CHAPTER II: MATERIALS AND METHODS

2.1 Specimen collection and evaluation

Blood samples were donated from ten healthy normolipidemic and ten diabetic hyperlipidemic volunteers after a 12 hours overnight fasting. Blood were collected in sterile tubes containing 1 mg/mL EDTA and 10 μM butylated hydroxytoluene (BHT) for LDL preparation, 2.5 mg/mL NaF for glucose analysis and clotted blood for lipid analysis (Young NL, *et al.*, 1985). Plasma and serum were separated immediately from cells by centrifugation at 2,000 rpm, 4° C for 15 minutes and stored in 4° C until analysis within 7 days.

All samples were measured the glucose levels and serum total cholesterol (TC), triacylglycerol (TG) by the glucose oxidase technique and enzymatic methods, respectively. High-density lipoprotein cholesterol (HDL-C) was measured by phosphotungstate precipitation of other non-HDL fractions and then measured the remaining cholesterol in the supernatant. All analyses were done by Hitachi 910 autoanalyzer with Boehringer Mannheim original reagents. Low-density lipoprotein (LDL) cholesterol was calculated by Friedewald's formula: [LDL-cholesterol]=[total cholesterol]-[HDL cholesterol]-([triglycerides]/5).

2.2 U937 cell preparation and proliferation assay

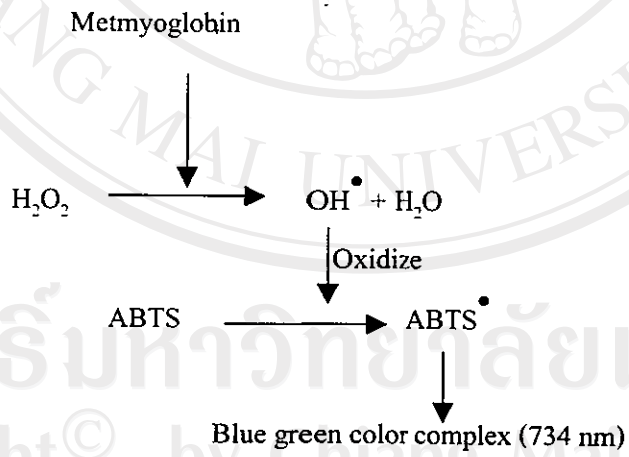
The myelomonocytic cell line, U937 was provided by Professor Dr. Watchara Kasinrerk, Department of Clinical Immunology, Faculty of Associated Medical Sciences. U937 cells were grown in 25 cm^2 T-flask in RPMI 1640 medium supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin, 2.5 μg/mL Fungizone and 10% heat-inactivated fetal calf serum (FCS) and kept in a fully humidified air atmosphere containing 5% CO_2 at 37°C (Frostegard J, *et al.*, 1990). Fresh medium was changed three times a week and cells were cultured at a density between 10^6 cells/ml. This culture medium was freshly prepared from a stock solution obtained from Flow Laboratories at the Department of Clinical Microbiology, Faculty of Associated Medical Sciences and stored at 4° C. For experiments, cells were washed three times with RPMI1640 without FCS. The cells viability was assessed by trypan blue exclusion method and the viability of the final preparation should be greater than 90% cell confluency.

2.3 Antioxidants preparation

The α-tocopherol and ascorbic acid were purchased from Sigma-Aldrich. Curcuminoids was provided by Dr. Songyot Anuchapreeda, Department of Clinical Microscopy, Faculty of Associated Medical Sciences, Chiang Mai University. Curcuminoids and α-tocopherol were initially solubilized in 10% DMSO at final concentration of 1 mg/mL, the next dilution was adjusted to the concentration of 0.5% DMSO. Ascorbic acid was dissolved in distilled water at final concentration of 1 mg/mL. All of antioxidant solutions were freshly prepared, protected from light and kept in the dark, at 4° C and used for antioxidant experiments within 30 minutes.

2.4 Total antioxidant capacity (TAC) of curcuminoids, α-tocopherol and ascorbic acid by ABTS method

The principle of the method is a colorimetric assay utilizes the quenching of 2,2 – azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) by oxidation of free radical cation which is formed in the presence of metmyoglobin having peroxidase activity according to the reaction below (Miller NJ, *et al.*, 1993).



