

Determination of Phyllanthin and Gallic Acid In Herbal Hepatoprotective Formulation By TLC-Densitometry Analysis

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ABSTRACT

Introduction: *Phyllanthus niruri*, *Andrographis paniculata* and *Embilica officinalis* are most important and high value medicinal plant known for its hepatoprotective activity. Several analytical methods including HPTLC and HPLC are reported for respective phytoconstituents. However, there is no simultaneous estimation of phyllanthin and gallic acid reported. The Objective of present work is to develop and validate HPTLC method for simultaneous determination of phyllanthin and gallic acid in polyherbal hepatoprotective formulation. **Method:** The method is employed TLC alumina plates precoated with silica gel 60F-254 as a stationary phase and toluene: ethyl acetate: formic acid (5:3.5:0.5 v/v/v) as a mobile phase. The standard markers namely phyllanthin and galic acid were identified in polyherbal formulation containing *P.niruri* and *E.officinalis* by R_f value. Densitometric analysis of phyllanthin and gallic acid was carried out at 254nm. **Results:** The precision of method was confirmed by relative standard deviation (RSD) which was lower than the 2%. This method was found to give proper separation of phyllanthin and gallic acid. The linear regression data for calibration plot shown good relationship with $r^2=0.998$ (phyllanthin) and 0.9991 (Gallic acid) in the concentration range 5-9 µg/band. The method was validated for precision, accuracy, sensitivity, and specificity. **Conclusion:** statistical analysis proves that method is accurate and reproducible. The method is economical and can be employed for routine analysis of marketed polyherbal hepatoprotective formulation containing *P.niruri* and *E.officinalis*.

Key Words: Gallic acid, Hepatoprotective, TLC, Phyllanthin

INTRODUCTION

The genus *Phyllanthus* (Euphorbiaceae) are distributed in all tropical regions of the world from Africa to Asia, South America and the West Indies. *Phyllanthus niruri* is the most widely species found along roads and valleys, and on riverbanks and near lakes in tropical areas. Other species found in India are *P. fraternus*, *P. urinaria*, *P. virgatus*, and *P.maderaspatensis* and *P. debilis*. The genus *Phyllanthus* has a long history of use in the treatment of diabetes, intestinal parasites and liver, kidney and bladder problems. ^[1-2] *P. niruri* is highly valued in the treatment of liver ailments and has been shown to possess anti-hepatitis B virus surface antigen activity in both *in vivo* and *in vitro* studies. ^[3-4] Phyllanthin and hypophyllanthin are lignans,

have been shown to be anti-hepatotoxic against carbon tetrachloride and galactosamine-induced hepatotoxicity in primary cultured rat hepatocytes.^[5] The roots, leaves, fruits, milky juice and whole plants are used in medicinal preparations.

E.officinalis is reported to possess antioxidant, anticarcinogenic, antiulcer, hepatoprotective,, immunomodulatory activity.

Many herbal formulations, which are combinations of different herbal extracts, are used for the treatment of liver diseases. A large number of marked polyherbal formulations are reported for the hepatoprotective activity some of the formulations *viz* Livex, Liv-52, Hepatomed, Jigrin, Hepex, Jaudex, and Livol.^[6-8] The most commonly herbs *viz* *Boerhaavia diffusa*, *Eclipta alba*, *Picrorrhiza kurroa*, *Oldenlandia corymbosa*, *Asteracantha longifolia*, *Apium graveolens*, *Cassia occidentalis*, *Cichorium intybus*, *Embelia ribes*, *Tinospora cordifolia*, *Andrographis paniculata* Nees, *Phyllanthus niruri*, *Phyllanthus emblica*, and *Trachyspermum ammi* are reported to have hepatoprotective activity. Amongst these plants *Andrographis paniculata* (AP) Nees, *Phyllanthus niruri* (PN) Linn and

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Phyllanthus emblica (PE) Linn are used for polyherbal hepatoprotective formulations.^[9-11]

Thus, an appropriate analytical procedure for the quantitative determination of lignans in different *Phyllanthus* species is of considerable importance. Several analytical procedures involving HPLC^[12-13] and HPTLC have been described.^[14-16]

