JOURNAL OF NATURAL PRODUCTS

Volume 71, Number 9 September 2008

Full Papers

Antiurolithic Effect of Lupeol and Lupeol Linoleate in Experimental Hyperoxaluria

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Received June 28, 2007

The present study was undertaken to explore the efficiency of the pentacyclic triterpene lupeol (**1**) and its ester derivative, lupeol linoleate (**2**), in experimental hyperoxaluria. Hyperoxaluria was induced in male Wistar rats with 0.75% ethylene glycol (EG) in drinking water for 28 days. Hyperoxaluric animals were supplemented orally with **1** and **2** (50 mg/kg body wt/day) throughout the experimental period of 28 days. The renal enzymes were assayed as markers of renal tissue integrity. The redox status and oxalate metabolism in animals under oxalate overloading was also assessed. Microscopic analysis was done to investigate the abnormalities associated with oxalate exposure in renal tissues. Increase in oxidative milieu in hyperoxaluria was evident by increased lipid peroxidation (LPO) and decreased enzymic and nonenzymic antioxidants. Decrease in the activities of renal enzymes exemplified the damage induced by oxalate, which correlated positively with increased LPO and increased oxalate synthesis. Renal microscopic analysis further emphasized the oxalate-induced damage. These abnormal biochemical and histological aberrations were attenuated with test compound treatment, with **2** more effective than **1**. From the present study, it can be concluded that **1** and **2** may serve as candidates for alleviating oxalate toxicity.

Urinary stones affect 10−12% of the population in industrialized countries.[1] Their incidence has been increasing over the past few years with the age of onset decreasing.[2] Although oxalate, an important stone-forming constituent, is excreted mainly through the kidneys, it is harmless to the renal epithelial cells at low concentration. Oxalate exposure imposes an oxidant stress on the renal cells by generation of reactive species and accumulation of lipid peroxides.[5] Reactive oxygen species damage the membrane and help it anchor the crystals, serving as a substratum for stone growth; this initiates a self-perpetuating cycle ultimately leading to stone formation.[6] Reactive oxygen species generated during metabolism of oxalate and by oxalate itself are considered as the major contributors for the renal damage in lithogenesis. It has long been recognized that antioxidants may contribute to protection against stone formation. This premise has been supported by earlier findings, where administration of vitamin E and lipoic acid were able to counteract oxalate-induced oxidative changes.[7,8]

Crataeva nurvala Buch.-Ham (Capparidaceae) is a medicinal plant, from which the bark decoction was used against calcium oxalate urolithiasis in experimental rats.[9] Lupeol (**1**), isolated in our laboratory from the stem bark of this plant, was identified as the active compound for antiurolithic effects.[10] This compound has also been shown to exhibit other properties of pharmacological importance like antioxidative,[11] anti-inflammatory,[12] hepatoprotective,[13] and antilipidemic[14] activities. Recent investigations have shown that esterification of triterpene enhanced the efficiency of the parent drug, by increasing its bioavailability, penetration, and retention ability into the cell membrane.[15] The esterified derivative, lupeol linoleate (**2**), has been proved to be more effective than **1** in arthritis,[12] cardiotoxicity,[16] and hypercholesterolemia.[14] This prompted us to initiate a comparative study of the effect of lupeol (**1**) and its ester derivative, lupeol linoleate (**2**), in experimental hyperoxaluria.

Results and Discussion

Free radicals generated by oxalate are known to damage renal tissue, and experiments have shown that amelioration of free radicals might be an effective therapeutic strategy to control lithogenesis. Our previous studies with lupeol (**1**) have shown that it was able to normalize abnormal excretion of renal enzymes in urine during lithogenesis, suggestive of its reno-protective effect.[17]

Oxalate is known to damage the renal membranes and release the associated enzymes. Table 1 illustrates the activity of renal marker enzymes in the hyperoxaluric and treated groups along with the control groups. Activities of marker enzymes of renal cellular integrity such as alkaline phosphatase (ALP) and γ-glutamyl

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10.1021/np0703141 CCC: $40.75
Published on Web 08/22/2008

