

Evaluation of Protective Effect of Different Doses of Terminalia arjuna Bark Ethanollic Extract on Cisplatin Induced Oxidative Nephrotoxicity in Rats

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Abstract

Cisplatin (CP), a platinum compound, is one of the most active cytotoxic drugs used for cancer treatment. Nephrotoxicity is severe dose limiting side effect of this drug. Abnormal production of reactive oxygen species (ROSs) leading to oxidative stress has been implicated in kidney toxicity by Cisplatin. Here the study was aimed to evaluate nephroprotective effect of ethanollic extract of Terminalia arjuna bark (EETAB) at the doses (200 & 400 mg/kg, body weight) against Cisplatin (7.5 mg/kg, i.p) induced nephrotoxicity in rats. The evaluation was done by measuring % change in body weight, renal function tests such as Blood Urea Nitrogen (BUN), Serum Creatinine (Cr), Serum Total Protein (TP) and also Kidney SOD (Superoxide dismutase), CAT (Catalase), GSH (Reduced glutathione) and MDA (Malondialdehyde) levels altered by Cisplatin administration. Rats treated with EETAB2 (400mg/kg) significantly ($P < 0.001$) reduced the elevated levels of BUN, Cr, TP, MDA and significantly ($P < 0.001$) increased the levels of SOD, GSH, and CAT by restoring kidney architecture. In conclusion EETAB2 (400mg/kg) attractively showed the protection against Cisplatin nephrotoxicity.

Keywords: Cisplatin, Nephrotoxicity, Terminalia arjuna, Reactive oxygen species, Renal function tests.

Introduction

Cisplatin [cis-diamminedichloroplatinum (II)] is a platinum containing drug used in various cancers⁽¹⁾. Nephrotoxicity is one of the serious dose limiting side effect of the drug⁽²⁾, leading to acute kidney failure which is a major clinical problem seen in 20% of patients despite the use of hydration and diuretics^(3, 4). The concentration of Cisplatin in proximal tubule cells of S₃ segment is 5 times more when compared to serum^(5, 6). The mechanism by which Cisplatin targets the cancer cells is different from its action on proximal tubule cells⁽⁷⁾. Cisplatin is conjugated to glutathione in the proximal tubule cells and gets bio-transformed to a reactive thiol, which is a potent nephrotoxin. The pathway is γ -glutamyl transpeptidase and cysteine-S-conjugate β -lyase dependant⁽⁸⁾. Free radical generation in the renal tubule cells and its subsequent lipid peroxidation have been suggested to be a main cause for Cisplatin induced nephrotoxicity^(4, 9).

Terminalia arjuna (Family: Combretaceae) is an ancient Indian medicine used for different ailments⁽¹⁰⁾. So, far the activities reported are anti-atherogenic⁽¹¹⁾,

analgesic⁽¹²⁾, wound healing⁽¹³⁾, antioxidant⁽¹⁴⁾, hepatoprotective⁽¹⁵⁾, hypolipidemic⁽¹⁶⁾, antiulcer⁽¹⁷⁾, anti-inflammatory, immunomodulatory and antinociceptive activities⁽¹⁸⁾. The phytoconstituents of the Terminalia arjuna bark include triterpenoids (arjunic acid, arjunolic acid and terminic acid), glycosides (terminoside A and arjunetin), β -sitosterol, flavonoids (arjunolone, arjunone, bicalein, luteolin, gallic acid, quercetin and kampferol), tannins (punicallin, punicalagin, and casuarin)⁽¹⁰⁾. The flavonoid content in 100 g of Terminalia arjuna bark is 5698 ± 531 mg⁽¹⁹⁾.

Ethanol was selected as solvent for extraction since the extraction efficacy of components showing antioxidative properties are in the following order: ethanol > methanol > acetone⁽²⁰⁾.

Utilizing the antioxidant, free radical scavenging and anti-inflammatory activity of Terminalia arjuna bark, the present study was aimed to evaluate its protective effect on cisplatin induced oxidative nephrotoxicity in rats.

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Materials and Methods

Animals

Adult male Wistar albino rats weighing 170 to 200g were obtained from the animal facility of albino laboratories, Hyderabad. They were housed in polypropylene cages and maintained controlled temperature ($22 \pm 3^\circ\text{C}$) with a 12 hr light/dark cycle. During the experimental period they were to free access to food and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Vaagdevi College of Pharmacy, Warangal, India (1047/ac/07/CPCSEA).

Drug and chemicals

Cisplatin (50mg/50ml) was a marketed product obtained from Neon laboratories (code 66618), UREA/BUN kit of Reckon diagnostics Pvt. Ltd. Both Creatinine and Total protein test kit of CPC Diagnostics Pvt. Ltd and all other chemicals used were of analytical grade obtained commercially.

Plant collection

Terminalia arjuna stem bark was obtained from the nearby areas of Warangal forests. The obtained bark was authenticated by Dr. Vatsavaya S. Raju, Plant Botanist, Kakatiya University, Warangal and a voucher specimen (No.1873) has been deposited at the herbarium of department of botany.

