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Full Length Research Paper

Physiological and histological impact of *Azadirachta indica* (neem) leaves extract in a rat model of cisplatin-induced hepato and nephrotoxicity

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This study investigated the protective effect of *Azadirachta indica* (neem) leaves against cisplatin-induced hepato and nephrotoxicity. Neem leaves showed significant protection as evidenced by the decrease of elevated serum alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transpeptidase, alkaline phosphatase, total bilirubin, creatinine, uric acid and urea. This improvement of physiological function was associated with high protection against histopathological injury induced by cisplatin on liver and kidney. These results suggest that neem leaves pre, co and post-treatment can prevent the hepato and nephrotoxicity induced by cisplatin.

Key words: Cisplatin, *Azadirachta indica*, liver, kidney, rats.

INTRODUCTION

Cisplatin, cisplatinum or cis-diamminedichloridoplatinum (II) (CDDP), a platinum-based drug, is one of the most frequently used anti-neoplastic agents for various types of cancer. It has a potent anti-tumor action against wide range of malignancies, including testicular, ovarian, cervical, bladder and lung cancers as well as solid tumors resistant to other treatment regimens (Hanigan and Devarajan, 2003). Despite its clinical usefullness, cisplatin treatment has been associated with several toxic side effects, including nephrotoxicity, neurotoxicity and ototoxicity (Rabik and Dolan, 2007). In addition, alopecia, electrolyte disturbance, nausea and vomiting have been recorded in cisplatin (Lajolo et al., 2009).

Nephrotoxicity of cisplatin is the major drug, which limits its clinical use and appears to be a dose- limiting factor in its treatment. Use of higher doses with hydration and dieresis has been attempted to reduce nephrotoxicity and increase efficacy of cisplatin treatment (Borch and Markman, 1989). During the aggressive treatment protocols, higher doses of cisplatin that may be required for effective tumor suppression could also lead to hepatotoxicity, which is also encountered during low-dose repeated cisplatin therapy (Pratibha et al., 2006; Lee et al., 2008). Hepatotoxicity is a less- known aspect of cisplatin treatment, and there is little information about the underlying mechanism. It has been reported that oxidative stress through the generation of reactive oxygen species (ROS) (Chirino and Pedraza-Chaverri, 2009), decreased antioxidant defense system including antioxidant enzymes (Sadzuka et al., 1992) and non-enzymatic molecule reduced glutathione (GSH) are major alterations in cisplatin toxicity (Zhang and Lindup, 1993). In addition, functional and structural mitochondrial damage, apoptosis, perturbationin Ca^{2+} homeostasis (Martins et al., 2008), involvement of pro-inflammatory genes such as COX-2 and inducible nitric oxide synthase (iNOS) may play some important roles in the mechanism of cisplatin hepatotoxicity (Kim et al., 2004; Kart et al., 2010). Recently, several groups suggested that combined chemotherapy using cisplatin and plant extracts can reduce the side effects and enhance the antitumor efficacy (Kim et al., 2006).

Foods of plant origin with diverse medicinal properties have come under extensive study in the light of their antioxidant, antimutagenic, and anticarcinogenic effects (Khan and Mukhtar, 2008; Vinothini et al., 2009). In particular, *Azadirachta indica* (neem) is a widely prevalent and highly esteemed wonder tree of the Indian subcontinent and several of its beneficial properties are

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reported. Neem leaf consists of several valuable components and can be divided into two major classes: isoprenoids that include terpenoids containing limonoids, azadirone and its derivatives, c-secomeliacins, for example, azadirachtin. The nonisoprenoids include aminoacids, polysaccaharides, sulphurous compounds, polyphenolics like flavonoids and their glycosides for example, quercetin, dihydrochalcone, coumarin and tannins, aliphatic compounds (Bandyopadhyay et al., 2002). A portion of these compounds, present in the neem leaf, can be extracted with water (Sarkar et al., 2007).

In this paper, we studied the protective activity of *A. indica* leaves extract on cisplatin-induced hepatotoxicity and nephrotoxicity, in particular, on the level of cellular damage of liver and kidney. This was determined by measuring plasma liver and kidney function and by histochemical analysis of liver tissue.

