The protective effect of *Podophyllum hexandrum* on hepato-pulmonary toxicity in irradiated mice

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**Objective:** Present study reports the modulatory effect of a novel formulation (G-002M), prepared from three isolated molecules of *Podophyllum hexandrum* rhizomes on radiation induced oxidative stress in lung and liver tissues of strain A mice.

**Methods:** Mice, administered with G-002M formulation 1 h prior to lethal (9 Gy) radiation exposure dose, were sacrificed at different time intervals. Lung and liver tissues were processed to assess the expression of extracellular superoxide dismutase (EC-SOD) and immunohistochemistry and Western blotting techniques. Histological alterations along with the biochemical determinations, using as reported markers of hepatic and lung oxidative stress were recorded. Pre-administration of G-002M resulted in significant restoration of radiation induced decline in the expression of EC-SOD in both lung and liver tissues.

**Results:** In lungs, radiation mediated histological alterations like alveolar edema, interalveolar septa thickening, inflammation and infiltration of inflammatory cells into alveolar spaces were significantly prevented by G-002M pre-treatment. Histopathological features of liver in response to radiation such as steatosis, deformed cell structure, vacuolization of the cytoplasm and injury of hepatocyte membranes were also minimized by formulation pre-treatment. The formulation could successfully inhibit radiation induced malondialdehyde (MDA) content and counter the radiated induced depletion in SOD, catalase, glutathione reductase, glutathione S-transferase and glutathione, in serum, lung and liver tissues. Radiation induced alterations in serum aspartate aminotransferase, alanine transaminase, cholesterol, high-density lipoprotein, low-density lipoprotein, triglycerides, alkaline phosphatase, total protein, albumin and lactate dehydrogenase were maintained by G-002M pre-treatment.

**Conclusion:** These results demonstrated that the bioactive phyto-constituents of *Podophyllum hexandrum* could significantly protect lung and liver against radiation, predominantly by reduction in lipid peroxidation and elevation of EC-SOD along with a set of endogenous defense enzymes.

INTRODUCTION

Natural radiation mediated oxidative stress is carefully regulated by endogenous defence enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione S-transferase (GST), etc. The non enzymatic entities, i.e. glutathione (GSH), vitamin C, α-tocopherol etc, also play important role in minimizing this stress. Under adverse conditions, when the production of free radicals is in excess, there is a redox imbalance in the cell leading to oxidative stress which may lead to irreversible recovery.

Liver is the organ where antioxidant enzymes involved in the redox reactions in the cell leading to oxidative stress are synthesized and broken down. Being the main organ of metabolic reaction as well as detoxification, the reported radiosensitivity [1] and the slow regeneration capacity of liver cells makes the damages more threatened to life [2]; therefore, protection of liver from radiation induced damage is inevitable.

Presence of large concentrations of dissolved oxygen in lung enhances the probability of reactive oxygen species (ROS) generation [3]. This organ, being minimally regenerative, does not undergo fast recovery, hence can not tolerate high doses of radiation. Radiation mediated onset of complex reactions in lung is known to lead to pneumonitis, fibrosis and carcinogenesis. Extracellular (EC)-SOD, an antioxidant enzyme highly expressed in the lung, is mainly located in the extracellular matrix of tissue by heparin/matrix binding domain. The role of EC-SOD has been well established against radiation induced oxidative stress causing lung pathogenesis [4-6]. Oxidant/antioxidant imbalance mediated by extracellularly produced ROS which contributes to pathogenesis of acute lung injury can be significantly inhibited by EC-SOD. EC-SOD is also reported to inhibit inflammation [7, 8] and development of fibrosis [6]; however, knowledge related to lung radiobiology is still limited [9].

The search for development of radioprotectors started many decades ago, however, a success remained elusive till today. Only a thiol compound, namely...
WR-2721 (amifostine; S-2-(aminopropylamino) ethyl phosphorothioic acid) has been approved by the US Food and Drug Administration (FDA) as a radioprotector and chemoprotector. Basically, undesired toxicity of chemical radioprotectors and their analogues have barred them from clinical use and imposed the need to search the safe agents which could regulate the signaling cascades leading to major organs protection without causing any stress [10].

Use of natural resources in Ayurveda and Chinese medication system has prompted radiation biologists to study herbs against radiation. Various plants from different geographical locations have been evaluated for their radioprotective efficacy [11]. Among the high altitude plants screened for their protective potential against radiation, Podophyllum hexandrum was found to be a potent counter agent in crude [12-14] and semi-purified [15, 16] form. The extracts/formulations prepared from P. hexandrum rhizome have been found to deliver protection to the hematopoietic system [14, 16, 17], cellular macromolecules [18, 19] and the gastrointestinal system [20].

