Original Article

**Podophyllum hexandrum** Offers Radioprotection by Modulating Free Radical Flux: Role of Aryl-Tetralin Lignans

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We have evaluated the effect of variation in aryl-tetralin lignans on the radioprotective properties of *Podophyllum hexandrum*. Two fractionated fractions of *P. hexandrum* [methanolic (S1) and chloroform fractions (S2)], with varying aryl-tetralin lignan content were utilized for the present study. The peroxyl ion scavenging potentials of S1 and S2 were found to be comparable [i.e. 45.88% (S1) and 41% (S2)] after a 48 h interval in a time-dependent study, whereas in a 2 h study, S2 exhibited significant (*P* < 0.05) antioxidant activity in different metal ion + flux states. In the aqueous phase, S2 exhibited non-site-specific reactive oxygen species scavenging activity, i.e. 73.12% inhibition at 500 μg ml⁻¹. S1 exhibited 58.40 ± 0.8% inhibition (at 0.025 μg ml⁻¹) of the formation of reactive nitrite radicals, comparable to S2 (52.45 ± 0.825%), and also showed 45.01% site-specific activity (1000 μg ml⁻¹), along with significant (*P* < 0.05) electron donation potential (50–2000 μg ml⁻¹) compared to S2. Such activities of S1 could be attributed to the significantly (*P* < 0.05) higher levels of podophyllotoxin β-d-glucopyranoside (16.5 times) and demethyl podophyllotoxin glucoside (2.9 times) compared with S2. Together, these findings clearly prove that aryl-tetralin lignan content influences the radiation protective potential of the *Podophyllum* fractions to a great extent.

**Keywords:** antioxidant – aryl-tetralin lignans – biological radioprotection – hydroxyl ion podophyllotoxin glucopyranoside

**Introduction**

Cancer accounts for nearly 25% of all human deaths (1). Despite a number of medical advances, no sure-fire cure is available as yet (1). The most common treatment modality promising a cure appeared to be a combination of radiotherapy and chemotherapy. Following radiotherapy, the risk of normal tissue complication constitutes a significant clinical concern and limits the radiation dose that can be delivered to patients. Although radiotherapy is an effective treatment for malignant diseases, radiation morbidity does develop in certain patients with above-normal radiosensitivity (2), and certain complications can arise during treatment. Radiation exposure leads to the hydrolysis of water, thereby generating reactive oxygen species, which initiate chemical peroxidative processes that destroy biomolecules (3). The damage to normal tissues can be averted by the use of radioprotectors in advance (4). The past five decades have witnessed enormous research and development efforts worldwide to develop radioprotectors to...
guard normal cells against structural damage in operations such as radiotherapy, rescue missions and space exploration. However, only a single drug molecule, WR-2721, has so far received FDA approval. The interest in natural plant products for their medicinal value in treating different disorders is increasing worldwide (5–7). A number of recent investigations into radioprotectors show that the potential use of herbal plants and their products as modifiers of radiation response is receiving considerable attention (5,8–10). Herbal radioprotection is a multifaceted phenomenon, and thus there is a need to investigate the different modes of radioprotective action of herbal drugs.

The traditional medicinal uses of Podophyllum hexandrum (Division: Magnoliophyta; Family: Podophyllaceae) in the treatment of colds, constipation, septic wounds, burning sensation, erysipelas, mental disorders, rheumatism, plague, allergic and inflammatory conditions of the skin, cancer of the brain, bladder and lung, venereal warts, monocytoide leukemia, Hodgkin’s disease and non-Hodgkin’s lymphoma (11–13) make it a good candidate for further investigation. Our earlier studies on P. hexandrum utilizing a crude fraction (unstandardized) proved its potent radioprotective effect in a lower mammalian model system (14) and its ability to render protection to different targets (5). Recently, our group has also reported on malian model system (14) and its ability to render protection toized) proved its potent radioprotective effect in a lower mammalian model system (14) and its ability to render protection to different targets (5). Recently, our group has also reported on malian model system (14) and its ability to render protection to.

