



The effect of *Ginkgo biloba* against ferric nitrilotriacetate and/or low doses of gamma irradiation induced renal injury in rats

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ABSTRACT

The present study was designed to investigate the efficacy of *Ginkgo biloba* (GB) and low doses of gamma irradiation against ferric nitrilotriacetate (Fe-NTA) promote renal carcinogenesis in Wister rats. Renal carcinogenesis was initiated by single intraperitoneal injection of N-nitrosodiethylamine (DEN 200 mg/kg body weight) and promoted by twice weekly administration of Fe-NTA 9 mg Fe/kg body weight for 16 weeks. Animals (whole body) were exposed to 50 cGy per week for 19 weeks at a total dose of 9.5 Gy. The efficacy of GB was studied in terms of lipid peroxidation (LPO), renal anti-oxidant armory such as catalase, superoxide dismutase, glutathione peroxidase and glutathione (GSH) and plasma toxicity markers. Also, caspase 3 and renal Histopathological changes were detected. Administration of Fe-NTA and exposure to low doses of γ - radiation enhance renal LPx, with concomitant reduction in reduced GSH content and antioxidant enzymes. It induces serum toxicity markers, viz., blood urea nitrogen and creatinine. Histopathological alterations and renal caspase 3 enhancement were detected in kidney tissue. Chemo preventive effects of GB were associated with upregulation of antioxidant armory and down regulation of plasma toxicity markers. GB was also able to down regulate of caspase 3. Histopathological changes further confirmed the biochemical results. In conclusion: These results provide a powerful evidence for the preventive efficacy of GB against renal carcinogenesis induced by either low doses of γ - irradiation or Fe-NTA.

Introduction

Epidemiological studies have shown that increased body iron storage is associated with an increased risk of cancer and early death. Suzy and Frank [1] reported that iron can contribute to tumour initiation, tumour growth, tumour microenvironment and in metastasis. 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 ($\cdot OH$) and metal-oxygen complexes in biological systems, resulting in oxidative DNA damage. 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 renal carcinogenesis 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 $C_6H_9NO_6$. 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

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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 wellbeing, and the bioprospecting of new plant derived drugs.

Ginkgo biloba, a member of the family Ginkgoaceae, was cultivated in China in the mid 1700s. It is used medically today as a standardized preparation which contains 240 mg/g flavonoids (ginkgoflavone glycosides) and 60 mg/g terpenoids (ginkgolides and bilobalides) which are the most important active ingredients in the extract. The flavonoids act as free radical scavengers, especially for oxygen-derived free radicals, and to neutralize ferryl ion-induced peroxidation^[10]. The terpenoids are known as antagonists of platelet-activating factor, which implicates in the processes of platelet aggregation and arterial thrombosis, acute inflammation, allergic reactions and cardiovascular insufficiency^[11,12]. *Ginkgo biloba* is used to improve cardiovascular circulation and to lessen cerebrovascular insufficiency in western countries clinically^[13].

Radiation hazards present an enormous challenge for the biological and medical safety. The deleterious effects of ionizing radiation in biological systems are mainly mediated through the generation of reactive oxygen species (ROS) in cells as a result of water radiolysis^[14]. ROS and oxidative stress may contribute to cytotoxicity and to metabolic and morphologic changes in animals and humans^[15]. Nevertheless, the possibility that low doses of radiation may have beneficial effects has been the subject of considerable debate^[16]. Evidences accumulated over the past decade have indicated that living organisms, including humans, respond differently to low dose/low dose-rate radiation than they do to high dose/high dose-rate radiation^[17]. The radioadaptive response, radiation-induced bystander effects, low-dose radio-hypersensitivity, and genomic instability are specifically observed in response to low dose/low dose-rate radiation, and the mechanisms underlying these responses often involve biochemical/molecular signals that respond to targeted and non-targeted events^[17].

The objective of this work is to evaluate the role of *Ginkgo biloba* against ferric nitrilotriacetate and/or low doses of gamma irradiation induced renal carcinogenesis.

Materials and Methods

Chemicals

All chemicals and reagents used were of the highest purity available and purchased from either Merck (Darmstadt, Germany), or sigma Aldrich Chemie (Deisenhofen, Germany). Kits for BUN and creatinine were supplied by Diamond Diagnostics, Egypt. Kit for Caspase 3 was supplied by Enzo life sciences, New York, USA.

