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Ameliorative Effect of Ethanolic Extract of *Cichorium intybus* on Cisplatin - Induced Nephrotoxicity in Rats

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Abstract. In the study of the possible ameliorative effect of the *Cichorium intybus vs.* cisplatin-induced nephrotoxicity, no sign of toxicity was observed in rats on administration of ethanolic extract of *C. intybus* (500 mg/kg) with cisplatin (3 mg/kg). Oral administration of *C. intybus* extract reduced cisplatin-induced nephrotoxicity and also prevented elevated plasma creatinine, urea and nitrate, plasma and tissue MDA levels and restored antioxidant enzymes.

Keywords: cisplatin, *Cichorium intybus*, nephrotoxicity, antioxidant enzymes

Introduction

Cisplatin, or cis-diamminedichloroplatinum (II) (CDDP) is extensively used for the management of oncological disorders, particularly of ovary, testis, bladder, head and neck (Hamers *et al*., 1991). Although higher doses of cisplatin are more efficacious for cancer chemotherapy, but such therapy manifests non-haematological toxicities like nephrotoxicity (Bodenner *et al*., 1986). Formation of free radicals by CDDP (Matsushima *et al*., 1998), leading to oxidative stress, has been shown to be one of the main pathogenic mechanisms of these toxicities and side effects of nephrotoxicants (Greggi *et al*., 2001).

Antioxidant-based drugs/formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer have appeared during the last 3 decades (Devasagayam *et al*., 2004) attracting a great deal of interest in research on natural antioxidants. Subsequently, a worldwide trend has increased towards the use of natural phytochemicals present in berry crops, tea, herbs, oilseeds, beans, fruits and vegetables (Jiao and Wang, 2000). Several herbs and spices have been reported to exhibit antioxidant activity, including rosemary, sage, thyme, nutmeg, turmeric, white pepper, chilly pepper, ginger and several Chinese medicinal plants extracts (Ali *et al*., 2008; Lee and Shibamoto, 2000; Jiao and Wang, 2000; Deiana *et al*., 1999). The majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, ligands, catechins and isocatechins. In addition to the above compounds found in natural foods, vitamins C and E, carotene, α-tocopherol are known to possess antioxidant potential (Maliakel *et al*., 2008; Lee *et al*., 2003; Kikuzaki and Nakatani, 1993; Jitoe *et al*., 1992; Kikuzaki *et al*., 1991).

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Cichorium intybus Linn. (chicory) is a medicinally important plant that belongs to the family Asteraceae. (Nandagopal and Ranjitha, 2007; Varotto *et al*., 2000). The root of the chicory has been used as folk medicine in Pakistan for treating liver diseases and hepatic system disorders. A group of clinical researchers recently isolated a phenolic compound called esculetin from the extract of *C. intybus* root and further confirmed that it is a hepatoprotectant compound. This extract inhibited the oxidative degradation of DNA in the tissue debris of mice liver (Gilani *et al*., 1998). Ahmed *et al*. (1998) reported antiulcerogenic effect of the aqueous extract of *C. intybus*.

A decoction of dried root is a noted treatment for stomach acidity. The tonic property of *C. intybus* makes it an excellent mild bitter tonic for the liver, gall-bladder, digestive tract and is also used for cleaning the urinary tract (Khan and Aslam, 2004) and in fever, vomiting and as diuretic (Hussain *et al*., 2008). This herb is used for rheumatic conditions and gout as well. The leaves and flowers can aid in digestion. The bruised leaves are used externally as a poultice for the relief of swellings, skin lacerations and inflammations (Rehmat *et al*., 2006). Moreover, *C. intybus* reduces extra heat of various organs and acts as deobstruent, tonic and febrifuge (Aslam, 2006).

C. intybus extract has high antioxidant potential as reported previously by Nandagopal and Ranjitha (2007). Despite the above described role of *C.intybus* in the treatment of various diseases, no attempt has been as yet made to investigate its role as an antioxidant in prevention of drug-induced nephrotoxicity. The present study was designed to evaluate the role of altered antioxidant enzymes in the ameliorative effect of *C. intybus* on cisplatin-induced nephrotoxicity, using rat models.

