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BIOASSAY OF PHYLLANTHIN AND HYPOPHYLLANTHIN BY HPTLC IN *PHYLLANTHUS AMARUS* SCHUM & TH. (EUPHORBIACEAE) GROWING UNDER *IN VIVO* AND *IN VITRO* CONDITIONS.

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The present investigation is related to *Phyllanthus amarus* Schum. & Th. (Euphorbiaceae) which is one of the most widely used drug in Ayurvedic medicine to cure liver diseases. It contains two important lignans: (i) phyllanthin and (ii) hypophyllanthin, which are responsible for its antiviral activity. The present market demands of the herb needs increased production of the secondary bioactive metabolites which can be achieved by medicinal biotechnological methods. Thus, an effort was aimed to bioassay , (i) Phyllanthin and (ii) Hypophyllanthin under (I) *in vivo* conditions in different organs (root, stem and leaf) of plant in 30 days old and 90 days old crop and (II) *in vitro* grown in 10 weeks old friable callus and 10, 11 and 12 weeks old compact calli by HPTLC method. This is the first report of callus culture of *P.amarus* that successfully produced phyllanthin. Phyllanthin and hypophyllanthin have also been detected in roots of the *in vivo* grown plants. Optimum amount of phyllanthin in 10 weeks old friable callus appeared in MS basal medium with BAP (1mg/l), which is most suitable culture media for growth of callus and phyllanthin production.

Keywords: *P. amarus*,Phyllanthin, Hypophyllanthin, *in vivo,in vitro,* HPTLC

P. amarus "The Little Wonder Herb", has been used in Asian subcontinent since ages due to its curative actions against liver diseases. All the parts of the herb i.e., root, stem and leaf have astringent, deobstruent, stomachic, diuretic and febrifugal properties (Kritikar and Basu 2001). Present day researchers are focused on this species as a potential plant for the treatment of Hepatitis 'B' by increasing various antioxidants and reduce lipid peroxidation of hepatic cellular and intracellular membranes. Thus *phyllanthus amarus* protects liver damage due to free radicals in hepatitis- B (Padmaja *et al*. 2010). Phyllanthin and hypophyllanthin are therapeutically potent bioactive constituents serving as hepatoprotective agents by protecting hepatocytes (Padma and Setty 1999). Unfortunately, the herb is getting indiscrimately exploited from nature to prepare several hepatoprotective formulations by various pharmaceutical companies, rendering it 'Threatened'. Its traditional uses as natural remedy against several diseases viz. Jaundice, Hepatitis, Malaria, Asthma and its diuretic, antiviral and hypoglycemic properties (Calixto *et al*. 1998) provide evidence for a real biological activity, hence, studies were undertaken to bioassay its marker compounds (phyllanthin and hypophyllanthin) by HPTLC under *in vivo* and *in vitro* conditions to compare their profile. The tissue culture mediated approach has emerged as viable method for the production of medically active compounds because they are synthesized by cell cultures (Kumar 2000).

MATERIALS AND METHODS

P.amarus was analyzed at three different stages of growth i. e., (i) 90 days old mature plants for phyllanthin and hypophyllanthin, (ii) 30 days old plant and (iii) compact and friable calli for phyllanthin only. All together 10 samples were analyzed which have been designated as 90 days old plant [Root mature (RM), Stem mature(SM) and Leaf mature (LM)]; 30 days old plant [Root (R), Stem (S) and Leaf (L)]; 10 weeks old Friable Callus (10FC); 10 weeks old Compact Callus (10CC); 11 weeks old Compact Callus (11CC) and 12

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Table1. Peak height and area percentage of phyllanthin & hypophyllanthin in *P. amarus* (90 days old plant) grown under *in vivo* conditions.

