Journal of Pharmacology and Toxicology

ISSN 1816-496X

Academic Journals Inc.

www.academicjournals.com

Journal of Pharmacology and Toxicology 9 (1): 25-36, 2014
ISSN 1816-496X / DOI: 10.3923/jpt.2014.25.36

Prevention of Cisplatin-Induced Renal Toxicity in Swiss Albino Mice by Astaxanthine

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ABSTRACT

This study aims to explore amelioration of the renal toxicity induced by one of the commonly used antineoplastic agent, Cisplatin (CDDP). The therapeutic application of CDDP is limited by the induction of nephro-toxicity as a result of severe oxidative stress in renal tissues. The present study demonstrates that administration of astaxanthine (AST), a carotenoid, prevented the nephro-toxicity induced by CDDP by reducing oxidative stress in renal tissues. The renal toxicity was monitored by estimating levels of serum-biochemical parameters such as urea and creatinine. The oxidative stress in renal tissues were determined as depletion of antioxidant parameters and cellular DNA damage. The cellular DNA damages in kidney and tumor tissues were analysed using comet assay. Histopathological examinations were carried out to monitor the CDDP-induced alterations in the tissues of tumor and kidney. Histopathological studies revealed that administration of CDDP to tumor bearing mice caused morphological alterations in tumor and renal tissues. Also there was increase in the levels of serum- creatinine and urea, in these animals apart from cellular DNA damage and oxidative damage in the tissues. Administration of AST, following CDDP treatment, restored the serum parameters and prevented the morphological alterations in the renal tissues without protecting the tumor tissue. The comet assay results demonstrated that AST administration protected the cellular DNA from CDDP induced damages in the kidney cells without affecting the damage in tumor cells. The studies suggest the beneficial use of AST as an adjuvant to ameliorate the toxicity in Cisplatin-chemotherapy.

Key words: Cisplatin, astaxanthine, antineoplastic, oxidative stress, renal toxicity

INTRODUCTION

Cisplatin, cis-diamminedichlorido-platinum (II), a platinum based antineoplastic agent effective in treating different types of cancers including sarcomas, carcinomas, lymphomas and germ cell tumors (Kondagunta *et al*., 2005) has limited therapeutic applications due to renal toxicity. The anti-tumor agent cisplatin can cause severe nephrotoxicity, gastrointestinal toxicity and bone marrow toxicity (Arany and Safirstein, 2003; Ekborn *et al*., 2000, 2003; Kruidering *et al*., 1997). CDDP has been found to accumulate in the proximal tubular cells of the kidney. This disproportionate accumulation of CDDP contributes to CDDP-induced renal toxicity (Arany and Safirstein, 2003; Kuhlmann *et al*., 1997). The toxic effect of CDDP has been ascribed to the induction of oxidative stress mediated by the generation of free radicals in tissues. Several compounds with antioxidant activity offer protection to normal tissues from deleterious effects of ionising radiation, oxidative stress and cytotoxic effects of various chemotherapeutic agents (Nair *et al*., 2001).

AST is a naturally occurring α-hydroxy ketocarotenoid belongs to a large class of phytochemicals known as terpenes with powerful antioxidant properties, responsible for the pink-red pigmentation in variety of living organisms including various plants, algae etc. Being a strong natural antioxidant it shows good free radical scavenging activity and protects cell membranes from formation of lipid peroxides (Augusti *et al*., 2008; Durak *et al*., 2010). The objective of the present study was to explore whether AST could prevent CDDP-induced nephrotoxicity in tumor bearing animals. The effects of AST on CDDP-induced oxidative stress in terms of biochemical, cellular, morphological and molecular parameters were examined in a murine model.

MATERIALS AND METHODS

Chemicals: Astaxanthine was obtained as a gift for research from Parry, India. Thiobarbituric acid (TBA) and DTNB were from Sigma Chemical Company Inc., St Louis, MO, USA. Hydrogen peroxide was from Merck Specialities Pvt. Ltd., Mumbai. All other reagents were of analytical grade and purchased from reputed Indian manufacturers.

Animals: Swiss albino mice were maintained in the Breeding section, Pushpagiri Institute of Medical Sciences and Research Centre, Thiruvalla. The animals were kept in air-controlled room, fed with normal mice chow (Sai Feeds, Bangalore, India) and water *ad libitum*. All animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

Development of solid tumor: Animals weighing 25 g were grouped randomly with five animals each. Animals in the first groups were kept as control without tumor. Solid tumor was developed by transplanting Dalton's Lymphoma Ascites (DLA) cells subcutaneously in the hind limb of animals of the other four groups. The treatments were started after the size of tumor reached approximately 1.0 cm^3 (measured by Vernier calliper) in the tumor bearing animals.

