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The dual clastogenic and anti-clastogenic properties of quercetin is dose dependent

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1. ABSTRACT

Iron is a ubiquitous constituent of cytochromes, oxygen-binding molecules and a variety of enzymes because of its property to transition from ferric (Fe^{3+}) to ferrous (Fe^{2+}) state, leading to change in the redox potential. However, the same property accounts for free radical injury. In order to overcome harmful effects of iron we investigated the possible protective effect of quercetin (QCT), a flavonoid with antioxidant property, against the oxidative DNA damage caused by iron sulfate *in vivo*. We show that QCT exerts efficient anticlastogenic action in the context of iron sulfate treatment up to a dose of 500 mg/kg while it induces DNA damage at higher doses. These findings show that QCT has a dual effect; at low doses it ameliorates the oxidative damage produced by iron, and it is genotoxic and cytotoxic at a higher dose.

2. INTRODUCTION

Iron plays many essential roles in the body; however, when present in excess, it can induce damage in cells and tissues and lead to diseases (1). The deleterious effect of excess iron is related to its ability to generate reactive oxygen species. The toxicity of iron is mainly due to its Fenton and Haber–Weiss chemistry, where catalytic amounts of iron are sufficient to yield hydroxyl radicals ($OH^{\cdot}$) from superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), collectively known as 'reactive oxygen intermediates' (ROIs) (2). These species damage the cellular macromolecules causing cell death and tissue injury. Quercetin (QCT) is one of the several dietary flavonoids. It is a biologically and pharmacologically active polyphenolic compound that occurs naturally in plants, where it is involved in energy production and exhibit strong antioxidant properties. QCT is capable of preventing oxidant injury and cell death and thus protects the cells from oxidative damage (3) by several mechanisms such as scavenging oxygen radicals (4, 5) and protecting against lipid peroxidation (6, 7). It occurs naturally in apples, cranberries, blueberries, and onions at relatively high concentrations (8, 9). Many biological and pharmacological activities, that may be beneficial to human health, have been attributed to QCT including

antioxidant, anticarcinogenic, anti-inflammatory, and cardioprotective activities (10, 11). While these properties may account for the antimutagenic activity of flavonoids in experimental systems, results from different studies (12-14) demonstrated that QCT can also act as pro-oxidant at much higher doses. However, the molecular mechanisms of the mutagenicity of flavonoids are not well known. This indicates that QCT may have conflicting roles, i.e., as an antioxidant and as a pro-oxidant, depending on the concentration used. A recent study has demonstrated that the flavonoids, rutin and QCT, play a protective role in the context of the deleterious effects of free radicals in cirrhotic rats (15). Therefore, more studies are required *in vivo* to ascertain the beneficial/ toxic effects of QCT. The present study focuses on the protective role of QCT in pre-, simultaneous and post-treatments against genotoxicity induced by iron sulfate *in vivo*, as determined by bone marrow chromosomal aberration, micronucleus and comet assays.

3. MATERIALS AND METHODS

3.1. Chemicals

Iron sulfate (CAS 7782-63-0), quercetin (CAS 117-39-5), propidium iodide (P4170) **(**CAS 25535-16-4) and Giemsa stain (CAS 51811-82-6) were procured from Sigma, USA. Other chemicals such as ethylene diamine tetraacetic acid (EDTA) disodium (054448), triton-X-100 (2020130), tris base (2044122), and 4- (2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES, 75277) were purchased from SRL, India. Potassium chloride (Merck-7447-40-7), sodium chloride (Merck-7647-14-5), sodium hydroxide (Merck-1310-73-2), methanol (Merck-67-56-1), glacial acetic acid (Merck-64-19-7), sodium bicarbonate (Merck-144-55-8), low melting point (LMP) agarose (Merck-9012-36-6), glycerol (Merck-56-81-5), colchicines (Merck-64-86-8), modified May-Grünwald's eosine-methylene blue solution (200-659-6) and xylene (1330-20-7) were purchased from Merck, India.

3.2. Animal husbandry and study design

Adult male Wistar rats weighing 180-200 g were used in this study. The rats were housed according to the treatment groups in polycarbonate cages with steel wire tops and had access *ad libitum* to standard rat chow and clean drinking water. The rats were kept under standard laboratory conditions (23 ± 2ºC, 12 h light–dark cycle). All the animals were acclimated to the cage conditions for at least 2–3 days before the beginning of the experiments. All procedures were carried out as per CPCSEA guidelines for laboratory animal use as well as the prescriptions of the Institutional Animal Ethics Committee.

For pre-, simultaneous and post-treatments, rats were divided randomly into 9 groups of 6 each. Animals in Group 1 were used as control and administered distilled water. Animals in group 2 were administered $FeSO_4$ at a dose of 200 mg Fe/kg body weight through oral route. To observe the optimum level at which QCT expresses the best protective effect against genotoxicity induced by $FeSO_4$, animals in groups 3-9 were administered QCT at the doses 125, 250, 375, 500, 625, 750 and 875 mg/kg, respectively, through i.p. route. For the pre-treatment groups QCT was administered one hour before the administration of $FeSO_4$. For the simultaneous treatment groups $FeSO_4$ and QCT were administered to the animals simultaneously. For the post-treatment groups QCT was administered one hour after $FeSO_4$ treatment. The rats were sacrificed 24 h after each treatment. The dose of $FeSO_4$, 200 mg Fe/kg b.w., was selected on the basis of its effectiveness in inducing chromosomal aberrations and on the basis of data in published reports (16). The doses of QCT were also determined on the basis of published reports indicating antimutagenic effects (17,18).

3.3. Chromosomal analysis

Twenty four hour after the treatment, bone marrow cells were obtained from the rats according to Preston *et al*. (19). In order to stall progression of cells at metaphase the mitotic inhibitor colchicine, at 2 mg/kg b.w., was administered through i.p. route to the rats 2 h before animal sacrifice. The cells in bone marrow were obtained using a hypodermic syringe fitted with a 22 gauge needle by flushing 0.075 M KCl 2-3 times into the marrow cavity of femur until no bone marrow remained attached to the bone. After a few seconds, to allow the fragments to settle, the suspension was decanted. The tubes were centrifuged for 10 min at 1000 rpm. The supernatant was removed by gentle aspiration until a small volume remained above the pellet. The pellet was re-suspended in the remaining volume. KCl (0.075 M, 5 ml, pre-warmed to 37^{o}C) was added in drops under agitation. It was incubated for 20 min in water bath at 37^{o}C and centrifuged at 1,000 rpm for 10 min. The supernatant was removed and the pellet was re-suspended in the remaining volume. The cells were fixed in 0.5 ml absolute methanol: glacial acetic acid, 3:1, fixative prepared just before use, added in drops using a Pasteur pipette while continuously shaking the pellet so as to avoid formation of clots and allowed to stand at room temperature for 15-20 min. In order to ensure the proper fixation, the cells were kept suspended in the fixative at 4°C for a minimum period for one hour but preferably overnight. The contents, then, were again centrifuged at 1000 rpm for 10 min. This procedure was repeated two or three times, each time with fresh fixative. After final wash in the fixative, the cells were re-suspended in 0.2 ml of fresh fixative. The slides were washed, cleaned and immediately coded and kept at 4°C in distilled water. About 3-4 drops of cell suspension were dropped to each chilled and tilted slide to get good spreading, and the slides were briefly dried over flame. One hour thereafter the slides were stained in 5% Giemsa stain prepared in phosphate buffer (pH 6.8). Two slides per rat were prepared for each treatment and fifty well-spread complete metaphases were scored per slide. Cytogenetic analysis of the slides was performed in

a trinocular research microscope (Nikon 80i) using an oil immersion (100x) lens.

