



Journal of Medicinal Plants Studies

Part Based HPLC-PDA Quantification of Podophyllotoxin in Populations of *Podophyllum hexandrum* Royle “Indian Mayapple” from Higher Altitude Himalayas

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In the present study, a simple, sensitive, selective and reliable HPLC method based on photo diode array detector (PDA) has been developed and validated for the simultaneous determination of an important compound (podophyllotoxin) in leaves and rhizomes parts of four different populations of *Podophyllum hexandrum* Royle from North Indian Himachal Pradesh higher altitude Himalayas. The analysis was carried out on a RP-18e (LiCrosphere, 5 μ m, 250 x 4.0 mm) column, with isocratic elution of acetonitrile: water (40:60, v/v). The method was validated for accuracy, precision, limit of detection and quantification. The regression equation revealed a good linear relationship ($r^2 = 0.9999$ for podophyllotoxin) within test ranges. The limit of detection and limit of quantification for analyte in PDA was 50 and 110 μ g, respectively. The method showed good reproducibility for the quantification of podophyllotoxin in *P. hexandrum*.

Keyword: HPLC-PDA, Podophyllotoxin, Indian Mayapple, *Podophyllum hexandrum* Royle, Himachal Pradesh, Higher altitude Himalayas.

1. Introduction

Podophyllum hexandrum Royle (Berberidaceae) is a herbaceous, rhizomatous species of great medicinal importance, now endangered in India. The “*Podophyllum*” means footed leaf and “*hexandrum*” stands for six stamens. *P. hexandrum* growing in the inner ranges of Himalayas from Kashmir to Sikkim at an altitude of 2500-4500 m, Shalai hills east of Shimla, higher range of Kangra, Kullu, Rohtang and Chamba of Himachal Pradesh, that produces abundant quantities of lignans in the rhizomes^[1,2,3,4]. In Kashmir, it is commonly known as “wun-wangan” and

other common name of *P. hexandrum* is “bankakri”^[5].

Lignans are the main class of natural products which are found in genus *Podophyllum*. The rhizomes of *P. hexandrum* yield cytotoxic lignan podophyllotoxin and resin due to which *Podophyllum* possesses anti-tumour activity^[6]. The phenylpropanoid derived lignan podophyllotoxin, occurring in *Podophyllum* species, is used as a starting compound for the semisynthesis of the more water-soluble antitumor, testicular, small cell lung cancer and certain leukemias agents like etoposide (VP-16-213),

teniposide (VM-26) and etophos, which have FDA approval in the United States^[7,8,9,10]. Podophyllotoxin is also a precursor for the new derivative CPH-82 (reumacon) being tested in Europe in phase III clinical trials for arthritis^[11], and some other derivatives for the treatment of psoriasis and malaria were also reported^[12,13]. In addition, podophyllotoxin and podophyllin (*Podophyllum* resin) are considered as active constituents in dermatologic products for therapy of genital warts^[14]. Indian *Podophyllum* (*Podophyllum hexandrum*) contains three-times more resin and podophyllotoxin (4.3%) than the American species, *Podophyllum peltatum* (0.25%), which additionally contains α - and β -peltatins^[15]. Anti-oxidant and radioactive properties of *P. hexandrum* were also reported by many researchers^[16,17,18,19].

Therefore, arresting the decline of population of *P. hexandrum* in the wild, studying the structure of the remaining populations and also quantify the contents of active constituents such as podophyllotoxin in different populations is of critical importance. Various HPLC methods for the determination of podophyllotoxin in the plant materials were given by different workers^[20,21,22,23,24]. Beside this some other analytical methods for the quantification of lignans in the genus *Podophyllum* have also been reported in the literature including HPLC^[25-26].

However, our study on *P. hexandrum* aimed on four geographical locations of higher altitude Northern Himalayas, Himachal Pradesh, India. Moreover, the impact of geographical distance/altitude and phytochemical analysis of podophyllotoxin content and their relationship with each other in the diminishing wild populations of *P. hexandrum* of these study areas has not been reported so far, which we feel is very important for plants conservation aspect and also for herbal formulation point of view.

Because, it is well known, that a species without enough genetic diversity is thought to be unable to survive with changing environments or evolving competitors and parasites^[27].