Experiment design: The animal groups were treated as described below:

Group 1: Normal animals (without tumor)
Group 2: Control tumor-bearing animals (without any treatment)
Group 3: Tumor-bearing animals, administered p.o with AST (50 mg kg^{-1})
Group 4: Tumor-bearing animals, treated with CDDP i.p (12 mg kg^{-1})
Group 5: Tumor-bearing animals, treated with CDDP i.p (12 mg kg^{-1})+administered p.o with AST (25 mg kg^{-1})
Group 6: Tumor-bearing animals, treated with CDDP i.p (12 mg kg^{-1})+administered p.o with AST (50 mg kg^{-1})

A single dose of CDDP (12 mg kg^{-1} body weight) was administered intraperitoneally (i.p) in animals of group 4, 5 and 6. After one hour of cisplatin injection, two doses (25 and 50 mg kg^{-1}) of AST were given to animals in group 5 and 6 per orally (o.p) using oral needle as suspension.

Seventy two hours after the administration of CDDP the animals were sacrificed by cervical dislocation, blood was collected by cardiac puncture and serum was separated for biochemical analysis. The kidney and solid tumor were excised and washed with ice-cold Phosphate Buffered Saline (PBS) and tissue homogenates [10% (w/v)] were prepared in PBS.

Assessment of antioxidant status of kidney: The protective action of AST was analysed in the tissue homogenate by lipid peroxidation assay, glutathione (GSH) and glutathione peroxidase assay (GPx). The levels of lipid peroxides were estimated by thiobarbituric acid reactive substances (TBARS) assay, where malondialdehyde (MDA), the end-product of lipid peroxidation is quantified (Buege and Aust, 1978). The final result was expressed as nanomoles of malonedialdehyde per mg protein in the tissue homogenate. GPx activity was measured by method of Hafeman *et al.* (1974) based on the degradation of H_2O_2. GSH level was assayed (Moron *et al.*, 1979) based on the reaction with 5, 5'-Dithiobis-2-nitrobenzoic acid (DTNB). Protein levels were measured by the method of Lowry *et al.* (1951).

Serum biochemical analysis: Serum urea level was measured by Diacetyl monoxime (DAM) reagent method (Kassirer, 1971) and serum creatinine level was determined by alkaline picric acid method (Allen *et al.*, 1982) using reagent kit from Agappe Diagnostic Pvt. Ltd., Ernakulam, Kerala, India.

Assessment of cellular DNA damage: Alkaline single cell gel electrophoresis (comet assay) on tumor cells was carried out to determine cellular DNA damages. Single cell suspension (10^6 cells mL^{-1}) of the tumor mass was prepared in PBS and the cells were embedded in low melting agarose (0.8%) on normal melting agarose (1%) coated slides. After solidification these slides were kept in pre-cooled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 10, 1% DMSO and 1% Triton X) to ensure cell lysis for one and half hours and drained accurately. The slides were kept in alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, 0.2% DMSO, pH = 13) for 10-20 min and electrophoresed at 20 V for 30 min. The slides were then washed with distilled water and allowed to dry. The dried slides were stained with fluorescent dye, propidium iodide (50 μg mL^{-1}) and viewed under fluorescent microscope. The same procedure was used in the case of kidney tissue.

The comet parameters such as percentage DNA in tail, tail length, tail moment and olive tail moment were calculated by using software 'CASP' to analyze the DNA damages (Klaude *et al.*, 1996; Cerda *et al.*, 1997; Konca *et al.*, 2003).

Studies on morphology: Histopathological examinations of kidney and tumor from the animals of all groups were evaluated using light microscopy. The kidney and tumor tissues were excised from each group and fixed them in 10% formalin and embedded in paraffin wax. Sections of 5 micron thickness were made using a microtome and stained with haematoxylin-eosin carried out at pathological laboratory, Pushpagiri Institute of Medical Sciences and Research Centre, Thiruvlla, Kerala, India (Culling, 1963).

Statistical analysis: The results are presented as Mean±SD (standard deviation) of the studied group. Statistical analyses of the results were performed using ANOVA with Tukey-Kramer multiple comparisons test.

RESULTS

CDDP-induced cellular DNA damages in tumor and kidney cells: Figure 1a-c represents the cellular DNA comets obtained from tumor tissues of the tumor-bearing mice. It can be seen that the treatment with CDDP increases DNA comet parameters in tumor cells. The DNA comets from tumor cells treated with AST following CDDP also showed longer tails than that of untreated tumor cells. The DNA comet parameters of the kidney tissues of tumor-bearing animals are presented in Fig. 1d-f. It can be seen that CDDP administration cause damages to kidney tissues also and the DNA comet tail length is higher than that of untreated animals. This result shows the renal toxicity caused by the administration of cancer-chemotherapeutic drug, CDDP to the tumor-bearing mice. The cellular DNA comet parameters of tumor and kidney tissues of animals administered with AST following CDDP treatment was presented in Fig. 1c-f. This would indicate that the AST administration following CDDP treatment increases DNA comet parameters in tumor cells while decreases tail length in kidney cells.

