RADIO-RESPONSE TO LEUCOCYTES IN PERIPHERAL BLOOD OF MICE AGAINST GAMMA IRRADIATION AND THEIR PROTECTION BY ALSTONIA SCHOLARIS EXTRACT

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The present study deals with the protective effect of Alstonia scholaris extract against radiation-induced hematological alterations. Swiss albino male mice were selected from an inbred colony and divided into four groups. The first group received only double distilled water orally, served as vehicle control and the second group were administered the Alstonia scholaris extract at a dose of 100 mg/kg body weight per day dissolved in the double distilled water. The third group was administered the double distilled water, which served as irradiated control while the fourth group was administered the Alstonia scholaris extract once in a day for five consecutive days. Groups third and fourth were exposed to 7.5 Gy of gamma radiation after half an hour of 5th day of double distilled water or Alstonia scholaris extract administration, respectively. The animals were autopsied at 12 hours, days: 1st, 3rd, 7th, 15th, and 30th post-exposure for hematological evaluation. The extract was found to restore the total leucocytes and differential leucocytes (lymphocytes, monocytes, neutrophils, and non-neutrophilic granulocytes) count in the Alstonia scholaris extract pretreated animals as compared to the irradiated control group. The data clearly indicate that the Alstonia scholaris extract significantly reduced the deleterious bioeffects of radiation on peripheral blood.

Key words: Alstonia scholaris, radioprotection, hematological profile, Swiss albino mice

INTRODUCTION

Radiotherapy has become a routine treatment for various types of malignancies. However, severe side effects commonly arise from radiotherapy including nausea and vomiting, loss of appetite, decreased leucocytes count, and weakened immunofunction which often prevent patient from completing the treatment course [1]. Hemopoietic tissues mainly bone marrow and lymphoid are highly radiosensitive. The most marked effects are on the parent (stem) cell of the leucocytes, lymphocytes and platelets. Red cells are much less radiosensitive as their life cycle is much longer. The interaction of radiation with the components of living systems results in the generation of several oxygen free radicals which are responsible for many of the detrimental effects of radiation. They can attack virtually all components including DNA, protein, and cause membrane lipid peroxidation. They also impair the indigenous antioxidant defense mechanism.

Radiation protection is at a cross-road after radiation incidents and unacceptable tragedies such as those at Chernobyl and Three Mile Island. Radiation induced damage to the normal tissues can be partially reduced by the use of radioprotectors that reduce the damaging effects of radiation, including radiation-induced lethality [2]. Various researchers have investigated the possible application of radioprotective chemicals in the event of planned and unplanned exposure i.e., clinical oncology, radiation site cleanup, military scenarios, radiological terrorism, radiation accidents, etc. [3, 4].

The use of chemical agents to provide protection against radiation injury has been a major field of study, and historically the discovery of the radioprotective effects of cysteine in rats and mice by Patt et al., [5] paved the way of research on radiation protection in humans. Since then, there has been an explosion in studies on radioprotection, and compounds with varied structures and physiological functions have been tested for their radioprotective abilities over the past 60 years. However, the practical applicability on the majority of these synthetic compounds remained lim-
itted, owing to their toxicity at their optimum protective doses [6]. Therefore, a need has been felt to find non-toxic and effective alternatives to the synthetic compounds.

Plants have been used by humans throughout history to construct a myriad of products for economic and health benefits. Interest in evaluating medicinal plant and edible phyto-products for use in health benefits, especially cancer, is gaining ground for prioritization in the public health programs of many developing and developed countries [7]. Over the past decade and a half, these plants have been scientifically investigated for their effectiveness in radiation protection, and recent studies form around the world substantiate this effectiveness [8].

