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Original Article

High Performance Thin Layer Chromatography (HPTLC) Methods for Simultaneous Estimation and Quantification of Vasicine, Gallic acid, Piperine and Pterostilbene from Two *Ayurvedic* Formulations and Herbal Extracts

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ABSTRACT

Background: High Performance Thin Layer Chromatography (HPTLC) is an advanced analytical technique used for the identification and quantification of marker compounds in Ayurvedic formulations. **Aims and Objectives:** The purpose of this study is to create and validate an HPTLC method for the simultaneous estimation and quantification of four marker compounds: Vasicine (VS), Gallic Acid (GA), Piperine (PP), and Pterostilbene (PTS) from two formulations: Amarantha Gynorite syrup and Amarantha Gynorite capsule. **Materials and Methods:** A precoated silica gel aluminum plate (G60F254) was selected as the stationary phase to separate the four markers. The samples were developed using methanol. The mobile phase was composed of a mixture of Toluene, Ethyl acetate, Formic acid, and Glacial Acetic Acid (GAA) in a 2:1:1:0.75 v/v ratio. Quantification was performed at the isosbestic point of 280 nm utilizing the absorbance reflectance mode. **Results:** The Retention factor (R_f) values for VS, GA, PP, and PTS were determined to be 0.11, 0.34, 0.68, and 0.71, respectively. Linearity was achieved within the range of 5-50 µg/mL for all four compounds, with correlation coefficients falling within acceptable limits. Satisfactory recovery rates of 100.21%, 99.96%, 100.66%, and 99.28% were recorded for VS, GA, PP, and PTS, respectively. The analysis results were validated concerning accuracy and precision. The Limits of Detection (LOD) were found to be 0.662 ng, 1.573 ng, 1.152 ng, and 1.688 ng for VS, GA, PP, and PTS, while the Limits of Quantification (LOQ) were 2.018 ng, 4.769 ng, 3.492 ng, and 5.115 ng for the same compounds, respectively. The results obtained from the validation tests indicate that the developed simultaneous method is both accurate and reliable. **Conclusion:** The HPTLC method established in this study offers a quicker and more cost-effective approach for the simultaneous analysis of VS, GA, PP, and PTS from Amarantha Gynorite syrup and Amarantha Gynorite capsule.

Keywords: Gallic acid, HPTLC, Piperine, Pterostilbene, Simultaneous analysis, Vasicine.

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INTRODUCTION

Standardization refers to a procedure that includes the examination, validation, preservation, and enhancement of the quality, effectiveness, safety, and consistency of a medicinal substance within a complex infrastructure. It has been suggested that nearly 80% of pharmaceutical products are derived from natural sources (Kunle, 2012). Significant advancements have been observed in the Ayurvedic and herbal Research and Development (R&D) sector over the past few decades (Bhattacharya, 2019). Nevertheless, only a limited number of Indian pharmaceutical companies are engaging in the standardization of herbal medicinal products using advanced instrumentation (Sardana, 2012).

The Amarantha Gynorite capsule and syrup are proprietary Ayurvedic formulations created and produced by Ari Healthcare Private Limited, Pune, India, aimed at managing various menstrual issues such as dysmenorrhea, hypomenorrhea, oligomenorrhea, amenorrhea, menorrhagia, metrorrhagia, menometrorrhagia, dysfunctional uterine bleeding, and Polyendocrine Metabolic Ovarian Syndrome (PMOS). Both products include standardized hydroalcoholic extracts of Ashoka bark (*Saraca indica* Linn.), Vasa leaf (*Adhatoda vasica* Nees.), Bijaka stem (*Pterocarpus marsupium* Roxb.), and a mixture known as *Trikatu* (composed of *Zingiber officinale, Piper nigrum*, and *Piper longum* in equal ratios)

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as their primary ingredients, along with additional components. The key marker compounds that significantly contribute to the pharmacological effects of these formulations are Vasicine (VS), Gallic Acid (GA), Piperine (PP), and Pterostilbene (PTS) (Katole *et al*., 2021; Vidyasagar & Prashantkumar, 2006).

Vasicine, also known as peganine, is a type of quinazoline alkaloid and a chemical marker found in *Adhatoda vasica* Nees. Vasicine has activities that stimulate the uterus, reduce inflammation, provide antioxidant effects, and modulate the immune system. Gallic acid, or 3,4,5-trihydroxybenzoic acid, is one of the primary secondary metabolites in *Saraca asoca* Linn. Gallic acid is beneficial in treating uterine disorders, including bleeding and infections in uterine tissues. It also has anti-inflammatory and antioxidant properties. Piperine is an alkaloid present in *Piper nigrum* Linn. and *Piper longum* Linn. Piperine is known for its capability to enhance bioavailability, as well as its antioxidant, anti-inflammatory, antiplatelet, antihypertensive, hepatoprotective, and anticancer effects. It is effective in managing ovarian cancer, hypertension, and thyroid issues. Pterostilbene is a natural polyphenol that is a dimethyl ether derivative of resveratrol. Research has indicated that Pterostilbene is helpful in treating gynecological disorders, blood diseases, vascular problems, impaired insulin signaling, endometrial cancer, and ovarian cancer. Additionally, Pterostilbene aids in the regulation of gonadotropin hormone secretion (Bai *et al*., 2021; Karri, 2014; Chopra *et al*., 2016; Yu *et al*., 2021; Zhang *et al*., 2016; Ministry of AYUSH, n.d.).

