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Modification of gamma radiation induced response of peritoneal macrophages and splenocytes by *Hippophae rhamnoides* (RH-3) in mice

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Abstract

Alcoholic extract of *Hippophae rhamnoides*, RH-3, reported to render >80% survival against lethal whole body Co-60-gamma irradiation (10 Gy) in mice, was investigated for its immunostimulatory effects. In comparison with un-irradiated control, whole body irradiation did not reduce peritoneal macrophage counts at 24 h post-irradiation. RH-3 treatment (30 mg kg^{-1} body weight) alone or 30 min before whole-body irradiation enhanced viable counts of macrophages significantly ($P \leq 0.05$) compared with both un-irradiated control and irradiated groups. Whole-body irradiation reduced the number of viable splenocytes significantly ($P \leq 0.05$) compared with un-irradiated control at 24 h post-irradiation. RH-3 treatment alone or before whole-body irradiation appreciably countered radiation-induced decrease in splenocyte count. ^{3}H-thymidine uptake method revealed that whole-body irradiation reduced splenocyte proliferation significantly (159 ± 45 counts $min^{-1}/10^6$ cells; $P \leq 0.05$) in comparison with control (607 ± 142 counts min^{-1}) at 24 h after irradiation but RH-3 treatment before irradiation reduced the steep decrease and maintained it as 444 ± 153 counts-min^{-1}. After whole-body irradiation, the ratio of spleen weight/mouse weight decreased to 1.5 ± 04 compared with 2.9 ± 0.32 in un-irradiated control at 24 h post-irradiation. Similarly, total protein content in splenocytes also decreased to 48 ± 6 $\mu g/10^6$ cells in comparison with 368 ± 16 $\mu g/10^6$ cells of un-irradiated control. RH-3 treatment before irradiation countered radiation-induced decrease in both spleen weight/mouse weight ratio (4.0 ± 0.35) and total protein content (360 ± 13 $\mu g/10^6$ splenocytes). In the supernatant of peritoneal macrophage cultures exposed to 2 Gy Co-60-gamma radiation ex-vivo, the total nitrite content was enhanced significantly ($P < 0.05$) to 5.72 ± 0.09 μM in comparison with un-irradiated control (1.64 ± 0.09 μM). RH-3 treatment (30 $\mu g\ mL^{-1}$) before irradiation reduced total nitrite significantly (0.93 ± 0.3; $P \leq 0.05$) in comparison with irradiated control group. At 24 h after whole body irradiation, the CD4+/CD8+ ratio reduced to 1.5 in comparison with un-irradiated control (1.9) but RH-3 treatment before irradiation restored the ratio to 2.1. These findings explicitly reveal the immunostimulatory activity of RH-3, which may play an important role in the manifestation of its radioprotective efficacy.

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Introduction

Ionizing radiations having low linear energy transfer (LET) are known to cause bone-marrow suppression and depletion of peripheral blood lymphocytes thereby leading to immunosuppression (Pecaut et al 2001). The exposed animals become susceptible to opportunistic pathogens and some of these infections could be lethal (Hammond et al 1978). Most of the Chernobyl accident casualties were primarily due to radiation burns (Densow et al 1997), yet a sizable population of exposed victims suffered bone-marrow injuries and radiation-induced haematopoietic depression, which led to reduced immunocompetence. The irradiated individuals became highly vulnerable to secondary microbial infections, such as pneumonia (Guskova 1986). Some of the mature haematopoietic cells like macrophages are, however, fairly radio-resistant (Buescher & Gallin 1984) and provide protection to some extent in the post-irradiation scenario. Radiation exposure affects the proliferation of various stem cells, including haematopoietic cells. Survival after radiation exposure, therefore, depends largely on the survival of a critical number of these haematopoietic stem cells and their ability to

generate an effective level of immunocompetent system. Several immunostimulators have been reported to render a radioprotective effect by inducing colony stimulating factors (Neta 1997) and various cytokines (Golde & Gasson 1988). The toxicity of such immunomodulators to various systems has severely restrained their use in clinics. Therefore, development of a suitable, non-toxic immunostimulatory agent is an inevitable necessity to mitigate radiation injuries and subsequent infections and haemorrhage.