Figure 2 shows the graphical representation of cellular DNA comets from tumor and kidney tissues.

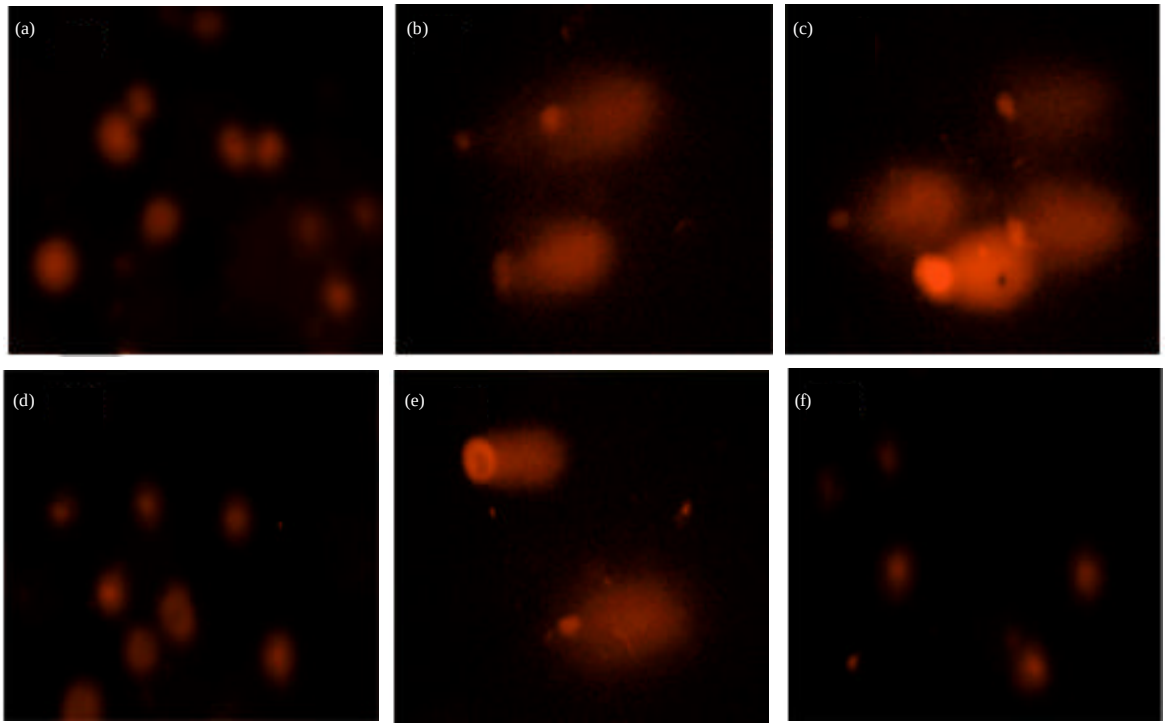

Fig. 1(a-f): Images of comet assay done in tumor and kidney cells of tumor-bearing mice (a) Tumor cells of tumor-bearing mice-no treatments, (b) Tumor cells of tumor-bearing mice treated with CDDP (12 mg kg^{-1}), (c) Tumor cells of tumor-bearing mice treated with CDDP (12 mg kg^{-1}) and AST (50 mg kg^{-1}), (d) Kidney cells of tumor-bearing mice-no treatments, (e) Kidney cells of tumor-bearing mice treated with CDDP (12 mg kg^{-1}) and (f) Kidney cells of tumor-bearing mice treated with CDDP (12 mg kg^{-1}) and AST (50 mg kg^{-1})

