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Nephroprotective Effects of Methanolic Extract of *Peucedanum grande* against Acute Renal Failure Induced by Potassium dichromate in Rats

M Aslam[1*], R Dayal[2], K Javed[3], SA Parray[4], S Jetley[5], M Samim[6]

[1]*Department of Ilmul-Advia, Faculty of Medicine (U), Jamia Hamdard, New Delhi-110062, India*
[2]*Head Chemistry Division (Rtd), Forest Research Institute, Dehradun, Uttranchal, India*
[3]*Department of Chemistry, Faculty of Science, Jamia Hamdard, New Delhi-110062, India*
[4]*Department of Phytochemistry & Pharmacognosy, F/O Pharmacy, Jamia Hamdard, New Delhi-110062, India*
[5]*Hamdard Institute of Medical Sciences and Research, New Delhi-110062, India*
[6]*Department of Chemistry, Faculty of Science, Jamia Hamdard, New Delhi-110062, India*

ABSTRACT
Peucedanum grande has been found to be associated with the several therapeutic properties. In the current study, we have used *P. grande* as an ameliorating agent against nephrotoxic effects of Potassium dichromate. Potassium dichromate ($K_2Cr_2O_7$) is a soluble hexavalent chromium compound that is extensively used in several industries. The rats were given pretreatment of *P. grande* orally at a dose of (60 and 120 mg/kg body weight) for five consecutive days 10 mg/kg b.wt was used as renal toxicant, and injected subcutaneously in the neck region in a volume of 1 ml/kg. The modulatory effects of *P. grande* on Potassium dichromate induced nephrotoxicity was investigated by assaying oxidative stress biomarkers, lipid peroxidation, serum kidney toxicity markers and by histopathological examination of kidney. *P. grande* pretreatment prevented deteriorative effects induced by Potassium dichromate through a protective mechanism that involved reduction of increased oxidative stress as well as by restoration of histopathological change against Potassium dichromate administration.

Keywords: *Peucedanum grande,* Mercuric chloride, Kidney, histopathology, oxidative stress.

INTRODUCTION

Chromium (Cr) is one of the essential trace elements which play role in glucose and lipid metabolism. [1] It is commonly used in various industries (e.g. steel, alloy, cast irons, chrome plating, paints, leather tanning, photography, metal finishes). Cr exists in various valence states however the tri-valent [Cr(III)] and hexavalent [Cr(VI)] forms are of biological significance. The Cr (VI) compounds are more toxic than Cr (III) as they are stronger oxidizing agents and are easily absorbed and transported across membranes via non specific anion carriers. [2-3] There are three possible routes inhalation, dermal contact and ingestion through which exposure to Cr(VI) compounds occurs and has been associated with skin, kidney and liver toxicities. [4] Cr(VI) is reduced through reactive intermediates like Cr(IV) and Cr(V) to the kinetically further more stable Cr(III) by intracellular reductants together with glutathione, vitamin C and NADPH-dependent flavoenzymes. [5-8]

***Corresponding author: Dr. Mohammad Aslam,**
Assistant Professor, Department of Pharmacology, Faculty of Medicine (U), Jamia Hamdard (Hamdard University), New Delhi, India-110062, India; **E-mail:** m.aslamjh@gmail.com

It has been demonstrated through in vivo and in vitro studies that the generation of reactive oxygen species occurs through this reduction process due to which various types of cellular damage occurs. [6-7] Occupational exposure to Cr occurs in workers who are associated with welding, chrome plating and chromium pigment works. Beside the exposure at workplace, environmental exposure from Cr(VI) containing waste sites and polluted water supplies has also raised alarm. Due to industrial application Cr(VI) has been detected in ground water and also found in drinking water.

Herbal plants have been used for the cure of several human diseases and are gaining more attention due to less toxicity and high efficacy. *Peucedanum grande* belongs to family Umbelliferae and have several names like Duku, Baphalle, Wild carrot, Hingupatri. [9]

In Unani system Peucedanum grande is known to its medicinal values like, diuretic (Mudir–e-Baul), emmenagogue (Mudir-e- Haiz), aphrodisiac (Muqawwi-e-Bah), demulscent (Mulattif), deobstruent (Mufatteh), urolithotriptic (Mufattite–Hissat-e-Gurda-Wa-Masana), anti-Inflammatory (Mohallil-e-Auram), antidote (Daaf-e-Sammyat), concoctive/maturative (Munzij), etc. [10-13]

The aim of this study was to determine if *P. grande* shows protection against Potassium dichromate induced renal toxicity. The extent of the protective effect of *P. grande* against nephroprotective effects were determined by studying serum markers enzymes and biochemical estimation of antioxidant enzymes of rats.

