Chapter 4

Clerodendron glandulosum.Coleb leaf extract attenuates pathophysiological changes and expression of VCAM-1 and P-selectin in thoracic aorta of atherogenic diet fed rats

INTRODUCTION

Cardiovascular diseases (CVD) are currently the leading cause of death in developed and developing countries worldwide (Murray and Lopez, 1997). Cardiovascular disease would be the leading cause of mortality and morbidity in the world by the year 2015 and people from Indian subcontinent are at higher risk. It is a multifactorial disease accompanied by factors like hereditary, hyperlipidemia, obesity, hypertension, environmental factors and life style variables like stress, smoking, alcohol consumption, etc. Atheroscelrosis related diseases are major cause of death in USA affecting over 60 million people. Atherosclerosis, characterized by the accumulation of lipids and fibrous elements in the large arteries is the most common contributor to the CVD related mortality and morbidity (Libby, 2002). Recent studies have documented that atherosclerosis is not merely a dyslipidemic condition but involves a series of inflammatory changes (Libby, 2002). Although elevated low density lipoprotein cholesterol (LDL) is thought to be the best indicator of lowering of atherosclerosis risk, patients treated with lipid-lowering drugs have not been able to achieve the lower LDL-cholesterol levels (Reiner, 2006) thus compounding the problems. Studies on correlation between plasma total cholesterol levels and incidences of the major coronary heart disease (CHD) have revealed that, a 10% increase in plasma total cholesterol is associated with about 27% increase in the incidences of CHD whereas, 10% decrease in plasma cholesterol level is associated with a 25% drop in the incidence level of CVD. A reduction of LDL cholesterol by 1 m mol/L is followed by a 23% reduction in CHD. An elevated level of low density lipoprotein (LDL) lead to higher concentration in the sub endothelial space where LDL undergoes oxidative modification by reactive oxygen

species (ROS), produced in endothelial cells, resident macrophages or smooth muscle cells. Once formed in the sub endothelial space, oxidized LDL may injure the endothelium and play a role in the increased adherence of leucocytes to the vascular wall. Adhesion molecules mediate the adherence and subsequent transmigration of leucocytes across the vascular endothelium. Selectins are adhesion molecules that mediate the initial rolling of inflammatory cells along endothelial cells; expression of P-selectin has been shown to precede macrophages and lymphocytes infiltration. E-selectin and P-selecitin are preferentially expressed in the endothelium overlying atherosclerotic plaque. Intercellular adhesion molecule (ICAM) and vascular adhesion molecule (VCAM) are thought to regulate attachment and trance endothelial migration of leucocytes. Oxidized LDL (Ox-LDL) induces monocyte chemo attractant protein-1 (MCP-1) expression in endothelial cells and macrophages and growth factor such as macrophage colony stimulating factor (M-CSF). Monocytes recruitment into the wall of blood vessel due to the expression of adhesion molecule and MCP-1. Subsequently, these monocytes undergo differentiation and become macrophages that now reside in the tunica-intima of a blood vessel. The macrophages express scavenger receptors to capture the internalized Ox-LDL particles, which ultimately result in the formation of "foam cells", a hall mark of the arterial lesions. These foam cells continue secreting pro-inflammatory cytokines that result in a disproportionate recruitment of additional immune cells. The Ox-LDL stimulates T-cell migration into the endothelium and induces antibody production. Both Th_1 (IFN-γ) and Th_2 cytokines (IL-10) as well as TGF-β are expressed within atherosclerotic lesion (Libby, 2002).

Several attempts such as use of cholesterol lowering, anti-platelate, beta blocker inhibitors, angiotensin converting enzyme inhibitors drugs have been used to control atherosclerosis (Mayo clinic health information) but they are often associated with undesirable side effects on a long term usage. In this behest recently use of herbal extracts and dietary supplements to combat atherosclerosis is in vogue (Amom *et al.*, 2011, Park *et al.*, 2010). Recently extracts of Arjuna- *Terminalia arjuna* (Subramaniam et al., 2011), black cumin- *Nigella sativa* (Al-Naqeep et al., 2011), kalli mooliyan- *Caralluma fimbriata* (Kamalakkannan et al., 2010) etc. have been documented to possess anti-atherogenic potential in experimental models.

The present study evaluates the potential of CG extract in mitigating *in vivo* pathophysiological changes during experimentally induced atherosclerosis and expression of cell adhesion molecules.

MATERIALS AND METHODS

Plant, preparation of extract and phytochemical analysis

As mentioned in chapter 1

Experimental animals

Male *Sprague Dawley* rats weighing 300±20 (Obtained from Sun pharmaceutical advanced research centre, Baroda, India) were maintained in clean polypropylene cages and fed with laboratory chow (M/S Pranav agro, Ltd Baroda, India) and water *ad libitum*. The experimental protocol was executed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and

approved by the animal ethical committee of the Department of Zoology, The M.S. University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

Induction of atherosclerosis

A total of 24 rats were divided into three groups of eight rats each. Group I served as control (CON) and was fed with standard laboratory chow (Pranav Agro Ltd, Baroda, India) and administered with 0.5 % CMC orally for 8 weeks. Groups II and III were given single dose of Vitamin D3 (600,000 unit/kg, *i.p.*) and later fed with an atherogenic (ATH) diet (3% cholesterol, 0.5% cholic acid, 0.2% 6-propyl 2-thiouracil, 5% sucrose, 10% lard, and 81.3% powdered laboratory chow) for 8 weeks (Cai *et al.*, 2005, Wu *et al.*, 2009, Pang *et al.*, 2010). Group III (ATH+CG) also received 200mg/kg CG extract orally (chapter 1) while, group II (ATH) received equal volume of vehicle (0.5% CMC) for 8 weeks.

At the end of the experimental period, blood was collected via retro-orbital sinus from overnight fasted rats in a micro centrifuge tubes. Serum separation was carried out by cold centrifugation (4°C) of the vials at 1500 rpm for 10 min. Later, animals were sacrificed by cervical dislocation under mild ether anesthesia and thoracic aorta of control and experimental rats were collected. Two small pieces of thoracic aorta from aortic arch were collected and used for preparation of fresh frozen sections and fixation for histopathological evaluations respectively. The remaining piece of thoracic aorta was stored at -80°C (Cryo Scientific Ltd, India) for further use.

