Prophylactic Use of *Alstonia Scholaris* (Sapthaparna) against Gamma Irradiation

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Summary

This study was carried out to assess the radioprotective effect of *Alstonia scholaris* against gamma irradiation in mice.

Adult Swiss albino mice were exposed to different doses of gamma radiation in the presence (experimental) or absence (control) of *Alstonia scholaris* extract (ASE). Dose tolerance of ASE, DRF, CFU colonies in spleen, LPx and GSH were studied.

The optimum dose for radioprotection was determined by administering 100, 250, 500, 750, 1000, 2000 mg/kg body weight of ASE consecutively for five days before irradiation. The dose of ASE found to be most effective against radiation was 100 mg/kg b.wt., because this dose significantly increased the survival time and reduced the mice mortality rate. The dose reduction factor was calculated as 1.80 on basis of survival data. Furthermore, irradiation of animals resulted in an elevation in lipid peroxidation (LPx) and a significant decline in glutathione in blood and liver. Conversely, administration of animals with ASE before irradiation caused a significant decline in LPx accompanied by a significant incresse in GSH concentration.

From the present study it is clear that ASE provides protection against radiation-induced deleterious effects of radiation.

Keywords: Gamma radiation, *Alstonia scholaris*, LPx, GSH, Swiss albino mice

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Introduction

Ionizing radiation has always been a part of the human environment. Along with natural radioactive sources present in the Earth's crust and cosmic radiation, man-made sources also contribute to our continuous exposure to ionizing radiation. Environmental radioactive pollution has resulted from past the nuclear weapons testing, nuclear waste disposal, accidents at nuclear power plants, as well as from transportation, storage, loss, and misuse of radioactive sources.

Radiation-induced damage is one of the deleterious effects of exposure to radiation, either accidentally or during radiotherapy. In contrast to other forms of radiation, ionizing radiation has the capacity to break chemical bonds; it imparts energy to living cells through random interaction with atoms giving rise to ions and reactive radicals. These in turn, cause molecular changes that may lead ultimately to biological injury. The development of effective and non-toxic radioprotective agents is therefore of considerable interest and a large number of chemical and biological agents having been screened in experimental and clinical trials to mitigate radiation injury caused by whole body exposure.

These radioprotective agents include cysteine[**1**], 2-MPG[**2**], WR-2721 (gammaphos)[**3**] and Diltiazem[**4**] which were tested for their radioprotective capacity and found to be radioprotector. Owing to their severe side effects such as nausea, vomiting, hypotension and neurotoxicity, these have not been found successful in the field of clinical radiotherapy[**5**].

Plants have played a significant role in maintaining health and improving the quality of human life for thousands of years and have served humans well as valuable components of medicines. Herbal medicine is based on the premise that plant contains natural substances that can promote health and alleviate illness. In recent time, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems.

One such plant, *Alstonia scholaris*, invites attention of the researchers worldwide for its pharmacological activities ranging from anti-malarial to antidiarrheal activities[6]. *Alstonia scholaris* belongs to family Apocynaceae grows throughout India, in deciduous and evergreen forests, also in plains. The plant is widely found in India in sub Himalayan region from the Yamuna eastward ascending to 3000 feet above sea level, abundantly found in West Bengal and South India[**7**].

It has wide occurrence also in the Asia-pacific region from India, Sri Lanka through main land South-East Asia and Southern China, through out Malaysia to Northern Australia and Solomon Islands. The common usage, wide acceptability in human beings, and diverse medicinal and antioxidative characteristics attributed to this plant stimulated us to obtain insight into possible radioprotective effect of *Alstonia scholaris* bark extract in mice exposed to whole-body lethal gamma irradiation.

Materials and Methods

Animal care and Handling

The animal care and handling were performed according to the guidelines set by the World Health Organization (Geneva, Switzerland) and the INSA (Indian National Science Academy, New Delhi, India). Swiss albino mice, 6-8 weeks old weighing 22±2 gm from an inbred colony were used. They were maintained under controlled conditions of temperature and light (14 and 10 hr of light and dark, respectively). The animals were provided with standard mice feed (procured from Ashirwad Industries, Chandigarh, India) and water *ad libitum*. Tetracycline water was also given once a fortnight as a preventive measure against infection. Four to six animals were housed in a polypropylene cage containing paddy husk (procured locally) as a bedding throughout the experiment. The Institutional Animal Ethical Committee approved the study.