Materials and Methods

Animals and diet. 24 Wistar male albino rats (200-260 g b.w.) were purchased from the animal house of International Center for Chemical and Biological Sciences (ICCBS), Karachi, Pakistan for the study. Animals were acclimatized to the laboratory conditions one week before the start of experiment and caged in a temperature controlled room (23±4 °C). Rats had free access to water and standard rat diet. The experiments were conducted in accordance with ethical guidelines of internationally accepted principles for laboratory use and care in animal research (Health Research Extension Act, 1985).

Crude extraction of *C. intybus* Linn. *C. intybus* Linn plant was collected from the Northern area of Pakistan and identified by experts. 10 kg of dried aerial parts of *C. intybus* plant was powdered, screened and soaked in ethanol (10 litre) for one week. The filtrate was separated and concentrated under vacuum using a rotary evaporator, as a dark green semisolid (yield 44.4%). The extract was standardized by quantification of a reference standard with HPLC system. The reference standard was gallic acid.

Study design. The animals were divided into four experimental groups (n=6). Each group received the following treatment:

Group I, control, remained untreated

Group II received cisplatin i.p. (3 mg/kg b.w.) for 5 alternate days

Group III received *C. intybus* extract orally (500 mg/kg b.w.) for 10 days

Group IV received *C. intybus* extract orally (500 mg/kg b.w.) for 10 days + cisplatin i.p. (3 mg/kg b.w.) for 5 alternate days (the extract was administered orally, 30 min prior to administration of cisplatin).

Sample collection. After 48 h of administration of the last dose to the treated groups, animals were anesthetized, decapitated and blood was sampled from head wounds in lithium heparin coated tubes. A portion of blood was used to get plasma. Kidneys were excised, trimmed of connective tissues, rinsed with saline to eliminate blood contamination, dried by blotting with filter paper and weighed. The tissues were then kept in freezer at 70 °C until used for analysis.

Histological examination. Left kidney was quickly removed, immersed in 10% formaline, dehydrated and embedded in paraffin, sectioned at 3 µm, stained with hematoxylin and eosin (H&E) and examined by light microscopy.

Biochemical Assay. *Assessment of renal functions*. Plasma samples were assayed for urea and creatinine, spectrophotometrically by Oxime method (Butler *et al*., 1981) and Jeff's method (Spierto *et al*., 1979), respectively.

Assessment of oxidative stress. Plasma sample was assayed for nitrate and MDA.

Plasma nitrate estimation by ion selective electrode (ISE) method. Plasma nitrate was estimated by ion selective electrode using ion meter 3345 (Jenway) which detected free ionic nitrate (NO_3^{-2}) in plasma/serum sample. In a clean glass test tube, 1.7 ml of deionized water was taken; 0.2 ml of ISAB(4M KCl) (ionic strength adjusting buffer) and 0.1 ml of plasma were added and mixed well. The electrode was rinsed, blotted dry and the result was recorded in mg/l.

Kidney homogenate preparation. Kidney homogenate was obtained by using a tissue homogenator, Ultra Taurax T-25 Polytron, at 4 °C. The homogenates (1:10 w/v) were prepared by using a 100 mmol KCl buffer pH 7 containing EDTA 0.3 mmol. All homogenates were centrifuged at 600 g for 60 min at 4 °C and the supernatant was used for biochemical assays.

Assessment of tissue lipid peroxides. 10 µl of BHT (butylated hydroxytoluene) (0.5 M in acetonitrile) was added to prevent homogenate from oxidation and the homogenate was stored at -70 °C until analysis was made for malonyldialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE).

Estimation of MDA. The MDA content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al*. (1979). Briefly, the reaction mixture, consisting of 0.2 ml of 8.1% sodium dodecyle sulphate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid, was added to 0.2 ml of 10% (w/v) of the homogenate. The mixture was brought up to 4.0 ml with distilled water and heated at 95 °C for 60 min. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of *n*-butanol and pyridine (15:1 v/v) was added and the mixture was centrifuged. The organic layer was taken out and its absorbance was measured at 532 nm and compared with those obtained from MDA standards (1,1,3,3- tetramethoxy-propane). The concentration values were calculated by measurement of absorption against standard absorption.

Estimation of 4-HNE. The 4-HNE content was measured by the method of Kinter (1996). Briefly, the assay mixture consisted of 2 ml of filtrate with 1 ml of 2,4-dinitrophenyl hydrazine which was kept for 1 h at room temperature. Sample was extracted with hexane, and the extract was evaporated at 40 °C. Cooled sample was measured at 350 nm against methanol blank. The standard was 4-HNE-DMA (dimethyle acetal).

