PROTECTIVE EFFECTS OF LUSIANTHRIDIN ON HEMIN-INDUCED LOW DENSITY
LIPOPROTEIN OXIDATION AND FOAM CELL FORMATION OF RAW 264.7 MACROPHAGE
CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ผลปกป้องของลูเซียนทรีดินต่อการออกซิเดชันของไลโปโปรตีนชนิดความหนาแน่นต่ำที่เหนี่ยวนำด้วย
ฮีมินและการก่อเกิดโฟมเซลล์ของแมโครฟาจชนิด *RAW 264.7*

น.ส.ซู วุทยิ ธันท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	PROTECTIVE EFFECTS OF LUSIANTHRIDIN ON HEMIN-INDUCED LOW DENSITY LIPOPROTEIN OXIDATION AND FOAM CELL FORMATION OF RAW 264.7 MACROPHAGE CELLS
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ผลปกป้องของลูเซียนทรีดินต่อการออกซิเดชันของไลโปโปรตีนชนิดความหนาแน่นต่ำที่เหนี่ยวนำด้วยฮีมินและการก่อเกิดโฟมเซลล์ของแมโครฟาจชนิด *RAW 264.7*. (PROTECTIVE EFFECTS OF LUSIANTHRIDIN ON HEMIN-INDUCED LOW DENSITY LIPOPROTEIN OXIDATION AND FOAM CELL FORMATION OF RAW 264.7 MACROPHAGE CELLS) อ.ที่ปรึกษาหลัก : รศ. ภญ. ดร.รัตยา ลือชาพุฒิพร, อ.ที่ปรึกษาร่วม : รศ. ดร.นพวรรณ ภู่มาลา มอราเลส,ดร.วิศรุต บูรณสัจจะ

การออกซิเดชันของไลโปโปรตีนชนิดความหนาแน่นต่ำ (แอลดีแอล, LDL) มีบทบาทสำคัญในการก่อให้เกิดโรคผนังหลอดเลือดแดงแข็งตัว ฮีมิน (iron(III)-protoporphyrin IX) เป็นสารจากการสลายตัวของฮีโมโกลบินและก่อให้เกิดภาวะเครียดออกซิเดชัน สามารถก่อให้เกิดภาวะแทรกซ้อนของหลอดเลือดในผู้ป่วยธาลัสซีเมียได้ ฮีมินถูกใช้เป็นตัวออกซิไดซ์สำหรับแอลดีแอลออกซิเดชัน ลูเซียนทรีดิน (LST) จาก *Dendrobium venustum* เป็นสารประกอบฟีโนลิกที่มีฤทธิ์ในการต้านอนุมูลอิสระ วัตถุประสงค์ของการศึกษานี้เพื่อประเมินผลของลูเซียนทรีดินต่อการป้องกันการเกิดออกซิเดชันของแอลดีแอลที่ถูกเหนี่ยวนำด้วยฮีมิน (he-oxLDL) และผลต่อการเกิดของโฟมเซลล์ในเซลล์แมโครฟาจชนิด RAW 264.7 เมื่อบ่มด้วย he-oxLDL ที่เติม LST การศึกษาดำเนินการโดยบ่มแอลดีแอลด้วย LST ที่ความเข้มข้นต่าง ๆ (0.25, 0.5, 1 และ 2 ไมโครโมลาร์) เป็นเวลา 30 นาที จากนั้นเติมฮีมินความเข้มข้น 5 ไมโครโมลาร์ เพื่อกระตุ้นการออกซิเดชัน และวัดการออกซิเดชันของไขมันในแอลดีแอลที่เวลาต่างๆ (0, 1, 3, 6, 12, 24 ชั่วโมง) ด้วยวิธีการวัดสารธิโอบาร์บิทูริกรีแอกทิฟ (TBARs) วัดการเคลื่อนที่บนอิเลกโทรโฟเรติก (REM) ส่วนประกอบของไขมันของแอลดีแอลวิเคราะห์โดย HPLC การก่อเกิดโฟมเซลล์ในแมโครฟาจชนิด RAW 264.7 ตรวจโดยการย้อมสี oil red O ผลการทดลองพบว่า LST ที่ความเข้มข้น 1 และ 2 ไมโครโมลาร์สามารถปกป้องการเกิดออกซิเดชันของไขมันใน he-oxLDL ได้ โดยมีผลยับยั้งการสร้าง TBARs ลด REM คงระดับของ cholesteryl arachidonate และ cholesteryl linoleate และลดระดับสารที่เกิดจากการออกซิไดซ์ของไขมัน นอกจากนี้ he-oxLDL ที่เติม LST 1 และ 2 ไมโครโมลาร์ ยังสามารถลดการเกิดโฟมเซลล์และลดปริมาณไขมันในเซลล์แมโครฟาจชนิด RAW 264.7 ได้อย่างมีนัยสำคัญเมื่อเทียบกับ he-oxLDL ที่ไม่ได้บ่มด้วย LST (p<0.05 และ p<0.0001 ตามลำดับ) โดยสรุปได้ว่าลูเซียนทรีดินสามารถป้องกันการเกิดออกซิเดชันของแอลดีแอลที่ถูกเหนี่ยวนำด้วยฮีมินได้และป้องกันการเกิดโฟมเซลล์ในเซลล์แมโครฟาจชนิด RAW 264.7

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Su Wutyi Thant : PROTECTIVE EFFECTS OF LUSIANTHRIDIN ON HEMIN-INDUCED LOW DENSITY LIPOPROTEIN OXIDATION AND FOAM CELL FORMATION OF RAW 264.7 MACROPHAGE CELLS. Advisor: Assoc. Prof. RATAYA LUECHAPUDIPORN, Ph.D. Co-advisor: Assoc. Prof. Noppawan Phumala Morales, Ph.D., VISARUT BURANASUDJA, Ph.D.

Oxidation of low-density lipoprotein (LDL) take parts in a crucial role in the pathogenesis of atherosclerosis. Hemin (iron (III)-protoporphyrin IX) is a degradation product of hemoglobin and cause oxidative stress-promoting vascular complications in thalassemia patients. Hemin was used as an oxidizing agent for LDL oxidation. Lusianthridin (LST) from *Dendrobium venustum* is a plant phenolic compound and possess antioxidant activity. The aim of this study is to evaluate the protective effect of lusianthridin on hemin-induced LDL oxidation (he-oxLDL) and foam cell formation in RAW 264.7 macrophage cell loaded with he-oxLDL treated with LST. Various concentrations of LST (0.25, 0.5, 1 and 2 μM) were preincubated with LDL for 30 min, then 5 μM hemin was added to initiate the oxidation and measured oxidative parameters at various times of incubation (0, 1, 3, 6, 12, 24 hr). Lipid peroxidation of LDL was measured by thiobarbituric reactive substance (TBARs) assay and relative electrophoretic mobility (REM). Lipid composition of LDL was analysed by using reverse phase HPLC. Foam cell formation with he-oxLDL in RAW 264.7 macrophage cells were detected by oil red O staining. The results demonstrated that LST at 1 and 2 μM were able to protect against lipid peroxidation in he-oxLDL by inhibition of TBARs formation, decreasing REM, preservation of cholesteryl arachidonate and cholesteryl linoleate level and decreased oxidized lipid products. In addition, exposure of he-oxLDL treated with 1 and 2 μM LST reduced the foam cell formation and decreased lipid content in macrophages significantly different from he-oxLDL untreated with LST ($p < 0.05$ and $p < 0.0001$, respectively). In conclusion, LST can protect LDL oxidation induced by hemin and showed the potential protective effect in foam cell formation in RAW 264.7 macrophage cell.

จุฬาลงกรณ์มหาวิทยาลัย
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CHULALONGKORN UNIVERSITY

LIST OF ABBREVIATIONS

α-tocopherol   Alpha-tocopherol

Acc   Acceleration

Apo   Apolipoprotein

BHT   Butylated hydroxytoluene

β-thal/HbE   β-thalassemia/hemoglobin E

CA   Cholesteryl arachidonate

CAT   Chloramphenicol acetyltransferase

CL   Cholesteryl linoleate

CO   Cholesteryl oleate

COX   Cyclooxygenase enzyme

Conc.   Concentration

CP   Cholesteryl palmitate

d   Density

Dec   Deceleration

DMEM   Dulbecco's modified eagle's medium

DMSO   Dimethyl sulfoxide

EDTA	Ethylene diamine tetraacetic acid
FBS	Fetal bovine serum
FC	Free cholesterol
Fe	Ferric
GPx	Glutathione peroxidase
HPLC	High Performance Liquid Chromatography
iNOS	Inducible nitric oxide synthase
hr	hour
he-oxLDL	hemin oxidized low-density lipoprotein
LDL	Low-density lipoprotein
LST	Lusianthridin
MDA	Malondialdehyde
Mg	Milligram
min	Minute
ml	Milliliter
μg	Microgram
μM	Micromolar

Na$_2$EDTA	Ethylenediamine tetra-acetic acid disodium salt
NaOH	Sodium hydroxide
nLDL	Native low-density lipoprotein
nmol	Nanomole
ox-LDL	Oxidized low-density lipoprotein
PBS	Phosphate buffer solution
PC	Polycarbonate
PUFA	Polyunsaturated fatty acid
REM	Relative electrophoretic mobility
RT	Retention time
SOD	Superoxide dismutase
TBARs	Thiobarbituric acid reactive substance
TRO	Trolox
VLDL	Very low-density lipoprotein
xg	Relative centrifugal force

CHAPTER 1

INTRODUCTION

1. Background and rational

Oxidation of low-density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis. Oxidation of LDL in arterial wall is important in the initial process of atherosclerosis due to the uptake of oxidized LDL [1]. LDL can be oxidized by enzymatically, lipoxygenase and myeloperoxidase[2] and non-enzymatically by transition metals (iron, copper) [3], hemin [4] and many other catalysts which can produce free radicals. The free radical can cause oxidation of LDL and then cause lipid peroxidation which is the autoxidation of polyunsaturated fatty acid (PUFA) chain of lipid and finally LDL become engulfed by scavenger receptor of macrophage, endothelial cell, and smooth muscle cell. Macrophages engulf oxidized low-density lipoprotein (ox-LDL) and this ox-LDL causes macrophage into foam cell which is the hallmark of atherosclerosis [2]. Oxidation of LDL can also find in some diseases like thalassemia in which iron overload occur in circulation. Hemin which may appear in the circulation of thalassemia patients can also be a possible physiological iron to induce LDL oxidation [5] .

Hemin, iron (III)-protoporphyrin IX is the degradation product of hemoglobin oxidation. Hemin may appear in plasma because of intravascular hemolysis and ferric component of hemin can cause oxidation of LDL. Hemin can be detected in the serum

of β-thalassemia/hemoglobin (β-thal/HbE) [6] and serum hemin catalyses rapidly free radical reaction and serves as pro-oxidant to induce LDL oxidation in *in vivo* and cause vascular complications [7]. The lipophilic antioxidants in LDL have the protective effect on LDL from oxidation [8]. Therefore, the prevention of LDL oxidation by antioxidants becomes the potential therapy in order to reduce lipid peroxidation and prevention of foam cell formation, the early characteristics of atherosclerosis.

Dendrobium species from Orchidaceae has been proved to show various biological activities including antioxidant activity and many of the epiphytic Orchids have been used as traditional Chinese medicine [9]. *Dendrobium* species produce several bioactive components such as bibenzyls, phenanthrenes, fluorenones and alkaloids and pharmacological activities from these constituents have been studied [10]. Lusianthridin which is extracted from *Dendrobium venustum* is a plant phenolic compound include in stilbenoids group (phenanthrenes). The antioxidant activity of lusianthridin had been proved and the EC_{50} value in DPPH assay is 22.3 μM compared with resveratrol, the positive control, with EC_{50} at 21.2 μM [11]. However, the effect of lusianthridin from *Dendrobium* species on LDL oxidation has not been investigated. Therefore, in this study, the effect of lusianthridin from *Dendrobium* species was investigated on hemin-induced LDL oxidation and hemin-oxidized LDL loaded RAW 264.7 macrophage cells.

2. Objective

The objectives of this study are

1. To evaluate the protective effect of Lusianthridin on hemin-induced low-density lipoprotein (LDL) oxidation

2. To evaluate the effect of Lusianthridin-treated hemin oxidized LDL loaded on foam cell formation in RAW 264.7 macrophage cell

3. Hypothesis

Lusianthridin is able to protect hemin-induced LDL oxidation in *in vitro* and lusianthridin-treated hemin oxidized LDL is able to reduce foam cell formation in RAW 264.7 macrophage cells.
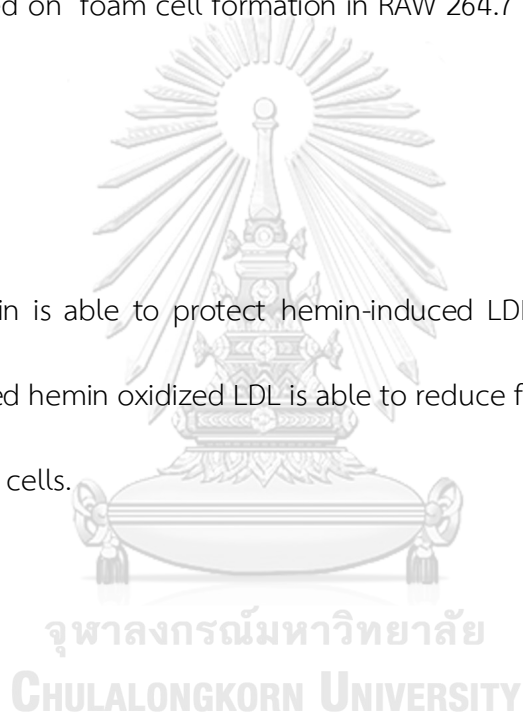
CHAPTER 2

LITERATURE REVIEW

1. Lipoprotein

Lipoproteins are lipid carrier molecules [12] and the major function of lipoprotein is to transport triglycerides and cholesterol between organs and tissues. There are four different types of lipoproteins in plasma which are based on their density, size, and lipid composition ratio. They are chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (ILDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) [13].

Table 1. Characteristics of lipoprotein [14].

