

Evaluating *Nothapodytes nimmoniana* population from three localities of Western Ghats using camptothecin as phytochemical marker and selection of elites using a new-content range chart method

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ABSTRACT

Background: *Nothapodytes nimmoniana* (Grah.) Mabb. is a high valued medicinal plant endemic to Western Ghats of India, distributed in fragmented populations. The plant is valued for potent anticancer drug camptothecin (CPT). **Objective:** The study compares and expounds variation in CPT content from leaves and stems of *N. nimmoniana* obtained from three populations of Western Ghats, India. The study also describes a method for categorizing these populations using content range chart (CRC) method for percent yield of CPT. **Materials and Methods:** A total of 60 samples were investigated including ten each of leaves and stems from three localities. Micro-extraction method was implemented to extract CPT. reversed phase ultra-performance liquid chromatography photo diode array technique was used to quantify CPT. **Results:** Leaf samples of an individual collected from Joida, yielded lowest CPT content (0.002 ± 0.000 g/100 g), whereas a stem sample from Amgaon, yielded highest CPT content (0.123 ± 0.006 g/100 g). The findings suggest great variation in individuals producing and accumulating CPT. Using this data along with earlier published work, five categories of CPT yielding plants were made viz. I: Very low: <0.020 , II: Low: $0.021-0.039$, III: Moderate: $0.040-0.059$, IV: High: $0.060-0.079$ and V: Very high: >0.080 . Based on CPT content in leaves, majority of individuals were under very low category (Ist) and on the other hand stem samples were in 'II' category. Besides, very few individuals were observed in category 'V'. **Conclusion:** The study expounds use of CRC method for identifying elite population and suggests the need for its conservation.

Key words: Camptothecin, categorization, micro-extraction, *Nothapodytes nimmoniana*, reversed phase ultra-performance liquid chromatography photo diode array

INTRODUCTION

Camptothecin (CPT) is a modified monoterpene indole alkaloid originally isolated from *Camptotheca acuminata* Decne. (Nyssaceae) by Wall *et al.*^[1] It was then identified from *Nothapodytes nimmoniana* Grah.(Mabb.) (Icacinaceae) and many other plants with unrelated orders.^[2,3] Hereto, *N. nimmoniana* has known to produce better yield of CPT

compared to other indigenous plants from Western Ghats, India.^[4] *N. nimmoniana* has become a popular medicinal plant with high-demand in trade and economic importance in India. The indiscriminate trading from the region has forced it in IUCN 'vulnerable' category.^[5]

Camptothecin along with its other derivatives have been studied and reported by many workers.^[5-18] The variations observed in CPT content from leaves and/or stem of *N. nimmoniana* among the population vary largely [Table 1]. It is well-understood that the content of CPT vary within and between the populations of *N. nimmoniana*. Even the extraction methods differ in these studies [Table 1]. Fulzele and Satdive^[7] have

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documented the dependence of CPT content on extraction methods. These factors have a direct impact on exhibiting variation in content but with uncertainty. Despite of thorough prospecting of CPT from plants obtained from different localities, we are deficient in distinguishing the populations based on common parameters. However, attempts made by some workers to study the populations cannot be sidelined.^[12,14]

Thus, a comprehensive common understanding on population of *N. nimmoniana*, selection of plant material, with a simple method for extraction and determination of CPT is essential. The present study covers such aspects and tries to evaluate populations of *N. nimmoniana* to categorize individuals based on different range of CPT content. This data collected will help in marking number of superior plants in a population to determine it as elite population.

MATERIALS AND METHODS

Chemical reagents and standards

Standard CPT (high-performance liquid chromatography [HPLC] grade) was obtained from Sigma-Aldrich (India). Acetonitrile, methanol, water were of HPLC grade.

