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PROTECTIVE ROLE OF *PHYLLANTHUS FRATERNUS* AGAINST CYCLOPHOSPHAMIDE INDUCED NEPHROTOXICITY IN MICE

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Abstract

Kidney is an important excretory organ of body, playing a dominant role in homeostasis. Cylophosphamide (CPA), a widely used chemotherapeutic drug, induces inflammation of urinary bladder and renal damage, thereby limiting the therapeutic use of the drug. *Phyllanthus fraternus* is a potent medicinal herb used to treat various human diseases including renal disorders. Therefore, the aim of this study was to evaluate the protective effects of aqueous exrtact of *Phyllanthus fraternus* (AEPF) on the CPA-induced nephrotoxicity in mice. Twenty four adult Parke's strain mice were divided into four groups: group I given only distilled water (control); group II administered with CPA (200 mg/kg b.w., intraperitoneally) once in a week for five weeks; group III administered CPA along with AEPF (400 mg/kg b.w., orally) and group IV with AEPF alone once in a week for five weeks. Histopathological changes, markers related to renal activity like kidney somatic index (KSI), creatinine, catalase, superoxide dismutase and lipid peroxidation were assessed in mice of all the groups. CPA caused marked alteration in KSI, creatinine, antioxidant enzymes and lipid peroxidation level. The KSI decreased while creatinine increased significantly after CPA treatment. These changes were almost restored following co-administration of AEPF. This might be due to decreased activity of oxidative stress. Thus this study suggests that AEPF have nephroprotective effect against CPA-induced oxidative stress. AEPF may serve as a promising medicinal herb in complementary chemotherapeutic modalities.

Key words: Nephroprotective, *Phyllanthus fraternus*, cyclophosphamide, oxidative stress.

INTRODUCTION

Cyclophosphamide (CPA) is a drug with a wide spectrum of clinical uses. Its effectiveness in the treatment of cancer (lymphoma, acute and chronic leukemias, multiple myeloma) and non-malignant diseases such as rheumatoid arthritis and vasculitis (Dollery, 1999) has been well established. However, this drug also induces acute inflammation of the urinary bladder (Walker and Sommerkamp, 1998), renal damage (Kopecna, 2001) and liver damage (Shaunak *et al*., 1988; Gustafsson *et al*., 1996), thereby limiting its therapeutic use. CPA is extensively metabolized by cytochrome P450 pathways. The therapeutic and toxic effect of CPA is a requirement for bioactivation by hepatic microsomal cytochrome P450 mixed function oxidase system (Lindley *et al.,* 2002). Metabolic activation through the predominant pathway

yields 4-hydroxycyclophosphamide that exist in equilibrium with aldophosphamide, which is degraded by β-elimination to form phosphoramide mustard and an equimolar amount of the toxic byproduct, acrolein (Lindley *et al.,* 2002; Pass *et al.,* 2005). Phosphoramide mustard brings about interstrand cross-links between opposite DNA strands and hampers their replication and transcription process that characterize the clinical activity of CPA (Paolo *et al.,* 2004). The therapeutic effect of CPA is thus attributed to phosphoramide mustard and acrolein is associated with unwanted side effects (Colvin, 1999). Bioconversion of CPA into these metabolites leads to the formation of high level of reactive oxygen species (ROS), which results in decreased antioxidant activity (Stankiewicz *et al.,* 2002). Excessive production of ROS could also culminate in oxidative stress (Scherz-Shouval and Elazar, 2007). The cytotoxic metabolites formed in the liver are distributed to different tissues by systemic circulation. Oxidative stress has been reported to play a role in CPA-induced tissue damage (Haque *et al*., 2003; Manda and Bhatia, 2003).

