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Radioprotective Properties of *Allium sativum* (Garlic) Extract on Cultured Human Lymphocytes against Electron Beam Radiation

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ABSTRACT

The radioprotective effects of naturally occurring compounds from herbs have been investigated *in vitro* and *in vivo* considering their ethnopharmacological role in prevention and treatment of cancer. *Allium sativum* supplementation in diet has been shown to be beneficial to cancer patients. The present study was designed to detect the radioprotective effect of garlic extract (GE) on cultured human peripheral blood lymphocytes. Garlic bulbs were extracted using ethanol and water separately followed by assays on antioxidant activities to assess the efficiency of radical scavenging capacity of various extracts. Lymphocytes were treated with different concentrations of GE for 2, 4, 6 and 24 hr periods. Cell survival was determined by tryphan blue dye exclusion assay, single strand DNA damage by alkaline comet assay and *in vitro* cytogenetic damages were evaluated by micronucleus assays. Ethanol boiled GE showed highest radical scavenging capacity and reducing property. Treatment of GE to lymphocytes before and after exposure to 4Gy of electron beam radiation (EBR) the percentage of tail DNA was reduced from 24.06±3.92 to 2.87±0.18. The elevated micronucleus formation in radiation control group (13.15±0.75) was significantly reduced in various concentrations of GE treated groups (10.35±0.44, 7.05±1.17, 6.42±0.47) respectively. Cells treated with GE at 10µg/mL showed maximum viability after exposure to EBR. Present investigations indicate that ethanol boiled GE shows good radiation protection at 10µg/mL concentration. However, increase in concentration above this dose though resulted in higher protection, increased cell toxicity was also noticed.

Keywords: Electron beam radiation, Radioprotection, DNA damage, Garlic.

INTRODUCTION

Ionizing radiation induces many forms of cellular damage, such as reproductive death, interphase death, cell division delay, chromosomal aberrations, mutation and transformation. These effects are conventionally attributed to an irreversible change during the process of repair of DNA replication.

A large number of drugs have been screened for their radioprotective activity. None of them could show clinical acceptability owing to their cellular toxicity at effective dosage. [1] It has been hypothesized that, chem physiological alterations resulting from the reactive oxygen species

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(ROS) during radiation exposure. [2] Protection and recovery from the consequences of ionizing radiation have been investigated at different cellular and molecular levels. DNA repair mechanism and protection offered by thiol compounds have been studied extensively. [3-4] Since alteration in cell cycle progression induces radiosensitivity of cells, agents that can interfere with cell cycle regulatory mechanism may be useful in providing radiosensitization or protection. Cell cycle checkpoints are considered as important molecular targets for modulation of radiation response. [5]

Search for radio protectors has been dominated by the study of sulfhydryl compounds such as Amifostin (WR 2721), which is considered as gold standard. It gives protection against many chemotherapeutic drugs induced DNA damage. Despite its protective nature amifostin also possesses toxicity. This necessitated the need for research into suitable radioprotectors.

Sulfur containing compounds are important in the biological systems. Several sulfur containing compounds are found in plants. [6] The protective nature of garlic has been attributed to the presence of organosulfur compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS), ajone, allicin, allylmercaptons and allyl methyl sulfides. [7] The present study was aimed at investigating the radioprotective effect of ethanol extract of garlic pods on cultured human peripheral blood lymphocytes against electron beam radiation (EBR).

MATERIAL AND METHODS

Preparation of *Alliium sativum* extract: [8] The pods of *Allium sativum* were collected from the local market. About 2 kilogram bulbs were cleaned in tap water followed by distilled water and outer coverings were removed. The pods were peeled out, chopped and shade dried for 15 days and was powdered. The powder was divided into 4 parts of 25gms each and the first part was subjected to soxhlet extraction with 99% ethanol for 72 hours, second part was socked in 70% ethanol for 24 hours at 4°C for cold ethanol extraction, third part was boiled for 20 minutes in 50mL distilled water to obtain boiled aqueous extract, and finally fourth part was kept for cold aqueous extraction which was maintained at 4°C for 24 hours. The boiled extract mixtures were evaporated to dry in a rotary flash evaporator and stored at 4°C till further use.

