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# Phytochemical Study of *Nothapodytes foetida* by LC-ESI-MS

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**Abstract** In the present study, hyphenated technique using dyad system of high performance liquid chromatography with diode array detection directly coupled with electrospray ionization mass spectrometry (LC-ESI-MS) has been employed for identification of camptothecines and some minor constituents of methanol extract from different parts of *Nothapodytes foetida* on the basis of systematic mass spectrum analysis without isolation and purification of these components. Also, a simple, precise, sensitive and reproducible LC-MS method has been developed for quantification of camptothecin (CPT), a potent cytotoxic isoquinoline alkaloid, in methanol extract from these different parts of *Nothapodytes foetida* using the same technique.

**Keywords:** *N. foetida*, LC-MS, camptothecin, camptothecinoids, hyphenated technique, quantitative analysis

## 1. INTRODUCTION

*Nothapodytes foetida* (Wt.) Sleumer, formerly known as *Mappia foetida* Miers, is a small, spreading, sub-canopy evergreen tree of family Icacinaceae naturally distributed in in the Indo-Malaysian region and China. In India, it is distributed in the wild forests of Western Ghats, some parts of west Bengal and Assam. This plant species has gained substantial commercial interest in recent years, as it is a prospective source of an expensive cytotoxic monoterpenoid isoquinoline alkaloid, camptothecin (CPT), used as an antitumor drug in the

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treatment of colon, head, breast and bladder cancers acting by stabilizing the DNA-protein complex by forming topoisomerase I-DNA adduct [1], [2]. A number of reports have indicated its therapeutic potential against colon cancer [3], ovarian cancer [4], HIV-1 [5], HSV-2 [6], parasitic trypanosomas & leishmania [7] and malaria [8]. CPT is believed to be the third most important alkaloid sought after by the pharmaceutical companies around the world. Various semi-synthetic analogs of CPT including topotecan and irinotecan have been synthesized as potential therapeutic agents against different types of cancer [9], [10]. *Nothapodytes foetida* has been reported for the highest concentration of CPT amongst the other known botanical sources [11]. This has lead to the large scale exploitation resulting serious threat of extinction of the species from its wild habitat. Phytochemical study of this plant species utilizing simple and reproducible analytical methods is of great significance.

Different analytical methods like high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), proton nuclear magnetic resonance spectroscopy (1H-NMR) and desorption electrospray ionization mass spectrometry (DESI-MS) have been reported for quantification of CPT from *N. foetida*. [12-18]. Furthermore, HPLC profiles have been developed for qualitative and quantitative analysis of active constituents in different parts of this plant[19], [20]. In recent years, hyphenated techniques involving coupling of a separation technique and an spectroscopic detection technology have received much attraction as the principal means to obtain structural information leading to the identification of phytochemicals present in a crude samples [21]. Aim of the present study was to evaluate the percentage of camptothecin and identify camptothecin-related alkaloids and other minor components in the methanol extracts from stem, young leaves, stem bark, seeds, fruits and roots of *Nothapodytes foetida* using reverse-phase high performance liquid chromatography coupled with on-line photodiode array detection and electrospray-ionization tandem mass spectrometry without isolation and purification of these phytochemicals.

## 2. MATERIALS AND METHODS

### 2.1 Experimental

Commercially available water and acetonitrile of HPLC grade (Merck, Darmstadt, Germany) were used as received for the present study. Reagent grade dichloromethane 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 UV light (254-366 nm)

and visible spots were marked. Then the non-visible spots were visualized using p-anisaldehyde solution as visualizing agent (9.2 mL of p-anisaldehyde in 338 mL of 95% EtOH, 3.75 mL of glacial CH3COOH and 12.5 mL of concentrated $H_2SO_4$) followed by heating the TLC plate for 05-10 min at 100-150$^oC$ giving blue to black spots. Silica gel 60-120 mesh was used for column chromatography using $CH_2Cl_2$-$CH_3OH$ gradient as eluent.

### 2.2. Plant Material and Extraction

For this study, the live plant material of *Nothapodytes foetida* was collected from botanical garden of Indian Institute of Integrative medicine (IIIM), Jammu (J&K) that was cultivated from the seeds obtained from the Mahabaleshwar forests of India. Stem, stem bark, seeds, roots, fruits and young leaves were collected. For stem bark collections, the outer bark at breast height was scrapped using a knife.