Track	Peak	Start Rf	Start ht	Max Rf	Max Ht	Ht %	End Rf	End Ht	Area	Area %
1	1	0.14	3.7	0.19	403.5	100.00	0.22	1.7	10504.7	100.00
2	1	0.10	0.6	0.14	86.3	9.35	0.16	68.7	2215.6	8.49
2	2	0.16	69.1	0.19	336.6	36.44	0.23	7.1	8893.7	34.08
2	3	0.23	7.2	0.25	54.3	5.87	0.28	0.6	1129.9	4.33
2	4	0.28	0.0	0.34	342.0	37.03	0.38	6.8	1379.6	43.60
2	5	0.40	26.0	0.43	47.8	5.17	0.44	41.4	201.8	4.61
2	6	0.44	41.5	0.46	56.7	6.14	0.49	0.2	277.1	4.89
3	1	0.07	3.0	0.10	34.0	9.46	0.13	0.0	600.4	6.06
3	2	0.16	0.0	0.21	83.9	23.34	0.24	0.8	506.1	25.29
3	3	0.28	0.1	0.30	28.7	8.00	0.31	27.1	408.8	4.12
3	4	0.31	27.2	0.32	44.2	12.30	0.35	24.1	116.7	11.27
3	5	0.35	24.1	0.39	76.9	21.41	0.42	23.3	2787.0	28.13
3	6	0.42	25.7	0.45	91.5	25.49	0.48	9.4	2489.8	25.13
4	1	0.01	3.2	0.02	15.1	6.69	0.04	0.0	105.6	1.78
4	2	0.04	0.9	0.07	26.3	11.61	0.08	0.1	417.3	7.05
4	3	0.08	0.0	0.10	10.8	4.79	0.11	0.5	131.1	2.21
4	4	0.12	0.4	0.19	104.6	46.23	0.23	1.4	3215.5	54.31
4	5	0.29	0.1	0.34	33.7	14.91	0.38	13.0	1317.3	22.25
4	6	0.44	19.2	0.46	23.5	10.37	0.48	8.7	547.7	9.25
4	7	0.48	9.0	0.49	12.2	5.40	0.51	0.5	186.5	3.15
5	1	0.31	0.5	0.36	402.1	94.41	0.39	4.2	11780.9	95.72

1*: Standard (Phyllanthin), **2*:** RM (Root Mature), **3*:** SM (Stem Mature), **4*:** LM (Leaf Mature) and **5*:** Standard (Hypophyllanthin).
***: Track.**

weeks old Compact Callus (12CC). All the compact calli have developed hairy roots. The several steps of investigated methodology are as follows:

***(I) In vivo* multiplication**: Plants were identified and authenticated at Botanical Survey of India, Sibpur, Howrah. The seeds were collected and sown to prepare nursery in the Polyhouse of Deptt. Of Botany, Patna University, Patna. Seedlings (3-4" long) were transplanted in the soil mix (soil: balu: FYM) in the ratio of 2:1:1. Harvesting was done after 30 days and 90 days (maturity time), parts (root, stem and leaf) were separated and dried under semi shade conditions.

***(II) In vitro* multiplication:** Calli were established in Murashige and Skoog (1962) medium fortified with BAP (1mg/l), 10% coconut milk and 3% sucrose. Nodal stem segments were used as explants and 10FC, 10CC, 11CC and 12CC week's old calli were taken out from the semisolid media, washed with luke warm water to remove agar and air dried to prepare samples.

(III) Sample preparation: Air dried samples were separately grinded to powder in pestle and postle, stored in sterilized specimen tubes and sealed to be used for analytical studies.

(IV) **HPTLC analysis:** All the ten samples were separately extracted with methanol. The extracts were applied with a Linomat IV applicator onto two different HPTLC plates (Merck, 0.2 mm thickness, F_{254}) i.e., one with separate samples of root, stem and leaf of 90

Table2. Peak height and area percentage of phyllanthin in *P. amarus* (30 days old plant and Calli at different stages of growth).

