ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Chemopreventive effect of daidzein on renal toxicity by targeting oxidative stress and inflammation

Hiba Hazim Hamid Al-Yousuf*

Department of Biotechnology, Institute of Genetic Engineering and Biotechnology for post graduate studies, Bagdad University

Manuscript Info

Manuscript History:

Received: 11 December 2014
Final Accepted: 22 January 2015
Published Online: February 2015

Key words:

Daidzein, Inflammation, Oxidative Stress and renal toxicity.

Corresponding Author

Hiba Hazim Hamid Al-Yousuf

Abstract

Flavonoid family is a rich source of polyphenolic compounds and hence possess strong antioxidant and anti inflammatory properties. The aim of this study was to determine the efficacy of Daidzein; a bio-active flavonoid as an anti-nephrotoxic agent. Several published reports advocate that supplementation with antioxidant can influence Fe-NTA induced renal damage. In the present study the wistar rats were subjected to concurrent prophylactic oral treatment of daidzein (25 and 50 mg/kg b.wt.) against the nephro-toxicity induced by intraperitoneal administration of Fe-NTA (9 mg Fe /kg b.wt.). Efficacy of chrysin against the renal toxicity was evaluated in terms of biochemical estimation of antioxidant enzyme activities, histopathological changes and expression / levels of molecular markers of inflammation. Daidzein ameliorated Fe-NTA-induced lipid peroxidation, xanthine oxidase activity, glutathione depletion, decrease in antioxidant (catalase, glutathione reductase, superoxide dismutase and glutathione peroxidase). Daidzein also attenuated expression of COX-2, i-NOS and levels of NFκB TNF-α, IL-6 and IL-1β and renal tissue damage which were induced by Fe-NTA. Histological findings also supported the protective effects of daidzein against Fe-NTA-induced renal damage. The results of the present study demonstrate that oxidative stress and inflammation are closely associated with Fe-NTA induced toxicity and chrysin shows the protective efficacy against Fe-NTA-induced renal toxicity possibly via attenuating the oxidative stress and inflammatory response.

INTRODUCTION

NTA can complex with metal ions such as Fe^{3+}or Cu^{2+} (Irwing et al., 1996). Fe-NTA is a complex between Fe^{3+} and NTA, is a strong nephrotoxic agent and a renal carcinogen. It has been documented that an iron-chelate of nitrilotriacetate, Fe-NTA induces acute and sub-acute renal injury in animals (Awai et al., 1979; Rehman et al., 2012). Published reports have documented that oxygen free radical was formed from redox-active iron and was detected in the serum of Fe- NTA-treated rats (Jacob, 1980). Surplus free form of iron causes free radical-mediated peroxidation of lipids on membrane and cause the generation of malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) (Jacob, 1980; Toyokuni 2002). This iron mediated oxidative stress is reported to be the main offender involved in the pathogenesis of iron induced cancer (Gupta et al., 2009; Liochev and Fridovich 2002; Rehman et al., 2012). It is considered that reactive oxygen species (ROS) induced by univalent reduction of molecular oxygen in presence of iron and forms superoxide anion (O^{2-}) via Fenton/Haber–Weiss or auto-oxidation reaction (Iqbal et al., 2009; Rafter 2002).

It is widely accepted that oxidative stress due to excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS) is reason for pathogenesis of diseases (Bashan et al., 2009). Peroxynitrite (ONOO−), the reaction product of the interaction between superoxide (•O2), and nitric oxide (NO•), is a strong pro-inflammatory and

cytotoxic RNS involved in acute and chronic inflammatory state. RNS also has been known to activate nuclear factor-kappa B (NF-κB), redox sensitive and pro-inflammatory transcription factor (Mariotto et al., 2007).
Various studies have investigated the link between Fe-NTA and nuclear factor kB (NFκB). NFκB regulates genes that are involved in the control of the immune responses and inflammatory responses. NFκB is presumed to be an important redox-sensitive transcriptional factor that regulates transcription of genes encoding inflammatory cytokines, adhesion molecules, and chemokines (Collins et al., 1995). TNF-α is a key element in a network of proinflammatory chemokines and cytokines activated in the kidney by cisplatin. Blockade of TNF-α action prevents the activation of this cytokine network and provides protection against cisplatin nephrotoxicity (Ramesh and Reeves, 2002).
Chemoprevention emerged as a new strategy to fight human pathologies, seek to prevent and reduce disease risk by ingestion or administration of natural or synthetic chemicals. In the search for new chemopreventive agents, many plant constituents have been evaluated for their chemopreventive activities against different diseases over the past few years (Shi et al., 2004). Soy products containing phytoestrogens have received much attention as dietary components to promote better health. Daidzein (3,4,5,7-tetrahydroxyflavone) is a naturally derived flavonoid found widely in soya. Daidzein is known for its antioxidant, anti-inflammatory, and chemopreventive properties (Masilamani et al., 2012; Messina, 2009; Mishra et al., 2009; Wijeratne and Cuppett 2007). Moreover, it is used in hypertension and inflammatory diseases (Jackman et al., 2007). Dietary soy protein lowers blood lipid concentrations and reduces the incidence of cardiovascular diseases in animals and humans (Torres et al., 2006).
The purpose of this work was to investigate the potential of dietary daidzein for chemoprevention to Fe-NTA induced renal toxicity and to study its role in modulating inflammatory markers in wistar rats.

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), Daidzein and proteinase K 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 University. Experiments were conducted according to protocols approved by Institutional animal ethical committee. The well ventilated animal rooms (room temperature set at 25^0C) 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 and water ad libitum.

Preparation of Fe-NTA

Preparation of Fe-NTA solution was done by method given by (Awai et al., 1979) as modified by Athar and Iqbal (1998). 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

To study the effect of pretreatment with daidzein on Fe-NTA mediated renal oxidative stress and serological studies, thirty male Wistar rats were randomly allocated to five groups of six rats each. Group I served as control and received only saline. Group III received pretreatment with daidzein by gavage (orally) once daily for 20 days at a dose of 20 mg/kg body weight and groups IV and V received pretreatment with daidzein by gavage once daily for 20 days at a dose of 40 mg/kg body weight. Half-an-hour after the last treatment with daidzein, the animals of the groups III and IV received only a single intraperitoneal injection of Fe-NTA at a dose of 9 mg/kg body weight. The animals of group II received single i.p. dose of Fe-NTA (dose of 9 mg/kg body weight) only on the 20^{th} day. After 12 h of i.p. injection of Fe-NTA, the animals of all the groups were sacrificed by cervical dislocation. Just before sacrifice, blood for separation of serum was collected from these animals.

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.

Postmitochondrial supernatant (PMS) preparation

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

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 H2O2 consumed per min per mg of protein.

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 × 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.

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 H2O2 (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 × 103M−1 cm−1.

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% .

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}.

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}.

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. To 0.5 ml of the protein-free filtrate
was added 3.5 ml of distilled water, 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.

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.

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^{O}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.