Renal disorders have always been remained a major area of concern for physicians since a long time. It is the 9th leading cause of death in United States (Javaid *et al.,* 2012). Incidence of kidney diseases leading to kidney failure is increasing day by day. A large number of chemicals in common use are potential renal toxins (Hall, 2011). It is prime target of several drugs, toxic xenobiotics or chemicals. The high rate of blood flow (20%- 25% of cardiac output) and presence of cellular transport systems causes accumulation of these compounds within the nephron epithelial cells (Pfaller and Gstraunthaler, 1998). The kidneys are poised to sense plasma concentrations of ions like sodium, potassium, hydrogen and compounds such as amino acids, creatinine, bicarbonate and glucose, they are therefore important regulators of blood pressure, glucose metabolism and erythropoiesis. Nephrotoxicity is an inherent adverse effect of many antibiotics, anticancer drugs and other synthetic molecules (Javaid *et al*., 2012). Metabolites of the drugs excreted from the kidneys may also cause cellular damage leading to kidney dysfunction.

Kidney disease is one of the most common cause of hospitalization and has not only a significant morbidity, but a high mortality as well. The high costs and complexity of kidney disorder treatments, places a heavy financial burden on the society. Acute renal failure is a common and serious renal problem having high morbidity and mortality rate in most of the countries (Begum, 2006).

Plants have always been an exemplary source of drugs. Many of the modern drugs that are currently available have also been derived directly or indirectly from herbal sources. Herbal medicines have been proved to be highly effective, economical and safe alternative tools for the treatment of various human diseases. India harbors the richest plant-based medical traditions in the world. According to an estimate, there are around 25,000 effective plant-based formulations used as folk medicine in curing many ailments and diseases (Kirtikar and Basu, 1975). Many of such medicinal plants are known to the rural communities in India and they frequently use their varied herbal preparations as an alternative medicine (Singh and Sharma, 2013).

Phyllanthus fraternus (Bhui-amla) is an ancient Indian traditional phytomedicine used for treatment of several human diseases including hepatic and renal disorders. It belongs to the Euphorbiaceae family in plant division and an annual herb, distributed in India, Pakistan, South Arabia, Africa and the West Indies (Abedin *et al*., 2001). It is also widely used as a folklore remedy for the treatment of various diseases of liver by traditional healers and tribal people (Kirtikar and Basu, 1975).

As *P. fraternus* extract has long been used by tribals for the treatment of renal disorders, this present study is designed to investigate the protective effect of *P. fraternus* against CPA-induced renal dysfunction in mice.

MATERIALS AND METHODS

Drug and Chemicals

Cyclophosphamide was obtained from Sigma Aldrich Ltd., New Delhi, India. The other chemicals and solvents used were of the highest purity of analytical grade.

Preparation of Plant Extract

P. fraternus plants were collected from the campus of Banaras Hindu University, Varanasi, and was authenticated by Prof. N. K. Dubey, Department of Botany, Banaras Hindu University, Varanasi. A voucher specimen (Euphor./14/2013) has been kept in the herbarium for future reference. Fresh aerial parts of plants were washed under running tap water, dried in shade at room temperature for a week and powdered mechanically. The powder (100 grams) was added in 400 ml deionized water and after stirring was kept overnight at room temperature (25± 2˚C). The method was same as followed described by Faremi *et al*. (2008) for aqueous extraction. The mixture was then centrifuged at 5000 rpm to separate the supernatant. The filtrate was evaporated to dryness at 45 ˚C with a rotary evaporator. The dried extract powder was collected and stored in a refrigerator at 4°C for further use.

Animal Model

All the experiments were performed in accordance with institutional practice and within the framework of revised animals (Committee for the Purpose of Control and Supervision of Experiments on Animals; CPCSEA) Act of 2007 of Govt. of India on animal welfare. The study was conducted on adult male Parke's strain mice (30 ± 3 g), which were obtained from Department of Zoology, Banaras Hindu University, Varanasi, India. The animals were fed with commercially available standard mice pellet feed and water was provided *ad libitum*. The mice were housed under conditions of controlled temperature (25 ± 2 °C) and acclimatized to a 12 h light: 12 h dark cycle.