MATERIAL AND METHODS

Plant material

The drugs were purchased from Ajmal and Brothers Khari Baoli, Delhi. The botanical identity of the purchased drugs was established as Duku (*Peucedanum grande* C.B Clark Seeds) rhizome at NISCAIR (National Institute of Science Communication and Information Resources), Dr. K. S. Krishnan Marg, Pusa Gate, New Delhi, 110012 under Ref. NISCAIR/RHM/F-3/2004Consult/-486/62.

Preparation of extract of *Peucedanum grande*

The 2 Kg dried seeds/fruits of the drug *Peucedanum grande* was extracted exhaustively extracted with methanol by using a Soxhlet apparatus over boiling water bath for 3 h. It was removed from the water bath and allowed to cool at room temperature and filtered. The plant material obtained after filtration was re-extracted twice by the same procedure. All three extracts were combined together (methanolic extracts). Methanol was recovered by distillation method under reduced pressure. The yield of extract was 12 % w/w in the terms of starting materials. The yield of extract was calculated with respect to starting material.

Chemicals

Reduced glutathione (GSH), 1,2-dithio-bis-nitrobenzoic acid (DTNB), 1-chloro-2, 4-dinitrobenzene (CDNB), bovine serum albumin (BSA), oxidized and reduced nicotinamide adenine dinucleotide phosphate (NADP), (NADPH), thiobarbituric acid (TBA), trichloroacetic acid (TCA) etc were obtained from Sigma-Aldrich, USA. India. All other reagents and solvents were of a high analytical grade.

Animals

Eight week old male wistar rats (150-200g) were obtained from the Central Animal House Facility of Hamdard University, New Delhi and were housed in a ventilated room at 25 ± 5°C under a 12 h light/dark cycle. The animals were acclimatized for one week before the study and had free access to standard laboratory feed (Hindustan Lever Ltd., Bombay, India) and water *ad libitum.* The study was approved by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) having Registration number and date of registration: 509/CPCSEA, May 28th, 2009. CPCSEA guidelines were followed for animal handling and treatment.

Rats were dived into four groups of 6 animals each.

Test drug (methanolic extract of *P. grande*) was administration in the form of suspension using 1% CMC as a suspending agent in distilled water.

- Animals of Group I (Control) received 1% CMC in distilled water (10 mg/kg/d) for 5 days
- Animals of group II (toxic) received 1% CMC water (10 mg/kg/d) for 5 days
- Animals of group III received lower dose 60 mg/kg/d suspended in the vehicle (10 ml/kg) for 5 days.
- Animals of group IV received W-INS (120 mg/kg/d suspended in the vehicle (10 ml/kg) for 5 days.

A single dose of potassium dichromate (20 mg/kg) Jonker *et al.*, 1993 was administered subcutaneously in neck region in a volume of 1 ml/kg, on fourth day to all the animals except of group 1. Group 1 received normal saline only. On the sixth day, blood samples were withdrawn from retro-orbital venous plexus and serum was separated for the estimation for urea nitrogen and creatinine consternations. Animals were sacrificed and their kidney were isolated for histopathological studies and post-mitchondrial supernatant (PMS).

Post-Mitochondrial Supernatant Preparation

Kidneys were removed quickly, cleaned free of extraneous material and immediately perfused with ice-cold saline (0.85% sodium chloride). The kidneys were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCI (1.17%) using a Potter Elvehjen homogenizer. The homogenate was filtered through muslin cloth, and was centrifuged at 800 X g for 5 min at 4°C by REMI Cooling Centrifuge to separate the nuclear debris. The aliquot so obtained was centrifuged at 12000 r.p.m. for 20 min at 4°C to obtain PMS, which was used as a source of enzymes. All the biochemical estimations were completed within 24 hr of animal sacrifice. [14]