The present study is focused on evaluating the radioprotective potential of G-002M, a formulation prepared by combination of three active principles isolated from the rhizome of P. hexandrum. Expression of EC-SOD, histological alterations, changes in the oxidative stress marker enzymes and serum biochemical profile are the integral parts of the current analysis.

MATERIALS AND METHODS
Reagents and antibodies
Goat anti-rabbit horseradish peroxidase (HRP) was procured from Santa Cruz (Cat. No. Sc-2030; CA, USA). Polyclonal antibody of EC-SOD (Cat. No. S4946), goat anti rabbit fluorescein-5-isothiocyanate (FITC) (Cat. No. F4018), SOD standard (Cat. No. S9636), dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA), thiobarbituric acid (TBA), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide phosphate (NADPH), bovine serum albumin (BSA) and all other required chemicals were obtained from Sigma Aldrich (St Louis, MO, USA). Mayer’s Hematoxylin and Eosin stain was purchased from Fisher Scientific (Pittsburgh, PA, USA).

Animals and gamma-ray irradiation
Strain A female mice (26 ± 2 g), 8-10 weeks old, maintained at standard laboratory conditions, fed with standard food pellet (Amrut Laboratory Animal Feed; Pune, India) and water ad libitum, were used for study. They were housed in polypropylene cages bedded with sterilized rice husk under the controlled environmental conditions (24 ± 2°C; 12 h alternating dark and light cycle). The experiments were conducted strictly adhering to the guidelines of the institutional animal ethics committee. Mice were lethally exposed to 9 Gy dose in 60Co gamma chamber (Cobalt Teletherapy Bhabhatron-II) at the dose rate of 0.925-0.828 Gy/min. Radiation dose calibration was done by Fricke’s dosimetry method.

Plant material and preparation of G-002M
The formulation (G-002M) was prepared by combining of three active molecules isolated from dried rhizomes of Podophyllum hexandrum. The plant material was collected from high altitude regions of Leh and Ladakh (Jammu and Kashmir, India) and was authenticated by plant taxonomist from the center of Plant Taxonomy, University of Kashmir, Srinagar, India. The shade dried rhizomes were crushed to obtain fine powder which was processed further and extracted with petroleum ether. The three active principles isolated after elaborate processing, were analyzed on HPLC for their chemical identification and purity. The active principles were identified as podophyllotoxin, podophyllotoxin-β-D-glucoside and rutin. All the molecules were in their > 97% purity. G-002M (3.5 mg/kg by weight of animal) was prepared freshly at the time of administration by dissolving in DMSO (10% of final concentration) which was diluted further in distilled water.

Experimental design
The animals were divided into four groups with three animals in each group: control group (C), G-002M group (D), irradiated group (R) and G-002M plus irradiated group (DR). The experiments were performed in triplicate having 3 animals in each group (3 x 3 = 9). Control group mice were administered 200 µl normal saline while G-002M group was administered the formulation only, intramuscularly. Irradiated group animals were irradiated with a dose of 9 Gy. In G-002M plus irradiated group, mice were administered with G-002M intramuscularly one hour prior to 9 Gy radiation exposure. The animals were dissected at different time intervals (24 h, 72 h and 5th day) by cervical dislocation and blood was collected by heart puncture. Lung and liver were excised out immediately after dissection and processed for biochemical and other studies.

Immunohistochemical staining
Mice lung and liver tissue, fixed with 10% buffered formalin, dehydrated through graded series of alcohol, paraffinized and were cut in 3-5 µm sections. Sections were deparaffinized, rehydrated, rinsed in distilled water and washed in Tris buffer saline (TBS). Immunohistochemistry was then performed as
described by Midgley et al [21]. To inactivate endogenous peroxidases, sections were incubated with 6% H₂O₂. Antigen retrieval done by heating the sections immersed in citrate buffer (pH 6) in a domestic microwave oven at 600 Watt. Slides were then washed in TBS for 3 min and immunostained using EC-SOD in a dilution of 1:200. After incubation with fluorescence labelled (FITC tagged) secondary antibody for 2 h, the sections were counterstained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) at room temperature and visualized under fluorescence microscope (Olympus; Model: BX 63).

