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Research Article

HPTLC ANALYSIS AND STABILITY STUDY OF PHYLLANTHIN BIOMARKER IN TABLET FORMULATION

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ABSTRACT

Phyllanthus amarus Schum and Thonn from the family Euphorbiaceae has been widely used in India and other tropical countries for the treatment of various ailments as hepatoprotective, antibacterial, antiviral, anti-inflammatory, antidiabetic, etc. Various activities in genus Phyllanthus are reported due to different lignans present. Preservation of herbal extract may cause degradation of the active constituents and thus reducing its pharmacological efficacies. So there is need for an appropriate analytical procedure to quantify the content of actives in the extract when stored before use. In the present work stability profile of herbal tablet containing *Phyllanthus amarus* extract was studied and quantitative estimation of phyllanthin biomarker was done with HPTLC. Tablet formulation of methanolic extract was formulated, evaluated and stored at different stabilty conditions. HPTLC method was developed and validated to quantify biomarker in fresh extract and in formulation using Hexane: Acetone: Ethyl acetate (7:2:1v/v/v). The results of HPTLC study indicated that tablet samples kept under long term condition consist of more phyllanthin as compared to the tablets samples stored under accelerated and room temperature after 6 months study. Therefore, *Phyllanthus amarus* extract should be stored at definite temperature and humidity conditions to get maximum concentration of biomarker phyllanthin.

Keywords: *Phyllanthus amarus,* methanolic extract, tablet formulation, HPTLC, stability study.

INTRODUCTION

Stability testing has remained an imperative aspect of drug development as it affects quality, safety, and efficacy of the drug product. It is carried out at distinct phases, including preformulation, formulation development, product development, and post marketing. Stability testing of herbal drugs is challenging because of inherent physicochemical complexity. *Phyllanthus amarus* Schum and Thonn from the family Euphorbiaceae has been widely used in India and other tropical countries for the treatment of various ailments. It has been reported to exhibit marked anti-hepatitis B virus surface antigen activity both in *in vivo* and *in vitro* studies. Furthermore, the plant has been reported to possess antibacterial, antiviral, anti-inflammatory, antidiabetic, and anticholesterol activities. Different parts of plant are reported to have various therapeutic activities such as expectorant, diaphoretic[1-3].

Many active phytochemicals, flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins, and saponins, have been identified from various parts of *Phyllanthus*. The lignin phyllanthin present in the plant have been shown to be antihepatotoxic against carbon tetrachloride and galactoseamine-induced hepatotoxicity in primary cultured rat hepatocytes[4-7]. Quantitative determination of lignans by an appropriate analytical procedure is important because some herbs are perishable in their fresh state and may deteriorate within a few days after harvest. One way to preserve the plant products is to dry them in order to conserve their desirable qualities, reduce storage volume, and extend their shelf life. Several analytical procedures involving high performance liquid chromatography (HPLC)[8-12] and high-performance thin-layer chromatography (HPTLC)[13,14] have been described for detection of these lignans. In the present study, we are reporting the stability study of crude plant material of *P. Amarus* and its effect on quantification of lignan phyllanthin.

MATERIALS AND METHOD

Chemicals

Standard Phyllanthin was procured from Cayman chemical company, USA. Methanol AR grade was used as solvent for extraction and sample preparation. Hexane, acetone, ethyl acetate, formic acid etc were of AR grade.

Collection of plant material

Fresh whole plant of *Phyllanthus amarus* (Euphorbeaceae) was collected from Dr. D. Y. Patil Ayurveda College, Pimpri, Pune. Plant was identified and authenticated by Botanical survey of India, Pune, where a voucher specimen (No: BSI/WRC/100-1/2017/4) has been kept. Plant material was collected, cleaned and washed under running water. Plant was then dried and grinded to coarse powder. Proximate analysis of the plant powder was studied[15].

