Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb

Document heading doi:10.1016/S2221-1691(12)60292-5

Micropropagation and production of camptothecin form in vitro plants of *Ophiorrhiza mungos*

A. G. Namdeo*, Priya T., B. B. Bhosale

Department of Pharmacognosy,Poona College of Pharmacy, Bharati Vidyapeeth University,Pune, Maharashtra – 411038, India.

ARTICLE INFO

Article history:
Received 25 June 2012
Received in revised form 5 July 2012
Accepted 7 August 2012
Available online 28 August 2012

Keywords:
Camptothecin
HPLC
Ophiorrhiza mungos (Family Rubiaceae)
micropropagation
in vitro plants

ABSTRACT

Objective: To explore the biotechnological potential of *Ophiorrhiza mungos* for micropropagation and camptothecin (CPT) production from *in vitro* grown plants.**Methods:** Surface sterilized explants of *O. mungos* were transferred aseptically in MS media containing various combinations of phytohormones for callus initiation and multiple shoot proliferation. The content of CPT was quantified in the methanolic extract of *O. mungos* plants and in in vitro grown plants by using high performance liquid chromatography (HPLC). **Results:** Maximum fresh weight and dry weight biomass of *O. mungos* callus was obtained from MS medium supplemented with IAA (2 ppm) + BAP (2 ppm) + GA (1 ppm). The maximum shoot proliferation (25) and elongation (6.5 cm) was found in MS medium supplemented with Picloram + Thidiazuron + Gibberellic Acid in 1:2:1 ratio after four weeks of incubation. The maximum content of CPT (0.0768 % w/w) was found in whole *in vitro* plants whereas the minimum CPT was observed in adventitious buds (0.0026 % w/w) as compared to the naturally grown *O. mungos* plants (0.0030% w/w).**Conclusions:** Present findings indicate that *O. mungos* plants respond favourably for *in vitro* propagation and also produce higher amount of CPT as compared to naturally grown plants.

1. Introduction

Camptothecin (CPT) is a water-insoluble cytotoxic monoterpene-derived indole alkaloid initially isolated from the Chinese tree *Camptotheca acuminata* (Nyssaceae). CPT derivatives such as irinothecan and topothecan have been widely used for the treatment of cancer over the world [1, 2]. CPT has also been isolated from other plants/trees like *Nothapodytes foetia*, *Merriliodendron megacarpum*, *Eravatamia heyneana* and *Ophiorrhiza species*. Various plants from *Ophiorrhiza species* (Family Rubiaceae) such as *O. pumila*, *O. mungos*, *O. rugosa*, *O. filistipula*, *O. prostrate*, *O. liukiuensis* etc. have been reported as prominent source of CPT [3-6]. *Ophiorrhiza species* are distributed throughout the Western Ghats of India (Wynaad, Anamalais, Travancore and Tinnevelly hills, Andamans and Nicobars islands, Assam). They are also available in Sri Lanka, China, Thailand, Peninsula, Malaysia, Sumatra, Java and Philippines. The enormous worldwide demand for CPT, overexploitation of natural resources of CPT, lower yields of CPT from intact plant, poor seed germination and lack of economically viable method of production has encouraged us to explore the biotechnological potential of *O. mungos* for micropropagation and CPT production.

2. Material and Methods

2.1. Plant Material

Plant material of *Ophiorrhiza* mungos was collected and authenticated from the Tropical Botanical Garden and Research Institute (TBGRI), Trivendrum, Kerala, India, in the month of September. The voucher specimen (674) was kept at the departmental herbarium of TBGRI, Trivendrum, India.

2.2. Surface sterilization of explants

One month old young fruits of *O. mungos* were surface sterilized with 70% alcohol for 3-5 min, rinsed with 6-7 times with sterile distilled water and then washed with freshly prepared 0.1% mercuric chloride solution in

*Corresponding author: Dr. Ajay G. Namdeo Associate Professor, Department of Pharmacognosy, Poona College of Pharmacy, Bharati Vidyapeeth University, Pune, Maharashtra – 411 038, India
Tel: 91-20-25437237, Fax: +91-20-25439383
E-mail: agnamdeo@gmail.com

sterile distilled water for 2-4 min. Finally, the fruits were washed 6-7 times with sterile distilled water to give surface sterilized explants for aseptic transfer.