The chromogen ABTS is incubated at 37° C with a peroxidase (metmyoglobin) and hydrogen peroxide to produce the ABTS radical cation. The blue-green ABTS radical is detected at 734 nm. Antioxidants in the sample will delay the formation of the radical cation to a degree which is proportional to their concentrations. Values are expressed as mM.

The total antioxidant capacity of curcuminoids, α-tocopherol and ascorbic acid were determined by ABTS method using Trolox, a water-soluble vitamin E analog as a standard. The results are expressed as Trolox equivalent capacity.

Procedure

1. UV-spectrophotometer (Shimadzu-160A) Operation are as follows :

MODE KINETIC

λ = 734 nm

UPPER = +2.00A LOWER = +0.00A

LAG T = 0 SEC RATE = 300 SEC

INTERVAL T = 60 SEC

- FACTOR = 1.000
- SAMPLE NO. = 1

GAIN x 10 NO.

2. Pipette the following solutions into a series of polystyrene microcuvette path length 1 cm :

Reagent	Blank (μL)	Test (μL)
Sample (or standard)	-	10
Metmyoglobin	40	40
100 μM ABTS	300	300
5 mM PBS	510	500

3. Transfer to UV-spectrophotometer, and 500 μM H_2O_2 250 μL and then mix immediately to start the reaction.

4. Calculate %inhibition of oxidation (see appendix D) of each sample and obtain the TAC equivalent to Trolox from calibration curve.

Calibration curve

1. Dilute stock 2.5 mM Trolox standard with 5 mM PBS to make various concentrations as follows.

Trolox (mM)	0.625	1.25	1.875
5 mM PBS (μL)	300	200	100
2.5 mM Trolox (μL)	100	200	300

2. Perform the test in the same way as sample.

3. Calculate the % inhibition of oxidation.

4. Plot the % inhibition versus concentration in mM of Trolox on a graph paper.

2.5 Cytotoxic effect of curcuminoids, α-tocopherol and ascorbic acid on U937 cells

Immediately prior to the experiments, the curcuminoids was filtered and sterilized through a 0.45 μm cellulose filter. U937 cells were cultured for 3 days and adjusted cell concentration to 1.0 x 10^6 cells/mL in completed RPMI-1640 medium and then the cells were treated with curcuminoids at the final concentrations to 0, 10, 20, 30, 40, 50, 60, 80 and 100 μg/mL. Cells were cultured at 37° C, in 5% CO_2 for 24 and 48 hours (Limtrakul P, *et al.*, 1997). At the end of 24 and 48 hours, U937 cells were washed twice with incompleted RPMI-1640 medium and then centrifuged at 2,000 rpm for 10 minutes. The pellet was resuspended and diluted to 1.0 x 10^5 cells/mL in PBS buffer (pH 7.2). The cell viability was observed by trypan blue exclusion method and counted cells as the percent of total cells.

Cytotoxicity tests of α-tocopherol and ascorbic acid were observed in the same way.

2.6 LDL preparation and protein determination

2.6.1 LDL preparation

Separation of LDL from other lipoproteins by single vertical discontinuous density gradient ultracentrifugation was performed. EDTA blood was collected and centrifuged at 2,000 rpm for 15 minutes. The plasma density was adjusted to 1.31 g/mL by addition of solid KBr (0.485 g/mL). Three mL of density-adjusted plasma was layered under 6.0 mL of 0.154 M NaCl,

placed in Beckman 70.1Ti fixed-angle rotor, and centrifuged in a Beckman L-60 ultracentrifuge at 50,000 rpm, 10° C for 150 minutes (Chung BH, *et al.*,1980). After centrifugation, the tubes were removed from rotor and three main lipoprotein fractions; VLDL in the upper layer; LDL in the middle layer; HDL in the bottom layer, were separated. The yellow LDL band located in the middle portion was collected by puncturing the tube and aspirating into a syringe. Residual KBr and plasma-derived small-molecular-weight contaminants in the LDL preparation were removed by dialyzed against deoxygenated PBS containing chloramphenicol (0.1 g/L) and EDTA (1.0 g/L) pH 7.4 in the dark at 4° C for 24 hours under nitrogen gas (Esterbauer H, *et al.*, 1992). LDL protein was determined by a Lowry's method using BSA as the standard. Isolated LDL was used immediately after preparation and sterilizing through a 0.45 μm cellulose filter. LDL preparation were stored at 4° C in the dark under nitrogen gas which can be used within 1 week (Jessup W, *et al.*, 1990 and Wheeler DC, et al., 1994).

