

Should Seed Be an Alternative Source of Camptothecin From *Nothapodytes nimmoniana* and *Chonemorpha fragrance*?

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ABSTRACT

The Southeast Asian forest is blessed with plant sources of anticancer molecules including camptothecin (CPT) but, the irrational harvest is among serious threat to its existence for use by the future generation. Among plant sources of CPT found in the region are *Nothapodytes nimmoniana* and *Chonemorpha fragrance* that are under heavy exploitation pressure for the alkaloid and other medicinal purposes. Under the natural conditions of forests, seeds produced by the plants show germination difficulties, and as a result lose viability few months after set. The unviable seeds can be used as an alternative source of CPT and other phytochemicals isolated from the species. In this study, evaluation of CPT yield of seeds and their parts was performed by high-performance thin layer chromatographic analysis to assess the feasible economic use of the seeds as an alternative source of the alkaloid. The results showed a manifold higher yield of CPT in seeds and their parts in *N. nimmoniana* over *C. fragrance*, suggesting seeds of the former that face more exploitation of its forest than the latter as a better alternative source of the alkaloid. The results also suggest that collection of the seed should be made before the onset of rain season. In this study, the economic implications of using seeds to isolate CPT are also discussed.

Key words: Conservation, Forest Management, Camptothecin, Seeds, *Nothapodytes nimmoniana*, *Chonemorpha fragrance*.

INTRODUCTION

Forestry is a vital source of natural anticancer molecules but, the irrational use in Southeast Asian countries is of serious concern on the availability to the future generations. The medicinal importance of a forest plant lies on the bioactive constituents it produces that have significant pharmacological activity and can be isolated with potentials for drug development.¹ The breakthrough on cytotoxicity of extract from plants led to the discovery of natural compounds with anticancer properties² and their development into drugs emanated from studies of extracts isolated from seeds.^{1,2} Biosynthesis and yield of the compounds vary with forest species and with many, seeds are an important source of the alkaloids. They offer a practical, cost-effective alternative to extraction from the natural population over chemical synthesis. Among high-value alkaloids isolated from native Southeast Asian forest plants is camptothecin (CPT) which is a monoterpene indole anticancer alkaloid from which many semisynthetic analog drugs used to treat varied human cancer types were developed.

Nothapodytes nimmoniana is an endangered medicinal forest tree and most convenient source of the alkaloid.³ The tree belongs to family Icacinaceae and is found in Sri Lanka, China (East and Mainland) Mainland

South East Asia, North Sumatra, Sundaic region in Taiwan, Isabela province, Luzon Philippines and India where the endemic distribution in the Western Ghats is under heavy exploitation for CPT.⁴ In the region, because one ton of the alkaloid can be isolated from 1000-1500 tons of *N. nimmoniana* wood chips, the natural population is overharvested for export of CPT. This led to declined forested area and tree individuals by 50-80% in the last decade.⁵ The loss in areas of patchy endemic distribution have remarkably declined to 1-2 individuals/hectare and the trend is ever on the increase.⁶

Chonemorpha fragrance is a seed-propagated ever-green vigorous growing endemic and endangered climbing shrub that produces stem of 30m or more and can climb the top of the tallest tree in a forest. The plant produces many phytochemicals attributed the many biological activities exhibited by extracts isolated from different parts of the species.⁷ Camptothecin was recently isolated from the climber with subcellular localization identified.⁸⁻⁹ Although the alkaloid is produced at low yield in *Chonemorpha fragrance*, it is among endangered natural sources that could face over harvesting pressure for CPT.

As CPT is isolated at higher yield from *N. nimmoniana* with greater quantity in stem and

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root of the tree than other natural sources, its natural population is under indiscriminate exploitation for the alkaloid. Annual demand for wood chips for domestic consumption and export reached 500-700 metric tons.⁶ The tree produces many and desiccations sensitive seeds bearing small embryo with low germination capacity and unviable seeds can be found in large numbers on forest floor few months after seed set.¹⁰⁻¹¹ Although *C. fragrans* produces CPT, albeit at low yield, the species is propagated via seeds. This, coupled with slow growth in nature makes natural population subject to overexploitation for CPT with consequence on declined number of individuals and current endangered status in the Western Ghats, India.

