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Chemopreventive effect of *Quercus infectoria* against chemically induced renal toxicity and carcinogenesis

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Abstract

In this study we have shown that *Quercus infectoria* attenuates Fe-NTA induced renal oxidative stress, hyperproliferative response and renal carcinogenesis in rats. Fe-NTA promoted DEN (*N*-diethyl nitrosamine) initiated renal carcinogenesis by increasing the percentage incidence of tumors and induces early tumor markers viz. ornithine decarboxylase (ODC) level and PCNA expression. Fe-NTA (9 mg Fe/kg body weight, intraperitoneally) enhances renal Malondialdehyde, xanthine oxidase and hydrogen peroxide generation with reduction in renal glutathione content, antioxidant enzymes, viz., glutathione peroxidase, glutathione reductase, catalase, glucose-6-phosphate dehydrogenase and phase-II metabolizing enzymes such as glutathione-S-transferase and quinone reductase. It also enhances blood urea nitrogen and serum creatinine. Fe-NTA also lead to increase in levels of some inflammatory markers viz NO and MPO and some proinflammatory cytokines viz PGE-2 and TNF-α. The chemopreventive efficacy of *Quercus infectoria* was studied in terms of xenobiotic metabolizing enzyme activities, LPO, redox status, serum toxicity markers, inflammatory and proinflammatory markers and cell proliferation in the kidney tissue. Oral administration of *Quercus infectoria* at doses of 75 and 150 mg/kg b wt effectively suppressed renal oxidative stress, inflammation and tumor incidence. Chemopreventive effects of *Quercus infectoria* were associated with up-regulation of xenobiotic metabolizing enzyme activities and down regulation of serum toxicity markers. Present study supports *Quercus infectoria* as a potent chemopreventive agent and suppresses Fe-NTA-induced renal carcinogenesis and oxidative and inflammatory response in Wistar rat.

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Chemoprevention, Carcinogenesis, Nephrotoxicity, PCNA and Inflammation.

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Introduction

Epidemiological studies have shown that increased body iron storage is associated with an increased risk of cancer and early death. Experimental studies have

demonstrated that iron overload dramatically potentiates chemical carcinogenicity [1]. Mechanisms whereby iron may act in carcinogenesis are induction of oxidative stress, facilitation of tumor growth and modification of the immune system. Metal ions react with superoxide anion ($O^{-}{}^{2}$) and H_2O_2 to produce highly reactive species such as hydroxyl free radical (·OH) and metal–oxygen complexes in biological systems, resulting in oxidative DNA damage. Since H_2O_2 itself is not toxic to cells, H_2O_2-induced oxidative DNA damage in cells has been thought to result from the formation of hydroxyl free radicals through Fenton reaction with iron [2].

The development of RCC has been linked with innumerable risk factors including environmental exposure to different toxicants [3]. Nitrilotriacetic acid (NTA) is an aminotricarboxylic acid with an empirical formula of $C_6H_9NO_6$. Nitrilotriacetate (NTA) can make complexes with metal ions such as Fe^{3+} or Cu^{2+}. Fe-NTA, a complex of $Fe^{3+}$and NTA, is a strong nephrotoxic agent and a renal carcinogen. It is an established fact that an iron-chelate of nitrilotriacetate, ferric nitrilotriacetate (Fe-NTA) induces acute and sub-acute renal injury in animals [4-6]. It is evidenced from previous reports that oxygen free radical was formed from redox-active iron and was detected in the serum of Fe-NTA-treated rats [7-8].

Medicinal plants have been used by all civilization as a basis of medicines since ancient times. In the recent times natural products have been used to prevent the toxicities induced by chemicals, drugs and carcinogenic xenobiotics. Plant based products are generally considered safe and proved to be effective against various human ailments and their medicinal uses have been gradually increasing in developed countries. Epidemiological studies have also proven that consumption of vegetables and fruit have caused lower incidence of cancers [9]. Interest in medicinal plants as a re-emerging health aid also has been encouraged by the rising costs of prescription drugs in the maintenance of personal health and well-being, and the bioprospecting of new plant derived drugs. Studies have shown that natural phytochemicals containing phenolic compounds derived from certain plants have the capability to prevent cancer.

Quercus infectoria Olivier (Fagaceae) is a small tree or a shrub mainly present in Asia Minor, Greece, Syria and Iran. The tree yield galls that emerge on its shoots as a consequence of assault of gall wasp *Cypnis gallae tincotoriae* [10]. The *Quercus infectoria* galls (QI) have a great medicinal value and have pharmacologically been deciphered to be astringent, antipyretic, anti-diabetic, anti-tremor, local anesthetic and anti-Parkinson [11-13].

Kaur et al., 2007[14] reported QI is capable of protecting against oxidative damage to lipids and proteins and also chelates metal ions that catalyze the generation of oxidants. The main constituents found QI are tannins (50-70%) and free gallic acid and ellagic acid [15-16].

No published information is available on the chemopreventive aspects of QI against renal carcinogenesis. Therefore, we examined its effects in amending the Fe-NTA nephrotoxicity and its chemopreventive efficacy against two stage renal carcinogenesis in wistar rats by studying its various potential molecular targets. We also studied the level of inflammatory markers and tumor promotion markers which are known to be dysregulated in cancer cells and which might be one of the targets of the chemopreventive action of QI.

Material Methods

Chemicals

Oxidized and reduced glutathione, Nitrilotriacetate (NTA), N-nitrosodiethylamine (DEN), nitrilotriacetic acid, H_2O_2, dithionitrobenzene (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reductase, reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavine adenine dinucleotide

(FAD) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the antibodies, chemicals and reagents used were of highest purity and standard commercially available. Rat TNF-α ELISA Ready Set Go, E-bioscience. (U.S.A), ELISA PGE2 EIA kit, Cayman chemical company, (U.S.A)

Animals

Young (8–10 weeks old), male wistar rats were housed in plastic (polypropylene) cages in animal house facility of Hamdard University. Experiments were conducted according to protocols approved by CPCSEA animal ethical committee, New Delhi, India, project number and date 547/CPCSEA, May 28th, 2009. The well ventilated animal rooms (room temperature set at 25°C) were maintained on 12 h light–dark cycles. They were acclimatized for one week before the study and had free access to standard laboratory feed (Hindustan lever Ltd, Mumbai, India) and water *ad libitum*.