2.6.2 LDL protein determination

LDL protein was determined by the Lowry's method based on the reactivity of the peptide nitrogen with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteau phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The Lowry's method is sensitive to pH change and therefore the pH of assay solution should be maintained at 10-10.5 (Lowry OH, *et al.*, 1951).

Procedure

1. Set up triplicate of eleven sets 13 x 100 mm test tubes in a rack.
2. Add 100 μL of sample and standard BSA (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μg) to these tubes.
3. Add 1.0 mL of Lowry stock reagent (see Appendix C) to each test tube.
4. Incubate for 30 minutes at room temperature.
5. Add 100 μL of Folin's reagent to each tube, vortex each tube immediately.
6. Incubate at room temperature for 30 minutes.
7. Determine absorbance of each sample at 600 nm.
8. Plot absorbance versus mg protein to obtain standard curve.

9. Set up triplicate assays for all "unknowns" and read the concentrations from standard curve.

2.7 LDL oxidation preparation and evaluation

LDL sample was dialyzed against deoxygenated PBS-free EDTA buffer containing chloramphenicol (0.1 g/L) pH7.4 in the dark at 4° C for 24 hours under nitrogen gas to remove EDTA (Hessler JR, *et al.*, 1983). The LDL were diluted with PBS free-EDTA to a standard concentration of 200 μg/mL protein. LDL oxidation was initiated by the addition of freshly prepared $CuSO_4$ at final concentration of 20 μM at 37° C for 2 hours. At the time points, LDL oxidation was stopped by butylated hydroxytoluene (BHT, a radical-trapping antioxidant) and EDTA to final concentrations of 40 μM and 5 mM, respectively (Heather MK, *et al.*, 2002).

LDL oxidation was evaluated by 3 different ways, (1) the mobility of ox-LDL on cellulose acetate electrophoresis, (2) measuring the increase in absorbance at 234 nm by continuous monitoring the formation of conjugated dienes and (3) measuring the amount of malonaldehyde (MDA) formation as thiobarbituric acid reactive substance (TBARs) over 2 hours of incubation.

2.7.1 Lipoprotein electrophoretic analysis

Electrophoresis was used to validate the purity of the LDL and ox-LDL fractions compared with plasma lipoprotein fractions. Cellulose acetate electrophoresis was performed on plasma samples compared with the LDL isolation (Ericson C, *et al.*, 1975). Cellulose acetate plates (76×90 mm) were soaked in 38 mM Tris, 46 mM Na-barbitone, 16 mM diethylbarbituric acid (pH 8.6) buffer for 30 minutes before sample application. Electrophoresis was performed in the same buffer at 180 V for 20 minutes. Plates were stained in 30 mL, 1% (w/v) Sudan black B in methanol/10 mL 1 M KOH for 4 hours, followed by destaining in 10% acetic acid in 50% methanol and wiping with distilled water.

2.7.2 Determination of conjugated diene formation

LDL sample was dialyzed against deoxygenated PBS-free EDTA buffer containing chloramphenicol (0.1 g/L) pH 7.4 in the dark at 4° C for 24 hours under nitrogen gas to remove EDTA. For the oxidation experiments, the LDL preparations were diluted with PBS-free EDTA buffer (pH 7.4) to contain 200 μg/mL of protein. The oxidation was started by adding of 20 μM $CuSO_4$ at the final concentration in LDL solution in 1-cm quartz cuvettes. The kinetics of the oxidation of LDL was determined by monitoring the absorbance changes in 234-nm at 37° C by a Shimadzu UV 1601A spectrophotometer, equipped with a 6-position automatic cell changer fitted with a Peltier element to keep the temperature at 37° C (Esterbauer H., *et al.*, 1989). The absorbance changes were recorded every 5 minutes for 2 hours after initiating oxidation with copper. Several characteristic indices were obtained from the resulting absorbance versus time curves. The lag time was determined to be the intercept of the slopes for the lag and propagation phases. The experiment included one control, using LDL without $CuSO_4$.

