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Camptothecine and methoxy camptothecine from callus cultures of *Miquelia dentata* Bedd.—A rare plant of the Western Ghats of India

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Miquelia dentata Bedd. (Family: Icacianceae) is a small climbing shrub producing anti-cancer alkaloid, camptothecine (CPT). The plant occurs sparsely in the southern parts of the forest of Western Ghats, India. Callus cultures of the plant were established using leaf explants on Murashige and Skoog medium supplemented with several plant growth regulators. The highest callus growth was obtained in the medium with 1.0 mg/L each of indole butyric acid (IBA) and benzyl adenine purine (BAP). The calli cultures produced CPT (5.4 µg/mg of the dry wt) and traces of methoxy camptothecine (MCPT).

Keywords: Callus culture, camptothecine (CPT), methoxy camptothecine (MCPT), *Miquelia dentata*, Icacinaceae

Camptothecine (CPT) is an important anti-cancer compound obtained from several plant species belonging to the Asterid clade[1]. It is a potent inhibitor of the intra-nuclear enzyme topoisomerase-I, which is required in DNA replication and transcription[2]. Several semi-synthetic drugs, such as, Hycamtin (topotecan) and Camptostar (irinotecan or CPT 11) are derived from CPT and are currently in clinical use against ovarian, small lung and refractory ovarian cancer[1,3-5]. Recently, CPT has also been reported from yet another plant, *Miquelia dentata* Bedd., belonging to the family Icacinaceae, sub-order, Asterid[6,7]. The fruits of the plant produce the highest ever reported content of CPT, ranging from 0.8 to 1.2 per cent by dry wt. This species is a slow growing climber and is sparsely distributed in the Western Ghats, India[8]. Despite the discovery of several species producing CPT, an inherent constraint is the sustainable extraction of CPT; most of the extraction is done by destructive felling of the woody trees and plants. The heavy demand for CPT has resulted in extensive harvesting of two of the most important plant species producing CPT, namely, *Campotheca acuminata* (in China) and *Nothapodytes nimmoniana* (in India). In fact, consequent to the indiscriminate harvesting, both the species are threatened; *N. nimmoniana* has been declared as vulnerable in India[9]. Against this background, several attempts have made to explore plant cell culture approaches for a more stable production of CPT from *C. accuminata*[10,11] *N. nimmoniana*[12] *and O. pumila*[13]. In the present study, we report the development of callus cultures from leaf explants of *M. dentata*, which are able to produce CPT and its analog, MCPT (methoxy campothecine). These findings are also discussed in context to the development of stable *in vitro* production system for CPT.

Callus cultures were established from explants collected from the nursery grown plants of *M. dentata* maintained at the College of Forestry, Sirsi, India. The plants were originally sourced from the Sampaje forest range of Madikeri forest division, Kodagu, Western Ghats, India. Young tender leaves, apical buds, internodal and nodal regions were cut into 1 cm^2 segments, washed with running tap water and treated with 1% Tween-20 detergent. The tissues were surface sterilized using sodium hypochlorite (50% v/v) and 71% (v/v) EtOH. The surface sterilized tissue segments were placed on Murashige and Skoog (MS) medium[14] supplemented with plant growth hormones. The concentrations of the various growth hormones used are given in Table 1. The explants were maintained under aseptic conditions at 25°C with a photoperiod of 12 h light (2000 lux)/12 h dark. Callus cultures formed were periodically sub-cultured to fresh MS medium containing the respective supplementation of plant growth hormones.