3.4. Micronucleus test

Bone marrow preparation was made for micronucleus test according to Schmid (20). Both femurs were removed and the bone marrow was flushed out immediately as a fine suspension into centrifuge tubes containing 2 ml of fetal calf serum. The bone marrow suspension was centrifuged at 1000 rpm for 10 min at 4ºC and the pellet was re-suspended in a drop of serum. A small portion of this suspension was placed on a clean slide, smeared and air-dried for 18 h. The slides were then stained and fixed. For each animal, three slides were prepared and the best slide was selected for scoring. The incidence of micronuclei (MN), observed in 2000 PCE/ rat, was calculated to find the clastogenic property of the test chemicals.

3.5. Single cell gel electrophoresis (SCGE/ Comet assay)

Comet assay was performed according to the method described by Buschini *et al.* (21) with slight modifications. Low melting point (LMP) agarose (0.8%) was prepared in saline and maintained at 39ºC to prevent solidification. Subsequently, 20 µl of whole blood obtained from each rat was treated with $FeSO_4$ and/or $FeSO_4$ (200 mg/kg) + QCT (125, 250, 375, 500, 625, 750 and 875 mg/kg) and was gently mixed with 250 µl of 0.8% LMP agarose. The resulting suspension was layered onto fully frosted slides. The slides were placed on ice for approximately 5 min to allow the agarose to solidify. Subsequently, the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA with fresh 1% Triton-X 100 and 10% DMSO) for one hour to eliminate non-nuclear components. The slides were further immersed in alkaline buffer (300 mM NaOH, 1 mM EDTA, pH = 13) for 20 min to allow the DNA to unwind and to subject alkali labile sites to single strand breaks. Electrophoresis was conducted for 30 min at 15 V and 200 mA (at a rate of 0.6. V/cm) using a compact power supply. The slides were gently washed with 0.4 M Tris (pH = 7.5) to remove the alkali and detergents. The slides were placed in a humid chamber until staining to prevent the gel from drying. The cells were stained with propidium iodide (20 µg/ml) and observed in a fluorescent microscope (Nikon, Japan). Images of the cells were captured using a digital camera. Approximately 50 images per slide were captured from different imaging fields and analyzed with the CASP software. For each image, two SCGE parameters, i.e. olive tail moment (OTM) and tail moment (TM) were analyzed. Olive tail moment = (tail mean−head mean) × tail % DNA/100; tail moment was = tail length × tail % DNA (tail intensity)/100.

3.6. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) (22) for multiple comparisons and expressed as the mean ± SD. Tukey post hoc test was employed to compare the differences between samples with the help of SPSS (version 16). The level of significance was set at $p<0.05$. Broken-line regression analysis was employed to determine the optimum level of QCT (23). The equation employed was Y= a+bX. Statistical analysis was conducted using Origin (version 6.1, Origin Software, San Clemente, CA, USA).

4. RESULTS

4.1. Chromosomal aberrations assay

The types of structural chromosomal aberrations, with special emphasis on breaks, exchanges forming dicentrics, fragments and sister chromatid union forming rings induced by $FeSO_4$ in the absence or presence of QCT are presented in the Table 1. Highly significant increase was recorded in respect of the total number of structural chromosomal aberrations in $FeSO_4$-treated animals compared to the untreated control ($p<0.001$). There were also significant differences in the total number of structural chromosomal aberrations between the controls and those treated with $FeSO_4$ and QCT in the pre-, simultaneous and post-treatments (for all the treatments $p<0.001$). In the presence of QCT (125, 250, 375 and 500 mg/kg), the number of chromosomal aberrations significantly ($p<0.001$) decreased when compared to animals treated with $FeSO_4$ alone. QCT exhibited significant ameliorating effect against $FeSO_4$ induced chromosomal aberrations in the simultaneous treatment groups (Figures 1-4). In animals treated with 625, 750 and 875 mg/kg doses of QCT, the chromosomal aberrations increased.

4.2. Micronucleus test

The cytotoxic potential of $FeSO_4$ was evaluated by counting the number of PCE among 1000 cells (PCE + NCE). The number of MNPCEs among 2000 PCE, indicative of genotoxicity, is presented in Table 2. The number of MNPCEs among 2000 PCE induced by $FeSO_4$ was significant ($p<0.001$) at a dose of 200 mg Fe/kg. QCT produced statistically significant decrease in the yields of MN induced by $FeSO_4$ in pre-, simultaneous and post-treatments at doses up to 625 mg/kg. All doses of QCT evaluated, except the two highest doses, were found to be effective in reducing the frequency of MN induced by $FeSO_4$. Significant reduction in the total MN yield (MN in PCE and NCE) was also observed. Lower doses of QCT were not found effective in reducing the frequency of MN. At 500 mg/kg dose QCT exhibited the most effective and significant inhibitory effect on MN in PCE ($p<0.001$). Similar to the chromosomal aberration study here also the protective potential of QCT was most prominent in the simultaneous treatments (Figures 5A, B and 6).

4.3. Single cell gel electrophoresis (SCGE/ Comet assay)

Table 3 summarizes the effects of $FeSO_4$ and the protective potential of QCT on the extent of DNA damage. Two SCGE parameters, i.e. tail moment (TM)

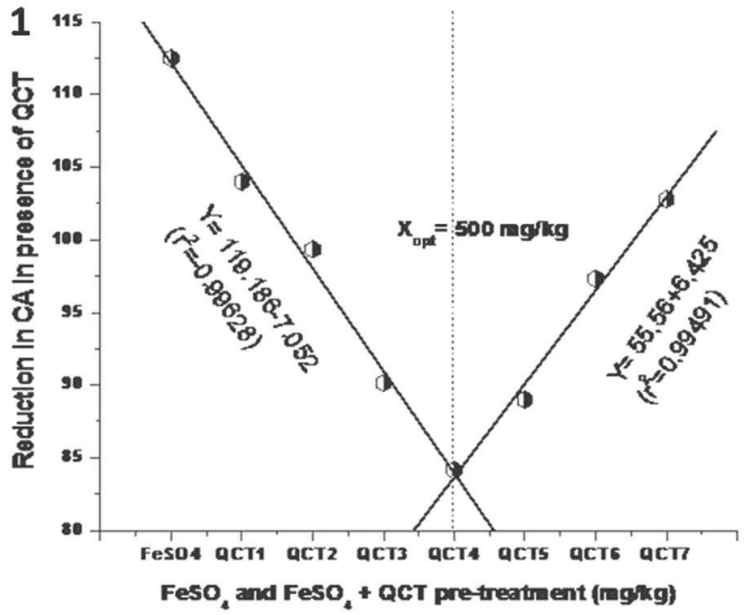

Figure 1. Broken-line relationship of pre-treatment of quercetin levels with FeSO4 to reduction in chromosomal aberrations in bone marrow cells of Wistar rats.

