MOLECULAR AND CHEMICAL CHARACTERIZATION OF *N NIMMONIANA* (ICACINACEAE) AND ITS RELATED GENERA: TOWARDS IDENTIFYING HIGH YIELDING SOURCES OF THE ANTICANCER ALKALOID, CAMPTOTHECIN

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Thesis submitted to the

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In partial fulfillment of the requirements for

the award of the degree of

Doctor of philosophy

In

CROP PHYSIOLOGY

BANGALORE OCTOBER 2008

DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES

DEDICATED TO MY
BELOVED TEACHER
Dr. R. UMA SHAANKER
AND FAMILY MEMBERS

MOLECULAR AND CHEMICAL CHARACTERIZATION OF *NOTHAPODYTES NIMMONIANA* (ICACINACEAE) AND ITS RELATED GENERA: TOWARDS IDENTIFYING HIGH YIELDING SOURCES OF THE ANTICANCER ALKALOID, CAMPTOTHECIN

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DEPARTMENT OF CROP PHYSIOLOGY

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BANGALORE

2008

BANGALORE

Certificate

This is to certify that the thesis entitled "Molecular and Chemical characterization of *N nimmoniana* (Icacinaceae) and its related genera: towards identifying high yielding sources of the anticancer alkaloid, camptothecin" submitted in partial fulfillment of the requirement for the degree of doctor of philosophy, in Crop Physiology to the University of Agricultural Sciences, Bangalore, is a record of research work carried out by Mr. Ramesha B.T under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associate ship, fellowship or any other similar titles.

Bangalore
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INTRODUCTION

I INTRODUCTION

Plants are the basis of traditional medicine systems that have been in existence for thousands of years. A clinical, pharmacological, and chemical study of these traditional plants derived medicines and forms the basis of most early medicines such as aspirin, digitoxin, morphine, quinine and pilocarpine. Plants continue to provide with invaluable compounds as starting point for development of new drugs. The potential for finding new compounds from plants is enormous as till date only about 1% of tropical species have been studied for their pharmaceutical potential (Butler 2004).

Even at the dawn of the twenty-first century, 11% of the 252 drugs considered as basic and essential by the World Health Organization were exclusively from flowering plants. (Rates 2001 and Raskin *et al,* 2002). In the past screening efforts, plant extracts were evaluated mainly in experimental animals. The National Cancer Institute (NCI) in the United States (Suffness and Douros 1982; Cragg *et al*, 1997) and the Central Drug Research Institute (CDRI) in India (Dhar *et al,* 1968; Dhawan *et al*, 1980; Bhakuni *et al,* 1969) sponsored the most extensive of the screening programs. More than 35,000 species were screened *in vitro* and later *in vivo* by NCI from 1960 to 1981. Taxol and camptothecin (Wall and Wani, 1996) were discovered in this program although several other compounds were unsuccessful in human studies. These chemicals derived from plants have demonstrated pharmaceutical activity against human ailments such as cancer and HIV. For example against cancer, taxanes from *Taxus sp*; terpenoid indole alkaloids, including Vincristine and Vinblastine from *Catharanthus roseus*; Camptothecins from *Camptothecca acuminata* showed activity. Similarly against HIV coumarins, including Calanolide A and B from *Calophyllum lanigerum* showed activity (Roberts 2007).

Several of these anti-tumor drugs (including taxols, camptothecins, maytansine, homoharringtonine, vinblastine, vincristine, indicine-N-Oxide, baccharin, podophyllotoxin derivatives and etoposide etc) sold today are simple synthetic modifications or copies of the naturally obtained substances and are being extracted from plant sources (Nalawade *et al*, 2003; Yan *et al*, 2003; Butler 2004). Considering the enormous potentiality of these

compounds, several laboratories worldwide have been working on them towards standardizing methodologies for their large-scale production.

Among the plant-derived compounds, camptothecin (CPT), a quinoline alkaloid, has been extensively used as a novel anti-tumor agent. CPT, a monoterpene indole alkaloid obtained from several plant sources. It was first isolated from extracts of *Camptotheca acuminata*, a tree native to China (Wall and Wani*,* 1968). Following the discovery that the primary cellular target of CPT is DNA topoisomerase-I (topo-I), a number of reports have indicated its therapeutic potential, against colon cancer, uterine cervical cancer and ovarian cancer (Masuda *et al*, 1992; Lilenbaum *et al*, 1995; Romanelli *et al*, 1998; Clements *et al,* 1999; Priel *et al,* 1991). Two clinically used anti-tumor compounds, topotecan and irrinotecan, are currently semi-synthesized using natural camptothecins. In fact the ever-increasing worldwide market of irinotecan and topotecan has currently reached one thousand million US dollars which represents approximately one ton of CPT in terms of raw material (Watase *et al*, 2000).

Besides this, there are 26 other camptothecin-derived drugs, which showed promising results and are in clinical trials. Camptothecin anologs have also been demonstrated to be potent antiviral, anti-HIV agents and chemosterilants. Thus, camptothecin will have broader uses and worldwide demand of camptothecin (CPT) is going to increase (Cragg and Newman, 2004).

Camptothecin has been isolated from several other species including *Merriliodendron megacarpum* (Gunashekara *et al,* 1979) and *Nothapodytes nimmoniana* (Govindachari and Vishwanath, 1972) both belonging to the family Icacinaceae, *Ophiorrohiza mungos* (Tafur *et al,* 1976) and *O. pumila* (Aimi *et al,* 1990) from the family Rubiaceae, *Eravatamia heyneana* (Gunasekara *et al,* 1979) belonging to Apocynaceae and *Mostuea brunonis* (Dai *et al,* 1999) belonging to the family Loganiaceae. However to date, the highest content of CPT has been reported from *Nothapodytes nimmoniana* (about 0.3 % on a dry weight basis) compared to any other botanical source (Ku and Tang*,* 1980). *Nothapodytes nimmoniana* Grahm formerly known as *Nothapodytes foetida* Sleumer and *Mappia foetida* Meirs is a small tree of the family Icacinaceae. The tree commonly referred to as "Stinking Tree" and is native to warmer

regions of South India. Despite its great demand as the only commercially available source of these medicines, CPT is still supplied exclusively from intact plants, mainly *C. acuminata* and *Nothapodytes foetida* (Icaccinaceae) (Watase *et al*, 2004). But recent results of investigation showed that, wild happy tree populations are in few provinces of China owing to the exhaustive harvesting of trees from the wild. Recently the tree has been declared vulnerable (Prop. 11.58).

Consequently there has been an unprecedented pressure on the natural populations of *Nothapodytes nimmoniana* in the Western Ghats. Indiscriminate felling of the trees for short-term gains could lead to the loss of elite individuals and populations which could otherwise serve as sources of high CPT. In fact it is estimated that just in the last decade, there has been at least 20% decline in the population leading to the red listing of the species (Hombe Gowda *et al*, 2002; Ravikumar and Ved, 2000). In addition to the difficulties of the practical total synthesis of these natural compounds and inconsistent supply of CPT from natural sources have raised a strong need for the establishment of more stable and profitable methods of producing CPT. Therefore it is essential to find alternative and consistent sources to meet the pharmaceutical demand. In order to ensure a reliable supply of CPT several attempts have been made to identify alternate sources that would help in combating the heavy demand on single species of plants and some of them yielded good results.

With this background the present study was specifically designed to screen populations of *N nimmoniana* in the Western Ghats to identify high CPT yielding individuals. Besides, the study intends to screen phylogenetically related genera of *N. nimmoniana* in the family Icacinaceae to yield alternate sources of CPT. The study is also proposed to isolate and screen endophytic fungi from *N nimmoniana* that produce CPT.

The specific objectives of the study are to:

1) Chemically profile populations of *Nothapodytes nimmoniana* in the Western Ghats for camptothecin and its related alkaloids.

2) Undertake molecular characterization of populations of *Nothapodytes nimmoniana* in the Central Western Ghats, India using Simple Sequence Repeat (SSR) markers.

3) Prospect phylogenetically related genera/species of *Nothapodytes nimmoniana* for camptothecin.

4) Prospect endophytic fungi from *Nothapodytes nimmoniana* for camptothecin.

REVIEW OF LITERATURE

II REVIEW OF LITERATURE

In this chapter the review of literature, pertaining to plants as source of medicine, natural products from plants used for cancer therapy are presented. A brief review is made on Camptothecin (discovery to clinic), sources of Camptothecin, with special emphasis on *Nothapodytes nimmoniana:* an Indian source of Camptothecin. An attempt to review the possible alternate sources of high valued secondary metabolites from plants with special emphasis on endophytic fungi has also been made. Besides a brief review of use of SSR markers in assessing population genetic variability of threatened plants species has also been presented.

1. Plants as source of medicine:

Plants are the basis of traditional medicine systems that have been in existence for thousands of years. Written records about medicinal plants date back at least 5000 years to the Sumerians; the first records written on clay tablets in cuneiform are from Mesopotamia. Archeological records suggest even earlier use of medicinal plants and continue into modern times (Swerdlow, 2000).

Even at the dawn of the twenty-first century, 11% of the 252 drugs considered as basic and essential by the World Health Organization were exclusively of flowering plant origin (Rates 2001). These chemicals derived from (Figure 2.1) plants have demonstrated pharmaceutical activity against human ailments such as cancer (for example, taxanes including paclitaxel from *Taxus sp,* vincristine and vinblastine from *Catharanthus roseus* and Camptothecins from *C acuminata*), malaria (for example, artemisinin from *Artemisia annua*), and HIV (for example, coumarins including Calanolide A and B from *Calophyllum lanigerum*) (Roberts 2007; Figure 2.1). Several of these drugs sold today are simple synthetic modifications or copies of the naturally obtained substances.

Some of the drugs/chemicals are still sold as plant based drugs requiring the processing of the actual plant material. Others have been chemically copied or synthesized by laboratories and no plant materials are used in the manufacture of the drug

(http://www.rain-tree.com/plantdrugs.htm). A good example of this is the plant chemical quinine, which was discovered in a rainforest tree (*Cinchona ledgeriana*) over 100 years ago. For many years the quinine chemical was extracted from the bark of this tree and processed into pills to treat malaria. Later, efforts were made to synthesize or copy this plant alkaloid into a chemical drug without using the original tree bark for manufacturing the drug. Today, all quinine drugs sold are manufactured chemically without the use of any tree bark. However, another chemical in the tree called quinidine which was found to be useful for various heart conditions could not be completely copied in the laboratory and the tree bark is still harvested and used to extract this plant chemical from it. Quinidine extracted from the bark is still used today to produce quinidine-based drugs. In the U.S. there are four patented brand-name heart drugs sold in pharmacies containing bark-extracted quinidine: Cardioquin, Quinaglute Dura-tabs, Quinidex Extentabs and Quin-Release.

Bioprospecting value of Biodiversity hotspots: It is estimated that only 5–15 % of the approximately 2, 50, 000 species of higher plants have been systematically investigated, chemically and pharmacologically (Balandrin *et al,* 1993). With more than 2, 50, 000 species of higher plants more useful drugs remains to be discovered. The potential of large areas of tropical rainforests remains virtually untapped. Various studies have attempted to assess the value of plant diversity for pharmaceutical uses. Hit probability for unit area of land in a biodiversity hotspot is proportional to the density of endemic species in the area. Western Ghats constitutes one of the unique biological regions of the world. Among the biodiversity hotspots, it ranks 5th in its bioprospecting value (Figure 2.2) (Pushpam Kumar, 2004). Fifty-six genera and 38 percent of all species of flowering plants are considered endemic to the Western Ghats. About 63% of India's evergreen woody plants are endemic to the Western Ghats. Nearly 650 species of plants in the Western Ghats are trees. With such a huge diversity of endemic plants including medicinal plants distributed all along Western Ghats, there is immense potential for bioprospecting for biologically active compounds in this region.

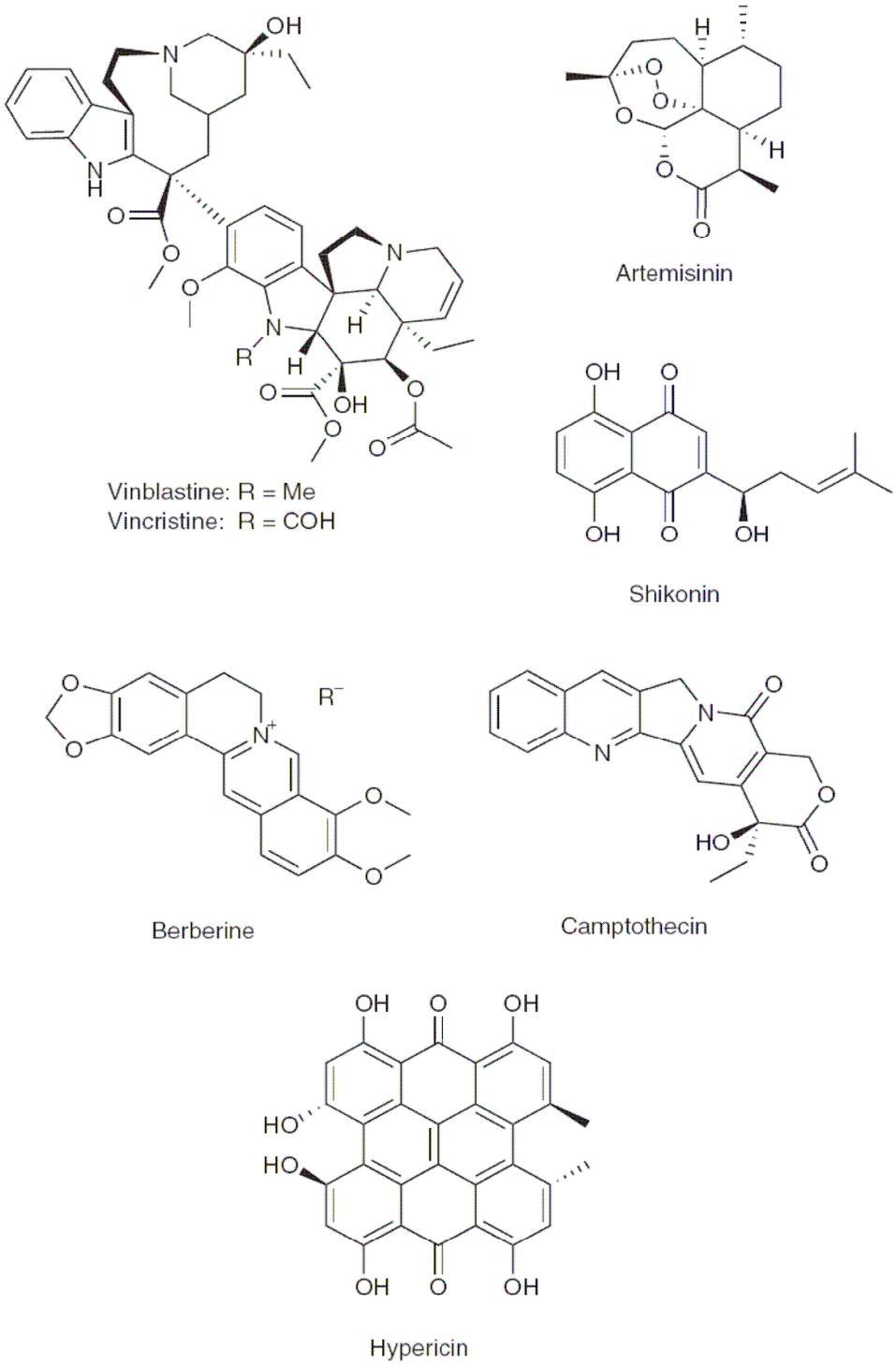


Figure 2.1: Structures of industrially relevant secondary metabolites**.**

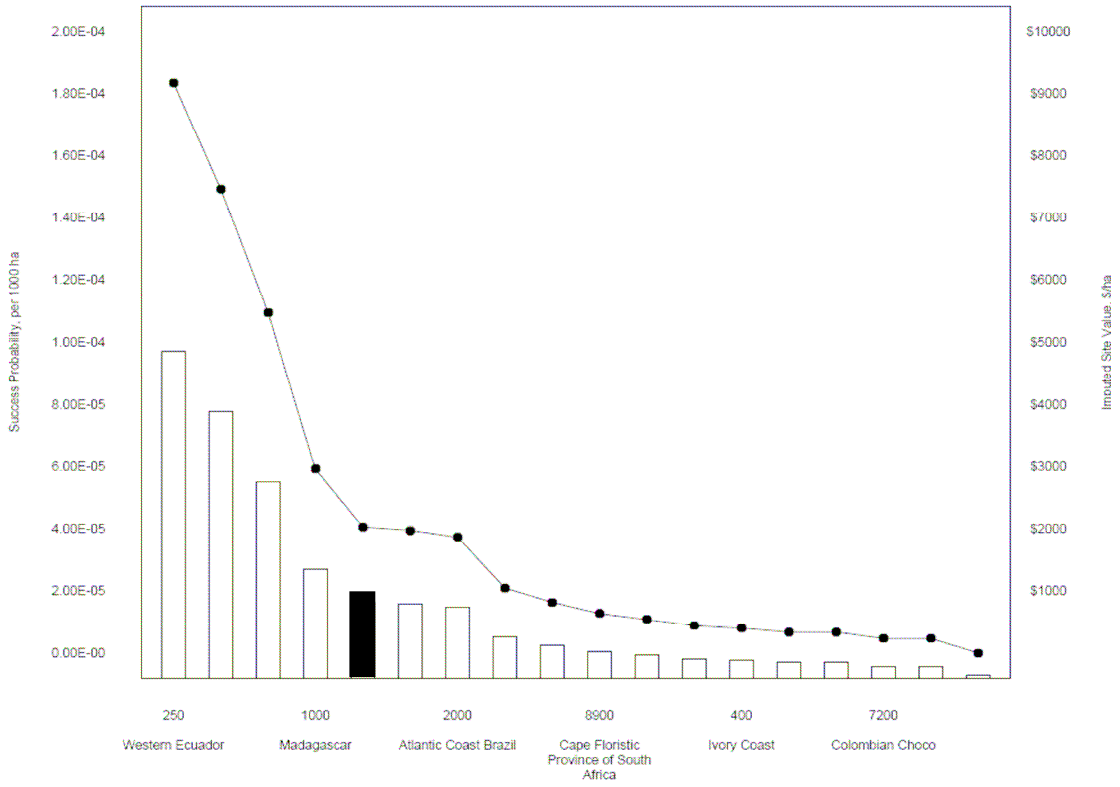


Figure 2.2: Bioprospecting value of different biodiversity hotspots. Filled histogram is Western Ghats, hotspot of biodiversity, India.

2. Anticancer natural products from plants

Cancer is second only to heart disease as a leading cause of death in the United States and it is estimated that about one out of every three Americans develop cancer at some point during his or her lifetime. Drugs from plants (natural product drugs) have played a dominant role in pharmaceutical care for the treatment of various diseases, especially cancer. The greatest recent impact of plant-derived drugs was probably felt in the antitumor area, where taxol, vinblastine, vincristine and camptothecin have dramatically improved the effectiveness of chemotherapy against some of the deadliest cancers (Raskin *et al,* 2002).

The search for anti-cancer agents from plant sources started in 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins. These discoveries prompted the United States National Cancer Institute (NCI) to initiate an extensive plant collection program in 1960, focused mainly in

temperate regions. This led to the discovery of many novel chemotypes showing a range of cytotoxic activities, including the taxanes and camptothecins, but their development into clinically active agents spanned a period of some 30 years, from the early 1960s to the 1990s.

Several plant-derived compounds are currently successfully employed in cancer treatment. One of the most significant examples is the vinca alkaloid family isolated from the periwinkle *Catharanthus roseus*, which is found in the rain forests of Madagascar. The introduction of the vinca alkaloid vincristine was responsible for an increase in the cure rates for Hodgkin's disease and some forms of leukemia. Vincristine inhibits microtubule assembly, inducing tubulin self-association into coiled spiral aggregates (DeVita *et al*, 1970; Noble 1990).

The two clinically active agent's etoposide and teniposide are semi synthetic derivatives of the natural product, epipodophyllotoxin. Epipodophyllotoxin is an isomer of podophyllotoxin, which was isolated as the active antitumor agent from the roots of various species of the genus *Podophyllum*. These plants possess a long history of medicinal use by early American and Asian cultures, including for the treatment of skin cancers and warts (Stahelin 1973; Liu 1989).

More recent additions to list of plant-derived chemotherapeutic agents are the taxanes and camptothecins. Paclitaxel (taxol) initially was isolated from the bark of *Taxus brevifolia* (Taxaceae), collected in Washington State as part of a random collection program by the U.S. Department of Agriculture (USDA). The taxanes paclitaxel and docetaxel, showed impressive antitumor activity against breast, ovarian and other tumor types in the clinic. Paclitaxel stabilizes microtubules, leading to mitotic arrest (Wani *et al,* 1971).

Like wise Camptothecin was discovered from *Camptotheca acuminata*. Camptothecins are monoterpene indole alkaloids and are lauded as one of the most promising anticancer drugs of the twenty-first century (Romanelli *et al*, 1998; Vladu *et al*, 2000). Irinotecan and Topotecan, of camptothecin (CPT) have been approved by the Food and Drug Administration of the United States of America (FDA) for treating colorectal and ovarian

cancer (Lilenbaum *et al*, 1995). Presently both taxanes and the camptothecins are used as precursors to semi synthesize various clinically useful antitumor drugs.

3. Camptothecin:

Camptothecin (CPT) is a potent antitumor agent first isolated by Monroe E. Wall and Mansukh C. Wani in 1958 from extracts of *Camptotheca acuminata* ("Xi Shu" or tree of joy), a deciduous tree native to China and Tibet, which has been extensively used in traditional Chinese medicine.

Discovery of CPT: The discovery of the potent antitumor activity of an extract of the leaves of *Camptotheca acuminata* Decne. (Nyssaceae) by Wall in 1958 was somewhat serendipitous. The Chico Station provided leaves of *Camptotheca acuminata* to the Biochemistry Division of the USDA's Eastern Utilization and Development Division (EURDD) research labs in Philadelphia (Technical Bulletin). One of whose objectives was to screen for plant steroidal sapogenins suitable for the synthesis of Cortisone. 7000 of the extracts were also tested for potential antitumor activity through collaboration with Dr. Jonathan Hartwell of the National Cancer Institute (NCI), Cancer Chemotherapy National Service Center (CCNSC). In 1958, Dr. Hartwell informed Dr Wall that, of all the extracts, only *Camptotheca acuminata* demonstrated activity in a number of assays including CA755 (adenocarcinoma) assay.

In July 1960, Wall joined the newly founded Research Triangle Institute as head of the Natural Products Laboratory and in 1962, was joined by Dr. Mansukh Wani; this was the beginning of a highly productive partnership that would last over four decades. This also led to a lifelong collaboration with the NCI and one of the early products of this collaboration was the isolation and structural elucidation of camptothecin as the active agent of *C. acuminata* in 1966 (Cragg and Newman, 2004).

Mode of action of CPT: The cellular target of the CPT is topoisomerase I (Topo-I), a nuclear enzyme responsible for DNA replication. CPT interacts only with cells, which are in the S-phase. CPT molecules bind to topoisomerase I-DNA complex and prevent the

replication process (Hseing *et al*, 1985). This means that CPT is toxic to cells that are undergoing DNA synthesis. Rapidly replicating cells such as a cancerous cell spend more time in the S-phase relative to the healthy tissues. Therefore, cancerous cells are killed with much higher efficiency than the healthy host tissues. Such selectivity of cytotoxicity is a promising property of CPT.

Topo-I unwinds supercoiled DNA ahead of active transcription/translation sites ("replicating forks"). The noncovalent complex of double stranded DNA and Topo-I, described as the "noncleavable complex," is in rapid kinetic equilibrium with the so-called "cleavable complex," which forms when Topo I creates a transient break in one DNA strand and concomitantly becomes covalently bound to the 3'-phosphoryl end of the mutilated nucleic acid. The intact DNA strand is allowed to unwind once and to pass through the break site, before Topo I re-ligates the cleaved DNA and re-establishes the double stranded configuration. These events constitute an obligatory stage of DNA replication/transcription, as the DNA must be unwound for the cell to express genetic information or to divide. Camptothecin interferes with the religation by binding to the DNA-enzyme binary complex resulting in a reversible enzyme-camptothecin-DNA tertiary complex (Figure 2.3). Consequently, the advancing DNA polymerases operating in the replicating fork soon "collide" with the stabilized cleavable complex and create an irreparable double-strand break. This event is fatal to the cell (Hseing *et al,* 1985; Liu *et al* 2000).

Structure-Activity Relationship Studies: Due to the S-phase specificity of CPT, continuous exposure to this drug must be maintained in order to achieve optimum therapeutic efficacy. Unfortunately, there is some difficulty for CPT to fulfill this requirement. Under physiological conditions (pH=7.4) CPT hydrolyses and converts to the "ring opened" inactive carboxylate form (Pizzolato and Saltz, 2003).

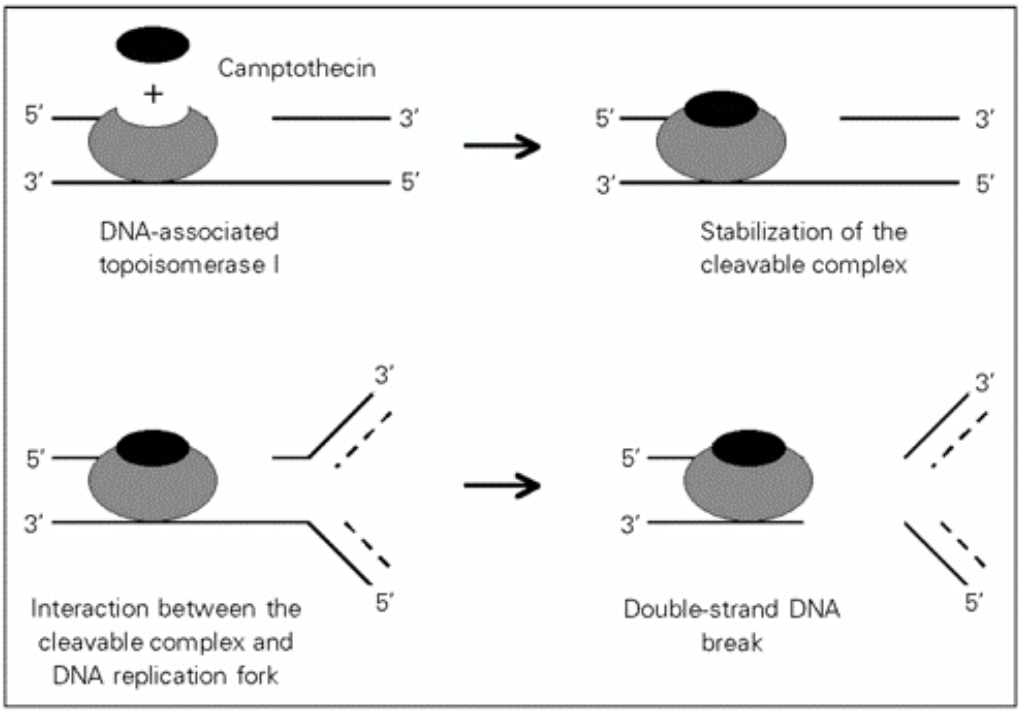


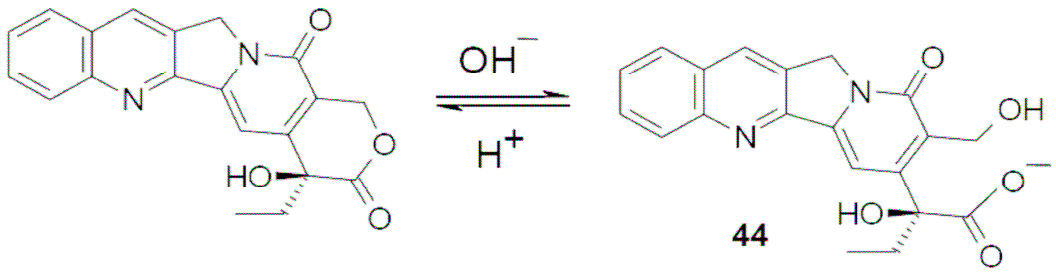
Figure 2.3: Mechanism of action of CPT.

The concentration of the lactone form is much smaller than carboxylate and depends on the environment into which the CPT is introduced. The low concentration of the lactone form under physiological conditions seriously limits the possibility of CPT application in cancer chemotherapy (Figure 2.4). Efforts to find more stable CPT analogues have been undertaken. The new analogues were obtained by modifying the camptothecin molecule.

In order to improve the antitumor efficacy of camptothecins, several approaches have been undertaken. This includes the development of pro-drugs, new formulations, synthesis of lipophilic and water soluble camptothecins. Particularly great deal of attention was paid to water-soluble analogues to facilitate intravenous drug administration.

Research in this direction has culminated in achieving a major milestone by successful commercial launching of two water soluble analogs namely **Irinotecan** (prodrug)

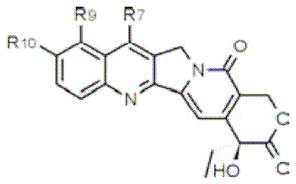
and T**opotecan** as approved drugs for the treatment of lung, cervical and metastatic ovarian cancers (Dai *et al,* 1999; Burke and Bom, 2000).



Lactone form (closed ring) Carboxylate form (open ring)

Figure 2.4: Forms of CPT in physiological fluids (lactone and carboxylate forms).

CPT analogues in clinical use: It was proved that camptothecin derivatives obtained by modification of CPT molecule at positions 7, 9 or 10 retain the anticancer activity. Some camptothecins obtained by such modifications are summarized in Table 2.1. Topotecan (TPT), Irrinotecan and SN-38 are approved as anticancer drugs and are used in chemotherapy of many human cancers (Kruszewski and Thomas, 2002). These compounds exhibit substantially higher stability in the human blood in comparison to Camptothecin as such.



A) Basic structure of CPT.

B)

Compound	R7	R9	R10
Camptothecin	H	H	H
Topotecan	H	$CH_2N(CH_3)_2$	OH
Irrinotecan (CPT-11)	C_2H_5	H	$O_2CNC_5H_4NC_5H_5$
SN-38	C_2H_5	H	OH
DB-202	$Si(CH_3)_2C(CH_3)_3$	H	H
DB-67	$Si(CH_3)_2C(CH_3)_3$	H	OH

Table 2.1: Structure of CPT (A) and its analogues (B) that are in clinical use.

Recent preclinical developments of CPT: Of the 2255 cancer clinical trials recorded, as of August 2003, at the website http://clinicaltrials.gov/ct/screen/AdvancedSearch, 121 or approximately 5.3% are listed as involving camptothecin-derived drugs, including 74 with irinotecan (CPT-11), 32 with topotecan and 12 with other miscellaneous analogues, either as single agents or in combination with other anticancer agents.

The topoisomerase I-interactive agents in clinical and preclinical development, as reported in the Prous *Ensemble* database, Of the 60 agents listed, 26 or approximately 43%, are camptothecin derivatives, which emphasizes the considerable continuing interest in enhancing the effectiveness of this class of molecules (Cragg and Newman, 2004).

Other pharmacological activities of CPT: CPTs have also been studied as potent inhibitors of replication, transcription, and packing of double stranded DNA-containing adenoviruses, papovaviruses, herpesviruses and the single-stranded DNA-containing autonomous parvoviruses (Pantazis *et al*, 1999). CPT inhibits viral functions by poisoning topo I, the host cell enzyme required for initiation and completion of viral functions. If properly developed, CPTs could prove to be powerful antiviral drugs for several DNA viruses, which are causative agents for a large number of diseases (Pantazis *et al,* 1999).

In the mid-1990s, CPT was also shown to have promising activity against parasitic trypanosomes and Leishmania (Bodley and Shapiro, 1995). More recently, researchers at the National Cancer Institute screened 2000 diverse compounds for functional inhibition of the hypoxia-inducible factor 1 (HIF-1), a master regulator of the cancer cells ability to survive under oxygen deprivation. Only four compounds exhibited HIF-1 inhibitory activity, and three of these were CPTs (Rapisarda *et al*, 2002). Hence, these drugs may have other desirable activities against solid tumors that are independent of topo-I poisoning (Pantazis *et al*, 1999).

Natural sources of CPT: Camptothecin was first isolated from a Chinese deciduous tree *Camptotheca acuminata* Decaisne (Nyssaceae) (Wall *et al*, 1966). Later CPT was isolated from a variety of plants including *Merriliodendron megacarpum* (Arisawa *et al,* 1981) and *Nothapodytes foetida* (Aiyama *et al*, 1988), both belonging to the family Icacinaceae, *Ophirrohiza mungos*

(Tafur *et al*, 1976) and *O. pumila* (Saito *et al*, 2001) from the family Rubiaceae, *Eravatamia heyneana* belonging to Apocynaceae (Gunasekera *et al*, 1979) and *Mostuea brunonis* belonging to the family Loganiaceae (Dai *et al* 1999). The relative concentration of CPT from the various species and tissues are presented in Table 2.2. The maximum reported content of CPT ranges from about 0.3 to 0.4 per cent. Compared to *Campotheca acuminata* which had the highest CPT content in the leaves, in *Nothapodytes nimmoniana* the CPT content was highest in the stem bark.

Table 2.2: CPT (% dry wt) content in different plant species and tissues.

Plant species	Tissue analyzed	CPT (% dw)	References	Chromatographic analysis
Camptotheca acuminata	Young leaves	0.4-0.5	Lopez *et al* 1994	HPLC
	Seeds	0.30	Lopez *et al* 1994	HPLC
	Bark	0.18-0.2	Lopez *et al* 1994	HPLC
	Young leaves	0.24-0.30	Li *et al* 2002	HPLC
	Hairy roots	0.1	Lorence and Craig 2004	HPLC
	Callus	0.20-0.23	Wiedenfeld *et al* 1997	HPLC
Camptotheca lowreyana	Young leaves	0.39-0.55	Li *et al* 2002	HPLC
	Old leaves	0.09-0.11	Li *et al* 2002	HPLC
Camptotheca yunnanensis	Young leaves	0.25-0.44	Li *et al* 2002	HPLC
	Old leaves	0.059	Li *et al* 2002	
Ervatamia heyneana	Wood, stem bark	0.13	Gunasekera *et al* 1979	HPLC
Merriliodendron megacarpum	Leaves and stem	0.053	Arisawa *et al* 1981	HPLC
Ophiorrhiza pumila	Young roots	0.1	Saito *et al* 2001	HPLC
	Hairy roots	0.1	Saito *et al* 2001	
Ophiorrhiza liukiuensis	Whole plant	0.012	Kitijima *et al* 2005	H^1 NMR
Ophiorrhiza mungos	Whole plant	0.0012	Tafur *et al* 1976	HPLC
Ophiorrhiza rugosa	Albino plants	0.1	Vineesh *et al* 2007	HPLC
	Plant grown *invitro*	0.03	Vineesh *et al* 2007	HPLC
Mostuea brunonis	Whole plant	0.01	Dai *et al* 1999	HPLC
Pyrenacantha klaineana	Stems	0.0048	Zhou *et al* 2000	HPLC
Nothapodytes foetida	Stem wood	0.14-0.24	Aiyama *et al* 1988	HPLC
	Shoot	0.075	Roja and Heble 1994	HPLC
	Plant	0.048	Yamazaki *et al* 2003	HPLC-DAD-ESI
Nothapodytes nimmoniana	Stem bark	0.3	Govindachari and Vishwanathan 1972	UV, IR, NMR and MS
	Wood	0.1	Govindachari 2002	HPLC
	Leaves	0.081	Padmanabha *et al* 2006	HPLC
	Stem bark	0.236	Padmanabha *et al* 2006	HPLC
	Root bark	0.33-0.77	Padmanabha *et al* 2006	HPLC
	Stem wood	0.14%	Padmanabha *et al* 2006	HPLC
	Root wood	0.18%	Padmanabha *et al* 2006	HPLC

4. Biosynthesis and translocation of CPT:

Although camptothecin is structurally grouped in quinoline alkaloids, it is biogenetically a modified monoterpenoid indole alkaloid (TIA). Hutchinson *et al* 1979 provided the evidence by demonstrating the incorporation of radioactive tryptophan, tryptamine, mevalonic acid and strictosidine and isolating radioactive CPT from the apical cuttings of young seedlings of *C. accuminata.*

CPT is derived from strictosidine, a product of condensation between indole tryptamine and terpenoid secologanin, catalysed by the enzyme strictosidine synthase (Kutchan, 1995). Strictosidine is converted into strictosamide and thereafter to CPT through several intermediary steps that are not yet elucidated. Figure 2.5 excerpts the elements of the pathway that is generally regarded to be involved in CPT biosynthesis. As seen from the figure, except the last few steps, most other genes involved in the biosynthetic pathway are well characterised and available (Yamazaki *et al*, 2004) raising the possibility of exploring a processes for CPT production.

As a secondary metabolite, synthesis of CPT may be induced by biotic or abiotic factors. In *Camptotheca acuminata,* discs when treated with fungal elicitors and methyl jasmonate led to an increase in the expression of a gene for a key enzyme in CPT biosynthesis, *tryptophan decarboxylase* (Lopez and Nassler, 1997).

At cellular level, CPT has been localized in mesophyll and sub-palisade layers of young leaves. CPT has also been localized in vacuoles of young and older leaves (Nolte, 1999). The precise mechanism of transport and storage of CPT has not yet been fully understood. One possible mechanism of transport of such an insoluble compound is its conversion into a more water-soluble form such as glucosides *eg.* Chaboside.

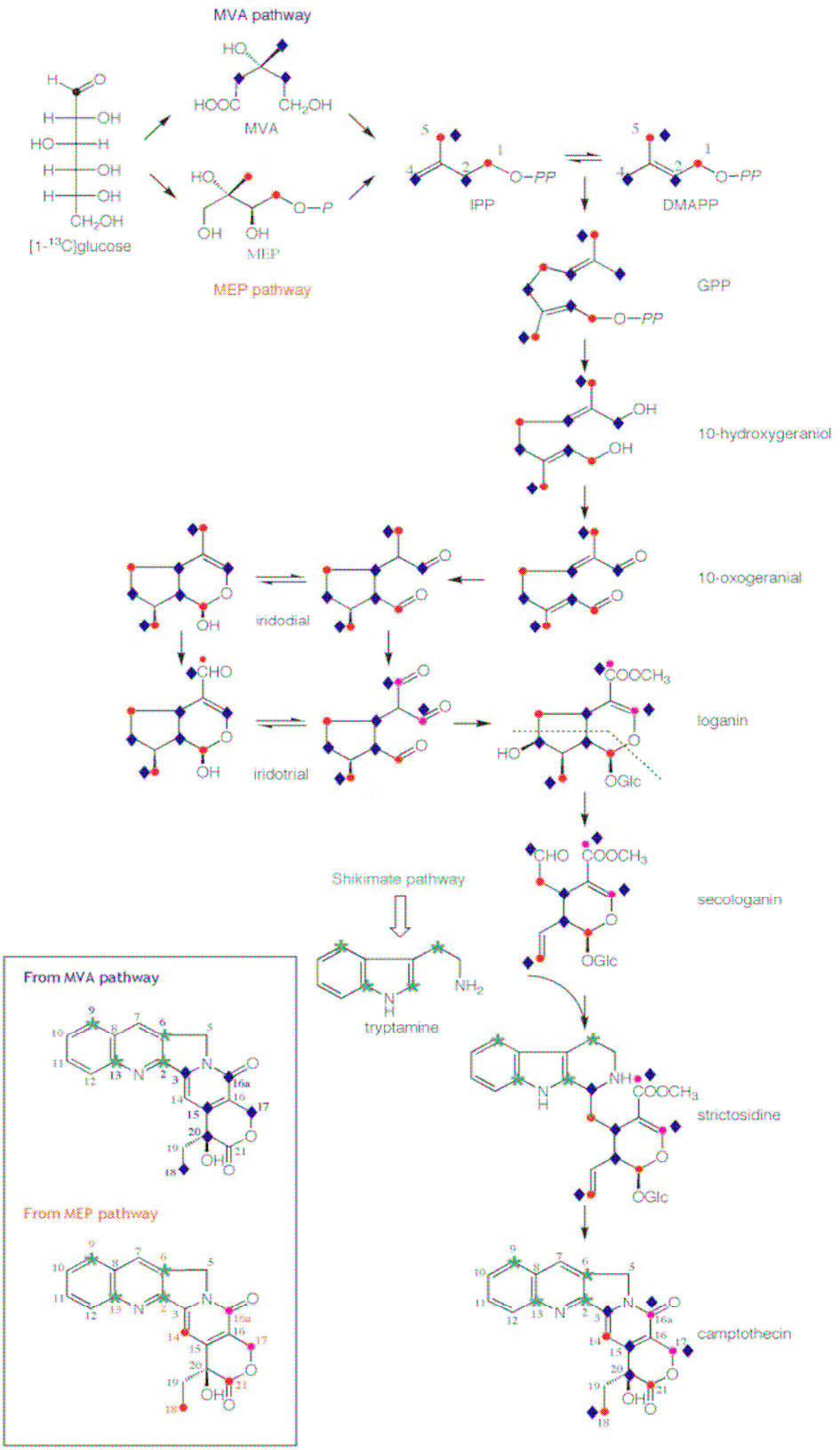


Figure 2.5: Biosynthesis of camptothecin from [1-13C] Glc (Yamazaki, Y. *et al.* 2004).

5. Demand for CPT:

There are 26 other camptothecin derived drugs, which showed promising results and are in clinical trials. Camptothecin anologs have also been demonstrated to be potent antiviral, anti-HIV agents and chemosterilants. Thus, camptothecin will have broader uses and worldwide demand of CPT has been increased (Cragg and Newman, 2004). The worldwide market of irinotecan and topotecan has currently reached one thousand million US dollars, which represents approximately one ton of CPT in terms of raw material. Despite its great demand as the only commercially available source of these medicines, CPT is still supplied exclusively from intact plants, mainly *Camptotheca acuminata* and *Nothapodytes foetida* (Icacinaceae) (Watase *et al* 2004).

Presently, CPT production relies primarily on the extracts from *Camptotheca acuminata*. Recent reports states that (comprehensive statistics of the seed company and research institutes) 1000 kg pure of CPT is traded per year. According to calculations the 1000 kg CPT comes from approximately 3,000,000 kg seeds of 750,000 happy trees (fifteen years old) or 9,000,000 kg branches or timber (come from 500,000 happy trees which are fifteen years old). Although the historical range of happytree's distribution is wide. According to the results of investigation, wild happytree populations are in few provinces of China owing to the exhaustive harvesting of trees from the wild. Recently the tree has been declared vulnerable (Prop. 11.58).

In India camptothecin is being isolated from various parts of *Nothapodytes nimmoniana* (formerly *Mappia foetida)* (Icacinaceae). CPT is mainly isolated from the stems of *N nimmoniana.*

Nothapodytes nimmoniana:

Nothapodytes nimmoniana, Graham, formerly known as *Nothapodytes foetida* Sleumer and *Mappia foetida* Meirs is a small tree belonging to the family Icacinaceae. *N. nimmoniana* commonly referred to as "Stinking Tree", is native to warmer regions of South India. The

tree is distributed in the shola forests in Nilgiris and present in both the Western Ghats and the Eastern plateau. It is also distributed in Sri Lanka, Myanmar, Indonesia and Thailand (Hombe Gowda *et al* 2002; Ku and Tang 1980; Wu *et al* 1980). The species exhibits a wide array of breeding systems including male, female, hermaphrodite, monoecious, andromonoecious, gynomonoecious and trimonoecious individuals (Hombe Gowda *et al* 2002).

***Nothapodytes nimmoniana* rich source of CPT:** *Nothapodytes nimmoniana* has gained international importance because of its pharmacologically important compound Camptothecin. It is the potential source of CPT and its derivatives than any other botanical sources known to occur in the country. The dried plant material has about 0.14-0.24% of CPT (Puri *et al* 1999). The stem bark, stem wood, root bark, root wood and leaves are significant sources of the anticancer alkaloid. Roja and Heble (1994) reported that shoots of mature trees of *M. foetida* contained 0.075% CPT and 0.013% of 9-methoxycamptothecin (9CPT). Traditionally, CPT has been extracted from root, root bark and fruits (Liu *et al* 1999). Fairly good amounts (0.10%) of these alkaloids are also found in the seeds (Puri *et al* 1999).

A recent study on different tissues for CPT content revealed that stem bark and root bark had high concentration of 0.23% and 0.29% respectively. Over all, bark tissue had significantly higher CPT than any other plant part. The leaf had lowest CPT of 0.08% (Padmanabha *et al* 2006).

Studies of Suhas *et al* 2007 provide one of the most exhaustive chemical screenings of *N. nimmoniana* for CPT. The study is perhaps the first to report at least 5 to 8 fold more CPT in *N. nimmoniana* than is hitherto reported. The study has demonstrated a significant population level variation in CPT content-a tool kit that can be exploited for developing clonally multiplied material from the identified high-yielding populations.

Threats: *Nothapodytes nimmoniana* has been exploited quite intensively from its natural populations in the Western Ghats forests. In many areas of western/south western Karnataka, the species has been very heavily extracted. The export data recorded at the Bombay port during 1994 shows the export of wood chips of this species to the tune of 54

tunes. Based on its assessed decline of more than 20 % over the last 10 years, this species has been assigned the threat status i.e. vulnerable at the regional level in south India (Ravi kumar and Ved, 2000).

6. Endophytic fungi:

Endophytes are microorganisms whose infection is generally inconspicuous, the host tissues infected are at least transiently symptomless. Microbial colonization can be demonstrated to be internal, either through histological means or by isolation from strongly surface disinfected tissue, or most recently, through direct amplification of fungal nuclear DNA from colonized plant tissue (Stone *et al.* 2000). They are quite ubiquitous and have been found in all plant species examined to date (Arnold *et al* 2000). Endophytes mediate interactions between host plants and their competitors, herbivores and pathogens (Carroll 1988; Clay 1990; Clay and Holah 1999).

In addition, fungal endophytes have been recognized as a repository of novel secondary metabolites. Some of which have beneficial biological activities (Bills and Polishook 1991; Strobel and Daisy 2003). A recent comprehensive study has indicated that 51% of bioactive substances isolated from endophytic fungi were previously unknown (Schutz 2001). Out of many bioactive molecules, paclitaxel and some of its derivatives represent the first major group of anticancer agents that are produced by endophytes. This compound is the world's first billion-dollar anticancer drug and is used in the treatment of several human cancers**.** Another molecule camptothecin **(**CPT), a monoterpenoid alkaloid found in *Nothapodytes nimmoniana* is one of the most sought after compounds for treatment against certain forms of cancer. Recently, Puri *et al* 2005 have isolated an endophytic fungus which produces CPT from *Nothapodytes nimmoniana.* Hence, the endophytic fungi are expected to be a potential source for new natural bioactive products.

7. SSR markers:

In recent years Microsatellite markers or simple sequence repeats (SSRs) have become a popular tool for genetic mapping (Weissenbach et. al., 1992), and analysis of paternity, and gene flow (Chase et. al., 1996). SSR's contain tandem repeats of simple motif sequences

distributed throughout the genomes and are capable of detecting a large number of alleles with high reproducibility (Litt and Luty 1989; Tautz 1989). These nucleotide repeats are often flanked by unique sequences which are conserved and hence it is possible that SSR primer developed for one species can be used to detect polymorphism at "homologous" loci of a related species. The high degree of polymorphism and co-dominance of microsatellites make them extremely informative (Beckman and Weber 1992). Hence they are markers of choice for 'DNA fingerprinting' applications and have been used to investigate questions relating to effective population size, population structure, migration and colonization rates and mating systems (Luikart and England 1999; Ouborg *et al* 1999; Sunnucks 2000; Morgan and Conner 2001; Clauss *et al* 2002).

Microsatellites, markers appear to be hyper variable, in addition to which their co-dominance and reproducibility make them ideal for genome mapping, as well as for population genetic studies (Dayanandan *et al*, 1998). Inter-SSRs are a variant of the RAPD technique, although the higher annealing temperatures probably mean that they are more rigorous than RAPDs. Chloroplast microsatellites (cpSSRs), are similar to nuclear microsatellites but the repeat is usually only 1 bp $(T)_n$.

Microsatellite variation results from differences in the number of repeat units. These differences are thought to be caused by errors in DNA replication (Moxon and Willis, 1999; Jarne and Lagoda, 1996). The DNA polymerase "slips" when copying the repeat region, changing the number of repeats (Jarne and Lagoda, 1996). Larger changes in repeat number are thought to be the result of processes such as unequal crossing over (Strand *et al*, 1993). Such differences are detected on polyacrylamide gels, where repeat lengths migrate different distances according to their sizes. Microsatellites, which detect variation at individual loci, have been thought of as the "new allozymes". Consequently much of their use has been in studies where allozymes have been used, *e.g.* diversity studies (Rosetto *et al*, 1999), gene flow and mating (Chase *et al*, 1996) systems and paternity analysis.

As with AFLPs, the great advantage of microsatellite analysis is the large number of polymorphisms that the method reveals. One locus in soybean (*Glycine max*) is reported to

have 26 alleles (Cregan *et al*, 1994). Furthermore, the ability of the method to differentiate individuals when a combination of loci is examined makes the technique very useful for gene-flow experiments, cultivar identification and paternity analyses (Hokanson *et al*, 1998). Since microsatellites only survey one locus at a time, they are not directly comparable to AFLPs. Comparisons that include microsatellites with other single loci markers, such as RFLPs and isozymes. For example, Rossetto *et al.* (1999) found that observed heterozygosity (H_o) for *Melaleuca alternifolia* microsatellites to be 0.724, much higher than the value for allozymes (H_o = 0.154). McCouch *et al* (1997) compared the number of alleles revealed by RFLPs and microsatellite loci in rice (*Oryza* sp.) and found 2-25 alleles per microsatellite loci compared with 2-4 alleles per RFLP loci, illustrating the large number of polymorphisms potentially highlighted by microsatellites.

Unlike AFLPs, microsatellites are co-dominant markers, thus heterozygotes can be readily identified. Microsatellite co-dominance will increase the efficiency and accuracy of population genetic measures. Furthermore, the identity of heterozygotes in the F_1 generation makes gene flow, hybridisation and paternity analyses simpler (Schlotterer and Pemberton, 1994). Since the method is DNA-based, this brings advantages, such as high-throughput and the ability to use dried leaf material. In comparison with allozymes, SSRs are thought to be selectively neutral is one of the assumptions of using markers in many analyses, which though not essential for phylogenetic studies.

MATERIAL AND METHODS

III MATERIALS AND METHODS

In this chapter, we present the materials and methods pertaining to **1)** Chemical profiling of different populations of *Nothapodytes nimmoniana* for camptothecin and its related alkaloids and **2)** Molecular characterization of populations of *Nothapodytes nimmoniana* in the Central Western Ghats, India using SSR markers **3)** Prospecting phylogenetically related genera/species of *Nothapodytes nimmoniana* for camptothecin and **4)** Prospecting endophytic fungi from *Nothapodytes nimmoniana* for camptothecin.

1. Chemical profiling of populations of *Nothapodytes nimmoniana* for CPT and its related alkaloids.

Nothapodytes nimmoniana: Ecology and distribution

Nothapodytes nimmoniana Graham formerly known as *Nothapodytes foetida* Sleumer and *Mappia foetida* Meirs is a small tree belonging to the family Icacinaceae (Figure 3.1). The genus *Nothapodytes* includes *N. obtusifolia* distributed in China, *N. montana* distributed in Thailand, north eastern Sumatra, western Java, western Sumbava and *N. pittosporoides,* distributed in China and Indonesia. It is also reported in Taiwan. *N. nimmoniana* commonly referred to as "Stinking Tree", is native to warmer regions of South India. It is reported in the western parts of Deccan peninsula, North Bengal and Assam. The tree is distributed in the shola forests in Nilgiris and present in both the Western Ghats and the Eastern plateau. It is also distributed in Sri Lanka, Myanmar, Indonesia and Thailand (Hombe gowda *et al,* 2002).

The species exhibits a wide array of breeding systems including male, female, hermaphrodite, monoecious, andromonoecious, gynomonoecious and trimonoecious individuals (Hombe gowda *et al,* 2002). The trees flower during July-August and most of the early flowering trees are dioecious, whereas late flowering trees are monoecious, hermaphrodite and a mixture of other breeding types (Hombe gowda *et al,* 2002) (Figure 3.1). The fruits ripe during November-December and germinate during May-June after the onset of monsoon rainfall (Figure 3.1).

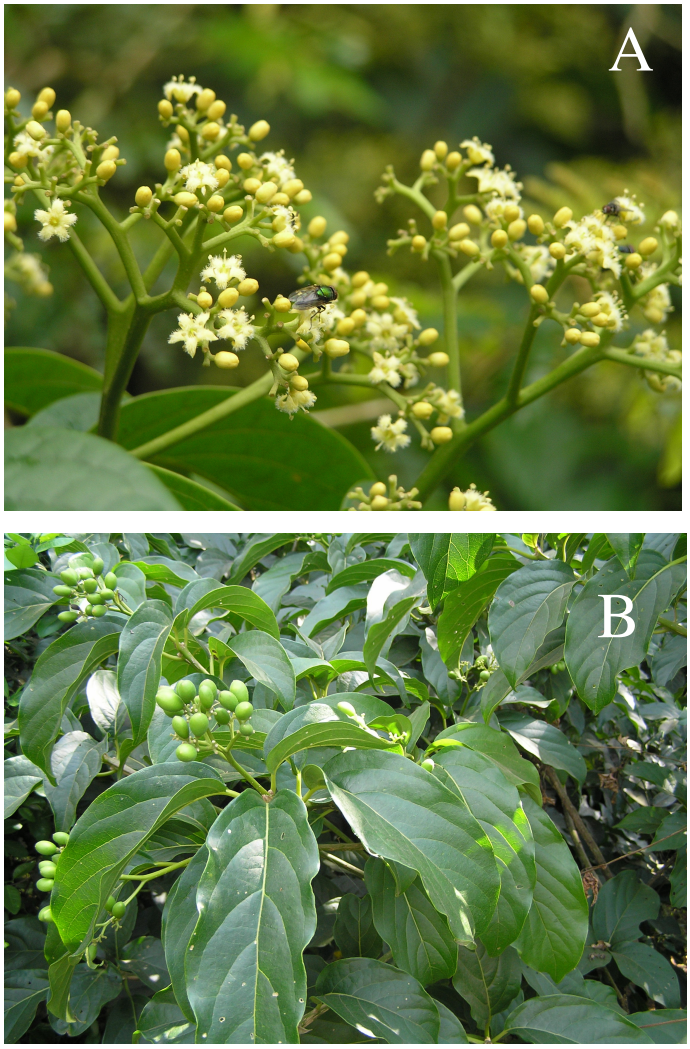


Figure 3.1*:* *Nothapodytes nimmoniana* tree showing A) tree under profuse flowering in October 2007 and pollinated by flies that are attracted to foetid smell. B) tree with immature fruits C) tree with mature fruits. (Photo credits: Dr. G Ravikanth).

