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Effect of hydroalcoholic extract of *Lens culinaris* against doxorubicin induced renal damage

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Abstract : The current study was designed to evaluate the nephroprotective potential of seeds of hydroalcoholic extract of seeds of *Lens culinaris* against doxorubicin- induced renal damage in male Albino Wistar rats. Nephrotoxicity was induced by single intra peritoneal injection of doxorubicin at a dose of 15mg/kg b w. Nephroprotective activity of hydroalcoholic extract of *Lens culinaris* was tested at two dose levels i.e., 200 and 400mg/kg b w. Nephroprotective activity was assessed by determining serum markers, urinary parameters, lipid peroxidation and antioxidant levels in renal tissue. Histological and immuno-histochemical studies had been carried out in the renal tissue. Doxorubicin had induced marked nephrotoxicity manifested by a significant increase in Serum creatinine, Blood urea nitrogen, Urinary total protein, lipid peroxidation and decrease in Urinary creatinine, catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH).
The administration of extract at both dose levels restored the levels of serum cretinine, urinary creatinine,urinary total protein, LPO SOD and GSH, CAT. The protection is almost equal at both dose levels. Histological and immune histochemicalstudies also substantiated the biochemical parameters. The preset study reveals that hydroalcoholic extract of seeds of *Lens culinaris* partially ameliorated doxorubicin- induced renal damage.
Keywords: Doxorubicin, *Lens culinaris,* Lipid peroxidation, Immuno-histochemical.

Introduction:

The kidney is an important organ actively involved in maintaining body fluid homeostasis by reabsorbing important material and excreting waste products. Despite its essential roles in maintaining homeostasis the kidney is a main target organ for xenobiotic toxicity because of its high perfusion rate and specialized uptake system[1]. Notably, various nephrotoxicants, including many clinical drugs, can induce kidney damage including acute kidney injury, tubulopathies, proteinuric renal diseases, chronic kidney injury, and renal failure. Among many clinical drugs doxorubicin, an anthracycline glycoside antibiotic that possesses a potent and broad spectrum antitumour activity[2]. Its clinical use has been limited largely due to nephrotoxicity and cardiotoxicity[3,4]. Doxorubicin-induced changes in the renal tissue of rats include increased glomerular capillary permeability and tubular atrophy[5]. Doxorubicin- induced renal damage believed to be mediated through free radical formation, iron-dependent oxidative damage of biological macromolecules, and membrane lipid peroxidation[6]. Owing to several limitations in modern medicine for treating drug- induced nephrotoxicity, seeking alternative treatments becomes a necessity.

Use of medicinal plants in renal failure goes back to ancient days. More than 221 plants have been screened for nephroprotective activity both in acute and chronic renal failure models[7]. To determine the

potential of herbal medicines and to promote their use it is essential to intensify the study of medicinal plants that find a place in folklore. Seeds of *Lens culinaris* (F:Fabaceae) commonly called as lentils was used as diuretic and for treatment of various kidney ailments in traditional and folklore medicine[8]. Hence, the present study was aimed to pharmacologically evaluate hydroalcoholic extract of seeds of *Lens culinaris* (HAELC) against doxorubicin- induced renal failure.

Materials and methods:

Collection of Seeds of *Lens culinaris*: *Lens culinaris* seeds were purchased from local market and authenticated by botanist Dr. K. Madhava Chetty, Asst. Professor, Dept. of Botany, S.V.University, Tirupati, Andhra Pradesh and a voucher specimen was deposited in Dept. of Botany, S.V.University, Tirupati.

Preparation of hydroalcoholic extract: Seeds of *Lens culinaris* powdered in a Wiley mill. The powdered seeds were macerated with ethanol and water (70:30) for 24 h. Macerated material was refluxed for 3h. and filtered. The procedure was repeated twice, filtrate was combined which was subjected to distillation under reduced pressure to obtain semisolid. This was used for phyto and pharmacological studies.

Preliminary phytochemical screening: Preliminary phytochemical screening was carried out for hydroalcoholic extract of seeds of *Lens culinaris* by following standard procedures[9].

Pharmacological studies:

The experimental protocol was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the Institutional Animal Ethical Committee (IAEC) (Registration No.: 1677/PO/a/12/CPCSEA/9).

