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Protective effect of lupeol and lupeol linoleate in hypercholesterolemia associated renal damage

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Abstract The association between hypercholesterolemia and kidney damage has been well known for last few decades. The oxidative stress and inflammatory responses are involved in renal injury, which is upregulated in hypercholesterolemic condition. The present study is aimed to evaluate the possible effect of lupeol and its ester derivative, lupeol linoleate in renal damage associated with hypercholesterolemic rats. Hypercholesterolemia was induced in male Wistar rats by feeding them with a high cholesterol diet (HCD) comprising 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) to HCD fed rats during the last 15 days. Increased levels of renal total cholesterol, triglycerides and phospholipids, along with altered serum biochemical parameters of tissue injury indices and elevated activities of renal marker enzymes (lactate dehydrogenase and alkaline phosphatase) were noted in HCD fed rats. Elevated lipid peroxidation levels coupled with decreased antioxidant status (enzymatic and non enzymatic antioxidants) were observed in hypercholesterolemic rats, which indicate the onset of oxidative changes in the renal tissue. Renal lysosomal acid hydrolase activities (ACP, β-Glu, β-Gal, NAG and Cat-D) and acute phase proteins like C-Reactive protein and fibrinogen were significantly increased in HCD fed rats, which further indicates the heightening of inflammation. In addition, histopathological findings also confirmed the renal damage in hypercholesterolemic condition. Lupeol and lupeol linoleate effectively reverted the above abnormalities and was comparable with that of the control. These observations highlight the protective effect of lupeol and its ester derivative in ameliorating the renal injury associated with hypercholesterolemia.

Keywords Hypercholesterolemia · Triterpene · Renal damage · Oxidative and inflammatory stress

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Introduction

Lipids have been reported to play a potential role in the pathogenesis of progressive glomerulosclerosis [1]. It has been demonstrated that the experimental hypercholesterolemia caused by cholesterol feeding leads to spontaneous arterial constriction and/or increased reactivity to various vasoconstrictors, thereby causing renal vasoconstriction and associated decrease in glomerular filtration rate [2, 3], which eventually results in ischemic renal failure. Romero et al. [4] reported that a cholesterol-rich diet leads to early focal glomerulosclerotic lesions, which exacerbate with a reduction in renal mass. Hence, hypercholesterolemia is an independent risk factor for the development of renal injury [5]. Moreover, hypercholesterolemia was found to cause heightening of inflammation [6] and also increase oxidative stress in renal tissue [7]. These may be additional mechanisms explaining the possible deleterious role of hypercholesterolemia in renal failure.

The currently available cholesterol-lowering drugs have one or more adverse and undesirable side effects [8]. Hence, an attempt was made to search for herbal products to have beneficial effect for lowering cholesterol in traditional medicine. *Crataeva nurvala* is one of the medicinal plants recorded

in the Indian system of Medicine. Lupeol, a pentacyclic triterpene was isolated from the stem bark of *Crataeva nurvala* in our laboratory [9] and has been shown to exhibit antiurolithic [9], anti-inflammatory [10] and cytoprotective [11] effects in experimental rat models. Esterification of triterpenes enhanced the efficiency of the parent drug by increasing its bioavailability, penetration and retention ability into the cell membrane [12]. 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 [10] and cardioprotective effects [11] than lupeol. Furthermore, our previous investigations on the effect of lupeol and lupeol linoleate in hypercholesterolemic condition have shown reduction in serum total cholesterol level by 51.87% and 62.6% respectively [13].

Nutrition-related factors (such as blood pressure, cholesterol) participate an important role in the development of cardiovascular diseases. High cholesterol diet (HCD) plays an important role in the initiation and progression of hypercholesterolemia induced atherogenesis. Hypercholesterolemia is known to induce the renal damage by augment lipid levels and subsequent increase oxidative stress and inflammation. Therefore, the hypolipemic, antioxidative and anti-inflammatory treatment to hypercholesterolemia would attenuate the progression of renal damage. The aim of the present investigation is to evaluate the possible effects of lupeol and lupeol linoleate on renal lipemic oxidative abnormalities and inflammation associated in the early stage of atherogenesis with initiative to identify a successful therapy.

Materials and methods

Drugs and chemicals

Lupeol was isolated from the stem bark of *Crataeva nurvala* as reported earlier in our laboratory [9]. The isolated lupeol was further esterified to lupeol linoleate by adding equimolar amounts of pyridine and linoleoyl chloride as reported by the method of Geetha and Varalakshmi [10]. Bovine serum albumin, 4-nitrophenyl-*N*-acetyl glucosaminide, *p*-nitrophenyl *β*-D-glucuronide and 1,1,3,3-tetraethoxy propane were obtained from Sigma Chemicals Co, St. Louis, USA. Cholesterol, cholic acid, reduced glutathione, dinitrobenzoic acid, hydrogen peroxide (H_2O_2) and all other chemicals and solvents used were obtained from Sisco Research Laboratories, Mumbai, India and were of analytical grade.

