

# Protection of DNA from Radiation by the Medicinal Plant *Holarrhena antidysentrica*

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## ABSTRACT

**Objective:** To investigate the radioprotecting ability of the extract of the medicinal plant *Holarrhena antidysentrica*, especially to protect DNA from gamma- radiation induced damages.

**Materials and Methods:** Hydro-alcoholic extract of dried roots of *Holarrhena antidysentrica* (HAE) was prepared, freeze dried and stored at 4°C. The DNA of the plasmid pBR<sub>322</sub> was exposed to gamma-radiation in presence and absence of HAE and subjected to agarose gel electrophoresis to study radioprotecting ability of the extract under *in vitro* conditions. The radioprotecting ability of the extract under *ex-vivo* conditions was examined by performing alkaline comet assay on human peripheral blood leucocytes exposed to the radiation with and without HAE. Mice were whole body exposed to gamma- radiation and single cell suspensions of spleen and bone marrow were subjected to alkaline comet assay to investigate the radioprotecting ability of HAE under *in vivo* conditions. The effect of HAE on radiation induced mortality was checked by monitoring the mortality of mice exposed to 10 Gray(Gy) whole body gamma-radiation after administering HAE.

**Results:** Exposure to gamma-radiation led to induction of DNA strand breaks, resulting in the relaxation of plasmid DNA from super coiled (ccc) form to open circle (oc) form and the presence of HAE during radiation exposure protected the DNA from the induction of strand breaks. The exposure of the human leucocytes to the radiation *ex vivo* resulted in the increase in the damage to cellular DNA measured as increase in the comet parameters such as percent DNA in tail, tail length and tail moment and olive tail moment and the presence of the extract during irradiation decreased all the comet parameters indicating *ex vivo* radioprotection of DNA. In whole body irradiated mice cellular DNA damage in bone marrow and spleen cells, seen as increased comet parameters were found reduced by administration of HAE prior to radiation exposure, indicating *in vivo* radioprotection of DNA. Investigations on survival of animals following acute lethal dose of 10 Gy whole body gamma- radiation

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showed that the administration of HA provided only a small extent of radioprotection and survival advantage.

**Conclusions:** The results indicate that under *in vitro*, *ex vivo* and *in vivo* conditions of radiation exposure, the extract HAE protects cellular DNA from deleterious effects of radiation and hence it may be useful to prevent genomic insults from radiation.

**Keywords:** *Holarrhena antidysenterica*; Gamma-radiation; DNA strand breaks; Radioprotection; Comet assay.

## INTRODUCTION

With the wide spread application of radioisotopes and radiation in nuclear industry for power production, in health care for therapeutic and diagnostic purposes, in agriculture for developing improved varieties of crops by mutation breeding, in food preservation, sterilization of medical products in health care, in industry for nondestructive testing etc, there is an increased need to have safe and effective means to protect not only the special high risk group (personnel handling the radioactive materials, radiation sources and equipments) but also the general population at large from health hazards of unintended ionizing radiation exposures. Ionizing radiation inflicts deleterious effects to living cells through the generation of reactive oxygen species that damage vital cellular targets such as DNA and membrane<sup>1</sup>.

Radiotherapy is one of the most effective treatments for cancer and 80% of the cancer patients require radiotherapy at sometime or, other, either for curative or palliative purpose<sup>2,3</sup>. During radiotherapy, cells of the normal tissues near the tumor site also suffer radiation damage and this becomes a serious problem in some cases<sup>2</sup>. The search for an effective and non-toxic radioprotector is a major concern in the medical, environmental and space sciences. Several compounds, derived from plants, possessing high antioxidant activity are reported to have radioprotecting properties<sup>4</sup>.

The endogenous biochemical mechanism of the protection includes the presence of cellular compounds like glutathione, and antioxidant enzymes like super oxide dismutase, catalase etc. Another line of cellular defense against deleterious consequences of radiation is the presence of DNA repair enzymes<sup>5</sup>.

In clinical radiation therapy, radioprotective compounds are of great importance because normal tissues should be protected against radiation injury while using higher doses of radiation to obtain better cancer control. In the recent past, many natural and synthetic compounds have been investigated for their efficacy to protect radiation induced damages in biological systems<sup>2</sup>. However, the inherent toxicity of most of the synthetic agents at the radioprotective concentrations warranted further research for a safe and effective radioprotector. In fact, as on today, no radio protective agent or agents either alone or in combination meet all the requisites of an ideal radioprotector<sup>6</sup>.

In this report we explore whether the hydroalcoholic extract of *Holarrhena antidysenterica* WALL can be used for deteriorating the adverse effects of gamma-radiation. *Holarrhena antidysenterica* (L.) WALL (Apocynaceae) is a medicinal plant, found throughout the Indian subcontinent. Stem bark of the plant, locally known as “kurchi”, has been extensively investigated

as it is traditionally used in the treatment of amoebic dysentery, diarrhoea, asthma, and bronchopneumonia<sup>7</sup>. Chloroform extract of *H. antidysenterica* was reported to be effective in treating malaria<sup>8</sup>. Ali and coworkers have reported the *in vitro* antioxidant activity of hydromethanolic extract of the seeds of *H. antidysenterica*. The methanolic extract of *Holarrhena antidysenterica* bark has been shown to possess significant skeletal muscle relaxant and CNS depressant activity in mice<sup>10</sup>. Efforts are made to study the DNA protecting ability of *Holarrhena antidysenterica* (L.) on plasmid DNA under *in vitro* conditions as well as on cellular DNA human blood leucocytes under *ex-vivo* conditions and under *in vivo* conditions on cellular DNA in mice murine tissues following whole body exposure to gamma-radiation. The comet assay was used for assessing DNA damage.