Assessment of antioxidant status. *Estimation of catalase*. Catalase activity was assayed by the method of Sinha (1972). Briefly, the assay mixture consisted of 1.96 ml phosphate buffer (0.01 M, pH 7.0), 1.0 ml hydrogen peroxide (0.2 M) and 0.04 ml (10%) homogenate in a final volume of 3.0 ml. 2 ml dichromate acetic acid reagent was added to 1 ml of reaction mixture, boiled for 10 min, cooled and changes in the absorbance were recorded at 570 nm.

Estimation of SOD. Levels of SOD in the cell free supernatant were measured by the method of Kono (1978). Briefly, 1.3 ml of solution A (0.1 mm EDTA containing 50 mM Na_2CO_3, pH 10.0), 0.5 ml of solution B (90 µm NBT i.e. nitro blue tetrazolium dye), 0.1 ml of solution C (0.6% TritonX-100 in solution A) and 0.1 ml of solution D (20 mmol hydroxylamine hydrochloride, pH 6.0) were mixed and the rate of NBT reduction was recorded for one min at 560 nm. 0.1 ml of the supernatant was added to the test and reference cuvette, which did not contain solution D. Finally, the percentage inhibition in the rate of reduction of NBT was recorded as described above. One enzyme unit was expressed as inverse of the amount of protein (mg) required for inhibiting the reduction rate by 50% in one minute.

***Statistical analysis*.** Results are presented as mean± SE. Statistical significance of the differences between the control and the test values were evaluated by Student's t-test. Statistical probability of P <0.01, <0 .05 was considered to be significant.

Results and Discussion

In the evaluation of effect of *C. intybus* on cisplatin-induced nephrotoxicity in rats, observations were made of four groups of rats i.e. control, cisplatin treated, *C. intybus* treated and *C. intybus* treated with cisplatin pretreated rats. Results are given as under:

Effects on body weight, kidney weight and relative kidney weight. Table 1 and Fig. 1 show effects of cisplatin, *C. intybus* extract and pretreatment of *C. intybus* extract with cisplatin on kidney and body weight, respectively. A marked decrease in rats body weight was observed in cisplatin-treated rats and CDDP + *C. intybus* group on the 10th day. Alone *C. intybus* extract also showed decrease in body weight but the decrease was less than that in cases of cisplatin treatment and CDDP + *C. intybus* pretreatment groups. Kidneys of rats treated with cisplatin were enlarged with significantly increased kidney weight ($P < 0.05$) and relative kidney weight; however, the results, were not significant as compared to the control. Sole treatment and pretreatment with *C. intybus* extract showed significantly increased relative kidney weight ($P < 0.05$).

Table 1. Effect of treatments on kidney weight and relative kidney weight of the study groups

Parameters	Control	Cisplatin (3mg/kg)	*C. intybus* (500mg/kg)	*C. intybus* +cisplatin
Mean kidney weight (g)	0.672 ±0.132	0.861** ±0.118	0.791 ±0.070	0.863 ±0.177
Mean relative kidney weight 10^{-3}	2.516 ±0.784	3.551 ±0.881	3.452** ±0.191	3.696** ±0.269

Values are mean±SE; significant difference between various groups by t-test; **P<0.05

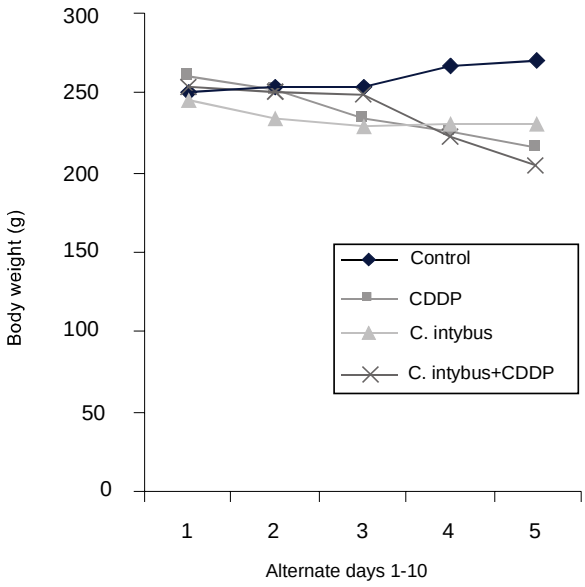

Fig. 1. Effect of treatments on body weight in the study groups.