2.3. Preparation of explants

For initiation of callus, disinfected fruit were cut into small pieces (6-8 mm). These small pieces were transferred in pre-sterilized culture medium and incubated under controlled environmental conditions for callus initiation. The trimmed explants were inoculated on Murashige and Skoog's (MS) [7], B5 and Gamborg medium [8] supplemented with different combinations of growth hormones. The explants were also inoculated in modified medium in which coconut water was used in place of synthetic hormones. The pH of media was adjusted to 5.6-5.8 with the help of 0.1N NaOH and 0.1N HCl prior to gelling with 0.8% agar, dispensed (15 mL) into culture tubes and was sterilized by autoclaving (121℃ at 15 psi for 15 min). The cultures were incubated under a regime of 16 h photoperiod (intensity 40 μE cm^2/min/sec) 25±2℃.

2.4. Subculture

The callus culture was sub-cultured every 3rd week on medium with similar composition and concentration of growth hormones for the development of friable callus.

2.5. Sample preparation and extraction

Naturally grown plant material (whole plant) and tissue culture grown material (callus) of *O. mungos* were collected, washed and dried at 55℃ in an air dryer for constant weight. Dried materials were powdered to pass a 20 mesh sieve. Dried powdered materials (2.0 g) were taken in 50 mL volumetric flask, percolated with 50 mL of methanol and vortexed for 2 min followed by sonication (33 MHz, Roop Telesonic, India) at room temperature for 15 min. The process was repeated thrice for complete extraction. After sonication, the methanolic extract was evaporated to dryness at 40℃ in vacuo using a Buchi Rotavapor (RE111, Switzerland). The dried extracts (50.0 mg) were transferred to 5 mL volumetric flask and the volume was made upto 5.0 mL with methanol to furnish the final concentration of 10 mg/mL.

2.6. High performance liquid chromatography

CPT was analyzed using an HPLC system model (Jasco PU 2080, Intelligent HPLC pump), UV/Vis detector (Jasco UV 2075) model operated at a wavelength of 360 nm. Data was integrated using a Jasco Borwin version 1.5, LC-Net II/ADC system. Separations were performed with Perfect SIL-100 ODS-3-C18 (250 mm × 4.6 mm, 5.0 μ) from MZAnalysetechnik GmbH, Germany. Kromasil C18 (250 mm × 4.6 mm, 5.0 μ) from Flexit Laboratories Pvt. Ltd. Pune, India at 25℃ with a flow rate of 1 ml/min using an isocratic elution of acetonitrile: water (45:55 v/v) solvent system. HPLC assay of different extracts yielded chromatograms with a retention time 4 min for CPT [9].

2.7. Preparation of standard solutions and calibration curve of CPT

A stock solution of CPT was prepared by dissolving 3 mg of CPT in 10 mL of methanol (300 μg /mL). The standard solutions were prepared by dilution of the stock solution with methanol to reach a concentration range of 3-15 μg/mL for CPT. Triplicate 20 μL injections were made six times for each concentration and chromatographed under the conditions described above. The peak area was plotted against the corresponding concentrations to obtain the calibration graphs.

3. Results

3.1. Callus initiation from O. mungos

Surface sterilized explants of *O. mungos* were transferred aseptically in MS media containing various combinations of phytohormones. Out of several media tried for callus initiation some media showed favorable response. Table 1 represents the influence of phytohormones on initiation and morphology of *O. mungos* callus. MS media supplemented with Picloram, BAP, IBA resulted in the formation of green friable callus. MS medium supplemented with IBA + BAP + GA (2:2:1), Piclo + BAP + GA (1:3:1), IBA + BAP + GA (2:2:2), IBA + BAP + GA (2:1:2) and IBA + BAP + GA (5:1:1) along with 3% sugar gave healthy, green, friable callus within 30 days of incubation however, some media like MS supplemented with Piclo + TDZ + GA showed browning of callus. Table 2 demonstrates the callus growth of *O. mungos* in different media containing various combinations of phytohormones.

