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Production of Secondary Plant Metabolite Phyllanthin in *Phyllanthus niruri* Linn. by Leaf Tissue Culture

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ABSTRACT

Phyllanthus niruri Linn. (Euphorbiaceae) is native of Brazil and indigenous to Indiaand commonly knownas"Pitirishi" or "Budhatri". The plant is reported to be used as a remedy for asthma, bronchitis, extreme thirst, anemia, jaundice and tuberculosis. Young leaves and flowers were cultured on White's media, MS media and Gamborg media (B_5) with different groups of phytohormones. MS media and Gamborg media were found to be suitable for the induction of callus culture. The outcome of the study showed that, out of the two media which were employed for the induction of callus, the Gamborg media supplemented with 2, 4-D (1mg/L) along with Kinetin (0.5 mg/L) as phytohormones showed faster initiation of callus culture. However MS Media, supplemented with 2, 4-D (1mg/L) and Kinetin (0.5mg/L) as the phytohormones, contributed to the maximum yield of the callus. Simultaneously, the amount of phyllanthin produced in different age groups of callus culture was estimated by HPLC. The maximum yield of phyllanthin was obtained in 12 week callus with MS media, supplemented with 2, 4-D (1mg/L) along with Kinetin (0.5mg/L). The callus also showed moderate In vitro antioxidant and anticancer activity. The described method can be successfully employed for the large-scale multiplication and maximize the yield of secondary metabolites.

Keywords: *Phyllanthus niruri*, callus, phytohormones, phyllanthin

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INTRODUCTION

Phyllanthus niruri (*P.niruri*) belongs to the family Euphorbiaceae, with a worldwide distribution; it is used in Brazilian folk medicine to treat urolithiasis. Among the Phyllanthus species, P. niruri is a small erect annual herb growing up to 30-40 cm in height and is indigenous to the Amazon rainforest and other tropical areas, including South East Asia, Southern India and China [1]. Its leaves are 7-12 cm long, alternate, sessile and oblong. It has small off-white-greenish flowers, which are solitary, auxiliary, pedicellate, apetalous and monoecious. P. niruri has a long history in herbal medicine systems such as Ayurveda, Traditional Chinese Medicine and Indonesian Jamu (formerly Djamu). The whole plant is used as remedies for many conditions such as dysentery, influenza, vaginitis, tumours, diabetes, diuretics, jaundice, kidney stones and dyspepsia. The plant is also useful for treating hepatotoxicity, hepatitis B, and hyperglycaemia, viral and bacterial diseases [2].P. niruri has been used in Ayurvedic medicine for over 2000 years and has a wide number of traditional uses for jaundice, gonorrhoea, frequent menstruation and diabetes. It is a well-known Indonesian traditional herbal medicine to treat various diseases. In Jamu preparations, the plant is used as antiviral and hepatoprotective agent. In Malaysia, P. niruri, known as Dukunganak, is used internally for diarrhoea, kidney disorders, gonorrhoea and cough [3]. Modern civilization is confronted with terminal diseases like cancer and AIDS in contrast to the earlier populations, which were devastated by infectious diseases. Large number of people dies every year due to the cancer. Nature has provided a rich storehouse of herbal remedies to cure all ailments of mankind. Searching for novel anticancer agent is a recent trend in modern medical research throughout the world. Anticancer compounds from plants are in high demand for clinical use. Unfortunately, because of low yields, extractions of phytopharmaceuticals from the plants have been considered uneconomical. In addition, most of such plants are slow growing and the accumulation pattern of their active substances is highly susceptible to geographical and environmental conditions [4] and thus, unpredictable. It is mostly difficult to obtain these products through chemical synthesis, because of lengthy and complicated procedures involved in obtaining pure products [5].

Plant tissue culture has been employed to solve these problems and improve the yield of useful secondary metabolites. Year 1975 was regarded as watershed in cultures of secondary product research In-vitro [6]. The advantages of the In-vitrosystems, viz. increased purity of end products, conversion of inexpensive precursors to expensive end products and the potential for feeding novel compounds necessitated the use of plant tissue culture (PTC) in the large scale production of fine chemicals and pharmaceuticals by the investor and companies.