Estimation of Blood Urea Nitrogen

Estimation of blood urea nitrogen was carried out by the diacetyl monoxime method. Protein-free filtrate was prepared by adding serum and equal amount of 10% TCA, then mixture was centrifuged at 2000 r.p.m. and supernatant was taken. To 0.5 ml of protein free filtrate, were added 3.5 ml of distilled water, 0.8 ml diacetylmonoxime (2%) and 3.2 ml sulphuric acid–phosphoric acid reagent (reagent was prepared by mixing 150 ml 85% phosphoric acid with 140 ml water and 50 ml of concentrated sulphuric acid). The reaction mixture was placed in a boiling water bath for 30 min and then cooled to room temperature. The absorbance was read at 480 nm. [15]

Estimation of Creatinine

Creatinine was estimated by the alkaline picrate method. Protein-free filtrate was prepared. To 1.0 ml serum were added, 1.0 ml sodium tungstate (5%), 1.0 ml sulfuric acid (0.6 N) and 1.0 ml distilled water. After mixing thoroughly, the mixture was centrifuged at 800 X g for 5 min. The supernatant was added to a mixture containing 1.0 ml picric acid (1.05%) and 1.0 ml sodium hydroxide (0.75 N). The absorbance at 520 nm was read exactly after 20 min. [16]

Lipid peroxidation

Lipid peroxidation (LPO) was measured in terms of malondialdehyde (MDA) formation which is the major product of membrane lipid peroxidation. The reaction mixture a total volume of 3 ml contained mainly TCA 1ml (10%) and TBA 1.0 ml. Test tubes having reaction mixture were kept in boiling water for about 45 minutes and transferred on to ice cooled water and then centrifuged at 2500 X g for 10 minutes. The malondialdehyde formation in each sample was detected as optical density observed at 532 nm. The results were expressed as nmole of MDA formed per minutes per gram of tissue using molar extension coefficient 1.56×10^5 M^{-1} cm^{-1}. [17]

Estimation of reduced glutathione

For reduced GSH, 1.0 ml of 10% PMS mixed with 1.0 ml of 4% sulphosalicylic acid, Then incubated at 4°C for a minimum time period of 1 h and then centrifuged at 4°C at 1200×g for 15min. Briefly reaction mixture having 0.4 ml supernatant, 2.2 ml phosphate buffer (0.1M, pH 7.4) and 0.4 ml DTNB (4 mg/ml) making a total volume of 3.0 ml. The

Table 1: Results of pre-treatment of *Peucedanum grande* on GSH, catalase, LPO and SOD on administration of $K_2Cr_2O_7$ in kidney of rats

Treatment regimen per group	GSH (n mol CDNB Conjugate formed/g tissue)	Catalase (nmol H_2O_2 consumed/min/mg protein)	SOD Unit/mg of protein	LPO (n mol TBARS / mg protein)
Group I (control 10 ml/kg)	0.324 ± 0.005	190.8 ±1.2	5.42 ± 0.16	3.58 ± 0.05
Group II ($K_2Cr_2O_7$)	0.220 ± 0.004***	139.6 ± 1.7***	3.70 ± 0.07***	7.00 ± 0.07***
Group III (*P. grande* D1+ $K_2Cr_2O_7$)	0.243 ± 0.002#	150.4 ± 1.8#	4.40 ± 0.07#	5.98 ± 0.19##
Group IV (*P. grande* D2+ $K_2Cr_2O_7$)	0.261 ± 0.002##	160.2 ± 2.5##	4.60 ± 0.14###	5.12 ± 0.22###

Results represent mean ± SE of six animals per group. Results obtained are significantly different from Control group (***P < 0.001). Results obtained are significantly different from $K_2Cr_2O_7$ treated group (#P < 0.05), (##P < 0.01) and (###P < 0.001). D1 = 60 mg/kg b wt; D2 = 120 mg/kg b wt.

