Canadian Journal of Pure and Applied Sciences
Vol. 10, No. 2, pp. 3835-3845, June 2016
Online ISSN: 1920-3853; Print ISSN: 1715-9997
Available online at www.cjpas.net

EFFECT OF *GINKO BILOBA* EXTRACT ON CYCLOPHOSPHAMIDE-INDUCED NEPHROTOXICITY AND OXIDATIVE STRESS IN ALBINO RATS

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ABSTRACT

Cyclophosphamide (CP) is an anticancer drug utilized for treatment of many types of cancer. Botanical remedies became a promising tool for alleviation of side effects of many drugs. Among these natural plants is *Ginko biloba* (GB) that possesses therapeutic efficiency against many diseases. The present work studied the effect of GB extract on CP-induced nephrotoxicity in albino rats. Adult male rats were divided into 4 groups; control, GB-administered, CP-treated, and CP plus GB exposed groups. Treating rats with CP for 4 weeks caused renal veins congestion, intertubular leucocytic infiltrations, tubular epithelium degeneration and atrophied glomeruli. Treatment with CP+ GB showed an improvement in the structure of kidney cortex. Histochemical results showed that treatment with CP induced extreme decrease in total carbohydrates and proteins while treatment with CP and EGB restored these components in the majority of the cells. Immunohistochemically, an increase in α-SMA expression was induced by CP whereas GB alleviated elevation of α-SMA caused by CP. Serum creatinine, urea and lipid peroxidation marker, MDA were significantly elevated by CP treatment while there was a decrease in SOD and CAT. Treatment with CP+GB restored the normal values of these parameters. In conclusion, *Ginko biloba* extract exhibited protection against cyclophosphamide-induced renal toxicity in albino rats.

Keywords: *Ginko biloba*, cyclophosphamide, rats, kidney, histology, histochemistry, immuno histochemistry, oxidative stress.

INTRODUCTION

Application of antineoplastic agents in cancer treatments poses toxic impacts on healthy cells, such as those of the gastrointestinal, hematopoietic and reproductive systems, alongside on tumour cells. Cyclophosphamide (CP) is a nitrogen mustard alkylating agent from the oxazophorines group beneficial in the treatment of many neoplastic ailments including Hodgkin's sickness, lymphomas, leukemia, Wegener's granulomatosis, in addition to a variety of bone and soft tissue sarcomas (Avendano and Menendez, 2008). However, treatment with CP was accompanied with numerous deleterious effects such as counting hemorrhagic cystitis, alopecia and skin hyperpigmentation (Sweetman and Martindale, 2007). Metabolism of CP could lead to the formation of several kinds of cytotoxic metabolites that might induce oxidative stress and cause the hepatotoxicity (Li *et al*., 2010). Another study, Kim *et al*. (2014) found that CP caused urotoxicity in rats evidenced by significant increases in bladder weight, edema, hemorrhage as well as increased urinary bladder epithelial cell apoptosis, protein expression of nuclear factor erythroid 2-related factor-2 (Nrf-2) and phase II enzymes. The two active metabolite of CP is phosphoramide mustard and acrolein. Acrolin is the highly reactive aldehyde extensively damages kidney and urinary bladder cells (Yousefipour *et al*., 2005). It was reported that CP induced glomerular and tubular dysfunction, glomerular proteinuria, tubular proteinuria, decreased glomerular filtration rate and decrease in concentration function (Senthilkumar *et al*., 2006; Sugumar *et al*., 2007).

Meanwhile, a large number of patients depend on botanical remedies to relief their pain and cure their illness and this number is on rising (Jalili *et al*., 2014). The unavoidable tedious pharmaceutical preparation raised vigilant towards benefits of the herbal medicines (Girish and Pradhan, 2008). Accordingly, herbal medicines derived from plant extracts are used to treat a wide variety of diseases. As has been cited by Ishita *et al*. (2004), plants were applied by herbalists and indigenous healers globally for many purposes such as blood purification, digestion, an anti-inflammatory and hepatoprotective agents. Nowadays, phyto components have acquired golden share attention among scientists owing to efficiency against different ailments such as cancer therapy and prevention (Subbaraj *et al*., 2013).