Preparation of Fe-NTA solution

Fe-NTA is prepared fresh immediately before its use as

per Awai et al.^[4]. To prepare Fe-NTA, ferric nitrate solution is mixed with fourfold molar excess of disodium salt of NTA and the pH is adjusted to 7.4 with sodium bicarbonate solution. The dose of NTA is 9 mg Fe/kg body weight twice a week for 20 weeks.

Radiation process

Animals were exposed to whole body gamma radiation using a Canadian gamma cell-40, (¹³⁷Cs) at the National Center for Radiation Research and Technology (NCCRT), Cairo, Egypt. The irradiation dose rate was 0.61Gy/min. Animals (whole body) were exposed to 50 cGy per week for 19 weeks at a total dose of 9.5 Gy.

Ginkgo biloba treatment

Ginkgo biloba extract was provided by Changsha Sunfull Biotech Co., Ltd., China, was dissolved in saline then orally administered to rats with dose of 100 mg/kg/day^[18], 3 times per week for 21 weeks starting from one week before Fe-NTA administration.

Experimental animals

Eighty adult Male albino rats (120-150 g) obtained from Al-Neil Company for drugs; Cairo, Egypt. Animals were housed in cages under good ventilation condition. The Animals were kept in isolated cages, under standard laboratory conditions including all hygienic measures with constant illumination, temperature and humidity.

Experimental design:

The animals were allowed 7 days for adaptation. The experimental animals were divided into eight groups (n = 10), namely (1) **Control** healthy animals received only saline injection intraperitoneally (0.85% NaCl) at a dose of 10 ml/kg body weight; (2) **(GB)**: Rats received *Ginkgo biloba* extract; (3) **(IR)**: Animals were exposed to γ -radiation; (4) **(Fe-NTA)**: Animals 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 were promoted with intraperitoneal injection of Fe-NTA at a dose level of 9 mg Fe/kg body weight, twice a week for 20 weeks; (5) **(GB + IR)**: Animals received *Ginkgo biloba* extract and were exposed to γ irradiation; (6) **(GB +Fe-NTA)**: Animals received *Ginkgo biloba* extract and were injected Fe-NTA; (7) **(Fe-NTA +IR)**: Animals were injected Fe-NTA and exposed to γ -radiation and (8) **(GB +Fe-NTA +IR)**: Animals received *Ginkgo biloba* extract and were injected Fe-NTA and exposed to γ -radiation.

Samples:

At the end of the experimental period (21 weeks) blood samples were taken from rats under diethyl ether anesthesia by heart puncture. Blood samples were collected in heparinized tubes, centrifuged at 3000 r.p.m. for 10 minutes. The whole blood was used for determination of antioxidant markers. Whereas, plasma was separated in vials for determination of other parameters. The kidneys of experimental animals were dissected out and divided into two parts, one part was kept in 10% formalin for histopathological studies and the other part was homogenized in (10% w/v) phosphate-buffered-saline (0.02 M sodium phosphate buffer with 0.15 M sodium chloride, pH 7.4).

Biochemical determinations

For antioxidant markers, glutathione (GSH) content was determined according the method of Beutler et al. [19], glutathione peroxidase (GPX) activity level was evaluated according method mentioned by Gross et al. [20], superoxide dismutase (SOD) and Catalase (CAT) activities were evaluated according to the method of Minami andYoshikawa [21] and Sinha [22], respectively. The estimation of lipid peroxidation (LPx) was done following the method described by Yoshioka et al. [23] and nitric oxide (NO) was determined according to Miranda et al [24]. Kidney tissue homogenates of each group was assayed for caspase-3 activity using rat caspase-3 ELISA kit.

Statistical analysis:

The SPSS (version 10) was used in data analysis. Data were analyzed with one-way analysis of variance (ANOVA) followed by a *post hoc* test (LSD alpha) for multiple comparisons. The data were expressed as mean \pm standard error (SE). P values < 0.05 were considered to be statistically significant.

Results

Effect of *Ginkgo biloba* on kidney caspase-3 activity

A significant decrease in activities of kidney caspase 3 observed in group subjected to Fe-NTA compared with the negative control group. Prophylactic and therapeutic treatment with GB significantly increases caspase3 activity (Fig. 1).

