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Research Article

Protective Efficacy of *Asparagus racemosus* Root Extract and Isoprinosine against Ionizing Radiation - induced Clastogenicity and Toxicity in Swiss Albino Mice

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ABSTRACT

Objective: We aimed to evaluate anticlastogenic and radioprotective potential of *Asparagus racemosus* root extract (ARE) and Isoprinosine (IPR) against electron beam radiation (EBR) induced clastogenicity and toxicity in Swiss albino mice. **Methodology:** In the pre-radiation study, the experimental animals were orally administered ARE - 200mg and IPR - 400mg/kg b.wt once daily for 15 consecutive days. The animals exposed to sublethal dose (6Gy) of whole body EBR. Chromosomal aberration analysis and micronucleus assay were carried out in the bone marrow cells of the experimental animals. The various types of aberrations were scored and the micronuclei in Polychromatic Erythrocytes (PCE) and Nomochromatic Erythrocytes (NCE) were recorded. Assessment of Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), was performed using mouse GM-CSF Picokine ELISA kit. Non-specific Alpha - esterase activity was determined by simultaneous azo dye coupling method. Dose Reduction Factor (DRF) was calculated to determine the protective role of ARE and IPR against EBR. **Result:** Treatment of mice with ARE-200 mg/kg b.wt and IPR-400mg/kg b.wt decreased the percentage of the total aberration compared to the irradiated group; significantly reduced ($P<0.05$) the frequency of Mn PCE and Mn NCE when compared with irradiation alone groups. Irradiation reduced the level of GM-CSF in the splenocytes which was enhanced by the pre-treatment with ARE and IPR. There was a significant increase in the number of alpha-esterase positive cells in the pre-treatment group compared to radiation control. Increase in survival percentage was observed in the pre-treated mice when compared to radiation alone group. The DRF value of 1.11 and 1.04 was observed respectively. **Conclusion:** The present study suggests that the antioxidant potential of ARE and IPR could be of extreme significance in offering radioprotection and may be useful in combating various free-radical and reactive oxygen species - mediated human pathological conditions.

Key words: *Asparagus racemosus* root extract, Isoprinosine, Electron beam radiation, Clastogenicity, Toxicity.

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INTRODUCTION

Ionizing radiation has been reported to induce DNA damage, which in turn causes mutagenesis and carcinogenesis that is dependent on the total dose, dose rate, and animal species.[1,2] In recent days, the need for nontoxic protective compounds is heightened due to radiation damage to normal tissues from occupational, therapeutic and accidental exposures and this has prompted interest in the discovery and development of medicinal plants and dietary compounds for protection against radiation-induced toxicity.[3-5]

Ionizing radiation is known to induce a wide array of molecular lesions in mammalian cells that can lead to diverse cellular responses namely cell inactivation, chromosomal rearrangements and mutations, eventually resulting in cancer and several genetic diseases. DNA damage occurs either by direct ionization or indirectly through generation of free radicals which attack DNA, thereby resulting in single-strand breaks and oxidative damage to sugar and base residues which may later be converted into DNA double strand-breaks (DSBs). It is reported that the unrepaired DSB will contribute to chromosomal aberrations. Aberrations like fragments, dicentrics and chromosomes with damaged kinetocore following division appear as micronuclei in the daughter cells. The micronucleus count gives an indirect measure of cytogenetic damage induced by any genotoxic agent and the inhibition of radiation-induced micronuclei indicates the radioprotective potential of any test agent.[6]

Cytogenetic damage induced by any genotoxic agent is indirectly measured by the micronucleus count. The inhibition of radiation induced micronuclei formation indicates the radioprotective efficacy of any test (herbal or chemical) agent. Radiation-induced aberrations in bone marrow-derived cell populations are known to persist for months after irradiation as evidenced by several studies evaluating bone marrow cells and bone marrow stromal cells after irradiation. Radioprotective compounds, which can selectively protect normal tissues against radiation injury, is immensely useful; in association with protecting the normal tissue, it will also permit use of higher doses of radiation to obtain better cancer control and possible cure. However, till date no ideal radioprotectors are available as most synthetic compounds, including the Food and Drug

