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Post-treatment effects of *Alstonia scholaris* extract against radiation-induced biochemical alterations in Swiss albino mice

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Background: This study has been undertaken to find out the possible radioprotective potential of the *Alstonia scholaris extract* (ASE). ***Materials and Methods:*** For experimental study, healthy Swiss albino male mice were selected from an inbred colony and divided in four groups. Group I (normal) did not receive any treatment. Group II was orally supplemented ASE once daily at the dose of 100 mg/ kg.b.wt/day for 5 consecutive days. Group III (control) received distilled water orally equivalent to ASE for 5 days then exposed to 7.5 Gy of gamma radiation. Group IV (experimental) was administered orally ASE for 5 consecutive days once daily and exposed to single dose of 7.5 Gy of gamma radiation. Mice were sacrificed at different autopsy intervals viz. 12 hrs. 1, 3,7,15 and 30 days, and their liver and blood were taken for various biochemical estimations viz. lipid peroxidation (LPO), reduced glutathione (GSH), protein and cholesterol. ***Results:*** Radiation induced augmentation in lipid peroxidation and cholesterol was significantly ameliorated by ASE extract and deficit produced in protein and glutathione content by radiation was checked. ***Conclusion:*** *Alstonia scholaris* extract pretreatment hence renders protection against radiation-induced biochemical alterations in mice. **Iran. J. Radiat. Res., 2010; 8 (3): 169-177**

Keywords: *Alstonia scholaris, gutathione, lipid peroxidation, radioprotection, mice.*

INTRODUCTION

Applications of ionizing radiation in different areas are constantly increasing including the use for medicinal and industrial purposes. Ionizing radiation passing through living tissue generates reactive oxygen species such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($O_{\cdot}$), nitric oxide (NO), hydrogen peroxide (H_2O_2) and peroxyl radicals in a biological system, by radiolysis of water that can damage several cellular components and biomolecules such as DNA, proteins, lipids, amino acids and carbohydrates [1-3]. The metabolism and physiological functioning of the specific body organs are also altered on exposure the organism to ionizing radiations. Radiations may damage the cell membranes in such a manner that these not only loose certain compounds but also are unable to take them from surrounding extra cellular medium causing biochemical changes [4].

The damaging effects of ionizing radiation lead to cell death and are associated with an increased risk for numerous genetically determined diseases [5]. With respect to radiation damage to human, it is important to protect humans from adverse effects induced by ionizing radiation. The use of radioprotectors represents an obvious strategy to improve the therapeutic index in radiotherapy. Although synthetic radioprotectors such as the aminothiols have yielded the highest protective factors, typically they are more toxic [6] than naturally occurring protectors [7]. In general, the best radioprotective agents also have been reported to result in the highest behavioral toxicity [8, 9]. Hence, the search for alternative sources, including bioactive principles of plant origin, has been an ongoing task worldwide.

In recent years, it has become well known that antioxidant phytochemicals are present in plants, fruits and vegetables [7, 10, 11]. Indeed, herbal medicine (phytomedicine) is generally considered a well-established

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form of complementary medicine. It is estimated that within the population of USA, use of complementary medicine has increased from 33.8% in 1990 to 42.1% in 1997 [12, 13]. In an attempt to find potent natural antioxidants, some herbal medicines have recently gained recognition as biological response modifiers [14, 15]. In particular, the use of herbal plants for their potential as possible modifiers of the radiation response is receiving considerable attention [16, 17].

Alstonia scholaris, a tree belonging to family Apocyanaceae, is a popular remedy in India for the treatment of various types of disorders in both the Ayurvedic and folklore systems of medicine. It is commonly known as the devil's tree and it has been widely used in the traditional system of medicine, reported to be of immense use in the treatment of various ailments [18]. The decoction of *Alstonia scholaris* bark has been used as a folk medicine in coastal part of Karnataka (India) to treat cold and other ailments like fever every year only in the monsoon season. The bark of the plant is most extensively used part and is reported to have anticancer properties by the people of different cultures and civilizations like India, Admiralty Islands and Thailand [19- 25]. The alcoholic extract of *Alstonia scholaris* has been recently reported to be antineoplastic and chemopreventive [20, 26, 27].