The levels of plasma creatinine and BUN were significantly increased after exposure to low doses of γ -radiation and Fe-NTA treatment either alone or combined compared with control group level (Table 1). Prophylactic treatment with GB significantly reduced the elevation in the plasma creatinine level compared with the previous treatments. In kidney tissue, a significant increase in MDA level was observed in γ -irradiated rats compared to control rats (Table 1). Also, the same observation was recorded after Fe-NTA treatment either alone or combined with γ -irradiated compared to control level. Prophylactic treatment with GB for 21 weeks showed a significant protection against Fe-NTA or γ -irradiation induced lipid peroxidation in kidney and offered a significant decrease in MDA level compared to γ -irradiated rats. Reverse directed observations were detected in NO levels when Fe-NTA or γ -irradiated rats treated with GB for 21weeks either alone or in combined.

Treatment of the experimental animal with *G. biloba* represents a non significant change in Kidney antioxidants markers (GSH, CAT, GSH-px and SOD) in Table 2. Whereas either γ - irradiation or Fe-NTA treatments led to a significant depletion in kidney antioxidants markers (GSH, CAT, GSH-px and SOD) when compared with their equivalent values in control rats. Pre-treatment with GB improved significantly kidney antioxidants markers compared to the Fe-NTA group (Table 2).

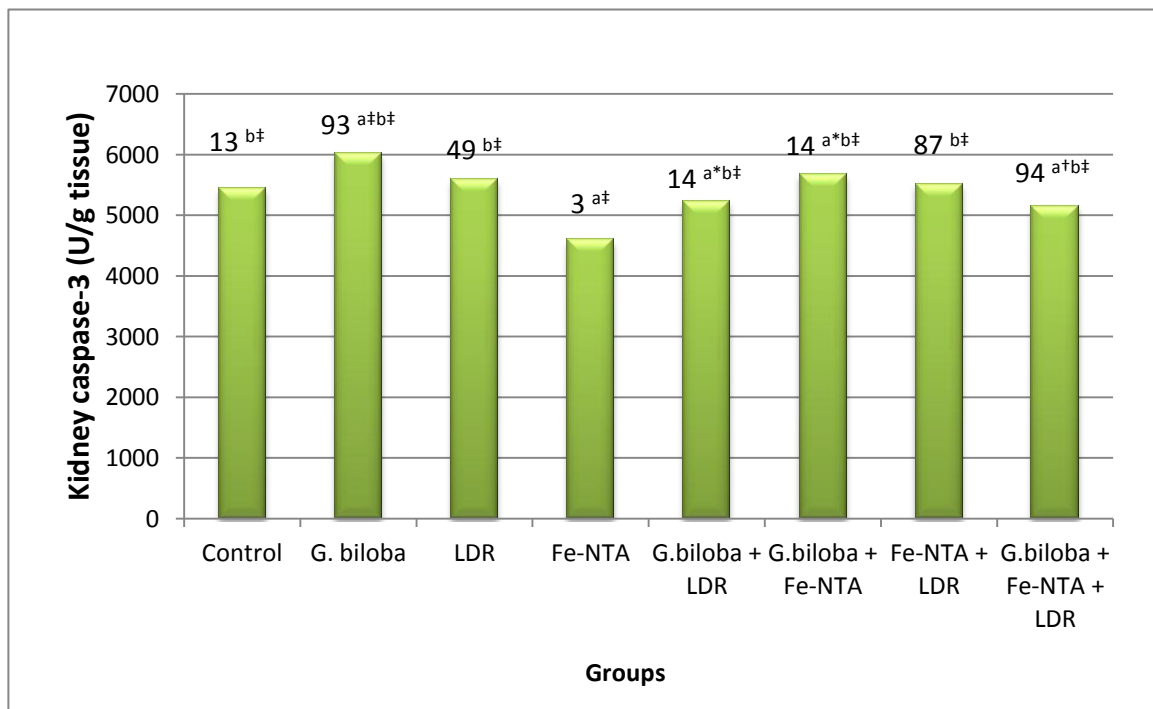


Fig. (1): Kidney caspase-3 activity. **a:** significantly different from control group. **b:** significantly different from Fe-NTA treated group. * Means are significantly different at the 0.05 level. †: Means are significantly different at the 0.01 level. ‡: Means are significantly different at the 0.001 level.

Table (1): Effect of GB and low doses of γ -irradiation on Kidney function parameters, nitric oxide and lipid peroxide levels in kidneys of Fe-NTA treated groups.

