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Original Article

VALIDATION OF ACCUMULATION OF CAMPTOTHECIN CONTENT, AN ANTI-CANCER ALKALOID IN *NOTHAPODYTES NIMMONIANA* GRAHAM. IN PHENOTYPIC VARIANTS: METHOD FOR IDENTIFYING HIGH-YIELDING SOURCES OF ALKALOID

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ABSTRACT

Objective: The study was aimed to find the CPT accumulation, in particular organ, via variable extraction method and *in vitro* propagation method both qualitatively and quantitatively.

Methods: In the present study, the seven phenotypic variants of *N. nimmoniana* such as dry leaf, fresh barks of plant, induced callus, somatic embryo and suspension culture (In exogenous medium and in pallet) in 100 mg/ml were analyzed for CPT content by TLC (Thin layer chromatography) and HPLC (High performance liquid chromatography).

Results: TLC and HPLC analysis confirm the presence of CPT in dry leaf, fresh barks of the plant, induced callus and somatic embryo. Out of which bark extracts possessed a maximum amount of CPT, i.e. 377.77 µg/g, which supposed to be the first choice of CPT source.

Conclusion: Results conclude that the CPT is present in all samples used except exogenous suspension culture, out of which soxhlet bark extract possesses the maximum amount of CPT.

Keywords: Camptothecin (CPT), Anti-cancer, High-performance liquid chromatography.

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INTRODUCTION

Nothapodytes nimmoniana (Grah.) Mabb. is a tree of up to 3-8 m tall from the family Icacinacae. It is an endangered medicinal tree from Western Ghats of India, specially to central and south Maharashtra Sahyadris, some parts of Assam, the Himalayan foot hills, Ceylon, Burma and Thailand. Some vernacular names of *Nothapodytes nimmoniana* are Ghanera, Durvasane mara, Kalgur, Kalagaura that has been used in various parts according to the local folklore knowledge. Due to excessive harvesting without any concern on habitat loss, the population of this species has been declined by 50-80 %, resulting in its vulnerable status [1].

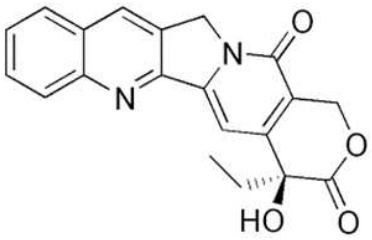

Fig. 1: Structure of camptothecin

Camptothecin (CPT) was first reported in the Chinese tree *Camptotheca acuminata* [2] and later discovered in *N. nimmoniana.* The potential of CPT in the treatment of cancer and various diseases has brought interest in the extraction of bioactive compounds and its process optimization from *N. nimmoniana*. This plant is a rich source of camptothecin–a monoterpene indole alkaloid (Fig. 1), supposed to be most promising anticancer drug [3]. CPT and their derivative are useful as anti-cancer agents in the treatment of tumors [4].

The well-known target of camptothecin is the enzyme, DNA topoisomerase-I. Many analogues of CPT, which possesses potential against various diseases have been synthesized [5]. CPT shows inhibitory prospective against HIV. Furthermore, it is also effective at lung, breast, uterine and cervical cancers [6-8]. Some derivatives of Camptothecin, including topoisan and irinotecan, is in routine practice for the treatment of colorectal and ovarian cancers. CPT reported to shows the cytotoxic effect against *Plasmodium falciparum,* and thus it can be an interesting molecule for new antimalarial drug discovery [12].

In the present study, we have extracted the CPT from seven phenotypic variants of *N. nimmoniana* in 100 mg/ml such as dry leaf, fresh leaf, the bark of the plant, induced callus, somatic embryo and suspension culture (in exogenous medium and in a pallet). The characterization, production and quantification of CPT have been validated by TLC analysis and quantification by HPLC.

MATERIALS AND METHODS

Collection of plant material and identification

The seeds of *N. nimmoniana* were collected from Chiplun part of Western Ghats, Maharashtra. As a part of our research, the plants were germinated and established in our departmental nursery [13]. The plant was authenticated using standard flora and cross-checked and further stored in the herbarium records at Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati, India as *N. nimmoniana* (J. Graham) with an accession number-BT/SGBAU-07.

