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Evaluation of radioprotective activities of *Rhodiola imbricata* Edgew – A high altitude plant

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Abstract

The present study reports the radioprotective properties of a hydro-alcoholic rhizome extract of *Rhodiola imbricata* (code named REC-7004), a plant native to the high-altitude Himalayas. The radioprotective effect, along with its relevant superoxide ion scavenging, metal chelation, antioxidant, anti-lipid peroxidation and anti-hemolytic activities was evaluated under both *in vitro* and *in vivo* conditions. Chemical analysis showed the presence of high content of polyphenolics (0.971 ± 0.01 mg% of quercetin). Absorption spectra analysis revealed constituents that absorb in the range of 220–290 nm, while high-performance liquid chromatography (HPLC) analysis confirmed the presence of four major peaks with retention times of 4.780, 5.767, 6.397 and 7.577 min. REC-7004 was found to lower lipid oxidation significantly ($p < 0.05$) at concentrations viz., 8 and 80 μg/ml respectively as compared to reduced glutathione, although the optimally protective dose was 80 μg/ml, which showed 59.5% inhibition of induction of linoleic acid degradation within first 24 h. The metal chelation activity of REC-7004 was found to increase concomitantly from 1 to 50 μg/ml. REC-7004 (10–50 μg/ml) exhibited significant metal chelation activity ($p < 0.05$), as compared to control, and maximum percentage inhibition (30%) of formation of iron-2,2′-bi-pyridyl complex was observed at 50 μg/ml, which correlated well with quercetin (34.9%), taken as standard. The reducing power of REC-7004 increased in a dose-dependent manner. The absorption unit value of REC-7004 was significantly lower (0.0183 ± 0.0033) as compared to butylated hydroxy toluene, a standard antioxidant (0.230 ± 0.091), confirming its high reducing ability. Superoxide ion scavenging ability of REC-7004 exhibited a dose-dependent increase (1–100 μg/ml) and was significantly higher ($p < 0.05$) than that of quercetin at lower concentrations (1–10 μg/ml), while at 100 μg/ml, both quercetin and REC-7004 scavenged over 90% superoxide anions. MTT assay in U87 cell line revealed an increase in percent survival of cells at doses between 25 and 125 μg/ml in case of drug + radiation group. *In vivo* evaluation of radio-protective efficacy in mice revealed that intraperitoneal administration of REC-7004 (maximally effective dose: 400 mg/kg b.w.) 30 min prior to lethal (10 Gy) total-body γ-irradiation rendered 83.3% survival. The ability of REC-7004 to inhibit lipid peroxidation induced by iron/ascorbate, radiation (250 Gy) and their combination [i.e., iron/ascorbate and radiation (250 Gy)], was also investigated and was found to decrease in a dose-dependent manner (0.05–2 mg/ml). The maximum percent inhibition of formation of MDA-TBA complex at 2 mg/ml in case of iron/ascorbate, radiation (250 Gy) and both i.e., iron/ascorbate with radiation (250 Gy) was 53.78, 63.07, and 51.76% respectively and were found to be comparable to that of quercetin. REC-7004 (1 μg/ml) also exhibited significant anti-hemolytic capacity by preventing radiation-induced membrane degeneration of human erythrocytes. In conclusion, *Rhodiola* renders *in vitro* and *in vivo* radioprotection *via* multifarious mechanisms that act in a synergistic manner. (Mol Cell Biochem **273**: 209–223, 2005)

Key words: anti-lipid peroxidation activity, linoleic acid, radiation, survival assay, *Rhodiola imbricata*

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Introduction

Development of non-toxic, effective radioprotectors for human applications has been an area of immense interest for radiation biologists. The synthetic radioprotectors developed so far leave much to be desired in view of their associated toxicity at effective doses [1]. The last decade has seen a transformational change in favour of natural products as a reliable alternative to molecular radioprotectors [2, 3].

High-altitude Indian medicinal plants like *Podophyllum hexandrum* and *Hippophae rhamnoides* which grow in extremely difficult environments (very low temperature, low oxygen tension, high UV flux etc.) have been reported to provide significant protection against ionising radiation in experimental animals [4–6].

Rhodiola, a high-altitude plant, is extensively being used in traditional folk medicine in China, Tibet, Serbia, Ukraine, Mongolia, Russia and Scandinavian countries, to increase physical endurance, work productivity, longevity and to treat fatigue, asthma, haemorrhage, depression, anaemia, impotence, gastrointenstinal ailments, infections, and nervous system disorders [7, 8]. The plant has been reported to possess antimutagenic [9], anticancer [10], antidepressant [11], tonifying [8], adaptogenic [12–14], antiageing, cardioprotective and central nervous system enhancing properties [15–17] DNA repair enhancing [18], anti-inflammatory [13, 19, 20], stimulative [20, 21], active oxygen scavenging [22], and antioxidant [23] properties, many of which are apparently considered essential for achieving radiation protection. The therapeutic activities of *Rhodiola sps*. have also been documented in asthenic conditions like sleep disturbances, decline in work performance, loss of appetite, irritability and hypertension that develops subsequent to intense physical or intellectual strain [24].

Recently *Rhodiola* has been used for treating conditions like high altitude sickness, hemorrhoids pain, scurvy, obesity, amenorrhoea, depression, schizophrenia, fatigue, headache, hypertension, insomnia, sexual dysfunction and even cancer [7]. The adaptogenic, cardiopulmonary protective and central nervous system enhancing activities of different species of *Rhodiola* have been attributed primarily to its ability to influence levels and activities of monoamines and opioid peptides like beta-endorphins [7, 25].

The chemical composition and pharmacological properties of *Rhodiola* species are to a great extent species-dependent, although some overlap in constituents and physiological properties has been reported [7]. The roots and rhizomes of *Rhodiola* species contain a plethora of bioactive compounds, including organic acids, flavonoids, tannins and phenolic glycosides. Roots of *Rhodiola* have been reported to contain six distinct groups of chemical compounds viz., phenylpropanoids [rosavin, rosin, rosarin (specific to *R. rosea*)]; flavonoids [rhodiolin, rhodionin, tricin, acetylrodalgin, catechins and proanthocyanins]; phenylethanol derivatives [rhodioloside (salidioside); tyrosol]; monoterpenes (rosaridin, rosaridol); triterpenes (beta-cytosterol, daucosterol) and phenolic acids (chlorogenic hydroxycinnamic, caffeic and gallic acid) [7]. The chemical constituents of different species of *Rhodiola* have been characterised using HPLC by several workers [26, 27], while several bioactive glycosides such as rhodiocyanosides [28], sacranosides [29] and phenolic components [23, 30] have been isolated from *Rhodiola sachalinensis*.

Rhodiola imbricata Edgew. (Crassulaceae), commonly known as rose root, is found in the high altitude regions (>4000 m) of Leh and Ladakh Jammu and Kashmir, India [31]. The present study reports the promising radioprotective potential of *Rhodiola imbricata*, along with its relevant superoxide ion scavenging, metal chelation, antioxidant, antihemolytic and anti-lipid peroxidation activities.

Materials and methods

Chemicals

Ferric chloride, sodium sulphite, ferrous sulphate, 2-2′-bi-pyridyl, potassium di-hydrogenorthophosphate, di-potassium hydrogen orthophosphate, potassium ferricyanide, trichloroacetic acid, di-sodium ethylene diamine tetra acetic acid, ascorbate, thiobarbituric acid (TBA), were purchased from Sigma Chemicals (St. Louis, MO, USA), while dimethylsulfoxide, hydrochloric acid and ethanol were obtained from BDH Chemical Co. (Toronto, Ontario, Canada) and James Burrough (F.A.D.) Ltd., UK respectively. The rest of the chemicals utilised for this study were of analytical reagent (AR) grade and were obtained from reputed local suppliers in India. Plastic-ware (micro-centrifuge tubes, pipette tips and 35 mm petridishes etc.) were obtained from Tarsons (Kolkata, India).

