Subcellular Localization and Quantification of Camptothecin in Different Plant Parts of *Chonemorpha fragrans*

Pradnya Kedari, Nutan Malpathak*

Department of Botany, University of Pune, Pune 411007, Maharashtra, India

*Corresponding Author: mpathak@unipune.ac.in

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Abstract *Chonemorpha fragrans* (*C. fragrans*), a liana belonging to family Apocynaceae, shows presence of widely used anticancer compound Camptothecin (CPT). CPT is a modified monoterpene indole alkaloid produced by very few species belonging to unrelated orders of angiosperms, especially plants belonging to the families Apocynaceae and Icacinaceae. In this work a sensitive high performance thin layer chromatography method (HPTLC) was used for detection and quantification of CPT. HPTLC analysis showed that highest amount of CPT was present in root methanolic extract (0.023% of dry weight) followed by stem bark methanolic extract (0.013% of dry weight). Also, the subcellular localization of an anticancer drug has been studied using autofluorescence property of CPT under Ultra violet (UV) light. Localization study revealed that out of all the organs studied roots accumulated highest levels of CPT followed by stem bark. Xylem tissues showed a major accumulation site for CPT. Presence of CPT in *in vitro* culture is an important finding as *in vitro* systems can be used for further experiments to reduce overexploitation of natural population. This is the first report regarding subcellular localization and quantification of CPT in sequential extracts of different plant parts in *C. fragrans*.

Keywords *Chonemorpha fragrans*, Apocynaceae, Anticancer, Camptothecin, HPLC, HPTLC, Localization

1. Introduction

C. fragrans (Moon) Alston. (Apocynaceae) is an unexplored medicinal plant belonging to Western Ghats. Scanty information has been available so far on the composition and activity of secondary metabolites in *C. fragrans*. Roots of this plant have wide importance in folk medicines. Recently an anticancer compound Camptothecin (CPT) has been reported from *C. fragrans* [1].

Anti-cancer activity of CPT is due to its ability to inhibit the eukaryotic DNA topoisomerase I [2]. Naturally occurring CPT is important source of chemotherapeutic agents for clinical treatment of cancer. It also inhibits retroviruses such as the human immunodeficiency virus (HIV) [3]. Chemical synthesis of camptothecin is reported but extraction from plant sources is cost effective method. This fact makes the plants more valuable as a source of CPT as there are limited sources available for CPT extraction. The literature survey reveals that the presence of any valuable product from plant becomes the reason for its overexploitation and so for its threatened status. It is essential to find out new sources as well as attempts have to be made in order to boost the CPT production in the available sources. The present study illustrates first time the presence of CPT in the *in vitro* cultures of *C. fragrans* established in our lab. Localization and quantification of anti-cancer alkaloid Camptothecin (CPT) *in vivo* and *in vitro* in the sequential plant extracts of *C. fragrans* has been investigated in this communication. These results have important implications for harnessing the high yielding individuals for clonal multiplication. Presence of such drug in the cultures will help to prevent the overexploitation as well as could prove an alternative source.

Extracts of root, stem bark, leaves, *in vitro* shoots and callus in methanol, ethyl acetate, chloroform and hexane were comparatively analyzed for CPT content using high performance thin layer chromatography (HPTLC) and high performance liquid chromatography techniques (HPLC). The quantity of CPT in the extract was estimated on the basis of linear calibration curves obtained using the concentration range of 5 to 50 ng with standard CPT. The subcellular localization of CPT, has been investigated in this work using autofluorescence of CPT in UV light which reveals the cellular site of accumulation of CPT in *C. fragrans*.

To our knowledge this is the first report of localization of CPT in *C. fragrans*, serial extraction of CPT and showing presence of CPT in the *in vitro* shoots and callus in *C. fragrans*.

2. Materials and Methods

2.1. Localization

Histochemical analysis was carried out using fresh vegetative organs. Fresh sections of meristem, leaf, petiole, internode, root and callus were analyzed under UV-fluorescent microscope (Olympus research microscope 10X.) for the presence of autofluorescence that CPT molecule emits when exposed to UV radiation.