The plant *Alstonia scholaris* belongs to the family Apocynaceae, grows throughout India, in deciduous and ever green forests and also in plains [9]. It is useful in fever, malarial fevers, abdominal disorders, dyspepsia, leprosy, skin diseases, puritus, tumours, chronic and foul ulcers, asthma, bronchitis, cardiopathy, helminthiasis, agalactia, and debility [10]. Several studies have demonstrated that this plant contains potent antioxidants and represent important sources of natural antioxidants [11, 12]. As there is paucity for the radiomodulatory activity of *Alstonia scholaris*, the present study was taken to explore the radioprotective efficacy of alcoholic extract of *Alstonia scholaris* (ACE) using various hematological parameters.

**MATERIALS AND METHODS**

**Animal care and handling**

Male Swiss albino mice (*Mus musculus*), 6-8 weeks old weighing 20-24 g, from an inbred colony were used for the present study. The animals were provided standard mice feed (procured from Ashirwad Industries Chandigarh, India) and water *ad libitum*, and were maintained under controlled conditions of temperature and light (light: dark, 10-14 hours). Four animals were housed in a polypropylene cage with locally procured paddy husk (*Oryza sativa*) as bedding throughout the experiment. Tetracycline-containing water (0.13 mg/ml) was provided once a fortnight as a preventive measure against infections. Animal care and handling were performed according to the guidelines set by the World Health Organization (WHO), Geneva, and the Indian National Science Academy (INSA), New Delhi. The Departmental Animal Ethical Committee approved the present study.

**Irradiation**

Cobalt teletherapy unit (ATC-C9) at the Cancer Treatment Centre, Radiotherapy Department, SMS Medical College & Hospital, Jaipur, was used for irradiation. Unanaesthetised animals were restrained in well-ventilated perspex boxes and exposed to gamma radiation at the source to surface distance of 77.5 cm to deliver the dose-rate of 1.32 Gy per minute.

**Plant material & extract preparation**

The bark of *Alstonia scholaris* (*Sapthaparna*) was collected after proper identification in herbarium of Botany Department (Voucher No. RUBL-19939). The plant bark was powdered in a mixture and the extract was prepared by refluxing with the double distilled water (DDW) for 36 hours (3 × 12 hours) at 40 °C. The liquid extract was cooled and concentrated by evaporating its liquid contents in an incubator. The extract was stored at low temperature until further use. The required dose for treatment was prepared by dissolving the drug pellets in double distilled water and administered by oral gavage with a micropipette (100 µl per animal) at a dose of 100 mg/kg body weight per animal.

**EXPERIMENTAL DESIGN**

**Selection of optimum dose of the *Alstonia scholaris* extract against irradiation**

Mice for this experiment were divided into six groups of 10 animals each and were administered ASE orally with 25, 50, 75, 100, 150, and 200 mg/per animal/day for five consecutive days, once daily. Thirty minutes after the last administration of ASE on day 5th, these were exposed whole-body to 8 Gy gamma radiation. All animals were observed till 30 days for any sign of radiation sickness, morbidity, mortality, and behavioral toxicity. The optimum dose was obtained on the basis of radiation sickness and survival of mice, and further studies were carried out using this dose of ASE.

**The LD50/30 and dose reduction factor**

The efficacy of any protective agent is evaluated by the determination of its dose reduction factor (DRF). The DRF of the ASE based on the lethal dose LD50/30 survival experiment was calculated after irradiating a large number of Swiss albino mice to different doses of gamma rays in the presence (experimental) or absence (control) of ASE. The percentage of mice surviving at each radiation dose till 30 days following such exposures was used to construct survival dose response curves. Regression analysis was done to obtain LD50/30, and dose reduction factor was computed as

\[ DRF = \frac{LD_{50/30} \text{ of experimental animals}}{LD_{50/30} \text{ of control animals}} \]
Modification of radiation response

A total of 70 animals used for the experiment were sorted into 4 groups. Mice of Group I (vehicle control, \(n = 5\)) were orally administered DDW at a dose of 100 mg/kg body weight, volume equal to ASE. Animals belonging to Group II (ASE alone, \(n = 5\)) were given daily ACE at a dose of 100 mg/kg/animal for 5 consecutive days. Animals of Group III (irradiated control, \(n = 30\)) were exposed to 7.5 Gy gamma rays alone 30 minutes after DDW treatment on day 5. Group IV (ASE experimental, \(n = 30\)) received ASE (100 mg/kg body weight per animal) as in Group 2. Half an hour after last administration of ASE, mice were exposed to above used radiation dose. These animals were observed daily for any sign of sickness, morbidity, behavioral toxicity, and mortality. A minimum of 5 animals from group III and IV were necropsied on 12 hours, day 1st, 3rd, 7th, 15th, and 30th post-treatment intervals to study hematological parameters.

