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Original Research Article

Protective effect of *Phyllanthus fraternus* against cyclophosphamide-induced nephrotoxicity in rats

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ABSTRACT

Background: Cyclophosphamide is commonly used against malignancies, such as leukemia and solid organ cancers. It can induce nephrotoxicity in cancer patients thereby complicating the initiation of chemotherapy. The study is to evaluate the effect of *Phylllanthus fraternus* against cyclophosphamide induced nephrotoxicity in albino rats.
Methods: In this study, a total of 20 albino rats were divided into four groups of five each. Group I (normal control) received i.p. injection of normal saline. While, group II, III and IV received single dose (200 mg/kg b/w) of cyclophosphamide i.p. on day 1. Group III and IV received 200 mg/kg and 400 mg/kg of aqueous extract of *Phyllanthus fraternus* respectively p.o. daily for 10 days. All the groups were fed with standard diet and water ad libitum. Serum levels of creatinine, urea and albumin were estimated. Histopathology of renal tissues was compared among the groups.
Results: The renal parameters significantly improved in groups III and IV after 10 days of treatment with the extract. The histopathology study also supported the finding.
Conclusions: The aqueous extract of *Phyllanthus fraternus* possesses protective effect against cyclophosphamide induced nephrotoxicity.

Keywords: Cyclophosphamide, Nephrotoxicity, Nephroprotective, *Phyllanthus fraternus*

INTRODUCTION

Nephrotoxicity is one of the major kidney problems caused by drug or toxin.[1] Drugs, radiocontrast agents and heavy metals are well known to be nephrotoxic.[2] Cyclophosphamide (CP) is an immunosuppressive and cytotoxic drug used in various clinical scenarios such as tissue transplantation, autoimmune disorders and neoplasia. It is an alkylating agent belonging to the nitrogen mustard subclass of anticancer drugs. In human, serum the half-life of cyclophosphamide is about 6.5 hours.[3] Tubular reabsorption of cyclophosphamide is very high. So, a very small fraction of it excreted through the kidneys.[4] Active metabolites of cyclophosphamide i.e., acrolein and phosphoramide mustard generated in the body act as free radicals that cause damage to renal microstructures.[5]

Herbal drugs are readily available, low cost, and with less side effects. Indian medicinal plants also provide a rich source for antioxidants that are known to prevent or delay different diseased states.[6] The medicinal plants also contain other beneficial compounds or ingredients which can be used as food.[7] They are the potential source of therapeutics aids and played a significant role in the health care system all over the world. They not only provide relief to diseased conditions but also help in maintaining health in day to day life. Studies done on nephroprotective properties of various medicinal plants like *Allium sativum, Zingiber officinale, Curcuma longa*,[1]

Butea monosperma, *Tinospora cordifolia* etc, used drug induced animal models of acute nephrotoxicity.[8-12]

Phyllanthus fraternus is a medicinal herb growing widely in most tropical and subtropical countries. The plant belongs to Euphorbiaceae family.[13] In India, *Phyllanthus fraternus* is mixed with other *Phyllanthus spp*., to make a polyherbal preparation called 'Bhumyamlaki', which is widely used against medical problems of the genito-urinary tract, anuria and hiccups.[14] Phytochemical analysis of plant extract reveals presence of alkaloids, tannins, saponin, terpenoid and steroid which are medicinally important bioactive compounds.[15,16] Aerial part of the plant shows greater antioxidant property by virtue of its higher polyphenolic content.[17]Aqueous extract of the plant has shown hepatoprotective effect against CP induced oxidative liver injury, which may serve as a promising medicinal herb in the protection against similar oxidative damage to other vital organs.[18]

The study was undertaken to evaluate the protective effect of aqueous extract of *Phyllanthus fraternus* leaves (AEPF) against cyclophosphamide induced nephrotoxicity in rats.

METHODS

The study was conducted in the Departments of Pharmacology and Pathology, RIMS, Imphal, India from November 2014 to July 2016.

