Prospecting for Camptothecines from *Nothapodytes nimmoniana* in the Western Ghats, South India: Identification of High-Yielding Sources of Camptothecin and New Families of Camptothecines

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Abstract

Camptothecin (CPT), a monoterpene alkaloid, is an important anti-cancer compound obtained from several plant sources including *Camptotheca acuminta* (from China) and *Nothapodytes nimmoniana* (from India). Currently, by far the highest levels of CPT (approximately 0.3% w/w) are reported from *Nothapodytes nimmoniana*, a small tree distributed in the Western Ghats, India. In recent years because of the heavy demand, there has been a serious threat of extinction of the populations of the tree in the Western Ghats forest of south India. Several studies have chemically profiled populations of the species in the Western Ghats to identify sources of high yield and therefore to enable the sustainable production and harvesting of CPT. In this study, using both high-performance liquid chromatography and liquid chromatography–mass spectrometry, we report for the first time the identification of trees that produce at least 5- to 8-fold more CPT than hitherto reported. Furthermore, we show for the first time the production of a few minor camptothecines, including 10-hydroxy camptothecin, in the stem and root bark extracts of the tree. These results have important implications for not only harnessing the high-yielding individuals for clonal multiplication but also for exploiting some of the minor camptothecines, which also have been shown to have important anti-cancer and anti-viral activity.

Introduction

Camptothecin (CPT), a monoterpene alkaloid, is an important anti-cancer compound obtained from several plant sources (1–9). It was first isolated from extracts of *Camptotheca acuminata*, a tree native to China (3). Since the discovery that the primary cellular target of CPT is DNA topoisomerase I (topo I), a number of reports have indicated its therapeutic potential (10), against colon cancer (11), AIDS (13), uterine cervical cancer, ovarian cancer (13), and malaria (14). Two clinically used anti-tumor compounds, topotecan and irinotecan, are currently semi-synthesized using natural camptothecins. In fact, the ever-increasing worldwide market of irinotecan and topotecan has currently reached one billion US dollars, which represents approximately one ton of CPT in terms of raw material (15,16). Though CPT has been reported from several sources [including *Merriliodendron megacarpum* (7,5), both belonging to the family Icacinaceae; *Ophirrohiza mungos* (17) and *O. pumila* (18) from the family Rubiaceae; *Eravatamia heyneana* belonging to Apocynaceae; and *Mostuea brunonis* belonging to the family Loganiaceae (7)], the highest concentration of CPT (approximately 0.3 % on a dry weight basis) has been reported from *Nothapodytes nimmoniana* (19). Consequently, there has been an unprecedented pressure on the natural populations of *Nothapodytes nimmoniana* in the Western Ghats. It is estimated that over the last decade there has been a 20% decline in the natural population of this species in Western Ghats (20,21). In fact, owing to this threat, *Nothapodytes nimmoniana* has been classified as a "Vulnerable" species (21).

In recent years, efforts have been made to identify high-yielding sources of *N. nimmoniana* that could be used in the clonal multiplication of trees or in developing appropriate in vitro production systems (22–28). In a previous study, Suhas et al. (29) analyzed 147 individuals from 11 populations in Western Ghats, south India, for their CPT content and found that approximately 23 of these individuals yielded CPT in excess of 1% by dry weight. In this study, we have determined the CPT content in stem and root bark tissues of 17 of these 23 individuals using liquid chromatography–mass spectrom-

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etry (LC–MS). Besides confirming the earlier estimates reported by Suhas et al. (29), we report here the presence of some new camptothecines in the root and stem bark samples. For the first time, we report the occurrence of certain camptothecines, namely 10-hydroxy camptothecin, diacetoxy-camptothecin, and acetoxy-camptothecin glycoside in *Nothapodytes nimmoniana*. We discuss these results in the light of its implications for the conservation and management of elite individuals of *Nothapodytes nimmoniana* for a sustained production of CPT.

Experimental

Study system

The study was conducted on *Nothapodytes nimmoniana* Graham (Icacinaceae) (formerly known as *Nothapodytes foetida* [Wight] Sleumer and *Mappia foetida* Meirs), a small broad-leaved tree commonly referred to as "stinking tree" because of the fetid smell of its fruits and seeds. The species is polygamous in nature with a wide array of breeding types including male, female, hermaphrodite, monoecious, andromonoecious, gynomonoecious, and trimonoecious trees. The species is distributed in the Western Ghats forest of south India, some parts of Assam in northeast India, and in the Himalayan foothills, besides being reported in Sri Lanka, Myanmar, and Thailand (30).