## MATERIALS AND METHODS

### Preparation of *Holarrhena antidysenterica* extract

Dried stem bark of *Holarrhena antidysenterica* were obtained from Amala Ayurvedic Hospital and Research Centre, Thrissur, Kerala, India and powdered. The powder was extracted, at room temperature with a mixture of ethanol and water in the ratio 7:3. Extract was filtered through Whatmann No.I filter paper and was evaporated using rotary evaporator at 45<sup>o</sup>C and the final liquid suspension was lyophilized to get a powder, hereafter referred as HAE (*Holarrhena antidysenterica* extract). The yield of the powder obtained was 13.5 %. The powder was dissolved in distilled water to obtain desired concentrations. The Hgh Performance Thin Layer Liquid Chromatography (HPTLC) fingerprinting of the extract was carried out with the solvent system Butanol-acetic acid-Water (5:1:4).

The HPTLC profile of HAE is presented in Scheme I.

### Exposure to gamma- radiation

Irradiation was carried out using a <sup>60</sup>Co-Theratron Phoenix teletherapy unit (Atomic energy ltd, Ottawa, Canada) at a dose rate of 1.88 Gy per minute.

### *In vitro* and *ex vivo* studies

#### Protection of plasmid pBR322 DNA by HAE against different doses of gamma-radiation (0-25 Gy)

The plasmid pBR322 (150 ng) in phosphate buffer (0.1 M), pH 7.4 was exposed to various doses of gamma-radiation (0-25 Gy) in the presence and absence of HAE (0-10 mg/ml) on ice. Exposure to gamma-radiation led to DNA strand breaks, resulting in the relaxation of plasmid DNA from supercoiled (ccc) form to open circle (oc) form. Both this forms are separated by electrophoresis in 0.8% agarose gels and stained with ethidium bromide to visualize the plasmid DNA under the uv light. After gamma-irradiation the DNA was electrophoresed in 0.8% agarose at 55 V for 2 hours and the DNA damage was analyzed by Digital Gel Documentation and Analysis Software, Biotech R&D Laboratories, Yercaud<sup>11</sup>.

#### Protection of plasmid pBR322 DNA by different doses of HAE against 25 Gy gamma- radiation

The plasmid pBR322 (150 ng) in phosphate buffer (0.1 M), pH 7.4) was treated with different concentrations of HAE (0-10mg/ml), exposed to 25Gy gamma-radiation and analyzed by agarose gel electrophoresis<sup>12</sup>.

### Alkaline single cell gel electrophoresis on human peripheral blood leucocytes exposed to gamma-radiation *ex vivo*

For studying the effect of HAE on human peripheral blood leucocytes, blood was collected from three healthy non-smoker volunteers having the mean age  $22 \pm 3$  years, by finger prick method, in heparinized eppendorf tubes and stored at ice temperature. HAE (10 mg/ml) was added to the blood 5-10 minutes before it is exposed to 4Gy gamma-radiation. Radiation induced damage to DNA in the blood leucocytes was measured as strand breaks following alkaline single cell gel electrophoresis.

Alkaline single cell gel electrophoresis was performed using method of Singh with minor modifications<sup>13,14</sup>. This assay determines damage to DNA in single cells – blood leukocytes or single cells prepared from mammalian tissues. Microscopic slides were coated with 1% normal melting agarose in phosphate buffered saline (PBS). To these coated slides 200 microliters of 0.8% low melting agarose (LMA) containing approximately  $10^5$  treated cells in 50 microliters were added at  $37^\circ\text{C}$  and the slides were placed at  $4^\circ\text{C}$ . After solidification of the LMA, the slides were placed in the chilled lysing solution containing 2.5 M NaCl, 100mM Na<sub>2</sub>-EDTA, 10mM Tris-HCl, pH 10, and 1% DMSO, 1% Triton X100 and 1% sodium sarcosinate, for 1 hour at  $4^\circ\text{C}$ . The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300mM NaOH, 1mM Na<sub>2</sub>-EDTA and 0.2% DMSO, pH13.0). The slides were equilibrated in the same buffer for 20 min and electrophoresis was carried out at 25 V, 180mA for 20 min. After electrophoresis the slides were washed gently with 0.4 M Tris-HCl buffer, pH 7.4, to neutralize the alkali. The slides were stained by lysing on the top with 50 $\mu\text{l}$  of

propidium iodide (PI, 20 $\mu\text{g}/\text{ml}$ ) and visualized using a Fluorescent microscope with bright field phase-contrast and epifluorescence facility. The images (50-60 cells/ slide) were captured and quantification of the DNA strand breaks of the images were done using the CASP software by which % DNA in tail, tail length, tail moment and Olive tail moment were obtained directly<sup>15</sup>. The parameter tail moment (TM) is the product of tail length and % DNA in tail and olive tail moment (OTM) is the product of the distance between the centre of gravity of the head and the centre of gravity of the tail and % DNA in tail.

