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Identification of Aryltetralin Lignans from *Podophyllum hexandrum* Using Hyphenated Techniques

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ABSTRACT

In the present study, with the appliance of hyphenated techniques involving high performance liquid chromatography with diode array detection directly coupled with ^{1}H nuclear magnetic resonance spectroscopy (LC-NMR) and electrospray ionization mass spectrometry (LC-ESI-MS), four known aryltetrahydronephthalene lignans (podophyllotoxin 4-O-glucopyranoside, 4'-demethylpodophyllotoxin, podophyllotxin and dehydropodophyllotoxin) have been well characterized from methanol extract of rhizomes of *Podophyllum hexandrum* Royle without involving the time consuming steps of isolation and purification of individual constituents of the extract. Also, a simple, sensitive and reproducible LC-MS method has been developed for the quantitative analysis of podophyllotoxin in crude methanolic extract from different parts of *Podophyllum hexandrum* Royle. The present work adds up to the existing significance of hyphenated techniques combining chromatographic technologies with spectroscopic techniques for both qualitative and quantitative analysis of natural product extracts or fractions without any need of reference standards for the markers.

Keywords: *Podophyllum hexandrum*, LC-MS, LC-NMR, podophyllaceae, phytochemical study, aryltetrahydronephthalene lignans, podophyllotoxin.

INTRODUCTION

Podophyllum hexandrum Royle (Berberidaceae) syn. P. emodi Wall bearing the common names Himalayan Mayapple or Indian Mayapple (as its fruits ripen in spring), is a perennial herb native to the lower elevations of Himalayan regions of Asian continent including Afghanistan, Pakistan, India, Nepal, Bhutan and Southwest China. [1-4] In India, this plant species is commonly distributed in alpine and sub-alpine Himalayan regions at altitudes ranging from 2000 to 4500 meters of Jammu and Kashmir, Sikkim, Himachal Pradesh, Uttaranchal and Arunachal Pradesh with certain morphological, biochemical and genetical variations. [5-6] It is considered as endangered and highly valuable medicinal plant, known as "Aindri", a divine drug in the traditional systems of medicines such as Ayurveda, Unani, Siddha and has been used as an intestinal purgative and emetic, salve for infected and necrotic wounds and inhibition of tumor growth. [7] The roots and rhizomes of *Podophyllum hexandrum* are known to contain several secondary metabolites including lignans, which have been used as folk remedies in the traditional medicines of several cultures. [8-9] These lignans exhibit numerous promising biological activities such as reverse transcriptase inhibition & anti HIV activity. [10-12] immunomodulatory activity, [13] effects on cardiovascular system, [14] anti-leishmaniosis, [15] effects on high density lipoproteins and hypolipemiant

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properties, [16] antiatherosclerotic, anti-diabetic, [17] 5-lipoxigenase inhibition, [18] antifungal, [19] antirheumatic, [20-21] antipsoriasis and antimalaria. [22] Podophyllotoxin, an active constituent of *Podophyllum hexandrum*, is well renowned for its anti-cancer, immunostimulatory, anti-fungal, anti-mitotic, [22] antiviral, [23] and radioprotective effect. [24] Podophyllotoxin is used as starting compound for the chemical synthesis of anti-cancer drugs etoposide, teniposide and etophos, used for the treatment of lung and testicular cancers as well as certain leukemias. [25-27] The limited availability of *Podophyllum hexandrum* Royle due to its long juvenile phase, poor fruit setting ability and time consuming collection of the plants results in difficulty in obtaining aryltetrahydronephthalene and related lignan markers from this plant. Also, keeping in view of the impact of geographical distance/altitude & environmental conditions on active constituents of declining populations of *P. hexandrum*, phytochemical study of this plant species employing simple analytical methods is of great significance.