Keeping these aspects in mind the present study was designed to carry out the tissue culture studies of the plant P. niruriin such a way to increase the yield of its secondary metabolite phyllanthin, continued by studies to investigate the antioxidant effects of alcoholic callus extract and anticancer effects of alcoholic callus and conventional extracts using established In-vitro models. So far, there are no reports on an efficient culture system for callus induction in this species. This work was, for the first time, undertaken to study the effect of

different type of explants and plant growth regulators & media's on the callus induction in *P.niruri*.

MATERIAL AND METHODS

Plant material

The plant P. niruri collected from Medicinal garden of "Manipal College of Pharmaceutical sciences", Manipal, Karnataka during the month of October was identified and authenticated by Dr. Gopalkrishna Bhat, Professor of Botany, PoornaPrajna College, Udupi, Karnataka. A sample specimen (MCOPS/PCOG/2008/23) was deposited in the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal.

The young leaves and stem were 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 minutes 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.

Establishment and maintenance of Callus cultures

The surface sterilized explants (leaves and stem)of *P.niruri* were transferred aseptically onto the MS [7], Gamborg [8] and White's media [9] supplemented with 2, 4-D, NAA, Kinetin at various concentrations in culture tubes. The tubes were plugged properly and incubated in dark at 25 ± 2^0C. The cultures were maintained at 25 ± 2^0C by sub culturing the fragile globular callus pieces onto the corresponding medium at an interval of 4 weeks.

Measurement of growth and frequency of callus induction

The growth indices of the cultures were determined on dry weight basis [10]. The growth index (G.I.) of the cultures was calculated by using the following formula.

$$\text{Growth Index (GI)} = \frac{\text{Final weight of Biomass}}{\text{Initial weight of Biomass}}$$

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

Preparation of extract of callus culture

The solvent was carefully selected according to type of active matter to be extracted. Known amount of callus was extracted with benzene. Maceration time was standardized to 45

minutes. The cell wall of the plant must be open to allow solvent access to the cytoplasmic phytoconstituents. The extract was concentrated under reduced pressure. The temperature was adapted to thermo sensibility of active principle that was around 70°C. The extract was then further boiled with 10% alkali solution for 2 h, which was followed by extraction with benzene. The extracted benzene was once again concentrated under reduced pressure. The extract was loaded on column and eluted with Toluene: Ethyl acetate (2:1) as solvent system. Phyllanthin was separated out on alumina column. The compound had R_f value of 0.3. The conformation of the compound was done by TLC method.

Chromatographic Analysis callus extract

Aluminum plate pre-coated with silica gel G (Merck) was used as a stationary phase. Standard phyllanthin solution was prepared in HPLC grade methanol. The solvent system used to identify phyllanthin was Toluene: Ethyl acetate (2:1).The extracts derived from callus culture and standard solution were applied 1.5cm away from the lower edge of the plate with the help of micro-capillary tube. The solvent was allowed to evaporate after each application by air-drying. The loaded plates were then placed vertically in the chamber previously saturated with solvent system for 30 min. After the solvent front moved upto a distance of about 80% of length, the plate was taken out, solvent front was marked and the plate was dried at room temperature. After development, the plates were observed under UV light to identify the position of spots if any, and then the plates were sprayed with vanillin-sulphuric acid reagent, followed by heating at 110 °C for 5 minutes. The R_f values were calculated.

After the confirmation of presence of phyllanthin by TLC, HPLC analysis was carried on Shimadzu HPLC (SCL-10AVP, Shimadzu Corp., Japan).A 1000 μg/ml solution of phyllanthin reference standard was prepared in methanol. It was further diluted to yield the final concentration of 2, 4, 6, 8 and 10 μg/ml solution.

