Chapter 5

Sida rhomboidea.Roxb and anti-atherosclerotic potential: *In vitro* evidence for the leaf extract in curbing LDL oxidation, macrophage differentiation, oxidized LDL induced foam cell formation and macrophages apoptosis

The present study attempts to evaluate the protective role of *Sida rhomboidea* leaf (SR) extract against *in vitro* LDL oxidation and Ox-LDL induced apoptosis of human monocyte derived macrophages (HMDMs)

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INTRODUCTION

Serum level of low-density lipoprotein (LDL) cholesterol is a crucial factor in the initiation and progression of atherosclerosis. During the last decade, LDL oxidation and LDL particle size have received extensive attention for their atherogenic potentials (Steinberg *et al.*, 1989). LDL particles are heterogeneous with varying density, size, charge, and composition. Small dense LDL particles seem linked to increased risk for Coronary artery diseases (CAD) (Regnstrom *et al.*, 1992). These LDL particles are considered as atherogenic due to their ability to penetrate the arterial wall and readily bind with intimal proteoglycans making the lipoproteins more susceptible to oxidation (Rajman *et al.*, 1999). Substantial evidence indicates that oxidized LDL is present *in vivo* within the atherosclerotic lesions but not in the normal arterial wall (Aviram and Fuhrman, 1998). Hence, oxidative changes in the arterial wall are crucial for the cellular uptake of LDL and initiation of plaque formation (Steinberg *et al.*, 1997). Further, scavenger receptors on macrophages recognize oxidized LDL and induce sub-endothelial lipid accumulation and foam cell formation, the earliest hallmark feature of atherosclerosis (Berliner *et al.*, 1995).

Secondly, ox-LDL stimulates endothelium to secrete monocyte chemotactic protein 1 (MCP-1) from endothelial cells, which facilitate infiltration of monocytes into the subendothelial space (Cushing *et al.*, 1990). Oxidized LDL can also induce migration and proliferation of smooth muscle cells and impede endothelial cell migration. Oxidized LDL promotes the differentiation of macrophage colony stimulating factor from endothelial cells and inhibits the motility of resident macrophage (Stocker and Keaney, 2003). Unlike the native LDL, ox LDL is immunogenic and cytotoxic to various cell types including endothelial cells (Hegyi *et*

al., 2001) and hence, disrupts endothelial integrity. Finally, oxLDL may interfere with endothelium-mediated relaxation and, stimulate platelet adhesion and aggregation through lowered endothelial nitric oxide (NO) production (Romics *et al.*, 1992). Some components of oxLDL, like oxysterols or phosphatidylcholine, may induce cell injury by both apoptotic and necrotic pathways (Galle *et al.*, 1999). Recent evidence suggests macrophage apoptosis to be a key event in the development of atherosclerotic lesions (Asmis and Begley, 2003) and seems to be a prevalent feature during all stages of atherosclerosis. Progressive macrophage apoptosis leads to the formation of apoptotic bodies that culminate in the accumulation of necrotic debris. Consequently, establishment of a necrotic core occurs that promotes inflammation, plaque instability and acute lesional thrombosis (Tabas, 2005).

MATERIALS AND METHODS

***Plant material and preparation of extract*:** as mentioned in chapter 1

Isolation of human LDL

Venous blood collected from fasting normo-cholesterolemic healthy volunteers as per standard norms by a pathologist and kept at room temperature for 45 min was centrifuged at 3000 rpm for 10 min at 4°C to obtain serum. LDL was isolated by heparin-citrate buffer (64 mM tri sodium citrate at pH 5.05 containing 50,000 IU/l heparin) precipitation method as described earlier by Ahotupa *et al.* (1998).

LDL oxidation kinetics

Briefly, 0.1 ml of LDL (100μg protein) diluted to 0.9 ml with PBS was incubated with or without 0.1 ml of SR extract (10-200 μg/ml) at 37°C for 30 min. Oxidation was initiated at the end of incubation period by adding 0.01 ml of freshly prepared

0.167 mM $CuSO_4$. The LDL oxidation kinetics were determined by continuously monitoring the change in absorbance for 180 min (at 37 °C) every 10 min at 234 nm in a UV/VIS Perkin Elmer spectrophotometer as described by Esterbauer *et al.* (1989).

Lag time (min) was determined from the intercepts of lines drawn through the linear portions of the lag phase and propagation phase. The rate of oxidation was determined from the slope of the propagation phase. The concentration of CD in the samples was calculated by using a molar extinction coefficient of 2.95×10^4 M^{-1} cm^{-1}. Maximum concentration of CD formed was calculated from the difference in the concentration of CD at zero time and at diene peak (absorption maxima) (Esterbauer *et al.*, 1989).

Measurement of malonaldehyde (MDA), lipid hydroperoxide (LHP) and protein carbonyl (PC) contents

Three sets of tubes were prepared for LDL oxidation. Copper mediated LDL oxidation in presence or absence of SR extract (10-200 µg/ml) for 24 h as mentioned above was evaluated. Later, oxidation was stopped by adding 0.01 ml of 10 mM EDTA to each tube and the samples processed for measurement of MDA, LHP and PC.

For MDA measurement, 0.1 ml aliquot was mixed with 1 ml TBA reagent (0.37% TBA, 15% TCA in 0.25 N HCl) and placed in water bath at 100°C for 30 min, cooled to room temperature and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm with a UV-VIS Perkin Elmer spectrophotometer and, MDA was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (Buege and Aust, 1978).

For LHP estimation, 0.1 ml aliquot mixed with 0.9 ml of Fox reagent (0.25mM ammonium sulphate, 0.1 mMxylenol orange, 25 mM H_2SO_4 and 4 mM BHT in 90% (v/v) HPLC-grade methanol) was incubated at 37 °C for 30 min. The absorbance was read at 560 nm and the LHP content determined by using the molar extinction coefficient of 4.3×10^4 M^{-1} cm^{-1} (Nourooz-Zadeh *et al.*, 1996).

For the estimation of PC, 0.1 ml of aliquot mixed with 0.2 ml of DNPH (in 2 M HCl) was incubated at room temperature for 60 min and, 0.6 ml of denaturing buffer (150 mM sodium phosphate buffer containing 3% SDS) was added and mixed thoroughly. Later, ethanol and heptane (1.8 ml of each) were added, the contents were mixed and centrifuged to precipitate protein. The protein precipitate was washed three times with 1.5 ml ethyl acetate/ethanol (1:1, v/v), dissolved in 1 ml denaturing buffer and read at 360 nm in a spectrophotometer. The carbonyl content was calculated using molar extinction absorption coefficient of 22.000 $M^{-1}cm^{-1}$ (Reznick and Packer, 1994)

Relative Electrophoretic Mobility (REM) Assay

Cu^{2+} mediated LDL oxidation was carried out in presence or absence of SR extract (10-200 μg/ml) for 24 h as mentioned above. Later, oxidation was stopped by adding 0.01 ml of 10 mM EDTA and, centrifuged to form a LDL pellet. The electrophoretic mobility of native or oxidized LDL (with or without SR extract) was detected by agarose gel electrophoresis (Reid and Mitchinson, 1993). Samples were loaded onto 0.6 % agarose gel and electrophoresed (100 V) in 50mM barbituric acid (pH 8.6) for 40 min. After electrophoresis, the gels were fixed in a solution containing 60% methanol, 30% water, and 10% glacial acetic acid for 30 min. The gels were then dried at 50°C for 40 min in a hot air oven and stained with 0.6% Sudan black B for 60

min and photographed. The result was expressed in terms of distance (meter) travelled by LDL from the point of origin.