To determine CPT content, a 14-wk-old calli were used for the extraction. The calli were thoroughly washed with sterile water. Fresh wt of the tissues was

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Table 1—MS media optimization for callus initiation in different tissue segments of *M. dentata*

Explants	Phytohormones (mg/L)	Callus response*
Leaf segment	BAP (0.5)+2,4-D (0.5)	-
	BAP (0.5)+2,4-D (1.0)	-
	BAP (0.5)+NAA (0.25)	-
	IBA (0.125)+BAP (1)	+
	IBA (0.125)+BAP (1.5)	+
	IBA (0.5)+BAP (0.5)	-
	IBA (0.5)+BAP (1.0)	++
	IBA (0.5)+BAP (1.5)	+
	IBA (1.0)+BAP (1.0)	+++
Apical bud	IBA (0.125)+BAP (1)	-
	IBA (0.125)+BAP(1.5)	-
Nodal segments	BAP (0.5)+2,4-D (1)	-
	BAP (0.5)+2,4-D (0.5)	-
	BAP (0.5)+NAA (0.25)	-

*Growth rate of callus: +, callus initiation; ++, good callus; -, absence of callius formation

BAP, 6-Benzyl aminopurine; IBA, Indole butyric acid; 2,4-D, 2,4-Dichloro phenoxy acetic acid; NAA, Napthalene acetic acid

recorded and then dried in an air dryer at 65°C for 2 d. The dried tissue was finely powdered and weighed. The fine tissue powder (400 mg) was mixed with 5 mL of methanol, sonicated and kept overnight in deep freezer at –20°C. After thawing, the methanol layer was collected and extracted with water/chloroform (18:20, v/v). The process was repeated thrice. The chloroform layer was collected and evaporated to dryness using a Buchi Rotavapor (RE111, Switzerland). The residue was finally dissolved in 500 µL of methanol and used for analysis[15]. Fresh fruits of *M. dentata* were also collected and dried in an air drier at 65°C for 3 d. Dried fruits were finely ground and used for the extraction. About 100 mg of fine tissue powder was extracted with 5 mL of 61% ethanol (v/v) in shaking water bath at 65°C for 3 h[16]. The extract was centrifuged at 10,000 rpm for 10 min. The supernatant was filtered using 0.22 µ filter and used for further chemical analysis. The extracted residues from the callus and the plant tissue were dissolved in methanol and along with the reference CPT standard (Sigma Chemical Company, St. Louis, USA) were co-chromatographed on aluminium-backed silica gel 60 F_{254} plates. Chloroform/ethyl acetate (1:1, v/v) were used as the mobile phase. The Rf values of the sample were matched with the reference compound, CPT. Compound detection was done using a UV trans-illuminator at 280 nm. CPT was further analyzed by reverse phase HPLC (Supelco 516, LC-10AS, Shimadzu, Japan) on a C18 column (250×4.6 mm^2, 5 µm). The HPLC conditions were: 254 nm as the detector wavelength, 1.5 mL/min flow rate and 20 µL sample loop. The mobile phase was adjusted as follows: 25% acetonitrile and 0.1% trifluro-acetic acid (TFA) in 75% water (v/v) in a binary mode[17]. The CPT standard was prepared using DMSO/methanol (1:3, v/v) ratio and the retention time (T_R) of CPT was 18.5 min. CPT levels were quantified in the plant samples by using the regression of peak areas against standard CPT and expressed as percent dry wt of tissue.

The mass of CPT and its analogs in the samples were analysed by LC-ESI-MS (LCMS-2020, Shimadzu, Japan). The LC was coupled to an ion trap mass analyzer and then to a detector (LCMS-2020AD). Mass analyzer was equipped with atmospheric pressure ionization source electrospray ionization (ESI). High purity nitrogen from a nitrogen generator was used as a carrier gas. LC conditions for the analysis were as follows: RPC-C18, 250×4.6 mm^2, 5 µ size (Phenomenex), Detector: UV-Visible, wave length: 254 nm, flow rate: 0.3 mL/min, injection volume: 5µL, mobile phase: pump A: 25% acetonitrile and pump B: 75% water+0.1% trifluro-acetic acid (TFA) (v/v) in an isocratic mode. The total analysis run time was 40 min. The conditions for mass spectrum during the analysis were set as follows: dry gas flow rate 10 L/min, nebulizer pressure 35 psi, nebulising gas flow 1.5 L/min, DL temp 250°C and the mass range was from 50-700 *m/z*. By applying these conditions, CPT and MCPT were observed at the positive mode of ESI-MS. CPT exhibited a molecular adduct $(M+H)^+$ at *m/z* 349 and MCPT at *m/z* 379.12.

