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Research Article

Development of validated HPTLC method for quantitation of phyllanthin, hypophyllanthin and picroside-II in marketed herbal formulation (Valiliv capsules)

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ABSTRACT

A rapid and sensitive high-performance thin-layer chromatographic (HPTLC) method was developed and validated for quantitative estimation of Picroside-II, Phyllanthin and Hypophyllanthin simultaneously in marketed formulation (Valliliv capsule) containing *Picrorhiza kurroa* and *Phyllanthus niruri.* The sample (Valliliv capsule) were chromatographed on silica gel 60F_{254}- TLC plates, using solvent system (Hexane: Ethyl acetate, 6:3 v/v) and scanned at 278nm. The linearity range for Picroside was 200-1600ng/spot with average recovery of 99.90%, Phyllanthin was 200-1600ng/spot with average recovery 100.41% and Hypophyllanthin 200-1600ng/spot with average recovery of 99.99% in formulation. The limit of detection and limit of quantification for Picroside-II, Phyllanthin and Hypophyllanthin were found to be 40ng/spot- 120ng/spot, 90-270ng/spot and 100ng/spot-300ng/spot. The developed method was successfully applied for the assay of market formulation containing *Picrorhiza kurroa* and *Phyllanthus niruri.*

Keywords: Fingerprinting; Hepatoprotective; HPTLC; Phyllanthin; TLC; Hypophyllanthin; Picroside; Method development; Validation

INTRODUCTION

The marketed herbal product "Valiliv" is a combination of two hepatoprotective herbs (Girish, et al., 2009; Ansari-1991, Iqbal-2008), namely Picrorhiza kurroa (Bhandari, 2008) and Phyllanthus niruri (Chatterjee, 2007). Picroside-II is active compound of Picrorhiza kurroa and Phyllanthin and Hypophyllanthin are active compounds of Phyllanthus niruri (Bhattacharjee, 2004).

MATERIAL AND METHODS

All the solvents of analytical grade were purchased from Merck, Phyllanthin (Kale, 2001); Hypophyllanthin and Picroside-II was procured from Natural Remedies Pvt, Ltd, Banglore India.

TLC Conditions

The TLC plates were 20x 20cm, Precoated with silica gel F_{254} TLC plates (E.Merck) (0.2mm thickness); spotting device was Camag Linomat V sample applicator, Camag (Muttenz, Switzerland); syringe was a 100µl (Hamilton); developing chamber was a CAMAG glass twin trough chamber (20x 10); densitometer was CAMAG TLC Scanner 3 linked to winCATS software. Experimental conditions: Temperature 25±2°C, relative humidity 40% (Gupta, 2008).

TLC fingerprinting profile

Sample solution: Preparation of sample solution was optimized to achieve good fingerprinting and also to extract the marker compounds effectively. Of these, the finally selected sample solution was discussed. (Masturah, et; al, 2007; Vinod, 1997).

Methanolic extract: Since all three actives were soluble in methanol, we prepared methanolic extract. Accurately weighed 2.0g of the powdered drug was extracted with methanol (30mlx4) under reflux on a water bath. The methanolic extract was filtered through Whatman I filter paper, filtrate were combined, concentrated under vacuum and the volume was made up to 25ml in a volumetric flask. This extract was used for TLC fingerprinting and quantitative analysis with marker compounds.

Standard Solutions of Picroside-II, Phyllanthin, and Hypophyllanthin

Stock solution of Picroside-II, Phyllanthin, and Hypophyllanthin was prepared by dissolving 5mg of each in methanol and making up the volume to 5.0ml. Working standard was prepared by dissolving 1ml of the stock solution to 10.0ml with methanol.

Solvent System: Hexane: Ethyl acetate (6:3 v/v).

