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Radioprotection by a herbal preparation of *Hippophae rhamnoides,* RH-3, against whole body lethal irradiation in mice

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Summary

***Purpose*: *Hippophae rhamnoides* L. has been well documented to have anti-oxidative, immunostimulative and regenerative properties and therefore a herbal preparation of *H. rhamnoides* coded as RH-3 was investigated for its radioprotective action.**
***Materials and methods:* RH-3 was administered intraperitonially (i.p.) to mice 30 minutes before whole body irradiation and whole body survival, spleen Colony forming units (CFU) and haematological parameters were studied. To investigate free radical scavenging and antioxidant potential, Fenton reaction, radiation mediated OH radical scavenging and chemically generated superoxide anions scavenging were studied *in vitro* while inhibition of lipid peroxidation was studied in liver homogenate of mice.**
***Results:* A dose of 30 mg/kg body weight of RH-3 rendered 82% survival as compared to no survival in irradiated control. The endogenous CFU counts in mouse spleen on 10^{th} post-irradiation day with and without RH-3 demonstrated radioprotective effect. Various hematological parameters also corroborated the radioprotective effect of RH-3. In a dose dependent manner, RH-3 inhibited Fenton reaction and radiation mediated generation of hydroxyl radicals *in vitro*, superoxide anion mediated Nitroblue tetrazolium (NBT) reduction and $FeSO_4$ mediated lipid peroxidation in liver.**
***Conclusion:* Free radical scavenging, acceleration of stem cell proliferation and immunostimulation are the radioprotective attributes, which require further investigations.**

Key words: Radioprotection, *Hippophae rhamnoides*, lethal irradiation, herbal radioprotector, antioxidant

Introduction

Development of effective and non-toxic radioprotective agents is of considerable interest for radiation medicine, space flights, nuclear industries and emergencies. A large number of chemical and biological agents have been screened and reviewed in this connection (Weiss et al. 1990, Maisin 1992). Several molecular drugs of synthetic and natural origin are being tried in experimental models and human clinical trials to mitigate radiation injury caused by whole body gamma radiation exposure ranging from sub-lethal to supra-lethal doses. Among molecular radioprotectors, WR-2721 (Weiss et al. 1990) and related compounds have been the most promising so far. However, severe side effects such as nausea, vomiting, hypotension and neurotoxicity (Landauer et al. 1987) associated with most of the radioprotective agents tried at therapeutic levels, have restrained their use. Infact no radioprotective agent available today meets all the requisites of an

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ideal radioprotector (Maisin 1998). In view of this, search for newer more effective agents is inevitably continuing. Recently a number of plant products have been evaluated for radioprotective action (Shimoi et al. 1994, 1996, Goel et al. 1998, Umadevi et al. 1999). Our hypothesis has been that plant extracts eliciting radioprotective efficacy contain a large number of active constituents like antioxidants, immunostimulants, cell proliferation promoters cytokines etc. Some of them may individually as well as combinely render protection against radiation induced pathology. In this process some toxic effects could be generated which could be countered by several other types of molecules present in the whole extract. Immense interest is, therefore generating in herbal drugs globally.

H. rhamnoides L. (F. Elaeagnaceae), commonly known as sea buckthorn and native to Europe & Asia, is a deciduous shrub, 2–4 m in height, very hardy and salt tolerant. It can withstand –43 to 40 °C 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 slopes and is a nitrogen fixing plant. In Tibetean and Indian systems of medicine, for centuries, *H. rhamnoides* has been exploited for treatment of sluggish digestion, stomach malfunctioning (Nikitin et al. 1989, Xiao et al. 1992), circulatory disorders, ischemic heart disease (Liu et al. 1988, Zhang 1987), burn and wound healing (Ianev et al. 1995, Nikulin et al. 1992), hepatic injury (Cheng 1992, Cheng et al. 1994) and neoplasia (Nikitin et al. 1989, Luginov et al. 1983). *H. rhamnoides* contains a large number of constituents like flavonoids (Wu et al. 1994, Zhang 1987, Liu et al. 1988), vitamin A, C, E, and K, tannins (Ianev et al, 1995, Spirodonov et al. 1997) sugars, fats and various trace elements like Se, Zn, Cu, and S (Ianev et al. 1995). Due to these molecules, it can act as strong antioxidant (Wang et al. 1992) and inhibitor of succinate oxidation (Spirodonov et al. 1997). Radiation exposure induces lipid peroxidation (Von Sonntag 1987) and subsequently disturbs ion homeostasis especially the Ca^{++} influx (Trump et al. 1992). Dilitiazam, a known Ca^{++} channel blocker, could therefore render significant radioprotection (Goel et al. 1996). *H. rhamnoides* has also been reported to control Ca^{++} influx from extracellular to intracellular component (Wu et al. 1994) and therefore it may also inhibit radiation induced calcium mediated cytotoxicity.

For radioprotection, stimulation of stem cell proliferation, free radical scavenging, antioxidant, calcium channel blocking, immunostimulation and DNA repair enhancement are considered essential. This plant since has been demonstrated to have such properties, RH-3, a preparation of *H. rhamnoides,* was therefore investigated for its radioprotective efficacy in experimental animals exposed to whole body lethal gamma irradiation. Free radical scavenging properties were also studied under *in vitro* conditions.