Sampling strategy

The samples were drawn from an earlier collection of 147 trees that were chemically profiled for camptothecin (29). These trees were sampled from 11 populations along the Western Ghats (from 80 N to 150 N latitude), one of the three megadiversity hotspots in India (Figure 1). Twenty-three of the trees from 4 populations were found to have CPT in excess of 1% either in their stem or root barks. These are by far the highest yields of CPT reported thus far (29).

For this study, we chose 17 of these 23 trees (9 for which highest CPT was reported from stem bark and 8 from root bark) for the high-performance liquid chromatography (HPLC)–LC–MS quantitation of camptothecin as well as the detection of other camptothecines in the tissue. Each of the trees was given a unique identification number and the details of collection (tissue collected), name of the site, latitude, and longitude of collection were recorded in a registry maintained at the School of Ecology and Conservation, University of Agricultural Sciences, GKVK, Bangalore, India. For each tree, the girth at breast height was recorded. For stem bark collections, the outer bark at breast height was scraped using a knife and a section of the inner bark (5 cm × 5 cm) was collected into a plastic bag and sealed. Similarly, for the root bark samples, exposed (surface) roots were scraped and the inner bark was collected into a separate plastic bag and sealed.

Extraction of CPT

One hundred milligrams of the dried plant material (6–8% moisture contents) was extracted in a centrifuge tube with 2 mL of methanol by sonication (2 × 30 s). The mixture was mixed with 18 mL water and 20 mL dichloromethane and the material was stirred vigorously for 5 min on a magnetical stirrer. Centrifugation for 10 min at 2000 rpm yielded two phases. The dichloromethane phase was separated and evaporated to dryness. The residue was dissolved in 1 mL of chloroform. These extracts were dried to give camptothecinoids residues. The residues were reconstituted in a chloroform–methanol (3:1) mixture, filtered through 0.2 µm filter paper, and analyzed by HPLC and LC–MS.

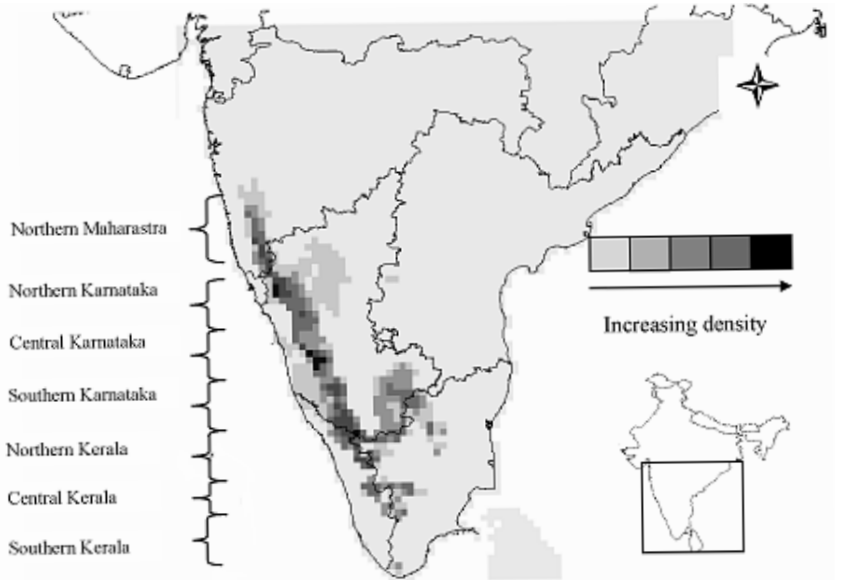

Figure 1. Density distribution map of *Nothapodytes nimmoniana* in the Western Ghats, India. The density distribution map was developed based on 64 points of occurrence of the species using a GIS platform. The different shades of gray indicate the relative concentration of records of the species in the Western Ghats (light to dark, indicating increasing concentration). The sites of origin of 17 trees sampled in the study are indicated on the map. The classification of the latitudinal gradient of the species into the different zones is purely for the purpose of discussion in the text.