The samples were passed through 0.22 micron filter (PVDF) using a syringe and 20μl each of test solution and different concentration of standard phyllanthin were injected to HPLC having Phenomenex C-18 reserved phase column. The mobile phase consisting of methanol: water (66:34) was degassed using vacuum and filtered through 0.22 micron filter and used for separating the content with flow rate of 1.5 ml/min. The chromatogram was scanned upto 30 min, which was detected at 230 nm [11]. Standard plot of phyllanthin was obtained by plotting concentration of phyllanthin (X-axis) versus respective peak area of the chromatogram (Y-axis). The amount of phyllanthin in test sample was determined from the linear regression equation of calibration graph plotted between concentration and area.

RESULTS

The explants (leaves and stems) of P. niruri were taken for induction of callus culture on White's media, MS media and Gamborg media (B5) with different groups of phytohormones.

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Only MS media and Gamborg media composition was found to be suitable for the induction of callus culture, White's media composition did not respond with either of explants(Table 1).

Table 1: Response of Various Medias

Media	Response
Murashige and Skoog media	Callus induced
Gamborg media (B5)	Callus induced
White's media	Not responded

The explants from leaves and stems were taken for the induction of callus culture in equal batches, but only leaves responded for callus culture. So, the leaves were taken as explants for further studies. MS, Gamborg and white media supplemented with various concentrations of phytohormones showed positive and negative result for the induction of callus (Table 2).

Table 2: Effect of Phytohormones in the Induction of Callus Culture

Media	Auxin (mg/L)	Cytokinin (mg/L)	Response
MS	2,4-D	Kinetin	
	0.1	1.0	Not Induced
	0.5	0.5	Induced
	1.0	0.5	Induced
	2.0	0.1	Not Induced
MS	NAA	Kinetin	
	0.1	1.0	Not Induced
	0.5	0.5	Induced
	1.0	0.5	Induced
	2.0	0.1	Not Induced
Gamborg(B5)	2,4-D	Kinetin	
	0.1	1.0	Not Induced
	0.5	0.5	Induced
	1.0	0.5	Induced
	2.0	0.1	Not Induced
White	2,4-D	Kinetin	
	0.1	1.0	Not Induced
	0.5	0.5	Not Induced
	1.0	0.5	Not Induced
	2.0	0.1	Not Induced

The frequency of callus induction of leaves of P. niruri is shown in Table 3. There was significant difference in callus induction frequencies of different hormonal concentration. MS media supplemented with hormones 2, 4-D (1.0 mg/L) and kinetin (0.5 mg/L) showed maximum % induction of callus followed by Gamborg media supplemented with hormones 2, 4-D (1.0 mg/L) and kinetin (0.5 mg/L) (Fig 1).The effect of different concentration of phytohormones on growth indices for different groups of callus culture are shown in Table 4. TLC studies of standard phyllanthin showed dark brown spots with an R_f value of 0.3 and the same results were obtained for the compound which was isolated from callus and conventional extracts.

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Table 3: Frequency of Induction of Callus with Leaf as an Explant

Media	Auxin	Cytokinin	%Induction of callus
MS	2,4-D (0.5mg/L)	Kinetin (0.5mg/L)	61
MS	2,4-D (1.0mg/L)	Kinetin (0.5mg/L)	92
MS	NAA (1.0mg/L)	Kinetin (0.5mg/L)	88
MS	NAA (0.5mg/L)	Kinetin (0.5mg/L)	70
Gamborg (B5)	2,4-D (0.5mg/L)	Kinetin (0.5mg/L)	67
Gamborg (B5)	2,4-D (1.0mg/L)	Kinetin (0.5mg/L)	90