Estimation of Antioxidants in *Allium sativum* extracts

DPPH Radical scavenging assay: In order to measure antioxidant activity, DPPH (2, 2'-diphenyl-1-picrylhydrazyl) free radical scavenging assay was employed. The assay was carried out as described by Brand *et al*. [9] DPPH solution (0.004% w/v) was prepared freshly in 99%ethanol and sample solutions were prepared (1000µg/ml) in ethanol and water respectively for ethanolic and aqueous extract. *Allium sativum* extracts (200 to 1000µg/mL) was added to 1ml of 0.004% ethanolic solution of DPPH. The mixture was allowed to stand at room temperature in dark for 20 minutes. The mixture was vortexed and the absorbance was read at 517nm using a spectrophotometer. Reduced glutathione was used as a reference standard. Control sample was prepared containing the same volume without any extract and 99% ethanol was used as blank. Lower absorbance of the reaction mixture i.e decolouration of the sample indicates higher free radical scavenging activity.

The radical scavenging activity was calculated from the equation:

$$\text{Percentage of radical scavenging activity} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100$$

Ferric ion reducing power assay (FRAP): The $FeCl_3$ - reducing power of the extract was determined by the method of Oyaizu [10] with a minor modification. Different concentration of GE solution (final concentration 0 - 400 mg/ml) was mixed with 1 ml phosphate buffer (0.2M, pH 6.6) and 0.5 ml potassium ferricyanide [$K_3Fe(CN6)$] (0.1%). Then mixture was incubated at 50°C for 20 minutes in a water bath. Exactly 0.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000rpm for 10 min. Finally, 1 ml of the supernatant solution was mixed with 1 ml of distilled water and 0.1 ml $FeCl_3$ (0.01%) and absorbance was measured at 700 nm in spectrophotometer. Reduced Glutathione (GSH) was used as standard and phosphate buffer as blank solution. Increased absorbance of the reaction mixture indicates stronger reducing power.

Lymphocyte isolation and culture: [11] Approximately, 1 ml venous blood was collected from healthy, nonsmoking, non-alcoholic volunteer by venipuncture. Peripheral blood mononuclear cells (PBMCs) were separated using lymphocyte separation media (LSM-Himedia, Mumbai). About 1 ml of fresh EDTA blood was mixed with equal volume of phosphate buffered saline (PBS) and was overlaid with 1 ml of LSM. Lymphocytes were separated by density gradient centrifugation. Separated lymphocytes were washed twice with PBS and cells were suspended in minimum volume of RPMI-1640 (Himedia) and counted. Exactly, 1×10^6 cells/ml cells were cultured in RPMI medium supplemented with 6µg/ml Phytohemagglutinin and 15% Fetal Bovine Serum (FBS) at 37°C in a 5% CO_2 incubator.

Cell viability assay: Cell viability was measured by trypan blue dye exclusion. Lymphocytes were mixed with equal volume of 0.4% Trypan Blue Dye for 3 minutes and cells were counted using haemocytometer. Viable and dead cells were scored under the microscope.

Analysis of DNA damage by comet assay: The alkaline comet assay was performed basically as described by Tice and co-workers. [12] Electrophoresis, which allowed for fragmented DNA migration was carried out for 30 min at 25 V and 300 mA. After electrophoresis, the slides were neutralized with 0.4M Tris, pH 7.4, stained

with 50µL of ethidium bromide (20µg/mL) and analyzed under a fluorescence microscope (Olympus. 40x objective). The extent of DNA damage was assessed from the DNA migration distance, which was derived by subtracting the diameter of the nucleus from the total length of the comet. Fifty randomly selected cells were examined for each replicate, for each sample or subject. The quantification of the DNA strand breaks of the stored images was performed using Comet score software by which the percentage of DNA in the tail, tail length and Olive tail moment (OTM) could be obtained directly.

