academicJournals

Vol. 7(44), pp. 3237-3241, 25 November, 2013
DOI: 10.5897/JMPR2013.4491
ISSN 1996-0875 ©2013 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Podophyllotoxin content in leaves and stems of *Podophyllum hexandrum* Royle from Indian Himalayan region

Hemant K. Pandey[1,2], Shyamal K. Nandi[1]* and Lok Man Singh Palni[1,3]

[1]G. B. Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Almora-263 643, Uttarakhand, India.
[2]Agro Division, Merino Industries Ltd, Achheja, Hapur-245 101, Distt. Ghaziabad, Uttar Pradesh, India.
[3]1-A/B, Riturain, Woldorf Compound, Nainital- 263 001, Uttarakhand, India.

Accepted 5 November, 2013

The rhizomes of *Podophyllum hexandrum* Royle, a perennial herb found in restricted areas of the Himalayan region, is a commercial source of podophyllotoxin, a highly valued aryltetralin used as precursor of some widely used anticancer drugs. This detailed investigation highlights an alternative source of podophyllotoxin from leaves and stems of three morphological variants of *P. hexandrum* with one leaf (1 L), two leaves (2 L) and three leaves (3 L), collected from seven populations (2800 to 3600 m) of Uttarakhand state in Indian central Himalaya. In general, podophyllotoxin content (on % dry wt.) varied significantly ($p < 0.05$) between the morphological variants, in both leaf and stems. The content of leaf ranged from 0.001 to 0.599%; maximum (0.599%) podophyllotoxin in 3 L (Dodital), and minimum (0.001%) in 1 L and 3 L (Kedarnath) plants were detected. The mean podophyllotoxin content (population wise) was significantly ($p < 0.05$) higher in Dodital population, with maximum value (0.229%) in Dodital and minimum (0.003%) in Kedarnath. The levels in stem samples were found in the range of 0.001 to 0.596%, similar to those found in the leaves. While maximum amount (0.596%) was estimated in 3 L plants from Dodital, the minimum (0.001%) level was found in 1 L (Kedarnath), 2 L (Garur Chatti) and 3 L (Kedarnath) plants. The mean podophyllotoxin content (population wise) was significantly ($p < 0.05$) higher in Dodital, Dayara and Bharnala populations, with maximum value (0.234%) in Dodital. Both the leaves and stems of 3 L plants (from Dodital) contained maximum levels of podophyllotoxin. The observed variation in content emphasizes the need for the selection of 'elites' based on higher toxin content; use of stem and leaves should be seen as a renewable source of podophyllotoxin.

Key words: Conservation, leaf, podophyllotoxin, renewable, medicinal herb.

INTRODUCTION

Podophyllum hexandrum Royle (Syn. *Podophyllum emodi* Wale; Indian May apple; Family Podophyllaceae), an alpine Himalayan herb distributed in the Indian sub-continent, is a source of high value aryltetralin lactone (-), podophyllotoxin, the starting compound for the preparation of semisynthetic compounds, namely etoposide, etopophos and teniposide, which are well known effective anti-tumour agents (Canel et al., 2000; Schacter, 1996;

*Corresponding author. E-mail: shyamal_nandi@rediffmail.com. Tel: +91 5962 241041. Fax: +91 5962 241150.

Van Uden et al., 1989). The rhizome of *P. hexandrum* contains three times more podophyllotoxin than the American species *Podophyllum peltatum* (Fay and Ziegler, 1985). Owing to steady decline in its natural populations, largely due to reckless harvesting for pharmaceutical purposes, *P. hexandrum* has been listed as “endangered” (Ved et al., 2003).

Several reports on the occurrence of different lignans, including podophyllotoxin from the underground rhizomes of *P. hexandrum* and *P. peltatum* exist in the literature (Kitchlu et al., 2011; Moraes et al., 2000; Nadeem et al., 2007; Naik et al., 2010; Pandey, 2002; Pandey et al., 2007; Purohit et al., 1998; Sharma et al., 2000; Zheljazkov et al., 2011). Harvesting of rhizomes adversely affects subsequent regeneration of plants. Although several studies have reported the occurrence of podophyllotoxin from the leaves of *P. peltatum* (Bastos et al., 1996; Canel et al., 2001; Cushman et al., 2005; Moraes et al., 2000, 2002, 2005; Zheljazkov et al., 2011), however, only two investigations thus far reported this compound from *P. hexandrum* leaves (Pandey, 2002; Sharma, 2013).