Plant extraction

The *Terminalia arjuna* bark was shade dried and powdered using grinder. About 1000g of bark powder was subjected to maceration using 1000 ml 80% v/v ethanol for about 10 days. After the 10th day supernatant should be decanted and filtered through Whatman No.1 filter paper. Filtered extract was concentrated under vacuum pressure at 45°C using rotating vacuum evaporator (Heidolph Manufacturers). The percentage yield of the extract was about 7.96%.

Phytochemical screening

Phytochemical screening of the ethanolic extract of *Terminalia arjuna* bark revealed the presence of triterpenoids, flavonoids, tannins, glycosides⁽²¹⁾.

Acute toxicity studies

Acute oral toxicity studies were performed as per Organization for Economic Cooperation and Development guidelines (OECD 423). Thirty Male Swiss albino mice (20-25 gm) were selected for acute toxicity study. The animals were fasted overnight and the fractions were administered orally at doses of 100, 400, 800, 1500 and 3200 mg/kg body weight. The animals were closely observed for the first 24 h for any toxic symptoms and for 72 h for any mortality⁽²²⁾.

Experimental design

Twenty four Wistar albino rats were selected and they were divided into four groups each containing six rats. Cisplatin was injected intraperitoneally (*i.p.*) at the dose of 7.5 mg/kg body weight for induction of nephrotoxicity in rats⁽²⁾. All the animals were sacrificed on 6th day of cisplatin administration. The experimental design was given below

Group I

Single intraperitoneal (*i.p.*) injection of 0.5 ml isotonic saline was given on 5th day and 0.5% sodium carboxy methyl cellulose (sod CMC) suspension was administered orally for 10 days (Normal Control).

Group II

Single intraperitoneal (*i.p.*) injection of cisplatin (7.5 mg/kg) was given on 5th day (Disease Control).

Group III

Ethanolic extract *Terminalia arjuna* Bark I (EETAB I: 200 mg/kg, p.o) + Cisplatin (7.5 mg/kg). Rats were administered (200 mg/kg) orally for 10 consecutive days in addition to cisplatin which was administered as a single intraperitoneal dose on the 5th day of the experiment 1 h prior to EETAB I dose.

Group IV

Ethanolic extract *Terminalia arjuna* Bark II (EETAB II: 400 mg/kg, p.) + Cisplatin (7.5 mg/kg). Rats were administered with ethanolic extract *Terminalia arjuna* bark (400 mg/kg) orally for 10 consecutive days in addition to cisplatin which was administered as a single intraperitoneal dose on the 5th day of the experiment 1 h prior to EETAB II dose.

At the end of the experiment (i.e. six days after cisplatin administration), body weights of Group II, Group III and Group IV rats were weighed.

Sample preparations

Serum sample

Blood sampling was withdrawn by retro orbital puncturing after anaesthesia. Blood was collected in eppendorf and left at room temperature for 10 minutes to clot, and centrifuged at 3000 rpm at -4°C . Separated serum was used for accessing renal function tests.

Tissue sample

All the animals were sacrificed by cervical dislocation under slight anaesthesia, kidneys were dissected out; left kidney separated and was immediately minced in ice cold phosphate buffer saline (PBS) (0.05M, pH 7) to obtain 1:9 (%w/v) whole homogenate. Homogenate obtained was centrifuged at 3000 rpm and separated supernatant was stored at -80°C for the estimation of antioxidants. The

right kidney fixed in 10% formalin and used for histopathological examinations.

Assessment of renal functions

Estimation of blood urea nitrogen

It was estimated using ENZOPAK UREA/BUN kit of Reckon diagnostics Pvt. Ltd.

Estimation of serum creatinine and serum total protein levels

Creatinine and total protein in serum were estimated using Identi -creatinine and -total protein test kit of jeev Diagnostics Pvt. Ltd, using auto analyser (Turbochem, INDIA).

Assessment of kidney oxidative stress estimation of renal lipid peroxidation (MDA)

The concentration of MDA levels in kidney homogenate was determined based up on the reaction with thiobarbituric acid ⁽²³⁾ taking lipid peroxidation as an index. MDA levels were measured spectrophotometrically at 532nm, using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nanomoles of MDA per g of tissue.

Estimation of reduced glutathione (GSH)

GSH concentration in the kidney homogenate was measured by the method of Ellman ⁽²⁴⁾ and was expressed as $\mu\text{g}/\text{mg}$ tissue.

Estimation of superoxide dismutase (SOD)

SOD in the kidney homogenate was estimated by the method of Misra and Fridovich ⁽²⁵⁾ and was expressed as U/mg of tissue. One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of epinephrine auto oxidation.

Estimation of catalase (CAT)

CAT was estimated by using the method of Aebi ⁽²⁶⁾. Catalase activity was expressed as $\mu\text{moles of H}_2\text{O}_2 \text{ utilized min}^{-1} \cdot \text{mg}^{-1}$ of protein.

