

CHAPTER 4

Anethum graveolens L. seeds
extract inhibits human LDL
oxidation and ox-LDL induced
apoptosis in macrophage

CHAPTER 4***Anethum graviolens* L. Prevents Formation of LDL Oxidation Products and Foam Cell Formation in RAW 264.7 Cells****INTRODUCTION**

Cardiovascular diseases (CVDs) have been reported as the greatest public health problem and have been accounted as the major causes of death globally. Approximately 17.5 million people died in 2012 and out of these 7.4 million died due to coronary heart disease and 6.7 million due to stroke (WHO, 2014). The CVDs include coronary artery disease, coronary heart disease, cardiovascular disease, and diseases of the aorta and the arteries, including hypertension and peripheral vascular diseases. A common cause of these disorders is the occurrence of atherosclerotic processes in various areas of the circulatory system (Kowalska *et al.*, 2015). A plaque formation in circulatory system is the root cause of these diseases and is commonly known as atherosclerosis (Galkina and Ley, 2009).

High blood levels of cholesterol and triglyceride initiates the atherosclerotic process and degenerative changes in blood vessels. Oxidative modification of Low Density Lipoprotein Cholesterol (LDL-C) hastens the process of atherosclerosis. Oxidised LDL (Ox-LDL) binds with receptors present on the plasma membranes of macrophages, internalised and accumulates in macrophages. These macrophages later on convert into foam cells. The foam cell formation is the hall mark for the atherosclerotic plaque formation (Ross, 1999; Weber *et al.*, 2008; Hansson and Libby, 2006).

Lowering plasma levels of lipids and protection against LDL oxidation have been shown effective strategy to prevent plaque formation. Several evidences from epidemiologic

studies have shown to contribute to prevent atherosclerosis by using antioxidants and lipid lowering agents. Several lipid lowering plants with strong antioxidant activity have been shown to reduced plaque formation (Rosenson, 2004). *Anethum graveolens* L. (AG) has shown lipid lowering activity (Jana and Shekhawa, 2010) and strong antioxidant properties (Singh *et al.*, 2005, Taher *et al.*, 2007, Bahramikia and Yazdanparast, 2008). Essential oils of AG have been reported to possess hypolipidemic and cardioprotective potentials (Hajhashemi and Abbasi, 2008). Studies on a detailed phytochemical analysis have established the presence of flavonoids (rutin, quercetin), hydroxycinnamic acid derivates (caffeic acid, chlorogenic acid), coumarins (scopoletin), sterols (beta sitosterol/stigmasterol) and mucilages (Ortan *et al.*, 2009).

The present study was designed to assess the effect of AG extract in preventing experimentally induced modifications of LDL. Also, the efficacy of AG in preventing ox-LDL uptake and subsequent formation of foam cells has also been assessed herein.

MATERIALS AND METHODS

Collection of Plant

As shown in chapter 1.

Preparation of Plant Extract

As shown in chapter 1.

Isolation of LDL

Venous blood was collected by a pathologist at blue cross pathology lab, Vadodara (IMA-BMWMC No. 1093) from fasting healthy volunteer (having normal levels of blood cholesterol) after taking prior written consent and the protocol was run as per the ethical guidelines for human studies. Blood samples were kept at room temperature for 45 min. Serum was separated by centrifugation at 3000 rpm for 10 min at 4°C. LDL was isolated from serum according to Ahotupa *et al.*, (1998). A mixture of 0.1 ml of serum and 1 ml of the heparin–citrate buffer (64 mM trisodium citrate at pH 5.05 containing 50,000 IU/l heparin) was vortexed and allowed to stand for 10 min at room temperature and later centrifuged (3,000 rpm for 10 min at 20°C) to remove insoluble lipoproteins. Precipitated LDL was suspended in 0.1 ml of phosphate-buffered saline (PBS; 0.1 M, pH 7.4, containing 0.9% NaCl). Protein level in isolated LDL was estimated by the method of Lowry *et al.* (1951).

LDL Oxidation Kinetics

LDL fraction (100 µg protein/mL) was suspended in 50 mM PBS buffer, pH 7.4 in a total volume of 4 mL. The reaction was initiated with the addition of 10 µM CuSO₄.