Extraction of *Phyllanthus amarus*

Course powder of *Phyllanthus amarus* (PA) were packed in thimble for Soxhlet extraction. The sample was extracted successively with petroleum ether, chloroform, ethyl acetate, methanol and water. Solvent filled in distillation tank and thimble clogged with cotton in order to avoid transfer of sample particles to the distillation tank. The packed material was extracted until

clear solution was observed in siphon tube. The extracts were concentrated using rota evaporator and percentage yield was calculated.

Phytochemical Screening[16]

Freshly prepared extracts were subjected to phytochemical screening to confirm presence of secondary metabolites.

HPTLC Method Development and Validation:

Standard preparation

A stock solution of 100µg/mL of phyllanthin was prepared in methanol and was used for analysis. 6µl of the above solution was applied on plate to obtain standard densitogram of standard phyllanthin.

Sample preparation

500 mg methanolic extract of *Phyllanthus amarus* was dissolved in 10 mL of methanol. (50 mg/mL).40µl of the above solution was applied on plate to obtain standard densitogram of extract. Presence of Phyllanthin in extract was confirmed by overlay spectra.

Chromatography

Chromatography was performed using commercially-prepared, pre-activated (110°C) silica gel 60 F254 TLC plates (10 × 10 cm). A Linomat IV (Camag, Muttenz, Switzerland) semi-automatic TLC applicator was used to apply samples and standards onto the TLC plate under a flow of nitrogen gas.

Developing Solvent System

A number of solvent systems were tried with variation. The satisfactory resolution obtained from extract in the solvent Hexane: Acetone: Ethyl acetate (7:2:1v/v/v).

Development of Chromatogram

After the application of sample, the chromatogram was developed in twin trough glass chamber 10×10 cm saturated with previously equilibrated mobile phase for 15min. The chromatographic conditions were optimized to obtain the best peak shape.

Scanning

The plates were fixed in the scanner stage (CAMAG TLC SCANNER) and scanning was done at UV 254nm. The peak table, peak display, spectrum mode was recorded.

Validation

The developed method was validated for parameters such as linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and robustness as per ICH guidelines Q2 (R1).

Tablet Formulation of *Phyllanthus amarus* Extract

200 mg *Phyllanthus amarus* methanol extract was mixed with equal quantity of silicon dioxide. Tablet was prepared by using varying types and proportions of disintegrate, glidant, binder and filler. All the ingredients were passed through sieve no-80 and mixed with silicon dioxide. The powder mixture had good flow property. The mixture was then punched using direct compression method. Tablets were compressed each of 500 mg weight on karnawati rotatory tablet compression machine fitted with 10 mm punches. Formulated tablet were evaluated for different parameters.

Quantification of Biomarker by HPTLC

To quantify the content of Phyllanthin in methanol extract of PA, extract was subjected to HPTLC. 500 mg of extract was dissolved in 10 ml of methanol to prepare stock solution of which 40 µL concentrations was spotted. Peak matching R_f of standard Phyllanthin then subjected for spectral scanning. Concentration of Phyllanthin in methanol extract was calculated using standard calibration curve of Phyllanthin standard.

Stability Study

Freshly prepared tablet formulations were kept for stability study in stability chamber as per ICH guidelines Q1A (R2) at accelerated (40°C /75 % RH) and long term conditions (30°C / 65 % RH).The changes were observed for the samples at an interval of 0, 3 and 6 months for all levels is real time, accelerated study and long term study. Tablets were evaluated for all the parameters as after 3 months and 6 months. HPTLC quantification of Biomarker Phyllanthin was performed after 3 and 6 month optimised chromatographic conditions and was compared with freshly prepared tablet densitogram.

RESULTS AND DISCUSSION

Proximate analysis of *Phyllanthus amarus* was performed as per standard procedure. The results were within the specified limits as shown in Table 1. Sequential extraction was carried out using various solvents as pet ether, chloroform, ethyl acetate, methanol and water and Percent yield was calculated as presented in Table 2. Preliminary phytochemical screening of all the extracts was carried out as shown in Table 3.

HPTLC method was developed for PA methanol extract using optimised chromatographic conditions as shown Table 4.R_f of Phyllanthin standard was found to be 0.44. Presence of phyllanthin was confirmed by overlaying spectra of phyllanthin standard and phyllanthin in extract as shown in Figure 1-3. Developed method was validated and results are shown in Table 5 and Figure 4.