2.2. Plant Material

Plant samples were collected from Kerala (10°-46' N latitudes and 75°-55' E). The plants were grown in the glasshouse through cuttings and maintained at the Botanic Garden, Department of Botany, University of Pune (Maharashtra, India). Leaf, stem, and root samples were collected from *C. fragrans* trees. *In vitro* shoots used for experiment were taken from the cultures maintained on B5 medium supplemented with 8.8µm BAP, were air dried and used to prepare the extracts. The callus used was obtained on B5 medium supplemented with 8.8µm BAP +3.2 µm NAA.

2.3. Extract Preparation

The plant parts of *C. fragrans* (i.e. leaves, stem bark, roots, *in vitro* shoots and callus) were collected and shade-dried. This shade dried material was crushed in to fine powder using grinder. 20 g sample was extracted repeatedly in 150 ml of hexane and methanol separately for 18 hrs and then the hexane extract residue was sequentially extracted in chloroform and ethyl acetate (Merck, India) using same procedure. The methanolic extract was called as crude extract. All the extracts were evaporated to dryness and redissolved in HPLC-grade methanol. These extracts were filtered using a 0.45µm cellulose nitrate membrane (Laxbro) and stored at -20°C till further analysis was carried out.

2.4. Analysis

2.4.1. Thin-Layer Chromatography (TLC)

Silica gel coated plates (Merck) were used for the TLC analysis. As standard solution, authentic CPT (Sigma) in DMSO: methanol (1:50) at a concentration of 0.01 mg/ml was used. The extracts of the plant samples and the standard solution were spotted on a TLC plate (10cm in height and 20 cm in width) and developed with chloroform: ethyl acetate (1:1 v/v) in a saturated chromatography chamber for about 20 min. The plates were visualized in UV light. The TLC analyses revealed that CPT was present.

2.4.2. High Performance Thin-Layer Chromatography (HPTLC)

Chromatographic analyses of the extracts were performed on TLC silica gel 60 F254 plates (10 × 20 cm; Merck,

Darmstadt, Germany). Aliquots (10 µl) of each extract were applied on the plates as bands using an Linomat V Automatic Sample applicator (Camag, Muttentz, Switzerland). Each 5 mm-wide band was separated from its neighboring bands by a distance of 11.4 mm. Plates were developed in a glass twin trough chamber (20 × 10 × 4 cm) (Camag) previously saturated (20 min) using ethyl acetate:chloroform (1:1 v/v) as the mobile phase. The development length was 80 mm (development time approximately 10 min). After development, plates were dried for 10 min. Plates containing methanol extracts and standard CPT (Sigma) were scanned using Camag TLC scanner operated by win CATS software (Camag) under the following conditions: scanning mode, re-emission fluorescence (mercury lamp); measurement wavelength- 366 nm; slit dimension- 4.00 × 0.03 mm.

2.4.3. Calibration curve of Camptothecin

10 µl of each concentration of the standard solutions of CPT was applied on TLC plate. The plate was developed in solvent system chloroform: ethyl acetate (1:1 v/v). After development, the plate was dried in air and scanned at 366 nm. Data was used to obtain CPT calibration curve.

2.4.4. HPLC Analysis

HPLC analysis of the methanolic extracts of *C. fragrans* was carried out as described in our earlier report [4] with slight modification. Isocratic analytical HPLC was performed with DIONEX (Germany) using RPC18 column, P680 HPLC pump, a 2ml injection loop (Rheodyne USA) and UVD 170 detection controlled with Chromeleon software (DIONEX softtron GmbH, Germany). The mobile phase for alkaloid elution was acetonitrile: water (40:60), at a flow rate 1.0 ml/min with a sample size of 20 µl and UV detection at 254 nm. A standard curve was obtained using authentic sample of camptothecin (Sigma Aldrich). The standard was prepared using DMSO: methanol (1:50 v/v). Co-chromatography of the extracts was performed with authentic samples for confirmation. Validation of quantitative method was performed for samples in 3 replications. All reagents were HPLC grade.

2.4.5. Statistical Analysis

Analysis of variance (one-way ANOVA) was performed to test the significance of differences between means obtained among the treatments in each experiment at the 5% level of significance ($P < 0.05$).

3. Results

The objective of the present study was to reveal the accumulation of the alkaloid camptothecin (CPT) in both *in vitro* as well as *in vivo* plant material of *C. fragrans*. CPT localized in the sections was characterized by a blue fluorescence similar to that shown by the authentic CPT (Sigma) (Figures 1-5). The accumulation of CPT was seen in various tissues of almost all the organs observed.