Irradiation

The Cobalt teletherapy unit (ACT- C9) at Cancer Treatment Center, Radiotherapy Department, SMS Medical College & Hospital, Jaipur was used for irradiation. Unanesthetized animals were restrained in well-ventilated Perspex boxes and exposed whole-body to gamma radiation.

Preparation of the Plant Extract

Alstonia scholaris was identified by a competent Botanist in Herbarium of Botany Department, University of Rajasthan, Jaipur (RUBL No. 19939). Fresh bark of the Alstonia *scholaris* was collected, cleaned, shade dried, powdered and extracted. The extract was prepared by refluxing with double-distilled water (DDW) for 36 hours. The cooled liquid extract was concentrated by evaporating its liquid contents to render it in powder form. An approximate yield of 22 %

extract was obtained. The extract was redissolved in DDW just before oral administration in mice. Henceforth in this article, the extract of *Alstonia scholaris* bark will be called ASE.

Experimental Design

The following experiments were conducted:

1. Determination of Acute Toxicity of ASE

The acute toxicity of ASE was determined according to Prieur *et al*[8] and Ghosh[9]. Briefly, the animals were fasted by withdrawing food and water for 8 hrs., and these were divided into various groups of eight each. Each group of animal was administered with various doses, as 100, 250, 500, 750, 1000, 2000 mg/kg body weight, of the freshly prepared extract of *Alstonia scholaris* orally once daily for five consecutive days. Animals were provided with food and water immediately after the drug administration. Mortality of the animals was observed up to 30 days post-treatment and acute LD_{50} of the extract was calculated by the method of Miller and Teinter[10].

2. Selection of Optimum Dose of ASE

A separate experiment was carried out to determine the optimum dose of ASE in which mice were divided into seven groups. One group received DDW, while others were administered with 100, 250, 500, 750, 1000, 2000 mg/kg body weight of ASE orally once daily for five consecutive days.

Half an hour after the last administration of DDW or ASE on the fifth day, animals of both the groups were exposed to the Cobalt-60 teletherapy unit (ATC-CA) at the Cancer Treatment Centre, Radiotherapy Department, SMS Medical College & Hospital, Jaipur (India). Unanesthetized mice in a batch of eight animals each time were restrained in a well-ventilated perspex box and they were whole-body irradiated to 8 Gy gamma radiation at a dose-rate of *(Gy/min.)* at a source-to-animal distance (mid point) of 77.5 cm. This allowed the preliminary screening of the optimum drug dose of ASE. Further studies have been performed using this dose of ASE.

3. Radioprotective effect of ASE

Mice for this study were divided into four groups: animals in Group-I were administered orally with DDW (volume equal to ASE) to serve as normal (vehicle treated), while animals in Group-II were given ASE orally in a dose of 100 mg/kg body weight. Animals of Group-III received an equal volume of DDW (as in Group-I) were exposed to 8.0 Gy rays. Animals in Group-IV (experimental) were given ASE (as in Group-II) 30 minutes before exposure to 8.0 Gy gamma radiation.

All animals were monitored for weight change, behavioral changes, mortality, food and water consumption, etc. until 30 days of irradiation or ASE treatment.

4. Endogenous spleen colony assay (CFU-S)

The endogenous spleen colony assay was done with the slight modification of the method of Till and Mac Culloch[11]. Five animals were irradiated to 10 G_y with or without ASE. These were sacrificed on 10^{th} day after single total body irradiation (TBI) and their spleen were removed, weighed, and fixed in Bouin's fluid and grossly visible nodules on the surface of the spleen were counted.

5. Calculation of Dose Reduction Factor (DRF)

In order to establish the survival-dose-response of Swiss albino mice to radiation in the presence or absence of ASE, two groups of animals were used. The mice of one group were given orally double-distilled water (DDW), equal to volume of ASE and were exposed to different doses (2.5, 5, 7.5, 10 Gy) of gamma radiation, while the animals of other group were given orally, the optimum dose of ASE for five consecutive days once daily, and were irradiated on the last day of ASE administration.

6. Biochemical determinants

The Animals for this experiment were divided into following four groups for estimation of Lipid Peroxidation (LPx) and Glutathione (GSH):

Group I: These animals were given orally DDW equivalent to the dose of ASE (i.e. 100 mg/kg b.wt./day) for five consecutive days and were considered as vehicle treated control.