Distribution of *Nothapodytes nimmoniana* in Western Ghats, India.

Nothapodytes nimmoniana is distributed all along the Western Ghats. The distribution is dense in northern parts of Western Ghats comprising the regions of Amboli in Maharastra and parts of Sirsi in Northern Karnataka. The distribution is sparse in southern parts of Western Ghats in Kerala; populations were located in only 3-4 regions such as Mananthwady, Periyar Tiger Reserve (PTR) and Kuttikanam. There was nearly an even distribution of the species in southern parts of Karnataka comprising parts of Hassan, Agumbe, BR Hills and Kemmanagundy etc. The species was not recorded in the region between Mananthawady and Periyar Tiger Reserve and in parts of Tamilnadu as far as the primary data sets were considered (Figure 3.2).

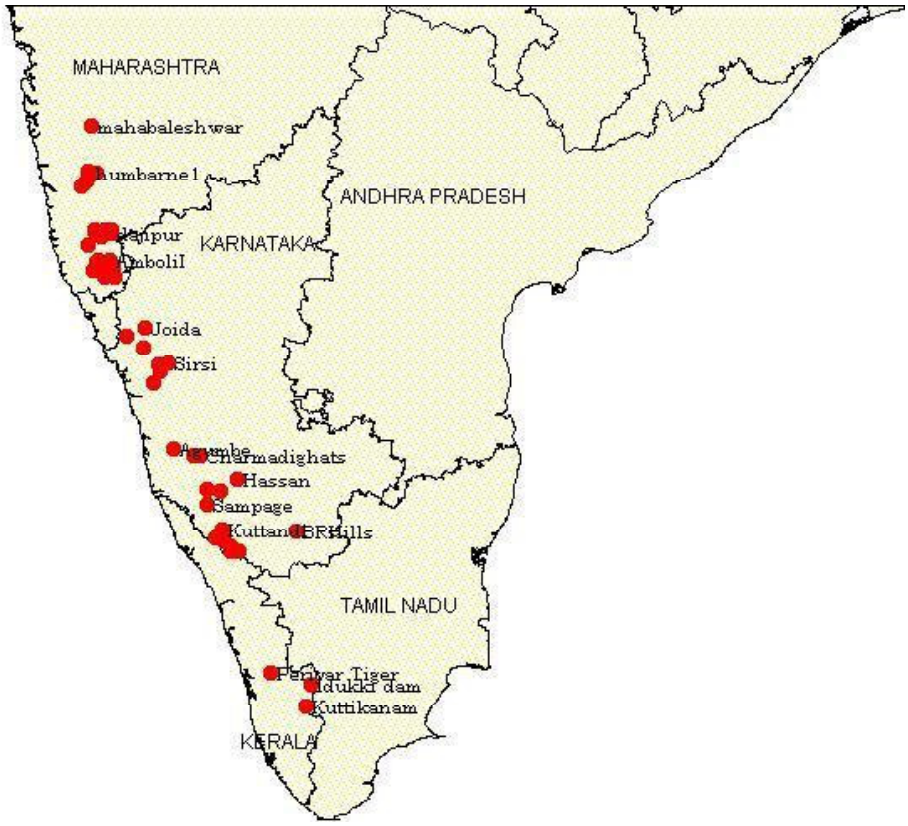


Figure 3.2: Map showing the distribution of *Nothapodytes nimmoniana* in Western Ghats of India. Map was developed based on primary points of occurrences (n=64 records) of *N. nimmoniana*.

3.1.1 Study sites and Sample collection

The samples were drawn from an earlier collection of 147 trees that were chemically profiled for camptothecin (Suhas *et al,* 2007). These trees were sampled from 11 populations along the Western Ghats (from 8^0 N to 15^0 N latitude), one of the three-megadiversity hotspots in India. Twenty-three of the trees from 4 populations were found to have CPT in excess of 1 per cent either in their stem or root barks. These individuals were sampled again and subjected for consistency analysis as well as detailed chemical profiling using LC-MS and LC-MS/MS chromatographic techniques to identify new Camptothecines if any.

We chose 17 of these 23 trees (9 for which highest CPT was reported from stem bark and 8 from root bark) for the HPLC/LC-MS quantification of camptothecin as well as in detecting other camptothecines in these tissues. 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 scrapped using a knife and a section of the inner bark (5cmX5cm) was collected into a plastic bag and sealed. Similarly for the root bark samples, exposed (surface) roots were scrapped and the inner bark was collected into a separate plastic bag and sealed.

3.1.2 Extraction of Camptothecines

CPT was extracted using the following two protocols and was further subjected for HPLC and LC-MS/MS analysis.

61% ethanol extraction: All samples were dried to constant moisture content at 60^0C for 96 hours in a hot air oven. The dried samples were ground to fine powder using a pestle and mortar. 0.1 g of fine tissue powder of each of the samples was extracted in 10mL of 61% ethanol at 60^0C for 90 min in a shaking water bath (Padmanabha *et al,* 2005). After cooling to room temperature, 1mL of the extract was centrifuged at 10,000 rpm for 10 min at 10^0C. The

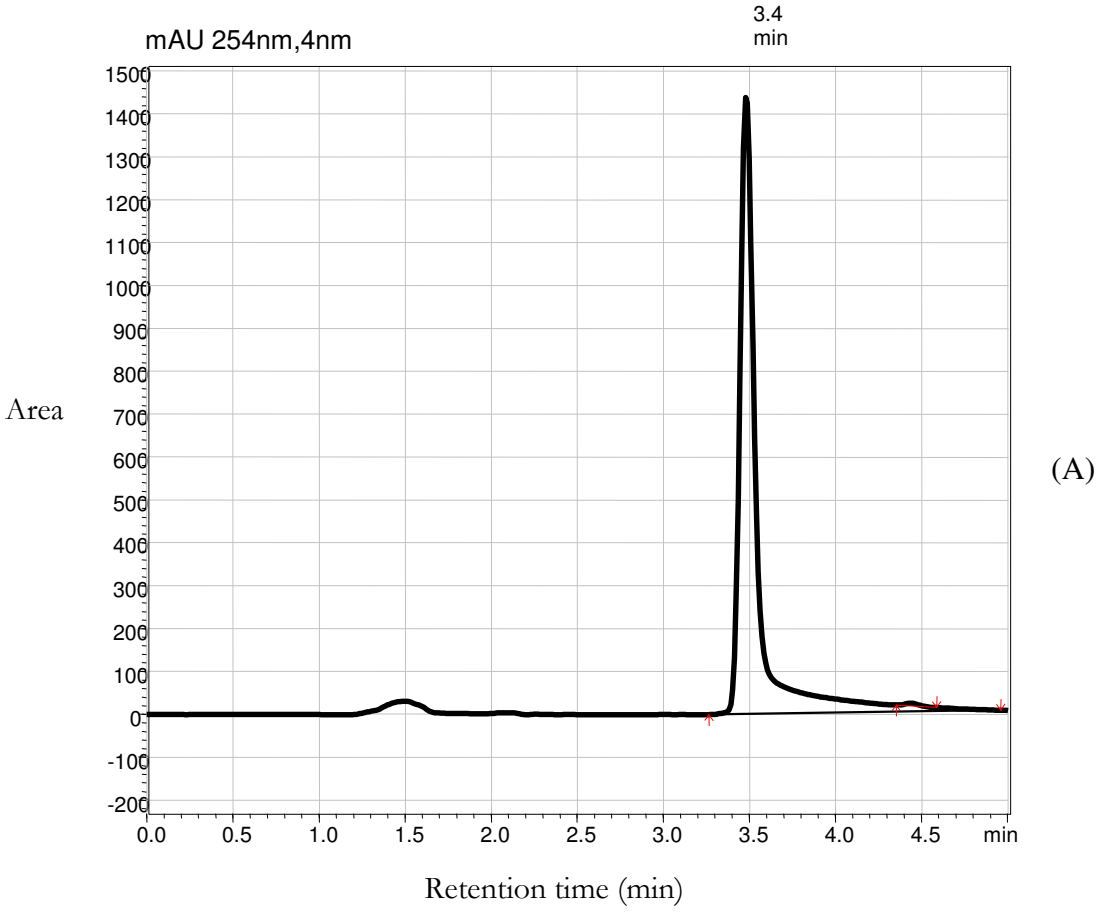
supernatant was passed through 0.2μm filter (Tarsons, India) and analyzed for CPT content using a HPLC. CPT accumulation was determined for different tissues.

Methanol extraction: 100 mg of the dried plant material (6-8% moisture contents) was extracted in a centrifuge tube with 2ml of methanol by sonication (2x30 sec). The mixture was mixed with 18 ml water and 20 ml dichloromethane and the material was stirred vigorously for 5 min on a magnetic 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 chloroform: methanol (3:1) mixture and filtered through 0.2 μm filter and analyzed by LC-MS and LC-MS/MS.

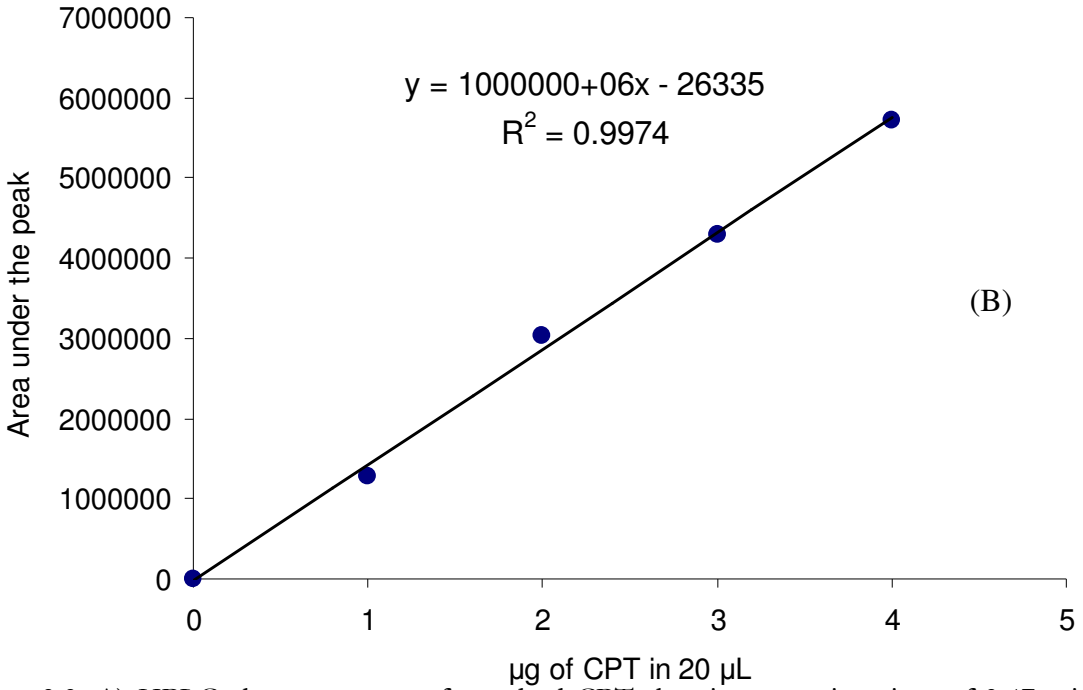
3.1.3 HPLC analysis

CPT was analyzed by reverse phase HPLC (LC-10AS, Shimadzu, Japan) on a C18 column (250x4.6mm, 5μm). The HPLC conditions were: 254 nm as the detector wavelength, 1.6mL/min flow rate and 10μL sample loop. The mobile phase was adjusted as follows: 40% acetonitrile and 60% water+0.1%Trifluro-acetic acid (TFA) in an isocratic mode (Yan *et al,* 2003). CPT (Sigma, 95% HPLC purified) standard was procured from Sigma Chemicals. The standard CPT was prepared using DMSO and methanol in 1:3 (v/v) ratio respectively.

The retention time of CPT was 3.4 min. For every five runs, the HPLC was re-standardized using the CPT standard. On an average, the coefficient of variation for the peak area for five consecutive runs of standard CPT was 0.55 per cent. Standard curve was developed by injecting following concentrations of standard CPT: 0.05mg/mL, 0.1 mg/mL, 0.15 mg/mL, 0.20 mg/mL and to 0.25mg/mL and best fit was obtained (R^2=0.99). By using the (y = 1000000+06x - 26335) equation of the standard curve amount of CPT in the sample was calculated (Figure 3.3). The data were subjected to relevant statistical treatment using the Statistica version 4.0 software package (Statsoft 1993).



(A)



(B)

Figure 3.3: A) HPLC chromatogram of standard CPT showing retention time of 3.47 min. B) Standard curve of standard CPT developed based on 0.05mg/mL (1µg in 20 µL of injection volume), 0.1 mg/mL (2µg in 20 µL of injection volume), 0.15 mg/mL (3µg in 20 µL of injection volume) and 0.20 mg/mL (4µg in 20 µL of injection volume).

3.1.4 LC-MS analysis

For HPLC-MS analysis, the same HPLC which was used for HPLC analysis was coupled to Ion Trap of camptothecin mass spectrometer (Enquire- 3000) from Bremen (Germany). The mass spectrometer was equipped with an atmospheric pressure ionization (API) 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 of 35 psi and drying gas temperature was set at 320^{o}C. The mass range was from 50-700m/z, ICC target value 8000, while the maximum accumulation time was 200ms.

By applying these conditions in the positive mode of ESI-MS, camptothecin exhibited a molecular adduct $(M+H)^+$ at m/z 349.1 (Figure 3.5). 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 quantification during the LC-MS studies, as this was the most intense peak in the spectrum.

Quantification of CPT was carried out using selective ion monitoring (SIM) detection of the molecule at m/z 349 $[M+H]^+$ (Figure 3.4 and 3.5). Quantification 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 1μg to 10μg each time before sample analysis. Linear calibration curve of CPT with in the concentration range of 1μg to 10μg (R^2 = curve co-efficient 0.999) was obtained (Figure 3.8). Validation of the method was carried out by spiking 10μg of standard camptothecin to 10mg 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 qualitative and quantitative analysis of camptothecin was carried out by LC-MS.

3.1.5 LC-MS scan analysis for new Camptothecines

The other minor camptothecinoids were identified on the basis of MS and mass fragmentation peaks. A number of molecular compounds having different retention times (Rt) but same molecular weight were identified. These compounds are isomeric entities of

camptothecinoids. Besides the 8 minor camptothecinoids detected in the accessions of *N nimmoniana* and there were number of other constituents that could not be identified only by LC-MS.

3.1.6 LC-MS/MS analysis of plant samples

Plant samples were also subjected for LC-MS/MS analysis based on MRM mode (Multi Reaction Monitoring). Analysis was done on Waters Quattro Premier Micromass system. The assay uses a reversed phase C18 HPLC column (150x2.1 mm, 5um) and mobile phase consisting of acetonitrile/water (60/40 v/v) containing 0.1% formic acid pumped at a flow rate of 0.3 mL/min. High quality argon gas was used for collision and nitrogen gas for desolvation. The conditions for mass spectrum analysis during the LC-MS/MS studies were set at a capillary voltage of 2.99 kV, cone at 18 V, source temperature at 100^{o}C, desolvation temperature at 300 ^{o}C and collision temperature at 25 ^{o}C. All the interface parameters were optimized by injecting standard solution of camptothecin during the LC-MS/MS experiments. Sample introduction and ionization was electrospray ionization in the positive ion mode. The MS was operated in the MRM mode.

Mass tuning was done at three transitions using MRM mode. The molecular ion peak at m/z 349 $[M + H]^{+}$ was taken for MS/MS studies. This parent ion upon ionization and fragmentation exhibited different daughter ions as follows: Parent 1 > daughter 1 349 > 305, Parent 2 > daughter 2 349 > 317, Parent 3 > daughter 3 349 > 262 (Figure 3.6 and 3.7). Using this technique both parent (molecular mass), and its daughter ions were monitored simultaneously (product ion) in a MRM mode. Among the daughter ions monitored the one with best intensity transition was used for quantification and rest for confirmation. The daughter ion peak at m/z 305 was taken up for quantification (Figure 3.7). Quantification of CPT in the fungal extracts from different isolates was done on the basis of the calibration curves established by injecting six concentrations of the CPT standard in the concentration range of 5 to 5000ppm. Linear calibration curve of 305 ion of CPT with in the concentration range of 50 to 5000ppm (R^2 = curve co-efficient 0.982) was obtained. Validation of the method was carried out by spiking 5ug of standard camptothecin to 100ppm of the plant extract and the recovery was within the range of 93.8 to 102.5%.

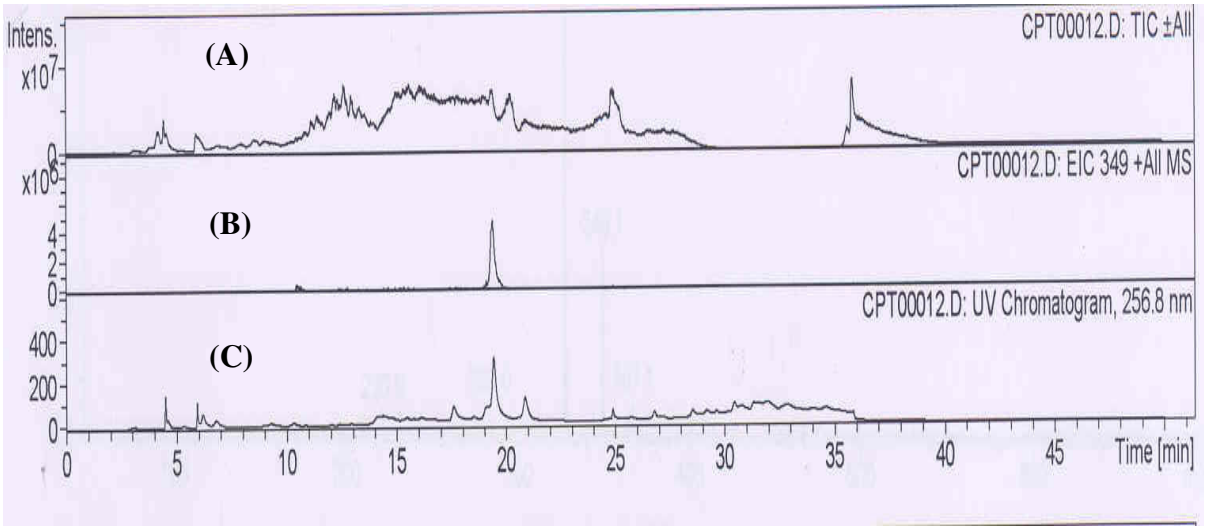


Figure 3.4**:** Total Ion Current (A) Trace, Extracted Ion Current (B) trace and UV-DAD chromatogram(C) of CPT in plant sample.

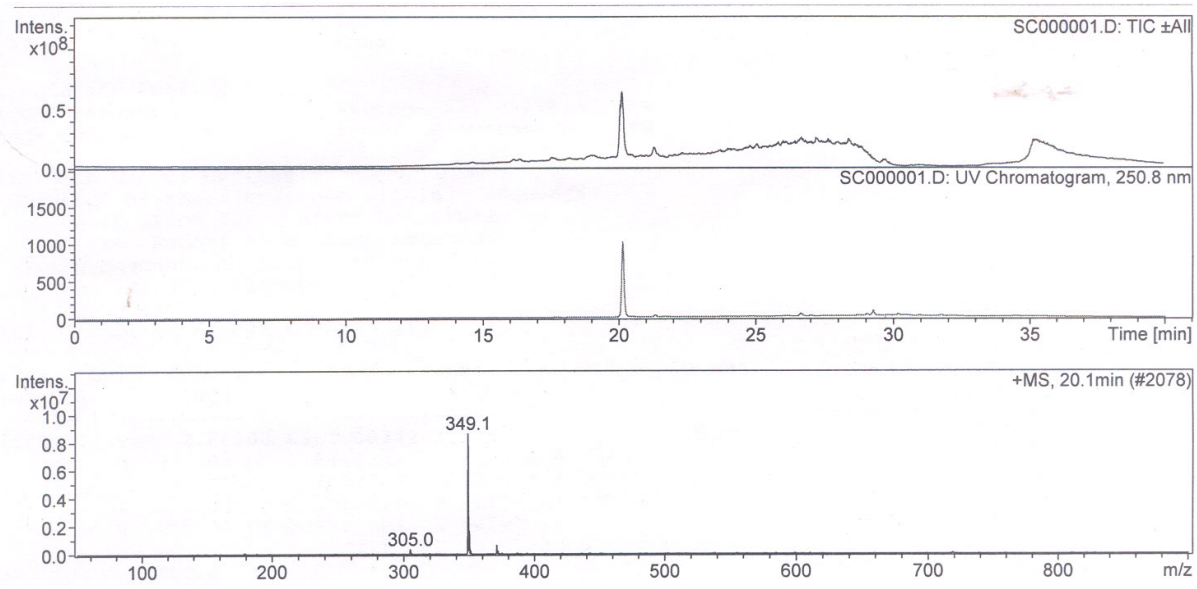


Figure 3.5: TIC, UV-DAD chromatogram and mass spectra of CPT.

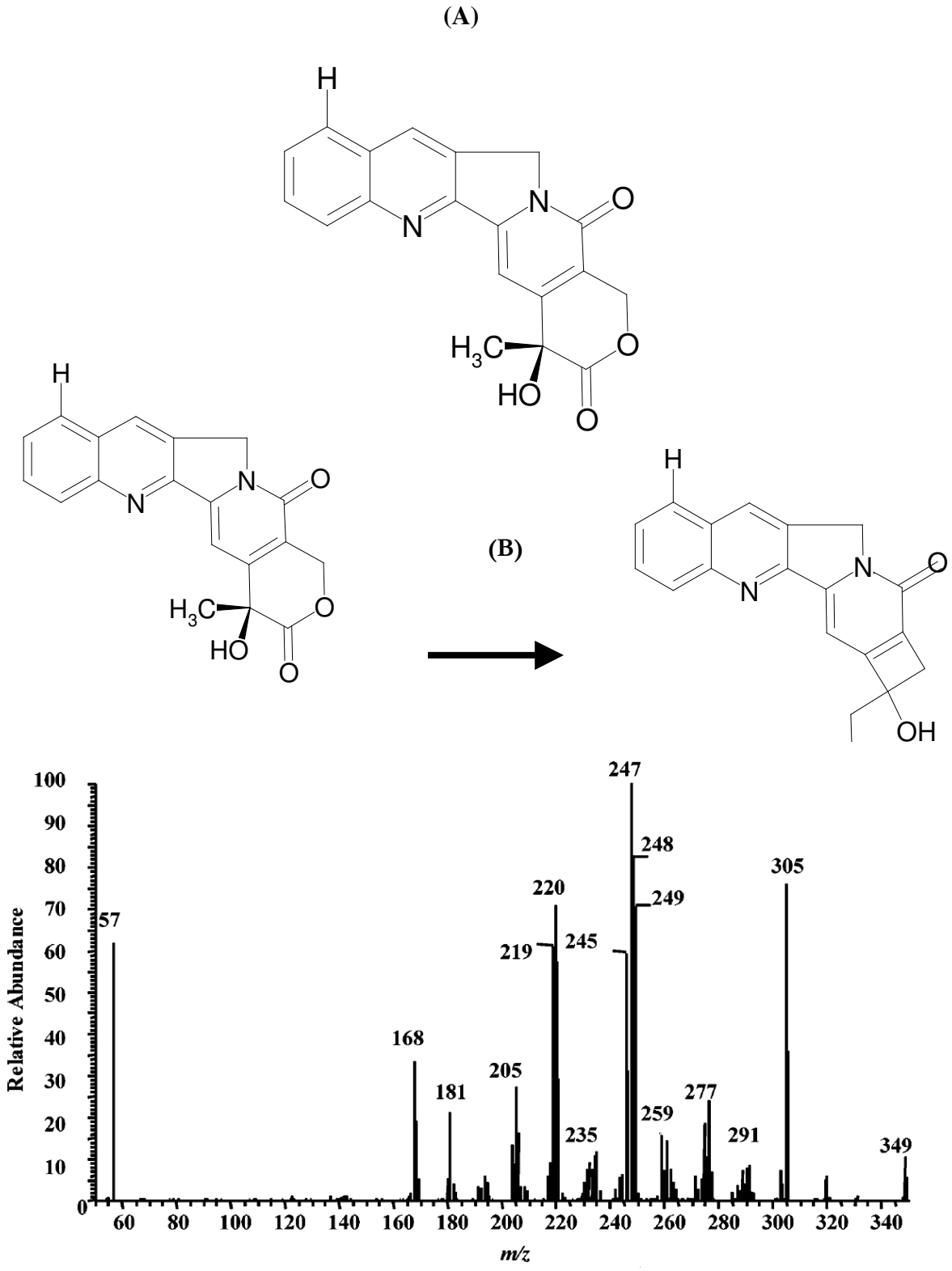


Figure 3.6: A: Structure of Camptothecin and a Schematic diagram showing LC-ESI-MS/MS fragmentation pattern of Camptothecin. **B:** ESI-MS/MS of Standard Camptothecin: the most abundant ion 305 was considered for quantification of Camptothecin in plants samples.

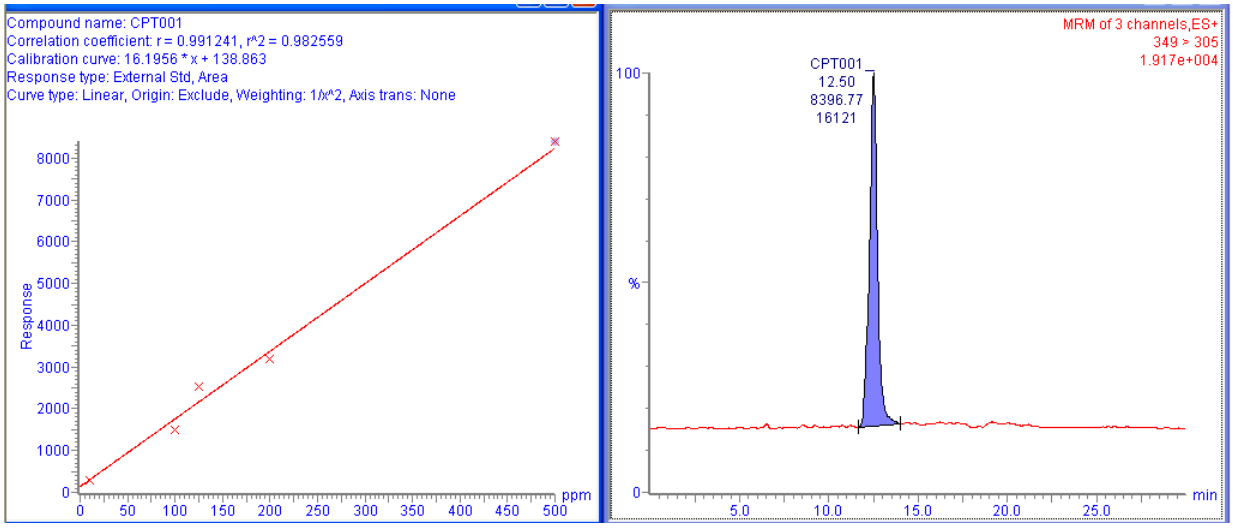


Figure **3.7**: ESI-MS/MS chromatogram of 305 ion peak of Camptothecin at 12.50 min retention time and standard curve developed based on the 305 ion.

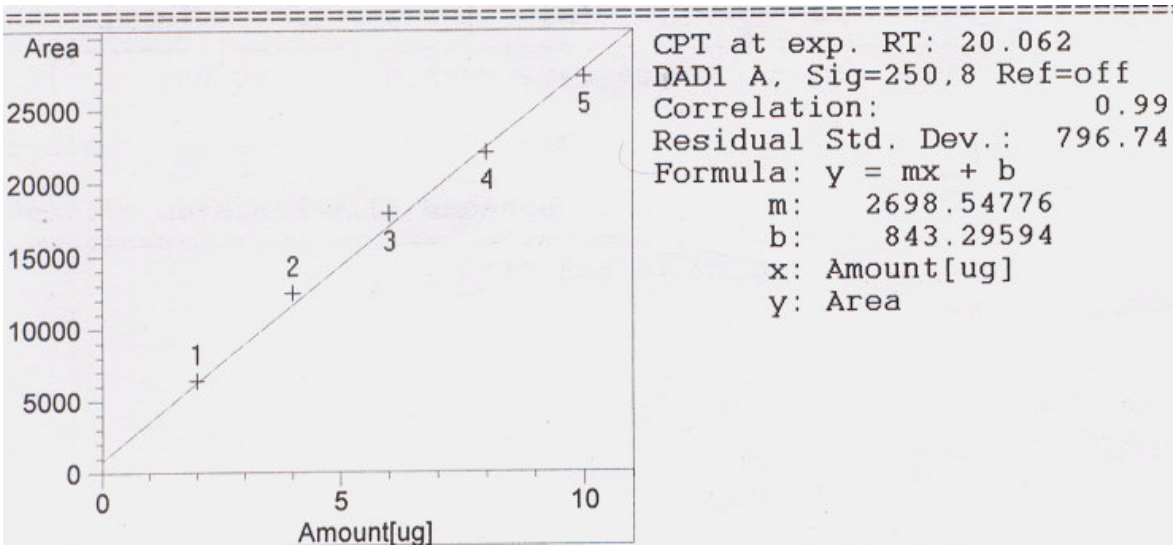


Figure **3.8**: LC-MS standard curve for Camptothecin.

3.1.7 Ecological niche modeling and chemical data: Analysis of the CPT content of the different populations was also done based on the habitat suitability predicted by the ecological niche model. Accordingly, sites were categorized as unsuitable, poorly suitable, moderately suitable and highly suitable and the mean percent CPT computed. Individuals with more than 1% CPT by dry weight were reclassified into different habitat suitability categories and number of individuals with more than 1% CPT in different categories were compared.

3.1.8 Development of Sustainable harvesting protocol for Camptothecin from *Nothapodytes nimmoniana*

An effort was made to develop strategies for sustainable harvesting of *N. nimmoniana* for an important metabolite camptothecin. The study has also been designed to study the amount of biomass production, CPT yield as well as on the CPT concentration at different stages of growth of *N. nimmoniana.*

The study was carried out in the seedlings, saplings and adult trees of *N. nimmoniana.* The seedlings were raised from seeds obtained from 10 to 15 years old trees of *N. nimmoniana* from Sirsi forest and Biligiri Rangaswamy Temple Wildlife Sanctuary (BR Hills). Seedlings were grown in a polyhouse and after one year, seedlings were removed from the polythene bags and observations on biomass and camptothecin (CPT) content were recorded. The saplings, which were 5 years old, were also collected for CPT estimation. Similarly leaves from coppice were also collected and CPT was estimated. Leaf samples were collected from 123 individuals of one and half year old seedlings, 29 individuals of three to four year old coppices, 22 individuals of five year old saplings and 30 individuals of 10 to 15 years old adult trees.

3.2 Molecular characterization of *Nothapodytes nimmoniana* populations using SSR markers.

3.2.1 Development of SSR markers for *Nothapodytes nimmoniana*

Microsatellite markers were developed using the selective hybridization technique (Figure 3.9 Zane *et al* 2000).

Isolation of genomic DNA from *Nothapodytes nimmoniana:* Mature leaf samples of *Nothapodytes nimmoniana* were collected and stored at 4^0 C until DNA was extracted. Good quality genomic DNA was isolated using C-TAB method as described below (Doyle and Doyle 1980).

I. 200 mg of leaf tissue was ground to fine powder using liquid nitrogen, in a sterile mortar and pestle. After mixing the powder with approximately 70 mg PVPP, the powder was transferred into the centrifuge eppendorff tube containing the 1.0 ml of extraction buffer (20 mM Na EDTA in 100 mM Tris HCI: pH 8.0) and 1 percent β-mercaptoethanol.
II. The contents were thoroughly mixed by inverting the tubes several times and incubated at 60^0 C for one hour with intermittent shaking. After incubation, it was cooled to room temperature and 0.5 ml of Chloroform: Isoamyl alcohol (24:1 v/v) was added and mixed gently by inverting the tubes 20-25 times until it formed an emulsion.
III. The mixture was centrifuged at 8000 rpm for 15 minutes and the clear aqueous phase was transferred to a new sterile tube. Centrifugation was repeated twice by adding Chloroform: Isoamyl alcohol (24:1 v/v) amounting to 1/7th volume of the supernatant.
IV. Finally, one volume of chilled isopropanaol was added and mixed gently and kept overnight for incubation at -40^0 C. After the incubation, it was subjected to centrifugation at 8000 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet was washed with 76 % ethanol and centrifuged at 5000 rpm for 5 minutes. The wash was repeated once again to completely remove the salts and polysaccharides. The supernatant was then drained out and only the pellet was retained. Ethanol was completely removed by leaving the tubes uncovered at 37^0 C for 30-40 minutes.
V. After drying, the pellet was re-suspended in 200μL of Tris EDTA buffer and treated with RNAase (5mg/ml).
VI. DNA was quantified using Nandrop spectrophotometer and accordingly working concentration of DNA was prepared by diluting the stock DNA to 20ng/uL.

Purification of DNA: Since the DNA preparations obtained by the above method contained considerable amounts of RNA, polysaccharides, phenolics and other impurities; the extract was subjected to further purification (Doyle and Doyle 1980). To the DNA samples, one tenth of the volume of 3M sodium acetate of pH 5.5 was added and mixed gently. Twice the volume of cold ethanol was added to it, mixed gently and kept on ice for further precipitation for about 2 hrs.

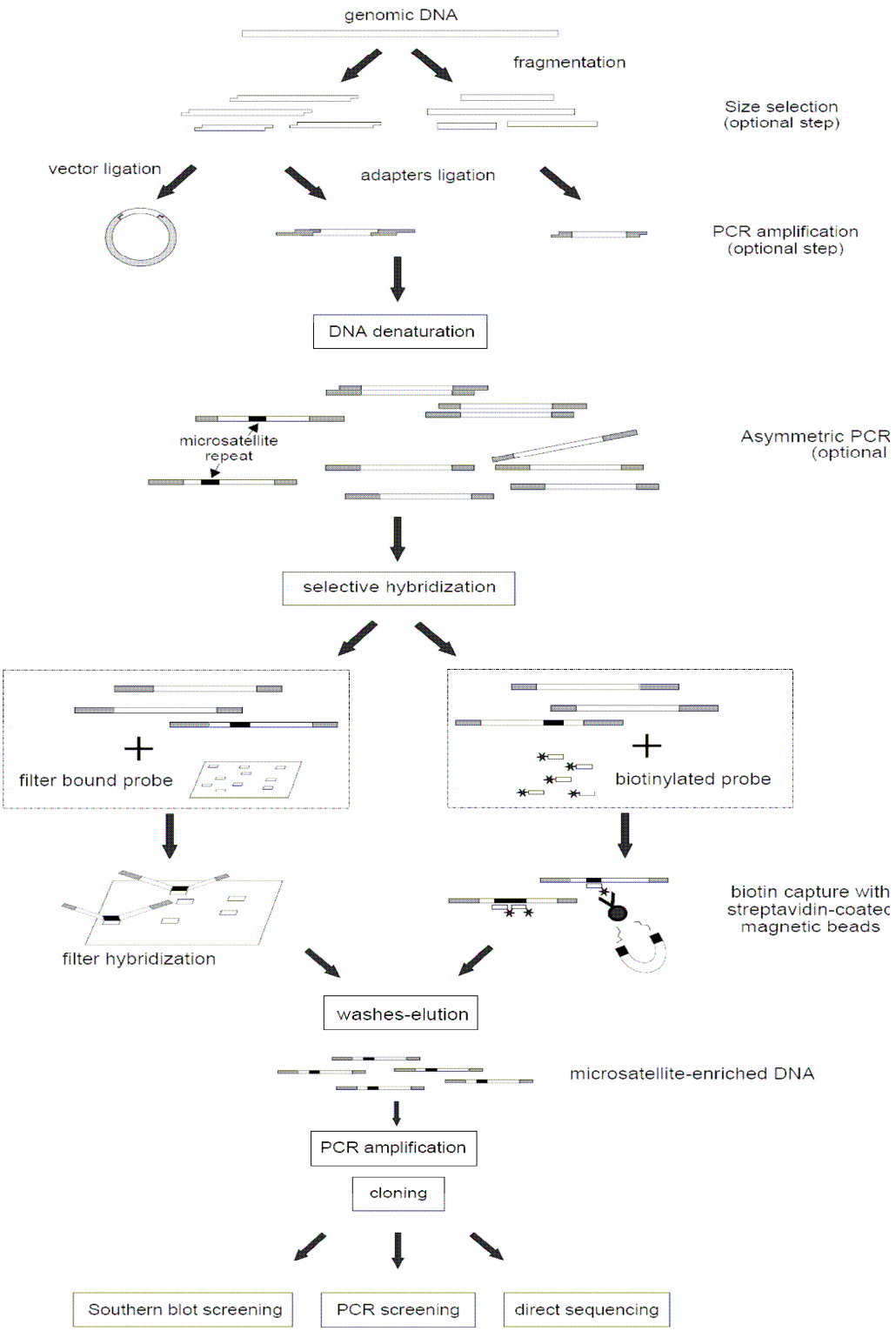


Figure 3.9: Selective hybridization protocol for microsatellite isolation. In the present study biotinylated repeat probes were used and captured using streptavidin coated magnetic beads (Schematic diagram adapted from Zane *et al* 2002).

This was centrifuged at 6000 rpm for 5 minutes to pellet out the DNA. It was washed with 70 % ethanol and the traces of ethanol were removed by air-drying for one to two hours. The pelleted DNA was re-dissolved in 100µl of sterile TE buffer, treated with 10µl RNAse and incubated at 35^0 C for 30 minutes. For longer use, it was stored at -20^0 C after ethanol precipitation.

Quantification of Genomic DNA: The method adopted for the quantification of DNA is based on the spectrophotometric measurement of UV absorbance at 260 nm in Nanodrop instrument. The ratio of OD 260 to OD 280 provides information about the purity of DNA sample, DNA samples with a ratio between 1.8 to 2.0 was chosen for further analysis.

Restriction digestion: About 2-3µg of good and intact genomic DNA was used in restriction digestion. Digestion mix was made by mixing the components as given in the protocol and the mix were incubated in a 37°C water bath for 30-60 min (Travis Glen, 2000).

Ligating Linkers to DNA Fragments: Following linkers (called 'SuperSNX') were ligated onto each end of each DNA fragment that provides the primer-binding site for subsequent PCR steps. The SuperSNX linkers also incorporates a GTTT "pig-tail" that facilitates non-template A addition by Taq DNA polymerase during PCR which then can be used for TA cloning (Travis Glen, 2000).

SuperSNX24 Forward: 5'GTTTAAGGCCTAGCTAGCAGAATC

SuperSNX24+4P Reverse: 5'pGATTCTGCTAGCTAGGCCTTAAACAAAA

Double stranded (ds) SuperSNX linkers were prepared by mixing equal volumes of SuperSNX24 and SuperSNX24+4p primers to get a final concentration of 10 µM each. NaCl was added to the solution to a final concentration of 100 mM. The resultant solution was heated to 95°C and was allowed to cool slowly to room temperature. The following ligation mix was added to 10µL of cut DNAs and the mixture was incubated at room temperature for 2 hrs or ideally at 16°C overnight. The ligation mixture consisted of the following components: 10.0µL reaction consists of 7.0µL ds SuperSNX linkers, 1.0µL 10x Ligase Buffer and 2.0µL DNA ligase (400 units/µL).

To ensure that the ligation is working, a PCR of the linker ligation was performed using SuperSNX-24 F as primer with 2.0 µL Linker ligated DNA fragments as template. The PCR

reaction mixture contained 2.5 µL of 10x PCR buffer, 2.5 µL BSA (250 µg/mL), 1.5 µL dNTP's (150 µM final), 2.0 µL $MgCl_2$ (2.0 mM final) and 0.2 µL Taq DNA Polymerase (5 units/µL) to a final volume of 25µL. PCR cyclic conditions were set as follows: 95°C for 2 min.; then, 20 cycles of 95°C for 20 sec., 60°C for 20 sec., 72°C for 1.5 min.

Dynabead Enrichment for Microsatellite-containing DNA Fragments

DNA fragments obtained in the previous step were subjected for hybridization with biotinylated oligos (microsatellite probes) and these hybridized fragments were captured using the magnetic dynabeads in a magnetic particle-collecting unit (Travis Glen *et al,* 2006). Hybridization reaction was carried out in a thermal with the cylicling conditions as given in the Travis Glen 2000.

50µL of Dynabeads were taken and resuspended in their original tube containing 250 µL of TE. Beads were captured using the Magnetic Particle Collecting (MPC) unit. The process repeated with TE and twice with 1xHyb Solution and finally resuspended in 150 µL of 1xHyb Solution. DNA+probe (hybridization mix) mix was added to the 150µL of washed, Dynabeads (i.e., to the 1.5mL tube). The mix was then incubated on a rotator at room temperature for 30 min and beads were captured using the MPC unit. The Supernatant obtained was removed by pipetting. Dynabeads that stuck to one end of the tube were washed two times with 400 µL 2xSSC, 0.1% SDS and two additional times using 400µL 1x SSC, 0.1% SDS at 45 or 50°C. In the final capture 200µL of TLE was added, vortexed, incubated at 95°C for 5 minutes and supernatant was quickly removed. To the resultant supernatant, 22 µL of 3 M NaOAc was added and mixed by flicking. The pellet thus obtained, was washed with 444 µL of 95% ethanol and was resuspended in 25 µL of TLE. Pure gold DNA was obtained by subjecting it for one round of amplification with SuperSNX-24F as primer.

Transformation of plasmid DNA: Using Fermentas TA cloning Kit (MBI, Fermentas), pure gold DNA was incorporated into a multiple priming site of pTZ57R plasmid (NEB: MBI Fermentas) vector. The transformed plates were left overnight at 37°C and the next day plates were scanned for blue and white colonies.

Colony PCR: Using sterile toothpicks, distinct white colonies were picked and added into a PCR tube containing master mix (25µL reaction contained 2.50 µL of 250µg/mL BSA, 2.50 µL

of 10X PCR reaction buffer, 0.625 μL (10 μM) M13 forward primer, 0.625 μL (10μM) M13 reverse primer, 1.50 μL 25 mM $MgCl_2$, 1.50 μL of 2.5mM dNTP's (2.5 mM each), 0.10 μL Taq DNA Polymerase and remaining volume was made up with dH_2O). The cyclic conditions were set at 95° for 3 minutes of initial denaturation followed by 35 cycles of 95° for 20 seconds, 50° for 20 seconds and 72° for 1 minute and 30 seconds. The PCR products were electrophoresed on a 1% agarose gel along with a 100bp ladder. The PCR products with different sizes ranging from 500-1000 bp were selected and further purified using eppendorfs DNA cleaning kit.

Sequencing of PCR products and editing the sequences: The purified PCR products were sequenced using Cycle sequencer (Bangalore Genei, India). Sequences were edited and imported into online SSR finder software to look for microsatellites.

Designing the primers: Sequences that contained microsatellites were short-listed. The primers were designed using the software Primer 3.0 (Available online). The criteria adopted to reduce the amplification of nonspecific bands in PCR reactions included a minimum primer annealing temperature (Ta) of 50°C, a maximum difference of 1°C in Ta between the two primers of an SSR locus, and G + C content ranging from 40 to 50%. The primers were synthesized by Sigma (Sigma-Aldrich, Bangalore). SSR primers thus obtained were used in evaluating the amplification success.

3.2.2 Genotyping *Nothapodytes nimmoniana* populations using SSR markers

Study sites and Sample collection

The study was conducted on *Nothapodytes nimmoniana* Graham. (Icacinaceae). Using both primary and secondary data sets, a spatially explicit distribution map was developed on a GIS platform (Figure 3.2). Based on the distribution map, we selected 6 representative sites from central Western Ghats for estimating population genetic variability of *Nothapodytes nimmoniana* (Figure 3.10 and Table 3.1). At each of the chosen sites, individuals were sampled from an area of at least 5 hectares.

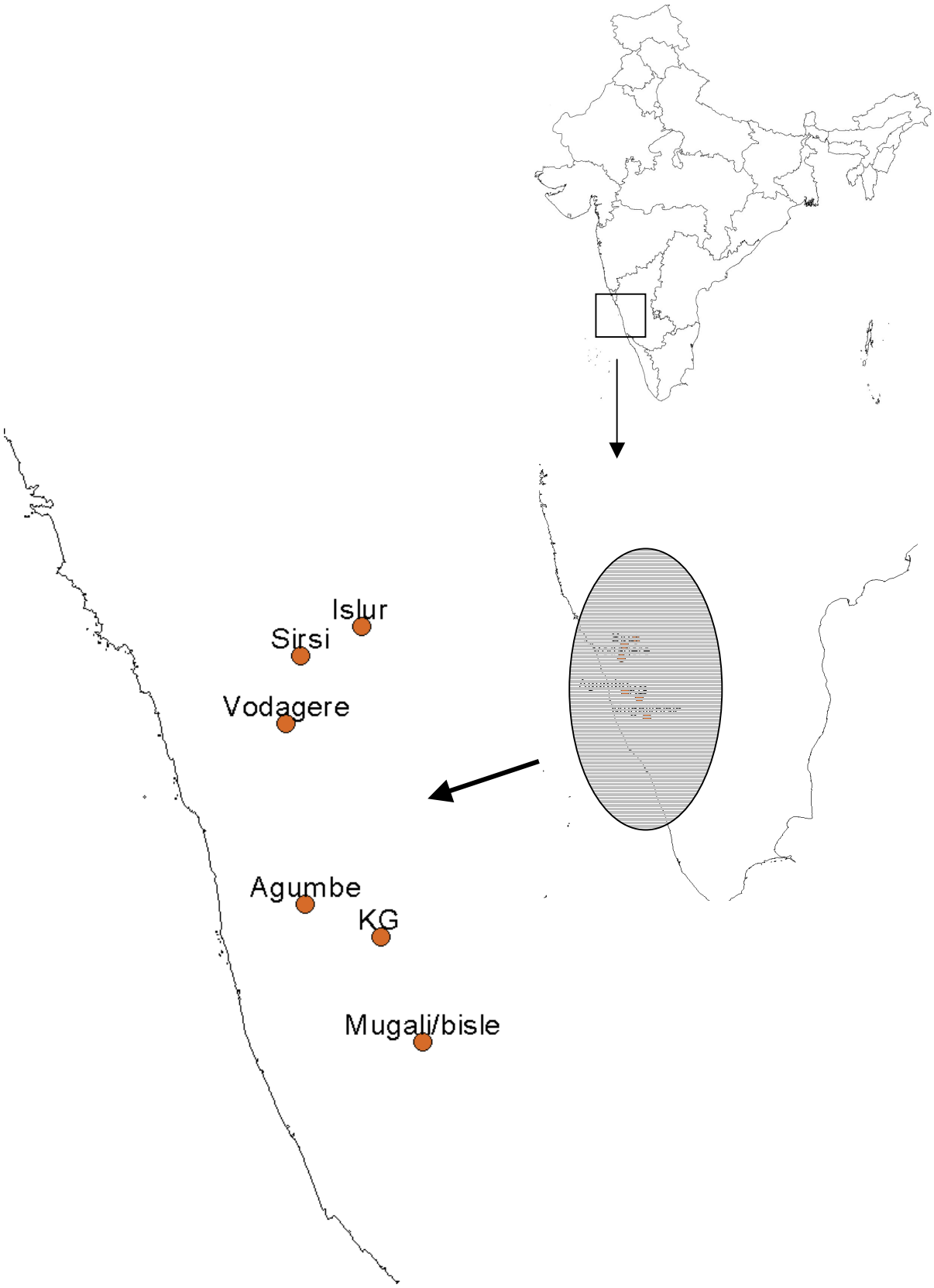


Figure 3.10: Map of study sites in central the Western Ghats of India. Distinct sites were chosen based on the distribution of Nothapodytes nimmoniana in the Western Ghats.

Based on the population size (number of individuals/site), on an average about 10 to 15 trees were sampled randomly from the respective sites (Table 2.1). Samples were washed with tissue paper and stored in polythene bags and transported to the laboratory. Data on number of adults/quadrant, regenerating individuals, girth and number of stems cut were recorded.

Table 3.1: Details of study sites (latitude, longitude and number of samples collected from each of the sites).

Sl no	Population name	Latitude °N	Longitude °E	Number of Samples collected
1	Sirsi	14.69	75.07	7
2	Kemmangundy	13.35	75.45	10
3	Agumbe	13.50	75.09	9
4	Islur	14.83	75.36	7
5	Vodagere	14.37	75	7
6	Mugali/bisle	12.85	75.65	10

Isolation of Genomic DNA: Samples were subjected for isolation of genomic DNA using C-TAB method as described previously.

PCR protocol: The primer nucleotide sequences were synthesized (Sigma-Aldrich, Bangalore, India). 22 primers were evaluated for their site-specific amplification using the respective annealing temperature as listed in the Table 3.2. The PCR amplification was carried out in 25µL volume reaction mixture containing 100 ng template DNA, 5pmol each forward and reverse primer, 1.5 mM $MgCl_2$, 0.5 U of *Taq DNA polymerase* and 100mM of each dNTPs. PCR cycles included initial denaturation at 94 °C for 2 minutes followed by 35 cycles of 40 sec denaturation at 94° C, 1 min annealing at 52° C and 2 min polymerization at 72° C. Finally, extended polymerization at 72° C was done for 10 minutes.

Agarose electrophoresis: To check for PCR success aliquots of 10µL PCR reaction products was elctrophoresed on 3% agarose gels at 100v for 45 min. The gel was stained with ethidium bromide and visualized under UV-transillmunitor. Samples that gave good amplification in the expected size range were further subjected for Polyacrylamide-Gel electrophoresis.

Polyacrylamide-Gel electrophoresis: 12% PAGE gel was prepared using acrylamide: bis-acrylamide (19:1) solution. Gel was removed from the casting apparatus and washed with 1X

TBE. The gel was allowed to pre-run in 1X TBE for about 30-60min at 90V. After pre-run samples were loaded and electrophoresed for about 12-15 hrs at 150-200mA.

Silver Staining of PAGE gels

Fixing the gel: Fixing solution was prepared using 10% ethanol and 0.5% acetic acid in a total volume of 200mL ultra pure distilled water. Gel plate was placed in the tray with the gel facing upward, covered with fix/stop solution and agitated well for 20 minutes. After fixing, fix/stop solution was saved to terminate the developing reaction in the last step. Finally the gel was washed twice for 5 minutes with 1 L ultrapure water with agitation.

Staining the gel: Gel was transferred to the plate containing staining solution (0.2% silver nitrate in 200mL of water) and was agitated for 30 minutes.

Rinse the gel: The gel was dipped briefly into the tray containing ultrapure water, drained and placed immediately into the tray containing developing solution (developing solution : 1.2% NaOH and 0.3% of 37% formaldehyde).

Developing the gel: The gel was **a**gitated by shaking until the bands became visible. Prolonged development time could lead to a high background. Developing reaction was terminated by adding the fix/stop solution.

Scoring Microsatellite Loci: SSR-PAGE gels were scored in co-dominant fashion. Bands in sample lanes were scored using the standard allele names. Bands that were diffused or too difficult to score were considered as missing data and in cases of multiple bands of varying intensity the most intense band was scored.

Population genetic analysis: SSR data generated based on co-dominant scoring was used and analyzed using the GenAlex Excel based programme (Peakall and Smouse, 2006). Following genetic parameters were calculated using the programme, numbers of alleles, allele frequencies, heterozygosity, F-statistics, genetic distances and AMOVA.

3.2.3 Niche modeling and Heterozygosity of *N nimmoniana* populations: Using the ecological niche model, the potential geographical distribution map of *Nothapodytes nimmoniana* was developed. The mid points (longitudes and latitude) of 64 primary locations of occurrence of the species comprised the input data. The program DIVA-GIS ver 5.2 was used to predict the distribution in the Western Ghats following Bioclim classic model (includes 19 bioclimatic

variables). Based on the habitat suitability, the model provides the information on highly suitable to unsuitable locations in the specified region.

In this study an attempt was made to compare the heterozygosity of *N nimmoniana* and degree of habitat suitability of respective sites/populations in the Western Ghats. Six populations were classified into three (low, medium and excellent) habitat suitability categories. Frequency distribution of heterozygosity values of the populations that falls into three (low, medium and excellent) habitat suitability categories was developed and tested for its significance using Kolmogorov-Smirnov test (Statsoft 1993).

3.2.4 Cross-amplification of SSR markers in phylogenetically related genera of *N nimmoniana*

SSR primers that were developed for *N nimmoniamna* were used in cross-amplification with its related genera in the family Icacinaceae. Following genera were short listed *Sarcostigma kleinii, Natsiatum herpaticum, Pyrenacantha volubilis, Apodytes dimidiata, Gomphandra tetrandra.* Leaf samples of all these species were collected from different locations in south India. Genomic DNA was isolated from these species and subjected for PCR amplification using SSR primers.

Table 3.2*:* SSR primers designed for *Nothapodytes nimmoniana.*

Sl No	Primer ID	Repeat	Annealing temperature (°C)	Primer sequences (5'--3') forward-reverse	Product size (bp)
1	Nn32	Dinuc	58	GCGCACTGCTCATACTCAAG	160
				ATTGCTTTTATGCGGCTTTG	
2	Nn42	Dinuc	58	TGAGGGCTGCTGTCTACAAA	229
				TCAGAACAAGCATTCATGTTACAAT	
3	Nn44	Dinuc	58	TCACAAATCTGATTAAGCCATCTT	160
				TTTCAAAAAGTGTTTTCTCAACGA	
4	Nn45	Dinuc	58	CAATTCAAGGAAAGGAGTTG	232
				TGTTCCTAAGTATTTATTGTTCCT	
5	Nn46	Dinuc	58	TTACTGCAGGTGGTGGTTCA	152
				GCTTTCTGCATGAGGTCCAC	
6	Nn48	Trinu	58	GGCGGTGATTATTGCGTTAT	177
				CCAGATTACCGCCATCCTTA	
7	Nn50	Dinuc	62	CCAGATTTACATCCGCATTCT	150
				GGGCACTCTCCCTCCTTATC	
8	Nn52	Dinuc	58	AAGGCGAGATCGCTGTATGT	200
				AAACAAAACCACGTCGAACC	
9	Nn54	Dinuc	62	ATCCTCGGCTCCTTCGAC	244
				CCCAAAAGCAAACACACAGA	
10	Nn57	Dinuc	58	CCATGACAAAGGGGAAGAGA	209
				GCCGTTCCTACAGACACACA	
11	Nn60	Trinu	58	TAGAAGAATCGACGGCCCTA	164
				ACTTCACACGCACACAGCTC	
12	Nn65	Trinu	58	GGATGCAAAGCTGAAGTTGA	214
				GTCTGCAAACCAAGGACCAT	
13	NnD4	Dinuc	58	TTTGATGATTTTATGCTCCTATGTG	217

				GAATTTTTCCAAGGGCTTTGT	
14	NnM14	Trinu	58	GGATGCATGCCGACATATAG	210
				ATTGCTGGGATTGAGGCTTA	
15	NnM17	Dinuc	58	CCGAACGTCGTGAAGAAAAT	166
				TAAATTCCTTCCACCGCAAC	
16	NnM18	Dinuc	58	AATCAACTGGCCTTTTGTGG	172
				GTGAAAATAGTATCACAACAAGTGATT	
17	NnM3	Dinuc	58	CAACAAGCTTGCATTGGTGA	231
				TGATCCCGATACAATGAGGA	
18	NnM5	Trinu	58	TCCACAAGTCCCAAATAGCA	211
				TGACAATTTTTGCCATTCCA	
19	NnM6	Dinuc	58	TGTTTGCCGGGATCTTATTC	226
				TCAGAATCGTTTTCTCTGAATCC	
20	NnM7	Dinuc	58	GCCACCAGTGATTCCAGAAA	231
				TGGATTTTTAGCAATATTCACAATG	
21	NnP6	Dinuc	58	AAAGGGTGCATTGTGTAAAAGAA	216
				CAAGGATTCCATGTTTGATTTTT	
22	NnT4	Trinu	58	GCACTGAGCATATGAACACGA	201
				CCACTTTCCATTTTGCTGGT	

Note: Dinuc: Dinucleotide repeat
Trinu: Trinucleotide repeat

3. Prospecting phylogenetically related species/genera of *N nimmoniana* for Camptothecin.

3.3.1 Mapping the distribution of related genera of *N nimmoniana* in Western Ghats: Information about the distribution of related genera was obtained from secondary data such as floras, herbaria, books, and other published sources including forest department records. Besides the secondary sources, primary survey in major forest divisions in the Western Ghats was also done to obtain data on distribution of *Nothapodytes nimmoniana* and related genera. The latitude and longitude of the sites were obtained using a global positioning system (GPS Model 2.03). The geographical coordinates, both from the primary and secondary data, were digitized using GIS Map-Info software.

