Seabuckthron (*Hippophae rhamnoides* L.) leaf extract ameliorates the gamma radiation mediated DNA damage and hepatic alterations

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*In vitro* assessment showed that *H. rhamnoides* (HrLE) extract possessed free radical scavenging activities and can protect gamma (γ) radiation induced supercoiled DNA damage. For *in vivo* study, Swiss albino mice were administered with HrLE (30 mg/kg body weight) for 15 consecutive days before exposing them to a single dose of 5 Gy of γ radiation. HrLE significantly prevented the radiation induced genomic DNA damage indicated as a significant reduction in the comet parameters. The lipid peroxidation, liver function enzymes, expression of phosphorylated NFκB (p65) and IkBa increased whereas the endogenous antioxidants diminished upon radiation exposure compared to control. Pretreatment of HrLE extract ameliorated these changes. Based on the present results it can be concluded that *H. rhamnoides* possess a potential preventive element in planned and accidental nuclear exposures.

**Keyword:** DNA damage, Gamma radiation, Hepatic alteration, Reactive oxygen species, Seabuckthron

The increasing application of γ radiation for medical and industrial purposes is leading to an increase in the number of incidents with destructive impacts on human beings. The damage caused by ionizing radiation is either by direct relations with target molecules or via the formation of free radicals, such as hydrogen radical (H·), hydroxyl radical (OH·), hydroperoxyl radical (HO₂·), hydrated electron (e_{aq}⁻), hydronium ion (H₃O⁺), superoxide anion (O₂⁻) etc. These free radicals react with cellular macromolecules and thereby causing cellular dysfunction and mortality and cause the DNA damage directly via strand breakage and indirectly by generating reactive oxygen species (ROS)³.

Several synthetic compounds have been developed as radioprotectors, but their practical uses were limited because of their adverse side effects³-⁵. Nowadays, the potential of natural radioprotectors have gathered considerable attention as possible alternatives to synthetics⁶,⁷. Recently, interest in the radioprotective properties of plants and plant compounds has escalated owing to their natural origin, cost-effectiveness and minimal side effects.

*Hippophae rhamnoides* L. (F. elaeagnaceae), commonly known as seabuckthorn, is a deciduous shrub, 2–4 m in height, very hardy and salt tolerant. It is native to Europe and Asia. It can survive –43 to +40 °C temperature and grows on acid alkaline soils with pH of 5.8–8.3 (optimal pH 6–7) and even nutritionally poor soils like river bank steep slope and is a nitrogen fixing plant⁸. In Tibetan and Indian systems of medicine, for centuries, *H. rhamnoides* has been exploited for treatment of lethargic digestion, stomach malfunctioning,⁸,¹⁰, circulatory disorders, burn and wound healing⁸,¹⁰ and hepatic injury¹¹,¹². *H. rhamnoides* contains a large number of constituents like flavonoids⁸,¹³, vitamin A, C, E, and K, tannins, sugars, fats and various trace elements like Se, Zn, Cu, and S¹⁰. Due to these molecules, it can act as a strong antioxidant⁸. Previously, in acute toxicity study, LD₅₀ of the seabuckthorn extract was observed >10 g/kg body weight when given orally¹³. The radioprotective efficacies of leaf extract of *H. rhamnoides* (HrLE) were also observed previously. The HrLE counteracted the radiation induced damage to haemopoietic system⁸,¹⁴. *H. rhamnoides* conferred the protection of gastrointestinal system against radiation induced damage¹⁵.

DNA damage is one of the major effects of radiation exposures. Therefore supercoiled plasmid
DNA was chosen for observing the defensive efficacies of HrLE against the radiation induced strand breakage. Further the lymphocytes in experimental animals were selected to substantiate the protective effect of HrLE against radiation induced DNA damage. Since liver is very active metabolic organ and reflects any metabolic as well as systemic changes after ionizing radiations thus, it was preferred as a target organ\(^1\). Therefore, the major aim of this study is to justify the radioprotective efficacy of HrLE against radiation induced DNA damage and hepatic alterations.

**Materials and Methods**

*Chemicals—*Trichloroacetic acid (TCA), thiobarbituric acid (TBA), (2,2-diphenyl-1-pircylhydrazyl) (DPPH), 5′,5′-dithio-bis (2-nitro benzoic acid) (DTNB) and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma (St Louis, MO, USA). Ethylene di amine tetra acetic acid (EDTA), benzoic acid) (DTNB) and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma (St Louis, MO, USA). Ethylene di amine tetra acetic acid (EDTA), as a target organ 1.