Estimation of creatinine in urine

Creatinine in urine was determined using Identi creatinine test kit of CPC Diagnostics Pvt. Ltd, using auto analyser (turbochem, INDIA).

Histopathological studies

10% formalin fixed kidney was sectioned at $5\mu\text{m}$ thickness and embedded in paraffin. Later they were stained with hematoxylin and eosin (H&E). The stained sections were examined under microscope for determination of tissue pathological changes. A maximum of 10 fields of each slide were examined and score for the severity of changes in architecture was given. The scoring was done as none (-), mild (+), moderate (++) , severe (+++) changes.

Statistical analysis

Results were expressed as mean \pm SD of six rats in each group. Statistical significance of any difference in each parameter among the groups was evaluated by one-way ANOVA,

followed by Dunnett's multiple comparison tests using Graph Pad Prism software (version 5.01). P value <0.05 was considered as statistically significant.

Results

Acute oral toxicity study

The ethanolic extract of *Terminalia arjuna* bark did not show any mortality and toxic manifestations up to the dose of 3200 mg/kg. According to OECD guidelines, for acute toxicity, an LD₅₀ dose of 2000 mg/kg and above is categorized as unclassified, and hence, the extract is found to be safe. Based on the acute toxicity studies, the doses 200 mg/kg and 400 mg/kg of the *Terminalia arjuna* bark ethanolic extract have been selected as therapeutic doses.

The effect of ethanolic extracts of Terminalia arjuna bark on body weight

Significant body weight loss ($P<0.01$) were seen in rats intoxicated with cisplatin compared to normal control. Moreover, ethanolic extracts of *Terminalia arjuna* bark (EETAB I & II) were significantly decreased the loss in body weight caused by cisplatin ($P<0.05$), but still significantly different from those of control rats as shown in figure 1.

The effect of ethanolic extracts of Terminalia arjuna bark on renal functional tests

A-The effect of ethanolic extract of Terminalia arjuna bark on BUN

Significant ($P<0.01$) rise in serum BUN were seen in rats intoxicated with cisplatin compared to normal control. Furthermore, EETAB II produced highly significant ($P<0.001$) reduction in the levels of BUN, which was elevated by cisplatin compared to EETAB I. (Figure2).

B-The effect of ethanolic extract of Terminalia arjuna bark on serum Creatinine levels

Significant ($P<0.01$) rise in serum creatinine levels were seen in rats intoxicated with cisplatin compared to normal control. Additionally, EETAB II produced very high significant reduction ($P<0.001$) in the levels of serum creatinine, which was elevated by cisplatin compared to EETAB I (Figure 3).

C-The effect of ethanolic extract of Terminalia arjuna bark on serum total protein levels

Figure 4 showed that there were very high significant ($P<.001$) rise in serum total protein levels in rats intoxicated with cisplatin compared to normal control. EETAB II significantly ($P<0.01$) reduced the increased levels of serum creatinine by cisplatin compared to EETAB I as shown in figure 4.

The effect of ethanolic extract of Terminalia arjuna bark on kidney MDA levels

Figure 5 showed that very high significant ($P < 0.001$) rise in kidney MDA levels were seen in rats intoxicated with cisplatin compared to normal control. Moreover, EETAB II was highly significantly ($P < 0.001$) reduced the increased levels of kidney MDA levels compared to EETAB I.

The effect of ethanolic extract of Terminalia arjuna bark on kidney antioxidant levels

A-The effect of ethanolic extract of Terminalia arjuna bark on kidney GSH levels

Very high significant ($P < 0.001$) reduction in kidney GSH levels were seen in rats intoxicated with cisplatin compared to normal control (Figure 6). The intended figure also showed that EETAB II significantly ($P < 0.001$) increased kidney levels of GSH, which was reduced by cisplatin compared to EETAB I ($P < 0.05$).

B-The effect of ethanolic extract of Terminalia arjuna bark on kidney SOD levels

Figure 7 showed that very high significant ($P < 0.001$) reduction in kidney SOD levels in rats intoxicated with cisplatin

compared to normal control (Figure 7). Furthermore, the intended figure demonstrated that EETAB II significantly increased kidney levels of SOD, which was reduced by cisplatin compared to EETAB I ($P < 0.01$).

C- The effect of ethanolic extract of Terminalia arjuna bark on kidney CAT levels

Very high significant ($P < 0.001$) reduction in kidney CAT levels were seen in rats intoxicated with cisplatin compared to normal control (Figure 8). EETAB II produced very high significant ($P < 0.001$) increase in kidney levels of CAT, which was reduced by cisplatin compared to EETAB I ($P < 0.01$) (Figure 8).

D- The effect of ethanolic extract of Terminalia arjuna bark on serum creatinine levels

Significant ($P < 0.001$) rise in serum creatinine levels were seen in rats intoxicated with cisplatin when compared with normal control (Figure 9). EETAB II produced very high significant ($P < 0.001$) reduction of the increased levels of serum creatinine by cisplatin when compared with EETAB I (Figure 9).

