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ETHANOLIC EXTRACT OF *AEGLE MARMELOS* MEDIATES ITS HYPOCHOLESTEROLEMIC EFFECT BY RETARDING CIRCULATORY OXIDIZED LDL FORMATION VIA 12/15 LIPOXYGENASE PATHWAY

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ABSTRACT
Hypercholesterolemia is one of the prominent risk factors for the development of cardiovascular disease (CVD) and when conjugated with ageing, the progression of CVD has devastating complications. *Aegle marmelos (AM)* plants have been traditionally used for curing many human ailments. The present study was designed to investigate the hypocholesterolemic effect of ethanolic extract of AM in aged male Wistar rats when fed with high cholesterol diet (HCD) and prerogative modulation of ROS mediated inflammation and its signalling leading to pro-atherogenic events. The lipid profile along with lipid peroxidation and the activity of antioxidant enzymes i.e., superoxide dismutase, glutathione peroxidase and catalase were measured in all the experimental groups. We have also assessed the levels of 12/15-Lipoxygenase and C - reactive protein which act as biomarker in the development of further complications. The findings of the study reveal that the ethanolic extract of *Aegle marmelos* leaves can effectively control initial events of atherosclerosis by restoring the high cholesterol induced impaired redox status and further ameliorate the Reactive Oxygen Species(ROS) mediated inflammation to near normal levels suggesting it to be a potential candidate as a hypocholesterolemic agent.

KEYWORDS: Aegle marmelos; Hypercholesterolemia; 12/15 Lipoxygenase, Cardio vascular disease (CVD).

INTRODUCTION
Atherosclerosis is characterized by excessive deposits of oxidized lipids, inflammatory cells, and calcium in the vessel wall (Hansson and Goran, 2005). Although the list of the causative factors of atherosclerosis keeps growing (Fruchart *et al.*, 2004), the widely accepted risk factors for this disease are hypercholesterolemia, hyperglycaemia, dyslipidaemia, diabetes, obesity, and smoking (Libby *et al.*, 2011). Increasing evidences indicate that aging is also an important risk factor for atherosclerosis and persists as an independent contributor when all other known factors are controlled. Premature or accelerated vascular aging can be promoted by cardiovascular risk factors, (Niemann *et al.*, 2011, Farhat *et al.*, 2008) and cellular senescence is also observed in patients with atherosclerosis (Bolton *et al.*, 2011). Atherosclerosis is therefore a disease of both cellular and organismal aging. However, the animal models for testing the hypocholesterolemic effect of the drug excludes the impact of ageing are limited. Hence in the present study aged rats, were fed with high cholesterol diet and the anti-atherosclerotic efficacy of AM extract was tested.

The role of oxidized LDL (oxLDL) in atherosclerosis is indisputable, and the appearance of oxLDL in the vessel wall is the hallmark of the development of atherosclerosis (Pourcet *et al.*, 2016). 12/15-LO was shown to oxidize low-density lipoprotein (LDL) directly in a cell-free system (Cathcart *et al., 1991).* Based on the ability of 12/15-LO to oxidize LDL, many studies have proposed that 12/15-LO plays a major role in the development of atherosclerosis *(*Cyrus *et al*., 2001; Yla-Herttuala *et al*, 1995). Forced expression of 15-LO in cultured fibroblasts and rabbit arteries also resulted in increased oxidation of LDL compared with their controls (Sigari *et al., 1997 ;* Yla-Herttuala *et al*, 1995). It is becoming more evident that ROS mediated inflammation plays an important role in the metabolic consequences of Hypercholesterolemia mediated Atherosclerotic complication, as well as other chronic degenerative conditions.

Aegle marmelos, known as bael in India, a plant of Rutaceae family, is one of the important plants in the Ayurveda. All parts of the plant such as leaves, roots, seed, bark and fruit possess different activities (Maity *et al*., 2009). Various studies in the recent past with

ethanolic extract of leaves, was reported to possess several biological properties that include lipid lowering activity in hyperlipidemic albino Wistar rats, (Vijaya *et al*., 2009) antibacterial and anti-proliferative activities, (Prema, 2004; Premchanien *et al*., 2004) dose dependent, reversible antifertility effect in male albino rats (Chauhan *et al*., 2007) and analgesic, antipyretic and anti-inflammatory activities (Arul *et al*., 2005).

In light of proven antioxidant nature of leaves of *Aegle marmelos* and the definitive role of oxidative stress in the pathogenesis of hypercholesterolemia, the current work exploits the potential of this herb to alleviate hypercholesterolemia in an experimental animal model in treatment and management of hypercholesterolemia. The current study proposes alternative approaches for the treatment of hypercholesterolemia with special reference to metabolic consequences driven by the role of 12/15 Lipoxygenase.

MATERIALS AND METHODS

Drugs and Chemicals

Aegle marmelos powdered leaf extract was purchased from R.R. Herbs, Chennai, Tamilnadu. Authentication of plant material was done by Prof. P. Jayaraman of the plant anatomy Research Centre, Tambaram, Chennai. All chemicals used were obtained from Sigma –Aldrich USA, Merck Chemical Supplies (Darmstadt, Germany), Sisco Research Laboratories (SRL, India) and fine chemicals, Mumbai, India. Diets were procured from Hindustan Lever Ltd., under commercial name "GOLD MOHUR RAT FEED" containing 5% fat, 21% protein, 55% nitrogen free extract, 4% fibre (w/w) with adequate vitamins and minerals.