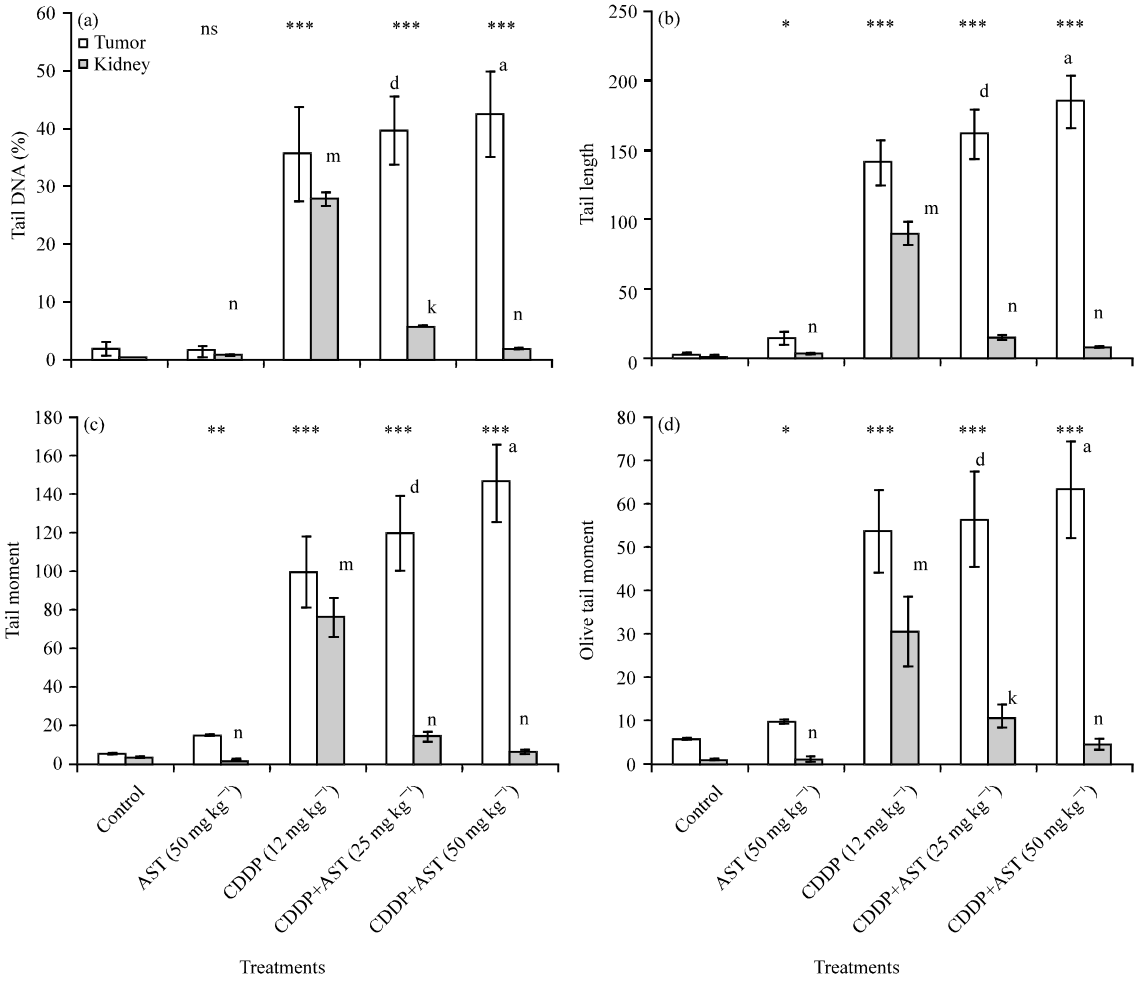

Fig. 2(a-d): Cellular DNA comet parameters such as (a) Percent tail DNA, (b) Tail length, (c) Tail moment and (d) Olive tail moment of tumor and kidney cells of tumor-bearing mice. 'ns', 'd' and 'n' indicates not significant ($p>0.05$); '*', 'a' and 'k': $p<0.05$; '**' $p<0.01$; '***' and 'm': $p<0.001$ when compared with respective controls (untreated control animals and CDDP (12 mg kg^{-1}) alone treated mice)

Study of morphology: The morphological changes in kidney and tumor tissues were evaluated by histopathological examination as described in the materials and methods. Figure 3 presents the histology of tumor tissues following various treatments. The treatment with CDDP cause decreases in the cellularity of tumor cells. AST administration following CDDP treatment also decreases cellularity of tumor tissues in a dose dependent manner.

Figure 4 represents the morphology of kidney tissues of mice bearing tumor. The treatment with CDDP cause decreases in the cellularity of the glomeruli, vacuolation of the nuclei of the lining cells and oedema of the lining of epithelial cells in the renal tubules. However, administration with AST showed near normal structural design as evident from Fig. 4.