Apolipoprotein B100 (ApoB) Fragmentation Assay

Copper mediated LDL oxidation was carried out in presence or absence of SR extract (10-200 µg/ml) for 24 h as mentioned above. Later, 0.01 ml of 10 mM EDTA was added in each tube to stop oxidation and centrifuged to form a LDL pellet. These LDL samples were denatured with 3% SDS, 10% glycerol, and 5% bromophenol at 95 °C for 10 min and cooled to room temperature. Later, LDL samples were loaded on 8 % SDS-polyacrelamide gels and electrophoresis was performed at 100 V for 60 min. The gels were stained with 2% coomassie brilliant blue solution for 6 hr at 4°C and de-stained (20% glacial acetic acid and 10% methanol in water) for 30 min. Later, gels were cleared in 15% and 10 % acetic acid for 10 min each, fixed in 10% glycerol and, photographed using canon power shot S70 digital camera (Lee et al., 2002).

Preparation of oxidized LDL

0.1 ml of LDL (100µg protein) diluted to 0.9 ml with PBS was incubated for 24 h at 37°C subsequent to initiation of oxidation by 0.01 ml of freshly prepared 0.167 mM $CuSO_4$. Analysis of MDA and CD was the carried out in the LDL samples. Samples with MDA 50±5 nmol/mg LDL protein and CD 80±8 nmol/mg LDL protein were used for further studies.

In vitro monocyte-macrophage differentiation

THP-1 cells (human monocyte cell line) were purchased from National Centre of Cell Sciences, Pune, India. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1 % antibiotic-antimycotic solution in a humidified incubator with 5% CO_2. THP-1 monocytes were differentiated into macrophages by

the addition of 50 nM phorbol 12-myristate 13-acetate (PMA) for 48 h (Park *et al.*, 2007). The differentiation protocol was carried out on cover slips placed in 6 well culture plates. At the end of incubation period, cover slips from each well were collected, washed in PBS twice and immersed in 4% paraformaldehyde solution for 10 min followed by incubation in 3% H_2O_2 (to block endogenous peroxidase) for 20 min. At the end of incubation, non-specific sites were blocked by addition of 1% fetal bovine serum (20 min, at 4°C in humidified chamber), followed by incubation with mouse anti-human F4/80 primary antibody at 4°C in humidified chamber overnight. For visualization, coverslips were incubated with rabbit anti-mouse IgG-HRP; 1:100 (Bangalore Genei Pvt Ltd, INDIA) secondary antibody for 4 hr in a humidified chamber (at 4°C). At the end of incubation, sections were thoroughly washed with PBS and final detection step was carried out using DAB detection system (Bangalore Genei Pvt Ltd, INDIA) and counter-stained with haematoxylin. Sections were examined under Leica DMRB microscope and photographed using a canon Power shot S 70 digital camera.

Macrophage mediated LDL oxidation

HMDMs ($1x10^5$/ml) underwent incubation in 1 ml of Ham's F-12 medium (without phenol red) containing LDL (100μg/ml) at 37°C for 24 hr. Cell free control wells were used for all conditions. Oxidation was arrested at the end of incubation by chilling the medium and adding 0.2 mM EDTA and 0.04 mM BHT. Later, 0.1 ml of each supernatant was used for the assay of MDA and CD as described earlier (Duell et al., 1998).

In vitro foam cell formation

THP-1 differentiated macrophages were grown in a 6 well plate and incubated with oxidized LDL (For preparation of Ox-LDL; see supplementary data) for 24 hr. Later, medium was decanted and cells were fixed in 4 % paraformaldehyde for 15 min. The cells were then washed twice with PBS, and stained in 1% Oil red O solution for 30 min. At the end of staining, excess oil red o was removed and 1 ml of glycerin added. Photographs were taken on Leica DMIL inverted microscope using canon power shot S 70 digital camera (Pang et al., 2010).

For quantitative evaluation of foam cells formation, another set of experiment was repeated using 6 well culture plate at 1.0 x 10^5cells/ml and stained with Oil red O as mentioned above. Excess stain was removed and intracellular Oil red O was recovered by addition of 1 ml of isopropenol in each well and absorbance was measured using Perkin-Elmer UV/Vis spectrophotometer.

Ox-LDL induced peroxyl radical generation in HMDMs

HMDMs (1x10^5/ml) pre-treated with SR extract (200µg/ml) for 30 min were incubated in presence of 100 µg/ml of Ox-LDL for 24 hr. Later, the cells incubated further for 60 min at 37°C with 0.0075mM dichlorodihydrofluoresceindiacetate (DCF-DA) for 30 min in dark (Silva *et al.*, 2010), were photographed with a canon power shot S70 digital camera in Leica DMRB florescence microscope.

Measurement of mitochondrial membrane potential

The changes in mitochondrial membrane potential were measured using the fluorescent cationic dye Rhodamine 123 (rho123) as per Pereira and Oliveira (2000).

HMDMs ($1x10^5$/ml) pre-treated with SR extract (200µg/ml) for 30 min, were incubated in presence of 100 µg/ml of Ox-LDL for 24 hr. The cells were then incubated with 0.001 mM rho123 for 10 min at 37 °C. The fluorescence was determined at excitation and emission wavelengths of 485 and 530 nm, respectively using a spectroflurometer (Jasco FP-6350).

Ox-LDL induced cytotoxicity of HMDMs

HMDMs ($1X10^4$) pre-treated with SR extract (10-200µg/ml) for 30 min were incubated in presence of 100 µg/ml of Ox-LDL for 24 hr. Further incubation of the cells was carried out in a culture medium containing 0.5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 160 min. Later, 0.15 ml of dimethyl sulphoxide was added to all the wells and incubated for 30 min at room temperature with constant shaking. Absorbance was read at 540 nm using a ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT) and subsequently % cell viability was calculated.

Ox-LDL induced chromatin condensation in HMDMs

HMDM cells ($1x10^5$/ml) pre-treated with SR extract (200µg/ml) for 30 min, were incubated in presence of 100 µg/ml of Ox-LDL for 24 hr. Single-cell suspensions of treated HMDMs were washed with PBS, fixed in 70% ethanol for 20 min, and washed again with PBS. Cells were then incubated with DAPI stain (0.6 μg/ml in PBS) for 5 min and washed with PBS for 5 min. Chromatin fluorescence was observed under a Leica DMRB 2000 florescence microscope. Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and chromatin condensation (Hsieh *et al.*, 2007).

Ox-LDL induced alterations in cell cycle distribution in HMDMs

HMDMs ($1x10^6$/ml) pre-treated with SR extract (200µg/ml) for 30 min, were incubated in presence of 100 µg/ml of Ox-LDL for 24 hr. At the end of incubation, cells were collected, washed twice with PBS, fixed overnight in cold 70% ethanol at 4°C and, re-suspended in PBS. Cells were incubated with RNase A for 45 min, and stained with propidium iodide (1 mg/ml) in the dark at 37 °C for 30 min (Pozarowski and Darzynkiewicz, 2004). The suspension was analyzed with a Flow Cytometer (MoFlo™ Cytomation, Modular Flow Cytometer). Apoptosis was determined based on the "sub-G1" peak.

Annexin-V/Propidium iodide staining of Ox-LDL treated HMDMs

Annexin-V FITC/PI double-staining assay was used to quantify apoptosis, according to the manufacturer's protocol (Sigma Aldrich, Ltd.USA). HMDMs ($1x10^6$/ml), pre-treated with SR extract (200µg/ml) for 30 min were incubated in presence of 100 µg/ml of Ox-LDL for 24 hr Later, cells from each well were centrifuged, washed with PBS and suspended in 0.1 ml binding buffer; 0.005 ml of Annexin V FITC Conjugate and 0.001 ml of propidium Iodide solutions were added to each cell suspension and incubated for 10 min at room temperature in the dark. Later, samples were analyzed on flow cytometer (MoFlo™ Cytomation, Modular Flow Cytometer) and CellQuest software. Double staining of cells with FITC-Annexin V and PI enables the discrimination of live cells ($FITC^-PI^-$), early apoptotic ($FITC^+PI^-$), late apoptotic ($FITC^+PI^+$) or necrotic cells ($FITC^-PI^+$).

Statistical analysis

Statistical evaluation of the data was done by one way ANOVA followed by Bonferroni's multiple comparison tests. The results were expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.

