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Anti-Atherosclerotic Activity of *Lagenaria Siceraria* in Experimentally Induced Atherosclerosis in C57BL6J Female Mice

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Abstract:
Atherosclerosis is one of the prominent cardiovascular disease which is preventable and is a leading cause of morbidity and mortality. Currently lipid lowering agents like statins offer some protection, but is associated with drawbacks. The authentic ancient Ayurvedic text books mention that Lagenaria Siceraria (LS) has a heart strengthening property. Hence we undertook this study with the aim of evaluating the anti-atherosclerotic and hypo-lipidemic effect of Ethanolic extract of Lagenaria siceraria(EELS). Atherosclerosis was induced in experimental C57 BL6J mice with high cholesterol diet and the effect of EELS on atherosclerosis, blood lipid levels and liver histopathology were observed. We observed that 3 doses of EELS (160,320 and 640) significantly ($p< 0.05$) reduced the histopatological grading of atherosclerosis; additionally the mean lesion area was significantly lower ($p<0.05$) in all the EELS treated groups. However, in both the histo-pathological grades and lesion area the results were not comparable to Atorvastatin. Similarly the histo-pathological grades of liver were improved significantly ($p<0.001$) in all the EELS treated groups than the disease control. The serum total cholesterol and triglyceride levels were reduced with EELS with 3 doses of EELS. The serum LDL and VLDL levels were significantly lowered with 3 doses of EELS and the effect was comparable to Atorvastatin. It can be concluded that the EELS in doses of 160,320 and 640 mg/kg showed a prominent anti-atherosclerotic effect in the mice (C57 BL6J).

Keywords: *ethanolic extract, Lagenaria Siceraria, atherosclerosis, C57 BL6J*

1. Introduction

Atherosclerosis is a multifactorial and preventable cause of cardiovascular diseases (CVD) with dyslipidemia as important risk factor (GazianoTA, Gziano JM 2008, Mahmoud Rafieian *et al* 2014, Witztum, J. L. 1991). Hence most of the strategies are based on reducing the lipid levels for protection. Amongst all classes, statins are the drugs of choice for management of hyperlipidemia and thereby prevent atherosclerosis (Langsjoen, P.H., 2003). However they have few adverse events like myopathy, hepatotoxicity (Pandit, A., 2012) along with drawback of long term administration and high cost of the therapies (Cholesterol Treatment trialists' (CTT) Collaboration, 2005).

Ayurveda regards atherosclerosis to be a disease of vata. Vata is said to be increased above normal in atherosclerosis. It also considers atherosclerosis as a complication of meda roga (obesity). Lagenaria siceraria, commonly known as 'bottle gourd' ('Dudhi' or 'Louki' in Hindi) belongs to family Cucurbitaceae which has been mentioned in the authentic ayurvedic text book Nighantu, to have vatal which means xxxx and strengthening effect on heart (Bapalal G). The potential properties of LS are hypoglycaemic (Prerona Shah et al,2011), anti-inflammatory agent (Prajapati, R. P. et al., 2010) and analgesic (Shah B. N. et al, 2010) and has been shown to have anti-hyperlipidemic action (Ghule B.V. et al., 2009, Katare C et al, 2011, Mahurkar N.M. et al, 2012, Uplangwar A, et al, 2012, Nainwal P. et al., 2011). However, there are no reports to show its effect directly on the atherosclerotic plaque, hence we planned the present study to evaluate the anti-atherosclerotic effect of EELS in C57BL6 mice fed with high cholesterol diet.

2. Objectives

i. To evaluate the anti-atherosclerotic effect of ethanolic extract of Lagenaria siceraria in high cholesterol diet induced atherosclerosis model in mice.
ii. To evaluate the anti hyperlipidemic effect of ethanolic extract of Lagenaria siceraria in high cholesterol diet induced atherosclerosis model in mice.
iii. To evaluate effect of ethanolic extract of Lagenaria siceraria on liver in high cholesterol diet induced atherosclerosis model in mice.

3. Methods

Approval from Animal Ethics Committee of the institution (number AEC/06//13 dated 15th May 2013) was obtained prior to the commencement of the study.

3.1. Study Drugs

Standardized dried of *Lagenaria siceraria* fruit were obtained from an M/S Gayatri Industries, Kodinar, Gujarat with analytical report no. 13012020 Batch no.133012020 and extractive value 15:1.

Three doses of EELS were selected 160, 320 and 640 mg/kg. Atorvastatin was obtained from Cipla Pvt. Ltd and was used in the dose of 2mg/kg. The drugs were administered in the form of solution prepared using distilled water as solvent which served as vehicle control and administered orally.

3.2. Animals

C57BL6J female mice weighing 25-30 gm obtained from animal house of ACTREC Kharghar were used in the present study. The experiment was conducted as per CPCSEA (*Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines. Food and water was provided ad libitum during this period.*

3.3. Method of induction of atherosclerosis (*Zhang, S. H. et al,1994)*

A special cholesterol rich diet obtained from VRK Nutrition, Pune containing 15.8% (wt/wt) fat, 1.25% (wt/wt) cholesterol, 0.5% (wt/wt) sodium cholate was used. The diet pellet were crushed to powdered form and was mixed with distilled water and given as gavage with mice feeding cannula. The quantity administered was 600mg/kg.