Preparation of herbal extract of Rhodiola imbricata

The fresh rhizome of *Rhodiola imbricata* was collected from Leh region (Jammu and Kashmir, India) at a height of over 4000 m during the months of August–October. The plant material was identified by Dr. O.P. Chaurasia, an Ethnobotanist at the Field Research Laboratory, Leh. A voucher specimen of the same is available at FRL, Leh. The plant material was frozen and kept at −80 °C, till the extraction was performed. A known quantity of the plant material was extracted with a mixture of ethyl alcohol and triple distilled demineralised water (70:30, v/v, three changes) at a temperature below 60 °C. The extract obtained was filtered initially through ordinary

filter paper, followed by filtration through Whatman 3 M paper and finally through 0.22 μm filter (Millipore, USA). The filtrate was concentrated in a rotary evaporator (R-134; Buchi, Switzerland) at 55–60 °C and finally freeze-dried in a Lyophilizer (Flexi-Dry MP, FTS Systems, USA). The extract (code named as REC-7004) was stored at a temperature of 4 °C till it was used. The yield on w/w basis was 0.4%. The extract was reconstituted by dilution with water immediately before use.

Determination of total phenolic content

The phenolic content was estimated in the alcoholic extract of *Rhodiola imbricata* (REC-7004) using Folin's ciocalteu reagent (FCR) based assay [32]. To an aliquot (10 μl), taken from a stock solution (1 mg/ml) of the extract, 10 ml of water and 1.5 ml of FCR were added. The mixture was kept for 5 min at room temperature, and then 4 ml of 20% sodium carbonate solution was added and the volume made up to 25 ml with double-distilled water. The mixture was kept for 30 min and absorbance of the colour developed was recorded at 765 nm using UV visible spectrophotometer (Electronics Corporation of India Ltd., Hyderabad, India).

UV-vis spectroscopy and HPLC analysis

The UV-vis spectrum was measured with an ECIL spectrophotometer using a 50% methanol:water solution as solvent. HPLC analysis was performed with a Hypersil C18 H reverse phase column (column size: $250 \times 4.6\,\mathrm{mm}^2$) using a Spectra Systems AS3000 HPLC machine, comprising of an auto-injector (Thermo-Finnigan, Finland) and a UV detector (Spectra System UV1000; Thermo-Finnigan, Finland). A stock solution of REC-7004 (1 mg/ml) was prepared, and 5 μl of this sample was injected. Elution was carried out for 30 min at a flow rate of 0.6 ml/min using the mobile phase [MeOH:H_2O; 50:50]. Detection of the peaks was done at a wavelength of 290 nm.

Screening for biological activity

Total antioxidant activity

The total antioxidant activity was evaluated using time-dependent study of linoleic acid degradation (Ferri-thiocyanate method) along with protection potential of the drug against the peroxyl radicals generated in the system containing linoleic acid emulsion. Appropriate quantity of drug (micro liters) and reduced glutathione in a triplicate set in screwed cap test tubes (with capacity of 25 ml each) were taken. In the above tubes, 4 ml of absolute ethanol, 4.1 ml of 2.52% of linoleic acid in absolute ethanol, and 8 ml of (0.05 M) potassium phosphate buffer pH 7.0 were added and the volume was made up to 20 ml by adding appropriate amount of double-distilled water. The stock solution was placed in an incubator at 40 °C in dark conditions along with a set for control with no drug (in triplicate).

Evaluation of lipid oxidation using ferri-thiocyanate method

At time intervals of 24, 48, 72, 96, 120 h (till the absorbance becomes constant), an aliquot of 0.1 ml of stock solution was taken and to it 9.7 ml of 75% ethanol and 0.1 ml each of 30% ammonium thiocyanate and 0.02 M ferrous chloride (in 3.5% hydrochloric acid) was added. The absorbance of the reaction mixture was measured at 500 nm, 3 min after the addition of ferrous chloride [33]. The procedure was carried out for every time interval until the absorbance of control reached the maximum.

Evaluation of thiobarbituric acid (TBA) values

The TBA values were also determined in continuation with FTC method after every 24 h of incubation. An aliquot of 1 ml of stock solution was taken and to it 2 ml of 20% trichloroacetic acid and 2 ml each of 0.8% TBA aqueous solution was added. The assay mixture was incubated for 24 h at 40 °C. After 24 h, the assay mixture was placed in hot boiling water bath for 10 min followed by cooling and centrifugation at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm [34].

Metal chelation activity

Metal chelation assay was performed using bi-pyridyl method [35]. Varied concentrations of REC-7004 (2 ml) were mixed with 4 ml of ferric chloride solution (5 μg/ml; 0.005 N HCl) and then incubated at room temperature for 10 min. Aliquots (2 ml) were taken from each sample and mixed with sodium sulphite (final concentration: 0.05 M) and 2,2-bi-pyridyl (0.2%). The solution was then re-incubated in hot water bath (Yorko, India) at 55 °C for 5 min. The tubes were cooled to room temperature and the absorbance recorded at 520 nm. The percentage inhibition of formation of metal 2,2′-bi-pyridyl complex (chromogen) was evaluated as:

$$\frac{\mathrm{O.D.}_{\mathrm{control}} - \mathrm{O.D.}_{\mathrm{sample}}}{\mathrm{O.D.}_{\mathrm{control}}} \times 100.$$

Reducing power

The reducing power of REC-7004 was evaluated by using potassium ferricyanide assay [36]. The different concentrations of REC-7004 (50 μl) were mixed with

200 μl each of 0.2 M phosphate buffer (pH 6.5) and 0.1% potassium ferricyanide and incubated at 50 °C for 20 min. About 250 μl of 10% of trichloroacetic acid was added to the above mixture and centrifuged at 3000 $\times$ g for 10 min at room temperature in a Sorvall table-top centrifuge [Sorvall (Kendro) Instruments, USA]. The resulting supernatant was then taken and mixed with 500 μl of double-distilled demineralised water and 100 μl of 0.1% ferric chloride, and further incubated at 37 °C for 10 min. The absorbance was recorded at 700 nm using a spectrophotometer (Electronics Corporation of India Ltd, Hyderabad, India).

Free radical scavenging activity

The free radical (superoxide-ion) quenching ability of REC-7004 was determined using nitrobluetetrazolium as a marker substrate [37]. Varied concentrations of REC-7004 were mixed with 1 ml of sodium pyrophosphate buffer (0.052 M, pH 8.3) and 0.1 ml of phenazine methnosulfate (186 μM). 300 μl of nitrobluetetrazolium (300 μM) was added to the above solution and final volume was adjusted to 3 ml. Thereafter, the reaction was initiated by adding 200 μl of NADH (780 μM) followed by incubation of reaction mixture at 37 °C for 90 s. The reaction was terminated by adding 1 ml of glacial acetic acid, and the resultant mixture was shaken with 4 ml of *n*-butanol and further allowed to stand for 10 min at room temperature. The *n*-butanol layer was separated by centrifugation and the colour intensity of chromogen was measured at 560 nm against *n*-butanol. The percentage inhibition of formation of formazan crystals was evaluated as:

$$\frac{\text{O.D.}_{\text{control}} - \text{O.D.}_{\text{sample}}}{\text{O.D.}_{\text{control}}} \times 100.$$

Irradiation

Lower doses of radiation (2 Gy to U87 cells), and whole-body lethal gamma radiation (10 Gy) to mice were delivered from a ^{60}Co gamma chamber (Model-220, Atomic Energy of Canada Ltd.), at a dose rate ranging between 0.64 and 0.59 Gy/min. Higher radiation doses (250 Gy) were delivered to mice liver homogenate from a ^{60}Co gamma chamber (Gamma Cell 5000, Board of Radiation and Isotope Technology, Mumbai) at a dose rate of 3.54 kGy/h. Dosimetry was carried out using Baldwin Farmer's secondary dosimeter and Fricke's Chemical Dosimetry method.