Western blot analysis
Proteins from frozen liver and lung tissue were isolated by homogenizing in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate , 1% Nonidet-P40, and a mixture of protease inhibitors). Proteins, quantified by Bradford method [22], were denatured, subjected to SDS-PAGE on 12% polyacrylamide gels and electrophoretically transferred to Whatman PROTRAN Nitrocellulose Transfer Membrane (Sigma) [4]. After blocking nonspecific sites with 5% (w/v) skimmed milk for 2 h, the membranes were incubated with anti-mouse EC-SOD antibody overnight at 4°C. These membranes, after washing three times, were incubated with HRP-conjugated goat anti-rabbit IgG for 2 h at room temperature. EC-SOD expression signal was detected using chemiluminescence detection system (Sigma). Densitometry was performed on the resulting autoradiograph using Image Lab software of BioRad Gel Documentation System (Gel Doc XR; Cat. No. 1708195).

Histological studies
For histological evaluation, mice were sacrificed on 5th day of experimentation. Tissues were fixed in 10% buffered formalin, dehydrated through graded series of ethanol, cleared in xylene and infiltrated with melted paraffin wax. Paraffin blocks were cut into 3-5 μm serial sections and stained with hematoxylin and eosin for microscopic examination.

Biochemical studies
The lung and liver tissues from differentially treated mice were isolated and homogenized in ice cold phosphate buffer saline to make 10% homogenate. The samples were centrifuged to obtain clear supernatants and were subjected to following biochemical analysis. Lipid peroxidation (LPx) was estimated by thiobarbituric acid reaction; malondialdehyde (MDA), the end product of lipidperoxidation, reacts with thiobarbituric acid to form pink coloured substances which were measured at 535 nm [23]. Superoxide dismutase activity was measured spectrophotometrically at 560 nm using NBT [24]. Catalase activity was assayed by the method of Sinha [25] using dichromate acetic acid reagent which reacts with the hydrogen peroxide to form blue precipitate of perchromic acid and decomposed to give green solution. Reduced GSH concentration was determined following the method described by Beutler [26]; the yellow colour obtained by reacting 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) with the GSH present in the sample was measured at 412 nm against the reagent blank. Glutathione reductase activity was determined by procedure of Carlberg and Mannervik [27]; addition of NADPH and oxidized glutathione (GSSG) resulted in changes in the enzyme activities which was measured at 340 nm. Glutathione S-transferase activity was estimated spectrophotometrically at 340 nm using 1-chloro 2,4-dinitrobenzene and GSH [28].

Protein determination
Protein contents were measured by Bradford method [22]. Standard curve was plotted by using different known concentrations of BSA as a standard.

Assessment of serum biochemistry profile
The mice were sacrificed on 5th day after treatment. Blood samples were collected in plain vials and serum was separated by centrifugation at 5000 rpm for 10 min at 4°C. The sera were stored at -20°C until analysis. The serum level of aspartate aminotransferase (AST), alanine transaminase (ALT), cholesterol (CHO), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), alkaline phosphatase (ALP), total protein (TP), lactate dehydrogenase (LDH) and albumin were measured in all the experimental mice by using a full-automatic Biochemistry Analyzer (Erba; Model No: EM-360).

Statistical analysis:
The results were expressed as mean ± SEM of three replicates. Statistical analysis was performed using analysis of variance (ANOVA) to test the significant difference between the groups. A value of P < 0.05 was considered as statistically significant.

RESULTS

Immunohistochemical studies
Figs.1A&B depict lung tissue sections from control mice stained with DAPI showing nucleus and FITC indicating prominent localisation of EC-SOD in alveolar parenchyma and matrix, respectively. In radiation exposed group FITC staining, indicating the presence of EC-SOD, was found diminished in comparison to controls, indicating loss in radiation mediated EC-SOD expression (Fig.1D). G-002M pre-treatment could significantly retain EC-SOD expression both in extracellular spaces and cell lining of bronchioles, expressed with more prominent staining (Fig.1F). Lungs of G-002M group mice showed localization of EC-SOD comparable to

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controls (Fig.1H). DAPI in this study was used to confirm the existence of cells in all the experimental groups (Figs.1ACEG).

Fig.2 indicates immunolabeling of EC-SOD in liver tissues. In control mice (Fig.2B), FITC staining in sinusoidal lining and vascular wall was quite visible indicating rich existence of EC-SOD in these areas. Radiation exposure in these animals has significantly descended the presence of EC-SOD which is indicated in the figure in the form of faintly stained sections (Fig.2D). In G-002M plus irradiated group, we could see enhanced intensity of the stain expressing increase in EC-SOD expression in comparison to radiation exposed group (Fig.2F). G-002M group animals showed intense staining of EC-SOD in liver sections which was comparable to controls (Fig.2H). Sections stained with DAPI (Figs.2ACEG) have confirmed the existence of nucleus in hepatocytes.

