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PHYTOCHEMICAL ASSESSMENT FROM CALLUS AND SHOOT CULTURES OF A POTENTIAL MEDICINAL HERB: *PHYLLANTHUS AMARUS* (Schum. & Thonn)

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This study was undertaken to evaluate the performance of cut Dendrobium pink sunshine under various holding solutions at post graduate lab Department of Horticulture, Faculty of Agriculture, Annamalai University, Tamil Nadu, India, during February 2016. The treatments with three replications were carried out in completely randomized design with 3 spikes in each replication. Nine chemical preservative solutions were used for extending the vase life and the treatments are T_1 8-HQC (200-ppm), T_2 Citric Acid (200-ppm), T_3 STS (200-ppm), T_4 GA (200-ppm), T_5 $Al_2(SO_4)_3$ (10-ppm), T_6 8-HQC (200-ppm) + 5% sucrose, T_7 Citric Acid (200-ppm + 5% sucrose, T_8 STS (200-ppm) + 5% sucrose, T_9 GA (200-ppm) + 5% sucrose,T_{10} $Al_2(SO_4)_3$ (200-ppm) + 5% sucrose and T_{11} Distilled water. Postharvest observations including bud open (%), flower drop (%), flower colour retention (days), flower diameter (cm), vase life and water uptake (ml). Among the treatments (T_{10}) $Al_2(SO_4)_3$ (200-ppm) + 5% sucrose recorded maximum performances in the postharvest studies followed by (T_8) STS (200-ppm) + 5% sucrose.

ABSTRACT

This study reports the phytochemical production from the callus and shoot cultures of *Phyllanthus amars*. Callus and shoot cultures were established from the nodal explants. Murashige and Skoog medium supplemented with N^6-benzylaminopurine was found best medium for multiple shoot regeneration from the node explant and further maintenance of the shoot cultures. Callus induction was favoured on medium supplemented with 4.50 µM 2,4-dichlorophenoxy acetic acid. *In vitro* regenerated shoots and callus cultures were dried at room temperature and after measuring the dry biomass subjected for phytochemical extraction by using methanol and water. The extracts were quantified tested for total phenolics and flavonoids. The methanolic extract of *in vitro* and *in vivo* shoot showed the antioxidant activity.

Keywords **:** Callus, shoot culture, phenolics, Phyllanthus, 2,4-Dichlorophenoxy acetic acid.

Introduction

The genus *Phyllanthus* (Phyllanthaceae) including more than 1000 species is distributed in Africa, America, Asia and Australian continents (Sarin *et al*., 2012). *Phyllanthus amarus* Schum. & Thonn. is a widely used plant in Indian Ayurvedic system of medicine and generally used in the treatment of problems related to stomach, liver, kidney, genitourinary system and spleen. Phytochemical studies have showed the presence of a number of valuable compounds including lignans, flavonoids, hydrolysable tannins (ellagitannins), polyphenols, triterpenes, sterols and alkaloids. Phyllanthin and hypophyllanthin are the most important bioactive lignans found in *Phyllanthus* species. The extracts and the compounds isolated from *P. amarus* have a wide spectrum of pharmacological activities including antiviral, antibacterial, antiplasmodial, anti-inflammatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective nephro-protective and diuretic properties (Patel *et al*., 2011; Nisar *et al*., 2018).

Exploitation of large quantity of plants in diverse medicinal uses can reduce local plant population. The application of plant tissue culture for production of medicinal compound can overcome the danger of extinction of plants from the nature due to excessive utilisation of plant for natural product. In culture production of natural compound can be carried out throughout the year without any climatic and environmental pressure while the production of medicinal compounds from natural plant population may lead to several climatic and environmental fluxes that may lead to change in their chemical profile and inconsistent production (Manivannun *et al*., 2015; Singh and Chaturvedi, 2012)

Micropropagation of *P. amarus* have been attempted from nodal explants (Shekhawat and Dixit, 2007; Sen *et al*., 2009), shoot tip (Bhattacharyya and Bhattacharyya, 2001) leaf and internode explants (Chitra *et al*., 2009). Despite having significant medicinal importance, *Phyllanthus amarus* has so far rarely used for metabolite production from *in vitro* cultures and requires *in vitro* culture and optimization of metabolite production for the development of commercially feasible process. The present study reports the establishment

of *in vitro* shoot and callus cultures from node sections and accumulation of phenolics, flavonoids and antioxidant activity in *in vitro* established shoot and callus cultures.

