Molecular and Cellular Biochemistry **295:** 189–198, 2007.
DOI: 10.1007/s11010-006-9288-2

Mitigating role of lupeol and lupeol linoleate on hepatic lipemic-oxidative injury and lipoprotein peroxidation in experimental hypercholesterolemia

V. Sudhahar,[1] S. Ashok Kumar,[1] P. Varalakshmi[1] and R. Sundarapandiyan[2]

[1]*Department of Medical Biochemistry, Dr. ALM. Postgraduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, 600 113, India;* [2]*Department of Pathology, Dr. ALM. Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, 600 113, India*

Received 5 March 2006; accepted 24 July 2006

Abstract

In the present study, the role of pentacyclic triterpenes, lupeol and its ester lupeol linoleate, was studied in relation to hepatic oxidative abnormalities and lipoprotein peroxidation in hypercholesterolemic rats. Hypercholesterolemia was induced in male Wistar rats by feeding them with high cholesterol diet (4% cholesterol + 1% cholic acid; HCD) for 30 days. Pentacyclic triterpenes, lupeol and lupeol linoleate were supplemented (50 mg/kg body wt/day) during the last 15 days. After the experimental period, there was a significant depression in hepatic activities of antioxidant enzymes, SOD (38.39%), CAT (25.03%) and GPx (30.26%) along with a marked fall in the levels of non-enzymic antioxidant molecules GSH (31.39%), vitamin C (46.07%) and vitamin E (42.28%), with a concomitant increase ($p < 0.001$) in lipid peroxidation and in the activities of serum alkaline phosphatase, lactate dehydrogenase and aminotransferases when compared to controls. Treatment with triterpenes decreased lipid peroxidation and reverted the activities of antioxidants ($p < 0.001$ and $p < 0.01$) and marker enzymes to near control. Histopathological findings further confirmed the hepatoprotective nature of triterpenes by showing the normal architecture in treated rats, as against the fatty cellular changes in HCD fed rats. Further, the susceptibility of apo-B containing lipoprotein to oxidation by copper and Fenton's reagent was increased in *in vitro* condition in HCD fed rats, whereas the lipoproteins were less susceptible to oxidation in triterpenes treated animals. Therefore, it may be concluded that lupeol and its ester afford protection against the hepatic abnormalities and lipoprotein peroxidation in hypercholesterolemic rats.

Key words: triterpenes, high cholesterol diet, antioxidant enzymes, LDL oxidation

Introduction

Dietary cholesterol is regarded as an important factor in the development of atherosclerosis and subsequent cardiovascular disease. Exogenous hypercholesterolemia 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

Address for offprints: P. Varalakshmi, Department of Medical Biochemistry, Dr. A.L.M. Postgraduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, 600 113, India (E-mail: drvlakshmi@yahoo.com.)

intracellular accumulation of lipids [1, 2]. Feeding of cholesterol diet produces severe hypercholesterolemic and vascular atherosclerotic lesion and increased oxidative stress in several tissues [3]. Recent findings support the hypothesis that oxidative stress is one of the causative factors that link hypercholesterolemia with the pathogenesis of atherosclerosis [4]. In hypercholesterolemia, the cholesterol content of erythrocyte, platelets, polymorphonuclearleucocyte and endothelial cells increases, which is reported to activate these cells and cause the enhanced production of oxygen free radicals leading to tissue injury [5].

Medicinal plants are natural sources yielding valuable herbal products, which are often used in the treatment of various ailments. *Crataeva nurvala* is one of the medicinal plants recorded in the Indian system of Medicine [6]. Lupeol, a pentacyclic triterpene was isolated from the stem bark of *C. nurvala* in our laboratory [7]. Triterpenes, widely distributed in edible and medicinal plants, are effective in protecting against liver disorders [8]. Triterpene celastrol has been shown to have a potent inhibitory effect against lipid peroxidation in liver mitochondria [9]. Lupeol possesses low density lipoprotein (LDL) protective activity during LDL oxidation studies performed in *in vitro* condition [10]. An ester derivative, lupeol linoleate has been shown to possess better antiarthritic [11] and cytoprotective [12] effects than lupeol. The effect of lupeol and lupeol linoleate were investigated in our previous experiments in hypercholesterolemic condition, which showed reduction in serum total cholesterol level by 51.87% and 62.6%, respectively [13]. Here, we have studied the individual effects of lupeol and lupeol linoleate on serum enzymes, hepatic lipid peroxidation, antioxidants level, plasma lipoprotein oxidation and hepatic tissue histology in early stage of atherogenesis.

Materials and methods

Drugs and chemicals

Lupeol was isolated from the stem bark of *C. nurvala* as reported earlier in our laboratory [7]. 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 [11]. Bovine serum albumin 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.

Experimental design

Animals were maintained as per national guidelines and protocols, approved by the institutional ethical committee (IAEC No. 02/ 038/ 03). Male albino rats of the Wistar strain (140 ± 10 g, 10-weeks old) 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. They were randomized into six groups comprising of six rats each. Group I served as vehicle control. Group II, V and VI were given a high-cholesterol diet (HCD) consisting of the normal rat chow along with 4% cholesterol and 1% cholic acid for 30 days. Treatment with lupeol and lupeol linoleate (50 mg/kg/day, orally) commenced 15 days after the start of the experimental period for Groups V and VI, respectively. Corn oil was used as the vehicle in the experimental study. Groups III and IV animals fed with normal rat chow were supplemented with lupeol and lupeol linoleate respectively during the last 15 days of experimental period, served as drug controls.

