Cellular and Molecular Biology [TM] **56**, OL1341-OL1349
DOI 10.1170/153

ISSN 1165-158X
2010 Cell. Mol. Biol.[TM]

INHIBITION IN RADIATION MEDIATED CELLULAR TOXICITY BY MINIMIZING FREE RADICAL FLUX: ONE OF THE POSSIBLE MECHANISMS OF BIOLOGICAL PROTECTION AGAINST LETHAL IONIZING RADIATION BY A SUB-FRACTION OF PODOPHYLLUM *HEXANDRUM*

M. L. GUPTA[1], V. GUPTA[1], S.K. SHUKLA[1], S. VERMA[1], S. SANKHWAR[1], A. DUTTA[1] AND K.A. SURI[2]

1 Institute of Nuclear Medicine and Allied Sciences, SK Mazumdar Marg, Timarpur, Delhi, India
Fax: +91-11-23919509; Email: drmanjugupta2003@yahoo.com
2 Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu-Tawi, India

Received September 1th, 2009; Accepted June 15th, 2010; Published September 11th, 2010

Abstract – The study has focused on exploring the mechanism of action of *Podophyllum hexandrum* sub-fraction (G-001M) exhibiting >90% protection in lethally irradiated mice. Currently, G-001M was assessed for antioxidant characteristics by evaluating DPPH, superoxide and hydrogen peroxide radical formation, anti-lipid per oxidation, metal chelation and total flavonoid content. To affirm cytoprotective efficacy of G-001M, plasmid DNA protection, blood WBC counts, marker for lipid peroxidation (MDA) and antioxidant status (GSH) in mice splenocytes and thymocytes were studied. G-001M, having high amount of total phenolic contents (200±10mg, w/w), exhibited dose dependent inhibition in DPPH and superoxide radical formation. Hydrogen peroxide radical scavenging was higher than standards. With pre-treatment of G-001M, plasmid DNA was also maximally restored to supercoiled form. Radiation modulated MDA and GSH values in splenocytes and thymocytes of mice altered significantly after 24 hrs and at later intervals, values were close to the controls. Radiation mediated losses in WBC counts were significantly regained ($p<0.001$) in G-001M pre-treated irradiated mice. The above findings explicitly conveyed that G-001M has successfully minimized radiation inflicted free radicals generation and their multiplication. This activity of G-001M could be undoubtedly among one of the major modes of action in extending whole body survival in lethally irradiated mice.

Key words: Radioprotection, Free radical scavenging, Polyphenols, Antioxidants, Lipid peroxidation, *Podophyllum hexandrum.*

INTRODUCTION

Multi-faceted use of nuclear energy has amplified the probabilities of accidental radiation exposures. Scientists, immediately after Hiroshima and Nagasaki disaster initiated their endeavour to save mankind against such unforeseen nuclear eventualities. Ionizing radiation damages biological system predominantly by generating free radicals which immediately after origin, in search of getting themselves stabilized, react with major cellular molecules and multiplies further forming a never ending chain of free radicals species. The influx of these radicals somehow keeps multiplying unconditionally till a potential antioxidant is administered exogenously to curb their growth (13). To meet the need, plethora of pure compounds were used in their original as well as in structurally modified forms. However, closeness in their effective and toxic doses kept them at distance from meaningful use.

Herbs, because of their long history of safe clinical use and multitasking in action have attracted the attention of radiobiologists to give a purpose full thought on screening of some plants rich in antioxidants besides being potential disinfectant, rejuvenators for immune system, savours for DNA, lipid and proteins along with having rich quantity of vitamins, amino acids, carbohydrates, minerals etc.. Out of various

Abbreviations: BHT: Butylated hydroxyl toluene; **DMSO:** Dimethyl sulphoxide; **DPPH:** 1, 1-diphenyl-2-picrylhydrazyl; **DTNB:** 5-5'-dithiobis-(2-nitrobenzoic acid; **EDTA:** Ethylene diamine tetra acetic acid; **$FeCl_3$:** Ferric chloride; **GSH:** Reduced glutathione; **MDA:** Malondialdehyde; **NBT:** Nitroblue tetrazolium chloride; **PBS:** Phosphate buffer saline; **TBA:** Thiobarbituric acid; **TCA:** Trichloroacetic acid.

plants screened in our group in last one decade, *Podophyllum hexandrum*, a high altitude plant, was found highly effective against lethal whole body irradiation in mice. Reports on medicinal usage of this herb such as cure for various bacterial and viral infections, metabolic disorders, leukaemia, lymphomas, venereal warts, rheumatism etc (3, 27) have amply supported its multidirectional use. Articles on whole extract (9) and various semi purified preparations (11, 12, 15) of *P. hexandrum* have conveyed that rhizomes of this plant acquire almost all the potentials of a good radio protector. Chemoprofiling of these preparations have shown that *P. hexandrum* is rich in flavonoids and lignans and both of these chemical groups individually are elaboratively reported for having excellent antioxidant properties (7, 12, 19, 30).

The current study has been taken up to understand the probable mode of action of G-001M in exhibiting protection against lethal irradiation. This sub fraction has been recently reported by our group for showing >90% protection against lethally irradiation with large therapeutic time window (30min to 4h) and protection to cellular DNA besides supporting for fast replenishment of immune system in irradiated mice (24). The HPLC analysis of G-001M has confirmed the presence of lignans such as Podophyllotoxin, Podophyllotoxin glucoside and Demethyl podophyllotoxin and flavonoids viz. Quercetin, Quercitrin, Rutin and Kaempferol.

To explore the possible mode of action of G-001M sub fraction, we have thoroughly assessed its antioxidant and free radical scavenging properties using various *in vitro* and *in vivo* model systems. Cytoprotective efficacy against lethal radiation mediated toxicity has also been affirmed during current study by assaying plasmid DNA protection, splenocytes and thymocytes GSH and MDA measurements and peripheral blood cell count.