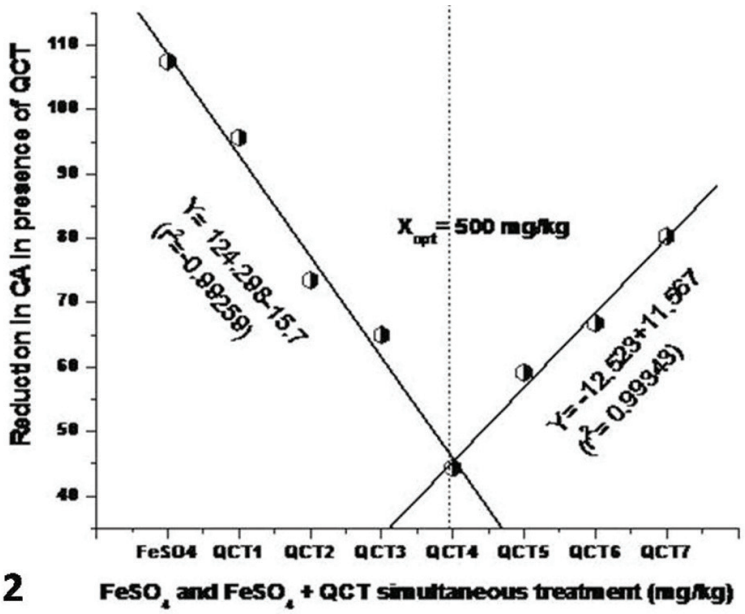

Figure 2. Broken-line relationship of simultaneous treatment of quercetin levels with FeSO4 to reduction in chromosomal aberrations in bone marrow cells of Wistar rats.

and olive tail moment (OTM) were analyzed using CASP software. Significantly higher levels of DNA damages in terms of TM and OTM were found in rats exposed to $FeSO_4$ compared with the untreated control (p<0.001). There were significant (p<0.001) differences in the extent of DNA damage in terms of TM and OTM between the control and those treated with QCT in the pre-, simultaneous and post-treatment groups, except 625, 750 and 875 mg/kg dose QCT groups. During simultaneous treatment the protective influence of QCT was most prominent against $FeSO_4$ induced DNA damage in terms of TM and OTM (Figures 7A, B; 8A, B; 9A,B; 10).

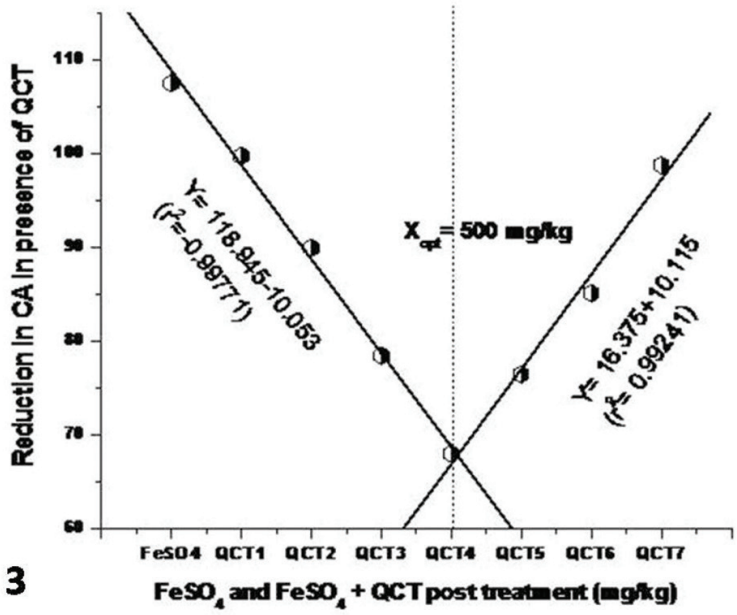

Figure 3. Broken-line relationship of post treatment of quercetin levels with FeSO4 to reduction in chromosomal aberrations in bone marrow cells of Wistar rats. Broken-line regression analysis was employed to determine the optimum level of QCT.

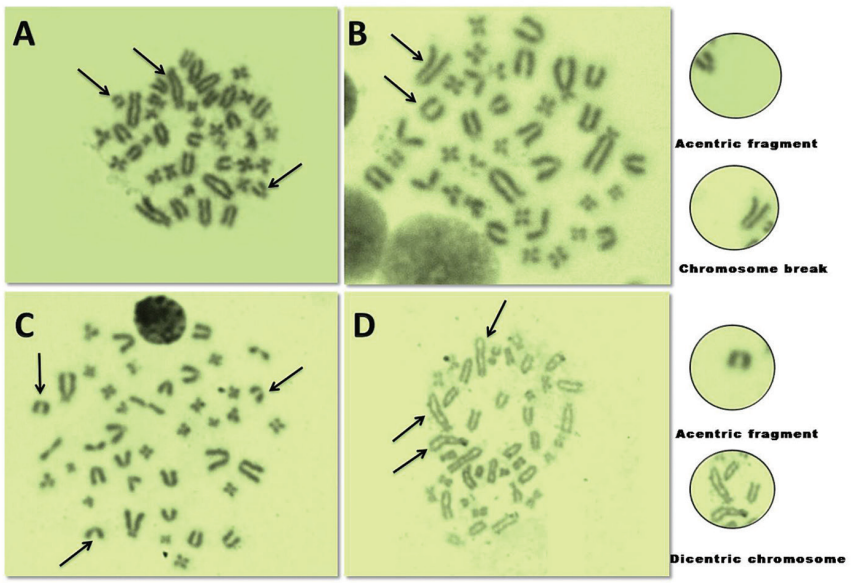

Figure 4. Photomicrographs illustrating the effects of Iron sulfate (FeSO4) on bone marrow cells of Wistar rats (A) Acentric fragment; (B) Chromosome break; Ring chromosome (C) Acentric fragment (D) Dicentric chromosomes.

