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Studies on podophyllotoxin content in different populations of *Podophyllum hexandrum* Royle in Himachal Pradesh

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Abstract
Podophyllum hexandrum Royle, a valuable medicinal herb, grows in the Himalayan alpine and subalpine zones. Underground part of the plant yield highly valued lignin, podophyllotoxin which is effective in treatment of lung cancer, a variety of leukemias, and other solid tumors. The root and rhizome of the plant is being indiscriminately harvested in large quantities from the wild to meet the ever increasing demand of the crude drug for pharmaceutical purposes, leading to steady decline in its natural populations. Due to this reckless harvesting from wild the species has come up in the list of 'critically endangered' species as per IUCN criteria.

During the present study, ten population of *Podophyllum hexandrum* from Himachal Pradesh at altitude ranging from 2050 m to 3636 m amsl were studied for active constituent. Plants of *Podophyllum hexandrum* were observed to have different number of leaves/ plant *viz* single leaf, two leaves, three leaves and four leaves per plant.

The podophyllotoxin content in different types of plants of different population ranged from 2.93% to 6.31% in roots, 2.22% to 8.96% in rhizomes and 2.85% to 6.95% in whole underground part i.e. rootstock.

During the present investigation, plants from Koksar, Chatru and Chanshal ghati population excelled well in terms of podophyllotoxin content and further studies are needed for testing their superiority under cultivated conditions.

Keywords: *Podophyllum hexandrum*, podophyllotoxin, HPLC, populations, root and rhizome

Introduction

In the present scenario quality and quantity of medicinal plants are serious issues for the pharmaceutical and dietary supplements industries. Traditionally, the raw material of most of medicinal plants has being regularly harvested from the wild. Increasing public demand for these products is creating a serious environmental problem as demand is outpacing the supply and endangering the survival of many of these species in the world. The Himalayan region is home of numerous highly valued medicinal plants including *Podophyllum hexandrum* Royle which is a herbaceous, rhizomatous species of great medicinal importance, now endangered in India (Alam *et al*., 2009) [3].

Podophyllum hexandrum Royle belonging to family Berberidaceae, is a valuable medicinal herb commonly known as the Himalayan May Apple, grows in the Himalayan alpine and subalpine zones. Underground part i.e. rhizome and roots of the plant yield podophyllotoxin, an active ingredient used as a starting compound for the chemical synthesis of etoposide and teniposide, compound that are effective in treatment of lung cancer, a variety of leukemias, and other solid tumors (Sharma *et al.*, 2010) [9].

Though podophyllotoxin is present in different plant species, but in sufficient amounts it is present only in some species of genus Podophyllum. Podophyllum comprises about 22 species (Airi *et al*., 1997) [1] and out of the different species screened for podophyllotoxin and related lignans, Indian Podophyllum (*Podophyllum hexandrum* syn. P. Emodii) and American Podophyllum (*Podophyllum peltatum*) have been found promising with reference to podophyllotoxin contents. It has long been used by the Himalayan natives and the American Indians. *Podophyllum hexandrum* of Indian origin contains three times more podophyllotoxin than its American counterpart *Podophyllum peltatum* (Alam *et al*., 2009) [3].

Podophyllotoxin derived from Podophyllum species after derivatization used for curing various diseases like venereal warts, testicular cancer, small cell lung cancer, brain tumour, adenocarcinoma, carcinoma, melanoma, neuroblastoma, AIDS-associated kaposis sarcoma, etc. The *Podophyllum hexandrum* is also traditionally used as cathartic, cholagogue, alterative

and bitter tonic (Anonymous, 1969; Watt, 1972; Stahelin and Von-Wartburg, 1991; Husain, 1993; Mascaux *et al.*, 2000) [4, 14, 12, 7, 8]. There has been massive extraction of its rootstock (official part) over the last several decades leading to destructive harvesting. This has led to severe reduction in its population density and the species is now listed in endangered plant species category.