Animals, diet and treatment

Animals were maintained as per national guidelines and protocols and approved by the institutional ethical committee (IAES No. 02/038/03). 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 under conditions of controlled temperature (25 ± 2°C) with 12 h/12 h day–night cycle. Since cholesterol diet produces severe hypercholesterolemic and vascular atherosclerotic lesions, feeding a HCD for 30 days was chosen as the experimental model of early phase atherogenesis. The animals were divided into six groups of six rats each. Group I served as vehicle control. Rats in group II, V and VI were fed a HCD comprising normal rat chow supplemented with 4% cholesterol and 1% cholic acid for 30 days. Addition of cholic acid enhances the hypercholesterolemic effect of cholesterol feeding. Groups V and VI were treated with lupeol and lupeol linoleate (50 mg/kg/day/orally), respectively for the last 15 days along with the HCD. Corn oil was used for dissolving lupeol and so corn oil is used as the vehicle in this study. Group III and IV rats received lupeol and lupeol linoleate alone for last 15 days of experimental period and served as drug controls.

At the end of the experimental period, all the animals were killed by cervical decapitation. Blood was collected for various analyses. Kidney was excised and homogenized in ice-cold 0.01 M Tris–HCl buffer, pH 7.4 to give a 10% homogenate.

Assay of tissue lipids

Lipids were extracted from the tissues according to the method of Folch et al. [14] using chloroform–methanol mixture (2:1 v/v). Total cholesterol was estimated by the method of Parekh and Junk [15]. The tissue extract was made to react with ferric chloride–uranyl acetate reagent followed by sulphuric acid–ferrous sulphate reagent. The colour developed was read at 540 nm. Triglycerides [16], free fatty acid [17] and phospholipids [18] were estimated by standardized methods, in the renal tissue.

Biochemical estimation in blood

Serum albumin was estimated by the method of Reinhold [19] and the units were expressed as g/dl in serum. Serum urea was estimated by the method of Natelson et al. [20], in which urea forms a coloured complex with diacetyl monoxime in acetic medium on heating and the colour developed was read at 480 nm. Uric acid [21] and creatinine [22] were measured by standard procedures.

Enzymatic indices of cellular integrity

Lactate dehydrogenase (LDH) was assayed by the method of King [23]. 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 colour complex in alkaline medium, which was measured at 420 nm. Alkaline phosphatase (ALP) activity was assayed using disodium phenyl phosphate as substrate [24] and expressed as μmoles of phenol liberated/min/mg of protein. Total protein is estimated by Lowry et al. [25].

Assessment of lipid peroxidation

Lipid peroxidation in the renal tissue was determined by the procedure of Högberg et al. [26]. Malondialdehyde (MDA), formed as an end product of peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with thiobarbituric acid (TBA) to generate a coloured product which absorbs at 532 nm. The ferrous sulphate and ascorbate induced lipid peroxidation system contained 10 mM ferrous sulphate and 0.2 mM ascorbate as inducers [27].

Determination of antioxidant levels

Antioxidant enzymes were assayed in kidney of experimental groups. Superoxide dismutase (SOD) was assayed by the method of Marklund and Marklund [28]. The degree of inhibition of the autooxidation of pyrogallol at an alkaline pH by SOD was used as a measure of the enzyme activity. Catalase activity was assayed by the method of Sinha [29]. Glutathione peroxidase (GPx) was assayed by the method of Rotruck et al. [30]. The utilization of glutathione was used to express the activity. Reduced glutathione (GSH) in the renal tissue was determined by the method of Moron et al. [31]. This method was based on the reaction of GSH with 5,5′ dithiobis (2-nitrobenzoic acid) to give a compound that absorbs at 412 nm. Ascorbic acid was estimated by the method of Omaye et al. [32]. Vitamin E was estimated by the method of Desai [33].

Assay of lysosomal enzymes

Acid phosphatase (ACP) was assayed by the method of King [24] using disodium phenyl phosphate as the substrate. *N*-Acetyl glucosaminidase (NAG) activity was assessed by the method of Maruhn [34] using 4-nitrophenyl-*N*-acetyl glucosaminide as the substrate and its activity was expressed as μmoles of *p*-nitrophenol formed/h/mg protein. *β*-Glucuronidase (*β*-Glu) was assayed by the method of Kawai and Anno [35]; the substrate for the enzyme reaction was *p*-nitrophenyl *β*-D-glucuronide. The activity of *β*-Galactosidase (*β*-Gal) was expressed as μmoles of *p*-nitrophenol liberated/h/mg protein, where the colour developed was read at 400 nm against a reagent blank in a photochem colorimeter [35]. Cathepsin-D (Cat-D) was estimated by the method of Etherington [36]. The incubation mixture contained the enzyme homogenate and buffered substrate (1.5% haemoglobin in sodium acetate buffer). The colour developed was read at 640 nm and the enzyme activity was assessed in terms of tyrosine liberated.