3.4. Procedure

After the period of acclimatization, the mice were kept fasting overnight and on day 0, blood was collected by retro orbital blood collection into vacutainers without any anticoagulants for the estimation of serum lipids. The animals were then assigned randomly to six experimental groups as shown in table 1. Each group had 6 animals. The animals were continued to feed on normal pellet diet, and in addition were given cholesterol rich diet daily through gavage with distilled water for a period of 14 weeks except for the animals in normal control group who received distilled water through gavage.

After 14^{th} week, blood sample was collected by retro orbital blood collection for measuring lipid levels. The mice were euthanized. Heart along with the opening of aorta (0.5 cm) was sent for histopathology. The pathologist was blind to the experimental study groups.

3.5. Variables Assessed were as Follows

i. Histo-pathological grading of atherosclerosis (Stewart Whitman, D. A, 2002).
ii. Extent of atherosclerosis estimated mean lesion area by using image analyser
iii. Estimation of Total serum cholesterol (Allain CC et al, 1974)
iv. Estimation of LDL done by autoanalyzer using Roche kits
v. HDL cholesterol (Burstein, et al., 1970)
vi. Estimation of VLDL by subtracting the values of LDL and HDL cholesterol from the total cholesterol
vii. Histopathological grading of fatty changes in liver-by using image analyser

3.6. Statistical Analysis

Statistical analysis was done using the Graph pad version 6.

3.6.1. Histopathological Assessment

i. The histopathological grades of aortic lesions among drug treated groups and disease control group were compared with each other using the Kruskal Wallis test followed by Dunn's test.
ii. One-way ANOVA (analysis of variance) with post hoc Tukey's test was used to compare the effects of the drugs on mean lesion area in individual aortic section to those observed in disease control group.

3.6.2. Serum Lipid Levels

i. To compare basal and final values of the lipid parameters as well as ratios of total cholesterol/HDL cholesterol within the same group Paired 't' test was used.
ii. One-way ANOVA with post hoc Tukey's test was used to compare the effects of the drugs on lipid profile to disease control groups.

4. Results

4.1. Histopathological assessment - Grades of atherosclerosis in the region of aortic opening

Table 2 shows the grades of atherosclerosis observed. There was statistically significant difference in the median grades of atherosclerotic lesions in the disease control group as compared to normal control group. It was significantly reduced in positive control as compared to disease control group. EELS in all three doses showed significant reduction in the grade of atherosclerosis as compared to disease control, however it was not comparable to Atorvastatin group.

Figure 1 shows the histo-pathological changes in the opening of aorta in all the study groups. The normal control i.e. Group 1 showed no atherosclerotic lesions or 'Grade 0'.The disease control i.e. Group 2 showed 'Grade 3' and 'Grade 4' atherosclerotic lesions. While Group 3

i.e. positive control group showed either no lesion or 'Grade 1'. Group 4 of the study receiving EELS 160 mg/kg exhibited the atherosclerotic lesions of 'Grade 1' and 'Grade 2'.Group 5 receiving. EELS 320 mg/kg, the atherosclerotic lesion of 'Grade 1'to 'Grade 2' were seen. Mice in Group 6 receiving EELS 640 mg/kg showed the atherosclerotic lesion of 'Grade 1'. Table 3 describes the grading of atherosclerotic lesions in each group.