In vitro evaluation of radioprotective efficacy

Cell culture

Human malignant glioma cells (U87) were grown in T-25 culture flasks (Nunc) in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS) supplemented with 1000 units/ml penicillin and 100 μg/ml streptomycin sulphate, in a humidified atmosphere in 5% CO_2 at 37 °C. The cells were sub-cultured at a 1:5 ratio once a week. For experimentation, the exponentially growing cells (1×10^3) were treated with desired concentrations of REC-7004 for different time periods, and/or radiation (2 Gy) under normal culture conditions.

MTT reduction assay

MTT reduction assay [38] was conducted to determine cell survival. In all, 1×10^3 cells were seeded onto 96-well microtitre plates after the corresponding treatments. A stock solution of MTT in PBS (5 mg/ml) was prepared and filtered through 0.22 μm membrane filters (Millipore, USA) and 15 μl of this solution was added to each well of microtitre plate and kept at 37 °C for 3 h to incubate the cells. After incubation, the microtitre plate was centrifuged at 2000 rpm for 10 min at room temperature and the supernatant from each well was carefully removed and cell viability was assessed by assaying for the ability of functional mitochondria to catalyze the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a dark blue formazan salt by mitochondrial dehydogenases. The formazan crystals were dissolved by adding 200 μl of DMSO/well of microtitre plate and absorbance was measured at 570 nm (reference wavelength: 630 nm). The absorbance of formazan formed in the untreated cells was considered as 100%, while the absorbance of treated cells was evaluated as % of survival with respect to untreated cells (used as control).

In vivo evaluation of radioprotective efficacy

Animals

Adult (6–8 weeks old) Swiss Albino Strain 'A' male mice (25 $\pm$ 2 g), that were in-bred in the animal house of the Institute of Nuclear Medicine and Allied Sciences, Delhi (India), were used for experimentation. The animals were maintained under controlled temperature (25 $\pm$ 2 °C; 12 h alternating dark and light cycle) and humidity (60.05–62% RH) in polypropylene cages. Standard food pellets (M/s Amrut Feeds Pvt. Ltd., Kolkata, India) and drinking water were provided *ad libitum* to the animals. Permission for use of animals was taken from the Institutional Animals Ethics Committee (IAEC), and all experiments were carried out strictly in accordance with the guidelines laid down by the

Indian National Science Academy (INSA) for the care and use of laboratory animals for research purposes.

Toxicity determination and evaluation of radioprotective potential in mice

Different doses of REC-7004 were administered to mice *via* intraperitoneal (i.p.) route and the non-toxic range was determined in a study ranging over a period of 2–3 days, recorded in terms of gross changes in behaviour, changes in neuromuscular co-ordination and respiratory distress. The radioprotective efficacy against 10 Gy gamma-radiation was determined using the standard 30-day total–body survival test.

Anti-lipid peroxidative activity in mice liver homogenate

Mice were randomly selected and sacrificed by cervical dislocation, dissected and the abdominal cavity was perfused with 0.9% saline. Whole liver was taken out and visible blood clots were carefully and maximally removed. A 10% homogenate was prepared in cold buffered saline (pH 7.4) using Potter Elvejam homogenizer and filtered to get a clear homogenate. A 10% liver homogenate (2 ml) was taken in a series of 35-mm petridishes to which desired amount of extract was added, and mixed gently to form a homogenous solution. Lipid peroxidation was initiated by adding 20 μl of ferric chloride (0.5 mM) with 200 μl of ascorbate (1 mM), and also by exposure to radiation (250 Gy) and both [i.e., iron/ascorbate and radiation (250 Gy)] followed by incubation of petridishes at 37 °C for 30 min. The homogenate (1 ml) was pipetted out for estimating lipid peroxidation levels in terms of thiobarbituric acid reactive substances [34].

Nanomoles of malonyldialdehyde (MDA)-TBA complex formed/h/g of tissue = O.D. (535 nm) × dilution factor/Molar extinction coefficient (1.56×10^{-6}).

Radio-modulatory activity on human erythrocytes

Preparation of erythrocyte suspensions

Washed erythrocyte suspensions were prepared as per the procedure adopted by Dacie and Lewis [39]. Whole-blood from normal adult male volunteers (taken after informed consent) was drawn using EDTA as an anticoagulant and centrifuged to remove the buffy coat layer. The cells were suspended in 0.14 M NaCl solution and then the packed cells were washed three times with the same solution.

Modification of radiation-induced haemolysis by REC-7004

Washed erythrocyte suspensions were pre-incubated with 2 mM sodium azide for 1 h at 37 °C in a shaking water-bath to inhibit any activity of the enzyme catalase. Equal volumes of erythrocyte suspension in PBS were taken and different concentrations (25–125 μg/ml) of REC-7004 were added 30 min prior to irradiation (250 Gy), and thereafter further incubated for 1 h at 37 °C. Untreated control samples contained erythrocyte suspension in PBS (final volume: 1 ml), while in the drug control samples different concentrations of REC-7004 were added to erythrocyte suspension. Both these control samples were not subjected to radiation, while radiation control samples were subjected to radiation (250 Gy). Anti-hemolytic activity of REC-7004 was evaluated in terms of membrane degeneration or lipid peroxidation activity [34].

Statistical analysis

Each experiment was performed in triplicate and repeated three times. All results are expressed as mean ± S.D. or as percentage. Statistical analysis of data was performed by using Student's *t*-test and $p < 0.05$ was considered significant. Data generated from lipid oxidation studies was subjected to Tukey's HSD test.

Results

Total polyphenolic content and HPLC fingerprinting

Evaluation of total polyphenolic content revealed that the extract is quite rich in polyphenols (0.971 ± 0.01 mg% of quercetin). Absorption spectra analysis revealed that REC-7004 contains constituents that absorb in the range of 220–290 nm. HPLC fingerprint analysis of the hydro-alcoholic extract (70% ethanol) showed several peaks, among which, four main peaks at retention times of 4.780, 5.767, 6.397 and 7.577 min exhibiting 13.51, 54.18, 16.11 and 10.36% coverage area respectively were prominent.

Antioxidant activity

The absorbance at 500 nm, evaluated using FTC method indicated peroxide levels at initial stage of experimentation, which decreased as antioxidant activity increased, and was studied over a period of 120 h in a metal free linoleic acid emulsion. Different concentrations (8, 80, 200, and 400 μg/ml) of REC-7004 showed different effects (evaluated using Tukey's HSD test and compared with control samples and reduced glutathione levels). It was observed that REC-7004 (8 and 80 μg/ml) showed significant lowering in the lipid oxidation maximally in first 24 h. Although higher concentration tends to induce peroxyl levels at initial stages, it showed significant antioxidant activity ($p < 0.05$) in last 48 h of experimentation. Figure 1 indicated the immense antioxidant potential of REC-7004 to lower lipid oxidation as compared to reduced glutathione. REC-7004 (80 μg/ml) showed significant lowering of TBA values ($p < 0.05$) over a period of 24 h (Fig. 2).