Group II: Animals of this group received only ASE (100 mg/kg b.wt./day) for five consecutive days and were considered as Drug treated control.

Group III: These animals were given orally DDW equivalent to the dose of ASE (i.e. 100 mg/kg b.wt./day) for 5 consecutive days. 30 minutes after the last administration, these were exposed whole-body to 5 Gy gamma radiation.

Group IV: Animals of this group received only ASE (100 mg/kg b.wt./day) for 5 consecutive days and were exposed on day 5^{th} to the similar dose of radiation as in Group III.

Glutathione (GSH) assay

The hepatic level of glutathione (GSH) was determined by the method of Moron *et al*[12]. The GSH content in blood was measured Spectrophotometrically using Ellman's reagent with 5-5, dithiobis-2-nitrobenzoic acid (DTNB) as a coloring reagent, according to the method of Beutler *et al*[13]. the absorbance was read at 412 nm using a UV-VIS systronic Spectrophotometer.

Lipid peroxidation (LPx) assay

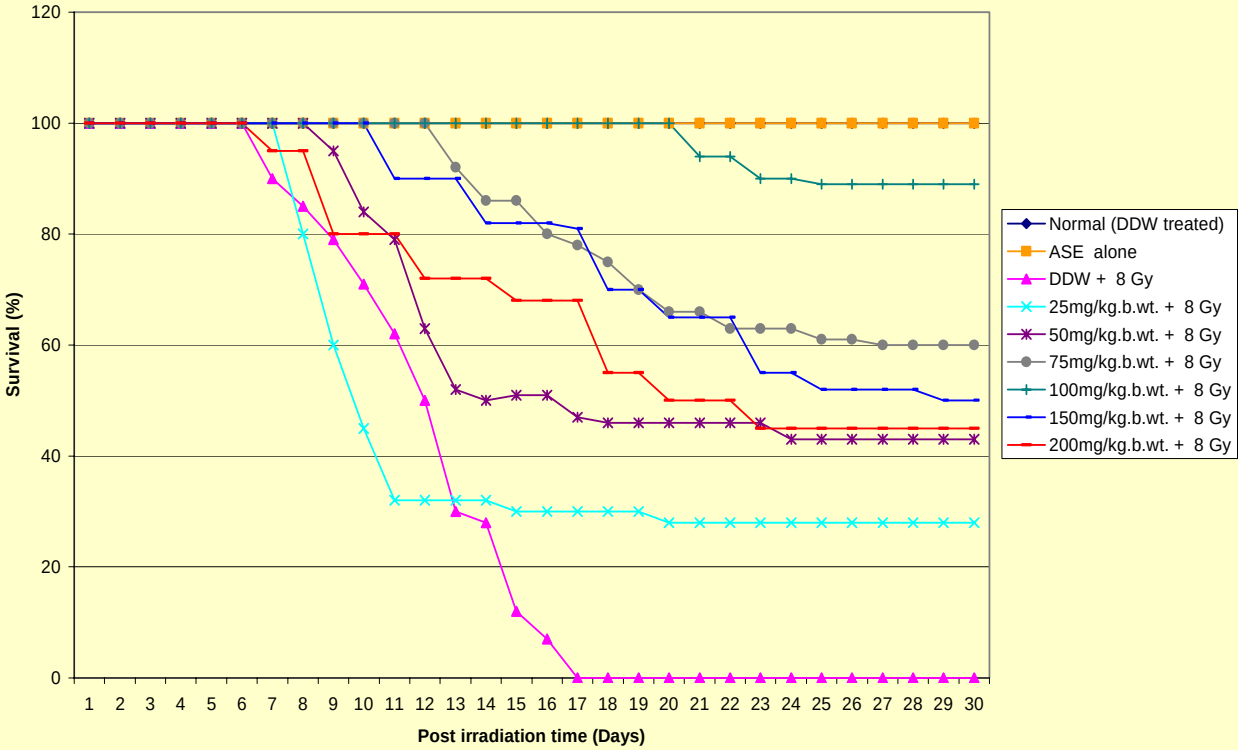
The lipid peroxidation (LPx) level in liver and serum was measured by the assay of thiobarbituric acid reactive substances (TBARS) using the method of Okhawa *et al*[14] in which the absorbance was read at 532 nm using a UV-VIS systronic Spectrophotometer.

