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Podophyllotoxin content in rhizome and root samples of *Podophyllum hexandrum* Royle populations from Indian Himalayan region

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The podophyllotoxin content in rhizome and root samples of *Podophyllum hexandrum* Royle (with leaf morphological variants, that is, 1, 2 and 3L), an endangered perennial herb and a source of highly valued aryltetralin lignan, collected from 17 different populations (2800 to 3600 m asl) spread across Uttarakhand State of Indian Central Himalaya were analyzed by high performance liquid chromatography (HPLC). In general, podophyllotoxin content (on percent dry weight) of both rhizomes and roots varied significantly ($p < 0.05$) between the morphological variants. The podophyllotoxin content of rhizomes ranged from 0.012 to 5.480%; maximum and minimum levels were recorded in 2L (Kedarnath population) and 3L variants (Ghangria population), respectively. The mean podophyllotoxin content (population-wise) varied significantly ($p < 0.05$) between different populations; in general, a positive correlation ($p < 0.01$) was observed between podophyllotoxin content and increase in altitude; population-wise maximum (2.053%) and minimum (0.045%) levels were recorded in Kedarnath and Dayara-1 populations, respectively. The levels in root samples ranged from 0.021 to 5.800%, similar to those in the rhizomes. While maximum amount (5.800%) was estimated in 2L plants from Kedarnath population, minimum (0.021%) level was found in 2L plants from Ghangria population. The mean podophyllotoxin content across morphological variants (population-wise) was significantly ($p < 0.05$) higher in Kedarnath population (maximum: 2.090%); a positive correlation ($p < 0.01$) was found between the podophyllotoxin content and increase in altitude. Amongst all the morphological variants analyzed from different areas, 2L plants of Kedarnath population (highest altitude; 3600 m) exhibited maximum podophyllotoxin content, both in the rhizomes and roots. The observed chemo-diversity amongst morphological variants (leaf number) and populations could be used for selecting elites for multiplication and commercial and/or conservation purposes.

Key words: Alpine, cultivation, *Podophyllum hexandrum*, May apple, podophyllotoxin.

INTRODUCTION

Podophyllum hexandrum Royle (Syn. *P. emodi* Wale; Indian May apple; Family Podophyllaceae), a perennial herb distributed of high altitude (2000 to 4000 masl) areas of the Himalayan region, is a source of high value

compound of non-alkaloid nature, podophyllotoxin [Chemical name: 5,8,8a,9-Tetrahydro-9-hydroxy-5(3,4,5-trimethoxyphenyl) furo [3',4':6,7] naphtho [2,3,d]-1,3-dioxol-6 (5aH)-one; MW: 414.40]. It is the starting compound for the preparation of semi-synthetic compounds, namely, etoposide, etopophos and teniposide, and is useful in the treatment of lung cancer and refractory testicular cancer, stomach and pancreatic cancers, and myeloid leukemias (Van Uden et al., 1989; Stahelin and von Wartburg, 1991; Schacter, 1996; Ekstrom et al., 1998; Holm et al., 1998; Ajani et al., 1999; Lee and Xiao, 2005). It is also a precursor to CPH 82 (Reumacon) under trial for rheumatoid arthritis in Europe (Calstrom et al., 2000). In addition, podophyllotoxin has also been reported as a potent antiviral agent (Beutner and von Krogh, 1990).

The rhizomes of *P. hexandrum* are known to contain three times more podophyllotoxin than the American species *Podophyllum peltatum* (Fay and Ziegler, 1985). The reported total synthesis of podophyllotoxin has been found to be uneconomical. Further, while some attempts have been made for podophylloxin production using callus and suspension cultures of *P. peltatum* (Kadakade, 1982) and *P. hexandrum* (Chattopadhyay et al., 2001), respectively, large scale exploitation of natural populations continues to be the main source for pharmaceutical companies. In India, the compound is primarily obtained from rhizomes of *P. hexandrum* with concomitant decline in its natural populations. The species is currently in the negative list of exports of the Ministry of Commerce, Government of India (Lakhanpal, 1998), and also listed as “endangered” (Ved et al., 2003).

