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Evaluation of leaves of *Lawsonia inermis* for nephroprotective activity

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ABSTRACT

The present study was aimed to evaluate the nephroprotective potential of ethanol extract of leaves of *Lawsonia inermis* in cisplatin-induced nephrotoxicity in male Albino rats. Leaves of *Lawsonia inermis* were powdered and ethanol extract was prepared by hot extraction method. Preliminary phytochemical screening was carried out. Further ethanol extract was subjected to GC-MS analysis which revealed the presence of various bioactive phytoconstituents. Nephroprotective activity of extract was evaluated at 200 and 400mg/kg b. w. in male Albino rats in both curative and prophylactic regimens. Nephrotoxicity was induced in rats by single intra peritoneal injection of cisplatin at a dose of 5mg/kg b. w. Nephroprotective activity was determined by estimating serum markers, urinary parameters, lipid peroxidation and antioxidant levels in renal tissue. It was observed that Cisplatin-induced marked nephrotoxicity manifested by a significant increase in Serum marker levels, Urinary total protein, lipid peroxidation and decrease in creatinine clearance, glutathione, catalase and superoxide dismutase levels. Treatment with extract significantly attenuated drug-induced nephrotoxicity in cisplatin model by restoring the biochemical and oxidative stress markers in dose dependent passion in both regimens. Histological studies also substantiated the biochemical parameters. Thus the findings of the present study provided a corroborative scientific evidence for folklore use of *Lawsonia inermis* as a nephroprotective agent.

KEYWORDS: Nephroprotective, *Lawsonia inermis*, Cisplatin, Serum markers.

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INTRODUCTION

India has a rich culture of medicinal herbs and species, which includes more than 2000 species, but only very few have been studied chemically and pharmacologically for their potential medicinal value[1]. In India, about 80% of the rural population uses medicinal herbs or indigenous systems of medicine[2].

Traditional system of medicine continued to be widely practiced on many accounts. "Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drug for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicine for a wide variety of human ailments"[3].

Medicinal plants may serve as a vital source of potentially useful new compounds for the development of effective therapy to combat a variety of ailments including kidney problems[4]. Herbal therapy to treat severe renal disorders requires systematic investigation of properties like acute renal failure, nephritic syndrome and chronic interstitial nephritis.

Many medicinal plants which have claimed to treat renal disorder showed significant nephroprotection. Tribal people of Maharashtra and Kerala use leaves of *Lawsonia inermis* in the treatment of various kidney ailments but scientific studies are not yet undertaken to verify these claims[5]. Hence, the present study designed to screen the nephroprotective activity of leaves of *Lawsonia inermis.*

MATERIALS AND METHODS

Collection of Leaves of Lawsonia inermis: *Lawsonia inermis* leaves were collected from Pannur, Chittor Dt., A.P. and authenticated by botanist Dr. K. Madhava Chetty, Asst. Professor, Dept. of Botany, S.V.University, Tirupati and a voucher specimen (No. 1307) was deposited in Sri Venkateswara University Botany Dept., Tirupati.

Preparation of extract: Leaves of *Lawsonia inermis* were shade dried and powdered in a Wiley mill and the powdered leaves were defatted with petroleum ether and then macerated with ethanol for 24 h. Macerated material was refluxed for 3h and then filtered. The procedure was repeated twice and filtrates were combined and subjected to distillation under reduced pressure.

Preliminary phytochemical screening: Preliminary phytochemical screening of ethanol extract was carried out as per standard procedures[6].