Histology of kidney. After treatment for alternate days, cisplatin-treated rats showed glomeruli congestion, marked ATN in proximal and distal tubules, decrease in the height of epithelial cells, shedding of atypical cytoplasm and loss of brush boarder Fig. (2b). Wide opening of lumen and marked congestion in vessels (++++) were also seen. Pretreatment with *C. intybus* extract markedly prevented congestion in glomeruli and vessels (+) and other alterations Fig. 2(d). Treatment with *C. intybus* extract alone showed slight congestion in vessels and glomeruli (Fig. 2(c)). Histology of kidney of control group is given in Fig. 2(a) and histological activity index (HAI), in Table 2.

Plasma urea level. Cisplatin treated rats showed marked increase in level of plasma urea (35.7±3.46, P<0.01) as compared to the control (Fig. 3). *C. intybus* extract alone showed slightly increased urea level but the result was not significant. *C. intybus* extract partially prevented increase in urea level

in CDDP + *C. intybus* group (27.0±3.60, P<0.01) as compared to the control.

Plasma creatinine level. Figure 4 shows a significant effect on plasma creatinine level in cases of cisplatin and pretreatment with *C. intybus* extract. Creatinine level was significantly increased (1.03 ± 0.13, P < 0.01) in cisplatin-treated rats as compared to the control. Pretreatment with *C. intybus* extract partially prevented increase in plasma creatinine level in CDDP + *C. intybus* group(0.36±0.28, P<0.05). *C. intybus* extract alone produced no significant change.

Plasma MDA level. Increased level of plasma MDA was observed in cisplatin treated rats but the result was not significant. A significant decrease in plasma MDA was noted after only *C. intybus* extract treatment (3.26 ± 0.91, P< 0.01). The increased MDA level was prevented by pretreatment with *C. intybus* (10.03 ± 4.71, P < 0.05) in CDDP + *C. intybus* group (Fig. 5) as compared to control.

Table 2. Histological activity index (HAI)

Groups	Congestion in vessels	Congestion in glomeruli	Congestion in tubules	Interstitial inflammation	Interstitial edema
Control	-	-	-	-	-
Cisplatin	+4	+3	+4	+1	-
C. intybus	+1	+1	-	-	-
Cisplatin +*C. intybus*	+1	+1	-	-	-

