Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2582-2845.8982

ISSN: 2582 – 2845

Ind. J. Pure App. Biosci. (2023) *11*(3), 9-16

Indian Journal of Pure & Applied Biosciences

Peer-Reviewed, Refereed, Open Access Journal

Research Article

Quantification and FTIR Analysis of Precursor Mediated Alkaloid Production in Cell Suspension Culture of *Gnidia glauca* (Fresen.) Gilg

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Received: 11.02.2023 | Revised: 9.04.2023 | Accepted: 16.04.2023

ABSTRACT

The current investigation focuses on developing a reproducible protocol for callus induction and for studying the precursor L-phenylalanine impact on product enhancement for alkaloid production and measurement of pH. Alkaloids are nitrogen-containing compounds; individual alkaloids were purified and synthesized commercially as excellent substances with recognized and experimentally verified pharmacological attributes because of their antimicrobial, anticancer properties and other therapeutic values. The current studies explore the impact of Precursor on alkaloid production by cell suspension culture in G. glauca, resulting in an increase in the cell growth observed and quantified by HPLC, TLC analysis, and the presence of functional groups identified by FTIR profiling. Additionally, this finding offers a protocol for biomass production of alkaloids from medicinally important endangered plants aiding in the conservation of the plants as well as producing commercially valuable secondary metabolites.

Keywords: *G. glauca (Fresen.) Gilg, Alkaloids, HPLC, TLC, FTIR.*

INTRODUCTION

Plant cell and tissue culture technologies were first established in the late 1960s as a vital tool for the production of natural products. *Invitro* propagation methods are one of the alternative strategies for conserving medicinally important endemic plant species and synthesizing plant secondary metabolites. The secondary metabolites, such as terpenoids, alkaloids, saponins, phenolic acids, and other metabolites, play a pivotal role in plant defence mechanisms (Nowakowaska et al., 2019). The Thymelaeceae family encompasses 45 genera and 800 endemic and threatened plant species with important biological value, the *Gnidia glauca* (Fresen.)

Cite this article: Ruksana, F., Kumar, V., Vishala, E., Poornima, D. V., Anuradha, C. M., Prathap, H. M., Savitharani, M., & Manjunatha, T. (2023). Quantification and FTIR Analysis of Precursor Mediated Alkaloid Production in Cell Suspension Culture of *Gnidia glauca* (Fresen.) Gilg, *Ind. J. Pure App. Biosci. 11*(3), 9-16. doi: http://dx.doi.org/10.18782/2582-2845.8982

Gilg is an endangered medicinal shrub that belongs to the Thymelaeceae family with potent biological activity (Bhandurge et al., 2013). Plant commercialization is now dependent on successful *invitro* propagation; throughout the world, numerous commercial and national institutions are in support of in *vitro* plant cell and tissue cultures for rapid plant multiplication, pathogen extermination, genetic manipulation, and bioactive plant metabolite production (Kumar Reddy et al., 2011).

Plants are ample sources of secondary metabolites abundant in around 3000 different types of alkaloids. Indole alkaloids, also known as monoterpenoid indole alkaloids or terpenoid indole alkaloids, are derived from tryptamine, tropane and steroidal alkaloids (Panda et al., 1997). Alkaloids are nitrogen-containing compounds; since the 20th century, people have used plant alkaloid extracts as herbal remedies for over a thousand years. However, individual alkaloids were purified and synthesized commercially as excellent substances with recognized and experimentally validated therapeutic potential (Zalak Sharma et al., 2022).

This study examines the growth of alkaloids in cultured cells and the factors that might influence both synthesis and storage, which may, in certain circumstances, vary from those that occur in plants. Also, it reports that in vitro cell cultures seem to be specific structures, in contrast to the naturally occurring secretory cells that exist in a whole plant (Ratnadewi, 2017).

MATERIALS AND METHODS

Chemicals

L-phenylalanine was purchased from Sigma-Aldrich, HPLC (High-performance liquid chromatography) grade solvents (Himedia, India; SRL, India), and the analytical grade solvents were used and freshly prepared when required, glass wares procured from (Borosil, India).