Figure 1.
EC-SOD immunostaining in the lung tissue sections.
(B) Controls showing bronchiole (*), alveoli with large air spaces and EC-SOD deep staining in extracellular portions.
(D) Irradiated group showing diffuse staining in bronchiolar (*) and alveolar cells.
(F) G-002M pre-treated irradiated mice showing bronchioles (*) and over-expression of EC-SOD in extracellular spaces.
(H) Enhanced expression of EC-SOD in G-002M alone group.

Nucleus (DAPI) staining in different groups:
(A) controls;
(C) irradiated;
(E) G-002M pre-treated irradiated;
(G) G-002M alone.
The lung sections were studied under fluorescence microscope (x200).
Figure 2.
Images showing EC-SOD immunostaining in liver tissue sections.
(B) Controls showing intense immunostaining of EC-SOD.
(D) Irradiated groups showing diffused staining in the sinusoidal lining and vascular wall.
(F) G-002M pretreated irradiated group showing restored EC-SOD expression.
(H) G-002M group showing prominent EC-SOD staining.
Nucleus (DAPI) staining in different groups:
(A) controls;
(C) irradiated;
(E) G-002M pre-treated irradiated;
(G) G-002M alone.
The liver sections were observed under fluorescence microscope (x400).

EC-SOD expression by Western blotting
Figs.3A&B demonstrate EC-SOD expression by Western blotting in lung and liver tissues, respectively, of mice sacrificed on 5th study day. In radiation exposed group we observed significant decrease in EC-SOD band intensity. The same was confirmed by densitometry and represented in the same figure in histogram form. G-002M pre-treatment could significantly enhance EC-SOD expression in lungs (Fig.3A) when compared with irradiated group. However, band intensity in this group was still less than control. In G-002M group, we found also insignificantly increased intensity of EC-SOD band compared to controls.

Also in liver study we found more or less similar results. Formulation pre-treatment could significantly enhance the expression of EC-SOD when compared to irradiated group (Fig.3B). In liver we could also see that G-002M treatment alone had increased EC-SOD existence in comparison to controls.

Histological studies
Figs.4A-D depict lung histology of controls, irradiated, G-002M pre-treated irradiated, and G-002M alone groups of mice, respectively. In control tissue sections we observed visible thin walled alveoli and numerous bronchioles lined with ciliated epithelium. In radiation exposed mice, the lung architecture was found severely affected.
Expression of EC-SOD in lung and liver tissues detected by Western blotting. Exposure of gamma radiation (9 Gy) resulted in significant loss of EC-SOD in both the tissues. [A] EC-SOD expression in lung of controls (C), G-002M administered (D), irradiated (R), and G-002M pretreated irradiated (DR) mice; [B] EC-SOD expression in liver of control, G-002M administered, irradiated, and G-002M pretreated irradiated mice.

Alveolar edema, hemorrhage and interalveolar septa thickening were commonly seen. Lung inflammation and infiltration of inflammatory cells into interstitium and residual alveolar spaces due to damaged endothelium was also seen (Fig.4B). In G-002M pre-treated mice, thickening of alveolar wall was mild (Fig.4C). Interstitial edema was also significantly reduced in this group. Infiltration of inflammatory cells was much less in comparison to radiation exposed mice. Damage to endothelial cells was also found significantly reduced. G-002M group mice showed normal alveoli, bronchioles and no pathological alterations in lung architecture (Fig.4D).

Histological examination of the control liver section had shown hexagonal hepatic lobule having hepatocytes, central vein, vascular channels, i.e., sinusoids along with its empty spaces and Kupffer cells, lining the sinusoids (Fig.5A). Portal tract consisted of portal vein, hepatic artery and bile ducts were also apparent in the control liver sections. Exposure of lethal dose of gamma radiation resulted in severe steatosis, deformed cell structure, vacuolization of the cytoplasm, injury of hepatocyte membranes, and cloudy swelling of sinusoidal and endothelial cells (Figs.5C&D). Portal vessels were also found to be expanded and bile ducts were highly damaged in radiation exposed mice. In G-002M pretreated group the effect of radiation was significantly less than irradiated animals. Steatosis which was severe in radiation exposed group was still visible in formulation treated group but significantly less. Cell morphology, vacuoles formation in cytoplasm, swelling of sinusoidal, endothelial cells and hepatocyte membrane injury was more or less corresponding to controls (Figs.5E&F). Bile ducts and portal vessels were also minimally damaged than irradiated group. Liver architecture was comparable to controls in G-002M group mice on 5th day of experimentat (Figs.5G&H).