MATERIALS AND METHODS

Preparation of neem leaves extract

Fresh matured leaves of neem tree were collected in August from the garden in Obour City, Cairo. The samples were identified in Botany Department, Faculty of Science, Helwan University. The leaves were cleaned, dried and powdered, the powder was used for the preparation of crude methanolic extract according to the procedure described by Manikandan et al. (2008) with some modification. Air-dried powder (100 g) of *A. indica* leaves were extracted by percolation at room temperature with 70% methanol alcohol and kept in refrigerator for 24 h. Leaves extract of *A. indica* was concentrated under reduced pressure (bath temperature 50°C) and dried in a vacuum evaporator. The residue was dissolved in distilled water, filtered and used in experiments.

Animals and experimental design

To study the protective effect of neem leaves extract on cisplatin-induced hepatic and renal toxicity. Male albino rats weighing 120 to 150 g were obtained from The Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). Rats were provided with water and balanced diet *ad libitum*. The experiments were approved by the state authorities and Egyptian rules on animal protection were followed. Forty two adult rats were randomly divided into seven groups, six rats of each. Group I served as untreated control. Groups II and III were received an oral administration of 500 mg/kg neem leaves extract for 5 and 10 consecutive days, respectively via epigastric tube. Group IV received a single intraperitoneal injection of cisplatin (5 mg/kg, Oncotec Pharma Produktion GmbH) and left for 5 days. Groups V and VI received the same dose of the extract for 5 days post- and pre- a single intraperitoneal injection of 5 mg/kg cisplatin, respectively. Group VII received an oral administration of 500 mg/kg neem leaves extract for 5 days pre- and post- a single intraperitoneal injection of 5 mg/kg cisplatin. The animals of all groups were killed by fast decapitation; blood samples were collected, and allowed to stand for half an hour and then centrifuged at 500 g for 15 min at 4°C to separate serum and stored at -70°C for the different biochemical measurements. Liver and kidney were dissected out and fixed immediately for light microscopy study.

Histological examination

Pieces of liver and kidney were fixed in 10% formal saline, embedded in paraffin, and sectioned. The sections were stained with ordinary haematoxylin and eosin.

Biochemical estimations

Liver function test

Colorimetric determination of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) was estimated by measuring the amount of pyruvate or oxaloacetate produced by forming 2, 4-dinitrophenylhydrazine according to the method of Reitman and Frankel (1957). The color of which was measured at 546 nm. γ-glutamyl transpeptidase (γGT) and alkaline phosphatase were assayed in liver homogenate using kits provided from Biodiagnostic Co. (Giza, Egypt) according to the method described by Szasz (1969) and Belfield and Goldberg (1971), respectively. Also, total bilirubin (TB) in serum was assayed according to the method of Schmidt and Eisenburg (1975).

Kidney function test

Uric acid, urea and serum creatinine were assayed in serum using kits provided from Biodiagnostic Co. (Giza, Egypt).

Statistical analysis

Data were presented as mean ± standard error. One-way ANOVA was carried out, and the statistical comparisons among the groups were performed with Duncan's test using a statistical package program (SPSS version 17.0).

RESULTS

Earlier experimental studies have shown that a minimum dose of cisplatin (5 mg/kg body wt. i.p) was sufficient to induce hepato and nephrotoxicity in rats (Babu et al., 1995). Although a number of studies have demonstrated some side-effects of the chemotherapeutic drug cisplatin, the present study is the first *in vivo* study used in *A. indica* leaves extract on cisplatin-induced hepatotoxicity and nephrotoxicity.