Cell suspension culture establishment

In the present investigation, precursor L-phenylalanine (200 µM) was dissolved in 1N sodium hydroxide (NaOH), and prior to adding it to the media, the prepared precursor solution was filter sterilized. Precursor concentrations (50, 100, and 200µM) were optimized individually for the enhancement of alkaloids in cell suspension cultures of *G, glauca,* L-phenylalanine 200 µM was the optimal precursor concentration and their respective combinations. A white friable callus of *Gnidia glauca* was induced on solid Murashige and Skoog (MS medium, 1962). Containing 2 mg/L 2, 4-D (2, 4-dichloro phenoxy acetic acid) and 0.2 mg/L isopentenyl adenine (2iP) for cell suspension culture establishment (Hari et al., 2018), the cells were collected by filtration at each culture duration 6, 12, 18, and 24 days respectively, for the measurement of cell growth, pH, and quantitative profiling of alkaloids.

UV-VIS (UV-Visible spectrophotometric) analysis of alkaloid

UV- Visible spectrometric analysis is a widely employed analytical technique for the Quantification of chemical compounds due to their biochemical and molecular properties. (Lozada ramirez et al., 2021). The Quantification of alkaloids was evaluated by using a UV-visible spectrophotometer (systronics119). The phenylalanine-treated 200 µM 12th day cell suspension cultured samples were prepared in 80% methanol and subjected to UV- Visible spectrometric analysis. The results were recorded as a unique and distinct absorption spectrum.

RP-HPLC analysis of alkaloid Quantification

HPLC is the most comprehensive technique for the separation of individual components that occur in the plant (Thirumal et al., 2017). The alkaloid content of *G. glauca* in cell suspension cultures was subjected to HPLC Agilent-1260 system integrated with DAD (diode array detector), C18 column, and methanol: water (35:65) was the mobile phase with 1% acetonitrile, at 30ºC column temperature, and 1ml/min flow rate, with 20 µl injection volume, colchicine, and caffeine was used as alkaloid standards, baseline resolution was attained for all the analytes under the

proposed analytical condition (Oliveira et al., 2019).

TLC analysis (Thin layer chromatography)

TLC (Thin layer chromatography) is widely employed as a fingerprint technique for natural product extract analysis and is applicable for identifying and quality control drug preparations and other applications in pharmaceutical industries (Sharif et al., 2016). Separation of phenolic compounds in *G. glauca* was achieved by TLC (thin layer chromatography) analysis with PRISMA optimized solvent system. The optimized mobile phase was n-butanol: acetic acid: dichloromethane (5:1:4), and the samples were prepared in 80% methanol. Colchicine was used as an alkaloid standard, and The RF value was calculated by following the formula

$$RF = \frac{\text{Distance moved by the solute front}}{\text{Distance moved by the solvent front}}$$

Fourier transform Infrared (FTIR) analysis

The most effective method for functional group analysis in a particular sample is FTIR spectroscopy Bruker alpha eco ATR (attenuated total reflectance) optics, Germany. Fourier-transformed infrared spectrometer (FTIR) spectra were employed to determine the structural characteristics of the selected *G. glauca* plant suspension cultured callus sample (sahayaraj et al., 2015). Since the suspended samples enhance the intensity of spectral bands while minimizing interference from water and other organic solvents, they were used in the FTIR analysis of L-phenylalanine treated at a concentration of 200 µM the 12th day (Kamnev et al., 2021). FTIR spectra were captured with an ATR and a reflection crystal (ZnSe). With OPUS (v.5.5, Bruker Optics, Germany at 20º C for processing, and frequencies range between 4000 to 600 cm^{-1}.

Statistical analysis

Each experiment was performed individually thrice under identical circumstances. The probability and significance difference between paired samples was estimated by using a t-test in the Microsoft Excel program.

