Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(2): 3748-3752
Received: 21-01-2018
Accepted: 23-02-2018

Seema Sharma
Department of Forest Products, University of Horticulture and Forestry, Solan, Himachal Pradesh, India

Yash Pal Sharma
Department of Forest Products, University of Horticulture and Forestry, Solan, Himachal Pradesh, India

Chitra Bhardwaj
Department of Forest Products, University of Horticulture and Forestry, Solan, Himachal Pradesh, India

Correspondence
Seema Sharma
Department of Forest Products, University of Horticulture and Forestry, Solan, Himachal Pradesh, India

Development and validation of Hplc-Uv method for quantification of podophyllotoxin in *Podophyllum hexandrum* Royle

Seema Sharma, Yash Pal Sharma and Chitra Bhardwaj

Abstract
Podophyllum hexandrum Royle, (Podophyllaceae) a critically endangered Himalayan herb is a rich source of a neurotoxin compound-podophyllotoxin, which has antitumour activity and after derivatization to etoposide, etoposide phosphate and teniposide is used for treatment of various malignant conditions. A simple, precise and accurate HPLC method with UV-detection was developed and validated for quantification of podophyllotoxin in *Podophyllum hexandrum.* The method was validated according to the ICH guidelines which includes the parameters like accuracy, precision, linearity, range, LOD, LOD and robustness. Seperation was achieved on a reverse-phase C18 column using methanol: water: 62: 38 (v/v) as mobile phase in isocratic mode with a flow rate of 0.9 ml/min and detection was done at 280 nm. The method showed high correlation coefficients (R > 0.998) for standard subjected to the entire procedure. Value of RSD % of area for Inter-day and Intra-day precision of the developed method was 0.68 % and 0.66 % respectively. Detection and quantitation limits of the developed method were 0.026µg/ml and 0.106µg/ml respectively.

Keywords: podophyllotoxin, *Podophyllum hexandrum*, podophyllaceae, HPLC, method development, validation

1. Introduction
Podophyllum hexandrum Royle (Podophyllaceae) commonly known as bankakri is a herbaceous, rhizomatous plant of great medicinal value, which is now critically endangered in India and is listed in appendix-II of CITES [1, 2]. *Podophyllum hexandrum* is distributed in Himalayas from Kashmir to Sikkim at an altitude of 2500-4000m. Plants containing podophyllotoxin analogues have been used as folk medicine in traditional medicines for use in treatmemt of cancer, cold, cough, cuts, wounds, fever, ulcers, tumors *etc*.[3] In modern times, species is significantly valued due to its anticancerous properties of major active constituents *i.e.* podophyllotoxin. The phenylpropanoid derived lignan podophyllotoxin, occurring in *Podophyllum* species, is used as a starting compound for the synthesis of etoposide (VP-16-213), teniposide (VM-26) and etophos, which are used in the treatment of specific types of cancers like testicular, small lung cancer *etc.* Podophyllotoxin is also used as a precursor for new derivative CPH-82 (reumacon) being tested in Europe in phase III clinical trials for arthritis [4].
There are few methods in the scientific literature for determination and quantification of lignan podophyllotoxin in *Podophyllum hexandrum.* An HPLC method[5] was developed for determining the podophyllotoxin in roots of *Podophyllum hexandrum* using Lichrospher 100 5-RP-18e column, 1ml/min flow rate, detection wavelength at 230nm with mobile phase acetonitrile:water::40:60, v/v and validated for linearity, LOD and LOQ parameters. In another HPLC method[6] the quantitation of podophyllotoxin in *Podophyllum emodi* var. *chinensis* was performed in Kromasil-C18, solvent A, 25 m M phosphate buffer, pH 2.5, solvent B, methanol, gradient, 50/50/70% B at 0/13/33 min; flow rate, 0.8 mL/ min. detection UV 270 nm. Some other HPLC methods [7, 8] were developed and validated with different instrumentation and chromatographic conditions. The chromatographic instrumentation and conditions for HPLC validated method in our study are not reported earlier.
The scope of the present work was to develop a simple, accurate and precise method for podophyllotoxin determination in *Podophyllum hexandrum* using HPLC with a UV detector. Additionally, the method needed to be sensitive with a low limit of detection (LOD) and limit of quantitation (LOQ), where low concentrations of podophyllotoxin could be determined as its concentration in different parts of *Podophyllum hexandrum* is low. The objective of this work was therefore to develop and validate a simple, precise, accurate, robust and linear (with wide dynamic range) method for the determination of podophyllotoxin in *Podophyllum*

hexandrum. HPLC with a UV detector and isocratic elution method were used in the current work for podophyllotoxin determination in *Podophyllum hexandrum*. The method is simple with the reversed phase mode being used with isocratic elution and using a UV detector, which is available in most analytical laboratories. Validation of the method was conducted in accordance with the guidelines of ICH [9] which include linearity and range, accuracy, precision, robustness, limit of detection and limit of quantitation.