Drugs and chemicals

Cyclophosphamide (CYPHOS^{TM}) was purchased from Getwell Pharmaceuticals, Haryana, India. Biochemical estimation and analyzing kits for serum creatinine, urea and albumin were purchased from Avantor Performance Materials India Ltd, Uttarakhand, India. All other chemicals and solvents used were of analytical grade. Standard pellet diets were procured from Amricon Agrovet Private Limited, India.

Plant material

The fresh plant of *P. fraternus* was collected from the Lamphel area, Imphal, Manipur in the month of August. The plant was identified and authenticated by Dr. P. K. Singh, Professor, Department of Life Sciences, Manipur University, Imphal. A voucher specimen was kept in the University herbarium for reference (Voucher no. 000874).

Preparation of extract

The plant leaves were washed and shade dried. The leaves were powdered by mixer grinder and stored in an airtight container for future use. Preparation of aqueous extract was done by the method described by Verma SCL and Agrawal SL.[19] The powdered leaves were extracted with distilled water using soxhlet apparatus. The greenish brown extract obtained was filtered, spread in an evaporating dish and dried on a hot water bath. The dried extract was scraped out, weighed and stored in glazed porcelain jar for use in the experiment. The yield was 13.5%.

Phytochemical studies

Phytochemical tests using standard techniques confirmed the presence of tannins, alkaloids, flavonoids, terpenoids, steroids and saponins.[20,21]

Acute toxicity testing

Acute toxicity test was carried out as per the OECD guidelines 423 in female albino rats (3 rats per step).[22] The rats were fasted for overnight with water *ad libitum*. Then, aqueous extract of *Phyllanthus fraternus* was administered to the fasted rats at a dose of 300 mg/kg by feeding tube. Food was withheld for further 3-4 h and observed once in every 30 min during the first 24 h and thereafter, daily for a period of 14 days for any mortality. As there was no mortality, the procedure was repeated with higher dose of 2000 mg/kg and animals were observed for mortality and toxic symptoms. It was observed that the dose of 2000 mg/kg caused no mortality or toxic symptoms among the tested animals and considered safe. Two doses of 200 mg/kg (1/10th of the maximum test dose) and 400 mg/kg of AEPF were used as working doses for the experiment.

Selection of animals

The young adult Wistar albino rats of either sex weighing 150 to 210 g age between 3 months and 1 year procured from the Animal House, RIMS, Imphal, India were used for the study. The animals were kept in polypropylene cages in room temperature under 12h light:dark cycle for 1 week in the animal room of Department of Pharmacology, RIMS, Imphal for acclimatization. They were fed with standard pellet diet with free access to water.

Inclusion and exclusion criteria

Baseline serum levels of creatinine, urea and albumin were estimated and normal reference ranges were calculated assuming the values are normally distributed.[23] The value ranges are of 0.4-1.3 mg/dl, 10-42 mg/dl and 3.5-4.5 g/dl respectively. Animals with higher serum levels of creatinine and urea more than the above ranges and lower serum albumin levels below the given reference range were excluded.

Experimental design

In the study, doses of 200 and 400 mg/kg body weight of AEPF were selected as working doses based on acute toxicity data. The animals were divided into 4 groups (I, II, III and IV) of 5 animals each (Table 1). On day 1, the

group I animals were given 0.5 ml/100 g of normal saline (NS) intraperitoneally (i.p.) as single injection.

Table 1: Allotment of animals to different groups and treatments given.