**Collection and preparation of H. rhamnoides leaves—**Hippophae rhamnoides leaves were obtained from Ladakh, India during the month of September. The dried leaves were extracted sequentially with petroleum ether, chloroform and 70% aqueous (aq.) acetone. Removal of solvent from the aq. acetone mixture was shaken vigorously and incubated at room temperature for 15 min and the supernatant of HrLE was further diluted with 50% aq. ethanol and centrifuged. The extract below 50 °C gave a sticky residue which was acetone. Removal of solvent from the aq. acetone was added to 0.3 mL of the 50% aq. ethanol solution of HrLE at various concentrations. The mixture was shaken vigorously and incubated at room temperature for 30 min before the absorbance was measured spectrophotometrically (Smartspec™ plus, BIORAD, Hercules, CA, USA) at 517 nm and DPPH solution (0.2 mM) was used as a control. Butylated hydroxyanisole (BHA) was used as a positive control 17.

**Hydroxyl radical scavenging activity:** Hydroxyl radical scavenging activity was determined according to the method described by Singh *et al* 17. Briefly, different concentrations of HrLE were taken in 1 mL ethyl alcohol. Iron-EDTA solution (1 mL, 0.1% ferrous ammonium sulphate and 0.26% EDTA), 0.5 mL of EDTA (0.018%) and 1 mL of the dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 M PBS, pH 7.4) were added to these tubes and the reaction was initiated by adding 0.5 mL of 0.22% ascorbic acid, followed by heating in a water bath at 80-90 °C for 15 min. The reaction was terminated by the addition of 1 mL of ice cold TCA (17.5% w/v). Nash reagent (3 mL, 75 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL acetyl acetone were mixed and raised to 1 L with distilled water) was added to all of the tubes and left at room temperature for 15 min and absorbance was taken at 412 nm against reagent blank. Mannitol was used as a positive control 18.

**Nitric oxide radical scavenging activity:** At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, and is quantified by the Griess Illosvoy reaction 18. The reaction mixture contained 10 mM SNP, PBS (pH 7.4) and increasing concentrations of the test solution in a final volume of 3 mL. After incubation for 150 min at 25 °C, 1 mL sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 mL of the incubated solution and allowed to stand for 5 min. Then 1 mL of naphthyl ethylenediamine dihydrochloride (NED) (0.1% w/v)
was added and incubated for 30 min at 25 °C. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. Quercetin was used as a positive control.

Ferric reducing antioxidant power (FRAP) assay: FRAP of HrLE was determined according to Sinha et al. Briefly, 1 mL FRAP reagent (300 mM acetate buffer, 10 mM TPTZ solution, 20 mM FeCl₃·6H₂O solution in a ratio of 10:1:1) and 10 µL of increasing concentrations of HrLE were mixed and kept in a water bath at 37 °C for 4 min. The optical density was measured at 593 nm. Concentration was calculated against a ferrous sulfate (FeSO₄) standard curve. Gallic acid was used as a positive control.

FRAP unit is equal to 100 µmol/dm³ Fe²⁺(ref 21).

Ferrous (Fe²⁺) chelation activity: The Fe²⁺ ion chelating activity was evaluated by a standard method with minor changes. The reaction was carried out in 4-(2-hydroxyethyl)-1 piperazine ethane sulfonic acid (HEPES) buffer (20 mM, pH 7.2). Briefly, increasing concentrations of HrLE were added to 12.5 µM FeSO₄ solution and the reaction was initiated by the addition of ferrozine (75 µM). The mixture was shaken vigorously and incubated for 20 min at room temperature. The absorbance was measured at 562 nm. EDTA was used as a positive control.

Reducing power assay: The ferric (Fe³⁺) reducing power of the HrLE was determined by the method of Oyaizu with slight modification. Increasing concentrations of HrLE were mixed with 0.5 mL of PBS (0.2 M, pH 6.6) and 0.5 mL potassium hexacyanoferrate (aq. 0.1%), followed by incubation at 50 °C in a water bath for 20 min. After incubation, 0.5 mL of 10% trichloroacetic acid (TCA) was added to terminate the reaction. Solution (1mL) was mixed with 1 mL distilled water, and 0.1 mL 0.01% FeCl₃ solution was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. Butylated hydroxytoluene (BHT) was used as a positive control.

