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Abrogation of DEN/Fe-NTA induced carcinogenic response, oxidative damage and subsequent cell proliferation response by *Terminalia chebula* in kidney of Wistar rats

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In an effort to identify a new chemopreventive agent, the present study was conducted to investigate the role of *T. chebula* in the prevention of ferric nitrilotriacetic acid (Fe- NTA) induced oxidative stress and renal tumorigenesis in Wistar rats. A single application of Fe-NTA (9 mg Fe/kg body weight, intraperitoneally) significantly induced oxidative stress and elevated the marker parameters of tumor promotion. However, the pretreatment of animals with different doses of *T. chebula* extract (25 and 50 mg/kg body weight) restored the levels of reduced glutathione (GSH) and cellular protective enzymes ($p < 0.05$). Concomitantly, malondialdehyde (MDA) formation and hydrogen peroxide content were also reduced significantly ($p < 0.05$) at both the doses. The promotion parameters tested (ornithine decarboxylase activity and DNA synthesis) were also significantly suppressed ($p < 0.05$). *T. chebula* also inhibited *N*-diethyl nitrosamine initiated renal carcinogenesis by showing reduction in the number of animals with renal cell tumors and percentage incidence of tumor as compared to the DEN initiated and Fe-NTA promoted rats. The study was further histologically confirmed. These results suggest a potential role of *T. chebula* in protection from Fe-NTA-induced renal carcinogenesis and oxidative damage.

1. Introduction

In an effort to identify a new chemopreventive agent, the present study was conducted to investigate the role of Terminalia chebula in the prevention of renal tumorigenesis. *T. chebula* Retzius (*T. chebula* Retz.)(Combretaceae) commonly known as "black myroblans" in English and "Harad" in Hindi is called the "king of medicines" and is always listed first in ayurveda because of its extraordinary powers of healing. It has been studied extensively for its homeostatic, antitussive, laxative, diuretic, antioxidative, antibacterial and cardiotonic activities (Barthakur and Arnold 1991; Sabu and Kuttan 2002; Malekzadeh et al. 2001). It is an excellent free radical scavenger, a property arising mainly from the presence of well-known antioxidants like ascorbate, gallic acid, ellagic acid, tannic acid, ß-sitosterol, ethyl gallate, chebulic acid, and mannitol (Naik et al. 2004). The plant is used extensively in the preparation of many ayurvedic formulations against chronic ulcers, leucorrhoea, pyorrhoea and fungal infections of the skin.

Nitrilotriacetic acid (NTA) is the constituent of various domestic and hospital detergents and is a common water contaminant (Nancharaiah et al. 2006). The iron complex of the chelating agent nitrilotriacetic acid (Fe-NTA) is a potent nephrotoxic agent. Iron has also been shown to participate in radical generating reactions and the high body stores of iron may increase the risk of cancer in humans (Stevens et al. 1988). The major target organ for Fe-NTA damage is the kidney and it is assumed that Fe-NTA mediated generation of free radicals plays an important role in renal tumorigenesis. In the kidney Fe-NTA is filtered through the glomeruli into the lumen of the renal proximal tubule where Fenton chemistry occurs mediating induction of lipid peroxidation and oxidative DNA damage (Aruoma 1989; Umemura et al. 1990).

This present study was designed to investigate the effect of *T. chebula* with anti-oxidant potential, on Fe-NTA-induced nephrotoxicity, hyperproliferative response and renal tumor promotion in a rat *in vivo* model.

2. Investigations and results

Treatment of rats with Fe-NTA at the dose level of 9 mg Fe/kg body weight in rats causes overproduction of cellular oxidants and modulation of antioxidant defense system. In our study, several doses of *T. chebula* were tested and maximum efficacy was found at 25 mg/kg body weight and 50 mg/kg body weight. Table 1 shows the effect of pretreatment of rats with *T. chebula* on Fe-NTA-mediated total renal glutathione content and on the activities of its metabolizing enzymes, viz., glutathione-S-transferase and glutathione reductase. Treatment with Fe-NTA

Table 1: Effect of pretreatment with *T. chebula* on Fe-NTA-mediated depletion of total reduced glutathione level and decrease in the activities of glutathione metabolizing enzymes, glutathione-*S*-transferase and glutathione reductase in kidney of rats

Treatment groups	Total reduced thiol (nmol GSH/g tissue)	Glutathione-S-transferase (nmol CDNB conjugate formed/min/mg protein)	Glutathione reductase (nmol NADPH oxidized/minute/mg protein)
Saline (control)	0.67 ± 0.01	350.8 ± 1.2	375.3 ± 4.6
Fe-NTA (9 mg Fe/kg body weight)	$0.48 \pm 0.01^{\#\#\#}$	$185.6 \pm 1.1^{\#\#\#}$	$239.0 \pm 7.5^{\#\#\#}$
T. chebula (25 mg/kg body weight) + Fe-NTA (9 mg Fe/kg body weight)	$0.57 \pm 0.01^{**}$	$223.1 \pm 3.2^{***}$	$283.4 \pm 8.7^{***}$
T. chebula (50 mg/kg body weight) + Fe-NTA (9 mg Fe/kg body weight)	$0.60 \pm 0.02^{***}$	$271.2 \pm 3.4^{***}$	$339.1 \pm 8.7^{***}$
T. chebula only (50 mg/kg body weight)	0.69 ± 0.02	352.6 ± 3.9	377.1 ± 3.9

Each value represents mean ± S.E., n = 6.
$p < 0.001$ compared with the corresponding value for saline treated control group.
** $p < 0.01$ and *** $p < 0.001$ compared with the corresponding value for Fe NTA treated group