In view of this, leaf and stem samples of *P. hexandrum* populations from alpine and sub-alpine areas of Garhwal and Kumaun regions of Indian central Himalaya have been analyzed as a possible sustainable and renewable source of podophyllotoxin.

MATERIALS AND METHODS

Plant

Morphological variants of *P. hexandrum* [with one leaf (1 L), two leaves (2 L) and three leaves (3 L)], were collected (in mid-September) from different locations in Garhwal and Kumaun regions (2800 to 3600 masl) of Uttarakhand state. The botanical identity of the plants was confirmed and voucher specimens deposited at G. B. Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Almora. The leaf and stem samples were sprinkled with a systemic fungicide, Bavistin (50% carbendazim, w/w; BASF, Mumbai) and brought to the laboratory. Plant parts (leaf and stem) were washed under running tap water, rinsed with distilled water (×3), allowed to air dry at room temperature (2 to 3 days) and powdered. Analyses were carried out on samples collected from 3 different plants for each population.

Extraction and purification

Podophyllotoxin analysis was carried out essentially following the method of Van Uden et al. (1989) with minor modifications as described earlier (Nadeem et al., 2007). In brief, the powdered samples (leaves/stem; 200 mg) were initially extracted with 4 ml of 80% (v/v) aqueous methanol (MeOH). Following sonication (1 h; Branson 2200; Branson Ultrasonics Corp., Danbury, USA), the samples were kept at room temperature (24 h), supernatant collected and residue re-extracted with 2 ml of 80% (v/v) aqueous MeOH (24 h). Both the supernatants were pooled and the final volume made up to 6 ml with 80% (v/v) aqueous MeOH. This supernatant was then partitioned (×3) with an equal volume of water and dichloromethane (1:1, v/v; 6 ml each time). The dichloromethane fractions were collected, combined and dried *in vacuo* (30°C) in a rotatory film evaporator (Polymix-RV-ML; Kinematica; Littau, Switzerland). The residue was then taken up in 1.0 ml of high performance liquid chromatography (HPLC) grade methanol for HPLC analysis.

HPLC analysis

Purified samples (20 µl) were subjected to HPLC system (Kontron Instruments, Milan, Italy) using RP-18 column (Lichrosorb, 250 × 4.6 mm id, 5 µm), and eluted isocratically with MeOH: H_2O (60:40, v/v; flow rate 1.0 ml/min). Detection was carried out at 290 nm using an online ultraviolet (UV) detector (Kontron). The podophyllotoxin content was calculated on the basis of peak area, using a standard curve made with the known quantities of reference compound (Sigma Chemicals Co., St. Louis, USA). The lower limit of detection of podophyllotoxin was found to be 25 ng.

Data analysis

Mean values of various treatments were subjected to analysis of variance (ANOVA) using statistical package for social sciences (SPSS) version 7.5. Significance level was determined (at $p < 0.05$) and significant difference was separated using Duncan’s multiple range test (DMRT).

RESULTS

Podophyllotoxin content of leaves and stems of *P. hexandrum* plants (variants with 1 to 3 L) from different populations is summarized (Table 1). In general, the content varied significantly ($p < 0.05$) between the morphological variants, in both leaf and stem. The content in leaves ranged from 0.001 to 0.599% (on dry wt. basis); maximum (0.599%) and minimum (0.001%) levels were found in samples of 3 L (Dodital), 1 and 3 L (both Kedarnath) plants, respectively. More specifically the content in leaves of 1, 2, and 3 L plants ranged from 0.001 to 0.077, 0.002 to 0.031% and 0.001 to 0.599%, respectively. The mean podophyllotoxin content in the leaves of variants amongst seven populations exhibited significantly ($p < 0.05$) higher value in Dodital population, which also showed maximum level (0.229%); minimum (0.003%) level was found in Kedarnath (Table 1).

