Seasonal variation in content of camptothecin from the bark of *Nothapodytes nimmoniana* (Grah.) Mabb., using HPLC analysis

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ABSTRACT

Objective: To study and compare seasonal variation in camptothecin (CPT) content from bark samples of *Nothapodytes nimmoniana* obtained from geographically and climatologically isolated populations. **Methods:** A standard High Performance Liquid Chromatography methodology was used to analyze and quantify CPT from bark samples of *N. nimmoniana.* **Results:** Sample collected from Amboli yielded highest CPT content 1.337 g/100 g dry bark powder during the monsoon compared to other localities in study. Monsoon (August) showed to accumulate higher levels of CPT in barks of *N. nimmoniana* as compared to summer (May). Amboli averaged highest accumulation of CPT compared to other localities under study. **Conclusion:** These findings indicate season to have control over accumulation of CPT. Locality Amboli has highest CPT content in all seasons and were the elite population during the study. The study also suggests the need for further investigation in lights of biosynthesis in the plant.

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INTRODUCTION

Forests are imperative in regulation of the water cycle and stabilizing soils. They also help in soaking carbon dioxide and balancing oxygen levels. In addition, forest provides habitat for diverse flora and fauna, offer cultural, spiritual, and recreational opportunities, and provide a variety of food, medicines, and wood. The forest cover of India is 19.27 % of the geographic area, corresponding to 63.3 million hectare.[1]

Over 50 % of all modern clinical drugs are of natural product origin.[2] Plant secondary metabolism is a paradigm for the metabolic diversity found in nature. Several tree species from Western Ghats is gaining importance due to its newly discovered pharmaceutical and curative properties. *Nothapodytes nimmoniana* (Grah.) Mabb., (Icacinaceae), (Syn.: *Nothapodytes foetida, Mappia foetida*) is one such plant. It is a rich source of potent alkaloid camptothecin (CPT) and 9-methoxycamptothecin.[3-5] The metabolites extracted from *N. nimmoniana* show anti *human immunodeficiency virus*, anti-neoplastic, and anti-malarial activity.[3] Many researchers have suggested High Pressure Liquid Chromatography to be one of the best analytical methods to detect and quantify plant metabolites.[6-9] CPT (mol. Formula $C_{20}H_{16}N_2O_4$ and mol. wt. 348.4) is an alkaloid originally isolated from a Chinese tree *Camptotheca acuminata* (Nyssaceae).[10] In this context, there has been an enormous demand world-wide for the alkaloid CPT. Herein, we present work on the effect of the season on content of CPT accumulation.

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MATERIALS AND METHODS

Sampling and sample processing

Field surveys were carried out in the Southern parts of Maharashtra State (MS), India (part of Western Ghats)

for locating populations of N. nimmoniana. Voucher specimen was deposited in the herbarium, Laboratory of Angiosperm Taxonomy, Department of Botany, Shivaji University, Kolhapur (MS) India (voch. no. SRP-NN 1/05). Western Ghats is one of the global biodiversity hot spots with discrete tropical climate and distinct forest types. Like other parts of India, Southern Maharashtra receives three seasons viz. monsoon, winter and summer. The peak periods being in the months of August, December and May selected as representatives for the three seasons. Five sites (Amba, Amboli, Chandoli, Naikwadi, and Panhala) were sampled with different geographical and cliamtological settings. Plants above 15 cm gbh were selected from each population. Samples collected were oven dried at 50 ± 2°C for 48 h and powdered. The powdered samples were stored in plastic containers in cool dry place until use.

Extraction

The samples were prepared by the method employed by Pai *et al*.; Fulzele and Satdive[5,11] with following modifications. The samples for HPLC analysis were prepared by exposing 1 g of dry bark powder suspended in 15 ml 90% aqueous methanol to microwave extraction technique. The extracts were filtered through filter paper and centrifuged at 10,000 × g for 10 min. The volumes of yielded supernatants were adjusted to 15 ml with the same solvent. The extracts were re-filtered through a 0.2 μ nylon filter and were used for the HPLC analysis.

Quantitation of CPT using HPLC

Instrumentation

The HPLC analysis was performed on Waters chromatographic system (Model no. 2690) consisting of a quaternary pump, manual injector, and UV detector. The Waters software system was used for HPLC data processing. Chromatographic separation was achieved on a Waters C 8 column (Symmetry, 5 μm, 4.6 mm× 250 mm).

Chromatographic conditions

Mobile phase consisting of A (acetonitrile) and B (water) was used for separation with 40 % A as to 60% B in an isocratic mode with injection volume 10 μL. The flow rate was 1.6 mL/min and the detection wavelength of the dual λ absorbance detector beam was set at 254 nm and 240 nm. The analysis time was 10 min for both, standards, and samples used for the analysis.

