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Influence of Geographical and Climatic Conditions on Camptothecin Content of *Nothapodytes nimmoniana*

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Abstract: Camptothecin, topoisomerase I-DNA inhibitor, has been evaluated in methanolic extract of various parts of *Nothapodytes nimmoniana* (J. Graham) Mabberly (Family Icacinaceae) collected in the month of February from different regions (Mahabaleshwar and Patan regions of Maharashtra state and Sirsi region of Karnataka state) of Western Ghats, India, using high performance liquid chromatography. Quantification was performed with the regression analysis and the method was validated as per International Conference of Harmonization (ICH) guidelines. In regard to various plant parts, maximum concentration of camptothecin was found in root (2.62%) collected from Mahabaleshwar, Patan (1.21%) and Sirsi (0.88%) regions followed by stem collected from Patan (1.45%), Sirsi (0.70%) and Mahabaleshwar (0.43%) regions. The lowest concentration of camptothecin was found in leaves collected from Sirsi (0.29) region, followed by Patan (0.37) and Mahabaleshwar (0.70%) region. Fruits collected from Mahabaleshwar region contain maximum concentration of camptothecin (0.63%) whereas fruits from Patan region contain minimum concentration of camptothecin (0.36%). There was a 2-fold higher concentration of camptothecin observed in roots from Mahabaleshwar region than roots from Sirsi and Patan region. So it is evident that geographical and climatic conditions have remarkable influence in the content of camptothecin in *N. nimmoniana*.

Keywords: *Nothapodytes nimmoniana; Icacinaceae; camptothecin; HPLC; Anti-cancer.*

1. Introduction

Nothapodytes nimmoniana (J. Graham) Mabberly (Syn. *Nothapodytes foetida* (Wight) Sleumer or *Mappia foetida* Meirs, vern. “Narkya”/ "Kalagur", Family Icacinaceae) is a small tree distributed in Western Peninsula from Konkan Southward i.e. Nilgiris, Anamalis, Pullneys, North Kanara and Konkan Ghats, broadly the Western Ghats of India, a global biodiversity hot spot. This

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tree is also distributed in the warmer regions of the Indian subcontinent, Sri Lanka, Myanmar and Thailand [1].

Camptothecin, a monoterpene indole alkaloid, was first discovered in the Chinese deciduous tree, *Camptotheca acuminata* [2]. The alkaloid has been reported from several plant species (*Ophiorrhiza species, Ervatamia heyneana, Merrilliodendron megacarpum*), with by far the highest yield found in *Nothapodytes nimmoniana* [3].

Camptothecin is regarded as one of the most promising anticancer drug of the twenty first century [4]. The cellular target of camptothecin is DNA topoisomerase I and numerous analogs have been synthesized as potential therapeutic agents [5]. CPT inhibits the replication of Human Immuno Deficiency Virus (HIV) *in vitro* and is also shown to be effective in the complete remission of lung, breast, uterine and cervical cancer [6-8]. The molecular and cytotoxic effects of camptothecin on *Plasmodium falciparum* proven that it is an interesting target for new anti-malarial drug development [9].

Looking to the enormous demand for camptothecin worldwide, there has been an indiscriminate extraction of trees from many parts of India, especially from the Western Ghats, a megadiversity forest range along the western coast of India. The continuous production of anti-cancer, anti-AIDS and other life saving drugs requires repeated collection of plant materials and consequently this leads to the serious problem of depletion of natural sources. Thus, *N. nimmoniana* has been classified as a 'vulnerable' species and considerable efforts are in progress to map its populations in India [10, 11]. Recently, several independent groups have addressed the need to conserve the species, to chemically characterize populations, to identify populations or individuals with high camptothecin yields and to explore the possibilities of identifying high yielding individuals for the development of *in vitro* production systems [12-15]. Generally the concentration of the active constituent present in the herbals may vary due to many factors like, time and period of collection, geographical and climatic conditions. Many times even the absence of active constituents may be observed with same plant collected from different region, which was difficult to assess earlier. Recent developments in analytical techniques made this task easier to identify and quantify the active constituents in the herbals [16-18].