LDL (100 µg protein/mL) was suspended in PBS (pH 7.4). LDL was incubated with AG extract (10–150 µg/ml) at 37°C for 30 min. The reaction was initiated with the addition of 0.167 mM CuSO₄. The LDL oxidation kinetics was determined by continuously monitoring (every 10 min) the absorbance for 180 min (at 37°C) at 234 nm in a UV/VIS Perkin Elmer spectrophotometer. 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 conjugated diene (CD) in the samples was calculated by using a molar extinction coefficient of $2.95 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$. 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).

LDL Oxidation Products

Copper-mediated LDL oxidation was carried out in presence or absence of AG extract (10-150 µg/ml) for 24 h. After 24 hour incubation, 10 µL of 10 mM EDTA was added in each tube to stop oxidation reaction and each sample was assessed for malondialdehyde (MDA), lipid hydroperoxide (LHP) and protein carbonyl (PC) as follows:

Malondialdehyde (MDA): MDA was analysed using Buege and Aust method (Buege and Aust, 1978). Samples were mixed with 1 ml TBA reagent (0.37% TBA, 15% TCA in 0.25N HCl) and incubated at 100°C for 30 min. Samples were allowed to attain to room

temperature and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm using Perkin Elmer spectrophotometer. MDA levels were calculated using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Lipid hydroperoxide (LHP): LHP was analysed using Nourooz-Zadeh *et al* method (Nourooz-Zadeh *et al.*, 1996). Samples were mixed with 0.9 ml of Fox reagent (0.25 mM ammonium sulphate, 0.1 mM xylenol orange, 25 mM H_2SO_4 , and 4 mM BHT in 90% (v/v) HPLC-grade methanol) and incubated at 37°C for 30 min. The absorbance was read at 560 nm and LHP content was determined using the molar extinction coefficient of $4.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Protein carbonyls (PC): PC was analysed using Reznick and Packer (1994) method. Samples were mixed with 0.2 ml of DNPH (in 2 M HCl). After incubation at room temperature for 60 min, 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) was added to precipitate protein and the contents were centrifuged. The protein was washed three times with 1.5 ml of ethyl acetate/ethanol (1:1, v/v), dissolved in 1 ml of denaturing buffer and read at 360 nm in a spectrophotometer. The carbonyl content was calculated using molar extinction absorption coefficient of $22.000 \text{ M}^{-1} \text{ cm}^{-1}$.

Cell mediated LDL Oxidation

RAW 247.6 cells ($1 \times 10^5/\text{ml}$) were incubated in medium (without phenol red) containing LDL (100 $\mu\text{g}/\text{ml}$) at 37°C for 24 h in presence and absence of AG extract. At the end of incubation, oxidation reaction was arrested by addition of stop buffer (adding 0.2 mM EDTA and 0.04 mM BHT). The supernatant was used for the assay of MDA described earlier (Patel *et al.*, 2011).

Preparation of Oxidized LDL and Culture of RAW 247.6 Cells

LDL (100 µg protein) was diluted to 0.9 ml with PBS and incubated for 24 h at 37°C. LDL was oxidized with 10 µl freshly prepared CuSO₄ (0.167 mM). Analysis of MDA and CD were carried out in the LDL samples. Samples with MDA 60 ± 5 nmol/mg LDL protein and CD 85 ± 5 nmol/mg LDL protein were used for further studies.

Macrophage cell line (RAW 247.6 cells) was purchased from National Centre of Cell Sciences, Pune, India. Macrophages were cultured in DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic solution at 37°C with 5% CO₂.

Cytotoxicity Assay

RAW 247.6 cells (1 x 10⁴ /mL) pre-treated with various concentrations of AG extract (10–100 µg/ml) for 30 min and treated with 100 µg/ml of Ox-LDL for 24 h. After treatment, cells were incubated with culture medium containing 0.5 mg/ml MTT for 160 min. Later, 0.15 ml of dimethyl sulphoxide was added to each well and incubated for 30 mins at room temperature. Absorbance was read at 540 nm using ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT) and % cell viability was calculated.