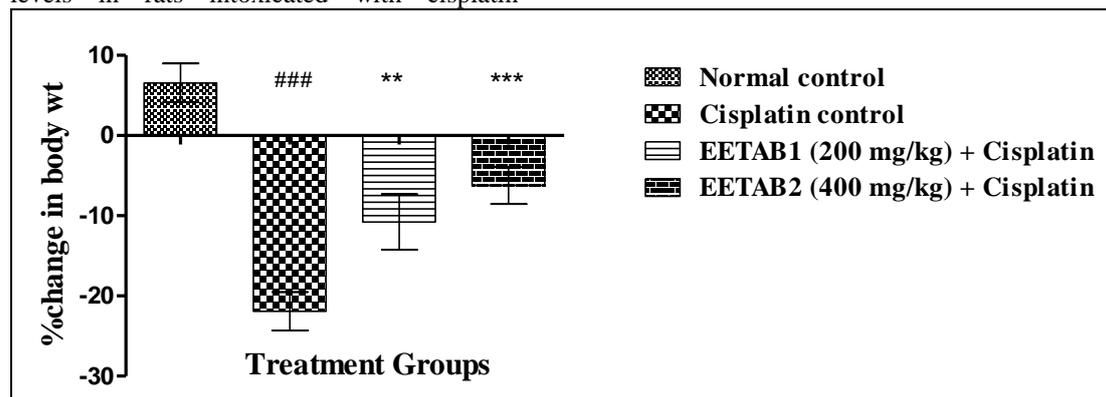


Figure (1): Effect of different doses of ethanolic extracts of Terminalia arjuna bark (EETAB I and II) on % change in body weight. $n=6$, ### $P < 0.001$ vs normal control, ** $P < 0.01$ vs Cisplatin control, *** $P < 0.001$ vs Cisplatin control.

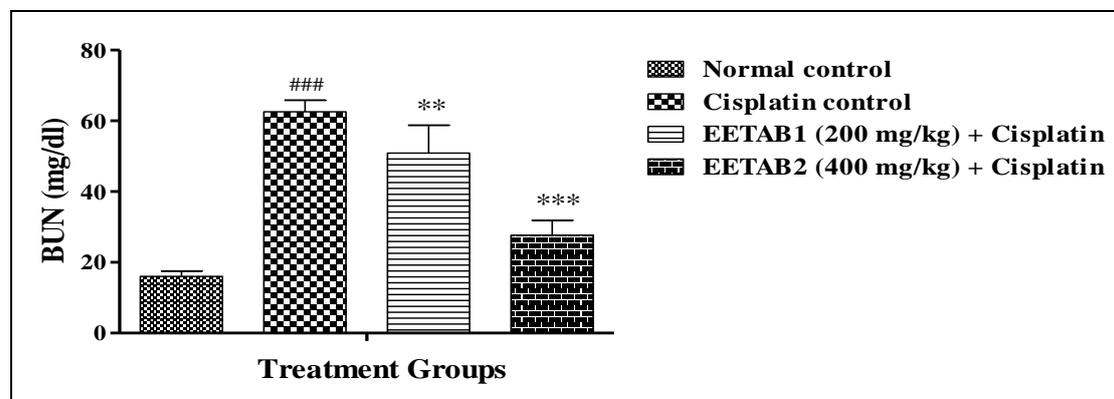


Figure (2): Effect of different doses of ethanolic extracts of Terminalia arjuna bark (EETAB I and II) on blood urea nitrogen. $n=6$, ### $P < 0.001$ vs Normal control, ** $P < 0.01$ vs Cisplatin control, *** $P < 0.001$ vs cisplatin control.

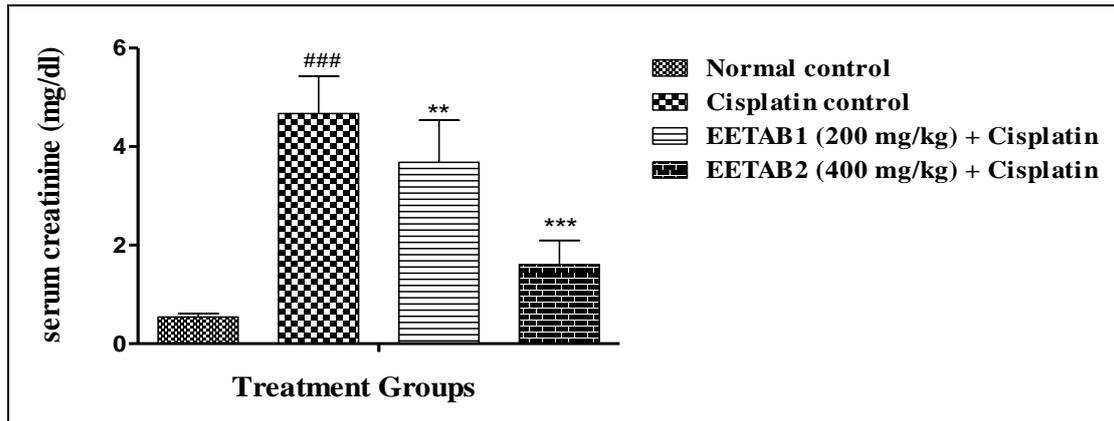


Figure (3): Effect of different doses of ethanolic extracts of *Terminalia arjuna* bark (EETAB I and II) on serum creatinine levels. n=6, ### $P < 0.001$ vs normal control, ** $P < 0.01$ vs cisplatin control, *** $P < 0.001$ vs cisplatin control.