RESULTS AND DISCUSSION

Determination of cell growth and measurement of pH

The measurement of cells growth was initiated at 6th-day old inoculum, in L-phenylalanine treated suspension cultured callus sample shows increased biomass production of alkaloids at a concentration of 200 µM, 1.43, 1.13, and 0.78 folds more in contrast to the untreated (control) sample and has significance ($p=0.035$) at level $p<0.05$, which was optimized, and results of cell growth were measured in terms of dry cell weight (DCW) are illustrated in table 1 and fig.1. In Precursor treated samples had a pH that has been lesser compared to control cultures. Furthermore, correlation analysis revealed a significant positive correlation between precursor concentration as well as the pH, between cell growth and pH compared to control cultures ($r=0.50$); in contrast to control cultures, this study reveals that the precursor concentration may positively affect alkaloid production in *G. glauca* (Fresen.) Gilg.

Table 1: Precursor effect on cell growth in *G. glauca* cell suspension culture

Sample	**measurement of Cell growth in terms of DCW (dry cell weight)**			
Control	**6th day**	**12th day**	**18th day**	**24th day**
	1.8 ± 0.025 mg/g DCW	1.05 ± 0.045 mg/g DCW	0.78 ± 0.055 mg/g DCW	0.60 ± 0.065 mg/g DCW
L-phenylalanine treated suspension cultured callus (200M+ 12th day)	2.3 ± 0.0124 mg/g DCW	3.15 ± 0.0230 mg/g DCW	2.0 ± 0.0251 mg/g DCW	(1.2 ± 0.0888 mg/g DCW

Note: values were represented in Mean ± SD of two replicates repeated in triplicates, DCW (dry cell weight)

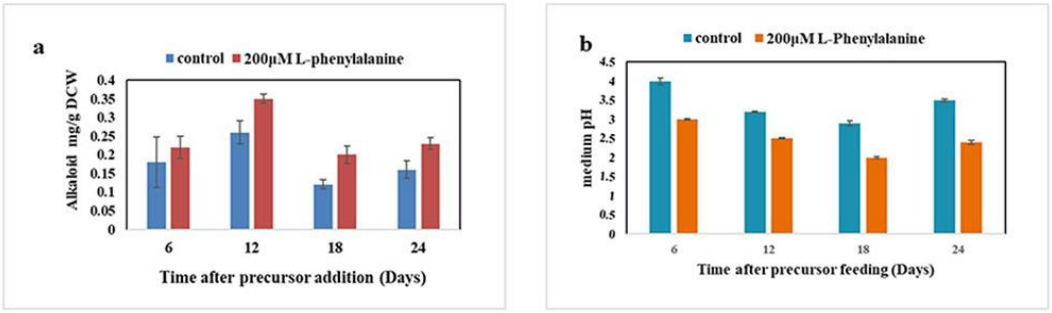

Fig.1. Results of cell growth and pH analysis expressed in terms of Mean ± SD. (a) cell growth analysis in L-phenylalanine treated cell suspension cultured call sample 200µM 12th day (b) measurement of medium pH in L-phenylalanine treated cell suspension cultured call sample 200µM 12th day.

Phytochemical screening for alkaloids

The suspension-cultured callus extracts were qualitatively evaluated for the abundance of alkaloids using standard procedures. Preliminary phytochemical screening was employed to determine the existence of alkaloids in *G. glauca* the cell suspension culture by performing Dragendorff's, Hager's, Wagner's, and Mayer's tests, respectively; analytical results were tabulated in Table 2.

Table 2: Phytochemical evaluation of alkaloids in *G. glauca* cell suspension cultured callus sample

Phytochemical test	Results	
	Control	L-Phenylalanine treated 200µM+12th day
I. Wagner's test	-	++
II. Dragendorff's test	-	++
III. Mayer's test	-	+
IV. Hager's test	-	+

Note: (-) indicates absent, (+) indicates (average), (++) indicates good

UV-Visible spectrophotometric analysis of alkaloids

UV-Visible spectroscopic estimation is the vital method for the Quantification of alkaloid content present in Precursor treated cell suspension cultured callus sample of *G. glauca* (Fresen.) Gilg. The results of UV-Visible spectrophotometric analysis are shown in Figure 2. Standard colchicine and caffeine exhibit absorption maxima at 351 nm and 272 nm, respectively, L- phenylalanine treated 200µM, 12th-day suspension cultured sample shows absorption maxima at 280 nm.