Table 2: Effect of pretreatment with *T. chebula* on Fe-NTA-induced depletion in the level of renal antioxidant enzymes in rats

Treatment groups	Catalase (nmol H_2O_2 consumed/min/mg protein)	Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)	Glucose-6-phosphate dehydrogenase (nmol NADP reduced/min/mg protein)
Saline (control)	70.5 ± 0.5	273.5 ± 6.9	40.3 ± 1.1
Fe-NTA (9 mg Fe/kg body weight)	$30.4 \pm 0.74^{\#\#\#}$	$154.8 \pm 1.6^{\#\#\#}$	$15.4 \pm 1.4^{\#\#\#}$
T. chebula (25 mg/kg body weight) + Fe-NTA (9 mg Fe/kg body weight)	$46.6 \pm 0.73^{***}$	$183.3 \pm 6.0^{**}$	$22.7 \pm 1.24^{**}$
T. chebula (50 mg/kg body weight) + Fe-NTA (9 mg Fe/kg body weight)	$63.9 \pm 0.67^{***}$	$237.6 \pm 10.1^{***}$	$29.7 \pm 1.6^{***}$
T. chebula only (50 mg/kg body weight)	72.8 ± 1.25	251.8 ± 6.7	39.4 ± 2.0

Each value represents mean ± S.E., n = 6.
$p < 0.001$ compared with the corresponding value for saline treated control group
** $p < 0.01$ and *** $p < 0.001$ compared with the corresponding value for Fe-NTA treated group

alone resulted in the depletion of renal glutathione and reduction in the activities of glutathione-S-transferase and glutathione reductase by 28%, 47% and 36% respectively of that of saline-treated control group. However, pretreatment of animals with *T. chebula* at 25 mg/kg body weight and 50 mg/kg body weight resulted in the recovery by 13–18%, 11–24% and 12–26% respectively, as compared with Fe-NTA treated group.

The effect of prophylactic treatment with *T. chebula* on Fe-NTA-induced reduction in the activities of renal antiox-

Table 3: Effect of pretreatment with *T. chebula* extract on Fe-NTA-mediated depletion of renal quinone reductase and enhancement of xanthine oxidase and renal microsomal lipid peroxidation in rats

Treatment groups	Quinone reductase (nmol of dichloroindophenol reduced/min/mg protein)	Xanthine oxidase (μg of uric acid formed/min/mg protein)	Lipid peroxidation (nmol MDA formed/h/g tissue)
Saline (control)	170.4 ± 1.6	0.2 ± 0.01	2.2 ± 0.15
Fe-NTA (9 mg Fe/kg body weight)	$82.0 \pm 1.3^{\#\#\#}$	$0.52 \pm 0.02^{\#\#\#}$	$4.0 \pm 0.12^{\#\#\#}$
T. chebula (25 mg/kg body weight) + Fe-NTA (9 mg Fe/kg body weight)	$100.1 \pm 3.7^{***}$	$0.31 \pm 0.01^{***}$	$3.4 \pm 0.06^{**}$
T. chebula (50 mg/kg body weight) + Fe-NTA (9 mg Fe/kg body weight)	$130.4 \pm 1.2^{***}$	$0.24 \pm 0.01^{***}$	$2.76 \pm 0.03^{***}$
T. chebula only (50 mg/kg body weight)	173.6 ± 1.1	0.19 ± 0.01	2.08 ± 0.08

Each value represents mean ± S.E., n = 6.
$p < 0.001$ compared with the corresponding value for saline treated control group.
** $p < 0.01$ and *** $p < 0.001$ compared with the corresponding value for Fe NTA treated group

Table 4: Effect of pretreatment with *T. chebula* on Fe-NTA-induced enhancement of blood urea nitrogen, serum creatinine and hydrogen peroxide in rats

Treatment groups	Blood urea nitrogen (mg/100 ml) IU/l	Creatinine (mg/100 ml) IU/l	H_2O_2 (nmol H_2O_2/g tissue)
Saline (control)	23.4 ± 0.23	2.42 ± 0.01	288.9 ± 2.61
Fe-NTA (9 mg Fe/kg body weight)	$52.0 \pm 0.85^{\#\#\#}$	$4.32 \pm 0.07^{\#\#\#}$	$523.3 \pm 7.1^{\#\#\#}$
T. chebula (25 mg/kg body weight) + Fe-NTA (9 mg Fe/kg body weight)	$32.0 \pm 0.4^{***}$	$3.68 \pm 0.03^{***}$	$406.1 \pm 10.3^{**}$
T. chebula (50 mg/kg body weight) + Fe-NTA (9 mg Fe/kg body weight)	$28.0 \pm 0.13^{* **}$	$2.84 \pm 0.09^{***}$	$348.3 \pm 6.4^{***}$
T. chebula only (50 mg/kg body weight)	21.9 ± 0.3	2.46 ± 0.09	278.0 ± 6.9

Each value represents mean ± S.E., n = 6.
$p < 0.001$ compared with the corresponding value for saline treated control group.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the corresponding value for Fe-NTA treated group

Table 5: Summary of tumor data of the effect of *T. chebula* extract on DEN initiated and Fe-NTA promoted renal tumors

Treatment groups	No. of animals treated	No. of animals studied histo-pathologically	No. of animals with renal cell tumors	Incidence of tumors (%)
Saline (alone)	20	19	0	–
DEN + Fe-NTA	20	11	9	81.8
Fe-NTA (alone)	20	16	4	25.0
T. chebula extract (D1) + DEN + Fe-NTA	20	14	6	42.8
T. chebula extract (D2) + DEN + Fe-NTA	20	15	4	26.6

Dose of DEN = 200 mg/kg body weight, dose of Fe-NTA = 9 mg Fe/kg body weight. Doses (D1 and D2) represent 25 and 50 mg/kg body weight of *T. chebula* extract

idant enzymes is shown in Table 2. Fe-NTA treatment alone caused reduction in the activities of renal antioxidant enzymes such as catalase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase by 57%, 43% and 62% respectively as compared to the saline-treated control group. Treatment with *T. chebula* at a lower dose of 25 mg/kg body weight and a higher dose of 50 mg/kg body weight caused recovery of the above enzymes by 23–48%, 10–30% and 18–36% respectively as compared with Fe-NTA treated control group.