Serum and aortic lipids: as mentioned in chapter 1.

Serum autoantibody titer

Serum autoantibody titer was determined as described earlier (Uusitupa *et al.*, 1996). A 96 well ELISA plates was coated with 5 μg/ml antigen in PBS (contained 0.27 mmol/l EDTA and 20 μmol/l butylated hydroxytoluene) overnight at 4°C. Plates were then, washed three times with PBS (containing 0.5% Tween 20) and twice with water. Non specific sites were blocked with 2% fetal bovine serum (Himedia India, Pvt, Ltd) and washed again as mentioned above. Serum samples (in PBS containing 1% bovine serum albumin, 0.27 mmol/l EDTA, 20 μmol/l butylated hydroxytoluene, and 0.05% Tween 20) were added on to the plates and were incubated overnight at 4°C followed by washing as mentioned above. Horseradish peroxidase–conjugated human anti-rat IgG (Bangalore Genei, India) was added (1:200) and plates were incubated at 4°C for 4 hours. After washing, 3, 3'-diaminobenzidine (DAB) and H_2O_2 (Bangalore Genei Pvt Ltd, INDIA) was added and the color was allowed to develop for 10 mins. The reaction was stopped by adding 2 mol/l H_2SO_4 and absorbance was read at 492 nm using ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT) and expressed as absorbance unit.

Isolation and characterization of LDL

LDL was isolated from serum samples of control and experimental rats by heparin-citrate buffer precipitation method as described earlier (Ahotupa *et al.*, 1998). One ml of heparin-citrate buffer (0.064 M tri sodium citrate at pH 5.05 containing 50,000 IU/l heparin) added to 0.1ml serum was mixed with a vortex mixer and allowed to stand for 10 min at room temperature. The insoluble lipoprotein sediment, obtained as a pellet by centrifugation at 3000 rpm for 10 min (at 20°C), was suspended in 1 ml PBS. The protein

content of LDL was estimated by the method of Lowry *et al.* 1951 using bovine serum albumin as standard. Oxidation state was evaluated by assaying malonaldehyde (MDA) (Buege and Aust, 1978) and baseline conjugated diene (CD) (Esterbauer *et al.*, 1989) levels in the LDL samples of control and experimental groups.

Ex-vivo susceptibility for LDL oxidation and LDL aggregation

Evaluation of *ex-vivo* susceptibility to oxidation involved incubating LDL with $CuSO_4$ and continuously monitoring (at 10 min interval for 200min) the formation of CD at 234nm (Esterbauer *et al.*, 1989). The lag time was used to represent the *ex vivo* susceptibility of LDL oxidation.

To determine LDL aggregation, LDL (100 mg protein/ml) was mixed by vortex at a fixed strength and absorbance at 680 nm was monitored every 10 seconds against a blank solution (Khoo *et al.*, 1988). The increase in absorbance was used as a measure of LDL aggregation and expressed as percentage (%) aggregation.

Gross microscopic evaluation of thoracic aorta

Aorta of control and experimental rats fixed in 4% buffered paraformaldehyde was dehydrated in graded alcohol series and embedded in paraffin wax using automated tissue processor. Sections of five-μm thickness cut on a microtome were stained with haematoxylin-eosin (HXE) for microscopic observation. The sections were photographed with a Canon power shot S70 digital Camera at 100 X magnification attached to a Leica microscope.

Determination of aortic elastin auto-florescence

For auto-florescence assay, a piece of thoracic aorta was frozen immediately after, sacrifice at -20°C and 8μm sections were cut on IEC Minotome Plus Cryostat, GMI, USA (at -20°C). Immediately after preparation of sections, they were examined under Leica DMRB florescence microscope using 488 nm filter and photographed using Canon power shot S70 digital camera.

Evaluation of aortic calcification

Paraffin wax sections of control and experimental rats were de-paraffinised, hydrated and rinsed in distilled water and incubated in von kossa stain solution (1% silver nitrate) under ultraviolet light for 20 min. The sections were then rinsed repeatedly in distilled water and placed in 5% sodium thiosulphate for 5 min and rinsed in distilled water again (to remove un-reacted silver) before counterstaining with 1% eosin for 5 min (Sheehan and Hrapchak, 1979). Slides were examined under Leica DMRB microscope and photographed with Canon power shot S70 digital Camera.

Immunohistochemical localization of macrophage surface marker (F4/80), VCAM-1 and P-selectin in thoracic aorta

Paraffin embedded sections of thoracic aorta of control and experimental rats were de-paraffinized in xylene and hydrated using graded series of alcohol and water. Sections were then washed in PBS and antigen retrieval step was carried out by immersing slides in sodium citrate buffer at 80°C for 10 min. Later, endogenous peroxides were removed by incubation of sections in 3% HO_2 for 20 min in dark. Non-specific binding sites were blocked by incubation of slides with 1% FBS, for 30 min. Localization of macrophage surface marker (F4/80), vascular cell adhesion molecule-1 (VCAM-1) and P-selectin was carried out using mouse anti-rat macrophage immunoglobulin (IgG) at a dilution of 1:100

(Santa Cruz Biotechnology, Inc, USA), rabbit anti-rat IgG at a dilution of 1:100 (Santa Cruz Biotechnology, Inc, USA) and, goat anti-rat P-selectin IgG at a dilution of 1:100 (Santa Cruz Biotechnology, Inc, USA) respectively for overnight at 4°C in a humidified chamber. The sections were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies for 4 hrs at room temperature. Rabbit anti-mouse IgG-HRP 1:100 (Bangalore Genei Pvt Ltd, INDIA) for macrophage marker, goat anti-rabbit IgG-HRP 1:100 (Bangalore Genei Pvt Ltd, INDIA) for VCAM-1, and rabbit anti-goat IgG-HRP 1:100 (Bangalore Genei Pvt Ltd, INDIA) for P-selectin were used. 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) as the chromagen and sections were counter-stained with haematoxylin. Sections were micro photographed with a canon Power shot S 70 digital camera under Leica DMRB microscope.