Biochemical indices of inflammation: appraisal of C-reactive protein and fibrinogen levels

C-reactive protein (CRP) was quantitatively determined by the reagent CRP-Turbilatex agglutination assay kit (SPINREACT, Spain). It is a quantitative turbidometric assay for the measurement of CRP in plasma. In short, antibody coated latex particles are agglutinated with the samples containing CRP; the agglutination causes an absorbance range dependent upon the CRP contents of the sample that can be quantified by comparison with a calibrator of known CRP concentration. The absorbance was read at 540 nm. Plasma fibrinogen was estimated by the method of Lempert [37]. Fibrinogen levels were expressed as mg/dl plasma.

Histopathological studies

Portions of kidney tissues were fixed in 10% formalin. The washed tissues were dehydrated in the descending grades of isopropanol and finally cleared in xylene before being embedded in molten paraffin wax. Sections were cut to 5 μm thickness, stained with haematoxylin and eosin (H&E). The sections were then viewed under a light microscope (Nikon microscope ECLIPSE E400, model 115, Japan) for histopathological changes.

Statistical analysis

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

Results

The role of lupeol and lupeol linoleate in combating the renal aberrations, accompanying diet-induced hypercholesterolemia were investigated. Table 1 represents the tissue lipid status in hypercholesterolemia-induced and treated group along with the control values. The levels of renal total cholesterol, free fatty acid, triglycerides and

Table 1 Effect of lupeol and lupeol linoleate on lipid profile in kidney in HCD fed rats

Lipids profile	Group I control	Group II HCD	Group III lupeol	Group IV lupeol linoleate	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
Total cholesterol	5.14 ± 0.54	7.28 ± 0.77^{a*}	5.15 ± 0.54	5.09 ± 0.52	6.16 ± 0.59$^{a\#b\#}$	5.64 ± 0.67^{b*}
Triglycerides	5.62 ± 0.58	7.07 ± 0.63^{a*}	5.60 ± 0.59	5.54 ± 0.58	6.17 ± 0.54$^{b@}$	5.74 ± 0.59^{b*}
Free fatty acid	2.05 ± 0.20	3.11 ± 0.34^{a*}	2.01 ± 0.19	2.04 ± 0.19	2.35 ± 0.23$^{a@b*}$	2.26 ± 0.22^{b*}
Phospholipids	14.35 ± 1.58	18.32 ± 1.90^{a*}	14.38 ± 1.57	14.29 ± 1.52	16.04 ± 1.16$^{b@}$	15.54 ± 1.15$^{b\#}$

Values are expressed as mean ± SD for six animals. Units: Total cholesterol, triglyceride, free fatty acids and phospholipids—mg/g of wet tissue. Comparisons are made among: a—groups I and II, III, IV, V, VI; b—groups II and V, VI. The symbols represent statistical significance at * $P < 0.001$, $^{\#}$ $P < 0.01$ and $^{@}$ $P < 0.05$

Table 2 Effect of lupeol and lupeol linoleate on serum biochemical parameters of tissue injury indices in HCD fed rats

Parameters	Group I control	Group II HCD	Group III lupeol	Group IV lupeol linoleate	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
Urea	26.25 ± 2.73	36.45 ± 4.10^{a*}	26.27 ± 2.43	25.98 ± 2.32	30.79 ± 3.35$^{a@b\#}$	28.38 ± 3.04^{b*}
Uric acid	1.84 ± 0.18	2.99 ± 0.32^{a*}	1.84 ± 0.17	1.82 ± 0.19	2.13 ± 0.22$^{a@b*}$	1.97 ± 0.19^{b*}
Creatinine	1.07 ± 0.10	1.68 ± 0.18^{a*}	1.06 ± 0.11	1.04 ± 0.10	1.26 ± 0.12$^{a@b*}$	1.14 ± 0.12^{b*}
Albumin	3.92 ± 0.42	2.73 ± 0.30^{a*}	3.91 ± 0.41	3.94 ± 0.42	3.44 ± 0.39^{b*}	3.88 ± 0.38^{b*}

Values are expressed as mean ± SD for six animals. Units: Urea, uric acid and creatinine—mg/dl; albumin—g/dl. Comparisons are made among: a—groups I and II, III, IV, V, VI; b—groups II and V, VI. The symbols represent statistical significance at * $P < 0.001$, $^{\#}$ $P < 0.01$ and $^{@}$ $P < 0.05$

phospholipids were significantly higher ($P < 0.001$) in HCD-fed rats, when compared with the controls. However, treatment with lupeol and lupeol linoleate decreases these levels considerably, indicating its hypocholesterolemic effect.