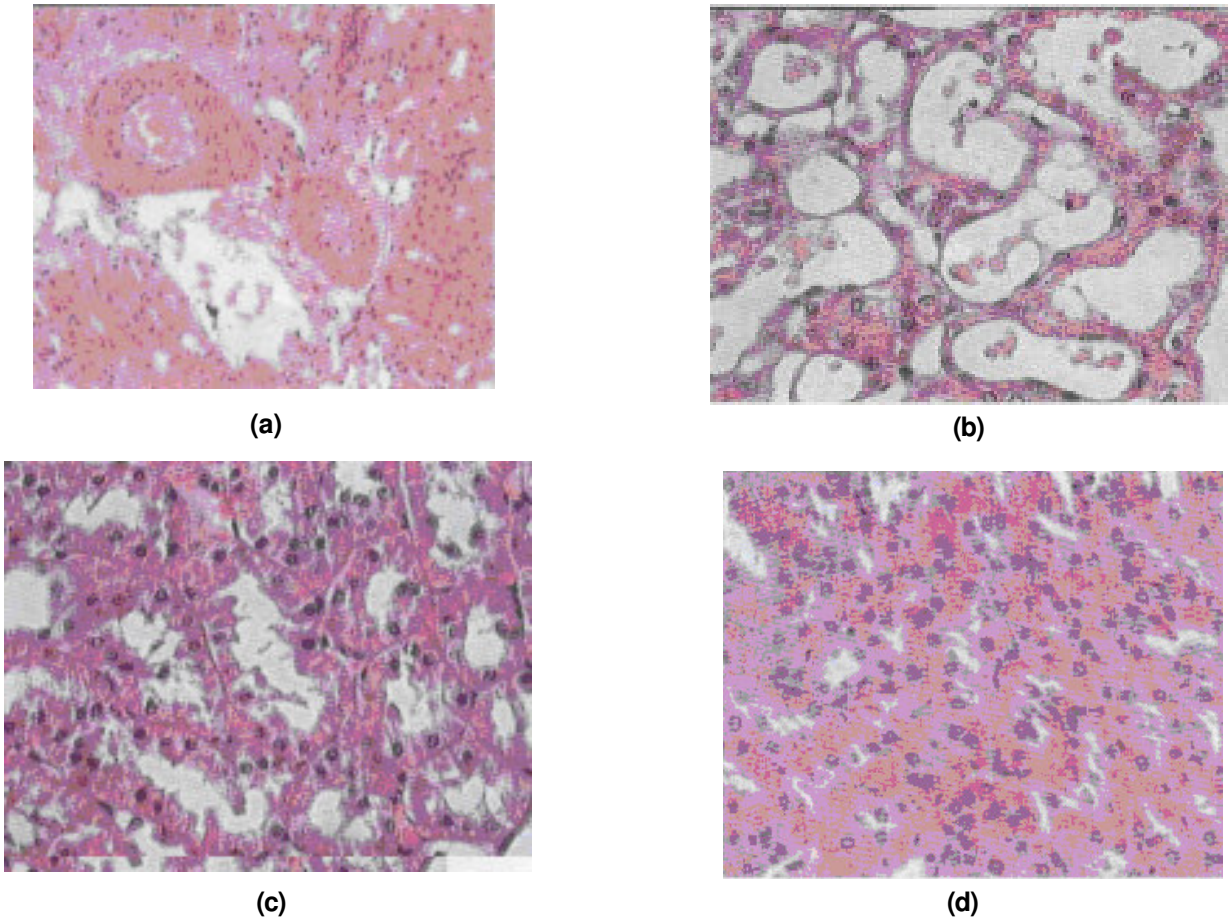

Fig. 2. (a-d) Histology of kidney in (a) control; (b) cisplatin treated rats; (c) *C. intybus* treated rats; (d) cisplatin + *C. intybus* pretreated rats.

(a) Normal kidney histology of control rats; (b) histological abnormalities after cisplatin administration for 5 alternate days; (c) *C. intybus* extract did not produce any histological alterations; (d) histological abnormalities and their prevention by *C. intybus* extract

Tissue MDA level. Figure 6 shows oxidative status in rat kidney after cisplatin treatment, *C. intybus* extract treatment and cisplatin with *C. intybus* extract treatment. Tissue MDA level increased in cisplatin treated rats. The correction of stress was indicated in CDDP + *C. intybus* group. *C. intybus* extract treatment alone showed significant decrease in MDA level ($1.20 \pm 0.35, P < 0.01$).

Tissue 4-HNE level. A significant increase in tissue 4-HNE was observed in cisplatin treated rats ($412.13 \pm 69.09, P < 0.01$) as compared to the control, indicating oxidative stress (Fig. 7). In CDDP + *C. intybus* group, 4-HNE level was not different as compared to the control group. *C. intybus* extract alone treatment produced no significant result.

Plasma nitrate level. Nitrate level in plasma was increased ($23.49 \pm 1.97, P < 0.01$) (Fig. 8) in cisplatin treated rats, indicating nitrosative stress. CDDP + *C. intybus* group showed slightly decreased nitrate level but results were not significant. *C. intybus* extract treatment solely showed no significant effect.

Tissue catalase level. Tissue catalase level decreased significantly ($25.8 \pm 2.5, P < 0.01$) in cisplatin treated group as compared to the control (Fig. 9). *C. intybus* extract prevented decrease in catalase level in CDDP + *C. intybus* group but results were not significant, while only *C. intybus* extract produced no significant effect.

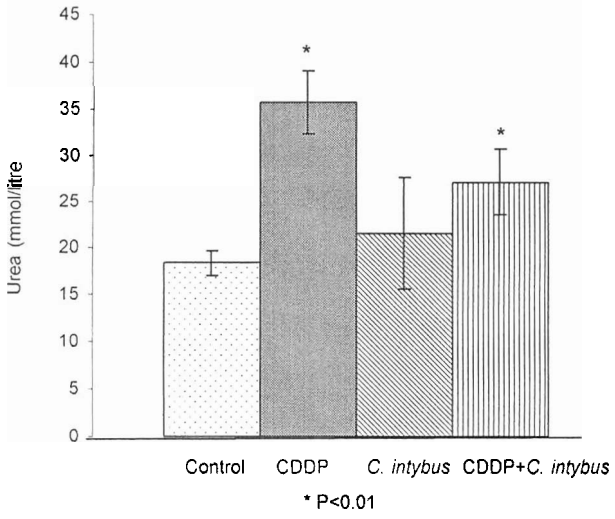

Fig. 3. Plasma urea level in the study groups.

