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ORIGINAL RESEARCH ARTICLE

Acyclic Isoprenoid Attenuates Lipid Anomalies and Inflammatory Changes in Hypercholesterolemic Rats

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Abstract The present study was aimed to explore the antihyperlipidemic and anti-inflammatory effect of acyclic isoprenoid on high fat diet fed rats. Hypercholesterolemia was induced by the diet comprising of the normal rat chow 84.3%, lard 5%, yolk powder 10%, cholesterol 0.2% and 0.5% bile salt were fed to the rats for the period of 8 weeks. The results showed that abnormally elevated levels of plasma lipid profiles. Three different doses of acyclic isoprenoid (20, 40 and 80 mg/kg b.w/day) were administered orally to hypercholesterolemia suffering rats for the period of 30 days. Among these three doses of acyclic isoprenoid, the dose 80 mg/kg b.w. was significantly decreased the plasma lipid profiles when compared to other two doses. The effect produced by acyclic isoprenoid (80 mg/kg b.w) was comparable to that of simvastatin. Therefore, 80 mg/kg b.w was fixed as a effective dose and used for further analyses. Acyclic isoprenoid administration reinstated the elevated levels of cardiac and inflammatory markers in both blood and serum of hypercholesterolemic rats. In addition, acyclic isoprenoid administration decreased activity of 3-hydroxy 3-methyl-glutaryl-CoA reductase and increased the activity of lecithin cholesterol acyl transferase. These findings suggest that the administration of acyclic isoprenoid was potentially ameliorated the cardiac marker enzymes and inflammatory markers in addition to its antihypercholesterolemic effect.

Keywords *Semecarpus anacardium* · Acyclic isoprenoid · Hypercholesterolemia · Inflammatory cytokines

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Introduction

Cardiovascular disease is a most important cause of global mortality, accounting for almost 17 million deaths once a year. Atherosclerosis is a main contributor for the pathogenesis of myocardial and cerebral infarction [1]. Elevated levels of plasma low-density lipoprotein cholesterol and triglycerides, accompanied by reduced high-density lipoprotein levels are often associated with an increased risk of coronary heart disease [1]. Atherosclerosis signifies a state of discriminating oxidative stress, characterized by lipid and protein oxidation [2]. Several studies point out that LDL oxidation is an early event of atherosclerotic process. In fact, oxLDL is cytotoxic to a variety of vascular cells which induces the synthesis of monocyte chemotatic protein-1, recruits inflammatory cells and stimulates the production of autoantibodies [3].

There are numerous drugs that may help to lower plasma lipid and low-density lipoprotein cholesterol by 20–40% and increase the high-density lipoprotein cholesterol [4]. Available drugs include fibrates and 3-hydroxy 3-methyl-glutaryl-CoA reductase inhibitors (HMG CoA reductase) among others. However, these synthetic medications

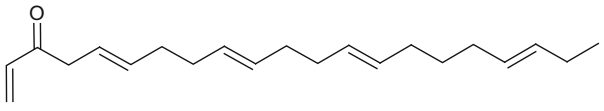
produce adverse effects. In view of this, the search for natural products with lipid lowering potential and with minimal or no side effects is warranted. Therefore, the use of medicinal plants became most important in the treatment of cardiovascular diseases is increasing due to its less adverse effects [5].

Semecarpus anacardium Linn, belongs to the family Anacardiaceae and distributed in sub-Himalayan region, tropical and central parts of India. The nuts are commonly known as marking nut and Bhallaatak-in Hindi, It has high priority and applicability in traditional system of medicines (Ayurvedic and Siddha system of medicine) either alone or as an ingredient of many polyherbal formulations for treating various ailments. [6]. The phytochemical analyses of its nuts revealed the presence of biflavonoids [7] and phenolic compounds [8], sterols and glycosides [9]. Other components isolated are, catechol [10, 11], tetrahydroamentoflavone (THA) [12], jeediflavanone [13], galluflavonone [14], semecarpetin [15] and anacardioflavonone [16] which illustrate a variety of medicinal properties. Some extracts of S. *anacardium* nuts have been found to exhibit anti-oxidants, anti-inflammatory, anti-microbial and bacterial activities [17, 18]. Recently, acyclic isoprenoid has been reported to possess anti-bacterial activity [19]. But, no study has been reported the effect of acyclic isoprenoid on atherosclerosis so far. Therefore, the present study was intended to assess the effect of cyclic isoprenoid on lipid anomalies and inflammatory changes in hypercholesterolemic rats. The structure of acyclic isoprenoid was given in Fig. 1.

Materials and Methods

Sources of Chemicals

Cholesterol was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Bile salt was purchased from Central Drug House Pvt. Ltd., New Delhi, India. The high fat diet components such as, egg yolk power was obtained from SKM Egg Products Export (India) Limited, Erode, Tamil Nadu, India and lard was obtained from locally available market in Chennai. Diagnostic kits used for the estimation of TC, triglyceride and HDL-c were obtained from Agappe Diagnostics Pvt. Ltd., Kerala, India. The standard drug simavastatin was purchased from Micro Labs Ltd Pondicherry, India. All other chemicals used were of analytical grade.



(5*E*,9*E*,13*E*,18*E*)-henicosa-1,5,9,13,18-pentaen-3-one

Fig. 1 Acyclic isoprenoid

Plant Materials

Semecarpus anacardium seeds were purchased from Ramasamy Chettiyar, Traditional & Herbal Medicine shop, Parris, Chennai, Tamil Nadu, India. The identity of the plant was confirmed by Prof. Raman, plant taxonomist, Centre for Advanced Studies in Botany, University of Madras and voucher specimens (MUCASB- H105) was deposited in the department herbarium.