Groups	Drugs given as single dose on day 1 (i.p.)	Drugs given as single daily dose for 10 days (p.o.)
I (Normal)	0.5 ml of 0.9% NS	2% gum acacia in DW (1 ml/100 g)
II (CP)	Cyclophosphamide (200 mg/kg)	2% gum acacia in DW (1 ml/100 g)
III (Test 1)	Cyclophosphamide (200 mg/kg)	AEPF suspension (200 mg/kg)
IV (Test 2)	Cyclophosphamide (200 mg/kg)	AEPF suspension (400 mg/kg)

Similarly, groups II, III and IV animals were administered CP (200 mg/kg) mixed in NS at a volume of 0.5 ml/100 g i.p. Animals in groups I and II were given 2% gum acacia in distilled water at dose of 1 ml/100 g orally for 10 days. Groups III and IV animals were made to receive 200 and 400 mg/kg of AEPF respectively suspended 2% gum acacia orally daily. AEPF was suspended in 2% gum acacia in distilled water (DW) in such a way that 1 ml contained the calculated doses. Both control and treated animals were observed for 10 days after the i.p. injection for the general appearance, behaviour and mortality.

Blood collection

Blood samples were drawn before any treatment was given to the animals to assess the baseline biochemical parameters. The animals were anaesthetized with ether and blood samples were collected by retro-orbital venous sinus puncture.[24] About 2 ml of blood from each animal were collected in a vacutainer from all groups and allowed to clot. The blood was then centrifuged at speed of 3000 rpm for 10 min. The serum separated was kept in refrigerator at 4°C. It was used for biochemical estimation of creatinine, urea and albumin. The samples were again taken on day 11.

Biochemical estimations

Serum biochemical parameters were assessed using commercially available test kits as per the standard methods described. Serum creatinine, urea and albumin were estimated by using Jaffe's kinetic method, Berthelot method and Bromocresol green end-point reaction.[25-27]

Determination of relative kidney-somatic index (kidney to body weight ratios)[28]

$$\text{Kidney-somatic index} = \frac{\text{weight of 2 kidneys}}{\text{weight of the rat at the end of experiment}}$$

Histopathological preparation and scoring

A midline abdominothoracic incision was performed under ether anaesthesia. Kidneys were quickly dissected out, washed in ice cold saline, dried on filter paper and weighed immediately. The tissues were fixed in 10% neutral buffered formalin for 48 hours. Renal tissues were prepared for histopathological examination using standard techniques.[29]

Tissue sections (5 µm thick) of the kidneys were prepared, stained with haematoxylin and eosin (H&E). The sections were observed under light microscope for histopathological changes. The histological scoring was performed based on the following parameters - tubular degeneration, necrosis, tubular dilatation, hyaline protein casts and interstitial leucocytic infiltration.30 These parameters were assessed and graded in the scale of 0 to 5 (0=normal histology, 1=degeneration only without necrosis, 2≤25%, 3=25-50%, 4=51-75%, 5≥75% degeneration with necrosis in 20 high power fields). The histological scores were compared among different treatment groups.

The animal carcasses were buried deep in the ground covered with lime and disinfectants after the experiment.[31]

Statistical analysis

The results of serum biochemical parameters and histological scoring were analyzed using one way ANOVA followed by Dunnett's t-test using SPSS version 21. P value less than 0.05 was considered significant.

RESULTS

Administration of CP to rats significantly decreased ($P<0.001$) the body weight and absolute kidney weights. Co-administration of CP with AEPF causes lesser body weight loss and increases the kidney weights significantly ($P<0.001$). However, there was no significant difference in kidney-somatic index (KSI) among various groups (Table 2).

The treatment of CP to rats significantly increased ($P<0.001$) the serum levels of creatinine and urea as compared to the control group. Oral administration of AEPF in CP treated rats significantly ($P<0.01$) decreases creatinine and urea levels compared with CP only treated group. Serum albumin level significantly decreased ($P<0.001$) in CP treated rats compared with normal group. But the levels were increased in AEPF treated groups significantly when compared with the CP only treated group (Table 3). The histopathological observations of effect of AEPF extract on CP-intoxicated rats are shown in Figure 1. The microscopic examination of kidneys in the normal control rats revealed normal renal glomeruli surrounded by Bowman's capsule.

Table 2: Changes in the body weight, weight of kidneys and relative kidney to body weight ratio of normal, CP and AEPF treated groups of rats.