Thus in continuation to our previous study i.e. A simple micro-analytical technique for determination of podophyllotoxin in *P. hexandrum* roots by quantitative RP-HPLC and RP-HPTLC^[28]. Now, we have reported here only the phytochemical aspect on the basis of most important active constituent such as podophyllotoxin content in different plant parts (leaves and rhizomes) of four populations with the help of high performance liquid chromatographic (HPLC) technique.

2. Materials

2.1 Chemicals

All the chemicals, including HPLC solvents, were of analytical grade purchased from J.T. Baker, USA. The standard, podophyllotoxin was purchased from Sigma, New Delhi, India.

2.1.1 Plant Materials

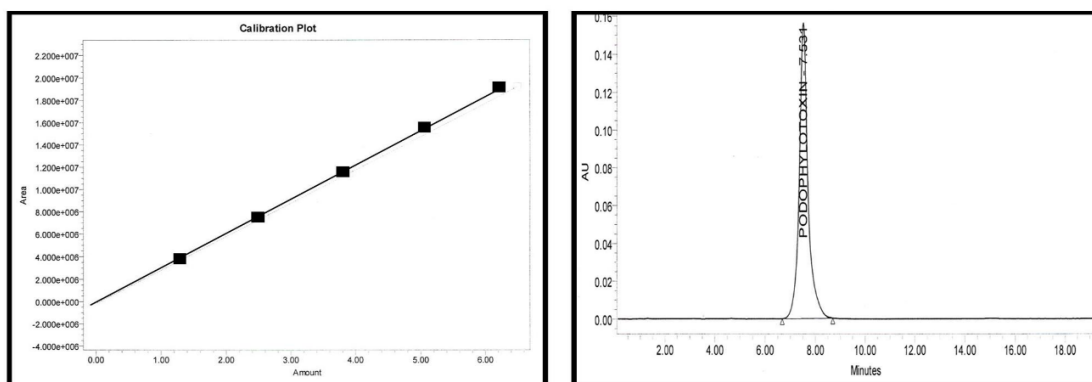
Plant materials for part basis HPLC analysis of four populations were collected from different altitude range of study areas of North Indian, Himachal Pradesh, higher altitude Himalayas during the months of July-September, 2008 (Table 1). Plants specimens were identified by the Botanical Survey of India (B.S.I., Northern Circle), Dehradun and specimens were deposited in the Herbarium, Department of Botany, Punjabi University, Patiala (Punjab), India.

2.1.2 Preparation of Sample Solutions

100mg of air dried plant material (leaves and rhizomes) of four populations were extracted three times with 20mL methanol for 6-8 hours. All the extracts are concentrated to dryness under reduced pressure 45°C. Dried extract then re-

dissolved in 2mL of HPLC mobile phase. Dried extract is filtered through 0.45 μ m

filter and degassed for one minute.



Figures 1. & 2. Calibration curve and chromatogram of standard podophyllotoxin.

2.1.3 Preparation of Standard Solutions

Stock solution of podophyllotoxin (1mg/5mL) was prepared in methanol and

different amounts were used for five points calibration curve.

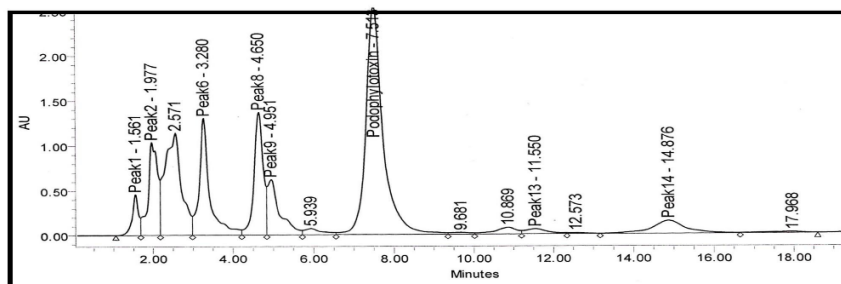


Figure 3. HPLC chromatogram of a rhizome sample PHR-1 (3300m) showing peak of podophyllotoxin.

2.2 HPLC Analysis

2.2.1 HPLC Instrumentation and Chromatographic Conditions

HPLC analysis was carried out Waters HPLC System 600 gradient pump; Waters 717 plus autosampler; 996 PDA detector; Empower Version 2 software. Separation was achieved on RP-18e (LiCrosphere, 5 μ m, 250 x 4.0 mm); Merck Made column. The mobile phase was consisted of acetonitrile: water (40:60, v/v) in isocratic elution with flow rate 1mL/min. Injection volume of standard and samples was 10 μ L

and run time was 20 min. The column temperature was kept 30 $^{\circ}$ C. The detection of analyte was carried out by using photodiode array detector with wavelength 240nm.

