Nephroprotective Activity of Aqueous Extract of
Phyllanthus fraternus and Aegle marmelos against
Cyclophosphamide-Induced Toxicity

6.1. Introduction

The kidney is an essential excretory organ of body. It plays a dominant role in
homeostasis by excreting the metabolic waste products and excess necessary
substances. It conserves necessary products depending on the needs of the body (Hall
et al., 2010; Nivetha and Prasanna, 2014). 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 (Pandith 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 et al., 2010). It is prime target of several
drugs, toxic xenobiotics or chemicals. The kidney is 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 adverse effect of many antibiotics, anticancer drugs and other synthetic
molecules (Pandith et al., 2012). Metabolites of the drugs excreted from the kidney
may also cause cellular damage leading to kidney dysfunction.

CPA induces acute inflammation of the urinary bladder (Walker and Sommerkamp,
1998), renal damage (Kopecna, 2001; Singh et al., 2014) and liver damage (Shaunak
et al., 1988; Gustafsson et al., 1996), thereby limiting the therapeutic use of the drug
(Sugumar et al., 2007). The major limitation of cancer chemotherapy is the injury of
normal tissue, leading to multiple organ toxicity (Bukowski, 1996; Shokrzadeh et al., 2014). The nephrotoxicity of CPA was evidenced by the proximal tubular damage, a significant reversible depolarization and a decrease in conductance (Kleta et al., 1996; Rossi, 1997). 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).

Life without natural products is unimaginable. The traditional medicines, despite their limitations, are addressing the health needs of millions of people worldwide. It is estimated that about 65-85% of the world population use traditional medicines for their primary health cares. It is also estimated that about 39% of all 520 newly approved drugs developed in past in twenty years belong to natural products (Arumugama et al., 2008).

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 25000 effective plant-based formulations used as folk medicine in curing many ailments and diseases. 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 (Kirtikar and Basu, 1993). During the present study, we investigated the protective role of aqueous extract of *Phyllanthus fraternus* and *Aegle marmelos* in kidney against the damage caused by CPA-induced, the results are reported in this communication.
6.2. Materials and Methods

6.2.1. Animals and Treatment: Adult (age 12-15 weeks) male laboratory mice of Parke’s strain weighing 30 ± 3 gm were used in the study. Mice were divided into 12 groups (Group I to XII), each group comprising six animals. In a group, all animals had more or less identical body weight (bw). Mice of each group were housed separately in polypropylene cage (430 mm X 270 mm X 150 mm) and the treatment was done as shown below:

Group I  Control mice received distilled water (intraperitoneally) once in a week for 5 weeks.  
Group II  AEPF (400 mg/kg bw, orally) once in a week for 5 weeks.  
Group III AEAM (600 mg/kg bw, orally) once in a week for 5 weeks.  
Group IV Silymarin 100 mg/kg bw, for 5 weeks (once in a week) orally.  
Group V Received CPA 200 mg/kg bw, for 5 weeks (once in a week) by intraperitoneal injection.  
Group VI-VIII Received CPA (200 mg/kg bw, intraperitoneally) and AEPF 200, 300 and 400 mg/kg bw, orally for 5 weeks (once in a week).  
Group IX-XI Received CPA (200 mg/kg bw, intraperitoneally) and AEAM 400, 500 and 600 mg/kg bw, orally for 5 weeks (once in a week).  
Group XII Received CPA (200 mg/kg bw, intraperitoneally) and silymarin (well known standard nephroprotective drug) 100 mg/kg bw, orally for 5 weeks (once in a week).

After recording the body weight, animals were sacrificed by dislocation of cervical vertebrae. The kidney was excised, blotted free of blood and fixed in Bouin’s solution for histopathological analysis by H & E staining. Blood was collected and serum was separated for analysis of biochemical parameters. Details of the procedure of the biochemical estimation and histopathology are described in materials and methods.
6.2.2. Statistical Analysis: The results were expressed as Mean ± SE (SEM), analyzed through one-way ANOVA, followed by the post hoc Dunnett’s test for comparison of various treatments using the SPSS 16.0. Differences were considered statistically significant at $p<0.05$.

