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Role of *Rubia cordifolia* Linn. in radiation protection

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The radioprotective potential of alcoholic extract of root of R. *cordifolia,* was studied by survival, hemopoietic cell protection and micronucleus assay. The LD_{50} value for the alcoholic root extract was found to be 1200 mg/kg body weight at 72 hr post irradiation. A significant radiation protection (67%) as assessed by increased animal survival was observed when *R. cordifolia* (RC) extract was administered intraperitoneally, 90 min. before the radiation exposure. Besides, the extract also inhibited radiation induced lipid peroxidation measured by the inhibition of thiobarbituric acid reactive substance (TBARS). The RC extract at a selected dose of 460 mg/kg body weight was effective in protecting the radiation induced suppression of endogenous colony forming units in spleen. A significant inhibition of radiation (2 Gy) induced micronuclei formation was observed when RC extract was administered 90 min prior to irradiation. Thus, it appears that the alcoholic root extract of *R. cordifolia* provides significant protection against radiation induced lipid peroxidation, hemopoietic injury and genotoxicity. The mechanism of action of RC extract appears to be through its anti-oxidant, metal chelation and anti-inflammatory property.

Keywords: Antioxidant, Micronuclei, Radiation protection, *Rubia cordifolia*

Ionizing radiation generates free radicals throughout the cell, resulting nonselective alterations in various cell targets and macromolecules, leading to acute and chronic deleterious biological effects[1]. The acute radiation morbidity may be reduced by several pharmacological interventions. Inflammation of tissues is a common phenomenon after radiation exposure. Although several polyherbal formulations, single plant extracts and purified phytochemicals, such as curcumin, quercetin, rutin, ellagic acid, gallic acid, several other polyphenols and flavonoids have been studied for their radioprotective property, search for newer molecules are in demand aiming for improved therapeutic potential[2].

Rubia cordifolia Linn., Family Rubiaceae, commonly known as *Manjistha* in Hindi, is widly used for the management of chronic wounds, discoloured skin, fever, microbial diseases, diabetes, mental disorders, paralysis, leprosy and also as a general blood purifier[3]. It grows wild at an altitude of 1,000-2,500 meters in the Himalayan ranges of Northern India, in Nilgiris, and in (a few) hilly areas of Central India. Presence of anthraquinones, hexapeptides, alizarin-glycosides and terpenes are the major constituents of this plant[4]. Some of the other known active phytomolecules of this plant are cordifoliol and cordifodiol, O-methyl deoxybouvardin (hexapeptide), rubiadin (1, 3-dihydroxy-2-methyl anthraquinone), furomollugin, mollugin, naphthohydroquinones and its dimmers, rubilactone (3'-carbomethoxy-4'-hydroxy-naphtho[1',2'-2,3] pyran-6-one[5,6].

R. cordifolia alcoholic extract (RC) significantly prevented cumene hydroperoxide induced lipid peroxidation[7]. The protective effect was found to be better in comparison with Vitamin E and PBQ (parabenzoquinine)[8]. It also showed significant iron chelation property and rubiadin was associated for its antioxidant property[9]. RC extract inhibited Platelet activating factor (PAF) induced aggregation of rabbit platelets[10]. It also inhibited the potato lipoxygenase activity[11] and showed anticancer property[12]. Since radiation induced biological changes are mediated through free radical generation by the activation of the inflammatory cascade, it is hypothesized that RC extract could significantly inhibit the radiation-induced hazards and therefore, may be used as a radioprotective agent. The present study has been undertaken to investigate the radioprotective potential of RC extract against radiation induced damage.

Materials and Methods

Chemicals—Thiobarbituric acid, potassium chloride, glacial acetic acid and methanol were obtained from Central Drug House, New Delhi and 1,1,3,3-tetra ethoxy propane (TEP) was obtained from Sigma

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Chemical Co, St. Louis, USA. All other reagents were of analytical grade and procured locally.

Preparation of extract and drug administration—The roots of *Rubia cordifolia* (250g, authenticated by pharmacognostical parameters) were exhaustively extracted with ethanol in a soxhlet extractor for 40 hr. The solvent free concentrated extract was used for all the experiments. To avoid the batch-to-batch variation the RC extract was subjected to HPLC finger print profile, which has already been standardized[13]. For *in vivo* experiments, the extract, was filtered through 0.2 μ Millipore filter and administered intraperitoneally.

Experimental animals, maintenance and whole body irradiation procedure—Eight to ten weeks old Swiss albino mice of strain A, weighing 25-40 g were used from the inbred colony. The animals were maintained under controlled temperature and humidity with sterile bedding, food (standard rat chow) and water *ab libitum*. Animal care and handling to protect the welfare of the experimental animals was done according to the guidelines issued by the "Institutional animal care and use committee".