Table 3 shows that Fe-NTA treatment enhances the activity of xanthine oxidase by 160% and susceptibility of renal microsomal membrane for iron-ascorbate induced lipid peroxidation by 82% whereas it causes a reduction in the activity of quinone reductase by 52% as compared with saline-treated controls. *T. chebula* treatment caused reduction in activity of xanthine oxidase and renal microsomal lipid peroxidation by 105–140 % and 26–57% respectively and increases in the level of quinone reductase by 11–29% at lower and higher doses of *T. chebula* as compared with Fe-NTA treated group.

The effect of pretreatment of rats with *T. chebula* on Fe-NTA-induced enhancement in the levels of blood urea nitrogen, serum creatinine and hydrogen peroxide is shown in Table 4. Fe-NTA treatment leads to about 111%, 73%,

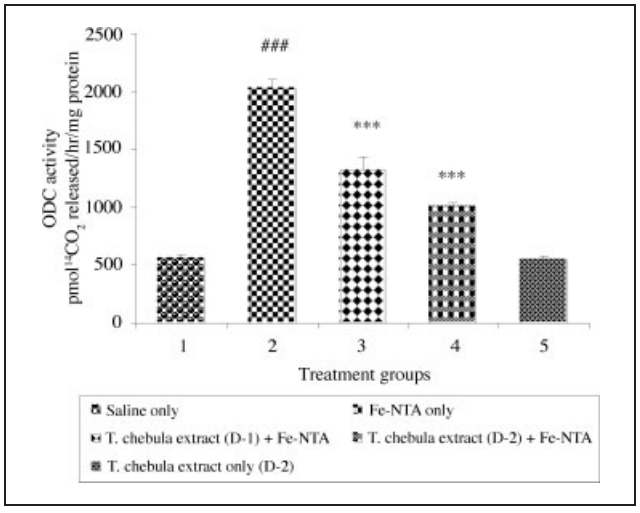


Fig. 1: Effect of pretreatment of rats with *T. chebula* on Fe-NTA-induced enhancement of renal ornithine decarboxylase (ODC) activity. Each value represents mean ± S.E. of six animals. ### Significant ($P < 0.001$) when compared with saline-treated control group. *** Significant ($P < 0.001$) when compared with Fe-NTA treated control group. Doses (D1 and D2) represent pretreatment 25 and 50 mg/kg body weight of *T. chebula*

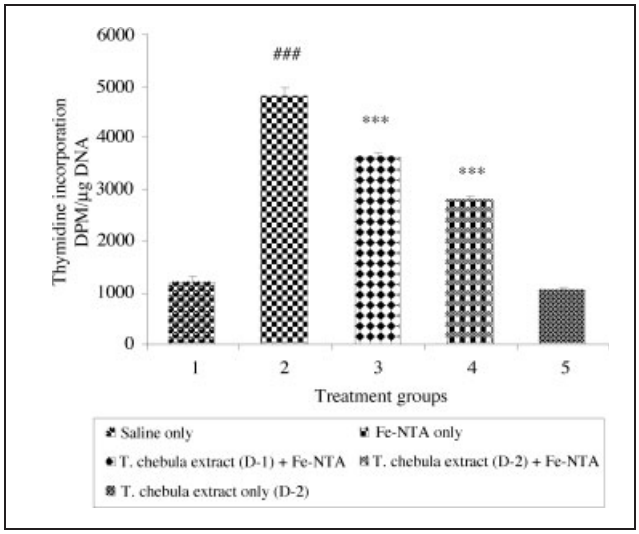
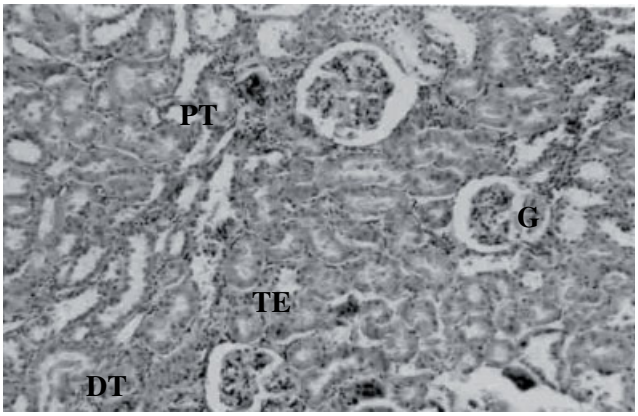


Fig. 2: Effect of pretreatment of rats with *T. chebula* on Fe-NTA-induced enhancement of [^{3}H] thymidine incorporation into renal DNA. Each value represents mean ± S.E. of six animals. ###Significant ($P < 0.001$) when compared with saline-treated control group. ***Significant ($P < 0.001$) when compared with Fe-NTA treated control group. Doses (D1 and D2) represent pretreatment with 25 and 50 mg/kg body weight of *T. chebula*



