

Enzymatic and Non Enzymatic Antioxidant Activity of *Tabernaemontana divaricate* R.Br. against DEN and Fe-NTA Induced Renal Damage in Wistar Albino Rats

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ABSTRACT

Plant derived drugs have been a part of the evolution of human healthcare for thousands of years. The present study was carried out to evaluate the antioxidant potential of ethanolic extract of *Tabernaemontana divaricata* on DEN initiated and Fe-NTA promoted renal damage in rats. Fe-NTA was induced after 10 days of DEN (200mg/kg body weight) initiation at a dose level of 9mg Fe-NTA/kg body weight twice a week for one month. The biochemical parameters were analyzed in serum and the antioxidant assays were carried out in kidney. Lipid peroxidation level was increased due to the administration of Fe-NTA, which caused the reduction of enzymatic antioxidant such as SOD, GPx, Catalase, G6PD, and also the non-enzymatic antioxidants vitamin C and GSH. The levels of urea, uric acid, creatinine, blood urea nitrogen were increased and protein level decreased on Fe-NTA intoxication. The secondary metabolites present in the plant increased the synthesis of antioxidant enzymes and its free radical scavenging properties helped to scavenge all free radicals thereby decreasing lipid peroxidation. Thus, the present study indicates that the plant may clinically valuable agent in the prevention of renal failure caused by DEN and Fe-NTA intoxication.

INTRODUCTION

Nephrotoxicity is one of the most common kidney problems and occurs when body is exposed to a drug or toxin (Sarang and Ameeta, 2001). Exposure to chemical reagents like ethylene glycol, carbon tetrachloride, sodium oxalate and heavy metals such as lead, mercury, cadmium and arsenic also induces nephrotoxicity. Punctual recognition of the disease and cessation of responsible drugs are usually the only necessary therapy (Paller, 1990). Nitrilotriacetic acid is a synthetic tricarboxylic acid which forms water-soluble chelate complexes with various metal ions, including iron, at neutral pH and has been used as a substitute for polyphosphates in detergents utilized both in developed and developing countries (Athar and Iqbal, 1998). Fe-NTA is a potent nephrotoxic agent and induces acute and

subacute renal proximal tubular necrosis by catalyzing the decomposition of H₂O₂-derived production of hydroxyl radicals, which are known to cause lipid peroxidation and DNA damage (Iqbal *et al.*, 2010). A number of therapeutic agents can adversely affect the kidney resulting in acute renal failure, chronic interstitial nephritis and nephritic syndrome because there is an increasing number of effective therapeutic drugs like aminoglycoside antibiotics, NSAID's, chemotherapeutic agents have been added to the therapeutic arsenal in recent years (Hoitsma *et al.*, 1991). India is endowed with a rich wealth of medicinal plants. These plants have made a good contribution to the development of ancient Indian materia medica (Sheetal and Singh, 2008). Phytochemicals are the natural bioactive compounds rich in plants. These phytochemicals work with nutrients and fibers to form an integrated part of defense system against various diseases and stress conditions (Dipak *et al.*, 2010). The most important bioactive constituents of plants are alkaloids, tannins, flavonoids, steroid, terpenoid, carbohydrate and phenolic compounds (Paramapojn and Gritsanapan, 2009).

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These secondary metabolites have been implicated for most of the plants therapeutic activities (Archana *et al.*, 2011). Medicinal plants are becoming increasingly popular in modern society as natural alternatives to synthetic chemicals. In the last few decades there has been an exponential growth in the field of herbal medicine (Ramesh *et al.*, 2011). A large number of herbs are traditionally used in different countries during in response to drug or toxin induced hepatic and renal disorders (El-Beshbishy, 2005). *Tabernaemontana divaricata* R.Br. (syn. *Ervatamia coronaria*) is a glabrous, evergreen, dichotomously branched shrub, belonging to the family Apocynaceae. This species has been extensively investigated and a number of chemical constituents such as alkaloids, triterpenoids, steroids, flavanoids, phenyl propanoids and phenolic acids were isolated from leaves, roots and stems of the plant (Iqbal, and Athar, 1998). Different extracts of this plant shows analgesic, antipyretic, vasodilator and CNS depressant effects and anti inflammatory as well as anti cancer activity (De *et al.*, 2003; Gupta *et al.*, 2004). The present study was designed to investigate the antioxidant activity of *Tabernaemontana divaricata* on DEN initiated and Fe-NTA promoted renal toxicity.