Assessment of levels of antioxidants in renal tissues: Figure 5 represents the MDA level of kidney tissues of mice. The administration of CDDP to tumor-bearing mice showed increase in the

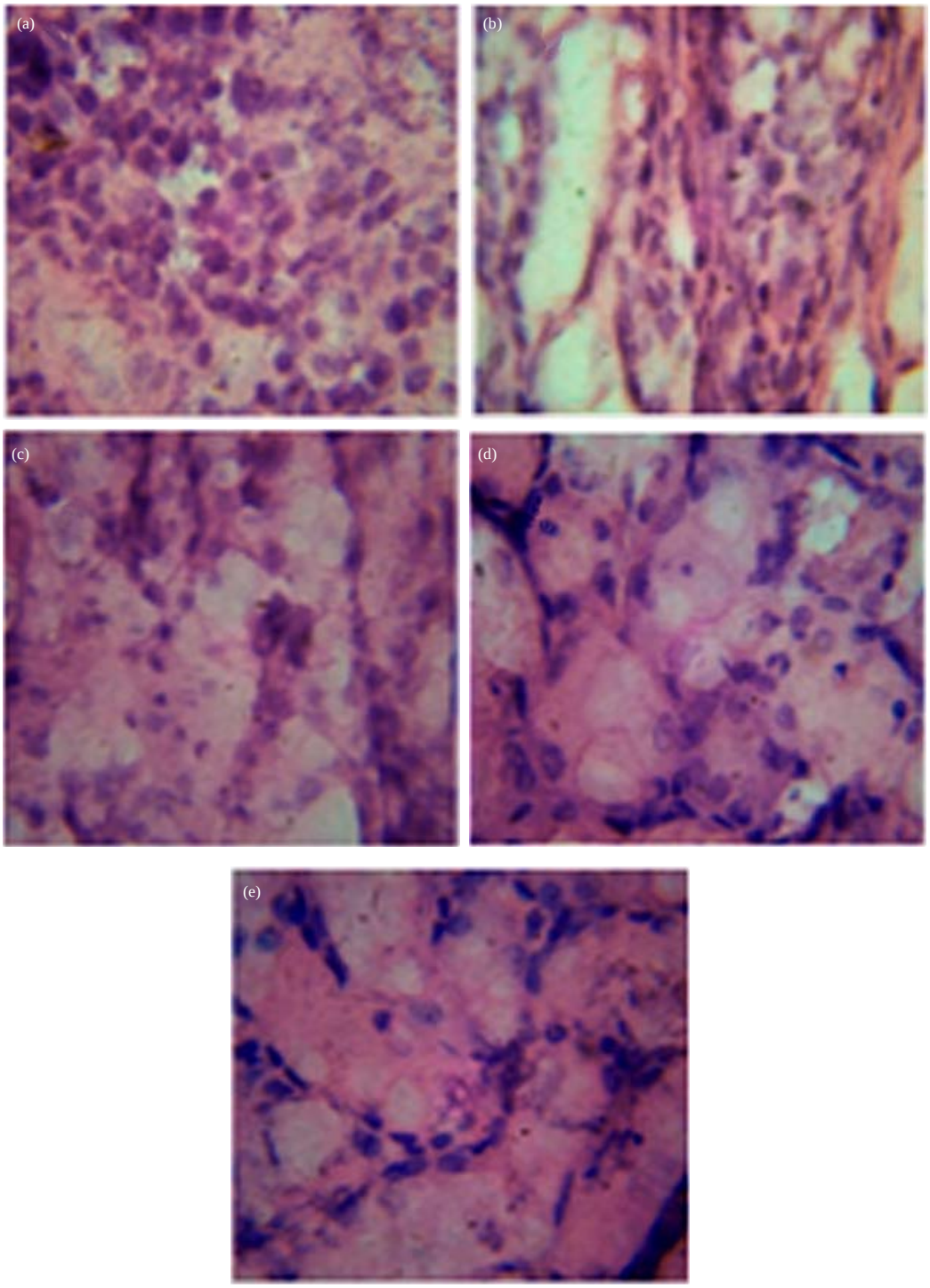

Fig. 3(a-e): Histology of tumor tissues of mice bearing tumor: Tumor tissues of mice (a) No treatments, (b) AST (50 mg kg^{-1}), (c) CDDP (12 mg kg^{-1}), (d) CDDP (12 mg kg^{-1}) and AST (25 mg kg^{-1}) and (e) CDDP (12 mg kg^{-1}) and AST (50 mg kg^{-1})