Cultivation of CPT-producing plants as cash crops is receiving increasing consideration in Southeast Asia where many endemic species produce the alkaloid.^{12,13} The over-exploitation of their natural population for domestic use and export of CPT is ever on the increase. Threats due to recalcitrant seeds produced by the plants and slow natural growth led to increasing the call for conservation by implementing strategies against exploitation at the local level.¹⁴⁻¹⁵ Promoting the use of non-destructive parts as seeds to isolate CPT could be a promising alternative. The recalcitrant seeds produced by the plants may be considered an alternative source of CPT when they lose viability as many could be found on forest floor few months after seed set. Although in the earlier studies, CPT yield in seed mass of *N. nimmoniana* found in India was quantified¹⁶⁻²¹ and new camptothecinoids isolated in Taiwan.²² The various parts of the seeds and variability in yield of the alkaloid across seed sources to (or "intending to") assessing workable economic use for CPT extraction were not analyzed. For *C. fragrans*, no study investigated the production or evaluation of CPT yield in the seeds and their parts. In this study, potentials and possibility of using seeds as an alternative source of CPT from *N. nimmoniana* and *C. fragrans* are evaluated and discussed.

MATERIALS AND METHODS

Seeds collection

Authenticated seeds of *C. fragrans* was obtained from Indian Institute of Horticultural Research Bengaluru, Karnataka India while *N. nimmoniana* seeds from three areas in the Western Ghats; One of the seed samples was provided by Sanjivini herbals Salem, Tamil Nadu India (S1). The other samples by Rishi herbals Bengaluru, Karnataka (S2) and Mr. N. Balakumar of Karthik Agro Traders Allagapuram Kathoor Tamil Nadu (S3). Dried seeds of both of the species were soaked in sterile distilled water overnight to soften seed coat for the ease of seed embryo axes removal from the seeds. The excised seed embryo axes were used for alkaloid extraction after dried to constant mass or further experiments²³ while seed coat dried to constant mass. Fruit wall of the seeds was removed using forceps before seed soaking in water.

Alkaloid Extraction and High-Performance Thin Layer Chromatographic analysis

Camptothecin extraction was performed using microwave irradiation method of Fulzele and Satdive¹⁷ with slight modifications. In brief, various components of the seeds comprising dried seed coat, fruit wall, seed embryo axes and whole seed mass of *C. fragrans* and *N. nimmoniana* were dried to constant mass. One gram of the dried samples was ground to fine powder using mortar and pestle and transferred into a 200 ml glass beaker, and 50 ml of 90% HPLC grade methanol added to the contents. The beaker containing the suspension were subjected to microwave oven extraction at 100 w for 3 mins. Obtained suspensions were not allowed to super-boil in the micro wave by the regular stoppage of the irradiation and to cool the beaker (with contents) in water for minutes. Contents were again subjected to microwave irradiation and cooling cycles to complete three rounds. Suspensions obtained from the extraction were allowed to

settle and upper aqueous phase transferred into 2 ml-capacity centrifuge tube, and then centrifuged at 14,000xg for 10 mins. Obtained supernatants were filtered using a Millipore filter (0.45 µm) into a fresh centrifuge tube. High-Performance Thin Layer Chromatographic (HPTLC) analysis was performed using modified method of Kulkarni *et al.*⁸ Briefly, 20 µl of the extracts were loaded to an HPTLC plates (Merck silica gels) using automated sample loading system (Linomat 5 Camag). The applied samples were allowed to dry for minutes and later developed in duplicates in a glass chamber saturated with ethylacetate:toluene (7:3) mobile phase solvent system. Developed chromatographic plates were dried and later scanned with Camag 3 densitometric scanner. The peaks, peak area and Rf of the spots recorded. Samples of pure CPT standard was procured from Sigma Aldrich and working stock solution prepared by dissolving 800 µg of CPT powder in 1ml chloroform:methanol solvent mixture (4:1). Different working concentrated dilutions were prepared from a standard stock solution. They were then spotted on a TLC plates (silica Merck), air-dried for minutes and allowed to develop in a saturated chromatographic glass chamber. The procedure was used for the CPT reference standard dilutions in a similar way to plant tissue extract. The experiment was carried out in duplicates, the fluorescence and Rf of CPT recorded at 366 nm. Camptothecin yield of the various tissues was evaluated from a standard curve drawn using different concentrations of the pure camptothecin reference standard.