Table 1.
Influence of phytohormones on initiation and morphology of *O. mungos* callus.

Media	Hormones	Conc. (ppm)	Morphology of callus
MS	Piclo+BAP+GA	1:3:1	Green, friable, high mass
MS	IBA+BAP+GA	5:1:1	Green, friable, high mass
MS	IBA+BAP+GA	2:2:1	Green, friable, high mass
MS	IBA+BAP+GA	2:1:2	Green, friable, high mass
MS	IBA+BAP+GA	2:2:2	Green, friable, high mass
MS	Piclo+TDZ+GA	1:1:4	Brown, friable, high mass

3.2. Shoot proliferation, organogenesis and root induction from O. mungos

Table 3 shows the effect of phytohormones on proliferation of shoots of *O. mungos* grown in MS medium. Shoot elongation was maximum in MS liquid medium supplemented with Piclo + TDZ + GA (1:2:1). The higher concentration of phytohormones affected the quality of shoots (browning / necrosis).

Table 2.
Callus growth of *O. mungos* in different media containing various combinations of growth hormones.

Media	Hormones	Conc. (ppm)	FW (mg)	DW (mg)
MS	IAA +BAP+GA	2:2:1	2090	360
MS	BAP + GA	4:8	730	100
MS	IAA+BAP+GA	1:2:1	760	60
MS	Piclo+TDZ+GA	1:3:1	1450	140
MS	Piclo+TDZ+GA	1:2:1	1620	120
1/2 MS	Piclo+TDZ+GA	1:3:1	920	50
1/2 MS	IBA+BAP+GA	1:2:3	800	60
G6	Piclo + TDZ + GA	1:2:1	230	40
G7	Piclo + TDZ + GA	4:3:1	1420	140
G7	Piclo + TDZ + GA	4:3:1	1420	140
G8	Piclo + TDZ + GA	5:3:1	310	40
B8	Piclo + TDZ + GA	5:3:1	80	10
B9	IBA + BAP + GA	4:1:1	370	60

Table 3.
Effect of phytohormones on proliferation of shoots of *O. mungos* grown in MS medium.

Media	Hormones	Conc. (ppm)	Length of shoots (cm)	Quality of shoots
MS	BAP + GA	8:12	5	Green
MS	IBA + BAP + GA	5:1:1	4	Green
MS	Piclo + TDZ + GA	1:2:1	6.5	Green

The organogenesis from *O. mungos* grown on MS media with different concentrations of phytohormones is represented in Table 4. The maximum number of shoots (25) was observed in MS medium supplemented with Piclo + TDZ + GA (1:2:1) whereas the MS medium containing BAP + GA (8:12) showed 18 shoots after 60 days of incubation.

Table 4.
Organogenesis from *O. mungos* grown on MS media with different concentrations of phytohormones.

Media	Hormones	Conc. (ppm)	Number	Length (cm)
MS	BAP + GA	8:12	18	1.4
MS	IBA + BAP + GA	5:1:1	12	1
MS	Piclo + TDZ + GA	1:2:1	25	1.6

Table 5.
Effect of IBA on root induction from *O. mungos* shoots using MS media.

Media	Hormones	Conc. (ppm)	Length of root (cm)	Number of roots	Color of root
MS	IBA + BAP + GA	4:1:2	12	22	Brown
MS	IBA + BAP + GA	1:2:1	10	14	Brown
MS	IBA + BAP + GA	1:3:1	09	16	Brown

The effect of IBA on root induction from *O. mungos* shoots using MS media is illustrated in Table 5. Rooting of individually excised shoots occurred after 60 days from MS media supplemented with IBA, BAP and GA added in different combinations. Maximum number (22) and maximum length (12 cm) of roots were observed in media supplemented with IBA + BAP + GA (4:1:2).