HPLC analysis

The HPLC system consisted of an Agilent 1100 instrument (Palo Alto, CA) 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 Merck (4.6 × 250 mm, 5 µm) column. The mobile phase consisted of a gradient of water and acetonitrile at a flow rate of 0.5 mL/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 nm.

Camptothecin stock solution (1 mg/mL) was prepared in an HPLC-grade chloroform–methanol (9:1) solvent mixture. The stock solution was stored in the refrigerator at 4°C. From the stock solution, working solution was prepared in the concentration range of 0.1 to 0.5 mg/mL and 10 µL (n = 6) of the solution was injected into the HPLC system for preparation of the standard curve (curve co-efficient 0.999849).

Under the previously mentioned conditions, camptothecin was eluted at a retention time of 20.1 min. The recovery studies were carried out by adding camptothecin at 100 µg/10 mg of the extract, and the recoveries were with in the range of 98.5% to 103.7%.

HPLC–MS analysis

For HPLC–MS analysis, the same HPLC which was used for the HPLC analysis was coupled to an Ion Trap of camptothecin MS (Enquire-3000) from Bremen (Germany). The MS was equipped with an atmospheric pressure ionization electrospray interface. High purity nitrogen from a nitrogen generator was used as a carrier gas. All the interface parameters were optimized by injecting standard solution of camptothecin during the HPLC–MS experiments. The conditions for mass spectrum analysis during the LC–MS studies were set at a dry gas flow rate of 11 L/min, nebulizer pressure 35 psi, and drying gas temperature at 320°C. The mass range was from 50–700 *m/z*; ICC target value 8000, while the maximum accumulation time was 200 ms.

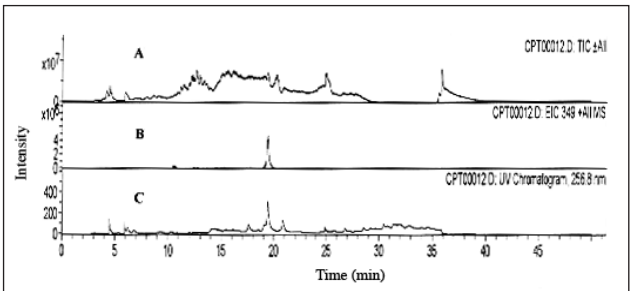

Figure 2. Total ion current trace (A), extracted ion current trace (B), and UV–DAD chromatogram (C) of CPT in plant sample.

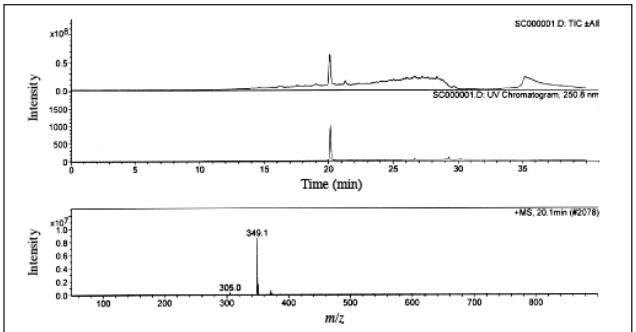

Figure 3. Total ion current, UV–DAD chromatogram, and mass spectra of CPT.

By applying these conditions in the positive mode of ESI–MS, camptothecin exhibited a molecular adduct $(M+H)^+$ at *m/z* 349.1 (Figure 3). The sodium adduct of CPT was also visible at *m/z* 371 $[M+Na]^+$. The molecular ion peak at *m/z* 349 was carried for quantitation during the LC–MS studies, as this was the most intense peak in the spectrum.

Quantitation of CPT in the extracts prepared from different stem and root barks was done on the basis of the calibration curves established by injecting five concentrations of the CPT standard in the concentration range of 1 µg to 5 µg each time before sample analysis. Quantitation of CPT was carried out using selective ion monitoring (SIM) detection of the molecule at *m/z* 349 $[M+H]^+$ (Figures 2 and 3). Linear calibration curve of CPT within the concentration range of 1 µg to 50 µg (R^2 = curve co-efficient 0.999) was obtained. Validation of the method was carried out by spiking 10 µg of standard camptothecin to 10 mg of the plant extract, and the recovery was within the range of 93.8% to 102.5%.

