5.1. Introduction

Natural products have been shown to be an excellent and reliable source for the development of new drugs (Newman and Cragg, 2007). Epidemiologic studies have showed that there is a association between the intake of phenolic foods and the protection from various diseases (Morton et al., 2000). These phenolic compounds have tremendous antioxidant and chemoprotective properties in vivo (Zhao et al., 2004). Phenolic compounds are the most promising anticarcinogenic agents in plants (Ren et al., 1997). EA has been reported to inhibit peroxynitrite-induced oxidation and nitration reactions (Ippoushi et al., 2009). EA is a potent dietary antioxidant found in variety of fruits, nuts and many other food sources.

EA is reported to exhibit antioxidant, antiproliferative, radical scavenging, antiapoptotic, antiatherogenic, antiasthmatic, estrogen receptor modulator and chemopreventive, in a variety of tissues and cells. It has been also shown to inhibit cancer caused by several types of chemical carcinogens including polycyclic aromatic hydrocarbons, N-nitrosamines, aflatoxin B1, and aromatic amines (Hannum, 2004; Heur et al., 1992; Mandal et al., 1987; Papoutsi et al., 2005; Yuce et al., 2007; Turk et al., 2008; Yu et al., 2005; Atessahin et al., 2007; Jung et al., 2010; Rogerio et al., 2008). EA also finds application in cosmetology as a skin whitening agent as it inhibits radiation induced melanogenesis. EA contains four hydroxyl groups and two lactone groups in which hydroxyl group is known to increase antioxidant activity in lipid peroxidation and protect cells from oxidative damage (de Boer et al., 2004) and few more reported that EA decrease hepatic cytochrome P450 activity and to increase the activities of several phase II enzymes (Ahn et al., 1996; Shepherd et al., 2000; Van der Logt et al., 2003).

Cyclophosphamide (CPM) is a well known bi-functiona alkylating agent widely used in cancer chemotherapy and expresses its genotoxicity when metabolically activated (Baumann and Preiss, 1973; Fleming, 1997). It is extensively used for the treatment of various cancers as well as an immunosuppressant in organ transplantation, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and other benign diseases (Perini et al., 2007; Uber et al., 2007). According to The International Agency for Research on Cancer (IARC) CPM is widely used as reference mutagen and has been classified as carcinogenic for animals and humans (IARC, 1987). Reactive Metabolites of
CPM chemically alkylates DNA as well as protein, producing cross-links which are responsible for its cytotoxic effect (Hales, 1982). Normal tissues injury or damage is the major limitation of using CPM, which gives rise to numerous side effects, CPM treatment also results in the production of reactive oxygen species (ROS), which cause peroxidative damage to Kidney and other vital organs (Patel, 1987). Antineoplastic and toxic effects like necrosis, apoptosis and oncosis of CPM are linked with two active metabolites i.e, phosphoramidase and acrolein. Further Tripathi and Jena (2008) reported CPM to be toxic in germ cells of mice, it has also been reported that CPM treatment for non-Hodgkin’s lymphoma leads to the induction of secondary cancers in bladder and kidney (Travis et al., 1995).

The important factor for therapeutic and toxic effects of cyclophosphamide is the requirement of metabolic activation by hepatic microsomal cytochrome P₄₅₀ mixed functional oxidase system (Sladek, 1971 and 1988). On activation CPM generates active alkylating metabolites such as 4-hydroxycyclophosphamide, acrolein and aldophosphamide mustard, which hamper with cellular DNA synthesis in fast dividing cells and ultimately lead to cell death (Roy et al., 1999). Bagley and Bostick, (2001) have reviewed the toxicity and pharmacology of cyclophosphamide.

In the present investigation, we have made an attempt to investigate the beneficial effects of EA against CPM-induced nephrotoxicity and genotoxicity. The experimental end points for nephrotoxicity included biochemical estimation of antioxidant enzymes and Histopathological measurement for the determination of oxidative stress. Genotoxicity end points included evaluation of micronuclei (MN), DNA fragmentation and alkaline unwinding assay in terms of DNA strand breaks. Our results reveal that EA decreased the CPM-induced oxidative stress and subsequent oxidative DNA damage and genotoxicity in Swiss albino mice.

