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Full Length Research Paper

RP-HPLC analysis for camptothecin content in *Nothapodytes nimmoniana*, an endangered medicinal plant

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Studies were carried out on analysis of secondary metabolites by RP-HPLC from different parts (both *in vitro* and *in vivo*) of *Nothpodytes nimmoniana*, an endangered medicinal plant. HPLC analysis showed the presence of camptothecin, a medicinally important alkaloid from *N. nimmoniana*. The plant parts used for the analysis include leaves, seeds, seed coat and leaf derived callus. Among all the plant parts used, seeds recorded maximum (0.040%) camptothecin content followed by dried leaf samples. Seed coat recorded least camptothecin content. The study indicated that almost all parts of the plant contain camptothecin.

Key words: *Nothapodytes nimmoniana*, Icacinaceae, HPLC, secondary metabolite, alkaloid, quinoline, camptothecin.

INTRODUCTION

India, being a tropical country, is rich in medicinal plant diversity and is one of the twelve-mega diversity hot spot regions of the world. *Nothapodytes nimmoniana* (Grah.) Mabb. (Synonyms, *Nothapodytes foetida* and *Mappia foetida*) belonging to family Icacinaceae, is an important anti-cancer medicinal plant. It is an endangered medicinal tree from Western Ghats of India. Because of destructive harvesting and habitat loss, the population of this species has declined by 50 - 80%. Recently, the tree has been assigned a vulnerable status (Padmanabha et al., 2006). It is an excellent source of quinoline alkaloids, camptothecin (CPT) and 9-methoxycamptothecin (9-OMeCPT) (Figure 1) which are used clinically as such or after derivatization as anti-cancer agents for the treatment of solid tumors (Govindachari and Viswanathan, 1972). Camptothecin was first reported in the Chinese tree *Camptotheca acuminata* (Wall and Wani, 1996)) and later discovered in *N. nimmoniana.* In *C. acuminata,* the camptothecin content was 0.005% of dried plant as against 0.14 - 0.24% in *N. nimmoniana* (Puri et al., 1999). Recent interest in the possible anti-cancer effects has focused the need for the extensive study of the bioactive compounds from *N. nimmoniana*.

MATERIALS AND METHODS

Study on RP-HPLC analysis of bioactive compounds from the medicinal plant *N. nimmoniana* was carried out in the Plant Tissue Culture Laboratory, Department of Plant Molecular Biology and Biotechnology, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, India during 2007 - 2008. The HPLC was executed in the FIST Laboratory, Department of Plant Molecular Biology and Biotechnology, CPMB, TNAU, Coimbatore.

Collection of plant material

The collection of plant material was done from Ooty and Yercaud in Tamil Nadu, India, during the months of October to March. The plant material was collected in the form of seedlings, leaves (both mature and immature) and seeds. Leaves, seeds and seed coat from the collected plant material were used for extraction of camptothecin from *N. nimmoniana*.

Surface sterilization and preparation of samples

The samples were surface sterilized thoroughly. The dried samples

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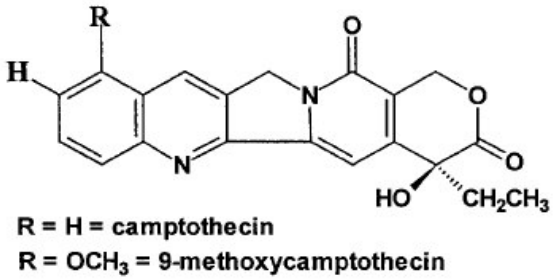

Figure 1. Chemical structure of camptothecin

were grounds to the fine powder using a pestle mortar. Some of the leaf samples were ground using liquid nitrogen.

Extraction

One hundred milligram of the fine tissue powder from each tissue sample was taken from each sample and extracted in 10 ml of 60% ethanol for 3 - 4 h in a shaking water bath. The extracts were then allowed to cool at room temperature. After cooling to room temperature, 1 ml of the extract from each sample was taken in a centrifuge tube of 1.5 ml capacity and centrifuged at 10,000 for 10 min at 10°C. The supernatant was passed through 0.2 µm filter before injecting in HPLC column.