RESULTS AND DISCUSSIONS

In the Southeast Asia, conservation of forest genetic resources, particularly those that produce bioactive molecules with anticancer properties is receiving increased recognition due to recent awareness on the threat to their availability for the future generation.²⁴ Besides a primary role in sexual propagation; plant seeds can be a valuable source of bioactive compounds, including anticancer alkaloid CPT. However, seeds are among neglected sources of the alkaloid due to their status in plants propagation and more preference given to the root, shoots/wood and leaves for alkaloid isolation. This has lead to a remarkable loss in plant genetic resources in forested areas due to exploitation for the molecule. In recent years, alternative collection measures are increasingly sought. Although establishing protected areas is a conservation strategy used to protect the genetic resources in some of the countries, it does not reduce irrational harvest of forest for CPT.²⁵

Hard seed coat and dormant embryos, coupled with difficult-to-get favorable environmental conditions of moisture, oxygen, and soil hinders seed germination with many of the forest plants. As a result, they lose viability within a short period after set, in particular, those that produce recalcitrant seeds. Thus, the seeds that represent a 'waste of propagules' become available for other economic use, including extraction of the important phyto-compounds as CPT.^{26,27} During the reproductive season, *N. nimmoniana* produces many seeds but, due to natural recalcitrance, majority lose viability before the onset of germination season when enough moisture become available to support germination, seedling cohorts establishment and forest generation.²⁸ The unviable seeds found in large numbers on the forest floor are of no use for propagating the tree, and overtime, they become attacked by fungi (personal communication with locals). *C. fragrans* produces a large number of seeds but, due to hard seed coat (Figure 1a), germination under the natural condition is poor. As a result, the majority do not germinate in a forest and over time, they lose viability. Promotion of seeds collection for alkaloid extraction and export is a significant step towards mitigating irrational harvest of the two species for CPT. For instance, in *N. nimmoniana* seedlings need 6-8 years to reach maturity and reproductive stage.⁶ A collection of the entire mature tree may not yield equivalence of CPT produced by seeds over the eight year period as the seeds are produced in large number by



Figure 1: a, *C. fragrance* seed mass; b, *N. nimmoniana* fruits; c, *N. nimmoniana* dried seed; d, decorticated *N. nimmoniana* seeds (Scale bar 2mm); e, excised seed embryonal axes of *C. fragrance* (Scale bar 2mm); f, excised seed embryonal axes of *N. nimmoniana* (Scale bar 1.5mm).

the tree in a season. Assessing the yield of the alkaloid in seeds and their parts could give an insight on potential economic use to isolate CPT.

Besides a primary role in plant reproduction/propagation, seeds could be an alternative source of CPT to other plant parts as leaves, shoots/wood, and roots, based on the pattern of accumulation of the alkaloid. However, the implication of differential alkaloid yield from the seeds and other plant parts is unknown in many forest trees.²⁹ To evaluate the potential of the seeds as an alternative source for extraction of CPT and as a substitute to the harvest of natural stands, alkaloid was extracted from the ground seeds of *N. nimmoniana* and *C. fragrance*, their various parts and obtained tissue extracts analyzed by HPTLC analysis (Figure 1a-f, 2b-c). The analysis showed manifold CPT content in seeds and their part in *N. nimmoniana* over *C. fragrance* (Figure 2a), suggesting that the former has more potential as an alternative source of the alkaloid than the latter. The highest yield of the alkaloid was found in seed mass of *N. nimmoniana* with greatest CPT yield of 0.16% CPT/g seed DW over 0.0097% in *C. fragrance*. The yield varied with seed source and highest

found in seeds obtained from Sanjivini herbals while least in those from Karthik Agrotaders Tamil Nadu. This showed the genotype-dependent effect on CPT content in *N. nimmoniana* seeds. Earlier studies on populations of the species in the Western Ghats region, India showed variability in CPT yield in wood and shoots of individuals populations in a forest.³⁰⁻³¹ The yield of the alkaloid in the seeds used in this study is less than reported for wood in the reports. Although the yield of the alkaloid from seeds of a plant depends on plant habit, climatic condition, genetic make up and proportion of species producing the alkaloid, and may not depend on latitude.³²⁻³³ In this study, CPT yield of *N. nimmoniana* seeds varied across sources (Figure 2a) which could be due to the influence of climatic condition as biosynthesis of plant secondary metabolites that includes CPT are influenced by environmental factors. In *C. acuminata* that produces higher CPT in seeds over seedlings, collected seeds in different geographical regions showed variation in CPT yield, and the variability reflected seed length, stem diameter and height.³⁴ From the results of this study, CPT yield in *N. nimmoniana* and *C. fragrance* was