Calculation of half maximal inhibitory concentration (IC₅₀)—The IC₅₀ values were calculated using the dose inhibition curve in the linear range by plotting the concentration (µg/mL) versus the corresponding scavenging effect ( % scavenging activity).

Irradiation of plasmid DNA—pET28a supercoiled plasmid DNA were irradiated using ⁶⁰Co γ chamber (Model no: GC1200, Strength 3.7 kCi; Cylindrical Sample Chamber with a diameter of 10.6 cm and height of 14.2 cm, Board of Radiation and Isotope Technology (BRIT), Mumbai, MH, India) at UGC-DAE CSR Kolkata Centre, Kolkata, India. Supercoiled pET28a plasmid DNA (300-350 ng) were irradiated (5 Gy) in micro centrifuge tube with the Tris-EDTA (pH 7.5) buffer. They were exposed to γ radiation at a dose-rate of 6.85 kGy/h in the presence and absence of different doses of HrLE.

Analysis of plasmid DNA damage—Radiation induced damage of the supercoiled plasmid DNA was determined by electrophoresis in 1% agarose gel. The ethidium bromide (10 µg/mL) stained DNA bands were photographed in Gel Doc (Molecular Imager® Gel Doc™ XR System, BIORAD, Hercules, CA, USA) and analysed using Image J software 1.45 S. The Strand Break Index (SBI) was calculated by the following formula:

\[ SBI = \frac{\text{Open circular DNA}}{\text{Total supercoiled DNA}} \times 100 \]

Irradiation of mice—Unanaesthetised animals were restrained in well-ventilated perspex boxes and whole body was exposed to γ radiation (5 Gy), at a dose-rate of 1 Gy/min and a source-to-surface distance of 77.5 cm. Mice were irradiated with ⁶⁰Co source at Saha Institute of Nuclear Physics, Kolkata, India.

Experimental design—The dose of HrLE was selected according to dose dependent study on the basis of detection of liver function enzymes. It was observed from the results that the 30 mg/kg body weight of HrLE groups gave optimum protection by diminishing the SGOT, SGPT and ALP level. Swiss albino mice were selected from a congenital colony and divided into 4 groups of 8 animals each. These groups were:

Control group: The animals of control groups were given distilled water through oral gavages once a day for 15 consecutive days.

IR group: Mice were given distilled water for 15 consecutive days before exposing them to a single dose of 5-Gy γ irradiation.

HrLE group: Mice were administered with HrLE (30 mg/kg body weight) orally for 15 consecutive days.
HrLE+IR group: Mice were administered with HrLE (30 mg/kg body weight) orally for 15 consecutive days. One hour after the administration of last dose, the animals were exposed to a single dose of 5-Gy γ irradiation.

All the animals were necropsied by cervical dislocation at 6 h of post-irradiation. Serum was collected for alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT). Lymphocytes were isolated from mice blood for assessment of DNA damage by comet assay and liver was collected for the estimation lipid peroxidation (LPO), superoxide dismutase (SOD) activity, catalase (CAT) activity, reduced glutathione (GSH) level, ferric reducing antioxidant power (FRAP), histology and western blot analysis.

**Measurement of serum ALP, GOT, GPT levels**—ALP, SGOT, SGPT levels were measured using spectrophotometric assay kits of Randox Laboratories Ltd. (Antrim, United Kingdom) according to the manufacturer’s instructions.

**Isolation of lymphocytes from mice blood**—Lymphocytes were isolated using procedure described earlier  with slight modification. Briefly, 3 mL of whole blood was diluted (1:1) with RPMI 1640 and layered on the top of lymphocyte separation medium in a centrifugation tube. After centrifugation for 15 min at 400×g, the white layer of lymphocytes at the boundary between blood plasma and the medium was carefully transferred using a Pasteur pipette into a tube containing 5 mL culture medium, RPMI 1640. The lymphocytes were then washed twice with RPMI 1640 and centrifuged at 250×g for 10 min. The cell pellet was resuspended in 6 mL of RPMI and 1 mL of the suspension was used for comet assay.

