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Effect of lupeol and lupeol linoleate on lipemic – hepatocellular aberrations in rats fed a high cholesterol diet

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Cholesterol feeding has been often used to study the etiology of hypercholesterolaemia-related metabolic disturbances. The aim of the present study is to investigate the effects of a pentacyclic triterpene, lupeol, and its ester derivative on hepatic abnormalities associated with hypercholesterolemic rats. Hypercholesterolaemia was induced in male Wistar rats by feeding them with a high cholesterol diet (HCD) containing normal rat chow supplemented with 4% cholesterol and 1% cholic acid, for 30 days. Lupeol and lupeol linoleate were supplemented (50 mg/kg body wt/day) during the last 15 days. Increased hepatic lipid profile along with abnormalities in lipid-metabolizing enzyme activities were seen in hypercholesterolemic rats. An apparent increase in the expression of Acyl-CoA cholesterol acyltransferase mRNA was seen in HCD fed rats. The activities of hepatic marker enzymes, which serve as indices of cellular injury, were altered in HCD fed rats. Treatment with triterpenes significantly modulated the abnormalities induced by hypercholesterolaemia. Also, an increased ($P > 0.001$) faecal excretion of cholesterol and bile acids were observed in lupeol and lupeol linoleate group when compared with HCD fed group. Therefore, it can be concluded that triterpenes treatment afforded substantial protection against the anomalies, which are manifested during the early stage of hypercholesterolemic atherogenesis.

Keywords: Hypercholesterolaemia / Lipid metabolism / Liver / Marker enzymes / Pentacyclic triterpenes

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1 Introduction

Cholesterol is an important constituent of tissues by virtue of its dual role both as a structural component of biological membranes, and as a precursor for steroid hormones and bile acids. Cholesterol feeding has often been used to elevate serum or tissue cholesterol levels to study the etiology of hypercholesterolaemia-related metabolic disturbances [1, 2]. Exogenous hypercholesterolaemia causes fat deposition in the liver and depletion of hepatocyte population. It causes the malfunction of liver, which is apparently presented through microvesicularstenosis due to the intracellular accumulation of lipids [3, 4]. The lipid composition of membranes can be modified by dietary fats [5]. ATPases are membrane-bound enzymic proteins that maintain the ionic gradients between aqueous intra- and extracellular phases, and their activities may be altered by increase in cholesterol levels [6].

Recently, much attention has been focused on the protective biochemical function of naturally occurring compounds in plants and on the mechanisms of their action. Lupeol is a naturally occurring triterpenes found in various fruits and vegetables of many medicinal plants [7–10]. *Crataeva nurvala* Buch Ham (Capparidaceae) is one of the medicinal plants recorded in the Indian System of Medicine [10]. From the stem bark of this plant, lupeol, a pentacyclic triterpene, is isolated which has been shown to exhibit antiurolithic [11], anti-inflammatory [12] and cytoprotective [13] effects in experimental rat models. Somova *et al*. [14] reported that the naturally occurring triterpenes, oleanolic acid and ursolic acid, which are structurally related to lupeol, possess hypoglycemic, antihyperlipidemic (anti-atherosclerotic) and antioxidant activities. Dietary linoleic acid is stated to have hypocholesterolemic effect [15].

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Abbreviations: ACAT, Acyl-CoA cholesterol acyltransferase; **ALP,** alkaline phosphatase; **CES,** cholesterol ester synthetase; **HCD,** high cholesterol diet; **LCAT,** lecithin/cholesterol acyl transferase; **LDH,** lactate dehydrogenase; **LPL,** lipoprotein lipase; **RPL-19,** rat ribosomal protein 19; **TL,** total lipases

Esterification of triterpenes enhanced the efficiency of the parent drug by increasing its bioavailability, penetration and retention ability into the cell membrane [16]. Therefore, lupeol was further esterified with linoleic acid to form lupeol linoleate. In our laboratory, the ester derivative, lupeol linoleate, was found to exhibit better anti-inflammatory [12] and cytoprotective effects [13] than lupeol. So, the biologically favourable properties of triterpenes like hypolipemic, antioxidative and cytoprotective effects are suggestive of their potential to protect the hepatocytes in hypercholesterolemic condition. Hence, the aim of the present investigation is to evaluate the beneficial effects of lupeol and its ester derivative on hepatic lipid profiles, faecal cholesterol and bile acid content, hepatic marker enzymes and lipid-metabolizing enzymes, which are influenced by early stage of hypercholesterolaemia.