Fig 1: Different Media Responses at 12Weeks Old Callus

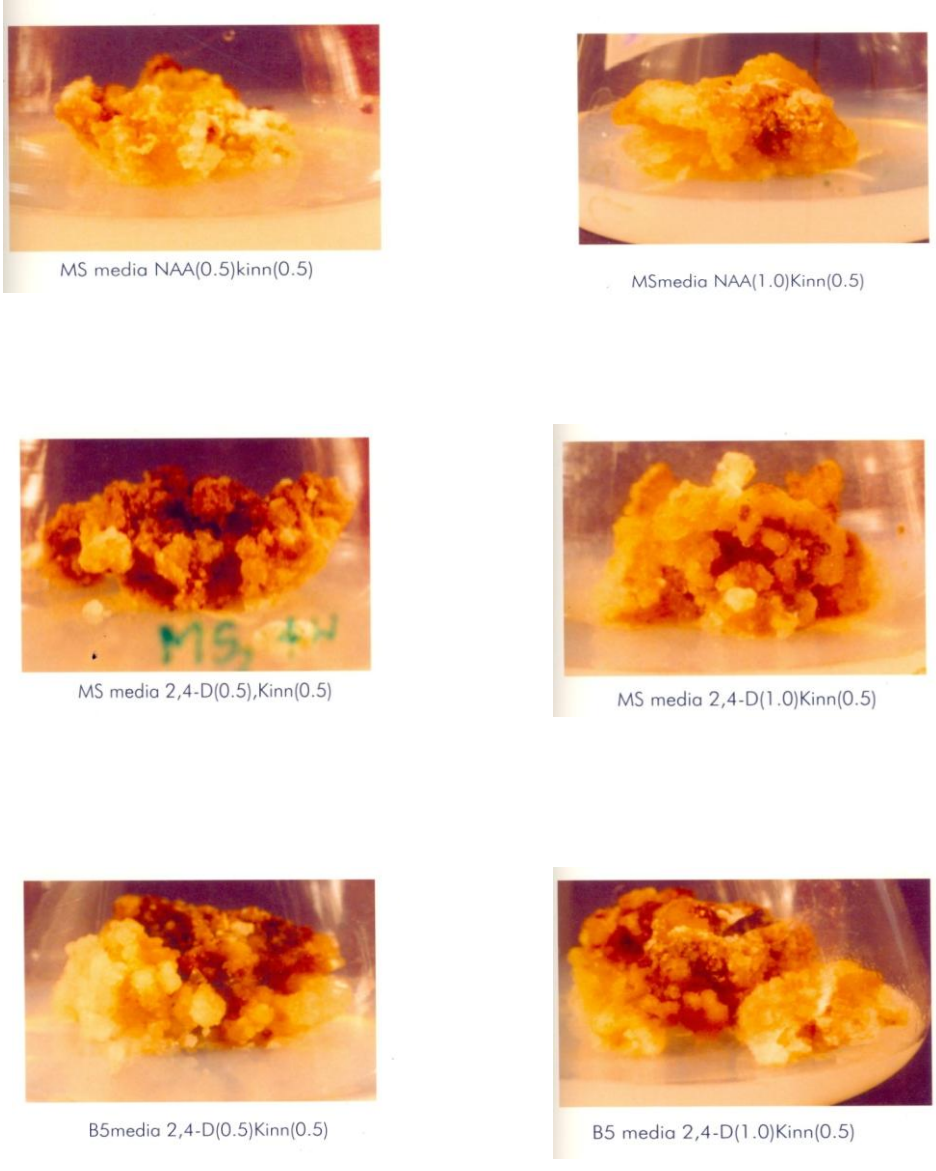

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Table 4: Growth Indices of Callus with Different Hormonal Groups

Medium	4 Week	8 Week	12 Week	16 Week	20 Week
MS media combinations					
2,4-D (0.5mg/L),Kinetin (0.5mg/L)	0.5	1.42	1.67	1.78	1.66
2,4-D (1mg/L),Kinetin (0.5mg/L)	0.6	1.71	2.12	2.30	1.86
NAA (1mg/L), Kinetin (0.5mg/L)	0.5	1.33	1.64	1.86	1.66
NAA (0.5mg/L), Kinetin (0.5mg/L)	0.4	1.24	2.03	2.22	1.80
Gamborg media combinations					
2,4-D(0.5mg/L),Kinetin(0.5mg /l)	0.4	1.28	1.66	1.76	1.57
2,4-D (1mg/L),Kinetin(0.5mg/L)	0.6	1.65	2.19	2.30	1.77