For each wash, the material was kept in the solvent for 24 h. The solvent was removed and concentrated under vacuum. The defatted plant material was then fractioned with chloroform or methanol (four washes, cold) following the standard procedure. The yield of the S1 fraction (methanolic) was 0.25%; S2 (chloroform), 3.9%.

HPLC-Based Standardization of Fractions

The fractionated extracts were analyzed on a Shimadzu LC-10 AT VP HPLC machine isocratically, utilizing on E. Merck RP-18 e column (250 × 4.0 mm, 5 μm) with a diode array detector (SPD M-10 A VP/RF-10 AXL fluorescent detector) and auto-injector SIL-10 AD VP. Elution was carried out with the mobile phase (MeOH:H₂O 60:40) for 30 min at a flow rate of 0.8 ml min⁻¹, and a wavelength of 240 nm was used for measurement. After isocratic analysis, the fractions were reprocessed using gradient analysis (MeOH:H₂O) at 35:65 (5 min), 65:35 (65 min) and 35:65 (75 min). HPLC profiles are illustrated in Fig. 1 a–c. The different lignans (99% purity) were isolated and characterized by a team of natural product chemists under the supervision of Dr S. C. Puri at the Regional Research Laboratory, Jammu. A standardized mixture of known concentrations of seven compounds (Fig. 1a)—dm2PTG (R = glycoside; R’ = H; R” = H), PDT (R = H; R’ = OH; R” = CH₃), dmdeoPTD (R = H; R’ = H; R” = CH₂), deoPTD (R = H; R’ = H; R” = CH₂) and cis-configuration [includes picroPTD (R = H; R’ = OH; R” = CH₃) as well as isopicroPTD (R = H; R’ = OH; R” = CH₂; isomeric form of picroPTD)]—was used to create standard curves (percentage area with respect to the quantity of pure compound based on isocratic analysis). The quantitative analysis of the aryl-tetralin lignans in different fractions was carried out using by co-spiking percentage area at a particular retention time of S1 and S2 indicated the corresponding quantity of a particular compound; Fig. 1b and c. Ten microliters of stock solution (1 mg ml⁻¹) of S1 and S2 (filtered via a 0.2 μm filter) was injected separately into the column and all the measurements were made while maintaining the column temperature at 30°C.

Ammonium Thiocyanate Assay-Based Study of Pro-antioxidant Activity in the Lipid Phase

The biphasic activity of S1 and S2 was evaluated using the ammonium thiocyanate assay, for which the linoleic acid pre-emulsion was prepared as described elsewhere (18). Linoleic

Methods

Plant Material Authentication and Preparation of Fractions

Live plant material of P. hexandrum Royle (syn. Podophyllum emodi Wall) was collected from the high-altitude regions of Sonmarg (>3000 m), Kashmir Himalayas, Jammu and Kashmir, and transplanted under natural conditions at the Regional Research Laboratory, Field station, at Bonera-Pulwama (<2000 m) for developing agro-technological protocols. The
Figure 1. Parent compound of family of aryl-tetralin lignans. (a) Podophyllotoxin skeleton. (b) HPLC profile of S1 (gradient analysis) and (c) HPLC profile of S2 (gradient analysis).

Acid was subjected to a slow autooxidation and exposed to radiation; peroxyl ion levels built up over time. A time-dependent study was performed: samples were exposed to supra-lethal radiation (0.25 kGy) from a $^{60}$Co gamma chamber (Gamma Cell 5000, Bhabha Radiation Isotope Technology, Mumbai) at a dose rate of 3.20 kGy h$^{-1}$ after 24 h of incubation. Aliquots were taken at different time intervals (27, 48 and 52 h) to monitor peroxyl flux assay. Similarly, aliquots were collected (+2 h) for Fe(II), Fe(III) and Cu(II) ± 0.25 kGy-induced lipid oxidation studies. In this monitoring assay, the peroxyl ions react with ammonium thiocyanate (30%) and ferrous chloride (0.1%) to form red-colored ferrothiocyanate readable at 500 nm.