However, no detailed radioprotective efficacy of this plant has been studied. Therefore, the present communication deals with the radiomodulatory effects of this plant in Swiss albino mice by taking some biochemical end points.

MATERIALS AND METHODS

Animal care and handling

Male Swiss albino mice (*Mus musculus*), 6-8 weeks old of 20-24 g. b. wt., from an inbred colony was used for the present study. The animals were provided standard mice feed (procured from Ashirwad Industries Chandigarh, India) and water *ad libitum*, were maintained under controlled conditions of temperature and light (Light: dark, 10 hrs: 14 hrs.). 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, Switzerland and the INSA (Indian National Science Academy), New Delhi, India. 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 and 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 (SSD) of 77.5 cm to deliver the dose -rate of 1.32 Gy/min.

Plant material and 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 hrs at 40^0 C. The liquid extract was cooled and concentrated by evaporating its liquid contents in vacuo and freeze dried. The extract was stored at low temperature until further use. Such extract was redissolved in DDW prior to the oral administration in mice.

Evaluation of radiomodulatory influence of bark extract of *Alstonia scholaris*

Experimental Design

Mice selected from the inbred colony were divided into four groups. Animals of

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Group I were administered double distilled water (DDW) once a day for five consecutive days to serve as a vehicle treated control, while Group II received bark extract of *Alstonia scholaris* orally (100 mg/kg b.wt/ day) for five consecutive days. Animals of Group III received DDW (volume equal to bark extract of Alstonia scholaris) to serve as irradiated control whereas animals of Group IV were administered ASE orally (100 mg/kg b.wt/day) for five consecutive days to serve as an experimental group. Thirty min after the above treatments on the fifth day, animals of Group III and IV were exposed to 7.5 Gy gamma radiations. These animals were observed daily for any sign of sickness, morbidity, behavioral toxicity and mortality. Mice from the above groups were necropsied on 12 hrs, days 1, 3, 7, 15 and 30 post-treatment. The following parameters were studied to assess the radioprotective effects of *Alstonia scholaris* extract:

Lipid peroxidation assay

The lipid peroxidation level in liver and blood was measured after 24 hrs. of irradiation by the assay of thiobarbituric acid reactive substances (TBARS) using the method of Ohkhawa *et al.* [28]. Briefly, homogenate was mixed with sodium dodecyl sulphate (SDS), pH 3.5, 20% tricholoroacetic acid (TCA). To the same, aqueous thiobarbituric acid (TBA), double distilled water was added, and heated at 95°C for 60 min. The mixture was cooled and added to n-butanol and pyrimidine (15:1 w/v). The absorbance was read at 532 nm using a UV-VIS Systronic Spectrophotometer (Mfd. at Ahmedabad, India).

Glutathione assay

The hepatic level of glutathione was determined after 24 hrs. of irradiation by the method of Moron *et al.* [29]. Briefly, liver homogenate was added to 20% TCA, centrifuged, and the supernatant was collected. The supernatant was mixed with 0.3M Na_2HPO_4 and 5-5, dithiobis-2-nitrobenzoic acid (DTNB) reagent, and allowed to stand for 10 min at room temperature. The absorbance was read against blank at 412 nm using a UV-VIS Systronic Spectrophotometer (Mfd. at Ahmedabad, India).

The glutathione content in the blood was measured spectrophotometrically using DTNB as a coloring reagent, according to the method of Beutler *et al.* [30]. Briefly, 0.2 ml of blood was mixed in 1.8 ml of double distilled water and added to the precipitating solution, centrifuged and supernatant was collected. This supernatant was mixed with 0.3 M disodium hydrogen sulphate and DTNB reagent, and was allowed to stand for 2 min. at room temperature. The absorbance was read at 412nm.

Total plasma protein

Total plasma protein was estimated by the method of Lowry *et al.* [31]. In this procedure, the final colour is a result of burette reaction of protein with copper ion in alkali and reduction of phosphomolybdic phosphotungustic reagents by the tyrosine and tryptophan present in the proteins. The absorbance was read at 540 nm using a UV-VIS Systronic and results were expressed as mg/100 ml.

Plasma cholesterol

Total cholesterol was estimated using Burchard method [32]. The absorbance was read at 550 nm against blank.