Statistical analysis

Statistical evaluation of the data was done by one way ANOVA followed by Bonferroni's multiple comparison test .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

Aortic and Serum lipids and, auto-antibody titer

Atherogenic dyslipidemia in the ATH rats were characterized by significantly elevated levels of TC, TG, LDL and VLDL along with concomitant decrement in the HDL level. Also, aortic lipid accumulation in these rats was marked by significantly elevated levels of TC and TG compared to CON rats. However, CG supplementation to ATH diet fed

rats was able to significantly minimize ATH diet induced serum and tissue hypercholesterolemia/hyperlipidemia and elevated serum HDL level (Table.1). In keeping with the results of serum and tissue lipid profiles in the ATH rats, auto-antibody titer against Ox-LDL was significantly elevated in these rats compared to CON rats (Table 1). However, CG supplementation to ATH diet fed rats recorded minimal changes in the auto-antibody titer compared to ATH rats (Table. 1)

In vivo LDL oxidation, and ex vivo aggregation and, oxidation assay

In vivo LDL oxidation was evaluated by quantifying MDA and CD contents in the isolated LDL from CON, ATH and ATH+CG groups. There was a 56.87 % and 53.05 % increment in MDA and CD contents respectively in the LDL samples isolated form ATH rats compared to 21.55 % and 27.03 % increment recorded in the ATH+CG rats compared to CON rats (Figure. 2). Further, *ex vivo* aggregation behavior and susceptibility to Cu^{2+} mediated oxidation of isolated LDL samples were carried out from all the experimental groups. LDL samples isolated from ATH rats showed maximum aggregation and highest susceptibility towards Cu^{2+} mediated oxidation compared to CON and ATH+CG group (Figure. 2). These results indicate at the ability of CG extract to prevent *in vivo* LDL oxidation and provide resistance towards *ex vivo* LDL oxidation.

Histopathological observations of thoracic aorta

In the present study, HXE stained sections of aorta of ATH rats revealed loosening of smooth muscle cells of tunica media and formation of a distinct necrotic core due to accumulation of lipids and foam cells (Figure.3). Auto-florescence of elastin fibers revealed extensive derangement in thoracic aorta of ATH rats (Figure.4). Calcification of thoracic aorta was evaluated by Von kossa staining that revealed pronounced calcium

deposition in tunica media and intima in aorta of ATH rats (Figure.5). ATH+CG rats revealed minimal evidence for atheromatous plaque formation along with moderate vascular injuries, calcium deposition and elastin derangement (Figure. 3, 4 & 5).

Immunohistochemical localization of macrophage marker and cell adhesion molecules

Atherogenic diet induced vascular endothelial inflammation in the thoracic aorta of ATH rats showed heightened expression of VCAM-1 and P-selectin along with accumulation of macrophages in the atheromatous plaques compared to their absence in the thoracic aorta of ATH+CG rats (Figure. 6). However, CG extract supplementation to ATH diet fed rats was capable of minimizing expression of VCAM-1 and P-selectin and accumulation of macrophages in the atheromatous plaque compared to ATH rats (Figure. 6).

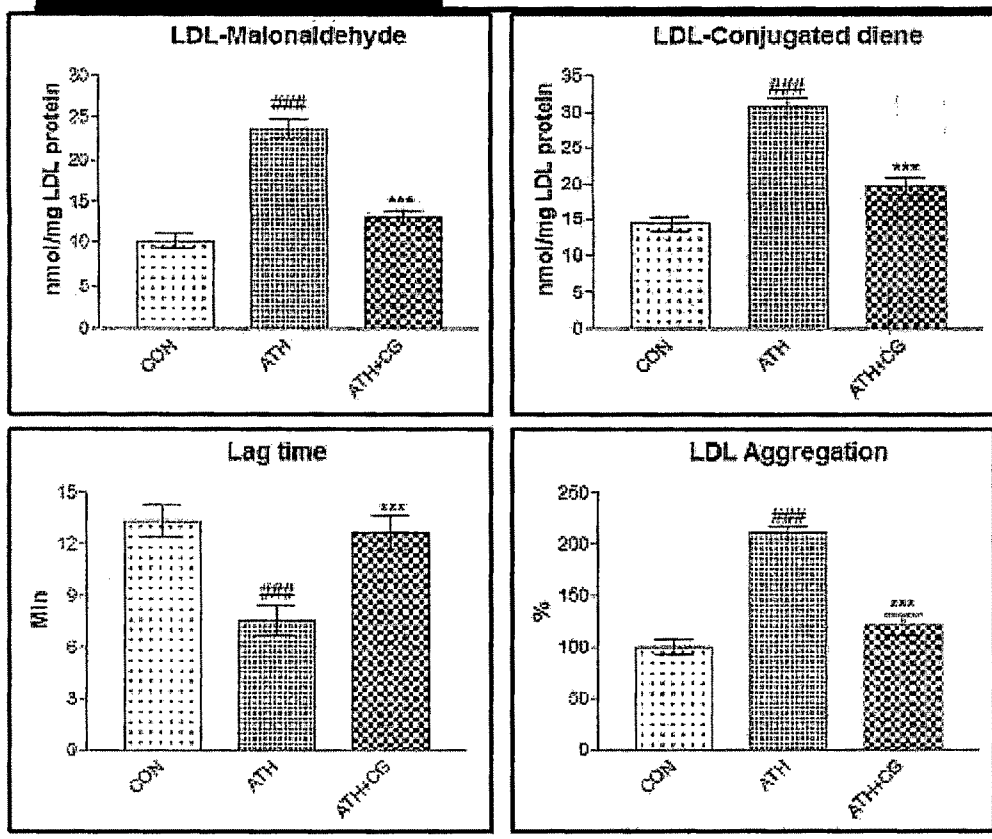
Table.1 Effect of *C.glandulosum*.Coleb on serum lipid profile, auto-antibody titer and tissue lipids in atherogenic diet fed rats.

