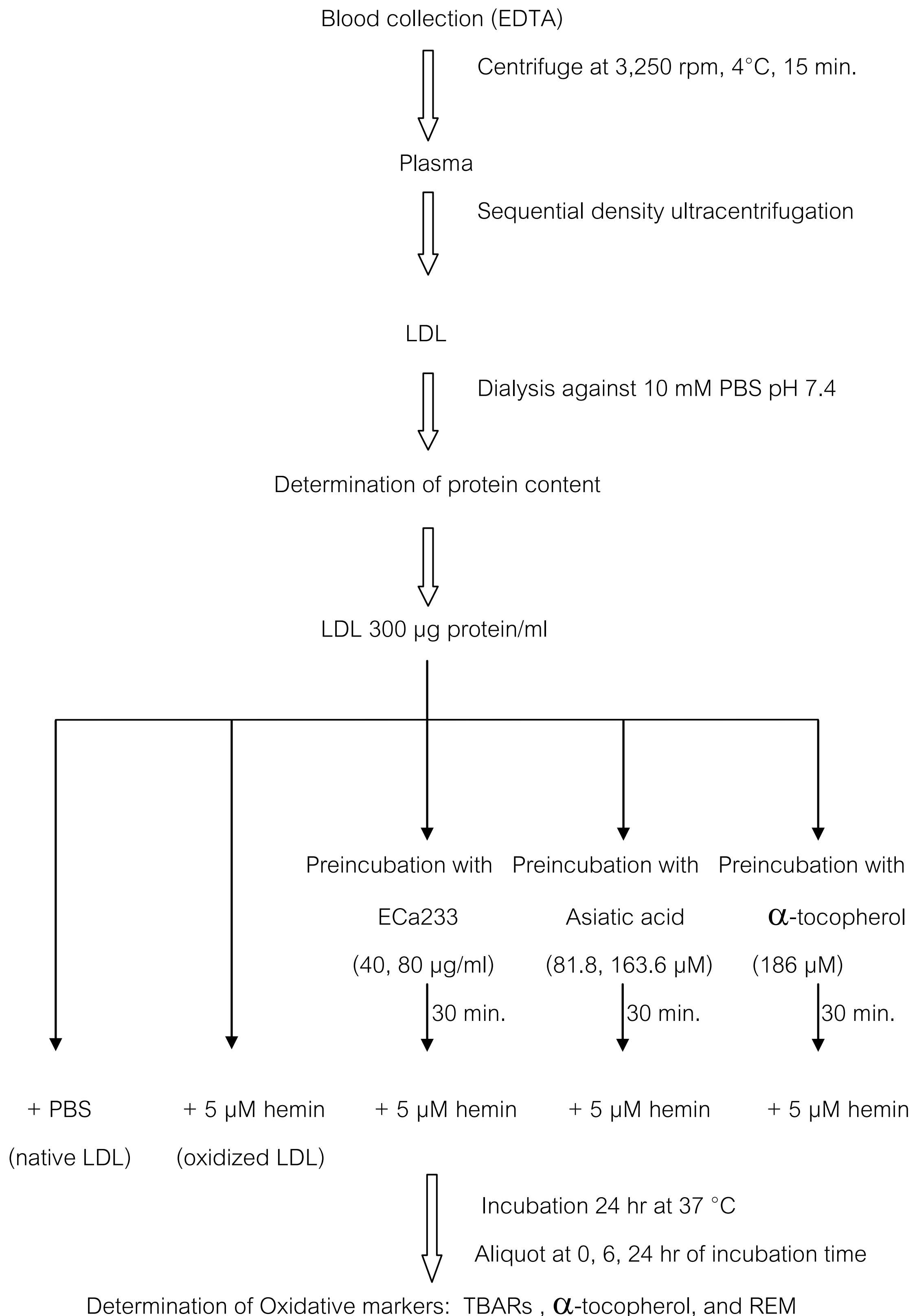
2.9 Determination of REM by gel electrophoresis (Bier, 1959)

Agarose gel (1%) was prepared in 60 mM barbital buffer pH 8.6. Gel was placed in the chamber contained with barbital buffer. Then 12 µl of sample was mixed with 2 µl of dye (glacial acetic acid + bromophenol blue) and pipetted to each well. Gel electrophoresis was run with power supply at 70 Volt 400 mAMP for 45 min. After that agarose gel was put into 20% TCA and washed out with washing solution (10% glacial acetic acid + 25% Methanol + 65% H_2O). Gel was colored with Coomassie blue and then destained with washing solution. Distances that oxidized LDL samples move from the origin relative to native LDL were measured.

3. Statistical Analysis

Data analysis was expressed in mean $\pm$ standard deviation. One way analysis of variance (ANOVA) was used to test the significant differences among groups and Student's independent t test was used to test the significant differences from he-oxLDL groups. Levene's test for equality of variences was used before test the equality of means. Differences were statistically significant at 95% confidence interval (p-value less than 0.05).

Study the effect of standardized extract of *Centella asiatica* ECa233, asiatic acid. and α-tocopherol on LDL oxidation



CHAPTER IV

RESULTS

1. The effect of hemin on LDL oxidation

1.1 TBARs formation

TBARs is the marker that determines final products of lipid peroxidation. The results showed that the TBARs values in nLDL were not significantly changed between 0 and 24 hr (3.29 $\pm$ 1.65 *vs.* 2.64 $\pm$ 0.76 nmol/mg protein) when incubated at 37°C for 24 hr. When LDL was incubated with 5µm of hemin, TBARs levels were significantly increased from 3.27 $\pm$ 1.59 nmol/mg protein to 17.27 $\pm$ 7.05 nmol/mg protein (Figure 4.1). These indicated that hemin can induce lipid peroxidation in LDL.