Group	Parameters	Creatinine (mg/dl)	BUN (mg/dl)	NO (nM/g wet tissue)	LPx (μ M/g wet tissue)
Control		0.36 \pm 0.024 ^{b‡}	29.48 \pm 1.12 ^{b‡}	26.3 \pm 0.57 ^{b‡}	181.99 \pm 9.2 ^{b‡}
GB		0.33 \pm 0.038 ^{b‡}	23.94 \pm 1.30 ^{b‡}	28.77 \pm 0.37 ^{b‡}	173.98 \pm 13.19 ^{b‡}
IR		0.54 \pm 0.019 ^{a‡}	47.22 \pm 1.80 ^{a‡b†}	14.77 \pm 2.34 ^{a‡b‡}	265.97 \pm 19.06 ^{a‡}
GB + IR		0.38 \pm 0.039 ^{b‡}	36.65 \pm 1.75 ^{b‡}	26.58 \pm 0.13 ^{b‡}	181.82 \pm 5.96 ^{b‡}
Fe-NTA		0.56 \pm 0.026 ^{a‡}	60.79 \pm 2.11 ^{a‡}	16.38 \pm 2.50 ^{a‡}	275.31 \pm 26.61 ^{a‡}
GB + Fe-NTA		0.48 \pm 0.011 ^{a†}	41.48 \pm 0.78 ^{a†b‡}	27.19 \pm 0.07 ^{b‡}	192.32 \pm 11.35 ^{b‡}
Fe-NTA + IR		0.6 \pm 0.034 ^{a‡}	63.76 \pm 7.35 ^{a‡}	15.79 \pm 0.32 ^{a‡}	275.81 \pm 13.75 ^{a‡}
GB + Fe-NTA + IR		0.5 \pm 0.038 ^{a†}	47.12 \pm 2.00 ^{a‡b†}	26.84 \pm 0.15 ^{b‡}	207.82 \pm 3.96 ^{b†}

a: significantly different from control group. b: significantly different from Fe-NTA treated group. * Means are significantly different at the 0.05 level. †: Means are significantly different at the 0.01 level. ‡: Means are significantly different at the 0.001 level.

Table (2): Effect of GB and low doses of γ -irradiation on Kidney antioxidant markers of Fe-NTA treated groups.

Groups	Parameters	GSH (mg/g wet tissue)	SOD (U/g wet tissue)	CAT (μ M/g wet tissue)	GSH-Px (Oxidized GSH/min/g wet tissue)
Control		111.74 \pm 0.49 ^{b‡}	7.92 \pm 0.92 ^{b‡}	547.22 \pm 25.42 ^{b‡}	0.51 \pm 0.032 ^{b*}
GB		115.31 \pm 3.28 ^{b‡}	7.25 \pm 0.67 ^{b‡}	587.78 \pm 34.55 ^{b‡}	0.52 \pm 0.041 ^{b*}
IR		98.98 \pm 1.16 ^{a‡b‡}	5.63 \pm 0.33 ^{a†}	466.67 \pm 14.02 ^{a*}	0.57 \pm 0.023
GB + IR		110.26 \pm 0.38 ^{b‡}	7.41 \pm 0.36 ^{b‡}	506.11 \pm 32.13 ^{b*}	0.54 \pm 0.012
Fe-NTA		69.29 \pm 0.38 ^{a‡}	4.13 \pm 0.37 ^{a‡}	421.67 \pm 23.94 ^{a‡}	0.59 \pm 0.012 ^{a*}
GB + Fe-NTA		110.81 \pm 0.21 ^{b‡}	7.42 \pm 0.45 ^{b‡}	498.33 \pm 4.00 ^{b*}	0.55 \pm 0.02
Fe-NTA + IR		98.98 \pm 1.16 ^{a‡b‡}	2.65 \pm 0.45 ^{a‡}	417.22 \pm 13.59 ^{a‡}	0.6 \pm 0.008 ^{a*}
GB + Fe-NTA + IR		110.19 \pm 0.28 ^{b‡}	5.43 \pm 0.39 ^{a†}	460 \pm 31.00 ^{a*}	0.57 \pm 0.013

a: significantly different from control group. b: significantly different from Fe-NTA treated group. * Means are significantly different at the 0.05 level. †: Means are significantly different at the 0.01 level. ‡: Means are significantly different at the 0.001 level.