3.3.2 Study sites and sample collection: Based on the maps developed efforts were made to collect samples from some of the sites in the Western Ghats. Samples were also collected from different herbaria collections like FRLHT herbaria, herbaria of French Institute, Pondichery and Suhas *et al*, 2007(Table 3.3).

3.3.3 Chemical profiling of related species for Camptothecin: Extraction and quantification of camptothecin (HPLC and LC-MS/MS) from all the related genera of *N nimmoniana* was done according to the protocol that is described in earlier sections.

3.3.4 *ndhF* gene phylogeny of Asterids

Several candidate chloroplast genes have been suggested for phylogenetic studies (Olmstead and Palmer 1994). *ndh*F has recently been demonstrated to be useful for phylogenetic studies at intra-familial levels compared with the *rbc*L gene which has been widely used at higher taxonomic levels (Olmstead and Sweere 1994; Kim and Jansen 1995). The utility of sequence change in the *rbc*L gene has been recently evaluated for the estimation of divergence time between woody-taxon pairs (Albert *et al,* 1994). In the present study, *ndhF* sequences were used to reconstruct phylogenetic relationships of different genera of Icacinaceae. DNA was extracted from fresh material using standard protocol as described previously (Doyle and

Doyle 1980). *ndhF* gene was amplified using the following pair of primers, first half of *ndhF*: 15F(5'ATGGAACAGACATATCAATAYGSRTG3')8R(5'ATAATCCGCACATATAAAA TGCGGTTC3'), Second half of *ndhF*: 7F(5' AGGTACACTTTCTCTTTGCGGTATTCC 3') and 2133R (5' CAGGAACAAGAGGGATCCACCGAA 3'). PCR amplification was carried out in 25 µL volume reaction mixture containing 25 ng template DNA, 2.5 µl 10× reaction buffer containing 15 µM MgCl2, 3 µM of each dNTP, 0.5 µM of each primer and 0.5 U unit *Taq* DNA polymerase (Bangalore Genei, India). The PCR was set for 3 min at 94◦C, followed by 35 cycles of 45 s at 94◦C, 45 s annealing at 45◦C and 2 min extension at 72◦C, and a final extension cycle of 8 min at 72◦C. Five microliters of each PCR reaction was run on a 1.5% agarose gel to check the quality of amplification. To purify the resulting DNA, 20µL of each PCR reaction was run on a 1.0% TBE agarose gel. Agarose blocks containing the DNA were excised from the gel with a scalpel over UV light and purified.

Table 3.3: List of phylogenetically related genera of *Nothapodytes nimmoniana* used in chemical profiling for Camptothecin. Place of collection and their respective geographical coordinates are given in the table.

Sl no	Species	Family	Angiosperm order	Place of collection	Geographical coordinates of collection	
					Latitude (N)	Longitude (E)
1	*Pyrenacantha volubilis*	Icacinaceae	Garryales	Puducherry	11.56	79.53
2	*Sarcostigma klenii*	Icacinaceae	Garryales	TBGRI,	8.45	77.1
				Yana, kigga	13.47	75.27
3	*Apodytes dimidiata*	Icacinaceae	Garryales	Periya. Kerala	11.5	76.26
				Yana, kigga	13.47	75.27
5	*Gomphandra tetrandra*	Icacinaceae	Garryales	TBGRI,	8.45	77.1
				Yana, kigga	13.65	75.33
6	*Gomphandra polymorpha*	Icacinaceae	Garryales	-		
7	*Natsiatum herpaticum*	Icacinaceae	Garryales	-		
8	*Strombansia zeylanica*	Olacaceae	Santalales	Yana, kigga	13.47	75.27
9	*Ophiorhiza pumila*	Rubiaceae	Gentianales	Nelkunda, Karnataka	14.69	75.07
10	*Tobermontana heyneana*	Apocynaceae	Gentianales	Yana, kigga	13.47	75.27
	R56	-	-	-		
	R59	-	-	-		
	R6/R10	-	-	-		

The purified double stranded DNA products were sequenced directly by automatic sequencing. *ndhF* sequences of different species/genera of Icacinaceae and its related family were obtained from Gene bank database. *ndhF* sequences of members from different orders like Cornales and Ericales were also obtained from the database. All the sequences were subjected for pair wise and multiple sequence alignment in MEGA using CLUSTAL W and X.

Phylogenetic analysis: The sequence data were used in phylogenetics analysis. Neighbor joining tree was developed using the MEGA 3.1 programme, with all changes weighted equally (transitions and transversions). Analyses of the complete data set were performed rooting the tree with member taxa from each of the order; Ericales and Cornales. To evaluate relative levels of support for individual clades, the bootstrap method was used with 500 replicates.

Mapping the occurrence of Camptothecin on Angiosperm Phylogenetic tree: Minimal phylogenetic tree was developed for Asterids based on *ndhF* gene sequences. Tree thus developed was overlaid with presence of camptothecin on different clades and clades that contain camptothecin were identified.

3.4 Prospecting endophytic fungi of *Nothapodytes nimmoniana* for camptothecin

3.4.1 Sample collection and preservation of samples for Isolation of endophytic fungi

The samples were drawn from an earlier collection of 147 trees that were chemically profiled for camptothecin. These trees were sampled from 11 populations along the Western Ghats (from 8^0 N to 15^0 N latitude), one of the three-megadiversity hotspots in India. Samples were collected from six of the trees from 2 populations (Central Western Ghats) that reported to produce CPT excess of 1 per cent by dry weight in the stem barks. These are by far the highest yields of CPT reported thus far (Suhas *et al* 2007). Details of the study sites are given in Table 3.4 and Figure 3.11.

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

Table 3.4: Sample used in isolation of endophytic fungi *Nothapodytes nimmoniana.*

Tree ID	Plant part	Host CPT content (%)
D1SB	Stem bark	1.89
D2SB	Stem bark	0.37
D5SB	Stem bark	1.48
D6SB	Stem bark	0.56
D8SB	Stem bark	0.46
D9SB	Stem bark	0.32
D10SB	Stem bark	1.31
D15SB	Stem bark	0.67
KG1SB	Stem bark	1.21
KG4SB	Stem bark	2.82
	Stem bark	2.82
	Stem bark	2.82
KG6SB	Stem bark	0.22
	Stem bark	0.22
KG10SB	Stem bark	1.99
	Stem bark	1.99
Cutting	Stem bark	0.46
Leaf petiole	Stem bark	0.40
Kod SB-1	Stem bark	0.14
NLSB-11	Stem bark	
NLSB-12	Stem bark	
NLSB-13	Stem bark	
Per SB-11	Stem bark	0.41
Per SB-12	Stem bark	0.41
Kod SB-1	Flower buds	-
Callus culture	-	-
Callus culture	-	-
Seedling raised Invitro	Seedling leaf	-
-	Stem	-
-	Stem apex	-
-	Leaf	-
-	Leaf	-
-	Stem	-

Note: (DSB: devimani stem bark sample, Karnataka, KGSB: Kemmannagundy stem bark sample, Karnataka NLSB: Nalmukh stem bark sample, Kerala, Per SB: Persia jn sample, Kerala , and Kodeyar stem bark sample, Kerala.).

Sciences, GKVK, Bangalore, India. For stem bark collections, the outer bark at breast height was scrapped using a knife and a section of the inner bark (5cmX5cm) was collected into a plastic bag and sealed. Similarly leaf samples from seedlings and young flower buds from flowered trees were collected, sealed in a plastic bag and transported to the laboratory. 24 samples of *Nothapodytes nimmoniana* were used in isolating endophytic fungi. Out of 24 two were calli material raised out of embryo, one cutting raised out of a tree from Bisle population, one flower bud from Kodeyar population and remaining all were stem bark

samples from Devimani Ghat, Kemmannagundy from Karnataka part of the Western Ghats. Nalmukh and Kodeyar from Kerala part of the Western Ghats.

Figure 3.11: Map of the study sites at the Western Ghats, India. From five sites, samples of *Nothapodytes nimmoniana* were collected for isolation of endophytic fungi.

3.4.2 Isolation of endophytic fungi from *Nothapodytes nimmoniana*: Stem bark, leaf petiole, flower buds and cuttings of *N nimmoniana* were used in isolation of endophytic fungi. The material was treated with 95% ethanol, and pieces of the inner bark of the stem were placed on aqueous agar and incubated at 28 ± 2 °C until fungal growth started. The tips of the fungal hyphae were then removed from the aqueous agar and placed on mycological medium (potato dextrose agar) or Sabouraud agar.

The pure cultures obtained were transferred to Sabouraud agar (dextrose 40 g/L; peptone 10 g/L; agar 20 g/L). In the process, 26 different endophytic fungi were isolated.

Each microbe was grown in liquid Sabouraud medium (100 mL) and screened for CPT production. To establish the *de novo* production of CPT by the isolated fungi, the growing mycelium was serially transferred several times to fresh mycological agar to eliminate the possibility of the fungal hyphae carrying residual camptothecin as 'contaminant' from the initial plant material.

3.4.3 Morphological characterization of fungal isolates: Traditional identification includes culture characteristics and the morphology of fruiting bodies and spores. Many of these fungi were non-sporulting hence a special protocol was used to induce sporulation. The fungi on agar plates were inoculated onto sterilized banana leaf bits (1 cm^2) impregnated on water agar medium. The plates were continuously monitored for spore formation by stereo and light microscopy. Microscopic slides were prepared, stained using lactophenol cotton blue (Vainio *et al*, 1998) and were examined under light microscope (Olympus, USA). Photographs were taken by using a digital camera (C-2100 ultra zoom, Tokyo, Japan) and identifications of the endophytic fungi were based on their morphology.

3.4.3 Molecular characterization of fungal isolates suing ITS (Inter Transcribed Spacer region):

DNA extraction from fungal mycelia:

The mycelium sample was collected from fresh fungal cells on the culture plates of Sabouraud agar (SBA). The freeze-dried mycelium sample was ground in liquid nitrogen and the resultant powder (10-50 mg) transferred into a 1.5-ml eppendorff tube. Total genomic DNA was extracted using a standard protocol (Cappiccino and Sherman, 1996). The DNA was quantified using nanodrop spectrophotometer. Two microliter of the DNA suspension was used for PCR amplification.

PCR amplification and ITS Sequencing: Amplification of the ribosomal ITS regions ITS1 and ITS2 flanking the 5.8S subunit was performed using a pair of primers ITS1 (5'-TCCGTAGGTGAACCTGCGG- 3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described previously (White *et al* 1990). The amplifications were performed in a eppendorf PCR set at the following profile: an initial step at 95°C for 5 min; 35 cycles at 95°C for 30 s,

54°C for 30s, and 72°C for 45s; a final extension step at 72°C for 10 min. The PCR-amplified nuclear ITS ribosomal RNA gene was purified from 2% agarose gel using the eppendorff gel extraction kit (Eppendorff) and sequenced directly.

BLAST and Phylogentic analysis using ITS sequence data:

To identify endophytic fungal isolates from *Nothapodytes nimmoniana*, ITS sequence of each of the isolate was compared with the sequence reported in the GenBank. Hits with maximum identity and BLAST score were listed against each of the isolates. Similarly Phylogenetic analyses were conducted using MEGA software (ver. 3.1). Phylogenetic trees were inferred from the alignments and analyzed by the neighbor-joining (NJ) method. One thousand bootstrap replicates were used to estimate the reliabilities of the nodes on phylogenetic trees. The distance matrix of neighbor- joining tree was calculated using Kimura's two-parameter model (Kimura 1980).

3.4.4 Extraction of Camptothecin from fungal mycelia: Mycelia and broth were separated by filtration. Mycelia were thoroughly washed with sterile distilled water and homogenized in a cell disintegrator. Both cell homogenate and cell-free broths were extracted four times with equal volume of chloroform: methanol (4: 1 v/v). The solvent was evaporated using a rotary evaporator leaving behind the organic residue, which was dissolved in DMSO: methanol (3:1 v/v) and analyzed by using HPLC. The rest of the residue was used for quantification of CPT by LC-MS/MS.

3.4.5 HPLC and LC-MSMS analysis of fungal extracts: HPLC and LC-MSMS protocol was followed as described in the previous section.

EXPERIMENTAL RESULTS

IV RESULTS

In this chapter the results pertaining to the following studies are presented. **1)** Chemical profiling of different populations of *Nothapodytes nimmoniana* for camptothecin and its related alkaloids and **2)** Molecular characterization of populations of *Nothapodytes nimmoniana* in the Central Western Ghats, India using SSR markers **3)** Prospecting phylogenetically related genera/species of *Nothapodytes nimmoniana* for camptothecin and **4)** Prospecting endophytic fungi from *Nothapodytes nimmoniana* for camptothecin.

4.1.1 Chemical profiling of different populations of *Nothapodytes nimmoniana* for CPT and its related alkaloids

Different populations of *N nimmoniana* showed significant variation in mean CPT content both in stem bark (one-way ANOVA, $P < 0.004$) and root bark ($P < 0.001$). Percent CPT in stem bark ranged from as low as 0.03 to as high as 2.7, with an overall mean of 0.7. The mean CPT content in the root bark ranged from 0.003 to 1.41%, with an overall mean of 0.48% (Figure 4.1.1). CPT content of stem bark was significantly positively correlated with that of the respective root bark ($n = 126$; $r = 0.320$, $P < 0.05$). Finally, the frequency distribution of CPT content over all populations was highly positively skewed (Figure 4.1.1). Twenty-three of the individuals showed CPT in excess of 1%.

CPT yields- LC-MS analyses

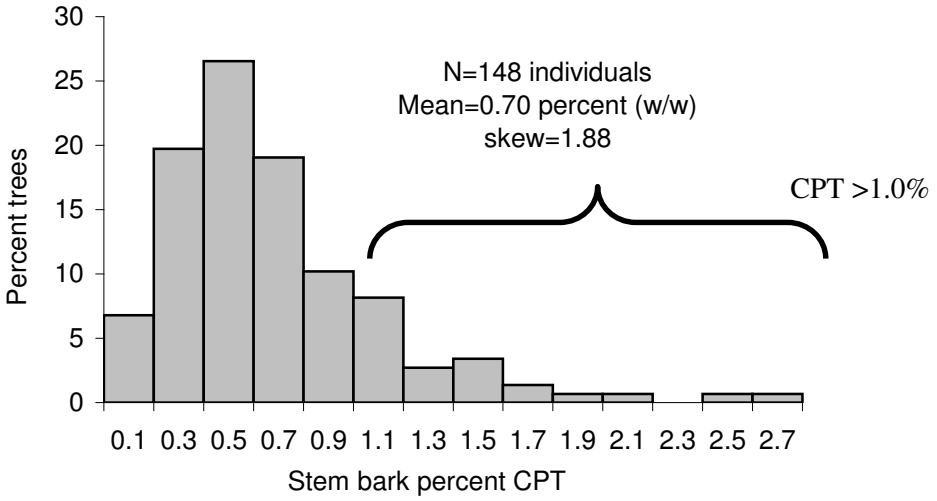
For this study, 17 of the 23 trees (9 for which highest CPT was reported by Suhas et al, 2007 from stem bark and 8 from root bark) for the HPLC/LC-MS quantification of camptothecin as well as in detecting other camptothecines in the tissue.

HPLC-MS analysis profile showed CPT peak at 20.17 min in stem bark sample. This peak passed through the flow cell of the DAD detector. The column eluate was directly transferred to MS detector without any split and mass of the eluate was read, in this case CPT (349). In all the samples, mass spectra showed presence of 349 peak indicating the presence of CPT and this was used in quantification of CPT in all the samples (Figure 4.1.3).

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 per cent (w/w). These results corroborate those obtained earlier for the same trees by Suhas et al (29) who quantified the CPT using HPLC analysis (Figure 4.2.1). 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 4.1.2).

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. The incredibly high yields of these individuals from several populations could not be attributed to their girth; the difference in the CPT yields among the individuals was not related to their stem girth ($r=0.16$: NS).

a)



b)

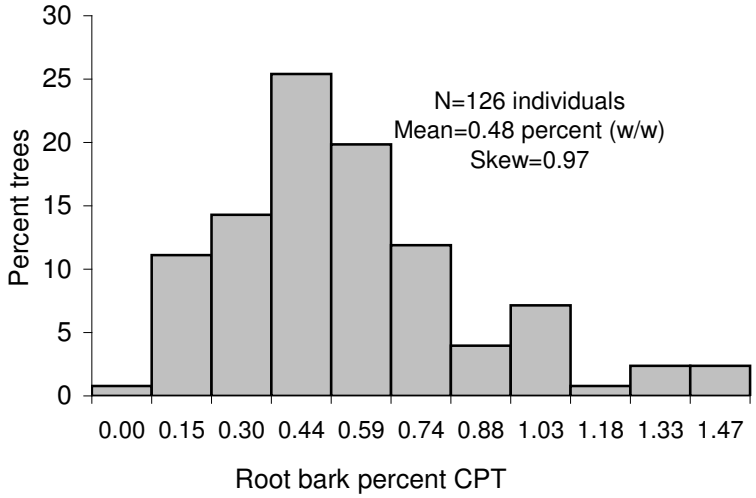


Figure 4.1.1: Frequency distribution of percent CPT (w/w) in stem bark (a) and root bark (b) of *Nothapodytes nimmoniana* (Adapted from Suhas et al 2007).

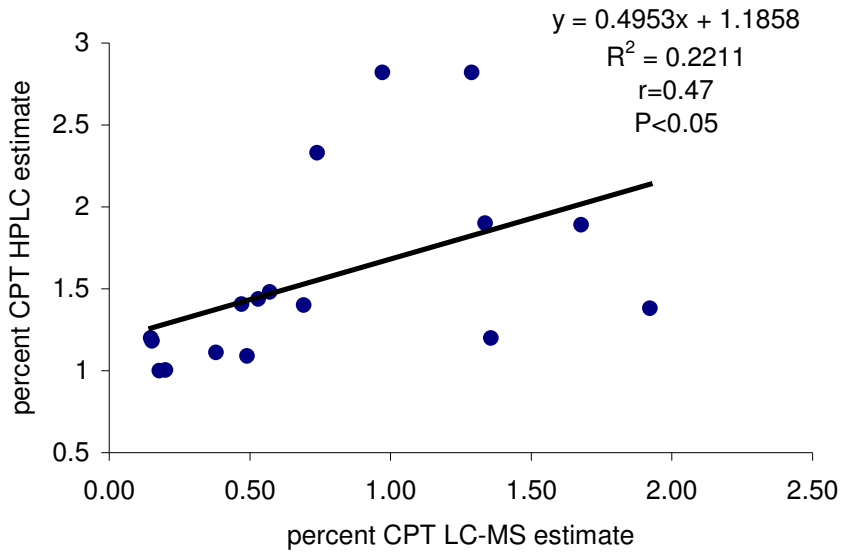
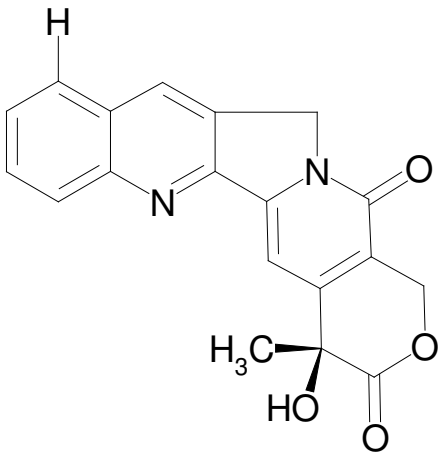


Figure 4.1.2: Correlation between HPLC and LC-MS CPT estimates in 17 accessions of *N. nimmoniana*.

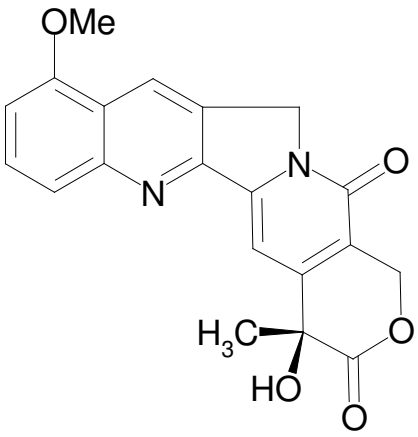
4.1.2 New class of Camptothecines/CPT related alkaloids from *Nothapodytes nimmoniana*

Liquid Chromatography (LC) combined with UV and mass spectrometric detection (LC-UV-MS), especially high-resolution mass spectrometry, offers the possibility to calculate elementary composition of a compound. In the present study, 17 samples of *N nimmoniana* were subjected for LC-MS scan analysis in the range 100-900 m/z. Using retention time, mass and mass of fragmentation peaks data, some of the molecules were identified. 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 (Uk) (Table 4.1.1 and Figure 4.1.3).

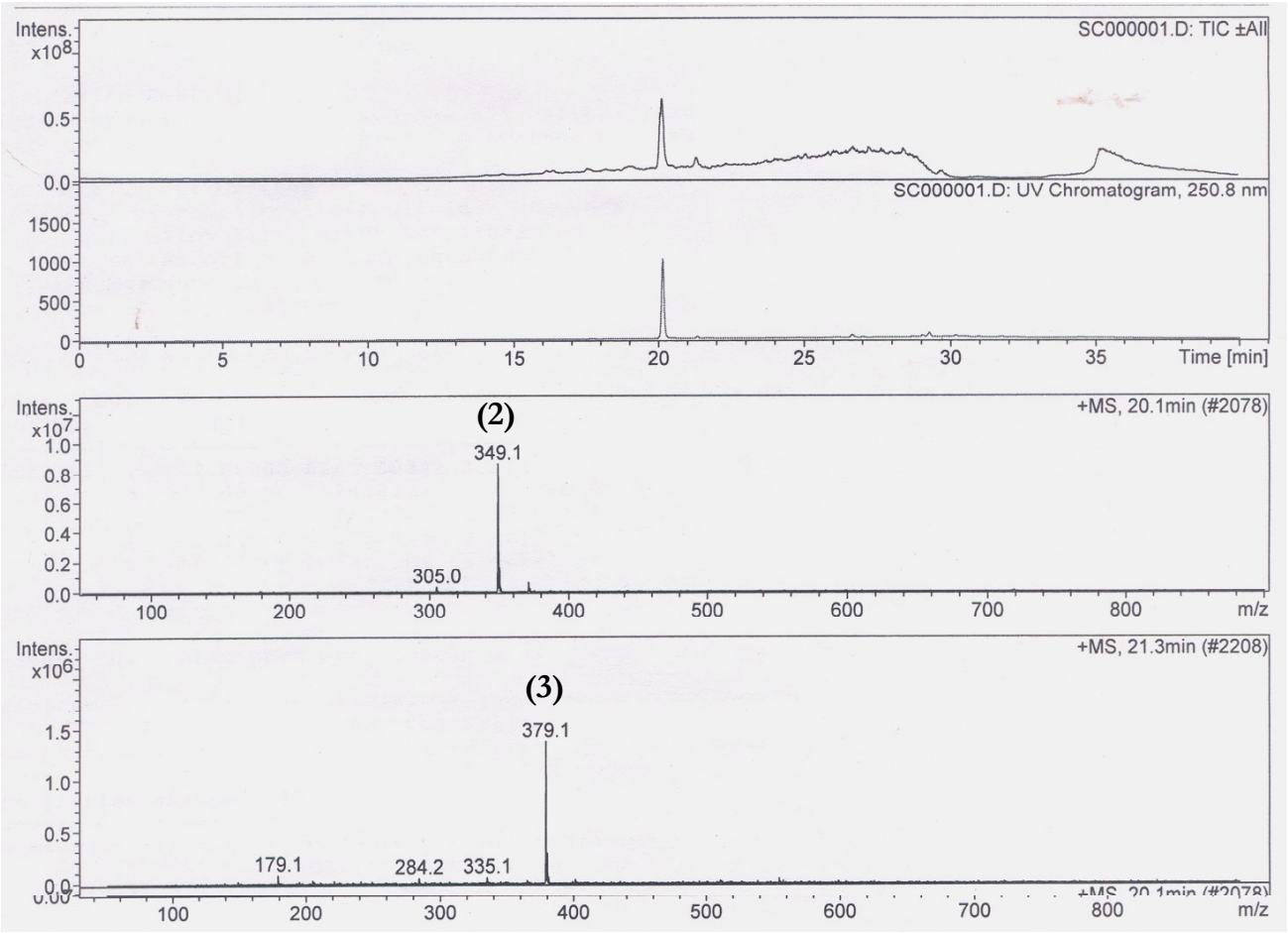
The ten camptothecinoids identified based on their retention time, MS and mass fragmentation peaks were diacetoxy camptothecin **(4)** (t_R 13.0min;m/z=431), diacetoxy-9 methoxy camptothecin **(5)** (t_R14.2min; m/z 461.2), acetoxy camptothecin glucopyranoside **(6)** (t_R 17.5 m/z 511.1), 9-methoxy mappicine 20-B-glucopyranoside **(7)** (t_R18.4min;

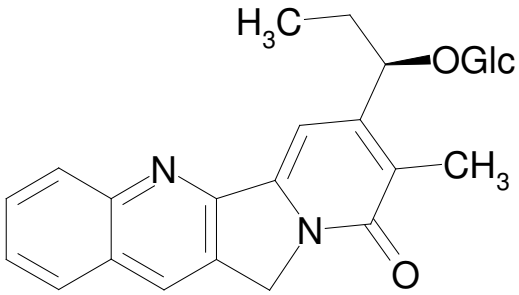


Camptothecin (**2**) (m/z=349.1)

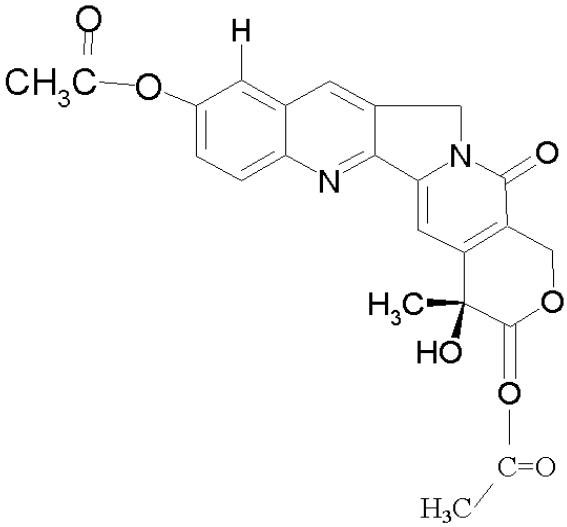


9-methoxy camptothecin (**3**) (m/z=379.1)

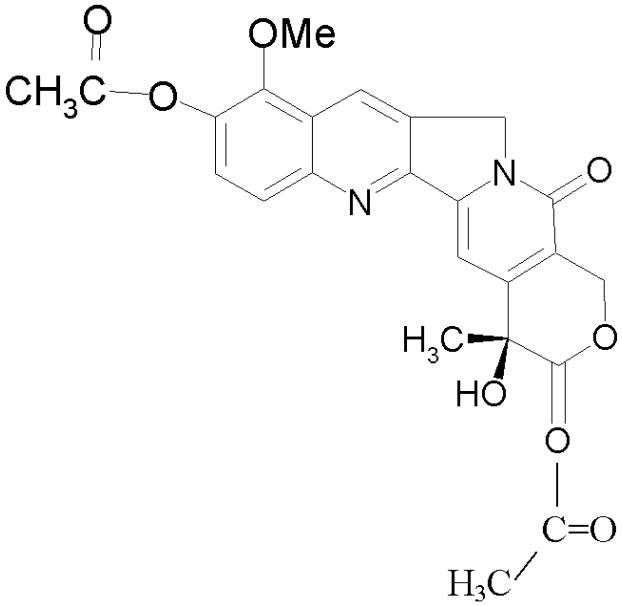




Mappicine-20-β-glucopyranoside (**1**) (m/z=469.2)

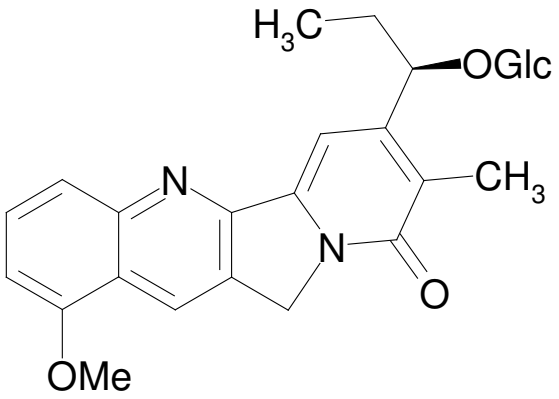


10, 20 Diacetoxy-camptothecin (**4**) (m/z=431.1)

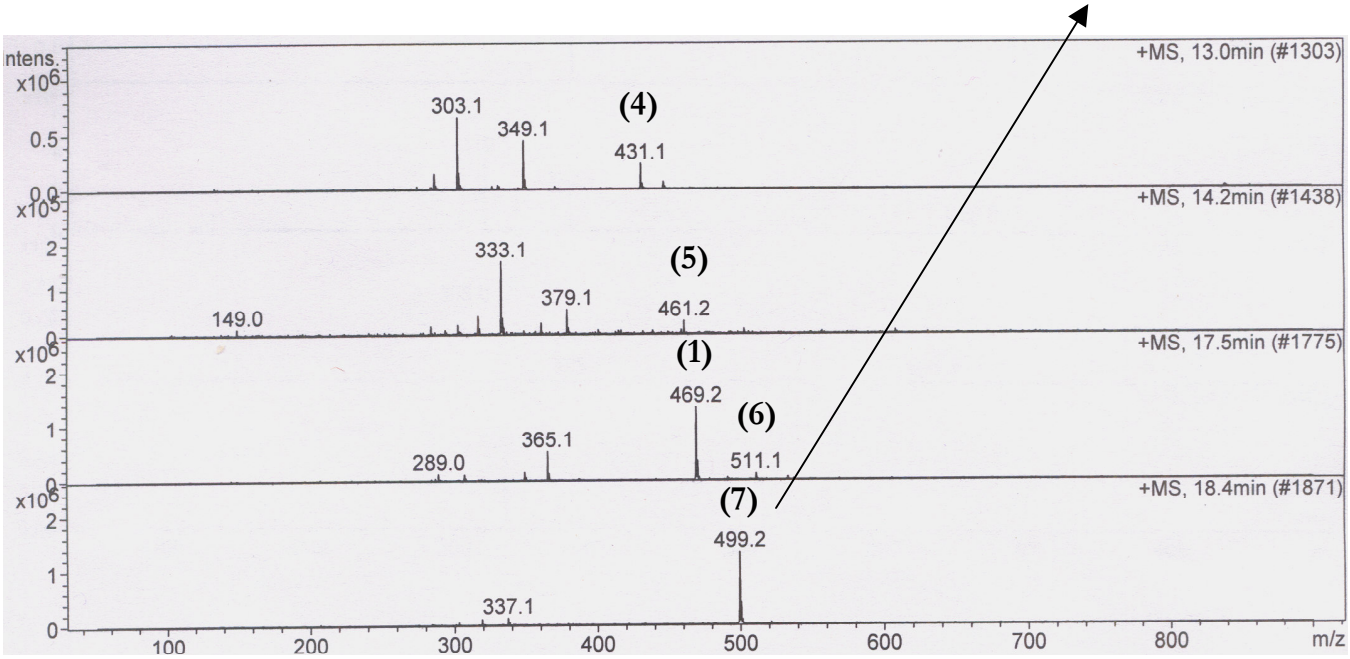


10, 20 Diacetoxy 9-methoxy camptothecin (**5**) (m/

Acetoxy-camptothecin-glycoside (**6**) (m/z=511)



9-Methoxy-mappacine-20-β-glucopyranoside (**7**)
(m/z=499.2)



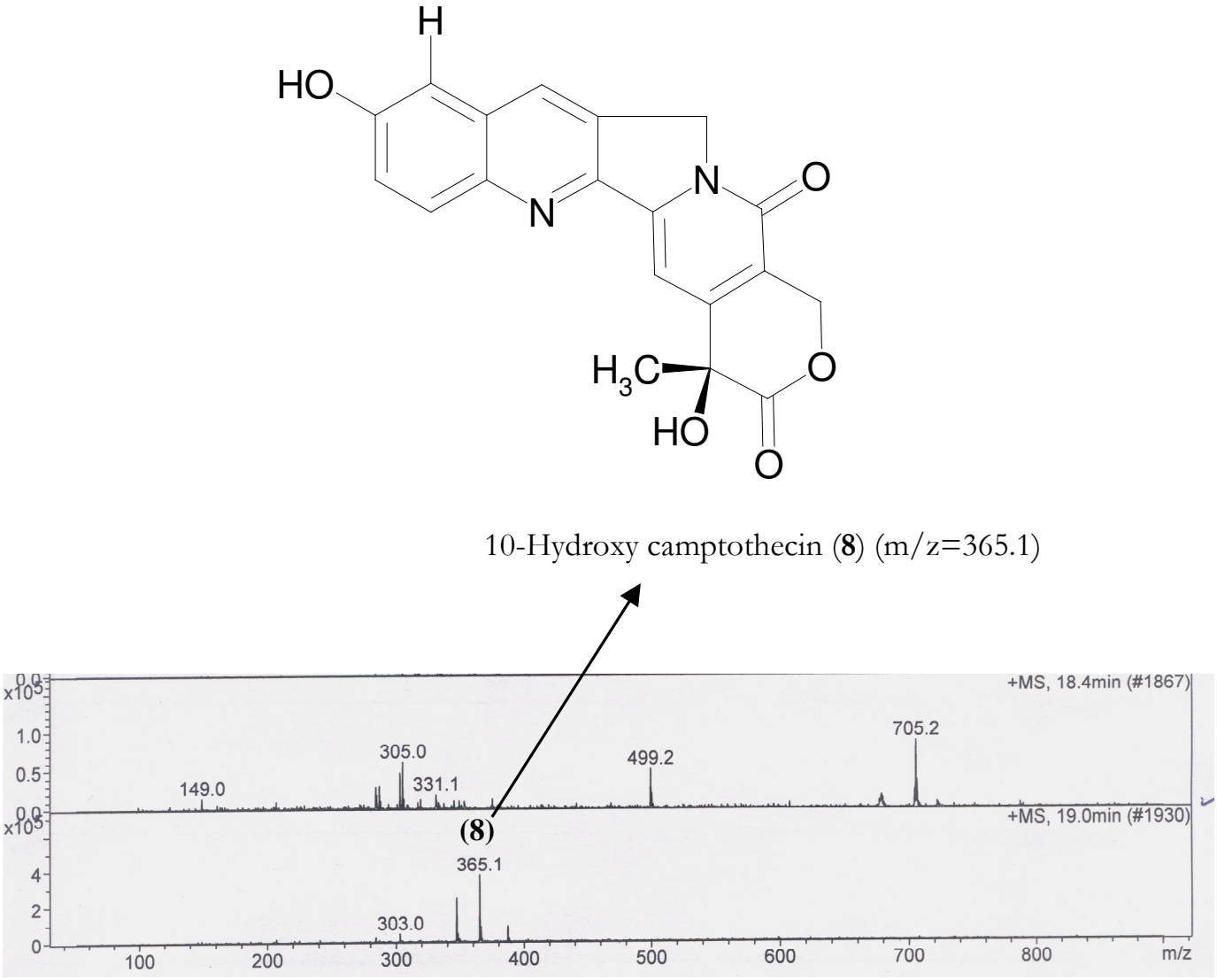


Figure 4.1.3: Mass spectra and chemical structure of Camptothecin, 9-methoxy camptothecin and other Camptothecines from *Nothapodytes nimmoniana*. Mappicine-20-β-glucopyranoside (1), Camptothecin (2), 9-Methoxy camptothecin (3), Diacetoxy-camptothecin (4), Diacetoxy-9-methoxy camptothecin (5), Acetoxy-camptothecin-glycoside (6), 9-Methoxy-mappacine-20-β-glucopyranoside (7) and 10-Hydroxy camptothecin (8). All the chemical structures were drawn in MDL ISIS Draw 2.5 software programme (http://en.bio-soft.net/chemdraw/ISISDRAW.html).

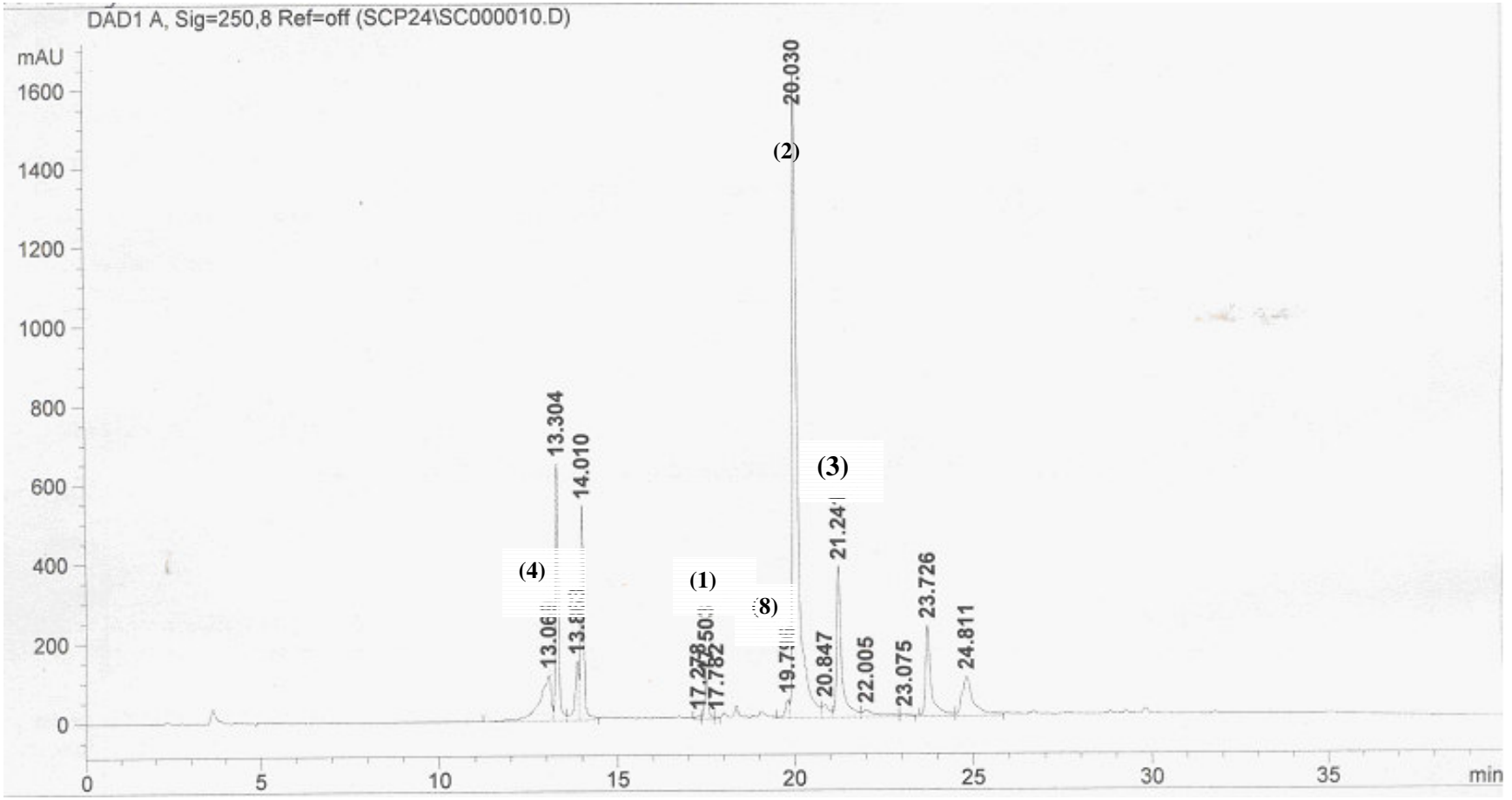


Figure 4.1.4: HPLC chromatogram of Camptothecines from accession number; 4. Mappicine-20-β-glucopyranoside (1), Camptothecin (2) 9-methoxy camptothecin (3), Diacetoxy-camptothecin (4), 10-Hydroxy camptothecin (8).

Table 4.1.1: Camptothecines obtained from the accessions of *Nothapodytes nimmoniana* (stem bark and root bark).

Compound No	t_R (min)	Mass	Other fragmentation peaks	Accession number	
				Stem bark	Root bark
Mappicine-20-β-glucopyranoside (1)	17.5	469.2	289, 307, 365.1, 207, 349, 319	2, 3, 12, 17	1, 9, 10, 11, 13
Camptothecin (2)	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 (3)	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 (4)	13.0	431.1	349.1, 303, 149,	4, 5, 7, 8	10
Diacetoxy-9-methoxy camptothecin (5)	14.2	461.2	379.1, 333.1, 415.2,	4, 5, 6, 7	-
Acetoxy-camptothecin-glycoside (6)	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 (7)	18.4	499.2	337.1,	2,3, 4, 8	7, 9, 10, 11, 13, 14, 15, 16
10-Hydroxy camptothecin (8)	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

m/z=499.2), mappicine 20-B-glucopyranoside **(1)** (t_R 17.5 min;m/z=496.2) along with major camptothecin **(2)** and 9-methoxy camptothecin **(3)** (Figure 5a and 5b) Mappicine glycopyranoside **(1)** and methoxy-mappicine **(7)** 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 analysed. Thus for the first time compound **(1), (4), (5), (6), (7)** and **(8)** in stem and root bark of *Nothapodytes nimmoniana* have been identified. However besides camptothecin, all other campothecinoids were relatively smaller in their content. For example in sample tree # 4, which had 7 of the ten known campothecines, the largest peak corresponded to camptothecin with all others being available in smaller concentrations (Figure 4.1.4).

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.

4.1.3 Ecological niche modeling for CPT accumulation:

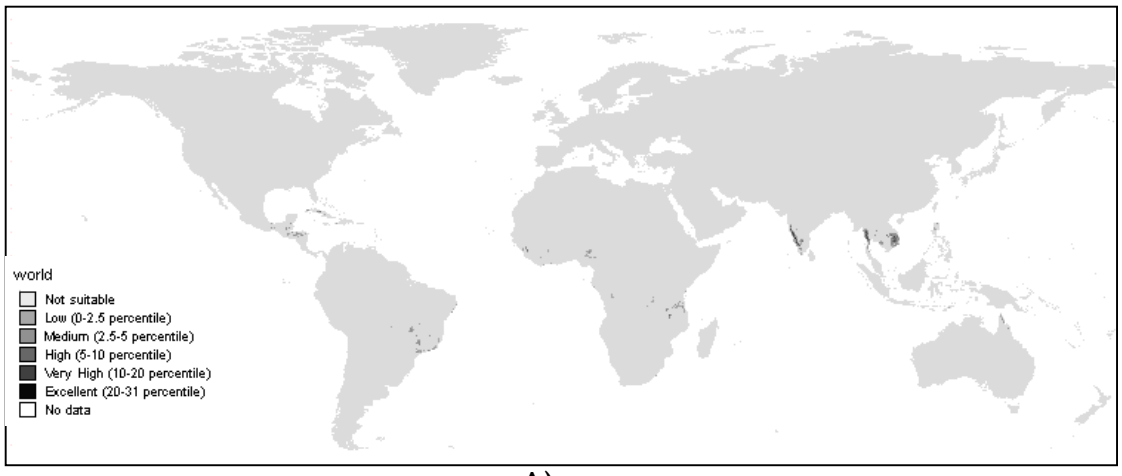
One of the key challenges in prospecting for high yielding sources of specific plant metabolites is to develop algorithms or approaches that can help predict hot-spots of distribution of the metabolite. Recently, a GIS based approach called the ecological niche model has been used to model the spatial distribution of a given species and offer predictions on the habitat suitability of the species. In the present study, attempt has been made to extend the use of ecological niche modeling tools to offer predictions on the spatial distribution of plant metabolites. An underlying assumption of this application is that, plants would be selected to accumulate secondary metabolites at sites predicted to be highly suitable for the given species compared to sites, which are not predicted to be suitable. In the present study ecological model was used to develop distribution maps based on the primary distribution points of *N nimmoniana* in the Western Ghats (Uma Shaanker et al 2008)

(Figure 4.1.5). The percent CPT content in the stem bark was correlated with the predictions of habitat suitability generated using the DIVA-GIS. The CPT content of individuals occurring in the different habitat suitability areas indicated that individuals in highly suitable areas accumulated significantly higher levels of CPT compared to those that occurred in unsuitable or poorly suitable areas (Figure 4.1.6). Furthermore, more than 60 percent of trees in the excellent habitats accumulated more than 1 percent CPT (Figure 4.1.6).

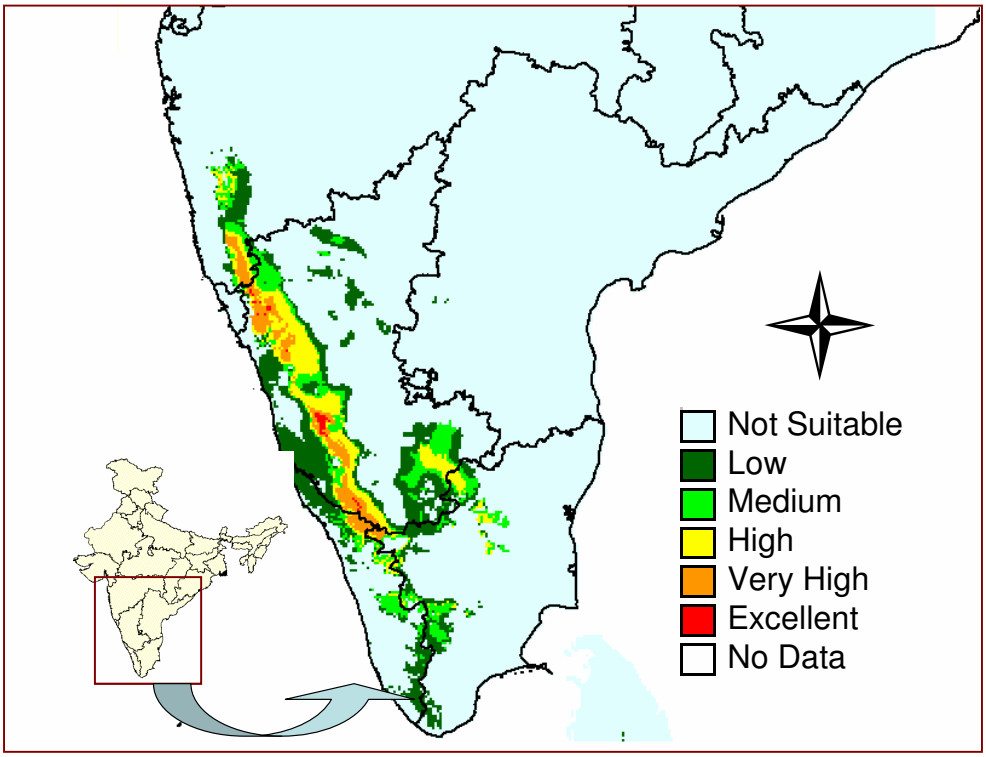
The frequency distribution of CPT content of individuals occurring in the different habitat suitability (low, medium and excellent) indicated that higher proportion of individuals in highly suitable areas accumulated higher levels of CPT compared to those that occurred in low and medium suitable areas (Figure 4.1.7).

Environmental variables influencing CPT production

In order to arrive at the possible environmental variables that influence the accumulation of CPT content in *N. nimmoniana,* we carried out a forward step-wise linear regression using the 19 climatic variables that are loaded with the DIVA-GIS program. The program is loaded with the data on the climatic variables for 30 years (1960-1990). The mean values of the climatic variables for each individual was taken and correlated with the CPT content of the same individual. Only two variables namely, mean temperature of the driest and wettest quarter of the year significantly explained the differences in stem bark CPT among the populations (Figure 4.1.8 and Figure 4.1.9).



A)



B)

Figure 4.1.5: A; Predicted distribution map of *Nothapodytes nimmoniana* B; Predicted distribution map of *Nothapodytes nimmoniana* in Western Ghats, India. The colors indicate the predicted areas of the species using DIVA-GIS.

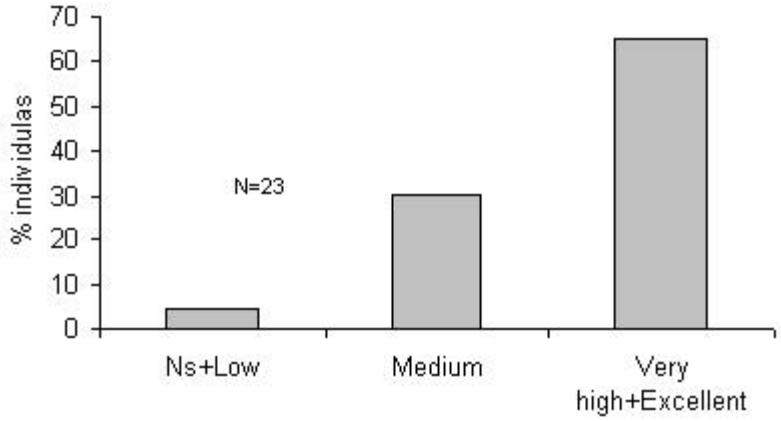


Figure 4.1.6: Graph showing percentage of individuals of *Nothapodytes nimmoniana* grouped into different habitat suitability categories yielding in excess of 1% CPT.

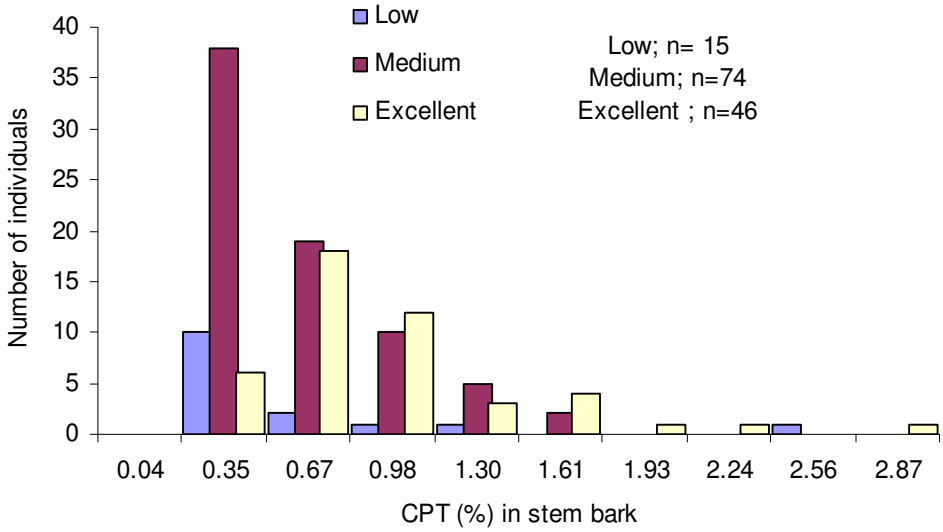


Figure 4.1.7: Frequency distribution of percent CPT content in 148 individuals of *N. nimmoniana* producing CPT in stem bark according to the different habitat suitability category.

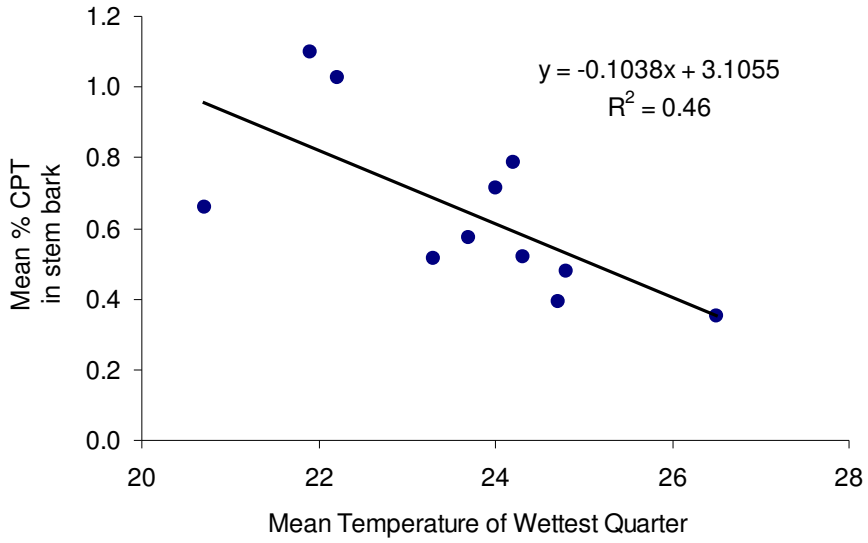


Figure 4.1.8: Relationship between CPT and mean temperature of wettest quarter

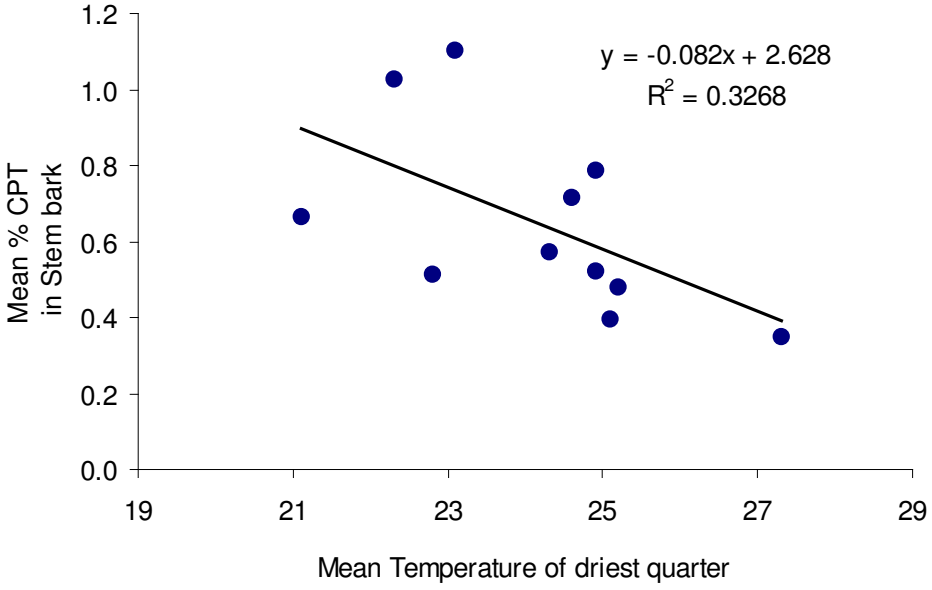


Figure 4.1.9: Relationship between mean CPT in stem bark and mean temperature of driest quarter.

