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# Quantification of Podophyllotoxin From *Podophyllum hexandrum* Using HPLC-UV-DAD

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## ABSTRACT

Gradient RP-HPLC method for the quantification of podophyllotoxin from *Podophyllum hexandrum* has been developed. In our study, qualification of podophyllotoxin was performed by Thin Layer Chromatography (TLC) with $R_f$ values of 0.85 (leaf) and 0.94 (root) when compared with the standard. UV-VIS spectrophotometeric studies showed electronic absorption at $\lambda_{max}$ value 284 nm. Gradient chromatographic separation of Podophyllotoxin was performed on a 50 mm × 2.1 mm (i.d.) Macherey-Nagel NUCLEODUR C18 Gravity HPLC column, packed with 1.5 $\mu$m particles equipped with a 0.5m pre-filter (Upchurch Scientific, Oak Harbor, WA, USA), using mobile phase methanol/water (1/1, v/v) and 100% methanol, both containing 0.1% ammonium hydroxide (25%) and 10 mmol/L ammonium acetate (pH 9). Van Deemter Equation and Fundamental Resolution Equation were validated to measure optimum velocity of the mobile phase and to check resolution of the peaks. DAD detector was used to profile the exact compositions and to quantify podophyllotoxin herein. Chromatographic purity of podophyllotoxin was found to be 2.001% in leaf and 2.08% in root sample of *Podophyllum hexandrum.* Further, the information generated is useful for various stake holders like pharmaceutical industries, research institutes and others.

**Keywords**:

DAD, Podophyllum hexandrum, Podophyllotoxin, RP-HPLC

## INTRODUCTION

Himalayan region are the richest source of diversity of medicinal and aromatic plant including *Podophyllum hexandrum* also known as Indian Mayapple, which is an endangered medicinal perennial herb belong to the family Berberidaceae. *Podophyllum hexandrum,* a moisture and shade loving erect, glabrous and succulent herb thriving from Himalayan zone at an altitude between 1300 to 4300 m above sea level. Extensive chemical investigation of Podophyllum species revealed the presence of a number of compounds like podophyllin, podophyllotoxin, querectin, 4-dimethypodophyllotoxin, kaempherol, picropodophyllotoxin, -peltatin and -peltatin (Singh and Shah 1994). The podophyllotoxin content in Indian Podophyllum is more of about 7-15% in comparison to other species like *Podophyllum peltatum* (4-8%), one of the most common species distributed in American sub-

continent (MacRae and Towers 1984). Recently podophyllotoxin 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 (Macrae and Towers 1984; Richter et al. 1987). The rhizome powder of the plant is used as laxative or to get rid of intestinal worms and also used as poultice to treat warts and tumorous growth on the skin. Semi synthetic derivatives of podophyllotoxin like etoposide (VP-16; Allevi et al. 1993), etopophos (Schacter 1996) and tenoposide (VM-26) are effective against the treatment of lung cancer, a variety of leukaemia, and other solid tumours (Pandey et al. 2007).

In the modern allopathic system of medicine, the plant has successfully used for the treatment of various disorders, monocytoid leukaemia, Hodgkin's lymphoma, bacterial and viral infections (Gowdey and Carpenter 1995), venereal warts (Beutner and Krog 1990) rheumatoid arthalgia associated with the numbers of the limbs and pyogenic infection of skin tissue, AIDS associated Kaposis sarcoma and different cancer of brain, lung and bladder (Blasko and Cordell 1998). In the search of novel, effective and non toxic radio protectants, number of plants product has been evaluated for the plant protection against lethal dose of radiation including Podophyllum hexandrum (Goel et al. 1998; Rajesh et al. 2005) and it is found that pre-radiation administration of the extracts of *Podophyllum hexandrum* mitigated radiation induced postnatal and physiological alternations and highly effective in control of both planned and unplanned radiation exposure (Goel et al. 2002). Recently, *Podophyllum hexandrum* extracts have been reported to offer radioprotection by modulating free radical flux involving the role of lignans presents (Chawla et al. 2006). Due to its anticancerous property podophyllotoxin is in increased demand throughout the world. Total synthesis of podophyllotoxin is an excessive process; however, availability of compound from natural resource is an important issue for pharmaceutical companies that manufacture these drugs (Canel et al. 2000). The present demand is more than 100 tonnes of this compound, however, as per estimation, it is suppose to supply only about 50-80 tonnes (Alam et al. 2009). To meet out this increasing demand of crude drug, the rhizomes of Podophyllum hexandrum are being indiscriminately harvested in large quantities from natural habitat. This could be the major threat for Podophyllum hexandrum to become an endangered species in Himalayan region.