Experimental animals and grouping

Healthy male albino rats of Wistar strain (n=30; weight, 130-250g), 12 weeks old (Young rats) and 120 weeks old (Aged rats) procured from Animal House Facility Taramani Campus University of Madras were housed in polypropylene cages, maintained under standardized condition (12 h light/dark cycles, 28±2°) with paddy husk bedding at the central animal house, and had access to food and water ad libitum. All experiments were approved by the Institutional Animal Ethical Committee of The University of Madras, Taramani Campus (IAEC No #01/19/13).

Experimental Design: Hypercholesterolemia was induced by a high – cholesterol diet (HCD) comprising of the normal rat feed supplemented with 4% cholesterol and 1% cholic acid (Kumaran *et al*., 2009) and *A marmelos* (200 mg /kg b.w) was given orally to the experimental rats (Vijaya *et al.*, 2009). The experimental animals were grouped as follows: i) Control group (n=6), in which the Young rats (12 weeks) were fed a standard diet for 45 days; ii) Aged control group (n=6), in which the Aged rats were fed a standard diet throughout the experimental period iii)Aged rats, high cholesterol group (HC, n=6), in which the animals were fed with HCD for 30 days, followed by standard diet for 15 days; iv) Aged rats, treatment group (HCD+AM extract 200mg/kg b.w (Vijaya *et al*., 2009), n=6), in which the animals were fed with HCD diet for 30 days, followed by standard diet with oral administration of AM extract for 15 days; v) Aged rats, Drug control group (AM extract alone 200mg/kg b.w, n=6), in which the animals were fed with standard diet for 45 days with oral administration of AM extract for last 30 days.

Sample preparation

At the end of the experimental period, rats were anesthetized with ketamine (22 mg kg−1, i/m) and blood samples were collected via tail vein into anticoagulant-containing and anticoagulant-free test tubes. Heart was excised immediately, immersed in ice-cold physiological saline, and weighed. Small sections from each tissue were kept aside for histological studies. A 10% tissue homogenate was prepared using Tris-HCl buffer (0.01 M, pH 7.4), followed by centrifugation at 12000 rpm for 10 min. The supernatant was used for the analysis of various parameters. The rest of the tissue was stored at −80 °C for gene and protein expression studies. The protein concentration of the tissue homogenate was determined by the standard method of Lowry et al. (1951) using bovine serum albumin as standard.

Evaluation of serum lipid profile

Total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL) in serum were analysed using commercial kits from spin react in semi-auto analyser (RZ Monza, Randox, UK). The values of total cholesterol, TG and HDL are expressed as mg/dl.

Determination of lipid peroxidation (LPO)

LPO was evaluated in the tissue homogenate. The concentration of malondialdehyde (MDA) was assayed in the form of thiobarbituric acid-reacting substances (TBARS) with the method described by Ohkawa *et al.*, (1979).

Enzymatic antioxidant activity

The activity of enzymes in the antioxidant system was evaluated in the tissue homogenate following previously reported methods. Catalase (CAT) activity was determined using the method of Sinha, (1972) and expressed as Units/ mg protein (µmol of H_2O_2 consumed/ min/ mg protein). Superoxide dismutase (SOD) activity was determined using the method of (Marklund and Marklund, 1974) and expressed as U/mg protein. Glutathione peroxidase (GPx) was determined as described by Rotruck *et al*. (1973) and expressed in terms of µg of reduced glutathione (GSH) consumed/min/mg protein. The enzyme activity was expressed as nmol of 1-chloro-2, 4-dinitrobenzene formed/min/mg protein.

Non-enzymatic antioxidant levels

The levels of non-enzymatic antioxidants in cardiac tissue homogenate samples were determined by

following previously reported methods. The GSH content was estimated by the method of Moron *et al.*, (1979). Ascorbate (vitamin C) was measured using the method of Omaye *et al*., (1979). α -tocopherol (vitamin E) was estimated by the method of Desai *et al*., (1984). The results of all the experiments are expressed as µg of α-Tocopherol /mg protein.

Gene expression studies

Gene expression studies by reverse transcription polymerase chain reaction (RT-PCR).Total RNA was isolated from the heart tissue using total RNA isolation reagent (TRIZOL, Invitrogen, Carlsbad, CA, USA). Oligonucleotide primer sequences of the selected genes for reverse transcription-polymerase chain reaction (RT-PCR) were synthesized by Sigma-Aldrich (St Louis, MO, USA) and Eurofins Genomics (India). The amplified products were separated by electrophoresis on 2% agarose gel and identified by ethidium bromide staining. Specificity was confirmed by the size of the amplified products with reference to a 100 bp DNA ladder (Bio vision, USA) and the band intensities were quantified by Quantity One Software (Bio-Rad, USA).

Immunohistochemistry

Immunostaining was done by the method of Khundakar et al. (2011). In brief, paraffin-embedded heart sections were deparaffinized in xylene, then rehydrated through a graded alcohol series. Antigen retrieval was performed by incubating slides in citrate buffer (pH 6.0) (10 mM) at 95 ◦C for 20 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min. To detect 12/15 Lipoxygenase immunoreactivity, sections were incubated under humid conditions overnight at 4°C with the anti-12/15 Lipoxygenase antibody. Next day, the slides were washed three times in PBS and were incubated with biotinylated donkey anti-goat secondary antibody (1:200; BioRad, USA) for 1 hour at room temperature. This step was followed by further wash in PBS and incubation of slides at room temperature with a Streptavidin Peroxidase Plus (Thermo Fisher Scientific, USA) that binds to the biotin present on the secondary antibody. After washing in PBS, the immunostaining reaction product was developed using 3, 3'-diaminobenzidine (Sigma Chemicals, St. Louis, MO, USA). After immunoreactivity, slides were dipped in distilled water, counterstained with Harris hematoxylin and finally the sections were dehydrated in xylene, mounted with DPX and cover slipped. Slides prepared for each case were examined by light microscopy.