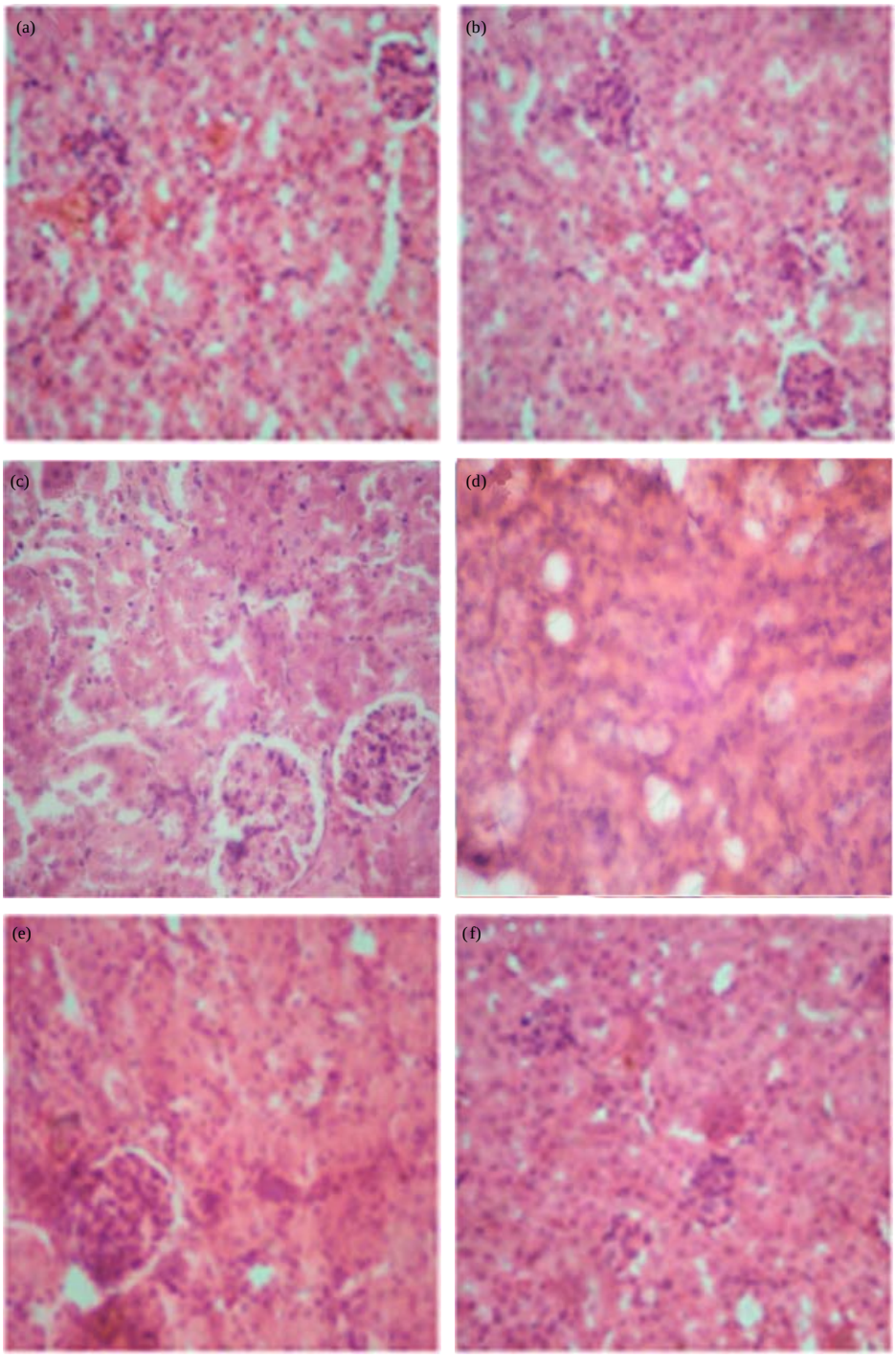

Fig. 4(a-f): Histology of kidney tissues of mice: Kidney tissues of mice (a) Without tumor, no treatments, (b) With tumor, no treatments, (c) Treated with AST (50 mg kg^{-1}), (d) Treated with CDDP (12 mg kg^{-1}), (e) Treated with CDDP (12 mg kg^{-1}) and Treated with AST (25 mg kg^{-1}) and (f) CDDP (12 mg kg^{-1}) and AST (50 mg kg^{-1})