2. Results and Discussion

Method development

During the optimization of the method different columns of different brands like Grace spherisorb ODS-2, Waters spherisorb ODS-2 and Waters Sunfire C-18 (4.6 x 250mm, 5µm) were tried. Seperation was attempted by using aqueous methanol and aqueous acetonitrile separately with different flow rate. When methanol was used in mobile phase better separation was achieved and sharp peak shape than acetonitrile. Different detecting wavelengths, ratios of solvents (methanol: water) with different flow rate of mobile phase were tried. After series of screening experiments, it was concluded that Waters Sunfire reversed – phase column C-18 (4.6 x 250mm, 5µm) with methanol : water :: 62:38, at a flow rate of 0.9 ml/min. in isocratic mode gave better separation with good resolution of adjacent peaks. Fig. 2a shows a chromatogram of a standard solution of podophyllotoxin with a retention time of about 9.455 minutes. Fig 2b shows a chromatogram of podophyllotoxin in a sample of *Podophyllum hexandrum* rootstock.

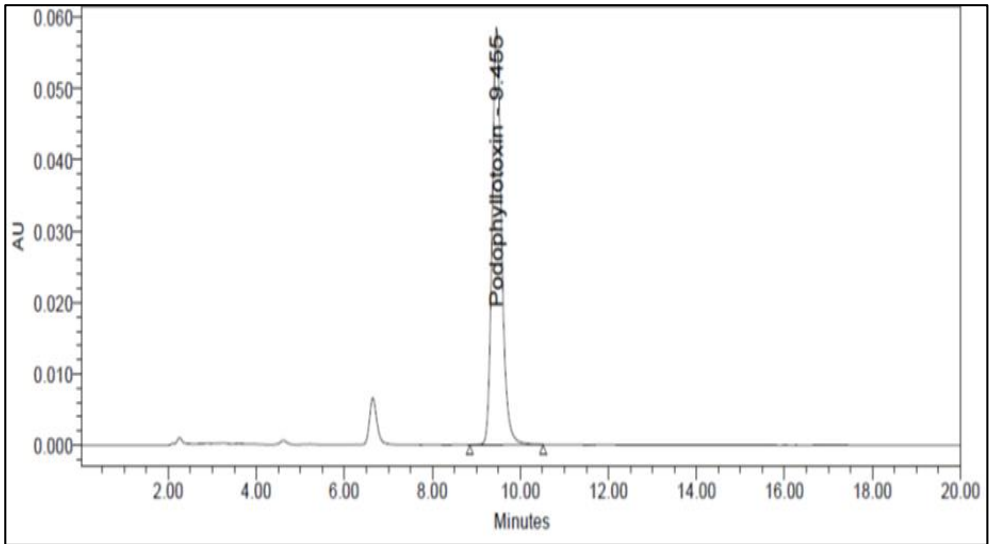

Fig 1: Linearity graph for Podophyllotoxin

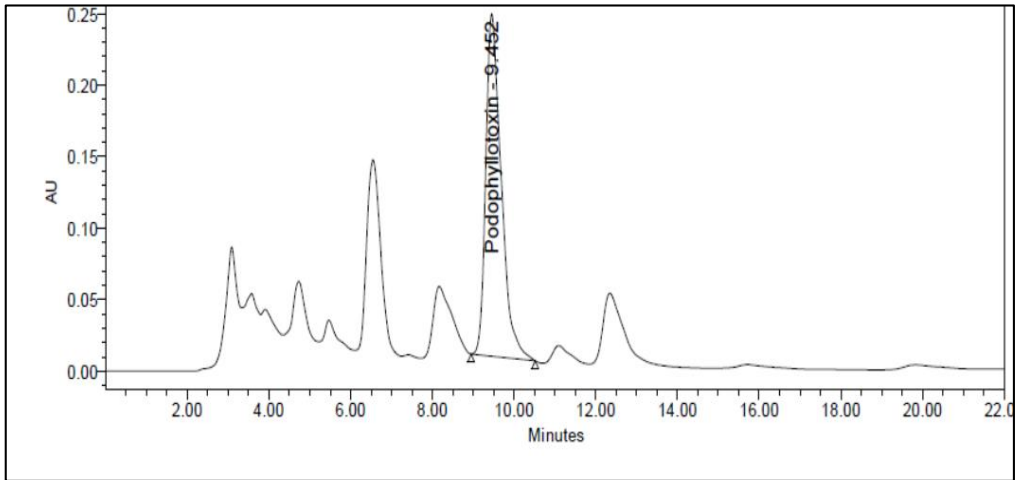

Fig 2a: HPLC chromatogram of Standard compound of Podophyllotoxin

HPLC Method Validation

Validation of the HPLC developed method was done for seven parameters as mentioned in ICH guidelines (2005) and procedure followed for testing these parameters was as per ICH guidelines (2005). Different parameters tested were Linearity, Range, Accuracy, Precision, Limit of Detection, Limit of Quantitation and Robustness.

