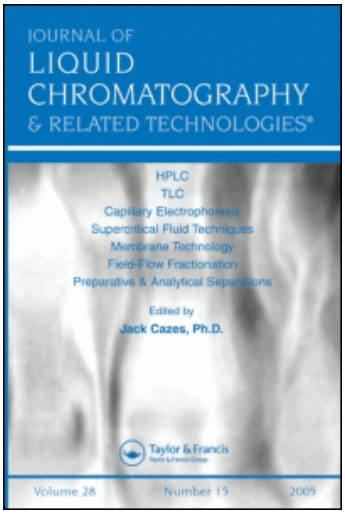

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A Rapid Determination of Podophyllotoxin in *Podophyllum hexandrum* by Reverse Phase High Performance Thin Layer Chromatography

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Abstract: A sensitive, selective, and precise RP-HPTLC method has been developed for the quantification of podophyllotoxin in *Podophyllum hexandrum*, collected from different locations of the western Himalaya of India, ranging from 3250 to 4100 meters above the mean sea level. The assay employed RP-18 F_{254} TLC plates (chromatoplates) as the stationary phase. The solvent system consisted of acetonitrile–water (4 : 6, v/v) and gave better resolution, with well separated, compact spots for podophyllotoxin (Rf value 0.41 $\pm$ 0.02). Densitometric analysis was carried out in the absorption remission mode at 217 nm.

Keywords: Podophyllum hexandrum, HPTLC, podophyllotoxin, podophyllin

INTRODUCTION

Podophyllum hexandrum Royle, a perrenial herb, native of India, belonging to the Berberidaceae family, is known to contain the highest content of podophyllotoxin.[1] The rhizomes of *P. hexandrum* are enriched with several lignans.[2,3] The structure of some lignans are shown (Fig. 1). The lignans occurring in *Podophyllum* possess anti-tumour activity,[4] podophyllotoxin

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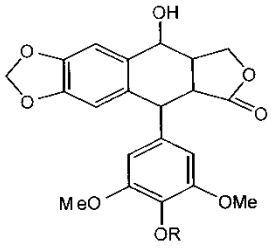
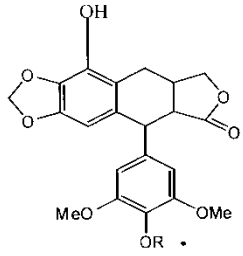




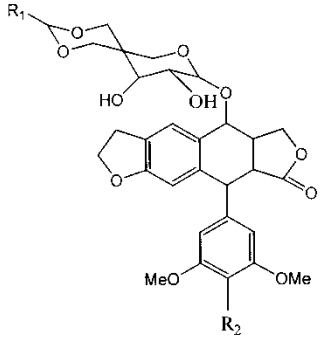
A R= Me

B R= H

C R= Me

D R=H

Structures of podophyllotoxin (A), 5-methoxypodophyllotoxin (B), α-Peltatin (D), β-Peltatin (C).



	R_1	R_2
Teniposide		OH
Etoposide	CH_3	OH

Figure 1. Structures of some lignans.

being the most cytotoxic compound. The natural lignan, podophyllotoxin, is used for the semi-synthesis of anti-neoplastic drugs, etoposide and teniposide. Their cytotoxic action is based on the inhibition of topoisomerase II,[5] while podophyllotoxin acts as an inhibitor of the microtubule assembly. Podophyllotoxin is also the precursor for a new derivative CPH-82 (reumacon) that is

being tested in Europe in phase III clinical trials for arthritis.[6] For the past five decades, podophyllin (*Podophyllum* resin) and podophyllotoxin are considered as active constituents in dermatologic products for therapy of genital warts.[7] It has been reported that different populations of this species show considerable variation in seed character, isoenzyme, and polypeptide photosynthetic rates and leaf morphology.[8]

Several HPLC methods[9–15] have been reported for quantification of podophyllotoxin, but, in all of them, there is a need for sample clean up to remove the interfering constituents of plant extracts, making the procedure lengthy and laborious for screening large number of samples. An immunoassay for podophyllotoxin has been reported.[16] Earlier, few efficient analytical procedures for specific secondary metabolites in other plants have been developed.[17–19] To the best of our knowledge, there is no report related to the HPTLC determination of podophyllotoxin in *P. hexandrum.* This work was aimed to develop a simple analytical tool for rapid, cost-effective, and reproducible analysis of podophyllotoxin content, and to characterise high yielding plant populations for further crop improvement and its multiplication. As part of our work on standardisation of indigenous medicinal plants, we report a simple, specific, and sensitive method for the estimation of podophyllotoxin, from the roots of *P. hexandrum* using HPTLC. The procedure is of importance in screening large number of samples for podophyllotoxin content, while evolving a package of practice in domestication and cultivation of the important endangered plant species.