2.7.3 Determination of Thiobarbituric acid reactive substances (TBARs) formation

The basic principle method is the reaction of one molecule of malonaldehyde and two molecules of TBA form a red malonaldehyde-TBA complex, which can be quantitated spectrophotometrically. However, this method has been criticized as being nonspecific and insensitive for the detection of low levels of malonaldehyde. Other TBA-reactive substances (TBARs) including sugars and other aldehydes could interfere with the malonaldehyde-TBA reaction. Abnormally, low values may result if some of the malonaldehyde reacts with proteins in an oxidizing system. In many cases, however, the TBARs test is applicable for comparing samples of a single material at different states of oxidation.

The amount of malonaldehyde was measured as TBARs. Briefly, 1.0 mL of 20% trichloroacetic acid was added to 500 μL of a solution containing 200 μg protein of LDL or LDL oxidation, and the mixture was vortexed. Thiobarbituric acid (1%) 1.0 mL was then added, mixed and incubated at 100°C for 30 minutes, cooled, and centrifuged at 1,000 *g* for 20 minutes. The absorbance of the supernatant at 532 nm was determined by using a Shimadzu UV 1601A spectrophotometer. The experiment included one controls, using $CuSO_4$ at the final concentration

of 20 μM. The amount of TBARs was expressed as malondialdehyde (MDA) equivalents, using an extinction coefficient of MDA at 532 nm 1.56 105 $M^{-1}cm^{-1}$ (Puhl, H, *et al.*, 1994).

2.8 Oxidative susceptibility of LDL oxidation in healthy with normolipidemic and diabetic with hyperlipidemic groups

The oxidative susceptibility of LDL oxidation were evaluated by 3 different ways, (1) measuring the percent cellular LDL uptake by U937 cell, (2) measuring the increase in absorbance at 234 nm by continuous monitoring the formation of conjugated dienes and (3) measuring the amount of malonaldehyde (MDA) formation as thiobarbituric acid reactive substance (TBARs) over 2 hours of incubation.

2.8.1 Determination of cellular LDL uptake by U937 cells

Immediately prior to the experiments, LDL and ox-LDL from healthy and diabetic groups were dialyzed against deoxygenated PBS-free EDTA containing chloramphenicol (0.1 g/L) pH7.4 in the dark at 4° C for 24 hours under nitrogen gas. The LDL and ox-LDL were diluted with PBS-free EDTA to a final concentration of 200 μg/mL protein and sterilized through a 0.45 μm cellulose filter.

U937 cells were washed three times with incompleted RPMI1640. They were then resuspended in fresh completed RPMI medium supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin, 2.5 μg/mL fungizone, without 10% heat-inactivated FCS and incubated at a fully humidified air atmosphere containing 5% CO_2 at 37° C for 24 hours (Frostegard J, *et al.*, 1990). Cells were adjusted to density 1 x 10^6 cells/mL, when cells were grown to 90% confluency, fresh cultures were initiated. These cells were seeded out in sterile screw capped tube with 1 mL in fresh completed RPMI medium, without 10% heat-inactivated FCS. LDL and ox-LDL were incubated with cells, and added to the culture medium to a final concentration of 100 μg protein/mL (Frostegard J, *et al.*, 1990). Incubation were then carried out for 24 hours at 37° C in a humidified atmosphere containing 5% CO_2. Control tube, from which U937 cells was incubated under identical conditions (Elaine JB, *et al.*,1997). At the end of the incubation period, the cells were washed twice in PBS with centrifugation at 2000 rpm for 10 minutes at 4° C. These cells were inoculated on glass-slide and fixed with neutral formalin. Cells were observed

under simple light microscope, original magnification x 100, stained with oil red O and Mayer's hematoxylin. The oxidative susceptibility of LDL oxidation were determined as cellular LDL uptake and comparison between healthy and diabetic groups were done.