Materials and methods

RH-3 preparation

Fresh whole berries were collected from Lahaulspiti (an altitude of about 3800 meters) in Himachal Pradesh, India and the plant was confirmed as *Hippophae rhamnoides* by comparison with the Voucher specimen (IHBT No. 1047) kept in herbarium of Biodiversity Centre at Institute of Himalayan Biosource Technology, Palampur, Himachal Pradesh, India. The berries were washed and shade dried. Known quantity of the dried material was extracted using absolute alcohol and tripled distilled water (50:50, v/v; three changes). Finally the extract was lyophilized, weighed and stored at 4 °C. RH-3 was a code name of this product prepared by Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India and was studied for its radioprotective properties in our Laboratory at Delhi. The preparation was diluted in triple-distilled water for desired concentration and the dose expressed in mg refers to weight of dried RH-3. HPLC-fingerprint analysis of the 50% alcoholic extract shows several peaks of flavonoids which ranged between 7 to 18 minutes retention time (see Figure HPLC).

Treatment

Desired doses of RH-3 were administered i.p. 30 minutes before whole body gamma radiation.

Animals

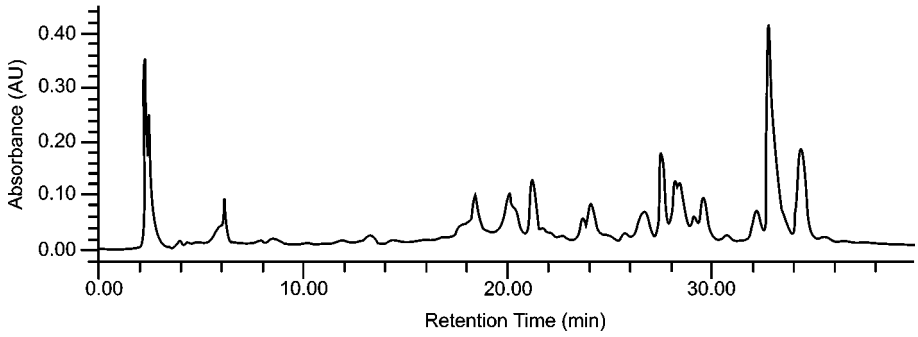
Male Swiss albino Strain 'A' inbred mice (12 weeks old, weighing 25 ± 2 g) were maintained on standard food pellets (Lipton India) and water *ad libitum*. Animal experiments were conducted according to 'INSA – Ethical guidelines for use of animals in Scientific Research published by Central Drug Research Institute, Lucknow, India.

Maximum tolerated dose

Acute toxicity of single dose administration of RH-3 was studied in terms of survival, changes in behavior, neuromuscular co-ordination and respiratory disorder etc. Period of observation for acute toxicity was 2 days for a single dose regimen.

Irradiation

Whole body irradiation was given through ^{60}Co gamma cell (Model 220 – Atomic Energy of Canada) at an absorbed dose rate of 0.66 Gy/min and fresh air was continuously circulated in the irradiation chamber to avoid generation of hypoxic conditions. Mice were kept in



HPLC-Figure: *Conditions:* Column: 250 mm × 4.6 mm; Column separation material: Novapack C18 H, Reverse phase; Solvent system: A: water with 1% 0.1 N phosphoric acid; B: acetonitrile with 1% 0.1 N phosphoric acid; Gradient: 5% to 25% B in 40 min; Sample: 12 mg/ml (Conc. of extract mg/ml); Injected volume: 10 µl of sample; Flow: 1.00 ml/min.; Detection: 254 nm

perforated plastic bottles for irradiation individually. For studying free radical scavenging *in vitro*, different solutions were exposed to various desired doses of gamma rays delivered by Gamma chamber 5000 (BRIT, India), having a dose rate of 1.78 Gy/sec during the course of experiment.

Survival studies

Animals were observed for survival daily up to 30 post-irradiation days. Data were presented as % survival after the expiry of 30 post-irradiation days. The body weight was also recorded every alternate day.

Haemopoietic Stem Cell assay

For endogenous colony forming unit assay (Till and McCulloch 1961), mice were sacrificed by cervical dislocation 10 days after various treatments. Spleen was removed and fixed in Bouin's solution for 24 h. Macroscopic colonies (CFU) visible per spleen were scored in each mouse.

Haematological studies

Hemoglobin, Total leukocyte count and Differential lymphocyte counts were studied in blood samples drawn from heart of mice sacrificed by cervical dislocation.

Free radical scavenging and antioxidant studies

These studies were undertaken to unravel the mode of action of RH-3. All spectrophotometric measurements were done with the help of chemito UV-Vis spectrophotometer. All the experiments were done in triplicate.

• *Protein:* Total proteins were estimated in 10% liver homogenate (Lowry et al. 1952).

• *Scavenging of hydroxyl radicals*: The radiation and $FeSO_4$ generated OH radicals quenched by RH-3 were estimated using 2-deoxyribose as the marker substrate (Gutteridge 1981). Each tube contained 0.5 ml of 2-deoxyribose and 0.5 ml of phosphate buffered saline (pH 7.4). In one tube 0.1 ml of 100 µM $FeSO_4$ was added and mixed thoroughly. Other tubes were exposed to desired doses of gamma radiation in the presence or absence of plant extract. Thereafter 0.5 ml of (1% Thiobarbituric acid, TBA) was added and vortexed. 0.5 ml of 10% Trichloro acetic acid (TCA) was added and vortexed again thoroughly and the whole solution was incubated at 100 °C for 15 minutes; Each tube was cooled and measured for the intensity of chromogen by recording at 532 nm.