Extraction and Isolation

500 g of S*emecarpus anacardium* seeds were crushed and soaked in a liter of methanol and kept in refrigerator for 3 days. Then, the filtrate was filtered through Whatman filter paper No 1 and this was repeated three to four times until the filtrate gave no coloration and concentrate using vacuum rotary evaporator at 40 °C. The methanolic concentrate was fractionated sequentially with petroleum, diethyl ether, chloroform and n-butanol. The n-butanolic fraction was evaporated to dryness and then checked it on thin layer chromatography with hexane and ethyl acetate (8:2 ratios) in which four spots were appeared. The n-butanolic concentrate was chromatographed on silica gel column (Merk 60-120 mesh size) and eluted successively with hexane and ethyl acetate in the ratio of 8:2. A total of 50 fractions were collected at an interval of 5 ml each and monitored by thin layer chromatography (precoated silica gel merk-60 F_{254} 0.25 mm thick plate). Fraction from 1 to 5 formed as a pale green coloured and showed single spot on TLC and pooled together in a cleaned vial and evaporated to dryness. This process was repeated until getting satisfactory yield of the compound. The structure of the compound was confirmed as acyclic isoprenoid derivatives on the basis of IR, ^{1}H NMR, ^{13}C NMR and high-resolution mass spectrometry (HRMS). Molecular formula of the compound is $C_{21}H_{32}O$. Yield of the compound was 300 mg/500 g of methanolic crude extract. The chemical structure of the acyclic isoprenoid was given in Fig. 1.

Spectral Description

The FTIR spectrum of the acylic isoprenoid shows absorption peak at 1754 cm^{-1} can be assigned as carbonyl group. The other peaks at 2924 and 2854 cm^{-1} were represented by the methyl, methylene stretching groups respectively. The ^{1}H NMR spectrum shows the triplet signal at δ 0.96 (3H, t, J = 7.2 Hz) which corresponds to the methyl protons present in the isoprenoid compound. The alkene protons appeared as a multiplet at δ 6.28–6.31

is due to adjacent of the carbonyl group. The ^{13}C NMR spectrum of the isoprenoid showed the presence of 21 carbon signals. The peak at δ 196.09 and 14.13 were identified as the carbonyl carbon and the methyl carbon group. The isoprenoid compound was assigned to the molecular formula is $C_{21}H_{32}O$ to the HRMS (EI) molecular ion peak at m/z = 301.2517 [M +] corresponding to calcd m/z = 301.2531.

IR (KBr) λ_{max} = 2924(–CH_3), 2854 (=CH), 1745 (C=O), 1460, 1161 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ = 0.96 (t, 3H *J* = 7.2 Hz), 1.36 (s, 2H), 1.63–1.69 (m, 2H), 2.01–2.09 (m, 2H), 2.29–2.42 (m, 8H), 4.17–4.38 (m, 2H), 5.33–5.39 (m, 6H), 5.76–5.79 (m, 4H,), 6.29–6.31 (m, 1H), 6.67–6.79 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 14.13 (C-21), 22.67 (C-16), 24.86 (C-20), 27.21 (C-17), 29.11 (C-15), 29.25 (C-11), 29.51 (C-12), 31.91 (C-7), 34.02 (C-8), 52.08 (C-4), 127.21 (C-1), 128.49 (C-14, C-18), 129.16 (C-6), 129.42 (C-13), 129.61 (C-9), 129.86 (C-10), 129.98 (C-19), 130.17 (C-5), 136.47(C-2), 196.09 (C-3). HRMS (EI) calcd for $C_{21}H_{32}O$ (M + H): 301.2531. Found 301.2517.

Experimental Procedure

Animals

Male albino rats of Wistar strain with body weight ranging from 180 to 200 g were procured from the Central Animal House Facility University of Madras Taramani Campus, Chennai, Tamil Nadu, India. They were maintained at an ambient temperature of 25 ± 2 °C and 12/12 h of light/-dark cycle. Animals were given standard commercial rat chow and water ad libitum and housed under standard environmental conditions throughout the study. The laboratory animal protocol used in this study was approved by the Institutional Animal Ethical Committee, University of Madras in accordance with the Indian National law on animal care and use (IAEC No. 01/013/2009).

Induction and Assessment of Hypercholesterolemia

High cholesterol diet was prepared according to the method of Xie et al. [20]. High fat diet comprising of normal rat chow 84.3%, 5% lard, 10% yolk powder, cholesterol 0.2% and 0.5% bile salt were fed to the rats for the period of 8 weeks. To confirm the induction of hypercholesterolemia, blood samples were collected in a heparin coated tubes from retro orbital sinus, then the blood was centrifuged and plasma was separated and used for estimation of cholesterol. The plasma cholesterol was determined and those rats with fasting plasma cholesterol greater than 250 mg/dl were used in the present study. The rats again fed with high fat diet (HFD) and treatment was started on the next day after hypercholesterolemia confirmation and this was considered as 1st day of treatment and it was continued for 30 days. The initial and final body weights of the various groups were recorded prior to induction of hypercholesterolemia and sacrifice.

Experimental Design

The animals were divided into 7 groups of 6 rats in each. They are as follows

Group I	Control rats fed with normal diet and received intra gastrically 0.5 ml olive oil for 30 days
Group II	Control rats fed with normal diet and received intra gastrically acyclic isoprenoid (80 mg/kg b.w) dissolved in 0.5 ml of olive during the last 30 days
Group III	Hypercholesterolemic control rats (Rats were fed with high fat diet comprising of normal rat chow 84.3%, lard 5%, yolk powder 10%, cholesterol 0.2% and 0.5% bile salt for the period of 8 weeks
Group IV	Rats were fed with high fat diet for the period of 8 weeks and received intra gastrically acyclic isoprenoid (20 mg/kg b.w) dissolved in 0.5 ml of olive oil during last 30 days
Group V	Rats were fed with high fat diet for the period of 8 weeks and received intra gastrically acyclic isoprenoid (40 mg/kg b.w) dissolved in 0.5 ml of olive oil during last 30 days
Group VI	Rats were fed with high fat diet for the period of 8 weeks and received intra gastrically acyclic isoprenoid (80 mg/kg b.w) dissolved in 0.5 ml of olive oil during last 30 days
Group VII	Rats were fed with high fat diet for the period of 8 weeks and received intra gastrically simvastatin (5 mg/kg b.w) dissolved in 0.5 ml of 0.9% saline during last 30 days

At the end of the experimental period, the animals were deprived of food overnight and sacrificed by cervical decapitation under pentobarbitone sodium (60 mg/kg) anaesthesia. Blood was collected in two different test tubes, one with anticoagulant for the separation of plasma and another without anticoagulant for the separation of serum. The plasma and serum were separated by centrifugation at 2000×*g* for 10 min and stored at -80 °C for assay of enzymes and other biochemical assays.