EXPERIMENTAL

Materials and Reagents

The plant material of *P. hexandrum* was collected from western Himalaya, an altitude ranging between 3250 to 4100 meters above the mean sea level, and was authenticated at the Biodiversity Department, IHBT, Palampur, H.P, India. RP-18 60 F_{254S} TLC plates used were from E. Merck, Germany. All the reagents were of analytical grade purchased from Ranbaxy Chemicals (Delhi, India), and were used without further purification.

Isolation and Characterisation of Podophyllotoxin Standard

Powdered plant material of 1 Kg was extracted with methanol. The methanol extract (98.70 gm) was then fractionated with hexane, chloroform, and ethyl acetate. The ethyl acetate fraction (12.20 gm) was chromatographed on silica gel and eluted with chloroform : methanol in increasing polarity.

The fraction obtained from chloroform : methanol, 95 : 5, was rechromatographed using dry column chromatographic techniques on a column (diameter 50 mm) packed with silica gel H (without binder and particle size 10–40 micron), and the volume of fractions collected was 50 mL. The column was eluted with dichloromethane : methanol in varying proportions. The fraction obtained from (2% MeOH in DCM) yielded podophyllotoxin, which was recrystallised with benzene-methanol.

The identity of the compound was confirmed by recording the melting point in a Mettler FP800 (central processor), ^{1}HNMR spectra in $CDCl_3$ on a Bruker DRX-300 MHz and IR spectra on JASCO FT/IR 5300. The purity of the compound was established by analysis with a scanner HPTLC (Camag, Multenz Switzerland). This Podophyllotoxin was used for developing the HPTLC method and as a standard for the analysis of Podophyllotoxin content in the root samples.

Preparation of Standard Solutions

Stock solutions of Podophyllotoxin (1 mg/mL) were prepared in methanol and different amounts (2–10 μL) of these were loaded on a TLC plate, using ATS4 for preparing calibration graphs.

Preparation of Sample Solutions

Air dried rhizomes of *P. hexandrum* (0.1 gm) were soaked in methanol for 12 hours, filtered, and evaporated. The concentrate was resinified with acidulated water, filtered, and then washed with water and dried for determination of resin content.

Instrumentation

The HPTLC workstation consisted of an Automatic TLC applicator ATS-IV (from CAMAG, Multenz Switzerland) equipped with Win CATS software (Version 1.2.3) and mercury, tungsten, and deuterium lamp for scanning of TLC plates. The separation was achieved on thin layer plates of precoated RP18 60 F_{254S} TLC plate, (20 × 20 cm, 0.2 mm thickness). Samples and standards were applied to the plate as 6 mm. wide bands with an automatic TLC applicator (ATS 4) with N_2 flow (Camag, Multenz Switzerland), 10 mm from the bottom and 10 mm from the sides. The application parameters were identical for all the analyses performed.

Chromatographic Conditions

The conditions were as follows: Test plate: precoated TLC plate RP18 60 F_{254S}, (E.Merck, Germany). Format: $20 \times 20\,cm^2$, 0.2 mm thickness. Spotting volume: 5 μL. Band width: 6 mm. Separation technique: ascending. Development chamber: Camag twin–trough glass chamber $20 \times 20\,cm^3$. Mobile phase: acetonitrile : water (4 : 6, v/v). Temperature: $25 \pm 5^{\circ}C$. Migration distance: 8 cm. Migration time: 15 minutes. Detection wavelength: 217 nm. Mode: absorbance/reflectance. Span: slit 6.00×0.45 mm. Scanning speed: 20 mm/s. Data resolution: 100 μm/step.

Callibration Curve

Stock solutions of podophyllotoxin (1 mg/mL) were prepared in methanol and different amounts (2–10 μL) of these were loaded on a TLC plate, using ATS4 for preparing Calibration graphs.