Material and Methods

Culture establishment

The shoots 5-6 cm long were collected from the plants growing in the Chhatrapati Shahu Ji Maharaj University campus, Kanpur, India (Fig. 3A). The branches and leaves were removed from the main shoot and washed under running tap water for about 15 minutes, followed by surface disinfection by immersing the washed shoots for about 10 - 12 minutes in solution having 2% cetrimide, 2% (v/v) sodium hypochlorite and 1-2 drop tween-20 and then washed with distilled water. The washed shoots were then carried out in Laminar-air-flow and dipped for 30 sec. in 70% alcohol followed by washing with sterile double distilled water. The shoots were again dipped in 0.05% (w/v) solution of mercuric chloride solution for about 3 minutes and then washed with autoclaved double distilled water.

The nodal section about 1.0-1.5 cm long were dissected from the washed shoots and inoculated on 0.8 (%) agar gelled Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with different concentrations (0.44, 4.4and 20.0 µM) of BAP or (0.46, 4.65 and 23.25 µM) kinetin for shoot organogenesis. For callus induction the nodal sections approximate 1.0cm long were cultured on agar gelled MS medium supplemented with different concentrations (0.45, 4.5, 13.6 and 22.67µM) of 2,4-dichlorophenoxy acetic acid (2,4-D). The regenerated callus was maintained by subculturing approximate 100mg healthy callus at an interval of four weeks on callus proliferation medium (CPM) composed of agar gelled MS medium supplemented with 4.5 µM 2,4-D.

The pH of all the culture medium was maintained to 5.8 ± 0.02 prior to autoclaving at 121ºC for 15 minutes. All the explants were inoculated in 25 X 150 mm glass culture tubes each having 10 ml medium. The cultures were incubated at 25±2ºC and illuminated 16h by cool white fluorescent tube lights with light intensity 25 µmol m^{-2} s^{-1}.

Growth Measurement

Callus growth was determined by measuring fresh and dry weight after four weeks of subculture of callus on different concentrations of 2,4-D containing MS medium. To measure fresh weight callus was taken out from the culture tube and washed with double distilled water and after blotting to dry water the fresh weight was measured. To obtain dry weight the calli were dried at 60ºC in hot air oven (3-4 hours) until a constant weight obtained followed by weight measurement.

Preparation of Plant Extracts

To extract phytochemicals, the callus and shoots were harvested between 35–40 days of culture and washed to remove medium attached on it. After recording the fresh weight separately, the callus and shoot were wrapped with aluminium foil and kept in dark at 25-30ºC for drying until a constant weight was achieved. The dried callus and shoots were subjected to extraction of phytochemicals. The extraction was performed through soxhlet extractor and using methanol as a solvent. Once the extraction completed the extract was kept for drying in dark at 25 – 30ºC and after complete drying the dry weight of the extract was measured. The extract was stored under dark at room temperature for further analysis.

Total Phenolic and Flavonoid Estimation

Folin-Ciocalteu method was applied to estimate total phenol of the culture extract (Manivannun *et al*., 2015). The estimation was performed by mixing 2.5 ml of sodium carbonate solution (7.5%) and 0.5ml of Folin-Ciocalteu reagent (diluted 10 times with double distilled water) in 1.0 ml (0.1 ml extract made to 1.0ml by distilled water) of extract. The reaction mixture was vortexed and incubated in dark at 45°C for 15 min to develop blue colour of molybdenum-tungsten complex. The absorbance of the blue colour was measured by spectrophotometer at 764 nm wavelength and the total phenol content was computed by comparing sample value to standard gallic acid and expressed as gallic acid equivalents (GAE).

The total flavonoid composition was determined based on the aluminium chloride calorimetric method (Manivannun *et al*., 2015). In 0.5 ml callus extract 1.5ml methanol was added and after mixing it well 0.1ml aluminium chloride solution (10% w/v), 0.1 ml potassium acetate (1 mol/l) and 2.8 ml double distilled water were added. The reaction mixture was kept at 25ºC for 30 minutes and absorbance of the reaction mixture was measured at 415 nm wavelength. A standard curve of various concentrations of quercetin in methanol was prepared and by comparing sample value with standard curve total flavonoid content was calculated and was expressed as quercetin equivalent (mg QE/g extract).

Antioxidant assay

The free radical scavenging activity was measured by the method of Manivannun *et al*. (2015). Fresh 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution was prepared by dissolving 6.4 mg of DPPH in 100 ml of absolute ethanol. In 50 µl of callus or shoot extract 2.95 ml of DPPH solution was added and the reaction mixture was incubated for 30 min at 25ºC in dark. After the incubation period absorbance of mixture was recorded at 515 nm and the scavenging activity of the DPPH radicals was calculated by the formula

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

Experimental design and statistical analysis

The experiments were repeated three times with randomized design. In all experiments on *in vitro* regeneration each treatment was consisted with twelve replications. The phytochemical analysis was performed with three replicates. The least significant difference (LSD) test was performed for comparison between the mean values of each treatment and one way analysis of variance test (ANOVA) was used for data analysis.