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Ginko biloba (GB), one of the oldest tree species has been used for many centuries as a traditional Chinese herbal medicine (Clostre, 1986) due to its ability to exhibit a variety of pharmacological properties. It is used for treatment of cerebral insufficiency, inflammation, Alzheimer's disease and diabetes complications (Lu *et al*., 2015) and certain peripheral vascular diseases (Diamond *et al*., 2000). In spite of isolating more than 40 components from Ginkgo leaves, mainly two are responsible for its medicinal effects; flavonoids and terpenoids (Bridi *et al*., 2001). The therapeutic mechanisms of action of Ginkgo leaf extract seems to be through its antioxidant, antiplatelet, antihypoxic, antiedemic, hemorrheologic and microcirculatory actions where flavonoid and terpenoid fractions work in a complementary manner (Mahadevan and Park, 2008). GB leaves extract contains 24 - 25% flavonoid glycosides and 6% terpenoids (Shen *et al*., 1998) possess different pharmacological activities including anticancer, neuroprotective, stress alleviating, cardioprotective, memory enhancing effects and possible effects on tinnitus and psychiatric disorders (Chao and Chu, 2004; Yang *et al*., 2005). GB leaves extract was shown to have hepatoprotective effect against thioacetamide (Al-Attar, 2012) and CCl_4 (Shenoy *et al*., 2001). Tang *et al*. (2009) reported that GB extract has a protective effect against glomeruloscleroses in diabetic nephropathy of mesangial cells. In the present work, the protective effect of GB leaves extract against CP-induced nephrotoxicity in albino rats was evaluated.

MATERIALS AND METHODS

Experimental animals, adult male Sprague-Dawley rats weighing 150±5g were accommodated in special plastic rodent cages and housed in a well ventilated animal room under standard conditions of 24°C temperature, 50% relative humidity and 12 hr light/12 hr dark cycles. Rodent diet and water were provided *ad libitum*. Rats were acclimatized to the laboratory environment for one week prior to experimentation. The study and all procedures were approved by the Animal Care and Bioethics Committee, Menoufia University, Egypt.

In this study, animals were divided into four groups. Group 1 control; Group 2, 10 rats were orally given 100 mg/kg b w/day EGb for four weeks. *Ginkgo biloba* leaves extract (EGb) was obtained in the form of Tanakan (EGb761) produced by Amriya for Pharmaceutical Industries, Egypt under license of Beaufour-Ipsen International, Paris, France. Group 3, 10 rats were orally given aqueous CP at a dose level 6.5 mg/ kg b w/ day (equivalent to the therapeutic human dose, Paget and Barnes, 1964) for four weeks. CP was obtained in the form of powder (Endoxan injection vial) manufactured by Baxter Oncology, Germany; Group 4, in this group, 10 rats were orally given CP (6.5 mg/kg b w) then after 2 hours they were orally given EGb (100 mg/kg b w) daily for four weeks.

Histological and histochemical investigation

Animals of different groups were dissected out at the end of the experiment and their kidneys were washed in normal mammalian saline, fixed in 10% phosphate buffered formalin (pH 7.4). Fixed materials were embedded in parablast (M.P. 56) and sections of 5µ thickness were cut using rotary microtome. Slides were stained with haematoxylin and counterstained with eosin for histological examination. For histochemical demonstration of total carbohydrates, periodic acid Schiff's technique (PAS) was applied (Kiernan, 1981). Total proteins were demonstrated following mercury bromophenol blue method of Pearse (1972). Sections were dehydrated, cleared and mounted in DPX.

Immunohistochemical study

Avidin-biotin peroxidase method was used for the immunohistochemical demonstration of alpha-SMA. Formalin-fixed affixed- slide sections were deparaffinized and blocked for endogenous peroxidase with 1.75% hydrogen peroxide in methanol for 20 min, antigen retrieval for 15 min using Biogenex Antigen Retrieval Citra solution in 90°C water bath for 30 min. Slides were left to cool for 20 min then blocked by normal horse serum for 5 min at 37°C. The monoclonal antibody was applied overnight in humid medium at room temperature followed by biotinylated secondary antibody for 15 min at 37°C and the ABC complex for 15 min at 37°C (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) was applied for 20 min at room temperature as chromogen. Slides were counterstained with hematoxylin, dehydrated and mounted. In negative control slides, the same procedures were applied with replacement of the monoclonal antibody by diluted normal bovine serum. α-SMA immune-staining was performed using polyclonal rabbit-anti-human (A3533 I g fraction; DAKO, Glostrup, Denmark). Area of α-SMA positive staining was assessed in predetermined high power field (40×) of the cortex (10 fields) then was captured by a digital camera (Kawai *et al*., 2009).

Biochemical studies

Sera were obtained by centrifuging blood samples and stored at 20°C until further processing. Creatinine was determined according to Henry (1974). Urea nitrogen was determined following method of Patton and Crouch (1977). Kidney was quickly removed from different animal groups, weighed and stored at -20°C then 10%W/V homogenate was prepared by grand 0.3 g of tissue in 3ml of saline. Malondialdehyde (MDA) was estimated by the method of (Placer *et al*., 1966), total superoxide dismutase (SOD) was measured by the

method of (Paoletti and Mocali, 1990) and catalase (CAT) activity was assayed according to the method of Xu *et al*. (1997).

STATISTICAL ANALYSIS

Statistical analysis of the results were conducted by calculating the means ± standard deviation. Analysis of variance [ANOVA] test was applied using the software SPSS V17 (SPSS, 1999). The means were compared by significant difference test at $p \leq 0.05$.