Plants are known to produce a plethora of secondary metabolites with a wide array of pharmacological activity and therefore have been used both in the traditional as well as the modern system of medicine. The isolated compounds or active principle and the molecular drugs derived from these plant extracts influence certain biochemical pathways and also interfere with other intracellular activities leading to the manifestation of toxic effects. The natural combination of compounds present in the whole extract of a plant often has a large number of molecules, of which some have principle pharmacological activity with respect to a particular ailment or disorder, while others may take care of the side effects produced by the active components. Radiation-induced immunosuppression is an important reason for radiation mortality. Therefore, in the current context of the development of a radioprotector, exploration of the immunomodulatory effects of plant products has become a subject of active research.

Hippophae rhamnoides L. (Elaegnaceae), commonly known as sea buckthorn, has been exploited in the Ayurveda for the treatment of sluggish digestion, stomach malfunctioning (Xiao et al 1992), burns and wounds (Nikulin et al 1992; Ianev et al 1995), hepatic injuries (Cheng 1992; Cheng et al 1994) and neoplasms (Cheng et al 1994). *H. rhamnoides* contains a large numbers of molecules, such as a flavones (Wu et al 1994) and flavonoids (Zhang 1987), vitamins A, C, E and K (Wu 1991) and tannins (Spirodonov et al 1997). *H. rhamnoides* has been known to act as a strong antioxidant (Zhang 1987) and inhibitor of succinate oxidation (Spirodonov et al 1997). An alcoholic extract of *H. rhamnoides* has been shown to render significant radioprotection (>82%) in mice if administered intraperitoneally before whole-body lethal Co-60 gamma irradiation (Goel et al 2002). In this study some aspects of the immunomodulatory potential of this radioprotective agent (RH-3) have been unravelled.

Materials and Methods

Reagents

RPMI 1640 and fetal bovine serum (FBS) were procured from Hyclone (Logan, UT), Brewer's thioglycolate medium from Becton Dickinson (MD), LPS (*Escherichia coli*, Serotype 0111: B4) from Sigma (St Louis MO), and *N*-(naphthyl)ethylene-diamine dihydrochloride, sulphanilamide, HEPES buffer, EDTA, dithiothreitol (DTT) and phenylmethylsulfonylfluoride (PMSF) from E. Merck (Germany). Streptomycin, gentamicin, penicillin, Trypan blue dye, phosphate-buffered saline (PBS), $NaNO_2$ and dimethyl sulfoxide (DMSO) were purchased from Hi-Media Mumbai (India), ^{3}H-thymidine was purchased from the Board of Radiation and Isotope Technology, Mumbai, India.

Herbal extract preparation

Fresh berries of *H. rhamnoides* were collected from Himalayan ranges as described earlier (Goel et al 2002). A known quantity of shade-dried berries was extracted by a combination of absolute alcohol and triple-distilled water (50:50, v/v) and the extract was lyophilized, weighed and stored at 4°C. The extract, so prepared, was designated as RH-3 in our laboratory. For evaluating the radioprotective efficacy, a known quantity of RH-3 was diluted in triple-distilled water and sterilized by passing through a 0.2-μm filter (Minisart NML).

HPLC characterization

An HPLC chromatogram of *H. rhamnoides* was obtained using a Novapack C18H silica column (Waters, HPLC system) and the mobile phase consisted of phosphoric acid and water. The HPLC profile of a 50% alcoholic extract showed several peaks of flavanoids, in the range 7–18 min retention time (Figure 1). This chromatogram was used as a fingerprint to monitor batch-to-batch variation, if any, in extract preparation. A batch of extract showing gross changes from this chromatogram was rejected.

Experimental animals

Under this research project, no tests were done on human beings. Inbred male Balb/c mice, 25 ± 3 g, 6–8 weeks old, maintained under controlled environment (25 ± 2°C), were provided with standard animal food pellet (Amrut laboratory animal feed, India) and allowed free access to water. Not more than five mice were housed in a polyvinyl cage. The animal experiments were conducted according to INSA-Ethical Guidelines for the use of Animals in Scientific Research, published by the Central Drug Research Institute, Lucknow, India. For these experiments, animals were taken with the approval of the Animal Experimentation Ethics Committee of the laboratory.