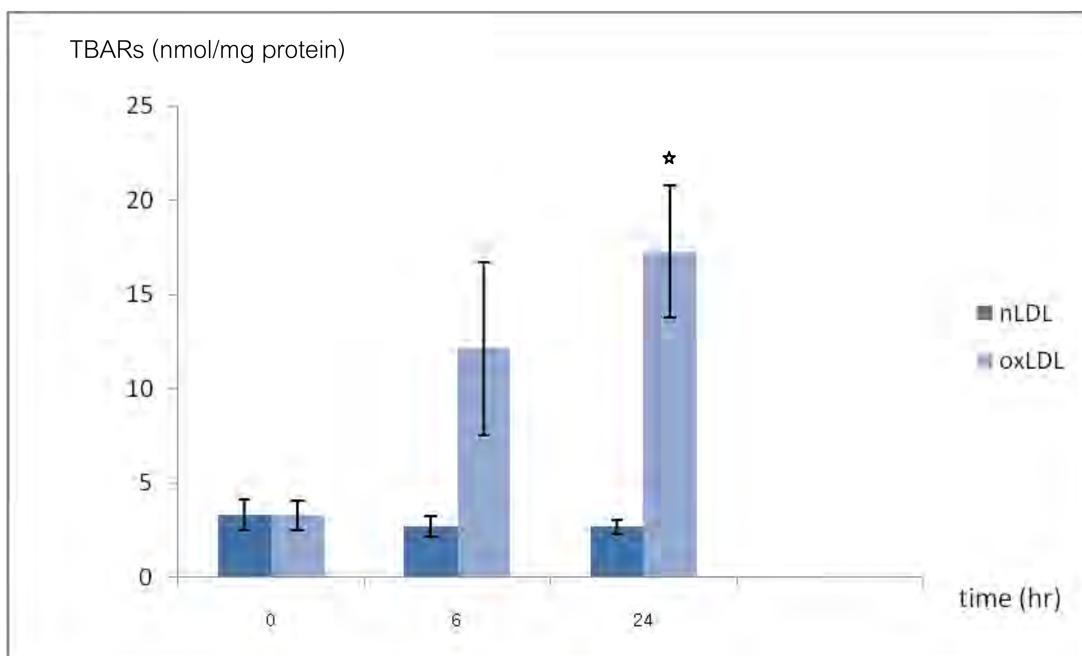


Figure 4.1 Effect of 5µM hemin on TBARs formation in LDL oxidation. (N=4)

⭑ $p<0.05$ significantly different compared to the nLDL control.

1.2 α-Tocopherol levels

α-Tocopherol is an important antioxidant for protection of oxidation in LDL. In nLDL, α-tocopherol levels were 34.90 ± 18.45, 34.01 ± 16.98 and 18.63 ± 23.65 nmol/mg protein at 0, 6 and 24 hr respectively. When incubation the LDL with 5 μM of hemin for 24 hr, α-tocopherol levels in oxLDL were decreased from 32.25 ± 14.54 nmol/mg protein to 8.46 ± 5.91 and 2.34 ± 4.67 nmol/mg protein at 6 and 24 hr, respectively (Figure 4.2). The results indicated that α-tocopherol was consumed to protect oxidation induced by hemin.

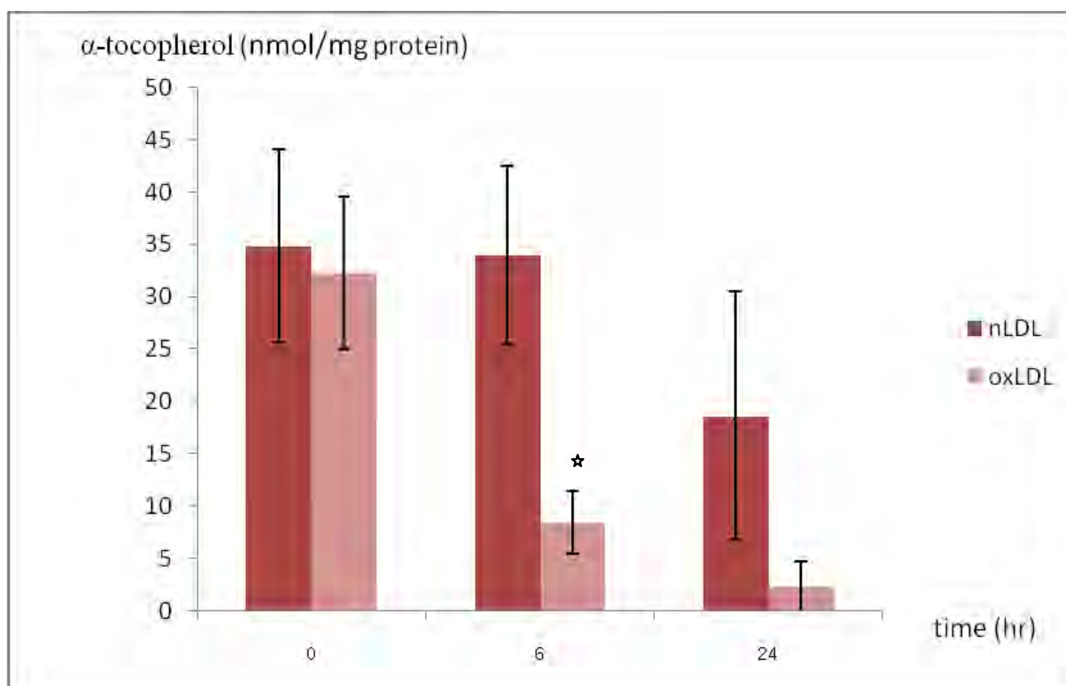


Figure 4.2 Effect of 5μM hemin on α-tocopherol levels in LDL oxidation (N=4), * $p<0.05$ significantly different compared to the nLDL control.

There is a reverse relationship between TBARs levels and α-tocopherol levels with the r value of -0.629 (p = 0.003) as shown in figure 4.3. When α-tocopherol level was rapidly decreased, TBARs level was increased.

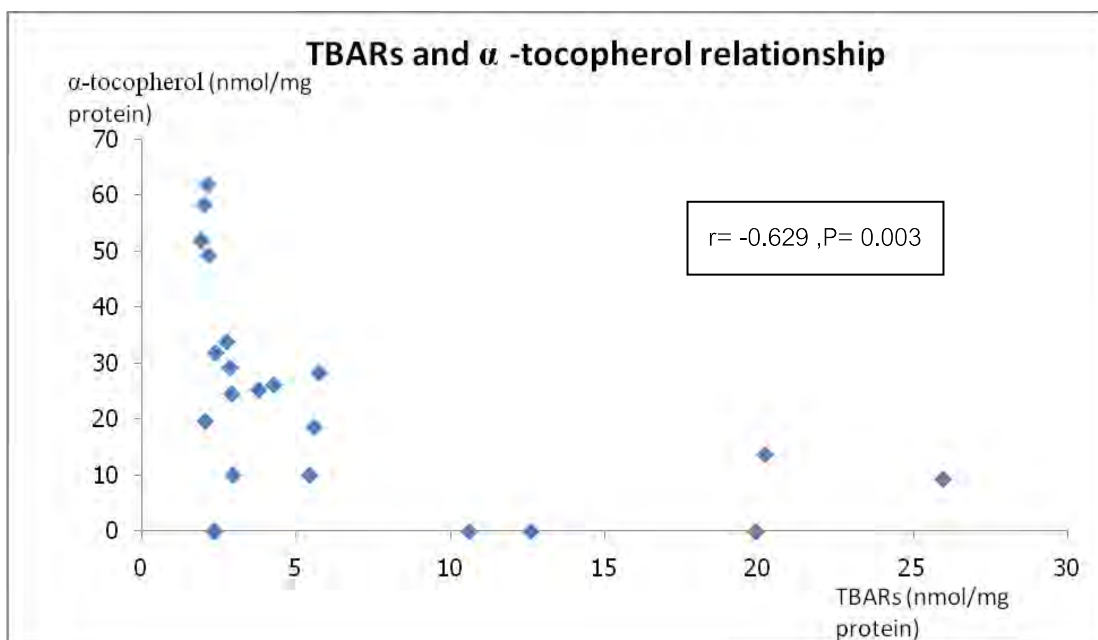


Figure 4.3 Relationship between TBARs levels and α-tocopherol levels in LDL incubated with 5 μM of hemin for 24 hr (N=4)