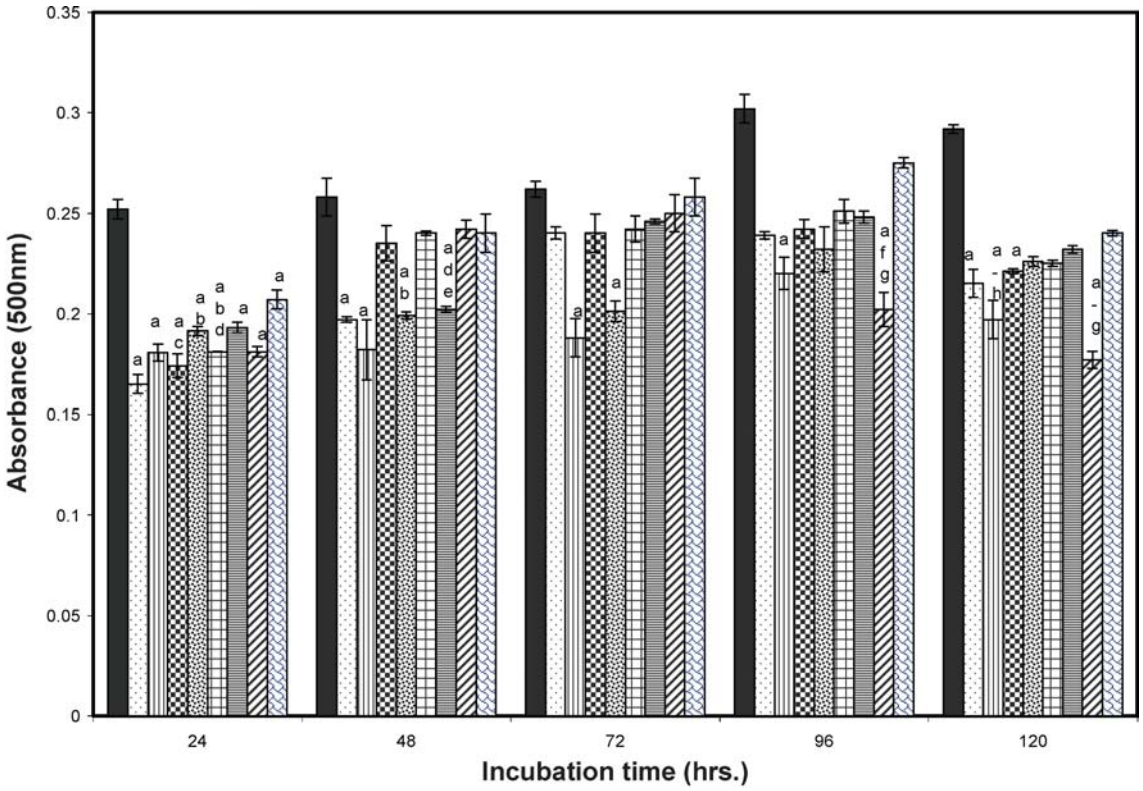

Fig. 1. Effect of REC-7004 on lipid oxidation occurring in a linoleic acid assay system. Values represent mean ± S.D. of three separate experiments. Peroxyl levels were estimated by ferrithiocyanate method, as described under Materials and methods. Letters a–h represent Tukey's HSD test results and $p < 0.05$ was considered to be significant. ■ Control with no drug, ▯ 8 μg/ml GSH, ▥ 8 μg/ml REC-7004, ▩ 80 μg/ml GSH ▩ 80 μg/ml REC-7004 ▦ 200 μg/ml GSH ▤ 200 μg/ml REC-7004 ▨ 400 μg/ml GSH ▨ 400 μg/ml REC-7004.

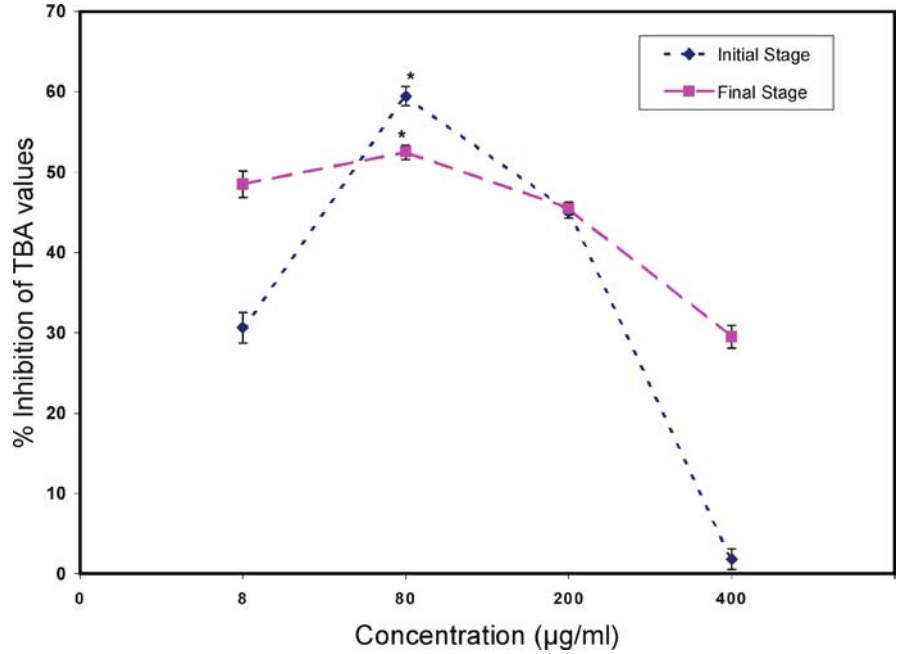

Fig. 2. Effect of different concentrations of REC-7004 on TBA values (at initial and final stage of experiment.) % inhibition of TBA values represents increased antioxidant activity. Maximal antioxidant activity ($^{*}p < 0.05$) with least possible induction of linoleic acid degradation was observed at 80 μg/ml (59.459%; 52.5%).

Metal chelation activity

The metal chelation activity of REC-7004 was found to increase concomitantly with increasing concentration (1–50 μg/ml). Although REC-7004, in the range of 10–50 μg/ml, exhibited significant metal chelation activity ($p < 0.05$) as compared to control (0% inhibition), maximum percentage inhibition of formation of iron-2,2′-bi-pyridyl complex was observed at 50 μg/ml (30%) which was very close to that observed with quercetin (34.9%), a standard bioflavonoid with metal chelation properties (Fig. 3).

Reducing power

The reducing power of REC-7004 was found to increase in a dose-dependent manner. The absorption unit values (concentration to attain the unit absorbance) were calculated using the graphical values following the linear response curve maximally. Lower absorption unit value indicates the higher reducting power of drug. The absorption unit value of REC-7004 was significantly ($p < 0.05$) low (0.0183 ± 0.0033), as compared to that of butylated hydroxy toluene (BHT), a standard synthetic antioxidant (0.230 ± 0.091; Fig. 4), indicating that REC-7004 has very high reducing power.

Free radical scavenging activity

Free radical scavenging ability of REC-7004 exhibited a dose-dependent increase (1–100 μg/ml) and was significantly higher ($p < 0.05$) than that of quercetin (standard) at lower concentrations (1–10 μg/ml), while at highest tested dose (100 μg/ml), quercetin and REC-7004, both exhibited more than 90% inhibition of formation of formazan crystals (Fig. 5).

MTT reduction assay

REC-7004 showed an increase in % survival of cells in a range of 25–125 μg/ml of dose treatments in case of drug + radiation group. Maximum survival was found to be at 125 μg/ml, i.e. 87.89%. However, on the other hand, drug only group showed a negative shift in % survival at higher dosed. Thus, the most optimal dose, which provided maximal % survival in both groups, i.e. drug only and drug + radiation was 25 μg/ml (91.145 and 82.59% respectively) which was found to be significant at $p < 0.05$ (Fig. 6).