Table 2 shows the serum biochemical parameters of tissue injury indices in control and experimental animals. HCD-fed rats (Group II) showed elevations ($P < 0.001$) in the concentrations of urea, uric acid and creatinine coupled with decreased ($P < 0.001$) levels of albumin. Figure 1 reveals abnormal activities of tissue enzymes that indicate cellular damage caused by HCD. A 1.49 and 1.52-fold increase in renal LDH and ALP activities is recorded in group II compared to controls. The therapeutic strategies that prevent the above anomalous effects may prove effective in reducing the mortality associated with ischemic tissue diseases. Normalizing tissue enzymes activity and altered biochemical parameters are observed in lupeol and lupeol linoleate treated HCD-fed rats, which highlights the renoprotective effect of these compounds.

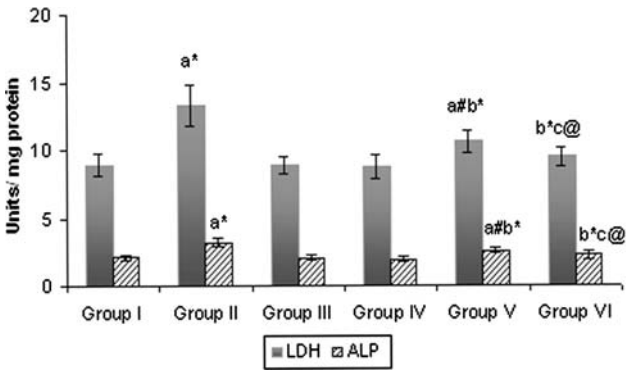

Fig. 1 Effect of lupeol and lupeol linoleate on renal marker enzymes of HCD fed rats. Units: LDH—μmoles × 10^{-1} of pyruvate formed/min; ALP—μmoles × 10^{-2} of phenol liberated/min. Comparisons are made as follows: a—between group I and groups II, V, VI; b—between group II and groups V, VI; c—between group V and group VI. *, $^{\#}$ and $^{@}$ represents statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively

The levels of lipid peroxidation in the control and experimental groups are presented in Table 3. Renal lipid peroxidation was increased by 2.03, 1.42 and 1.69-fold in basal, ferrous- and ascorbate-induced, respectively, of HCD-fed group II rats. However, lupeol and lupeol linoleate treatment restored the levels of lipid peroxidation to normal. Table 4 shows the antioxidant status of the control and experimental groups. There was a significant ($P < 0.001$) decrease in the activities of enzymic antioxidants, (SOD, CAT and GPx), which are known to play an important role in the defence machinery against harmful toxic oxygen free radicals in biological system, and the levels of non-enzymic antioxidants (GSH, vitamin C and Vitamin E) in HCD-fed group. All the above abnormalities were restored in lupeol

Table 3 Effect of lupeol and lupeol linoleate on lipid peroxidation in HCD fed groups compared with the control animals

Parameters	Group I control	Group II HCD	Group III lupeol	Group IV lupeol linoleate	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
Basal	1.56 ± 0.14	3.17 ± 0.27^{a}*	1.51 ± 0.13	1.45 ± 0.12	2.09 ± 0.21^{a}*^{b}*	1.89 ± 0.15^{b}*
FeSO4-induced	8.11 ± 0.69	11.50 ± 1.21^{a}*	7.96 ± 0.70	7.87 ± 0.75	9.40 ± 0.80$^{a@b}$*	8.91 ± 0.85^{b}*
Ascorbate induced	4.31 ± 0.38	7.31 ± 0.72^{a}*	4.29 ± 0.41	4.20 ± 0.38	5.55 ± 0.53^{a}*^{b}*	5.12 ± 0.55$^{a\#b}$*

Values are expressed as mean ± SD for six animals. Units: Lipid peroxidation—nmoles of MDA released/mg protein. Comparisons are made among: a—groups I and II, III, IV, V, VI; b—groups II and V, VI. The symbols represent statistical significance at * $P < 0.001$, $^{\#}$ $P < 0.01$ and $^{@}$ $P < 0.05$

Table 4 Effect of lupeol and lupeol linoleate on levels of antioxidants in HCD fed groups compared with the control animals