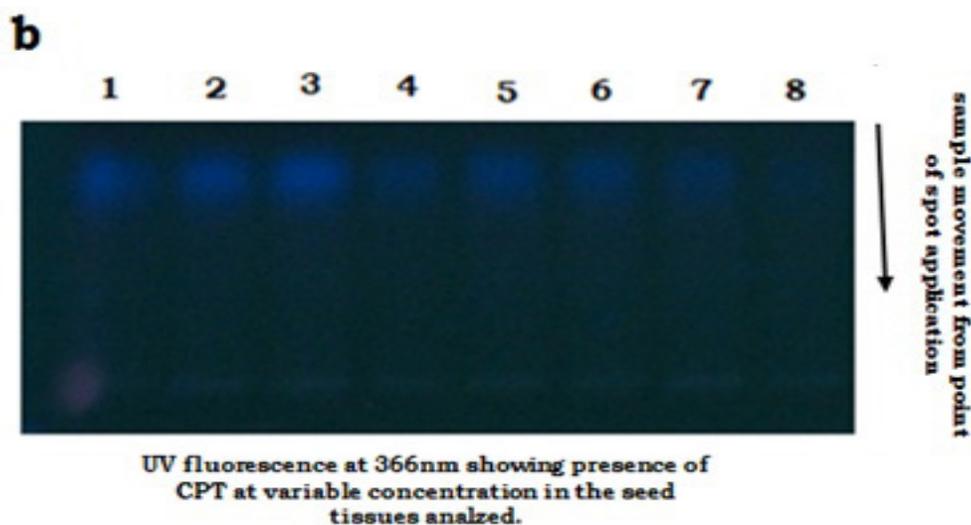
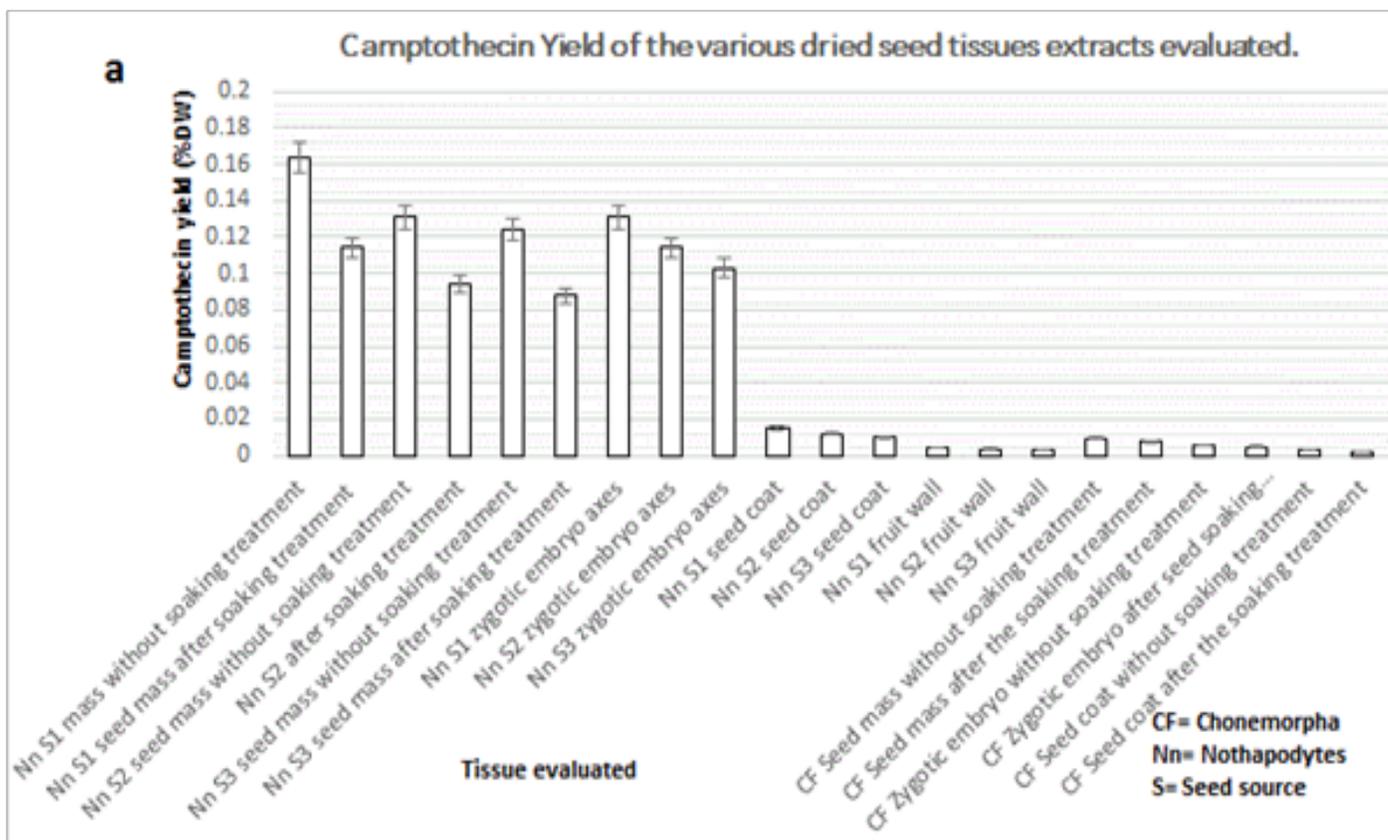