5.2. Results

5.2.1. Effects of pretreatment of EA on renal glutathione, its dependent enzymes and antioxidant enzyme system

Table 1 shows that CPM administration leads to significant depletion of renal GSH (p<0.001). In addition there was marked inhibition of GR enzyme by (p<0.001), GST
5.2.2 Effects of pretreatment of EA on renal toxicity marker enzymes

The effect of EA administration on CPM-mediated leakage of kidney toxicity markers (BUN, LDH and creatinine) were shown in Figure 1-3. Figures 1 and 2 showed that mice treated with CPM showed a significant increase in BUN (P < 0.001) and creatinine (P < 0.001) levels when compared with control. On treatment with EA marked inhibition was observed in BUN at both the doses (P < 0.001) (Figure 1) and in the creatinine level at both the doses (P < 0.01) (Figure 2). Similar type of results was observed for LDH levels (Figure 3). No significant difference was found in the only D2 group compared with control.

5.2.3 Effects of EA pretreatment on genotoxicity

Table 2 shows that there was higher induction of micronuclei in CPM treated group (p<0.001) and this level was decreased by prophylactic treatment of EA at low dose (p<0.05) and more effectively at higher dose (p<0.001). DNA damage is estimated in terms of smearing and lack of intact band control, only the EA treated groups at both doses showed less smearing and an intact band was also observed (Table 2). Similarly, in the DNA alkaline unwinding assay, a simultaneous significant decrease in F-value (p<0.001) on treatment with CPM was noted as compared to control group, whereas results indicate that there was significant increase in the F-value at both the doses of EA (p<0.05, p<0.001). The estimated PCE: NCE ratio in bone marrow preparations in the...
Table 2 shows a statistical decrease (p<0.001) in haematopoiesis as a result of CPM treatment as compared to control. The pretreatment of EA at both the dose significantly increased the PCE: NCE ratio, indicating reversal of the cytotoxic effects caused by CPM administration to mice. In the Figure 4, results indicate that there was significant DNA fragmentation only in the toxicant group as compared to the control group while there was less fragmentation in EA pre-treated groups. EA treatment restored the DNA integrity.

5.2.4. Histopathological Examination

The control mice showed normal glomerular and tubular histology while cyclophosphamide administered mice were found to have necrosis and desquamation of epithelial cells, peritubular and glomerular congestion and showed invasion of inflammatory cells in the cortical and medullary regions of kidneys. The nephrotoxicity was characterized by widespread degeneration of tubular architecture, tubular congestion, swelling and necrosis. In comparison, renal sections obtained from mice that were pre-treated with EA at a dose of 100 mg/kg body weight demonstrated marked reduction of the histological character of renal injury. Prophylactic treatment of EA (50 mg/kg b.wt.) was also associated with a significant reduction in injury, but this reduction was less marked than in the higher dose EA pretreatment groups (Figure 5).

5.3. Discussion

The kidney is the target of many xenobiotics toxicants, including drugs. There are many factors that contribute to the sensitivity of the kidney viz., presence of variety of metabolizing enzymes and xenobiotic transporters, large blood flow and concentration of solutes during urine production. Further physiological, anatomical and biochemical features of the kidney make it particularly sensitive to many toxins and drugs. Present investigation was carried out with the aim of evaluating the possible role of EA in modulating the in vivo genotoxicity and oxidative renal injury of cyclophosphamide. The active metabolites of CPM phosphoramide and Acrolein slow down the growth of cancer cells as they interfere with the cellular DNA.