Experimental Design

The animals were randomly divided into four groups of six mice each as follows:

Group I. Control group mice: Received distilled water for 5 weeks (once in a week) by intraperitoneal injection.

Group II. Nephrotoxic bearing mice (CPA-induced group): Received CPA 200 mg/kg body weight (b.w.) for 5 weeks (once in a week) by intraperitoneal injection.

Group III. Treatment group: Received CPA (200 mg/kg, b.w., intraperitoneally and AEPF 400 mg/kg b.w. for 5 weeks (once in a week) orally.

Group IV. Extract receiving group: Received AEPF 400 mg/kg b.w. for 5 weeks (once in a week) orally.

After 5-week all the animals were killed by cervical dislocation. The kidneys were excised immediately, rinsed in ice-cold physiological saline, further homogenized in 0.2 M phosphate buffer saline and aliquots of this homogenates were used for the antioxidant assays.

Estimation of Biochemical and Antioxidants assay

In kidney samples, the level of lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT) activity and creatinine in serum were quantified using commercially available diagnostic kits (Sigma-Aldrich, New Delhi, India).

Histopathological Studies

The kidneys were fixed in Bouin's solution for 12 hours and then embedded in paraffin wax using conventional methods. Sections of 5-µm were prepared, stained with haematoxylin-eosin and mounted in diphenylxylene. These sections were observed under the research microscope for histopathological changes and useful photomicrographs were taken.

Statistical Analysis

The results were statistically analyzed and expressed as mean ± SEM (Standard error mean) for six animals in each group. Difference between the groups were assessed by one-way analysis of variance (ANOVA), using SPSS 16.0 software, followed by Dunnett's test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Effect of AEPF on renal antioxidant profile

The results relating the protective effect of AEPF against the toxic effect of CPA administration on activities of antioxidant enzymes such as SOD, CAT and

Malonyldialdehyde (MDA) are shown in Fig 1. MDA is an end product of peroxidation of polyunsaturated fatty acids and related esters and is a marker of lipid peroxidation (LPO). Activities of antioxidant enzymes such as SOD and CAT were found to have reduced ($P<0.001$) while MDA level increased significantly ($P<0.001$) in CPA-induced group against the control group. This reduction was reversed significantly in SOD enzyme activity ($P<0.001$) and CAT activity ($p<0.01$) by the treatment of AEPF. Level of renal MDA was also significantly decreased ($P<0.01$) by administration of AEPF to mice treated with CPA. Alteration in the renal enzyme activities with the treatment of AEPF (400 mg/kg b.w.) alone remained unchanged as compared to the control group.

Effect of AEPF on kidney somatic index (KSI)

Protective effect of AEPF against CPA administration to mice on KSI is shown in Fig. 2. Administration of CPA to mice significantly decreased ($P<0.001$) the KSI. Administration of CPA with AEPF consistently restored the percentage of the KSI as compared to the CPA treated group. AEPF alone did not cause any significant change in the KSI as compared to the control group.

Effect of AEPF on Creatinine

Serum concentration of creatinine is the marker test for injuries and dysfunction of kidneys. The effect of CPA administration on the creatinine concentration is presented in Fig 2. The treatment of CPA to mice significantly increased ($P < 0.001$) the serum level of creatinine as compared to the control group. Toxicity of CPA was reduced with the treatment of AEPF and the level of serum creatinine almost returned to that of the control group. Only AEPF treatment did not show change in creatinine level.

Effect of AEPF on renal histopathology

The histopathological observations on nephroprotective effect of AEPF extract on CPA-intoxicated mice are shown in Fig.3. The microscopical examination of kidney in the control group mice revealed normal renal glomeruli surrounded by Bowman's capsule (Fig. 3 A). Sections from the kidney of mice treated with CPA show decrease of Bowman's capsule space, congestion of glomerular capillaries, inflammatory infiltrates, tubular brush border loss and intertubular hemorrhage (Fig. 3 B). Histopathological lesions observed were mild in glomerulus and renal tubules of the kidneys treated with CPA + AEPF when compared with the CPA-treated group (Fig. 3 C). Alteration in the renal histopathology with the treatment of AEPF alone remained almost unchanged as compared to the control group of mice (Fig 3 D).