High-Performance Thin Layer Chromatography (HPTLC) is a commonly used analytical method in herbal industries due to its low operational costs, high sample throughput, and minimal need for sample preparation. One of the key benefits of HPTLC is the ability to analyze multiple samples at once with a small volume of mobile phase, unlike High Performance Liquid Chromatography (HPLC), which helps reduce the time and cost associated with each analysis. The comparison of HPTLC chromatogram patterns appears to be a valuable approach for identifying the active compounds in plant extracts. In the course of reviewing the literature, there was no information found concerning the simultaneous analysis of VS, GA, PP, and PTS (Sachan *et al*., 2016; Attimarad *et al*., 2011; Garg *et al*., 2013; Tsering *et al*., 2014; International Council for Harmonisation [ICH], 2005).

To standardize the Amarantha Gynorite syrup and capsule, an effort was made to create a simultaneous determination of four distinct marker compounds utilizing the HPTLC method.

MATERIALS AND METHODS

Solvents & Chemicals

Solvents of analytical grade, including toluene, ethyl acetate, methanol, and glacial acetic acid, were acquired from Merck Life Science Pvt. Ltd., in Mumbai, India. The reference standards were obtained from Natural Remedies Ltd. in Bangalore, India. Table 1 contains the list of standards along with their respective potency.

Investigational Products

Ayurvedic formulations (Amarantha Gynorite capsule and Amarantha Gynorite syrup) were manufactured and provided by Ari Healthcare Pvt. Ltd., Pune, India. The ingredients of Amarantha Gynorite capsule and Amarantha Gynorite syrup are mentioned in Tables 2 and 3, respectively.

HPTLC Instrumentation

HPTLC method development and validation was performed on CAMAG system. The details of instruments and specification are given in Table 4.

Preparation of Solution

Preparation of Standard Solutions

Stock solutions of VS standard were created by dissolving 10.0 mg of VS in 10 mL of methanol, resulting in a concentration of 1.0 mg/mL. A working standard concentration of 100 µg/mL was generated by diluting 1 mL of the stock solution to 10 mL with methanol. Serial dilutions were made by measuring 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 mL of the working standard solution and adding methanol to achieve a total volume of 10 mL for standards containing 5, 10, 15, 20, 30, 40, and 50 µg/mL respectively. Likewise, working standard concentrations (100 µg/mL) for GA, PP, and PTS were prepared by dissolving 10 mg of each individual standard in 100 mL of methanol. A series of dilutions were conducted in the same way as with VS to achieve final concentrations ranging from 5-50 µg/mL.

Preparation of Sample Solutions

Sample solutions were created by placing 250 mg of Amaranth Gynorite capsule powder and 1000 mg of Amaranth Gynorite syrup solution into a 100 mL volumetric flask. Then, 50 mL of methanol was added, and the mixture was subjected to sonication in a bath sonicator for 30 min at room temperature. The flask was subsequently filled to the mark with methanol, and the extract was filtered using Whatman filter paper. The resultant filtrates were utilized as the sample solutions for the respective formulations.

Percentage Assay of Standard Solution from Formulations and Herbal Extracts

Amarantha Gynorite syrup, Amarantha Gynorite capsule and herbal extracts were analyzed to determine the contents of VS, GA, PP and PTS as per method described under chromatographic conditions using HPTLC. Every single analysis was repeated three times and results were expressed in percent assay.

Method Validation

The HPTLC method that was developed underwent validation in accordance with the guidelines established by the International

Council on Harmonization (ICH) Q2 (R1). All measurements were conducted in triplicate, with the following details provided.

Specificity

In line with the revised ICH Q2 (R1), specificity testing is essential to verify identification tests, assess impurities, and conduct the assay. During the specificity testing, specificity densitograms were generated for calibration curve (Figure 1), the standards, blank, and placebo formulations (Figures 2-4). No interference with the analyte was observed (Figure 5).

Linearity

According to ICH guidelines, a minimum of five concentrations is required to establish linearity. Linearity is indicated by the correlation coefficient, y-intercept, and slope of the regression line, along with a visual representation of the data. Linearity was assessed within the concentration range of 5-50 µg/mL for VS, GA, PP, and PTS (Figure 6). The relationship between peak area and concentration was analyzed using least square linear regression, determining the slope, intercept, and correlation coefficient for the calibration. The acceptable standard is an R^2 value of ≥ 0.99.

Precision

The evaluation of repeatability was conducted by utilizing a minimum of 9 measurements that encompass the specified range for the procedure (for example, 3 concentrations with 3 replicates each). Three replicate analyses were carried out at three varying concentration levels, specifically low (LQC: 50 µg/mL), medium (MQC: 100 µg/mL), and high (HQC: 150 µg/mL) for VS, during the same day at three different time points (Session 1, 2, 3). Intermediate precision was determined to examine the influence of random occurrences, such as different days, on the precision of the analytical method. Intraday and interday precision assessments were conducted by taking 9 measurements of 3 concentrations with 3 replicates each, at 3 time points on the same day and over 3 separate days, respectively. The aforementioned procedures were similarly repeated for GA, PP, and PTS. The accepted criterion was that the Relative Standard Deviation (RSD) should be ≤ 2%.

LOD & LOQ

The detection limit and quantification limit were established using the standard deviation of the response and the slope. Limit of Detection (LOD) is calculated as 3.3×σ/S. Limit of Quantification (LOQ) is calculated as 10×σ/S. Here, σ represents the standard deviation of the response estimated from the calibration curve, while S denotes the slope of the calibration curve. The values for LOD and LOQ were determined based on the standard deviation of the response and the slope of the calibration graph.