Preparation of Fe-NTA

Preparation of Fe-NTA solution was done by method given by Awai et al. (1979) [4] as modified by Athar and Iqbal (1998) [17]. Briefly, ferric nitrate (0.16 mM) solution was mixed with a four-fold molar excess of disodium salt of NTA (0.64 nM) and the pH was adjusted to 7.4 with a sodium bicarbonate solution. The solution was prepared immediately before each protocol.

Experimental Design

The treatment regimen for *Quercus infectoria* and the proposal of verifying its chemopreventive efficacy against renal carcinogenesis was based on the preliminary dose dependent pilot study carried out in our laboratory. To study the chemo-protective effects of *Quercus infectoria* on biochemical and serological changes induced by toxicity of Fe-NTA in rats, 30 male Wistar rats were randomly divided into five equal groups. *Quercus infectoria* was dissolved in double distilled water. Rats were initiated with intraperitoneal (IP) injection of DEN at a dose of 200 mg/kg b wt and promoted with Fe-NTA at a dose of 9 mg Fe/kg b wt IP. Selection of the dose regimen is based on our own preliminary experiments and is also based on previously published data from our laboratory [17-18]. Following treatment regimen was followed in our study

- Group I animals received only normal saline (0.9%) for seven consecutive days by oral gavage and thus served as untreated controls.
- Group II served as treated control and was administered a single dose of Fe-NTA on 20th day.
- Group III was pretreated with oral gavage of *Quercus infectoria* at a dose of 75 mg/kg b wt for 20 consecutive days followed by administration of Fe-NTA on 20th day.
- Group IV was pretreated with oral gavage of *Quercus infectoria* at a dose of 150 mg/kg b wt for 20 consecutive days followed by administration of Fe-NTA on 20th day.
- Group V received by oral gavage of *Quercus infectoria* only, at a dose of 150 mg/kg b wt for 20 consecutive days.

All animals were sacrificed exactly 12 h after Fe-NTA administration. Kidney tissue was processed for biochemical estimations. Blood was collected and serum was separated out and processed for serological studies.

To study the effect of pretreatment with *Quercus infectoria* on DEN initiated and Fe-NTA–promoted renal carcinogenesis, the rats were divided into six groups of 25 rats per group. The complete treatment regimen followed in tumor study is described below:

- Group I animals received only normal saline (0.9%) by oral gavage once daily for 16 weeks and served as controls.
- Group II also received only normal saline (0.9%) by oral gavage once daily for 16 weeks. In addition group II was given IP injection of DEN in saline on the very first day of experiment and ten days after the

injection, the animals were promoted with IP injection of Fe-NTA, twice a week for 16 weeks.

- Group III was given same treatment as group II and was also co-treated by oral gavage of *Quercus infectoria* once daily, at a dose of 75 mg/kg b wt, an hour prior to the treatment of Fe-NTA for a period of 16 weeks.
- Group IV was given same treatment as group II and was also co-treated by oral gavage of *Quercus infectoria* once daily at a dose of 150 mg/kg b wt, an hour prior to the treatment of Fe-NTA for a period of 16 weeks.
- Group V received only *Quercus infectoria* by oral gavage, once daily for 16 weeks. In addition group V was given IP injection of DEN in saline on the very first day of experiment.

At the end of 24 weeks, all the animals were sacrificed under light ether anesthesia. Their kidneys were quickly removed and processed for various molecular, histopathological and immunohistochemical studies.

Postmitochondrial supernatant (PMS) preparation

Post-mitochondrial supernatant of kidney samples was prepared by the method as described by Tahir and Sultana, 2011 [19].

Assay for catalase activity

The catalase activity was assessed by the method of Claiborne, 1985. In short, the reaction mixture was comprised of 0.05 ml of PMS, 1.0 ml of hydrogen peroxide (0.019 M), 1.95 ml of phosphate buffer (0.1 M, pH 7.4), in a total volume of 3 ml. Changes in absorbance were recorded at 240 nm, and the change in absorbance was calculated as nmol H_2O_2 consumed per min per mg of protein [20].

Estimation of lipid peroxidation level

The assay of lipid peroxidation (LPO) was done according to the method of Wright et al., 1981. The reaction mixture consisted of 0.58 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsome, 0.2 ml of ascorbic acid (100 mM) and 0.02 ml of ferric chloride (100 mM) in a total of 1 ml. This reaction mixture was then incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1 ml of TCA (10%). Following addition of 1.0 ml of TBA (0.67%), all the tubes were placed in a boiling water bath for a period of 20 min. The tubes were shifted to an 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 $1.56 \times 105M{-}1\ cm{-}1$ [21].

Estimation of GSH level

GSH was assessed by the method of Jollow et al., 1974. A quantity of 1.0 ml of 10% PMS mixed with 1.0 ml of (4%) sulphosalicylic acid was taken and then incubated at 4°C for a minimum time period of 1 h and then centrifuged at 4°C at 1200 × g for 15 min. The reaction mixture of 3.0 ml was composed of 0.4 ml of supernatant, 2.2 ml of phosphate buffer (0.1 M, pH 7.4) and 0.4 ml of DTNB (4 mg/ml). The yellow color developed was read immediately at 412 nm on the spectrophotometer (Perkin Elmer, lambda EZ201). The GSH concentration was calculated as nmol GSH conjugates/ g tissue [22].

Assay for glutathione peroxidase activity

The activity of glutathione peroxidase (GPx) was calculated by the method of Mohandas et al. (1984). The total volume of 2 ml was composed of 0.1 ml of EDTA (1 mM), 0.1 ml of sodium azide (1 mM), 1.44 ml of phosphate buffer (0.1 M, pH 7.4), 0.05 ml of GR (1 IU/ml), 0.05 ml of GSH (1 mM), 0.1 ml of NADPH (0.2 mM) and 0.01 ml of H_2O_2 (0.25 mM) and 0.1 ml of 10% PMS. The depletion of NADPH at 340 nm was recorded at 25°C. Enzyme activity was calculated as nmol NADPH oxidized/min/mg protein with the molar extinction coefficient of $6.22 \times 103M{-}1\ cm{-}1$ [23].