Collection and preparation of plant material

Three geographically isolated populations of *N. nimmoniana* were selected. Attributes of localities are presented in Table 2. Leaf and stem materials from ten individuals of *N. nimmoniana* from each locality were collected. Herbarium of plant was authenticated and deposited at Regional Medical Research Centre (ICMR), Belgaum, Karnataka, India for future reference (Voucher Number - *N. nimmoniana*: RMRC 1313). The plant materials were dried at room temperature and grounded to powder for further analysis.

Table 1: CPT (g/100 g) content in stem and leaves of *N. nimmoniana* determined by different workers with reference to extraction methods and sample origin from Western Ghats, India

Tissue analyzed	Sample size	Sample origin*	Extraction	CPT [#]	References
Stem	NM	Western Ghats	SL	0.060	[8]
Stem bark				0.080	
Leaves				0.010	
Shoot	NM	Mahabaleshwar	SL	0.075	[15]
Stem bark	NM	India	SL	0.270	[6]
Stem	NM	India	MW	2.670	[7]
Stem	62	Sirsi, Ulvi	ME	0.142	[12]
Stem bark				0.236	
Leaves				0.081	
Stem (J)	NM	Mahabaleshwar	SL	0.027	[4]
Leaves (J)				0.034	
Stem (M)				0.060	
Leaves (M)				0.010	
Bark				0.080	
Stem bark	147	Kerala	ME	1.100	[17]
Stem bark	17	Western Ghats	US	1.860	[14]
Leaves	NM	Ooty	ME	0.020	[16]
Stem bark	NM	Karnataka	NM	0.240	[9]
Stem				0.120	
Leaves				0.080	
Stem	NM	Pathan	US	1.450	[11]
Leaves				0.700	
Stem	NM	Mahabaleshwar	US	0.810	[10]
Leaves				0.700	
Stem bark	NM	Amboli	MW	1.337	[13]
Stem	NM	Belgaum	ASE	0.188	[18]

*Sample origin column represents the region of the highest yield reported and not the total sampling area; [#]g/100 g DW. Extraction methods-SL: Solid liquid extraction; ME: Micro extraction; US: Ultra sonication; MW: Microwave; NM: Not mentioned; CPT: Camptothecin; *N. nimmoniana*: *Nothapodytes nimmoniana*; ASE: Accelerated solvent extraction

Table 2: Localities attributes of sample collection from North Central corridor of Western Ghats, India

Localities	District	Longitude	Latitude	Altitude (M)	Forest type
Amgaon	Belgaum	N 15.68600°	E074.34985°	843	SE
Joida	North Canara	N 15.16837°	E074.48124°	600	SE
Amboli	Sindhudurg	N 15.96201°	E073.99564°	762	SE

SE: Semi evergreen

Micro-extraction

A total of 0.3 g of powdered leaves, and stem of each individual plant collected were extracted separately with 1.5 ml methanol in micro centrifuge tubes using micro-pestle. The mixture was warmed on a water bath at $60 \pm 2^\circ\text{C}$ for 5 min, cooled to room temperature and centrifuged at 5000X for 15 min to obtain 20% extract. The extracts were diluted to 1% for ultra-performance liquid chromatography (UFLC) analysis. The extracts were stored at 4°C until further use.

Quantification of camptothecin using reversed phase-ultra flow liquid chromatographic-photo diode array analysis

Instrumentation

The reversed phase UFLC photo diode array (RP-UFLC-PDA) analysis was performed on Shimadzu chromatographic system (Model no. LC-20AD) consisting of a quaternary pump, manual injector, degasser (DGU-20A5) and dual λ UV absorbance diode array detector SPD-M20A. The built in LC-Solution software system was used for data processing. Chromatographic separation was achieved on a Hibar RP-select B column (LiChrospher 60, $5 \mu\text{m}$, $4.6 \times 250 \text{ mm}$) for CPT.

Chromatographic conditions

Mobile phase consisting of acetonitrile: water (40:60) for CPT was used for separation with an injection volume of $20 \mu\text{l}$. A chromatographic condition of 1.0 ml/min flow rate at 254 nm was set for CPT. The retention time was observed 8 min for CPT.