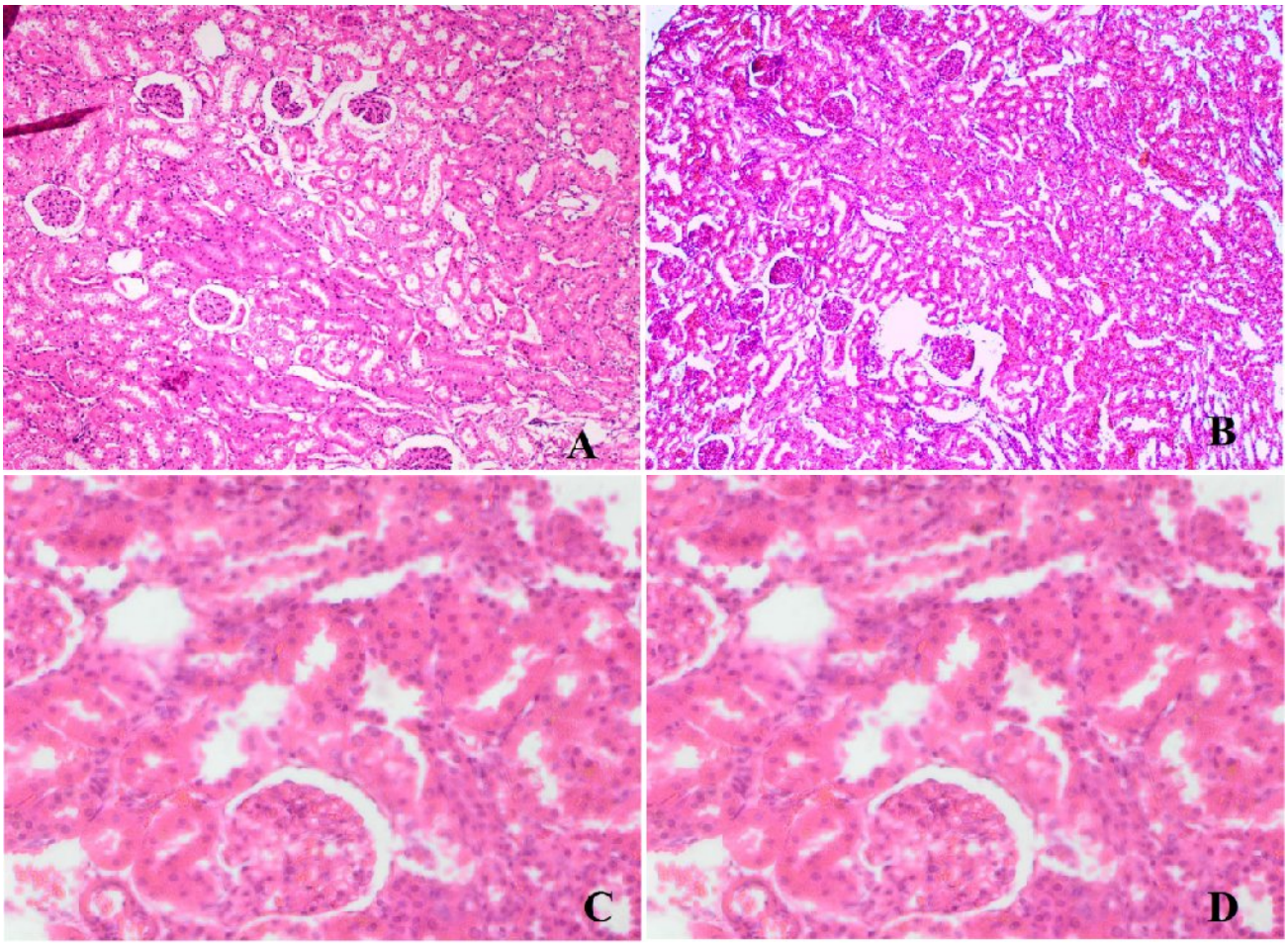

Fig. 1:Kidney histology of Rats (x 400 magnifications):
(A) Control group showed normal glomerular and tubular histology. The tubules were largely intact without the presence of any mononuclear infiltrates in the interstitium and blood vessels were also unremarkable. (B)**Toxicant (**Potassium dichromate) group showed acute tubular necrosis and glomerular widening. **(C) Low dose** of ***Peucedanum grande*** showed focal necrosis of the proximal convoluted tubular lining epithelial cells with areas of desquamation of the cells in the tubular lumina. **(D) High dose** of ***Peucedanum grande*** showed that focal areas of necrosis of the proximal tubular lining epithelial cells were still seen along with cellular swelling, desquamation and loss of brush border.

