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HPTLC and HPLC Analysis of Bioactive Phyllanthin from Different Organs of *Phyllanthus amarus*

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Abstract: One of the most widely used herbs in Ayurvedic medicine is *Phyllanthus amarus*, which is predominantly grown in Indian subcontinent. Since, the market surveillance of herbal drugs is reported to contain minimum 0.5% of phyllanthin, demands increased production of this herb, which could be achieved by standard agrotechniques. Moreover, since, it is also desirable to check for the effect of these improved measures on the bioactive compound-phyllanthin, an effort was aimed at investigating a standard strategy to quantify the principle compound. Hence, a detailed investigation was adopted to provide a comparative quantification of phyllanthin through High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) from different organs of *P. amarus* grown with improved agrotechniques. However, it revealed to be more effective through HPLC especially higher in the leaves as against the analysis through HPTLC.

Key words: Phyllanthin, *Phyllanthus amarus*, comparative quantification, HPTLC, HPLC

INTRODUCTION

The use of traditional medicine in both developing and developed countries has significantly increased in recent times. *Phyllanthus amarus* Schum. and Thonn. (Euphorbiaceae), popularly known as Bhumyamalaki in Sanskrit and Keezhaanelli in Tamil finds a reputed role in folk remedy for the treatment of a variety of ailments. Different parts of the plants are ethonobotanically reported to have various therapeutic activities such as, leaves as expectorant, diaphoretic and diuretic (Kirtikar and Basu, 2001). Besides these, the lignans-phyllanthin and hypophyllanthin present are hepatoprotective-protecting hepatocytes against carbon tetrachloride (CCl_4) and galactosamine induced cytotoxicity in rats (Syamasundar *et al*., 1985). It has been reported to exhibit marked anti-hepatitis B virus surface antigen activity both *in vivo* (Thyagajaran *et al*., 1988; Blumberg *et al*., 1990; Shead *et al*., 1992) and *in vitro* studies (Mehrotra *et al*., 1991; Unander and Blumberg, 1991). Furthermore, the plant has been reported to possess anti-bacterial (Verpoorte and Dihal, 1987), anti-crustacean (MacBae *et al*., 1988), anti-fungal and anti-viral activities (Huang *et al*., 2003), anti-inflammatory (Candida *et al*., 2005), anti-cancerous (Kumar and Kuttan, 2005), anti-diabetic and anti-cholesterol (Raphael *et al*., 2002), besides liver protective and detoxification (Khatoon *et al*., 2006).

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The multidimensional exploitation by the multinational pharmaceutical companies for manufacturing different therapeutic preparations needs continuous, uninterrupted and abundant supply of this plant. Since the availability of the plant is subject to seasonal variation (Rajasubramaniam and Saradhi, 1997) and considering the raised export and domestic demand, the cultivation of this herb for sustained and constant supply have to be promoted.

The qualities of the herbs tend to vary when grown under different ecological conditions. However, adopting standard cultivation practices could minimize the variation in quality and quantity. Hence, in this perception, an attempt has been made by one of the authors Annamalai (2002) to standardize the agrotechniques in two different soil conditions namely sandy loam soil and red soil for enhanced production of this herb (data not provided). Since, the quality of the herb is accessed in terms of a potential bioactive component -phyllanthin requires measures to be designed to simultaneously increase it with the biomass. Hence, the plants grown with standard agrotechniques in sandy loam soil (Annamalai *et al*., 2004) was screened for the performance of phyllanthin in different organs such as leaves, stems, seeds and roots of the plant using HPTLC and HPLC. Therefore, this investigation was performed to provide a comparative quantification of phyllanthin through HPTLC and HPLC from different plant organs of *P. amarus* L.

MATERIALS AND METHODS

Experimental Site, Period and Plant Material

Field experiment was carried out at the Botany Field Research Laboratory, extension lab of Center for Advanced Studies in Botany, University of Madras, Maduravoyal (13°04' N latitude and 80°14' E longitude) which is located at a distance of 13 Km east of Chennai, India. *Phyllanthus amarus* a potential anti-Hepatitis B viral agent grown during the period 2001 to 2002 in sandy loam soil with the pH of 6.8 to 8 by sowing about 250 g seeds ha^{-1}, where the seedling emerged in nursery beds were transferred to Exploded Block Design (EBD) using relatively large pot size of 600 m^2 $plot^{-1}$ (Annamalai *et al*., 2004) with narrow space intervals of 20×30 cm on the 30th day and was cultivated manually at the final stage (90 days old). Different organs such as the leaves, stems, seeds and roots of the plants were shade dried, powdered using a laboratory blender and extracted for phyllanthin.