***GC –MS analysis*:** The GC-MS analysis of ethanol extract of leaves of *Lawsonia inermis* (EELI) was carried out using a Clarus 500 Perkin-Elmer (Auto system XL) Gas chromatograph equipped and coupled to a mass detector Turbo mass gold-Perkin Elmer with turbomass ver 5.2.0 spectrometer with an Elite-5MS (5% Phenyl 95% dimethyl Polysiloxane), 30mx500μm id capillary column. The instrument was set to an initial temperature of 60°C. Then the oven temperature was rose upto 150°C at the rate of an increase of 6°C/min, and maintained for 2 min. Then the oven temperature was rose upto 280°C, and maintained for 5 min. Injection port temperature was ensured at 280°C and Helium flow rate at 1.0ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 1:10. Mass spectral scan range was set at 40-450 amu. The ion source temperature was maintained at 160°C and Interface temperature was at 180°C. The MS end time was 54.5min. Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology.

Pharmacological studies:

Animals:

Healthy Wistar strain albino rats aged between 2 - 3 months and weighing about 150-200g were used in the current study. They were maintained in a 12 h light/dark cycle and housed in a room at a temperature of 20 ± 2^{0}C and relative humidity $50 \pm$ 10%. The animals were provided with free access to standard rat pellet diet and water *ad libitum*. 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/22).

Acute toxicity studies

Oral acute toxicity studies were conducted for EELI at 2000 mg/kg body weight as per OECD 423 guidelines[7].

Evaluation of nephroprotective activity:

Nephroprotective effect of EELI was evaluated at two different dose levels *i. e.*, 200 and 400 mg/kg body weight. Cisplatin at a dose of 5mg/kg b.w i.p. was used to induce nephrotoxicity.

The experimental rats were randomly assigned into nine experimental groups (n=6 per each group) and the following treatment schedule was employed:

1. Group-I was given vehicle (water) orally from day 1-5 and was kept as a normal control.
2. Group-II was given single dose of cisplatin (5mg/kg/b.w.,i.p) on day 1 and vehicle orally from day 5 – day 9 and was kept as a curative control.
3. Group -III was given vehicle (water) from day 1 – day 5 and was administered with cisplatin (5mg/kg/b.w.,i.p) on 5th day and was kept as prophylactic control.

4. Group -IV was administered with cisplatin (5mg/kg/b.w.,i.p) on 1^{st} day and was given with low dose (200mg/kg/b.w.,p.o) of EELI from day 5 – day 9 and served as curative group.
5. Group-V was administred with cisplatin (5mg/kg/b.w.,i.p)) on day1 and was given with higher dose (400mg/kg/b.w.,p.o) of EELI from day 5 – day 9 and served as curative group.
6. Group-VI was given low dose (200mg/kg/b.w.,p.o) of EELI from day 1 – day 5 and administered with cisplatin (5mg/kg/b.w.,i.p) on day 5 and was kept as prophylactic group.
7. Group-VII was given high dose (400mg/kg/b.w.,p.o) of EELI from day1 –day 5 and administered with cisplatin (5mg/kg/b.w.,i.p) on day 5 and was kept as prophylactic group.
8. Group-VIII was administered with only high dose (400mg/kg/b.w.,p.o) of EELI from day 1 – day 5 to assess protective effect of *Lawsonia inermis.*

On day 5 from animals of group-I, VIII and on day 9 from remaining groups urine was collected using metabolic cages; the urine samples were subjected for estimation of urinary functional parameters. The animals were sacrificed on the respective following days by cervical decapitation and blood samples were collected by cardiac puncture and were used for estimation of serum markers[8].

Antioxidant studies:

Kidneys were homogenized in ice cold 0.05 M phosphate buffer p^H 7.8 to obtain a 20% (w/v) homogenate. The homogenate was subjected to centrifugation at 10,000 rpm for 15 min and the clear supernatant obtained was immediately used for the analysis of antioxidant enzymes. Anti-oxidant studies were performed by the estimation of levels of lipid peroxidation (LPO), reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD)[9-12].

Histological studies

The portion of the kidneys were dissected and fixed in 10% neural buffer formalin and processed to paraffin wax. Sections (5 microns) are stained with haematoxylin and eosin and are examined under light microscope.