Enzyme Linked Immunosorbent Assay (ELISA)

One of the very important markers of cardiac tissue damage is the assessment in the levels of C Reactive Protein (CRP). The levels of CRP were measured using ELISA as described by Hardie *et al.* (1996). High affinity 96 well microtitre plates were coated with 50 µl/well 0.05 M carbonate/bicarbonate buffer (pH 9.6) containing 20µg of protein sample, incubated overnight at 4°C. Plates were washed thrice with 0.15 M phosphate buffered saline (pH 7.4) and then blocked for 1hour at room temperature with 200 µl/well PBS containing 1% BSA. After 2 hours of incubation at room temperature, plates were washed twice before the addition of primary antibody 100µl per well (Rabbit anti-CRP) and incubated overnight at 4ºC, then washed four times with PBS. Thereafter, 100µl of horse radish peroxidase conjugated secondary antibody diluted 1:10000 in blocking buffer were added and incubated for 1 hour at room temperature. After further washing, and a final rinse with PBS, immune reactivity was visualized by the addition of 50µl/well 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate for 15 min at room temperature. The reaction was stopped by the addition of 50µl/well 2M H_2SO_4, optical densities were determined at 450 nm using a plate ELISA reader (BioTek, USA). The serum CRP levels are expressed as mg/dl.

Western blot analysis

The tissue homogenate prepared 50-100 ml of lysis buffer (with protease inhibitors), centrifuged and the supernatant was collected. Total tissue extracts containing 50-100 µg of protein were prepared in SDS sample buffer (Sigma-Aldrich) were separated by SDS-electrophoresis on 10%–12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes prior to immunodetection and subjected to western blot analysis. The antibodies against Nuclear Factor Kappa B (NF-Kb), Low Density Lipoprotein Receptor, Anti-(LDL-R), Inter Leukin 6 (IL6) and 12/15-Lipoxygenase (12/15-LO) was purchased from Santa Cruz Biotechnology, and was used to detect protein levels in the heart tissues. To verify the uniformity of protein load and transfer efficiency across the test samples, membranes were reprobed with Beta Actin (β-actin) (Cell Signaling Technology). Immuno-reactive bands were developed with Immobilon Western-Chemiluminescent HRP substrate (Millipore Corporation, Billerica, USA), visualized using an enhanced chemiluminescence system (Chemi-Doc, BioRad, USA) and presented in comparison to β-actin expression.

Statistical analysis

Statistical analysis was performed using SPSS 21 package. Values represent Mean ±SEM for six rats in each group and the significance of difference between mean values were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

RESULTS

AM extract improves serum lipid profile: An increased levels of serum TC, TG, LDL and VLDL were observed in aged rats when compared to Young Control group. Aged HCD fed rats exhibited a significant ($P<0.05$) increase in the serum TC, TG, LDL and VLDL levels and the cardiac risk ratio, when compared to Young control and the aged control group. However, HCD fed aged rats treated with AM extract exhibited a significant

(P<0.05) improvement in their serum lipid profiles to near-normal levels. AM extract treated aged group rats exhibited a well-maintained lipid profile compared to that of aged control group of rats (Table I).

AM extract prevents LPO in the cardiac tissue: LPO was determined by the mean concentration of MDA assayed in the form of TBARS. The cardiac tissue and haemolysate samples from Aged Control rats showed increase in the levels of MDA as compared to Young control group; whereas HC diet fed rats exhibited a significant (P<0.05) increase in the levels of MDA compared to those from the Aged control (Fig 1a). Contradictorily, in the HC+AM extract group, LPO was significantly (P<0.05) inhibited in the cardiac and haemolysate samples. Similarly, the AM extract alone treated group exhibited notably improved protection against LPO compared to the control rats.

AM extract improves enzymatic antioxidant activity and non-enzymatic antioxidants levels: It showed a decrease in the levels of these antioxidants in the Aged control group of rats in comparison to Young control group of rats and a significant (P<0.05) decrease in the mean activity of the enzymatic antioxidants CAT, SOD, and GPx was observed in cardiac tissue (Fig. 2) samples of HC diet fed rats when compared to control rats, whereas HC+AM Extract treated rats exhibited improved antioxidant activities. Similarly, there were decrease in the levels of these non‑enzymatic antioxidants GSH, ascorbate and α-tocopherol in aged control group of rats in comparison to young control group of rats as the mean levels of these non‑enzymatic antioxidants in the cardiac tissues of HC diet fed rats showed a significant decrease when compared with the aged control (P<0.05), whereas the mean concentration of ascorbate in the cardiac tissues of HC+AM extract rats was significantly (P<0.05) increased to near-normal levels. However, no significant difference was observed in the mean levels of GSH and α-tocopherol in the cardiac tissue samples of AM only extract treated rats as compared to young control group of rats establishing the anti-aging role of AM extract in reversing age induced deterioration of antioxidant status.

AM extract regulates lipid metabolism: We detected the key expression profiles of genes and proteins involved in lipid metabolism using PCR, western blot analysis and Enzyme Linked Immunosorbent Assay (ELISA) respectively. Notably, ageing alone demonstrated a higher expression profile in comparison to Young control group; Whereas HC+AM extract treated rats exhibited significantly decreased protein levels of CRP (Fig 1b), NF-κB (Fig 3); IL6 (Fig. 3); and 12/15-LO (Fig. 4); in cardiac tissue samples; however, HC diet fed rats exhibited a reverse effect in the expression pattern to treated and control groups and not much difference was observed in positive control (AM extract alone). These results may constitute key evidence for the elucidation of the mechanism of action of AM extract against lipid deposition in cardiac tissues.