MATERIALS AND METHODS

Collection of plant

The whole plant of *Tabernaemontana divaricata* R.Br. was collected in and around Coimbatore district, Tamil Nadu, India. The plant was authenticated by Botanical Survey of India, TNAU Campus, Coimbatore. The voucher number is BSI/SRC/5/23/2009-2010/Tech.-987. Fresh whole plant material was cut into small pieces, air dried and powdered using mixer.

Preparation of plant extract

50g of powdered plant material *Tabernaemontana divaricata* is weighed and extracted with 250ml of ethanol for 72 hours using soxhlet apparatus. The extract was concentrated and used for further studies.

Experimental animals

Adult male albino rats weighing about 120-150 g were obtained from the animal house of Karpagam University, Coimbatore and were used for the study. Rats were housed in polycarbonate cages in a room with a 12-hour day-night cycle, at constant temperature of 22°C and humidity of 45-64%. During the experimental study rats were fed on pellets with free access to tap water. All the experiments were carried out according to the guidelines and the study was approved by IAEC, Government of India.

Induction of Nephrotoxicity

The toxicity induction was initiated with a single intraperitoneal injection of Diethyl nitrosamine (DEN) at a dose level of 200mg/kg body weight in saline. Ten days after initiation the animals were promoted with intraperitoneal injection of Ferric

nitrosacetate (Fe-NTA) at a dose level of (9 mg Fe/kg body weight) twice a week for 4 weeks. Fe-NTA dose was selected according to (Gupta *et al.*, 2007).

Experimental protocol

The animals were divided into 6 groups of six animals each.

Group I : Control animals.

Group II : DEN initiated and Fe-NTA promoted toxic rats.

Group III: Toxicity induced rats treated with plant extract (200mg/kg bw)

Group IV: Toxicity induced rats treated with plant extract (400mg/kg bw)

Group V : Control animals treated with plant extract only (200mg/kg bw).

Group VI: Control animals treated with plant extract only (400mg/kg bw).

The animals were weighed and dosed through oral intragastric tube every day. The test drug was fed orally for 4 weeks. The experiment was terminated by overnight fasted mg of the rats at the end of thirtieth day. The rats were sacrificed by cervical dislocation after giving mild anesthesia using chloroform. Kidneys were immediately dissected out, washed and stored in 0.9% ice cold saline for various enzymatic, non enzymatic antioxidant analysis, lipid peroxidation and histopathology studies.

Biochemical parameters

Biochemical parameters such as Urea by using the diagnostic kit based on the DAM method (Coulambe, 1965), Uric acid (Natelson *et al.*, 1951), Creatinine by using the diagnostic kit based on the alkaline picrate method (Bonses and Tausky, 1945) and Blood urea nitrogen (Brod and Sirota, 1946) were analyzed.

Antioxidant activity assays

The enzymatic and non enzymatic antioxidant assays were carried out in kidney tissues. The enzymatic antioxidants such as superoxide dismutase (Das *et al.*, 2000), catalase (Sinha, 1972), GPx (Rotruck *et al.*, 1973), G6PD (Balinsky and Bernstein, 1963) Non enzymatic antioxidants such as vitamin C (Moron, 1979) and reduced glutathione (Ohkawa *et al.*, 1979). The lipid peroxidation (Gupta *et al.*, 2008) was also estimated.

Histopathological examination

The histopathological examination of the kidney was carried out by staining the tissue sections using haematoxylin and eosin were examined and photographed by a histopathologist using a light microscope.

Statistical analysis

The results are expressed as Mean \pm standard deviation (S.D). Difference between the groups was assessed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test using the SPSS 16.0 version software package for windows. The values were considered statistically significant if P value was less than 0.05 (P<0.05).