### *In vivo* studies: Estimation of DNA damage *in vivo*, in tissues of whole body gamma-irradiated mice

For analyzing the *in vivo* radioprotective effect of HAE on cellular DNA of murine tissues, animals were divided into four groups.

- I. Double distilled water (DDW)+ Sham irradiation
- II. DDW+4 Gy irradiation
- III. 200 mg/kg body wt HAE+ Sham irradiation
- IV. 200 mg/kg body wt HAE+ 4 Gy irradiation

Animals were sacrificed by cervical dislocation 1 hour after irradiation; bone marrow cells were collected by flushing the femur bones of the animals with PBS. Spleen was also taken out, minced and single cell-suspensions ( $10^6$  cells/ml) were made in PBS. All the samples were stored on ice in dark and alkaline single cell gel electrophoresis was carried out.

### Effect of HAE on the survival of mice exposed to lethal dose of whole body gamma- radiation (10 Gy)

Swiss Albino male mice (20-25 g body weight) were divided into 4 groups (each group with 10 animals). Animals in the groups I and II were orally administered with distilled water (0.2 ml) and those in Group III and IV were given HAE (200 mg/kg body weight in 0.2 ml distilled water). After one hour of the oral administration, Groups II and IV were exposed to 10 Gy whole body gamma-radiation. After the radiation exposure, oral administration of DDW for Groups I and II and HAE for Groups III and IV were continued for 7 consecutive days. The percentage survival in each group was recorded.

### Statistical Analysis

Statistical analysis on the results was performed using Microsoft Excel and Micrococcal Origin softwares. The data were expressed as mean  $\pm$  SD. The error bars in the figures indicate the standard error of mean. Statistical analyses of the results were performed using ANOVA with Tukey-Kramer multiple comparisons.

## RESULTS

### Protection of plasmid pBR322 DNA by HAE against different doses of gamma-radiation (0-25 Gy)

*In vitro* DNA protecting ability of the plant extract was performed by exposing the plasmid pBR322 to radiation with and without the presence of the extract (Fig 1a). Exposure to gamma - radiation caused strand breaks in DNA resulting in relaxation of the plasmid DNA from supercoiled (ccc) form to open circular (oc) form. It was seen that HAE reduced ionizing radiation-induced disappearance of ccc form of the plasmid DNA considerably. Figure 1a and 1b demonstrate the effect of different

concentrations of HAE on plasmid pBR322 DNA against gamma-radiation induced strand breaks at 25 Gy. The percentage of ccc form remaining after the irradiation is plotted against the concentration of HAE. From these results, it is evident that there is a slight dose dependent protection seen in the HAE treated samples. (See fig. 1a & 1b)

### Protection of plasmid pBR322 DNA by different doses of HAE against 25 Gy gamma - radiation

Figure 2 presents the effect of HAE on induction of strand breaks in plasmid pBR322 DNA by increasing doses of gamma - radiation (0-25 Gy). It can be seen that HAE offered some protection to plasmid DNA by preventing the conversion of supercoiled (ccc) form to open circular (oc) form. The dose modifying factor (D.M.F) was calculated at 50% protection and it was found to be 2.55. DMF is the ratio of the radiation doses to bring down the percentage of ccc DNA to the same extent in presence and absence of the radioprotector. (See fig. 2)

### Alkaline single cell gel electrophoresis using human peripheral blood leucocytes *ex vivo*

The exposure of the human leucocytes to the radiation *ex vivo* resulted in the increase in the damage to cellular DNA measured as increase in the comet parameters such as percent DNA in tail, tail length and tail moment and olive tail moment as can be evidenced from the data presented in figure 3. Our *ex vivo* results suggest that the presence of the extract during the radiation exposure to human blood leucocytes could decrease the radiation induced damage to DNA. Gamma-irradiation resulted in increase of the comet parameters such as % DNA in tail, tail length, tail moment and olive tail moment due to strand breaks, All the comet parameters were found to be decreased when

the blood leucocytes were treated with HAE. (See fig. 3)

#### Estimation of DNA damage in the murine tissues of whole body irradiated mice *in vivo*

To examine the radioprotective effect of HAE on cellular DNA under *in vivo conditions of irradiation*, alkaline comet assay was performed on, bone marrow and spleen cells of mice exposed to radiation after administering HAE. Exposure to 4 Gy whole body irradiation induced strand breaks in the cellular DNA of murine tissues such as bone marrow and spleen. The data presented in figures 4 and 5, revealed that all the comet parameters (percent DNA in tail, tail length and tail moment and olive tail moment) increased in the radiation alone treated group, implying induction of DNA strand breaks due to radiation exposure. The values for all the comet parameters were elevated in mice exposed to gamma-rays in comparison to their un-irradiated controls. When animals were exposed to gamma-radiation, tail moment increased to  $1.003 \pm 0.034$  from  $0.163 \pm 0.044$ , % DNA in tail increased to  $7.38 \pm 0.447$  from  $1.32 \pm 0.150$  and tail length increased to  $5.00 \pm 0.787$  from  $3.04 \pm 0.273$ . But the administration of HAE brought down these parameters to  $0.56 \pm 0.169$ ,  $4.43 \pm 0.835$ ,  $3.30 \pm 0.638$  respectively, in the irradiated group in the case of bone marrow cells. (See fig. 4)