Micronucleus Assay: [13] Triplicate cultures containing 1 × 10^6 cells were irradiated and treated with each concentration of GE. The cells were allowed to attach for another 24 h. Thereafter, the cultures were treated with 3 mg/ml of cytochalasin-B and were incubated for another 24 h. The micronuclei were prepared according to the method described by Fenech and Morley with minor modifications. The results were confirmed by repetition of the experiment.

Statistical analysis: The results were expressed as mean ± standard deviation. Comparison between the control and treated groups were done by analysis of variance (ANOVA) followed by Tukey's test. The comparison between different time intervals was performed by One Way ANNOVA followed by Bonferroni test. In all these test criterion for statistical significance was $P<0.05$.

RESULTS

Following statistical tools it was possible to reach appropriate inference about the significance change in antioxidant level, cell viability, difference in micronucleus formation and significant reduction in DNA damage between radiation control group and lymphocytes treated with GE treatment group.

Radical scavenging activity (RAS) of the GE is shown in Figure 1. Among the extracts tested, boiled ethanol extract reacted faster than other extract and it was the most effective DPPH scavenger, while aqueous boiled extract showed lowest RAS. FRAP assay also showed similar result. The effective concentration of garlic boiled ethanol extract was lower (687.7±2.7) when compare to other extract. Hence for further study we selected garlic ethanol extract for radioprotection assays (Figure 1 and Table 1).

Results of comparative *in vitro* cell viability at 2, 4, 6 and 24 hour showed significantly ($P<0.001$) decreased in cells exposed to different EBR doses (2, 4, 6, 8 and 10Gy). Cell viability was found to be approximately 50% at 6 Gy EBR dose. Hence we selected 4Gy as sublethal dose for our experiments (Table 2). In cells treated with various concentrations of GE, we found that 10µg/mL concentration was non-toxic to lymphocytes and there was no significant ($P>0.05$) reduction in cell viability. As the GE concentration increased to 50 and 100µg/mL 33% and 50% of the lymphocytes were lysed at 50 and 100µg/mL concentration respectively, 100µg/mL was found to be IC_{50} value of GE for cultured lymphocytes (Table 3). For radioprotection study we selected 4Gy EBR dose as it is sub lethal dose for lymphocytes. Lymphocytes treated with 3 different concentrations of garlic ethanol extract was exposed to 4Gy EBR and then viability, micronucleus and DNA damage was assessed; which is considered as pretreatment group. In post treatment group, lymphocytes were initially exposed to 4Gy radiation and then treated with 3 different concentrations of garlic extract and the same parameters as mentioned above were studied (Table 2 and Table 3).

In pretreatment groups compared with radiation control, there was significant increase in cell viability (93.56±0.88) observed, but at 50 and 100µg/mL GE dose significant increase in cell viability was not noticed (Table 4). Surprisingly post treatment group has not showed increase in cell viability when compared to radiation control (Table 4); instead percentage cell viability decreased significantly in groups post treated with 100µg/mL GE (Table 4).

Comet parameters (percentage of tail DNA and OTM) were increased with increase in radiation dosage. This is directly proportional to the increased ssDNA damage (Figure 2 and 3). Lymphocytes treated with 100µg/mL of GE showed up to 5.94 percentage of DNA in tail at 4th hour and it was reduced at 24 hour up to 4.06. At 50µg/mL concentration there was no significant ($P<0.05$) difference observed in percentage of tail DNA at different time interval. There was no DNA damage observer at 10µg/mL of GE when compared with the untreated group. Similar results were observed for OTM value in GE treated groups (Figure 4 and 5).