Table 6.
CPT content analysis by HPLC at different stages of in vitro and of naturally grown plants of *O. mungos*.

Stages	CPT (% w/w)
Callus	0.0181
Plantlets	0.0254
Adventitious buds	0.0026
Multiple shoots with branches	0.0208
Plant with multiple shoots	0.0227
Plant with roots	0.0324
Whole in vitro grown plant	0.0768
Naturally grown plant	0.0030

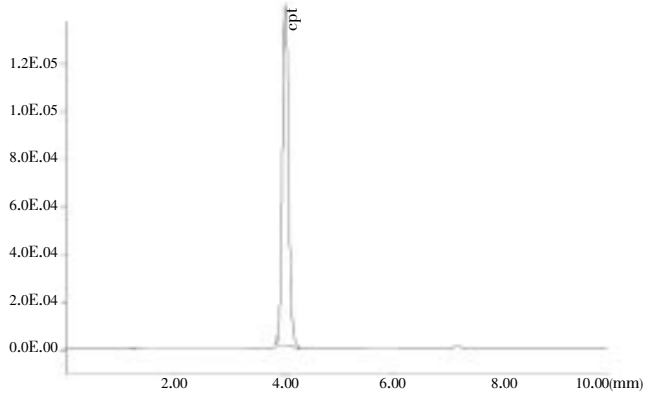


Figure 1. HPLC chromatogram of CPT.

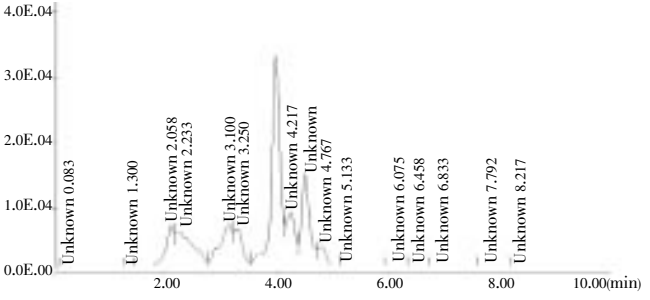


Figure 2.HPLC chromatogram of naturally grown samples of *O. mungos* spiked with CPT.