HPLC analysis

The standard camptothecin was purchased from Sigma Aldrich Inc, USA, minimum 95% HPLC powder, empirical formula- $C_{20}H_{16}N_2O_4$ and formula weight- 348.35. Camptothecin standard solutions within the range from 1 - 100 µg/ml concentrations were prepared for HPLC analysis. The camptothecin standard was prepared by dissolving in a solution of DMSO and absolute HPLC grade methanol in a ratio of 5: 50 (v/v). The standard sample solutions for HPLC were filtered using 0.2 µ syringe filter before injection. The analysis of extracts was done in High Performance Liquid Chromatographic system (HPLC) equipped with LC8A pump, SPD-M 10 A vp photo array detector in combination with class LC 10 A software (Shimadzu). The presence of camptothecin in the samples was detected by comparing with the retention time of the standard sample. The area of the standard was compared with area of the sample and the amount of camptothecin in the extracts was calculated. The chromatographic conditions for the analysis were as follows: mobile phase: acetonitrile: water (60: 40), column: ODS (Octadecyl silane) C18, 5 µ size, 250 X 4.6 mm (Supelco), Detector: SPD-M 10 A vp photo array detector, wave length: 254 nm, flow rate: 1.0 ml/min, injection volume: 25 µl, retention time: 6.4 min

Amount of camptothecin present in dry weight of sample was calculated using the following formula, given by Scott (1996) and expressed in µg per g of sample dry weight.

$$Cp_{(s)} = \frac{Ap_{(s)}}{Ap_{(st)}} \times Cp_{(st)}$$

$Cp_{(s)}$ is the concentration of the solute in the mixture.

$Ap_{(s)}$ is the area of the peak for the sample in HPLC chromatogram.
$Ap_{(st)}$ is the area of the peak for the standard in HPLC chromatogram.
$Cp_{(st)}$ is the concentration of standard used for injecting in HPLC.

HPLC injections for each of the samples and standards were done at least in duplicate. The linearity of detector response for standards of camptothecin was observed by injecting 50 µl from solutions of standards ranging in concentration from 1.0 µg -100 µg/ml (Elsohly et al., 1995) and plotting the standard curve. The amount of camptothecin present in different extracts was calculated by comparing the peak area of the sample to the peak area of the standard and expressed as camptothecin content in µg per g of dry weight of the sample, except for the leaf extracts from liquid nitrogen ground sample. The camptothecin content from the leaf extract obtained after grinding the samples in liquid nitrogen was expressed in µg per g of fresh weight.

RESULTS AND DISCUSSION

Camptothecin standard (Sigma) was run in HPLC and the chromatogram is shown (Figure 2). The single peak of camptothecin was obtained at the retention time of 6.4 min. The extract of leaves, seeds and seed coat collected from *in vivo* plant of *N. nimmoniana* showed similar peaks at retention time of 6.4 min, corresponding to camptothecin. The seed extracts from *in vivo* grown plants recorded the maximum content of camptothecin (401.3 µg/g). The camptothecin content recorded from leaf samples (ground in liquid nitrogen) was 159.3 µg/g. The leaf samples dried in hot air oven recorded 202.6 µg/g dry weight camptothecin content. The seed coat recorded the lowest level of accumulation (107.4 µg/g) (Figures 3 - 5 and Table 1).

Camptothecin in leaves, seeds and seed coat of *in vivo* grown plants at a retention time of 6.4 min was identified using standard camptothecin. Govindachari and Viswanathan (1972) had already reported presence of CPT and 9-methoxy-CPT from *in vivo* parts of *N. foetida* plant. In the present study, camptothecin was detected at a flow rate of 1.0 ml min^{-1} and by using the mobile phase in an isocratic mode that is, without altering the solvent pumping throughout the course of HPLC run. Similar isocratic mode, but at a flow rate of 1.6 ml min^{-1} was used by Padmanabha et al. (2006) for extraction of camptothecin from *in vivo* parts of *N. nimmoniana* plant. They detected the presence of camptothecin at a retention time of 3.5 min. Padmanabha et al. (2006) reported 0.081% camptothecin content from leaf samples. In the present study, the content of CPT in fresh leaves, dry leaves, seed and seed coat samples was 0.016, 0.020, 0.040 and 0.011% respectively. This variation may be due to genetic differences resulting from geographical differences or effect of a different culture environment such as due to plant growth regulator or temperature. Leaf extracts from *in vivo* grown plants showed higher content of camptothecin than leaf calli extracts. This might be due to the altered physical conditions provided in culture. Similar results were reported by Becker (Becker, 1970)) in *Pimpinelle anisum, Foenicum vulgare* and *Mentha piperita*. The callus cultures did not produce appreciable