Ox-LDL Induced Foam Cell Formation

RAW 247.6 cells treated with AG extract were incubated in presence of 100 µg/ml of Ox-LDL for 24 h. Later, medium was removed 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. Cells were observed under Leica DMIL inverted microscope and photographs were taken using canon power shot S 70 digital camera (Jadeja *et al.*, 2011).

Intracellular Oxidative Stress

As shown in chapter 3.

Mitochondrial Membrane Potential Assay

As detailed in Chapter 3.

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 \pm 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 using 10 μ M CuSO₄ showed significant ($p < 0.001$) increased CD formation and reduction in lag time while AG (20, 50, 100, and 150 μ g/ml) co-supplementation showed significant reduction in CD formation and also delayed the lag time in a dose-dependent manner (Figure 4.1).

LDL Oxidation Products

LDL oxidation with 10 μ M CuSO₄ showed significant ($p < 0.001$) increase in the formation of MDA, LHP and PC as compared to the control group. However, AG co-supplementation (20, 50, 100, and 150 μ g/ml) significantly reduced MDA, LHP and PC formation in a dose dependently as compared to LDL+ 10 μ M CuSO₄ group (Figure 4.2).

Cell mediated LDL Oxidation

Cell mediated LDL oxidation reaction was induced by incubating the RAW 247.6 cells and LDL showed significant ($p < 0.001$) increased MDA levels as compared control group. The co-supplementation of AG extract showed significant decreased MDA levels as compared to the Ox-LDL group (Figure 4.3).

Ox-LDL Induced Foam Cell Formation

RAW 247.6 cells treated with Ox-LDL showed significant accumulation of Ox-LDL and foam cell formation as compared to control group while AG co-supplementation showed significantly reduced foam cell formation as compared to the Ox-LDL group (Figure 4.4).

Intracellular Oxidative Stress

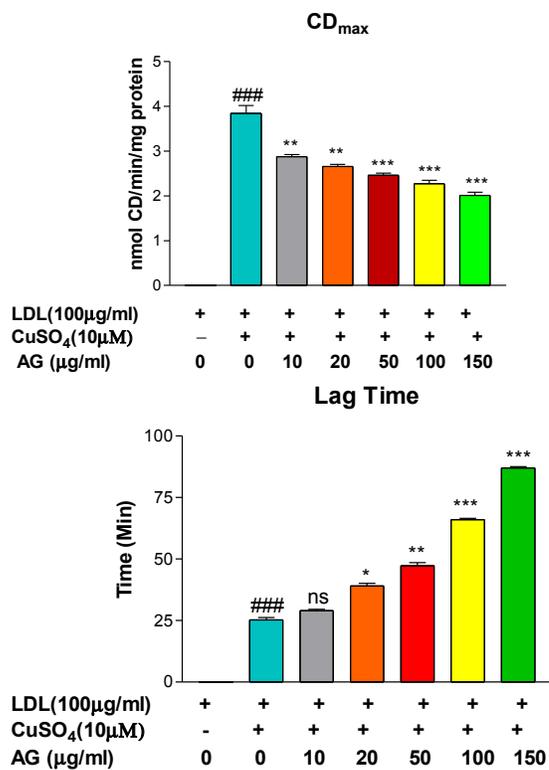
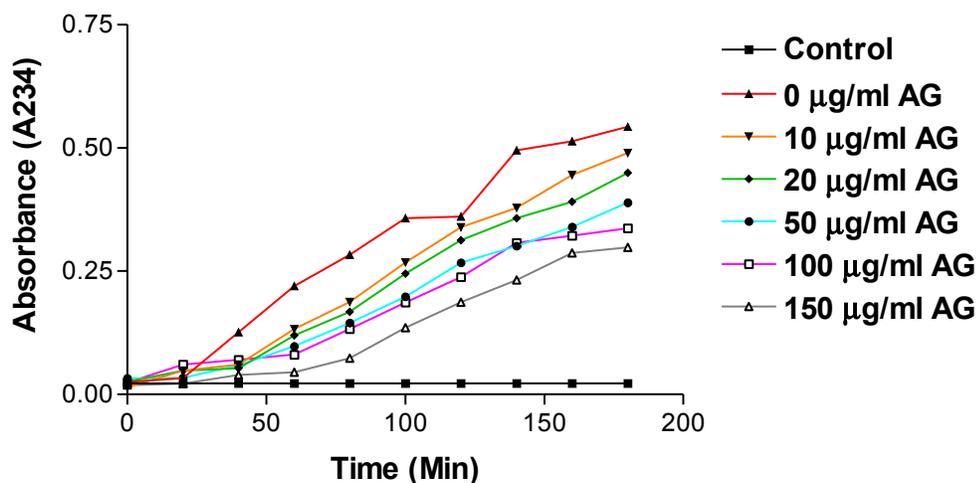
Ox-LDL treatment to RAW 247.6 cells for 24 h showed considerable increased green fluorescent cells as compared to control group indicating oxidative stress caused by Ox-LDL. While AG co-supplementation showed remarkably decreased number of green fluorescent cells in Ox-LDL+AG group as compared to Ox-LDL group. These results are indicating protective role of AG against to Ox-LDL induced oxidative stress (Figure 4.5).