The presence of different lignans, particularly podophyllotoxin, in the rhizomes of *P. hexandrum* (Purohit et al., 1998; Sharma et al., 2000; Pandey et al., 2007; Nadeem et al., 2007; Naik et al., 2010; Kitchlu et al., 2011) and *P. peltatum* (Moraes et al., 2000; Canel et al., 2001; Zheljazkov et al., 2011) have been reported. Harvesting of this perrennating underground organ, for the analyses or commerce, adversely affects growth in subsequent years.

In the acute paucity of quality planting material of *P. hexandrum,* large scale production of propagules, either by conventional and/or biotechnological means (Nadeem et al., 2000) assumes importance for commercial plantations to meet the pharmaceutical demand. Identification of elites, in terms of high podophyllotoxin content, is a pre-requisite for meeting the aforementioned objectives. In view of this, *P. hexandrum* populations from alpine and sub alpine areas of Garhwal and Kumaun regions of Uttarakhand State in the Indian Central Himalaya (ICH) have been analyzed for podophyllotoxin content in the rhizomes and roots.

MATERIALS AND METHODS

Plant

The rhizome and root samples of *P. hexandrum* Royle [morphological variants with 1-leaf (1 L), 2-leaf (2L) and 3-leaf (3L)] were collected between mid-September to mid-October during 2000 from different geographical locations of Garhwal and Kumaun regions (2800 to 3600 m asl) of Uttarakhand in ICH (Table 1). It must be mentioned that the 4-leaf (4L) variant could not be located in any of the populations examined in this study. The botanical identity of the plants was confirmed and voucher specimens deposited in the G.B. Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Almora. The samples were sprinkled with a systemic fungicide, Bavistin (50% carbendazim w/w; BASF, Mumbai) and brought to the laboratory. The rhizome and roots were carefully separated, washed under running tap water to remove the adhering soil particles, rinsed with distilled water (thrice) and then allowed to air dry at room temperature. After drying, the samples were ground to a fine powder using mortar and pestle and packed in airtight polythene bags for storage at 4°C before analyses. The chemical analyses were carried out using composite samples prepared from three different plants of each of the seventeen populations examined.

Extraction and purification

Analyses for the estimation of podophyllotoxin were carried out following the method of Van Uden et al. (1989). The powdered rhizome and root samples (in triplicate; 200 mg) were extracted with 4 ml of 80% (v/v) aqueous methanol (MeOH). Following sonication (Branson, USA) for 1 h, the samples were allowed to stand at room temperature (24 h), the supernatant collected and the residue re-extracted with 2 ml of 80% (v/v) aqueous MeOH (24 h). Both supernatants were pooled and the final volume made up to 6 ml with 80% (v/v) aqueous MeOH. The pooled supernatant was then partitioned (thrice) with an equal volume of water and dichloromethane mixture (1:1, v/v; 6 ml each time), and the dichloromethane fractions were collected, dried in vacuo (30°C) in a rotatory film evaporator (Kinematica, Switzerland). The dried extracts were individually dissolved in 1.0 ml of HPLC grade MeOH and subjected to HPLC analyses.

High performance liquid chromatography (HPLC)

Purified samples (20 µl) were subjected to HPLC analyses (Kontron Instruments Ltd, Italy) on a RP-18 column (Lichrosorb, 250 × 4.6 mm id, 5 µm) eluted in an isocratic mode with MeOH:H_2O (60:40, v/v) at a flow rate of 1.0 ml/min. Detection was carried out at 290 nm using an online UV detector (Kontron). The podophyllotoxin content was estimated on the basis of peak area, using a standard curve made with known quantities of reference compound (Sigma Chemicals Co., USA) (Nadeem et al., 2007). The lowest limit of detection of podophyllotoxin under these experimental conditions was 25 ng.

