INTRODUCTION

*Notaphodytes nimmoniana* (Grah.) Mabb. is a tree of up to 3-8 m tall from the family Icacinaceae. 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 *Notaphodytes 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].

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 the 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 bark of the plant, induced callus and somatic embryo and suspension culture (in exogenous medium and in 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.

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.
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 mortar 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 (2×10⁴ approximately). 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 Rf values of the CPT were calculated by putting 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 transilluminator. 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 Rf 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.
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 present 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

| No. | Name of the sample                  | Peak area     | Crude extract (µg/g)±SEM |
|-----|-------------------------------------|---------------|-------------------------|
| 1   | Fresh Leaf                          | 234082        | 26.54±0.50±0.11224      |
| 2   | Dry Leaf                            | 1770418       | 2.02±0.56±0.17094       |
| 3   | Soxhlet bark extract                | 3307929       | 3.77±0.71±0.52307       |
| 4   | Fresh Callus                        | 51941         | 5.89±0.14±0.17066       |
| 5   | Fresh Somatic Embryo                | 15349         | 1.74±0.58±0.08064       |
| 6   | Suspension Cell Culture             | 16504         | 1.87±0.59±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.
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, nun-millimetre, R:Retardation factor, TDI-Thidiazuron, MS=Mangeshie and Skoog

CONFLICT OF INTERESTS

Authors have no conflict of interest.

REFERENCES

1. Padmanabh BV, Chandrasekhar M, Ramesh BT, Hombegowda HC, Gunaga RP, Suhas S, et al. Pattern of accumulation of camptothecin anticancer alkaloids in Notaphyodes nimmoniana, Graham in Western Ghats, India, implications for high-yielding sources of alkaloids. Curr Sci 2006;90:95-100.

2. Wall ME, Wani MC. Plant antitumor agents II: the structure of two new alkaloids from Camptotheca acuminata. J Org Chem 1968;34:1364-7.

3. Nalawade SM, Abhay PS, Lee CY, Kao CL, Tsay HS. Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. Bot Bull Acad Sci 2003;44:79-98.

4. Govindachari TR, Viswanathan N. Alkaloids of Mappia foetida. Phytochemistry 1972;1:1352-9.

5. Wall ME, Wani MC. CPT and Analogs: From Discovery to Clinic, CRC Press: Boca Raton; 1995.p. 21-41.

6. Priet E, Showalter SD, Blair MG. Inhibition of human immunodeficiency virus (HIV-I) replication in vitro by noncytotoxic doses of CPT, a topoisomerase inhibitor. AIDS Res Hum Retroviruses 1991;7:65-72.

7. Takeuchi A, Dohashi K, Fujimoto S, Tanaka K, Suzuki M. A late phase II study of CPT-II in uterine, cervical cancer and ovarian cancer. Jpn J Cancer Chemother 1991;18:1661.

8. Potmesil M. CPT from bench research to hospital. Cancer Res 1994;54:1431-9.

9. Lilenbaum RC, Ratanj MJ, Miller AA, Hargis JB, Hollis DR, Rosner GL, et al. Phase I study of pacitaxel and topotecan in patients with advanced tumors: a cancer and leukemia group B study. J Clin Oncol 1995;3:1220-7.

10. Romanelli SP, Perego P, Pratesi N, Carenini M, Zunino TF. In vitro and in vivo interaction between cisplatin and topotecan in ovarian carcinoma systems. Cancer Chemother Pharmacol 1998;41:385-90.

11. Vladu B, Jan W, Manikumar MG, Mansukhlika C, Wani ME, Wall ME, et al. 7-and 10-Substituted camptothecin: dependence of topoisomerase I-DNA cleavable complex formation and stability on the 7 and 10-substituents. Mol Pharmacol 2000;57:243–51.

12. Bleyl AF, Cumming JN, Shapiro TA. Effect of CPT, a topoisomerase I inhibitor, on Plasmidium falciparum. Biochem Pharmacol 1998;55:709-11.

13. Patil A, Patil S, Mahure S, Kale A. UV, FTIR, HPLC confirmation of Camptotheca acuminata. A late study. J Clin Oncol 1995;13:2230-7.

14. Takeuchi A, Dohashi K, Fujimoto S, Tanaka K, Suzuki M, Terashima Y, et al. A late phase II study of CPT-II in uterine, cervical cancer and ovarian cancer. Jpn J Cancer Chemother 1991;18:1661-89.