Table 1: **List of standards.**

Sl. No.	Name of Standard	Batch Number	Purity (%)
1.	Vasicine	T22E155	98.2
2.	Gallic Acid	T12K021	99.0
3.	Piperine	T13D040	98.0
4.	Pterostilbene	H220659	95.0

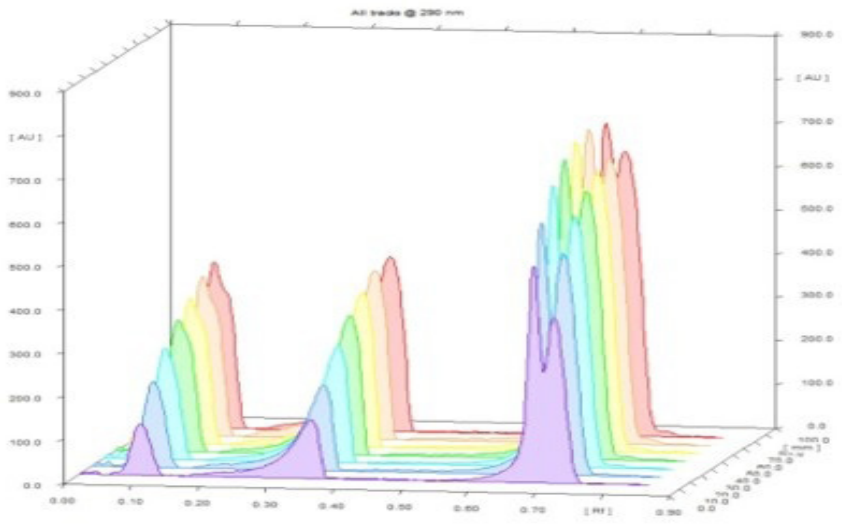

Figure 1: Densitogram of calibration curve.

Table 2: Active ingredients of Amarantha Gynorite capsule: each capsule contains.

Sl. No.	Ingredient Name	Scientific Name	Quantity
1.	*Ashoka* Bark Extract	*Saraca indica* Linn.	150 mg
2.	*Kumari* Leaf Extract	*Aloe vera* Tourn.ex Linn.	75 mg
3.	Pushyanuga Churna	*Ayurvedic* Classical Formulation	50 mg
4.	*Bijaka* Stem Extract	*Pterocarpus marsupium* Roxb.	50 mg
5.	*Lodhra* Bark Extract	*Symplocos racemosa* Roxb.	50 mg
6.	Tankana Bhasma	*Ayurvedic* Classical Formulation	25 mg
7.	Kasisa Bhasma	*Ayurvedic* Classical Formulation	25 mg
8.	*Latakaranja* Seed Extract	*Caesalpinia bonducella* (Linn.) Roxb.	25 mg
9.	*Vasa* Leaf Extract	*Adhatodha vasica* Nees.	25 mg
10.	*Krishna Jeerak* Fruit Extract	*Carum carvi* Linn.	15 mg
11.	*Ajamoda* Fruit Extract	*Apium graveolens* L.	15 mg
12.	*Trikatu* Extract	*Ayurvedic* Classical Formulation (*Zingiber officinale, Piper nigrum, Piper longum*)	10 mg

Table 3: Active ingredients of Amarantha Gynorite syrup: each 5 mL contains.

Sl. No.	Ingredients	Latin Name	Quantity
1.	*Ashoka* Bark Extract	*Saraca indica* Linn.	150 mg
2.	*Kumari* Leaf Extract	*Aloe vera* Tourn.ex Linn.	75 mg
3.	Pushyanug Churna	*Ayurvedic* Classical Formulation	100 mg
4.	*Bijaka* Stem Extract	*Pterocarpus marsupium* Roxb.	50 mg
5.	*Lodhra* Bark Extract	*Symplocos racemosa* Roxb.	50 mg
6.	Tankana Bhasma	*Ayurvedic* Classical Formulation	25 mg
7.	Kasisa Bhasma	*Ayurvedic* Classical Formulation	25 mg
8.	*Latakaranja* Seed Extract	*Caesalpinia bonducella* (Linn.) Roxb.	25 mg
9.	*Vasa* Leaf Extract	*Adhatodha vasica* Nees.	25 mg
10.	*Krishna Jeerak* Fruit Extract	*Carum carvi* Linn.	15 mg
11.	*Ajamoda* Fruit Extract	*Apium graveolens* L.	15 mg
12.	*Trikatu* Extract	*Ayurvedic* Classical Formulation (*Zingiber officinale, Piper nigrum, Piper longum*)	10 mg

Accuracy

Accuracy was determined throughout the defined range of the analytical method by incorporating known quantities of the analyte into a synthetic mixture of drug product components and the combined dosage form. According to ICH guidelines, accuracy must be evaluated using at least 9 measurements across a minimum of three concentration levels that encompass the specified range, which means 3 concentration levels tested in triplicate (i.e., 3 concentrations, each replicated 3 times). The accuracy of the method is expressed as the percentage recovery of the known amount of analyte added to the sample. Accuracy should be indicated by the percentage recovery determined by assaying the known amount of analyte in the sample. Accuracy must be assessed using a minimum of 9 determinations spread across 3 concentration levels that cover the specified range. In this study, percentage recovery was computed by executing recovery tests in triplicate at three concentration levels: 80%, 100%, and 120%, by adding known amounts of standard solutions of vasicine, gallic acid, piperine, and pterostilbene. The samples were subsequently analyzed, and the obtained results were compared with the expected values. The acceptable criteria for recovery were determined to be between 98% and 102%.

Robustness

The robustness of the analytical method was assessed to demonstrate its reliability amid intentional changes in method parameters. The evaluation of robustness aimed to validate the dependability of the analytical technique concerning deliberate variations in various method parameters. To determine the robustness of the analytical method, the following factors were examined: modifications in the mobile phase ratio and changes

in chamber saturation time. The acceptable criteria set for these evaluations was a pooled Relative Standard Deviation (RSD) of ≤ 3% for each change examined.

Stability testing (Intraday and Interday Assessments)

The stability of the sample solutions was evaluated over various time periods, including intraday (after 6 and 12 hr) and interday (after 24 hr), by storing them separately at 4°C and 25°C. The assessment of stability involved comparing the chromatographic attributes of the stored solutions with those of freshly prepared solutions, thereby covering both intraday and interday evaluations.

