Objective: CD8 T cells make up a large amount of lymphocytes in atherosclerotic plaques, however their role in atherosclerosis has not been thoroughly studied. Since ApoB100 is a key antigen in atherosclerosis and a plausible vaccination target, we were interested in the role of ApoB100 specific CD8 T cells in atherosclerosis. To be directly relevant for human vaccination HLA-A2 transgenic (HLA-A2) and human ApoB100 transgenic (hApoB100tg) mice were used.
Methods: HLA-A2 restricted CD8 T cell epitopes derived from human ApoB100 were in silico predicted using The "Proteasomal cleavage/TAP transport/MHC class I combined predictor"of the immune epitope database. Best 6 epitopes were synthesized and binding to HLA-A2 assesed with T2 cell binding assays. To assess induction of peptide specific CD8 T cells, HLA-A2 transgenic mice were vaccinated with peptide pulsed DCs. Peptide recall responses were measured with flow cytometry. To study whether vaccination with ApoB100 derived CD8 T cell epitopes could influence atherosclerosis, male hApoB100tg HLA-A2tg LDLrKO mice (n=10-11) were vaccinated with peptide pulsed bone marrow derived DCs and boosted a week later with with αCD40 and Poly(I:C) and peptides. Animals were fed western type diet for 11 weeks following DC vaccination, after which they were sacrificed to assess aortic root lesion development.
Results: All predicted HLA-A2 restricted CD8 T cell epitopes stabilized HLA-A2 in T2 cell binding assays, indicating binding of all peptides to HLA-A2. For 5 of 6 peptides peptide specific recall response were measured a week after vaccination with peptide pulsed DCs. Vaccination with these CD8 T cell epitopes did not lead to reduced atherosclerosis. 10 weeks after the booster vaccination still peptide specific CD8 T cell responses could be detected, indicating that the vaccination was succesful.
Conclusions: We have identified 5 HLA-A2 restricted ApoB100 derived CD8 T cell epitopes. Vaccination with these peptides did induce long lasting ApoB100 specific CD8 T cells, however did not affect atherosclerosis in male hApoB100tg HLA-A2tg LDLrKO mice.

## P3.049
## MOLECULAR MECHANISMS OF CARDIAC REMODELING IN THE ISCHEMIC HEART: UNDERSTANDING THE ROLE OF FIBROBLASTS

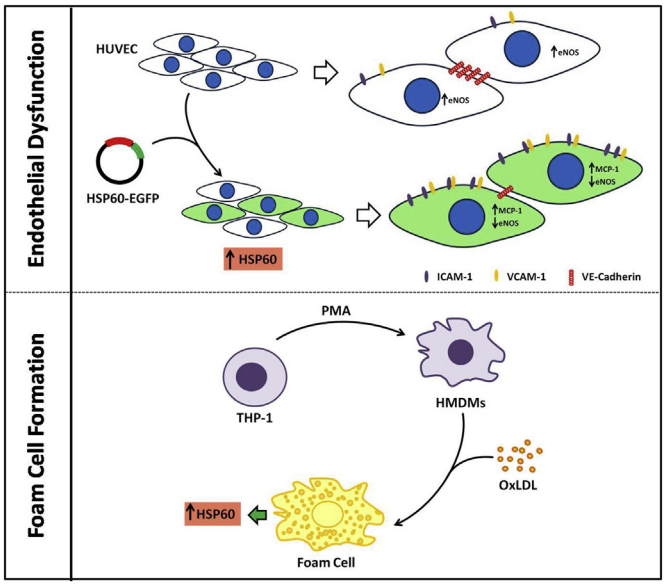
Haisam Shah[1,2], Shafkat Choudhury[1,2], Hangjun Zhang[1,2], Meghan McFadden[1,2], Yuqing Zhou[1,2], Boris Hinz[1], Anthony Gramolini[1,2], Scott Heximer[1,2]. [1] *University of Toronto, Toronto, ON, Canada;* [2] *Ted Rogers Centre for Heart Research, Toronto, ON, Canada*

Atherosclerosis of the coronary arteries is a common cause of ischemic heart disease and heart failure (HF), conditions that are often characterized by cardiac remodeling and fibrosis. Cardiac fibrosis is thought to be primarily mediated by myofibroblasts, a population of 'activated' cardiac fibroblasts (CFs), characterized by α-smooth muscle actin (αSMA) expression. We posit that studying the proteomic changes of CFs during ischemia-induced cardiac remodeling will help to identify novel biomarkers and potential therapeutic targets for fibrosis and HF. M-mode, B-mode, and pulse-wave doppler echocardiography demonstrated cardiac dysfunction in mice that underwent left anterior coronary descending artery (LAD) ligation, a model of chronic ischemic injury. Specifically, ischemic hearts exhibited decreases in ejection fraction, fractional area change, stroke volume, and cardiac output seven days post-myocardial infarction (MI) compared to sham controls. To study the role of fibroblasts, we isolated CFs from ischemic hearts and sham controls, ensuring that our isolation protocol yielded primarily positive signals for known CF markers, including collagen type I and Vimentin. To identify CF isolation conditions that had minimal impact on myofibroblast differentiation, αSMA protein expression was evaluated using immunoblotting to establish a kinetic profile for CF differentiation in culture. No αSMA expression was observed after one day, however level progressively increased after three and five days in culture. Thus, for all subsequent proteomic experiments, minimal cell culture was used following the acute dissociation of CFs from infarcted and remote cardiac regions of seven-day post-MI and sham control mice. Global proteomic analysis by liquid chromatography tandem mass spectrometry (LC/MS-MS) revealed differential profiles for each treatment group and region-specific differences in the protein expression of CFs isolated from MI and sham hearts ($p < 0.01$). In conclusion, this study shows that proteomic analysis of freshly isolated CFs from ischemic hearts may provide valuable insight into the role CFs play in the development and progression of fibrosis, cardiac remodeling, and heart failure.