Evaluation of toxicity and radioprotective potential

REC-7004, upto 1400 mg/kg b.w., was tolerated well by mice without any adverse manifestations, except for the mice

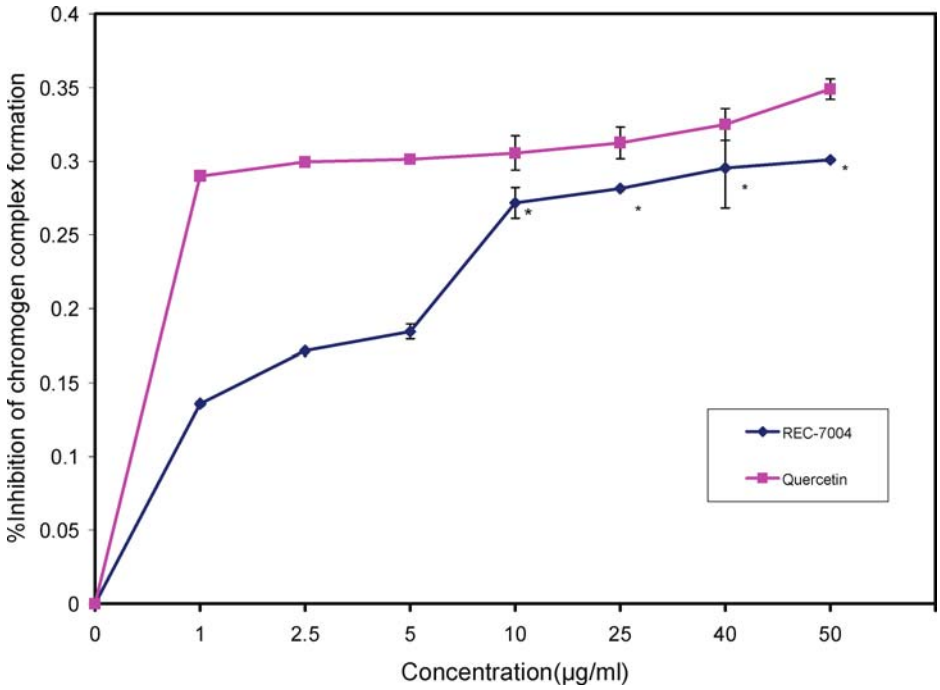

Fig. 3. Effect of different concentrations of REC-7004 (1–50 μg/ml) on chelation of iron. The percentage inhibition of iron-2,2′-bi-pyridyl (chromogen) complex containing all reagents but without REC was considered as 0% inhibition (control). REC-7004 (10, 25, 40, 50 μg/ml) exhibited significant % inhibition of chromogen complex formation with respect to control ($^*p < 0.05$).

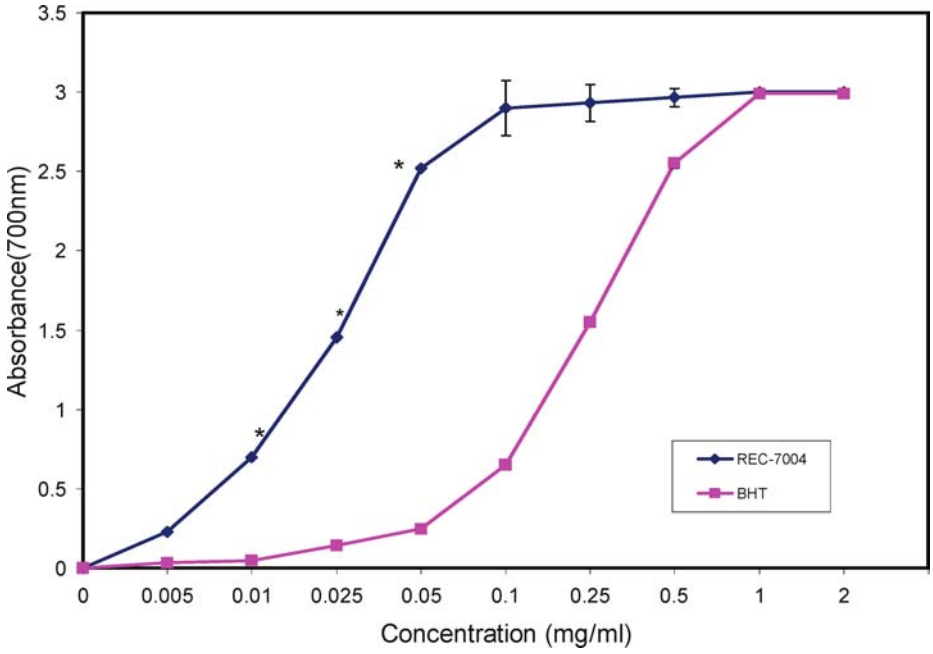

Fig. 4. Evaluation of reducing power of REC-7004. The absorbance at 700 nm was recorded in triplicate and each experiment was repeated thrice. The values are expressed as mean ± S.D. BHT, a standard synthetic antioxidant was used as control. REC-7004 with respect to control ($^{*}p < 0.01$).

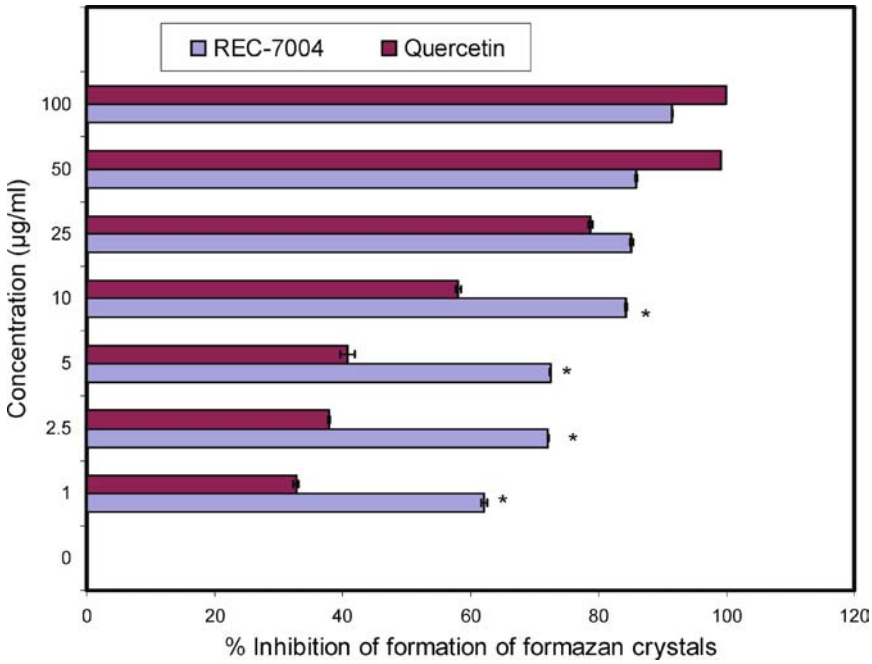

Fig. 5. Effect of different concentrations of REC (1–100 μg/ml) on scavenging of superoxide ions. The percentage inhibition of formation of formazan crystals containing all reagents but without REC-7004 was considered as 0% inhibition (control). REC-7004 (1–10 μg/ml) exhibited significant % inhibition of formation of formazan crystals with respect to control ($^{*}p < 0.05$).

being a little drowsy for 3–5 min, while more higher doses of REC-7004 manifested mortality in a dose-dependent manner. Mice exposed to 10 Gy gamma-radiation died within 12th post-irradiation day. Intraperitoneal administration of REC-7004 (maximally effective dose: 400 mg/kg b.w.) 30 min prior to 10 Gy total-body gamma-irradiation rendered 83.3% survival ($p < 0.01$), as compared to the 10 Gy irradiated group.