The present work reports the development of HPLC method for the identification and quantification of podophyllotoxin from Podophyllum hexandrum, where the information may be useful for pharmaceutical industries as well as for researchers.

## MATERIALS AND METHODS

### *Chemicals and Solutions*

Acetonitrile (HPLC grade), HPLC grade water (Ranbaxy, Mumbai), Chloroform, n-Hexane, Ethyl acetate, Ethanol, Methanol (Merk), 25% NH4OH, 0.1% HCOOH, 10mM NH4OAC in water were used in the present study. Podophyllotoxin standards of 98% purity were purchased from Sigma-Aldrich chemicals USA.

### *Samples and Samples preparation*

Samples were obtained from regional centre of Dr. Yashwant Singh Parmar University of Horticulture and Forestry located at Ralla, Manali, Himachal Pradesh. Leaf and Root tissue of *Podophyllum hexandrum* were crushed in liquid Nitrogen. One gram of each sample powder was put in 10 ml of falcon tube, vortexed for 10 minutes. Kept the tubes as such overnight and centrifuged at 3000 rpm for 10 minutes at room temperature and then collected the methanolic supernatant ready for RP-HPLC and LC-MS analysis.

### *TLC analysis*

To perform thin layer chromatography analysis, leaf and root tissues of Podophyllum hexandrum were crushed in grinder. One gram of each sample powder was put in 10 ml of falcon tube each. 10 ml of absolute methanol in each falcon tube was added and vortexed for 10 minutes. Kept the tubes as such for overnight and centrifuged at 3000 rpm for 10 minutes at room temperature, later, collected the supernatant. This supernatant