2 Materials and methods

2.1 Chemicals

BSA, cholesterol oleate and L-α-phosphatidyl choline were obtained from Sigma chemicals (St. Louis, USA). Cholesterol, cholic acid and all other chemicals and solvents used were obtained from Sisco Research Laboratories (Mumbai, India) and were of analytical grade.

2.2 Isolation and esterification of lupeol

Lupeol was isolated from the stem bark of *C. nurvala* as reported earlier from our laboratory [11]. In brief, *C. nurvala* stem bark powder (500 g) was extracted exhaustively with petroleum ether by cold percolation and the resulting extract was subjected to column chromatography on silica gel (100–200 mesh), eluting successively with hexane, hexane–benzene and benzene. The fractions collected with benzene gave a single component, lupeol (yield, 0.7%) mp at 215°C, which was identified by comparison with an authentic sample. The isolated lupeol was further esterified to lupeol linoleate as reported earlier [12]. Briefly, lupeol was dissolved in dichloromethane, to which pyridine and linoleoyl chloride were added in equivalent moles. The solution was stirred overnight at room temperature. The product obtained was washed with water, dilute acid and then bicarbonate. The dichloromethane extract obtained was dried over anhydrous sodium sulphate and concentrated. The product obtained was lupeol linoleate.

2.3 Animal model

Animals were used after obtaining prior permission and handled according to the University and institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (IAEC No. 02/038/03), Ministry of Social Justice and Empowerment, Government of India. Male albino rats of the Wistar strain (140 ± 10 g) were purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai. The animals were housed in spacious cages and given food and water *ad libitum* under standard conditions of controlled temperature (25 ± 2°C), with 12 h/12 h day/night cycle.

2.4 Experimental protocol

The animals were divided into six groups of six rats each. Group I served as vehicle control. In Groups II, V and VI, the rats were fed a high cholesterol diet (HCD) containing normal rat chow, supplemented with 4% cholesterol and 1% cholic acid for 30 days. Groups V and VI rats were also treated with lupeol and lupeol linoleate (50 $mg^{-1}kg^{-1}day^{-1}$ orally), respectively, for the last 15 days along with the HCD. In Groups III and IV, the rats received lupeol and lupeol linoleate for the last 15 days, which acted as drug controls. Corn oil was used as a vehicle to dissolve triterpene, and for administration. At the end of the experimental period, all the animals were sacrificed by cervical decapitation. Liver tissues were immediately excised and rinsed in ice-cold physiological saline and the following experiments were done from tissue homogenates.

2.5 Estimation of tissue lipids

Lipids were extracted from the tissues according to the method of Folch *et al.* [17] using chloroform-methanol mixture 2:1 v/v. Total cholesterol [18], triglycerides [19] and phospholipids [20] were estimated by standardized methods. Free cholesterol was precipitated as its digitonin according to the method of Sperry and Webb [21] and cholesterol in the precipitate was estimated by the method of Parekh and Jung [18] and free fatty acid was estimated by standardized method [22], in the liver tissue.

2.6 Estimation of faecal cholesterol and bile acids

Faecal samples were collected from the animals of each group for the last 5 days of the experimental period and were dried and powdered. Faecal sterols and bile acids were extracted by the method of Jayakumari and Kurup [23]. Lyophilized samples were extracted with 1 N NaOH in 95% ethanol at 80°C for 2 h. The residue was extracted once with 95% ethanol. The fraction containing sterols were pooled and extracted with petroleum ether, after diluting with an equal volume of water. Cholesterol content was estimated by the above-mentioned method [18]. For the iso-

lation of bile acids, the alkaline aqueous solution left after extraction with petroleum ether was acidified to pH 1.0, after removing the residual ethanol in a water bath. Bile acids were extracted with chloroform/methanol 2:1 v/v and estimated by the method of Levin *et al.* [24].