Chemicals

Reagents and HPTLC plates used were obtained from Merck. Methanol was HPLC grade while, hexane, ethanol and ethyl acetate were of analytical grade. All chemicals were obtained from Sigma Chemicals, Bangalore. The authentic Phyllanthin obtained from SPIC- Pharma, Chennai, India was plotted to estimate the calibration curve against which, the recovery of phyllanthin from different organs of *P. amarus* was compared.

Sample Extraction

Method of Row *et al*. (1966) was followed to extract and purify phyllanthin. Fine powdered material (1 kg) of each organ (leaves, stems, seeds and roots) was thoroughly mixed with lime (300 g) and water (300 mL) and dried overnight at the room temperature condition. The bright yellow semi-solid residue (56 g of leaves, 51.46 g of stems, 41 g of seeds and 53 g of roots, respectively) obtained was distilled with petroleum ether and left until separation of yellow residue. The supernatant was discarded and the residue was boiled again with alcohol (400 mL) for 10 min, cooled to room temperature and filtered. This step was

repeated twice and the pooled filtrates approximately 300 to 350 mL of all the organs each was concentrated to 25% separately of its original volume, under reduced pressure and analyzed through HPTLC and HPLC.

Chromatographic Conditions and Detection of HPTLC

HPTLC was performed using the methodology of Sane *et al.* (1997). Chromatography was performed on pre-activated (at 110°C) silica gel MERCK $60F_{254}$ HPTLC plates (20×10 cm; 0.25 mm layer thickness) using hexane and ethyl acetate (2:1 v/v) mobile phase. Standard compounds and samples (5 μL) were applied to the layer as 8 mm wide bands, positioned 10 mm from the bottom of the plate, using an automated TLC applicator Linomat IV (Camag, Muttenx, Switzerland) with nitrogen flow providing delivery from the syringe at a speed of 10 μL^{-1}. Each TLC plate was developed to a height of about 10 cm under the laboratory conditions of 25- 30°C with 40-50% relative humidity. Developed plates were dried in a stream of air and then immersed in a freshly prepared mixture of vanillin (1 g) in 100 mL of concentrated sulphuric acid: ethanol (5:95 v/v). After drying, the plates were heated at 110°C for 25 min to develop the colour of the spots. For quantitative determination, the corresponding spots were scanned using a Camag TLC Scanner 3 at 254 nm with a slit size of 6×0.4 mm. Stock solution (1 mg mL^{-1}) of the standard compound (Phyllanthin) was prepared in methanol and different concentrations were spotted onto TLC plates in order to prepare the calibration graphs.

Chromatographic Conditions and Detection of HPLC

HPLC was performed using the methodology of Murali *et al.* (2001). About 1 mg of the concentrated sample was dissolved in 1 mL of methanol and 20 μL was injected to determine the phyllanthin content. The experiment was performed in LC-10AD Shimadzu (Japan) processing Merck, an instrument equipped with a Shimadzu SPD-M10 AVP Photodiode Array Detector (PAD) in order to determine peak purity. μBondapak C18 column (25 cm×4.6 mm) was used for isocratic resolution of phyllanthin using methanol : water (66:34 v/v) as the mobile phase at a flow rate of 1.0 mL min^{-1}. Using the detector SPD-10A Schimadzu (Japan) absorbance was recorded at 254 nm to calculate the percentage of phyllanthin.

Statistical Analysis

All the data obtained in triplicates were subjected to one way Analysis of Variance (ANOVA) and the significant difference among the means were compared by Duncan's Multiple Range Tests (DMRT) at a threshold p-value of 0.05 to test the differences between the samples of the organs of herb using SPCC/PC + Students statistical software.

RESULTS

An attempt made to analyze the bioactive compound phyllanthin under HPTLC and HPLC produced certain interesting results to be compared. The performance of HPLC was proved to be significant in producing higher resolution and increased peaks compared to HPTLC. Interestingly, among the different organs analyzed by HPTLC, elution peak was observed only for leaves which showed about 0.63% (w/w) (Fig. 1b) while no trace of phyllanthin was observed in any of the organs such as roots, seeds or stems of *P. amarus* (Fig. 1c-e) against the standard compound (Fig. 1a), which indicated the presence or absence of phyllanthin to be compared with.