Track	Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	1	0.34	1.9	0.38	214.7	100.00	0.42	4.4	4960.0	100.00
2	1	0.32	8.0	0.37	380.9	100.00	0.40	0.4	9788.5	100.00
3	1	0.32	2.8	0.37	388.2	100.00	0.40	0.2	10088.9	100.00
4	1	0.32	0.4	0.35	31.9	100.00	0.40	0.2	765.6	100.00
5	1	0.39	13.3	0.43	49.1	100.00	0.47	1.1	1716.9	100.00
6	1	0.40	1.7	0.44	28.8	100.00	0.47	6.5	768.2	100.00
7	1	0.41	4.9	0.45	33.8	100.00	0.48	3.3	792.3	100.00
8	1	0.34	0.7	0.39	250.2	100.00	0.44	0.3	5653.3	100.00
9	1	0.35	3.7	0.39	250.1	100.00	0.43	2.1	5629.5	100.00
10	1	0.33	2.7	0.37	41.7	100.00	0.41	0.1	1275.3	100.00
11	1	0.34	4.8	0.37	44.4	100.00	0.41	0.8	1257.1	100.00
13	1	0.37	6.1	0.39	12.5	100.00	0.41	0.1	226.8	100.00
14	1	0.35	7.2	0.38	26.2	100.00	0.40	8.6	627.6	100.00
15	1	0.34	3.6	0.37	21.3	100.00	0.40	3.6	541.9	100.00
16	1	0.35	2.2	0.38	23.5	100.00	0.41	0.0	576.6	100.00
17	1	0.34	4.5	0.37	24.4	100.00	0.41	0.0	710.1	100.00
18	1	0.36	0.1	0.41	262.9	100.00	0.45	0.3	5936.0	100.00

1*: Standard (Phyllanthin), **2*- 3*:** Leaf (L), 30 days old, **4*- 5*:** Stem (S), 30 days old, **6*- 7*:** Root (R), 30 days old, **8*- 9*:** Standard (Phyllanthin), **10*- 11*:** (10FC), 10 weeks old Friable Callus, **12*- 13*:** (10CC) 10 weeks old Compact Callus, **14*- 15*:** (11CC), 11weeks old Compact Callus, **16*- 17*:** (12CC) 12weeks old Compact Callus and **18*:** Standard (Phyllanthin).

days old plant along with standards of phyllanthin and hypophyllanthin, the second one with separate samples of root, stem and leaf of 30 days old plant, 10 weeks old friable and compact calli, 11 and 12 weeks old compact calli along with standard phyllanthin. The standards used were of Sigma Co. Ltd., USA. After drying, the plates were developed with the solvent (Toluene: Ethyl acetate:: 6.5: 3.5), video documented using Camag Reprostar III Documentation Unit and then densitometrically scanned by CAMAG TLC Scanner-3 using WinCat 3.2 Software at 280 and 282 nm. The Rf values of test samples were compared with the markers. Peak purity was ascertained using spectrum analysis of the standards with those of the samples in the eluted plate. Peaks were recorded under UV light at 280 and 282 nm of wavelength. The peak height, area percentage, Rf values and percentage of phyllanthin and hypophyllanthin were estimated for all the samples.

OBSERVATIONS

90% calli were obtained out of which 70% were compact, pale yellow in colour which turned green and roots started emerging out and 20% were scattered, friable and creamish white in colour.

Estimation of phyllanthin and hypophyllanthin (w/w) has been presented in

Table 3. HPTLC Profiles of phyllanthin and hypophyllanthin in *P. amarus*

Sl.No. of samples	Organs of Herb	Phyllanthin (%)	Hypophyllanthin (%)	Growth stage of Samples (Days/Weeks)
1	Leaf (LM)	0.364 ± 0.0005	0.415	90 days
2	Stem (SM)	0.109 ± 0.0002	0.062	
3	Root (RM)	0.076 ± 0.0005	0.377	
4	Leaf (L)	0.0165±0.0005	-	30 days
5	Stem (S)	0.00019 ± 0.0045	-	
6	Root (R)	0.00065 ± 0.0045	-	
7	10 FC	0.00099± 0.0005	-	10 weeks
8	10 CC	0.00023± 0.0006	-	10 weeks
9	11 CC	0.00049 ± 0.00045	-	11 weeks
10	12 CC	0.00052 ± 0.0005	-	12 weeks