Several analytical methods including HPLC have been reported in the literature for the determination and quantification of lignans in the genus *Podophyllum*. [28-31] In recent years, hyphenated techniques have received much attention as the principal means for unequivocal identification and characterization of known and novel metabolite structures without the necessity of isolation and purification of the individual constituents from plant extracts using dyad or sometime triad systems involving the combination of effective separation technique with spectroscopic analysis e.g., LC-MS, GC-MS, LC-FTIR, LC-NMR, etc.. [32-34] The aim of the present study was to utilize hyphenated techniques involving dyad system of combination of high performance liquid chromatography with ^{1}H nuclear magnetic resonance spectroscopy (LC-NMR) and tandem mass spectroscopy (LC-ESI-MS) for structural characterization of aryltetrahydronephthalene lignans present as minor constituents in methanolic extract from rhizomes of *Podophyllum hexandrum* Royle and to evaluate the percentage of podophyllotoxin in the different parts of this plant species via LC-ESI-MS. The availability of this information about the plant extracts results good starting condition for the selective and therefore economic identification & interpretation of novel structures using the known structures of analogous basic skeleton as substructural templates.

MATERIALS AND METHODS

Experimental

Commercially available methanol, water and acetonitrile of high performance liquid chromatography (HPLC) grade (Merck, Darmstadt, Germany) were used as received for the present study. Reagent grade n-hexane, ethyl acetate and methanol (Ranbaxy, Gurgaon, India and Rankem, Mohali, India) were used for extraction and column chromatography. TLC was performed on 0.25 mm silica gel 60 F254 plates. After development of TLC plate, the plate was kept under the exposure of 254 nm UV light and visible spots were marked. Then the chromatogram was immersed in 10% solution of H_2SO_4 in CH_3OH followed by heating for 10 minutes on a hot plate at nearly 100°C. The grey or purple spots thus developed clearly indicated carbonized lignans. Silica gel 60-120 mesh was used for column chromatography using n-hexane-EtOAc gradient as eluent. ^{1}H NMR chemical shifts and coupling constants *J* are given in ppm and Hz, respectively.

Plant Material and Extraction

For the present study, live plant material of *Podophyllum hexandrum* Royle (Synonym *Podophyllum emodi* Wall) i.e., rhizomes, fruits, leaves and roots were collected from the high-altitude regions of Sonmarg (>3000m, Kashmir Himalayas, Jammu and Kashmir) during August, 2008 and it was authenticated by a plant taxonomist from the centre of Plant Taxonomy, University of Kashmir, Srinagar (J&K). Specimens were deposited in the repository of Indian Institute of integrative medicine, Jammu, India. The extraction procedure was kept as simple as possible to allow its facile application. The rhizomes, fruits, leaves and roots were washed thoroughly with the running tap water to remove the extraneous material and were dried under partial shade in a well ventilated room. The dried samples (leaves weighing 75 g, roots weighing 50 g, fruits weighing 50 g & rhizomes weighing 50 g) were powdered with a grinder and subjected to petroleum ether fractionation (40-60°C) in a percolator, giving total four washes with petroleum ether. For each wash, the material was kept in the solvent for 24 hours. The solvent was removed under vacuum. The defatted plant material was then fractioned with methanol (four washes, cold) and extracts were concentrated to dryness under reduced pressure at 40°C to afford dried crude extracts (1.32 g extract from leaves, 1.18 g extract from roots, 1.16 g extract from fruits & 1.30 g extract from rhizomes). Each dried extract (2 mg) was then redissolved in 2 mL of HPLC-grade methanol and filtered through 0.45µm filter.

Preparation of Standard Solutions

Podophyllotoxin stock solution (1 mg/mL) was prepared in HPLC-grade methanol. From the stock solution, different amounts were injected into the LC system to create the five point calibration curve.

Liquid chromatography-tandem mass spectrometry (LC–ESI-MS) analysis

HPLC analysis was conducted using Agilent 1100 series HPLC system consisting of quaternary pump and Sedex 75 ELSD detector connected in series with PDA detector to enhance its detection capability. LC-ESI-MS analysis was performed using Bruker Daltonics Esquire 3000 ion trap mass spectrometer (MS) with an electrospray interface coupled with Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, U.S.A.). The chromatographic system was equipped with a binary pump, an auto sampler, an automatic

electronic degasser, an automatic thermostatic column oven, a diode array detector and a computer with chemstation software for data analysis.