At the end of the experimental period, all the animals were sacrificed by decapitation. Blood samples were collected for biochemical analysis and the liver was excised. Liver was homogenized in ice-cold 0.01 M Tris–HCl buffer, pH 7.4 to give a 10% homogenate. A section of the liver was kept aside for histological processing.

Enzymatic indices of cellular integrity

Protein content in the serum and rat hepatic tissue was determined by the method of Lowry *et al*. [14]. Lactate dehydrogenase (LDH) was assayed by the method of King [15]. 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 dinitrophenyl hydrazine 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 [16] and expressed as μmoles of phenol liberated/min/mg of protein. Activities of aminotransferases – aspartate and alanine transaminases, AST and ALT, respectively [17], were determined and expressed in terms of μmoles of pyruvate liberated/min/mg of protein at 37 °C.

Assessment of lipid peroxidation

Lipid peroxidation in the liver tissue was determined by the procedure of Hogberg *et al*. [18]. 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 colored 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 [19].

Determination of enzymic antioxidant activities

Antioxidant enzymes were assayed in hepatic tissue of experimental groups. The enzyme superoxide dismutase (SOD) was assayed by the method of Marklund and Marklund [20]. The unit of enzyme activity is defined as the amount of enzyme required to give 50% inhibition of pyrogallol autooxidation. Catalase activity was assayed by the standard method [21]. Glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al*. [22]. It is based on the reaction between glutathione, remaining after the action of GPx and 5,5′-dithio-bis (2-nitrobenzoic acid) to form a complex that absorbs maximally at 412 nm. The activity of GPx was expressed in terms of μg of GSH consumed/min/mg protein.

Estimation of non-enzymatic antioxidants

Reduced glutathione (GSH) in the liver was determined by standard method [23]. This method is based on the reaction of GSH with 5,5′ dithiobis (2-nitrobenzoic acid) to give a compound that absorbs at 412 nm and expressed as μg/mg protein. Ascorbic acid was estimated by the method of Omaye *et al*. [24]. Vitamin E was estimated by the method of Desai *et al*. [25].

Histopathological studies

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

LDL (+VLDL) oxidation studies

Separation of apo B-containing lipoproteins (LDL + VLDL)

Apo-B lipoprotein separation was done according to the method of Wilson and Spiger [26]. About 2 ml of plasma was mixed with 2 ml of physiological saline and 0.36 ml of heparin-manganese chloride. It was then shaken well and incubated at 4 °C overnight. The contents were centrifuged at 10,000 rpm for 20 min and the supernatant containing the HDL fraction was separated, while the precipitate containing LDL + VLDL mixture was washed repeatedly with saline. Finally, the residue with the apo B-containing lipoproteins LDL + VLDL was suspended in 4 ml of phosphate buffered saline (PBS; 7 mmol/l phosphate buffer with 0.89% saline, pH 7.4) containing 1% bovine serum albumin. Aliquots of this suspension were taken for further studies.

Oxidation of the isolated lipoproteins with Fenton's reagent and copper ions

A known aliquot of lipoproteins was incubated with 2 mmol/l hydrogen peroxide, 50 μmol/l ferrous sulfate and 7 mmol/l PBS. Another lipoprotein aliquot was incubated with 50 μmol/l copper sulfate and 7 mmol/l PBS. The reaction mixtures were incubated at 28 ± 3 °C for 2 different time periods i.e., 3 h and 6 h. Simultaneously, a blank containing lipoprotein aliquot and PBS was also incubated for 3 h and 6 h. The lipid peroxidation products were estimated as micromoles of malondialdehyde formed/mg protein, at zero time and at the end of 3 h and 6 h by the method of Hogberg *et al*. [18].

Statistical analysis

The values are expressed as mean ± standard deviation (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 inter-group 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.

Results

In the present study, high cholesterol diet fed rats for a period of 30 days was chosen as the experimental model of early phase atherogenesis. The role of triterpenes on the oxidative aberrations in the liver and plasma lipoprotein oxidation associated with diet-induced hypercholesterolemia has been investigated.

The serum activities of LDH, ALP, AST and ALT were shown to be significantly increased (1.89, 1.78, 2.13 and 1.81-fold, respectively) in group II animals, when compared with group I. The enzymic abnormalities were significantly normalized upon treatment with lupeol and lupeol linoleate (Table 1).

Table 2 represents the abnormal elevation of LPO in the hepatic tissue of HCD fed rats. Group II animals showed a

Table 1. Effect of lupeol and lupeol linoleate on HCD induced marker enzymes in serum (Values are expressed as mean ± S.D. for six animals)

Serum enzymes (Units/min/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
Lactate dehydrogenase	5.36 ± 0.58	10.14 ± 1.08 a*	5.36 ± 0.56	5.31 ± 0.57	7.15 ± 0.77 a*b*	5.94 ± 0.50 b*c#
Alkaline phosphatase	2.52 ± 0.22	4.49 ± 0.40 a*	2.51 ± 0.27	2.46 ± 0.28	3.04 ± 0.22 a#b*	2.88 ± 0.29 a@b*
Aspartate amino transferase	0.61 ± 0.06	1.30 ± 0.13 a*	0.62 ± 0.05	0.58 ± 0.061	0.81 ± 0.07 a*b*	0.67 ± 0.06 b*c#
Alanine amino transferase	0.94 ± 0.09	1.70 ± 0.18 a*	0.89 ± 0.09	0.90 ± 0.08	1.25 ± 0.12 a*b*	1.16 ± 0.12 a#b*

Units: Lactate dehydrogenase: μmoles$\times 10^{-1}$ of pyruvate formed; Alkaline phosphatase: μmoles$\times 10^{-2}$ of phenol liberated; Aspartate and alanine amino transferase: μmoles$\times 10^{-2}$ of pyruvate liberated.
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.