4.4. Optimum level of QCT

The broken-line regression analysis of the QCT levels against CAs, MNPCEs and DNA damage induced by $FeSO_4$ showed that the reduction in these values were best attained at 500 mg/kg of QCT. The equations and optimum level of QCT are given below.

4.4.1. Chromosomal aberrations

Y= 119.186-7.052, X ≤ 500 mg/kg (r^2=-0.99628); Y= 55.56+6.425, X ≥ 500 mg/kg (r^2= 0.99491) X_{opt} = 500 mg/kg QCT (Pre-treatment with QCT)
Y= 124.298-15.7, X ≤ 500 mg/kg (r^2=-0.99259); Y=-12.523+11.567, X ≥ 500 mg/kg

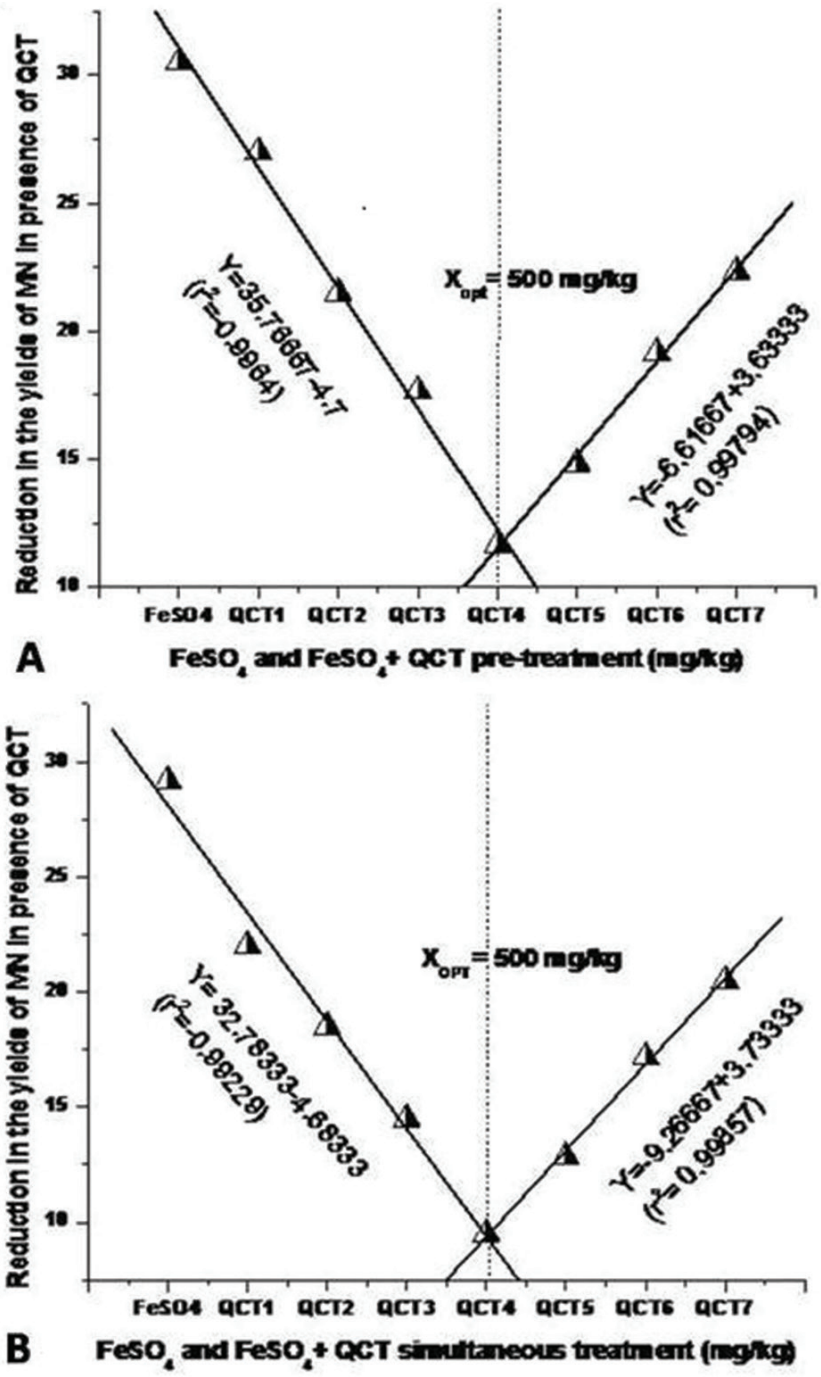

Figure 5. Broken-Line relationship of pre and simultaneous treatment of quercetin - mediated modification of iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats.

(r^2=0.99343) X_{opt} = 500 mg/kg QCT (Simultaneous treatment with QCT)
Y=118.945-10.053, X ≤ 500 mg/kg (r^2=-0.99771); Y= 16.375+10.115, X ≥ 500 mg/kg
(r^2=0.99241) X_{opt} = 500 mg/kg QCT (Post treatment with QCT)

4.4.2. Micronucleus assay

Y= 35.76667-4.7, X ≤ 500 mg/kg (r^2=-0.9964); Y=-6.61667+3.63333, X ≥ 500 mg/kg
(r^2= 0.99794) X_{opt} = 500 mg/kg QCT (Pre-treatment with QCT)
Y= 32.78333-4.68333, X ≤ 500 mg/kg (r^2=-0.99229);

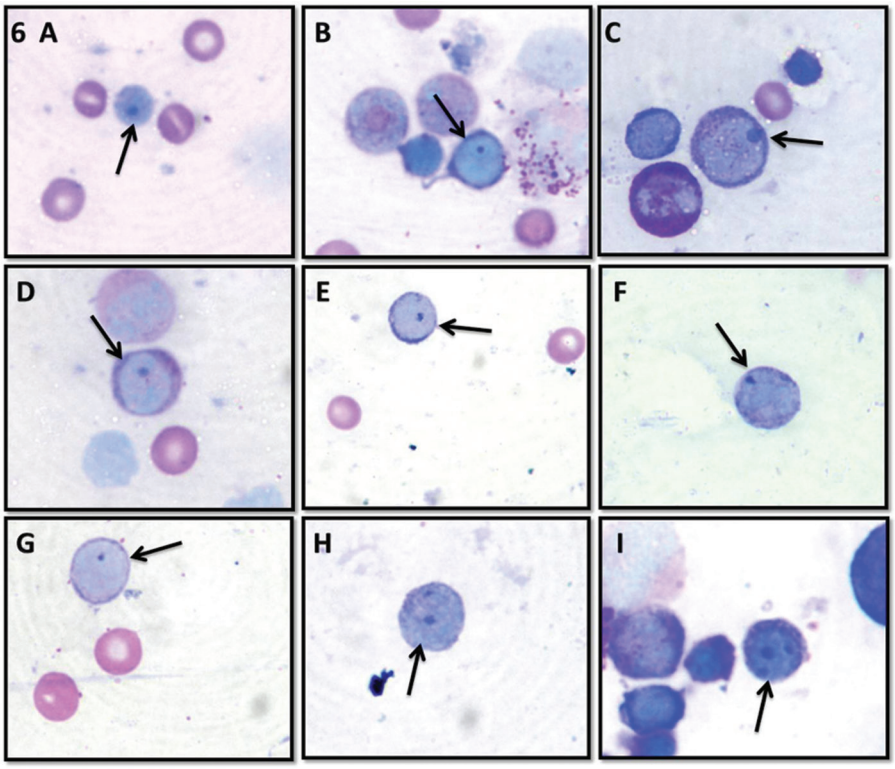

Figure 6. Representative images of polychromatic erythrocytes (PCEs) containing micronuclei in bone marrow smear of Wistar rats after 24 h of iron sulfate.

