Original Research

D-limonene suppresses doxorubicin-induced oxidative stress and inflammation via repression of COX-2, iNOS, and NFκB in kidneys of Wistar rats

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Abstract

D-limonene is a naturally occurring monoterpene and has been found to posses numerous therapeutic properties. In this study, we used D-limonene as a protective agent against the nephrotoxic effects of anticancer drug doxorubicin (Dox). Rats were given D-limonene at doses of 5% and 10% mixed with diet for 20 consecutive days. Dox was give at the dose of 20 mg/kg body weight intraperitoneally. The protective effects of D-limonene on Dox-induced oxidative stress and inflammation were investigated by assaying oxidative stress biomarkers, lipid peroxidation, serum toxicity markers, proinflammatory cytokines, and expression of nuclear factor kappa B (NFκB), cyclo-oxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) and Nitrite levels. Administration of Dox (20 mg/kg body weight) in rats enhanced renal lipid peroxidation; depleted glutathione content and antioxidant enzymes; elevated levels of kidney toxicity markers viz. kidney injury molecule-1 (KIM-1), blood urea nitrogen (BUN), and creatinine; enhanced expression of NFκB, COX-2, and iNOS and nitric oxide. Treatment with D-limonene prevented oxidative stress by restoring the levels of antioxidant enzymes, further both doses of 5% and 10% showed significant decrease in inflammatory response. Both the doses of D-limonene significantly decreased the levels of kidney toxicity markers KIM-1, BUN, and creatinine. D-limonene also effectively decreased the Dox induced overexpression of NF-κB, COX-2, and iNOS and nitric oxide. Data from the present study indicate the protective role of D-limonene against Dox-induced renal damage.

Keywords: Chemoprevention, doxorubicin, D-limonene, nephrotoxicity

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Introduction

Doxorubicin (Dox) due to its unmatched efficacy and broad-spectrum effects is a highly successful chemotherapeutic agent for malignant neoplasms but its usefulness is limited by acute and chronic dose related, cumulative, and essentially irreversible toxicities. Published reports show Dox to induce generation of both reactive oxygen species (ROS) and reactive nitrogen species (RNS), which engage in the interplay of a number of processes including redox cycling of the quinone moiety of Dox, disturbance of iron metabolism and Dox.[1] Since, quinone moiety is capable of undergoing one-electron redox reactions by redox cycling. In this process, ''Dox quinone'' is converted to ''Dox semiquinone'' by accepting an electron from an oxidant; in the presence of oxygen, this semiquinone is converted back to its native ''Dox quinone,'' producing superoxide (O^{2-}) as a byproduct.[2,3] O^{2-} can damage biological components directly, as well as being converted to more reactive ROS/RNS. Further, Dox-induced toxicity has also been believed to be mediated through formation of free radicals, lipid peroxidation of membranes, and iron-dependent oxidative damage to biological macromolecules.[4–7]

In view of the fact that, widespread free radical production is likely to overcome antioxidant security, the activation of free radical-induced hostile reactions could be protected by antioxidants that are expected to reduce the intracellular level of ROS. This increase in oxidative stress and depletion of endogenous antioxidants triggers immense immune response.[7,8]

Inflammation is an intricate process mediated by the activation of various immune cells. Macrophages play a

central role in mediating many different immune-pathological phenomena during inflammation, including the overproduction of pro-inflammatory cytokines and inflammatory mediators, such as interleukin IL-1β, IL-6, tumor necrosis factor-alpha (TNF-α), and nitric oxide (NO) synthesized by inducible NO synthase (i-NOS), and prostaglandin PGE-2 synthesized by cyclo-oxygenase-2 (COX-2).[9,10] Many studies have reported increased tissue content of inflammatory mediators together with inflammatory cell infiltration, signifying that inflammation plays a significant role in Dox-induced renal injury.[11,12] Dox increases levels of the proinflammatory cytokine TNF-α in the periphery, which can migrate into tissues and stimulate local inflammation and oxidative stress,[13] eventually leading to cellular apoptosis and, possibly appearance of side effects.[8]

Various studies have investigated the link between Dox and nuclear factor kappa B (NFκB). NFκB regulates genes that are involved in the control of the inflammatory responses and immune responses. NFκB is recognized to be an important redox-sensitive transcriptional factor that regulates transcription of genes encoding inflammatory cytokines, adhesion molecules, and chemokines. TNF-α is a vital member in a network of proinflammatory chemokines and cytokines activated in the kidney by Dox. Inhibition of TNF-α action prevents the activation of this cytokine network and provides defense against Dox nephrotoxicity.[14] NO levels have also been modulated by the use of Dox treatment.[8] Dox treatment has also been reported to induce COX-2 expression *in vitro* and *in vivo* models.[15]

Dietary natural products have shown protection against various degenerative diseases including cancer and in the present era, they are therapeutically importance because of the virtue of their anti-oxidant and anti-inflammatory potential. Epidemiological findings also implicate that substantial intake of fruits and vegetables in the diet have protective effects against different types of pathologies.[16] Natural products with anti-oxidant and anti-inflammatory activities are known to exhibit protection against Dox-induced toxicities. Sylimarin,[17] Lycopene,[18] *Zingiber officinale*,[19] *Solanum torvum*,[20] and Resveratrol[21] were found to protect against Dox-induced nephrotoxicity.

D-limonene, a monocyclic monoterpene, is a naturally occurring substance derived from orange peels.[22] D-limonene is a major constituent of numerous citrus oils. D-limonene is widely used as a flavor and fragrance additive in soaps, foods, perfumes, chewing gum, and beverages.[23] When orally administrated, D-limonene is rapidly and almost completely absorbed from the GI tract of both animals as well as human.[24,25]

The goal of this study was to examine the effects of D-limonene pretreatment on Dox toxicity in the kidneys. The results of this investigation show that D-limonene pretreatment protects against Dox-induced renal-toxicity while substantially decreasing oxidative stress, proinflammatory cytokines, and the expression of some proteins involved in inflammation.

Materials and methods

Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), reduced nicotinamide adenine dinucleotide phosphate (NADPH), NADP+, FAD, ethylene diamine tetra acetic acid (EDTA), thiobarbituric acid (TBA), pyrogallol, poly-L-lysine, xanthine, glucose-6-phosphate, bovine serum albumin (BSA), dichlorophenolindophenol, 5,50-dithio-*bis*-(2-nitrobenzoic acid), *d*-D-limonene, 1-chloro-2,4-dinitrobenzene and glutathione reductase (GR) were obtained from Sigma (Sigma Chemical Company). H_2O_2, magnesium chloride, sulphosalicylic acid, perchloric acid, TCA, Tween-20, Folin–Ciocalteau reagent, sodium potassium tartarate, di-sodium hydrogen phosphate, sodium di-hydrogen phosphate, and sodium hydroxide were purchased from E. Merck Limited. All other chemicals and reagents were of the highest-purity grade commercially available.