**Alkaline single cell gel electrophoresis (comet assay)**—Radiation-induced DNA double strand breakage in mice lymphocytes was evaluated using single cell gel electrophoresis (comet assay) based on the method of Singh et al. In brief, special slides (frosted) were covered with 1% normal melting agarose (NMA) in PBS and allowed to solidify. Removal of the cover slip from the agar layer was followed by the addition of a second layer of low melting agarose (LMA) containing approximately 2x10⁷ cells at 37 °C and cover slips were placed immediately. After solidification of the LMA, the cover-slips were removed and chilled lysing solution containing 2.5 M NaCl, 100 mM di sodium EDTA (Na₂-EDTA), 10 mM Tris–HCl at pH 10, and 1% DMSO, 1% Triton X100 were applied and kept overnight at 4 °C. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300 mM NaOH), 1 mM Na₂-EDTA and 0.2% DMSO, pH ≥13.0. The slides were equilibrated with the same buffer for 20 min, and electrophoresis was carried out at 25V, 180 mA for 20 min. After electrophoresis, the slides were washed gently with 0.4 M Tris–HCl buffer, pH 7.4, to remove the alkali. The slides were stained with EtBr (20 µg/mL) and visualized using a bright field phase-contrast microscope with epi-fluorescence facility (Leica DC 300 FX, Wetzler, Germany) in 400X magnification. The quantification of the DNA strand breaks of the stored images were done using the Comet Score software by which percent of DNA in tail, tail length, tail moment and Olive tail moment were obtained directly.

**Biochemical estimation from liver tissue homogenate**—

**Catalase activity:** Catalase activity was measured by monitoring the decrease in absorbance resulting from the elimination of H₂O₂ by the action of catalase. The standard reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 30 mM H₂O₂, and 3 μL liver homogenate for a total volume of 1 mL. The reaction was run at 20 °C and only the initial linear rate was used to estimate the catalase activity. The enzyme activity was determined from its ability to decompose 1 μmol of H₂O₂/min as 1 U activity at 240 nm, that is, using ε for H₂O₂ as 43.6 M⁻¹cm⁻¹.

**Superoxide dismutase activity:** Superoxide dismutase activity was determined using the slightly modified pyrogallol autoxidation method. Briefly, liver homogenate was added to 62.5 mM tris-cacodylic acid buffer followed by the addition of 4 mM pyrogallol. The autoxidation of pyrogallol was monitored at 420 nm.

**Lipid peroxidation:** Thiobarbituric acid reactive substance (TBARS) in the homogenate was estimated using standard protocol. Briefly, the homogenate was incubated with 15% TCA, 0.375% TBA and 5 N HCl at 95 °C for 15 min; the mixture was cooled, centrifuged and the absorbance of the supernatant was measured at 535 nm against appropriate blank. The amount of lipid peroxidation was determined by using ε = 1.56 x 10⁵ M⁻¹cm⁻¹ and expressed as amount of produced TBARS in nmol/g tissue.
Reduced glutathione: Liver homogenate was treated with 0.1 mL of 25% TCA, and the resulting precipitate was pelleted by centrifugation at 3900xg for 10 min. Free endogenous sulphhydril was assayed in a total of 3 mL volume by adding 2 mL of 0.5 mM DTNB prepared in 0.2 M phosphate buffer (pH 8) to 1 mL of the supernatant. The GSH reacts with DTNB and forms a yellow complex with DTNB. The absorbance was read at 412 nm.\(^{32}\)

Ferric reducing antioxidant power: FRAP level of liver homogenate was determined. Briefly, 1 mL FRAP reagent (300 mM acetate buffer, 10 mM TPTZ solution, 20 mM FeCl\(_3\)·6H\(_2\)O solution in a ratio of 10:1:1) and 10 µL liver homogenate were mixed and kept in water bath at 37 °C for 4 min. The optical density was measured at 593 nm. Concentration was calculated against a FeSO\(_4\) standard curve.

Histological analysis of liver tissue—For histology, a small portion of liver tissue was cleaned, and immediately preserved in a fixative containing 10% buffered formaldehyde. The liver blocks were cut 5 µm in thickness, processed and embedded in paraffin wax. Paraffin blocks were fixed in a fixative containing 10% formaldehyde. The liver slices were then processed and embedded in paraffin wax. Paraffin blocks were cut 5 µm in thickness, processed and stained with hematoxylin (H) and eosin (E) for histopathological evaluation of liver lesions. The stained slide for each group was observed using light microscope (Olympus 207444, Tokyo, Japan) at 200X magnification. The photomicrograph was taken using the digital Camera (Canon Power Shot S70).