2.3 Method Validation

2.3.1 Calibration Curve

Stock solution containing of analyte podophyllotoxin 1mg/5mL was prepared in methanol and different amounts (5, 10, 15, 20 and 25 μ L) of these were used for preparation of five point calibration curve (Figure 1).

2.3.2 Limit of Detection and Limit of Quantification

Again stock solution containing analyte podophyllotoxin 1mg/1mL was prepared and diluted to six appropriate concentrations i.e. in the range of 50-1000 µg/mL and each solution was injected in triplicate. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at signal-to-noise ratio. The signal-to-noise ratio was calculated using Empower Version 2 Software. The LOD and LOQ values were

experimentally verified by injections of standard solutions of the compound at the LOD and LOQ concentrations.

2.3.3 Selectivity

The selectivity of the method was determined by analysis of standard compound and samples. The peak of standard podophyllotoxin (Figure 2) within the plant samples was identified by comparing their retention times and spectra with those of the standard.

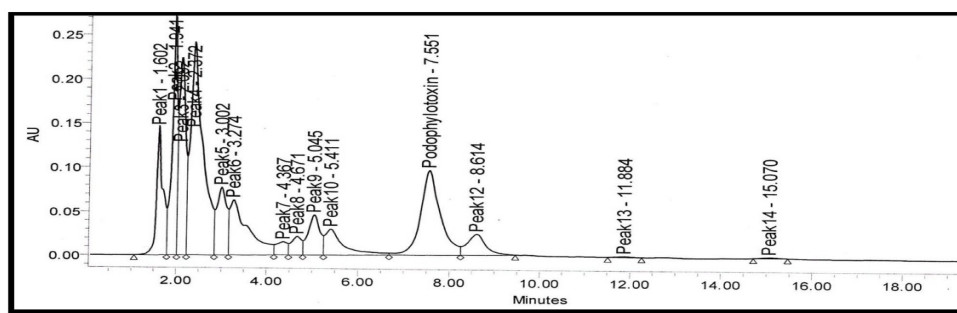


Figure 4. HPLC chromatogram of a leaf sample PHL-1 (3300m) showing peak of podophyllotoxin.

2.3.4 Accuracy

Recovery test was used to evaluate the accuracy of the method. For the percent recovery experiments, three different concentrations of reference compound (50, 100 and 150 µg/mL of compound podophyllotoxin was spiked to the three sets of *P. hexandrum* samples (100mg/2mL). The spiked samples were extracted with

optimized solvent system, i.e. methanol and analyzed by the proposed method.

2.3.5 Precision

The precision of the method was evaluated by adding different concentrations of reference compounds to the samples and comparing amounts determined from their chromatograms with the amount actually added.

Table 1. Plant materials collected from different regions of Himachal Pradesh higher altitude Himalayas.

S. No.	Plant Name	Location	Altitude (m)	Plant Parts & Sample Code	
				Leaves	Rhizomes
1.	<i>Podophyllum hexandrum</i> Royle "Indian Mayapple"	Marhi (H.P.)	3300	PHL-1	PHR-1
2.		Rohtang (H.P.)	3978	PHL-2	PHR-2
3.		Koksar (H.P.)	3160	PHL-3	PHR-3
4.		Kukumseri (H.P.)	2730	PHL-4	PHR-4

Table 2. Regression equation, limit of detection, limit of quantification and linearity range of podophyllotoxin.

Compound	Regression equation	Linearity range (µg/mL)	r ²	LOD (µg)	LOQ (µg)
Podophyllotoxin	$Y=2.978903e^{+006} X-1840603e^{+005}$	50-1000	0.9999	50	110

Table 3. Contents (%) of podophyllotoxin in leaves and rhizomes of four populations of *P. hexandrum* Royle.