PN, PE and AP contain phyllanthin, gallic acid and andrographolide respectively as active constituent, is well known for its hepatoprotective activity from ancient times. Therefore, it was thought to develop herbal formulation containing these three drugs. This formulation was subjected to standardization by TLC-densitometric method using phyllanthin and gallic acid as marker compound.^[17]

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^[18]. TLC or High Performance Thin Layer Chromatography (HPTLC) is primarily used as an inexpensive method for separation, qualitative identification, or for the semi quantitative visual analysis of samples. TLC is thus often described as a pilot method for HPTLC. 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. 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.^[19]

Several analytical methods are reported for estimation of phyllanthin from phyllanthus plant species. As there is no official HPTLC protocol for quantization of phyllanthin and gallic acid from polyherbal formulations therefore in the present paper we report an HPTLC method for quantitative analysis of phyllanthin and gallic acid from hepatoprotective Polyherbal formulation which provides good resolution of the peaks. This method will be utilize for routine analysis of marketed polyherbal hepatoprotective formulation containing *P.niruri* and *E.officinalis*.

MATERIAL AND METHODS

Instrumentation: A Camag HPTLC system (Muttentz, Switzerland) equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scanner 3, winCATS software and Hamilton (Reno, Nevada, USA) Syringe (100 μ L).

Material and reagents: HPLC grade alcohol, ethyl acetate, methanol, toluene, formic acid were obtained from S.D. Fine Chem Ltd (Mumbai, India). The biomarkers phyllanthin

(Natural Remedies, Bangalore) and Gallic acid (Hi media, Mumbai) were used as working standards.

Preparation of standard stock solution

A stock solution containing 1mg ml⁻¹ phyllanthin and gallic acid was prepared in methanol.

Preparation of sample for phyllanthin

Polyherbal tablet formulation was developed at laboratory scale. It contains a spray dried aqueous extract of *Phyllanthus niruri* and *E.officinalis*. Laboratory developed twenty tablets were weighed and crushed into fine powder. An accurately weighed 200 mg of powder was extracted with 50 ml alcohol on water bath; filtered through Whatmann filter paper no.42, evaporated on hot plate and residue was repeatedly washed (25x3) with hot water. The aqueous solution was successively extracted with (25x3) petroleum ether (60-80). The petroleum ether layer was discarded and aqueous layer was further extracted with ethyl acetate (25x3) in separator. Finally aqueous layer was discarded and ethylacetate solution was evaporated to dryness.^[20] The Phyllanthin content was quantified by TLC-densitometric scanning.

Sample Preparation for Gallic acid

An amount of 200 mg of powder was extracted with 50 ml of methanol on water bath. Methanol evaporated under vacuum, then filtered through whatman paper No 42 and filtrate was evaporated to dryness.^[24] The gallic acid content was analyzed by HPTLC.

Chromatographic conditions

Chromatograph was performed on 10x10 cm aluminum backed TLC plate coated with 0.2 mm layer of silica gel 60F₂₅₄ (E. Merck Ltd, Darmstadt, Germany) stored in a dessicator, application was done by Hamilton micorsyringe (Switzerland), mounted on a Linomat V applicator. Spotting was done on the TLC plate, ascending development of the plate, migration distance 80 mm (distance to the lower edge was 10 mm) was performed at 25 \pm 2^oc with toluene: ethyl acetate: formic acid (5: 3.5: 0.5 v/v). as a mobile phase for phyllanthin and gallic acid, (Fig 1) in a camag chamber previously saturated for 30 min. samples were applied as 6 mm width at a spraying rate of 15s μ L⁻¹. The average development time was 15 minutes. After development the plate was dried at 50^oC in an oven for 5 minutes. Densitometric scanning was then performed with a Camag TLC Scanner 3 equipped with winCATS Software Version 1.3.0 at λ_{max} = 254 nm using Deuterium light source, the slit dimensions were 6.00 X 0.45 mm.