Cytokine ELISA—The levels of TNF-α and IL-6, the two major pro-inflammatory cytokines were measured by standard protocol\(^{36}\) using a sandwich ELISA Kit purchased from Endogen Inc. (Rockford, IL, USA).

Western blot analysis—Whole cell lysate of liver tissue was prepared with the help of radioimmunoprecipitation assay (RIPA) buffer (0.1% sodium dodecyl sulfate, 0.5% deoxycholic acid, 1% Igepal, 150 mM NaCl and 50 mM Tris–HCl)\(^{35}\). Protein concentration was determined by the method of Lowry et al\(^{37}\). Equal amounts of protein (50 µg) in each lane were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane following electrophoresis. The membrane was blocked with 3% bovine serum albumin solution and kept overnight at 4 °C. Immunoblotting was done as described previously by Sinha et al\(^{20}\), using monoclonal antibody to mouse IkBα total NF-κB (p65) (Cell Signaling Technology, Inc. Danvers, MA, USA). Anti β-Actin (mouse) (Santa cruz Biotech, USA) was used as loading control for tissue homogenates respectively. Immunoblot pictures were taken by Gel Doc EZ and the images were analyzed using Image Lab\(^{TM}\) Software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis—The values are given as mean ± SE. One-way analysis of variance (ANOVA) with Tukey’s post hoc test was performed for statistical evaluation of the data and for the determination of the level of significance in various groups. In all cases, a value of P<0.05 was considered significant.

Results

In vitro scavenging activity of HrLE—The DPPH scavenging activity, hydroxyl radical scavenging activity, reducing activity and nitric oxide scavenging activity of HrLE were similar with their respective standards and there were no significant difference between the above mentioned scavenging properties of HrLE and their standards. The iron chelating activity and FRAP activity of HrLE were lower than the standards (Fig.1). There was no significant difference between the IC\(_{50}\) values of HrLE of DPPH scavenging activity (22.72 ± 2.66 µg/mL) and BHA (23.49 ± 2.78 µg/mL). The IC\(_{50}\) values of hydroxyl radical scavenging activity of the HrLE was little superior (15.44 ± 0.75 µg/mL) than standard (13.77 ± 0.95 µg/mL) and there was no significant difference between them. HrLE also inhibited nitric oxide (NO) generation in a dose dependent manner (Fig. 1c) with the IC\(_{50}\) value being 15.89 ± 0.99 µg/mL. Quercetin was used as a reference standard which has IC\(_{50}\) value of 17.64 ± 2.04 µg/mL. HrLE has subordinate FRAP level than gallic acid of different concentration (Fig. 1d). At 100 µg/mL concentrations, the FRAP value of HrLE and standard gallic acid was 3.80 ± 0.12 and 4.79 ± 0.21, respectively. Ferrozine produced a violet complex with Fe\(^{2+}\). In presence of a chelating agent like HrLE, complex formation was interrupted and as a result the violet colour of the complex was decreased. The formation of the ferrozine-Fe\(^{2+}\) complex was inhibited in the presence of the HrLE and standard EDTA (Fig. 1e). The IC\(_{50}\) values of metal chelating activity of the HrLE and EDTA were 42.67 ± 1.24 and 24.83 ± 1.87 µg/mL, respectively.
HrLE reduced radiation induced supercoiled DNA damage—Agarose gel electrophoresis study of supercoiled pET28a DNA exposed to γ radiation (5 Gy) in absence and presence of different doses of HrLE were shown (Fig. 2a). The upper bands are the open circle (Form II) and the lower bands represent the super-coiled (Form I) form of plasmid DNA. Single strand breakage of supercoiled plasmid DNA occurred at 5 Gy dose of γ-radiation. HrLE protected the γ radiation induced plasmid DNA breakage in a dose dependent manner.

The strand break index (SBI) was calculated after measuring the band intensity (measured by ImageJ software). It was observed that the SBI value of supercoiled DNA was significantly augmented ($P < 0.05$) after exposure of 5 Gy dose of γ radiation than the control group (Fig. 2b). HrLE confined the supercoiled DNA damage by diminishing the SBI value significantly ($P < 0.05$) than the IR group in a dose dependent manner.