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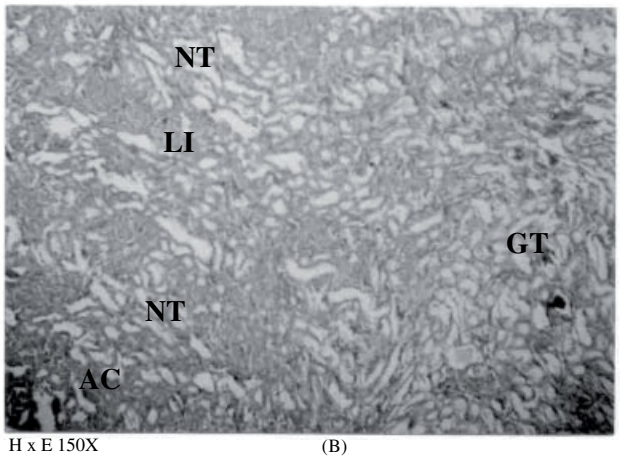
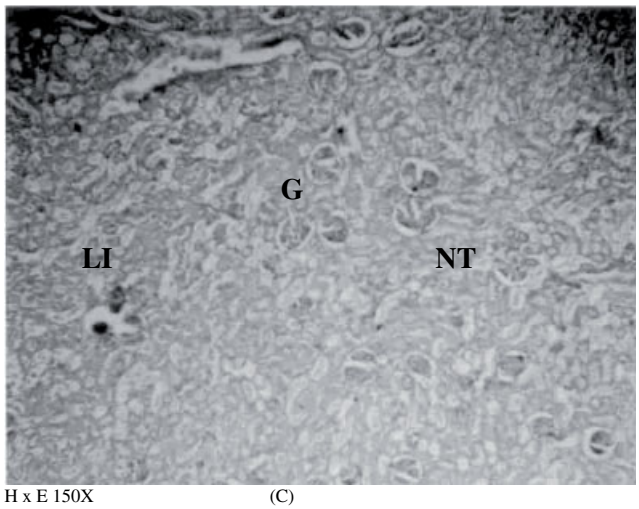
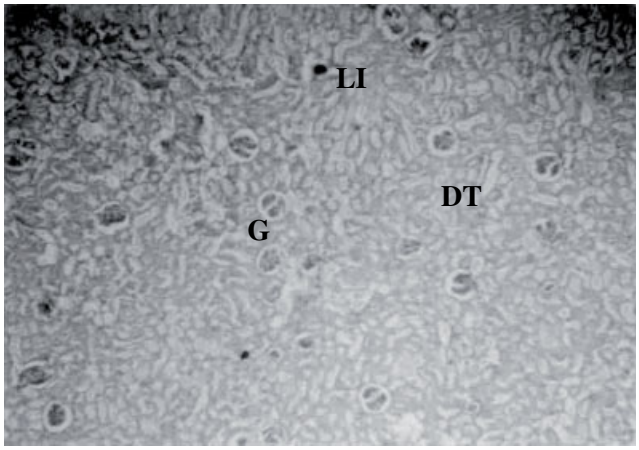


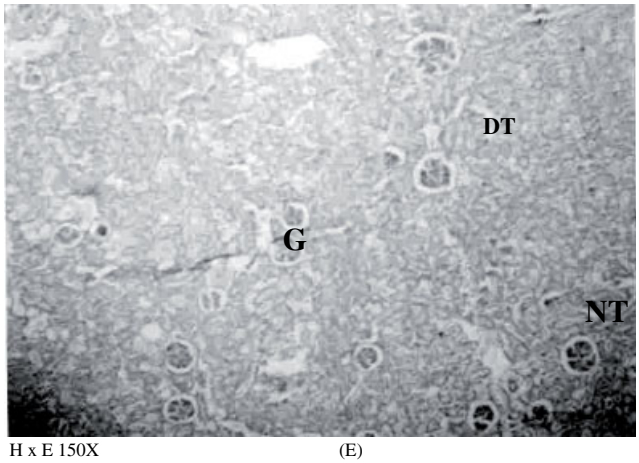
H x E 150X (B)



H x E 150X (C)



H x E 150X (D)



H x E 150X (E)

Fig. 3: (a) Normal saline treated, (b) DEN + Fe-NTA treated (c) Only Fe-NTA treated (d) T. chebula (D1) + DEN + Fe-NTA treated (e) T. chebula (D2) + DEN + Fe-NTA treated. All slides were stained with hematoxylin and eosin.
Dose of DEN = 200 mg/kg bodyweight, dose of Fe-NTA = 9 mg/kg bodyweight TC (D1) = 25 mg/kg bodyweight and TC (D2) = 50 mg/kg bodyweight.
Abbreviations: AC = adenocarcinoma; G = glomerulus; LI = leucocytic infiltration; NT = necrotic tissue; PT = proximal tubule; DT = distal tubule; TE = tubular epithelium; GT = ghost tissue; HC = hyperchromatism

and 81% enhancement in the values of blood urea nitrogen, serum creatinine and H2O2 respectively, as compared with saline-treated controls. Prophylaxis with *T. chebula* at both doses resulted in 85–102%, 21–56% and 40–60% reduction in the values of blood urea nitrogen, serum creatinine and H2O2 respectively as compared with Fe-NTA treated group.

The summary of the percentage incidence of renal cell tumors (RCTs) in different treatment groups is given in Table 5. The saline alone group did not showed any tumors. The treatment with Fe-NTA of the DEN-initiated animals enhanced the occurrence of RCTs by 81.8% in the animals studied whereas treatment with Fe-NTA of the uninitiated animals led to the development of RCTs in 25% of the animals studied. The tumor incidence was decreased in the group of animals pretreated with *T. chebula* extract at the lower dose of 25mg/kg body weight by 42.8% whereas in the group receiving the higher dose of 50 mg/kg body weight, the tumor incidence was reduced by 26.6%.