Validation

The method was validated for precision, reproducibility, and accuracy (Table 1). Precision of the instrument was checked by repeated scanning of the same spot of podophyllotoxin (concentration: 2000 ng) three times, and the coefficient of variation (CV) was calculated. Variability of the method was tested by performing the recovery studies at two levels by addition of 50% and 100% of podophyllotoxin to one of the samples. To 0.1 gm of root powder (containing 5 mg of podophyllotoxin), known amounts of standard podophyllotoxin were added (2.5 and 5 mg) and estimated as described. The percentage recovery, as well as the average percentage recovery was calculated (Table 2).

Table 1. Method validation parameters for the estimation of podophyllotoxin by the HPTLC method

Serial no.	Parameter	Result
1	Instrumental precision (CV, %) (n = 3)	0.085
2	Repeatability (CV, %) (n = 3)	1.2490
3	Limit of detection (μg/spot)	2 μg
4	Limit of quantification (μg/spot)	5.031 μg
5	Specificity	Specific
6	Linearity (correlation coefficient)	0.98643
7	Range (μg/spot)	4–10 μg
8	Robustness	Robust

Table 2. Recovery study of podophyllotoxin by the HPTLC method (n = 3)

Serial No.	Amount of podophyllotoxin present in root powder (mg)	Amount of podophyllotoxin added (mg)	Amount found in mixture* (mg)*	Recovery* (%)
1	5.11	2.5	7.120 ± 0.051	93.56
2	5.11	5.0	9.856 ± 0.023	97.48

*average percentage recovery = 95.52%.

Estimation of Podophyllotoxin from *P. hexandrum* Roots

The samples were dissolved in analytical grade methanol and different amounts (5 μL) of these were spotted on a TLC plate, and the analyses were performed in triplicate.

RESULTS AND DISCUSSION

One of the major therapeutic areas where natural products have made a major impact on longevity and quality of life, is in the chemotherapy of cancer. In fact, most of the important anti-neoplastic drugs are natural products,[20] from plants or microorganisms.[21] In a serious exploration of indigenous medicinal plants for compounds with anticancer activity, Podophyllotoxin from *P. hexandrum*, was found to have significant tumour inhibitory activity. Podophyllotoxin was isolated (melting point 183–184°C) and the identity of the compound was established from the following spectral data:

UV: λ_{max}: 217 nm

IR: 3600-3100, 1770, 1628, 1595, 1510

^{1}HNMR: 7.03 (1H, s, H-5), 6.42 (1H, s, H-8), 6.29 (2H, s, H-2′, H-6′), 5.90 (2H, d, J = 5.25, $-OCH_2O$), 4.75 (1H, d, J = 8.8, H-4β), 4.50 (2H, m, H-1), 4.07 (1H, t, H-3β), 3.73 (3H, s, 4′-OMe), 3.67 (6H, s, 3′, 5′-OMe), 2.74 (2H, m, H-2, H-3′).

The melting point, UV_{max}, IR, and ^{1}HNMR spectral data were in agreement with the reported data for podophyllotoxin.[3]

The purity of the compound was established by the TLC Chromatogram, which showed a single peak (Fig. 2) and the UV absorption spectra that were recorded (on the CAMAG TLC scanner, Multenz Switzerland) at the start, middle, and end positions of the band completely overlapped (Fig. 3).

The requirements of the thin layer chromatographic method to be used for quantification of podophyllotoxin in resin is that it must be able to resolve

