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Development and validation of HPTLC Method For Quantification of Betanin from Peel and Pulp of Hylocereus Polyrhizus Extract

Komal Patel*[1], Paresh Patel[2],Umang Gajjar[3,] Ankit Chaudhary[4]

[1]Research Scholar, Faculty of Pharmacy, Ganpat University, Kherva, Mehsana, Gujarat

[1] Assistant Professor, Department of Pharmaceutical Chemistry and Quality Assurance Faculty of Saraswati Institute of Pharmaceutical Sciences, Dhanap, Gandhinagar, Gujarat

[2] Professor Dean and Head of Department of Pharmaceutical Chemistry and Quality Assurance, Ganpat University, Kherva, Mehsana, Gujarat

[3]Professor,Head of Department pharamcognosy,Saraswati Institute of Pharmaceutical Science, Dhanap, Gandhinagar,382355 India.

[34]Professor and Principal, Saraswati Institute of Pharmaceutical Science, Dhanap, andhinagar,382355 India.

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KEYWORDS

HPTLC,

Dragon fruit, Validation, Quantification

ABSTRACT:

This study details the development of validated a TLC-densitometry method to measure betanin in extracts of Hylocereus spp. (dragon fruit). Chromatographic separation occurred on TLC aluminum plates coated with silica gel 60 F_{254}. A mobile phase system was optimized for good resolution of betanin. The method produced sharp, well-defined spots with an Rf value specific to betanin, ensuring clear separation from other components. Densitometric detection was done at a visible wavelength suitable for betanin, in reflection or absorbance mode, allowing for reliable measurement. The method showed good linearity across a concentration range of 10–50 µg/ml, with a high correlation coefficient ($R^2 \geq 0.99$). The limit of detection (LOD) and limit of quantification (LOQ) were at acceptable sensitivity levels, making the method suitable for detecting low amounts of betanin in plant samples. The validated method showed satisfactory accuracy, precision, repeatability, and specificity, in line with ICH guidelines. The measured amount of betanin in the fruit extract was 0.6 ± 0.0032 gm/100 gm, indicating its notable presence as a bioactive compound. In conclusion, the TLC-densitometric method is simple, quick, cost-effective, and reliable for measuring betanin in Hylocereus spp. It can be effectively used for routine quality control, standardization, and screening of betanin in herbal raw materials and products.

Introduction

Hylocereus Polyrhizus, known as Dragon fruit, is a fascinating tropical plant that offers many health benefits. This fruit, also called pitahaya, has gained popularity in production and export. In 2018, the US, Canada, Japan, and the EU consumed nearly 17,000 tons of it. The demand for Dragon fruit mainly comes from its components, including glucose, betanin, betalains, vitamins, organic acids, soluble dietary fiber, phyto albumins, and essential minerals [1]. Calcium (8.5 mg), phosphorus (22.5 mg), vitamin B1 (0.04 mg), vitamin B2 (0.05 mg), vitamin B3 (0.16 mg), vitamin C (20.5 mg), water (87 g), protein (1.1 g), fat (0.4 g), fiber (3 g), carbohydrates (11 g), and iron (1.9 mg) are all present in 100 g of the fruit.. These components have useful properties for medical and diuretic purposes [2,3]. Dragon fruits are rich in potassium, magnesium, zinc, and phosphorus, along with smaller amounts of iron, calcium, and copper. All these minerals contribute positively to health due to their antioxidant properties. Extracts from the stems, petals, peels, and pulps of dragon fruit have shown beneficial biological effects against diseases like cancer, diabetes, obesity, and

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hyperlipidemia, as well as against harmful microbes such as bacteria, fungi, and viruses [4,5].