Fig. 1 shows the effect of pretreatment of animals with *T. chebula* on Fe-NTA mediated induction of renal ODC activity. Treatment with Fe-NTA caused 259% induction in the ODC activity as compared with saline-treated control. The pretreatment of rats with *T. chebula* at two doses caused inhibition in the elevation of ODC activity by 124% at a dose of 25 mg/kg body weight and 181% at a dose of 50 mg/kg body weight as compared with Fe-NTA treated control group.

Fig. 2 shows the effect of prophylaxis of rats with *T. chebula* on Fe-NTA mediated enhancement in the incorporation of [^{3}H] thymidine into renal DNA. Fe-NTA alone

treatment caused increase in the incorporation of [^{3}H] thymidine into renal DNA by 302% as compared with saline-treated control. However, *T. chebula* pretreatment caused reduction in the enhancement of DNA synthesis by 97–168% as compared with Fe-NTA treated group.

Fig. 3 shows the histopathological findings of renal tumor tissue, initiated with DEN and promoted with Fe-NTA and its inhibition by *T. chebula* extract. The control group did not showed any morphological changes. By contrast, the kidneys of rats promoted with Fe-NTA showed marked pathological changes as evidenced by tubular dilatation, necrosis of proximal tubules, flattened epithelium, swelling of kidneys and congested blood vessels. The renal section from DEN initiated and Fe-NTA promoted rats showed prominent adenocarcinoma, tubular brush border loss with presence of ghost tissue, prominent adenocarcinoma, leucocytic cell infilteration and hyperchromatism. Pretreatment with *T. chebula* extract at both doses resulted in alleviation of Fe-NTA induced renal pathological deterioration as evident in Fig. 3(d) and (e).

3. Discussion

Experimental works on several plants has been carried out to evaluate their efficacy against chemically induced toxicity (Sehrawat et al. 2006; Jahangir et al. 2005; Prasad et al. 2004). Chemopreventive agents work by inhibiting the development of invasive cancer either by blocking initiation or reversing the progression of premalignant cells (Hong and Sporn 1997; Wattenberg 1997). The anti-oxidant and anti-tumor property of *T. chebula* may be related to the presence of tannins and related compounds as the major phenolics of *T. chebula* extract. The antioxidants are known to play a key role in reducing cancer cell proliferation (Chinery et al. 1998), and tannins are known as strong lipid peroxidation inhibitors (Okuda et al. 1983). Some of the major constituents of *T. chebula* extract such as gallic acid and chebulagic acid have been reported to have antigrowth and cytotoxic effects (Lee et al. 1995). Also ellagic acid, generated by the hydrolysis of ellagitarmins in plants, is well known as a natural dietary antioxidant and chemopreventive agent (Perchellet et al. 1992). It is a potent antagonist of the mutagenicity of various aromatic hydrocarbons and a possible prototype of a new class of anti-cancer drug molecules (Das et al. 1985). Presence of these potent antioxidants in the extract may therefore be responsible for the overall antiradical and antioxidant activity of the plant. *T. chebula* ameliorated Fe-NTA mediated inhibition of the activities of antioxidant enzymes viz., glutathione peroxidase, glutathione reductase, catalase, glucose-6-phosphate dehydrogenase and phase II metabolizing enzymes such as glutathione-S-transferase and quinine reductase. The action of phase II enzymes on the substrates generated by the action of phase I enzymes on innocuous or hazardous chemicals leads to their solubilisation and excretion (Shimada 2006).

T. chebula has established antioxidant property that might have counteracted the oxidant effects of Fe-NTA by scavenging free radicals. Antioxidant and detoxification enzymes can block carcinogenesis by acting as inhibitors of environmental carcinogens and mutagens (Nagao et al. 1986). Hence, enhancement of these enzymes by a natural or synthetic component may result in the amelioration of carcinogen induced toxicity. Thus it suggests that *T. chebula* ameliorated Fe-NTA mediated renal damage. There was also dose dependent decrease in the Fe-NTA mediated susceptibility of renal microsomal membrane for iron-ascorbate induced lipid peroxidation as shown by ameliorated malondialdehyde levels. The increased activities of xanthine oxidase, H_2O_2 and the depleted levels of renal glutathione content were also recovered on treatment with *T. chebula.* Thus showing that *T. chebula* has the ability to detoxify the foreign agents that pose threat to the integrity and proper functioning of the cell since it restores the levels of endogenous thiol group containing moieties, the reduced glutathione. ODC is a rate limiting enzyme of polyamine biosynthesis and the activity of ODC has been reported to increase at the time of acceleration of cell proliferation and development (Megosh et al. 1995). An enhancement in both renal ODC activity and [^{3}H] incorporation suggests a strong proliferative and tumor promoting potential of Fe-NTA. Most of the inhibitors of ODC induction and blockers/or inhibitors of DNA synthesis tested to date also protect against tumor promotion (Wei et al. 1998; Peter et al. 1992). A sharp decrease in Fe-NTA mediated induction in ODC activity and decline in the enhancement of [^{3}H] thymidine incorporation with the pretreatment of *T. chebula* further suggests the anti-tumor potential of *T. chebula.* The improvement in kidney function in Fe-NTA treated rats as evident from a sharp decrease in the levels of blood urea nitrogen and serum creatinine, marker parameters of kidney damage suggests the effectiveness of the prophylaxis of *T. chebula* extract. Also, in this study, the kidneys of DEN initiated and Fe-NTA promoted rats showed characteristic pathological findings such as adenocarcinoma, necrotic tissue, ghost tissue and hyperchromatism which was attenuated on *T. chebula* pre-treatment, thus showing its efficacy against Fe-NTA induced renal carcinogenesis via inhibition of tumor formation.