Estimation of protein

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

Estimation of kidney injury molecule-1

Kidney injury molecule-1 (KIM-1) levels were measured by a commercially available ELISA-based kit (RAT KIM-1 ELISA Kit; Adipo Bioscience, Inc.) following the manufacturer's instructions.

Cytokine Analysis (NFκB, TNF-α, 1L-6 and 1L-1β)

Serum levels of NFκB, TNF-α and proinflammatory cytokines: IL-6 and IL-1β were analyzed. 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 COX-2 antibody raised in rabbit (1:200 diluted in tris buffered saline), anti-NFkB (1:200, Thermo Fisher Scientific, USA), anti-PCNA (1:150, Thermo Fisher Scientific, USA), anti-iNOS (1:100, Thermo Fisher Scientific, USA) for overnight 4^{0}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 Dunnet's multiple comparisons test. All data points are presented as the treatment mean ± standard error of the mean (S.E.).

Results

Effect of Daidzein on the renal GSH content

Pretreatment of daidzein before the Fe-NTA administration was found effective in restoring the endogenous anti-oxidant GSH. There was significant depletion in the level of GSH content in group II & when compared with group I ($p<0.001$). Pretreatment with Daidzein in group III group IV shows significant increase in the level of GSH content ($p<0.05$, $p<0.01$) as compared with group II. There was no significant difference in the GSH content between group I and V (Table 1).

Effect of Daidzein supplementation and Fe-NTA on the activities of glutathione dependent enzymes in renal tissue

Fe-NTA treatment caused a significant decrease in the activities of GPx ($p<0.001$), GST ($p<0.001$) and GR ($p<0.001$) in Group II as compared to Group I. The higher dose of Daidzein (50 mg.kg b. wt.) significantly attenuated the activities of GPx ($p<0.001$), GST ($p<0.01$) and GR ($p<0.001$) in Group IV as compared to Group II. However, the activities of these enzymes in Group V did not change significantly as compared to Group I (Table 1).

Effect of Daidzein pretreatment and Fe-NTA on the xanthine oxidase activity in kidney

The activity of XO was significantly increased ($P<0·001$) in Group II as compared to Group I. Daidzein pretreatment significantly decreased the activity of XO in Group IV ($P<0·001$) as compared to Group II. Group V exhibited no significant change in the activity of XO as compared to Group I (Table 2).

Effect of Daidzein on Fe-NTA induced lipid peroxidation in rat kidney

Daidzein inhibits lipid peroxidation caused by Fe-NTA application in terms of MDA formation, a well known biomarker of oxidative stress. Administration of Fe-NTA leads to significant elevation in the level of MDA in the group II to that of the control group I ($p<0.001$). Pre-treatment with Daidzein at both the doses was found significantly ($p<0.05$, $p<0.001$) effective in amelioration of MDA formation. There was no significant change observed in the level of MDA between control and only Daidzein treated animals (Table 2).

Effect of Daidzein on the antioxidant enzymes

The effect of Daidzein pre-treatment on Fe-NTA induced depletion in the activity of different antioxidant enzymes was examined and the results were shown in table 2. We have observed that there was a significant ($p<0.001$) difference in the activity of different antioxidant enzymes between Group I and Group II. However pretreatment with Daidzein in the Group III & IV significantly restored the activity of antioxidant enzymes when compared with the only Fe-NTA treated group. There was no significant difference observed between the group I and V.

Effect of Daidzein on TNF-α and NFκB

We have assessed the effect Daidzein on Fe-NTA induced renal NFκB levels and TNF-α production. We found that there was a significant difference in the level of proinflammatory cytokine in control group as compared to Fe-NTA treated group ($p<0.001$). Pre-treatment with Daidzein significantly inhibit their production in the group III & IV when compared with the Fe-NTA treated group II. There was no significant difference found between group I and V (Figure 1).

Effect of Daidzein on Fe-NTA induced changes in serum toxicity parameters

Fe-NTA-treated groups showed ($P<0.001$) significant increase in serum BUN, Creatinine and Kim-1 levels, when compared with the control group. Both the doses of Daidzein administered were found effective in restoring levels of these serum toxicity markers BUN ($p<0.01$,$p<0.001$), Creatinine ($p<0.01$,$p<0.001$) and Kim-1 ($p<0.01$,$p<0.001$) when compared with Fe-NTA-treated groups (Table 3).

Effect of Daidzein on Fe-NTA induced changes in IL-6 & IL-1β

Fe-NTA-treated groups showed ($P<0.001$) significant increase in interleukin (IL-6 & IL-1β), levels studied when compared with the control group. Both the doses of Daidzein administered were found effective in restoring levels of these interleukins Il-6 ($p<0.05$, $p<0.001$), IL-1β ($p<0.01$, $p<0.001$) when compared with Fe-NTA-treated groups.

Effect of Daidzein on the Fe-NTA-induced renal Immunohistochemical expression of COX-2 and i-NOS

Hepatic expression of the above mention proteins are shown in the figures respectively. Brown colour clearly indicates the more number of cells having, COX-2 and i-NOS expression in the group II as compare to that of group I. Pretreatment with Daidzein in the group III results in reducing the number of cells showing expression of COX-2

and i-NOS. However there was no significant difference observed in the expression of these proteins in group IV as compared to group I. For immunohistochemical analysis, brown colour indicates specific immunostaining of COX-2 and i-NOS and light blue colour indicates haematoxylin staining. Original magnification: 40X (Figure 3 & 4).

Effect of Daidzein on the hepatic histological alterations

Effect of orally administered Daidzein was seen on hepatic histological changes caused by Fe-NTA administration were characterized by severe activation of Kupffer cells, degenerated hepatocytes, neutrophil infiltration and moderate enlargement of sinusoids. Daidzein pre-treatment diminished hepatic alterations by partially preserving the kidney architecture.