Figure 1: Dragon fruit

In recent years, the advancement of chromatographic and spectral fingerprints has played a pivotal role in the quality control of complex herbal medicines [6]. High Performance Thin Layer Chromatography has become a routine analytical technique due to its advantages of reliability in quantification of analytes at micro and even in nanogram levels and cost effectiveness. It has proved as a very useful technique because of its low operating cost,high sample throughput and need for minimum sample clean-up. The major advantage of HPTLC is in reducing analysis time and cost per analysis. Thin Layer Chromatography has been known as the fast tool for the detection of compounds. Another advantage of TLC is the capability to detect more compounds than High Performance Liquid Chromatography, although the resolution is poorer. In this regard, the compounds which cannot be eluted still can be detected. Moreover, the compounds having no UV absorption, e.g. sugar, still can be detected by reagent spraying. The TLC chromatogram pattern comparison seems to be promising for fingerprinting the active compounds in plant extracts. Thus, it can be used as a tool in the quality con- trol in order to warranty that the active compounds are extracted. By means of data analysis system and optimized experimental conditions, HPTLC is also feasible for development of chromatographic fingerprint methods to determine and identify complex herbal Extracts just like HPLC and GC. Furthermore, the colorful picture, like HPTLC image provides extra intuitive parameters of visible color and/or fluorescence and unlike HPLC and GC, HPTLC can simultaneously determine different samples on the same plate. Such an approach causes the HPTLC method to maintain its innate advantage as well as get over a limitation of developing distance and plate efficiency.

Literature survey revealed that no method has been reported for quantitation of Betanin from peel and pulp of the extracts of *H. Polyrhizus.* Hence a densitometric HPTLC method has been developed in the present work for quantitation of Betanin from Aqeous extract of peel and pulp of the of *H. Polyrhizus*. The method was found suitable for rapid screening of plant material for their quantitative assessment and can be performed without any special samplepretreatment[7].

2. MATERIAL AND METHOD

2.1 Reagents and standards

All chemicals and solvents used were of analytical grade and obtained from E-Merck (Darmstadt, Germany). Stock solutions (mg/ml) of standards were prepared daily in methanol. From this, solution was applied using Linomat applicator on TLC aluminum plates precoated with silica gel 60 F_{254} (10 · 10 cm, 0.2 mm thick) obtained from E. Merck Ltd. (Mumbai, India).

2.2 Plant material

The collection of dragon fruit took place at Smart Bazar, strategically chosen for its accessibility and availability of the sought-after fruit. The plant was originally authenticated by Dr. Nainesh R Modi, an esteemed Associate Professor in the school of science, Gujarat, India. A herbarium sample of this plant is preserved and deposited with Department of Botany. The fruit parts of the plant were manually separated, Undertake the careful separation of the peel from the flesh, employing either a knife or manual extraction. This process mandates a delicate touch to prevent any unintended damage to the flesh. Vigilance in this phase is essential to preserve the inherent qualities of both components.

2.3 Preparation of standard solution

Standard biomarker betanin was weighing precisely 10 mg, was placed into a 100 mL volumetric flask.

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Approximately 25 mL of diluent was added, and the solution was sonicated until dissolved. The final volume was adjusted to the mark with diluent and mixed thoroughly. Subsequently, 1.0 mL of the above solution was transferred into a 10 mL volumetric flask, and the volume was adjusted to the mark with diluent and mixed to give concentration 10 µg/ml..

2.4 Preparation of plant extracts

The initial step involved the transformation of the homogenous paste derived from the peel and pulp into a desiccated state, carried out at ambient room temperature. A container served as the setting for the creation of a 5% (w/v) suspension. This suspension was crafted by meticulously blending hot, distilled water that had been subjected to boiling, with 50 g of dried fruit epicarp (peel) and endocarp (pulp).The mixture was maintained at a temperature of 37 °C, with a rotational speed of 200 rpm for a duration of 4 hours, facilitated by a rotary flask evaporator. Subsequent to this period, the suspension was subjected to agitation and subsequently cooled to the ambient room temperature before embarking on the filtration process. Four layers of No. 1 Whatman filter paper functioned as the conduit for filtering the suspension, ensuring the separation of the extract from any particulate matter. The resulting aqueous extract was ready for HPTLC analysis.

2.5 Optimized Chromatographic conditions

Chromatography was performed on a 10 · 10 cm preactivated HPTLC Silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). Aliquots of each of the extracts were separately applied (Samples and standard) to the plate as 8 mm wide band with an automatic TLC applicator Linomat-V with N_2 flow (CAMAG, Switzerland), 8 mm from the bottom. Densitometry scanning was performed on TLC scanner IV at 254 nm. The plates were prewashed by methanol and activated at 60 °C for 5 min prior to chromatography. The slit dimension was kept at 6. 0.45 and 20 nm s^{-1} scanning speed was employed. The mobile phase consisted of methanol 10 ml was used per chromatography. Linear ascending development was carried out in 10 · 10 cm twin glass chamber saturated with the mobilephase.

