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Quantitative determination of phyllanthin in *Phyllanthus amarus* by high-performance thin layer chromatography

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ABSTRACT

A simple, precise and rapid high-performance thin-layer chromatographic method has been developed for the estimation of phyllanthin and is the important lignans of *Phyllanthus amarus*. Separation of phyllanthin was carried out on silica gel 60 F_{254} layers eluted with hexane: ethyl acetate (2:1), and the analytes were visualized through colour development with 10% concentrated sulphuric acid in ethanol. Scanning and quantification of spots was performed at 200 nm. The proposed method being precise and sensitive can be used for the detection, monitoring and quantification of phyllanthin from *Phyllanthus amarus*.

KEYWORDS: HPTLC, *Phyllanthus amarus*, Phyllanthin, Lignans.

RESUMEN

Un método simple, preciso y rápido de cromatografía de capa fina de alto rendimiento ha sido desarrollado para la estimación de phyllantina y los lignanos importante de Phyllanthus amarus. La separación de phyllantina se llevó a cabo en capas de silica gel 60 F254 eluidas con hexano: acetato de etilo (2:1), y los analitos fueron visualizados mediante el desarrollo de color con un 10% de ácido sulfúrico concentrado en etanol. Los análisis y cuantificación de los puntos se realizó a 200 nm. El método fue validado.

PALABRAS CLAVE: **HPTLC, Phyllanthus amarus, phyllantina, lignanos**

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INTRODUCTION

The plants of the genus Phyllanthus (Euphorbiaceae) are widely distributed in most tropical and subtropical countries, and have long been used in folk medicine to treat kidney and urinary bladder disturbances, intestinal infections, diabetes and hepatitis B. In recent years, the interest in these plants has increased considerably. Substantial progress on their chemical and pharmacological properties, as well as a few clinical studies of some Phyllanthus species has been made. Several compounds including alkaloids, flavonoids, lignans, phenols and terpenes were isolated from these plants. The bioactivity of this plant proposed us to investigate its chemical components. The pharmacological activity of Phyllanthus species is mainly a result of the presence of lignans and polyphenols, especially flavonols (Calixto et al , 1998).

Phyllanthus amarus although considered from the farm point of view as a weed, is a valuable medicinal plant used by herbalists (Oudhia and Tripathi, 2002.). The roots, leaves, fruits, milky juice and whole plants are used in medicinal preparations. The bark yields a bitter taste called phyllanthin (Shakila and Rajeswari, 2006). The Structure of Phyllanthin is given in Figure 1. In particular, the herb *P. amarus* is known for its anti-hepatitis B virus (HBV) activity (Venkateswaran et al., 1987; Thyagarajan et al., 1988 ; Blumberg et al., 1990 ; Thamlikitkul et al., 1991; Munshi et al., 1993 ; Jayaram and Thyagarajan, 1996). Though considerable work has been done on exploring the effectively of the plant against HBV, no such report has yet been published on its utility against hepatitis C virus (HCV).

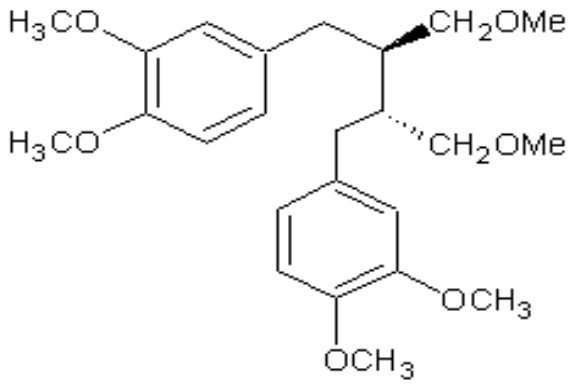
Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity (Pothier, 1996; Gupta *et al.*, 1996, 1998, 1999a, b; Gupta and Verma, 1996; Saxena *et al.*, 2000; Srivastava *et al.*, 2000). TLC or High Performance Thin Layer Chromatography (HPTLC) is primarily used as an inexpensive method for separation, for qualitative identification, or for the semiquantitative visual analysis of samples. TLC is thus often described as a pilot method for HPLC (Rozylo and Janicka, 1991, 1996). However, recent reviews show that the TLC and HPTLC techniques can be used to solve many qualitative and quantitative analytical problems in a wide range of fields, including medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology and environmental analysis (Weins and Hauck, 1996, Kalasz and Bathori, 1997). The use of TLC/HPTLC have expanded considerably due to the development of forced flow (FF) and gradient TLC methods, stationary and mobile phase selection, as well as new quantitative methods (Poole and Poole, 1994, Sherma, 1994). In the present paper we report HPTLC method that provide good resolution of the peak associated with phyllanthin from those of closely related compounds.