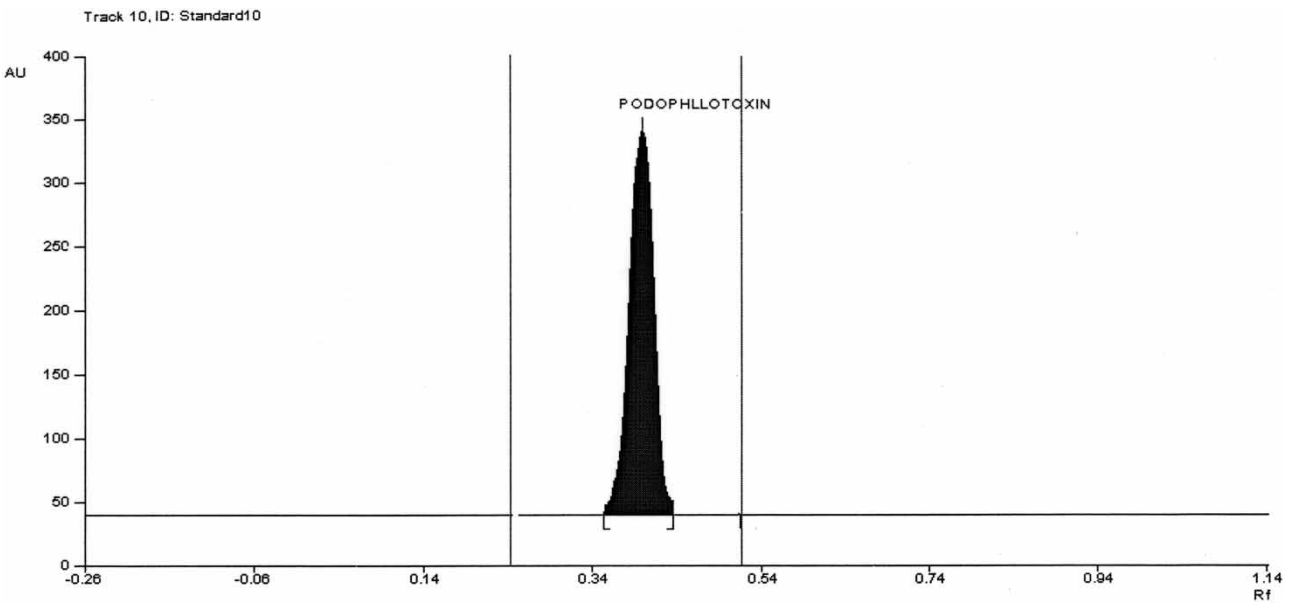


Figure 2. TLC chromatogram of podophyllotoxin standard, 1 μg/spot.

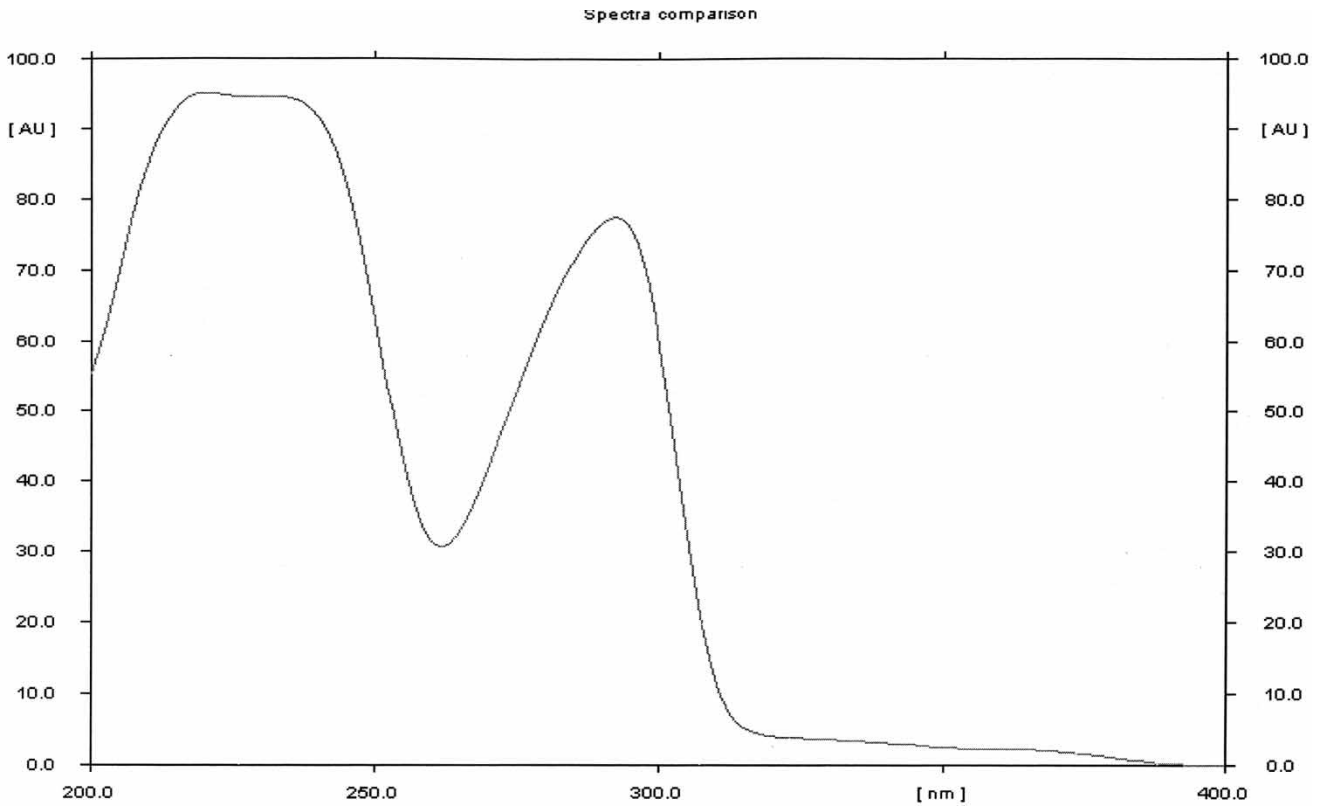


Figure 3. UV spectrum of standard podophyllotoxin.