Hydroxycyclophosphamide and deschloroethyl cyclophosphamide are the two intermediate compounds that lead to the formation of phosphoramide, the main agent
responsible the mutagenic effect of CPM (Ren et al., 1997). Phospharamide causes the induction of crosslink and strand lesion in DNA (Hengstler et al., 1997). Studies have indicate that CPM has a pro-oxidant nature, and production of oxidative stress after CPM administration leads to decrease in the activities of antioxidant enzymes and increase in lipid peroxidation in serum and different tissues of mice and rats (Haque et al., 2003; Selvakumar et al., 2005). In our study also CPM treatment resulted in significant increases (P < 0.001) in the renal MDA with decrease in GSH (free thiols).

GSH and its oxidized counterpart glutathione disulphide represent a major redox buffer system of the cell. GSH can act either as a non-enzymic antioxidant by direct interaction of –SH group with ROS or it can be implicated in the enzymatic detoxification reaction for ROS, as a cofactor or coenzyme. The depletion of GSH content may be attributed to the direct conjugation of CPM and its metabolites with free or protein bound –SH groups and significant decreases (P < 0.001) in the GSH-Px and CAT activity in comparison to the control levels (Table 1). Moreover, Cooper et al.,(1986) Kehrer and Biswal, (2000) suggested that CPM treatment results in inflammation thus disturbing the overall renal redox cycling. CPM was also shown to cause decrease levels of other antioxidant enzymes like glutathione reductase and glutathione-S-Transferase. EA dose dependently restored the level of all the renal antioxidant enzymes to normal and this phenomenon was in agreement with the previously published reports by Tripathi and Gena (2009) and Bhatia et al., (2006). Simultaneously the EA pre-treatment also restored the depleted levels of GGT and XO in these mice.

The marked increase in the levels of serum BUN and creatinine is a marker for the nephrotoxicity and kidney damage Cagler et al., (2002). CPM also induced renal damage characterized by increases in the level of LDH, BUN and creatinine. CPM-induced elevations in the levels of LDH, BUN and creatinine observed in our study are in agreement with the previously published reports by Cagler et al. (2002). CPM administration leads to elevated levels of serum toxicity markers of the Kidney (BUN, Creatinine and LDH), that is, index of renal dysfunction. The elevation in the activities of these enzymes in the serum might be due to the leakage of these cytosolic enzymes into the circulatory system resulting from kidney damage after CPM administration. This is
indicative of the onset of renal damage due to kidney dysfunction and disturbance in the biosynthesis of these enzymes, with alteration in the membrane permeability. Administration with EA prevented Cyclophosphamide-induced renal toxicity, as indicated by a steep decrease in serum BUN, creatinine levels and LDH activity, possibly by maintaining the renal cellular membrane integrity. This is an indicator of possible nephro-protective efficacy offered by EA compared with the untreated and CPM-intoxicated groups.

Histopathological studies also provided supportive confirmation for the biochemical parameters depicted by the photomicrographs. Kidney tissue (Fig.5) of CPM-administered rat shows tubular necrosis and desquamation of epithelial cells, invasion of inflammatory cells in the cortical and medullary regions of kidneys was also prominent. Lumen of tubules was observed to have collection of eosinophilic granular material. Another characteristic feature of CPM induced toxicity is tubular renal epithelial cell hypertrophy which was observed in CPM-treated group and was in agreement with previously published report by Abrahama et al., (2007). The key histological finding of this study was that EA pretreatment influenced the recovery of kidney architecture induced by CPM.

Experimental evidences have strengthened the concept that, DNA damage plays a crucial role in the initiation and subsequent promotion of carcinogenesis induced by different genotoxicants. Errors in the DNA molecules cause chromosomal aberrations. Micronuclei are well characterized biomarkers of structural and numerical chromosomal damage. The micronuclei in young erythrocytes arise mainly from chromosomal fragments that are not incorporated into the daughter nuclei at the time of cell division in the erythropoietic blast cells (Salamone and Heddle, 1983). Decrease in the PCE:NCE ratio is responsible for the induction of bone marrow cytotoxicity (Al-Majed et al., 2006). Results of our study reveal that CPM treatment shows cytotoxicity and has highly clastrogenic nature. CPM was found to induce micronuclei formation as it decreased PCE:NCE ratio and EA effectively brought this PCE: NCE ratio to the normal level. CPM also results in the formation of DNA strand breaks as a result of interaction of acrolein with DNA and cause DNA fragmentation due to necrosis and MnPCEs formation.
Prophylactic treatment of EA significantly and dose dependently restores normal level all these hallmarks of genotoxicity.