Figure 1

Treatment protocol

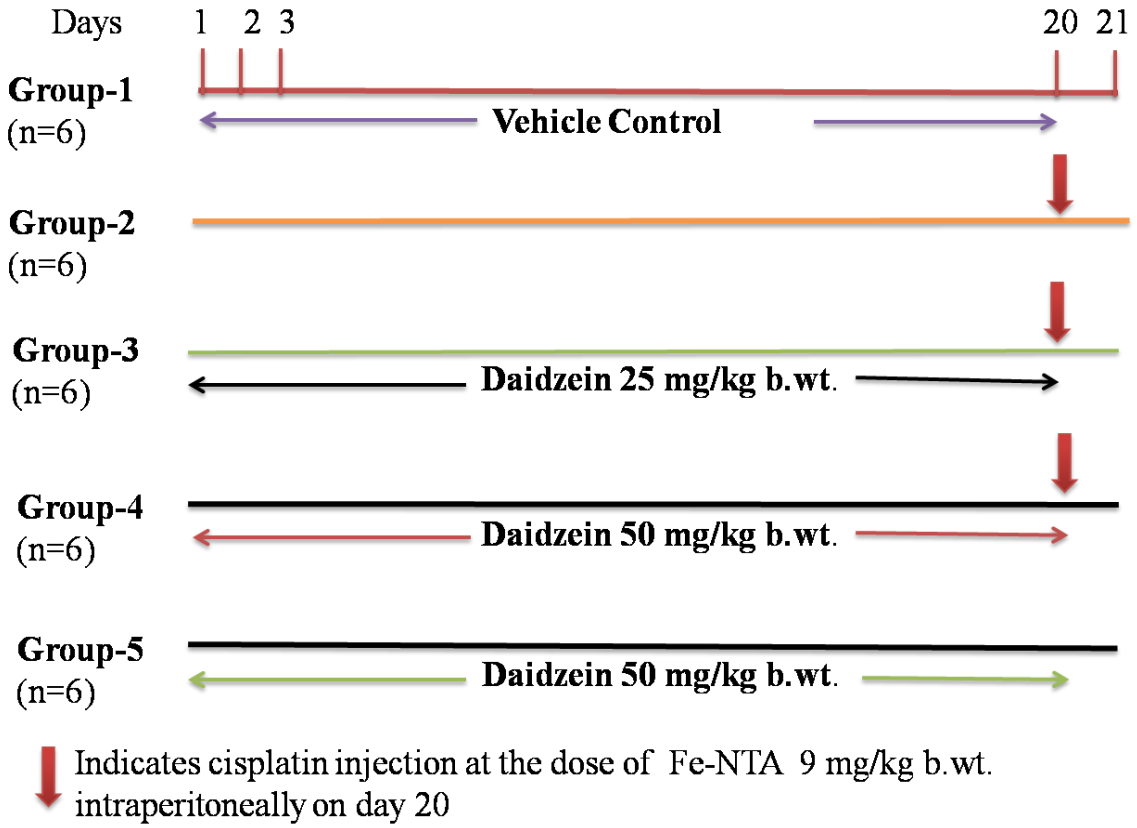

Figure 2

Effect of daidzein pre-treatment on Fe-NTA induced increase in NFκB. Values are expressed as mean ± SEM. (n = 6). $^{***}p<0.001$ shows significant difference in Group II (Cisplatin 7.5 mg/kg b.wt) when compared with Group I. $^{\#}p<0.05$ shows significant difference in the Group III (Cisplatin 7.5 mg/kg b.wt + chrysin 25mg/kg b.wt) when compared with Group II and $^{\#\#}p<0.01$ also shows significant difference in Group IV (Cisplatin 7.5 mg/kg b.wt + chrysin 50 mg/kg b.wt) as compared to Group II. There was no significant difference between Group I and Group V.

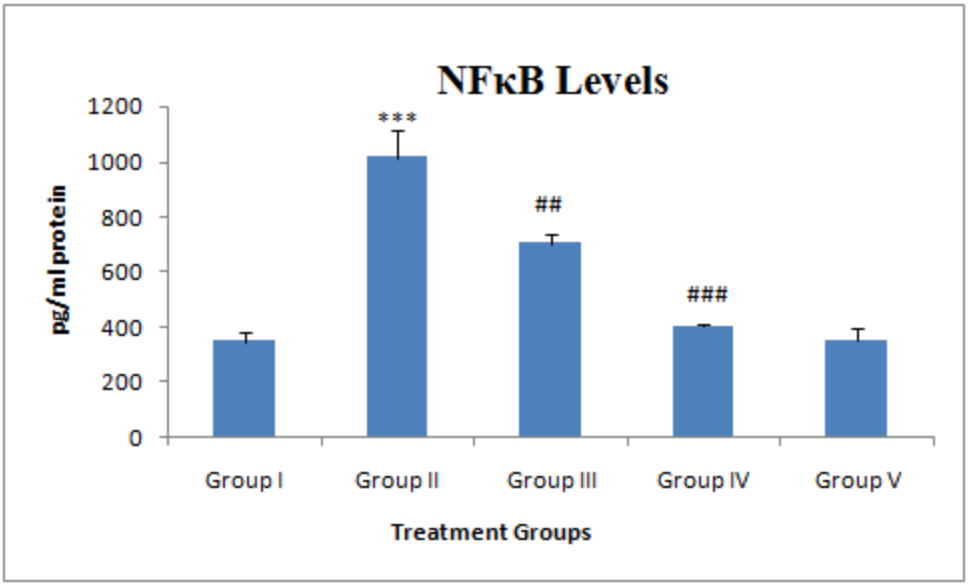

Figure 3
Effect of daidzein pretreatment on Fe-NTA-induced renal expression of COX-2. Representative photomicrographs (magnification ×40), (I) only vehicle (II) Fe-NTA 9 mg/kg b.wt (III) Daidzein 25mg/kg b.wt + Fe-NTA 9 mg/kg b.wt (IV) Daidzein 50 mg/kg b.wt + Fe-NTA 9 mg/kg b.wt (V) only Daidzein 50 mg/kg b.wt. Brown color indicates COX-2 specific staining and blue color indicates haematoxylin staining. Daidzein treated group (Group II) shows more COX-2 immunopositive staining as compared with vehicle treated group (Group I).Daidzein pretreatment (Group III & IV) reduces COX-2 expression as compared to Group II. However there was no significant difference in the COX-2 immunostaining in Group V as compared to Group I.