MATERIALS AND METHODS

Chemicals

pUC18 DNA, Butylated hydroxyl toluene (BHT), ascorbic acid, α-tocopherol, quercetin, gallic acid, tricarboxylic acid, thiobarbituric acid, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), potassium chloride, dimethylsulphoxide (DMSO), nitro blue tetrazolium chloride (NBT), xylene cynol and ferric chloride were purchased from sigma (St. Louis, USA).

Plant material and extract preparation

The rhizomes of *P. hexandrum* Royale were collected from the high-altitude regions (>3000 m) of India. G-001M was prepared and chemically characterized as per the details described in earlier published report (24).

In vitro analysis of G-001M

Free radical scavenging activity

The free radical scavenging activity of G-001M was based on its ability to stabilize DPPH radicals as described by Molyneux (18). Briefly, 1 ml of varying concentration (0.5–500 µg/ml) of G-001M/standard antioxidants (BHT and Quercetin) was added to each 1ml of 0.1mM DPPH in methanol. The mixtures were left in the dark for 30 minutes, and the resultant purple colour was measured at 517 nm against the control.

Lipid per-oxidation assay

Swiss Albino Strain 'A' male mice weighing 30 $\pm$ 2gm were maintained under standard laboratory conditions (12h light/dark cycle; 25 $\pm$ 1 ºC). Experiments strictly adhered to the guidelines of Institute Animal Ethical Committee (IAEC). After decapitation, carefully removed brain tissue was homogenized with cold 0.15M KCl to make 10% homogenate using Teflon homogenizer. *In vitro* lipid per-oxidation was determined by the method of Beuge and Aust, 1978. Briefly, 0.5 ml of brain homogenate was added to various concentrations of the G-001M and incubated for 30min. Per-oxidation was terminated by the addition of 2ml TBA-TCA-HCl reagent [15% w/v trichloroacetic acid (TCA) and 0.375% w/v thiobarbituric acid (TBA)]. The solution was heated for 15min to complete the reaction. The pink colour obtained was measured spectrophotometrically at 535nm.

Superoxide scavenging activity

The superoxide scavenging activity of G-001M was determined by method of Kakkar (14). Alkaline dimethyl sulphoxide (DMSO) was used as a superoxide generating system. To 0.5 ml of different concentrations of G-001M, 1ml of alkaline DMSO and 0.2ml of 20mM nitro blue tetrazolium chloride (NBT) in phosphate buffer pH 7.4 was added. Ascorbic acid, α-tocopherol and Quercetin were used as a reference compounds. The absorbance was measured on at 560nm for NBT reduction.

Iron chelation activity

The metal chelating activity of the G-001M and standard were estimated by the method of Benzie & Strain (1) with slight modifications. The reaction mixture containing 1ml 0.05% o-phenanthroline in methanol, 2ml 200µM ferric chloride ($FeCl_3$) and 2ml various concentrations of the test sample was incubated at ambient temperature for 10min and the absorbance of the same was measured at 510nm. Quercetin and α-tocopherol were used as reference compounds.

Hydrogen peroxide radical scavenging activity

The ability of G-001M to scavenge hydrogen peroxide was determined by Ruch (22). Various concentrations of 0.5 ml of the test sample or standards were added to 1ml of H_2O_2 (40 mM) solutions in PBS. The absorbance of hydrogen peroxide was measured at 230 nm, after 10 min against a blank solution.

DNA damage study with pUC18

The plasmid DNA damage assay was carried out according to the protocol described by Shukla (26) with

some modifications. Briefly, pUC18 DNA (300ng) in phosphate buffer (pH 7.4) with different concentrations (50-100 µg/ml) of G-001M, was exposed to 150 Gy of radiation. BHT was used a standard. DNA samples were kept on ice during irradiation. The DNA samples were stabilized by adding a 0.1-volume of 10x TE (100 mM Tris-HCl, 10 mM EDTA), and stored at 4°C until they were analyzed. Plasmid DNA samples were mixed with 6x loading buffer and electrophoresed on a 1.2% agarose gel in 0.5x TBE buffer (0.045 M Tris borate, 0.005 EDTA). After electrophoresis, the gel was stained with 0.5 µg/ml ethidium bromide for 30 min, visualized under a transilluminator and photographed.

In vivo analysis of G-001M

G-001M administration

Required quantities of G-001M were administered in mice through intramuscular route in a maximum volume of 0.2ml. Dimethyl sulphoxide (10% DMSO) was used as a solvent.

Irradiation

Whole body radiation was performed in ^{60}Co gamma chamber (Model-220, Atomic Energy of Canada Ltd.) at a dose rate of 0.47cGy/second. Mice were irradiated (10Gy) with constant supply of fresh air. Dosimetry was carried out using Baldwin Farmer's secondary dosimeter and Fricke's chemical dosimetry method.

Splenocytes and thymocytes isolation

Animals were sacrificed by cervical dislocation at various time periods from 2hrs to 10th day. Thymus and spleen were excised as a whole and their splenocytes and thymocytes were estimated as mentioned earlier (24). MDA and GSH was then estimated in splenocytes and thymocytes by the methods described below.

Estimation of splenocyte and thymocyte MDA

Malondialdehyde (MDA), a marker for lipid per-oxidation was estimated according to the method of Beuge and Aust (6). Briefly, tissue homogenate was mixed with TCA-TBA- HCl and was heated for 15 min in a boiling water bath. The pink colour extracted was read at 531 nm to measure the amount of MDA formed in each sample. The MDA results were expressed as the mmol/mg protein.