Hematological study

For this study, blood was collected from the orbital sinus of animals from each group in a vial containing 0.5 M EDTA. Total leucocytes count and the percentage of different types of leucocytes (lymphocytes, monocytes, neutrophils, and non-neutrophilic granulocytes) were determined by adopting standard procedures.

Statistical analysis

The results from all the groups at various necropsy intervals were expressed as mean ± standard error of the mean to evaluate whether the mean of the sample drawn from experimental (ASE treated) deviated significantly from respective control (irradiation control). Student’s “t” test was used by the method of Bourke et al., [13]. The significance level was set at different levels as \(p < 0.05\), \(p < 0.01\), and \(p < 0.001\).

RESULTS

No noticeable signs of behavioral change and sickness or mortality were observed in Vehicle control as well as in ASE-treated groups. Animals exposed to 7.5 Gy gamma rays exhibited epilation, ruffled hair, watering of eyes, diarrhea, lethargic nature, and weight loss. No animal could survive in the 7.5 Gy irradiated alone group beyond day 15th. Animals pretreated with ASE did not exhibit mortality or any symptoms of radiation sickness. General health, activity, food, and water intake were found to be normal in ASE pretreated irradiated animals.

Mice treated with ASE at doses of 25, 50, 75, 100, 150, and 200 mg/kg body weight per day for 5 consecutive days prior to irradiation exhibited of 28, 43, 60, 88, 50, and 48 percent of survival, respectively. The dose 100 mg/kg body weight was found to be the optimum dose on the basis of above data (figs. 1 and 2), and the further studies were carried out using this dose of ASE.

The \(L_{D_{50/30}}\) values for irradiated control and experimental animals obtained from the survival data were 5.4 and 9.77, respectively. The dose reduction factor of \textit{Alstonia scholaris} against gamma radiation was calculated on the basis of the survival experiment and measured as 1.80 (fig. 3). The total leucocytes count was expressed as number of leucocytes per mm\(^3\) of blood. A marked decline (55.18\%) in total leucocytes count was observed at 12 hours (2609 ± 98.03) which remained the lowest at the 7th day (1913 ± 121.43) in irradiated Group III. In ASE pretreated irradiated animals of Group IV, these cells were scored as

![Figure 1. 30 days survival of mice pretreated with different doses (once in a day for 5 consecutive days) of ASE against exposure to 8.0 Gy gamma radiation](image-url)
significantly higher than the corresponding control throughout the study. However, an initial depression in counts was observed but day 7th onwards, such number increased gradually and normal counts could not be regained even on the last autopsy interval i. e. day 30 (fig. 4).

In vehicle treated controls, the lymphocytes count was 66.08 ± 1.62%, whereas in the ASE alone treated group, lymphocytes were 66.15 ± 1.04%. The above difference in levels of lymphocytes was not significant between the two groups. However, in the radiation alone treated group, maximum decrease (53.68%) in lymphocytes count was observed on day 3rd, followed by gradual increment till day 15th while no animal could survive beyond this day. In ASE pretreated animals, such decrease was significantly ($p < 0.01$) lesser in comparison to irradiated control during entire period of study (fig. 5).
In irradiated control, the percentage of neutrophils decreased till day 3rd, thenceforth increased up to their survival (i.e. day 15th) but remained below normal. In animals treated with ASE prior to irradiation, the number was regained without attaining normal by the last autopsy interval and a significant difference was observed throughout the study (fig. 6).