Results and Discussion

Adventitious Shoot Induction and Proliferation

An efficient method for phytochemical production from *in vitro* established shoot and callus cultures of *P. amarus* is established. Phytochemical production from *in vitro* cultures has been established in several medicinal plants including *Stevia rebaudiana* (Janarthanam *et al*., 2010), *P. tenellus* (Nikule *et al*., 2020), *Spilanthus acmella* (Singh and Chaturvedi, 2012). In the present study the *in vitro* culture

was established from the node explants (Fig. 3B). The nodal sections cultured on MS medium supplemented with cytokinin showed multiple shoot induction while cytokinin free MS medium occasionally showed shoot regeneration with only a single shoot. Shoot began to initiate within a week of culture on the medium supplemented with BAP while on medium devoid of growth regulator shoot induction observed in second week of culture initiation (Fig. 3C). Of the two cytokinin BAP and Kn, the shoot induction and multiplication was significantly high on medium supplemented with BAP and of the different concentrations of BAP tested the highest frequency (100%) of shoot induction and number of shoots per explant (8.23 ± 0.65) was recorded on the medium having 4.4µM BAP. (Table 1; Fig.3C). BAP induced shoot multiplication has been reported in *Phyllanthus fraternus*. Where the shoot tip explants induced high frequency multiple shoots on MS medium supplemented with 1 mg/l BAP and 0.5 mg/l kn (Upadhyay *et al*., 2015). On increasing the concentration of BAP decrease in shoot induction frequency and number of shoots was recorded. Shoot regeneration with BAP containing medium has been reported in several medicinal plants including *Withania somnifera* (Singh *et al*., 2017), *Phyllanthus amarus* (Chitra *et al*., 2009), *Scrophularia kakudensis* (Manivannan *et al*., 2015).The regenerated shoots could be maintained by subculturing on MS medium supplemented with 4.4 µM BAP.

Callus Induction

In this study the callus was induced from the nodes cultured on MS medium supplemented with 2,4-D. All the concentration of 2,4-D were effective for callus induction. However, the percent frequency and callus growth differ significantly on different concentration of 2,4-D used in the culture medium. The maximum frequency of callusing was recorded on medium supplemented with 13.6 µM 2,4-D while the optimum callus growth was recorded on the medium having 4.5µM 2,4-D. The regenerated callus was friable, white, light green and cream in colour (Fig. 3D). On medium with13.6 µM2,4-D though the frequency of callusing was maximum but callus growth was slow and differ significantly with callus growth on medium with 4.5µM 2,4-D(Table 2). This showed that an optimum concentration of 2,4-D is required for optimum callus induction and growth. The observation that the addition of optimum growth regulator concentration for callus regeneration has also been shown in other plant species including *Dendrocalamus strictus* (Singh *et al*., 2019), *Withania somnifera* (Gaurav and Kumar, 2019). The callus cultures were maintained by subculturing on MS medium having 4.5µM 2,4-D with an optimum about 3.0 gm increase in fresh weight of callus in four weeks (Table 2).

Phytochemicals from callus and shoot culture

The phytochemical analysis has shown that callus and shoot cultures accumulate phenolic and flavonoid compounds but the quantification of phenolics and flavonoid compounds showed that the accumulation of these phytochemical was significantly high in regenerated shoots than the callus (Fig. 1). The accumulation of phenolics and flavonoids in *in vitro* regenerated shoot was similar to that of the phenolics and flavonoid accumulation in shoots collected from the field grown intact plant (Table 3). Similar observation is reported in several medicinal plants including *Eryngium alpinum* (Kikowska *et al*., 2020). Production of phyllanthin, phyltetralin, hypophyllanthin and niranthin, the anticancerous and hepatoprotective polyphenolic lignans from the cultures of has been demonstratedin *P. tenellus* (Nikule *et al*., 2020). In this study the ability to scavenge free radicals in the extracted phytochemical was confirmed by the DPPH test (Fig. 2). Antioxidant activity from the extracts of *in vitro* cultures is also reported in *Scrophularia kakudensis* (Manivannan *et al*., 2015) *Phyllanthus* spp. (Muthusamy *et al*., 2016). Thus, this study showed that *in vitro* regenerated biomass could be utilised for phyto pharming without effecting natural biodiversity.

