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Original article

Development and validation of a high-performance thin-layer chromatographic method for the quantitative analysis of vitexin in *Passiflora foetida* herbal formulations

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ABSTRACT

The aim of this study is the development of validated HPTLC method for the quantification of vitexin from *Passiflora foetida* commercial herbal formulations. The developed method was validated, in accordance with ICH guidelines for precision, accuracy, specificity and robustness. The plate was developed using ethyl acetate:methanol:water:formic acid 30:4:2:1(%, *v*/*v*/*v*/*v*) on 20 × 10 cm glass coated silica gel 60 F_{254} plates and the developed plate was scanned and quantified densitometrically at λ = 340 nm. Linear regression analysis revealed a good linear relationship between peak area and amount of vitexin in the range of 100–700 ng/spot. The amount of vitexin in nine commercial herbal formulations was successfully quantified by the developed HPTLC method. The developed and validated high performance thin layer chromatographic method offers a new sensitive and reliable tool for quantification of vitexinin in various herbal formulations containing *Passiflora foetida.*

1. Introduction

Traditional systems of medicines and medicinal plants play an important role in many Asian countries as a common remedy for many diseases. Medicinal plants are rich sources of bioactive compounds and thus serve as an important raw material for drug production and have become a target for the search of new drugs (Aseervatham et al., 2011). Passiflora is the largest genus in the Passifloraceae family and comprises nearly 500 species *Passiflora foetida* L. (*P. foetida*) (Stinking passion flower) is South American in origin, which has been spread to many tropical areas (Sasikala et al., 2011).

Its leaves, fruit and flower contain components such as tannins, coumarin alkaloids, flavonoids, tyrosine, and glycine (Reddy et al., 2006). The ethanobotanical views of *P. foetida* L. suggest that decoction of flower can be used to treat asthma, bronchitis and whooping cough (Saraswathy et al., 2014; Mijin et al., 2017). In various traditional systems of medicine, the plant can be used for treating urinary infection, anxiety, migraine, nervousness, and insomnia. Leaves and root decoction is emmenagogue, used in hysteria and leaf paste is applied on the head for giddiness and headache and skin disease (Zibadi and Watson, 2004; Akhondzadeh et al., 2001). Traditionally, the plant has been used for its properties like antiproliferative, sedative, anti-anxiety, antibacterial, leishmanicidal, antispasmodic, emetic, dressing for wounds and antiulcer (Puricelli et al., 2003; Dhawan et al., 2004).

The major phytoconstituents of *Passiflora foetida* are alkaloids, phenols, glycosides, flavonoids, cyanogenic compounds, passifloricins, polypeptides and alpha-pyrones (Ingale and Hivrale, 2010; Elsas et al., 2010; Gadioli et al., 2018). Vitexin (Fig. 1), a natural flavonoid compound identified as apigenin-8-C-β-D-glucopyranoside, a chemical compound found in the passion flower, possess to have anticancer (Yang et al., 2013) antioxidant (Borghi et al.,

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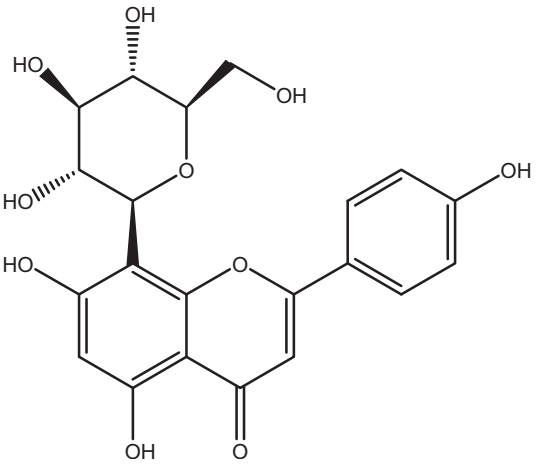

Fig. 1. The chemical structure of vitexin.

2013), anti-viral (Krcatovic et al., 2008) anti-inflammatory (Bahareh et al., 2017), anti-thyroid, anti-arteriosclerotic (Gaitan et al., 1995), antihypertensive (Prabhakar et al., 1981) and antihepatotoxic (Hoffmann-Bohm et al., 1992) properties.