• *Scavenging of superoxide anions:* The superoxide quenching ability of RH-3 was estimated using nitrobluetetrazolium (NBT) as the marker substrate (Kakkar et al. 1984). In each tube 1.2 ml of sodium pyrophosphate buffer (0.052 M, pH 8.3), desired concentration of plant extract and 0.1 ml of 186 µM phenazine methosulfate were taken followed by addition of 300 µl of 300 µM nitrobluetetrazolium and total volume was adjusted to 3 ml. Thereafter reaction was initiated by adding 200 l of NADH (780 µM) and the solution was incubated at 37 °C for 90 seconds. The reaction was stopped by adding 1 ml of glacial acetic acid. The resultant mixture was shaken with 4 ml of n-butanol and allowed to stand for 10 minutes at room temperature. By centrifugation butanol layer was separated and color intensity of chromogen in the butanol was measured at 560 nm against butanol. The percentage of inhibition by extract was calculated by considering the optical density of the chromogen in the absence of extract as 0% inhibition of NBT reduction.

• *Lipid peroxidation:* Randomly selected 6–8 weeks strain 'A' mice were sacrificed by cervical dislocation, dissected and abdominal cavity were perfused with 0.9% saline; whole liver was taken out and visible blood clots were carefully and maximally removed and weighed amount of liver was processed to get a 10% homogenate in cold buffered saline (pH 7.4); using potter elvejam homogenizer and filtered through a mesh to get a clear homogenate. 2 ml of 10% liver homogenate

was taken in a series of 35-mm petri dishes to which desired amount of RH-3 was added and mixed gently to form a homogeneous solution. Lipid peroxidation was initiated by adding 100 µl of ferrous ammonium sulfate (0.5 mM) and thereafter petri dishes were incubated at 37 °C for 30 minutes. 100 µl of homogenate was pipetted out for estimating lipid peroxidation levels in terms of Thiobarbituric Acid Reactive Substances (TBARS) following method of Beuge and Aust (1978).

Table 1. Estimation of maximum tolerated dose: Survival of strain 'A' mice was observed up to 48 hours after administration of single dose of RH-3.
(* n 5 for each dose of RH-3)

Dose in mg/kg b.w.	* % Survival
25	100
35	100
40	100
45	80
55	60
60	40
100	0

Analysis of data & Statistical analysis

All experiments were repeated thrice and the data were analyzed statistically and expressed as mean ± MSE; student-t-test was applied for significance and P value < 0.05 was considered significant.

Results

Maximum tolerated dose

Single doses of RH-3 up to 40 mg/kg b.w. was tolerated by mice without any apparent adverse manifestation, except being little drowsy for 3 to 5 minutes. However, RH-3 beyond 45 mg/kg b.w. manifested mortality within 48 h in a dose dependent manner (Table 1).

Survival studies

Survival of mice up to 30 post-irradiation days has been shown in (Figure 1a and 1b). All irradiated animals without RH-3 treatment were dead with in 15 days. On 30th post-irradiation day maximum survival (81.7%) was achieved by administration of 30 mg/kg

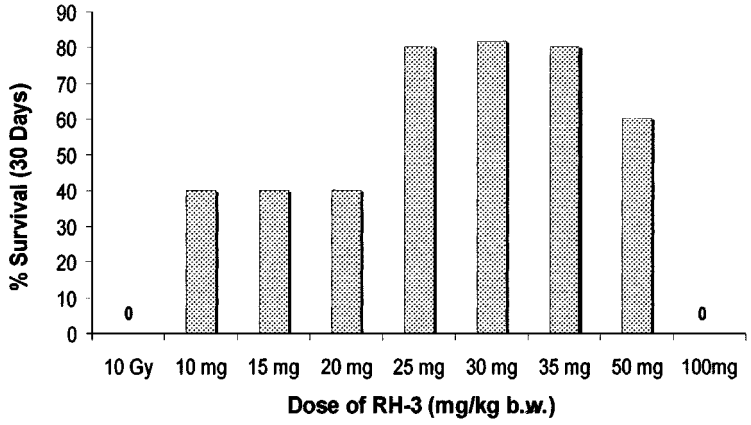


Fig. 1a. Radioprotective effect of different doses of RH-3 (i.p., –30 min. Single dose) on survival in strain a male mice against whole body irradiation (10 Gy).