6.3. Results

6.3.1. Effect of AEPF and AEAM on the KSI, Serum BUN and Creatinine against CPA-Induced Toxicity in Mice: The effects of different doses of AEPF and AEAM on KSI, BUN and creatinine against CPA-induced nephrotoxicity of mice are shown in figure 6.1. Intoxication of CPA significantly ($p<0.001$) decreased the KSI of mice. Treatment of mice with different doses of AEPF and AEAM with CPA-treated mice restored the KSI, more marked effects were observed at the higher doses of extracts. CPA-induced the nephrotoxicity in mice with reference to increased serum BUN and creatinine levels in mice. The concentration of serum creatinine and BUN levels were significantly elevated ($p<0.001$) in the CPA-treated mice compared to the control group. Treatment to mice with both extracts significantly reduced the elevated levels of serum BUN and creatinine in dose related fashion. At low doses of AEPF (200 and 300 mg/kg) and AEAM (400 and 500 mg/kg), the effect was only marginal, whereas higher doses of AEPF (400 mg/kg) and AEAM (600 mg/kg) provided the best protection against CPA-induced nephrotoxicity. The silymarin, which was used as standard nephroprotective drug, with CPA treated group showed protection against CPA-induced nephrotoxicity. Mice treated with silymarin or a higher dose of extracts alone as well as co-treatment of silymarin and CPA subjected to the biochemical parameter analysis revealed that the values were near to the control group. Hence, the aqueous extract did not show any toxic effects on kidney.
6.3.2. Effect of AEPF and AEAM on the Kidney Antioxidants Activities against CPA-Induced Toxicity in Mice: Malondialdehyde (MDA) level is widely used as a marker of free radical mediated lipid peroxidation injury of the kidney. SOD and CAT are antioxidant enzyme and measured as an index of antioxidant status of tissues. As shown in Figure 6.2, MDA level of kidney was significantly increased ($p<0.001$) in the CPA-treated group compared to the control group. Treatment of mice with AEPF and AEAM significantly reduced the elevated MDA levels of kidney. SOD and CAT activities in kidney were lower in the CPA treated group as compared to the control. Treatment of the different doses of extracts significantly elevated ($p<0.001$) the level of SOD and CAT. At lower doses of AEPF (200 and 300 mg/kg) and AEAM (400 and 500 mg/kg), the effect was only marginal, whereas at the higher doses of AEPF (400 mg/kg) and AEAM (600 mg/kg), effectively prevented the CPA-induced kidney damage. Mice treated individually with AEPF, AEAM and silymarin showed near normal level of MDA, SOD and CAT comparable to the control group (Figure 6.2).

6.3.3. Histopathological Study of Kidney: Histopathological photomicrographs of kidney sections from various treatment groups are shown in Figure 6.3. In microscopical examination of kidney sections of the control group, mice revealed normal glomeruli surrounded by capsule (group I). However, treatment of extracts and silymarin alone also showed normal histopathology of kidney (group II to IV). The kidney section of CPA intoxicated group exhibited decrease of Bowman’s capsule space, congestion of glomerular capillaries, tubular brush border loss and intertubular haemorrhage (group V). Kidney sections from mice treated with different doses of extracts with CPA administration showed marked protection against CPA toxicity, characterized by regenerative changes in glomeruli cells (group VI to XI). The silymarin with CPA treated kidney shows histopathological protection against CPA renal tubular damage which was used as standard nephroprotective for comparison with the test component (group XII).
6.4. Discussion

Nephrotoxicity is an undesired side effect of chemotherapy. Most chemotherapeutic drugs target pathways that are essential to dividing cells (Onyemaechi et al., 2010). CPA is a potent drug used in the treatment of a wide range of cancer (Ratain and Plunkett, 1997). However, the severe toxic side effects are the major limitations in its usage. The importance of reactive oxygen metabolites in CPA-induced renal damage is now well documented (Nitha and Janardhanan, 2008).