Numerous methods have been reported on the quantification of vitexin in different plants by chromatographic methods like HPTLC (Aussavashai et al., 2016; Shivraj and Se, 2015; Jin et al., 2010), HPLC (Chang-He et al., 2011; Sagaradze et al., 2017; Liu et al., 2004) and UHPLC-DAD (Paula et al., 2018). Aussavashai et al., reported the quantification of vitexin in *Passiflora foetida* leaves using HPTLC. The method found to have low linearity range with high R_f value (Bahareh et al., 2017). The other two HPTLC methods by Gaitan et al. (1995) and Prabhakar et al. (1981) was on the simultaneous quantification of vitexin along with various other flavonoids was reported on some other plant species.

In this context it is necessary to develop HPTLC analytical method for vitexin, through which the standardization of this important herb can be established. More over the research will help in evaluating the usefulness of this flavonoid as a chemical marker of the analyzed species. The goal of the present research was to develop an analytical technique for the qualitative and quantitative determination of the flavonoid vitexin, in some herbal formulations containing *Passiflora foetida* as main ingredient using HPTLC.

2. Experimental

2.1. Standard and chemicals

Reference standard vitexin was purchased from Sigma-Aldrich, St. Louis, MO, USA. Acetone, methanol, ethyl acetate, distilled water and other chemicals used were of analytical reagent (AR) grade. Different commercial passiflora formulations were collected from different shops in Turkey.

2.2. Preparation of standard solution

Accurately weighed 10 mg of standard vitexin (purity ≥98%) was dissolved in methanol in a 10-mL volumetric flask; 1 mL accurately measured was transferred to another 10 mL volumetric flask and completed to volume with methanol to get a concentration of 100 μg/mL. Different volumes of working standard, i.e., 1, 2, 3, 4, 5, 6 and 7 (100, 200, 300, 400, 500, 600 and 700 ng, respectively), were applied on TLC. The calibration curve was plotted in the range of 100–700 ng/spot, using data of peak areas against the corresponding amount per spot. The standard solution was stored in refrigerator at 4 °C.

2.3. Preparation of extract for HPTLC analysis

The samples (5 g) from each sample were extracted by maceration in methanol (3× 100 mL) at room temperature. Extracts were separately evaporated under reduced pressure using rotary vacuum evaporator. The concentrated extracts were transferred to 50 mL volumetric flask and made up the volume with methanol and kept in refrigerator for analyses.

2.4. Chromatographic conditions

HPTLC analysis was performed on 10 × 20 cm glass-backed HPTLC plates coated with 0.2 mm layers of silica gel 60 F_{254} (Merck, Darmstadt, Germany). The standard and sample solutions were applied to the TLC plates as 6 mm bands using a Camag Automatic TLC Sampler 4 (ATS4) sample applicator (Switzerland) fitted with a Camag microlitre syringe. A constant application rate of 150 nl/s was used. Linear ascending development of the plates to a distance of 8 cm was performed with ethyl acetate:methanol:water:formic acid 30:4:2:1 (%, *v*/*v*/*v*/*v*) as mobile phase in a Camag Automatic Developing Chamber 2 (ADC2) previously saturated with mobile phase vapor for 30 min at 22 °C.

2.5. Detection and quantification

The developed plate was scanned and quantified densitometrically at 340 nm. Linear regression analysis revealed a good linear relationship between peak area and amount of vitexin in the range of 100–700 ng/band.

2.6. Method validation

The validation of analytical procedures of HPTLC method was performed according to the guidelines of international conference on harmonization (ICH). The linearity of the method for vitexin was checked between 100 and 700 ng/spot and concentration was plotted against peak area.

Accuracy, as recovery, was determined by the standard addition method. Pre-analyzed samples of vitexin (200 ng/spot) were spiked with extra amounts of vitexin (0, 50, 100 and 150%) and the mixtures were reanalyzed. Percentage recovery and relative standard deviation (RSD, %) were calculated for each concentration level.

Precision was assessed by determination of repeatability and intermediate precision. Repeatability of sample was determined as intra-day variation whereas intermediate precision was determined by assessment of inter-day variation for analysis of vitexin at three different amounts (300, 400 and 500 ng/spot) in six replicate.