	CON	ATH	ATH+CG
Serum			
Cholesterol (mg/dl)	60.37±3.95	320.11±19.17^{C}	104.2±9.40^{c}
Triglycerides (mg/dl)	50.37±4.43	103.20±7.76^{B}	61.00±6.00^{a}
High density lipoprotein (mg/dl)	28.30±1.55	18.11±0.93^{B}	32.46±1.77^{c}
Low density lipoprotein (mg/dl)	42.14±3.21	322.64±18.89^{C}	83.94±7.89^{c}
Very low density lipoprotein (mg/dl)	10.07±0.97	20.64±1.11^{B}	12.20±0.89^{a}
Ox-LDL Auto-antibody titer (Optical density)	0.244±0.013	0.605±0.011^{C}	0.301±0.011^{c}
Thoracic aorta			
Cholesterol (mg/g)	5.64±0.45	22.09±1.00^{C}	8.04±0.56^{c}
Triglycerides (mg/g)	6.21±0.42	14.13±0.71^{C}	7.88±0.60^{c}

Where, CON; rats fed with standard laboratory chow, ATH; rats fed with atherogenic diet and ATH+CG; rats fed with atherogenic diet and orally treated with *C.glandulosum*.Coleb extract (200mg/kg BW) for 8 weeks. Data expressed as Mean±S.E.M for n=8. Where, $^{A}p<0.05$, $^{B}p<0.01$ and $^{C}p<0.001$ compared to CON and $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$ compared to ATH.

Figure.2 Effect of *C.glandulosum*.Coleb on LDL malonaldehyde and conjugated diene contents, lag time during Cu^{2+} mediated oxidation and *ex vivo* LDL aggregation.

	CON	ATH	ATH+CG
LDL-MDA (nmol/mg LDL protein)	10.190±0.890	23.630±1.110^{c}	12.990±1.000^{c}
LDL-CD (nmol/mg LDL protein)	14.360±0.980	30.590±1.210^{c}	19.680±1.210^{c}
Lag time (min)	13.360±0.890	7.560±0.880^{c}	12.650±1.000^{c}
LDL aggregation (%)	100.0±7.650	210.69±7.000^{c}	120.980±9.98^{c}



Where, CON; rats fed with standard laboratory chow, ATH; rats fed with atherogenic diet and ATH+CG; rats fed with atherogenic diet and orally treated with *C.glandulosum*.Coleb extract (200mg/kg BW) for 8 weeks. Data expressed as Mean±S.E.M for n=8. Where, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared to CON and $^{**}p<0.01$ and $^{***}p<0.001$ compared to ATH.

Figure.3 Photomicrographs of rat thoracic aorta from control (CON), atherogenic diet fed (ATH) and atherogenic diet fed and treated with *C.glandulosum*.Coleb extract (ATH+CG) depicting elastin auto florescence (200X) and stained with haematoxylin and eosin (100X; HXE) and, von kossa (200X) stains.

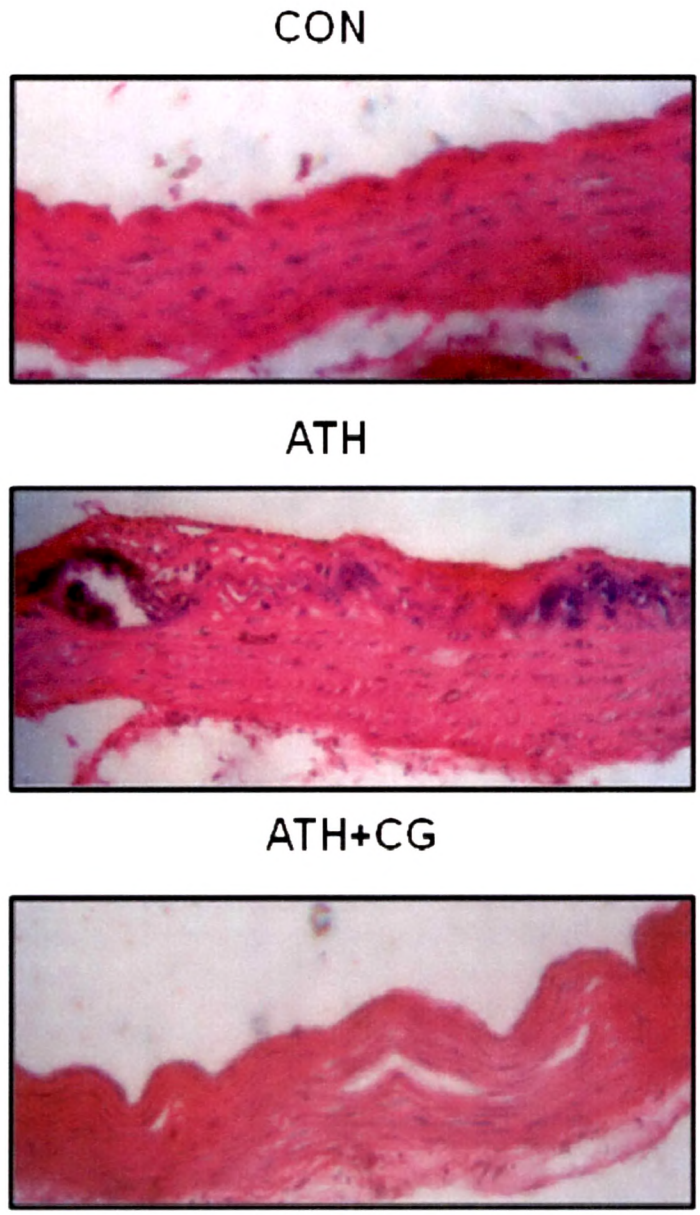


Figure.4 Photomicrographs of rat thoracic aorta from control (CON), atherogenic diet fed (ATH) and atherogenic diet fed and treated with *C.glandulosum*.Coleb extract (ATH+CG) depicting elastin auto florescence (200X).