2.8.2 Determination of conjugated diene formation

The lag time of LDL oxidation were determined in the topic as 2.7.2. for comparison between healthy and diabetic groups.

2.8.3 Determination of thiobarbituric acid reactive substances (TBARs) formation

The MDA concentrations were determined in the topic as 2.7.3. for comparison between healthy and diabetic groups.

2.9 Effect of curcuminoids on inhibition of Cu^{2+} mediated LDL oxidation

2.9.1 Comparison between healthy with normolipidemic and diabetic with hyperlipidemic groups

Effect of curcuminoids on inhibition of LDL oxidation in healthy and diabetic groups were evaluated by 3 different ways, (1) measuring the percent cellular LDL uptake by U937 cell, (2) measuring the increase in absorbance at 234 nm by continuous monitoring the formation of conjugated dienes and (3) measuring the amount of malonaldehyde (MDA) formation as thiobarbituric acid reactive substance (TBARs) over 2 hours of incubation.

2.9.1.1 Determination of cellular LDL uptake by U937 cells

To evaluate the ability of curcuminoids to inhibit copper Cu^{2+}-induced LDL oxidation, LDL was prepared as the topic 2.8.1. The oxidation was started by adding the final concentration of freshly prepared 20 μM $CuSO_4$ in the presence or absence of curcuminoids at final concentration of 30 μg/mL at 37°C for 2 hours. Prior to the cellular uptake LDL experiments, all sample preparations were passed through a Sephadex G-25 column to remove $CuSO_4$ and curcuminoids. The cellular LDL uptake were determined as the topic 2.7.1. to compare in healthy and diabetic group. The experiment included three controls, cells control (U937 cells alone),

native LDL control (LDL without $CuSO_4$ and antioxidant), and ox-LDL control (LDL in the presence of $CuSO_4$ without antioxidant)(Table 1).

Table 1. Experimental design for studying the effect of curcuminoids on inhibition of LDL oxidation determined by U937 cellular LDL uptake.

Reagents	Cur	Cell control	LDL control	ox-LDL control
210 µg/mL LDL	1900 µL	-	1900 µL	1900 µL
1 mg/mL Curcuminoids	60 µL	-	-	-
1mM $CuSO_4$	40 µL	-	-	40 µL
PBS	-	-	100 µL	60 µL
Incubated 37°C for 2 hours				
Passed through a Sephadex G-25 column				
Transfer to sterile screw capped tube	↓ 500 µL	↓ -	↓ 500 µL	↓ 500 µL
Completed RPMI 1640 medium without FCS	400 µL	900 µL	400 µL	400 µL
10^7 cells/mL U937 cells	100 µL	100 µL	100 µL	100 µL
Incubate at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. Cell stained with oil red O and Mayer's hematoxylin.				

Cur = Curcuminoids

2.9.1.2 Determination of conjugated diene formation

LDL preparations from healthly and diabetic groups were diluted with PBS-free EDTA buffer (pH 7.4) to contain 200 µg/mL of protein. The oxidation was started by adding to the final concentration of freshly prepared 20 µM $CuSO_4$ in the presence of curcuminoids at the final concentration of 30 µg/mL. The lag times of conjugated diene formation were determined as the topic 2.7.2. The experiment included native LDL control and ox-LDL control (Table 2).

Table 2. Experimental design for studying the effect of curcuminoids on inhibition of LDL oxidation determined by conjugated dienes formation.

Reagents	Cur	LDL control	ox-LDL control
210 µg/mL LDL	950 µL	950 µL	950 µL
1 mg/mL Curcuminoids	30 µL	-	-
1mM $CuSO_4$	20 µL	-	20 µL
PBS		50 µL	30 µL
The formation of conjugated dienes determined by spectrophotometer at 234 nm			

Cur = Curcuminoids

2.9.1.3 Determination of Thiobarbituric acid reactive substances (TBARs) formation

For the oxidation experiments, the LDL preparations from healthly and diabetic groups were diluted with PBS-free EDTA buffer (pH 7.4) to contain 200 µg/mL of protein. The oxidation was started by adding the final concentration of freshly prepared 20 µM $CuSO_4$ in the presence of curcuminoids at the final concentration of 30 µg/mL. At time points (2 hours) each aliquots was taken and the oxidation was stopped by butylated hydroxytoluene (BHT, a radical-trapping antioxidant) and EDTA to final concentrations of 40 µM and 5 mM, respectively. The TBARs was measured as the topic 2.7.3. The experiment included native LDL control and ox-LDL control (Table 3).