Robustness of the proposed TLC densitometric method was determined to evaluate the influence of small deliberate changes in the chromatographic conditions like mobile phase composition, mobile phase volume, duration of mobile phase saturation and activation of HPTLC plates during the determination of vitexin.

Limit of detection (LOD) and limit of quantification (LOQ) were determined by standard deviation (SD) method. They were calculated from the slope of the calibration (S) curve and SD of the blank sample using following equations:

$LOD = 3.3SD/S$

$LOQ = 10SD/S$

The standard deviation of the response was determined based on the standard deviation of *y*-intercepts of regression lines.

Specificity of the proposed TLC densitometric was confirmed by analyzing and comparing the R_f values and UV spectra of the spot for vitexin in the samples with that of the standards.

2.7. Quantification of vitexinin in methanolic extract

The test samples were applied and chromatograms were obtained under the same conditions as for analysis of standard vitexin. The area of the peak corresponding to the R_f value of vitexin standard was recorded and the amount present was calculated from the regression equation obtained from the calibration plot.

3. Results and discussion

3.1. Method development

The mobile phase composition was optimized to establish a suitable and accurate densitometric HPTLC method for analysis of vitexin. The mobile phase ethyl acetate:methanol:water:formic acid 30:4:2:1 (*%, v/v/v/v*) resulted in well resolved peak, compact and symmetrical at R_f value of 0.49 ± 0.01 (Fig. 2). UV spectra measured for the bands showed maximum absorbance at approximately 340 nm.

3.2. Method validation

The calibration plot of peak area against amount of vitexin was linear in the range 100–700 ng/spot. Linear regression data for the plot confirmed the good linear relationship (Table 1). The correlation coefficient (R^2) was 0.9966 which was highly significant ($P < 0.05$). The linear regression equation was Y = 13.88x + 1459, where Y represents the UV absorption while X is the concentration of vitexin (Table 1).

Accuracy was expressed as percentage recovery. The accuracy of the method, as recovery, was 97.83–99.33%, with RSD values in the range 0.45–1.32. These results indicated the accuracy of the method (Table 2).

Table 1
Linear regression data for the calibration curve of vitexin (n = 6).

Linearity range (ng/spot)	100–700
Regression equation	Y = 13.88x + 1459
Correlation coefficient	0.9966
Slope ± SD	13.88 ± 0.1894
Intercept ± SD	1459 ± 73.76
Standard error of slope	0.07625
Standard error of intercept	3.34
95% confidence interval of slope	4.513–5.143
95% confidence interval of intercept	820–987.2

Precision was expressed as percentage coefficient of variation (% CV) of measured concentrations for each calibration level. Results from determination of repeatability and intermediate precision, expressed as SD (%) are shown in Table 3. RSD was in the range 0.36–0.66 for repeatability and 0.47–0.93 for intermediate precision. These low values indicated that the method is precise.

Results of robustness are shown in Table 4. Low values of % RSD (0.35–0.38) were obtained after introducing small deliberate change into the densitometric TLC procedure proved the robustness of the proposed HPTLC method. LOD and LOQ of the proposed method was found to be 6.51 and 15.12 ng/spot, for vitexin, which indicated that the proposed method can be used in wide range for detection and quantification of vitexin effectively.

The peak purity of vitexin was assessed by comparing the overlaid spectra at peak start, peak apex and peak end position of the spot. The overlaid spectra of vitexin standards and samples were given in Fig. 7.

3.3. Quantification of vitexinin in methanolic extract

Vitexin peaks from methanolic extract of samples were identified by comparing their single spot at R_f = 0.20 ± 0.01 (Figs. 3–6) values with those obtained by chromatography of the standard under the same conditions. The vitexin content in methanol extract of different passiflora samples were quantified using the obtained linear regression equation (Fig. 8). The result of the analysis proved that the content of vitexin in passiflora samples can be quantified