Role of ROS Quenching Potential

The non-site-specific and site-specific reactive oxygen species (hydroxyl ion) quenching potential of S1 and S2 was evaluated.
as described elsewhere (19). In the non-site-specific assay, fenton-reaction-mediated hydroxyl ion production predominates and thereby induces deoxy-d-ribose degradation. Fe(II) directly attacks deoxy-d-ribose, leading to the formation of malondialdehyde (MDA). In the monitoring assay, MDA reacts with thiobarbituric acid in the presence of trichloroacetic acid to produce chromogen complex (pink color) readable at 532 nm.

Reactive nitrite species (nitric oxide) quenching potential was evaluated using the procedure standardized earlier in our laboratory, and described by Sagar et al. (14). The nitric oxide quenching ability of S1 and S2 was evaluated as a decrease in percentage absorbance of the complex formed by diazotization of nitrite (generated using sodium nitroprusside) with sulphanilamide and subsequent coupling with naphthylethylendiamine leading to a colored complex (violet) readable at 546 nm (20).

Electron Donation Ability Directly Related to Optical Density Increase

The electron donation capacity of both the fractionated fractions was evaluated as reductant power using a modified potassium ferricyanide method (21), as described elsewhere (15). The green chromogen complex formed as a final product of the reaction between herb fraction-induced reduced potassium ferrocyanide and exogenously supplied ferric ions. Electron donation capacity has a direct relation to the increase in optical density of the complex recorded at 700 nm.

Correlation Analysis

A correlation analysis was carried out between the maximal efficacy of each fraction in terms of mean protective potential against different stresses ± flux—induced lipid oxidation, ROS/RNS quenching ability or electron donation ability and the ratio of change in aryl-tetralin content.

Statistical Analysis

Significant differences between means of antioxidant activity (time-dependent study) in the lipid phase were determined using analysis of variance (ANOVA) followed by Tukey’s HSD test at \( P < 0.05 \) using SPSS software ver.10. Student’s \( t \)-test was used to obtain significant differences between the means of triplicates of other tests. \( P < 0.05 \) was considered to be significant.

Results and Discussion

Standardization of S1 and S2

‘Antioxidants’ work primarily by donating an electron to the free radical, chelating transition metals, boosting natural antioxidant enzymes, protecting cellular organelles against respiratory burst or enhancing detoxification enzymes, thereby reducing the amplification of free-radical-induced oxidative stress (5). The failure of biological system owing to radiation flux is primarily mediated by free radical burst (7,22). In this study we examined the correlation between aryl-tetralin lignans content and the radioprotective properties of two fractionated extracts of *P. hexandrum* (S1 and S2). HPLC fingerprinting carried out for standardization of the phytofractions is illustrated in Fig. 1b and c.

Modulation of Autooxidation of Lipid Component

Autooxidation of the phospholipid content of biological membranes generates highly reactive hydrogen peroxide radicals (LOOHs) that are further decomposed into a variety of products, including aldehydes, secondary ketones and alcohols (23). The ability to scavenge the peroxyl radical (LOO-) determines the potency of a fraction in the lipid phase. To mimic the slow and progressive degradation of lipid membranes initiated by radiation, an ammonium thiocyanate assay was used. Peroxyl ions were generated in an ex vivo system using a linoleic acid pre-emulsion (containing Tween-20) that degrades slowly over a period of time (3 days). After 24 h of incubation, a supra-lethal radiation exposure was given to half of the aliquots to understand the defensive ability of each fraction against radiation-induced free radical flux over a period of time. At 52 h, a decrease in optical density was observed, indicating the end point of the experiment. The maximal peroxyl ion scavenging potential of S1 (at the 48 h time interval) observed at 1000 \( \mu \text{g ml}^{-1} \) (45.88%; Fig. 2a) was comparable to that of S2 (41%; 1000 \( \mu \text{g ml}^{-1} \); Fig. 2b). The observed ability could be associated with the bioactive compounds of the fraction, i.e. aryl-tetralin lignans, which are most likely acting in synergism due to their miscibility in the lipid phase. The higher levels of deoPDT (1.59-fold higher), isopicroPDT (34-fold) in S1, compared with S2, indicated a perfect positive correlation (\( r = +1 \)) between levels of aryl-tetralin lignans and mean anti-lipid oxidation ability. Other lignans present in different plant species are also well known for their ability to act as antioxidants (24).