the principal compound. The solvents showing the highest selectivity for the separation of principal lignan were acetonitrile and water as strength adjusting solvent. On RP-18 60 F_{254S} plates better resolution was obtained with a migration distance of 50%. Different composition of the mobile phases were tested and the desired resolution of podophyllotoxin was achieved by using acetonitrile–water (40 : 60) as mobile phase. The analysis of the samples was performed in triplicate (Table 1). The Rf of Podophyllotoxin was found to be 0.41 $\pm$ 0.02 (Fig. 4). The Regression equation conformed to linearity (Fig. 5) with standard deviation 3.48%, the equation being:

$$Y = 6817.010 + 531.288 * X, \quad r = 0.97781$$

The spot for podophyllotoxin was ascertained by comparing the Rf values (Fig. 2) and spectra of the spot with that of the standard podophyllotoxin (Fig. 6).

Application of the Method

The podophyllotoxin content of four different samples of *P. hexandrum* was estimated by the above validated HPTLC method (Table 3). The amount of

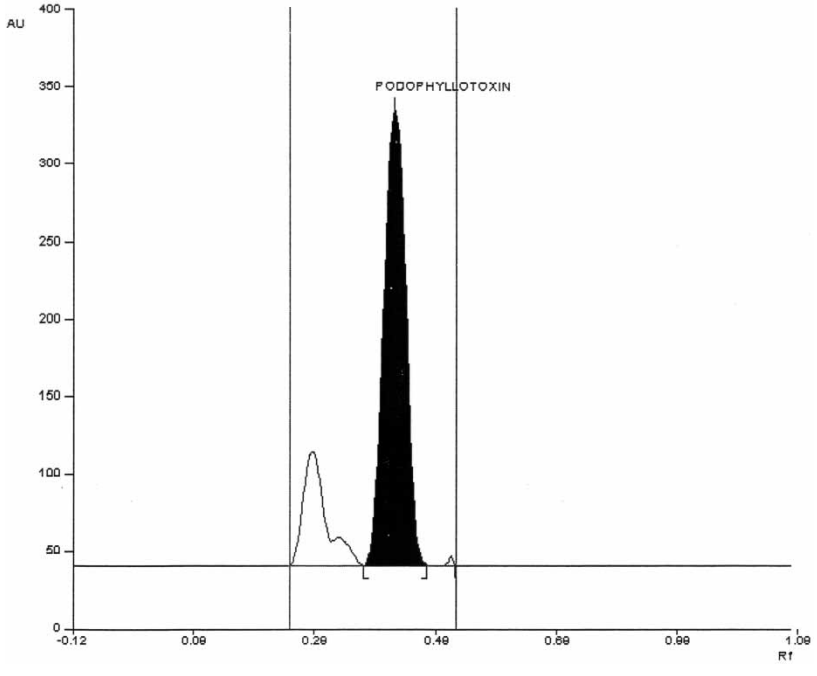


Figure 4. TLC chromatogram of resolution of podophyllotoxin in the sample.