Estimation of splenocyte and thymocyte GSH

Reduced glutathione (GSH) was measured by the method of Beutler (2). To 0.1 ml of sample, 0.9 ml distilled water and 1.5 ml of precipitating reagent (3.34 g metaphosphoric acid, 0.4g EDTA and 60.0g sodium chloride) was added. The mixture was centrifuged for 15 min at 4,000 rpm at 4°C. In 1.0 ml supernatant, 4.0 ml of phosphate solution (0.3 M disodium hydrogen phosphate) and 0.5 ml 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was added. The developed yellow colour complex was recorded at 412 nm on a spectrophotometer.

Total protein estimation

The protein content in splenocytes and thymocytes suspension was determined by the method described by Bradford (5).

Total leukocyte count

0.5 ml of whole blood was drawn from mice through cardiac puncture and collected in an EDTA glass vial. For Total Leukocyte Count, 20 µl of collected blood was mixed with 400 µl of Turk's Fluid (2% acetic acid, 2-3 drops of methylene blue) and counted on Neubauer's chamber.

Statistical analysis

In vitro results were analyzed using Student's t-test. Experimental results were Mean ± S.E.M. of three parallel measurements. Linear Regression analysis was used to calculate the IC_{50} value. The results of in vivo experiments were also analyzed by using Student's t-test. Experiments were done in triplicate with 3 animals in each group and the results have been presented as Mean ± S.E.M. A value of $P < 0.05$ was considered as statistically significant.

RESULTS

Chemical characterization of G-001M by HPLC

HPLC figures 1(a) and 1(b) have confirmed the presence of lignans podophyllotoxin, podophyllotoxin glucoside and demethyl podophyllotoxin and flavonoids quercetin, quercitrin, rutin and kaempferol in G-001M.

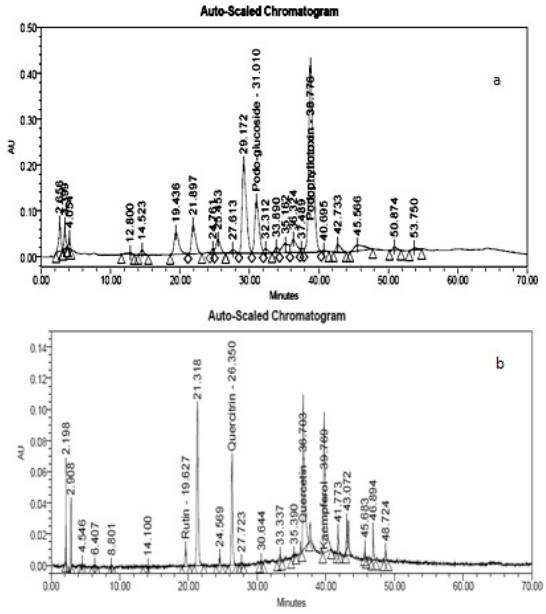

Figure 1. HPLC chromatogram of G00-1M (a) represents chromatogram of Lignans using methanol: water gradient over a period of 70 min and ;(b) shows chromatograph of Flavonoids using acetonitrile containing 0.05% of TFA: water containing 0.05% TFA gradient over a period of 70 min.

In vitro studies

DPPH radical scavenging activity

The free radical scavenging activity of G-001M was studied by its ability to bleach stable radical DPPH. Fig. 2 illustrates significant decrease in the concentration of DPPH radical due to scavenging ability of *P.hexandrum.* The G-001M exhibited a significant dose dependent inhibition of DPPH activity; with 50% inhibition (IC_{50}) at a concentration of 15.05µg/ml. The IC_{50} value of BHT and Quercetin (used as standards) was found to be 14.56 and 7.69µg/ml respectively.

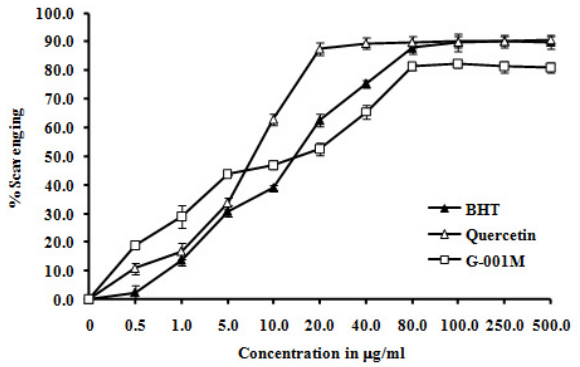

Figure 2. Free radical scavenging activity of different concentrations of the *P. hexandrum* subfraction (G-001M, 0.5-250µg/ml) by 1, 1-diphenyl-2-picrylhydrazyl radicals. The % inhibition in formation of free radicals by G-001M was compared with reference standards BHT and Quercetin. Results are expressed as mean ± S.E. of three parallel measurements.

Anti-lipid per-oxidation activity

The anti-TBARS activity of G-001M was evaluated on the basis of lipid per-oxidation activity. *P.hexandrum* sub fraction exhibited maximum scavenging activity at 500µg/ml and the minimum scavenging activity at 0.5µg/ml (Fig. 3). The IC_{50} value of G-001M and α-tocopherol (standard) was found to be 7.72 and 0.94 µg/ml respectively.

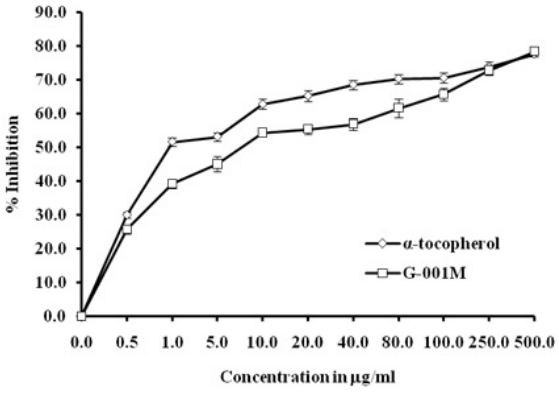

Figure 3. Effect of different concentrations of alcoholic extract of *P. hexandrum* subfraction (G-001M) on lipid per-oxidation of brain homogenate. α-tocopherol, was used as a standard antioxidant. Results are expressed as mean ± S.E. of three parallel measurements.