Lipoprotein class	Size (nm)	Density (g/mL)	Major lipid and apoprotein
Chylomicron	100-500	0.93	Dietary TGs, B-48
VLDL	30-80	0.93-1.006	Endogenous TGs, B-100
IDL	25-50	1.006-1.019	CEs and TGs , B-100
LDL	18-28	1.019-1.063	CEs , B-100
HDL	5-15	1.063-1.210	CEs , A

TG. Triglyceride, CE. Cholesteryl ester

2. Low-density lipoprotein (LDL)

Low-density lipoprotein (LDL) is a major carrier of cholesterol and it has spherical in shape. The density of LDL is 1.019-1.063 g/ml and it has 18-28 nm in diameter. The lipophilic core of LDL particle is composed of molecule of cholesteryl ester (CE) and molecule of triglyceride (TG). The chemical structure of cholesteryl ester contains part of cholesterol and part of polyunsaturated fatty acids (PUFA) such as arachidonic acid, linoleic acid, oleic acid, and palmitic acid and the most abundant cholesteryl ester in LDL is cholesteryl linoleate (CL) [15]. The surface layer of LDL is composed of free cholesterol (FC), phospholipid and apo lipoprotein B 100 (Apo B 100) which is major apolipoprotein in LDL that contains 4536 amino acids. Furthermore, LDL contains lipophilic antioxidants that protect polyunsaturated fatty acid from the attack of free radical and oxidation. The most important antioxidant in LDL is alpha tocopherol (vitamin E). Other antioxidants including carotenoid, γ-tocopherol and ubiquinol 10 have lower activity in the protection of LDL from oxidation [16].
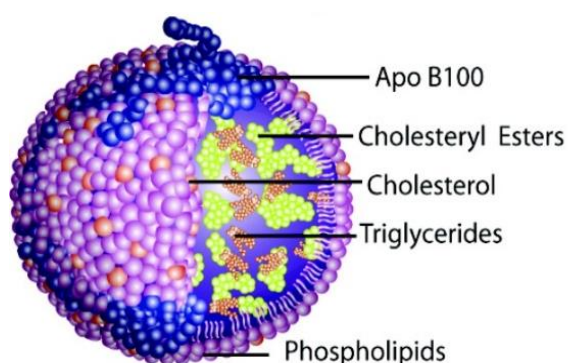
Figure 1.Structure of low-density lipoprotein [13].

2.1. Low-density lipoprotein (LDL) oxidation

Low-density lipoprotein (LDL) oxidation is caused by the action of enzymes such as lipoxygenase and myeloperoxidase. Lipoxygenase directly oxidize the polyunsaturated fatty acid of LDL and myeloperoxidase oxidize chloride ions and produce the formation of reactive substance species called HOCl (3-chlorotyrosine) and this HOCl cause oxidation of LDL [2]. Myeloperoxidase (MPO)-mediated oxidation of LDL results primarily in the modification of tyrosine residues of Apo B [17]. Moreover, LDL can be oxidized non-enzymatically by transition metal ions by the production of free radical. Oxidation of LDL by transition metal ions occur in three phases: an initial lag phase (consumption of endogenous antioxidant), a propagation phase (oxidation of unsaturated fatty acids to lipid hydroperoxides) and decomposition phase (hydroperoxides are converted to reactive aldehydes) [18]. Moreover, activated macrophages produce myeloperoxidase enzyme which produce reactive species, thereby oxidizes protein and lipid inside LDL. Finally, LDL interact with the scavenger receptors present on the endothelial cells, smooth muscle cell, and macrophages [19].

Endothelial Cells

Smooth Muscle Cells　　　　　　　　　**Activated Macrophages**

Redox active metal ions (Cu^{2+}, Fe^{2+})　　Lipoxygenase　　　Myeloperoxidase
　　　　　　　　　　　　　　　　　(metal ion dependent)　(metal ion independent)

　　　　　　　　　　　　　　　　　　　　　　　　　　　NO

　　　　　　　　　— NO

　　　　　　　　　　　　　PUFAs　　　　　　　　　　— Nitrite

LDL:　　　　　　　　　　　　　　Hydroperoxides

　1) Consumption of endogenous　　　　　　Reactive species:
　　　antioxidant　　　　　　　　　　　　-　HOCL
　　　　　　　　　　　　　　　　　　　-　Chloramine
　2) Lipid peroxidation in LDL　　　　　-　Tyrosyl radicals
　　　　　　　　　　　　　　　　　　　-　NO_2
　3) Formation of reactive

　　　aldehydes ◄　　　　　　　　　　Protein and lipid
　　　　　　　　　　　　　　　　　　oxidation in LDL

Aldehydes react with lysine residues
in apoB-100

　　　　　　　　　　　OxLDL ◄

　　　　　　　Scavenger receptors
　　　　　　　Endothelial cells
　　　　　　　Macrophages
　　　　　　　Smooth muscle cells

Figure 2. Mechanism of low-density lipoprotein oxidation [20].

2.2. Lipid peroxidation process

LDL oxidation is free radical-driven lipid peroxidation chain reaction. Lipid peroxidation process is initiated by the attack of free radical on double bond of polyunsaturated fatty acid (PUFA) and this cause the removal of hydrogen atom from a methylene group and this step is called the initiation step of lipid peroxidation process. The molecular rearrangement of the resulting unstable carbon radical result in unstable configuration called conjugated diene. This conjugated diene reacts quickly with molecular oxygen and then the peroxyl radical is formed. This peroxyl radicals attract a hydrogen atom from the adjacent PUFAs and this reaction causes lipid hydroperoxide and another lipid radical. This lipid radical also attracts hydrogen atom from the adjacent radical and this reaction is called chain reaction, propagation step of lipid process. Lipid hydroperoxide fragment to shorter chain aldehyde including malondialdehyde (MDA) and 4 hydroxynonenal [21]. These reactive aldehydes attach to amino groups of apo B-100 and change protein into increased negative charge. This condition decreases the affinity of LDL to LDL receptor which recognize a specific domain of positive charge on apo B-100. Increasing negative charge on surface of apo B-100 can cause increase recognition by scavenger receptors of macrophage, endothelial cell, and smooth muscle cell [22]. The macrophage engulfs oxidized low-density lipoprotein (ox-LDL) and this ox-LDL cause macrophage into foam cell which is the hallmark of atherosclerosis.

Unsaturated fatty acids + Free radical (OH$^\bullet$) **Initiation**

$$\downarrow - H^\bullet$$

Lipid radical

$$\downarrow + O_2$$

Lipid peroxyl radical + Another unsaturated lipid **Propagation**

additional radical reaction

Lipid hydroperoxide + Newly formed lipid radical

Lipid hydroperoxide produce oxidation products such as **Termination**

aldehyde, ketones and another oxidized lipid products
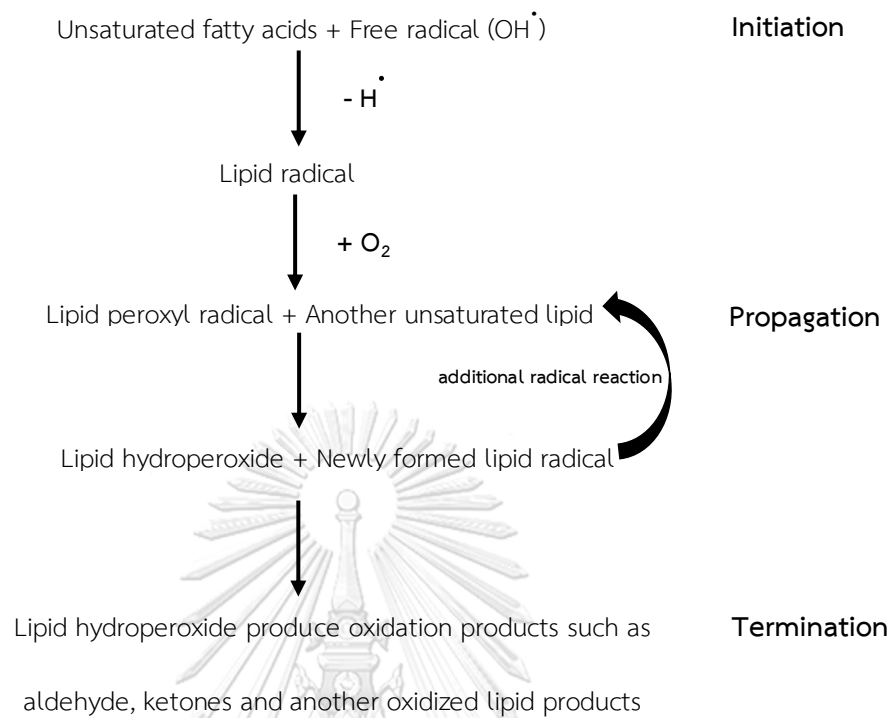
Figure 3. Lipid peroxidation process [21] .

2.3. Changes occur in LDL particle caused by oxidation

The following changes occur in LDL particles during oxidation [23]

- Loss of antioxidants

- Loss of poly unsaturated fatty acids (PUFA)

- Generation of lipid peroxidation products (lipid peroxides, hydroxides, aldehydes, ketones and hydrocarbons)

- Modification of apolipoprotein

Changes in apolipoprotein B-100 cause extensive alteration in the protein composition and structure of LDL [24]. Moreover, apo B-100 can also oxidized directly by the action of oxidizing agents, HOCl generated by myeloperoxidase. Modification of protein causes alteration of electrophoretic mobility, as well as the biological properties of LDL [25]. Alteration in electrophoretic mobility become considerable attention due to the potential role in recognition by the scavenger receptors of smooth muscle cells, endothelial cells, and macrophages [23].

2.4. The role of oxidized LDL in atherosclerosis

Oxidized LDL take part in the evaluation of atherosclerosis. Modification of LDL causes recognition by scavenger receptors of macrophages. Macrophages engulf the oxidized LDL until it became filled with fatty droplets and it became the foam cells. These foam cells engorged macrophages accumulate below the lining of vessel

and finally produce visible fatty streak which is the earliest form of atherosclerotic plaque. Therefore, the first stage of plaque formation became by the accumulation of cholesterol-rich deposit under the endothelium. Smooth muscle cells within the blood vessel also migrate from the muscular layer of blood vessel to the top of lipid accumulation. This process of migration is activated by chemicals released at the inflammatory site and that smooth muscle cells continue to divide and became enlarge. Therefore, lipid rich core and smooth muscle overlying form mature plaque and this process can narrow opening through blood flow [26].

Moreover, oxidized LDL can bind to its receptor, oxidized low density protein receptor (LOX1) on the endothelium. Due to the binding of this receptor, it induces the secretion of monocyte chemotactic protein 1 (MCP-1) and macrophage stimulating factor (MCSF) from endothelium. This induces the adhesion of monocyte to the endothelium and this effect also induce adhesion of vascular adhesion molecule (VCAM-1) and intracellular adhesion molecule (ICAM-1) that lead to leucocyte adhesion to vascular endothelium. Furthermore, oxidized LDL also impair the secretion of nitric oxide (NO) from the endothelium. NO take parts in the vasoprotective function of the endothelium and NO also reduce the expression of chemoattractant protein MCP-1, VCAM-1 and ICAM-1. Due to the inhibition of NO secretion, it facilitates leucocyte adhesion on endothelium that can lead to the formation of foam cell and it can contribute the evolution of atherosclerosis lesion [27].

3. Inhibitor of LDL oxidation

Overproduction of free radicals can cause oxidative damage and it can be regarded as an important event in the development of diseases, including arthritis, thyroid, cancer, and atherosclerosis [28]. In order to reduce oxidative damage- radical scavenging antioxidant inhibit free radicals and reduce oxidative damage [29]. The following antioxidants which can inhibit LDL oxidation by their antioxidant properties. Vitamin C, also known as ascorbic acid, act as hydrophilic antioxidant and found in tissues of living organisms and it is important for normal function of metabolic processes [30]. Moreover, functional groups in flavonoids attract to free radical and it has been reported the positive impact on LDL oxidation [31]. Furthermore, consumption of carotenoid can strongly inhibit LDL oxidative damage [32] and vitamin E (α-tocopherol) is also consumed by inhibiting free radicals of decreasing in LDL [33].

4. Hemin-induced LDL oxidation

Hemin is the amphipathic molecule, and it is a protoporphyrin IX with a coordinated Fe^{3+}. It has been detected in the serum of β-thal/HbE patients. Hemin appear in plasma following intravascular hemolysis and also hemin can also directly release from red blood cells without cell lysis [5]. Hemin participates in LDL oxidation in *vivo* because of hemoglobin oxidation and decomposition. Free hemoglobin release

into the blood plasma during intravascular hemolysis and can rapidly oxidized to methaemoglobin and cleavage into ferriheme (i.e. hemin) and globin [34]. 80 % of hemin bind with lipoprotein (HDL and LDL) when hemin appear in plasma and the remaining 20 % bind with albumin, hemopexin and antioxidant [35]. Hemin overload and antioxidant depletion in tissue and circulation of β-thalassemia cause oxidative stress and this oxidative stress cause atherogenesis-related vascular complication in β-thalassemia patients [6].

Hemin can be used as *in vitro* inducer of LDL oxidation [36]. Hemin can induce free radical and lipid peroxidation at micro molar concentration [5]. Incorporation of hemin into LDL cause the delivery of pro-oxidant Fe^{3+} to the hydrophobic core of LDL and initiate lipid peroxidation [37] and produce lipid peroxidation products and cause oxidative modification in apolipoprotein component of LDL and produce hemin-oxLDL complex which transform LDL particle to proatherogenic form which accumulate in macrophage and cause foam cell that is the characteristics of atherosclerosis lesion [7].

Oxidation of LDL induced by hemin in *in vitro* mimics oxidative stress on LDL of thalassemia patient and can also cause atherosclerosis as complication [7]. Therefore, hemin is used to induce LDL oxidation in this study.

5. Vitamin E (α-tocopherol)

The most abundant antioxidant in LDL is vitamin E. The role of vitamin E is to destroy free radicals that cause a chain reaction, mostly in highly unsaturated lipids. α-Tocopherol in suppressing lipid peroxidation is usually attributed to its scavenging of chain-carrying lipid peroxyl radicals and change into α-tocopheryl radicals. These α-tocopheryl radicals are reactive poorly when compare with other radicals and it can rapidly degrade into other products [38].

6. *Dendrobium* species

Dendrobium is one of the largest and most important genera in the family Orchidaceae [39]. They can be found in Asian countries, Australia, and nearly 150 species of this genus have been found in Thailand [40]. They are widely used in traditional Chinese medicine to reduce fever, hyperglycemia and as a tonic [9]. Several classes of secondary metabolites from *Dendrobium* plants including phenanthrenes, bibenzyls, alkaloids, fluorenones, sesquiterpenes, coumarins and polysaccharides have been reported to possess various pharmacological activities, such as antitumor, anti-inflammatory, antiplatelet aggregation, antidiabetic, antimalarial, antifibrotic, neuroprotective, antioxidant activity and immunoregulatory activities [40].

The antioxidant and free radical scavenging activity of *Dendrobium* species have been studied. Methanolic extract of *Dendrobium tosaense* and *Dendrobium*

monilforme had the DPPH radical scavenging activity at 95.9 and 83.4 % respectively [10]. Several compounds which is isolated from *Dendrobium secundum* showed appreciable DPPH radical scavenging activity [41]. In another study, antioxidant and anti-inflammatory effects of seven isolated compounds including two new compounds from *Dendrobium parishii* had been investigated in lipopolysaccharide and hydrogen peroxide treated RAW 264.7 cells. Antioxidant activities of all seven isolated compounds were evaluated by using DPPH, ORAC, and deoxyribose assays and found that one of the new bibenzyl compounds showed highest antioxidant activity. This new compound was tested for its anti-inflammatory and antioxidant effects in RAW 264.7 macrophage cells by using hydrogen peroxide and lipopolysaccharide to induce inflammation and oxidative stress. The results showed that this new compound can inhibit inflammation by reducing the expression of iNOS and COX-2 in lipopolysaccharide treated RAW 264.7 cells. It can also significantly decrease reactive oxygen species in dose-dependent manner (12.5-50 µg/mL) and increase antioxidant enzyme (CAT, SOD and GPx) in hydrogen peroxide treated RAW 264.7 cells [42].