**Biochemical studies**

**Lipid peroxidation:** Fig.6 indicates MDA formation in liver and lung of controls, G-002M, radiation exposed and G-002M pre-treated groups. In radiation exposed group MDA formation in liver was found approximately 2 folds increased at 24 h (P < 0.001; radiation vs controls) while in lungs increase in MDA at the same time interval was not significant. In G-002M group, MDA at the same time interval, was corresponding to controls. In G-002M pre-treated group, MDA concentration in liver at 24 h was observed to be significantly declined (P < 0.001; radiation vs G-002M pre-treated). At 72 h study, MDA was found still increased in comparison to controls in both liver and lung but the values got decreased in liver when compared to 24 h irradiated group. In G-002M pre-treatment samples, MDA concentration at 72 h was less in both the organs when compared with irradiated group. On 5th day study (Fig.6), there was a sharp decline in liver MDA concentration of radiation exposed group. However, in lungs the values further increased. In G-002M pre-treatment group, MDA in both liver and lung was corresponding to controls at this time point also.

**Superoxide dismutase:** Fig.7 depicts SOD level in mice serum, liver and lung of different experimental groups processed at different time intervals. At 24 h of study, SOD level was found significantly declined in both serum (P < 0.001) and liver (P < 0.01) of irradiated mice when compared with controls, however,
Figure 4. Histological examination of lungs in differentially treated mice on 5th day of experiment. (A) Control group showing normal architecture with alveoli (arrow) and no pathological changes; (B) irradiated mice showing edema (*), wall thickenings (arrow) and infiltration of inflammatory cells (arrow head); (C) G-002M pre-treated irradiated mice showing normal alveoli (arrow head), minimum interstitial edema, no inflammatory infiltration and bronchioles with large air spaces (arrows) as compared to irradiated group; (D) G-002M group mice showing normal architecture. The lung sections were studied under light microscope (x100).

Figure 5. Histological examination of liver in differentially treated mice on 5th day of experimentation. (A) Control showing normal hepatocytes, central vein and sinusoids; (B) inset image showing magnified view indicating the normal hepatocytes (arrows); (C) irradiated mice showing steatosis and structural anomalies; (D) magnified view showing steatosis (*), deformed cell structure, vacuolization of the cytoplasm, injury of hepatocyte nucleus and membranes (arrow head); (E) G-002M pre-treated irradiated mice showing mild steatosis nearly normal structure; (F) inset shows magnified view indicating mild steatosis (*) and normal hepatocyte nucleus (arrows); (G) G-002M group mice showing normal hepatocytes and no pathological changes; (H) inset image showing magnified view indicating normal hepatocytes by arrows. The sections were studied under light microscope (A, C, E and G x100; B, D, F and H x400).
the fall was not significant in lungs. The values of SOD at 72 h in the same group had fallen faster in comparison to 24 h in liver and blood. At this time point, decline in SOD concentration was noticed in lung tissue also. Formulation treatment had enhanced SOD level in all the three tissues under study, when compared with irradiated group (Fig.7). On 5th day fall in blood, liver and lung SOD in radiation exposed group was severe. G-002M pre-treatment though could enhance SOD concentration when compared with corresponding irradiated group, but fall was apparent in comparison to controls (Fig.7). G-002M administration alone did not alter SOD expression either in liver or lung and blood at any time point of study.

Catalase activity: Concentration of catalase in serum, liver and lung of differentially treated experimental mice is expressed in Fig.8. We observed continuous regression in catalase activity in serum, liver and lung of irradiated mice, measured at 24h, 72h and 5th day of study. G-002M pre-treatment, though significantly countered the fall in catalase activity at all the time points in all studied tissues (serum, liver and lung), still the values are not comparable to controls. G-002M treatment alone did not induce any variation in catalase activity studied at all the time points and in all studied tissues.

Reduced glutathione: Fig 9 expresses the level of GSH in differentially treated mice sacrificed at 24 h, 72 h and 5th day. In irradiated group, we found constant fall in GSH concentration in blood, liver and lung, studied at different time intervals. G-002M pre-treatment could significantly enhance GSH level when compared in serum, liver and lung of irradiated animals. However, the values of GSH were still less on 5th day of study when compared with controls. G-002M alone did not induce any effect on GSH level of treated mice at any time interval.