## P3.050
## HEAT SHOCK PROTEIN (HSP) 60 OVEREXPRESSION IS ASSOCIATED WITH ATHEROGENIC CHANGES IN HUVEC AND THP-1 CELLS

Kavita Shirsath, Ranjitsinh Devkar. *The Maharaja Sayajirao University of Baroda, Vadodara, GJ, India*

Objective: Growing body of evidence suggests that HSP60 is strongly associated with atherosclerosis, but its precise mechanism of promoting the same remains unclear. Since, human HSP60 and its bacterial counterpart are genetically conserved, it tends to show immune-crossreactivity leading to auto-immune reactions. Under normal physiological conditions, HSP60 is encoded by nucleus and expressed in mitochondria. However, in cells stressed with classical atherogenic risk factors, it is transported to the cytosol and plasma membrane. Our study investigates the association of HSP60 in aggravating atherogenic changes in Human umbilical vein endothelial cells (HUVEC) and human monocytes (THP-1) cells.
Methods: Briefly, HUVEC were transfected with HSP60-EGFP plasmid using Lipofectamine 3000 and internalization and successful expression of the same was assessed by microscopic observation. Further confirmation was obtained by RT-PCR and western blotting of HSP60. Later, mRNA expression of cell adhesion molecules (VCAM1, ICAM1 and VE-Cadherin), monocyte chemoattractant protein-1 (MCP-1) and endothelial nitric oxide synthase (eNOS) were assessed in transfected cells. In the second part of the study, THP-1 were differentiated into human monocyte derived macrophages (HMDMs) using phorbol 12-myristate 13-acetate (PMA) and assessed for uptake of oxidized low density lipoprotein (OxLDL) by Oil red O staining. Further, expression of HSP60 in OxLDL treated HMDMs was assessed by RT-PCR and western blotting.
Results: Upregulation of candidate genes (VCAM1, ICAM1 and MCP-1) and downregulation of VE-Cadherin and eNOS in HSP60 overexpressing HUVEC, implied towards their atherogenic transformation; a condition comparable with endothelial dysfunction. Also, significant uptake of OxLDL and consequent upregulation of HSP60 was observed in HMDMs.
Conclusions: Results obtained revealed that HSP60 is a key player in mediating atherogenic changes in vascular endothelium and OxLDL-mediated foam cell formation.




## P3.051
## BLOOD BIOMARKERS AND ELECTRICAL MONITORING IN ADVANCED HEART FAILURE

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# *Cuminum cyminum* methanolic extract prevents oxidative modification of low density lipoproteins: Preliminary evidence on its anti-atherosclerotic potential

Ranjitsinh Devkar*, Kiran Lagu, Jaymesh Thadani, Kavita Shirsath

## ABSTRACT

The significance of oxidative modification of LDL in the pathogenesis of atherosclerosis and the lack of efficient treatment intervention has led researchers to develop an effective therapy based on natural anti-oxidants. The present study provides preliminary evidence in support of the anti-atherosclerotic potential of methanolic extract of *Cuminum cyminum* L. (CC). We found that CC inhibited $Cu^{2+}$-mediated LDL oxidation as demonstrated by the *ex vivo* LDL oxidation kinetic study, the LDL oxidation products (malondialdehyde, lipid hydroperoxide and protein carbonyl), and ApoB fragmentation assay. It can be concluded that, CC efficiently alleviates experimentally induced oxidative changes and modifications of LDL. Since oxidative changes in LDL are prerequisite to onset of atherogenic changes, this study provides preliminary evidence on anti-atherosclerotic potential of CC.

**Keywords:** *Cuminum cyminum*, LDL oxidation, ApoB fragmentation.

## INTRODUCTION

Atherosclerosis is a multifactorial disease primarily characterized by deposition of fatty substances in the arterial wall leading to the narrowing of its lumen. The elevated levels of plasma low density lipoproteins (LDL) caused by dysregulated lipid metabolism is one of the key factors contributing to complex etiology of atherosclerosis [1]. Furthermore, the impairment of anti-oxidant defense mechanisms along with the generation of reactive oxygen species (ROS) causes oxidative modification of LDL forming oxidized LDL (Ox-LDL), which is taken up by macrophages to form foam cells initiating inflammatory events. As the disease progresses, the foam cells undergo apoptotic death, further contributing to the inflammatory milieu of the subendothelial space by recruiting more macrophages to the site [2]. Thus, it is evident that oxidative stress acts as the initiator of atherosclerosis and hence, it appears that antioxidants that inhibit LDL oxidation may be effective as therapy against atherosclerosis.

Active antioxidant constituents of have been in focus of research for their therapeutic potentials includinganti-atherosclerotic properties. Till date, our lab had reported anti-atherosclerotic potential of *Clerondendron glandulosum* [3], *Sida rhomboidea*. Roxb [4], *Coriandrum sativum* L. [5] and *Murraya koenigii* [6]. *Cuminum cyminum* (fam. Apaiaceae) is an annual herb whose seeds are widely used in Indian culinary and are also reported for their medicinal properties. Cumin seed extract (CC) have been reported to be effective against skin rashes, kidney and gallbladder stones, nausea toothaches, hiccoughs, dyspepsia, diarrhea, epilepsy, jaundice, flatulence and indigestion. Furthermore, essential oil of CC has been reported to have diuretic, carminative, emmenagogic, antibacterial, antifungal, antioxidant, anticancer, antidiabetic and antispasmodic properties [7, 8]. Besides being anti-oxidant, CC has been reported for its lipid lowering activity [9]. This study investigates efficacy of CC in preventing oxidative modifications of LDL by assaying LDL oxidation products and ApoB fragmentation. The aim was to gather preliminary evidence on therapeutic potential of CC in an experimentally simulated condition wherein lipotoxicity is at the epicenter of pathophysiology.

## MATERIALS AND METHODS

### Chemicals and reagents

Sodium carbonate, folin's reagent, acetic anhydride, trisodium citrate, ammonium sulphate, xylenol orange, potassium acetate, sodium chloride, copper sulphate, ethylene di-amine tetrachloro acetic acid

(EDTA), thiobarbituric acid (TBA), trichloroaceticacid (TCA), butylated hydroxyanisole (BHT), sodium dodecyl sulphate (SDS), di-nitrophenyl hydrazine (DNPH), heptane, ethyl acetate, chloroform, iron (III) chloride, phosphomolybdic acid, acrylamide, bisacrylamide, ammonium persulphate, tetramethylethylenediamine (TEMED), glycerol, bromophenol blue and coomassie brilliant blue R250 were purchased from Sisco Research Laboratories (Mumbai, India). Hydrochloric acid (HCl), sulphuric acid ($H_2SO_4$), and glacial acetic acid were purchased from Suvidhanath Laboratories, (Vadodara, India). Tris base and glycine were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

**Preparation of plant extract**

CC seeds were collected from Spencer's mall, Vadodara, Gujarat, India, identified and authenticated by Dr. Vinay Raole, Department of Botany and voucher specimen (accession number 379) was submitted to departmental herbarium (BARO), The M. S. University of Baroda, Vadodara, Gujarat, India. 100 g dried seeds were powdered, defatted (in 70% petroleum ether overnight) and extracted with 80% methanol in a soxhlet apparatus to obtain a flavonoid rich extract. The resultant extract was concentrated in a rotary evaporator, followed by hydrolysis in a waterbath (at 60°C) for 24 h that yielded two phases. The organic phase (flavonoid rich) was used for further investigation [10]. The final yield was 8.3 g (w/w).

**Qualitative phytochemical analysis**

CC extract was subjected to qualitative phytochemical analysis for detection of major chemical groups such as phenolic acids (phosphomolybdic acid test), alkaloids (Dragendorff's test), flavonoids (Shinoda test), glycosides (Keller-kilani test), terpenoids and steroids (Liebermann-Burchardt test), saponins (Frothing test) and proteins and amino acids (copper sulphate test) as described previously [11, 12].

