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Research Articles

Radioprotective Activities of *Podophyllum hexandrum:* Current Knowledge of the Molecular Mechanisms

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This is the second article published in the Trees for Life Journal *which deals with radioprotection (see http://www.tfljournal.org/article.php/2007042416194363 by Sancheti and Goyal). While it is not a major focus of this journal, radioprotection has interest for our readership and is attracting ever more attention within the scientific community. No synthetic radioprotective chemicals are available that are perfectly safe and effective. While a variety of plant extracts have been evaluated for their protective capacity, this work is still in its infancy. The following review highlights the radioprotective effects of the Himalayan Mayapple. It also illustrates the degree to which mechanism-based studies are beginning to support the purely observational work that initially brought to light the radioprotective effects of certain plants.*

Abstract

Radiation mediated free radical flux interferes with oxidation/reduction-based physiological mechanisms. These free radicals react with a number of biomolecules including Deoxyribonucleic acid (DNA), lipids and proteins. The rate and selectivity of these free radical mediated reactions depend upon the concentration, half-life and state of delocalization of electrons in the free radicals and the free radicals' oxidizing ability.

Podophyllum hexandrum Royle (Himalayan Mayapple) was known as *Aindri* ("a divine drug") in ancient times. It has been reported to be used through the ages and in modern times as a cure for allergic and inflammatory conditions of the skin; biliary fever; burning sensation; cold; constipation; cancer of the brain, bladder and lung; erysipelas; Hodgkin's disease; insect bites; mental disorders; monocytoid leukemia non-Hodgkin's lymphoma; rheumatism; septic wounds; plague; and venereal warts. It has served as a commercial source of podophyllotoxin and related aryltetralin lignans and several other bioactive constituents. Podophyllotoxin finds use as a precursor for the semi-synthetic topisomerase inhibitors in the treatment of leukemias, lung and testicular cancers, dermatological disorders like warts, rheumatoid arthritis, psoriasis and malaria. It also has numerous applications in modern medicine by virtue of its free radical scavenging capacity. An extract of *P. hexandrum* has been shown to provide approximately 80% whole-body radioprotection in mice.

The present review highlights the state of knowledge about the radioprotective mechanism of *P. hexandrum* at different levels of organization in living organisms. Further, an insight into its mode of action at the molecular level, including the studies of the expression patterns of various proteins associated with inhibition of apoptosis in the spleen of male Swiss albino strain 'A' mice by immunoblotting, has been presented. In conclusion, the studies clearly demonstrated that *P. hexandrum* extract provides protection from gamma-radiation by the modulation of expression of proteins associated with cell death attributed to its ability to modulate free radical flux.

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Introduction

Radiation is one of the most severe causes of oxidative stress mediated by free radical flux. This flux interferes with oxidation/reduction-based physiological mechanisms existing inside the mammalian body system. The rate and selectivity of free radical mediated reaction depends primarily upon several factors: the concentration of radicals, the state of delocalization of

electrons, the half-life of free radicals and weak bonds of nearby bio-molecules. Radiation protection is an area of great significance due to its possible applications in planned radiotherapy as well as unplanned radiation exposure (1,2). Research in the development of radio-protectors worldwide has focused on screening a variety of chemical and biological compounds. Various drugs from natural or synthetic origin, i.e., antioxidant cyto-protective agents, immuno modulators, vitamins and DNA binding molecules, have been evaluated extensively for their radioprotective potentials in both *in vitro* and *in vivo* models (1,3,4,5,6). However, the fact remains that there is not a single radioprotective drug available which meets all the prerequisites of an ideal radioprotector, i.e., produces no cumulative or irreversible toxicity, provides effective long-term protection, remains stable for a number of years without losing shelf life, and can be easily administered (7,8). In view of this, the search for less toxic and more potent radioprotector drugs continues.

Herbal drugs have been utilized since ancient times for curing various diseases and other disorders. Even today, more than 70% of the world's population still depends on plant-based remedies to meet their health care needs (9). Various plants have been reported to be beneficial for free radical-mediated conditions in humans such as arthritis, atherosclerosis, cancer, Alzheimer's disease, Parkinson's disease, aging and inflammatory disorders. It is, therefore, logical to expect that plants may contain groups of compounds that can protect against radiation-induced reactive oxygen species (ROS) and reactive nitrogen species (RNS) mediated damage (7,8).