Superoxide anion radical scavenging activity

G-001M at various concentrations ranging from 0.5-250µg/ml inhibited the superoxide radical formation. The values were compared with same doses of different standards such as ascorbic acid, α-tocopherol and Quercetin (Fig. 4). The IC_{50} value of ascorbic acid, α-tocopherol, Quercetin and G-001M, was found to be 2.76, 4.41, 3.84 and 6.82µg/ml respectively. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture.

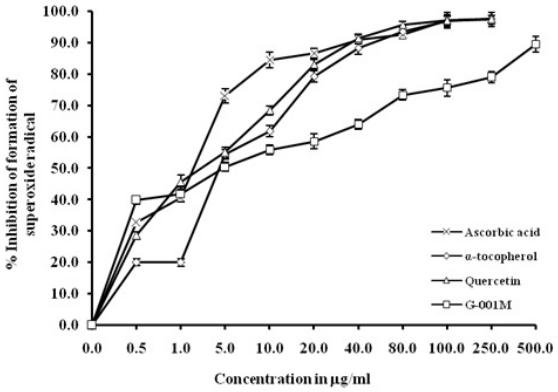

Figure 4. Superoxide anion radical scavenging activity of different concentrations of the *P. hexandrum* subfraction (G-001M). The % inhibition in formation of superoxide radical by G-001M was compared with reference standards ascorbic acid, α-tocopherol and quercetin. Results are expressed as mean ± S.E. of three parallel measurements.

Metal chelation activity

Ferrous ion chelation activity of G-001M was compared with standards Quercetin and α-tocopherol. The IC_{50} value of Quercetin, G-001M and α-tocopherol was found to be 2.87, 16.38 and 9.57µg/ml, respectively (Fig. 5).

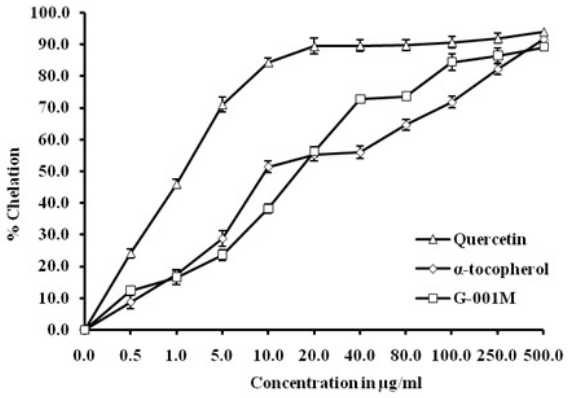

Figure 5. Metal chelating activity of different concentration of *P. hexandrum* subfraction (G-001M) and standards Quercetin and α-tocopherol. Results are expressed as mean ± S.E. of three parallel measurements.

Hydrogen peroxide radical scavenging assay

Fig. 6 shows the strong H_2O_2 scavenging activity of *P. hexandrum* sub fraction when compared to the α-tocopherol. The IC_{50} of G-001M and α–tocopherol was found to be 0.71

and 3.57 µg/ml. These results showed that G-001M had effective H_2O_2 scavenging activity.

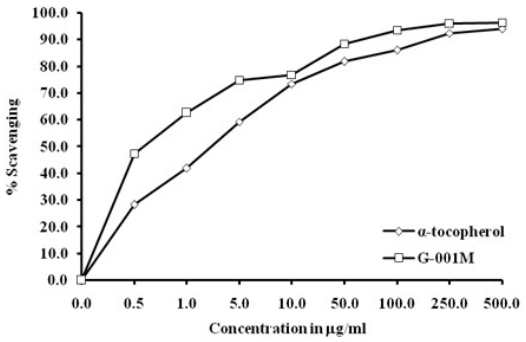

Figure 6. Effect of different concentrations of *P. hexandrum* subfraction (G-001M) on hydrogen peroxide scavenging activity. The % scavenging of hydrogen peroxide by G-001M was compared with α-tocopherol which is used as a reference standard. Results are mean ± S.E. of three parallel measurements.

Total phenolic content

Evaluation of total phenolic content in the extract revealed high amounts of phenols which was 200.0 ± 10.0 mg/g (w/w) total phenol in terms of gallic acid equivalent.

Plasmid DNA damage assay

The control DNA represents about 95% supercoiled and 5% open circular forms. 150 Gy radiation resulted in significant amounts of double and single strand breaks (2.8 fold) with compared to control as represented as linear and open circular forms shown in the Fig. 7. G-001M at concentrations of 50 to 100µg/ml protected 2.5 fold DNA damage as compared to radiation control, represented as disappearance of linear, decrease in open circular and restoration of super coiled forms.

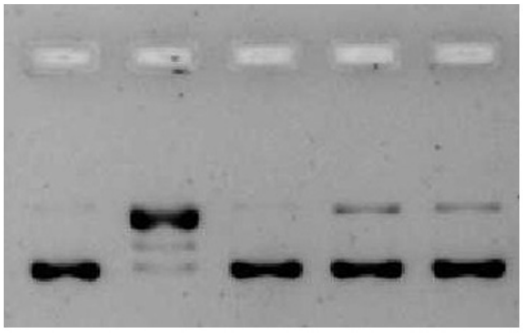

Figure 7. Effect of varied concentrations of G-001M on radiation induced strand breaks and conversion of fast migrating supercoiled plasmid DNA to slow migrating open circular form. Lane 1 300ng Control pUC18; Lane 2 pUC18 + 150Gy; Lane 3 pUC18+ 100µg/ml BHT+ 150Gy; Lane 4 pUC18+ 50µg/ml G-001M+ 150Gy; Lane 5 pUC18+ 100µg/ml G-001M+ 150Gy.

