SIMULTANEOUS ESTIMATION OF CORILAGIN, GALLIC ACID AND ELLAGIC ACID BY HPTLC METHOD

TUSHAR CHHAGAN KAKADE
Dept. of Pharmaceutical Chemistry
Poona College of Pharmacy,
Pune Maharashtra 411038

Dr. L. SATHIYANARAYANAN
Guide, Dept. of Pharma chemistry
Poona College of pharmacy, Pune,
Maharashtra.

ATUL RATHOD
Co-guide, Dept. of Pharma chemistry
Poona College of pharmacy, Pune,
Maharashtra.

Dr. V.R.PATIL
Principle of HLMC College of Pharmacy,
Faizpur, Maharashtra.

KAKASAHEB R. MAHADIK,
Dept. of Pharmaceutical Chemistry, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Erandwane, Pune-411038, Maharashtra, India.

ABSTRACT

A simple, fast and precise high performance Thin layer chromatographic method has been developed for the simultaneous determination of corilagin, gallic acid and ellagic acid. Usi1223ng Silica gel 60 F_{254s} HPTLC plates, optimized solvent system of n-butanol:water:Methanol:Formic acid (6:1:0.1:0.8; v/v/v/v) in a twin trough chamber saturated for 30 min. Validation was carried out by testing its specificity, linearity (300-1300 ng/spot), precision (1.36%, 0.83% and 0.81 %), limits of detection (53.37ng/spot, 50.03ng/spot & 59.29ng/spot) and quantification (161.74ng/spot, 151.61ng/spot & 179.67ng/spot) for corilagin, gallic acid & ellagic acid respectively.

Keywords: *HPTLC, validation, corilagin, gallic acid and ellagic acid,Phyllanthus amarus etc.*

INTRODUCTION

Corillagin (fig:1), 1-O-Galloyl-3,6-hexahydroxydiphenol—D-Glucopyranose is used as Anti-inflammatory agent. Gallic acid (fig:2), 3,4,5-Trihydroxybenzoic acid is used as Anti-cancer & Anti-oxidant agent. Ellagic acid(fig:3) **:** 2,3,7,8-tetrahydroxy(1)benzopyrano(5,4,3-cde)(1)benzopyran-5,10- dione; 4,4',5,5',6,6'-Hexahydroxydiphenic acid 2,6,2',6'-dilactone is used as Anti-cancer,HIV-inhibitor,Anti-oxidant, Anti-mutagenic, Anti-microbial agent.

Litrature survey reveals that Corilagin estimation by HPLC-MS[8],MS[30],Ellagic acid estimation by HPLC[9], Gallic acid estimation by RP-HPLC[13] ,HPTLC[19,20,21,23], ESI-MS-MS[18], GC-MS[24], LC-MS-MS[25], Ellagic acid & Gallic acid estimation by RP-HPLC[10, 11, 12], Corilagin & Gallic estimation by GC-MS[28], Corilgin,Gallic acid & Ellagic acid estimation by HPLC[15], HPLC,1H-NMR,ESI-MS[16] was done. Above litrature survey reveals that no method has been reported for simultaneous estimation of Corillagin, Gallic acid & Ellagic acid by HPTLC.