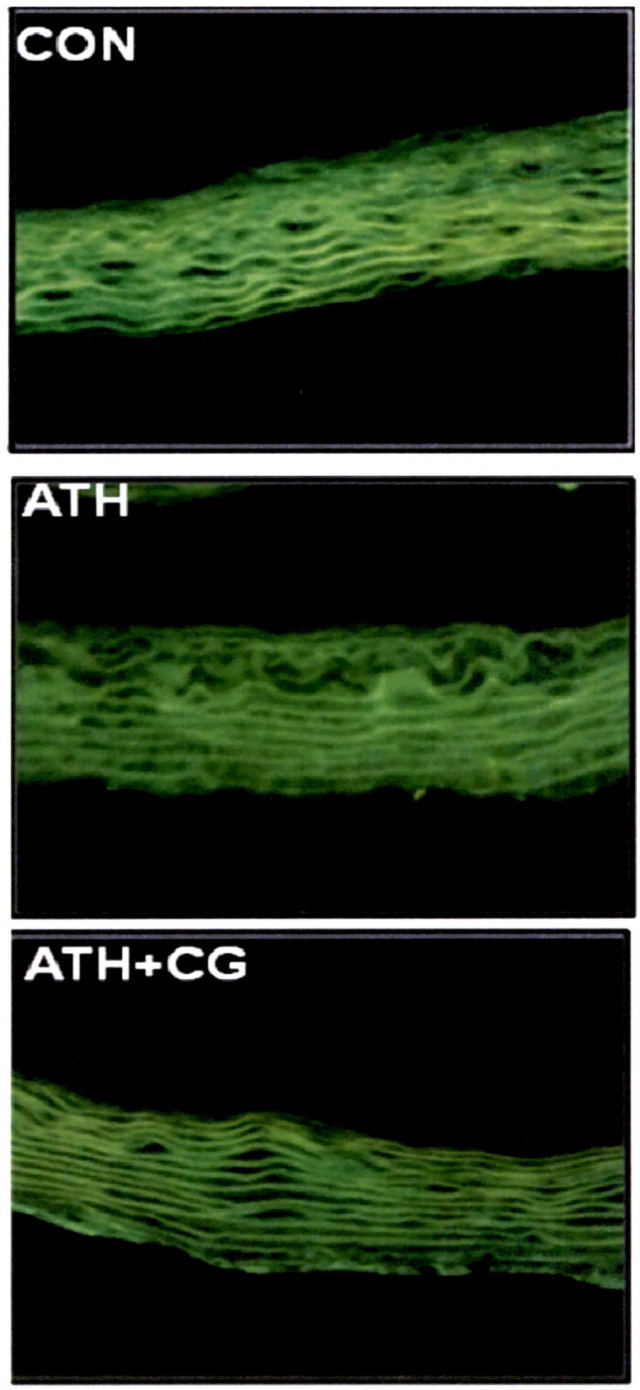


Figure.5 Photomicrographs of rat thoracic aorta from control (CON), atherogenic diet fed (ATH) and atherogenic diet fed and treated with *C.glandulosum*.Coleb extract (ATH+CG) stained with von kossa (100X) stains.

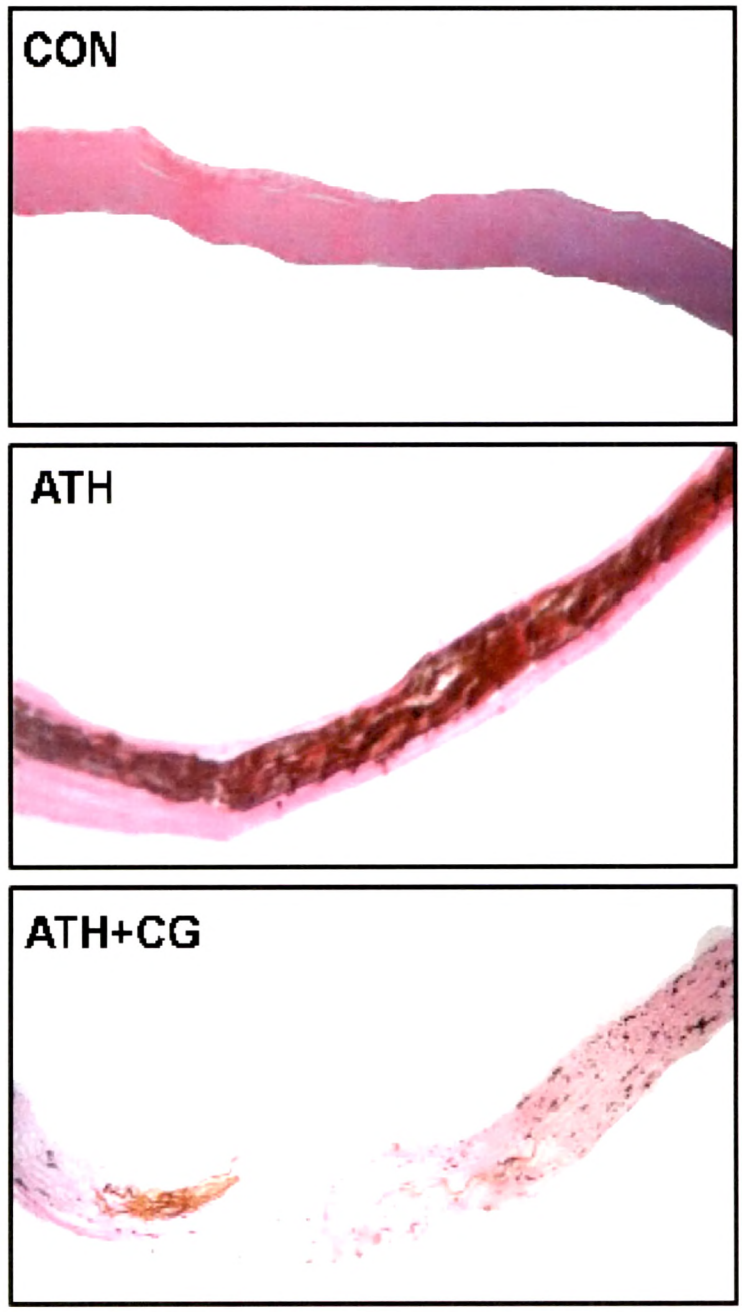


Figure.6 Photomicrographs of rat thoracic aorta showing immunolocalization of macrophage surface marker (F4/80) (100X) from control (CON), atherogenic diet fed (ATH) and atherogenic diet fed and treated with *C.glandulosum*.Coleb extract (ATH+CG).