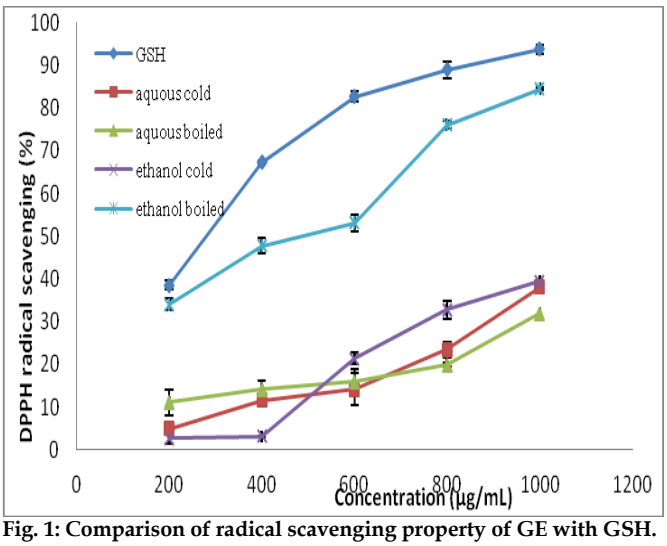

Fig. 1: Comparison of radical scavenging property of GE with GSH.

Table 1: EC_{50} values of different GE

Concentration 400µg/ ml	EC_{50} values of FRAP Assay (µg/ml)
Standard GSH	597.2±2.404
Ethanol cold extract	1112.4±1.831
Ethanol hot extract	687.7±2.736
Aqueous cold extract	1335±2.361
Aqueous hot extract	1668±1.895

Table 2: Percentage cell viability of cultured lymphocytes at different doses of EBR

	Control	2 Gy	4Gy	6Gy	8Gy	10Gy
2 h	97.00±2.00	87.35±0.77	76.34±1.23	55.34±0.34	36.98±0.98	1.18±0.01
4 h	97.00±2.00	87.33±0.61	76.31±1.12	53.14±0.45	36.13±0.81	0.00
6 h	97.03±1.50	86.67±0.71	76.39±1.11	52.01±0.71	35.41±0.17	0.00
24 h	98.14±1.10	85.39±0.93	75.98±1.98	50.35±0.65	34.45±0.56	0.00

The results are expressed as mean ±standard deviation. The results were statistically significant (P<0.001).

Table 3: Percentage of cell viability of cultured lymphocytes treated with different doses of GE

	Control	10 µg/ml	50 µg/ml	100 µg/ml
2 h	97.00±2.00	96.13±2.90	84.81±1.60	51.45±3.40
4 h	98.00 ±1.98	96.82±2.10	80.33±4.90	48.45±4.30
6 h	97.18±1.00	97.18±1.20	79.91±1.30	46.18±1.39
24 h	98.24±2.80	98.34±2.00	73.01±3.50	45.34±3.50

The results were statistically significant in between various concentrations of GE (P<0.001).

Table 4: Percentage cell viability of cultured lymphocytes exposed to 4Gy EBR with pre and post treatment of GE.

	Without radiation	Radiation control	10µg/ml	50µg/ml	100µg/ml
			Pre treatment		
2 h	97.52±1.45	76.34±1.23	92.93±1.87	75.44±1.57	68.24±0.34
4 h	97.13±0.98	76.31±1.12	92.89±0.55	75.87±1.08	68.21±0.56
6 h	98.89±1.13	76.39±1.11	93.78±1.14	74.87±1.18	67.91±0.78
24 h	99.89±1.34	75.98±1.98	93.56±0.88	74.26±0.92	67.25±0.83
			Post treatment		
2 h	97.52±1.45	76.34±1.23	74.35±1.04	60.08±1.89	52.14±0.45
4 h	97.13±0.98	76.31±1.12	74.19±0.78	60.78±0.79	52.66±1.34
6 h	98.89±1.13	76.39±1.11	75.89±0.67	60.71±2.81	53.96±1.47
24 h	99.89±1.34	75.98±1.98	75.83±1.07	62.97±1.45	60.57±0.67

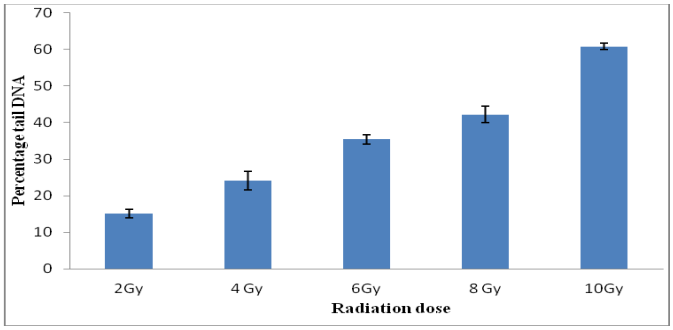

Fig. 2: Percentage tail DNA in lymphocytes exposed to different doses of EBR. The results were statistically significant (P<0.001).