Radiation-Induced Peroxyl Flux

In case of flux only (0.25 kGy + 2 h), S2 exhibited significant \( (P < 0.05) \) inhibition of lipid oxidation (54.08%) compared with S1 (13.2%) at 2.5 \( \mu \text{g ml}^{-1} \), which could be attributed to the greater ratio of isopicroPDT/PDTG in S2 (0.155) compared to S1 (0.013) (Tables 1 and 2).

Metal-ion-Induced Amplification of Free Radical Flux

**Fe(II) and Flux-Induced Stress**

In the presence of Fe(II) and flux, both S1 and S2 exhibited similar protection (>30%) at 2.5 \( \mu \text{g ml}^{-1} \), in accordance with their metal chelation activity observed in an Fe(II) control. Fe(II) ions are physiological amplifiers of radiation-induced oxidative stress and our earlier reports support this
observation based on different high-altitude plant species (5,15) (Tables 1 and 3).

**Fe(III) and Flux-Induced Stress**

S1, in the presence of Fe(III) exhibited significant \( P < 0.05 \) pro-oxidant activity (79.2%) compared to S2; the addition of flux resulted in an increase in antioxidant activity (Tables 1 and 4). Such biphasic activity could be due to the conversion of Fe(III) to Fe(II) by donation of an electron at lower concentrations, similar in action to ascorbic acid (25). An increase in antioxidant activity with the addition of flux can also be visualized in terms of a rapid conversion of moieties (contributing

![Figure 2](image)

**Table 1.** Aryl-tetralin lignans present in S1 and S2 fractions

| S. no | Phyto-constituents identified | Relative percentage of the major constituents | Relative ratio of the lignans in S1/S2 |
|-------|------------------------------|---------------------------------------------|--------------------------------------|
|       |                              | S1: 27.02068                  | S2: 27.02068                  | 2.93                                      |
| 1     | 4-Demethyl podophyllotoxin glycoside | 5.843573 | 5.843573 | 2.93 |
| 2     | Podophyllotoxin-β-D-glucopyranoside | 51.39492 | 42.02375 | 16.58 |
| 3     | Podophyllotoxin                   | 27.50243 | 14.26229 | 26.14 |
| 4     | 4-Demethyl deoxy podophyllotoxin | 14.0032  | 5.53672 | 26.14 |
| 5     | Picropodophyllotoxin               | 0               | 0               | 0       |
| 6     | Ispicropodophyllotoxin            | 0.714151 | 6.551496 | 1.47 |
| 7     | Deoxypodophyllotoxin              | 0.541736 | 4.608124 | 1.59 |

*P < 0.05 for variation between fractions.*
to pro-oxidant activity) in the presence of oxidant species. This, in turn, indicates the latent effect of the solvent system utilized for their extraction. Such conversion exhibited an overall increase in metal chelation effect.

**Cu(II) and Flux-Induced Stress**

An increase in antioxidant activity in the presence of Cu(II) and flux was also observed, compared with Cu(II) only. This effect could be attributed to the metal-ion oxidation of bioactive constituents, as has been reported by other researchers (26). Upon comparison of S1 and S2, it was found that S2 exhibited significantly higher inhibition (64.2%) in the combined stress state, and this finding was similar to the case in which flux alone was used (Tables 1, 4 and 5).