2.7 Enzymatic indices of cellular integrity

Lactate dehydrogenase (LDH) was assayed by the method of King [25]. The method is based on the ability of LDH to convert lactate to pyruvate with the help of the coenzyme nicotinamide adenine dinucleotide (NAD). The pyruvate formed was made to react with DNPH in HCl. The hydrazone formed turns into an orange coloured complex in alkaline medium, which was measured at 420 nm. Alkaline phosphatase (ALP) activity was assayed using disodium phenyl phosphate as substrate [26] and expressed as micromoles of phenol liberated/min/mg of protein. The aminotransferases, aspartate and alanine transaminases, AST and ALT, respectively [27], were determined and expressed in terms of μmoles of pyruvate liberated/min/mg of protein at 37°C.

2.8 Assay of transmembrane ATPases activity

Na^+,K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase were determined by the method of Bonting [28], Ohnishi *et al*. [29] and Hjerten and Pan [30], respectively. The enzyme activity is expressed as a function of inorganic phosphorus liberated during the breakdown of ATP. Protein content in the rat hepatic tissue and plasma were determined by the method of Lowry *et al.* [31].

2.9 Assay of lipid-metabolizing enzymes

Total lipases (TL) were assayed by the method of Bier [32]. The substrate for the enzyme reaction was *p*-nitrophenyl acetate and the colour developed was read at 420 nm and the activity expressed as micromoles of *p*-nitrophenol liberated/h/mg protein. Lipoprotein lipase (LPL) was assayed by the method of Baginsky [33]. The colour developed was read at 430 nm. The LPL activity was expressed as μmoles of free fatty acids liberated/h/mg protein. Lecithin:cholesterol acyl transferase (LCAT) activity was assayed by the method of Legraud *et al*. [34] with modifications by Hitz *et al*. [35], wherein the colour developed was read at 540 nm. LCAT activity was assessed in terms of nanomoles of cholesterol esterified/h/mg protein. Cholesterol ester synthetase (CES) was assayed by the method of Kothari *et al*. [36] and the colour developed was read at 540 nm. Enzyme activity was expressed as nanomoles of cholesterol esterified/h/mg protein. Cholesterol ester hydrolase (CEH) activity was estimated by the method of Kothari *et al*. [37] with slight modification by Kritchevsky and Kothari [38]. In this method, the free cholesterol liberated from cholesterol oleate was precipitated. The precipitate was processed and then dissolved in 3.0 mL of uranyl acetate reagent and the cholesterol content was estimated as described earlier. The enzyme activity was measured as nanomoles of cholesterol liberated/h/mg protein.

2.10 Reverse transcriptase-PCR (RT-PCR) analysis

To determine the expression of Acyl-CoA cholesterol acyltransferase (ACAT) mRNA in each group, total RNA was isolated from hepatic tissue using one-step RNA isolation kit (Trizol, Medox Biotech, India). One-step RT-PCR was performed in a total volume of 50 μL according to the manufacturer's instructions (Quagen one-step RT-PCR kit, Germany). Reverse transcription was performed at 50°C for 60 min followed by PCR amplification in an eppendorf master cycler thermocycles. The amplification was operated for initial denaturation step at 94°C for 15 min; then 39 cycles at the following conditions: 94°C for 15 s (denaturation), 62°C for 40 s (annealing), and 72°C for 30 s (extension) and further extension at 72°C for 5 min. The following primer pair was used; Sense 5′- CCT CCC GGT TCA TTC TGA TA -3′; antisense 5′- ACA CCT GGC AAG ATG GAG TT -3′ [61]. The expected product size of ACAT was 370 bp. The PCR products were run on a 2% agarose gel in Tris-borate-EDTA buffer. Rat ribosomal protein 19 (RPL-19) was used as an internal standard.

2.11 Statistical analysis

The values are expressed as mean ± SD for six animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using SPSS software package for Windows. Post hoc testing was performed for intergroup comparisons using the least significance difference (LSD) test, significance at *P*-values <0.001, <0.01, <0.05 have been given respective symbols in the tables.