Glutathione reductase: Fig.10 depicts GR activity of serum, liver and lung tissues of differentially treated experimental mice. Radiation exposure significantly declined GR activity at all the time points of study. Fall was continuous up to 5th day. G-002M pre-treatment could enhance the activity of this enzyme in comparison to irradiated mice; however, the concentration still could not reach the control level even on 5th day of study. G-002M treatment alone did not alter GR activity at any time point of study in any of the studied tissues.

Glutathione S-transferase: GST activity in serum, liver and lung of experimental mice is shown in Fig.11. Radiation has significantly declined the activity of this enzyme at all the time intervals of study in all the three tissues. The regression of GST in this group was constantly increased upto 5th day of the study. G-002M treatment alone did not induce any alteration in GST activity in any of the tissue studied currently. G-002M pre-treatment could significantly counter the activity of this enzyme at all the time points in all the tissues; however, the values were not comparable to the controls at any time interval even on 5th day of study.

Serum biochemistry profile

Table 1 reflects the levels of CHO, HDL, LDL, TG, ALP, AST, ALT, LDH, TP and albumin in sera of differentially treated experimental mice. We observed that radiation exposure could significantly decrease the level of CHO, HDL, LDL, TG and ALP on 5th day of experimentation. The values of AST (148.80 ± 8.66 U/l and 112.07 ± 7.8 in irradiated and control groups, respectively; P < 0.01), ALT (86.65 ± 3.15 IU/l and 62.5 ± 5.01 in irradiated and control groups,

![Figure 6](image-url)

**Figure 6.** Effect of G-002M on radiation (9 Gy) induced lipid peroxidation in liver and lung tissues of mice. Mice were dissected at different time intervals. Experiments were performed in triplicate with 3 animals in each group. Tissue homogenates were prepared from excised lungs and liver. MDA values measured at 535 nm, are expressed as nanomoles of MDA formed/mg of protein. Error bars are SEM for n = 9. *R vs C of the respective tissue; **24 h DR vs R of the respective tissue; ***72 h DR vs R of the respective tissue; ****5th day DR vs R of the respective tissue. *P < 0.01, **P < 0.001, NS = not significant.
Figure 7. Level of SOD in differentially treated mice. Mice were dissected at different time intervals. Experiments were performed in triplicate with 3 animals in each group. Homogenates were prepared from excised lungs and liver tissues. SOD activity measured at 560 nm, is expressed as U/mg protein in tissues and U/µl in serum. Error bars are SEM for n = 9. *R vs C of the respective tissue; **24 h DR vs R of the respective tissue; †72 h DR vs R of the respective tissue; ‡5th day DR vs R of the respective tissue. *P < 0.01, **P < 0.001.

Figure 8. Catalase activity of differentially treated mice. Mice were dissected at different time intervals. Experiments were performed in triplicate with 3 animals in each group. Tissues homogenates were prepared from excised lungs and liver. The enzyme activity measured at 570 nm, is expressed as U/mg protein in tissues and U/ml x 10 in serum. Error bars are SEM for n = 9. *R vs C of the respective tissue; †24 h DR vs R of the respective tissue; ‡72 h DR vs R of the respective tissue; §5th day DR vs R of the respective tissue. *P < 0.01, **P < 0.001.

Figure 9. GSH level in differentially treated mice. Mice were dissected at different time intervals. Experiments were performed in triplicate with 3 animals in each group. Tissues homogenates were prepared from excised lungs and liver. Glutathione concentration measured at 412 nm, is expressed as µM GSH/mg protein in tissues and µM GSH/ml x 10⁵ in blood. The experiment was performed in triplicate with 3 animals in each group. Error bars are SEM for n = 9. *R vs C of the respective tissue; †24 h DR vs R of the respective tissue; ‡72 h DR vs R of the respective tissue; §5th day DR vs R of the respective tissue. *P < 0.01, **P < 0.001, NS = not significant.
respectively; \( P < 0.01 \) and LDH (462.67 ± 16.75 U/l and 301 ± 18.18 in irradiated and control groups, respectively; \( P < 0.001 \)) got increased after radiation exposure. TP and albumin were not found altered in any of the experimental group. With G-002M pre-treatment, the radiation induced increased level of LDH, ALT and AST reduced to normal level, while CHO, HDL, LDL, TG and ALP got normalized. However in our study we found that TG was still higher than normal on 5th day of study. G-002M treatment alone did not induce any alteration in any of the studied parameter.

