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3. MATERIALS AND METHODS

3.1 Herbal preparations

Study was carried out with two composite herbal preparations from leaves of *Hippophae rhamnoides* SBL-1 and SBL-2. SBL-1 was aqueous in nature and soluble in water and SBL-2 was soluble in 25 % DMSO. At the outset both the preparations were compared with each other and with three other radioprotective herbal preparations viz., RDII (preparation from *Rhodiola imbricata*, soluble in 20 % alcohol), RTc (preparation from *Tinospora cardifolia*, soluble in 20 % alcohol) and RPI (preparation from *Podophyllum hexandrum*, soluble in 20 % alcohol).

3.2 Chemicals

2-Deoxy D-ribose, ferrozine (extra pure), gentian violet, magnesium chloride (MgCl₂), 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT), potassium bicarbonate (KHCO₃), RPMI-1640, thiourea and urea were from Hi-media, India; glacial acetic acid (CH₃COOH), 99.99 % pure, glucose, 1-chloro-2,4-dinitrobenzene (CDNB), formaldehyde, p-nitrophenol (pNP), 2,4 dinitrophenylhydrazine (2,4 DNPH), ortho phosphoric acid, proteinase K, magnesium sulphate (MgSO₄), N,N,N,N-Tetramethylethylenediamine (TEMED), TRIS-HCl were from Merck, Germany; chloroform, diethyl ether, DPX, dimethyl sulphoxide (DMSO), Giemsa stain, hydrogen peroxide (H₂O₂), methanol, potassium chloride (KCl), sodium hydroxide (NaOH), tannic acid, sodium citrate, xylene were from Qualigens, India; acrylamide, brilliant blue G, Folin-Ciocalteau reagent, ferric chloride (FeCl₃), glycine, glutathione reduced (GSH), glutathione oxidized (GSSG), nitro blue tetrazolium (NBT), nicotine diamine dihydrochloride (NADH), butanol, hydrochloric acid (HCl), potassium dichromate, potassium ferricyanide, sodium chloride (NaCl), sucrose, trichloroacetic acid (TCA), triethanolamine (TEA) were from Sisco Research Laboratory India; agarose, ammonium persulphate (APS), bovine serum albumin (BSA), citric acid, commassie brilliant blue, bromophenol blue (BPB), ethylene diamine tetraacetic acid (EDTA), ferrous sulphate (FeSO₄), TRIS base, n-lauryl sarcosine, ethidium bromide, and agarose were from Sigma, USA; TMB stabilized substrate for HRP and glycerol were from Promega, USA.
3.3 DNA, kits and antibodies

Primers for TNF like weak inducer of apoptosis (TWEAK) and fibroblast growth factor inducible molecule 14 (Fn14) were synthesized using services of Sigma, USA. Tumor growth factor-β (TGF-β), interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) enzyme linked immunosorbant assay (ELISA) kits were procured from BD Biosciences, USA. High mobility group box 1 (HMGB1) ELISA kit was from IBL Hamburg (Germany). All other antibodies were from Santa Cruz USA. Qiagen RNA isolation kit and one step reverse transcriptase-polymerase chain reaction (RT-PCR) kit was procured from Qiagen, USA. Puregene™ DNA isolation kit (D-6000A) was procured from Gentra systems, USA.

3.4 Instruments

UV-VIS Spectrophotometer (Biomate5, Thermofisher, USA), Gamma Cell-220 (Canada), electrophoresis unit (Tarson), ELISA Reader (Bio-Tek, USA), microcentrifuge (Remi Motors Ltd.), refrigerated centrifuge (Heraeus Biofuge Stratus, Thermofisher Scientific, Germany), homogenizer (Remi motors ltd.), SDS-PAGE gel assembly (Bio-Rad, USA), western blotting apparatus (Bio-Rad), Clamped Homogenous Electric Field- MAPPER (CHEF MAPPER, Bio-Rad, US), Night Hawk gel documentation system (USA), CO₂ incubator (Jouan, Germany), autoclave (Scientific Systems, India).

3.5 Animals

Swiss albino Strain 'A' inbred male mice, 8-10 weeks old, weighing 28 ± 2 g, bred and maintained at Experimental Animal Facility, Institute of Nuclear Medicine and Allied Sciences (INMAS), India, were used throughout the study. All animal experiments were conducted at INMAS, Delhi as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). All animals were housed in polyvinyl cages (not more than six animals per cage) under controlled temperature (22 ± 2 °C) and humidity (60 ± 5) for 12 h light/ 12 h dark cycle with free access to food (Golden Feed, Delhi, India) and water.
3.6 Experimental work with animals

For experimental work animals were divided into various groups. Depending upon the objective of the experiment the number of animals per group was decided. Each group of animal was subjected to different treatments as per the plan. In every experiment at least one group of animals was kept as untreated control which was administered sterile water or vehicle intraparitoneally (i.p.) only; and at least one group was kept as drug control, which was administered drug (dissolved in appropriate solvent) i.p. only. The other groups of animals were given different treatments such as; (i) whole body exposure to $^{60}$Co-gamma radiation (ii) drug treatment i.p. followed by whole body exposure to $^{60}$Co-gamma radiation. The specific details of different treatments are described in the “Results Section” along with corresponding experiment. For all i.p. injections volume of drug or vehicle was equal and was 0.2 ml.

3.7 Splenocyte culture

Mice were sacrificed humanely and spleen was excised and kept in ice cold culture medium (RPMI-1640) under sterile conditions. The spleen was minced with frosted slides to release the splenocytes. The splenocyte suspension was washed once in culture medium and resuspended in isotonic ammonium chloride (0.83 %) to lyse the red blood cells (RBCs). Cell suspension was centrifuged at 1000 rpm for 10 minutes and the pellet was resuspended in culture medium. After washing, the cells ($5 \times 10^6$) were resuspended in 1 ml RPMI-1640 culture medium containing 10 % fetal bovine serum (FBS). The cultures were incubated at $37 \pm 2 \, ^\circ C$ in a 5 % CO$_2$/95 % air humidified incubator for 24-72 h. Cells were harvested and viability of cells was determined either by trypan blue assay or MTT assay.

3.7.1 Trypan blue assay

Trypan blue assay was done by the method of Strober (2001).