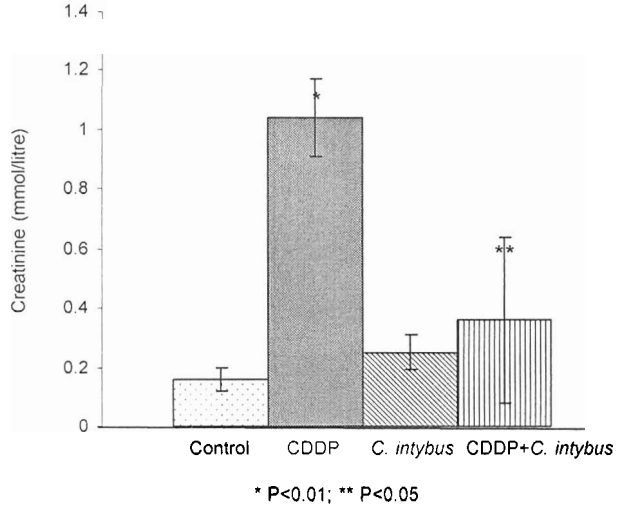

Fig. 4. Plasma creatinine level in the study groups.

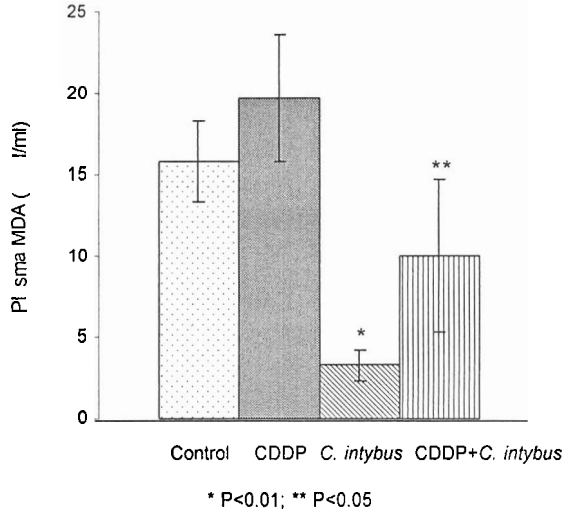

Fig. 5. Plasma MDA level in the study groups.

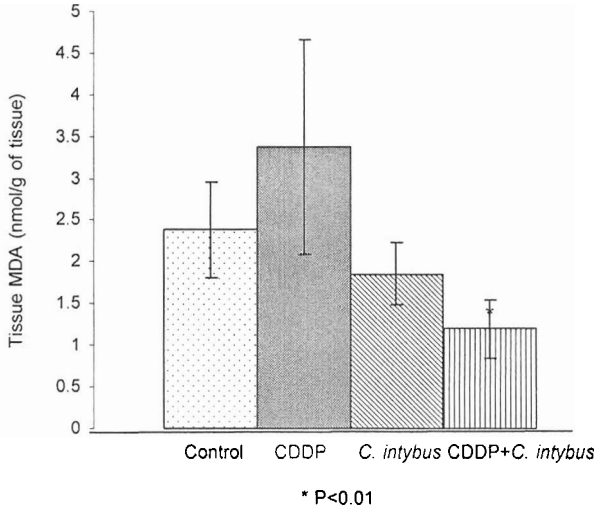

Fig. 6. Tissue MDA level in the study groups.

Tissue SOD level. Figure 10 shows a significant effect on cisplatin treated (17.61 ± 4.19, $P < 0.05$) rats. Cisplatin treatment decreased the SOD level significantly. In CDDP + *C. intybus* group the results were not different as compared to the control group. *C. intybus* extract alone produced no significant effect.

Reactive oxygen species (ROS) are constantly generated in our body. Production of ROS is essential for a number of biochemical reactions involved in the synthesis of prostaglandins, hydroxylation of proline and lysine, oxidation of xanthine and other oxidative processes. Excessive oxidation leads to impairment of cell functions and development of morbid conditions (Ames *et al*., 1993).

There is evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is increasing interest in the protective biochemical functions of natural antioxidants contained in spices, herbs and medicinal plants (Noda *et al*., 1997).

In the present study, the rats treated with cisplatin showed a decrease in body weight (Fig. 1). Mora *et al*. (2003) suggested that CDDP-induced weight loss might be due to gastrointestinal toxicity and reduced ingestion of food.