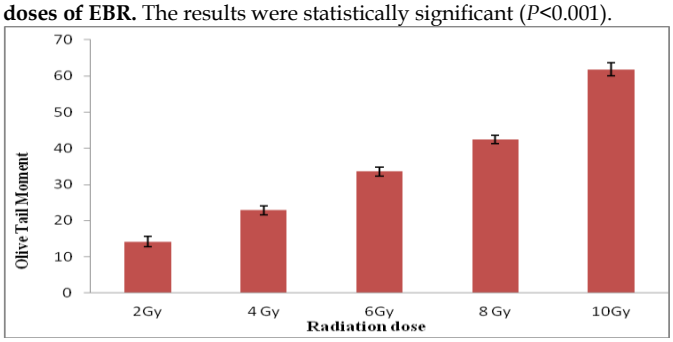

Fig. 3: Olive tail moment (arbitrary scale) in lymphocytes exposed to different doses of EBR. The results were statistically significant (P<0.001).

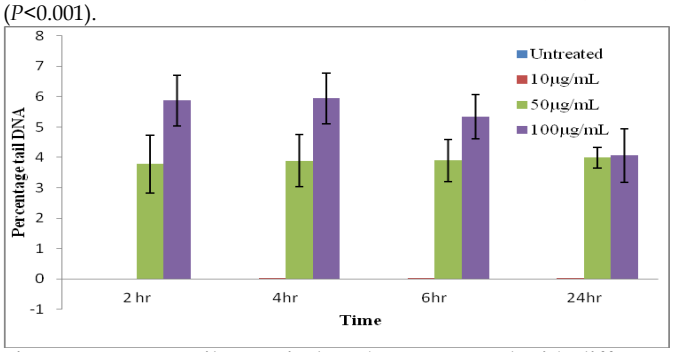

Fig. 4: Percentage tail DNA in lymphocytes treated with different doses of GE. The results were statistically significant (P<0.001).

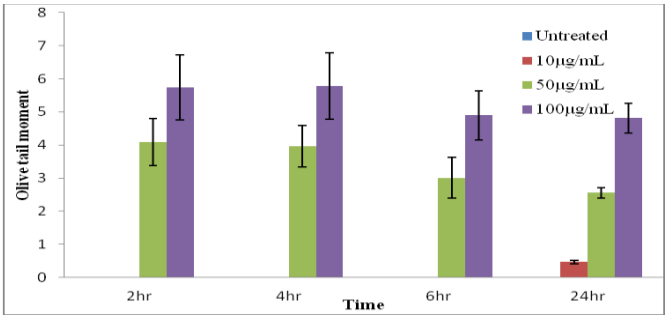

Fig. 5: Olive tail moment in lymphocytes treated with different doses of GE. The results were statistically significant (P<0.001).

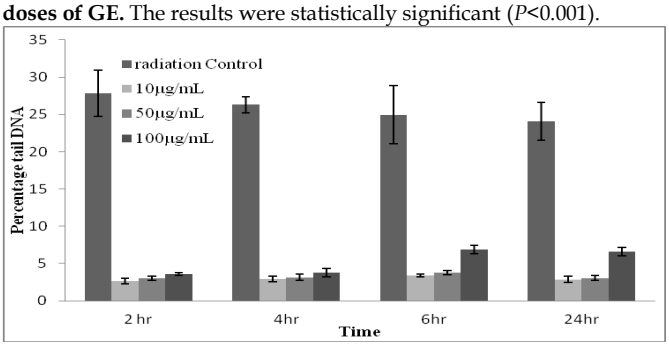

Fig. 6: Percentage of DNA damage when lymphocytes were treated with GE before exposure to EBR. The results were statistically significant (P<0.001).