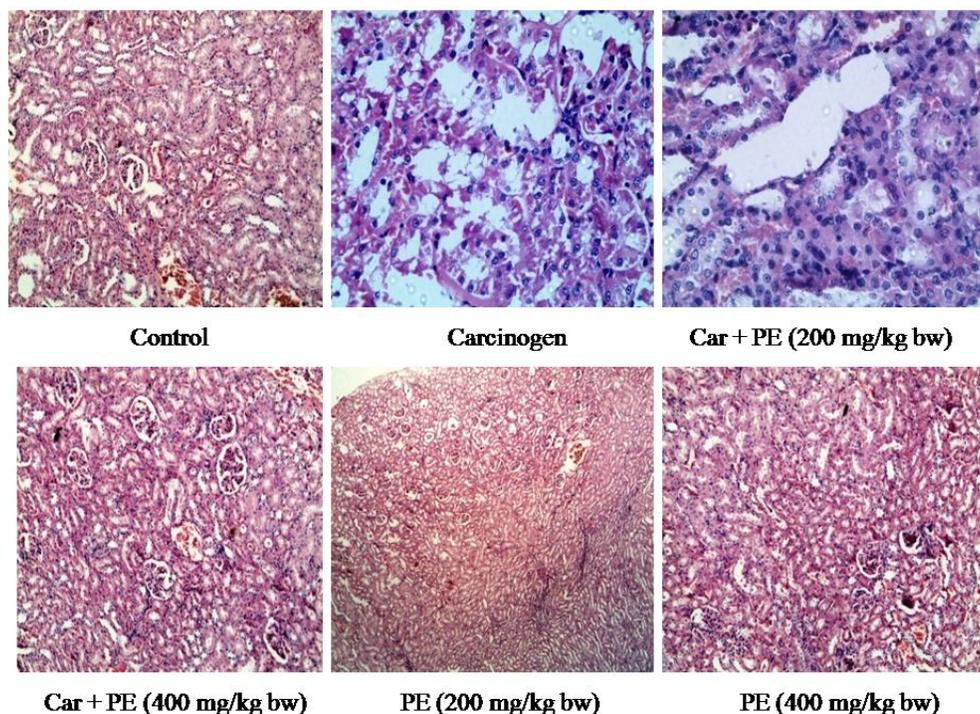


Fig. 1: Histopathology of kidney in control and experimental rats.

Table 1: Concentration of Urea, Uric acid, Creatinine and Blood urea nitrogen in serum of control and experimental groups.

Particulars	Control (Group I)	Fe-NTA (Group II)	Fe-NTA + plant extract (200mg/kg) (Group III)	Fe-NTA + plant extract (400mg/kg) (Group IV)	Plant extract alone treated (200mg/kg) (Group V)	Plant extract alone treated (400mg/kg) (Group VI)
Urea (mg/dl)	22.46±0.10 ^a	30.41±0.08 ^b	26.23±0.10 ^c	26.02±0.10 ^d	24.22±0.09 ^c	20.26±0.23 ^f
Uric acid (mg/dl)	4.17±0.11 ^a	5.8±0.14 ^b	5.10±0.11 ^c	4.59±0.05 ^d	4.11±0.22 ^a	4.01±0.12 ^a
Creatinine (mg/dl)	0.31±0.02 ^a	0.61±0.014 ^b	0.42±0.17 ^c	0.41±0.01 ^c	0.52±0.02 ^d	0.43±0.02 ^c
BUN (mg/dl)	10.36±0.09 ^a	14.35±0.16 ^b	12.47±0.17 ^c	12.21±0.11 ^d	11.44±0.1 ^c	12.31±0.22 ^c

Values are expressed as Mean ± SD for four animals in each group.