Administration of RH-3 and vehicle

For in-vivo experiments the desired quantity of RH-3 was dissolved in triple-distilled water and each mouse received a dose of 30 mg kg^{-1} (based on body weight) of RH-3 intraperitoneally 30 min before whole-body irradiation (10 Gy). Sham-treated mice received normal saline only. For ex-vivo experiments, RH-3 was dissolved in triple-distilled water to make a stock solution of 1 mg mL^{-1}. It was further diluted in incomplete serum-free RPMI-1640

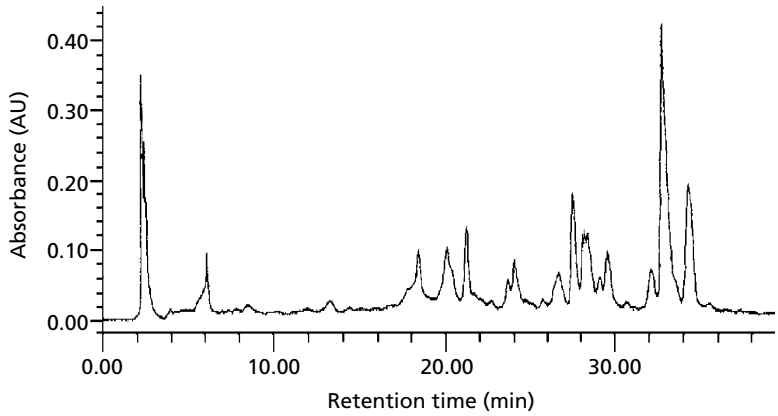

Figure 1 HPLC fingerprint of RH-3. Conditions, 250 mm × 4.6 mm; column separation material, Novapack C18H reverse phase; solvent system, A — water with 1% 0.1 N phosphoric acid; B — acetonitrile with 1% 0.1 N phosphoric acid; gradient, 5–25% B in 40 min; sample, 12 mg mL^{-1} (concn of extract); injected volume, 10 μL of sample; flow: 1.00 mL min^{-1}; detection, 254 nm.

medium to achieve the desired concentration for irradiation and ex-vivo experimentation.

Experimental groups

The mice and ex-vivo cultured cells used for experiments were divided into different groups: untreated un-irradiated control (n = 5) × 3 (0.2 mL vehicle (i.p.) and sham irradiated); irradiated control (n = 5) × 3 (0.2 mL vehicle (i.p.) and 10 Gy whole-body gamma-irradiation); RH-3 treated (n = 5) × 3 (30 mg kg^{-1} RH-3 in 0.2 mL vehicle (i.p.), 30 min before sham irradiation); and RH-3 + irradiated (10 Gy) (n = 5) × 3 (30 mg kg^{-1} RH-3 in 0.2 mL vehicle (i.p.), 30 min before whole-body gamma-irradiation (10 Gy)). Where n = number of the mice and the number outside parentheses depicts the number of times the experiment was repeated.

Irradiation

Each mouse was placed individually in a plastic container, and exposed to whole-body irradiation in a Co-60 gamma cell (model 220, dose rate 0.8 Gy min^{-1}; Atomic Energy Commission, Canada). To avoid hypoxia, a fresh-air supply in the irradiation chamber was maintained through a tube connected to an air pump. The cells in the cultures were also exposed to different doses of radiation in a similar fashion except that no aeration was given in the irradiation chamber. Dosimetry was carried out with Baldwins Farmer secondary dosimeter and Fricke's chemical dosimeter (Massay 1966).