RESULTS

HPLC analyses of rhizome samples of 17 different populations from the Garhwal and Kumaun regions

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revealed a wide variation in podophyllotoxin content among the morphological variants (1 to 3L) (Table 1). In general, the content varied significantly ($p < 0.05$) between the leaf variants, and the podophyllotoxin levels ranged from 0.012 to 5.480% (on dry weight basis) between populations. Maximum (5.480%) podophyllotoxin content was found in Kedarnath (2L), followed by Harkidun (2L) and Goi-2 (2L) populations, while the minimum (0.012%) level was found in Ghangria (3L) population. The podophyllotoxin content in rhizomes of 1, 2, and 3L plants ranged from 0.020 to 1.293%, 0.021 to 5.480% and 0.012 to 0.925%, respectively. The mean podophyllotoxin content in the rhizomes across leaf variants of seventeen populations exhibited significantly ($p < 0.05$) higher value for Kedarnath (2.053%, maximum), Murapara (1.072%) and Phurkia-2 (1.009%) populations, while the minimum (0.045%) level was found in Dayara-1 population (Table 1). The regression analysis suggests that the podophyllotoxin content of rhizomes was positively correlated with increase in the altitude ($y=0.002x – 5.6228$, $R^2=0.6346$; $p < 0.01$).

The podophyllotoxin content of root samples also varied significantly ($p < 0.05$) between morphological variants, and the levels ranged from 0.021 to 5.800% (on dry weight basis) among different populations (Table 1). Maximum (5.800%) podophyllotoxin content was found in Kedarnath (2L), followed by Dodital-2 (2 and 3L; 2.407 and 1.793%, respectively), while the minimum level (0.021%) was found in Ghangria (2L) populations. The content in roots of 1, 2, and 3L plants ranged from 0.025 to 1.377%, 0.021 to 5.800% and 0.028 to 1.793%, respectively. The mean podophyllotoxin content in the roots of the leaf morphological variants of seventeen populations exhibited significantly ($p < 0.05$) higher values for Kedarnath (2.090%, maximum), Dodital-2 (1.859%) and Auli (0.486%) populations, while the minimum (0.025%) level was found in Ghangria population (Table 1). The regression analysis suggests that the podophyllotoxin content was positively correlated with increase in the altitude ($y=0.0018x – 4.9423$, $R^2=0.4326$; $p < 0.01$).

DISCUSSION

It has been observed in the present investigation that, in general, samples collected from the higher altitudes (alpine regions) contained greater levels of podophyllotoxin in comparison to samples from the lower altitude (sub-alpine). However, in a few cases samples from the sub-alpine region also contained higher amounts of the active principle. It is interesting that amongst the morphological variants (1 to 3L), 2L plants of Kedarnath population contained maximum podophyllotoxin content, both in rhizome (5.480%) and root (5.800%) samples. Population-wise estimates indicated maximum podophyllotoxin content (rhizome-2.05%, root-2.09%) in Kedarnath plants; for both rhizomes and roots, a positive correlation was observed between the content and increase in the altitude. The plants (all the three morphological variants) from Kedarnath region, therefore, need particular attention.

Podophyllotoxin content of *P. hexandrum* rhizomes varies considerably, and values up to 8.26% have been reported (Purohit et al., 1998; Sharma et al., 2000). Levels in the range of 3.02 to 9.53% have been reported from 28 populations occurring at various altitudes (1570 to 4300 m) in the Northwest Himalaya (Naik et al., 2010). Similar levels were also reported for some populations collected from the trans-Himalayan region (Kitchlu et al., 2011). An earlier study from this laboratory found levels ranging from 0.36 to 1.08% in 8 populations (2740 to 3350 m) of the Kumaun region of ICH, exhibiting a positive correlation between the content and increasing altitude (Nadeem et al., 2007).

The occurrence of morphological variants with 1 to 4 leaves (1 to 4L plants) has been reported with an inverse relationship between podophyllotoxin content and the leaf number; the 4L plants contained lowest levels of the toxin as compared to 3, 2 and 1L plants (Purohit et al., 1998). In addition, *P. hexandrum* populations do exhibit marked variation in seed character, isozyme patterns and photosynthetic rates (Bhadula et al., 1996; Purohit et al., 1998).