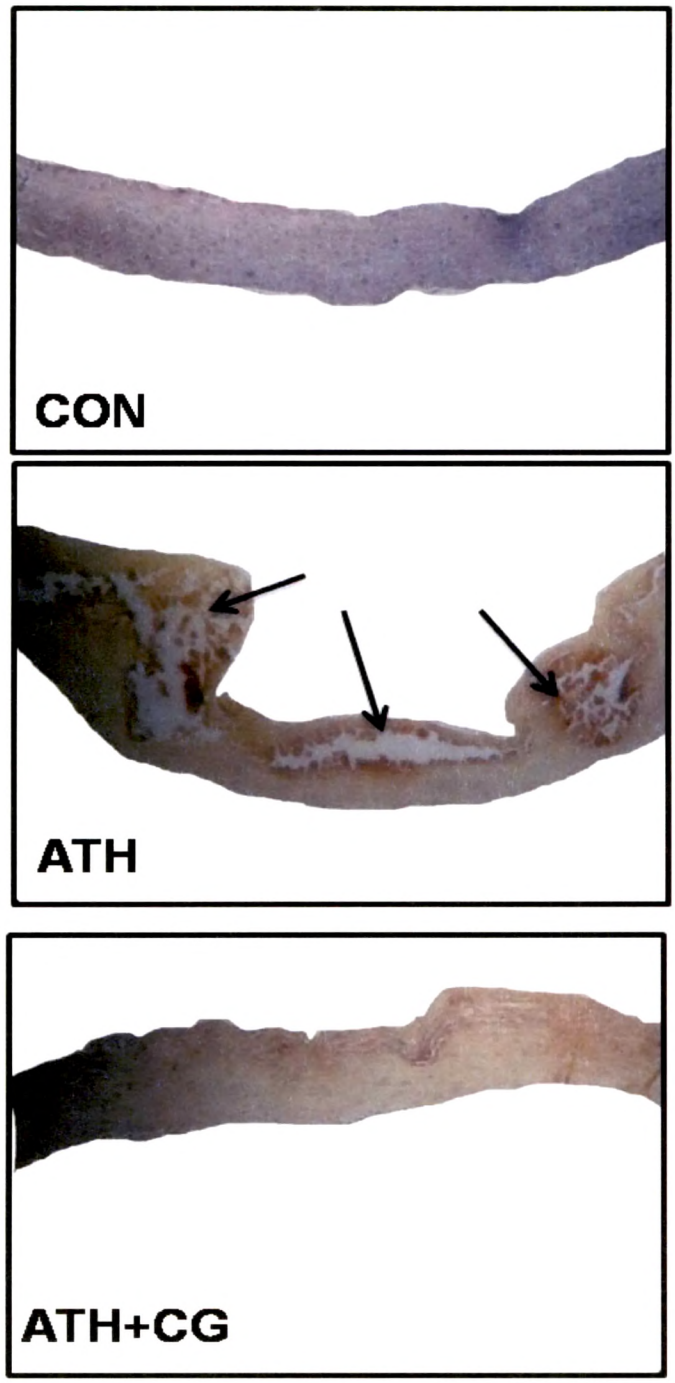


Figure.7 Photomicrographs of rat thoracic aorta showing immunolocalization of macrophage surface marker (F4/80), vascular cell adhesion molecules-1(VCAM-1) and p-selectin (100X) from control (CON), atherogenic diet fed (ATH) and atherogenic diet fed and treated with *C.glandulosum*.Coleb extract (ATH+CG).