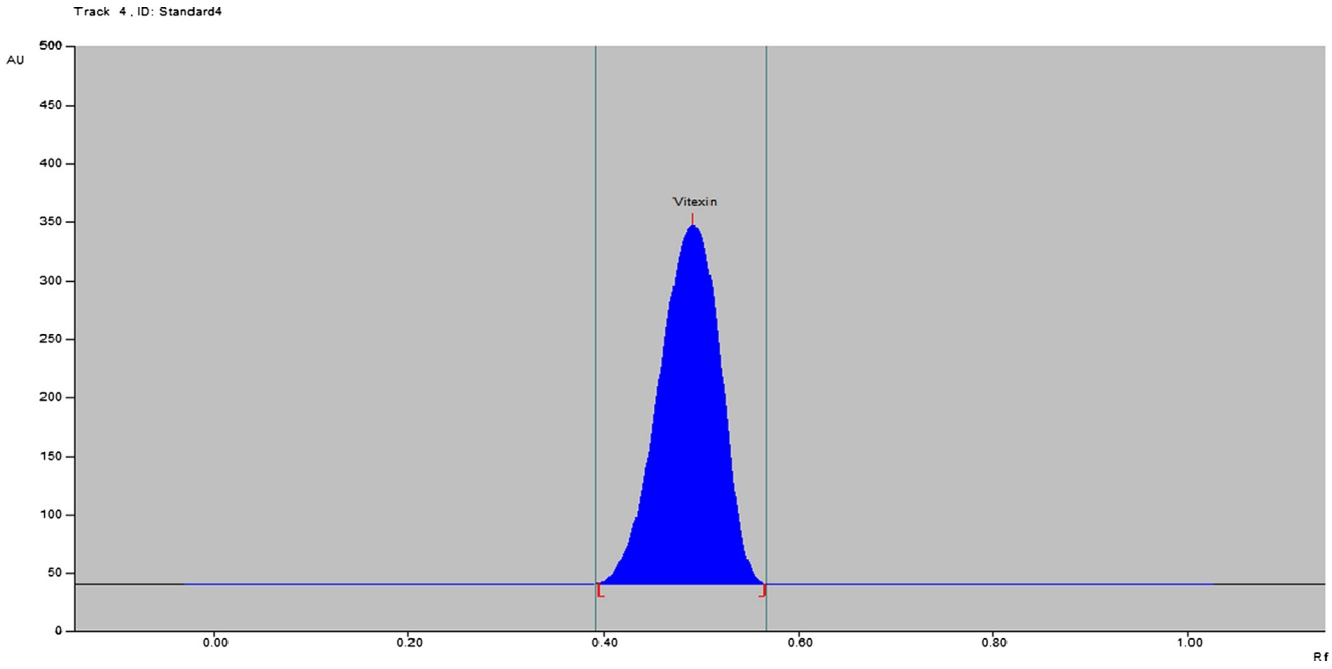

Fig. 2. HPTLC densitogram of standard Vitexin.

Table 2
Accuracy of the proposed method (n = 6).

Excess drug added to analyte (%)	Theoretical content (ng)	Conc. found (ng) ± SD	% Recovery	% RSD
0	200	195.67 ± 2.58	97.83	1.32
50	300	294.83 ± 2.32	98.28	0.79
100	400	396.50 ± 3.83	99.13	0.97
150	500	496.67 ± 2.25	99.33	0.45

Table 3
Precision of the proposed method.

Conc. (ng/spot)	Repeatability (Intraday precision)			Intermediate precision (Interday)		
	Area ± SD (n = 6)	Standard error	% RSD	Area ± SD (n = 6)	Standard error	% RSD
300	5693.00 ± 21.73	8.87	0.38	5698.17 ± 34.34	14.02	0.60
400	7061.17 ± 25.11	10.25	0.36	7062.83 ± 33.50	13.68	0.47
500	8431.50 ± 55.99	22.86	0.66	8496.50 ± 79.27	32.37	0.93

Table 4
Robustness of the proposed HPTLC method.

Conc. (ng/spot)	Mobile phase composition (Ethyl acetate:Methanol:Water: Formic acid)			Results		
	Original	Used		Area ± SD (n = 6)	% RSD	R_f
500	30:4:2:1	30.2:3.8:2:1	+0.2	8396.50 ± 87.20	1.04	0.48
		30:4:2:1	0.0	8429.83 ± 56.12	0.67	0.49
		29.8:4.2:2:1	−0.2	8429.83 ± 35.63	0.42	0.51