Calculations, calibration curves and linearity

Camptothecin was accurately weighed and dissolved in few drops of DMSO by warming and the volume was made with methanol to produce a standard stock solution (0.5 mg/ml). The stock solutions of CPT was prepared and serially diluted with respective solvents to obtain working concentrations for plotting calibration curves. Seven different concentration levels of CPT (0.001, 0.01, 10, 20, 40, 80, and $100 \mu\text{g/ml}$) were used during the study. All the solutions and analytes were stored in microfuge tubes at 4°C until further use.

System suitability, limit of detection and limit of quantification

The system suitability test was assessed by three replicates of standard CPT at a particular concentration ($40 \mu\text{g/ml}$). The peak areas were used to evaluate repeatability of the method, and analyzed for resolution and tailing factors. The limit of detection (LOD) and limit of quantification (LOQ) were determined with the signal: noise method. Signal: noise ratios of 3.3 and 10 were used for estimating the LOD and LOQ, respectively.

Statistical analysis

Data were expressed as mean values of three readings and relative standard deviations (RSD). The experiments were repeated at least twice, and the representative results were taken. The statistical analyses were carried out using Office Excel 2007 software manufactured by Microsoft Corporation compatible to Windows 7 Operating System.

RESULTS AND DISCUSSION

A 10 individual from three localities were selected for the study. Table 2 represents details on the localities under study with their attributes. The flowering individuals were marked as mature plants and were considered suitable for sampling. Stem and leaf materials from each localities were collected to make a total sample size of 60.

Camptothecin was extracted using simple solid: liquid micro extraction technique with temperature (60°C) using a water bath as described in the experimental section. The micro extraction performed was less laborious, easy, without sophisticated instrumental requirement. The material required for extraction was very less (0.3 g) and the method can be implemented for a number of samples at a time. Quantitative determination of CPT was achieved using RP-UFLC-PDA method, and the results were expressed as g/100 g on dry weight basis. Calibration curves were constructed against its area under curve to obtain a regression equation with a coefficient of determination (R^2) above 0.980 [Figure 1a]. This was used to estimate CPT content from stem and leaf samples obtained from different localities. The RSD values for both analytes were found $<2\%$ indicating precision and reproducibility of the method. Validation of the method was carried out by spiking known amount of CPT standard to an equal volume of sample extract to obtain recovery within the range of 95-100% for both.

Reversed phase UFLC profiles with retention time of $5.933 \pm 0.051 \text{ min}$ for CPT in standards and samples were obtained as the final output [Figure 1b and c]. All samples were detected above LOD ($0.202 \mu\text{g/ml}$) and quantifications above LOQ ($0.612 \mu\text{g/ml}$) for CPT determined using signal: noise equation. Analysis of *N. nimmoniana* collected from three geographically isolated populations yielded a range of CPT from 0.016 ± 0.001 to 0.123 ± 0.006 in stems and from 0.002 ± 0.000 to $0.033 \pm 0.003 \text{ g/100g}$ in leaves on dry weight basis [Table 3]. Variation in content of CPT can be mainly attributed to genetic makeup, age and maturity of the plant, and by far means analytical method involved in extracting and determining. It is evident that though there is significant variation in CPT content among the individuals in populations, it did not relate to the size,