Table 3. Experimental design for studying the effect of curcuminoids on inhibition of LDL oxidation determined by TBARs formation.

Reagents	Cur	LDL control	ox-LDL control
210 μg/mL LDL	950 μL	950 μL	950 μL
1 mg/mL Curcuminoids	30 μL	-	-
1mM $CuSO_4$	20 μL	-	20 μL
PBS	-	50 μL	30 μL
Stop reaction by addition of BHT 40 μM and EDTA 5 mM			
Transfer to test tube	↓	↓	↓
	500 μL	500 μL	500 μL
20% Trichloroacetic acid	1.0 mL	1.0 mL	1.0 mL
1% Thiobarbituric acid	1.0 mL	1.0 mL	1.0 mL
Incubate at 100°C for 30 minutes Read absorbance at 532 nm			

Cur = Curcuminoids

2.9.2 Comparison with α-tocopherol and ascorbic acid

Effect of curcuminoids on inhibition of LDL oxidation in healthly and diabetic groups were evaluated by 3 different ways, (1) cellular LDL uptake percentage by U937 cells, (2) measuring the increase in absorbance at 234 nm by continuous monitoring of conjugated dienes formation and (3) measuring the amount of malonaldehyde (MDA) formation as thiobarbituric acid reactive substance (TBARs) over 2 hours of incubation.

2.9.2.1 Determination of cellular LDL uptake by U937 cells

For the oxidation experiments, the LDL preparations from healthly and diabetic groups were diluted with PBS-free EDTA buffer (pH 7.4) to contain 200 μg/mL of protein. Oxidation was started by adding freshly prepared $CuSO_4$ to the final concentration of 20 μM in the presence

or absence of curcuminoids, α-tocolpherol and ascorbic acid at final concentration of 30 μg/mL at 37° C for 2 hours. Prior to the cellular uptake LDL experiments, all sample preparations were passed through a Sephadex G-25 column to remove $CuSO_4$ and curcuminoids. The cellular LDL uptake were determined as the topic 2.9.1.1 to compare between curcuminoids with α-tocopherol and ascorbic acid in healthy and diabetic groups. The experiment included three controls, cells control, native LDL control and ox-LDL control (Table 4).

Table 4. Experimental design for studying the effect of curcuminoids compared with α-tocopherol and ascorbic acid on inhibition of LDL oxidation determined by U937 cellular LDL uptake.

Reagents	Cur	E	C	Cell control	LDL control	ox-LDL control
210 μg/mL LDL	1900 μL	1900 μL	1900 μL	-	1900 μL	1900 μL
1 mg/mL Curcuminoids	60 μL	-	-	-	-	-
1 mg/mL α-Tocopherol	-	60 μL	-	-	-	-
1 mg/mL Ascorbic acid	-	-	60 μL	-	-	-
1mM $CuSO_4$	40 μL	40 μL	40 μL	-	-	40 μL
PBS	-	-	-	-	100 μL	60 μL
Incubated 37° C for 2 hours						
Passed through a Sephadex G-25 column						
Transfer to sterile screw capped tube	↓ 500 μL	↓ 500 μL	↓ 500 μL	↓ -	↓ 500 μL	↓ 500 μL
Completed RPMI 1640 medium without FCS	400 μL	400 μL	400 μL	900 μL	400 μL	400 μL
10^7 cells/mL U937 cells	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL
Incubate at 37° C in a humidified atmosphere containing 5% CO_2 for 24 hours. Cell stained with oil red O and Mayer's hematoxylin.						

Cur = Curcuminoids , E = α-Tocopherol and C = Ascorbic acid

2.9.2.2 Determination of conjugated diene formation

The oxidation was started by adding freshly prepared $CuSO_4$ to the final concentration of 20 μM in the presence of curcuminoids, α-tocopherol and ascorbic acid at final concentrations of 30 μg/mL. The lag times of LDL oxidation were determined as the topic 2.9.1.2 to compare between curcuminoids with α-tocopherol and ascorbic acid in healthy and diabetic groups. The experiment included native LDL control and ox-LDL control (Table 6).