Principle: Dead cells take up the trypan blue dye while the live cells exclude it. The non-stained viable cells therefore, can be distinguished from darkly stained dead cells.
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Procedure:
1. One volume of the cell suspension was mixed with one volume of trypan blue solution (0.4 % w/v in 0.9 % NaCl).
2. Haemocytometer chamber was filled with cell suspension and cells were counted using light microscope.
3. The percentage of viable cells were calculated using the formula:

   \[
   \% \text{ Viability} = \left( \frac{\text{No. of unstained cells}}{\text{Total no. of cells}} \right) \times 100
   \]

3.7.2 MTT assay

MTT assay was done by the method of Mosmann (1983).

Principle: The soluble tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells to form insoluble purple formazan dye crystals. The amount of tetrazolium reduction is proportional to the number of cells.

Procedure:
1. The cells were centrifuged to remove the medium and the fresh medium containing MTT (5 \( \mu \text{g/ml} \)) was added to the wells. The incubation was for 3 h to allow the formation of coloured formazan crystals.
2. Cell suspension was centrifuged for 10 minutes at 1000 rpm and the supernatant was removed.
3. Pellet containing coloured formazan was dissolved in fixed volume of isopropanol and the absorbance was recorded at 570 and 630 nm.
4. Standard graph [prepared by plotting cell number vs. absorbance (A)], was used to calculate the actual cell number.

3.8 \(^{60}\text{Co}\)-gamma irradiation

Gamma-Cell 5000 (GC-5000), Board of Research Isotope Technology (BRIT); India, was used as \(^{60}\text{Co}\)-gamma radiation source for achieving total absorbed dose of 100 Gy or more. Such studies were performed mainly with tissue homogenates and different type of reaction mixtures, which were prepared to evaluate efficacy of herbal
drugs *in vitro*. Samples were suspended in tubes and placed in the gamma chamber for pre-calculated time. Radiation dose rate during the whole study period varied from 2.6 KGY/h to 1.9 KGY/h (due to disintegration of $^{60}$Co, half life = 5.27 years). No air circulation was provided during irradiation.

Gamma Cell- model 220, Atomic Energy Commission, Canada was used for mice whole body exposure to $^{60}$Co-gamma radiation. For irradiation, single animal was kept in perforated cage and during irradiation fresh air was continuously circulated in the radiation chamber to avoid hypoxia. Radiation dose rate during the whole study period varied from 0.42 rad/sec to 0.28 rad/sec (due to disintegration of $^{60}$Co, half life = 5.27 years).

3.9 Biochemical assays

3.9.1 Biochemical assays with herbs *in vitro*

Biochemical assays were performed to estimate total polyphenols, flavonoids, tannins, reducing power, hydroxyl radical scavenging activity and superoxide radical scavenging activity of all herbal preparations. Before testing, the herbal preparations were dissolved in their respective solvents (section 3.1) and stock solutions were prepared. To avoid interference of herbal suspension's colour in the colorimetric assays, appropriate drug controls were kept in each experiment. Each experiment was repeated three times with three replicates in each group.

a. *Estimation of total polyphenolic content*

Total polyphenolic content was determined using Folin-Ciocalteau method as per the protocol of Singleton and Rossi (1965).

Principle: Reduction of phosphomolybdic/phosphotungstic acid complex by polyphenols produces soluble reaction product which is blue in colour, having absorption maxima at 765 nm. Colour intensity is proportional to polyphenol concentration.
Procedure:
1. One volume of the herbal suspension was mixed with 5 volumes of Folin-Ciocalteu reagent (50%).
2. The reaction was allowed for 5 minutes at room temperature.
3. One volume of Na$_2$CO$_3$ (5%) was added to 10 volume of the reaction mixture and reaction was allowed in dark for 10 minutes at room temperature.
4. Absorbance was recorded at 765 nm using UV-VIS spectrophotometer.
5. To carry out concentration dependent study each herbal preparation was tested at concentration ranging from 0 to 500 µg/ml in steps.
6. The gallic acid (C$_7$H$_6$O$_5$) was used to prepare the standard graph.

\(b.\) *Estimation of flavonoid content*

Total flavonoids in each herbal preparations were quantified by colorimetric method using aluminium chloride as per protocol described by Zhishen et al., (1999).

Principle: Aluminum chloride forms acid stable complex with one of the following groups i.e. C-4 keto group, C-3 hydroxyl group or C-5 hydroxyl group of flavones and flavonols. The complex is soluble in NaOH, produces yellow colour, which has absorbance maxima at 410 nm. Intensity of colour produced is directly proportional to flavonoid concentration.

Procedure:
1. To one volume of herbal suspension, four volumes of distilled water was added followed by addition of 1/7th volume of NaNO$_2$ (5%).
2. Reaction mixture was allowed to stand at room temperature for 5 minutes.
3. AlCl$_3$ (10%) was added in reaction mixture and one minute later 2 volumes of NaOH (1 M) was added and total volume was made up to 1 ml with Milli Q (Millipore corporation, Germany) water.
4. Absorbance of resultant mixture was recorded at 410 nm using UV-VIS spectrophotometer.
5. To carry out concentration dependent study each herbal preparation was tested at different concentration ranging from 0 to 500 µg/ml in steps.
6. Quercetin (C$_{15}$H$_{10}$O$_7$) was used to prepare standard graph.
c. *Estimation of tannin content*

Tannin content was evaluated by method of Hagerman and Butler (1978).

Principle: The condensed and hydrolysable tannins are first precipitated by standard protein, BSA at pH 4.9. The precipitate is then dissolved at pH higher than 4.9 in the presence of a suitable detergent to obtain red coloured iron phenolate complex which is then quantified spectrophotometrically at 510 nm. Intensity of colour produced is proportional to tannin content.

Procedure:
1. Powdered herbal preparation was dissolved in methanol.
2. To one volume of the herbal suspension, two volumes of BSA [0.1 % in acetic acid buffer (CH₃COOH =0.2 M): (NaCl =0.17 M) buffer, pH 4.9], was added, samples were immediately mixed and maintained at 4 °C for 24 h.
3. Reaction mixture was centrifuged at 4100 x g for 15 minutes.
4. Supernatant was discarded and the precipitate was dissolved in buffer containing SDS (1 %) and TEA (5 %).
5. To three volumes of reaction mixture one volume FeCl₃ (0.01 M in 0.01 M HCl) was added.
6. Absorbance of the resultant mixture was recorded at 510 nm using UV-VIS spectrophotometer.
7. To carry out concentration dependent study each preparation was tested from 0 to 500 µg/ml in steps.
8. Tannic acid (C₇₆H₅₂O₄₆) was used to prepare standard graph.