7. Lusianthridin

Lusianthridin is the plant phenolic compound and it include in stilbenoids group (phenanthrenes). In this study, it was extracted from *Dendrobium venustum*.
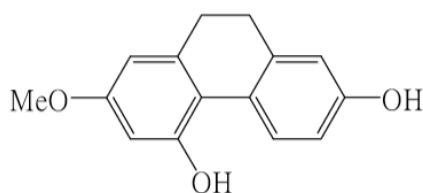
Figure 4. Chemical structure of lusianthridin [40].

Lusianthridin which is extracted from *Dendrobium formosum* had been evaluated the glucose uptake stimulated without toxicity in L6 skeletal muscle cells [43]. The activity of lusianthridin from *Dendrobium brymerianum* showed cytotoxic properties in human lung cancer lines with IC_{50} value of 65 ± 3.51 μg/ml. In addition, lusianthridin showed anti-migratory at nontoxic concentrations [44]. The cytotoxic activities of both *in vitro* and *in vivo* of lusianthridin have been already evaluated. It has potent antitumor effects against A549 human lung carcinoma, SK-OV-3 human ovary adenocarcinoma, and HL-60 human promyelocytic leukemia with EC_{50} values ranging from 0.11 to 9.8 μg/ml compare with the positive control, 5-fluorouracil, with EC_{50} values ranging from 0.21-5.1 μg/ml [39].

In one study of lusianthridin showed DPPH radical scavenging activity with IC_{50} 21.40 ± 0.61 μg/ml and FRAP value 1071.46 ± 46.58 Trolox/g dry wt. compared with trolox, IC_{50} 16.9 ± 0.47 μg/ml and proved that phenanthrenes also had high level of

antioxidant [9]. Moreover, in another study, phenanthrenes which was extracted from *Ephemerantha lonchophylla* from another Orchidaceae had the inhibitory activity of lipoprotein oxidation [45]. In addition, it has been proved that methanolic extract from the whole plant *Dendrobium venustum* showed significant antimalarial and anti-herpetic activities [40]. However, the effect of lusianthridin from *Dendrobium venustum* on LDL oxidation has not been investigated yet and lusianthridin is phenanthrenes which may reduce oxidative stress in LDL oxidation. In this study, the preliminary antioxidant activity of lusianthridin was investigated with DPPH assay and FRAP assay. The result showed that the IC_{50} of lusianthridin in DPPH assay was 24.53 ± 3.17 µM, compared with IC_{50} of vitamin C was 65.9 ± 9.05 µM. The FRAP valve of lusianthridin was 240.76 ± 2.45 µM ferrous equivalents, compared with the FRAP valve of vitamin C was 51.01 ± 1.21 µM ferrous equivalents. The percent scavenging activity of lusianthridin in DPPH assay is 65.6 % at 50 µM.

8. Conceptual Framework

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┌─────────────────────────────────────────┐
│       Low-Density Lipoprotein (LDL)       │
└─────────────────────────────────────────┘
                    │
              Hemin │  ◄── Inhibit? ── ┌──────────────┐
                    │                  │ Lusianthridin │
                    ▼                  └──────────────┘
┌─────────────────────────────────────────┐
│            Oxidation of LDL               │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│  •  Lipid peroxidation                    │
│  •  Oxidative modification of lipid       │
│     and Apolipoprotein of LDL             │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│  ox-LDL is taken by scavenger receptor    │
│  of macrophage                            │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│            Foam cell formation            │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│              Atherosclerosis              │
└─────────────────────────────────────────┘
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9. Research design: Experimental research

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┌─────────────────────────────────────────┐
│     Blood sample from healthy volunteers  │
└─────────────────────────────────────────┘
                    │
                    ▼
          ┌──────────────────┐
          │ Plasma separation │
          └──────────────────┘
                    │
                    ▼
          ┌──────────────────┐
          │  LDL separation   │
          └──────────────────┘
                    │
                    ▼
          ┌──────────────────┐
          │  Dialysis of LDL  │
          └──────────────────┘
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1. *In vitro* lipid peroxidation (TBARS, electrophoresis, determination of lipid composition & vitamin E content)	RAW 264.7 macrophage cell
Positive control: Trolox Test sample: Lusianthridin (LST) • Group 1: native LDL (LDL + PBS) • Group 2: oxidized LDL (LDL + hemin) • Group 3: LST (0.25, 0.5, 1 & 2 µM) + LDL + hemin • Group 4: trolox (0.5 & 1 µM) + LDL + hemin (time point: 0,1,3,6,12,24 hr)	1. MTT assay to determine the nontoxic effect of Lusianthridin and hemin oxidized LDL • A range of concentrations of lusianthridin • Different range of protein concentrations of hemin-oxidized LD 2. Foam cell formation assay • The hemin-oxidized LDL treated with LST or trolox for 24 hr was used in the foam cell formation in RAW 264.7 macrophage cells

Chapter 3

MATERIALS AND METHODS

1. Materials

1.1. Chemicals

The following chemicals were purchased from Sigma Chemical Co., St. Louis, U.S.A.: Ethylene diamine tetra acetic acid disodium dehydrate (Na_2EDTA), potassium bromide, sodium bicarbonate, sodium azide, potassium chloride, disodium phosphate, potassium dihydrogen phosphate, butylated hydroxyl toluene, sodium dodecyl sulphate, trichloroacetic acid, thiobarbituric acid, dimethyl sulfoxide (DMSO), vitamin E, cholesteryl oleate, cholesteryl arachidonate, cholesteryl linoleate, cholesteryl palmitate and oil red O powder.

Other chemicals were purchased from other sources as follow: Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, and penicillin-streptomycin were purchased from Gibco, U.S.A. Sodium chloride and trichloroacetic acid were purchased from Merck Germany. Absolute ethanol, butan-1-ol (n-butanol), isopropanol, acetonitrile, and methanol were purchased from RCI Lab Scan Co., Ltd Thailand. Agarose powder, Coomassie blue solution and Tris-acetate-EDT (TAE) buffer were purchased from Bio-Rad Laboratories, Hercules, California, U.S.A. and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich.

The RAW 264.7 macrophage cell line (ATCC TIB71) was purchased from ATCC (Bethesda, MD, USA).

Lusianthridin was isolated from *Dendrobium venustum* and the purity were evaluated using NMR spectroscopy. The compound with > 98% purity was used in this study.

1.2. Instruments

- Refrigerated centrifuge (Falcon:6300)

- CP100 NX ultracentrifuge (Hitachi)

- Microplate reader (CLARIO Star)

- Biological Safety Cabinet 1300 A2 (Thermo Scientific)

- CO_2 incubator (STERI-CYCLE-i160)

- Nikon Ts2 Inverted microscope

- Electrophoresis system AE-8130 (ATTA, Japan)

- HPLC system (Waters 2690 Separation Module, Waters, USA) with Waters 2487 Dual Wavelength Detector and Jasco FP-2020 Plus Intelligent Fluorescence Detector

2. Methods

2.1. Subjects

The study was performed in human LDL obtained from healthy volunteers. Normal pooled plasma was obtained from three females at the age of 27 years. The followings are the inclusion and exclusion criteria.

Inclusion criteria

- Age between 18 to 55 years

- No prior or ongoing medical conditions

- Non-smokers, non-alcoholics,

- Not donating blood in the last one month

- Not taking any medications at least 2 weeks before participated in the study

- Appreciate to participate in the study with sign

Exclusion criteria

- Female who are pregnant or breast-feeding

- Female who are during or for 5 days after your period

Volunteers need to sign the informed consents before participating in this study. The protocol was approved by Institutional Review Board, Faculty of Medicine, Chulalongkorn University (COA No. 493/2020).

2.2. Plasma preparation

Blood was obtained from three female healthy volunteers (age 27 years) fasted overnight. The 30 ml of blood sample from each volunteer was collected in tube containing EDTA (final concentration: 1 mg/ml blood) as anticoagulant and then the 15 ml of plasma from each volunteer was separated by centrifugation at 2454 xg for 15 min at 4°C and stored at -80°C until LDL separation. The pooled plasma were collected for 5 times and used in independent experiments (n=5). The same volunteers were used for the whole experiments.

2.3. Low-density lipoprotein (LDL) separation

Low-density lipoprotein was separated by sequential density gradient ultracentrifugation method. LDL (density: 1.019-1.063 g/ml) was separated from plasma with salt solutions. Firstly, two salt solutions with different density were prepared.

1. Mock solution: 1.142 g sodium chloride and 10 mg Na_2EDTA was dissolved in 100 ml of water (d = 1.0063 g/ml)

2. Concentrated salt solution: 35.4 g potassium bromide,15.3 g sodium chloride and 10 mg Na_2EDTA was dissolved in 100ml of water (d = 1.346 g/ml)

From two stock solutions, the salt solutions that are used for LDL separation was prepared from the following formula

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

Where,

X   = desired density

A   = volume of mock solution

B   = volume of concentrated salt solution

Y   = density of mock solution

Z   = density of concentrated salt solution.

LDL was separated by ultracentrifugation according to the following procedure by using Hitachi ultracentrifuge CP100-NX (P100AT2 rotor, k value 18), 60,000 rpm at 16°C [46]. 4.7 PC thick walled tubes were used and eight tubes can be used for each centrifugation. 13.6 ml of plasma (1.7 x 8) was needed for each centrifugation and around 3.5 ml of LDL was obtained from each round of centrifugation.

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Pooled Plasma (1.7 ml) + Salt solution (1.7 ml) (d = 1.032 g/ml)

Centrifuge 5 hr 30 min (Acc 5, Dec 7)

Upper Layer

(Chylomicron & VLDL)

Lower layer (1.7 ml) + Salt solution (1.7 ml)

(d = 1.107 g/ml)

Centrifuge 8 hr (Acc 9, Dec 7)

Upper Layer (LDL)

Lower layer

1.019 < d < 1.1063
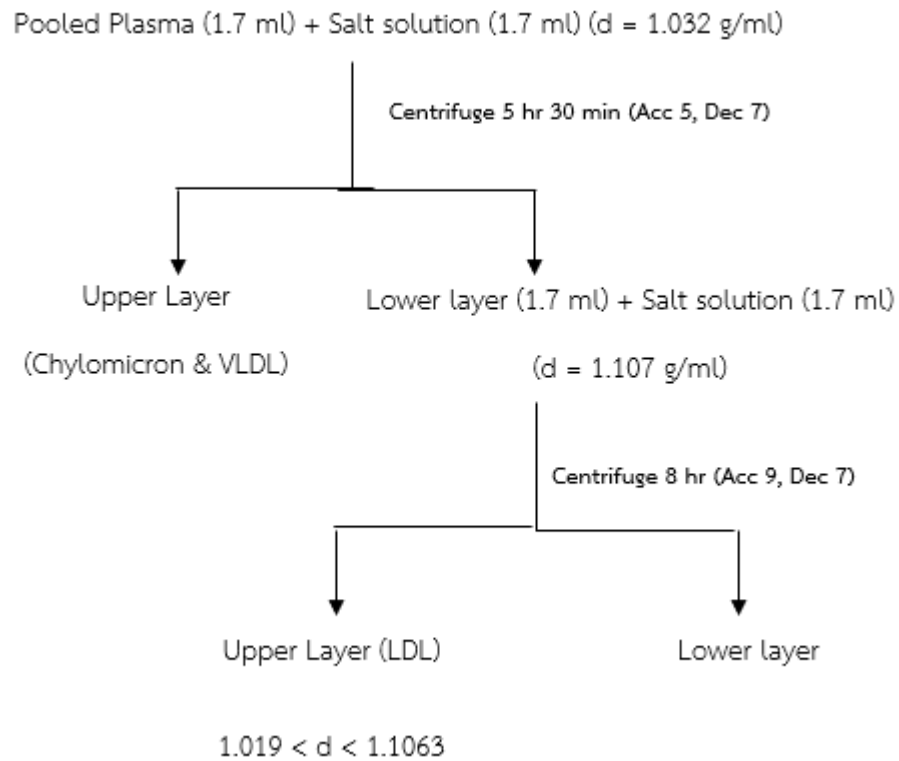
Acc : Acceleration

Dec : Deceleration

Figure 5. Separation of low-density lipoprotein by ultracentrifugation.

2.4. Preparation of hemin

The stock solution of hemin was prepared by dissolving hemin with 500 µl of 0.1 M NaOH solution and then added 4.5 ml of 10 mM PBS. The concentration of hemin solution was calculated by reading absorbance at λ = 385 nm using molar coefficient ε = 58.4 mmol $L^{-1}cm^{-1}$.

2.5. Hemin-induced LDL Oxidation

LDL (400 mg/ml) was pre-incubated with the various concentrations of lusianthridin or trolox as the positive control about 30 min in shaking incubator at 37℃, then added hemin (5 µM) to induce oxidation of LDL for 24 hr. The aliquots were collected at various time of incubation (0, 1, 3, 6, 12, 24 hr). The reaction was terminated by adding 100 µM EDTA and 5 mM BHT.

2.6. Parameter measurements

2.6.1. Thiobarbituric reactive substances (TBARs)

TBARs was used as an oxidative marker of lipid peroxidation process. The oxidative breakdown of unsaturated fatty acids reacted with thiobarbituric acid (TBA) and produced pink coloured adduct [47].

Lipid peroxidation of LDL was terminated by 5 µl of 100 mM butylated hydroxytoluene (BHT). After that, 100 µl of 10 % trichloroacetic acid (TCA) and 50 µl of 5 mM ethylene diamine tetra acetic acid (EDTA) was added and mixed well. Then

50 µl of 8 % sodium dodecyl sulphate (SDS) and 150 µl of 6 % thiobarbituric acid (TBA) was added and mixed again. This mixture was heated in water bath at 95°C about 1 hr. After that, this mixture was cooled in ice-cold water about 10 min and then 400 µl of n-butanol was added and mixed again. This mixture was centrifuged at 1500 xg, 10 min and the sample were measured by spectrofluorometer, excitation and emission wavelength at 515 nm and 553 nm, respectively. Tetramethoxypropane (TMP) was used as standard. The results are presented as nanomole thiobarbituric reactive substance (TBARs) per milligram protein LDL [48].

2.6.2. Relative Electrophoretic Mobility of LDL

The negative charge modification in LDL was determined by agarose gel electrophoresis and it was used as indicator of protein oxidation. Native LDL (nLDL) and he-oxLDL was run on 1 % agarose gel in TAE (Tris-acetate-EDTA) buffer and run at constant voltage (70 V) about 45 min. The protein on gel was stained in coomassie blue solution about 30 min and then washed with water [49]. Then the distance away from origin was measured and expressed as relative electrophoretic mobility (REM).