The percentage amount of phyllanthin present in different age groups of callus supplemented with different combination of hormones was estimated by performing HPLC analysis of callus extracts (Fig. 2, Fig.3), which are shown in the tables Table 5 and Table 6. Among different groups of media the callus induced by MS media supplemented with 2,4-D (1mg/L) along with Kinetin (0.5mg/L) showed maximum amount of phyllanthin.

Table 5: Effect of MS Media Supplemented with Different Concentrations of Phytohormones

MS Media supplemented with 2,4-D (1mg/L) and Kinetin (0.5 mg/L)		
S.No.	**Age of the callus culture**	**Concentration of phyllanthin (%w/w)**
1	4 Week	0.15037
2	8 Week	0.37778
3	12 Week	0.80523
4	16 Week	0.71628
5	20 Week	0.55667
MS Media supplemented with 2,4-D (0.5mg/L) and Kinetin (0.5 mg/L)		
6	4 Week	0.20566
7	8 Week	0.31505
8	12 Week	0.70354
9	16 Week	0.64136
10	20 Week	0.49198
MS Media supplemented with NAA (1 mg/L) and Kinetin (0.5 mg/L)		
11	4 Week	0.20965
12	8 Week	0.27964
13	12 Week	0.65889
14	16 Week	0.61710
15	20 Week	0.45797
MS Media supplemented with NAA (0.5 mg/L) and Kinetin (0.5 mg/L)		
16	4 Week	0.18488
17	8 Week	0.32597
18	12 Week	0.73205
19	16 Week	0.69423
20	20 Week	0.51604

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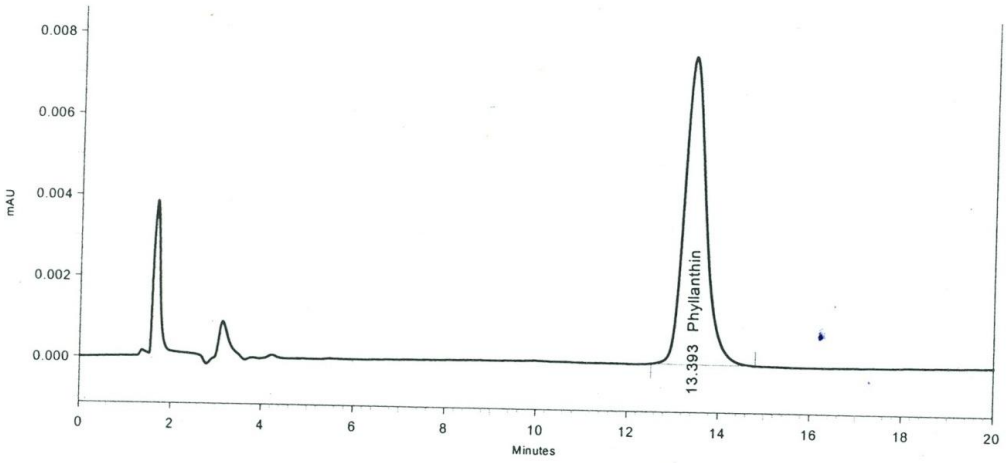
Table 6: Effect of Gamborg Media supplemented with Different Concentrations of Phytohormones

MS Media supplemented with 2,4-D (1 mg/L) and Kinetin (0.5 mg/L)		
S.No.	**Age of the callus culture**	**Concentration of phyllanthin (%w/w)**
1	4 Week	0.18688
2	8 Week	0.32257
3	12 Week	0.73336
4	16 Week	0.66020
5	20 Week	0.49870
MS Media supplemented with 2,4-D (0.5 mg/L) and Kinetin (0.5 mg/L)		
6	4 Week	0.20965
7	8 Week	0.25695
8	12 Week	0.66389
9	16 Week	0.54502
10	20 Week	0.42385