Values not sharing common superscript letters^(a-d) differ significantly at p<0.05 (DMRT)

RESULTS

The urea, uric acid, creatinine and blood urea nitrogen levels were elevated in Fe-NTA induced renal toxicity rats in groups II, when compared to group I normal rats. There was significant reduction of renal marker levels in group III and IV rat with oral administration of ethanolic extract of *T.divaricata* and there was no significant change in above parameters in rats treated with plant extract alone and is similar to control groups. The maximum protection was existed by the 400mg/kg of *T.divaricata* treatment (Table 1). From the table 2 it can be observed that there was a significant (P<0.05) decrease in serum protein, in rats exposed to Fe-NTA (Group II) when compared to normal control rats (Group I). It was restored to near normal level when treated with *T. divaricata* simultaneously (Group III & IV).

A significant reduction in the activity of antioxidant enzymes namely superoxide dismutase (SOD), catalase (CAT), glucose 6 phosphatase dehydrogenase (G6PD) and glutathione peroxidase (GPx) in kidney tissues were noted in rats intoxicated with Ferric nitrilotriacetate (Fe-NTA) (Group II) when compared

to control (group I). The simultaneous treatment with ethanolic extract of *T.divaricata* along with Fe-NTA (group III& IV) was found to increase the activity of SOD, CAT, G-6-PD and GPx when compared to group II rats. There was no significant difference between groups V & VI when compared to group I. this shows the safety nature of the plant extract (Table 3).

Table 4 represents the level of vitamin C glutathione and LPO in kidney of control and experimental animals. The depletion of vitamin C and GSH in the kidney of rats treated with Fe-NTA (group II) were restored to near normal levels in rats treated with *T.divaricata* [group III & IV]. In group V & VI the level of glutathione and vitamin C is near normal when compared to control. This shows the safety nature of the plant extract. From the figure 1 it is found that the histopathology of group I untreated normal rats showed no pathological changes. Group II kidney of Fe-NTA intoxicated rats showing focal renal tubular atrophy. Group III and IV kidney of nephrotoxic animal treated with ethanolic extract of *T. divaricata* showed normal histology. Group V and VI kidney showed normal histological structure.

Table 2: Concentration of protein in serum and kidney of control and experimental groups.

Particulars	Control (Group I)	Fe-NTA (Group II)	Fe-NTA + plant extract (200mg/kg) (Group III)	Fe-NTA + plant extract (400mg/kg) (Group IV)	Plant extract alone treated (200mg/kg) (Group V)	Plant extract alone treated (400mg/kg) (Group VI)
Serum Protein (g/dl)	6.55±0.17 ^a	4.86±0.10 ^b	6.51±0.03 ^c	7.32±0.042 ^d	6.54±0.10 ^a	6.92±0.03 ^c
Kidney Protein (mg/g)	3.72±0.58 ^a	2.72±0.15 ^b	2.88±0.17 ^c	3.55±0.44 ^d	3.59±0.46 ^a	3.89±0.11 ^c

Values are expressed as Mean ± SD for four animals in each group.

Values not sharing common superscript letters ^(a-d) differ significantly at p<0.05 (DMRT)

Table 3: The activities of enzymatic antioxidants SOD, Catalase, GPx and G6PD in kidney of control and experimental groups.

Particulars	Control (Group I)	Fe-NTA (Group II)	Fe-NTA + plant extract (200mg/kg) (Group III)	Fe-NTA + plant extract (400mg/kg) (Group IV)	Plant extract alone treated (200mg/kg) (Group V)	Plant extract alone treated (400mg/kg) (Group VI)
Superoxide dismutase (Units/g tissue)	1.64±0.06 ^a	1.17±0.03 ^b	1.27±0.18 ^c	1.45±0.03 ^d	1.57±0.02 ^e	1.67±0.03 ^a
Catalase (μ moles of H ₂ O ₂ utilized/ min/ mg/protein)	29.19±0.24 ^a	15.64±0.14 ^b	25.44±0.18 ^c	26.38±0.39 ^d	27.38±0.23 ^e	29.49±0.33 ^a
GPx (μg of GSH/mg of protein)	39.52±0.20 ^a	20.23±0.12 ^b	32.58±0.14 ^c	34.43±0.21 ^d	37.43±0.27 ^e	38.71±0.14 ^f
G-6-P-D(0.01 O.D/min/mg protein)	0.33±0.03 ^a	0.06±0.01 ^b	0.24±0.02 ^c	0.34±0.03 ^d	0.32±0.01 ^a	0.36±0.02 ^d

Values are expressed as Mean ± SD for four animals in each group.

