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Clearing the Clutter: Antiatherosclerotic activity of *Eucalyptus camaldulensis* crude extract

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Abstract: Plant components have been extensively evaluated for their pharmacological activities. This study provides scientific rationale towards the therapeutic effect of *Eucalyptus camaldulensis* aqueous bark extract against induced atherosclerosis and hyperlipidemia in pigeons. Phytochemical components of Eucalyptus bark extract possess a great antioxidant activity that potentially reduced the risk of heart diseases. A total of 42 Pigeons of both sexes were distributed into negative control (fed normal grain diet), hyperlipidemic control (fed HFD 1% animal fat oil and 0.1% cholesterol for 3 months), test groups of variable doses (0.05, 0.1, 0.2 to 0.4 gms/kg BW for 21 days) and the group received atorvastatin daily after induction used. At the end of the experiment biochemical and histological evaluation has been performed. After HFD induction the serum levels of liver enzyme AST, glucose, urea, cholesterol, LDL, VLDL, and TG were significantly increased with the reduction in HDL levels. The atherogenic index was also found significantly raised. Microscopic examination of the liver and aorta showed the appearance of lipid-filled foam cells all over the liver parenchyma and intima after the HFD induction. Thus it was concluded that Eucalyptus aqueous bark extract can be effective against atherosclerosis and hyperlipidemia.

Keywords: Atherosclerosis, high-fat diet (HFD), *Eucalyptus camaldulensis.*

INTRODUCTION

Ischemic heart disease becomes the most lethal cause of death nowadays, according to WHO around 8.5 million of world by 203 wide deaths were reported in 2012 which will estimate to be above 52 million killings of individuals 2030. Atherosclerosis is defined as arterial illness due to deposition of lipid, permeation of blood cells and increment of VSMCs. Lipid deposition in initima (Glass and Witztum, 2001); smooth muscles (Libby, 2021; Luscher and Barton, 1997) and macrophages aggregation cause lesion production (Orford *et al.,* 2000, Tedgui and Mallat, 2001). The atherosclerotic process is triggered through a buildup of intimal lipoproteins activation of the endothelium (Ross, 1999; Kuiper *et al.,* 2007), results in further employment of leucocytes and growth factors (Ross, 1999; Kuiper *et al.,* 2007). Hypertension, diabetes mellitus and smoking are the risk issues involve in the manifestation of adhesion molecules (Libby, 2021), results in nitric oxide production which regulates endothelial activity (Tedgui and Mallat, 2001). The migration and recruitment of VSMCs are mainly involved in the pathogenesis of atherosclerosis (Marais, 2021). Thus these cells not only have structural and functional roles (maintain vascular tone) but are also involved in the evolution of atherosclerosis and hypertension (Orford *et al.,* 2000). The deposition of lipid filled lipoprotein and necrotic cells are the primary characteristic feature of fatty streaking in injured cells (Koo *et al.,* 2018). Upon entering into the intima, monocyte derived macrophages lead to oxidized LDL cholesterol-filled foam cells production (Ross, 1999).

There have been extensive studies provided the proves of use of different animal models selections for the explorations of atherosclerosis progression and treatment strategies, which may be from the use of avian to primates and rodents to hamsters (Veseli, 2017). Avian models include pigeon, chicken, Japanese quail, turkey and parrots. The atherosclerotic model of the pigeon was extensively reviewed (Clair, 1983). The atherosclerotic pigeon models have various similarities with human atherosclerosis that may conclude as following:

1. Pigeons are naturally hypercholesterolemic
2. They are predominantly HDL-C carrier but HFD administration results in high levels of LDL and VLDL.
3. The atherosclerosis underline processes are similar to human
4. Apolipoprotein E, Apolipoprotein B48 and chylomicron vesicles are not evident in pigeon atherosclerosis
5. They are a good example of both natural and induced atherosclerosis (Moghadasian *et al.,* McManus, 2001).