RESULTS

LDL oxidation kinetics

LDL oxidation kinetics was studied to assess Cu^{2+} mediated oxidation of LDL and formation of CD in presence or absence of SR extract. Various dose ranges of SR extract (20-200µg/ml) were selected for the study. It was observed that, presence of SR extract significantly prevented Cu^{2+} induced oxidation of human LDL in a dose dependent manner. Co-presence of SR extract significantly increased lag time ($p<0.05$) for LDL oxidation and decreased the overall rate of LDL oxidation ($p<0.05$) in dose dependent manner (Figure, 1& 2). The same was evident in the form of significant decrement in formation of CD in samples that had presence of SR extract.

Measurement of MDA, LHP and PC contents

The measured concentrations of MDA, LHP and PC indicate the degree of Cu^{2+} mediated LDL oxidation and, significant increment ($p<0.05$) in their contents contents were recorded when LDL was incubated with Cu^{2+} compared to Cu^{2+} free control LDL. In contrast, co-presence of SR extract (10-200µg/ml) successfully minimized the production of MDA, LHP and PC in a dose dependent manner (Figure, 3).

Relative Electrophoretic Mobility and ApoB fragmentation

Figure.4 shows the pattern of REM and ApoB fragmentation of Ox-LDL in presence or absence of SR extract. Co-presence of SR extract (10-200µg/ml) significantly

prevented ApoB fragmentation as noted by the intact ApoB protein band compared to fragmented band in the Cu^{2+} treated LDL sample (Figure,4). Moreover, Cu^{2+} treated LDL moved a distance of 4.3 mm from the origin compared to 1.3mm of native LDL while, SR (10-200μg/ml) treated LDL samples recorded decrement in REM in the range of 3.9 to 1.4 m in a dose dependent manner (Figure, 4).

Macrophage mediated LDL oxidation

HMDMs incubated with LDL sample in presence or absence of SR extract (200μg/ml) indicated the effect of SR extract on macrophage mediated LDL oxidation. It was observed that incubation of LDL with HMDMs for 24 hr significantly increased the contents of MDA (58.33%) and CD (51.38%). However, co-presence of SR extract significantly attenuated macrophage mediated LDL oxidation as noted by the significantly lower indices of MDA (37.5%) and CD (30.66%) compared to LDL incubated with HMDMs (Figure, 5).

Monocyte to macrophage differentiation

Treatment of THP-1 monocytes with PMA for 24 hr resulted in differentiation of monocytes to macrophages that were characterized by their adherent property on differentiation. Macrophage differentiation in presence of SR extract resulted in significant lower number of F4/80 positive cells, indicating a strong inhibitory potential of SR on macrophage differentiation *in vitro* (Figure, 6).

In vitro foam cell formation

Incubation of monocyte derived macrophage cells with Ox-LDL (100 μg/ml) for 24 hr resulted in significant uptake of Ox-LDL, leading to higher intracellular cholesterol accumulation compared to Ox-LDL deprived cells (Figure, 7). Addition of SR extract

to Ox-LDL treated macrophages significantly reduced intracellular cholesterol accumulation (Figure, 7).

Ox-LDL induced peroxyl radical generation and nuclear condensation in HMDMs

Exposure of HMDMs to Ox-LDL resulted in significant increment in the peroxyl radical generation ($p<0.05$) as evidenced by the higher number of DCF-DA positive cells; however, co-presence of SR extract in cultures of HMDM exposed to Ox-LDL minimized peroxyl radical generation (Figure. 8). As shown in Figure.6, exposure of HMDMs to Ox-LDL resulted in appearance of more number of cells with condensed nuclei (45 % vs. 8%) compared to untreated cells. However, co-presence of SR extract in Ox-LDL exposed HMDMs minimized the degree of nuclear condensation (15% vs.45%).

Ox-LDL induced mitochondrial depolarization and cytotoxicity in HMDMs

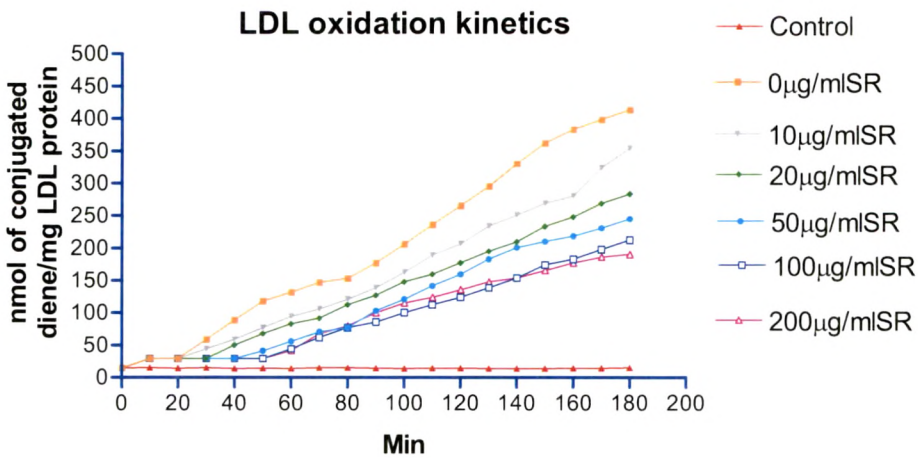
Mitochondrial membrane potential of Ox-LDL exposed HMDMs evaluated in presence or absence of SR extracts (20-200μg/ml) using Rhodamine 123 florescence dye showed significant decrement in the mitochondrial membrane potential of Ox-LDL exposed HMDMs compared to Cu^{2+} free controls. However, Ox-LDL induced membrane depolarization was significantly attenuated in presence of SR extract in a dose dependent manner (Figure.9). Exposure of HMDMs to Ox-LDL resulted in significant decrement in cell viability ($p<0.05$). However, Ox-LDL induced decrement in cell viability ($p<0.05$) was significantly minimized by the co-presence of SR extract in Ox-LDL exposed HMDMs (Figure, 9).

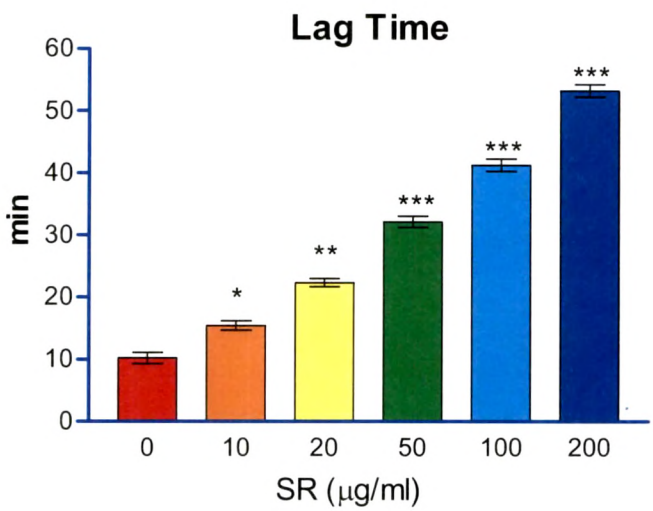
Ox-LDL induced alteration in the cell cycle distribution and apoptosis in HMDMs

Flow cytometric analysis of propidium iodide stained control and treated cells revealed that Ox-LDL exposed HMDMs recorded 20.11 % cells in the sub G0 phase

compared to 4.43 % cells in untreated HMDMs. However, Ox-LDL exposed HMDMs in presence of SR recorded only 8.56 % cells in sub G0 phase (Figure. 10). In the annexine V-FITC/ propidium iodide staining assay, Ox-LDL treated HMDMs recorded 28.89% Annexine V positive cells compared to 5.45 % in untreated and 12.01 % in Ox-LDL exposed HMDMs in presence of SR (Figure, 11)

