

Chemical profiling assisted quality assessment of podophyllotoxin from *Podophyllum hexandrum* by LC-APCI-3Q-MS/MS

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ABSTRACT

A fast and sensitive HPLC coupled with APCI-3Q mass spectrometer was utilized to assist the quality assessment of podophyllotoxin from *Podophyllum hexandrum*. More than ten peaks were separated and detected on a SeQuant TM ZIC®-HILIC LC column along with an Oasis MCX 96-well solid-phase extraction cartridge with 35 min. *P. hexandrum* phytoextract was monitored by a APCI-3Q 5500 mass spectrometer with both positive and negative APCI ionization modes. Multiple reaction monitoring was used for quantification using the precursor to product ion pairs of m/z 414.4 to 437.5 adduct with Sodium, m/z 414.4 to 432.4 adduct with water and m/z 414.4 to 828.8 dimer. The assay had a calibration range from 0.2 to 5 µg/ml and a lower limit of quantification of 0.68 µg/ml. The inter- and intra-day precisions were <0.4 per cent and the accuracies were within the range of 99.9–100.1 per cent. The mean recovery ranged from 82.0 to 98.7 per cent and internal standard normalized matrix effect from 0.813 to 0.899. The analyte was stable under all tested conditions. This ICH Q2b validated method can successfully be employed in the absence of reference standards for the marker and is particularly useful in view of the scarcity of the chemical standard of podophyllotoxin.

Keywords: Podophyllotoxin (PPT); *Podophyllum hexandrum*; ICH; APCI-3Q; LC- MS/MS

INTRODUCTION

Podophyllum species possess a number of compounds like podophyllin, podophyllotoxin, querecetin, 4-dimethylpodophyllotoxin, kaempferol, picropodophyllotoxin, α -peltatin and β -

peltatin (Singh et al 1994). The podophyllotoxin content in Indian *Podophyllum* is more (7-15%) in comparison to other species notably *Podophyllum peltatum* (4-8%) the most common species in American subcontinent (MacRae and Towers 1984). Podophyllo-

toxin has acquired great importance and high medicinal status due to its effectiveness as antimitotic, anticancer and immunostimulatory activity (Kalpan 1942, Lokie et al 1978, Pugh et al 2001) especially for curing uterine tumors (Richter et al 1987).

P hexandrum extracts have been reported to offer radioprotection by modulating free radical flux involving the role of lignans present (Chawla et al 2006). Total synthesis of podophyllotoxin is an excessive process and availability of compound from natural resource is an important issue for pharmaceutical companies that manufacture these drugs (Canel et al 2000). Its annual supply is at present estimated at 80 tonnes while the demand is more than 100 tonnes. To meet this ever increasing demand of crude drug the rhizomes of *P hexandrum* are being indiscriminately collected in large quantities (Airi et al 1997). As a result *P hexandrum* is reported as an endangered species in Himalayan region and its quality assessment becomes even more important. Traditionally developed methods with thin layer chromatography (TLC), high performance thin layer chromatography (HP-TLC) and high performance liquid chromatography (HPLC) for podophyllotoxin quantification from *P hexandrum* are longer, time consuming and costly leading to higher material cost and increased energy consumption and labour and are even hazardous for nature with use of several non-green solvent systems (Sharma 2015, Cairnes et al

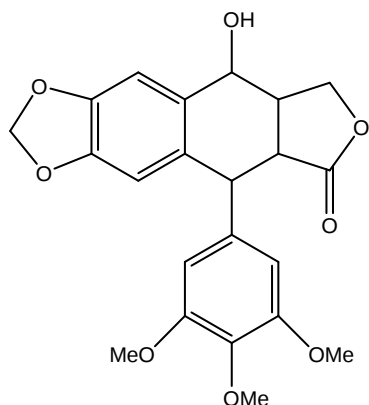
1981). In the present work quality assisted time saving APCI-3Q-MS/MS profiling of podophyllotoxin from *P hexandrum* roots and leaves collected from Kullu-Manali valley of Himachal Pradesh, India is presented.

MATERIAL and METHODS

Chemicals and solutions

Acetonitrile (Merck, HPLC grade), HPLC grade water (Ranbaxy, Mumbai), chloroform, n-hexane, ethyl acetate, ethanol, 25 per cent NH_4OH , 0.1 per cent HCOOH , 10 mM NH_4OAC in water were taken. All chemicals were of analytical grade. The standard podophyllotoxin was purchased from Sigma-Aldrich Chemicals and was of at least 99 per cent purity.

