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Production and Optimization of Asiaticoside in Callus Cultures of *Centella asiatica* L. Urb., Family: Umbelliferae

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Abstract

This work has been chosen to make an effort to increase the content of asiaticoside, one of the principal constituents of *Centella asiatica* a weakly aromatic smelling plant utilized as a medicine in India since time immemorial for various ailments, through callus culture, using different hormone combinations than the ones already reported. Static cultures of *C. asiatica* were established from different explants [leaves and stem tips]. Media and growth hormone concentration was optimised for the cultures obtained from the different explants which were taken for the initiation of callus culture in equal batches. A maximum growth was observed in 12 week old callus culture derived from leaves supplemented with NAA [1.0 mg/L], Kinetin [0.5mg/L]. Out of all the combinations 12-week-old callus cultures developed on medium supplemented with NAA [1.0 mg/L], Kinetin [0.5 mg/L] was found to show maximum yield of asiaticoside in comparison to callus cultures grown on medium supplemented with other hormonal combinations.

Key words: *Centella asiatica*, NAA, Kinetin, callus, HPTLC, asiaticoside

Introduction

In common with most traditional phytotherapeutic agents, *Centella asiatica* is claimed to possess a wide range of pharmacological effects, being used for wounds healing, mental disorders, atherosclerosis, fungicidal, antibacterial, antioxidant and anticancer purposes. *C. asiatica* has also been reported to be useful in the treatment of inflammations, diarrhea, asthma, tuberculosis and various skin lesions and ailments like leprosy, lupus, psoriasis and keloid (Suguna *et al*.,1996; Oyedeji *et al*.,2005; Jayashree *et al*.,2003; Wong *et al*.,2006; Hussein *et al*.,2007; Wattanathorn *et al*.,2008).

Triterpene is a major and the most important component of *C. asiatica*, regarded as a marker constituent in terms of quality control. The triterpenes obtained from *C. asiatica* are mainly pentacyclic triterpenic acids and their respective glycosides, belonging to ursane or oleanane type including asiatic acid, asiaticoside, madecassic acid, madecassoside, brahmoside, brahmic acid, brahminoside, thankuniside, isothankuniside, centelloside, madasiatic acid, centic acid, cenellic acid, betulinic acid, indocentic acid, etc. (Brinkhaus *et al*.,2000; Siddiqui *et al*.,2007).

In view of its versatile medicinal properties, the requirement of *C. asiatica* in pharmaceutical industries has been sharply increasing, thus leading to the over exploitation of this herb. It has already been listed as threatened species by the International Union for Conservation of Nature and National Resources [IUCN] and an endangered species (Cheng-Jian and Lu-Ping, 2007). Therefore application of tissue culture approaches for rapid multiplication of elite clones and germplasm conservation is of vital importance In recent years, *C. asiatica* regeneration has been achieved by using leaf derived callus, stem segments and nodal segments as explants, shoot tip and suspension cultures, providing a prerequisite for the generation of bioactive secondary products from this species (Banerjee *et al*.,1999). Furthermore, there is a wide scope of application in increasing the content of asiaticoside, the principal constituent responsible for the varied medicinal effects.

The present work, to our knowledge, reports for the first time, a simple and rapid method for the *in vitro* production and optimization of the triterpenoid saponin glycoside, asiaticoside in callus cultures of various explants [stem and leaf] in *Centella asiatica*.

Materials and Methods

Collection and sterilization of plant material

The young leaves and stem tips of *C. asiatica* were collected [September-January] from medicinal garden of "Manipal College of Pharmaceutical Sciences", Manipal, washed thoroughly with tap water and followed by washing with Tween-20 [10 % v/v], for 2-3 minutes. The residue of surfactant solution was completely removed with washing under tap water followed by distilled water. Then different explants were surface sterilized with ethanol [70 % v/v] for 1 minute, followed by mercuric chloride [0.1% w/v] for 4 - 5 min, under laminar flow. After this, the explants were washed thrice with sterile double distilled water and transferred onto the solidified medium [culture tubes] for establishment of static cultures. The work was carried out at Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India during the months of September through January, 2003 - 04.

Chemicals

2,4-D [2,4-dichloro phenoxy acetic acid], Gamborg media, Indole acetic acid, Kinetin, Agar, BAP [benzyl amino purine], MS Media [Murashige and Skoog media], Naphthalene acetic acid and White's media were procured from Himedia Laboratory Ltd, Mumbai, India. Asiaticoside from Regional Research Laboratory, Jammu, India. Acetone, Chloroform, Petroleum Ether [60-80], Sucrose, Dichloromethane, Mercuric chloride, Methanol and Sodium Hydroxide from Merck Ltd., Mumbai.