The atherosclerosis pathophysiology of the pigeons was found to have resembled other animal models as well as humans. The very primary feature developed in induced

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atherosclerosis pigeons was the evident development of macrophage foam calls (Jerome and Lewis, 1985; Taylor and Lewis, 1986). LDL/VLDL receptor and scavenger receptors are expressed on the macrophages for the lipoproteins uptake (Henson *et al.,* 1989; Adelman and Clair, 1988).

The strategic therapy or prevention trials for atherosclerosis involve effective regression of plaques and cholesterol levels (Levine *et al.,* 1995). Nowadays plants have been extensively reviewed for their medicinal properties (Wiley, 1990). Although the toxic potentials of many plants have unexplored, phytochemicals that are derived from the plant contain a variety of medicinal properties for mankind against many diseases including IHD/CH (Dorcas, 2015). *Eucalyptus camaldulensis* or the River Red Gum is one of the 800 genera of Eucalyptus plants. It has worldwide plantation but mainly found in Australia.

The *Eucalyptus camaldulensis* phytochemical analysis shows that all the parts of the plant were rich in saponins and tannins, alkaloids, anthraquinone though cardiac glycosides are not present (Ghareeb *et al.,* 2018; Roger, 1999). *Eucalyptus* oil has various uses such as antifungal, antiseptic, sterilizing, aromatic and food seasoning (Shaighal *et al.,* 2012; Mehraban *et al.,* 2005; Essien and Akpan, 2004; Chao and Young, 1998). *Eucalyptus camaldulensis* possesses great variety of pharmacological properties that make this herb valuable for the research purpose therefore we selected eucalyptus aqueous extract for the investigation against atherosclerosis (Aleksic Sabo *et al., 2019;* Amabye *et al., 2016*).

MATERIALS AND METHODS

Columba livia (pigeon) of both sexes was used with an approximate bodyweight of 200-260 gms and housed in an animal house in the Physiology Department of the University of Karachi with free access to water and a regular grain diet. Before any pretest, all animals were familiarized to test environment for one week. The weight, age and physical changes in each pigeon were recorded and then divided into different groups for the experiment.

Eucalyptus camaldulensis selected and identified parts of air-dried bark used for extract preparation. 50 grams of dried bark added with 250 ml of water and left for 3 days in a flask covered with aluminum foil. The mixture was filtered and evaporated at 40^{o}C by using a rotary evaporator and refrigerated at 4^{o}C in the vials (Mann *et al.,* 2008).

The total of 42 pigeons of both sexes were equally divided into 7 groups (of 6 pigeons each) which were designated as A (negative control), B (positive control), C (extract treatment group) and D (Lipitor 10 mg daily) group. The pigeons in group A were gavaged 10 ml distilled water daily throughout the experiment while the pigeons in group B, C and D were induced hyperlipidemia with 0.1% cholesterol and animal rendered fat oil in a 1:10 ratio for 3 months along with normal grain diet. Group C was categorized into C1, C2, C3 and C4 according to dose regimen (0.05, 0.1, 0.2, 0.4 gms/kg of body weight), administered aqueous bark extract for three weeks.

After the completion of trial periods overnight fasting blood samples were drawn from the brachial artery/heart (Campbell and Dein, 1984) and serum was prepared for biochemical assays. The pigeons were sacrificed and tissue specimen (liver and aorta) was removed, fixed in 3.7% normal buffer formalin.

Assays of biochemical parameters
Plasma levels of TG and cholesterol were determined by utilizing commercial kits methods (Dialab, Wiener Neudorf, Austria). High-density lipoprotein concentration determined through precipitation of VLDL and low-density lipoprotein with magnesium chloride and phosphotungstic acid. VLDL and LDL levels were calculated by Friedewald equation given below

- LDL = Total cholesterol - (HDL + VLDL)
- VLDL = Triglycerides/5

Atherogenic index (AI) was calculated by the Friedwald formula too (Friedwald *et al.,* 1972)

- AI=HDL/TC

The plasma albumin, creatinine, Aspartate Aminotransferase, Alkaline phosphatase, and Alanine Aminotransferase were evaluated spectrophotometrically (NV-202) by enzymatic kits.