Experimental animals: Wistar strain Male Albino rats weighing 150-200gm were selected for study. The animals were maintained under controlled environmental conditions with 12:12 hour light/dark cycle in polypropylene cages. They had free access to standard pellets as basal diet and water *ad libtum*.

Acute toxicity studies: The prepared hydro alcoholic extract was subjected to acute toxicity studies at a dose of 2000mg/kg body weight according to the OECD 423 guidelines[10]. The animals were observed for 14 days and any changes in body weights of the rat, changes in skin and fur, eyes and mucous membranes, salivation, nasal discharge, urination and behavioral, neuromuscular, cardiovascular changes, lethargy, sleep and coma were noted.

Evaluation of nephroprotective activity:

Experimental design:

After acclimatization rats were randomly assigned into six groups of six each.

Group-I: Normal control (Only vehicle from day1 to day8)
Group-II: Vehicle day 1 to day 8+ Doxorubicin (15mg/kg b. w.) on day 4
Group-III: Lower dose (200mg/kg b. w.) of hydroalcoholic extract from day 1 to day 8+ Doxorubicin (15mg/kg b. w.) on day 4
Group-IV: Higher dose (400mg/kg b. w.) of hydroalcoholic extract from day 1 to day 8+ Doxorubicin (15mg/kg b. w.) on day 4
Group-V: Cystone (5ml/kg) from day1 to day 8+ Doxorubicin (15mg/kg b.w.) on day 4
Group-VI: Only higher dose (400mg/kg b. w.) of hydro alcoholic extract from day 1 to day 8

Prior to the termination of experiment urine was collected with the help of metabolic cages and urine samples were subjected for estimation of urinary functional parameters. On day 9 animals were sacrificed by cervical decapitation and blood samples were collected by retro-orbital puncture and were used for estimation of serum markers. Kidneys were isolated immediately and left kidney was used for assessing antioxidant parameters and right kidney for histological and immuno-histochemical studies.

Estimation of serum markers:

Nephroprotective activity was evaluated in terms of biochemical parameters such as Blood Urea nitrogen (DAM method), Serum creatinine (Jaffe's Alkaline picrate method), Urinary total protein (Turbidity method) and Urinary creatinine (Alkaline picrate method) by using commercial kits[11].

Estimation of antioxidant activity:

Kidneys were homogenized with ice cold 0.05 M potassium phosphate buffer (pH 7.4) to make 10 % homogenates. The homogenates were centrifuged at 6000 rpm for 15 mins at 4 °C. The homogenized kidney was used for estimation of levels of lipid peroxidation, superoxide dismutase, catalase and reduced glutathione using standard methods prescribed[12-15].

Histological studies:

Kidneys were rinsed with normal saline and then fixed in 10% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination. The sections were 2 -5 mm thick, deparaffinized, hydrated and stained with haematoxylin and eosin. The renal sections were examined for the extent of damage to glomeruli, tubules and interstitium as well as for capillary congestion and hemorrhage.

Immuno-histochemical studies:

Sections of formalin-fixed, paraffin-embedded kidneys were obtained on polyl- lysine coated slides. Sections were deparaffinized in xylene, then rehydrated through a graded alcohol series. Antigen retrieval was performed by incubating slides in citrate buffer (pH 6.0) (10 mM) at 95 ◦C for 20 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min. To detect active (cleaved) caspase-3 sections were incubated under humid conditions overnight at 4 ◦C with the following anti-caspase-3 monoclonal antibody (1:400;

Thermo Fisher Scientific, USA). Next day, the slides were washed three times in Tris buffers (pH 6.0) and were incubated with a biotinylated Goat Anti-Polyvalent Plus (Thermo Fisher Scientific, USA) for 30 min at room temperature. This step was followed by further wash in Tris buffer and incubation of slides at room temperature with a Streptavidin Peroxidase Plus (Thermo

Fisher Scientific, USA) that binds to the biotin present on the secondary antibody. After washing in Tris buffer, the immunostaining reaction product was developed using 3,3-diaminobenzidine (DAB Plus substrate, Thermo Fisher Scientific, USA). After immunoreactivity, slides were dipped in distilled water, counterstained with Harris haematoxyline and finally the sections were dehydrated in xylene, mounted and cover slipped. Slides prepared for each case were examined by light microscopy[16].