Histopathological Studies

Histological examination of kidney sections derived from normal control rats is shown in **Fig. 2A**. The circular areas observed in this photograph are the renal Malpighian corpuscle. It is composed of glomerulus surrounded by Bowman's capsule with thin glomerular basement membranes and patent capsular space. Numerous tubules (proximal and distal) lie in the area adjacent to the glomerulus. Treatment of the experimental animals with *Ginkgo biloba* (GB) represents a no significant change in kidney tissue structure (**Fig. 2B**). However exposure of the experimental animals to 50 cGy per week for 19 weeks either alone or combined with GB recording some bleeding lesion in the kidney cortical structure (**Figs. 2 C, D**). Carcinogenesis of rats by Fe-NTA recording bleeding lesion in all kidney cortical structure, absence of renal Malpighian corpuscle and accumulation of inflammatory cells (**Figs. 2 E, Ea, Eb**). Exposure of Fe-NTA to cGy per week for 19 weeks represents some improvement in renal tissue structure (**Fig. 3G**). In the

meantime, normal renal tissue structure was detected when Fe-NTA (**Fig. 3F**) or Fe-NTA irradiated groups (**Fig. 3H**) treated with GB.

Discussion

Renal cell carcinoma (RCC) is widespread around the world. Phytochemicals have been found to be the most helpful alternative to delay or thwart carcinogenesis. Ginkgo has long been used in Oriental medicine. EGb761, a standard extract of the *Ginkgo biloba*, is widely used as a medicinal supplement^[25]. For example, it is used clinically in patients with cognitive function disorders, peripheral blood flow insufficiency, tinnitus, and vertigo^[25,26].

Context with the finding of Gupta et al. and Summya et al.^[27,28] the kidney of ferric nitrilotriacetate-treated rats showed characteristic morphological changes including bleeding lesion in all kidney cortical structure, absence of renal Malpighian corpuscle and accumulation of inflammatory cells.