2.6.3. Determination of lipid composition

Lipid composition and **α**-tocopherol were analysed by reverse phase HPLC using UV-Vis detector at 210 nm for free cholesterol and cholesteryl esters, 234 nm for oxidized lipid products and fluorescence detector, excitation 295 nm and emission 370 nm for **α**-tocopherol.

Lipid in 100 µl of LDL was extracted by adding 250 µl ice-cold methanol and 1500 µl ice-cold hexane and then vortex about 30 second and 1 minute, respectively. The hexane layer was transferred into another test tube and dried under nitrogen and then redissolved in 100 µl of acetonitrile. After that, the sample was injected into Hypersil BDS C-18 stainless steel column (5 µm; 4.6 x 250 mm) (Thermo scientific USA) with the mobile phase of 46 % acetonitrile: 51 % isopropanol: 3 % water. The 20 µl of sample was injected and the flow rate was 1 ml/min. The temperature of column was controlled at 50°C. The standard solution of free cholesterol, cholesteryl arachidonate, cholesteryl linoleate, cholesteryl oleate, cholesteryl palmitate and **α**-tocopherol were prepared and the amount of lipid composition can be calculated from the chromatogram [50].
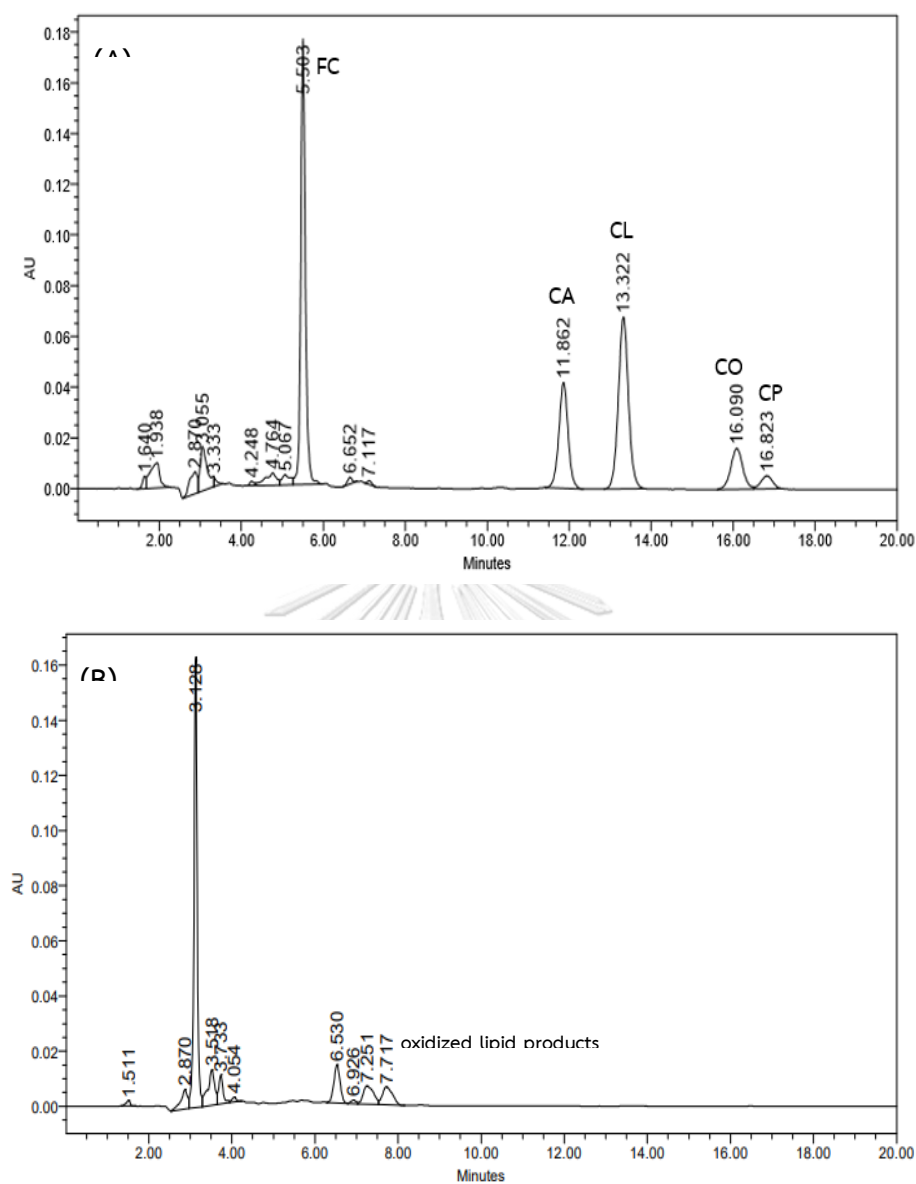
Figure 6. (A) Typical HPLC chromatogram of free cholesterol (FC) and a series of cholesteryl esters standards: cholesterol arachidonate (CA), cholesteryl linoleate (CL), cholesterol oleate (CO) and cholesterol palmitate (CP) at 210 nm, (B) oxidized lipid products at 234 nm determined by reverse phase HPLC using UV monitor.

2.7. Cell culture

The RAW 264.7 macrophage cell line (ATCC TIB71) was used. RAW 264.7 macrophage cells were cultured in high glucose DMEM medium (Gibco, USA) supplemented with 10 % fetal bovine serum (Gibco, USA) and 1 % penicillin and streptomycin (Gibco, USA). The cells were incubated at 37°C in humidified atmosphere 5 % CO_2 and 95 % air and grown to 70 % - 80 % confluence [51].

2.7.1. Cell viability assay

The cell viability of RAW 264.7 macrophage cells was determined by MTT assay, tetrazolium salt reduction, indicator of cell growth. Viable cells with active metabolism convert yellow tetrazolium MTT into a purple-colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose the ability to convert MTT into formazan. Therefore, color formation serves as a useful and convenient marker of only the viable cells [52].

The cells were seeded overnight in 24 well plate. The cells were then treated with various concentrations of lusianthridin or he-oxLDL and incubated about 24 hr at 37°C, 97 % humidity and 5 % CO_2. After that, the medium was removed and added 250 μl PBS and 25 μl MTT solution (final concentration: 0.5 mg/ml) and then mixed well. Then the cells were incubated at 37°C, 97 % humidity and 5 % CO_2 about 3 hr. MTT solution was removed after 3 hr of incubation and 250 μl of 100 % DMSO was

added to dissolve formazan crystals. The absorbance was measured at 570 nm by microplate reader [53].

$$\% \text{ cell viability} = \frac{\text{Optical density of sample}}{\text{Optical density of control}} \times 100$$

2.7.2. Foam cell formation detection

Foam cell formation was detected by oil red O staining method. RAW 264.7 macrophage cells were seeded in 24 well plate. Then, the cells were treated with nLDL, he-oxLDL and he-oxLDL incubated with lusianthridin and trolox and then incubated 24 hr at 37°C, 97 % humidity and 5 % CO_2. Then the cells were washed with PBS and then fixed with 4 % formaldehyde about 15 min. After that, cells were washed with PBS again. Then added 60 % isopropanol and incubated 5 min. The cells were then stained with oil red O solution and incubated about 6 min at 37°C. The cells were washed with 60 % isopropanol and then washed with PBS (2 times). Finally, the red staining cells were observed under the inverted microscope (Nikon) [54] . Lipid content can also be measured by extracting the stained cells with 250 µl of 100 % isopropanol about 5 min with gentle shaking. After that, 200 µl of aliquot was added into 96 well plate and the absorbance was measured at 492 nm by spectrophotometer. 100 % isopropanol was used as background control.

3. Data analysis

The statistical analysis was performed using SPSS version 22. All experimental values were be expressed as means ± standard errors of mean (SEMs). Differences between groups were be analyzed using one-way ANOVA followed by Tukey post hoc test and student t test. P-values less than 0.05 were accepted as statistically significance.

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CHAPTER 4

RESULTS

1. The effect of hemin on TBARs formation

TBARs is one of the oxidative markers in lipid peroxidation process. TBARs level in native LDL (nLDL) was detected very low amount during 24 hr incubation period (0.07 ± 0.13 vs 0.02 ± 0.13 nmol/mg protein). TBARs level in LDL incubated with 5 µM hemin was increased from 0.17 ± 0.09 nmol/mg protein to 18.39 ± 0.86 nmol/mg protein during 24hr. The result showed that hemin can induce lipid peroxidation in LDL.
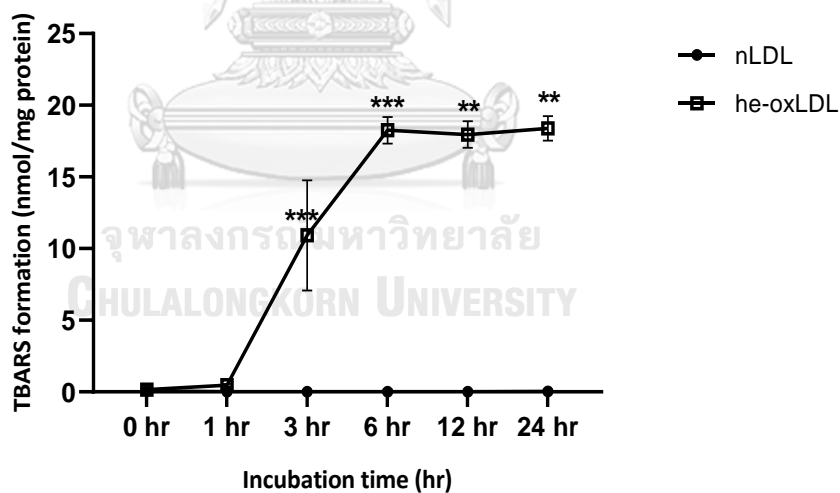

Figure 7. Effect of hemin on TBARs formation in LDL oxidation. Data was presented as mean ± SEM (n=5). [**]p<0.001, [***]p<0.0001 compared to respective nLDL.

2. The effect of lusianthridin on TBARs formation

TBARs formation in nLDL was not change significantly during 24 hr. TBARs level of he-oxLDL were 0.17 ± 0.09, 0.46 ± 0.08 and 10.92 ± 3.85 nmol/mg protein at 0,1 and 3 hr, respectively. Maximum TBARs formation of he-oxLDL occurred at 6 hr was 18.25 ± 0.92, then 18.39 ± 0.86 nmol/mg protein at 24hr. 0.25 µM lusianthridin could not reduce TBARs formation. 0.5 µM decreased TBARs formation only 3 hr, 1 µM decreased until 6 hr and 2 µM reduced TBARs formation until 12 hr. However, trolox, the positive control, reduced TBARs formation until 6 hr.

Lusianthridin 2 µM inhibited TBARs formation 95.2 % at 12 hr and it still inhibited 54.7% in TBARs formation at 24 hr. Trolox inhibited TBARs formation 95.9 % at 6 hr whereas lusianthridin inhibited 95.8 % at 6 hr. Therefore, it could be concluded that lusianthridin could protect hemin-induced LDL oxidation and more potent than trolox, the positive control.
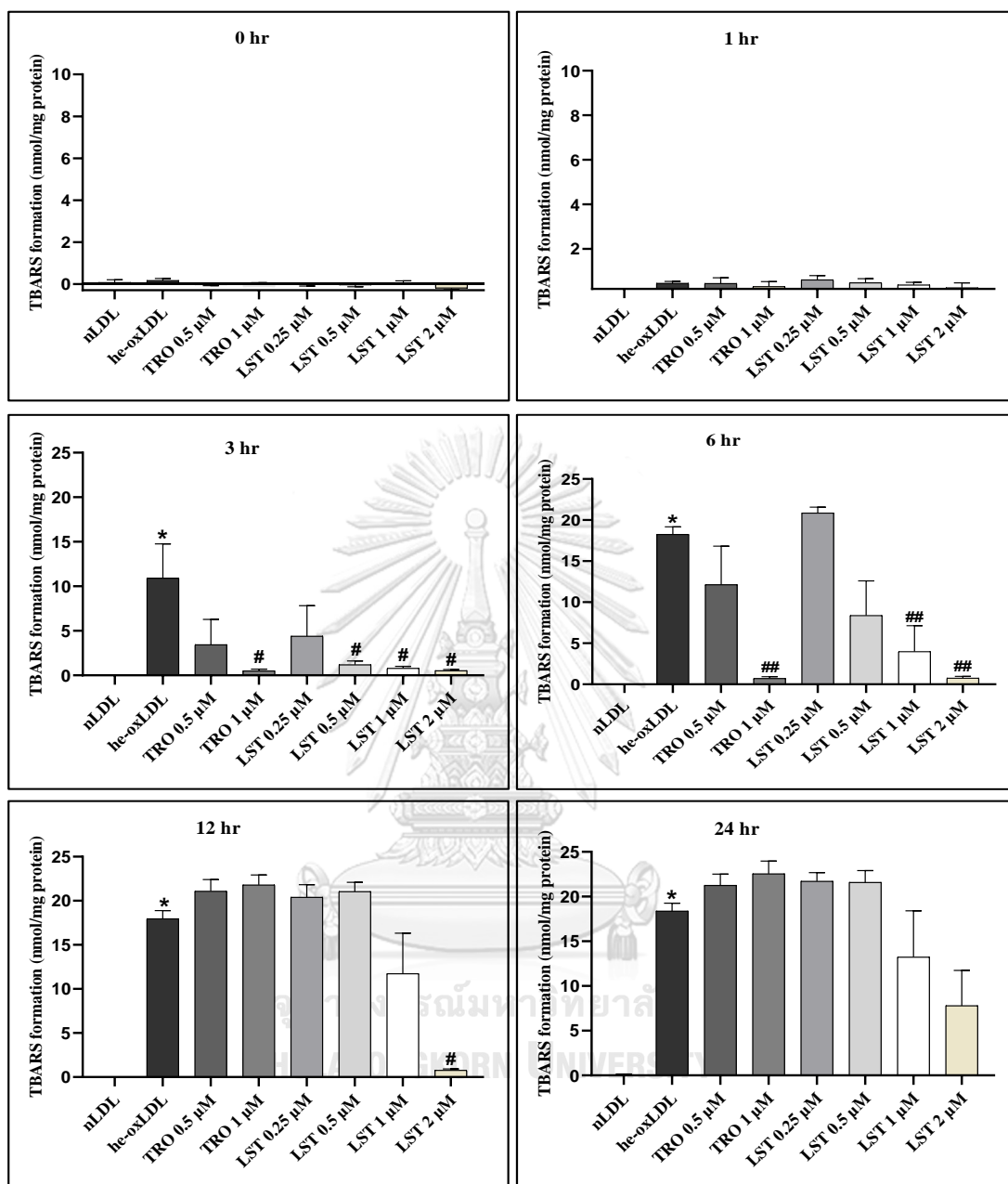
Figure 8. Effect of lusianthridin and trolox in hemin-induced LDL oxidation at various times from 0 to 24 hr. Data were presented as mean ± SEMs (n=5). *p<0.05 compared to nLDL, #p<0.05, ##p<0.001 compared to he-oxLDL.