Control hepatocytes were normal polygonal with oval-shaped nuclei (Figure 1A). *A. indicia* leaves extract (500 mg/kg) treatment for 5 and 10 days preserved the liver parenchyme; that is, the appearance of the hepatocytes, sinusoids and Kupffer cells was similar to the control morphology (Figure 1C and D, respectively). In contrast, there was cellular damage in the liver of cisplatin-injected animals (5 mg/kg). The hepatic tissue showed degenerated hepatocytes, vascular congestion in sinusoids, focal collection of inflammatory cells appeared markedly distributed in the necrotic foci, loss of hepatic tissue structural pattern and inflammatory cell infiltration in portal triad region in liver. The hepatocytes showed pyknotic nuclei, karyorrhexis and karyolysis with irregular nuclear membrane (Figure 1B). The liver of the rats with

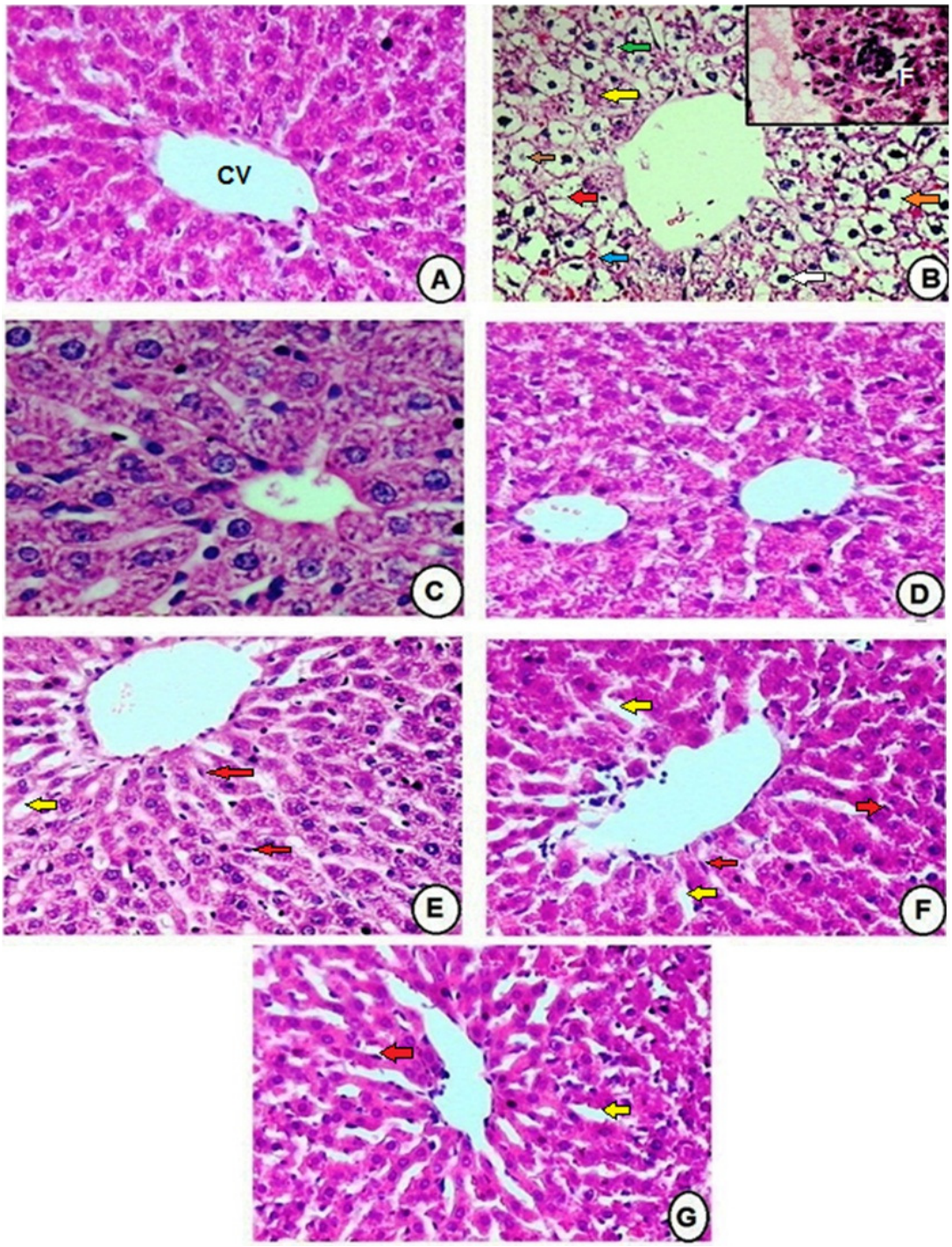

Figure 1. Neem leaves extract protects liver from cisplatin-induced injure. A; control liver section with the normal architecture of hepatic lobule. The central vein (CV) lies at the center of the lobule surrounded by cords of hepatocyte. B; rats treated with cisplatin shows loss of hepatic tissue structural pattern and hydropic degeneration (⇦) in the hepatocytes, granulated cytoplasm(⇦), the pyknotic nuclei(⇦), Karyorrhexis (⇦), Karyolysis (⇦), pale stained nuclei (⇦), congested blood sinusoid (⇦) and focal necrosis associated with lymphocytic infiltration (f). C and D; rats treated with neem leaves extract for 5 and 10 days, respectively. E, F and G; rats treated with a single dose of cisplatin followed by a daily dose of neem leaves extract for 5 days, neem leaves extract for 5 days before injection of a single dose of cisplatin and neem leaves extract for 5 days before and after injection of a single dose of cisplatin, respectively, showing the nearly normal polyhedral hepatocyte, the dilated blood sinusoids (⇦) and activated Von Kupffer cell (⇦) (H & E X 200).

Table 1. Effect of *Azadirachta indica* leaves methanol extract administrated for 5 and 10 days (500 mg/kg b. wt.) on the levels of ALT, AST, γ-GT, ALP and TB in serum of albino rats injected with cisplatin (5 mg/kg b. wt., i.p.)-induced hepatotoxicity.