2.58-fold rise in basal LPO in hepatic tissue, as well as a 67.86% and 74.17% increase in LPO following addition of exogenous inducers such as ascorbate and ferrous sulphate, respectively, compared with control animals. The LPO levels were considerably lowered with the treatment of triterpenes (Groups V and VI).

Changes in the activities of hepatic enzymic antioxidants SOD, CAT and GPx are given in Table 3. The HCD fed rats showed a decrease in the activities of SOD (38.39%), CAT (25.03%) and GPx (30.26%) in the hepatic tissue. Treatment with lupeol significantly enhanced the activities of these enzymes (Group V). The effect of lupeol linoleate in this context was even better than lupeol, since the activities of these enzymes were brought almost nearly to that of the controls (Group VI).

Table 4 displays the hepatic levels of non-enzymic antioxidants in HCD fed rats with and without treatment. In group II, a significant decrease ($p < 0.001$) in GSH, ascorbate and tocopherol levels was observed in the hepatic tissue as compared to control rats. There was a significant increase in the level of these antioxidants in the treated groups, thereby indicating that treatment with triterpenes protect against oxidative stress induced by the depletion of enzymic and non-enzymic antioxidants.

Histopathological changes in hepatic tissue of control and experimental animals have been presented in Fig. 1. Control animals depict normal hepatic architecture and normal hepatocytes (Fig. 1a). Hypercholesterolemic rats showed marked fatty changes in the hepatocytes (Fig. 1b). Lupeol and lupeol linoleate administered rats (drug controls) show normal hepatic architecture (Fig. 1c, d). The HCD fed rats treated with lupeol (Fig. 1e) show a partial reduction of fatty changes and the lupeol linoleate treated rats (Fig. 1f) show very occasional fatty globule in hepatocytes and almost normal architecture of liver.

Figure 2a represent the MDA levels, as an indicator of oxidation of LDL + VLDL in the HCD fed and treated samples, initially (0 h), and after 3 h and 6 h, induced by copper ions, and the MDA levels induced by Fenton's reagent are given in Fig. 3a.

Here, the mean inhibition ratio (%) [27] has been computed for the triterpenes, wherein

$$\text{Mean inhibition ratio } (\%) = \frac{\text{(MDA) in group II - (MDA) in group V or VI}}{\text{(MDA) in group II}} \times 100$$

In the present work, lupeol-treated groups showed marked resistance to LDL oxidation induced by copper seen as mean inhibition ratio of 47.73% after 3 h and 60.08% after 6 h. Lupeol linoleate treated groups showed resistances

Table 2. Effect of lupeol and lupeol linoleate on HCD induced lipid peroxidation in hepatic tissue (Values are expressed as mean ± S.D. for six animals)

Lipid peroxidation	Group I Control	Group II HCD	Group III Lupeol alone	Group IV Lupeol linoleate alone	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
Basal	1.33 ± 0.10	3.43 ± 0.41 a*	1.32 ± 0.10	1.29 ± 0.11	2.02 ± 0.15 a*b*	1.69 ± 0.14 a#b*c#
$FeSO_4$-induced	5.32 ± 0.46	8.93 ± 0.84 a*	5.26 ± 0.53	5.18 ± 0.52	6.32 ± 0.66 a#b*	6.09 ± 0.58 a@b*
Ascorbate induced	3.91 ± 0.30	6.81 ± 0.64 a*	3.85 ± 0.33	3.79 ± 0.34	4.88 ± 0.47 a#b*	4.43 ± 0.47 a@b*

Units: Lipid peroxidation – nmoles of MDA released/mg protein.
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.

Table 3. Effect of lupeol and lupeol linoleate on enzymatic antioxidant status in hepatic tissue of HCD fed groups compared with the control animals (Values are expressed as mean ± S.D. for six animals)

Antioxidant enzymes	Group I Control	Group II HCD	Group III Lupeol alone	Group IV Lupeol linoleate alone	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
SOD	12.53 ± 1.08	7.72 ± 0.75 a*	13.60 ± 1.03	12.74 ± 1.14	9.60 ± 0.57 a*b#	10.93 ± 0.85 a#b*c@
CAT	381.11 ± 34.69	285.71 ± 28.25 a*	385.48 ± 29.15	387.62 ± 35.86	359.49 ± 26.07 b*	370.41 ± 27.11 b*
GPx	22.17 ± 2.14	15.46 ± 1.46 a*	21.95 ± 2.03	23.39 ± 2.28	18.73 ± 1.43 a#b#	19.91 ± 2.02 b*

Units of enzyme activity: Superoxide dismutase (SOD): Units/mg protein, One unit is equal to the amount of enzyme that inhibits the autooxidation reaction by 50%, Catalase (CAT): μmoles of H_2O_2 consumed/min/mg protein, Glutathione peroxidase (GPx): μg of GSH consumed/min/mg protein.
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.

by 52.79% and 62.14% after 3 h and 6 h, respectively (Fig. 2b). Similar resistance to Fenton's reagent induced LDL oxidation was seen with lupeol by 48.57% and 65.45% and lupeol linoleate by 60.05% and 72.64% after 3 and 6 h, respectively (Fig. 3b).