System suitability

The system suitability test was assessed by three replicate injections of the standard solutions at a particular concentration. The peak areas of which were used to evaluate repeatability of the proposed method and their peaks were analyzed for resolution.

Calculations, calibration curves and linearity

CPT was accurately weighed and dissolved in few drops of Dimethyl sulfoxide (DMSO) by warming and the volume was made accordingly with methanol to produce a standard stock solution (mg/mL). The stock prepared was warmed to dissolve CPT completely and avoid turbidity. The stock solution was then serially diluted with methanol to prepare the working solutions for the calibration curves at five concentration levels (25, 250, 500, 750, and 1000 μg/mL). All the solutions were stored in microfuge tubes at 4°C.

RESULTS

The selected sites have fairly rich distribution of the plant population, dense patch of individuals were seen at Chandoli, Amboli, and Naikwadi where as localities Amba and Panhala had scattered patches. Flowering was observed from August to October. Fruit setting continues until December, where during later part of the month fruit ripens. Poaching marks in the form of cut trees were observed at the localities Chandoli and Amboli, indicating arguably high-risk of these plants to be vanished from the areas in the near future. The other localities were well preserved and still untouched.

Quantitation of CPT using HPLC

HPLC analysis of different concentrations of standard CPT yielded profiles with a retention time of 5.389 (±0.3) min. Sharp and clear peaks of standards were obtained. A linear calibration curve for the CPT within the concentration range of 25-1000 μg (R^2= curve coefficient 0.981) was obtained by plotting concentration of CPT against Area Under Curve for respective concentration peak. The calibration equation (y= 26437x+ 2E+ 06) obtained for the curve was used to calculate the CPT concentration.

Fifteen samples were chemically profiled for understanding seasonal effect on CPT content [Figure 1]. Profiles for the samples collected from 5 localities during 3 seasons were evaluated and compared with the retention time of standard CPT. Validation of the method was carried out by spiking 100 μl (750 μg) of standard CPT to 100 μl of the extract of bark collected during the summer (May) from Naikwadi and the recovery was within the range of 95-100%.

CPT content during monsoon (August)

The peaks obtained from HPLC analysis were sharp enough to identify CPT content [Figures 2a and e]. The profiles also show other peaks clustering at different retention times. Sample collected from Amboli yielded highest (1.337 g/100 g dry bark powder) CPT content followed by Naikwadi, Amba and Panhala [Figure 1]. Sample from Chandoli yielded

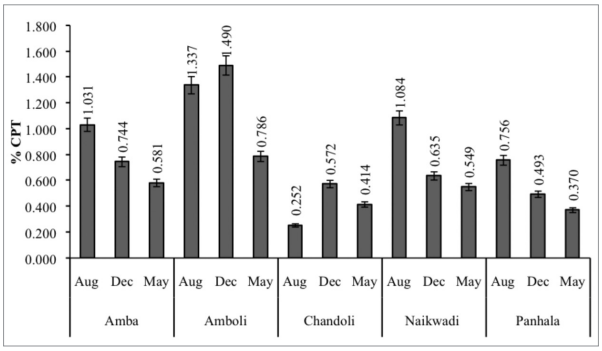

Figure 1: Content of camptothecin (%) from bark extracts of *N. nimmoniana* collected from various localities during different seasons

very low amount (0.252 g/100 g dry bark powder) of CPT as compared to other localities within this season.

CPT content during winter (December)

The peaks of CPT in the HPLC profile obtained by injecting the extracts of the bark samples obtained from different localities during the winter (December) are presented in [Figure 2f and j]. A range from 0.490 to 1.490 % CPT accumulation in bark samples collected in the season from the localities under study. Highest percent accumulation of CPT was observed in samples collected from Amboli (1.490 %) during the season, followed by the samples collected from Amba > Naikwadi > Chandoli > Panhala [Figure 1].

CPT content during the summer (May)

The peaks obtained for the HPLC analysis carried out for the extract of the samples obtained during the summer (May) were sharp and isolated [Figure 2k and o]. Bark sample collected from the locality Amboli showed highest percent accumulation of CPT in the season, followed by samples collected from Amba > Naikwadi > Chandoli > Panhala [Figure 1]. The amount was comparatively less than that recorded during the monsoon (August) and winter (December) for all the localities except Chandoli.