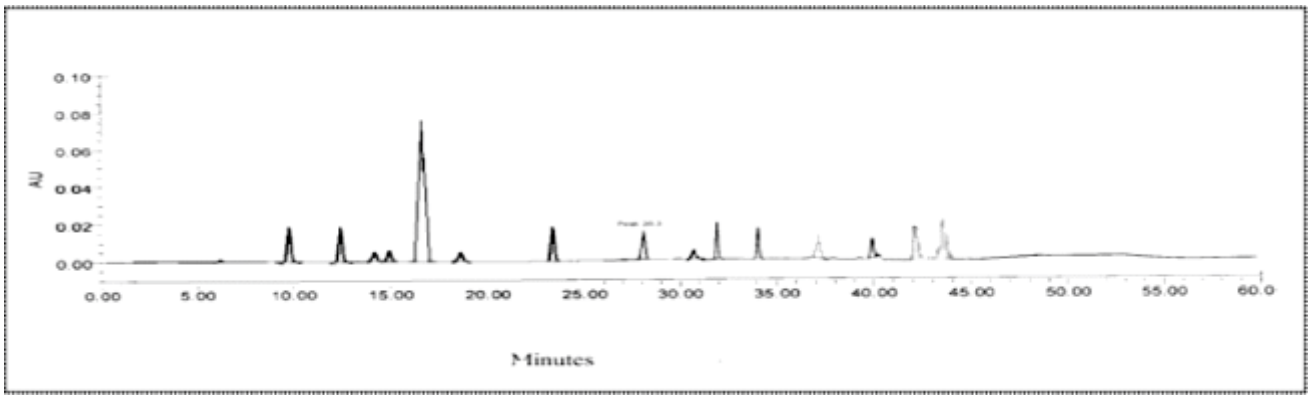


**Fig. 1.** HPLC Chromatogram for root samples of Podophyllum hexandrum.

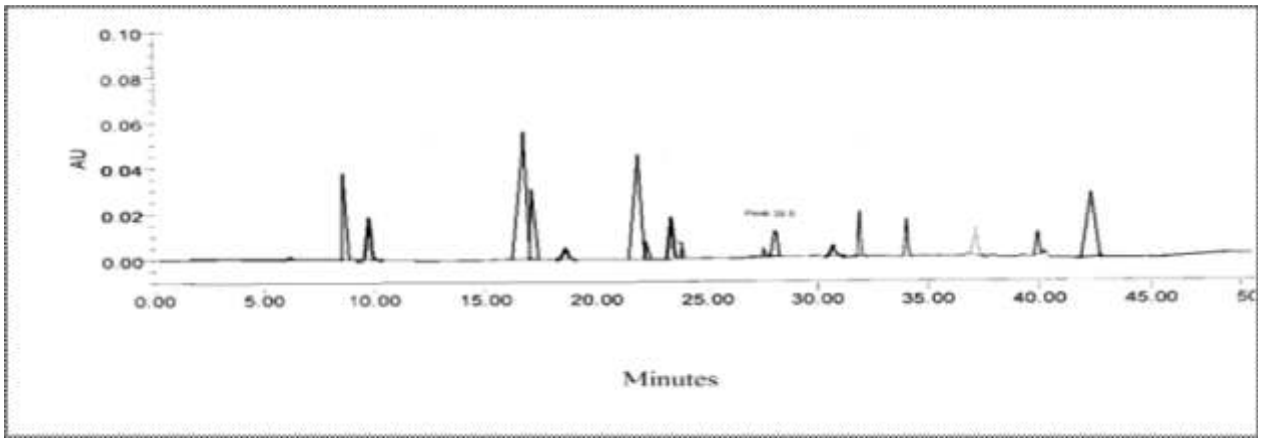


**Fig. 2.** HPLC chromatogram for leaf samples of Podophyllum hexandrum

**Table 1.** HPLC data for p-toxin

| Sample name | Retention time (R.T) (minutes) | HPLC Purity (%) |
|---|---|---|
| Blank | 28.4 | - |
| Leaf | 28.5 | 2.001 |
| Root | 28.3 | 2.08 |

mixture was used for TLC spotting. For TLC profiling, standardized solvent of chloroform and methanol at 9:1 was used.

***HPLC analysis***

Podophyllotoxin analysis was performed by High Performance Liquid Chromatography (HPLC). The HPLC equipment (Make: Agilent, Technologies, Germany) consisted of a 1200 series binary pump (G1312B), a 1200 series gradient pump (G1310A) and a degasser (G1379B) connected to an autosampler. Gradient chromatographic separation of Podophyllotoxin was performed on a 50mm×2.1mm (i.d.) Macherey-Nagel NUCLEODUR C18 Gravity HPLC column, packed with 1.5 $\mu$m particles equipped with a 0.5_m pre-filter (Upchurch Scientific, Oak Harbor, WA, USA). The injection volume was 10$\mu$L and the column

oven temperature was set to 30°C. Mobile phase 'A' was methanol/water (1/1, v/v), mobile phase 'B' was 100% methanol, both containing 0.1% ammonium hydroxide (25%) and 10 mmol/L ammonium acetate (pH 9). A gradient elution was performed with 100% 'A' for 0.5 min, a linear increase to 50% 'A' until 4.5 min, followed by 0% 'A' from 4.6 until 5.5 min and re-equilibration from 5.6 to 6.5 min with 100% 'A'. The flow rate was set to 0.5.

Due to the use of crude samples of podophyllotoxin and the fast LC separation, the influence of the matrix on signal intensities was analysed. Solvent system like Acetonitrile, methanol, water and 25% NH4OH shows a variation in the quantification of podophyllotoxin from Podophyllum hexandrum. The presence of Podophyllum hexandrum extract caused a mean signal reduction (±S.D.) of 20±5%, calculated with the EZ – Chrome 6.0 version software, 1200 series Agilent Technologies.

## RESULTS AND DISCUSSION

From the previously reported results, it was concluded that podophyllotoxin present in higher amount in plant population collected from Ralla region of North Indian, Himachal Pradesh, (Sharma 2013). All these plants were collected from the higher altitude range (2500-4000 m) of Himalayas, Himachal Pradesh and there is no impact of altitude on the content of podophyllotoxin. It was reported that, content of podophyllotoxin decreased in rhizomes as the altitude increased and this phenomenon is reversed in case of leaves of *P. hexandrum* (Sharma 2013). 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 (Airi et al. 1997).

A single laboratory validation (SLV) method was developed with RP-HPLC technique. HPLC chromatograms for root sample and leaf sample are presented in Fig. 1 and Fig. 2, respectively. Thin layer chromatogram for the leaf and root samples of *Podophyllum hexandrum* shows Rf value 0.87, 0.85 for the leaf and root respectively. Results showed that the leaf sample analysed resulted in 2.001% podophyllotoxin content, whereas root sample resulted in 2.08% podophyllotoxin content in *Podophyllum hexandrum* (Table 1). Therefore, the proposed protocol is a simple, short and low cost effective HPLC method and may be useful for determination of podophyllotoxin in Podophyllum hexandrum for various experimentation.

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