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Radiation Protection by Nanosilver-Glycyrrhizic Acid Complex Prepared by Green Nanotechnology

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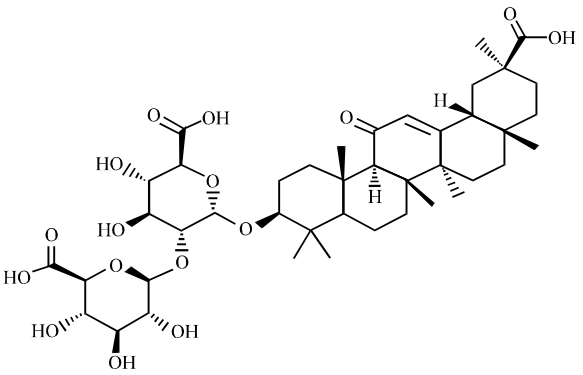
ABSTRACT

Silver nanoparticles were prepared from silver nitrate using Glycyrrhizic Acid (GLY), under sonication. The resultant golden yellow solution containing glycyrrhizic acid-silver nanoparticle complex (GLY-Ag) of particle size of less than 100 nm was analyzed for antioxidant activity by DPPH free radical scavenging activity and it was shown that GLY-Ag possesses higher free radical scavenging activity than GLY. Oral administration of GLY-Ag offered significant protection against gamma radiation induced cellular DNA damage as shown by the results of comet assay performed in bone marrow cells and blood leukocytes of mice exposed to various doses of whole body gamma radiation (0-6 Gy). GLY-Ag was also found to offer protection against the genotoxic effects of ionising radiation as shown by the results of micronucleus assay and chromosomal aberration analysis in whole body gamma irradiated mice (0-2 Gy). The complex offered significant protection to the hemopoeitic system from radiation injury (6 Gy) as shown by bone marrow cellularity, total blood count and endogenous spleen colony formation. Oral administration of GLY-Ag enhanced the survival of animals following a lethal dose of whole body gamma irradiation (8 Gy).

Key words: Silver nanoparticle, radioprotector, DNA damage, whole body gamma-irradiation, glycyrrhizic acid

INTRODUCTION

Ionizing radiation in the cellular milieu generates several reactive oxygen species such as superoxide, hydrogen peroxide, hydroxyl radical etc. These free radicals react with cellular macromolecules such as nucleic acids, lipids and proteins resulting in damage to genomic DNA, peroxidation of membrane lipids, protein oxidation and altered gene expression. These molecular alterations cause activation of cytotoxic and cytoprotective cellular signaling pathways (Schmidt-Ullrich *et al*., 2000). Multiple organ dysfunctions occur due to total-body exposure to ionizing radiation (TBI) in humans and animals as a consequence of toxicity to the hematopoietic, gastrointestinal or cerebrovascular systems, depending on the total dose of radiation absorbed (Mettler and Voelz, 2002). Extracts of several medicinal plants, synthetic drugs, phytoceuticals and nutraceuticals were investigated for their radioprotective efficacy and varying degrees of success have been reported (Maurya *et al*., 2006). In view of their potential application during both planned radiation exposure (e.g., radiotherapy) and unplanned radiation exposure (e.g., in the nuclear industry, natural background radiation emanating from the earth or other sources) development of effective radioprotectors and radiorecovery drugs is of paramount importance (Nair *et al*., 2001; Coleman *et al*., 2004).



Scheme 1: Glycyrrhizic acid (GLY)

Since most radiation damages arise from the interaction of radiation induced free radicals with the biomolecules, compounds with the ability to scavenge free radicals can prevent radiation damage. Glycyrrhizic Acid (GLY) (Scheme 1) is a major bioactive triterpene glycoside present in the root extracts of (*Glycyrrhiza glabra*) possessing a wide range of pharmacological properties such as anti-inflammatory (Fujisawa *et al*., 2000), anti-ulcer (Dehpour *et al*., 1995), anti-hepatotoxic (Ito *et al*., 1997), antiviral (Cinatl *et al*., 2003) and radioprotective (Gandhi *et al*., 2004; Lin *et al*., 1996) activities.

Nanoparticles due to the electron clouds that surround them could have high reactivity with free radicals (Bhatia, 2008). Silver nanoparticles have applications from electronics and catalysis to biology, pharmaceutical and medicine (Atiyeh *et al*., 2007; Khaydarov *et al*., 2009). Fish oil, which contains carboxylate and amine functional groups, can act as a reducing agent as well as surfactant for the synthesis of silver nanoparticles (Khanna and Nair, 2009). In the present work silver nanoparticles were prepared using the phytoceutical, glycyrrhizic acid (GLY) and the resultant complex of glycyrrhizic acid-silver nanoparticle (GLY-Ag) was explored for its antioxidant and radiation protecting properties.

MATERIALS AND METHODS

Animals: Male Swiss albino mice of 8-10 weeks old, weighing 22-25 g was obtained from the Small Animal Breeding Section (SABS), Mannuthy, Thrissur, Kerala. They were kept under standard conditions of temperature and humidity in the Centre's Animal House Facility. The animals were provided with standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water ad libitum. All animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

Chemicals: Acridine orange, colchicine and low melting point agarose were obtained from Sigma Chemical Company Inc., St Louis, MO, USA. EDTA (Ethylene Diamine Tetra Acetic acid), silver nitrate, ammonium nitrate, Zinc sulphate hepta hydrate and Tungsto silicic acid were from Merck Specialities Pvt. Ltd., Mumbai, India. Analytical grade silver nitrate was obtained from Qualigen India Ltd. All other chemicals were of analytical grade procured from reputed Indian manufacturers.

Sonochemical preparation of silver nanoparticle-glycyrrhizic acid complex: The solutions (1% in triple glass distilled water) of silver nitrate and glycyrrhizic acid were mixed in different proportions and sonicated at a frequency of 20 kHz. 250 w for 20 min, at 25°C in dark. The appearance of golden yellow colour indicated the formation of silver nanoparticles. The golden yellow colour developed was stable for the solution mixture containing 8 mM glycyrrhizic acid and 20 mM silver nitrate and this was designated as glycyrrhizic acid-silver nanoparticle (GLY-Ag) (Scheme 2). Scanning Electron Microscopic (SEM) analysis of GLY-Ag was done using JEOL Model JSM-6390 LV at Sophisticated Test and Instrumentation Centre, CUSAT, Kerala, India.