Table 2: Results of pre-treatment of *Peucedanum grande* on serum marker enzymes

Treatment regimen per group	BUN(mg/dl)	Creatinine(mg/dl)
Group I (control 10 ml/kg)	8.6 ± 1.24	0.119 ± 0.007
Group II ($K_2Cr_2O_7$)	81.84 ± 5.40***	0.796 ± 0.238***
Group III (*P. grande* D1+ $K_2Cr_2O_7$)	19.4 ± 0.019##	0.288 ± 0.019##
Group IV (*P. grande* D2+ $K_2Cr_2O_7$)	24.2 ± 0.28###	0.250 ± 0.27###

Results represent mean ± SE of six animals per group. Results obtained are significantly different from Control group (***P < 0.001). Results obtained are significantly different from $K_2Cr_2O_7$ treated group (##P < 0.01) and (###P < 0.001). D1 = 60 mg/kg b wt; D2 = 120 mg/kg b wt.

yellow color developed was read immediately at 412 nm on spectrophotometer (Perkin Elmer, lambda EZ201). The reduced glutathione concentration was calculated as nmol GSH conjugates/g tissue. [18]

Assay for superoxide dismutase activity

Superoxide dismutase (SOD) activity was assayed by the method of Stevens et al (2000). The assay mixture consisted of 50mM, pH 10.4 glycine buffer, 20 mg/ml epinephrine solution, and cytosolic fraction (10% w/v) in a total volume of 1.0 ml. Enzyme activity was recorded at 480 nm and the activity was calculated as uM epinephrine oxidized /min/mg protein. [19]

Assay for Catalase activity

In case of catalase activity, the reaction mixture comprised of 0.05 ml PMS, 1.0ml hydrogen peroxide (0.019M), 1.95 ml phosphate buffer (0.1M, pH 7.4), in a total volume of 3 ml. Changes in absorbance were recorded at 240 nm and the change in absorbance was calculated as nmol H_2O_2 consumed per min per mg protein. [20]

Histopathological examination

The kidneys were quickly removed after sacrifice of rats and were fixed in 10% neutral buffered formalin solution for histopathological processing. Sections were stained with hematoxyline and eosin before being observed under an Olympus microscope at x 400 magnification.

Statistical analysis

Differences between groups were analyzed using analysis of variance followed by Dunnet's multiple comparisons test. All data points are presented as the treatment groups mean ± S.E.

RESULT

Effect of *Peucedanum grande* on blood urea and creatinine level

Protective effect of *P. grande* on serum BUN and creatinine level was observed. Significant protection ($p < 0.01$, $p < 0.001$) in these marker enzymes were observed in the *P. grande* pre-treatment group and found to be effective in the normalization of these markers when compared to $K_2Cr_2O_7$ treated group.

Effect of *Peucedanum grande* on lipid peroxidation

A significant ($p < 0.001$) increase of the MDA formation was found in the $K_2Cr_2O_7$ treated group when compared with control group. It has been observed that pre-treatment with *P. grande* at both doses D1 and D2 leads to significant ($p < 0.01$ and $p < 0.001$ respectively) prevention of membrane damage by reducing the elevated level of LPO in liver when compared to only $K_2Cr_2O_7$ treated group.

Effect of *Peucedanum grande* on GSH

Protective effect of *P. grande* on GSH level was observed. GSH level was depleted significantly ($p < 0.001$) in $K_2Cr_2O_7$ treated group as compared to the control group. The GSH level in *P. grande* pre-treated groups is increased significantly ($p < 0.05$, $p < 0.01$) as compared to $K_2Cr_2O_7$ treated group.

Effect of *Peucedanum grande* on SOD

The kidney superoxide level decreased in $K_2Cr_2O_7$ treated rats as compared to control animal ($P < 0.001$). The SOD level in *P. grande* pre-treated groups is increased significantly ($p < 0.05$, $p < 0.001$) as compared to $K_2Cr_2O_7$ treated group.

Effect of *Peucedanum grande* on catalase activity

The $K_2Cr_2O_7$ treatment diminished the level of catalase in the kidney compare to vehicle treated control ($P < 0.001$). Pre-treatment of rats with *P. grande* (p.o.), (s.c.) dose dependently enhanced the reduced level of catalase significantly ($p < 0.05$, $p < 0.01$).

Effect of *Peucedanum grande* pre-treatment on the histology of rats

Normal glomerular and tubular histology was seen both in cortical and medullary regions of kidney in control rats. $K_2Cr_2O_7$ was found to cause severe glomerular and peritubular congestion. There was also severe invasion of inflammatory cells seen in both cortical and medullary section from the $K_2Cr_2O_7$ treated group. In addition to these features of $K_2Cr_2O_7$ nephrotoxicity, necrosis of tubular structure was seen in proximal and distal portions. The inner cortical and outer medullary regions exhibited more damage than the inner medullar regions of the kidney. In contrast, renal sections obtained from rats that were pre-treated with *P. grande* at a dose of 60 mg/kg b.wt. showed partial reduction of the histological features of renal injury (figure 1). Pre-treatment of *P. grande* at 120 mg/kg b.wt. was associated with more reduction in injury almost similar to control rat kidney.

