CHAPTER 5

Anti-atherosclerotic potential of Anethum graveolens L. seeds extract in high cholesterol diet fed atherosclerotic rats

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Anti-atherosclerotic Potential of *Anethum graviolens* L. Seed Extract

INTRODUCTION

The pathogenesis of atherosclerosis is a complicated process consisting of a complex sequence of events that culminates in plaque like deposits, hardening of arteries, narrowing of lumen which leads to reduced blood supply to key organs and body systems (Saffi *et al*., 2015). Heightened levels of oxidative stress, cholesterol, inflammation etc. causes endothelial dysfunction and forms basis of plaque formation (Petersen *et al*., 2016).

Cholesterol lowering drugs such as statins are widely used to block the rate limiting enzyme HMG-Co-A reductase and lower cholesterol synthesis (Grundy, 2002). There are major concerns regarding uses of high dose of statins for aggressive reduction of cholesterol levels because it causes adverse effects (Jadeja *et al*., 2012). Rhabdomyolysis is uncommon but can be life threatening consequence of statins (Antons *et al*., 2006; Fadini *et al*., 2010). Owing to the multi-factorial nature of atherosclerosis, a multi targeted approach can be the most effective with minimum side effects. Herbal extract singly or in combination with other herbal supplements have been extensively reported in tackling life style related disorders including atherosclerosis due to their multi-proned mode of action (Patel *et al*., 2013; Jadeja *et al*., 2012, Thounaojam *et al*., 2012). This includes polyherbal formulation for synergistic effects which imply towards multiple targets as seen in onset or progression of atherosclerosis.

The present study was designed to assess *in vivo* anti-atherosclerotic potential of AG. This perception was drawn after obtaining favourable results on the efficacy of AG in preventing LDL modifications, ox-LDL uptake and foam cell formation and lowering experimentally induced inflammation in macrophages (Chapter 4).

MATERIALS AND METHODS

Collection of Plant and Preparation of Plant Extract

Collection of plant and preparation of extract were same as in chapter 4.

Experimental Animals

Male *Sprague dawley* rats (10 weeks old; weighing 325 ± 20 gm) were obtained from Zydus research centre, Ahmedabad and maintained at the Department of Zoology, Faculty of Science, The M.S. University of Baroda, Vadodara, India. Rats were 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

The atherosclerosis was induced in rats by single intraperitoneal injection of Vitamin D3 (600,000 unit/kg) followed by feeding them with high cholesterol diet (3% cholesterol, 0.5% cholic acid, 0.2% 6-propyl 2-thiouracil, 5% sucrose, 10% lard, and 81.3% powdered laboratory chow) (Cai *et al*., 2005; Huang *et al*., 2004). There were four groups (n=8), control rats were given single dose of 0.9% saline (0.1 ml) intraperitoneally, maintained on standard laboratory chow and simultaneously administered with 0.5% CMC (0.1 ml) orally daily for 8 weeks (Group I: NC). Rats were given single dose of

Vitamin D3 (600,000 unit/kg) intraperitoneally and later, fed with a high cholesterol diet and simultaneously administered with 0.5% CMC (0.1 ml) orally daily for 8 weeks (Group II: ATH). Rats were given single dose of Vitamin D3 (600,000 unit/kg) intraperitoneally and later fed with a high cholesterol diet and simultaneously administered with 200 mg/kg of AG extract by oral feeding for 8 weeks daily (Group III: ATH+AG).

After 8 weeks, blood was collected from overnight fasted rats (12 h) via retro-orbital sinus puncture in 2 ml centrifuge tubes. Serum was separated by cold centrifugation (4°C) at 1500 rpm for 10 min. Later, animals were sacrificed by cervical dislocation under mild ether anesthesia and thoracic aorta of control and experimental animals were collected. Two small pieces of thoracic aorta from aortic arch were collected and processed for paraffin wax histology. The remaining piece of thoracic aorta was stored at –80°C (Cryo Scientific Ltd., India) for further use.