Assay of Plasma Lipid Profile and Cardiac Marker Enzymes

The levels of total cholesterol, triglyceride, high density lipoprotein cholesterol (HDL-c) creatine kinase-MB (CK-

MB) and lactate dehydrogenase (LDH) were estimated by using biochemical kits (Agappe Diagnostics Pvt. Ltd., Kerala, India). For the determination of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol Friedewald's formula was used which states: VLDL cholesterol = Triglyceride/5 and LDL cholesterol = Total cholesterol- (VLDL + HDL cholesterol). Atherogenic index was calculated by the following formula AI = TC-HDL-c/HDL-c.

Assessment of HMG-Co A Reductase, LCAT and Fibrinogen

The activity of HMG-CoA reductase and LCAT in the plasma and liver was assayed by the method of Philipp and Shapiro [21] and Hitz et al. [22] respectively. Plasma fibrinogen was estimated by the method of Lempert [23].

Assessment of Tumour Necrosis Factor-α (TNF-α) and C-reactive protein (CRP)

The levels of TNF-α and CRP were measured using enzyme linked immunosorbent assay (ELISA) kits (MyBiosource, California, US and SPINREACT, Spain). The analyses were performed according to instructions of the manufacturer's.

Hematological Indices

Erythrocyte Sedimentation Rate (ESR) was measured by the Westergren method. Total white blood cell (WBC) and platelet counts were performed on whole blood samples using a Sysmex KX-24 blood cell counter (Transasia Biomedicals Ltd., Mumbai, India). Blood smears were made from EDTA-treated samples and the differential leucocyte count was done manually.

Oil Red O Staining of Thoracic Aorta

Thoracic aorta tissues were rapidly dissected and fixed in liquid nitrogen and stored until use at −80 °C. The frozen tissues was processed with a cryomicrotome (Cryotome FSE Cryostat; Thermo Electron Corp., Cambridge, MA, USA) using sections 5 µm thickness and stained with oil red-O. Then, stained aortic tissue samples were examined and photographed under a light microscope to assess the presence of lipid droplets in the aorta and atherosclerotic plaque lesions. Digital images were obtained using an Olympus BX51 microscope equipped with a Camedia C3040ZOOM digital camera (Olympus America Inc., Melville, NY, USA). All images were taken under 40× magnification.

Statistical Analysis

The results are expressed as mean ± standard deviation (SD). Differences between groups were assessed by ANOVA using the SPSS software package for Windows. Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) P values < 0.05 were considered as significantly altered.

Results

Effect of Acyclic Isoprenoid on Lipid Profile in HFD Fed Rats

Table 1 portrays the levels of plasma cholesterol, triglyceride, lipoproteins and atherogenic index in control and experimental rats. The levels of plasma cholesterol, triglyceride, low density lipoprotein cholesterol (LDL-c), very low density lipoprotein cholesterol (VLDL-c) and atherogenic index were significantly increased whereas; the levels of high density lipoprotein cholesterol (HDL-c) were significantly decreased in hypercholesterolemic rats when compared to control rats. Administration of acyclic isoprenoid to hypercholesterolemic rats significantly reversed the all these altered parameters to near normal level in a dose dependent manner. Acyclic isoprenoid at a dose of 80 mg/kg body weight showed a highly significant effect in lowering cholesterol than the other two doses (20 and 40 mg/kg body weight). Therefore, effective dose was fixed at 80 mg/kg body weight and used for further biochemical analysis. The effect produced by acyclic isoprenoid (80 mg/kg b.w) was comparable to that of simvastatin.

Body Weight Gain, Food Intake and Food Efficiency

A significant increase in body weight was observed in hypercholesterolemic rats when compared to control rats whereas food consumption and feed efficiency were not altered in all the groups tested. Elevated body weight gain and organs weight gain in hypercholesterolemic rats was brought back to near normal after treatment with acyclic isoprenoid and simvastatin. The administration of acyclic isoprenoid to normal rats produced no significant changes in the body weight gain, food intake and food efficiency when compared with normal control rats. (Table 2).

HMG-CoA Reductase Activity

The activity of HMG-CoA reductase in plasma and liver normal and experimental rats are shown in Fig. 2. Increased activity of HMG-CoA reductase was observed in

Table 1 Effect of acyclic isoprenoid on the levels of plasma lipid profiles in control and experimental animals

Groups	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	Athrogenic index
Group I (normal control)	88.5 ± 8.57	134.83 ± 12.30	41.5 ± 2.66	20.03 ± 7.44	26.96 ± 2.46	1.14 ± 0.26
Group II (normal control + acyclic isoprenoid 80 mg/kg b.w)	89.16 ± 5.38	132 ± 12.08	43.16 ± 2.78	19.6 ± 5.97	26.4 ± 2.41	1.07 ± 0.16
Group III (hypercholesterolemia induced)	283.5 ± 21.35[b]	320.33 ± 20.04[b]	23.16 ± 2.13[b]	196.26 ± 19.53[b]	64.06 ± 4.00[b]	11.37 ± 1.89[b]
Group IV (hypercholesterolemia + acyclic isoprenoid 20 mg/kg b.w)	250.33 ± 22.77[c]	277.16 ± 20.00[c]	29 ± 2.09[c]	165.9 ± 20.96[c]	55.43 ± 4.00[c]	7.71 ± 1.38[c]
Group V (hypercholesterolemia + acyclic isoprenoid 40 mg/kg b.w)	190.33 ± 18.21[c]	222.5 ± 23.18[c]	34.83 ± 2.40[c]	111 ± 16.28[c]	44.5 ± 4.63[c]	4.49 ± 0.74[c]
Group VI (hypercholesterolemia + acyclic isoprenoid 80 mg/kg b.w)	137.66 ± 13.29[c]	149.5 ± 11.37[c]	40.5 ± 2.81[c]	67.26 ± 15.31[c]	29.9 ± 2.27[c]	2.42 ± 0.53[c]
GroupVII (hypercholesterolemia + simvastatin 5 mg/kg b.w)	124.16 ± 10.14[c]	133.5 ± 8.26[c]	41.83 ± 4.49[c]	55.63 ± 13.20[c]	26.7 ± 1.65[c]	3.02 ± 0.55[c]

Values are given as mean ± SD for six animals in each group (n = 6). Values are considered significantly different at $p < 0.05$ with post hoc LSD test $^{*}P < 0.05$

Atherogenic Index = TC-HDL-c/HDL-c

Comparisons are made between—[a]Control versus acyclic isoprenoid alone treated control rats; [b]control versus hypercholesterolemic rats; [c]hypercholesterolemic rats versus acyclic isoprenoid treated hypercholesterolemic rats; [d]acyclic isoprenoid treated hypercholesterolemic rats versus simvastatin treated hypercholesterolemic rats