4.1.4 Sustainable harvesting of camptothecin from *N nimmoniana:*

Leaf samples were collected from 123 individuals of one and half year old seedlings, 29 individuals of three to four year old coppices, 22 individuals of five year old saplings and 30 individuals of 10 to 15 years old adult trees of *Nothapodytes nimmoniana* from Sirsi and Biligiri Rangaswamy Temple Wildlife sanctuary (BRT). All these samples were analyzed for CPT using HPLC and total camptothecin concentration in leaf tissue was expressed on a dry weight basis. To confirm the HPLC results, some of these samples were also subjected for LC-MS analysis at RRL, Jammu. In all the samples, mass spectra showed presence of 349 indicating the presence of CPT.

Mass spectra of all the leaf samples showed three signatures at m/z 371 $[M+Na]^+$, $(M+H)^+$ at m/z 349.1 and a fragment of CPT m/z 305 $(M+H-CO_2)^+$ (result of partial fragmentation of CPT). The molecular ion peak at m/z 349 was carried for quantification during the LC-MS studies, as this was the most intense peak in the spectrum (Figure 4.1.10 to Figure 4.1.14).

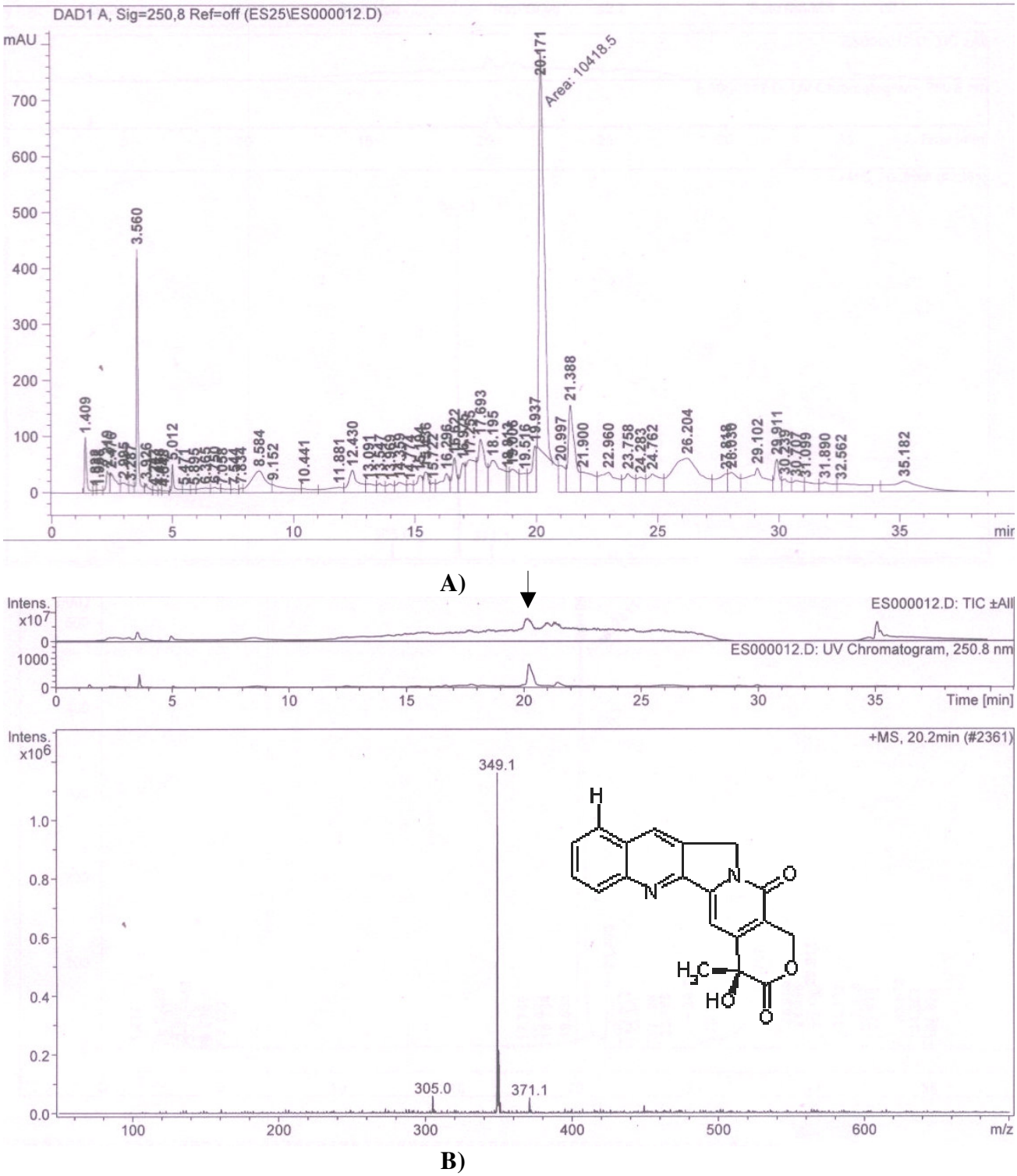
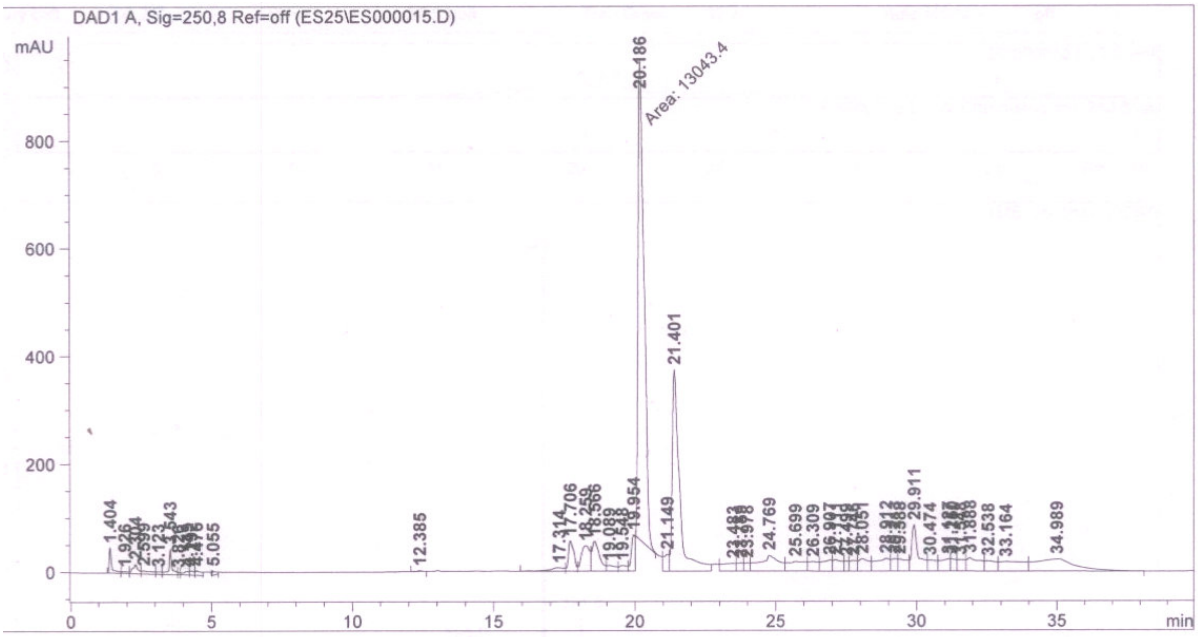
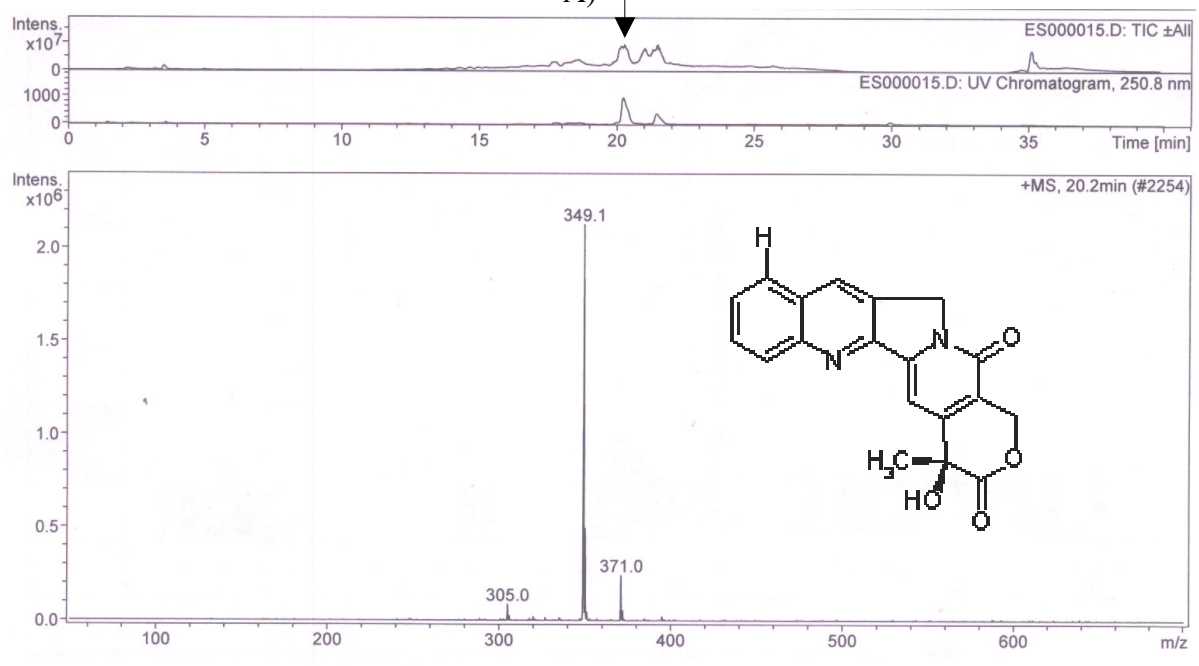


Figure 4.1.10: A; HPLC chromatogram of leaf of coppice plant of *N nimmoniana* showing CPT peak at 20.17 min. B; TIC, UV-DAD chromatogram and mass spectra of CPT peak (indicated an arrow) in coppice leaf sample 1 (HPLC=0.36% LC-MS=0.17%).



A)



B)

Figure 4.1.11: A; HPLC chromatogram of leaf of one year old seedling of *N nimmoniana* showing CPT peak at 20.17 min. B; TIC, UV-DAD chromatogram and mass spectra of CPT peak (indicated an arrow) in one year old seedling leaf sample2 (HPLC=0.9% LC-MS=0.85%).

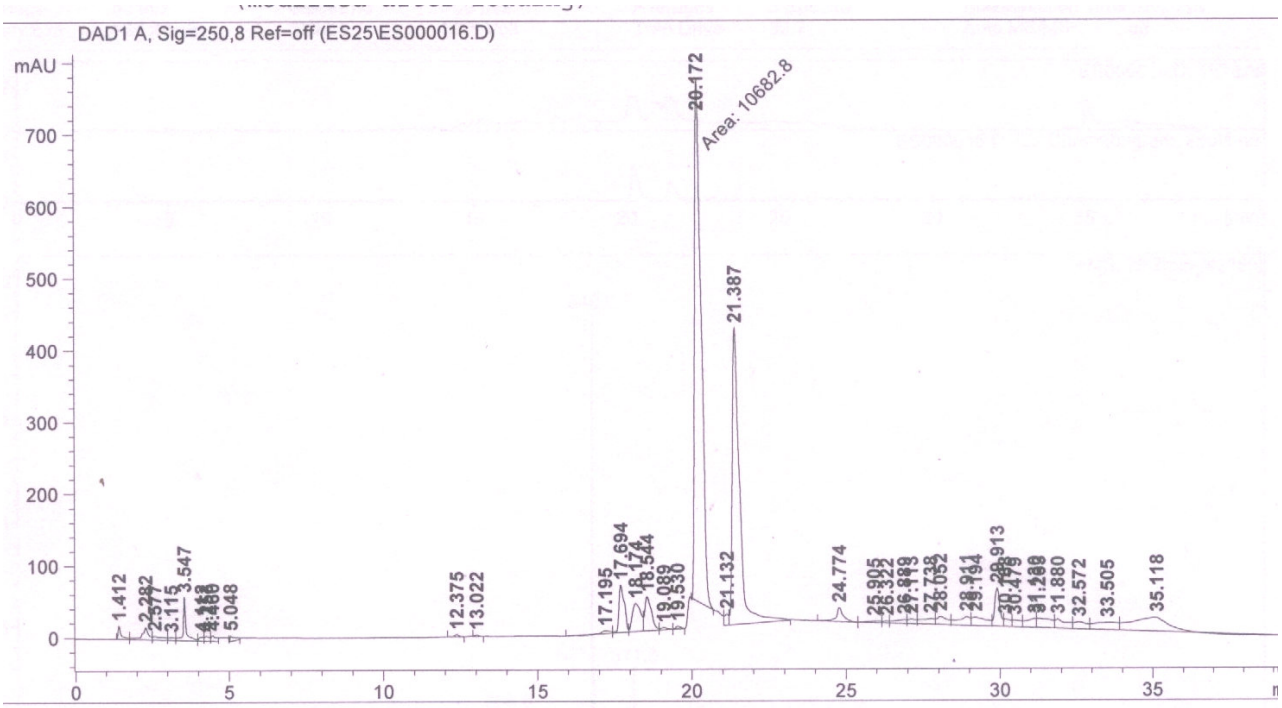


Figure 4.1.12: A; HPLC chromatogram of leaf of one year old seedling of *N nimmoniana* showing CPT peak at 20.17 min. B; TIC, UV-DAD chromatogram and mass spectra of CPT peak (indicated an arrow) in one year old seedling leaf sample3 (HPLC=0.92% LC-MS=0.70%).

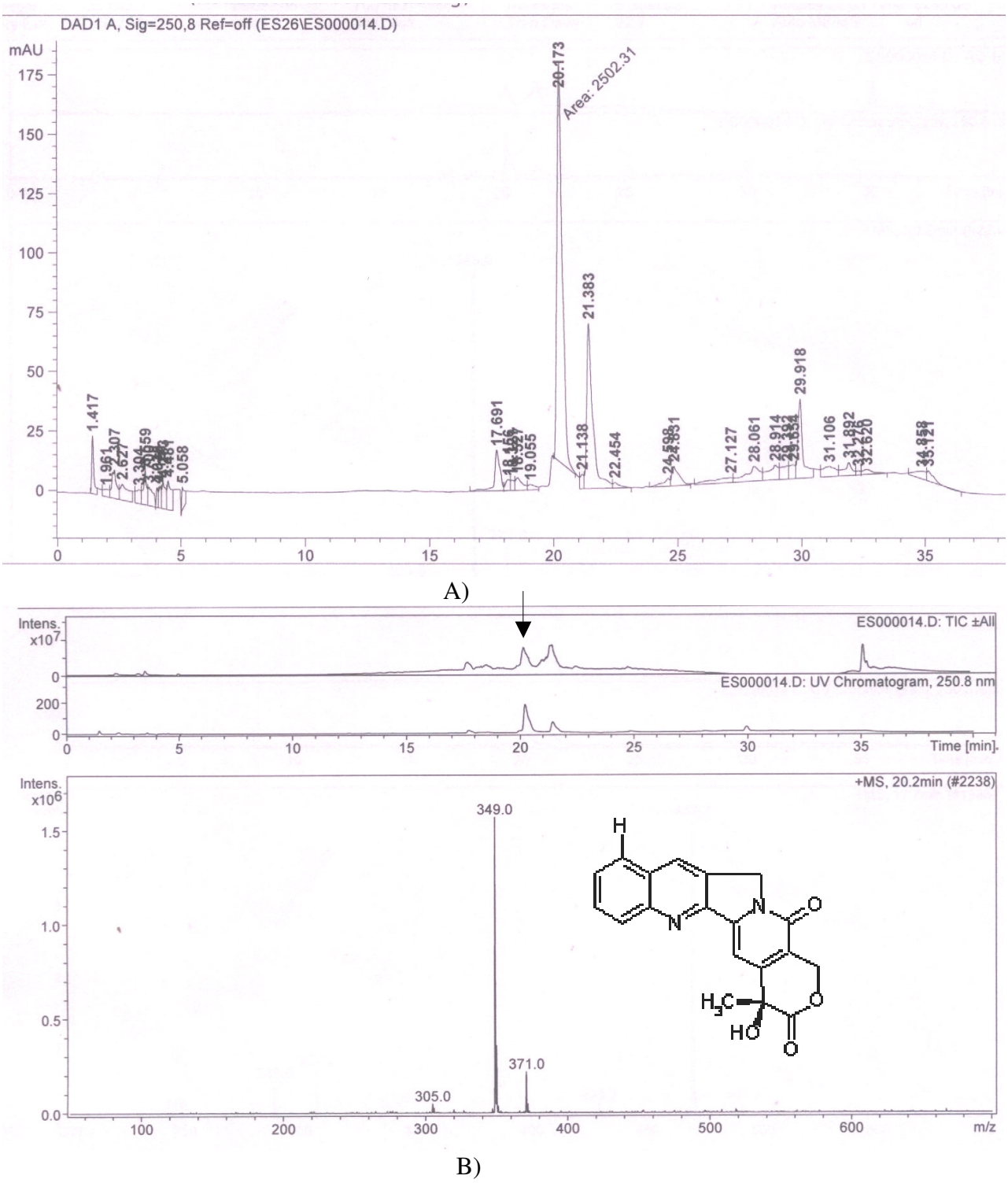


Figure 4.1.13: A; HPLC chromatogram of leaf of one year old seedling of *N nimmoniana* showing CPT peak at 20.17 min. B; TIC, UV-DAD chromatogram and mass spectra of CPT peak (indicated an arrow) in one year old seedling leaf sample4 (HPLC=0.23% LC-MS=0.06%).

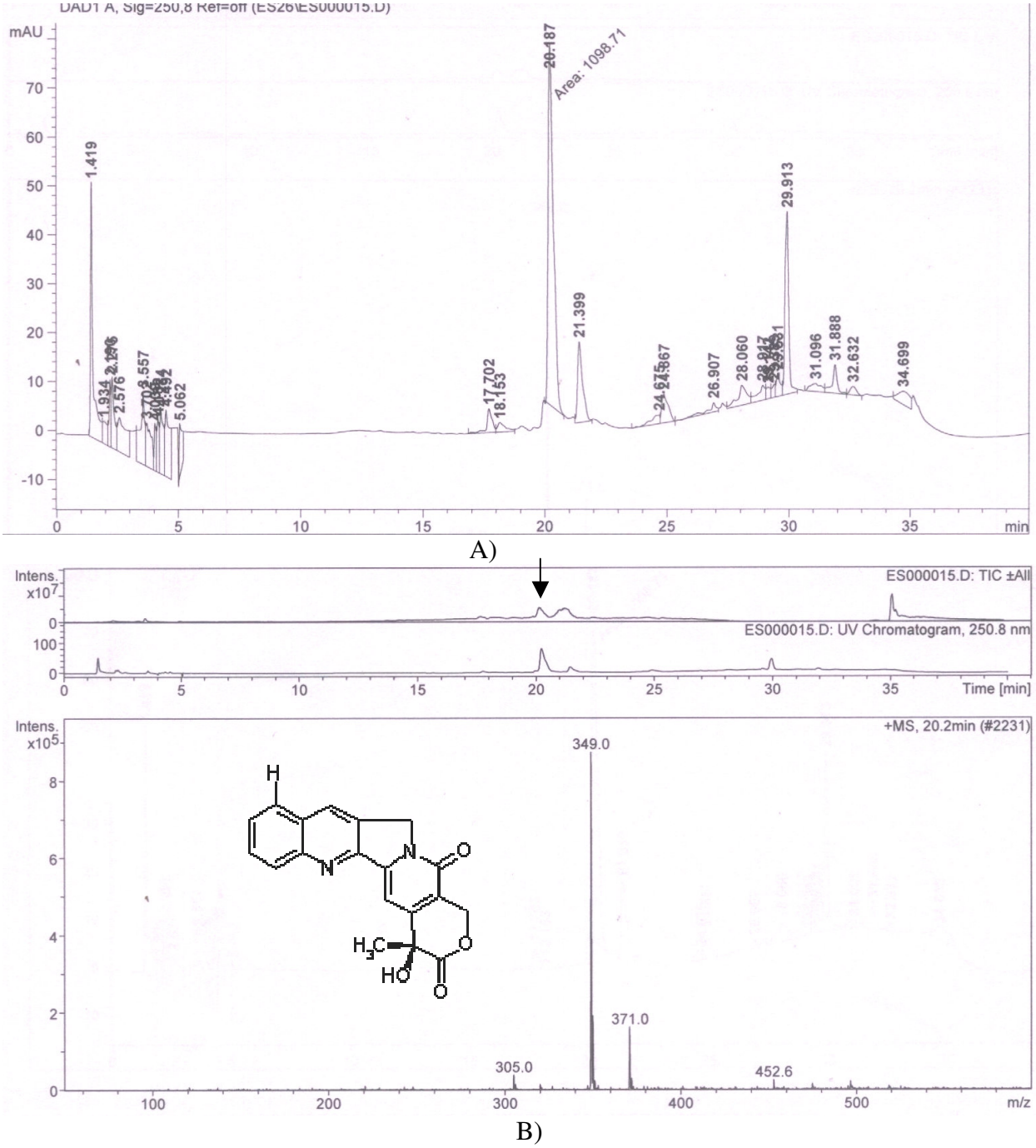


Figure 4.1.14: A; HPLC chromatogram of leaf of one year old seedling of *N nimmoniana* showing CPT peak at 20.17 min. B; TIC, UV-DAD chromatogram and mass spectra of CPT peak (indicated an arrow) in one year old seedling leaf sample5 (HPLC=0.2% LC-MS=0.04%).

Variation in the Leaf Camptothecin content:

There was a significant positive correlation between HPLC and LC-MS CPT estimates in some of the leaf samples of one-year-old seedlings (Figure 4.1.15).

In seedlings of BRT population, the leaf percent CPT varied from 0.027 to 0.92. The mean percent CPT in seedlings from BRT Sanctuary was 0.30 ± 0.17 and seedlings from Sirsi had a mean percent CPT of 0.29 ± 0.20. Over all, the mean CPT in seedlings across both the populations was 0.29 % (Figure 4.1.16 Figure 4.1.17 and Figure 4.1.20).

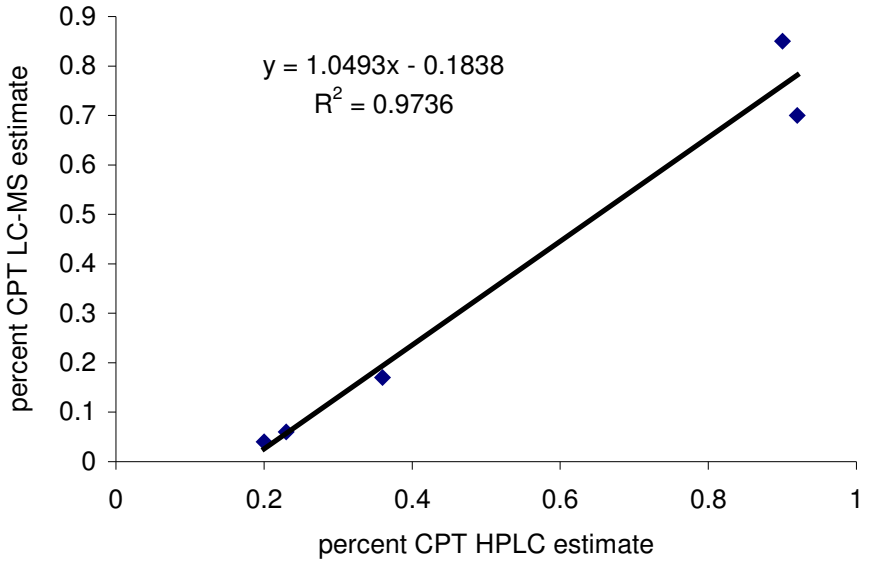


Figure 4.1.15: Association between HPLC and LC-MS CPT estimates in leaves of coppice and one year old seedling systems of *N nimmoniana*.

Further, there were higher percentages of seedlings producing more than 0.2% CPT (Figure 4.1.16 Figure 4.1.17). In fact, some seedlings had leaf CPT of more than 1% (Figure 4.1.16 Figure 4.1.17). The leaf CPT content in the trees did not exceed 0.2% (Figure 4.1.19).

In the leaves of three to four years old coppices, the percent CPT varied from 0.12 to 0.48 with a mean value of 0.26 ± 0.10 (Figure 4.1.18). Leaf percent CPT of saplings ranged from

0.022 to 0.176 with a mean of 0.082 ± 0.042 (Figure 4.1.20). In adult trees of BRT, the percent CPT varied from 0 to 0.071 with a mean value of 0.009 ± 0.017 (Figure 4.1.19 and 4.1.20). Mean leaf per cent CPT was maximum in seedlings followed by coppices. The adult tree leaves had the lowest CPT content (Figure 4.1.20).

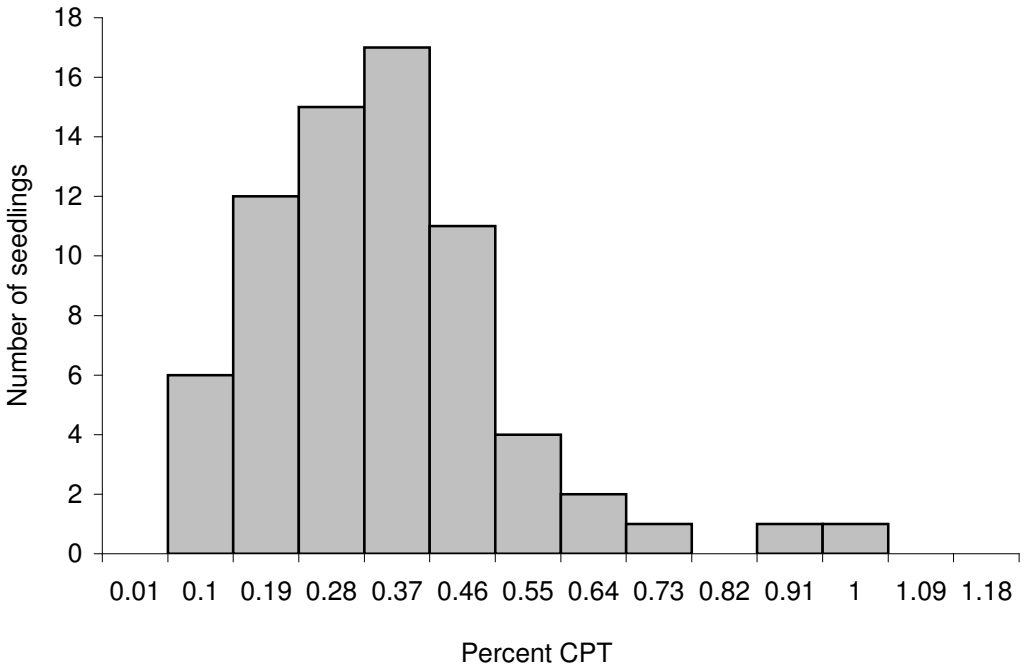


Figure 4.1.16: Frequency distribution of CPT content in one-year-old seedlings population from BR hills (n=70).

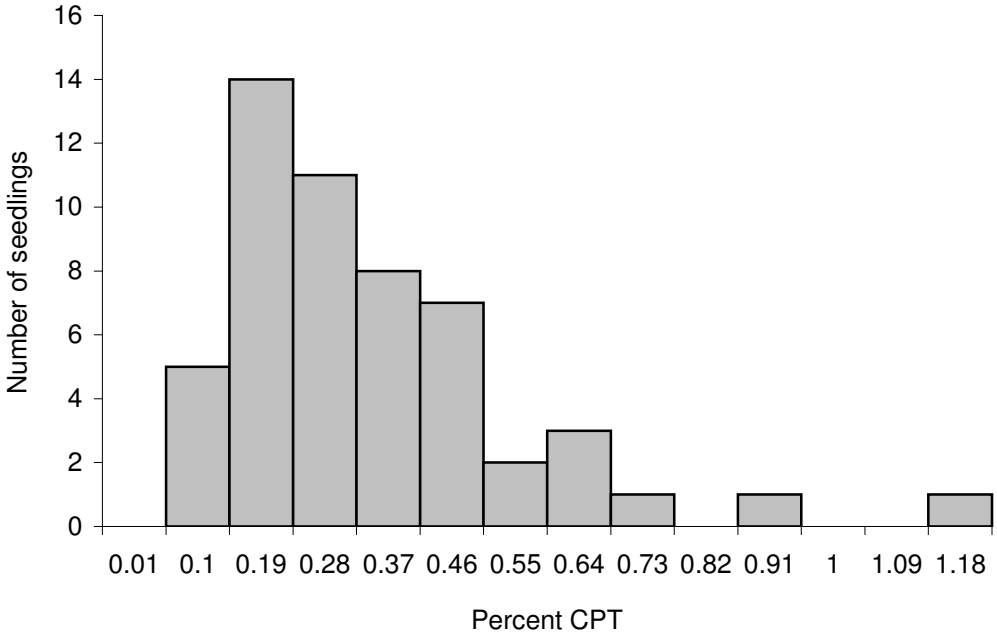


Figure 4.1.17: Frequency distribution of CPT content in one-year-old seedlings population from Sirsi (n=53).

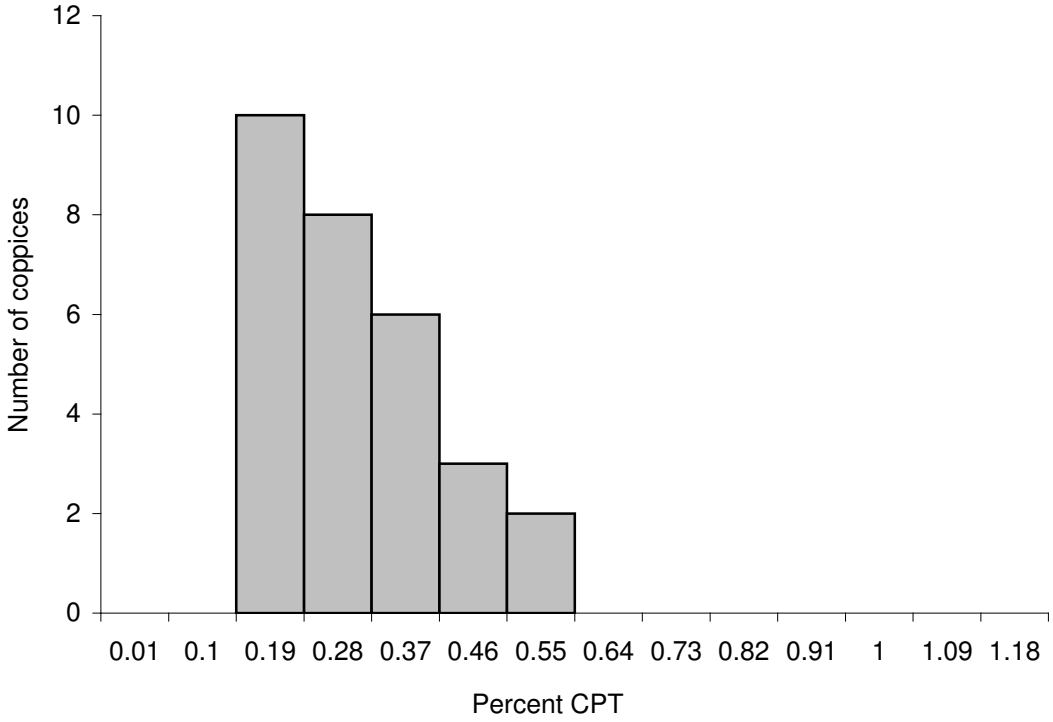


Figure 4.1.18: Frequency distribution of CPT content in coppice population from Sirsi (n=29).

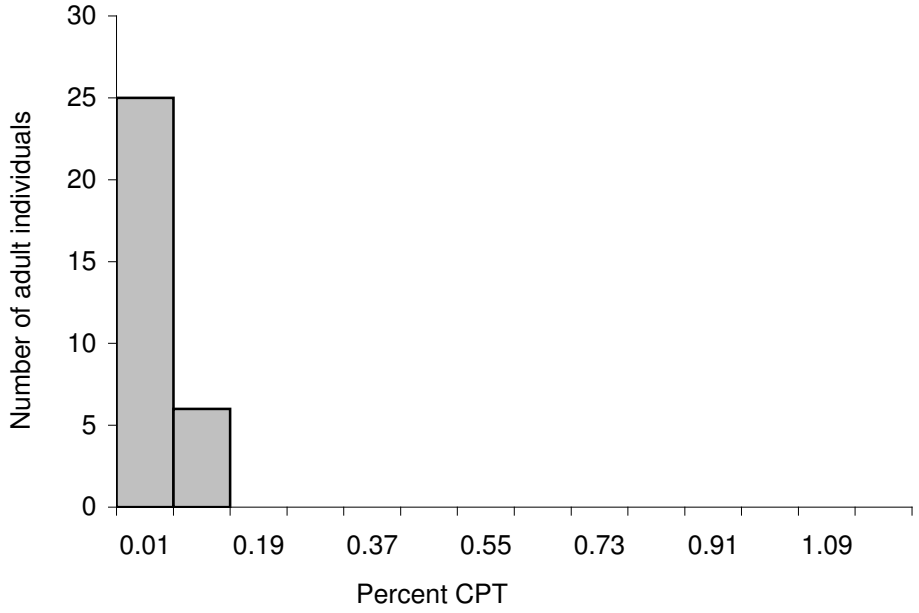


Figure 4.1.19**:** Frequency distribution of CPT content in adult's population from BR hills (n=31).

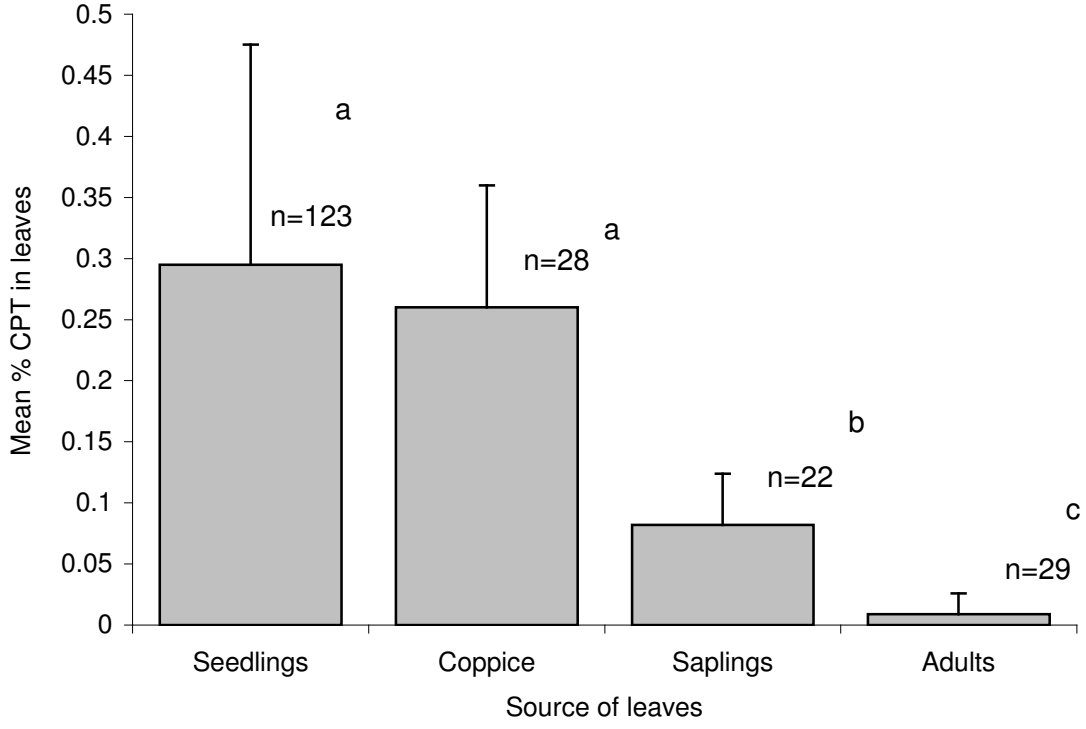


Figure 4.1.20: CPT in leaves of seedlings, coppice, saplings and adults of *N. nimmoniana.* Histograms with dissimilar letters indicate significance at 5% (Students t test).

Amount of biomass and Camptothecin production in *N. nimmoniana* seedlings.

Since the seedlings showed highest leaf percent CPT, they were further analyzed for amount of biomass and total CPT yield. One-year-old seedlings of *N. nimmoniana* produced a total biomass of 16.53 g per seedling on dry weight basis. Among the different tissues measured, root wood contributed a maximum biomass of 4.61 ± 1.34g per seedlings with a mean percent CPT of 0.26 ± 0.13 followed by a shoot wood, which had a biomass of 4.58 ± 1.13g per seedlings with a mean percent CPT of 0.25 ± 0.11. The leaf biomass produced per seedlings was 3.32 ± 0.73g with a mean CPT value of 0.30 ± 0.17. Total amount of Camptothecin produced by one-year-old seedling was 46.78 mg. The root and shoot wood contributed 11.99 mg and 11.45 mg of CPT respectively (Table 4.1.2).

Table 4.1.2: Biomass, percent CPT and CPT yield (mg) in different tissues of *N. nimmoniana* seedlings.

	Biomass per seedling± SD (Dry weight in g)	% CPT± SD	CPT yield (mg)
Leaf	3.32 ± 0.73	0.30 ± 0.17	9.96
Shoot bark	1.67 ± 0.34	0.28 ± 0.16	4.68
Shoot wood	4.58 ± 1.13	0.25 ± 0.11	11.45
Root bark	2.34 ± 0.50	0.37 ± 0.12	8.68
Root wood	4.61 ± 1.34	0.26 ± 0.13	11.99
Total	16.53	0.29	46.78

4.2 Molecular characterization of populations of *Nothapodytes nimmoniana* in the Central Western Ghats, India using SSR markers.

4.2.1 Development of SSR markers for *Nothapodytes nimmoniana.*

Genomic DNA of *N nimmoniana* was isolated and subjected for restriction digestion using RsaI. A continuous smear around 500-1000bp indicated successful digestion (Figure 4.2.1). A digested DNA fragment was purified using eppendorff gel cleaning kit and to the ends both the ends of DNA fragments linkers were ligated. To confirm ligation and increase the yield of linker-ligated products, PCR was done by using the linker-ligated DNA fragments as template. Again continuous smear around 500-1000bp showed successful ligation of linkers to the DNA fragments (Figure 4.2.2A).

SSR sequences from *N nimmoniana* were captured using the biotynlated oligos $(CA)_n$, $(CAA)_n$ and $(AAG)_n$. Enrichment for following SSR repeats has resulted in a small smear in 1.5% agarose gel indicating presence of complimentary SSR repeats in *N nimmoniana*. However, the intensity of the smear was light indicating presence of few SSR repeats in the species (Figure 4.2.2B).

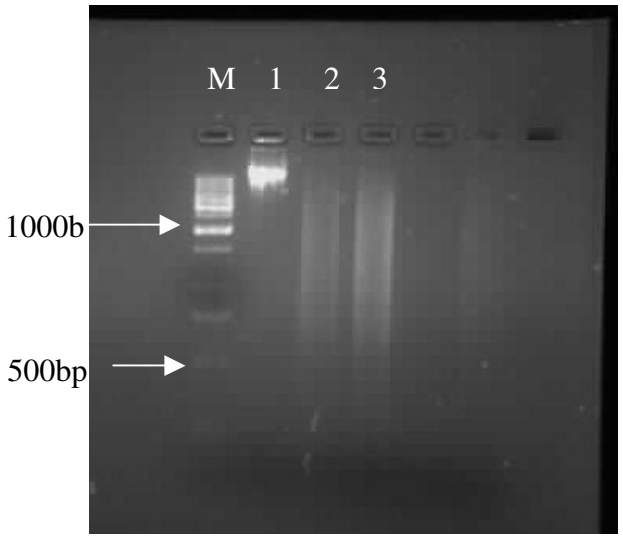


Figure 4.2.1: Restriction digestion of *Nothapodytes nimmoniana* genomic DNA using RsaI (M; Marker, 1: Uncut DNA, 2&3: samples).

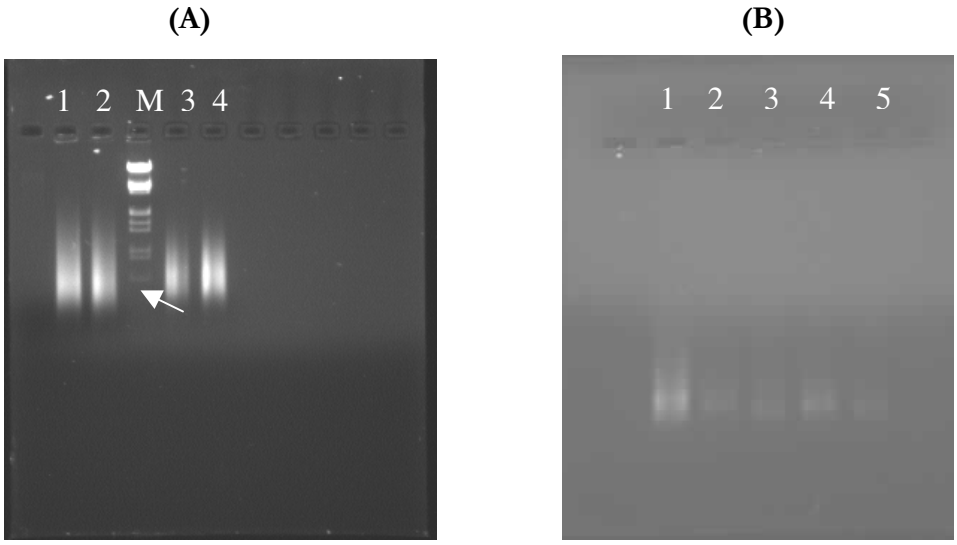


Figure 4.2.2: A; 1.5 % agarose gel showing smear around 500bp. Arrow indicates 500bp fragment of the 1Kbp ladder. 1, 2, 3 and 4 are samples. B; Smear around 500bp in samples 1, 2, 3 and 4 indicated recovery of pure gold DNA (microsatellite enriched sequences).

Transformation of plasmid DNA with inserts:

Pure gold DNA was purified and then ligated into a TA vector (pTZ57R). Vector ligated with the pure gold DNA insert was transformed into competent *E coli* cells. Overnight incubation of plates at 37°C has resulted in appearance of blue and white colonies. Less than 10% of the colonies were blue indicating high degree of transformation (Figure 4.2.3). All the distinct white colonies were individuals picked and transferred to a 96-well plate. All the white colonies were subjected for colony PCR using M13 primers. Almost all the white colonies gave good amplification in the size ranging from 400-700bp (Figure 4.2.4). Some colonies, which were partially blue, also had a band in the expected range.

Sequencing the inserts:

Different sized fragments were carefully chosen, eluted from the gel and sequenced. Thirty-seven distinct clones were sequenced and evaluated for presence of microsatellites using the online free programme SSR finder (http://bioinfo.agri.gov.il/cgi-bin/GE_SSR_Finder.pl). Of the sequences that were scanned for presences of microsatellites only 28 clones (75%) were found to contain microsatellites of adequate size. Only clones with microsatellites size

of more than 15 bases were considered for designing primers. Of these 28 clones, 22 had microsatellite sequences and a flanking region of adequate size for the design of forward and reverse primers (Figure 4.2.5A&B). Primers were designed for these 22 sequences using the default parameters of primer 3 available online (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

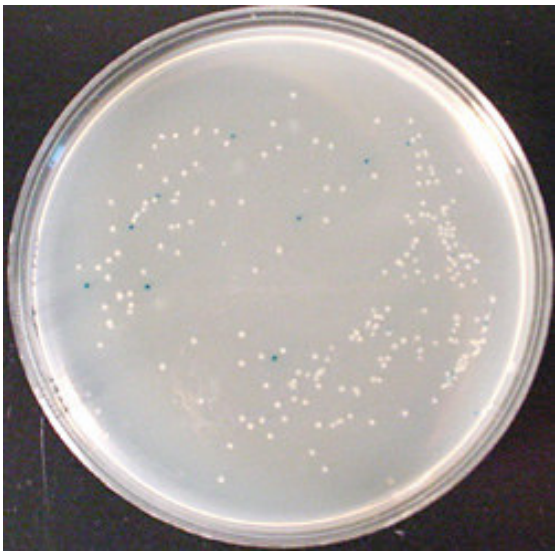


Figure 4.2.3: *E coli* cells transformed with pTZ57R plasmid. Blue (without insert) and white transformed *E coli* colonies (with insert) on antibiotic selection media.

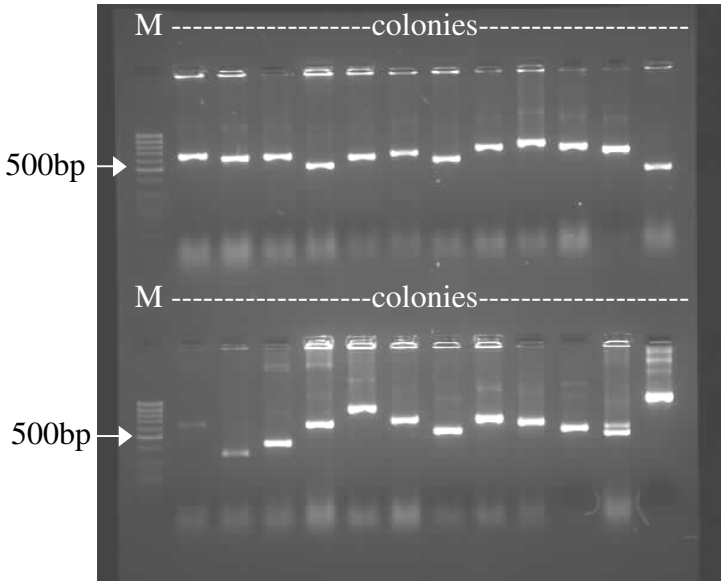


Figure 4.2.4: 1.5 % Agarose gel showing insert length variation. Inserts were amplified using M13 F and R primers (M; 100 bp ladder).

(A)

Clone no: 50

>ACTCGTTACATTCTGGGCAAGTTTAAACTTAAGTTTCTACTTTGATATTGAAT
CAGT**C**ATTACCTCTGCCAGGATG**C**ATGCCGACATATAGTGAACAAATTTTGGA
CATTGCATATGATCTCAGTTCACCATAGCAGAAAAGGATATAGCTACTGATGG
AAATCAAATTGGAGATATCAACGTATAAGTAGATTGGATTCACATTAGGTTCA
ATTCACACTTTTAACTTCG**CAACAACAACAACAACAACAACAACAACA**CTAAGC
CTCAATCCCAGCAATAGGGGTCGGTTATATGAATCTTTCTTTTTCGTTCAGTAC
GATTTAAGGTCGTCGACTCAGCTAATTGGT

(B)

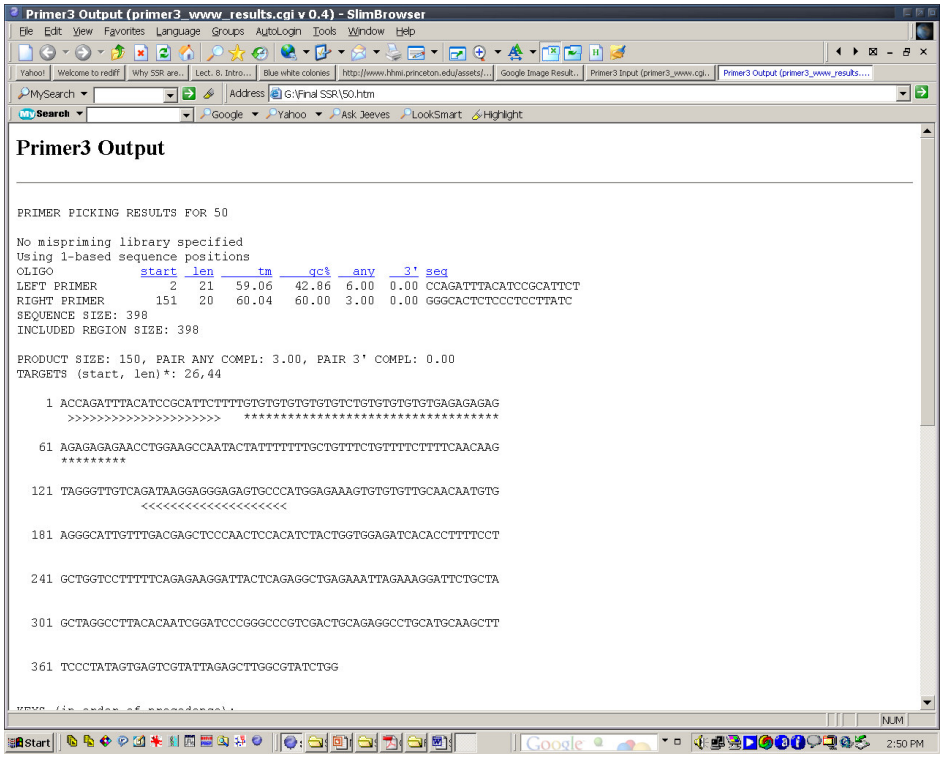


Figure 4.2.5: A; Nucleotide sequence of clone no 50 with CAA repeat of size 29 bases. B; primer3 output for clone no: 50 with forward and reverse primers indicated.

4.2.2 Population genetic variability of *N nimmoniana* populations in the Central Western Ghats.

Standardization of SSR amplification:

Microsatellite DNA loci or simple sequence repeats (SSRs) have become important sources of genetic information for a variety of purposes (Webster and Reichart 2004). To amplify microsatellite loci by PCR, primers must be developed from the DNA that flanks specific microsatellite repeats. In the present study 22 SSR primers were developed for *Nothapodytes nimmoniana* and primer details are listed in the table 3.2 (materials and methods section). All these primers were evaluated for amplification success against one individual of *N nimmoniana* and were separated on 3.5% agarose gels. Out of 22 primers evaluated for amplification success, 11 primers gave good amplification in the expected range. For further genotyping studies over different populations, 5 primers that were polymorphic on 3.5% agarose gels were selected. For example at one of the loci Nnm7, different alleles were observed in the range of 221-241bp with all of them occurring in the expected range (Figure 4.2.6).

Fifty individuals of *N nimmoniana* over 6 populations were evaluated for allelic diversity at 5 selected loci. The amplification products were separated initially on 3.5% agarose gels and further resolved on 10% PAGE gel. The PAGE gels were silver stained and captured using UV transilluminator. Amplification profile over six populations of *N nimmoniana* at 5 loci reveled good allelic variation (Figure 4.2.7 and Figure 4.2.8).

Population genetic was variability assessed using the SSR markers. Out of 22 SSR primers, 11 primers revealed target specific amplification with amplified product in the expected range. Five out of eleven revealed good polymorphism. Using the genetic data on six SSR primers over six populations of *Nothapodytes nimmoniana* following genetic variability parameters were estimated.

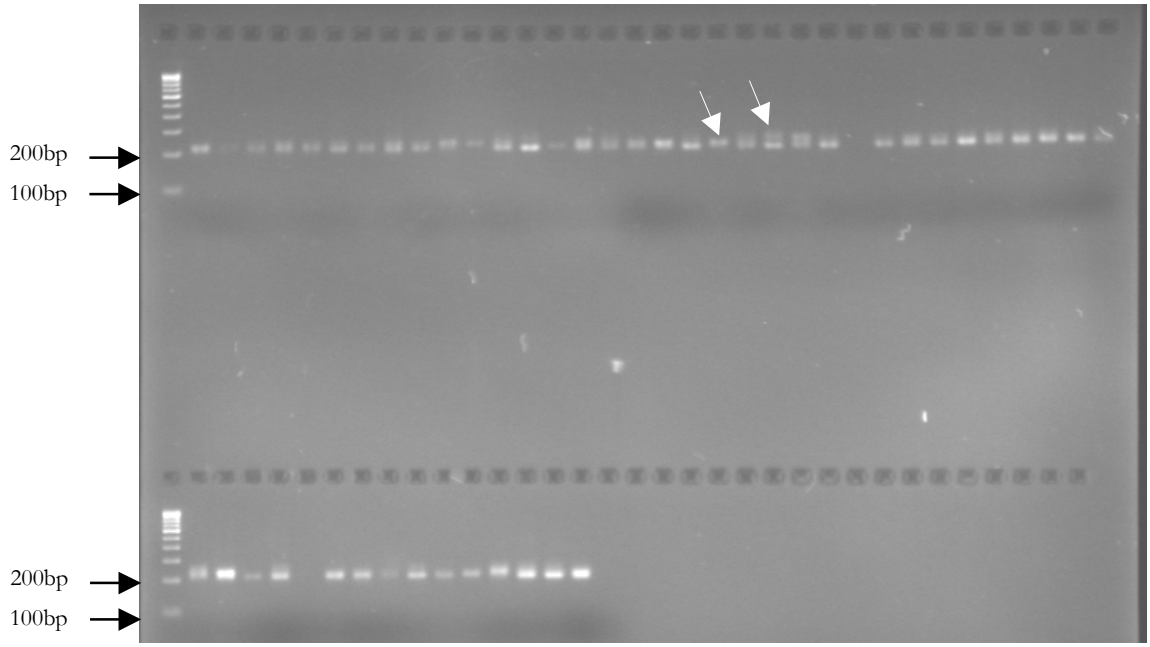


Figure 4.2.6: 3.5 % agarose gel showing SSR amplification over different populations of *Nothapodytes nimmoniana,* at Nnm7 SSR locus. White arrows indicate different alleles in the population.