Mitochondrial Membrane Potential Assay and Cell viability

Ox-LDL treatment to RAW 247.6 cells showed a significant alteration in mitochondrial membrane potential and cell viability. Ox-LDL treated cell showed significant reduction in mitochondrial potential and cell viability as compared to the control group. AG co-supplementation significantly prevented the decreased mitochondrial membrane potential and cell viability as compared to the Ox-LDL treated group (Figure 4.6 and Figure 4.7).

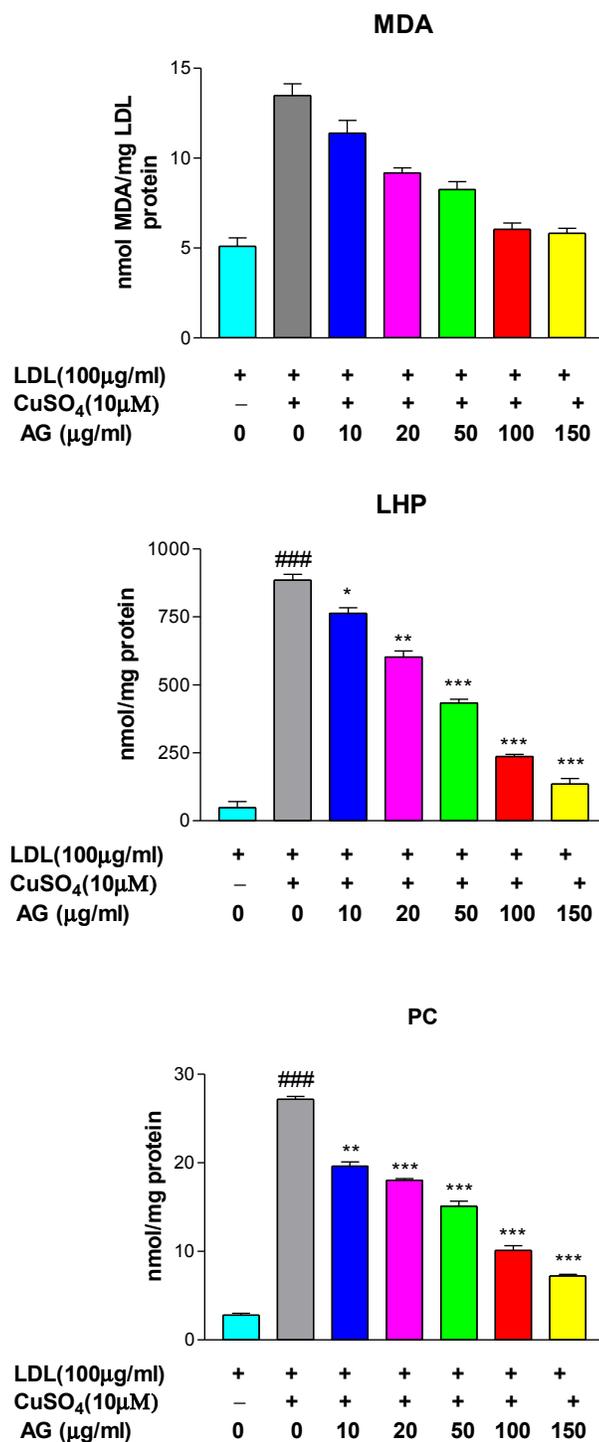
TABLES AND FIGURES

Figure 4.1: Effect of AG on Copper-Mediated LDL Oxidation Kinetics

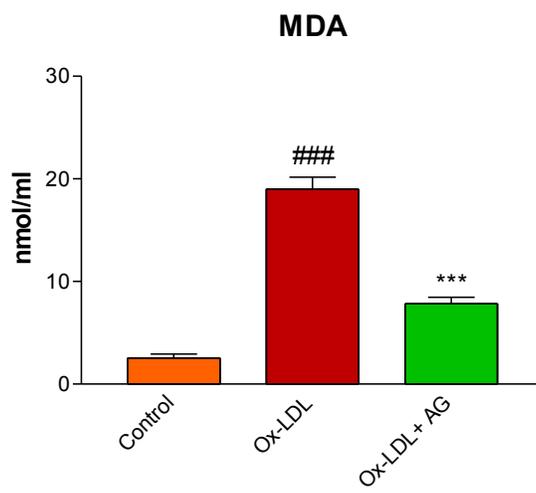


Data expressed as mean \pm S.E.M. for n=3. ### p<0.001 compared to LDL alone and *p<0.05, ** p<0.01, *** p<0.001 and ns = non significant compared to LDL+ CuSO₄.