**Isolation of LDL**

LDL was isolated using heparin–citrate buffer precipitation method [13]. Briefly, blood was collected under the supervision of a pathologist from normocholesterolemic healthy adult volunteers (n=6) under fasting condition after taking prior consent and as per the standard guidelines at Blue cross pathology lab (IMA-BMWMC No. 1093). Serum was obtained after centrifugation (3000 rpm for 10 min at 4°C) and 0.1 ml was mixed with 1 ml heparin–citrate buffer (64 mM trisodium citrate at pH 5.05 containing 50,000 IU/l heparin), vortexed and allowed to stand for 10 min at room temperature. Later, the mixture was centrifuged at 3,000 rpm for 10 min at 20 °C and the resultant pellet was re-suspended in 0.1 ml of phosphate-buffered saline (PBS; 0.1 M, pH 7.4, containing 0.9 % NaCl). The protein concentration of the obtained LDL was estimated [14] using bovine serum albumin as a standard.

**LDL oxidation kinetics**

100 µg/ml of LDL was incubated with or without 0.1 ml of various concentrations of CC extract (10–300 µg/ml) at 37 °C for 30 min and subsequently, 10 µl freshly prepared 0.167 mM Copper sulfate solution. Absorbance was recorded by continuous monitoring (at 10 min interval for 180 min at 234 nm using UV-Vis Perkin Elmer spectrophotometer) to record LDL oxidation kinetics. The lag time was determined from the intercepts of lines drawn through the linear portions of the lag phase and propagation phase, whereas the rate of oxidation was determined from the slope of the propagation phase. The concentration of conjugate diene (CD) in the samples was calculated by using a molar extinction coefficient of 2.95 x $10^4$ $M^{-1}cm^{-1}$. Maximum concentration of CD formed was calculated from the difference in the concentration of CD at time 0 min and at absorption maxima [15].

**Determination of LDL oxidation products**

The LDL was oxidized in presence or absence of CC extract (10–300 µg/ml) for 24 h as mentioned above. Later, 10 µl of 10 mM ethylene diamine tetra acetic acid (EDTA) was added to stop oxidation reaction and the samples (in triplicates) were subjected to quantification of LDL oxidation products viz. malondialdehyde (MDA), lipid hydroperoxide (LHP) and protein carbonyl (PC) as follows:

For MDA estimation, 0.1 ml of oxidized LDL (Ox-LDL) was mixed with 1 ml TBA reagent (0.37 % TBA, 15 % TCA in 0.25N HCl) and incubated (at 100 °C for 30 min), cooled and centrifuged at 3000 rpm for 10 min. The absorbance was read at 532 nm (in UV–Vis Perkin Elmer spectrophotometer) and the amount of MDA was calculated using a molar extinction coefficient of 1.56 x $10^5$ $M^{-1}cm^{-1}$ [16].

For LHP estimation, 0.1 ml of Ox-LDL was 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 recorded at 560 nm and LHP content was determined using the molar extinction coefficient of 4.3 x $10^4$ $M^{-1}$ $cm^{-1}$ [17].

For estimation of PC**,** 0.1 ml of aliquot was 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. This was followed by addition of ethanol and heptane (1:1; 1.8 ml each) and the contents were centrifuged at 3,500 rpm to precipitate protein. The protein was washed thrice with 1 ml ethyl acetate/ethanol (1:1, v/v), dissolved in 1 ml of denaturing buffer, and read at 360 nm using UV–Vis Perkin Elmer spectrophotometer. The PC content was calculated using molar extinction absorption coefficient of 22.0 x $10^3$ $M^{-1}cm^{-1}$ [18].

**Apolipoprotein B100 (ApoB) fragmentation assay**

ApoB fragmentation assay was carried out to check the oxidative fragmentation of ApoB of LDL. Briefly, LDL was oxidized in presence or absence of CC extract (10–300 µg/ml) for 24 h and the reaction was stopped using 10 mM EDTA as mentioned above. LDL obtained after centrifugation was denatured with sample buffer (3 % SDS, 10 % glycerol, and 5 % bromophenol) at 95 °C for 5 min and cooled to room temperature. All the LDL samples were subjected to electrophoretic separation using 8 % SDS-PAGE. The gels were stained (2 % Coomassie brilliant blue R250 solution) and de-stained (20 % glacial acetic acid and 10 % methanol in water) followed by its imaging using Bio-Rad gel documentation system [19].

**Statistical analysis**

All the experiments were performed in triplicates. 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 and the statistical analysis was carried out using Graph Pad Prism version 3.0.

## RESULTS

### Phytochemical constitution of CC extract

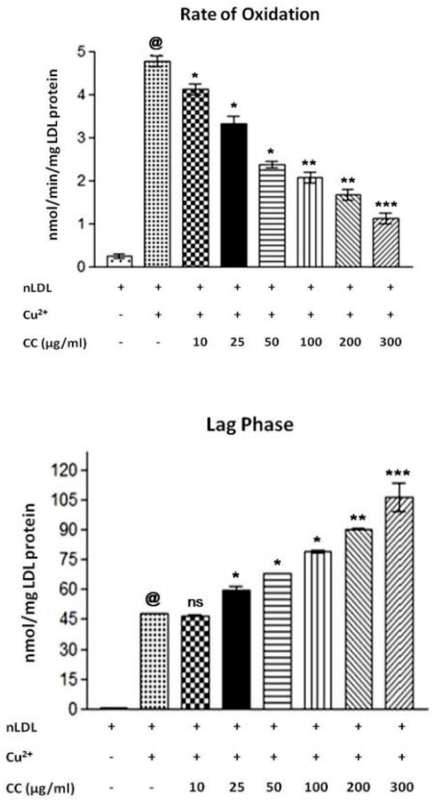
The results of the preliminary phytochemical analysis are shown in Table 1.

**Table 1:** Qualitative assessment of phytochemical constituents of CC extract.

| Phytochemical | Test results |
|---|---|
| Alkaloids | Absent |
| Phenolic compounds | Highly present |
| Phytosteroids | Poorly available |
| Proteins and amino acids | Absent |
| Saponins | Absent |
| Glycosides | Present |
| Flavonoids | Present |

### $Cu^{2+}$-induced LDL oxidation

We observed a dose-dependent decrease in the rate of $Cu^{2+}$-mediated oxidation of LDL in presence of CC (Fig. 1). Further, a significant increase in the lag period and decrease in the $CD_{max}$ values of LDL oxidation was observed in samples containing CC (Fig. 1). Moreover, increase in LDL oxidation products (MDA, LHP and PC) was recorded on addition of $CuSO_4$ but CC extract supplementation recorded a dose dependent decrement in the same (Fig. 2). Absence of ApoB band suggested its fragmentation in Ox-LDL group whereas, its presence was recorded in the sample containing CC (Fig. 3).