Correlation for the content of CPT and the altitudinal differences of the sites of the collection had no significance. Further, it was seen that the correlation coefficient during each season was varying widely with *P* values > 0.05 level (August: *R*= 0.578, *P*= 0.307), (December: *R*=−0.243, *P*= 0.6939), (May: *R*= −0.4781, *P*= 0.4153). The results were in accordance with Padmanabha *et al*.[12] wherein they have revealed that the difference in content of CPT yield among different sites could not be attributed to either geographical location (latitude) or altitudinal differences. More likely, environmental (seasonal) and genetical background of the population are the regulating factors.

Seasonal variation in CPT content

Seasonally, monsoon (August) showed a rise in the yield of CPT content in all localities tested except Amboli and Chandoli where winter (December) demonstrated high yield [Figure 2]. Difference between highest and lowest content of CPT for each of the locality was more than 50%. Locality Chandoli, Amba, and Panhala showed 55.94, 55.34, and 51.06 % difference respectively between highest- lowest content of CPT. Similarly, difference between highest to lowest content of CPT during the respective seasons among all the localities were: August: 81.15 % difference between Amboli (1.337 %) and Chandoli (0.252 %), December: 66.91 % difference between Amboli (1.490 %) and Panhala (0.493 %), May: 52.93 % difference between Amboli (0.786 %) and Panhala (0.370 %).

DISCUSSION

The content of CPT quantified by HPLC method illustrated a pattern where monsoon averaged highest followed by winter and summer (August > December > May). Quantitation of 15 extracts of dried, powdered bark material gave values from 0.252to 1.490 % of CPT [Figure 2]. The lowest content of percent CPT was lower than that reported (0.4%) by Ramesha *et al*.[13] Over all production of CPT was highest in all seasons in locality Amboli, whereas other localities in order from high to low content of % CPT after Amboli are Amba, Naikwadi, Panhala, and Chandoli.

Thus, it is fascinating to know the underlying reasons for high production of CPT. Though, content of CPT has been less studied from genetical point of view, we make here an attempt to know the seasonal (environmental) effect on yield of CPT. Seasons have shown to play a key role in regulation of many physiological processes, and ultimately rate of metabolism.[14] It is well-known that the secondary metabolites are influenced, either qualitatively or quantitatively, by the age of the plant, variety, and climatic conditions.[15,16] The probability of difference due to developmental stages in the plant under study was minimized by selecting mature trees over 16 gbh. Seasonal changes are quantitative and qualitative, but the possibility cannot be entirely ruled out that particular biochemical pathways can be switched on or off as a result of environmental influence.[17]

CPT with all other terpinoid indole alkaloid's (TIAs), are derived from the common precursor strictosidine, which is the product of a condensation reaction between indole tryptamine and terpenoid secologanin.[18] Tryptophan (Trp) biosynthesis is proved to be essential in primary and secondary metabolism in *C. acuminata* another CPT yielding plant. The biosynthesis is governed by enzyme Trp synthase, which is made of two subunits (A and B). The abundance of Tryptophan synthase subunit B (TSB)