Peritoneal macrophage primary cell culture

Mouse peritoneal macrophages were elicited as per the procedure described by Edelson & Cohn (1976). Briefly 4% Brewer's thioglycolate medium (Becton Dickinson, MD, USA) was injected into the peritoneal cavity of male Balb/c mice. Peritoneal exudate cells were obtained 72 h after injection by flushing the peritoneal cavity with ice-cold incomplete serum free RPMI 1640 medium using a 22G1 needle. Peritoneal lavage was pooled and collected in a 50-mL conical centrifuge tube and centrifuged at 1500 rev min^{-1}, (10 min, 4°C) and the pellet was resuspended in complete RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal calf serum, FBS (Hyclone, Logan, UT, USA) containing 15 mM HEPES buffer, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin. The cells were counted and checked for viability by the Trypan blue dye exclusion method. Cells (1×10^5/well in a final volume of 200 μL) were seeded in flat-bottom 96-well polystyrene microtitre plate (Costar, Corning, NY) and incubated at 37°C for 3 h to allow adherence to the plate. The non-adherent cells were removed by repeated washing with incomplete RPMI 1640 medium. Cells were identified by the presence of macrophage membrane marker (Mac-1) by staining them with anti-Mac-1 (fluorescein isothiocyanate (FITC)-conjugated antibody) as per the method described earlier by Victor & De la Fuente (2000). Peritoneal macrophages in culture were treated with different doses of RH-3, 30 min before irradiation (2 Gy). These cells were further cultured for estimation of total nitrite.

Splenocyte primary cell culture

The spleens were excised from mice aseptically and kept in incomplete RPMI 1640 medium. The single-cell suspension was prepared as per the procedure described by Ly & Mishell (1974). The spleens were crushed between two frosted-end slides and the suspension was layered on incomplete serum free RPMI 1640 medium in a 60-mm Petri dish for 10 min to allow cellular debris to settle down. The suspension was carefully transferred to a 15-mL conical centrifuge tube and centrifuged at 1500 rev min^{-1}, (10 min, 4°C). Red blood cells were removed by centrifuging the pellet in RBC lysing buffer (0.1 M NH_4Cl). Monocytes were removed by adherence method to plastic as described earlier. The cell suspension was again centrifuged at 1500 rev min^{-1}, (10 min, 4°C) and

the pellet was resuspended in complete RPMI 1640 medium. Cells were counted and viability was assessed by the Trypan blue dye exclusion method. Cells (1×10^5 cells/well in a final volume of 200 μL) were seeded in flat-bottom 96-well polystyrene microtitre plate and cultured for the proliferation assay.

Viability assay

Viable counts of macrophages and splenocytes were measured by the Trypan blue dye exclusion method described by Yu et al (2003). Cells from the different experimental groups were taken 24 h after irradiation as described in the sections on cell isolation and culture. The cell suspensions were mixed with equal volume of dye and incubated at room temperature for 5 min. The cells were washed to remove the free dye. Viable cell counts were taken by using a haemocytometer under 40× magnification of the microscope. Cells with dye were excluded and only transparent cells were taken and counted as viable cells.

Total nitrite estimation

Peritoneal exudate cells were obtained 72 h after injecting 4% Brewer's thioglycolate medium into the peritoneal cavity of the mice. As described earlier, the peritoneal lavage was collected and washed. The cells were counted and cultured for 3 h in microtitre plates. The cultured cells were checked for viability and then flow-cytometrically examined for the presence of macrophage membrane markers (Mac-1). RH-3 treatment and irradiation was given to these cultured cells ex-vivo and cells were further cultured for 48 h. The macrophage culture supernatant was used for estimating the stable NO metabolite (i.e. nitrite (NO_2^-)) by Griess assay as described by Green et al (1982). An equal volume of the culture supernatant (100 μL) from various treatment groups was mixed with Griess reagent (100 μL) of 1% sulphanilamide/0.1% *N*-(naphthyl)ethylene-diaminedihydrochloride and the absorbance was measured at 550 nm by ELISA reader (BIO-TEK instrument Inc., Canada). The amount of nitrite was calculated from a $NaNO_2$ standard curve.

^{3}H-Thymidine incorporation studies

Splenocytes from the different experimental groups were cultured and pulsed with 0.5 μCi ^{3}H-thymidine/well as described by Terasima & Tolmach (1963). The cells were harvested after 18 h on the glass fibre filter mats (Wallac) by using a cell harvester (Skatron Instruments) and counted by using a liquid scintillation counter (Wallac Instruments). Increase in counts per minute indicated proliferation of splenocytes.