DISCUSSION

Phyllanthus fraternus modulates several biological functions and exhibits diuretic, antidiabetic, hypotensive, hypoglycemic, antihyperlipemic, antihepatotoxic and antioxidant activities due to its appreciable free radical-scavenging property (Kushwah *et al*., 2010). The nephroprotective activity of *P. fraternus* is probably due

to the presence of flavanoids (Lakshmi *et al.,* 2012; Talele et al., 2012). The present study on male parke's strain mice revealed that CPA treatment led to a decrease in the KSI whereas CPA + AEPF alleviate the toxicity. The animals treated with AEPF alone show an increase in KSI. Thus the observations made in KSI increase with CPA + AEPF suggest that, AEPF can alleviate CPA-induced toxicity in these animals.

Serum creatinine test is a useful method for evaluating function of kidneys. This study shows that CPA administration led to a marked increase in serum level of creatinine, whereas AEPF nearly restored CPA-induced toxicity and the levels of creatinine could be brought down that seen in the control group. Other researchers have also shown that different plant's extract can significantly reduce the renal injuries, induced through CPA intoxication (Senthilkumar *et al.,* 2006; Haque *et al*., 2003). The results suggested that AEPF prevented CPA-induced toxicity and that the levels of creatinine could be altered to those seen in control group.

Free radicals and reactive oxygen species mediate the propagation of peroxidation of polyunsaturated fatty acids. This cascade can be prevented through enzymatic and nonenzymatic antioxidants. Increased MDA concentration of renal tissues in CPA treated mice may be the result of an increase in oxidative stress. MDA, the final metabolite of peroxidized polyunsatured fatty acids (Ohkawa *et al*., 1979), considered as a late biomarker of oxidative stress (Draper *et al*., 1993; Kim *et al*., 2000; Dotan *et al*., 2004), not only translates ROS into active chemicals but also magnifies the function of ROS through the chain reaction, inducing alterations in cellular and functional impairment (Cheeseman, 1993) and serves to indicate the presence of free radicals and lipid peroxide formation (Banerjee *et al*., 2003; Hazarika *et al*., 2003).

There are a number of evidences implicating the existence of oxidative stress and reactive oxygen species with CPA-induced toxicity in animal models. The mean activity of antioxidant enzymes CAT, SOD were found to be significantly lowered in the CPA-treated group as compared to that of the control group. The lowered activities of these antioxidant enzymes in CPA treated mice have been reported by *in-vivo* experimental models (Shanmugarajan *et al*., 2008).

The increase in LPO, as assessed by the elevated levels of MDA following CPA administration has been well documented in the studies (Sai *et al*., 1992; Khan *et al*., 2000). Free radical-induced increase in LPO is accompanied by contemporaneous decline in the activities of cellular antioxidants. This may be owing to the inactivation of cellular antioxidants by lipid peroxides and ROS, which are produced due to CPA intoxication (Shanmugarajan *et al*., 2008). The CPA treated animals in this study, were also administered with AEPF, showed a decrease concentration in MDA level, which indicates the ameliorating effects of AEPF against the oxidative stress, induced by CPA in kidneys. The present study demonstrates the nephroprotective, curative and antioxidant effects of AEPF against CPA-induced kidney injury in mice.

Cytoarchitectural distortion in the renal histopathology with CPA-treated mice was found to be almost normal with CPA + AEPF and normal to that of control with

AEPF alone. The histopathological results in this study is in consonance with the reports of Ahmad *et al.* (2012), Yaman and Balikci (2010) and Kumar *et al.* (2001). Thus the nephroprotective effect of AEPF is also confirmed by histopathological examination of kidneys of control and treated mice. The results of this study indicate that aqueous extract of *Phyllanthus fraternus* have good potentials for use in kidney damage.