*d. Reducing power estimation*

The reducing power of the given herbal preparations was evaluated using method developed by Yen and Duh (1993).

Principle: Reduction of Fe³⁺/ferricyanide complex to ferrous form (Fe²⁺) by reducing agents, forms Pearl's Prussian blue colour (absorption maxima at 700 nm). Intensity of colour is proportional to reducing power.
Procedure:
1. Herbal suspension, potassium ferricyanide (1 %) and phosphate buffer (0.2 M, pH 6.5) were mixed in 5:2:2 ratios (v/v/v).
2. Reaction mixture was heated in water bath (50 °C) for 20 minutes.
3. Mixture was cooled down to room temperature and to one volume of reaction mixture 0.1 volume of TCA (10 %) was added.
4. Reaction mixture was centrifuged for 10 minutes at 3,000 rpm and supernatant was collected.
5. To one volume of supernatant, five volumes of distilled water and one volume of ferric chloride (0.1 %) were added.
6. Reaction was allowed at 37 °C for 10 minutes and absorbance of green coloured chromogen was recorded at 700 nm.
7. To carry out concentration dependent study each herbal preparation was tested from 0 to 500 µg/ml in steps.

**Estimation of hydroxyl radical (·OH) scavenging activity**

Hydroxyl radical scavenging activity of given herbal preparation was estimated by deoxy D-ribose assay developed by Halliwell et al., (2004).

**Principle:** The ·OH (generated either by Fenton reaction or by gamma irradiation)) when reacts with deoxy D-ribose, forms malondialdehyde (MDA). MDA upon reaction with TBA forms TBA-MDA-TBA complex (pink coloured) having absorption maxima at 532 nm. An agent having ability to scavenge ·OH inhibits the formation of MDA and hence inhibits the colour formation. ·OH scavenging activity is expressed in terms of percentage inhibition of MDA formation.

**Procedure:**
(i) Estimation of ·OH scavenging activity (·OH generated by Fenton reaction)
1. To one volume of herbal suspension, five volume PBS, one volume FeSO₄·7H₂O (10 mM), and five volume deoxy D-ribose (5 mM) were added.
2. Reaction was allowed at 37 °C for 30 minutes. This was followed by addition of five volumes of TBA (1 % w/v in 0.05 M NaOH) and five volumes of TCA (1
% to the reaction mixture. The reaction mixture was heated in boiling water bath for 15 minutes.

3. The resultant mixture was allowed to cool at room temperature and centrifuged at 2000 x g.

4. Absorbance of supernatant was recorded at 532 nm in a UV-VIS spectrophotometer.

5. Percentage inhibition of MDA formation was calculated by the formula:

\[
\frac{(A_{\text{reference}} - A_{\text{sample}})}{A_{\text{reference}}} \times 100
\]

6. A graph showing percent inhibition of MDA formation versus concentration of drug (\(\mu g/ml\)) was plotted. The concentration of drug required for 50 % inhibition (IC\(_{50}\)) was read from the graph and was recorded as IC\(_{50}\) for each test solution.

(ii) Estimation of \(^\cdot\)OH scavenging activity (\(^\cdot\)OH generated by irradiation)

1. To one volume of herbal suspension, five volumes of PBS and five volume of deoxy D-ribose (5 mM) were added.

2. After 10 minutes, the reaction mixture was exposed to \(^{60}\)Co-gamma radiation (200 Gy).

3. Steps 2 to 5 were same as that described part (i) of the section (given above).

f. Determination of superoxide radical scavenging activity

The superoxide radical (O\(_2^-\)) scavenging activity of given herbal preparation was determined by the method described by Nishikimi et al., (1972).

Principle: Mixture of NADH and phenazene methosulphate (PMS) generates O\(_2^-\) by univalent oxidation of reduced PMS. Nitro blue tetrazolium (NBT) in the presence of O\(_2^-\) gets reduced to form stable formazan (purple colour), having absorption maxima at 560 nm.

Procedure:

1. Herbal suspension, sodium pyrophosphate buffer (0.052 M, pH 8.2), PMS (186 \(\mu M\)) and NBT (300 \(\mu M\)) were mixed in 1:12:1:3 ratios (v/v/v/v).
2. Reaction was initiated by addition of NADH (780 µM).
3. Reaction mixture was incubated at 37 °C for 90 seconds.
4. 1 ml acetic acid was added to stop the reaction.
5. 4 ml n-butanol was added to the reaction mixture, allowed to stand for 10 minutes at room temperature and centrifuged at 2000 rpm for 10 minutes.
6. Intensity of chromogen in butanol was recorded at 560 nm.
7. Reaction mix without herbal preparation was treated as reference.
8. Percentage inhibition of superoxide radicals was calculated by the following formula:

\[
\left( \frac{A_{\text{reference}} - A_{\text{sample}}}{A_{\text{reference}}} \right) \times 100
\]

9. A graph showing percent inhibition of NBT reduction versus concentration of drug (µg/ml) was plotted. The concentration of drug required for 50 % inhibition was read from the graph and was recorded as IC50 for each test solution.

3.9.2 Estimation of damage to biological membranes

To study the efficacy of different herbal drugs to counter radiation induced damage to biological membranes, two assays (red blood cell haemolysis and lipid peroxidation) were performed with all the drugs. Red blood cells (RBC) haemolysis was studied with RBCs isolated from freshly drawn blood from healthy mouse. Lipid peroxidation was performed with liver excised from healthy mouse.

a. Estimation of RBC haemolysis

RBC haemolysis was determined by the method of Kuang et al., (1994).

Principle: Disturbance in RBC membrane structure and/or permeability of membrane increase the flux of water inside the cell which in turn leads to RBC haemolysis giving red colour (absorption maxima at 540 nm). Percentage inhibition of RBC haemolysis is calculated by decrease in red colour in comparison to untreated control.
Procedure:
The whole blood was collected in sterile heparin coated containers and centrifuged at 1000 x g for 10 minutes at 4 °C. Supernatant and whitish buffy coat was removed with a pipette. Pellet containing RBCs was washed three times with normal saline, (0.9 % w/v, pH 7.2) and centrifuged for 10 minutes at 1000 × g, 4 °C. The pellet was resuspended in PBS (pH 7.4) and incubated for 5 minutes under air atmosphere at 37 °C.