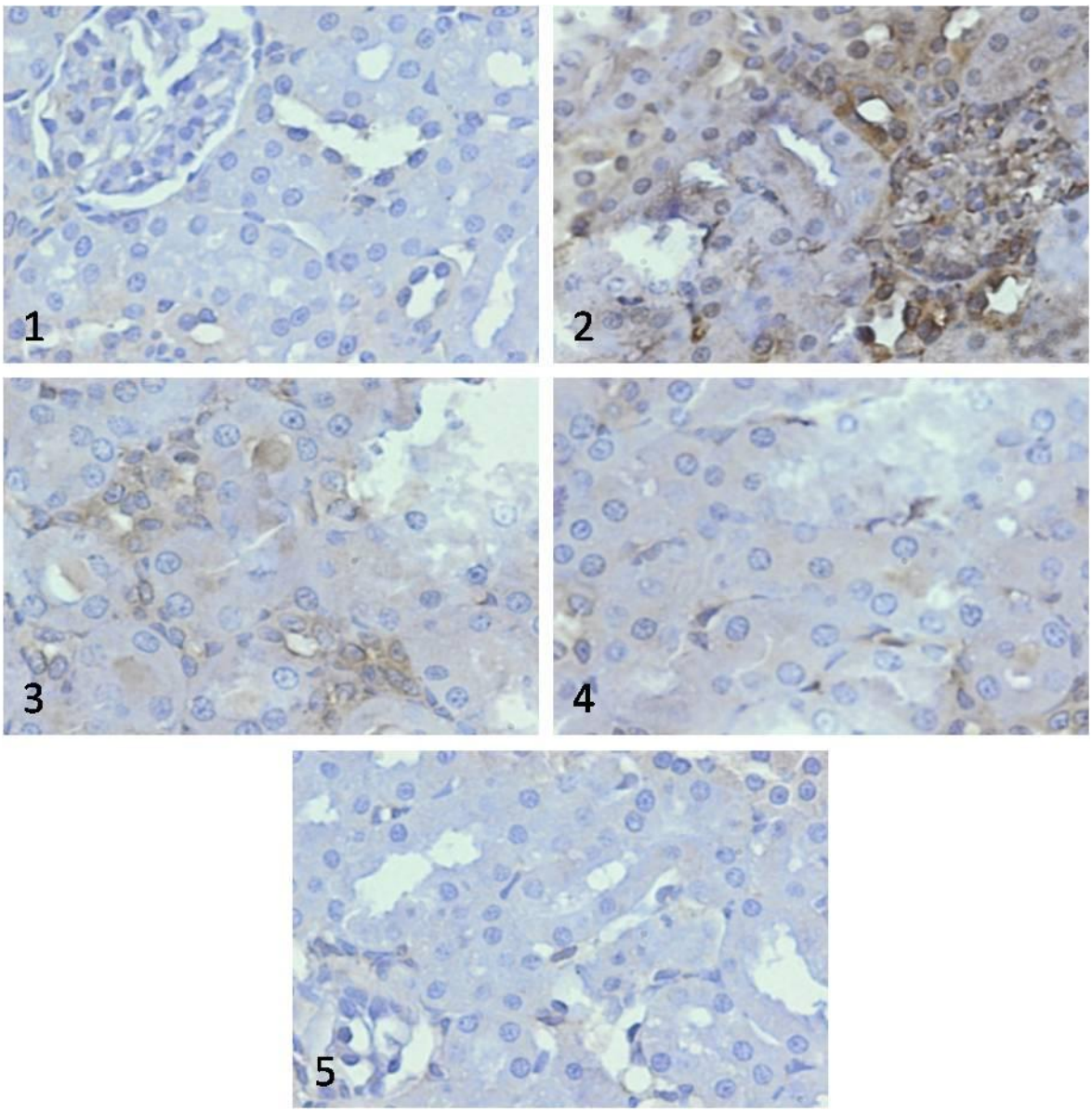

Figure 4
Effect of daidzein pretreatment on Fe-NTA-induced renal expression of iNOS. Representative photomicrographs (magnification ×40), (I) only vehicle (II) Fe-NTA 9 mg/kg b.wt (III) Daidzein 25mg/kg b.wt + Fe-NTA 9 mg/kg b.wt (IV) Daidzein 50 mg/kg b.wt + Fe-NTA 9 mg/kg b.wt (V) only Daidzein 50 mg/kg b.wt. Brown color indicates iNOS specific staining and blue color indicates haematoxylin staining. Daidzein treated group (Group II) shows more iNOS immunopositive staining as compared with vehicle treated group (Group I).Daidzein pretreatment (Group III & IV) reduces iNOS expression as compared to Group II. However there was no significant difference in the iNOS immunostaining in Group V as compared to Group I.

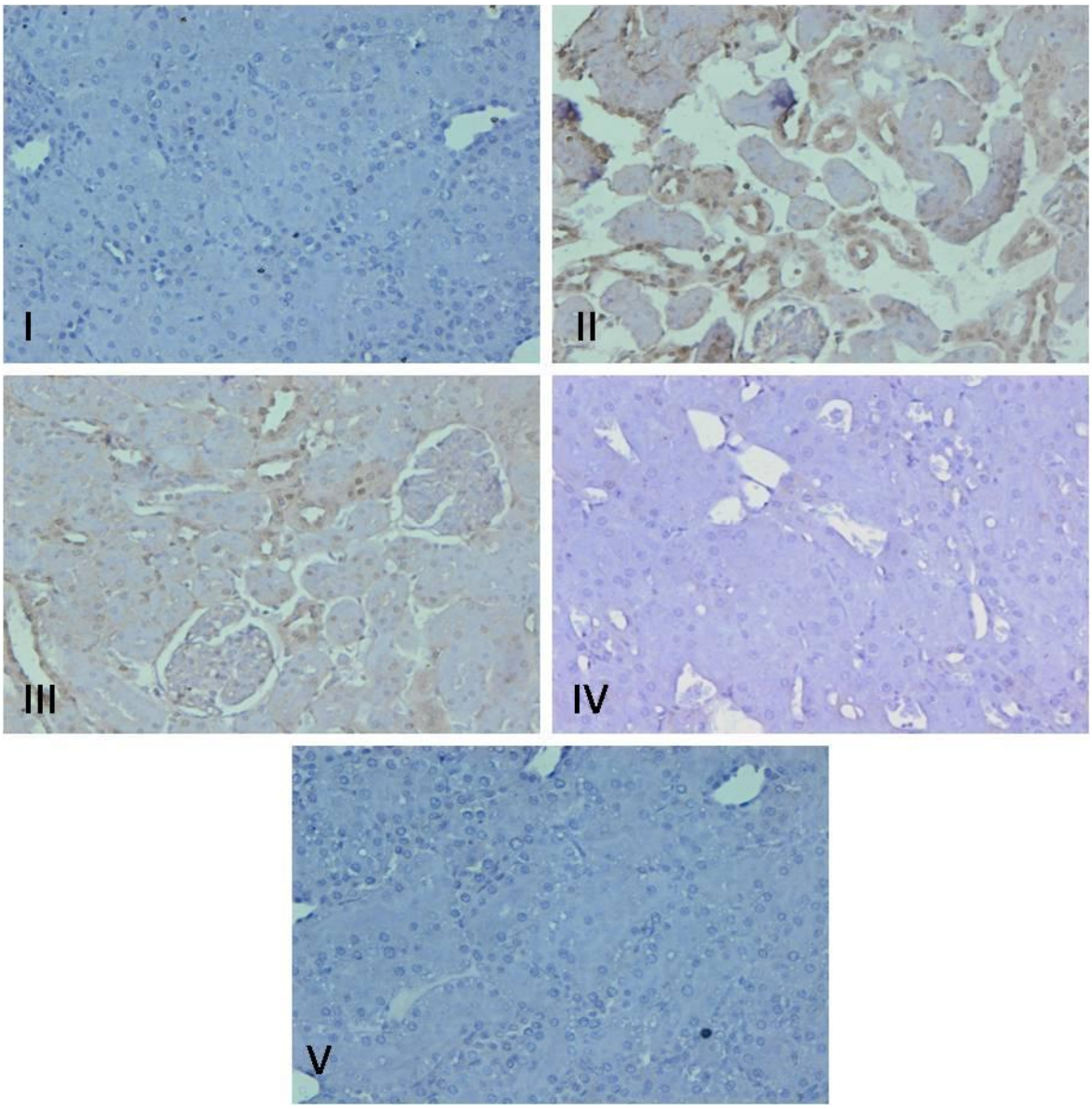

Figure 5
Effect of daidzein pre-treatment on Fe-NTA induced increase in TNF-α. Values are expressed as mean ± SEM. (n = 6). $^{***}p<0.001$ shows significant difference in Group II (Fe-NTA 9 mg/kg b.wt) when compared with Group I. $^{\#}p<0.05$ shows significant difference in the Group III (Fe-NTA 9 mg/kg b.wt + Daidzein 25mg/kg b.wt) when compared with Group II and $^{\#\#}p<0.01$ also shows significant difference in Group IV (Fe-NTA 9 mg/kg b.wt + Daidzein 50mg/kg b.wt) as compared to Group II. There was no significant difference between Group I and Group V.