In a recent study, podophyllotoxin content in the leaves and stems of morphological variants (1 to 3L plants) of *P. hexandrum* collected from seven populations (2800 to 3600 m) of the same region varied significantly ($p < 0.05$) between the variants, both in leaf and stem; the content ranged from 0.001 to 0.60%, and maximum content (0.60% in both leaf and stem) was found in 3L plants collected from Dodital (3100 m) population (Pandey et al., 2013). In another study, a maximum of 0.30% podophyllotoxin was reported in leaf samples collected from high altitude populations of Himachal Pradesh (Sharma, 2013). These values were, however, lower compared to contents up to 5.2% reported from leaf samples of *P. peltatum* (Moraes et al., 2005).

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 has been reported to influence the toxin content in *P. hexandrum* (Sharma et al., 2000; Pandey et al., 2007). In this investigation, analyses were carried out on samples collected from wild populations; hence, the exact age of the sampled plants could not be ascertained. It is possible that variations arise due to the presence of different chemotypes in natural populations and also on the method of extraction (Bastos et al., 1996; Canel et al., 2001). Further, both biotic and abiotic factors, including soil conditions affect lignan yield in *P. peltatum* (Moraes et al., 2005). While the morphological variants (1 to 3 L) of *P. hexandrum*

Table 1. Podophyllotoxin content in rhizome and root samples of morphological variants of *P. hexandrum* collected from seventeen populations across Garhwal and Kumaun regions of Uttarakhand in the Indian Central Himalaya.

Population (Place)	Altitude (m)	Plant type[#]	Podophyllotoxin content* (% of DW)			
			Rhizome	Population average[$] (Rhizome)	Root	Population average[$] (root)
Bharnala	2800	1 L	0.319±0.004[a]	0.187±0.035[cd]	0.388±0.006[a]	0.334±0.021[b]
		2 L	0.157±0.004[b]		0.380±0.005[a]	
		3 L	0.085±0.006[c]		0.262±0.002[b]	
Manjhi	2850	1 L	0.549±0.004[a]	0.246±0.076[cd]	0.297±0.008[b]	0.392±0.057[b]
		2 L	0.123±0.004[b]		0.260±0.003[c]	
		3 L	0.064±0.004[c]		0.618±0.022[a]	
Ghangria	2850	1 L	0.042±0.003[b]	0.049±0.012[d]	0.025±0.004[a]	0.025±0.002[b]
		2 L	0.093±0.003[a]		0.021±0.004[a]	
		3 L	0.012±0.002[c]		0.030±0.003[a]	
Goi-1	2850	1 L	0.044±0.003[c]	0.188±0.036[cd]	0.044±0.003[b]	0.078±0.019[b]
		2 L	0.278±0.003[a]		0.153±0.003[a]	
		3 L	0.243±0.003[b]		0.037±0.003[b]	
Murapara	2900	1 L	1.293±0.004[a]	1.072±0.057[b]	0.193±0.004[c]	0.255±0.025[b]
		2 L	0.997±0.008[b]		0.352±0.006[a]	
		3 L	0.925±0.030[c]		0.221±0.006[b]	
Dayara-1	2900	1 L	0.020±0.001[a]	0.045±0.009[d]	0.041±0.002[c]	0.086±0.012[a]
		2 L	0.079±0.002[a]		0.121±0.002[a]	
		3 L	0.036±0.002[b]		0.097±0.002[b]	
Goi-2	2950	1 L	0.656±0.004[b]	0.804±0.227[bcd]	0.320±0.004[b]	0.280±0.068[b]
		2 L	1.652±0.012[a]		0.491±0.004[a]	
		3 L	0.103±0.005[c]		0.028±0.002[c]	
Dayara-2	3000	1 L	0.561±0.002[a]	0.206±0.089[cd]	0.275±0.004[a]	0.223±0.014[b]
		2 L	0.021±0.002[c]		0.182±0.004[c]	
		3 L	0.037±0.002[b]		0.213±0.004[b]	
Garur Chatti	3000	1 L	0.046±0.003[c]	0.208±0.045[cd]	0.055±0.003[c]	0.227±0.066[b]
		2 L	0.359±0.004[a]		0.486±0.003[a]	
		3 L	0.219±0.003[b]		0.140±0.002[b]	
Auli	3000	1 L	0.047±0.002[c]	0.142±0.028[d]	0.077±0.003[a]	0.486±0.190[b]
		2 L	0.137±0.004[b]		0.137±0.004[b]	
		3 L	0.242±0.002[a]		1.245±0.003[c]	
Dodital-1	3000	1 L	0.087±0.004[c]	0.193±0.034[cd]	0.464±0.004[b]	0.457±0.006[b]
		2 L	0.322±0.004[a]		0.436±0.004[c]	
		3 L	0.170±0.003[b]		0.470±0.003[a]	
Khatyia[+]	3000	1 L	0.544±0.003[a]	0.409±0.049[bcd]	0.639±0.003[a]	0.359±0.076[b]
		2 L	0.465±0.008[a]		0.321±0.004[b]	
		3 L	0.218±0.004[a]		0.118±0.002[c]	