Experimental design

To study the effect of prophylactic treatment with D-limonene on Dox-induced oxidative stress and inflammatory responses in the kidney, five groups each of six male Wistar rats were kept in different cages as per the different dose and modulator combinations requirement. The rats of group I (control group) received normal diet for 20 consecutive days. Group III received diet with 5% D-limonene for 20 consecutive days. Groups IV and V received D-limonene 10% in diet for 20 consecutive days. Groups II, III, and IV were given a single intraperitoneally injection of Dox at the dose of 20 mg/kg body weight on 20th day. All the rats were anaesthetized with mild anesthesia and killed by cervical dislocation after 24 h of the Dox injection[26–29] (Figure 1).

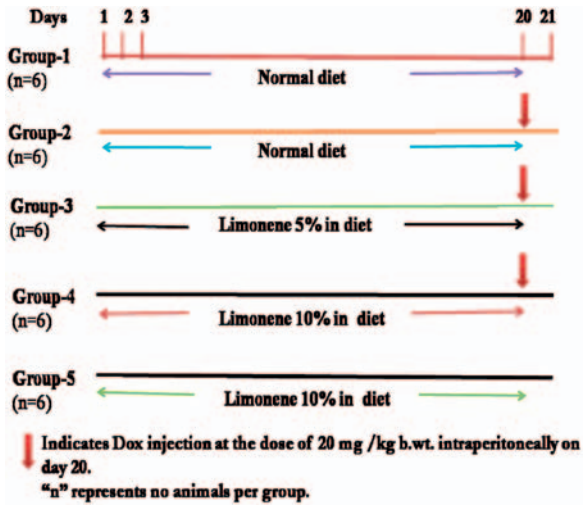

Figure 1 Schematic representation of experimental design. (A color version of this figure is available in the online journal)

Postmitochondrial supernatant preparation and estimation of different parameters

Kidneys were removed quickly, cleaned free of irrelevant material and immediately perfused with ice-cold saline (0·85% NaCl). The kidneys (10% w/v) were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) using a Potter Elvehjen homogenizer. The homogenate was filtered through muslin cloth, and centrifuged at 3000 rpm for 10 min at 48°C in a Remi Cooling Centrifuge (C-24 DL) to separate the nuclear debris. The aliquot so obtained was centrifuged at 12,000 rpm for 20 min at 4°C to obtain post mitochondrial supernatant (PMS), which was used as a source of various enzymes.

Assay for catalase activity

Catalase activity was assayed by the method of Claiborne.[30] The reaction mixture consisted of 1.95 mL phosphate buffer (0.1 M, pH 7.4), 1.0 mL hydrogen peroxide (0.10 mM), and 0.05 mL 10% PMS in a final volume of 3 mL. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed/min mg protein.

Assay for glutathione-S-transferase activity

Glutathione-*S*-transferase (GST) activity was estimated by the method of Habig et al.[31] The reaction mixture consisted of 1.525 mL phosphate buffer (0.1 M, pH 7.4), 0.2 mL reduced glutathione (1 mM), 0.025 mL 1-chloro 2, 4-dinitrobenzene (CDNB) (1 mM), and 0.250 mL PMS (10% w/v) in a total volume of 2.0 mL. The changes in the absorbance was recorded at 340 nm and enzymes activity was calculated as nmol CDNB conjugate formed $min^{-1} mg^{-1}$ protein using a molar coefficient of $9.6 \times 10^3 M^{-1} cm^{-1}$.

Activity of reduced glutathione (GSH)

Reduced glutathione was determined by the method of Jollow et al.[32] Sample of PMS (1.0 mL) was precipitated with 1.0 mL of sulphosalicylic acid (4%). The samples were kept at 4°C for 1 h and then centrifuged at 3000 rpm for 20 min at 4°C. The assay mixture contained 0.4 mL supernatant, 2.2 mL phosphate buffer (0.1 M, pH 7.4), and 0.4 mL 5, 5′-dithio bis-[2-nitrobenzoic acid] (10 mM) in a total volume of 3.0 mL. The yellow color developed, was read immediately at 412 nm on spectrophotometer and GSH was expressed as n mol GSH/g tissue.

Activity of glutathione peroxidase

Glutathione peroxidase (GPx) activity was estimated by the method of Mohandas et al.[33] The reaction mixture consisted of 1.49 mL phosphate buffer (0.1 M, pH 7.4), 0.1 mL EDTA (1 mM), 0.1 mL sodium azide (1 mM), 0.05 mL GR (1 IU/mL), 0.05 mL GSH (1 mM), 0.1 mL NADPH (0.2 mM), 0.01 mL H_2O_2 (0.25 mM), and 0.1 mL 10% PMS in a total volume of 2 mL. The disappearance of NADPH at 340 nm was recorded at 25°C. Enzyme activity was calculated as n mol NADPH oxidized per minute per mg protein using molar extinction coefficient of $6.22 \times 10^3 M^{-1} cm^{-1}$.

Assay of xanthine oxidase

The activity of xanthine oxidase (XO) was assayed by the method of Stripe and Della Corte.[34] The reaction mixture consisted of 0.2 mL PMS that was incubated for 5 min at 37°C with 0.8 mL phosphate buffer (0.1 M, pH 7.4). The reaction was started by adding 0.1 mL xanthine (9 mM) and kept at 37°C for 20 min. The reaction was terminated by the addition of 0.5 mL ice-cold perchloric acid (10% v/v). After 10 min, 2.4 mL of distilled water was added and centrifuged at 4000 rpm for 10 min and µg uric acid formed per minute per mg protein was recorded at 290 nm.

Measurement of superoxide dismutase activity

The superoxide dismutase (SOD) activity was measured by the method of Marklund and Marklund.[35] The reaction mixture consisted of 2.875 mL Tris–HCl buffer (50 mM, pH 8.5), pyrogallol (24 mM in 10 mM HCl) and 100 µL PMS in a total volume of 3 mL. The enzyme activity was measured at 420 nm and was expressed as units/mg protein. One unit of enzyme is defined as the enzyme activity that inhibits auto-oxidation of pyrogallol by 50%.