2.6 Chromatographic Detection

After sample application plates were developed in a Camag twin through a glass tank pre-saturated with the mobile phase (10 ml) methanol for 20 min. The plate was developed in Camag horizontal developing chamber (10 · 10 cm) at the room temperature up to 7 cm. Ascending mode was used for the development of Thin Layer chromatography. After development, plates were dried with a hair dryer and The plate was observed after 30 min under Camag UV cabinet (254 and 366 nm).Quantitative analysis of the compound was done by scanning the plates at 254 nm using Camag TLC TLC scanner IV equipped with win-CATS-V 1.2.3 software (Camag). The identification of Betanin was confirmed superimposing the UV spectra of the samples and standards within same R_f 0.605 (Figure 2).

A densitometry HPTLC analysis was also performed for The development of characteristic fingerprint profile, which may be used as a marker for quality evaluation and standardization of the drugs.

2.7 Calibration curve of Betanin

The content of Betanin compound was determined by using a calibration curve established with a standard concentration range from 10 to 50 µg/ml. A stock solution of standard betanin (100 µg/ml) was prepared in methanol. The different volumes of stock solution 1, 2, 3, 4, and 5 ll were spotted on HPTLC plate to obtained concentration 10, 20, 30, 40, and 50 µg/ml. respectively (band width 6 mm, distance between tracks 15 mm) using automatic samplespotter. Each concentration peak area was plotted against the concentration of betanin spotted or injected. The linear regression of standard curve was determined with $R^2 \pm SD = 0.994 \pm 5.05\%$. The linear regression line is $y = 24.125x - 28.154$ (Fig. 2). The regression data have shown a good linear relationship over the concentration range of 10–50 µg/ml. The linearity of calibration graphs and adherence of the system to Beer's law are validated by high value of correlation coefficient and the SD for intercept value is noticed to be less than 2% (RSD 0.28%). No significant difference is observed in the slopes of standard curves (ANOVA; $p <$

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0.05).

2.8 Validation of HPTLC method

2.8.1 Precision

ICH guidelines were followed for the validation of the analytical method developed for precision, repeatability and accuracy. Instrumental precision, intra-day precision and inter- day precision of the method were determined. Instrumental precision was measured by replicate (n = 6) applications of same Betanin solution. Intra-day assay precision was evaluated by analysis of replicate (n = 6) applications of freshly prepared standard solution of same concentration (10– 50 µg/ml), on the same day. Intermediate precision was evaluated by analysis of replicate (n = 6) applications of standard solution of the same concentration (10– 50 µg/ml) on six different days. The repeatability of sample application and measurement of peak area have been expressed in termsof % CV.

2.8.2 Limit of detection and limit of quantification

For the evaluation of limit of detection and limit of quantification different concentrations of the standard solutions of Betanin were applied along with chloroform as blank and determined on the basis of signal to noise ratio. LOD was determined at an S/N of 3:1 and LOQ at an S/N of 10:1.

2.8.3 Specificity

The specificity of the method was ascertained by analyzing standard Betanin and extracts. The spot for Betanin in the sample was confirmed by comparing the R_f and spectra of the spot with that of sample. The peak purity of Betanin was assessed by comparing the spectra at three different levels, i.e., peak start, peak middle and peak end positions of the spot/ bands.

2.8.4 Robustness

The estimation was performed by varying the selected parameters (mobile phase composition, mobile phase volume and duration of mobile phase saturation) within certain limits (±10%) and there has been no notable alteration found in method performance and in results obtained. The results were indicated by the %RSD between the data at each variablecondition.

2.8.5 Accuracy

The accuracy of the method was measured by performing recovery experiments at three different levels (50%, 100% and 150% addition of betanin) using the standard addition method. The known amounts of betanin standard (20 µg/ml) were added by spiking. The values of % recovery and average value of % recovery for betanin were calculated.