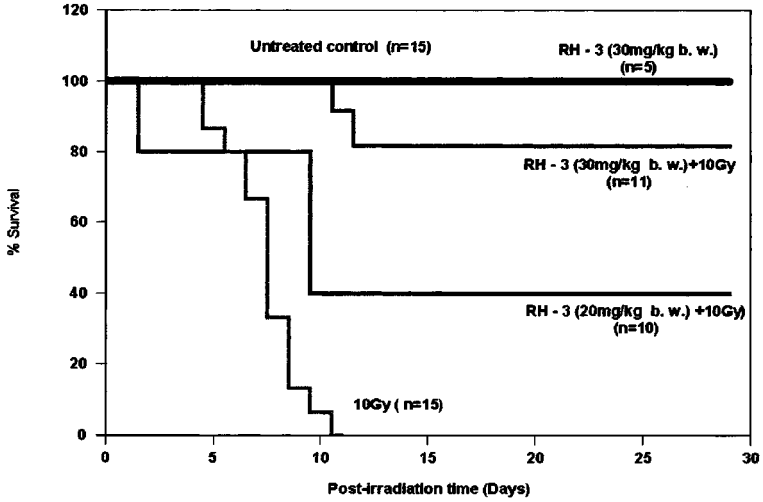


Fig. 1b. Radioprotective effect of different doses of RH-3 (i.p, –30 min.) on day-wise survival against radiation (10 Gy) induced mortality in strain A mice.

Table 2. CFU counts observed on 10th post-irradiation day in spleen of animals given different doses of radiation or 30 mg/kg b.w. i.p. of RH-3 or both. Time interval between drug administration and irradiation was 30 minutes.

Treatment RH-3	Radiation (Gy)	Mice No	Mean CFU counts/Spleen*
–	5	6	13.58 ± 0.74
+	5	6	18.33 ± 0.96
–	7.5	12	2.25 ± 0.82
+	7.5	6	14.0 ± 1.17
–	10	12	0.37 ± 0.09
+	10	6	6.0 ± 0.88

* MSE – Mean standard error.

b.w. of RH-3, 30 minutes before 10 Gy irradiation. Doses of RH-3, less than 25 mg or more than 35 mg/kg b.w. were less effective for 30 days post-irradiation survival. Effect of time interval between administration of RH-3 and irradiation was also studied on 30 days post-irradiation survival and it was observed that 30 minutes interval was most optimal for radioprotective effect.

Haemopoietic stem cells

Effect of various radiation doses on colony forming capacity and its modulation by pre-irradiation administration of RH-3 (30 mg/kg b.w., –30 min.) has been depicted in Table 2. CFU counts in spleen decreased with increasing radiation dose in the absence of RH-3. RH-3 administration to unirradiated rats did not influence CFU counts. Pre-irradiation treatment with RH-3 rendered protection against all radiation doses used here and was found to be significantly higher ($p < 0.05$) than the corresponding irradiated groups without RH-3 treatment. The CFU counts however declined with increasing radiation doses even in presence of RH-3.

Haematological studies

• *Haemoglobin:* Changes in the amount of haemoglobin (Hb) in mice in different treatment groups have been shown at different post-irradiation intervals (Figure 2). RH-3 alone decreased the haemoglobin level to 12.8% ± 3.7 up to 10th post-treatment day and thereafter on 15th day it was observed to be equivalent to untreated control (15 g%). In irradiated group (10 Gy) haemoglobin decreased continuously attaining the value of 10.8 ± 1.14 g% by 10th day as compared to 15 g% in untreated control; data for 15th day could not be collected since all the animals in this group were dead by that time. In RH-3 + 10 Gy group the haemoglobin level remained low (11.86 ± 0.49) up to 7 days but thereafter it started recovering with time and increased to 12.4 ± 0.29 g% in 15 days.

• *Total leucocyte counts (TLC):* Treatment with any single dose of RH-3 alone increased TLC up to 7 days and thereafter it reverted to control value (Figure 3). In irradiated group TLC decreased sharply up to 10 days and data for 15 days could not be collected since by that time all untreated irradiated animals were dead. RH-3 treatment 30 minutes before irradiation did not exhibit any protection over untreated irradiated group up to 7 days but the recovery became evident thereafter and was quite steep during successive three days. Within 15 days TLC became very close to unirradiated control.

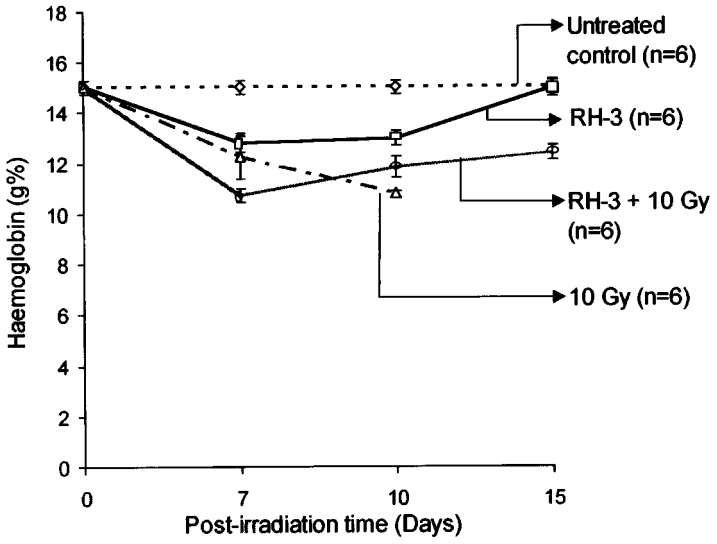


Fig. 2. Effect of RH-3 (30 mg/kg b.w. i.p., – 30 min.) on haemoglobin in peripheral blood of strain 'A' male mice given various treatments. Bars represent ± MSE