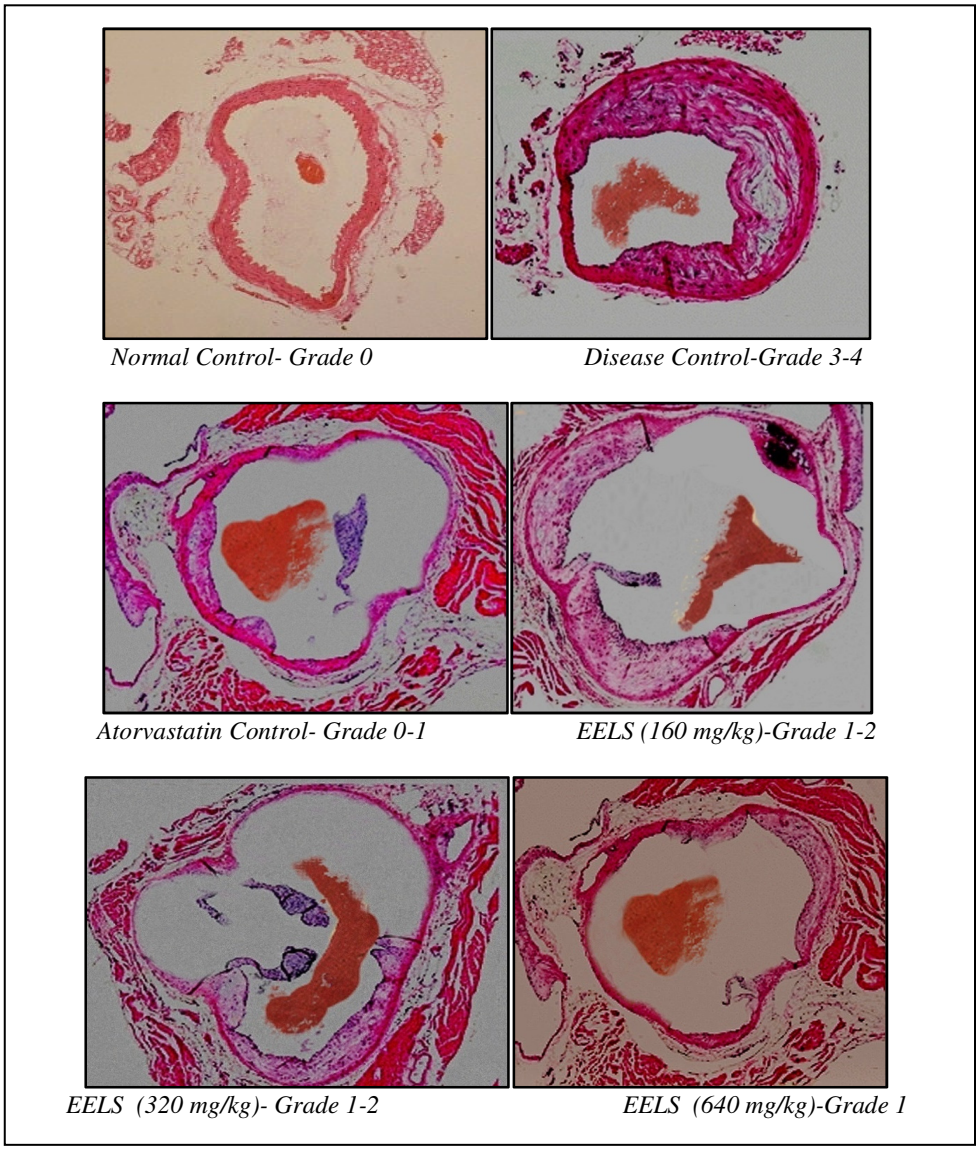

Figure 1: Histopathological changes in aortic opening

4.2. Extent of Atherosclerosis Measured as Mean Lesion Area

The mean lesion area was significantly ($p < 0.001$) reduced in Atorvastatin group when compared with disease control group as shown in Table 4. Animals given EELS 160 mg/kg, 320 mg/kg and 640 mg/kg showed a significantly lesser ($p < 0.001$) mean lesion area when compared with disease control group. However, the reduction in lesion area in EELS groups was not comparable with Atorvastatin group.

4.3. Serum Lipid Levels Assessment

4.3.1. Serum total Cholesterol Levels

The serum total cholesterol levels were comparable in all the groups at baseline. A significant rise at day 98 was observed in the disease control group. Atorvastatin and all EELS treated groups showed significant lowering of ($p < 0.001$) the total cholesterol level as compared to disease control group at 98 day as shown in figure 3. The reduction in total serum cholesterol in the EELS treated groups was comparable to Atorvastatin.

4.3.2. Serum Triglyceride Levels

All the groups had comparable serum triglycerides at day 0. It increased significantly in the disease control group as shown in figure 4. While mice in Atorvastatin group and all EELS groups showed significant ($p < 0.001$) lowering of triglyceride level as compared to the disease control group. However the lowering by all three doses of EELS groups was not comparable to Atorvastatin.

4.3.3. Serum LDL Levels

Figure 5 shows that disease control group showed significantly higher levels of serum LDL levels. Mice in atorvastatin group and the three EELS groups showed significantly lower ($p < 0.001$) LDL levels as compared to the disease control group at day 98. The LDL level in EELS 640mg/kg group was comparable to Atorvastatin at day 98. However, the LDL with EELS 160mg/kg and 320mg/kg treated groups was significantly higher than the Atorvastatin group at day 98.

4.3.4. Serum HDL Levels

Serum HDL levels of animals given cholesterol rich diet were significantly higher ($p < 0.001$) as compared to the normal control group at 98 day as shown in figure 6. However the animals in Group 3 i.e. positive control group and Group 4; 5; 6 i.e. test formulation groups showed significantly lower ($p < 0.001$) HDL levels as compared to Group 2 i.e. disease control group. The HDL levels of Atorvastatin and all the EELS treated groups were comparable.

4.3.5. Serum VLDL levels

Serum VLDL levels of all the groups were comparable at baseline. The figure 7 shows a significant increase ($p < 0.05$) in VLDL levels at day 98 in the disease control group. Mice in atorvastatin group and the three EELS groups showed significantly lower ($p < 0.001$) VLDL levels as compared to disease control group. However the reduction in all the three groups of EELS was not comparable to Atorvastation.