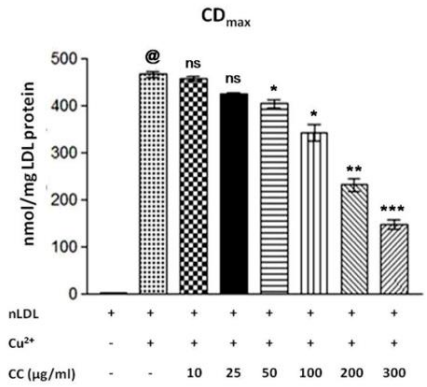




**Figure 1:** Effect of CC on LDL oxidation kinetics (lag time period, rate of oxidation and maximum conjugated diene formation; CDmax) in $Cu^{2+}$ induced LDL oxidation. Data were expressed as mean ± S.E.M. for n=3.@p<0.001 compared to nLDL, ns non-significant, *p<0.05, **p<0.01, ***p<0.001 compared to Ox-LDL.

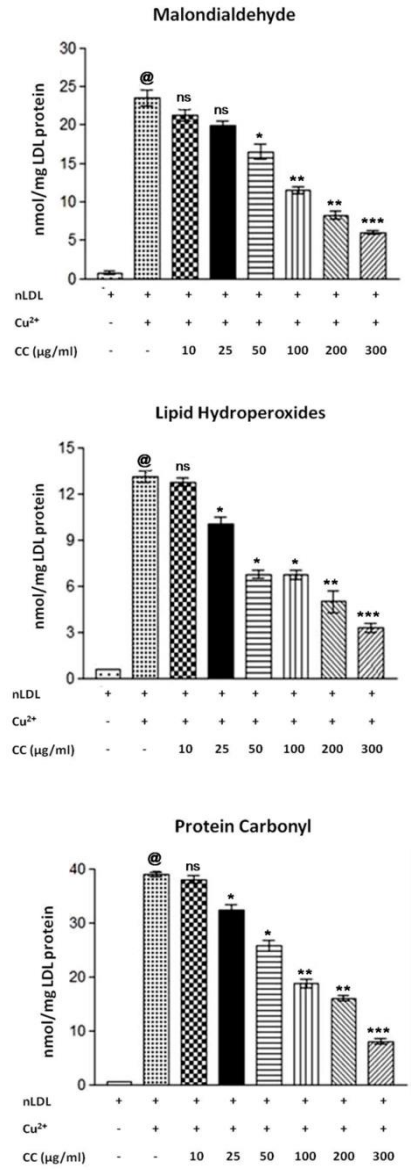



**Figure 2:** Effect of CC on LDL oxidation end products (malondialdehyde, lipid hydroperoxides and protein carbonyls) in $Cu^{2+}$ induced LDL oxidation. Data were expressed as mean ± S.E.M. for n=3. @p<0.001 compared to nLDL, *p<0.05, **p<0.01, ***p<0.001 compared to Ox-LDL

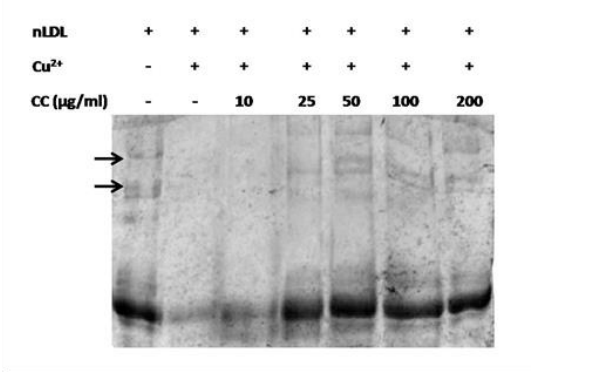



**Figure 3:** Effect of CC on ApoB fragmentation in $Cu^{2+}$ mediated oxidized LDL. Arrow indicates ApoB protein band in SDS-PAGE.

## DISCUSSION

Progression of atherosclerosis in vivo is preceded by elevated levels of circulating cholesterol coupled with low grade inflammation [20] or environmental pollutant [21]. The sequence of events often overlap and make the study challenging. Oxidative modification of LDL is one such event that has been extensively studied and prevention of the same by a therapeutant is believed to retard the atherosclerotic cascade. Covalent modification lipids lead to harmful intermediate products that are toxic to endothelial cells, monocytes, neutrophils, etc. and restrict macrophage mobility [1, 22]. Oxidation of lipid moieties also produce lipid peroxides that cause rearrangement of fatty acid double bonds and production of conjugated dienes (CD). Also, the resultant aldehydes and ketones produced by fatty acid fermentation cause ApoB fragmentation [1].

Copper mediated LDL oxidation is a widely used protocol to obtain prima facie evidence on potential of test compound in preventing LDL oxidation. In our study, an extended lag phase recorded in presence of CC indicates at its ability in delaying $Cu^{2+}$ mediated LDL oxidation and minimizing the resultant CD. Further, a dose dependent decrement in LDL oxidation products (MDA, LHP, PC) in presence of CC is in agreement with the previous observation. Overall, CC appears to play a protective role as evidenced by significantly less formed LDL oxidation products.

Oxidative deletion of peptide bonds in an LDL molecule leads to its alteration and ApoB fragmentation. This process also form covalent adducts and increases the net negative charge of the molecule. Since the same is recognized by the scavenger receptors of the macrophages that follows foam cell formation [23], reduction/prevention of ApoB fragmentation is an important credential in a test compound. In our study, the electrophoretic profile showed disappearance of the ApoB band in Ox-LDL sample however, a dose-dependent recovery of the ApoB band was seen in CC supplemented groups. These results are attributable to high content of flavonoids and phenolic compounds in CC reported herein. Hence, CC is thought to be instrumental in preventing oxidative modification of LDL molecules due to free radical scavenging activity of the phenolic compounds and flavonoids present in its extract.

## CONCLUSION

It can be concluded from this study that CC is a promising prospect as an anti-atherosclerotic agent and needs a detailed study to establish the same.

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Research Article

# *Cuminum cyminum* Prevents Lipotoxicity and Apoptosis but Cuminaldehyde Fails to Do So: A Study on Mouse Macrophage (RAW 264.7) Cells

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## Abstract:

Aim: The present study aims to compare the cytoprotective effect of Cuminum cyminum L. (CC) extract and cuminaldehyde (CA) against lipotoxicity induced by oxidized low density lipoprotein (Ox-LDL) in mouse macrophage (RAW 264.7) cells.