Therefore, this study, suggests the nephroprotective effect of AEPF against CPA-induced oxidative stress, which may serve as a promising medicinal herb in complementary chemotherapeutic modalities.

CONCLUSION

The protective role of aqueous extract of *Phyllanthus fraternus* was evaluated in this study. AEPF may contribute its protective effect against damaging activity of CPA. The protective potential may involve scavenging potential and antioxidant capacity to ameliorate the CPA-induced toxicity. This study substantiated the scientific evidence in favours of its pharmacological use in renal injuries. However further studies are essential to elucidate the exact mechanism of nephroprotector activity.

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FIGURE LEGENDS

Fig. 1 Protective effect of aqueous extract of *P. fraternus* on CPA- induced toxicity in the kidney. SOD, Superoxide dismutase; CAT, Catalse; LPO, Lipid peroxidation. Each bar represents the mean ±SE, n=6; $^{*}p<0.001$, $^{**}p<0.01$ indicate statistically significantly different from control group.

Fig. 2 Protective effect of aqueous extract of *P. fraternus* on CPA- induced toxicity in the serum. Kidney somatic index ; Creatinine. Each bar represents the mean ±SE, n=6; $^{*}p<0.001$, $^{**}p<0.01$ indicate statistically significantly different from control group.

Fig. 3 Representative photomicrographs of kidney sections (400X: A-D). The kidney section from control animals (Fig.3 A) showed normal renal glomeruli (G) surrounded by capsule. The kidney sections from the toxic mice (Fig. 3 B) showed decrease of Bowman's capsule space, congestion of glomerular capillaries, inflammatory infiltrates (C), tubular brush border loss and intertubular hemorrhage (D). The treatment of animals with CPA + AEPF at 400 mg/kg (Fig. 3 C) showed mild histopathological lesions in glomerulus and renal tubules of the kidney. Treatment of AEPF alone remained unchanged (Fig. 3 D).