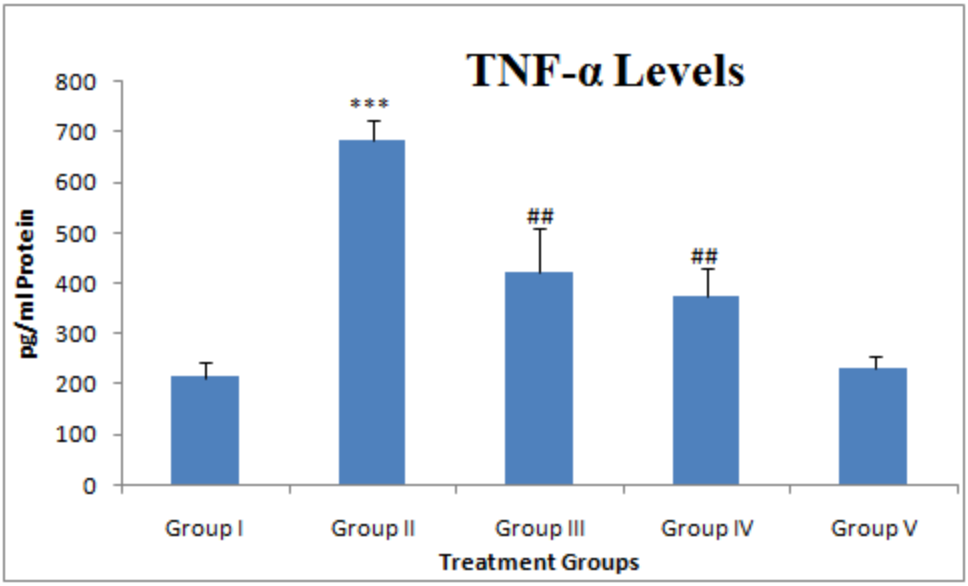

Figure 6
Effect of daidzein pre-treatment on Fe-NTA induced increase in IL-6. Values are expressed as mean ± SEM. (n = 6). $^{***}p<0.001$ shows significant difference in Group II (Fe-NTA 9 mg/kg b.wt) when compared with Group I. $^{\#}p<0.05$ shows significant difference in the Group III (Fe-NTA 9 mg/kg b.wt + Daidzein 25mg/kg b.wt) when compared with Group II and $^{\#\#}p<0.01$ also shows significant difference in Group IV (Fe-NTA 9 mg/kg b.wt + Daidzein 50mg/kg b.wt) as compared to Group II. There was no significant difference between Group I and Group V.

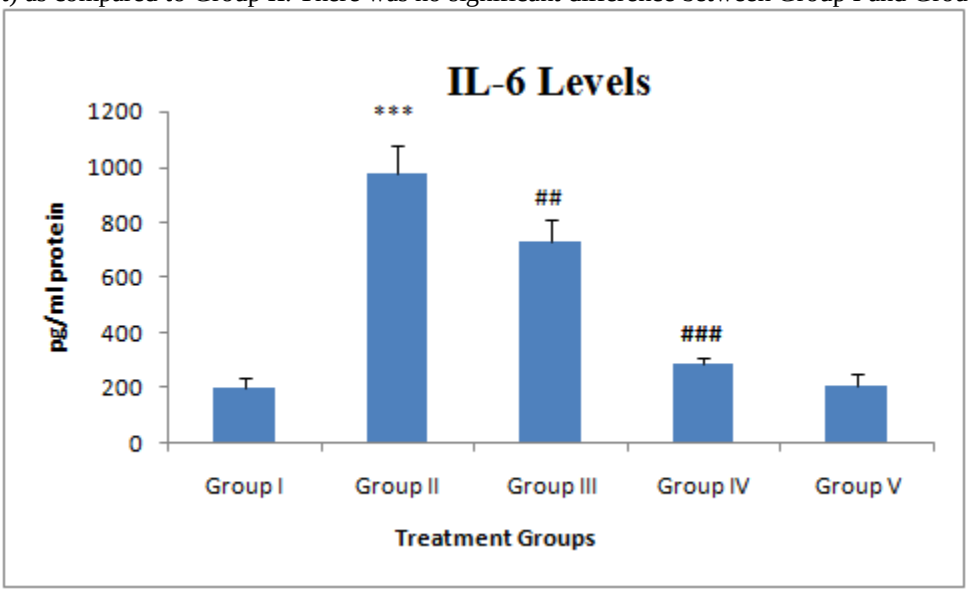

Figure 7
Effect of daidzein pre-treatment on Fe-NTA induced increase in IL-1β. Values are expressed as mean ± SEM. (n = 6). $^{***}p<0.001$ shows significant difference in Group II (Fe-NTA 9 mg/kg b.wt) when compared with Group I. $^{\#}p<0.05$ shows significant difference in the Group III (Fe-NTA 9 mg/kg b.wt + Daidzein 25mg/kg b.wt) when compared with Group II and $^{\#\#}p<0.01$ also shows significant difference in Group IV (Fe-NTA 9 mg/kg b.wt +

Daidzein 50mg/kg b.wt) as compared to Group II. There was no significant difference between Group I and Group V.