Results

1. Determination of ASE tolerance

No adverse effects were observed in terms of sickness, body weight changes, mortality and visible abnormality throughout the study in animals treated with different drug doses (125, 250, 500, 750, 1000 & 2000 mg/ kg b.wt/ day) of ASE for 5 consecutive days. These mice were observed first for 12 hrs and then for 30 days post-treatment. However, after the termination of the experiment, no sickness and mortality were observed in any of the above group, which indicates that even the high dose of ASE (i.e. 2000 mg/kg b.wt / day) is well tolerable in Swiss albino mice (Fig-1).

Fig. 1 : 30 days survival of mice pretreated with different doses (once in a day for 5 consecutive days) of Alstonia scholaris extract (ASE) against exposure to 8 Gy gamma radiation

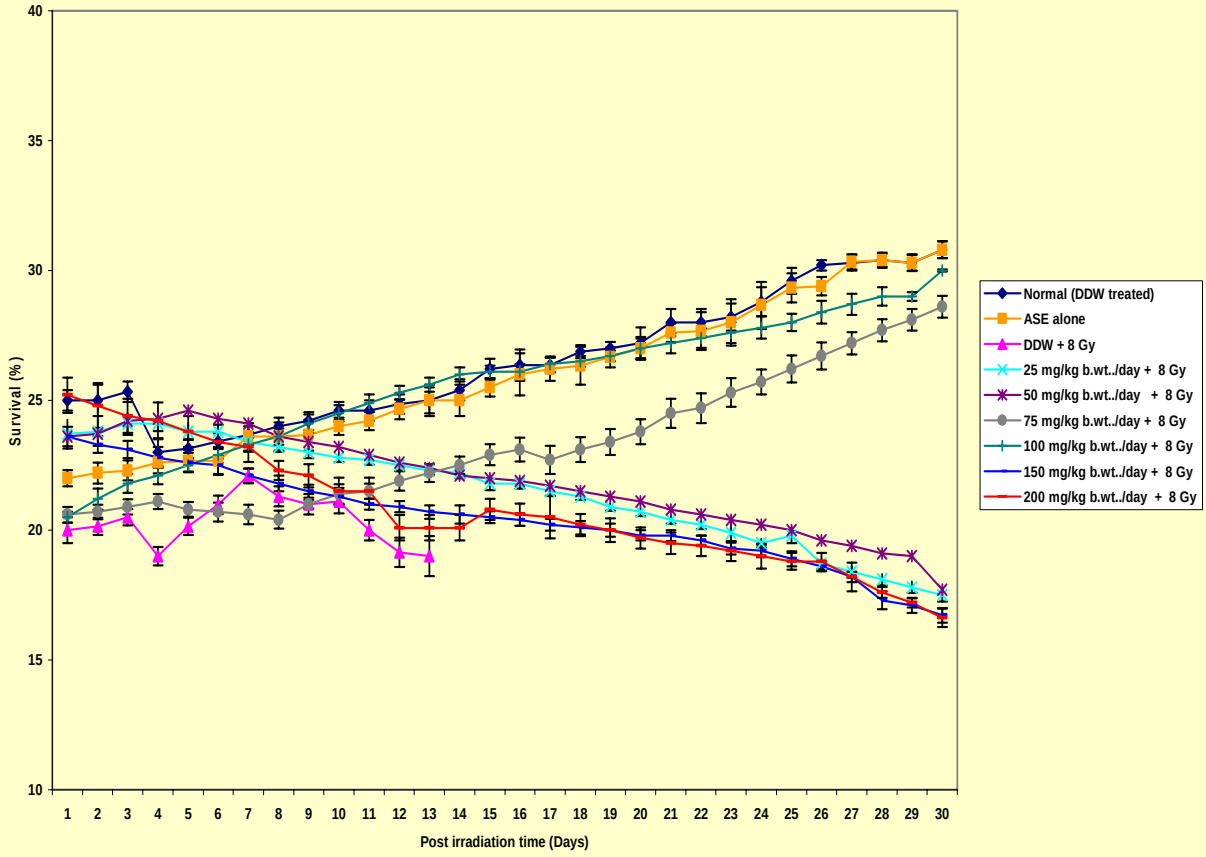


2. Selection of optimum dose of ASE against irradiation

The optimum dose of ASE against lethal gamma radiation (i.e. 8 Gy) in Swiss albino mice was selected on the basis of survival experiment, where number of deaths and surviving animals were recorded up to 30 days of irradiation. Mice treated with ASE at doses of 25, 50, 75, 100, 150, 200 mg/kg b.wt./ day for 5 consecutive days