Similar trend was observed in the case of spleen cells (Fig. 5). The % DNA in tail was found to be  $6.02 \pm 0.637$ , tail length  $7.362 \pm 1.901$ , tail moment  $0.659 \pm 0.047$  and olive tail moment  $1.84 \pm 0.724$  in control irradiated group whereas the presence of the extract were brought down these levels to  $0.81 \pm 0.298$ ,  $3.40 \pm 0.863$ ,  $0.07 \pm 0.023$  and  $0.70 \pm 0.134$  respectively. (See fig. 5)

#### Effect of HAE on the survival of mice exposed to lethal dose of whole body gamma-radiation (10 Gy)

Figure 6 presents the data on the effect of HAE administration on the survival of animals following acute lethal dose of 10 Gy whole body gamma-irradiation. No lethality occurred in the un-irradiated Groups I and III but the animals in the irradiated groups started dying from 5th day in case of Group II and from 6<sup>th</sup> day in case of Group IV. On day 6, the mortality in Group II was 60% while it was only 50% in Group IV. On the 10<sup>th</sup> day there was 100% mortality in the control irradiated group (Group II) while the mortality was only 90% in the extract administered group (Group IV). This would imply that the administration of HA provided only a small amount of radioprotection and survival advantage at the lethal dose of 10 Gy. (See fig. 6)

## DISCUSSION

The damages in cellular DNA induced by ionizing radiation are of prime biological significance. Most of the damages induced by radiation to living cells are due to the generation of aqueous free radicals. Thus, any compound capable of reducing the free radical activity could be useful as radioprotector<sup>16</sup>. In the present study we are trying to find out whether the extract of *H. antidysenterica* can be used as a good radioprotector for human applications. We have shown the *in vitro* radioprotection of plasmid DNA by HAE. Plasmid in native form is predominantly in super coiled form. DNA when exposed to  $\gamma$  radiation will suffer strand breakage. This in turn leads in opening of the supercoiled form to circular form. Presence of HAE could protect the plasmid pBR322 from radiation induced damage. Ionizing radiation induces a variety of lesions in DNA such as single strand breaks, double strand breaks, DNA-DNA and DNA-protein cross links together with

damage to nucleotide bases. Comet assay is an elegant method to measure DNA damage, particularly strand breaks, and repair at the level of single cells<sup>17</sup>. It is also called single cell gel electrophoresis and the term comet assay comes from the characteristic shape seen when the cellular DNA in the nucleus migrates in an electric field based on the extent of damage suffered by the DNA. Neutral comet assay can be used to assess double strand breaks in DNA while alkaline comet assay estimates the total number of breaks – both single and double stranded breaks, and alkali labile sites. The alkaline single cell gel electrophoretic analysis of murine tissues such as bone marrow and spleen of the whole body irradiated mice; *in vivo*, showed the increased comet parameters indicating radiation induced damages like formation of alkali labile sites and single and double strand breaks etc. The comet parameters in the extract treated animals showed a slight decrease. Similar results were obtained in the case of human peripheral blood leucocytes when exposed to gamma-radiation *ex vivo*.

Mortality studies done by administering HAE to the lethally irradiated mice (10 Gy) also did not give any promising results. Only we could say that the administration of HAE could increase the life span of irradiated animals to few more days than the radiation alone treated animals. On the 11<sup>th</sup> day there is only 10 % protection was observed in the HAE administered group. In conclusion, the present results do reveal that the extract HAE protects DNA from deleterious effects of ionizing radiation under *in vitro*, *ex vivo* and *in vivo* conditions of radiation-exposure and hence it may be useful to prevent genomic insults from radiation. However following whole body exposure to lethal dose of gamma-radiation HAE could provide only a little survival advantage, indicating that it cannot be used as a good

radioprotector in instances of whole body exposures although it has several other beneficial applications.

In past, there are several attempts to devise an ideal radioprotector, although many of them showed good radioprotecting ability in laboratory experiments, they failed in their application to human beings due to the high toxicity<sup>2,18</sup>. The enormous heritage of vast natural dietary and time tested medicinal resources could be worth exploring the possibility of developing efficient, economically viable and clinically acceptable radioprotectors for human application.