A species without enough genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites. Considering the importance, threat perception and need for sustainable supply of its rootstock, there is need of not only multiplying its stock (by organized cultivation) but also assessing the relative active content concentration in its populations in different agroclimatic regions and also in different morpho types. Such studies shall hopefully identify the best stocks/morpho types, which can be further multiplied (through cultivation). Further, studies on population genetic diversity and the structure of population within a species may not only illustrate the evolutionary process and mechanism but also information useful for biological conservation of *Podophyllum hexandrum.*

Material and Methods

Plant material

Mature plants of *Podophyllum hexandrum* were collected from alpine and sub alpine regions of Himachal Pradesh. Ten geographical distinct locations ranging from altitude 2050m to 3636m amsl were selected on the basis of survey as shown in table 1.

The selected plants in various sites were uprooted and washed under running tap water to remove soil particles. The washed plant material was then separated into roots and rhizome and were placed inside perforated paper envelopes. These small pieces of root and rhizome were dried under shade at room temperature and were then used for estimation of podophyllotoxin content.

Table 1: Sites studies for evaluation of podophyllotoxin

S. No.	Name of site	Altitude (m) amsl
1.	Chanshalghatti (Shimla)	3636
2.	Nichar (Kinnaur)	2890
3.	Katgaun(Kinnaur)	2855
4.	Koksar (Lauhal&Spiti)	3160
5.	Chatru (Lauhal&Spiti)	3389
6.	Jagrauta (Chamba)	2270
7.	Topi (Chamba)	2200
8.	Kalatop (Chamba)	2423
9.	Paulanhi (Chamba)	2510
10.	Manali (Kullu)	2050

Podophyllotoxin Analysis

Chemical and reagents used

The podophyllotoxin content in roots and rhizome of plant of different treatments was estimated through HPLC in the laboratory of Department of Forest Products. For extraction of podophyllotoxin, Methanol AR grade of CDH brand was used. HPLC analysis was performed by using HPLC grade solvents i.e Methanol and Water Merck brand. TLC was developed using TLC Silica gel 60 F254 plates of Merck brand. Podophyllotoxin and other lignans were visualized on TLC plates by the following reagents.

1. Iodine
2. Sulfuric acid: methanol (50:50)
3. Nitric acid: acetic acid (3:10)

Standardization of analytical method

Analytical method for estimation of Podophyllotoxin was standardized on Waters HPLC unit using Waters 515-HPLC pump with Sun fire C-18 column (4.6 x 250mm, 5µl), Rheodyne injector, Waters 2487 dual λ absorbence detector. Before injecting the sample of pure podophyllotoxin solution, HPLC system was calibrated by running it with HPLC grade methanol for overnight. HPLC was then standardized by injecting 20 µl methanolic solution of pure podophyllotoxin of 1 ppm concentration which showed single peak of podophyllotoxin at retention time of 7.1 min. The mobile phase used was HPLC grade methanol: water (62: 38) at a flow rate of 0.9 ml/min. Monitoring was done at 280 nm. For preparation of standard curve of podophyllotoxin solution of 50ppm (Fig 1), 100ppm and 200ppm concentration of pure podophyllotoxin was prepared in HPLC grade methanol. 20µl solution of each concentration was injected in HPLC and area under curve was recorded at 280 nm wavelength. For each concentration 20 µl volume of podophyllotoxin solutions were injected in triplicate. Mobile phase used was methanol: water (62: 38) at a flow rate of 0.9 ml/min. Monitoring was done at 280 nm. A graph constructed between concentration of podophyllotoxin in ppm and area under curve showed almost a linear straight line as shown in fig 2.