1. Linearity

To evaluate the linearity of the current method for determination of Podophyllotoxin, different calibration standards of Podophullotoxin were analysed by HPLC-UV, and responses were recorded. Seven standard concentration were made as 42.5 µg/ml, 85 µg/ml, 170 µg/ml, 340 µg/ml, 510 µg/ml, 680 µg/ml and 850 µg/ml. Each concentration was injected in HPLC system in triplicate to study the linearity of the developed method. Linearity of the developed method was found in range from 42.5 µg/ml - 850 µg/ml with correlation coefficient value 0.999. Regression equation found was Y = 1.68e+004 X + 2.61e+005. The calibration curve was

constructed by plotting the mean peak area versus the concentration of each analyte as givent in Fig 1. The result demonstrated the linearity of this method over a wide range (Table. 1).

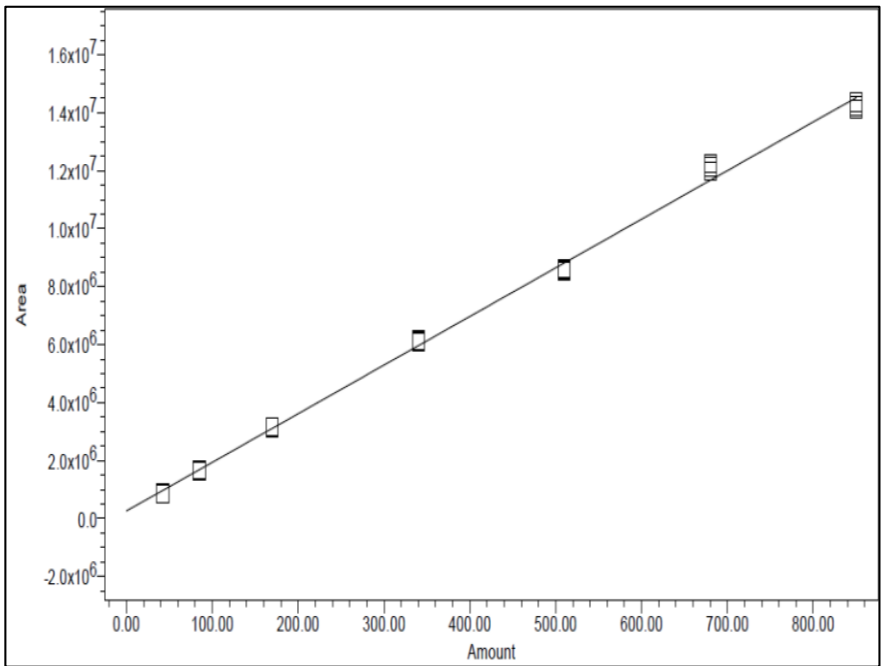

Fig 2b: HPLC Chromatogram of *Podophyllum hexandrum* sample

Table1: Results of Linearity and range

Phyto-constituent	Linearity(µg/ml)	Regression equation	Correlation coefficien(R)	Ret. Time(minutes)[a]	Range(µg/ml)
Podophyllotoxin	42.5-850	Y = 1.68e+004 X + 2.61e+005	0.998	9.455±0.057	42.5-850

[a]Mean±SD(n=18)

2. Range
The specified range was derived from interval between upper and lower values (including these values) of linearity. It was established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The range for Podophyllotoxin was found from 45.5-850µg/ml (Table.1).

3. Accuracy To study the accuracy of the method, recovery tests were carried out by adding three standard concentration levels as 85 µg/ml, 170 µg/ml and 340 µg/ml of podophyllotoxin to the known amounts 42.5µg/ml of podophyllotoxin standard. The injections were injected in three replications for each concentration. The standards were evaluated by calculating the proportion of the quantity of spiked concentration to the quantity of known initial concentration. Percent (%) was taken as measuring unit for recovery study. The results showed that the current method has good recovery (96.6-99.9%) (Table.3).