Table 5. Experimental design for studying the effect of curcuminoids compared with α-tocopherol and ascorbic acid on inhibition of LDL oxidation determined by conjugated dienes formation.

Reagents	Cur	E	C	LDL control	ox-LDL control
210 μg/mL LDL	950 μL	950 μL	950 μL	950 μL	950 μL
1 mg/mL Curcuminoids	30 μL	-	-	-	-
1 mg/mL α-Tocopherol	-	30 μL	-	-	-
1 mg/mL Ascorbic acid	-	-	30 μL	-	-
1mM $CuSO_4$	20 μL	20 μL	20 μL	-	20 μL
PBS	-	-	-	50 μL	30 μL
The formation of conjugated dienes determined by spectrophotometer at 234 nm					

Cur = Curcuminoids , E = α-Tocopherol and C = Ascorbic acid

2.9.3 Determination of Thiobarbituric acid reactive substances (TBARs) formation

For the oxidation experiments, the LDL preparations from healthy and diabetic groups were diluted with PBS-free EDTA buffer (pH 7.4) to contain 200 μg/mL of protein. The oxidation was started by adding freshly prepared $CuSO_4$ to the final concentration of 20 μM in the presence of curcuminoids, α-tocopherol and ascorbic acid at final concentrations of 30 μg/mL. At time points (2 hours) aliquots were taken and oxidation was stopped by addition of butylated hydroxytoluene (BHT, a radical-trapping antioxidant) and EDTA to final concentrations of 40 μM and 5 mM respectively. The TBARs was measured as the topic 2.7.3 to compare between

curcuminoids with α-tocopherol and ascorbic acid in healthy and diabetic group. The experiment included native LDL control and ox-LDL control (Table 6).

Table 6. Experimental design for studying the effect of curcuminoids compared with α-tocopherol and ascorbic acid on inhibition of LDL oxidation determined by TBARs formation.

Reagents	Cur	E	C	LDL control	ox-LDL control
210 μg/mL LDL	950 μL	950 μL	950 μL	950 μL	950 μL
1 mg/mL Curcuminoids	30 μL	-	-	-	-
1 mg/mL α-Tocopherol	-	30 μL	-	-	-
1 mg/mL Ascorbic acid	-	-	30 μL	-	-
1mM $CuSO_4$	20 μL	20 μL	20 μL	-	20 μL
PBS	-	-	-	50 μL	30 μL
Stopped by addition of BHT 40 μM and EDTA 5 mM					
Transfer to test tube	↓	↓	↓	↓	↓
	500 μL	500 μL	500 μL	500 μL	500 μL
20% Trichloroacetic acid	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL
1% Thiobarbituric acid	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL
Incubate at 100°C for 30 minutes					
Read absorbance at 532 nm					

Cur = Curcuminoids , E = α-Tocopherol and C = Ascorbic acid

2.10 Effect of curcuminoids on ox-LDL determined by U937 cellular LDL uptake

2.10.1 Comparison between healthy with normolipidemic and diabetic with hyperlipidemic groups

The ox-LDL were prepared as the topic 2.7, then diluted with PBS-free EDTA buffer (pH 7.4) to contain 200 μg/ml of protein. Curcuminoids was added to the ox-LDL a final concentration of 30 μg/mL and incubated at 37° C for 2 hours. All sample preparations were passed through a Sephadex G-25 column to remove $CuSO_4$ and curcuminoids. The cellular LDL uptake were determined as the topic 2.9.1.1 to compare between healthy and diabetic groups. The experiment included three controls, cells control, native LDL control and ox-LDL control (Table 7).

2.10.2 Comparison with α-tocopherol and ascorbic acid

Curcuminoids, α-tocopherol and ascorbic acid were added to the ox-LDL at the final concentration of 30 μg/mL and incubated at 37° C for 2 hours. All sample preparations were passed through a Sephadex G-25 column to remove $CuSO_4$ and antioxidants. The cellular uptake LDL were determined as the topic 2.9.1.1 to compare between curcuminoids with α-tocopherol and ascorbic acid in healthy and diabetic groups. The experiment included three controls, cells control, native LDL control and ox-LDL control (Table 7).