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Administration (FDA), USA, approved aminothiol S-2-(3-aminopropyl-amino) ethyl phosphorothioic acid, [WR-2721, amifostine, ethiophos (USA), or gammaphos (former USSR)], are toxic at their optimal doses. Obviously, there has been a limited success of these agents in clinical applications.[7-9]

Plant-derived drugs provide an alternative to the synthetic compounds and are considered either non-toxic or less toxic than their synthetic counterparts. Herbs and their phytoconstituents, particularly with free radical scavenging, antioxidant properties, and immunostimulatory effects have been evaluated for their radioprotective efficacy. In the past two decades, preclinical studies have shown that some commonly used medicinal plants and their phytoconstituents possess radioprotective properties. Therefore, screening of natural products presents a major avenue for the discovery and development of novel radioprotective drugs.[10-15]

Asparagus racemosus belongs to family *Asparagaceae* commonly known by the name Shatavari, is one of the recognized drugs in Ayurveda, which is known to prevent aging, increase longevity, impart immunity, and improve mental function. Reports indicate that the pharmacological activities of *A. racemosus* root extract include antiulcer, antioxidant and immunomodulatory activities. This plant is grouped under the rejuvenator herbs which improves health by increasing immunity, imparting longevity as well as protection against stress.[16-18]

Isoprinosine has been shown to enhance production of cytokines such as IL-1, IL-2 and IFN γ. It increases the proliferation of lymphocytes in response to mitogenic or antigenic stimuli, increases active T-cell rosettes and induces T-cell surface markers on prothymocytes.[19]

However, till date, no evidence is available on the anticlastogenic and radioprotective activity of *Asparagus racemosus* root extract and Isoprinosine against ionizing radiation-induced clastogenecity. Therefore, the present study is intended to investigate the anticlastogenic and radioprotective activity of *Asparagus racemosus* root extract and Isoprinosine using chromosomal aberration and micronucleus assays.

MATERIALS AND METHODS

Collection of Plant Material

Asparagus racemosus roots were collected from the Western Ghats region of Karnataka during March 2015 and were identified by a Taxonomist from Mangalore University, Karnataka.

Preparation of Root Extracts

Ethanolic Extract

Asparagus racemosus (AR) roots were washed in distilled water, air dried and then dried in hot air oven at 40°-50°C for a week. The dried plant material was powdered using mixer grinder and subjected to Soxhlet extraction with 99% ethanol for 48 hours. The mixture was evaporated to dryness in a rotary flash evaporator and stored in a refrigerator. The condensed extracts were used for preliminary screening of phytochemicals.

Procurement of Isoprinosine

Isoprinosine tablets were procured from Brandmedicines, European Union. (product code: 78/158)

Ethical Clearance

The Institutional Animal Ethics Committee of K.S Hegde Medical Academy, Nitte – Deemed to be University, has approved this study. (Ref. KSH-EMA/IAEC/19/2015 dated 27.11.2015)

Pre - radiation Study

Animal Care and Handling

The guidelines set by WHO (World Health Organization, Geneva, Switzerland) was followed for animal care and handling. Swiss albino mice 6-8 weeks old, weighing 20-25 g taken from an inbred colony were used for the study. Six animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The animals were maintained under controlled conditions of temperature and light. They were provided with standard mice feed and water *ad libitum*.

Pre - Treatment

The animals were housed in the animal house and prior exposure to sublethal radiation dose, they were administered *Asparagus racemosus* ethanolic root extract (ARE) 200mg and Isoprinosine (IPR) 400mg/kg b.wt orally via gavage once daily for 15 consecutive days. Meanwhile, control group and radiation control group were also maintained which were orally administered with distilled water. ARE and IPR were made into suspension using distilled water.

Food and water intake were recorded daily, whereas, body weight was recorded once in a week throughout the study period.

Irradiation

The animals were restrained in well- ventilated perspex box and exposed to sublethal dose (6Gy) of whole-body electron beam radiation at a distance of 100 cms from the beam exit point of the linear accelerator and at a dose rate of 3Gy/min.

Analysis of Cytogenetic damage

Chromosomal Aberration Analysis

After the experimental period, 0.2-0.3ml of 0.025% of colchicine was injected intraperitoneally into the mice and left for one and a half hours. The experimental animals were sacrificed and the femur bone was removed and cleaned well. 0.5 ml of KCl was taken in a syringe and bone marrow cells were flushed into a Petri dish, the suspension was mixed well and the analysis carried out according to the method of George and Kuttan.[20] Chromosomal aberrations were scored under a light microscope. A total of 500 metaphase plates were scored per group and each aberration type was calculated in percentage.