The present study shows the protective effect of EA against CPM induced genotoxicity and nephrotoxicity. In conclusion our study provides evidence that EA pretreatment attenuates CPM-induced oxidative stress and the subsequent DNA damage in mice. The anti-genotoxic potential of EA might be due to its antioxidant property. Future investigation targeting molecular pathways involved in modulatory action of EA on CPM induced genotoxicity and nephrotoxicity are needed.
Table 1 Results of pretreatment of Ellagic acid on MDA formation, antioxidant and related enzymes like GSH, GST, GR, GPX, Catalase, XO and -GGT on Cyclophosphamide induced renal redox imbalance

<table>
<thead>
<tr>
<th>Treatment regimen per group</th>
<th>GSH (n mol GSH /g tissue)</th>
<th>GST (n mol CDNB conjugate formed/min/mg protein)</th>
<th>GR (n mol NADPH Oxidized/min/mg protein)</th>
<th>GPX (n mol NADPH Oxidized/min/mg protein)</th>
<th>Catalase (nmol H2O2 consumed/min/mg protein)</th>
<th>MDA (nmol MDA formed /g tissue)</th>
<th>XO (µg of uric acid formed/min mg protein)</th>
<th>-GGT (nmoles p nitroaniline formed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (control)</td>
<td>0.65±0.04</td>
<td>130.4±10.23</td>
<td>219.9±8.42</td>
<td>280.0±14.0</td>
<td>151.9±6.98</td>
<td>2.83 ± 0.32</td>
<td>0.206±0.01</td>
<td>488.0 ± 18.6</td>
</tr>
<tr>
<td>Group II (CPM only)</td>
<td>0.26±0.03***</td>
<td>42.10±2.62**</td>
<td>102.6±2.82***</td>
<td>141.2±6.73***</td>
<td>54.51±4.40***</td>
<td>5.76 ± 0.11***</td>
<td>0.480 ± 0.03***</td>
<td>836.2 ± 10.9***</td>
</tr>
<tr>
<td>Group III (CPM + EA D-1)</td>
<td>0.43±0.03**</td>
<td>93.65±9.10**</td>
<td>138.0±11.4**</td>
<td>194.9±6.43**</td>
<td>118.2±3.26**</td>
<td>4.30 ± 0.36**</td>
<td>0.340 ± 0.01**</td>
<td>681.7 ± 42.3**</td>
</tr>
<tr>
<td>Group IV (CPM + EA D-2)</td>
<td>0.51±0.03***</td>
<td>104.2±5.98**</td>
<td>180.5±23.3**</td>
<td>230.8±19.6**</td>
<td>96.58±6.09**</td>
<td>3.13 ± 0.18**</td>
<td>0.30 ± 0.00**</td>
<td>569.8 ± 18.5**</td>
</tr>
<tr>
<td>Group V (only EA D2)</td>
<td>0.63±0.04</td>
<td>129.9±9.46</td>
<td>202.5±7.22</td>
<td>202.1±4.67</td>
<td>148.7±0.02</td>
<td>2.74 ± 0.36</td>
<td>0.25 ± 0.04</td>
<td>484.1 ± 20.7</td>
</tr>
</tbody>
</table>

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 Cyclophosphamide treated group (#P < 0.05), (##P < 0.01), (ns P= not significant) and (###P<0.001). EA= Ellagic acid; D1= 50mg/kg/b wt; D2 = 100mg/kg/b wt.
Table 2  Results of modulatory effect of Ellagic acid on Genotoxicity Markers (MnPE/100 PCE, PCE/NCE, DNA strand Breaks) on CPM induced damage.