1.3 Relative Electrophoretic Mobility (REM)

REM is another marker that involves in oxidative reaction of protein. It is the distance which oxLDL moves relatively to that of nLDL. After incubation of LDL with 5µm of hemin at 37 °C for 24 hr, the results showed that REM values were significantly increased from 1.04 to 1.36 and 1.30 at 6 hr and 24 hr, respectively (Figure 4.4). These indicated that hemin can induce protein oxidation in LDL.

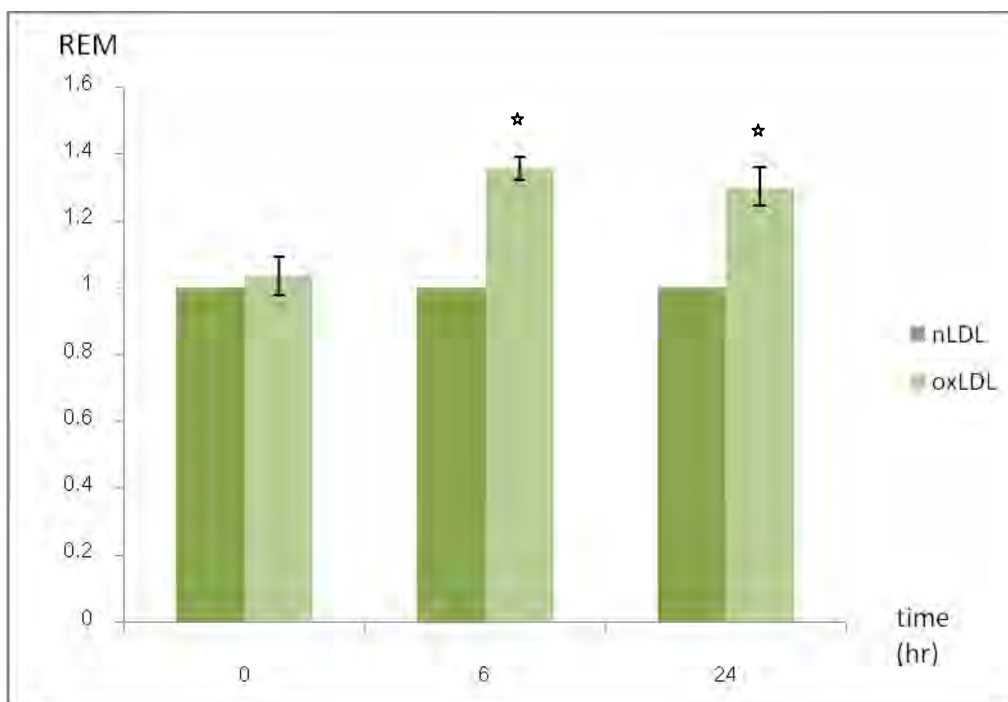


Figure 4.4 Effect of 5µM hemin on REM in LDL oxidation (N=4) ⋆$p<0.05$ significantly different compared to the nLDL control

2. The effect of ECa233, asiatic acid and α-tocopherol on hemin induced LDL oxidation

2.1 TBARs

Figure 4.5 and 4.6 showed TBARs levels of native LDL (nLDL), and oxidized LDL (oxLDL) when pre-incubation with tested compounds: ECa233 (CA), asiatic acid (AA), and α-tocopherol (α-TC) as a positive control at incubation time 0, 6 and 24 hr. The results indicated that TBARs levels were increased at 6 and 24 hr. The percentage of inhibition of TBARs formation at 6 and 24 hr was shown in figure 4.7. Both ECa233 and asiatic acid can decrease the TBARs formation at 6 hours of incubation. The percentage of inhibition of α-tocopherol was approximately 90% while those of both test compounds were 70-75%. Since the percent inhibition of both compound were not significant different from that of α-tocopherol. This results suggested that the protective effect of ECa233 and asiatic acid on lipid peroxidation are comparable to α-tocopherol control (figure 4.7 a). In addition, the percentage of inhibition of TBARs formation at 6 hr of ECa233, asiatic acid, and α-tocopherol were more than those at 24 hr of incubation. The results showed that ECa233 and asiatic acid can protect lipid peroxidation at least for 6 hours but slightly protect the lipid peroxidation in the *in vitro* LDL oxidation induced by 24 hour- incubation of hemin.

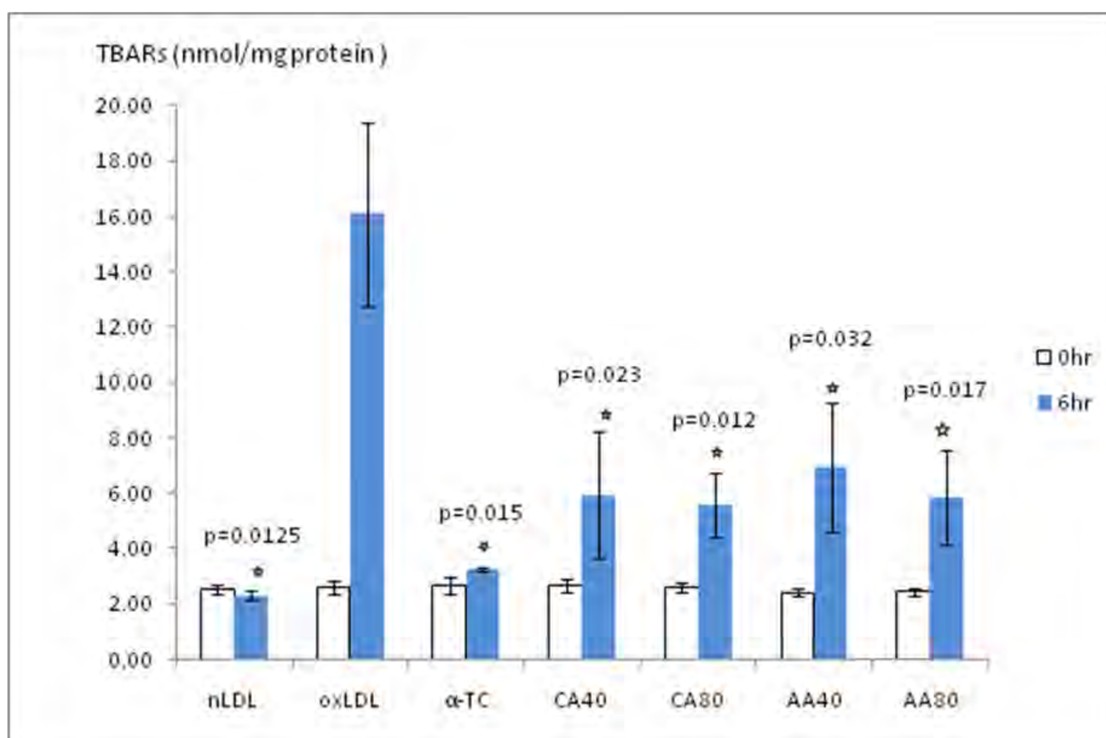


Figure 4.5 Effect of α -tocopherol, ECa233, and asiatic acid on TBARs levels of oxLDL at 6 hr of incubation with hemin 5 μM.