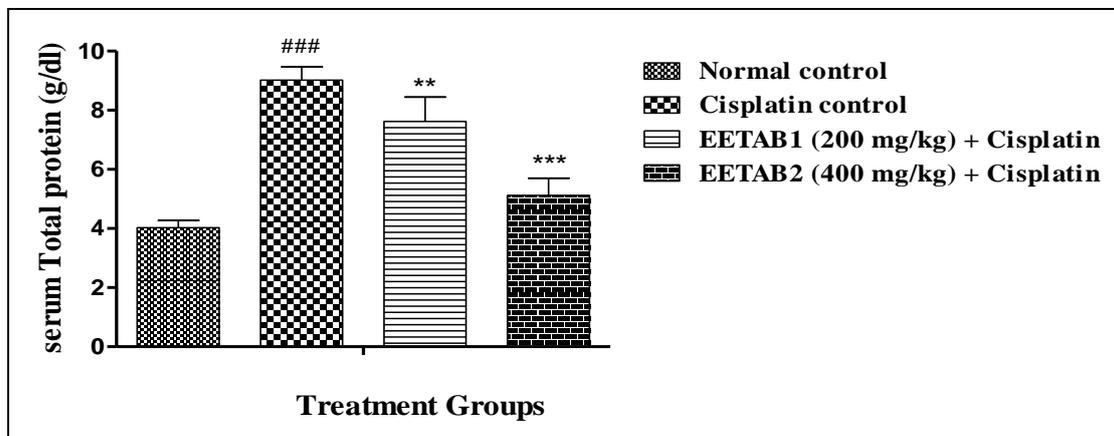
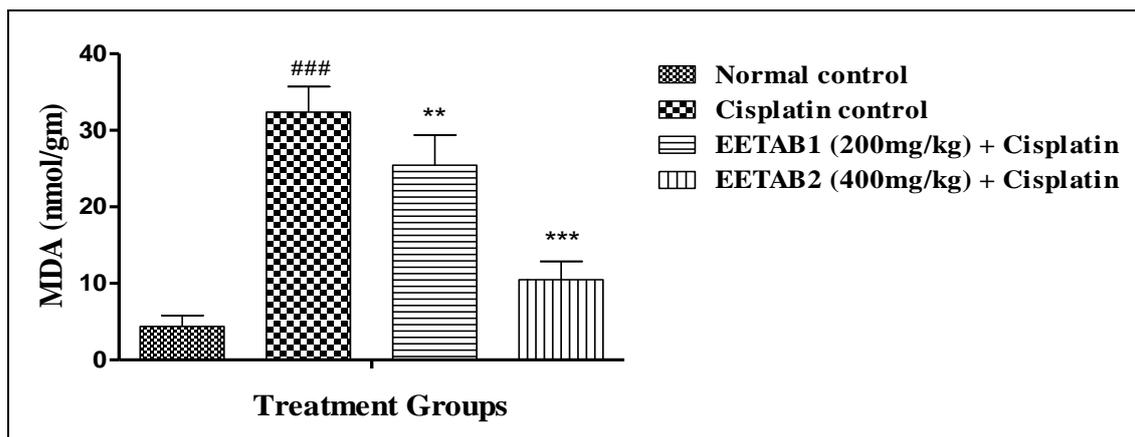


Figure (4): Effect of different doses of ethanolic extracts of *Terminalia arjuna* bark (EETAB I and II) on serum Total protein levels. n=6, ### $P < 0.001$ vs normal control, ** $P < 0.01$ vs cisplatin control, *** $P < 0.001$ vs cisplatin control.



Figure(5): Effect of different doses of ethanolic extracts of *Terminalia arjuna* bark (EETAB I and II) on kidney MDA levels. n=6, ### $P < 0.001$ vs normal control, ** $P < 0.01$ vs cisplatin control, *** $P < 0.001$ vs cisplatin control.