RESULTS

i. Histological results

Examination of control rats' and rats given GB kidney cortex demonstrated typical histological features (Fig.1A). Treating rats with CP for 4 weeks caused numerous histological alterations. The renal veins were augmented and congested with blood (Fig.1B). Intertubular broad leucocytic infiltrations were obviously observed between the tubules (Fig. 1C). Large edematous lesions were seen and the epithelial cells of numerous tubules exposed ill-defined limits and contained dark pyknotic nuclei (Fig. 2A). The glomeruli were atrophied and Bowman's capsule appeared with large vacuole (Fig. 2B). Animals treated with CP+ GB showed an improvement in the structure of kidney cortex. The tubules and the glomeruli appeared with normal structures (Fig. 2C).

ii. Histochemical results

a. Total carbohydrates

The glomerulus, the parietal layer of Bowman's capsule, the basement membrane and brush borders of renal tubules of control rats showed normal PAS positive reaction as did those from rats administered GB (Fig. 3A). Following treatment with CP, the glomerulus, the parietal layer of Bowman's capsule and the brush boarders of the tubules suffered extreme decrease in PAS- positive reaction (Fig. 3B). However, kidney cortex of rats treated with both CP and EGB appeared with nearly normal PAS positive reaction (Fig. 3C).

B. Total proteins

Both in control and GB-administered rats, the total protein materials demonstrated in the renal tubule epithelial cells cytoplasm in the form of small bluish irregular particles, in addition to positive stainability manifested in the nuclear envelope, chromatin materials and nucleoli (Fig. 4A). In the kidney cortex of rats treated with CP most cells lining tubular epithelia manifested reduction in protein content (Fig. 4B). Animals subjected to the dual treatment i.e. CP and EGB exhibited restoration of protein material in that a large number of tubular cells contained considerable amounts of proteins in comparison to CP- alone treated rats (Fig. 4C).

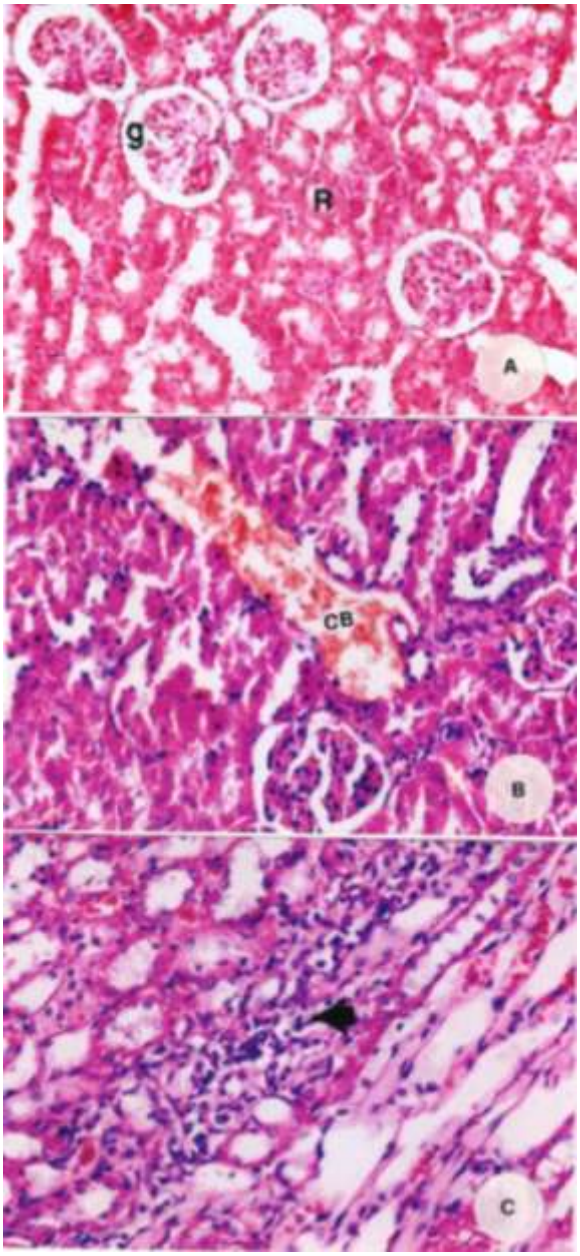

Fig.1. Kidney cortex histology. X400
A: A control rat showing normal tubular epithelium (R) and renal corpuscle (g).
B: A rat treated with CP for 4 weeks exhibiting disruption of many tubules with loss of nuclei (arrow), renal corpuscle condensation and enlarged congested blood vessel (CB).
C: Cortex of another rat treated with CP for 4 weeks exhibiting leucocytic infiltration (arrowhead) and hemorrhage (H).