Plant materials (roots and leaves of *P hexandrum*) were collected from Kullu-Manali valley of Himachal Pradesh during the months of November (2014) and January and June (2015). Plant specimens were identified and confirmed at Department of Forest Products, Dr YS University of Horticulture and Forestry, Nauni, Solan, HP. Plant tissues (roots and leaves) of *P hexandrum* (separately one by one) were pulverized under liquid nitrogen after air-drying in shade to get their powder. One gram of each sample powder was put in 10 ml of falcon tube with methanol and vortexed for 10 minutes. The tubes were kept as such overnight, centrifuged at 3000 rpm for 20 minutes at



Podophyllotoxin

room temperature, the methanolic supernatant after filtering the extract was collected and was used for further qualitative and quantitative analysis. The step was repeated several times to get the bulk material and to avoid the wastage.

Screening studies by thin-layer chromatography

To perform thin-layer chromatography analysis of leaf and root tissues of *P. hexandrum* above methanolic supernatant mixture was used for TLC spotting in triplicate. Chloroform and methanol (8:2) solvent system was standardized for the TLC analysis to calculate R_f value of root and leaf extracts as compared to standard podophyllotoxin spot.

Chemical profiling by liquid chromatography

Podophyllotoxin analysis was performed by HPLC. This equipment

consisted of a 1200 series binary pump (G1312B), a 1200 series gradient pump (G1310A) and a degasser (G1379B) (Agilent Technologies, Germany) connected to an auto-sampler with chemstation 6.0 version software package. Gradient chromatographic separation of podophyllotoxin was performed on a SeQuant TM ZIC[®]-HILIC HPLC column along with an Oasis MCX 96-well solid-phase extraction cartridge with 35 min run time gradient (C-8: 4.0×175 mm, $1.5 \mu\text{m}$ particle size). The injection volume was 15 μl and the column oven temperature was set to 25°C . CH_3CN /water mobile phase combination was performed in gradient mode and re-equilibration for 5 minutes. The flow rate was optimized and set to 0.8 ml/min.

APCI-3Q-MS/MS analysis of podophyllotoxin

MS/MS studies were performed on triple quadrupole, APCI-3Q-EMD/MS and Q Trap 5500 mass spectrometer. To achieve a fast separation infusion MS sample introduction method to the APCI source was used. A hybrid triple quadrupole linear ion trap mass spectrometer APCI 5500 Q-Trap equipped with a Turbo V source ion spray operating in positive and negative modes was used for detection (Agilent, Germany). High purity nitrogen was produced by a nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany). To minimize contamination of the mass spectrometer the

Table 1. APCI-3Q-MS/MS data for podophyllotoxin analysis

Sample	Mass or ratio (m/z)	Standard PPT	PPT-Na adduct	PPT-H ₂ O adduct	PPT dimer
<i>Podophyllum Hexandrum</i>	[M] ⁺ , [M+1] ⁺ , [M-1] ⁺	415.2	437.5	432.4	828.8

column flow was directed only from 2.0 to 5.0 min into the mass spectrometer using a diverter valve. Otherwise CH₃CN with a flow rate of 2.50 µl/min was delivered into the mass spectrometer. The Turbo Ion Spray source was operated in the positive ion mode using the settings: ion spray voltage= -4500 V, ion source heater temperature= 750°C, source gas 1= 45 psi, source gas 2= 40 psi and curtain gas setting= 25 psi. Analytes were monitored in the multiple reaction monitoring (MRM) mode, mass transitions and MS parameters. Quadrupoles Q1 and Q3 were working at unit resolution. The major fragment ion masses observed in the mass spectra are summarised in Table 1. The compound identification was possible on the basis of different molecular ionization pathways. Since the molecular mass for podophyllotoxin is 414.405 here APCI-MS/MS mass of podophyllotoxin in the leaf as 415.2 as (M+1)⁺ peak and 415.2 (M+1)⁺ in the root were found.

RESULTS and DISCUSSION

Chromatographic content of podophyllotoxin was found to be 2.01 per

cent (TLC, R_f= 0.85) in leaf and 4.28 per cent in root sample (Fig 1) of *P hexandrum* which showed that content of podophyllotoxin was less in leaf as compared to root. As the altitude increased this phenomenon was reversed in case of leaves. 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 (Table 2).