Podophyllum hexandrum

Podophyllum hexandrum Royle (Himalayan Mayapple), is an herb that grows at about 4000 meters of altitude in the Himalayan region (Figure 1) (10). *P. hexandrum* has been investigated extensively for its radioprotective capabilities in recent years, including free radical scavenging, time and dose-dependent inhibition of apoptosis (programmed cell death) and cell cycle arrest-related activities in both *in vitro* and *in vivo* models (7,8). The plant is an endangered species and included in the Red Book. *In vitro* propagation for mass multiplication of *P. hexandrum* has been recently reported (11). Besides its traditional uses (10), methanolic, hydro-alcoholic and chloroform extracts of *P. hexandrum* have been reported to render approximately 70-95% radioprotection in mice when administered 1-2 hours before lethal whole-body 10Gy radiation (12,13,14). To achieve radioprotection, recovery of damaged cells after radiation exposure and minimization of cell death by inhibition of apoptosis is an inescapable necessity (15,16,17,18,19). *P. hexandrum* has been reported to contain a number of bioactive molecules including flavonoids and lignans (10,20,21,22).

Many flavonoids and lignans are already known for their antioxidant action and anti-apoptotic potential, and thus contribute towards radioprotection (21). Several mechanisms have been proposed to explain the radiation protection observed, following administration of *P. hexandrum* extract, such as free radical scavenging, stabilization of mitochondrial cell potential, regulation of cell cycle activities and time dependent apoptosis (12,13,14,15,16,20,21,22,23,24,25,26,27,28,29,30,31,32,33, 34,35).

P. hexandrum exhibited both anti-cancer (pro-oxidant ability) and radioprotective (antioxidant ability) effects respectively depending upon the dose administered

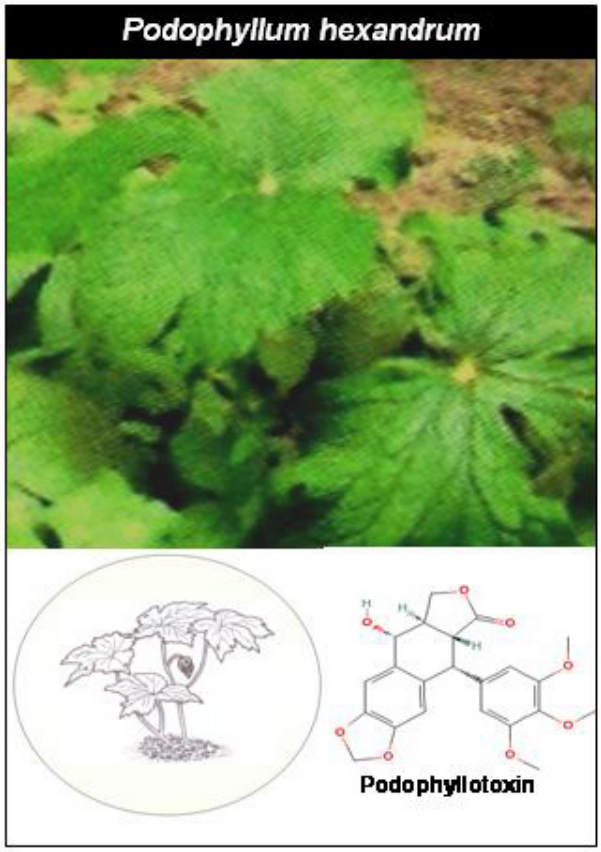

Figure 1: *Podophyllum hexandrum* (syn. *P. emodi*); common names - *Aindri*, Himalayan Mayapple, Devil's apple, and Duck's foot; low to the ground with glossy green, drooping, lobed leaves on its few stiff branches, with a pale pink flower and bright red-orange bulbous fruit; propagated by seed or by dividing the rhizome; tolerant to cold temperatures but not to dry conditions. In Eastern Asia, it is found from Afghanistan to China and through the Himalayan ranges.

(1,20,21,24,29). The pro-oxidant effects are observed in the presence of metal ions by virtue of the presence of some aryltetralin lignans (22), further supporting DNA fragmentation ability at the *in vitro* level (35). At specific radioprotective doses, it exhibited significant antioxidant effect as summarized in Table 1.