Table 1. The apportionment of structural aberrations in bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of quercetin pre, simultaneous and post treatments

Treated Groups	At 24 h total cells Studied/n	Dose (mg/kg body weight)	Pre treatment with QCT total aberrations (X±SD)	Simultaneous treatment with QCT total aberrations (X±SD)	Post treatment with QCT total aberrations (X±SD)
Negative control	600/6	---	1.5±1.22	1.66±1.21	2.0±0.89
$FeSO_4$	600/6	200 mg Fe/kg	112.5±33.97**	107.5±21.71**	107.6±15.20**
$FeSO_4$+QCT_1	600/6	125 mg/kg	104±11.52**(a)	95.66±24.91**(a)	99.83±15.94**(a)
$FeSO_4$+QCT_2	600/6	250 mg/kg	99.33±17.69**(b)	73.50±11.84**(a)	90.0±13.16**(a)
$FeSO_4$+QCT_3	600/6	375 mg/kg	90.16±22.42**(c)	65.0±8.41**(b)	78.50±10.36**(b)
$FeSO_4$+QCT_4	600/6	500 mg/kg	84.16±20.74**(c)	44.33±12.69**(b)	68.0±7.01**(b)
$FeSO_4$+QCT_5	600/6	625 mg/kg	89.0±19.42**(c)	59.16±12.35**(b)	76.50±6.22**(b)
$FeSO_4$+QCT_6	600/6	750 mg/kg	97.33±14.05**(b)	66.83±12.0**(b)	85.16±5.41**(a)
$FeSO_4$+QCT_7	600/6	875 mg/kg	102.8±25.02**(b)	80.33±15.80**(a)	98.83±8.47**(a)

About 600 cells were analysed per treatment for the total number of structural aberrations. Data are expressed as the X±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. $FeSO_4$: Iron sulfate, QCT: Quercetin, X: Mean, SD: Standard deviation. Statistically significant different compared to control: *p<0.05; **p<0.001. Statistically significant different from $FeSO_4$ groups: (a) p<0.05; (b) p<0.005; (c) p<0.001

Y= -9.26667+3.73333, X ≥ 500 mg/kg (r^2= 0.99857) X_{opt} = 500 mg/kg QCT (Simultaneous treatment with QCT)

Y= 36.43333-4.66667, X ≤ 500 mg/kg (r^2=-0.99511); Y=-3.96667+3.36667, X ≥ 500 mg/kg (r^2= 0.99302) X_{opt} = 500 mg/kg QCT (Post treatment with QCT)

Table 2. Pre, simultaneous and post treatment of quercetin-mediated modification of iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats

Treated Groups	At 24 h total PCE/n	Dose (mg/kg body weight)	Pre-treatment with QCT X±SD	Simultaneous treatment with QCT X±SD	Post treatment with QCT X±SD
Negative control	12000/6	---	2.0±1.6	1.5±0.8	1.66±0.9
$FeSO_4$	12000/6	200 mg Fe/kg	30.5±3.1**	29.16±3.9**	31.0±2.9**
$FeSO_4$+QCT1	12000/6	125 mg/kg	27.0±2.9**(a)	22.0±3.2**(a)	28.16±2.2**(a)
$FeSO_4$+QCT2	12000/6	250 mg/kg	21.5±3.3**(b)	18.5±2.9**(b)	22.16±1.9**(b)
$FeSO_4$+QCT3	12000/6	375 mg/kg	17.6±1.8**(b)	14.5±2.2**(c)	18.16±2.8**(b)
$FeSO_4$+QCT4	12000/6	500 mg/kg	11.66±1.4**(c)	9.5±2.4**(c)	12.66±1.4**(c)
$FeSO_4$+QCT5	12000/6	625 mg/kg	14.83±2.6**(c)	12.8±3.1**(c)	16.83±2.6**(b)
$FeSO_4$+QCT6	12000/6	750 mg/kg	19.16±2.2**(b)	17.16±2.6**(b)	19.0±2.0**(b)
$FeSO_4$+QCT7	12000/6	875 mg/kg	22.33±3.5**(b)	20.5±3.3**(b)	23.16±3.6**(a)

Values are expressed as mean±SD of 6 Wistar rats in each group and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. *p* values difference between the nine groups of rat bone marrow MNPCEs results statistically significant different compared to control: *p<0.05; **p<0.001 and statistically significant different from $FeSO_4$ groups: (a) p<0.05; (b) p<0.005; (c) P<0.001. $FeSO_4$: Iron sulfate, QCT: Quercetin, PCE: Polychromatic erythrocyte, MNPCEs: Micronucleated polychromatic erythrocytes, X: Mean, SD: Standard deviation

Table 3. Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood cells of Wistar rats induced by iron sulfate alone and in combination of pre, simultaneous and post treatments with different doses of quercetin for 24 h

Treated groups	At 24 h total cells/n	Dose (mg/kg b.w.)	Pre-treatment with QCT		Simultaneous treatment with QCT		Post treatment with QCT	
			Olive tail moment (X±SD)	Tail moment (X±SD)	Olive tail moment (X±SD)	Tail moment (X±SD)	Olive tail moment (X±SD)	Tail moment (X±SD)
Negative control	300/6	---	0.98±0.9	1.86±1.2	1.33±0.8	1.77±0.7	1.24±0.4	1.79±1.1
$FeSO_4$	300/6	200 mg Fe/kg	32.66±3.4**	36.22±3.7**	31.57±2.8**	37.74±3.3**	33.24±4.1**	38.67±3.1**
$FeSO_4$+QCT1	300/6	125 mg/kg	25.62±3.1**(a)	27.04±2.8**(a)	26.85±3.7**(a)	30.18±3.1**(a)	24.20±3.7**(a)	28.78±3.5**(a)
$FeSO_4$+QCT2	300/6	250 mg/kg	17.72±2.8**(b)	23.12±3.1**(a)	18.28±2.2**(b)	24.84±2.9**(b)	18.45±2.3**(b)	23.79±2.9**(b)
$FeSO_4$+QCT3	300/6	375 mg/kg	13.94±1.9**(c)	15.96±2.3**(b)	12.21±2.1**(c)	15.71±2.4**(b)	12.63±3.1**(c)	16.34±3.7**(b)
$FeSO_4$+QCT4	300/6	500 mg/kg	5.96±1.1*(c)	7.66±1.9*(c)	4.94±0.9*(c)	6.98±2.1*(c)	6.56±2.1*(c)	8.83±2.2*(c)
$FeSO_4$+QCT5	300/6	625 mg/kg	9.64±2.4**(c)	14.12±2.4**(c)	7.82±1.3*(c)	10.91±2.0**(c)	10.21±1.6**(c)	13.68±2.5**(c)
$FeSO_4$+QCT6	300/6	750 mg/kg	11.40±2.1**(c)	17.30±3.2**(b)	8.99±2.4**(c)	12.59±2.2**(c)	15.74±2.3**(c)	19.45±3.0**(b)
$FeSO_4$+QCT7	300/6	875 mg/kg	16.33±3.2**(a)	24.9±3.8**(a)	11.93±2.9**(c)	14.60±4.0**(c)	17.64±3.3**(b)	23.14±3.1**(a)