HrLE prevented radiation induced genomic DNA damage in lymphocytes—Damage to cellular DNA in mice lymphocytes upon the whole body exposure to 5 Gy γ-radiation in the presence and absence of HrLE is given in Fig. 3 (I). Exposure of lymphocytes to 5 Gy γ radiation resulted in an increase in comet parameters which was evident from Fig. 3 (II).

![Fig. 1](link)

Fig. 1—*In vitro* free radical scavenging activities of HrLE. (a) DPPH radical scavenging activities of the HrLE and the reference compound BHA. (b) Hydroxyl radical scavenging activities of the HrLE with the reference compound mannitol. (c) The NO scavenging activity of HrLE and the quercetin as reference was represented. (d) FRAP activity of HrLE with standard gallic acid. (e) Fe$^{2+}$ chelating assay. (f) The reducing abilities of HrLE with the standard BHA. (Values are mean ± SE from triplicate experiments).

![Fig. 2](link)

Fig. 2—(a) Agarose gel electrophoresis pattern of pET28a DNA exposed to γ radiation (5 Gy) in absence and presence of different doses of HrLE. (b) Strand break index (SBI) value of different lane of the fig 2A. $P < 0.05$ was considered significant (Values are mean ± SE from triplicate experiments). Significant ($P < 0.05$) differences were observed in between * lane 1 vs lane 2, ^ lane 2 vs lane 3, # lane 2 vs lane 4, @ lane 2 vs lane 5.

Pretreatment of HrLE to γ radiation exposure resulted in significant decrease in the comet parameters.
**Determination of liver function test from serum**—The normal functional status of liver was assessed by estimating the levels of liver enzymes ALP, SGPT and SGOT (Table 1). The serum ALP was much elevated by γ irradiation compared to normal values. This elevation was reduced by treatment with HRLE in HRLE+IR group. Serum GPT and GOT levels were also elevated by γ radiation as compared to normal levels. Treatment with HRLE ameliorated these levels significantly ($P<0.05$).

**Biochemical estimation from liver tissue homogenate**—Mice exposed to 5 Gy irradiation, showed the significantly ($P<0.05$) reduced levels of catalase activity in comparison to control. In HRLE+IR group the catalase activity was significantly superior to irradiated group (Fig. 4a). Therefore, HRLE pretreatment restored the catalase activity after radiation exposure. The SOD activity after radiation exposure of mice showed the significantly ($P<0.05$) decreased levels in comparison to control. SOD activity in the HRLE+IR group, significantly ($P<0.05$) increased compared to IR groups (Fig. 4b). Therefore, HRLE conserved SOD activity of mice liver homogenate even after the radiation exposure. γ radiation (5 Gy) caused a significant ($P<0.05$) increase in the level of TBARS compared to control group. In HRLE+IR group, pre-treatment with HRLE ameliorated the effect of radiation exposure as TBARS level significantly ($P<0.05$) compared to irradiated group (Fig. 4c). Thus, HRLE elicited radiation induced hepatic lipid peroxidation. γ radiation induced significant ($P<0.05$) reduction in GSH level compared to control group. Pre-administration of HRLE to radiation exposed mice showed significant increase in HRLE content compared to irradiated group ($P<0.05$) (Fig. 4d). Therefore, hepatic glutathione content was restored after radiation exposure by the HRLE pretreatment. It was observed that γ irradiation (5 Gy) induced significant ($P<0.05$) decrease in FRAP level compared to control group. Interestingly, pre-administration of HRLE prior to radiation resulted significant ($P<0.05$) increase in FRAP level compared to irradiated group (Fig. 4e). Thus, the total

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Table 1—Liver function enzyme in mice serum [Values are mean ± SE from 8 observation each]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>IR</th>
<th>HRLE</th>
<th>HRLE+IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (KA)</td>
<td>8.74±0.085</td>
<td>12.62±0.04*</td>
<td>8.64±0.079</td>
<td>9.23±0.090**</td>
</tr>
<tr>
<td>SGPT (IU/L)</td>
<td>10.52±0.39</td>
<td>19.49±0.24*</td>
<td>11.78±0.57</td>
<td>12.79±0.46**</td>
</tr>
<tr>
<td>SGOT (IU/L)</td>
<td>26.76±2.56</td>
<td>38.5±4.26*</td>
<td>27.66±1.76</td>
<td>24.83±3.78**</td>
</tr>
</tbody>
</table>

*P values: *control vs IR; **IR vs HRLE+IR
The antioxidant pool reduced after irradiation whereas HrLE significantly ameliorated the change.