Groups	Initial BW (g)	Final BW (g)	Weight diff. (g)	Weight of 2 kidneys (g)	Kidney somatic Index ($\times 10^{-3}$)
I	174.60 ± 21.40	190.80 ± 19.00	16.20 ± 4.54	1.27±0.03	6.70±0.38
II	182.00 ± 24.60	126.25 ± 20.61**	-55.75 ± 6.29***	0.71±0.02***	5.75±0.53
III	182.50 ± 16.05	141.00 ± 18.70*	-41.50 ± 18.28***	0.87±0.03***††	6.21±0.24
IV	179.00 ± 23.12	149.75 ± 25.53	-29.25 ± 5.37***†	0.97±0.02***††	6.58±0.66

Values are expressed as mean±SEM (n=5), One way ANOVA followed by Dunnett's t-test (SPSS 21), *P <0.05, **P <0.01, ***P <0.001 with respect to Normal gr; †P <0.05, ††P <0.01 with respect to CP gr; Group I-Normal control , Group II-CP (200 mg/kg), Group III-CP+AEPF (200 mg/kg) and Group IV-CP+AEPF (400 mg/kg); (-)=weight loss.

Table 3: Effect of aqueous extract of *Phyllanthus fraternus* leaves on serum creatinine, urea and albumin in cyclophosphamide induced nephrotoxic rats after 10 days treatment.

Groups	S. Creatinine (mg/dl)	S. Urea (mg/dl)	S. Albumin (g/dl)
Baseline	0.77±0.06	17.50±0.9	3.88±0.12
I	0.76±0.07	18.40±0.75	3.85±0.15
II	2.55±0.10**	55.50±0.96**	2.16±0.11**
III	2.00±0.08**†	40.00±2.16**††	2.95±0.11**†
IV	1.55±0.10**††‡	32.00±1.83**††‡‡	3.24±0.10*††

Values are expressed as mean±SEM (n=5) One way ANOVA (SPSS 21), *P <0.05, **P <0.001 with respect to Normal gr; †P <0.01, ††P <0.001 with respect to CP gr; ‡P <0.05, ‡‡P <0.01 with respect to gr III; Baseline Group–before start of experiment, Group I-Normal control , Group II-CP (200 mg/kg), Group III-CP+AEPF (200 mg/kg) and Group IV-CP+AEPF (400 mg/kg).

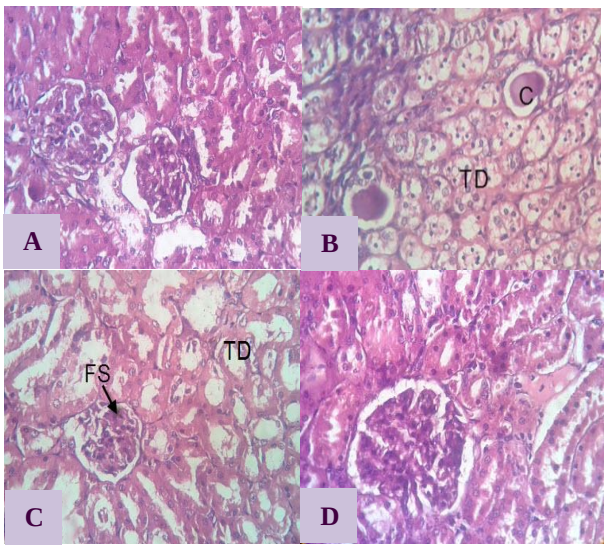

Figure 1: Photomicrograph of kidney tissues in (A) Normal, (B) CP (200 mg/kg) treated, (C) CP+AEPF (200 mg/kg) treated and (D) CP+AEPF (400 mg/ kg) treated rats. C-cast, TD-tubular degeneration and dilatation, FS-focal sclerosis of glomeruli. Stain: H and E. 40x.