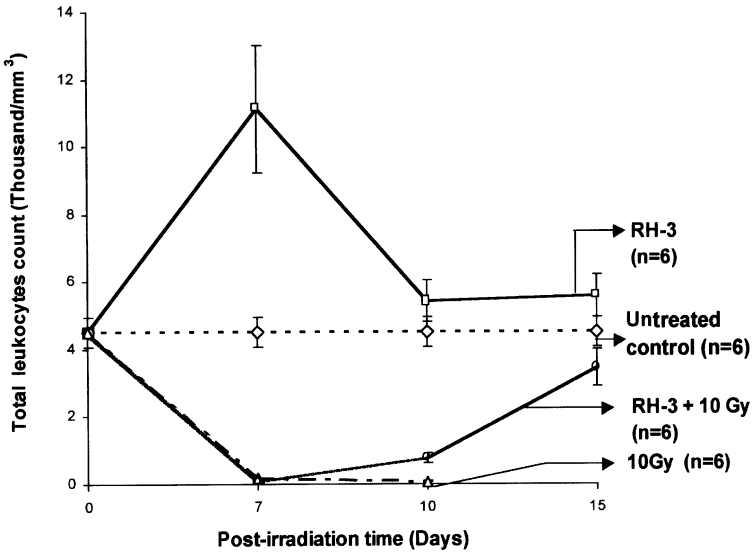


Fig. 3. Effect of RH-3 (30 mg/kg b.w., i.p., – 30 min.) on total leucocyte counts in peripheral blood of strain 'A' male mice given various treatments. Bars represent ± MSE.

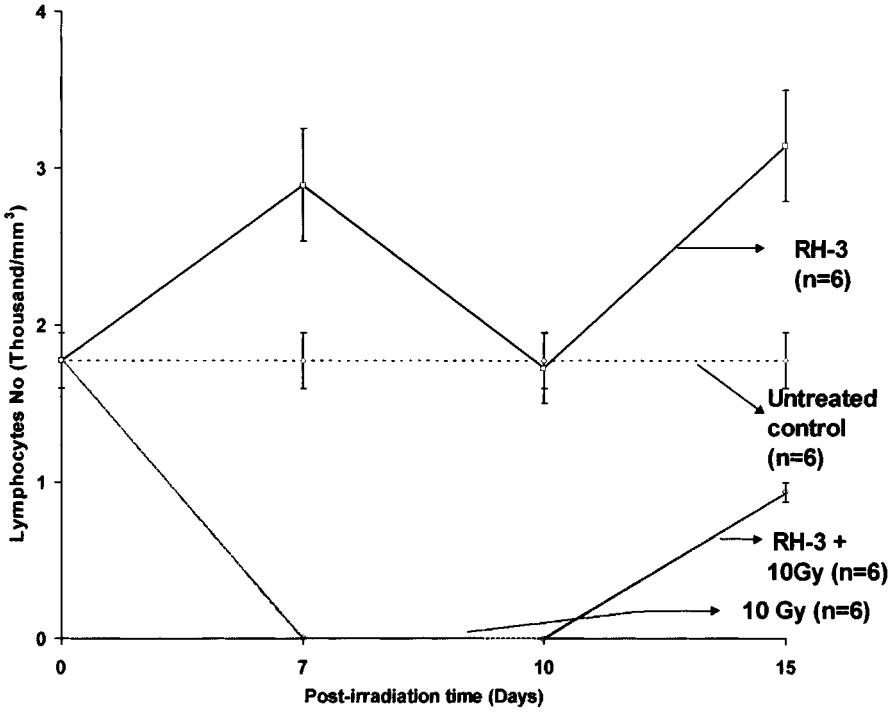


Fig. 4a. Effect of RH-3 (30 mg/kg b.w., i.p., – 30 min.) on number of lymphocytes in peripheral blood of strain 'A' male mice given various treatments. Bars represent ± MSE.

• *Differential leucocyte counts (DLC):* The effect of RH-3 in protecting lymphocyte, polymorphs and monocytes against radiation damage has been depicted in Figures 4a, b and c. In the group treated with RH-3 alone the number of lymphocytes, polymorphs and monocytes increased upto 7 days but reverted to normal value within 10 post-treatment days. Lymphocytes and monocytes however again showed an upward trend as seen on 15th post-treatment day. In irradiated group (10 Gy), the number of lymphocyte, polymorphs and monocytes decreased sharply up to 7 days as compared to control values. There was no recovery and all animals died with in 15 post-irradiation days. On 7th and 10th post irradiation days the TLC was so low that DLC could not be studied. In RH-3 + 10 Gy group, the lymphocyte and monocytes decreased sharply up to 7 days as happened to irradiated untreated animals. There was no sign of significant recovery up to 10 days. However on 15th day lymphocytes, monocytes and polymorphs were seen to have significant recovery.