Plasma urea and creatinine levels significantly increased after administration of cisplatin (3 mg/kg) on 5 alternate days (Fig. 2(b), 3, 4), showing insufficiency of renal function.

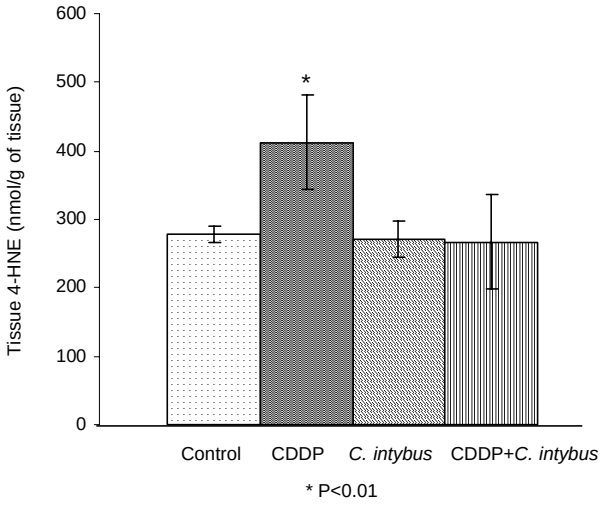

Fig. 7. Tissue 4-HNE level in the study groups.

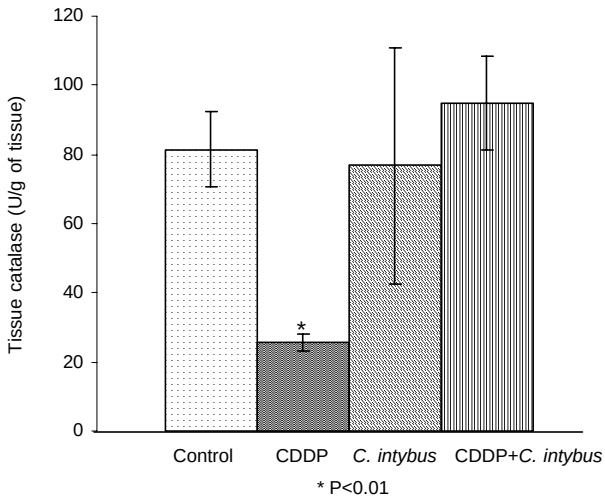

Fig. 9. Tissue catalase level in the study groups.

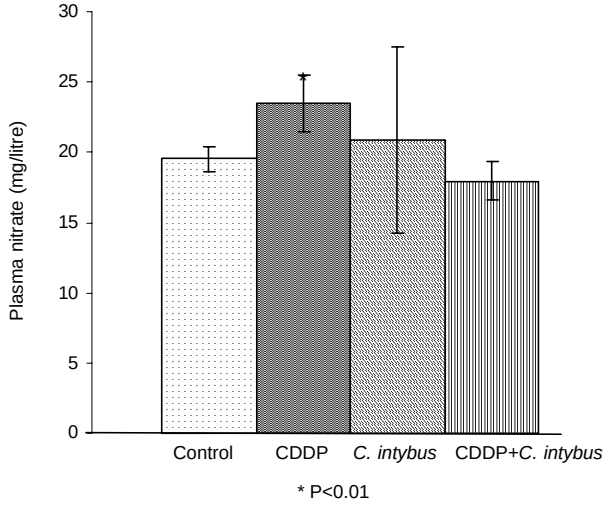

Fig. 8. Plasma Nitrate level in the study groups.

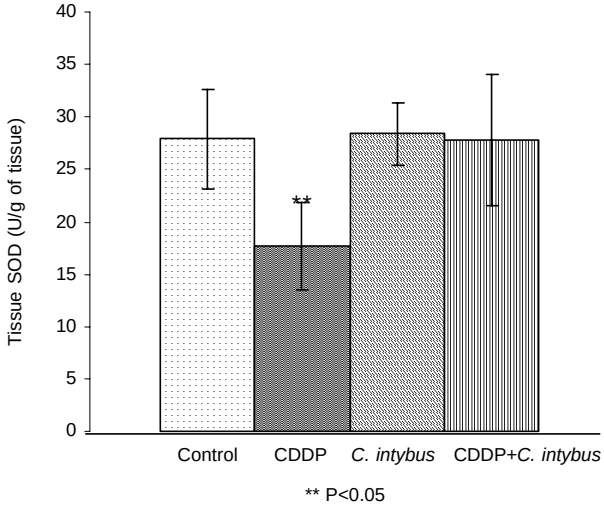

Fig. 10. Tissue SOD level in the study groups.

