Prevention of radiation-induced damages by aqueous extract of *Ganoderma lucidum* occurring in southern parts of India

Thulasi G. Pillai[1], Veena P. Salvi[2], Dharmendra Kumar Maurya[2], Cherupally Krishnan Krishnan Nair[2] and K. K. Janardhanan[1,*]

[1]Amala Cancer Research Centre, Thrissur 680 555, India
[2]Radiation Biology and Health Sciences Division, Bhabha Atomic Research Centre, Mumbai 400 085, India

Our previous studies have demonstrated that aqueous extract of *Ganoderma lucidum* occurring in South India possessed significant antioxidant activity. The present study was aimed at evaluating the radioprotective properties of the aqueous extract of this mushroom. Single-cell gel electrophoresis (comet assay), protection of radiation-induced plasmid pBR322 DNA strand breaks and inhibitions of lipid peroxidation (TBARS assay) were employed to determine the level of protection offered by the extract. The results indicate that aqueous extract of *G. lucidum* possessed significant radioprotective activity. The findings suggest the potential use of this mushroom extract for the prevention of radiation-induced cellular damages.

Keywords: Comet assay, *Ganoderma lucidum*, lipid peroxidation, medicinal mushroom, radioprotection.

RADIATION protection has significant importance in radiotherapy of cancer, nuclear accidents and even in nuclear warfare. Radiation-induced cell damage results from either damage to cell membrane or DNA[1,2]. Lesions in DNA can be induced either by direct ionization of DNA or indirectly through the reaction of aqueous free radicals leading to base damage, intra- or inter-strand cross-linking and single- or double-strand breaks[1,3]. Unrepaired or misrepaired DNA damage leads to genetic instability, mutations and chromosomal aberrations[1,3,4]. This may lead to the death of progeny after several mitotic cycles; this type of cell death called as 'mitotic or clonogenic death' or 'mitotic catastrope' is the most common in solid tumours exposed to radiation[3,5–7]. Protection of normal tissues against this cellular damage is important in radiotherapy. The major problem associated with cancer radiotherapy is the severe side effects and damage to normal tissues.

Ionizing radiation is one of the well established and widely used therapeutic modalities either for curative or palliative treatment of tumours in man. The cellular responses include arrest in cell-cycle progression at cell-cycle check points and induction of DNA repair. But the balance of survival and death signals determines the homoeostasis of normal cell systems. In radiotherapy of cancer, normal tissues need to be protected while cancers are exposed to high doses of radiation. A large number of compounds, natural and synthetic, have been evaluated for this purpose[8]. However, most of them failed clinically because of toxicity and side effects. Hence search for an ideal radioprotector is a compelling urgency.

Ganoderma, commonly known as reishi, is highly ranked in Oriental folklore. In Chinese medicine, reishi has been considered as a panacea for all types of diseases. Reishi has attracted significant attention in recent years due to its large number of pharmacological properties[9]. The fruiting bodies of this mushroom contain a variety of chemical substances. However, it has been reported that the physiological effects and distinguishing properties of *Ganoderma* are strain-dependent[10]. Recent investigations carried out in our laboratory have shown that *G. lucidum* occurring in the southern part of India possessed significant antioxidant, antitumor and anti-inflammatory activities[11]. We have examined the radioprotective effect of the aqueous extract of this medicinal mushroom and the findings are reported in this communication.

Tris base, high melting agarose, low melting point agarose, Na_2-EDTA, TritonX-100, sodium sarcosinate, DMSO and propidium iodide were obtained from Sigma Chemicals (St. Louis, Missouri). Plasmid pBR322 DNA was obtained from Bangalore Genei (Bangalore, India). All other chemicals used were of analytical grade and procured locally.

The extract of *G. lucidum* was obtained from mushrooms collected from the outskirts of Thrissur, Kerala, India. The type specimen was deposited in the herbarium of Centre for Advanced Studies in Botany, University of Madras, Chennai, India (HERB. MUBL.3175).