Statistical analysis:

The results were expressed as mean $\pm$ standard error. Parametric data which include all the biochemical parameters were analysed using one way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison tests. The significance was considered at p-value less than 0.05.

RESULTS

Preliminary phytochemical studies: Phytochemical screening of the EELI revealed the presence of carbohydrates, alkaloids, flavonoids, glycosides, tannins and other phenolic compounds.

GC-MS analysis: Twenty eight compounds were identified in GC-MS analysis of EELI (Figure 1). The active principles with their molecular formula, retention time, peak area and biological activity (as per Dr. Duke's ethnomedicinal database) are mentioned in Table 1.

Pharmacological studies

Acute toxicity studies:

There was no morbidity and animals did not show any changes in behavior. Hence, the ethanol extract was found to be safe at 2000 mg/kg, b.w.dose.

Effect of EELI on cisplatin-induced nephrotoxicity:

Administration of cisplatin resulted in significant increase ($p<0.05$) in BUN, serum creatinine, urinary total protein, lipid peroxidation and decrease in Creatinine clearance, reduced glutathione, catalase and superoxide dismutase levels when compared to normal animals. However administration of the ethanol extract of leaves *Lawsonia inermis* at both doses of 200 and 400 mg/kg ,b.w. significantly reversed the effects caused by cisplatin in dose dependent passion in both curative and prophylactic regimen (Table 2 and 3)

Renal histological examination revealed that cisplatin-induced renal damage which was indicated by the presence of degenerative tubules, degenerative glomeruli and glomerular atrophy with areas of hemorrhages and elongated tubules. Whereas dose dependent regenerative changes were observed in groups treated with ethanol extract (Figure 2).