Statistical Analysis :

Values were represented as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) and group means were compared using the Tukey-Kramer Multiple Comparison Test using Graph Pad Prism software. P values < 0.05 were considered significant.

Results:

Preliminary phytochemical studies: Phytochemical screening of the HELC revealed the presence of carbohydrates, proteins, saponins, flavonoids, tannins and phenolic compounds.

Acute toxicity studies: Animals which received extract at 2000mg/kg b. w. observed for 14 days had not shown any clinical signs of toxicity and mortality.

Effect of hydro alcoholic extract of seeds of *Lens culinaris* on serum and urinary markers:

Group	BUN (mg/dl)	SC (mg/dl)	U_{TP} (mg/24hrs)	U_{cr} (mg/24hr)
I	14.50±0.78	1.06±0.05	1.62±0.15	17.63±0.53
II	35.28±1.34 *a	2.53±0.18 *a	4.54±0.22 *a	1.67±0.22 *a

III	31.73±0.52 ns, b	1.68±0.16 * b	2.24±0.15 *b	13.17±0.74 *b
IV	31.62±0.99 ns,b	1.65±0.18 *b	3.23±0.12 *b	9.51±0.41 *b
V	12.23±0.48 * b	1.0±0.05 * b	1.55±0.20 * b	18.42±0.71 * b
VI	11.96±0.66 ns, a	0.91±0.04 ns, a	1.09±0.07 ns, a	18.83±0.40 ns, a

Each value represents Mean ± S.E.M from 6 animals in each group. * represents P<0.05, ns : not significant. a: Group-II & VI compared with Group-I. b: Group-III, IV & V compared with Group-II.

Effect of hydro alcoholic extract of *Lens culinaris* on anti-oxidant levels against doxorubicin induced nephrotoxicity:

Group	LPO nmol/g of wet tissue	SOD U/mg of wet tissue	CAT U/mg of protein	GSH µmol/g of wet tissue
I	1.15±0.26	42.63±0.60	42.37±0.74	80.68±0.59
II	7.73±0.39 *a	16.80±0.35 *a	11.88±0.42 *a	34.91±0.32 *a
III	4.33±0.26 *b	29.30±0.99 *b	17.23±0.79 *b	84.60±0.73 *b
IV	5.08±0.34 *b	20.33±0.75 *b	14.48±0.51 ns, b	77.77±0.13 *b
V	2.81±0.19 *b	35.23±0.83 *b	33.45±0.87 *b	64.54±0.54 *b
VI	1.53±0.19 ns, a	43.55±0.58 ns, a	44.13±0.13 ns, a	79.97±0.92 ns, a

Each value represents Mean ± S.E.M from 6 animals in each group. * represents P<0.05, ns : not significant. a: Group-II & VI compared with Group-I. b: Group-III, IV & V compared with Group-II.

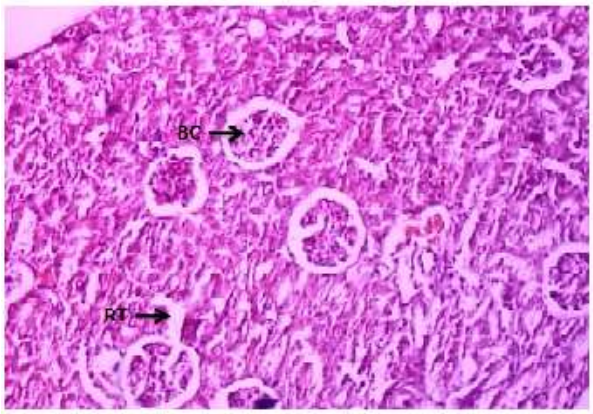
Administration of high dose of HELC for 8 days does not show any deteriorative effects on kidney. Intraperitonial single administration of (5mg/kg b.w.) of doxorubicin – induced the nephrotoxicity which was manifested by elevated levels of serum markers, increased excretion of protein in urine, reduced excretion of creatinine in urine, increased levels of LPO, SOD, Catalase and reduced levels of GSH

Animals administered with extract at 200mg/kg b. w. i.e. Group-III animals showed decrease in serum creatinine, urinary total protein and increase in urinary creatinine when compared with disease control i.e. Group-II animals. But Blood urea nitrogen levels were not restored in Group-III animals. In group-IV animals serum creatinine, urinary total protein and urinary creatinine levels reduced when compared with group-II animals. But decrease in levels of above parameters is less when compared with group-III animals (Table:1).