Free radical scavenging activity: The free radical scavenging activity of glycyrrhizic acid (GLY) and glycyrrhizic acid-silver nanoparticle (GLY-Ag) complex was determined by the method of Von Gadow *et al.* (1997) with some minor modifications. The DPPH (1,1diphenyl-2-picryl-hydrazyl) radical scavenging activity of glycyrrhizic acid (GLY) or glycyrrhizic acid-silver nanoparticle (GLY-Ag) complex (equivalent to 0.2 mM) was measured. The percent of inhibition of DPPH reduction (decolourization) was calculated according to the formula:

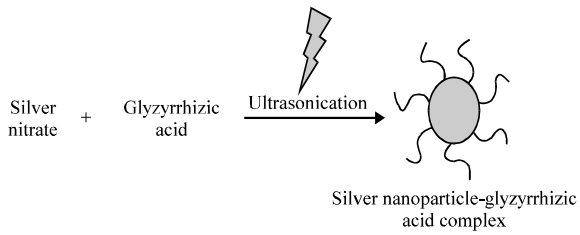
$$\text{Inhibition (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

where, $A_{control}$ is absorbance of control (DPPH alone) at 515 nm and A_{test} is absorbance of test (complex+DPPH) at 515 nm.

Exposure to gamma radiation: Irradiation was carried out using a ^{60}Co-Theratron Phoenix teletherapy unit (Atomic energy Ltd., Ottawa, Canada) at a dose rate of 1.88 Gy min^{-1}. For all experiments Swiss albino mice were divided into 8 groups and treated as follows:

- Distilled water+Sham irradiation
- Distilled water+1, 2, 4, 6 or 8 Gy ^{60}Co-γ-rays
- GLY+Sham irradiation
- GLY+1, 2, 4, 6 or 8 Gy ^{60}Co-γ-rays
- GLY-Ag+Sham irradiation
- GLY-Ag+1, 2, 4, 6 or 8 Gy ^{60}Co-γ-rays

Animals were administered with GLY or GLY-Ag (the animal received 50 mg GLY kg^{-1} b.wt.) or distilled water and one hour after administration, the animals were exposed to whole body gamma irradiation.



Scheme 2: Preparation of silver nanoparticle-glycyrrhizic acid complex

For studying the extent of DNA damage by means of alkaline single cell gel electrophoresis, a radiation dose of 2, 4 and 6 Gy was used because any significant DNA damage detectable by comet assay is induced from 2 Gy dose onwards. The dosage selected for micronucleus and chromosomal aberration studies were 1 and 2 Gy dose since at these low radiation dose only the DNA damage will get expressed as micronucleus or chromosomal aberrations. The 6 Gy dose has been selected as the dose for monitoring the efficiency of the complex to offer protection to the hemopoetic system since this can be efficiently studied at this dose. A lethal dose of 8 Gy gamma irradiation was used to study the potential of the complex to offer survival advantage to irradiated animals.

Protection of cellular DNA-in mouse tissues *in vivo*: Animals were administered with GLY or GLY-Ag (the animal received 50 mg GLY kg^{-1} b.wt.) or distilled water (3 animals per group) and one hour after administration the animals were exposed to whole body 2, 4 or 6 Gy gamma irradiation. Immediately after irradiation the animals were sacrificed and blood and bone marrow was collected to perform alkaline single cell gel electrophoresis or comet assay using method given by Singh, with minor modifications (Singh and Stephens, 1997; Chandrasekharan *et al*., 2009). After electrophoresis silver staining was carried out. The comets were visualized using Olympus BX-41 microscope and more than 50 comets images were captured (Plate 1) and analyzed using the software 'CASP' which gives percentage of DNA in tail, tail length, tail moment and olive tail moment directly. 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 the head and the centre of the tail and percentage of DNA in tail (Konca *et al*., 2003). Results are given as Mean±standard deviation.

Micronucleus assay: Animals were administered with GLY or GLY-Ag (the animal received 50 mg GLY/kg body weight) or distilled water (4 animals per group) and one hour after

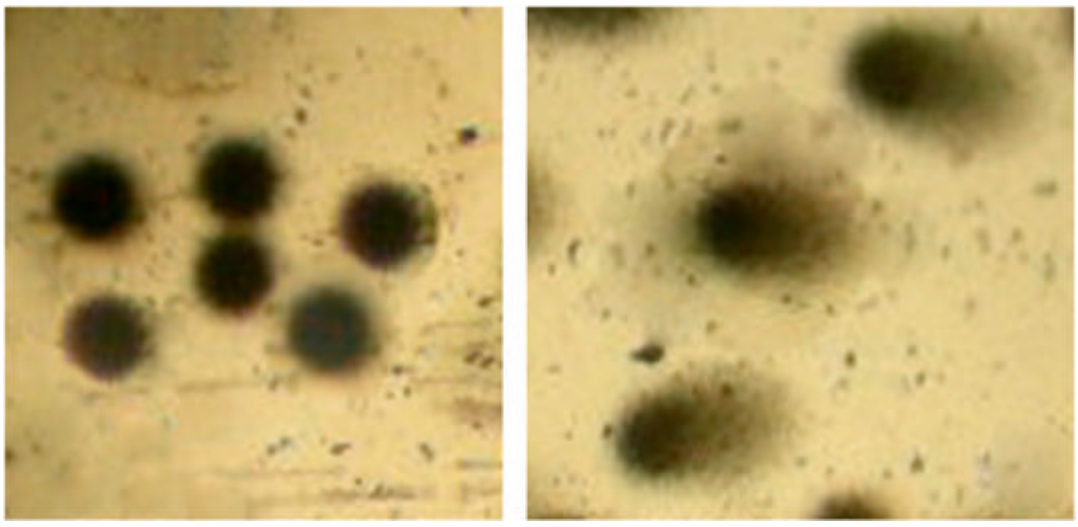


Plate 1: Unirradiated or irradiated cells following alkaline single cell gel electrophoresis. The unirradiated cells are disc shaped and have no cellular DNA damage while the irradiated cell form a comet shape indicative of cellular DNA damage

administration, the animals were exposed to whole body 1 or 2 Gy gamma irradiation. The micronucleus assay with mouse peripheral blood reticulocytes as reported by Hayashi *et al.* (1990) using Acridine Orange (AO)-coated slides was carried out to evaluate the chromosomal damage. 2000 reticulocytes (identified by their reticulum structure with red fluorescence) (Plate 2) were observed and percentage of micronucleated (round in shape with a strong yellow-green fluorescence) reticulocytes were scored.