### ACKNOWLEDGEMENT

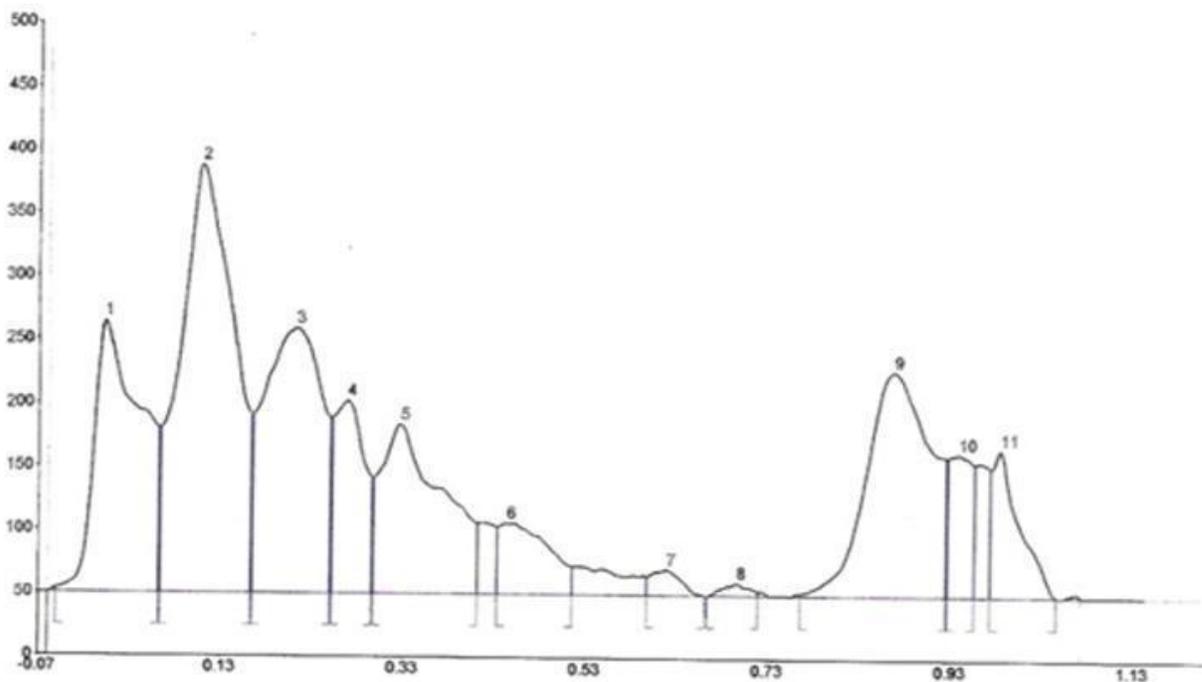
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*Holarrhena antidysenterica* Wall



Scheme I. HPTLC profile of the plant extract *Holarrhena antidysenterica* Wall

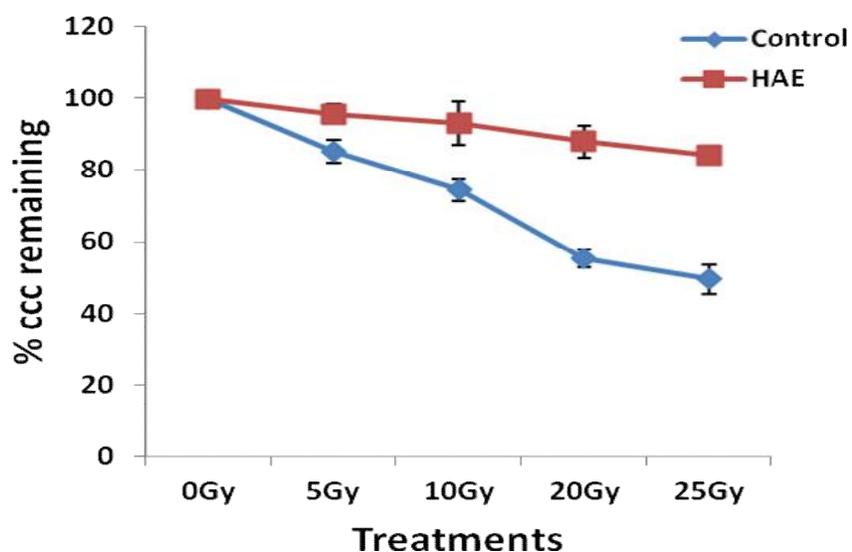
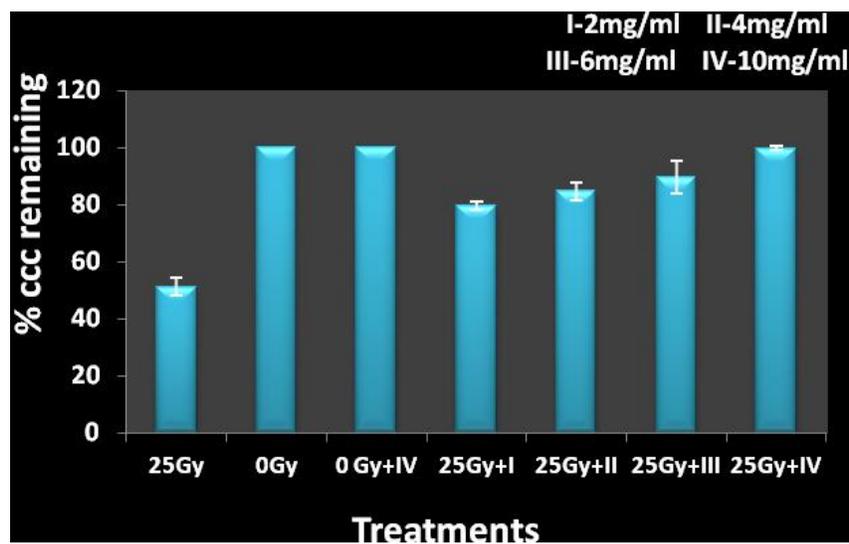