Estimation of lipid peroxidation

The assay for microsomal lipid peroxidation was done following the method of Wright et al.[36] The reaction mixture in a total volume of 1.0 mL contained 0.58 mL phosphate buffer (0.1 M, pH 7.4), 0.2 mL homogenate, 0.2 mL ascorbic acid (100 mM), and 0.02 mL ferric chloride (100 mM). The reaction mixture was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by addition of 1.0 mL 10% trichloroacetic acid (TCA). Following addition of 1.0 mL 0.67% TBA, all the tubes were placed in boiling water-bath for 20 min and then shifted to crushed ice-bath before centrifuging at 4500 rpm for 10 min. The amount of malondialdehyde formed in each of the samples was assessed by measuring optical density of the supernatant at 535 nm using spectrophotometer (Milton Roy 21 D) against a reagent blank. The results were expressed as nmol MDA formed per hour per gram tissue at 37°C using molar extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$.

Measurement of GR activity

The GR activity was measured by the method of Carlberg and Mannervik.[37] The assay system containing 1.65 mL phosphate buffer (0.1 M, pH 7.6), 0.1 mL EDTA (0.5 mM), 0.05 mL oxidized glutathione (1.0 mM), 0.1 mL NADPH (0.1 mM), and 0.1 mL PMS (10%) in a total volume of 2.0 mL. The enzyme activity was recorded at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as µmol NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22 \times 10^3 M^{-1} cm^{-1}$.

Measurement of quinone reductase activity

The quinone reductase (QR) activity was determined by the method of Benson et al.[38] The 3 mL reaction mixture consists of 2.13 mL Tris–HCl buffer (25 mM, pH 7.4), 0.7 mL BSA, 0.1 mL FAD, 0.02 mL NADPH (0.1 mM), and 50 µL PMS (10%). The reduction of dichlorophenolindophenol

(DCPIP) was recorded calorimetrically at 600 nm and the enzyme activity was calculated as μmol of DCPIP reduced/min/mg protein using molar extinction coefficient of $2.1 \times 104\,M^{-1}cm^{-1}$.

Assay for lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity was estimated in serum by the method of Kornberg.[39] The assay mixture consisted of 0.2 mL of serum, 0.1 mL of 0.02 M NADH, 0.1 mL of 0.01 M sodium pyruvate, 1.1 mL of 0.1 M (pH 7.4) phosphate buffer, and distilled water in a total volume of 3 mL. Enzyme activity was recorded at 340 nm, and activity was calculated as nmol NADH oxidized/min/mg protein.

Measurement of NO

Production of NO was evaluated by measuring the level of nitrite (an indicator of NO) in the supernatant using a colorimetric reaction with Griess reagent. Briefly, 100 μL of supernatants from different groups was mixed with 100 μL Griess reagent [0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4]. After incubation at room temperature in the dark for 10 min, total nitrites were measured spectrophotometrically at 540 nm. The concentration of nitrite in the sample was determined from a $NaNO_2$ standard curve.[40]

Assay for hydrogen peroxide

Hydrogen peroxide (H_2O_2) was assayed by H_2O_2-mediated horseradish peroxidase-dependent oxidation of phenol red by the method of Pick and Keisari.[41] 2.0 mL of supernatant, suspended in 1.0 mL of solution containing phenol red (0.28 nm), horseradish peroxidase (8.5 units), dextrose (5.5 nm), and phosphate buffer (0.05 M, pH 7.0) was incubated at 37°C for 60 min. The reaction was stopped by the addition of 0.01 mL of NaOH (10 N) and then centrifuged at $800 \times g$ for 5 min. The absorbance of supernatant was recorded at 610 nm against a reagent blank. The quantity of H_2O_2 produced was expressed as nmol H_2O_2/h/gm tissue based on the standard curve of H_2O_2-mediated oxidation of phenol red.

Assay for BUN and creatinine

BUN and creatinine levels were measured by the commercially available kit from Reckon digonistics, Pvt. Ltd. India, following manufacturer's instruction.

Kidney injury molecule-1 levels

KIM-1 Levels were measured by Elisa Plate Reader (Benchmark plus, BioRad) by the commercially available kit from Adipo Biotech (CA, USA) following manufacturer's protocol.

Cytokine analysis

Serum levels of proinflammatory cytokines, TNF-α and PGE2, were analyzed in serum. Serum was separated from blood and the levels of earlier-mentioned cytokines were evaluated in it by Elisa Plate Reader (Benchmark plus, BioRad) following the instructions of the manufacturer.

Histological investigation

For histopathology study, the kidneys were removed and immediately fixed in freshly prepared 10% neutral-buffered formalin at 4°C. Then, the skin was embedded in paraffin wax. A vertical section of skin (5-μm thick) was cut and stained with hematoxylin and eosin (H&E). The leucocytes infiltration and epidermal thickness were observed as an indicator of histological changes with microscope (fluorescent microscope, Olympus) at least in six different regions.

Measurement of protein

The protein concentration in all samples was determined by the method of Lowry et al.[38,42] using BSA as the standard.

Statistical analysis

The data from individual groups are presented as the mean ± standard error of the mean (SEM). Differences between groups were analyzed by using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test and minimum criterion for statistical significance was set at $P < 0.05$ for all comparisons.

Results

d-limonene attenuates the serum toxicity parameters for renal toxicity

Protective effect of D-limonene on serum BUN, LDH, kidney injury molecule-1 (Kim-1), and creatinine level was observed. Significant change in these parameters was found in the Dox-treated group II as compared with group I ($P < 0.001$). Pretreatment with D-limonene was found significantly effective in normalization of these kidney markers when compared with Dox-treated group (Table 1 and

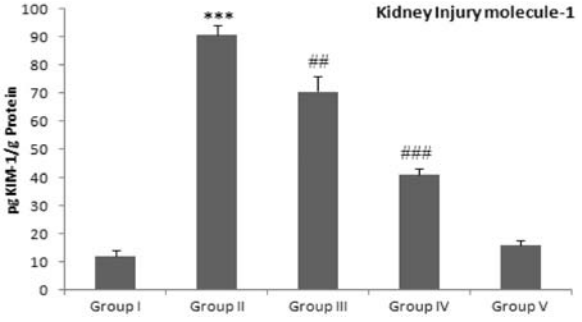

Figure 2 Group I: Control group (normal diet); group II: doxorubicin-treated group (20 mg/kg body weight); group III: dose 1 of D-limonene (5% in diet) + doxorubicin (20 mg/kg body weight); group IV: dose 2 of D-limonene (10% in diet) + doxorubicin (20 mg/kg body weight); group V: only dose 2 of D-limonene (10% in diet). Effect of prophylactic treatment of D-limonene against Dox-induced Kim-1 level in kidney of Wistar rats. Data were expressed as mean ± SD ($n = 6$) and measured as pg Kim-1/g protein. Kim-1 level was significantly increased (***$P < 0.001$) in Dox-treated group (group II) when compared with group I. Pretreatment with D-limonene significantly attenuated the level of Kim-1 in group III (##$P < 0.01$) and group IV (###$P < 0.001$) when compared with group II

Figure 2). D-limonene alone (group V) did not show any significant difference as compared with control (group I).