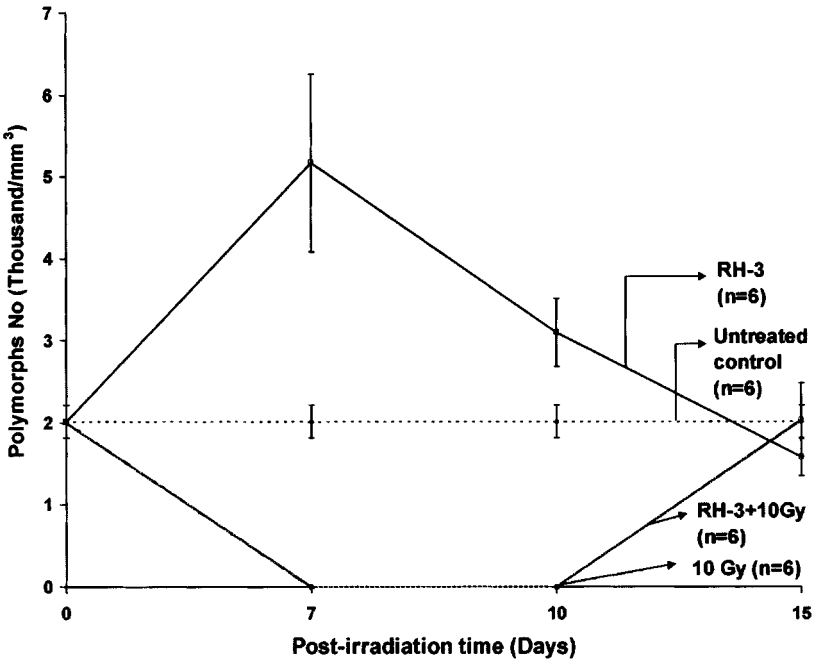


Fig. 4b. Effect of RH-3 (30 mg/kg b.w., i.p., – 30 min.) on number of polymorphs in peripheral blood of strain 'A' male mice given various treatments. Bars represent ± MSE.

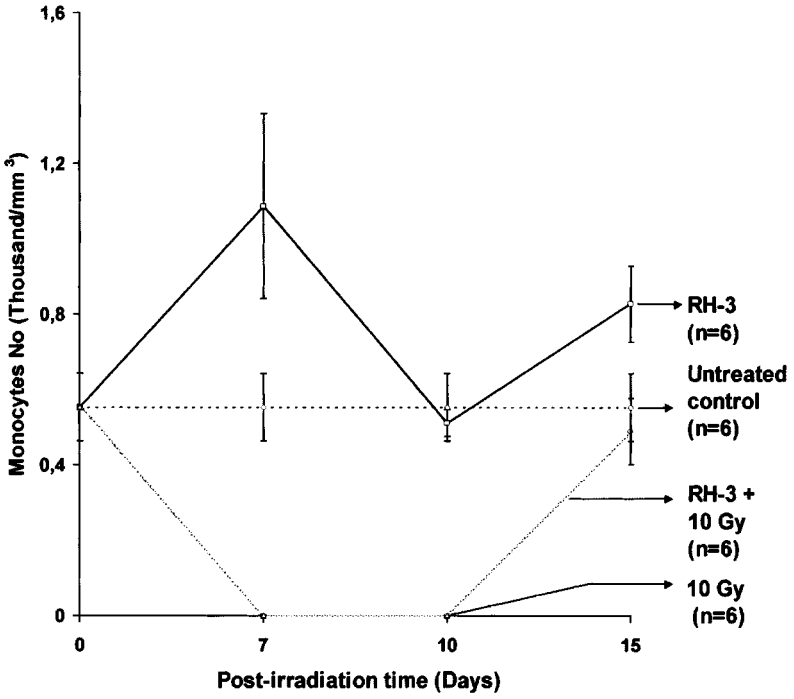


Fig. 4c. Effect of RH-3 (30 mg/kg b.w., i.p., – 30 min.) on monocytes in peripheral blood of strain 'A' male mice given various treatments. Bars represent ± MSE.

Effect of RH-3 on free radical scavenging

These studies were conducted under *in vitro* conditions.

• *OH radical scavenging:*

– *Fenton reaction mediated OH radical generation:* The effect of varied concentrations of RH-3 on scavenging of Fenton reaction mediated OH radical as determined by inhibition of 2-deoxyribose degradation has been depicted in Figure 5. RH-3 rendered inhibition of 2-deoxyribose degradation in a dose dependent manner; maximum effect (75.71%) was achieved at a concentration of 2 mg/ml ($P < 0.05$).

– *Fixed radiation induced OH radical generation and scavenging by varied herbal concentrations:* The effect of different concentrations of RH-3 on inhibition of 2-deoxyribose degradation by a radiation dose

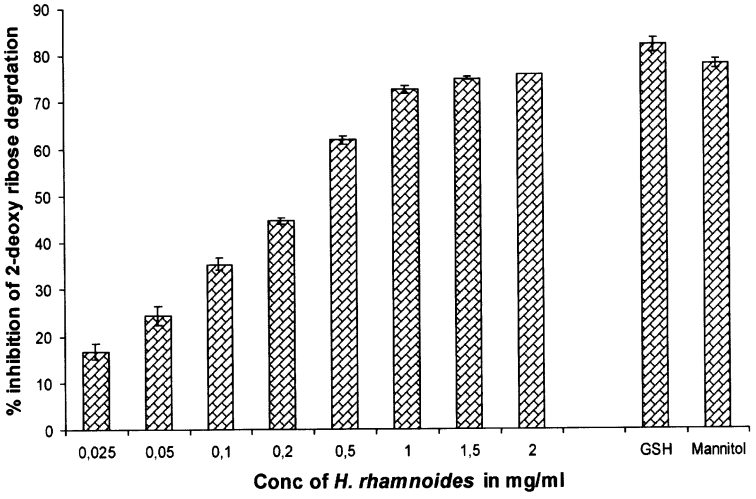


Fig. 5. Effect of RH-3 on scavenging of Fenton reaction generated OH radicals *in vitro* system. On top of each bar the ± MSE has been indicated.