Tables (1-3). Maximum concentration of phyllanthin was quantified in mature crop i.e., LM (0.364%) at 0.19 Rf and 280 nm (Tables 1&3). Hypophyllanthin has been detected in roots (0.377%) of mature crop also. It was maximum in LM (0.415%) and minimum in SM (0.062%) at 0.36 Rf and 280 nm (Tables 1&3). In 30 days old *in vivo* grown plants and *in vitro* grown calli, maximum concentration of phyllanthin was recorded in sample L (0.0165%) while 0.00019% and 0.00065% were estimated in samples R and S respectively (Tables 2& 3) at 0.38 Rf and 282nm against the standard phyllanthin. Concentration of phyllanthin observed in 10FC was 0.00099% at 0.39 Rf, where as 10CC, 11CC and 12CC exhibited minimum concentration in rooted compact calli where roots have emerged out (Table 3).

DISCUSSION

Plant tissue culture methods may be utilized to screen explants of different plants to assess the production of bioactive compounds *in vitro* and results compared with its *in vivo* counter parts. Moreover, if the levels are higher under *in vitro* system, then their synthesis could be commercialized to isolate useful chemicals. Uses of herbal drugs have increased many fold in many years which has caused environmental unbalance, hence investigations should be targeted to estimate and enhance the secondary metabolites under *in vitro* conditions. Sexena *et al*. (2000) undertook the studies to screen sec. metabolites by growing them in tissue culture and their identification by Thin Layer Chromatography. With the above mentioned objectives the finding of the present work are discussed. The investigated herb has well documented history of hepatoprotective potentials which has been thoroughly investigated and established in recent years.

The defined marker chemicals i.e., lignans (phyllanthin and hypophyllanthin) in *P.amarus* were extracted in methanol and for HPTLC analysis the mobile phase used was hexane: acetone: ethyl acetate (v/v/v). Arvind *et al*. (2006) have recommended methanolic extraction for lignans, hexane: acetone: ethyl acetate to be used as mobile phase for better resolution and HPTLC technique for rapid screening of germplasms of *Phyllanthus*

species for the determination of chemical profile and quantification. Aeri *et al*. (2000) and Christian *et al*. (2006) have recommended HPTLC technique for maximum extraction, recovery, better resolution and peak purity for phyllanthin and hypophyllanthin while Annamali and Laxmi (2009) made a comparison of HPTLC and HPLC methods of analysis to assay phyllanthin from *P. amarus.* HPTLC method should be used for bioassay of secondary. metabolites because it can also detect the metabolites in trace amounts. The assay can also be applied without any special pretreatment of the sample; moreover, large number of samples can be analyzed in a single run without compromising accuracy.

***In vivo*:** The results of *in vivo* grown mature plants (90 days old) shows optimum accumulation of phyllanthin in leaves which is 0.364% (w/w) followed by stem (0.109%) and least in roots (0.076%) at 0.19 Rf (Table- 1& 3). Similar trend was recorded in 30 days old plant i.e., 0.0165% (w/w), 0.00019% and 0.00065% in leaf, stem and root respectively at 0.38 Rf (Tables-2 & 3). Bioassay of phyllanthin for *in vivo* grown plants points to the fact that: (1) phyllanthin is present in all the organs of the plant but its concentration is optimum in the leaves. Similar results have been obtained by Arvind *et al*. (loc.cit) and Srivastava *et al*. (2008) in the four species of *Phyllanthus* i.e., *amarus, maderaspatensis, urinaria* and *virgatus,* (2) the presence of phyllanthin in 30 days old plant is indicative of the fact that the synthesis of this metabolite starts at very early stage of plant growth and (3) roots also accumulate, the bioactive component though in meager amount. A thorough investigation by the earlier workers (Annamali 2000, Annamali *et al*. 2004) revealed that phyllanthin accumulation can be increased in the plant organs by replenishing the nutrition. Sane *et al*. (1997) and Murali *et al*. (2001) have discussed the enhancement effect in the light of maintenance of ideal conditions i.e., the period of harvest around 90 days from the date of sowing.