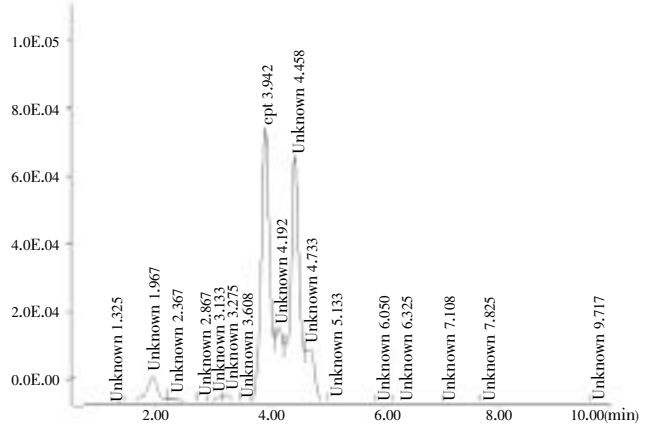


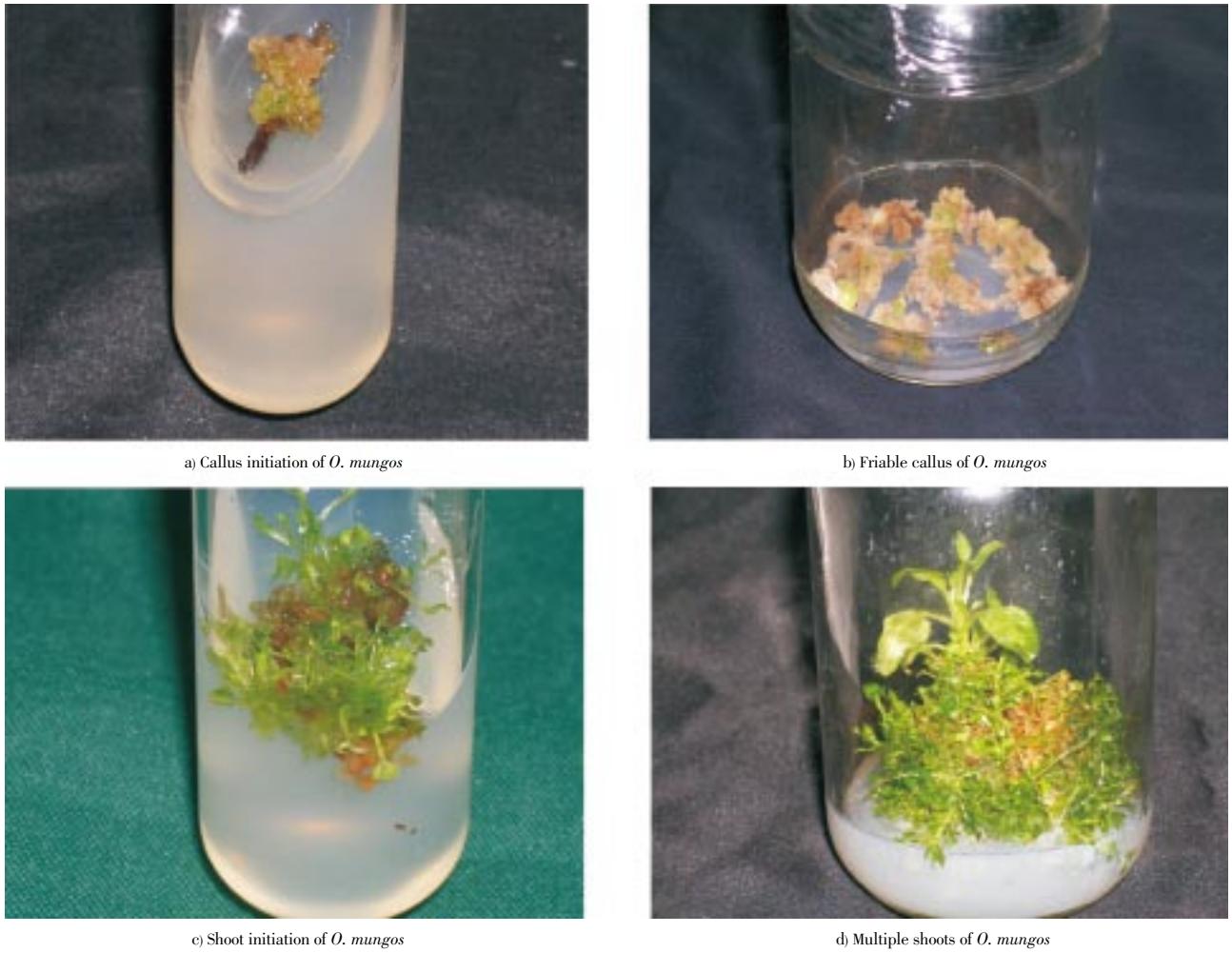
Figure 3. HPLC chromatogram of in vitro grown samples of *O. mungos* spiked with CPT.

3.3. Quantification of camptothecin in natural plants and in in vitro grown plants

For optimization of HPLC procedure initially, acetonitrile and water in different ratios was tried. Mobile phase consisting of acetonitrile:water (45:55 v/v) gave a symmetrical, sharp peak at retention time (tR) of 4.0 min for CPT. HPLC chromatogram of authentic/ standard CPT is presented in Figure 1 .