Podophyllotoxin content of stem samples was in the range of 0.001 to 0.596%, similar to those found in the leaves (Table 1). The levels in stem portions of 1, 2, and 3 L plants ranged from 0.001 to 0.305%, 0.001 to 0.291% and 0.001 to 0.596%, respectively. Maximum amount (0.596%) was detected in 3 L plants collected from Dodital, while the minimum (0.001%) level was detected in 1 and 3 L, and 2 L plants collected from Kedarnath and Garur Chatti, respectively. The mean podophyllotoxin content (population wise) was significantly ($p < 0.05$) higher among Dodital, Dayara and Bharnala populations, with maximum (0.234%) in Dodital and minimum (0.003%) value in Kedarnath (Table 1). It was found that 1 and 3 L plants of Kedarnath population showed minimum

Table 1. Podophyllotoxin content in leaf and stem samples of morphological variants of *P. hexandrum* collected from different populations of Garhwal and Kumaun region of Indian central Himalaya.

Population (Place)	Altitude (m)	Leaf Type[#] (L)	Podophyllotoxin content* (% of DW)			
			Leaf	Population Avg.[$] (Leaf)	Stem	Population Avg. [$] (Stem)
Bharnala	2800	1	0.005±0.001[b]	0.040±0.016[b]	0.305±0.009[a]	0.118±0.047[ab]
		2	0.010±0.002[b]		0.025±0.003[b]	
		3	0.106±0.004[a]		0.025±0.004[b]	
Dayara	3000	1	0.067±0.004[a]	0.029±0.010[b]	0.201±0.011[b]	0.166±0.042[a]
		2	0.009±0.001[b]		0.291±0.007[a]	
		3	0.010±0.001[b]		0.007±0.001[c]	
Auli	3000	1	0.004±0.001[b]	0.007±0.002[b]	0.008±0.002[b]	0.022±0.013[b]
		2	0.002±0.001[b]		0.002±0.001[b]	
		3	0.015±0.003[a]		0.056±0.004[a]	
Dodital	3100	1	0.077±0.004[b]	0.229±0.093[a]	0.088±0.004[b]	0.234±0.091[a]
		2	0.011±0.002[c]		0.017±0.003[c]	
		3	0.599±0.009[a]		0.596±0.006[a]	
Garur Chatti	3200	1	0.009±0.001[b]	0.027±0.011[b]	0.044±0.003[b]	0.035±0.009[b]
		2	0.002±0.001[b]		0.001±0.000[c]	
		3	0.071±0.005[a]		0.059±0.003[a]	
Phurkia[+]	3260	1	0.018±0.001[b]	0.029±0.004[b]	0.021±0.003[a]	0.018±0.003[b]
		2	0.031±0.004[a]		0.027±0.004[a]	
		3	0.039±0.004[a]		0.006±0.002[b]	
Kedarnath	3600	1	0.001±0.000[b]	0.003±0.001[b]	0.001±0.000[b]	0.003±0.001[b]
		2	0.007±0.001[a]		0.009±0.002[a]	
		3	0.001±0.000[b]		0.001±0.000[b]	

[#]1, 2 and 3 denote the number of leaves (L); *Values are from composite samples of 3 different plants and represent mean of 3 separate HPLC analyses; [$]average of 1, 2 and 3 L variants for each population; ; [+]Kumaun region, the other 6 locations are from Garhwal region of Uttarakhand. All values are mean ± standard error. Mean values followed by the same letter(s) in a column are not significantly different ($p < 0.05$) based on DMRT. Avg = average, DW = dry weight.

(0.001%) content, in both leaf and stem samples.

DISCUSSION

In this study, the level of podophyllotoxin in the leaves and stems of *P. hexandrum* was found to be up to 0.599% (% of dry wt. basis). In an earlier study, similar levels of podophyllotoxin were detected in leaves and stems of this species (Pandey, 2002). However, these values are lower as compared to up to 5.80% reported from rhizomes/roots collected from the same region (Pandey, 2002). Recently, Sharma (2013) reported analysis of leaves samples from 4 high altitude populations (2730 to 3978 m; Himachal Pradesh state in Indian Himalaya) of *P. hexandrum,* and maximum podophyllotoxin content (0.30%) was found in Marhi (3300 m) population. The podophyllotoxin content detected in the present investigation is nearly 2-fold (0.599%) compared to that reported by Sharma (2013). On the other hand, leaves of *P. peltatum* contain high level of podophyllotoxin (5.2%) in comparison to rhizomes (Bastos et al., 1996; Canel et al., 2001; Moraes et al., 2000, 2002); subsequently, comparable levels of podophyllotoxin were reported from leaves and rhizomes (Cushman et al., 2005; Moraes et al., 2005). Levels up to

2.5% have also been estimated in leaves of the same species (Zheljazkov et al., 2011).