Measures were calculated for 50 cells per rats (six rats per group).Values are expressed as mean±SD and were analyzed by using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. Statistically significant different compared to control: *p<0.05; **p<0.001 and statistically significant different from $FeSO_4$ groups: (a) p<0.05; (b) p<0.001. $FeSO_4$: Iron sulfate, QCT: Quercetin, X: Mean, SD: Standard deviation

4.4.3. Single cell gel electrophoresis (SCGE/ Comet assay)

4.4.3.1. Tail moment

Y=38.704-6.508, X ≤ 500 mg/kg (r^2=-0.99531); Y=-10.488+ 3.283, X ≥ 500 mg/kg (r^2=0.0.98429) X_{opt} = 500 mg/kg QCT (Pre-treatment with QCT)

Y= 39.14-6.79, X ≤ 500 mg/kg (r^2=-0.99716); Y=-5.971+2.214, X ≥ 500 mg/kg (r^2=0.98785) X_{opt} = 500 mg/kg QCT (Simultaneous treatment with QCT)

Y= 38.495-6.493, X ≤ 500 mg/kg (r^2=-0.99545);

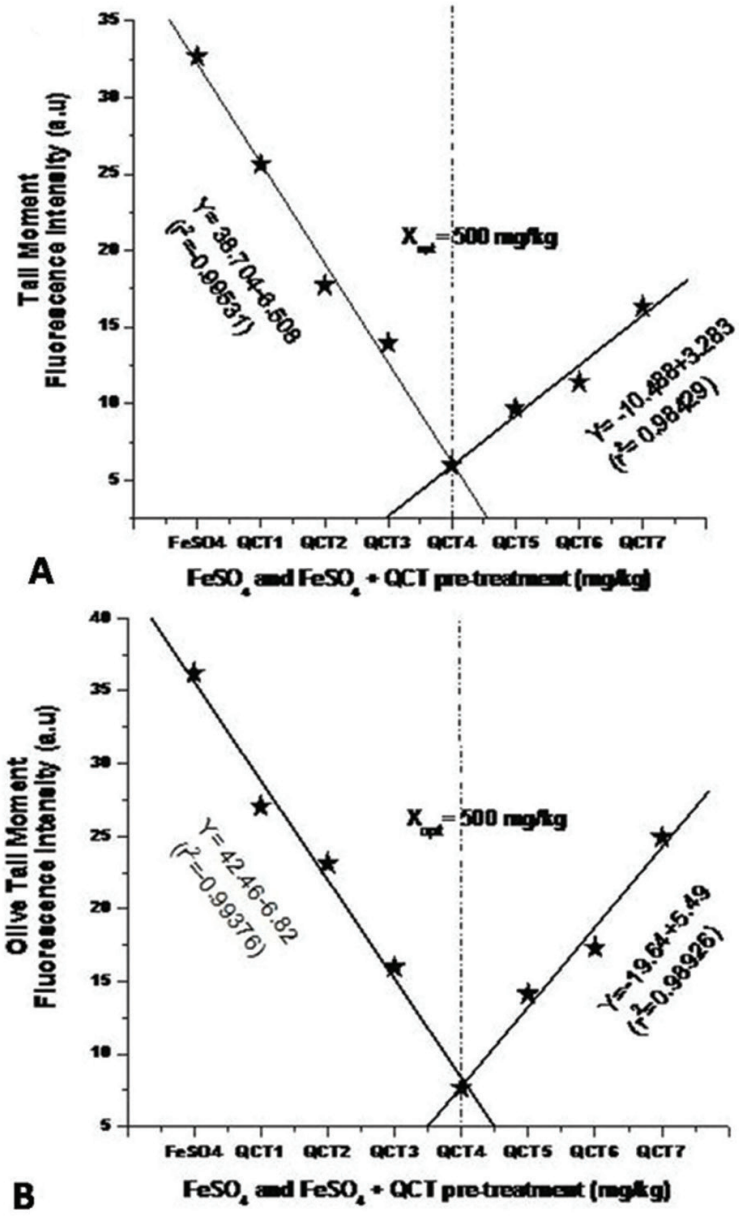

Figure 7. A. Broken-line relationship of pre-treatment of quercetin levels with FeSO4 to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: Tail moment. B. Broken-line relationship of pre-treatment of quercetin levels with FeSO4 to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

Y=-12.663+3.877, X ≥ 500 mg/kg
(r^2=0.98515) X_{opt} = 500 mg/kg QCT (Post treatment with QCT)

4.4.3.2. Olive tail moment

Y=42.46-6.82, X ≤ 500 mg/kg (r^2= 0.99376); Y=-19.64+5.49, X ≥ 500 mg/kg
(r^2=0.98926) X_{opt} = 500 mg/kg QCT (Pre-treatment with QCT)
Y=45.887-7.599, X ≤ 500 mg/kg (r^2=-0.99612); Y=-4.681+2.454, X ≥ 500 mg/kg
(r^2=0.9798) X_{opt} = 500 mg/kg QCT (Simultaneous treatment with QCT)
Y=44.918-7.212, X ≤ 500 mg/kg (r^2=-0.99599); Y=-15.38+4.87, X ≥ 500 mg/kg
(r^2=0.9967) X_{opt} = 500 mg/kg QCT (Post treatment with QCT)

The equations for QCT doses to CAs, MNPCEs and DNA damage induced by $FeSO_4$ employed to