After 12 hours of irradiation, the number of monocytes reduced significantly in irradiated group (1.02 ± 0.14%) when compared with vehicle control group (3.08 ± 0.27%). The maximum decrease was observed on day 3rd in irradiated mice which was 6.8 folds lower than Sham-irradiated mice. However, pretreatment with ASE significantly increased the monocytes count in irradiated mice (fig. 7).

Non-neutrophilic granulocytes (eosinophils and basophils) showed a significant decline after radiation exposure which was found maximum on the day 7th.
(66.88%) but thereafter gradual reparation started and counts elevated to 59.30% of normal value. In ASE pretreated animals, such decrease was significantly lesser in comparison to control during entire period of study except for day 15th (fig. 8).

**DISCUSSION**

The radioprotective effect of ASE was demonstrated in the present study by determining its dose reduction factor (i.e., 1.80) on the basis of survival data of mice after irradiation. The maximum survival of animals (88%) was observed at 100 mg/kg body weight per day for five consecutive days before irradiation to 8 Gy. However, at higher concentrations of ASE, the protection was found to be lesser. A similar modulation of radiation effects at low doses, instead of higher non-toxic doses of radioprotector has been reported with MPG (2-mercaptopropionylglycine), ginseng, and serum thymic factor (FTS) [14-16]. FTS at higher concentrations, however, was not effective. It was suggested that the high doses of FTS was not attributed to its toxicity, but it may be due to negative feedback reaction or to the down-regulation of radioprotective cytokines receptors in target cells [16]. Similarly, in the present study it is postulated that the lesser radioprotection at higher doses of ASE may be due to the negative feedback reaction or to the down-regulation of radioprotective cytokines receptors in target cells.

It has been evident that high-dose gamma radiation-induced depletion of the hematopoietic tissues coupled with compromised immunity are the main contributing factors to the hematopoietic syndrome and death in mammals. Furthermore, chemotherapy- and/or radiotherapy-induced damage to the circulatory system of cancer patients persists as a difficult clinical problem. Rapidly dividing cells of the blood vascular system, especially leucocytes and erythrocytes, are highly prone to radiation-induced damage, because reactive oxygen species impacts the blood system and decreases its cellular components, including reticulocytes, considerably.

The present work describes the marked decrease in the white blood cells (WBC) count in mice subjected to gamma irradiation. This is in agreement with the findings of earlier workers [17, 18]. Irradiation-induced leucopenia has likewise been reported by Mishima et al. [19] in gamma ray irradiated mice. The leucocyte number showed a drastic decline during the first 24 hours. This initial phase of rapid decrease is due to direct killing of lymphocytes while the slower fall at later intervals is due to the reduced number of new lymphocytes entering in to the peripheral blood. Mature lymphocytes are considered to be the most radio-sensitive type of blood cells. The peripheral lymphocytes exhibited the maximum depletion at day 1st in the current investigation elucidating an early cell killing effect of radiations on this cell type, which is the most radiosensitive in peripheral blood. ASE pre-irradiation administration rendered a significant increase in the number of total leucocytes. The increase in the colony forming unit (CFU) counts in spleen associated with the increase in leucocytes number indicated the immuno-stimulatory role of ASE and could therefore be attributed to the already known immuno-modulatory activity [20] present in *Alstonia scholaris*.

Monocytes count also followed the similar pattern as the total leucocytes and lymphocytes in irradiated control group. As nearly all the cells are ultimately affected by radiation because they originate from the stem cells of the bone marrow. ASE pretreatment significantly increase in monocytes count throughout the study as compared to irradiated control. Increase percentage of monocytes count after gamma radiation has also been reported by Arora et al. [21].