Parameters (units/mg protein)	Group I control	Group II HCD	Group III lupeol	Group IV lupeol linoleate	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
Enzymatic antioxidant						
SOD	6.52 ± 0.56	4.40 ± 0.33^{a}*	6.63 ± 0.65	6.66 ± 0.63	5.45 ± 0.50$^{a\#b\#}$	5.83 ± 0.57$^{a@b}$*
CAT	177.70 ± 14.59	109.96 ± 9.98^{a}*	175.03 ± 20.95	179.79 ± 17.59	146.33 ± 14.29$^{a\#b}$*	160.60 ± 15.34^{b}*
GPx	20.92 ± 1.90	15.68 ± 1.77^{a}*	20.34 ± 1.67	21.59 ± 1.79	18.42 ± 1.30$^{b\#}$	19.16 ± 1.35^{b}*
Non enzymatic antioxidant						
GSH	11.50 ± 0.97	8.11 ± 1.01^{a}*	11.54 ± 1.07	11.60 ± 0.77	9.93 ± 0.69$^{a\#b\#}$	11.14 ± 1.10^{b}*$^{c@}$
Vit C	1.57 ± 0.12	1.08 ± 0.11^{a}*	1.57 ± 0.10	1.58 ± 0.12	1.38 ± 0.10$^{a\#b}$*	1.45 ± 0.13^{b}*
Vit E	0.96 ± 0.078	0.61 ± 0.059^{a}*	0.95 ± 0.064	0.97 ± 0.074	0.82 ± 0.083$^{a\#b}$*	0.92 ± 0.077^{b}*$^{c@}$

Values are expressed as mean ± SD for six animals. Units: Superoxide dismutase (SOD)—units/mg protein, one unit is equal to the amount of enzyme that inhibits the auto-oxidation reaction by 50%; catalase (CAT)—µmoles of H_2O_2 consumed/min; glutathione peroxidase (GPx)—µg of GSH consumed/min; reduced glutathione (GSH), vitamin C (Vit C) and vitamin E (Vit E)—µg. Comparisons are made among: a—groups I and II, III, IV, V, VI; b—groups II and V, VI; c—groups V and VI. The symbols represent statistical significance at * $P < 0.001$, $^{\#}$ $P < 0.01$ and $^{@}$ $P < 0.05$

and lupeol linoleate treated HCD-fed groups, which might be due to the antioxidative effect of these compounds.

Atherosclerosis is recognized as a chronic inflammatory disease of the arterial wall, wherein an inflammatory response seems to be a key event leading to the formation of atheromatous lesions [38]. In the present study, Fig. 2 shows the levels of CRP and fibrinogen in plasma. Elevated levels of CRP and fibrinogen (5.09 and 2.17-fold, respectively) were observed in hypercholesterolemic rats. On the other hand, supplementation of lupeol and its ester exerts anti-inflammatory effect, as evident from the restoration of acute phase proteins to normal. Lysosomes are essential for controlled intracellular digestion of cellular components by different pathways, such as autophagy, heterophagy and endocytosis. The activities of lysosomal enzymes in inflammation exudates serve as good markers to assess the intensity of inflammation in experimental groups. The lysosomal enzymes—ACP, Cat-D, β-Glu, β-Gal and NAG activity were significantly ($P < 0.001$) increased in the kidney tissue of HCD-fed rats (Table 5). Lupeol and lupeol linoleate treatment restores the membrane stability and the above enzyme activities. In this context, lupeol linoleate exhibits better effect than lupeol.

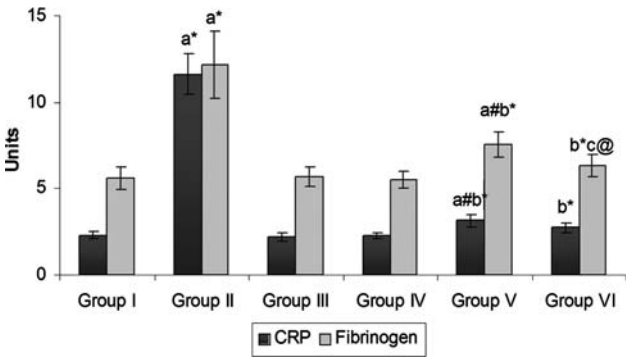

Fig. 2 Effect of lupeol and lupeol linoleate on plasma CRP and fibrinogen levels in control and experimental animals. Units: CRP—mg/l; Fibrinogen—mg × 10/dl. Comparisons are made as follows: a—between group I and groups II, V, VI; b—between group II and groups V, VI; c—between group V and group VI. *, $^{\#}$ and $^{@}$ represents statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively

Histopathological changes in kidney tissues have been presented in Fig. 3. The normal histological findings in the control as well as lupeol and lupeol linoleate alone given groups were presented in Fig. 3a, c and d. HCD-fed rat

Table 5 Effect of lupeol and lupeol linoleate on activities of lysosomal hydrolase in renal tissue of HCD fed animals