Tablets were prepared using optimised formulation and were evaluated for hardness, thickness, weight variation, friability, disintegration time and dissolution. The results are presented in Table 6 &7 0.135% phyllanthin was present in 100gms of powder. Formulated tablet were subjected to different storage condition and were evaluated for 0, 3 and 6 months.

HPTLC quantification of biomarker phyllanthin was done. Concentration of phyllanthin after 3 months and 6 months was calculated based on the initial concentration present in fresh extract. After real time study concentration of Phyllanthin decreased up to 80% in 3 months and up to 90% in 6 months. Similarly, in case of accelerated Study (40^0c/75% RH) concentration of Phyllanthin get reduced to 50% after 6-month exposure. Concentration of Phyllanthin was reduced to 15% and 25% in 3 months and 6 months respectively, when tablets were exposed to long term stability study (30^0c/65% RH). Stability results are presented in Table 8.

Table 1: Proximate analysis PA Powder

Test	Observed value in %	Standard Value
Ash Value	8.5	NMT 11%
Acid insoluble ash	0.13	NMT 3.0%
Alcohol soluble extract	2.13	NMT 6%
Water soluble extract	6.62	NMT 12%
Loss on drying	3.76	NMT 10%

Table 2: Extractive yields

Plant used	Extraction method	Percentage yield				
Phyllanthus amarus powder	Soxhlet Extraction	Pet ether	Chloroform	Ethyl Acetate	**Methanol**	Water
		0.945	1.16	0.729	**1.454**	0.889

Table 3: Preliminary Phytochemical Screening

Sr. No.	Chemical test	Pet Ether	Chloroform	Ethyl acetate	Methanol	Water
01	Test for Carbohydrate					
	Fehling test	-	-	-	-	+
	Molish test	-	-	-	-	-
	Benedict's test	-	+	+	+	+
02	Test for Amino acid					
	Ninhydrin	-	-	-	+	
03	Test for Glycoside					-
	Keller-killani	-	+	-	-	
	Legal test					
04	Test for flavonoid	-	-	+	+	
05	Test for Alkaloid's					
	Mayer's reagent	+	+	+	-	
	Wagner's reagent	+	-	+	+	
	Dragendroff's reagent	-	-	-	-	+
06	Test for Tannin and Phenolic					
	5% Ferric Chloride	-	-	+	+	
	Lead Acetate	-	+	+	-	
07	Proteins	-	-	-	+	-
08	Test for saponin's	+	+	+	-	+
	Foam test					

Table 4: Optimised chromatographic condition

Stationary Phase	Silica gel 60 F_{25} plates
Mobile Phase	Hexane: Acetone: Ethyl acetate (7:2:1v/v/v)
Plate Size	10 cms X 10 cms
Mode of application	Band
Development Chamber	Twin trough chamber with glass slide
Saturation time	15 mins
Separation technique	Ascending
Migration distance	80 mm
temperature	RT
Scanning mode	Absorbance/Reflectance
Slit dimension	5 X 0.45 mm
Scanning Wavelength	275 nm

Table 5: Results of validation study

Parameter	Results
Linearity	25 – 125 µg/band
Precision	
Interday precision	0.757
Intraday precision	1.153
Accuracy	
80 %	99.06 %
100 %	101.65 %
120%	101.68 %
LOD	1.05 µg/band
LOQ	3.185 µg/band
Robustness	Robust
Specificity	Specific

Table 6: Optimised Tablet Formulation

Ingredients	Quantity(mg)
Extract	200
Silicon dioxide	100
Crosspovidone	80
Acacia gum	80
Starch	40
Total	500

Table 7: Evaluation of tablet formulation

Parameter	Results
Hardness	6.4 kg/cm^2
Thickness	3.36 mm
Diameter	12.81mm
Weight variation	NMT 2%
Friability	0.7%
Disintegration time	15.30 min
Dissolution study	6 hours