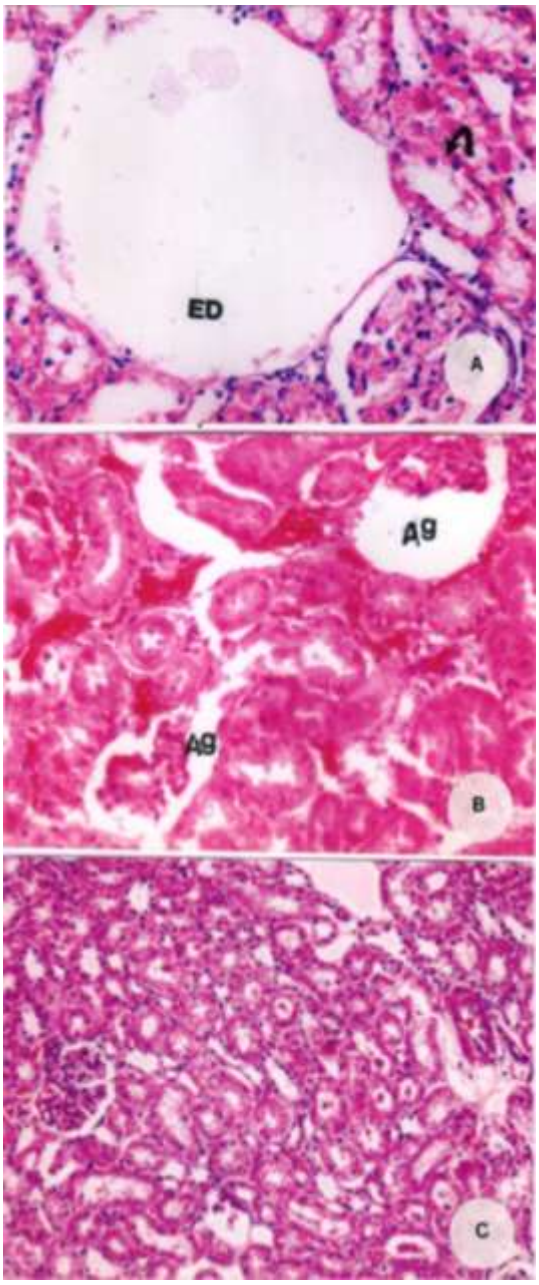

Fig. 2. A and B Kidney cortex of rats treated with CP. X 400

A: degenerated cells having ill-defined borders and pyknotic nuclei (curved arrow) andlarge edemouts lesion (ED)

B: showing cytoplasmic basophilia and atrophied glomeruli (Ag). Nuclei lost their staining characteristics.

C: CP-Ginko treated animals exhibiting improvement in cell condition where there are normal cytoplasm and nuclei stanability and well-defined cell membranes. X 200

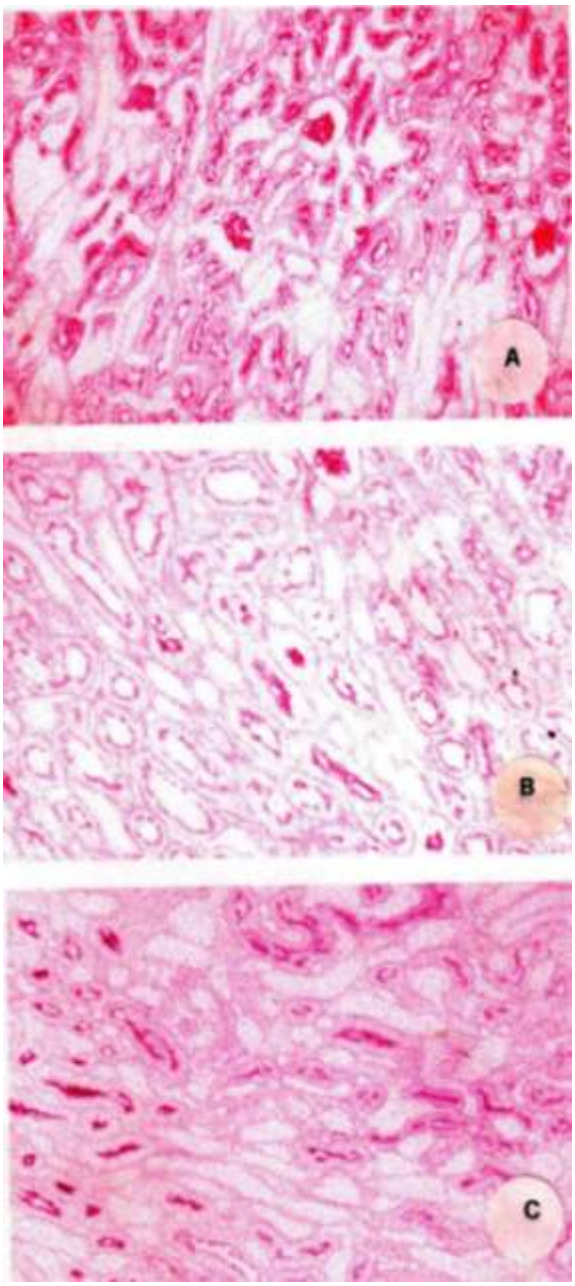

Fig. 3. PAS- positive material in the kidney cortex, X400.