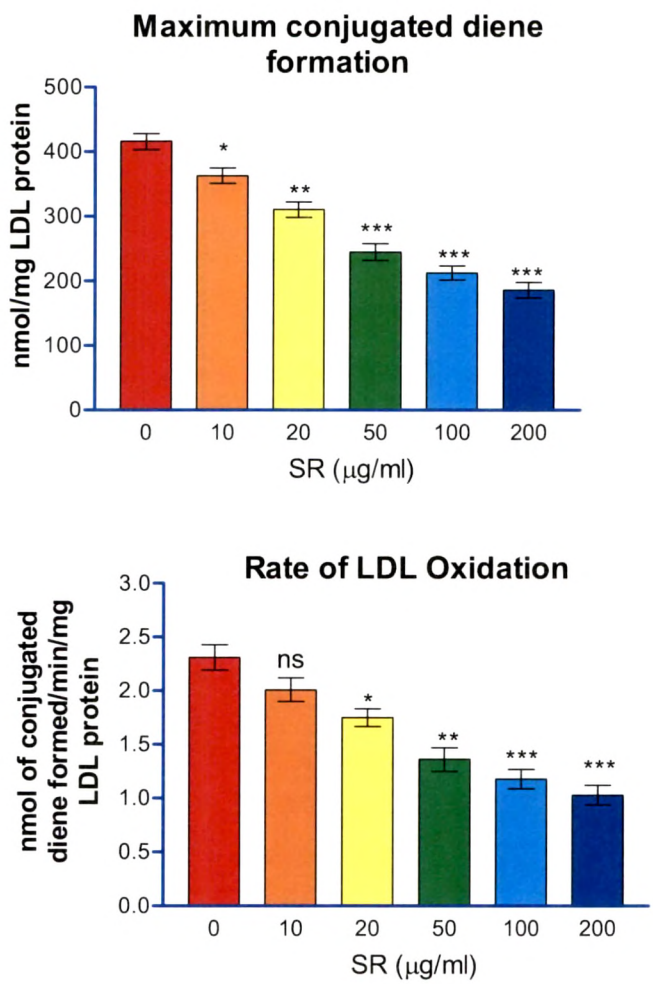
Figure. 1 Effect of *S.rhomboidea.*Roxb extract on oxidation kinetic and lag time during Cu^{2+} mediated LDL oxidation.





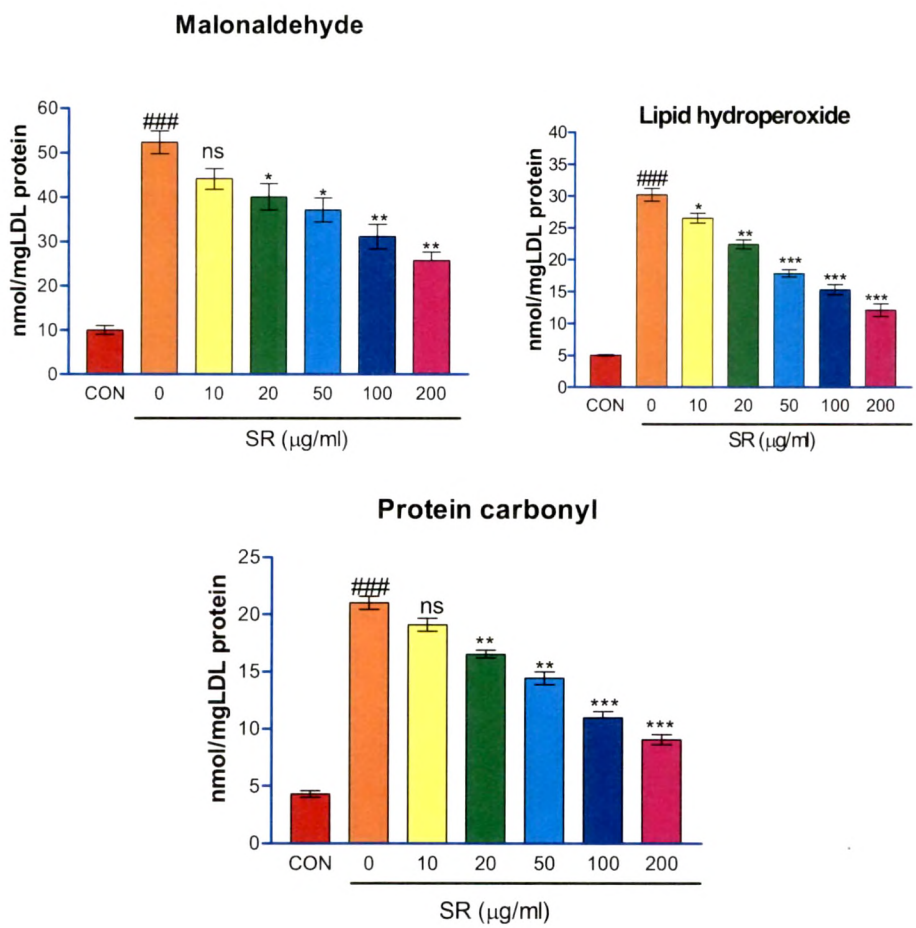
Data expressed as mean±S.E.M for n=3. *p<0.05, **p<0.01,***p<0.001 and ^{ns}non-significant compared to 0µg/ml SR.

Figure. 2 Effect of *S.rhomboidea.*Roxb extract on maximum conjugated diene formation and rate of oxidation during Cu^{2+} mediated LDL oxidation.



Data expressed as mean±S.E.M for n=3. *p<0.05, **p<0.01,***p<0.001 and ^{ns}non-significant compared to 0µg/ml SR.

Figure.3 Effect of *S.rhomboidea.*Roxbextract on MDA, LHP and PC content in Cu^{2+} mediated LDL oxidation.



Data expressed as mean±S.E.M for n=3. ###p<0.001 compared to CON (Cu^{2+} free LDL control) and *p<0.05, **p<0.01,***p<0.001 and, ^{ns}non-significant compared to 0µg/ml SR.

Figure.4 Effect of *S.rhomboidea.*Roxb extract on REM and ApoB fragmentation of Cu^{2+} mediated oxidised LDL.