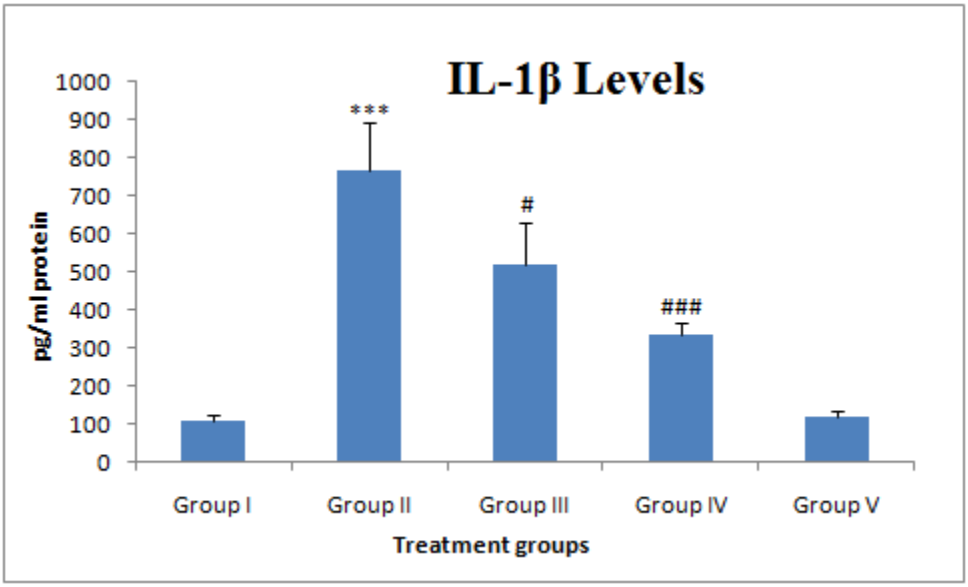

Figure 8
Hematoxylin and eosin stained sections of rat kidney showing effect of daidzein pretreatment on Fe-NTA-induced renal histological alterations. Representative photomicrographs (magnification ×40), (I) only vehicle (II) Fe-NTA 9 mg/kg b.wt (III) Daidzein 25mg/kg b.wt + Fe-NTA 9 mg/kg b.wt (IV) Daidzein 50 mg/kg b.wt + Fe-NTA 9 mg/kg b.wt (V) only Daidzein 50 mg/kg b.wt.

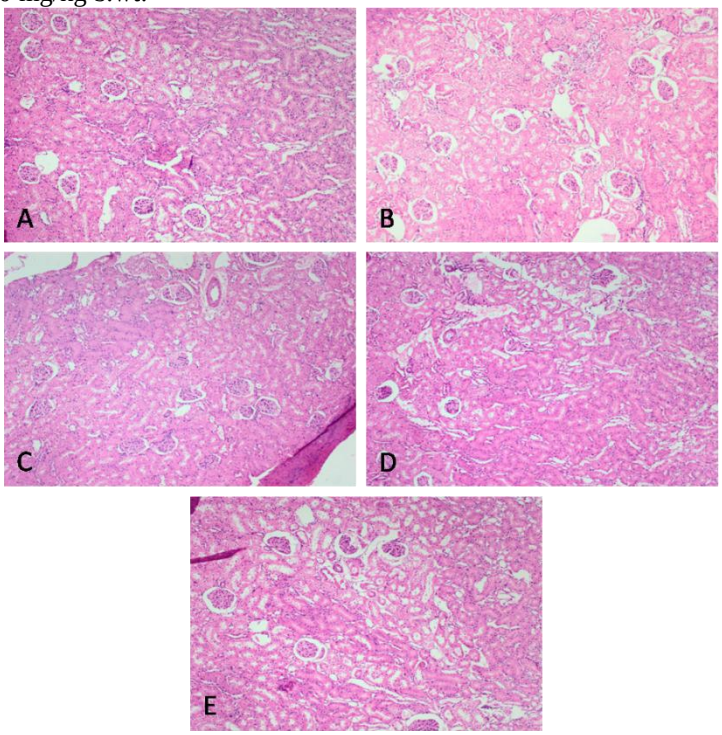

Table.1 Results of pretreatment of Daidzein on antioxidant enzymes like GSH, GST, GR and GPX on Fe-NTA induced imbalance in renal tissue.

Treatment regimen per group	**Catalase** (nmol H_2O_2 consumed /min/mg protein)	**XO** (µg uric acid / min/mg protein)	**SOD** (Unit per mg/ protein)	**MDA** (nmoles of MDA formed/g tissue)
Group I	23.70±2.60	0.66±0.08	22.67±1.0	7.22±0.91
Group II	10.01±1.52**	1.59±0.60***	13.22±2.9**	19.12±1.5***
Group III	14.3±1.43$^{\#}$	0.99±0.80^{ns}	14.3±1.3^{ns}	13.31±1.9$^{\#}$
Group IV	19.10±2.1$^{\#\#}$	0.84±0.09$^{\#\#\#}$	16.97±1.2$^{\#\#}$	9.11±1.02$^{\#\#\#}$
Group V	22.60±3.5	0.66±0.09	20.22±2.2	7.15±0.7

Results represent mean ± SE of six animals per group. Results obtained are significantly different from group I (***P < 0.001) (***P < 0.001). Results obtained are significantly different from group II (##P < 0.01) and (###P<0001). Group I – control; group II (toxicant) – Fe-NTA; group III – Fe-NTA + Daidzein 20mg/kg b.wt; group IV – Fe-NTA + Daidzein 40mg/kg b.wt; group V – Daidzein 40mg/kg b.wt.

Table: 2 Effects of Daidzein and Fe-NTA on the activities of Catalase (CAT), Superoxide dismutase (SOD), Xanthine oxidase (XO) and Lipid peroxidase (LPO).

Treatment regimen per group	**GSH** (n mol GSH /g tissue)	**GST** (n mol CDNB Conjugate formed/min/mg protein)	**GR** (n mol NADPH Oxidized/min/ mg protein)	**GPX** (n mol NADPH Oxidized/min/ mg protein)
Group I	0.55±0.01	339.5±22.1	356.4±16.9	250.0±15.29
Group II	0.31±0.01***	171.2±14.5***	190.3±15.7***	100.6±9.64**
Group III	0.37±0.01$^{\#}$	223.0±13.2^{ns}	235.9±14.2^{ns}	140.1±8.61$^{\#}$
Group IV	0.49±0.01$^{\#\#}$	290.5±18.4$^{\#\#}$	331.6±19.6$^{\#\#\#}$	226.9±12.11$^{\#\#\#}$
Group V	0.53±0.02	337.5±21.1	355.8±17.2	249.7±6.8

Results represent mean ± SE of six animals per group. Results obtained are significantly different from group I (***P < 0.001) (***P < 0.01). Results obtained are significantly different from group II (#P<0.05) (ns P=non significant) (##P < 0.01) and (###P<0001). Group I – control; group II (toxicant) – Fe-NTA; group III – Fe-NTA + Daidzein 20mg/kg b.wt; group IV – Fe-NTA + Daidzein 40mg/kg b.wt; group V – Daidzein 40mg/kg b.wt

Table.3 Results of pretreatment of Daidzein on Fe-NTA induced serum toxicity markers.

Treatment regimen per group	BUN (mg / 100 ml) IU/L	Creatinine (mg / 100 ml) IU/L	KIM-1 (µg/g serum $_{Cr}$)
Group I	18.42±1.11	1.33±0.40	15.0±1.30
Group II	48.41±3.58***	3.98±0.80***	88.3±4.10***
Group III	33.83±2.54##	2.12±0.51##	39.2±3.7##
Group IV	22.14±2.43###	1.84±0.18###	23.4±4.2###
Group V	19.10±1.70	1.36±0.60	14.4±6.4

Results represent mean ± SE of six animals per group. Results obtained are significantly different from group I (***P < 0.001). Results obtained are significantly different from group II (##P < 0.01) and (###P<0001). Group I – control; group II (toxicant) – Fe-NTA; group III – Fe-NTA + Daidzein 20mg/kg b.wt; group IV – Fe-NTA + Daidzein 40mg/kg b.wt; group V – Daidzein 40mg/kg b.wt

Discussion

In the present study, we investigated the efficacy of Daidzein on Fe-NTA induced renal oxidative stress and inflammation. Daidzein ameliorates oxidative stress and inflammation induced by Fe-NTA in renal tissue. Several investigators have shown that ROS are closely related to inflammation induced by Fe-NTA (Rehman and sultana, 2012; Rehman et al., 2013). Medicinal plants as component of diet are used throughout the world for centuries to treat many diseases, and a major portion of the world population relies on naturally occurring agents for their health solutions. Plant-based antioxidants in food are not only efficient, but are also relatively safe. Natural antioxidants have become increasingly essential as therapeutic agents against oxidative stress and in inflammatory disorders such as cancer.

