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Estimation of anti-hepatic viral compounds in *Phyllanthus amarus in vitro* cultures

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ABSTRACT

***Phyllanthus amarus* Schum. and Thonn (Euphorbiaceae) is recognized commonly as 'Bhumyamlaki' in the Indian system of medicine and has been traditionally used for treating a variety of ailments, including hepatic disorders. Anti-hepatic viral compounds such as *phyllanthin* and *hypophyllanthin* were evaluated in different types of *in vitro* cultures of *Phyllanthus amarus* by High Performance Liquid Chromatography (HPLC). Among the cultures, *in vitro* plantlets regenerating from the nodal segment recorded higher amounts of *phyllanthin* and *hypophyllanthin*.**

Key Words: *Phyllanthus amarus*, HPLC, phyllanthin and hypophyllanthin

INTRODUCTION

A large number of chemical compounds including fragrances, flavours, pigments, natural sweeteners, antimicrobials and pharmaceuticals are obtained from plants. In most cases, these compounds belong to a broad metabolic group, collectively referred to as secondary products. Plant cell cultures can be established from an array of plant species, including most that produce secondary products of commercial value (Berlin, 1984).

Phyllanthus amarus (Euphorbiaceae) finds a reputed place, especially, in the Indian Pharmacopoeia (Kamboj, 2000). It has been traditionally used in the treatment of a variety of ailments, including hepatic disorders (Nadkarni, 1976). It is a potential diuretic, hypotensive and hypoglycaemic drug (Raphael, 2002). It has immense medicinal properties by virtue of containing several phytochemicals, *viz*., securinine, norsecurinine, epibubbialine and isobbubialine (Foo and Wang, 1992), lignans like phyllanthin and hypophyllanthin (Row *et al*, 1966), phenolics like gallic acid; polyphenolics like ellagic acid, phenazine and phenazine derivatives (Foo, 1995). About 300 million people worldwide are estimated to be carriers of the Hepatitis B virus. The plant has therapeutic potential for treating Hepatitis B virus by inhibiting polymerase activity and decreased episomal DNA content.

It has also been shown to exhibit antihepatotoxic activity against carbon tetrachloride and galactosamine in primary cultured rat hepatocytes (Syamasunder *et al*, 1985). Knowledge about *Phyllanthus amarus* especially on its anti-viral property, has elicited a great interest in this plant, and has triggered its large-scale collection from natural flora. Availability of this plant is subject to seasonal variations, which leads to uncertainty in supply of the plant material when required (Rajasubramanian and Pardha saradhi, 1997).

In vitro secondary metabolite extraction has been a precision tool for studying organic compounds in plants even when present in trace quantities. The study of cell suspension culture, hairy root culture and other *in vitro* cultures is an ideal method to investigate the rare compounds and, especially, many active, unknown compounds within a short period.

In this background, the study was taken up to explore a lignans from *in vitro* culture of *Phyllanthus amarus*. In field grown crops, it takes about six months for extraction of these lignans whereas this time lag is just three months in *in vitro* cultured plantlets.

MATERIAL AND METHODS

Indirect organogenesis

Murashige and Skoog (1962) medium was used for induction of callus from leaf bits, stem pieces, shoot tips and nodal segments of *Phyllanthus amarus*. Sucrose (3.0%), agar (0.8%), cytokinins, viz., kinetin (3.0 mgl^{-1})

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and BAP (3.0 mgl^{-1}) and auxins, viz., 2, 4-D (4.0 mgl^{-1}) and NAA (0.4 mgl^{-1}) were added to the medium.

Cultures containing 2, 4-D was inoculated under darkness by covering culture racks with a black cloth and the remaining cultures were incubated at 25±2°C in light: dark cycle of 16:8 h, respectively.

Direct organogenesis

For direct organogenesis by axillary shoot proliferation or by adventitious shoot formation, the explants, *viz*., shoot tip and nodal segments, were inoculated onto MS basal medium supplemented with BAP(2.0 mgl^{-1}) along with GA_3 (1.0 mgl^{-1}).