Objectives:

i) To assess comparative Cytoprotective potential of CC and CA against Ox-LDL induced cytotoxicity.

ii) To study efficacy of CC and CA in preventing Ox-LDL induced apoptosis

Methods: Protective effect of CC extract and CA aganist Ox-LDL induced cytotoxicity in RAW 264.7 cells was assessed by MTT assay. DCFDA stain was used to check the generation of ROS followed by analysis of apoptotic genes by quantitative RT-PCR.

Results: CC extract was found to be non-toxic up to 300 µg/ml but CA showed significant toxicity from 50 to 300 µg/ml. Cells treated with Ox-LDL recorded 80 % decrement in cell viability as compared to the control cells. But Ox-LDL+CC treated group accounted for improved cell viability (88 %) which was comparable to that of control. However, Ox-LDL+CA treated cells did not record any improvement in cell viability (19 %). DCF-DA staining revealed that the presence of CC could minimize intracellular oxidative stress but similarly this was persistent in CA supplemented group. Furthermore, mRNA expression of apoptotic genes revealed that Ox-LDL induced upregulation of Bax and downregulation of Bcl-2 genes were not recorded in Ox-LDL+CC treated group.

Conclusion: It can be concluded that CC extract efficiently prevented Ox-LDL induced lipotoxicity and apoptosis and has an anti-atherosclerotic potential. The failure of CA emphasizes the importance of naturally occurring polyherbal formulations over pure compounds in imparting bioactivity and for therapeutic applications.

**Keywords:** Cuminum cyminum L., Cuminaldehyde, LDL oxidation, macrophage, apoptosis, oxidative stress.



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# *Cuminum cyminum* prevents lipotoxicity and apoptosis but cuminaldehyde fails to do so: A study on mouse macrophage (RAW 264.7) cells

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Running Title: *Cuminum cyminum* prevents lipotoxicity and apoptosis in RAW 264.7 cells

**ABSTRACT**

The present study compares cytoprotective effect of *Cuminum cyminum* L. (CC) extract and cuminaldehyde (CA) against lipotoxicity induced by oxidized low density lipoprotein (Ox-LDL) in mouse macrophage (RAW 264.7) cells. CC extract was found to be non-toxic up to 300 µg/ml but CA showed significant toxicity from 50 to 300 µg/ml. Cells treated with Ox-LDL recorded 80 % decrement in cell viability as compared to the control cells. But Ox-LDL+CC treated group accounted for improved cell viability (88 %) which was comparable to that of control. However, Ox-LDL+CA treated cells did not record any improvement in cell viability (19 %). DCF-DA staining revealed that presence of CC could minimize intracellular oxidative stress but the same was persistent in CA supplemented group. Further, mRNA expression of apoptotic genes revealed that Ox-LDL induced upregulation of Bax and downregulation of Bcl-2 genes but such changes were not recorded in Ox-LDL+CC treated group. It can be surmised that CC extract efficiently prevented Ox-LDL induced lipotoxicity and apoptosis and has anti-atherosclerotic potential. Failure of CA emphasizes the importance of naturally occurring polyherbal formulations over pure compounds in imparting bioactivity and for therapeutic applications.

**Key words**

*Cuminum cyminum* L.; Cuminaldehyde; LDL oxidation; Macrophage; Apoptosis; Oxidative stress

## 1. Introduction

Metabolic disorders such as atherosclerosis, non-alcoholic steatohepatitis, etc have a strong basis of macrophage activation resulting from lipid overload (quote our old ref.). Hyperlipidemia with increased levels of plasma low density lipoproteins (LDL) caused by dysregulated lipid metabolism is one of the key factors contributing to complex etiology of diseases such as atherosclerosis [1]. Development of atheroma involves multiple causative agents that can be broadly categorized as exogenous viz. *Chlamydia pneumonia*, cytomegalovirus [2-3], diesel exhaust [4-5] or endogenous factors such as persistent low grade inflammation [6], physiological stress, lipotoxicity, etc. to name a few. Thus, it is evident that intracellular oxidative stress is the initiator of cell death and natural antioxidants that can inhibit LDL oxidation or improve cellular viability by preventing apoptosis may be an effective therapeutic alternative to synthetic drugs. A strong association between dietary plants and a decreased risk of cardiovascular disease has been attributed to the active antioxidant constituents of these plants. In this context, our lab has previously studied the anti-atherosclerotic potential of *Clerondendron glandulosum* [7], *Sida rhomboidea* Roxb. [8], *Coraindrum sativum* L. [9] or *Murraya koenigii* [10].

*Cuminum cyminum* (CC; commonly called cumin) is a small annual herb (family Apaiaceae) that produces highly nutritional oleaginous seeds widely used for various culinary and medicinal purposes. Seeds are rich source of essential oils that imparts a distinctive flavor and strong aroma. In Ayurvedic medicine, CC is considered to be effective against skin rashes, kidney and gallbladder stones, nausea toothaches, hiccoughs, dyspepsia, diarrhea, epilepsy, jaundice, flatulence, and indigestion [11]. Further, essential oils of CC seeds have been reported to have diuretic, carminative, emmenagogic, antibacterial, antifungal, antioxidant, anticancer, antidiabetic and antispasmodic properties [12].

Several studies had revealed that the bioactive components present in CC contribute to its therpeutic effects [13-14]. Further, chemical composition of the essential oils of CC revealed cuminaldehyde (CA) as the principle active constituent (40-45%) [14]. Besides being an anti-oxidant, CC has a lipid lowering activity [15], but its therapeutic potential against lipotoxicity related diseases such as atherosclerosis has not been reported. This study investigates the efficacy of CC against Ox-LDL fed mouse macrophage (RAW 264.7) cells in preventing Ox-LDL induced intracellular oxidative stress and apoptosis with implications of its possible anti-therapeutic potential.

## 2. Materials and methods

*2.1 Chemicals and reagents*

Folin's reagent, sodium chloride, copper sulphate, ethylene di-amine tetrachloro acetic acid (EDTA), thiobarbituric acid (TBA), trichloroaceticacid (TCA), butylated hydroxyanisole (BHT), sodium dodecyl sulphate (SDS), di-nitrophenyl hydrazine (DNPH), heptane, ethyl acetate, glycerol, bromophenol blue and coomassie brilliant blue R250 were purchased from Sisco Research Laboratories (Mumbai, India). Hydrochloric acid (HCl), sulphuric acid ($H_2SO_4$) and glacial acetic acid were purchased from Suvidhanath Laboratories, (Vadodara, India). Barbituric acid and Na-barbiturate were purchased from National Chemicals (Vadodara, India). Acridine orange (AO), ethidium bromide (EtBr), phosphate buffer saline (PBS), RPMI-1640, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), 3- ( 4 , 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT), Dulbecco's modified eagles medium (DMEM) and antimicrobial-antimycotic solution were purchased from Himedia Laboratories (Mumbai, India). Aluminium chloride, dichloro-dihydro-fluoresceindiacetate (DCF-DA), rhodamine-123 (RHO) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich Inc. ( St. Louis, USA). RNase A, propidium iodide (PI) and trizol reagent were purchased from Invitrogen (Thermo Fischer, USA). MMLV iScript cDNA synthesis kit Dream Taq green PCR master mix was purchased from Bio-Rad Laboratories (California, USA).