Table 1. Effect of Lupeol (**1**) and Lupeol Linoleate (**2**) on the Renal Marker Enzymes in Experimental Hyperoxaluria[a]

enzyme	group I	group II	group III	group IV	group V	group VI
ALP	1.71 ± 0.06	1.21 ± 0.05[b,e]	1.72 ± 0.07	1.68 ± 0.07	1.53 ± 0.06[c,d]	1.65 ± 0.07[c,e]
γ-GT	8.75 ± 0.36	6.27 ± 0.30[b,e]	8.73 ± 0.35	8.79 ± 0.35	8.28 ± 0.33[c,e]	8.62 ± 0.34[c,e]

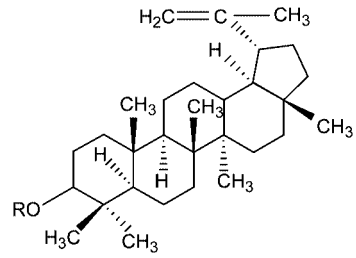
[a] Values are expressed as mean ± SEM for 6 animals in each group. Group I: control. Group II: EG. Group III: **1** alone. Group IV: **2** alone. Group V: EG + **1**. Group VI: EG + **2**. Unit: ALP: μmol of phenol liberated/min/mg protein. γ-GT: nmol × 10 *p*-nitroaniline liberated/min/mg protein. [b] Comparison between group I and groups II, V, and VI. [c] Comparison between group II and groups V and VI. [d] $p < 0.01$. [e] $p < 0.001$.

Table 2. Effect of Lupeol (**1**) and Lupeol Linoleate (**2**) on Oxalate-Metabolizing Enzymes of Liver and Kidney in Hyperoxaluric Rats[a]

enzyme	group I	group II	group III	group IV	group V	group VI
liver						
LDH	2.36 ± 0.09	2.76 ± 0.10[b,f]	2.35 ± 0.10	2.33 ± 0.09	2.42 ± 0.10[c,e]	2.39 ± 0.09[c,f]
XO	1.31 ± 0.05	2.48 ± 0.10[b,g]	1.31 ± 0.05	1.28 ± 0.05	1.54 ± 0.06[b,e,c,g]	1.39 ± 0.05[c,g]
GAO	2.76 ± 0.11	5.36 ± 0.24[b,g]	2.77 ± 0.10	2.75 ± 0.10	3.61 ± 0.10[b,e,c,g]	3.12 ± 0.10[c,g d,e]
kidney						
LDH	2.08 ± 0.09	3.34 ± 0.15[b,g]	2.05 ± 0.09	2.01 ± 0.08	2.41 ± 0.10[b,e,c,g]	2.26 ± 0.10[c,g]
XO	0.92 ± 0.04	1.46 ± 0.07[b,g]	0.93 ± 0.04	0.90 ± 0.04	1.09 ± 0.05[b,e,c,g]	0.97 ± 0.04[c,g]

[a] Values are expressed as mean ± SEM for 6 animals in each group. Group I: control. Group II: EG. Group III: **1** alone. Group IV: **2** alone. Group V: EG + **1**. Group VI: EG + **2**. LDH, XO: units/mg protein (1 unit = the amount of enzyme that bring about a change in OD of 0.01 min). GAO: nmol of glyoxylate formed/mg protein. [b] Comparison between group I and groups II, V, and VI. [c] Comparison between group II and groups V and VI. [d] Comparison between group V and group VI. [e] $p < 0.05$. [f] $p < 0.01$. [g] $p < 0.001$.

phosphatase (γ-GT) were significantly ($p < 0.001$) decreased in hyperoxaluric rats when compared with the controls, in accordance with an earlier report.[7] The above decrease may be due to the leakage of these enzymes from the tissue or decreased translocation of the enzyme from the cytosol to the membrane. Renal membrane damage by oxalate might also be one of the factors leading to the decreased activity of the renal enzymes. Treatment with **1** and **2** was able to increase the activities of these renal enzymes, which might be due to the transmembrane stabilizing effects of these triterpenes. The stabilizing effect of **1** and **2** could be because of the increase in the surface area/volume ratio of the cells, which could be brought about by an expansion of membrane or shrinkage of the cell through an interaction of these compound with membrane protein.[18] Alleviation of this abnormal decrease in the renal enzyme activity by **2** in the present study is in line with the earlier observation where these triterpenes were able to prevent the increased urinary excretion of renal enzymes.[17]