Parameters (units/mg protein)	Group I control	Group II HCD	Group III lupeol	Group IV lupeol linoleate	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
ACP	3.28 ± 0.35	5.92 ± 0.68^{a}*	3.22 ± 0.36	3.24 ± 0.37	4.12 ± 0.51$^{a\#b}$*	3.77 ± 0.39^{b}*
NAG	39.03 ± 4.46	52.18 ± 4.77^{a}*	38.02 ± 3.72	37.93 ± 2.41	44.45 ± 4.46$^{a@b\#}$	40.99 ± 4.06^{b}*
β-Glu	40.96 ± 3.75	54.32 ± 5.77^{a}*	40.27 ± 4.14	38.30 ± 3.60	45.29 ± 4.49$^{b\#}$	42.51 ± 4.66^{b}*
β-Gal	24.56 ± 2.77	36.39 ± 4.32^{a}*	24.69 ± 2.66	24.51 ± 2.77	29.14 ± 2.44$^{a@b}$*	27.85 ± 3.09^{b}*
Cat-D	0.29 ± 0.029	0.51 ± 0.05^{a}*	0.30 ± 0.029	0.29 ± 0.025	0.36 ± 0.038$^{a\#b}$*	0.32 ± 0.026^{b}*$^{c@}$

Values are expressed as mean ± SD for six animals. Units: ACP—μmoles × 10^{-2} of phenol liberated/min; NAG, β-Glu, β-Gal—μmoles × 10^{-2} p-nitrophenol liberated/h; Cat-D—μmoles × 10^{-2} tyrosine released/min. Comparisons are made among: a—groups I and II, III, IV, V, VI; b—groups II and V, VI; c—groups V and VI. The symbols represent statistical significance at * $P < 0.001$, $^{\#}$ $P < 0.01$ and $^{@}$ $P < 0.05$

shows mild tubular epithelial denudation with casts in the renal tissue (Fig. 3b), which may be attributed to lipemic-oxidative injury. Lupeol treated rat kidney (Fig. 3e) shows substantially low tubular damage, whereas near normal renal architecture was seen in lupeol linoleate treated rats (Fig. 3f), when compared to HCD-treated rats.