Chromosome aberration assay: Animals were administered with GLY or GLY-Ag (the animal received 50 mg GLY kg^{-1} b.wt.) or distilled water (4 animals per group) and one hour after administration, the animals were exposed to whole body 1 or 2 Gy gamma irradiation. At 22 h after irradiation all the animals were injected i.p. with 2 mg kg^{-1} b.wt. colchicine and sacrificed 2 h later by cervical dislocation. Both femurs were dissected out and metaphase plates were prepared by air-drying method (Devi *et al.*, 1998). Chromosomal aberrations like breaks, rings and dicentrics (Plate 3) as well as cells showing polyploidy, pulvarisation and severe damage (SDC, cells with 10 or more aberrations of any type) were scored under light microscope. Data are presented as Mean±(SD).

Effect on hemopoetic system damage and recovery: Animals were administered with GLY or GLY-Ag (the animal received 50 mg GLY kg^{-1} b.wt.) or distilled water (8 animals per group) and one hour after administration, the animals were exposed to whole body 6 Gy gamma irradiation.

Twenty four hours following irradiation, 4 animals from each group were sacrificed by cervical dislocation. The bone marrow cells were collected from both femurs into phosphate buffered saline

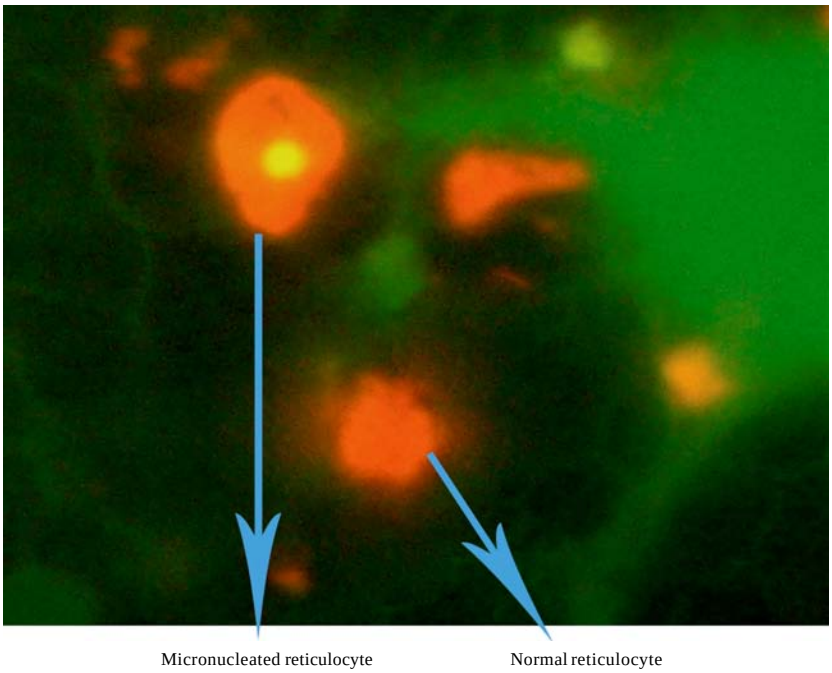


Plate 2: Micronucleated and normal reticulocyte observed after staining with acridine orange

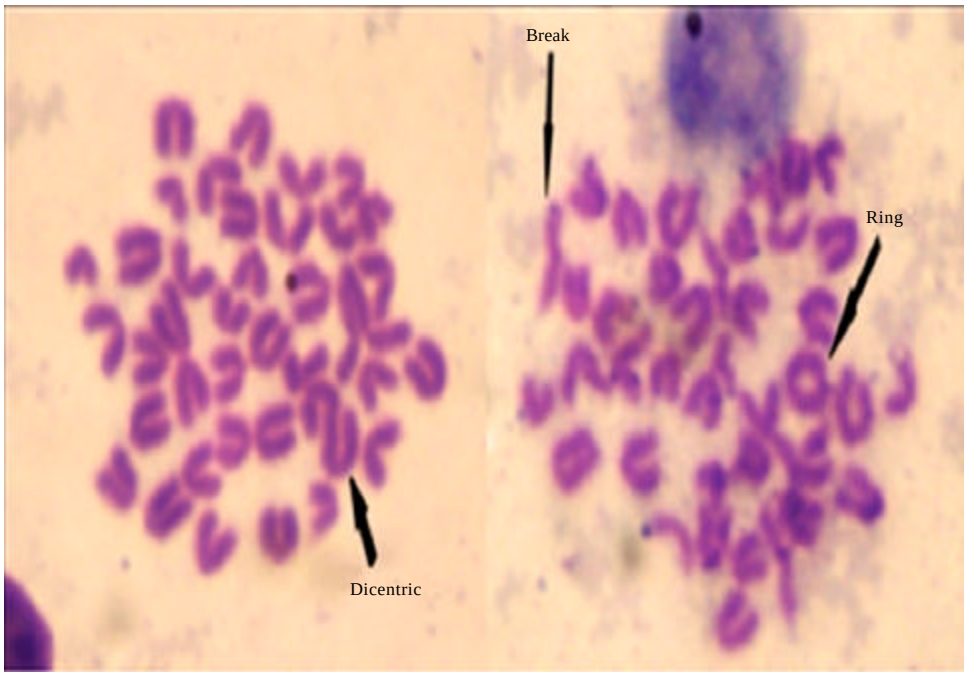


Plate 3: Chromosomal damages in mouse bone marrow cells

(pH 7.4) containing 2% fetal calf serum. The number of cells was determined with a hemocytometer and expressed as total cells ($\times 10^6$)/femur. Blood was collected from each of the animals by heart puncture into heparinized tubes and blood count was performed using an auto hematology analyzer (BC-2800 Vet, SHENZHEN MINDRAY).

The remaining animals (n = 4) were sacrificed on the 12th day postirradiation by cervical dislocation and the spleen was excised out and fixed in Bouin's solution containing 1.2% saturated picric acid, 30-40% formalin and glacial acetic acid in the ratio 15:5:1 and the spleens were analyzed for colony formations (Till and Culloch, 1961). Formation of endogenous spleen colonies is an index of hematopoietic stem cell proliferation.