2.8.6 System suitability

System suitability tests were performed to verify whether resolution and repeatability were adequate for the analysis. System suitability was determined by applying freshly prepared standard solution of Betanin concentration 30 µg/ml, 6 times to the same chromatographic conditions then scanned and densitograms were recorded. The measured peak areas for Betanin and their retention factor were noted for each concentration of Betanin and values of the mean peak area, the standard deviation (SD) and the %CV were calculated.

2.8.7 Estimation of Betanin in herbal extracts

50 gm dried powder of dragon fruit pulp gives approx. 12.5 gm of aqueous extract after Soxhlation. From that 5 gm diluted with methanol for sample preparation. {means 5000 mg in 10 ml ≈ 500 µg in 1 µl sample}.

3. RESULTS AND DISCUSSION

3.1 Chromatographic fingerprint

TLC fingerprint analysis has emerged as a rational approach for the quality determination and authentication of traditional herbal medicines. This technique employs various chromatographic methods to generate specific recognition patterns for medicinal plants. These fingerprint profiles can be used not only to detect the presence or absence of specific marker compounds but also to evaluate the relative ratio of all detectable analytes within a sample. Among the available techniques [8] , High-Performance Thin-Layer Chromatography (HPTLC) stands out due to its simplicity, cost-effectiveness, and low sample and solvent requirements. Although it has certain limitations—such as a shorter developing distance and

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lower plate efficiency when compared to High-Performance Liquid Chromatography (HPLC) and Gas Chromatography **(GC)—**HPTLC remains a reliable and efficient method for herbal analysis. Importantly, some of these limitations can be overcome by developing fractions of different polarities on two or more separate TLC plates. The image-like output of HPTLC, when combined with digital scanning and densitometry analysis**,** enhances the reliability and reproducibility of herbal fingerprints. As a result, HPTLC is increasingly recognized as a powerful tool for constructing chromatographic fingerprints of herbal medicines, providing sufficient information for the comprehensive identification**,** authentication, and differentiation of closely related species[9,10.]

To the best of our knowledge, there are limited reports on the quantification of betanin in *H. polyrhizus* using HPTLC. Therefore, this study presents a novel, rapid, and reliable method that yielded sharp, well-resolved peaks with high reproducibility. The developed HPTLC method is suitable for routine quality control and standardization of betanin in *H. polyrhizus* fruit extracts and formulations containing its bioactive pigments.

HPTLC fingerprint patterns have been evolved for extracts of *H. Polyrhizus*. Betanin standard was quntitated accurately using silica gel F_{254} HPTLC pre-coated plates with mobile phase methanol:water (98: 02 v/v), the R_f value was about 0.605 The chromatographs of betanin and extract of peel and pulp of *H. Polyrhizus* are shown in figure 2-5 . The R_f value of betanin matched with the R_f value of extract was about 0.605 shown in peak in Figure. 4–5.

3.2 TLC densitometric quantification of betanin using HPTLC

A simple and precise High-Performance Thin-Layer Chromatography (HPTLC) method was developed for the quantification of betanin in the fruit extract of *Hylocereus polyrhizus* (red dragon fruit). The chromatographic conditions were optimized to ensure accurate identification and quantification of betanin as a marker compound.

The TLC chamber was saturated with the mobile phase— methanol: water (98: 02 v/v), upper layer)— for 20 minutes at room temperature. After sample application and development, the TLC plates were visualized at 540 nm, the known absorption maximum (λmax) of betanin.

A photograph of the developed TLC plate showed clear bands corresponding to betanin in both the standard and the fruit extract samples. The identity of betanin in the sample was confirmed by comparing the chromatogram of the extract with that of the reference standard solution (Figure 2). Furthermore, the spot in the sample extract exhibited the same retention factor (Rf = 0.605) as that of the betanin standard (Figure 4,5).

3.3 Method Validation

The developed HPTLC densitometric method for the estimation of betanin in *Hylocereus polyrhizus* was validated in accordance with standard analytical parameters, including linearity, precision, repeatability, accuracy, sensitivity (LOD & LOQ) and robustness.