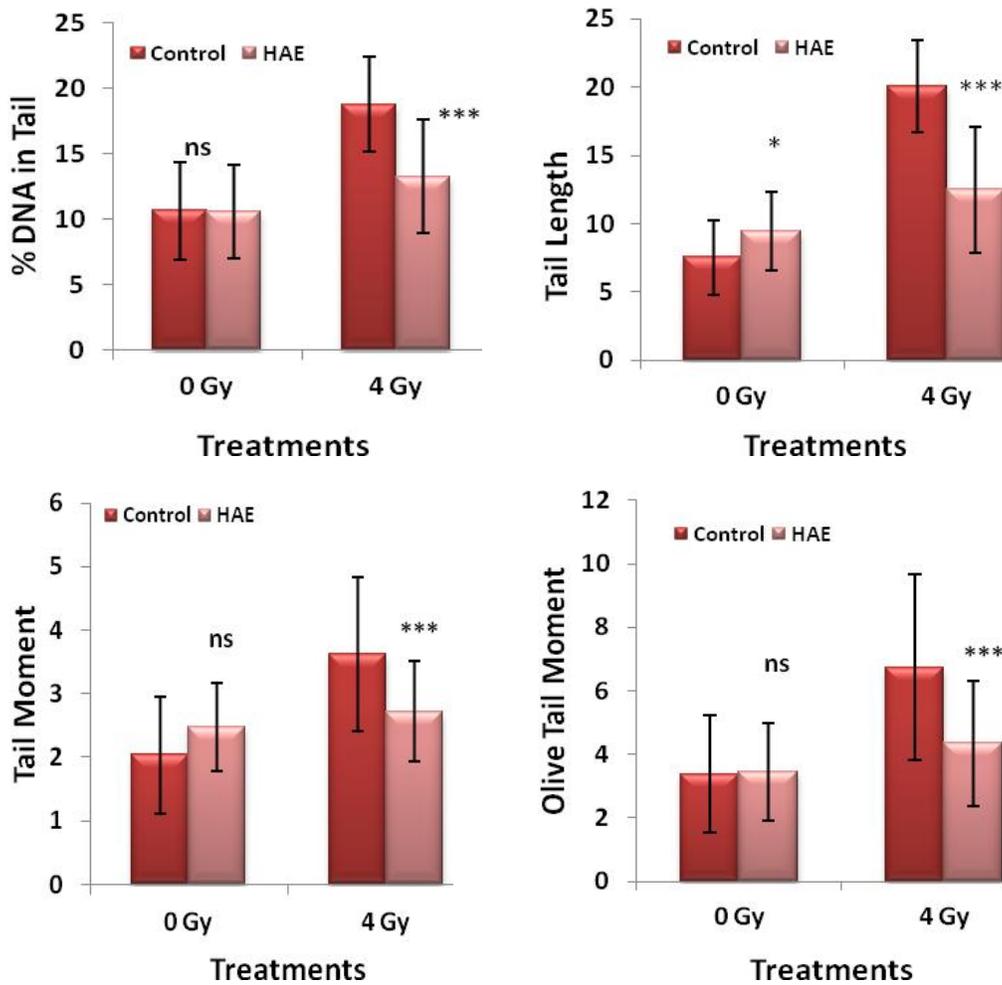
Fig. 1a: Effect of different doses of gamma-radiation on plasmid pBR 322 DNA in presence and absence of HAE