Group	Parameter				
	ALT (U/L)	AST (U/L)	γ-GT (U/L)	ALP (IU/L)	Bilirubin (mg/dl)
I	54.54±0.92	62.00±0.36	949.80±41.57	3.12±0.52	1.66±0.13
II	32.54±0.95^{a}	57.33±1.05^{a}	886.78±50.34	1.85±0.21^{a}	1.38±0.02
III	27.96±1.21^{a}	54.00±1.32^{a}	818.10±32.51^{a}	1.39±0.22^{a}	1.06±0.13^{a}
IV	68.98±1.89^{a}	76.50±0.43^{a}	1266.56±18.95^{a}	4.35±0.07^{a}	2.23±0.14^{a}
V	49.56±0.59^{b}	7.50±1.25^{b}	979.30±31.69^{b}	2.27±0.17^{ab}	1.95±0.11^{a}
VI	39.02±0.66^{ab}	52.50±0.43^{ab}	971.22±19.98^{b}	2.02±0.34^{ab}	1.83±0.08^{b}
VII	35.87±0.50^{ab}	57.50±0.43^{b}	1009.80±43.17^{b}	1.31±0.18^{ab}	1.56±0.04^{b}

pre-, post- and co-treatment of the same dose of the extract with cisplatin shows some regions of recovery. *A. indicia* leaves extract has a high potential in healing liver parenchyma and regeneration of liver cells; that is, the appearance of the hepatocytes and sinusoids cells was near to control morphology. The dilation of sinusoids and activation of Kupffer cells are seen (Figure 1E, F and G, respectively).

Serum ALT, AST, γ-GT, ALP and total bilirubin (TB) levels were significantly elevated ($P < 0.05$) in the cisplatin-treated animals compared to the control group. The increase of serum ALT and AST levels were 0.26 and 0.23-fold, respectively. In addition, serum γ-GT, ALP and TB were increased significantly in cisplatin injected group by 0.49, 0.39 and 0.34-fold, respectively when compared to control group (Group I) indicating this hepatotoxicity of cisplatin. Pre-, post- and co-treatment of animals with methanolic extract of *A. indica* significantly reduced the elevated levels of serum liver function parameters. The extract treatment was able to lower the serum ALT, AST, γ-GT, ALP and TB to almost control value (Table 1). The data so far obtained in this study demonstrated that cisplatin caused significant increase in serum ALT and AST activities. The observed increase in serum aminotransferase activities could be a secondary event following cisplatin-induced liver damage with consequent leakage from hepatocytes.