For whole body irradiation, the animals were placed in small perspex boxes and irradiated individually using ^{60}Co source (Eldorado, Atomic Energy, Canada) at a dose rate of 2.5Gy/min. Dosimetry was based on air ionization measurements made with Philips vibrating reed electrometer. Absorbed dose measurement was made with the ferrous ammonium sulfate dosimeter.

Acute toxicity of RC extract—To determine the LD_{50}, single doses of RC extract (460 mg to 1500 mg) were intraperitoneally administered to animals of experimental group, After 72 hr, percent survival was determined. Further, for the determination of effective time point of drug administration, RC extract was given on different time points (0.5 h to 4 hr) before irradiation and after 6 hr of radiation, lipid peroxidation was estimated in all the animals. The time showing maximum protection against radiation-induced lipid peroxidation was used for the remaining 3 experiments to assess the effect of RC extract.

Effect of RC extract on mouse survival exposed to gamma radiation (10Gy)—To study the effect of RC extract on radiation induced mortality, mice were randomly divided into sham and experimental groups (12 mice/group). Animals of sham (control) group received the drug vehicle, while all the other experimental groups received three different doses of RC extract viz., 115, 230 and 460 mg/kg body weight, 90 min before lethal dose of radiation (10Gy). Animal survival was monitored daily, and reported as percentage of animals survived through 30 days after irradiation.

Estimation of lipid peroxidation as thiobarbituric acid reactive substances (TBA-RS) in liver—The experimental groups and the irradiation procedure was similar as in the previous experiments. After 6 hr of post irradiation, mice were killed by cervical dislocation and liver was collected. Liver homogenate (10%) was prepared in a glass-Teflon homogenizer by using phosphate buffer saline (*p*H 7.4). Total protein content was estimated by the standard method of Lowry *et al.*[14] and TBARS was estimated according to the method of Okhawa *et al.*[15] with minor modifications[16]. The value of TBARS was calculated on the basis of standard curve, drawn by using 1,1,3,3, tetra ethoxy propane (TEP).

Hemopoietic stem cell survival assay and micronucleus assay—To assess the effect of RC extract on endogenous colony forming units (eCFUs), the animals were divided as sham and experimental groups. The RC extract at (460 mg/kg body weight was selected and administered 90 min prior to 5 and 7.5 Gy of gamma radiation. After 10 days of irradiation, all the animals were sacrificed and spleen of each animal was excised and fixed in Bouin's solution The number of macroscopically visible spleen colonies (nodules of diameter of 0.5 mm or larger) on the surface, per spleen was counted according to the method of Till and Mc Culloch[17].

To assess the effect of RC extract on radiation induced formation of micronuclei in the bone marrow erythrocytes of mice[18], the animals were divided into different groups as explained earlier for lipid peroxidation study. These animals were given the RC extract in different doses through intraperitoneally and after 90 min, they were exposed to 2 Gy of radiation. After 24 hr of irradiation, animals were sacrificed by cervical dislocation and bone marrow from both the femurs was flushed out in the form of suspension into a centrifuge tube containing fetal calf serum (FCS). The cells were dispersed by gentle pipetting and centrifuged at 1500*g* for 10 min at 4°C. The cell pellet was resuspended in 2-3 drops of FCS and smears were prepared on glass slides, air dried, and stained with Giemsa. The micronuclei containing erythrocytes were scored by counting 1000 cells/animal[19].

Statistical evaluation—The result was expressed as mean ± SD of 6 animals in each group. Student's "*t*" test was performed to assess the level of significance between the two groups.

Results

Effective prior time of drug administration—Effective time of drug administration, prior to radiation was found to be 90 min through intraperitoneal route (Fig. 1).

Acute toxicity of RC extract—After 72 hr of single dose of intraperitoneal administration of the RC extract, 100% survival was noted only up to the dose of 460 mg/kg body weight. While, on higher doses, mortality was observed in a dose dependent manner. Based on the data, LD_{50} was found to be 1200 mg/ kg body weight under the present experimental conditions.