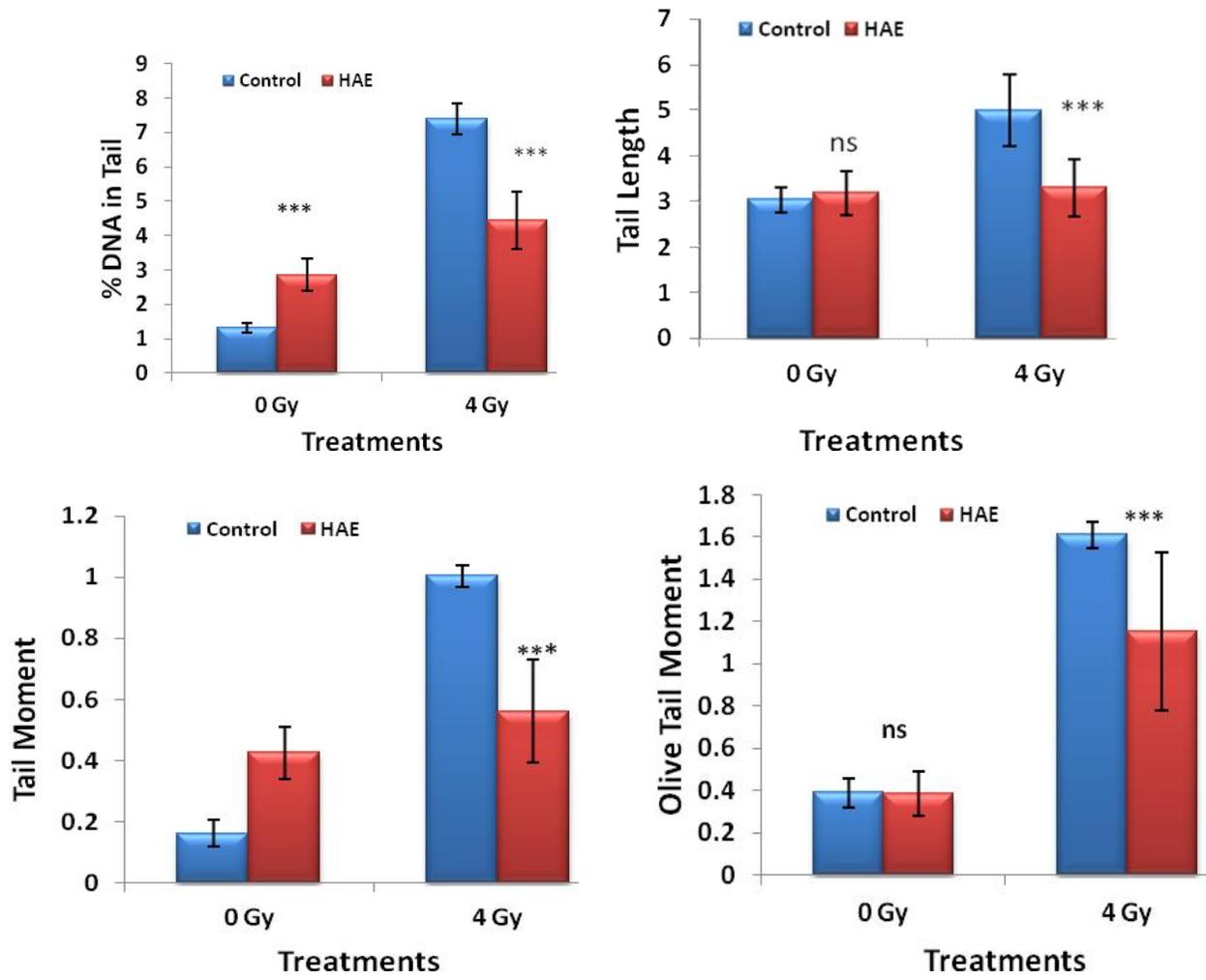
**Fig. 1b:** Effect of HAE on plasmid pBR322 DNA exposed to different doses of - radiation. 1: 0 Gy control. 2: 0 Gy, HAE, 3: 5 Gy control. 4: 5 Gy, HAE. 5: 10 Gy control. 6: 10 Gy, HAE. 7: 20 Gy control. 8. 20 Gy, HAE. 9. 25 Gy control. 10. 25 Gy, HAE



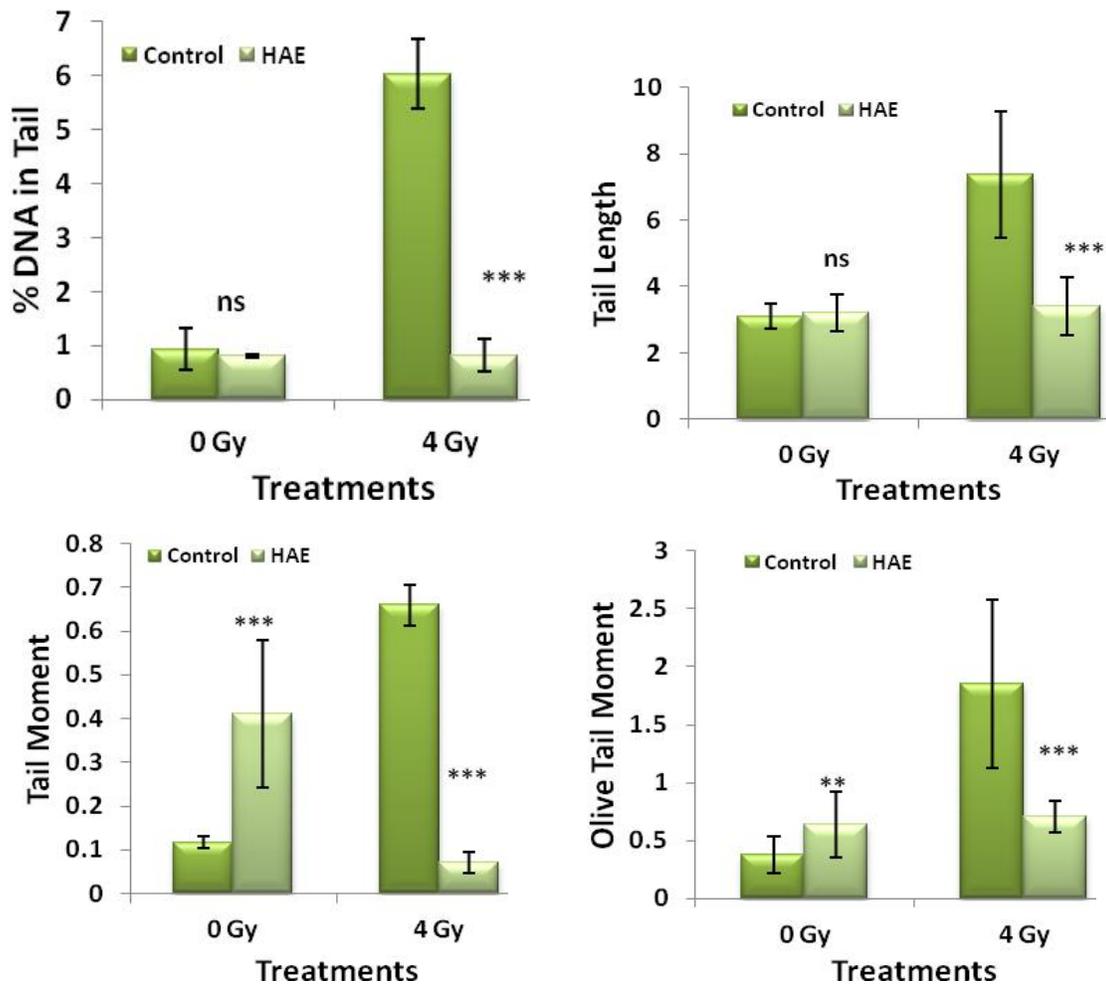
**Fig. 2:** Effect of various concentrations of HAE on pBR 322 DNA exposed to 25 Gy gamma-radiation. I-0 Gy, 0 HAE, II-0 Gy, 10 mg/ml HAE, III- 25 Gy, 0 HAE, IV-25 Gy, 2 mg/ml HAE, V-25 Gy, 4 mg/ml HAE, VI-25 Gy, 6 mg/ml HAE,VII- 25 Gy, 10 mg/ml HAE