MATERIALS AND METHODS

Chemicals and Reagents

Standard Phyllanthin was purchased from Natural Remedies Pvt. Ltd. (Bangalore, India). Methanol was of HPLC grade and was used as a solvent for the preparation of standards and samples. Hexane and ethyl acetate (2:1) were used as mobile phase for HPTLC analysis. All solutions used for the analysis were filtered through 0.45 µm membrane filter using Millipore filtration unit.

Figure 1: Structure of Phyllanthin



Materials

The *Phyllanthus amarus* whole plants were collected from the experimental area of the University, JNKVV, Jabalpur (M.P) India. One sample was collected from the garden which was grown in normal condition while the other sample was obtained from the stress condition i.e. water was not given and it faced the various environmental conditions. The samples were dried at room temperature, subsequently milled into powder and stored in air-tight stopped glassware before subjected to physical and chemical analysis.

Preparation of Standard solution

Stock solution was prepared by dissolving 1 mg of Phyllanthin standard in 5ml methanol. Stock solution was further diluted to prepared working solutions with methanol in the concentration range 200 – 1200 µg/ml. 10 µl of each working standard solution were spotted on the TLC plate and calibration curve was generated by linear regression based on the peak areas.

Preparation of Sample solution

Air-dried of 1.0 gram sample was extracted in a soxlet for 10 hours separately at room temperature (25 ± 5º C) with 10 ml of methanol. Filtered and repeat the same process (2 x 10 ml) with methanol. Combined all the alcoholic extracts and dried under vacuum. Filtered through 0.45 µm membrane and made upto 1ml of methanol prior to HPTLC analysis.

HPTLC Chromatography

The samples were spotted with a Camag 100µL microsyringe on a pre-coated silicagel aluminum plates 60 F_{254} (20 cm x 10cm) with 250 µm thickness, (E. Merck, Darm stadt, Germany) using a Camag Linomat IV (Muttenz, Switzerland) applicator. Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag Muttenz, Switzerland) using mobile phase consisting of Hexane: Ethyl acetate (2:1). The length of the chromatogram run was 8 cm. After air drying, the TLC plates were scanned with a Camag TLC scanner-III in absorbance mode at λ_{max}, controlled by WINCATS software 1.4.1 version. Freshly prepared 10% Sulphuric acid reagent is used. After drying, the plate was heated at 110^0C for 10 min to develop the colour of the spots.

Estimation of Phyllanthin

To estimate the content of Phyllanthin in *Phyllanthus amarus* plant samples aliquots of 10 µl were subjected to HPTLC. The HPTLC plates were developed to a distance of 8 cm from the point of application, dried and scanned at λ_{max} = 200 nm. The content of Phyllanthin was calculated by linear regression and mean percentages were calculated from six replicate.

RESULTS AND DISCUSSION

The HPTLC method reported by Deb and Mandal (1996) suffers from poor resolution of phyllanthin and hypophyllanthin from other closely related compound. While the mobile phase reported by Sane et al (1997) gave resolved peak of phyllanthin and hypophyllanthin, the content of the latter was reported higher than of the former. In this study the variation of phyllanthin in Hexane: Ethyl acetate (2:1) gave good resolution of phyllanthin from other closely related lignans. Quality of the extracts can only be assured by the use of validated analytical methods for identification and quantification of the active ingredients. The result revealed that plants which were grown under extreme climatic stress conditions have accumulated higher concentration of Phyllanthin as compared to that grown at normal condition (Table 1). This can be justified in terms that accumulation of secondary metabolites is favored during stress conditions than in optimal conditions. So, global warming is going to favor the accumulation of higher quantity of lignans in *Phyllanthus amarus*. Similar types of studies were made by Sharma et al (1993) and Murali et al. (2001) in *Phyllanthus amarus*. The HPTLC method for the quantitative estimation of Phyllanthin was validated with regard to their specificity, precision, accuracy and linearity. Quantification of the marker may also reflect the quality of raw material in general and quantitative composition of other phytoconstituents in particular. Biomarker profiling is being highlighted in quality control and standardization of herbal medicine to establish the quality control approaches with chemoprofiling techniques with the lead from some therapeutically potent medicinal plants. Biomarker profiling of a maximum number of medicinal plants used in therapy is required to establish the quality control development based on this new emerging technique, which is being utilized by people globally for drug development from natural resources (Mukherjee, 2002, Mukherjee, 2003).