prior to irradiation exhibited 28, 43, 60, 88, 50 and 48 per cent survival respectively. The dose100 mg/kg b. wt. was found to be the optimum dose based on the above data, and the further studies were carried out using this dose of ASE. (Fig-2)

Fig. 2 : Variation in body weight (gm) of Swiss albino mice pretreated with different doses of ASE (once daily for 5 consecutive days) against exposure to 8Gy gamma radiation.



3. Endogenous spleen colony assay (CFU-S)

The group of 8 animals was irradiated to 10 Gy with or without ASE. These were sacrificed on 10^{th} day after single total body irradiation (TBI) and their spleen was removed, weighed, and fixed in Bouin's fluid, and grossly visible nodules on the surface of the spleen were counted. Number of CFU-S as compared to irradiated control was found to be significantly higher in ASE treated mice. Furthermore, a considerable loss in spleen weight following irradiation was noticed at day 10, however, a significant increase in such weight was evident in ASE pretreated irradiated animals. (Table-1)

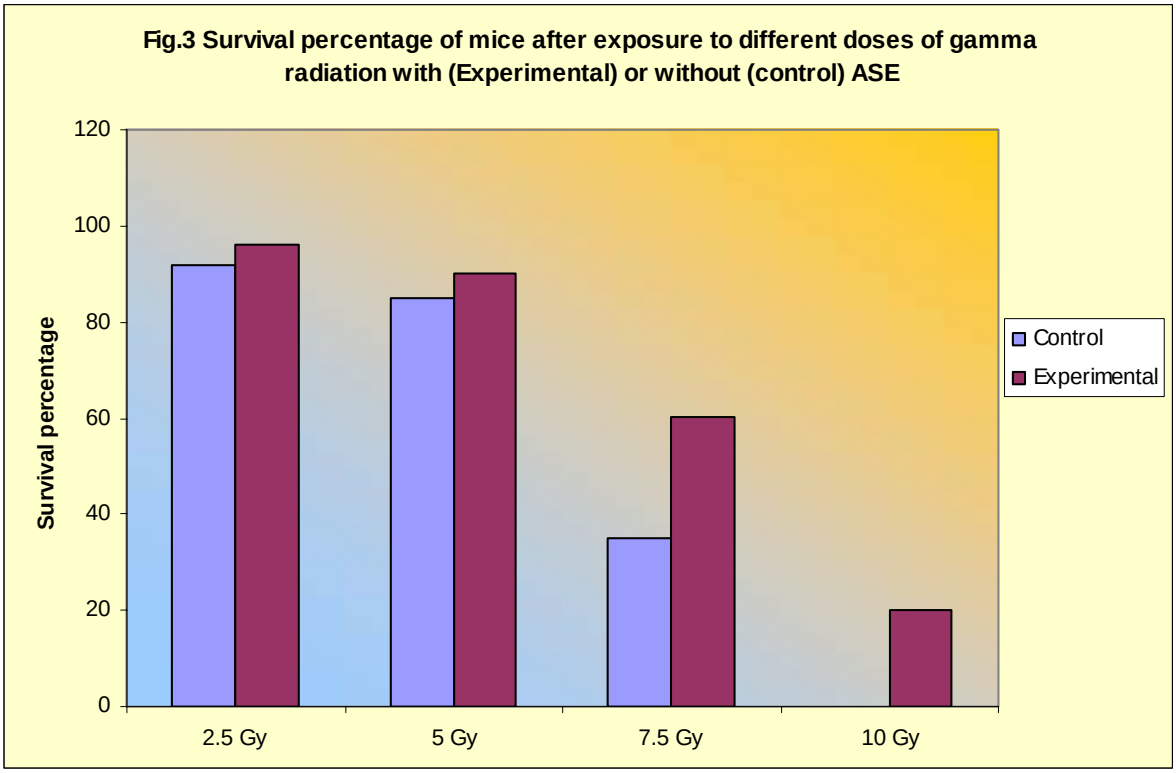
Radiation Dose	Group	Spleen Weight (mg)	No. of macroscopic Colonies
10 Gy	Control	N.S.	N.S.
	Experimental	30.8±0.35	39±0.66