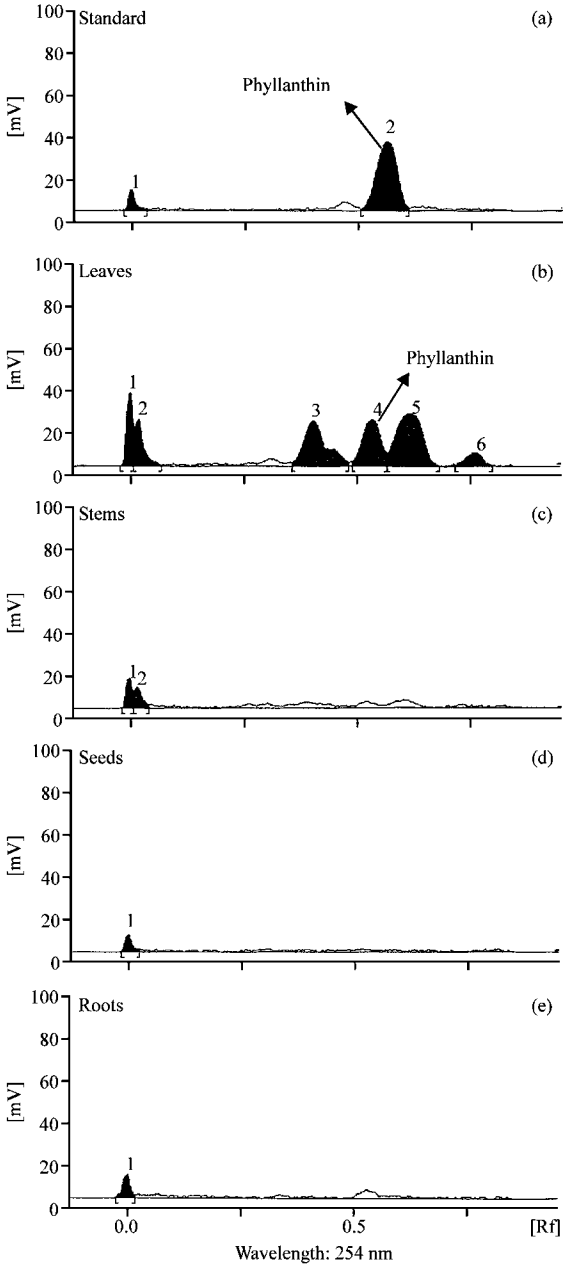

Fig. 1: HPTLC finger print of phyllanthin in different organs of *P. amarus* (panel a indicates standard(s) phyllanthin compound, panel b, c, d and e represents leaves, stems, seeds and roots, respectively

However, HPLC analysis showed a different pattern, in which, peaks denoting phyllanthin was observed from all organs (Table 1) of the plant indicating the purity of the compound to be very high. Perhaps, it has given a clear indication about the efficiency in highlighting the compound even at trace levels in terms of the peaks. Hence, by comparing