Table 2 Effect of acyclic isoprenoid on body weight, organs and adipose weight, food intake and food efficiency of control and experimental animals

Group	Body weight (g)		Organs and tissue weight(g)				Food intake (g/day)	Food efficiency
	Initial	Final	Heart	Liver	Kidney	Adipose tissue		
Group I (control)	189.5 ± 7.42	235.5 ± 13.30	1.62 ± 0.13	12.30 ± 1.18	2.94 ± 0.28	5.83 ± 0.47	24.11 ± 2.31	9.86 ± 1.28
Group II (acyclic isoprenoid alone 80 mg/kg)	192.16 ± 6.58	239 ± 12.49	1.58 ± 0.11	12.17 ± 1.02	2.66 ± 0.25	5.27 ± 0.41	23.88 ± 2.21	8.10 ± 0.75
Group III (HFD induced Hypercholesterolemia)	197.16 ± 8.72	283.83 ± 8.30[b]	2.51 ± 0.16[b]	17.99 ± 1.62[b]	3.79 ± 0.30[b]	11.28 ± 1.09[b]	25.07 ± 2.78	9.63 ± 1.22
Group IV (HFD + acyclic isoprenoid 80 mg/kg)	193.83 ± 5.52	241.66 ± 14.43[c]	1.95 ± 0.20[c]	14.46 ± 1.15[c]	3.14 ± 0.29[c]	8.38 ± 0.77[c]	25.55 ± 2.14	7.74 ± 0.55
GroupV (HFD + simvastatin 5 mg/kg)	194.5 ± 6.31	234.66 ± 15.17	1.77 ± 0.14	13.56 ± 1.15	2.87 ± 0.19	7.78 ± 0.69	26.85 ± 2.26	10.63 ± 0.93

Values are given as mean ± SD for six animals in each group (n = 6). Values are considered significantly different at $p < 0.05$ with post hoc LSD test $^{*}P < 0.05$

Food efficiency = Body weight gain/food intake

Comparisons are made between—[a]Control versus acyclic isoprenoid alone treated control rats; [b]control versus hypercholesterolemic rats; [c]hypercholesterolemic rats versus acyclic isoprenoid treated hypercholesterolemic rats; [d]acyclic isoprenoid treated hypercholesterolemic rats versus Simvastatin treated hypercholesterolemic rats

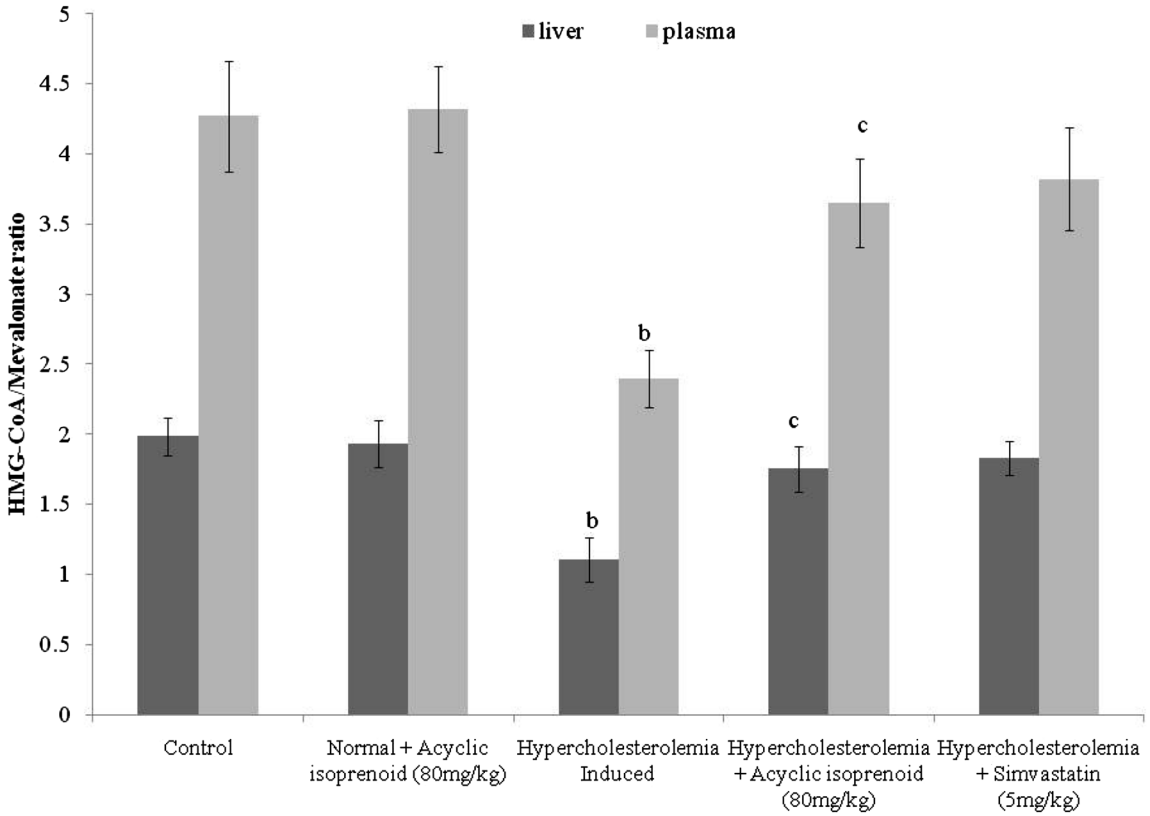


Fig. 2 Effect of acyclic isoprenoid on the activity of HMG-CoA reductase in the plasma and liver of control and experimental rats. Values are given as mean ± SD for six animals in each group (n = 6). Values are considered significantly different at $p < 0.05$ with post hoc LSD test $^{*}P < 0.05$. Comparisons are made between—[a]control versus acyclic isoprenoid alone treated control rats; [b]control versus Hypercholesterolemic rats; [c]hypercholesterolemic rats versus acyclic isoprenoid treated hypercholesterolemic rats; [d]acyclic isoprenoid treated hypercholesterolemic rats versus simvastatin treated hypercholesterolemic rats

both plasma and liver of high fat diet fed rats. Treatment with acyclic isoprenoid and simvastatin to high fat diet fed rats significantly decreased the activity of HMG-CoA reductase in plasma and liver tissues on comparison with untreated high fat diet fed rats. Lower ratio of HMG-CoA to mevalonate indicates higher activity of the enzyme.