After separating the multiple shoots, each individual shoot was sub-cultured onto half strength MS medium containing two auxins, IAA (0.5 mgl^{-1}) and IBA (0.5 mgl^{-1}). The Cultures were maintained in a growth room at 24±2°C under 16 h light and 8 h dark photoperiodic regime.

Estimation of anti-hepatic viral compounds

For estimation of lignans, different types of cultures were used as follows:

Cultures used for estimation of anti-hepatic viral compounds

Treatment (Nature of culture)	Source of culture	Culture medium on which the culture was initiated
T_1 (Multiple shoot clumps with basal callus)	Shoot tip	MS + BAP (2.0 mgl^{-1}) + GA_3 (1.0 mgl^{-1})
T_2 (Multiple shoot clumps with basal callus)	Nodal segment	MS + BAP (3.0 mgl^{-1}) + GA_3 (1.0 mgl^{-1})
T_3 (Micro shoots with roots)	Shoot tip	½ MS + IBA (0.5 mgl^{-1}) + IAA (0.5 mgl^{-1})
T_4 (Micro shoots with roots)	Nodal segment	½ MS + IBA (0.5 mgl^{-1}) + IAA (0.5 mgl^{-1})
T_5 (Green callus)	Shoot tip	MS + BAP (3.0 mgl^{-1}) + Kin (3.0 mgl^{-1})
T_6 (Green callus)	Nodal segment	MS + BAP (3.0 mgl^{-1}) + Kin (3.0 mgl^{-1})
T_7 (White callus)	Stem pieces	MS + 2,4-D (4.0 mgl^{-1}) + NAA (0.4 mgl^{-1})
T_8 (White callus)	Leaf bits	MS + 2,4-D (4.0 mgl^{-1}) + NAA (0.4 mgl^{-1})

Analysis of anti-hepatic viral compounds

The above *in vitro* grown materials were dried and ground to a fine powder using a mortar and pestle. Each powdered sample (1 g) was macerated with lime (300 mg) and HPLC grade water (2.5 ml) at room temperature and kept in a shaker for 18 hours. Thirty ml of methanol containing 3% Potassium hydroxide was added to the macerated material and kept in boiling water-bath for 30 min. The material was filtered the residue washed 3 times with 5 ml methanol and the volume of the combined filtrate and washings was made up to 50 ml. A sample (10 µl) of this solution was injected in to HPLC column and the lignans were estimated.

A Varian Chromatographic system comprising of L.C.8A Model dual pump and UV detector was employed. A µ Bondapak C_{18} column (30 cm X 3.9 mm) with isocratic run of solvent system of methanol: water (66:4), v/v at the rate of 1.8 ml/min flow rate and UV detection at 230 nm was used for resolving and analysing phyllanthin and hypophyllanthin. The quantification was carried out using external standards of phyllanthin and hypophyllanthin (Sigma Aldrich chemicals) and values were expressed as percentage.