RESULTS

Validation of the method

The developed method was validated for following parameters;

Linearity

The stock solution (1mg/ml) of phyllanthin and gallic acid was prepared in methanol. Different concentration of the stock solution for phyllanthin and gallic acid (5-9 µg) were located to a plate to provide 5, 6,7,8,9 µg of phyllanthin and gallic acid/band separately. Each volume was applied three times on the TLC plate. Peak area data and the corresponding amounts were treated with linear least- square regression analysis. The calibration graphs were constructed by plotting peak area against amount of drug (µg spot⁻¹). The correlation coefficient, r^2 , intercept and slope of phyllanthin was found to be 0.9998, 544.79, and 0.2145 respectively while for gallic acid was 0.9991, 32314.5 and 1.974. **Table 1**

Precision

The repeatability of sample application and measurement of peak area were expressed in terms of % R.S.D. and found to be 1.08 and 0.98 for phyllanthin and gallic acid

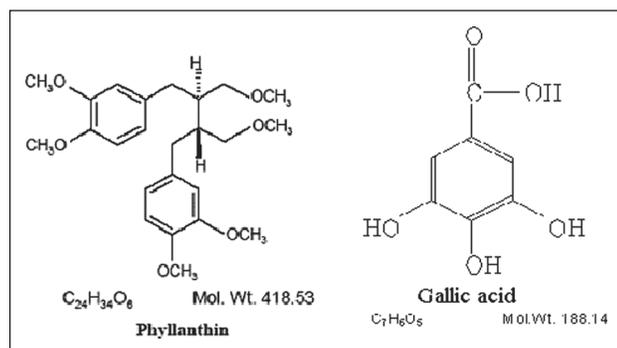


Figure 1: Structure of phyllanthin and gallic acid

Table 1: Summary of validation parameters

Parameters	Phyllanthin	Gallic acid
Linearity range (µg spot ⁻¹)	5 – 9	5-9
Correlation coefficient	0.998	0.9991
Slope	0.2145	1.974
Intercept	544.79	32314.54
Standard deviation	7.71	155.51
Precision (R.S.D. %)	1.08	0.98
LOQ & LOD (ng)	359.86 and 118.75	787.79 and 259.95
Robustness	Robust	Robust
Specificity	Specific	Specific

respectively. The results depicted in Table 2 showed that no significant intra- and inter-day variation was observed in the analysis of phyllanthin at three different concentration levels. The % R.S.D. for intra- and inter-day analysis was found to be < 2% in all the cases. **Table 1**

Sensitivity

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times. The signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting known concentrations of phyllanthin and gallic acid until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations. These were calculated by the use of the equations $LOD = 3 \times \delta/s$ and $LOQ = 10 \times \delta/s$; Where, δ is the standard deviation of the peak area of the drugs ($n=3$) and s is the slope of the corresponding calibration plot. LOQ and LOD for phyllanthin were 359.86 and 118.75 ng, respectively. For gallic acid LOQ and LOD were found to be 787.79 and 259.95 ng respectively. (**Table 1**).

Accuracy (% recovery)

To ensure the accuracy of the proposed method, recovery studies for phyllanthin and gallic acid were performed by standard addition method at 80,100 and 120% concentration levels of the phyllanthin. The mean percentage recovery for phyllanthin and gallic acid was found to be 98.21 and 97.19 respectively (**Table 3**).

Specificity

It was observed that the phytoconstituents of the other aqueous extracts of herbs present in the formulation and pharmaceutical excipients did not interfere with the peak of phyllanthin and gallic acid. Therefore, the method was specific. The overlap in the spectrum of standard phyllanthin and gallic acid sample extracted from formulation showed good correlation.

UV Spectrum: The standard spot with the same R_f was located in the fingerprint of the formulation and confirmed

Table 2: Percentage amount of Phyllanthin and gallic acid present in Hepatoprotective formulation.