Discussion

The drugs available in the modern medicinal system are definitely good enough to provide symptomatic relief, but are associated with undesirable side effects. In recent years there has been considerable emphasis in rediscovering biomolecules with hypolipidemic property from medicinal plants. The results of the present work revealed collapse of oxidative defence system in hepatic tissue and lipoprotein oxidation in the hypercholesterolemic condition, while treatment with lupeol and its ester derivative afforded considerable protection.

Hypercholesterolemia is associated with increased production of oxygen radicals and increased oxidation of LDL cholesterol [28, 29]. This oxidised LDL may directly induce endothelial dysfunction [30], which may also occur in hepatic tissue. Gupta *et al*. [1] demonstrated that hypercholesterolemia causes malfunctioning of liver through microvesicularsteatosis, due to the intracellular accumulation of lipids. This might be the possible reason for tissue damage. Such damage causes enzymes like LDH, ALP, ALT and AST to leak from the injured hepatic cells into the bloodstream. The activities of these hepatic enzymic markers of cellular damage were elevated in the serum of HCD fed rats. Lupeol and its ester treatment significantly decreased the marker enzymes and this may be due to the hypocholesterolemic property of triterpenes. Our earlier result showed that the triterpenes reduced serum total cholesterol level by 51.87% and 62.6%, respectively [13].

Lipid peroxidation of biological membranes can cause alterations in fluidity, reduction in membrane potential, increased permeability to H^+ and other ions and eventual membrane rupture, leading to the release of cell and organelle contents. Cytotoxic aldehydes resulting from LPO can block macrophage action, inhibit protein synthesis, inactivate enzymes, cross-link proteins, and can lead to the generation of thrombin [31]. Hence, lipid peroxidation can play a crucial role in inflammation, cancer and cardiac diseases [32]. A cholesterol-rich diet results in increased LPO by the induction of free radical production, followed by hypercholesterolemia [33]. The relationship between LPO and hypercholesterolemia has been recognized earlier. Lipid peroxidation levels were found to be increased in the liver, aorta and serum of hypercholesterolemic rabbits [34]. Corroborating with this study, high cholesterol fed rats in

Table 4. Effect of lupeol and lupeol linoleate on antioxidant status in hepatic tissue of HCD fed groups compared with the control animals (Values are expressed as mean ± S.D. for six animals)

Non-enzymic antioxidants	Group I Control	Group II HCD	Group III Lupeol alone	Group IV Lupeol linoleate alone	Group V HCD + lupeol	Group VI HCD + lupeol linoleate
Reduced glutathione	15.93 ± 1.41	10.93 ± 1.04 a*	15.91 ± 1.39	15.99 ± 1.49	13.86 ± 1.14 a#b#	14.02 ± 1.42 a@b*
Vitamin C	1.91 ± 0.11	1.03 ± 0.12 a*	1.93 ± 0.16	1.94 ± 0.18	1.67 ± 0.13 a@b*	1.82 ± 0.17 b*
Vitamin E	1.23 ± 0.12	0.71 ± 0.06 a*	1.22 ± 0.12	1.24 ± 0.10	1.09 ± 0.10 a#b*	1.15 ± 0.09 b*

Non-enzymic antioxidants are expressed as: Reduced glutathione (GSH), ascorbate and α-tocopherol: μg/mg protein.
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.

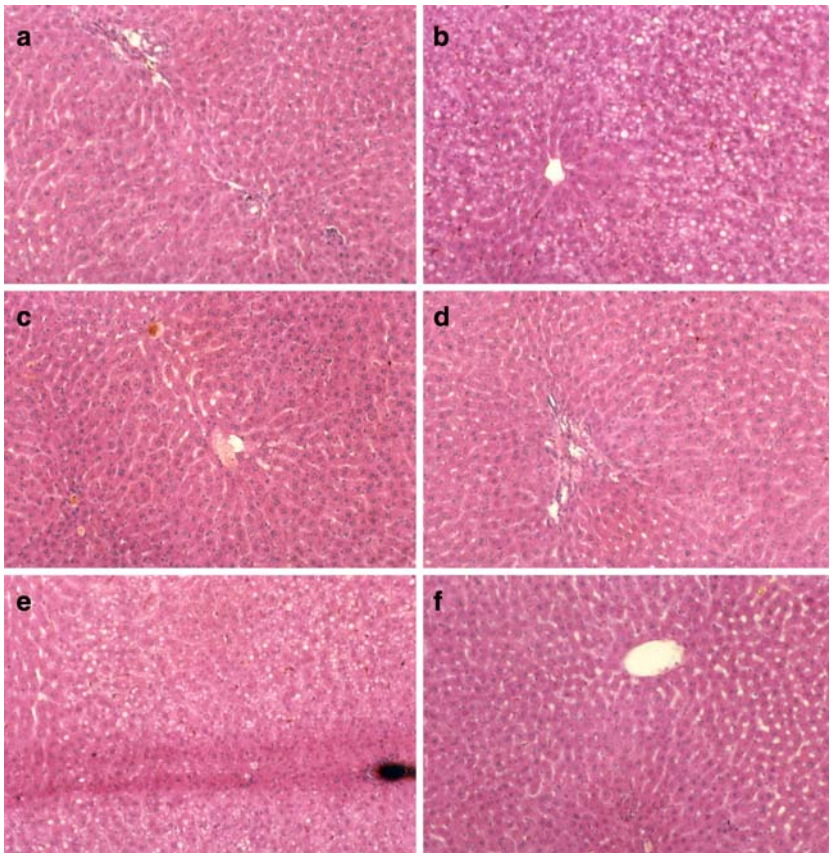

Fig. 1. Histopathological observation of liver tissue: (a) Group I: Control animal shows normal hepatic architecture; (b) Group II: HCD fed rats show marked fatty changes of hepatocytes; (c, d) Group III and IV: lupeol and lupeol linoleate supplemented rats showing near normal hepatic architecture; (e) Group V: lupeol treated hypercholestereolemic rats showing a partial reduction of fatty changes; (f) Group VI: lupeol linoleate-treated hypercholesterolemic rats showing very occasional fatty globule in hepatocytes and almost normal architecture of liver. (H&E, 50×).

the present study, also showed increased LPO in the hepatic tissue. Supplementation of lupeol and lupeol linoleate caused a significant decrease in lipid peroxidation level. An earlier report showed that the triterpene celastrol also has a potent inhibitory effect against lipid peroxidation in liver mitochondria [35].