Spleen weight

Spleens of mice from the different experimental groups were excised, blot dried and weighed separately. The ratio of spleen weight (mg) to animal weight (mg) was recorded.

Protein content

The total protein contents in mouse splenocytes were quantified following the method of Bradford (1976). A single-cell suspension of splenocytes was made in PBS. Splenocytes (1×10^6) were taken from each experimental group and spun at 1500 rev min^{-1} at 4°C for 10 min. The cell pellets were resuspended in 1 mL of cell lysis buffer (PBS + 0.1 mM EDTA + 0.1 mM DTT + 1 mM Na_3VO_4 + 1 mM PMSF) and immediately spun at 10 000 rev min^{-1} for 15 min at room temperature. For protein estimation, 100 μL of each of cell lysate and Bradford reagent (0.1% Coommassie G250 in ethanol–acetic acid, 95:5%) were mixed and the plate was read at 595 nm by a microtitre plate reader (BIO-TEK instrument Inc., Canada) equipped with 620 nm reference wavelength. The amount of total protein in various samples was calculated with the help of a standard curve drawn for bovine serum albumin.

CD4+ and CD8+ cell enumeration

CD4+ and CD8+ T cell populations in mouse splenocytes were quantified by FACS scan as described earlier (Ledbetter et al 1980; Dialynas et al 1983). Briefly, 1×10^6 splenocytes were taken and labelled by anti-mouse CD4/8 FITC-labelled antibody in FACS labelling buffer (PBS + 0.1% BSA + Tween 20) for 30–40 min at 4°C in the dark. The cells were washed, resuspended in FACS buffer and analysed flow-cytometerically on a Beckton Dickinson FACS scan system. The data was analysed using Cell Quest and Mod-fit software.

Analysis of data

All experiments were repeated at least thrice and data from different experiments were pooled and presented as mean ± s.d. Non-parametric multiple comparison test (Kruskal–Wallis test) was performed using SPSS software. $P < 0.05$ was considered significant. The individual differences between two treatments were then compared using post-hoc Dunn's test; $P \leq 0.05$ was considered significant.

Results

Peritoneal macrophage cell survival

Whole-body irradiation (10 Gy) did not affect the viability of resident macrophages, which remained comparable with that of un-irradiated control (Table 1). RH-3 treatment alone significantly ($P \leq 0.05$) enhanced the viability of peritoneal macrophages as compared with un-irradiated control groups. However, RH-3 treatment before whole-body irradiation did not alter viability of peritoneal macrophages in comparison with those subjected to irradiation alone (Table 1).

Splenocyte survival

Whole-body irradiation (10 Gy) reduced the viability of mouse splenocytes significantly ($P \leq 0.05$) as compared with

Table 1 Effect of RH-3 treatment (30 mg kg^{-1} body weight, −30 min., 10 Gy) on various parameters of mouse peritoneal macrophages and splenocytes

Cell/tissue	Parameter	Untreated control	Radiation control	RH-3 treated	RH-3 + radiation
Macrophages	Viable count ($\times 10^6$)/mouse	1.30 ± 0.08	1.20 ± 0.06	5.80 ± 0.35**	1.30 ± 0.15
Splenocytes	Viable count ($\times 10^6$)/mouse	15.0 ± 1.9	2.5 ± 0.8*	21.4 ± 2.6**	$5.3 \pm 0.7^{\#}$
Splenocytes	Tritiated thymidine uptake (counts min^{-1} per 10^6 cells)	607 ± 142	159 ± 45*	538 ± 69	$444 \pm 153^{\#}$
Spleen	Spleen weight/mouse weight	2.90 ± 0.32	1.5 ± 0.4*	4.00 ± 0.35**	$2.10 \pm 0.15^{\#}$
Splenocytes	Total protein content (μg per 10^6 cells)	368 ± 16	48 ± 6*	340 ± 11**	$360 \pm 13^{\#}$
Splenocytes	Percent CD4+ population	27.2 ± 1.2	16.4 ± 5.1*	41.7 ± 8.0**	$43.7 \pm 10.5^{\#}$
Splenocytes	Percent CD8+ population	14.40 ± 0.02	10.8 ± 1.9*	24.5 ± 8.0**	$20.8 \pm 2.0^{\#}$