Validation of the method

The composition of the mobile phase for HPTLC was optimized by testing different solvent mixtures of varying polarity. The best results were obtained using Hexane: Ethyl acetate (2:1). The selected mobile phase produced highly symmetrical peaks showing good resolution. The compound with an R_f value of 0.24 ± 0.03 was identified as Phyllanthin (**Figure 2**). The specificity of the method was ascertained by analyzing standards and samples. The spot for Phyllanthin in the sample was confirmed by comparing the R_f value (**Figure 3 & 4**). Spectral studies revealed that the peaks obtained from both Phyllanthin standard and test samples were identical, because they had similar pattern as shown in **Figure 5**. Linearity was evaluated by determining six standards working concentrations containing 200 – 1200 µg/ml of Phyllanthin. Peak area and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficient. The regression equation was y = 6.1485 x + 4678.6 (where y is the response and x the amount of Phyllanthin. Linearity was found over the concentration range 2 – 12 µg / spot with a correlation coefficient of 0.985. The linearity of the calibration curve was validated by a high value correlation coefficient. The recovery rate was 98.5% as calculated by addition of known amounts of pure compound to the plant extract.

Figure 2. Chromatogram obtained from Phyllanthin standard.

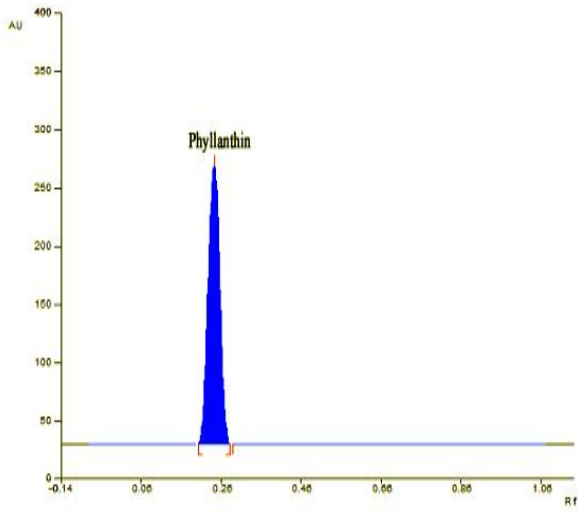
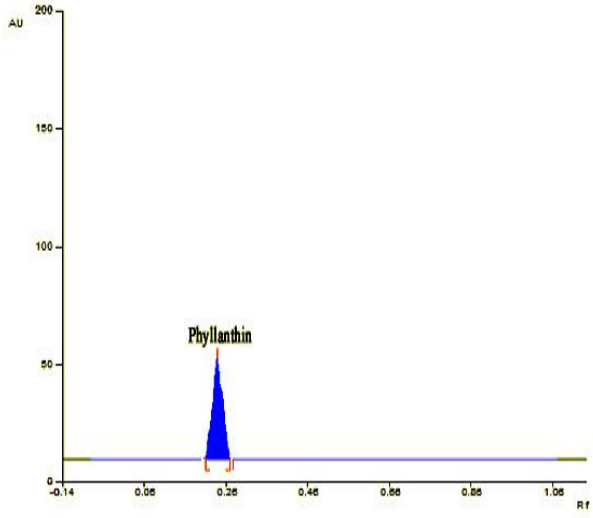


Figure 3. Chromatograms obtained from Garden normal sample.



The method was specific because the other compounds present in their constituents did not interfere with the peak of Phyllanthin. The comparing spectrum of Phyllanthin in the standard and in the sample was found to be similar or overlaped. The limits of detection and quantification of Phyllanthin were found to be 60 and 120 ng/spot respectively. Accuracy was measured by analysis of standard solutions spotted at different concentrations several times on the same day. The % RSD values were found to be less than 2% which is acceptable.

Figure 4. Chromatograms obtained from Field stress sample.