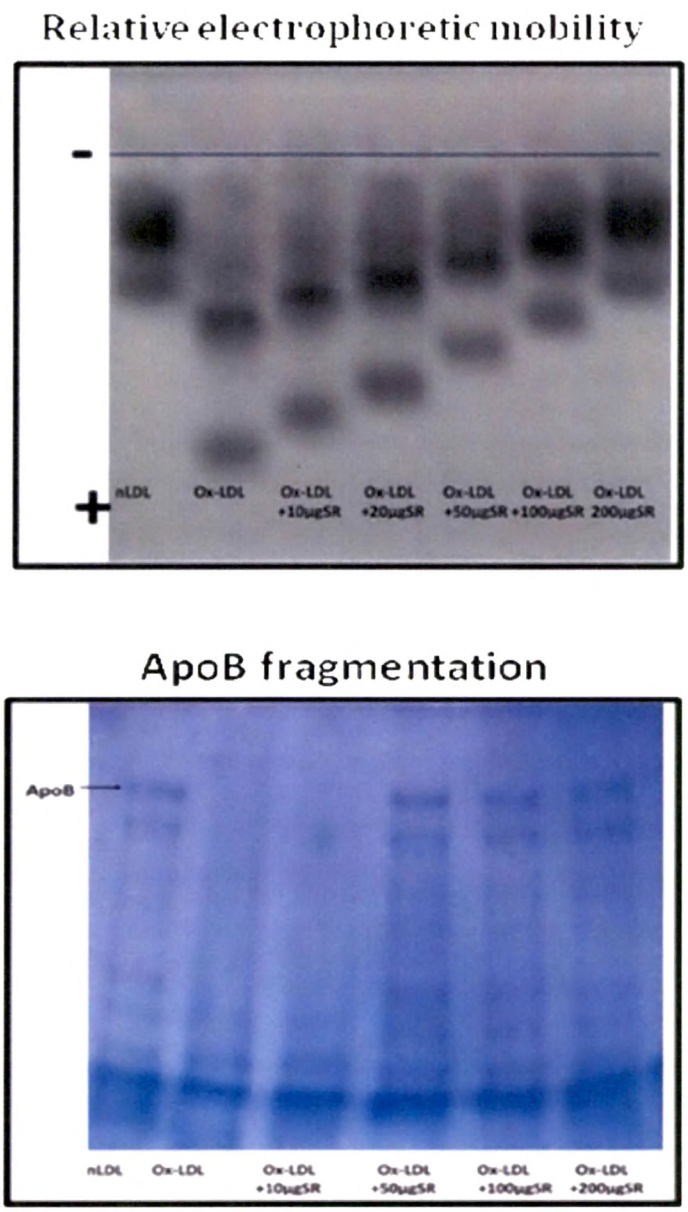
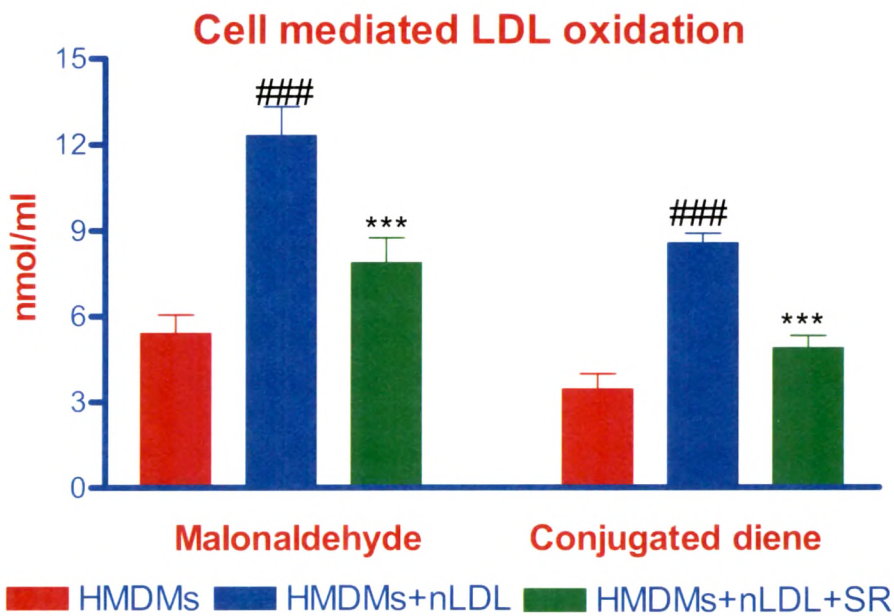
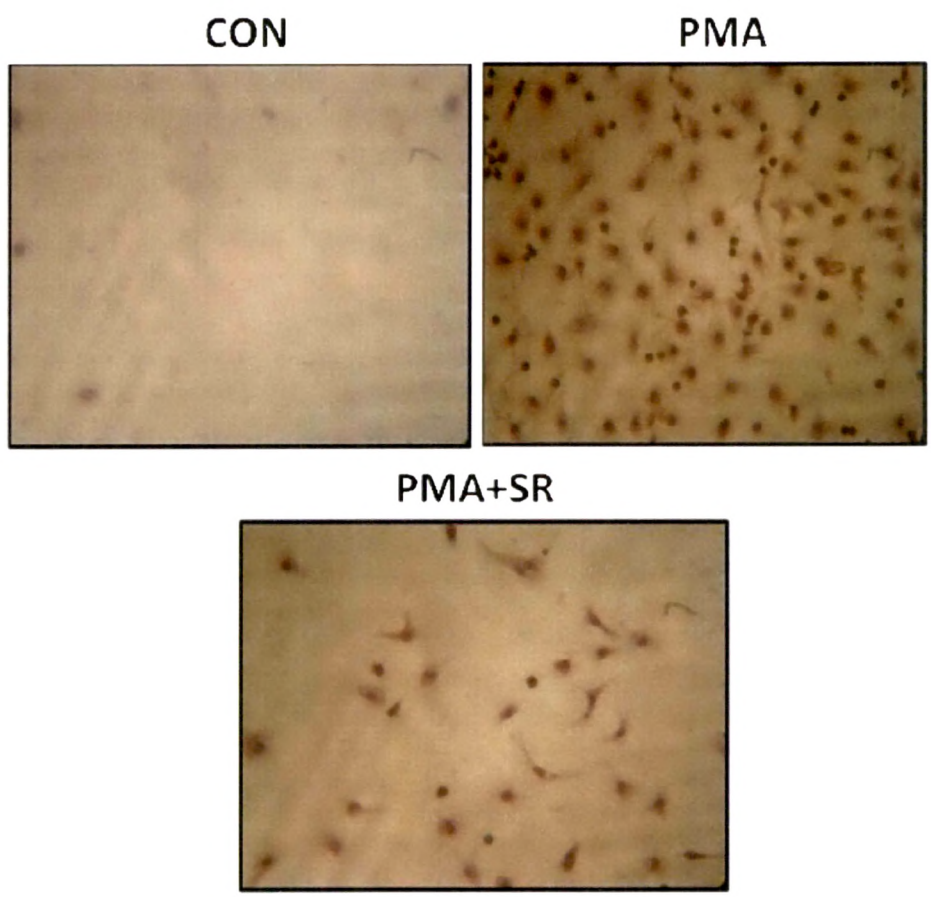


Figure.5 Effect of *S.rhomboidea.*Roxb extract on LDL malonaldehyde and conjugated diene content during cell mediated LDL oxidation.



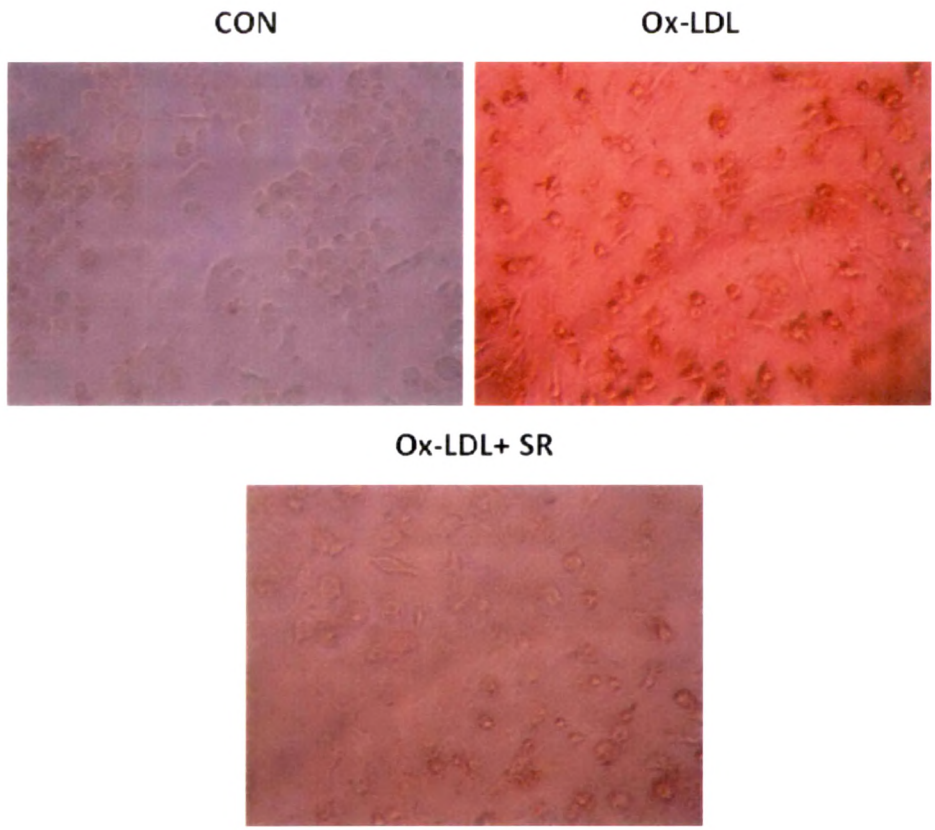
Data expressed as mean±S.E.M for n=3. $^{###}p<0.001$ compared to HMDMs and ***$p<0.001$ compared to HMDMs+nLDL.

Figure.6 Effect of *S.rhomboidea*.Roxb extract on *in vitro* monocyte to macrophage differentiation (40X).