Table 6 indicates CPT content analysis by HPLC at different stages of in vitro grown plants of *O. mungos* and compared with naturally grown whole plants. The maximum content of CPT (0.0768 % w/w) was found in whole in vitro plants whereas the minimum CPT was observed in adventitious buds (0.0026 % w/w) as compared to the naturally grown *O. mungos* plants (0.0030% w/w). Other plant/ plant parts developed in vitro showed CPT content in following increasing order *O. mungos* callus, (0.0181 % w/w); multiple shoots with branches, 0.0208 % w/w), plant with multiple shoots, (0.0227 % w/w); plantlets, (0.0254 % w/w) and *O. mungos* plant with roots, (0.0324 % w/w). Figure 2 and Figure 3 the CPT content HPLC chromatogram of in vitro grown samples of *O. mungos* spiked with CPT.

4. Discussion



a) Callus initiation of *O. mungos*

b) Friable callus of *O. mungos*

c) Shoot initiation of *O. mungos*

d) Multiple shoots of *O. mungos*

Figure 4. Different stages of in vitro grown material of *O. mungos*.
a) Callus initiation of *O. mungos ;* b) Friable callus of *O. mungos*; c) Shoot initiation of *O. mungos*; d) Multiple shoots of *O. mungos*

The method developed for the callus initiation and regeneration of multiple shoots from fruit explants of *O. mungos* was found to significantly enhance CPT as compared to naturally grown plant. Callus consists of undifferentiated mass of cells developed on a semi-solid medium that can be initiated from any viable explants of intact plant. Auxin/ cytokinin ratio plays an important role in callus initiation, proliferation of shoot and organogenesis under in vitro conditions. Maximum fresh weight and dry weight biomass of *O. mungos* callus was obtained from MS medium supplemented with IAA (2 ppm) + BAP (2 ppm) + GA (1 ppm). Callus initiated on MS medium with NAA alone or in combination with BA or Kn upon subculture developed adventitious shoots. MS medium with 8.83 μM BA and 2.46 μM IBA was the best with a mean of 20.1 shoots within 40 days [6]. Concentrations of cytokinins specially, BA favored callus proliferation. Callus mediated shoot proliferation and organogenesis has been well accomplished in number of medicinal plants like Tylophora indica, Cassia angustifolia and Saussurea obvallata [6]. Callus mediated shoot regeneration can be utilised for the induction of somaclonal variation for the improvement of this valuable medicinal

plant.

Direct shoot proliferation and organogenesis is regarded as the most reliable method for clonal propagation as it maintains the genetic uniformity among the progenies. In our study, direct shoot proliferation (25 shoots) and elongation (6.5 cm) was found in MS medium supplemented with Picloram + Thidiazuron + Gibberellic Acid in 1:2:1 ratio after four weeks of incubation. Direct shoot proliferation of shoots and organogenesis in different plants of *Ophiorrhiza species* has been reported [6, 10]. Pratap [6] reported organogenesis from leaf and internode explants of *Ophiorrhiza* prostrate. Leaf explants of *O. rugosa* var. decumbens were used for multiplication of albino plants in MS medium supplemented with 4 mg/l BA [3]. Similarly, plantlets of *O. rugosa* were successfully regenerated from auxillary meristems on medium supplemented with BA 4ppm + NAA 0.05 ppm [5]. Internode explants O. mongoose Linn cultured on MS medium with 8.83 μM BA and 2.46 μM IBA yielded the highest number of shoots [6]. Shoot multiplication on leaf and node explants from germinated seeds of O. alata was successful on half-strength MS medium supplemented with varying amounts of Kn and NAA [19]. They also reported that leaf explants on 1/2 MS medium containing 9.3 M Kn and 0.54 M NAA showed maximum multiple-shoot induction and the greatest number of shoots per explant. The shoots proliferation and organogenesis has been reported in different explants/species, e.g. from internodes of Bacopa monniera [6], and leaf explants of Drosera binata [6].