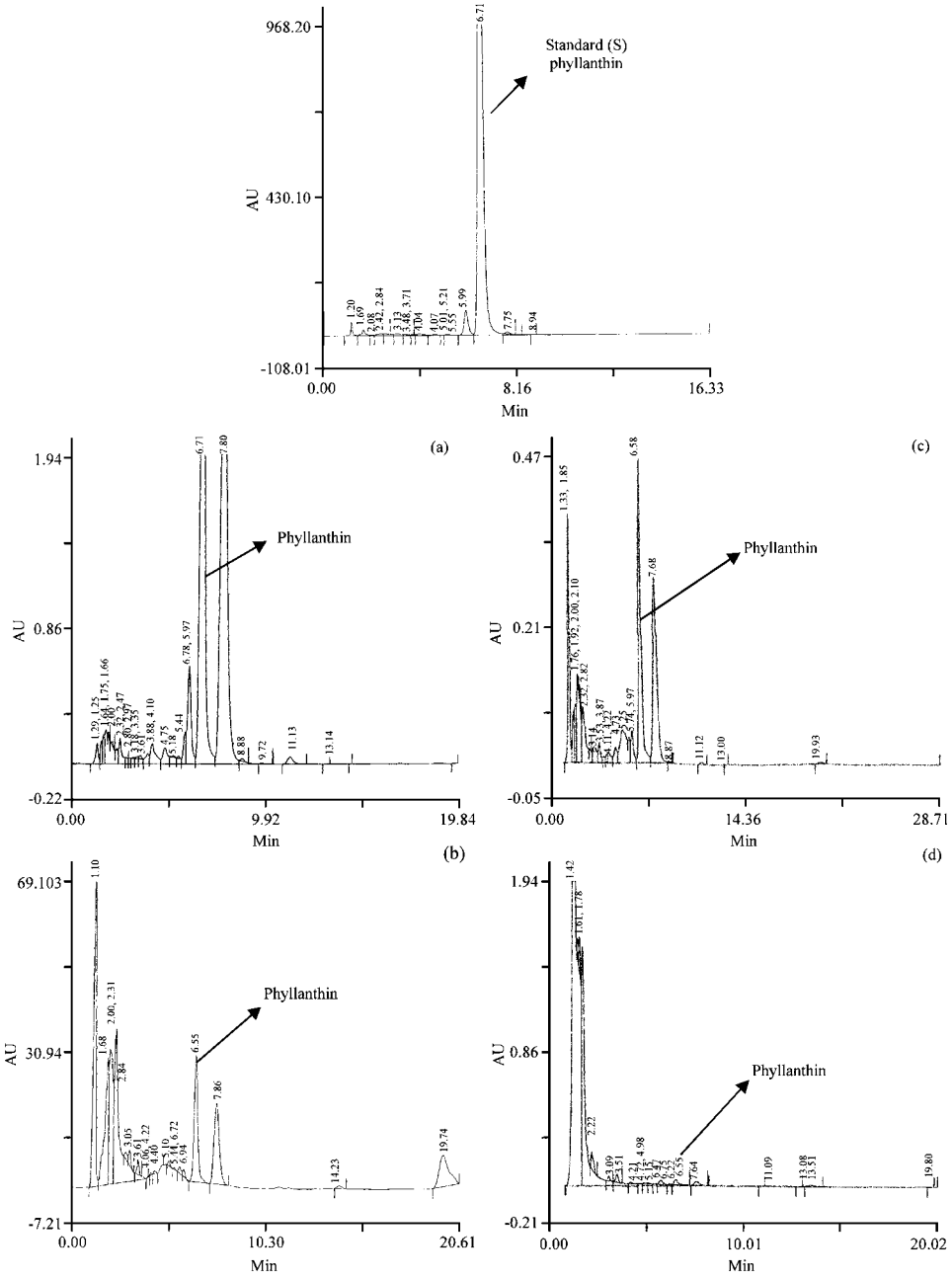

Fig. 2: HPLC profiles of phyllanthin in different organs of *P. amarus* (panel S indicates standard(s) phyllanthin compound, panel a, b, c and d represents leaves, stems, seeds and roots, respectively

with the standard graph (Fig. 2a), the highest peak was observed in the leaves (Fig. 2b), which quantified to 0.8335% (w/w), while the smaller peak with better resolution was quantified to 0.0016% in roots (Fig. 2, Table 1). Although, phyllanthin content was

Table 1: HPTLC and HPLC profile of phyllanthin in *P. amarus*

	Phyllanthin (%)	
Organs of herb	HPTLC	HPLC
Leaves	0.627±0.0050*	0.8335±0.0068*
Stems	-	0.0455±0.0045
Seeds	-	0.0543±0.0032
Roots	-	0.0016±0.0005

*Indicates statistical significance at $p<0.05$ level in relation to other organs of the herb

negligible in the roots, a comparative performance was achieved in terms of peaks for the other organs such as stems and seeds, where a considerable amount of 0.0455% (w/w) and 0.0543% (w/w), respectively was recorded (Fig. 2c, d, Table 1).

DISCUSSION

Medicinal plants have been traditionally used for treating liver diseases since centuries. Several leads from plant sources have been found as potential hepatoprotective agents with diverse chemical structures. Although, a big list of hepatoprotective phytomolecules was reported in the scientific literature, only a few were potent against various types of liver damages, of which, phyllanthin and hypophyllanthin have largely attracted the scientific community (Negi *et al*., 2008). Perhaps, interest in *P. amarus* has increased in recent years based on the efficacy of the herb against Hepatitis B virus because all parts of the wonder plant are medicinally important. Different species of *Phyllanthus* are considered to be very effective hepatoprotective agents in the Indian indigenous systems of medicine and are considered bitter, astringent, stomachic, diuretic, febrifuge, deobstruant and antiseptic. Still ayurvedic practitioners prescribe fresh juice of 'Bhuiamlki' for jaundice. Various species of *Phyllanthus* are being sold in India under the trade name 'Bhuiamlki'. In fact, during a market surveillance of herbal drug, it was observed that almost all the commercial samples either comprise of *Phyllanthus amarus* Schum and Thonn. or *Phyllanthus maderaspatensis* Linn. or mixture of *Phyllanthus amarus*, *Phyllanthus fraternus* Webster. and *Phyllanthus maderaspatensis* (Khatoon *et al*., 2006).