3 Results

In the present study, HCD feeding for 30 days was chosen as the experimental model of early phase atherogenesis. The role of lupeol and lupeol linoleate in countering the hepatocellular aberration accompanying diet-induced hypercholesterolaemia has been investigated.

Table 1 shows the effect of lupeol and lupeol linoleate on hepatic lipid profile in experimental animals. Increased

Table1. Effect of lupeol and lupeol linoleate on hepatic lipid profile in HCD fed rats (Values are expressed as mean ± SD for six animals)

Lipids (mg/g of wet tissue)	Group I control	Group II HCD	Group III lupeol alone	Group IV lupeol linoleate alone	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
Total cholesterol	4.5 ± 0.4	7.5 ± 0.8[a)*]	4.5 ± 0.4	4.4 ± 0.4	5.5 ± 0.6[a)†b)*]	5.2 ± 0.5[a)‡b)*]
Free cholesterol	3.2 ± 0.3	4.5 ± 0.5[a)*]	3.2 ± 0.3	3.2 ± 0.3	3.7 ± 0.4[a)†b)*]	3.3 ± 0.3[b)*]
Triglycerides	5.9 ± 0.6	10.1 ± 1.1[a)*]	5.9 ± 0.5	5.8 ± 0.6	7.2 ± 0.8[a)†{b)*]	6.3 ± 0.7[b)*c)‡]
Free fatty acid	2.8 ± 0.3	3.8 ± 0.4[a)*]	2.8 ± 0.3	2.8 ± 0.3	3.6 ± 0.3[b)*]	3.0 ± 0.3[b)*]
Phospholipids	23 ± 2	38 ± 4[a)*]	23 ± 2	23 ± 2	29 ± 3[a)*b)*]	26 ± 2[b)*c)‡]

a) Comparisons are made between Groups I and II, V and VI.
b) Comparisons are made between Groups II and V, VI.
c) Comparisons are made between Groups V and VI.
*, † and ‡ represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

Table 2. Effect of lupeol and lupeol linoleate on the levels of faecal cholesterol and bile acids in HCD fed rats (Values are expressed as mean ± SD for six animals)

Parameters (mg/day *per* rat)	Group I control	Group II HCD	Group III lupeol alone	Group IV lupeol linoleate alone	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
Cholesterol	27 ± 2	241 ± 21[a)*]	29 ± 3	31 ± 3	336 ± 26[a)*b)*]	360 ± 24[a)*b)*]
Cholic acids	11 ± 1	21 ± 2[a)*]	11 ± 1	11 ± 1	41 ± 4[a)*b)*]	43 ± 4[a)*b)*]
Deoxy cholic acid	10 ± 1	22 ± 2[a)*]	10 ± 1	10 ± 1	31 ± 2[a)*b)*]	33 ± 3[a)*b)*]

a) Comparisons are made between Groups I and II, V and VI.
b) Comparisons are made between Groups II and V, VI.
c) Comparisons are made between Groups V and VI.
* represents statistical significance at $P < 0.001$

Table 3. Effect of lupeol and lupeol linoleate on the marker enzymes in hepatic tissue (Values are expressed as mean ± SD for six animals)

Marker enzymes (U/mg protein)	Group I control	Group II HCD	Group III lupeol alone	Group IV lupeol linoleate alone	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
LDH	14 ± 1	18 ± 2[a)*]	14 ± 1	14 ± 1	15 ± 2[b)†]	15 ± 1[b)†]
ALP	1.5 ± 0.2	3.0 ± 0.3[a)*]	1.5 ± 0.2	1.4 ± 0.1	1.8 ± 0.2[a)‡b)*]	1.6 ± 0.2[b)*]
ALT	0.1 ± 0.01	0.2 ± 0.02[a)*]	0.1 ± 0.02	0.1 ± 0.01	0.2 ± 0.02[a)*b)*]	0.2 ± 0.01[a)‡b)*c)‡]
AST	0.1 ± 0.01	0.3 ± 0.03[a)*]	0.1 ± 0.01	0.1 ± 0.01	0.2 ± 0.02[a)†b)*]	0.2 ± 0.01[b)*]