Procedure

For chromatography with Picroside-II, Phyllanthin, and Hypophyllanthin (API Edi.I, 1999), 10µl of sample

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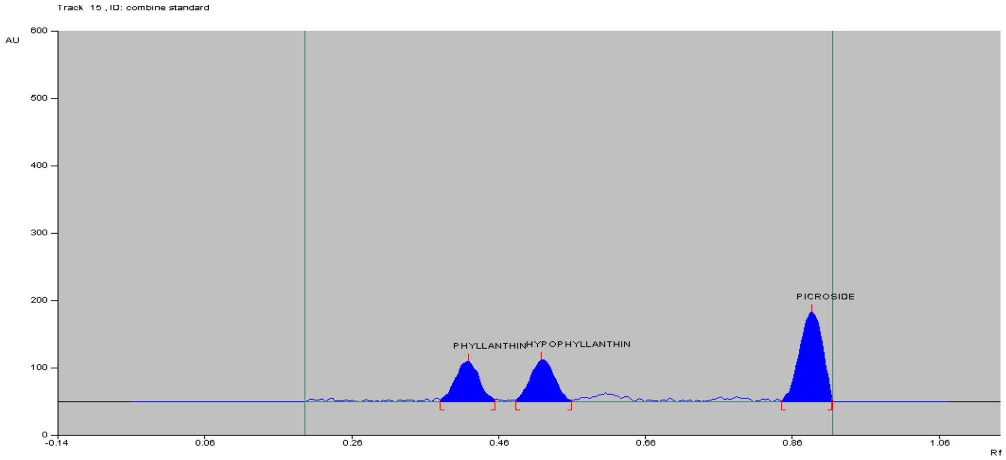

Figure 1: Shows densitogram of standard showing Phyllanthin, Hypophyllanthin and Picroside-II simultaneously

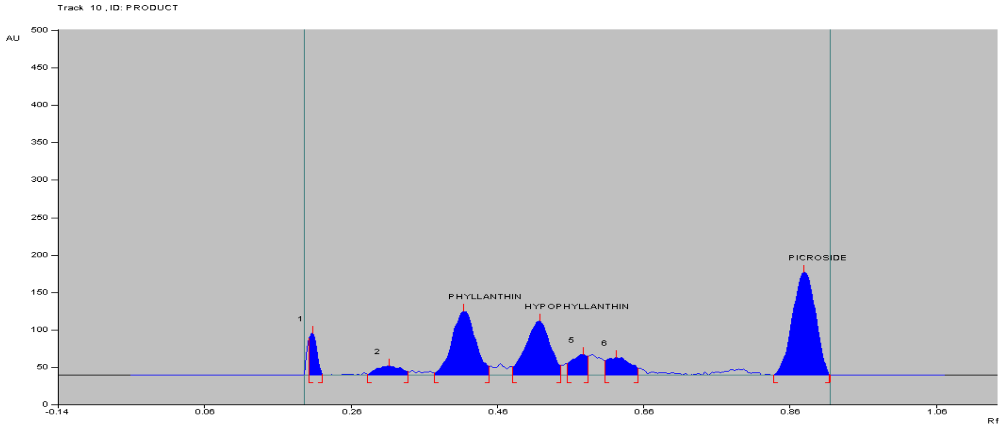

Figure 2: Shows densitogram of product (Valiliv) showing Phyllanthin, Hypophyllanthin and Picroside-II simultaneously