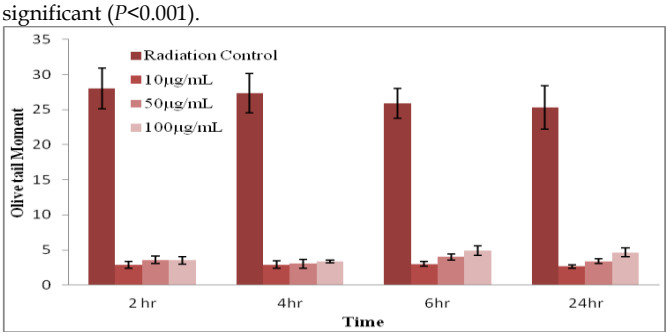

Fig. 7: Olive tail moment when lymphocytes were treated with GE before exposure to EBR. The results were statistically significant in between radiation control and GE treated groups (P<0.001).

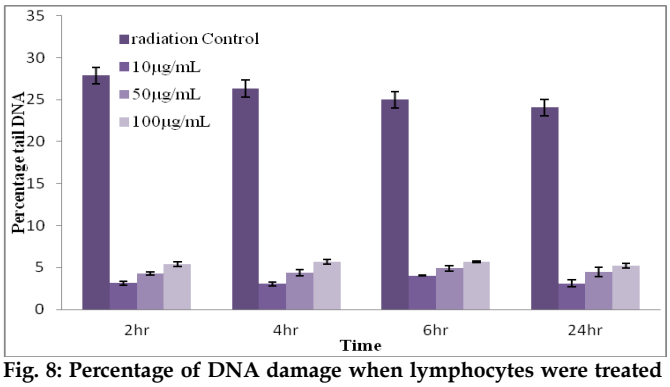

Fig. 8: Percentage of DNA damage when lymphocytes were treated with GE after exposure to EBR. The results were statistically significant in between radiation control and GE treated groups (P<0.001).

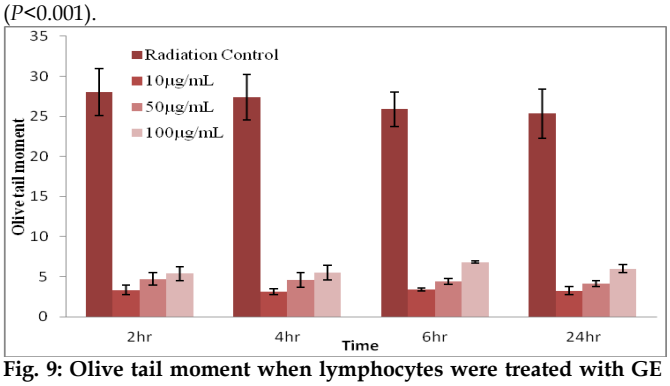

Fig. 9: Olive tail moment when lymphocytes were treated with GE after exposure to EBR. The results were statistically significant in between radiation control and GE treated groups (P<0.001).

Table 5: Percentage of micronucleus formation

Radiation Dose (Gy)	Percentage of Micronucleated cells
Control (no irradiated)	2.3±0.239
2Gy	6.65±0.244
4Gy	13.15±0.750
6Gy	16.62±0.286
8Gy	21.6±0.420

Percentage of micronucleus formation out of 1000 cells, when cells were treated with different doses of EBR. The results were statistically significant (P<0.001).

Table 6: Percentage of micronucleus in lymphocytes

Allium sativum Dosage	Percentage of Micronucleated cells
Control (Untreated)	2±0.151
10µg/mL	2.55±0.244
50µg/mL	2.67±0.332
100µg/mL	5.25±0.220

Percentage of micronucleus in lymphocytes treated with different doses of GE. The results were statistically significant in between radiation control and GE treated groups (P<0.001).

Table 7: Percentage of micronucleus in lymphocytes treated with different doses of GE before and after exposure to 4Gy of EBR

	Percentage of Micronucleus	
Control (without radiation and GE treatment)	2.000±0.151	
Radiation control (exposed to 4Gy radiation)	13.150±0.750	
	Pre treatment	Post treatment
10µg/mL	10.35±0.444	11.32±0.923
50µg/mL	7.05±1.177	7.82±0.349
100µg/mL	6.42±0.474	7.22±0.815

The results were statistically significant in between radiation control and GE treated groups (P<0.001).