The data (mean ± s.d.) were pooled from three independent repeats of each experimental group. No. of mice (n) in each experimental group was 5. *$P < 0.05$, radiation control vs untreated control; **$P < 0.05$, RH-3 treated vs untreated control; $^{\#}P < 0.05$, RH-3+radiation vs radiation control.

un-irradiated control. RH-3 treatment alone (30 mg kg^{-1}) enhanced the viability of splenocytes to some extent in comparison with un-irradiated control. This increase was less marked than with peritoneal macrophages (Table 1). RH-3 treatment before irradiation (30 mg kg^{-1}, −30 min, 10 Gy) countered radiation-induced depletion in the splenocyte population significantly ($P \leq 0.05$) though the population remained substantially less than in the un-irradiated control group (Table 1).

Splenocyte proliferation

The ^{3}H-thymidine uptake method revealed that whole-body irradiation (10 Gy) reduced mouse splenocyte proliferation (159 ± 45 counts min^{-1}) significantly ($P \leq 0.05$) compared with un-irradiated control (607 ± 142 counts min^{-1}). RH-3 treatment alone did not alter the control value appreciably (538 ± 69 counts min^{-1}). However, RH-3 treatment before irradiation countered the radiation-induced decrease in proliferation (444 ± 153 counts min^{-1}) significantly ($P \leq 0.05$) compared with the irradiated control (Table 1).

Ratio of spleen weight/mouse weight

Whole-body irradiation (10 Gy) also reduced the spleen/mouse weight ratio (1.5 ± 0.4) significantly ($P \leq 0.05$) compared with control (2.9 ± 0.32). RH-3 treatment alone enhanced the spleen/mouse weight ratio significantly (4.0 ± 0.35; $P \leq 0.05$). RH-3 treatment before irradiation recovered the spleen/mouse weight ratio (2.1 ± 0.05) over the irradiated group (Table 1).

Total protein content

Whole-body irradiation (10 Gy) reduced the total protein content ($48 \pm 6\,\mu$g) of mouse splenocytes significantly ($P \leq 0.05$) compared with the un-irradiated control ($368 \pm 16\,\mu$g). RH-3 treatment alone did not reduce protein content ($340 \pm 11\,\mu$g) significantly ($P \leq 0.05$) compared with control. RH-3 treatment before irradiation did not permit the radiation-induced loss of spleen protein content and maintained it at a level comparable with that of the un-irradiated control (Table 1).

Effect on CD4+/CD8+ cell population

Whole-body irradiation (10 Gy) depleted both CD4+ and CD8+ T-cell populations significantly ($P \leq 0.05$) compared with un-irradiated control observed at 24 h after irradiation. RH-3 treatment (30 mg kg^{-1}) alone enhanced the population of both CD4+ and CD8+ T-cells significantly ($P \leq 0.05$) compared with that of the un-irradiated control group. However, RH-3 treatment before whole-body irradiation (30 mg kg^{-1}, −30 min, 10 Gy) enhanced both these cell populations significantly ($P < 0.05$) compared with those of the irradiated control (Table 1). Whole-body irradiation (10 Gy) also reduced the CD4+/CD8+ ratio (1.5) significantly ($P \leq 0.05$) compared with the un-irradiated control (1.9) observed at 24 h post irradiation. RH-3 treatment (30 mg kg^{-1}) alone also reduced it (1.7) but by an insignificant amount. However, RH-3 treatment before whole-body irradiation maintained the ratio (2.1) at a value comparable with un-irradiated control, contrary to irradiated control.