3. The effect of hemin on lipid composition

The effect of hemin on lipid composition was shown in figure 9. The level of free cholesterol (FC), cholesteryl arachidonate (CA) and cholesteryl linoleate (CL) of nLDL was not changed during 24 hr. However, CA and CL were decreased in he-oxLDL at 24 hr incubation. CA and CL are the major substrates for lipid peroxidation and hence, we can be said that hemin can induced lipid peroxidation in LDL.
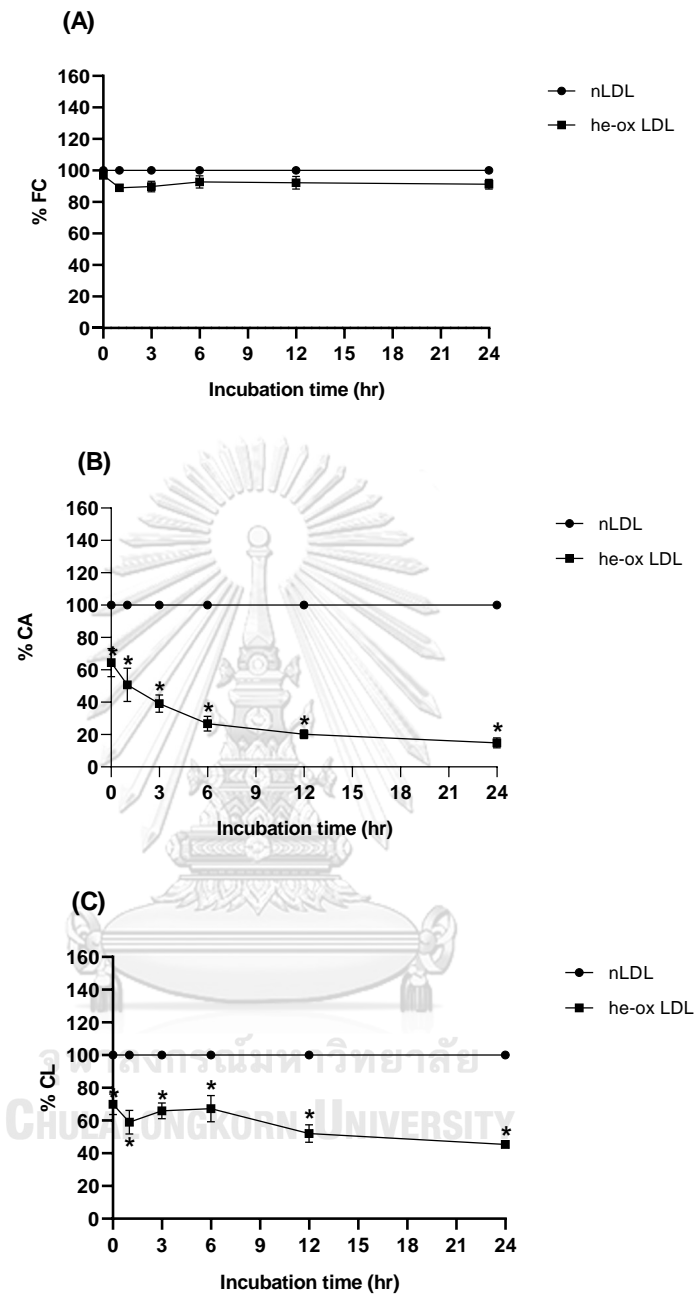
(A)

(B)

(C)

Figure 9. Effect of hemin on (A) FC, (B) CA and (C) CL level in LDL oxidation. Data

were presented as mean ± SEM (n=4). *p<0.05 compared to respective nLDL.

4. The effect of lusianthridin on lipid composition

The level of free cholesterol (FC), cholesteryl arachidonate (CA) and cholesteryl linoleate (CL) in nLDL were not changed significantly. Decreasing of cholesteryl esters of unsaturated fatty acids is a marker of lipid peroxidation in lipoproteins. In he-oxLDL, the CA level decreased from 50.71 ± 10.31 % at 1 hr to 14.83 ± 3.06 % at 24 hr. The CL level of he-oxLDL at 24 hr decreased from 58.97 ± 7.22 % at 1 hr to 45.37 ± 2.33 % at 24 hr. Free cholesterol (FC) level remained steady for 24 hr. Lusianthridin 0.25 and 0.5 µM could not protected against the reduction of CA, however, 1 and 2 µM preserved 51.85 ± 12.58 % and 71.87 ± 7.15 % of CA and 75.26 ± 9.6 % and 87.72 ± 2.54 % CL at 24 hr, respectively. Trolox 1 µM preserved both CA and CL level until 6 hr, 78.53 ± 8.54 % and 87 ± 4.4 %, respectively. Therefore, the result indicated that lusianthridin had protective effect against in changes of lipid composition.

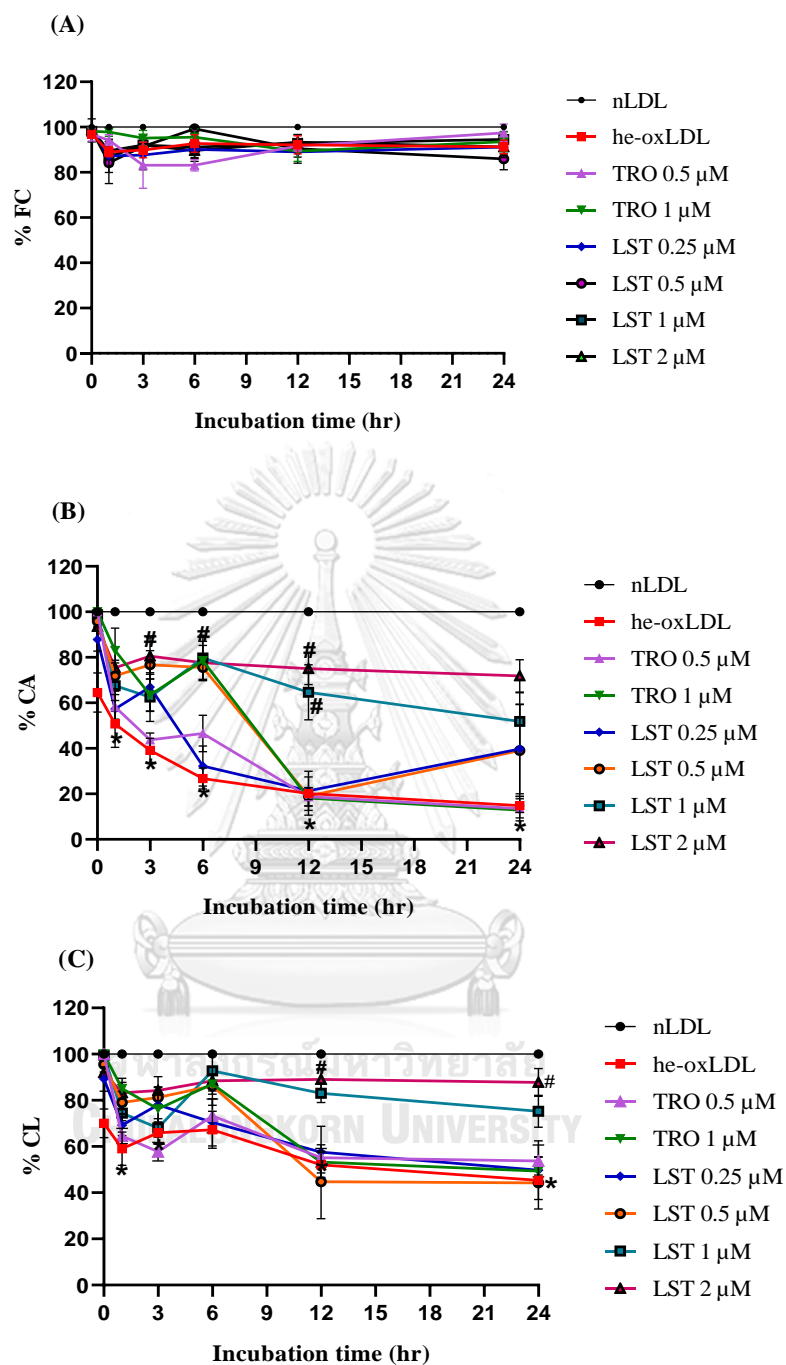**(A)**

(B)

(C)

Figure 10. Effect of lusianthridin and trolox on (A) FC, (B) CA and (C) CL level in hemin-induced LDL oxidation at various times from 0 to 24 hr. Data were presented as mean ± SEM (n=4). $^*p<0.05$ compared to nLDL, $^#p<0.05$ compare to he-oxLDL.

5. The effect of lusianthridin on oxidized lipid products

Corresponding to the decreasing of CA and CL level, the oxidized lipids were produced. Oxidized lipid products were detected at 7.7 min during LDL oxidation. From the result of figure 11, the level of oxidized lipid products in he-oxLDL were significantly increased from 6 hr until 24 hr. Trolox could not reduce oxidized lipid products whereas lusianthridin (0.25, 0.5, 1 and 2 µM) reduced oxidized lipid products in concentration dependent manner. Hence, the result showed that lusianthridin had protective effect against hemin-induced LDL oxidation.
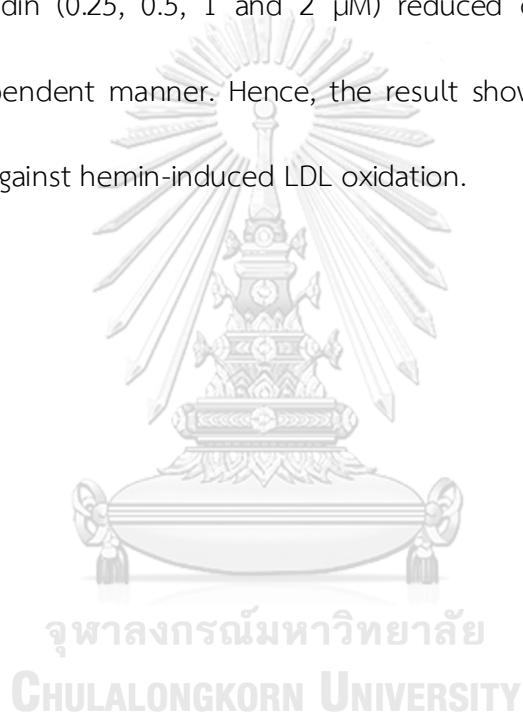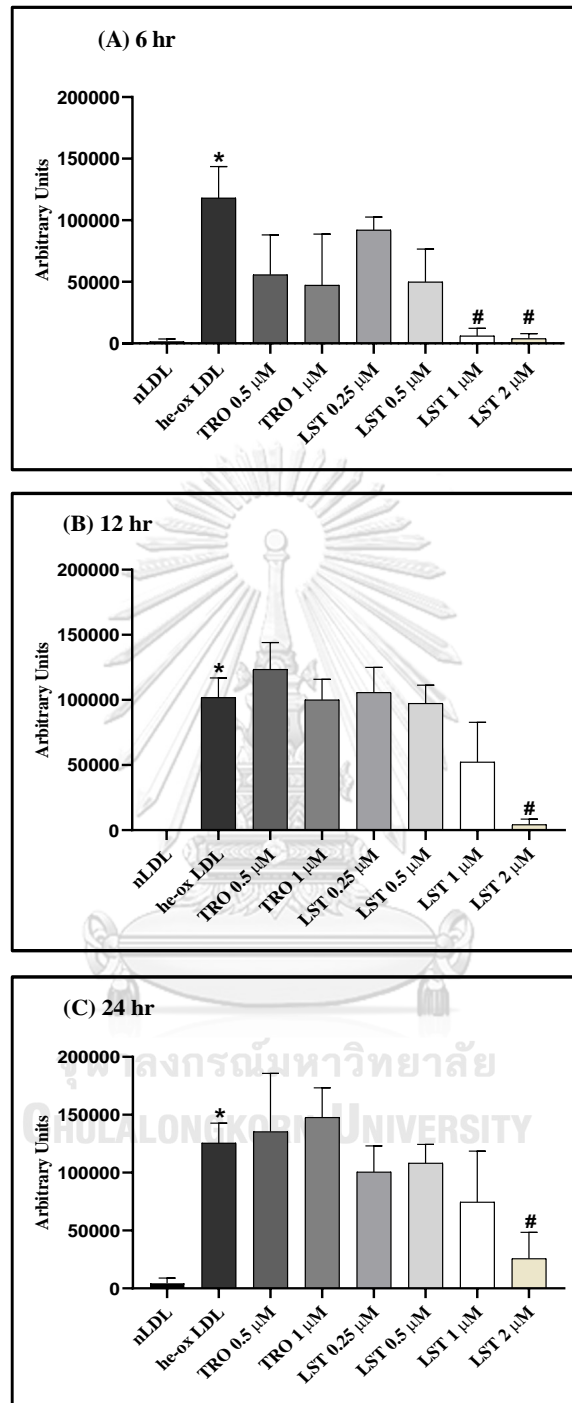
Figure 11. Effect of lusianthridin and trolox on oxidized lipid product at (A) 6 hr, (B) 12 hr and (C) 24 hr. Data were presented as mean ± SEMs (n=4). *p<0.05 compared to nLDL, $^{#}$p<0.05 compared to he-oxLDL.

6. The effect of hemin on α-tocopherol

α-Tocopherol is the most abundant and major endogenous antioxidant in LDL. The effect of hemin on α-tocopherol was shown in the figure 12. α-tocopherol level in nLDL was not change during 24 hr incubation period. However, α-tocopherol level in he-oxLDL was rapidly decreased and undetectable at 1 hr of incubation period. The depletion of α-tocopherol indicated that α-tocopherol was consumed during LDL oxidation induced by hemin.

Figure 12. Effect of hemin on α-tocopherol level in LDL oxidation. Data was presented as mean ± SEM (n=4). [*]p<0.05, [**]p<0.001 compared to respective nLDL.

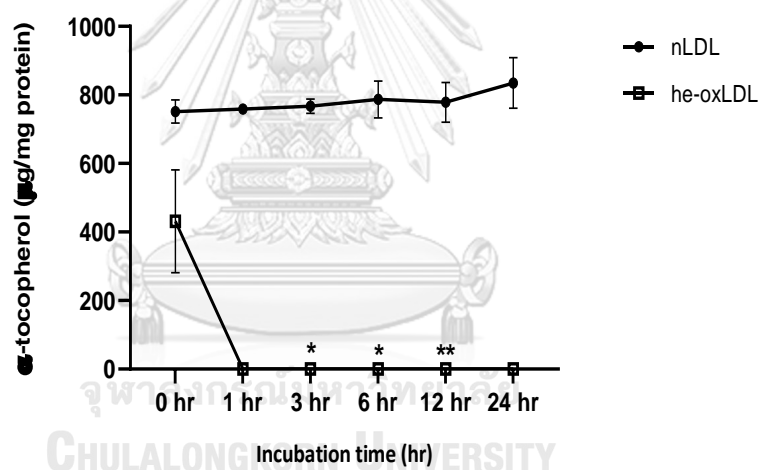7. The effect of lusianthridin on **α**-tocopherol

α-Tocopherol is the major endogenous lipid soluble antioxidant in LDL oxidation. The level of **α**-tocopherol in native LDL was approximately 700 µg/mg protein, it was rapidly decreased in the presence of hemin and was undetectable within 1 hr. Lusianthridin 2 µM and trolox 0.5 and 1 µM delayed the consumption of endogenous **α**-tocopherol, the remaining of **α**-tocopherol at 1 hour were 20.4 %, 18.1 % and 75.9 %, respectively. It should be noted that the level was not changed over 24 hour without hemin.