4.4. Histopathology of Liver

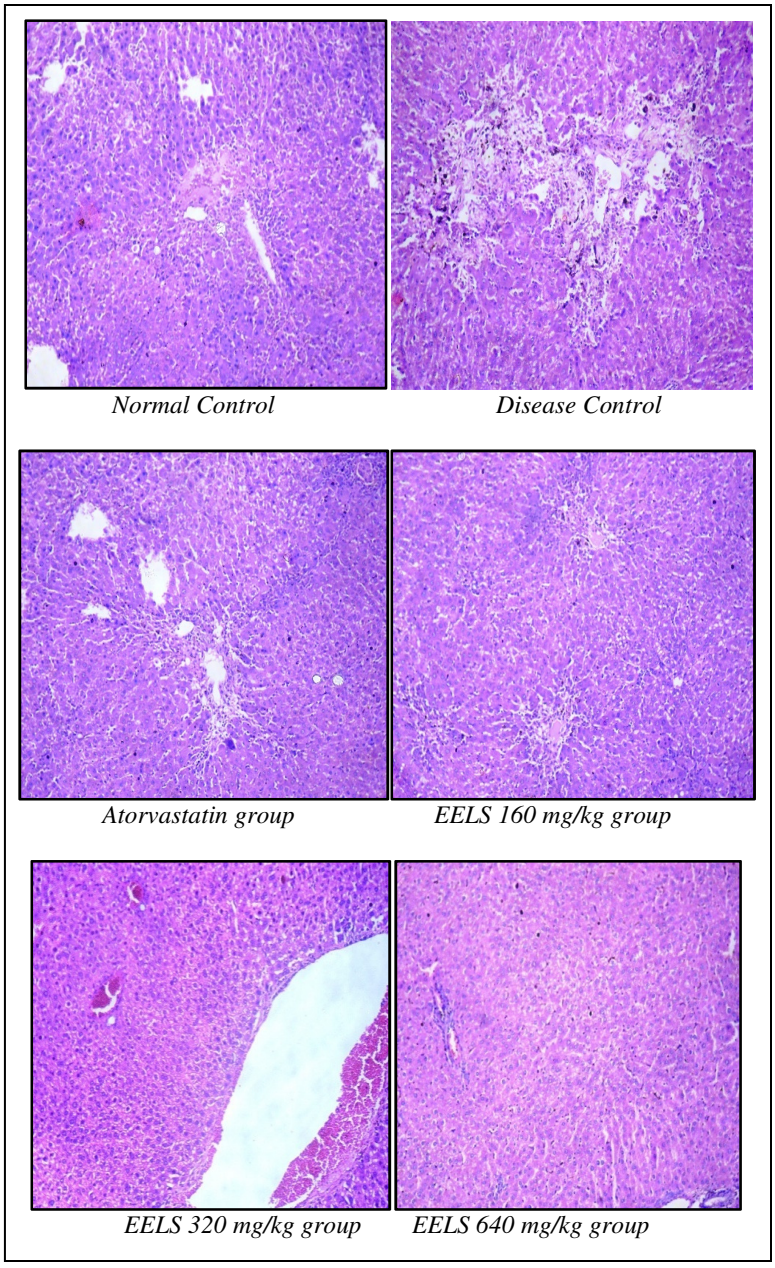

Figure 2: shows histopathological changes in liver

Table 5 shows number of animals showing the grades of fatty liver. More number of animals in EELS treated groups showed significantly lesser or no fatty changes as compared to disease control and Atorvastatin treated groups. Additionally figure 2 shows lesser fatty changes in the liver of mice treated by EELS and the highest dose group mice showing the best response.

5. Discussion

In this study, we found that the ethanolic extract of Lagenaria siceraria showed a significant reduction in the development of high cholesterol diet induced atherosclerosis in the aortic opening of C57BL6J mice. Among many risk factors and patho-physiology for development of atherosclerosis the most important factor is the rise in total cholesterol and mainly LDL cholesterol. Pathophysiology of atherosclerosis has revealed that oxidative reduction of LDL cholesterol by free oxygen radicals marks the initiation of atherosclerosis (Georgia Vogiatzi and Dimitris Tousoulis, C.S., 2009). Consequently the strategies involved aimed at lowering these lipid levels with agents such as statins. However the use of statins is associated with adverse effects. Hence we turned to traditional medicines.

Lagenaria siceraria has been described to have heart strengthening properties. It is a nutraceutical and is available in different extracts like methanolic, ethanolic, ether, chloroform, aqueous etc.(Prajapati, R. P. et al. 2010). The dose used in the present study was 160 mg/kg, 320 mg/kg and 640 mg/kg that was derived from previous study showing hypolipidemic effect of Lagenaria siceraria (Upaganlawar, A. et al, 2012). So far all the studies have evaluated its efficacy as a lipid lowering agent and has been shown to have anti-hyperlipidemic action ((Prajapati, R. P. et al, 2010, Ghule B.V. et al, 2009, Katare C et al, 2011, Mahurkar N.M. et al, 2012, Uplangwar A, et al, 2012, Nainwal P. et al., 2011). However, there is no study that has evaluated its effect on the atheromatous plaque. Hence we undertook this study to evaluate the effect of three doses (160, 320 and 640mg/kg) of EELS on atherosclerosis induced by high cholesterol diet in C57BL6J mice.