DISCUSSION

Potassium dichromate ($K_2Cr_2O_7$) is a chemical compound widely used in metallurgy, chrome plating, chemical industry, textile manufacture, wood preservation, photography and photoengraving, refractory and stainless steel industries and cooling systems. [21] The oxidation state and solubility of chromium (Cr) compounds determine their toxicity. In contrast to Cr(III), which is a naturally occurring form and an essential trace element for humans and others mammals, Cr(VI) compounds are highly toxic. [22] $K_2Cr_2O_7$ is a hexavalent form of Cr and has been demonstrated to induce oxidative stress and carcinogenic in nature. [23-25] The kidney is the principal route of Cr excretion and it has been reported that acute exposure induces an increase in Cr kidney content on $K_2Cr_2O_7$-treated rats. [26] Exposition to Cr (VI) produced anatomical lesions at the level of the proximal tubular cells [27] and lipid peroxidation in human kidney. [28] The effect of methanolic extract *P. grande* (p.o.) on the levels of LPO and GSH in kidney PMS is shown in Table 2. $K_2Cr_2O_7$ treatment alone raised renal LPO and exhausted renal GSH as compared to their vehicle treated control. However, co-administration of *P. grande* methanolic extract (p.o.) with $K_2Cr_2O_7$, (s.c.) dose dependently enhanced GSH contents and LPO (Table 2). Interestingly, evidences suggest that reactive oxygen species (ROS) are involved in Cr (VI)-induced cell injury. [29] Cr reduction intermediates [Cr (V) and Cr (IV)], may be toxic as they involve ROS production [30] which may be generated during physiological conditions. In vitro, chromate reduction via hydrogen peroxide (H_2O_2) has been shown to produce hydroxyl radical (OH^-) via a Fenton-like reaction. [30- 31] In *in-vivo* experiments have been shown that $K_2Cr_2O_7$ exposition induces oxidative and nitrosative stress measured as protein carbonyl content and 3-nitrotyrosine (3-NT) immunostaining. [32, 26] The role of oxidative stress in the renal damage induced by $K_2Cr_2O_7$ has been supported by the fact that some antioxidants such as a-tocopherol, ascorbic acid, and glutathione (GSH) [33-34] and the previous induction of heme oxygenase-1 [32] are able to ameliorate $K_2Cr_2O_7$-induced nephrotoxicity and oxidative damage.

Antioxidant enzyme activity was also modulated. Reduced glutathione neutralizes the hydroxyl radical and plays a major role against inflammatory responses and oxidative stress. [35] A significant restoration of reduced glutathione, to normal levels in the dose dependent manner in *P. grande* pre-treated groups was observed. Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it catalyzes the decomposition of hydrogen peroxide to water and oxygen. However, pretreatment of *P. grande* (p.o.) dose dependently enhanced catalase activity significantly. Superoxide dismutases (SOD) are a class of enzymes which catalyze the dismutation of superoxide into hydrogen and peroxide oxygen. As such, they are an important antioxidant defence in nearly all cells exposed to oxygen. In the present study, potassium dichromate treatment caused nephrotoxicity as evidenced by marked elevation in blood urea and creatinine. Pretreatment of methanolic extract (60 mg/kg) (p.o.) inhibited the rise in blood urea and serum creatinine. There were inhibition in the rise of BUN and creatinine in serum with high dose (120 mg/kg). The present finding clearly indicates the protective effect of *P. grande* at both

doses (60 mg & 120 mg/kg) on $K_2Cr_2O_7$ toxicity in rats (Table 2). $K_2Cr_2O_7$ treatment alone diminished the levels of SOD and catalase respectively in the kidney compared to their vehicle treated control. Co-administration of methanolic extract (p.o.) with $K_2Cr_2O_7$ (s.c.) resulted in the dose dependent increase in the reduced levels of these antioxidant enzymes (Table 2). The main histological finding of this study was that $K_2Cr_2O_7$ group showed acute tubular necrosis and glomerular widening, however pre-treatment with *P. grande* protected the kidney architecture from damage induced by $K_2Cr_2O_7$. We can conclude that mechanism of the protective action of *P. grande* against nephrotoxicity of $K_2Cr_2O_7$ might be due to its free radical scavenging activity.

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