Measurement of SOD activity

The SOD activity was measured by the method of Marklund and Marklund 1974. The reaction mixture consisted of 2.875 ml Tris–HCl buffer (50 mM, pH 8.5), pyrogallol (24mM in 10mM HCl) and 100 µl PMS in a total volume of 3 ml. The enzyme activity was measured at 420 nm and was expressed as units/mg protein. One unit of enzyme is defined as the enzyme activity that inhibits auto-oxidation of pyrogallol by 50% [24].

Measurement of quinone reductase (QR) activity

The QR activity was determined by the method of Benson et al., 1980. The 3ml 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 PMS (10%). The reduction of dichlorophenolindophenol (DCPIP) was recorded calorimetrically at 600 nm and the enzyme activity was calculated as µmol of DCPIP reduced/min/mg protein using molar extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} [25].

Glutathione reductase activity

GR activity was determined by the method of Carlberg and Mannervik. The reaction mixture consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml GSH (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. Enzyme activity was quantified at 25 °C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized per min per mg protein using a molar extinction coefficient of 6.22× 103 M−1 cm−1 [26].

Glutathione-S-Transferase activity

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. Changes in absorbance were recorded at 340 nm, and enzymatic activity was calculated as nmol CDNB conjugate formed per min per mg protein using a molar extinction coefficient of 9.6×103 M−1 cm−1 [27].

Blood urea nitrogen level

Estimation of blood urea nitrogen (BUN) was done by the method of Kanter (1975). Protein-free filtrate was prepared by adding serum and an equal amount of 10% TCA; then the mixture was centrifuged at 2000 r.p.m. and the supernatant was obtained.

0.8 ml of diacetylmonoxime (2%) and 3.2 ml of sulfuric acid–phosphoric acid reagent (reagent was prepared by mixing 150 ml of 85% phosphoric acid with 140 ml of water and 50 ml of concentrated sulfuric acid). The reaction mixture was placed in a boiling water bath for 30 min and then cooled. The absorbance was read at 480 nm [28].

Serum creatinine level

Creatinine was estimated by the method of Hare (1950). Protein-free filtrate was prepared. To 1.0 ml of serum were added 1.0 ml of sodium tungstate (5%), 1.0 ml of sulfuric acid (0.6 N) and 1.0 ml of distilled water. After mixing thoroughly, the mixture was centrifuged at 800 g for 5 min. The supernatant was added to a mixture containing 1.0 ml of picric acid (1.05%) and 1.0 ml of sodium hydroxide (0.75 N). The absorbance at 520 nm was read exactly after 20 min [29].

Assay for lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity was estimated in serum by the method of Korenberg, 1955. The assay mixture consisted of 0.2 ml of serum, 0.1 ml of 0.02 M NADH, 0.1 ml of 0.01 M sodium pyruvate, 1.1 ml of 0.1 M (pH 7.4) phosphate buffer and distilled water in a total volume of 3 ml. Enzyme activity was recorded at 340 nm, and activity was calculated as nmol NADH oxidized/min/mg protein [30].

Assay for Hydrogen Peroxide

Hydrogen peroxide (H2O2) was assayed by H2O2 mediated horseradish peroxidase– dependent oxidation of phenol red by the method of Pick and Keisari, 1981. Microsomes (2.0 ml) 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 800g for 5 min. The absorbance of the supernatant was recorded at 610 nm against a reagent blank. The quantity of H2O2 produced was expressed as nmol H2O2 per hour per g tissue based on the standard curve of H2O2 oxidized phenol red [31].

Nitrite Estimation

Nitrite assay was done using Griess reagent by the method of Green et al. 1982 with some modifications. In brief, 100 µl of Griess reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in water) was added to 100 µl of PMS incubate for 5–10 min at room temperature protected from light. Purple/magenta color began to form immediately. Absorbance was measured at 546 nm, nitrite concentration was calculated using a standard curve for sodium nitrite, and nitrite levels were expressed as l mol/mg protein [32].

γ-glutamyl transpeptidase Activity

GGT Activity was done by Orlowski and Meister, 1973. The reaction mixture in a total volume of 0.1ml contained 0.2ml serum, which was incubated with 0.8ml of the substrate mixture (containing 4 mM Gammaglutamyl *p*-nitroanilide, 40 mM glycine and 11 mM $MgCl_2$ in 185 mM Tris HCl buffer, pH 8.25) at 37°C. At 10 minutes after initiation of the reaction 1.0 ml of Trichloroacetic acid (TCA) (25%) was added abd mixed to terminate the reaction. The solution was centrifuged and the supernatant fraction was read at 405 nm. The enzyme activity was calculated as nmol p-nitroaniline formed min^{-1} (mg protein)$^{-1}$ using a molar extinction coefficient of *p*-nitroaniline as 1.74×10^3 M^{-1} cm^{-1} [33].

Myeloperoxidase quantification (marker for early inflammation)

The Myeloperoxidase (MPO) activity or direct neutrophil quantification was determined by the method of Bradley et al., 1982. With some modifications and expressed as units/ min/mg protein. One unit of MPO activity was defined as that degrades 1 mmol of peroxide per min [34].

Measurement of Xanthine Oxidase activity

The activity of xanthine oxidase (XO) was assayed by the method of Stirpe & Della Corte, 1969. The reaction mixture consisted of 0·2ml PMS which was incubated for 5 min at 37°C 0·8ml phosphate buffer (0·1 M, pH 7·4). The reaction was started by adding 0·1ml xanthine (9mM) and kept at 378C for 20 min. The reaction was terminated by the addition of 0·5ml ice-cold perchloric acid (10% (v/v)). After 10min, 2.5 ml distilled water was added and the mixture was centrifuged at 4000 x g for 10min. The absorbance of clear supernatant was read at 290 nm. The activity was expressed as uric acid formed /mg protein [35].