Figure 4.2: Effect of AG on Formation of LDL Oxidation Products (MDA, LHP and PC)



Data expressed as mean \pm S.E.M. for n=3. ### p<0.001 compared to LDL alone and * p<0.05, ** p<0.01, *** p<0.001 and ns = non significant compared to LDL+ CuSO₄

Figure 4.3: Effect of AG (150 µg/ml) on Cell Mediated LDL Oxidation

Data expressed as mean \pm S.E.M. for n=3. ### p<0.001 compared to control and *** p<0.001 compared to OX-LDL

Figure 4.4: Effect of AG (150 $\mu\text{g/ml}$) on Foam Cell Formation Assay using Oil Red O Staining

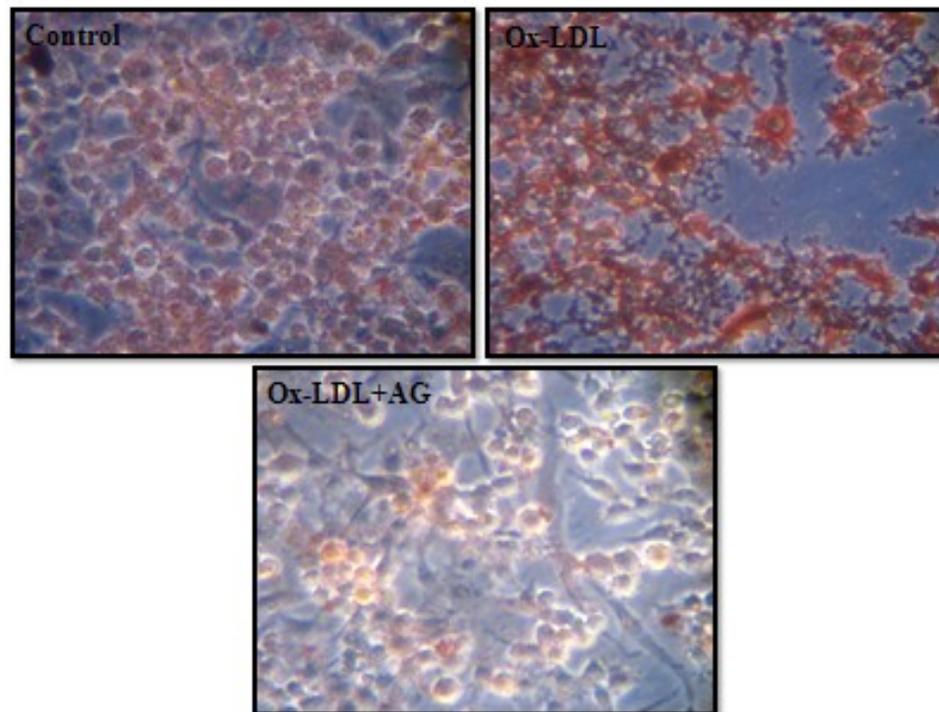


Figure 4.5: Effect of AG (150 $\mu\text{g/ml}$) on Peroxyl Radical Generation (DCF-DA Staining)