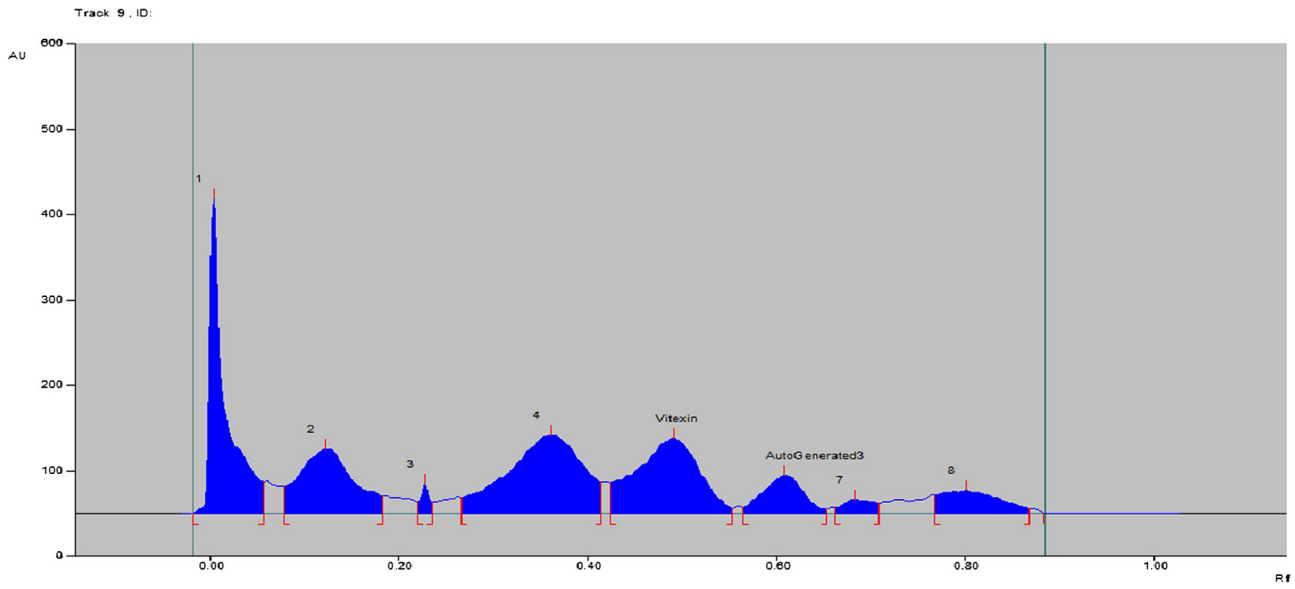

Fig. 3. HPTLC densitogram of *Passiflora foetida* containing herbal formulation (HS).

using the newly developed HPTLC method (Table 5). The method was found to be sensitive enough for the successful analysis of vitexin even in low quantities. It was also demonstrated that peaks of vitexin in samples were well resolved and did not merged with any impurity or any other constituent of drug as well as formulation

4. Discussion

The developed HPTLC procedure allows a simple but effective solution for the estimation of vitexin in various herbal formulations with a short analysis time and with satisfactory UV detection. The methods reported by Aussavashai et al., on the quantification of vitexin in *P. foetida* leaves using HPTLC using ethyl acetate: methanol:distilled water:formic acid (50:2:3:6 (v/v) with R_f 0.70 and linearity range of 2.5–17.5 mg/mL. Comparing to this earlier method, our method has a much better R_f (0.49) and linearity range of 100–700 ng/spot which make the method more sensitive. When comparing with the HPLC method on vitexin, the HPTLC method have the advantage of simultaneous analysis of several analytes using small amounts of mobile phase, with minimum analysis time and cost.