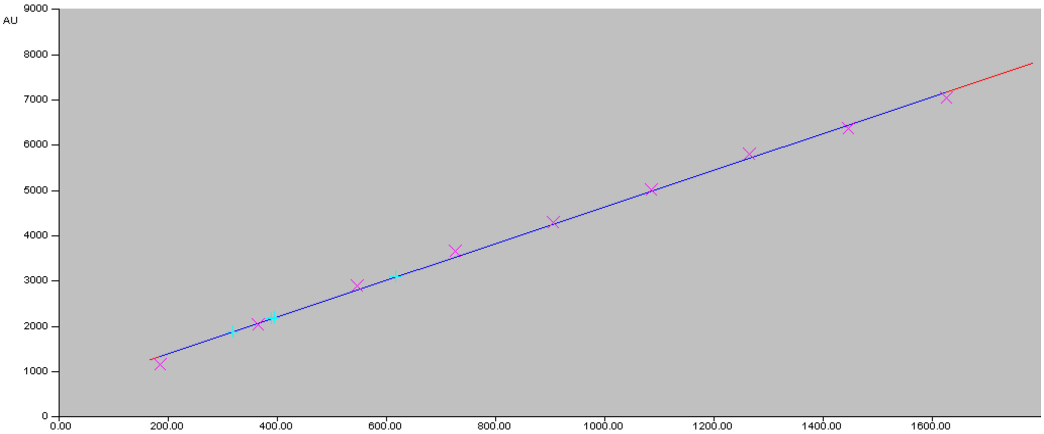

Figure 3: Calibration curve of Hypophyllanthin with standard deviation 2.10%

solution along with the standards were applied on a TLC plate and was developed in solvent system to a distance of 8cm. The plates were observed under UV 254nm and 366nm. The Rf values of resolved bands were noted. The identity of the bands of Picroside-II, Phyllanthin, and Hypophyllanthin in the sample track was confirmed by overlying their UV absorption spectra with those of the respective reference standards using a Camag TLC Scanner 3 with winCATS software. The purity of each of these separated bands in the sample extract track was check by comparing the absorption spectra recorded at start, middle, and end positions of each of the band.

TLC densitometric quantification of picroside-II, phyllanthin, and hypophyllanthin

Sample solution: sample solution described under the previous section was used for simultaneous quantification of Picroside-II, Phyllanthin, and Hypophyllanthin (Sane RT, 1997).

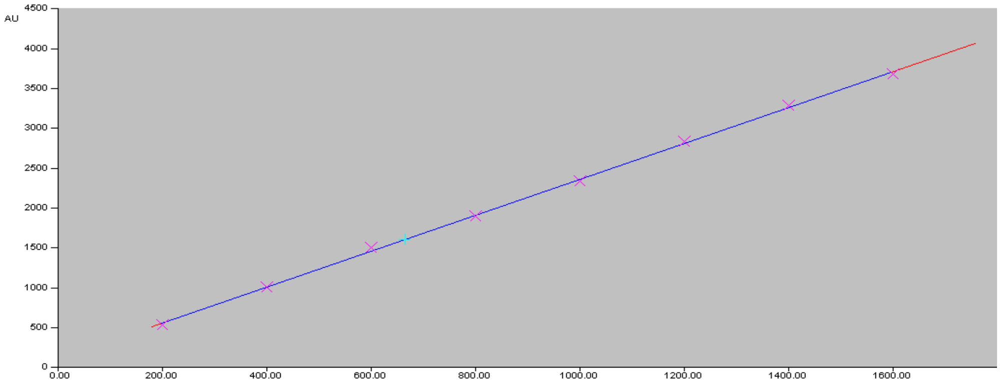

Figure 4: Calibration curve of Hypophyllanthin with standard deviation 1.29%

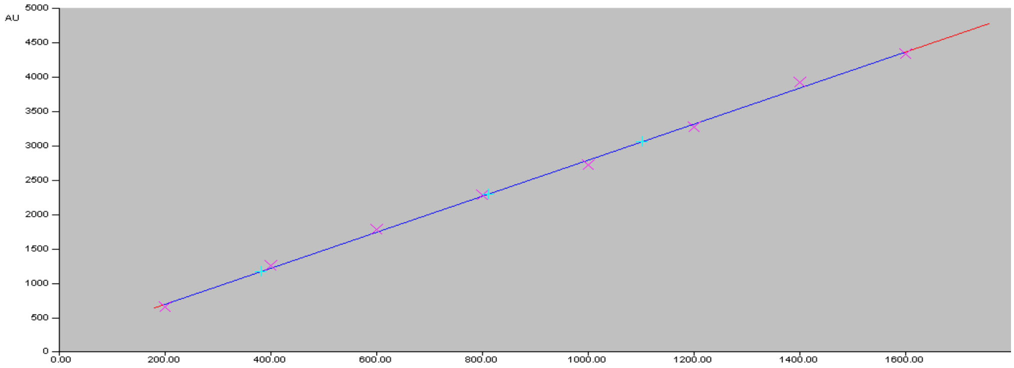

Figure 5: Calibration curve of Hypophyllanthin with standard deviation 2.17%

Table 1: Shows results of Simultaneous Quantitative Study

S.No.	Compound	Quantity in Product (mg/capsule)	Quantity in Product (%w/w)
1.	Picroside-II	0.01756mg	0.00373%
2.	Phyllanthin	0.04198mg	0.00893%
3.	Hypophyllanthin	0.05263mg	0.01119%