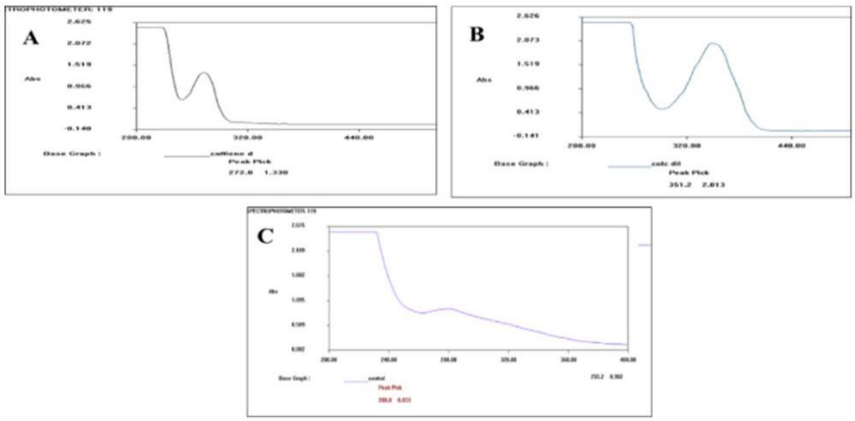

Fig.2. UV-Visible spectrometric analysis of alkaloids from *G. glauca* (Fresen.) Gilg suspension culture sample A, standard caffeine; B, standard colchicine; C, L-phenylalanine 200 µM 12th day

Thin layer chromatography analysis

The Quantification of alkaloids was evaluated by thin layer chromatography (TLC) technique. Precursor-treated (L-phenylalanine treated 200µM 12th day) samples were prepared in 80% methanol subjected to TLC analysis, and n-butanol: acetic acid: dichloromethane in the ratio of (50:10:40) was used as mobile phase, (sannabommaji et al., 2019), as a result, a characteristic band was observed, under UV-transilluminator. Those bands were represented as B-1, B-2, and B-3, and the degree of retardation factor of colchicine was 0.50, and the phenylalanine treated 200µM+12th day sample exhibited three distinct bands; the Rf value of B-1was,0.35 is B-2 was 0.46, B-3 was 0.62 respectively, were depicted in fig.6.

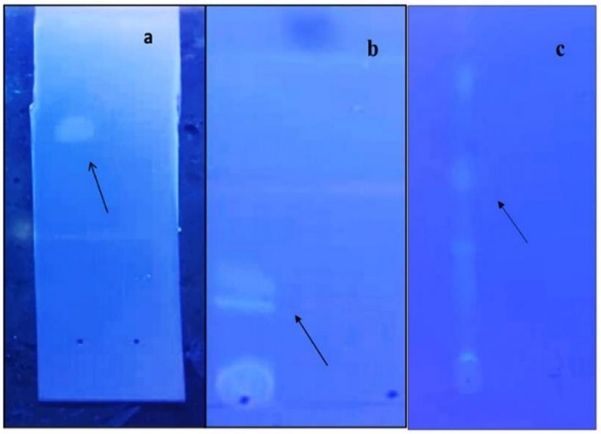

Fig. 3. TLC analysis of alkaloid Quantification (a) standard colchicine, (b) control sample, (c) phenylalanine treated 200µM 12th day sample

Alkaloid Quantification by RP-HPLC Method

The results of alkaloid Quantification have been performed by the RP-HPLC method, are illustrated in Fig. 4. The retention time (Rt) of *G. glauca* (Fresen.) Gilg suspension-cultured samples (200 µM L-phenylalanine treated, 12th day) were 13.79, 4.924 showing absorption at 280 nm. Compared with standard colchicine and standard caffeine, with retention time was 14.358 and 3.740, respectively, and the quantified alkaloid content in the suspension sample of *G. glauca* 200 µM L-phenylalanine treated, 12th-day sample was 58.43 µg/ml compared to a control sample of alkaloid content was 38.38 µg/ml, respectively.