Fe-NTA has been shown to increase the level of MDA. Lipid peroxidation and the associated membrane damage is implicated in the pathophysiology of a number of diseases, including renal carcinogenesis. Elevated lipid peroxidation has been reported upon Fe-NTA exposure as well. In the present study too Fe-NTA treatment enhanced the levels of MDA formation in renal tissue and Daidzein significantly ameliorated this increase. The observed prevention of MDA formation is in accordance with previous reports were in Daidzein has been demonstrated to inhibit MDA (Lateef at al., 2012; Plos one 2013).

Single i.p. dose of Fe-NTA induced significant depletion in the renal GSH content and its metabolizing enzymes. Depletion in GSH levels, a natural cellular antioxidant, is indicator of the insult by toxic foreign agent (Fe-NTA). GR maintains GSH reduced where as GPx utilizes it for decomposition of lipid peroxides/ hydro peroxides and other ROS. Substantial decrease in renal GSH is noted with concomitant decrease in GR, GPx, QR, and GST levels on Fe-NTA administration (Rehman and sultana, 2012; Jahangir and sultana, 2005; Khan et al., 2004). A dose dependent marked but partially significant recovery of the depleted GR, GPx, QR, GSH, and GST on pretreatment of animals with Daidzein was observed.

Further, the serum toxicity markers like BUN and creatinine showed significant elevation in Fe-NTA treated rats in accordance with earlier reports (Rehman et al., 2013). However, prophylactic treatment of rats with daidzein significantly restored the serum toxicity marker level to normal. Rats treated with daidzein had BUN and creatinine significantly lower than those receiving only Fe-NTA. These results suggested that daidzein may defend against Fe-NTA induced renal toxicity and might serve as a novel agent to limit renal injury.

Kim-1 has been recommended a useful and one of the most sensitive biomarker for renal proximal tubule injury in preclinical and clinical studies of drug safety evaluation, heavy metal related renal injury and the monitoring of renal disease status (Zhou et al., 2008). Our study showed Kim-1 levels were increased Fe-NTA treated thus consistent with earlier reports (Prozialeck et al., 2007; Zhou et al., 2008). Daidzein effectively inhibits the abnormal increase in Kim-1 levels.

Various published reports have revealed oxidative stress to be the major cause of the inflammation stimulating the release of several proinflammatory cytokines. It also plays a major role in the development of renal nephropathy (Rivero et al., 2009). Redox status has also been shown to influence NF-κB regulation and hence several genes involved in cell transformation, proliferation, and angiogenesis. Although, relationship between ROS and NF-κB is complex, ROS are believed to be implicated as second messengers in the activation of NF-κB via TNF-α and other proinflammatory cytokines (Reuter et al., 2012: Rehman et al., 2013). Inhibition of NF-κB is a good strategy to control carcinogenesis and tumor promotion. Since NF-κB is sensitive to ROS it is activated by strong oxidants like Fe-NTA. Thus Fe-NTA toxicity too can, at least to a certain extent, be controlled by NF-κB inhibitors. Further there

is enough evidence that COX-2 inhibition represents a suitable target for prevention of various proliferative diseases including cancer (Hull, 2005). COX-2 is also under the transcriptional instructions of NF-κB by virtue of which it makes the environment conducive for tumor promotion. Daidzein pretreatment significantly suppressed Fe-NTA induced COX-2 expression.

Fe-NTA exposure induced the expression of proinflammatory cytokines TNF- α, IL-6 and IL-1β which are also under direct transcriptional regulation of NF-κB. These cytokines have an important role in inflammation, as well as proliferation. Thus, inhibition in their secretion by daidzein seems to play an important role in its protective effect against renal toxicity.

Our results are in agreement with the findings of Rehman et al. (2013) who have showed daidzein to inhibit these cytokines in Fe-NTA stimulated proinflammatory response in rat model. Thus, Inhibition of proinflammatory cytokines by daidzein treatment may be now accepted as yet another approach to control the renal toxicity.

References

Griffiths RR, Bigelow GE and Liebson IA. (1986). Human coffee drinking: Reinforcing and physical dependence producing effects of caffeine. J Pharmacol Exp Ther **239**: 416–425.

Rehman M U, Sultana S (2011). Attenuation of oxidative stress, inflammation and early markers of tumor promotion by caffeic acid in Fe-NTA exposed kidneys of Wistar rats. Mol Cell Biochem, **357**, 115-24.

Masilamani MJ, Wei, Sampson H A (2012). Regulation of the immune response by soybean isoflavones. Immunol Res, **54**(1–3), 95–110.

Messina M, (2010). A brief historical overview of the past two decades of soy and isoflavone research. J Nutr, **140**, 1350S–1354S.

Mishra P, Kar A, Kale RK (2009). Prevention of chemically induced mammary tumorigenesis by daidzein in pre-pubertal rats: the role of peroxidative damage and antioxidative enzymes. Mol Cell Biochem, **325**,149–157.

Wijeratne SS, Cuppett SL (2007). Soy isoflavones protect the intestine from lipid hydroperoxide mediated oxidative damage. J Agric Food Chem, **55**, 9811–9816.

Jackman KA, Woodman OL, Sobey CG (2007). Isoflavones, equol and cardiovascular disease: pharmacological and therapeutic insights. Curr Med Chem, **14**, 2824–2830.

Shi T, Liou LS, Sadhukhan P, et al (2004). Effects of resveratrol on gene expression in renal cell carcinoma. Cancer Biology & Therapy, **3**, 882.

Torres N, Torre-Villalvazo I, Tovar AR, (2006). Regulation of lipid metabolism by soy protein and its implication in diseases mediated by lipid disorders. J Nutr Biochem, **17**,365–373

Ramesh G, Reeves WB, (2002). TNF-alpha mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. J. Clin. Invest, **110**, 835–842.

Collins T, Read MA, Neish AS, et al (1995) Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. FASEB J, **9**, 899.

Mariotto S, Suzuki Y, Persichini T, (2007). Cross-talk between NO and arachidonic acid in inflammation. Curr. Med. Chem, **14**, 1940–1944.

Rivero, A., C. Mora, M. Muros, J. García, H. Herrera and J.F. Navarro-González, 2009. Pathogenic perspectives for the role ofinflammation in diabetic nephropathy. Clin. Sci. (Lond.), **16**, 479–492.