Histopathological Protocol
After fixation in formalin buffer solution liver and aorta were processed in pure paraffin wax for overnight. After fixation tissues were transferred to microtome sectioning of about 5um thickness and stained with hematoxylin and eosin (H&E) stain. The stained sections were then transferred to slide while covered with coverslip and examined under a microscope (200,400X) and photomicrographs would be obtained.

Ethical approval
Ethics approval was obtained from the DRC Department of Physiology, Animal Ethics Committee, University of Karachi and all experiments involving animals were conducted under the strict guidelines of said committee.

STATISTICAL ANALYSIS

All the values were represented as Mean±S.E.M. difference between control and tested parameters. For this

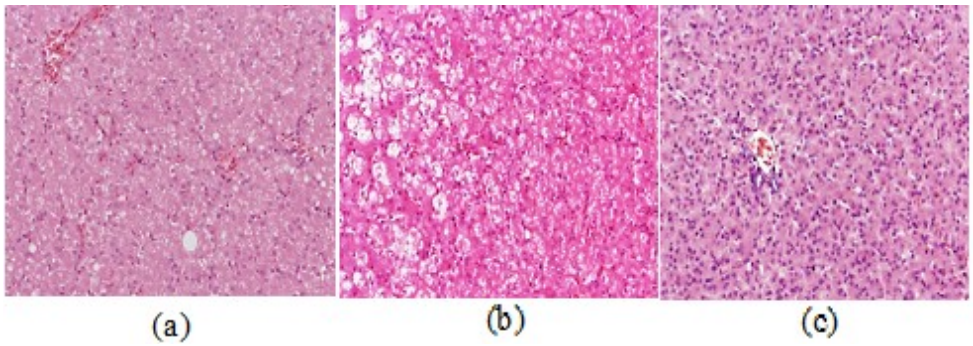

Fig. 1: Histopathological examination of 5µm cut liver section showing (a) normal architecture, (b) fatty liver with the appearance of micro and macro steatosis, decongestion and loss of cytoplasmic content and (c) eucalyptus treatment with the significant improvement.

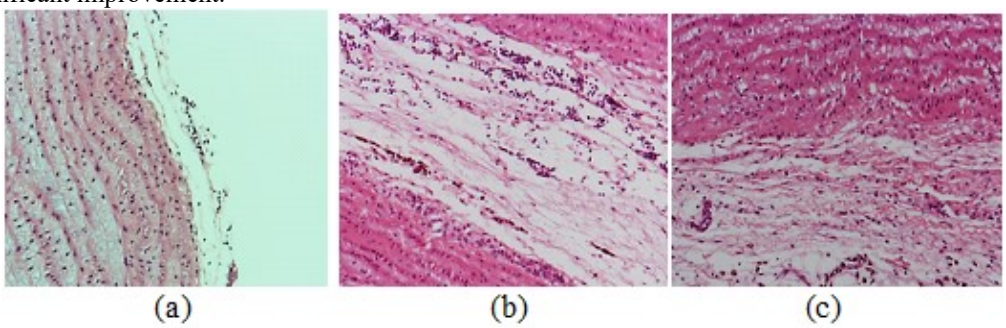

Fig. 2: Histopathological examination of 5µm cut aortic section showing (a) normal intima with lumen surface, (b) aortic lumen with the significant infiltration of macrophages and VSMC and (c) eucalyptus treatment with the nonsignificant healing.