Micronucleus assay

The mouse bone marrow micronucleus test was carried out according to the method described by Schmidt.[21] The bone marrow from the femur of the experimental animals was flushed into a centrifuge tube containing 5% Bovine serum albumin (BSA). The slides were stained with May-Grunwald's and Giemsa stain. 500 Polychromatic (PCE) and Normochromatic erythrocytes (NCE) were observed and counted under light Microscope. The micronuclei in PCE and NCE were recorded and micronuclei per 500 PCE and NCE cells were calculated.

Assessment of Granulocyte - Macrophage Colony-Stimulating Factor (GM-CSF) production

Mice were sacrificed and spleen tissue was isolated using ice-cold phosphate buffer saline. Single-cell suspensions were prepared by pressing isolated spleens through a cell strainer as described previously.[22]

To assess whether ***A. racemosus*** root extract and **Isoprinosine** could induce the production of GM-CSF, an enzyme-linked immunosorbent assay (ELISA) was performed in the prepared spleen cell suspension using mouse GM-CSF Picokine ELISA kit from Boster Biological Technology according to manufacturer's instructions.

Determination of alpha esterase activity

Bone marrow was collected from the femur into the medium containing 2% bovine serum albumin and made into a single cell suspension. The number of cells was determined using hemocytometer.

Bone marrow cells from the above preparations were smeared on clean glass slides and stained with Fast blue base solution and Hematoxylin to determine the non-specific α- esterase activity by simultaneous azo dye coupling method following the Sigma Aldrich procedure no.91.[23]

The alpha (α)- esterase positive cells were scored microscopically.

Determination of dose reduction factor (DRF)

DRF was calculated to determine the protective role of ARE and IPR against lethal Electron Beam Radiation [EBR]. For this, animals were divided into following groups (n=10 per group).

- Radiation alone group

Three groups of ten animals were maintained. They were orally administered. 1ml / 100g.b.wt. of distilled water orally for 15 consecutive days. One hour after of the last administration, they were exposed to 7, 9 and 11Gy of EBR respectively.

- ARE and IPR + radiation group

Six groups of ten animals were maintained. They were orally treated with the optimal dose of 200 mg/kg. b. wt. of ARE and 400 mg/ kg. b.wt. of IPR once daily for 15 consecutive days and after the last dose, i.e., on the 15th day, animals were exposed to 7, 9 and 11 Gy of EBR respectively.

The animals of all the groups (radiation alone, ARE and IPR + radiation) were observed daily for up to 30 days post-irradiation for signs of radiation sickness and mortality. The DRF was calculated according to the method of Miller and Tainter[24] as follows:

$$\text{Dose reduction factor} = \frac{\text{LD 50/30 of treated group + irradiation}}{\text{LD50/30 of irradiation alone animals}}$$

Statistical Analysis

The data are expressed as Mean± SD. Analysis of variance (ANOVA) was used to make the statistical comparison between the groups followed by Tukey's multiple comparison tests using Prism 3.0 software. Dose reduction factor was calculated from the ratio of LD_{50}/30 of treated group + radiation to radiation alone group.

The criterion for statistical significance was taken as $P < 0.05$.

RESULTS

Effect of ARE and IPR Pre-Treatment on Chromosomal Aberrations and Micronucleus Formation

In the present study, control mice showed less percentage of aberrant cells while radiation treatment (whole body 6Gy radiation) showed an increase in the percent of aberrant cells. A corresponding increase was found in all the individual aberrations. Treatment of mice with ARE at 200 mg/kg b.wt and IPR at 400mg/kg bwt resulted in a decreased percentage of total aberration and number of aberrations per cell compared to the irradiated group. There was a decrease in all types of aberrations (Table 1). Metaphase chromosome preparation from bone marrow cells of control and treatment groups is shown in Figure 1.

Pretreatment with ARE and IPR significantly reduced ($P<0.05$) the frequency of Mn PCE and Mn NCE when compared with irradiation alone groups (Table 2). Micronuclei formation in the bone marrow preparation of experimental animals is shown in Figure 2A-NCE and Mn NCE and Figure 2B-PCE and Mn PCE.