High performance liquid chromatography (HPLC) is an advanced form of liquid chromatography used in separating the complex mixture of molecules encountered in chemical and biological systems, in order to understand better the role of individual molecules. HPLC is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It also allows you to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. The advantages of HPLC over other forms of liquid chromatography are several. It allows analysis to be done in a shorter time and achieves a higher degree of resolution, that is, the separation of constituents is more complete. In addition, it allows stationary columns to be reused a number of times without requiring that they be regenerated, and the results of analysis are more highly reproducible (± 1%). A further advantage of HPLC is that it permits both instrumentation and quantitation to be automated and highly sensitive [19-22]. Several methods have been described for the measurement of camptothecin and its analogue (topotecan and irinotecan) using total fluorescence analysis, HPLC using ultraviolet detection or fluorescence with thin layer chromatography. For the detection and estimation of camptothecin concentration in biological fluids a sensitive HPLC method has been developed method in which the best mobile phase was methanol−10 mM phosphate (75:25 v/v, pH 4) [23].

The present study was aimed to determine the concentrations of medicinally important alkaloid camptothecin in various parts of *N. nimmoniana* collected from different regions of Western Ghats using HPLC analysis.

2. Materials and Methods

2.1. Plant Material

Plant materials of *N. nimmoniana* were collected from the local areas of Mahabaleshwar, Patan region of Maharashtra state and Sirsi region of Karnataka state, India, in February. The samples were authenticated by Chief Botanist, Botanical Survey of India (BSI) and voucher specimen (NNASP1) was kept at departmental herbarium of BSI. Drug materials were stored at room temperature in an air tight container.

2.2 Chemicals

All chemicals used were of analytical grade. Reference standard of camptothecin (purity 95% w/w) was purchased from Hi media (Mumbai, India). Solvents were purchased from Merck, Mumbai, India.

2.3 High performance liquid chromatography

Quantification of camptothecin was performed by following the method of Fulzele and Satdive [24]. Isocratic analytical HPLC assay was performed on a Jasco 900 instrument and 20 µL of supernatant extracts was loaded onto ODS (5 µm; Inertsil) column (150×4.6 mm). Acetonitrile: water (45:55) was used as mobile phase at a flow rate of 1 mL/min and camptothecin was detected at 360 nm by UV detector (UV-975, Jasco). The peak areas corresponding to camptothecin were integrated by comparison with external standard calibration curves.

2.4 Preparation of standard solution of Camptothecin

A stock solution of camptothecin was prepared by dissolving 5 mg of standard in chloroform-methanol mixture (3:1), and making up the volume to 5 mL with methanol. From this stock solution, standard solutions of 10 µg/mL to 50 µg/mL were prepared by transferring aliquots (0.1 to 0.5 mL) of stock solution to 10 mL volumetric flasks and adjusting the volume with methanol.

2.5 Calibration curve for camptothecin

20 µL of standard solutions of camptothecin was injected in triplicate in column. The peaks were detected at 360 nm. Calibration curves of camptothecin were prepared by plotting peak area *vs.* concentration.

2.6 Sample preparation and extraction

Whole plant materials of *N. nimmoniana* were collected from different regions, thoroughly washed, segregated into different parts (roots, stems, leaves and fruits) and dried at 55ºC in an air dryer for 48 h. Dried plant parts were powdered separately with Wiley mill (Model 4276-M, Thomas Scientific, USA) to pass 20 mesh sieve and stored in sealed plastic bags. 500 mg of the various powders were taken in 5 mL volumetric flask, mixed with 5 mL of MeOH and vortexed for 2 min followed by sonication (33 MHz, Roop Telesonic, India) at room temperature for 5 min. The process was repeated thrice for complete extraction. After sonication, methanolic extracts were combined and evaporated to dryness *in vacuo*. For determination of camptothecin content, the concentrate was

transferred into polypropylene micro-centrifuge tubes, mixed with HPLC grade MeOH (1 mL), vortexed for 20s followed by centrifugation at 5000 rpm for 10 min. 20 µL of the clear supernatant was applied directly onto HPLC column.

3. Statistical Analysis

Analysis of variance (One-way ANOVA) was performed to test the significance of differences between means obtained among the treatments in each experiment at the 5% level of significance ($p<0.05$).