Molecular mechanism of *P. hexandrum* mediated radioprotection

Cytoprotective properties

We have recently reported that the mode of protection by *P. hexandrum* at the molecular level includes the modulation of protein expression [associated with cell death (apoptosis) and DNA repair)] by *P. hexandrum* administration 200 mg/kg.b.wt. i.p. 2 hrs. before 10 Gy irradiation (17,18,19). The most striking observations indicated that as a result of *P. hexandrum* extract treatment in mice, the expression of heat shock proteins, especially HSP-70 along with its transcriptional factor HSF-1 (heat shock transcription factor; a protein regulates HSP-70 synthesis) was increased significantly as compared to the mice treated with radiation (10 Gy) only. Heat shock proteins are cytoprotective in nature and have been reported to be increased under various environmental stresses such as increased temperature, ionizing radiation and chemical toxicants (17). The overexpression of HSF-1 suggested transcriptional level induction of heat shock proteins in the spleen of mice treated with *P. hexandrum* extract. The expression of phosphorylating enzymes (proteins regulating phosphate group transfer to other functional proteins) such as PkC, MAPKAP Kinase-2 was also observed to increase in the mice spleen after *P. hexandrum* treatment (with or without irradiation), as compared to irradiated control suggesting the possible role of phosphorylating enzymes in the signal transduction pathways that is initiated upon *P. hexandrum* treatment (17,18,19).

Apoptosis inhibitory activities

A strong inhibition of Apoptosis Inducing Factor (AIF - a cell death promoting protein) expression in the group of mice showed overexpression of HSP-70 upon *P. hexandrum* treatment (17). On the basis of a previous study (36), it was suggested that *P. hexandrum* treatment initiates an antagonistic interaction between AIF and HSP-70 expression and thus suppresses the apoptotic molecular cascade thereby protecting DNA fragmentation. The anti-apoptotic effect of HSP-70 can be attributed to the ability of *P. hexandrum* to provide protection to chromatin from proteases and nucleases, which are well known apoptotic markers (17,18,19).

Cell proliferation activities

The role of nuclear factor kappa beta (NFκβ) has also been studied extensively in apoptotic regulation NFκβ-Iκβ complex and was found to be stable in the cytoplasm. However, under radiation stress $I\kappa\beta_2$ gets phosphorylated by enzymes like Pkc and consequently free NF-κβ moves towards the nucleus, binds with DNA, and initiates a variety of transcription at times leading to induced cell death (37,38). The expression of NF-κβ was found to be induced in the cytoplasm upon *P. hexandrum* treatment (+/- irradiation), but it was found to be translocated into the nucleus. *Podophyllum hexandrum* treatment inhibited the translocation of free NFκβ from the cytoplasm to the nucleus and could explain its anti-inflammatory effects. This effect may have further lead to down-regulation of p53 and up regulation of Bcl-2 protein expression, which may have possible contributions toward reduction of apoptosis and enhanced cell proliferation. Increased expression of Bcl-2, and HSP70, observed following *P. hexandrum* treatment (+/- irradiation), suggests a role of *P. hexandrum* in stabilization of the mitochondrial membrane potential leading to inhibition of membrane permeability (36,39). This prevents release of cytochrome C, leading to inhibition of apoptosis and thereby offering protection against radiation-mediated damage to the cells in conformity with the earlier observations (40). The induced expression of Ras-GAP directly demonstrated the activation of Ras-related protein R-Ras in the animals treated with *P. hexandrum* extract (+/- irradiation), indicating enhanced cell proliferation.

DNA Repair Studies

The expression of proliferating cell nuclear antigen (PCNA), an important cell proliferation marker in the cell system, was found to be enhanced in the spleen of mice treated with *P. hexandrum* (with or without irradiation), further demonstrating improved cell proliferation and eventually survival. *P. hexandrum* treatment (with or without irradiation) induced the molecular expression of the Poly (ADP-ribosyl) DNA polymerase (PARP) and down-regulated the expression of caspases and their transcription regulators (i.e., Caspase Activated DNAase), further indicating improved DNA repair and significant inhibition of the apoptosis in the mouse model system (17,18). A summary of the radioprotective action