* p<0.05 significant different from oxLDL

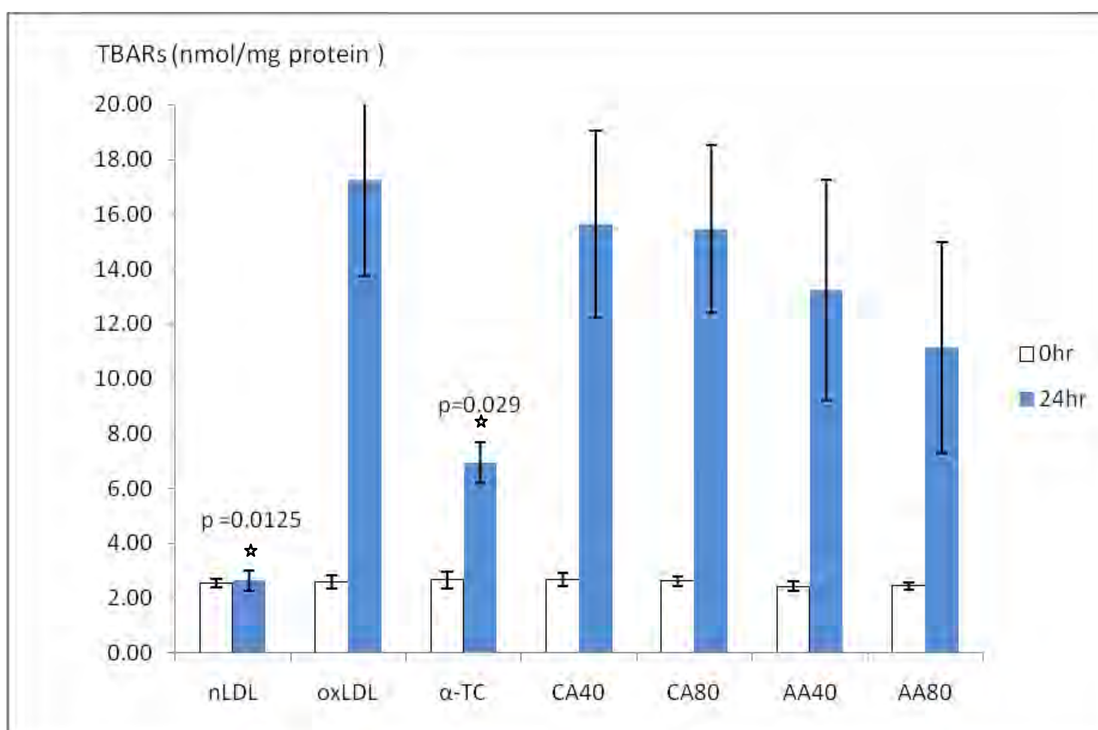
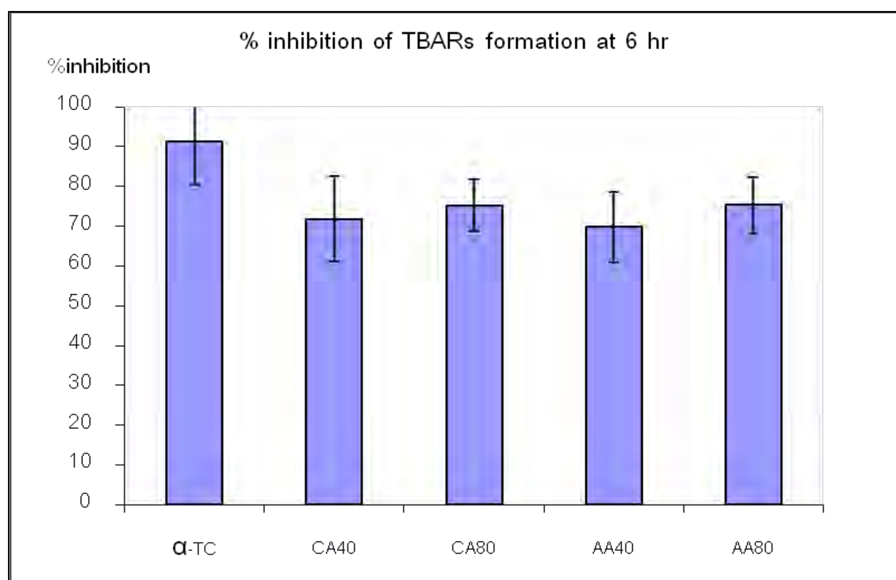


Figure 4.6 Effect of α -tocopherol, ECa233, and asiatic acid on TBARs levels of oxLDL at 0 and 24 hr of incubation with hemin 5 μM. *p<0.05 significantly different compared to the oxLDL.

(a)



(b)

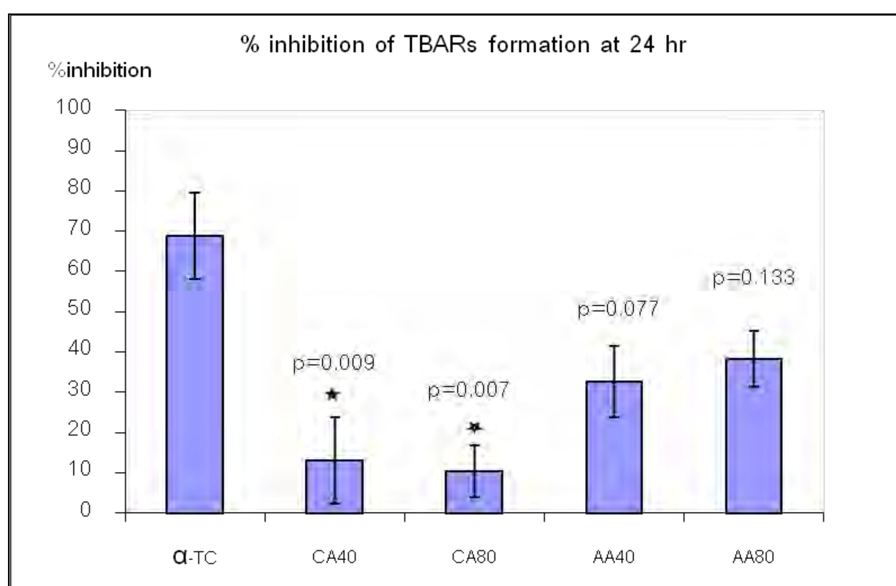


Figure 4.7 The percent inhibition of α-tocopherol, ECa233, and asiatic acid on TBARs formation at 6 hr (a) and 24 hr (b) of incubation with hemin 5 μM. * $p<0.05$ significantly different compared to the α-TC.