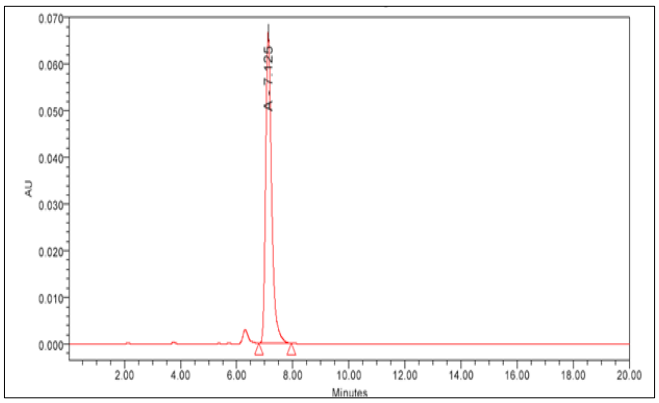

Fig 1: HPLC chromatogram of pure podophyllotoxin solution of 50ppm

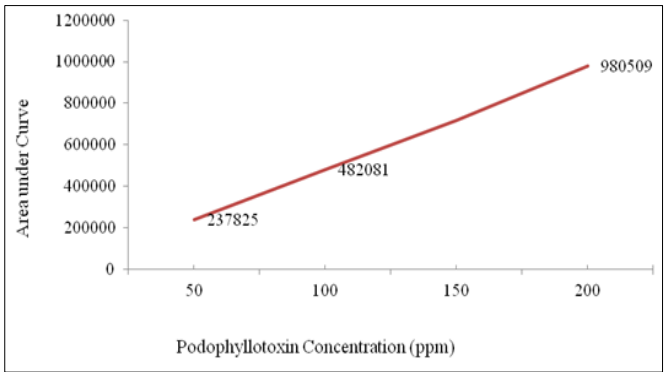

Fig 2: Standard curve of podophyllotoxin

Extraction and Determination of Podophyllotoxin

The oven dried (100 ºC) plant material was powdered in a pestle and mortar and weighed (1.0 g) for each sample of roots and rhizomes of *Podophyllum hexandrum*. The powdered plant material was extracted with methanol (100 ml) in a soxhlet apparatus for 8 hours. After extraction, solvent was distilled off and extracts were completely dried under reduced pressure. The residue of each sample was then dissolved in HPLC grade methanol and made to a total

volume of 10 ml in volumetric flasks. From the above solutions of roots and rhizomes, 1 ml volumes were pipetted out in 10 ml volumetric flasks for further dilution in 9 ml methanol (HPLC grade) and final volume of each was made to 10 ml. 20µl of this solution was injected in HPLC and observation on area under curve was recorded. The percentage of the podophyllotoxin in the sample was calculated by the following formula:

$$\text{Podophyllotoxin (\%)} = \frac{\text{Test area}}{\text{Standard area}} \text{ X } \frac{\text{Wt. of standard}}{\text{Standard dilution}} \text{ X } \frac{\text{Test dilution}}{\text{Test weight}} \text{X } 100$$

Evaluation of podophyllotoxin content in rootstock of different populations.

The plants of each population were grouped into three types i.e. single leaved plant, two leaved plant, three leaved plants. Three plants in each group were randomly selected in each population and were used for estimation of podophyllotoxin content with the help of HPLC as described earlier. The three type of plants were found in eight populations viz 1. Nichar; 2. Katgaun; 3.Koksar; 4. Jagrauta; 5. Topi; 6. Kalatop; 7. Paulanhi; 8.Manali. In two populations viz 1. Chanshal Ghati; 2. Chatru only single leaved and two leaved plants were found.

Statistical Analysis

Data regarding the estimation of Podophyllotoxin content were subjected to statistical analysis under RBD as described by Gomez and Gomez (1984) [6].

Results

Variation in podophyllotoxin content in different populations

The plants of different populations, on the basis of number of leaves/ plant were grouped into different Types i.e Single leaved, Two leaved, Three leaved and Four leaved plant. The podophyllotoxin content was estimated in roots, rhizomes and rootstocks of different types of plant of different populations the data was found statistically significant and is presented in table 2. The podophyllotoxin content ranged from 2.93 percent to 6.31 percent in roots and 2.22 to 8.96 in rhizome of different type of plants from different populations (Table 2).