Animals which received lower dose of HAELC (Group-III) exhibited significant and remarkable increase of SOD, CAT, GSH and reduced LPO levels when compared with animals received high dose of HAELC (Group-IV) (Table:2).

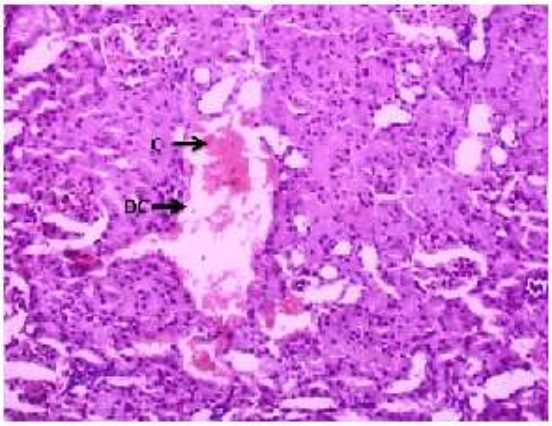
Histological studies:

Kidney sections of rat which received doxorubicin caused a marked necrosis in proximal tubules, degeneration of the tubular epithelial cells and glomeruli. The treatment with HAELC caused moderate regenerative changes. (Fig:1 to Fig:6)



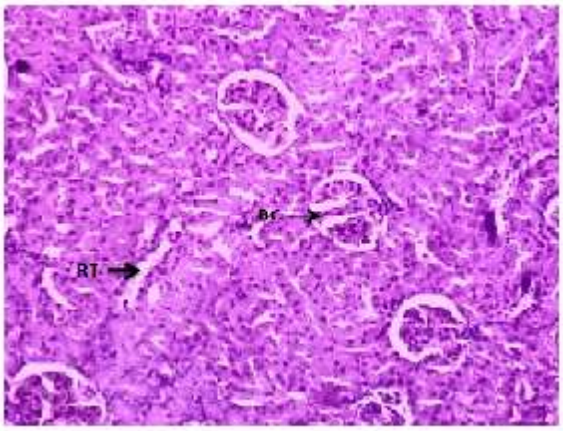
Group-I Kidney 10X

Fig:1 Section of rat kidney showing normal architecture



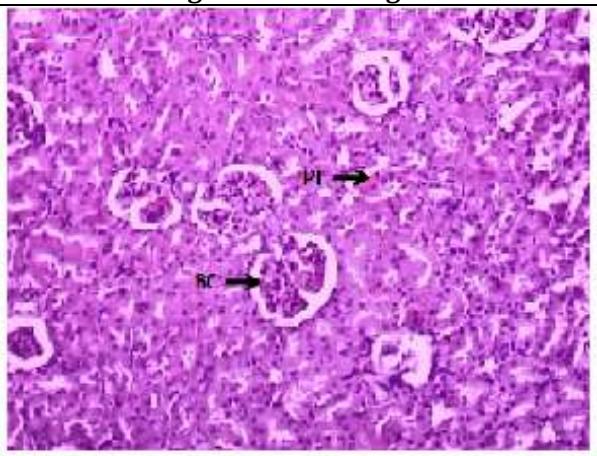
Group-II Kidney 10X

Fig:2 Section of rat kidney showing congestion and marked degenerative changes



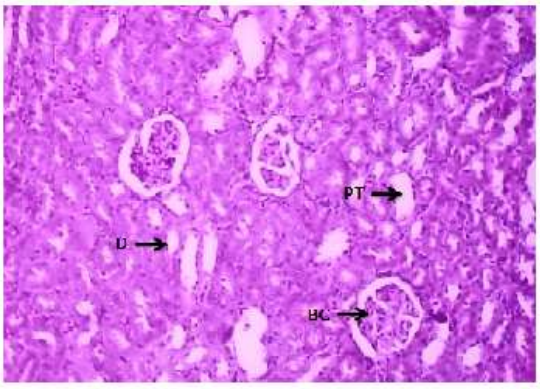
Group-III kidney 10X

Fig:3 Section of rat kidney showing regenerative changes



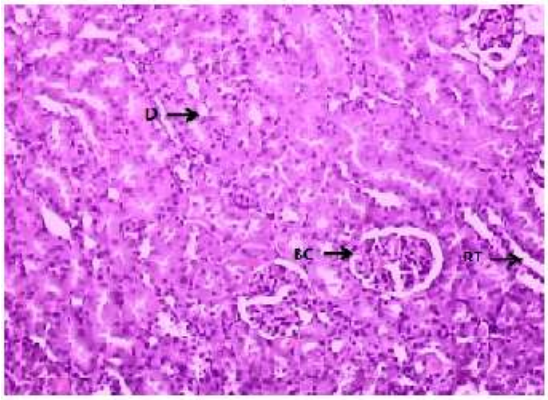
Group-IV kidney 10X