Number of alleles: The number of alleles detected over all loci across different populations was either 4 or 5. The mean number of alleles across different populations and over all loci was 3.6±0.15. Maximum of four alleles were observed in Vodagere population, followed by Agumbe (3.8±0.37), Mugali (3.6±0.24), and Sirsi (3.6±0.50). In Kemmannagundy and Islur least number of different alleles were observed (Table 4.2.1).

Effective numbers of alleles (Ne): Effective numbers of alleles (those that are required to maintain current level of heterozygosity) was also estimated over all populations. In Sirsi, Kemmannagundy, Agumbe and Vodagere populations, estimated Ne was 2.81-2.83 followed by 2.45 and 2.66 in Islur and Mugali respectively. Over all populations, effective number of alleles was 2.7±0.1 (Table 4.2.1).

Allele frequency: At locus 1 (Nnm6), 6 different alleles were observed. Allele one was most frequent in all populations of *Nothapodytes nimmoniana.* Some of the alleles at this locus were

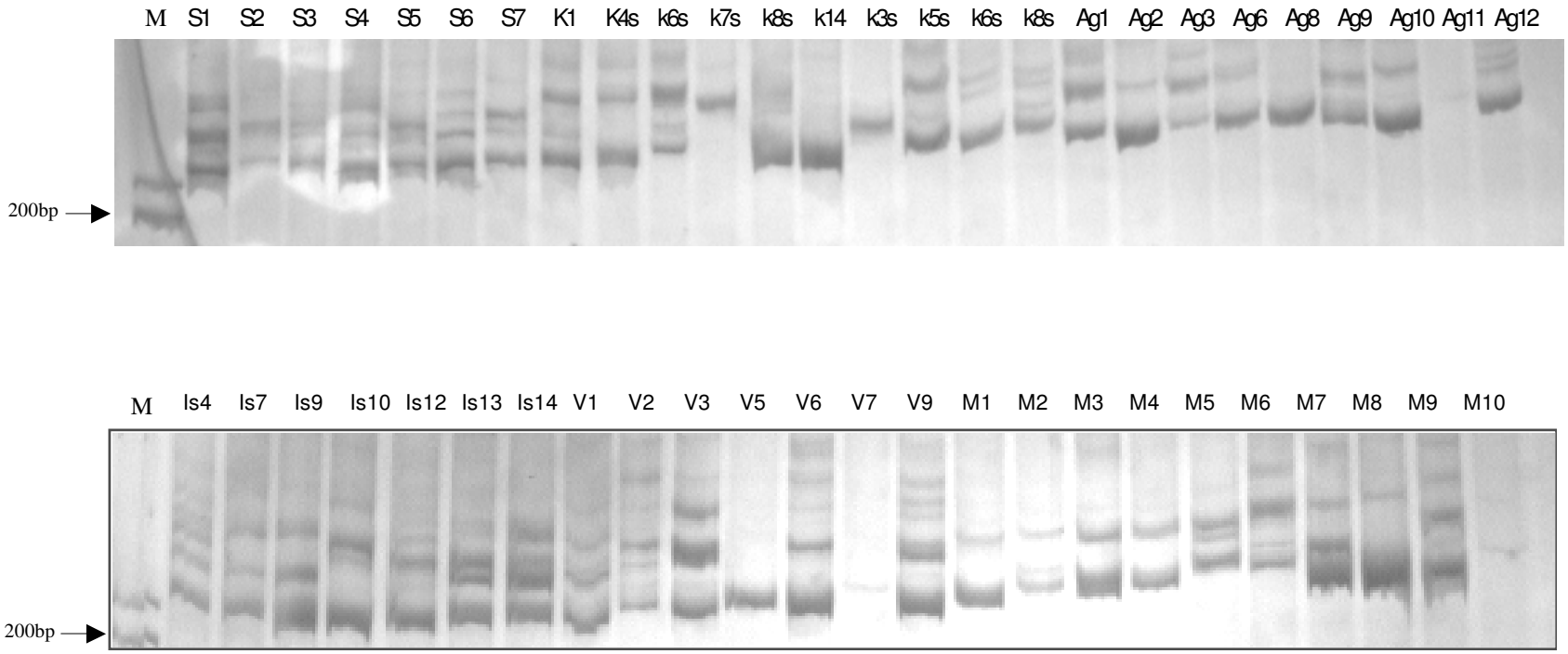


Figure 4.2.7: Nnm7 SSR primer amplification profile (range: 221 to 241 bp) of six different populations of *Nothapodytes nimmoniana* resolved by electrophoresis on a 10% polyacrylamide gel (S; Sirsi, K; Kemmannagundy, Ag; Agumbe, Is;Islur, V; Vodagere, M; Mugali). The M label stands for molecular weight standard.

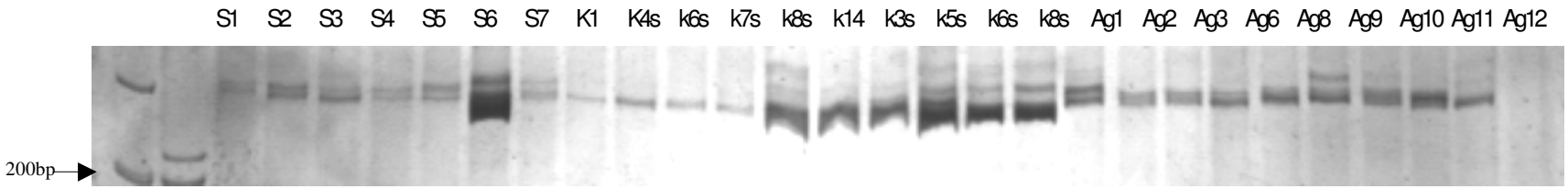


Figure 4.2.8: Nn54 SSR primer amplification profile (range: 230 to 254 bp) of six different populations of *Nothapodytes nimmoniana* resolved by electrophoresis on a 10% polyacrylamide gel (S; Sirsi, K; Kemmannagundy, Ag; Agumbe). M; 100bp ladder.

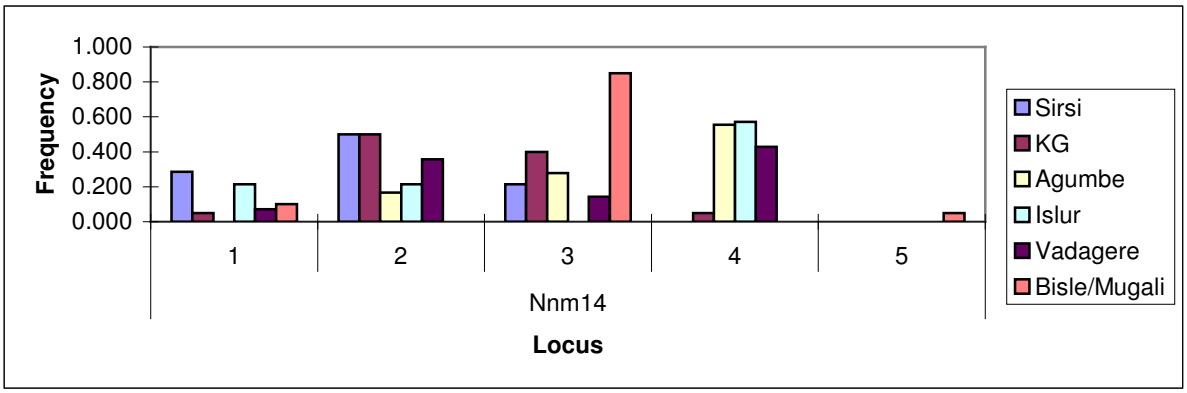
observed in only one or two populations. Allele number 5 was observed in Sirsi and Islur populations. At locus3 (Nnm14) 5 different alleles were observed and allele 3 was most frequent in Mugali population. Allele 5 was observed only in Mugali population. Some of the common alleles at locus 4 (NnT4) and locus 5 (Nn 54) were found in 50% of the individuals over all populations of *Nothapodytes nimmoniana*. Alleles that are specific to one or two populations were allele 6 at Nnm7, allele 1 and 5 at Nn54 in less than 20% of the individuals indicating their uniqueness (Figure 4.2.9).

Table 4.2.1: Population genetic parameters of six different populations of *Nothapodytes nimmoniana* based on 5 SSR primers.

Populations	**Na**	**Ne**	**Ho ± SD**	**He ± SD**
Sirsi	3.6±0.50	2.82±0.43	0.83±0.11	0.61±0.05
Kemmannagundy	3.4±0.6	2.81±0.49	0.58±0.08	0.599±0.06
Agumbe	3.8±0.37	2.83±0.28	0.51±0.13	0.63±0.03
Islur	3.2±0.20	2.45±0.09	0.82±0.10	0.59±0.01
Vodagare	4±0.31	2.81±0.17	0.74±0.09	0.64±0.02
Mugali	3.6±0.24	2.66±0.39	0.56±0.12	0.578±0.08
Over all populations and all loci	3.6±0.15	2.7±0.1	0.67±0.04	0.60±0.01

Na: Observed number of alleles, **Ne:** Expected number of alleles, **Ho:** Observed heterozygosity, **He:** expected heterozygosity and **SD:** Standard deviation.

Heterozygosity: The mean observed heterozygosity over all populations at all loci was 0.67±0.04. Mean observed hetrozygosity (over all populations and at all loci) was more than expected heterozygosity based on Hardy Weinberg equilibrium 0.60±0.01. Among different populations Sirsi (0.83±0.11), Islur (0.82±0.10) and Vodagere (0.74±0.09) had highest observed heterozygosity and in all the three cases Ho was more than expected heterozygosity. Kemmannagundy, Agumbe and Mugali populations had least observed heterozygosity. In summary populations in the northern part of the Western Ghats, maintained higher heterozygosity compared to the ones in the southern Western Ghats (Table 4.2.1).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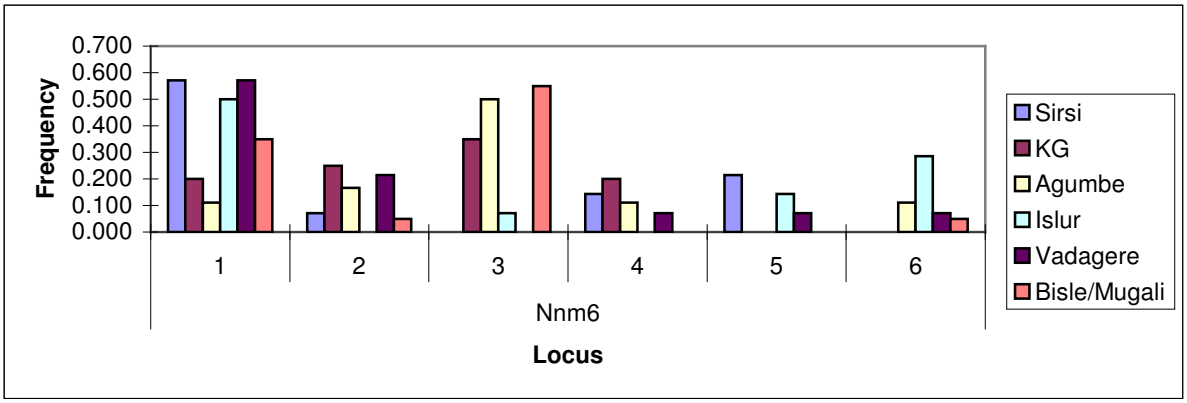
Agumbe
Islur
Vadagere
Bisle/Mugali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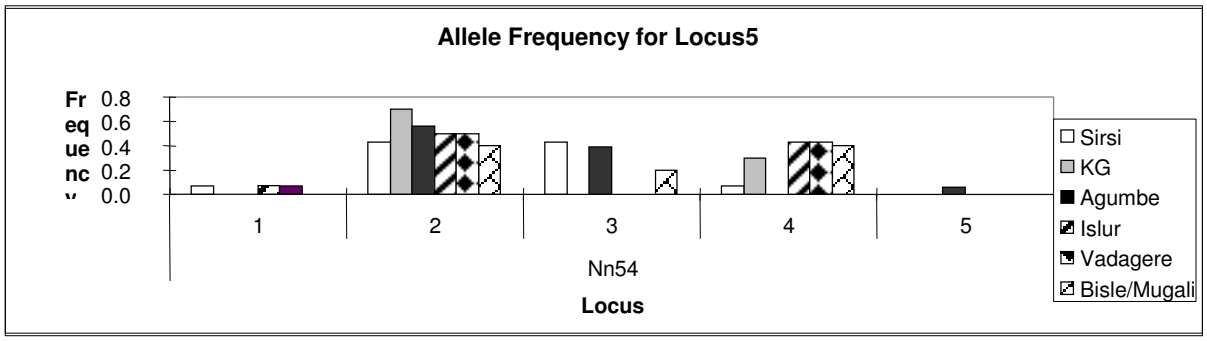
Allele Frequency for Locus5
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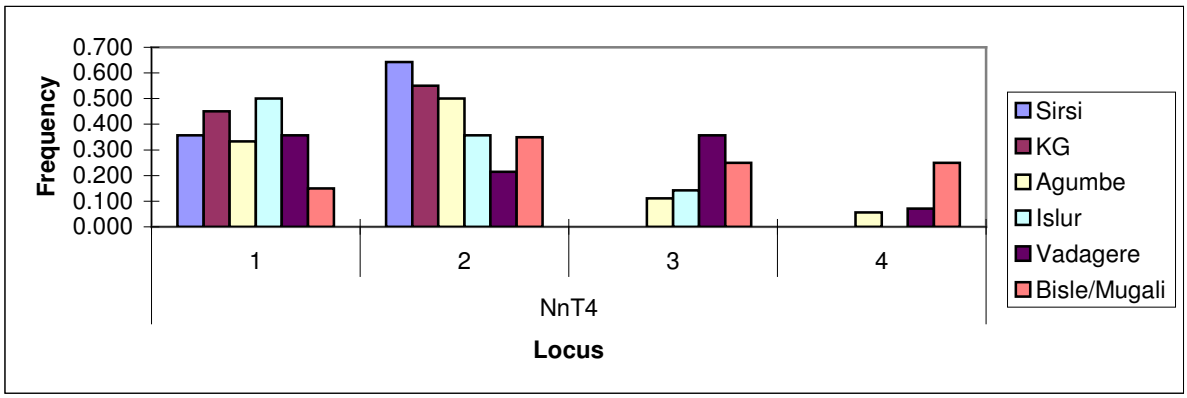
Vadagere
Bisle/Mugali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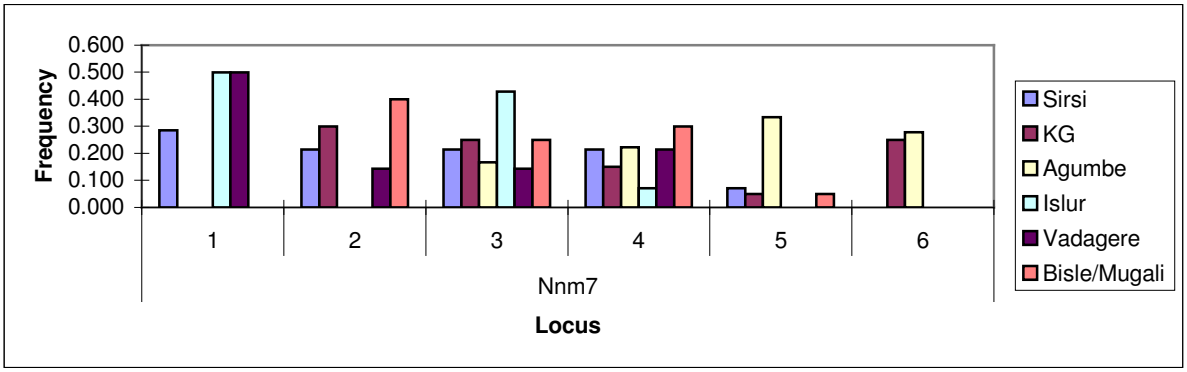


Figure 4.2.9: Allele frequency pattern over different populations of *Nothapodytes nimmoniana* based on 5 different SSR primers.

AMOVA: An AMOVA of the 6 populations indicated that a large percentage of total genetic variation (83) was within populations, with only about (17% P<0.01) was among populations (Table 1.2).

Table 4.2.2: Analysis of Molecular Variance (AMOVA) based on 5 SSR primer data over different populations of *Nothapodytes nimmoniana* in the Western Ghats.

Variable	df	SS	MS	Est. Var.	%	Stat	Value	Probability
Among Populations	5.00	139.53	27.91	1.30	0.17			
Within Pops	94.00	600.59	6.39	6.39	0.83	Rst	0.17	0.01
Total	99.00	740.12	34.30	7.69				

Association between Ho and latitude of populations: Levels of heterozygosity at each populations was correlated with their respective latitude of collections. There was a positive and significant correlation between observed heterozygosity of the population and latitude, indicating populations in the northern part of the Western Ghats are genetically more diverse as compared to ones in the southern part (Figure 1.13: P<0.01).

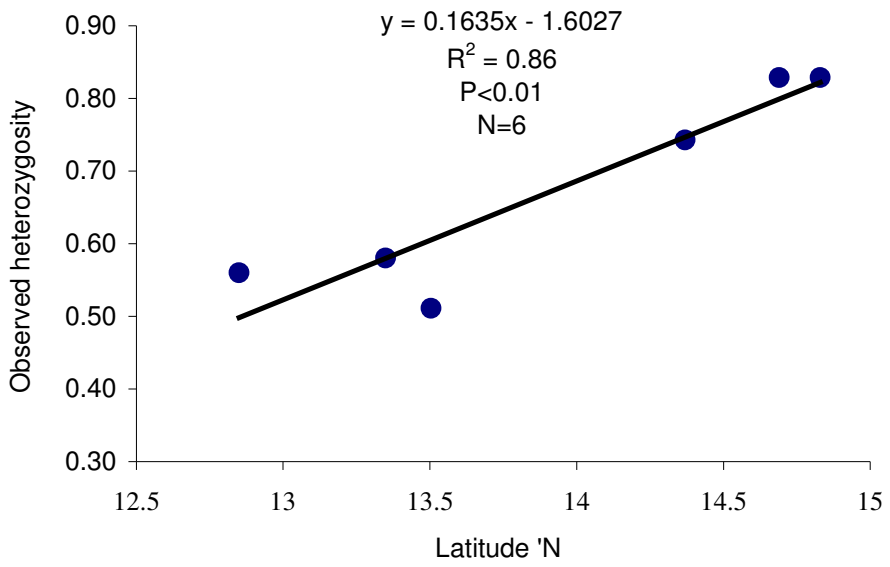


Figure 4.2.10: Correlation between latitude and observed heterozygosity of *Nothapodytes nimmoniana* populations in the central Western Ghats.

4.3 Prospecting phylogenetically related genera/species of *Nothapodytes nimmoniana* for Camptothecin.

4.3.1 Mapping the related genera of *Nothapodytes nimmoniana* in the Western Ghats, India.

Based on the secondary data obtained from herbaria sheets and floras the distribution maps of all the related genera of Icacinaceae members in the Western Ghats were developed. *Apodytes beddomei* was found in Kemmanagundi, parts of Shiridi Ghats in Karnataka and in the belt of Mananthawady and Silent valley in Kerala. This genus was also found in southern Western Ghats in parts of Thirunevelli etc (Figure 4.3.1). *Sarcostigma kleineii* was found in Makut of Coorg and is reported in many parts of Kerala like Gudalur, Devala, Udumanparai and Thenmala etc. This genus is restricted to Kerala part of the Western Ghats (Figure 4.3.2).

The genus *Gomphandra* has two species, which are restricted to the Kerala part of the Western Ghats. In Karnataka it is found in parts of Bababudangiri hills. *Gomphandra polymorpha* is densely distributed in parts of Kennakadu, Karimbam, Thovai, Annamalai, etc of Kerala. Similarly species *Gomphandra coriaceae* is sparsely distributed in southern parts of Kerala and parts of Tamil Nadu in Quilon (Figure 4.3.3).

Pyrenacantha volubilis is distributed in south-east of Andhra Pradesh in Hajjipuram and in Kannangudi in east and in many parts of Tamil Nadu in east (Figure 4.3.4). The genus *Natsiatum herpaticum* is very sparsely distributed in eastern part of Tamil Nadu and Andhra Pradesh (Figure 4.3.5A).

Miquelia dentata is distributed in regions of Chimmini dam, Idukki and Silent valley in Kerala and in south-east of Karnataka in Bettagiri (Figure 4.3.5B). In summary except genus *Nothapodytes nimmoniana* which is widely distributed in the Western Ghats, rest of the Icaccinaceae genera have a relatively restricted distribution in the Western Ghats.

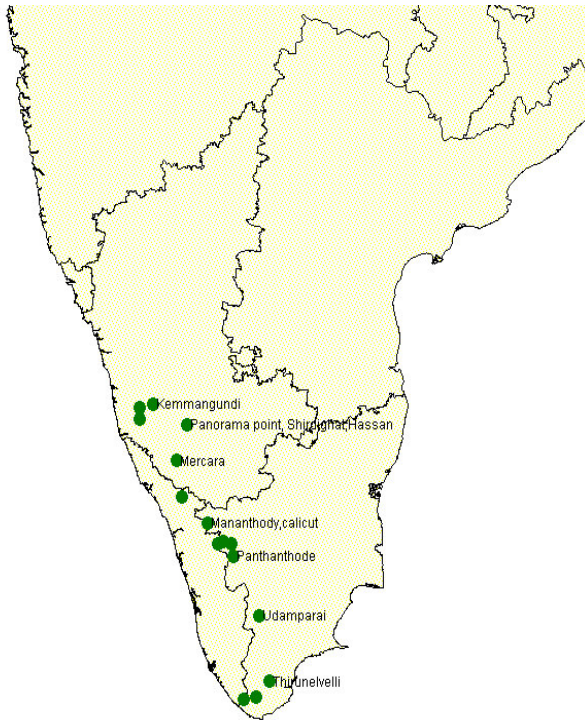
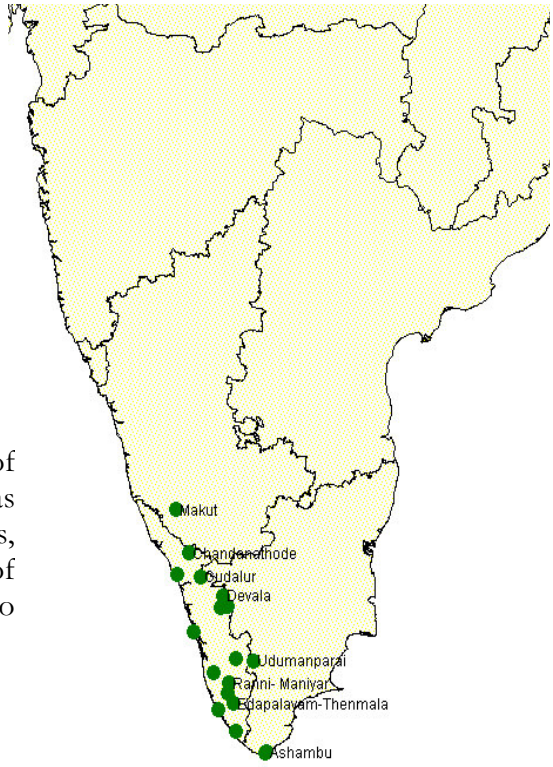
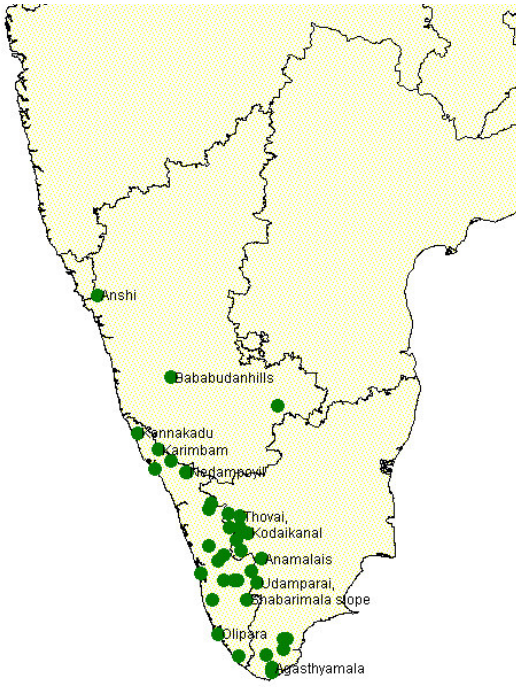


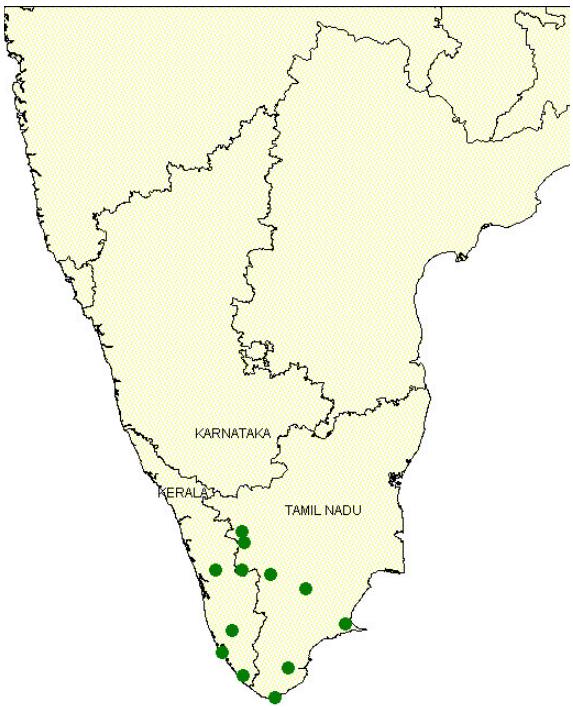
Figure 4.3.1 : Map showing the distribution of *Apodytes beddomei* in the Western Ghats. Map was developed based on the data from herbaria sheets, floras and other records. Pressed herbaria specimen with collection details is also shown.

Figure 4.3.2 : Map showing the distribution of *Sarcostigma klenii* in the Western Ghats. Map was developed based on the data from herbaria sheets, floras and other records. Pressed herbaria image of *Apodytes beddomei* with collection details is also shown.





Gomphandra polymorpha



Gomphandra coriaceae

Figure 4.3.3: Map showing the distribution of *Gomphandra* in Western Ghats of India. Map was developed based on the data from herbaria sheets, floras and other records. Pressed herbaria specimen with collection details is also shown.

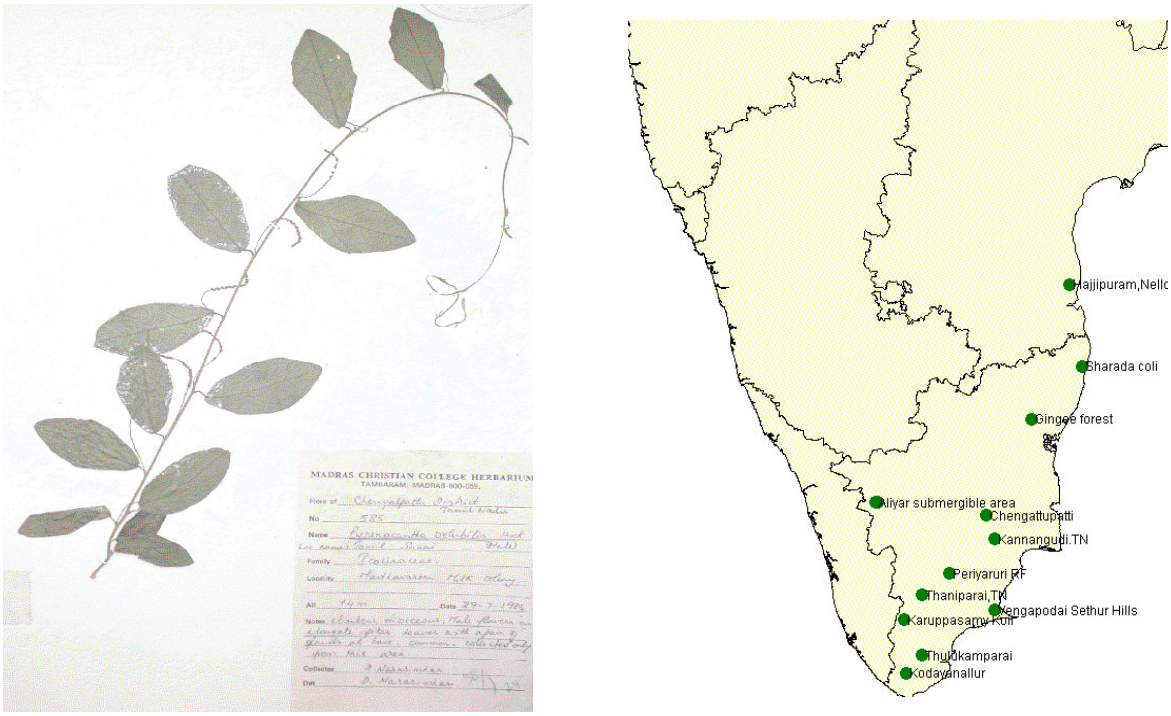


Figure 4.3.4: Map showing the distribution of *Pyrenacantha volubilis* in parts of Tamil Nadu, India. Map was developed based on the data from herbaria sheets, floras and other records. Pressed herbaria specimen with collection details is also shown.

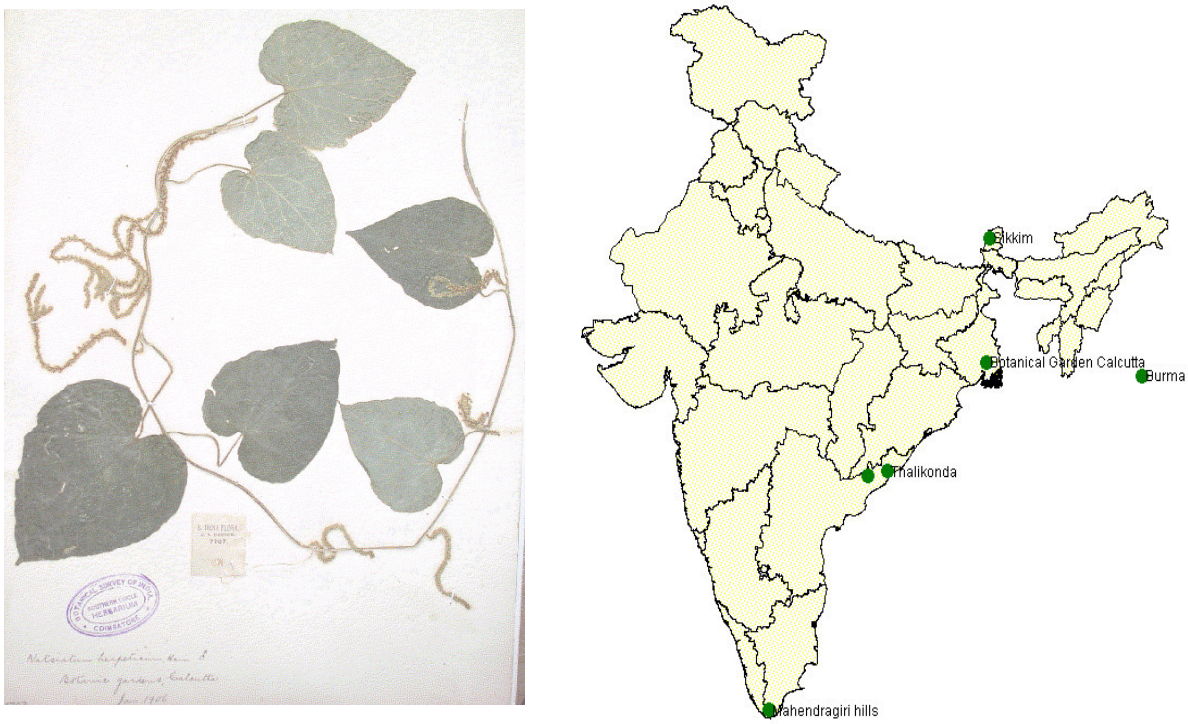


Figure 4.3.5A: Map showing the distribution of *Natsiatum herpaticum* in India. Map was developed based on the data from herbaria sheets, floras and other records. Pressed herbaria specimen with collection details is also shown.

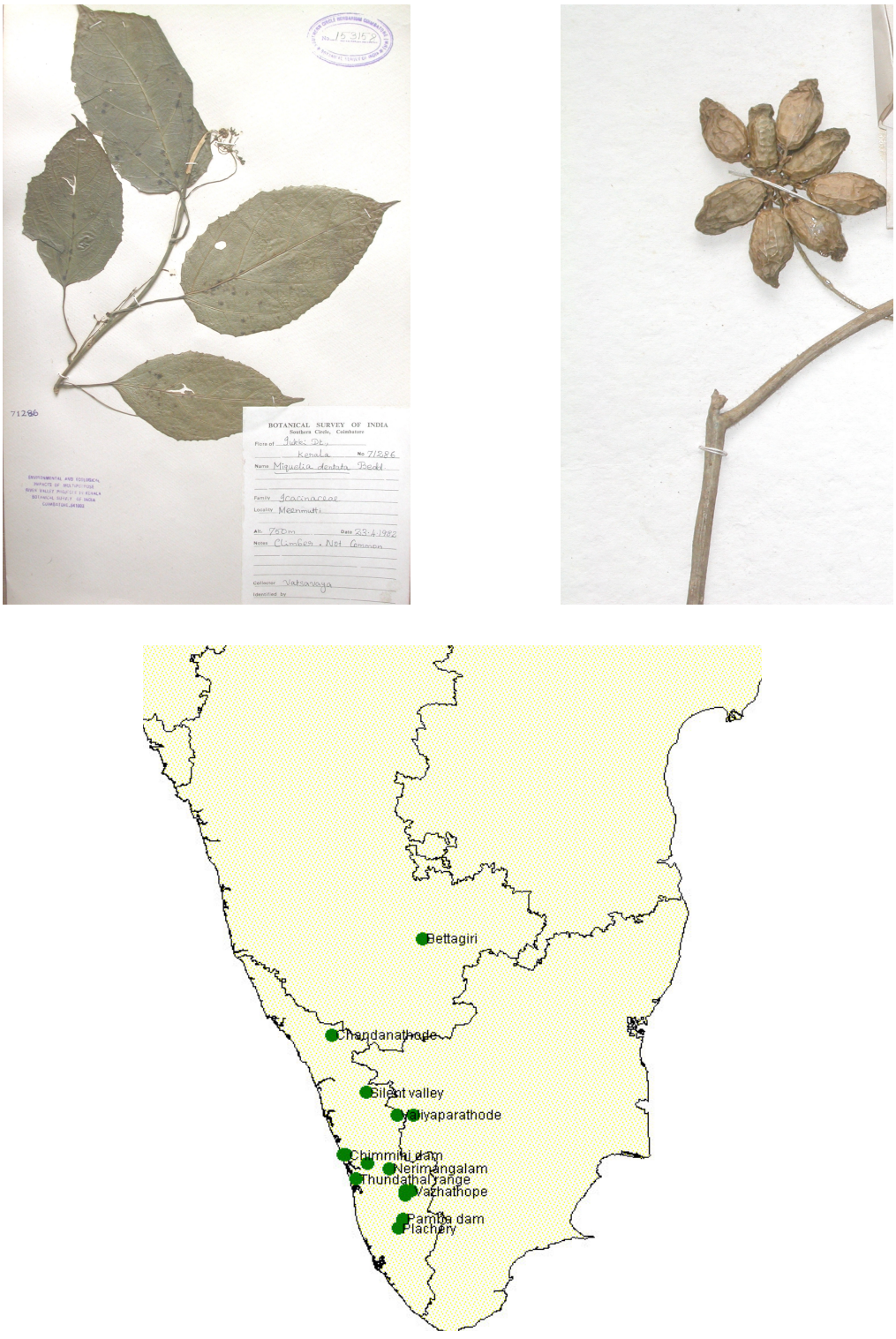


Figure 4.3.5B: Map showing the distribution of *Miquelia dentata* in Western Ghats of India. Map was developed based on the data from herbaria sheets, floras and other records. Pressed herbaria specimen with collection details is also shown.

4.3.2 Chemical profiling of related genera of *Nothapodytes nimmoniana* in the Western Ghats, India.

In the present study, different phylogenetically related genera of *N nimmoniana* were selected and screened for Camptothecin. About 8 different genera were short listed for the study and spatially explicit distribution maps were developed for the study. Using the distribution maps samples of all these genera including different plant parts were collected. All these samples were subjected for CPT analysis.

HPLC results

Presence and quantity of CPT was been confirmed by HPLC analysis. HPLC profile of all the related genera extracts showed the presence of CPT except in *Strombansia zeylanica* (Figure 4.3.6). Among the Icaccinaceae genera that were screened for CPT *Natsiatum herpaticum* fruits had maximum CPT of 0.026% followed by *Sarcostigma klenii* leaf (0.018%) and *Gomphandra polymorpha* fruits (0.011%). The genera *Pyrenacantha volubilis, Apodytes dimidiata* and *Gomphandra tetrandra* had CPT less than 0.01% in both the leaf and stem bark samples analyzed. In *Apodytes dimidiata* CPT was detected only in the stem bark sample and was not detected in the leaf sample. Across different plants parts of different genera analyzed, CPT was higher in stem bark than in leaf or fruits (Table 4.3.1).

Two genera *Ophiorhiza pumila* and *Tobermontana heyneana* belonging to the family Rubiacaeae and Apocynaceae respectively were also screened for CPT. CPT was detected in both the parts of *Ophiorhiza pumila* and *Tobermontana heyneana*. *Ophiorhiza pumila* root had more CPT (0.1%) followed by stem bark of *Tobermontana heyneana* (Figure 4.3.6 and Table 4.3.1). Leaf sample of *Ophiorhiza pumila* had more CPT (0.052%) as compared to leaf samples of other species screened.

Among the 13 different species belong to three different families (Icacinaceae, Rubiaceae and Apocynaceae) screened for CPT herbarium specimens of one of the specimens consistently had more CPT in the fruits (Figure 4.3.7). HPLC profile of R56 and R59 (for proprietary reasons the specimen name has been coded) showed string signals presence of

more CPT (Figure 4.3.7). Leaves of the herbarium specimen of this species had very less CPT and it was not detected in one of the sample (data not given).

Efforts were also made to collect fresh specimens of R56 from the Western Ghats, India. Leaf and very young fruit samples of R56 were collected from the Western Ghats during June 2007. HPLC analysis of these samples showed a CPT peak and the concentration was 0.011 and 0.008% in two fruit samples respectively (Figure 4.3.8). This concentration is 100 times less than the concentration that is present in the matured fruits of herbarium specimens.

Four months after initial collection, again the fruit samples of 9 different plants of R56 were collected from same site (during October 2007). To see the pattern of accumulation of CPT with in a fruit, fruit coat and embryo were separated and analyzed using HPLC. HPLC profile showed the presence of CPT in both fruit coat and embryo with considerable variation in the quantity (Figure 4.3.9 and 4.3.10). CPT content in fruit coat ranges from 0.03%-0.14% with a mean of 0.069±0.035%. Similarly in the embryo it ranged from 0.31-0.83% with a mean CPT content of 0.55%±0.25% (Table 4.3.2).

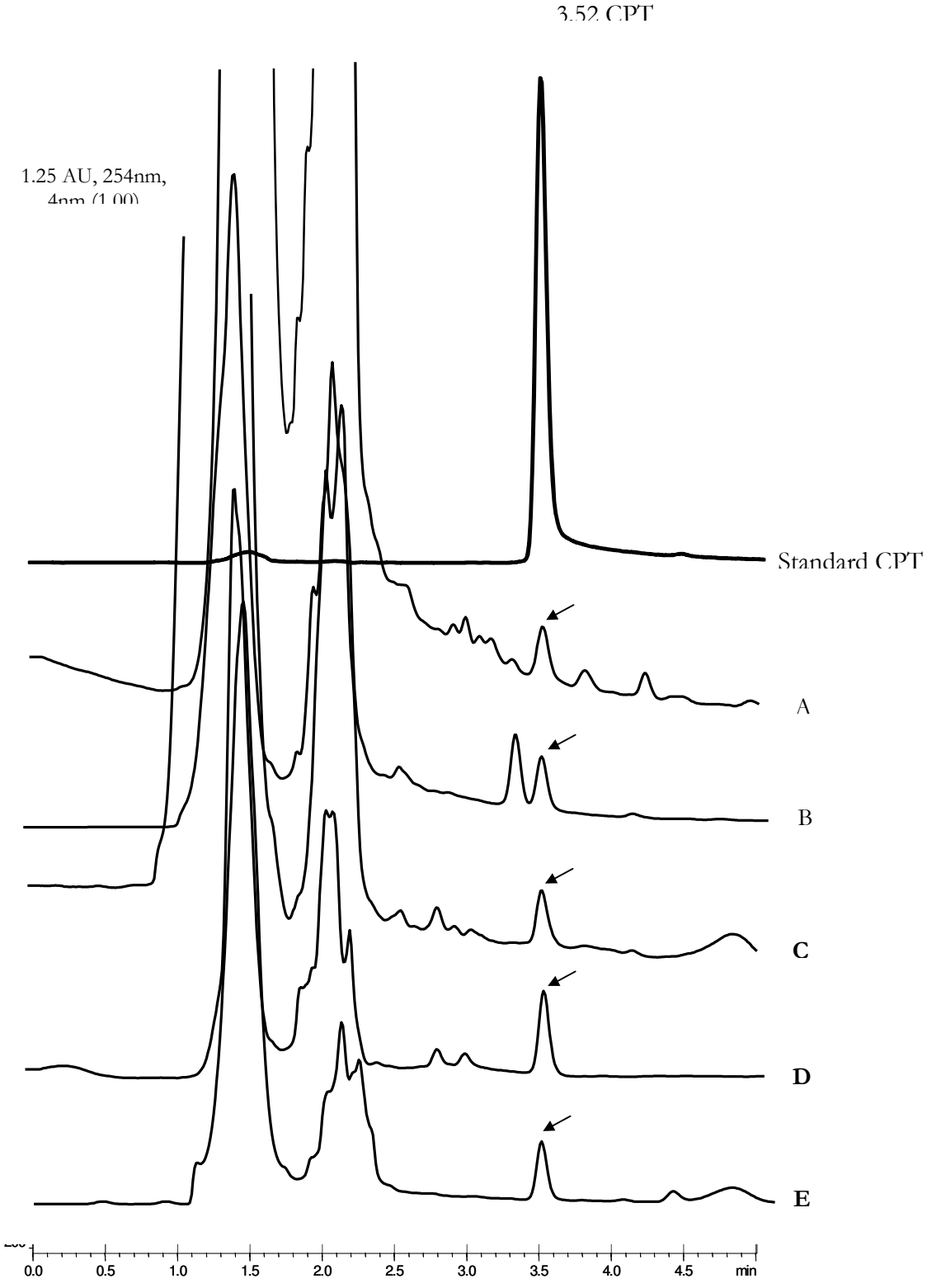


Figure 4.3.6: HPLC profile of different genera of Icaccinacae and others. HPLC profile of standard Camptothecin indicated a peak at 3.52 min, similarly in different plant samples CPT peak is indicated with an arrow. A; *Gomphandra tetrandra* stem bark B; *Sarcostigma kleinii* leaf C; *Pyrenacantha volubilis* leaf D; *Ophiorhiza pumila* stem E; *Natsiatum herpaticum* fruits.

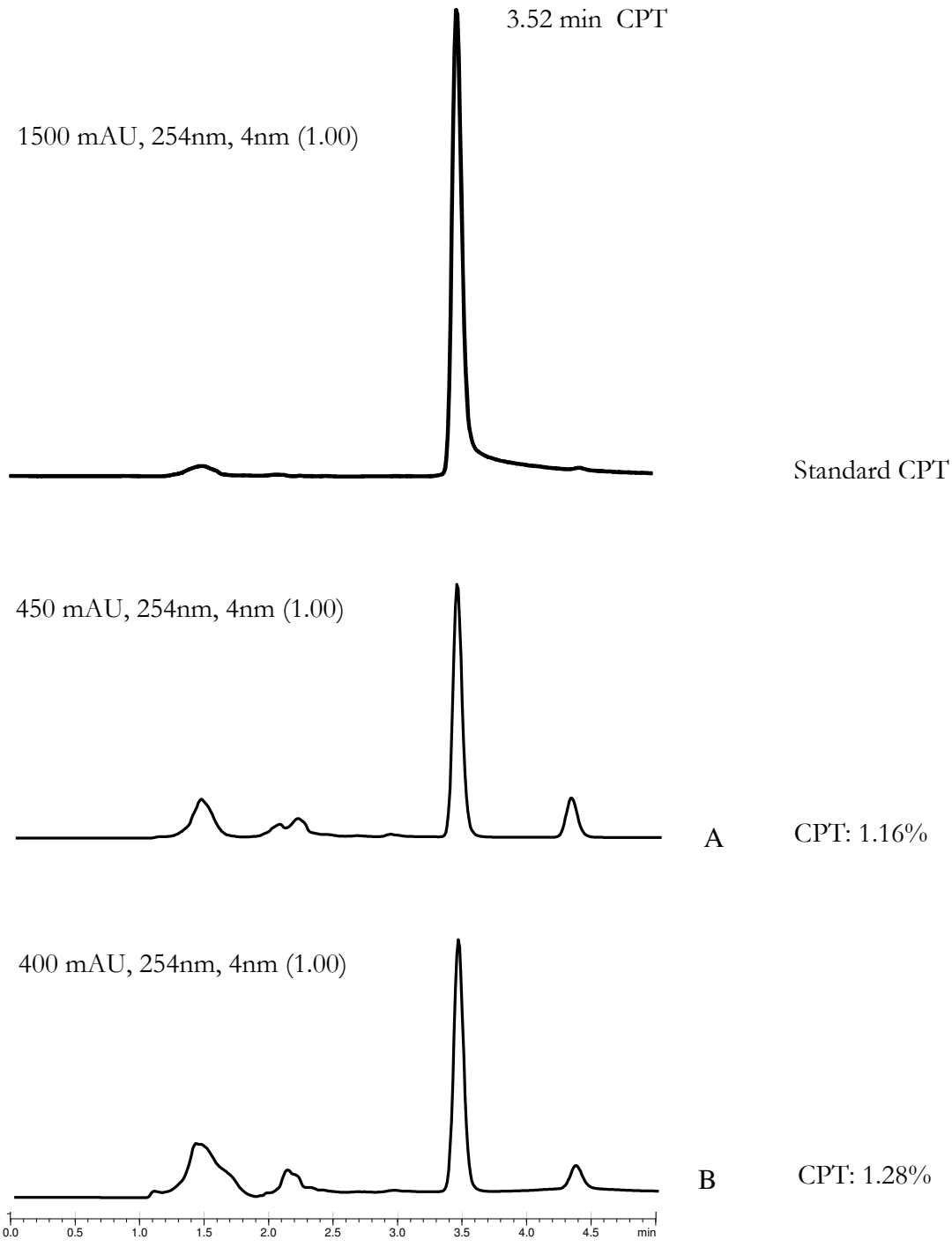


Figure 4.3.7: HPLC chromatograms showing CPT peak in standard at 3.52 min and in samples. A; R56 (two fruits) B; R59 (one fruit).

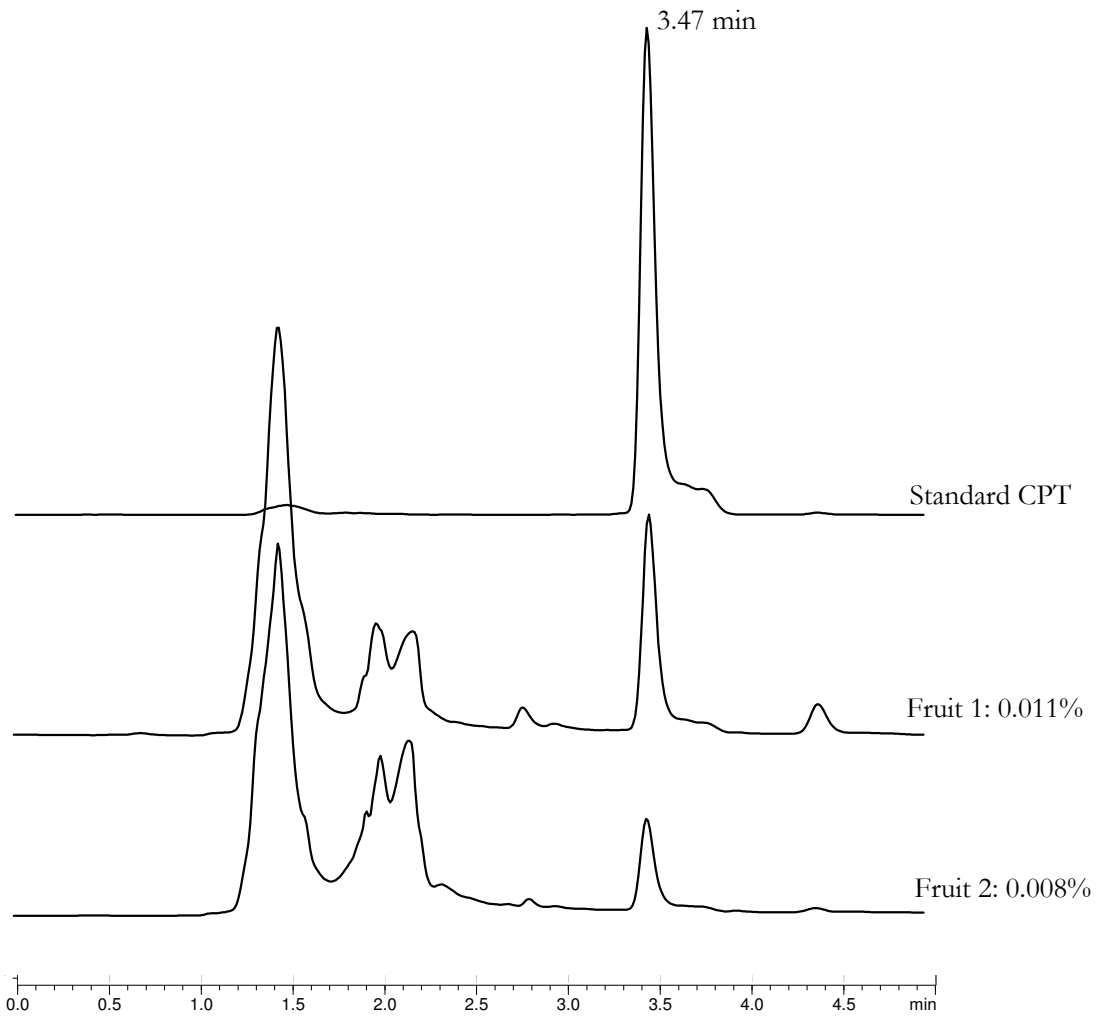


Figure 4.3.8: HPLC chromatograms standard CPT showing CPT peak at 3.47 min, similarly in imature fruit samples (fruit1 and fruit2) of R56 collected during June 2007 from the Western Ghats, India.

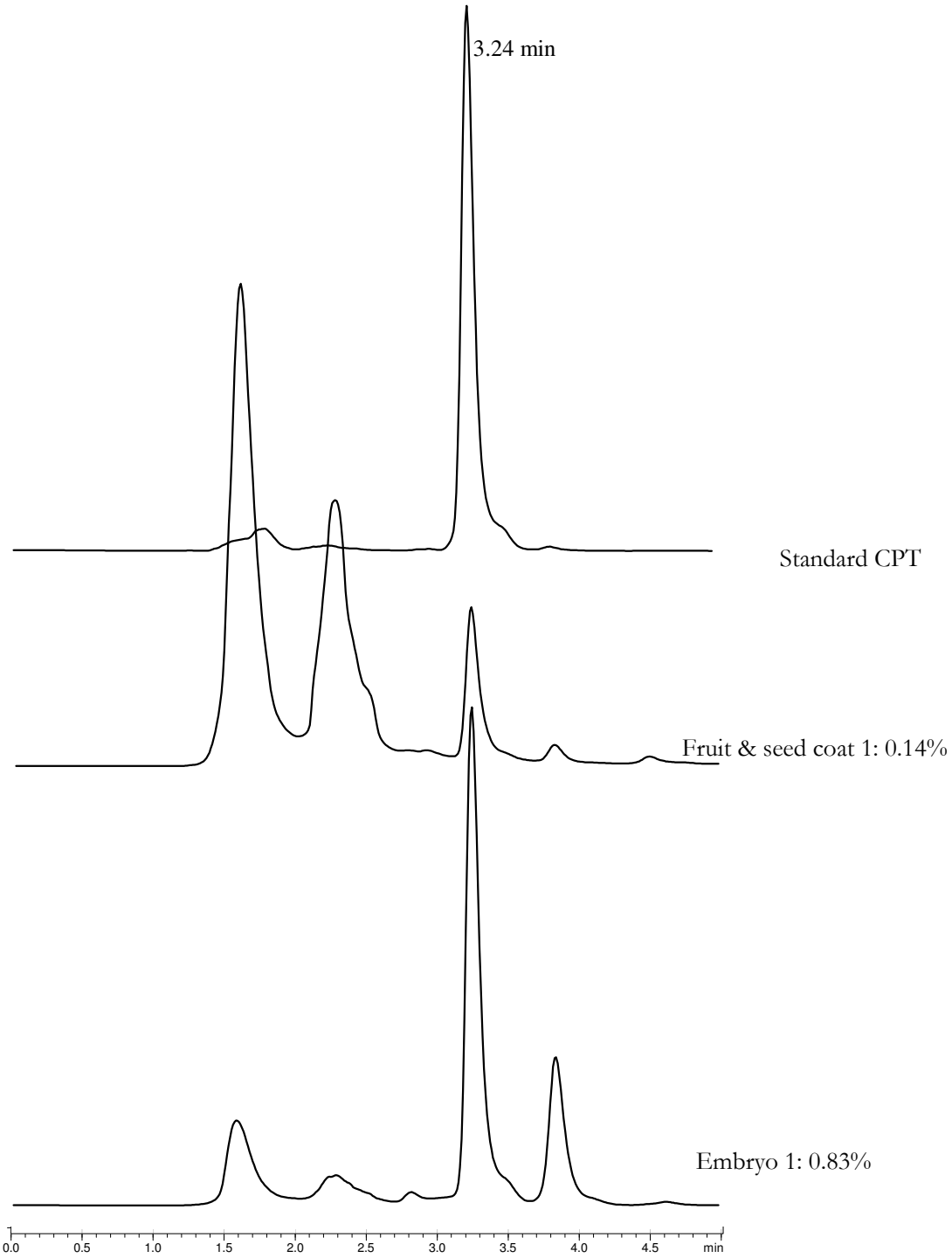


Figure 4.3.9: HPLC chromatograms of standard CPT showing CPT peak at 3.24 min. Partially matured fruit samples of R56 collected during October 2007 from the Western Ghats, showed CPT of 0.14% in fruit coat and seed coat and 0.83% in its embryo (sample no: 1).