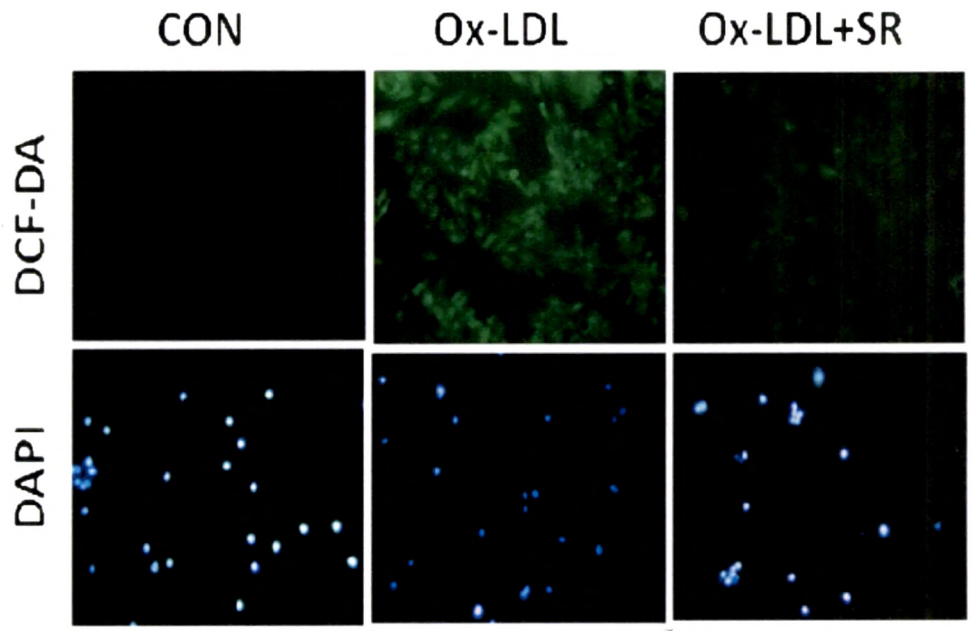
Where, CON; undifferentiated monocyte (THP-1 cells), PMA; THP-1 cells treated with phorbol-12-myristate-13-acetate, PMA+SR; THP-1 cells treated with phorbol-12-myristate-13-acetate in presence of *S.rhomboidea*.Roxb extract (200 µg/ml).

Figure.7 Effect of *S.rhomboidea*.Roxb extract on *in vitro* foam cell formation (40X).



Where, CON; untreated human monocyte derived macrophages, Ox-LDL; human monocyte derived macrophages treated with oxidized LDL (100µg/ml) and Ox-LDL+SR; human monocyte derived macrophages treated with oxidized LDL (100µg/ml) in presence of *S.rhomboidea*.Roxb extract (200 µg/ml).

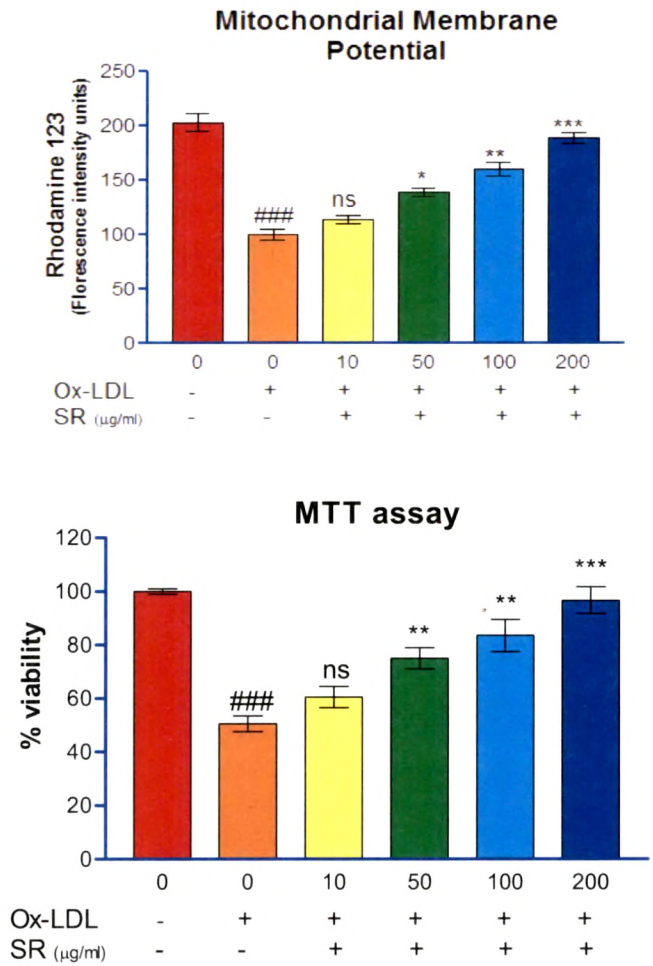
Figure.8 Effect of *S.rhomboidea.*Roxb extract on peroxyl radical generation (DCF-DA staning) and mitochondrial membrane potential (Rhodamine 123 florescence) in Ox-LDL treated HMDMs.



Control; HMDMs cells, Ox-LDL; HMDMs exposed to Ox-LDL and Ox-LDL+ SR; HMDMs exposed to Ox-LDL in presence of 200µg/ml SR extract.

Data expressed as mean±S.E.M for n=3. $^{###}p<0.001$ compared to nLDL and $*p<0.05$, $**p<0.01$, $***p<0.001$ and ^{ns} non-significnat compared Ox-LDL.

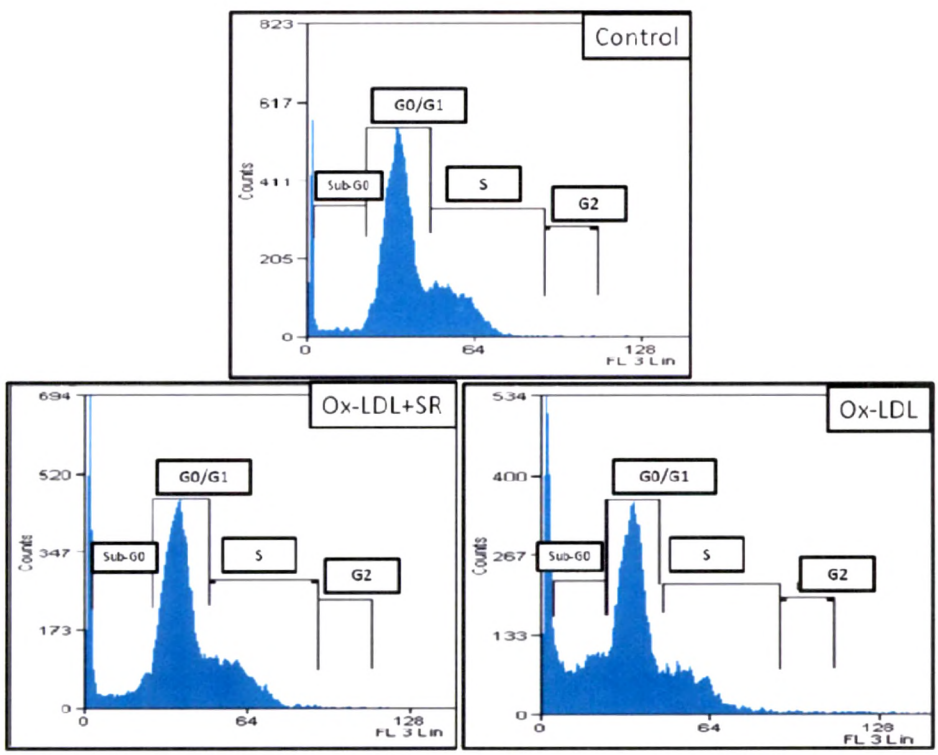
Figure.9 Effect of *S.rhomboidea.*Roxb extract on nuclear condensation (DAPI staining) and cell viability (MTT assay) in Ox-LDL treated HMDMs



Control; HMDMs cells, Ox-LDL; HMDMs exposed to Ox-LDL and Ox-LDL+ SR; HMDMs exposed to Ox-LDL in presence of 200µg/ml SR extract. Note the presence of cells with normal nuclei (arrow) and cells with condensed nuclei (arrow head).