Table 1: Effect of cytokinin on shoot induction and multiplication from node

Growth Regulator (GR)	Concentration of GR (µm)	Percent response (%) (Mean ± SD)	Number of shoots per explants (Mean ± SD)	Length of Shoots (cm) (Mean ± SD)
BAP	0.00	42.80 ± 5.09	1.10 ± 0.10 [a]	1.05 ± 0.41 [a]
	0.44	82.02 ± 3.93[a]	3.20 ± 0.57 [b]	1.96 ± 0.34 [b]
	4.4	100.00[b]	8.23 ± 0.65[c]	1.12 ± 0.40 [a]
	20.0	87.47 ± 6.23[a]	4.42 ± 0.62[b]	0.88 ± 0.24 [a]
Kn	0.46	62.00 ± 6.23[c]	2.21 ± 1.10[b]	1.65 ± 0.64 [b]
	4.65	84.07 ± 7.59[a]	2.50 ± 0.54[b]	0.85 ± 0.14 [a]
	23.25	77.64 ± 4.32[d]	1.20 ± 0.38 [a]	0.54 ± 0.13[c]

Values represent the mean ± SD of three experiments each with twelve replicates. Means followed by same letter(s) within a column are not significantly different ($P \leq 0.05$)

Table 2: Effect of 2,4-D on callus induction and growth from node explant

Conc. of 2,4-D (µM)	Per cent callusing (Mean ± SD)	Fresh weight (gm) (Mean ± SD)	Dry weight (Mean ± SD)
0.00	0.00	0.00	0.00
0.45	57.5 ± 3.81[a]	1.95 ± 0.09[a]	0.17 ± 0.031[a]
4.50	69.9 ± 1.83[b]	3.21 ± 0.14[b]	0.43 ± 0.023[b]
13.6	81.02 ± 3.46[c]	2.52 ± 0.32[c]	0.33 ± 0.021[c]
22.67	76.2 ± 3.65[b]	2.47 ± 0.22[c]	0.31 ±0.020[c]

Values represent the mean ± SD of three experiments each with twelve replicates. Means followed by same letter(s) within a column are not significantly different ($P \leq 0.05$)

Table 3: Phenolics and flavonoids quantification from different plant material

Plant material	Phenolics (mg GE/g DW) (Mean ± SD)	Flavonoids (mg QE/g DW) (Mean ± SD)
In vitro shoot	12.87 ± 1.16[a]	09.52 ± 1.42[a]
Callus	08.12 ± 0.83[b]	05.22 ± 0.46[b]
In vivo shoot	10.21 ± 1.37[a]	09.91 ± 0.81[a]

Values represent the mean ± SD of three experiments each with three replicates. Means followed by same letter(s) within a column are not significantly different ($P \leq 0.05$)