Normal Spleen Weight = 38.80±0.76 mg

ASE treated spleen weight = 39.2±0.66 mg

4. Calculation of Dose Reduction Factor (DRF)

In order to establish the survival-dose-response of Swiss albino mice to radiation in the presence or absence of ASE, two groups of animals were used. The mice of one group were given orally double-distilled water (DDW), equal to volume of ASE and were exposed to different doses (2.5, 5, 7.5, 10 Gy) of gamma radiation, while the animals of other group were given orally, the optimum dose of ASE for five consecutive days once daily, and were exposed on 5th day to similar doses of gamma radiation (as in Group-I). On the basis of survival data in different groups, with or without ASE, DRF was calculated as 1.80. (Fig-3)



5. Biochemical determinants

Lipid Peroxidation (Lpx)

Administration of ASE when compared with DDW treatment did not alter the lipid peroxidation (LPx) in Groups III and IV. ASE pretreatment significantly reduced LPx induction in the ASE + irradiation group, thereby protecting liver and blood against radiation induced LPx. (Fig 4, 5)

Fig. 4: Variations in Lipid peroxidation (LPx) in blood serum of Swiss albino mice after exposure to 5 Gy gamma rays with or without *Alstonia scholaris* bark extract (ASE)

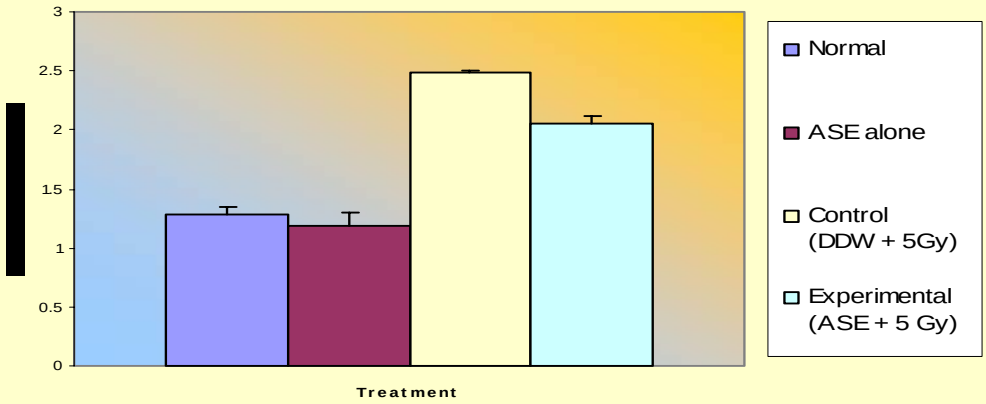
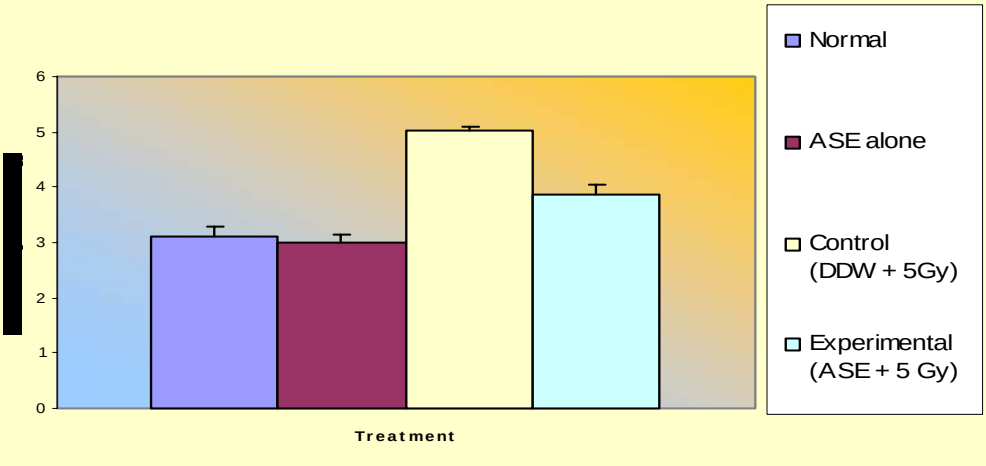