Male Swiss albino mice, 8–10 weeks old and weighing 20–25 g, were selected from an inbred group maintained under standard conditions of temperature (25 ± 2°C) and humidity. All the experiments were conducted strictly according to the guidelines prescribed by the Ethical Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), constituted by the Animal Welfare Board, Government of India.

^{60}Co-gamma rays in a Gamma Cell 220 (AECL, Canada) at a dose rate of 5.3 Gy/min and Junoir Theratron unit (AECL, Ottawa, Canada) with a dose rate of approximately 0.35 Gy/min at 38 cm were used for irradiation purposes depending on the type of dose given for the experiments.

Sporocarps of *G. lucidum* were dried at 40 to 50°C and several batches of 100 g powder were extracted with distilled water at 80°C for 8–10 h. The extracts were combined, filtered, concentrated and evaporated at low temperature using rotary vacuum evaporator. The residue thus obtained was used for the experiments. The yield was 8%.

The plasmid pBR322 DNA (250 ng in 10 µl in 0.01 M sodium phosphate buffer) was exposed to various doses

*For correspondence. (e-mail: kkjanardhanan@yahoo.com)

of γ-rays in the presence and absence of the drug. After irradiation, the DNA was electrophoresed in 1% agarose gel using 0.8 mM Tris borate/2 mM EDTA buffer, pH 8.3. The ethidium-bromide-stained DNA bands were photographed and analysed using the Bio. Rad Gel.Doc system.

Rat liver microsomal fractions were isolated using standardized protocol in our laboratory[12,13]. The protein concentration of the microsomal fraction was determined using Bradford's method[14].

The damage to microsomal membrane by gamma irradiation was assessed in terms of lipid peroxidation using the method of Beuge and Aust[15], with some modification[16]. Microsomal membranes were suspended in 250 µl of 10 mM potassium phosphate buffer, pH 7.4 to have a protein equivalent of 200 to 300 µg, and exposed to various doses of γ-radiation up to 350 Gy. For studying the protection of lipid peroxidation in microsomal membranes, the membrane samples were exposed to 350 Gy gamma radiation in the absence or presence of the extract. After radiation exposure, 750 µl TBA reagent (0.375% thiobarbituric acid (TBA), 0.25 M HCl, 15% trichloroacetic acid, and 6 mM EDTA) was added. The reaction mixture was incubated at 85°C for 20 min, cooled to ambient temperature and centrifuged at 12,000 *g* for 10 min at 25°C. TBARS (Thiobarbituric acid reacting substances) in the supernatant was estimated by measuring the absorbance at 535 nm using a Varian DMS 200 UV–visible spectrophotometer. The extent of lipid peroxidation is expressed as nmol of TBARS per mg of protein[12,13].

Alkaline single-cell gel electrophoresis was performed using the method by Singh[17], with minor modifications[16,18]. In order to estimate DNA damage in blood leukocytes, 10 µl heparinized whole blood was mixed with 200 µl of low-melting point agarose at 37°C and layered on frosted slides pre-coated with 200 µl high-melting point agarose. After solidification of agarose, the cover slips were removed and the slides were kept in pre-chilled lysing solution containing 2.5 M NaCl, 100 mM Na_2-EDTA: pH 10.0, 10 mM Tris HCI, 1% sodium sarcosinate with freshly added 1% Triton X-100 and 1% DMSO at 4°C for 1 h. The slides were removed from the lysis solution and placed on a horizontal electrophoresis tank filled with alkaline buffer (300 mM NaOH, 1 mM Na_2-EDTA, 0.2% DMSO, pH > 13.0). The slides were equilibrated in the same buffer for 20 min. Electrophoresis was carried out for 20 min at 25 V, 300 mA using a compact power supply. After electrophoresis, the slides were stained by layering on the top with 50 µl of propidium iodide (20 µg/ml) and visualized using a Carl Ziess Axioskop microscope with bright field, phase contrast and epi-fluorescence facility (HBO 50 high pressure mercury lamp), 40X camera adaptor lens. The integral frame grabber used in this system (Cvfb01p) was a PC-based card made in the Electronics Division, Bhabha Atomic Research Centre, Mumbai and it accepted colour composite video output of the camera.