The methanol extract of phyllanthin from the leaves of *Phyllanthus niruri* L. showed oral antihyperuricemic activity in potassium oxonate- and uric acid-induced hyperuricemic rats by significantly reversing the plasma uric acid level of hyperuricemic animals to its normal level but however, in a dose-dependent manner, comparable to that of allopurinol, benzbromarone and probenecid which are used clinically for the treatment of hyperuricemia and gout (Murugaiyah and Chan, 2006). The protective effect of *P. amarus* extract and phyllanthin was studied on CCl4-induced toxicity in human hepatoma HepG2 cell line had also indicated that CCl4 treatment may cause a significant decrease in cell viability, in addition to alleviating the changes induced such as the initiated lipid peroxidation (LPO) and leakage of enzymes like alanine transaminase (ALT) and lactate dehydrogenase (LDH) with a significant decrease in glutathione (GSH) levels (Krithika *et al*., 2009). Owing to these importances, demands a detailed analysis of the active ingredient responsible for these effects in different parts of the plant species. In this context, the present study has been aimed and carried out to obtain an interesting observation. Although, number of investigators has developed methods to isolate and crystallize phyllanthin, the major bioactive principle of *P. amarus*, no steps have yet been taken so far to screen for the increased production of this compound from different organs of the plant.

Although, on one hand the results obtained in the present investigation was in conformity with the observation of Sharma *et al.* (1993), according to whom the whole plant, when dried and extracted yielded about 0.71% (w/w) by HPLC analysis, on the other hand it resulted in about 12% increase when compared to their observation yielding 0.83%. A sensitive, selective and robust high-performance TLC (HPTLC) method using chiral TLC plates for qualitative and quantitative analysis of phyllanthin, the active lignan of *Phyllanthus* species revealed the effectiveness in achieving good separation and provided the well-separated compact bands of the lignan and recovered about 99.98, 100.51, 99.22 and 98.74%, respectively especially in the leaves of four *Phyllanthus* species, i.e., *P. amarus*, *P. maderaspatensis*, *P. urinaria* and *P. virgatus* (Srivastava *et al.*, 2008). However, this is observed to be many fold higher compared to our results, which recorded only 0.63% through HPTLC. Perhaps, this is in superior to the results of researchers, according to whom, a simple high-performance thin-layer chromatography (HPTLC) densitometric method developed for the quantification of phyllanthin with other compounds in the whole plant of *P. amarus* revealed to exhibit the level of 0.37% (w/w) with the average recovery of 99.09%.

Since, a thorough investigation by the earlier workers have revealed the fact that phyllanthin enables its applicability as a marker compound in other drugs, an increase in its content could well be attributed to the kind of experiment that was conducted in the field by replenishing the nutrition that had greatly supported the plants for the better production in terms of vegetative growth such as shoot and root length, number of compounds and phyllanthoid branches and number of leaves per phyllanthoid branch, however data not provided but supported by the evidence of Annamalai (2002) and Annamalai *et al.* (2004). Perhaps, a major factor that could have contributed to this incremental effect could be the ideal conditions optimized for the conducive growth of the plants and period of harvest (around 90 days from the date of sowing) suitable to yield high phyllanthin compared to plants grown in wild conditions as reported by Sane *et al.* (1997) and Murali *et al.* (2001).

Besides these, the significant elution factor and recovery of high percentage of phyllanthin could also be attributed to the remarks made by Unander and Blumberg (1991), whose work revealed that high soil fertility would substantially increase the number and length of compound branches, resulting in less apical dominance in few species of *Phyllanthus*. It has also been evidenced by the work of Bowen (1980) and Benoit *et al.* (2005) that in addition to supply of the nutrients, the property of soil that inherit its characteristics along with the prevailing environmental conditions of the ecosystem could increase the plant growth and ultimately crop yields by secreting growth promoting substances. Moreover, the emphasize on the yield of phyllanthin in the present study could also be attributed to the fact contributed by the mobile solvents used, which actually produced best resolved peaks in the method of HPLC method as evidenced by Sane *et al.* (1997), who standardized based on the results obtained by Deb and Mandal (1996), who suffered less yield of phyllanthin due to the poor resolution.

Hence, keeping the efficacy and demand for all parts of the wonder plant in view, the present study perused in this direction to optimize the strategy for enhanced production through high resolution has proved to be effective. However, HPLC produced significant resolution and yielded high content of 0.8335% in the leaves alone compared to HPTLC, unlike that evidenced in the whole plant material by Sane *et al.* (1997). Thus, leaving the focus contingent entirely on the standard methodology devised by Annamalai (2002), which

if suggested to be adopted by the farmers could well produce high yield and in turn good economic return and thereafter for better exploitation by the Pharmaceutical industries.

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