In vivo studies

Splenocytes MDA and GSH

Splenocytes MDA and GSH values at different intervals of treatment and with and without the administration of G-001M are depicted in Fig. 8 and 9 respectively. In radiation alone group, increase in MDA (0.118±0.007 and 0.105±0.002 vs. 0.08±0.009; irradiated vs. control) and decline in GSH values after 2hrs and 5hrs of irradiation (0.64±0.006 and 0.66±0.008 vs. 1.42±0.01) was apparently significant. In this group maximum increase in splenocytes lipid per-oxidation and decrease in GSH occurred at 24hrs (0.157±0.004) (0.97±0.01). In radiation alone group MDA in splenocytes at 48hrs and on 5th day was comparable to the controls but GSH in these cells at the same time point remained declined. In G-001M pre-treated irradiated group MDA of splenocytes declined during 2hrs and 5hrs interval but not significantly when compared with irradiated group. However, at later intervals, in this group, fall in MDA values was more pronounced to the respective irradiated group (Fig. 8). The splenocytes GSH in G-001M pre-treated group were found significantly up-regulated at 5hrs and 24hrs (0.92±0.008 and 1.54± 0.02 vs. 0.66± 0.08 and 0.97± 0.01; G-001M pre-treated vs. irradiated, Fig. 9) of the treatment.

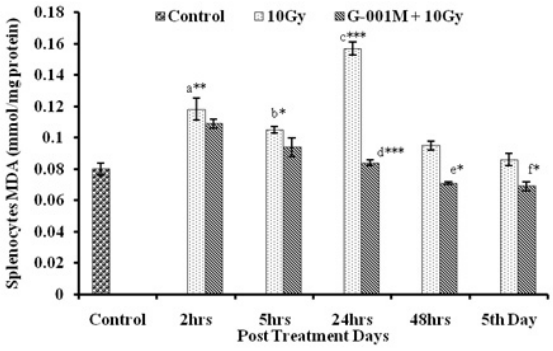

Figure 8. Effect of subfraction of *P. hexandrum* (G-001M) on Splenocyte MDA of whole body irradiated (10Gy) Swiss Albino mice. Animals were sacrificed at different time intervals. Experiments were done in triplicate with 3 animals in each group. a= Control vs. 2hrs 10Gy; b=Control vs. 5hrs 10Gy; c=Control vs. 24hrs 10Gy; d= 24hrs 10Gy vs. G001M + 10Gy; e= 48hrs 10Gy vs. G001M + 10Gy; f=5th day 10Gy vs. G001M + 10Gy; (Significance was set at **P*<0.05, ***P*<0.01, ****P*<0.001).

Thymocytes MDA and GSH

Thymocytes MDA (Fig. 10) and GSH (Fig. 11) was analysed at different intervals of treatment and with and without the

administration of the formulation. In radiation only group, significant increase in thymocytes MDA was registered at 2h and 5h post irradiation (0.2±0.003 and 0.12±0.004 vs. 0.07±0.01, $p<0.001$, control vs. irradiation) and in this group at later intervals thymus was found regressed (Fig. 10). With G-001M pre-treatment, though there was a reduction in thymocytes MDA when compared with radiation alone group values but the decrease was not significant at initial intervals of study. However, G-001M administration minimized lipid per-oxidation in thymocytes at 24h, 48h and 5th day. In radiation only group thymocytes GSH decreased significantly at 2h and 5h of irradiation (0.052±0.01 and 0.021±0.001 vs. 1.41±0.01, $p<0.001$, control vs. irradiation). G-001M pre-treatment could significantly elevate GSH values at these time points (0.49±0.007 and 0.62±0.008 vs. 0.052±0.01 and 0.021±0.001). The level of thymocytes GSH increased further at 24h and 48h intervals and was found comparable to the untreated controls on 5th day (Fig. 11) of treatment.

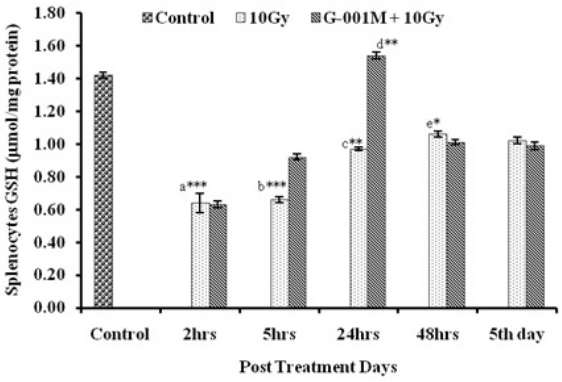

Figure 9. Effect of subfraction of *P. hexandrum* (G-001M) on Splenocyte GSH of whole body irradiated (10Gy) Swiss Albino mice. Animals were sacrificed at different time intervals. Experiments were done in triplicate with 3 animals in each group. a= Control vs. 2hrs 10Gy; b=Control vs. 5hrs 10Gy; c=Control vs. 24hrs 10Gy; d=24hrs 10Gy vs. G001M+10Gy, e=Control vs. 48hrs 10Gy (Significance was set at **P*<0.05, ***P*<0.01, ****P*<0.001).

White blood cells count

In lethally irradiated (10Gy) mice, white blood cells (Fig. 12) declined sharply ($p< 0.001$, Control vs. Radiation only) at 5hrs of experimentation. Fall in radiation only group continued further till the animals survived ($p< 0.001$, Control vs. Radiation only). In G-001M pre-treated irradiated group also abatement in total counts at least up to 48 hrs of exposure was not very different from radiation only group ($p>0.05$) but hereafter amplification in the counts started. On 10th day of study the difference in counts of radiation alone and pre-treated group was highly significant ($p<0.001$). In radiation alone group all the animals died within 11 days of exposure.