3.3.1 Linearity

To assess linearity, standard solutions of betanin was prepared at various concentration levels. . Linearity was evaluated by applying each concentration (10-50µg/mL) of betanin in triplicates per sample and five such samples were evaluated (n = 3 × 5).Five point calibration curve was constructed by plotting peak area against concentration.

3.3.2 Precision and Repeatability

Precision was assessed at five concentration levels, and the method showed low % coefficient of variation (%CV) values for intra-day (0.48–1.72%) and inter-day (0.52–1.80%) studies, confirming excellent precision and repeatability (Table 2).

3.3.3 Accuracy (Recovery Studies)

Accuracy was verified through recovery studies by spiking known amounts of betanin into pre-analyzed samples. The percentage recovery ranged from 98.64% to 99.66%, indicating the method's high accuracy and reliability (Table 4).

3.3.4 Sensitivity

The detection limit (LOD) quantification limit (LOQ) for betanin were determined to be 0.096 µg and 0.291 µg, respectively, demonstrating that the method is sufficiently sensitive for the detection and quantification of betanin in fruit extracts (Table 5).

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3.3.5 Robustness

The robustness of the method was confirmed by a %RSD of 0.32% between peak area values, indicating that betanin remains stable during sample preparation and analysis. Robustness testing, involving minor deliberate changes in the mobile phase composition, did not significantly affect the results, confirming method reliability under variable conditions.(Table 6).

3.4 Quantification of betanin in Extract

12 µg betanin in 1 µl sample therefore 12 µg in 500 µg aq. extract ≈ 2.4 µg in 100 µg aq. extract ≈ 0.3 gm in 12.5 gm aq. Extract Means 50 gm dried powder of dragon fruit pulp contains 0.3 gm betanin
Means approx. 0.6% betanin present in selected sample dragon fruit

Conclusion

A reliable and effective HPTLC method was developed and validated for measuring betanin in Hylocereus spp. (dragon fruit) extract. The method showed good peak resolution and selectivity, with no interference from other compounds, ensuring its effectiveness for quantifying betanin. The recovery values fell within acceptable limits, demonstrating the method's reliability. The clear peaks seen in the densitograms further proved the method's ability to separate betanin from other components in the extract. The quantification results indicated a significant amount of betanin in the fruit extract, showcasing its potential as a valuable natural pigment and antioxidant. Given betanin's known health benefits, including antioxidant, anti-inflammatory, and liver-protective effects, measuring it accurately is important for the standardization and quality control of herbal products and functional foods. In summary, the proposed HPTLC method is simple, sensitive, cost-effective, and suitable for routine analysis of betanin in natural sources. It can be a dependable tool for assessing quality and standardizing betanin in various herbal and nutraceutical products.

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Authors Contributions

All authors have contributed equally.

Disclosure statement

The authors declare that they do not have conflict of interest

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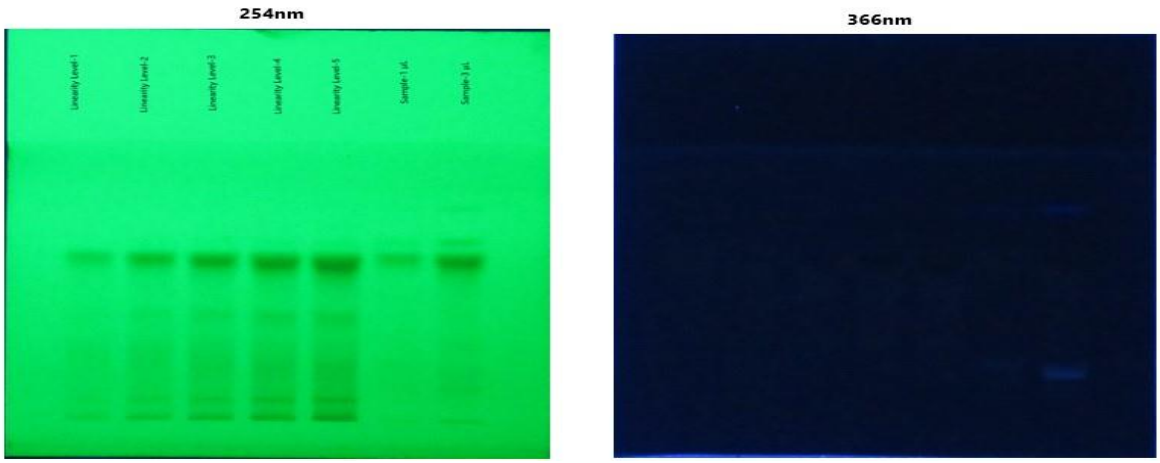