2.2 α -tocopherol level

α -tocopherol level in oxLDL was rapidly decreased until 6 hr and further decreased until undetectable at 24 hr of incubation (Figure 4.8). However, α -tocopherol cannot preserved when incubated with ECa233 (CA 40 - 80 μg/ml) and asiatic acid (AA 81.8-163.6 μM). Since in the tube that α –tocopherol (186 μM) was added, α –tocopherol level was very high (Table 1). These results showed that *Centella asiatica*, asiatic acid and α -tocopherol did not protect the decrease of α -tocopherol in LDL oxidation.

The percent decrease from baseline of ECa233, and asiatic acid at 6 and 24 hr of incubation was shown in figure 4.9. α-tocopherol levels was rapidly decreased (about 80-90%) in both ECa233 (40-80 μg/ml) and asiatic acid (81.8-163.6 μM) at 6 hours and further decreased until 24 hr of incubation (about 95%).

2.3 REM value

Figure 4.10 (a – b) showed gel picture of gel electrophoresis at 0, 6 and 24 hr of incubation The value of REM in oxLDL was increased at 24 hr.(figure 4.11). However REM values were lower in ECa233 (CA 40-80 μg/ml), α-tocopherol (186 μM) and asiatic acid (AA 81.8-163.6 μM). The results indicated that only α-tocopherol had a potential to protect the increase of REM in LDL oxidation while ECa233 and asiatic acid cannot protect the protein oxidation.

Table 1. The effect ECa233, asiatic acid and α-tocopherol on α-tocopherol levels at 0, 6, 24 hr of incubation. Values were presented as mean±S.D. (N=4)

Tested compounds	α-tocopherol levels (nmol/mg protein)		
	Time of incubation		
	0 hr	6 hr	24 hr
nLDL	38.90 ± 15.78	34.01 ± 16.98	26.33 ± 20.34
oxLDL	32.25 ± 14.54	5.95 ± 7.04 *	2.34 ± 4.67 *
α-TC	180.467 ± 91.77 *	272.20 ± 273.08 *	105.69 ± 152.45
CA 40	32.46 ± 27.38	3.98 ± 7.96 *	2.22 ± 4.44 *
CA 80	34.42 ± 15.58	4.45 ± 8.89*	2.69 ± 5.38 *
AA 40	36.79 ± 21.37	5.84 ± 11.69 *	3.73 ± 7.46 *
3AA 80	32.63 ± 14.83	7.62 ± 8.89 *	2.63 ± 5.27 *

*$p<0.05$ significantly different compared to nLDL, (student's t test)

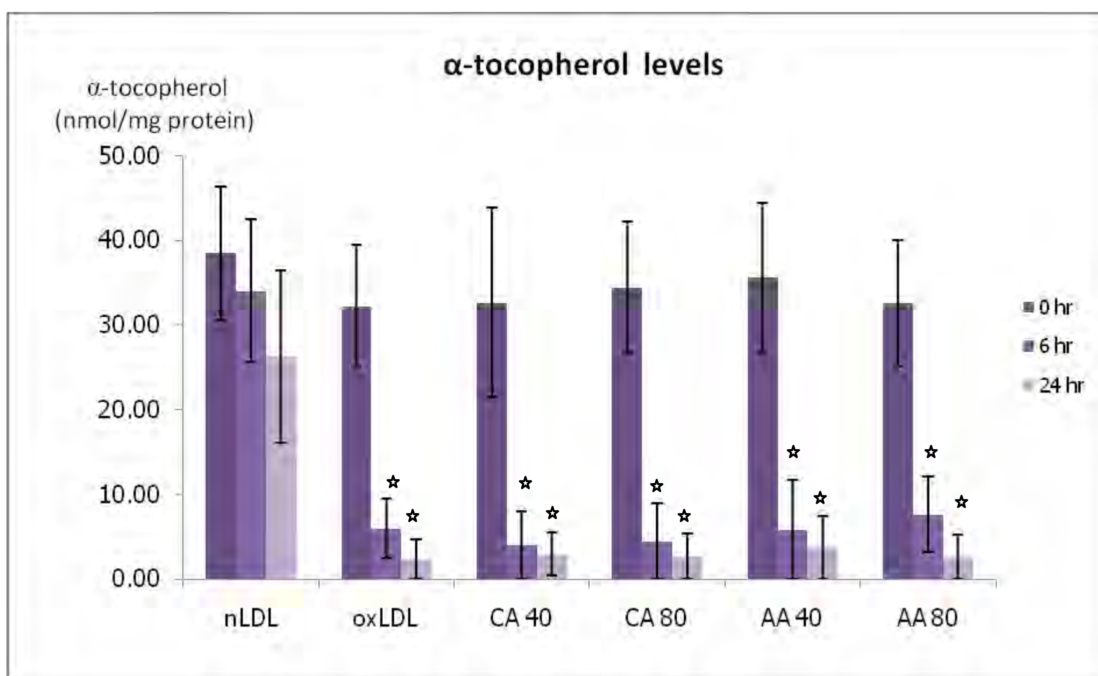


Figure 4.8. α-tocopherol levels in hemin- induced LDL oxidation when pre-incubated with ECa233, and asiatic acid at 0, 6 and 24 hr of incubation (N=4).

☆ $p<0.05$ significantly different compared to nLDL at same time of incubation.