In summary the data of this study suggest that *T. chebula* facilitates a number of responses relevant to cancer chemoprevention and inhibits Fe-NTA induced renal carcinogenesis in Wistar rats.

4. Experimental

4.1. Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), bovine serum albumin (BSA), 1,2-dithio-bis-nitrobenzoic acid (DTNB), 1-chloro-2, 4-dinitro benzene (CDNB), nitrilotriacetic acid (NTA), reduced nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate, Tween-20, 2,6- dichlorophenolindophenol, thiobarbituric acid (TBA) were obtained from Sigma chemicals Co (St Louis, MO, USA). DL [^{14}C] ornithine (specific gravity 56mCi mmol) and [^{3}H] thymidine (specific gravity 82mCi mmol) were purchased from Amersham Corporation (Little Chalfort, UK). All other chemicals were of the highest purity and commercially available.

4.2. Plant material

Total extract of *T. chebula* in semi-solid form was purchased from Saiba industries, Mumbai, India. The extract is claimed to possess all the active ingredients of the plant. Methanolic fraction was used for the present study after preliminary in vitro tests.

4.3. Preparation of Fe-NTA solution

Fe-NTA was prepared fresh immediately before its use by the method of Awai et al. (1979). To prepare Fe-NTA, ferric nitrate (0.16 mmol/kg body weight) solution was mixed with a fourfold molar excess of disodium salt of NTA (0.64 mmol/kg body weight) and the pH was adjusted to 7.4 with sodium bicarbonate solution.

4.4. Animals

Four to six weeks old male Wistar rats (130–150 g) of were obtained from the Central Animal House of Hamdard University, New Delhi, India. They were housed in polypropylene cages in groups of six rats per cage and were kept in a room maintained at 25 ± 2 °C with a 12 h light/dark cycle. They were allowed to acclimatize for one week before the experiments and

were given free access to standard laboratory feed (Hindustan Lever Ltd., Bombay, India). Animals received enough food to permit normal growth and maintenance of age-appropriate body weight. Food was stored in designated restricted areas that are cool, clean, dry and free of vermin. Animal facilities and other areas in contact with laboratory animals were cleaned and disinfected often to keep them free of dirt, debris, and biological or chemical contaminants.

4.5. Treatment regimen

To study the effect of pretreatment of animals with *T. chebula* on Fe-NTA induced renal oxidative stress and ODC induction, 30 male Wistar rats were randomly allocated to five groups of six rats in each. The animals of group I served as control and received normal saline (0.85% NaCl). The animals of group II received only a single intraperitoneal injection of Fe-NTA at a dose of 9 mg Fe/kg body weight. Group III received pretreatment with *T. chebula* by gavages once daily for 7 days at a dose of 25 mg/kg body weight. Groups IV and V received pretreatment with *T. chebula* extract once daily for seven consecutive days at a dose level of 50 mg/kg body weight. One hour after the last treatment with *T. chebula* the animals of group III and IV received a single intraperitoneal injection of Fe-NTA (9 mg Fe/kg body weight). All the animals were sacrificed under light ether anesthesia 12 h after last treatment. Kidneys were quickly removed and washed with ice-cold saline. Immediately before sacrifice, blood was collected from the retro-orbital sinus for the estimation of serum creatinine and blood urea nitrogen (BUN).

To study the effect of pretreatment with *T. chebula* on Fe-NTA mediated [^{3}H] thymidine incorporation into renal DNA, the grouping of animals and schedules for prophylaxis were same as described above. One hour after the last treatment with *T. chebula,* the animals of group II, III and IV received only a single intraperitoneal injection of Fe-NTA at a dose of 9 mg Fe/kg body weight. Eighteen hours after the treatment with Fe-NTA the rats received [^{3}H] thymidine (30 μCi/animal) by intraperitoneal injection. Two hours later, they were sacrificed under light ether anesthesia and their kidneys were quickly removed.

To study the effect of pretreatment with *T. chebula* extract on DEN (*N*-diethylnitrosamine) initiated and Fe-NTA promoted renal carcinogenesis, the animals were divided into five groups of 20 rats per group. Group I received only saline injection intraperitoneally (0.85% NaCl) at a dose of 10 ml/kg body weight. Animals of groups II, IV and V were initiated with a single i.p. injection of DEN at a dose level of 200 mg/kg body weight in saline. Ten days after initiation, the animals in groups II, III, IV and V were promoted with an intraperitoneal injection of Fe-NTA at a dose of 9 mg Fe/kg body weight, twice a week for 16 weeks. Groups IV and V received oral treatment with *T. chebula* extract by gavage once daily at a dose of 25 and 50 mg/kg body weight respectively 30 min prior to the treatment with Fe-NTA for a period of 16 weeks, twice a week. At the end of 24 weeks, the animals were sacrificed under light ether anesthesia and their kidneys were quickly removed and preserved in 10% neutral buffered formalin for histopathological studies. Haematoxylin and eosin preparations of processed sections were prepared for microscopic examination.