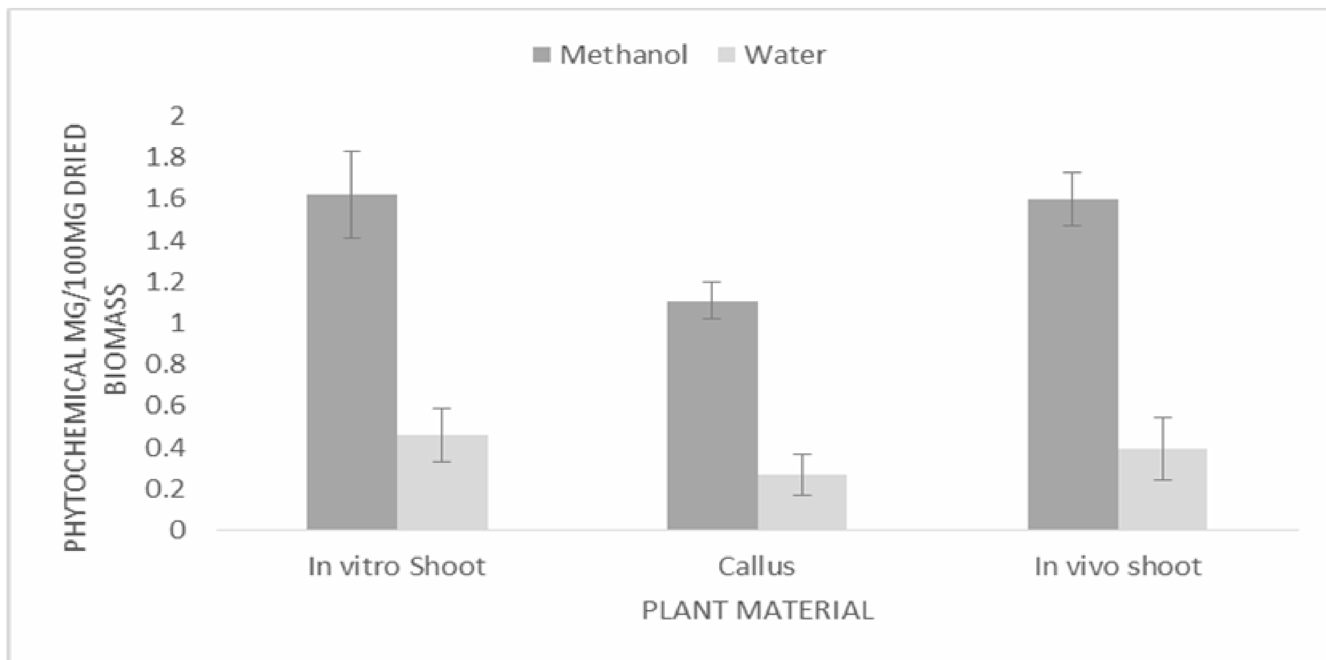

Fig. 1: Amount of Phytochemical extracted from different plant material.

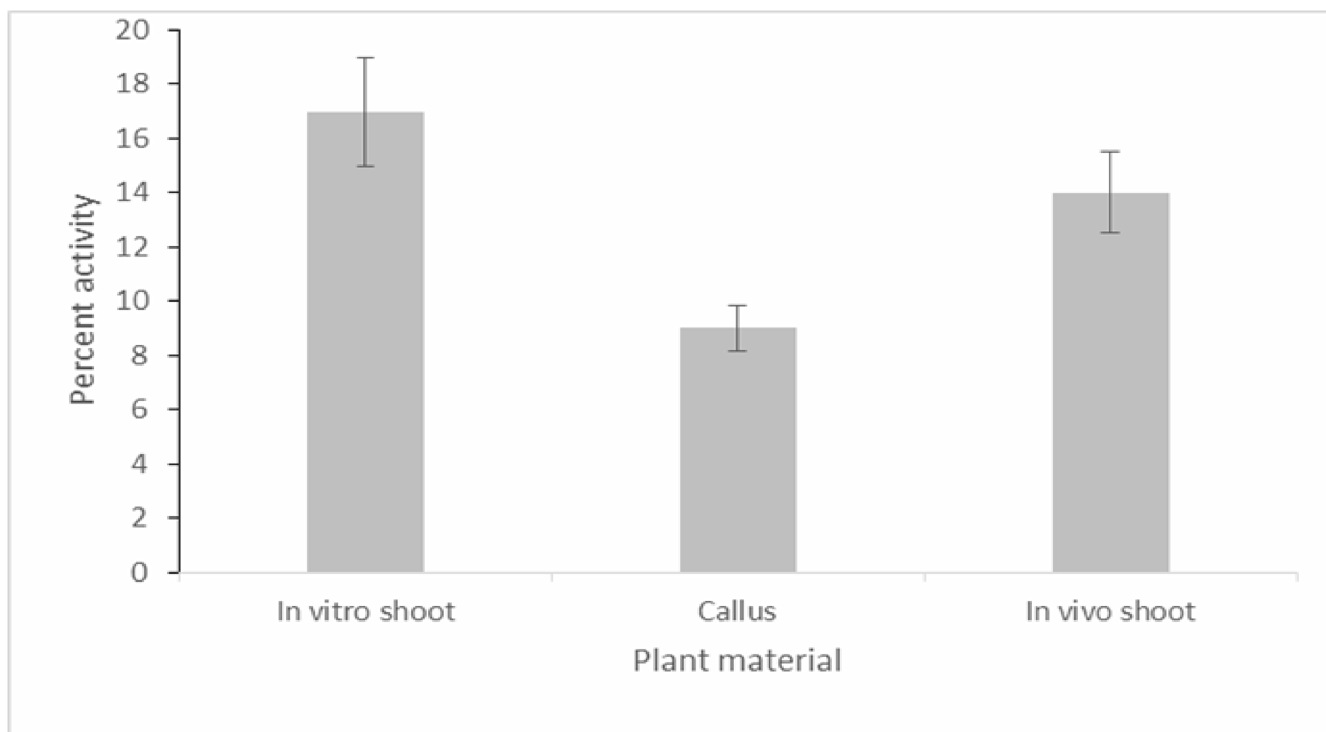

Fig. 2: Antioxidant activity tested by DPPH in extracts of different plant material

Figure 3: Callus and shoot regeneration: A Plant growing in university campus, B: node cultured for shoot initiation, C Multiple shoot induction, D: Callus induction and growth

Conflict of Interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

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