Standard CPT sample

The standard CPT was purchased from Sigma Aldrich Inc, USA, minimum 95% HPLC grade powder. A stock solution of CPT was prepared by dissolving 100 mg in chloroform: methanol mixture (3:1) [14] and making up the volume to 1 ml with methanol. From

this stock solution, standard solutions of 20 µg/ml to 100 µg/ml were prepared by transferring aliquots (0.2 to 1.0 ml) of stock solution to 2 ml centrifuge tube and adjusting the volume with methanol up to 1 ml.

Preparation of experimental samples

500 mg each of fresh and dry leaves of *N. nimmoniana* was weighed and crushed in a mortal and pestle with HPLC grade methanol (5 ml). The plant bark was sun-dried and crushed to a mesh size of 0.1 to 0.5 mm. About 10 g of powdered material was kept in a thimble into the solvent extractor and extracted it with 95% methanol for 9 h. Further, 200 ml of extract was allowed to evaporate at room temperature; it takes approximately 7-8 d to get the brown gummy solid material measuring about 0.76 gm.

Young callus induced by inoculating fresh leaf of *N. nimmoniana* on MS basal medium with 2,4-D (2, 4-Dichlorophenoxyacetic acid) 0.5 mg/l and BAP (6-Benzylaminopurine) 3.0 mg/l. Callus induction were observed after 35-40 d of incubation. Further subculturing of callus on similar fresh medium resulted in fragile white callus. Direct somatic embryos were observed from the leaf explants of *N. nimmoniana* cultured on the MS basal medium containing various combinations of TDZ ranging from 0.5 to 3.0 mg/l. After 25 d of incubation, induction was observed and at 60 d, well-matured embryos were seen on four different concentrations, including 0.5, 1.5, 2.0 to 3.0 mg/l.

Similarly, fresh fragile callus (approx 4 g) of *N. nimmoniana* was used to establish the suspension culture. The callus was kept in the MS basal salt medium (400 ml) with the sterile 1 mg/l pectinase enzyme for the separation of cells for 10-15 d. The suspension was subcultured by adding separated cell form above system and kept again for 30 d for growth of cells (2×10^4approximately). About 50 ml of suspension was taken into 50 ml centrifuge tube and centrifuged at 2000 rpm for 5 min at room temperature. The quantity of CPT in filtered supernatant (in exogenous medium) and in the pellet (100 mg of total cells) were determined using HPLC (13).

Experimental sample stock extract (100 mg/ml) was dissolved in HPLC grade 1 ml methanol and sonicated for about 10 min. It was further centrifuged at 4000 rpm for 10 min at room temperature. The supernatant was collected in a fresh tube and transferred to HPLC vial by using 0.25 µm syringe filter.

Qualitative estimation by TLC

The confirmation of CPT in experimental extracts was done by TLC. The TLC plate was prepared on 15x10 cm glass plates coated with silica gel slurry, dried in room temperature and kept in the oven for activation at 110 °C for 1 hour. About 20 µl of extract was spotted onto the silica plate in line spotting. The experimental plates were developed in a chromatographic chamber, saturated by solvent system (100 ml) Ethyl acetate: Methanol (3:1) [13,15]. The plate was allowed to develop for 10-15 min, air dried and observed under UV transilluminator (Cleaver) at 365 nm. The R_f values of the CPT were calculated by the given formula and compared with standard CPT.

$$R_f = \frac{\text{Distance traveled by sample}}{\text{Distance traveled by Solvent}}$$

Quantification by HPLC

Preparation of the calibration curve for camptothecin

Standard solutions of CPT in 20, 40, 60, 80 and 100 µg/ml was injected in HPLC column. The peaks were detected at 365 nm. Calibration curves of CPT were prepared by plotting peak area vs. concentration. The CPT concentration in various samples was obtained by plotting the values in the standard graph.

HPLC analysis

The experimental sample solutions for HPLC were filtered using 0.2 µm syringe filter before injection. The instrument was purchased from Agilent Technologies (Model–1260 Infinity) and column used was ZORBAX Eclipse plus C18 (4.6 × 100 mm, 3.5). Acetonitrile: Water (25:75) solvent system with flow rates 1 ml/min was selected [13]. Detection of CPT was done at 365 nm; isocratic gradient elution was followed for a sample volume of 10µl. The flow rate was kept at 1 ml/min. Standard CPT from Sigma-Aldrich (1 mg/ml) was used as the reference compound.