Table 1: Hypercholesterolemia effect on body weight and beak length

Parameters	Negative control A (n=6)	Positive control B (n=6)
Body weight (g)	246 ± 4.21	350 ± 23.25[a]
Beak length (mm)	5 ± 0.25	12 ± 0.34[a]

Table 2: Hypercholesterolemia effect on blood glucose, albumin, urea and creatinine

Parameters	Negative control A (n=6)	Positive control B (n=6)
Blood glucose (g/dl)	243±14.89	360.33±3.63[a]
Albumin (g/dl)	4.6±0.37	3.75 ± 0.45
Serum urea (mg/dl)	20±1.2	63.83±0.65[a]
Creatinine (mg/dl)	0.41±0.17	0.20 ± 0.03

Table 3: Hypercholesterolemia effect on lipid profile and AI.

Parameters	Negative control A (n=6)	Positive control B (n=6)
Total Cholesterol (mg/dl)	120.01±9.17	565.13±41.7[a]
Triglycerides (mg/dl)	70.75±5.97	148.86±15.43[a]
HDL (mg/dl)	69.70±4.19	13.39±4.98[a]
VLDL (mg/dl)	14.15±1.19	29.77±3.08[a]
LDL (mg/dl)	36.17±9.96	503.97±38.55[a]
AI (TG/HDL)	1.03±0.11	5.35±0.91[a]
AI (HDL/TC)	0.59±0.05	0.55±0.05[a]

study, One way ANOVA was used while significance acceptance was at $p<0.05$. One way ANOVA trailed by 2 sided Dunette's test used for the determination of significant difference among groups, significance acceptance was at $P<0.01$ and $p<0.005$. SPSS version 20.0 was used to scrutinize the results.

Table 4: Hypercholesterolemia effect on liver enzymes

Parameters	Negative control A (n=6)	Positive control B (n=6)
AST (U/L)	48.81±6.79	64.01±8.46
ALP (U/L)	69.45±7.66	88.02±13.66
ALT (U/L)	17.69±5.04	54.32±4.79a

Table 5: Effect of different EC extract doses and Lipitor on BW and BL in hypercholesterolemic pigeons

Parameters	Eucalyptus extract treated				Lipitor treated
	C1 (n=6)	C2 (n=6)	C3 (n=6)	C4 (n=6)	D (n=6)
	0.05g/kg	0.1g/kg	0.2g/kg	0.4g/kg	10mg
Body weight (g)	263±12.01^{b,c}	240±8.9^{b,c}	246±17.06^{b,c}	238±16.81^{b,c}	264±15.5
Beak length (mm)	10±0.57^{b,c}	11±0.42	12±0.40	11±0.56	12.5±0.42 ^{a,b,c}

Table 6: Effect of different EC extract doses and Lipitor on lipid profile and AI in hypercholesterolemic pigeons

Parameters	Eucalyptus extract treated				Lipitor treated
	C1 (n=6)	C2 (n=6)	C3 (n=6)	C4 (n=6)	D (n=6)
	0.05g/kg	0.1g/kg	0.2g/kg	0.4g/kg	10mg
Total Cholesterol (mg/dl)	304.62±20.07^{a,b,c}	402.05±17.64^{a,b,c}	339.62±42.60^{a,b,c}	226.15±18.35^{a,b,c}	271.795±38.87^{a,b,c}
Triglycerides (mg/dl)	77.30 ± 6.64^{b,c}	73.55 ± 6.90^{b,c}	71.05 ± 8.23^{b,c}	54.59 ± 6.54^{b,c}	65.21±5.70
HDL (mg/dl)	45.11± 3.33	57.54± 10.94	65.10± 6.91^{b,c}	66.37± 10.11^{b,c}	45.20±4.62
VLDL (mg/dl)	15.46± 1.32^{b,c}	14.71± 1.38^{b,c}	14.21± 1.64^{b,c}	10.92± 1.31^{b,c}	13.04±1.14
LDL(mg/dl)	244.05± 21.8^{a,b,c}	329.81± 24.71^{a,b,c}	260.31± 41.64^{a,b,c}	148.87± 15.58^{a,b,c}	213.54±39.51^{a,b,c}
AI (HDL/TC)	0.55±0.008^{a,b,c}	0.146±0.034^{a,b,c}	0.26±0.032^{a,b,c}	0.294±0.0417^{a,b,c}	0.35±0.066^{a,b,c}