Fig. 5: Variations in Lipid peroxidation (LPx) in liver of Swiss albino mice after exposure to 5 Gy gamma rays with or without *Alstonia scholaris* bark extract (ASE)



Glutathione (GSH) assay

No significant difference of the hepatic and blood GSH contents was observed between normal and ASE treated animals. However, a statistically significant ($p<0.001$) decreased in GSH was evident in radiation treated control animals. ASE treated irradiated animals showed a significant increase in GSH content (blood & liver) with respect to irradiated control, but the value remained below normal. (Fig.-6,7)

Fig. 6 : Reduced Glutathione (GSH) level in blood of Swiss albino mice after exposure to 5 Gy gamma rays with or without *Alstonia scholaris* bark extract (ASE)

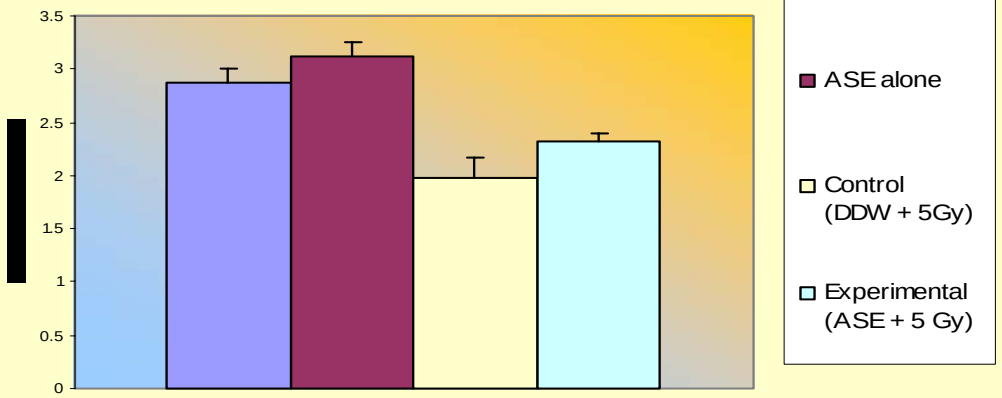
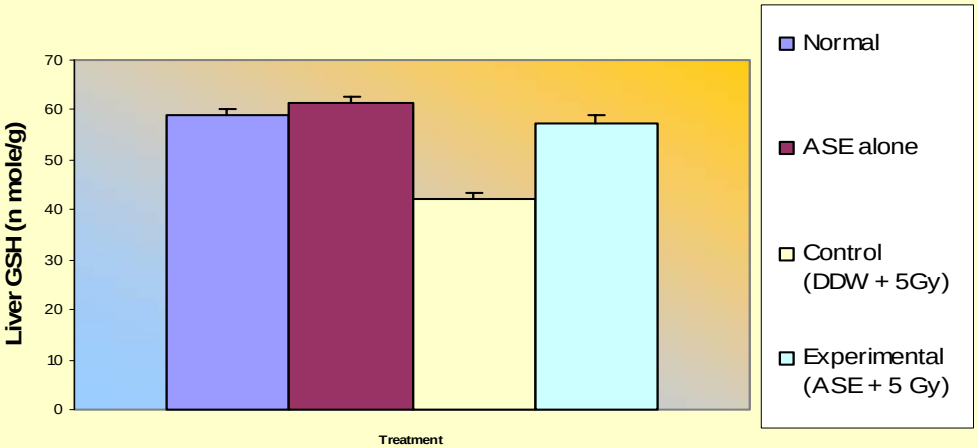


Fig. 7 : Reduced Glutathione (GSH) level in liver of Swiss albino mice after exposure to 5 Gy gamma rays with or without Alstonia scholaris bark extract (ASE)



Discussion

There is a continual interest worldwide to screen for non-toxic radioprotectors that can be used against harmful effects of radiation in occupation as well as in therapeutic settings for mankind. Despite extensive work done in this field, not a single compound has emerged so far as an effective non-toxic radioprotector for practical purposes[15]. Therefore, screening of natural products of plant origin represents a major avenue for the discovery of new radioprotective drugs.