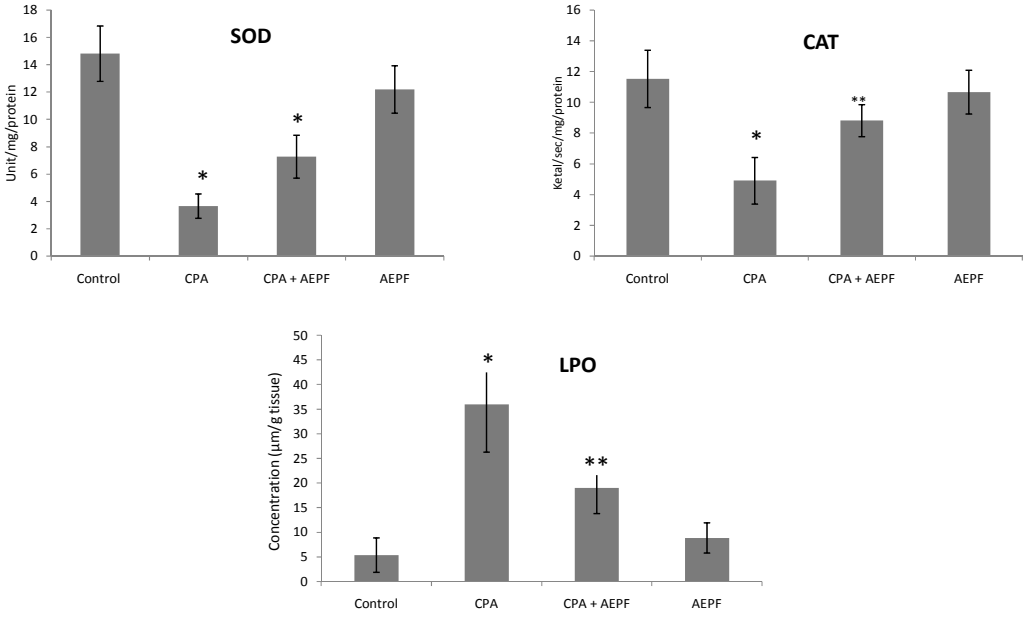

Fig. 1

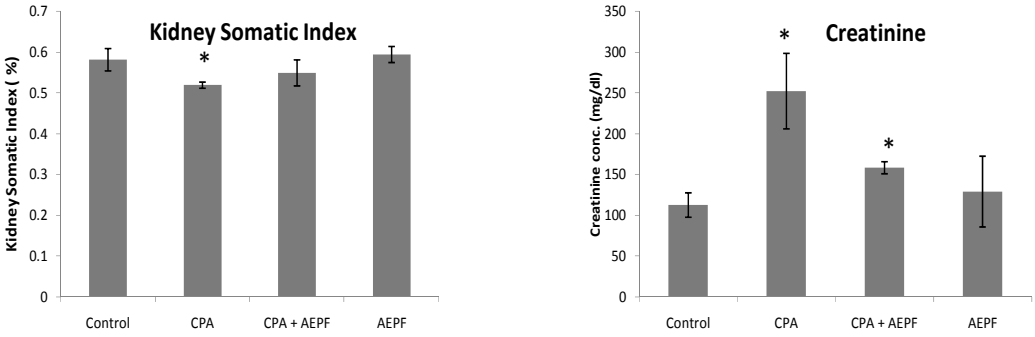
Kidney Somatic Index
Kidney Somatic Index (%)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control
CPA
CPA + AEPF
AEPF
Creatinine
Creatinine conc. (mg/dl)
350
300
250
200
150
100
50
0
*
*
Control
CPA
CPA + AEPF
AEPF

Fig. 2

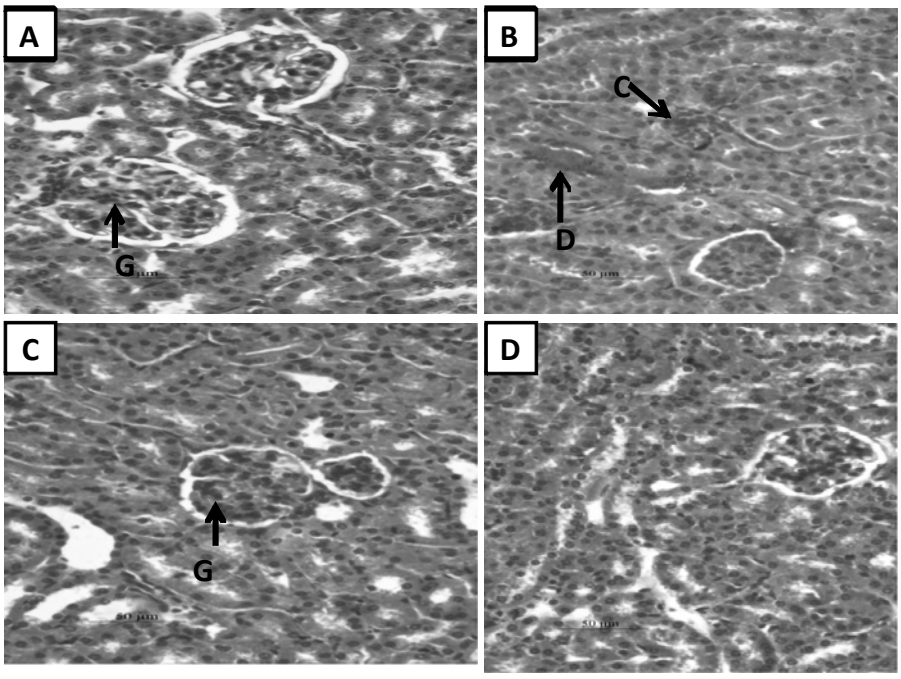
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Fig. 3