The LC separations were achieved using an RP-18 column (4.0 mm × 100 mm, 5µm particle size) (Merck, Darmstadt, Germany). Elution was carried out with the mobile phase of MeOH-H_2O at a flow rate of 0.8 mL/min. The gradient started with 35% methanol (5 min isocratic), the percentage of methanol was increased to 60% (10 min isocratic) and subsequently decreased again to 10%. The total analysis run time was 30 min. The LC column temperature was maintained at 30°C and the chromatograms were recorded at 240.8 nm.

The MS was equipped with an atmospheric pressure ionization electrospray interface. High purity nitrogen from a nitrogen generator was used as a carrier gas. The parameters for mass spectrum analysis were set as: drying gas flow rate of 11 L/min, drying gas temperature 320°C, nebulizer pressure 35 psi, capillary voltage 4000 volt, capillary exit voltage 112.6 volt and the temperature of heated inlet capillary was 220°C. The mass range was set from 50-900 m/z, ICC target value 8000, while the maximum accumulation time was 200 min. All the interface parameters were optimized by injecting standard solution of podophyllotoxin during the experiments. A 5µl aliquot of extract solution was injected; MS spectra were acquired selecting positive mode scanning from 50 to 900 m/z with ion accumulation time of 50955 micro seconds for each spectrum. The mass spectra were recorded in the centroid mode and referenced to 100% intensity of the base peak.

Liquid chromatography - ^{1}H nuclear magnetic resonance spectroscopy (LC-NMR) analysis

Experiments were performed using Bruker Peak Sampling Unit (BPSU-36) with loop storage mode. The LC-NMR system included Avance 500 NMR spectrometer (Bruker-Biospin, Switzerland) coupled to Agilant 1100 HPLC system with G1314A variable wavelength detector, RP-18 column, quaternary pump G1311A and 4mm flow probe (active cell volume 120µL) with Z145 gradient. Elution was carried out with the mobile phase of CH_3CN-D_2O (35:65, v/v) at the flow rate of 0.6 mL/min for 37.05 minutes and chromatograms were recorded at 254 nm. One dimensional NMR spectra were acquired with 20 ppm spectral window and TD 16 K. Solvent peaks were suppressed and number of scans accumulated was 512 to obtain appropriate signals to noise ratio.