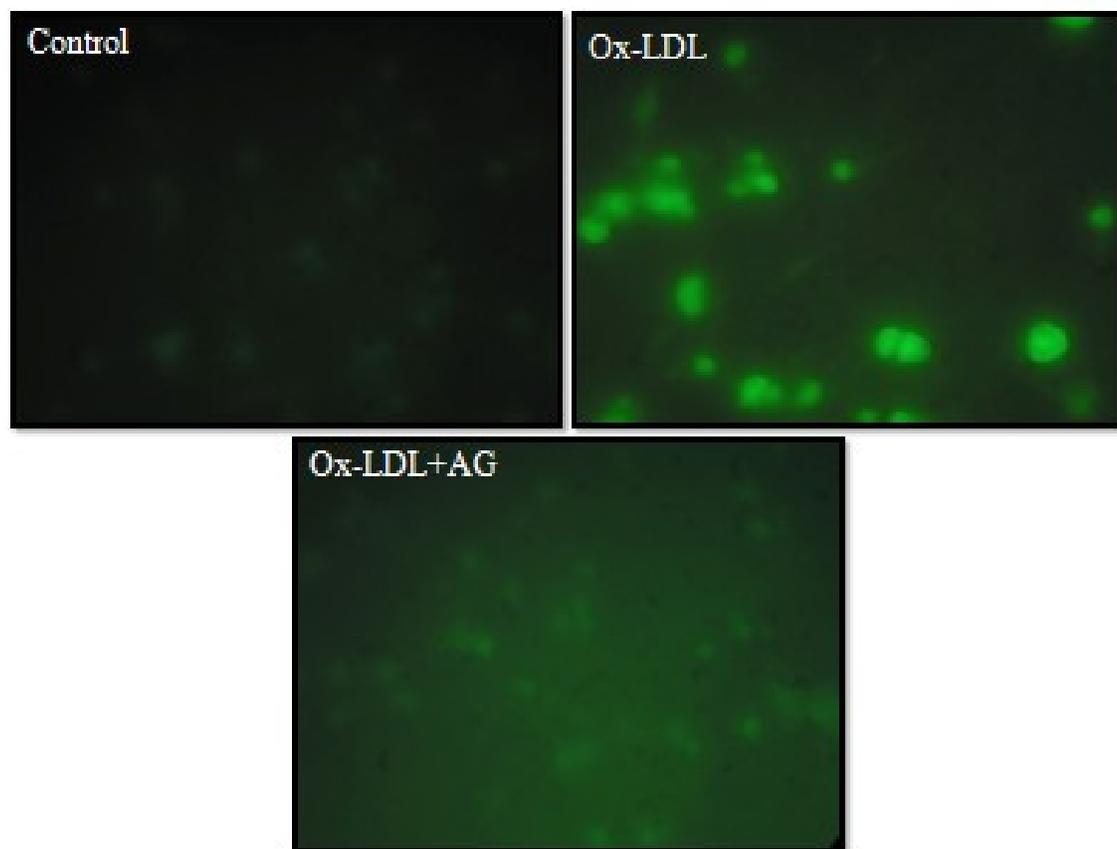
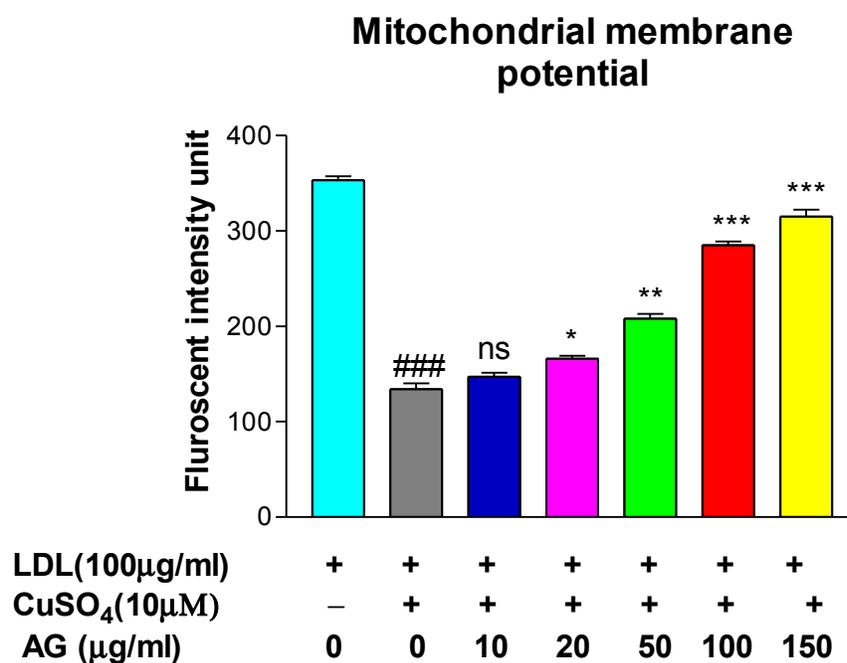
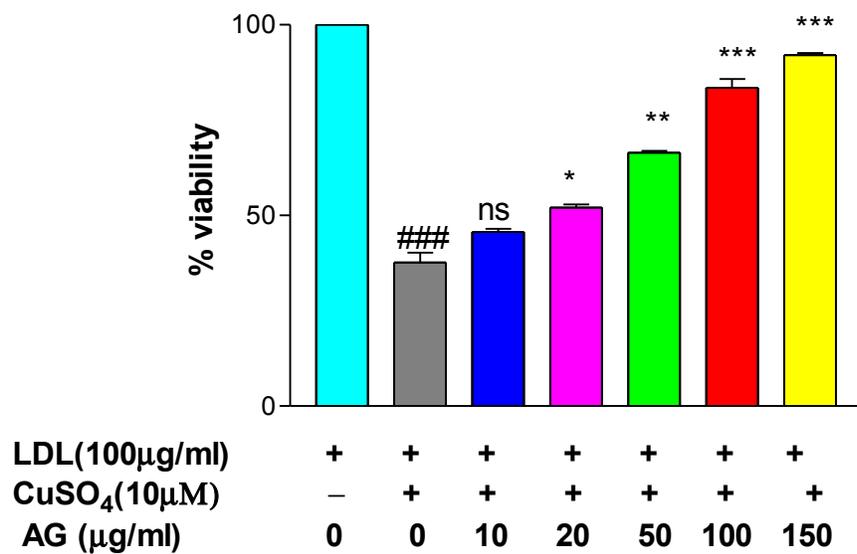