Table 2. Summary of validation parameters of RP-HPLC method

Parameter	Experimental value
Linearity and range (µg/ml)	10-80
Correlation coefficient	0.9997
Accuracy (% recovery)	99.92-100.14
Precision (% RSD)*	
Intra-day	0.250
Inter-day	0.328
Ruggedness (% RSD)*	0.516
Robustness (% RSD)*	
Change in wavelength	0.015
Change in flow rate	0.031
LOD ^a (µg/ml)	0.68
LOQ ^b (µg/ml)	1.05

*Values expressed as mean of five determinations, ^aLOD= Limit of detection,

^bLOQ= Limit of quantitation

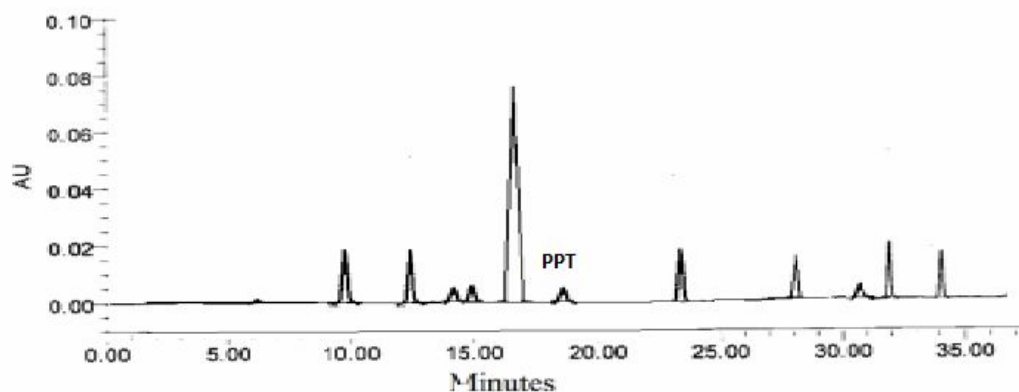


Fig 1. HPLC chromatogram for the *Podophyllum hexandrum* root extract

By studying the MS pattern and molecular ion peaks as revealed in corresponding mass spectra, a number of podophyllotoxin and related lignan marker compounds were identified in various extracts. To ensure and assess the quality of PPT, product ion pairs of m/z 414.4 to 437.5 adduct with sodium, m/z 414.4 to 432.4 adduct with water and m/z 414.4 to

828.8 dimer PPT were observed as molecular ion peak with highest signal abundance in their MRM mode of APCI soft mass spectrum using software modulations (Figs 2-5). Due to the high sensitivity of MS instrument this method was particularly advantageous for the samples of limited quantity. Using this methodology detailed structural information