Table 1. CPT content from different parts of *Nothapodytes nimmoniana*.

Plant samples	Amount of camptothecin present in dry weight of sample (µg/g)	Camptothecin content in percent
***In vivo* parts of the plants**		
$Leaf_1$	202.6	0.020
$Leaf_2$	159.3	0.016
Seed	401.3	0.040
Seed coat	107.4	0.011
***In vitro* parts of the plants**		
Callus	143.4	0.014

$Leaf_1$ represents data from the leaf sample dried in hot air oven before grinding. $Leaf_2$ represents data from the leaf sample dried in liquid Nitrogen without drying.

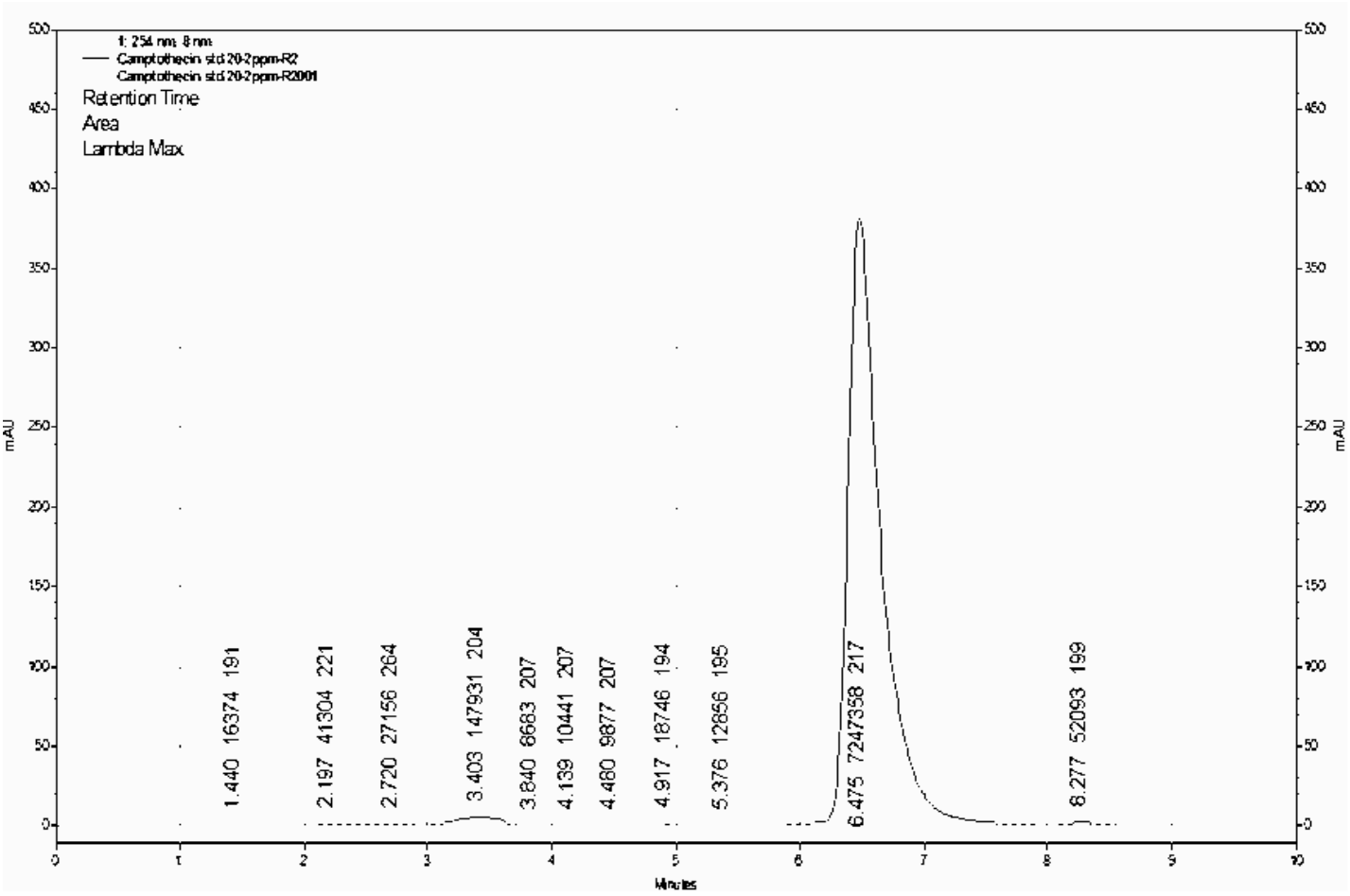

Figure 2. HPLC chromatogram for CPT standard at 10 ppm concentration.

amount of secondary metabolites comparable to that of intact plants. This might be because secondary metabolite production is lesser in cultures due to lesser organization. Secondary metabolites accumulate less in undifferentiated tissues than in differentiated tissues. The production of secondary metabolites in *in vitro* cultures may be enhanced by using some specialized techniques like hairy root cultures, precursor feeding or use of