AM extract improves expression of 12/15 Lipoxygenase: The expression of 12/15 Lipoxygenase in experimental rat heart tissues by immunohistochemistry is shown in (Fig. 5). The hypercholesterolemic rats are positively stained and it depicts as positive expression for 12/15 Lipoxygenase (G3). No observed stained cells for 12/15 Lipoxygenase were found in young control rats (G1), whereas, mild positivity of 12/15 Lipoxygenase expression was seen in normal diet fed aged rats (G2). The results also revealed that the number of 12/15 Lipoxygenase positive stained cells in the heart of HCD fed aged rats (G3) were markedly higher than aged control group (G2). In contrast, AM extract treatment decreased the number of positive stained cells remarkably in HCD fed aged rats (G4), when compared with HCD alone fed rats (G3). Aged rats upon supplementation with AM extract (G5) showed lesser expression to Aged control (G2) also depicting the role of AM extract in attenuating the effect of Aging.

Table 1

Groups	Group1	Group2	Group3	Group4	Group5
Total Cholesterol	**95.43±8.7**	**106.32±9.1[a]**	**148.56±11.1[b]**	**116.34±9.7[c]**	**99.85±8.7[b]**
Triglyceride	**65.41±5.7**	**69.32±6.3[a]**	**110.58±8.7[b]**	**89.21±6.7[c]**	**75.42±6.3[b]**
HDL	**27.01±2.3**	**21.41±2.5[a]**	**12.63±1.3[b]**	**18.87±2.0[c]**	**21.31±1.5[b]**
LDL	**55.33±5.2**	**77.47±5.5[a]**	**113.80±9.2[b]**	**79.62±6.5[c]**	**63.45±5.2[b]**
VLDL	**13.08±1.2**	**13.86±1.1[a]**	**22.11±1.9[b]**	**17.84±1.5[c]**	**15.08±1.3[b]**

Figure captions and legends

Table I. Administration of A marmelos extract improves serum lipid profile.

Values are expressed as (mg/dl) each value represents mean ± SEM for six rats in each group. Values are statistically significant at the level of p< 0.05, where 'a' - compared with Group I, 'b' –compared with Group II, 'c' – compared with Group III; Group I- Young control; Group II- Aged control; Group III- Aged HCD; Group IV- Aged HCD +A marmelos; Group V- Aged +A marmelos alone.

Figure 1(i). ***A marmelos extract prevents lipid peroxidation (LPO)***

LPO was determined by the mean concentration of malondialdehyde assayed in the form of thiobarbituric acid-reacting substances and is measured in the heart tissue samples of the experimental groups. Each value

represents mean ± SEM for six rats in each group. Values are statistically significant at the level of p< 0.05, where 'a' - compared with Group I, 'b' –compared with Group II, 'c' – compared with Group III; Group I- Young control; Group II- Aged control; Group III- Aged HCD; Group IV- Aged HCD +A marmelos; Group V- Aged +A marmelos alone.

Fig 1(ii). ***Effect of Aegle marmelos on inflammatory marker expression by ELISA***

Each value represents mean ± SEM for six rats in each group. Values are statistically significant at the level of p< 0.05, where 'a' - compared with Group I, 'b' – compared with Group II, 'c' – compared with Group III; Group I- Young control; Group II- Aged control; Group III- Aged HCD; Group IV- Aged HCD +A marmelos; Group V- Aged +A marmelos alone.

Fig 2. ***A marmelos extract is a potent antioxidant.***

Units - SOD- is expressed in units/mg protein where 1 unit is equal to the amount of enzyme required to prevent 50% of auto oxidation of pyrogallol/min/mg protein. CAT- µmoles of H_2O_2 consumed/min/mg protein. GPx-µ moles of GSH oxidized/min/mg protein. GSH- nmoles/mg protein. Vitamin C- µg of ascorbate /mg protein. Vitamin E- µg of α-Tocopherol /mg protein - Values are statistically significant at the level of p< 0.05, where 'a' - compared with Group I, 'b' –compared with Group II, 'c' – compared with Group III; Group I- Young control; Group II- Aged Control; Group III- Aged HCD Group IV- Aged HCD + Aegle marmelos; Group V- Aged + A marmelos alone.

Fig (3). Effect of Aegle marmelos leaf extract on mRNA and protein expression of NF KB, IL6 and 12/15LO in the heart of HCD fed experimental rats.

Each value represents mean ± SEM for six rats in each group. Values are statistically significant at the level of p< 0.05, where 'a' - compared with Group I, 'b' – compared with Group II, 'c' – compared with Group III; Group I- Young control; Group II- Aged control; Group III- Aged HCD; Group IV- Aged HCD +A marmelos; Group V- Aged +A marmelos alone.

Fig (4). ***Effect of Aegle marmelos leaf extract on mRNA and protein expression of 12/15LO in the Heart of HCD fed experimental rats***

Each value represents mean ± SEM for six rats in each group. Values are statistically significant at the level of p< 0.05, where 'a' - compared with Group I, 'b' – compared with Group II, 'c' – compared with Group III; Group I- Young control; Group II- Aged control; Group III- Aged HCD; Group IV- Aged HCD +A marmelos; Group V- Aged +A marmelos alone.

Fig (4).*Effect of Aegle marmelos leaf extract on protein expression of LDL-R in the Heart of HCD fed experimental rats*

Each value represents mean ± SEM for six rats in each group. Values are statistically significant at the level of p< 0.05, where 'a' - compared with Group I, 'b' – compared with Group II, 'c' – compared with Group III; Group I- Young control; Group II- Aged control; Group III- Aged HCD; Group IV- Aged HCD +A marmelos; Group V- Aged +A marmelos alone.