Ornithine decarboxylase (ODC) activity

ODC was evaluated according to O'Brien et al., 1975. 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. The kidneys were homogenized in Tris–HCl buffer (pH 7.5, 50 mM) containing EDTA (0.1 mM), pyridoxal phosphate (0.1 mM), PMSF (1.0 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (0.1 mM) and Tween-80 (0.1%) at 4°C. In brief, the reaction mixture contained 400µl enzyme 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), Brig 35 (0.02%) and [^{14}C] ornithine (0.05 /µCi) in a total volume of 0.495 ml. After adding buffer and co-factor mixture to blank and other test tubes, the tubes were closed immediately with a rubber stopper containing 0.2 ml ethanolamine and methoxyethanol mixture in the central well and kept in a water bath at 37°C. After 1 h of incubation, the enzyme activity was inhibited by injecting 1.0 ml citric acid solution (2.0 M) along the

sides of glass tubes and the incubation was continued for 1 h to ensure complete absorption of $^{14}CO_2$. Finally, the central well was transferred to a vial containing 2ml ethanol and 10 ml toluene-based scintillation fluid was added. Radioactivity was counted in a liquid scintillation counter (LKB Wallace-1410). ODC activity was expressed as pmol $^{14}CO_2$ released/h mg protein [36].

Estimation of protein

The protein concentration in all samples was determined by the method of Lowry et al., 1951, using BSA as standard [37].

Cytokine Analysis

Serum levels of proinflammatory cytokines: IL-6, TNF-α, and PGE_2 were analyzed. After the completion of tumor promotion for 24 weeks, the animals were anesthetized and blood withdrawn from retro-orbital sinus. Serum was separated from blood and the levels of above-mentioned cytokines were evaluated in it by ELISA following the instructions of the manufacturer.

Immunohistochemistry

The processed renal tissues were obtained and preserved in the 10% paraformaldehyde overnight followed by dehydration in 30, 20 and 10% sucrose solution successively uptil 3 days and was fixed after that in formaldehyde fixative until immunochemical staining. 5–15 lm thick sections of paraffin embedded tissues were cut using grading type lieca microtome and boiled in 0.1 M citrate buffer (pH 6.0) for 5 min for antigen retrieval process and then incubated in 0.3% H_2O_2 in methanol followed by incubation in blocking buffer containing 0.1 M PBS, 0.04% Triton X-100 and 10% NGS (normal goat serum). Sections were incubated in antibodies anti rat PCNA (1:150,Thermo Fisher Scientific, USA), for overnight 4°C. After rinsing in buffer, sections were processed using a three layer peroxidase staining kit from Thermo scientific system. The peroxides complex was visualized with 3,3-diaminobenzidine (DAB Plus substrate, Thermo Fisher Scientific, USA). Lastly the slides were counterstained with haematoxylin for 5s. Slides were then cleaned in BDH, gradually dehydrated with ethanol and cover slipped in mounting medium and photographed under Olympus microscope (BX51).

Statistical analysis

Differences between groups were analyzed using analysis of variance (ANOVA), followed by Turkey multiple comparisons test. All data points are presented as the treatment mean ± standard error of the mean (S.E.).

Results

Effect of QI on Fe-NTA induced oxidative stress and Hyperproliferation.

Effect of pretreatment of QI on Fe-NTA induced changes on glutathione content and glutathione-dependent enzymes in rat kidney is shown in table-1. Reduced glutathione (GSH) was depleted in group II due to Fe-NTA administration in comparison to controls, but was restored to normal at higher dose ($p < 0.01$) of QI. There was a concomitant and significant decrease in the activity of glutathione dependent antioxidant enzymes viz. GST ($p < 0.001$). This decrease in their activity was attenuated substantially by the prophylactic treatment of high dose of QI as evident from table 1. Elevated levels of MDA were also observed on treatment with Fe-NTA ($p<0.001$), QI substantially attenuated the elevated levels of MDA (Table 1).

Table 2 shows that there was a marked reduction ($p < 0.001$) noted in catalase, QR and SOD activity after Fe-NTA administration in group II. Their activity was maintained at normal level by the pretreatment of QI at higher dose catalase ($p<0.01$), QR ($p<0.01$) and SOD ($p < 0.001$). It also shows Fe-NTA induced Xanthine Oxidase (XO) and H_2O_2 substantially ($p < 0.001$) in group II when compared with group I, but both were modulated by the administration of QI at

both doses and higher dose being more effective than lower dose.

Serological chemistry revealed that Fe-NTA induced acute nephropathy as evident by the significant increase of kidney toxicity markers viz. BUN, LDH and creatinine into serum compared to control rats ($p < 0.001$) (Table 3). Prophylactic treatment of QI at a higher dose prevented the Fe-NTA induced elevation in the serum levels of BUN, LDH and creatinine ($p < 0.001$). The cytotoxicity marker LDH and GGT, showed elevation in their levels ($p < 0.001$) due to Fe-NTA induced cytotoxicity, but release in serum was suppressed by the treatment of QI at both doses significantly ($p <0.05$) and ($p < 0.01$), as evident from Table 3. However there was no significant difference between group I and group V in all the parameters studied in table 3.

Apart from inducing oxidative stress, Fe-NTA is also known to cause tumor promotion by inducing cellular proliferation. Fe-NTA exposure significantly ($P<0.001$) increased renal ODC which is a hallmark of tumor promotion and is greatly induced during tumorigenesis. Intraperitoneal application of Fe-NTA significantly elevated ODC activity However, QI pretreatment in both the doses (75 & 150 mg/kg BW) down regulated Fe-NTA-induced ODC activity (Figure 1).

Further, quantification of PCNA, well known cell proliferation markers by immunohistochemistry has been used to characterize the proliferation of cells in many fields, such as in tumor studies. The semi-quantitative expression of PCNA protein in all the groups of long term renal tumorigenesis study is given in Figure 2. According to Figure the number of PCNA positive cells increased substantially in group II indicating the proliferative potential of Fe-NTA. Higher dose of QI markedly suppressed the proliferation of tubular epithelium cells as revealed in Figure 3.

Effect of QI on Fe-NTA induced proinflammatory and inflammatory cytokines.