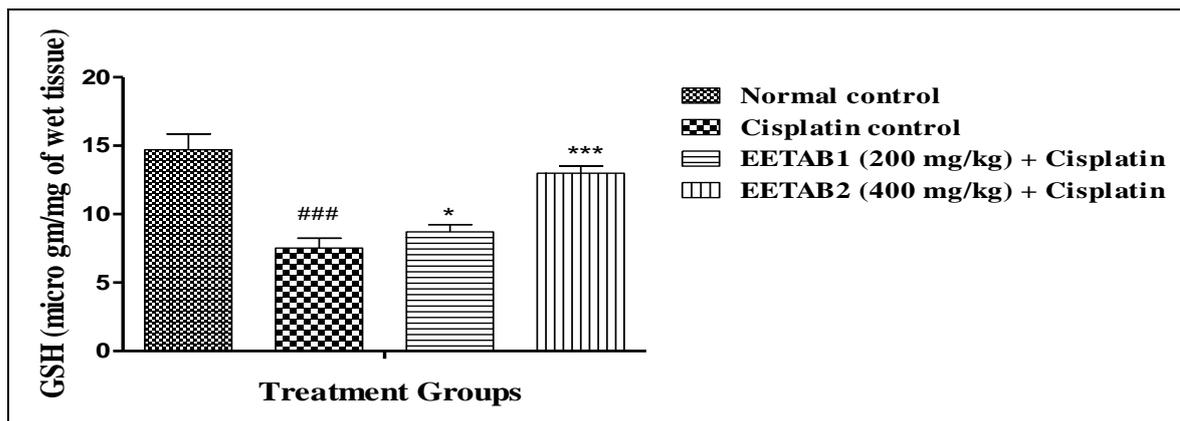


Figure (6): Effect of different doses of ethanolic extracts of *Terminalia arjuna* bark (EETAB I and II) on kidney GSH levels. n=6, ### $P < 0.001$ vs normal control, * $P < 0.05$ vs cisplatin control, *** $P < 0.001$ vs cisplatin control.

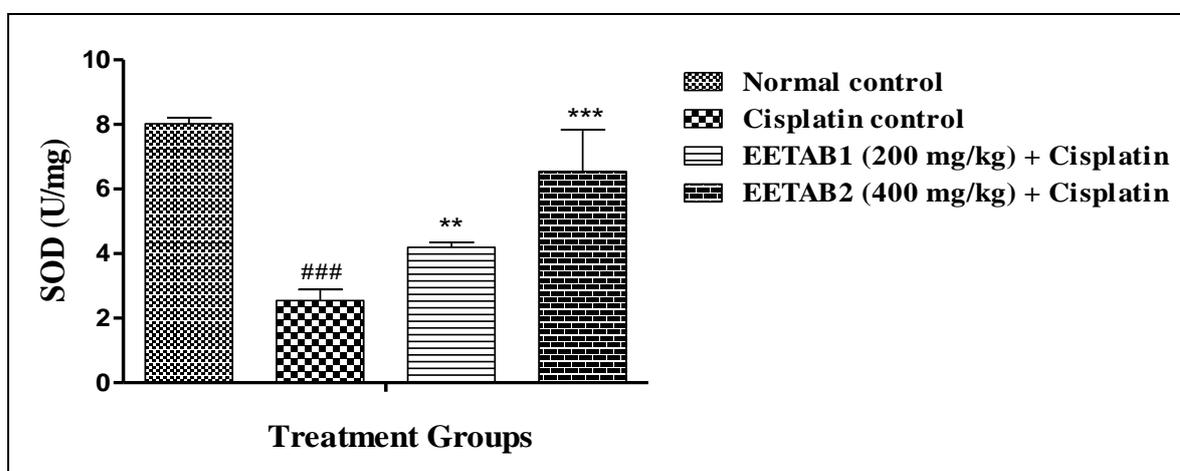


Figure (7): Effect of different doses of ethanolic extracts of *Terminalia arjuna* bark (EETAB I and II) on kidney SOD levels. n=6, ### $P < 0.001$ vs normal control, ** $P < 0.01$ vs cisplatin control, *** $P < 0.001$ vs cisplatin control.