**Reactive Oxygen Species and Amplification in the Aqueous Phase**

Radiation-induced, partially reductant, products of oxygen can be amplified by fenton-mediated reactions, showing the redox status of a biological system. With a view to mimicking such a chemical system, which is operational in our body, a deoxyribose assay was used (27) to evaluate the non-site-specific (Fe^{2+} + H_{2}O_{2} + EDTA) hydroxyl ion scavenging activity in an aqueous system. The hydroxyl ion scavenging potential
was found to increase in line with an increase in concentration (1–500 μg ml⁻¹) of S2 or S1 (Fig. 3a). The maximum percentage inhibition of S1 and S2 was 65.89 ± 0.96% (500 μg ml⁻¹) and 73.12 ± 0.97% (500 μg ml⁻¹), respectively; at 1000 μg ml⁻¹, S1 and S2 achieved equivalent scavenging potential. These results were in agreement with the ability of fractions to scavenge peroxyl radicals generated in the lipid phase. In the site-specific assay, the maximum percentage inhibition of S1 was 45.01 ± 0.6% (1000 μg ml⁻¹), significantly (P < 0.05) higher than that of S2 (19.1 ± 0.2% at 1000 μg ml⁻¹) (Fig. 3b). This study shows that S1 possesses higher chelation potential, which can be attributed to its higher aryl-tetralin lignan content (r = +1) compared with S2 (Table 1). The higher levels of 4-demethylpodophyllotoxin glycoside (2.9-fold) and podophyllotoxin β-D-glucopyranoside (16.5-fold) in S1 could further explain the augmented activity in the aqueous phase as both compounds possess a Lipinski score of 2/4, and therefore S1 possesses lower permeation properties, and thereby improved efficacy in the aqueous phase. Several researchers have reported that numerous phyto-constituents, such as silymarin, catechin and luteolin chelate metal ions, thereby offering radioprotection (5,28,29).

Reactive Nitrite Species

Nitric oxide readily diffuses across cells and is considered an important molecule in cellular signaling. Both S1 and S2 exhibited a dose-dependent increase in nitric oxide scavenging...
potential from 0.0625 to 0.025 µg ml⁻¹ (Fig. 4). S1 exhibited maximal nitric oxide scavenging potential (58.40 ± 0.8%) at 0.025 µg ml⁻¹, which was comparable to the activity exhibited by S2 (52.45 ± 0.825%). There are several reports suggesting increased nitric oxide synthase during radiation-induced oxidative stress (22), and some researchers have reported the role of nitric oxide in the pathogenesis of the inflammatory response in radiation enteritis (30). Giannopoulou et al. (2003) reported that amifostine exhibits antiangiogenesis activity (31) by reducing the levels of nitric oxide; they concluded that reduction in the levels of nitric oxide reduces the inflammatory responses associated with radiation. Our observation could be attributed to the aryl-tetralin lignan content, since it has been previously reported that podophyllotoxin is toxic in mammalian systems at a high concentration, but acts as an immunostimulant at lower doses (32). The relative difference between the activities of the two fractions was not very high, indicating that here the total lignan content accounts for nitric oxide scavenging activity.

**Electron Donation Ability in the Aqueous Phase**

S1 and S2 exhibited equivalent electron donation potential between 1 and 10 µg ml⁻¹. In the concentration range 25–2000 µg ml⁻¹, S1 exhibited significantly ($P < 0.05$) higher optical density than S2 (Fig. 5). The electron donation potential of S1 could be attributed to the higher concentrations (2- to 16-fold) of glycoside derivatives of podophyllotoxin. These glycosides possess higher molecular weight (>500 Da) and thereby have Lipinski scores less than 4, indicating their increased solubility in aqueous phase (33). It is likely that these compounds were acting in synergism with the polyphenolic constituents. These results agree with our earlier findings with high-altitude *P. hexandrum* (15). In addition, these results are in agreement with earlier studies (based on electron donation potential) with different extracts by other workers (34). This suggests that some of the compounds in the phytotfractions evaluated in fact are electron donors and react with free radicals and, thereby, terminate radical chain reactions.

In conclusion, S1 was found to have significantly higher ability to tackle radiation-induced oxidative stress than S2, leading us to conclude that enriched aryl-tetralin lignan content influences radioprotective potential to a great extent. S1 and S2, the fractions evaluated in the present study, were prepared using rhizomes obtained with farming methods under fairly standardized conditions. The study indicates the potential future of agro-technological practices for the production of biologically active enriched secondary metabolites from endangered or rare medicinal plant species for commercial exploitation.

**Acknowledgments**

Raman Chawla and Shikha Singh are grateful to CSIR/UGC for the award of fellowships. Research grants received from DRDO under the CHARAK 'Herbs for Health of the Armed Forces' program are duly acknowledged.

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Received February 5, 2006; accepted May 19, 2006