Effect on 8 Gy gamma-radiation induced mortality: Animals were administered with GLY or GLY-Ag (the animal received 50 mg GLY kg^{-1} b.wt.) or distilled water (10 animals per group) and one hour after administration, the animals were exposed to whole body 6 Gy gamma irradiation. The animals in the groups were provided with standard diet and water ad libitum and their survival was monitored. The animals were checked on a daily basis to record the mortalities if any.

Statistical analysis: The results are presented as Mean±SD of the studied groups. Statistical analyses of the results were performed using ANOVA with Tukey-Kramer multiple comparisons test.

RESULTS

Sonochemical preparation of silver nanoparticle by glycyrrhizic acid: Sonication of silver nitrate and glycyrrhizic acid resulted in the formation of a golden yellow coloured solution which was termed as GLY-Ag. Glycyrrhizic acid reduced silver nitrate under ultrasonication and resulted in formation of silver nanoparticle-glycyrrhizic acid complex (GLY-Ag).

The Scanning Electron Micrograph (SEM) of silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) is shown in Fig. 1. The SEM analysis confirmed GLY-Ag as nanoparticles well below 100 nm. Figure 1a gives the SEM image of the nanoparticles at a magnification of 10000X and the encircled portion in this shows nanoparticles of less than 100 nm. Figure 1b gives the SEM image of the nanoparticles at a magnification of 3000X which shows almost uniformly distributed nanoparticles.

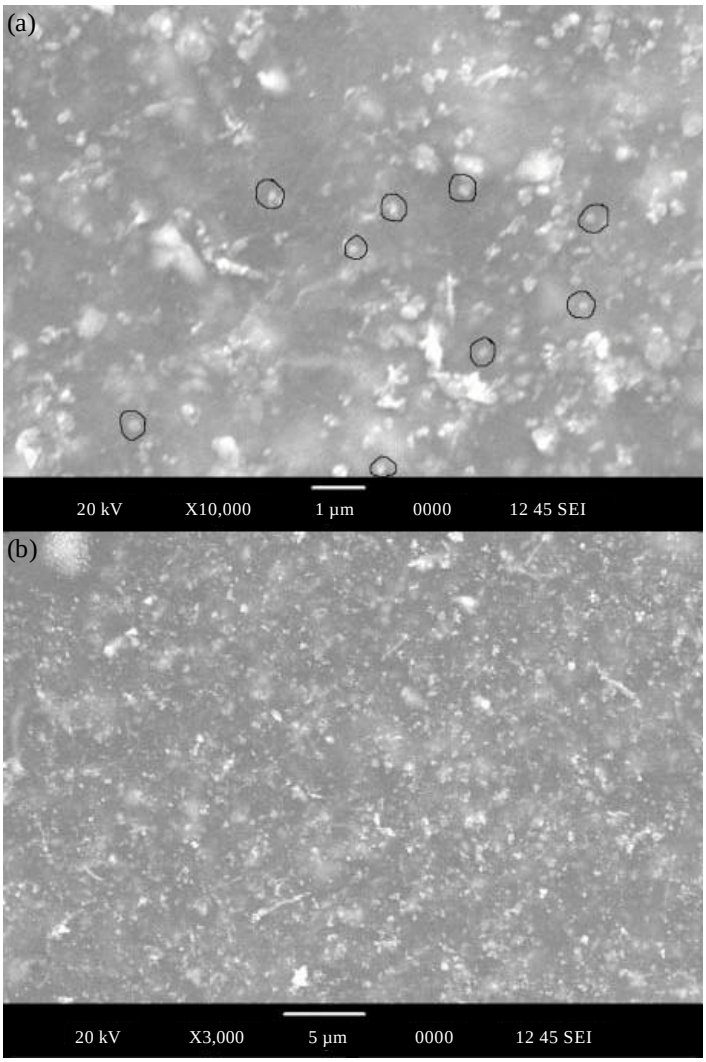


Fig. 1(a-b): Scanning Electron Micrograph (SEM) of silver nanoparticle-glycyrrhizic acid complex (GLY-Ag). The encircled portion shows nanoparticles (less than 100nm)

Free radical scavenging activity: The DPPH is a stable radical, having characteristic absorption at 515 nm. The DPPH radical reacts with compounds having antioxidant activity and gets reduced. The reduction of DPPH results in a decrease in A515. GLY at a concentration of 0.2 mM caused 10.91% and GLY-Ag at a concentration of 0.2 mM (GLY equivalent) caused 14.71% reduction of DPPH free radical (Table 1). The result indicated that GLY-Ag possess greater free radical scavenging activity than GLY.

Protection of cellular DNA-in mouse tissues *in vivo*: Figure 2 and 3 gives the results of comet assay on blood leukocytes and bone marrow cells of Swiss albino mice exposed to 2, 4 or 6 Gy whole body gamma radiation one hour after administration of GLY or GLY-Ag.