Quantitation of DNA strand breaks of the stored images was done using the imaging software CASP by which the percentage DNA in tail, tail length, tail moment, and olive tail moment could be obtained directly[19]. The tail length of the comet indicated the extent of damage, because the smaller molecules move faster on the agarose gel. Thus, longer tails indicated that the strand breaks were more frequent and DNA was fragmented into several small molecules. The tail moment was a commonly accepted unit of DNA damage that normalizes the difference in the size of the nucleus studied (e.g. blood leukocytes)[16,18]. It is the product of the per cent DNA in the tail of the comet and tail length. For olive tail moment, distance of centre of gravity of DNA is considered instead of usual tail length.

The presence of aqueous extract of *G. lucidum* along with plasmid pBR322 DNA during irradiation protected DNA from radiation-induced lesions (Figure 1; Table 1). During exposure to ionizing radiation the plasmid DNA suffered strand breaks, which converted the supercoiled (ccc) form of plasmid DNA to open circular form (oc). The radiation-induced conversion of supercoiled form to open circular form was considerably reduced in the presence of the mushroom extract. Exposure of plasmid DNA to 50 Gy gamma irradiation resulted in complete damage of the (ccc) form. The aqueous extract of *G. lucidum* at a concentration of 50 µg/ml along with the DNA during irradiation, rendered protection to the plasmid DNA to an extent of 89.53%.

Exposure of mice liver microsome to gamma radiation resulted in peroxidative damage to membranous lipids. The peroxidation of membrane lipids in mice liver microsomal membrane estimated as nmol of malonaldehyde equivalent TBARS, increased with increasing doses of γ-rays up to 350 Gy. The effect of mushroom extract on radiation-induced lipid peroxidation in the microsomes is repre-

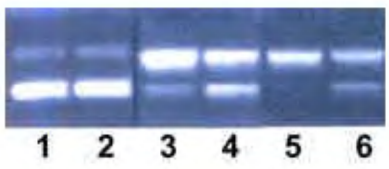

Figure 1. Protection of plasmid pBR 322 against gamma radiation-induced strand breaks by aqueous extract of *Ganoderma lucidum*.

Table 1. Protection of plasmid pBR 322 against gamma radiation-induced strand breaks by aqueous extract of *Ganoderma lucidum*. Lanes 1–6 are shown in Figure 1

Lane		Percentage SC from remaining
1	0 Gy, no extract	100
2	0 Gy, aqueous extract	100
3	25 Gy, no extract	74.758
4	25 Gy, aqueous extract	89.534
5	50 Gy, no extract	61.835
6	50 Gy, aqueous extract	72.035

sented in Figure 2. The aqueous extract of *G. lucidum* prevented 98% of lipid peroxidation.

Damage to cellular DNA was studied by alkaline comet assay (Figure 3). Exposure of leukocytes to γ-radiation resulted in an increase of comet attributes such as % DNA in the tail, tail length, tail moment and olive tail moment from 1.97 ± 0.21, 9.7 ± 0.34, 0.30 ± 0.04 and 0.73 ± 0.06 to 6.66 ± 0.37, 21.72 ± 0.62, 2.01 ± 0.16 and 2.72 ± 0.14 respectively. The aqueous extract of the mushroom reduced the increase in % DNA in tail (4.30 ± 0.56), tail length (16.83 ± 1.20), tail moment (1.03 ± 0.18) and olive tail moment (1.66 ± 0.21) respectively (Figure 4). This indicated that the aqueous extract of *G. lucidum* protected DNA against radiation-induced single-strand breaks.