Reuter S, Gupta SC, Chaturvedi MM et al (2010). Oxidative stress, inflammation, and cancer: How are they linked? Free Radical Biology & Medicine, **49**,1603–1616.

Hull MA, (2005). Cyclooxygenase-2: how good is it as a target for cancer chemoprevention? Eur J Cancer, **41**, 1854–1863.

Jahangir T, S Sultana, (2006). Modulatory effects of Pluchea lanceolata against chemically induced oxidative damage, hyperproliferation and two-stage renal carcinogenesis in Wistar rats. Mol Cell Biochem, **291**. 175–185.

Khan N, Sharma S, Sultana S, (2004). Attenuation of potassium bromate induced nephrotoxicity by coumarin (1,2-benzopyrone) in Wistar rats: chemoprevention against free radical-mediated renal oxidative stress and tumor promotion response. Redox Rep, **9**(1),19–28.

Rehman MU and S Sultana, (2011). Attenuation of oxidative stress, inflammation and early markers of tumor promotion by Caffeic acid in Fe-NTA exposed kidney of Wistar rats. Mol Cell Biol, **357**(1-2),115-24.

Rehman MU, Tahir M, Khan AQ et al., (2013). Chrysin suppresses renal carcinogenesis via amelioration of hyperproliferation, oxidative stress and inflammation: plausible role of NF-κB. Toxicol Lettr., **216**(2-3):146-158.

Rehman MU, Tahir M, Lateef A, et al., (2012). Methanolic extract of Bacopa monnieri protects against KBr_{O3}-induced renal damage in Wistar rats by restoring antioxidant enzyme activities and suppressing inflammation and apoptosis. J Pharm Res, **5**(11):5231-5238.

Khan R, A. Quaiyoom AQ, Lateef A, et al., (2013). Glycyrrhizic acid as a chemopreventive agent against of 1,2-dimethylhydrazine induced colon tumorigenesis in Wistar rats. PLoS One, **8**(2),e56020.
Lateef A, Khan AQ, Tahir M et al., (2013) Androgen deprivation by flutamide modulates uPAR, MMP-9 expressions, lipid profile, and oxidative stress: amelioration by daidzein. Mol Cell Biochem. **374**(1-2), 49-59.
Irwing HM, Miles NH Miles MG, (1966). Analogues of nitrilotriacetic acid, and the stabilities of their proton and metal complexes. J Chem Soc A, 1268–1275
Awai M, Narasaki M, Yamanoi Y, (1979). Induction of diabetes in animals by parenteral administration of ferric nitrilotriacetate. A model of experimental hemochromatosis. Am J Pathol, **95**,663–673
Hamazaki S, Okada S, Ebina Y, et al., (1985). Acute renal failure and glycosuria induced by ferric nitrilotriacetate in rats. Toxicol Appl Pharmacol, **77**:267–274
Liu M, Okada S. et al (1993). Radical promoting free iron level in serum of rats treated with other iron chelate complexes. Acta Med Okayama, **45**:401–408
Zhang D, Okada S, et al 1995. An improved simple colorimetric method for the quantization of non-bound iron in serum. Biochem Mol Biol Int, **35**:635–641
Jacobs A., 1980. In: Jacobs A, Worwood M (eds) Iron in biochemistry and medicine, II. Academic Press, New York, pp 427–459
Chen L, Wang Y et al (2001) Molecular mechanisms by which iron induces nitric oxide synthesis in cultured proximal tubule cells. Exp Nephrol **9**(3):198–204
Toyokuni S, (2002). Iron and carcinogenesis: from Fenton reaction to target genes. Redox Rep, **7**(4):189–197
Toyokuni S, 1996. Iron-induced carcinogenesis: the role of redox regulation. Free Radic Biol Med, **20**:553–566
Liochev SI, Fridovich I, (2002). The Haber–Weiss cycle-70 years later: an alternative view. Redox Rep, **7**:55–57
Gupta A, Sharma S, Kaur I, et al., (2009). Renoprotective effects of sesamol in ferric nitrilotriacetate (Fe-NTA)-induced oxidative renal injury in rats. Basic Clin Pharmacol Toxicol, **104**:316– 321
Iqbal M, Okazaki Y, Okada S, (2009). Curcumin attenuates oxidative damage in animals treated with a renal carcinogen, ferric nitrilotriacetate (Fe-NTA): implications for cancer prevention. Mol Cell Biochem, **324**:157–164
Rafter JJ, (2002). Scientific basis of biomarkers and benefits of functional foods for reduction of disease risk: cancer. Br J Nutr, **88**:219–224.
Athar M, Iqbal M, (1998). Ferric nitrilotriacetate promotes Ndiethyl nitrosoamine-induced renal tumorigenesis in rat: implications for the involvement of oxidative stress. Carcinogenesis, **19**:1133–1139.
Tahir M, Sultana S, (2011). Chrysin modulates ethanol metabolism in Wistar rats: a promising role against organ toxicities. Alcohol and Alcoholism **46** (4), 383–392.
Wright, J.R., H.D. Colby., and P.R. Miles, 1981. Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. Arch Biochem Biophys, **206**:296–304
Jollow DJ, Mitchell JR, Zampaglione, N (1974). Bromobenzene induced liver necrosis: protective role of glutathione and evidence for 3,4-bromobezene oxide as the hepatotoxic metabolite. Pharmacol, **11**,151
Habig WH, Pabst MJ, Jakoby WB (1974). Glutathione-S-transferases: the first enzymatic step in mercapturic acid formation. J Biol Chem., **249**,7130–7139
Carlberg I, Mannervik B (1975). Glutathione level in rat brain. J Biol Chem **250**, 4480–4575
Stripe F, Della Corte E (1969). The regulation of rat liver xanthine oxidase. J Biol Chem, **244**, 3855–3863
Claiborne A, (1985). Catalase activity. In: Greenwald RA (ed) CRC handbook of methods in oxygen radical research. CRC Press, Boca Raton., pp 283–284
Mohandas M, Marshall JJ, Duggin GG, (1984). Differential distribution of glutathione and glutathione related enzymes in rabbit kidney. Cancer Res., **44**:5086–5091
Kanter MW (1975). Clinical chemistry. The Bobber Merill Company Inc, New York
Hare RS (1950). Endogenous creatinine in serum and urine. Proc Soc Exp Biol Med, **74**, 148–151
Lowry OH, Rosebrough NJ, Farr A, et al., (1951). Protein measurement with the Folin phenol reagent. J Biol Chem, **193**,265–275.
Zhou Y, Vaidya VS, et al., (2008). Comparison of kidney injury molecule-1 and other nephrotoxicity biomarkers in urine and kidney following acute exposure to gentamicin, mercury, and chromium. Toxicological Sciences, **101**,159.
Prozialeck WC, Vaidya VS, (2007). Kidney injury molecule-1 is an early biomarker of cadmium nephrotoxicity. Kidney International, **72**, 985–993.