Fig (5). ***Immuno-histological photomicrographs of 12/15Lipoxygenase stained sections of Heart Magnification (20X)***

Immuno histological analysis of Lipoxygenase expression in the rat myocardium. (G1) Normal appearance of myocardial tissue in control rats; (G2) Aged healthy myocardium from positive control rats with expression of Lipoxygenase; (G3) Relatively significant expression of 12/15 Lipoxygenase in high-cholesterol (HC)-fed aged rats; and (G4) HC+ Aegle maremlos treatment group; (G5) Aged healthy myocardium with Aegle maremlos treatment alone with no expression at all; Aegle maremlos prevented HC-induced damage to the myocardium. Scale bar, 50 µm.

Figure 1 a.

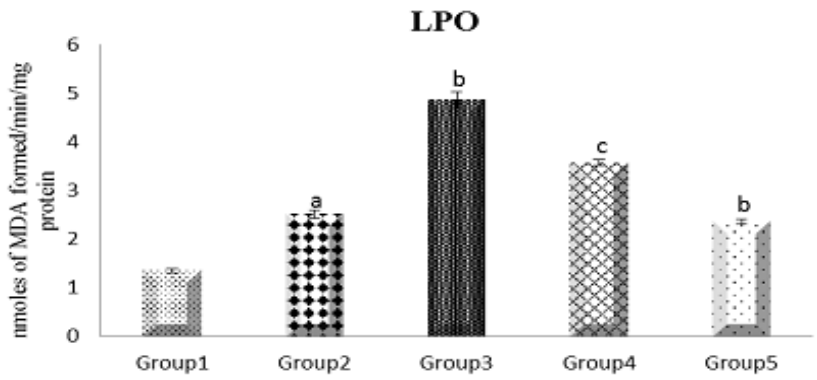


Figure 1 b.