Table 2. Effect of lusianthridin and trolox on **α**-tocopherol level (µg/mg protein) in hemin-induced LDL oxidation.

	Incubation time (hr)					
	0	1	3	6	12	24
nLDL	751.5 ± 34	758.7 ± 11	766.8 ± 21	787.0 ± 54	778.4 ± 58	834.8± 74
he-oxLDL	431.4 ± 149.7*	0.0	0.0	0.0	0.0	0.0
LST 0.25 µM	758.4 ± 79	0.0	0.0	0.0	0.0	0.0
LST 0.5 µM	894.3 ± 146.6[#]	0.0	0.0	0.0	0.0	0.0
LST 1 µM	863.2 ± 125[#]	0.0	0.0	0.0	0.0	0.0
LST 2 µM	655.8 ± 229.6	154.5 ± 154.5	0.0	0.0	0.0	0.0
TRO 0.5 µM	860.6 ± 113.5[#]	137.3 ± 137.3	0.0	0.0	0.0	0.0
TRO 1 µM	760 ± 34.2	575.7 ± 198	0.0	0.0	0.0	0.0

Data was presented as mean ± SEMs (n=4). *$p < 0.05$ compared to nLDL, [#]$p < 0.05$ compared to he-oxLDL.

8. The effect of hemin on relative electrophoretic mobility(REM)

Oxidation of LDL also cause physiochemical changes. Modification of negative charge occurred in LDL during oxidation and this change become recognized by macrophage and altered biological properties of macrophage. Alteration in relative electrophoretic mobility (REM) was used as indicator for protein oxidation. The result from the figure 13 showed that the level of REM of he-oxLDL increased during 24 hr incubation. Therefore, the result indicated that hemin can induced lipid peroxidation and cause negative charge in LDL.
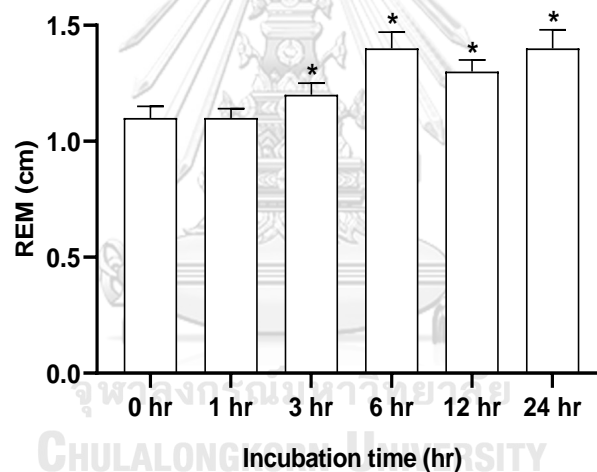
Figure 13. Relative electrophoretic mobility (REM) of he-oxLDL. Data was presented as mean ± SEMs (n=5). *p<0.05 compared to nLDL.

9. The effect of lusianthridin on relative electrophoretic mobility

REM of nLDL was not changed during 24 hr of incubation. However, REM of he-oxLDL were 1.2 ± 0.05, 1.3 ± 0.07 and 1.39 ± 0.08 at 3, 6 and 24 hr, respectively. Lusianthridin 1 µM reduced REM (1.07 ± 0.05, 1.12 ± 0.05 and 1.16 ± 0.05) and 2 µM of lusianthridin reduced REM (1.05 ± 0.07, 1.05 ± 0.04 and 1.09 ± 0.07) at 6, 12 and 24 hr whereas 0.25 µM and 0.5 µM lusianthridin could not reduce REM. Trolox 1 µM, the positive control, reduced REM (1.08 ± 0.05) until 6 hr. Therefore, the result indicated that lusianthridin protected against protein oxidation than trolox, the positive control.
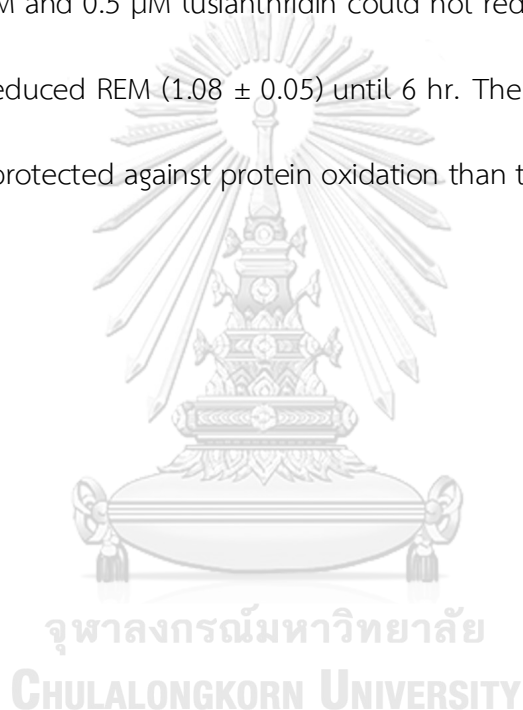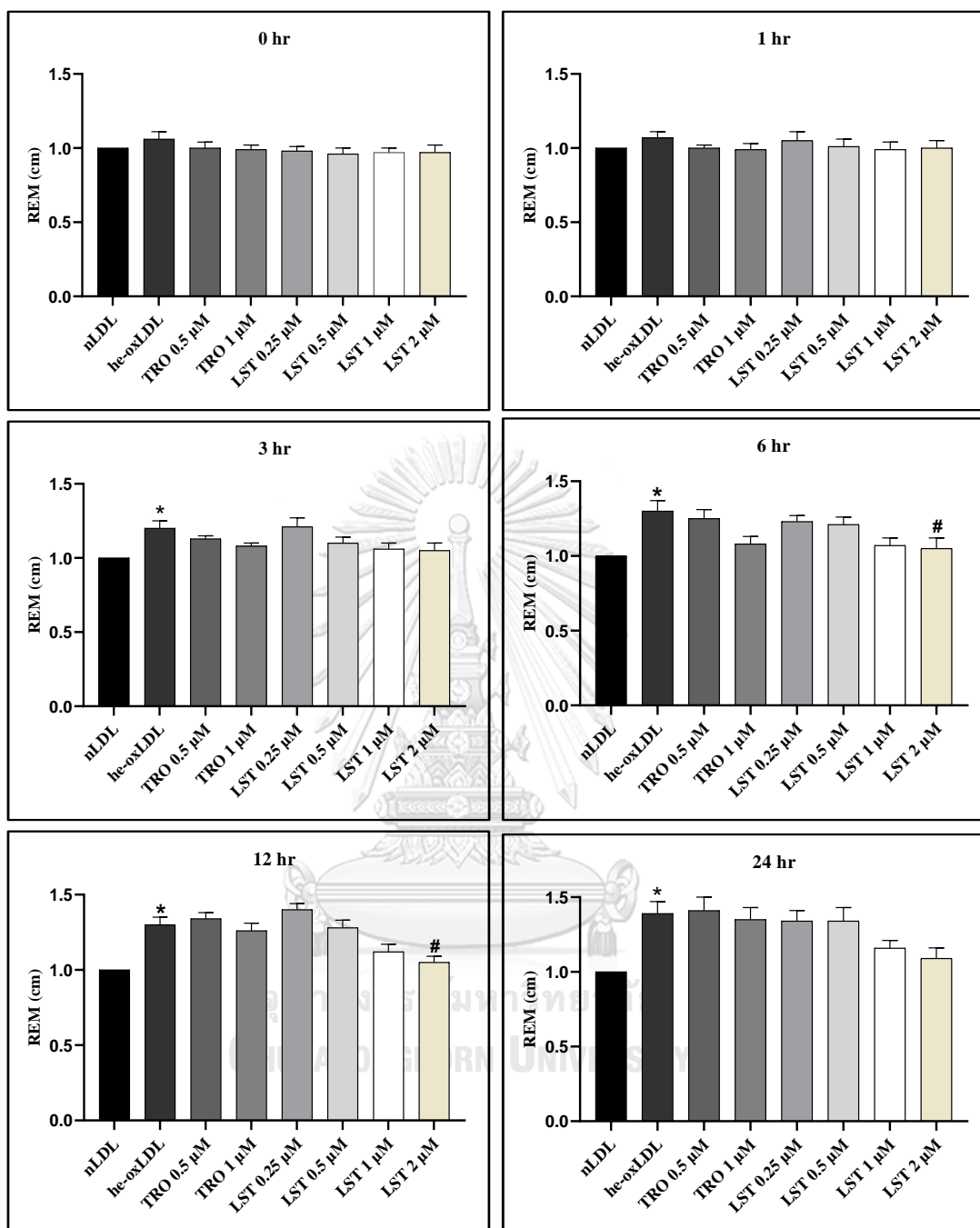
Figure 14. Effect of lusianthridin on relative electrophoretic mobility (REM) in hemin-induced LDL oxidation at various times from 0 to 24 hr. Data were presented as mean ± SEMs (n=5). *p<0.05 compared to nLDL, #p<0.05 compared to he-oxLDL.

10. Cell viability assay of RAW 264.7 macrophage cells

10.1. Effect of lusianthridin on RAW 264.7 macrophage cell viability

The effect of lusianthridin on RAW 264.7 macrophage cell viability was performed to determine nontoxic concentration of lusianthridin. The cell viability was significantly decreased at concentration of 50 and 100 µM. The cell viability of remaining concentrations (0.5, 1, 2 ,5, 10, 20 µM) were not significant difference (84 %, 82 %, 79 %, 76 %, 80 %, and 78 % respectively) compared with control. This result showed that lusianthridin in low concentrations had no cytotoxic effect on RAW 264.7 macrophage cell. Therefore, 0.5, 1, and 2 µM of lusianthridin was chosen to preincubate with he-oxLDL and then investigate for foam cell formation assay.

.
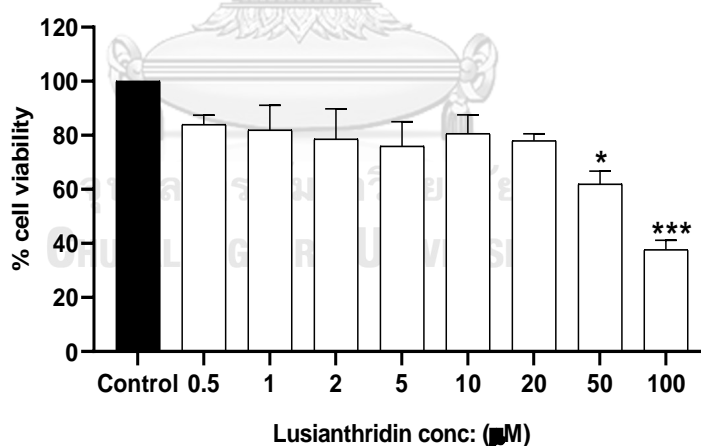
Figure 15. Effect of lusianthridin on cell viability of RAW 264.7 macrophage cell. Data were presented as mean ± SEMs (n=3). *p<0.05, ***p<0.0001 compared to control.

10.2. Effect of hemin-oxidized LDL on RAW 264.7 macrophage cell viability

The cell viability of hemin-oxidized LDL on RAW 264.7 macrophage cells was accessed by MTT assay to observe cytotoxic effect of he-oxLDL. RAW 264.7 macrophage cells were treated with 50, 100, 150 and 200 µg/ml of he-ox-LDL. The result showed that he-oxLDL at 200 µg/ml showed 62 % viability. Both 50 and 100 µg/ml survived the cell nearly 80 % and he-oxLDL at 150 µg/ml showed nearly 70 % of cell viability. The result indicated that 200 µg/ml of he-oxLDL decreased cell viability compared with control and other concentrations of he-oxLDL. 50, 100, and 150 µg/ml had no cytotoxic effect on RAW 264.7 macrophage cells. Therefore, the final concentration of 100 µg/ml he-oxLDL was chosen to induce foam cell formation assay.
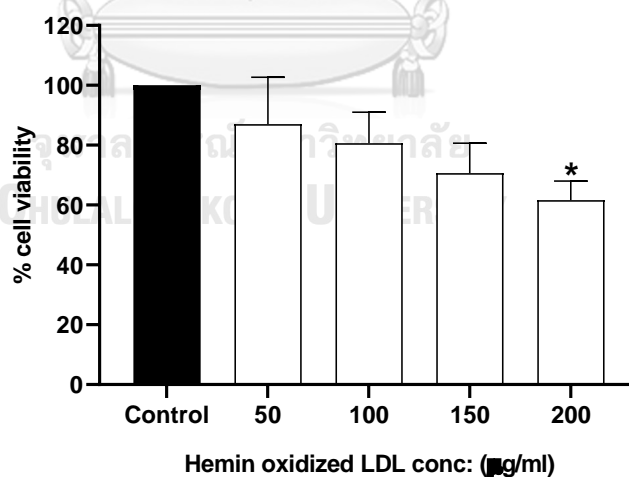
Figure 16. Effect of hemin-oxidized LDL on cell viability of RAW 264.7 macrophage cell. Data was present as mean ± SEM (n=3). $^{*}p<0.05$ compared to control.