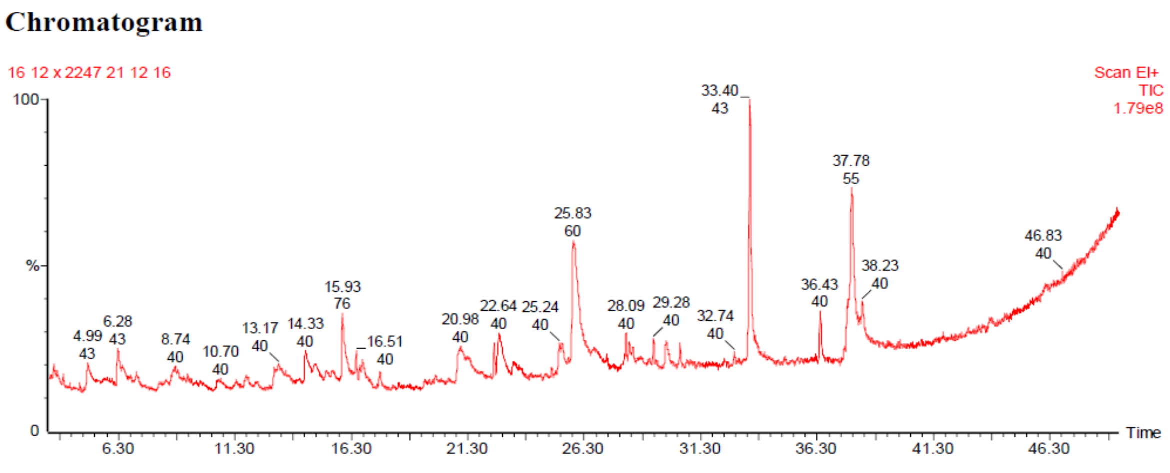

Figure 1: GC-MS Chromatogram of EELI

Table 1: GC-MS analysis of EELI

S.No.	Peak name	Retention time	Peak area	%Peak area	Biological activity*
1.	Name: 1-Butanol, 3-methyl-, acetate Formula: $C_7H_{14}O_2$ MW: 130	4.63	203127	0.2537	Methyl donar, catechol,o-methyl-transferase
2.	Name: Methanamine, N-hydroxy-N- methyl- Formula: C_2H_7NO MW: 61	4.99	2617520	3.2695	Antitumor ,GABA-nergic, nephritic
3.	Name: 1,2-Cyclopentanedione Formula: $C_5H_6O_2$ MW: 98	6.28	1574271	1.9664	Nf
4.	Name: 2-Cyclopenten-1-one, 2-hydroxy- Formula: $C_5H_6O_2$ MW: 98	6.48	362102	0.4523	Nf
5.	Name: 2,4-Dihydroxy-2,5-dimethyl-3(2H)- furan-3-one Formula: $C_6H_8O_4$ MW: 144	7.06	700383	0.8748	Hepatotropic,hepatotoxic,anti HIV, hemostat, antidote
6.	Name:1,2-Cyclooctanedione Formula: $C_8H_{12}O_2$ MW: 140	8.35	279528	0.3492	Nf
7.	Name: Pyrimidine-4,6-diol, 5-methyl- Formula: $C_5H_6N_2O_2$ MW: 126	10.58	760311	0.9497	Nf
8.	Name: 4H-Pyran-4-one, 2,3-dihydro-3,5- dihydroxy-6-methyl- Formula: $C_6H_8O_4$ MW: 144	11.78	1323981	1.6538	Antidote,5HT inhibitor,Anti5HT,Anti HIV integrase.
9.	Name: Benzaldehyde, 4-methyl- Formula: C_8H_8O MW: 120	14.33	3407697	4.2565	Methyl donar, catechol-o-methyl-transase inhibitor.
10.	Name: 2-Furanone, 3,4-dihydroxytetrahydro Formula: $C_4H_6O_4$ MW: 118	15.25	829038	1.0355	Nf
11.	Name: Phthalic anhydride Formula: $C_8H_4O_3$ MW: 148	15.93	4922389	6.1485	Nf
12.	Name: E-11,13-Tetradecadien-1-ol Formula: $C_{14}H_{26}O$ MW: 210	16.51	770371	0.9623	Anticancer, emetic, euphoric
13.	Name: Undecanoic acid, ethyl ester Formula: $C_{13}H_{26}O_2$ MW: 214	16.68	421114	0.5260	Nf
14.	Name: 1(3H)-Isobenzofuranone Formula: $C_8H_6O_2$ MW: 134	16.77	1045828	1.3063	Antidote, hypokinatic, histamic, HMG-co-A inhibitor
15.	Name: 3-Hexadecene, (Z)- Formula: $C_{16}H_{32}$ MW: 224	22.42	999843	1.2489	Nf
16.	Name: D-Allose Formula: $C_6H_{12}O_6$ MW: 180	22.64	4997839	6.2427	Diuretic, CNS depresent, urine-deodorant, DNA Protective.
17.	Name: 1,4-Naphthalenedione Formula: $C_{10}H_6O_2$ MW: 158	23.25	558509	0.6976	Nf