Units: LDH: μmoles × 10^{-1} of pyruvate formed; ALP: μmoles × 10^{-2} of phenol liberated; ALT, AST: μmoles × 10^{-2} of pyruvate liberated.
a) Comparisons are made between Groups I and II, V and VI.
b) Comparisons are made between Groups II and V, VI.
c) Comparisons are made between Groups V and VI.
*, † and ‡ represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

levels of liver total cholesterol, free cholesterol, triglycerides, free fatty acid and phospholipids ($P < 0.001$) were observed in HCD fed rats (group II). Treatment with lupeol and lupeol linoleate significantly reduced the level of lipids (Groups V and VI). These results imply the hypolipidemic effect of triterpenes.

Table 2 depicts the effect of triterpenes on faecal cholesterol and bile acids in hypercholesterolemic rats. The concentration of faecal cholesterol, cholic acid and deoxy cholic acid were significantly higher ($P < 0.001$) in hypercholesterolemic group than control. Lupeol and lupeol linoleate treated groups showed still higher ($P < 0.001$) faecal cholesterol and bile acids excretion than HCD fed rats.

The activities of hepatic marker enzymes in control and experimental groups is presented in Table 3. The hepatic enzymes, LDH, ALP, aspartate aminotransferase and alanine aminotransferase were increased significantly ($P < 0.001$) in HCD fed rats (group II). Administration of lupeol and lupeol linoleate reverted the above changes to near control.

Table 4. Effect of lupeol and lupeol linoleate on hepatic ATPases activities (Values are expressed as mean ± SD for six animals)

Hepatic ATPases	Group I control	Group II HCD	Group III lupeol alone	Group IV lupeol linoleate alone	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
Na^+,K^+-ATPase	0.6 ± 0.06	0.4 ± 0.04[a)*]	0.6 ± 0.06	0.7 ± 0.06	0.56 ± 0.06[a)‡b)*]	0.7 ± 0.06[b)*]
Ca^{2+}-ATPase	0.6 ± 0.05	0.4 ± 0.04[a)*]	0.6 ± 0.05	0.6 ± 0.06	0.5 ± 0.04[a)†b)*]	0.5 ± 0.05[a)‡b)*]
Mg^{2+}-ATPase	0.6 ± 0.06	0.4 ± 0.04[a)*]	0.6 ± 0.06	0.6 ± 0.06	0.5 ± 0.05[a)†b)*]	0.6 ± 0.06[a)†b)*c)†]

Units of enzyme activity: ATPases: micromoles of Pi formed/min/mg protein.
a) Comparisons are made between Groups I and II, V and VI.
b) Comparisons are made between Groups II and V, VI.
c) Comparisons are made between Groups V and VI.
*, † and ‡ represent statistical significance at P <0.001, P <0.01 and P <0.05, respectively.

Table 5. Activities of lipid-metabolizing enzymes in plasma and hepatic tissues of control and experimental rats (Values are expressed as mean ± SD for six animals)

Enzymes (U/mg protein)	Group I control	Group II HCD	Group III lupeol alone	Group IV lupeol linoleate alone	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
Plasma						
TL	2.3 ± 0.2	3.6 ± 0.4[a)*]	2.3 ± 0.2	2.3 ± 0.2	2.8 ± 0.3[a)†b)*]	2.7 ± 0.3[a)‡b)*]
LPL	5.0 ± 0.5	3.4 ± 0.4[a)*]	5.1 ± 0.5	5.1 ± 0.5	4.4 ± 0.4[a)‡b)*]	4.6 ± 0.5[b)*]
LCAT	7.6 ± 0.7	5.5 ± 0.6[a)*]	7.5 ± 0.7	7.6 ± 0.7	6.6 ± 0.6[a)‡b)†]	6.8 ± 0.6[a)‡b)†]
CES	4.2 ± 0.3	6.0 ± 0.4[a)*]	4.2 ± 0.5	4.2 ± 0.5	4.9 ± 0.4[a)†b)*]	4.6 ± 0.4[b)*]
CEH	5.9 ± 0.4	4.4 ± 0.5[a)*]	5.8 ± 0.5	5.9 ± 0.6	5.2 ± 0.4[a)‡b)†]	5.4 ± 0.5[b)*]
Liver						
TL	2.5 ± 0.2	4.8 ± 0.5[a)*]	2.4 ± 0.2	2.5 ± 0.2	3.6 ± 0.4[a)*b)*]	3.3 ± 0.3[a)*b)*c)‡]
CES	14 ± 1	23 ± 3[a)*]	14 ± 2	14 ± 2	19 ± 2[a)*b)*]	19 ± 2[a)*b)*]
CEH	19 ± 2	14 ± 2[a)*]	19 ± 2	19 ± 2	18 ± 2[b)†]	18 ± 2[b)*]