Histological examination of the kidney revealed that cisplatin (5 mg/kg) induced a nephrotoxicity, as judged by the histopathological changes including glomerular congestion and degeneration, dilatation in Bowman's Space, tubular degeneration and dilation, necrosis with small pyknotic nuclei was separated from the prominent basophilic basement membrane, loss of brush border of epithelial cells in renal tubular epithelium, the lumen was filled with debris and necrotic cells (Figure 2A and B). Administration of oral administration of 500 mg/kg neem leaves extract for 5 and 10 consecutive days showing the same structure of the renal corpuscles and tubules as in the control group (Figure 2C and D, respectively). Pre-, post- and co-treatment of the same dose of the extract with cisplatin dramatically improved the cisplatin nephrotoxicity, and less histological damage was observed in renal tubules. The tubular regenerations were observed (Figure 2E, F and G, respectively).

Serum uric acid, urea and creatinine levels were significantly elevated ($P < 0.05$) in the cisplatin-treated animals compared to the control group. The increase of serum uric acid, urea and creatinine levels were 0.20, 0.37 and 0.48-fold, respectively. Pre-, post- and co-treatment of animals with methanolic extract of *A. indica* significantly reduced the elevated levels of serum uric acid, urea and creatinine. The extract treatment was able to lower the serum uric acid, urea and creatinine to almost normal level (Table 2).

DISCUSSION

The present investigation showed many histopathological abnormalities in the liver including inflammatory infiltration, marked disruption of hepatic cords and dilated blood sinusoids. Many hepatocytes showed hydropic degeneration and pyknotic nuclei indicating necrosis. The liver is known to accumulate significant amounts of cisplatin, second only to the kidney (El-Sayyad et al., 2009), thus hepatotoxicity can be associated with cisplatin treatment (Liao et al., 2008). Clinical evidence of cisplatin-induced liver injury has been demonstrated by elevated activities of serum enzymes and bilirubin levels, and the development of jaundice (Moriya et al., 2000). Cell death can result from naturally occurring apoptosis (physiological apoptosis) or from irreparable cell injury (pathological apoptosis) as described by Farber (1994). Cisplatin is thought to kill cells primarily by forming DNA adducts, causing G2 arrest in the cell cycle, triggering apoptosis (Kishimoto et al., 2000). Administration of neem leaves extract protected liver form cisplatin toxicity this observation was also found in several experiments where in rabbits, neem fruits caused decreased serum activities of alkaline phosphatase and an improvement of ALT, AST and bilirubin values (Boeke et al., 2004).