Sample	Amount found ng spot ⁻¹		% w/w	
	Phyllanthin	gallic acid	Phyllanthin	gallic acid
Hepatoprotective Formulation	0.41	3.060	41.63	61.21

Table 3: Recovery studies for Phyllanthin and gallic acid

	Amount added %	Amount found (ng) mean \pm S.D	% Recovery	Average recovery
Phyllanthin	80	5529.473 \pm 2.27	98.78	98.21
	100	6839.523 \pm 3.89	97.73	
	120	8242.177 \pm 2.00	98.12	
Gallic acid	80	5357.447 \pm 3.01	95.69	97.19
	100	6879.657 \pm 1.56	98.28	
	120	8200.423 \pm 2.31	97.62	

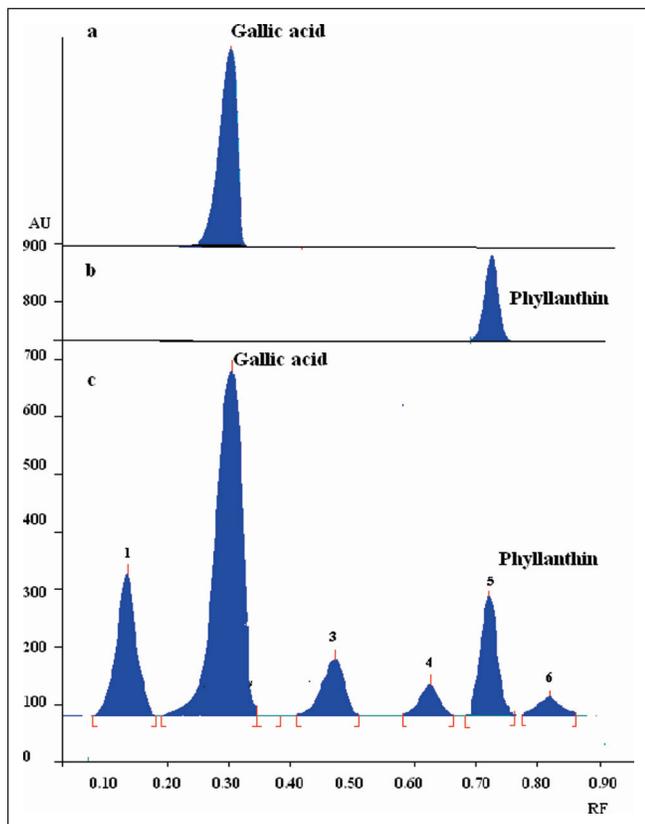


Figure 2: Chromatogram of standard gallic acid (a) (R_f : 0.30 \pm 0.02); standard phyllanthin (b) (R_f : 0.72 \pm 0.02); Polyherbal tablet formulation (c). Mobile phase: toluene: ethyl acetate: formic acid (2.5: 5.5: 0.5% v/v)

by superimposable spectra. At the shorter wavelength in the UV spectrum of the spots in standard and formulation are different but the shape is identical at longer wavelength. A peak purity spectrum of Phyllanthin and gallic acid was found to superimposable (**Figure 3.A & B**).

Analysis of the marketed formulation

Six spots at R_f 0.13, 0.30, 0.49, 0.63, 0.72 and 0.82 (Fig.2) were observed in the chromatogram of herbal drug samples extracted from tablet. There was no interference in analysis of phyllanthin and gallic acid from the other components present in the tablet formulation. These components appear in the chromatogram at significantly different R_f values.

The total phyllanthin and gallic acid content was found to be 41.63 and % (w/w) in tablet formulation. Statistical evaluation of the results was performed with regard to accuracy and precision using Student's t -test and the F -ratio at 95% confidence level. The low % R.S.D. value indicated the suitability of this method for routine analysis of phyllanthin and gallic acid in pharmaceutical dosage form. **Table 2**

CONCLUSION

Herbal medicine is still used for primary healthcare because of better cultural acceptability, better compatibility with the human body and lesser side effects. So the multi-component herbal formulations can be standardized with newer techniques such as High performance thin layer chromatography. It emphasizes an integral formulation of pharmacologically active and phytopharmaceutically characteristic component of samples with similar and different attributes.^[21] This is the most significant method which can be used for routine herbal drug analysis and for quality assurance. The proposed HPTLC method was found to be rapid, simple and accurate for quantitative estimation of phyllanthin and gallic acid present in formulation prepared at lab scale. The recovery values of phyllanthin and gallic acid was found to be about 98.21 and 97.19 shows the reliability and suitability of the method.