Table 1: Effect of ARE and IPR pre-treatment on radiation induced chromosomal aberrations in swiss albino mice.

Treatment	Chromatid break (%)	Chromosome break (%)	Exchange (%)	Ring (%)	Fragmentation (%)	Polyploidy (%)	Total aberration (%)
Control	0.5	0	0	0.75	0.5	1.0	2.75
RC(6Gy)	8.5	10.5	9	15	8	2.0	53
ARE	4	4	4	7	2.5	1.5	23
IPR	5	4	5	8	3	2.0	27.0

Figure 1: Metaphase chromosome preparation from bone marrow cells of control and treatment groups.

Table 2: Effect of ARE and IPR pre-treatment on radiation induced micronuclei formation in swiss albino mice.

Treatment	MnPCE/ 500PCE (mean± SD)	MnNCE/ 500NCE (mean± SD)
Control	5.5±0.70	0±0
RC(6Gy)	34±9.9	4.5±0.70
ARE	6.5±2.1	1±0.12
IPR	24.5±2.1	2.5±0.70

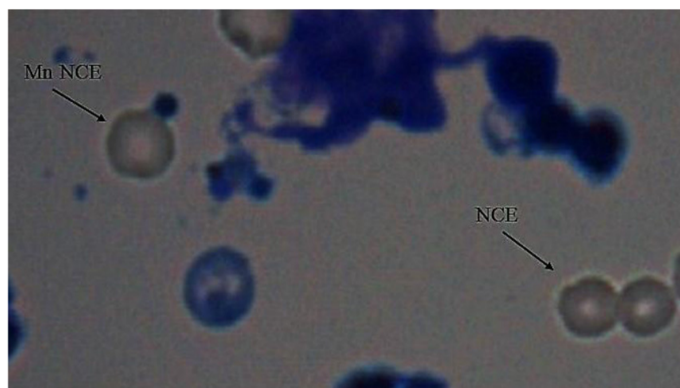

Figure 2A: Normochromatic Erythrocytes (NCE) and Micronucleated Normochromatic Erythrocytes (MnNCE).

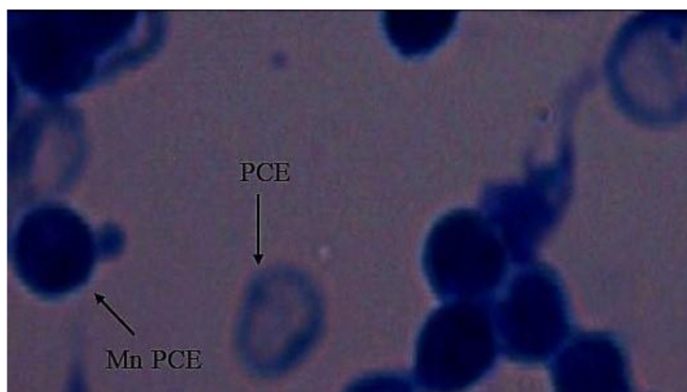

Figure 2B: Polychromatic Erythrocytes (PCE) and Micronucleated Polychromatic Erythrocytes (MnPCE).

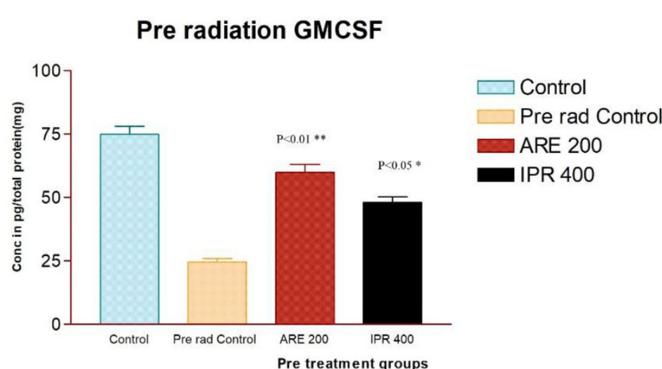

Figure 3: Effect of ARE and IPR pre-treatment on Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF).