Podophyllotoxin content in rhizomes of *P. hexandrum* varies considerably and values up to 8.26% have been reported from samples collected from Uttarakhand and Himachal Pradesh states in Indian Himalaya (Purohit et al., 1998; Sharma et al., 2000). Furthermore, levels from 3.02 to 9.53% have been reported from 28 populations occurring at various altitudes (1570 to 4300 m) in Himachal Pradesh (Naik et al., 2010). Similar levels were also reported from the rhizome buds of various populations collected from Zanskar valley of Jammu and Kashmir state in Indian Himalaya (Kitchlu et al., 2011). Recently, Sharma (2013) reported podophyllotoxin levels ranging from 3.44 to 5.87% in rhizomes collected from Himachal Pradesh, and no relationship with content and altitude was observed. It must be mentioned that similar levels (5.80%) were also found in rhizomes/roots collected from Kedarnath area (3600 m) in Uttarakhand state (Pandey, 2002). An earlier study from this laboratory found levels ranging from 0.36 to 1.08% in 8 populations (2740 to 3350 m) of Kumaun region of Uttarakhand in Indian central Himalaya, indicating a positive correlation between podophyllotoxin content and altitude (Nadeem et al., 2007).

The variation in podophyllotoxin content in different populations of *P. peltatum* and *P. hexandrum* can be ascribed to genotypic differences (Bastos et al., 1996; Moraes et al., 2000; Nadeem et al., 2007; Naik et al., 2010). The age of the plant do influence active ingredient content in rhizomes of *P. hexandrum* (Pandey et al., 2007; Sharma et al., 2000). In this investigation, analyses were carried out on samples from wild populations, thus the age of sampled plants could not be ascertained. It is possible that variations arise due to the presence of different chemo types in natural populations as also on the method of extraction (Bastos et al, 1996; Canel et al., 2001). Both biotic and abiotic factors, including soil conditions also affect the lignan yield in *P. peltatum* (Moraes et al., 2005).

P. hexandrum populations do exhibit marked variation in seed character, isozyme patterns, photosynthetic rates and leaf morphology. An earlier study reported that the morphological variants (1 to 4 L plants) exhibited an inverse relationship between podophyllotoxin level and leaf number; the 4 L plants contained lower levels of toxin in comparison to 3, 2 and 1 L plants (Purohit et al., 1998). While the morphological variants (1 to 3 L) contained varied levels of the toxin, both the leaves and stems of 3 L plants (from Dodital) contained maximum level highlighting the need for selection of 'elites' based on higher toxin content. In an effort to encourage cultivation and ensure conservation, propagation of elites of this species has already been taken up and nurseries established near natural habitats.

Conclusion

At present, podophyllotoxin is extracted for commercial purposes from the rhizomes and roots of *P. hexandrum*. This investigation reports, for the first time, a detailed investigation on analysis of podophyllotoxin in leaves and stems of morphological (leaf) variants of this 'endangered' species. This may be considered an attractive alternative, in comparison to the practice of destructive collection of rhizomes and roots, as a renewable source of the compound for pharmaceutical purposes.

ACKNOWLEDGEMENTS

Financial support to Dr H Pandey from a project funded by the Department of Biotechnology is duly acknowledged. Dr Anil Kumar and Mr Lalit Giri are thanked for help in HPLC and statistical analysis, respectively. The Ministry of Environment and Forests, Government of India provided fundamental support to the Institution.

REFERENCES

Bastos JK, Burandt CL, Nanayakkara NPD, Bryant L, McChesney JDJ (1996). Quantitation of aryltetralin lignans in plant parts and among different populations of *Podophyllum peltatum* by reverse phase high performance liquid chromatography. J. Nat. Prod. 59:406-408.

Canel C, Dayan FE, Ganzera M, Khan IA, Rimando A, Burandt CL, Moraes RM (2001). High yields of podophyllotoxin from leaves of *Podophyllum peltatum* by in situ conversion of podophyllotoxin 4-o-β-D glucopyranoside. Planta Med. 67:97-99.