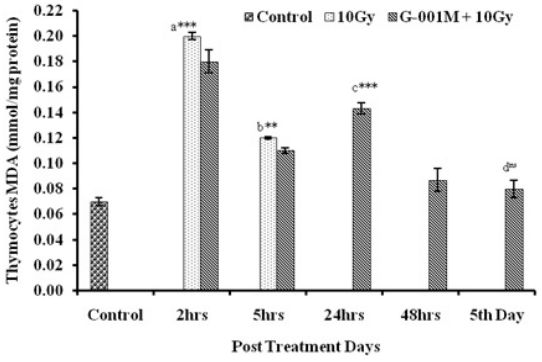

Figure 10. Effect of subfraction of *P. hexandrum* (G-001M) on Thymocytes MDA of whole body irradiated (10Gy) Swiss Albino mice. Animals were sacrificed at different time intervals. Experiments were done in triplicate with 3 animals in each group. Thymus in 10Gy only group was found fully regressed at 24hr and onward hence could not be processed for study. a= Control vs. 2hrs 10Gy; b=Control vs. 5hrs 10Gy, c= Control vs. 24hr G001M+10Gy, d=Control vs. 5th day G001M+10Gy (Significance was set at ***P*<0.01, ****P*<0.001, ns- non significant).

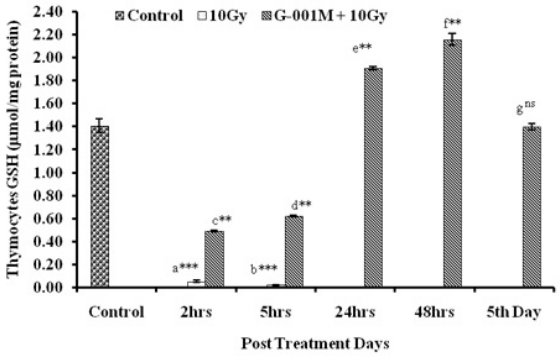

Figure 11. Effect of subfraction of *Podophyllum hexandrum* on thymocytes GSH of whole body irradiated (10Gy) Swiss Albino mice. Animals were sacrificed at different time intervals. Experiments were done in triplicate with 3 animals in each group. Thymus in 10Gy only group was found fully regressed at 24hr and onward hence could not be processed for study. a= Control vs. 2hrs 10Gy; b=Control vs. 5hrs 10Gy; c= 2hrs 10Gy vs. G001M+10Gy; d=5hrs 10Gy vs. G001M + 10Gy, e= Control vs. 24hr G001M + 10Gy, f= Control vs. 48hr G001M + 10Gy, g= Control vs. 5th day G001M + 10Gy (***P*<0.01, ****P*<0.001, ns- non significant).

DISCUSSION

Podophyllum hexandrum was initially evaluated a decade ago in our group for its radio protective efficacy with a concept that the plants

growing at extreme climate of high altitude acquire certain biomolecules to sustain life in highly adverse weather conditions. Chemoprofiling of this plant (7, 10, 12, 24 and 28) added vital support to this idea. Reports on long term use of this plant in Ayurveda system of medicine further strengthen our thought (27). The flavonoids: quercetin, quercitrin, rutin and kaempferol, richly found in this herb (HPLC data) are amply reported for scavenging free radicals (8, 25). The lignans podophyllotoxin, podophyllotoxin glucoside, dimethyl podophyllotoxin, the important constituent of G-001M, (Fig. 1 a & b) are also well documented as free radical scavengers, immunomodulators, DNA savour and enhancers of endogenous defence enzymes (10, 12, 17, 20, 23 and 32). Recently published study (24) on radio protective efficacy of G-001M has shown better results than earlier preparations of *P. hexandrum*. Low concentration of G-001M rendering high degree of survival against 100% death, an acceptable window in between effective dose and toxic doses (ED: MTD: LD 50: LD1000, 1:10:15:18) besides, fast recovery in health and body weight of treated and irradiated animals in comparison to their lethally irradiated counterparts which succumbed to death within 12 days of radiation exposure are some of the important features of G-001M.

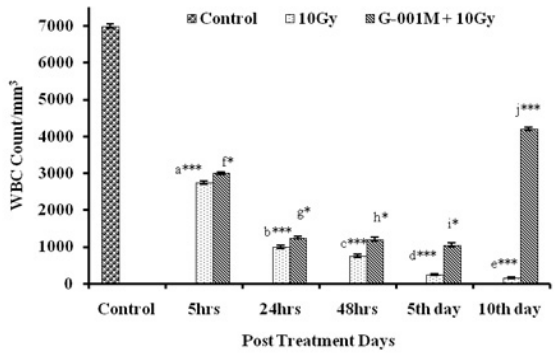

Figure 12. Effect of *P. hexandrum* subfraction (G-001M) on WBC count of whole body irradiated (10Gy) Swiss Albino mice. Animals were sacrificed at different time intervals. Experiments were done in triplicate with 3 animals in each group. Significance was set at $p<0.05$. a=Control vs. 5hrs 10Gy; b=Control vs. 24hrs 10Gy; c= Control vs. 48hrs 10Gy; d= Control vs. 5th day 10Gy; e= Control vs. 10th day 10Gy; f=5hrs 10Gy vs. G001M + 10Gy; g= 24hrs 10Gy vs. G001M + 10Gy; h= 48hrs 10Gy vs. G001M + 10Gy; i=5th day 10Gy vs. G001M + 10Gy; j=10th day 10Gy vs. G001M + 10Gy (*P<0.05, ***P<0.001).

Antioxidants are amply reported to scavenge free radicals by electron donation, chelation of transitional metal ions**,** up regulation of endogenous defence entities and thus preventing not only free radical generation but also inhibiting their chain propagation (4, 29). Significant decline in DPPH concentration with G-001M treatment in dose dependent manner is among one of the major support in favour of its strong antioxidant status. This characteristic of G-001M can be correlated with the presence of large quantity of polyphenols which has been confirmed through HPLC and HPTLC analysis of this sub-fraction. Polyphenols are always shown among one of the major constituents in most of the herbal preparations and they are also amply defined for their radical scavenging potential (19, 30).