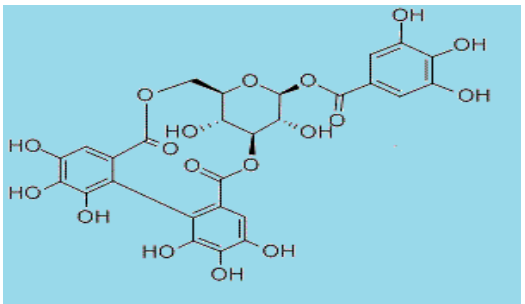


FIG:1STRUCTURE OF CORILAGIN

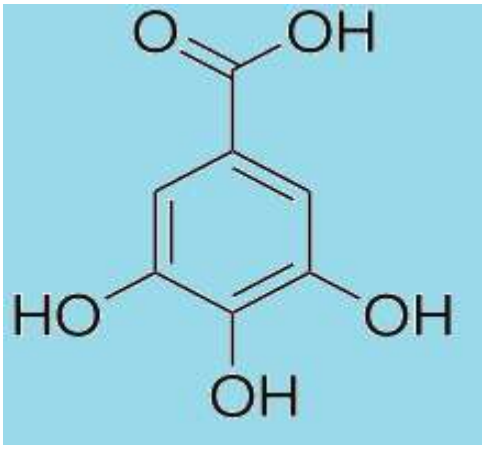


FIG:2 Structure Of Gallic Acid

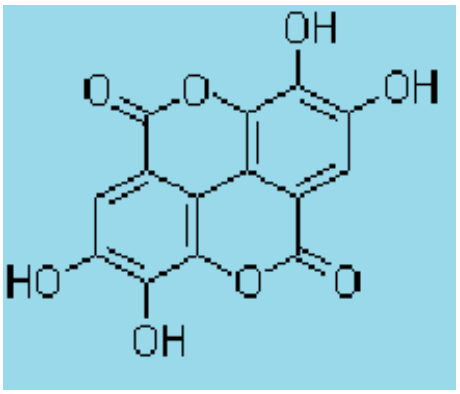


FIG:3 Structure Of Ellagic Acid

Experimental *Material*

Working standards of corilagin (>98% w/w), Gallic acid (>99% w/w), and Ellagic acid (>95% w/w) markers are obtained from Natural Remedy Pvt. Ltd, Bangalore India.The commercial crude powder of *Phyllanthus amarus* was collected from Green Pharmacy, Pune and The formullation containing *Phyllanthus amarus* was procured from the local market. All chemicals and reagents of analytical grade were purchased from Merk Chemicals, Mumbai, India.

Selection of analytical wavelength

Stock solutions of drugs were prepared in methanol separately. UV spectrum of 100 µg mL^{-1} of each individual drug was taken.

Instrumentation and chromatographic conditions

The hptlc plates were prewashed with methonal and activated at 110^0c for 5min prior to chromatography.The sample were spoted in the form of bands 6mm width with a CAMAG 100µL sample syringe(HAMILTON, BONADUZ, SWITZERLAND) on silica gel precoated HPTLC aluminium plate 60 F_{254} [(20x10 cm) with250µm thickness; E Merk, Darmstadt, Germany, supplied by ANCHROM technologies, Mumbai] using a CAMAG linomat V applicator (Switzerland). A constant application rate of 0.1µL/s was used and space between two bands of 6mm. linear ascending development was carried out in 20cm x 10cm twin through glass chamber (CAMAG, MUTTENZ, Switzerland) saturated with mobile phase. The mobile phase was consisted of n-Butanol: water: methanol: formic acid(6:1:0.1:0.8, v/v/v/v) and 20ml was used per chromatographic run. The optimized chamber saturation time with mobile phase was 30min using saturation pad at room temperature (25°C ± 2). The length of chromatogram run was 80mm and run time was 45min. Densitometric scanning was performed using a CAMAG TLC scanner III in the reflectance absorbance mode and operated by winCATs software (V 1.1.4, Camag). The slit dimension was kept at 5mm x 0.45mm and the scanning speed was 10mm/s. The source of radiation used was deuterium lamp emitting a continuous UV spectrum between 200 and 400nm. All determinations were performed at ambient temperature with a detection wavelength of 283nm.Concentration of the compound chromatographed were determined from the intensity of defused light. Evaluation was by peak area with linear regression.

Standard solutions and calibration graphs

Stock standard solution containing corilagin(100µg/ml), Gallic acid (100µg/ml) and Ellagic acid (100µg/ml) was prepared by dissolving 1mg each of accurately weight markers in methanol up the volume to 10mL with methanol. Different volumes of stock standard solution wete applied on the HPTLC plate to obtained working standard in the concentration range of 300-1300ng/spot for corilagin and 300-1300ng/spot for gallic acid and 300-1300ng/spot for Ellagic acid, respectively. Each concentration was applied six times on the HPTLC plate. The plate was then developed using the previously described mobile phase.The peak areas were ploted against the currosponding concentration to obtained the calibration graph. Linear calibration curves were generated using least-squares linear-regression analysis.