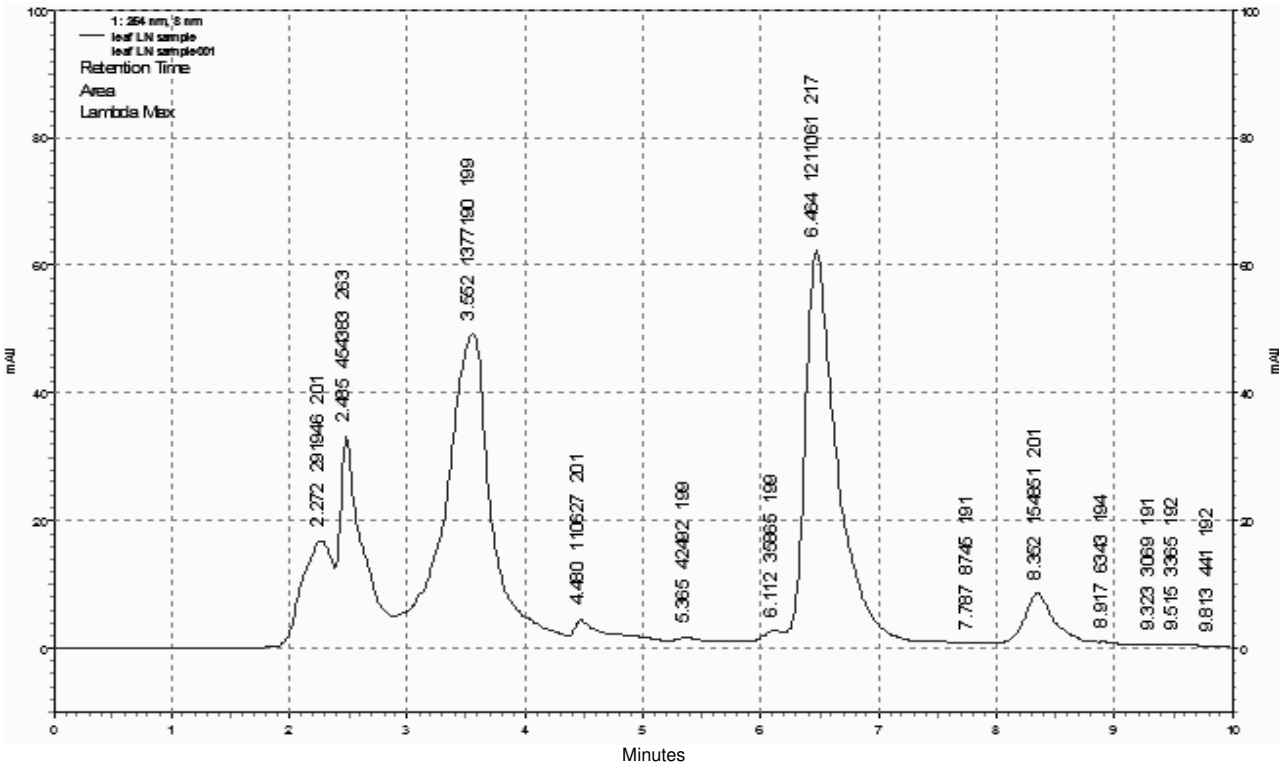

Figure 3. HPLC chromatogram showing presence of CPT in leaf samples of *Nothapodytes nimmoniana.*