1. Different concentration of herbal preparation (0 to 500 μg/ml) was added to RBC suspension and allowed to stand for 10 minutes.
2. Reaction mixture was exposed to ⁶⁰Co-gamma radiation (200 Gy).
3. After 30 minutes, aliquots of the reaction mixture were diluted with NaCl (0.15 M) and centrifuged at 2000 rpm for 10 minutes to separate the RBCs.
4. Supernatant was taken and absorbance was recorded at 540 nm.
5. For reference, completely haemolysed RBC suspension was prepared by treating RBCs with distilled water and absorbance was recorded at 540 nm.
6. Percentage inhibition of RBC haemolysis was calculated using following formula:-

\[
\left(\frac{A_{\text{Reference}} - A_{\text{Sample}}}{A_{\text{Reference}}}\right) \times 100
\]

7. A graph showing percent inhibition of RBC haemolysis versus concentration of drug (μg/ml) was plotted. The concentration of drug required for 50 % inhibition was read from the graph and was recorded as IC₅₀ for each test solution.

b. Estimation of lipid peroxidation

Lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) by the method of Ohkhawa et al., (1979).

Principle: Cellular membrane has lipid protein bilayer subunits. Malondialdehyde (MDA) is product of lipid peroxidation produced from the breakdown of polyunsaturated fatty acids. MDA upon reaction with TBA forms TBA-MDA-TBA complex (pink coloured), having absorption maxima at 532 nm.
Procedure:
1. Animals were sacrificed, liver was excised, rinsed with water, blot dried and weighed.
2. Liver homogenate was prepared in phosphate buffer (pH 7.2).
3. Total protein content was estimated by the Bradford assay (3.9.2.c).
4. 10% liver homogenate was prepared in PBS.
5. Different concentration of herbal preparation (0 - 500 μg/ml) was added to liver homogenate and allowed to stand for 10 minutes.
6. Reaction mixture was exposed to $^{60}$Co-gamma radiation (100 Gy).
7. Tissue homogenate, TBA (0.67%) and TCA (10%) were mixed in 1:1:1 ratios (v/v/v).
8. Reaction mixture was placed in a boiling water bath for 15 minutes.
9. Samples were allowed to cool at room temperature and then centrifuged for 10 minutes at 1000 x g.
10. Absorbance of supernatant was recorded at 535 nm against the water blank.
11. MDA concentration was calculated using molar extinction coefficient of MDA $1.52 \times 10^5 \text{ nM}^{-1} \text{ cm}^{-1}$. Results were expressed as nmoles MDA /mg protein.
12. A graph showing percent inhibition of MDA formation versus concentration of drug (μg/ml) was plotted. The concentration of drug required for 50% inhibition was read from the graph and was recorded as IC$_{50}$ for each test solution.

C. Estimation of total protein content

Protein was estimated using method of Bradford (1976).

Principle: The Commassie brilliant blue G-250 dye when mixed with phosphoric acid and ethanol in a fixed ratio (Bradford reagent) has an absorbance maximum at 465 nm. Binding of protein to Bradford reagent causes shift in the absorption maxima to 595 nm.
Procedure:

1. This assay was done in triplicate. Sample was suitably diluted with diluent to make volume up to 0.1 ml.
2. In 0.1 ml sample, 1 ml Bradford reagent was added and mixed.
3. Absorbance was recorded at 595 nm after 5 minutes.
4. Bovine serum albumin (BSA) was used to prepare the standard graph.

3.9.3 Biochemical assays after drug treatment in vivo

Biochemical assays such as total thiols, ferric reducing ability of plasma (FRAP), catalase (CAT) activity, lipid peroxidation, superoxide dismutase (SOD) activity, glutathione-s-transferase (GST) activity, glutathione reductase (GR) activity, alkaline phosphatase, myeloperoxidase and free iron content were performed in blood, liver and spleen excised from animals treated with drug and/or radiation as described in section 3.6 and 3.7. Animals were sacrificed humanely at different time points after treatment; tissues were excised and processed as described below:

Preparation of samples

a. Isolation of plasma/serum

Blood was drawn from the retro-orbital sinus of anesthetized animals and serum was separated from other blood components. For isolation of plasma, blood was collected in EDTA coated tubes. Plasma was separated from other blood components by centrifugation.

b. Preparation of tissue homogenate

Tissue homogenate of liver as well as spleen was prepared in buffer using homogenizer. For further experimental work, the tissue homogenate was divided into two parts. One part was aliquoted in several smaller volumes (capacity 0.5 ml) and kept at -80 °C until use. Another part was centrifuged and supernatant was collected. Supernatant was aliquoted in several smaller volumes (capacity 0.5 ml) and kept at -80 °C.
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Ferric reducing ability of plasma (FRAP)

FRAP was evaluated by the method of Benzie and Strain (1996). This assay is based on the reduction of ferric ions by various plasma constituents such as vitamin C, vitamin E, bilirubin and uric acid.

Principle: This method is based on the principle that at low pH, ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex, gets reduced to ferrous form (Fe^{2+}), in the presence of reducing agents, and has absorption maxima (blue colour) at 593 nm. Intensity of the colour is proportional to FRAP.

Procedure:
1. Plasma, distilled water and FRAP reagent (25 ml of 300 mM acetate buffer pH 3.6, 2.5 ml of 10 mM of 2,4,6-tripyridyl-s-triazine solution in 40 mM / liter HCl, 2.5 ml of 20 mM of FeCl_{3.6}H_{2}O ), were mixed in 1:3:30 ratio (v/v/v).
2. After 5 minutes absorbance was recorded at 593 nm.
3. FeSO_{4} was used as standard to plot graph.

Estimation of total thiols

Total thiols were estimated in tissue homogenate by the method of Sedlak and Lindsay (1968).

Principle: Thiols react with 5-5'-dithio-bis, 2-nitrobenzoic acid (DTNB) by cleaving disulfide bond to form 2-nitro-5-thiobenzoate (NTB'), which further ionizes to form NTB^{2-} in water at neutral and alkaline pH. This NTB^{2-} ion has absorption maxima (yellow colour) at 412 nm. Intensity of chromogen is proportional to thiol content.

Procedure:
1. In 1.6 ml reaction mixture, the sample homogenate, TRIS buffer (0.2 M, pH 8.2) and DTNB were mixed in 5:10:1 ratio (v/v/v).
2. Total volume was made up to 10 ml with absolute methanol.
3. Test tube were closed and allowed to stand for 30 minutes at room temperature with occasional shaking.
4. Reaction mixture was centrifuged at 3000 x g for 5 minutes at room temperature.
5. Absorbance of supernatant was recorded at 412 nm.
6. Glutathione was used to plot standard graph.

iii. Lipid peroxidation

Lipid peroxidation was estimated in tissue homogenates as described earlier section 3.9.2b (step 4 to 9).

iv. Glutathione reductase (GR) activity

GR activity was evaluated by the method of Carlberg and Maunervik (1975).