18.	Name: 8-Pentadecanone Formula: $C_{15}H_{30}O$ MW: 226	24.89	241853	0.3021	Nf
19.	Name: Ethyl à-d-glucopyranoside Formula: $C_8H_{16}O_6$ MW: 208	25.83	19126550	23.8907	Anti cancer,depressant, diuretic, DNA protective.
20.	Name: E-14-Hexadecenal Formula: $C_{16}H_{30}O$ MW: 238	28.09	1519580	1.8981	Anti cancer, cancer protective,emetic, cytochrom-p450-2E1-inhibitor.
21.	Name: Tetradecanoic acid Formula: $C_{14}H_{28}O_2$ MW: 228	28.23	1321094	1.6502	Urinary –acidulant, inhibitor the production of uric acid.
22.	Name: Benzyl Benzoate Formula: $C_{14}H_{12}O_2$ MW: 212	28.37	329931	0.4121	Nf
23.	Name: 3,7,11,15-Tetramethyl-2-hexadecen-1-ol Formula: $C_{20}H_{40}O$ MW: 296	29.28	866306	1.0821	Nf
24.	Name: 5,5,8a-Trimethyl-3,5,6,7,8,8a- hexahydro-2H-chromene Formula: $C_{12}H_{20}O$ MW: 180	29.82	2634592	3.2908	Antidiuretic ,ACE inhibitor,anticancer ,antidote,antihepatitic,antiinflamation,acetylcoA-carboxylase-inhibitor.
25.	Name: 2-Hydroxy-2-(1-nitro-ethyl)-indan-1,3-dione Formula: $C_{11}H_9NO_5$ MW: 235	32.74	408154	0.5098	Nf
26.	Name: n-Hexadecanoic acid Formula: $C_{16}H_{32}O_2$ MW: 256	33.40	14635522	18.2810	Urinary –acidulant, urine acidifier GABA-nergic, inhibit production of Tumor Necrsis factor.
27.	Name: Phytol Formula: $C_{20}H_{40}O$ MW: 296	36.43	2875628	3.5919	Cancerpreventive.
28.	Name: 2-Methyl-Z,Z-3,13-octadecadienol Formula: $C_{19}H_{36}O$ MW: 280	37.78	10325374	12.8973	Nf

*Bio-activity was predicted using Dr. Duke's Phytochemical and Ethnobotanical Databases

Table 2: Effect of EELI against Cisplatin- induced nephrotoxicity on serum and urine parameters

Group	BUN (mg/dl)	SC (mg/dl)	U_{TP} (mg/24hrs)	Cl_{cr} (ml/h/100gb.w)
I	23.87 ± 0.19	0.79 ± 0.04	4.04 ± 0.11	4.34 ± 0.12
II	$56.50 \pm 2.86^{*}$	$1.64 \pm 0.07^{*}$	$6.75 \pm 0.38^{*}$	$2.53 \pm 0.05^{*}$
III	$58.49 \pm 2.47^{*}$	$1.77 \pm 0.02^{*}$	6.58 ± 0.49*	$2.55 \pm 0.07^{*}$
IV	42.79 ± 1.43^{a}	1.31 ± 0.02^{a}	2.54 ± 0.18^{a}	5.43 ± 0.35^{a}
V	30.41 ± 2.56^{a}	1.24 ± 0.01^{a}	2.12 ± 0.02^{a}	7.36 ± 0.58^{a}
VI	46.36 ± 1.23^{b}	1.44 ± 0.08^{b}	3.50 ± 0.20^{b}	5.71 ± 0.55^{b}
VII	28.75 ± 2.93^{b}	1.26 ± 0.06^{b}	2.28 ± 0.11^{b}	7.92 ± 0.28^{b}
VIII	30.89 ± 2.93^{ns}	0.85 ± 0.04^{ns}	5.26 ± 0.38^{ns}	4.57 ± 0.10^{ns}

Each value represents the Mean ± S.E.M from 6 animals in each group. *: $p<0.05$; ns: not significant.

a: Group-II , III and VIII compared with Group-I

b: Group- IV and V compared with Group-II

c: Group- VI and VII compared with Group-V

Table 3: Effect of EELI on anti-oxidant levels on Cisplatin-induced nephrotoxicity in rats