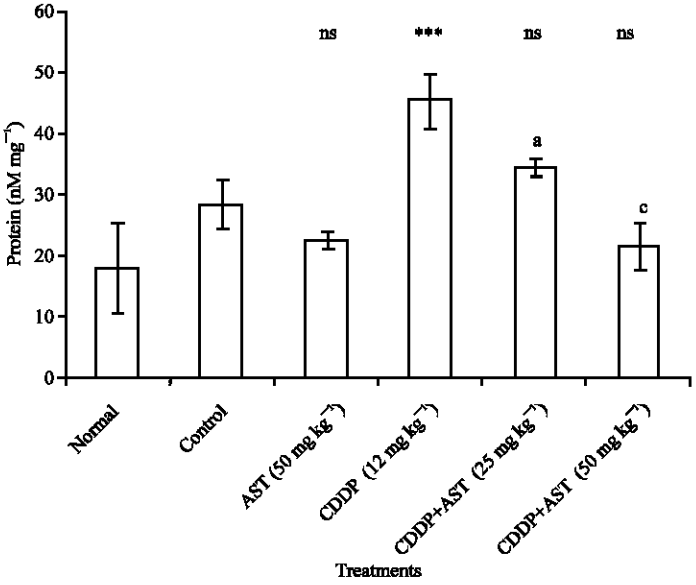

Fig. 5: Effect of AST on MDA level of kidney tissues of mice. Normal: Without tumor, no treatments, Control: Bearing tumor, no treatments AST (50 mg kg^{-1}); CDDP (12 mg kg^{-1}): Bearing tumor treated with AST (50 mg kg^{-1}) CDDP (12 mg kg^{-1}): Bearing tumor treated with CDDP (12 mg kg^{-1}) CDDP+AST (25 mg kg^{-1}): Bearing tumor treated with CDDP (12 mg kg^{-1}) and AST (25 mg kg^{-1}) CDDP+AST (50 mg kg^{-1}): Bearing tumor treated with CDDP (12 mg kg^{-1}) and AST (50 mg kg^{-1}). 'ns' and'd' indicates not significant ($p>0.05$); '**' and 'b' indicates $p<0.01$ and '***' and 'a' indicates $p<0.001$ when compared with respective controls (untreated control animals and CDDP (12 mg kg^{-1}) alone treated mice)

peroxide formation of membrane lipids in kidney tissue. However, the administration of two different doses of AST to CDDP treated tumor bearing mice showed a concentration dependent inhibition of lipid peroxidation.

A significant decrease in the levels of GSH and GPx were absent in the kidney tissues of the mice bearing tumor following treatment with CDDP as illustrated in Fig. 6a-b. It can be seen in the figure that the administration of AST increased the levels of the antioxidants GSH and GPx in the kidney tissues of CDDP treated tumor-bearing mice. The results showed that AST had a concentration dependent restorative effect on the CDDP-induced depletion of antioxidants.

Serum biochemical parameters: It can be seen in the Table 1 that the administration of CDDP to tumor-bearing mice caused elevation of the levels of urea upto 100.74±9.51 and creatinine upto 1.08±0.07 in the serum (while the untreated control animals showed only 32.27±4.76 for urea and 0.60±0.05 for creatinine). When these animals were treated with AST following CDDP administration, the elevated level of urea in the serum was significantly reduced to 54.57±13.79 and creatinine level in the serum was decreased to 0.65±0.06.

DISCUSSION

Renal toxicity is the major side effect and therapeutic limitation of CDDP, one of the commonly used antineoplastic agent (Yao *et al*., 2007). The kidney being the major route of excretion, CDDP

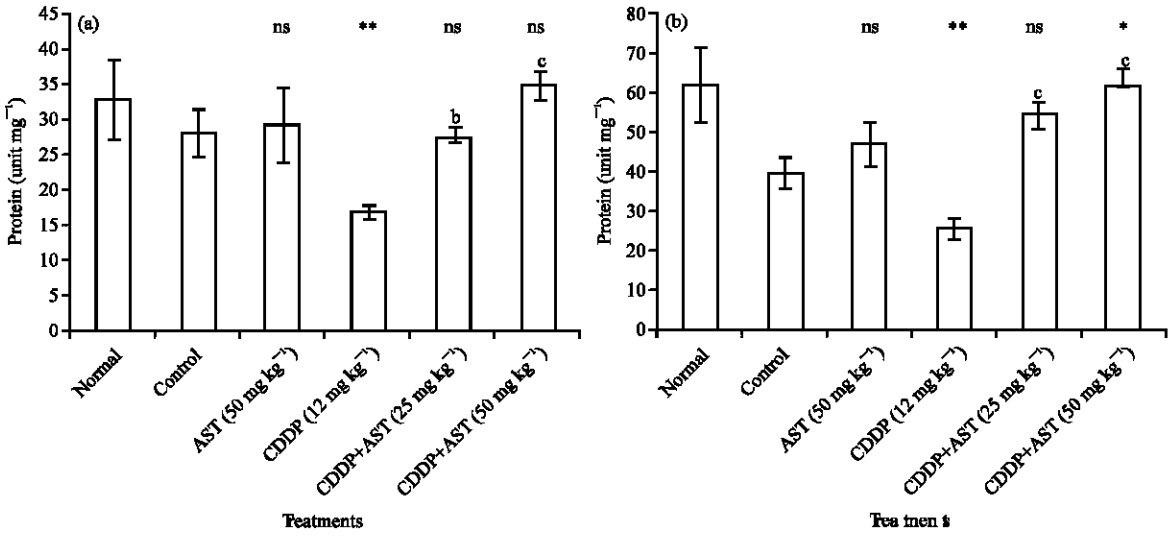

Fig. 6(a-b): Effect of AST on (a) GSH level and (b) GPx level of kidney tissues of mice ('ns' indicates not significant ($p>0.05$); '*' indicates $p<0.05$; '**' and 'b' indicates $p<0.01$and '***' and 'c' indicates '$p<0.001$' when compared with respective controls (untreated control mice and CDDP (12 mg kg^{-1}) alone treated mice)