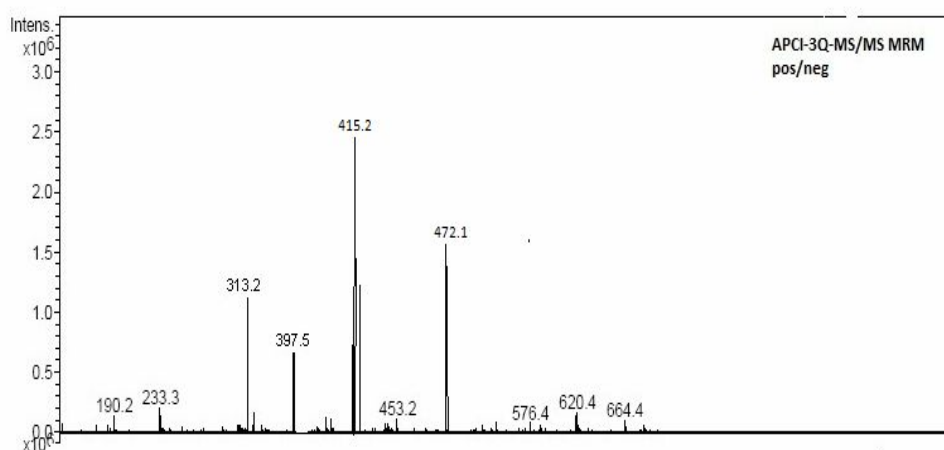


Fig 2. APCI-3Q-MS spectra for the standard PPT

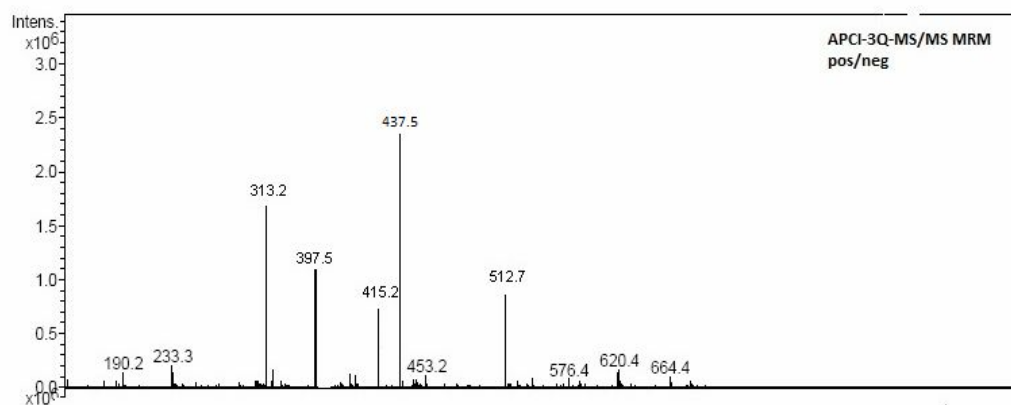


Fig 3. APCI-3Q-MS spectra for the *Podophyllum hexandrum* showing PPT-Na adduct

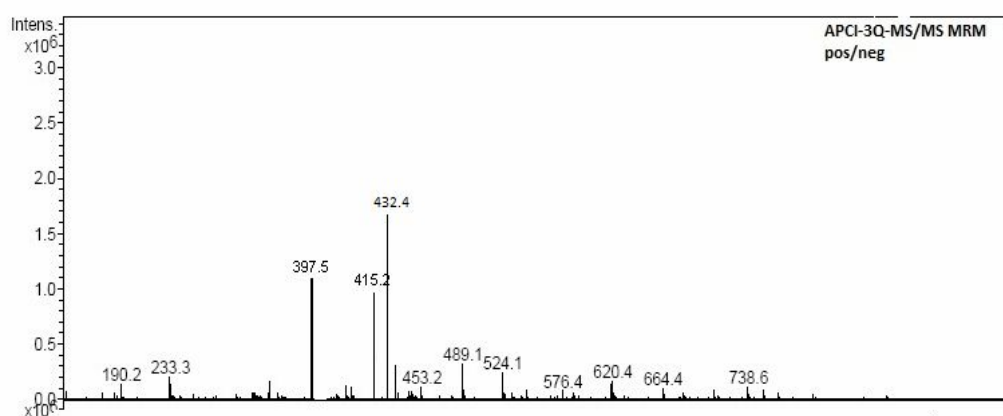


Fig 4. APCI-3Q-MS spectra for the *Podophyllum hexandrum* showing PPT-H₂O adduct

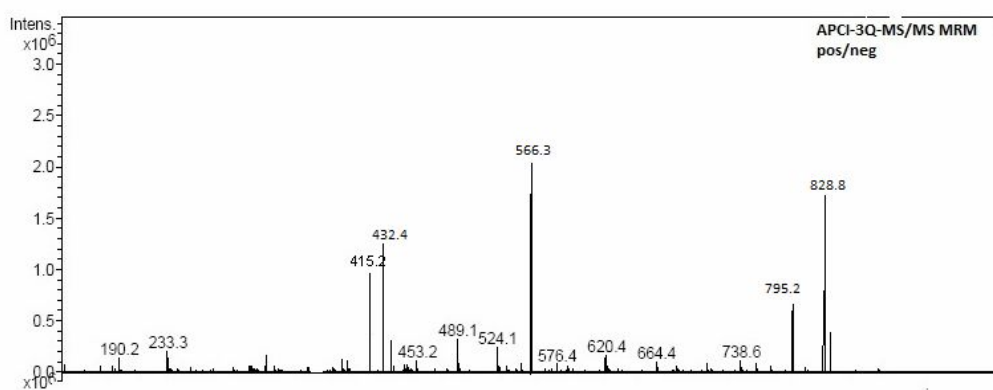


Fig 5. APCI-3Q-MS spectra for the *Podophyllum hexandrum* showing PPT dimer

was obtained for lignans, lignan glycosides and other secondary metabolites. HPLC analysis provides a reproducible retention time using standardised conditions for development of lignan database. UV spectra collected on line provided evidence for general classification and electronic data of each compound.

CONCLUSION

This LC-APCI-3Q-MS/MS method for the chemical profiling of podophyllotoxin from *P. hexandrum* developed is based on MS finger printing of the standard marker. The method can be employed in the absence of reference standards for the marker and is particularly useful in view of the scarcity of the chemical standard of podophyllotoxin. Application of MRM mode to analyse the content of podophyllotoxin from *P. hexandrum* is also presented. Besides the data obtained can be further employed to investigate the genotoxicity induced by *Podophyllum* species.

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