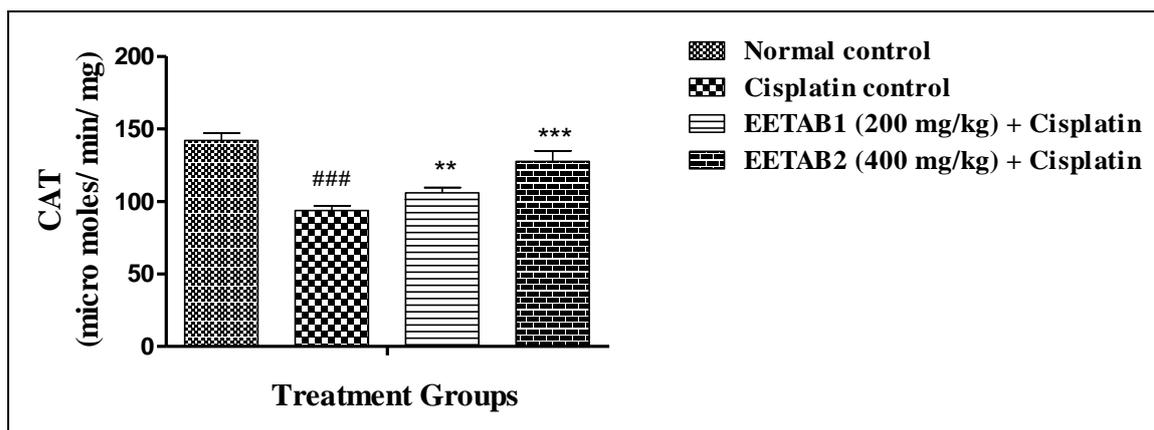


Figure (8): Effect of different doses of ethanolic extracts of *Terminalia arjuna* bark (EETAB I and II) on kidney catalase levels. n=6, ### $P < 0.001$ vs normal control, ** $P < 0.01$ vs cisplatin control, *** $P < 0.001$ vs cisplatin control.

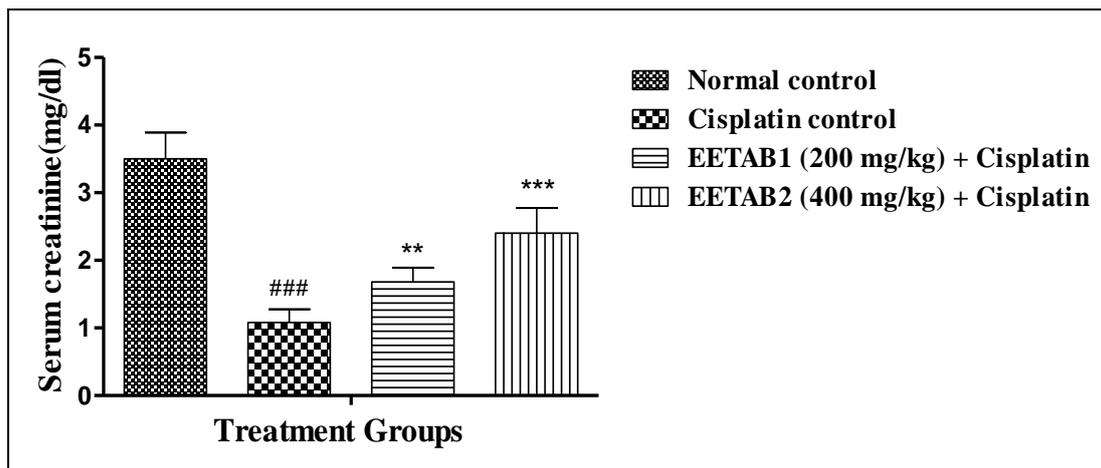
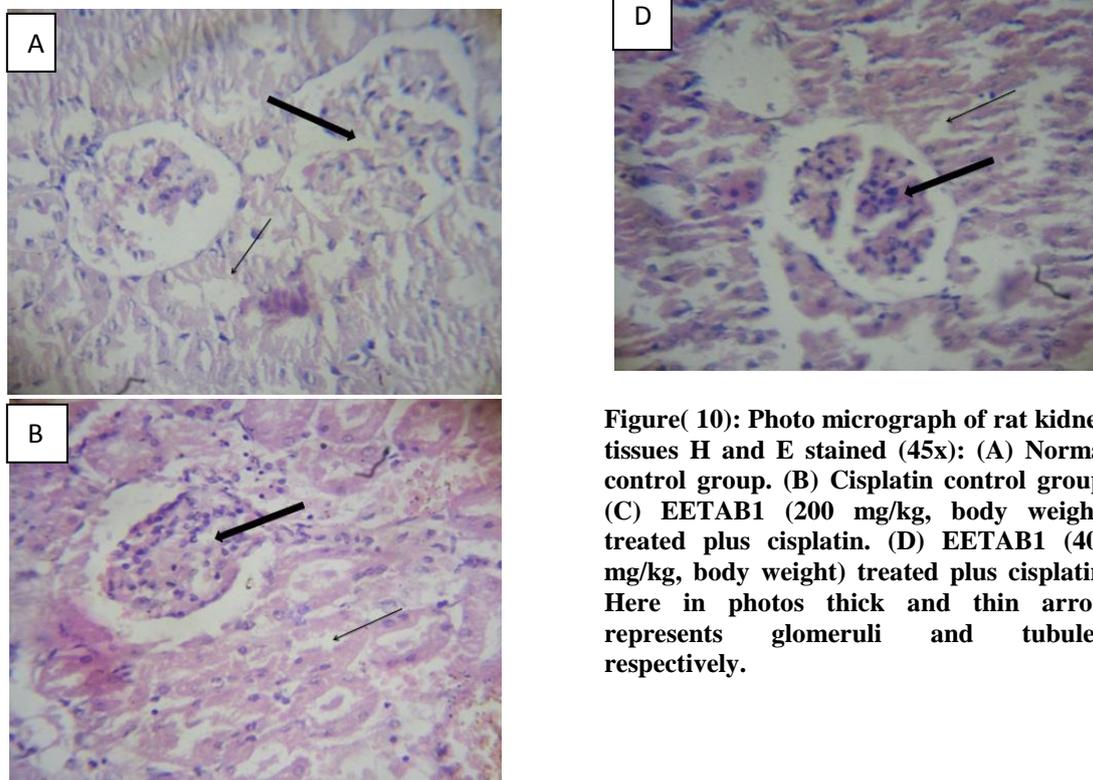


Figure (9): Effect of different doses of ethanolic extracts of *Terminalia arjuna* bark (EETAB I and II) on serum creatinine levels. n=6, ###P < 0.001 vs normal control, **P<0.01 vs cisplatin control, ***P< 0.001 vs cisplatin control.