Treatment of mice with ASE before irradiation to different doses of gamma-radiation reduced the symptoms of radiation sickness and mortality. Irradiation of animals to 8 Gy in the present study resulted in radiation sickness within 3-5 days of exposure. The symptoms including reduction in food and water intake, irritability, weight loss, lethargy, diarrhoea and ruffling of hairs. Similar symptoms have been observed in mice after gamma irradiation by others also[16, 17, 18]. Whole-body irradiation primarily affects rapidly proliferating germinal epithelium, gastro-intestinal epithelium, bone marrow and progenitor cells. Bone marrow, spleen progenitor cells and gastro-intestinal epithelium cells are crucial for the sustenance of life, and any damage to these cells will impair normal physiological host defense processes drastically, causing an adverse impact on survival.

The results from the present study indicate that pretreatment of *Alstonia scholaris* extract (ASE) protects the mice from the lethal effects of ionizing radiation. The radioprotective effect of ASE has been demonstrated by the increased body weight and survival rate. A significant radioprotection was achieved when ASE was given orally as 100 mg/kg b.wt for 5 consecutive days prior to irradiation. In the present study, a significant loss in body weight was evident in irradiated control animals, however, ASE pretreated irradiated animals showed recovery in body weight within 30 days of irradiation and only 11% mortality was evident in such animals.

Pretreatment of mice with ASE provided protection against radiation-induced sickness and mitigated suffering. Similarly, plants such as *Ocimum sanctum*[19], *Panex ginseng*[20], *Triphala*[21], *Emblica officinalis*[22], *Rosemarinus officinalis*[23] *and Aloe vera*[24] have been reported to provide protection against sickness induced by radiation.

Glutathione acts as one of the major detoxifiers in the body, but it must be in the reduced form to work efficiently. It is also considered to be the versatile body's self generated antioxidants and executes its radioprotective function through free radical scavenging restoration of the damage molecule by hydrogen donation, reduction of peroxides and maintenance of protein thiols in the damage state [25, 26]. The present study demonstrates a significant reduction in liver and blood GSH following radiation exposure. This could be due to the enhanced utilization of the antioxidant system as an attempt to detoxify the free radicals generated by radiation.

Depletion of intracellular GSH has been implicated as one of the causes of radiation-induced damage, while increased levels of intracellular GSH are responsible for radioprotective action. ASE pretreatment helped to restore GSH levels when compared with the concurrent DDW + irradiation group. This resulted in the inhibition of radiation-induced lipid peroxidation, thereby protecting against radiation-induced damage. Ionizing radiation induces lipid peroxidation, which can lead to DNA damage and cell death [27, 28, 29]. The administration of ASE before irradiation significantly reduced the amount of lipid peroxidation (LPx) as compared to the irradiated control group. The inhibition of LPx by ASE may also have been responsible for the observed radioprotection. Some of the plants extract like *Zingiber officinalis*[30], *Rosemarinus officinalis*[23], *Emblica officinalis*[22], *Aloe vera*[24] have also been reported for their radioprotective action in similar manner.

The basic effect of radiation on cellular membranes is believed to be the peroxidation of membrane lipids. Radiolytic products, including hydroxyl and hydroperoxyl radicals can initiate lipid peroxidation [31, 32]. In the present study, although *Alstonia scholaris* treatment alone did not significantly alter the lipid peroxidation level in irradiated mice, but it significantly lowered the radiation-induced lipid peroxidation in terms of malonaldehyde in biomembranes. Inhibition of lipid peroxidation in biomembranes can be caused by antioxidant.

Thus, the present study suggests that deleterious effects of radiation are reduced by *Alstonia scholaris* bark extract, which in turn reflected in the terms of increased survival, significant decline in LPx level and significant enhancement in GSH content in ASE pretreated experimental animals as compared to irradiated control. The radioprotective property of ASE

may be attributed to its components such as alstonine, echitamine chloride, villastonine, scholaricine, which show strong free radical scavenging activity.

Conclusion

From the present study, it is clear that ASE provides protection against the radiation-induced sickness and mortality in mice and its free radical scavenging and antioxidant activities may be responsible for the observed radioprotection. Furthermore, experimental studies are planned to explore how ASE may modulate the radiation-induced damage in radiation fractionation, for its utility in cancer treatment and its effects on cancer growth.

Acknowledgement

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