Proinflammatory cytokines like TNF-α, and PGE2 whose secretion is known to be enhanced by Fe-NTA, play an important role in tumorigenesis [38]. Significant levels of TNF-α ($p<0.001$) and PGE2 ($p<0.001$) could be detected in serum of rats exposed to tumor promotion with Fe-NTA (Figures 4 & 5). QI pretreatment at both the doses significantly restores the increased level of all the three proinflammatory cytokines studied to normal. Further, treatment with Fe-NTA resulted in the significant increase in the MPO ($p<0.001$) activity when compared with control. Both the doses of QI significantly brought the level MPO to normal low (Figure 6). QI alone did not show any significant difference as compared with control. Similar pattern as above was observed in NO estimation (Figure 7).

Histopathological observations and tumor incidence

The histopathological examination in the renal tissue of all the groups is shown in Figure 2. The tissue sections from kidney of rats treated with DEN and Fe-NTA, either with or without pre-treatment of QI, were examined for the degree of infiltration of leukocytes, tumor cells and hyperchromatism. Sections from control rat kidneys demonstrated normal tubular architecture with normal convoluted tubules and glomeruli within the cortex. Renal sections of rats in the Group II that were DEN initiated and chronically promoted with Fe-NTA for 16 weeks were abnormal and contained numerous tumor nodules. In this group there was an evidence of cell regeneration, and simple tubule hyperplasia, affecting proximal convoluted tubules in the deep cortex, extending into the outer region of medulla. There was also focal collection of leukocytic infiltratory cells and adenocarcinomas with hyperchromatism and enlargement of nuclei in the tubular epithelium. Scattered tubules within the

tumor nodules were congested. Most of the tumor nodules were in the cortical area of the kidney and, in some of the nodules, entrapped and atrophic glomeruli were seen. Sections from the kidney away from the tumor were also markedly abnormal and demonstrated large dilated congested convoluted tubules, which were lined by somewhat flattened, cuboidal epithelial cells. A significant increase in the number of congested convoluted tubules, congested glomeruli, atrophic glomeruli, and nodular proliferation was detected in the group II tumor bearing kidneys compared with untreated controls. QI treatment significantly ameliorated these histopathological changes in group III and IV. Renal sections pretreated with higher dose of QI revealed normal kidney architecture similar to control rats (Figure 2).

Discussion

Chemoprevention includes multiple intervention methods, either pharmacological or dietary agents to inhibit, arrest, or reverse carcinogenesis at various stages. Development of dietary agents as potent cancer chemopreventive agents is recommended due to their safety, low toxicity, and general acceptance as dietary supplements [39]. Fruits, vegetables, vitamins, common beverages and several herbs with diverse pharmacological properties have been shown to be rich sources of such chemopreventive agents [40]. Epidemiological studies have shown that consumption of fruit and vegetables have caused lower incidence of cancers [41]. Measuring the effects of these agents in cancer chemoprevention studies in human populations has now become a vital objective of experimental cancer research. Accordingly, many new classes of chemical compounds are being evaluated in clinical trials as cancer preventive agents for several malignancies. Further lots of scientific efforts are currently focused on exploration of safe and effective phytochemicals for the management of renal cancer. The central finding in the present study is that QI inhibit Fe-NTA dependent renal carcinogenesis, hyperproliferative response and diminish oxidative stress. The medicinal and pharmacological value of QI has been previously reported extensively. Previously published reports have also shown QI to possess antioxidant and anti-inflammatory responses in various previous studies [12, 42].

According to earlier findings, generation of ROS have been induced by administration of Fe-NTA and its role has been well documented in the initiation, promotion and progression phase of tumorigenesis [6,40,43]. ROS leads to oxidation of almost all biomolecules such as lipids, DNA, RNA and proteins, thus playing a key role in cancer. Several evidences demonstrate that the involvement of oxidative stress in Fe-NTA induced renal carcinogenesis [6, 43-45]. Treatment of Fe-NTA to animals lead to the induction of lipid peroxidation, reflecting the formation of activated species in rat kidney. On the other hand, renal GSH, activities of antioxidants and phase II metabolizing enzymes was found to be significantly depleted. Similar to the reported protective effects of QI in various experimental models [14,42,46], we also observed a dose-dependent decrease in Fe-NTA mediated oxidative stress in kidney of QI treated animals which is manifested by decrease in lipid peroxidation and reversal of enzymatic and non-enzymatic antioxidant molecules. Thus QI effectively inhibited Fe-NTA induced nephrotoxicity. In the present study, the modulation in the effect of Fe-NTA on renal lipid peroxide, enzymatic and non-enzymatic molecules by QI confirms its antioxidant properties. However, the exact mechanism of QI as an antioxidant is still not completely understood, it might be due to presence of several polyphenolic compounds like ellagic acid, gallic acid etc. Moreover, the serum toxicity markers like LDH, BUN and creatinine showed marked elevation in Fe-NTA treated rats in accordance with previous published reports [47]. However,

prophylactic treatment of rats with QI appreciably restored the serum toxicity marker level to normal. Rats treated with QI had LDH, BUN and creatinine significantly lower than those receiving only Fe-NTA. These results suggested that QI may protect against Fe-NTA induced renal toxicity.

Histopathological inspection of the kidneys of animals treated for 16 weeks with Fe-NTA revealed more varying tubular necrosis, massive inflammatory response and more numerous renal cell tumors [45]. All these pathological alterations were corrected by QI treatment.

Unsynchronized cell proliferation is a crucial feature of carcinogenesis. We have studied the expression of Proliferating cell nuclear antigen protein (PCNA) and level of ornithine decarboxylase enzyme (ODC) both studied as markers of hyperproliferation. Howard et al., 2001 [48] reported PCNA detection by immunohistochemistry can be used to characterize the proliferation of cells in various aspects viz tumor studies. PCNA expression reaches its maximum during the synthesis phase (S phase) [49]. ODC is a rate limiting enzyme in the biosynthesis of polyamines, spermidine, spermine, and putrecines. Increased level of ODC activity has been consistently detected in transformed cell lines [50] and plays a significant role in tumor promotion [36]. In the present study, we have observed the steep increase in the level of ODC and also increased expression of PCNA protein after 16 weeks of Fe-NTA treatment but both were inhibited dose dependently by QI, suggesting its anti-tumor and antiproliferative activity potential. Inhibition of ODC activity and PCNA reveals that QI may intercept tumor promoting and harmful functions of polyamine biosynthesis and arachidonic acid metabolism.