*2.2 Preparation of plant extract*

CC seeds were obtained from Spencer's mall, Vadodara, Gujarat, India, identified and authenticated by Dr. Vinay Raole, Department of Botany and voucher specimen (Accession number 379) was submitted to departmental herbarium (BARO), The M. S. University of Baroda, Vadodara, Gujarat, India. 100 g of powdered dry seeds were soaked in 1000 ml methanol:water (80:20 v/v) at room temperature in glass stopper vessel and allowed to stand for 72h. The resultant extract was filtered through a muslin cloth and the filtrate was concentrated in a rotary evaporator at 50˚C to obtain a thick semisolid brown colored paste. The final yield obtained was 9.5g (w/w). The extract was stored at -20˚C for further use.

*2.3 Isolation of LDL and its oxidation*

LDL was isolated using heparin–citrate buffer precipitation method [16]. Briefly, serum was collected following standard protocols from normocholesterolemic healthy adult volunteers (n=6) under fasting condition after taking prior consent at Blue cross pathology lab (IMA-BMWMC No. 1093). 1 ml heparin–citrate buffer (64 mM trisodium

citrate at pH 5.05 containing 50,000 IU/l heparin) was added to 0.1 ml serum, vortexed and allowed to stand for 10 min at room temperature. Later, the mixture was centrifuged at 3,000 rpm for 10 min at 20 °C and the resultant pellet was re-suspended in 0.1 ml of phosphate-buffered saline (PBS; 0.1 M, pH 7.4, containing 0.9 % NaCl). The protein concentration of the obtained LDL was estimated [17] using bovine serum albumin as a standard. For oxidation, 100 µg/ml of LDL was incubated with 10 µl freshly prepared 0.167 mM copper sulfate solution at 37 °C for 24 h. To stop the oxidation, 10 µl of 10 mM ethylene diamine tetra acetic acid (EDTA) was added and the oxidation was confirmed by assaying malonaldehyde (MDA) [18] and conjugated dienes (CD) [19]. The Ox-LDL thus obtained, was used in further experiments.

*2.4 Maintenance of cell line*

RAW 264.7 (Murine macrophage) cell line was purchased from National Centre of Cell Sciences, Pune, India. The cells were maintained in DMEM supplemented with 10 % FBS and 1X antibiotic-antimycotic solution (Himedia Laboratories, Mumbai, India) in a humidified atmosphere with 5 % $CO_2$ at 37 °C. Exponentially growing cells were used for all the experiments.

*2.5 Cytotoxicity assay*

Briefly, the cells were seeded in a 96 well-plate at a density of 1 x $10^4$ cells/ml and allowed to grow overnight. Thereafter, the cells were treated with CC (10–300 µg/ml) or CA (10–300 µg/ml) for 24 h. In another experimental set, the cells were pretreated with CC (10–300 µg/ml) or CA (10–300 µg/ml) for 30 min followed by treatment with 100 µg/ml of Ox-LDL for 24 h. Thereafter, cells were washed with PBS, followed by addition of 0.5 mg/ml MTT to each well and incubation at 37°C for 4 h. The purple formazan crystals formed were dissolved in 150 µl DMSO and the absorbance was recorded at 490 nm using ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT). The results were represented as percentage cell viability with respect to control.

*2.6 Intracellular oxidative stress*

The intracellular oxidative stress was measured by using 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), a ROS-sensitive dye [20]. Briefly, the cells were pretreated with 300 µg/ml CC or 300 µg/ml CA for 30 min followed by treatment with 100 µg/ml of Ox-LDL for 24 h. Thereafter, the cells were washed with PBS and stained with 7.5 µM DCF-DA for 30 min in dark. The cells were washed thoroughly with PBS and observed using Leica DMRB

florescence microscope. Images were acquired using canon power shot S70 digital camera and intensity was quantified using Image J software (NIH, USA).

*2.7 RNA Isolation and Semi-quantitative PCR analysis*

RNA was isolated using Trizol reagent (Invitrogen, USA) according to manufacturer's protocol and cDNA was reverse-transcribed using MMLViScript cDNA synthesis kit (Bio-Rad Laboratories, USA). The expression level of apoptotic markers (*bcl-2, bax*) mRNA was analyzed by semi-quantitative RT-PCR using Dream Taq green PCR Master Mix (Bio-Rad Laboratories, USA) in a Bio-Rad (T1000) Thermal Cycler instrument. All the necessary primers were synthesized from Integrated DNA Technology (IDT, Iowa, USA) and their sequences are given in Tables 1. The results were expressed as the fold change in mRNA expression with respect to control cells. GAPDH was used an endogenous control. Data were analyzed using Image Lab Software provided with Bio-Rad Gel-doc System.

Table **1**: PCR primers sequences of the genes analyzed.

| **Sr. No.** | **Gene** | **Forward Primer** | **Reverse Primer** |
|---|---|---|---|
| 1 | *gapdh* | 5'-TCCCCTTAGTTCGAGGGAT- 3' | 5'-ACATCACCCCCATCACTCAT-3' |
| 2 | *bcl-2* | 5'-GTTTGGTTTTCCAGGGTTCT-3' | 5'-TGGGAAATCATTTGCATTCT-3' |
| 3 | *bax* | 5'-ATCTTGCTGGCCCTAAGTTT-3' | 5'-ACAATCCAAAGTGGACCTA-3' |

*2.8 Statistical analysis*

All the experiments were performed in triplicates. Statistical evaluation of the data was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests. The results were expressed as mean ± S.E.M and the statistical analysis was carried out using Graph Pad Prism version 6.0 for Windows.

## 3. Results

*3.1 Cytotoxicity of CC and CA in Raw 264.7 cells*

The cytotoxicity of CC and CA on Raw 264.7 cells was assessed by MTT assay. As shown in Fig.**1a**, the cells treated with different concentrations of CC retained viability comparable to that of control cells. However, CA was non-toxic upto 25 µg/ml but higher concentrations caused a significant reduction in cell viability in a dose-dependent manner (Fig. **1b**).

*3.2 Ox-LDL induced cytotoxicity in Raw 264.7 cells*

Further, we found that treatment with Ox-LDL significantly decreased the cell viability. However, pretreatment with CC significantly improved viability in a dose-dependent manner at a concentration of 50 µg/ml and above (Fig.**1c**). Also, no significant improvement in the viability was observed in cells pretreated with CA (Fig. **1d**).

