Indian Journal of Novel Drug Delivery

IJNDD

An Official Publication of Karnataka Education and Scientific Society

Short Communication

# Effect of Subculturing and Phytohormones on Accumulation of Asiaticoside in Callus Cultures of *Centella asiatica* (L.) Urban

HASANPASHA N. SHOLAPUR*, FATIMA SANJERI DASANKOPPA

KLES' College of Pharmacy, Vidyanagar, Hubli – 580031, INDIA.

**ARTICLE DETAILS**

*Article history:*
Received on 17 April 2011
Modified on 22 June 2011
Accepted on 29 June 2011

*Keywords:*
*Centella asiatica*,
Callus culture
Subculture
Asiaticoside

**ABSTRACT**

Accumulation of asiaticoside (an important bioactive compound) was determined in parent, first subcultured & second subcultured calluses obtained from leaf explants of *Centella asiatica* (L.) Urban. In first subcultured callus the accumulation of asiaticoside was 0.125 mg/g dry mass, using leaf explants in presence of napthelene acetic acid (NAA 1mg/l) and benzyl amino purine (BAP 2mg/l), was the highest among all the callus cultures studied, but this accumulation of asiaticoside was lower than that obtained from whole plant material (0.179 mg/g dry mass).



## INTRODUCTION

*Centella asiatica* (L.) Urban belonging to family apiaceae also known "Brahmi" in classical ayurveda is a perennial herb with high medicinal values in traditional and modern medicine especially as brain tonic, anti-tubercular, anti-leprotic, for the treatment of skin diseases, rheumatism, elephantiasis and also as memory enhancer[1]. Phytochemical studies revealed the presence of terpenoids the most important among them is asiaticoside[2,3]. Tissue culture studies have been extensively carried out on *Centella asiatica* (L), which includes rapid regeneration[4-10], Hairy root culture[11], Enhanced production of asiaticoside by hairy root culture using elicitors[12], Enhanced production of asiaticoside in whole plant culture using elicitors[13], Somatic embryogenesis[14], Callus culture[15], Establishment of callus & cell suspension culture[16] & Production of asiaticoside in-vitro and in-vivo[17]. Gene expression and centelloside production in non-differentiated tissues[18]. However no reports are found regarding the effect of subculturing on accumulation of asiaticoside in callus culture of *Centella asiatica* (L.) since subculturing plays a vital role in accumulation secondary metabolites.

***Author for Correspondence:***

*Email:* hasanpashas@gmail.com

The aims of the present study were to 1) initiate callus and to determine the asiaticoside content in *Centella asiatica* (L.) 2) to study the effect of subculturing on accumulation of asiaticoside in callus cultures of *Centella asiatica* (L.) & 3) to study the effect of combination of plant hormones, auxin (NAA) and cytokinins (BAP, Kinetin) on accumulation of asiaticoside in callus cultures of *Centella asiatica* (L.).

## MATERIALS AND METHODS

### Materials

Bavistin (fungicide) was purchased from BASF, India ltd., Mumbai. Phytohormones, MS solid basal medium, sucrose and agar were purchased from Himedia ltd Mumbai. Asiaticoside as authentic sample was procured from Sigma Aldrich.

### Collection of plant material

*Centella asiatica* (L.) Urban plant material was collected from the University of Agricultural Sciences, Dharwad, India, in the month of May. A voucher specimen bearing number 04PG351 was deposited in the Herbarium of K.L.E.S College of Pharmacy, Hubli.

### Preparation of explants for callus initiation

Plant material collected was first made free from dust and soil and then washed under running tap water followed by distilled water. The plant material was soaked in 150 mg/l bavistin for 25 to 30 mins and was taken to aseptic area. Leaf explants were excised from natural plant and

were surface sterilized with 70% v/v alcohol for 30 seconds, rinsed with sterile distilled water for 2 to 3 times. Further they were surface sterilized with 0.1% w/v mercuric chloride solution for 2 to 3 minutes and rinsed with sterile distilled water for 2 to 3 times.

**Inoculation of explants for callus initiation**

For callus formation the sterile explants were placed aseptically in culture tubes containing 12 ml of sterile MS solid basal medium with ventral side of leaf explant facing the medium, media was supplemented with different concentrations & combinations of hormones like naphthalene acetic acid (NAA), kinetin (Kn) and benzyl amino purine (BAP), 30 g/l sucrose and 8 g/l agar. The cultures were incubated (16 h photoperiod) under fluorescent light (3000 lux) (all media had been adjusted to pH 5.8 and autoclaved for 20 min at 121$^{o}$C) at 25 ± 2$^{o}$C. After culturing for 30 days, portions of callus from explants were transferred and subcultured to the same medium with similar combinations and concentrations of hormones. Cultures were harvested at the interval of 30 days, 60 days & 90 days after processing and dried.