Lupeol (**1**) R = H

Lupeol linoleate (**2**) R =

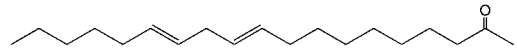


Table 2 presents the activities of oxalate-metabolizing enzymes in the pathologic and treated conditions, compared with the controls. A significant increase ($p < 0.001$) in the activities of lactate dehydrogenase (LDH), xanthine oxidase (XO), and glycolic acid oxidase (GAO) was observed in the liver of hyperoxaluric rats. Activities of LDH and XO in the kidney were also increased during hyperoxaluria. The upsurge in the oxalate-metabolizing enzymes might be attributed to the increased availability of their substrate and result in an increased oxalate load on the system. Significant reductions in the activities of these oxalate-metabolizing enzymes were observed with **1** and **2** treatment to hyperoxaluric groups (groups V and VI). GAO is a flavoprotein that catalyzes the two-step oxidation of glycolate to oxalate, with glyoxylate as an intermediate. LDH, a cytosolic enzyme, is also involved partly in the synthesis of oxalate. It catalyzes the coupling of oxidation and reduction of glyoxylate, resulting in the formation of glycolic acid and oxalate.[19] An increase in $FAD^+/FADH_2$ and $NAD^+/NADH$ ratio due to the interruption of the electron transport pathway and inhibition of the oxidative phosphorylation might be the possible reason for the increased activities of the GAO and LDH observed in the present study.[20] Increased activity of GAO along with the increased LDH and XO activities in kidney and liver have already been reported in hyperoxaluria.[19] Apart from increasing the concentration of oxalate, GAO and XO release H_2O_2 and/or superoxide anions as end products.[21] Oxidation of critical sulfhydryl groups of xanthine dehydrogenase, present in normal tissue, converts it into XO, which produces reactive intermediates.[22] Decreases in the antioxidants during the hyperoxaluric state further favor the conversion of xanthine dehydrogenase to XO.[21] This abnormal elevation in the enzyme activity was corrected to near normalcy with **1** and **2** administration. Moreover, lupeol linoleate (**2**) significantly ($p < 0.05$) decreased the activity of GAO more than lupeol (**1**).

The enzymic antioxidant activities in various groups are represented in Table 3. In this study, a significant ($p < 0.001$) reduction in the activities of the various antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)] was observed in the kidney of hyperoxaluric rats. SOD, CAT, and GPx are the important enzymic machinery of the antioxidant system. Oxalate is known to inhibit CAT, which causes the accumulation of H_2O_2.[21] Moreover, excessive accumulation of

Table 3. Effect of Lupeol (**1**) and Lupeol Linoleate (**2**) on Enzymatic Antioxidant Status in Kidney of Hyperoxaluric Rats Compared with the Control[a]

parameter	group I	group II	group III	group IV	group V	group VI
SOD	5.10 ± 0.20	3.92 ± 0.17[b,g]	5.04 ± 0.22	5.00 ± 0.21	4.67 ± 0.19[c,f]	4.82 ± 0.21[c,g]
CAT	163.2 ± 6.32	115.3 ± 4.19[b,g]	164.2 ± 6.9	164.9 ± 7.23	141.6 ± 5.5[b,e,c,f]	155.3 ± 6.91[c,g]
GPx	8.62 ± 0.32	5.54 ± 0.23 [b,g]	8.31 ± 0.36	8.32 ± 0.31	7.11 ± 0.19 [b,f,c,g]	8.01 ± 0.35[c,g,d,f]

[a] Values are expressed as mean ± SEM for 6 animals in each group. Group I: control. Group II: EG. Group III: **1** alone. Group IV: **2** alone. Group V: EG + **1**. Group VI: EG + **2**. Units: SOD: units/mg protein (1U = amount of enzyme required to bring about 50% inhibition of autoxidation of pyrogallol). CAT: μmol of H_2O_2 utilized/min/mg protein. GPx: μg of GSH utilized/min/mg protein. [b] Comparison between group I and groups II, V, and VI. [c] Comparison between group II and groups V and VI. [d] Comparison between group V and group VI. [e] $p < 0.05$. [f] $p < 0.01$. [g] $p < 0.001$.