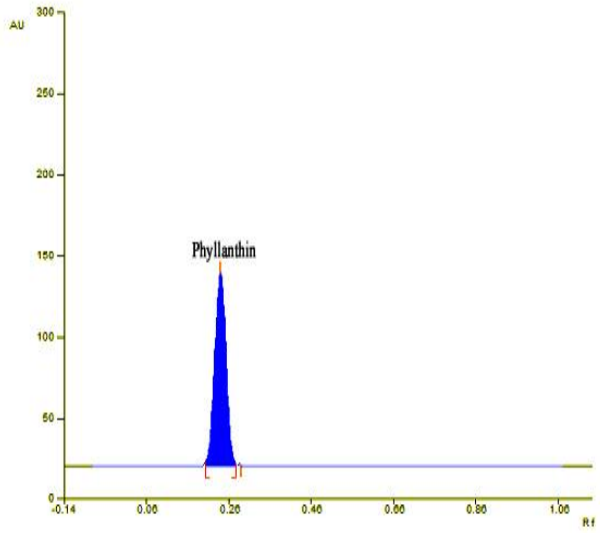
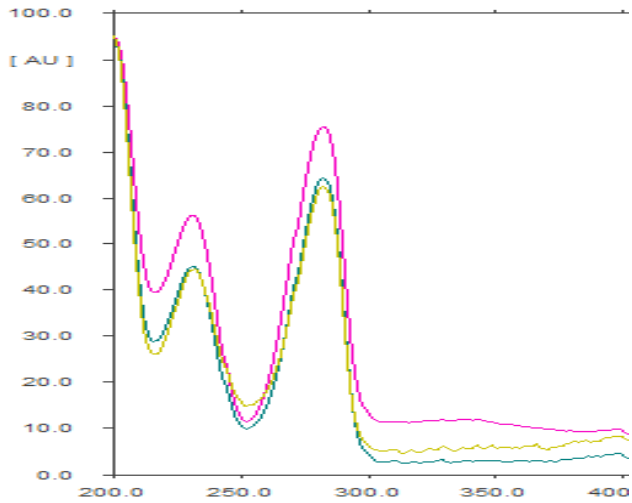


Figure 5. Over lay absorption spectra of Phyllanthin peaks from standard and sample.



CONCLUSION

Simple HPTLC method has been developed for the quantitative determination of Phyllanthin in *Phyllanthus amarus* plant. The methods developed are easy, sensitive and statistically validated for linearity, accuracy and precision. Hence this method can be adopted for the routine analysis

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Table 1. Estimation of Phyllanthin in *Phyllanthus amarus*

S.No.	Sample	Estimated amount (%)[a]	RSD (%)
1.	Garden normal sample	0.1018 ± 0.0001	0.150
2.	Field stress sample	0.1773 ± 0.0002	0.117
[a] Mean values (n=6) ± SD			

REFERENCES

Blumberg BS, Millman I, Venkateswaran PS and Thyagarajan SP, 1990. Hepatitis B virus and primary hepatocellular carcinoma: treatment of HBV carriers with *Phyllanthus amarus*. *Vaccine* 8: S86 – S92.

Calixto JB, Santos AR, Cechinel Filho V, Yunes RA, 1998. A review of the plants of the genus Phyllanthus: their chemistry, pharmacology, and therapeutic potential. *Med Res Rev*. 18: 225 - 258.

Deb S. and Mandal SK, 1996. TLC-densitometric determination of phyllanthin and hypophyllanthin in *Phyllanthus amarus* (Bhumiamalaki) and in polyherbal formulations. *Indian Drugs*, 33: 415 - 416.

Gupta AP, Gupta MM, Kumar S. 1998. Estimation of *Tropane* alkaloids using high performance thin layer chromatography. *J Ind Chem Soc* **76**: 277 – 278.

Gupta AP, Gupta MM, Kumar S. 1999a. High performance thin layer chromatography of asiaticoside in *Centella asiatica. J Ind Chem Soc* **76**: 321 – 322.

Gupta AP, Gupta MM, Kumar S. 1999b. Simultaneous determination of curcuminoids in *Curcuma* samples using high performance thin layer chromatography. *J. Liq. Chromatogr. Relat. Technol.* **22**: 1561– 1569.

Gupta MM, Jain DC, Verma RK, Gupta AP. 1996. A rapid analytical method for the estimation of artemisinin in *Artemisia annua. J. Med. Arom. Plant Sci* 18: 5 – 6.