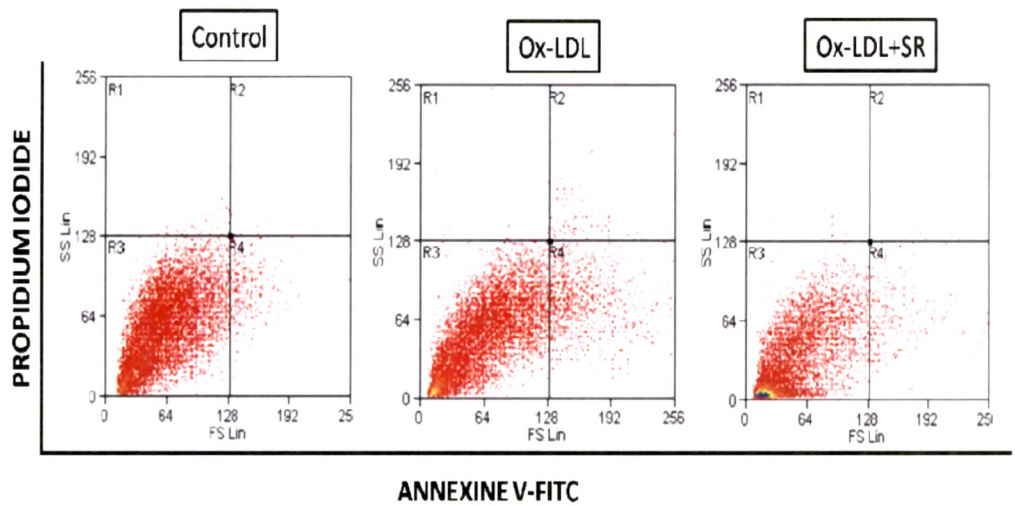
Data expressed as mean±S.E.M for n=3. $^{###}p<0.001$ compared to nLDL and *p<0.05, **p<0.01, ***p<0.001 and ^{ns} non-significnat compared Ox-LDL.

Figure.10 Effect of *S.rhomboidea.*Roxb extract on cell cycle distribution in Ox-LDL treated HMDMs.



Control; HMDMs cells, Ox-LDL; HMDMs exposed to Ox-LDL and Ox-LDL+ SR; HMDMs exposed to Ox-LDL in presence of 200µg/ml SR extract.

Figure.11 Effect of *S.rhomboidea.*Roxb extract on Ox-LDL induced HMDM apoptosis evaluated by Annexin-V FITC/propidium iodide staining.



Control; HMDMs cells, Ox-LDL; HMDMs exposed to Ox-LDL and Ox-LDL+ SR; HMDMs exposed to Ox-LDL in presence of 200µg/ml SR extract.

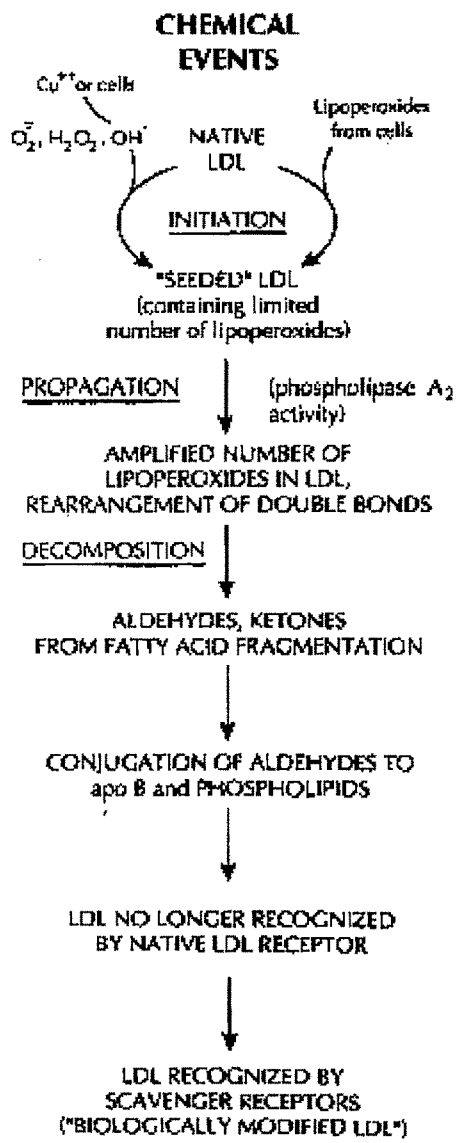
R1: necrotic cells ($FITC^-PI^+$), R2: late apoptotic ($FITC^+PI^+$), R3: live cells ($FITC^-PI^-$) and R4: early apoptotic ($FITC^+PI^-$).

DISCUSSION

Though cell types such as monocytes, macrophages, neutrophils, endothelial cells, smooth muscle cells and fibroblasts have all an inherent ability to oxidize LDL, the most likely cell types that participate in LDL oxidation *in vivo* are macrophages, endothelial cells and smooth muscle cells. Oxidation of LDL is a free radical-mediated process, resulting in numerous structural changes. Polyunsaturated fatty acids (PUFAs) are particularly prone to free radical attack because of their structural vulnerability (presence of double bond) that ends in a chain reaction leading to oxidative damage. Oxidation of LDL occurs in three phases (Esterbauer *et al.*, 1989): Initial lag phase, propagation phase and decomposition phase. The reaction is initiated by reactive free radicals, e.g., the hydroxyl radical, that abstracts a hydrogen atom (H•) from a methylene group (-CH2-) of PUFAs, leaving behind an unpaired electron on the carbon (-•CH-) (Betteridge, 2000). The remaining carbon-centred radicals undergo molecular rearrangement resulting in a conjugated diene (CD) (Abuja *et al.*, 1995). During the initiation phase of LDL oxidation, presence of endogenous antioxidants such as α tocopherol within the LDL particle that scavenge proxy radicals suppresses the rate of oxidation. A rapid propagation phase consequent to depletion of antioxidants that ends in the formation of lipid peroxides follow this phase, referred as the lag phase of oxidation. The propagation phase followed by a decomposition or degradation phase marks the cleavage of double bonds of PUFA, resulting in the formation of aldehydes. The major aldehydes produced include malondialdehyde (MDA), 4-hydroxynonenal (HNE) and hexanal (Figure.12). These oxidation products cross-link with amino groups on Apo B-100 leading to its

fragmentation (Young and McEneny, 2001). The difficulty in tracing this sequence of events *in vivo* entails the need for an *in vitro* assessment using a variety of protocols.

Figure. 12 LDL oxidation kinetics



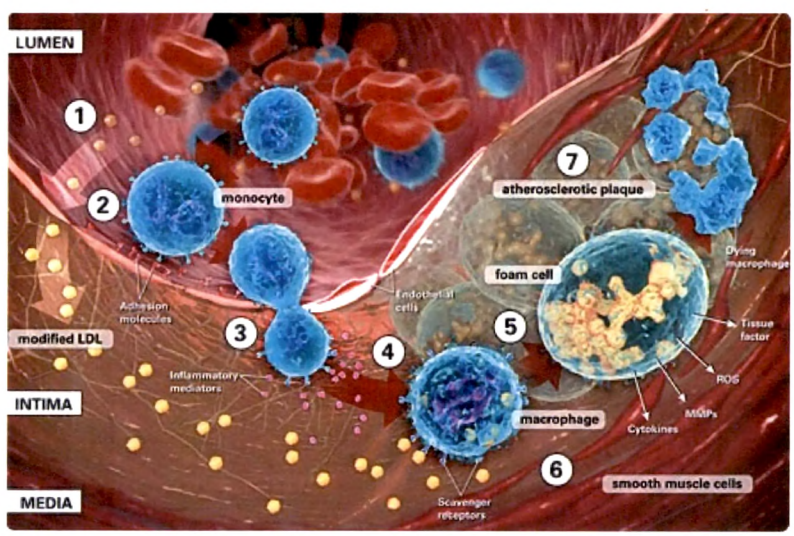
From: J Clin Invest. 1991 December; 88(6): 1785–1792.