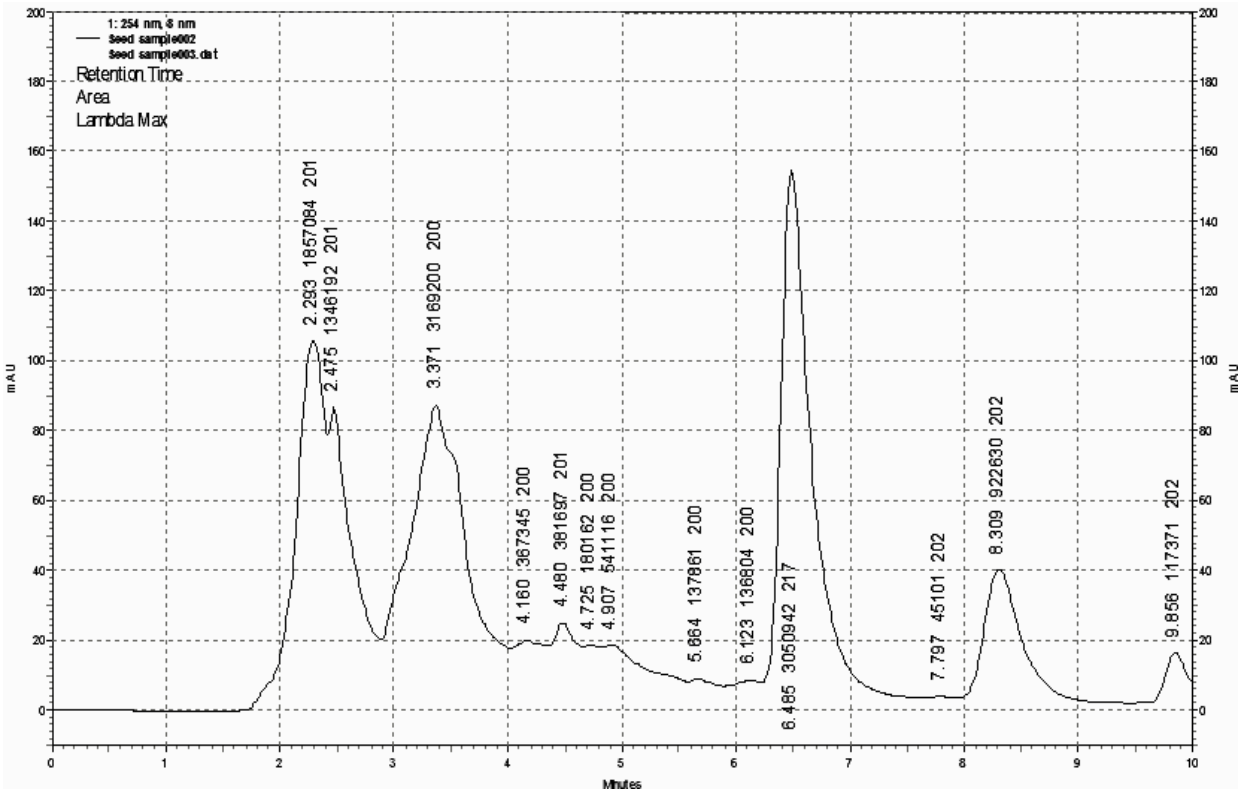

Figure 4. HPLC chromatogram showing presence of CPT in seed samples of *Nothapodytes nimmoniana.*