It was found that the podophyllotoxin content in roots of the two leaved plant obtained from Chanshal Ghati region (altitude 3636 m) was high (6.31%) (Fig. 3) as compared to the root samples collected from other site. The lowest value of podophyllotxin content (2.93%) in root samples was obtained from two leaved plant of Manali (altitude 2050 m). In rhizome, high value of podophyllotoxin content (8.96%) was recorded in two leaved plant of Koksar (altitude 3160 m) (Fig. 4). The minimum podophyllotoxin content (2.22%) was recorded in two leaved plant of Manali (altitude 2050m). In rootstock, podophyllotoxin content ranged from 2.85 percent to 6.95 percent in different type of plants from different populations. Maximum podophyllotoxin content (6.95%) in rootstock was recorded in one and two leaved plants of Chatru (altitude 3389 m) (Fig. 5 & 6) whereas minimum value of podophyllotoxin content (2.85%) in rootstock was recorded in two leaved plant of Manali (altitude 2050 m).

Table 2: Variation in podophyllotoxin content in different populations

SITE	No. of leaves	Roots (%)	Rhizome (%)	Rootstock (%)
Chanshalghati	1	5.63	5.64	5.63
	2	6.31	4.89	6.09
Nichar	1	4.00	4.43	4.09
	2	5.03	5.94	5.15
	3	3.32	3.57	3.46
Katgaon	1	4.16	3.61	4.16
	2	4.09	4.83	4.25
	3	4.49	4.28	5.82
Koksar	1	4.64	6.90	5.10
	2	5.13	8.96	6.06
	3	4.58	6.67	5.19
Chatru	1	6.16	8.24	6.95
	2	5.29	6.50	5.54
Jagrauta	1	3.34	3.29	3.32
	2	5.10	4.43	4.99
	3	4.37	3.95	4.42
Topi	1	3.19	2.87	3.04
	2	5.59	5.29	5.66
	3	5.96	5.87	5.92
Kalatop	1	4.76	5.12	3.59
	2	4.61	4.61	4.64
	3	5.17	5.42	5.22
Paulanhi	1	4.83	4.24	4.67
	2	5.16	3.35	4.68
	3	4.09	3.80	4.00
Manali	1	3.18	2.90	3.16
	2	2.93	2.22	2.85
	3	3.69	3.83	3.71
$CD_{0.05}$		1.57	1.556	1.703

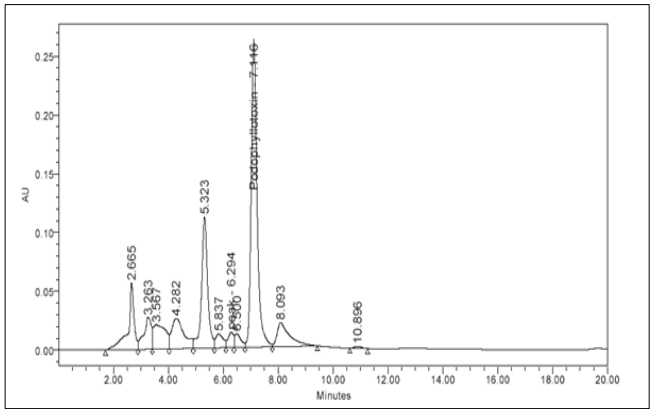

Fig 3: HPLC chromatogram in roots of the two leaved plant obtained from Chanshalghati region