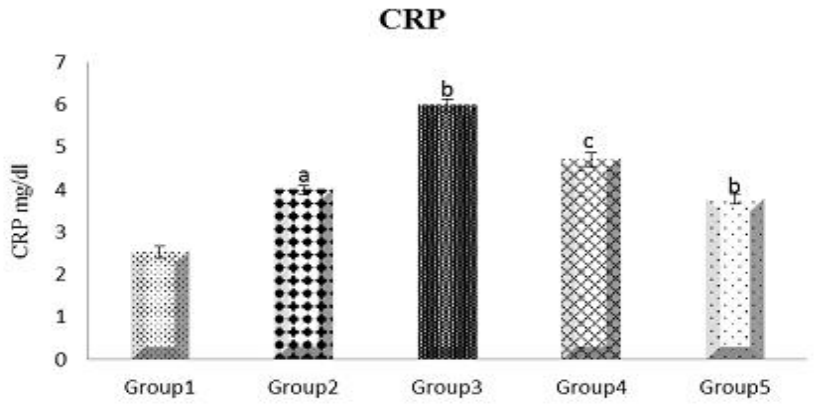


Figure 2

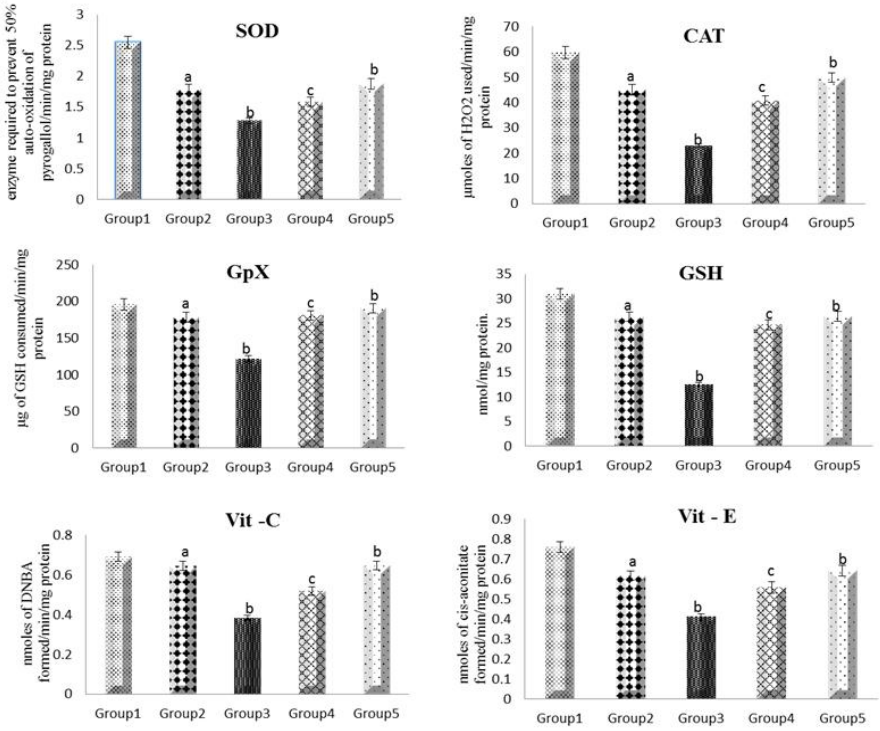


Figure 3

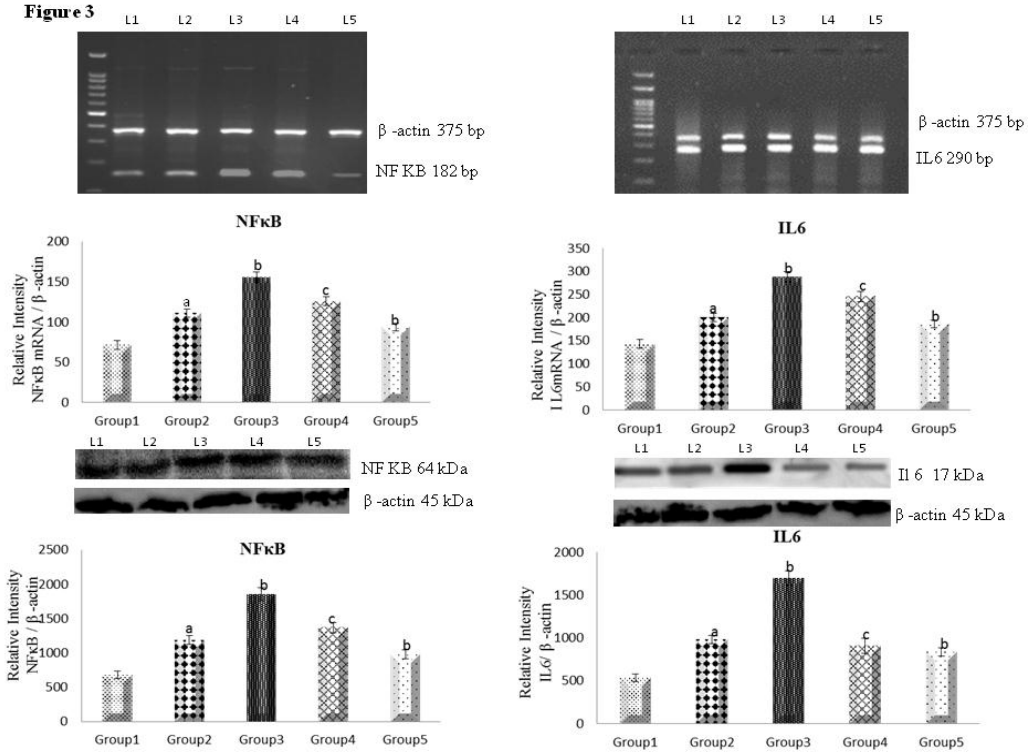


Figure 4

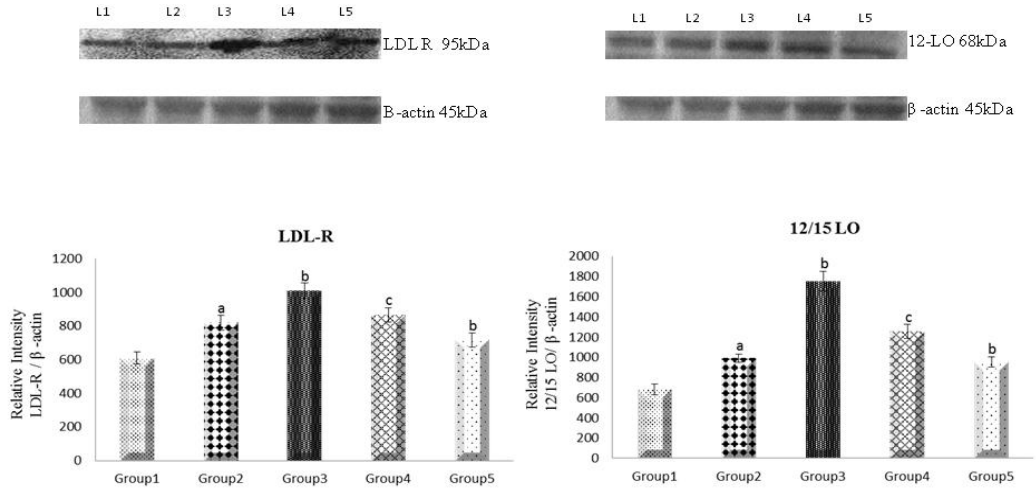
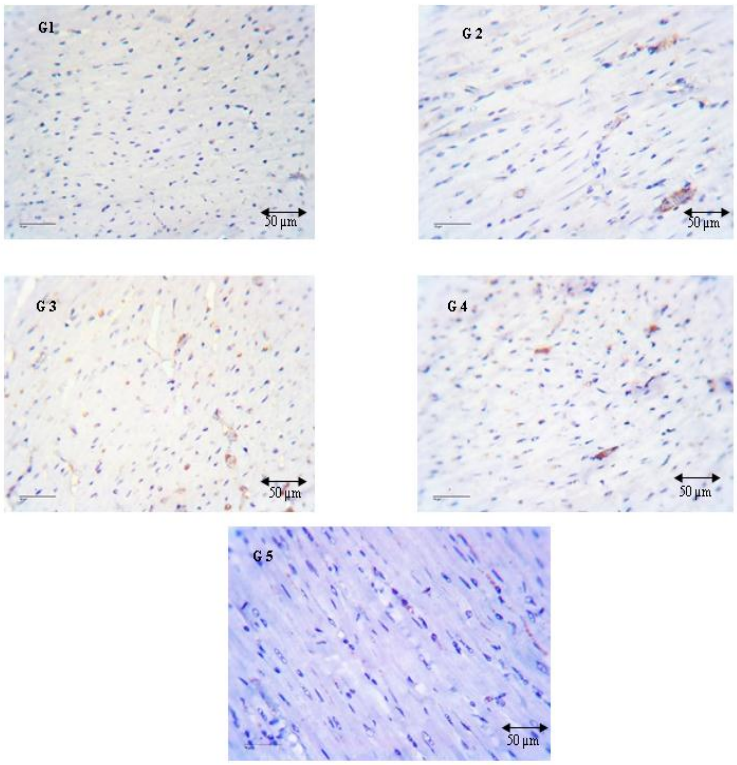
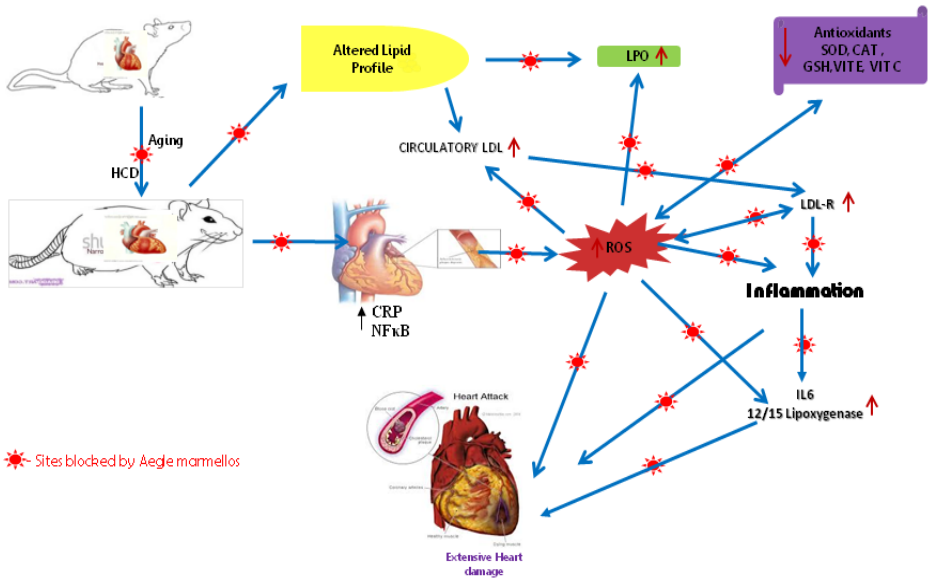