Fe-NTA induced oxidative stress and tumor promotion are intricately associated with inflammation as well [40, 6]. Distorted iron stores causes inflammatory conditions characterized by augmented macrophage activity and increased neutrophil levels [51]. Fe-NTA-induced free radicals lead to buildup of leukocytes in the tissue involved, and thus causes tissue injury also indirectly through activated neutrophils. Since the neutrophil infiltration is an important episode for inflammation, increase in MPO activity due to Fe-NTA may cause inflammation and damage in the organ. Federico et al. [52] reported that in most of the pathological conditions characterized by oxidative insult, there is an increase of nitrite level. NO increases renal injury [53] probably by means of its reaction with the superoxide radical ($O_2^{\bullet-}$) generating the very cytotoxic peroxynitrite [54]. In the present study, we show that Fe-NTA-treated rats exhibit evidence of inflammation with elevated renal MPO and NO levels and that QI partially prevented Fe-NTA-induced renal inflammation. Thus, QI attenuates these deleterious effects of Fe-NTA on the renal system. Fe-NTA was also found to induce the production of TNF-α and PGE-2[38]. Both of these are important proinflammatory cytokines and their expression is under the transcriptional regulation NFκB. A suppressive effect of QI on cytokine production may be implicated in its antitumor and anti-inflammatory activities.

Conclusion

From the results of the present study, it could be inferred that galls of QI possess potent antioxidant, nephroprotective and antitumor promoting activity. Protective efficacy of the extract against Fe-NTA induced toxicity and carcinogenesis probably implicates its antioxidant and free radical scavenging activity. By quenching the oxidants and thwarting ODC induction, PCNA expression, and attenuating the level of inflammatory and proinflammatory cytokines, QI extract may intercept the growth promoting and mutagenic functions of polyamines and arachidonic acid metabolism. The extract is also capable of increasing/maintaining the levels of antioxidant molecules and enzymes. The preliminary

chemical examination of alcoholic extract of QI has shown the presence of a number of polyphenols, which may be responsible for the antioxidant nephroprotective and anticancer activities.

Conflict of Interest

Authors declare that there is no conflict of interest.

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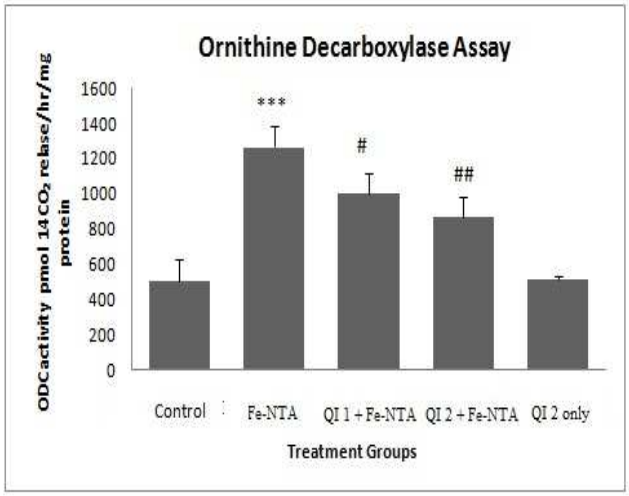
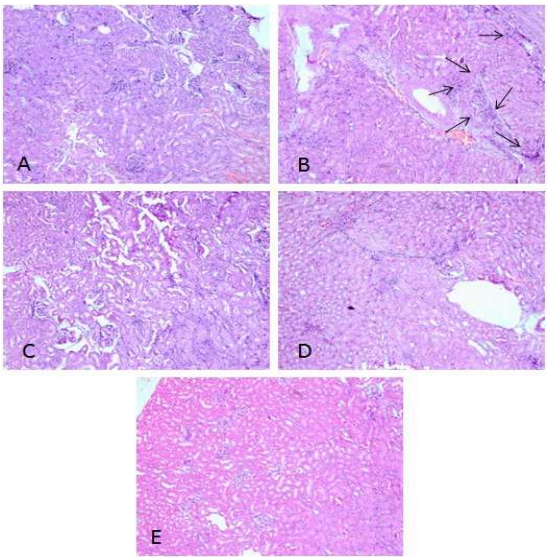
Figure 1: Effect of *Q .infectoria* pre-treatment on kidney ODC in Fe-NTA induced ODC levels:



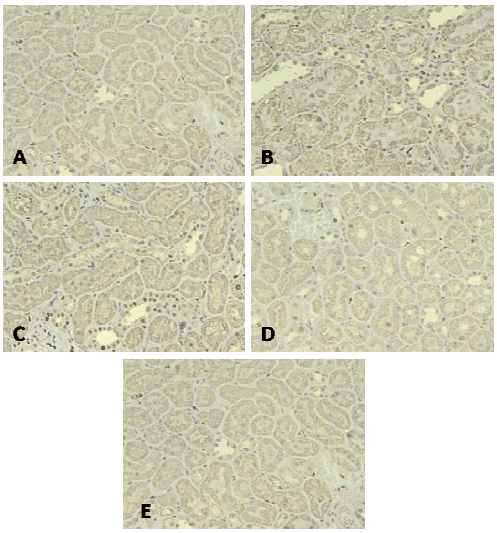
ODC level was significantly (***p< 0.001) elevated in Fe-NTA treated group II as compared to group I control. Pretreatment with *Q. infectoria* attenuates the ODC level significantly in the Fe-NTA + D1 & Fe-NTA + D2 groups in comparison with Fe-NTA (alone) treated group II (##p < 0.01, ###p < 0.001).