Total camptothecin concentration in plant tissues was expressed on a dry weight basis. The quantitative analysis of camptothecin was carried out by LC–MS. The other minor camptothecinoids were identified on the basis of MS and mass fragmentation peaks. A number of molecular compounds having different retention times (t_R) but the same molecular weight were identified. These compounds are isomeric entities of camptothecinoids. Besides the 8 minor camptothecinoids detected in the accessions of *N. nimmoniana*, there were a number of other constituents that could not be identified only by LC–MS. These unidentified molecules will be further subjected to LC–NMR studies for their characterization.

Results and Discussion

CPT yields: LC–MS analyses

The LC–MS estimate of CPT in the 17 trees studied ranged from as low as 0.4% to 1.86%. Six of the 17 trees had CPT in excess of 1% (w/w). These results corroborate those obtained earlier for the same trees by Suhas et al. (29), who quantitated

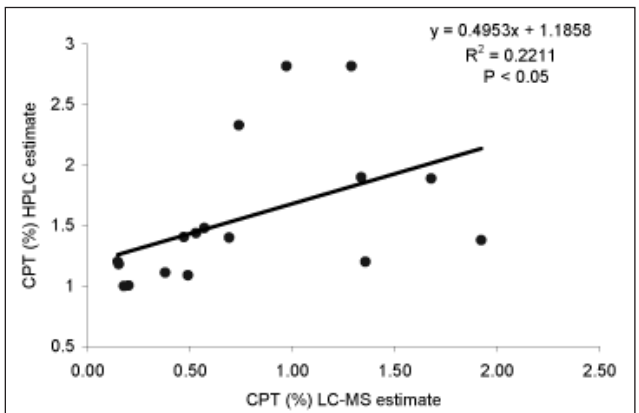

Figure 4. Association between HPLC and LC–MS CPT estimates in 17 accessions of *N. nimmoniana*.

Table I. Camptothecines Obtained from the Accessions of *Nothapodytes nimmoniana* (Stem Bark and Root Bark)

Compound	R_t (min)	Mass	Other fragmentation peaks	Accession number	
				Stem bark	Root bark
Mappicine-20-β-glucopyranoside	17.5	469.2	289, 307, 365.1, 207, 349, 319	2, 3, 12, 17	1, 9, 10, 11, 13
Camptothecin	20.1	349.1	305, 447.3, 284.2, 149.0	2, 3, 4, 5, 6, 7, 8, 12, 17	1, 9, 10, 11, 13, 14, 15, 16
9-Methoxy camptothecin	21.2	379.1	335.2, 516.4, 474.3, 305, 379.2	2, 3, 4, 5, 6, 7, 8, 12, 17	1, 9, 10, 11, 13, 14, 15, 16
Diacetoxy-camptothecin	13.0	431.1	349.1, 303, 149	4, 5, 7, 8	10
Diacetoxy-9-methoxy camptothecin	14.2	461.2	379.1, 333.1, 415.2	4, 5, 6, 7	–
Acetoxy-camptothecin-glycoside	17.5	511.1	469.2, 365.1, 289.0, 307.1, 349.1, 149, 189	2, 4, 6, 7, 8	–
9-Methoxy-mappacine-20-β-glucopyranoside	18.4	499.2	337.1	2,3, 4, 8	7, 9, 10, 11, 13, 14, 15, 16
10-Hydroxy camptothecin	19.0	365.1	303, 305	5, 6, 17	10
Uk1*	24.6	577.6	–	3	–
Uk2	23.7	385.1	305	4, 5	–
Uk3	24.8	335	–	4, 5, 17	10
Uk4	28.6	315		4	–
Uk5	18.4	705.2	499.2, 331.1, 305, 149	5	–
Uk6	26.8	782.5	665.2, 433.2, 335.1, 207.1, 466.4, 431.2, 373.1, 303	5, 6, 7	–
Uk7	18.5	345.1	305	6	–
Uk8	23.7	425.2	335.1, 305.1, 217	6	–
Uk9	24.6	782.5	690.8, 516.5, 448.4, 335.1	6	–
Uk10	24.6	448.3,	73.0, 231	7	–
Uk11	26.7	310.9	433.0, 782.5	7	–
Uk12	14.0	477.3	379.6, 333.0	8	–
Uk13	20.9	728.4	684.4, 340.4, 596.4, 552.4, 387.1, 337.1, 305.1	8	–
Uk14	23.7	602.4,	453.1, 385, 305	8	–
Uk15	24.6	453.2	393, 333.1	8	–
Uk16	26.1	468.3	586.3, 528.3	8	–
Uk17	27.7	628.4	482.3	8	–
Uk18	28.1	702.4	656.4, 584.4	8	–
Uk19	28.6	686.4	540.4	8	–
Uk20	29.1	642.4	–	8	–
Uk21	29.6	598.4	157.1	8	–
Uk22	24.3	407.2	335.1	12	9
Uk23	18.3	553.2	499.3, 337.1, 263.1	17	–
Uk24	20.8	772.4	728.5, 684.5, 640.4, 596.4, 552.4, 349.1	17	–
Uk25	22.0	658.9	379.1, 319.1	17	15, 16
Uk26	22.0	319.1	–	–	9, 10, 13
Uk27	20.7	684.4	640.4, 596.4, 552.4	–	10
Uk28	20.7	596.4	552.4, 508.4	–	11
Uk29	26.53	393.1		–	11
Uk30	24.3	482.4	415.2, 335.1	–	13
Uk31	22.5	437.2	397.1, 313.0	–	14
Uk32	24.5	570.5	526.5, 335.1	–	14
Uk33	26.0	443.2	395.1	–	14
Uk34	26.7	413.2	373.1, 331.1, 382.2, 315, 147	–	14, 15
Uk35	17.3	636.4	363.1, 149	–	15
Uk36	23.6	305.1	–	–	15
Uk37	24.5	498.5	335.1	–	15
Uk38	27.7	263	–	–	15
Uk39	24.5	602.4	474.4, 335.1	–	
Uk40	26.6	431.2	373.2, 289.2	–	16
Uk41	28.4	433.2	333.1, 247, 216	–	16