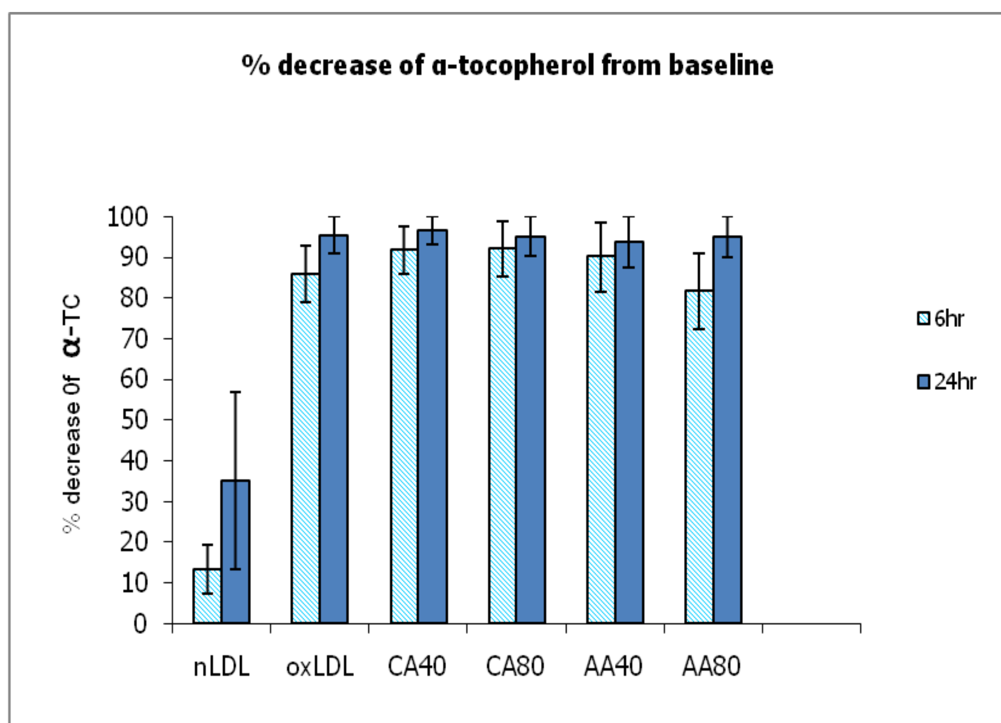
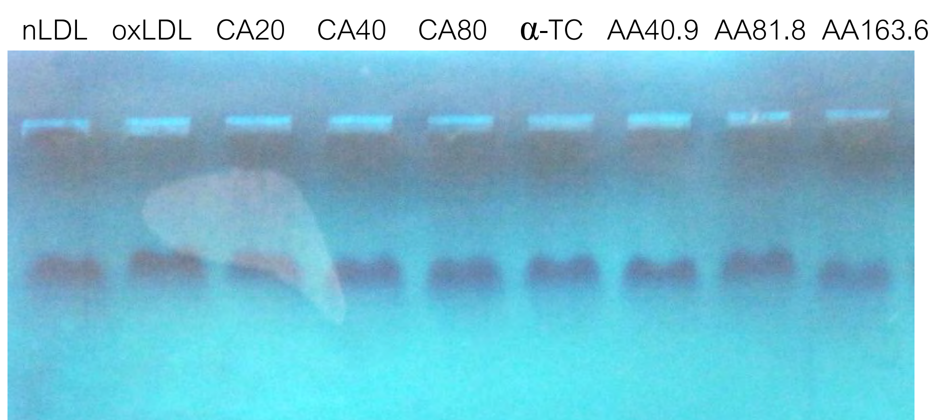


Figure 4.9 The percent decrease from baseline of α-tocopherol levels in hemin- induced LDL oxidation pre incubation with ECa233, and asiatic acid at 6 and 24 hr of incubation. (N=4)

a) 0 hr



b) 24 hr

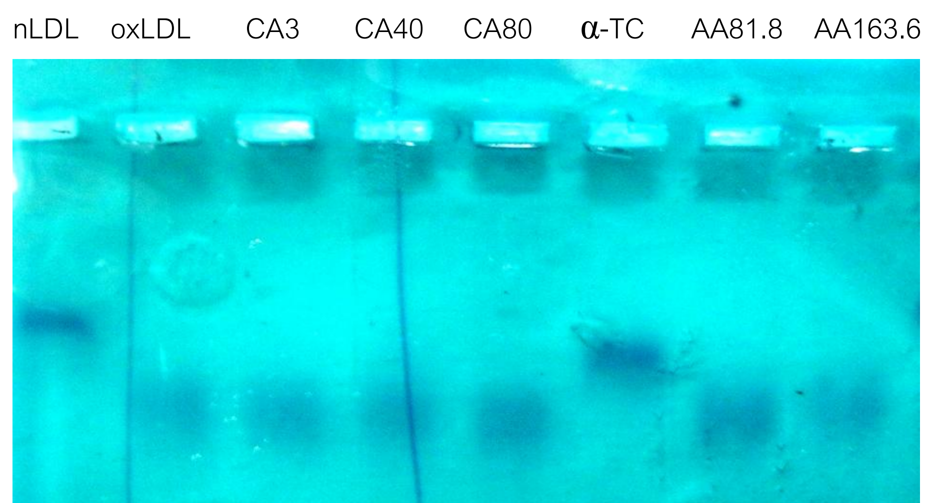


Figure 4.10 gel electrophoresis of he-oxLDL at 0 and 24 hr of incubation

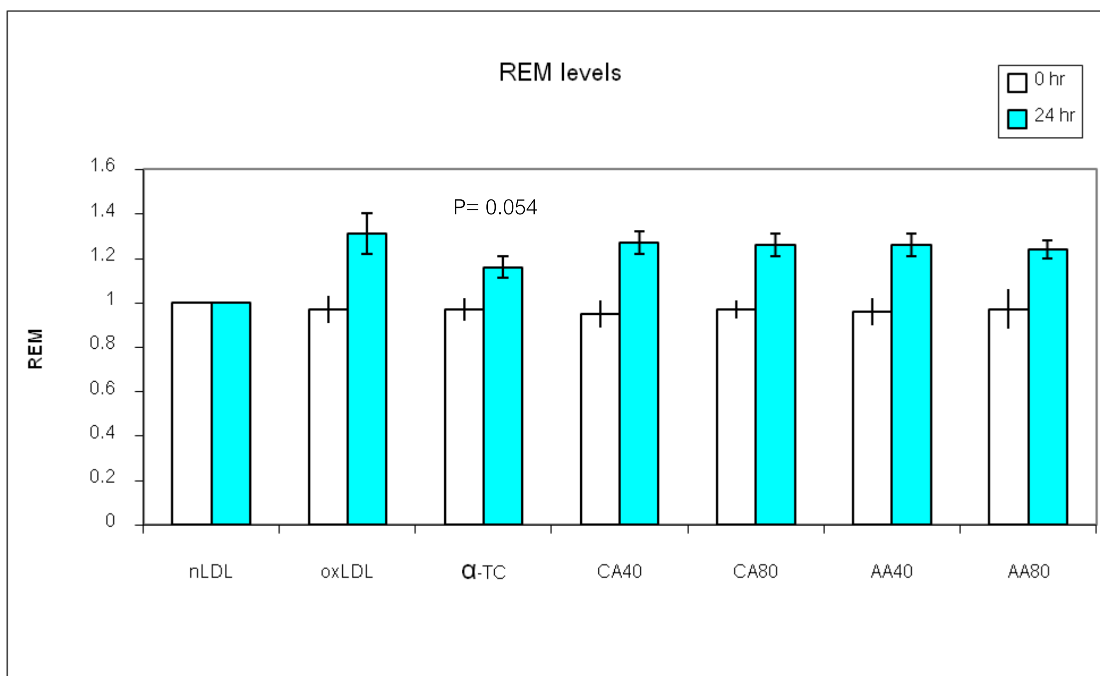


Figure 4.11 REM levels of he-oxLDL pre-incubated with α -tocopherol, ECa233, and asiatic acid at 0 and 24 hr of incubation with hemin 5 μM. Significantly different compared to the oxLDL.