Quantitation by HPLC revealed maximum content of CPT (0.0768 % w/w) in in vitro plants whereas the minimum CPT was observed in adventitious buds (0.0026 % w/w) as compared to the naturally grown *O. mungos* plants (0.0030% w/w). Our result agrees with the results of other workers that higher concentrations of CPT were observed from in vitro grown plants than naturally grown Ophiorrhiza plants [6-11]. Vineesh [3] reported that CPT production increased with respect to BA concentration. The highest CPT level was recorded from normal and albino plants in medium supplemented with 6 ppm BA was 0.311 and 1.04 mg/g DW respectively. Similarly, Roja [5] reported CPT production by in vitro cultures and plant regeneration in *Ophiorrhiza species*.

The present study revealed that phytohormones play an important role in initiation of callus from fruit explants of *O. mungos* and a protocol has been established for rapid proliferation of shoots and organogenesis. HPLC analysis revealed higher production of CPT in in vitro/ albino plants as compared to naturally grown plants. This protocol can be utilized for conservation of an important CPT producing species and also provide an ample opportunity to take this plant for extensive research for mass cultivation on plants and enhanced CPT production through different biotechnological strategies like precursor feeding, elicitation, hairy root culture and large scale cultivation in bioreactor system.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

The authors are thankful to Department of Biotechnology, Ministry of Science and Technology, New Delhi, for funding RGYI research project to Dr. Namdeo and Dr. S. S. Kadam Vice-Chancellor, Bharati Vidyapeeth University, Pune and Dr. K. R. Mahadik, Principal, Poona College of Pharmacy for providing all necessary facilities

References

[1] Namdeo AG, Sharma A, Sathiyanarayann L, Mahadik KR. HPTLC Densitometric Evaluation of Tissue Culture Extracts of Nothapodytes foetida Compared to Conventional Extracts for Camptothecin Content and Antimicrobial Activity. *Planta Med* 2009; **75**: 1-7.

[2] Namdeo AG, Sharma A, Fulzele DP, Mahadik KR "Influence of Geographical and Climatic Conditions on Camptothecin Content of Nothapodytes nimmoniana" Rec. *Nat. Prod.* 2010; **4**:1, 64-71.

[3] Vineesh VR, Jelly CL, Fijesh PV, Jaimsha VK, Padikkala J, " Effect of N6 - Benzyl amino Purine and Naphthalene acetic acid on camptothecin production through in vitro propagation of *Ophiorrhiza* rugosa Wall. Var. decumbens (Gardn. ex. Thw.) deb and Mondal" Nat. *Prod. Rad.* 2007; 6:**5**, 405-409

[4] Thuluvath PS. "Investigations on an endangered medicinal plant *Ophiorrhiza* mungos for its biotechnological and pharmacological profile" Master of Pharmacy thesis, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Pune, 2011.

[5] Roja G. "Micropropagation and production of camptothecin from in vitro plants of *Ophiorrhiza* rugosa var. decumbens. *Nat. Prod. Res*. 2008; **22**, 12.

[6] Prathap RV, Vijaykumar G, Narmada KM, "In-vitro organogenesis of *Ophiorrhiza* mongoose linn from root and internode explants" Inl. *J. Pharma Bio Sci.* 2011; **2**: 2, 509-519.

[7] Murashige T and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue. Physiology Plantarum 1962. 15: 473.

[8] Gamborg OL, Miller RA, Ojima K Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 1968; **50**: 151-158.

[9] Fulzele DP, Satdive RK, "Distribution of anticancer drug CPT in Nothapodytes foetida" *Fitoterapia* 2005; **76**, 643-648.

[10] Pornwilai Y, Piyarat C, Suchada S. Micropropagation and hairy root culture of *Ophiorrhiza* alata Craib for camptothecin production. Biotechnol Lett, 2011; August.

[11] Raveendran VV, Vijayan FP, Padikkala J. Antitumor activities of an anthraquinone fraction isolated from in vitro cultures of *Ophiorrhiza* rugosa var decumbens. Integr Cancer Ther. 2011.