Among the different explants, only the leaf segments showed signs of callus induction. Callus initiation started after 8 wk of plating the explant. The cell induction and division started after 90 d of incubation. The highest growth of callus was observed on MS media supplemented with 1.0 mg/L each of BAP and IBA along with 3% sucrose (Table 1). The callus initiation and growth was completed in 4 wk. Callus was white, friable and light creamy (Fig. 1). Overall the callus growth was very slow. The callus was maintained by periodic subculturing on MS medium.

TLC analysis showed the presence of CPT in the callus culture of *M. dentata*. The Rf values of the

sample matched with the standard CPT, which showed a blue fluorescence under UV light. The HPLC analysis also confirmed the presence of CPT in the callus culture. The retention time (25 min) and the UV absorption maxima (254 nm) of the sample were similar to that of the standard (Fig. 2). Further the mass spectra analysis confirmed the presence of m/z 349 $[M+H]^+$ ion of CPT ([†]Suppl Fig. 1). The amount of CPT recovered from the callus culture was 5.4 µg/mg dry wt of the sample along with the traces of MCPT. This estimate is much higher compared to that has been reported by some earlier studies from other plants producing CPT. For example, Sakato & Misawa[10] reported a production of 2.5 µg CPT per g dry wt from callus cultures of *C. acuminata* (Nyssaceae); while others reported 9.5 µg CPT per g dry wt from callus cultures of *N. nimmoniana*[15] and 0.003 mg CPT per g dry wt of callus from *Chonemorpha grandiflora* (Apocyanaceae)[18]. Two earlier studies have also reported the production of traces of 9-methoxy CPT from callus cultures of plants producing CPT[12,15]. In summary, our results clearly show that the callus culture of *M. dentata* is able to produce CPT besides traces of MCPT. Although the amount is less than that of the fruits, the finding is potentially important as it provides a tool to further optimise the production of CPT *in vitro* system including cell culture system for the production of CPT.

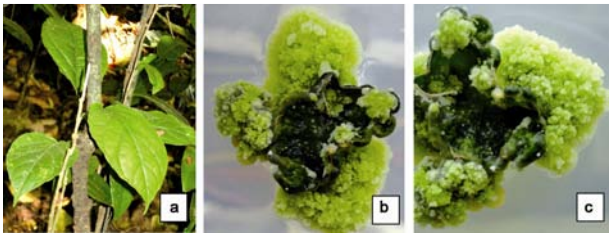

Fig. 1 (a-c)—Natural habit of *M. dentata* (a); *In vitro* regenerated green friable mass of callus from the leaf segments of *M. dentata* (b & c).

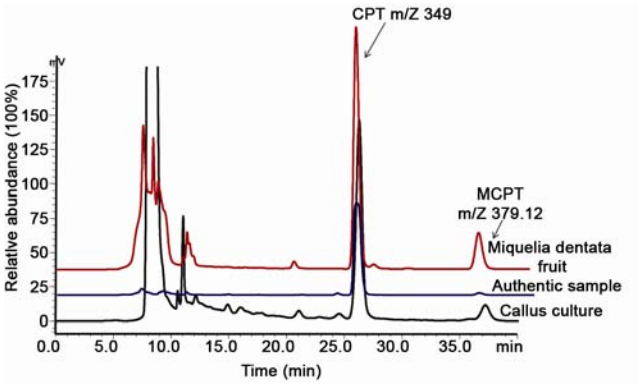

Fig. 2—Total chromatogram (TIC) of different samples, *viz.*, fruits and tissue callus culture of *M. dentata* with the authentic CPT (standard CPT obtained from Sigma Chemicals). The chromatograms were overlaid on each other for clarity. Intensity of the peaks does not correlate with concentration of metabolites.

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