CHAPTER V

DISCUSSION AND CONCLUSION

The oxidative state of oxidized LDL was evaluated by measurement the TBARs formation which indicates the lipid peroxidation. Besides the lipid peroxidation, the degree of protein modification was also evaluated by determining the REM of LDL by using gel electrophoresis, The level of α –tocopherol in LDL was also measured to evaluate lipid peroxidation in hemin oxidized LDL.

The results found that TBARs formation, the final product of lipid peroxidation was increased, according to depletion of α -tocopherol levels, indicating that hemin is a powerful in vitro inducer of oxidation in low density lipoprotein. The consequent was the modification of LDL and apolipoprotein that lead to have more negative charge. Then REM in gel electrophoresis was increased. The results demonstrated that hemin can induce oxidation in LDL. The increase of TBARs formation and the increase of REM in he-oxLDL indicate the elevation of lipid peroxidation and protein oxidation, respectively.

Hemin has been detectable in β-thalassemia/hemoglobin E disease (β-thal/Hb E). Chemiluminescence is a method for screening of free radical generation *in vitro* and its intensity (CL) was about 20 fold enhanced in serum of β-thal/Hb E, indicating its extensive pro-oxidant activity and the CL showed a good correlation with serum hemin. The authors suggested that serum hemin readily catalyzed free radical reactions and it may contribute a major pro-oxidant in blood circulation of β-thal/Hb E (Phumala N, et al. 2003). The correlation between endogenous hemin and pro-oxidant activity in serum of β-thal/Hb E was also found by using the technique of low temperature electron high spin resonance spectroscopy to characterize and quantify high spin ferric heme or hemin. In addition, the specific site of oxidative damage in lipoprotein can be identified that hydrophobic region at the core of thalassemic lipoprotein was a target site, due to the hydrophobic property of hemin (Morales NP, 2006). In the core of LDL contained mainly cholesteryl esters which are susceptible to lipid peroxidation.

The effect of ECa233 on lipoprotein oxidation showed that ECa233 and asiatic acid have an antioxidative activity (Figure 4.7) by protecting lipid peroxidation in LDL that induced by hemin (he-oxLDL). Though the potency of protective effect on TBARs formation and depleting of α-tocopherol was not in the dose dependent manner, ECa 233 in the concentration of 40 and 80 μg/ml was able to protect lipid peroxidation at least 70% of inhibition when incubation for 6 hours. However, only 15-20% of inhibition was detected at 24 hours indicating that the protection cannot prolong to 24 hours (Figure 4.7). In addition, this study found that the anti-lipid peroxidation of ECa 233 and asiatic acid was comparable to α-tocopherol, a positive control, but the duration of protective effect was shorter than that of α-tocopherol.

Lipid peroxidation is one outcome of free radical-mediated tissue injury and is an indicator of oxidative damage. The study on the role of *Centella asiatica* in improving antioxidant status demonstrated that supplementation of *Centella asiatica* extract was effective in reducing lipid peroxidation in rat brain regions (Subathra, et al. 2005). MDA levels were significantly decreased in aged brain. The reduction of lipid peroxide may involve the electron and H^+ donating capacity of flavonoids in *Centella asiatica*, which sound to contribute termination of chain reaction. Many studies showed that flavonoids interact with cell membranes, improving fluidity, thus protecting them from lipid peroxidation.

Phenolic compounds quercetin and catechins in *Centella asiatica* have been reported to have antioxidant properties by scavenging reactive oxygen species, inhibition of free radicals production, and chain-breaking activity. They may be hydrogen donating by scavenging lipid alkoxyl and peroxyl radical. Treatment with *Centella asiatica* increase the level of α-tocopherol in aged rat that could possibly be attributable to polyphenolic compounds found in *C. asiatica*. Previous reports suggested that polyphenols may regenerate α-tocopherol by reducing α-tocopheroxyl radical (Subathra, et al. 2005). In contrast, the result of present study did not show the regeneration of α-tocopherol by ECa233 and asiatic acid.

Another study on the effect of *Centella asiatica* extract and powder on oxidative stress in rats showed that *C. asiatica* (extract and powder) may improve H_2O_2-induced oxidative damage by decreasing lipid peroxidation (Hussin, et al. 2007).

The present study showed that ECa233 and asiatic acid protected the lipid peroxidation of he-oxLDL to the same extent as that of α-tocopherol. In some studies the antioxidative activity of *C. asiatica* and the activity of leaf and root of *C. asiatica* had been reported to be as good as that of α-tocopherol (Zainol, et al. 2003). It was suggested that phenolic contents contributed to the antioxidative activities of *C. asiatica*. (Potential mechanism of this compound is the stability of the aroxy radical formed in the structure.) However, the duration of antioxidative action of ECa233, as shown in the present study, was not prolonged to 24 hours. Since percent inhibition of TBARs formation in he-oxLDL at 6 hour of ECa233 and asiatic acid were much more higher than those at 24 hour of incubation. In addition, the REM value that indicated the degree of protein oxidation could not reverse by either ECa233 or Asiatic acid and still continue to modify LDL. So according to the current knowledge and the result of present study, suggesting that the antioxidative property of ECa233 and asiatic acid may be due to hydrogen donating for scavenging free radicals, and then delay the lipid peroxidation for at least 6 hours. They did not regenerate α-tocopherol, may be act like a chain breaking antioxidant but less reactive than α-tocopherol.

In conclusion, ECa233 and asiatic acid can protect lipid peroxidation at least for 6 hours and protective effect on lipid at 24 hour was trivial. Further studies on the free radical scavenging properties of ECa233 should be done to gain insight into its antioxidative property.