Oxidative stress, defined as a disruption of the balance between oxidative and antioxidative processes, plays an important role in the pathogenesis of hypercholesterolemic atherogenesis [36]. Oxygen free radicals (OFRs) are found to be produced during hypercholesterolemic atherogenesis [5]. Superoxide has been shown to play a major role in the oxidative stress produced in the circulation during atherogenesis. The increase in levels of OFRs could be due to their overproduction and/or decreased destruction. Hepatic tissue contains enzymes that contribute to the antioxidant defense mechanism. In the present study, the decreased activities of hepatic SOD, catalase and GPx were shown in high cholesterol diet fed rats. The decreased activities of these enzymes might be due to increased levels of OFRs. Triterpenes treatment enhanced the activities of SOD, catalase and GPx. Hence increase in catalase and GPx activities can prevent damage by detoxifying reactive oxygen species. The increased level of these enzymes in triterpenes treated animals may be due to the suppression of superoxide production. According to Yamashita *et al*. [37] lupeol showed a significantly 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 lupeol structure.

Apart from enzymic antioxidants, non-enzymic antioxidants play a vital role in protecting cells from oxidative changes. GSH is one of the important antioxidant in living systems, which is involved in numerous biochemical pathways within the cells. It also plays a key role in liver detoxification reactions by maintaining the structural integrity of cells and organelle membranes and its ability to form conjugates with reactive xenobiotic metabolites [38]. Tocopherol, a known biological antioxidant, is concentrated

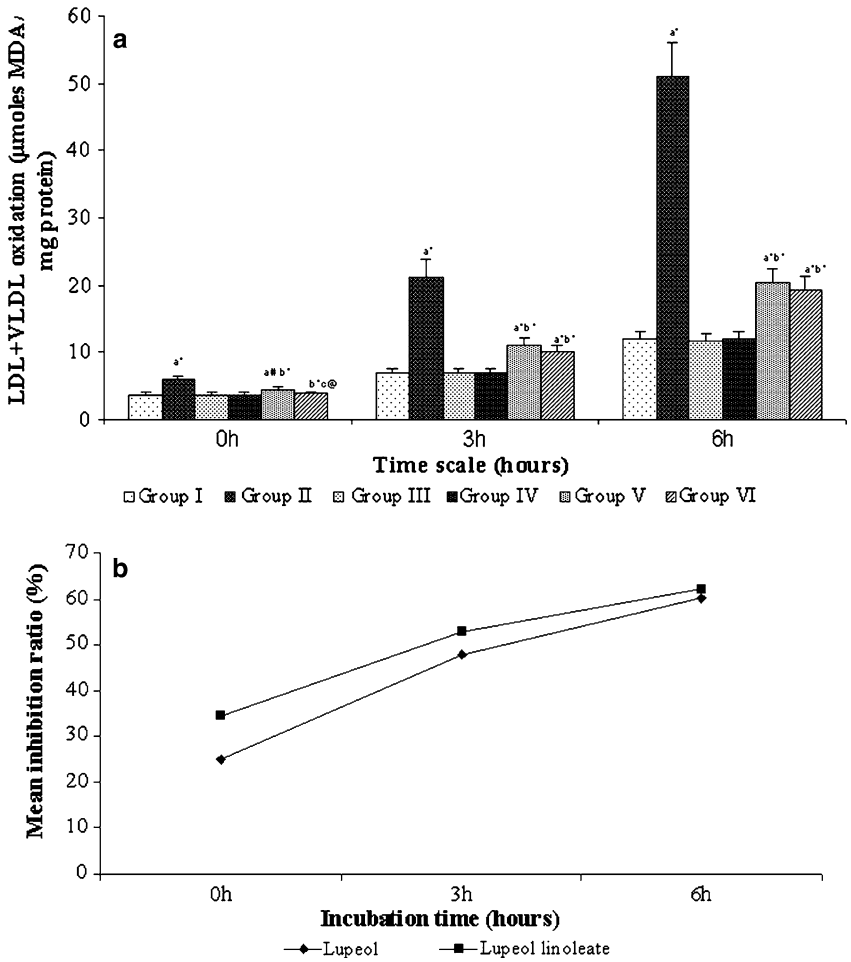

Fig. 2. (a) Effect of lupeol and lupeol linoleate on controlling the *in vitro* susceptibility of lipoproteins peroxidation induced by copper ions in HCD fed rats. Comparisons are made as follows: a – between group I and groups II, V, VI; b – between group II and groups V, VI; c – between group V and group VI. *, # and @ represents statistical significance at $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively. (b) Inhibition ratio (%) of lupeol and its ester of copper induced lipoprotein oxidation.

in the hydrophobic interior of cell membranes. In addition to protecting cellular structure from the attack of free radicals [39], it also protects against ROS through the maintenance of cellular protein thiol levels [40]. Ascorbic acid is the most widely cited form of water-soluble antioxidant, which prevents oxidative damage to the cell membrane induced by aqueous radicals. It also reduces and regenerates oxidised α-tocopherol and lipid peroxides [40]. With the downplay of hepatic antioxidant systems, normal levels of antioxidant molecules namely GSH, ascorbic acid and tocopherol were restored in the treated groups. In an earlier work, the restoration of hepatic non-enzymic antioxidants by triterpenes treatment was reported in cadmium-induced hepatotoxicity [12].