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of lipid per-oxidation reaction. Generation of these radicals could be declined by polyphenols intervention and the same has been shown in current study. Significant inhibition in MDA formation after G-001M insertion has reaffirmed minimization in OH radicals generation which are potentially capable in initiation of lipid per-oxidation.

Superoxide anion is yet another species of free radicals that has been implicated in several pathophysiological processes, specifically due to its transformation into more reactive species such as hydroxyl radical which ultimately initiates lipid per-oxidation. Numerous reports have explained that antioxidant properties of flavonoids are also effective via scavenging of superoxide anion radical (21). In the present study, G-001M has been appreciably successful in scavenging superoxide ions generated by alkaline DMSO after reacting with NBT. The scavenging potential of G-001M was found comparable to the tocopherol and quercetin which were used as standards. Hydrogen peroxide, able to penetrate biological membranes, though it is not very reactive, but it can be severely toxic to cell because of its reactive nature with Fe^{2+} ions available in plenty in body fluid after radiation exposure (16, 31). G-001M at 500µg/ml concentration showed maximum H_2O_2 scavenging activity. In the present study it is shown G-001M was able to remove free ferrous ion from the extracellular milieu almost equal to α-tocopherol (standard).

The induction of single-strand or double-strand breaks in plasmid DNA results in the conversion of fast-migrating supercoiled DNA into the more slowly migrating open circular form and a linear form with intermediate migration. Evaluating these changes in plasmid is a sensitive method for assessing DNA damage. In the present study, exposure of plasmid DNA to 150 Gy of radiation resulted in significant amounts of both single-strand and double-strand DNA breaks as seen by the loss of intact supercoiled DNA and the formation of open circular and linear forms. G-001M in a dose dependent manner mitigated the effects of radiation in damaging plasmid DNA. The result of significant decline in plasmid DNA damage further confirmed antioxidant nature of G-001M.

Phenolic contents, predominantly due to the presence hydroxyl groups, found in plants, have always been a great strength for their high free radical scavenging ability. In addition, it has been also reported that phenolic compounds strongly associated with antioxidant activity, play important role in stabilizing lipid peroxidation (31). Presence of rich quantity of phenolic contents (200$\pm$2.3mg /mg of G-001M) may have contributed directly to the anti-oxidative action of G-001M. In earlier studies (24) reducing power of G-001M was also found comparable to BHT standard used in the study. Reducing potential of G-001M is again attributed to the presence of significant quantity of poly-phenols in this formulation.

Fast replenishment in WBC count in G-001M treated irradiated group is in consonance with other studies conducted with many other preparations of *P. hexandrum* (11, 23). The upsurge in WBC count has been principally attributed to reduced loss of hematopoietic stem cells. This could also be correlated directly and indirectly to the free radical scavenging potential of G-001M, resulting into decline in DNA damage and cellular apoptosis. G-001M administration induced decline in MDA formation and enhanced GSH release in both the cell lineages of thymus and spleen has precisely conveyed a significant improvement in radiation mediated declined hematopoietic and immune system. Appreciable fall in lipid peroxidation and increase in endogenous defence enzyme, chiefly due to G-001M mediated free radical flux inhibition, also might have extended vital support to the whole body protection in G-001M pre-treated and lethally irradiated animals.

CONCLUSION

Based on current study it will be pertinent to conclude that protective potential of G-001M against debilitating effects of lethal radiation is predominantly because of its free radical scavenging potential. However, at this stage it may not be wise to affirm that this is the only mode of action for rendering >90% survival in lethally irradiated animals till other simultaneously going on molecular marker studies are completed.

Acknowledgements - This work was supported by a grant from Defence Research & Development Organisation (DRDO). Authors extend their thanks to Dr R P Tripathi, Director INMAS, and Dr BS Dwarakanath, Divisional Head, for administrative support. Thanks are also due to Director, IIIM for providing the sub-fractionated and chemically characterized plant material (G-001M). Experimental support by Mr. Pradeep Kumar Gupta, summer trainee, is also highly appreciated. S S acknowledges CSIR for the award of fellowship.

REFERENCES

1. Benzie, I.F.F. and Strain, J.T., The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Anal. Biochem. 1996, 239: 70-76.
2. Beutler, E., Duron, O. and Kelly, B.M., Improved method for the determination of blood glutathione. J. Lab. Clin. Med. 1963, 61: 882-888.
3. Beutner, K.R., Podophyllotoxin in the treatment of genital warts. Curr. Probl. Dermatol. 1998, 24: 227-232.
4. Blokhina, O., lainen, E.V. and Fagerstedt, K.V., Antioxidant, Oxidative damage, and oxygen deprivation stress: a review. Annals of Botany. 2003, 91: 179-194.
5. Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976, 72: 248- 254.
6. Buege, J.A. and Aust, S., Microsomal lipid peroxidation. In: Methods in enzymology LII, Biomembranes. Academic Press, London, 1978.
7. Chawla, R., Arora, R., Kumar, R., Sharma, A.K., Prasad, J., Singh, S., Sagar, R.K., Chaudhary, P., Shukla, S. and Qazi, G.N., Antioxidant activity of fractionated extracts of high altitude *Podophyllum hexandrum* in radiation protection. Mol. Cell. Biochem. 2005, 273: 193-208.
8. Di Carlo, G., Mascolo, N., Izzo, A.A. and Capasso, F., Flavonoids: Old and new aspects of a class of natural therapeutic drugs. Life. Sci. 1999, 65: 337–353.
9. Goel, H.C., Prasad, J., Sharma, A. and Singh, B., Antitumor and radio protective action of Podophyllum hexandrum. Indian J. Exp. Biol. 1998, 36: 583-587.
10. Gupta, M.L., Agarwal, P.K., Kumar, I.P., Devi, M., Soni, N.L. and Tripathi, R.P., Modulation of gamma radiation-inflicted damage in swiss albino mice by an alcoholic fraction of P. hexandrum rhizome. J. Med. Food. 2008, 11: 486-92.
11. Gupta, M.L., Sankhwar, S., Verma, S., Devi, M., Samanta, N., Agarwala, P.K., Kumar, R. and Singh, P.K., Whole body protection to lethally irradiated mice by oral