15. Sharma S, Kumar A, Namdeo AG. Pharmacognostical and phytochemical analysis of Notaphyodes nimmoniana stem. Int J Pharm Pharm Sci 2012;4:455-9.

16. Lokes P, Balasubramanaya S, Anuradha M. Cost effective quantification of camptothecin and a comparative study of its content in Notaphyodes foetida and Ophiorrhiza mungosa sourced from selected geographical locations. Oriental Pharm Exp Med 2014;4:87-92.

17. Takeuchi A, Dohashi K, Fujimoto S, Tanaka K, Suzuki M, Terashima Y, et al. A late phase II study of CPT-II in uterine, cervical cancer and ovarian cancer. Jpn J Cancer Chemother 1991;18:1661-89.

18. Potmesil M. Camptothecins: from bench research to hospital. Cancer Res 1994;54:1431-9.

19. Van-Hengel AJ, Harkes MW, Wichers HJ, Hesselink PG, Buitema RM. Characterization of callus formation and camptothecin production by cell lines of Camptotheca acuminata. Plant Cell Tissue Organ Cult 1992;30:174-85.

20. Aiyama R, Hisako N, Nokata K, Shinohara C, Sawada SA. A late study. J Nat Prod 1979;42:729-32.

21. Roja G, Heble MR. The quinoline alkaloids camptothecin and 9-methoxycamptothecin from tissue cultures and mature trees of Notaphyodes nimmoniana. Phytochemistry 1998;27:3663-4.

22. Ghaempari G, Chavan R, Lele U, Bhatt A, Bhave, Malpure N, et al. Camptothecin accumulation in Ophiorrhiza rugosa var. prostrata from Northern Western Ghats. Curr Sci 2010;30:2–1.

23. Namdeo AG, Sharma A. HPLC analysis of camptothecin content in various parts of Notaphyodes foetida collected on different periods. Asian Pac J Trop Biomed 2012;2:389–93.

24. Falade D, Satlivke RR. Comparison of techniques for the extraction of the anti-cancer drug camptothecin from Notaphyodes foetida. J Chromatogr A 2005;1063:9-13.

25. Roja G, Heble MR. The quinoline alkaloids camptothecin and 9-methoxycamptothecin from tissue cultures and mature trees of Notaphyodes nimmoniana. Phytochemistry 1994;36:65–6.
25. Hsiao HY, Cheng TJ, Yang GM, Huang IJ, Chen RL. Determination of camptothecins in DMSO extracts of *Notohapodytes foetida* by direct injection capillary electrophoresis. Phytochem Anal 2008;19:136–40.

26. Wu TS, Leu YL, Hsu HC, Ou FP, Chen CC, Chen CF, *et al*. Constituents and cytotoxic principles of *Notohapodytes foetida*. Phytochemistry 1995;39:383-5.

27. Liaoa N, Zhang P, Ao M, Wang J, Shi Y, Yu L. 9-methoxycamptothecin from *Notohapodytes foetida* induces apoptosis in Murine sarcoma 180 cells. Z Naturforsch C 2011;66:471-6.

28. Wiedenfeld H, Furmanowa M, Roeder E, Guzeoska J, Gustowski. Camptothecin and 10-hydroxy camptothecin in callus and plantlets of *Camptotheca acuminate*. Plant Cell Tissue Organ Cult 1997;49:213-8.

29. Pirillo A, Verotta L, Garibaldi P, Torregiani E, Bombardelli E. Constituents of *Notohapodytes foetida*. J Chem Soc Perkin Trans 1995;1:583.

30. Kavitha P, Vasantha Kumar T, Rajasekharan PE, Abdul Kareem VK, Rao VK. Camptothecin and 9-methoxy camptothecin, anti-cancer alkaloids in *Notohapodytes nimmoniana* from Western Ghats, India. J Med Aromat Plant Sci 2010;32:129.

31. Thengane SR, Kulkarni DK, Shrikhande VA, Joshi SP, Sonawane KB, Krishnamurthy KV. Influence of medium composition on callus induction and camptothecin(s) accumulation in *Notohapodytes foetida*. Plant Cell Tissue Organ Cult 2003; 72:247-51.

32. Fulzele DP, Satdive RK, Pol BB. Growth and production of camptothecin by cell suspension cultures of *Notohapodytes foetida*. Planta Med 2001;67:150–2.

33. Ciddi V, Shuler ML. Camptothecin from callus cultures of *Notohapodytes foetida*. Biotechnol Lett 2000;22:129–32.

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