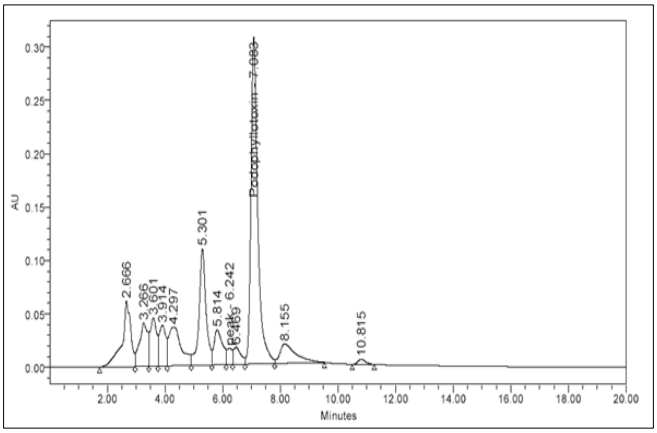

Fig 4: HPLC chromatogram in rhizome of two leaved plant at Koksar

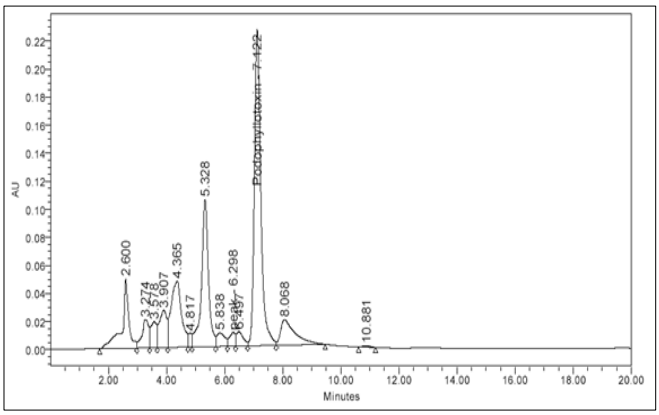

Fig 5: HPLC chromatogram of roots of one leaved plant at Chatru

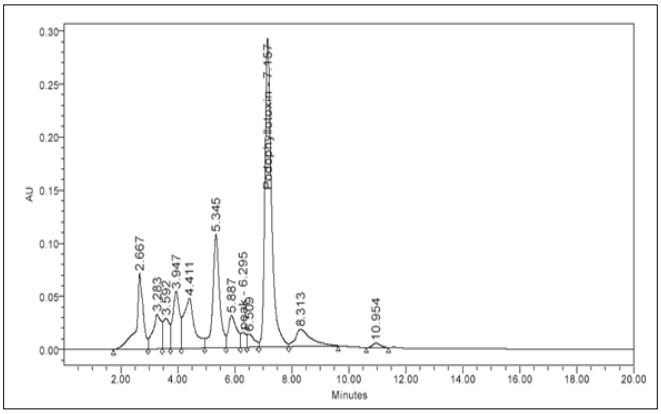

Fig 6: HPLC chromatogram of rhizome of one leaved plant at Chatru

Discussion

Podophyllotoxin content was estimated through HPLC in roots, rhizomes and whole underground part separately of different type of plants from different populations. The podophyllotoxin content ranged from 2.93 percent to 6.31 percent in roots and 2.22 percent to 8.96 percent in rhizomes of plants collected from different populations. Overall in underground part i.e. rootstock the podophyllotoxin content was recorded more than 6 percent in single leaved plant of Chatru (6.95%), two leaved plants of Chanshal Ghati (6.09%) and two leaved plants of Koksar (6.06%) populations. Significant difference in active content i.e. podophyllotoxin content (5.97% to 2.19%) in samples collected from different ecozones from Kashmir Himalayas was observed by Sultan *et al*. (2008) [13], Kullu and Lahaul Spiti area by Sharma (2013) [10] and Shah (2006) [11]. Alam *et al*. (2009) [3] also observed variation in podophyllotoxin content in *Podophyllum hexandrum* collected from 11 forest division of Himachal Pradesh with maximum podophyllotoxin content from the samples collected from Lahaul forest division. During the present investigation, the samples from Lahaul forest division i.e. Koksar and Chatru recorded higher podophylltoxin content. So the present findings are in agreement with the earlier findings of various workers (Alam *et al*., 2008; Sultan *et al*., 2008; Chopra *et al*., 1958) [2, 13, 5]. The aim of the present study was to identify the population/ morphotypes where *Podophyllum hexandrum* is found in North-Western Himalayas having the highest concentration of podophyllotoxin content so that these could be used for further multiplications and cultivation. During the present investigation, plants from Koksar, Chatru and Chanshal Ghati population excelled well in terms of podophyllotoxin content and further studies are needed for testing their superiority under cultivated conditions.

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