Establishment of Callus Culture and Optimization of Asiaticoside Content

The surface sterilized explants of *Centella asiatica* were transferred aseptically onto MS / B_5 / White's media in culture tubes. The tubes were plugged properly, covered with aluminum foil, and incubated in dark at 25 ± 2 °C. The cultures were maintained at 25 ± 2 °C by sub-culture of fragile globular callus pieces onto corresponding media supplemented with several combinations and concentrations of plant growth regulators, at an interval of 4 weeks.

Of the explants [leaves and stem tips] taken for the initiation of callus culture in equal batches, only leaf explants grown on MS medium, showed good response in initiation of callus cultures. Hence the leaves were taken as explants for further studies.

Measurement of growth and frequency of callus induction

The growth indices of the cultures were determined on dry weight basis. The growth index [G.I.] of the cultures was calculated by using the following formula.

$$Growth\ index = \frac{Final\ weight\ of\ biomass}{Initial\ weight\ of\ biomass}$$

The frequency of the callus induction was calculated based on the percentage of explants capable of being developed into the callus.

Extraction of callus culture

2g of callus was percolated with methanol; the resulting extract was then refluxed with acetone for 1 h. The acetone extract was then evaporated to dryness and the residue was dissolved in 10 ml methanol [HPLC grade] and this was used for analytical purpose.

Analysis

Thin layer Chromatography [TLC]

The glass plate coated with silica gel-G [Merck, India] was used with n-butanol: ethyl acetate: water [4:1:5] as solvent system and standard solution of asiaticoside [100µg/ml].

High-Performance Thin Layer Chromatography [HPTLC]

Quantitative estimation of asiaticoside in the test samples was done by HPTLC [CAMAG, Switzerland] analysis under the following conditions: Stationary Phase: HPTLC pre-coated plates silica gel 60 F_{254} [Merck KgaA, 64271 Darmstadt, Germany] of thickness,200µm. The samples were applied using a CAMAG Linomat V applicator and developed in a CAMAG Twin Trough Chamber.

Densitometric scanning was then done using CAMAG TLC Evaluation software Win CATS V4, 06, S/N: 0606A003/SC 3 V1.14, S/N: 060318 and detected using Deuterium lamp in Absorption/Reflection measurement mode.

Standard Preparation

A solution of asiaticoside [1.0 mg/ml] was prepared in methanol. It was further diluted to yield the final concentration of 10, 20, 40, 60, 80, 100, 200, 300, 400, 500 and 1000 µg/ml.

Plant extract

5g of the dried leaves of *C. asiatica* was extracted with methanol for 5 h. The resulting extract was then concentrated to 50 ml. 1ml of the resulting solution was again diluted to 10 ml.

Procedure

The test sample & standard dilutions were passed through 0.22-micron filter [PVDF] using a syringe and they were then applied as bands onto the HPTLC plate using a Linomat 5 applicator. The plate was then kept in the twin trough chamber for 15 min, for saturation of the plate after which it was kept in the solvent system [n-butanol: ethyl acetate: water [4:1:5]] for development. After developing, the plate was dried thoroughly and then scanned at 205 nm. The amount of asiaticoside in test sample was then determined by comparing peak areas of the standard and test samples, respectively.

Results and Discussion

MS media supplemented with sucrose [3 % w/v] and two different combinations of [i] NAA [1.0 mg/L], Kinetin [0.5 mg/L] and [ii] NAA [0.1mg/L], Kinetin [1.0mg/L], were found suitable for the initiation of the callus [Table 1]. MS media with NAA [1.0 mg/L] and Kinetin [0.5mg/L] combination induced the callus formation in the 4th week; callus was white in colour, soft and fragile [Figure 1].

Table -1: Selection of suitable growth hormones

S. No.	Hormone Combination [mg/L]							Response
	2,4-D	Kn	Dc	NAA	Pc	BA	Zn	
1	2	-	-	-	-	-	-	-
2	2	0.2	-	-	-	-	-	+
3	4	0.2	-	-	-	-	-	-
4	-	**0.5**	-	**1**	-	-	-	+ +
5	-	0.5	-	2	-	-	-	-
6	2	0.5	-	-	-	-	-	-
7	4	0.5	-	-	-	-	-	-
8	4	-	-	-	-	-	-	-
9	-	**1**	-	**0.1**	-	-	-	++
10	-	0.5	-	0.5	-	-	-	+
11	0.2	1	-	-	-	-	-	-
12	-	1	-	0.5	-	-	-	-
13	-	1	-	1	-	-	-	-
14	-	-	0.5	-	-	-	-	-
15	-	0.5	0.5	-	-	-	-	-
16	-	0.5	-	-	1	-	-	-
17	-	1	-	-	2	-	-	
18	-	-	-	-	2	-	0.5	-
19	1	0.5	-	-	-	-	-	+
20	4	0.5	-	-	-	-	-	-
21	-	-	-	0.5	-	1	-	-