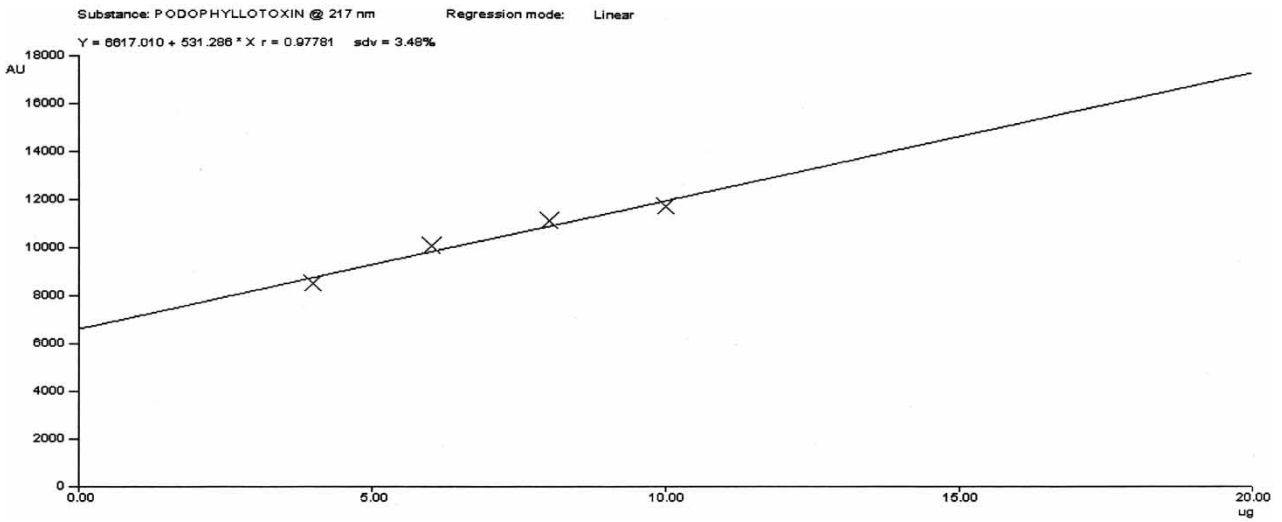


Figure 5. Calibration curve of standard podophyllotoxin.

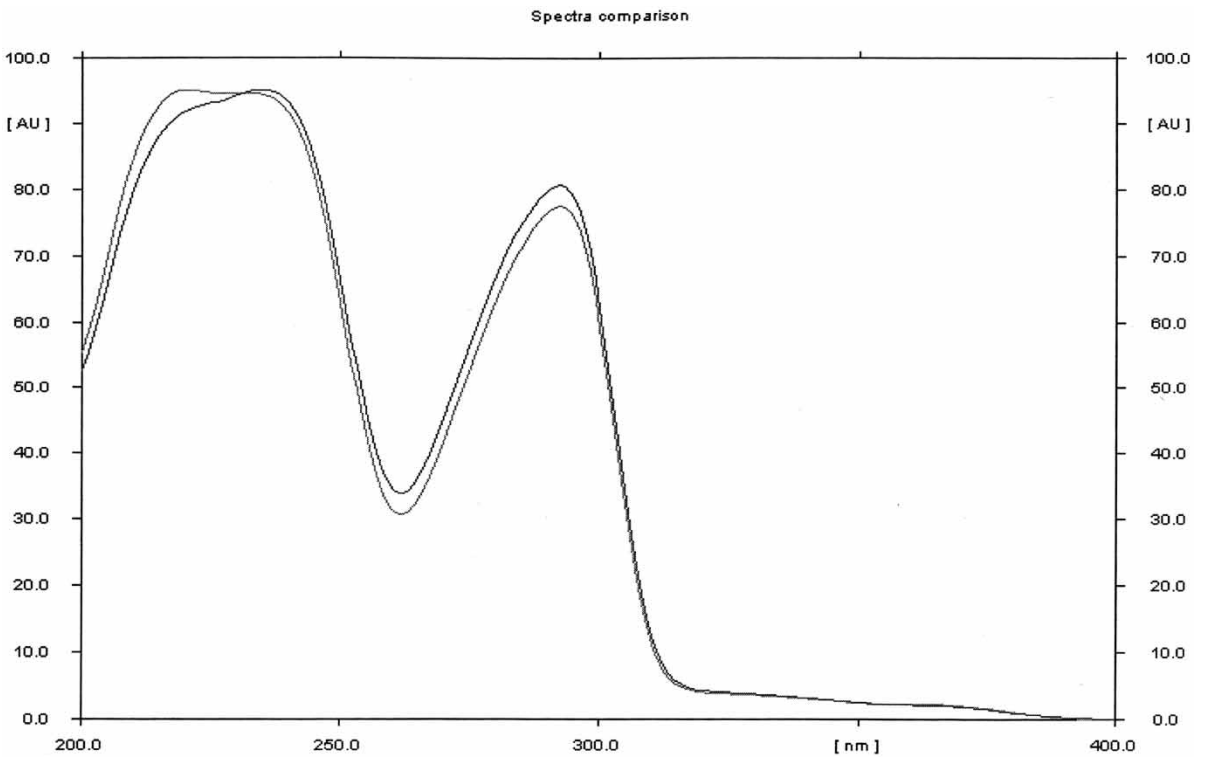


Figure 6. Spectral matching of standard podophyllotoxin and sample.