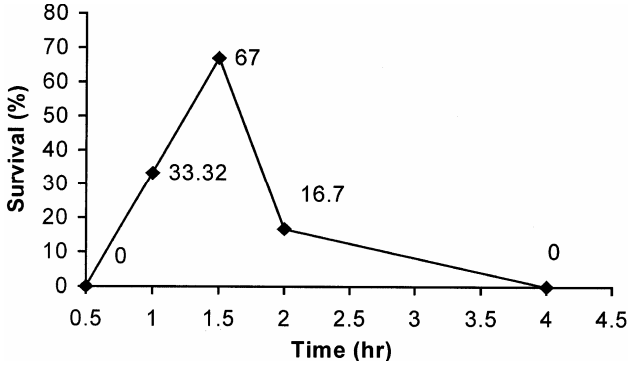

Fig. 1—Animal survival after single dose of RC extract (460 mg/kg body weight) at various time points before exposure to 10 Gy gamma radiation.

Effect of RC extract on radiation induced mortality—Animals exposed to 10 Gy alone (sham group) resulted in 100% mortality by 30 days. A dose dependent increase in the percent survival was observed when RC extract was administered 90 min prior to radiation treatment. A minimum mortality (37%) was noticed in the group receiving 460 mg /kg body weight of extract (Table 1).

Effect of RC extract on radiation induced lipid peroxidation and survival—RC extract significantly inhibited the radiation induced lipid peroxidation, analyzed after 6 hr of radiation. The effect was proportional to the concentration of extract administered and it was 60% at the dose of 460mg extract/kg body weight (Table 1). The extract by itself did not show any change in TBARS value when given to control animals.

Protection of endogenous colony forming units—Treatment with single dose of RC extract (460 mg/kg body weight) had no effect by itself on eCFUs in spleen, as the number of spleen nodules was same as in the sham treated control animals. Whereas, radiation alone significantly suppressed colony formation in a dose dependent manner. There was 75 and 83% reduction in the colony counts at the dose of 5 and 7.5Gy, respectively. Interestingly, pretreatment with

Table 1—Effect of different doses of *R. cordifolia* extract on mouse survival and lipid peroxidation after ^{60}Co γ radiation on survival[a] and lipid peroxidation[b]

[Values are mean ± SD from 12 mice in each groups]

Treatment groups	[a]Survival		[b]Lipid peroxidation	
	In numbers	%	TBARS in nmol/100 mg protein	Inhibition (%)
RC extract alone (460 mg/kg body weight)	12/12	100.0	75 ± 4	-
Radiation alone (10 Gy)	0/12	0.0	594 ± 42	-
Radiation (10 Gy) + *R. cordifolia* (mg/kg body weight)				
115	1/12	8.3	508 ± 36	15*
230	4/12	33.3*	401 ± 26	33*
460	8/12	66.6**	241 ± 29	60**

P value * < 0.05 ; ** < 0.01, compared to the corresponding data of sham control group, who received only radiation

[a]Survival was monitored up to 30 days.

[b]In the case of lipid peroxidation, experiment was made separately, where animals were sacrificed after 6 hr of irradiation. (n=6, values are mean ± SD)

RC extract (90 min, before irradiation) significantly (P <0.01) protected this suppression. (Table 2).

Protection against radiation induced micronuclei (MN) formation—RC extract by itself did not induce any additional micronuclei formation when administered at a dose of 460 mg/kg body weight, compared to the untreated control. The number of micronuclei (MN) bearing cells were in the range of 1.32% in the 2Gy exposed, There was a significant (P <.01) reduction in the MN bearing cells when RC extract was administered 90 min prior to 2 Gy gamma radiation (Table 3).

Table 2—Effect of *R. cordifolia* extract on radiation-induced suppression of eCFUs (endogenous colony forming units in spleen) in mice

[Value are mean ± SD from 6 different components]

Treatments	ECFUs (count/spleen)
Sham control[a]	26±3
R.C extract alone (460mg /kg. body weight)	25±3
Radiation (5 Gy) alone	6±1
7.5 Gy alone	4±2
R.C extract (460mg /kg body weight)+ radiation[b] (5 Gy)	21±2*
R.C extract (460mg/kg body weight)+ radiation[b] (7.5 Gy)	13±2*

*P < 001 compared to sham control group, who received radiation alone.
[a] only drug vehicle was given to animals and eCFU's was counted after 10 days.
[b] animals were pretreated with the RC extract at a dose of 460 mg/kg body weight 90 min before irradiation and then sacrificed after 10 days.

Discussion

RC extract enhanced the survival of irradiated animals. There may be several mechanisms, responsible for the increased animal survival and one of them may be due to protection against gastrointestinal (GI) death, which takes place within 10-15 days of radiation as it has been reported with several other plants having poly phenols such as *Podophyllum hexandrum*, *T. cordifolia*, *Ocimum sanctum*, etc.[20-24] RC extract possesses poly hydroxyl substituted anthraquinones, which may be responsible for this protective property[9]. Interestingly, best protection was found to be at the dose of 460 mg/kg body weight in our experimental conditions (higher doses of RC extract has not been tried because, it showed some degree of mortality by itself. Although $LD_{50(30)}$ was at 1200 mg because of no mortality, the dose of 460 mg/kg body weight has been chosen for all experiments.