Sample preparation

1. Estimation of corilagin ,gallic acid and ellagic acid in commercial crude powder of Phyllanthus amarus Linn.

500*mg of powder of plant material was extracted separately with (4×25)ml of water:methanol(70:30, v/v).The extract was centrifuge at 5000rpm for 10min and the supernatant was filtered through a 0.45µm nylon syringe filter before chromatographic analysis and analysed for the drug content. The analysis was repeated six times.*

*1.**Analysis of commercial formulation Phyllanthus amarus:** To detesrmines the content of corilagin, Gallic acid,Ellagic acid in containing, the contents of twenty tablets were weighed, their mean weight determined and they were finely powdered.*

The weight of powder equivalent to tablet content was transferred into 50mL volumetric flask containing 20mL water: Methanol (70:30, v/v), sonicated for 30 min and diluted to 50 mL with water. Methanol [70:30, v/v]. The resulting solution was centrifuged at 3000 rpm for 15 min and supernatant was analysed for the said markers. The filtered solution was spotted on the HPTLC plate followed by development and scanning.

Method validation The optimized HPTLC method was validated with respect to the following parameters as per the ICH guidelines [7]

Precision Precision study was carried out for the repeatability of sample application and measurement and the result was expressed as % RSD of peak areas. Variability of the method was studied by analyzing by aliquots of standard solution of Corilagin (300, 700, 1300 ng/spot). Gallic acid (300, 700, 1300 ng/spot). And Ellagic acid(300, 700, 1300 ng/spot). On the same day (intraday precision) and on the different days (inter-day precision), and the results were expressed as % RSD

Robustness

Robustness of the method was checked by making intentional changes in the parameters. Small change in the mobile phase composition was tried (Formic acid ± 0.01 ml). the amount of mobile phase was varied in the range of ± 5%. The plates were prewashed with methanol and activated at 110 ℃ ± 5 for 5, 10, 15 min respectively prior to chromatography. Time from the spotting to chromatography and from chromatography to scanning was varied from 0, 30, 60, 90 min. robustness was done at the three different concentration level 300, 700, 1300 ng/spot for Corilagin, Gallic acid, Ellagic acid, Respectively

Limit of detection (LOD) and limit of quantitation (LOQ) The detection limit of an indivisual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in the sample that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ of Corillagin, Gallic acid and Ellagic acid were determined by calibration curve method. LOD and LOQ were calculated by using following equation.

$$LOD = \frac{3.3 \times Sy.x;}{S} \qquad LOQ = \frac{10.0\ Sy.x;}{S}$$

Where, Sy.x is a standard deviation of reciduals from line; S is slope

Specificity

The identity of the spot of the markers in sample was confirmed by comparing the R_f and spectra of the spot with that of the standard. The peak purity of the markers was aassessed by comparing their respective spectra at peak start (S), peak apex (M) and peak apex (E) positions of the spot.

3 ***RESULTS AND DISCUSSION:***

Optimization of procedure: *Initially, mobile phase was selected on the basis of previous reports of Gallic acid and Ellagic acid.Acommon mobile phase consisting of toluene, ethyl acetate, methanol and formic acid was tried initially. Several modification were tried* on trails, addition of water was found to be sutaible for the moment of Corilagin whereas Formic acid have effect on the peak shape of Corilagin. Hence the final mobile phase was optimized as n-butanol :Water:Methanol:Formic acid (6:1:0.1:0.8, v/v/v/v) which was found to give desirable R_f value. The optimized mobile phase can able to give symmetrical, well resolved reproducible peaks with good shape and baseline separation. The R_f values obtained were 0.44,0.79 and 0.63 for Corilagin, Gallic acid and Ellagic acid was taken.

Selection of analytical wavelength

283nm was selected as scanning wave length (fig:4). The identities of the bands from the sample extracts and commercial formulations were confirmed by overlapping the densitograms of standerd with that of samples.