Effect of D-limonene and Dox on the antioxidant enzymes

The effect of D-limonene pre-treatment on Dox-induced depletion in the activity of different antioxidant enzymes was examined and the results were shown in Table 3. We have observed that there was a significant ($P < 0.001$) difference in the activity of different antioxidant enzymes between control group and the only Dox-treated positive control group. However, pretreatment with D-limonene in the groups III and IV before Dox administration significantly restored the activity of antioxidant enzymes when compared with the only Dox-treated group. There was no significant difference observed between the groups I and V (Tables 2 and 3).

Effect of D-limonene and Dox on the renal GSH content

Pretreatment of D-limonene before the Dox administration was found effective in restoring the endogenous anti-oxidant GSH. There was significant depletion in the level of

Table 1 Results of pretreatment of Limonene on Dox-induced serum toxicity markers

Treatment regimen per group	BUN (mg/100 ml) IU/L	Creatinine (mg/100 ml) IU/L	LDH (*n* mol NADH oxidized/ min/mg protein)
Group I	19.55 ± 1.13	1.40 ± 0.07	200.0 ± 16.3
Group II	49.61 ± 2.68*	3.86 ± 0.08*	431.7 ± 44.1*
Group III	30.88 ± 2.77†	2.83 ± 0.11‡	336.4 ± 23.7§
Group IV	25.64 ± 4.63†	1.84 ± 0.18†	285.7 ± 27.0†
Group V	21.18 ± 1.96	1.49 ± 0.07	190.4 ± 19.4

Results represent mean ± SE of six animals per group. Group I: control; group II (toxicant): Dox; group III: Dox + Lim (5%); group IV: Dox + Lim (10%); group V: Lim ( ). Lim, Limonene. Results obtained are significantly different from group I (*$P < 0.001$). Results obtained are significantly different from group II (†$P < 0.0001$), (‡$P < 0.01$) and (§$P < 0.05$).

Table 2 Results of pretreatment of Limonene on biochemical parameters in Dox-induced renal toxicity

Parameters	Group I	Group II	Group III	Group IV	Group V
MDA (nmol of MDA formed/g tissue)	2.19 ± 0.04	5.12 ± 0.24*	3.90 ± 0.40†	3.10 ± 0.09‡	2.18 ± 0.07
H_2O_2 (nmol of H_2O_2/g tissue)	186.5 ± 9.9	515.5 ± 39.2*	321.5 ± 20.4‡	223.1 ± 22.80‡	184.6 ± 26.41
XO (μg uric acid/min/mg protein)	0.19 ± 0.01	0.41 ± 0.01*	0.33 ± 0.02†	0.24 ± 0.01‡	0.19 ± 0.01
Catalase (nmol H_2O_2 consumed /min/mg Protein)	362 ± 42.2	105.8 ± 6.36*	302.4 ± 20.5‡	296.0 ± 25.1‡	360.4 ± 2.01
QR (nmol NADPH oxidized/min/mg protein)	266.9 ± 14.4	142.6 ± 5.86*	215.5 ± 8.60‡	249.0 ± 13.0‡	253.9 ± 6.13
SOD (IU/L)	175.5 ± 4.60	102.6 ± 5.60*	146.7 ± 4.51†	160.7 ± 3.31‡	177.9 ± 4.36

Results represent mean ± SE of six animals per group. Group I: control; group II (toxicant): Dox; group III: Dox + Lim (5%); group IV: Dox + Lim (10%); group V: Lim (10%). Lim, Limonene. Results obtained are significantly different from group I (*$P < 0.001$). Results obtained are significantly different from group II (†$P < 0.01$), (‡$P < 0.001$) and (§$P < 0.05$).

Table 3 Results of pretreatment of Limonene on antioxidant enzymes like GSH, GST, GR and GPX on Dox induced renal redox imbalance

Treatment regimen per group	GSH (*n* mol GSH/g tissue)	GST (*n* mol CDNB conjugate-formed/min/ mg protein)	GR (*n* mol NADPH oxidized/min/mg protein)	GPX (*n* mol NADPH oxidized/min/mg protein)
Group I	0.61 ± 0.03	120.4 ± 4.38	297.4 ± 21.5	328.6 ± 13.63
Group II	0.35 ± 0.01*	66.67 ± 5.31*	174.9 ± 8.6*	169.9 ± 13.84*
Group III	0.47 ± 0.01†	108.1 ± 7.68‡	204.8 ± 5.36‡	268.2 ± 9.66†
Group IV	0.55 ± 0.02†	111.6 ± 6.57†	290.9 ± 14.8†	290.3 ± 6.69†
Group V	0.61 ± 0.02	120.9 ± 6.8	299.0 ± 15.8	322.8 ± 11.24

Results represent mean ± SE of six animals per group. Group I: control; group II (toxicant): Dox; group III: Dox + Lim (5%); group IV: Dox + Lim (10%); group V: Lim ( ). Lim, Limonene. Results obtained are significantly different from group I (*$P < 0.001$). Results obtained are significantly different from group II (†$P < 0.001$), (‡$P < 0.01$) and (§$P < 0.05$).

GSH content in group II when compared with group I ($P < 0.001$). Pretreatment with D-limonene in groups III and IV shows significant increase in the level of GSH content ($P < 0.01$) as compared with group II. There was no significant difference in the GSH content between group I and V (Table 3).

Effect of D-limonene pretreatment and Dox on XO activity in renal tissue

The activity of XO was significantly increased ($P < 0.001$) in group II as compared with group I. D-limonene pretreatment significantly decreased the activity of XO in group III ($P < 0.01$) and group IV ($P < 0.001$) as compared with group II. Group V exhibited no significant change in the activity of XO as compared with group I (Table 2).

Effect of D-limonene pretreatment and Dox on QR activity

QR reflected significant decrease in the enzyme activity in renal tissue of group II when compared with group I (Table 2). D-limonene significantly restores the level of QR in both the groups III and IV when compared with group II. There was no significant change in group V compared with control group I.

Effect of D-limonene on Dox induced MDA levels in rat renal tissue

D-limonene inhibits lipid peroxidation caused by Dox administration in terms of TBARS (MDA), a well known biomarker of oxidative stress. Administration of Dox leads to significant elevation in the level of MDA in the group II to that of the acetone-treated group I ($P < 0.001$). Pre-treatment with D-limonene before Dox administration was found significantly ($P < 0.001$) effective in amelioration of MDA formation. There was no significant change observed in the level of MDA between control and only D-limonene-treated animals (Table 2).