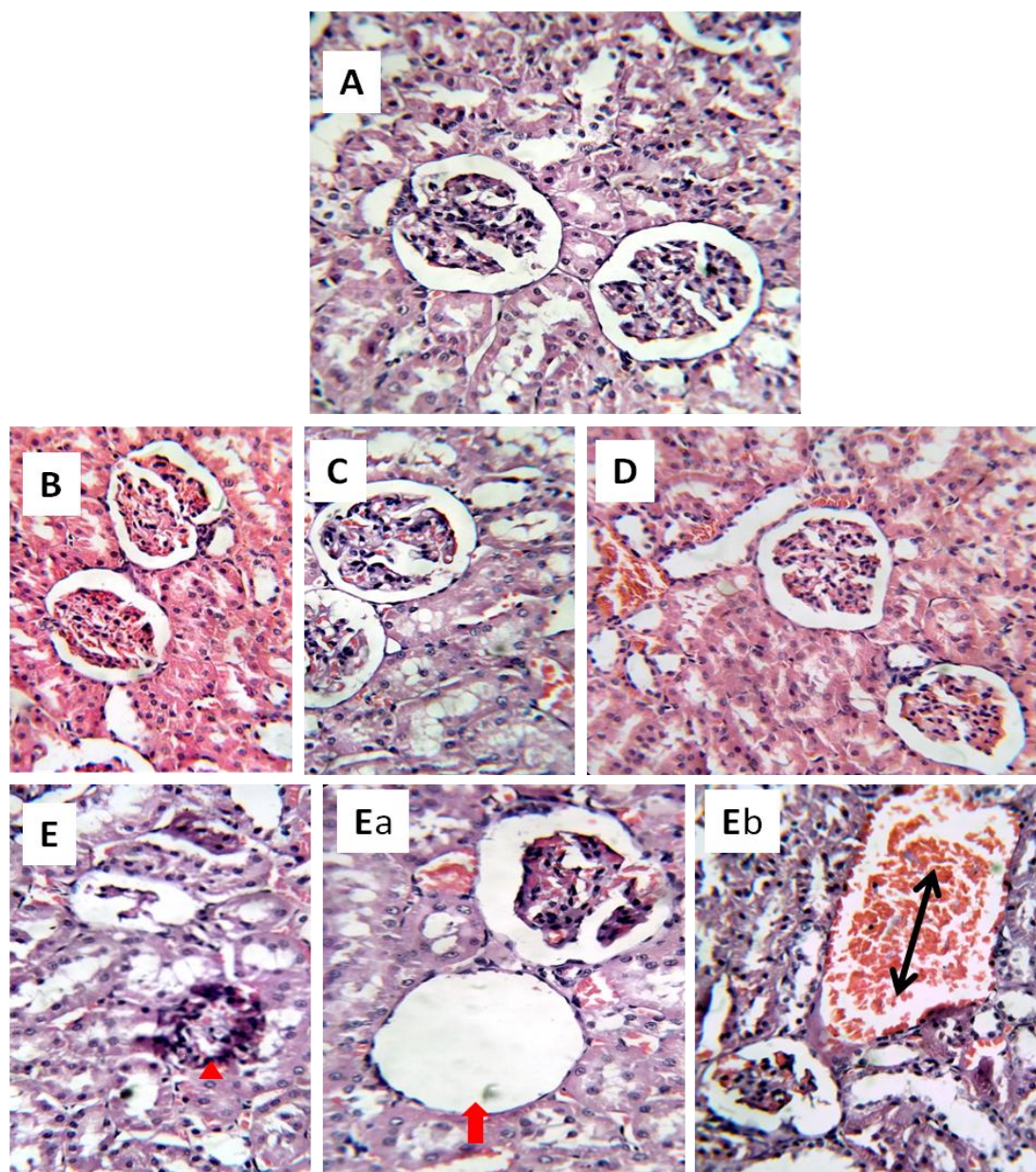


Fig. 2: Photomicrographs of sections in rat kidney stained with H&E. **A:** Rat kidney shows cortical part of a control rat. **B:** GB treated rat kidney show a no significant change. **C:** Rat kidney exposed to γ - radiation shows the appearance of some bleeding lesion. **D:** GB irradiated rat kidney shows the appearance of some bleeding lesion. **E, Ea and Eb:** Fe-NTA recording bleeding lesion in all kidney cortical structure (\ddagger), absence of renal Malpighian corpuscle (\uparrow) and accumulation of inflammatory cells (\blacktriangle). (All Photomicrographs are at X400).

In the other hand context with the finding of Samir et al ^[29] the histopathological examination of kidney, γ -irradiation induced congestion, interstitial haemorrhages and degenerative changes due to ROS production and pro-inflammatory cytokines expression as oxygen species.

Administration of Fe-NTA and iron deposition within kidney tissues which is found to be the main generator of ROS, leading to oxidative damage which further enhances renal lipid peroxidation with concomitant reduction in reduced GSH content and antioxidant enzymes. It induces serum toxicity markers, blood urea nitrogen, creatinine and lactate dehydrogenase ^[28].

Also, Radiation –induced increases in the levels of free iron in the cell (i.e., iron that is not bound to various proteins), which can promote ROS generation ^[30]. In our cumulative γ - irradiation treatments led to a significant depletion in kidney antioxidants markers (GSH, CAT, GSH-px and SOD) when compared with their equivalent values in control rats. Atef et al ^[31] proposed that GB confers a beneficial radio-preventive effect against irradiation damage induced in rat. In the other hand, we have demonstrated GB inhibits several aspects of Fe-NTA promoted tumor induction response in kidney ^[32]. All the pathological alterations were down regulated by GB pretreatment probable by its modulation of inflammatory mediators and antioxidation ^[33].