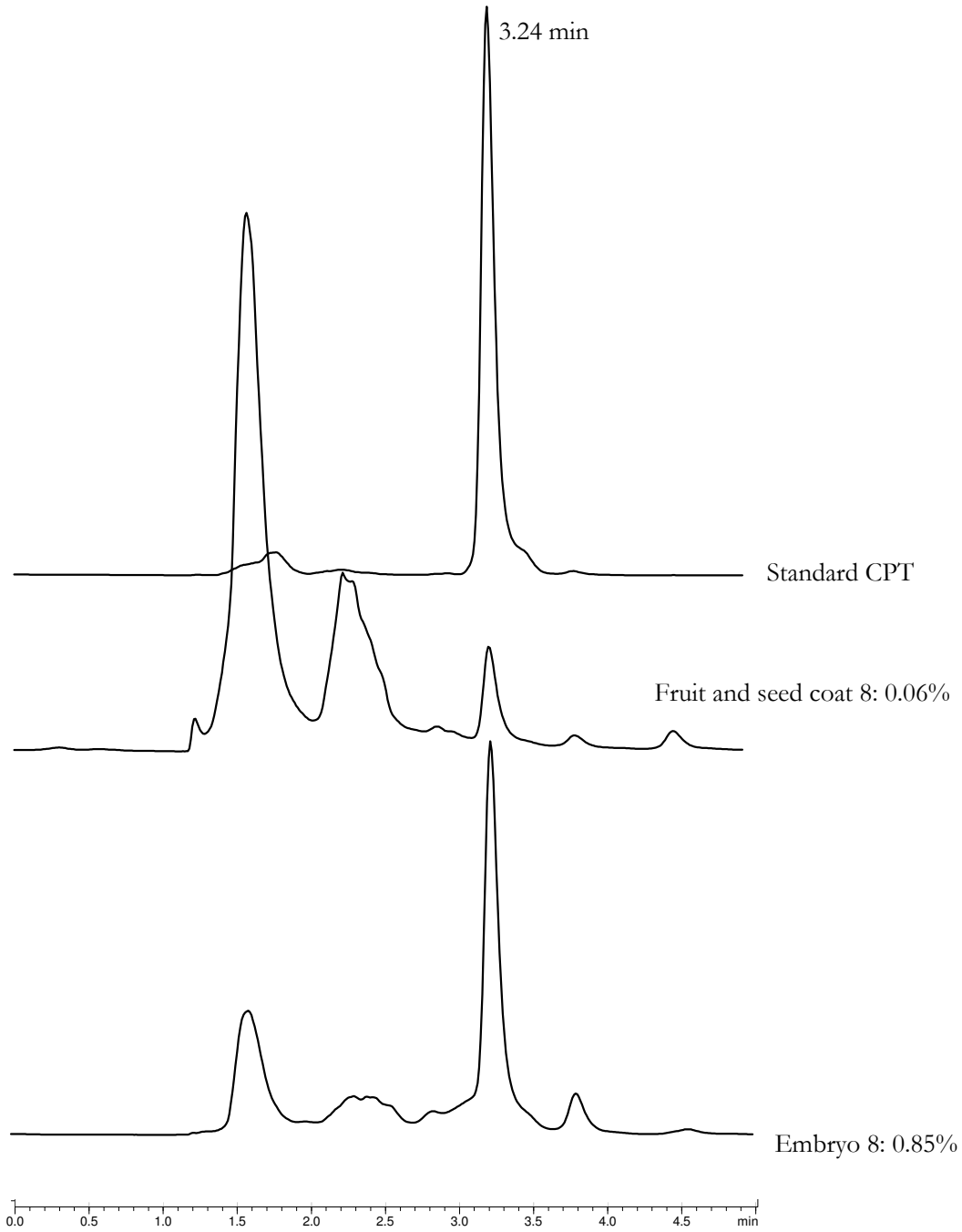


Figure 4.3.10: HPLC chromatograms of standard CPT showing CPT peak at 3.24 min. Partially matured fruit samples of R56 collected during October 2007 from the Western Ghats, showed CPT of 0.06% in fruit coat and seed coat and 0.85% in its embryo (sample no: 8).

Table 4.3.2: Percent CPT content in fruit and seed coat and respective embryos in partially matured fruits of R56 collected during October 2007 from Western Ghats.

	Percent CPT based on HPLC

Sl no	Fruit and seed coat	Embryo
Fruit 1	0.14	0.83
Fruit 2	0.03	-
Fruit 3	0.04	0.33
Fruit 4	0.06	0.31
Fruit 5	0.06	0.33
Fruit 6	0.1	0.78
Fruit 7	0.09	0.46
Fruit 8	0.06	0.85
Fruit 9	0.04	-
Mean	0.069	0.556
SD	0.035	0.253

LC-MS/MS results

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a versatile system which combines both selectivity and sensitivity, and it is generally considered as the most reliable technique to quantify chemical compounds in crude extracts. The method is highly sensitive and helps in detection of nano grams quantity of compound of interest in plants extracts. In the present study LC-MS/MS (done at RRL, Jammu) was used to quantify CPT from various plants extracts.

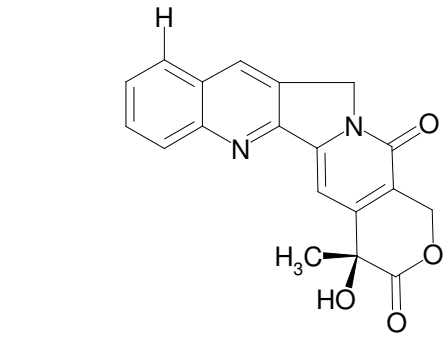
The same plant extracts (13 different species belong to three different families (Icacinaceae, Rubiaceae and Apocynaceae) were used in detection and quantification of CPT using LC-MS/MS. The daughter ion peak at m/z 305 was taken up for quantification (Figure 4.3.11). In some cases quantification was done based on all the three transitions (ions).

LC-MS-MS profile of standard CPT showed appearance of m/z 305 ion at 8.11 min. Similarly in the samples *Pyrenacantha volubilis* leaf, *Ophiorhiza pumila* stem and *Sacrostigma kleinii* leaf ion eluted at 8.09 min (Figure 4.3.11). LC-MS/MS profile of samples R56 and R59 showed appearance of all the three ions (m/z 305, m/z 263, m/z 247) of parent molecule at 8.09 min. CPT in these two extracts has been quantified based on all the three transitions (Figure 4.3.12).

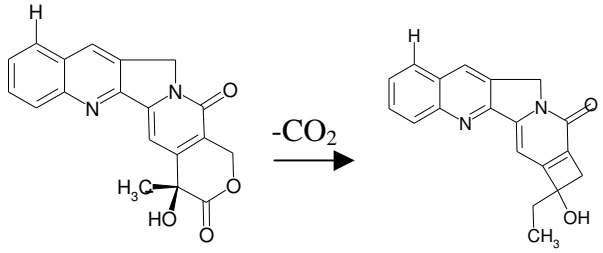
LC-MS-MS results showed that, among the Icacinaceae genera screened for CPT *Sacrostigma kleinii* leaf, had maximum CPT (0.14%) followed by *Pyrenacantha volubilis* leaf (0.04%) and then in *Natsiatum herpaticum* fruits (0.01%). Least CPT was found in *Gomphandra tetrandra* stem bark (0.0008%). Stem samples of *Ophiorhiza pumila* and *Tabermontana heyneana* had CPT of 0.02% each (Table 4.3.1).

LC-MS-MS estimation also showed presence of significant amount of CPT in fruit samples of R56 (1.61%) and R59 (0.45%). Along with these two samples one more fruit sample R6/R10 also showed high CPT of 0.9% (Table 4.3.1). In summary among all the samples screened, fruit samples of R56 had highest CPT compared to other species.

A



CPT



CPT 349 $[M+H]^+$ m/z 305 $[M+H]^+$

B

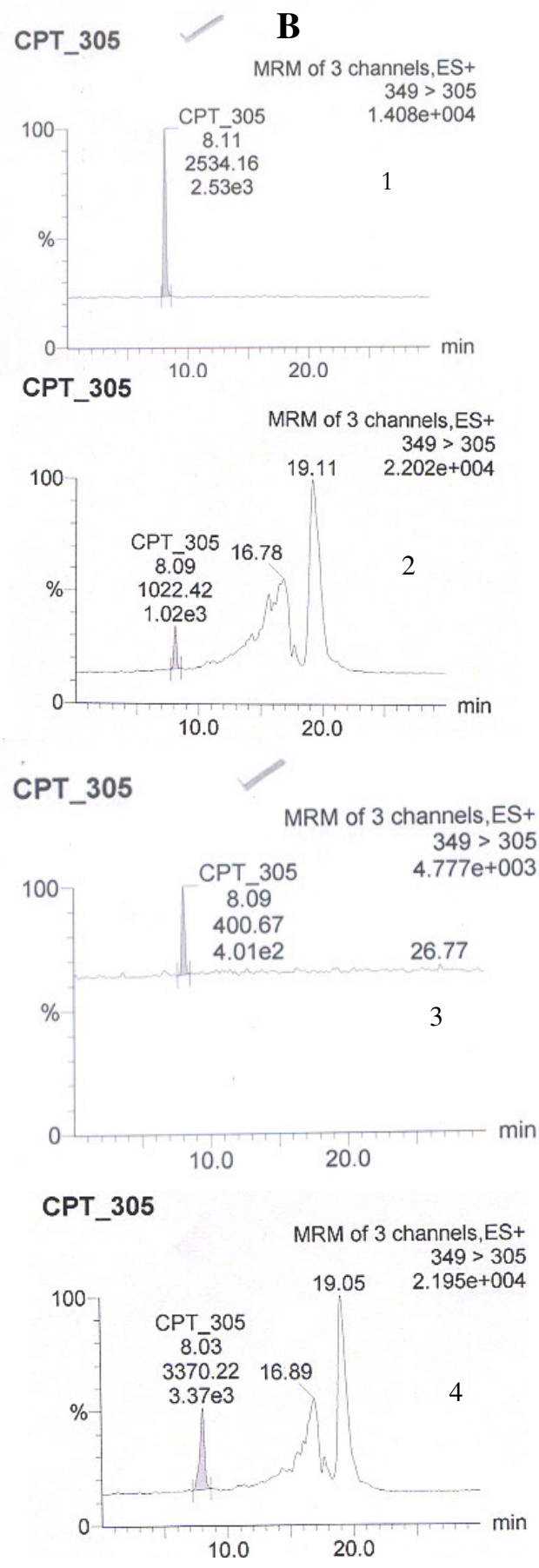
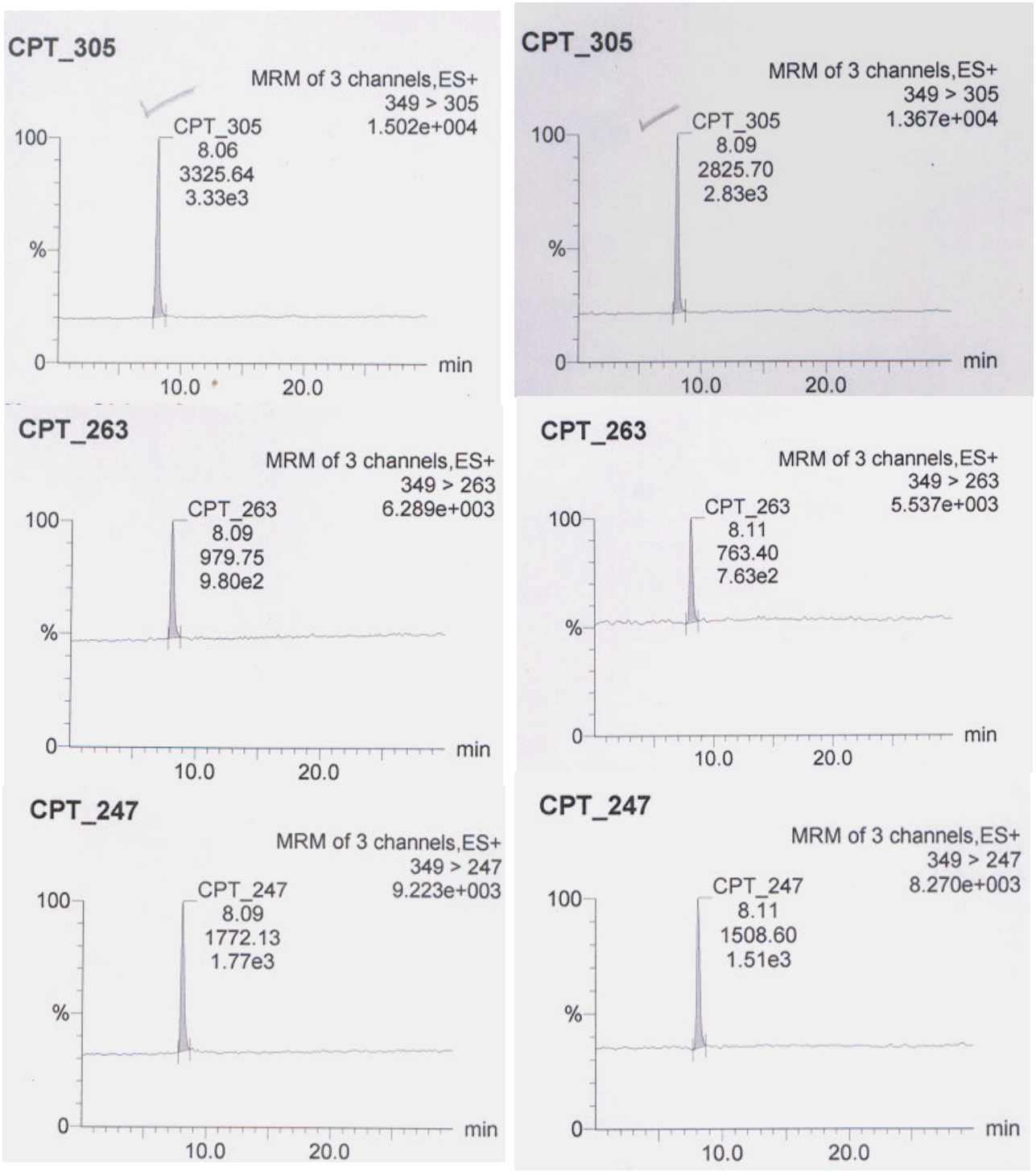


Figure 4.3.11: A: Structure of Camptothecin and a Schematic diagram showing LC-ESI-MS/MS fragmentation pattern of Camptothecin. **B:** ESI-MS/MS chromatograms of different genera of Icaccinaceae and others. ESI-MSMS chromatogram of standard Camptothecin showing a daughter ion of 305 at 8.11 min, similarly in plant samples: 1; standard CPT, 2; *Pyrenacantha volubilis* leaf, 3; *Ophiorhiza pumila* Stem, 4; *Sarcostigma kleinii* leaf.



R56 **R59**

Figure 4.3.12: ESI-MS/MS chromatograms of R56 and R59 samples. ESI-MSMS chromatograms of three daughter ions of Camptothecin 305, 263 and 247 eluting at 8.09 and 8.11 min in both the samples respectively.

Table 4.3.1: List of genera/species of Icaccinaceae, Rubiaceae, Apocyanaceae and Olacaceae with Percent CPT content based in HPLC and LC-MS/MS. (**Note:** ND: Not detected. NA: Not analyzed.).

Sl no	Species	Plant Part	No of individuals	HPLC (% CPT by dw)	LC-MSMS (% CPT by dw)
1	*Pyrenacantha volubilis*	Leaf	6	0.0064±0.0059	0.04%
		Stem	6	0.0071±0.0048	NA
2	*Sarcostigma klenii*	Leaf	1	0.0042	NA
		Leaf	1	0.018	0.14%
		Stem bark	1	0.0037	NA
		Fruits	1	0.00036	NA
3	*Apodytes dimidiata*	Leaf	2	ND	ND
		Stem bark	2	0.0051±0.0014	NA
		Leaf	1	ND	NA
		Stem bark	1	0.00076	NA
5	*Gomphandra tetrandra*	Leaf	1	0.00045	NA
		Stem bark	1	0.006	0.0008%
6	*Gomphandra polymorpha*	Fruits	1	0.011	NA
7	*Natsiatum herpaticum*	Fruits	1	0.026	0.01%
8	*Strombansia zeylanica*	Stem bark	1	ND	NA
9	*Ophiorhiza pumila*	Leaf	1	0.052	NA
		Stem	1	0.052	0.02%
		Root	1	0.14	NA
10	*Tobermontana heyneana*	Leaf	1	0.006	NA
		Stem bark	1	0.1	0.02%
11	R56,	Fruit	1	1.16	1.61
12	R59	Fruit	1	0.62	0.45
13	R6/R10	Fruit	1	1.28	0.9

4.3.3 *ndhF* phylogeny of Icacinaceae and others:

In the present study to construct minimal phylogeny of CPT producing plants *ndhF* gene sequences of different species/ genera were downloaded from the Gene bank and subjected for the Phylogenic analysis.

For some of the genera of Icacinaceae there were no ndhF sequences available in the database, these genera were subjected for *ndhF* gene amplification. PCR amplification profile of these species showed amplified products in the expected range (Figure 4.3.13) and in some case there were more than one band, in that case one prominent band in the expected range was considered.

Phylogenetic tree: In the strict consensus tree of the *ndhF* analysis, Icacinaceae. (represented by 15 genera and 16 sequences) together with Garryales form a monophyletic group as sister to the other euasterids I. With in this monophyletc clade, *Natsiatum herpaticum*, *N nimmoniana* and *Mappia racemosa* clustered together with a bootstrap value of 96 and remaining genera formed a different cluster. This cluster also included *Pyrenacantha, Icacina, Miquelia, Phytocrene and Stachyanthus* (Figure 4.3.14).

Emmotum and *Apodytes* form a separate clade within Icaccinaceae. This group in turn clustered with genera of the families Rubiaceae, Apocynaceae, Loganiaceae and Gelsemiaceae. Within Aquifoliales there were two separate sub groups, which belonged to the families Cardiopteridaceae and Stemonuraceae. Genera Pennantia is well supported with a separate group in Apiales.

In summary, Icacinaceae are polyphyletic. The genera included belong to both euasterids I and II and should be rearranged into three different orders: Garryales, Aquifoliales, and Apiales.

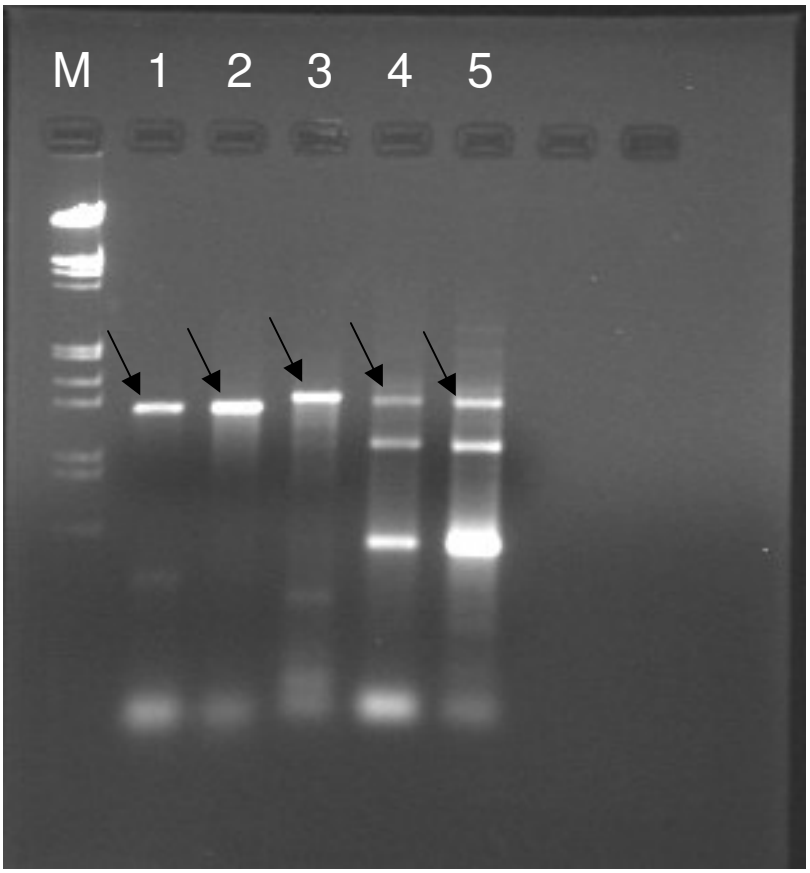


Figure 4.3.13: Amplification profile of chloroplast ndhF gene of *Miquelia dentata* and *Nothapodytes nimmoniana.* M: Lambda double digest ladder, 1: *Miquelia dentata* : 1st half ndhF, 2: *N nimmoniana :* 1st half ndhF, 3: *Miquelia dentata* : 2nd half ndhF and 4 5 : *N nimmoniana* : 2nd half ndhF.

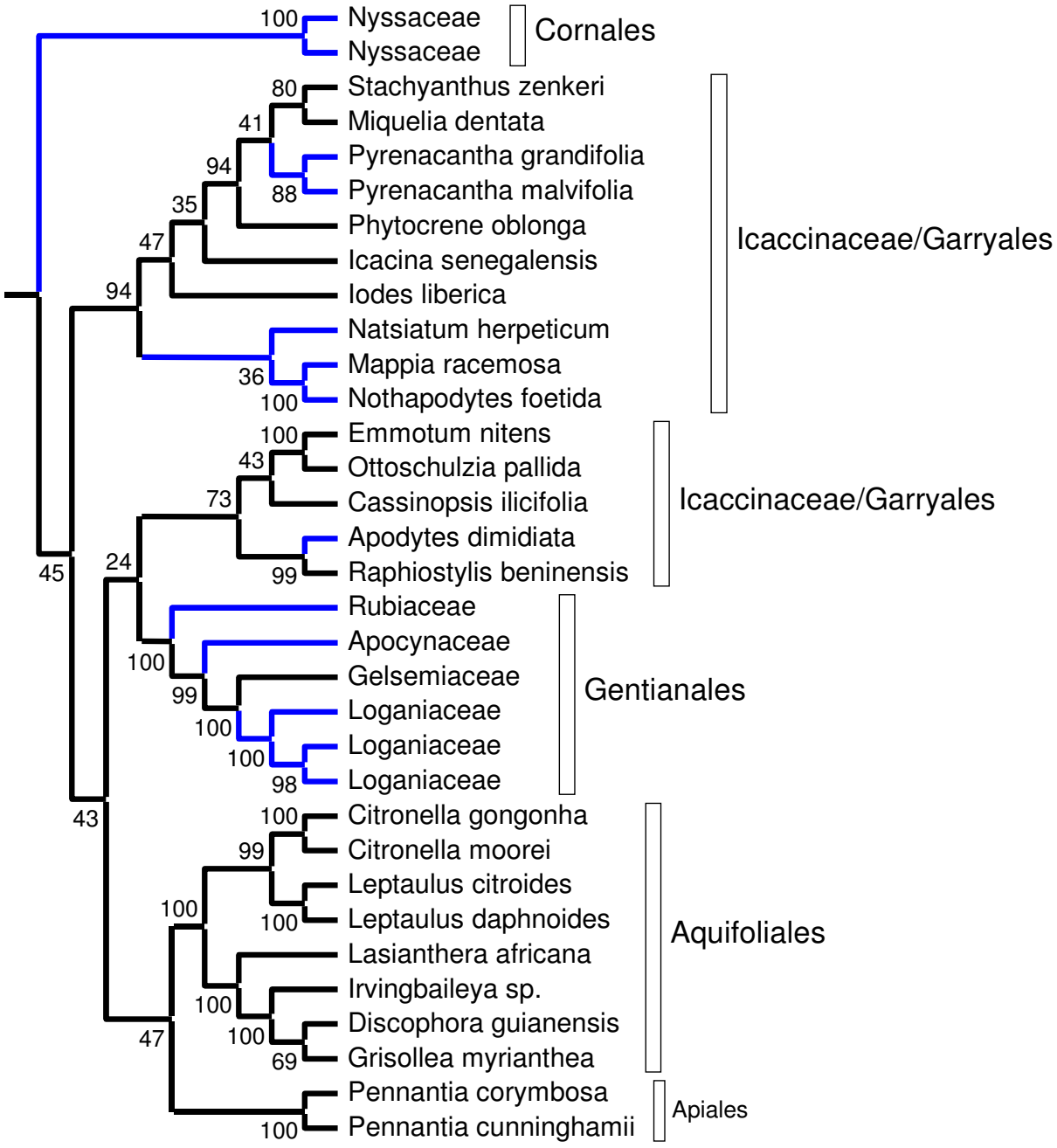


Figure 4.3.14: Minimal phylogeny of Asterids developed based on ndhF sequences.

4.4 Prospecting endophytic fungi from *Nothapodytes nimmoniana* for Camptothecin.

4.4.1 Endophytic fungi from different plant parts and populations of *N nimmoniana* :

Individuals of *Nothapodytes nimmoniana* that differ in CPT in stem bark were used for isolation of endophytic fungi with a presumption that individuals might also differ in their endophytic fungal composition. With this hypothesis twenty-six endophytes were isolated from 24 trees and 4 tissue samples (Table 4.4.1). These were designated as putative endophytes. There were no isolates obtained from explants that were unsterilized. All the fungal isolates started appearing on the 3rd day after inoculation on to the agar plates. Isolates significantly differed in their rate of colony formation on the agar plates that indicated their uniqueness. (Figure 4.4.1). Maximum of three isolates were obtained from a single stem bark sample of Kemannagundy, Nalmukh and Persia population. Leaf and stem sample of in vitro-raised seedling did not give any endophytes however *N nimmoniana* seed tissue that was used for callus induction showed fungal initiation after three months of inoculation on to the MS media. There was no correlation observed between host CPT content and number of different isolates obtained from the same tissue.

4.4.2 Camptothecin production by endophytic fungi.

HPLC analysis:

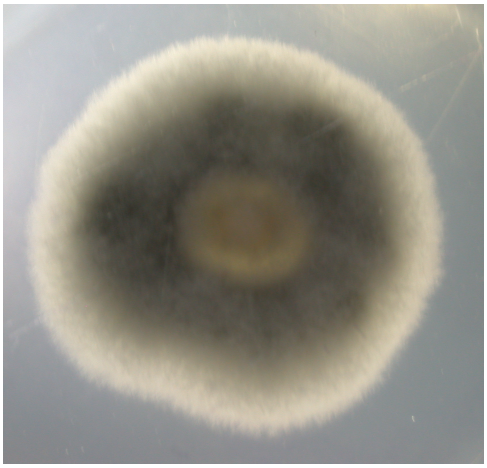
All endophytic fungal isolates were subcultured to ensure an axenic culture; each fungal culture has been continuously maintained since the original isolation, with transfer to fresh medium every 4-5 weeks (Figure 4.4.1). CPT estimation was done when isolates were in 3rd subculture (3rd generation away from the host). Twenty-six endophytic fungal isolates from *N. nimmoniana* were cultured in shake flasks as described earlier and CPT estimated at the end of the growth period in both broth and hyphae (Figure 4.4.1). CPT presence was confirmed by matching retention time of authentic CPT with that of endophytic fungal extract. Some of the isolates showed good CPT signal in hyphae as well as broth, its presence was confirmed in all the 26 isolates.

Table 4.4.1: Sample used in isolation of endophytic fungi from *Nothapodytes nimmoniana.*

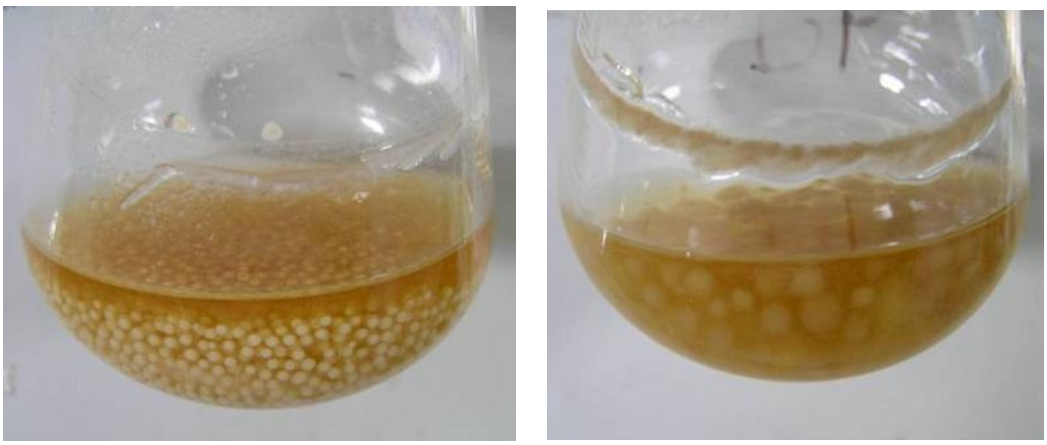
Tree ID	**Plant part**	**Host CPT content (%)**	**Isolate code**	**Day at which fungal initiation started after inoculation**
D1SB	Stem bark	1.89	UAS005	15th
D2SB	Stem bark	0.37	-	
D5SB	Stem bark	1.48	-	
D6SB	Stem bark	0.56	-	
D8SB	Stem bark	0.46	-	
D9SB	Stem bark	0.32	-	
D10SB	Stem bark	1.31	UAS004	8th
D15SB	Stem bark	0.67	UAS026	11th
KG1SB	Stem bark	1.21	UAS006	5th
KG4SB	Stem bark	2.82	UAS003	6th
	Stem bark	2.82	UAS007	7th
	Stem bark	2.82	UAS008	11th
KG6SB	Stem bark	0.22	UAS009	3
	Stem bark	0.22	UAS010	7
KG10SB	Stem bark	1.99	UAS011	7
	Stem bark	1.99	UAS012	5
Cutting	Stem bark	0.46	UAS013	7
Leaf petiole	Stem bark	0.40	UAS014	7
Kod SB-1	Stem bark	0.14	UAS018	5
NLSB-11	Stem bark		UAS020	20
NLSB-12	Stem bark		UAS021	11
NLSB-13	Stem bark		UAS022	7
Per SB-11	Stem bark	0.41	UAS023	28
Per SB-12	Stem bark	0.41	UAS025	11
Kod SB-1	Flower buds	-	UAS024	7
Callus culture	-	-	UAS001	90
Callus culture	-	-	UAS002	90
Seedling raised Invitro	Seedling leaf	-	-	-
-	Stem	-	-	-
-	Stem apex	-	-	-
-	Leaf	-	-	-
-	Leaf	-	-	-
-	Stem	-	-	-

Note: DSB: Devimani stem bark sample, Karnataka, KGSB: Kemmannagundy stem bark sample, Karnataka NLSB: Nalmukh stem bark sample, Kerala, Per SB: Persia Junction sample, Kerala , and Kodeyar stem bark sample, Kerala.

A)



B)



C)

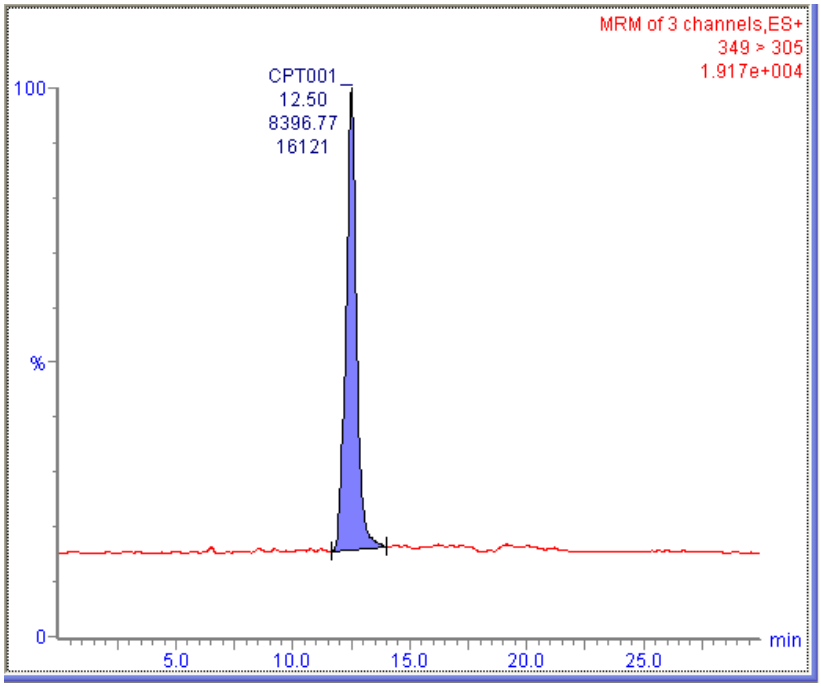
Figure 4.4.1: A; Plates showing the emergence of fungal hyphae from cut ends of stem bark of *N nimmoniana*. B; Plate showing the growth of sub-cultured endophytic fungi. C) Growth of endophytic fungi in shake flask culture.

Quantification of the compound was made in the samples in which CPT was detected. CPT was expressed as μg per 100mg of hyphae or μg per 100 mL of the broth. CPT content in the hyphae ranges from 0.10μg to 9.8μg. Highest CPT 9.8μg was found in hyphae of UAS 011 and lowest was found in UAS 006 (0.10μg). Six isolates (UAS 004, UAS 011, UAS 016, UAS 018 UAS 019 and UAS 020) produced CPT more than 1μg per 100mg of dry hyphal biomass. Four isolates produced more than or equal to 0.5μg of CPT in the hyphae. Similarly in the broth, the highest CPT content in broth was 0.30 μg and in many of the isolates CPT was not detected in the broth. In six of 26 isolates CPT was detected in the broth and amount was more than or equal to 0.1 μg. UAS 018 isolate produced more CPT (2μg) in the hypahe as well as broth (0.3μg) (Table 4.4.2).

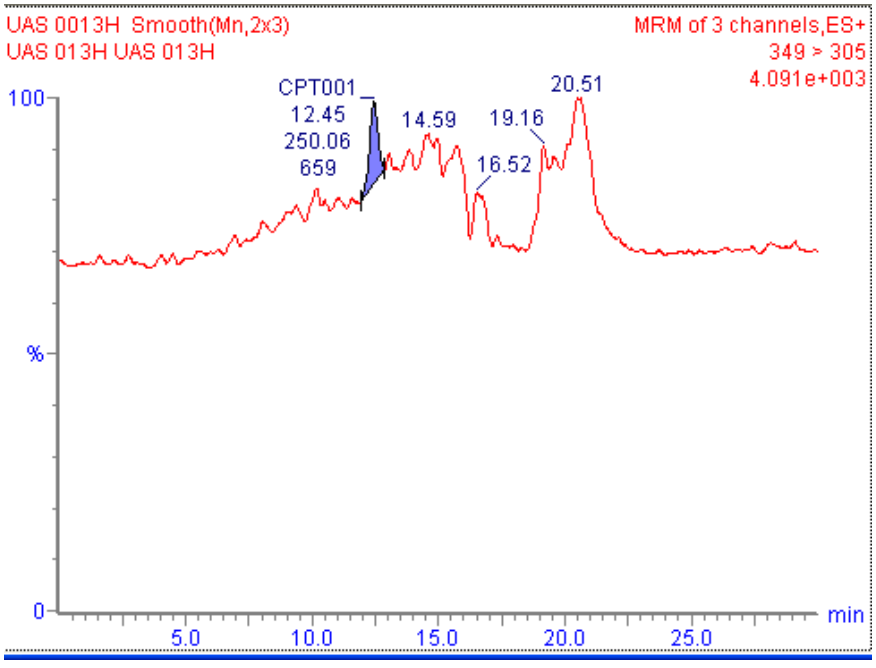
LC-MSMS analysis:

The same extracts were subjected for LC-MSMS analysis. Detection and quantification of CPT was made based on multiple ion-monitoring mode in LC-MSMS. The mass spectrum of fungal camptothecin was identical to the published spectrum (Van Hengel et al 1992), having a molecular ion peak at m/z 349 with characteristic fragments. CPT presence and its quantity in fungal extracts were confirmed by appearance of characteristic ions after fragmentation of parent molecule m/z 349. Three ions were observed upon fragmentation m/z 305 (M - CO2), m/z 247 (m/z 275 - CO) and m/z 263. Some of these extracts produced clear 305 signals like UAS 017, UAS 021, UAS13, UAS 019 and UAS 018 (Figure 4.4.2). In all the isolates CPT was detected, but quantification was possible in few of the samples because of amount of CPT in rest of the samples was below limit of quantification in LC-MSMS.

UAS 005 had the maximum CPT 13μg followed by UAS 025 (7.1μg) and UAS 013 (4.1μg). Isolates UAS 012 and UAS 006 had 2.9 and 2.3 μg of CPT respectively. UAS 001, UAS 009, UAS 014, UAS 015, UAS 018, UAS 019 and UAS 022 isolates produced CPT less than 1μg. HPLC CPT estimates of hyphae were correlated with LC-MSMS CPT estimates. There was no correlation observed between HPLC CPT estimates and LC-MSMS estimates (Table 4.4.2).



A)



B)

Figure 4.4.2: A) LC-MSMS chromatogram of standard CPT showing 305 peak at 12.50 min. B) LC-MSMS chromatogram of endophytic fungal extract (UAS013) showing 305 peak at 12.45.

Table 4.4.2: CPT in hyphae and broth of 26 endophytic fungal isolates from stem bark of *Nothapodytes nimmoniana* based on HPLC and LC-MSMS**.** CPT in mycelia extracts of 26 endophytic fungal isolates from stem bark of *Nothapodytes nimmoniana* based on LC-MSMS. Based on multiple ions monitoring system in LC-MSMS CPT was quantified based on three molecular ions 305, 247 and 263. D; CPT detected in LC-MS/MS but quantity was below limit of quantification.

	HPLC estimates		**LC-MSMS estimates**
Isolate code	**µg/100mg (hyphae)**	**µg/100mL (broth)**	**µg/100mg (hyphae)**
UAS 001	0.40	0.10	0.068
UAS 002	0.50	0.00	D
UAS 003	0.80	0.00	D
UAS 004	1.50	0.00	D
UAS 005	0.00	0.10	13
UAS 006	0.10	0.00	2.3
UAS 007	0.10	0.00	D
UAS 008	0.20	0.00	D
UAS 009	0.60	0.00	0.5
UAS 010	0.40	0.00	D
UAS 011	9.80	0.10	D
UAS 012	0.30	0.10	2.9
UAS 013	0.40	0.00	4.1
UAS 014	0.10	0.00	0.2
UAS 015	0.50	0.00	0.93
UAS 016	1.10	0.00	D
UAS 017	0.60	0.00	D
UAS 018	2.10	0.30	0.85
UAS 019	1.80	0.00	0.13
UAS 020	6.20	0.00	D
UAS 021	0.20	0.10	D
UAS 022	0.20	0.00	0.23
UAS 023	0.40	0.00	D
UAS 024	0.20	0.00	D
UAS 025	0.10	0.00	7.1
UAS 026	0.00	0.00	D

4.4.3 ITS taxonomy and phylogeny of endophytic fungi from *N nimmoniana*.

The ITS PCR product lengths of all the isolates range from 500- 720bp (Figure 4.4.3); thus there was a considerable length variation observed across the isolates.

A comparative analysis of the ITS sequence alignments for the herein studied endophytic fungal isolates was carried out using CLUSTALW software (Thompson et al. 1994). Analysis of the ITS region among the different strains revealed sequence variability. Each of the sequence was processed and subjected for BLAST analysis. BLAST analysis results are given in the Table 4.4.3; against each isolate a BLAST hit with highest BLAST score, percent identity, gene bank accession number and species name was given. Taxonomic identity for each of the isolate was given in the Table 4.4.3. Seventeen out of 26 isolates were classified as Fusarium; remaining isolates belong to five different genera of fungi. Fusarium isolates belongs to two different species *Fusarium solani* (*Nectria haematococca; sexual stage of Fusarium solani*) and *Fusarium oxysporum* (*Gibberella moniliformis: sexual stage of Fusarium oxysporum*). Isolate UAS 002 and UAS 014 had a ITS similarity of 98 and 100% with *Diaporthe conorum and Phomopsis sp.* MAFF 665006 respectively. Except *Irpex* (isolate UAS009) and *Phanerocheta*; (UAS021) belongs to Basidiomycetes fungi and remaining isolates belongs to Ascomycetes group of fungi.

Phylogenetic analysis of isolates revealed three different clusters. The cluster I contained 17 *Fusarium* isolates. There are three species of *Fusarium* from this cluster, which includes *Fusarium oxysporum* isolates, *Fusarium solani* isolates and *Fusarium beoforme* and the cluster was supported with a bootstrap value of 75%. The cluster II contained *Diaporthe* and *Phomospsis* isolates and this cluster was well supported by bootstrap value of 63%. Further one isolate of *Botryosphaera* was in a separate cluster III and was closely clustered with *Diaporthe* isolates. This isolate separated with a bootstrap value of 94%. The cluster IV contains isolates belongs to Basidiomycetes fungi and there are three isolates in the cluster, namely *Galactomyces sp, Irpex lacteus* and *Phanerochaeta tuberculata*. *Irpex lacteus* and *Phanerochaeta tuberculata* are very closely clustered into a small sub-cluster with a bootstrap of 99%

indicating their high degree of ITS similarity. ITS marker was able to distinguish different isolates and were clearly segregated into separate clusters (Figure 4.4.4).

The isolates clustered clearly into separate groups based on ITS similarity and there was no geographical association of the isolates observed. Isolates coming from different populations of *N. nimmoniana* did not cluster into a single group. Isolates that are obtained from different plant parts of *N. nimmoniana* clustered separately like, *Diaporthe* and *Phomopsis* were isolated from leaf petioles, *Galactomyces* from flower buds of *N. nimmoniana*, remaining all other isolates were originated from stem bark of *N. nimmoniana* indicating the tissue specificity of endophytic fungal isolates in *N. nimmoniana*.

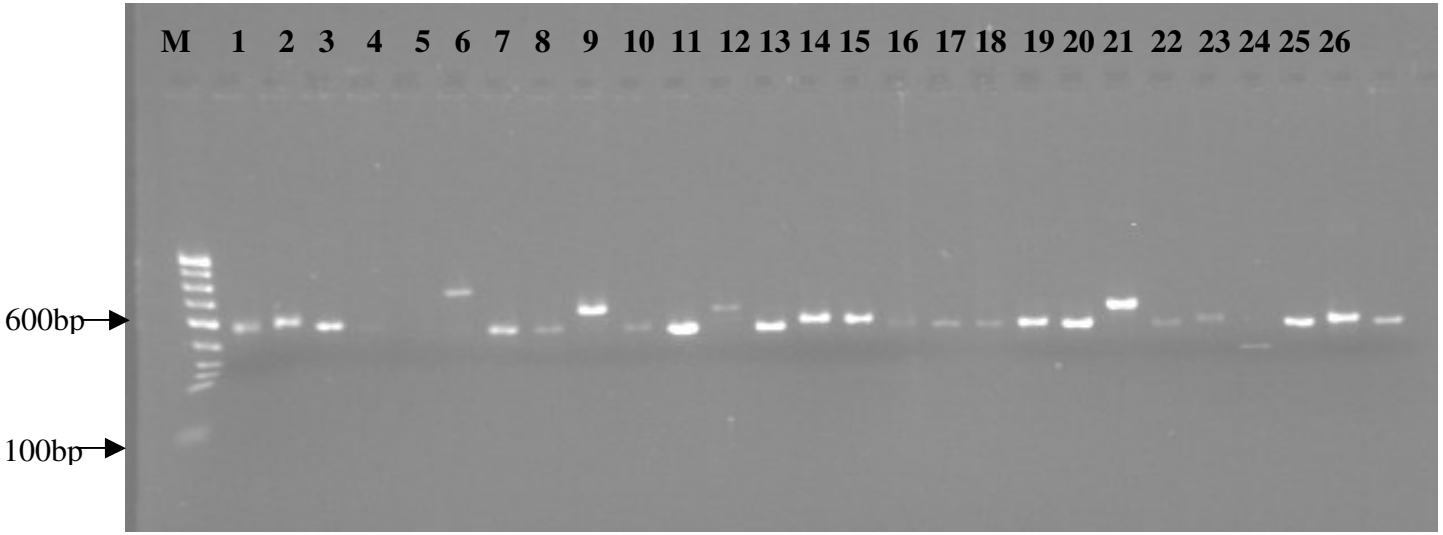


Figure 4.4.3: ITS amplification of 27 endophytic fungal strains by ITS1 and ITS4 primers.M; 100bp marker, 1-26 are endophytic fungal strains.

Table 4.4.3: BLAST results of 26 endophytic fungal isolates with gene bank accession number of the closest hit with isolates ITS sequences.

Isolate code	Genbank Accession Number	BLAST score	% Identity	Closest match in the Gene bank database
UAS001	DQ486878.1	452	93	**Fusarium solani isolate**
UAS002	AB201443.1	809	98	*Diaporthe conorum*
UAS003	DQ094708.1	890	99	*Fusarium solani* strain FRC#s588
UAS004	AF178402.1	1017	99	*Fusarium sp.* NRRL 22354
UAS005	EF483929.1	624	99	*Gibberella moniliformis* strain bxq512
UAS007	DQ279794.1	973	99	*Fusarium oxysporum f. sp. gladioli isolate*
UAS008	EF158027.1	141	93	*Fusarium oxysporum* strain GT6
UAS009	AF479666.1	849	100	*Irpex lacteus* SFC 951007-39
UAS010	AY662326.1	549	95	*Gibberella moniliformis* strain KSU 12911
UAS011	DQ166550.1	995	99	*Fusarium sp.* JJ002
UAS013	AF455450.2	969	99	*Gibberella sacchari* isolate wb396
UAS014	AB107890.1	827	100	*Phomopsis sp.* MAFF 665006
UAS015	DQ316078.1	844	100	*Botryosphaeria parva* isolate CMW_14029
UAS017	DQ297569.1	975	99	*Fusarium subglutinans* isolate ZMS-547-14
UAS018	DQ780422.1	720	98	**Fusarium oxysporum *isolate Endophyte 60***
UAS019	AB277550.1	174	100	*Fusarium equiseti*
UAS020	AY864891.1	434	99	*Fusarium oxysporum f. cubense*
UAS021	AY219356.1	169	100	*Phanerochaete tuberculata*
UAS022	AY864891.1	557	99	*Fusarium oxysporum f. cubense*
UAS024	DQ667968.1	242	98	*Galactomyces sp.* 3S-28C
UAS025	DQ780422.1	940	100	*Fusarium oxysporum isolate* Endophyte 60
UAS026	DQ780458.1	406	97	*Nectria haematococca isolate* Saprobe 74

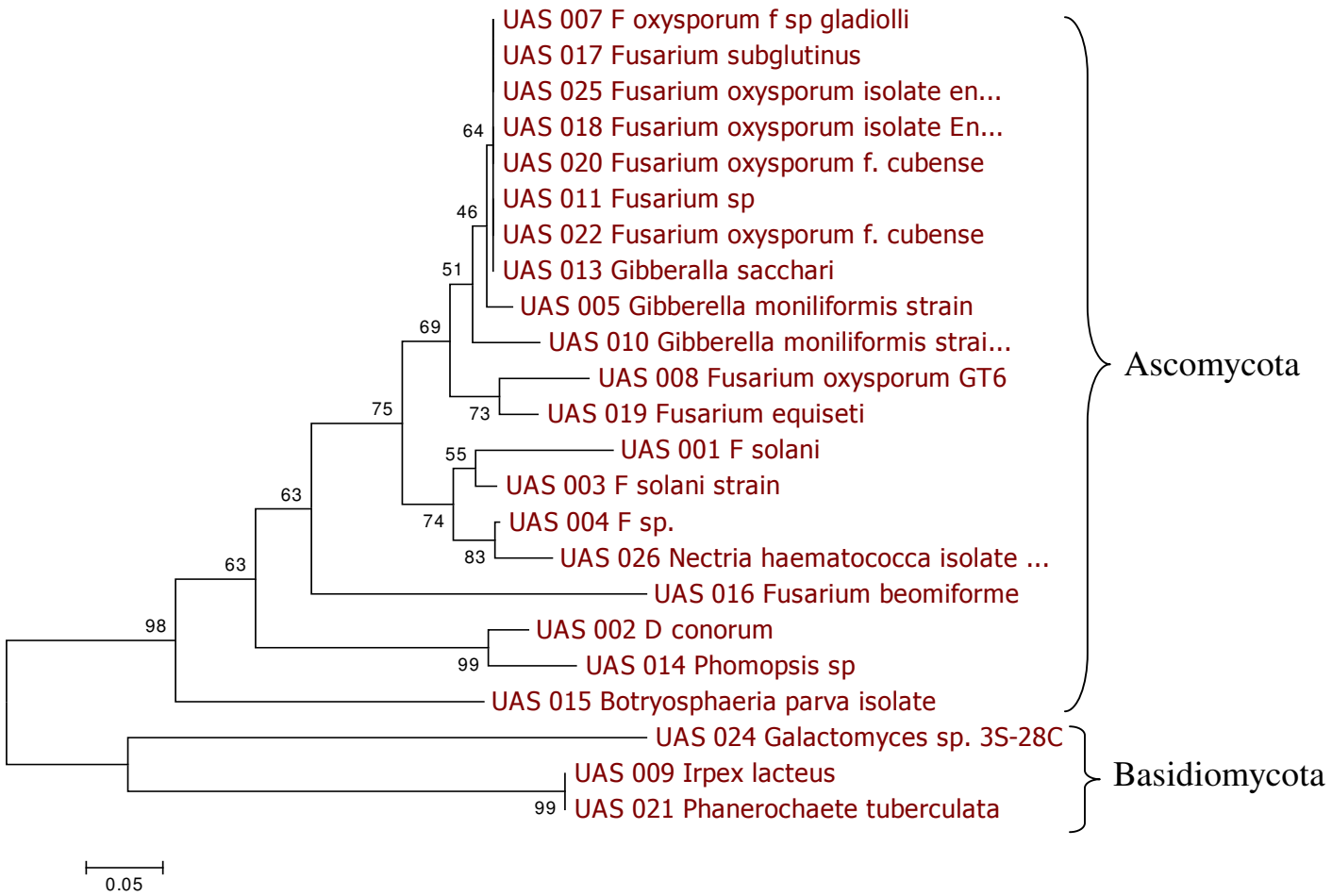


Figure 4.4.4: Neighbor-joining phylogenetic tree of 23 endophytic fungal isolates of *Nothapodytes nimmoniana*, against each isolate their closet species in the gene bank database is indicated. Numbers at the nodes indicate the bootstrap values. Lower bar indicate relative genetic distance.

DISCUSSION

V Discussion

It is estimated that over 50 per cent of all drugs (and their derivatives and analogs) in clinical use, are derived from higher plants. According to the WHO, about 80 per cent of the people in developing countries still rely on traditional medicine for their primary health care, and about 85 per cent of such medicines involve the use of plant extracts. In other words, an incredibly large number of people (about 3.5 to 4 billion) in the world rely on plants as source of drugs (Farnsworth 1988; Raskin *et al* 2002).

In recent years, with the advent of newer tools including high throughput screening for bioactive molecules, there is a resurgence of interest in mining higher plants for a variety of metabolites. In fact, nowhere has the effort been more pronounced than in the National Cancer Institute USA, which has screened over 35,000 plants for anti-neoplastic effects (Daniel and Fransworth, 2001). Plant based natural products have played a significant role in the development of contemporary cancer chemotherapy. A number of novel anti-tumor compounds, including taxols, camptothecin and its derivatives, maytansine, tripdiolide, homoharringtonine, vinblastine, vincristine, indicine-N-Oxide, baccharin, podophyllotoxin derivatives and etoposide etc are being extracted from plant sources (Nalwade *et al* 2003; Yan *et al* 2003). Considering the enormous potentiality of these compounds, several laboratories worldwide have been striving to intensively mine them and standardize methodologies for their large-scale production.

Among the plant-derived compounds camptothecin, a pyrrolo quinoline alkaloid, has been extensively used as a novel anti-tumor compound. CPT is lauded as one of the most promising anticancer drugs of the twenty-first century (Lorence and Craig, 2004). CPT exhibits a broad spectrum of anti-tumor activity both under *in vitro* and *in vivo* conditions (Valdu *et al* 2000). CPT and its analogues in the presence of topoisomerase-I produce DNA damage by binding to and stabilizing a covalent DNA-topoisomerase-I complex in which one strand of DNA gets broken (Nalwade *et al* 2003; Yan *et al* 2003; Li *et al* 1994; Wall *et al* 1966; Craig *et al* 1997).

Irrinotecan and Topotecan two water-soluble derivatives of CPT, have been approved by the FDA for treating small cell lung cancer, colorectal and ovarian cancer (Masuda *et al* 1992; Abigers *et al* 1995; Lilenbaum *et al* 1995; Romanelli *et al* 1998; Clements *et al* 1999). These have also been approved by FDA for the treatment of AIDS (Priel *et al* 1991).

Camptothecin was first discovered in the Chinese deciduous tree, *Camptotheca acuminata* (Nyssaceae) (Wall *et al,* 1966). The other plant species from which CPT is isolated are *Merriliodendron megacarpum* (Arisawa *et al* 1981) and *Nothapodytes nimmoniana* Graham (Govindachari and Vishwanathan, 1972) both belonging to the family Icacinaceae, *Ophirrohiza mugos* (Tafur *et al* 1976) and *O. pumila* (Aimi *et al* 1990) from the family Rubiaceae, *Eravatamia heyneana* (Gunashekera *et al* 1979) belonging to Apocynaceae and *Mostuea brunonis* (Dai *et al* 1999) belonging to the family Loganiaceae (Table 5.1). However to date, the highest content of CPT has been realized from *Nothapodytes nimmoniana* (about 0.3 % w/w) (Govindachari and Vishwanathan, 1972).

The market demand for irinotecan and topotecan has been ever increasing and has currently reached approximately one thousand million US dollars, which represents approximately one ton of camptothecin in terms of natural material (Watase *et al* 2004; Raskin *et al* 2002). Most of this demand is currently met from plantations of *C. acuminata* that have been extensively established in China.

In India, however *N. nimmoniana* remains the main source of CPT. Based on current market price, it is estimated that *Nothapodytes nimmoniana* available along the northern part of the Western Ghats *per se* is worth over US$ 350 million (Ganeshaiah and Uma Shaanker, unpublished). While official records are not available, it is reliably learnt that the tree is extensively harvested from the Western Ghats and the billets exported for commercial extraction of CPT. In fact, it is estimated that in the last decade alone, there has been at least 20% decline in the population leading to the red listing of the species (Hombe Gowada *et al* 2002; Ravi Kumar and Ved, 2000). Indiscriminate felling of trees for short-term gains could perhaps lead to the loss of elite individuals and populations that otherwise could potentially serve as sources of high CPT.

With no synthetic source of this alkaloid and with an increasing global demand, it has become imperative that the demand for camptothecin is met from a sustainable supply rather than the current destructive harvesting. Among the various approaches, prospecting for populations and or individuals of the species for higher yields of the alkaloid could potentially help in establishing high yielding clonal orchards and in developing *in vitro* production systems and thereby relieving the pressure on natural populations. Another approach is to identify alternate sources of Camptothecin, by screening phylogenetically related species/genera/family of the taxa in which the compound has been already reported. This would help in combating the heavy demand on single species of plants. Yet another approach could be sourcing the endophytic fungi of the host plants, which also reportedly mimic the host plants by producing same metabolites.