* Uk: unidentified molecules.

the CPT using HPLC analysis. There was a significant positive correlation between the LC–MS estimates with the HPLC estimates reported by Suhas et al. (29); (r = 0.47, $p < 0.05$, Figure 4). Together, these results are significant in that, for the first time, nearly 5- to 8-fold higher CPT yields than hitherto reported in *Nothapodytes nimmoniana* have been recovered from individual trees. For example, the estimates are nearly 3- to 8-fold more than what has been hitherto reported (19,31). For that matter, these are the highest estimates ever reported of CPT from any plant source. The incredibly high yields of these individuals from several populations could not be attributed to their girth; the differences in the CPT yields among the individuals was not related to their stem girth (r = 0.164:NS). Suhas et al. (29) showed that there was a significant population effect on the accumulation of CPT (ANOVA, $p < 0.01$) and that even after removing girth effects, if any, there were significant differences amongst the population. It would be interesting to assess the underlying reasons for the high production and if such high levels are indeed genetically determined. The finding has immense potential to develop clonally multiplied material to lead to a sustained production technology for the supply of camptothecin. Subject to further confirmation, these "elite" trees could be focused for conservation and judicious utilization for clonal multiplication, also for deriving tissue material for in vitro production systems, as was done for several other systems such as taxane from *Taxus wallichiana* (32,33) and for podophyllotoxin from *Podophyllum peltatum* (34).