Table 4. Effect of Lupeol (**1**) and Lupeol Linoleate (**2**) on Antioxidant Status in the Kidney of Hyperoxaluric Animals Compared with the Control[a]

parameter	group I	group II	group III	group IV	group V	group VI
GSH	4.74 ± 0.18	3.09 ± 0.13[b,e]	4.75 ± 0.19	4.76 ± 0.19	4.27 ± 0.16[c,e]	4.59 ± 0.18[c,e]
Vit C	1.86 ± 0.07	1.16 ± 0.05[b,e]	1.86 ± 0.07	1.89 ± 0.08	1.60 ± 0.07[b,d,c,e]	1.74 ± 0.08[c,e]
Vit E	1.04 ± 0.04	0.72 ± 0.03 [b,e]	1.04 ± 0.05	1.06 ± 0.04	0.91 ± 0.05 [b,d,c,e]	0.97 ± 0.04[c,e]

[a] Values are expressed as mean ± SEM for 6 animals. Group I: control. Group II: EG. Group III: **1** alone. Group IV: **2** alone. Group V: EG + **1**. Group VI: EG + **2**. Units: GSH, Vit C, and Vit E; μg/mg protein. [b] Comparisons between group I and groups II, V, and VI. [c] Comparisons between group II and groups V and VI. [d] $p < 0.05$. [e] $p < 0.001$.

Table 5. Effect of Lupeol (**1**) and Lupeol Linoleate (**2**) on Lipid Peroxidation Level in the Kidney of Hyperoxaluric Animals Compared with the Control[a]

LPO	group I	group II	group III	group IV	group V	group VI
basal	1.86 ± 0.07	2.51 ± 0.11[b,g]	1.83 ± 0.07	1.85 ± 0.05	2.14 ± 0.10[c,g]	1.95 ± 0.08[c,g]
ascorbate	4.48 ± 0.18	6.32 ± 0.27[b,g]	4.50 ± 0.16	4.45 ± 0.21	5.11 ± 0.21[b,e,c,g]	4.95 ± 0.22[c,g]
ferrous	12.27 ± 0.52	17.21 ± 0.77 [b,g]	12.14 ± 0.54	12.20 ± 0.56	15.11 ± 0.66[b,f,c,e]	13.38 ± 0.57[c,g,d,e]

[a] Values are expressed as mean ± SEM for 6 animals in each group. Group I: control. Group II: EG. Group III: **1** alone. Group IV: **2** alone. Group V: EG + **1**. Group VI: EG + **2**. Units: nmol of MDA/mg protein. [b] Comparison between group I and groups II, V, and VI. [c] Comparison between group II and groups V and VI. [d] Comparison between group V and group VI. [e] $p < 0.05$. [f] $p < 0.01$. [g] $p < 0.001$.

H_2O_2 targets the active sites of enzymes, especially the −SH group, which might result in the inactivation/decreased activity of crucial enzymes such as SOD and GPx. An increase in H_2O_2 gives rise to intermediates such as hydroxyl radicals, which are also involved in the oxidative modification of numerous proteins, aggravating the oxidative stress on the renal cells. Addition of CAT and SOD mimetics to oxalate-exposed cells reversed the damage induced by oxalate, by maintaining a near normal redox state.[23,24] Treatment with **1** and **2** significantly enhanced the activities of these enzymes (groups V and VI) when compared to hyperoxaluric rats. In the case of GPx, **2** showed a significantly ($p < 0.01$) greater effect than **1**. An earlier report has shown that lupeol (**1**) showed a suppressive effect on *N*-formyl-methionyl-leucyl-phenylalanine- and arachidonic acid-induced superoxide generation in human neutrophils. This effect might be due to the $-CH_3$ group present in the C-17 position of the lupeol structure.[25] This could be the possible mechanism behind the protective effect of **1** and **2** in hyperoxaluria.