Effect of ARE and IPR pre-treatment on Granulocyte Macrophage Colony Stimulating Factor and alpha esterase activity

Effect of ARE and IPR pre-treatment on Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) is represented graphically in Figure 3. From the present study, it is evident that irradiation reduced the level of GM-CSF in the splenocytes which was significantly enhanced by the pre-treatment with *Asparagus racemosus* root extract ($P<0.01$) and Isoprinosine ($P<0.05$) compared to pre-radiation control.

The effect of ARE and IPR pre-treatment on alpha-esterase positive cells is shown in Table 3. The numbers of alpha esterase positive cells in irradiated control animals were found to be reduced compared to the control group. In the case of ARE and IPR pre-treated animals, there was a significant increase in the number of alpha-esterase positive cells compared to radiation control. $P<0.05$ was observed in the pre-treatment groups compared to pre-radiation control.

Dose Reduction Factor of ARE and IPR

Increase in survival percentage was observed in ARE treated and irradiated mice when compared to radiation alone group. The $LD_{50}/30$ was found to be 9 Gy for radiation alone. Although, it was increased to 10 Gy after ARE treatment resulting in increased $LD_{50}/30$ value by 1.0Gy. The DRF of 1.11 was observed (Figure 4).

IPR administered group also exhibited an increase in survival of mice when compared with radiation alone group. The $LD_{50}/30$ was found to be 9 Gy for radiation alone. Although, it was increased to 9.4 Gy after IPR treatment resulting in increased $LD_{50}/30$ value by 0.4 Gy. The DRF of 1.04 was observed (Figure 5).

Table 3: Effect of ARE and IPR pre-treatment on alpha esterase activity in irradiated mice.

Treatment	α- Esterase positive cells/1000cells (Mean ± SD)
Control	317 ± 8.0
Pre - radiation Control	91 ± 3.0
ARE 200 + radiation	171 ± 4.8
IPR 400 + radiation	119 ± 5.1

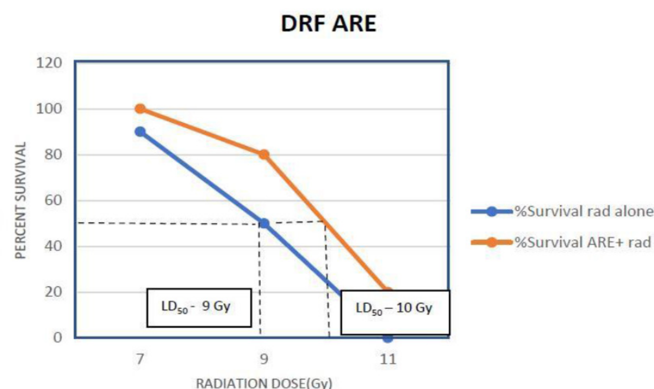

Figure 4: DRF-ARE.

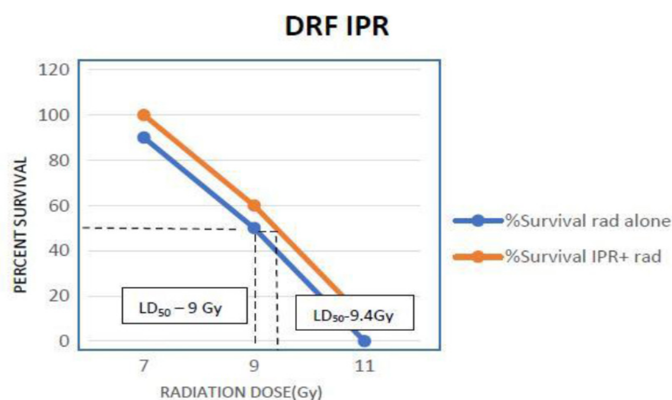

Figure 5: DRF-IPR.

DISCUSSION

Chromosomal aberrations (CAs) are observed as breaks, dicentrics, acentrics, fragments, rings, and translocations in cells when they are exposed to ionizing radiation during the G0 or G1 phases of the cell cycle. These CAs are used as biomarkers of radio sensitivity or radiation damage following medical, accidental and occupational exposure. There is evidence which suggest that some of these CAs may be strongly linked with different cancer types.[25-27] In addition, radiation is well-known to generate free radicals which cause chromosomal damages. The use of certain materials may aid in decreasing the genotoxicity caused by radiation and may inhibit mutagenesis and carcinogenesis.[28]

In the present study, it was observed that the frequency of aberrant cells: chromatid breaks, chromosome breaks, centric rings, exchanges, acentric

fragments, and polyploidy significantly increased in bone marrow cells of animals exposed to 6 Gy electron beam radiation (EBR). A significant increase in the percent aberrant metaphases as well as different aberrations, is known to be produced during radiation exposure. Chromosomal damage is manifested as breaks and fragments, which would later appear as micronuclei in the rapidly proliferating cells.