Activity of Lecithin Cholesterol Acyl Transferase (LCAT)

Hypercholesterolemic rats showed significant decrease in the activity of LCAT in the plasma and liver tissue homogenate compared to normal control rats (Fig. 3). However, treatment with acyclic isoprenoid and simvastatin to hypercholesterolemic rats normalized the activity of LCAT compared to untreated hypercholesterolemic rats.

Cardiac Marker Enzymes

Figure 4 depicts the levels of serum creatine kinase-MB and lactate dehydrogenase (LDH) in normal and experimental rats. Hypercholesterolemic showed significant increase in the activities of CK-MB and LDH in the serum compared to normal control rats. Oral administration of acyclic isoprenoid and simvastatin to hypercholesterolemic rats normalized the activities of these enzymes in serum compared to untreated hypercholesterolemic rats.

Levels of Inflammatory Cytokines

Table 3 shows the levels of pro-inflammatory (TNF-α) and acute phase proteins (CRP and fibrinogen) in the plasma of control and experimental animals. A significant increase in TNF-α, CRP and fibrinogen levels were observed in HFD fed rats when compared to control rats and these alterations were restored to near normal upon treatment with acyclic isoprenoid and simvastatin.

Hematological Indices

The mean values of inflammatory markers in blood samples of control and experimental animals are shown Table 4. The mean values of the ESR, platelet count, WBC count and differential leucocyte count—neutrophils and

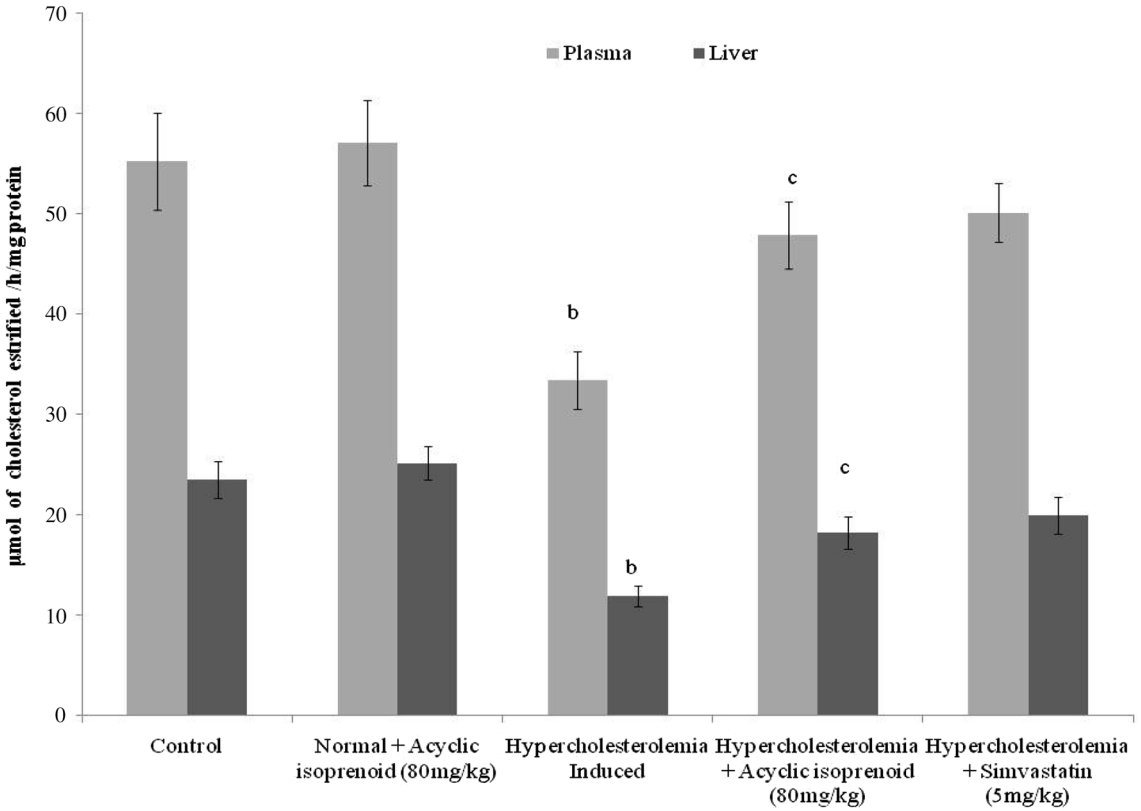


Fig. 3 Effect of acyclic isoprenoid on the activity of LCAT in the plasma and liver of control and experimental rats. Values are given as mean ± SD for six animals in each group (n = 6). Values are considered significantly different at $p < 0.05$ with post hoc LSD test *$P < 0.05$. Comparisons are made between—[a]control versus acyclic isoprenoid alone treated control rats; [b]control versus hypercholesterolemic rats; [c]hypercholesterolemic rats versus acyclic isoprenoid treated hypercholesterolemic rats; [d]acyclic isoprenoid treated hypercholesterolemic rats versus Simvastatin treated hypercholesterolemic rats

monocytes were significantly increased whereas lymphocyte count was significantly decreased in the blood samples of hypercholesterolemic rats when compared to control rats. However, oral administration of acyclic isoprenoid and simvastatin to hypercholesterolemic rats reinstated these blood levels of inflammatory markers to near normalcy. No significant differences were observed between the mean eosinophils counts in the blood samples of the five groups of rats, while basophils were completely absent in all the groups tested.

Oil Red O Staining of Thoracic Aorta

Figure 5 shows photomicrographs of oil red O stained aortic specimens. The histology of aorta is normal in the control and acyclic isoprenoid alone treated group (Fig. 5a, b).In high fat diet group, aorta shows widespread deposition of lipid droplets and inflammatory cells (Fig. 5c). Aorta of high fat diet treated with acyclic isoprenoid and simvastatin showed scattered droplets of fat when compared with untreated high fat diet fed rats (Fig. 5d, e).