Effect on total nitrite content

Peritoneal macrophages in culture were irradiated ex-vivo with different radiation doses ranging from 0.25 to 10 Gy. A dose of 2 Gy enhanced nitric oxide free radicals significantly ($P < 0.05$), as reflected in the total nitrite levels ($5.72 \pm 0.09\,\mu$M) in ex-vivo peritoneal macrophage culture supernatant when compared with un-irradiated control ($1.64 \pm 0.09\,\mu$M). Other doses, however, remained ineffective for generation of total nitrite in macrophage supernatant. Stimulated production of nitrite in peritoneal macrophages by LPS treatment was further enhanced by

irradiation (2 Gy). Higher doses of RH-3 (15 or 30 μg mL^{-1}) inhibited nitrite formation significantly ($0.93 \pm 0.3\,\mu$M; $P < 0.05$) in comparison with both irradiated and un-irradiated control. However, a lower dose of RH-3 (1 μg mL^{-1}) could not inhibit generation of nitrite appreciably (Figure 2) as compared with both un-irradiated and irradiated control.

Discussion

Radiation damage is a multifaceted event and therefore a radio-protective agent has to act in a multidirectional way. *H. rhamnoides* used for this study was collected from an altitude of 3000–4000 m where a sub-zero temperature range existed in winter and high-radiation flux in the environment posed a challenge to the survival of living systems. In response to this kind of extreme environment, the successful survivor plant species must have developed defensive systems through evolutionary mechanisms. Various molecules of antioxidative (Zhang 1987), anti-inflammatory (Cheng et al 1994) and immunostimulatory nature (Zhen et al 1990) present in *H. rhamnoides* could have rendered the capability to counter the climatic adversity. It was expected that such molecules together could make this plant extract a most effective radioprotector.

Administration of RH-3 alone significantly enhanced the total number of macrophages and splenocytes (Table 1). Berries of *H. rhamnoides* (source of RH-3) have already been reported to contain several flavonoids and flavones (Wu et al 1994) that could have stimulated the precursors of macrophages and splenocytes to proliferate. Flavonoids, such as catechin, have been demonstrated to promote the expression of IL-6 mRNA and GM-CSF mRNA in mice spleen (Liu et al 2004). Through such molecules it was expected that RH-3 could accelerate the cell cycle progression, proliferation and differentiation of haematopoietic progenitors in the bone marrow of irradiated mice, as has been demonstrated by some other plant extracts (Kajimura et al 1996; Choi et al 2002; Jeong et al 2003). The increase in spleen weight (Table 1) due to RH-3 treatment could be accounted for by the enhanced growth of splenocytes. Data on DNA synthesis and total protein content (Table 1) did not reveal any appreciable difference with respect to control. This implied that the increase in spleen weight could be assigned to an increase in the number of splenocytes. It also indicated that the RH-3 concentration used during this study was non-toxic.

The antioxidant potential of RH-3 was evident by the inhibition of nitric oxide free radicals, which in turn reduced the nitrite content in resident peritoneal macrophages. Other plants growing under similar adverse climatic conditions to *H. rhamnoides* have also been demonstrated to elicit such an activity (Mittal et al 2001; Miyamoto et al 2002). During this study the immunostimulatory potential of RH-3 was also confirmed by the enhancement of the CD4+ and CD8+ T cell population (Table 1).

Irradiation did not adversely affect the survival of peritoneal macrophages because of their terminally differentiated status (Buescher & Gallin 1984). Contrary to peritoneal macrophages, splenocytes have proliferative potential and were radiosensitive (Table 1). Our results corroborated the findings reported by other workers (Slonina et al 2000). During our experiments the enhancement of nitrite generation (Figure 2) was a consequence of radiation-induced oxidative stress as has already been reported (Ibuki & Goto 1997). Such oxidative stress damaged DNA directly leading to a cascade of reactions that has far-reaching cytotoxic effects (Popanda et al 2003). This was corroborated by reduction in thymidine incorporation in whole-body irradiated splenocytes (Table 1). In fact, irradiation depleted the number of surviving