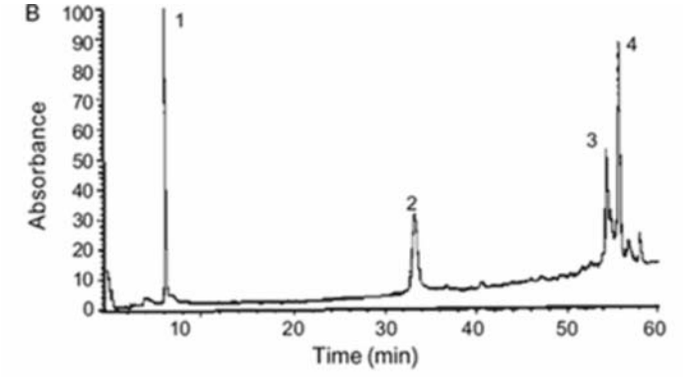
RESULTS AND DISCUSSION

The lignans were detected and quantified in the *in vitro* cultures of *Phyllanthus amarus*. Among the various cultures, *in vitro* grown plants recorded highest phyllanthin (0.921%) and hypophyllanthin (0.396 %) on ½ MS medium containing IBA (0.5 mgl^{-1}) and IAA (0.5 mgl^{-1}) as compared to the field grown plants. Phyllanthin (0.709 w/w dry basis) and hypophyllanthin (0.271 w/w dry basis) content was estimated by the method of Anupum Sharma *et al* (1993) in fieldgrown *Phyllanthus niruri.* Similar finding was also reported by Mahalakshmi *et al* (2006) in *Phyllanthus amarus* genotypes. This was supported by the findings of Ara Kirakosyan (2003) in *Hypericum perforatum* and Sharma Tripti, (2006) in *Artemisia annua*. The contents of phyllanthin (0.714 %) and hypophyllanthin (0.261%) was low in leaf-bit derived white callus on MS medium supplemented with 2,4-D (4.0 mg l^{-1}) and NAA (0.4 mgl^{-1}). The level of hypericin in *Hypericum perforatum* callus was very low, representing only 0.11% of that found in field-grown plants (Kirakosayan, 2003). Callus initiated from stamens of *H. perforatum* showed only traces of hypericin or pseudohypericin (Kirakosyan *et al*, 2000). In general, an increase in the level, of auxins such as 2, 4-D in the medium stimulates dedifferentiation of cells and consequently diminishes the level of secondary metabolites. This is the reason that auxins are commonly added to the medium for callus induction, but used at low concentrations or omitted altogether for production of metabolites. Zenk *et al* (1977) reported that cytokinins stimulated alkaloid synthesis which was induced by removing auxin from the medium of a cell line of *Catharanthus roseus*. This was

supported by the findings of Shiio and Ohta (1973) along with Takahashi and Yamada (1973). They reported that lower concentrations of auxins viz., IAA, NAA and 2,4-D promoted nicotine synthesis in tobacco cell cultures while higher concentrations inhibited nicotine synthesis.

In the present study, the quantity of phyllanthin and hypophyllanthin was found to be higher in *in-vitro* grown plants than in the callus. Thus, it seems that in many cases morphological differentiation may be necessary to obtain higher yields of secondary metabolites. Hiraoka and Tabata (1974) investigated the correlation between stage of morphological differentiation and tropane alkaloid producing ability in *Datura meteloides*, and found that roots forming shoots produced higher amounts of tropane alkaloids. Dhar and Pal (1988) demonstrated that more pyrethrin was being synthesized in *Chrysanthemum cinerariaefolium in vitro* shoots than the roots and its content was even lower in undifferentiated callus culture.

Chromatographic analysis of the regenerated plants of *Phyllanthus amarus* showed antiviral content higher than that found in field-grown plants, suggesting that an *in vitro* culture system could be used for this plant which may significantly reduce the cost, time and resources required for field-production of antiviral compounds, as well as enable growers to produce high quality, standard antiviral products.



3rd Peak - Hypophyllanthin (Retention time 54.33 minutes)
4th Peak - Phyllanthin (Retention time 56.87 minutes)

Fig 1. HPLC chromatogram of *Phyllanthus amarus*

Table 1. Estimation of anti-hepatic viral compounds from different types of cultures

Sl.No	Nature of culture & Source of culture	Phyllanthin (%)	Hypophyllanthin (%)
1.	Multiple shoot clumps with basalcallus (Shoot tip)	0.745	0.298
2.	Multiple shoot clumps with basal callus (Nodal segment)	0.723	0.289
3.	Microshoots with roots (Shoot tip)	0.892	0.325
4.	Microshoots with roots (Nodal segment)	0.921	0.396
5.	Green callus (Shoot tip)	0.716	0.294
6.	Green callus (Nodal segment)	0.731	0.309
7.	White callus (Stem bit)	0.728	0.314
8.	White callus (Leaf bit)	0.714	0.261
Mean		0.771	0.311
SEd		0.027	0.016
CD (*0.05*)		0.054	0.034

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