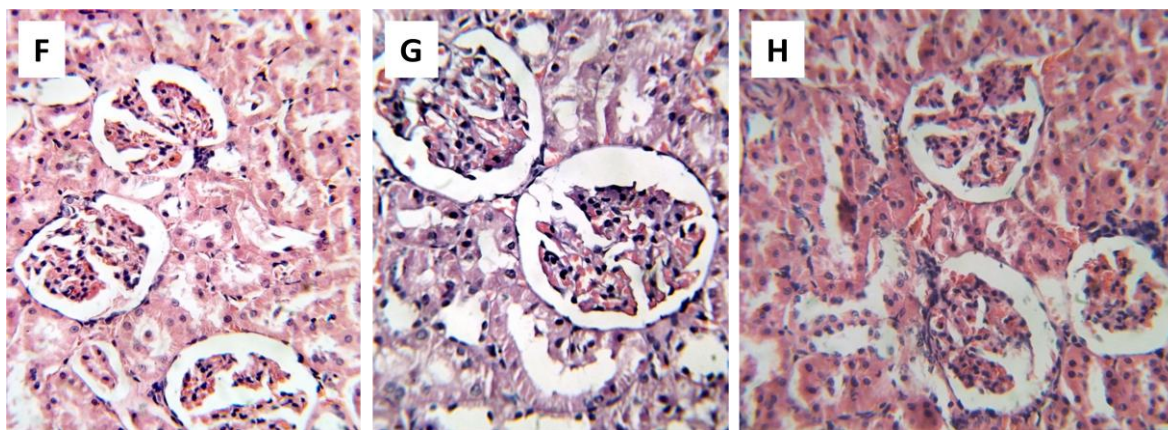


Fig. 3: Photomicrographs of sections in rat kidney stained with H&E. **F:** normal renal tissue structure was detected when Fe-NTA group treated with GB. **G:** Exposure of Fe-NTA to cGy per week for 19 weeks represents some improvement in renal tissue structure. **H:** Normal renal tissue structure was detected when Fe-NTA irradiated groups treated with GB. (All Photomicrographs are at X400)

In the same direction, it is evident from the present results that prophylactic treatment of GB abrogated GSH and antioxidant armory which was earlier exhausted in scavenging the ROS produced either by irradiation or Fe-NTA treatments. In our study, GB was able to restore SOD, CAT, and GPx levels in kidney tissues. Lin and Chang [34] reported similar elevation in SOD and CAT activities in the epidermis and in the liver of rats after GB treatment. In addition, the depletion of GSH redox cycle seems to be an essential factor that permits lipid peroxidation and ROS accumulation in the Cisplatin treated rats [35]. GPx metabolizes H_2O_2 to water by using GSH as a hydrogen donor, resulting in the generation of GSSG. GB was able to decrease GSSG/GSH ratio, probably by reversing the depletion of GSH concentration.

There was concomitant increase in plasma toxicity markers like BUN and creatinine in Fe-NTA treated group as compared to control. Our results are in good agreement with those previously reported [6]. On the contrary, lower BUN and creatinine levels were found in the rats administered with GB as compared to Fe-NTA treated group. Hence the decrease in the levels of these enzymes and attenuation of antioxidant armory may be possibly by nephroprotective efficacy of GB [36].

In our research, the NO levels was decreased following Fe-NTA administration context with the finding of Gupta et al. [37] home proposed Fe-NTA administration markedly increased the BUN and serum creatinine level which was coupled with a marked lipid peroxidation, reduced activity of glutathione and decreased total nitric oxide levels of rat kidneys coupled with significant morphological alterations. Many researches has shown that the catalytic activity of iNOS is enhanced by excess non-haem iron that causes the release of nitric oxide which combines with already present superoxide anion to form a toxic mediator 'peroxynitrite' and results in cellular membrane damage by lipid peroxidation and oxidation [38]. GB inhibited inflammatory iNOS expressions in LLC-PL1 cells, the classic target of anti-

inflammatory pathways. However, Gulec et al. [39] previously reported that GB protected against Fe-NTA induced nephrotoxicity by suppressing adenosine deaminase activities, and GB showed a tendency to inhibit NO levels but with no statistical significance. Although not all the results are uniform, an effect on iNOS by GB seems reasonable and accurate.

Context with finding of Mei et al. [40], a significant decrease in levels of kidney caspase 3 was observed in group subjected to Fe-NTA. Caspase 3 seems to play an important role in Fe-NTA-dependent loss of cell viability because a relatively specific peptide inhibitor of caspase 3. Fe-NTA induced notable ROS, which played a protective role in apoptosis by inhibiting Caspase-3 activation. Fe-NTA-induced ROS increased mRNA and protein level of anti-apoptosis Bcl-2 and decreased mRNA protein level of pro-apoptosis gene Bax, As a result, maintaining mitochondrial membrane potential [40]. The prophylactic and therapeutic treatment with GB significantly increases caspase 3 level due to its inhibition to ROS [41].

In this study, GB only caused a modest reduction in caspase-3 expression *in vivo*. This might reflect the incomplete suppression of caspase cascade by GB. Accordingly Luo et al. [42] revealed that GB protected against neuron cell death with only a minor effect on caspase-3 expression. ROS production can be boosted by inducing mitochondrial dysfunction and caspase cascade activation via the disrupted respiratory chain or by depleting and inactivating glutathione and thus shifting the cellular redox status. Our research suggested that caspase cascade was not a major pathway for GB's protection against Fe-NTA renal carcinogenesis.

From the previous discussed results it could be concluded that GB associated with upregulation of antioxidant armory, down regulate plasma toxicity markers, down regulate caspase 3 and normalize renal tissue structure in rats affected by Fe-NTA and / or gamma irradiation.

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