Figure 1 shows the effective time point for radioprotective property, which was found to be 90 min before irradiation, when administered through intraperitoneal route. This phenomenon could be associated to the pharmacokinetics and bioavailability of RC extract in mice. Similarly, in an earlier study on *Ocimum sanctum*, where a maximum radioprotective effect was obtained when it was injected through intraperitoneal route 30 min before irradiation[25].

Table 3—Effect of *R. cordifolia* extract against radiation induced micronuclei formation

[Values are mean ± SD of 6 different experiments]

Treatments	Total cell count	Micronuclei counts	Micronucleated cells (%)
Sham control[a]	1206	1	0.08 ± 0.008
R.C extract alone (460 mg/kg)	1366	1	0.07 ± 0.006
Radiation (2Gy)	1282	17	1.32 ± 0.002
Radiation (2 Gy) + *R. cordifolia* (mg/kg body weight)[b]			
115	1224	16	1.29 ± 0.001*
230	979	12	1.22 ± 0.002*
460	1183	10	0.81 ± 0.004**

P <0.05; ** < 0.01, compared to sham control group, who received radiation alone
[a] only drug vehicle was given to animals.
[b] animals were pretreated with the RC extract at a dose of 460 mg/kg body weight intraperitoneally, 90 min. before irradiation and then the animals were sacrificed after 24 hr.

Cytogenetic damage due to radiation has been associated with free radical production. The *in vivo* protection by RC extract, against radiation-induced cytogenetic damages may be because of its free radical scavenging potential. It could also because of direct scavenging/neutralization of the free radicals or by induction of the endogenous antioxidants enzymes[26] such as catalase, superoxide dismutase and glutathione S transferase[27]. Besides, some plant products show their role through metal chelation also, as metal ions are directly related to (responsible for) the free radical production[23]. RC extract significantly chelates iron[13] maintains the level of reduced glutathione content[8], and also scavenges the hydroxyl radicals[9]. Therefore the protective role of RC against radiation induced cytogenetic damage may be attributed to its multi functional role, as described above.

Another possibility involved in radioprotection of RC extract could be through increased hemopoiesis. It is known that spleen of rodents presents a suitable environment for erythroid differentiation[28] and increased number of spleen colonies in the RC extract treated animals support this proposed mechanism. Number of eCFUs,, which can be counted between 8-11 days after irradiation, indicate the number of pluripotoent colony forming cells, the hemopoietic stem cell pool. The increased number of spleen colonies, after RC extract treatment, could be because of enhanced survival due to less DNA damage in the spleen cells. Or it may be due to increased migration of colony forming cells from other sources, such as bone marrow to the spleen. Further radioprotective effect could also be due to enhanced regeneration of the hematopoietic stem cells, or may be due to enhanced post-irradiation repair[29]. Since RC extract has shown significantly higher number of eCFU counts in the 5Gy-irradiated animals than the 7.5 Gy exposed animals, it appear that RC extract could be enhancing the post radiation repair process. It may also be inhibiting the irradiation induced cell apoptosis, leaving more active stem cells in the spleen, because apoptosis is mediated through free radical and inflammatory cascade[26,30], as reported with other medicinal plants e.g. Lycium chinense (LC)[31] and Acanthopanax senticosus Harms (Shigoka)[32].

Inflammatory reaction is a classical feature of radiation exposure and the pathological changes within the intestinal muscle, which may be characterized by high production of cytokines in the early stages of radiation induced damage. This is supported by the observations that alpha-2-recombinant interferon, anti-inflammatory agents, steroids, antioxidants and enterosorbtive preparations of biological nature have shown significant protection[33]. IL-1 alpha-induced suppression of eCFUs was mediated through induction of TNF-α[34]. Inhibitors of prostaglandin synthesis (PG) have also shown to enhance leucopoiesis, which is impaired by sub-lethal fractionated irradiation[35]. The reported inhibitory role of RC extract on lipoxygenase activity[11] could also attribute to its radioprotective action, because the products of this enzymes has a role in inflammation, which is involved in radiation induced injury[26,30,36].

Thus it could be concluded that alcoholic extract of *Rubia cordifolia* root extract, possesses significant radioprotective potential and its mechanism of action could be through more than one pathway as it contains several phytomolecules in the extract. The possible proposed pathways behind its radioprotective effect may be attributed to its antioxidant, metal chelation and anti-inflammatory pathways.

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