A: Control rat showing intense reaction in glomeruli and tubular epithelium.

B: CP-treated rat exhibiting marked depletion.

C: CP- Ginko- treated rat showing increase in PAS reactivity in tubular cells compared to CP treatment.

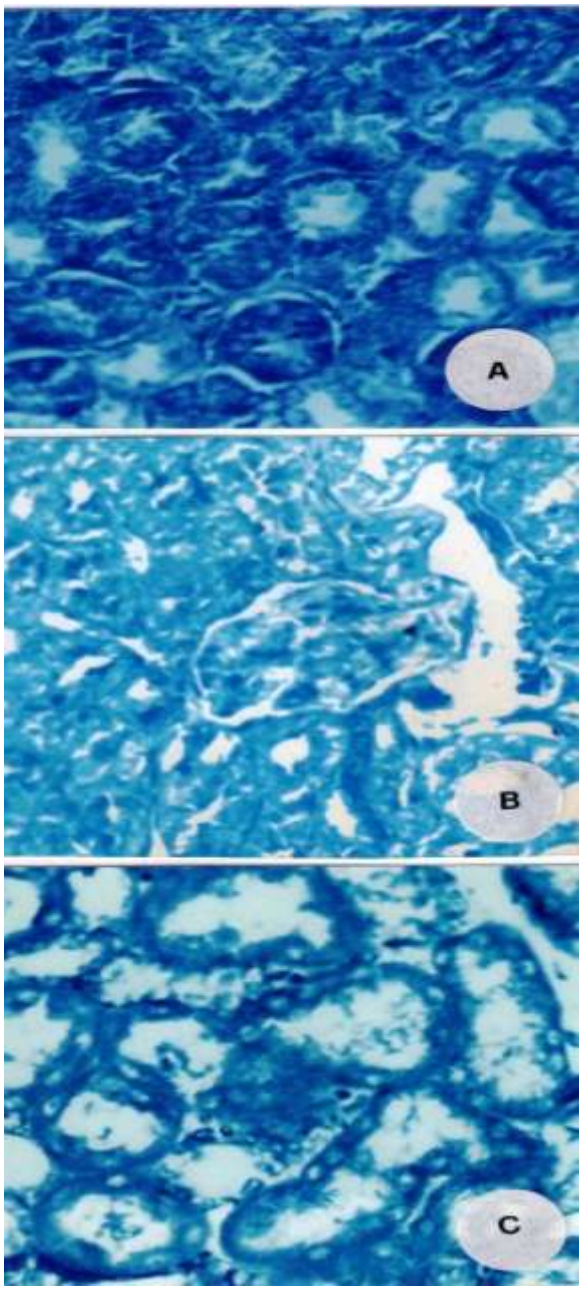

Fig. 4. Bromophenol blue staining for protein in kidney cortex, X 400..
A: Control rat showing dense blue proteinic inclusions in tubules cells.
B: A noticeable decrease in CP-administered rats.
C: Restoration of most protein content in CP-Ginko group.