Statistical Analysis

Data were expressed as mean ± SD, and linear regression was used for calibration.

RESULTS

Section of Absorbance

The absorbance for each standard solution, namely VS, GA, PP, and PTS, was measured by scanning within the 400-200 nm range. All standard absorbance readings exhibited an isosbestic point, which was utilized for simultaneous analysis. The combined UV spectra of the four compounds displayed an isosbestic point at 290 nm. Consequently, 290 nm was chosen as the wavelength for the analysis. The specifics are illustrated in Figure 7.

Analytical Method Development

Optimization of chromatographic conditions: Based on the polarity and solubility of VS, GA, PP, and PTS, numerous preliminary experiments were conducted to select the mobile phase. The details of these trials are shown in Table 5. For

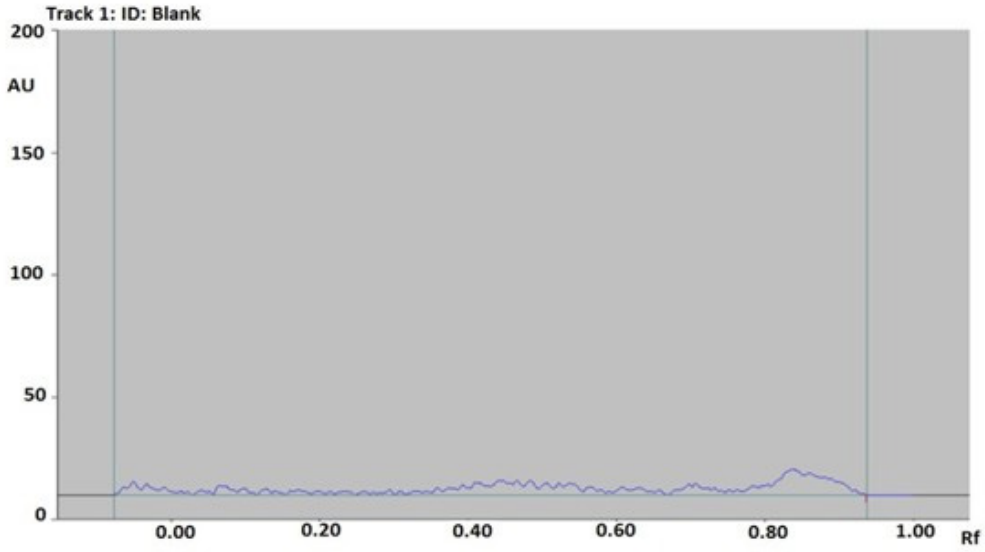

Figure 2: Densitogram of blank solution.

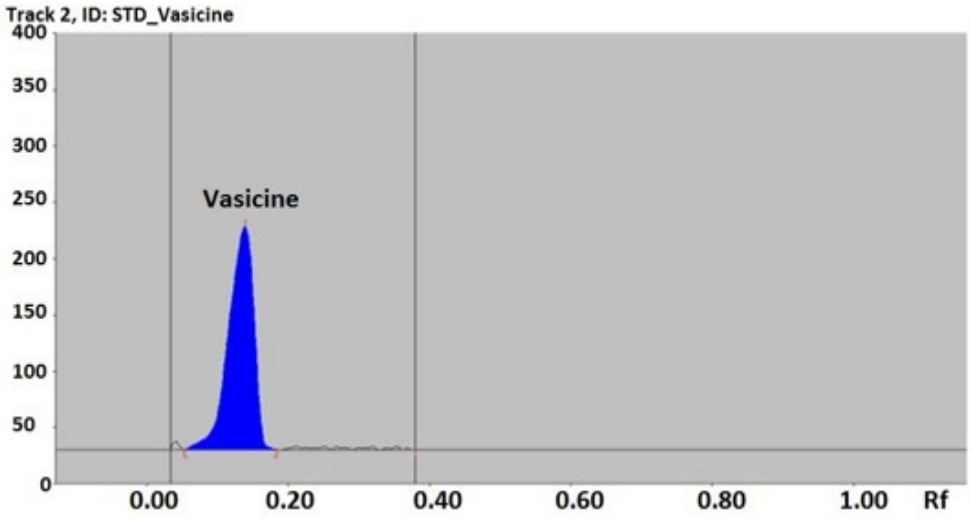

Figure 3: Densitogram of vasicine Rf 0.11±0.03.