Copper mediated LDL oxidation is the most common and accepted *in vitro* protocol as it resembles the *in vivo* sequence of events during atherosclerosis by forming endogenous lipid hydroperoxides (LOOH) via a redox reaction (Burkitt *et al.*, 2001). The resultant oxidized LDL exhibits chemical and biological properties similar, if not identical, to macrophage-modified LDL (Esterbauer *et al.*, 1992). Subsequent decomposition of LOOH forms malondialdehyde and 4-hydroxynonenal (Mertens and Holvoet, 2001), which can then interact with positively charged ε-amino groups of lysine residues in ApoB-100. This renders the LDL molecules negatively charged and with less affinity for the LDL receptors and, an increased affinity for the scavenger receptor (Mertens and Holvoet, 2001). An extended lag phase recorded in presence of SR extract indicates its efficacy in delaying Cu^{+2} induced LDL oxidation and minimizing the formation of CD. Further, minimal formation of MDA, LHP and PC in presence of SR proves its protective role. These results are attributable to a potent metal chelating property of SR extract reported earlier by our research group (Thounaojam *et al.*, 2010b)

Pathogenesis of *in vivo* induction of atherosclerosis is characterized by marked elevation in circulating titres of plasma LDL (Krauss, 1987) and, atherogenesis *per se,* is initiated in hypercholesterolemic patients by the accumulation of LDL in the arterial wall and its conversion to Ox-LDL and impaired endothelial function (Napoli and Lerman, 2001). During the progression of atherosclerosis, circulating monocytes migrate across endothelium at the site of injury and differentiate into resident macrophages. Subsequently, cell types (macrophages, endothelial cells and smooth muscle cells) present in the thoracic aorta oxidize the native LDL retained in the arterial wall (Han and Pak, 1999). Macrophages differentiated from monocytes

subsequently accumulate large amount of cellular cholesterol via scavenger receptor (SRB1) mediated uptake of Ox-LDL (Figure.13; Shashkin *et al.*, 2005).

Figure. 13 Monocyte recruitment, its differentiation to macrophage and foam cell formation during atherosclerosis.



From: http://www.laddmcnamara.com/Statin-Drugs.html

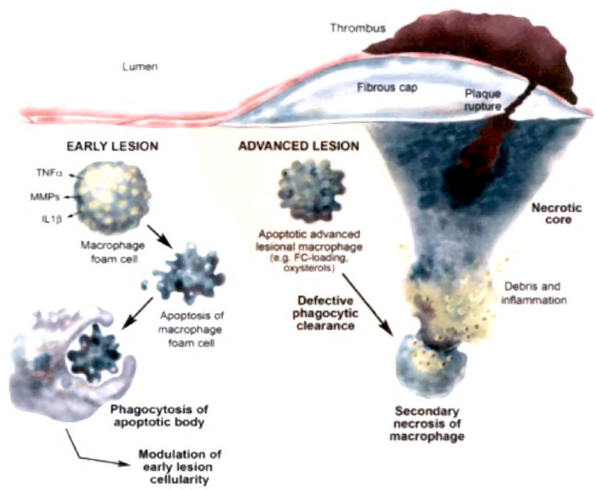
These lipid-laden cells, now termed as foam cells subsequently undergo apoptosis and secondary necrosis in the arterial wall forming a necrotic core of atheromatous plaque (Tabas, 2005). In the present study, SR extract could effectively prevent *in vitro* monocyte to macrophage differentiation and the consequent Ox-LDL induced foam cell formation. In the present study, assessment of cell mediated *in vitro* oxidation of native LDL by human monocyte derived macrophages (HMDMs) indicates the potential of SR extract in minimizing the production of MDA and CD during cell mediated LDL oxidation.

Formation of peroxy radicals either by abstraction of hydrogen or by formation of LHP intermediates is the main causative agent in the induction of

peroxyl radical mediated macrophage apoptosis (Asmis and Begley, 2003). In the present study, an analysis of peroxyl radical formation in Ox-LDL exposed HMDMs was performed by the use of peroxyl radical specific stain, DCF-DA (5-and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate. DCF-DA is a cell membrane permeable florescent dye that once inside the cell gets hydrolyzed by cellular esterases to form DCF, which remains trapped intracellularly due to its membrane impermeability. DCF then reacts with intracellular ROS to form the fluorescent product, 2', 7'- dichlorofluorescein. The results clearly suggest the preventive action of SR extract on Ox-LDL induced peroxyl radical formation in HMDMs *in vitro*. The potent free radical scavenging property of SR extract (Thounaojam *et al.*, 2010b) due to its high content of polyphenols and flavonoids could be responsible for the herein observed effects.

Mitochondrial integrity and functioning constitute the epicentre of metabolic performance of cells including macrophages (Newsholme and Newsholme, 1989). Hence, a mitochondrial membrane specific dye, Rhodamine 123, finds application in our study to evaluate the status of mitochondrial membrane potential of Ox-LDL exposed HMDMs. Rhodamine 123 is a chemical compound and a dye, used as a marker of mitochondrial membrane potential. The fluorescent property of Rhodamine dyes enables easy detection with spectrofluorometers. The evaluation provides substantial support to the competence of SR extract in preventing mitochondrial membrane depolarization and consequent dysfunctioning, attesting to the efficacy of SR extract to prevent mitochondrial oxidative stress (Chapter 2).

Figure. 14 In vivo foam cell formation, macrophage apoptosis and secondary necrosis during atherosclerosis.



From: Arterioscler Thromb Vasc Biol. 2005;25:2255–2064.

In vivo studies by other workers have led to a hypothesis of defective clearance of apoptotic macrophages in advanced lesions of atherosclerosis (Tabas, 2005). Several lines of evidence strongly link Ox-LDL to apoptosis and secondary necrosis of foam cells (Figure.14). After uptake by scavenger receptors, Ox-LDL inhibits the efflux of cholesterol from the cells, resulting in high intracellular cholesterol load (Krieger, 1998). Reports also suggest that, elevated levels of intracellular cholesterol play a critical role in the regulation of Ox-LDL-mediated cell apoptosis (Ryan *et al.*, 2005). Hence, it is pertinent to develop a novel therapeutic strategy to tackle the pathophysiological progression of atherosclerosis. The present study clearly indicates efficacy of SR extract in preventing Ox-LDL induced nuclear condensation, cell cycle arrest and apoptosis in HMDMs. Reports of lipid lowering interventions of herbal origin that prevent macrophage apoptosis by reverse

cholesterol transport and massive cholesterol efflux have appeared in literature (Kaplan *et al.*, 2001). In this context, our published observation of lipid lowering potential of SR extract (chapter 1) on the ability of SR extract to inhibit *in vitro* LDL oxidation, foam cell formation and cholesterol accumulation lend credence to its role in promoting cholesterol efflux and inhibiting foam cell formation.

Summary

The present study evaluates protective role of *S.rhomboidea*.Roxb (SR) against *in vitro* LDL oxidation, monocyte to macrophage differentiation, Ox-LDL induced foam cell formation and macrophage apoptosis. Copper and cell mediated LDL oxidation, Ox-LDL induced peroxyl radical generation, mitochondrial activity, foam cell formation and apoptosis in human monocyte derived macrophages were assessed in presence of SR extract. Results clearly indicate that SR is capable of reducing LDL oxidation and formation of intermediary oxidation products. Results also suggest successful attenuation of peroxyl radical formation, mitochondrial dysfunction, nuclear condensation and apoptosis in Ox-LDL exposed HMDMs in presence of SR. Further, SR extract significantly reduced monocyte to macrophage differentiation and foam cell formation. This scientific report is the first detailed *in vitro* investigation that establishes the anti-atherosclerotic potential of SR extract.

Schematic summary