Group	LPO (nmol/g of wet tissue)	GSH (µmol/g of wet tissue)	CAT (units/mg of protein)	SOD (units/mg of wet tissue)
I	2.678 ± 0.09	29.82 ± 0.69	23.08 ± 0.17	20.92 ± 0.17
II	3.93 ± 0.04*	16.19 ± 0.42*	16.09 ± 0.23*	14.71 ± 0.47*
III	4.10 ± 0.28*	16.26 ± 0.36*	15.63 ± 0.21*	13.11 ± 0.53*
IV	3.01 ± 0.19^{a}	27.69 ± 0.98^{a}	21.31 ± 0.76^{a}	23.65 ± 1.20^{a}
V	2.82 ± 0.20^{a}	31.35 ± 2.58^{a}	22.95 ± 1.47^{a}	28.34 ± 1.99^{a}
VI	3.16 ± 0.16^{b}	27.34 ± 0.86^{b}	20.66 ± 0.14^{b}	21.76 ± 1.12^{b}
VII	1.37 ± 0.10^{b}	37.92 ± 1.76^{b}	22.85 ± 1.14^{b}	27.01 ± 0.38^{b}
VIII	2.696 ± 0.11^{ns}	38.47 ± 5.39^{ns}	23.01 ± 0.84^{ns}	24.64 ± 1.50^{ns}

Each value represents the Mean ± S.E.M from 6 animals in each group. *: $p<0.05$; ns: not significant.

a: Group-II , III and VIII compared with Group-I

b: Group- IV and V compared with Group-II

c: Group- VI and VII compared with Group-III

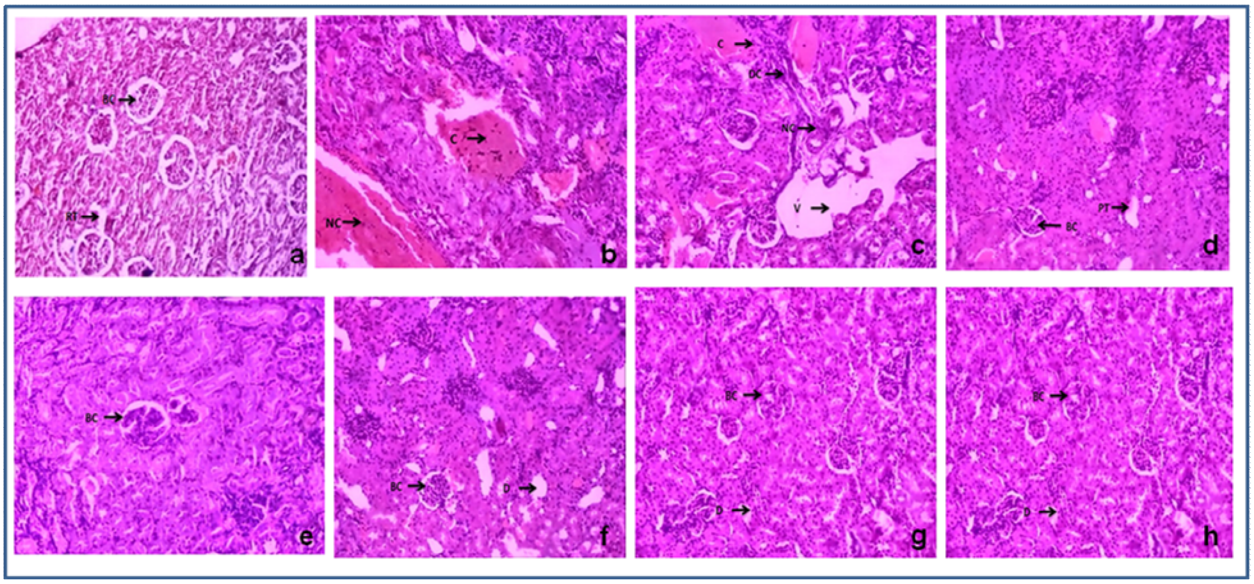

Figure 2: Histomicrographs of rat kidney 2(a)-Normal treated with vehicle; 2(b)-Cisplatin Curative control showing marked degenerative changes; 2(c)- Cisplatin prophylactic control showing marked degeneration and necrosis 2(d)-Lower dose treated in curative regimen showing mild regeneration; 2(e)-Higher dose treated in curative regimen showing marked regeneration; 2(f)-Lower dose treated in prophylactic regimen mild renal damage; 2(g)-Higher dose treated in prophylactic regimen showing regenerative changes; 2(h)- Only higher dose treated showing normal organization. BC-Bowman's capsule; RT-renal tubule; PT-Proximal tubule; D-Degeneration; NC-Necrotic changes; RC-Regenerative changes.