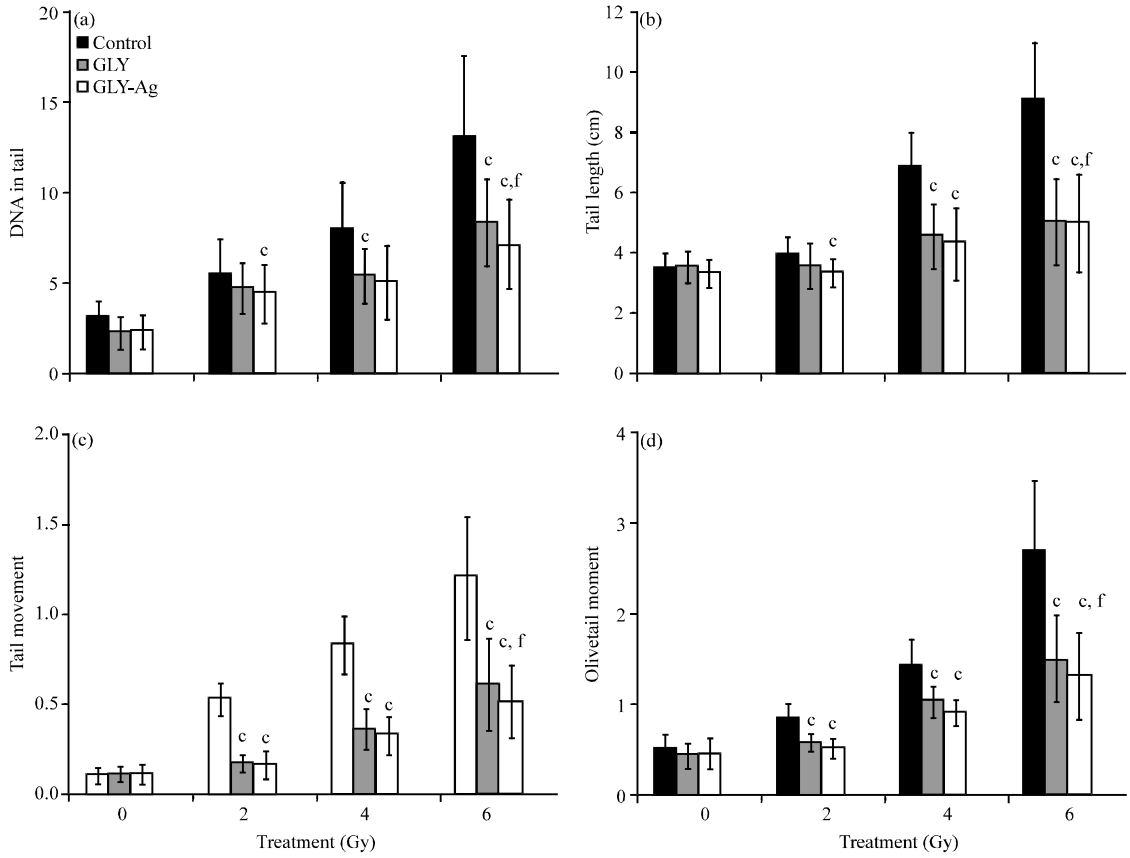


Fig. 2(a-d): Effect of oral administration of glycyrrhizic acid (GLY) or silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) on DNA damage in murine blood leukocytes induced by whole body exposure to gamma radiation (0-6 Gy) analyzed by comet assay. The animals were administered orally with the complex one hour prior to gamma irradiation exposure and were sacrificed immediately after radiation exposure to obtain blood (n = 3 each groups). The percentage DNA in tail, tail length, tail movement and olive tail moment are presented as Mean±SD (c: $p<0.001$ compared to respective radiation, d: $p<0.05$ compared to respective GLY, f: $p<0.001$ compared to respective GLY)

Table 1: DPPH free radical scavenging activity of GLY-Ag complex. GLY: Glycyrrhizic acid, GLY: Ag-Glycyrrhizic acid and silver nanoparticle complex

Concentration 0.2 mM (Equivalent to GLY)	Inhibition of DPPH free radicals (%)
GLY	10.73±0.38
GLY-Ag	14.71±0.16

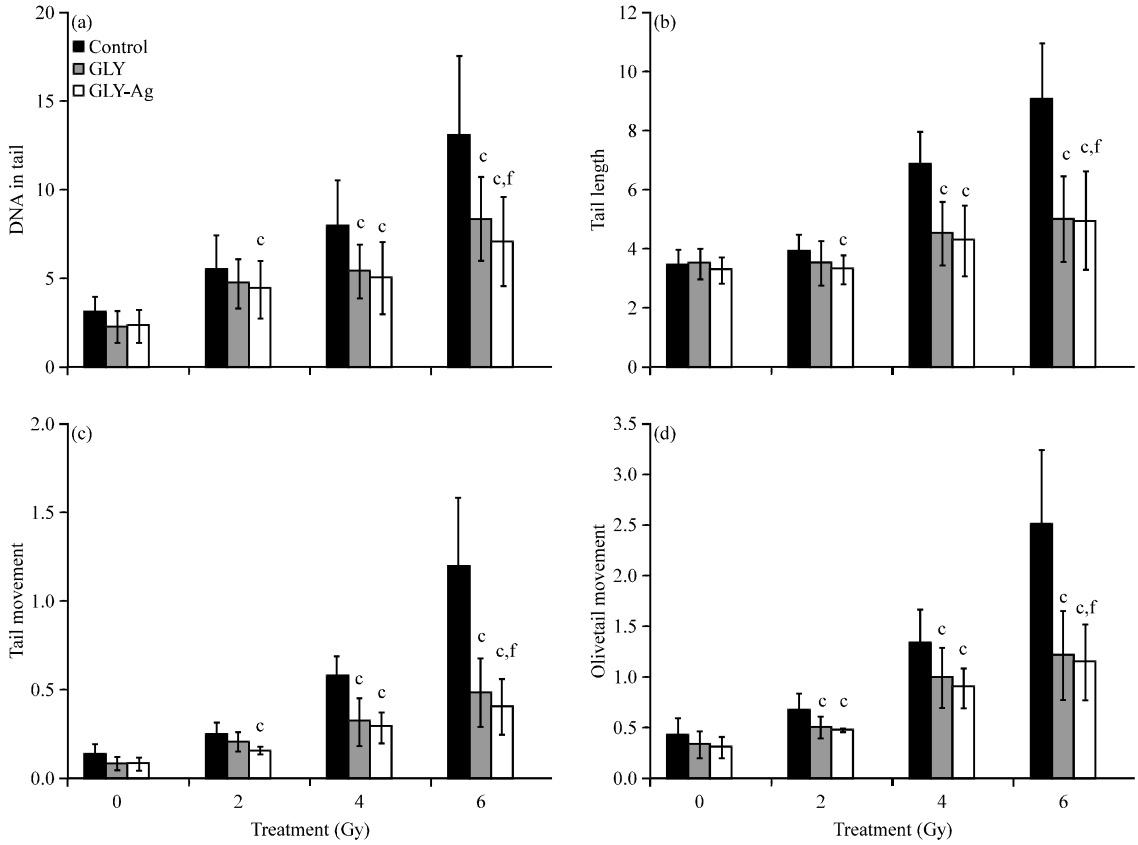


Fig. 3(a-d): Effect of oral administration of glycyrrhizic acid (GLY) or silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) on DNA damage in murine bone marrow cells induced by whole body exposure to gamma radiation (0-6 Gy) analyzed by comet assay. The animals were administered orally with the complex one hour prior to gamma irradiation exposure and were sacrificed immediately after radiation exposure to obtain bone marrow (n = 3 each groups). The percentage DNA in tail, tail length, tail moment and olive tail movement are presented as Mean±SD (c: $p<0.001$ compared to respective radiation, f: $p<0.001$ compared to respective GLY)

Whole-body exposure of animals to gamma radiation (2, 4 or 6 Gy) resulted in an increase in the comet parameters (% DNA in tail, tail length, tail moment and olive tail moment) of peripheral blood leukocytes and bone marrow cells of mice. The comet parameters indicate the extent of strand breaks in cellular DNA and an increase in these parameters are because of the radiation induced damage to cellular DNA. It can be seen from the figures that there was significant increase in the comet parameters and thus DNA damage in control irradiated animals in a radiation dose

dependant manner. The oral administration of glycyrrhizic acid (GLY) or silver nanoparticles-glycyrrhizic acid (GLY-Ag) to mice one hour prior to whole-body irradiation protected the cellular DNA of these tissues since there was a decrease in the comet parameters (Fig. 2, 3).