Table 7: Effect of different EC extract doses and Lipitor on plasma glucose, urea, albumin and creatinine in hypercholesterolemic pigeons

Parameters	Eucalyptus extract treated				Lipitor treated
	C1 (n=6)	C2 (n=6)	C3 (n=6)	C4 (n=6)	D (n=6)
	0.05g/kg	0.1g/kg	0.2g/kg	0.4g/kg	10mg
Blood glucose (g/dl)	287.66±0.71^{a,b,c}	274.0±1.72 ^{a,b,c}	296.33±1.47^{a,b,c}	293.66±1.47 ^{a,b,c}	264±1.06 ^{a,b,c}
Albumin (g/dl)	3.26 ± 0.32	4.33±0.56	4.37 ±0.39	4.03± 0.67	2.23±0.02 ^{a}
Serum urea (mg/dl)	52.0±0.577	43.16±0.477	31.33±0.49	29±1.31	25.20±0.42
Creatinine (mg/dl)	0.32±0.08	0.28±0.18	0.21± 0.06	0.42 ± 0.16	0.30±0.01

Table 8: Effect of different EC extract doses and Lipitor on liver enzymes in hypercholesterolemic pigeons

Parameters	Eucalyptus extract treated				Lipitor treated
	C1 (n=6)	C2 (n=6)	C3 (n=6)	C4 (n=6)	D (n=6)
	0.05g/kg	0.1g/kg	0.2g/kg	0.4g/kg	10mg
AST (U/L)	67.60±9.98	71.07±8.13	72.07±8.70	55.84±9.00	49.1±8.9
ALP (U/L)	71.78±7.99	73.36±6.54	70.46±8.03	64.96±7.82	55.16±0.47
ALT (U/L)	50.40±6.29^{a}	51.24± 8.53^{a}	46.58 ±4.37^{a}	45.36±5.20^{a}	34.0±0.577 ^{a,b,c}

*a=P<0.05 (compared with negative control), b=P<0.01 and c=P<0.005 (compared with positive control)

RESULTS

Induction of hyperlipidemia with HFD in pigeons for three months causes an increase in plasma TC, TG, VLDL, LDL levels and a drastic rise in the atherogenic index (table 3). The body weight and beak length was also significantly increased with the higher levels of cholesterol (table 1). In this study, high levels of cholesterol were observed after high-fat diet induction that evident in the significant increase in dietary cholesterol and LDL levels. The significantly higher levels of cholesterol were observed in hyperlipidemic pigeons of about 565.13 g/dl in contrast to the control group.

The serum concentration of AST and ALP enzyme levels has nonsignificant rise while ALT levels increased significantly after HFD induction (table 4). Creatinine and

albumin levels non-significantly changed among different groups. The glucose levels also observed a significant rise in comparison to control groups (table 2).

The results regarding the antihyperlipidemic response of aqueous bark extract of Eucalyptus with different doses showed a significant reduction in the plasma cholesterol, triglycerides, low-density lipoprotein, very low-density lipoprotein levels and atherogenic index along with an increase in high-density lipoprotein levels concerning the respective control group (table 6). In this experiment, it was observed that treating the HFD induced pigeon with 0.4 g/kg of BW for 21 produced the most significant reduction in lipid profile parameters, body weight and beak length (table 5).

However, treating the hypercholesterolemic pigeons produced non-significant changes in the liver enzymes (AST, ALP & ALT), urea, creatinine, and albumin levels (tables 7 and 8), except the substantial decline in glucose and ALT compared to respective control group with the administration of 0.4 g/kg of BW.

Atorvastatin administration to high fed diet fed pigeons resultant of substantial decline in TC, TG, VLDL and LDL levels along with a reduction in atherogenic index, while serum HDL-c levels were elevated (table 6).