Abbreviations:- 2,4-D: 2,4 - dichlorophenoxy acetic acid; Kn: Kinetin; Dc: Dicamba; NAA: Naphthalene Acetic Acid; Pc: Picloram; BA: Benzyl adenine; Zn: Zeatin.
+ : Medium response; + +: Good response.

Table- 2: Frequency of callus induction

Media	Auxin	Cytokinin	% Induction*
MS	2,4-D [1.0 mg/L]	Kinetin [0.5 mg/L]	53 %
MS	NAA [1.0 mg/L]	Kinetin [0.5 mg/L]	92%
MS	NAA [1.0 mg/L]	Kinetin [1.0 mg/L]	81%

Average of 10 inoculations.

Table- 3: Content of asiaticoside in callus cultures using different phytohormone combinations

S.No.	MS media supplemented with NAA [1.0mg/L], Kinetin [0.5 mg/L]		MS media supplemented with NAA [0.1mg/L], Kinetin [1.0mg/L]	
	Age	Percentage of asiaticoside [%]*	Age	Percentage of asiaticoside [%]*
1	4 Week	0.003	4 Week	0.0019
2	8 Week	0.0041	8 Week	0.0026
3	12 Week	0.0047	12 Week	0.0030
4	Plant extract	0.002	Plant extract	0.002

*Average of three readings

Table -4: Growth indices of callus cultures of *Centella asiatica*

Media	4 Week*	8 Week*	12 Week*
NAA [0.1], Kinetin [1.0]	0.5	1.34	2.02
NAA [1.0], Kinetin [0.5]	0.6	1.71	2.13

* Average of three readings

Figure 1: 4 week old callus from *Centella asiatica* leaf explant

Figure 3: 12 week old callus from *Centella asiatica* leaf explant

Figure 2: 8 week old callus from *Centella asiatica* leaf explant

Figure 7: Profuse rooting in 12 week old callus from *Centella asiatica* leaf explant

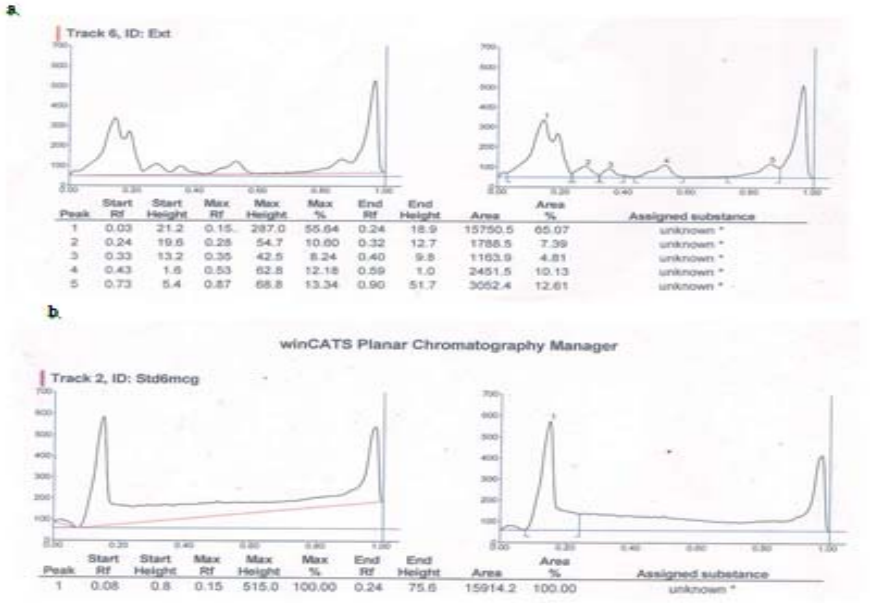

Figure 4: HPTLC spectra of the methanolic extract of *Centella asiatica* in comparison with standard compound asiaticoside. a] Methanolic extract of *Centella asiatica* and b] Asiaticoside