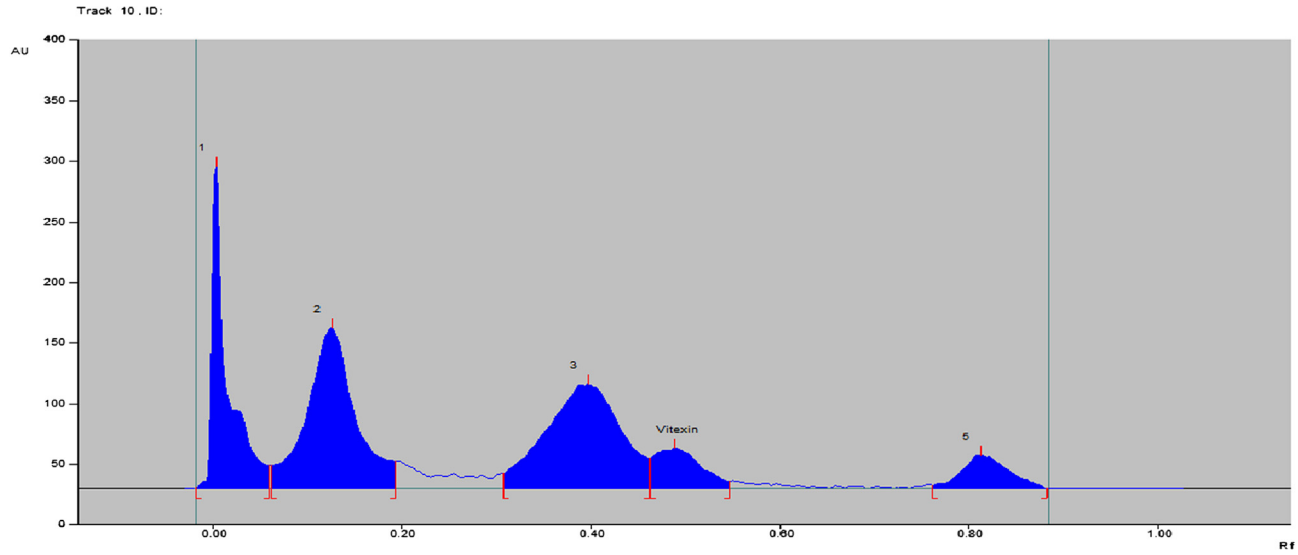

Fig. 4. HPTLC densitogram of *Passiflora foetida* containing herbal formulation (ET).

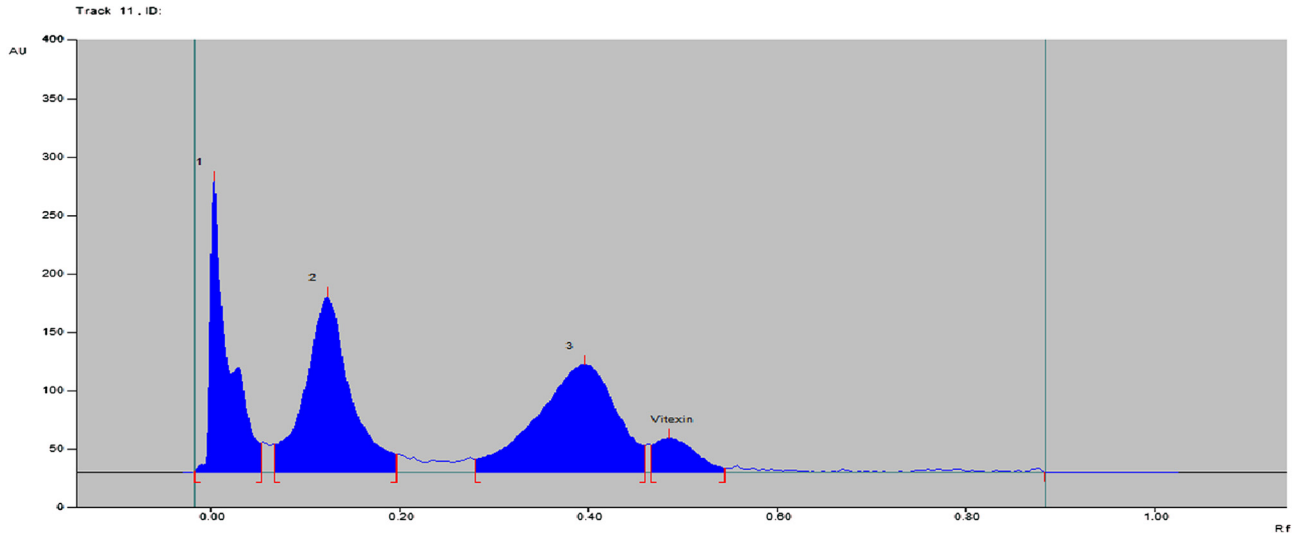

Fig. 5. HPTLC densitogram of *Passiflora foetida* containing herbal formulation (DT).