Statistical analysis

The result for all the groups at various necropsy intervals were expressed as mean ± Standard error (S.E.). To find out whether mean of sample drawn from experimental (Group-IV) deviates significantly from respective control (Group-III), Student's *t-test* was performed by the method of Bourke *et al.* [33]. The significance was set at different levels as $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULTS

A preliminary drug tolerance study and the selection of the optimum dose of *Alstonia scholaris* extract were described in our

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previous study [34]. For this, the animals pretreated with different 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, against 8 Gy gamma radiation. The 100 mg/kg b. wt. was found to be the optimum dose based on the above data; hence the further studies were carried out using this dose of ASE.

No noticeable signs of behavioral change, sickness or mortality were observed in Sham irradiated/ ASE-treated group. Animals exposed to 7.5 Gy gamma rays exhibited epilation, ruffled hair, watering of eyes, diarrhea, lethargicity 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, activeness, food and water intake were found to be normal in ASE pretreated irradiated animals.

Lipid peroxidation, estimated from MDA production in the microsomal fraction of liver homogenate and blood, was significantly elevated ($p < 0.001$) in the radiation alone group; whereas, the ASE pretreated irradiated group showed a significant decrease in MDA formation in liver and blood (figure 1).

Reduced glutathione (GSH), measured as acid soluble sulfhydryl group (-SH), in liver homogenate and blood showed a significant decline ($p < 0.001$) after exposure to 7.5 Gy gamma radiation. However, ASE pretreated irradiated (Group-IV) animals exhibited a significant elevation ($p < 0.001$) in glutathione (blood and liver) as compared to Group -III, but the values remained below normal (figure 2).

No significant difference in plasma protein was observed in ASE alone treated animals (Group-II) as compared to vehicle control (Group-I). In irradiated control (Group-III), there was a significant reduction up to 3 days; thereafter it increased till their survival i. e. day 15th. In ASE pretreated group, protein level was significantly higher than corresponding control throughout the experiment by restoring the normal value at the end of experiment (i.e. day 30th) (figure 3).

No significant alterations in plasma cholesterol level were observed between vehicle treated control and ASE treated animals. However, a statistically significant ($p < 0.001$) elevation in cholesterol level was noted in irradiated control (Group-II) animals as compared to Group-I. ASE pretreated irradiated (Group-IV) animals exhibited a significant decline ($p < 0.001$) in cholesterol level as compared to Group –III at by attaining almost normal value on 30th day of irradiation (figure 4).

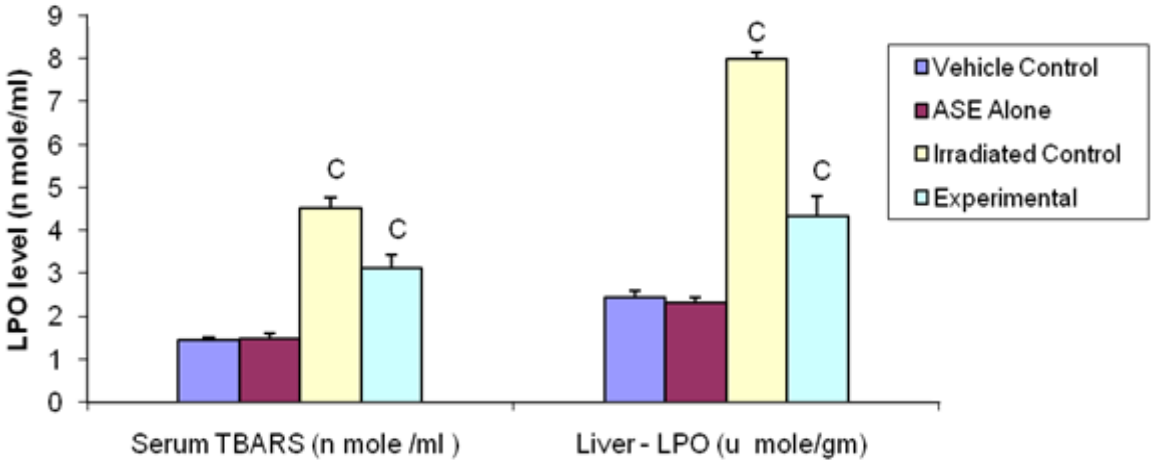


Figure 1. Lipid peroxidation (LPO) level in serum and liver of Swiss albino mice after exposure to 7.5 Gy gamma rays with (Experimental) or without (Control) Alstonia scholaris extract (ASE).