Figure 2: Effect of *Q. infectoria* treatment on renal histological alterations caused by DEN and Fe-NTA application



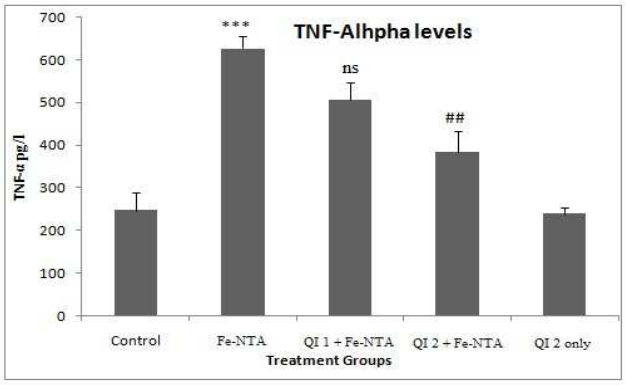
(A) Kidneys showed normal architecture with no signs of infiltration and tubular or glomerular damage. (B) DEN + Fe-NTA treated kidney showing areas of massive inflammatory cell invasion, hyperchromatism, glomerular and tubular congestion. (C) DEN + Fe-NTA + *Q. infectoria* (75 mg/kg BW) treated rats showed mild inflammation and cell invasion as compared to group B (D) DEN + Fe-NTA + *Q. infectoria* (150 mg/kg BW) treated animals showed almost normal renal histology (E) Shows the kidneys of the animals treated with the higher dose of *Q. infectoria* only with no significant change as compared with control. (10X magnification)

Figure 3: Representative photomicrographs of PCNA determined by immunohistochemistry



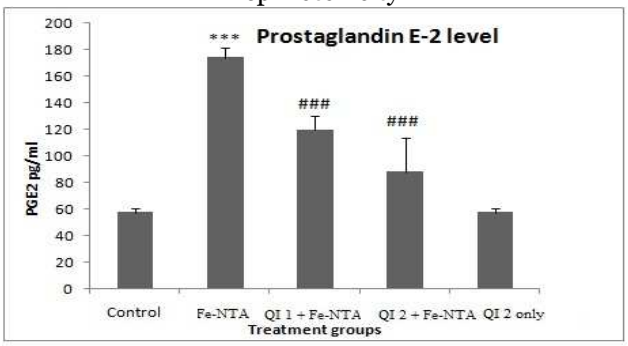
(A) No expression of PCNA was observed in case of control rats (B) DEN + Fe-NTA administration increased the number of PCNA positive cells in cortical and tubular region of renal sections of animals (C) DEN + Fe-NTA + *Q. infectoria* (75 mg/kg BW) treated animals showed slightly lesser number of PCNA positive cells as compared to group B. (D) DEN + Fe-NTA + *Q. infectoria* (150 mg/kg BW) treated animals showed significantly lesser number of PCNA positive cells (E) Only *Q. infectoria* treatment did not show any change in PCNA reactivity as compared to control.(40X magnification)

Figure 4: Effect of *Q. infectoria* pretreatment on kidney TNF-α in Fe-NTA induced nephrotoxicity:



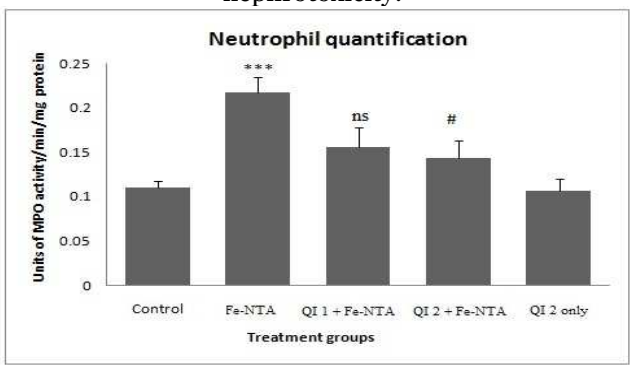
Values are expressed as mean ± SEM of six rats per group. TNF- α level was significantly (***p < 0.001) elevated in Fe-NTA treated group II as compared to group I control. Pretreatment with *Q.infectoria* attenuates the TNF- α level significantly in the IV (Fe-NTA + *Q.infectoria*) group only in comparison with Fe-NTA treated group II (##p < 0.01).

Figure 5: Effect of *Q.infectoria* pretreatment on kidney PGE-2 levels in Fe-NTA induced nephrotoxicity



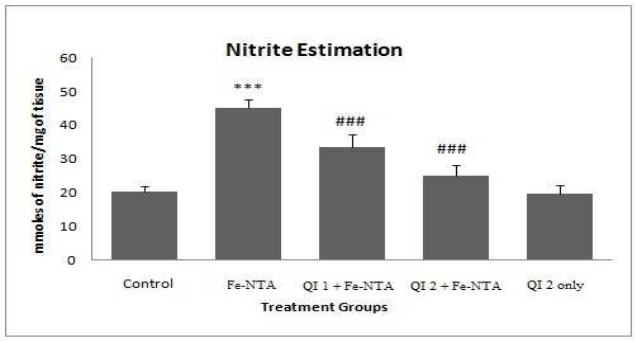
Values are expressed as mean ± SEM of six rats per group. PGE-2 level was significantly (***p < 0.001) elevated in Fe-NTA treated group II as compared to group I control. Pretreatment with *Q.infectoria* attenuates the PGE-2 level significantly in the III & IV (Fe-NTA + *Q.infectoria*) groups in comparison with Fe-NTA treated group II (###p < 0.001)

Figure 6: Effect of *Q.infectoria* pretreatment on kidney MPO levels in Fe-NTA induced nephrotoxicity:



Values are expressed as mean ± SEM of six rats per group. MPO level was significantly (***p < 0.001) elevated in Fe-NTA treated group II as compared to group I control. Pretreatment with *Q.infectoria* attenuates the MPO level significantly in the IV (Fe-NTA + *Q.infectoria*) group only in comparison with Fe-NTA treated group II (#p < 0.05).

Figure 7: Effect of *Q.infectoria* pretreatment on kidney Nitrite levels in Fe-NTA induced nephrotoxicity



Values are expressed as mean ± SEM of six rats per group. NO level was significantly (***p < 0.001) elevated in Fe-NTA treated group II as compared to group I control. Pretreatment with *Q.infectoria* restored the NO level significantly in the III & IV (Fe-NTA + *Q.infectoria*) groups in comparison with Fe-NTA treated group II (###p < 0.001, ###p<0.001)

Table 1: Results of pretreatment of *Q.infectoria* on glutathione and dependent parameters like GSH, GST, GR, GPX and LPO on TCE induced renal redox imbalance