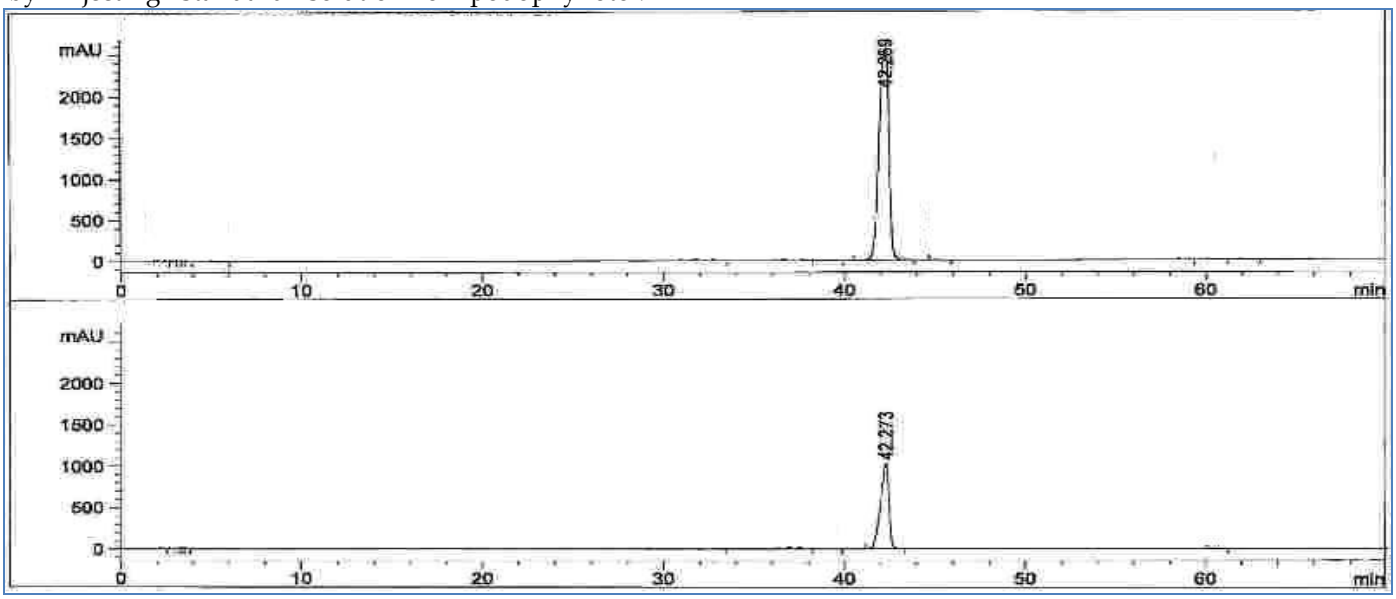

Fig. 1: HPLC chromatogram of standard podophyllotoxin.

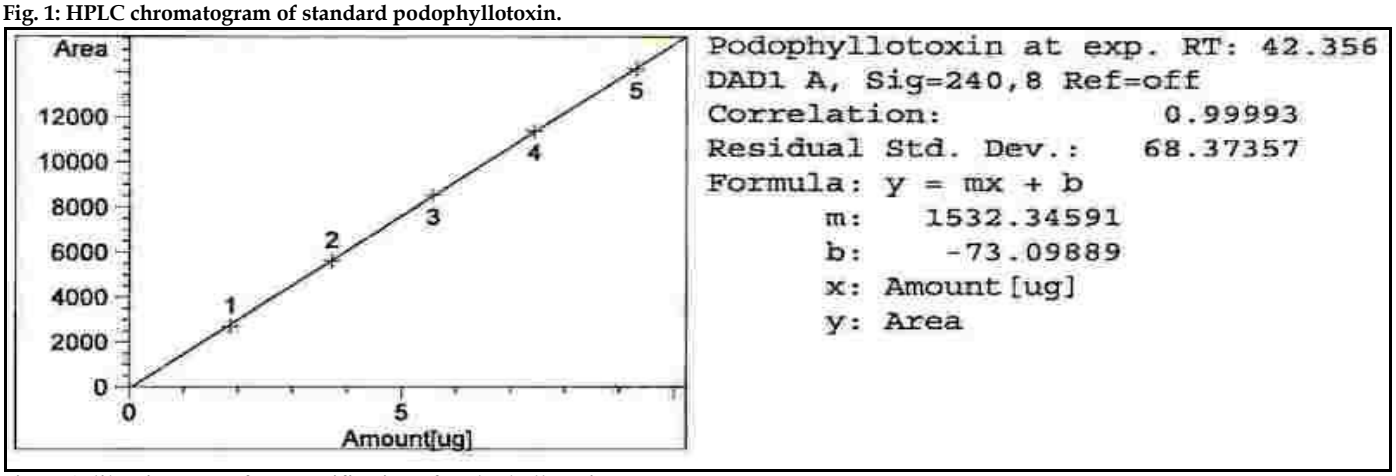

Fig. 2: Calibration curve for quantification of podophyllotoxin.