Values not sharing common superscript letters ^(a-d) differ significantly at p<0.05 (DMRT)

Table 4: The non-enzymatic antioxidants GSH, Vitamin C and Lipid peroxidation level in kidney of control and experimental groups.

Particulars	Control (Group I)	Fe-NTA (Group II)	Fe-NTA + plant extract (200mg/kg) (Group III)	Fe-NTA + plant extract (400mg/kg) (Group IV)	Plant extract alone treated (200mg/kg) (Group V)	Plant extract alone treated (400mg/kg) (Group VI)
GSH(μg/mg of protein)	48.48±0.25 ^a	22.58±0.15 ^b	40.4±0.23 ^c	44.38±0.17 ^d	48.05±0.02 ^e	48.29±0.07 ^a
Vitamin C (μg/mg of protein)	9.35±0.11 ^a	4.37±0.06 ^b	5.39±0.07 ^c	7.51±0.05 ^d	8.37±0.05 ^e	9.23±0.10 ^f
LPO (n Moles of MDA formed/gm of tissue)	69.21±0.14 ^a	98.74±0.21 ^b	73.18±0.27 ^c	71.79±0.07 ^d	70.40±0.06 ^e	69.57±0.05 ^f

Values are expressed as Mean ± SD for four animals in each group.

Values not sharing common superscript letters ^(a-d) differ significantly at p<0.05 (DMRT)

DISCUSSION

Oxidative stress and inflammation are closely associated with tumor promotion (Khan and Sultana, 2005). Toxicity to liver and kidney is one of the most common problems and occurs when body is exposed to a drug or toxin (Chander and Chopra, 2005). Free radical-mediated oxidative damage has been widely implicated as a potential mediator in a wide range of tissue impairments (Agarwal *et al.*, 2007; Gutierrez and Navarro, 2010). The antioxidant activity may be due to the inhibition of the formation of radicals or scavenging of the formed radical (Chesson and Collins, 1997). Traditional medical practitioners utilized plants for the treatment of several diseases since time immemorial. Recent studies have shown that plants have several active agents or compounds due to which they show more synergistic effect than any single active compound (Khan *et al.*, 2004).

Urea, uric acid, creatinine and blood urea nitrogen are still today as clinical markers for renal function. Significant elevation of urea, uric acid and creatinine levels in blood serum in rats treated with Fe-NTA indicate renal insufficiency (Umemura *et al.*, 1990). Iron deposition within kidney tissue is often related to generation of reactive oxygen species, leading to oxidative damage, LPO and concomitant increase in serum toxicity markers and depletion of renal GSH content Fe-NTA induces oxidative

stress in the kidney and decreases antioxidant defenses, as indicated by the fall in GSH level and in the activities of glutathione peroxidase and catalase (Jelenka *et al.*, 2004).

The increase in the SOD and CAT activities produced by treatment with herbal extract effectively eliminates the superoxide and peroxides produced by Fe-NTA intoxication. In this study, it was observed that a dose- dependent decrease in the Fe-NTA mediated oxidative stress in kidney of *T. divaricate* pretreated rats, which is manifested by a decrease in the susceptibility of microsomal membrane for LPO as well as H₂O₂ generation. Studies with a number of models show that the metabolism of xenobiotics often produced GSH depletion, which increases the sensitivity of organ to oxidative and chemical injury (Sivaprasad *et al.*, 2003).

CONCLUSION

In conclusion, the findings reported in the study indicated that the oral administration of *T.divaricate* to Fe-NTA intoxicated rats exhibited significant antioxidative property. Although promising results have been obtained, more concerted efforts are still needed for the isolation, characterization and biological evaluation for the active principles of the extract.

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