Principle: This assay is based on the oxidation of NADPH to NADP⁺ catalyzed by glutathione reductase. One GR activity unit is defined as the amount of enzyme catalyzing the reduction of one micromole of GSSG per minute at 25 °C, pH 7.6. One molecule of NADPH is consumed for each molecule of GSSG reduced. Therefore, the reduction of GSSG is determined indirectly by measuring the consumption of NADPH, as demonstrated by a decrease in absorbance at 340 nm (A₃₄₀) as a function of time.

Procedure:
1. The reaction was set up by mixing sodium phosphate buffer (0.10 M, pH 7.6), EDTA (0.5 mM), GSSG (1 mM) and NADPH (0.1 mM) and tissue supernatant in 7:1:1:1:1 ratio (v/v/v/v/v).
2. Absorbance was recorded every 10 seconds at 340 nm for next 3 minutes.
3. Enzyme activity was calculated by using molar extinction coefficient of NADPH and expressed as mU/mg protein

\[
mU/ml = \frac{A_{340/minute}}{6.22 \times 10^{-3} \text{nM/ml}}
\]

Where, \(A_{340/minute}\) is the absorbance change/minute at 340 nm;
\(6.22 \times 10^{-3} \text{nM/ml}\) is the molar extinction coefficient of NADPH.
v. **Glutathione-s-transferase (GST) activity**

Glutathione transferase activity was estimated spectrophotometrically using 1-chloro, 2,4-dinitrobenzene (CDNB) as substrate by the method of Habig et al., (1971).

**Principle:** GST catalyzes the conjugation of CDNB with reduced glutathione (GSH) to produce CDNB-GSH complex which has absorption maxima at 340 nm.

**Procedure:**
1. Reaction was set up by mixing phosphate buffer (10 mM, pH 6.5), GSH (30 mM) and CDNB (30 mM) in 10:1:1 ratios (v/v/v).
2. The mixture was incubated at 37 °C for 2 minutes.
3. 0.1 ml of diluted tissue supernatant was added to start the reaction.
4. Absorbance was recorded every 10 seconds at 340 nm for next 3 minutes.
5. The specific activity of GST was expressed as mM conjugate (GSH-CDNB) formed min⁻¹ mg⁻¹ protein using an extinction coefficient of 9.6 mM⁻¹ cm⁻¹.

\[
\text{mU/ml} = \frac{A_{340}/\text{minute}}{9.6 \times \text{mM}^{-1} \text{cm}^{-1}}
\]

Where, \( A_{340}/\text{minute} \) is the absorbance change/minute at 340 nm;

9.6 mM⁻¹ cm⁻¹ is the molar extinction coefficient of CDNB-GSH complex.

vi. **Catalase activity**

Catalase activity was assayed spectrophotometrically according to hydrogen peroxide (H₂O₂) degradation protocol of Claiborne, 1985.

**Principle:** This assay is based on breakdown of H₂O₂ into water (H₂O) and oxygen gas (O₂) by catalase. H₂O₂ has absorption maxima at 240 nm. Change in \( A_{240} \) is considered index of catalase activity.
Procedure:
1. Reaction was set up by mixing H₂O₂ (10 mM) and phosphate buffer (60 mM) in 10:1 ratio (v/v).
2. Reaction was started by addition of tissue supernatant (0.05 ml) and absorbance was recorded every 10 seconds at 240 nm for next 3 minutes.
3. The extinction coefficient of 0.04 mM⁻¹ cm⁻¹ was used to determine the specific activity of catalase. Results were expressed as mM H₂O₂ consumed min⁻¹ mg⁻¹ protein.

\[
mU/ml = \frac{A_{240}/\text{minute}}{0.04 \text{ mM}^{-1} \text{ cm}^{-1}}
\]

Where, \(A_{240}/\text{minute}\) is the absorbance change/minute at 340 nm; 0.04M⁻¹cm⁻¹ is the molar extinction coefficient of catalase.

vii. Superoxide dismutase activity

Tissue homogenates were prepared as described in section 3.9.2. Assay reaction was set up as per protocol described in earlier section 3.9.1(f).

viii. Alkaline phosphatase (ALP) activity

The assay was performed according to the method of Kojima and Sakurada, 1976.

Principle: p-nitrophenyl phosphate (colourless) is hydrolysed by alkaline phosphatase (pH 10, 37 °C) into yellow coloured p-nitrophenol, which has absorbance maxima at 405 nm. Colour intensity is proportional to p-nitrophenol.

Procedure:
1. Sodium carbonate buffer (0.1 M, pH 10), p-nitrophenyl phosphate (5 mM) and tissues samples were added in 9:1:10 ratios (v/v/v) (total volume 2 ml).
2. Reaction was allowed for 5 minutes at room temperature, absorbance was recorded at 405 nm.
3. Concentration of alkaline phosphatase was estimated in terms of production of p-nitrophenol. p-nitrophenol was used to prepare standard graph.
ix. **Myeloperoxidase (MPO) activity**

The assay was performed according to the method of Hillegass et al., 1990.

**Principle:** In the presence of H₂O₂ as oxidizing agent MPO catalyzes the oxidation of o-dianisidine (reduced, colourless) into brown coloured product (oxidized o-dianisidine), which has absorbance maxima at 470 nm.

**Procedure:**
1. Tissue homogenate was prepared in the potassium phosphate buffer (50 mM, pH 6.0), containing HTAB (0.5 %), centrifuged for 2 minutes at 14,000 rpm and supernatant was collected.
2. Phosphate buffer (50 mM, pH 6.0), o-dinasidine (16 mM), H₂O₂ and samples were added in 1:1:2 ratios (v/v/v), in total volume of 910 μl.
3. Absorbance change was recorded at at 460 nm at 10 seconds interval for 5 minutes.
4. One mU of myeloperoxidase caused absorbance change of 0.0113/min at 460 nm. Results were expressed as mU/mg protein.

\[ \text{MPO (mU/ml)} = \frac{\text{A}_{460}/\text{minute}}{0.0113/\text{minute}} \]

Where, \( \text{A}_{460}/\text{minute} \) is the absorbance change/minute at 460 nm.

x. **Ferrozine assay for free iron**

The assay was performed according to the method of Sohal et al., 1999.