In this experiment, histopathological examination also showed the tissue damage by feeding the pigeons HFD, there was marked changes were observed in liver and aorta sections when stained with the heamatoxyline-eosin stain. Induction of hyperlipidemia in HFD fed pigeons causes severe fatty liver changes due to fat droplets deposition in hepatocytes (figs. 1a, 1b). The aortic sections were also showed significant reduction in lumen diameter with the induced hyperlipidemia and the appearance of foam cells and proliferation of intimal VSMC (figure 1c). However, treatment groups of pigeons with varying doses of Eucalyptus extract showed insignificant alterations in liver sections includes, mild vascular congestion, necrosis, degenerative hepatocytes with broadening of some hepatocytes (ballooning shape)with two nuclei and some of them with pyknotic nuclei (Lumei, 1987). On the other hand, there were not such remarkable alterations observed in aortic sections that can be address in the future investigation with further elaborated setup (figs. 2a, 2b and 2c).

DISCUSSION

In this study we have successfully developed hyperlipidemia model using Columba livia (pigeons), a well-studied animal model for evaluation of CVD. It has been proposed that WC pigeons may serve as the best suited animal model for studying early atherosclerosis lesions. The elevated levels of LDL with the induction of HFD may be due to decreased receptor expression and higher levels of TG supplemented with Cholesterol ester (Padro *et al.,* 2015). The serum concentration of AST, ALT, and ALP enzyme are attributed to the liver damage with a subsequent rise in dietary cholesterol and tissue injury as a consequence of myocardial infarction whereas ALT is a consistent marker for liver mutilation and higher ALT provide evidence against damage of hepatobiliary system (Kim *et al.,* 2015). The lipid-lowering ability of extract may be due to the presence of its plant sterol content. These phytotherapeutic products more specifically sterols responsible for anti-hypercholestrolemic activity by inhibition of intestinal absorption of cholesterol (Bahmani *et al.,* 2015; Saravanan and Ignacimuthu, 2015; Timothy and Elliot, 2006) thus increase the fecal loss of steroids and a rise in uptake of LDL receptor activity (Tilvis and Miettinen, 1986). The exact mechanism through which plant extract causes the reduction the liver enzymes is may be due to their antioxidant activity, thus we can say that eucalyptus extract is potent hepatoprotective agent against various liver diseases (Zhang *et al.,* 2013).

The inhibitory effects of Atorvastatin was due to HMG-CoA reductase enzyme caused alteration of HMG-CoA to mevalonate, thus result into reduction in blood cholesterol and upregulation of LDL receptors causing the plasma LDL clearance (Blum, 1994). Such fatty deposition in liver characterized the imbalance mechanism between triglyceride production and its elimination that will lead to steatosis, which showed the characteristic histological fibrosis and necrosis in liver (Leclercq *et al.,* 1998**).** Hyperlipidemia also affects the structural integrity of liver cells causes the alteration in membrane permeability thus elevates the serums liver enzymes levels attributed for further damage. The production of oxygen free radicals due hypercholesterolemia may be the main cause of liver damage. In atherosclerosis, oxidized LDL production play a critical role in apoptosis and degenerative cell death.

CONCLUSION

Induction of hypercholesterolemia in pigeons represents the drastic alteration in biochemical and histological events. The pigeons as atherosclerosis model pose various benefits like they have similar biochemical and cellular event of atherosclerosis progression and development to human. They are less economical easy to handle and more specifically they tend to develop spontaneous atherosclerosis. It could be concluded that Eucalyptus aqueous bark extract possess antihyperlipidemic and antiatherosclerotic effects. The extract decreased the cholesterol level significantly in contrast to their placebo group. However no significant alteration would be reported for liver enzymes. Thus results indicate that the Eucalyptus bark extract possess antihyperlipidemic and anti-atherosclerotic activity, but these data provide further

consequence to study the fractional activity for the scope towards drug development with minimum side effects.

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