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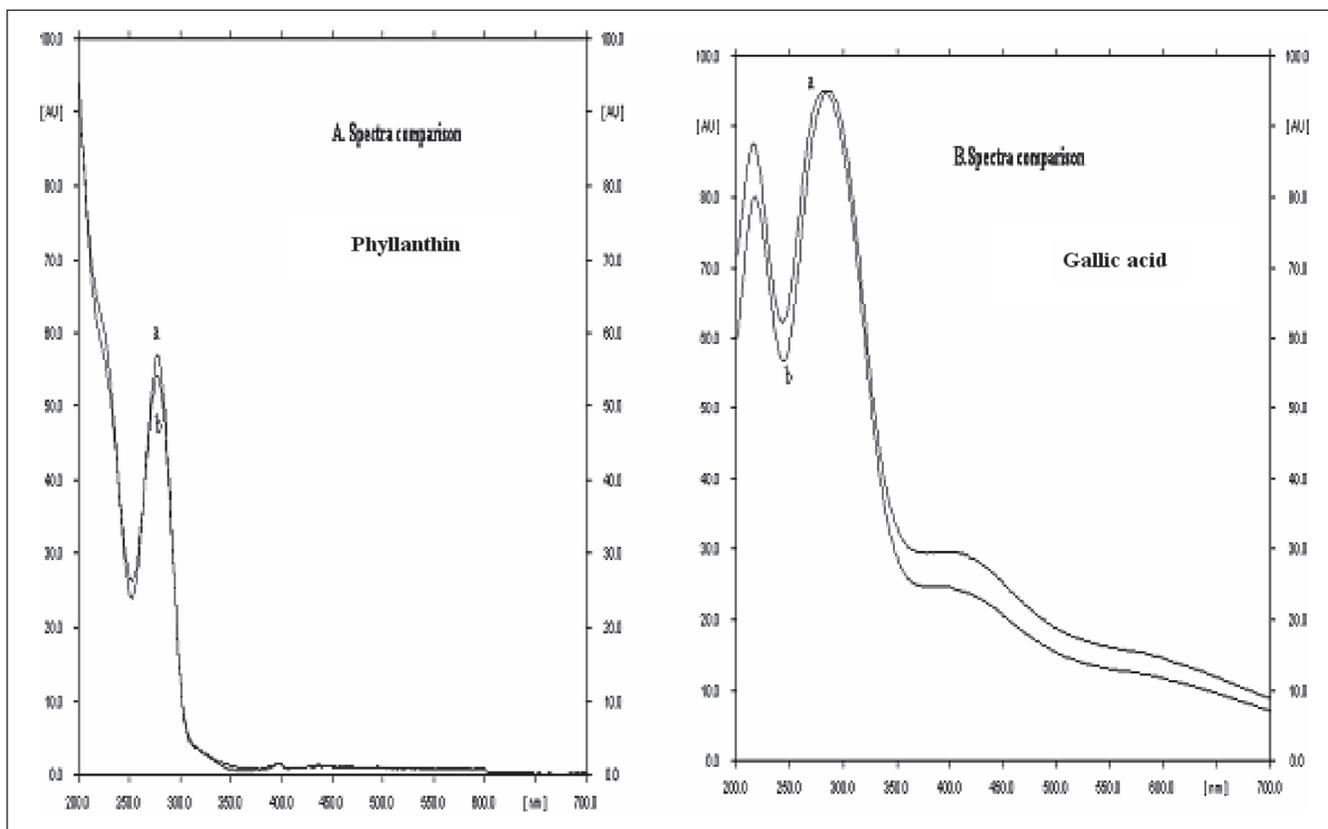


Figure 3: A: Peak purity spectrum of a) standard Phyllanthin (R_f 0.72) and b) Phyllanthin present in formulation (R_f 0.72). **B:** Peak purity spectrum of standard gallic acid (R_f 0.55) and gallic acid present in formulation (R_f 0.55).

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