Serum Lipids

Serum triglyceride (TG), total cholesterol (TC) and high density lipoprotein (HDL) contents were estimated using biochemical diagnostic kits (Reckon Diagnostics Ltd., Baroda, India) using a semi auto-analyser (Micro lab 300 L, Merck). LDL and VLDL were calculated (Friedewald *et al*., 1972).

Isolation of LDL from Rats and MDA Assay

LDL was isolated from serum samples of control and experimental rats by heparin-citrate buffer precipitation method as described earlier (Ahotupa *et al*., 1998). The protein concentration of LDL was estimated by the method of Lowry *et al*. (1951) using BSA as

standard. Oxidation state was evaluated by assaying malondialdehyde (MDA) levels in the LDL samples of control and experimental groups as mentioned above and the absorbance was measured at 532 nm with UV/VIS Perkin Elmer spectrophotometer and MDA was calculated using a molar absorption coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ (Buege and Aust, 1978).

Microscopic Evaluation of Thoracic Aorta

Thoracic aorta of control and experimental rats were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax using automated tissue processor. 5-7 µm sections were cut (on a Leica RM 2155 Microtome), stained with hematoxylin and eosin (H&E). Another set of sections of aorta from control and experimental rats were incubated in Von kossa stain solution (1% silver nitrate) under ultraviolet light for 20 min. The sections were then rinsed repeatedly in distilled water, placed in 5% sodium thiosulphate for 5 min and rinsed in distilled water again (to remove un-reacted silver). The sections were then counterstained with 1% eosin for 5 min (Hsieh *et al*., 2007). All sections were examined under a Leica DMRB microscope (100 X) and photographed with a canon power shot S70 digital camera at 100 X magnification.

Immunohistochemistry of Thoracic Aorta

Paraffin embedded sections of thoracic aorta of control and experimental rats were deparaffinised in xylene and hydrated using graded series of alcohol and water. Sections were then washed in phosphate buffer saline (PBS) and antigen retrieval step was carried out by immersing slides in sodium citrate buffer at 80°C for 10 min. Later, endogenous

peroxidases were removed by incubation of sections in 3% H_2O_2 for 20 min in dark. Non-specific binding sites were blocked by incubation of slides with 1% fetal bovine serum (FBS) for 30 min. Localization of vascular cell adhesion molecule-1 (VCAM-1) and P-selectin was carried out by incubating sections with rabbit anti-rat IgG at a dilution of 1:100 (SantaCruz Biotechnology, Inc.) and goat anti-rat P-selectin IgG at a dilution of 1:100 (Santa Cruz Biotechnology, Inc.), respectively for overnight at 4°C in a humidified chamber. At the end of incubation, slides were washed with PBS and then, the sections were incubated with respective horseradish peroxidise (HRP) conjugated secondary antibodies for 4 h at room temperature. Goat anti-rabbit IgG-HRP 1:100 (Bangalore Genei Pvt Ltd.) for VCAM-1, and rabbit anti-goat IgG-HRP 1:100 (Bangalore Genei Pvt Ltd.) for P-selectin were used. At the end of incubation, sections were thoroughly washed with PBS and final detection step was carried out using diaminobenzydine (DAB) detection system (Bangalore Genei Pvt Ltd.) and counterstained with haematoxylin. Sections were examined under Leica DMRB microscope and photographed using a canon Power shot S70 digital camera (Thounaojam *et al*., 2012).

RESULTS

Serum Lipids

Significant elevation in serum lipid profile (TC, TG, VLDL and LDL levels), while decrement in HDL level were observed in high fat diet fed rats as compared to the control rats. However, co-supplementation of high fat diet +AG showed remarkable decrement in TC, TG, VLDL and LDL levels and increment in HDL level [Table 5.1 and Figure 5.1].