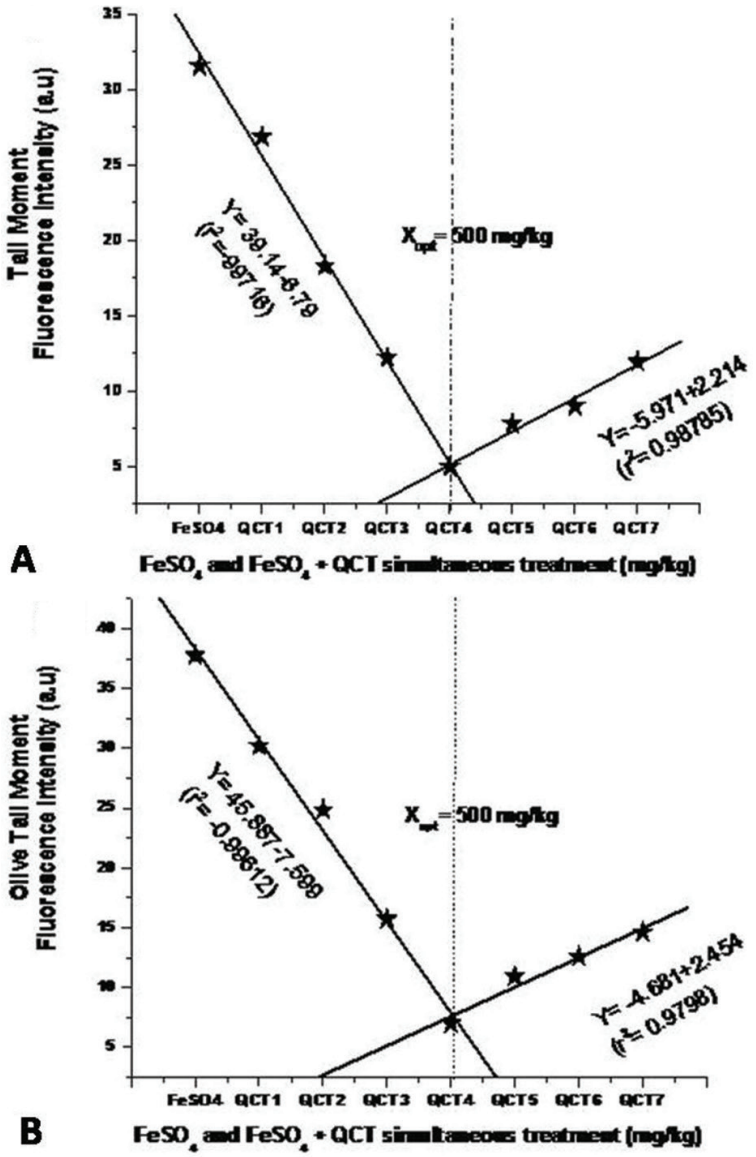

Figure 8. A. Broken-line relationship of simultaneous treatment of quercetin levels with FeSO4 to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment. B. Broken-line relationship of simultaneous treatment of quercetin levels with FeSO4 to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

calculate the optimum level of QCT, at which it showed maximum protective effect is given in the respective Figures (Figure 1-3, 5A, B, 7A, B, 8A, B and 9A, B).

5. DISCUSSION

A large body of the literature supports the notion that antioxidants play a key role in preserving health. These antioxidants are able to scavenge free radicals and help to reduce the extent of oxidative stress-induced DNA damages. Excess of iron is believed to cause oxidative stress and is understood as an increase in the steady state concentration of reactive oxygen and nitrogen species (24-28). The toxicity of superoxide anion ($O_2^{\cdot}-$) and hydrogen peroxide (H_2O_2) arises from their iron-dependent conversion into the extremely reactive hydroxyl radical ($OH^{\cdot}$) (Haber– Weiss reaction) leading to tissue damage, mutation and carcinogenesis (29). Okada (29) summarizes several studies reporting on the relationship between iron and cancer in humans and experimental animals. To mitigate such oxidative DNA damage, flavonoids have been identified as satisfying most of the criteria to be considered as antioxidants: the flavonoids inhibit the enzymes responsible for $O_2^{\cdot}-$ production (30,31); the low redox potentials of flavonoids thermodynamically allow them to reduce highly oxidising

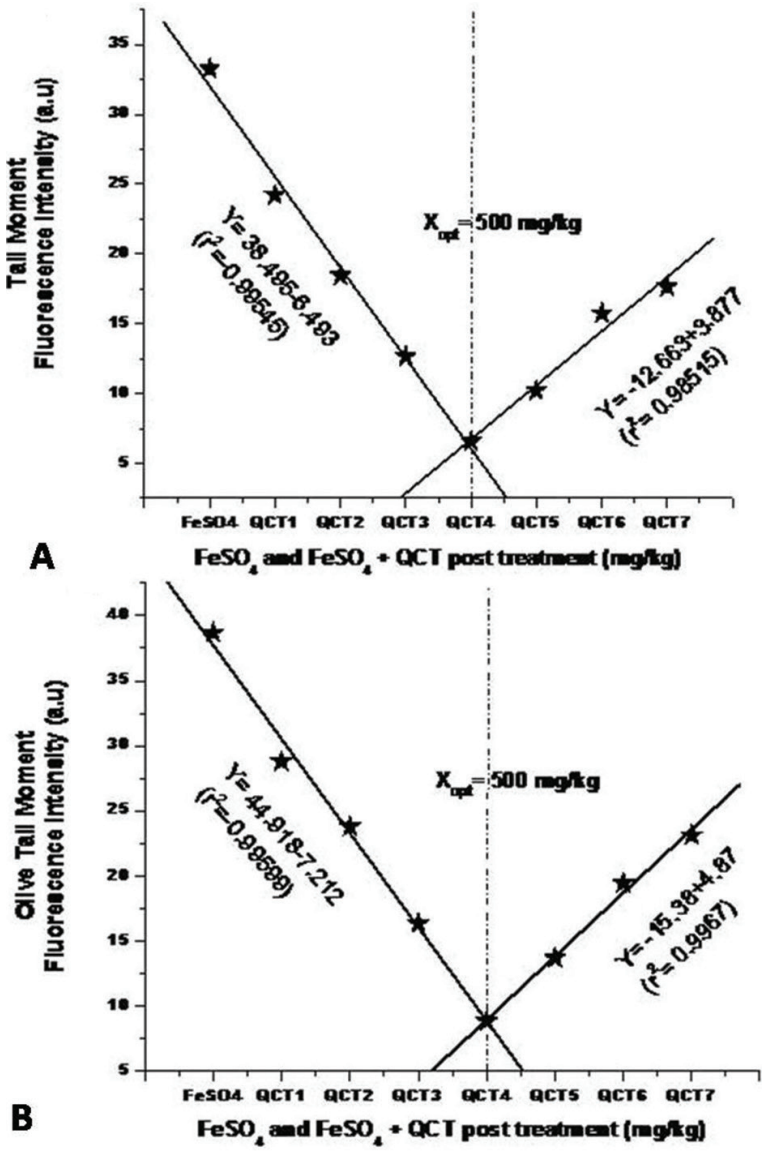

Figure 9. A. Broken-line relationship of post treatment of quercetin levels with FeSO4 to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: Tail moment. B. Broken-line relationship of post treatment of quercetin levels with FeSO4 to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: Olive Tail moment.