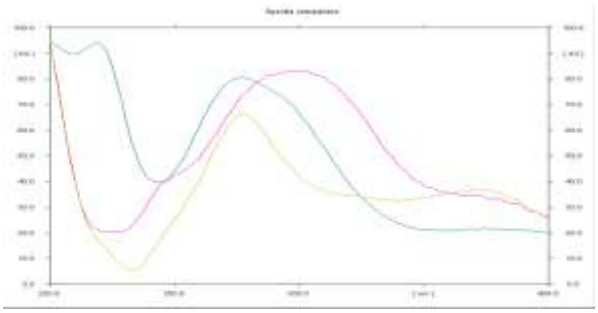
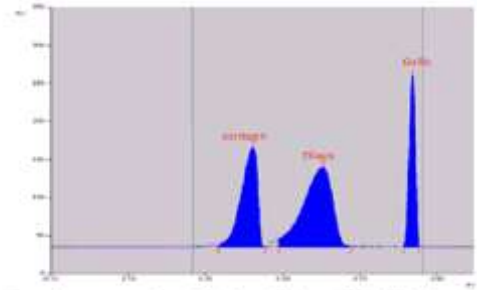


Fig4. *In situ HPTLC spectral overlain of Corilagin, Gallic acid and Ellagic acid*



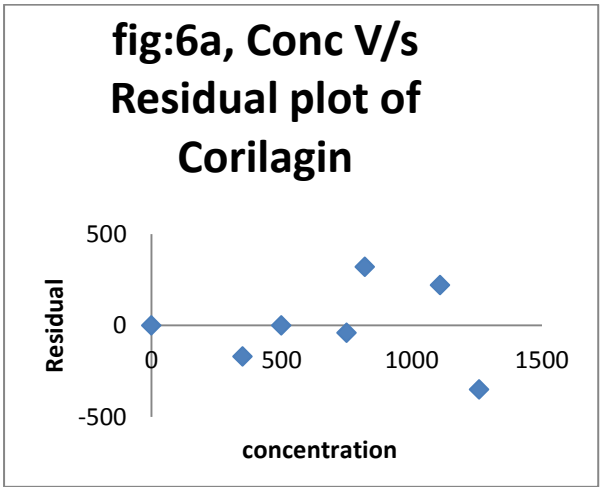
Densitogram of corilagin, Gallic acid & Ellagic acid 600ng/spot

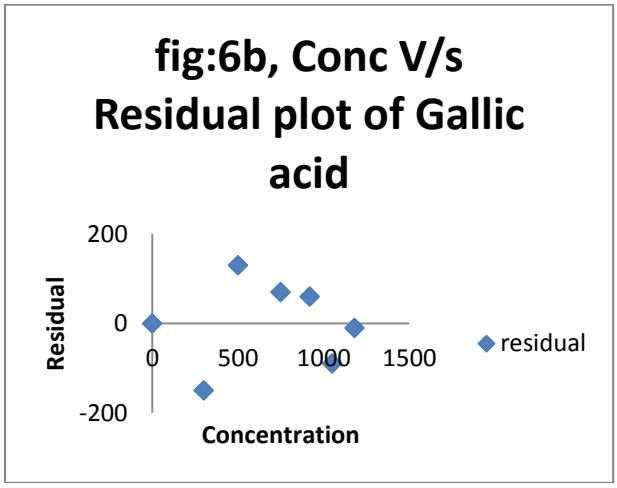
LINEARITY: Linear relationships were observed by ploting drug concentration against peak areas for each compound. Corilagin, Gallic acid and Ellagic acid show linear response in the concentration

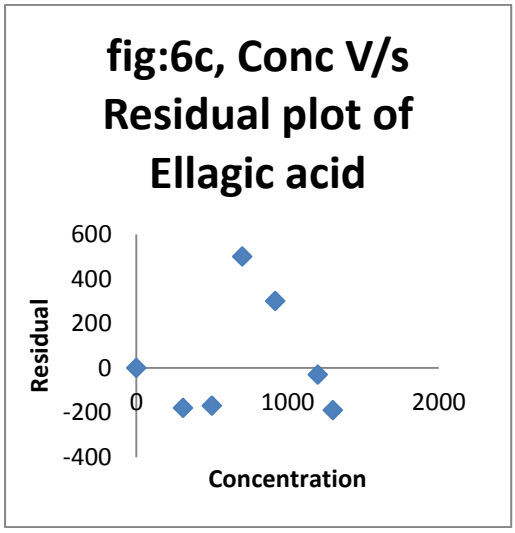
range of 300-1300ng/spot, respectively. The corresponding linear regression equation was y=15.46x-1369, y=6.888x+1962 and y=15.98x-1895 with square of correlation coefficient (r^2) of 0.9981, 0.9984 and 0.9977 for corilagin, allic acid and Ellagic acid respectively. No significant difference was observed in the slope of standard curve (Table:1).Residual analysis was performed to ascertain linearity (fig:6).