**Growth kinetics of callus:**

Growth studies were carried out on fresh mass (FM) basis. The fresh mass of the calluses were measured after removing agar. Biomass increase on fresh mass basis is expressed as the percent increase each over the initial value. Three replicates were used to assess the growth condition and to measure the callusing rate (%). Data was collected after 5, 10, 15, 20, 25, 30 & 35 days of inoculation for growth of callus. Each mean data collected was based of 10 replicates repeated 3 times each.

**Extraction of callus**

For the determination of asiaticoside, air dried whole plant material and calluses were separately subjected for extraction with methanol at room temperature. Extraction was carried out, by refluxing the samples for 20 minutes with 30 ml methanol on water bath and the procedure was repeated twice with fresh methanol. All the methanolic extracts were filtered, combined, concentrated, vacuum dried, weighed and used for identification of asiaticoside by TLC.

**Identification and estimation of asiaticoside in callus**

For TLC analysis the crude extracts were dissolved in small volume of methanol & the methanolic extracts co-chromatgraphed with authentic sample (Asiaticoside as authentic sample) to obtain the chromatograph using a solvent system ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:27). Asiaticoside was detected and identified by spraying with Libermann burchard spray reagent & by comparing the $R_f$ values[19].

Quantitative determination of asiaticoside was performed on Shimadzu 2010 system, by injecting 10µl of each standard solution and extracts using RP C-8 column (250x4mm Lichrocart, 5µ Lichrosphere). The mobile phase (Acetonitrile:Water 3:7) was pumped isocratically at a flow rate of 1ml/min and asiaticoside was UV detected at 210.4nm. The peaks were identified by comparing the retention time of the sample with authentic sample (Asiaticoside as authentic sample)[20]. The quantitative assessment of purified fraction of asiaticoside in callus culture by HPLC was based on comparison of peak areas of the standard and the sample, 3 replicates were taken & repeated 3 times each.

**RESULTS AND DISCUSSION**

**Initiation of callus:**

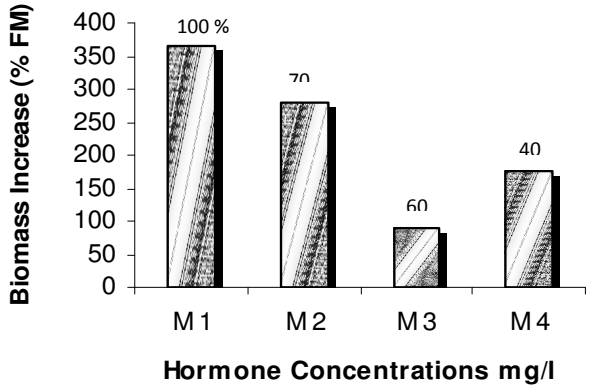
Callus response was investigated using the leaf explants of *Centella asiatica* (L.) Urban by inoculating these explants in MS (murashige and skoog) basal medium[21] supplemented with different concentrations & combinations ratio of auxin like naphthalene acetic acid (NAA) & cytokinins like kinetin (Kn) and benzyl amino purine (BAP). Initiation of callus was found in almost all the concentrations & combination ratio of hormones used. The results of callusing achieved are shown in Table 1. Best result for callus initiation (100% of explants) and maximum biomass increase was found with M1 (1 mg/l NAA + 2 mg/l BAP) (Fig.1). The callus turned light green & friable after 30days.

**Growth kinetics of callus**

Growth studies were carried out on fresh masses (FM). The growth curve revealed that the undifferentiated cells were in the exponential phase up to 60$^{th}$ day of incubation (data not show). The fresh mass of the calli increased after 30 days of incubation up to 60 days by 364 % in comparison with initial fresh mass(Fig.1).

**Table 1:** Effect of hormone concentration on callusing

| Media | Hormone Concentration in mg/l | Period of callus initiation (days) | Nature of callus |
|---|---|---|---|
| M1 | 1 NAA + 2 BAP | 15 days | Dark green nodular friable callus |
| M2 | 0.5 NAA + 2.5 KINETIN | 20 days | Green nodular callus |
| M3 | 1 NAA + 0.5 KINETIN | 25 days | Light green nodular callus |
| M4 | 1 NAA + 1 KINETIN | 25 days | Light green callus with brown patches |




M1= NAA 1 mg/l + BAP 2 mg/l,
M2= NAA 0.5 mg/l + Kinetin 2.5 mg/l,
M3 = NAA 1 mg/l + Kinetin 0.5 mg/l and
M4 = NAA 1 mg/l + Kinetin 1 mg/l.