Discussion

Elevated blood cholesterol and serum triglycerides levels are major risk factor for the development of coronary heart disease [24, 25]. In the present study, the rats were fed with high-fat diet showed higher concentration of serum cholesterol and triglycerides compared to rats fed with normal diet. However, there was an obvious reduction in serum cholesterol and triglycerides levels in rats fed with the high-fat diet supplemented with acyclic isoprenoid and simvastatin for a period of 30 days. It is also extensively accepted that an elevated plasma level of low density lipoprotein cholesterol is a major risk factor for coronary heart disease [26]. The levels of high density lipoprotein cholesterol are thought to reflect the rate of removal of excess peripheral cholesterol and raised levels are thereby associated with reduced risk of atherosclerosis. We observed that serum levels of low density lipoprotein cholesterol were significantly reduced whereas the levels of high density lipoprotein cholesterol were significantly increased in animals received the high fat diet additionally supplemented with acyclic isoprenoid and simvastatin

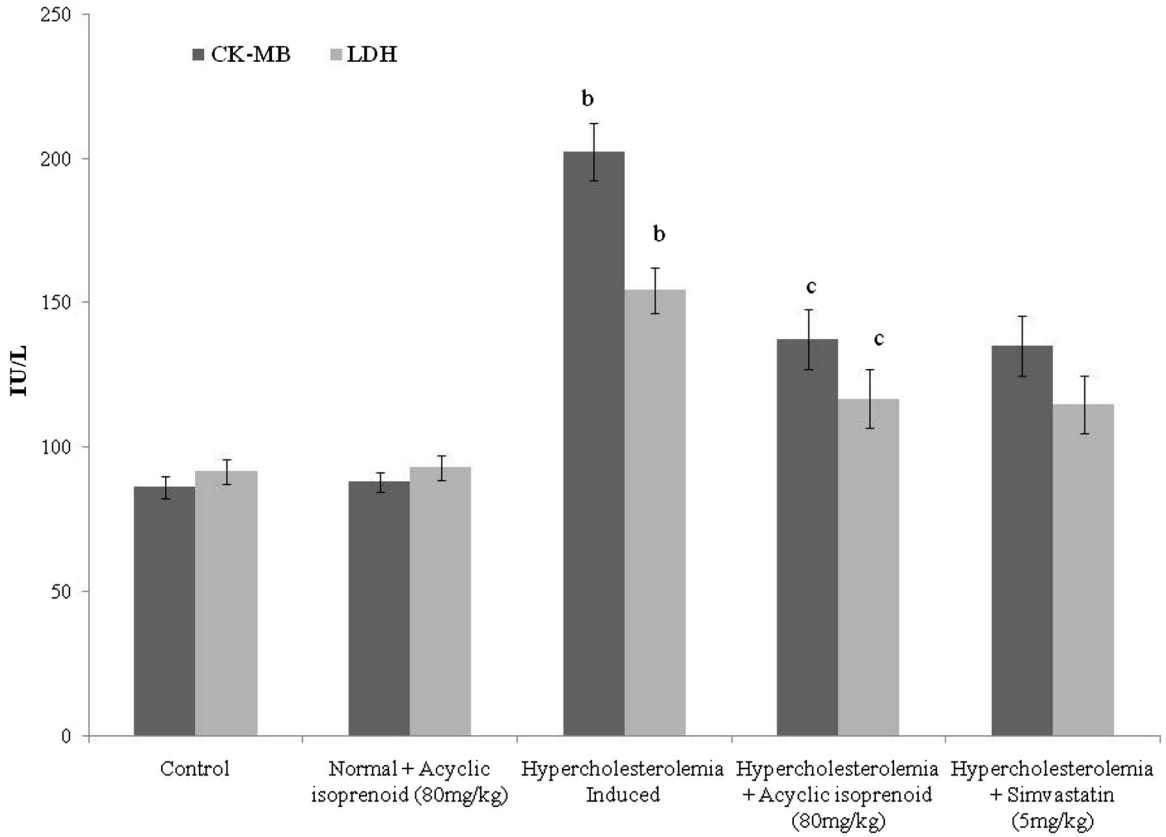


Fig. 4 Effect of acyclic isoprenoid on the levels of cardiac marker enzymes in the plasma of control and experimental rats. Values are given as mean ± SD for six animals in each group (n = 6). Values are considered significantly different at $p < 0.05$ with post hoc LSD test $^*P < 0.05$. Comparisons are made between—[a]control versus acyclic isoprenoid alone treated control rats; [b]control versus hypercholesterolemic rats; [c]hypercholesterolemic rats versus acyclic isoprenoid treated hypercholesterolemic rats; [d]acyclic isoprenoid treated hypercholesterolemic rats versus Simvastatin treated hypercholesterolemic rats

Table 3 Effect of acyclic isoprenoid on the levels of TNF-α, CRP and fibrinogen in control and experimental animals

Parameters	Group I (control)	Group II (acyclic isoprenoid alone 80 mg/kg)	Group III (HFD induced hypercholesterolemia)	Group IV (HFD + acyclic isoprenoid 80 mg/kg)	Group V (HFD + simvastatin 5 mg/kg)
TNF-α (pg/ml)	11.90 ± 1.09	11.26 ± 1.18	51.84 ± 3.54[b]	23.16 ± 2.13[c]	20.95 ± 1.48
CRP (mg/l)	2.53 ± 0.22	2.12 ± 0.17	6.87 ± 0.61[b]	3.44 ± 0.32[c]	2.93 ± 0.28
Fibrinogen (mg/dl)	67.82 ± 4.62	64.71 ± 5.16	192.53 ± 16.66[b]	91.39 ± 6.16[c]	87.81 ± 6.31

Values are given as mean ± SD for six animals in each group (n = 6). Values are considered significantly different at $p < 0.05$ with post hoc LSD test $^*P < 0.05$

Comparisons are made between—[a]control versus acyclic isoprenoid alone treated control rats; [b]control versus hypercholesterolemic rats; [c]hypercholesterolemic rats versus acyclic isoprenoid treated hypercholesterolemic rats; [d]acyclic isoprenoid treated hypercholesterolemic rats versus simvastatin treated hypercholesterolemic rats

compared to rats received normal diet. The atherogenic index (AI), defined as the ratio of TC-HDL-c/HDL-c, is a diagnostic indicator of the risk of atherosclerosis development. The serum atherogenic index was significantly increased in high fat diet fed rats compared to control rats fed with normal diet. The atherogenic index was significantly reversed to near normal in high fat diet fed rats after treatment with acyclic isoprenoid and simvastatin.