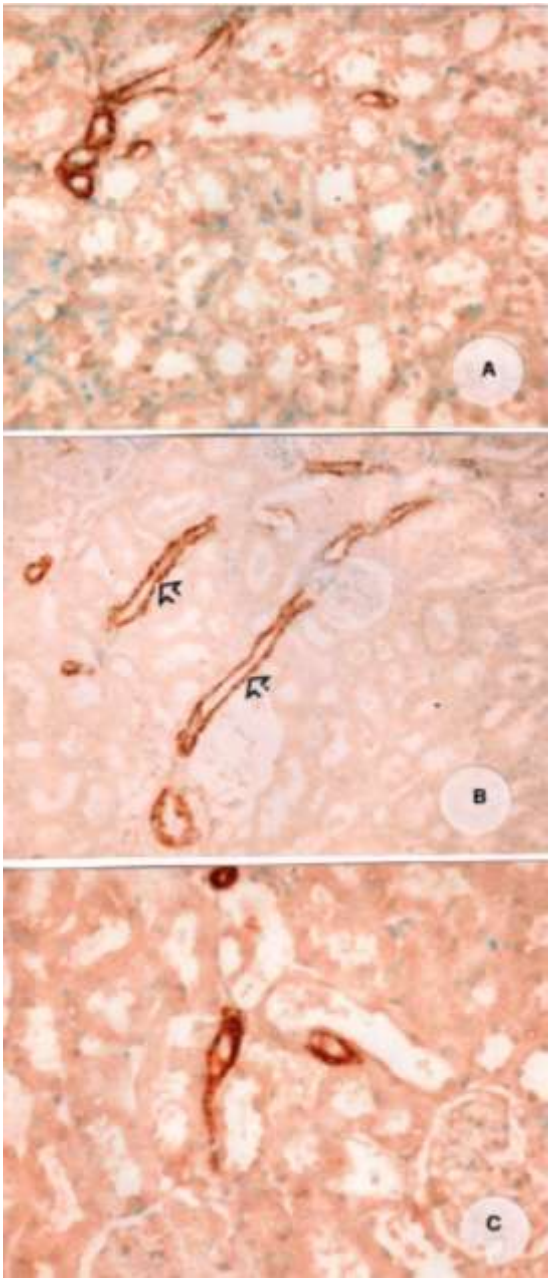

Fig. 5. Immune- staining of α-SMA in kidney cortex of rats. X400
A: Normal cortex showing α-SMA immune- staining in smooth muscle of renal arterioles.
B: an increase in expression of α-SMA positive fibroblastic renal cells (arrow heads); following CP exposure.
C: decrease of α-SMA expression after Ginko treatment with CP. X400

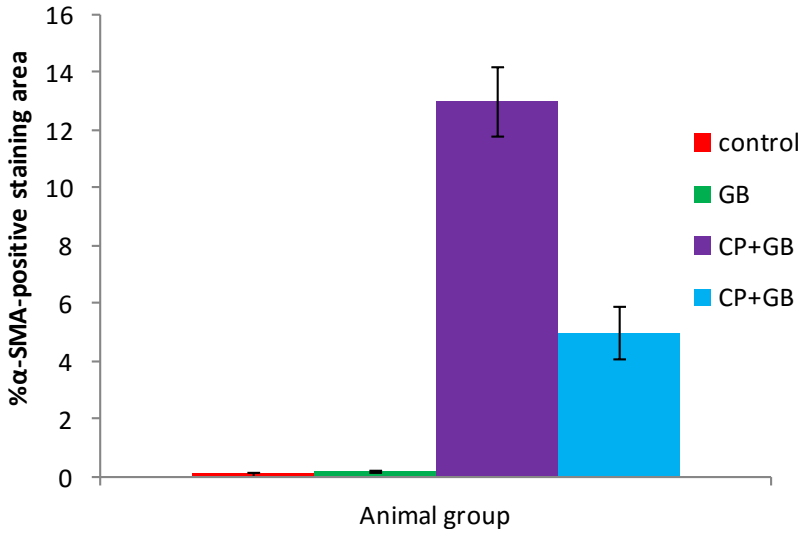

Fig. 5D. % α-SMA-positive staining area in different animal groups.

Table 1. Malondialdehyde (MDA) and enzymes activities in kidney of different animal groups.

Parameter	Control	GB	CP	CP+GB
MDA(mmol/ml)	17.3±1.2	16.6±14	31.1±1.7*	20±2.1
SOD (U/g wet weight)	1.8±0.5	2.1±0.6	0.7±0.03*	1.2±0.2
CAT (µmol/sec/ml)	3.6±0.9	3.7±1.1	2.2±0.03*	2.9±0.6

Data are expressed as mean ± SD, (*) significant at $P < 0.05$

Immunohistochemical results

Examination of kidney cortex of control animals and animals given EGB revealed that α-SMA was expressed in the smooth muscle cells of renal arterioles and rarely evident in the renal interstitium (Fig. 5A). A rise in the expression of α-SMA positive fibroblastic cells was observed in the kidneys of CP-treated rats (Fig. 5B). Rats treated with EGB and CP showed reduction in expression of α-SMA in the arterioles and interstitial tissue compared to treatment with CP alone (Fig. 5C). Quantification of α-SMA expression in renal tissue showed that the percentage of α-SMA-positive staining area significantly ($P < 0.05$) decreased in the CP+GB group compared with GB group (Fig. 5D).

Biochemical results

Results in Figure 6a manifested significant elevation in creatinine in the sera of rats treated with CP compared with that of control or GB- exposed rats. Treating animals with EGB and CP induced a significant decrease in creatinine when compared with CP group. Similarly, blood urea exhibited a significant increase after 4 weeks of treatment with CP while treatment with GB produced no change in urea. In animals treated with EGB and CP, urea was significantly decreased in comparison with the group of animals given CP (Fig. 6 b).

Data in Table 1 revealed that CP induced a significant increase in MDA and significant decrease in the antioxidant enzymes, SOD and CAT in comparison with control and GB groups.. On the other hand, treating rats with CP+GB caused a reduction in MDA and increase in SOD and CAT in comparison with CP group.