Five grams of the dried plant material (6-8% moisture contents) was extracted by centrifugation for 10 min. at 2000 rpm in a centrifuge tube with 80 mL of methanol. The supernatant liquid was then quickly decanted from the tube and evaporated to dryness under vacuum. Each residue (2 mg) was redissolved in 2 mL of HPLC grade methanol and filtered through 0.2 µm filter paper and analyzed by LC-MS. The methanol extract from roots was divided into two fractions (8:2, v/v). Column chromatography of the first fraction of this methanol extract using $CH_2Cl_2$-$CH_3OH$ gradient as eluent yielded pure camptothecin (CPT) that was used as standard for LC-MS study. Purity of CPT was checked by TLC and then further confirmed by HPLC (Area 100.0 %). The second fraction was evaporated to dryness under vacuum and analyzed by LC-MS after filtration through 0.2 µm filter paper.

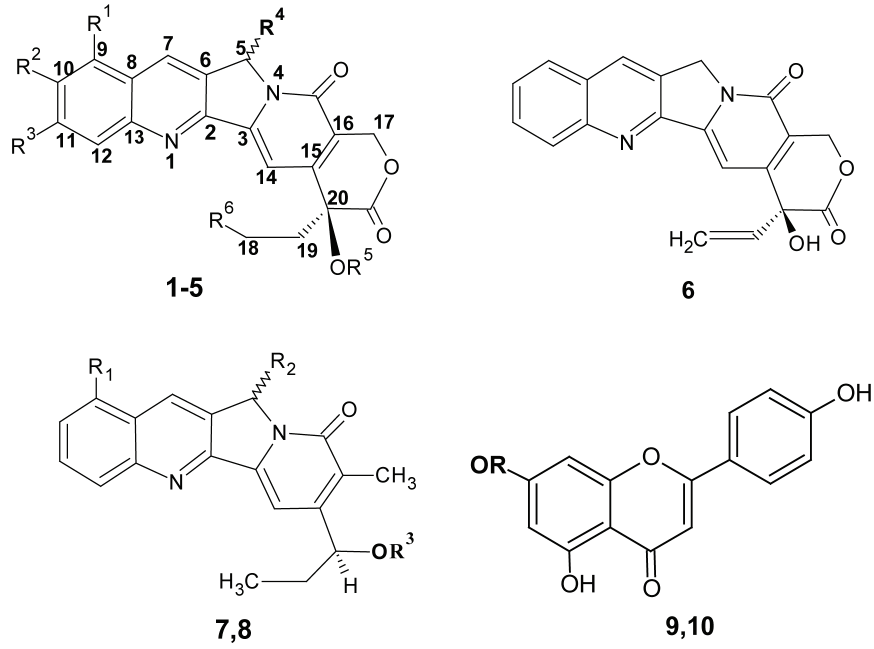
### 2.3. Preparation of Standard Solutions

Camptothecin 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.

### 2.4. 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.




1. **$R^1$= $R^2$ = $R^3$ = $R^4$ = $R^5$ = $R^6$ = H**
2. **$R^1$= OMe, $R^2$ = $R^3$ = $R^4$ = $R^5$ = $R^6$ = H**
3. **$R^1$= $R^3$ = $R^4$ = $R^5$ = $R^6$ = H, $R^2$ = OH**
4. **$R^1$= OMe, $R^2$ = $R^3$ = $R^6$ = H, $R^4$ = OH, $R^5$ = Ac**
5. **$R^1$= OMe, $R^2$ = $R^3$ = $R^5$ = $R^6$ = H, $R^4$ = OH**
6. **$R^1$= OMe, $R^2$ = H, $R^3$ = β-D-Glu**
7. **$R^1$= H, $R^2$ = OH, $R^3$ = β-D-Glu**
8. **R = β-D-Glu**
9. **R = H**

**Figure 1:** Structure of some camptothecins identified fron *N. foetida*

The LC separations were achieved using RP-18 column (4-mm×100-mm, 5µm particle size) (Merck, Darmstadt, Germany). The mobile phase consisted of a gradient of water and acetonitrile at a flow rate of 0.5mL/min. The gradient used started with 10% acetonitrile (5 min isocratic) and over a period of 35

min the percentage of acetonitrile was increased to 98% (10 min isocratic) and subsequently decreased again to 10%. The total analysis run time was 50 min. The LC column temperature was maintained at 30°C and the chromatograms were recorded at 250.8nm.

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 to 700 *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 CPT during experiment.