The amount of hypophyllanthin was maximum in leaf (0.415%) and minimum in stem (0.062%) unlike phyllanthin which was minimum in root (0.076%) in mature crop. Hypophyllanthin has been detected by previous workers Elfahim *et al.* (2006) and Arvind *et al*. (loc. cit) (in earial plant parts) and by Gupta *et al*. (2005) in whole plant hast. In the present report, hypophyllanthin have been detected in root also (0.377%) Table-3. The result clearly indicates that roots are associated with biosynthesis of hypophyllanthin and by growing hairy roots in tissue culture, hypophyllanthin could be extracted in appreciable amount. Stray reports are available on this metabolite. Hypophyllanthin has been detected only in *P.amarus* by Arvind *et al*.(loc. cit.). However Calixto *et al*. (loc. cit.) reported 0.32-0.37% in dry herb in 'CIM- Jeevan' cultivars of *P.amarus*. 17 different lignans have been reported by Elfahim *et al*. (loc. cit) in *P.niruri* and hypophyllanthin is one of them. According to Ganeshaiah *et al*. (1998) the species *amarus* and *niruri* are synonyms and its detection in *niruri* species confirms the theory that "*P.niruri* 'A Taxonomic hurdle or hurdled by taxonomist'. The available literature and present findings are complementary to each other and it appears that hypophyllanthin is specific to *amarus* species. By practicising standard agro technological methods of propagation, amounts of phyllanthin and hypophyllanthin could be increased under *in vivo* conditions.

***In-vitro*:** The data for phyllanthin under *in vitro* growth conditions have been presented in (Table-2&3). The interesting observations includes: (i) MS media with BAP (1mg/l) is most suitable for callus formation (ii) Friable calli accumulates optimum concentration of phyllanthin (iii) 10 weeks is the optimum time for the synthesis of phyllanthin and (iv) The compact callus had meager amount of phyllanthin and as soon as root initiation starts, its concentration declines as evidenced by 10 CC where it is only 0.00023% (Table- 3). For commercial purpose phyllanthin could be extracted only from friable calli and its reduction in 10CC may be due to root initiation where nutrients are redistributed (part of the

callus and part of the roots) or the other possible reasons may be that with root initiation, the biosynthetic pathway of phyllanthin may be disturbed, distorted or a temporary brake is possible which is restored after the emergence of roots as observed in 11CC and 12CC where there is gradual enhancement in the amount i.e. 0.00049% and 0.00052% (Table- 3) have been witnessed. This is the first report of phyllanthin accumulation in nodal callus.

In the present study the callus was initiated on MS media with BAP (1mg/l) from the nodal segments of the stem because due to the presence of lateral buds, callus initiation is very fast. Friable callus appeared in clumps after 6 weeks varying in colour from dark brown to yellow which were analyzed after 10 weeks of growth. The presence of maximum concentration of phyllanthin at this stage indicates that maximum stress occurs at this stage of growth and after this the callus starts losing its colour and texture and this may be the reason for reduction in phyllanthin concentration. So, in order to procure phyllanthin from nodal callus, 10 weeks is the most suitable period.

However, further in depth investigation is required for rapid growth of callus, reproducibility of result establishment of suspension cultures to enhance the production of phyllanthin along with bioassay of hypophyllanthin under *in vitro* conditions.

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