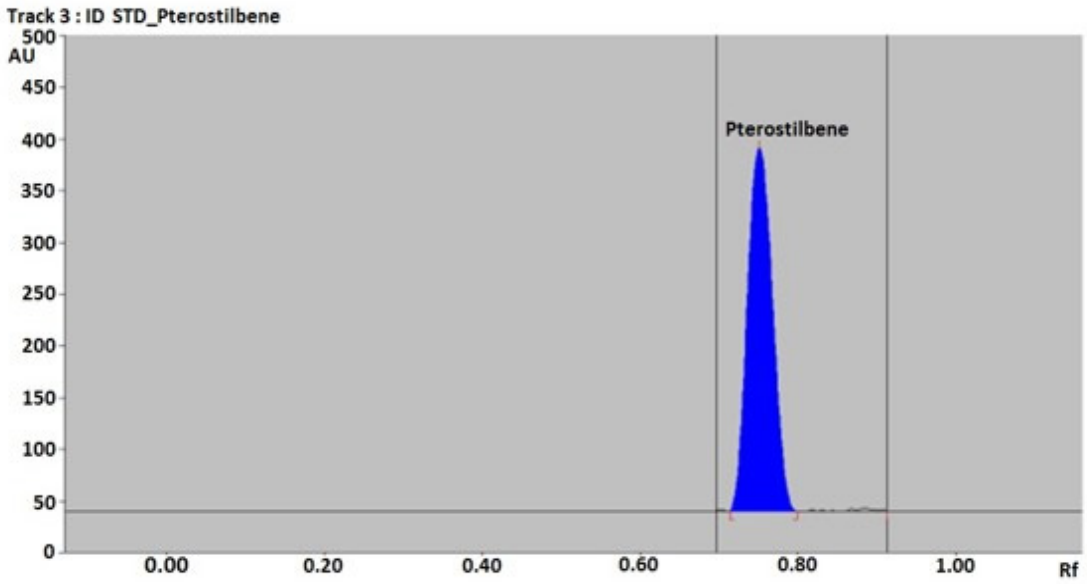

Figure 4: Densitogram of pterostilbene Rf 0.71 ± 0.03.

Table 4: Details of instruments of HPTLC.

Sl. No.	Components	Specification
1.	Make and Model	CAMAG
2.	Sample applicator	Linomat 5
3.	Scanner	TLC Scanner 4
4.	Chamber	Twin Trough chamber
4.	Sampling mode	Linomat applicator (Manual)
5.	Detection mode	Ultraviolet detector
6.	Application Syringe	Hamilton (100µL)
7.	Software	Wincats (Version_1.4.8.2031)

the HPTLC method, the chromatographic parameters were fine-tuned to achieve optimal resolution and peak shape for VS, GA, PP, and PTS. Various mobile phases in different ratios were tested, and a mobile phase composed of toluene: ethyl acetate: methanol: glacial acetic acid [7:3:1:1.5] (v/v/v/v) was chosen as the best option for obtaining well-resolved bands of VS, GA, PP, and PTS, as listed in Table 6.

Estimation of Marker compounds from Herbal Extracts and *Ayurvedic* formulations

The percent assay of VS, GA, PP, and PTS in herbal extracts (Vasa leaf, Ashoka bark, Trikatu, and Bijaka stem) and formulations (Amarantha Gynorite syrup and capsule) was calculated using a formula. Results are presented in Table 7.

For Extracts

$$\%\ \boldsymbol{Assay} = \frac{\text{Peak Area of Sample} \times \text{Concentration of Standard} \times \text{Purity of Standard}}{\text{Peak Area of Standard} \times \text{Concentration of Sample}} \times 100$$

For Formulation

$$\%\ \boldsymbol{Assay} = \frac{\text{Sample Peak Area} \times \text{Concentration of Standard} \times \text{Mean Weight} \times \text{Purity of Standard}}{\text{Peak Area of Standard} \times \text{Concentration of Sample} \times \text{Label Claim}} \times 100$$

Specificity

The specificity of the HPTLC method was demonstrated by separating the analyte from other potential components, including the blank and placebo of Amarantha Gynorite syrup and capsule. Application volume of 10 µL of potential components was injected and the densitogram was recorded [Figures 8 and 9]. Peaks of these components were not found at R_f of 0.11, 0.34, 0.68 and 0.71 of VS, GA, PP and PTS [Figures 2-4 and Figures 10-12]. Hence, the proposed method was specific.

Linearity

The calibration graph displayed a linear relationship within the concentration range of 5-50 µg/mL. The linearity was confirmed by analyzing the regression line and evaluating the Regression coefficient (R2). The linear regression data for the calibration curve can be found in Table 8.

Precision

Intraday Precision studies were conducted at three concentration levels (LQC, MQC, HQC) within the same day at different intervals. Details are mentioned in Table 9. Inter-day Precision studies were performed on three consecutive days using the same homogeneous sample and are listed in Table 10. The results were statistically analyzed for standard deviation and percent relative standard deviation (coefficient of variance), with a percentage RSD of peak areas less than 2%, indicating the developed method's precision.

Table 5: Selection of mobile phase composition for standardization of standards.

Trial. No.	Mobile Phase	Composition Ratio (v/v/v/v)	Applied Volume (µL)	Observation
1	Tol: EA: FA	7:3:0.5	5,10, 15 & 20	All bands were observed except Vasicine.
2	Tol: EA: MeOH: FA	8:3:1.5:2	5 & 10	All bands were observed except Vasicine.
3	EA: MeoH: Diethyl Amine	6:1:0.5	5 & 10	All bands were observed except Gallic acid. Injection volume was optimized.
4	Choloform: MeOH	8:2	10	Only two bands were detected i.e Piperine and Pterostilbene
5	Tol: EA: MeOH: Diethyl Amine	7: 3: 1: 1.5	10	All bands were observed except Gallic acid.
6	Tol: EA: MeOH	6: 3:1	10	All four bands were detected but its shows tailing. Mobile phase compositions have to optimize for better separations.
7	Tol: EA: MeOH: GAA	7: 4: 1: 1.5	10	All bands were detected but Piperine and Pterostilbene overlaying each other Hence mobile phase have to optimize.
8	Tol: EA: MeOH: GAA	7: 3: 1: 1.5	10	All bands are well separated

Table 6: HPTLC optimized chromatography conditions.

Sl. No.	Parameters	Optimized chromatographic conditions
1.	Stationary Phase	Precoated silica gel aluminum plate G 60 F254
2.	Plate and Chamber size	10 X 10 and 10 X 20
3.	Mobile Phase compositions	Tol: EA: MeOH: GAA (7:3:1:1.5 v/v/v/v)
4.	Chamber saturation time	30 min
5.	Sample application a) Application volume b) Band Length c) Distance between the tracks	10 µL 6 mm 10 mm
6.	Temperature (°C)	25-28
7.	Relative humidity (%)	55-60
8.	Technique of separation	Ascending
9.	Detection wavelength	290 nm

Limit of Detection & Quantification

The limit of detection and quantification were determined based on the standard deviation of the response and the slope of regression line. The calculated values of Limit of Detection (LOD) and Limit of Quantitation (LOQ) for vasicine, gallic acid, piperine and pterostilbene are shown in Table 11.