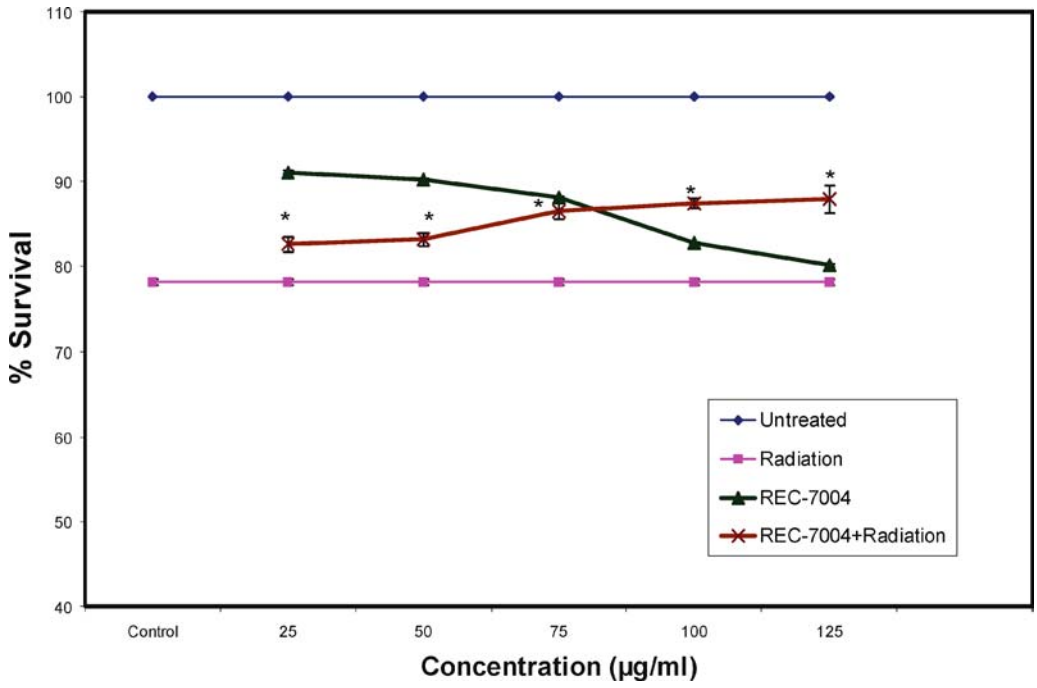

Fig. 6. Effect of REC-7004 (25–125 μg/ml) on % survival of U87 cells against radiation (2 Gy) for a period of 48 h using MTT assay [studied]. All the concentrations showed significant ($^*p < 0.05$) % survival in drug + radiation group, while absorbance of untreated control was considered as 100% survival. The maximally optimal dose was found to be 25 μg/ml (91.145 and 82.59% survival in drug only and drug + radiation group respectively).

Anti-lipid peroxidation activity

The ability of REC-7004 to inhibit lipid peroxidation, induced by iron/ascorbate, radiation (250 Gy) and both [i.e. iron/ascorbate and radiation (250 Gy)], in terms of formation of nanomoles of MDA-TBA complex was also investigated and was also found to decrease in a dose-dependent manner (0.05–2 mg/ml). The maximum percentage inhibition of formation of MDA-TBA complex at 2 mg/ml (highest tested dose with least lipid peroxidation activity) in case of iron/ascorbate, radiation (250 Gy) and both, i.e. iron/ascorbate with radiation (250 Gy) was 53.78, 63.07, and 51.76% respectively and was found to be comparable to that of quercetin (81.23, 78.20, and 80.52% respectively) and significant ($p < 0.05$) as compared to control (0% inhibition) (Figs. 7–9).

Modification of radiation-induced hemolysis of human erythrocytes by REC-7004

The anti-hemolytic ability of REC-7004 (10–1000 ng/ml) was also studied in terms of lowering in erythrocyte MDA values at different concentrations, and was evaluated by comparing drug + radiation group with radiation control. In a range of 10–1000 ng/ml of drug concentrations incubated with 200 μl of blood, one hour prior to radiation (250 Gy) exposure, clearly showed that at 100, 500 and 1000 ng/ml, 0.145, 0.107, and 0.089 activity (expressed as nanomoles of TBARS formed/h/ml of blood), was exhibited by REC-7004, which was significantly ($p < 0.05$) lower as compared to radiation control. The most optimal dose at which minimum drug-induced lipid per-oxidation, as well as, significant ($p < 0.05$) protection was observed was 1 μg/ml (Fig. 10).

Discussion

Radiation-induced oxidative stress leads to macromolecular structural damage. Antioxidant agents are able to prevent the occurrence of such deleterious processes, mainly due to their free radical scavenging and divalent ion chelating properties [40, 41]. These antioxidants catalyze the free radicals into stable products and also bind to the transition metals and clear them from the extra cellular *milieu* thereby preventing them from reacting with hydrogen peroxide to form ferryl, perferryl species, which can initiate lipid peroxidation [42].

In the present study, the ability of REC-7004 to inhibit lipid oxidation, chelate metal ions, electron donation potential, and free radical scavenging potential was evaluated in a cell free system to assess the inherent ability of the drug to act at different fronts in case of radiation-induced oxidative stress. Among four different concentrations tested, it was observed that lower doses (8 and 80 μg/mL) tend to inhibit lipid

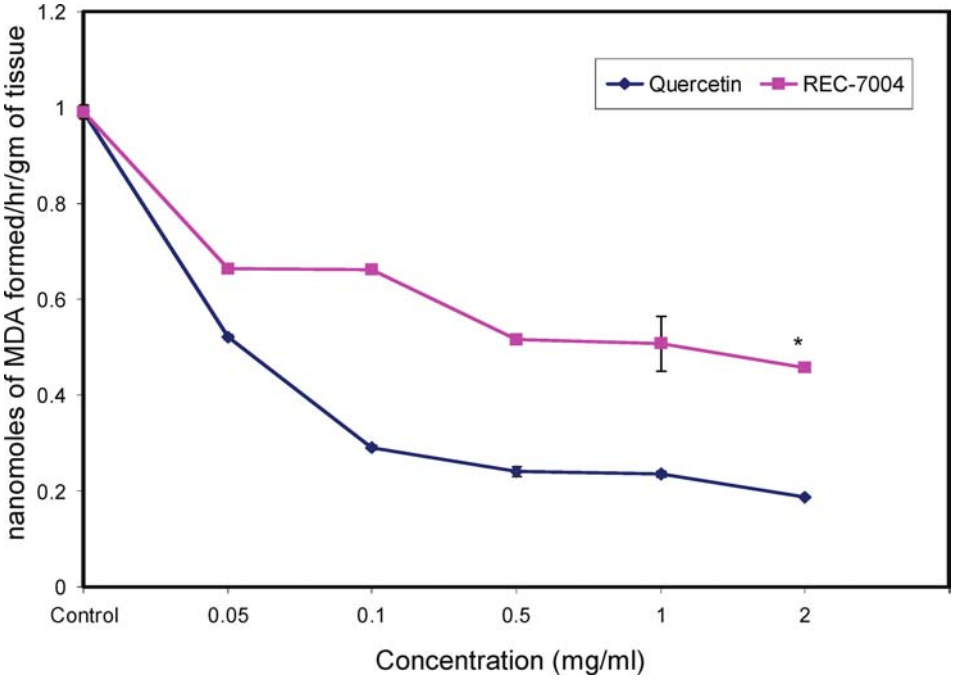

Fig. 7. Effect of different concentrations of REC-7004 on iron/ascorbate mediated lipid peroxidation evaluated in liver homogenate of strain 'A' mice. Each experiment was performed in triplicate and was repeated three times and the lipid peroxidation activity is expressed as nanomoles of MDA (malonialdehyde) formed $\times 10^6$. Lipid peroxidation in control represents 0% inhibition (maximal acitvity). Maximal decrease in activity at 2 mg/ml (53.78%) with respect to control ($^* p < 0.05$).