XO and H2O2 level restored to normal by d-limonene pretreatment

There was significant enhancement of renal microsomal XO ($P < 0.001$) and H_2O_2 ($P < 0.001$) levels in Dox treatment group. Marked reduction was noted in D-limonene-treated groups (III and IV) at both the doses in both enzymes. No significant difference was observed in group I and group V (Table 2).

Effect of D-limonene and Dox treatment on proinflammatory cytokines

We have assessed the effect D-limonene on Dox-induced renal TNF-α and PGE-2 production quantitatively (Figures 3 and 4). We found that there was a significant difference in the level of proinflammatory cytokines between control group and Dox-treated group ($P < 0.001$). Pre-treatment with D-limonene significantly inhibit their production in the group III when compared with the only Dox-treated group II. There was no significant difference found between group I and IV.

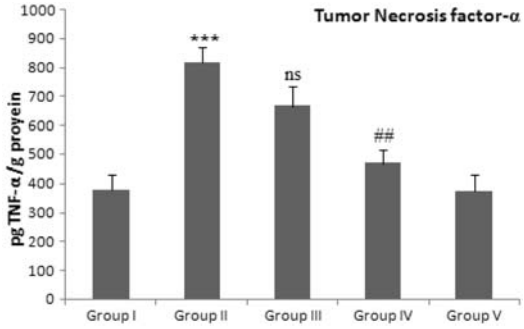

Figure 3 Group I: Control group (normal diet); group II: doxorubicin-treated group (20 mg/kg body weight); group III: dose 1 of D-limonene (5% in diet) + doxorubicin (20 mg/kg body weight); group IV: dose 2 of D-limonene (10% in diet) + doxorubicin (20 mg/kg body weight); group V: only dose 2 of D-limonene (10% in diet). Effect of prophylactic treatment of D-limonene against Dox-induced TNF-α level in kidney of Wistar rats. Data were expressed as mean ± SD ($n = 6$) and measured as pg TNF-α/g protein. TNF-α level was significantly increased (***$P < 0.001$) in Dox-treated group (group II) when compared with group I. Pretreatment with D-limonene significantly attenuated the level of TNF-α in group III (ns P = not significant) and group IV (##$P < 0.01$) when compared with group II

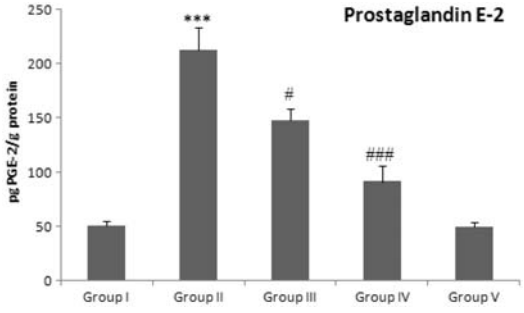

Figure 4 Group I: Control group (normal diet); group II: doxorubicin-treated group (20 mg/kg body weight); group III: dose 1 of D-limonene (5% in diet) + doxorubicin (20 mg/kg body weight); group IV: dose 2 of D-limonene (10% in diet) + doxorubicin (20 mg/kg body weight); group V: only dose 2 of D-limonene (10% in diet). Effect of prophylactic treatment of D-limonene against Dox-induced PGE-2 level in kidney of Wistar rats. Data were expressed as mean ± SD ($n = 6$) and measured as pg PGE-2/g protein. PGE-2 level was significantly increased (***$P < 0.001$) in Dox-treated group (group II) when compared with group I. Pretreatment with D-limonene significantly attenuated the level of PGE-2 in group III (#$P < 0.05$) and group IV (###$P < 0.001$) when compared with group II

Effect of D-limonene on the NO production

Administration of Dox resulted in the elevated renal NO production in the group II as compared with the group I ($P < 0.001$). We observed that pre-treatment with D-limonene was significantly effective in reducing NO production in group III and IV when compared with the group II ($P < 0.01$ and $P < 0.001$). There was no significant difference observed between group I and V as far as NO production is concern (Figure 5).

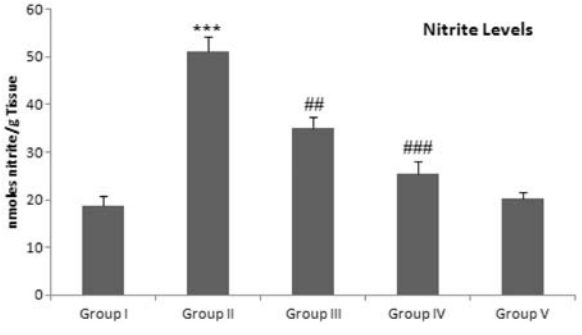

Figure 5 Group I: Control group (normal diet); group II: doxorubicin-treated group (20 mg/kg body weight); group III: dose 1 of D-limonene (5% in diet) + doxorubicin (20 mg/kg body weight); group IV: dose 2 of D-limonene (10% in diet) + doxorubicin (20 mg/kg body weight); group V: only dose 2 of D-limonene (10% in diet). Effect of prophylactic treatment of D-limonene against Dox-induced Nitrite level in kidney of Wistar rats. Data were expressed as mean ± SD ($n = 6$) and measured as nm nitrite/g tissue. Nitrite level was significantly increased (***$P < 0.001$) in Dox-treated group (group II) as compared with group I. Pretreatment with D-limonene significantly attenuated the level of Nitrite in group III (##$P < 0.01$) and group IV (###$P < 0.001$) when compared with group II

Effect of D-limonene on the Dox-induced renal Immunohistochemical expression of NF-κB, COX-2, and iNOS

Renal expression of the above-mentioned proteins is shown in the figures respectively. Brown color clearly indicates the more number of cells having NF-κB, COX-2, and i-NOS expression in the group II when compared with that of group I. Pretreatment with D-limonene in the group III results in reducing the number of cells showing expression of NF-κB, COX-2, and i-NOS. However, there was no significant difference observed in the expression of these proteins in group V as compared with group I. For immunohistochemical analysis, brown color indicates specific immunostaining of NF-κB, COX-2, and i-NOS and blue color indicates Hematoxylin staining. Original magnification: 40 × (Figures 6–8).

Effect of D-limonene pretreatment and Dox on renal histology

Effect of orally administered D-limonene was seen on renal histological changes caused by Dox injection with reference to neutrophil infiltration figure. We found that Dox administration caused damage in renal histo-architecture in group II when compared with the group I. Pretreatment with D-limonene in the groups III and IV diminished Dox-induced neutrophil infiltration as well as. There was no distinguished change observed between the groups I and V as far as these histological alterations are concerned (Figure 9).