A 10µl aliquot of extract solution was injected, MS spectra were acquired selecting positive ion mode scanning from 50 to 700 *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.

## 3. RESULTS AND DISCUSSION

The quantitative analysis of CPT (**1**) was carried out by LC-ESI-MS. Under the LC-MS conditions mentioned in experimental section, purified CPT was eluted at retention time (Rt) of 20.1 min. (Figure 2) and by applying these condition in positive ion mode of ESI-MS, the same peak exhibited a molecular adduct at *m/z* 349.1 $(M+H)^+$.

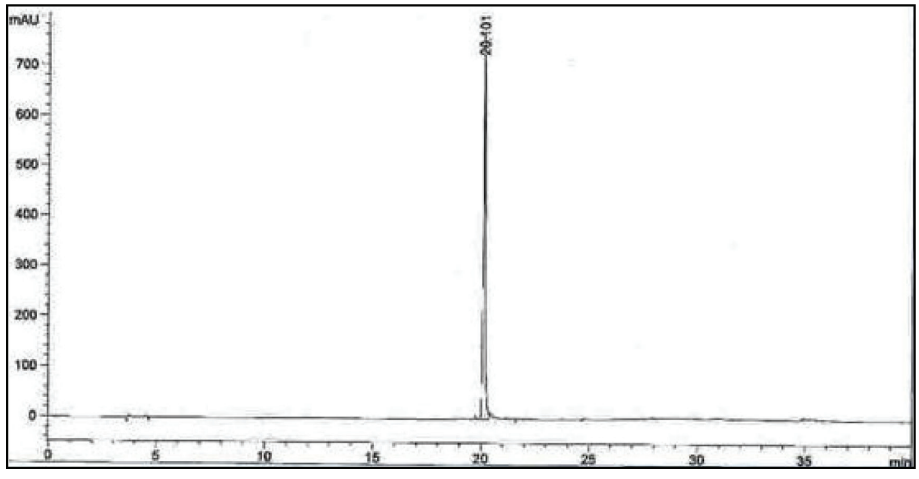



**Figure 2:** HPLC chromatogram of purified CPT

Quantitation of CPT in the extracts prepared from different parts of the plant (stem, stem bark, seeds, roots, fruits and young leaves) was done on the basis of the calibration curve established by injecting five different concentrations of CPT standard in the concentration range of 1µg/µL to 10µg/µL each time before sample analysis. Quantitation of CPT was carried out using selective ion monitoring (SIM) detection of the molecular ion peak at *m/z* 349.1 $[M+H]^+$. Linear calibration curve of CPT within the concentration range of 1µg to 10µg ($R^2$ = curve coefficient 0.9999) was obtained (Figure 3). Validation of the method was carried out by spiking 10µg of standard CPT to 10 mg of the dried plant extract. The spiked sample was extracted with methanol and analyzed by the proposed method. The recovery was within the range of 92% to 99%. The precision of the method was assessed by adding different concentrations of CPT standard to the samples and comparing the amounts determined from their chromatograms with the actually added amounts. Total CPT concentration in plant tissues has been expressed on a dry weight basis (Table 1).

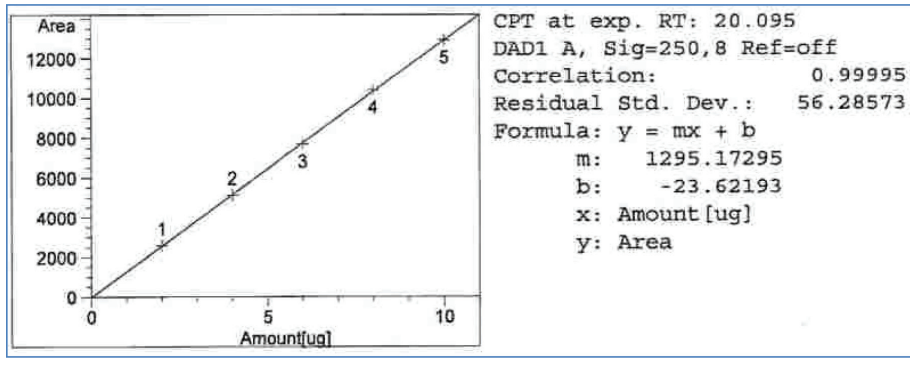



**Figure 3:** Calibration curve for quantification of CPT.

**Table 1:** CPT (% dry wt.) content in different parts of plant tissues of *Nothapodytes foetida*.

| Plant Tissues | CPT (% dry wt.) | Chromatographic analysis |
|---|---|---|
| Stem | 0.15-0.28 | LC-MS |
| Stem bark | 0.2 | LC-MS |
| Seeds | 0.1 | LC-MS |
| Roots | 1.0-2.0 | LC-MS |
| Fruits | 0.031 | LC-MS |
| Young leaves | 0.081 | LC-MS |