11. RAW 264.7 macrophage foam cell formation detection

Both oil red O staining and analysis of cellular lipid density by isopropanol were performed to observe the effect of lusianthridin on foam cell formation. Oil red O (ORO) is the dye which stain the lipid droplet inside the cells. The RAW 264.7 macrophage cells exposed to he-oxLDL showed foam cell phenotype which is characterized by lipid droplet formation (figure 18 (b)). Lipid content in cell incubated with he-oxLDL was significantly different with nLDL (figure 17). Exposure of he-oxLDL pre-treated with 1 and 2 µM lusianthridin reduced the foam cell formation and decreased lipid content significantly different ($p<0.05$ and $p<0.0001$, respectively) from he-oxLDL. However, low concentration of lusianthridin 0.25 and 0.5 µM could not reduce foam cell. Trolox 1 µM, the positive control, also reduced foam cell formation ($p<0.05$). Therefore, it can be concluded that lusianthridin had the protective effect in the formation of foam cell.
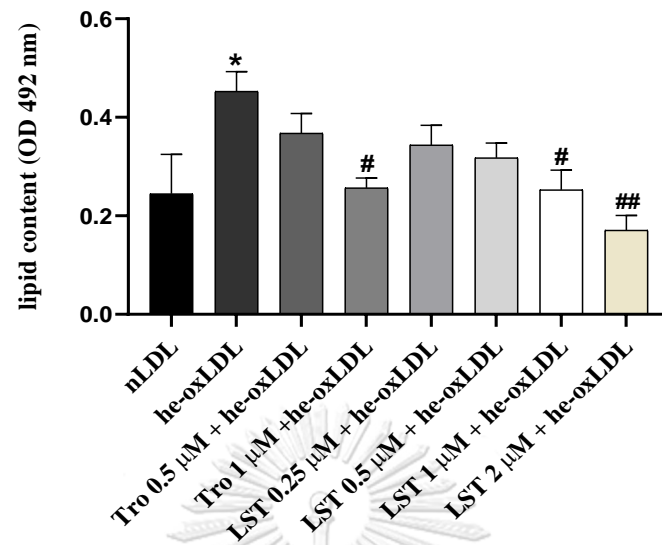
Figure 17. Determination of lipid content in RAW 264.7 macrophage cell exposed to

he-oxLDL. Data was presented as mean ± SEM (n=4). *p<0.05 compared nLDL,

$p^{\#}$<0.05, $p^{\#\#}$<0.0001 compared to he-oxLDL.
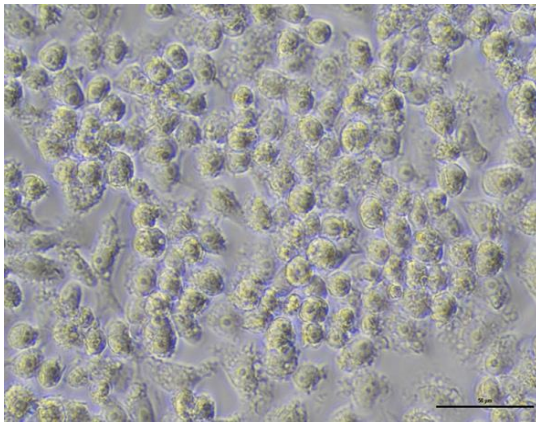
(a)
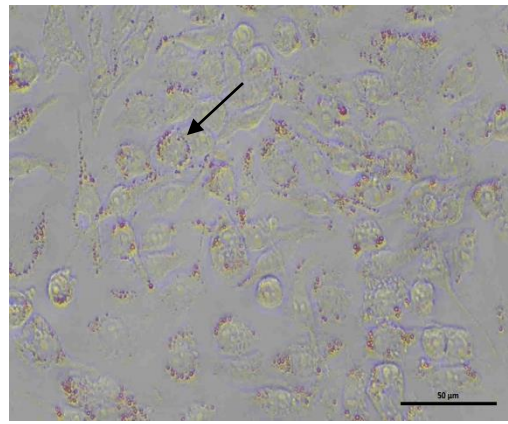
(b)
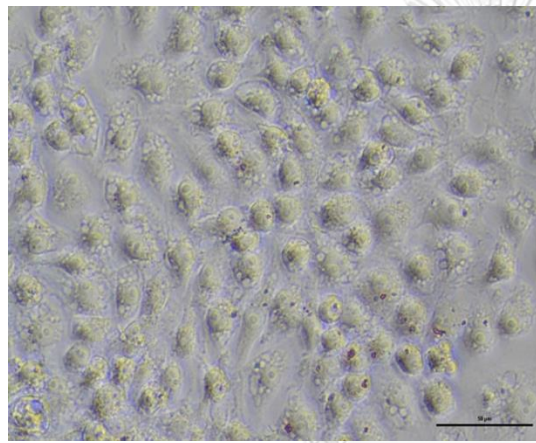
(c)
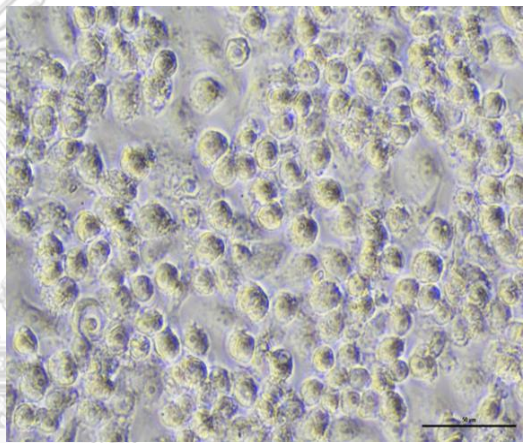
(d)
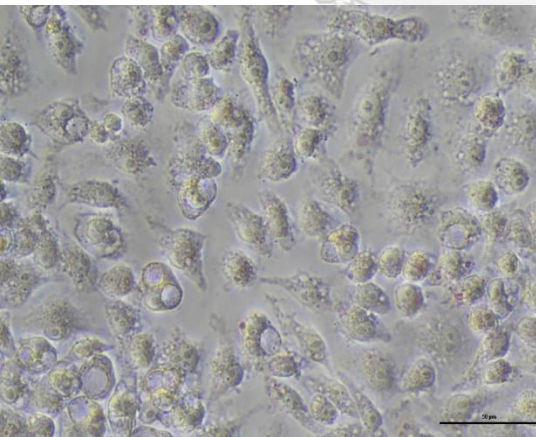
(e)
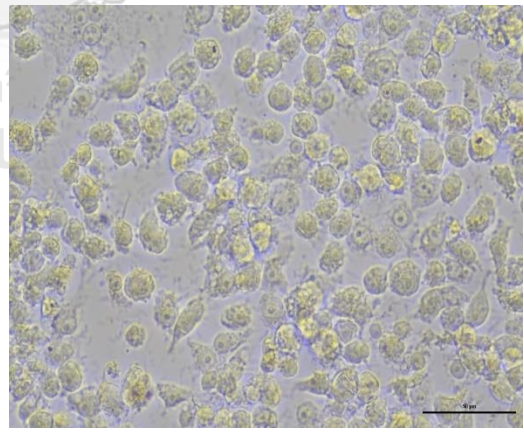
(f)
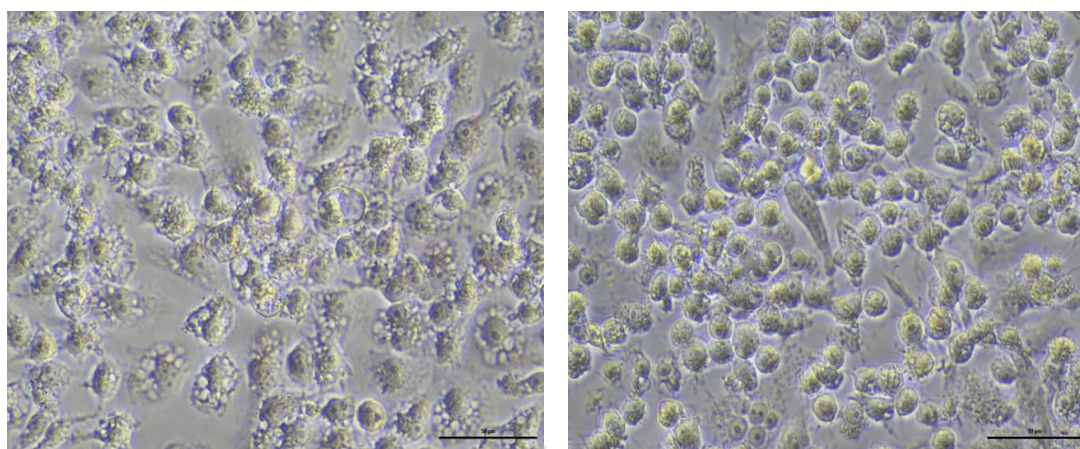
<div align="center">(g)</div> <div align="center">(h)</div>

Figure 18. Representative photographs of oil red O staining in RAW 264.7macrophage cells (x 40 magnification), (a) nLDL, (b) he-oxLDL, (c) TRO 0.5 μM, (d) TRO 1 μM, (e) LST 0.25 μM, (f) LST 0.5 μM, (g) LST 1 μM, (h) LST 2 μM.

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CHAPTER 5

DISCUSSION AND CONCLUSION

Nowadays, atherosclerosis is the most common cause of disease and it is developed all over the world. Modification of LDL is one of the crucial factors in the initiation of atherosclerosis and oxidative stress is one of the important mechanisms in the progression of atherosclerosis. Antioxidants become the potential treatment for the prevention of LDL oxidation. Plants which contain phenanthrene derivative have many biological activities including antioxidant activity. *Dendrobium* species are one of the largest and most important genera in the family Orchidaceae which contain phenanthrene derivatives showed antioxidant activity. According to the result of this study, lusianthridin, a phenanthrene derivative from *Dendrobium venustum* become potential compound for LDL oxidation. In this study, hemin was used as an oxidant to induce LDL oxidation. Hemin can be found in the serum of thalassemia patients and it has been used as a prooxidant in many studies [7]. Miller et al., proved that hemin bind to LDL and initiate lipid peroxidation process of LDL [4]. Hemin release iron (Fe^{3+}) to the hydrophobic core of LDL after incorporation into LDL particles. Fe^{3+} exerts the toxicity through the series of reaction with reactive oxygen species called the Fenton reaction which produces highly toxic hydroxy radical [55]. This hydroxyl radical reacts with cholesteryl ester of lipoprotein and initiate lipid peroxidation process. The most sensitive molecules that undergo peroxidation are membrane phospholipids

containing polyunsaturated fatty acids (PUFAs), including arachidonic, linoleic, linolenic, eicosapentaenoic, and docosahexaenoic acids. Therefore, due to ROS reacting with PUFAs, deprotonation occurs at the double bond, followed by oxygen introduction, promoting lipid peroxide radical formation, which undergoes oxidation forming lipid hydroperoxide (LOOH). LDL oxidation induced by hemin produced oxidative products including malondialdehyde (MDA) which cause oxidatively modified apolipoprotein of LDL [56]. Moreover, changes in lipid composition of LDL also occurred especially in CA and CL comprise polyunsaturated fatty acids (PUFA) which cause more oxidation than monounsaturated and saturated fatty acids [57]. Modification of apolipoprotein which produce negative charge and becomes unrecognized by LDL receptors. Apolipoprotein become recognized and engulfed by scavenger receptors of macrophage and produced foam cell formation. In this study, preparation of he-oxLDL system was used to evaluate the protective effect of lusianthridin on hemin-induced LDL oxidation in *in vitro* and in RAW 264.7 macrophage cells [7]. Oxidative parameters of lipid peroxidation process such as MDA and REM were measured in this study. Moreover, level of lipid composition of LDL and foam cell formation which was caused by modification of apolipoprotein were also measured.

In the present study, hemin cause not only increased in TBARs formation (figure 7) but also modified apolipoprotein of LDL (figure 13). Increasing in TBARs formation might be due to the depletion of CL [58], which is the most abundant and major target of lipid peroxidation process [15]. Increasing REM means that hemin induced protein

oxidation of LDL. Moreover, **α**-tocopherol was rapidly depleted due to its property of chain breaking antioxidant effect to protect against lipid peroxidation. Therefore, **α**-tocopherol suppress the aldehydes formation, according to entrapping free radical intermediates in lipid peroxidation process [59]. When **α**-tocopherol was undetectable, MDA was rapidly increased and lipid compositions of LDL were changed, especially the depletion in cholesteryl arachidonate (CA) and cholesteryl linoleate (CL) together with the increase of oxidized lipid products [58]. CL in LDL core is the most abundant and main target of lipid peroxidation process [15]. Decreasing of CA and CL level and increased in FC level of he-oxLDL might be due to the hydrolysis of ester bond because of the lipoprotein separation procedure using high speed of ultracentrifugation (60,000 rpm). The rotor speed, sample volume, and ultracentrifugation time that include in lipoprotein separation procedure might be influence lipid composition result [60]. According to the result of present study, it can be said that indicated *in vitro* oxidation of LDL was induced by hemin.

When treated he-oxLDL with lusianthridin, 2 µM lusianthridin can inhibit TBARs formation and decreased REM until 12 hr indicating that lusianthridin was able to protect against lipid peroxidation process and also apolipoprotein oxidation in LDL. Interestingly, 2 µM lusianthridin also preserved 71.87 % of CA and 87.72 % of CL level and it seems that lusianthridin could inhibit lipid peroxidation then preservation of CL and CA, the major target of lipid peroxidation [15]. The prevention of depletion of CA and CL level cause reduction of oxidative lipid products and this correction occurred

at lusianthridin 2 μM. The results indicated that lusianthridin has good antioxidant properties. Although, **α**-tocopherol level was rapidly decreased, lusianthridin can inhibit TBARs formation, decreased protein oxidation and preserved lipid content, effectively. From our findings, it can be postulated that lusianthridin act as antioxidant and protect against lipid peroxidation in hemin-induced LDL oxidation.

Additionally, modification of apolipoprotein in LDL oxidation induced by hemin may lead to macrophage foam cell formation. Foam cell formation is the accumulation of lipids and it is the early stage of atherosclerosis. Normally, LDL receptor transport LDL which is the major carrier of cholesterol across the cell membrane. In this process, apolipoprotein of LDL interact with cysteine-rich binding site of LDL receptor and this interaction result in endocytosis of LDL. The modification of apolipoprotein causes the depletion of positive charge of lipoprotein. This condition can decrease the interaction with cysteine binding site of LDL receptor and cause increased in plasma cholesterol and initiate atherosclerosis [61]. Oxidized LDL cause a number of proatherogenic effects such as endothelial cells and monocyte stimulation which cause increased inflammatory cytokine, chemokine, adhesion molecules and stimulation of scavenger receptors of macrophages, lectin like low-density lipoprotein receptor, LOX-1, CD36 and SR-A1 [2, 62]. Oxidized LDL with modification of apolipoprotein was unrecognized by lipoprotein receptor (LDLR) whereas it become engulfed by scavenger receptors of macrophages (LOX-1, CD36 and SR-A1). Therefore, it is assumed that foam cell formation occur due to modification of apolipoprotein.

The detection of foam cell formation on RAW 264.7 macrophage cells by measuring cell lipid content confirmed that nLDL did not produce foam cell formation. Lusianthridin 1 and 2 µM have the potential protective effect on foam cell formation. We found that both 1 and 2 µM of lusianthridin were significantly different with he-oxLDL ($p < 0.05$ and $p < 0.0001$, respectively) and hence it can reduce foam cell formation.

From the result of foam cell formation accessed by oil red O (ORO) staining photographs, we found that RAW 264.7 macrophage cells incubated with he-oxLDL about 24 hr showed the appearance of foam cells with ORO stained lipid droplets (Fig.18 (ii)); in contrast, there was no lipid droplets in untreated cells and treatment with lusianthridin. ORO staining time is important in foam cell formation examination. In the present study, fixation time of cell and staining time with ORO was shorted compared with the previous studies [63-65], in order to obtain the better photographs. Fowler et al., proved that the more staining period (15, 30 min or 1 hr) produced the less staining lipid and this effect might be due to the fact that the lipid was dissolved in isopropanol instead of stained by oil red O [66]. 60 % isopropanol was used in foam cell detection assay according to the method of Suowen et al., in order to assist lipid infiltrating into macrophages and it is also used in destaining step to remove nonspecific staining to produce a clear background [54]. We found that lusianthridin 2 µM has more effect on foam cell formation than trolox, the positive control. Therefore,

lusianthridin had protective effect on hemin-induced in both *in vitro* lipid peroxidation and in RAW 264.7 macrophage cells.