C57BL6J mice were chosen as they are known to develop both spontaneous and diet induced atherosclerosis. Female gender mice were chosen as they are more prone to develop lesion than male and they develop larger atherosclerotic lesions as compared to male; additionally they are more reliable for developing atherosclerosis (Georgia Vogiatzi and Dimitris Tousoulis, C.S., 2009). The advantage of this model is that the atherosclerosis can be induced in comparatively shorter duration of time, easily reproducible, popular and is well accepted. (Daugherty, A. & Rateri, D.L., 2005, Daugherty Alan, Stewart W., 2002).

The development of lesion in aortic opening is sequential i.e. lesions are first developed in aortic root (aortic opening) in first three months, then aortic arch in next three month (i.e. six month) and the development of lesion in abdominal aorta take 9-12 months (Nainwal P. et al., 2011). Therefore, we decided to use feeding for short duration of 3 months and looked for lesions in the aortic opening. It results in lesions with predominant foam cell appearance and minimal or no fibrosis. Hence, it was decided to grade the lesions using grading system proposed by Stewart and Doughty, 2003, which is based on the size and extent of the lesion rather than composition. Additionally, we quantified the lesions using image analyzer, which can perform morphometric analysis to estimate the extent of these lesions.

There was reduction in the development of atherosclerotic lesion with all the three doses of EELS as compared to disease control group. All the EELS treated groups showed significantly lower grades of atherosclerosis when compared to disease control group. A graded dose response was observed with 160,320 and 640mg/kg dose of EELS. Out of the three doses of L. siceraria, 640 mg dose showed the most prominent reduction in development of atherosclerosis, while 160 and 320 mg/kg doses group the effect wasn't comparable to Atorvastatin. Though, we observed a trend towards reduction in atherosclerotic lesion. Thus, it may be appropriate to evaluate the responses with higher doses of EELS.

We also estimated hyperlipidemia as it is an established risk factor for atherosclerosis (Ross, R.. and Harker, L.,1976, Kerenyi, L. et al, 2006) and lowering lipids is associated with plaque stabilization and therefore decreases coronary events (Ray, K. K., & Cannon, C. P. 2005). We observed that L. siceraria in highest dose reduced the lipid levels similar to statins. The hypolipidemic effect seen this study was comparable with previous studies done by Vijaykumar M, et al. (2010) in wistar rats. The plausible mechanism proposed in that study was antioxidant property of L. siceraria. The antihyperlipidemic effect of EELS can also be attributed to its various constituents viz sterols, flavonoids, saponins, and polyphenolics. The sterols are responsible for reduction in absorption of cholesterol. The flavonoids increase the activity of lecithin acyl transferase, which plays an important role in the incorporation of free cholesterol in HDL and transferring it back to VLDL and LDL which is taken back in liver cells and is excreted in bile. Saponins bind with cholesterol in intestine and inhibits its absorption; also it causes increase in lipoprotein lipase activity which help in faster removal of fatty acid from circulation, which indirectly decreases total cholesterol levels (Mohale D.S.et al, 2008). The other possible mechanisms for anti-atheroslcerotic effect due to reduced generation of oxygen free radicals, lipid peroxidation, glutathione peroxidase and catalase (Singh R.B. et al 2002). LDL oxidation is an important step in induction of atherosclerosis (Witztum, J. L. and Steinberg D. 1991). L siceraria may prevent this by free radical scavenging activity (Deshpande J.R. et al, 2008). Thus all the above mentioned mechanisms may contribute to anti-atherosclerotic effect of L. siceraria. The HDL levels were found be raised in the mice treated with high cholesterol diet. This is in consonance with report by Ali et al, 2014. The raised HDL was reduced by Atorvastatin and all EELS doses. The HDL rise has been linked to hepatic expression of SR-BI receptors which plays a key role in HDL level regulation.

Rajput MS et al, 2014 observed that ethanolic extract of L. siceraria causes significant increase in tail bleeding time and plasma recalcification time. Additionally, they also reported that it provides significant protection against ADP induced pulmonary thromboembolism and inhibition of platelet aggregation induced by ADP in vitro. It is a known fact that platelets play a critical role in this phenomenon (Robert A. et al, 2003). The significant antithrombotic potential has been correlated to inhibition of ADP-mediated platelet aggregation and the involvement of various non-cellular chemical mediators of blood.

The histopathology of liver revealed the lowest fatty infiltration in the liver of the mice treated with EELS. The highest dose of EELS has shown no fatty infiltration as compared to mild fatty infiltration seen with statin. There are reports of hepatoprotective potential of EELS (Deshpande J.R. et al., 2008). This may be an advantage of EELS over statins. This effect needs further exploration.

The rabbit model of atherosclerosis is a better model than mice, but because of technical and financial difficulties mice model was used for this study. We suggest the effects of EELS should be confirmed in rabbit model of atheroslcerosis and hypercholesterimia.with higher doses as well.