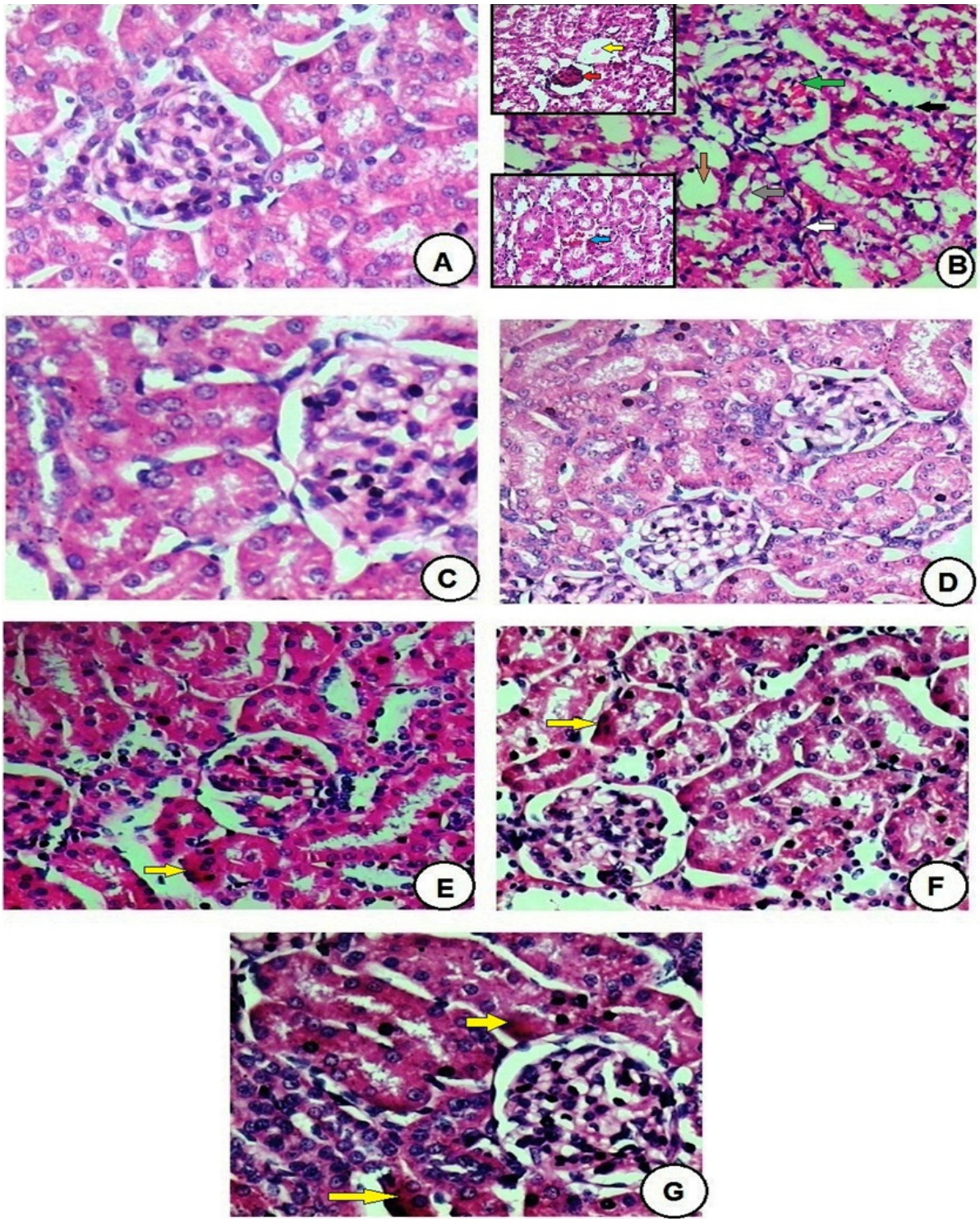

Figure 2. Neem leaves extract protects kidney from cisplatin-induced injure. A; control kidney sections with normal renal corpuscle and renal tubules. B; rats treated with cisplatin with congested (⇦), shrinked (⇦) and completely degenerated (⇦) glomeruli, debris (⇦) in the lumen of some renal tubules, pyknotic nuclei (⇦), degeneration (⇦) and dilation (⇦) of renal tubules and hemorrhage (⇦) in the interstitial space of renal tubules. C and D; rats treated with neem leaves extract for 5 and 10 days, respectively. E, F and G; rats treated with a single dose of cisplatin followed by a daily dose of neem leaves extract for 5 days, neem leaves extract for 5 days before injection of a single dose of cisplatin and neem leaves extract for 5 days before and after injection of a single dose of cisplatin, respectively, showing normal renal corpuscle and renal tubule more or less like normal structure with the regeneration (⇦) of some renal tubules (H & E X 200).

Table 2. Effect of *Azadirachta indica* leaves extract on the levels of uric acid, urea and creatinine in blood serum of albino rats injected with cisplatin-induced nephrotoxicity.

Group	Parameter		
	Uric acid (mg/dl)	Urea (mg/dl)	Creatinine (mg/%)
I	63.70±0.77	3.84±0.17	0.44±0.02
II	60.27±0.35[a]	3.64±0.14	0.48±0.01
III	58.22±0.59[a]	3.64±0.07	0.35±0.02
IV	76.71±1.32[a]	5.28±0.06[a]	0.65±0.04[a]
V	63.93±1.79[b]	3.90±0.12	0.43±0.01[b]
VI	63.93±1.68[b]	3.67±0.23	0.42±0.02[b]
VII	61.64±0.50[b]	3.23±0.18[ab]	0.43±0.02[b]

Values are means ± SE (n = 6). a: significant change at $P < 0.05$ with respect to Group I. b: significant change at $P < 0.05$ with respect to Group IV.