free radicals such as $O_2^{\cdot -}$, $RO^{\cdot}$ and $HO^{\cdot -}$ (32); and a number of flavonoids chelate trace metals (33). Besides scavenging, flavonoids may stabilize free radicals by forming complexes with them (34). *In vitro* and *in vivo* studies have reiterated that flavonoids possess anti-inflammatory, antioxidant, antiallergenic, hepato-protective, antithrombotic, antiviral and anticarcinogenic activities (35). We, thus, set out to observe the optimum level of QCT, at which it exhibits maximum level of protection against free radical-induced damages due to $FeSO_4$ toxicity in rat bone marrow cells by using chromosomal aberrations, micronucleus assay and whole blood cells of Wistar rats using single cell gel electrophoresis. The precise mechanism of the protective action of QCT on iron-mediated cytotoxicity is due to the scavenging of superoxide anions that produce highly toxic hydroxyl radicals via the Haber-Weiss reaction. This is based on the fact that like other polyphenolics, the biological effects of QCT are generally attributed to their antioxidant activities in scavenging ROS and chelating iron (36,37). Chromosomal aberrations, yields of MNPCE and DNA damage induced by $FeSO_4$ (200 mg Fe/kg) decreased markedly in the pre-treatment, simultaneous and post-treatment with QCT, except the three highest doses (625, 750 and 875 mg/kg doses of QCT). The multiple protective activities of QCT arise from its strong

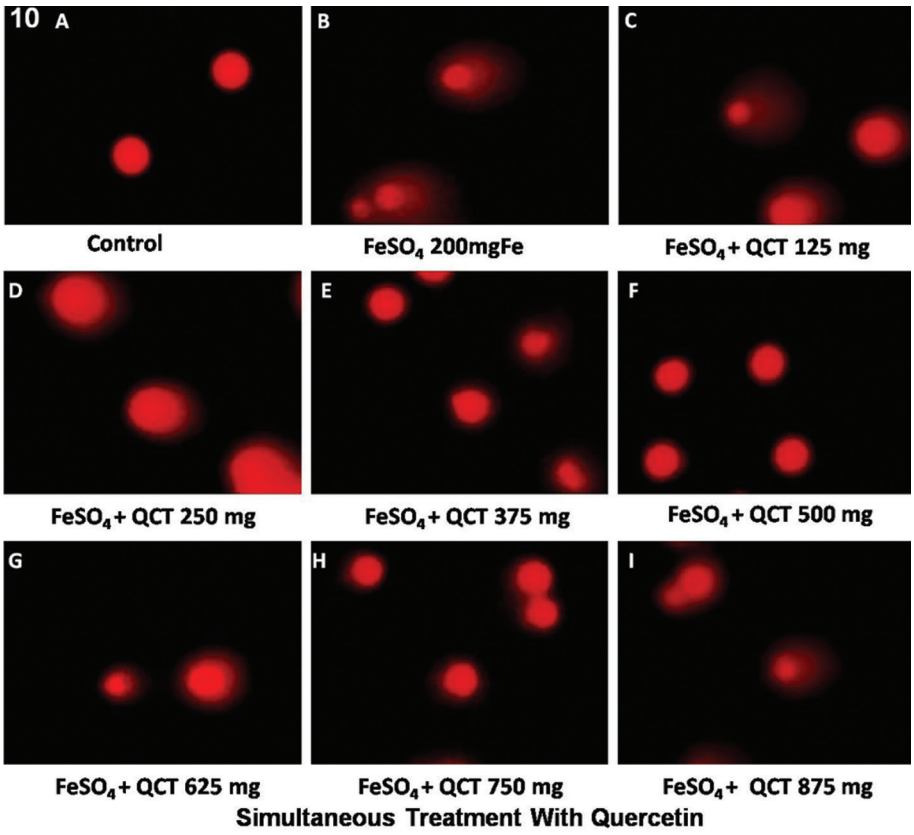

Figure 10. SCGE (Comet) photographs illustrating the effects of iron sulfate and quercetin (QCT) simultaneous treatment on the extent of DNA damage. [A] control (DMSO); [B] treated with FeSO4 at the dose of 200 mgFe/kg; [C, D, E, F, G, H, I] treated with QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg along with FeSO4 at 200 mg Fe/kg respectively.

antioxidant properties (38-41). It has also been recently observed that QCT may behave as a cytotoxic agent and as a mutagen at much higher doses (42). Thus, the ineffectiveness of QCT at higher doses is attributed to its pro-oxidant activities (43). In the present study we also observed these apparently contradictory effects of QCT that it can act as both antioxidant and pro-oxidant, depending on concentration and the source of the free radicals (44,45). Among pre-, simultaneous and post treatments, the protective effect of QCT was most prominent in the simultaneous treatment. The results suggest that QCT decreases the number of chromosomal aberrations, yields of MNPCE and DNA damage induced by $FeSO_4$, but it could not completely protect cells from the damage. The most efficient anticlastogenic effect of QCT was observed at 500 mg/kg dose, at which it showed the best protective effect against genotoxicity and clastogenicity induced by $FeSO_4$. An effectively significant protection by QCT against the formation of oxidative DNA damage generated by $FeSO_4$ was observed in the present study. Protection against H_2O_2 was also attained for myricetin, QCT and rutin in Caco-2 and HepG2 cells (46) and for $FeSO_4$ and luteolin in murine and human leukaemia cell lines (47-48). With regard to the other genotoxicants, QCT and rutin showed antigenotoxic effects on DNA damage induced by mitomycin C in a concentration-dependent manner (49).

The antigenotoxic and protective role of QCT has been reasonably confirmed in the present study. One possible mechanism involved in the protection against iron-induced cytotoxicity and genotoxicity during simultaneous treatment with QCT may be the interception of free radicals before they induce any damage. It was also observed in the present study that QCT acts as a strong inhibitor of iron-dependent $OH^{\cdot}$ generation and help protection against $OH^{\cdot}$ induced DNA damage in bone marrow and blood cells of Wistar rats.

6. CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

7. ACKNOWLEDGEMENTS

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Abbreviation: $FeSO_4$- iron sulfate; QCT- quercetin; ROS- reactive oxygen species; CAs- chromosomal aberrations; MNPCEs- micronucleated polychromatic erythrocytes; MN- micronuclei, h- hour; KCl- potassium chloride; PI- propidium iodide; EDTA- ethylene diamine tetraacetic acid; DMSO- dimethyl sulfoxide; LMP- low melting point agarose; b.w.- body weight; ip- intraperitoneal; po- oral.

Key Words: Iron, Quercetin, Chromosomal Aberration, Micronucleus Test, Comet Assay, DNA Damage

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