4.6. Tissue preparation

Kidneys were removed quickly, cleaned free of extraneous material and immediately perfused with ice-cold saline (0.85% sodium chloride). The kidneys were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCI (1.17%) using a polytron homogenizer (Kinematica A.G.). The homogenate was filtered through a muslin cloth, and was centrifuged at 800 × g for 5 min at 4 °C by Eltek Refrigerated Centrifuge (model RC 4100 D) to separate the nuclear debris. The aliquot so obtained was centrifuged at 12000 × g for 20 min at 4 °C to obtain PMS, which was used as a source of enzymes. A portion of the PMS was centrifuged for 60 min by ultracentrifuge (Beckman L7-55) at 10,500 × g at 4 °C. The pellet was washed with phosphate buffer (0.1 M, pH 7.4) containing KCI (1.17%).

4.7. Biochemical determinations

4.7.1 Total reduced thiol

Total reduced thiols were determined by the method of Jollow et al. (1974). A 1.0 ml postmitochondrial supernatant fraction (PMS) (10%) was mixed with 1.0 ml sulphosalicylic acid (4%). The samples were incubated at 4 °C for at least 1 h and then centrifuged at 1200 g for 15 min at 4 °C. The reaction mixture contained 0.4 ml of the filtered sample, 2.2 ml phosphate buffer (0.1 M, pH 7.4) and 0.4 ml DTNB (4 mg/ml) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm on a spectrophotometer (Milton Roy Model-21 D). The reduced glutathione concentration was calculated as nmol GSH/g tissue.

4.7.2. Glutathione S-transferase (GST) activity

GST activity was assayed by the method of Habig et al. (1974). The reaction mixture consisted of 2.5 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml GSH (1 mM), 0.2 ml CDNB (1 mM) and 0.1 ml of the cytosolic fraction (10%) in a total volume of 3.0 ml. The changes in absorbance were recorded at 340 nm and enzymatic activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of $9.6 \times 10^3\,M^{-1}\,cm^{-1}$.

4.7.3. Glutathione reductase (GR) activity

GR activity was assayed by the method of Carlberg and Mannervick (1975). The reaction mixture consisted of 1.65 ml phosphate buffer (0.1 M, p H7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml PMS (10%) in a total volume of 2.0 ml. Enzyme activity was quantitated at 25 °C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using a molar extinction coefficient of $6.22 \times 10^3\,M^{-1} \cdot cm^{-1}$.

4.7.4. Lipid peroxidation (LPO)

LPO was estimated following method of Wright et al. (1981). The reaction mixture in a total volume of 1.0 ml contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsome, 0.2 ml ascorbic acid (100 mM), 20 μl ferric chloride (100 mM). The reaction mixture was incubated at 37 °C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1 ml of trichloroacetic acid TCA (10%). Following addition of 1.0 ml TBA (0.67%), all the tubes were placed in a boiling water bath for a period of 20 min. The tubes were shifted to ice bath and then centrifuged at 2500 × g for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm. The results were expressed as the nmol MDA formed/h/g tissue at 37 °C by using a molar extinction coefficient of $1.56 \times 10^5\,M^{-1} \cdot cm^{-1}$.

4.7.5. Blood urea nitrogen (BUN)

BUN was determined by the diacetyl monoxime method of Kanter (1975). Protein free filtrate was prepared. To 0.5 ml of protein free filtrate, 3.5 ml of distilled water, 0.8 ml diacetylmonoxime (2%) and 3.2 ml sulphuric acid-phosphoric acid reagent were added (reagent was prepared by mixing 150 ml 85% phosphoric acid with 140 ml water and 50 ml of concentrated sulphuric acid). The reaction mixture was placed in a boiling water-bath for 30 min and then cooled. The absorbance was recorded at 480 nm.

4.7.6. Creatinine

Creatinine was estimated by the alkaline picrate method of Hare (1950). Protein free filtrate was prepared. To 1.0 ml serum was added, 1.0 ml sulphuric acid (0.6 N) and 1.0 ml distilled water. After mixing thoroughly, the mixture was centrifuged at 800xg for 5 min. The supernatant was added to a mixture containing 1.0 ml picric acid (1.05%) and 1.0 ml sodium hydroxide (0.75 N). The absorbance at 520 nm was recorded after 20 min.

4.7.7. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) was assayed by H_2O_2 mediated horseradish peroxidase dependent oxidation of phenol red by the method of Pick and Keisari (1981). 2.0 ml of microsomes were suspended in 1.0 ml of solution containing phenol red (0.28 nm), horseradish peroxidase (8.5 units), dextrose (5.5 nm) and phosphate buffer (0.05 M, pH 7.0) and were incubated at 37 °C for 60 min. The reaction was stopped by the addition of 0.01 ml of NaOH (10 N) and then centrifuged at 800 × g for 5 min. The absorbance of the supernatant was recorded at 610 nm against a reagent blank. The quantity of H_2O_2 produced was expressed as nmol H_2O_2 per hour per gram tissue based on the standard curve of H_2O_2 oxidized phenol red.

4.7.8. Glutathione peroxidase (GPx) activity

GPx activity was measured by the method of Mohandas et al. (1984). The reaction mixture consisted of 1.44 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 0.05 ml glutathione reductase (1 IU/ml), 0.05 ml reduced glutathione (1 mM), 0.1 ml NADPH (0.2 mM) and 0.01 ml H_2O_2 (0.25 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. The disappearance of NADPH at 340 nm was recorded at 25 °C. Enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using a molar extinction coefficient of $6.22 \times 10^3 M^{-1} \cdot cm^{-1}$.

4.7.9. Catalase activity

Catalase activity was assayed by the method of Claiborne (1985). The reaction mixture consisted of 1.95 ml phosphate buffer (0.1 M, pH 7.4), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml 10% PMS in a final volume of 3 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed/min/mg protein.