Table 3. Podophyllotoxin content in different samples of *P. hexandrum* by the HPTLC method

	TLC densitometry values			Podophyllotoxin content in samples
Sample	1	2	3	Average ± SE
PT1	5.031	5.122	5.197	5.116 ± 0.028
PT2	6.334	6.360	6.290	6.328 ± 0.025
PT3	6.088	6.001	6.122	6.070 ± 0.139
PT4	7.062	7.065	7.227	7.118 ± 0.072

podophyllotoxin in the samples was found to vary from 5.11 to 7.11% (w/w) (Table 4). Resin and podophyllotoxin contents in the rhizomes of different populations are shown (Fig. 7). The maximum resin content was found in the population obtained from an altitude of 3250 meters and lowest in population obtained from 3900 meters above the mean sea level, where as podophyllotoxin content was highest in the sample obtained from 3250 meters and lowest in sample from an altitude of 4000 meters. Samples collected from 3250 meters altitude contained higher contents of podophyllin as well as podophyllotoxin. A low content of podophyllin was observed in plant populations collected from 3900 meters altitude, whereas podophyllotoxin content was low in samples collected from 4000 meters. From these observations, it can be concluded that altitude does play a role in biosynthesis of desired secondary metabolites, but it may also be due to other agro-ecological factors such as plant morphology, seasonal variations, location, temperature, etc.

The method applied here is simple, inexpensive as compared to HPLC (no use of HPLC grade solvents, HPLC columns), and the quick scanning of 15 plant extracts in a single 20 × 20 cm. RP_{18S} TLC plate in about 30 minutes.

Table 4. Variation in resin and podophyllotoxin content in samples

Altitude a.s.l. (meters)	Sample	Resin content (%)	Podophyllotoxin (%)
4,000	PT-1	6.34	5.11
4,100	PT-2	6.76	6.32
3,900	PT-3	6.97	6.07
3,250	PT-4	7.71	7.11

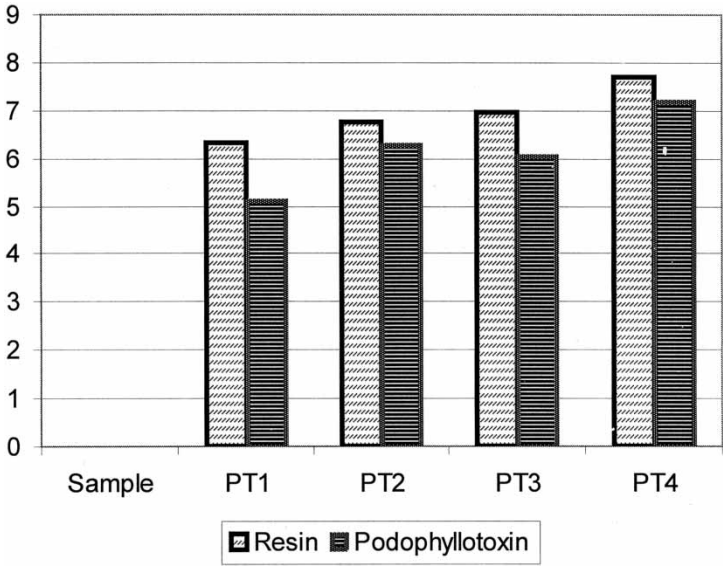


Figure 7. Variation in resin and podophyllotoxin in samples.

The proposed HPTLC method can be used for the estimation of podophyllotoxin in a large number of samples in a plant breeding programme.

CONCLUSION

Thin layer Chromatography is suitable for the separation of lignans found naturally in *Podophyllum hexandrum*, especially, podophyllotoxin. RP-HPTLC methods provides simple, rapid, and accurate quantitative analysis for the determination of podophyllotoxin. The advantage of TLC for this analysis is the high sample throughput, which results from the small amount of sample preparation required and simultaneous separation of several samples.

The small amount of sample preparation required for the analysis of these compounds by TLC makes the method very attractive, as compared to alternative methods of analysis by HPLC.