The liver produces urea in the urea cycle as a waste product of the digestion of protein. If kidney is not functioning normally, these metabolites remain in the blood. BUN is an indicator of renal health. If glomerular filtration rate and blood volume decrease, then BUN will increase. The creatinine is a breakdown product of creatine phosphate in muscle. Serum creatinine is also an important indicator of renal health because it is an easily measured by-product of muscle metabolism and excreted unchanged by the kidney (Ueda et al., 2000). BUN and creatinine are removed from the blood by the kidney, primarily by glomerular filtration and via proximal tubular secretion. If the filtration in the kidney is deficient, the level of creatinine will increase in blood (Ueda et al., 2000). Therefore, creatinine levels in blood and urine may be used to calculate the creatinine clearance, which correlates with the glomerular filtration rate. In the renal disorders induced by CPA, renal tubular cells suffer cytotoxic injuries, ranging from mild sub-lethal changes to a necrotic death (Fishman et al., 2012). Increasing the serum levels of BUN and creatinine in current study may be because of nephrons damaging potential of CPA which has affected the glomerular filtration rate (Kos et al., 2013). Furthermore, decreasing the nephrotoxicity markers of kidney with extracts of AEPF and AEAM is the good references to nephroprotective potential of this plant against oxidative damage of kidney. The present results show that the AEPF and AEAM possessed significant protective effect against CPA-induced nephrotoxicity and the effect was found to be in a dose dependent manner.
During cellular respiration ROS are collectively produced via the sequential biological reduction of the molecular oxygen (Scandalios, 2002). Thus, the endogenous production of ROS is an unavoidable consequence of aerobic respiration (Touyz, 2000; Wilcox, 2002). The exogenous sources by which ROS are generated include exposure to cigarette smoke, environmental pollutants, consumption of alcohol in excess, exposure to ionizing radiation, etc (Gustafsson et al., 2000; Van Vleet and Schnellmann, 2003; Guelman. et al., 2005; Novitskiy. et al., 2006).

Oxidative stress has been defined as a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage (Sies, 1991). Thus, oxidative stress is imposed on cells as a result of two factors i) a reduction in antioxidant enzyme activity ii) an increase in the ROS. ROS may damage cell membrane (by reacting with lipids in cellular membranes), DNA, protein, tumor suppressor genes and enhanced expression of proto-oncogenes (Suji and Sivakami, 2006; Sicinska et al., 2006).

CPA-induces oxidative stress causing damage to intracellular organelles and alter their functions which lead to inhibition of protein synthesis, lipid peroxidation and mitochondrial damage (Meerson et al., 1982). Many plants and their products are reported to protect the body from deleterious effects of free radicals (Nair et al., 2001; Fylaktakidou et al., 2004; Nascimento et al., 2014).

In the present study, we found that CPA administration caused enhanced oxidative insult in the kidney tissue as evidenced by the increased renal MDA level and depletion in the activities of antioxidant enzymes such as SOD and CAT. These findings are attributed to the fact that, CPA is able to generate ROS and thereby inhibiting the activity of antioxidant enzymes. The increased ROS that attacks the cell membrane lipids leads to increased tissue lipid peroxides as manifested by increased MDA level. Over accumulation of lipid peroxides in tissue causes over consumption and depletion of SOD, CAT and inhibition of antioxidant enzymes. CPA caused degeneration in mice kidney due to oxidative stress (Senthilkumar et al., 2006).
Conclusive evidence demonstrates that supplementation with AEPF and AEAM protect against the free radical-mediated oxidative stress in kidney of animals suffering from CPA-induced injury. Thus, it appears that the orally administered AEPF and AEAM protects against CPA-induced toxicity possibly through the inhibition of increased MDA.

A major defence mechanism involves the antioxidant enzymes, including SOD and CAT, which convert active oxygen molecules into non-toxic compounds. Besides the increase in MDA levels, we found that CPA-treated mice showed a significant decrease in SOD activity. The decrease in SOD activity could be due to the increased production of ROS, as evident from the increased MDA levels after CPA treatment. However, these results have indicated that superoxide radicals can also inhibit CAT activity and the increased H₂O₂ levels resulting from CAT inhibition could finally inhibit SOD activity. Thus increased amount of MDA could be due to both increase in CPA-induced ROS formation and SOD, CAT inhibition (Gultekin et al., 2001). In contrast, plant extracts treatment were found to significantly increase SOD and CAT enzyme activity in the kidney, which could explain the decrease in LPO levels (Dasgupta et al., 2004; Zhang et al., 2014). Results suggest that administration of CPA significantly altered the status of the antioxidant defence system in the kidney. AEPF and AEAM treatment to the CPA administration restored the activities of all the cellular antioxidant enzymes as well as level of cellular metabolites. However, it is not clear how this plant extract increases SOD activity. Increased SOD activity by extracts possibly may be either enhanced enzyme transcription or decreased superoxide production.