Significance Level: c = $p<0.001$

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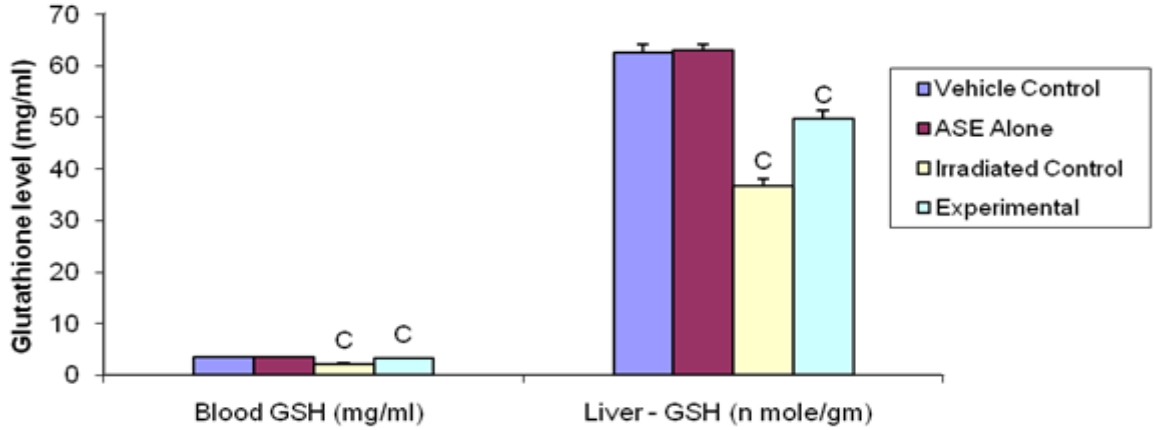


Figure 2. Reduced glutathione (GSH) level in blood and liver of Swiss albino mice after exposure to 7.5 Gy gamma rays with (Experimental) or without (Control) Alstonia scholaris extract (ASE).

Significance Level: c = p<0.001

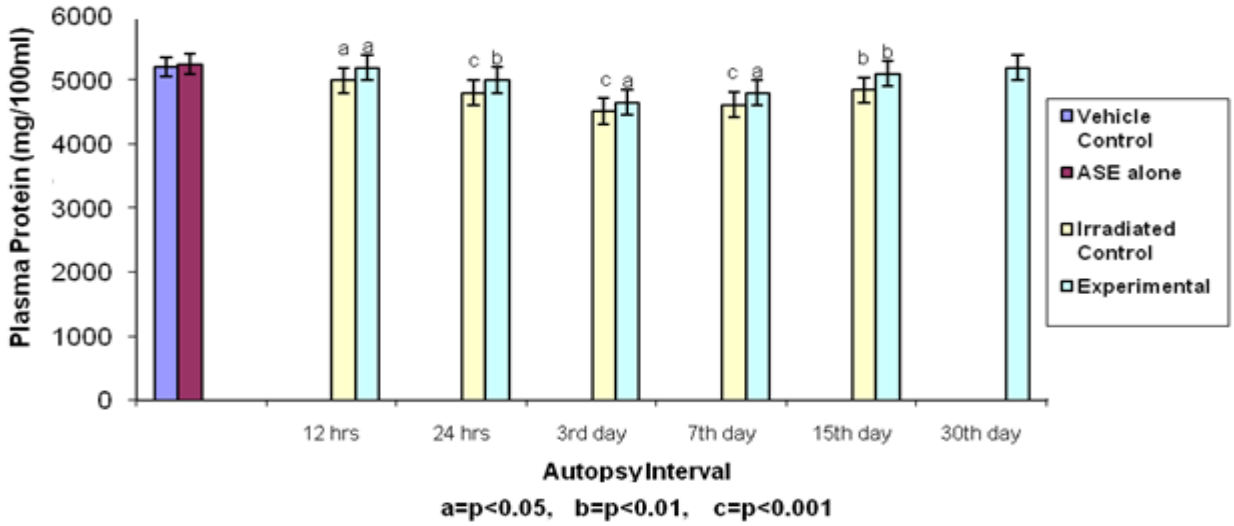


Figure 3. Variations (mean+S.E.) in Plasma Protein (mg/100ml) in peripheral blood of mice after exposure to 7.5 Gy gamma radation with (Experimental) or without (Control) Alstonia sholaris extract (ASE).