These results clearly indicated the ability of GLY or GLY-Ag to protect from the radiation-induced cellular DNA damages, under *in vivo* conditions.

Table 2 shows the extent of micronucleated reticulocytes in whole body gamma irradiated mice. Whole body gamma irradiation (1 or 2 Gy) resulted in significant increase in micronucleated reticulocytes. At 24 h post irradiation the percentage of micronucleated reticulocyte was 2.48±0.44 and 3.44±0.72 and at 48 h it increased to 5.0±0.4 and 6.48±1.72, respectively in 1 Gy and 2 Gy irradiated animals. Administration of glycyrrhizic acid (GLY) or silver nanoparticles-glycyrrhizic acid (GLY-Ag) prior to radiation exposure resulted in significant decrease in micronucleus induction (Table 2).

Bone marrow cells of unirradiated control animals showed only a few aberrant cells while radiation treatment (whole body 1 or 2 Gy radiation) resulted in a significant increase in the percent aberrant cells. A corresponding increase was found in all the individual aberrations. Treatment of mice with glycyrrhizic acid (GLY) or silver nanoparticles-glycyrrhizic acid (GLY-Ag) one hour prior to whole-body irradiation resulted in significant decrease in the percent aberrant cells and number of aberrations per cell compared to the control irradiated group. There was a decrease in all types of aberrations, as well as polyploidy and cells with pulverisation (Table 3, 4).

Table 2: Effect of oral administration of glycyrrhizic acid (GLY) or silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) on micronucleus induction in mouse peripheral blood reticulocytes by whole body irradiation (1-2 Gy). 2000 peripheral blood reticulocytes and percentage of micronucleated reticulocytes was scored

		0 Gy	1 Gy	2 Gy
Control (h)	24	0.16±0.35	2.48±0.44	3.44±0.72
	48	0.48±0.52	5.00±0.40	6.48±1.72
GLY (h)	24	0.24±0.21	1.20±0.28[b]	1.83±0.76[c]
	48	0.32±0.17	2.00±0.49[c]	3.76±0.77[c]
GLY-Ag (h)	24	0.24±0.35	1.12±0.33[c]	1.83±0.21[c]
	48	0.40±0.28	1.84±0.22[c]	3.52±0.91[c]

n = 4, b: p<0.01 compared to respective radiation, c: p<0.001 compared to respective radiation

Table 3: Effect of oral administration of glycyrrhizic acid (GLY) or silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) on the induction of individual chromosomal aberrations and number of chromosomal aberrations per cell in mouse bone marrow by whole body irradiation (1-2 Gy)

Treatment	B (%)	R (%)	D (%)	A/cell
Normal	5.00±4.242	0.50±0.70	1.50±2.12	0.07±0.07
1 Gy	18.35±3.350	3.90±1.38	7.34±2.65	0.29±0.07
GLY+1 Gy	10.31±2.300[c]	2.40±1.48[a]	2.93±1.22[c]	0.15±0.05[c]
GLY-Ag+1 Gy	10.12±0.900	2.13±0.64[a]	2.60±1.55[c]	0.14±0.03[c]
2 Gy	22.67±1.870	7.74±3.03	10.00±1.68	0.40±0.06
GLY+2 Gy	15.53±4.290[b]	3.26±0.57[c]	3.61±0.95[c]	0.22±0.05[c]
GLY-Ag+2 Gy	11.65±4.980[c]	5.35±1.26[a]	3.48±1.38[c]	0.20±0.07[c]

B: Break, R: Ring, D: Dicentric, A: Aberratious, n = 4, a: p<0.05 compared to respective radiation, b: p<0.01 compared to respective radiation, c: p<0.001 compared to respective radiation

Table 4: Effect of oral administration of glycyrrhizic acid (GLY) or silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) on the induction of chromosomal aberrations such as polyploidy, SDC and pulverization in mouse bone marrow cells by whole body irradiation (1-2 Gy)

Treatment	Pulvurization	Polyploidy	SDC
Normal	0.00±0.00	0.00±0.00	0.00±0.00
1 Gy	0.99±0.68	1.94±0.60	0.97±0.66
GLY+1 Gy	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
GLY-Ag+1 Gy	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
2 Gy	1.12±0.67	5.15±0.50	1.52±1.24
GLY+2 Gy	0.00±0.00[c]	1.08±0.49[c]	0.00±0.00[c]
GLY-Ag+2 Gy	0.00±0.00[c]	1.51±0.378[c]	0.44±0.63[a]

n = 4, a: p<0.05 compared to respective radiation, c: p<0.001 compared to respective radiation

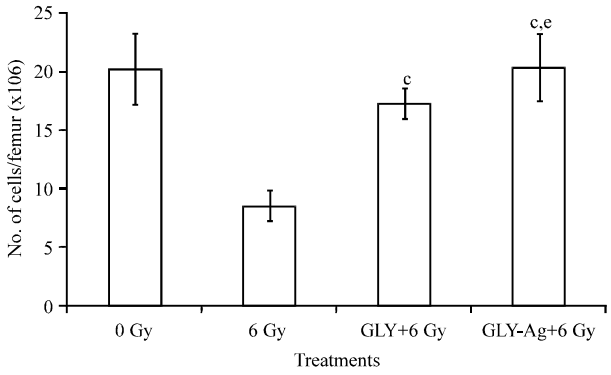


Fig. 4: Effect of oral administration of glycyrrhizic acid (GLY) or silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) on bone marrow cellularity in mice exposed to 6 Gy whole body gamma radiation (n = 4) c: p<0.001 compared to respective radiation, e: p<0.01 compared to respective GLY

Effect on hemopoetic system damage and recovery: The exposure of animals to 6 Gy whole body gamma radiation resulted in a significantly decreased bone marrow cellularity and total blood count. This decrease was found to be minimised in animals administered with glycyrrhizic acid (GLY) or silver nanoparticles-glycyrrhizic acid (GLY-Ag) complex one hour prior to radiation exposure (Fig. 4, 5).