Serum MDA Assay

There was significant ($p<0.001$) increment in circulating levels of MDA in high fat diet fed rats as compared to the standard laboratory diet fed rats. However, AG co-supplementation accounted for significantly reduced levels of MDA [Table 5.2 and Figure 5.2].

Microscopic Evaluation of Thoracic Aorta

Hematoxylin and eosin staining of thoracic aorta of standard laboratory diet fed rats showed normal histo-architecture with intact intima. There was a remarkable change observed in intima of high fat diet fed rats. There were degenerative changes such as plaque formation between endothelial lining and intimal layer and rupturing of smooth muscle layers observed. However, less degree of degenerative changes were observed in the photomicrographs of thoracic aorta of rats fed with AG along with high fat. This included reduced plaque formation and derangement of smooth muscle layer. Von kossa staining of aorta of high fat fed rats showed significant deposition of calcium as compared to normal standard laboratory diet fed rats. While AG co-supplementation

along with high fat diet showed remarkable reduced levels of calcium deposit [Figure 5.3].

Immunohistochemistry of Thoracic Aorta

The immunohistochemistry of thoracic aorta of high fat diet fed rats showed significant increased expression of cell adhesion molecules (CAMs; VCAM-1 and P-selectin) on endothelial lining as compared to standard laboratory diet fed rats. But, in AG co-supplemented rats, there was remarkable decrease in expression of the cell adhesion molecules [Figure 5.4].

TABLES AND FIGURES

Table 5.1: Effect of AG on Serum Lipid Profile of Rats Fed with Normal and High Fat Diet

Parameters	Groups		
	NC	ATH	ATH + AG
TC	54.50 ± 1.31	266.80 ± 5.04###	149.50 ± 3.14***
TG	33.67 ± 2.02	90.8 ± 1.86###	58.17 ± 1.68***
VLDL	6.73 ± 0.40	18.170 ± 0.37###	11.63 ± 0.33***
LDL	21.67 ± 2.76	243.00 ± 5.15###	122.0 ± 4.41***
HDL	20.50 ± 1.20	5.00 ± 0.68###	16.67 ± 1.35***

Data are expressed as mean ± S.E.M for n=6. Where, ### $p < 0.001$ compared to NC and ***$p < 0.001$ compared to ATH.

Figure 5.1: Effect of AG on Serum Lipid Profile of Rats Fed with Normal and High Fat Diet

Data are expressed as mean ± S.E.M. where n=8. ### $p<0.001$ compared to ATH and *** $p<0.001$ compared to ATH+AG

Figure 5.1 (Continued)

Data are expressed as mean ± S.E.M. where n=8. ### $p<0.001$ compared to ATH and *** $p<0.001$ compared to ATH+AG

Table 5.2: Effect of AG on Serum MDA Levels of Rats Fed with Normal and High Fat Diet

Parameters	Groups		
	NC	**ATH**	**ATH + AG**
MDA	3.48 ± 0.65	28.39 ± 0.71###	9.18 ± 0.27***

Data are expressed as mean ± S.E.M. where n=8. ### $p<0.001$ compared to ATH and *** $p<0.001$ compared to ATH+AG

Figure 5.2: Effect of AG on Serum MDA Levels of Rats Fed with Normal and High Fat Diet

Data are expressed as mean ± S.E.M. where n=8. ### p<0.001 compared to ATH and *** p<0.001 compared to ATH+AG

Figure 5.3: Effect of AG on Histopathology of Thoracic Aorta of Rats Fed with Normal and High Fat Diet

Figure 5.4: Effect of AG Extract on expression of VCAM-1 in thoracic aorta of Rats Fed with Normal and High Fat Diet

Figure 5.4: Effect of AG on the expression of P-selectin in thoracic aorta of Rats Fed with Normal and High Fat Diet

DISCUSSION

Atherosclerosis develops through a series of complex overlapping physiological events that often remains untreated in whole animal experiments. Events such as formation of ox-LDL, foam cell formation and LDL uptake, inflammatory changes and apoptosis are therefore studies via *in vitro* protocols (Patel *et al*., 2013). The same has been elucidated in chapter 4 using AG extract and based on favourable findings; anti-atherosclerosis potential of AG has been addressed herein.