Fig.2: HPLC Chromatogram of Phyllanthin Standard



Detector A (230nm) Name	Retention Time	Area	Theoretical plates / meter	Asymmetry (10%)
Phyllanthin	13.393	250144	16115.97	1.23

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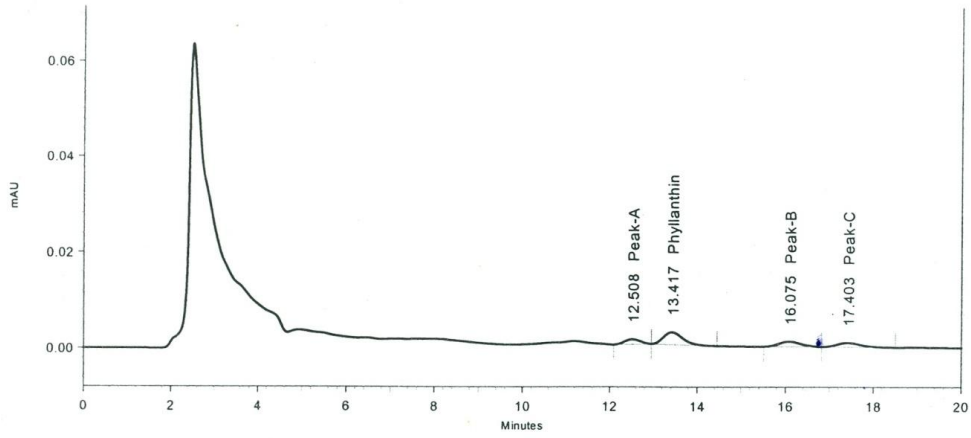
Fig.3: HPLC Chromatogram of P. niruri leaf callus



Detector A (230nm) Name	Retention Time	Area	Theoretical plates / meter	Asymmetry (10%)
Peak-A	12.508	25665	21100.36	1.02
Phyllanthin	13.417	78779	17299.62	1.37
Peak-B	16.075	36234	19401.76	1.16
Peak-C	17.403	33261	20023.97	1.37

DISCUSSION

In this present study an attempt has been made on the establishment of tissue culture studies of the plant P. niruri which is well known for its wide range of curative properties against various ailments like cancer, diabetes etc. Phyllanthin is one of the secondary metabolite (Bioactive compound) of the plant P. niruri which is claimed to be responsible for many of its pharmacological properties. The amount of phyllanthin present in the plant was reported to be 0.80% of its total constituents.

Since the technique of plant tissue culture serves as a tool in manipulating the production of bio medicinal, an attempt has been made in this current study to increase the production of bioactive compound phyllanthin in the plant P. niruri. The study comprised of several trials with an aim of induction of callus culture of the plant in various types of media, which were supplemented, with differing composition of phytohormones in order to increase the production of its secondary metabolite phyllanthin. Simultaneously the amount of phyllanthin produced in different age groups of callus culture was estimated by employing HPLC methods with phyllanthin as the reference standard. The outcome of the study showed that, out of the different medias which were employed for the induction of callus, the Gamborg media which was supplemented with 2,4-D (1mg/L) along with Kinetin (0.5mg/L) as

phytohormones showed faster initiation of callus culture. However MS Media, which was supplemented with 2,4-D (1mg/L) along with Kinetin (0.5mg/L) as the phytohormones, contributed to the maximum yield of the callus.

Traditional approaches to induction of callus by manipulating the relative ratio of phytohormones have been successfully used in the current investigation. Estimation of phyllanthin content in different age groups of callus cultures revealed that the 12 week old callus culture induced in MS media which was supplemented with phytohormones 2,4-D (1mg/L) along with Kinetin (0.5mg/L) had the maximum yield of phyllanthin (0.805%).

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