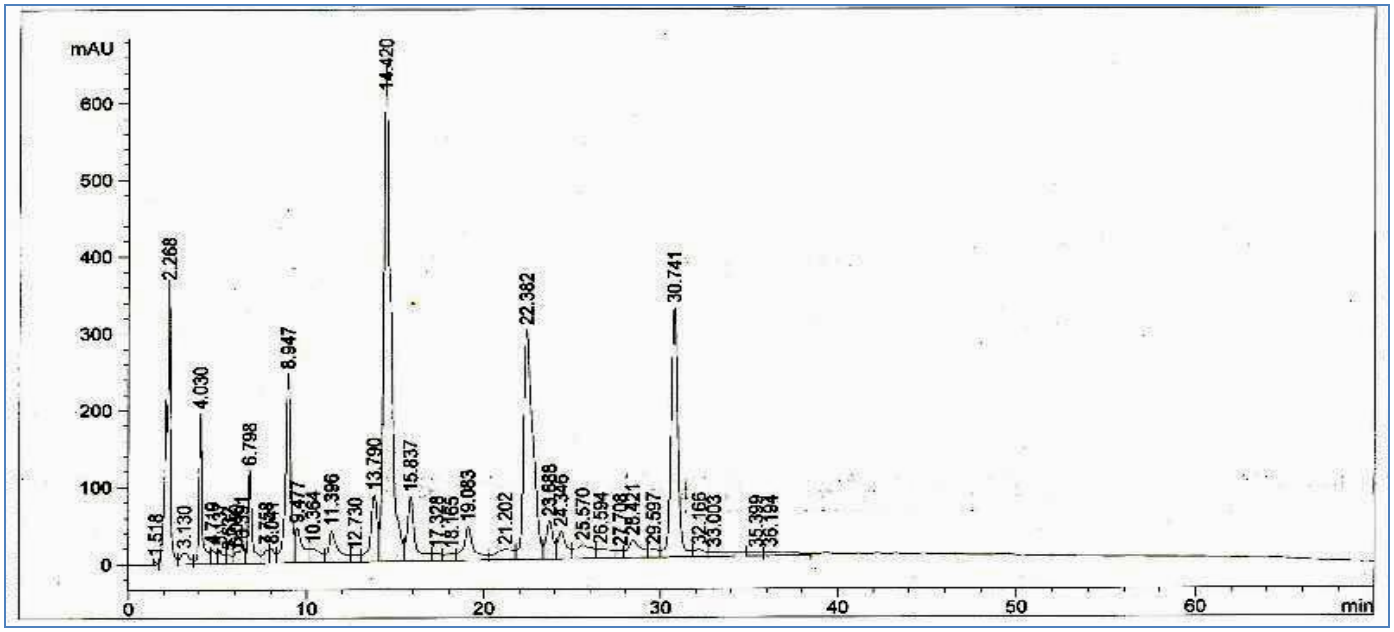

Fig. 3: HPLC chromatogram of methanolic extract from rhizomes of *Podophyllum hexandrum*.

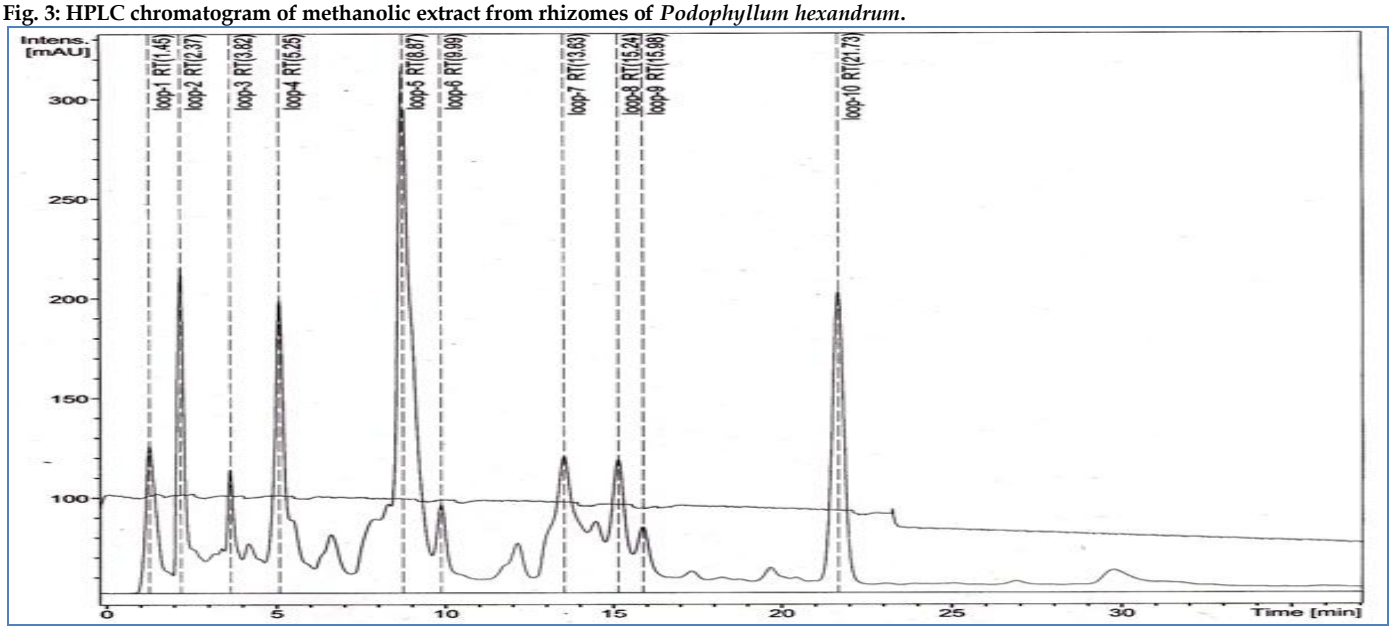

Fig. 4: LC-NMR profile of methanolic extract from rhizomes of *Podophyllum hexandrum*.