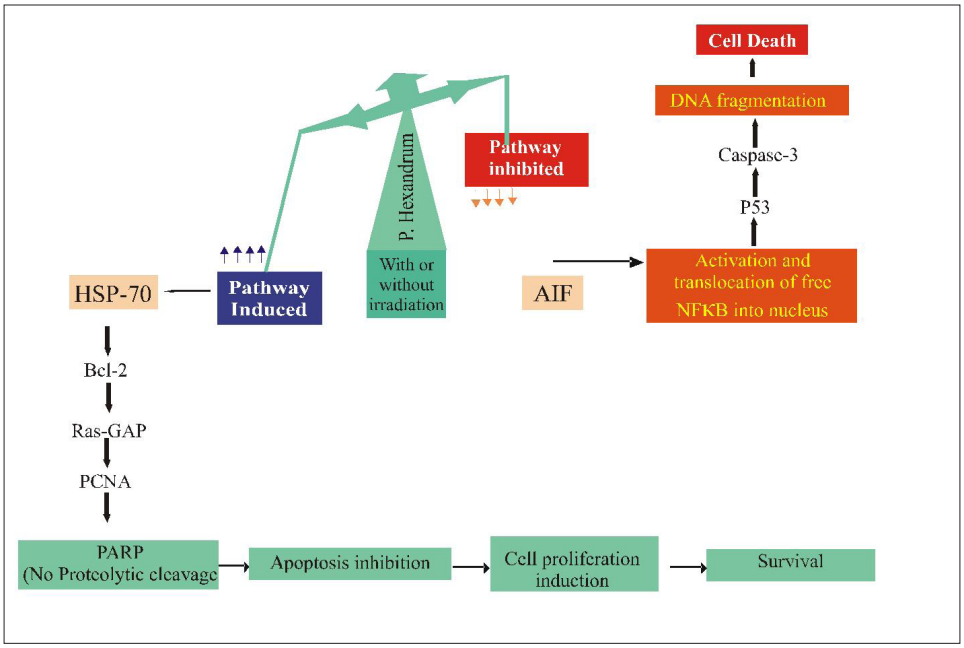

Figure 2: Possible molecular mechanisms of radioprotection provided by *P. hexandrum*

modulated via modulation of expression of proteins is shown in Figure 2.

Future Prospects

In view of its acceptable therapeutic index and a significant dose-reduction factor, *P. hexandrum* is a promising candidate plant for development of radioprotective drugs. Since it is an endangered plant, the interesting lead of usage of *Trametes hirsuta* (an endopyte) for mass production of precursors of radioprotective formulation (41) needs to be further pursued for upscaling. Further studies to characterize the total profile of gene expression (up or down regulation) by cDNA micro-array and the effects of *P. hexandrum* on protein expression (proteomic profiling) will help unravel the intricate mechanisms of signal transduction pathways operating at the molecular level. Such data will help in the development of novel radioprotectors for human use.

Conclusion

In conclusion, *P. hexandrum* acts in a holistic manner at various levels. Its administration on mice prior to irradiation leads to a dramatic enhancement (~80%) in survival. At the molecular level, it induces pro-survival protein and DNA repair protein expression while down-regulating the expression of proteins associated with induction of apoptosis. These activities are congruent with the in vitro free radical modulating ability of rhizome extracts of *P. hexandrum*.

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Table 1: Radioprotective Studies using *Podophyllum hexandrum*

Radioprotective Studies	Major Inferences	References
Survival Study	Serendipitously discovered while evaluating anti-cancerous properties of *Podophyllum hexandrum*. More than 80% radioprotection observed in a 30-day survival study using Strain 'A' Swiss Albino mice against 10 Gy exposure with dose reduction factor of 1.33. Radioprotective effects of podophyllotoxin, an active constituent of *Podophyllum hexandrum* were reported in yeast (*Sacchromyces cerevisae*).	1, 6, 15
Gastroprotection	The aqueous crude extract (200mg/Kg b.wt., -2hrs) rendered gastroprotection by: a) threefold increase in number of surviving crypts b) 2.7-fold increase in villi cellularity c) limiting the radiation-induced apoptosis in crypt cells d) modulation of the antioxidant defense system in jejunum and ileum at 12th post-irradiation day e) enhanced levels of glutathione-S-transferase (GST) and superoxide dismutase (SOD) observed at in vivo level.	1, 36
Neuroprotection	Sub-lethal (2 Gy) dose was given to rats in utero on day 17 of gestation and pre-irradiation treatment of extract exhibited mitigation of neuro-physiological alternations	19, 38
Hepatoprotection	In vitro studies, using human hepato carcinoma cell lines further revealed its ability to stabilize the state of mitochondrial oxidative burst, decreased TBARS, time-dependent inhibition of gamma radiation-induced leakage of electrons in the mitochondrial electron transport chain (ETC) via reduction in ROS and NO generation and simultaneous enhancement in the thiol status via neo-synthesis. It also significantly inhibited the radiation-induced enhanced complex I (NADH: UQ reductase) activity. On the other hand, the flow and leakage of electrons from complex I/III (NADH: cytochrome C oxidoreductase) and complex II/III (succinate: cytochrome C oxidoreductase) was maintained. The above studies are in line with modulating ability of extract on antioxidant defense in mice.	20, 21, 30
Reproductive System	Extract administration exhibited significant increases in testis weight, repopulation of the seminiferous tubules and the resting primary spermatocytes and the stem-cell survival index was maintained. Increased levels of sperm counts and reduction in the abnormalities of sperm morphology revealed its ability to protect the testicular system. The extract has also exhibited its ability to modulate antioxidant enzymes in the male reproductive system.	1, 37
Molecular level Investigations	Studies at the molecular level revealed interesting data, e.g., anti-inflammatory activity (reduction of interferon-gamma, interleukin-6 and tumour necrosis factor-alpha secretion in lipopolysaccharide-induced inflammation in isolated macrophages); enhanced MAPKAP (mitogen-activated protein kinase-activated protein) kinase-2 activation along with HSF-1 (heat-shock transcription factor-1) leading to up-regulation of HSP-70 (heat-shock protein-70) with concomitant strong inhibition of AIF (apoptosis-inducing factor) expression; DNA degradation, and translocation of free NF-κβ (nuclear factor kappa beta) from cytoplasm to nucleus leading to decreased expression of tumor suppressor protein p53 and there is a simultaneous increase in Bcl-2 (B-cell chronic lymphocytic lymphoma 2), Ras-GAP (Ras-GTPase-activating protein) and PCNA (proliferating cell nuclear antigen). Such mechanisms have resulted in enhanced survival status.	26, 27, 28