Statistical analysis

All data were subjected to statistical analysis following Completely Randomized Design. The means were subjected to±SEM.

RESULTS

TLC analysis

TLC plate developed at 365 nm was analyzed under UV trans-illuminator. A total of seven samples along with standard CPT (20 µl) were loaded on TLC plate as line spots from Lane 1-8 (Shown in fig. 3). Lane 1 shows standard CPT band at R_f 0.83, which is common in all test sample bands from Lane 2-6. The results confirm the presence of CPT in all test samples (fresh leaf, dry leaf bark, callus, somatic embryo and suspension culture with cells) except seventh sample of suspension culture (exogenous type) without cells. Fig. 3 also revealed that CPT concentration was highest in methanolic bark extract, extracted in soxhlet apparatus, followed by dry leaf, fresh leaf, callus, somatic embryo and least in suspension culture.

HPLC analysis

The actual amount of CPT present in the samples was confirmed by HPLC using the standard CPT graph (fig. 5). The CPT content in the 100 mg/ml methanolic extracts of fresh leaf, dry leaf, bark, fresh callus, somatic embryo and suspension culture of *N. nimmoniana* were analyzed and quantified by HPLC method. The chromatogram obtained shows homogenous peaks of CPT in all samples except exogenous suspension culture with baseline separation at similar retention time 7.05 min, same as that of standard CPT. The exact retention time of test samples and standard CPT along with overlapping peaks confirms the presence of CPT in all samples. Although the variation in peak size related with types of explants used for experimentation and extraction method. The chromatogram confirms that the CPT is absent in Exogenous Suspension Culture (fig. 4).

The estimations of CPT present in samples were performed on HPLC by preparing a calibration graph (fig. 5: Area v/s CPT amount in µg/ml) with Y-equation = 1.33513x+1.97716 (error 0.00005). The CPT peaks of all samples including standard CPT were noted down as per fig. 5.

The amount of CPT present in test samples was calculated by putting the peak area value in automated generated calculator in the instrument. The results confirm that CPT content is maximum in Bark soxhlet extract i.e. 377.77715±2.52307 µg/g with the largest peak area of 3307929 on the chromatogram, (generated by Agilent-EZ Chrom Elite HPLC-based software) which was followed by dry leaf, fresh leaf, callus, somatic embryo and suspension culture as shown in table 1. During the analysis, one of the derivatives of CPT, 9-methoxy CPT was visible in chromatogram at a retention time of 13.25 min. The fig. 4 confirms that 9-methoxy CPT was present in fresh leaf, dry leaf and soxhlet extract, were as in another experimental sample it was missing.

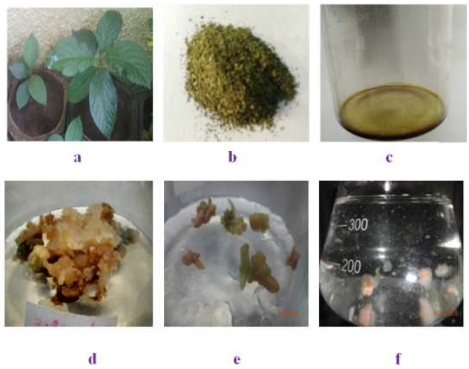

Fig. 2: Various phenotypic explants used for comparative CPT extraction and quantification, a. Fresh leafs; b. Dried leaf powder; c. Methanolic extract of bark; d. Young callus; e. Somatic embryos and f. Suspension culture