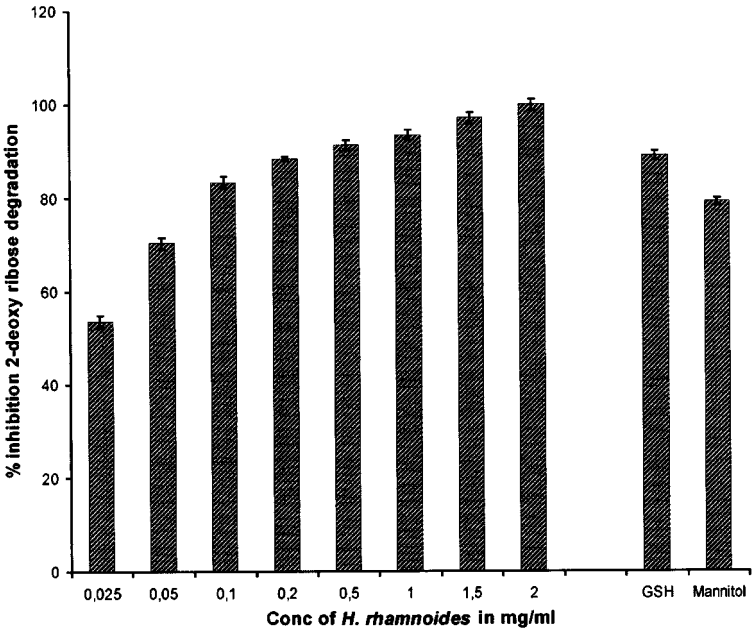


Fig. 6. Effect of RH-3 on scavenging of radiation generated OH radicals *in vitro* system. On top of each bar the ± MSE has been indicated.

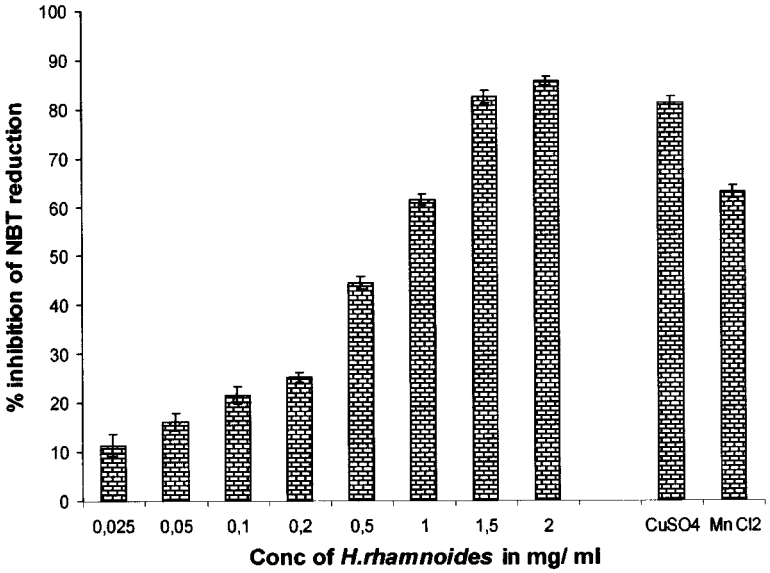


Fig. 7. Effect of RH-3 on scavenging chemically generated superoxide anions *in vitro* system as observed by % inhibition of nitro blue tetrazolium reduction. On top of each bar the ± MSE has been indicated.

(200 Gy) has been depicted in Figure 6. RH-3 inhibited 2-deoxyribose degradation in a dose dependent manner. At a concentration of 0.2 mg/ml of RH-3 about 90% inhibition was achieved, though maximum inhibition (97.09 %) was achieved at a concentration of 2 mg/ml ($P < 0.05$).

• *Superoxide anion scavenging:* Figure 7 shows that increasing dose of RH-3 inhibited superoxide anions in a proportionate manner. A concentration of 2 mg/ml rendered maximal inhibition (85.66%; $P < 0.05$) of superoxide anion mediated formazan production.

• *Inhibition of lipid peroxidation:* Inhibition of Fenton reaction mediated lipid peroxidation by RH-3 as expressed by inhibition of TBARS formation has been depicted in Figure 8. RH-3 elicited a dose dependent inhibition of lipid peroxidation, maximal inhibition (68.92 %) being achieved at a concentration of 2 mg/ml ($P < 0.05$).

Discussion

The present studies revealed that pre-irradiation administration of single dose of RH-3 (30 mg/kg b.w.) rendered 81.7% survival in mice against 10 Gy whole body irradiation. Untreated irradiated mice suffered 100% mortality within 10–15 days (Goel et al. 1998). The maximum protective effect was achieved at subtoxic level of RH-3. Infact, most of the molecular and herbal radioprotective agents often render maximum radioprotective effect at subtoxic level approaching maximum tolerated dose level (Riklis et al, 1990, Schuchter et al. 1993, Goel et al. 1996, Umadevi et al. 1999).