Accuracy

Accuracy indicates the percent recovery of a known analyte amount in a sample. It was assessed through recovery studies in triplicates at three concentration levels: 80%, 100%, and 120% using vasicine, gallic acid, piperine, and pterostilbene. Results are shown in Table 12.

Robustness

The robustness of the method was evaluated by making slight changes to chromatographic conditions, including mobile phase composition and chamber saturation time. A standard solution concentration of 10 µg/mL was applied in triplicate. The effects on the Retention factor (R_f) and peak area were assessed by calculating the (% RSD). The results indicated that the developed method is robust, as detailed in Table 13.

DISCUSSION

The Indian market is filled with Ayurvedic medicines offered in various forms such as tablets, capsules, granules, syrups, and several others aimed at addressing menstrual irregularities and PCOS. Most of these Ayurvedic products are made using raw herbs. It is crucial to standardize herbal formulations to ensure the quality of the medications. In addition to fundamental testing, identifying and measuring active ingredients, along with the final dosage form, is vital for ensuring consistency from one batch to another. Standardization also plays a significant role in the selection of raw materials, as well as in the processing and production of formulations (Sachan *et al.*, 2016; Attimarad *et al.*,

2011; Garg *et al.*, 2013; Tsering *et al.*, 2014; International Council for Harmonisation [ICH], 2005).

HPTLC is a rapid and accurate method for phytochemical profiling and quantification of compounds in plants. It provides better resolution and estimation of active constituents, allowing for efficient analysis. With the growing demand for herbal medicines and cosmetics, standardization of active raw materials and finished products is essential (Sachan *et al.*, 2016; Attimarad *et al.*, 2011; Garg *et al.*, 2013; Tsering *et al.*, 2014; International Council for Harmonisation [ICH], 2005).

To create a complete formula, we prepare Amarantha Gynorite syrup and Amarantha Gynorite capsules using standard herbal extracts. Analyzing this herbal combination is complex and takes a lot of time and resources, such as infrastructure, manpower, and materials. This research developed a single HPTLC method that is simple, cost-effective, and saves time for analyzing important compounds like Gallic Acid (GA), Vasicine (VS), Pterostilbene (PTS), and Piperine (PP) from both Amarantha Gynorite syrup and capsules.

In the current High-Performance Thin-Layer Chromatography (HPTLC) method, a stationary phase consisting of a precoated silica Gel aluminum plate (G60F254) was selected for the separation of four marker compounds. The individual standards and samples were developed utilizing methanol. Among various mobile phases tested, the optimized mobile phase was determined to be a mixture of toluene, ethyl acetate, formic acid, and Glacial Acetic Acid (GAA) in a volumetric ratio of 2:1:1:0.75, with a detection wavelength established at 280 nm, corresponding to the isosbestic point. The Retention factor (R_f) values for the compounds VS, GA, PP, and PTS were calculated as 0.11, 0.34, 0.68, and 0.71, respectively, as illustrated in Figures 2-4, 10-11.

Linearity was observed across the concentration range of 5-50 µg/mL for the aforementioned compounds, with correlation coefficients falling within acceptable limits. During the accuracy recovery studies, the overall recovery percentages were calculated

Table 7: Percent assay of herbal extracts and *Ayurvedic* formulations.

Sl. No.	Standards	Extracts (% Assay)	Gynorite Syrup (% Assay)	Gynorite Capsule (% Assay)
1.	VS	1.12	0.81	0.64
2.	GA	1.09	0.76	0.58
3.	PP	1.61	0.98	0.45
4.	PTS	2.12	1.07	1.27

Table 8: Linear regression data for calibrations curve (*n* = 7).

Sl. No.	Parameter	Results			
		VS	GA	PP	PTS
1.	Linearity Range (µg/mL)	5 - 50	5 - 50	5 - 50	5 - 50
2.	Correlation coefficient (R^2)	0.9958	0.9995	0.9966	0.9984
3.	y-intercept	91.731	402.02	381.94	438.75
4.	Slope	835.41	3182.2	897.81	780.11

Table 9: Intra-day precision studies.

	VS			GA			PP			PTS		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Amount (µg/mL)	50	100	150	50	100	150	50	100	150	50	100	150
Peak Area	4853.8	9918.4	15311.8	22430.8	45961.9	68608.4	20304.7	39083.3	59674.2	21362.9	45126.2	67689.3
	4818.4	9814.9	15221.2	22312.6	44918.4	68657	20126.2	38464.2	59890.5	21864.2	46023.6	67135.3
	4920.4	10008	15134.5	22642.1	45226.4	68308.9	19892.1	39061.9	59183.5	21141.2	45412.5	68001.3
Average Peak Area	4864.2	9913.9	15222.5	22461.8	45368.9	68524.7	20107.6	38869.8	59582.7	21456.1	45520.7	67608.6
SD	51.79	96.93	88.66	166.93	536.15	188.52	206.93	351.42	362.26	370.40	458.39	438.59
% RSD	1.06	0.977	0.582	0.743	1.181	0.275	1.029	0.904	0.608	1.726	1.006	0.648