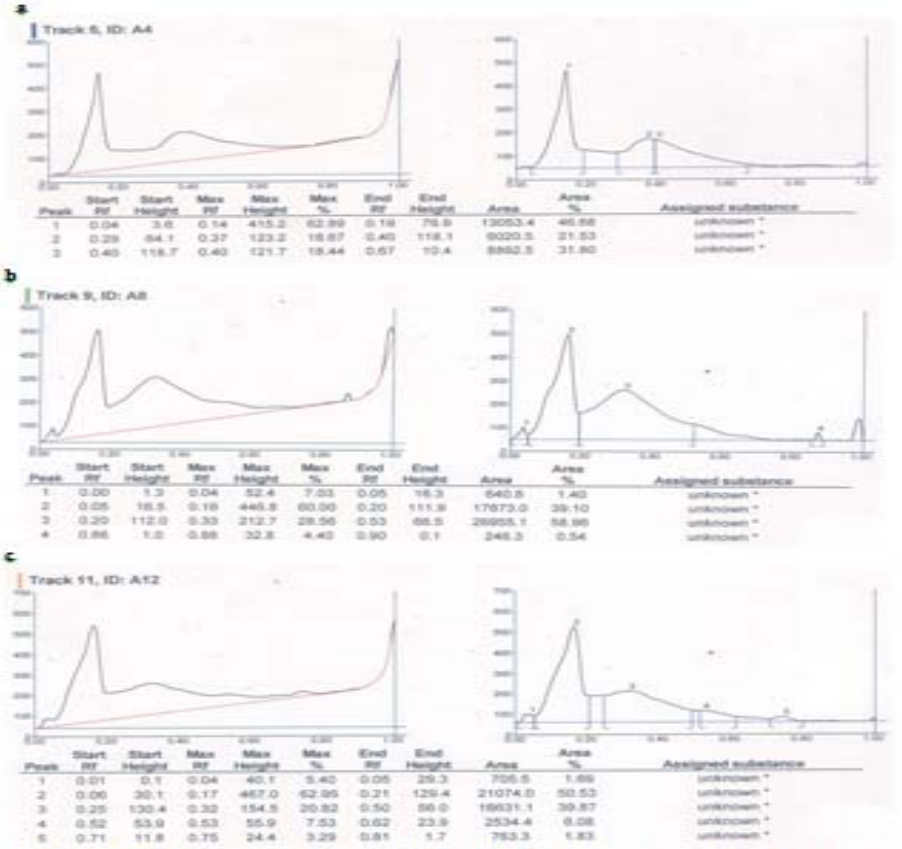

Figure 5: HPTLC spectra of 4, 8 and 12 week old calli of *Centella asiatica* using a combination of NAA [1.0 mg/L] and Kinetin [0.5 mg/L]. a] 4 week; b] 8 week and c] 12 week.