Radiation protection by chemicals at cellular and subcellular level likely reflects both wholesome effect of scavenging of radiation-induced free radicals and the repair of damaged targets and molecules. However, repair of damage and recruitment of cells to substitute apoptotic and necrotic cells are other very important manifestations of an ideal radioprotector. The recruitment of cells may contribute towards recovery of a number of tissues like bone marrow, intestine and skin etc. This could be possible due to proliferative stimulation rendered to the stem cells of the tissues/systems by certain molecules like cytokines, growth factors etc present in the herbal preparation. Administration of such chemicals has directly been shown to render significant radioprotection (Neta et al. 1994). RH-3 is a herbal preparation of *H. rhamnoides* and contains a plethora of molecules of diversified nature ranging from antioxidant to proliferative and immunostimulatory nature. Therefore, it became imperative to investigate the effect of RH-3 through various parameters for understanding the important facets of mode of action of a natural mixture of molecules available in RH-3.

H. rhamnoides 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 (Ianev et al. 1995). The antioxidant potential was therefore monitored *in vitro* by estimating OH radicals generated by irradiation or Fenton reaction. RH-3 inhibited OH radical by scavenging them, thus confirming its antioxi-

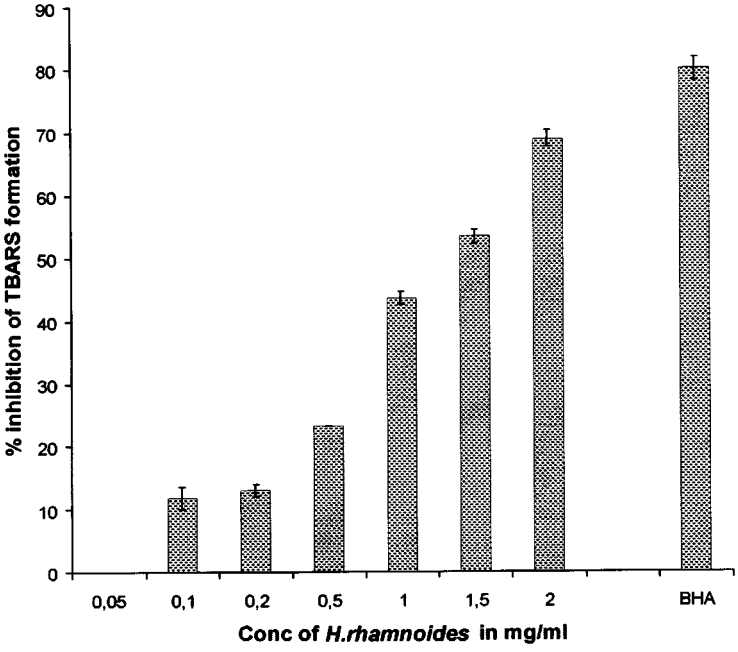


Fig. 8. Effect of RH-3 on inhibition of FeSO4 mediated lipid peroxidation in mice liver extract as observed by % inhibition of thiobarbituric acid reactive substance (TBARS) formation. On top of each bar the ± MSE has been indicated.

dant capabilities. The extraction was since done with polar solvents it is possible that antioxidants like vitamin A & E might not be there though vitamin C, tannins and other antioxidant molecules may be present. Therefore the antioxidant capabilities of this extract under present study could be a partial manifestation only.

The unirradiated mice spleen did not have any shortage of stem cells due to the absence of a cytocidal agent like radiation. Treatment with RH-3 possibly did not induce any proliferative stimulation in spleen and therefore CFU counts remained comparable to the untreated control. The enhanced CFU counts in spleen of RH-3 treated irradiated mice in comparison to untreated irradiated control, evidently indicate its radioprotective manifestation. The presence of RH-3 constituent molecules in cellular milieu at the time of irradiation and induction of damage could offer radioprotection by scavenging of free radicals. However some of the constituent molecules of RH-3 may not be getting immediately metabolized and persist even after irradiation. Such molecules could have influenced repair process at least the fast repair.

The reduction in the quantity of hemoglobin is a reflection of bone marrow stem cells activity; its depression, stimulation and recovery could be seen in the later part of second post-irradiation week. Administration of RH-3 before lethal irradiation ensured faster recovery in TLC in general and lymphocytes, polymorphs and monocytes in particular. Increase in TLC by RH-3 alone (Fig. 4a, b, c) is an interesting observation and could be an attribute responsible for immunostimulation. Some molecules enhanced bone marrow proliferation in a specific direction elevating TLC and down regulating RBC production resulting in the decrease of haemoglobin (Figure 3). Further, iron chelation studies done in our laboratory (unpublished data) could also be responsible for decreased haemoglobin.

The radioprotective effect generated by RH-3 at molecular level in terms of free radical scavenging as studied through in-vitro studies (Figure 5–8) could explain the cellular survival, proliferation enhancement, immunostimulation and ultimately the whole body survival. However, it is not revealed by this study whether enhancement of repair of DNA damage is also contributed by any component of RH-3 and it needs to be investigated further.

Acknowledgements

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