administration of semi purified fraction of *Podophyllum hexandrum* and post irradiation treatment of *Picrorhiza kurroa*. Tokai J. Exp. Clin. Med. 2008, 33: 6-12.
12. Gutpa, M.L., Tyagi, S., Flora, S.J.S.., Agarwala, P.K., Chaudhary, P., Puri, S.C., Devi, M., Sharma, A., Haksar, A., Qazi, G.N. and Tripathi, R.P., Protective efficacy of semi purified fraction of high altitude *Podophylum hexandrum* rhizomes in lethally irradiated swiss albino mice. Cell Mol. Biol. 2007, 53: 29-41.
13. Halliwell, B., Gutteridge, J.M.C. (1990) Role of free radicals and catalytic metal ions in human diseases: an overview. *Meth. Enzymol* 186: 1-84
14. Kakkar, P., Das, B. and Vishwanathan, P.N., A modified spectrophotometric assay of superoxide dismutase. Indian J. Biophys. Biochem. 1984, 21: 130–133.
15. Lata, M., Prasad, J., Singh, S., Kumar, R., Singh, L., Chaudhary, P., Arora, R., Chawla, R., Tyagi, S., Soni, N.L., Sagar, R.K., Devi, M., Sharma, R.K., Puri, S.C. and Tripathi, R.P., Whole body protection against lethal ionizing radiation in mice by REC-2001: a semi-purified fraction of *Podophyllum hexandrum.* Phytomed. 2009, 16 : 47-55.
16. Miller, M.J., Sadowska-Krowicka, H., Chotinaruemol, S., Kakkis, J.L. and Clark, D.A., Amelioration of chronic ileitis by nitric oxide synthase inhibition. J. Pharmacol. Exp. Ther. 1993, 264**:** 11-16.
17. Mittal, A., Pathania, V., Agrawala, P.K., Prasad, J., Singh, S. and Goel, H.C., Influence of *Podophyllum hexandrum* on endogenous antioxidant defense system in mice: a possible role in radioprotection. J. Ethnopharmacol. 2001, 76: 253–262.
18. Molyneux, P., The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity Songklanakarin. J. Sci. Technol. 2004, 26: 212-219.
19. Rice-evans, C.A., Miller, N.J. and Paganga, G., Antioxidant properties of phenolic compounds. Trends in Plant Science. 1997, 2: 152-159.
20. Rios, J. L., Giner, R. and M, Prieto., New findings on the bioactivity of lignans. Studies in Nat. Prod. 2002, 26: 183-292.
21. Robak, J. and Gryglewski, R.J., Flavonoids are scavengers of superoxide anion. Biochem. Pharmacol. 1988, 37: 837-841.
22. Ruch, R.J., Cheng, S.J. and Klaunig, J.E., Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis. 1989, 10: 1003–1008.
23. Sagar, R.K., Chawala, R., Arora, R., Singh, S., Krishna, B., Sharma, R.K., Puri, S.C., Singh, P., Kumar, R., Sharma, K.A., Singh, S., Prashad, J., Gupta, V., Ahmed, B., Singh, K.L., Khan, H.A., Gupta, M.L. and Quazi, G.N., Protection of the Hemopoietic System by Podophyllum hexandrum against Gamma Radiation-Induced Damage. Planta Med. 2006, 72: 114-120.
24. Sanghmitra, S., Gupta, M., Gupta, V., Verma, S., Suri, K.A., Devi, M., Sharma, P., Khan, E.A., Alam, M.S. and Tripathi, R.P., Podophyllum hexandrum- mediated survival protection and restoration of other cellular injuries in lethally irradiated mice. eCAM. 2009, 16: 47-55.
25. Sestili, P., Guidarelli, A., Dachà, M. and Cantoni, O., Quercetin Prevents DNA Single Strand Breakage and Cytotoxicity Caused By tert-Butylhydroperoxide: Free Radical Scavenging Versus Iron Chelating Mechanism. Free Rad. Biol. Med. 1998, 25: 196-200.
26. Shukla, S.K., Chaudhary, P., Kumar, I.P., Samanta, N., Afrin, F., Gupta, M.L., Sharma, U.K., Sinha, A.K., Sharma, Y.K. and Sharma, R.K., Protection from radiation-induced mitochondrial and genomic DNA damage by an extract of *Hippophae rhamnoides*. Environ. Mol. Mutagen. 2006, 47: 647-656.
27. Singh, J. and Shah, N.C., Podophyllum: A review. Curr. Res. Med. Arom. Plants. 1994, 16: 53-83.
28. Stahelin, H. and Von, Wartburg., A. From podophyllotoxin glucoside to etoposide. Prog. Drug. Res. 1989, 33 :169-267.
29. The antioxidant: Richard A passwater. Keat Publishing Incorporation, 1997.
30. Urquiaga, I. and Leighton, F., Plant Polyphenol Antioxidants and Oxidative Stress. Biol. Res. 2000, 33 N.2.
31. Yen, G.C., Duh, P.D. and Tsai, C.L., Relationship between antioxidant activity and maturity of peanut hulls. J. Agric. Food. Chem. 41, 1993: 67–70.
32. Ying-Quian, L., Yang, L. and Tian, M., Podophyllotoxin: Current Perspectives. Curr. Bioactive Compounds. 2007, 3: 37-66.