**Principle:** Free iron (Fe³⁺) is reduced into the bivalent form (Fe²⁺) by ascorbic acid. Ferrous ions (Fe²⁺) when reacts with ferrozine a coloured complex form. The intensity of the color formed is proportional to the iron concentration in the sample.

**Procedure:**
1. To one volume of tissue homogenate one volume of HCl (10 mM) was added.
2. Reaction mixture was heated for 30 minutes at 85 °C.
3. After cooling down reaction mixture was centrifuged at 10000 rpm for 5 minutes.
4. In five volumes of supernatant, one volume TCA (40 %) was added.
5. Reaction mixture was heated at 85 °C for 15 minutes. After cooling, reaction mixture was centrifuged at 10000 rpm for 5 minutes.

6. In one volume of supernatant, ten volumes of ferrozine solution (1.05 M in sodium acetate, pH 4.8) was added.

7. Absorbance was recorded at 570 nm.

8. FeCl₃ was used to prepare standard graph.

3.10 Haematological analysis

Hematological analysis in mouse blood was done using automated haematology analyser sysmex 4500 (Japan) as per instruction of manufacturer. Differential leukocyte count was performed manually by preparing blood smear and then staining with Leishmann stain. Different types of cells were observed in microscope under oil immersion.

3.11 Immunological assays

Enzyme linked immunosorbent immunoassay (ELISA) was used to estimate TNF-α, IL-10, TGF-β, HMGB1 and total IgG in the serum. ELISA was also used to estimate GM-CSF and VEGFR-3 in serum as well as in spleen. The spleen was suspended in ice-cold PBS containing detergent and then homogenized at 4 °C. The homogenate was kept at -80 °C for 20 minutes. Thereafter, tissue was thawed at 4 °C. Freeze thaw cycle was repeated three times. Homogenate was centrifuged at 10000 rpm for 20 minutes at 4 °C, and the supernatants were collected, aliquoted and stored at -80 °C for cytokine analysis. The method described in section 3.9.2 was used to isolate serum.

Principle: The basic principle of ELISA is to use an enzyme conjugated antibody to quantify antigen/or an antibody. First the antigen antibody reaction is allowed. The enzyme conjugated to antibody is then used to convert a suitable colourless substrate to a coloured product, which is quantified colorimetrically.

The TNF-α, IL-10 and TGF-β were quantified by sandwich ELISA using kits from (BD biosciences, US) as per manufacturer’s instructions. HMGB-1 was quantified using kit from Shino Test Corporation, Germany, as per manufacturer’s instructions.
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Material and Methods

Total IgG levels were determined using direct ELISA protocol (Hornbeck et al., 1991).

1. Wells of microplates were coated with 50 μl serum, covered and incubated for 16 h at 4 °C.
2. Wells of microplate were washed three times, blotted onto absorbent paper and blocked by adding 200 μl blocking buffer (5 % BSA in PBS) per well, incubated for 2 h at room temperature.
3. Plate was washed three times with wash buffer and antibody conjugated with enzyme was added (100 μl/well), incubated for 2 h at room temperature.
4. Plate was washed four times with wash buffer and substrate solution was dispensed in microwell plate (100 μl/well).
5. After development of colour, reaction was stopped by adding 100 μl of stop solution to the wells and absorbance was recorded at 490 nm.
6. Standard graph was plotted using pure IgG (Sigma Chemical Co., US).

Indirect ELISA was used to quantify the levels of GM-CSF and VEGFR-3 in serum as well as spleen (Hornbeck et al., 1991).

1. 96 well microplate was coated with 50 μl serum/spleen samples (diluted in carbonate buffer), covered and incubated for 16 h at 4 °C.
2. Plate was washed with wash buffer and blotted onto absorbent paper.
3. Wells were blocked with 200 μl blocking buffer (5 % serum in PBS) per well, covered and incubated for 2 h at room temperature.
4. Plate was washed two times and 100 μl of diluted primary antibody (GM-CSF or anti VEGFR-3) was added to each well, covered, incubated for 2 h at room temperature.
5. Plate was washed four times and 100 μl of enzyme conjugated secondary antibody was added into microwell plate, covered, incubated for 2 h at room temperature.
6. Plate was washed four times and 100 μl substrate solution was dispensed per well.
7. After development of colour 50 μl of stop solution was added to the wells and absorbance was recorded at 490 nm.
8. Standard graph was plotted using pure GM-CSF and VEGFR-3 proteins.
3.12 Assays for estimating DNA damage

The micronuclei assay was used to study the DNA damage in bone marrow and in peripheral blood. The pulsed field gel electrophoresis (PFGE) was used to study DNA damage in spleen.

3.12.1 Micronuclei assay

Micronuclei assay was performed by the method of Schmid (1975), standardized in our laboratory.

Procedure:

1. Mice were sacrificed humanely. The femur bones of hind legs were dissected out, cleaned and kept in ice cold saline in a petridish.

2. Femur head was removed and both the femurs were flushed with 2 ml normal saline and volume was made up to 5 ml, mixed and centrifuged at 1000 rpm for 10 minutes.

3. Supernatant was discarded and pellet was washed three times with normal saline.

4. The washed cell pellet was mixed with 2-3 drops of fetal calf serum (FCS) and mixed gently.

5. Cell smear from bone marrow or heparinized blood was prepared on glass slides and air dried for 24 h. 6-7 slides were prepared from each mouse.

6. Protocol for staining of slides is given Table 3.1.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Steps/Reagent</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Methanol (slides were fixed)</td>
<td>10 minutes</td>
</tr>
<tr>
<td>2.</td>
<td>Giemsa stain (stained)</td>
<td>2-3 minutes</td>
</tr>
<tr>
<td>3.</td>
<td>Distilled water (washing)</td>
<td>2 minutes</td>
</tr>
<tr>
<td>4.</td>
<td>Distilled water (washing)</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Air dry</td>
<td>30 minutes</td>
</tr>
<tr>
<td>6.</td>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>7.</td>
<td>DPX mounting and air drying</td>
<td></td>
</tr>
</tbody>
</table>
Slides were visualized under light microscope and observations were recorded using camera.

3.12.2 Pulse field gel electrophoresis (PFGE) to study DNA damage

PFGE was performed using Bio-Rad protocol.