Figure 2 High-Performance Thin-Layer Chromatography (HPTLC) plate post-development under ultraviolet (UV) light at 254 nm and 366 nm

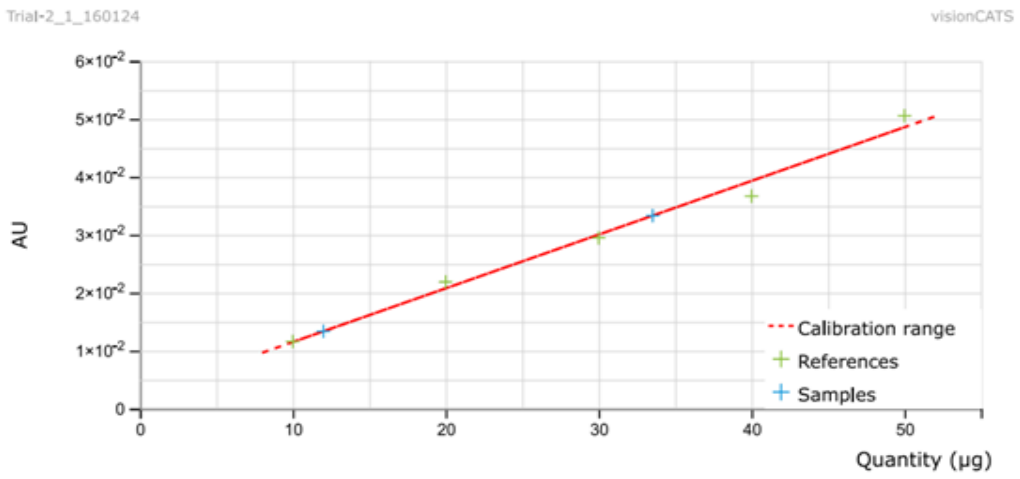

Figure 3. Calibration curve of Betanin

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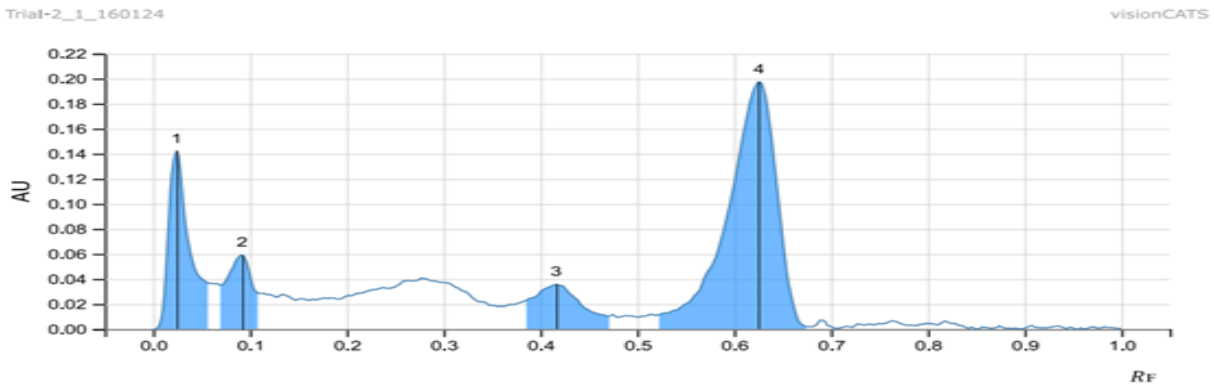