Hypercholesterolemia induced hepatic abnormalities are further confirmed by histopathological findings. In the present study, hypercholesterolemic rats showed marked fatty changes in the hepatocytes. Similar fatty changes have been observed by investigators [41] in hepatic tissue of hypercholesterolemic induced rats. Treatment with lupeol showed partial reduction of fatty changes. Similarly occasional fatty globules in hepatocytes and almost normal architecture was seen in lupeol linoleate treated HCD fed rats. Esterification of lupeol improved the efficiency of the parent drug by increasing its bioavailability, penetration and retention ability into the cell membrane [42]. This mechanism may be cited here for the protection afforded by lupeol linoleate.

Low density lipoprotein, a major cholesterol-carrying lipoprotein fraction in human blood plasma, is known to be a risk factor for the development of atherosclerosis [36] and also were shown to accumulate within the arterial wall. It is well established that increased level of LDL causes an increased risk for atherosclerosis [43]. When apolipoprotein

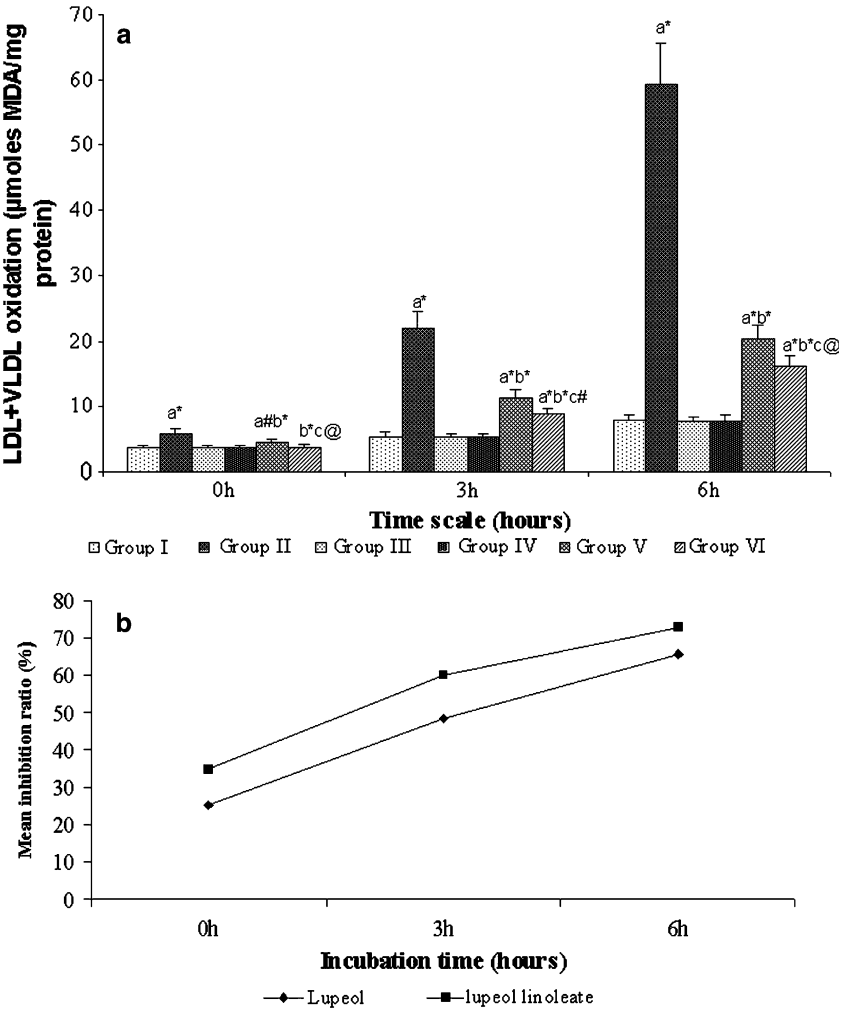

Fig. 3. (a) Effect of lupeol and lupeol linoleate on controlling the *in vitro* susceptibility of lipoproteins peroxidation induced by Fenton's reagent in HCD fed rats. Comparisons are made as follows: a – between group I and groups II, V, VI; b – between group II and groups V, VI; c – between group V and group VI. *, # and @ represents statistical significance at $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively. (b) Inhibition ratio (%) of lupeol and its ester of Fenton's reagent induced lipoprotein oxidation.