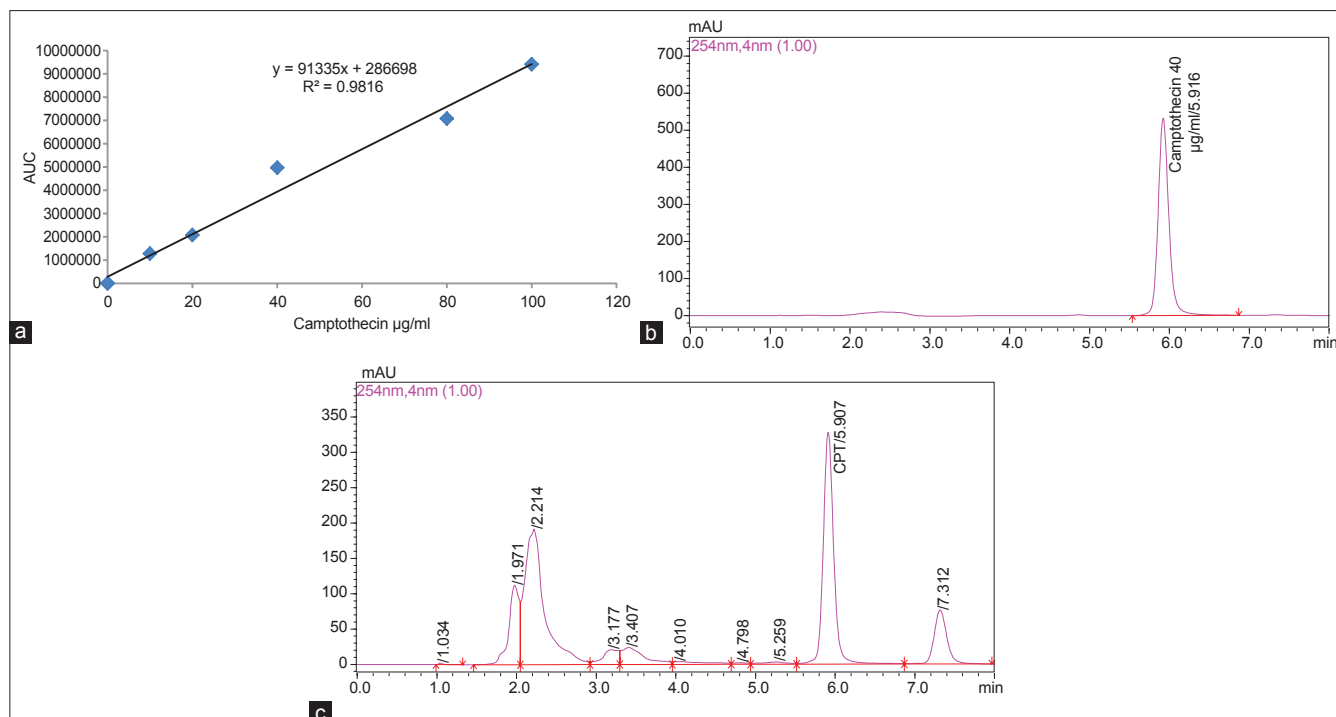


Figure 1: (a) Six-point calibration curve of standard camptothecin (CPT); ultra-performance liquid chromatography photo diode array profiles. (b) Standard CPT 40 µg/ml; (c) Individual no. 8, stem sample collected from Amgaon with highest CPT content

Table 3: Content of CPT (g/100 g DW) from leaves and stem parts of *N. nimmoniana* sampled from three different populations

Individual number	Amgaon		Amboli		Joida	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
1	0.017±0.001	0.038±0.002	0.022±0.001	0.051±0.003	0.004±0.000	0.068±0.003
2	0.033±0.002	0.036±0.002	0.012±0.001	0.048±0.002	0.002±0.000	0.024±0.001
3	0.007±0.000	0.028±0.001	0.016±0.001	0.060±0.003	0.002±0.000	0.069±0.003
4	0.023±0.001	0.029±0.001	0.014±0.001	0.023±0.001	0.010±0.000	0.028±0.001
5	0.033±0.002	0.061±0.003	0.004±0.000	0.051±0.003	0.007±0.000	0.033±0.002
6	0.017±0.001	0.059±0.003	0.004±0.000	0.015±0.001	0.003±0.000	0.016±0.001
7	0.020±0.001	0.038±0.002	0.008±0.000	0.030±0.001	0.016±0.001	0.057±0.003
8	0.017±0.001	0.123±0.006	0.013±0.001	0.067±0.003	0.029±0.001	0.072±0.004
9	0.008±0.000	0.040±0.002	0.013±0.001	0.059±0.003	0.016±0.001	0.023±0.001
10	0.012±0.001	0.075±0.004	0.024±0.001	0.065±0.003	0.017±0.001	0.040±0.002

CPT: Camptothecin, DW: Dry weight, *N. nimmoniana*: *Nothapodytes nimmoniana*

age or geographical location.^[17] This suggests that we may find a high yielding plant in each population. Further, the variation in CPT content may be due to different extraction methods [Table 1].