Studies in animals have established that tubular injury plays a central role in the reduction of glomerular filtration rate in acute tubular necrosis. Two major tubular abnormalities could be involved in the decrease in the glomerular function in cisplatin treated rats through obstruction and backleak of glomerular filtrate (Ozen *et al*., 2004).

Alterations in glomerular function in cisplatin-treated rats may also be secondary to reactive oxygen species (ROS) (Leena and Alaraman, 2005), which induce mesangial cells contraction, altering the filtration surface area and modifying the ultrafiltration coefficient factors that decrease the glomerular filtration rate (Ozen *et al*., 2004). In accordance with the previous findings, our result confirmed increased level of urea and creatinine in cisplatin treated group. A significant change in urea and creatinine levels, after pretreatment with *C. intybus* in cisplatin treated rats, provided an evidence of prevention of toxicity developed by cisplatin treatment (Fig. 2d, 3, 4).

Cisplatin induced oxidative stress is associated with increased level of MDA, 4-HNE and NO_3^{-2} (Fig. 5-8). Free radicals are known to play an important role in cisplatin-induced nephrotoxicity. The free radicals and reactive oxygen species induce oxidative stress in kidneys (Uslu and Bonavida, 1996).

MDA and 4-HNE (4-hydroxy-2-nonenal) are the end products produced by the decomposition of w_3 and w_6 polyunsaturated fatty acids (Yildirim *et al*., 2003). Due to cisplatin administration, platinum sulphydryl group complexes formed are taken up by renal cells and stabilized by intracellular GSH for several hours. In case of intracellular GSH depletion the complexes undergo rapid transformation to receive metabolites. This depletion seems to be the prime factor that permits lipid peroxidation and impairs antioxidant enzymes (Ban *et al*., 1994).

NO is able to react with O_2^- to produce $ONOO^-$, which is a powerful oxidant, more reactive than its precursors, and has been implicated in an increasing, acute renal ischemia (Ganther and Lawrence, 1997). The increase in renal $ONOO^-$ induced by cisplatin may be secondary to the increase in NO and O_2^- production. The O_2^- increase in nephrotoxicity due to cisplatin may be simply the consequence of the mitochondrial dysfunction (Chirino *et al*., 2004; Ozen *et al*., 2004) and the decrease in superoxide dismutase activity (Kadikoylu *et al*., 2004). The present investigation indicate that *C. intybus* extract produced no sign of toxicity in rats. Survival rate of rats in CDDP + *C. intybus* group showed protective effect of *C. intybus*. Alone *C. intybus* lead to marked decrease in tissue MDA level and slight decreased plasma MDA level. High intake of *C. intybus* may be responsible for less production of ROS.

Decreased antioxidant enzymes levels (SOD, catalase) observed in this study after cisplatin administration (Fig. 9-10), might be due to the loss of copper and zinc, which are essential for enzyme activity (Sharma, 1985). The decrease in SOD activity is insufficient to scavenge the superoxide anion produced during the normal metabolic process. The superoxide anion can cause initiation and progression of lipid peroxidation (Sheena *et al*., 2003).

Similarly, decreased catalase activity in cisplatin treated rats decreases the activity of kidney to scavenge the toxic H_2O_2 and lipid peroxides. Restoration of renal oxidative enzymes by pretreatment with *C. intybus* extract suggests the extract is capable of protecting the antioxidant enzymes.

The data discussed in this study shows that cisplatin treatment increased the MDA, 4-HNE and NO^-_3 and decreased SOD and catalase level, which implicated the nephrotoxic effect and produced the imbalance between oxidative status and antioxidant enzymes in kidney. Pretreatment with *C. intybus* counteracted these deleterious effects. The active phytochemicals other than gallic acid may have been responsible for antioxidant activity. However, alone *C. intybus* showed less production of ROS and decreased body weight, which intimates that high doses of *C. intybus* could interfere with the important biological functions. It is, therefore, suggested that the therapeutic doses of *C. intybus* could be adjusted for preventing cisplatin-induced nephrotoxicity. Moreover, combined therapy of *C. intybus* extract offers protective effect.

Acknowledgement

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