Plant Parts	Sample Codes	Collection Area	Podophyllotoxin (%)
L E A V E S	PHL-1	Marhi (3300m)	0.30
	PHL-2	Rohtang (3978 m)	0.22
	PHL-3	Koksar (3160 m)	0.05
	PHL-4	Kukumseri (2730 m)	0.097
R H I Z O M E	PHR-1	Marhi (3300m)	5.87
	PHR-2	Rohtang (3978 m)	3.44
	PHR-3	Koksar (3160 m)	4.6
	PHR-4	Kukumseri (2730 m)	4.7

PHL-1: *P. hexandrum* Leaf-1; PHL-2: *P. hexandrum* Leaf-2; PHL-3: *P. hexandrum* Leaf-3; PHL-4: *P. hexandrum* Leaf-4; PHR-1: *P. hexandrum* Rhizome-1; PHR-2: *P. hexandrum* Rhizome-2; PHR-3: *P. hexandrum* Rhizome-3; PHR-4: *P. hexandrum* Rhizome-4.

Table 4. Recovery and precision data for analyte podophyllotoxin from *P. hexandrum* Royle.

Analyte	Amount present in plant material (µg)	Amount of standard added to sample (µg)	Average amount found in mixture (µg)	Average recovery percentage (%)	Mean (%)	RSD (%)
Podophyllotoxin	587.0	50	635.0	96.0	96.6	0.68
		100	683.5	96.5		
		150	733.0	97.3		

3. Results and Discussion

After trials of different compositions of water-acetonitrile as of mobile phase solvents to resolve the podophyllotoxin in the leaf and root extracts, the complete resolution (Figures 2, 3 and 4), could be achieved using acetonitrile–water as the solvent system (40:60, v/v) with a flow rate of 1mL/min, maintaining this composition up to the run-time reached 20 min. Under these HPLC conditions, the mean retention times (*R_t*) for podophyllotoxin, respectively, was 7.5 min. Calibration curve was constructed for PAD data by regression analysis of plots of peak area response (units) against amount of podophyllotoxin. The developed HPLC method was applied for the quantitative evaluation of podophyllotoxin in leaves and rhizomes parts of four populations of *P. hexandrum* collected from different altitude locations of North Indian higher altitude Himachal Pradesh, Himalayas. Calibration curve showed good linear regression ($r^2 = 0.9999$). The LOD (S/N=3) and LOQ (S/N =10) for podophyllotoxin were 50µg and 110µg respectively (Table 2). In all the (leaves and rhizome) samples, podophyllotoxin was recorded in the range of (0.05-0.30%) in leaves and (3.33-5.87%) in rhizomes. Maximum content of podophyllotoxin was recorded in rhizomes as compared to the leaves samples. In rhizome parts of all plant

samples, maximum content of podophyllotoxin was recorded in sample (PHR-1) 5.87% (Figure 3) collected from Marhi (3300 m) and lesser amount (3.44%) was observed in sample (PHR-2) collected from Rohtang (3978 m). Whereas, in all the leaves samples the maximum content 0.05% (Figure 4) was recorded in sample (PHL-1), collected from Marhi (3300 m) and lesser content was recorded in the sample (PHL-3) collected from Koksar (3160 m). All values are summarized in Table 3. The method was subjected to analysis of quality and validation parameters. The precision and recovery of the method were estimated to be better (96.6%) and RSD (0.68%) in *P. hexandrum* (Table 4).

4. Conclusion

From all the results, it is concluded that podophyllotoxin present in higher amount in plant population collected from Marhi (3300 m) region of North Indian, Himachal Pradesh, higher altitude Himalayas. All these plants were collected from the higher altitude range (2500-4000m) of Himachal Pradesh Himalayas and there is no impact of altitude on the content of podophyllotoxin. It is found in the sample (PHR-1) collected from Marhi (3300m) has more quantity of podophyllotoxin as compared to the sample PHR-2 from Rohtang (3978m). This indicates that, content of podophyllotoxin

decreased in rhizomes as the altitude increased and this phenomenon is reversed in case of leaves of *P. hexandrum*. The applied HPLC method is specific and can be referred for the simultaneous analysis of other active constituents in *P. hexandrum* plant and its products with good sensitivity, precision, and repeatability.

5. Acknowledgements

The authors are grateful to Honourable Donna Rougeau, Advanced Nutrients Ltd., Abbotsford (Canada, Woodland USA-or-Sophia, Bulgaria), Honourable Mohan Kumar, Director, Plant Genome Sciences Pvt. Ltd., Mumbai (India) and Dr. R.C. Gupta, Senior Professor & Former Head, Department of Botany, Punjabi University Patiala (Punjab) India and Dr. Bikram Singh, Senior Scientist & Head, NPP Division, IHBT (CSIR), Palampur (H.P) India for providing necessary research facilities and support.

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