Preparation of calibration curve

Standard solutions of Picroside-II, Phyllanthin and Hypophyllanthin (200ng to 1600ng) were applied (band width: 6mm, distance between the tracks: 12mm) in triplicates on a TLC plate using automatic sample spotter. The plates were developed in a twin trough chambers (20 X10 cm) up to a distance of 8cm using a solvent system of Hexane: Ethyl acetate (6:3 v/v) at 25±2°C temperature and 40% humidity. The plates were dried at room temperature and scanned at 278nm in absorbance mode using deuterium lamp. The areas of the resolved peaks were recorded. Calibration curve of Picroside-II, Phyllanthin and Hypophyllanthin was obtained by plotting peak areas vs. applied concentrations of Picroside-II, Phyllanthin, and Hypophyllanthin respectively.

Simultaneous quantification of Picroside-II, Phyllanthin, and Hypophyllanthin in sample

The solvent system was optimized to Hexane: Ethyl acetate (6:3 v/v). 10µl each of suitably used sample solution along with the marker compounds were applied in triplicate on a TLC plates. The plate was developed in the given solvent system and scanned as mentioned above. The peak areas and absorption spectra were recorded and the amount of Picroside-II, Phyllanthin, and Hypophyllanthin were calculated using their respective calibration curves (Ahuja, 2001).

Validation of the Method

ICH guidelines were followed for the validation of the analytical method developed for precision, accuracy and repeatability (J Ermer, 2005).

Precision

Three sets of three different concentration standard solutions were prepared. The intra day and inter day precision of the developed TLC method was determined by preparing the samples of the same batch in nine determinations with three concentrations and three replicate each on the same day. The inter-day precision was also determined by assaying the sample in triplicate per day for consecutive three days.

Instrumental precision

Instrumental precision was checked by repeated scanning of the same spot of Picroside-II, Phyllanthin, and Hypophyllanthin and expressed as relative standard deviation (% RSD).

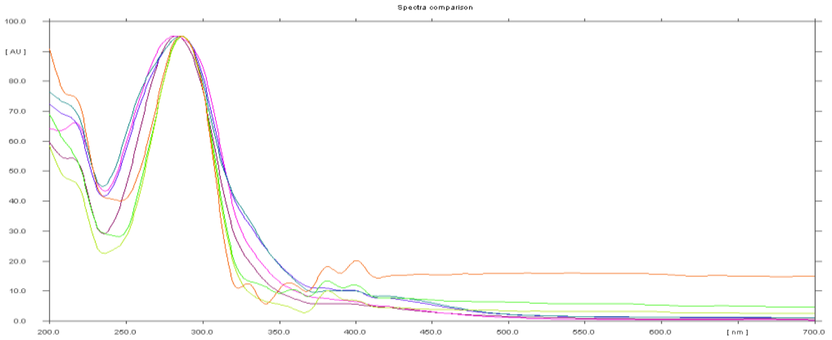

Figure 6a: Shows specificity of Picroside in product and raw herb with standard at peak start, peak apex and peak end position

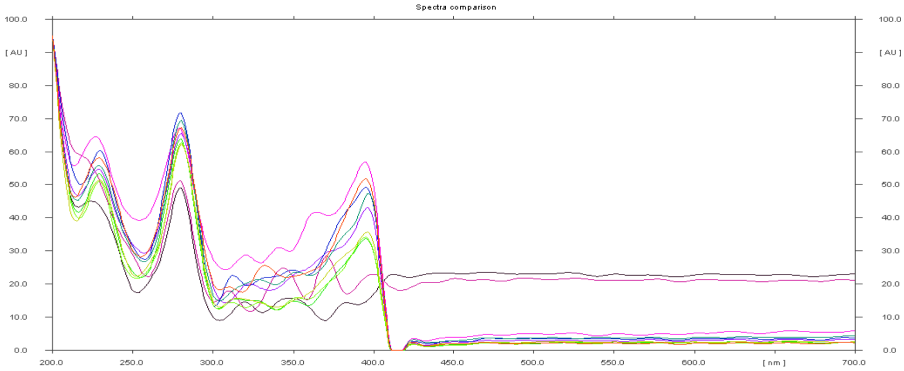

Figure 6b: Shows specificity of Phyllanthin in product and raw herb with standard at peak start, peak apex and peak end position