Parameters	Corilagin	Galic acid	Ellagic acid
Linearity range	300-1300 ng spot^{-1}	300-1300 ng spot^{-1}	300-1300 ng spot^{-1}
R^2 ± S.D.	0.9981 ± 0.0007	0.9984 ± 0.002	0.9977± 0.001
Slope ± S.D.	15.46 ± 0.3342	6.888± 0.1394	15.98±0.3838
Intercept ± S.D	-1359± 290.7	1962± 121.4	-1895±333.9
Confidence limit of slope[a]	14.54 to16.39	6.501 to7.275	14.92 to 17.05
Intercept[a]	-2166 to-552.2	1626 to 2300	-2822 to -968.4
Sy.x[b]	279.6	116.8	321.1

[a] 95% Confidence interval
[b] Standard deviation of residuals from line







Precision

The repeatability of the sample application and measurement of peak area were expressed as % RSD of corilagin, gallic acid and ellagic acid, respectively. The result of intermediate precision experiments are shown in table:2. The developed method was found to be precise as the RSD value of repeatability and intermediate precision studies were < 2% , respectively as recommended by ICH guidelines. Separation of the drug was found to be similar when analysis was performed using different chromatographic system on different days.

Precision studies of proposed HPTLC method Table:2

		Repeatability (intra-day)		Intermediate precision (inter-day)	
Drugs	**Conc. (µg mL^{-1})**	**Found conc. ± S.D.**	**%. R.S.D.**	**Found conc. ± S.D.**	**% R.S.D.**
Corilagin	300	299.46 ± 1.08	0.36	294.01 ± 2.05	0.70
	700	695.13 ± 3.72	0.53	689.19 ± 4.20	0.61
	1300	1342.02 ± 5.81	0.43	1325.94 ± 18.12	1.36
Gallic acid	300	301.04 ± 0.95	0.32	302.06 ± 0.68	0.23
	700	700.71 ± 5.86	0.84	701.28 ± 4.65	0.66
	1300	1290.43 ± 15.23	1.18	1299.41 ± 10.73	0.83
Ellagic acid	300	292.78 ±1.34	0.46	297.45± 1.67	0.56
	700	704.86 ±2.77	0.39	702.53± 2.89	0.41
	1300	1311.37 ± 12.53	0.96	1302.16± 10.55	0.81

Robustness

The standard deviation of the peak areas were calculated for each parameter and the %RSD was found to be less than 2%. The low values of the %RSD as shown in table 3 indicated the robustness of the method.

Robustness testing of HPTLC method Table:3

Parameters	S.D. of peak area			%R.S.D.		
	Corilagin	Gallic acid	Ellagic acid	Corilagin	Gallic acid	Ellagic acid
Mobile phase composition (formic acid ± 0.01 ml)	3.18	3.25	2.73	0.65	0.43	0.43
Amount of mobile phase (±5 %)	2.94	3.63	1.57	0.50	0.66	0.18
Time from spotting to chromatography	2.33	3.40	2.75	0.41	0.48	0.45
Time from chromatography to scanning	2.87	4.52	2.55	0.47	0.89	0.36
Plate pretreatment	1.98	2.78	2.11	0.34	0.29	0.28

Limit of detection and limit of Quantitation

The LOD & LOQ was found to be 53.37, 161.74 ng/spot for corilagin, 50.03, 151.61 ng/spot for Gallic acid and 59.29, 179.67 ng/spot for ellagic acid, respectively.

Specificity

The peak purity of Corilagin, Gallic acid and Ellagic acid was assessed by comparing their respective spectra at the peak start, apex and peak end positions of the spot i.e., r (S, M) = 0.999467, r (M, E) = 0.998935, r (S, M) = 0.999786, r (M, E) = 0.996751 and r (S, M) = 0.999787, r (M, E) = 0.996050 respectively. A good correlation (r^2 = 0.999, r^2 = 0.996 and r^2=0.996) was also obtained between the standard and commercial crude powder sample spectra of Corilagin, Gallic acid and Ellagic acid. A good correlation (r^2 = 0.998, r^2 = 0.999 and r^2=0.998) was also obtained between the standard and commercial formulation sample spectra of Corilagin, Gallic acid and Ellagic acid .A good correlation(r^2=0.998, r^2=0.999 and r^2=0.998) was also obtained between the standard and commercial formulation 2 sample spectra of corilagin,Gallic acid and Ellagic acid (fig:6a, 6b, 6c).