_HrLE inhibited radiation induced alteration in liver._—The histopathological investigations of the liver sections showed that radiation exposure resulted in morphological changes leading to inflammation (Fig. 5). Compared to control, these morphological changes include inflammatory cellular infiltration, intense inflammatory response around the central vein, hepatocytes containing pyknotic, multilobed, dense and haematoxylin rich nuclei. Some of the hepatocytes found swelled and membranes appeared much disrupted, sinusoidal spaces increased which is the indication of hepatic inflammation. Treatments with HrLE prior to irradiation prevented radiation induced inflammation.

**Fig. 4**—(a) Effect of HrLE on radiation (5 Gy) induced alteration of catalase activity (µmol H$_2$O$_2$ reduced/mg protein) in murine liver homogenates. (b) Effect of HrLE on radiation (5 Gy) induced alteration of superoxide dismutase activity (unit/mg protein) in murine liver homogenates. (c) Effect of HrLE on radiation (5 Gy) induced lipid peroxidation in terms of TBARS (nmol/g tissue) in murine liver. (d) Effect of HrLE on radiation (5 Gy) induced alteration of reduced glutathione (nmol/mg protein) in mice liver homogenates. (e) Effect of HrLE on radiation (5 Gy) induced alteration of Ferric Reducing Antioxidant Power (FRAP Unit) in murine liver homogenates. All tests were performed in triplicate (Values are mean ± SE). *P <0.05 was considered significant. Statistical comparison: * control vs. IR, ^IR vs. HrLE+ IR.

**Fig. 5**—Light micrograph of mice liver sections. Sections were stained with haematoxylin and eosin. Magnification: 200X. (CV=Central Vein, HC=Hepatocytes, S=Sinusoidal space, I=Inflammatory cellular infiltration)
Expression of TNF-α and IL-6—In IR group serum TNF-α and IL-6 expression was significantly higher ($P < 0.05$) as compared to control. On the contrary, the HrLE treated groups, have significantly ($P < 0.05$) decreased cytokine expression as compared to IR group (Fig. 6a and 6b).

Inhibition of IR induced NF-κB (p65) activation by HrLE—Both the phosphorylated IκBα and NF-κB expression were augmented in the IR group compared to control which indicated activation of the NF-κB pathway (Fig. 7). This expression was markedly reversed by HrLE treatment along with irradiation as shown by significantly ($P < 0.05$) decreased expression of both phosphorylated IκBα and NF-κB.

Discussion

In the present investigation the in vitro radical scavenging activity of HrLE was examined with reference standards and also the protective effect of HrLE was evaluated against radiation induced supercoiled plasmid DNA damage, genomic DNA damage and hepatic alterations in murine system by detecting hepatic stress markers.

The γ radiation generates oxidative stress in the body mainly by radiolysis of water and produces hydroxyl radical (OH•) which in turn initiates the production of other ROS like superoxide anion (O₂⁻), hydrogen radical (H•), hydroperoxyl (HO₂) radical, hydrated electron (eaq), hydronium ion (H₃O⁺), hydrogen peroxide etc. both in the extracellular fluid and the intracellular fluid. Therefore in vitro radical scavenging activity is an important parameter for the selection of a good antioxidant. The in vitro characterization of HrLE showed that it can scavenge DPPH, hydroxyl radicals, NO efficiently in...
comparison to standards. HrLE possessed high reducing power and iron chelating activity. Therefore its role under in vivo condition might prove fruitful to tackle the radiation-induced oxidative stress and handle the secondary oxidative damage. Iron has been shown to act as a secondary initiator of oxidative stress. The initial damage is further aggravated by Fenton reaction generated hydroxyl radicals in presence of superoxide and hydrogen peroxide. FRAP assay denotes a total antioxidant pool except the thiol group. High FRAP value indicates a total scavenging power by HrLE. Relation between the antioxidant property and radiation protection by plant flavonoids and phenols was reported earlier. The excellent antioxidant profile qualifies its benchmark for further use in animal model.

In view of this, assessing the radiation induced supercoiled DNA damage by gel electrophoresis remains as a most reliable parameter. It is now well established that radiation produces a wide spectrum of DNA lesions, which include damage to nucleotide bases (base damages), DNA single-strand breaks (SSBs) and double-strand breaks (DSBs). The results of the present experiments on radiation induced DNA damage using supercoiled plasmid DNA as a model clearly justify that HrLE at a very low quantity can protect DNA from undergoing strand breakage due to γ radiation exposure.