DISCUSSION

Acute renal failure is a common and serious renal problem having high morbidity and mortality rate in most countries (Begum *et al.*, 2006). The present study has investigated the efficacy of EGB against the toxicological disorders induced by CP in the kidney using rat model. It is evident from the results of the present investigation that

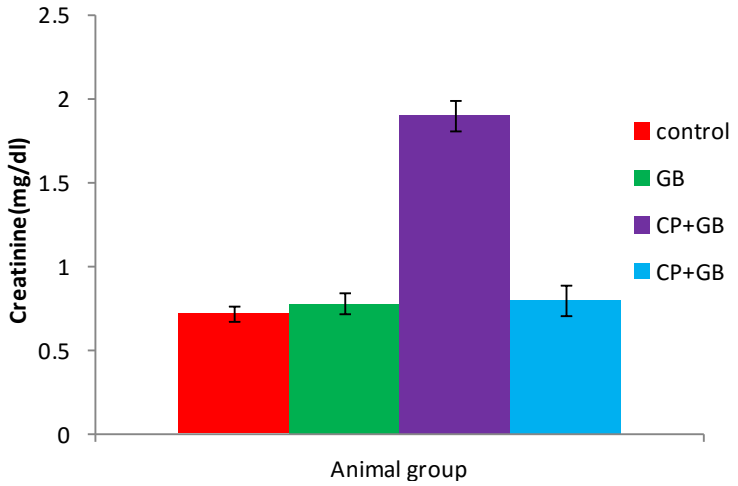

Fig. 6a.Change in creatinine in different animal groups.

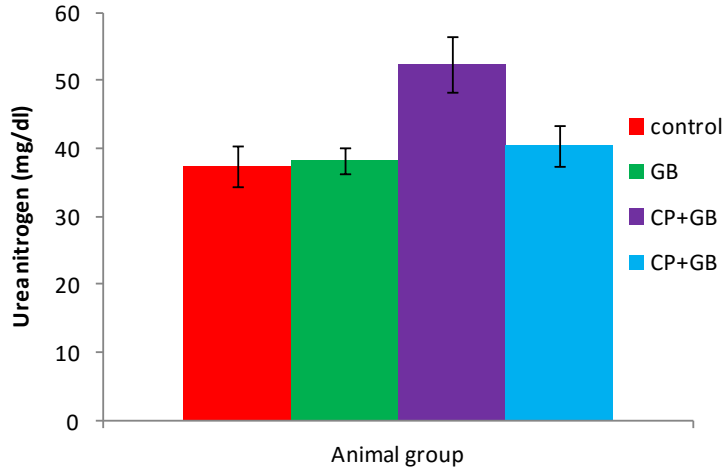

Fig. 6b.Change in urea nitrogen in different animal groups.

supplementation of EGB extract in conjunction with CP alleviated renal toxic effects induced by CP. Treating animals with CP leads to an increase in creatinine and urea. CP administration induced many histological alterations in the kidney including congestion of renal blood vessels, leucocytic infiltrations and degeneration of renal tubules as well as atrophy of glomeruli. These findings are in agreement with that announced by many investigations. Singh *et al*. (2014) reported that CP administration markedly elevated serum creatinine level, whereas *P. fraternus extract* alleviated CP-induced toxicity and creatinine levels were decreased compared to controls. Similarly, Estakhri *et al*. (2013) found a significant increase in creatinine and blood urea nitrogen levels and histopathologic change in renal tissue upon CP treatment. However, Sugumar *et al*. (2007) demonstrated in rat model that, CP induced histological renal damage while the plasma creatinine remains unaltered. Abraham

and Rabi (2009) reported nephrotoxicity due to CP in rats manifested in reduction of glomerular filtration rate along with tubular dysfunction. Histologically the CP treated rat kidneys showed glomerular nephritis, interstitial edema and cortical tubular vacuolization.

Histochemically, there was a decrease in PAS-positive material in kidney of CP-treated rats. Similarly, Nicol and Prasad (2006) observed significant reduction in carbohydrates in liver of mice administered CP. Sweetman and Martindale (2007) reported that disturbance in carbohydrate metabolism is one of the adverse effects of CP treatment. In this study, the total protein was also decreased in kidney of CP-treated rats. Impairment of DNA, RNA and protein biosynthesis was observed in mice treated with CP (Al-Bekairi *et al*., 1991). CP caused rough endoplasmic reticulum degeneration in hepatocytes (Zimnoch *et al*., 2002), that might be responsible for the lowered activity of such cells in protein synthesis (El-Banhawy *et al*., 1993).

Currently, immunohistochemical results revealed increase in α-SMA expression in kidney of CP-treated rats. Similar results were recorded by Zhang *et al*. (1995) that might be attributed to renal disease progression. Groma *et al*. (1997) demonstrated increased α-SMA expression in types of glomerulonephritis. The present elevation in α-SMA expression may be attributed to kidney injury induced by CP.