Table 4 delineates the changes in the levels of various nonenzymic antioxidants in the kidney. Induction of hyperoxaluria mediated by ethylene glycol led to the depletion of cellular nonenzymic antioxidant components in kidney (group II). Marked reduction in the level of glutathione (GSH) in the kidney might be responsible for the low levels of the other antioxidants such as vitamins E and C in the kidney tissue ($p < 0.001$), which were remarkably depleted when compared to the control animals. Treatment with **1** and **2** (groups V and VI, respectively) was efficient in restoring the antioxidant potential of the tissues, by maintaining the cellular GSH level. GSH, an essential tripeptide, plays a pivotal role against oxidative insult by acting as an endogenous scavenger of free radicals.[26] Vitamin C and vitamin E are the two important vitamins involved in the defense of a cell against reactive species. These vitamins are mutually inter-related, and appropriate levels of these molecules are essential for the normal functioning of the cell. The recycling of these nonenzymic antioxidants between their oxidized and reduced form is essential for maintenance of normal cellular function. The overall decrease in these nonenzymic antioxidants increases the oxidative stress imposed on renal cells. With the downplay of renal oxidative challenge, normal levels of antioxidant molecules, namely, GSH and vitamins C and E, were restored in the **1**- and **2**-treated groups. Triterpene treatment substantially augmented the levels of antioxidants and of the antioxidant enzyme system. In line with the present findings, Sudharsan et al. also reported that adminstration of these triterpenes increased the level of nonenzymic antioxidants.[27]

The levels of lipid peroxides are presented in Table 5. The lithogenic group II animals showed 0.35-, 0.41-, and 0.40-fold increase in basal-, ascorbate-, and ferrous-induced LPO, respectively, when compared to the control animals. Increased LPO may be due to the free radicals produced by oxalate and during its metabolism. Exposure to oxalate is known to increase the lipoperoxyl radicals that damage the membrane through the LPO cycle, resulting in damage of the receptors and proteins associated with membranes. Increased LPO has been observed in urine of stone patients as well as in hyperoxaluric rats, which corroborates well with our present findings.[7,28] The observed decrease in the antioxidant enzymes in the present study might also be a cause for the increased LPO. Treatment of the hyperoxaluric animals with **1** and **2** (groups V and VI) restricted the oxalate-induced peroxidative changes in the kidney tissue to a great extent. The increase in oxidative stress in cardiac tissues was also normalized with triterpene administration, which is in agreement with our present findings.[27]

Triterpenes have been reported to render protection against oxidative assault caused by various pro-oxidants such as ADP/Fe^{2+} and CCl_4.[29,30] The antiperoxidation effect might be attributed to the ability of these triterpenes to reduce overall concentration of oxalate and to alter the environment conducive for crystal formation. Esterification of lupeol (**1**) to **2** improved the efficiency of the parent drug, by increasing its bioavailability, penetration, and retention ability into the cell.[14] We consider that this mechanism might lie behind the increased efficiency of **2**, when compared to unmodified **1** in the present study.

Histopathological changes in the renal tissue of control and experimental animals are presented in Figure S1 (Supporting

Information). Control animals depict normal glomeruli with tubules (Figure S1a). Group II animals treated with ethylene glycol showed crystal deposition, indicating increased oxalate along with mild diffuse mononuclear infiltration (Figure S1b). Rats administered with **1** and **2** (treated controls) showed normal kidney architecture, indicating the nontoxic nature of these compounds (Figures S1c and S1d). Rats supplemented with **1** (Figure S1e) and **2** (Figure S1f) showed near normal glomeruli and tubules. The above results show that **1** and **2** are effective in alleviating the renal abnormalities in hyperoxaluric rats by regulating oxidative stress and protecting the membrane integrity.