Similarly, the irradiated animals administered with GLY or GLY-Ag complex one hour prior to gamma irradiation showed a significantly high number of colonies formed in the spleen (endogenous spleen colony formation) indicating a faster hematopoietic stem cell proliferation in these animals (Fig. 6).

Effect on 8 Gy gamma-radiation induced mortality: Figure 7 shows the survival of animals following exposure to a single lethal dose of 8 Gy gamma radiation. There was 100 % mortality in the control irradiated group by 9th day while in the group of animals orally administered with glycyrrhizic acid (GLY), there was 20% survival and in the silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) treated group there was 30% survival even beyond 30th day post-irradiation.

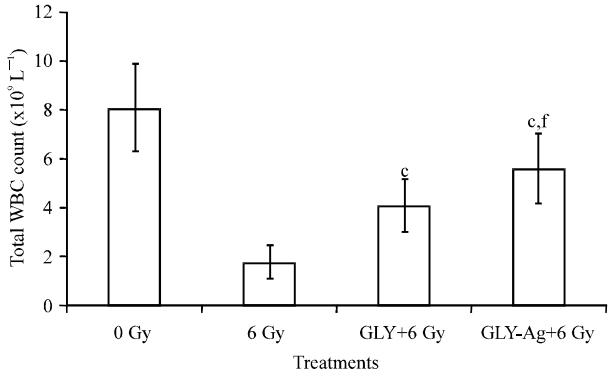


Fig. 5: Effect of oral administration of glycyrrhizic acid (GLY) or silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) on total WBC count in mice exposed to 6 Gy whole body gamma radiation (n = 4) c: $p<0.001$ compared to respective radiation, f: $p<0.001$ compared to respective GLY

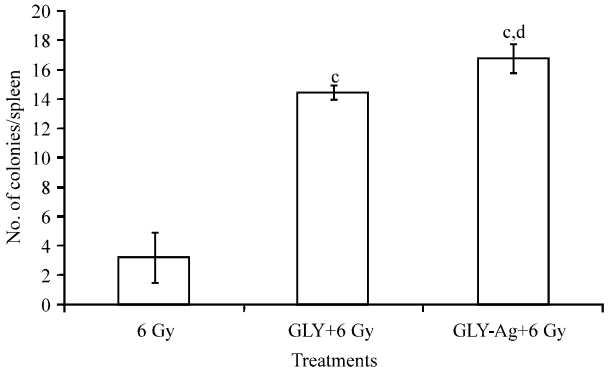


Fig. 6: Effect of oral administration of glycyrrhizic acid (GLY) or silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) on endogenous spleen colony formation in mice exposed to 6 Gy whole body gamma radiation (n = 4) c: $p<0.001$ compared to respective radiation, d: $p<0.05$ compared to respective GLY

DISCUSSION

Gamma radiation induces damages in biological systems either by direct hit or indirectly through generating free radicals such as $OH^{\cdot}$, $H^{\cdot}$, eAq-, $HO_2^{\cdot}$, H_3O+, etc. (Draganic and Draganic, 1971) and these radicals cause damage to vital cellular macromolecules, mainly DNA and membranes. These free radical-biomolecular interactions results in hematopoietic, gastrointestinal or central nervous system dysfunctions or syndromes in mammals depending upon the dose of radiation. Survival after irradiation actually results from the recovery of several target systems, such as the bone marrow, gastrointestinal tract, skin and hemostatic systems (Widel *et al*., 2003). The hematopoietic system is known to be one of the most radiosensitive systems and its damage may lead to the development of hematopoietic syndrome and result in death. Death from the so called hematopoietic syndrome results from impairment of bone marrow hematopoietic function that results in leucopenia, erythropenia and thrombocytopenia which ultimately predispose to infection, hemorrhage and death (Chen *et al*., 2006). An accelerated ability to regenerate new haemopoietic

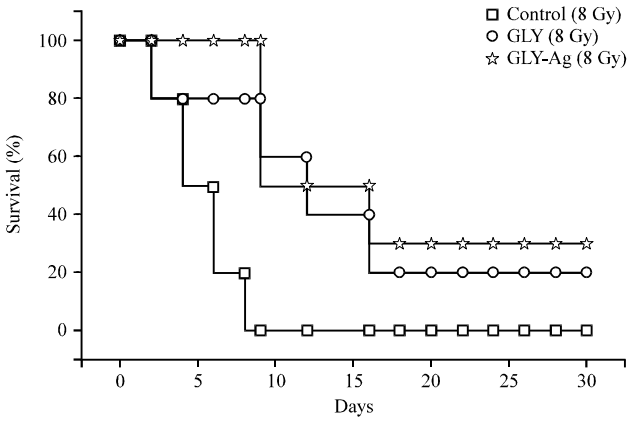


Fig. 7: Effect of oral administration of glycyrrhizic acid (GLY) or silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) on 8 Gy whole body gamma irradiation induced mortality in Swiss albino mice (n = 10). glycyrrhizic acid (GLY) or silver nanoparticle-glycyrrhizic acid complex (GLY-Ag) was orally administered one hour prior to whole body gamma irradiation

elements, especially those that are important in controlling microbial infections, such as granulocytes, allows the host to resist opportunistic infections better and, hence, enhances survival. Survival brought about by radioprotectors after potentially lethal irradiation is primarily due to their effect on hematopoietic cells (Floersheim *et al.*, 1988). Various mechanism such as prevention of damage through inhibition of free radical generation or their intensified scavenging, enhancement of DNA and membrane repair, replenishment of dead hematopoietic and other cells and stimulation of immune cell activity are considered important for radioprotection.

The chemicals that can scavenge free radicals generated during irradiation reduce the occurrence of various radiation damages. Thus agents that can prevent the formation of free radicals or destroy free radicals by reacting with them, thereby inhibiting their reaction with biomolecules, can function as radio-protectors. Since free radicals are short-lived, it is necessary for such radio-protective molecules to be present in the cellular milieu in sufficient concentration at the time (Coogle, 1983).