Table 1. cont'd.

		1 L	0.507±0.003[b]		0.296±0.005[b]	
Phurkia-1[+]	3000	2 L	0.349±0.003[c]	0.512±0.048 [bcd]	0.509±0.005[a]	0.351±0.040[b]
		3 L	0.679±0.003[a]		0.246±0.004[c]	
		1 L	0.118±0.002[c]		1.377±0.349[c]	
Dodital-2	3100	2 L	0.920±0.005[a]	0.389±0.133[bcd]	2.407±0.007[a]	1.859±0.180[a]
		3 L	0.129±0.002[b]		1.793±0.013[ab]	
		1 L	0.976±0.006[c]		0.491±0.004[a]	
Phurkia-2[+]	3260	2 L	1.425±0.014[a]	1.009±0.116[b]	0.090±0.002[c]	0.338±0.063[b]
		3 L	0.626±0.006[b]		0.434±0.004[b]	
		1 L	0.284±0.007[c]		0.357±0.005[b]	
Harkidun	3400	2 L	2.087±0.012[a]	0.940±0.288[bc]	0.759±0.006[a]	0.425±0.088[b]
		3 L	0.449±0.006[b]		0.159±0.003[c]	
		1 L	0.412±0.007[b]		0.310±0.006[b]	
Kedarnath	3600	2 L	5.480±0.045[a]	2.053±0.857[a]	5.800±0.032[a]	2.090±0.928[a]
		3 L	0.268±0.005[c]		0.159±0.004[c]	

[#]1, 2 and 3 denote the number of leaves (L) in morphological variants; *Values are based on analyses of composite samples from 3 different plants of individual populations, and represent mean of 3 separate HPLC analyses; [$]average of 1L, 2L and 3L variants for each population; [+]From Kumaun region, the other 14 locations are from Garhwal region of Uttarakhand. All values represent mean± standard error. Mean values followed by the same letter(s) in a column are not significantly different ($p < 0.05$) based on DMRT.

from different populations contained varying levels of the toxin, the 2L plants from Kedarnath (highest altitude) contained maximum levels, both in the rhizomes and roots. These observations emphasize the need for selection of 'elites' based on higher toxin content. In view of the ever growing demand for podophyllotoxin and severe pressure on the natural populations, concerted efforts should now be directed towards selective propagation of elites. Propagation of *P. hexandrum*, both by conventional as well as *in vitro* methods reported from this laboratory (Nadeem et al., 2000), can be employed for mass scale multiplication of quality propagules for systematic cultivation in locations closer to their natural habitats (in alpine and sub-alpine regions) near village clusters. Modest efforts in this direction have been initiated and propagation of elites taken up and nurseries established in near natural habitats. Such steps would not only result in increased yields and assured supply, but would also pave the way towards conservation of this valuable medicinally important endangered species.

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Conflict of interest

Authors have not declared any conflict of interest.

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