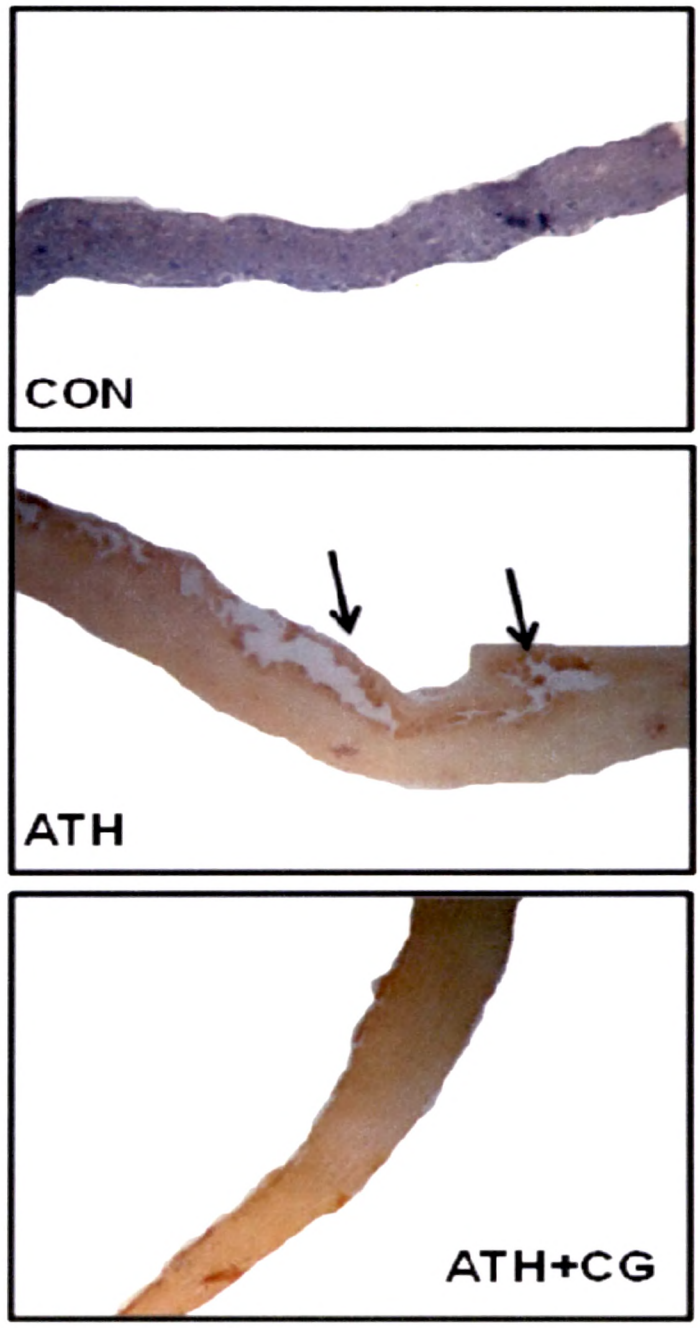
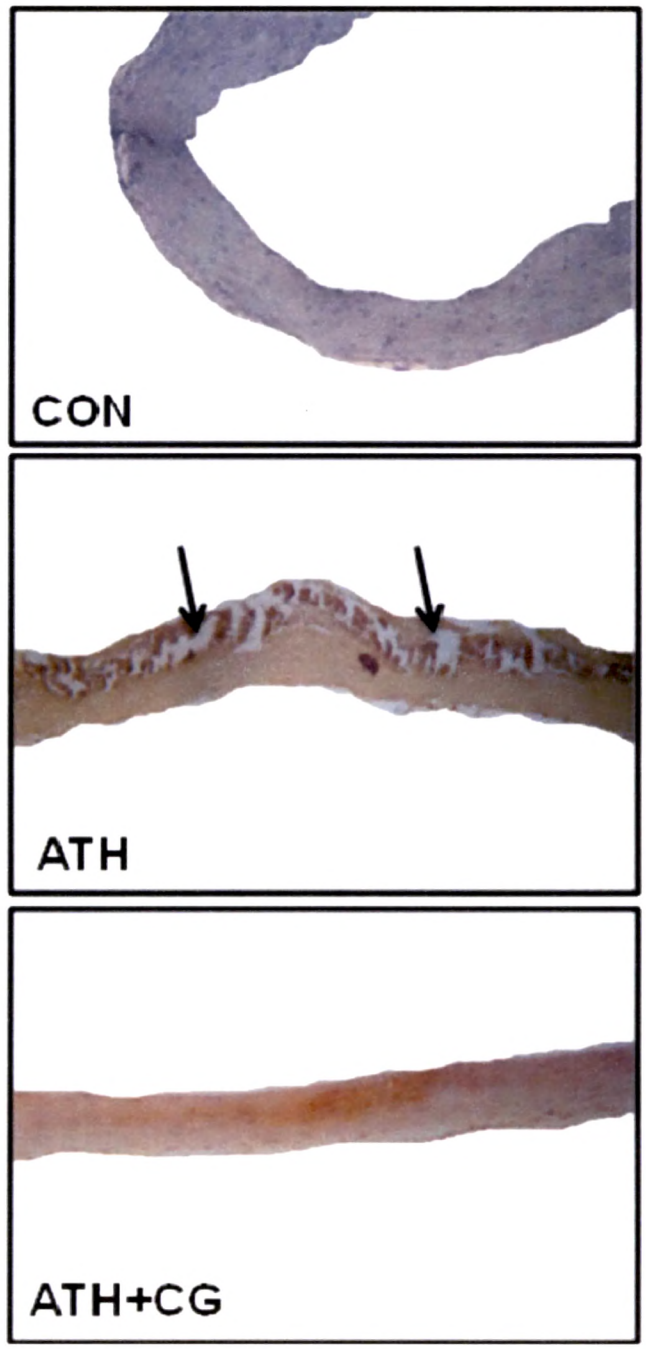


Figure.8 Photomicrographs of rat thoracic aorta showing immunolocalization of macrophage surface marker (F4/80), vascular cell adhesion molecules-1(VCAM-1) and p-selectin (100X) from control (CON), atherogenic diet fed (ATH) and atherogenic diet fed and treated with *C.glandulosum*.Coleb extract (ATH+CG).



DISCUSSION

Elevated cholesterol levels leading to dyslipidemia is one of the most important risk factors associated with atherosclerosis. Oxidative modification of LDL has been reported to trigger the onset and progression of atherosclerosis in experimental animals and humans (Palinski *et al.*, 1989, Maggi *et al.*, 1994). Atherogenic diet feeding to vitamin D_3 treated male *Sprague Dawley* rats for 8 weeks leads to development of dyslipidemia, that is marked by significant increment in the serum and tissue cholesterol, serum LDL and VLDL along with significant decrement in the serum HDL levels (Cai *et al.*, 2005, Wu *et al.*, 2009, Pang *et al.*, 2010) and the same was observed in our study. A previous study from our lab had also reported elevation in serum HDL_f level following CG treatment and the same was attributable to elevated activity levels of serum LCAT (chapter 1). In the present study, CG treatment to ATH rats significantly attenuated dyslipidemia along with an increment in HDL levels possibly due to an increase in LCAT activity.

In vivo LDL oxidation is a free radical driven chain reaction that brings about changes in LDL composition and characteristics. Studies have reported that, during LDL oxidation PUFA get modified into various oxidation products i.e. MDA and CD (Ahotupa *et al.*, 1998), thus increasing the susceptibility of Ox-LDL towards aggregation (Maor *et al.*, 1997). Elevated indices of MDA and CD in LDL isolated from ATH rats, along with higher aggregation characteristics and susceptibility to undergo oxidation provide ample evidence of *in vivo* LDL oxidation in the ATH group. However, LDL samples isolated from ATH+CG group recorded minimal formation of MDA and CD and lesser susceptibility of LDL towards oxidation or aggregation. These *in vivo* observations

are in agreement with our report on protective role of CG against *in vitro* LDL oxidation (chapter 5) and provide compelling evidence on the ability of CG in protecting oxidation of LDL within a living system.