Table 10: **Inter-day precision studies.**

	VS			GA			PP			PTS		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Amount (µg/mL)	50	100	150	50	100	150	50	100	150	50	100	150
Peak Area	4798.3	9641.5	14023.6	21907.2	43701.2	65892.3	19454.1	38993.7	57520.5	20175.5	44676.3	65873.5
	4753.1	9786.2	14345.5	21523.8	44052.6	65423.1	19821.8	38179.3	57240.8	20631.1	43987.6	66159.6
	4890.5	9741.3	14535.2	21646.1	44121.2	67404.2	19907.7	38806.5	58741.3	20421.3	44253.6	66371.8
Average Peak Area	4813.96	9723	4301.4	21692.3	43958.3	66239.8	19727.8	38659.8	57834.2	20409.3	44305.8	66134.9
SD	70.02	74.06	258.63	195.8	225.31	1035.27	240.94	426.55	797.9	228.03	347.30	250.06
% RSD	1.45	0.76	1.80	0.91	0.512	1.57	1.22	1.10	1.37	1.11	0.783	0.378

Table 11: **Results of Limit of Detection (LOD) and quantification.**

Sl. No.	Parameters	VS	GA	PP	PTS
1.	LOD	0.662 ng/mL	1.573 ng/mL	1.152 ng/mL	1.688 ng/mL
2.	LOQ	2.018 ng/mL	4.769 ng/mL	3.492 ng/mL	5.115 ng/mL

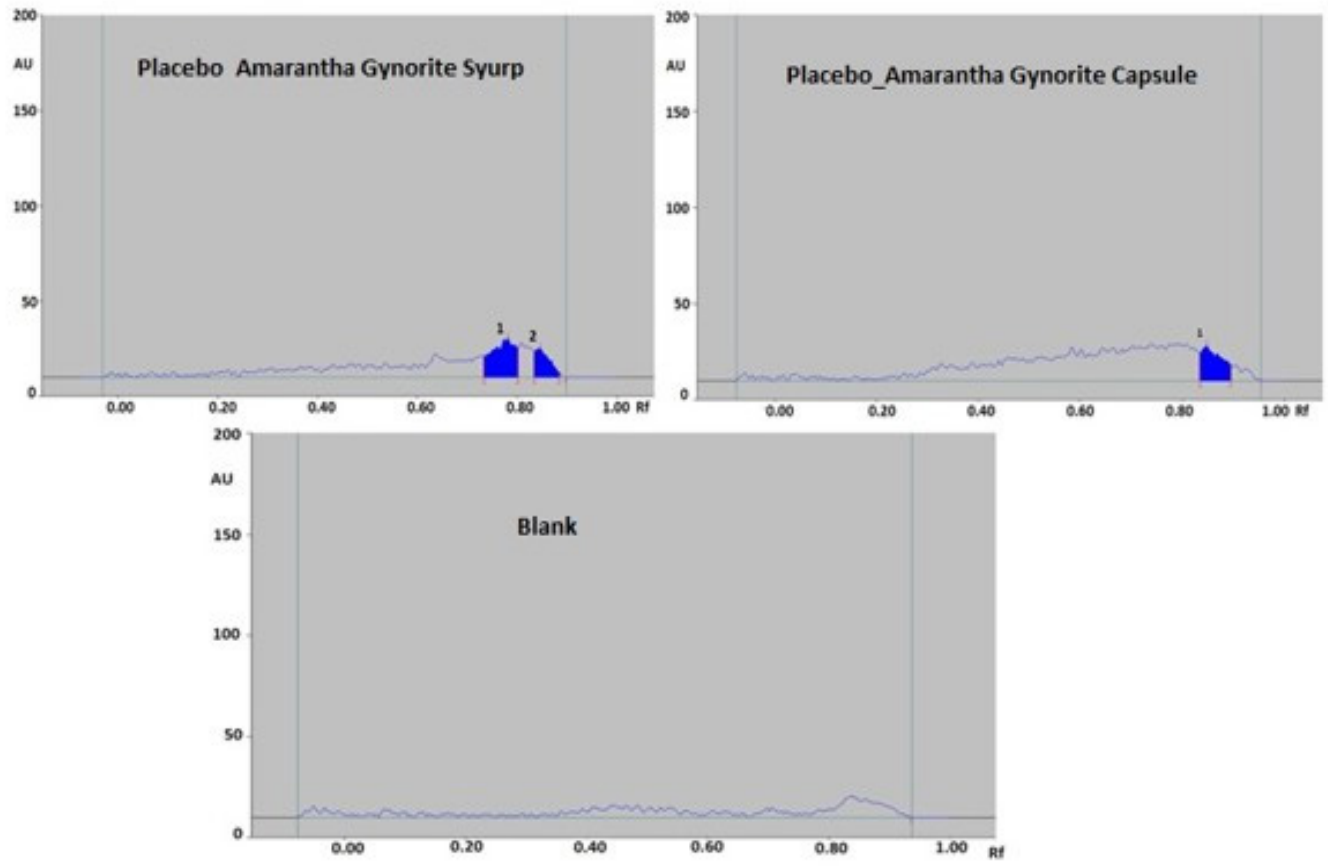

Figure 5: Densitogram of placebo syrup, placebo capsule & blank.