Figure 5



SCHEME OF STUDY



DISCUSSION

In this study, we investigated the potential of *Aegle marmelos* leaf extract as a therapeutic agent for hypercholesterolemia and retarding the early events in atherosclerotic plaque progression. Our study demonstrated that rats fed with HC diet exhibited increased lipid levels in the serum. Lipoproteins are the vehicle for transporting plasma lipids to the blood. (Yu *et al., 2005*) reported that serum TC and TG are increased significantly in rabbits receiving a high-fat and cholesterol diet, but decreased in rabbits receiving the same diet supplemented with ellagic acid. Similarly in the current study on treatment with AM extract the cholesterol levels were controlled substantiating AM leaf extract to be a better hypocholesterolemic agent. Methanolic extract of bark of *Aegle marmelos* and ethanolic extracts of *Aegle marmelos* leaves have been exploited for their hypocholesterolemic effect in animal models (Agarwal *et al*., 2012; Vijaya *et al*., 2009).

Oxidative stress is known to play a role in the etiology of a spectrum of diseases and it has been demonstrated that hypercholesterolemia increases the overproduction of free radicals due to increased mitochondrial respiration and lowered antioxidant status (Thiruchenduran *et al.*, 2011). Our results demonstrated an increased LPO as MDA levels, thereby confirming the induction of free radical production in HC diet fed rats. Our results are in accordance with those of previous studies reporting that increased LPO was associated with the heart tissues, aorta and serum of hypercholesterolemic rats (Hussein *et al*., 2016; Abduallah *et al*., 1998). Salvayre *et al*. (2006), have stated that oxidative stress is an early event in the evolution of hyperlipidaemia and appropriate support for enhancing anti-oxidant supply in hyperlipidemic subjects may prevent the course of the disease. Treatment with AM leaf extract on effectively prevented the HC induced LPO and a similar hypocholesterolemic activity of AM leaf extract had already been reported by Vijaya *et al. (2009).* In this regard, AM leaf extract may be beneficial in mitigating the progression of atherosclerosis.

Notably oxidative stress is characterized not only by increased free radical production, but also by reduced enzymatic activities (Salvayre *et al.,* 2006). The antioxidant enzymes CAT, SOD and GPx are involved in free radical scavenging, disposal of superoxide anions and hydrogen peroxide. These activities constitute the first line of cellular defence against oxidative injury. CAT specifically enables disposal of H2O2 by the erythrocyte, thereby protecting against ROS (Agar *et al*., 1986). Our results demonstrated a significant decrease in the mean activities of CAT, SOD, GPx, GSH, Vit C and Vit E in the cardiac tissues of HC rats. The observed antioxidant boosting potential of the *Aegle marmelos* leaf extract might be attributed to its radical scavenging activity and the anti-adipogenic activity by its multi-bioactive classes of compounds including halfordinol, ethyl ether, Aegeline and esculetin that have been reported to have anti-adipogenic activity (Karmase *et al*., 2013a; Karmase *et al.,* 2013b). Moreover Bhatti *et al*. (2013) have shown that *Aegle marmelos* leaf extract has ameliorated the alloxan induced oxidative stress during diabetes mellitus and attenuated cardiomyopathy in rats. It was demonstrated that an improvement in the function of the antioxidant system following administration of lupeol and lupeol linoleate in hypercholesterolemic rats (Sudhahar *et al*., 2006) and the presence of these compounds along with Aegeline in the leaf extract of Aegle marmelos would have been responsible for the hypocholesterolemic effect. Cells can withstand against chronic oxidative stress by boosting the activities of the antioxidant enzymes. The results of the present study also demonstrate that *Aegle marmelos* leaf extract possibly acts by regulating the activities of these antioxidant enzymes.