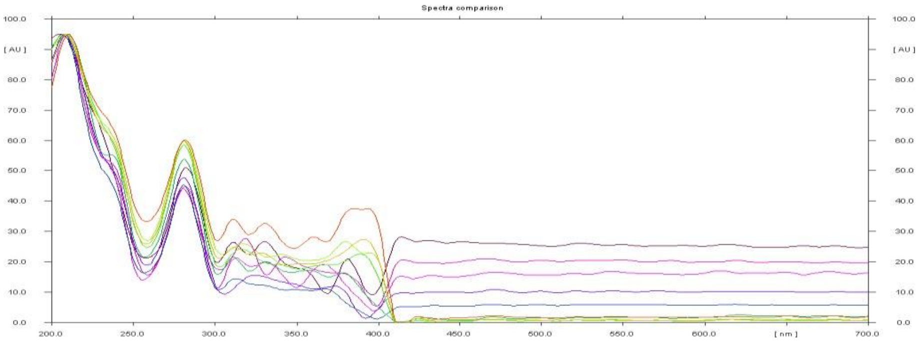

Figure 6c: Shows specificity of Hypophyllanthin in product and raw herb with standard at peak start, peak apex and peak end position

Table 2: Data obtained for linearity of Picroside-II, Phyllanthin and Hypophyllanthin

Regression equation	Linearity range	Standard deviation	Regression coefficient
Picroside-II			
Y = 3158.18+3.054* X	200-1600 ng	Sdv = 2.10 %	r = 0.994
Phyllanthin			
Y = 104.362 + 2.252* X	200-1600 ng	Sdv = 1.29 %	r = 0.999
Hypophyllanthin			
Y = 165.733 + 2.624* X	200-1800 ng	Sdv = 2.17 %	r = 0.999

Specificity

Specificity of the proposed method was evaluated by comparing the R_f value of standard with that of sample. The peak purity of standards was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot.

Linearity

For linearity study standard solution was applied over plate in increasing concentration. The intercept, correlation coefficient, standard deviation was calculated from regression equation.

Table 3: Statistical data of accuracy of Picroside-II, Phyllanthin, and Hypophyllanthin

S.No.	Picroside-II in sample (ng)	Std. added (ng)	Total added conc.	Found conc.	% Recovery
1	380	300	680	678.61	99.79
2	380	300	680	677.01	99.56
3	380	300	680	675.99	99.41
4	380	380	760	759.16	99.88
5	380	380	760	758.52	99.81
6	380	380	760	762.57	100.33
7	380	450	830	829.48	99.93
8	380	450	830	834.69	100.56
9	380	450	830	833.00	100.36
Average					99.908
Phyllanthin					
S.No.	**Phyllanthin in sample (ng)**	**Std. added (ng)**	**Total added conc.**	**Found conc.**	**% Recovery**
1	234.19	190	425	425.14	100.03
2	234.19	190	425	423.95	99.75
3	234.19	190	425	427.25	100.53
4	234.19	234	468	469.99	100.42
5	234.19	234	468	470.68	100.57
6	234.19	234	468	471.23	100.69
7	234.19	281	515	516.69	100.23
8	234.19	281	515	514.52	99.97
9	234.19	281	515	505.63	98.18
Average					100.41
Hypophyllanthin					
S.No.	**Hypophyllanthin in sample (ng)**	**Std. added (ng)**	**Total added conc.**	**Found conc.**	**% Recovery**
1	384	306	690	686.27	99.45
2	384	306	690	689.78	99.96
3	384	306	690	687.01	99.56
4	384	384	768	774.99	100.90
5	384	384	768	775.65	100.99
6	384	384	768	761.06	99.09
7	384	460	844	846.39	100.28
8	384	460	844	840.24	99.55
9	384	460	844	845.47	100.17
Average					99.99