Table 1: Radioprotective Studies using *Podophyllum hexandrum* (cont'd)		
Radioprotetive Studies	**Major Inferences**	**References**
Comparative Studies on Fractionated Extracts of High-Altitude *Podophyllum hexandrum* and semi-purified extract of Low-altitude *Podophyllum hexandrum*	On the basis of antioxidant screening using radioprotective studies of *Podophyllum hexandrum*, aqueous, aqua-alcoholic, chloroform and alcoholic extracts were selected. The polarity of solvent system to be used was established. Further, the comparative analysis with semi-purified extract of low-altitude *Podophyllum hexandrum* revealed that the semi-purified extract along with alcoholic and chloroform extract exhibited immense potential. The semi-purified extract exhibited significant inhibitory ability against 20 Gy induced double and single strand breaks by virtue of its free radical scavenging potential. The extract acted as a pro-oxidant in the presence of metal ions supported by its DNA fragmentation ability. The extract also exhibited apoptosis induction ability at selected doses. The selected extracts exhibited significant ($p<0.05$) nitric oxide scavenging potential and antioxidant activity in the lipid phase.	2, 9, 25, 40
Hemopoietic Modulation	The studies are further supported by hemopoietic modulatory ability of the semi-purified extract. The semi-purified extract exhibited significant recovery in hemoglobin content as compared to irradiated group. Total leukocytes content was fairly high on 30th post-treatment day and also the differential leukocyte count was restored to an extent. The activity was found to be attributed to the hydroxyl ion scavenging activity of the extract. The extract prophylactic treatment causes the recovery of the animals on 10th day from bone-marrow aplasia caused due to lethal exposure of gamma radiation (10Gy). In a recent study using a hydro-alcoholic extract of *Podophyllum hexandrum*, the enhanced expression of heme-oxygenase-1 and pro-survival multi domain Bcl-2 proteins revealed that the extract-induced modulation of hemopoietic system is linked to the expression levels of these proteins. The above studies are in coherence with the immunostimulatory ability and cytoprotective potential of the extract. The extract also exhibited an inherent potential to chelate iron at *in vitro* level indicating its ability to reduce the amplification process.	5, 14, 16, 24, 35
Role of secondary metabolites of *Podophyllum hexandrum* in radiation protection	The comparative studies using the comparable levels of aryltetralin lignans exhibited an indicative correlation between the pro-antioxidant behavior of the extracts of *Podophyllum hexandrum* and the content of individual lignans. The study was just a step towards revealing the role of lignans in radiation protection. In addition, a novel galactoside of quercetin was identified in aquo-alcoholic extract of High-altitude *Podophyllum hexandrum* and the extract exhibited significant ($p<0.05$) protein protection and peroxyl radical scavenging activity in *ex vivo* model. These studies indicated the probable role of polyphenolics in addition to lignans in radiation protection. These leads revealed that the ratio of lignans:polyphenolics might play a critical role in providing overall radiation protection. The endophytic *Trametes hirsuta* is reported as a novel alternative source of podophyllotoxin and related aryltetralin lignans. An isolated study using endophyte *Trametes hirsuta* exhibited the independent synthesis of podophyllotoxin and related aryl-tetralin lignans. The isolated lignans, even in combinations of two or more lignans, exhibited significant antioxidant activities relevant to radiation protection. The studies at the compound levels are still in infancy and require augmenting for the development of an effective radioprotective alternative for human use.	10, 11, 33

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