Treatment Regimen groups	GSH (n mol GSH /g tissue)	GST (n mol CDNB conjugate formed/min/mg protein)	GR (n mol NADPH Oxidized/min/g protein)	GPX (n mol NADPH Oxidized/min/ mg protein)	MDA (nmol MDA formed /g tissue)
Group I (control)	0.48±0.01	327.5±11.58	346.4±6.89	133.6±9.64	2.21±0.09
Group II (Fe-NTA 9mg Fe/kg BW)	0.33±0.01***	163.3±4.55***	188.3±5.71***	250.0±15.29***	4.46±0.2***
Group III (Fe-NTA 9mg Fe/kg BW+Q.I D-1)	0.38±0.01^{ns}	214.0±4.97^{ns}	235.7±3.37$^{\#\#}$	180.1±8.61$^{\#\#}$	3.36±0.05$^{\#\#}$
Group IV (Fe-NTA 9mg Fe/kg BW+Q.I D-2 BW+Q.I D-2)	0.42±0.01$^{\#\#}$	263.5±8.42$^{\#\#}$	323.8±9.46$^{\#\#\#}$	136.9±12.11$^{\#\#\#}$	2.78±0.09$^{\#\#\#}$
Group V (Q.I D-2 only)	0.49±0.02	314.5±25.15	365.8±7.72	130.7±6.8	2.08±0.05

Results represent mean ± SE of five animals per group. Results obtained are significantly different from Control group ($^{***}P < 0.001$). Results obtained are significantly different from Fe-NTA treated group ($^{\#}P < 0.05$), ($^{\#\#}P < 0.01$), (^{ns} P= not significant) and ($^{\#\#\#}P<0001$). *Q.infectoria*; D1= 75mg/kg/b wt; D2 = 150mg/kg/b wt.

Table 2: Results of pretreatment of *Q.infectoria* on Fe-NTA induced changes in following parameters catalase, QR, SOD, and H_2O_2.

Treatment Regimen groups	Catalase (nmol H_2O_2 consumed /min/mg protein)	QR (nmol NADPH oxidized/min/mg protein)	SOD (Units/mg protein)	H_2O_2 (nmoles H2O2/g tissue)	XO (µg of uric acid formed/min mg protein)
Group I (control)	52.31±1.76	382.3±26.32	174.9±4.93	601.7±24.33	0.23±0.09
Group II (Fe-NTA 9mg Fe/kg BW)	31.09±1.76***	152.5±21.67***	116.6±6.64***	866.0±10.62***	0.45±0.004***
Group III (Fe-NTA 9mg Fe/kg BW+Q.I D-1)	40.08±2.60$^{\#}$	204.6±14.79^{ns}	134.4±2.94^{ns}	769.5±18.71$^{\#}$	0.36±0.02$^{\#\#}$
Group IV (Fe-NTA 9mg Fe/kg BW+Q.I D-2)	45.96±3.01$^{\#\#}$	264.3±11.55$^{\#\#}$	151.6±2.83$^{\#\#\#}$	713±14.40$^{\#\#}$	0.30±0.005$^{\#\#\#}$
Group V (Q.I D-2 only)	52.46±3.80	384.8±16.22	176.9±4.71	603±11.33	0.23±0.02

Results represent mean ± SE of five animals per group. Results obtained are significantly different from Control group ($^{***}P < 0.001$). Results obtained are significantly different from Fe-NTA treated group ($^{\#}P < 0.05$), ($^{\#\#}P < 0.01$), (^{ns} P= not significant) and ($^{\#\#\#}P<0001$). *Q. infectoria*; D1= 75mg/kg/b wt; D2 = 150mg/kg/b wt.

Table 3: Result of Fe-NTA induced increase in BUN, LDH, Creatinine and GGT and subsequent attenuation by *Quercus infectoria*.

Treatment Regimen groups	BUN (mg / 100 ml) IU/L	Creatinine (mg / 100 ml) IU/L	LDH (n mol NADH oxidized / min/ mg protein)	γ-GGT (nmoles *p* nitroaniline formed/min/mg protein)
Group I (control)	33.90±0.16	1.80±0.10	200.0±14.72	395.2±3.34
Group II (Fe-NTA 9mg Fe/kg BW)	48.99±1.35***	3.97±0.18***	368.2±33.81***	591.3±8.74***
Group III (Fe-NTA 9mg Fe/kg BW+Q.I D-1)	40.23±2.05$^{\#\#}$	2.67±0.11$^{\#\#}$	285.5±15.39$^{\#}$	552.0±11.9$^{\#}$
Group IV (Fe-NTA 9mg Fe/kg BW+Q.I D-2)	39.30±0.48$^{\#\#\#}$	2.51±0.02$^{\#\#\#}$	241.2±18.37$^{\#\#}$	468.6±9.54$^{\#\#}$
Group V (Q.I D-2 only)	31.99±1.21	1.63±0.09	203.1±9.25	395.3±2.35

Results represent mean ± SE of five animals per group. Results obtained are significantly different from Control group ($^{***}P < 0.001$). Results obtained are significantly different from Fe-NTA treated group ($^{\#}P < 0.05$), ($^{\#\#}P < 0.01$), (^{ns} P= not significant) and ($^{\#\#\#}P<0001$). *Q.infectoria*; D1= 75mg/kg/b wt; D2 = 150mg/kg/b wt.

Table 4: Summary of tumor data of the effects of Q. *infectoria* treatment on DEN initiated and Fe- NTA- induced renal cell tumors.

Treatment regimen per group	Number of animals treated	Number of animals studied Histopathologically	Number of animals with renal cell tumors	Incidence of renal cell tumors (%)
Group I	20	18	0	0
Group II	20	14	11	78.57
Group III	20	15	9	60.00
Group IV	20	15	5	33.33
Group V	20	20	0	0
Group VI	20	15	5	33.33

Group I (control): Normal Saline; Group II (toxicant): DEN + Fe-NTA; Group III: DEN + Fe-NTA + *Quercus infectoria* (75 mg/kg b wt); Group IV: DEN + Fe-NTA + *Quercus infectoria* (150 mg/kg b wt); Group V: DEN only and Group; VI:Fe-NTA only. QI – *Quercus infectoria*; Fe-NTA – Ferric-Nitrilotriacetate. Toxicant group showed highest percentage of tumor incidences which was abrogated by the administration of *Quercus infectoria* in groups III and IV. Whereas, group V (DEN only) and group VI (Fe-NTA only) did not develop significant no of tumors.

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