Myelocytes, precursor cells to the granulocytes, are somewhat less sensitive than agranulocytes. The radio-sensitivity differs for myelocytes in different stages of maturation as mitosis is inhibited in young extent. In this study, non-neutrophilic granulocytes...
(acidophils and basophils) were significantly decreased in irradiated control group throughout the study. Their reduction in numbers may reflect the time of their maturation and release from the bone marrow in the peripheral blood. These data agree with the findings of Kafaty et al. [22]. In ASE pretreated group, a significant increment in their counts was observed as compared to irradiated control group. It is also in collaboration of earlier findings of Samarth [23] and Sancheti and Goyal [24] while using other medicinal plants.

The pattern of differential leucocytes count (DLC) in terms of neutrophils remained unchanged for the vehicle and drug treated control groups. There was a drastic decrease in the neutrophils count in the radiation only group because radiation sets up an emergent response that causes a sudden drop in the neutrophil count in the blood. The source of replacement cells is depleted due to mitotic inhibition of the young myelocytes, which may be another reason. Micke et al. [25] reported a significant decrease of human neutrophilic granulocytes at 3.5 and 4.0 Gy sub-lethal radiation dose. The drug + radiation group, however, showed a nearly similar pattern to the irradiated control group but there was a significant recovery as compared to the radiation-only group. The bioactive constituents of present in ASE were possibly being metabolized and sequestered in the blood and initiating a cascade of pathways at a molecular/cellular level to augment neutrophils recovery. Similar way of protection was observed by Arora et al. [21] while using Podophyllum hexandrum.

Alstonia scholaris extract has also been found to inhibit radiation-induced lipid peroxidation and the decline in reduced glutathione (GSH) levels in irradiated mice in our previous study [26]. The depletion of intracellular glutathione has been implicated as one of the causes of radiation-induced damage, while increased levels of intracellular GSH are responsible for a radioprotective action. Increase in GSH and decrease in lipid peroxidation by ASE seem to be an important mechanism in protecting hematopoietic system against radiation-induced alterations.

The present study demonstrates, radio-modulatory activity of ASE, and suggests that the ASE should be explored as a type of radio-protectant and radio-therapeutic agent. Our results show that ASE increases survival after radiation primarily by enhancing peripheral blood cells production especially leucocytes and lymphocytes. Because of the effect of ASE on stimulating and proliferation of bone marrow progenitor’s cells, it can be effective for protection and treatment of radiation-induced hematopoietic injury.

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РАДИЈАЦИОНИ ОДЗИВ НА ГАМА ОЗРАЧИВАЊЕ У ЛЕУКОЦИТИМА ПЕРИФЕРНЕ КРВИ МИШЕВА И ЉУХОВА ЗАШТИТА ЕКСТРАКОМ *ALSTONIA SCHOLARIS*

Рад се бави заштитним ефектима екстракта биљке *Alstonia scholaris* употребљеног против радијацијом индукуваних хематолошких промена. Муждаци узгајаних швајцарског белог миша изабрали су и подељени у четири групе. Првој групи, која је служила као контрола, орално је аплицирана два пута дестилисана вода, док је другој групи додељивана дневна доза екстракта од 100 mg/kg телесне тежине разближено у два пута дестилисаног воде. Трвој групи, која представља озрачну контролу групу, аплицирана је само два пута дестилисана вода, док је четворој групи аплициран екстракт једном дневно у току пет узастопних дана. Петог дана, трећа и четврта група изложене су гама зрачењу дозе 7.5 Gy, пола часа по администрирању два пута дестиловане воде или *Alstonia scholaris* екстракта, респективно. Ради хематолошких испитивања, животиње су жртвоване у подне, првог, трећег, седмог, петнаестог и тридесетог дана по излагању зрачењу. Показало се да код животиња претстригираних *Alstonia scholaris* екстрактом, екстракт обнавља укрупне леукоците и диференцијалне леукоците (лимфоците, моноците, неутрофилне и не-неутрофилне гранулоците), у поређењу са озраченом контролном групом. Подаци јасно указују да *Alstonia scholaris* екстракт значајно умањује штетне биоензиме зрачења на периферну крв.

Кључне речи: *Alstonia scholaris*, радиозащитен, хематологички ефекти, швајцарски бели миши