1. Spleen single cell suspension was prepared as described in section 3.7. The spleen cell suspension (2 x 10^7 cells/ml) was mixed with low melting agarose (2 %, 50 °C) in 1:1 ratio and pipetted onto ice cold plastic moulds.
2. After complete solidification, the agarose blocks were transferred to 1 ml lysis solution.
3. Plugs were incubated in lysis buffer at 50 °C for 24 h. Subsequently, the blocks were washed four times with wash buffer containing PMSF for 3 h followed by washing with TE buffer to remove PMSF.
4. Plugs were stored in EDTA (0.5 M) at 4 °C until use.
5. Electrophoresis was carried out in agarose gel (1 % molecular grade).
6. The agarose blocks were cut into equally sized plugs and inserted into the wells.
7. The wells were sealed with 1 % low melting agarose.
8. Plugs containing Schizosaccharomyces pombe (S. pombe) artificial chromosomes DNA ladder were loaded in one lane as DNA size standards.
9. The electrophoresis was performed in a CHEF-Mapper apparatus at 14 °C in TAE buffer (0.5 x, pH 8.3) at 1.5 V/cm. After completion of electrophoresis, gel was removed from the tank and stained with ethidium bromide.
10. Picture was captured using gel documentation system.
11. Densitometric analysis of gel was done using NIH imageJ software (http://rsb.info.nih.gov/nih-image).

3.13 DNA/RNA studies

3.13.1 Nucleic acid quantification

Principle: Purines and pyrimidines in nucleic acid show absorption maxima at 260 nm. One absorbance unit at 260 nm corresponds to 50 μg/ml of double stranded DNA. Proteins absorb at 280 nm. The ratio of A260/A280 is used to assess the purity of
the DNA sample. For pure DNA, the observed $A_{260}/A_{280}$ ratio is near 1.8, elevated ratios indicate the presence of RNA. One absorbance unit at 260 nm corresponds to 40 µg/ml of RNA. Phenolate ion and other organic compounds absorb at 230 nm. The ratio of $A_{230}/A_{260}$ more than 0.5 indicates the presence of contamination.

Procedure:
1. Nucleic acid samples were suitably diluted and absorbance at 260 nm as well as 280 nm was recorded.
2. Ratio of $A_{260}/A_{280}$ was taken to check purity of nucleic acid.
3. DNA concentration was calculated using the formula:

   $$\text{DNA concentration (µg/ml)} = A_{260} \times 100 \times 50 \text{ µg/ml}$$

3.13.2 Agarose gel electrophoresis

Procedure:
1. 1% agarose gel containing ethidium bromide (EtBr) was casted in TRIS acetate buffer (TAE). Electrophoresis was carried out at room temperature under submerged gel conditions using TAE buffer (pH 8.0) at 50 V/cm.
2. The gel pictures were captured using gel documentation system (Night Hawk, UK).
3. The densitometry and data analysis were performed using UVP software, Labworks 4.0 (UK).

3.14 Study on changes in key molecular entities

Changes in expressions of TWEAK and Fn14 were studied using RT-PCR. Changes in expressions of CD4, CD8, CD14, bcl-2 and bax were studied using western blotting.

3.14.1 RT-PCR studies

RNA from liver was extracted using RNA isolation kit (Qiagen, US) as per manufacturer's instructions. RNA purity was checked by $A_{260}/A_{280}$ ratio as described in section 3.13. RNA quantification was done spectrophotometrically by formula:
RNA concentration (µg/ml) = A_{260} x 100 (dilution factor) x 40 µg/ml. RT-PCR was performed using one step RT-PCR kit (Qiagen, US) as per manufacturer's instructions. Assays were performed in triplicates using iCycler (Bio-Rad, US). Primer sequences for TWEAK and FN14 were as detailed in Jakubowski et al., 2005.

Murine TWEAK primer sequences were as follows:

forward, 5'-CGAGCTATTGCAGCCCATTAT-3';
backward, 5'-ACCTGCTTGTGCTCCATCCT-3'.

Murine Fn14 primer sequences were as follows:

forward, 5'-CTAGTTTCCTGGTCTGGAGAAGATG- 3';
backward, 5'-CCCTCTCCACCAGTCTCCTCTA-3'.

Table 3.2: Steps for RT-PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>Number of cycles</th>
<th>Temperature</th>
<th>Time</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>One</td>
<td>50 °C</td>
<td>30 minutes</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>2</td>
<td>One</td>
<td>95 °C</td>
<td>10 minutes</td>
<td>Activation of Taq polymerase and deactivation of reverse transcriptase</td>
</tr>
<tr>
<td>3</td>
<td>Thirty</td>
<td>Step 1</td>
<td>95 °C</td>
<td>45 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Step 2</td>
<td>58.5 °C</td>
<td>45 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Step 3</td>
<td>72 °C</td>
<td>1 minute</td>
</tr>
<tr>
<td>4</td>
<td>One</td>
<td>72 °C</td>
<td>10 minutes</td>
<td>Final extension</td>
</tr>
</tbody>
</table>

After final extension samples were run on agarose gel to check the amplified product. To further confirm the formation of desired product sequencing of PCR amplicons was done using commercial services. The sequences of PCR amplicons were compared with TWEAK and FN14 sequence available in NCBI database (www.ncbi.nlm.nih.gov/blast.cgi) using BLAST search engine.
3.14.2 Western blotting

Protein was isolated from spleen using Protein isolation kit (Qiagen, US) following manufacturer's instructions. Protein was quantified using Bradford assay described in section 3.9.2c. For sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) protein samples were diluted 1:1 (v/v) with 2x SDS sample buffer and heated for 3 to 5 minute at 100 °C in a sealed screw-cap microcentrifuge tube. Protein was loaded carefully into the wells of precasted stacking gel (6 %) and separating gel (12 %). The vertical gel electrophoresis was carried out at 26 °C ± 2 °C using TRIS HCl/SDS buffer (pH 8.8). The voltage used for electrophoresis in stacking gel was 50 V, while after migration of protein tracking dye in separating gel, voltage was maintained at 70 V. After the bromphenol blue tracking dye reached the bottom of the separating gel, electrophoresis was stopped, gel was removed and kept in ice cold transfer buffer. For protein transfer the PVDF membrane having same dimensions as that of the gel was dipped in methanol for 15 seconds, placed in purified water for 2 minutes and transferred to pre-chilled transfer buffer for 15 minutes. Fiber pads (2 per gel), filter papers were kept separately in pre-chilled transfer buffer. Gel was assembled with treated PVDF membrane as per manufacturer's instruction and set for transfer at constant voltage of 100 V for 40 minutes. After transfer the gel holder cassette from tank was removed and membrane was removed carefully and placed in blocking buffer for 16 h at 4 °C. The membrane was washed three times for 5 minutes each with TRIS buffered saline with tween 20 (TBST). Membrane was treated for 2 h at room temperature with primary antibody diluted in the blocking buffer. Membrane was washed three times with TBST and incubated with secondary antibody conjugated to HRP. After washing in TBST membrane blot was developed by incubation with the substrate. Band formed were quantified by densitometry using NIH imageJ software (http://rsb.info.nih.gov/nih-image).