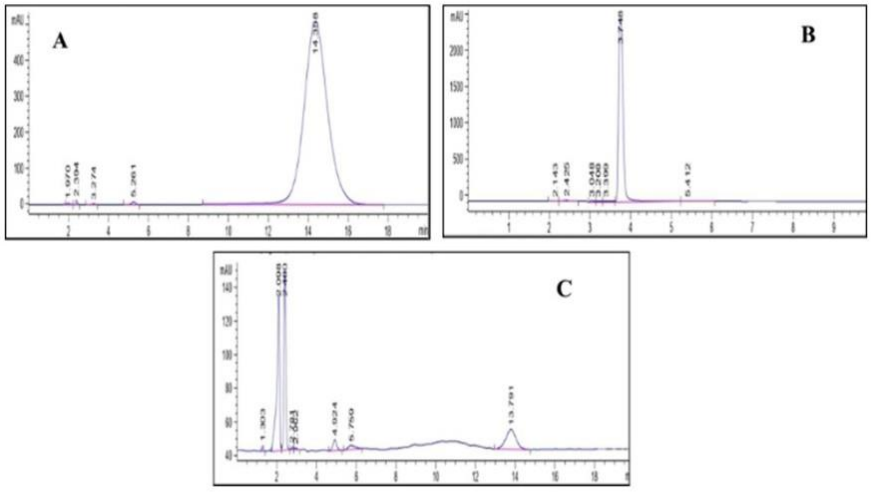

Fig.4. RP-HPLC chromatogram A; standard colchicine, B; standard caffeine, C; L-phenylalanine 200 µM 12thday sample

Fourier transforms infrared analysis (FTIR)

The results of FTIR analysis of the L-phenylalanine treated sample of *Gnidia glauca* (Fresen.) Gilg is shown in Fig. 5 and Table 3. The FTIR absorption spectrum peak values and the functional groups (Vijendra et al., 2020), in which the highest wavenumber, 3541.21 cm^{-1} was, implying the occurrence of primary amine (N-H stretch), At 651.61 cm^{-1} could be C-Br stretch identified as Halo compounds. for α C-H or β C-H ring at 776.10 cm^{-1} ascertained as Heteroaromatic compounds, at 926.98 61 cm^{-1} for C-H was observed detected as alkenes. A strong C-N stretch 1022.4 61 cm^{-1} was identified as amine. α-unsaturated or cyclic tertiary alcohols (C-O stretch) were identified at 1136.78 cm^{-1}. A band was observed at 1261.28 cm-1 enumerated as C-O stretch tentatively identified as an aromatic ester. C-O-H bend and C=O stretch observed at 1348.26 cm^{-1} found to be alkanes, C-O-H bend and strong C=O stretch at 1392.95 cm^{-1} identified as carboxylic acids, C-C stretch observed at 1424.07 cm^{-1} was found to be aromatics. Aromatic hydrocarbons identified at 1595.64 cm^{-1} as C-C stretch, 2151.84 cm-1 for N=N=N Stretch were found to be azide compounds, strong C=C, C-H stretch observed at 2359.32 cm-1 identified as alkynes; mono/di-substituted. O-H stretching was noticed at 2722.41 cm^{-1} and was ascertained as alcohol. At 2805.40 cm^{-1}, detected as C-H, O-H stretch may be carboxylic acids and alkanes, and a medium C-H stretch was observed at 2940.26 cm^{-1} identified as alkanes. A strong and broad O-H stretch H-bonded was observed at 3241.10 cm^{-1} for O-H stretch H-bonded. The occurrence of N-H and C-H stretching vibrations are characteristics of alkaloids.