Arivazhagan et al. (2000) was found also, that chemically-induced carcinogenesis with accompanying high levels of lipid peroxidation and low levels of glutathione (GSH), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and gamma glutamyl transpeptidase (GGT) in rats could be effectively reduced with leaf extract. A five-day pre-treatment with leaf extract decreased the formation of lipid peroxides and enhanced the levels of antioxidants and detoxifying enzymes in the stomach, the liver and circulation. In addition, livers of paracetamol-induced rats were normal in appearance and histology after leaf extract administration. The extract caused a reduction of paracetamol-induced high serum levels of ALT, AST and GGT (Bhanwra et al., 2000).

The non hepatotoxic nature of neem was proved in the study performed by Haque et al. (2006) who found unaltered and normal activities of serum ALT, AST, ALP as well as retained architecture of liver after neem treatment. Also, Yanpallewar et al. (2003) reported the hepatoprotective role of neem leaves extract against paracetamol-induced hepatic damage in albino rats as indicated by stable serum activity of ALP, ALT, AST and histopathological observations of liver tissues. In addition, Peer et al. (2008) reported the cardioprotective effect of neem leaves extract on isoprenaline-induced myocardial infarction in rats as evidenced by significant decrease in serum cardiac marker enzymes, lactate dehydrogenase and AST.

The *in vivo* mechanisms of cisplatin nephrotoxicity are complex and involve oxidative stress, apoptosis, inflammation, and fibrogenesis (Yao et al., 2007). Cisplatin nephrotoxicity primarily causes tubule-interstitial lesions. In animal models cisplatin damages the proximal tubules, specifically the S3 segment of the outer medullary stripe. Mitochondrial swelling and nuclear pallor occur in the distal nephron. The glomerulus has no obvious morphologic changes (Vickers et al., 2004).

In this study, the impairment of kidney function by cisplatin is recognized as the main side effect and the most important dose limiting factor associated with its clinical use. Several investigators (Naziroglu et al., 2004; Atessahin et al., 2005) reported that the alterations induced by cisplatin in the kidney functions were characterized by signs of injury, such as increase of creatinine and urea levels in plasma. In the present study, it was shown that administration of cisplatin to rats caused a reduction in glomerular filtration rate, which correlated with increased creatinine and urea in plasma. These biochemical parameters were well correlated with the renal histological results. These observations indicated that cisplatin induced nephrotoxicity and the results are in accordance with those of aforementioned workers.

Several protective agents have been evaluated against cisplatin-induced nephrotoxicity in experimental and clinical studies. They include diethyldithiocarbamates, glutathione, glycine, methionine, procaine and procainamide (Husain et al., 1998). However, none of these compounds has proved to be clinically efficacious as complete protection in patients. A large number of sulfur-containing compounds have been shown to reduce the nephrotoxicity of cisplatin without inhibiting its antitumor effect in patients with ovarian cancer, non–small-cell lung cancer, metastatic breast cancer, and metastatic colon cancer (Kintzel, 2001).

It is important that the protective agent is present in renal tissue before damage occurs, may be preventing this damage. The acute renal failure indicated by increased creatinine and urea occurred before the development of tubular necrosis. These parameters are markers of glomerular filtration rate. Our results showed that extracts of *A. indica* reduced the rise of urea and creatinine induced by cisplatin as well as renal damage. Free radicals formation is one of the mechanisms of nephrotoxicity induced by cisplatin and antioxidants have protective effect against renal toxicity induced by this drug (Maliakel et al., 2008). Constituents of *A. indica* such as flavonoids (quercetin) have antioxidant activity (Bandyopadhyay et al., 2002), so this plant may inhibit lipid peroxidation by scavenging free radicals and increasing intracellular concentration of glutathione.

In conclusion, the use of *A. indica* leaves extract is a promising renal and hepatoprotective agent and this protective activity of *A. indica* leaves extract may be due to its antioxidant and normalization of impaired kidney and liver function activity. This was manifested by improvement in the biochemistry and histology of kidney and liver of the studied animals injected with cisplatin-induced kidney and liver adverse effect.

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