Tissue sections from the kidneys of rats treated with CP showed decreased Bowman's capsule space with focal sclerosis, loss of tubular brush border, epithelial cell necrosis and shedding, luminal cast and congestion of glomerular capillaries. However, no interstitial inflammatory cell infiltration was seen. Tissue sections from the groups co-treated with CP and AEPF revealed reduced tubular damages and decreased focal sclerosis when compared with that of CP only treated groups (Figure 1). As histological scoring system gives lucid inter-group comparision, the average group scores showed significant differences. Maximum scores were noted in cyclophosphamide treated group. Extract treatment showed dose dependant improvement in histological scores (Table 4).

Table 4: Histological scoring for severity of renal tissue damage.

Groups	Histological scoring
I	0.20±0.20
II	4.80±0.20*
III	3.60±0.25*†
IV	2.40±0.25*††‡

Values are expressed as mean±SEM (n=5) One way ANOVA (SPSS 21),*P < 0.001 with respect to Normal gr; †P < 0.05,††P < 0.001 with respect to CP gr; ‡P < 0.05 with respect to gr IV; Group I – Normal control , Group II – CP (200 mg/kg), Group III – CP + AEPF (200 mg/kg) & Group IV – CP + AEPF (400 mg/kg).

DISCUSSION

Cyclophosphamide causes cell damage and apoptosis resulting in body weight loss and shrinkage of kidneys.[32] Aqueous extract of *Phyllanthus fraternus* treatment may have prevented these damaging effects and retards the loss of body and kidney weights.

Serum creatinine concentration is a useful marker for injuries and dysfunction of kidneys.[33] In our study, CP administration led to a marked increase in serum level of creatinine. Co-treatment of AEPF with CP brought down elevated levels of creatinine remarkably. The elevation in serum creatinine is attributed to increased muscle catabolism of creatine to creatinine and decreased elimination of creatinine due to destruction of nephrons.[34] The decrease of creatinine level in our study showed that AEPF treatment might have prevented both the processes. Urea, a by-product of amino acid catabolism, also rises in the blood in a similar way i.e. increased accumulation and decreased elimination in urine.[35] The fall in serum urea level in AEPF treatment group might be attributed to reduced generation and/or increase excretion.

Serum albumin levels are maintained if the glomerular integrity is intact. In acute toxic nephropathy model using short study period, renal albuminuria may be a major cause of hypoalbuminemia owing to its long plasma half-life. Reduced serum albumin level signifies damage to glomerular barrier leading to renal albumin loss.[36] The prevention of decline in the albumin level in AEPF treated rats might signify its protective role in glomerular injury. Histopathological study and comparision of renal sections is considered the gold standard parameter in evaluating nephrotoxic studies and to assess the preventive interventions taken up.[37] Nephrotoxic features seen in CP treated rats are consistent with other similar studies using CP as inducing agent.[38,39] The extract treatment prevented the pathological changes in the renal architecture. However, absence of inflammatory cell infiltrates suggests the immunosuppressive effect of CP. This adds to our understanding that the oxidative stress from the toxic metabolites of CP is the main cause of renal structure damage. Free radicals and reactive metabolites of CP like acrolein generated in the body of rat depleted the antioxidant reserve and mediated the destruction of renal glomeruli and tubules. Enriching antioxidant levels in renal tissue might have prevented the progression of tissue destruction and restore normal renal architecture.[40] Phytochemical analysis of AEPF revealed the presence of flavonoids, alkaloids, steroids, tannins and terpenoids which are also reported in previous screening studies.[15,16] The nephroprotective activity of *P. fraternus* is probably due to the presence of flavanoids which is a good antioxidant.[41] The biochemical and histopathology provided substantial evidences of protection offered by the plant. Therefore, our study suggests the potential role of AEPF as nephroprotective agent and may serve as a promising herbal medicine for use in kidney ailments.

CONCLUSION

The study shows that aqueous extract of *Phyllanthus fraternus* provides protection against cyclophosphamide induced nephrotoxicity. The protective potential may be due to free radical scavenging and antioxidant capacity of the plant extract by virtue of its flavonoid constituents. Further studies will be promising to elucidate mechanism of the nephroprotection.

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