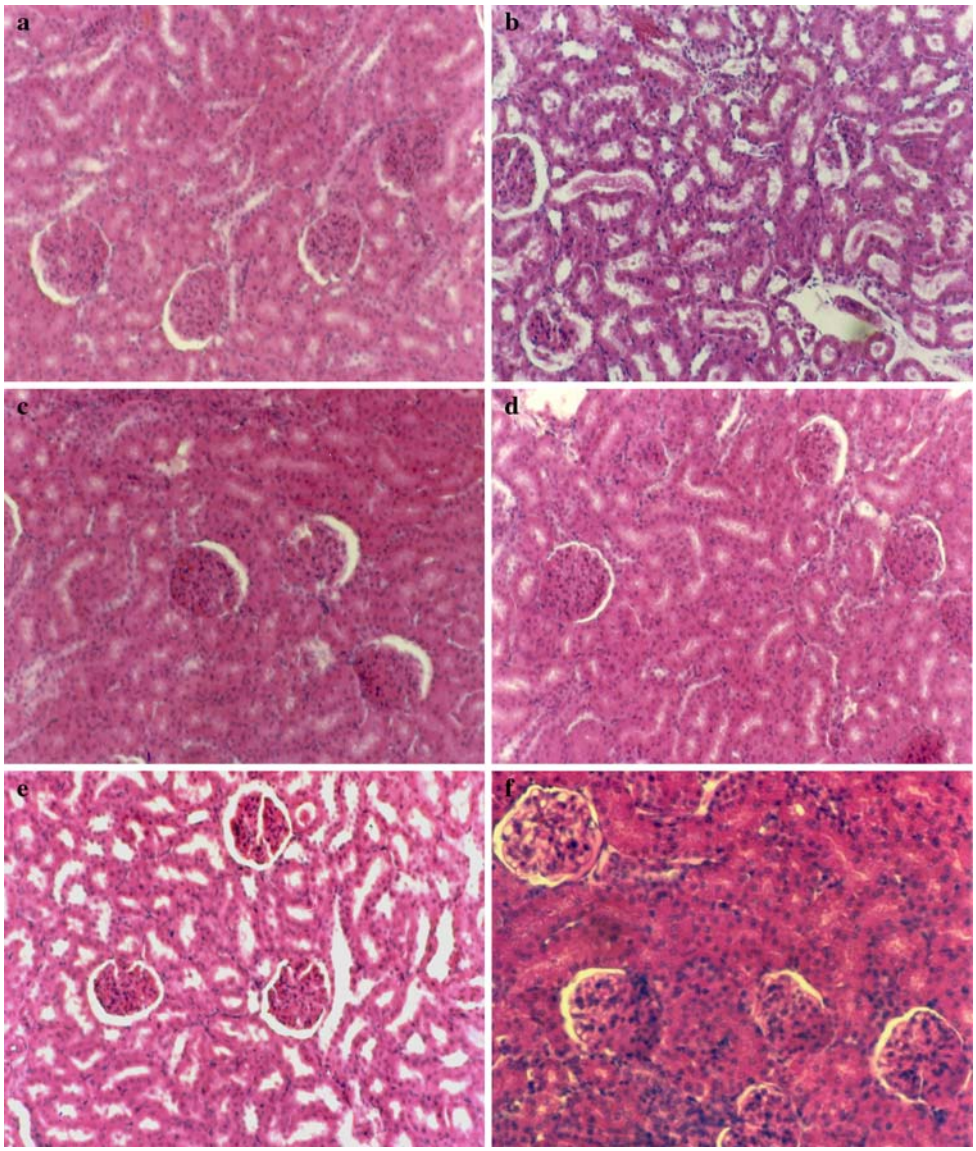

Fig. 3 Histopathological observation of kidney in control and experimental animals. (**a**) Control rat kidney shows normal glomeruli and tubules; (**b**) HCD fed rat shows mild tubular epithelial denudation with casts; (**c**, **d**) lupeol and lupeol linoleate supplemented rats show normal architecture; (**e**) HCD fed group treated with lupeol low tubular damage; (**f**) HCD fed rat treated with lupeol linoleate shows near normal architecture (H&E, 50×)

Discussion

The glomerulus has many structural features that resemble arteries, which are commonly involved in atherosclerosis and moreover, glomerular mesangial cells are found to be structurally similar to arterial smooth muscle cells. Hyperlipidemia has been shown to accelerate the induction and progression of renal injury, leading to glomerulosclerosis [39]. We have previously reported increased serum lipid abnormalities and cardiac and hepatic oxidative stress in HCD-fed rats [13, 40]. Napoli and Lerman [41] reported that increased accumulation of cholesterol in serum and tissue increases the free radical production. Recent reports also show that hypercholesterolemia is associated with increased formation of oxidized LDL and renal inflammation [42, 43]. Hence, cholesterol reduction and decreasing oxidative and inflammatory stresses can decrease the abnormalities associated with hypercholesterolemia. The results of the present work revealed the abnormally elevated lipid levels and collapse of oxidative defense systems and abnormal levels of inflammation in the hypercholesterolemic conditions, while treatment with lupeol and lupeol linoleate afforded considerable protection.

The levels of total cholesterol, triglycerides, fatty acid and phospholipids were increased in renal tissue of HCD-fed rats in the present study. These results agree with earlier finding [44]. Increased level of triglycerides and free fatty acids is might be due to the augmentation in the level of dietary cholesterol that increases the synthesis of free fatty acid and triglycerides in liver [45]. However, treatment with lupeol and its ester decreases the renal lipid levels, which may be due to their hypocholesteroloemic effect. The normalizing effect of lupeol and lupeol linoleate on the elevated serum concentrations of cholesterol, triglycerides, LDL and VLDL and depressed HDL levels, was previously reported by us [13], which may be due to their effect of decreasing the cholesterol absorption. This is complemented by the present observation of lupeol and lupeol linoleate exerting a favourable influence on the abnormal levels of renal lipids in HCD-fed rats.

Elevated levels of serum urea and creatinine may serve to indicate developing glomerulopathy, whereas lowered serum albumin levels in the HCD-fed rats may imply hepatic damage as well as abnormal renal glomerular function as a result of severe hyperlipidemia induced by the diet. Hyperuricemia has been reported to be an independent predictor of cardiovascular risk [46]. Increased serum urate concentration has been associated with increased risk of CHD events [47]. Although the mechanisms by which uric acid play a pathogenic role in cardiovascular disease is unclear, hyperuricemia is known to be associated with deleterious effects on endothelial function, oxidative metabolism, platelet adhesiveness, haemorheology and aggregation [48]. In the present study, increased levels of serum urea, uric acid, creatinine and subsequent decreased levels of albumin were observed in HCD-fed rats. Similar observations were reported earlier in hypercholesterolemic rats [49]. All the above abnormalities were reverted to near normal after lupeol and lupeol linoleate supplementation, which may be due to their hypocholesterolemic activity [13].

Hypercholesterolemia enhances the vulnerability of the microcirculation to the deleterious effects of ischemia and other inflammatory stimuli and leads to ischemic tissue damage. Enzymic markers of cellular damage with concomitant inflammation showed a sharp increase in the activities of ALP and LDH in the renal tissue of HCD-fed rats. Increases in tissue enzyme activities have been reported in experimental inflammatory conditions [50]. However, treatment with lupeol and lupeol linoleate decreases the activities of tissue enzymes considerably, which may be due to their anti-inflammatory effect [51].