Comet assay revealed a significant (P<0.001) decrease in DNA damage when cells were treated with various concentrations of GE. The group treated with 10µg/mL GE showed 3.2%, the group with 50µg/mL GE showed 4.3% and the group with 100µg/mL GE showed 5.2% of DNA in comet tail (Figure 6, 7). There was no significant difference observed in between pre and post treatment. Similar results were observed in post treatment also (Figure 8, 9) and (Figure 3-9).

Micronucleus formation also decreased significantly (P<0.001) in cells treated prior and after radiation exposure compared to radiation control group. In this case cells treated with 100µg/mL GE showed 6.425±0.474 % micronucleus per 1000 cells where radiation control group showed 13.150±0.750 micronucleated cells (Table 7) and (Table 5-7).

DISCUSSION

***In vitro* antioxidant studies** Free-radical reactions are responsible for wide range of chemo and radiotherapy induced side effects and antioxidants are responsible for protecting non-malignant cells from free radical damage. The findings of *in vitro* antioxidant studies on different extracts of garlic showed good free radical scavenging property. In present study, ethanol boiled extract showed high radical scavenging activity (84.51%) compare to other extracts. (Table-2 and Graph-1).In FRAP assay ethanol boiled extract also showed higher reducing ability(687.7±2.736) when compare to other extracts (Table-3).The present study showed that ethanol boiled extract has high radical scavenging activity; this may be because of the presence of more organosulfur containing compounds in garlic. [14] Hence this extract was selected for further studies.

Radioprotective properties of *Allium sativum* extract

In this study, the effect of ethanol extract of Garlic was assessed *in vitro* using human peripheral blood lymphocytes. S-allyl cysteine sulphoxide (SACS) is the first compound isolated from garlic (alliin). It is known that sulphydril compounds are capable of protection against tissue damaging effects of irradiation. Mechanism of effects supposed to be free radical extinction. Since garlic has been reported to contain more than 30 sulphydril compounds, which may play a role of radioprotective agent.

The present study showed that 10µg/mL of AE extract gave significant (P<0.001) protection against lymphocytes exposed to EBR. Cell viability was increased up to 93.56±0.88 group treated with 10µg/mL compared to radiation control. Percentages of DNA damage decreased in both pre and post treatment with ethanol extract. Micronucleus formation also decreased from 13.150±0.750 to 6.425±0.474 lymphocytes treated with 100µg/mL garlic extract.

The earlier reports of Jaiswal and co-workers, [15] the garlic oil gave protection against radiation induced mortality *in vivo* and results of Baer *et al*. [16] the DAS, an active principal component of garlic which provides protection against mouse colon also showed similar findings. Lau [17] also demonstrated radiation protective effect of garlic on cultured human lymphocytes. Our results are also in line with the previous findings. Other

sulphydryl compounds like triazole derivative showed modulatory effect on radiation induced clastogenic damages *in vivo*. The micronucleus frequency was reduced in bone marrow cells and also reduced the DNA fragmentation in mice hepatic cells. [18]

Earlier studies showed that radioprotection acts only at a particular dose and above which may not be protective and can even be toxic. [19] The reason behind it may be after a particular concentration, a compound may start manifesting its toxic effects. The active principle of garlic extract has been reported to stimulate B and T lymphocytes. [20] Higher dose over 50µg/mL caused growth suppression in cultured human blood lymphocytes in present study. The antioxidant property of GE might be the reason for radiation protection, but further studies are needed to know the exact protective mechanism.

The study may be concluded that, garlic extract may play a role as radioprotective agent since garlic is a rich source of many active biological compounds. Garlic is also known for its anticancer activity by killing cancer cells. Present results showed that garlic has both radioprotective properties. So it may be useful to those individuals who are exposed to radiation during chemotherapy. However, further research in this direction is needed.

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