LC-MS analysis of stem, young leaves, stems bark, seeds, fruits and root bark tissues of *Nothapodytes foetida* led to the identification of different phytochemicals (Table 2) and a number of as yet unidentified camptothecines. A number of compounds having different retention time but the same molecular weight were identified from these different plant tissues. These compounds were isomeric entities of camptothecinoids detected in the accessions of *N. foetida.*

**Table 2:** Different phytochemicals identified from *Nothapodytes foetida.*

| S. No. | Compound | Plant Tissue | Rt (min.) | m/z |
|---|---|---|---|---|
| 1 | Camptothecin | All | 20.1 | 349.1 $[M+H]^+$ |
| 2 | 9-Methoxy- | stem bark | 29.8 | 379.2 $[M+H]^+$ |
| | camptothecin | | | |
| 3 | 10-Hydroxy-camptothecin | stem bark | 28.0 | 365.0 $[M+H]^+$ |
| 4 | 5-hydroxy-9-methoxy- | seeds | 21.9 | 437.1 $[M+H]^+$ |
| | O-acetyl-camptothecin | stem bark | 22.4 | |
| 5 | 5-Hydroxy-9-methoxy- | leaves | 2.8 | 395.0 $[M+H]^+$ |
| | camptothecin | stem bark | 5.0 | |
| | | stem | 27.1 | |
| 6 | 18,19-Dehydrocamptothecin | Seeds, leaves | 3.0 | 347.0 $[M+H]^+$ |
| 7 | 9-Methoxy-mappicine- | leaves | 24.5 | 498.3 $[M+H]^+$ |
| | 20-O-glucopyranoside | seeds | 24.5 | |
| | | stem | 24.5 | |
| 8 | 5-hydroxy-mappicine- | leaves | 30.3 | 484.2 $[M+H]^+$ |
| | 20-O-glucopyranoside | | | |
| 9 | Apigenin-7-O- | stem | 25.9 | 433.0 $[M+H]^+$ |
| | glucopyranoside | | | |
| 10 | Apigenin | stem bark | 25.0 | 293.0 $[M+Na]^+$ |
| | | seeds | 30.4 | |
| 11 | Cinnamyl O-β- | leaves | 2.5 | 296.9 $[M+H]^+$ |
| | glucopyranoside | stem | 25.4 | |
| 12 | Omega-Hydroxypropioguaiacone | stem bark | 2.62 | 196.8 $[M+H]^+$ |

| | | | | |
|---|---|---|---|---|
| 13 | Scopoletin | leaves | 28.0 | 193.0 $[M+H]^+$ |
| 14 | Sitosterol-βD- | leaves | 29.5 | 576.8 $[M+H]^+$ |
| | glucopyranoside | stem bark | 29.8 | |
| 15 | 3-Hydroxystigmast-5-en-7-one | stem | 17.5 | 430.1 $[M+H]^+$ |
| | | | 3.5 | 451.3 $[M+Na]^+$ |
| 16 | Stigmastenone | leaves, fruits, seeds, | 2.5 | 413.1 [M]+ |
| | | stem, stem bark | 3.5 | |
| 17 | Trigonelline | leaves | 3.3 | 137.9 $[M+H]^+$ |
| 18 | Pumiloside | fruits | 28.0 | 513.1 $[M+H]^+$ |
| | | stem bark | 5.0 | |

In the present study, hyphenated technique involving dyad system of reverse-phase high performance liquid chromatography and electrospray-ionization tandem mass spectrometry (LC-ESI-MS) has been applied for the phytochemical study of methanol extracts from stem, young leaves, stem bark, seeds, fruits and roots of *Nothapodytes foetida* leading to the unanimous identification of some camptothecins and other minor components on the basis of systematic mass spectrum analysis including fragmentation patterns and comparison with literature data, without involving the time consuming and hectic separation process of these individual constituents. There were a number of other components that could not be identified only by LC-MS. Also, an LC-MS method has been developed for the quantitative analysis of CPT in crude methanolic extract from these different parts of the plant using the same technique.

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