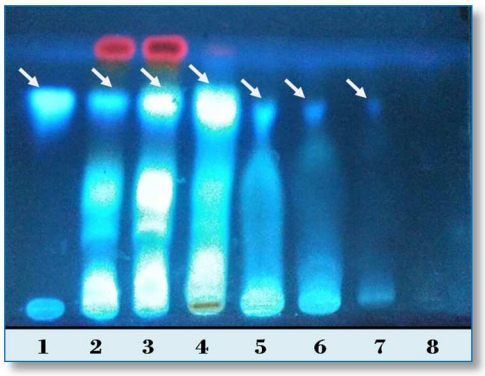

Fig. 3: TLC analysis of camptothecin isolated from fresh leaf, dry leaf bark, callus, somatic embryo and suspension culture of *N. nimmoniana* along with standard Camptothecin (Lane 1: Standard CPT, Lane 2: Fresh Leaf, Lane 3: Dry Leaf, Lane 4: Bark, Lane 5: Fresh Callus, Lane 6: Somatic Embryo, Lane 7: Cells of Suspension Culture, Lane 8: Exogenous Suspension Culture).

DISCUSSION

Nowadays, Camptothecin and their derivatives are important anticancer plant drugs [16, 17]. The plants reported producing CPT include *Camptotheca acuminata, N. nimmoniana*, *Tabernaemontana heyneana* and *Ophiorrhiza rugosa* [1, 16-21]. Many researchers have taken initiative for the extraction, purification and characterization of Camptothecin from various parts of *N. nimmoniana* [22]. In the light of above the research, our study deals with TLC confirmation and HPLC quantitative analysis of CPT isolated from seven different phenotypic variants of *N. nimmoniana* collected from Western Ghats of Maharashtra.

As TLC is used for identification and confirmation of herbal drugs, it shows a complete fingerprint of the compounds presents in the mixture. The TLC separation of CPT in *N. nimmoniana* has been discussed by many workers [15, 23, 24]. The CPT isolated from phenotypic variants of *N. nimmoniana* confirms the band to significant R_f 0.83 as earliest reports [13, 15]. Confirmation and quantification of CPT from the high-yielding variants of *N. nimmoniana* were done by HPLC analysis. In earlier reports, the CPT was estimated to be 0.4–0.5% in *C. acuminata*[18], 0.23%-0.33% in *N. nimmoniana* [1] and 0.00013% in *T. heyneana* stem bark [20]. Similarly about 0.075% CPT in shoots of mature trees of *N. nimmoniana* [24]. Initially 0.081% camptothecin content from leaf samples with retention time 3.5 min at a flow rate of 1.6 ml/min reported [1], which was modified in earlier studies [13], with 7.05 min retention time (flow rate-1 ml/min) used in the present study. This change in retention time could be due to change in solvent system and flow rate.

In *in vivo* study, the presence of CPT and 9-Methoxy CPT in *N. nimmoniana* was confirmed [4]. The 9-Methoxy camptothecin is one of the important antitumor compounds in *Nothapodytes foetida* [25] reported to inhibited Topoisomerase-I [26]. In the comparative antitumor activity of CPT and 9-Methoxy CPT against murine sarcoma S180 tumour cells, 9-Methoxy CPT shows lower IC_{50} as compared to CPT [27]. The low yield of 9-Methoxy CPT has been reported in *N. foetida* and *C. acuminata* [28, 29]. Furthermore, low 9-MCPT content as compared to CPT isolated from distinctive plant parts of *N. nimmoniana* collected from different geographical regions of Western Ghats of India [30]. Our study further confirmed the presence of CPT in various phenotypic variants along with its presence in different plant parts, as is visible in HPLC chromatogram (fig. 4).