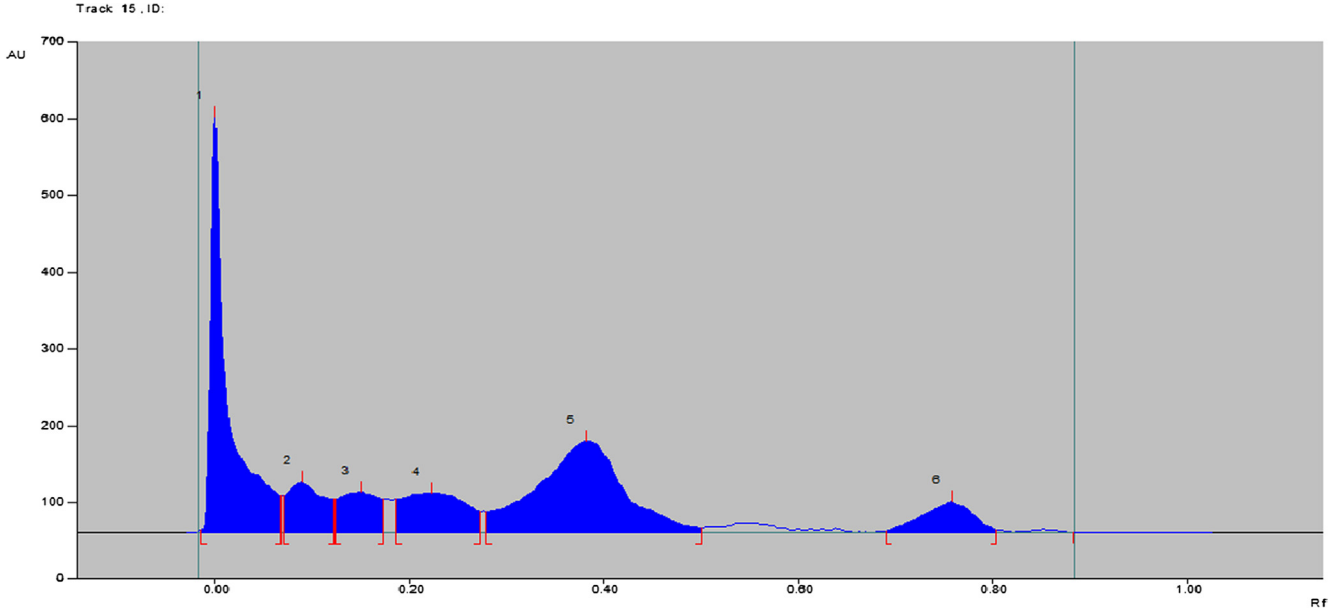

Fig. 6. HPTLC densitogram of *Passiflora foetida* containing herbal formulation (MC).