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APPENDIX

Table 2 .The effect *Centella asiatica*, asiatic acid and α-tocopherol on TBARs formation at 0 hr of incubation time

condition	conc. (mcg/ml)	MDA (nmol/mg protein)				Mean	S.D.
		N1	N2	N3	N4		
nLDL	-	2.48	2.84	2.68	2.12	2.53	0.31
oxLDL	-	2.89	2.73	2.84	1.90	2.59	0.46
α−TC	80	2.53	2.29	3.54	2.24	2.65	0.61
CA	40	2.46	2.62	3.37	2.28	2.68	0.47
	80	2.45	2.78	2.99	2.24	2.62	0.33
AA	40	2.25	2.32	2.91	2.21	2.42	0.33
	80	2.27	2.36	2.83	2.33	2.44	0.26

Table 3 .The effect *Centella asiatica*, asiatic acid and α-tocopherol on TBARs formation at 6 hr of incubation time

condition	conc. (mcg/ml)	MDA (nmol/mg protein)				Mean	S.D.
		N1	N2	N3	N4		
nLDL	-	2.04	2.38	2.84	2.00	2.31	0.39
oxLDL	-	19.90	6.20	18.01	20.19	16.07	6.65
α-TC	80	3.24	3.07	3.21	3.40	3.23	0.14
CA	40	2.61	3.89	4.47	12.78	5.94	4.63
	80	4.88	3.60	4.84	8.92	5.56	2.32
AA	40	3.77	3.05	7.72	13.18	6.93	4.64
	80	3.23	3.00	10.04	7.14	5.85	3.38

Table 4 .The effect *Centella asiatica*, asiatic acid and α-tocopherol on TBARs formation at 24 hr of incubation time

condition	conc. (mcg/ml)	MDA (nmol/mg protein)				Mean	S.D.
		N1	N2	N3	N4		
nLDL	-	2.31	2.35	3.77	2.14	2.64	0.76
oxLDL	-	19.94	12.59	10. 3	25.97	17.28	7.04
α-TC	80	5.94	6.95	5.86	9.03	6.95	1.48
CA	40	19.6	9.92	9.75	23.2	15.64	6.85
	80	14.8	12.04	10.73	24.27	15.46	6.11
AA	40	15.65	4.59	9.51	23.23	13.25	8.05
	80	3.62	8.23	10.91	21.76	11.13	7.34

Table 5. The effect *Centella asiatica*, asiatic acid and α -tocopherol on % inhibition of TBARs at 6 hr of incubation

Tested compound	conc. (mcg/ml)	% inhibition of TBARs formation				Mean	S.D.
		N1	N2	N3	N4		
α-TC	80	93.28	81.94	97.56	92.30	91.27	6.63
CA	40	96.81	60.47	89.25	40.74	71.82	25.96
	80	84.10	68.06	86.81	61.96	75.23	12.11
AA	40	90.31	82.46	67.83	38.54	69.78	22.8
	80	93.34	83.77	52.53	71.74	75.34	17.59

Table 6. The effect *Centella asiatica*, asiatic acid and α -tocopherol on % inhibition of TBARs at 24 hr of incubation

Tested compound	conc. (mcg/ml)	% inhibition of TBARs formation				Mean	S.D.
		N1	N2	N3	N4		
α-TC	80	79.41	55.08	69.53	71.09	68.78	10.11
CA	40	1.93	26.07	12.83	11.62	13.11	9.9
	80	29.15	5.37	0.00	7.13	10.41	12.85
AA	40	24.33	78.13	16.33	11. 0	32.57	30.83
	80	92.57	42.58	0.00	17.67	38.20	40.23

Table 7. The effect *Centella asiatica*, and asiatic acid on α -tocopherol levels at 0 hr of incubation

condition	conc. (mcg/ml)	ปริมาณ α -tocopherol (nmol/mg protein) N1	N2	N3	N4	Mean	S.D.
nLDL	-	29.65	34.02	28.36	61.87	37.29	16.40
oxLDL	-	24.57	33.86	18.64	51.93	32.25	14.54
α-TC	80	176.90	68.35	293.03	183.59	180.47	91.76
CA	40	19.09	33.15	14.34	63.96	32.46	22.36
	80	25.23	35.56	20.98	55.9	34.42	15.58
AA	40	34.39	32.24	16.72	59.26	35.65	17.60
	80	25.99	32.90	18.564	53.07	32.63	14.83

Table 8. The effect *Centella asiatica*, α-tocopherol and asiatic acid on α-tocopherol levels at 6 hr of incubation

condition	conc. (mcg/ml)	ปริมาณ α -tocopherol (nmol/mg protein) N1	N2	N3	N4	Mean	S.D.
nLDL	-	19.67	32.60	26.14	58.35	34.01	16.97
oxLDL	-	0	10.037	0	13.780	5.95	7.04
α-TC	80	164.185	77.087	170.740	676.780	272.20	273.08
CA	40	0	0	0	15.930	3.98	7.97
	80	0	0	0	17.790	4.45	8.90
AA	40	0	0	0	23.380	5.85	11.60
	80	0	13.685	0	16.790	7.62	8.89

Table 9. The effect *Centella asiatica*, asiatic acid and α-tocopherol on α-tocopherol levels at 24 hr of Incubation

condition	conc. (mcg/ml)	ปริมาณ α -tocopherol (nmol/mg protein)				Mean	S.D.
		N1	N2	N3	N4		
nLDL	-	0	30.79	25.24	49.291	26.33	20.34
oxLDL	-	0	0	0	9.346	2.34	4.67
α-TC	80	0	0	99.303	323.469	105.69	152.54
CA	40	0	0	0	8.880	2.22	4.44
	80	0	0	0	10.756	2.69	5.38
AA	40	0	0	0	14.913	3.73	7.45
	80	0	0	0	10.53	2.63	5.27

Table 10. The effect *Centella asiatica*, asiatic acid and α-tocopherol on REM levels at 0 hr of incubation

condition	conc. (mcg/ml)	REM					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	1.00	1.00	1.00	1.00	1.00	1.00	0.00
oxLDL	-	1.04	1.00	1.00	0.92	0.91	0.97	0.06
α-TC	80	1.04	1.00	0.97	0.93	0.91	0.97	0.05
CA	40		1.00	0.97	0.95	0.86	0.95	0.06
	80		1.00	1.00	0.95	0.91	0.97	0.04
AA	40	1.04	1.00	0.94	0.90	0.91	0.96	0.06
	80	1.11	1.00	0.97	0.92	0.86	0.97	0.09

Table 11. The effect *Centella asiatica*, asiatic acid and α-tocopherol on REM levels at 24 hr of incubation

condition	conc. (mcg/ml)	REM					Mean	S.D.
		N1	N2	N3	N4	N5		
nLDL	-	1.00	1.00	1.00	1.00	1.00	1.00	0.00
oxLDL	-	1.28	1.37	1.42	1.19	1.30	1.31	0.09
α-TC	80	1.14	1.18	1.17	1.23	1.10	1.16	0.05
CA	40		1.29	1.28	1.31	1.20	1.27	0.05
	80		1.29	1.31	1.24	1.20	1.26	0.05
AA	40	1.21	1.32	1.28	1.20	1.30	1.26	0.05
	80	1.21	1.26	1.31	1.23	1.20	1.24	0.04

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