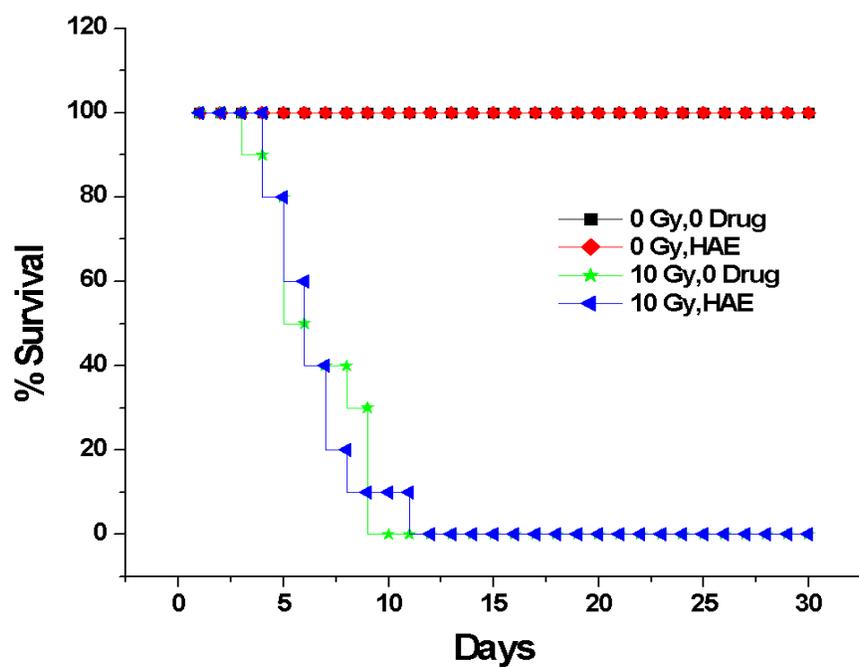
**Fig. 3:** Effect of HAE on comet parameters of the human peripheral blood leucocytes exposed to gamma-radiation (4 Gy) under *ex vivo*. The comet parameters - percentage DNA in tail, tail length, tail moment and olive tail moment - are presented as mean ± sd. (ns indicate not significant) p > 0.05 and \*\*\* indicate p < 0.001 when compared with respective control)



**Fig. 4:** Effect of HAE administration on comet parameters of bone marrow cells of mice exposed to whole-body gamma-radiation (4 Gy). Mean of the percentage DNA in tail, tail length, tail moment and olive tail moment are presented as mean± sd. (ns indicate not significant)  $p > 0.05$  and \*\*\* indicate  $p < 0.001$  when compared with respective control.



**Fig. 5:** Effect of of HAE administration on comet parameters of spleen cells of mice exposed to 4 Gy whole-body gamma-radiation. Mean of the percentage DNA in tail, tail length, tail moment and olive tail moment are presented as mean± sd. (ns indicate not significant) p>0.05 and \*\*\* indicate p <0.001 when compared with respective control)



**Fig. 6:** Effect HAE administration on mortality of mice following exposure to 10 Gy whole-body gamma-radiation.