Thus, we herein suggest an easy method for extraction and determining CPT content and also propose a content range chart (CRC) method, to classify high yielding plants within and among the populations [Table 4]. This is helpful in scoring superior plants and populations. The content ranges in the chart were prepared using results of RP-UFLC-PDA determination of 60 samples (stems and leaves) from the present investigation and also by

considering observations made in earlier reports published by different workers [Table 1].^[6-18] In the present study, CPT content ranged from 0.002 to 0.123 g/100g dry weight including leaf and stem materials [Table 3]. Whereas, different reports hereto suggests CPT range from 0.010 to 0.700 g/100g in leaf and 0.027-2.67 g/100g in stem [Table 1].^[6-18] Considering sustainability, conservation and judicial issues data of root and root bark were not considered during the study. Thus overall it is evident from the earlier reports that, stem yields higher CPT content than leaves and out of 12 reports 5 suggest CPT content over 1.000 g/100 g and 5 suggest between 0.100 and 0.99 [Table 1]. Similarly, for leaves 2 out of 7 had

Table 4: Distribution of number of individual plants in CRC of CPT content (g/100 g DW) in each population

Locality	Part	Number of plants as per CRC of CPT content (g/100 g DW)					Total
		I	II	III	IV	V	
Amgaon	Leaves	6	4	0	0	0	10
	Stems	0	5	2	2	1	10
Amboli	Leaves	8	2	0	0	0	10
	Stems	1	2	4	3	0	10
Joida	Leaves	9	1	0	0	0	10
	Stems	1	4	2	3	0	10

I: Very low - <0.020; II: Low - 0.021-0.039; III: Moderate - 0.040-0.059; IV: High - 0.060-0.079; V: Very high - >0.080; CPT: Camptothecin; CRC: Content range chart; DW: Dry weight

content 0.700 g/100g, another 2 had 0.080 g/100g and rests were below 0.034 g/100g [Table 1]. This data may give a picture about elite individual however populations of *N. nimmoniana* cannot be rated as the elite. Thus in the present study we considered our results along with the reference of published data to project a method for scoring elite populations. The chart range set are in the view that most of the individual will fall in I to IV, of CRC and very few individuals may be found in category V [Table 1].

In present investigation 5 CRC groups were identified, ranging from <0.02 to a maximum of >0.08 [Table 4]. As a result in present study majority of individuals fall in Ist CRC for leaf extracts and IInd CRC for stem extracts. Stem were found to be better options over leaf materials for CPT accumulation, also supported by findings of Padmanabha *et al.*^[12] Furthermore, individual wise locality Amgaon had a superior individual falling in Vth CRC, and Amboli had elite population with more number of individual falling in IIIrd CRC. The CRC groupings for earlier reports suggest most individuals under Vth category with variation in content from 0.080 to 2.670 [Table 1].

CONCLUSION

The study is very useful in evaluating and categorizing the plants into different yielding ranges provided a standardized methodology is followed. CRC method helps to categorize plants into different content yielding groups. Wherein, we suggest ten or more mature plants at a particular location may be considered as a population in *N. nimmoniana*. Geographical barriers rather than political boundaries are to be considered for defining different populations. Keeping in view conservation aspect, sustainable parts and its availability should be considered during designing the experiment. This along with simple micro extraction method and easy isocratic HPLC system for determination

of CPT can give us a perfect panorama of elites in the population of *N. nimmoniana* along the Western Ghats, India. It should be kept in view that CRC may vary from species to species, it is also well understood that the proposed method may improve owing to its utility.

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