So far we found only one clinical study evaluating the anti-dyslipidemic and anti-oxidant properties of fresh juice of Lagenaria siceraria in

dyslipidemic subjects (Katare C.,et al, 2014). In this study Lagenaria siceraria was reported to be effective as hypolipidemic. This study demonstrated anti-atherosclerotic effect of L. siceraria which could possibly be attributed to diverse mechanisms as discussed earlier. Additionaly, diabetes has been reported to exert an effect similar to lipids on the atheromatous plaque (Kanter J.E., et al, 2007). Also homocysteine and high-sensitivity C-reactive protein have been reported to be involved in the process (Fruchart J.C.et al, 2004). Additionally the role of epigenetic factors and the role of PPAR-gamma has been reviewed. Thus, there are lipid independent mechanisms in the initiation and progress of atheromatous plaque. In the light of this emerging information, we feel that it will be interesting to evaluate the effect of Lagenaria siceraria on these targets. We need to evaluate the effect of higher but safer doses of EELS and confirm the activities in long term and multiple models of atherosclerosis.

This is first in-vivo study to see the effect of Lagenaria siceraria directly on the atherosclerosis and hence no literature was available to compare the results of atherosclerotic change. Further, the results of this study needs to be supported with clinical studies for confirming and exploiting the antiatherosclerotic effect of this nutraceutical.

- Conclusion: To conclude, the EELS prevented the development of atherosclerosis in the murine model of experimentally induced atherosclerosis by high cholesterol diet.

Group(n=6/group)	Diet	Study Drugs	Doses per day
Normal Control	Normal	DW*	1 ml
Disease Control	Cholesterol Rich Diet	DW*	1 ml
Positive Control		Atorvastatin	8 mg/kg
Test Formulation 1		EELS**	160 mg/kg
Test Formulation 2		EELS**	320 mg/kg
Test Formulation 3		EELS**	640 mg/kg

Table 1: Experimental groups

*DW- distilled water

**EELS- Ethanolic Extract of Lagenaria Siceraria

Sr.No	Group (n=6/grp)	Median grade of atherosclerosis
1	Normal control	0
2	Disease control	4 (3-4)$^{@}$
3	Atorvastatin 8mg/kg	0 (0-1)*
4	EELS 160mg/kg	2 (1-2)$^{*, \$}$
5	EELS 320mg/kg	1 (1-2)$^{*, \$}$
6	EELS 640mg/kg	1 (0-1)$^{*, \$}$

Table 2: Grades of atherosclerotic lesions in various treatment groups

- Mann Whitney U test: $^{@}$ $p<0.01$v/s normal control
- Kruskal Wallis H test followed by Dunn's test: $^{*}p<0.05$ v/s disease control, $^{\$}p < 0.05$v/s positive control.

Grade	Description
0	Normal artery: No intimal thickening or fibrosis, normal media, intact Internal elastic lamina
1	Focal intimal thickening and proliferation without disruption of the internal elastic lamina
2	Intimal thickening of approximately 50% of the perimeter of the lumen, with or without disruption of the internal elastic lamina
3	Concentric intimal thickening, with or without disruption of the internal elastic lamina; without appreciable luminal stenosis (estimated at <10%)
4	Concentric arterial disease: Intimal thickening and proliferation with disruption of the internal elastic lamina and the medial elastic tissue; mild or moderate luminal stenosis (estimated at <50%)
5	Concentric arterial disease with severe luminal stenosis (estimated at >50%)

Table 3: Gradation system for aortic lesions

Sr.No	Group (n=6/grp)	Mean lesion of atherosclerosis (mm^2)
1	Normal control	0
2	Disease control	$0.025^{@} \pm 0.003$
3	Atorvastatin 8mg/kg	$0.0057^{*} \pm 0.002$
4	EELS 160mg/kg	$0.015^{*,\$} \pm 0.001$
5	EELS 320mg/kg	$0.011^{*,\$} \pm 0.0005$
6	EELS 640mg/kg	$0.009^{*,\$} \pm 0.001$

Table 4: Mean lesion area in treatment groups
All figures indicate mean ± SD

- Unpaired t test: $^{@}$ $p<0.001$ v/s normal control
- ANOVA followed by Tukey's test: $^{*}p<0.001$ v/s disease control, $\$p<0.001$ v/s positive control.

Sr.No	Group (n=6/grp)	Histopathological grade (no. of animals)			
		Grade 0	Grade I	Grade II	Grade III
1	Normal control	0	0	0	0
2	Disease control	0	0	2[@]	4[@]
3	Positive control	0	4[*]	2[*]	0
4	Test Formulation 1	3[*]	3[*]	0	0
5	Test Formulation 2	4[*]	2[*]	0	0
6	Test Formulation 3	5[*]	1[*]	0	0

Table 5: Grading of fatty changes in liver

- Mann Whitney U test: @p<0.05 v/s normal control
- Kruskal Wallis H test followed by Dunn's test: *p<0.05 v/s disease control