Fig: 4 Section of rat kidney showing regenerative changes



Group-V Kidney 10X

Fig: 5 Section of rat kidney showing marked regenerative changes

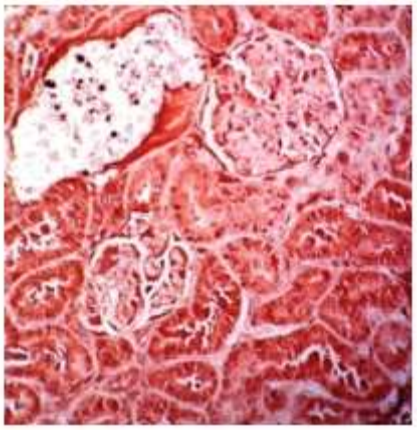


Group-VI Kidney 10X

Fig: 6 Section of rat kidney showing normal cytoarchitecture

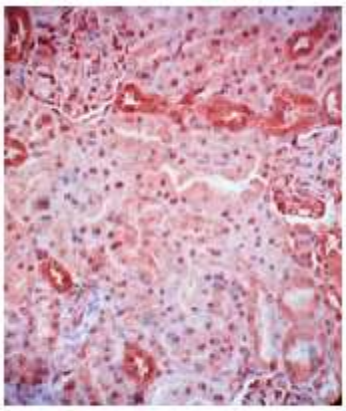
Immuno- histochemical studies:

Section of rat kidneys which received doxorubicin alone showed very mild expression of caspase-3 in damaged renal tissue. While the kidney sections of rats treated with HAELC at both dose levels showed moderate expression of caspase-3.



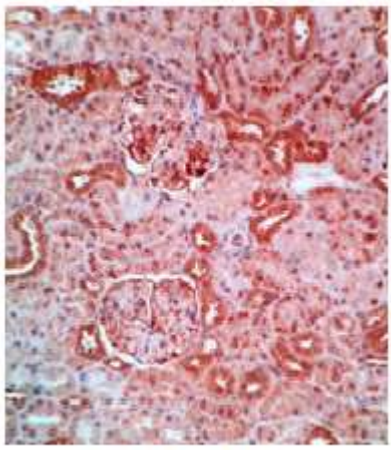
Group-I

Fig:7 Section of rat kidney showing marked expression of caspase-3.



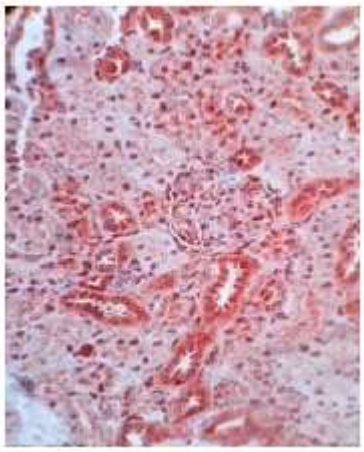
Group-II

Fig:8 Section of rat kidney showing very less expression of caspase-3 indicating renal damage



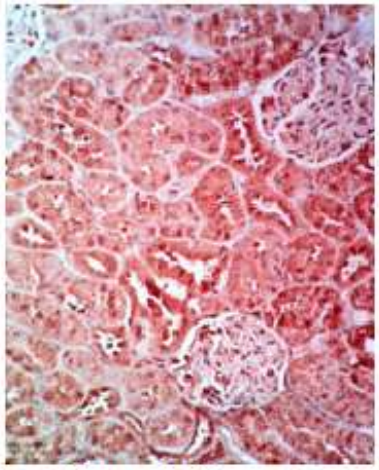
Group-III

Fig:9 Section of rat kidney showing moderate expression of caspase-3.