**DISCUSSION**

Free radicals generated by ionizing radiation induce damage to the critical macromolecules of the cell. The ROS such as superoxide, peroxynitrite, hydroxyl radical, hydrogen peroxide, etc lead to pathogenesis of many organs including lung and liver [29-32]. Chances of lung and liver cancer are also known to be augmented by over exposure of ROS [33]. Various reports have conveyed that treating liver and lung with radiation exposure induced fibrosis, malignancies and pneumonitis [30, 34]. Though in routine process, radiation mediated damages to the tissues are taken care by natural defense mechanisms; however, in severe conditions exogenous support to minimize
radiation induced damage is inevitable [2]. *P. hexandrum* has been extensively reported by our group to render protection against radiation mediated damages to various vital organs of lethally irradiated mice [14, 16-18]. In the present study, mode of extending radioprotection by *P. hexandrum* (G-002M) to lung and liver tissues of lethally irradiated mice could be via increased expression of antioxidant defensive enzymes resulting into minimal tissue injuries, has been experimentally evidenced.

In lungs, EC-SOD, the key antioxidant enzyme, is well reported to prevent free radicals mediated tissue damage [4, 7, 8]. Since lungs have large quantity of connective tissues and vessels, the enzyme EC-SOD is highly expressed as compared to the other tissues. This enzyme contains a heparin or matrix binding domain, which enables it to bind to the matrix and to cell surfaces in tissue. This matrix-binding domain is sensitive to proteolysis [35]. In the current study, whole body exposure to lethal dose of gamma radiation to mice has resulted in significant loss of EC-SOD from extracellular matrix of the lung (Figs.1D&3A). This indicates that radiation has either triggered the release of some proteases in extracellular matrix which catalyze the proteolysis of heparin binding domain or over-utilized this enzyme during superoxide radical scavenging process. Protection against functional and tissue damage in the lungs has been demonstrated earlier also by administration of manganese (Mn)SOD and SOD mimetic [9]. In consonance, present study confirms protection delivered to the lung tissues of lethally irradiated mice by pre-administration of G-002M. Observations demonstrate that this protection could have been due to G-002M mediated enhanced expression of EC-SOD (Figs.1F&3A) along with the up-regulation of other endogenous antioxidant enzymes in blood and lung tissues (Figs.7,8,10,11). Current study also is in corroboration to the previous findings where EC-SOD has been shown to extend protection to the lung inflammation and fibrosis induced by oxidative stress [6, 7, 8, 35].

In irradiated animals the values of MDA at 24 h were comparable to controls which may be due to abundant availability of EC-SOD in the lungs. EC-SOD is amply reported for inhibition in generation of superoxide radicals which prevents the formation of secondary radicals required to initiate lipid peroxidation. However on 3rd and 5th day marked increase in MDA formation was noticed which may be due to ROS mediated damage to vascular endothelial cells resulting into release of free iron from red blood cells. Free iron is known to aggravate Fenton reaction leading to enhanced lipid peroxidation. The glucoside and rutin present in G-002M, known for their free radical scavenging potential [36], might have certainly lowered ROS production leading to minimize lipid peroxidation. G-002M pre-administration also protected radiation induced lung injuries like inflammation and edema which are known to be caused predominantly due to free radicals mediated vasculature damage leading to excessive release of cellular fluid into alveolar spaces.

Radiation has also been amply reported for causing significant loss of antioxidant enzymes in liver leading in acceleration of lipid peroxidation which may result into oxidative damage to the tissues [2, 13, 29]. Enhanced oxidative stress under severe conditions may lead to hepatic fibrosis by degeneration of hepatic cells and their replacement by fibrotic tissues. This has been confirmed in our current investigation through biochemical and histological analysis. Whole body exposure to lethal radiation in our experimental mice

Table 1. Levels of serum cholesterol (CHO), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), total protein (TP), albumin, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) of various experimental groups of strain A mice

<table>
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<tr>
<th></th>
<th>Control group</th>
<th>G-002M group</th>
<th>Irradiated group</th>
<th>G-002M+irradiated group</th>
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<tr>
<td>CHO (mg/dl)</td>
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<td>TP (g/dl)</td>
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<tr>
<td>Albumin</td>
<td>3.59 ± 0.19</td>
<td>3.25 ± 0.12</td>
<td>3.09 ± 0.11</td>
<td>3.18 ± 0.09</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>112.07 ± 7.8</td>
<td>125.36 ± 9.64</td>
<td>148.8 ± 8.66</td>
<td>111.66 ± 2.17</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>62.5 ± 5.01</td>
<td>60.25 ± 4.56</td>
<td>86.65 ± 3.15</td>
<td>68.66 ± 4.61</td>
</tr>
<tr>
<td>ALP (IU/l)</td>
<td>112 ± 14.48</td>
<td>102.36 ± 12.25</td>
<td>41.66 ± 6.94</td>
<td>69.33 ± 5.43</td>
</tr>
<tr>
<td>LDH (IU/l)</td>
<td>301 ± 18.18</td>
<td>298.45 ± 12.3</td>
<td>462.67 ± 16.75</td>
<td>325 ± 7.07</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of serum collected individually from the groups of experimental animals. Experiments were performed in triplicates having 3 animals in each group. *Irradiated group vs controls, **G-002M + irradiated group vs irradiated group. *P < 0.01, **P < 0.001
has also shown loss of EC-SOD along with other antioxidant enzymes in the liver (Figs.2D,3B,7,8,10,11) which has aggravated lipid peroxidation leading to damage of the hepatocyte membranes and changes into nuclear morphology (Figs.5C&D). G-002M pre-treatment could successfully up-regulate/maintain the release of these antioxidants and also declined MDA formation resulting into minimal damage to the liver tissues as revealed by our histological analysis (Figs.5E&F).