Figure 2: (a) Camptothecin yield of the tissues evaluated; (b) representative UV fluorescence of CPT at 366nm in some of the seed tissue samples analyzed..

affected by pre-soaking treatment in water overnight as shown by the lower CPT content of the seeds subjected to overnight pre-soaking (Figure 2a). This can be explained by the leaching of the alkaloid in water as CPT is water-soluble; ongoing study with cell suspension culture of the species showed that high amount of the alkaloid is leached into culture media (unpublished). Also, from the results, it can be suggested that collecting seeds of the two species (particularly, those that lost viability) from forest should be done before the onset of rain season for greater CPT yield.

For evaluating CPT yield in various seed parts of the two species, fresh fruit wall, seed coat, and seed embryo axes were dried separately, the

alkaloid was extracted and HPTLC analysis performed (Figure 2b-c). In a similar way to seed mass, CPT yield was manifold higher in various seed parts of *N. nimmoniana* over *C. fragrans*. The yield varied with seed source in *N. nimmoniana* with the highest found in seeds obtained from Sanjivini herbals Tamil Nadu when compared to the other sources (Figure 2a). A comparison of various parts showed seed embryo contained higher CPT over seed coat and fresh fruit wall. However, combined CPT yield of the seed parts was near to seed mass (Figure 2a). The little differences observed may have been caused by extraction technique conditions and pre-soaking treatment of the seeds in water. Compared to the seed embryo of *C. fragrans* where greatest of 0.0053% CPT yield was

found, CPT yield of seed embryo axes of *N. nimmoniana* was relatively higher with 0.131% CPT. The yield varied with seed embryo axes seed source as well (Figure 2a). Camptothecin content of seed coat was manifold higher in *N. nimmoniana* over *C. fragrance* and varied with seed coat seed sources (Figure 2a). The yield was lower in the seed coat of *C. fragrance* seeds subjected to pre-soaking treatment in water overnight. This further established the effect of seeds soaking in water to reduced CPT yield in the two species. There was very low level accumulated CPT in fresh fruit wall of *N. nimmoniana* seeds and was variable with seed sources as well (Figure 2a).

From results of this study and the reported literature on CPT yield of seeds, their parts, and in various plant parts of the two species¹⁵ it can be inferred that seeds represent a practical alternative source of CPT, especially the ones that have lost viability. Thus, a strategy for conservation of the two species that involves using the unviable seeds (as they become a waste in the forest after germination season passed) for extraction of CPT, and as raw material for export, is an important conservation strategy. The introduction of saplings into new sites to set up forested areas and legislations regulating overharvest of the endangered natural population is an important step. Driven by demand for CPT in the international trade, commercial farmers in some states in India have developed plantations of *N. nimmoniana* to (or “intending to”) cultivating the tree parts for export while in other Southeast Asian countries, no such efforts are made. For *C. fragrance* effort towards forestation or establishment of commercial cultivation is none existent so far, possibly due to the low CPT yield but, the population is continuously exploited for other medicinal uses.

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CONFLICT OF INTEREST

The author do not have conflict of interest on the manuscript contents.

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