Towards this end, in recent years, attempts have been made to chemically characterize populations of *N. nimmoniana* along the distrubutional range of the species in the Western Ghats, India, with an ultimate aim of identifying populations/individuals with high CPT yields. High yielding sources can be used to produce material for clonal multiplication and to develop cell lines with high CPT yield. Attempts have also been made to identify the ecological correlates of camptothecin accumulation and identify ecological niche of *N. nimmoniana* in Western Ghats and identify the "hot-spots" of CPT accumulation. The latter can guide collection of accessions for conservation as well as for use in sustainable models of extraction of CPT.

An attempt has been made to prospect camptothecin from phylogenetically related species/genera of *N. nimmoniana* in the family Icacinaceae. Identification of alternate sources would lead to sustainable supply of camptothecin and decrease pressure on single species. Besides this, an attempt to isolate and screen endophytic fungi from *N. nimmoniana* has also been carried out. Identification of endophytic fungal strains that produce camptothecin under *in vitro* condition is also discussed.

Basic patterns of accumulation of Camptothecin in *Nothapodytes nimmoniana*

Though CPT has been reported in over nine species, the basic patterns of accumulation of CPT are well documented only in *Camptotheca acuminata* and to a lesser extent in *Nothapodytes nimmoniana*. Yan *et al* (2003) reported highest levels of CPT in leaves of *Camptotheca acuminata.* CPT content was at least 10-folds higher in young leaves (Yan *et al* 2003). In fact, the high concentration of CPT in leaves has reportedly lead to the poisoning of goats; even the honey bees foraging on the floral rewards are also known to be affected (Yan *et al* 2003). Though precise mechanism of transport and storage of CPT is not fully understood, it is conjectured that CPT is synthesized in leaves and sequestered in old and dead tissues (Yan *et al* 2003). At cellular level, CPT is localized in mesophyll and sub-palisade layers of young leaves (Yan *et al* 2003). CPT has also been reported to be localized in vacuoles of young and older leaves (Nolte 1999).

The basic patterns of accumulation of CPT in *Nothapodytes nimmoniana* have been characterized with respect to age and sex of plant and plant parts (Padmanabha *et al*, 2006). Among the various plant parts, inner root bark is reported to yield the highest CPT content followed by inner stem bark. The average CPT content in the inner root bark is about 0.33±0.21% compared to 0.23±0.15% in inner stem bark (Figure 5.1). The CPT content in the root and stem wood is significantly lower than that of the respective inner bark tissue. While the root wood contained 0.18±0.09%, the stem wood contained only 0.14±0.12% CPT. Seeds on an average contained only about 0.17% CPT (Padmanabha *et al* 2006). The CPT content in two-year-old seedlings was highest in the root tips (0.4%) followed by leaves and stem (0.2%). The CPT content did not differ between the old and the young leaves. There was no difference in the CPT content between the sexes (Padmanabha *et al* 2006). These studies reaffirm the earlier findings of Govindachari and Vishwanathan (1972) who reported highest yields of CPT from roots of *Nothapodytes nimmoniana.* In fact traditionally, CPT has been extracted from root, root bark and fruits (Roja and Heble 1994). Fairly good amounts (0.10%) of the alkaloid have also been reported from seeds (Roja and Heble 1994). Quite obviously, because of the relatively low levels of CPT in leaves, extraction of CPT from these trees has been mostly destructive involving the felling of the trees.

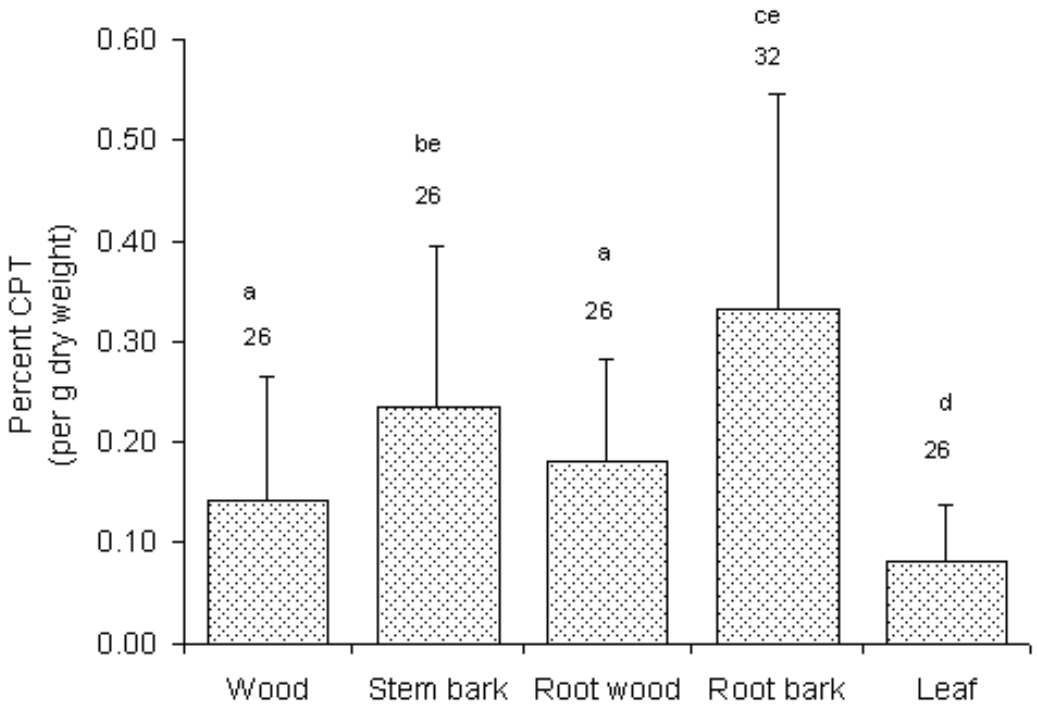


Figure 5.1: Mean percent CPT per gram dry weight in different tissues at three sites in the Western Ghats. The numbers on the histograms indicate number of trees used in the analysis. Respective histograms with dissimilar letters indicate a significant difference in CPT content (*t test* $p < 0.05$) (redrawn from Padmanabha *et al*, 2006).

Section I

Chemical profiling of populations of *Nothapodytes nimmoniana* for CPT

While *Nothapodytes nimmoniana* forms one of the richest sources of CPT, commercial production of the alkaloid is still limited for want of high yielding lines. Prospecting for high yielding individuals or populations across the distributional range of the species could help in using the identified high yielding lines for clonal multiplication and commercial production of CPT. Towards this end, recently, Suhas *et al*, (2007) chemically profiled populations of *Nothapodytes nimmoniana* along the Western Ghats, a mountain chain running parallel to the west coast of south India, considered as one of the 34-mega-biodiversity 'hot-spots' of the world (Myers *et al* 2000). Based on primary and secondary data sources, the occurrence of

the species in the Western Ghats was digitized on a GIS platform. While the species occurs along the length of the Western Ghats, it is clear that the distribution is not uniform; certain parts, namely the southern and central Western Ghats have a greater density of records of distribution. Based on the relative distribution, Suhas *et al*., (2007) analysed 11 populations from 8° to 15° N latitude. For each of the 11 populations, 10 to 15 trees were sampled randomly and the CPT estimated in the inner stem and root bark tissues respectively.

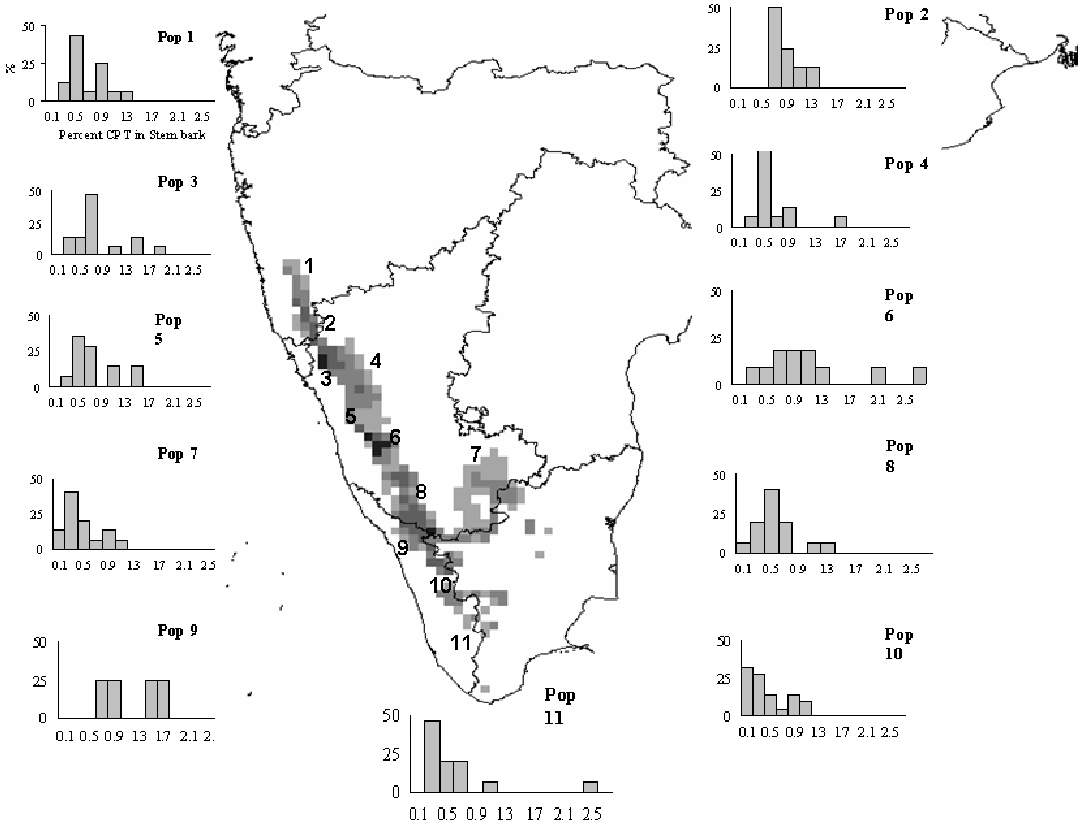


Figure 5.2: Frequency distribution of CPT (%, w/w) in stem bark of *N. nimmoniana* populations (Adapted from Suhas *et al*, 2007).

Significant variation existed among populations in their mean CPT content both in stem bark (one-way ANOVA, $P < 0.004$) and root bark ($P < 0.001$). Per cent CPT in stem bark ranged from as low as 0.03 to as high as 2.7, with an overall mean of 0.7. The mean CPT content in the root bark ranged from 0.003 to 1.41%, with an overall mean of 0.48%. The northern Kerala populations had the highest CPT content both in their stem bark (1.10 ±0.462%) and root bark (0.93 ± 0.359%). CPT content of stem bark was significantly positively correlated with that of the respective root bark ($n = 126$; $r = 0.320$, $P < 0.05$). Finally, the frequency distribution of CPT content over all populations was highly positively skewed (Figure 4.1.1 in results section).

Suhas *et al*., (2007) found no clear relation between CPT content and girth size of trees. In seven of the 11 populations, there was no relation; however of the remaining four, in three there was significant positive relation ($r = 0.678$, $r = 0.762$, $r = 728$; all $P < 0.05$), while in one it was negatively related ($r = –0.728$; $P < 0.05$). Thus the differences in CPT content among populations and individuals could not be attributed to possible age or size class differences. Suhas *et al*. (2007) also showed that even after normalizing for girth differences if any, among the trees, the CPT content expressed as CPT/girth was significantly different among the populations ($P = 0.0016$). The mean CPT content of populations was not correlated with latitude, longitude or altitude of their occurrence and collection. CPT content in *Camptotheca acuminata* was found to vary significantly across latitude (Liu *et al* 1999 and 2000).

In this study, the results obtained by Suhas *et al*, 2007 have been confirmed using LC-MS/MS analysis. The results provide one of the most exhaustive chemical screenings of *N. nimmoniana* for CPT. The study assumes significance in that it is perhaps the first to report at least 5 to 8 fold more CPT in *N. nimmoniana* than is hitherto reported. Of the 148 individuals assayed, 23 yielded more than 1 per cent CPT. These estimates are nearly 5 to 8 fold more than what has been reported hitherto in the literature (Govindachari and Viwanathan 1972). The study has demonstrated a significant population level variation in CPT content -a tool kit that can be exploited for developing clonally multiplied material from the identified high-yielding populations. While it will be important to examine if these differences reflect intrinsic genetic predisposition of populations to synthesize and accumulate CPT,

preliminary analyses do indicate a genetic basis. Clearly, more studies will be required to critically examine this issue. Populations in the northern Western Ghats had the highest mean CPT and least intra-population variation both based on the stem and root bark analysis. These populations could be important source material for developing high yielding clonal materials. Further, it will be important to study the heritability of the accumulation patterns across generations by analyzing the parent–offspring regression in the accumulation of CPT. It would be interesting to investigate the proximate/ultimate reasons for the enormously high levels of CPT produced by these trees, as a first step towards domesticating the species for obtaining high CPT yields.

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 per cent (w/w). The incredibly high yields of these individuals from several populations could not be attributed to their girth (r=0.164: NS). 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 supply of camptothecin. Subject to further confirmation, these "elite" trees could be focused for conservation and judicious utilization for clonal multiplication as also for deriving tissue material for *in vitro* production systems as was done for several other systems such as taxane from *Taxus wallichiana* (Poupat *et al* 2000) and for podophyllotoxin from *Podophyllum peltatum* (Rita *et al*, 2002).

New class of Camptothecins/CPT related alkaloids from *Nothapodytes nimmoniana*

New families of camptothecines were identified from *Nothapodytes nimmoniana*. Some of the camptothecins are at various stages of clinical development. This 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, search for high yielding lines of 10-hydroxy CPT could be useful as the compound can serve as precursor for irinotecan and topotecan. 10-HCPT has been shown to be more potent and less toxic than CPT (Zhang *et al*, 1998; Wiedenfeld *et al*, 1997) and exhibited a strong apoptosis-inducing effect on human hepatoma Hep G2 cells (Zhang *et al*, 1999; Zhang and Xu, 2000). 10-HCPT itself is in

clinical trails against colon cancer and in Colo-205 cells. 10-HCPT significantly repressed the cell proliferation at a relatively low concentration (5-20 nM) compared to previous studies. These results implied the potent antitumor activity of 10-HCPT at low-doses clinically (Ping *et al*, 2005).

In fact, studies that could lead to the identification of high yielding individuals of mappicine, first reported in the species by Govindachari and Viswanthan 1972 could be potentially important and interesting. The alkaloid, so far reported only from *N. nimmoniana* along with its ketone analogue nothapodytine B have been shown to have potent antiviral activity against herpes viruses (HSV) and human cytomegalovirus (HCMV). Hossain *et al* 2003 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 (Pedro *et al*, 2003). Recently, Hossain *et al*, 2003 discovered that certain analogues of mappicine are potent inhibitors of HIV-1 RT-associated RNAase-H. In this regard and because of the fact that these are associated with 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.

Modeling habitat suitability for CPT production

One of the key challenges in prospecting for high yielding sources of specific plant metabolites is to develop algorithms or approaches that can help predict hot-spots of distribution of the metabolite. Prediction of hot-spots and its subsequent validation can not only help focus efforts in collecting material from such sites but also serve to prioritize sites at which plants can be domesticated or conserved. Unfortunately few studies have seriously modeled the conditions that might help predict the spatial distribution of metabolites.

Recently, a GIS based approach called the ecological niche model has been used to model the spatial distribution of a given species and offer predictions on the habitat suitability of the species. Using specific algorithms, the model iteratively identifies habitats over a landscape that best match the climatic variables corresponding to sites of known occurrence of the species. Accordingly habitats are classified from those that are highly suitable (highest match) to those that are not suitable (least match) for the potential occurrence or invasion of the species (Ganeshaiah *et al*, 2003; Hijmans and David 2004). The ecological niche models have been used successfully in a variety of scenario including in locating rare and threatened species and in rationalizing the choice of habitats for species re-introduction (Hijmans and David, 2004).

In this study, attempts have been made to extend the use of ecological niche modeling tools to offer predictions on the spatial distribution of plant metabolites. An underlying assumption of this application is that, plants would be selected to accumulate secondary metabolites at sites predicted to be highly suitable for the given species compared to sites which are not predicted to be suitable. Thus, one would expect that a phytochemical such as santalols is best produced in sites suitable for the growth of sandal trees and not in those which are predicted to be unsuitable. Recently, Prakash Kumar (2007) modeled the distribution of *Withania somnifera* in south India and showed that individuals in sites predicted to be highly suitable accumulated higher levels of withaferin-A and withanolide-A compared to individuals in sites that were predicted to be unsuitable or poorly suitable.

Figure 4.1.5 (results section) shows the predicted habitat suitability for *N. nimmoniana* in the Western Ghats. It is evident that not all regions in the Western Ghats are uniformly suitable for the species. In fact within the Western Ghats, certain areas (in red) are highly suitable and others (in grey) are unsuitable. In fact, two distinct sites in the central and northern Western Ghats are predicted to be excellent in their match to the habitat requirements of the species. Analysis of the CPT content of individuals occurring in the different habitat suitability areas indicated that individuals in highly suitable areas accumulated significantly higher levels of CPT compared to those that occurred in unsuitable or poorly suitable areas (Figure 4.1.7 in results section). Furthermore, over 60 per cent of the trees that accumulated greater that 1 percent CPT were all from regions predicted to be highly suitable (Figure 4.1.6

in results section). In summary these results, for the first time, have demonstrated the utility of the ecological niche models in predicting the spatial richness of plant metabolites and hold several important implications.

Further, a forward step-wise regression for CPT content using 19 climatic variables averaged over 30 years for each of the sites of collection was conducted. Only two variables namely, mean temperature of the driest and wettest quarter of the year significantly explained the differences in stem bark CPT among the populations; for root bark CPT, only one variable, namely, the mean monthly temperature was significant. Similar studies conducted in *C. acuminata* showed that CPT content varied significantly with several environmental variables such as temperature, evaporation capacity as well as precipitation. Low temperature and precipitation was found to increase the CPT content (Yan *et al,* 2003).

The results raise interesting prospects for further research on how the habitat suitability or otherwise can influence the accumulation of a secondary metabolite. Do ecologically good habitats serve as areas in which the species are genetically predisposed to synthesizing secondary metabolites and other defense compounds that can lead to a potentially higher fitness of the populations? The outputs of the ecological niche model provide a powerful handle and direction to further explore newer populations of *N. nimmoniana* in areas/regions that have not yet been sampled from in search for higher CPT yields. The results have important implications for intelligent prospecting for economically important secondary metabolite such as CPT.

Development of sustainable extraction approach

The ever-increasing worldwide market of irinotecan and topotecan, the derivatives of CPT, has currently reached one thousand million dollars. This represents approximately one ton of CPT in terms of raw material (Watase *et al*, 2004). Realizing the demand for CPT, plantations of *C. acuminata* have been established in China since 1993 to supply material for CPT extraction (Liu *et al* 1999). In India, the major source of CPT continues to be *N. nimmoniana.* However since there are no commercial plantations, all of the demand is sourced from the trees extracted destructively from the natural populations of *N. nimmoniana.* This of course is not sustainable in the long run, because of loss of standing populations of the trees in the

distributional range of the species. For example, assuming an average concentration of about 0.3 per cent CPT, about 333.3 tons of wood chips (equivalent to about 22,200 adult trees will be required). While no detailed inventory of the distribution and abundance of the tree are available in the Western Ghats, it is conjectured that the estimated demand may not be met solely by sourcing trees from their natural populations. Clearly strategies need to be evolved that can ensure a sustained supply of CPT from *N. nimmoniana*.

Several approaches could be deployed to ensure the sustainable extraction of *N. nimmoniana*. For example, establishment of captive plantations using clonally multiplied material from high yielding lines could greatly contribute to the rising demand for the compound without jeopardizing the naturally occurring populations. In fact towards this end, efforts have been made to identify high yielding lines and populations from the distributional range of the species. The discovery of population variability for CPT accumulation in *N. nimmoniana* holds immense promise in developing high yielding clonal orchards and other captive plantations (Suhas *et al* 2007).

Extraction of renewable plant parts such as leaves and fruits instead of bark could be one of the possible approaches to sustain the extraction. However because of the extremely low levels of CPT in the leaves and fruits of trees, this is not economically attractive.

The CPT content of leaves could be strongly related to the age of the plant just as was shown in *C. acuminata* (Liu *et al* 1999). Thus leaves of seedlings could accumulate relatively higher levels of CPT than those of juveniles and adults (Figure 5.3). For example, Turner (1995) surveyed 41 woody species and reported that immature leaves contained a 70% higher phenolic and tannin content than mature leaves. In *Cynoglossum officinale* young leaves contained 190 times more pyrrolizidine alkaloids than older leaves (Van Dam *et al.* 1994). The same trend has also been observed in *C. accuminata,* where an inverse relationship between leaf age and CPT concentration has been reported (Liu, 2000). Similarly, in this study, highest CPT content was found in the seedlings of *N. nimmoniana* (Figure 5.3). It is estimated that one year old seedlings producing about 15 to 20 g of leaf biomass can easily yield about 50 mg of CPT. Though the economics of extraction need to be further analysed,

it appears that by simple ratooning of the seedling crop once a year, CPT could be extracted on a sustainable basis.

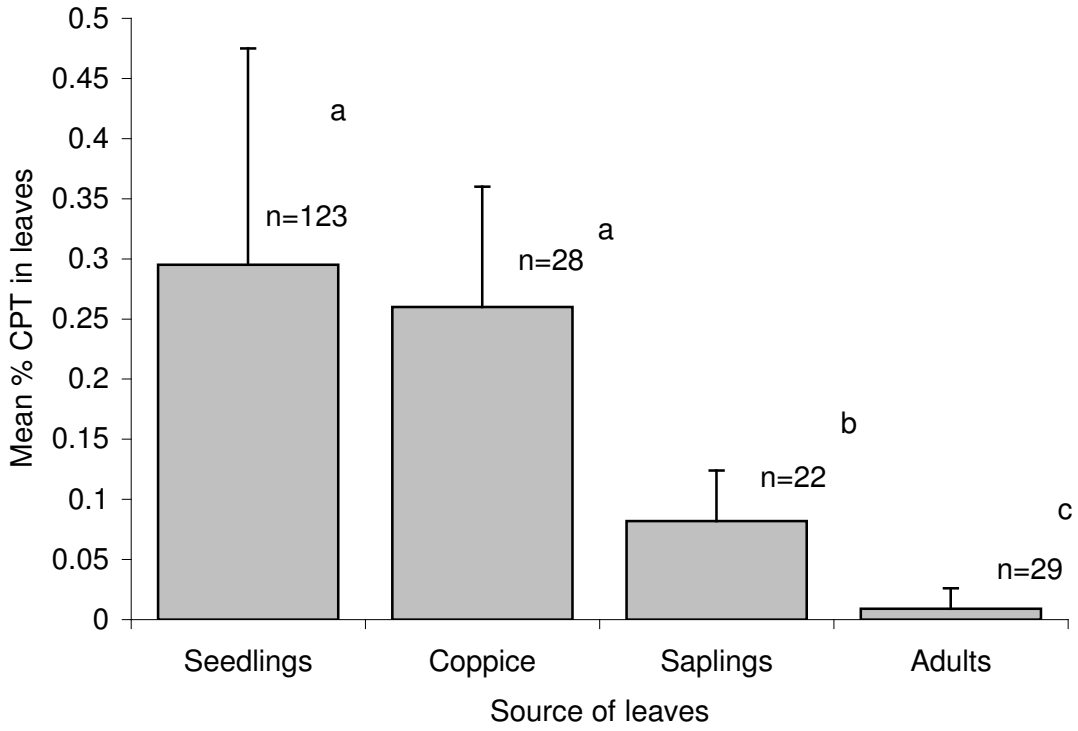


Figure 5.3: CPT in leaves of Seedlings, Coppice, Saplings and adults of *N. nimmoniana*. Histograms with dissimilar letters indicate significance at 5% (Students *t test*).

Sustainable extraction of CPT could also be arrived by exploring several in vitro production systems. For example, stabilization of cell cultures from high yielding individuals could help develop *in vitro* production systems. However the economic viability of this approach will depend upon optimizing several protocols including the sustained growth of the cell culture, in vitro elicitation of CPT, CPT yields etc.

Section II

Population genetic variability of *N nimmoniana* populations in the central Western Ghats, India.

In the present study SSR markers for *N. nimmoniana* have been developed. SSR primers were developed using selective hybridization technique with biotinylated repeat probes, which were captured using streptavidin coated magnetic beads (Zane *et al* 2002). Thirty-seven clones were sequenced and 22 primers were designed with a success rate of 59%.

This study for the first time reports the SSR primer information for *N nimmoniana*, which can be used in detailed analysis of population genetics structure and mating system of *N nimmoniana* in the Western Ghats. In the present study these markers were used to evaluate levels of genetic variability of few populations of *N nimmoniana* in the central Western Ghats, The markers were also used in evaluating the success of cross-amplification of the markers against different genera of Icacinaceae.

Population genetic variability of *N nimmoniana* in central Western Ghats

Population genetic variability of six populations of *N nimmoniana* was evaluated using six SSR primers; mean number of observed alleles over all populations of *N nimmoniana* was 4-5 and mean number of alleles per locus ranged from 3.4 to 4. Similarly the mean expected (*He*) and observed (*Ho*) heterozygosities for *N nimmoniana* were 0.60 and 0.67, respectively. High levels of genetic diversity was observed, a feature typical of highly out crossing species, with the same mean observed and expected heterozygosities across loci.

There was a significant positive association observed between latitude and heterozygoity of the populations, populations in the north were genetically more diverse compared to those in the southern Western Ghats. The north Western Ghats populations were denser favoring exchange of gene pool and have contributed to higher genetic diversity.

The mean pair wise genetic differentiation between the populations is low (F_{ST} = 0.095), indicating that the genetic diversity is maintained within rather than among populations and there is no isolation by distance. This observation was also supported by the AMOVA, which indicated that a large percentage of total genetic variation (83) within populations, with little amount (17% P<0.01) among populations. This observation is similar to that reported by Hamrick *et al.* (1992) on tropical forest tree populations. The low population differentiation in *N nimmoniana* could be due to the extensive seed mediated gene flow between the populations, as seeds are dispersed by the birds which can carry the seeds to long distances.

Ecological niche modeling and heterozygosity of *N nimmoniana* populations:

In this study, attempts were also made to extend the use of ecological niche modeling tools to offer prediction on the genetic variability of the species. An underlining assumption here was that, sites predicted to be highly suitable for a species should also have greater variability compared to sites that are unsuitable.

Analysis of heterozygosity occurring in different suitability areas indicated that individuals in highly suitable areas were genetically more variable. The frequency distribution of observed heterozygosity from the low habitat suitability areas was positively skewed and the Ho was negatively skewed for excellent habitat sites. The distribution was significant (Ks test). This analysis has indicated that, individuals of the populations that are found in excellent habitat suitability category are genetically more diverse among themselves as compared to the ones in the low habitat suitability category (Figure 5.4: Ks test P<0.01 Low vs Excellent). The study has demonstrated for the first time that ecological niche modeling tool can be also be used in predicting the genetic status of the populations of tropical tree species such as *N nimmoniana*.

There are very few studies that have attempted to address niche modeling and genetic diversity in tropical tree species. This study forms the basis and has major implications in terms of conservation of *N nimmoniana* populations that are genetically diverse.

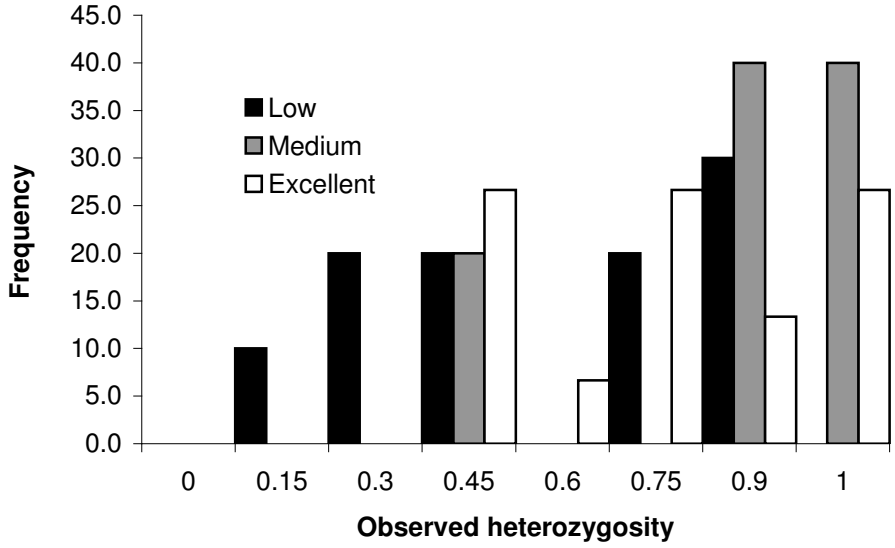


Figure 5.4: Frequency distribution of observed heterozygosity values for populations from different degree habitat suitability.

Section III

Prospecting phylogenetically related genera/species of *Nothapodytes nimmoniana* for Camptothecin

In order to ensure a reliable supply of Camptothecin, several attempts have been made to identify alternate sources that would help in combating the heavy demand on single species of plants and some of them yielded good results. One of the approaches was to search or screen for such compounds in phylogenetically related species/genera/family of the taxa in which compound has been already reported (Wink 1992; Wink, 2003).

In the present study the related genera of *N. nimmoniana* in the family Icaccinaceae was screened with an assumption that related genera are known to share similar biosynthetic pathways, suggesting a commonality of certain compounds or produce same class of compounds. These are often found in the same phylogenetic group made up of different genera and/or monophyletic clades. However, this is not always the case, and if the compound is ubiquitous, similar pathways may also be detected in totally disparate taxa.

Mapping the distribution of related genera of *N nimmoniana*:

As a first step towards prospecting for CPT in members of Icacinaceae, was made an attempt to map the distribution of phylogenetically related species of *N nimmoniana* in the Western Ghats. The distribution of seven related genera using secondary data sources was mapped. None of the related genera/species is as widely distributed as *Nothapodytes nimmoniana*. In fact very few records of the related genera were recorded indicating that these species may be quite rare and sparse in their distribution in the Western Ghats. Unlike *Nothapodyetes nimmoniana,* several of the species were indeed found to be distributed in Eastern Ghats of the country. To date only two other members of Icacinaceae, namely, *Merriliodendron megacarpum* (0.05%) and *Pyrenacantha kleinii* (0.00004%) have been assayed for the CPT content (Zhou *et al*, 2000). The sparseness of their distribution coupled with the lack of access, constrained the collection of these genera for estimating their CPT content in this study. However efforts were made to access the related genera and explore them for possible sources of high yields of CPT, just as was accomplished in the search for phylogenetically profitable sources for the exploitation of taxol (Swapna *et al*, 2002).

Chemical profiling of related genera of *N nimmoniana* for Camptothecin:

Present study has demonstrated the usefulness of phylogenetics approach in locating alternate taxa as source of complex bioactive molecules. In this case CPT an important pharmaceutical precursor. Five genera that belong to Icacinaceae and three other genera coming from families Apocynaceae, Rubiaceae and Olacaceae were screened for the CPT content using both HPLC and LC-MSMS analysis. The presence of CPT has been confirmed in five new genera besides the previous reports. Similarly CPT has also been reported from few other members out side the family Icacinaceae *Ophiorhiza pumila* belongs to the family Rubiaceae, *Tabermontana heyneana* belongs to the family Apocynaceae and *Mostuea brunonsis* belongs to the family Loganiaceae (Saito *et al* 2001; Gunasekara *et al* 1979; Dai *et al* 1999).

Same extracts were subjected for CPT quantification using HPLC and LC-MS/MS. There was a good correlation observed between HPLC and LC-MSMS estimates indicating the reliability of HPLC estimates ($r=0.91;p<0.01$). Many of these new genera had very less CPT either in their leaves or fruits or stem. The results confirmed the earlier results by Zhou *et al* and others (Saito *et al* 2001; Gunasekara *et al* 1979; Dai *et al* 1999) where in most of the genera had CPT of less than 0.1% by dry mass (*Pyrenacantha kleinii:* 0.00004%, *Merriliodendron megacarpum*: 0.05% and *O pumila* : 0.1% in stem).

An important finding from this study was that the highest level of CPT is found in dried fruits of R56 (1.61% by dry mass), approximately equal to what has been reported in stem bark of *Nothapodytes nimmoniana* (Suhas *et al*, 2007), approximately 5 times more than in seeds and leaves of *C acuminata* (0.3% by dry mass in Yamazaki *et al* 2003) and approximately 4 times more than in cotyledons of *N nimmoniana* (0.4% by dry mass in Devanand and Satdive, 2005). Again this is the highest ever-reported estimate of CPT from any plant source. The R56 has been confirmed in both fresh and herbarium material.

Partially matured fruit samples of R56 had approximately 20 times more (0.8% by dry mass) CPT compared to immature fruit samples (0.01% dry mass) collected June of 2007. This indicated fully matured fruit samples of R56 accumulated more CPT. Similarly with in the fruit; embryo accumulated approximately 10 times more CPT ($0.5\%\pm0.25\%$) compared to fruit coat and other parts ($0.069\pm0.035\%$) (Figure 5.5 t test $p<0.01$). In contrast to this in *N nimmoniana* Devanand and Satdive, (2005) have reported maximum concentration of camptothecin in cotyledons, which was 1.6-fold higher than that of the root and 4.5-fold higher than that in the leaves of the same trees. Immature seeds of the tree accumulated alkaloid concentrations that were twice that of the mature seed of the same tree. These results obtained not only demonstrate differential regulation of accumulation of the CPT but also suggest some interesting features pertaining to de novo biosynthesis, conversion, transport, and degradation. Results indicate that in seeds of R56 de novo biosynthesis of CPT must also have been stimulated, similarly in cotyledons of *C acuminata* it was reported that there was high levels of CPT and HCPT (Yan *et al*, 2003).

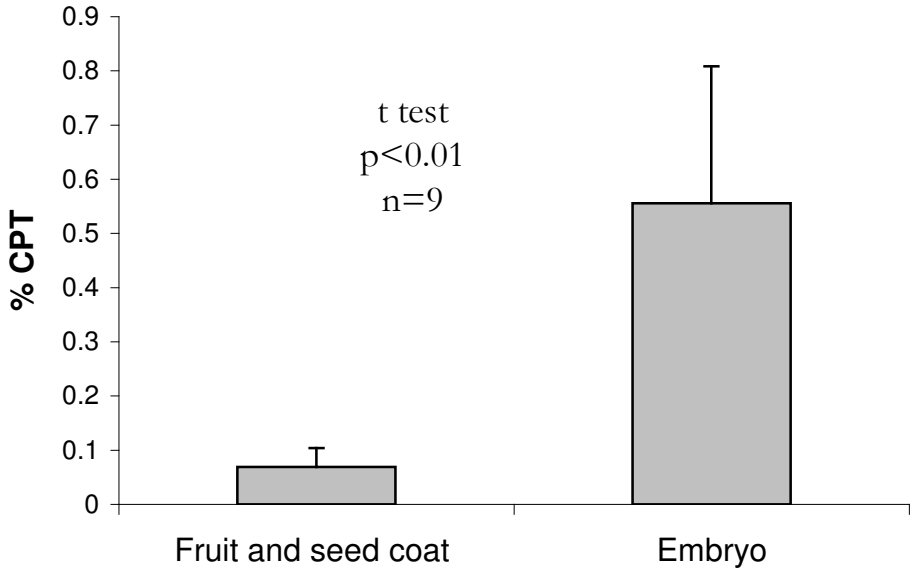


Figure 5.5: Mean CPT content in fruit and seed coat and embryo of different fruit samples of R56 collected during October 2007 from Western Ghats.

In general, seeds, buds and young tissues tend to sequester or synthesize large amounts of defense chemicals during vulnerable stages of the plant life cycle. Young leaves have been shown to contain much higher amounts of CPT than older leaves (Lopez *et al*, 1994; Liu *et al*, 1999; Yan *et al*, 2003). Endosperm, the nutritive part of the seed, contains the highest levels of the animal toxin CPT. Similarly in cotyledons and embryonic axis of young seedlings CPT and HCPT reached their maximums (Yan *et al*, 2003). Further, the fresh samples of R56 started accumulating more CPT as the seed started maturing, indicating role of CPT as a defense chemical. The accumulation of these secondary metabolites at this vulnerable stage may imply an important role for these two defensive compounds in juvenile stages of development.

Phylogeny of CPT containing plants:

Five new genera were found to contain CPT in this study. Together with this data and what has been reported in the literature for the other species, phylogenetic tree of CPT producing plants were developed. So far CPT has been reported from 12 different taxa of Angiosperms (Figure 5.6). Pattern of distribution of particular secondary metabolite over an angiosperm phylogenetic tree depends on a correct interpretation of a phylogenetic tree. Karehed (2001) showed that Icacinaceae did not belong to the order Celastrales (Lorence and Craig, 2004), instead the family contained several lineages associated with either Garryales in the lamiid clade or Aquifoliales in the campanulid clade (Figure 5.6). Phylogenetic data revealed that CPT accumulation is limited to the Asterids. Within the Asterids, CPT is restricted to the orders Cornales, Garryales and Gentianales. Order Cornales being the ancestral ones to contain CPT. Subsequently CPT is absent in order Ericales and appeared in the orders: Garryales and Gentianales. It can be argued in two ways. First, it is apparent that ancestral members of a group evolved the biosynthetic capacity to produce CPT. This could be one evidence for this argument that camptothecin biosynthesis is acquired only in taxa also being able to produce other monoterpene- indole alkaloids, namely within early diverging asterids (Cornales) and lamiids (Garryales and Gentianales) as shown in (Figure 5.6). The absence of such a trait in phylogenetically derived groups such as Ericales, Solanales and Lamiales, as well as to the campanulid clade is probably due to differential gene expression, in that the corresponding genes are not lost but switched off. This is necessary to discuss aspects such as monophyly of biosynthetic potential or parallel/ convergent evolution of such pathways (Wink, 2003). The benzyl-isoquinoline alkaloids are biosynthesized from (S)-nor-coclaurine and investigations of its synthase have revealed that this group has a monophyletic origin. In accordance it is reasonable to assume that the monoterpene-indole alkaloids have a monophyletic origin, as they are biosynthesized from strictosidine. Since secondary metabolites play a vital role as defence and signal compounds, their occurrence apparently reflects adaptations and particular life strategies embedded in a particular phylogenetic framework.

However in second, an instance particularly secondary metabolite may occur in several unrelated clades and/or plant families. The erratic secondary metabolite distribution can be

due to simple convergence, in that the genes that encode a particular biosynthetic pathway evolved independently in several parts of a phylogeny.

Use of Phylogenies in Bioprospecting:

In the search for alternatives to production of desirable medicinal compounds from plants, phylogenetic approach has been a good choice, specifically, screening phylogenetically related genera/species of plants in which the target compound has already been reported.

In this case, Camptothecin, a monoterpene indole alkaloid: is a precursor for the chemical synthesis of Topotecan and Irrinotecan drugs and is tremendously important to the pharmaceutical industry (Lorence and Craig, 2004). This approach has helped in discovering CPT from various genera of Asterids. The low concentration of Camptothecin present in some of these species (*Sarcostigma kleinii, Natsiatum herpaticum, Pyrenacantha volubilis, Apodytes dimidiata, Gomphandra polymorpha, Gomphandra tetrandra, Ophiorhiza pumila* (Rubiaceae) and *Tobermontana heyneana* (Apocynaceae) precludes its use as a source of Camptothecin for commercial production. Since Camptothecin has now been found in high concentrations in R56, it might prove useful to screen for CPT in other members of the genus R56, as well as, in other taxa of the Apocynaceae and Rubiaceae.

Section IV

Prospecting endophytic fungi from *Nothapodytes nimmoniana* for Camptothecin:

Endophytes are microbial entities that live within living tissues of plants. The term endophyte is applied to fungi (or bacteria), which live within plant tissues, and cause no apparent infections. They are considered to be mutualists because they receive nutrition and protection from their hosts, while the hosts receive benefits through increased resistance to herbivores, pathogens and drought etc (Masheswari, 2006). Many are capable of synthesizing bioactive compounds (anticancer, antidiabetic, insecticidal and immunosuppressive compounds) that can be used by the plant for defense against pathogenic fungi and bacteria. Some of these compounds have proven useful for novel drug discovery (Strobel and Daisy, 2003). Recently these endophytic fungi from plants have been widely accepted as an

important source of some of the known novel metabolites. A large number of compounds with new structures and various bioactivities are continuously isolated (Stierle *et al* 1993; Tan and Zou, 2001; Schulz *et al* 2002; Strobel and Daisy 2003). It is known that many bioactive agents produced by plants such as Taxol, could also be produced by endophytic fungi and form an important alternate source for these novel and high value metabolites (Strobel *et al* 1993).

The discovery of fungal endophytes that produce camptothecin has significant biological and commercial implications. Commercially, the fungal culture can be scaled to provide adequate camptothecin production to satisfy new drug development and patient treatment needs. This production reduces the need to harvest wild populations of the source plants, preserving these species. In a recent study, Puri *et al* 2005 were successful in isolating endophytic fungi from *Nothapodytes nimmoniana* that produced camptothecin (CPT), ranged between 5mg per 100 g of mycelial biomass. Though there was promise in utilizing the endophytic fungus for the production of CPT, it would be unviable to produce on a commercial scale because of the low yield. In this direction, a comprehensive study was required to isolate different fungal strains from *N. nimmoniana* that were capable of producing higher concentrations of this compound and a fungus that can produce Camptothecin even after several generations of sub-culture.

Suhas *et al* (2007) reported substantial variability in the production of CPT in trees of *Nothapodytes nimmoniana* from the Western Ghats, India. It is likely that the underlying variability could be associated with the endophytic fungal load.

The present study was designed to isolate, screen and identify high CPT producing endophytic fungal strains. 26 Endophytic fungi were isolated from these high yielding trees of *N. nimmoniana*. The high yielding trees reportedly produce more than 1 % CPT (Suhas *et al*, 2007). Isolation of endophytic fungi from these high yielding trees would help in identifying strains, which also yield higher CPT. The discovery that fungi can bio-synthesize camptothecin increasingly lends support to the possibility of horizontal gene transfer between *N. nimmoniana* and its corresponding endophytic fungal isolates (Young *et al*, 1992).

Isolation of endophytic fungi from *N nimmoniana*:

Twenty-six endophytes were isolated from 24 trees, 4 tissue samples coming from five different sites. Endophytic fungal species from of *N nimmoniana* were more abundant in the Kemannagundy, Nalmukh and Persia (more than three isolates) compared to other sites. These populations are relatively in the wet zone compared to the other populations like Devimani, which occurs in northern part of the Western Ghats (Dry zone). This site-specific disparity might be attributed to abiotic conditions such as humidity and density of vegetation. The humid environment could be more congenial for the horizontal transmission of fungus from plant to plant and survival of the fungi. (Arnold *et al* 2000; Arnold and Edward 2003; Tan and Zou, 2000). Most of these (16 out of 26) isolates were obtained from stem bark tissue of *N nimmoniana,* than leaf or other parts.

Some of the isolates showed tissue specificity that was indicated by their rate of appearance on water agar media. Isolates from stem bark tissue from Kemmannagundy population appeared quickly as compared to ones from Devimani population. Similarly in *vitro* raised seedling did not give any endophytes; it could be because of complete sterilization of the explant tissue during initial stages of inoculation. It has been shown in one of the study where in vitro raised plants of Banana were also free of non-pathogenic micro- organisms such as endophytes (Pereira *et al* 1999).

Host CPT content and endophytic fungi frequency:

In the present study, the correlation between host CPT content and abundance of endophytic fungi was evaluated and it indicated there was no correlation (G test $p<0.05$). In contrast to this, several studies have attempted to address the effect of host chemistry on endophyte species composition, however few studies have showed that there exists an association (Arnold *et al* 2000; Arnold and Edward, 2003). Among foliar endophytes, frequent association between particular fungal species and hosts (Arnold *et al* 2000), and variable growth rates of endophytes on media containing leaf extracts of various host species (Arnold and Edward, 2003) suggest that endophytic fungi are sensitive to gross leaf chemistry.

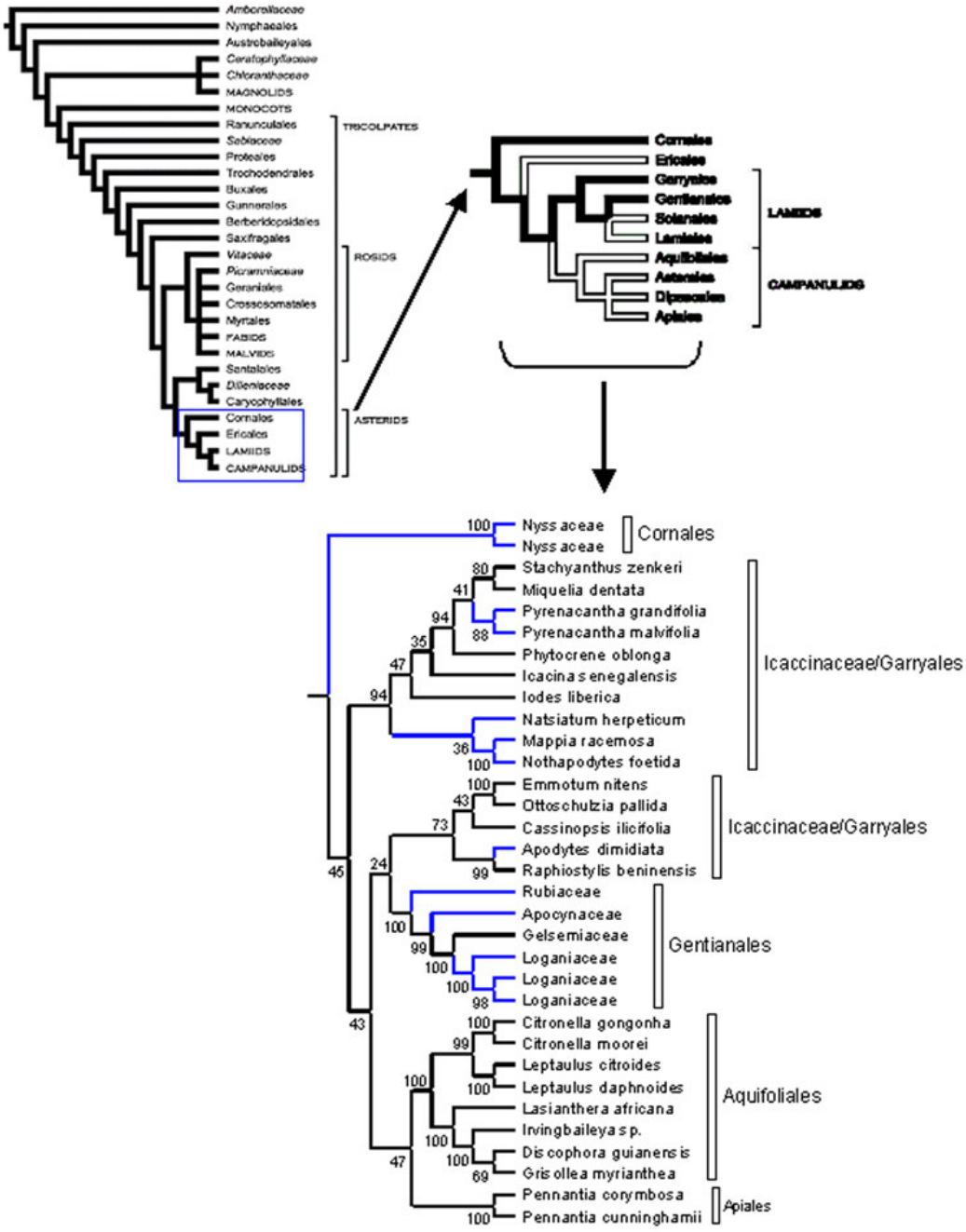


Figure 5.6 : Distribution of CPT in different orders of Angiosperms. The top tree shows the phylogeny of different orders of Angiosperms adopted from Olmsted et al 2004. Small tree is blow up of the Asterids with different orders showing the presence of CPT. The lower panel showing the minimal phylogeny of Asterids developed based on ndhF sequences (present study). Species in the blue clades contain CPT and some are new reports of CPT derived from the present study.

Production of Camptothecin by endophytic fungal isolates:

HPLC analysis of 26 endophytic fungal extracts indicated production of CPT. 26 Isolates exhibited more variation in the amount of CPT produced (both in mycelia and broth) under in vitro conditions. CPT content of all the endophytic fungal strains cultured in shake flasks was estimated for three consecutive generations i.e. second, third (data given) and fourth generation (*unpublished data*). The isolates produced CPT even after several generations of sub-culture away from the host; however the isolates showed more variation in amount of CPT produced from one generation to the other (*unpublished data*). The consistent production of CPT by endophytic fungal isolates of *N. nimmoniana* further supports the theory of Young and co-workers (1992) that during the course of evolution, the symbiotic endophytes developed machinery to biosynthesize and tolerate high levels of secondary metabolites in order to better compete and survive in association with the medicinal plant. Previous workers have reported the production of the antileukemic and antitumor drug taxol from the endophytes of *Taxus* spp. like *Taxomyces andreanae* and *Pestalotiopsis microspora* (Stierle *et al*, 1993). Others workers have reported the production of anti-fungal compounds by endophytes.

In the present study the data for third generation has been presented. These results were also confirmed by LC-MS/MS results, where 12 isolates showed presence of CPT both in HPLC and LC-MS/MS. Quantity of CPT was confirmed for 5 out of 12 isolates screened. However, there was no correlation observed between HPLC and LC-MS/MS results in terms of amount of CPT produced. According to LC-MS/MS isolates UAS006 and UAS013 produced maximum CPT of 2.3 and 4.1 μg/100mg of CPT respectively. Amna *et al* 2006 reported a fungus from *N nimmoniana* that produced 0.40μg/100mg. CPT produced by isolates UAS006 and UAS 013 is 5 to 10 times more than what has been reported by Amna *et al* 2006. Besides this several studies have also attempted to isolate and screen endophytic fungi from plants that has been reported to contain CPT. For example, Lin *et al* 2007 isolated 174 endophytic fungi from *C acuminata* (host plant reported to contain CPT) and the study has not reported even a single isolate that produced CPT. However 4% of these isolates has been reported to show the cytotoxicity in vitro studies. Similarly Liu and Reinscheid, (2007) have reported two endophytic isolates that does not produce CPT but are

reported to be not sensitive to externally applied CPT in culture medium. Indicating endophytic fungi has the mechanism to live with such a toxic chemical.

Association between host CPT and endophytic fungal CPT content

These 26 endophytic fungi were isolated from high yielding trees reportedly producing more than 1 % CPT (Suhas *et al*, 2007). The high CPT production in these trees may be because of presence of efficient endophytic strains. The CPT produced in the fungal isolates was however not correlated with the host tree CPT content indicating that the isolated fungal endophytes do not reflect its host CPT content. On the contrary, some of the studies indeed have reported positive correlation between host metabolite concentration and that produced by in vitro cultured endophytes. For example, endophyte-infected locoweed populations produced swainsonine, and the swainsonine level of endophyte strains in vitro was highly correlated with the swainsonine level of their host plant populations (Braun *et al* 2003). This indicates that endophyte has equally contributed to the overall metabolite yield in the plant.

Molecular taxonomy and phylogeny of endophytic fungi

Twenty-six endophytic fungal isolates of *N nimmoniana* were characterized using ITS region of ribosomal DNA**.** Many of these isolates were non-sporulating, and hence morphologically difficult to identify. It has often been reported that the ITS regions of the rRNA gene are often highly variable with respect to nucleotide composition and that this characteristic can be used to distinguish both morphologically distinct fungal species and strains of the same fungal species (Beltrame-Botelho *et al* 2005). DNA sequence comparisons of region containing the two internal transcribed spacer (ITS) regions (ITS1 and ITS2) and the 5.8S rRNA gene are useful in determining relationships between fungal genera and species (White *et al* 1990; Tanabe *et al* 2004).

In the present study, using a set of universal primers containing ITS1, ITS2 and 5.8S rRNA genes, the fungal DNA were amplified. There was considerable degree of amplified product length variation observed across 26 different isolates. The 26 isolates belonged to five different genera indicating low diversity of endophytic fungi as compared to any tree species like *A indica*, which is reported to have isolates belonging to 15 different genera of fungi The

low fungal diversity in *N nimmoniana* could be due to the presence of Camptothecin, which is reported to have antifungal properties (Liu and Reinscheid, 2007) and fungal species that are not sensitive to CPT can only inhabit *N nimmoniana.*

Different genera were clearly segregated into different clusters based on the length polymorphism, indicating length variation is species specific. BLAST analysis of ITS1, ITS2 and 5.8S rRNA sequence data revealed, 26 isolates belong to five different genera of endophytic fungi and seventeen of them are *Fusarium* species. Three genera were reported from Ascomycota (*Fusarium, Phomopsis and Botryosphaeara).* Of these *Fusarium* is the most common genus and dominant endophyte isolated from different tissues and sites of *N. nimmoniana* in the Western Ghats. Most of them are *F. oxysporum* isolates (10 of them) and remaining belongs to *F solani* and *F beofromi. F oxysporum* as an endophyte reported to be present in many different hosts. Lin *et al* (2007) and Liu and Reinscheid, (2007), also have isolated endophytic fungi from a host reported to have CPT (*C. acuminata*) and many of these isolates were identified as *Fusarium* and *Phomospsis.* Remaining two isolates belongs to Basidiomycetes and one of the isolate that was identified as *Botryosphaeara parva* is also reported from *C. acuminata* as an endophyte (Lin *et al* 2007). *Fusarium* and *Phomospsis* indicate that two phylogenetically different hosts *N nimmoniana* and *C acuminata,* which contain CPT seems to inhabit same set of endophytic fungi. This indicated some degree of similarity in composition of endophytic fungi from hosts that are similar in their chemistry. In contrasts to this a totally different endophytic fungi has been reported by Puri *et al* (2005) that also produces CPT, however the study was not exhaustive and resulted in recovery of only one isolate that yielded CPT.

Phylogenetic analysis of 26 endophytic isolates using ITS sequence data revealed three different clusters. Three clusters were occupied by species belongs to the genera *Fusarium*, *Phomopsis* and others *(Irpex, Botryopsphaera and Galactomyces* etc). There was no geographical association observed between isolates from where they are derived, indicating their consistency in colonization of *N nimmoniana* by same set of endophytes. This was observed in many cases, for example a species of *Phomopsis* dominated the endophyte assemblages of teak, irrespective of the location of the host trees (Suryanarayanan *et al* 1998). In another

case, among the endophytes, the genus *Pestalotiopsis* dominated the endophyte assemblage of *T. arjuna* collected from different locations.