The cellular membrane and DNA are the two main targets of radiation-induced lethal effects and mutagenicity. Formation of lipid peroxides in the tissues exposed to γ-radiation is one of markers of membrane damage. Exposure of human leucocytes to 4 Gy radiation causes severe damage as is reflected from the comet assay. The aqueous extract of the mushroom, *G. lucidum* causes decrease in the comet attributes. It also protects the membrane from damage and at the same time protects the plasmid DNA. Administration of *G. lucidum* extract has been reported to provide significant relief from the side effects of chemotherapy[20].

The present study indicates that the aqueous extract of *G. lucidum* has significant radioprotective activity. DNA constitutes the primary vital target for cellular inactivation of living systems by ionizing radiation. Ionizing radiation damage to cellular DNA are mainly strand breaks, elimination of bases and sugar damage. When the plasmid is exposed to γ-radiation, the ccc form of the molecule is converted to the oc form, with a difference in the mobility in the agarose gel because of the induction of strand breaks

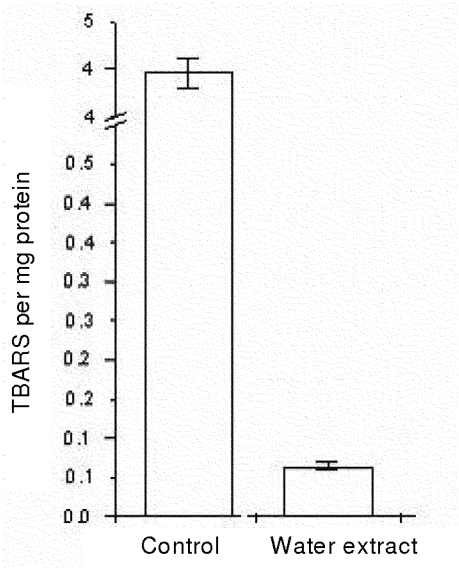

Figure 2. Protection of microsomal membrane against gamma radiation-induced lipid peroxidation at 350 Gy by aqueous extract of *G. lucidum*.

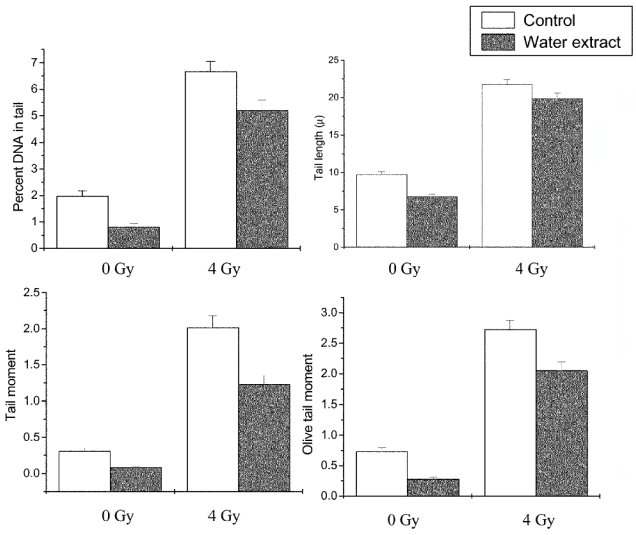

Figure 3. Protection of cellular DNA of human peripheral blood leukocytes exposed to gamma radiation in the presence of aqueous extract of *G. lucidum* as assayed by comet assay.

