A simple microanalytical technique for determination of podophyllotoxin in *Podophyllum hexandrum* roots by quantitative RP–HPLC and RP–HPTLC

Indiscriminate collection of medicinal plants has led to many of them becoming rare, threatened or endangered. *Podophyllum hexandrum* Royle (Berberidaceae) is a herbaceous, rhizomatous species of great medicinal importance, now endangered in India. It is distributed in restricted pockets throughout the alpine Himalayan region[1]. The rhizomes of *P. hexandrum* yield cytotoxic lignan podophyllotoxin and resin due to which *Podophyllum* possesses anti-tumour activity[2,3]. Moreover, important drugs used for the treatment of testicular and small-cell lung cancer, namely etoposide (VP-16-213) and teniposide (VM-26) are produced by semi-synthesis from the plant-derived lignan, podophyllotoxin[4]. Their cytotoxic action is based on the inhibition[5] of topoisomerase II, while podophyllotoxin acts as an inhibitor of the microtubule assembly. Podophyllotoxin is also a precursor for the new derivative CPH-82 (reumacon) being tested in Europe in phase III clinical trials for arthritis[6]. In addition, podophyllotoxin and podophyllin (*Podophyllum* resin) are considered as active constituents in dermatologic products for therapy of genital warts[7]. The demand for plant material, however, has endangered the common source of podophyllotoxin, *P. hexandrum*.

Podophyllotoxin is still extracted from the roots of *P. hexandrum* and *P. peltatum* collected from the wild. Chemical synthesis is also possible, but not economical. Therefore, there is an increasing interest in additional sources for supply of podophyllotoxin[8] and future production of these drugs depends upon cultivation of *P. hexandrum* or the use of tissue/cell culture techniques that provide only small quantities of plant material for analysis. A prerequisite to this endeavour is the development of a reliable procedure to determine rapidly the content of podophyllotoxin in minimum possible amount of plant material. Most methods for determining podophyllotoxin use High Performance Liquid Chromatography (HPLC)[9–13]. Other methods include enzymatic techniques[14]. Methods that use a small volume of sample are desirable when estimation has to be done where the collection of larger volume is not feasible. The present study was therefore undertaken using RP–HPLC and RP–TLC techniques for the determination of podophyllotoxin in dry cell mass obtained by tissue culture or single plant basis. Moreover, comparison between HPLC–PDA and HPTLC data was carried out in order to achieve an accurate, specific and reproducible method.

The underground parts of *P. hexandrum* were obtained from Koksar, Kullu, Himachal Pradesh, India located at an altitude of 3250 m amsl. The plant material ranging from 1 g to 2 mg was weighed and extracted thrice with methanol. The extracts were combined, filtered and dried under vacuum. The concentrate was redissolved in HPLC-grade methanol for quantitative

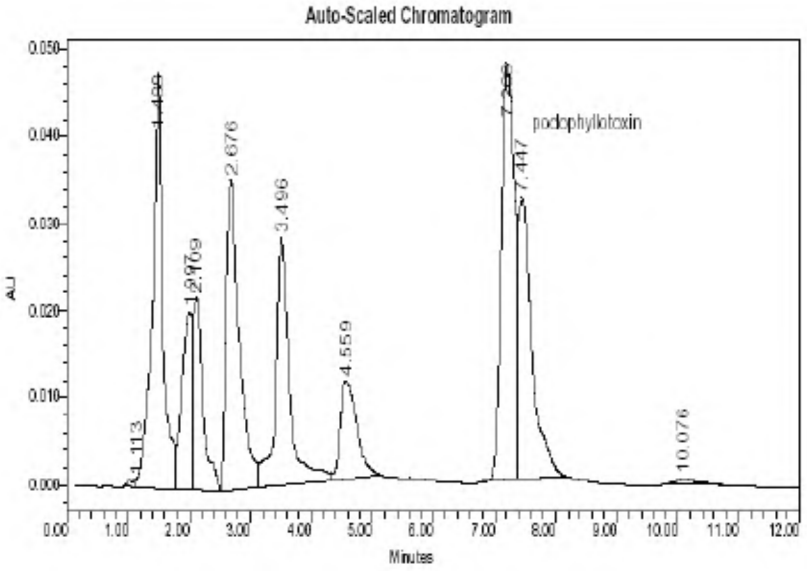

Figure 1. HPLC of methanolic extract sample of roots of *P. hexandrum*.