Discussion

In this study, we have observed the protective effects of D-limonene against Dox-induced renal toxicity in Wistar rats. Dox-induced renal toxicity is well documented.[18,20,43] However, the exact mechanism underlying Dox-induced renal toxicity is still unclear, but it may be due to ROS generated by Dox, which leads to the condition of oxidative stress. Therefore, natural compounds with antioxidant properties are gaining much attention. This study was carried out to elucidate the effect of D-limonene on Dox-induced renal toxicity in rats, and to assess its role in the modulation of the inflammatory pathway

The most widely accepted mechanism of Dox toxicity implicates the formation of a Dox semiquinone free radical by the action of NADPH dependant reductases. In presence of oxygen this semi-quinone form yields super oxide radicals (O^{2-}). Free radicals can also be produced by a non-enzymatic mechanism that involves reactions of iron–Dox complex that can reduce oxygen to H_2O_2 and other ROS.[19,44,45]

The antioxidant status of kidney is significantly lowered in the Dox treatment animals due to the decreased SOD, CAT, GPx activities, and GSH level. D-limonene administration restored the kidneys antioxidant status and completely protect against renal damage. The importance of thiol-mediated detoxification of anticancer drugs that produce toxic electrophiles has been of considerable interest to many investigators. GSH, a non-protein in the cell is involved in the xenobiotic metabolism. The enhanced GSH level in D-limonene-treated animals partially explains its mechanism of protection. The elevated levels of GSH could effectively provide thiol group for the possible GSH-mediated detoxification reactions of GPx and GST. Administration of D-limonene enhanced the activities of renal GPx and GST.[45,46] Moreover, the enhanced SOD activity in the D-limonene-treated group might be involved in the scavenging of O^{2-} generated from the Dox. The elevated renal GST activity in the D-limonene plus Dox treatment further supports the protective mechanism.[45,46] Thus, the enhanced renal antioxidant status results from the treatment of D-limonene could explain the nephroprotective effect.

Lipid peroxidation is a marker of oxidative stress, number of studies have reported that elevation in the level of malondialdehyde (MDA), a lipid peroxidation product, was observed after Dox-treatment.[19,20] Our results agreed with the above mentioned previous findings which showed that there is remarkable increase in the level of MDA in rats treated with Dox and pretreatment with D-limonene significantly reduced the level of MDA.

Serum BUN and creatinine are all considered as reliable, important, well-documented markers of renal function for investigating drug induced nephrotoxicity in animals and man, and Dox toxicity has been shown to increase their level.[47,48] In accordance with previous studies, Dox strongly induced both BUN creatinine and LDH[1,20] in our study, whereas our modulator viz., D-limonene decreased their level, thus showing substantial protection bestowed to rats against Dox nephrotoxicity.

Kim-1 is a type 1 transmembrane protein that is usually undetectable in healthy kidney tissue. Transcript levels for the gene that encodes Kim-1/Havcr1 are strongly up-regulated in dedifferentiated proximal tubule epithelial cells in kidney after ischemic or toxic injury. Kim-1 has been recommended to serve as a useful and most sensitive biomarker for renal injury in preclinical and clinical studies of drug safety evaluation, and the monitoring of renal disease status.[49] In our study, Kim-1 levels were elevated in the

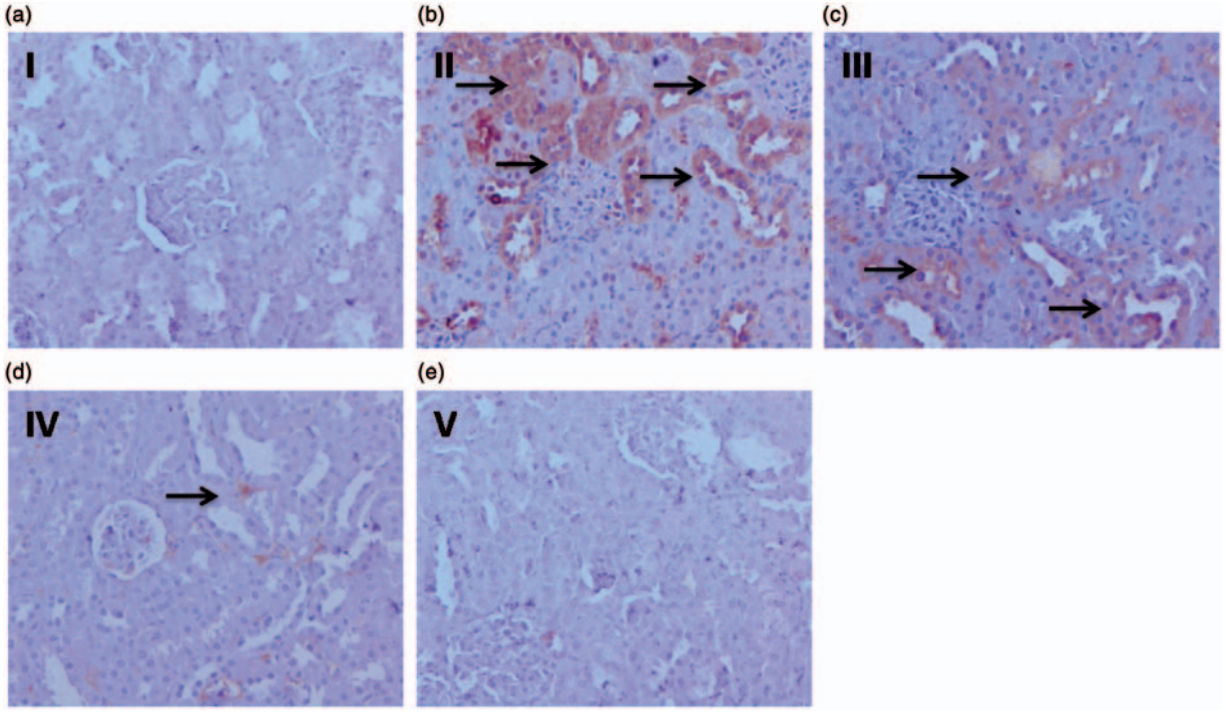

Figure 6 Effect of prophylactic treatment of D-limonene against Dox-induced NF-κB expression. Photomicrographs of renal sections of rat depicting (a) Control group given normal diet (group I), (b) Dox-treated group (20 mg/kg body weight) (group II), (c) dose 1 of D-limonene (5% in diet) + Dox (20 mg/kg body weight) (group III), (d) dose 2 of D-limonene (10% in diet) + Dox (20 mg/kg body weight) (group IV), and (e) only dose 2 of D-limonene (10% in diet) (group V). (a) There is almost no expression of NF-κB in the renal sections of control group. (b) Dox administration increased strongly NFκB expression in renal sections. (c) There was partial inhibition of NF-κB expression as evidenced by weak immunostaining in the rat kidneys treated with lower dose of D-limonene (5%). (d) In contrast, there was almost complete suppression of NF-κB in rats treated with higher dose of D-limonene (10%), this was evident from the figure, as the tubular structures within the inner cortical regions do not show any substantial immunostaining. (A color version of this figure is available in the online journal)