To further substantiate the radioprotective effect, the alkaline comet assay using mouse lymphocytes was performed to determine the severity of radiation induced DNA damage and level of protection obtainable by HrLE. HrLE counteracted the γ radiation induced DNA damage significantly as indicated by the significant decrease in all the comet parameters.

The western blot results clearly indicated the augmentation of phosphorylation of IkBα, NF-κB (p65), serum TNF-α and IL-6 levels were by radiation. γ-radiation was associated with the generation of ROS, which could activate redox-sensitive transcription factor, NF-κB. NF-κB is one of the crucial markers of the inflammatory responses. It mediates inflammatory responses through the regulation of inflammatory cytokines, for example, tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-6; adhesion molecules; inflammatory enzymes, for example, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase-2 (iNOS2).

In present investigation, 5 Gy γ radiation augmented all major hepatic functions. lipid peroxidation was enhanced, whereas the activities of antioxidant enzymes (SOD and CAT), GSH levels and FRAP values diminished. The liver stress markers like SGOT, SGPT and ALP augmented after radiation. These are the signs of hepatic injury and leakage of these enzymes that occurred after radiation. The histological finding corroborated these results. These hepatic alterations may have occurred due to over activities of radiation induced free radicals; the later reacted with unsaturated lipids generating hydroperoxides and inducing lipid peroxidation. It was correlated with the previous findings that lipid peroxidation caused degradation of fine structures, alteration of integrity, fluidity, permeability and functional loss of biomembranes. The reactive species reduced the antioxidant defense mechanism and exhausted the intracellular concentration of GSH and related enzymes. The results of biochemical, histological and western blots confirm the histopathology and inflammation. The membrane damage and subsequent leakage of enzymes from cells due to altered membrane permeability make enzymes available in the serum. In the present study, HrLE treatment prior to radiation was found to defend the liver from radiation induced hepatic lipid peroxidation. In addition, SOD, CAT and GSH levels were maintained after irradiation when the mice were pretreated with HrLE. Bioactive components from the HrLE polyphenols influence endogenous antioxidant defense system after whole body irradiation in mice. Polyphenol enriched fractions of *Moringa oleifera* plant can also confer the hepatoprotection. Furthermore, HrLE increased total antioxidant pool in mice liver as FRAP level elevated after the supplementation of HrLE, which help to ameliorate the oxidative stress. Thus, it prevented systemic and cellular oxidative stress.

The protective action depend on either the ability of the HrLE to act as a scavenger of radiation induced reactive species or elevating the activity of the antioxidant pool of body. HrLE contains molecules of diversified nature ranging from antioxidant to proliferative and immunostimulatory nature. HrLE has been well reported to contain several antioxidant molecules like vitamin A, C, E and K, tannins and flavonoids. It also contains certain trace elements like Se, Zn, Cu and S which are part of metallo enzymes and some of which are known to manifest antioxidant
activity and radioprotection. Recently it was reported that the *H. rhamnoides* extract have several numbers of flavonoids and flavones like quercetin, isorhamnetin, kaempherol, and ellagic acid etc. The HPLC study of HrLE also related with the previous reported that the activity and radioprotection. Isorhamnetin, kaempherol, and ellagic acid confer good radioprotective efficacies. Thus, free radicals generated through radiation, can be ameliorated by scavenging action of HrLE may have caused the reduction of radiation mediated ROS generation, which can indirectly inhibit the expression of phosphorylated IκBα and p65 and confined the liver from radiation mediated inflammation. This is a proof that the radioprotective effect of HrLE against radiation induced physiological responses at the molecular level which has never been reported earlier.

In conclusion, it is reported for the first time that HrLE mediated a central role to protect the liver from radiation hazards by preventing lipid peroxidation, inflammation and elevating the total antioxidant pool of the body. It was also found HrLE protected supercoiled DNA and genomic DNA against radiation induced damage. Therefore, the present study showed the capability of HrLE against the radiation mediated systemic stress in *in vitro* and mammalian model. HrLE may also be used as a supplement for the patients who undergo total body irradiation during different clinical maneuvers. However, this needs further investigation before clinical trials.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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