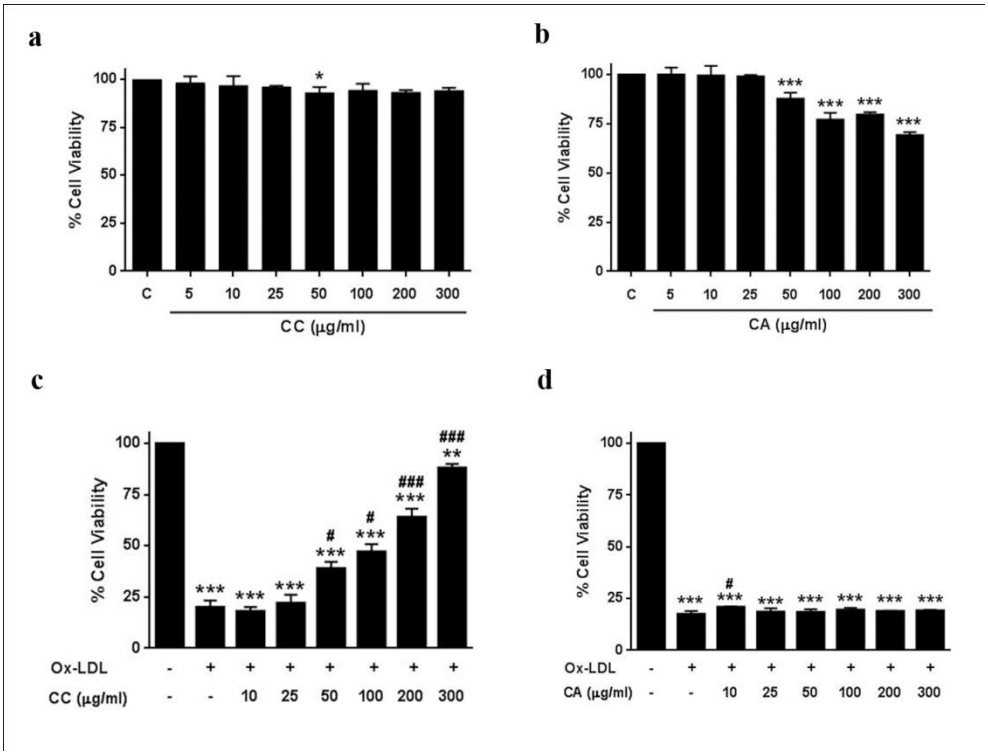


Figure **1**: Effect of CC and CA on Ox-LDL induced cytotoxicity in RAW 264.7 cells. Cells were treated with (**a**) CC or (**b**) CA for 24 h or pretreated with (**c**) CC or (**d**) CA for 30 min followed by Ox-LDL for 24h. Untreated cells were used as control. Percentage viability was determined by MTT assay. Data were expressed as mean ± S.E.M. for n=3. *p<0.05, **p<0.01, ***p<0.001 compared to control cells. #p<0.05, ##p<0.01, ###p<0.001 compared to Ox-LDL treated cells.

*3.3 Ox-LDL mediated generation of intracellular oxidative stress*

The generation of reactive oxygen species (ROS) by Raw 264.7 cells was monitored using redox sensitive dye, DCF-DA. Treatment with Ox-LDL led to a drastic increase in the levels of intracellular ROS (~27 fold) as compared to control cells. However, pretreatment of the cells with CC prevented the Ox-LDL induced increase in intracellular oxidative stress. On the contrary, CA pretreatment failed to prevent Ox-LDL mediated ROS generation (Fig. **2**).

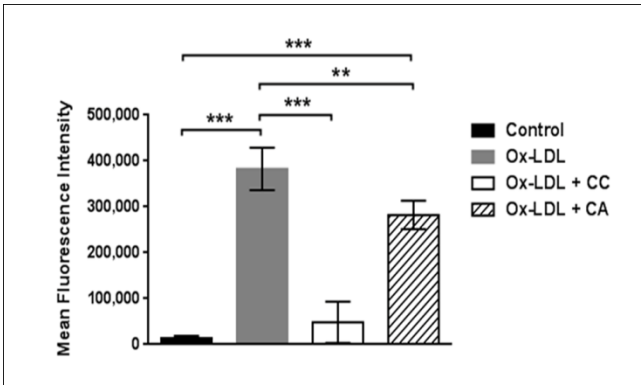


Figure **2**: Effect of CC and CA on Ox-LDL induced intracellular oxidative stress in RAW 264.7 cells. Cells were treated with ox-LDL (100 µg/ml) with or without pretreatment of CC and CA and untreated cells were used as control. Cells were subjected to DCF-DA staining for detection of intracellular ROS and intensity was quantified from the representative images using Image J software. Data were expressed as mean ± S.E.M. for n=3. **p<0.01, ***p<0.001.

*3.4 Ox-LDL mediated apoptosis in Raw 264.7 cells*

Ox-LDL treated cells showed an increased expression of *bax* (pro-apoptotic) and decreased expression of *bcl-2* (anti-apoptotic) mRNA leading to a significant increased Bax/Bcl-2 ratio indicating apoptosis. Whereas, presence of CC significantly prevented the Ox-LDL mediated modulations of apoptotic genes but, the same was less pronounced in CA treated group (Fig. **3**, Table **2**).

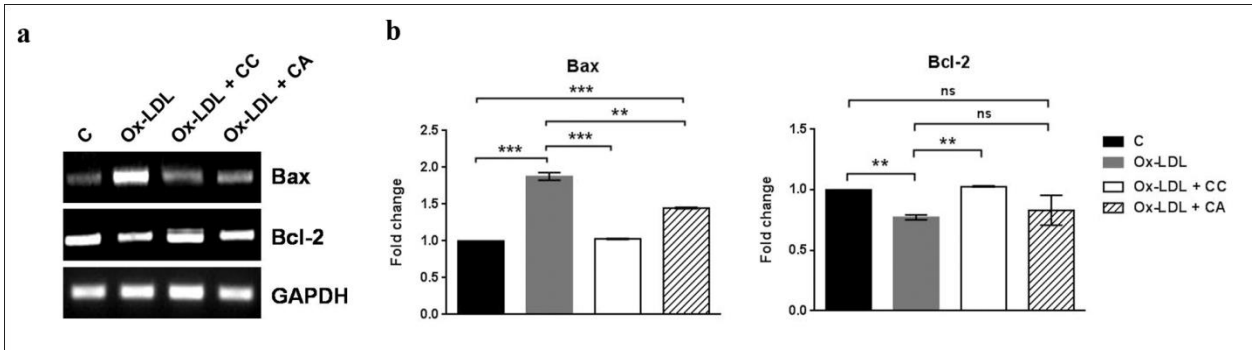


Figure **3**: Effect of CC and CA on Ox-LDL induced apoptosis in RAW 264.7 cells. Cells were treated with ox-LDL (100 µg/ml) with or without pretreatment of CC and CA and untreated cells were used as control. Semi-quantitative PCR was carried out using the cDNA synthesized from RNA of the cells. (**a**) Representative image of the agarose gel electrophoresis (**b**) Graphical representation of the band intensity quantified using Image J software. GAPDH was used as control. Data were expressed as mean ± S.E.M. for n=3. *p<0.05, **p<0.01, ***p<0.001, ns-non-significant.