4. Results and Discussion

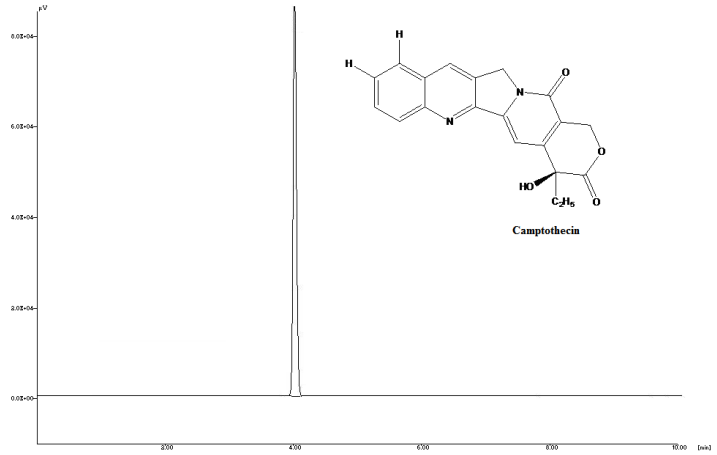
For the analysis of raw herbal materials and herbal preparations, HPLC is superior to other instrumental analytical techniques because it is simple, fast, sensitive and automated technique with higher degree of resolution. The HPLC technique is, therefore suggested for the determination of camptothecin in *N. nimmoniana*. The HPLC chromatograms of different extracts of *N. nimmoniana* shown similar retention time for the compounds (4.0 min) (Figure 1a). The method was validated and the standard deviation proved that the accuracy and reproducibility was found to be satisfactory for the quantitative assay.

The concentration of camptothecin in methanolic extracts of *N. nimmoniana* was calculated by the regression equation (Correlation coefficient $r^2 = 0.998$, $y = 113140\times + 453.5$). HPLC analysis of camptothecin in of *N. nimmoniana* root collected from Western Ghats illustrated in Figure 1b. The maximum concentration of camptothecin was found in roots collected from Mahabaleshwar region (2.62%) followed by roots collected from Patan (1.21%) and Sirsi (0.88%) region whereas the minimum concentration of camptothecin was found in leaves collected from Sirsi (0.29%) region. The pattern of compound accumulation in fruits collected from Mahabaleshwar region (0.63%) was higher as compared to Sirsi region (0.38%) and Patan (0.36%). Stems collected from Patan region (1.45% w/w) showed higher amount of it than Sirsi (0.70% w/w) and Mahabaleshwar region (0.43% w/w). There was more than 2-fold higher concentration of camptothecin observed in roots collected from Mahabaleshwar region than roots collected from Sirsi and Patan region (Figure 2). Among different sources of it, *N. nimmoniana* has been reported to contain much higher content of camptothecin [25]. Govindachari and Vishwanathan (1972) reported 0.06-0.1% camptothecin from *N. nimmoniana* [3] whereas lower yield of it was found to be 0.005 % in *Camptotheca acuminata* and 0.00013% in *Ervatamia heyneana* [26, 27]. *N. nimmoniana* trees cultivated in north-western agro-climatic region of Jammu, India, accumulated 0.1% dry weight camptothecin in roots and seeds, whereas bark produced lower concentrations of camptothecin [28]. The root bark (0.33%) of *N. nimmoniana* yielded the highest camptothecin content followed by stem bark (0.23%) whereas leaves yielded the lowest camptothecin content (0.081%) [12]. The mean camptothecin content of root bark of male trees of *N. nimmoniana* (0.605%) from Ulvi region was significantly greater than that for the male trees in Sirsi (0.339%) and Joida (0.135%) region of Karnataka state [12].

Several factors such as worldwide changes in seasonal patterns, weather events, temperature changes, biotic and abiotic stresses may affect the production of secondary metabolites in plants [29-31]. The samples of five oat cultivars located in different parts of Norway during 1985-1990 were shown considerable differences in deoxynivalenol (DON) content. The mean DON concentration was found to be highest in 1988 and 1989. These years were both characterized by heavy rainfall. The lowest DON concentration was found in 1987 and 1990, two years with drought weather [32]. Solar radiation (UV radiation), not only mutate but also induce the *de novo* synthesis of many secondary metabolites and UV-B exposure causes temporary changes in phenolic content in plant epidermal cells [33-37]. Azadirachtin content in the seeds of *Azadirachta indica* varied from 200 to 16,000 ppm (µg/g of the seed kernel). The highest content of azadirachtin was recorded in the neem tree

populations growing in the southern part of India [38]. The results of the present study showed that concentration of camptothecin was affected by geographical origin and climatic conditions.