Figure 4. Densitometric chromatogram of standard betanin

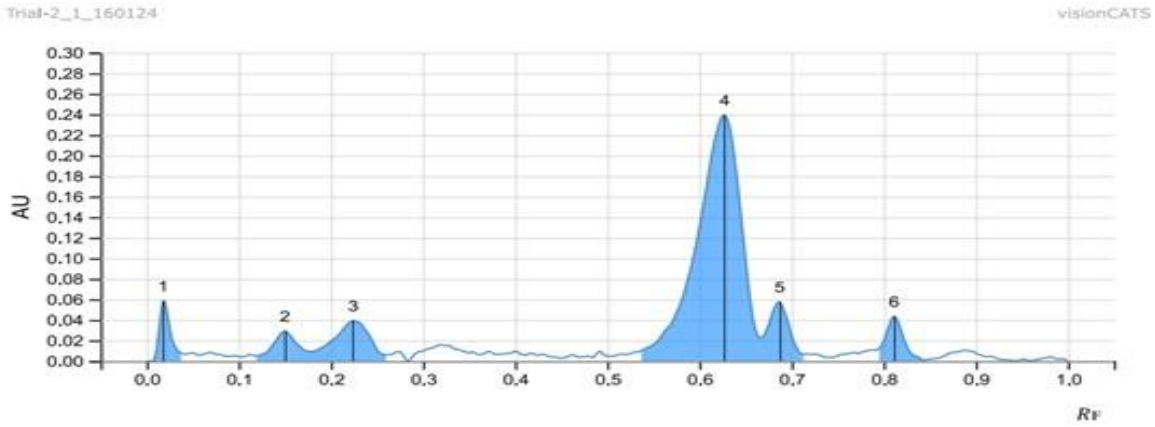

Figure 5. Densitometric chromatogram of *H. Polyrhizus* extract

Table 1: Method validation parameter

Parameter	Result (Betanin)	Acceptance Criteria
Selectivity	Selective	–
Specificity	Specific, no interference observed	No interference from matrix
Linearity and Range	10– 50 µg/ml	$R^2 > 0.995$
Correlation coefficient (r^2)	0.992875	0.9–1.1
regression equation	$Y = 9.273*10^{-10} + 2.219*^{10-3}$	–
Limit of detection (LOD)	0.096	–
Limit of quantification (LOQ)	0.291	–
% Recovery	97.12% to 99.68%	98–102%
Repeatability (%RSD, n = 6)	0.13%	RSD ≤ 2%
Precision (%CV) – Intra-day (n = 6)	0.07–0.15%	CV ≤ 2%

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Precision (%CV) – Inter-day (n = 6)	0.09–0.16%	CV ≤ 2%

Table 2 : Intermediate precision data for betanin

	Inter-day precision		Intra-day precision	
Conc (µg/ml)	**Average AUC ± SI (n=6)**	**%RSD**	**Average AUC ± SD (n=6)**	**%RSD**
20	466 ± 0.71	0.15	464 ± 0.74	0.16
30	671 ± 0.85	0.12	673 ± 0.86	0.12
40	896 ± 0.69	0.07	894 ± 0.80	0.09

Table 3: Repetability data for betanin (n=6

Average AUC - 673	**SD** - 0.92	**%RSD** - 0.14

Table 4: Recovery study data Proposed HPTLC Method of Betanin

Concentration of drug taken (µg/ml)	Concentration of drug added (µg/ml)	Total amount of drug (µg/ml)	Amount of drug found(µg/ml)	Amount of drug recovered ± SD	%Recovery ± %RSD
20	10	30	29.88	9.80 ±0.079	99.66 ± 0.80
20	20	40	39.456	19.51 ±0.21	98.64± 1.09
20	30	50	49.563	29.59± 0.27	99.126 ± 0.94

Table 5: LOD and LOQ of Proposed HPTLC Method of Betanin

Parameter	Betanin
LOD	0.096
LOQ	0.291

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Table 6: Robustness data of HPTLC method of Betanin

Parameter varied	Average AUC ±SD	%RSD
Mobile phase (Methanol) composition(±0.1mL)	672 ± 0.92	0.13
Amount of mobile phase (± 5 %)	674 ± 1.5	0.22
Time from band application to chromatography (+10min)	674± 1.87	0.27
Actual wavelength 254 nm (± 2 nm)	672± 2.2	0.32

Table 7: Amount of Betanin found in *H. Polyrhizus* extract

Plant Material	Betanin (gm/100 gm) (mean ± SD) (n = 3)
Fruit Extract	0.6 ± 0.0032