These finding correlated with the renal histopathological examination revealed that, decrease of Bowman’s capsule space, congestion of glomerular capillaries, tubular brush border loss and intertubular haemorrhage in the CPA treated group. At higher doses of AEPF and AEAM kidney showed almost normal architecture of glomeruli and tubular epithelial cells as similar to that of control group and standard drug (silymarin) treated group.
In animals received AEPF and AEAM alone, showed no change in serum markers and antioxidant enzyme levels as compared to control group. These results are also supported by histopathological studies. Thus it is suggested that AEAM does not show any deteriorative effect on kidney.

The nephroprotective activity of extracts has been compared against CPA-induced toxicity in kidney. Although both extracts were found with marked level of protection but AEAM had shown better efficacy than AEPF, as depicted by its level of percentage protection (Table 6.1).

6.5. Conclusion

CPA administration caused oxidative insult in the kidney. AEPF and AEAM treatment to the toxin administration normalizes that stress in the kidney. The results of this study indicate that extracts have good potentials for use in kidney damage.
Figure 6.1: Protective effect of aqueous extracts of *P. fraternus* and *A. marmelos* in KSI, serum BUN and creatinine against CPA-induced nephrotoxicity. 'a' indicates the significant difference between the control and CPA treated groups, 'b' indicates the significant difference between the CPA treated and AEPF and AEAM treated groups. Each bar represents the Mean ±SE, n=6.

CPA + P: Cyclophosphamide + Aqueous extract of *P. fraternus*
CPA + A: Cyclophosphamide + Aqueous extract of *A. marmelos*
CPA + SIL: Cyclophosphamide + Silymarin
Figure 6.2: Protective effect of aqueous extracts of *P. fraternus* and *A. marmelos* in LPO, SOD and CAT against CPA-induced nephrotoxicity. ‘a’ indicates the significant difference between the control and CPA treated groups, ‘b’ indicates the significant difference between the CPA treated and AEPF and AEAM treated groups. Each bar represents the Mean ±SE, n=6.

CPA + P : Cyclophosphamide + Aqueous extract of *P. fraternus*

CPA + A : Cyclophosphamide + Aqueous extract of *A. marmelos*

CPA + SIL: Cyclophosphamide + Silymarin
Figure 6.3: Representative photomicrographs of kidney sections (40X: A-H). The kidney sections from control animals showed normal renal glomeruli surrounded by capsule (group I). In the AEPF, AEAM and silymarin alone treated kidney showed normal renal glomeruli (group II-IV). The kidney sections from the toxic-induced mice (group V) showed decrease of Bowman’s capsule space (arrow), congestion of glomerular capillaries (arrow), tubular brush border loss (+) and intertubular haemorrhage (star). The treatment of animals with CPA + AEPF and AEAM showed mild histopathological lesions in glomerulus and renal tubules of the kidney (group VI-XI). CPA + silymarin treated kidney showed normal renal glomeruli surrounded by capsule (group XII).
Table 6.1: Percentage protection of various biochemical parameters and antioxidants activities after treatment with aqueous extracts of *P. fraternus* and *A. marmelos* against CPA-induced nephrotoxicity in mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th><em>P. fraternus</em></th>
<th><em>A. marmelos</em></th>
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<tbody>
<tr>
<td>KSI</td>
<td>12.06</td>
<td>12.06</td>
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<tr>
<td>BUN</td>
<td>69.15</td>
<td>75.67</td>
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<tr>
<td>Creatinine</td>
<td>67.30</td>
<td>67.84</td>
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<td>MDA</td>
<td>8.63</td>
<td>51.74</td>
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<tr>
<td>SOD</td>
<td>44.77</td>
<td>68.37</td>
</tr>
<tr>
<td>CAT</td>
<td>6.70</td>
<td>49.54</td>
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