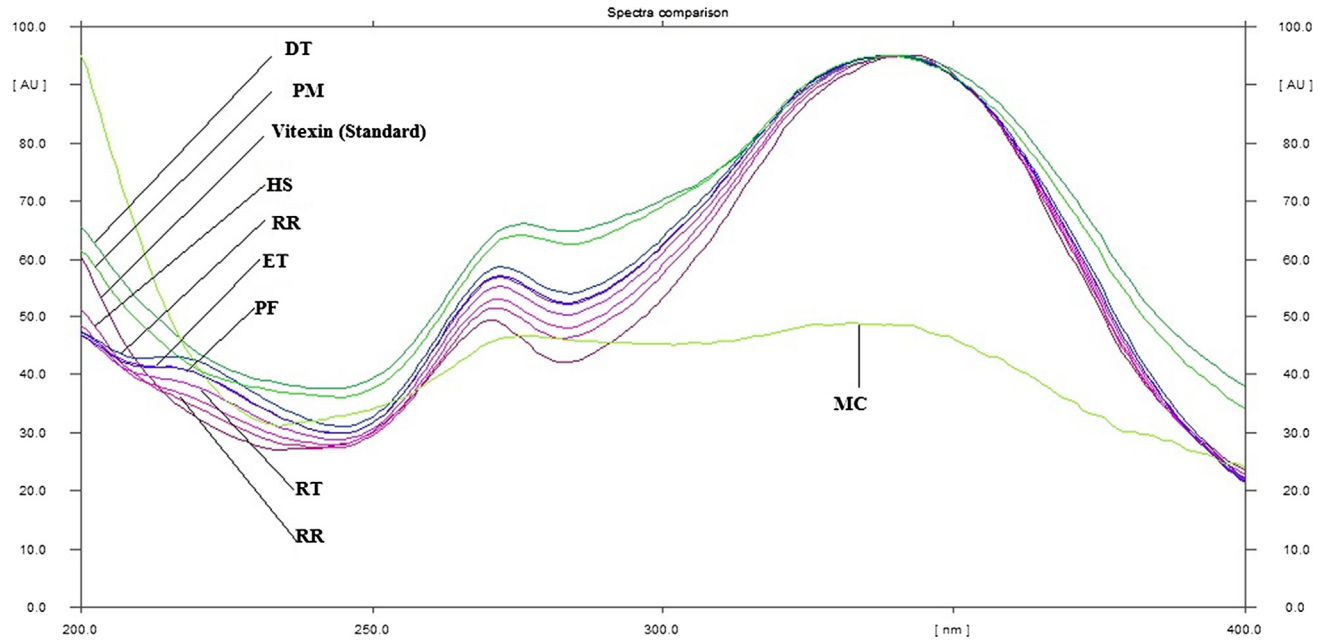

Fig. 7. Overlay UV absorption spectra of standard vitexin and *Passiflora foetida* containing herbal formulations.

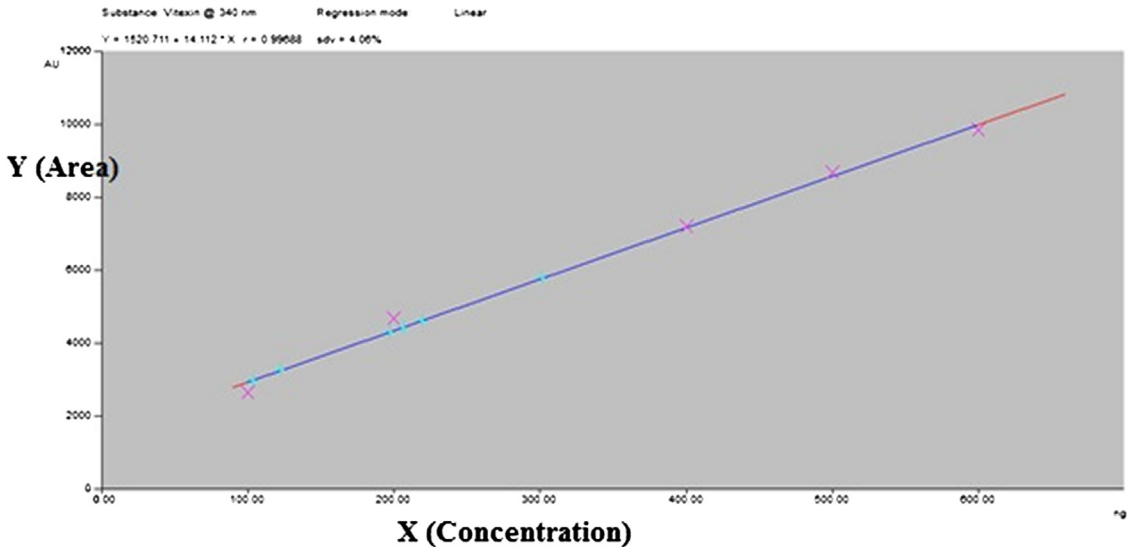

Fig. 8. The linearity graph of standard vitexin.

Table 5
Contents of vitexin in herbal formulations (n = 3).

Samples	Contents mean ± SD (% w/w)
DT	0.012 ± 0.002
PF	0.051 ± 0.004
PM	0.043 ± 0.004
ST	0.021 ± 0.003
ET	0.027 ± 0.004
MC	–
RT	0.060 ± 0.005
HS	0.071 ± 0.006
RR	0.067 ± 0.003

5. Conclusion

The HPTLC method developed for quantification of vitexin was found to be simple, accurate, reproducible and sensitive. Statistical data prove that the method is reproducible and selective for the analysis of vitexin with added advantages of short time, minimal sample preparation, in addition to the low cost.

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