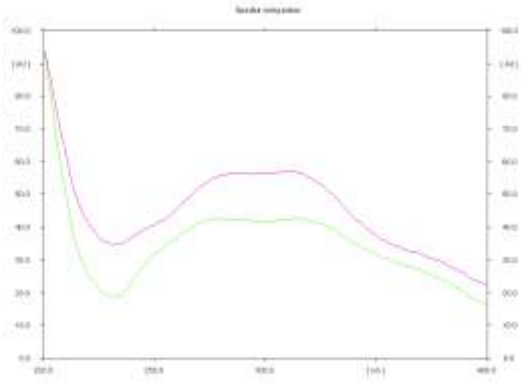


Fig6a.In situ HPTLC spectral overlain of Corilagin obtained from standard and commercial crude powder of *Phyllanthus amarus*

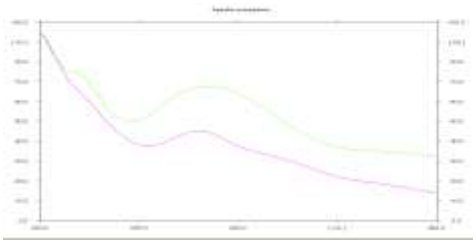


Fig6b: In situ HPTLC spectral overlain of Gallic acid obtained from standard and commercial crude powder of *Phyllanthus amarus.*

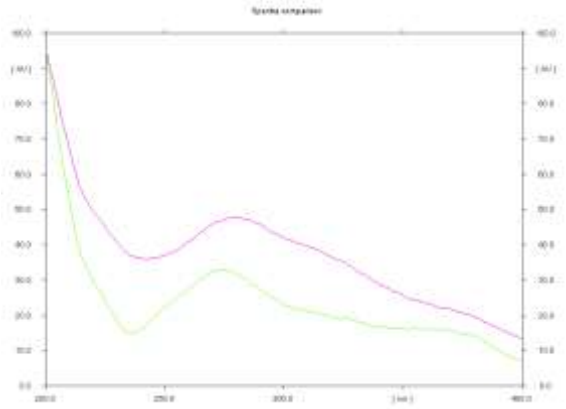


Fig6c. In situ HPTLC spectral overlain of Ellagic acid obtained from standard and commercial crude powder of *Phyllanthus amarus.*

Analysis of the commercial crude powder and commercial formulation of phyllanthus amarus Analysis of the amount of corilagin, Gallic acid and Ellagic acid studied application of the developed method in commercial crude powder and commercial tablets, using the reference comparison method.The content of corilagin, gallic acid and ellagic acid were found to be 0.030%, 0.035% and 0.072% for commercial crude powder of *Phyllanthus amarus* and 0.013%, 0.080% and 0.104% in commercial formulation, respectively.(fig:7, 8 & 9-a,b,c)

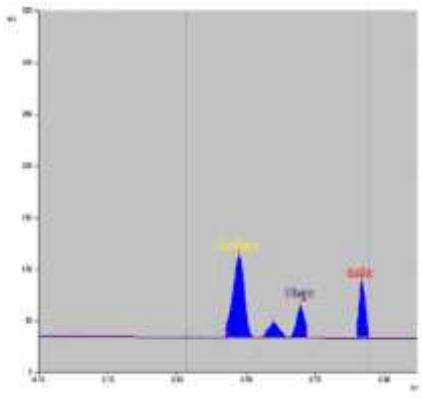


Fig:7.Densitogram of commercial crude powder of *Phyllanthus amarus*. Corilagin (R_f 0.45), Gallic acid (R_f 0.80) and Ellagic acid (R_f 0.66)