3.1. Roots

The difference between sections of *in vivo* and *in vitro* materials can be seen prominently in the root sections (Figure 1A-F). The *in vivo* root section showed prominent fluorescence in trichomes, epidermis as well as 3-4 layered hypodermis (Figure 1A,C,E). The *in vitro* sections showed fluorescence in epidermis but trichomes were absent (Figure 1B,D,F). The endodermis also shows the presence of CPT in *in vivo* as well as *in vitro* sections. In mature section the root hair showed presence of CPT and also number of layers showing the fluorescence was more in epidermis. The xylem elements showed prominent fluorescence in both *in vivo* as well as *in vitro* sections. Root tip also shows the presence of CPT in both the sections (Figure 1A).

Figure 1. Localization of CPT in **A, C, E:** *in vivo* root; **B, D, F:** *in vitro* root

3.2. Stem

Stem showed accumulation of CPT in single layered epidermal as well as in the xylem region. Primary as well as secondary cortex does not show presence of prominent fluorescence. Stem also showed the presence of trichome with a prominent fluorescence suggesting presence of CPT. Trichomes were dense in *in vivo* sections where as *in vitro* sections showed negligible number of trichomes (Figure 2).

Figure 2. Localization of CPT in **A, C:** *in vivo* stem; **B, D:** *in vitro* stem

3.3. Meristem

Apical as well as axillary meristem showed prominent fluorescence at the tip region suggesting accumulation of CPT in both *in vivo* as well as *in vitro* sections (Figure 3A-F). The trichomes were prominently present and showed the presence of CPT in *in vivo* section; but in *in vitro* sections trichomes were absent unlike in the leaf, stem sections. The trichomes were uniserrate and multicellular (Figure 3F)

Figure 3. Localization of CPT in **A, C:** *in vivo* auxiliary meristem, **E:** *in vivo* apical meristem; **B, D:** *in vitro* apical and auxiliary meristem, **F:** trichomes

3.4. Leaf

In vivo leaves were covered with dense layer of trichome on abaxial and adaxial surface of leaves as well as petiole (Figures 4A, 5A). *In vitro* leaves and petiole showed less or no trichome on its surface (Figure 4B, 5B). In case of leaves we also could see that the mature sections show abundance of CPT content compared to young tissue (Figure 4C). Epidermal layer showed presence of CPT in both *in vivo* and *in vitro* sections. In the petiole sections we could observe that the xylem tissue showed characteristic blue colour suggesting the presence of CPT (Figure 5D).

Figure 4. A, C: *in vivo* midrib; B, D: *in vitro* midrib

Figure 5. A, C: *in vivo* Petiole; B, D: *in vitro* Petiole

3.5. Quantification of CPT

Quantification of CPT using HPLC and HPTLC (Figure 6-9) shows that the highest amount of CPT is present in root methanolic extract ($0.026\% \pm 0.0005^A$ of dry weight) followed by bark methanolic extract ($0.014\% \pm 0.00043^A$). Whereas *in vitro* cultures shoots shows $0.002\% \pm 0.00004^A$ of its dry weight and callus shows $0.001\% \pm 0.00062^A$ of its dry weight. Hexane extracts showed no or negligible presence of CPT for all the plant parts.

Figure 6. HPTLC of Methanolic extracts A,B: Methanolic roots; C,D: Methanolic stem bark; E,F: Methanolic leaves; G,H: Methanolic shoots; I,J: Methanolic callus; K,L: Camptothecin.

Figure 7. HPTLC of Stem bark extracts A,B: Methanolic crude; C,D: Ethyl acetate; E,F: Chloroform; G,H: Hexane; I,J: Camptothecin

Figure 8. Representative HPLC chromatograms of extracts from A) Callus B) co-chromatogram of methanolic callus extract