Table 1: Podophyllotoxin (% dry wt.) content in different parts of plant extracts of *Podophyllum hexandrum*.

Plant extracts	Podophyllotoxin (% dry wt.)	Chromatographic analysis
Rhizomes	0.2	LC-MS
Roots	0.5	LC-MS
Fruits	0.031	LC-MS
Young leaves	0.081	LC-MS

RESULTS AND DISCUSSION

Quantitative analysis

The ESI-MS/MS spectrum of purified podophyllotoxin used as standard for the present study was recorded, that exhibited fragmentation pattern similar to that reported in literature. [28-29] The quantitative analysis of podophyllotoxin was carried out by LC-MS. Under the LC-MS conditions mentioned in experimental section, purified podophyllotoxin was eluted at retention time (Rt) of 42.3 min. (Fig. 1) and the same peak exhibited a molecular adduct at *m/z* 437.1 (sodium adduct of podophyllotoxin) in positive ionization mode of ESI-MS. Quantitation of podophyllotoxin in the extracts prepared from different parts of the plant (rhizomes, fruits, leaves and roots) was done on the basis of the calibration curve established by injecting five concentrations of the podophyllotoxin standard in the concentration range of 1µg/µL to 10µg/µL each time before sample analysis. Quantitation of podophyllotoxin was carried out using selective ion monitoring (SIM) detection of the molecular ion peak at retention time of 42.3 min with *m/z* 437.1 $[M+Na]^+$. Linear calibration curve of podophyllotoxin within the concentration range of 1µg to 10µg (R^2 = curve co-efficient 0.9999) was obtained (Fig. 2). Validation of the method was carried out by spiking 10µg of standard podophyllotoxin to 10 mg of the dried plant extract. The spiked sample was extracted with methanol and analyzed by the proposed method.

The precision of the method was assessed by adding different concentrations of podophyllotoxin standard to

the samples and comparing the amounts determined from their chromatograms with the actually added amounts. Total podophyllotoxin concentration in plant tissues has been expressed on a dry weight basis (Table 1).

LC-ESI-MS Analysis

In the HPLC profile of methanolic extract from rhizomes of *Podophyllum hexandrum* (Fig. 3), there were peaks with good separation, out of which four major peaks were analyzed and MS data for these peaks was obtained. These peaks correspond to retention time of 6.8 min., 8.9 min., 14.4 min. and 19.1 min.

LC-NMR analysis

For the characterization of the components present in the rhizome extract, an on-flow run was performed in the loop storage mode of LC-NMR that resulted different peaks (Fig. 4), out of which, peaks at retention time 5.25 min. (Loop-4), 8.87 min. (Loop-5), 13.63 min. (Loop-7) and 21.73 min. (Loop-10) were analyzed and the NMR spectrum of these individual peaks showed typical spin systems of an aryl tetralin lignans: aromatic signals at δ 6.31-7.29 ppm, methoxy signal at δ 3.52-4.10 ppm, aliphatic protons in the range δ 2.7-3.1 ppm, an OCH_2O group attached to aromatic ring at δ 5.85-6.00 ppm, indicating typical aryl tetrahydronaphthalene skelton (Fig. 5).