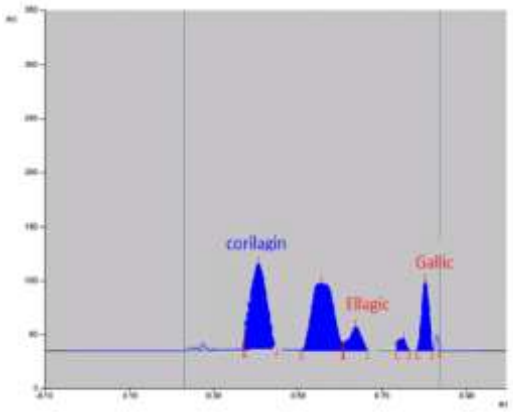


Fig:8 Densitogram of Commercial Formulation of *Phyllanthus amarus*. Corilagin (R_f 0.44), Gallic acid (R_f 0.80) and Ellagic acid (R_f 0.64)

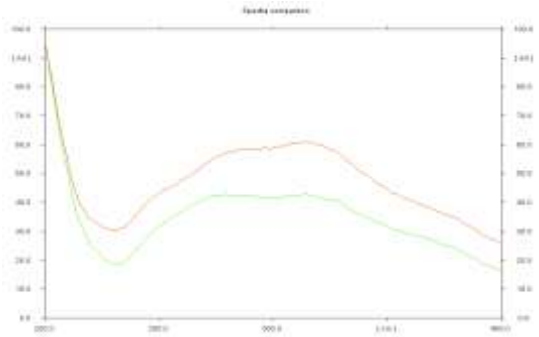


Fig:9a In situ HPTLC spectral overlain of Corilagin obtained from standard and commercial Formulation

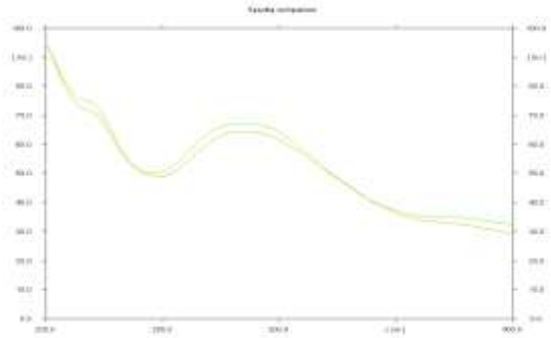


Fig:9b In situ HPTLC spectral overlain of Gallic acid obtained from standard and commercial Formulation

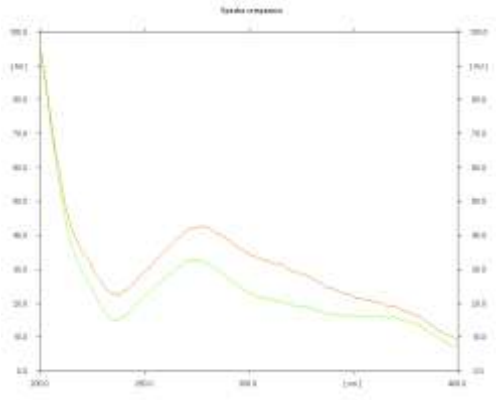


Fig:9c In situ HPTLC spectral overlain of Ellagic acid obtained from standard and commercial Formulation

Table:4 Estimation of drug content in samples

Samples **Drug Content* (% w/w)** Corilagin	Gallic acid	Ellagic acid
Commercial crude powder 0.030%	0.035%	0.072%
Marketed Formulation		
Formulation 0.013%	0.080%	0.104%

*Mean ± Standard deviation (n=3)

Result & Discussion

Analysis of the amount of corilagin, Gallic acid and Ellagic acid studied application of the developed method in commercial crude powder and commercial tablets, using the reference comparison method. The content of corilagin, Gallic acid and Ellagic acid were found to be 0.030%, 0.035% and 0.072% for commercial crude powder of ***Phyllanthus amarus*** and 0.013%, 0.080% and 0.104% in commercial formulation, respectively.

CONCLUSION

We established a HPTLC method for simultaneous estimation of the constituents corilagin, Gallic acid and Ellagic acid.The proposed method was found to be suitable for estimation of this markers in polyherbal formulation as it is proved to be précised, reproducible,reliable and robust. Hence, this method can be used for as a rapid analytical tool in routine analysis to monitor loss or variation of the content of the markers in various herbal formulation

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