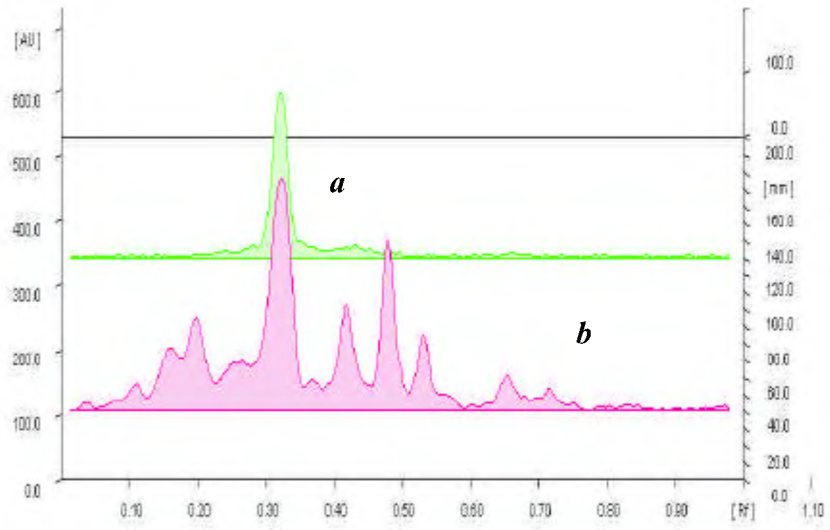

Figure 2. HPTLC; 3D overlay chromatogram of (***a***) standard track and (***b***) resin sample of roots of *Podophyllum hexandrum*.

analysis. Methanol extract of the plant material ranging from 1 g to 10 mg was resinified with acidulated water. The precipitated resin was redissolved in HPLC-grade methanol for podophyllotoxin analysis.

HPLC analysis was carried on Waters HPLC system (600 Gradient pump; 7725i Rheodyne Injector; 996 PDA Detector; Millennium^{32} version 3.05.1), all samples were filtered through 0.2 µm (Millipore) membranes. Column used was Lichrospher^{R} 100 5-RP-18e (250 mm × 4 mm × 5 µm), flow rate: 1 ml/min, run time: 20 min, detector wavelength 230 nm. HPLC-grade solvents were filtered through a 0.45 µm Millipore filter. The solvent system used was as solvent A: acetonitrile, solvent B: water in 4 : 6 ratio. The analysis was performed at 21 ± 1°C (Figure 1).

Podophyllotoxin content analysis in extracts and resins was performed by the external standard method, using pure podophyllotoxin as standard. Stock solutions of 1 mg/ml were further diluted to 0.03, 0.06, 0.125, 0.25, 0.50 and 1 µg/ml for the formation of calibration curves. Each determination was carried out in triplicate. The regression equation for methanol extract was $y = 0.979x–0.0175$, regression coefficient 0.9941, RT 7.20 ± 0.9, limit of detection (LOD) 30 pg, limit of quantitation (LOQ) 110 pg and for resin the parameters were $y = 0.0154x–0.0058$, 0.9687, 7.21 ± 0.5, 100 pg and 615 pg respectively.

HPTLC analysis was carried out using automatic TLC sampler ATS-4 (CAMAG, Switzerland) equipped with Win CATS software (version 1.2.3). Sample bands of width 6 mm were spotted on RP18 F_{254} TLC plates (20 cm × 20 cm with 200 µm thickness; E. Merck, Germany). Slit dimension 6 mm × 0.45 mm, scanning speed 10 mm/s, mobile phase, acetonitrile : water (50 : 50, v/v) was employed. Densitometric scanning was performed on Camag TLC scanner 3 in the absorbance–reflection mode at 217 nm (Figure 2). The spot for podophyllotoxin was ascertained by comparing the Rf values and spectra of the sample with those of the standard podophyllotoxin.

The regression equation for methanol extract was $y = 1294.733 + 564.314x$, regression coefficient 0.9883, Rf 0.32 ± 0.02, LOD 51 pg, LOQ 872 pg and for resin the parameters were $y = 1208.32 + 4253.044x$, 0.9784, 0.32 ± 0.03, 133 ng and 136 ng respectively.

Podophyllotoxin in methanol extract was calculated from 1 g to 2 mg of plant material, which ranged from 4.25 to 5.22% and 4.15 to 5.12% by HPLC and HPTLC respectively. Beyond this limit, there were difficulties in extraction and consistency of results. In case of podophyllin, consistent results could be obtained up to 10 mg of plant powder. Podophyllotoxin content in resin was estimated as 4.91–5.57% and 4.90–5.58% by HPLC and HPTLC respectively. All data represent an average of three analyses which showed consistency of results. The amount of podophyllotoxin estimated by both the methods was found to be in linearity.

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected, but not necessarily quantitated as an exact value. The limit of quantitation is the lowest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy. Linear correlation was obtained for each set of observations by HPLC and HPTLC. Since the trend observed for podophyllotoxin is similar, a linear regression between these two techniques was performed. A good correlation was obtained and the high correlation coefficient for both the procedures was recorded. Finally, a HPTLC method could be routinely used to analyse large number of samples. In fact, it requires a simple approach and a short analysis time.

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NIDHI MISHRA
RUCHI ACHARYA
AJAI P. GUPTA
BIKRAM SINGH*
VIJAY K. KAUL
P. S. AHUJA

Department of Natural Plant Products, Institute of Himalayan Bioresource Technology, Box No. 6, Palampur 176 061, India
**e-mail: bikram_npp@rediffmail.com*