B100, the major protein of circulating LDL, is chemically modified with reagents that derivatize lysine, it is not recognized by LDL receptor. But it is taken up by the cells of arterial wall, especially macrophages, in a non-regulatory manner, through scavenger–receptor pathway. This process leads to the formation of foam cells, the hallmark of atherosclerotic lesion. Among other effects, intimal accumulation of monocytes, macrophages-foam cell formation, induction of endothelial dysfunction as well as the development of atheromatous lesions are supposed actions of modified LDL or in other words they are atleast supported by its activity [44, 45]. LDL isolated from human and animal atherosclerotic lesion exhibits evidence of oxidative damage [46]. Some of the factors that are likely to promote LDL oxidation in the arterial wall are metal ions, lipoxygenase, reactive nitrogen species and myeloperoxidase [47]. Catalysis of oxidation by copper ions [48] and Fe^{2+} ions are generally accepted and widely used for the determination of oxidizability of LDL. In this test, the production of TBARS is measured, which is often used for characterization of the atherogenicity of LDL. The beneficial effects of cholesterol-lowering drugs have mainly been related to their hypocholesterolemic effects. However, the reduction of LDL oxidation may provide an additional mechanism for preventing the progression of atherosclerosis. The inhibition ratio exerted by triterpenes strongly suggests a protective effect on LDL oxidation, which may be due to their hypocholesterolemic and antioxidant effects. It has been suggested that the oxidation of lipids and proteins in lipoprotein, leads to the impairment of lipid transport and to cell injury and thereby contributes to the development of various diseases [49]. Metal ions may be possible mediators of LPO [50]. It

has been suggested that the triterpene celasterol acts as a very potent inhibitor of ADP and Fe^{2+} induced LPO in mitochondria [9]. Andrikopoulos *et al*. [10] have found that lupeol and other triterpenes protect against copper ions induced LDL oxidation, performed in *in vitro* conditions.

In conclusion, the present findings emphasize that administration of lupeol and lupeol linoleate play a positive role, by suppressing the free radical production and protecting the hepatic tissue from lipemic-oxidative injury. Further, the favorable effect of lupeol and lupeol linoleate on *in vitro* lipoprotein peroxidation is presented. Triterpenes with hypocholesterolemic, antioxidative and anti-inflammatory properties may protect against deleterious effects in hypercholesterolemic atherogenesis. Of the two triterpenes tested, lupeol linoleate appeared to be even more effective than lupeol. Further studies are in progress to clarify the mechanism underlying the protective role of triterpenes in early stage of atherogenesis.

References

1. Gupta PP, Tandon HD, Ramalingaswami V: Cirrhosis of the liver in rabbits induced by a high cholesterol diet – an experimental model. Indian J Med Res 64: 1516–1526, 1976
2. Assy N, Kaita K, Mymin D, Levy C, Rosser B, Minuk G: Fatty infiltration of liver in hyperlipidemic patients. Dig Dis Sci 45: 1929–1934, 2000
3. Balkan J, Kanbagli O, Hatipoglu A, Kucuk M, Cevikbas U, Aykac-Toker G, Uysal M: Improving effect of dietary taurine supplementation on the oxidative stress and lipid levels in the plasma, liver and aorta of rabbits fed on a high-cholesterol diet. Biosci Biotechnol Biochem 66: 1755–1758, 2002
4. Young IS, McEneny J: Lipoprotein oxidation and atherosclerosis. Biochem Soc Trans 29: 358–362, 2001
5. Prasad K, Kalra J: Oxygen free radicals and hypercholesterolemic atherosclerosis: effect of vitamin E. Am Heart J 125: 958–973, 1993
6. Nadkarni KM: Indian Materia Medica, vol I, 3rd edn. Bombay: Popular Book Depot. pp. 371–372, 1976
7. Baskar R, Meenalakshmi Malini M, Varalakshmi P, Bhima Roa B.K.: Effect of lupeol isolated from *Crataeva nurvala* stem bark against free radical-induced toxicity in experimental urolithiasis. Fitoterapia 67: 121–126, 1996
8. Liu J, Liu Y, Mao Q, Klaassen CD: The effects of 10 triterpenoid compounds on experimental liver injury in mice. Fundam Appl Toxicol 22: 34–40, 1994
9. Sassa H, Takaishi Y, Terada H: The triterpene celastrol as a very potent inhibitor of lipid peroxidation in mitochondria. Biochem Biophys Res Commun 172: 890–897, 1990
10. Andrikopoulos NK, Kaliora AC, Assimopoulou AN, Papapeorgiou VP: Biological activity of some naturally occurring resins, gums and pigments against in vitro LDL oxidation. Phytother Res 17: 501–507, 2003
11. Geetha T, Varalakshmi P: Anti-inflammatory activity of lupeol and lupeol linoleate in adjuvant-induced arthritis. Fitoterapia 69: 13–19, 1998
12. Sunitha S, Nagaraj M, Varalakshmi P: Hepatoprotective effect of lupeol and lupeol linoleate on tissue antioxidant defence system in cadmium-induced hepatotoxicity in rats. Fitoterapia 72: 516–523, 2001
13. Sudhahar V, Kumar SA, Varalakshmi P: Role of lupeol and lupeol linoleate on lipemic-oxidative stress in experimental hypercholesterolemia. Life Sci 78: 1329–1335, 2006
14. Lowry OH, Rosebrough NJ, Farr AI, Randall RJ: Protein measurement with folin-phenol reagent. J Biol Chem 193: 265–275, 1951
15. King J: The dehydrogenases or oxidoreductases – lactate dehydrogenase. In: D Van (ed) Practical Clinical Enzymology. Nostrand Company Limited, London, 1965, pp 83–93
16. King J: The hydrolases – acid and alkaline phosphatases. In: D Van (ed) Practical Clinical Enzymology. Nostrand Company Limited, London, 1965, pp 191–208
17. King J: The transferases-alanine and aspartate transaminases. In: D Van (ed) Practical Clinical Enzymology. Nostrand Company Limited, London, 1965, pp 121–138
18. Hogberg J, Larson RE, Kristoferson A, Orrenius S: NADPH-dependent reductase solubilized from microsomes by peroxidation and its activity. Biochem Biophys Res Commun 56: 836–842, 1974
19. Devasagayam TP: Lipid peroxidation in rat uterus. Biochim Biophys Acta 876: 507–514, 1986
20. Marklund S, Marklund G: Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47: 469–474, 1974
21. Sinha AK: Colorimetric assay of catalase. Anal Biochem 47: 389–394, 1972
22. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG: Selenium: biochemical role as a component of glutathione peroxidase. Science 179: 588–590, 1973
23. Moron MS, Depierre JW, Mannervik B: Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim Biophys Acta 582: 67–78, 1979
24. Omaye ST, Turnbull JD, Sauberlich HE: Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. Meth Enzymol 62: 3–11, 1979
25. Desai ID: Vitamin E analysis methods for animal tissues. Meth Enzymol 105: 138–147, 1984
26. Wilson DE, Spiger MJ: A dual precipitation method for quantitative plasma lipoprotein measurement without ultracentrifugation. J Lab Clin Med 82: 473–482, 1973
27. Deepa PR, Varalakshmi P: Atheroprotective effect of exogenous heparin-derivative treatment on the aortic disturbances and lipoprotein oxidation in hypercholesterolemic diet fed rats. Clin Chim Acta 355: 119–130, 2005
28. Davi G, Alessandrini P, Mezzetti A, Minotti G, Bucciarelli T, Costantini F, Cipollone F, Bon GB, Ciabattoni G, Patrono C: In vivo formation of 8-Epi-prostaglandin F2 alpha is increased in hypercholesterolemia. Arterioscler Thromb Vasc Biol 17: 3230–3235, 1997
29. Napoli C, Postiglione A, Triggiani M, Corso G, Palumbo G, Carbone V, Ruocco A, Ambrosio G, Montefusco S, Malorni A, Condorelli M, Chiariello M: Oxidative structural modifications of low density lipoprotein in homozygous familial hypercholesterolemia. Atherosclerosis 118: 259–273, 1995
30. Keaney JF Jr, Guo Y, Cunningham D, Shwaery GT, Xu A, Vita JA: Vascular incorporation of alpha-tocopherol prevents endothelial dysfunction due to oxidized LDL by inhibiting protein kinase C stimulation. J Clin Invest 98: 386–394, 1996
31. Halliwell B, Gutteridge JMC: Role of free radicals and catalytic metal ions in Human disease: an overview. Methods in enzymology. Academic Press, San Diego, 1990, pp 1–85