In several published studies, a number of possible mechanisms for the observed decrease in the frequency of CAs have been described although the specific mechanism responsible for the observed effect of ARE and IPR in reducing the frequency of chromosome aberrations was not evaluated in this study. Since Electron Beam Radiation (EBR) is known to induce cellular damage mediated through oxidative processes, it is possible that the significant reduction in observed chromosomal damage in the ARE and IPR pre-treatment group compared to the radiation alone group is the result of the direct antioxidant properties of ARE and IPR or that it occurs indirectly through the induction of antioxidants like glutathione and increases in enzymes like superoxide dismutase and catalase.[29,30]

Radioprotectors are known to offer protection to cells by scavenging the Reactive Oxygen Species (ROS) generated by ionizing radiation which is mediated through free radical scavenging mechanism and another mechanism includes hydrogen atom donation which facilitate direct repair at sites of DNA damage. Radioprotectors scavenge the ROS generated by ionizing radiation before they can interact with biochemical molecules, thus reducing the deleterious effects of radiation. Therefore, antioxidant and free radical scavenging properties may be suggested to be the likely mechanism of radiation protection.[31,32]

The induction of different types of lesions in the DNA by ionizing radiation namely, single and Double strand breaks (DSB), base damage, as well as DNA cross-links (DNA-DNA and DNA-Protein) is well established. Amongst these, DNA DSBs have been considered the critical lesion for the radiation-induced chromosome break and cell death. Several studies have established a correlation between the induction of cell death and chromosome aberrations and the frequency of micronuclei formation. Acentric fragment(s) or sometimes the whole chromosome (with defective kinetocore) will lead to the formation of micronuclei. An increase in the micronuclei frequency during the interphase after the first post-treatment mitosis is observed due to the induction of cytogenetic damage by radiation and clastogenic agents on the mitotic cell. Moreover, it is understood that nonrepair/ misrepair of the DNA double-strand breaks contributes to the chromosomal aberrations which could be quantitatively analyzed by the scoring of micronuclei formation in the cells.[33,34]

Therefore, the micronucleus assay is a very useful parameter used for evaluating cytogenetic damage and is very extensively used in the screening of cyto-protective / radio-modifying potential of natural and synthetic products. Our study demonstrated that ARE and IPR were effective in counteracting the clastogenic effect of radiation, thereby offering radioprotection as assessed by the micronucleus assay. It is interesting to note that ARE and IPR were very effective in ameliorating the effect of radiation at 200 and 400 mg/kg b.wt in mice. In the present study, we report for the first time the radio protective and anticlastogenic potential of ARE and IPR. Although at this stage, the exact mechanism of action of ARE and IPR on radioprotection is far from being understood, the available information on the antioxidant potential of ARE and IPR obtained from the present investigation supports the free radical scavenging mediated pathways.[35]

The earlier report on the phytoconstituents of the roots of *Asparagus racemosus* indicated the presence of saponins, flavonoids, tannins, free amino acids, carbohydrates, vitamin-C, and sterols. Therefore, the presence of some of these constituents may have rendered radio protective and anticlastogenic effect. Besides, the roots may also have other unidentified bioactive compounds. Therefore, although ARE and IPR is capable of imparting a radio protective effect, at this stage it is not possible to attribute the observed effect to any one of the bioactive principles of the roots as the degree of protection will depend on the effect of these agents either singly or collectively against radiation-induced cytogenetic damage.[36]

Granulocyte Macrophage Colony Stimulating Factor **(GM-CSF)** is one of the major hematopoietic growth factors; belongs to a family of glycoproteins. It is mainly produced by T lymphocytes or non-hematopoietic cells and stimulates proliferation and function of a wide variety of myeloid progenitor cell.[37]