Table 7. Experimental design for studying the effect of curcuminoids compared with α-tocopherol and ascorbic acid on ox-LDL determined by U937 cellular LDL uptake.

Reagents	Cur	E	C	Cell control	LDL control	ox-LDL control
210 μg/mL LDL	1900 μL	1900 μL	1900 μL	-	1900 μL	1900 μL
1mM $CuSO_4$	40 μL	40 μL	40 μL	-	-	40 μL
PBS	60 μL	60 μL	60 μL	-	60 μL	60 μL
Incubated 37°C for 2 hours						
LDL oxidation	970 μL	970 μL	970 μL	-	1000 μL	1000 μL
1 mg/mL Curcuminoids	30 μL	-	-	-	-	-
1 mg/mL α-Tocopherol	-	30 μL	-	-	-	-
1 mg/mL Ascorbic acid	-	-	30 μL	-	-	-
Incubated 37°C for 2 hours						
Passed through a Sephadex G-25 column						
Transfer to sterile screw capped tube	↓ 500 μL	↓ 500 μL	↓ 500 μL	↓ -	↓ 500 μL	↓ 500 μL
Completed RPMI 1640 medium without FCS	400 μL	400 μL	400 μL	900 μL	400 μL	400 μL
10^7 cells/mL U937 cells	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL
Incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. Cell stained with oil red O and Mayer's hematoxylin.						

Cur = Curcuminoids , E = α-Tocopherol and C = Ascorbic acid

LDL uptake

The cells were washed with RPMI 1640 medium (serum free) twice. Cells in culture were fixed on slides with 10% formaldehyde for 10 min followed by washing twice with PBS. The cells were pretreated with 60% 2-propanol for 2 min and then stained with 0.2% oil red O (Sigma) in 60% 2-propanol for 10 min. The slides were washed with 2-propanol and then with PBS. Finally, the cells were treated with Mayer's hematoxylin for 5 min to stain nuclei (Mori M, *et al.*, 2001). After washing with distilled water, the slide was mounted in glycerin jelly, then U937 cellular LDL uptake were observed under simple light microscope, original magnification x 100.

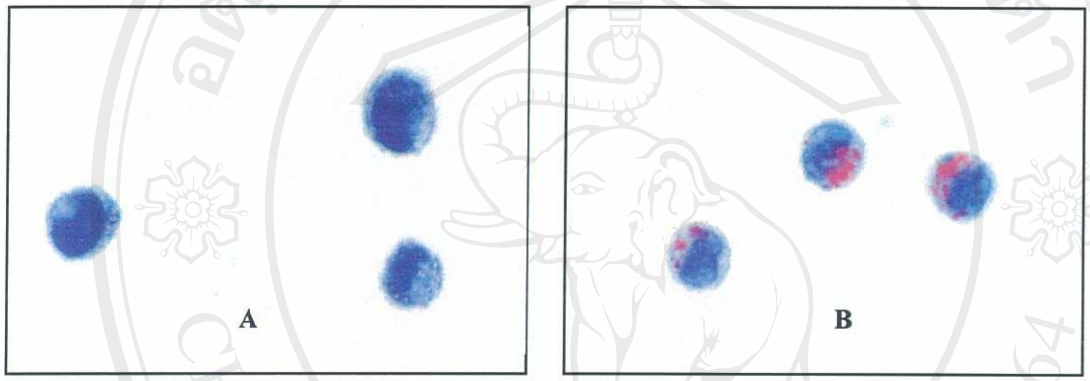


Figure 1. U937 cells stained with oil red O and Mayer's hematoxylin (A = negative control, B = positive control). The cells were observed under light microscope. Lipid droplets was stained in pink or orange with oil red O, localized to peripheral regions in the cytoplasm of the U937 cells and nucleus was stained in blue with Mayer's hematoxylin (original magnification, x100).

2.12 Statistics

Statistic methods used include mean±SD. Student's *t* test and *P* values <0.05 were considered statistically significant.