3.15 Study of histological changes

Histopathological changes in spleen and liver were studied by staining the tissue sections with haematoxylin and eosine. Liver and spleen were fixed in 10 % formalin.
Tissues were then processed for dehydration, clearing and impregnated into wax blocks using standard protocol summarized in Table 3.3.

**Table 3.3: Tissue processing steps for histology.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydration</td>
<td>30 % alcohol</td>
<td>1 hour</td>
</tr>
<tr>
<td></td>
<td>50 % alcohol</td>
<td>1 hour</td>
</tr>
<tr>
<td></td>
<td>70 % alcohol</td>
<td>overnight</td>
</tr>
<tr>
<td></td>
<td>90 % alcohol</td>
<td>1 hour</td>
</tr>
<tr>
<td></td>
<td>100 % alcohol (I)</td>
<td>2 hour</td>
</tr>
<tr>
<td></td>
<td>100 % alcohol (II)</td>
<td>Overnight at room temperature</td>
</tr>
<tr>
<td>Clearing</td>
<td>Xylene I</td>
<td>1 hours</td>
</tr>
<tr>
<td></td>
<td>Xylene II</td>
<td>1 hours</td>
</tr>
<tr>
<td>Wax embedding</td>
<td>Paraffin wax I</td>
<td>Overnight at 55 °C</td>
</tr>
<tr>
<td></td>
<td>Paraffin wax II</td>
<td>1 hour at 55 °C</td>
</tr>
<tr>
<td></td>
<td>Paraffin wax III</td>
<td>1 hours at 55 °C</td>
</tr>
<tr>
<td></td>
<td>Cooling of block</td>
<td></td>
</tr>
</tbody>
</table>

3.15.1 Section cutting

1. From paraffin wax embedded tissues 4 μ tissue sections were cut, floated on to purified water at 37 °C.
2. Floating tissue sections were picked up on microscope slides and drain thoroughly, and air-dried at 37 °C overnight.
3. Sections were then incubated at 60 °C until wax melted to improve adhesion.

3.15.2 Staining

Sections were stained using haematoxylene eosine stain. Detailed protocol involving steps are given in Table 3.4.
Table 3.4: Steps for staining histological sections with haematoxylin eosine.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Reagents</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Xylene</td>
<td>3 minutes</td>
</tr>
<tr>
<td>2</td>
<td>Absolute alcohol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>3</td>
<td>Harris haematoxyline</td>
<td>5 minutes</td>
</tr>
<tr>
<td>4</td>
<td>Absolute alcohol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>5</td>
<td>Acid alcohol (1 % HCl in 70 % alcohol)</td>
<td>1 minute</td>
</tr>
<tr>
<td>6</td>
<td>Tap water</td>
<td>30 seconds</td>
</tr>
<tr>
<td>7</td>
<td>Ammonia (2-3 drops)</td>
<td>Until blue color appear</td>
</tr>
<tr>
<td>8</td>
<td>Purified water</td>
<td>2 minutes</td>
</tr>
<tr>
<td>9</td>
<td>2 % eosin</td>
<td>3 minutes</td>
</tr>
<tr>
<td>10</td>
<td>Tap water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>11</td>
<td>Absolute alcohol</td>
<td>1 minute</td>
</tr>
<tr>
<td>12</td>
<td>Absolute alcohol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>13</td>
<td>Xylene</td>
<td>5 minutes</td>
</tr>
<tr>
<td>14</td>
<td>DPX Mounting and air dry</td>
<td></td>
</tr>
</tbody>
</table>

Slides were visualized under light microscope and observations were recorded using camera.

3.16 Statistical analysis

Results are expressed as the mean ± standard deviation of three different experiments. Polynomial regression was used to fit data points to make curve best fit. Pearson correlation coefficients were calculated to determine relation between free radical scavenging activity and inhibition of damage. R² values more than 0.7 were considered significant. To compare the two groups two tailed paired t-test was performed; \( p \leq 0.05 \) was considered significant. \( p \) values \( \leq 0.05 \) were considered 95 % significant while \( p \) values \( \leq 0.01 \) were considered 99 % significant. To do all statistical analysis Microsoft Word Excel was used.
Summary of Work Executed

Comparison of 2 different herbal preparations from leaves of *Hippophae rhamnoides* (SBL-1, SBL-2), *in vitro* and *ex vivo* studies

- Comparison of radioprotective potential of two different herbal preparations (*in vitro* studies)
- Comparison of radioprotective potential of two different herbal preparations (*ex vivo* studies)
- Phytochemical analysis

Comparison of radioprotective potential of SBL-1 and SBL-2 on the basis of *in vivo* biochemical studies

- Ferric reducing ability of plasma
- Total thiols
- Superoxide dismutase
- Catalase
- Lipid peroxidation

The most effective preparation, SBL-1, was selected and examined for protection of haemolymphoid system at lethal radiation doses (10 Gy)

Blood

- Whole blood
- Haematological analysis
- Lymphocytes
- Polymorphs
- Total leukocyte counts
- Platelets
- Hemoglobin
- Serum/Plasma
- DNA Damage
- Micronuclei
- Immunological entries/Inflammation
- HMGB1
- TNF-α
- GM-CSF
- sVEGFR3
- TGF-β
- IL-10
- MPO
- Free iron
- Total IgG

Bone Marrow

- DNA Damage
- Histological changes
- DNA Damage by PFGE
- Micronuclei
- Apoptotic bodies
- Protection against radiation induced oxidative stress

Spleen

- Cellular protection based on molecular markers
- Protection against radiation induced oxidative stress
- CD14
- CD8
- CD4
- Bcl-2
- Bax
- Catalase
- Superoxide dismutase
- Total thiol
- Free iron
- Lipid peroxidation

Liver

- Histological changes
- Liver regeneration based on molecular marker
- Protection against radiation induced oxidative stress
- CD14
- CD8
- CD4
- Bcl-2
- Bax
- Catalase
- Superoxide dismutase
- Total thiol
- lipid peroxidation