It could be assumed that the protective effects of lusianthridin on hemin-induced LDL oxidation might be come from the presence of phenanthrenes group and phenolic compound since lusianthridin is a plant phenolic compound include in stilbenoids group (phenanthrenes). The presence of hydroxy group on the structure of phenanthrene might be essential for antioxidant activity [67] and this activity might be reduce oxidation of LDL. Furthermore, phenolic compound had the ability of scavenging free radicals. The hydroxy group (-OH) attached with the benzyl ring of phenolic compounds transfer a hydrogen atom from its hydroxyl group and then scavenge the free radicals [68, 69]. Moreover, phenolic compounds have the Fe chelating activity because Fe is known as prooxidant which can cause lipid peroxidation by the Fenton reaction [69]. Fe^{3+} generate the toxic hydroxyl radical [55] which react with cholesteryl ester in hydrophobic core of lipoprotein and initiate lipid peroxidation. Hemin released Fe^{3+} after binding to LDL particles and cause lipid peroxidation. Hence, it could be said that lusianthridin inhibited lipid peroxidation of LDL induced by hemin due to the reduction of Fe^{3+}.

Trolox, the positive control, is a chain breaking antioxidant and contain hydroxyl-bearing aromatic system, one phenolic and one heterocyclic ring which is responsible for antioxidant property. Trolox donate hydrogen atom from the hydroxy group to the peroxyl radical and converts into lipid hydroperoxide and trolox radical

and then terminate the chain reaction [70]. Moreover, the study of phenanthrenes which was extracted from *Ephemerantha lonchophylla* from Orchidaceae family had the inhibitory activity of lipoprotein oxidation [45]. Moreover, Brassicaphenantherene A, the crude extract from Brassica rapa campestris showed the inhibitory of LDL oxidation in TBARs assay at the concentration at 2.9 μM [71]. Therefore, it could be assumed that phenanthrenes compounds has the inhibitory in LDL oxidation. In this study, lusianthridin could reduce the foam cell formation and this effect might be due to the structural activity of lusianthridin and the inhibition of lipid peroxidation effect of lusianthrindin.

In conclusion, this study demonstrated that lusianthridin, phenanthrenes from *Dendrobium venustum* possess anti-lipid peroxidation activity and protected against lipid damage and LDL oxidation induced by hemin. Lusianthridin also showed the potential decrease in macrophage foam cell formation. Taken together lusianthridin might be the beneficial in the prevention of disease related in LDL oxidation and further studies could be performed in atherosclerosis models.

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APPENDIX

1. **Extraction, isolation and identification of lusianthridin**

(A) Plant material

Samples of *Dendrobium venustum* were purchased from Jatujak market Bangkok, in May 2012. Authentication was performed by comparison with herbarium specimens at the Department of National Park, Wildlife and Plant Conservation, Ministry of National Resources and Environment.

(B) Extraction and isolation

The dried and powered whole plant (2 kg) was macerated with MeOH (3 x 10L) to afford a methanol extract (164g) after removal of the solvent. This material was subjected to vacuum-liquid chromatography (VLC) on silica gel (n-hexane-ethyl acetate gradient) to give 8 fractions (A-H). Fraction G was selected for further investigations as it showed antimalarial and antiherpetic effects. Fraction G (16.3 g) was fractionated by CC over (silica gel; CH_2Cl_2 ethyl acetate gradient) and then further purified on Sephadex LH 20 (acetone) to afford lusianthridin (618 mg).

(C) Identification and purity

The structure of the isolate was determined by comparing its spectroscopic data with those reported and the purity was evaluated using NMR spectroscopy.

2. The effect of lusianthridin on TBARs formation (nmol/mg protein)

Condition	Incubation time (hour)					
	0	1	3	6	12	24
nLDL	0.07 ± 0.13	0.03 ± 0.06	0.04 ± 0.13	0.002 ± 0.16	0.08 ± 0.12	0.02 ± 0.13
he-oxLDL	0.17 ± 0.09	0.46 ± 0.08	10.92 ± 3.85	18.25 ± 0.92	17.96 ± 0.92	18.39 ± 0.86
LST 0.25 µM	0.03 ± 0.07	0.61 ± 0.19	4.41 ± 3.42	20.86 ± 0.71	20.4 ± 1.42	21.72 ± 0.95
LST 0.5 µM	0.05 ± 0.08	0.48 ± 0.18	1.2 ± 0.41	8.39 ± 4.21	21.06 ± 1.04	21.59 ± 1.32
LST 1 µM	0.002 ± 0.15	0.38 ± 0.11	0.81 ± 0.19	3.99 ± 3.15	11.74 ± 4.58	13.24 ± 5.17
LST 2 µM	0.2 ± 0.07	0.25 ± 0.22	0.53 ± 0.12	0.76 ± 0.2	0.79 ± 0.13	7.8 ± 3.39
TRO 0.5 µM	0.04. ± 0.04	0.45 ± 0.25	3.44 ± 2.85	12.14 ± 4.68	21.1 ± 1.3	21.27 ± 1.24
TRO 1 µM	0.005 ± 0.06	0.31 ± 0.22	0.52 ± 0.18	0.72 ± 0.21	21.81 ± 1.11	22.55 ± 1.41

3. The effect of lusianthridin on free cholesterol (FC) level (µg/mg protein)

Condition	Incubation time (hour)					
	0	1	3	6	12	24
nLDL	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
he-oxLDL	96.9 ± 1.33	89 ± 2.25	89.8 ± 3.44	92.7 ± 3.89	92.1 ± 4.05	91.2 ± 3.06
LST 0.25 µM	88.5 ± 5.15	87.2 ± 4.59	87.6 ± 6.49	90.1 ± 5.36	89 ± 4.99	91.1 ± 4.65
LST 0.5 µM	98.5 ± 1	84.2 ± 9.21	91.4 ± 3.43	99.2 ± 0.48	90.4 ± 3.72	85.9 ± 4.88
LST 1 µM	98.1 ± 1.03	86.8 ± 6.89	92 ± 4.45	91.16 ± 4.64	92.8 ± 3.83	94.5 ± 3.5
LST 2 µM	98.8 ± 1.08	89.5 ± 5.13	92 ± 4.45	89.64 ± 3.66	93.3 ± 3.43	91.2 ± 3.14
TRO 0.5 µM	96.7 ± 2.9	94.2 ± 2.46	83.1 ± 10.27	91.63 ± 2.53	97.4 ± 2.28	95.5 ± 3.94
TRO 1 µM	98.1 ± 1.68	97.8 ± 1.93	95.1 ± 3.42	95.5 ± 3.85	89.3 ± 4.49	93.5 ± 4.38

4. The effect of lusianthridin on cholesteryl arachidonate (CA) level (µg/mg protein)

Condition	Incubation time (hour)					
	0	1	3	6	12	24
nLDL	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
he-oxLDL	64.5 ± 8.67	50.7 ± 10.31	39.1 ± 5.35	36.7 ± 4.6	20.2 ± 2.68	14.8 ± 3.06
LST 0.25 µM	87.8 ± 5.14	57.3 ± 7.79	66.9 ± 10.7	32.2 ± 8.76	21.3 ± 8.65	39.7 ± 19.74
LST 0.5 µM	95.8 ± 2.23	71.8 ± 5.75	76.7 ± 6.18	75.5 ± 5.22	19 ± 8.36	39 ± 20.22
LST 1 µM	98.7 ± 1.14	67.5 ± 11.26	62.5 ± 10.77	79.7 ± 10.01	64.6 ± 12.15	51.8 ±12,58
LST 2 µM	93.5 ± 5.75	75.4 ± 9.04	80.5 ± 8.01	77.6 ± 7.62	75.1 ± 7	71.8 ± 7.15
TRO 0.5 µM	100 ± 0	58.2 ± 5.46	43.7 ± 3	46.5 ± 8.02	18.7 ± 4.02	13.5 ± 5.4
TRO 1 µM	100 ± 0	83 ± 9.86	63.4 ± 6.96	78.5 ± 8.54	18.1 ± 3.51	12.8 ± 3.39

5. The effect of lusianthridin on cholesteryl linoleate (CL) level (µg/mg protein)

Condition	Incubation time (hour)					
	0	1	3	6	12	24
nLDL	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
he-oxLDL	69.9 ± 6.27	58.9 ± 7.22	65.9 ± 4.76	67.2 ± 8.02	52 ± 5.42	45.3 ± 2.33
LST 0.25 µM	89.2 ± 5.36	69.2 ± 4.6	77.9 ± 7.85	70.4 ± 10.2	57.5 ± 11.25	49.6 ± 12.72
LST 0.5 µM	95.6 ± 3.39	79 ± 4.81	81.2 ± 4.35	8.39 ± 4.21	44.7 ± 16.06	44.26 ± 11.38
LST 1 µM	99.5 ± 0.39	74.3 ± 8.2	68 ± 11.66	92.6 ± 4.71	83 ± 9.03	75.2 ± 9.6
LST 2 µM	91.9 ± 7.24	83.2 ± 5.12	84.1 ± 5.29	88.3 ± 6.03	89.1 ± 5.83	87.2 ± 2.54
TRO 0.5 µM	100 ± 0	64.5 ± 3.35	57.8 ± 4	73.2 ± 7.39	55.1 ± 4.03	53.7 ± 6.9
TRO 1 µM	100 ± 0	85 ± 4.57	76.1 ± 6.07	87 ± 4.4	53.24 ± 4.76	49.4 ± 6.06

6. The effect of lusianthridin on oxidized lipid products (Arbitrary Units)

Condition	Incubation time (hour)					
	0	1	3	6	12	24
nLDL	2763.7 ± 2763.7	9885 ± 5718.9	0 ± 0	1822.2 ± 1822.2	0 ± 0	4464.2 ± 4464.2
he-oxLDL	2110.7 ± 2110.7	0 ± 0	69765.2 ± 21977.9	118065.8 ± 25571.1	101866.8 ± 15061.9	125598.8 ± 17074.9
LST 0.25 µM	0 ± 0	5334.7 ± 5334.7	0 ± 0	92092 ± 10478.7	105790 ± 19226.7	100501 ± 22577.2
LST 0.5 µM	12884.2 ± 12884.2	10735 ± 6197.8	0 ± 0	49921.2 ± 26670.4	97355 ± 14039.1	108319 ± 16086.2
LST 1 µM	3892.2 ± 3892.2	6990.5 ± 6990.5	0 ± 0	6137 ± 6137	52347 ± 30468.7	74664.2 ± 43989.3
LST 2 µM	6797.5 ± 6797.5	0 ± 0	5883.7 ± 5883.7	3957.7 ± 3957.7	4219.5 ± 4219.5	25801.5 ± 22557.7
TRO 0.5 µM	0 ± 0	0 ± 0	0 ± 0	55690.2 ± 32415.9	123507.3 ± 20572.6	135439.5 ± 50284.9
TRO 1 µM	0 ± 0	4213.7 ± 4213.7	0 ± 0	47282.7 ± 41462.8	100113 ± 15665.8	147818 ± 25419.2

7. The effect of lusianthridin on α-tocopherol level (µg/mg protein)

Condition	Incubation time (hour)					
	0	1	3	6	12	24
nLDL	751.5 ± 34	758.7 ± 11	766.8 ± 21	787 ± 54	778.4 ± 58	834.8 ± 74
he-oxLDL	431.4 ± 149.7	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LST 0.25 µM	758.4 ± 79	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LST 0.5 µM	894.3 ± 146.6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LST 1 µM	863.2 ± 125	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LST 2 µM	655.8 ± 229.6	154.5 ± 154.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0
TRO 0.5 µM	860.6 ± 113.5	137.3 ±137.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0
TRO 1 µM	760 ± 34.2	575.7 ± 198	0 ± 0	0 ± 0	0 ± 0	0 ± 0

8. The effect of lusianthridin on relative electrophoretic mobility

Condition	Incubation time (hour)					
	0	1	3	6	12	24
nLDL	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
he-oxLDL	1.06 ± 0.05	1.07 ± 0.04	1.2 ± 0.05	1.3 ± 0.07	1.3 ± 0.05	1.39 ± 0.08
LST 0.25 µM	0.98 ± 0.03	1.05 ± 0.06	1.21 ± 0.06	1.23 ± 0.04	1.4 ± 0.04	1.34 ± 0.07
LST 0.5 µM	0.96 ± 0.04	1.01 ± 0.05	1.1 ± 0.04	1.21 ± 0.05	1.28 ± 0.04	1.34 ± 0.09
LST 1 µM	0.97 ± 0.03	0.99 ± 0.05	1.06 ± 0.04	1.07 ± 0.05	1.12 ± 0.05	1.16 ± 0.05
LST 2 µM	0.97 ± 0.05	1 ± 0.05	1.05 ± 0.05	1.05 ± 0.07	1.05 ± 0.04	1.09 ± 0.07
TRO 0.5 µM	1 ± 0.04	1 ± 0.02	1.13 ± 0.02	1.25 ± 0.06	1.34 ± 0.04	1.41 ± 0.09
TRO 1 µM	0.09 ± 0.03	0.99 ± 0.04	1.08 ± 0.02	1.08 ± 0.05	1.26 ± 0.05	1.35 ± 0.08

9. The effect of lusianthridin on RAW 264.7 macrophage cell viability

Condition	% cell viability (Mean)	SEM
Control	100	0.0
LST 0.5 µM	84	3.4
LST 1 µM	82	8.9
LST 2 µM	79	11.1
LST 5 µM	76	9.1
LST 10 µM	80	7.1
LST 20 µM	78	2.3
LST 50 µM	62	4.9
LST 100 µM	37	3.4

10. The effect of hemin-oxidized LDL on RAW 264.7 macrophage cell viability

Condition	% cell viability (Mean)	SEM
Control	100	0.0
he-oxLDL 50 µg/ml	87	15.6
he-oxLDL 100 µg/ml	81	10.3
he-oxLDL 150 µg/ml	71	9.9
he-oxLDL 200 µg/ml	62	6.4

11. The effect of lusianthridin on foam cell formation

Condition	Lipid content (Mean)	SEM
nLDL	0.245	0.08
he-oxLDL	0.453	0.04
he-oxLDL + LST 0.25 µM	0.344	0.04
he-oxLDL + LST 0.5 µM	0.318	0.03
he-oxLDL + LST 1 µM	0.253	0.04
he-oxLDL + LST 2 µM	0.171	0.03
he-oxLDL + TRO 0.5 µM	0.368	0.04
he-oxLDL + TRO 1 µM	0.257	0.02

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