Another most important change during *in vivo* LDL oxidation is the formation of oxidized phospholipids through peroxidation of LDL-PUFA. This modification renders Ox-LDL molecule more antigenic that result in the formation of auto-antibodies against Ox-LDL (Lourida *et al.*, 2002). Furthermore, antibodies against epitopes of ox-LDL have been found in several studies in both human and rabbit plasma, and in atherosclerotic lesions (Palinski et al., 1989, Rosenfeld *et al.*, 1990, Yla-Herttuala *et al.*, 1994). In the present study, ATH diet fed rats recorded significantly elevated indices of Ox-LDL auto-antibodies indicating at an atherogenic condition of the animals. Significant reduction in Ox-LDL auto-antibody titer in CG supplemented rats is in agreement with attenuation of *in vivo* LDL oxidation and can be comparable with previously reported CG mediated prevention of *in vitro* LDL oxidation (chapter 5).

During the onset and progression of atherosclerosis, monocyte recruitment and its subsequent differentiation to macrophages are the key events. Ox-LDL is reported to be a promoter of monocyte to macrophage differentiation that triggers early events during atherosclerosis (Bobryshev, 2006). Once differentiated, macrophages express Ox-LDL specific scavenger receptor that eventually results in Ox-LDL uptake and foam cell formation (Gillotte-Taylor *et al.*, 2001). We have previously reported CG extract induced prevention of Ox-LDL uptake and subsequent foam cell formation (chapter 5). Significant decrement recorded in monocyte to macrophage differentiation *in vitro* further justifies potent anti-atherogenic potential CG extract.

Monocyte recruitment and its adhesion at the site of vascular injury are prerequisite for pathogenesis of atherosclerosis (Zheng *et al.*, 2005). The adhesion of monocytes to the inflamed endothelium and its subsequent migration into the arterial wall is facilitated by expression of various cellular adhesion molecules on the surface of endothelial cells (Bobryshev *et al.*, 2006). In this regard, selectins are the first set of adhesion molecules to be expressed. It has been demonstrated that L-selectin is expressed on the surface of monocytes while P and E-selectins are expressed on the luminal surface of an activated endothelium (Quehenberger, 2005). However, the selectin-mediated adhesion of monocytes is very loose and is generally followed by expression of another set of adhesion molecules. The strong attachment of monocytes to the luminal surface of the endothelium is mediated by the interactions of the integrins (expressed on the surface of monocytes) with ligands belonging to the immunoglobulin super family, i.e. intercellular adhesion molecule-1 (ICAM-1) and VCAM-1(Quehenberger, 2005, Natarajan and Cai, 2005). Higher expression of VCAM-1 on the aortic endothelium of hyperlipidemic animals and humans has been well documented (Davies *et al.*, 1993, Iiyama *et al.*, 1999). In the present study, immunohistochemical localization of VCAM-1 and P-selectin revealed augmented expression in the aortic endothelium of ATH rats. However, CG supplementation significantly minimized expression of these cell adhesion molecules.

Arterial calcification represents an advanced and complicated state of atherosclerotic lesion that is responsible for hypertensive condition and increases myocardial after load (Safar *et al.*, 2003, Speer and Giachelli, 2004). Arterial calcification can be induced by treatment of vitamin D and feeding atherogenic diet to the

experimental rats (Tang *et al.*, 2007). Also, previous studies have also shown that, Ox-LDL promotes arterial calcification (Tang *et al.*, 2006) and the same was observable in ATH rats. However, minimal arterial calcification was evident in CG extract treated rats. These observations are attributable to CG extract induced prevention of *in vitro* and *in vivo* LDL oxidation.

Advanced atheromtous plaque formation starts with the aggregation of foam cells in the sub-endothelium of thoracic aorta. Later, destruction of these foam cells along with extracellular accumulation of lipids and cellular debris contributes to initiation of plaque formation. It has been well established that, foam cell undergoes apoptotic cell death in the arterial wall (Natarajan and Cai, 2005, Bobryshev, 2006) .Later, as a result of defective efferocytosis of dead foam cells, apoptosis of these cells is followed by secondary necrosis (Lusis, 2000, Schrijvers *et al.*, 2005), a feature that symbolizes late stage of atherosclerosis (Tabas, 2010). In the present study, histopathological evaluation of thoracic aorta of ATH rats depicted characteristic necrotic core formation and subsequent derangement of elastin fibers. However, these set of changes were minimal in ATH+CG group. These results can be correlated with presently recorded prevention of *in vivo* LDL oxidation and lowered expression of cell adhesion molecules (VCAM-1 and P-Selectin).

It can be summarized from the results obtained herein that, CG extract is capable of ameliorating experimental atherosclerosis by down regulation of vascular cell adhesion molecules and macrophage differentiation. Further studies are required to translate these pre-clinical findings to a clinical level.

Summary

Present inventory evaluates the anti-atherogenic potential of *C.glandulosum*.Coleb leaf extract (CG) using *in vivo* and *in vitro* experimental models. Serum markers of low density lipoprotein (LDL) oxidation, cholesterol, triglycerides, lipoproteins, auto-antibody titer, *ex vivo* LDL oxidation, LDL aggregation, aortic lipids, histopathological evaluations and immunolocalization of macrophage surface marker (F4/80), vascular cell adhesion molecule-1 (VCAM-1) and P-selectin were performed in CON (rats treated with single dose of saline (*i.p.*) and fed with laboratory chow), ATH (rats treated with single dose of vitamin D_3 (600000 IU, *i.p*) and fed with atherogenic diet) and ATH+CG (rats treated with single dose of vitamin D_3 (600000 IU, *i.p.*) and fed with atherogenic diet and simultaneously treated with 200mg/kg CG extract, *p.o.*) for 8 weeks. CG extract supplementation to atherogenic diet fed rats significantly prevented increment in serum cholesterol, triglycerides, and lipoproteins, markers of LDL oxidation, auto-antibody titer and aortic lipids. Also, Also, LDL isolated from ATH+CG rats recorded mimimal aggregation and susceptibility to undergo *ex vivo* LDL oxidation. Microscopic evaluation of thoracic aorta of ATH+CG rats reveled prevention of atheromatous plaque formation, accumulation of lipid laden macrophages, calcium deposition, distortion/defragmentation of elastin, accumulation of macrophages and, down regulation of cell adhesion molecules (VCAM-1 and P-selectin) expression. Further, *in vitro* monocyte to macrophage differentiation was significantly attenuated in presence of CG extract (200µg/ml). It can be concluded from the present study that, CG extract is capable of controlling induction