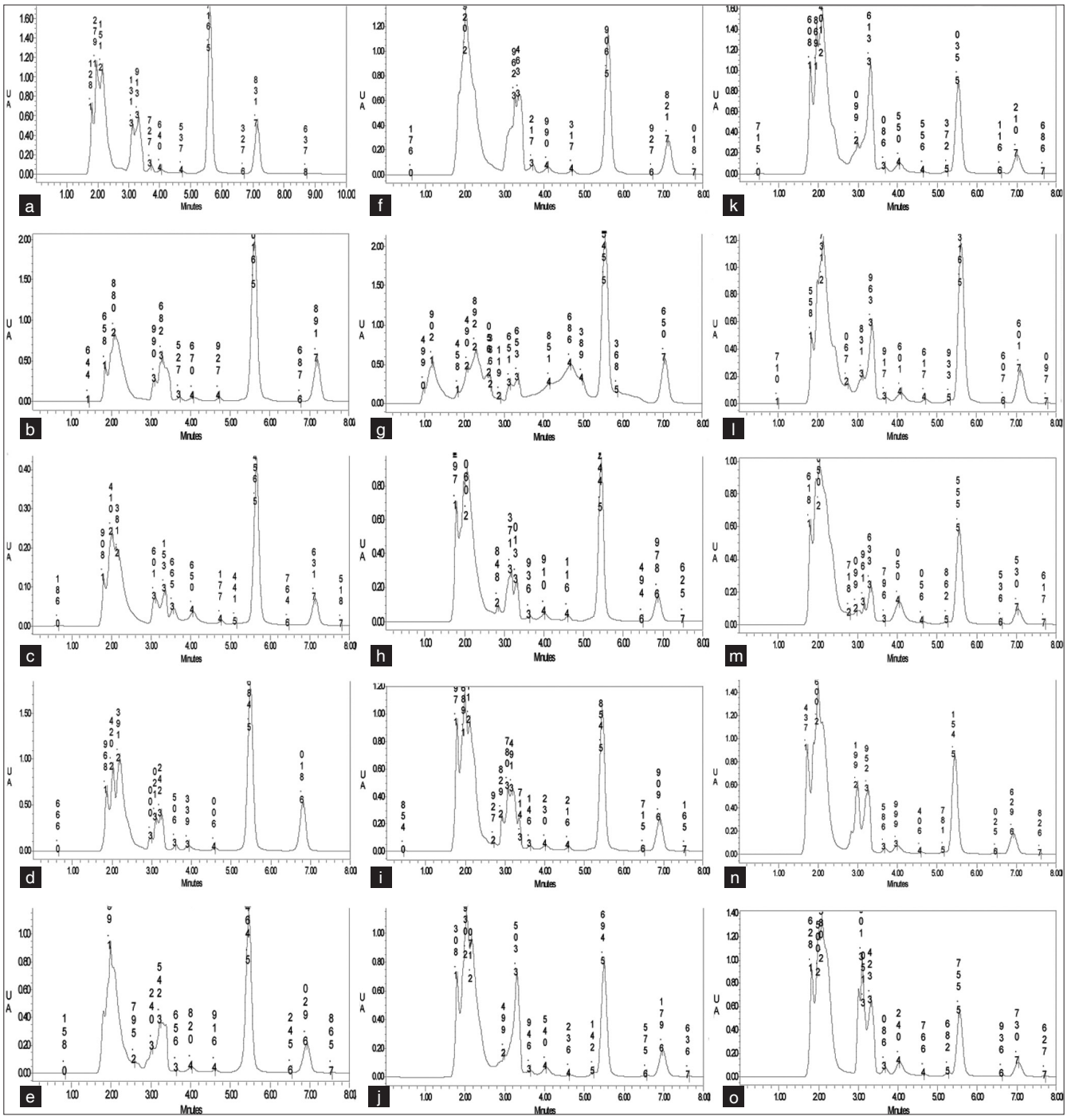

Figure 2: High Pressure Liquid Chromatography (HPLC) profiles of samples collected during (a-e): August; (f-j): December; (k-o): May and where (a, f, k): Amba; (b, g, l): Amboli; (c, h, m): Chandoli; (d, i, n): Naikwadi; (e, j, o): Panhala are localities

messanger Ribo Nucleic Acid (mRNA) and protein were paralleled to production of CPT in *C. acuminata* and was most abundant in vascular tissues, especially, cambium, primary xylem, and primary phloem.[19] TSB is highly expressed in *C. acuminata* during early seedling developmental stages corresponding to accumulation of CPT, consistent with the idea that Trp biosynthesis (primary) and secondary TIAs pathway are coordinately regulated.

Trp once formed is converted to tryptamine which plays a vital role in TIAs biosynthesis by acting a bridge between primary and secondary metabolism. The enzyme required for this conversion is Tryptophan decarboxylase (TDC). The enzyme is extensively studied in *Catharanthus roseus* and it is well-understood that it is encoded by a single copy gene[20] and its elicitation occurs at transcriptional level.[19] Whereas, Lopez-Meyer and Nessler[21] have showed that

TDC in *C. acuminata* is coded by 2 genes(TDC1 and TDC2) and out of which TDC1 is developmentally regulated and TDC2 serves as part of defense mechanism. TDC1 had highest expression in apex, young stem and bark, which also contains the highest level of CPT. Biosynthesis of TIAs has been well studied in *C. roseus*,[22] *C. acuminate,*[23] and *Ophiorrhiza pumila*,[24] which indicate a level of complexity to the multicellular nature of TIAs biosynthesis. It has also been proposed that the intermediates of TIAs are translocated from interior sources to epidermis and ultimately to the sink (laticifers and idioblasts).

This serves a rationale to our study, where we find monsoon (August) to accumulate higher levels of CPT in barks of *N. nimmoniana* as compared to summer (May). The plant under study is deciduous in nature and pre-monsoon showers elicit the sprouting and blossoming of the tree, which progresses to elevate primary metabolism. Previous work on TIAs and CPT biosynthesis signify primary metabolism to be directly linked to secondary metabolism, which in turn is related to TIA and CPT biosynthesis. However, more detailed studies are required for explaining apparent discrepancy in biosynthesis and accumulation.

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