Units: TL, μmoles of *p*-nitrophenol liberated/h; LPL, μmoles of free fatty acids liberated/h; LCAT, nanomoles of cholesterol esterified/h; cholesterol ester synthase (CES), nanomoles of cholesterol esterified/h; cholesterol ester hydrolase (CEH), nanomoles of cholesterol liberated/h.
a) Comparisons are made between Groups I and II, V and VI.
b) Comparisons are made between Groups II and V, VI.
c) Comparisons are made between Groups V and VI.
*, † and ‡ represent statistical significance at P <0.001, P <0.01 and P <0.05, respectively.

The activities of ATPases in response to the HCD fed and triterpene treated groups are given in Table 4. The activities of Na^+,K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase were decreased (33, 38 and 36%, respectively) in HCD fed rats (group II). There was a significant increase in the activities of ATPases with triterpenes treatment; thereby indicating that triterpenes protect ATPases from the HCD-induced depletion.

Table 5 portrays the effect of triterpenes on lipid-metabolizing enzymes in HCD fed rats. The untreated HCD fed group exhibited altered activities (P <0.001) of the enzymes that are responsible for the transport and metabolism of lipids. Lupeol and lupeol linoleate influenced these enzymes favourably, which may be attributed to the hypocholesterolemic nature of triterpenes.

Figure 1 represents the mRNA levels of ACAT in control and experimental groups. A single transcript (370 bp) was observed in all the groups. RT-PCR analysis of ACAT showed a significant (P <0.001) increase in the HCD fed rats, while the expression pattern of ACAT mRNA in triterpenes-treated (Groups V and VI) animals was decreased when compared to HCD fed animals.

4 Discussion

Increased cholesterol consumption can induce fatty liver, hepatic steatosis or hypertrophy of the liver [39]. Lowering cholesterol level may lower the risk of cardiovascular disease [40], and enormous efforts have been expended to achieve this aim. In general, the blood cholesterol concentration in the body is regulated by cholesterol biosynthesis, removal of circulating cholesterol, dietary cholesterol absorption and the excretion of cholesterol and bile acids in feces [41]. So, investigation of naturally occurring com-