Table 4: Statistical data of precision of Picroside-II, Phyllanthin, and Hypophyllanthin

S. No.	Application of sample (µl)	Concentration obtained		
		Picroside-II (ng)	Phyllanthin (ng)	Hypophyllanthin (ng)
1	2	3242.56	2311.00	697.10
2	2	2911.08	2354.14	693.77
3	2	2124.09	2259.72	708.06
Average		2759.24	2275.28	699.06
% RSD		1.786	1.403	1.068
1	4	5223.27	4478.28	1394.21
2	4	5112.37	4526.23	1394.61
3	4	5156.63	4579.57	1387.55
Average		5164.09	4528.02	1392.12
% RSD		0.927	1.119	0.285
1	6	7796.69	6792.33	4790.11
2	6	7777.00	6809.91	4759.53
3	6	7780.89	6811.99	4749.77
Average		7784.86	6804.74	4766.47
% RSD		0.134	0.159	0.442

Table 5: Statistical data of precision of Picroside-II, Phyllanthin and Hypophyllanthin

S. No.	Application of sample (µl)	Area of standard Picroside - II	Area of standard Phyllanthin	Area of standard Hypophyllanthin
1	6	4373.95	1743.06	1323.43
2	6	4414.46	1788.25	1332.59
3	6	4395.46	1743.10	1356.54
4	6	4397.78	1747.60	1361.42
5	6	4420.67	1739.05	1366.45
6	6	4389.08	1730.08	1347.45
Average		4398.56	1748.52	1338.55
% RSD		0.3868	1.163	1.25

Table 6: Detection Limit (LOD) and Quantitation Limit (LOQ) of Picroside-II, Phyllanthin and Hypophyllanthin - Based on Signal-to-Noise

Name of the plant constituents	LOD (ng)	LOQ (ng)
Picroside-II	40ng/spot	120ng/spot
Phyllanthin	90ng/spot	270ng/spot
Hypophyllanthin	100ng/spot	300ng/spot

Table 7: Statistical data of robustness of Picroside-II, Phyllanthin and Hypophyllanthin

Parameter	Initial condition	Change in condition	Effect found
Mobile phase composition	Hexane : Ethyl Acetate (6:3)	Hexane : Ethyl Acetate (6.5 : 3.5)	No change in Rf value
Development distance	8 cm	6cm	No Change in Rf value
Saturation time	20 min.	30 min.	No change
Extraction time	20 min.	30 min.	No change

Accuracy (% Recovery)

The recovery study was carried out by estimating the marker compounds in the pre analyzed sample of the product spiked with marker compound respectively at three concentration levels. Three replicate estimation were carried out for each concentration level.

System Precision

System precision was calculated by analyzing one conc. level of standards 6 times.

Limits of detection (LOD) and Limits of quantification (LOQ)

Limits of detection and limits of quantification were determined by calculation of the signal-to-noise ratio. Signal-to-noise ratios of approximately 3:1 and 10:1 were used for estimating the detection limit and quantification limit respectively.

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters such as mobile phase composition, saturation time and scanning time.

CONCLUSION

From the experimental data and results obtained, it can be concluded that the HPTLC method was found to be simple, precise, specific, sensitive and accurate for the qualitative and quantitative estimation of the active ingredients in the herbal Hepatoprotective product valiliv Capsule. The method was developed for the first time on HPTLC to estimate active ingredients in herbal product. The fingerprinting profile and spectral scanning was used for the qualitative study and densitometric scanning was used for the quantitative study.

TLC densitometric method was developed for the qualitative and quantitative estimation of bioactive compounds (**Picroside-II, Phyllanthin and Hypophyllanthin**) in polyherbal formulation (Valiliv Capsule) using HPTLC. We developed fingerprint profile of *Picrorrhiza kurroa and Phyllanthus niruri.* By using this method we estimated the quantity of Picroside-II, Phyllanthin and Hypophyllanthin individually and were also estimated simultaneously in raw material and product.

This method was found to be simple, precise, specific, sensitive and accurate and can be used for the identification and quantification of the marker compounds in the raw herb and product.

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