Figure 9. % amount of CPT obtained from different plant parts of *C. fragrans*

4. Discussion

In recent years because of the heavy demand, there has been a serious threat of extinction of the populations of the trees producing CPT in the Western Ghats (8° N to 21° N latitudes) forests of south India [5]. Also increase in the clinical application of CPT and its analogues have increased the need to develop methods for higher production. As biosynthesis and accumulation of alkaloids are tissue-specific and cell type-specific [6,7] studies revealing the site of accumulation in a plant provide knowledge about the highest accumulating organ which could be used for further studies. In case of *C. fragrans* the young plant material showed less fluorescence whereas mature tissue showed more fluorescence in large number of cells, suggesting that maturity contributes towards high accumulation of CPT. So mature tissue was further considered for extraction of CPT. Presence of CPT in *in vitro* cultures indicates that *in vitro* systems can be used for further experiments so as to reduce overexploitation of natural population. Localization study has led to the conclusion that the root and stem bark portions are more useful to extract CPT. Quantification of CPT in this plant was comparable with the levels in current CPT sources in use. In all the organs studied, roots accumulated highest levels of CPT followed by stem bark. Tissue-wise, CPT tends to accumulate more in the root bark than root wood, possibly due to the high activities of an enzyme tryptophan synthase, responsible for the key intermediate tryptophan in the CPT biosynthesis pathways [8,9] HPLC and HPTLC data showed that *C. fragrans* has significant level of CPT production in both *in vivo* as well as *in vitro* plant parts, manipulations of which could give enhanced metabolite production.

5. Conclusion

In *C. fragrans* CPT is accumulated in all the plant parts, especially in trichomes, epidermal region and vascular elements. Maturity contributes towards the accumulation of

CPT. Quantification studies showed that in all the plant parts studied roots accumulates highest amount of CPT followed by stem bark. *In vitro* shoots also showed presence of CPT which is considered as important finding as *in vitro* systems can further be used for manipulation studies like elicitation, precursor feeding and transformation to increase CPT production in *in vitro* cultures. This is the first report of localization of CPT, and quantification in serial extracts of *in vivo* and *in vitro* plant parts in *C. fragrans* giving basic information regarding extractability and site of accumulation of CPT.

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REFERENCES

- [1] A. V. Kulkarni, N. P. Malpathak. Micropropagation of *Chonemorpha fragrans* (Moon) Alston: An endangered medicinal plant, *Indian Drugs*, Vol.42, 1001-1003, 2006.
- [2] E. Kjeldsen, J. Q. Svejstrup, Gromova II, J. Alsner, O. Westergaard. Camptothecin inhibits both the cleavage and relegation reactions of eukaryotic DNA topoisomerase, *J Mol Biol*, Vol.228, 1025-1030, 1992.
- [3] E. Priel, S. D. Showalter, D. G. Blair. Inhibition of human immunodeficiency virus (HIV-1) replication *in vitro* by noncytotoxic doses of camptothecin, a topoisomerase I inhibitor, *AIDS Res Hum Retroviruses*, Vol.7, 65-72, 1991.
- [4] A. V. Kulkarni, A. A Patwardhan, U. Lele, N. P. Malpathak. Production of camptothecin in cultures of *Chonemorpha grandiflora*, *Pharmacognosy Res*, Vol.2, 296-299, 2010.
- [5] B. T. Ramesha, T. Amna, G. Ravikanth, R. P. Gunaga, R. Vasudeva, K. N. Ganeshaiah, R. Umashaanker, R. K. Khajuria, S. C. Puri, G. N. Qazi. Prospecting for Camptothecines from *Nothapodytes nimmoniana* in the Western Ghats, South India: identification of high-yielding sources of camptothecin and new families of camptothecines, *J Chromatogr Sci*, Vol.46, 362-368, 2008.
- [6] V. De Luca, B. St Pierre. The cell and developmental biology of alkaloid biosynthesis, *Trends Plant Sci*, April 5, 168-173, 2000.
- [7] N. Samanani, C.Y. Edry weightard, J.F. Peter. Cell type-specific protoberberine alkaloid accumulation in *Thalictrum flavum*, *J Plant Physiol*, Vol.159, 1189-1196, 2002.
- [8] Z. Liu, S. B. Carpenter, W. J. Bourgeois, Y. Yu, R. J. Constantine, M. J. Falcon, J. C. Adams. Variations in the secondary metabolite camptothecin in relation to tissue age and season in *Camptotheca acuminata*, *Tree Physiol*, Vol.18, 265-270, 1998.
- [9] H. Lu, T. D. McKnight. Tissue-specific expression of the β -subunit of tryptophan synthase in *Camptotheca acuminata*, an indole alkaloid producing plant, *Plant Physiol*, Vol.120, 43-52, 1999.