Canel C, Moraes RM, Dyan FE, Ferreira D (2000). Molecules of interest: Podophyllotoxin. Phytochem. 54:115-120.

Cushman KE, Maqbool M, Lata H, Bedir E, Khan IA, Moraes RM (2005). Podophyllotoxin content and yield of American may apple leaves in sun and shade. Hort. Sci. 40:60-63.

Fay DA, Ziegler HW (1985). Botanical source differentiation of *Podophyllum* resin by high performance liquid chromatography. J. Liquid Chromatogr. 8:1501-1506.

Kitchlu S, Ram G, Koul S, Koul K, Gupta KK, Ahuja A (2011). Podophyllum lignans array of *Podophyllum hexandrum* Royle populations from semi-desert alpine region of Zanskar valley in Himalayas. Ind. Crops Prod. 33:584-587.

Moraes RM, Bedir E, Barrett H, Burandt CL, Canel C, Khan IA (2002). Evaluation of *Podophyllum peltatum* L. accessions for podophyllotoxin production. Planta Med. 68:341-344.

Moraes RM, Burandt CL, Ganzera M, Li X, Khan I, Canel C (2000). The American may apple revisited *Podophyllum peltatum*, still a potential cash crop? Ecol. Bot. 54(4):471-476.

Moraes RM, Momm HG, Silva B, Maddox V, Easson GL, Lata H, Ferreia D (2005). Geographic information system method for assessing chemo-diversity in medicinal plants. Planta Med. 71:1157-1164.

Nadeem M, Palni LMS, Kumar A, Nandi SK (2007). Podophyllotoxin content, above- and below ground biomass in relation to altitude in *Podophyllum hexandrum* populations from Kumaun region of the Indian central Himalaya. Planta Med. 73:388-391.

Naik PK, Alam MA, Singh H, Goyal V, Parida S, Kalia S, Mohapatra T (2010). Assessment of genetic diversity through RAPD, ISSR and AFLP markers in *Podophyllum hexandrum*: a medicinal herb from the northwestern Himalayan region. Physiol. Mol. Biol. Plants. 16:135-148.

Pandey H (2002). Studies in relation to cotyledonary senescence and tissue culture of medicinally important alpine herbs. PhD thesis, Kumaun University, Nainital, India.
Pandey H, Nandi SK, Kumar A, Palni UT, Palni LMS (2007). Podophyllotoxin content in *Podophyllum hexandrum* Royle plants of known age of seed origin and grown at a lower altitude. Acta Physiol. Plant. 29:121-126.
Purohit AN, Lata H, Nautiyal S, Purohit MC (1998). Some characteristics of four morphological variants of *Podophyllum hexandrum* Royle. Plant Genet. Resour. Newsl. 114:51-52.
Schacter L (1996). Etoposide phosphate: what, why, where and how? Sem. Oncol. 23:1-7.
Sharma T, Singh BM, Sharma NR, Chauhan RS (2000). Identification of high podophyllotoxin producing biotypes of *Podophyllum hexandrum* from north-western Himalaya. J. Plant Biochem. Biotechnol. 9:49-51.
Sharma V (2013). Part based HPLC-PDA quantification of podophyllotoxin in populations of *Podophyllum hexandrum* Royle "Indian Mayapple" from higher altitude Himalayas. J. Med. Plant. Stud. 1:176-183.
Van Uden W, Pras N, Visser JF, Malingre TM (1989). Detection and identification of podophyllotoxin produced by cell cultures derived from *Podophyllum hexandrum* Royle. Plant Cell Rep. 8:165-168.
Ved DK, Kinhal GA, Ravikumar K, Prabhakaran V, Ghate U, Vijaya Sankar R, Indresha JH (2003). Conservation Assessment and Management Prioritization for the Medicinal plants of Himachal Pradesh, Jammu & Kashmir and Uttaranchal. Foundation of Revitalization of Local Health Traditions (FRLHT), Bangalore, India. pp. 1-24.
Zheljazkov VD, Cantrell CL, Astatkie T (2011). Variation in podophyllotoxin concentration in leaves and rhizomes of American mayapple (*Podophyllum peltatum* L.). Ind. Crops Prod. 33:633-637.