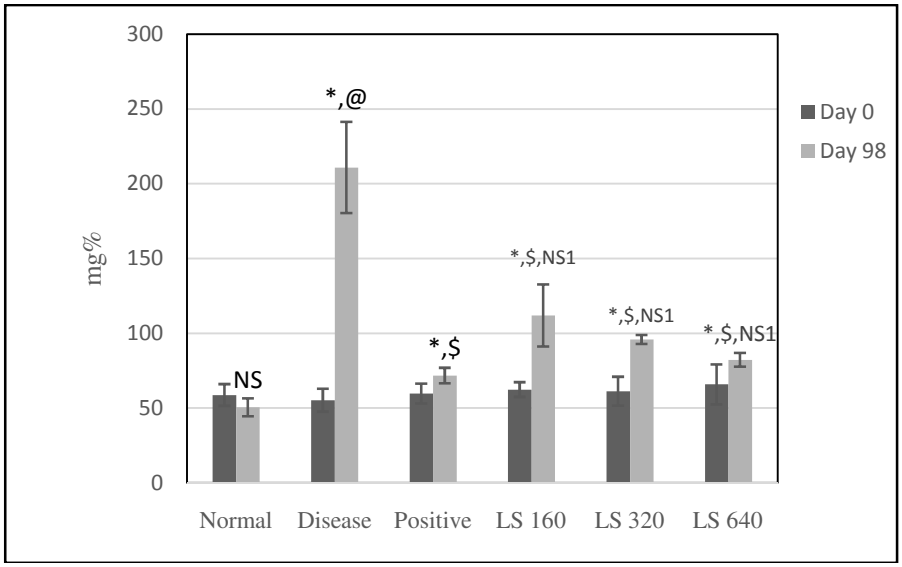

Figure 3: Serum Total Cholesterol levels

- Wilcoxon sign rank test: [*] P < 0.05 v/s day 0; NS – Not significant v/s Day 0
- Mann Whitney U test: [@] p < 0.05 v/s normal control on Day 98
- ANOVA followed by Tukey's test: [$] p < 0.05 v/s disease control on Day 98, NS1 – Not significant as compared to positive control on Day 98

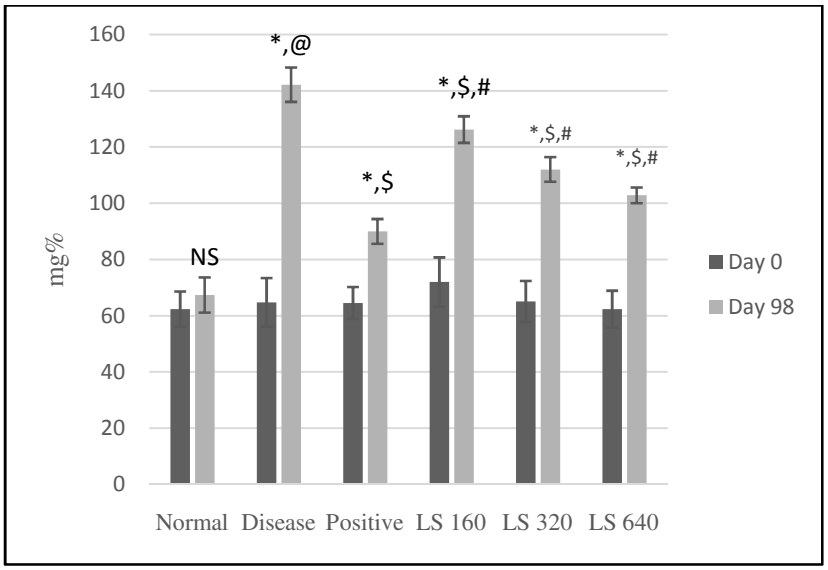

Figure 4: Serum Triglyceride levels

- Wilcoxon sign rank test: * P < 0.05 v/s day 0; NS – Not significant v/s Day 0
- Mann Whitney U test: @ p < 0.05 v/s normal control on Day 98
- ANOVA followed by Tukey's test: $ p < 0.05 v/s disease control on Day 98, # Significant as compared to positive control on Day 98