Figure 4.6: Effect of AG on Mitochondrial Membrane Potential (Rhodamine 123 Staining)



Data expressed as mean \pm S.E.M. for n=3. ### p<0.001 compared to LDL alone and * p<0.01, ** p<0.05, *** p<0.001 and ns = non significant compared to LDL+ CuSO₄

Figure 4.7: Effect of AG on Cell Viability



Data expressed as mean \pm S.E.M. for n=3. ### p<0.001 compared to LDL alone and * p<0.01, ** p<0.05, *** p<0.001 and ns = non significant compared to LDL+ CuSO₄

DISCUSSION

Atherosclerosis has been considered as a chronic inflammatory process and ox-LDL plays an important role in pathogenesis of atherosclerosis (Ross, 1999). Ox-LDL is engulfed by the macrophages and they convert into foam cells (Witztum and Steinberg, 1991). Fruits, vegetables, herbs and spices with strong antioxidant activity are capable of preventing oxidative stress and related alterations (Sankhari *et al.*, 2012; Patel *et al.*, 2013). Previous studies from our lab had shown that functional foods (Sankhari *et al.*, 2012), therapeutic herbs (Thounaojam *et al.*, 2010; Jadeja *et al.*, 2012) and spices (Patel *et al.*, 2013, Gajaria *et al.*, 2014) are able to prevent oxidative modification of LDL molecule as evidenced by assay of LDL oxidation products such as malondialdehyde, protein carbonyls and lipid hydroperoxide. Also, the ingredients of natural origin further contribute in prevention of ox-LDL uptake in macrophages and prevent foam cell formation.