DISCUSSION

Cisplatin is a potent anti-tumour drug. Cisplatin-based combination chemotherapy regimens are extensively used as front-line therapy in the treatment of ovarian germ cell tumors, testicular cancer, epithelial ovarian cancer, lung cancer, head and neck cancer, advanced cervical cancer, and malignant melanoma[13]. Although cisplatin has been a mainstay for therapy of cancer, its use is mainly limited by two factors: Acquired resistance to cisplatin and severe side effects in normal tissues which include neurotoxicity, ototoxicity, nausea and vomiting, and nephrotoxicity[14]. The proposed mechanisms of cisplatin (*cis*-$PtCl_2$ $(NH_3)_2$)-induced nephrotoxicity are: 1. Generation of reactive oxygen species (ROS) that bind covalently to tissue macromolecules[15]. 2. Binding of heavy metal present in cisplatin to sulphhydryl group present in GSH and cause reduction in GSH which is the primary event to cause biochemical change[10,16].

Literature reveals that many number of medicinal plants showed significant protection against Cisplatin-induced nephrotoxicity[17]. Tribal people of Maharashtra and Kerala use leaves of *Lawsonia inermis* in the treatment of various kidney ailments but scientific studies are not yet undertaken to verify these claims. Hence present study was focused on the curative and prophylactic effect of ethanol extract of leaves of *Lawsonia inermis* on the renal damage induced by Cisplatin in male albino Wistar rats. In the present study, Cisplatin at a dose of 5mg/kg, b. w. induced nephrotoxicity which was manifested by marked elevation of Serum markers and urinary parameters and decreased antioxidant levels in renal tissue which is also evidenced in earlier studies[18,19].

This may be due to the decrease in the glomerular filtration rate or may be due to the increase of the reactive oxygen species which induce mesangial cells contraction, altering the filtration surface area and modifying the ultra filtration coefficient factors[20,21]. In curative regimen, treatment with extract at 200 and 400mg/kg b w. reversed the nephrotoxic effects induced by cisplatin in dose dependent manner.

The nephrotoxicity is a rapid process due to the reaction with the proteins in renal tubules. The renal damage is produced within one hour after administration[22]. Hence the presence of protective agents like *Boerhaavia diffusa*, *Hygrophila spinosa*, *Scoparia dulcis* extracts in the renal tissues reduce the toxic effects of cisplatin[23-25]. This is the rationale behind the prophylactic regimen. In present study ethanol extract of leaves of *Lawsonia inermis* (200 and 400 mg/kg b.w) has showed dose dependent nephroprotective effect which may be due to the presence of phytochemicals in the extract protected from the damage induced by cisplatin.

Renal histological examination revealed that cisplatin caused renal damage which is evidenced by the presence of degenerative glomeruli, degenerative tubules whereas moderate degenerative changes were observed in groups treated with lower dose. In higher dose treated animals marked regenerative changes were observed in both curative and prophylactic regimens.

Our preliminary phytochemical studies revealed the presence of anti-oxidant principles like flavonoids and terpenoids which may play significant role in nephroprotective activity of *Lawsonia inermis*. Further GC-MS analysis revealed the presence of about 28 phyto-constituents which most of them were associated with various biological activities including antioxidant activity. Presence of these phytoconstituents may responsible for nephroprotective activity of leaves of *Lawsonia inermis*.

CONCLUSION

The findings of current study suggest that leaves of *Lawsonia inermis* can be used as effective nephroprotective agent against cisplatin-induced nephrotoxicity. Further the present study provided a corroborative scientific evidence for folklore use of *Lawsonia inermis* as a nephroprotective agent.

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