Table 8: Quantification of Biomarker

Storage Condition	% Phyllanthin detected after 3 months	% Phyllanthin detected after 6 months
Freshly Prepared tablet	100 %	100 %
Real time Study 3 Month.	18.62 %	11.87 %
Long – term Study(30^{0}c /65 % RH) 3 Month	85.7 %	77.58 %
Accelerated Study (40^0 c / 75 % RH) 3 Month	66.66%	45.56 %

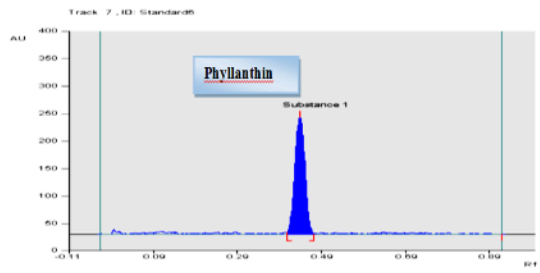


Figure 1: Typical Densitogram of Phyllanthin Standard.(Rf- 0.44)

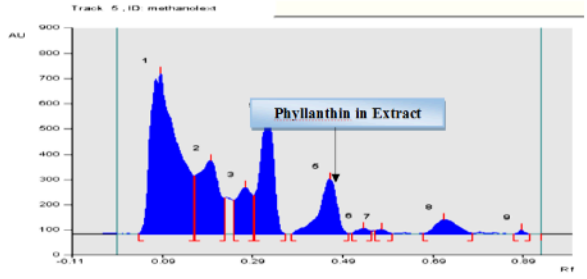


Figure 2: Typical Densitogram of PA methanol extract(Rf-0.45)

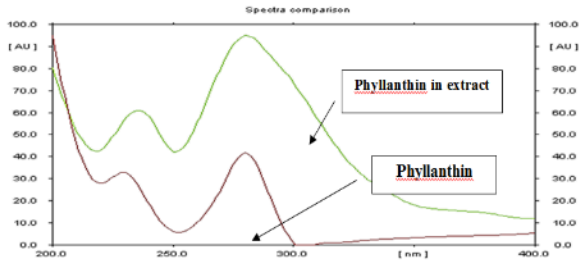


Figure 3: Overlay Spectra of Phyllanthin standard and Phyllanthin in extract

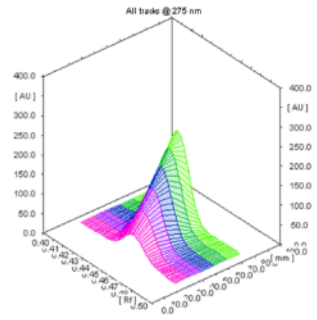


Figure 4: Densitogram for Linearity of Phyllanthin

CONCLUSION

Different solvents of increasing polarity as pet ether, ethyl acetate, chloroform, methanol, water were used for extraction of the *Phyllanthus amarus.* Percent extractive yield and preliminary Phytochemical screening obtained showed presence of majority of secondary metabolites in methanol extract. HPTLC analysis was performed for Phyllanthin standard using hexane: acetone: ethyl acetate (7:2:1 v/v/v) as mobile phase and silica gel 60 F254 TLC plates as stationary phase. Retention Factor for Phyllanthin standard was found to be 0.44. Optimised chromatographic conditions were used for quantification of Phyllanthin in the methanol extract of *Phyllanthus amarus*. The HPTLC method developed was validated as per ICH guidelines and was found to be accurate and precise. In house optimised tablet formulation was prepared and evaluated for hardness, thickness, weight variation, disintegration and dissolution. All the parameters were in acceptable limit. HPTLC analysis of tablet using optimised conditions showed presence of phyllanthin biomarker. Formulation was subjected to different storage condition as real time, accelerated condition (40^0c/75%RH) and long term study 30^0c/65% RH. Study revealed considerable decrease in the concentration of Phyllanthin after 3 months and 6 months HPTLC evaluation during real time study and accelerated study. Therefore, it may be concluded that either the formulation of *P. amarus* should be used as fresh or stored under long term condition up to 3 months to get the maximum concentration of Phyllanthin.

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