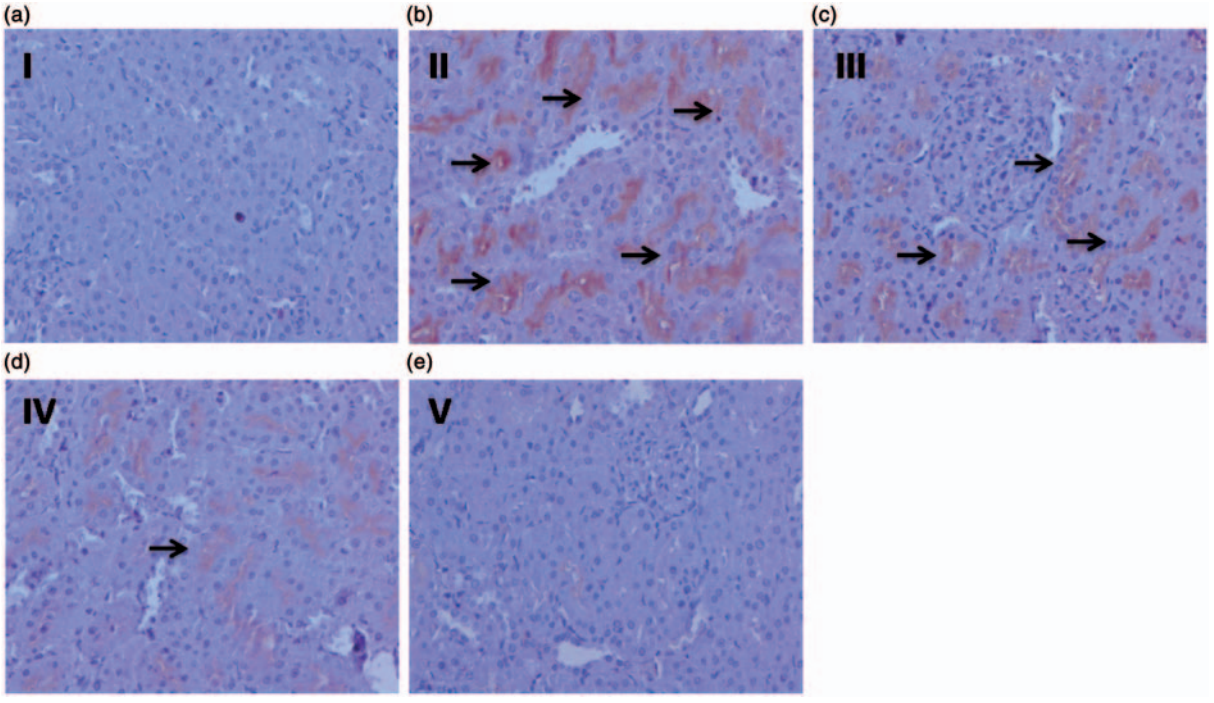

Figure 7 Effect of prophylactic treatment of D-limonene against Dox-induced COX-2 expression. Photomicrographs of renal sections of rat depicting (a) Control group given normal diet (group I), (b) Dox-treated group (20 mg/kg body weight) (group II), (c) dose 1 of D-limonene (5% in diet) + Dox (20 mg/kg body weight) (group III), (d) dose 2 of D-limonene (10% in diet) + Dox (20 mg/kg body weight) (group IV), and (e) only dose 2 of D-limonene (10% in diet) (group V). Photomicrographs of Immunohistochemical detection of COX-2 in renal tissue (a) COX-2 staining of control kidneys, (b) COX-2 staining in Dox-treated group was very intense, (c) D-limonene (5%) pretreated kidney sections show partial inhibition of COX-2 when compared with Dox-treated group, (d) D-limonene (10%) treatment showed there was almost complete suppression of COX-2, and (e) renal section of the groups treated with higher dose of D-limonene only (10%) with no significant change when compared with control. (A color version of this figure is available in the online journal)

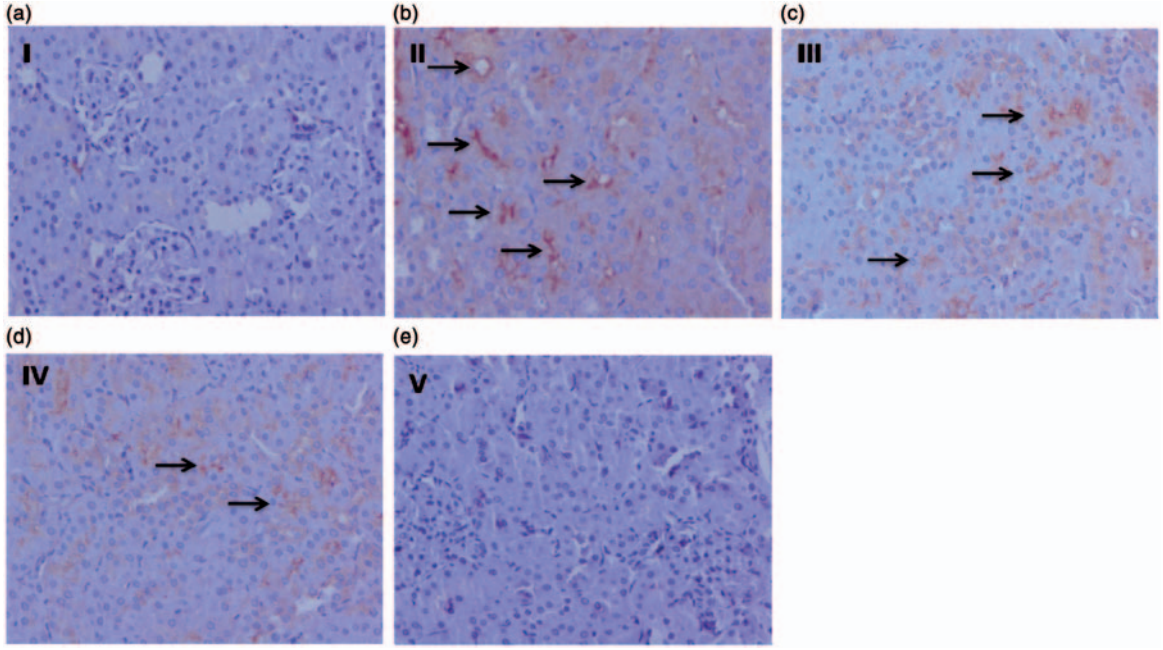

Figure 8 Effect of prophylactic treatment of D-limonene against Dox-induced iNOS expression. Photomicrographs of renal sections of rat depicting (a) Control group given normal diet (group I), (b) Dox-treated group (20 mg/kg body weight) (group II), (c) dose 1 of D-limonene (5% in diet) + Dox (20 mg/kg body weight) (group III), (d) dose 2 of D-limonene (10% in diet) + Dox (20 mg/kg body weight) (group IV), and (e) only dose 2 of D-limonene (10% in diet) (group V). Representative photomicrographs of iNOS determined by immunohistochemistry. (a) Renal sections of control group. (b) Dox administration increased strongly iNOS expression in renal sections. (c) There was partial inhibition of iNOS expression as evidenced by weak immunostaining in the rat kidneys treated with D-limonene (5%). (d) In contrast, there was almost complete suppression of iNOS in rats treated with higher dose of D-limonene (10%). (A color version of this figure is available in the online journal)