Table II. CPT (% dry wt) Content in Different Plant Species and Tissues

Plant species	Tissue analyzed	CPT (% dry wt)	Ref.	Chromatographic analysis
Camptotheca acuminata	Young leaves	0.4–0.5%	39	HPLC
	Seeds	0.30%	39	HPLC
	Bark	0.18–0.2	39	HPLC
	Young leaves	0.24–0.30	4	HPLC
	Hairy roots	0.1	1	HPLC
	Callus	0.20–0.23	38	HPLC
Camptotheca lowreyana	Young leaves	0.39–0.55	4	HPLC
	Old leaves	0.09–0.11	4	HPLC
Camptotheca yunnanensis	Young leaves	0.25–0.44	4	HPLC
	Old leaves	0.059	4	
Ervatamia heyneana	Wood and stem bark	0.13	7	HPLC
Merriliodendron megacarpum	Leaves and stem	0.053	5	HPLC
Ophiorrhiza pumila	Young roots	0.1	6	HPLC
	Hairy roots	0.1	6	
Ophiorrhiza mungos	Whole plant	0.0012	17	HPLC
Ophiorrhiza rugosa	Albino plants	0.1	37	HPLC
	Normal plant grown	0.03	37	HPLC
Mostuea brunonis	Whole plant HPLC	0.01	8	
Pyrenacantha klaineana	Stems	0.0048	9	HPLC
Nothapodytes foetida	Stem wood	0.14–0.24	2	HPLC
	Shoot	0.075	28	HPLC
	Plant	0.048	40	HPLC–DAD–LC–MS ESI
Nothapodytes nimmoniana	Stem bark	0.3	19	UV, IR, NMR, and MS
	Leaves	0.081%	31	HPLC
	Stem bark	0.236%	31	HPLC
	Root bark	0.333–0.775%	31	HPLC
	Stem wood	0.14%	31	HPLC
	Root wood	0.18%	31	HPLC

New class of camptothecins/CPT-related alkaloids from *Nothapodytes nimmoniana*

LC–MS analysis of stem and root bark tissues of *Nothapodytes nimmoniana* accessions indicated the presence of a total of 10 camptothecinoids and a number of as yet unidentified campothecines (Tables I and II and Figures 5A and 5B). The 10 camptothecinoids identified, based on their retention times (t_R), MS, and mass fragmentation peaks were: diacetoxy camptothecin (t_R 13.0 min; m/z = 431), diacetoxy-9 methoxy camptothecin (tR 14.2 min; m/z 461.2), acetoxy camptothecin glucopyranoside (t_R 17.5; m/z 511.1), 9-methoxy mappicine 20-β-glucopyranoside (t_R 18.4 min; m/z = 499.2), and mappicine 20-β-glucopyranoside (t_R 17.5 min; m/z = 496.2), along with major camptothecin and 9-methoxy camptothecin (Figure 5A and 5B). Mappicine glycopyranoside and methoxy-mappicine reported here are normally products obtained upon hydrolysis during the isolation process. Many of these compounds were derived only from few of the 17 accessions of *N. nimmoniana* analyzed, and their concentrations were highly variable among the individuals assessed. Except diacetoxy-9 methoxy camptothecin and acetoxy camptothecin glucopyranoside, which were not detected in the root bark, all other campothecines were common to the stem and root bark analyzed. Thus, we have identified for the first time the compounds mappicine 20-β-glucopyranoside, diacetoxy camptothecin, diacetoxy-9 methoxy camptothecin, acetoxy camptothecin glucopyranoside, 9-methoxy mappicine 20-β-glucopyra-noside, and in the stem and root bark of *Nothapodytes nimmoniana* (8). However, besides camptothecin, all other campothecinoids were relatively smaller in their content. For example, in sample tree #4, which had seven of the ten known campothecines, the largest peak corresponded to camptothecin, with all