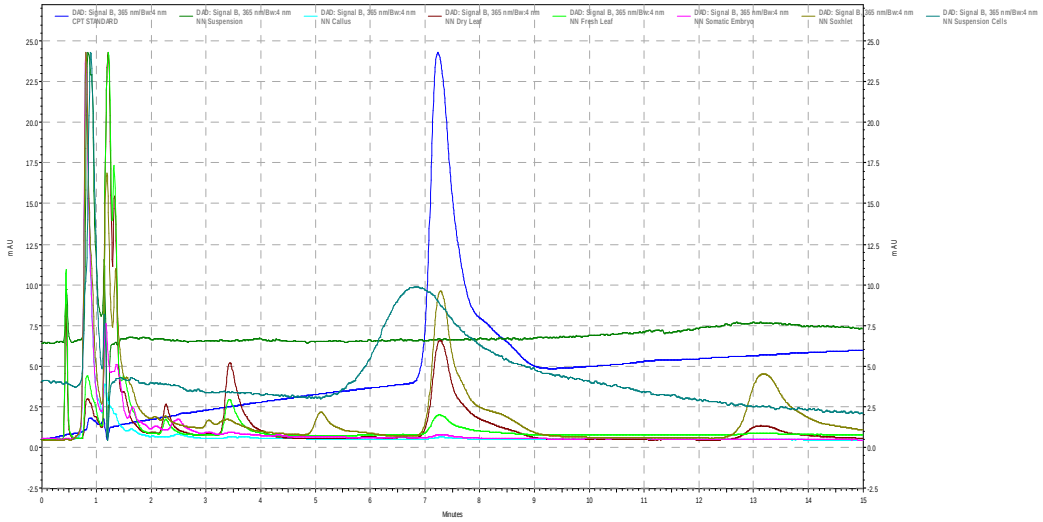

Fig. 4: HPLC determination of Camptothecin isolated from fresh leaf, dry leaf, bark, callus, somatic embryo and suspension culture of *N. nimmoniana* along with standard (CPT Retention time = 7.05 min)

Table 1: Quantitative estimation of CPT by HPLC, isolated from fresh leaf, dry leaf bark, callus, somatic embryo and suspension culture of *N. nimmoniana* along with standard camptothecin

S. No.	Name of the sample	Peak area	Crude extract (µg/g)±SEM
1	Fresh Leaf	234082	26.54505±0.11224
2	Dry Leaf	1770418	202.4064±1.01784
3	Soxhlet Bark extract	3307929	377.7715±2.52307
4	Fresh Callus	51941	5.89014±0.17066
5	Fresh Somatic Embryo	15349	1.74058±0.08064
6	Suspension Cell Culture	16504	1.87159±0.24507
7	Exogenous Suspension culture	-	-

Note: Standard stock 100 mg/ml for test samples solutions. All the data was statistically analyzed±SEM represent standard error of mean and n=3.

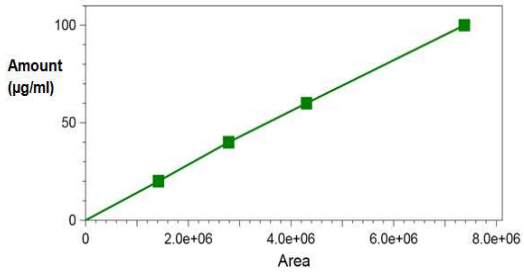

Fig. 5: Calibration graph of CPT standard (20 µg/ml to 100 µg/ml) with Y-equation = 1.33513x+1.97716 (error 0.00005)

In tissue culture efforts for CPT accumulation in callus culture, showed the traces of both CPT and 9-methoxy CPT [24, 31], as we have demonstrated and confirmed CPT was present in the small amount in callus culture, somatic embryo and suspension culture in this paper, but 9-MCPT was absent. This may be due to different hormones used in tissue culture. Initially, production of CPT by callus culture and suspension culture from *N. nimmoniana* has been established [32], which suggest the lower yield of CPT in *N. nimmoniana in vitro* (0.0003%-0.01%)[24, 33].

CONCLUSION

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, without altering the solvent pumping throughout the course of HPLC run.

In the present study, the content of CPT in bark extract was found to be highest (377.7715±2.52307 µg/g) as compared to other experimental samples. This variation may be due to physiological variation from different culture environment viz. plant growth regulator or temperature or stress.

The callus and somatic embryo cultures did not produce the appreciable amount of secondary metabolites comparable to that of intact plants. This might be because secondary metabolite production is lesser in cultures due to the 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 mutational techniques for appreciable results.

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ABBREVIATION

mg–milligram, CPT–Camptothecin, 9-MCPT-9-methoxy Camptothecin, TLC–Thin Layer Chromatography, HPLC–High Performance Liquid Chromatography, Min–Min, µg–microgram, µl–microliter, ml–milliliter, cm–centimeter, rpm–round per min, µm–micrometer, %-Percentage, mg/l–milligram/liter, mm–millimeter, R_f-Retardation factor, TDZ-Thidiazuron, MS = Murashige and Skoog

CONFLICT OF INTERESTS

Authors have no conflict of interest.

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