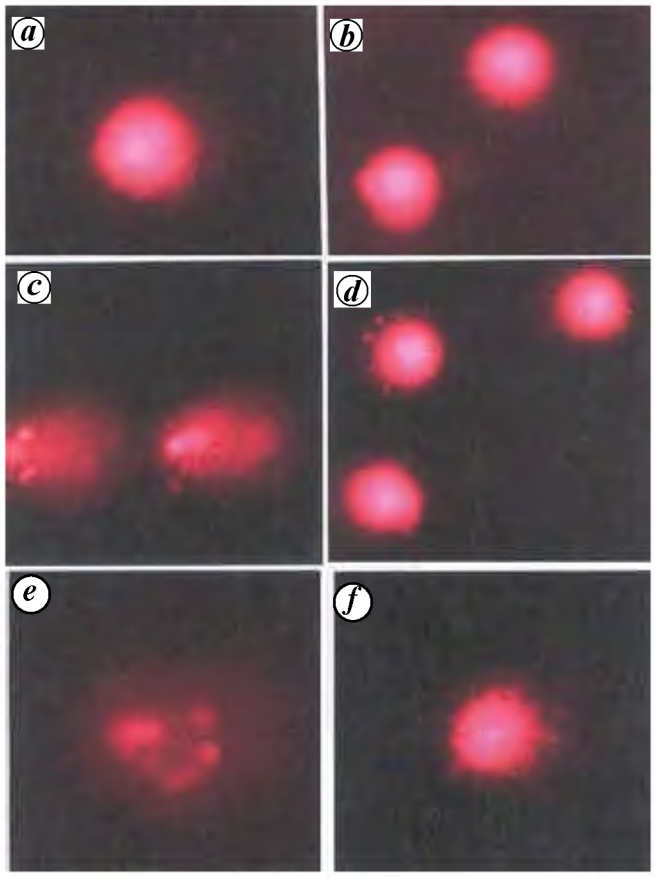

Figure 4. Protection of radiation-induced DNA damage of human lymphocytes by aqueous extract of *G. lucidum*: comet assay. ***a***, Control, 0 Gy; ***b***, 0 Gy + extract (50 μg/ml); ***c***, 2 Gy; ***d***, 2 Gy + extract (50 μg/ml); ***e***, 4 Gy; ***f***, 4 Gy + extract (50 μg/ml).

in the DNA. The damages by ionizing radiation to DNA can cause loss of viability of the cells exposed to radiation. The alkaline comet assay is an elegant and effective technique to monitor the extent of DNA damage and its protection. When human leukocytes are exposed to γ-radiation *ex vivo*, the cellular DNA undergoes damage, as reflected in the increase of the comet parameters (% DNA, tail length, tail moment and olive tail moment). The presence of aqueous extract of *G. lucidum* during irradiation of cells decreases the comet parameters, indicating its significant role in protection. One of the deleterious consequences of DNA damage from exposure to ionizing radiation is the induction of cancer. Protecting cellular DNA from radiation damage might result in the prevention of the cancers induced by radiation. Fungal polysaccharides of comparable structure and function as those found in *Ganoderma*, have undergone rigorous clinical trials. Based on such indirect experimental evidence, it is hypothesized that this medicinal mushroom polysaccharide might render significant relief from the side effects of both chemotherapy and radiotherapy[21].

The result of the present investigation reveals the potential of *G. lucidum* in radiation protection not only in radiotherapy, but also in accidental radiation exposure. The findings also suggest the possibility of using this medicinal mushroom extract as adjunct therapy in cancer radiotherapy and chemotherapy.

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Stable transformation of *Xylella fastidiosa* with small repW shuttle vector pUFR047

Vanamala Anjaiah[1,2,*] and Dean W. Gabriel[1]

[1]Plant Molecular and Cell Biology Program and Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA
[2]Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics, Patancheru 502 324, India

***Xylella fastidiosa* (*Xf*) is a fastidious, xylem-inhabiting, Gram-negative bacterium that causes serious plant diseases in a wide range of plant species. The most serious diseases are Pierce's Disease (PD) of grape and Citrus Variegated Chlorosis (CVC). Functional genomic analyses of *Xf* have been severely limited by lack of a stable replicative shuttle vector. Plamsid pUFR047, small, stable, wide host range, conjugative and repW shuttle vector have been successfully transferred into *Xf* strains by electroporation. The vector replicated in a stable manner for over thirty generations of growth in the absence of antibiotic selection in *Xf* strains.**

Keywords: Citrus variegated chlorosis, electroporation, Pierce's disease, shuttle vector.

XYLELLA FASTIDIOSA (*Xf*) is a xylem-inhabiting, Gram-negative bacterium that causes serious diseases in a wide range of plant species[1]. Two of the most serious of these

*For correspondence. (e-mail: a_vanamala@yahoo.com)