Hypercholesterolemia has been long associated with development of atherosclerosis and cholesterol lowering is an important therapeutic credential of test compound (Steinberg, 2005). In the present study, AG co-supplementation could induce favourable changes in the lipid profile of high fat diet fed rats. Increment in HDL accounts for reverse cholesterol transport (Ono, 2012) and the same can be accredited as the possible mechanism in our study due to AG induced increment in HDL levels. These observations are further supported by favourable changes in serum MDA levels. Studies have reported that during oxidation of LDL, PUFA gets modified into MDA and PC (Ahotupa *et al*., 1998) that increase susceptibility of ox-LDL to aggregate in atherogematous plaque (Maor *et al*., 1997). Hence, AG induced lowering of MDA observed herein is of high relevance in the chain of high fat diet fed changes. These results are attributable to high content of flavonoids and polyphenols in AG extract.

Microscopic evaluation of thoracic aorta reveals AG induced minimal damage to tunica intima. A careful scrutiny also indicated decrement in the size of lipid core. These results are in consequence of the potential of AG extract in preventing plaque formation. To

establish the underlying mechanism resulting from adhesion of monocyte to the site of vascular injury, immuno-localisation of cellular adhesion molecules was assessed on the surface of endothelial lining of thoracic aorta. Previous reports have suggested a strong correlation between monocyte recruitment and expression of ICAM-1, VCAM-1 and P-selectin (Bobryshev, 2006; Zheng *et al*., 2005)

During atherosclerosis onset, selectin is expressed first on luminal surface of activated endothelium. L-selectin is also expressed on monocyte followed by ICAM and VCAM-1 (Quehenberger, 2005; Natarajan and Cai, 2005). In the present study, the decreased expression of P-selectin, ICAM and VCAM-1 in thoracic aorta provides ample testimony to antiatherosclerotic potential of AG. Arterial hardening due to calcification is the hall mark of advanced high fat diet fed changes that results in hypertensive condition and increased myocardial afterload (Safar *et al*., 2003; Speer and Giachelli, 2004). Other studies have advocated ox-LDL induced arterial calcification (Jadeja *et al*., 2012) and the same was observed in our study.

However, reduced calcification following AG treatment further corroborates antiatherosclerotic potential of AG. It can be concluded that AG is capable of ameliorating experimentally induced atherosclerosis by favourable changes in lipid profile, prevention of plaque formation and reduction in hardening of thoracic aorta. These results also highlight the importance of functional food such as AG in management of atherosclerosis and warrants further studies at clinical levels.

SUMMARY

Atherosclerosis is caused by heightened levels of oxidative stress, cholesterol, inflammation induced endothelial dysfunction and formation of plaque beneath endothelial lining of aorta. The present study was design to evaluate the antiatherosclerotic effect of AG extract using atherosclerosis animal model of rats. Twenty-four rats were assigned to the control, high cholesterol diet (ATH) and ATH + AG group that were fed with high cholesterol diet co-supplemented with AG. Serum levels of triglyceride (TG), total cholesterol (T-CHO), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) and malondialdehyde (MDA) were detected at the end of experiments. Vascular cell adhesion molecules (VCAM-1), and P-selectin were investigated by immunohistochemistry of thoracic aorta of rats. Serum levels of TG, TC, LDL-C and VLDL-C levels were significantly increased in ATH group as compared to Control group while AG co-supplementation showed significant decrement as compared to ATH group. HDL level also significantly increased in ATH + AG as compared to ATH group. Immunohistochemistry of thoracic aorta also suggested that the expression of VCAM-1 and P-selectin was significantly reduced as compared to ATH group. Based on the results of present study, it can be concluded that the AG extract prevented the atherosclerotic changes by lowering serum lipid levels, oxidation of lipids and suppressing the inflammatory response.