Gupta MM, Verma RK. 1996. Combined thin layer chromatography– densitometry method for the quantitative estimation of major alkaloids in poppy straw samples. *Ind. J. Pharm. Sci* **58**: 161 – 163.

Jayaram S and Thyagarajan SP, 1996. Inhibition of HBsAg secretion from Alexander cell line by *Phyllanthus amarus*. Indian J. Pathol. Microbiol., 39: 211 – 215.

Kalasz H and Bathori, M. 1997. Present status and future perspectives of thin-layer chromatography. *LC-GC Int.* **10:** 440 – 445.

Mukherjee PK. *Quality Control of Herbal Drugs— An Approach to Evaluation of Botanicals*. New Delhi: Business Horizons; 2002.

Mukherjee P.K. 2003. Plant products with hypochlesterolemic potentials. In: Taylor SL, ed. *Advanced in Food and Nutrition Research*. Vol. 47. New Delhi: Elsevier Science.

Munshi A, Mehrotra R, Ramesh R and Panda SK, 1993. Evaluation of anti- Hepadnavirus activity of *Phyllanthus amarus* and *Phyllanthus maderaspatensis* in duck hepatitis B virus carrier Pekin ducks. J. Med. Virol., 41: 275 – 281.

Murali B, Amit A, Anand MS, Dinesh TK and Samiulla DS, 2001. An improved HPLC method for estimation of phyllanthin and hypophyllanthin in Phyllanthus amarus. J. Natural Remedies 1: 55 - 59.

Oudhia P, Tripathi RS, 2002. Medicinal weed flora of brinjal (*Solanum melongena* L.) fields in Chhattisgarh (India). Crop Res. 20: 482 – 488.

Poole CF and Poole SK. 1994. Instrumental thin-layer chromatography. *Anal. Chem.* **66:** 27A – 37A.

Pothier J. 1996. Thin layer chromatography in plant science. In: *Practical Thin Layer Chromatography—a Multidisciplinary Approach*, Fried B, Sherma J (eds). CRC Press: Boca Raton, FL.

Rozylo JK. and Janicka M. 1991. Thermodynamic description of liquid-solid chromatography process in the optimization of separation conditions of organic compound mixture. *J. Liq. Chromatogr.* **14:** 3197 – 3212.

Rozylo JK and Janicka M. 1996. Different planar techniques for prediction of solute retention in

column liquid chromatography. J. *Planar Chromatogr*. **9:** 418 – 424.

Sane RT, Chawla JL, Kuber VV. 1997. Studies on *Phyllanthus amarus*-Part-II. Indian Drugs, 34: 580 - 584.

Saxena S, Jain D.C, Gupta M.M, Sharma R.P, 2000. High performance thin layer chromatographic separation of hepatoprotective diterpenoids from *Andrographis paniculata. Phytochem Anal* **11**: 34 – 36.

Shakila A, Rajeswari R. 2006. Effect ofGA3, certain chemicals and media on germination, growth and yield of *Phyllanthus niruri*. Plant Arch. 6: 121 – 125.

Sharma A, Singh RT and Handa SS 1993. Estimation of phyllanthin and hypophyllanthin by high performance liquid chromatography in Phyllanthus amarus Phytochem. Anal. 4: 226 - 229.

Sherma J. 1994. Planar chromatography. Anal. Chem. **66:** 67R – 83R.

Srivastava S, Gupta MM, Verma RK and Kumar S. 2000. Quantification of 1, 3 benzodioxanes derivatives from *Piper mullesua*. J Assoc Off Anal Chem **83**: 1484 – 1488.

Thamlikitkul V, Wasuwat S and Kanchanapee P. 1991. Efficacy of Phyllanthus-Amarus for Eradication of Hepatitis B Virus in Chronic Carriers. J. Med. Assoc. Thailand, 74: 81 – 85.

Thyagarajan SP, Subramanian ST, Thirunalasundari, Venkateswaran PS and Blumberg BS, 1988. Effects of Phyllanthus amarus on chronic carriers of hepatitis B virus. Lancet 2: 764 – 766.

Venkateswaran PS, Millman I and Blumberg BS, 1987. Effect of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: *In vivo* and in viro studies. Proc. Natl. Acad. Sci. USA, 84: 274 – 278.

Weins, C. and Hauck, H.H., 1996. Advances and developments in thin layer chromatography. *LC-GC Int.* **9:** 710–717.