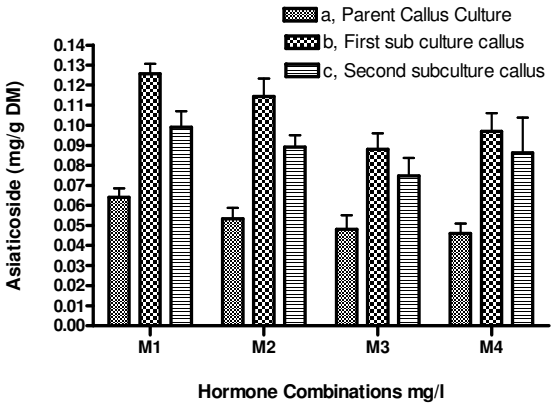
**Figure 1:** Effect of Auxin (NAA) and Cytokinins (BAP, Kinetin) plant hormones in combination on biomass increase and Frequency of callus induction. The numbers on above the parentheses columns indicate frequency of callus induction (%)

### Identification and estimation of asiaticoside in callus

TLC studies of the callus extracts upon co-chromatography with authentic samples (standard asiaticoside) showed brown spots upon treatment with Libermann burchard spray reagent. The $R_f$ values of the extracts were found to be the same as that of authentic samples ($R_f$ values of standard asiaticoside 0.20) indicating the presence of asiaticoside.

Asiaticoside in methanolic extracts of callus cultures was identified by comparing the HPLC profiles and it was found that the retention time ($R_t$) of the authentic sample & that of callus cultures were 8.5 min.

HPLC quantification & effect of subculturing on accumulation of asiaticoside in methanolic extracts of parent callus culture, first subcultured callus & second subcultured calluses grown on MS media supplemented with auxin (NAA) and cytokinins (BAP, Kinetin) plant hormones in combination are reported in Fig.2.




M1= NAA 1 mg/l + BAP 2 mg/l
M2= NAA 0.5 mg/l + Kinetin 2.5 mg/l
M3 = NAA 1 mg/l + Kinetin 0.5 mg/l and
M4 = NAA 1 mg/l + Kinetin 1 mg/l.

**Figure 2:** Effect of subculturing & plant hormones auxin (NAA) and cytokinins (BAP, Kinetin) in combination on asiaticoside accumulation quantified by HPLC in methanolic extracts of parent callus culture (30days), first subcultured callus (60 days) & second subcultured calluses (90 days).

Highest asiaticoside content in leaf calluses, 0.125 mg/g dry mass was obtained in the first subcultured callus with MS medium supplemented with 1mg/l NAA and 2 mg/l BAP (M1) of 60 days, but decreased in the longer culture period to 0.099 mg/g Dry Mass, [M1c]. Asiaticoside yield corresponding to other hormone combinations and culture periods fluctuated between 0.046 and 0.114 mg/g dry mass (M4a and M2b).

## CONCLUSION

Highest asiaticoside content was detected in first subculture calluses with MS medium supplemented with 1mg/l NAA and 2 mg/l BAP (M1). As first subculture medium, M1 also produced a good yield of asiaticoside. However the asiaticoside content was higher in whole plant 0.179 mg/g dry mass in comparison to the first subculture leaf calluses 0.125 mg/g dry mass.

Generally the present results agree with those of Toker et al, 2003[22], first subcultured callus of *Ecballium elaterium* accumulated more of the secondary metabolites compared to parent callus culture & second subcultured calluses. The present findings which reveal the presence of asiaticoside in leaf callus cultures are in consistent with the observations of Nath & Buragohain, 2005, who detected asiaticoside in callus & cell suspension culture of *Centella asiatica* (L.) Urban of Indian origin, but the quantity of accumulation is less than that found in naturally grown plant which is in contrast to the observations made by Nath & Buragohain, 2005. In contrast to the present observations & those of Nath & Buragohain, 2005, Kim et al, 2004 & Aziz et al, 2007, 0reported absence of asiaticoside in undifferentiated cells of Korean *Centella asiatica* (L.) Urban. This study has provided a baseline to obtain maximum production of asiaticoside from first subcultured callus. Further studies for adopting these conditions as well as to study the effect of various precursors in increasing the yield of asiaticoside in callus and suspension culture are in progress.

## ACKNOWLEDGMENTS

Authors are thankful to Dr. B Fakruddin and Dadapeer peerzade of University of Agriculture Sciences, Dharwad for their valuable guidance and help.