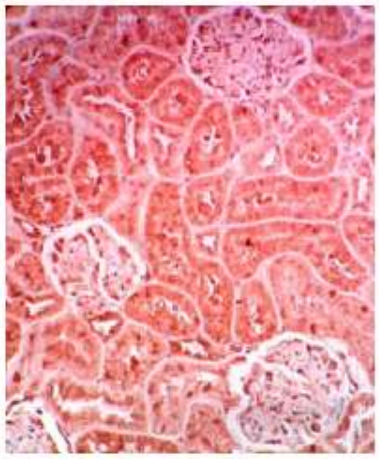
Group-IV

Fig:10 Section of rat kidney showing moderate expression of caspase-3.



Group-V

Fig:11 Section of rat kidney showing improved expression of caspase-3.



Group-VI

Fig:12 Section of rat kidney showing marked expression of caspase-3.

Discussion:

One of the most common manifestation of nephrotoxic damage is renal failure which characterized by decline in glomerular filtration rate with resulting azotaemia[17,18]. Doxorubicin (DXN), an anthracyclin antibiotic represents a class of anticancer agents. It shows broad spectrum anti-tumour activities in certain human cancers including breast cancer, small cell carcinoma of the lung and acute leukaemia[19]. The optimal use of doxorubicin is limited by a number of side effects; the most important are cardiotoxicity, haematotoxicity and nephrotoxicity[20,21].

Improvement of doxorubicin induced nephrotoxicity was previously tried with many plant extracts such as *Zingiber officinale* Roscoe, *Solanum torvum* which are partially succeeded in preserving normal renal function and structure probably through their antioxidant effects[22,23]. So in the present study *Lens culinaris* was selected to screen the nephroprotective activity against DXN- induced nephrotoxicity.

In the current study, the dose of Doxorubicin used corresponds to the dose that is currently being used in clinical practice[24]. As mentioned in earlier reports in the present study also administration of doxorubicin at a dose of 15 mg/kg significantly increased serum creatinine, BUN and urinary protein and decreased urinary creatinine levels[25,26]. Animals treated with hydroalcoholic extract of *Lens culinaris* at both 200 and 400 mg/kg b. w. showed nephroprotective effect which is evident from significant decrease in serum creatinine, urinary creatinine and urinary total protein. Unfortunately treatment with extract failed to restore BUN levels.

Free radical scavenging enzymes such as catalase, superoxide dismutase are the first line cellular defence enzymes against oxidative injury, decomposing O_2 and H_2O_2 before their interaction to form the more reactive hydroxyl radical (OH^-). The equilibrium between these enzymes is an important process for the effective removal of oxygen stress in intracellular organelles[27]. During doxorubicin induced nephrotoxicity, free radicals can also be produced by a non-enzymatic mechanism that involves reactions of iron–DXN complex that can reduce oxygen to H_2O_2 and other ROS[28]. In good agreement with earlier studies, in our study also doxorubicin significantly decreased the levels of SOD, CAT and GSH and increased the levels of LPO[23,29]. Animals treated with extract at dose of 200mg/kg b wt. showed significant increase in SOD, CAT and GSH levels and decrease in LPO levels than the animals treated with 400mg/kg b. w. These results are also substantiated by histological and immunohistological studies.

Biochemical, antioxidants, histological and immunohistological studies suggested that increasing the dose of HAELC from 200 to 400mg/kg b.w. in doxorubicin induced nephrotoxicity did not protected the kidney from its toxic effects. This may be due to the pro-oxidant effect of extract at higher dose. Phytochemical analysis of plant revealed the presence of quercetin, myricetin, kaempferol, apigenin and luteolin[30] . These flavonoids are previously reported to mediate induction of reactive oxygen species at high concentration[31]. So presence of above constituents may serve as prooxidants and which leads to the low activity of extract at high dose.

Conclusion:

The findings of the present study suggest that hydroalcoholic extract of seeds of *Lens culinaris* partially ameliorated oxidative stress and doxorubicin induced renal damage.

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