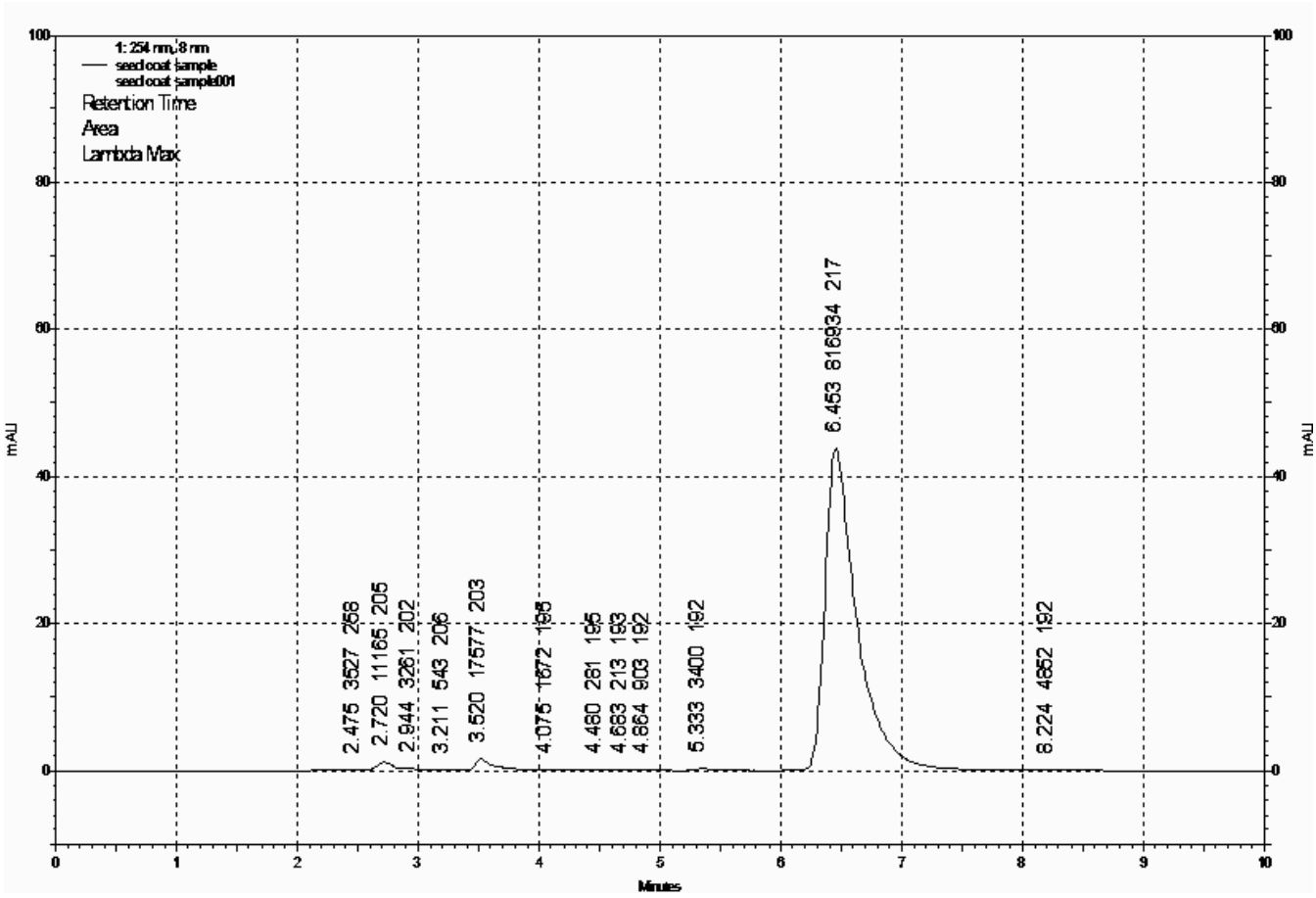

Figure 5. HPLC chromatogram showing presence of CPT in seed coat samples of *Nothapodytes nimmoniana.*

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