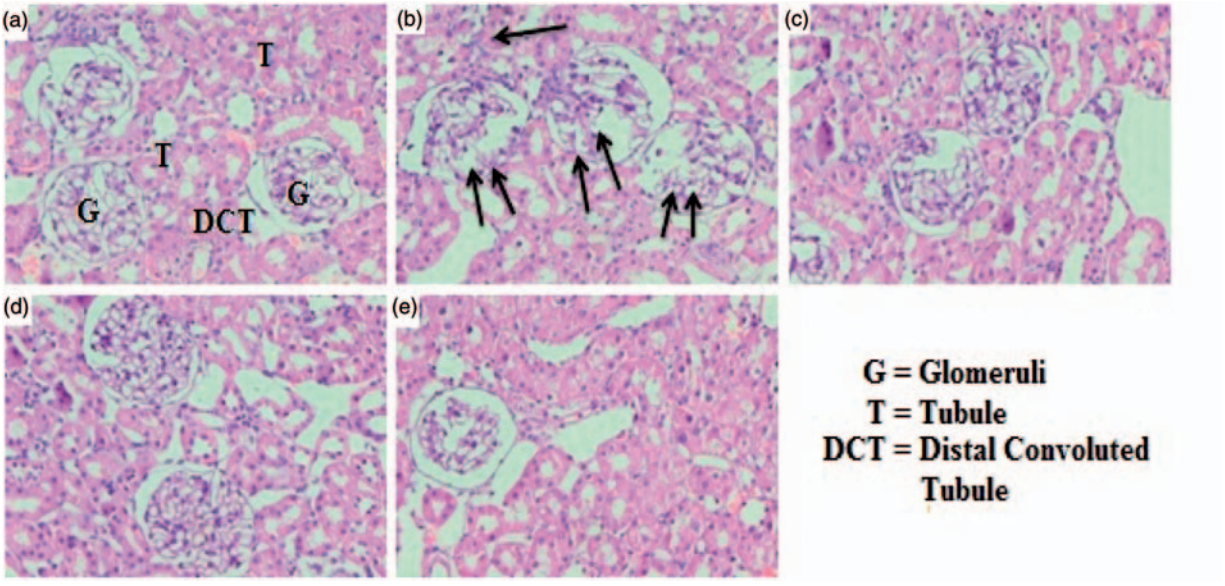

Figure 9 Effect of prophylactic treatment of D-limonene against Dox-induced histological alterations. Photomicrographs of histological sections of rat depicting (a) vehicle-treated control group (group I), (b) Dox-treated group (20 mg/kg body weight) (group II), (c) dose 1 of D-limonene (5% in diet) + Dox (20 mg/kg body weight) (group III), (d) dose 2 of D-limonene (10% in diet) + Dox (20 mg/kg body weight) (group IV), and (e) only dose 2 of D-limonene (10% in diet) (group V). (A color version of this figure is available in the online journal)

Dox-treated group, thus consistent with earlier reports.[49,50] However, D-limonene administration markedly suppressed its levels, thereby providing protection against Dox toxicity.

Oxidative stress and inflammation is implicated in Dox-induced renal toxicity. Dox administration leads to oxidative damage as well as increases in TNF-α levels.[11,51] This TNF-α induces the local generation of reactive nitrogen species (RNS) through NO synthase induction, and therefore intensifies the oxidative stress responsible for organ injury. Recent published papers identify TNF-α as part of the innate immune system in response to different forms of stresses which is initiated by binding of TNF-α to its

receptor-1 causing the classical activation of NFκB and increasing the expression of genes required to control infection and injury.[52]

Elevated production of NO results in peroxynitrite formation by reaction of NO and superoxide anion. Peroxynitrite is a powerful and aggressive cellular oxidant and causes the formation of 3-nitro-L-tyrosine.[53] The inducible nitric oxide synthase (iNOS) is involved in the inflammatory process. Published reports have shown that high NO production is involved in Dox toxicity.[54,55] High NO production might also be related to the inflammatory answer of the tissue against Dox. Some reports have also shown that NFκB enhancer elements regulate cytokine-mediated induction of the inducible NOS gene.[56] NFκB is a redox-sensitive transcription factor that has been shown to be activated by oxidizing agents such as hydrogen peroxide and ionizing radiation.[57,58] Thus, it is possible that the increased NO production by Dox is a result of TNF-α-mediated NFκB activation of iNOS expression.

This work confirmed the former findings, whereas Dox administered rats showed a significant increase in both TNF-α and NFκB expressions. Moreover, the elevation of iNOS in renal tissues supporting a role of TNF-α in mediating Dox effects in the kidney. Furthermore, the levels TNF-α and total nitrite were significantly increased in Dox group as compared with normal rats in accordance with previous findings.[51,54,55] D-limonene treatment effectively restored the level of all these inflammatory markers.

The aforementioned results further substantiated the histological data which exhibited the protective effects of D-limonene against Dox-induced distorted renal architecture with intense inflammatory cell infiltration in the renal tissue. In conclusion, the results of this study demonstrate that oxidative stress and apoptosis are closely associated with Dox-induced toxicity and D-limonene shows the protective efficacy against Dox-induced renal toxicity, possibly via attenuating the oxidative stress and inflammatory tissue damage. We did not find any toxicity of D-limonene in present study as has been established by other published reports. D-limonene does not possess mutagenic, carcinogenic, or nephrotoxic risk to humans and hence it is considered to be a chemical with fairly low toxicity.[59,60] It could be used as an adjuvant therapy with Dox but the exact mechanism of D-limonene is not fully defined yet. Hence, further studies are warranted to elucidate the exact mechanism of action of D-limonene.

Author contributions: The contributions of the authors to this study were as follows: M.U.R. and S.S. designed the experiment and wrote the manuscript; M.U.R., M.T., A.Q.K., R.K., O.-O.-H., A.L., and M.Z. conducted the experimental work; S.K., S.R., and N.A. helped in animal handling, data analysis, and editing of manuscript.

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