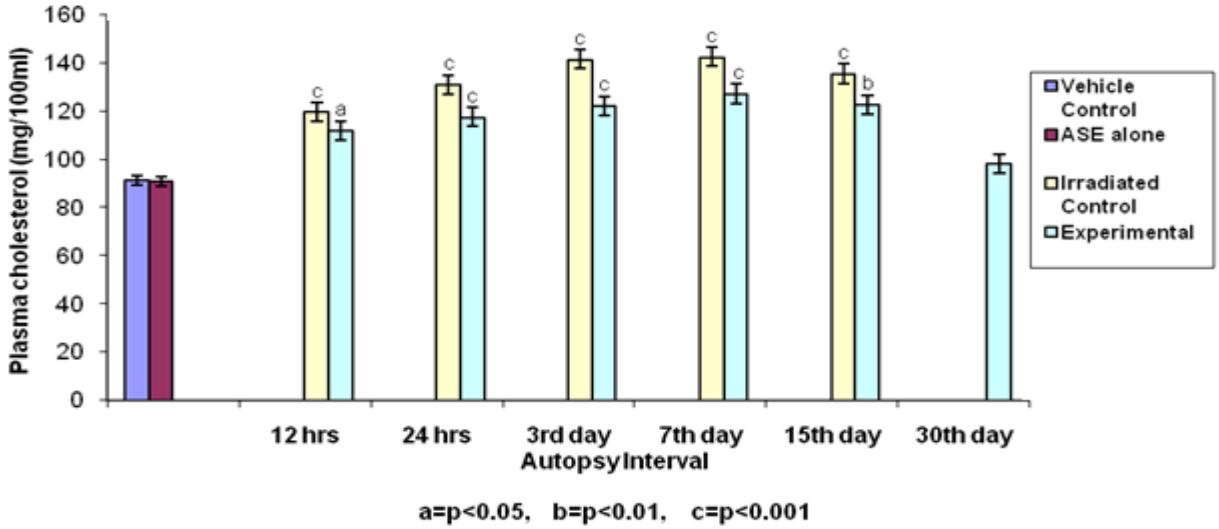


Figure 4. Variations (means+S.E.) in plasma cholesterol (mg/100ml) in peripheral blood of mice after exposure to 7.5 Gy gamma radiation with (Experimental) or without (Control) Alstonia scholaris extract extract (ASE).

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DISCUSSION

Ionizing radiation act either directly or by secondary reactions to produce biochemical lesions that induce a variety of physiological dysfunctions. Radiations also induce oxidative stress through the generation of reactive oxygen species (ROS) resulting in imbalance of the pro-oxidant and antioxidant activities, ultimately resulting in cell death [35]. Numerous attempts have been made to investigate different means for controlling and protection from radiation hazards using chemical, physical and biological means. Chemical protection is a useful strategy for the protection of individuals against radiation-induced biological lesions.

Irradiation of animals to 7.5 Gy gamma rays, in the present study, resulted in radiation sickness within 3-5 days after exposure. The symptoms included reduction in the food and water intake, weight loss, diarrhea, ruffling of hairs and irritability. The similar symptoms have been observed in mice after gamma irradiation by others also [36, 37]. None of the animals could survive after day 15th of irradiation. Death observed at this dose is mainly attributed to gastrointestinal and hematopoietic syndromes. Both cell linings, the alimentary tract and circulatory leucocytes, are relatively short lived and their orderly renewal depends on a population of constantly dividing stem cells. Therefore, any damage to these cells impairs normal physiological host defense processes drastically, causing an adverse impact on survival [38]. In this study, ASE pretreatment significantly reduced mortality induced by gamma irradiation. This may be due to protection of intestinal epithelium, which would have allowed proper absorption of the nutrients. The protection of circulating leucocytes may boost up the immune response against radiations [39]. Furthermore, antibacterial activity [40] of ASE may play some role in protecting mice. Magnitude of radioprotective effect of ASE was demonstrated by determining the LD50⁄30 (DRF = 1.80) in our earlier report [34]. This indicates that ASE showed protection against radiation-induced damage.