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REFERENCES

1. Chartterjee, R. Indian Podophyllum. Econ. Bot. **1952**, *6*, 343–354.
2. Jackson, D.E.; Dewick, P.M. Tumour inhibitory aryltetralin lignans from *Podophyllum pleianthum*. Phytochemistry **1984**, *24* (10), 2407–2409.
3. Jackson, D.E.; Dewick, P.M. Aryltetralin lignans from *Podophyllum hexandrum* and *Podophyllum pleianthum*. Phytochemistry **1984**, *23*, 1147–1152.
4. Jackson, D.E.; Dewick, P.M. Biosynthesis of the lignans α- and β-peltatin. Phytochemistry **1986**, *25*, 2089–2092.
5. Richter, A.; Strausfeld, U.; Knippers, R. Effects of VM-26 (teniposide), a specific inhibitor of type II topoisomerase, on SV-40 DNA replication in vivo. Nucleic Acid Res. **1987**, *15*, 3455–3468.
6. Carlstrom, K.; Hedin, P.J.; Jansson, C.; Lerndal, T.; Lien, J.; Weitaft, T.T.; Axelson, T. Endocrine effects of podophyllotoxin derivatives drug CPH 82 (Reumacon) in patients with rheumatoid arthritis. Scandinavian J. Rheumatol. **2000**, *29*, 89–94.
7. Stoerh, G.P.; Peterson, A.L.; Taylar, W.J. Systemic complication of local podophyllin therapy. Ann. Int. Med. **1978**, *89*, 362–03.
8. Purohit, M.C.; Bahuguna, R.; Maithani, U.C.; Purohit, A.N.; Rawat, M.S.M. Curr. Sci. **1999**, *77*, 1078–1080.
9. Bastos, J.K.; Burandt, C.L., Jr.; Nanayakara, N.P.D.; Bryanat, L.; McChesney, J.D. Quantitation of aryltetralin lignans in plant parts and among different populations of *Podophyllum peltatum* by reverse phase high-performance liquid chromatography. J. Natl. Prod. **1996**, *59*, 406–408.
10. Moraes, R.M.; Bedir, E.; Barrett, H.; Burandt, C.L., Jr.; Camilo, C.; Khan, IA. Evaluation of *Podophyllum peltatum* Accessions for podophyllotoxin production. Planta Med. **2002**, *68*, 341–344.
11. Camilo, C.; Dayan, F.E.; Ganzera, M.; Khan, I.A.; Rimando, A.; Burandt, C.L., Jr.; Moraes, R.M. High yield of podophyllotoxin from leaves of *Podophyllum peltatum* by in situ conversion of podophyllotoxin 4-O-β-D-glucopyranoside. Planta Med. **2001**, *67*, 97–99.
12. Uden, W.U.; Pras, N.; Malingre, T.M. On the improvement of the podophyllotoxin production by phenylpropanoid precursor feeding to cell cultures of *Podophyllum hexandrum* Royle. Plant Cell Tiss. Organ Cult. **1990**, *23*, 225–227.
13. Smollny, T.; Wichers, H.; Rij, K.de.T.; Zwan, A.van.; Shasavari, A.; Alfermann, A.W. Accumulation of Podophyllotoxin and related lignans in cell suspension cultures of *Linum album*. Phytochemistry **1998**, *48* (6), 975–979.
14. Heyenga, A.G.; Lucas, J.A.; Dewick, P.M. Production of tumour-inhibitory lignans in callus cultures of *Podophyllum hexandrum*. Plant Cell Rep. **1990**, *9*, 975–979.
15. Uden, W.V.; Pras, N.; Viser, J.F.; Malingre, T.M. Detection and identification of podophyllotoxin produced by cell cultures derived from *Podophyllum hexandrum* Royle. Plant Cell Rep. **1989**, *8*, 165–168.
16. Yoo, K.J.; Porter, J.R. Immunoassay of podophyllotoxin. J. Natl. Prod. **1993**, *56* (5), 715–721.
17. Gupta, A.P.; Gupta, M.M.; Kumar, S. Simultaneous determination of curcuminoids in *Curcuma* samples using high performance thin layer chromatography. J. Liq. Chrom. & Rel. Technol. **1999**, *22* (10), 1561–1569.

18. Gupta, A.P.; Verma, R.K.; Gupta, M.M.; Kumar, S. Estimation of plumbagin using high performance thin layer chromatography. J. Med. Arom. Plant Sci. **1999**, *21*, 661–663.
19. Gupta, A.P.; Gupta, M.M.; Kumar, S. HPTLC of asiaticoside in *Centella asiatica*. J. Indian Chem. Soc. **1999**, *76*, 277–278.
20. http://pharmacology.unmc.edu/cancer/alkaloid.htm (accessed July 2004).
21. http://pharmacology.unmc.edu/cancer/antibio.htm (accessed July 2004).

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