a)



b)

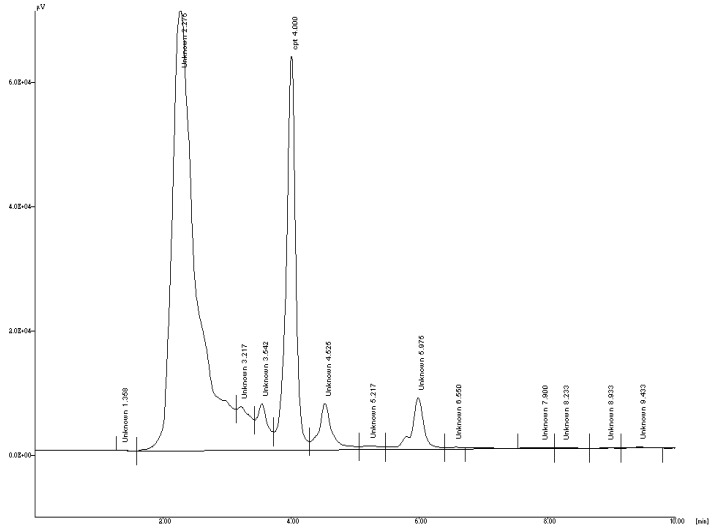


Figure 1. a) HPLC chromatogram of camptothecin authentic sample.
b) HPLC chromatogram of root of *N. nimmoniana* collected from Sirsi spiked with camptothecin.

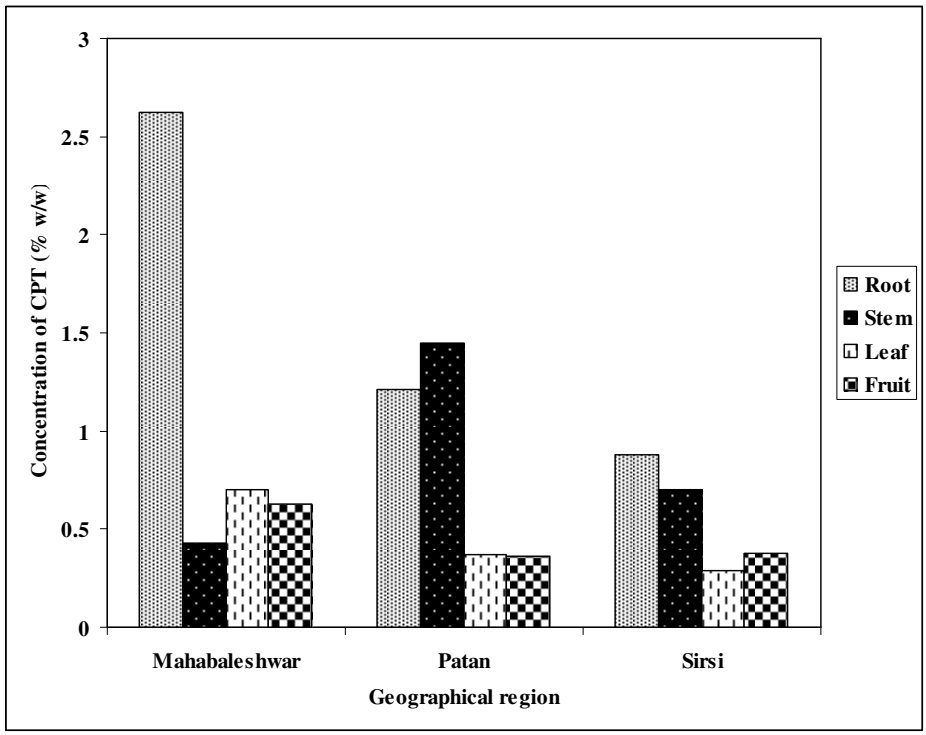


Figure 2. HPLC analysis of camptothecin in different parts of *N. nimmoniana* collected from different regions of Western Ghats**.**

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