Oxidative stress is also known to play a major role in hypercholesterolemic atherogenesis [52]. Hyperlipidemia leads to a rise in glomerular and tubulointerstitial generation of reactive oxygen radicals. Oxygen radicals were generated by enhanced xanthine oxidase activity [53], and concurrent glomerulosclerosis and chronic tubulointerstitial injury were noticed during hyperlipidemia. Certainly, hypercholesterolemia was found to induce or exacerbate glomerular injury in mammals [54]. Maintenance of normal cellular functions in the presence of oxygen largely depends on the efficacy of the tissue protection against free radical-mediated oxidative stress. Decline in the activities of renal SOD, CAT and GPx were pronounced in HCD-fed rats in the present study. On the other hand, the non-enzymic antioxidants such as GSH, vitamin C and vitamin E were also decreased in hypercholesterolemic rats. Both these effects may be due to the increased levels of oxidative stress. However, treatment with lupeol and lupeol linoleate enhanced the levels of this antioxidant defence system status to a noticeable extent. Yamashita et al. [55] reported that lupeol has shown a significant suppressive effect on arachidonic acid induced superoxide generation in human neutrophils, which largely substantiates our present observation.

Lysosomal hydrolytic enzymes are released by the rupture of membranes, which in turn initiates the synthesis of inflammatory mediators, such as thrombaxane, prostaglandins and leukotrienes. The accentuated levels of lysosomal enzymes were observed in hypercholesterolemic rats. These results were consistent with earlier study, which reported that experimental induction of atherosclerosis in human and animals are associated with increased arterial lysosomal hydrolase activities [56]. One of the possible explanations is that the injury produces an increased

intracellular level of lysosomal hydrolases in many tissues [57], including the arterial wall [58]. Thus, the elevated activity of lysosomal enzymes can often be considered as an indication of a greater exposure of the artery to injury [59]. Therefore, it has been proposed that drugs which are capable of stabilizing the lysosomal membrane can reduce inflammation [60]. In that line, treatment of HCD-rats with lupeol and lupeol linoleate maintained the activities of lysosomal enzymes, which may be due to its anti-inflammatory effect. An earlier study reported that lupeol and lupeol linoleate exert their effects, either by modifying the lysosomal membrane or by inhibiting the release of lysosomal enzymes, and thereby prevents the discharge of acid hydrolase [61].

Inflammatory processes play a central role in the pathogenesis of atherosclerosis [62]. The proinflammatory risk factors like oxidized low-density lipoproteins lead to activation of local and systemic inflammation that trigger the production of inflammatory cytokines, adhesion molecules and acute phase proteins from the cells involved in atherosclerotic process [63]. Increased levels of acute phase proteins, CRP and fibrinogen were observed in hypercholesterolemic condition. Lindahl et al. [64] reported that elevated levels of CRP and fibrinogen are strongly associated with the long term risk of death from cardiac causes, in patients with unstable coronary artery disease. CRP may directly contribute to the atherosclerotic process, by causing adverse changes in cultured endothelial cells [65] and by inducing the expression of adhesion molecules, VCAM-1 and ICAM-1, selectins, chemokines and monocyte chemotactic protein-1 [65, 66]. Elevation in plasma fibrinogen level has been identified as an independent risk indicator for ischemic heart disease and the severity of atherosclerosis in healthy populations [67] by the stimulation of smooth muscle cell proliferation and migration [68]. The anti-inflammatory effect of lupeol and lupeol linoleate treatment decreased the levels of CRP and fibrinogen, when compared to HCD-fed rats. As the development of inflammatory process is correlated with the release of lysosomal enzymes and acute phase proteins, the reduction in their release and levels of acute phase proteins confirm its therapeutic usefulness, as an anti-inflammatory agent.

The renal abnormalities induced by an HCD are further confirmed by histopathological findings. Mild tubular epithelial denudation with casts in the renal tissue was observed in HCD-fed rats, which can be attributed to lipemic-oxidative injury. Campos et al. [69] have demonstrated that hypercholesterolemia predisposes renal tubular cells to hypoxic injury, wherein hypercholesterolemia mediates a direct effect on epithelial tubular cells, which thus become more susceptible to ischemic injury. However, lupeol treated rat kidney shows low tubular damage, when compared to HCD fed rats, while near normal architecture was seen in lupeol linoleate treated rats. From the two compounds tested, lupeol linoleate was found to be more effective than lupeol. Esterification of lupeol enhances the efficiency of the parent drug by increasing its bioavailability, penetration and retention ability into the cell membrane [12]. We consider that the same mechanism must be underlying in the protection afforded by lupeol linoleate, which was more efficient than that of unmodified lupeol in the present study.

In conclusion, hypercholesterolemia resulted in substantial alterations in biochemical variables and marker enzymes with a concomitant increase in oxidative stress and inflammation in the renal tissue. Lupeol and lupeol linoleate supplementation effectively restored the abnormalities, suggesting that these compounds would be beneficial in mitigating the renal injury associated with hypercholesterolemic condition. From the two compounds tested, lupeol linoleate was more effective than lupeol.

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