Table 1: Effect of AST on serum markers of nephrotoxicity in CDDP treated tumor-bearing mice

Treatments	Urea (mg dL^{-1})	Creatinine (mg dL^{-1})
Normal	37.99±1.860	0.59±0.04
Control	32.27±4.760	0.60±0.05
AST (50 mg kg^{-1})	43.16±5.880	0.59±0.02
CDDP (12 mg kg^{-1})	100.74±9.510	1.08±0.07
CDDP+AST (25 mg kg^{-1})	68.43±10.98***	0.79±0.06***
CDDP+AST (50 mg kg^{-1})	54.57±13.79***	0.65±0.06***

(a) Normal: Mice without tumor, no treatments (b) Control: Mice bearing tumor, no treatments (c) AST (50 mg kg^{-1}): mice bearing tumor treated with AST (50 mg kg^{-1}) d) CDDP (12 mg kg^{-1}): mice bearing tumor treated with CDDP (12 mg kg^{-1}) (e) CDDP+AST (25 mg kg^{-1}): mice bearing tumor treated with CDDP (12 mg kg^{-1}) and AST (25 mg kg^{-1}) (f) CDDP+AST (50 mg kg^{-1}): mice bearing tumor treated with CDDP (12 mg kg^{-1}) and AST (50 mg kg^{-1}). Note: Values are expressed as Mean±SD (n = 5) where ***indicates $p<0.001$ when compared with respective control (CDDP (12 mg kg^{-1}) treated mice)

gets accumulated in this organ. The proximal tubule cells are selectively damaged by cisplatin. The mechanism for this renal cell injury has been the focus of intense investigation for many years and recent studies suggest that inflammation, oxidative stress and apoptosis are the major reasons for renal injury (Kuhlmann *et al*., 1997).

The cytotoxic effect of CDDP is due to cellular DNA damages. CDDP forms a platinum complex inside the cell which binds to DNA and this CDDP -DNA crosslinks cause cytotoxic lesions in tumors and other dividing cells. The interstrand cross-linking of DNA causes the cells to undergo apoptosis or programmed cell death (Siddik, 2003; Fuertes *et al*., 2003).

There are two possible mechanisms by which cisplatin entail renal toxicity. One mechanism is by forming conjugate with glutathione and metabolizing through Gamma-Glutamyl

Transpeptidase (GGT) and cysteine S-conjugate β-lyase-dependent pathways to form a reactive thiol, a potent nephrotoxin. The other mechanism is by the cleavage of GSH with the help of an enzyme GGT. This results in the depletion of tissue GSH level and thereby weakens the defensive mechanism of cells against oxidative stress (Merouani *et al.*, 1996; Townsend and Hanigan, 2002; Townsend *et al.*, 2003). Cisplatin also increases the production of TNF-α (Tumor Necrosis factor-alpha) in many cells including renal cells. TNF-α plays a pathogenic role in generating acute renal failure by stimulating cytokine and chemokine expression in the kidney (Zhang *et al.*, 2007; Ramesh and Reeves, 2002).

The highest concentration of cisplatin is found in cytosol, mitochondria, nuclei and microsomes in kidney tissues. In mitochondria, cisplatin induces oxidative stress resulting in the peroxidation of membrane lipids (Huang *et al.*, 2001; Fujieda *et al.*, 2011). The present work shows the ability of a caroteinoid, AST to mitigate the renal toxicity induced by cisplatin by activating the defence mechanism of cells against oxidative stress.

The treatment of mice with CDDP results in increased DNA damage in normal as well as tumor tissues, but AST administration prevented cellular DNA damages preferentially in the kidney. There was no reduction in the CDDP-induced DNA damages in tumor cells. Also the histopathology observations demonstrate that the CDDP-induced tissue alterations are prevented by AST in kidney tissues. At the same time AST treatment did not have any effect on the CDDP-induced alterations in tumor tissues.

CONCLUSION

The present study suggests the use of AST as a useful adjuvant for renal protection during therapy with CDDP. Compelling evidence has been proved to reveal that administration of AST to the tumor-bearing mice after CDDP treatment offered protection to renal tissue from CDDP- induced lesions without offering any protection to the tumor. The mechanism of renal protection by AST could be ascribed to its antioxidant activities.

ACKNOWLEDGMENT

SS express gratitude to UGC, Government of India for the award of JRF.

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