Feeding the rats with a high fat diet fed rats led to increase the body weight and accumulation of fat in

Table 4 Effect of acyclic isoprenoid on the values of inflammatory markers in blood samples of control and experimental animals

Parameters	Group I (control)	Group II (acyclic isoprenoid alone 80 mg/kg)	Group III (HFD induced hypercholesterolemia)	Group IV (HFD + acyclic isoprenoid 80 mg/kg)	Group V (HFD + simvastatin 5 mg/kg)
Total WBC ($\times 10^3 \times mm^3$)	5.78 ± 0.64	6.43 ± 1.26	14.18 ± 1.09[b]	8.01 ± 0.58[c]	7.4 ± 0.42
Platelet count ($\times 10^3 \times mm^3$)	1.81 ± 0.17	1.73 ± 0.16	5.96 ± 0.54[b]	2.88 ± 0.23[c]	2.65 ± 0.19
ESR (mm/h)	12.89 ± 1.15	12.09 ± 1.16	59.57 ± 5.70[b]	26.79 ± 2.53[c]	24.65 ± 2.29
Neutrophils (%)	55.78 ± 3.76	54.99 ± 2.82	65.26 ± 3.79[b]	60.31 ± 2.47[c]	58.69 ± 3.18
Lymphocytes (%)	40.02 ± 3.2	41.21 ± 3.43	28.06 ± 2.68[b]	34.42 ± 1.80[c]	36.13 ± 1.83
Monocytes (%)	2.28 ± 0.20	1.98 ± 0.15	4.9 ± 0.384[b]	3.48 ± 0.27[c]	3.26 ± 0.32
Eosinophils (%)	1.92 ± 0.14	1.82 ± 0.12	1.78 ± 0.14	1.79 ± 0.15	1.92 ± 0.13
Basophils (%)	ND	ND	ND	ND	ND

Values are given as mean ± SD for six animals in each group (n = 6). Values are considered significantly different at $p < 0.05$ with post hoc LSD test $^*P < 0.05$

ND not detected

Comparisons are made between—[a]control versus acyclic isoprenoid alone treated control rats; [b]control versus hypercholesterolemic rats; [c]hypercholesterolemic rats versus acyclic isoprenoid treated hypercholesterolemic rats; [d]acyclic isoprenoid treated hypercholesterolemic rats versus Simvastatin treated hypercholesterolemic rats

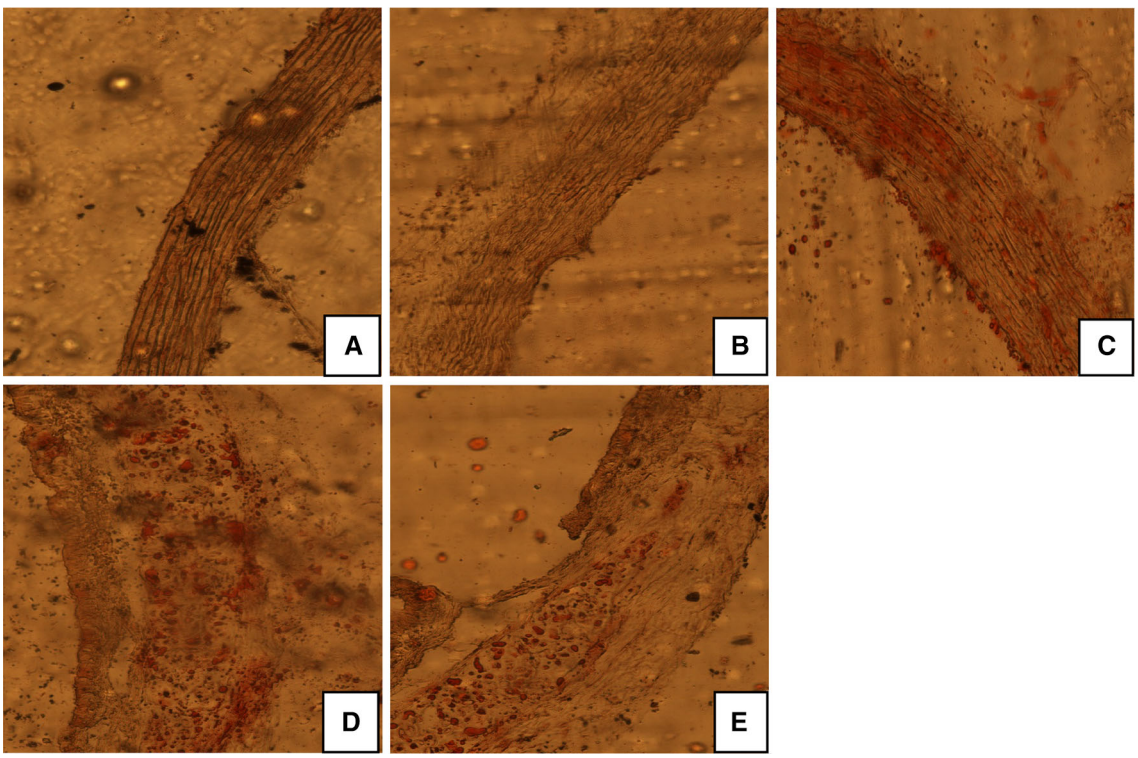


Fig. 5 Oil red O staining of aorta of control and experimental rats (×40). Control (**a**), normal + acyclic isoprenoid (**b**), high fat diet induced (**c**), high fat diet induced + acyclic isoprenoid (**d**), high fat diet induced + simvastatin (**e**)

visceral organs. These findings are in agreement with the results of Naderali et al. [27]. The increased body weight could probably due to intake of enriched high fat diet. The increase in the amount of fat in the diet have been shown to be associated with the risk of obesity and hyperlipidemia in human and rodents by changing the cholesterol and triglyceride levels in plasma and tissues. The increased body weight is characterized by an increased mass of fat or

white adipose tissue. White adipose tissue is a specialized loose connective tissue that is extensively laden with adipocytes which are differentiated cells, specialized in the storage of fat [28]. The increased prevalence of excessive visceral obesity is associated with various diseases, particularly obesity-related cardiovascular diseases and type 2 diabetes mellitus [29]. However, hypercholesterolemic rats fed with HFD and co- administration with acyclic isoprenoid and simvastatin significantly decreased the body weight, liver and adipose tissue weight. The body weight and organ weight reducing effect may be attributed to inhibition of HMG CoA reductase activity which is the key enzyme in cholesterol biosynthesis pathway.