4.7.10. Glucose-6-phosphate dehydrogenase (G6PD) activity

The activity of G6PD was determined by the method of Zaheer et al. (1965). The reaction mixture consisted of 0.3 ml Tris-HCl buffer (0.05 M, pH 7.6), 0.1 ml NADP (0.1 mM), 0.1 ml glucose-6-phosphate (0.8 mM),

0.1 ml $MgCl_2$ (8 mM), 0.3 ml PMS (10%) and 2.1 ml distilled water in a total volume of 3 ml. The changes in absorbance were recorded at 340 nm and enzyme activity was calculated as nmol NADP reduced/min/mg protein using a molar extinction coefficient of $6.22 \times 10^3\ M^{-1} \cdot cm^{-1}$.

4.7.11. Xanthine oxidase (XO) activity

The activity of XO was assayed by the method of Athar et al. (1996). The reaction mixture consisted of 0.2 ml PMS which was incubated for 5 min at 37 °C with 0.8 ml phosphate buffer (0.1 M, pH 7.4). The reaction was started by adding 0.1 ml xanthine (9 mM) and kept at 37 °C for 20 min. The reaction was terminated by the addition of 0.5 ml ice cold perchloric acid (10% v/v). After 10 min, 2.4 ml of distilled water was added and centrifuged at 4000 rpm for 10 min. The clear supernatant was read at 290 nm. The result was expressed as uric acid formed/min/mg protein.

4.7.12. Quinone reductase (QR) activity

The activity of QR was determined by the method of Benson et al. (1980). The 3 ml reaction mixture consisted of 2.13 ml Tris-HCl buffer (25 mM, pH 7.4), 0.7 ml BSA, 0.1 ml FAD, 0.02 ml NADPH (0.1 mM) and 50 µL (10%) PMS. The reduction of dichlorophenolindophenol (DCPIP) was recorded calorimetrically at 600 nm and enzyme activity was calculated as nmol of DCPIP reduced/min/mg protein using molar extinction coefficient of $2.1 \times 10^4\ M^{-1} \cdot cm^{-1}$.

4.7.13. Ornithine decarboxylase (ODC) activity

ODC activity was determined using 0.4 ml renal 105,000×g supernatant fraction per assay tube by measuring release of $^{14}CO_2$ from DL-[^{14}C] ornithine by the method of O'Brien et al. (1975). The kidney was homogenized in Tris-HCI buffer (pH 7.5, 50 mM) containing EDTA (0.4 mM), pyridoxal phosphate (0.32 mM), PMSF (0.1 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (4.0 mM) and Tween® 80 (0.1%) at 4 °C using a poltroon's homogenizer (Kinematica AGPT 3000). In brief, the reaction mixture contained 400 Wl enzymes and 0.095 ml co-factor mixture containing pyridoxal phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), Brij 35 (0.02%) and DL-[^{14}C] ornithine (0.05 µ Ci) in total volume of 0.495 ml. After adding buffer and co-factor mixture to blank and others tubes, the tubes were closed immediately with a rubber stopper containing 0.2 ml ethanol amine and methoxyethanol mixture (2 : 1) in the central well and kept in water-bath at 37 °C. After 1 h of incubation, the enzyme activity was arrested by injecting 1.0 ml citric acid solution (2.0 M) along the sides of glass tubes and the solution was continued for 1 h to ensure complete absorption of CO_2. Finally, the central well was transferred to a vial containing 2.0 ml ethanol and 10.0 ml toluene based scintillation fluid. Radioactivity was counted in a liquid scintillation counter (LKB Wallace-1410). ODC activity was expressed as pmol CO_2 released/hr/mg protein.

4.7.14. DNA synthesis

The isolation of renal DNA and incorporation of [^{3}H] thymidine in DNA was done by the method of Smart et al. (1986). Kidney's were quickly removed and cleaned free of extraneous material homogenate (10% w/v) was prepared in ice-cold water. The precipitate thus obtained was washed with cold trichloroacetic acid (TCA) (5%) and incubated with cold perchloric acid (PCA) (10%) at 4 °C overnight. After incubation it was centrifuged and the precipitate was washed with cold PCA (5%). The precipitate was dissolved in warm PCA (10%) followed by incubation in boiling water bath for 30 min, and filtered through Whatman 50. The filtrate was used for [^{3}H] thymidine counting in a liquid scintillation counter (LKB-Wallace-1410) by adding the scintillation fluid. The amount of DNA in the filtrate was estimated by diphenylamine method of Giles and Myers (1965). The amount of [3 H] thymidine incorporated was expressed as DPM/µg DNA.

4.7.15. Protein estimation

The protein concentration in all samples was determined by the method of Lowry et al. (1951). Peptide bonds form a complex with alkaline copper sulphate reagent, which gives a blue colour with Folin's reagent. Briefly, 0.1 ml PMS was diluted to 1 ml water and protein was precipitated with equal volume of TCA (10%), kept overnight 4 °C and centrifuged at 800 × g for 5 min. The supernatant was discarded. The pellet was dissolved in 5 ml of NaOH (1 N). Finally 0.1 ml of aliquot was further diluted to 1 ml with water and then 2.5 ml of alkaline copper sulphate reagent containing sodium carbonate (2%) copper sulphate (1%) and sodium potassium tartarate (2%) was added. Ten minutes after addition of alkaline copper sulphate reagent to allow complex formation, 0.25 ml of Folin's reagent was added. After 30 min a blue colour developed that was read at 660 nm. Serum albumin (BSA 0.1mg/ml) was used as a standard.

4.8. Statistical analysis

Differences between groups were analysed using analysis of variance (ANOVA) followed by Dunnet's multiple comparison tests. All data points are presented as the treatment group ± standard error of the mean.

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