<table>
<thead>
<tr>
<th>Treatment regimen per group</th>
<th>MnPE / 100 PCE</th>
<th>PCE/NCE</th>
<th>DNA strand Breaks (F-Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (control)</td>
<td>4.80±0.86</td>
<td>1.26±0.04</td>
<td>0.87±0.03</td>
</tr>
<tr>
<td>Group II (CPM only)</td>
<td>20.4±1.28***</td>
<td>0.70±0.04***</td>
<td>0.37±0.01***</td>
</tr>
<tr>
<td>Group III (CPM + EA D-1)</td>
<td>15.4±0.87#</td>
<td>0.90±0.03#</td>
<td>0.51±0.03#</td>
</tr>
<tr>
<td>Group IV (CPM + EA D-1)</td>
<td>9.2±1.85###</td>
<td>1.03±0.05###</td>
<td>0.76±0.02###</td>
</tr>
<tr>
<td>Group V (only EA D2)</td>
<td>3.2±0.80</td>
<td>1.27±0.05</td>
<td>0.87±0.02</td>
</tr>
</tbody>
</table>

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 Cyclophosphamide treated group (#P < 0.05), (##P < 0.01), (ns P= not significant) and (###P<0.001). EA= Ellagic acid; D1= 50mg/kg/b wt; D2= 100mg/kg/b wt. PCEs, polychromatic erythrocytes; NCEs, normochromatic erythrocytes; MnPEs, micronucleated polychromatic erythrocytes.
Figure 1 Effect of pre treatment of Ellagic acid on cyclophosphamide induced increase in serum blood urea nitrogen level (BUN):

![Graph showing BUN levels for different treatment groups.](image)

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 Cyclophosphamide treated group (#P < 0.05), (##P < 0.01), and (###P<0.001). EA= Ellagic acid; D1= 50 mg/kg/b wt; D2 = 100mg/kg/b wt.
Figure 2 Effect of pre treatment of Ellagic acid on cyclophosphamide induced increase in serum creatinine level:

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 Cyclophosphamide treated group (#P < 0.05), (##P < 0.01), and (###P<0.001).EA= Ellagic acid; D1= 50 mg/kg/b wt; D2 = 100mg/kg/b wt.
Figure 3 Effect of pre treatment of Ellagic acid on cyclophosphamide induced increase in serum Lactate Dehydrogenase level:

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 Cyclophosphamide treated group (#P < 0.05), (##P < 0.01), and (###P<0.001). EA= Ellagic acid; D1= 50 mg/kg/b wt; D2 = 100mg/kg/b wt.
Figure 4 Agarose (1.5%) gel electrophoresis of DNA obtained from mice Kidney

Lane 1-control; Lane 2- CPM only; Lane 3- EA 1+CPM; D1; Lane 4-EA 2+CPM; D2; Lane 5- EA D2 only. From gel picture, it is marked that CPM treatment introduced DNA fragmentation by smearing of DNA when compared to control. There is decrease in DNA smearing as a result of EA pretreatment at both the doses in mice kidney.

Cyclophosphamide – CPM, Ellagic acid – EA; D1 = 50 mg/kg b.wt. D2 = 100 mg/kg b.wt.
Figure 5 Photomicrographs of Kidney histology of Swiss albino mice (400X magnifications):

(A) Kidney section from control group shows normal renal histo-architecture. (B) Kidney section from only CPM treated group shows loss of normal renal architecture, inflammatory cells infiltration, and fatty changes with cell swelling. (C) Kidney section of the EA pretreated groups at lower dose of EA (50 mg/kg b.wt.) moderately prevented the cytotoxic damage induced by CPM; as indicated by moderate swelling of the hepatic cells. (D) Liver section of the EA pretreated groups at higher dose of EA (100 mg/kg b.wt.) restored the morphology of the kidney from the damage induced by CPM. (E) Higher dose of EA administration did not showed any kind of histological abnormalities in the kidney tissue. cyclophosphamide – CPM, EA, Ellagic acid; D1= 50 mg / kg b. wt. D2 = 100 mg / kg b. wt. (40x magnification)