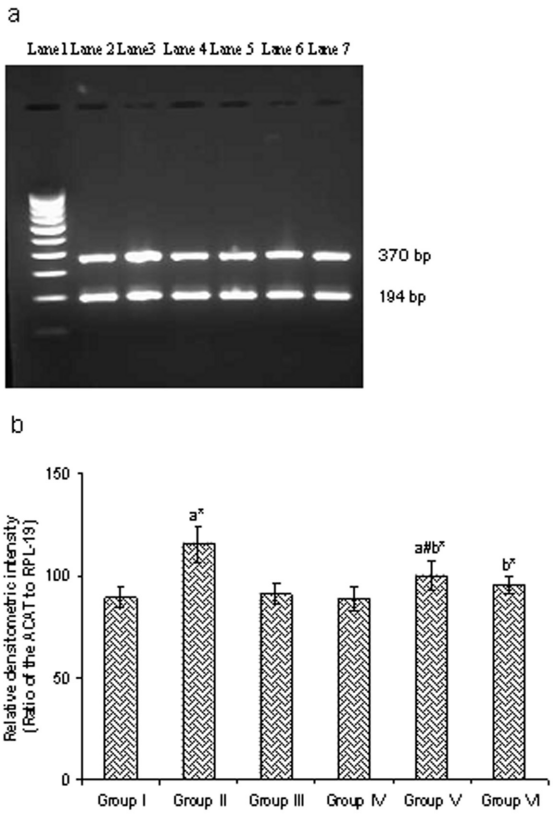

Figure 1. Effect of lupeol and its ester derivative on the expression pattern of ACAT mRNA levels in liver of HCD fed rats. (a) The 370 and 194 bp fragments represent ACAT transcript and RPL-19 as internal standard, respectively; lane 1: 100 bp DNA ladder, lane 2: control, lane 3: HCD fed group, lanes 4 and 5: lupeol and lupeol linoleate alone treated groups, respectively, lane 6: HCD + lupeol, lane 7: HCD + lupeol linoleate. (b) Relative densitometric intensity levels of ACAT mRNA expression compared to values for RPL-19 mRNA. Values are expressed as mean ± SD for six animals. Comparisons are made between: a: Groups I and II, V and VI; b: group II and V, VI; c: group V and VI. *, # and @ represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

pounds as regulators of cholesterol homeostasis has particularly gained therapeutic importance for the treatment of hyperlipidemia. The present study was aimed to investigate the effect of triterpenes on lipid profile, lipid metabolism and modulation of some biochemical variables associated with hypercholesterolemic rats.

Cellular cholesterol homeostasis is very important for the prevention of cardiovascular disease. In the present study, the levels of total cholesterol, free cholesterol, triglycerides, phospholipids and free fatty acids were increased in HCD fed rats. Dietary cholesterol has been reported to increase the synthesis of fatty acids and triacylglycerols in rat liver, thereby leading to an accumulation of triacylglycerols in the liver [42]. Treatment with triterpenes markedly decreased the levels of hepatic lipid profile, when compared with the hypercholesterolemic group. This supports the hypolipidemic effect of triterpenes. Our earlier report showed that lupeol and its ester reduced serum total cholesterol level by 51.87 and 62.6%, respectively [43]. Phytosterols decreased the absorption and incorporation of dietary and biliary cholesterol into micelles, owing to their structural similarity with cholesterol [44]. The triterpene alcohols found in the sheanut unsaponifiable material are structurally similar to phytosterols in comprising a tetracyclic ring of steroid nature. Schmidt *et al.* [45] reported that sheanut oil spread, containing triterpene alcohols like α-amyrin, β-amyrin, lupeol and germanicol was able to reduce the serum total and LDL cholesterol. The observed decrease in hepatic cholesterol by triterpenes in the present study can also be attributed to the same property as they bear similarity with phytosterols.

Cholesterol is excreted by vertebrates *via* two quantitatively important routes, the sterol and bile acid pathways. Aoyama *et al*. [46] suggested that the bile acid pathway is quantitatively important and changes in the rate of this pathway should influence tissue cholesterol levels. Increased excretion can consequently reduce the pool of bile acids in liver and enhance cholesterol catabolism to bile acids in liver [47]. A significant increase in cholesterol and bile acids excretion was observed in the triterpenes-treated groups, compared to HCD fed rats. Reduced absorption of cholesterol together with enhanced degradation in response to lupeol and lupeol linoleate administration may significantly contribute to the decrease in cholesterol content in serum and liver. Increased excretion of cholesterol and bile acids were seen in HCD fed rats as compared to their controls, which may be due to the higher intake of cholesterol.

Hypercholesterolaemia has been shown to promote ischemic tissue damage, by enhancing the vulnerability of the microcirculation to the deleterious effects of ischemia and other inflammatory stimuli [48]. The activities of hepatic enzymic markers of cellular damage and inflammation such as LDH, ALP, ALT and AST were elevated in HCD fed animals. High levels of AST are found in heart, liver, skeletal muscle and kidney, whereas ALT is abundant in liver. Liver alterations produce considerable increases in both enzymes, particularly on ALT. The evaluation of ALP is of great value in the differential diagnosis of liver alterations [49]. Following injury, accumulation of IL-6 can lead to the production of adenosine, a potent vasodilator and anti-inflammatory mediator catalyzed by ALP, and subsequent protection from ischemic injury [50]. Such an induction of ALP activity may substantiate the increased activity of this enzyme in atherogenic group. Treatment with triterpenes restored the enzyme activities, which may be attributed to its salient anti-inflammatory property [12].