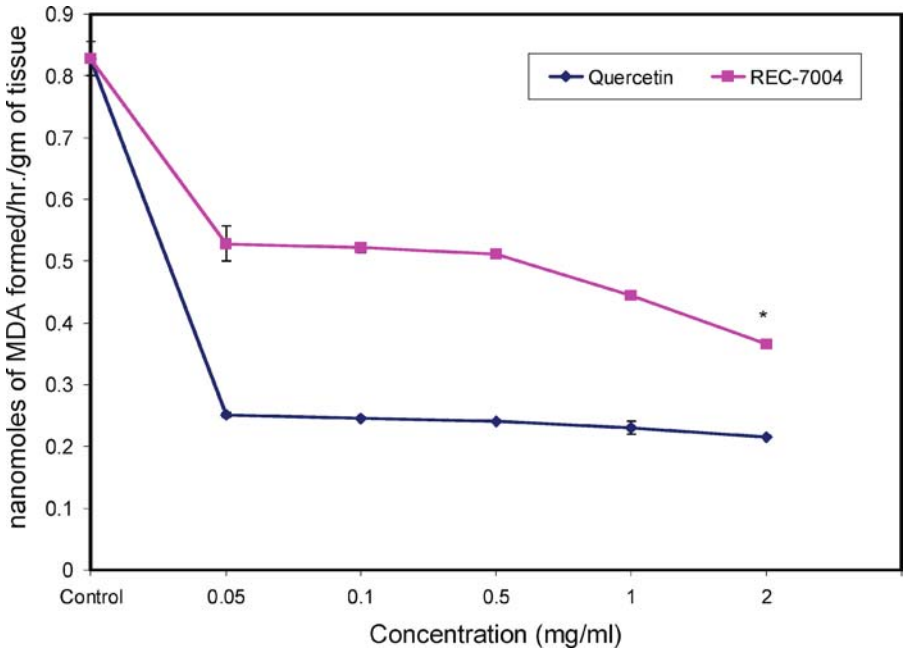

Fig. 8. Effect of different concentrations of REC-7004 on radiation (250 Gy) mediated lipid peroxidation evaluated in liver homogenate of strain 'A' mice. Each experiment was performed in triplicate and was repeated three times and the lipid peroxidation activity is expressed as nanomoles of MDA (malonialdehyde) formed $\times 10^6$. Lipid peroxidation in control represents 0% inhibition (maximal activity). Maximal decrease in activity at 2 mg/ml (63.07% inhibition) with respect to control ($^* p < 0.05$).

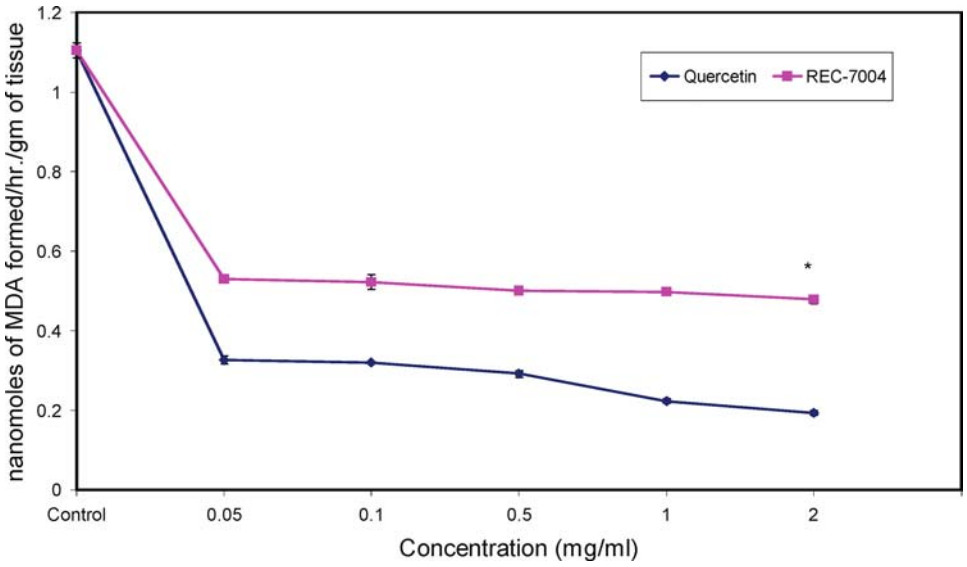

Fig. 9. Effect of different concentrations of REC-7004 on iron/ascorbate + radiation (250 Gy) mediated lipid peroxidation evaluated in liver homogenate of strain 'A' mice. Each experiment was performed in triplicate and was repeated three times and the lipid peroxidation activity is expressed as nanomoles of MDA (malonialdehyde) formed $\times 10^6$. Lipid peroxidation in control represents 0% inhibition (maximal activity). Maximal decrease in activity at 2 mg/ml (51.076% inhibition) with respect to control ($^*p < 0.05$).

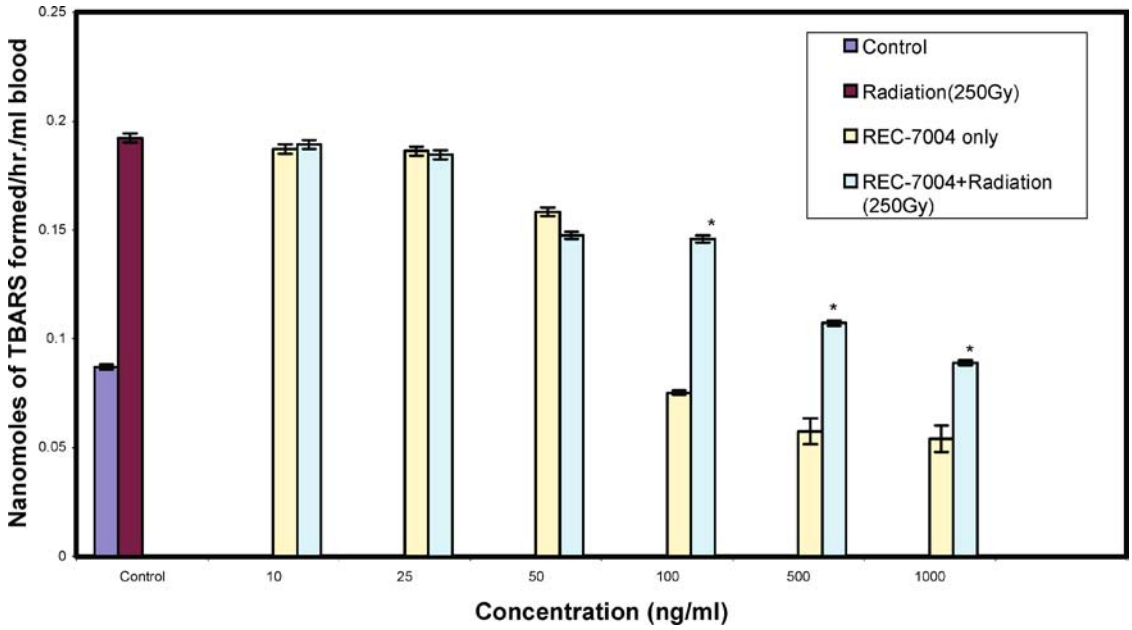

Fig. 10. Effect of different concentrations of REC-7004 (10 ng–1000 ng/ml) on radiation (250 Gy) induced hemolysis of human erythrocytes (evaluated as nanomoles of MDA formed/h/ml of blood). Each experiment was performed in triplicate and was repeated three times. Maximum hemolysis, observed in radiation control represents 0% inhibition (maximal activity). Maximal protection was observed at 1 μg/ml with respect to control ($^*p < 0.05$).