Radiation injuries is a complex clinical challenge and its management requires careful use of mitigation as well as therapeutic agents, administered at suitable times following the exposure. Available evidence from literature supports the concept that various colony stimulating factors viz. G-CSF, GM-CSF etc. significantly enable recovery from radiation-induced hematopoietic injury and thus, enhance the survival of individuals experiencing acute radiation syndrome.[38]

Naphthyl acetate esterase activity in the bone marrow is an indicator of maturation of stem cells to monocytes-macrophages. A number of bone marrow positive cells for non-specific esterase were found to increase after ARE and IPR treatment which indicates the maturation of cells of monocyte/macrophage lineage. One of the mechanism may be by the induction of proliferation of bone marrow stem cells either directly or indirectly stimulating the release of factors that are involved in the regulation of hemopoiesis. ARE and IPR were found to enhance the number of esterase positive cells that support the above data.[39]

The DRF is defined as the ratio of radiation doses required to produce the same biologic effect in the absence and presence of the radioprotector. The protective capacity of an agent (chemical or plant extract) is expressed as Dose Reduction Factor (DRF).[40]

The extent of protection against radiation damage is most commonly assessed either by comparing percentage survival between the treated and the control groups at a selected lethal radiation dose, or by computing a dose reduction factor (DRF) for the drug under study. To determine the DRF, groups of treated and control animals are exposed to several levels of radiation and observed for survival from day 1 to day 30. The LD_{50} of radiation is determined for the control group and the treatment groups.[41]

In animal studies, DRF is determined by irradiating the experimental animals with and without administering the test agent at a range of radiation doses and then comparing the endpoint of interest. Unfortunately, DRFs have not been reported often for plant-derived compounds. In mice model, DRF for naturally occurring compounds are not likely to be greater than 1.3 whereas high DRFs (>2.0) may be obtained with synthetic drugs.[42]

Assessment of DRF for 30-day survival (LD_{50}/30) allows for a comparison of potential agents that protect against radiation-induced hematopoietic mortality in mouse model. Naturally occurring antioxidant compounds namely, vitamin E (alpha-tocopherol), demonstrated a DRF of 1.11 (95% CI) when given within 15 min after TBI in CD2F1 mice.[43] In our study, we demonstrated that ARE + radiation afforded a better DRF (1.11) than IPR + radiation with DRF value of 1.04, when given 15 days orally before total body irradiation.

In our present study, the radio protective effect of ARE and IPR was demonstrated by the LD_{50}/30 values and DRF of 1.11 and 1.04 respectively, which is apparently quite high and therefore demonstrates the efficacy of ARE and IPR as potent radio protectors.

CONCLUSION

From the present study, it can be concluded that in order to understand the mechanism of radioprotection exhibited by ARE and IPR, its bioactive components need to be isolated, purified and characterized. The antioxidant potential of ARE and IPR in reducing radiation-induced clastogenicity

may also be due to the induction of phase II antioxidative enzymes such as superoxide dismutase, catalase etc., Therefore, there is a need for extensive exploration of the radio protective efficacy of ARE and IPR and its active principles by using human cell lines. In addition to its anti-clastogenic property, the present observation of the antioxidant potential of ARE and IPR could be of extreme significance in offering radioprotection and may be useful in combating various free-radical and reactive oxygen species-mediated human pathological conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ANOVA: Analysis of Variance; **ARE:** Asparagus racemosus Ethanol; **α:** Alpha; **b.wt:** Body weight; **CA:** Chromosomal Aberration; **DNA:** Deoxyribo nucleic acid; **DRF:** Dose reduction Factor; **DSB:** Double Strand Break; **EBR:** Electron Beam Radiation; **ELISA:** Enzyme Linked Immunosorbent Assay; **FDA:** Food and Drug Administration; **GM-CSF:** Granulocyte Macrophage Colony Stimulating Factor; **Gy:** Gray; **IPR:** Isoprinosine; **Kg:** Kilogram; **KCl:** Potassium Chloride; **mg:** milligram; **NCE:** Normochromatic Erythrocytes; **PCE:** Polychromatic Erythrocytes; **ROS:** Reactive Oxygen Species; **TBI:** Total Body Irradiation; **WHO:** World Health Organization; **WR-2721:** Walter Reed Army Institute.

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