Nanoparticles such as cerium oxide nanoparticles, yttrium oxide nanoparticles, carbon nanoparticles, etc. were found to possess antioxidant properties and several investigations have reported the ability of these nanoparticles to offer protection against radiation induced damages (Schubert *et al.*, 2006; Tarnuzzer *et al.*, 2005; Das *et al.*, 2007; Rzigalinski *et al.*, 2003; Ali *et al.*, 2004; Daroczi *et al.*, 2006; Trajkovic *et al.*, 2005, 2007; Mirkov *et al.*, 2004). In the present study silver nanoparticle complex of glycyrrhizic acid (GLY-Ag) was prepared and examined for its potential to offer protection against radiation induced damages to hematopoetic system. GLY was shown to possess free radical scavenging activity which was further improved when complexed with silver nanoparticles.

Cellular DNA get damaged by a variety of lesions such as single strand breaks, double strand breaks, DNA-DNA and DNA-protein cross links, nucleotide damages, etc. on exposure to gamma radiation. Radiation induced loss of viability of cells has been attributed to unrepaired lesions in DNA. Thiol compounds such as amifostine (WR1065), phosphonol (WR255591), N-acetyl-L-cysteine (NAC), captopril and mesna have been shown to exhibit antioxidant properties and reduce radiation-induced histone H2AX phosphorylation. However, except amifostine and phosphonol, all

the other compounds offered no protection with respect to cell survival in terms of colony forming ability in human microvascular endothelial cells (HMEC) (Kataoka *et al*., 2007). Also, it has been reported that clonogenic cell survival (SF2) and measurements of immunofluorescence intensity by digital image analysis of phosphorylated histone H2AX as a surrogate marker of DNA double-strand breaks did not have any correlation (Mahrhofer *et al*., 2006). Thus, the phosphorylation of H2AX cannot be considered as a predictor for radioprotective activity of a compound or phosphorylated H2AX has limitations in representing the DNA strand breaks (Chandrasekharan *et al*., 2009). Alkaline Comet assay is a powerful and sensitive technique to monitor DNA strand breaks and alkali labile DNA lesions and is widely used to study genotoxicity, cellular DNA lesions such as single strand breaks or double strand breaks, apoptosis and DNA repair (Sandeep and Nair, 2010). The results on alkaline comet assay performed in murine system (bone marrow cells and blood leukocytes) indicated that GLY-Ag does not possess genotoxicity as it did not induce any DNA damage by itself and that it protected cellular DNA from radiation induced damages when administered to animals one hour prior to the radiation exposure. The results on micronucleus assay and chromosomal aberration analysis also showed the ability of the complex to offer protection against genotoxic damages of ionising radiation.

Unrepaired lesions in DNA results in loss of viability of cells. The whole-body gamma irradiation of mice is known to result in the depletion of hematopoietic organs owing to the intensive destruction of cells (Mendiola-Cruz and Morales-Ramirez, 1999). Exposure to gamma radiation resulted in a drastic decrease in the bone marrow cellularity and blood cell count and violated the ability of the cells to proliferate indicating hemopoetic system damage. Normal bone marrow contains cells capable of forming macroscopically visible colonies in the spleens of irradiated animals. Colonies developing in the spleens of mice irradiated with doses between 6 and 8 Gy have been used to study the behaviour of colony-forming cells in situ (Till and Culloch, 1963). The results on bone marrow cellularity, blood count and spleen colony formation assay showed that the administration of GLY or GLY-Ag helped to protect the cells of the hemopoetic system from radiation induced depletion and it helped to enhance the hemopoetic system recovery following radiation. The results of *in vivo* alkaline comet assay on different tissues of gamma irradiated mice clearly indicated the radioprotecting efficiency of GLY or GLY-Ag to offer protection to cellular DNA of the hematopoetic system (bone marrow cells and blood leukocytes). The radiation induced genotoxicity as assessed by the micronucleus assay and chromosomal aberration analysis was also found to be significantly lower in GLY or GLY-Ag treated animals. The administration of GLY or GLY-Ag also enhanced the survival of animals following a lethal gamma radiation exposure where GLY-Ag offered a higher level of protection than GLY.

It has been reported that carbon nanoparticles scavenge Reactive Oxygen Species (ROS), behaving as a "free radical sponge" (Injac *et al*., 2008) and cerium oxide nanoparticles possess anti-inflammatory, radioprotective and longevity enhancing capabilities (Rzigalinski *et al*., 2006). Our earlier reports showed the radiation protection property of silver nanoparticles, glycyrrhizic acid and silver nanoparticle-glycyrrhizic acid complex where silver nanoparticles were prepared using tri-sodium citrate as initial surfactant cum reducing agent and sodium formaldehyde sulfoxylate as secondary reducing agent (Chandrasekharan and Nair, 2010; Chandrasekharan *et al*., 2011; Khanna *et al*., 2007). The present study demonstrates radiation protecting potential of glycyrrhizic acid-silver nanoparticle complex obtained by the reduction of silver nitrate by glycyrrhizic acid under sonication. The radioprotecting ability of GLY or GLY-Ag may attribute to the free radical scavenging property as shown by the DPPH free radical scavenging activity.

CONCLUSIONS

Glycyrrhizic Acid (GLY), the major bioactive triterpene glycoside of licorice root (*Glycyrrhiza glabra*) reduced silver nitrate to silver nanoparticles forming glycyrrhizic acid-silver nanoparticle complex (GLY-Ag) of less than 100 nm in particle size. GLY-Ag was found to possess enhanced free radical scavenging activity than GLY. It offered significant protection against gamma radiation induced cellular DNA damage as shown by the results of comet assay performed in bone marrow cells and blood leukocytes of mice exposed to various doses of whole body gamma radiation (0-6 Gy) under *in vivo* conditions. GLY-Ag was also found to offer protection against the genotoxic effects of ionising radiation as shown by the results of micronucleus assay and chromosomal aberration analysis in whole body gamma irradiated mice (0-2 Gy). The complex offered significant protection to the hemopoeitic system from radiation injury (6 Gy) as shown by bone marrow cellularity, total blood count and endogenous spleen colony formation. The results of survival analysis following a lethal dose of gamma radiation, which is considered as the gold standard test for radioprotection, further confirmed the potential of GLY-Ag as a radioprotector. GLY-Ag possesses higher radiation protecting activity than that of GLY and this increased protecting ability of this complex may be due to the additive free radical scavenging property of both glyzyrrhizic acid and silver nanoparticles, in the complex.

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