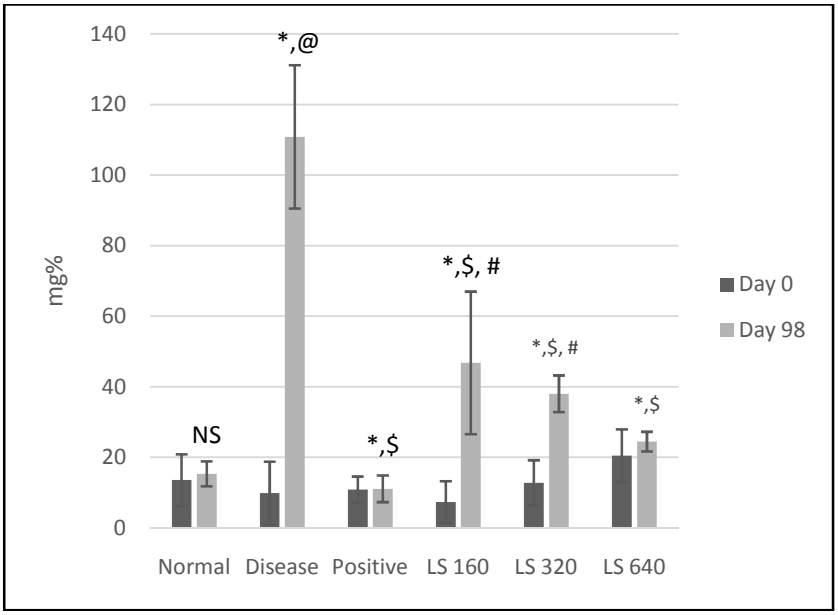

Figure 5: Serum LDL levels

- Wilcoxon sign rank test: * P < 0.05 v/s day 0; NS – Not significant v/s Day 0
- Mann Whitney U test: @ p < 0.05 v/s group 1 on Day 98
- ANOVA followed by Tukey's test: $ p < 0.05 v/s disease control on Day 98, # significant as compared to positive control on Day 98

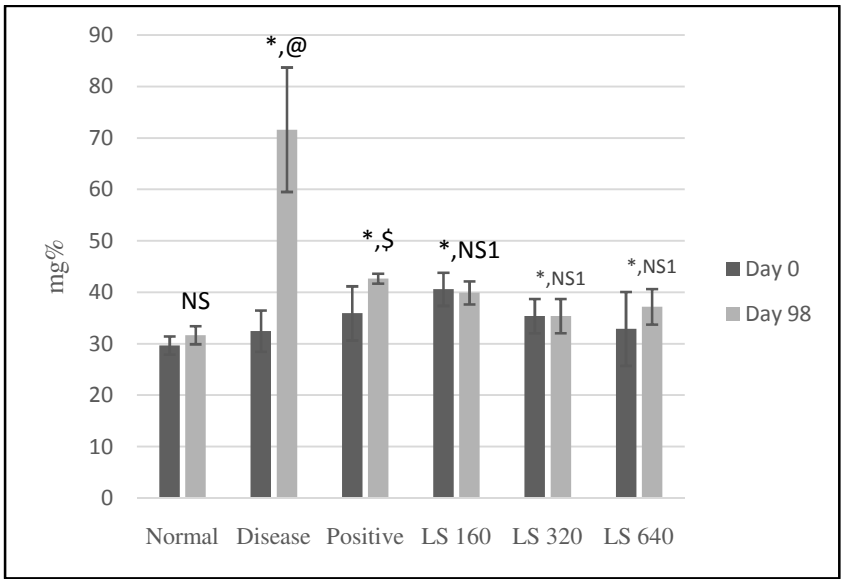

Figure 6: Serum HDL levels

Wilcoxon sign rank test: * P < 0.05 v/s day 0; NS – Not significant v/s Day 0
Mann Whitney U test: @ p < 0.05 v/s normal control on Day 98
ANOVA followed by Tukey's test: $ p < 0.05 v/s disease control on Day 98,
NS1 – Not Significant as compared to positive control on Day 98

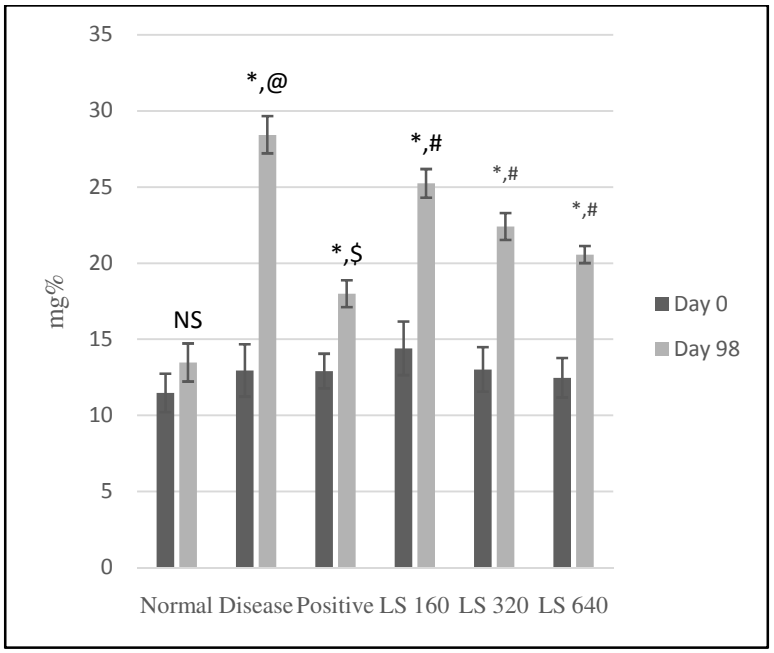

Figure 7: Serum VLDL levels

- Wilcoxon sign rank test: * P < 0.05 v/s day 0; NS – Not significant v/s Day 0
- Mann Whitney U test: @ p < 0.05 v/s normal control on Day 98
- ANOVA followed by Tukey's test: $ p < 0.05 v/s disease control on Day 98, # significant as compared to positive control on Day 98.

6. Acknowledgement

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