HMG-CoA reductase is a rate-controlling enzyme in the mevalonate pathway, the metabolic pathway that produces cholesterol and other isoprenoids. Inhibition of HMG-Co A reductase decreases cholesterol synthesis and its inhibitors are very effective in lowering serum cholesterol and of low density lipoprotein cholesterol in most of animal species, including humans [30]. We observed significant increase in the activity of HMG-CoA reductase in the plasma and liver of hypercholesterolemic rats. However, treatment of acyclic isoprenoid and simvastatin to hypercholesterolemic rats significantly inhibited hepatic cholesterol biosynthesis by blocking the HMG-CoA reductase. The obstruction of cholesterol synthesis by an inhibitor of HMG-Co A reductase, results in lower intracellular supply of cholesterol, thereby triggering an over-expression of hepatic of low density lipoprotein receptors and enhancing the clearance of circulating LDL particles which was also observed in acyclic isoprenoid and simvastatin treated animals.

Lecithin cholesterol acyltransferase (LCAT) is an enzyme responsible for the conversion of cholesterol to cholesterol esters on the surface of high density lipoproteins (HDLs). The esterification of cholesterol by LCAT leads to the remodeling of the lipoprotein- HDL and results in the formation of large HDL particles that are known to offer protection against cardiovascular disease (CVD). Decreased activity of LCAT inhibits the esterification of cholesterol in high fat diet fed rats. This leads to high concentration of lipids and lipoproteins in circulation which are at high risk of atherosclerosis and myocardial infarction. Increased oxidative stress may be resulted in the deficiency of LCAT in hypercholesterolemic rats. Acyclic isoprenoid and simvastatin treatment increased the activity of LCAT in hypercholesterolemic rats which increases the concentration of good cholesterol (HDL) which are known to be involved in transport of tissue cholesterol to liver for its excretion. Therefore, the observed increase in LCAT might be due to the blocking of HMG-CoA reductase in acyclic isoprenoid and simvastatin treated hypercholesterolemic rats.

Serum creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) are diagnostic markers of myocardial infarction. These marker enzymes are released from the heart into the blood during myocardial damage due to the accumulation lipids in the myocardium which increases the oxidative stress and thereby damaged the myocardial cells. Once the myocardial cells damaged or destroyed, the cell membrane becomes permeable or may rupture which results in the leakage of these enzymes. This accounts for the increased activities of serum CK-MB and LDH in hypercholesterolemia rats [31]. Treatment with acyclic isoprenoid and simvastatin normalized the activities of these enzymes in hypercholesterolemic rats by blocking the HMG-CoA reductase which reversed the deficiency of LCAT to normal levels.

Atherosclerosis is an inflammatory disease which increases the vascular risk in association with increased basal levels of several cytokines and acute phase reactants [32]. In the present study, we observed the elevated levels of CRP, fibrinogen and TNF-α in hypercholesterolemia suffering rats when compared with control rats. These results corroborate with previous report [33]. Plasma CRP an acute phase reactant has proven remarkably robust as a marker of cardiovascular risk, produced primarily by the liver in response to inflammatory cytokines such as TNF-α, IL-6. CRP may be a more specific predictor of plaque vulnerability and hence future cardiovascular events rather than the extent of atherosclerosis [34]. TNF-α as well as IL-6 affect the coagulation system and metabolism of lipids, causing a procoagulant state and dyslipidemia [35]. Epidemiological studies suggest that fibrinogen predicts coronary diseases better than cholesterol [36]. Fibrinogen contributes to atherogenesis through multiple mechanisms. Elevated levels of plasma fibrinogen observed in HFD fed rats. This may be due to high concentration of triglycerides which accelerates coagulation and impairs fibrinolysis and this could be the reason for the elevated levels of fibrinogen in hyperlipidemic rats. However, administration of acyclic isoprenoid and simvastatin to HFD fed rats significantly decreased the plasma fibrinogen by lowering the plasma triglyceride.

Apart from CRP, other inflammatory markers such as the ESR, total WBC count and platelet count have been shown to be positively correlated with cardiovascular disease and with acute coronary syndrome [37–39]. The ESR appears to be a strong predictor of coronary heart disease [40]. In the present study, the mean values of the ESR, WBC and platelet counts were significantly increased in hypercholestrolemic rats when compared to normal rats. However, the mean levels/counts of ESR, WBC and platelet counts were brought back to near normal level in HFD fed rats upon treatment with acyclic isoprenoid and simvastatin. Previous studies have demonstrated that

neutrophils, eosinophils, and monocytes counts are predictive of coronary artery disease [41]. However, an inverse relation was noted between lymphocyte count and cardiovascular risk [42]. In the present study, administration of acyclic isoprenoid and simvastatin to hypercholesterolemic rats significantly decreased the mean counts of neutrophils and monocytes and increased the mean counts of lymphocytes when compared to untreated hypercholesterolemic rats. No significant difference was observed in the mean counts of eosinophils in all the groups tested. These findings are agreement with Ramesh et al. [41] who reported that the eosinophils count were virtually unrelated to coronary atherosclerosis. Lupeol is a Triterpene compound which has been reported to have antihyperlipedimic and anti-inflammatory potential in high cholesterol diet fed rats [43–45]. Lycopene, carotene and beta carotene are triterpenoidal compounds which are more or less similar to the structure of isoprenoid and reported to have antioxidant,antihyperlipidemic and anti-inflammatory potential in high fat diet fed rats and rabbits which corroborate our findings [46–51]

Conclusion

Based on the results obtained, it can be concluded that treatment of acyclic isoprenoid normalized the levels of circulating lipids in hypercholesterolemic rats by inhibiting the activity of the HMG CoA reductase and increasing the activity of LCAT which reflected on the reduction of creatine kinase -MB, LDH and inflammatory cytokines. The results of biochemical tests together with oil red O staining observations of aorta suggest that acyclic isoprenoid treatment lowers the cholesterol by inhibiting the activity of the HMG CoA reductase which in turn reduces the inflammatory cytokines. The effect produced by acyclic isoprenoid on various parameters was comparable to that of simvastatin- an antihypercholesterolemic drug. Further studies need to explore the molecular mechanism of action of acyclic isoprenoid in lowering cholesterol. Previous studies have demonstrated that oral administration of *Semecarpus anacardium* (crude extract) to high fat diet—and streptozotocin induced type 2 diabetic rats significantly decreased the lipid profiles and inflammatory markers which have also been supported our findings [52]. In addition, Vijayalashmi et al. [53], has already been proved that administration of nut milk extract to normal rats at the dose of 2000 mg/kg b.w produced no mortality.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest concerning this article.

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