## REFERENCES

[1] Anonymous. The wealth of India: A Dictionary of Indian Raw Materials & Industrial Products, First supplement series (Raw materials). Vol.3. New Delhi: CSIR (Publications and information directorate);1992.

[2] Dutta T, Basu UP. Isothankunic acid – a new triterpene acid from *Centella asiatica* (Urban). Bull. Nat. Inst. Sci. India. 1968;37:178-184.

[3] Singh B, Rastogi RP. A reinvestigation of the triterpenes of *Centella asiatica* III. Phytochemistry. 1969; 8: 917-921.

[4] Aziz ZA, Lowe RC. Micropropagation of medicinal species of *Centella asiatica.* J Exp Biol. 1999;59(32-39):19-20.

[5] Banerjee S, Zehra M, Kumar S. *In vitro* multiplication of *Centella asiatica,* a medicinal herb from leaf explants. Current Science. 1999;7:147- 148.

[6] Josekutty PC. Callus culture and micropropagation of *Hydrocotyle asciatica [Centella asiatica* (L.)Urban], a medicinal plant. Int.J.Exp.Bot. 1998; 63(1-2):275-278.

[7] Nath S, Buragohain AK. *In vitro* method of propogation of *Centella asiatica* (L) Urban by shoot tip culture. J Plant Biochem Biot. 2003;12:167-169.

[8] Patra A, Rai B, Rout GR, Das P. Successful plant regeneration from callus cultures of *Centella asiatica* (Linn.) Urban. J. Plant Growth Regul.1998; 24:13-16.

[9] Shashikala CM, Shashidhara S, Rajashekharan PE. Invitro regeneration of *Centella asiatica* L. Plant Cell Biotec Mol Bio. 2005; 6(1&2): 53-56.

[10] Tiwari KN, Sharma NC, Tiwari V, Singh BD. Micropropagation of *Centella asiatica* (L), a valuable medicinal herb. Plant Cell Tiss Org. 2000; 63:179-185.

[11] Paek YW, Hwang SJ, Park BH, Hwang B. Multiplication and transformation of medicinal plants for the production of secondary metabolites, establishment of hairy root cultures of *Centella asiatica.* J Plant Biol.1996; 39(3): 161-66.

[12] Kim OT, Bang KH, Shin YS, Lee MJ, Jung SJ, Hyun DY, Kim YC, Seong NS, Cha SW, Hwang B. Enhanced production of asiaticoside from hairy root cultures of *Centella asiatica* (L.) Urban elicited by methyl jasmonate. Plant Cell Rep. 2007; 26: 1941-1949.

[13] Kim OT, Kim MY, Hong MH, Ahn JC, Hwang B. Stimulation of asiaticoside accumulation in the whole plant cultures of *Centella asiatica* (L.) Urban by elicitor. Plant Cell Rep. 2004; 23:339-344.

[14] Paramageetham C, Babu GP, Rao JVS. Somatic embryogenesis in *Centella asiatica* L. an important medicinal & neutraceutical plant of India. Plant Cell Tiss Org. 2004;79: 19-24.

[15] Rao KP, Rao SS, Sadandandan M. Tissue culture studies of *Centella asiatica.* Indian J Pharm Sci. 1999;29:393-94.

[16] Nath S, Buragohain AK. Establishment of callus & cell suspension cultures of *Centella asiatica*. Biol Plantarum. 2005;49(3): 411-413.
[17] Aziz ZA, Davey MR, Power JB, Anthony P, Smith RM, Lowe KC. Production of asiaticoside & madecassoside in *Centella asiatica* in-vitro and in-vivo. Biologia Plantarum. 2007;51(1): 34-42.
[18] Mangas S, Moyano E, Osuna L, Cusido RM, Bonfill M, Palazón J. Triterpenoid saponin content and the expression level of some related genes in calli of Centella asiatica. Biotechnol Lett. 2008; 30(10):1853-9.
[19] Singh B, Tyagia A, Agrawal S. HPTLC determination of Asiaticoside in *Centella asiatica* (L). Indian Drugs. 2005; 42(2): 87-89.
[20] Anonymous. Indian Herbal Pharmacopoeia. Revised New Edition. Mumbai: Indian Drugs Manufacturers Association; 2002.
[21] Murashige T, Skoog F. A revised medium for rapid growth & bioassays with tobacco tissue cultures. Physiol. Plant. 1962;15: 473-497.
[22] Toker G, Memisoglu M, Toker MC, Yesilada E. Callus formation and Cucurbitacin B accululation in *Ecballium elaterium* callus cultures. Fitoterapia. 2003; 74: 618-623.