Anethum graviolens L. (AG) has established antioxidant potential due to the presence of flavonoids (rutin, quercetin), hydroxycinnamic acid derivatives (caffeic acid, chlorogenic acid), coumarins (scopoletin) and sterols (beta sitosterol/stigmasterol) which are strong antioxidants (Ortan *et al.*, 2008). *In vitro* studies on ox-LDL induced cell death on macrophages have shown that antioxidants are effective in preventing the alteration caused by Ox-LDL. Hence, the present study was conducted to assess the effect of AG on CuSO₄ mediated LDL oxidation (Ox-LDL) and Ox-LDL induced changes on macrophages *in vitro*. CuSO₄ mediated LDL oxidation showed significantly increased conjugate diene formation (CD_{max}) and reduced lag time formation while AG treatment showed that significant reduction of CD_{max} and increased lag time. Oxidation of LDL

altered the lipid and the protein moieties of LDL and results into fragmentation of TBARS and protein carbonyl (Ahotupa *et al.*, 1998). Also, the LDL-protein apoB, undergoes fragmentation and due to the derivatization of the lysine residue and its electrophoretic mobility is altered (Holvoet *et al.*, 1995). In our study, the recorded increased levels of MDA, LHP and PC following LDL oxidation are in agreement with previous reports. However AG co-supplementation significantly reduced formation of LDL oxidation products. It can be inferred that CuSO₄ mediated LDL oxidation is prevented by AG due to its strong antioxidant potential. Since, oxidative modification of LDL is a key event during onset of atherosclerosis (Itabe, 2009), prevention of the same can be assumed to reduce the risk to a great extent that needs further validation in 'whole-organism' study.

Macrophages actively take up and clear Ox-LDL in an effort to prevent plaque formation but in that process, end up becoming bulky and transform into foam cells (Suowen *et al.*, 2010). In our study, RAW 264.7 cells (mouse macrophages) exposed to Ox-LDL showed foam cell formation as evidenced by Oil Red O staining. However, AG co-supplementation showed significantly reduced number of oil red O positive cells thus indicating decreased LDL uptake. These results provide strong experimental evidence of AG mediated prevention of foam cell formation. Ox-LDL is cytotoxic as it triggers apoptosis in macrophages via oxidative stress pathway (Witztum and Steinberg, 1991). DCF-DA staining of Ox-LDL treated RAW cells showed higher number of cells with green fluorescence which indicated heightened oxidative stress while AG co-supplementation showed significant reduction in fluorescence. Further, MMP and cytotoxicity assay (MTT assay) revealed poor functional status of mitochondria and

reduced cell viability following Ox-LDL treatment. However, AG co-supplementation significantly increased MMP and cell viability. Overall, it can be concluded that AG extract is instrumental in preventing foam cell formation, intracellular oxidative stress, improves functional status of mitochondria and contributes to the improvement in cell viability possibly due to its antioxidant and free radical scavenging potentials. These results further contribute towards the existing database of therapeutic properties of AG and provide strong indications of its anti-atherosclerotic potential. The same has been investigated in chapter 5 via an *in vivo* protocol using rodent model of atherosclerosis.

SUMMARY

Present study was designed to evaluate the effect of *Anethum graviolens* L. extract (AG) on LDL oxidation and foam cell formation *in vitro*. We investigated the effects of AG extract on the oxidation of LDL and the uptake of lipid in RAW 264.7 macrophages. Initially we isolated LDL from blood donated healthy donors and subjected to oxidation using copper (II) sulfate (CuSO_4) as an oxidative inducer in presence and absence of AG extract. AG extract prevented LDL oxidation and it was observed by increased lag time and decreased Conjugate diene formation. AG successfully reduced levels of Malondialdehyde (MDA), Lipid hydroperoxide (LHP) and Protein carbonyls (PC). Oxidised LDL was incubated with RAW 264.7 macrophage cells in presence and absence of AG extract. AG extract successfully prevented lipid accumulation and reduced foam cell formations. AG extract also reduced levels of intracellular oxidative stress, mitochondrial membrane potential assay and cell viability. These results demonstrate the protective effect of AG on LDL oxidation and lipid accumulation in macrophages.