32. Geetha RK, Vasudevan DM: Inhibition of lipid peroxidation by botanical extracts of *Ocimum sanctum*: in vivo and in vitro studies. Life Sci 76: 21–28, 2004
33. Wissler RW: Theories and new horizons in the pathogenesis of atherosclerosis and the mechanisms of clinical effects. Arch Pathol Lab Med 116: 1281–1291, 1992
34. Gokkusu C, Ademoglu E, Turkoglu UM, Oz H, Oz F: Thymosin alpha 1 protects liver and aorta from oxidative damage in atherosclerotic rabbits. Life Sci 59: 1059–1067, 1996
35. Sassa H, Takaishi Y, Terada H: The triterpene celastrol as a very potent inhibitor of lipid peroxidation in mitochondria. Biochem Biophys Res Commun 172: 890–897, 1990
36. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL: Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 320: 915–924, 1989
37. Yamashita K, Lu H, Lu J, Chen G, Yokoyama T, Sagara Y, Manabe M, Kodama H: Effect of three triterpenoids, lupeol, betulin, and betulinic acid on the stimulus-induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophils. Clin Chim Acta 325: 91–96, 2002
38. Klaassen CD, Bracken WM, Dudley RE, Goering PL, Hazelton GA, Hjelle JJ: Role of sulfhydryls in the hepatotoxicity of organic and metallic compounds. Fundam Appl Toxicol 5: 806–815, 1985
39. Bertoni-Freddari C, Fattoretti P, Caselli U, Paoloni R, Meier-Ruge W: Vitamin E deficiency as a model of precocious brain aging: assessment by X-ray microanalysis and morphometry. Scanning Microsc 9: 289–301, 1995
40. Freeman BA, Crapo JD: Biology of disease: free radicals and tissue injury. Lab Invest 47: 412–426, 1982
41. Deepa PR, Varalakshmi P: Protective effects of certoparin sodium, a low molecular weight heparin derivative, in experimental atherosclerosis. Clin Chim Acta 339: 105–115, 2004
42. Nikiema JB, Vanhaelen-Fastre R, Vanhaelen M, Fontaine J, De Graef C , Heenen M: Effects of antiinflammatory triterpenes isolated from *Leptadenia hastata* latex on keratinocyte proliferation. Phytother Res 15: 131–134, 2001
43. Goldstein JL, Brown MS: The low-density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem 46: 897–930, 1977
44. Rosenfeld ME: Oxidized LDL affects multiple atherogenic cellular responses. Circulation 83: 2137–2140, 1991
45. Ross R: Cell biology of atherosclerosis. Annu Rev Physiol 57: 791–804, 1995
46. Mahley RW: Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 240: 622–630, 1988
47. Heinecke JW: Oxidants and antioxidants in the pathogenesis of atherosclerosis: implications for the oxidized low density lipoprotein hypothesis. Atherosclerosis 141: 1–15, 1998
48. Esterbauer H, Ramos P: Chemistry and pathophysiology of oxidation of LDL. Rev Physiol Biochem Pharmacol 127: 31–64, 1996
49. Gutteridge JM: Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem 41: 1819–1828, 1995
50. Stohs SJ, Bagchi D: Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med 18: 321–336, 1995