Histopathological studies

In normal control hematoxylin and eosin stained kidney sections revealed normal glomerulus and tubules with regular anatomy in (Figure 10A). Rats treated with cisplatin showed degenerating glomeruli and tubules (Figure 10B). In EETAB I (200 mg/kg, body weight) treated rats large number degenerating tubules were observed (Figure 10C). However rats treated with EETAB II (400mg/kg, body weight) revealed predominant morphology which is nearer to normal with occasional degenerating tubules (Figure 10D).



Figure(10): Photo micrograph of rat kidney tissues H and E stained (45x): (A) Normal control group. (B) Cisplatin control group. (C) EETAB1 (200 mg/kg, body weight) treated plus cisplatin. (D) EETAB1 (400 mg/kg, body weight) treated plus cisplatin. Here in photos thick and thin arrow represents glomeruli and tubules, respectively.

Table (1): Histopathological examination and scoring of rat kidney injury.

| Groups | Tubular cell swelling | Tubular casts | Tubular dilation | Epithelial necrosis | Glomerular Hyper cellularity |
|-------------------|-----------------------|---------------|------------------|---------------------|------------------------------|
| Normal control | - | - | - | - | - |
| Cisplatin control | +++ | +++ | +++ | +++ | +++ |
| EETAB1 | ++ | ++ | ++ | ++ | ++ |
| EETAB2 | + | + | + | + | + |

Severity of the histopathological changes in kidney were scored using scale of none (-), mild (+), moderate (++), and severe (+++) damage.

Discussion

Cisplatin is an effective agent used against various solid tumours; nephrotoxicity is the severe dose limiting side effect of the drug⁽²⁷⁾. In the present study, rats were intoxicated with cisplatin (7.5 mg/kg). Noticeable increase in the levels of blood urea nitrogen, serum creatinine, serum total protein and urine creatinine levels were observed. Administration of EETAB2 significantly ($P<0.001$) attenuated the levels of the blood urea nitrogen, serum creatinine, serum total protein and urine creatinine levels to nearer normal values (Figure 2, 3, 4 and 9). Increased content of serum creatinine in rats intoxicated with cisplatin may be due to the up regulation of guanine acetate methyl transferase (GAMT) enzyme⁽²⁸⁾. Free radical generation and lipid peroxidation by cisplatin is responsible for its renal toxicity. Cytotoxic effect exerted by free radicals is the cause for peroxidation of membrane phospholipids finally leading to change in permeability and loss of membrane integrity⁽²⁹⁾. In the present study, intraperitoneal administration of cisplatin resulted in increased levels of MDA in kidney. EETAB2 treated rats showed significant ($P<0.001$) reduction in kidney MDA levels (Figure 5). Reduction in MDA levels may be due to inhibition of lipid peroxidation by β -sitosterol present in bark⁽³⁰⁾. Reduction of GSH levels in cisplatin intoxicated rats may be due to its direct conjugation with the drug⁽³¹⁾. Activities of antioxidant enzymes such as CAT, SOD were reduced significantly in GSH depletion condition⁽³²⁾. In the present study rats intoxicated with cisplatin showed decline in kidney cellular antioxidants such as GSH, SOD and CAT. Administration of EETAB II significantly ($P<0.001$) restored the enzyme levels (Figure 6, 7 and 8). It has been reported that the bark of *Terminalia arjuna* contains compounds that may have beneficial effects, for example flavonoids have antioxidant-; and sitosterol has anti-inflammatory-properties⁽³³⁾. The significant reduction in the body weights were observed in rats intoxicated with cisplatin

which may be due to gastric toxicity caused by cisplatin^(34,35). Administration of EETAB I or II to rats intoxicated with cisplatin resulted in increment in body weight compared to cisplatin-treated animals, this may be due to the symptomatic relief provoked by the intended extract against oedema-induced by cisplatin.

Conclusion

Intoxication of rats with cisplatin increased oxidative stress and kidney damage, which is illustrated by substantial increase in pathological parameters with concurrent decrease in antioxidant parameters. Here the present data reveals nephroprotective activity of ethanolic extract of *Terminalia arjuna* bark (400 mg/kg, BW) on Cisplatin induced oxidative nephrotoxicity by normalising pathological and antioxidant parameters. Further investigation need to be carried out for the individual phytoconstituents which are responsible for nephroprotection.

Conflict of Interests

No conflict of interests.

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