It has been demonstrated that increased intracellular generation of ROS plays an important role in chronic inflammatory responses to atherosclerosis. Endothelial cells exposed to accelerated ROS and disturbed flow, expresses the pro-inflammatory signals which appears to emanate from integrins, including activation of NF-κB. The transcription of NF-κB dependent genes influences the levels of ROS in the cell and in turn the levels of NF-κB activity are also regulated by the levels of ROS. ROS interacts with NF-κB at various places within the signalling pathway. Active NF-κB induces transcription of cell adhesion molecules as well as several chemokines and cytokines including Interleukin 6(IL-6), an important pro inflammatory cytokine. IL-6 has been shown to enhance fatty lesion development in mice (Huber *et al.*, 1999). Given the generally pro-inflammatory gene targets of NF-κB, it is not surprising that many elements of upstream signalling leading to NF-κB activation have been found to be atherogenic and targeting these will give a potential therapeutic outcome in this study. AM extract has succesfully down regulated the expression of these important mediators of Cardiac damage targeting these inflammatory cytokines expression. Among the numerous inflammatory biomarkers, the largest amount of published data supports a role for C-reactive protein (CRP) as a robust and independent risk marker in the prediction of primary and secondary adverse cardiovascular events (Li *et al.,* 2015). In addition to being a risk marker, there is much evidence indicating that CRP may indeed participate in atherogenesis (Li *et al.,* 2015). CRP has been found co-localized with LDL and macrophages in atherosclerotic lesions in humans and experimental animal models (Bhakdi *et al*., 1999; Sun *et al*., 2005; Meuwissen *et al*., 2006; Torzewski *et al*., 2000). CRP is shown to up regulate interleukin-6 and other interleukins in human aortic endothelial cells via nuclear factor-κB. Also, the classic dogma that CRP is produced exclusively in liver is challenged by the data on the extra hepatic production of CRP in different cells including atherosclerotic lesions (Griselli *et al*., 1999). Aging itself is characterized by a chronic low grade inflammatory status, the so called inflame-ageing appears to be associated with age related diseases like

atherosclerosis (Sikora *et al.*, 2010). Therefore the observed increased levels of CRP in aged HCD fed rats might be due to the combinational stress induced by ageing and HCD. AM leaf extract on treatment effectively prevented the age and HC-induced elevation in CRP levels in treatment groups of rats which is in concordance with the findings of Dhanalakshmi *et al*., 2014. During uncontrolled LDL productions as in high cholesterol diet intake leads to higher exertion on LDL receptor and so as its expressions in hypercholesterolemic condition and our studies are concomitant with Takeshi et al., 1999, who have demonstrated a decrease incidence of atherosclerosis in heterozygous LDL receptor deficient mice. Our present study demonstrated that AM extract downregulated the expression of LDL receptor, IL 6, NF-κB and 12/15-.LO. NF-κB is a pleiotropic transcription factor that control immunity and inflammation, is activated by many factors. The role of lipoxygenases in activating NF-κB cannot be undermined as suppression of NF-κB activation and NF-κB dependent gene expression by 5-lipoxygenase inhibitor (Kazmi *et al*., 1995). Further increased expression of 15-Lipoxygenase is found in early fatty streak lesions which up regulates the NF-κB mediated cell adhesion molecules (Dwarkanath *et al.*, 2008) and 12/15 LO to mice did not show infiltrates of macrophages when fed with HCD (Sears *et al*.,2009). 12/15 lipoxygenase is a non-heme iron dioxygenase critical in generating HPETE (Hydro peroxy eicosa tetraenoic acid). Pharmacological inhibition of this enzyme in hypercholesterolemic rabbits has led to significant attenuation of atherosclerosis. And over expression of 12-LO in PC 3 Prostrate cancer cells resulted in constitutive activation of NF-κB via its metabolite 12-HETE (Kandouz *et al.*, 2003). Hence, AM extract is shown to ameliorate LPO by inhibiting 12/15 lipoxygenase and NF-κB. The results of the present study demonstrated the advantages of the administration of AM extract for the prevention of cardiac abnormalities induced by HC diet. The cardio protective effect of AM extract was demonstrated by the improvements in the serum lipid profile, antioxidant system, lipid metabolism. These preliminary findings support the AM extract as a potent anti-hypercholesterolemic, anti-inflammatory and a powerful source of antioxidant and cardio protective agent.

CONCLUSION

In conclusion, high cholesterol diet in combination with ageing increases oxidative stress in the heart, as evidenced by the levels of macromolecular damage by increase in expression profile of 12/15 Lipoxygenase and a decrease in the antioxidant status. This in turn causes loss of cardiomyocyte integrity and supplementation of *Aegle maremlos* leaf extract significantly augments the antioxidant status by scavenging the macromolecular damage and thereby culminates the HCD induced oxidative stress during aging. AM extract also prevents inflammation by maintaining the levels of inflammatory cytokines. Thus, this preliminary study shows that the anti-inflammatory activity of *Aegle marmelos* leaf extract, which might be due to the presence of aegeline and other phytoconstituents, can be exploited to alleviate cardiomyocytes damage induced by CVD. However, further investigation of the extract will give more insights into the molecular mechanisms involved.

ABBREVIATIONS

AM Aegle Marmelos leaf extract
CVD Cardiovascular disease
HCD High Cholesterol Diet
LO Lipoxygenase
ROS Reactive oxygen species
HETE Hydroxyeicosatetraenoic acids
LDL Low density lipoprotein
Oxd LDL Oxidized low density lipoprotein

Conflict of interest

The authors declare that they have no potential conflict of interest, including any financial, personal or other relationships, with other people or organizations.

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