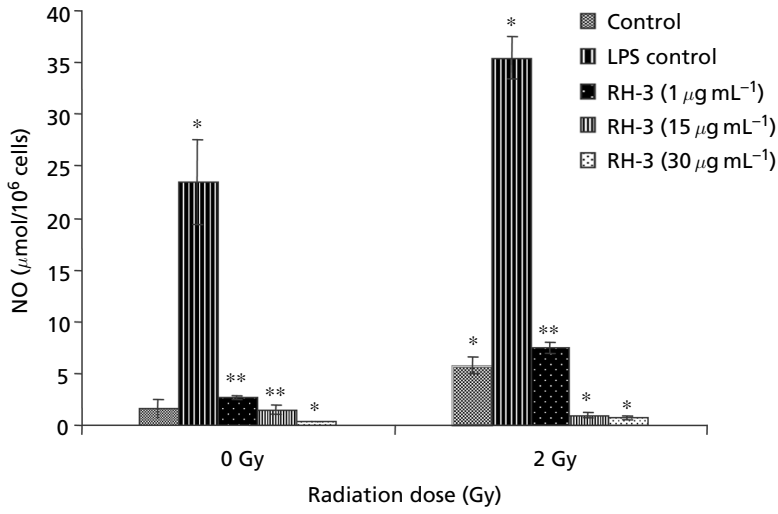

Figure 2 Effect of RH-3 treatment (30 mg kg^{-1}, −30 min, 2 Gy) on gamma radiation-induced nitrite generation in mouse peritoneal macrophages 48 h after irradiation. Data are mean ± s.d of three repeats for each experimental group. *$P < 0.01$ vs un-irradiated control; **$P < 0.05$ vs 2 Gy irradiated control group.

splenocytes (Popanda et al 2003), enhanced apoptosis in lymphocytes due to irradiation (Schmitz et al 2003) and affected cell cycle progression in the surviving fraction. Counts of CD4+ and CD8+ T cells and the CD4+/CD8+ ratio (Table 1) were also reduced after irradiation as has already been reported by several other workers (Zhu 1992; Pecaut et al 2001).

Reduction in total protein content by whole-body irradiation during our experiments (Table 1) may be attributed to oxidative damage (Halliwell et al 1992) leading to conformational changes and loss of certain functional groups in the proteins (Traverso et al 1996). The conformational and structural changes did not facilitate interaction of protein with chromophores to make protein–chromophores complex, which made the basis of colorimetric quantification (Sherman et al 1979). Depletion in splenocytes and reduction in protein content after whole-body irradiation (Table 1) has been observed by others also (Hau et al 1996) and this could account for decrease in spleen weight.

RH-3 treatment before whole-body irradiation did not elicit any change in macrophage survival at 24 h because macrophages by themselves are radio-resistant. The growth-stimulating effect shown by RH-3 treatment alone is not reflected in this group. It is expected that under high oxidative stress generated by lethal irradiation (10 Gy), growth stimulatory molecules present in RH-3 would take more time to stimulate the precursors of macrophages to proliferate and differentiate. Therefore, the enhanced proliferation was not observed in the 24 h post-irradiation period. Contrary to this, enhancement of the splenocyte survival by RH-3 treatment before whole-body irradiation revealed its cytoprotective potential (Table 1). RH-3 administration before irradiation protected DNA by several modalities, such as free radical scavenging, antioxidant action and compaction of DNA (Olive & Banath 1995; Goel et al 2003; Popanda et al 2003). This could have permitted the survival of a sufficient number of splenocytes, which could replenish the lost cells. The enhanced proliferation was further confirmed by increased uptake of labelled thymidine in the irradiated group treated with RH-3.

Pre-irradiation administration of RH-3 could also protect protein molecules against radiation-induced oxidative stress (Table 1). The immunostimulation as revealed by enhancement of the CD4+ and CD8+ T cell population and their ratio (Table 1) has also been reported for other herbal extracts (Gohla et al 1988; Kajimura et al 1996). All these manifestations of RH-3 make it a potential agent for radioprotection in man.

Conclusions

These findings explicitly reveal the immunostimulatory activity of RH-3, which may play an important role in the manifestation of its radioprotective efficacy. However, RH-3 needs to be investigated further at a cellular and molecular level before it can be considered for clinical applications.

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