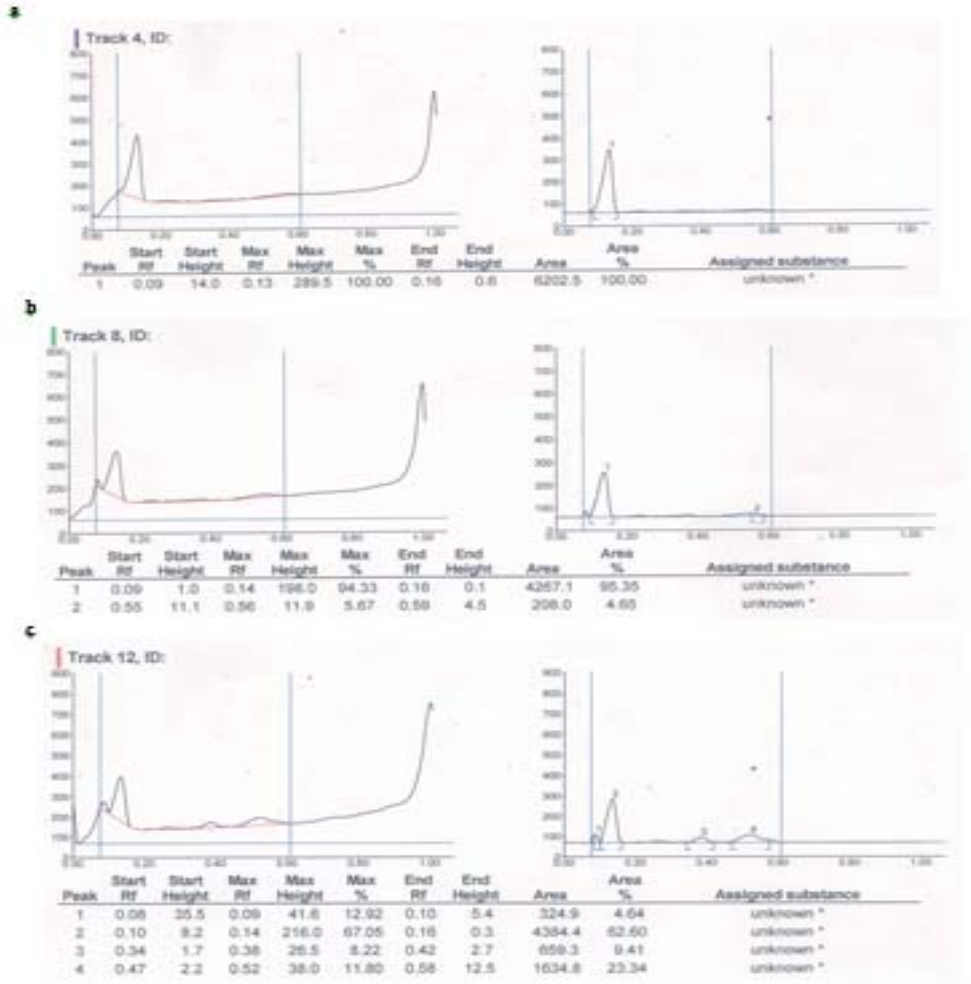

Figure 6: HPTLC spectra of 4, 8 and 12 week old calli of *Centella asiatica* using the combination of NAA [1.0 mg/L] and Kn [0.5 mg/L]. a] 4 week; b] 8 week and c] 12 week.

In MS media with NAA [0.1 mg/L] and Kinetin [1.0 mg/L] combination the callus induction was delayed, starting on the 46th day [8 week old callus, Figure 2]. The callus was light brownish in colour and hard compared to the previous combination. There was no significant difference in callus induction frequencies with different hormonal concentration [Table 2]. It was also found that young leaves were more suitable than the mature ones for initiation of callus cultures. This may be due to the fact that they are delicate and show more active growth than mature leaves. Growth indices for different groups of callus culture studied showed that a maximum growth was observed in 12 week old callus culture derived from leaves supplemented with NAA, 1.0 mg/L and Kinetin, 0.5mg/L [Figure 3, Table 4]. A similar study reveals the optimisation of callus culture of milk thistle, *Sylibum marianum*, for biomass production as a potential source of milk clotting peptidases (Cecilia *et al*., 2006). On TLC analysis, the callus extracts of *Centella asiatica* of different age groups upon co-chromatography with authentic sample on silica gel G plates with n-butanol: ethyl acetate: water [4 : 1: 5] as solvent system revealed a violet spot [R_f value-0.14]. The detection of asiaticoside was observed in different age groups of callus cultures developed on MS media supplemented with NAA [1.0 mg/L], Kinetin [0.5 mg/L] & on callus cultures derived from MS media supplemented with NAA [0.1 mg/L], Kinetin [1.0 mg/L].

Quantitative estimation of asiaticoside in the test samples were done by comparing the peak areas of the standard and test samples, respectively. The extracts obtained from different age groups

of callus cultures were analyzed by HPTLC [Figures 4 – 6]. Out of all the combinations 12-week-old callus cultures developed on medium supplemented with NAA [1.0 mg/L], Kinetin [0.5 mg/L] was found to show maximum yield of asiaticoside in comparison to callus cultures grown on medium supplemented with other hormonal combinations [Table 3]. Rao *et al*.,(1999) has described the tissue culture studies of *Centella asiatica* using NAA [2.0 mg/L] and Kinetin [0.5 mg/L] without any supplements. Other authors have reported micropropagation of *C. asiatica* using leaf explants (Nath *et al*.,2000). Owing to the high content of auxin in our particular combination, profuse rooting was also observed [Figure 7]. These results suggest that MS medium supplemented with sucrose [3 % w/v] and NAA [1.0 mg/L], Kinetin [0.5 mg/L] was found to be optimal for the bio-production of asiaticoside. Recently some authors have established enhanced production of asiaticoside via hairy root culture of *C. asiatica* using an elicitor, methyl jasmonate Kim *et al*.,2007). Hairy root cultures are expensive and involve laborious procedures, whereas callus cultures are economical, simple and rapid.

Concluding Remarks

The type of explants from which the callus was obtained could be of importance for the production and optimization of asiaticoside, an important constituent of *Centella asiatica*. Further investigations are required to see whether this callus culture technique could be useful for large scale multiplication of this important medicinal plant, increase the content of other secondary metabolites as well as for its *ex-situ* conservation.

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