The present results showed that CP caused oxidative stress in the kidney as indicated by increase in MDA and decrease in SOD and SOD. It was reported that the CP induced renal damage is explained on the basis of oxidative stress and glutathione depletion (Abraham, 2011). CP exhibited activity following metabolic activation by liver enzymes. The first step in this activation involves hydroxylation and formation of 4-hydroxycyclophosphamide. This metabolite breaks into two cytotoxic metabolites named crolein and phosphoramide mustard (Hales, 1982). Phosphoramide possesses an antineoplastic effect and acrolein produces free radicals by interacting with the body's antioxidant defense system. These free radicals are highly reactive and cause oxidation of various enzymes (Senthilkumar *et al*., 2006). The toxicity of cyclophosphamide is the cumulative effect of the two mechanisms: (a) Firstly the decrease in the levels of nucleophiles such as GSH on interaction with acrolein and (b) secondly the formation of peroxynitrite by coupling of O and NO (Kim *et al*., 2012). A single i.p administration of CP to mice increased malondialdehyde level with depletion in glutathione content, antioxidant enzymes activities, glutathione peroxidase, glutathione reductase, catalase and quinone reductas (Rehman *et al*., 2012). Thus, the renal toxicity recorded in this work may be attributed to the oxidative stress induced by CP.

Concerning the effect of EGB, the obtained results showed that rats administered EGB alone had no toxicological impacts whether histological, histochemical, immunohistochemical or biochemical. However, treatment with CP and EGB revealed marked improvement in the kidney structure and restored the levels of creatinine and urea toward normal limits. These results are in agreement with that of Abd-Ellah and Mariee (2007) who recorded that EGB prevented adriamycin-induced nephrotoxicity and improve serum lipid profile, total protein, urea and creatinine clearance. EGB was found to be effective against cisplatin-induced nephrotoxicity evidenced by improvement in histological structure of the kidney and decrease in blood urea nitrogen and creatinine levels (Okuyan *et al*., 2012).

Combined treatment with CP and EGB caused an elevation in tissue content of carbohydrates and total proteins in comparison to CP treatment alone. Al-Attar (2012) reported that EGB improved levels of glycogen and total proteins in thioacetamide injected mice. Sakr *et al*. (2011) observed that EGB improved carbohydrates and protein contents of ovarian tissue of rats exposed to fungicide, topsin. Expression of α-SMA was decreased in CP+ EGB treated rats. This is due to the antifibrotic effect of EGB. He *et al*. (2006) observed that EGB was effective against liver fibrosis induced by CCl_4 through its ability to suppress transforming growth factor B_1 (TGF-B_1) expression and TGF-B_1 initiated hepatic stellate cell activation, inhibit HSC proliferation and down regulate α-SMA. Additionally, Lu *et al*. (2015) indicated decrease in the creatinine, BUN, and urine protein, accumulation of glycogen and collagen and α-SMA by *Ginkgo biloba* leaves extract thus preventing renal fibrosis in diabetic nephropathy

Many investigations have reported that EGB could be helpful in both therapy and prevention of diseases and other degenerative processes associated with oxidative stress (Kusmic *et al*., 2004). GB treatment restored the normal values of lipid peroxidation marker, MDA and antioxidant enzymes, SOD and CAT. Similarly, Song *et al*. (2013) indicated reno-protective effect exerted by *Ginkgo biloba* treatment as it restored the levels of creatinine, BUN, MDA, NO, SOD and CAT in kidneys after cisplatin injection and this renoprotection might be mediated by antioxidant and anti-inflammatory activities. Moreover, Akdere *et al*. (2014) recorded decreases in tissue damage by *Ginkgo biloba* Egb761 extract application before renal ischemia-reperfusion evidenced by decrease in MDA and increase in SOD and CATcompared to renal ischemia-reperfusion injury rats. Haung *et al*. (2000) stated that GB extract could counteract the function of ROS, directly scavenge superoxide anion, hydroxyl radicals, peroxyl radical species and nitric oxide. Moreover, EGB has an SOD like activity and a hydroxyl radical scavenging activity (Wu *et*

al., 2002). Pener *et al*. (2005) concluded that EGB with its potent free radicals scavenging and antioxidant properties seems to be a highly promising agent in protecting hepatic tissue against oxidative damage and in preventing hepatic fibrosis and dysfunction due to obstructive jaundice. EGB also protected brain, lung, liver and kidney tissue against mercury (II)-induced oxidative damage in rats (Sener *et al*., 2007). Recently, Sakr *et al*. (2015) reported that EGB improved CP induced marked reduction in antioxidant enzymes activities (CAT and SOD) and elevation in oxidative stress marker (MDA) in testes of mice. Thus, it is concluded from the present work that EGB ameliorates the renal damage induced by CP through its antioxidant activity.

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Received: April 20, 2016: Accepted: May 9, 2016