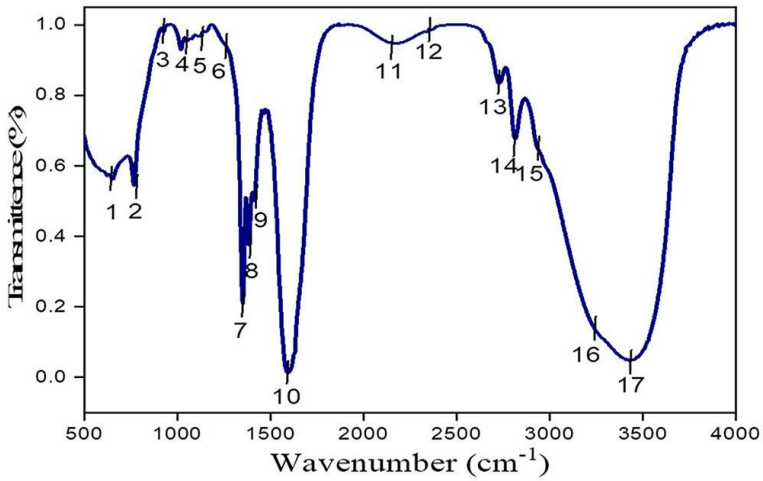

Fig.5. FTIR Spectrum of Precursor treated cell suspension cultured callus sample of Glauca (Fresen.) Gilg

Table 3- FTIR analysis in cell suspension culture of *G. glauca* (Fresen.) Gilg

Peak No.	Wave number in cm-1	Bond assignments	Functional groups
1	651.61 (s)	C-Br stretch	Halo compounds
2	776.10 (s)	α CH or β CH ring	Heteroaromatic
3	926.98 (w)	C-H	Alkenes
4	1022.68 (m)	C-N stretch	Amine
5	1136.78 (s)	C-O stretch	Cyclic tertiary alcohols or α-unsaturated
6	1261.28 (s)	C-O stretch	Aromatic ester
7	1348.26 (s)	C = O and C-O-H bend	carboxylic acids
8	1392.95 (m)	C = O and C-O-H bend	carboxylic acids
9	1424.07 (m)	C-C Stretch	aromatics

10	1595.64 (m)	C-C stretch	aromatic hydrocarbons
11	2151.84 (s)	N=N=N stretching	azide
12	2359.32 (s)	C-H stretch, (C = C)	alkynes; mono/di-substituted
13	2722.41 (m)	O-H stretching	Alcohol
14	2805.40 (s)	C-H, O-H stretch	carboxylic acids, alkanes,
15	2940.26 (m)	C-H stretch	alkanes
16	3241.10 (s)	O-H stretching	alcohols
17	3541.21 (m)	N-H stretch	Primary amine

Note: (S)= strong, (m) = medium, (W) =weak

CONCLUSION

From the results of the present study could be concluded that the precursor-mediated cell suspension culture of *Gnidia glauca* (Fresen.) Gilg was one of the vital and alternative approaches for the enhancement of alkaloid content; a total of 64.5 mg/L of alkaloid was produced on the 12th day. In this way, the alkaloid content of the cells was elicited more than two folds in contrast to the control culture. UV-Visible, HPLC, and TLC methods estimated alkaloids' quantification. These studies have offered a protocol and knowledge for the product enhancement of commercially and economically valuable plant secondary metabolites.

Acknowledgement:

Ruksana F. sincerely thanks to the Department of Studies in Biochemistry, Davangere University, for providing a laboratory facility for executing my research work and to the minority welfare department Government of Karnataka for financial assistance during my research work.

Funding:

The authors declare that no funding was received to carry out this research work from any agency/source.

Conflict of interest:

The author has no conflict of interest.

Author contribution:

RF: performed work, VK: manuscript revision and design, VE: suspension culture analysis, DVP: FTIR interpretation, ACM: Data analysis PHM: HPLC analysis, SRM: Statistical analysis, MT: UV-Visible analysis, we ensure and hereby declared that all authors have read and approved the manuscript.

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