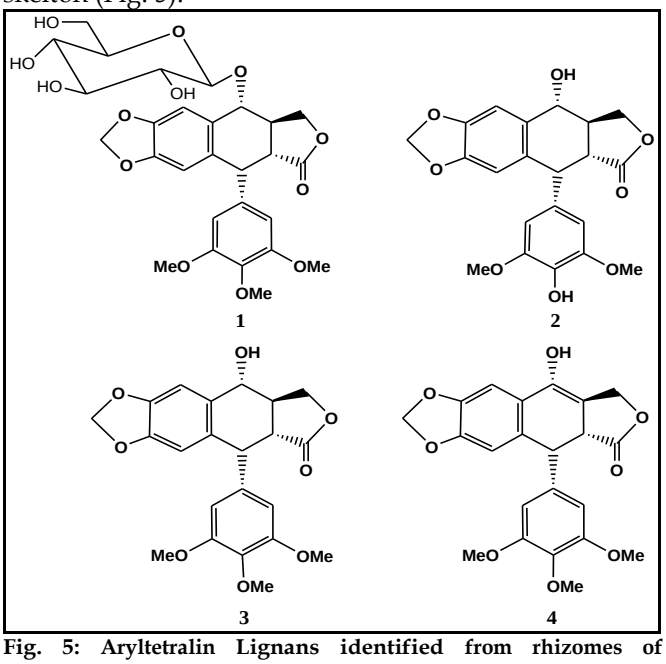

Fig. 5: Aryltetralin Lignans identified from rhizomes of *Podophyllum hexandrum*.

Podophyllotoxin 4-O-glucopyranoside (1)

LC-NMR (500 MHz, CH_3CN-D_2O, Loop-4, Rt 5.25 min.): δ 2.68-2.80 (m, 1H), 3.00-3.04 (m, 1H), 3.29-3.39 (m, 1H), 3.60-3.70 (m, 14 H), 4.08-4.11 (m, 1H), 4.49 (d, 2H, *J* = 5.31 Hz), 4.70 (d, 1H, *J* = 9.87 Hz), 5.09 (d, 1H, *J* = 7.0 Hz), 5.86 (s, 2H), 6.34 (s, 2H), 6.54 (s, 1H), 7.04 (s, 1H).

LC-ESI -MS (Rt 6.8 min.): 599.1 $[M+Na]^+$

4′-Demethylpodophyllotoxin (2)

LC-NMR (500 MHz, CH_3CN-D_2O, Loop-5, Rt 8.87 min.): δ 2.79-2.82 (m, 1H), 2.95-3.08 (m, 1H), 3.59-3.61 (m, 6H), 3.94-3.98 (m, 1H), 4.41-4.47 (m, 2H), 4.67 (m, 1H), 5.86 (s, 2H), 6.05 (s, 1H), 6.31 (s, 2H), 6.38 (s, 1H), 7.03 (s, 1H).

LC-ESI -MS (Rt 9.0 min.): 423.0 $[M+Na]^+$

Podophyllotxin (3)

LC-NMR (500 MHz, CH_3CN-D_2O, Loop-7, Rt 13.63 min.): δ 2.90-3.15 (m, 2H), 3.62 (s, 6H), 3.69 (s, 3H), 3.96-4.10 (m, 1H), 4.58-4.66 (m, 2H), 4.71 (m, 1H), 5.85 (s, 2H), 6.32 (s, 2H), 7.03 (s, 1H).

LC-ESI -MS (Rt 14.4 min.): 437.1 $[M+Na]^+$

Dehydropodophyllotoxin (4)

LC-NMR (500 MHz, CH_3CN-D_2O, Loop-10, Rt 21.73 min.): δ 3.52-3.69 (m, 9H), 4.80 (s, 2H), 5.98-6.00 (m, 2H), 6.35 (s, 2H), 6.65 (s, 1H), 7.29 (s, 1H).

LC-ESI -MS (Rt 19.1 min.): 435.0 $[M+Na]^+$

In the present study, hyphenated techniques involving dyad system of LC-NMR and LC-MS have been applied for the structural information leading to the identification of minor aryltetrahydronapthalene lignans present in methanolic extract from rhizomes of *Podophyllum hexandrum* Royle. Also, an LC-MS method has been developed for the quantitative analysis of podophyllotoxin in crude methanolic extract from different parts of *Podophyllum hexandrum* Royle based on LC-MS/MS fingerprint of pure podophyllotoxin. The experiments demonstrate the advantage of directly coupled LC-NMR and LC-MS with short analysis time compared to off-line analysis, where the individual lignans have to be isolated separately and purified prior to the identification by spectroscopic analysis and thus allowing the systematic screening of crude extract of *Podophyllum hexandrum* without involving hectic purification steps.

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