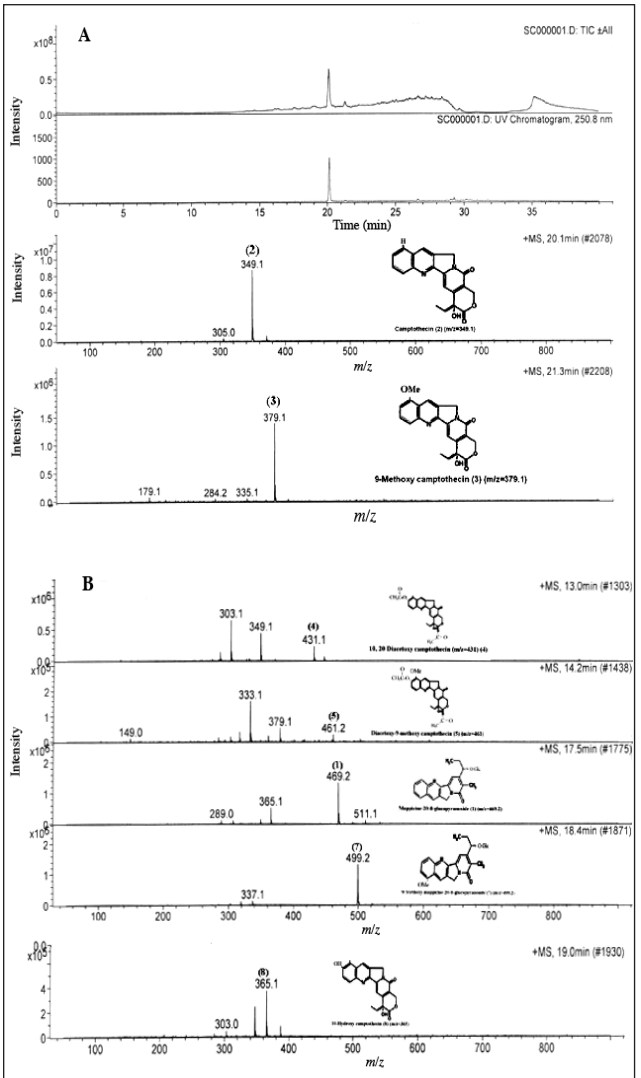

Figure 5. Mass spectra of camptothecin and 9-methoxy camptothecin from *Nothapodytes nimmoniana* (A); mass spectra and chemical structure of camptothecines in *Nothapodytes nimmoniana* (B).

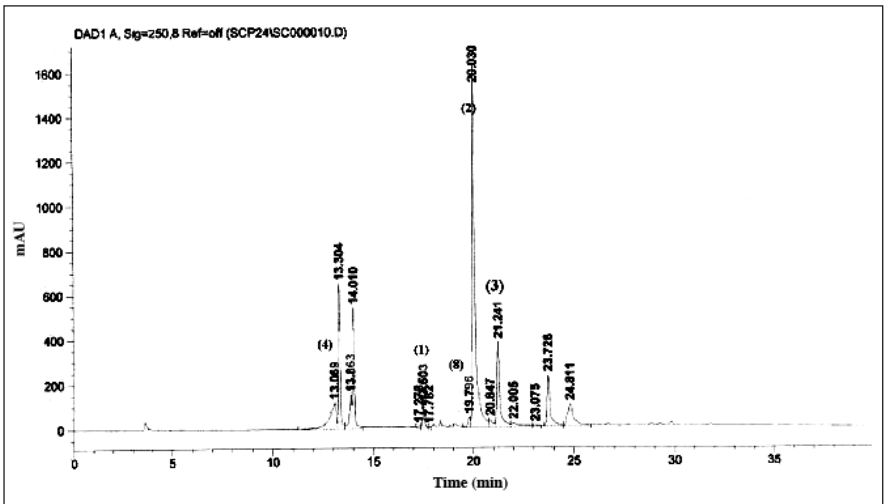

Figure 6. HPLC chromatogram of camptothecines from accession number. Mappicine-20-β-glucopyranoside (1), camptothecin (2), 9-methoxy camptothecin (3), diacetoxy-camptothecin (4), and 10-hydroxy-camptothecin (8).

others being available in smaller concentrations (Figure 6).

The identification of the new family of camptothecines in *Nothapodytes nimmonina*, some of which are currently at various stages of clinical development, raises the hope of further intensifying the screening of populations of the species in the Western Ghats with the aim of discovering high-yielding individuals. For example, high-yielding lines of 10-hydroxy CPT could be useful, as the compound can serve as a precursor for irinotecan and topotecan. In fact, studies that could lead to the identification of high-yielding individuals of mappicine, first reported in the species by Govindachari and Viswanthan (19), could be potentially important and interesting. The alkaloid, so far reported only for *N. nimmoniana* along with its ketone analogue nothapodytine B, have been shown to have potent antiviral activity against herpes viruses and human cytomegalovirus. Hossain (35) reported the generation of a 128-member library of mappicine analogues (64 racemates) and a 48 member library of nothapodytine B analogues by solution phase parallel synthesis, based on a radical cascade annulation (36). Recently, Hossain et al. (35) discovered that certain analogues of mappicine are potent inhibitors of HIV-1 RT-associated RNase H. In this regard, and because of the fact that these are associated with the absence of significant cytotoxicity, mappicine analogues are believed to represent an interesting new class of anti-retroviral agents.

These results have important implications for not only harnessing the high-yielding individuals for clonal multiplication but also for exploiting some of the minor camptothecines, which also have been shown to have important anti-cancer and anti-viral activity.

Acknowledgements

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