Lipid peroxidation is another important event related to cell death and has been reported to cause severe impairment of membrane functions through increased membrane permeability and membrane protein oxidation, DNA damage, cytotoxicity and eventually cell death [41-44]. The presence of antioxidants in the plants suppresses the formation of free lipid radicals and thus prevents the formation of endo-peroxidation. In the present study, Increase in TBARS is correlated with a decrease in body weight, organ weight and protein value in irradiated group. Since lipid peroxidation is a good biomarker of damage that occurs due to radiation and so the inhibition of lipid peroxidation is suggestive of radioprotective action of the ASE supplementation prior to irradiation.

ASE pre-treatment in present study significantly lowered the radiation-induced lipid peroxidation in terms of malondialdehyde. Prevention of lipid peroxidation by *Alstonia scholaris* extract via decrease in malondialdehyde level has been reported by Arulmozhi *et al.* [45]. The inhibition of lipid peroxidation in biomembranes can be caused by antioxidants [46, 47]. The presence of antioxidant activity in *Alstonia scholaris* may reduce lipid peroxidation as observed by Jagetia *et al.* [48].

Under normal conditions, the inherent defense system including glutathione and antioxidant enzymes protects against the oxidative damage. The present study demonstrates a significant reduction in GSH content following 7.5 Gy radiation exposures. This could be due to the enhanced utilization of the antioxidant system as an attempt to detoxify the free radicals generated by radiation. The lower depletion of GSH content in ASE pretreated irradiated animals could be due to the higher availability of GSH, which increases the ability of the cell to cope up with the free radicals produced by radiation. The

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increased GSH level suggests that protection by ASE may be mediated through the modulation of cellular antioxidant levels. The antioxidant activity of a compound has been attributed to various mechanisms among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [49]. Generally, flavonoids are the important class of antioxidants; hence, the medicinal plants containing flavonoids and phenolic compounds posses' antioxidant activity. *Alstonia scholaris* contain flavonoids, alkaloids and triterpenoids [40] which are responsible for antioxidant activity of this plant in present study.

The amount of total plasma protein in the present experiment decreased after irradiation. Reduction in rate of the protein synthesis may be due to unfavourable condition like unavailability of one or more essential enzymes and/or reduction in the sites of protein synthesis [50]. The decrease of protein noted may be due to its lysis by radiation or may be at the synthesis level, or also may be the depression of enzymes involved in the activation of amino acids and transferring to t-RNA [51] or by the inhibition of release of synthesized polypeptides from polysomes [52]. A decrement in RNA and protein content of small intestine has also been reported by Gajawat *et al.* (2001) [53] in mice after whole-body gamma irradiation.

Increased plasma protein concentration recorded in our study in ASE supplemented irradiated mice appears a protective effect. This proves an improvement in the ribosomal activities, which enhanced the protein synthesis and it can be treated as antiradiation effect. Grant [54] suggested that protection of protein is due to the hydrogen atom donation by the protector. Increase in protein concentration with supplementation of various medicinal plant extract after gamma irradiation was reported earlier too [55].

Plasma cholesterol level showed a significant rise in present experiment after irradiation. This may be due to the increased ability of the liver to biosynthesize cholesterol [56] as well as to the decreased activity of cholesterol 7-hydroxylase, the key enzyme involved in degradation of cholesterol in the liver, as mentioned by Chupukcharoen *et al.* [57]. The post-irradiation increase in cholesterol level in seminal vesicle has also been found by Jacob and Maini [58] in mice irradiated at 5.0 Gy dose. In ASE pretreated animals decrement in cholesterol level was reported at all autopsy intervals as compared to irradiated control. The findings of a significant depletion of cholesterol in protected mice is in corroboration with those of Gajawat *et al.* [53] who observed vitamin C or/ and E as a protector against radiation induced increase in cholesterol.

CONCLUSION

The protection afforded with ASE in the present study may prove to be beneficial for the clinical use of such medicinal plant as radio-protector. The radioprotective manifestations need to be further investigated in other model systems to assess its potential utility for human applications. The identification and characterization of individual constituents of ASE would be a necessary step in this direction.

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