Table 3: Recovery studies of podophyllotoxin from spiked standard concentrations

Phytoconstituent	Initial quantity (µg/ml)	Added quantity (µg/ml)	Total quantity (µg/ml)	Total observed quantity [a] (µg/ml)	Recovery (%)
Podohpyllotoxin	42.5	85	127.5	123.25 ± 0.443	96.67
	42.5	170	212.5	210.01 ± 1.003	98.83
	42.5	340	382.5	382.17 ± 1.786	99.91

4. Precision
To study the precision of the method inter-day and intra-day precisions were determined.

1. Intra-day precision: One fixed concentration which was 85 µg/ml of podophyllotoxin analyte was selected and injected in six replications during a single day for Intra-day precision. The Relative standard deviation % (R.S.D.%) was taken as measurement of precision which was found 0.66% (Table 2)
2. Inter-day precision: Standard concentration 85 µg/ml of podophyllotoxin was selected and injected by duplicating the same concentration for six days successively for inter-day assessment. The Relative standard deviation % (R.S.D.%) was taken as measurement of precision which was found 0.68%

Table 2: Results of Precision, LOD and LOQ

Phyto-constituent	Precision		LOD (µg/ml)	LOQ (µg/ml)
	Intra-day (RSD %)	Inter-day (RSD %)		
Podophylotoxin	0.66 %	0.68 %	0.026	0.106

5. Limits of quantitation and limits of detection (LOQ & LOD)

LOQ and LOD of podophyllotoxin using this method was determined by injecting different standard concentrations (0.026 µg/ml, 0.053 µg/ml, 0.106 µg/ml, 0.425 µg/ml) concentrations and injecting these into HPLC system. LOQ of the developed method was selected to be the concentration that gave S/N ratio between 10 and 20, and LOD was selected as concentration that gave S/N ration between 3 and 10. Results showed that the LOQ and LOD of podophyllotoxin was 0.106 µg/ml, 0.026µg/ml, respectively. The low LOQ and LOD permit the determination of podophyllotoxin in *Podophyllum hexandrum* (Table 2).

6. Robustness

Robustness of the developed method was investigated by testing the influence of small changes in HPLC conditions as change in flow rate (±0.05%) and change in mobile phase composition. A fixed standard concentration was (85µg/ml) selected for robustness study. The selected concentration was injected in triplicate, with standard HPLC conditions, with change in mobile phase flow from standard 0.9 ml / min to 0.85 ml / min and 0.95 ml/min. and with change in mobile phase composition from standard methanol : water (62:38, v/v) to methanol : water (60:40, v/v) and methanol : water (61:39, v/v). The % RSD of the retention time was calculated for mean value of each factor. Results obtained are mentioned in Table 4 which indicates that separation was not affected by slight changes in the chromatographic conditions.

Table 4: Robustness studies of podophyllotoxin

Factor I	Podophyllotoxin	Factor II	Podophyllotoxin
Flow rate (ml/min)	**Retention Time**	**Mobile Phase (Methanol: Water:: 62:38, v/v)**	**Retention Time**
0.85	10.21	60:40	11.31
0.9	9.74	62:38	9.74
0.95	9.16	61:39	10.46
Mean	9.709	Mean	10.506
*S.D.	0.174	*S.D.	0.261
%RSD	1.796	% RSD	2.487

Applicability of developed method

Rootstocks of cultivated *Podophyllum hexandrum* were sourced and used for testing of the developed method. The results were shown in table 5. The developed method show the range of podophyllotoxin in samples from 0.412 % to 1.114 %. The minimum podophyllotoxin content was found in rootstock of one year plants which increased with age and recorded maximum in three year old plants.

Table 5: Quantification of podophyllotoxin in underground parts of *Podophyllum hexandrum*

Age	Rootstock
One year	0.412 (0.642)
Two years	0.961 (0.980)
Three Years	1.114 (1.055)
$CD_{0.05}$	0.031

Values in the parentheses are transformed values using $\sqrt{x}$ transformed values

3. Materials and Methods

Chemical and Standards

The chemicals used were of analytical and HPLC-grade which was used for extraction of samples and HPLC method development and validation. HPLC-grade methanol and HPLC water was purchased from Merck (Darmstadt, Germany). Standard compound podophyllotoxin was purchased from Sigma Aldrich, USA. Extraction of samples was done with AR Methanol of CDH brand.