Membrane cholesterol has been shown to modulate the activities of numerous membrane-associated processes such as glucose transport, anion transport and other events, its effect being either inhibition or activation [51]. ATPases are membrane-bound enzymic proteins that maintain the ionic gradients between aqueous intra- and extracellular phases. In the present study, reduction in the activities of Na^+,K^+ ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in HCD fed animals may be due to oxidation of membrane lipids and proteins. Na^+,K^+-ATPase is an integral oligomeric membrane-bound enzyme, which plays a key role in the active transport of monovalent cations across membranes. The activity of this enzyme is very sensitive to the action of various bioregulators and is dependent on the lipid state of the membrane [52, 53]. Membrane Ca^{2+}-ATPase is responsible for fine tuning of intracellular calcium [54]. Lupeol and lupeol linoleate treatment enhanced the activities of these ATPases. This suggests that triterpenes protect attenuation in ion pump ATPases due to hypercholesterolaemia, apparently by decreasing the cholesterol level and limiting the degree of oxidation of membrane lipids and proteins.

LCAT is an enzyme involved in the transesterification of cholesterol, the maturation of HDL and the flux of cholesterol from cell membranes into HDL [55]. Plasma LCAT activity is decreased in HCD fed rats. This result is consistent with previous reports on the hypercholesterolemic condition [56]. The decreased activity of this enzyme may be attributed to the low availability of its substrate HDL in hypercholesterolemic rats. LPL is anchored to the luminal surface of the vascular endothelium and its primary function is to hydrolyze core triglycerides in lipoproteins and convert them into remnant particle. An earlier study has shown that plasma LPL activity has been found to be depressed in hyperlipidemic rats [57]. This decline in the activity of LPL in plasma of the untreated hypercholesterolemic rats may be one of the reasons for the high levels of triglycerides in these animals. In the present study, the activities of TL, CES and CEH were increased in plasma and hepatic tissue of HCD fed rats. The amount of free cholesterol within the cell is regulated by the activity of CES and CEH. Brecher and Chobanian [58] reported an increase in CES activity in atherosclerotic rabbit aorta. Decreased CEH activity may be one of the factors responsible for the rise in free cholesterol levels in the hypercholesterolemic group. Treatment with triterpenes restored the activities of these lipid-metabolizing enzymes to near normal levels. This modulatory effect may be due to their ability to decrease cholesterol absorption and triglyceride level, and increase HDL cholesterol level in hypercholesterolemic condition.

ACAT is the enzyme primarily responsible for the esterification of cholesterol in all mammalian cells and is implicated in the intestinal mucosal cholesterol absorption and synthesizes the cholesterol esters both to flow into very low-density lipoproteins (VLDL) and to store in fatty cells [59]. It is presumed to play a crucial role in foam cell formation from macrophages and vascular smooth muscle cells in the arterial wall under pathophysiological state [60]. An increased mRNA expression of ACAT in RT-PCR was observed in hypercholesterolemic conditions in this study. This result corroborated with earlier findings, where the increased ACAT mRNA expression occurs in hypercholesterolemic rats [61]. The drug which could down-regulate the expression of the enzyme can lower plasma cholesterol and triglyceride levels by inhibiting absorption and storage of metabolic fatty acid, and subsequently reduce VLDL production in liver which might directly block atherosclerotic lesion formation reducing the possibility of vascular attacks. In the present study, the triterpenes treatment lowered the expression of ACAT in HCD fed rats. Recently, Lee *et al*. [62] reported that structurally similar pentacyclic triterpenes oleanolic acid, ursolic acid and betulinic acid, isolated from the leaves of *L. lusidus* TURCZ, exhibited ACAT inhibitory activities.

In conclusion, the results show that triterpenes are effective in alleviating the hepatocellular abnormalities in hypercholesterolemic rats by regulating lipid metabolism and protecting membrane integrity. Further, hypocholesterolemic effect of triterpenes may be attributed to a decrease in cholesterol absorption and increase in the faecal excretion of cholesterol and bile acids. Of the two compounds tested, lupeol linoleate appeared to be more effective than lupeol.

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