Table **2**: Quantification of the Bax/Bcl-2 ratio.

| **Experimental Group** | **Bax Intensity (arbitrary units)** | **Bcl-2 Intensity (arbitrary units)** | **Bax/Bcl-2** |
|---|---|---|---|
| Control | 73.380 | 150.249 | 0.488 |
| Ox-LDL | 149.159 | 147.345 | 1.012* |
| Ox-LDL + CC extract | 74.963 | 161.525 | 0.464# |
| Ox-LDL + CA | 93.866 | 126.918 | 0.740 |

*p<0.05 compared to control, #p<0.05 compared to Ox-LDL

## 4. Discussion

Lipid metabolites can induced chronic inflammation by promoting macrophage infiltration and its subsequent activation in various target tissue in metabolic disease such as atherosclerosis. Pro-inflammatory and pro-apoptotic properties of lipids and their derivatives have been elaborately reported to induce lipotoxicity [21]. The blueprint of onset and progression of atherosclerosis may vary but culminates in foam rich fatty streak formation containing lipid

laden macrophages. These intermediate lesions undergo progressive transformation towards plaque formation with inputs from endothelial damage and smooth muscle cell migration [22]. Hence, increased plasma LDL levels remain a major risk factor that promotes atheroma wherein oxidative modification of LDL in the subendothelial space is a key event. The uptake of oxidized LDL (Ox-LDL) and foam cell formation triggers apoptotic death [21]. An inability of macrophages in handling scavenger receptor mediated internalized lipid overload triggers series of lipotoxic events culminating in macrophage apoptosis. Hence, a test compound that improves cell viability and reduces apoptotic changes in macrophages by preventing Ox-LDL uptake qualify as an anti-atherosclerotic agent.

Previous studies from our lab had reported therapeutic potential of herbs [7, 9] and functional food [10] in preventing formation of LDL oxidation products, cytotoxicity and apoptosis of macrophages. In the present study, CC and its abundant active compound CA were put to scrutiny to assess their merits in alleviating Ox-LDL induced lipotoxicity in mouse macrophage (RAW 264.7) cells.

MTT assay is a widely used technique to assess cell viability indices when treated with a test compound. Ox-LDL induced lipotoxicity was assessed in our study using MTT assay where formation of purple colored formazan indicates metabolically active cells [23]. Also, the color intensity is a reflection of functional status of mitochondria and a weak color intensity, therefore indicates poor cell viability. In our study, both CC and CA were non-toxic to RAW 264.7 cells upto 25 µg/ml dose. However, CA accounted for approximately 10-25% cell loss between 50-300 µg/ml, reason for the same could not be ascertained. Ox-LDL (100 µg/ml) treatment accounted for >75% cell loss but a dose-dependent improvement was observed in Ox-LDL+CC group. Further, 300 µg/ml CC could restore ~95% cell viability. These results were encouraging and hence, prompted us to assess CA in a similar experimental scenario. In contrast to our previous observations, presence of CA failed to improve cell viability as the observed percentage cell viability was similar to that of Ox-LDL treated group.

Atherogenic processes involve modulation of intracellular oxidative pathways, cell adhesion, migration, proliferation and differentiation [24]. Macrophage mediated Ox-LDL uptake have a more subtle impact on atherogenic processes by production of intracellular ROS that accounts for oxidative stress. In our study, a lipophilic non-fluorescent $H_2$DCF-DA (dichloro-dihydro-fluoresceindiacetate) stain was used to evaluate intracellular oxidative stress [20]. Observations revealed that healthy cells showed weak fluorescence, whereas Ox-LDL treated cells showed a drastic increase in fluorescence intensity. Presence of CC in Ox-LDL treated group accounted for weak fluorescence comparable to that

of control suggesting CC mediated decrement of oxidative stress. These results are attributable to the reported anti-oxidant and free radical scavenging potential of CC extract [13, 25]. Unlike Ox-LDL+CC group, prominent fluorescence intensity observed in Ox-LDL+CA treated group again indicates the inability of CA in lowering intracellular oxidative stress.

Pathological removal of damaged or unwanted foam cells in atherosclerosis by triggering apoptosis is well established [21]. The link between elevated Ox-LDL induced cell death and/or intracellular oxidative stress amounting to apoptosis of macrophages, is crucial in atherosclerosis [24]. Bcl-2 (B-cell lymphoma-2) gene, localized in outer mitochondrial membrane, plays an important role in promoting cellular survival and inhibiting actions of proapoptotic genes (Bax). Ox-LDL induced mitochondrial damage resulting in release of cytochrome-c accounts for intracellular oxidative stress and ROS that triggers apoptotic cascade [26-27]. Higher mRNA levels of Bax and lower levels of Bcl-2 observed in Ox-LDL treated group justify the prominent DCF-DA fluorescence and poor cell viability observed herein. Significant decrement of Bax in Ox-LDL+CC treated group and an increment in Bcl-2 suggests that presence of CC prevents Ox-LDL induced apoptosis of macrophages and is in agreement with improved cell viability observed in this treated group. However, Ox-LDL+CA failed to induce a similar change in Bax and Bcl-2 thus explaining the reason for poor cell viability. These findings are *prima facia* evidence on anti-atherosclerotic potential of CC. Previous studies in our lab had established the ability of CC in preventing experimentally induced oxidative modification of LDL [28]. Collectively, the observations provide strong evidence on anti-atherosclerotic potential of CC extract. But the inability of CA in imparting cytoprotection against Ox-LDL induced lipotoxicity is also a key finding that emphasizes the importance of use of crude extracts in traditional system of medicine. Studies in Ayurveda and alternative medicine have emphasized on use of heterogenous mixtures or poly-herbal formulations because the "lesser" compounds play a subtle, yet key role in providing stability and improving efficacy of the active ingredients [29]. Hence, the inability of CA to qualify as an anti-atherosclerotic agent is possibly attributable to the absence of such "lesser" compounds that needs further scrutiny.

## 5. List of Abbreviations

LDL- Low density lipoprotein; nLDL- native LDL; Ox-LDL- Oxidized LDL; DFC-DA- Dichlorodihydrofluorescein diacetate

## 6. Conflict of Interest

The authors confirm that this article content has no conflict of interest. Authors are thankful to Gujarat State Biotechnology Mission (GSBTM), Gandhinagar, Gujarat, India for providing financial support for the study in the form of Financial Assistance Program (FAP)-2010 (GSBTM/MD/PROJECTS/SSA/457/2010-2011) to RD.

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