Isolates from *N nimmoniana* showed more tissue specificity, where most of the isolates were derived from stem bark samples and all belong to the genus *Fusarium*. Similarly *Diaporthe* and *Phomopsis* were isolated from leaf petioles, *Galactomyces* from flower buds of *Nothapodytes nimmoniana*. *Fusarium* and *Phomopsis* are predominantly isolated from twig xylem and stem bark of many hosts, but not from leaf or root (Kumar & Hyde 2004).

Phylogeny and CPT content

To identify high CPT producers and distinguish them from the low producers, CPT data of the isolates was correlated with the ITS phylogeny by computing the mean CPT for each cluster. Mean CPT of different cluster did not differ significantly, indicating there was no phylogenetic association.

Prospects of endophytic fungi from *N nimmoniana*:

Isolates UAS006 and UAS013 produced maximum CPT as compared to any other strains and one of them was identified as *F oxysporum* (*G sacchari* its telomaprh). UAS006 did not give any BLAST hits indicating it is a unique isolate and also producing more CPT under in vitro conditions. These results offers promise for industrial up scaling and further commercialization of the technology to produce CPT under in vitro conditions, which would not only help in conserving the host species from overexploitation but also meet the market demand. This fungal library (26 isolates) from *Nothapodytes nimmoniana* could also be used to prospect for interesting metabolites that may be potentially be useful. Besides CPT endophytic fungi may be potentially useful in screening them for other related Camptothecines like 10-OHCPT, Mappicine and others.

In this regard, besides CPT, many authors have also reported different biological activities for *Fusarium, Phomopsis, Irpex* and *Botryosphaera* endophytes. For example Endophytic *Fusarium oxysporum* strains isolated from healthy banana plants inoculated into tissue culture banana plantlets have shown control of the banana weevil and the nematode, indicating use of *F oxysporum* isolates as biocontrol agents. An interesting study by Pamphile *et al* (2004),

demonstrated the effectiveness of endophytic fungi as (especially *F. oxysporum* strains) vectors for the introduction of characteristics of biotechnological or agricultural interest into tropical host plants. In their study a blue precipitate was clearly observable in the mycelium of *F. verticillioides* GUS^+ transformants colonizing maize roots, indicating that the co-transformant expressed the gene in the plant. Similarly many of the *Phomospsis* isolates also reported to have anticancer properties and was demonstrated by Li *et al* (2005) and culture extract of *Irpex lacteus* being able to inhibit the growth of *C. albicans*, *C. glabrata*, *C. parapsilosis*, *B. cereus*, *E. coli*, *S. typhimurium,* and *S. aureus* (Rosa *et al* 2003). This has indicated a promise in prospecting *N nimmoniana* isolates for other compounds which are potentially useful. Some isolates like *F oxysporum* could also be used in metabolic engineering studies and isolating genes responsible for production of CPT, as this fungus is reported to be genetically highly tractable (Strobel and Daisy, 2003).

SUMMARY

VI SUMMARY

Western Ghats constitutes one of the unique biological regions and biodiversity hot spots of the world. Among the biodiversity hotspots, it ranks 5th in its bioprospecting value (Pushpam Kumar, 2004). Many plants from these regions are the basis of age-old traditional medicine systems. With high richness medicinal plants distributed all along Western Ghats, there is immense potential for bioprospecting biologically active compounds in this region. Drugs from plants have played a dominant role in pharmaceutical care for the treatment of various diseases, especially cancer. The greatest recent impact of plant-derived drugs was probably felt in the antitumor area, where taxol, vinblastine, vincristine and camptothecin have dramatically improved the effectiveness of chemotherapy against some of the deadliest cancers.

Among the plant-derived compounds camptothecin (CPT), a pyrrolo quinoline alkaloid, has been extensively used as a novel anti-tumor compound. CPT, a monoterpene alkaloid is an important anti-cancer compound obtained from several plant sources. However to date, the highest content of CPT has been reported from *Nothapodytes nimmoniana* (about 0.3 % on a dry weight basis) compared to any other botanical source (Ku and Tang, 1980). *N. nimmoniana*, is a small tree of the family Icacinaceae and constitutes the major and perhaps the only source in India.

In India *Nothapodytes nimmoniana* is extensively harvested from the Western Ghats and the billets exported for commercial extraction of CPT. In fact, it is estimated that in the last decade alone, there has been at least 20% decline in the population leading to the red listing of the species (Ravi Kumar and Ved, 2000). Indiscriminate felling of trees for short-term gains could perhaps lead to the loss of elite individuals and populations that otherwise could potentially serve as sources of high CPT. Therefore it is essential to find alternative and consistent sources to meet the pharmaceutical demand. In order to ensure a reliable supply of Camptothecin, several attempts are underway to identify alternate sources.

With this background the present study was specifically planned to screen populations of *N nimmoniana* in the Western Ghats to identify high CPT yielding lines/

individuals. This would lead to the development of clonal systems from high CPT yielding lines or could be used in sustainable production of CPT.

Chemical profiling of populations of *Nothapodytes nimmoniana* for CPT

In this study, 17 trees (9 for which highest CPT was reported from stem bark and 8 from root bark) were selected and screened for the HPLC/LC-MS quantification of camptothecin as well as in detecting other camptothecines in the tissue. For the first time, nearly 5 to 8 fold higher CPT yields than hitherto reported in *Nothapodytes nimmoniana* have been recovered from individual trees. Further new families of camptothecines were identified from *Nothapodytes nimmoniana.* 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. 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.

Sustainable harvesting of camptothecin from *N nimmoniana:*

Leaf samples were collected from 123 individuals of one and half year old seedlings, 29 individuals of three to four year old coppices, 22 individuals of five year old saplings and 30 individuals of 10 to 15 years old adult trees of *Nothapodytes nimmoniana* from Sirsi and Biligiri Rangaswamy Temple Wildlife sanctuary (BRT). All these samples were analyzed for CPT using HPLC and total camptothecin concentration in leaf tissue was expressed on a dry weight basis. There were higher percentages of seedlings producing more than 0.2% CPT. In fact, some seedlings had leaf CPT of more than 1%. The leaf CPT content in the trees did not exceed 0.2%. Thus, it was found that the mean leaf percent CPT was maximum in seedlings followed by coppices. The adult tree leaves had the lowest CPT content.

Molecular characterization of populations of *Nothapodytes nimmoniana* in the Central Western Ghats, India using SSR markers

For the first time 22 microsatellite markers have been developed for *Nothapodytes nimmoniana.* Out of 22 SSR primers 11 primers revealed target specific amplification with

amplified product in the expected range. Five out of eleven revealed good polymorphism. Using the genetic data on six SSR primers over six populations of *Nothapodytes nimmoniana* following genetic variability parameters were estimated. About 50 individuals of *N nimmoniana* over 6 populations evaluated for allelic diversity at 5 selected loci. Amplification profile over six populations of *N nimmoniana* at 5 loci reveled good allelic variation. The number of alleles detected over all loci across different populations ranged from 4-5. Mean number of alleles detected across different populations and over all loci was 3.6±0.15. The mean observed heterozygosity over all populations at all loci was 0.67%±0.04%. Mean observed hetrozygosity (over all populations and at all loci) was more than expected heterozygosity based on Hardy Weinberg equilibrium 0.60±0.01. In summary populations in the northern part of the Western Ghats, maintained more heterozygosity compared to the populations in the southern Western Ghats.

Prospecting phylogenetically related genera/species of *Nothapodytes nimmoniana* for Camptothecin

Based on the secondary data obtained from herbaria sheets and floras the distribution maps of all the related genera of Icacinaceae members in the Western Ghats were developed. For about 8 different genera spatially explicit distribution maps have been developed. Using the distribution maps samples of all these genera including different plants parts were collected. All these samples were subjected for CPT analysis. HPLC profile of all the related genera extracts showed the presence of CPT except in *Strombansia zeylanica.* Among the Icacinaceae genera that were screened for CPT *Natsiatum herpaticum* fruits had maximum CPT of 0.026% followed by *Sarcostigma klenii* leaf (0.018%) and *Gomphandra polymorpha* fruits (0.011%). In *Apodytes dimidiata* CPT was detected only in the stem bark sample and was not detected in the leaf sample. Across different plants parts of different genera analyzed for CPT, it was more in stem bark than in leaf or fruits

Phylogeny of Icacinaceae

In the present study a minimal phylogeny of CPT producing plants was constructed using ndhF gene sequences of different species/ genera and subjected for the phylogenic analysis. The analysis showed that Icacinaceae are polyphyletic. The genera included belong to both euasterids I and II and should be rearranged into three different orders: Garryales, Aquifoliales, and Apiales.

Prospecting endophytic fungi from *Nothapodytes nimmoniana* for Camptothecin.

Individuals of *Nothapodytes nimmoniana* that differ in CPT in stem bark were used for isolation of endophytic fungi with a presumption that individuals might also differ in their endophytic fungal composition. All endophytic fungal isolates were subcultured to ensure an axenic culture; each fungal culture has been continuously maintained since the original isolation, with transfer to fresh medium every 4-5 weeks. CPT estimation was done when isolates were in 3rd subculture (3rd generation away from the host). CPT presence was confirmed by matching retention time of authentic CPT with that of endophytic fungal extract. Some of the isolates showed good CPT signal in hyphae as well as broth, its presence was confirmed in all the 26 isolates. Quantification of the compound was made in the samples in which CPT was detected. CPT content in the hyphae ranged from 0.10µg to 9.8µg. Highest CPT 9.8µg was found in hyphae of UAS 011 and lowest was found in UAS 006 (0.10µg). Six isolates (UAS 004, UAS 011, UAS 016, UAS 018 UAS 019 and UAS 020) produced CPT more than 1µg per 100mg of dry hyphal biomass. Four isolates produced more than or equal to 0.5µg of CPT in the hyphae. The same extracts were subjected for LC-MSMS analysis. CPT presence and its quantity in fungal extracts were confirmed by appearance of characteristic ions after fragmentation of parent molecule m/z 349. Three ions were observed upon fragmentation m/z 305 (M - CO2), m/z 247 (m/z 275 - CO) and m/z 263. Some of these extracts produced clear 305 signals like UAS 017, UAS 021, UAS13, UAS 019 and UAS 018 (Figure 4.4.2). In all the isolates CPT was detected, but quantification was possible in few of the samples because of amount of CPT in rest of the samples was below the limit of quantification in LC-MSMS.

The ITS PCR product lengths of all the isolates ranged from 500- 720bp. There was a considerable length variation observed across the isolates, with a 200bp difference between the shortest to the longest fragment. Length variation seems to be species/isolate specific and indicating a greater diversity of isolates from *N nimmoniana* based on ITS length polymorphism. Seventeen out of 26 isolates were classified as *Fusarium*; remaining isolates belong to five different genera of fungi. *Fusarium* isolates belongs to two different species *Fusarium solani* (*Nectria haematococca; sexual stage of Fusarium solani*) and *Fusarium oxysporum* (*Gibberella moniliformis: sexual stage of Fusarium oxysporum*).

Phylogenetic analysis of isolates revealed three different clusters. The cluster I contains 17 *Fusarium* isolates. The *Diaporthe* and *Phomopsis* isolates formed the II cluster and one isolate of *Botryosphaera* was in the III cluster. The IV cluster contained isolates belonging to Basidiomycetes fungi. The isolates clustered clearly into separate groups based on ITS similarity and there was no geographical association of the isolates observed.

REFERENCES

VII REFERENCES

Abigers D., Catimel G. and Kozimor R. 1995. Population pharmacokinetics and pharmacodynamics of irinotecan (CPT-11) and the active metabolite SN-38 during phase-I trials. *Ann Oncol.* **6:** 141-151.

Aimi, Hoshino N. H., Nishimura M., Sakai S. and Haginiwa J. 1990. Chaboside, first natural glycocamptothecin found from *Ophirrohiza pumila. Tetrahedron Lett.* **31**: 5169-5172.

Aiyama R., Nagai H. Nokata K., Shinohara C. and Sawada S. 1988. A camptothecin derivative from *Notapodytes foetida*. *Phytochemistry.* **27**: 3663–3664.

Arisawa M., Gunasekera S. P., Cordell G. A. and Farnsworth N. R. 1981. Plant anticancer agents-XXI. Constituents of *Merrilliodendron megacarpum*. *Planta Med.* **43**: 404–407.

Arnold Elizabeth A. and Edward Allen Herre. 2003. Canopy cover and leaf age affect colonization by tropical fungal endophytes: Ecological pattern and process in *Theobroma cacao* (Malvaceae). *Mycological Research*. **95(3):** 388-398.

Arnold A. E., Maynard Z., Gilbert G. S., Coley P. D. and Kursat T. A. 2000. Are tropical fungal endophytes hyperdiverse? *Ecology Letters.* **3:** 267-274.

Amna T., Puri S. C., Verma V., Sharma J. P., Khajuria R. K., Musarrat J., Spiteller M. and Qazi G. N. 2006. Bioreactor Studies on the endophytic Fungus *Entrophospora infrequens* for the Production of an anticancer alkaloid Camptothecin. *Canadian Journal of Microbiology*. **52(3)**: 189-196.

Balandrin M. F., Kinghorn A. D. and Farnsworth N. R. 1993. In Human Medicinal Agents from Plants. *Am. Chem. Soc. Symposium Series.* No. 534.

Beckman J. S. and Weber J. L. 1992. Survey of human and rat microsatelites, *Genome.* **12:** 627-631.

Beltrame-Botelho I. T, Gaspar-Silva D, Steindel M, Davila A.M. and Grisard E.C., 2005. Internal transcribed spacers (ITS) of *Trypanosoma rangeli* ribosomal DNA (rDNA): a useful marker for inter-specific differentiation. *Infect Genet Evol.* **5:**17–28.

Bills G. F. and Polishook J. D. 1991. Microfungi from *Carpinus caroliniana*. *Canadian Journal of Botany*. **69:** 1477-1482.

Bodley A. L. and Shapiro T A. 1995. Molecular and cytotoxic effects of camptothecin, a toposiomerase I inhibitor, on trypanosomes and Leishmania. *Proc. Natl. Acad. Sci.* **92**: 3726–3730.

Braun K., Romero J., Liddell C. and Creamer R. 2003. Production of swainsonine by fungal endophytes of locoweed. *Mycological Research*. **107:** 980-988.

Bryant J. P. and Julkunen T. R. 1995. Ontogenic development of chemical defense by seedling resin birch: energy cost of defense production. *J. Chem. Ecol.* **21:** 883--895.

Burke T. G. and Bom D. 2000. Camptothecin design and delivery approaches for elevating Anti-Topoisomerase I activities *In vivo*. *NY Acad Sci.* **922**: 36-45.

Chase M. R., Moller C., Kesseli R. and Bawa K. S. 1996. Distant gene flow in tropical trees. *Nature.* **383:** 398-399.

Clauss M. J, Cobban H. and Mitchell-olds T. 2002. Cross-species microsatellite markers for elucidating population genetic structure in *Arabidopsis* and *Arabis* (Brassicaeae), *Molecular Ecology*. **11:** 591-601.

Clay K. and Holah J. 1999. Fungal endophyte symbiosis and plant diversity in successional fields. *Science.* **285:** 1742-1744.

Clay K. 1990. Fungal endophytes of grasses. *Annual Review of Ecology Systems*. **21:** 275-297.

Clements M. K., Jones C. B., Cumming M. and Doud S. S. 1999. Antiangiogenic potential of Camptothecin and Topotecan, *Cancer Chemother Pharmacol.* **44(5):** 411-6.

Cragg G. M. Newman D. J. and Snader K. M. 1997. Natural products in drug discovery and development. *J Nat Prod.* **60:** 52–60.

Cragg G. M. and Newman D. J. 2004. A Tale of two tumor targets: Topoisomerase I and Tubulin. The Wall and Wani contribution to cancer chemotherapy. *J. Nat. Prod.* **67**: 232-244.

Cregan P. B., Bhagwat A. A., Akkaya M. S. and Rongwen. J. 1995. Microsatellite fingerprinting and mapping in soybean. *Methods in Molecular Cell Biology.* **5:** 49-61.

Dai J. R., Cardellina J. H. and Boyd M. R. 1999. 20-Oß-Glucopyranosyl camptothecin from *Mostuea brunonis*: a potential camptothecin pro-drug with improved solubility. *J. Nat. Prod.* **62**: 1427–1429.

Daniel S. F and Fransworth N. R. 2001. The Value of Plants Used in Traditional Medicine for Drug Discovery. *Environmental Health Perspective.* **109:** 69-75.

Dayanandan S, Rajora O. P. and Bawa K. S. 1998. Isolation and characterization of microsatellites in trembling aspen (*Populus tremuloides*). *Theoretical and Applied Genetics.* **96:** 950-956.

DeVita V. T., Serpick A. A. and Carbone P. O. 1970. Combination chemotherapy in the treatment of advanced Hodgkin's disease. *Ann Intern Med.* **73**: 881-895.

Devanand P. F. and Ramesh K. S. 2005. Distribution of anticancer drug camptothecin in *Nothapodytes foetida*. **76:** 643-648.

Fransworth N. R. 1988. Ethnobotany and search for new drugs. *Biodiversity Wislson.* **24:** 83-97.

Ganeshaiah K. N., Narayani B., Nilima N., Chandrasekara K., Swamy M. and Uma Shaanker R. 2003. Predicting the potential geographical distribution of sugarcane wooly aphid using GARP and DIVA GIS. *Current Science.* **85(11)**: 1526-1528.

Govindachari T. R. and Viwanathan N. 1972. Alkaloids of *Mappia foetida*. *Phytochemistry*. **11:** 3529-3531.

Govindachari, T. R. 2002. Five decades in the study of natural products, *Proc.Indian Acad.Sci.* **114:** 175-195.

Gunashekera S. P., Badawi M. M., Cordell G. A., Farnsworth N. R. and Chitnis M. 1979. Plant anticancer agents-X. Isolation of camptothecin and 9-methoxycamptothecin from *Ervatamia heyneana*, *J. Nat. Prod.* **42**: 475–477.

Hijmans R. J. and David M. S. 2001. Geographic distribution of wild potato species, *American Journal of Botany*. **88:** 2101-2112.

Hokanson S. C., Szewc-McFadden A. K., Lamboy W. F. and McFerson J. R., 1998., Microsatellite (SSR) markers reveal genetic identities, genetic diversity and relationships in a Malus x domestica Borkh. core subset collection. *Theoretical and Applied Genetics*. **97:** 671-683.

Hombe Gowda, H. C., Vasudeva R., Georgi P Mathachen., Uma Shaanker R. and Ganeshaiah K. N. 2002. Breeding types in *Nothapodytes nimmoniana* Graham. *Current Science*. **83(9):** 1077-1078.

Hossain M. M., Zhang W., Curran D. and Parniak M. A. 2003. Mappicine inhibitors of hiv-1 reverse transcriptase-associated ribonuclease-H. *Antivi Ther*. **8:** 14.

Hseing Y. H., Hetzberg R., Hecht S. and Liu L. F. 1985. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase-I. *J. Biol. Chem*. **260**: 14873-14878.

Hutchinson C. R., Heckerdorf A. H., Straughn J. L., Daddona P. E. and Cane D. E. 1979. Biosynthesis of camptothecin: definition of strictosamide as the penultimate biosynthetic precursor assisted by ^{13}C and 2H NMR spectroscopy. *J. Am. Chem. Soc.* **101:** 3358-3366.

Jarne P. and Lagoda P. J. L. 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution*. **11:** 424-429.

Karehed J. 2001. Multiple origin of the tropical forest tree family Icacinaceae. *American J. Bot.* **88(12):** 259-2274.

Kitijima M., Norie F., Furnie Y., Hiroshi S., Kazuki S., Noria A. and Hiromitsu T. 2005. Camptothecins and two new monoterpene glucosides from *Ophiorrhiza liukiuensis*. Chem. Pharm. Bull. **53(10)**:1355—1358.

Kruszewski S. and Thomas B. G. 2002. Properties of camptothecin analogues-promising Topoisomerase I inhibitors determined by Fluorescence spectroscopy methods. *Polish J Med Phys and Eng.* **8(3):** 183-192.

Ku K. Y. and Tang T. C. 1980. *Zhing cao yao*, **11:** 476.

Kumar D. S. S. and Hyde K. D. 2004. Biodiversity and tissue specificity of endophytic fungi from Tripterygium wilfordii. *Fungal Diversity*. **17:** 69–90.

Kutchan T. M. 1995. Alkaloid biosynthesis-the basis for metabolic engineering of medicinal plants. *Plant Cell.* **7:**1059-1070.

Li S., Yi Y., Wang Y. Zhang Z. and Beasley R. S. 2002. Camptothecin accumulation and variations in Camptotheca. *Planta Med.* **68**: 1010–1016.

Li S. Y. and Adair K. T. *Camptotheca acuminata* Decaisne Xi Shu (Chinese Happy tree) a promising anti-tumor and anti-viral tree for the 21st century. A Henry M. Rockwell Monograph (ed. Stephen, F.), Austin state University, Stephen F. Texas, 1994.

Lilenbaum R. C., Ratain M. J., Miller A. A., Hargis J. B., Hollis D. R., Rosner G. L., O'Brien, S. M., Brewster L., Green M. R. and Schilsk R. L., 1995. Phase-I study of paclitaxel and topotecan in patients with advanced tumors: A cancer and leukemia group B study. *J Clin Oncol.* **13:** 2230-2237.

Lin X., Lu C., Huang Y., Zheng Z., Su W. and Shen Y. 2007. Endophytic fungi from a pharmaceutical plant, *Camptotheca acuminata*: isolation, identification and bioactivity. *World J Microbiology Biotechnology*. **23:** 1037-1040.

Litt M. and Luty J. A. 1989. A hypervariable microsatellite revealed by in vitro amplification of a di-nucleotide repeat within the cardiac muscle action gene, *Am. J. Hum.Genet*. **44:** 397-409.

Liu L. F., 1989. DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem*. **58**: 351-375.

Liu W. and Reinscheid. 2004. Camptothecin-resistant fungal endophytes of *Camptotheca acuminata. Mycological Progress*. **3(3):** 189–192.

Liu L. F., Desai S. D., Li T. K., Mao Y., Sun M. and Sim S. P. 2000. Mechanism of action of camptothecin. *Ann NY Acad Sci*. **922**: 1-10.

Liu, Z., 2000. Drought-induced in vivo synthesis of camptothecin in Camptotheca acuminata seedlings. *Physiologia Plantarum*. **110:** 483-488.

Liu Z., John C. A., Viator H. P., Roysell J., Constantin J. and Stanley B. C. 1999. Influence of nutrient availability, spacing and coppicing on stomatal conductance, Abscisic acid and alkaloid levels in *Camptotheca acuminata* seedlings, *Physiol. Plant*. **105:** 402-408.

Lopez M. and Craig L. N. 2004. Camptothecin, over four decades of surprising findings. *Phytochemistry*. 65(20): 2731-2841.

Lopez M., Nessler C. L. and McKnight T. D. 1994. Sites of accumulation of the antitumor alkaloid camptothecin in *Camptotheca acuminata. Planta Med*. **60:** 558–560.

Lopez M. and Nessler C. L. 1997. Tryptophan decarboxylase is encoded by two autonomously regulated genes in *Camptotheca acuminata* which are differentially expressed during development and stress. *Plant J*. **11:** 1167–1175.

Lorence A. and Craig L. N. 2004. Camptothecin: over four decades of surprising findings. *Phytochemistry*. **65(20)**:2731-2841.

Luikart G. and England P. 1999. Statistical analysis of microsatellite DNA data, *Trends in Ecology and Evolution*. **14:** 253-256.

Masuda N., Fukuoka M. and Kusunoki Y. 1992. CPT-11: a new derivative of camptothecin for the treatment of refractory or relapsed small-cell lung cancer. *J Clin Oncol*. **10:** 1225-1229.

McCouch S. R., Chen X., Panaud O., Temnykh S., Xu Y., Cho Y. G., Huang N., Ishii T. and Blair M. 1997. Microsatellite marker development, mapping and applications in rice genetics and breeding. *Plant Molecular Biology*. **35:** 89-99.

Morgan and Conner J. 2001. Using genetic markers to directly estimate male selection gradients. *Evolution*. **55:** 272-281.

Moxon E. R. and Wills C. 1999. DNA microsatellites: agents of evolution. *Scientific American*. Jan: 72-77.

Myers Norman., Russell A. Mittermeier., Cristina G. Mittermeier., Gustavo A. B. da Fonseca and Jennifer Kent. 2000. Biodiversity hotspots for conservation priorities. *Nature*. **403:** 853-858.

Nalawade S. M., Abhay P. S., Kao C. L. and Tsay H. S. 2003. Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. *Bot. Bull. Acad. Sin*. **44:**79-98.

Noble R. L., 1990. The discovery of the Vinca alkaloids-chemotherapeutic agents against cancer. *Biochem Cell Biol*. **68**:1344-1351.

Nolte B. A. 1999. Micro-analysis and localization of camptothecin in *Camptotheca acuminata*. M.S. Thesis, Texas A and M University, College Station, TX.

Ouborg N., Piquot Y. and Van G. 1999. Population genetics, molecular markers and the study of dispersal in plants. *Journal of Ecology.* **87:** 551-568.

Padmanabha B. V., Chandrashekar M., Ramesha B. T., Hombe Gowda H. C., Rajesh P. Gunaga, Suhas S., Vasudeva R., Ganeshaiah K. N. and Uma Shaanker R. 2006. Patterns of accumulation of camptothecin, an anti-cancer alkaloid in *Nothapodytes nimmoniana* Graham., in the Western Ghats, India: Implications for identifying high-yielding sources of the alkaloid. *Current Science* **90:** 95-100.

Pantazis P., Han Z., Chatterjee D. and Wyche J. 1999. Water-insoluble camptothecin analogues as potential antiviral drugs. *J. Biomed. Sci.* **6:** 1-7.

Pedro M., Branco. and Paula. S. 2003. Natural product-like combinatorial libraries. *J. Braz. Chem. Soc.* **14(5):** 675–712.

Pereira J. O., Carneiro M. L., Vieira. and Azeved. J. L. 1999. Endophytic fungi from Musa acuminata and their reintroduction into axenic plants. *World Journal of Microbiology & Biotechnology* **15:** 37-40.

Ping Yueh Hsin., Hsin-Chen Lee., Jen-Y Lee., Pin-Ho Wu., Li-Kang H. O., Ching-Wen Chi., Ming-Fong L. U. and Jane-Jen Wang. 2006. Anticancer effects of low-dose 10 hydroxycamptothecin in human colon cancer. *Oncology Reports.* **15:** 1273-1279.

Pizzolato J. F. and Saltz L. B. 2003. The camptothecins. *Lancet.* **361:** 2235–2242.

Poupat Christiane., Ingrid H., Francoise G., Alain A., Daniel G. and Pierre P. 2000. Neutral and basic Taxoids in the needles of *Taxus* species. *Planta Medica.* **66:** 580-584.

Prakash Kumar U. M., 2007. Chemical profiling for few plant metabolites (Withanolide-A and Withafern-A) including anticancer compounds in forest species of Western Ghats, India. Thesis submitted to University of Agricultural Sciences.

Priel E., Showalter S. D. and Blair D. G. 1991. Inhibition of human immunodeficiency virus (HIV-1) replication in vitro by noncytotoxic doses of camptothecin, a topoisomerase I inhibitor. *AIDS Res Hum Retroviruses.* **7:** 65–72.

Prop. 11.58. Consideration of Proposals for Amendment of Appendices-II.

Puri S. C., Handa G., Gupta R. K., Srivastava T. N., Somal, P. and Sharma S. N. 1999. Quantitation of camptothecin in *Nothapodytes foetida. J Indian Chem. Soc.* **76**: 370-371.

Puri S. C., Verma V., Amina T., Qazi G. N. and Spiteller M. 2005. An endophytic fungus from Nothapodytes foetida that produces camptothecin. J Nat. Prod. **68 (12):** 1717-1719.

Pushpam Kumar., 2004. Valuation of Medicinal plants for pharmaceutical uses, *Current Science.* **86**: 7-10.

Maheshwari R. 2006. What is an endophytic fungus? *Current Science.* **90(10):** 1309.

Rapisarda A., Urachimeg B., Scudiero D. A., Selby M., Sausville E. A., Shoemaker R. H. and Melillo G. 2002. Identification of small molecule inhibitors of hypoxia-inducible factor I transcriptional activation pathway. *Cancer Res.* **62**: 4316-4324.

Raskin Ilya., David M. Ribnicky., Slavko Komarnytsky., Nebojsa Ilic., Alexander Poulev., Nikolai Borisjuk., Anita Brinker., Diego A. Moreno., Christophe Ripoll., Nir Yakoby., Joseph M.O Neal., Teresa Cornwell., Ira Pastor and Bertold Fridlender. 2002. Plants and human health in the twenty-first century, *Trends in Biotechnology.* **12:**522-531.

Rates S. M. K., 2001. Plants as sources of drugs. *Toxicon.* **39**: 603–613.

Ravi Kumar and Ved D. K. 2000. 100 Red listed medicinal plants of conservation concern in Southern India, *Foundation for revitalization of local Health Traditions.* **6:** 261-263.

Rita M. Moraes., Hemanth L., Ebru B., Muhammed M. and Kent C. 2001. The American May apple and its potential for podophyllotoxin production. *Trends in new crops and new uses*. **36:** 527-632.

Roberts, 2007. Production and engineering of terpenoids in plant cell cultures. *Nature Chemical Biology*. **3(7)**: 387-395.

Roja G. and Heble M. R., 1994. The quinoline alkaloids Camptothecin and 9-methoxycamptothecin from tissue cultures and mature trees of *Nothapodytes foetida, Phytochemistry*. **36:** 65-66.

Romanelli S. P., Perego G., Pratesi N., Carenini M. and Tortoreto Z. F. 1998. In *vitro* and *in vivo* interaction between cisplatin and topotecan in ovarian carcinoma systems. *Cancer Chemother. Pharmacol.* **41:** 385-390.

Rossetto M, Slade R. W., Baverstock P. R., Henry R. J. and Lee L. S. 1999. Microsatellite variation and assessment of genetic structure in tea-tree (*Melaleuca alternifolia*-Myrtaceae). *Molecular Ecology*. **8:** 633-643.

Saito K., Sudo H., Yamazaki M., Koseki-Nakamura M., Kitajima M., Takayama H. and Aimi N. 2001. Feasible production of camptothecin by hairy root culture of Ophiorrhiza pumila. *Plant Cell Rep*. **20:** 267–271.

Schlotterer C. and Pemberton J. 1994. The use of microsatellites for genetic analysis of natural populations. In: Schierwater B, Streit B, Wagner GP, DeSalle R (eds.). *Molecular Ecology and Evolution*: Approaches and Applications, Birkhäuser Verlag Basel, Switzerland, pp. 203-214.

Schulz B., Boyle C., Draeger S., Rommert A. K. and Krohn K. 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycol. Res.* **106:** 996-1004.

Schutz B. 2001. Endophytic fungi: a source of novel biologically active secondary metabolites. British Mycological Society, International Symposium Proceedings, University of Wales, Swansea.

Stahelin H., 1973. Actvity of a new glycosidic lignan derivative (VP-16-213) related to podophyllotoxin in experimental tumors. *Eur J Cancer*. **9**: 215-221.

Stierle A., Strobel G. A. and Stierle D. 1993. Taxol and taxane production by *Taxomyces andreanae*. *Science*. **260:** 214-216.

Stone J. K., Bacon C. and White W. J. F. 2000. An overview of endophytic microbes: endophytism defined. *Microbial Endophytes Marcel Dekker, New York*. Pp: 3-29.

Strand M., Prolla T. A., Liskay R. M. and Petes T. D. 1993. Destabilisation of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature*. **365:** 274-276.

Strobel G. and Daisy B. 2003. Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews*. **67:** 491-502.

Suhas S., Ramesha B. T., Ravikanth G., Rajesh P. Gunaga., Vasudeva R., Ganeshaiah K. N. and Uma Shaanker R., 2007. Chemical profiling of *Nothapodytes nimmoniana* populations in the Western Ghats, India for anti-cancer compound, camptothecin. *Current Science*. **92:** 1142-1147.

Sunnucks P. 2000. Efficient genetic markers for population biology. *Trends in Ecology and Evolution*. **15:** 199-203.

Swerdlow J., 2000. Nature's Medicine. Plants That Heal, *National Geographic Society*.

Swapna M., Biswajt G., Timir, B. J. and Sumita. J. 2002. Variation in content of taxol and related taxanes in Eastern Himalayan population of Taxus wallichiana. *Planta Med*. **68:** 757–759.

Tafur S., Nelson J. D., DeLong D. C. and Svoboda G. H. 1976. Antiviral components of *Ophiorrhiza mungos* isolation of camptothecin and 10-methoxycamptothecin. *Lloydia.* **39**: 261-262.

Tan R. X. and Zou W. X. 2001. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep*. **18:** 448-459.

Tanabe H., Kawasaki M., Mochizuki T. and Ishizaki H. 2004. Species identification and strain typing of *Fonsecaea pedrosoi* using ribosomal RNA gene internal transcribed spacer regions. *Nippon Ishinkin Gakkai Zasshi* **45:** 105–112.

Tautz D. 1989. Hyper variability of simple sequences as a general source for polymorphic DNA markers, *Nucleic Acids Res*. **17:** 6463-6467.

Van Dam N. M., Verpoorte R. and Van der Meijden E. 1994. Extreme differences in pyrrolizidine alkaloid levels between leaves of *Cynoglossum officinale*. *Phytochemistry*. **37:** 1013--1016.

Vineesh V. R., Fijesh P. P., Jelly louis P., Jaimsha P. P. and Jose Padikkala. 2007. In vitro production of camptothecin (an anticancer drug) through albino plants of *Ophiorrhiza rugosa var. decumbens*. *Current Science*. **92:**1216-1218.

Vladu B., Woynarowski Jan., Manikumar G., Mansukhlal C., Wani M. C., Wall M. E., Daniel D., Von Hoff and Wadkins R. M. 2000. 7 and 10-Substituted Camptothecins: dependence of Topoisomerase I-DNA Cleavable Complex Formation and Stability on the 7- and 10-Substituents. *Molecular Pharmacology*. **57**: 243–251.

Wall M. E., Wani M. C. and Cook C. E. 1966. Plant antitumor agents-I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from Camptotheca acuminata. *J Am Chem Soc.* **88**: 3888-90.

Wani M. C., Taylor H. L., Wall M. E., Coggon P. and McPhail A. T. 1971. Plant antitumor agents-VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J Am Chem Soc*. **93**:2325-2327.

Watase I., Sudo H., Yamazaki M. and Saito K. 2004. Regeneration of transformed *Ophiorrhiza pumila* plants producing camptothecin. *Plant Biotechnology*. **21(5)**: 337–342.

Weissenbach J., Gyapay G., Dib C., Vignal A., Morissette J., Millasseau P., Vaysseix G. and Lathrop M. 1992. A second-generation linkage map of the human genome. *Nature*. **359:** 794-801.

White, T. J., Bruns T., Lee S. and Taylor J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif.

Wiedenfeld H., Furmanowa M., Roeder E., Guzewska J. and Gustowski W. 1997. Camptothecin and 10-hydroxycamptothecin in callus and plantlets of *Camptotheca acuminata*. *Plant Cell Tiss. Org. Cult.* **49**: 213–218.

Wink, M., 1992. The role of quinolizidine alkaloids in plant insect interactions. In: Bernays, E.A. (Ed.), Insect- Plant Interactions. CRC Press, Boca Raton, pp. 133–169.

Wink M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry*. **64:** 3-19.

Wu T. S, Leu Y. L., Hsu H. C., Ou L. F., Chen C. C., Chen C., Ou J. C. and Wu Y. C. 1995. Constituents and cytotoxic principles of *Nothapodytes foetida*. *Phytochemistry*. **39:** 383–385.

Yamazaki Y., Kitajima M., Arita M., Takayama H., Sudo H., Yamazaki M., Aimi N. and Saito K. 2004. Biosynthesis of camptothecin. *In silico* and *in vivo* tracer study from [1-^{13}C] Glucose. *Plant Phys.* **134:** 161-170.

Yamazaki Y., Urano A., Sudo H., Kitajima M., Takayama H., Yamazaki M., Aimi N. and Saito K. 2003. Metabolite profiling of alkaloids and strictosidine synthase activity in camptothecin producing plants. *Phytochemistry.* **62:** 461–470.

Yan X. F., Yang W., Yu T., Zhang Y. and Dai. 2003. Variation in camptothecin content in *Camptotheca acuminata* leaves. *Bot. Bull. Acad. Sin.* **44:** 99-105.

Young D. H., Michelotti E. J., Sivendell C. S. and Krauss N. E. 1992. Anti-fungal properties of taxol and various analogues. *Experientia.* **48:** 882–885.

Zane L., Bargelloni L. and Patarnello T., 2002. Strategies for microsatellite isolation: a review. *Mol Ecol.* **1(1):** 1-16.

Zhang R., Li Y., Cai Q., Liu T., Sun H. and Chambless B. 1998. Preclinical pharmacology of the natural product anticancer agent 10-hydroxycamptothecin: an inhibitor of topoisonerase I. *Cancer Chemother. Pharmacol.* **41:** 257–267.

Zhang X. and Xu B. 2000. Differential regulation of P53, c-Myc, Bcl-2, Bax and AFP protein expression, and caspase activity during 10-hydroxycamptothecin-induced apoptosis in Hep G2 cells. *Anti-cancer Drugs.* **11:** 747–756.

Zhang X., Chen Q. and Xu B. 1999. Apoptosis induction and cell cycle perturbation in human hepatoma Hep G2 cells by 10-hydroxycamptothecin. *Anti-cancer Drugs.* **10:** 569–576.

Zhou B. N., Hoch J. M., Johnson R. K., Mattern M. R., Eng W. K., Ma J., Hecht S. M., Newman D. J. and Kingston D. G. I. 2000. Use of COMPARE analysis to discover new natural product drugs: isolation of Camptothecin and 9-methoxycamptothecin from a new source, *J. Nat. Prod.* **63:** 1273–1276.

Appendices

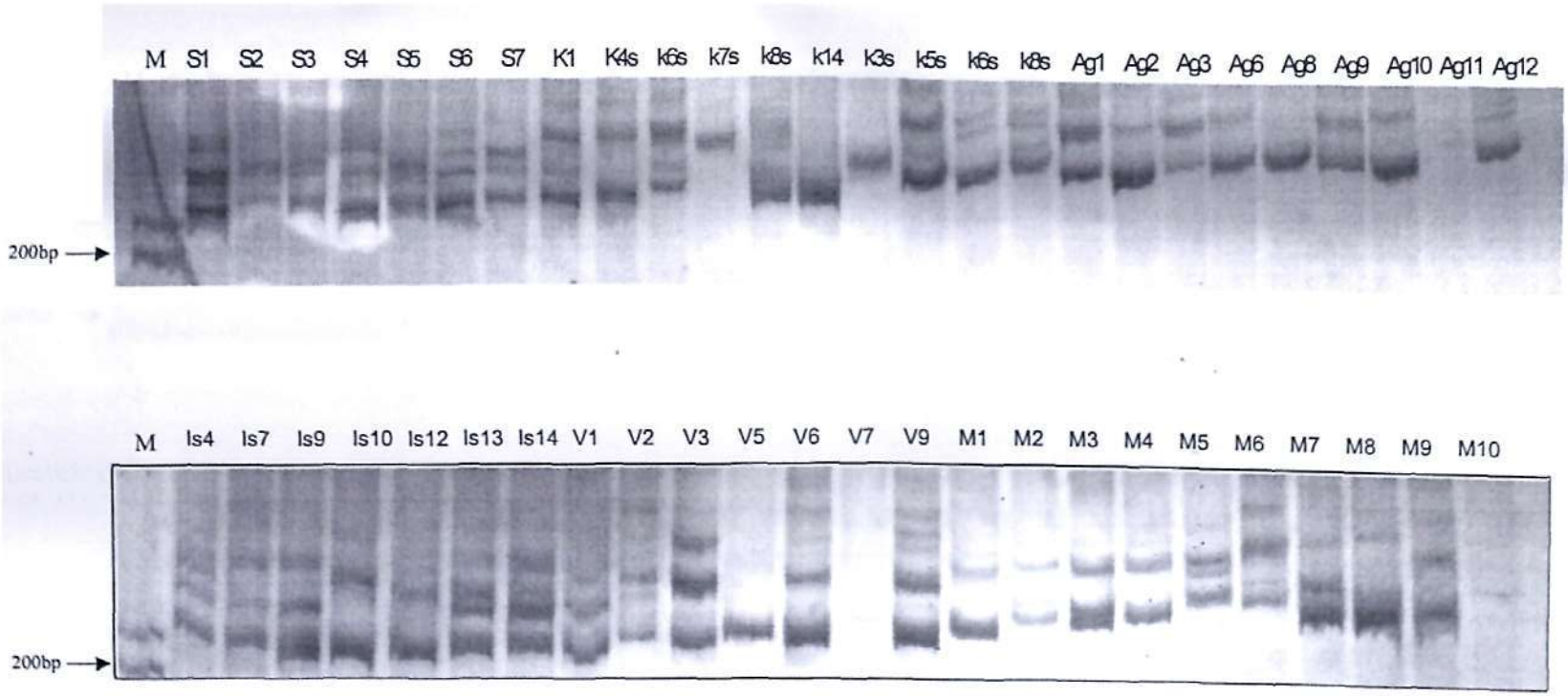


Figure 1: Nnm7 SSR primer amplification profile (range: 221 to 241 bp) of six different populations of *Nothapodytes nimmoniana* resolved by electrophoresis on a 10% polyacrylamide gel (S; Sirsi, K; Kemmannagundy, Ag; Agumbe, Is;Islur, V; Vodagere, M; Mugali). The M label stands for molecular weight standard.

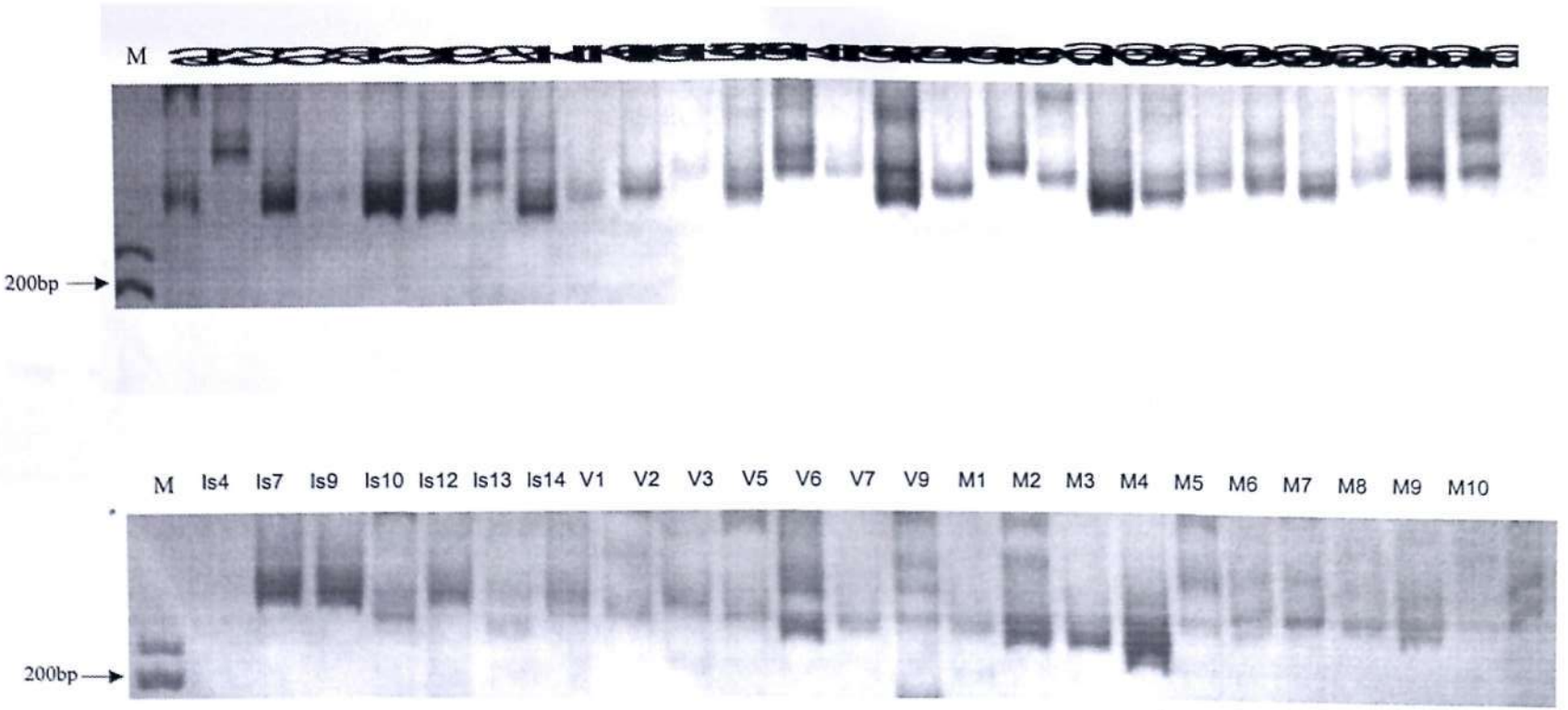


Figure 2: Nnm6 SSR primer amplification profile (range: 216 to 236 bp) of six different populations of *Nothapodytes nimmoniana* resolved by electrophoresis on a 10% polyacrylamide gel (S; Sirsi, K; Kemmannagundy, Ag; Agumbe, Is;Islur, V; Vodagere, M; Mugali). The M: 100bp ladder.

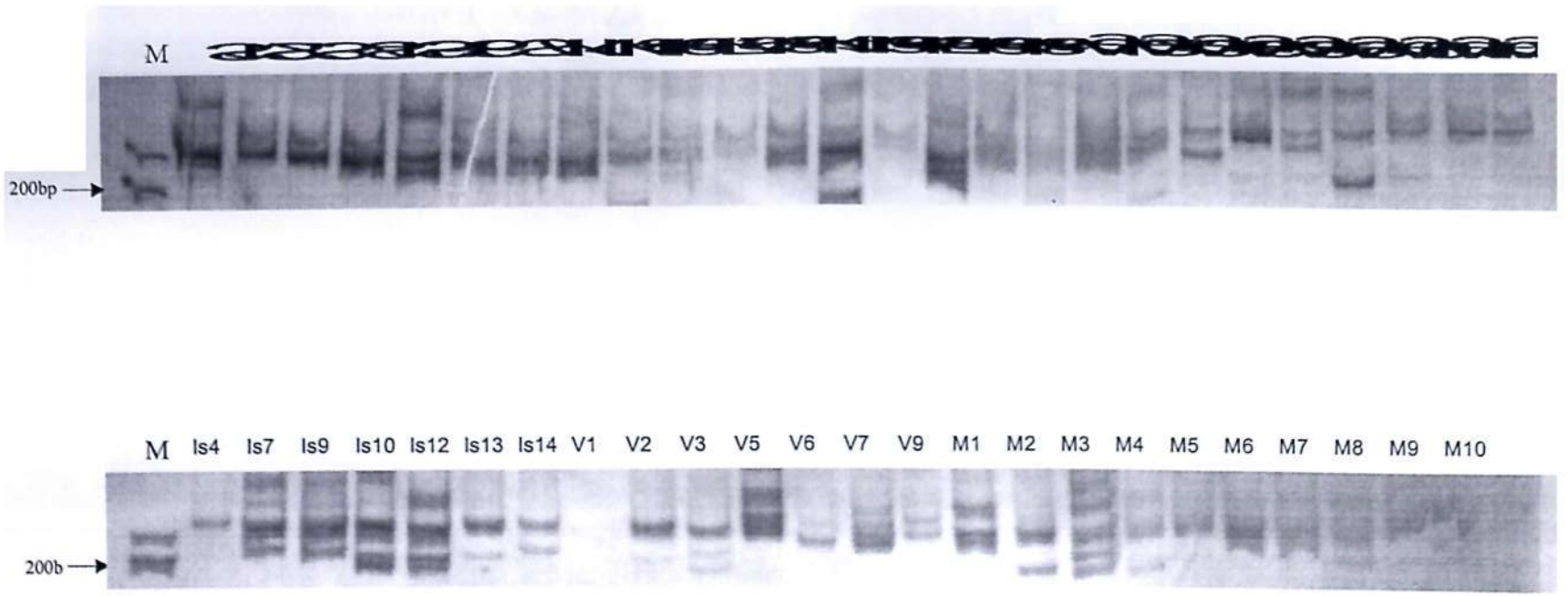


Figure 3: Nnm14 SSR primer amplification profile (range: 200 to 220 bp) of six different populations of *Nothapodytes nimmoniana* resolved by electrophoresis on a 10% polyacrylamide gel (S; Sirsi, K; Kemmannagundy, Ag; Agumbe, Is;Islur, V; Vodagere, M; Mugali). The M label stands for molecular weight standard.

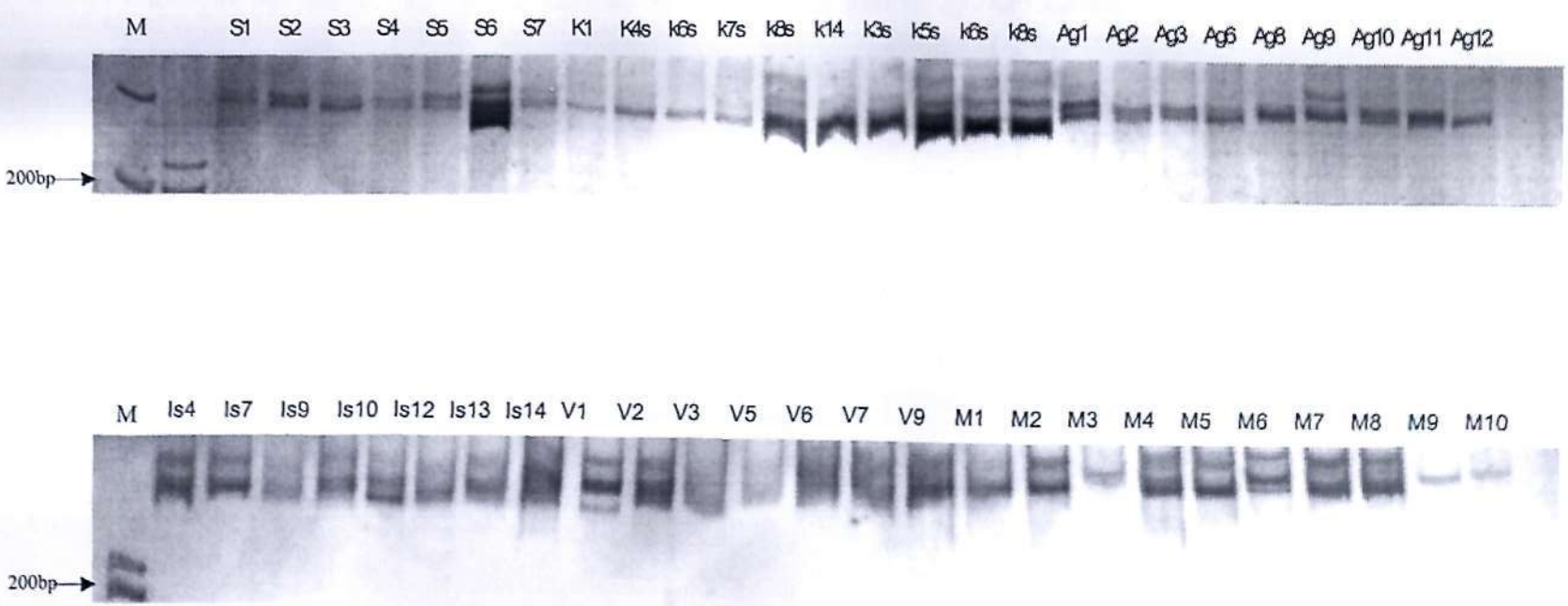


Figure 4: Nn54 SSR primer amplification profile (range: 230 to 254 bp) of six different populations of *Nothapodytes nimmoniana* resolved by electrophoresis on a 10% polyacrylamide gel (S; Sirsi, K; Kemmannagundy, Ag; Agumbe, Is;Islur, V; Vodagere, M; Mugali). M; 100bp ladder.

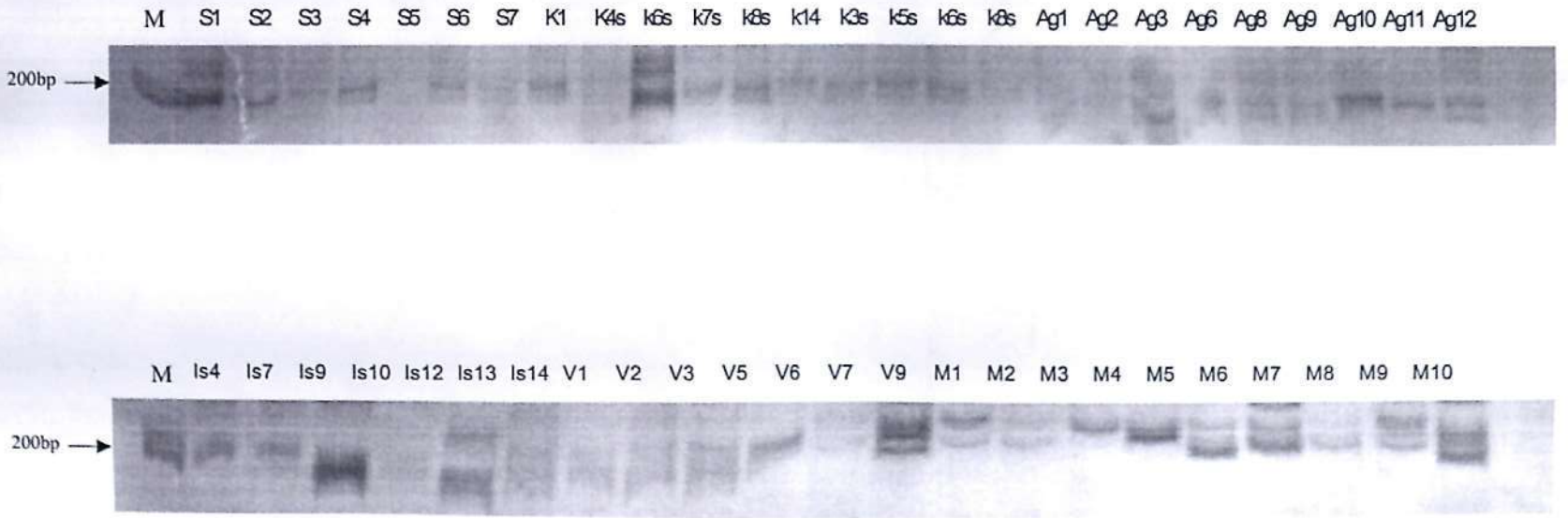


Figure 5: NnT4 SSR primer amplification profile (range: 190 to 210 bp) of six different populations of *Nothapodytes nimmoniana* resolved by electrophoresis on a 10% polyacrylamide gel (S; Sirsi, K; Kemmannagundy, Ag; Agumbe, Is; Islur, V; Vodagere, M; Mugali). The M : 100bp ladder.