oxidation significantly as shown in Fig. 1. An overall inhibition of 59.5 and 52.5% of TBA values (at initial and final stage of experiment respectively) exhibited the immense potential of REC-7004 (80 μg/mL) to tackle peroxyl radicals (Fig. 2). Iron is an essential micro-nutrient required for normal cellular functioning, as it has the ability to interact with oxygen (O_2), due to its favourable oxidation–reduction potential and its abundance in nature, which have resulted in its evolutionary selection for a diverse array of biological functions. However, the useful catalytic properties of iron also pose a threat

to cellular systems via generation of reactive oxygen species (ROS). REC-7004 was also found to possess significant ($p < 0.05$) metal chelation activity in a range of 1–50 μg/ml concentrations tested (Fig. 3). This could be attributed to its high polyphenolic content (0.971 ± 0.01/mg% of quercetin). Poly-phenols are composed of one (or more) aromatic rings bearing one (or more) hydroxyl groups and are capable of scavenging free radicals by forming resonance stabilised phenoxy radicals [43]. Although living systems must have iron to survive, metal-catalyzed generation of superoxide ($O_2^{\bullet -}$), and hydroxyl radical ($^{\bullet}OH$), as well as production of ferryl (FeO^{2+}) and perferryl ($Fe^{3+}O_2^-$) radicals, can cause oxidative stress. Consequently, living systems have evolved strategies to procure adequate iron for cellular function and homeostasis without major damage to biological macromolecules. However, increased ROS coupled with increased iron levels can result in disordered activation and expression of antioxidant processes. Exposure to ionising radiation leads to free radical mediated macromolecular damage and also enhances the free cytosolic pool of iron [44], which acts as a secondary initiator and catalyzes $OH^{\bullet}$ radical formation thereby accelerating lipid peroxidation [45, 46]. Thus, the redox state and concentration of iron ions in the cellular *milieu* plays a crucial role in amplification of damage [47] as they interact with biological membranes to generate alkoxy and peroxy radicals thereby inflicting further damage to the cellular system [48]. The polyphenolic components present in REC-7004 possibly contributed to its dualistic antioxidant potential i.e., donation of hydrogen atoms (ability to act as a chain breaking antioxidant) and also chelation of transition metal ions (secondary antioxidant activity). It can, therefore, be expected that REC-7004 can modulate the concentration of free iron ions in the biological system. Similar observations have been reported with radioprotective herbal extracts of *P. hexandrum*, *O. sanctum* and *T. cordifolia* [49–51].

REC-7004 was also found to possess nearly 12 times higher reducing power than that of the synthetic antioxidant BHT, used as a standard, on the basis of their respective unit absorption values. The increased absorbance indicates higher reducing power, and consequently higher antioxidant potential (Fig. 4), pointing to immense potential to donate electrons and thereby modulate the free radical mediated reactions. Several workers have reported that natural plant products act as electron donors, react with free radicals, terminate radical chain reactions and, therefore, are able to boost the natural antioxidant defence mechanism [2, 52, 53]. This lends support to the findings of the present study that polyphenols (0.971 ± 0.01 mg%), possibly along with other constituents, play a vital role in enhancing the overall electron donation capacity of REC-7004.

The ability of REC-7004 to scavenge free radicals was also investigated using nitroblue tetrazolium (NBT) as a marker substrate since it gets reduced by superoxide ions to formazan crystals. The percentage inhibition of formation of formazan crystals (91.54%; 100 μg/ml) by REC-7004 indicated its ability to scavenge superoxide ions (Fig. 5). These findings could also be attributed to the high polyphenolic content of REC-7004 (0.971 ± 0.01 mg%). Song and co-workers have confirmed the presence of the polyphenol kaempferol (5,7,4′-trihydroxylflavonol), a representative flavonol, from the roots of *Rhodiola* sps., using NMR spectroscopy. These workers have reported the protective activity of kaempferol against tacrine-induced hepatotoxicity. Kaempferol-6′-O-acetate, a derivative of kaempferol, has also been reported to modulate TNF-α-induced cell death [54, 55].

The radio-protective survival study indicated the ability of REC-7004 to protect Strain 'A' mice with a survival rate of 83.3% with a very high therapeutic window as well as no effect in the activity profiles of mice. This finding exhibited the immense radio-protective potential of REC-7004, which requires further investigation at both *in vitro and in vivo* levels to evaluate the site-specific mechanism involved. The antioxidant and related radioprotective effects of REC-7004 could be attributed to its ability to reduce oxidative stress by chelating metal ions, scavenging free radicals, and inhbiting lipid oxidation. The antioxidant and radioprotective effect observed in the present study could be attributed to the presence of kaempferol in *Rhodiola* extract, which is also well known for such effects [4, 56]. Another compound known as salidroside, a phenylpropanoid glycoside compound present in *Rhodiola* extract, has been reported to also possess anti-inflammatory activity [57]. It is now known that anti-inflammatory activity is a key activity that plays a crucial role in rendering radiation protection along with the associated synergistic effect(s) of other biological activities [2].

The free radicals generated during oxidative stress induced by radiation exposure target the biological membranes, resulting in generation of lipid hydroperoxides, aldehydes and ketones [58–60]. The lipid peroxidation induced by iron/ascorbate-induced stress, radiation-induced stress and both taken together as a single combined stress was also studied since these experimental conditions mimic the biological state maximally. In case of combined stress, REC-7004 inhibited 51.76% lipid peroxidation activity at a dose of 2 mg/ml (Figs. 7–9), while the inhibitory ratios evaluated as ratio of activity at 0.05–2 mg/ml in the respective stresses were found to be 1.44, 1.44, and 1.10 while in case of quercetin the ratios were 2.79, 1.16, and 1.68 respectively. A lower ratio is an indication of effectiveness of drug to work even at lower concentration. In case of iron/ascorbate and combined (radiation and iron/ascorbate) stress, REC-7004 was effective in lowering the activity even at lower concentrations as compared to that of quercetin, although its percentage inhibition of lipid peroxidation was lower in both cases as compared to that of

standard (Figs. 7 and 9). REC-7004 effectively lowered the generation of lipid hydroperoxides, thereby, preventing the damage to biological membranes. The activity could be due to polyphenolic compounds, which have been reported to exhibit the ability to donate their hydrogen atom in the initial stage of lipid peroxidation to compete with polyunsaturated fatty acids, thereby breaking the propagation chain [43, 61].

Human erythrocytes are particularly susceptible to oxidation by oxygen radicals since they are very rich in Fe^{2+}-containing molecules, primarily haemoglobin. Agents like hydrogen peroxide and ionising radiation cause oxidative damage *via* generation of a diverse array of reactive oxygen species, which bring about lipid peroxidation, protein degradation and a progressive loss of deformability in a dose and time-dependent manner [34]. The effect of REC-7004 in terms of reducing the radiation-induced hemolysis by lowering lipid peroxidation in human erythrocytes was studied, and the results (Fig. 10) showed that radiation significantly increased MDA production, and this could be lowered by pretreatment with REC-7004 (0.089 activity at 1 μg/ml). Several other workers have shown that phyto-constituents inhibit oxidative hemolysis in human erythrocytes [62, 63].

The erythrocyte protective property could be explained by the ability of this extract to interfere with the antioxidant system of erythrocytes either by inhibiting the antioxidant enzymes or by increasing the consumption of antioxidant compounds such as vitamin E and glutathione that are present in erythrocyte membrane. These results are consistent with the anti-lipid peroxidation activity observed in liver homogenate. Furthermore, the results obtained in the present study utilising REC-7004 are in line with the results obtained in our earlier *in vivo* experiments with *Podophyllum hexandrum* [64].

The overall multi-faceted radioprotective effect of REC-7004 can be attributed to the potent antioxidant, anti-lipid peroxidation, anti-hemolytic, metal chelation and superoxide scavenging activities. The synergistic effect of the different constituents present in *Rhodiola* needs to be systematically determined. Detailed studies at *in vivo* and *in vitro* level, including molecular studies, are underway to unravel the site-specific mechanisms involved.

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