Marginal decline in GSH level in G-002M pre-treated irradiated mice (Fig.9) had confirmed less consumption of this endogenous antioxidant which might be due to reduction in radiation induced ROS generation. Reduction in ROS formation could be attributed to proton donating and free radical stabilizing property of the flavonoid and glucoside present in our formulation. The similar kind of facts has been elaborately mentioned in our earlier study [36]. Significant availability of GSH and SOD, the key determinant of tissue integrity, in G-002M pretreated mice was able to inhibit extracellular and intracellular ROS generation resulting into decreased tissue injuries. In our study, antioxidant enzymes’ decline rate in G-002M pre-treated group, was significantly less than irradiated group; however, the values were lower than controls even on 5th day of the study. Delay in recovery may be predominantly because of lethal radiation induced severe damage to the major organ systems and slow cell turnover rate in the liver resulting into its compromised functionality. Reformation in tissue pathology and EC-SOD level, analyzed in the liver during current investigation, are also in consonance with antioxidant enzymes’ status. Studies on antioxidant status with lethal dose of radiation are very limited, therefore we found it difficult to corroborate.

Variations in the level of hepatic enzymes like AST, ALT and LDH are also used as diagnostic indicators of hepatic injury [37]. In the current investigation, levels of all the three enzymes in sera of radiation exposed animals were found elevated which could be due to radiation induced protein and lipid modification of the hepatocytes resulted in increased permeability and cellular leakage of these enzymes. As reported earlier and in our current study also, significant decrease in the level of serum CHO, HDL, LDL and TG after radiation exposure, could be due to altered liver lipid metabolism and oxidative damage of lipids in membrane of hepatocytes. These changes are confirmed in our histological findings where steatosis has been visualized in the liver of irradiated mice (Fig.5C). The protective action of G-002M on liver structural damage might be due to scavenging of oxidation-initiating radicals and minimizing modification in the protein and lipid backbone of cellular membrane. Significant protection to radiation induced hepatotoxicity and alterations in liver architecture like cellular morphology, vacuolization of cytoplasm and hepatocytes’ membrane damage (Figs.5E&F) by G-002M pre-treatment is due to reduced oxidative stress. Hepatoprotective role of P. hexandrum by elevating a set of endogenous antioxidant enzymes has been reported earlier also [13, 16, 38].

In the current study we have demonstrated that the P. hexandrum formulation (G-002M) has radioprotective potential and is able to provide significant protection against radiation mediated cytotoxicity in lung and liver tissues by significantly maintaining the level of EC-SOD and other antioxidant enzymes and minimizing lipid peroxidation. Podophyllotoxin, present in the formulation, temporarily induces cell cycle arrest (G2/M) by inhibiting tubulin polymerization, therefore, leading to minimal DNA damage and providing sufficient time for repair. The two other constituents such as podophyllotoxin-β-D-glucoside and rutin have also been shown in our other studies for significantly stabilizing free radicals by donating hydrogen atoms from their attached hydroxyl groups [36]. In addition, the immunomodulatory action of glucoside supports in fast recovery of the immune system by enhancing the level of anti-inflammatory cytokines and granulocytes growth stimulating factors. The three active principles of G-002M, having different properties, present in the formulation might have acted in synergism to extend radioprotection against lethal doses of radiation in mice. Further studies, besides antioxidant, anti lipid peroxidation, DNA damage repair property and antioxidant proteins status in G-002M treated samples should be done to explore the other possible role of these phyto-constituents of Podophyllum hexandrum by which it renders radioprotection, are underway.

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COMPETING INTERESTS
The authors declare that they have no conflict of interests.
REFERENCES


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