Samples and Sample preparation

Underground parts of cultivated *Podophyllum hexandrum* of different ages (one year, two year and three year old plants) were procured from seed raised plants cultivated at Medicinal and Aromatic Plants Farm, Shilly (30˚54'32.40" N latitude and 77˚09'04.29" E longitude, elevation 1342m) Department of Forest Products, UHF, Solan (H.P.) India. Plant material was separately dried in shade till constant weight, then ground mechanically to form uniform particle size. Accurately weighed (1gm) samples replicated eigth times were extracted with methanol in soxhlet apparatus for two hours each. After extraction, the solvent distilled off to complete dry and dry extracted residue was used for sample preparation for HPLC analysis. The samples were prepared with mobile phase (methanol: water: 62:38, v/v), filtered through a 0.45µm filter and injected into HPLC for analysis.

HPLC Method

HPLC equipment was from Waters HPLC unit with Waters HPLC pump 515 and dual λ absorbance detector 2487. Empower software II was used for data acquisition and processing. The Sunfire C-18 (4.6 x 250mm, 5µm) was from Waters Corp. (Milford, MA, USA).

Chromatographic conditions

UV detection was employed at 280 nm, isocratic elution was used at a flow rate of 0.9 ml/min. and injection volume was set to 20 µl. The total run time of standard was 22 minutes with retention time of podophyllotoxin was 9.45 ± 0.017 min. (mean ± standard deviation of triplicate analysis).

Preparation of standard solutions

i. Preparation of stock and linearity solutions

Podophyllotoxin stock solution was prepared in methanol: water (62:38, v/v) for linearity study. Working solution concentrations of 42.5, 85, 170, 340, 510 and 680µg/ml were made.

ii. Solutions for Recovery study of Podophyllotoxin

For determination of recovery of podophyllotoxin from *Podophyllum hexandrum*, three solutions of 85 µg/ml, 170 µg/ml and 340 µg/ml were prepared and spiked to known concentration *i.e.* 42.5 µg/ml. Then the spiked concentrations were injected in HPLC equipment for analysis.

iii. Solutions for the LOD and LOQ

To determine the LOD and LOQ of podophyllotoxin using this method, solutions of low concentrations were made by serial diluting the lowest concentration (42.5 µg/ml, already made for linearity study) till we obtained S/N ration upto about 3.

All the data regarding the HPLC method development and validation was processed by Empower - II Software.

Conclusion

A simple, accurate, precise and selective HPLC method was developed and validated for the determination of podophyllotoxin in *Podophyllum hexandrum*. The method is

linear for the determination of podophyllotoxin with a wide dynamic range (42.5 – 850 µg/ml). This method also proved to be accurate where the % recovery of podophyllotoxin is within 96.6–99.9 %. Precision of the method is confirmed by a RSD value less than 3% of replicate injections of podophyllotoxin. The method showed good separation of podophyllotoxin from other compounds in *Podophyllum hexandrum* with good resolution. The low LOD and LOQ of podophyllotoxin enable the detection and quantitation of podophyllotoxin in *Podophyllum hexandrum* at low concentrations.

References

1. Samant SS, Pal M. Diversity and conservation status of medicinal plants in Uttaranchal State. *Indian For*. 2003; 9:1090-108
2. CITES. 2015. https://cites.org/sites/default/files/eng/app/2015/E-Appendices-2015-02-05.
3. Samant SS, Pant S, Singh M, Lal M, Singh Ashok, Sharma A, *et al*. Medicinal Plants in Himachal Pradesh, North Werstern Himalaya, India. Int. J Bio. Sc. Manag. 2007; 3:234-251.
4. Carlstorm K, Hedin PJ, Jansson C, Lerndal T. Sc. J Rheum. 2000; 29:89-94.
5. Mishra N, Acharya R, Gupta A, Singh B, Kaul VK, Ahuja PS. A simple microanalytical technique for determination of podophyllotoxin in *Podophyllum hexandrum* roots by quantitative RP-HPLC and RP-HPTLC. *Curr. Sc*. 2005; 9:1372-1373.
6. Liu CL, Jiao BH. LC determination of *Podophyllum* lignans and falvanoids in *Podophyllum emodi* Wall. var. *chinensis* Sprague. *Chromatographia*. 2006; 9:603-607.
7. Sharma V. Part based HPLC-PDA quantification of podophyllotoxin in populations of *Podophyllum hexandrum* Royle Indian mayapple from higher altitude Himalayas. J. Med. Plants St. 2013; 3:176-183.
8. Gupta DK, Verma MK, Lal S, Anand R, Khajuria RK, Kitchlu S. *et al*. Extraction studies pf *Podophyllum hexandrum* using conventional and non-conventional methods by HPLC-UV-DAD. J Liq. Chr. Rel. Tech. 2014; 37:259-273.
9. ICH Q2. (R1), Validation of Analytical Procedures: Text and Methodology. International Conference on Harmonization, IFPMA, Geneva, 2005.