To conclude, the present work has demonstrated the escalated oxidative abnormalities associated with hyperoxaluric conditions and also underscores the positive role played by **1** and **2** in mitigating the biochemical anomalies. The results obtained indicate that lupeol linoleate (**2**) is more effective in reverting the abnormalities associated with hyperoxaluria than lupeol (**1**).

Experimental Section

General Experimental Procedures. Lupeol (**1**) was isolated from the stem bark of *Crataeva nurvala* as reported earlier in our laboratory.[10] Lupeol linoleate (**2**) was prepared according to the method of Geetha and Varalakshmi.[12] Bovine serum albumin and 1,1,3,3-tetraethoxypropane malondialdehyde were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals and solvents were of analytical grade. Animals were maintained as per national guidelines and protocols, approved by the institutional animal ethical committee (IAEC). Male albino rats of Wistar strain (120 ± 20 g) were purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India. The animals were housed under the conditions of controlled temperature (25 ± 2 °C) with 12 h/12 h day−night cycle. The rats were divided into six groups of six rats each. Group I served as control. Group II received ethylene glycol (0.75% in drinking water) for 28 days to induce a chronic low-grade hyperoxaluria and generate calcium oxalate deposition in kidneys. Rats in groups III and IV were given **1** and **2**, respectively. Groups V and VI received ethylene glycol for 28 days and were simultaneously treated with **1** and **2** (50 mg/kg body wt/day/orally), respectively. Infrared spectral studies were performed in our laboratory to confirm the structure of lupeol linoleate (**2**). At the end of the experimental period, all the animals were sacrificed by cervical decapitation. The kidneys and liver were excised and homogenized in ice-cold 0.01 M Tris-HCl buffer, pH 7.4, to give a 10% homogenate. Tissue homogenates were suitably processed for the assessment of enzymes, lipid peroxidation, and antioxidant status. A section of the kidney tissue was set aside for histopathological processing.

Assay of Renal Marker and Oxalate-Metabolizing Enzymes. Alkaline phosphatase (ALP) activity was followed by measuring the phenol liberated from disodium phenyl phosphate by the color reaction with Folin's reagent in an alkaline pH.[31] γ-Glutamyl transferase (γ-GT) was measured by the method of Orlowski and Meister.[32] Lactate dehydrogenase (LDH) was assayed by the method of Liao and Richardson[33] using glyoxylate as substrate. GAO was assayed by the method of Lui and Roels.[34] XO was assayed by the method of Fried and Fried.[35]

Assay of Lipid Peroxidation. LPO was assayed by measuring the release of malondialdehyde (MDA) in the tissue homogenate following the procedure of Hogberg et al. The ferrous sulfate and ascorbate induced LPO system contained 10 mM ferrous sulfate and 0.2 mM ascorbate as inducers. The methods are as described in our earlier study.[14]

Assessment of Enzymic and Nonenzymic Antioxidant Activities. Antioxidant enzymes were assayed in kidney homogenates of experimental groups. SOD was assayed by the method of Marklund and Marklund. CAT activity was assayed by the method of Sinha. GPx was assayed by the method of Rotruck et al. Protein content was estimated by the method of Lowry et al. GSH in the kidney tissue was determined according to the method of Moron et al. Vitamin C was estimated by the method of Omaye et al. Vitamin E was estimated by the method of Desai et al. The methods are detailed in our earlier study.[14]

Histopathological Studies. Portions of kidney tissues were fixed in 10% formalin, processed, and stained with hematoxylin and eosin. They were viewed under a light microscope (Nikon Eclipse E400, model 115, Japan) for histopathological changes.

Statistical Analysis. All data were expressed as mean ± standard error mean (SEM) for six animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the 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, and <0.05 have been given respective symbols in the tables.

Acknowledgment. Financial support by the University Grant Commission, India, to P.V. (project no. HS 7) is gratefully acknowledged.

Supporting Information Available: Histopathological observation of kidney tissues in different experimental groups (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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NP0703141