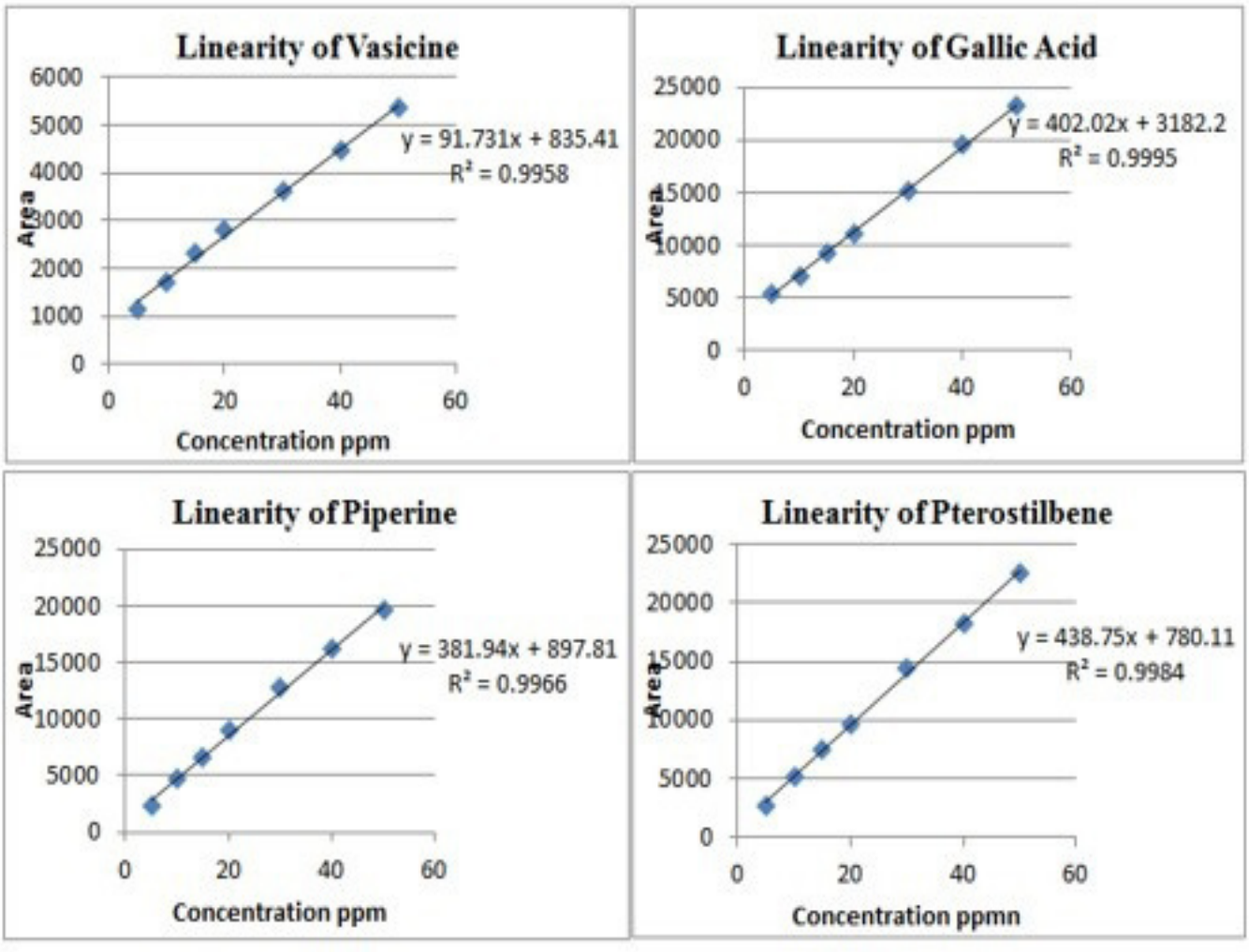

Figure 6: Linearity graph of vasicine, gallic acid, piperine and pterostilbene.

Table 12: Accuracy study.

Standard Solutions	Percent Level	Peak Area	% Recovery	Average % Recovery	%RSD
VS	80	7541.1	100.73	100.21	1.076
	100	10334.4	100.63		
	120	11411.2	99.28		
GA	80	35154.5	99.28	99.96	1.093
	100	45617.6	102.0		
	120	52600.3	98.60		
PP	80	35008.7	98.88	100.66	0.9038
	100	45189.8	101.49		
	120	54557.5	101.62		
PTS	80	32632.4	98.42	99.28	1.15
	100	37253.03	99.255		
	120	43797.4	100.15		

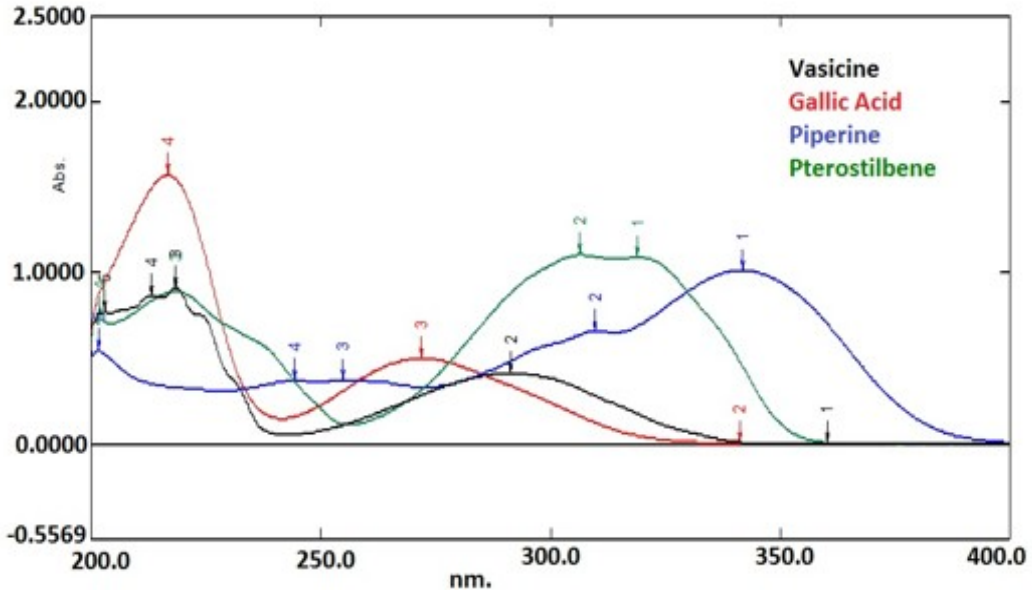

Figure 7: Overlay UV spectra of vasicine, gallic acid, piperine and pterostilbene.

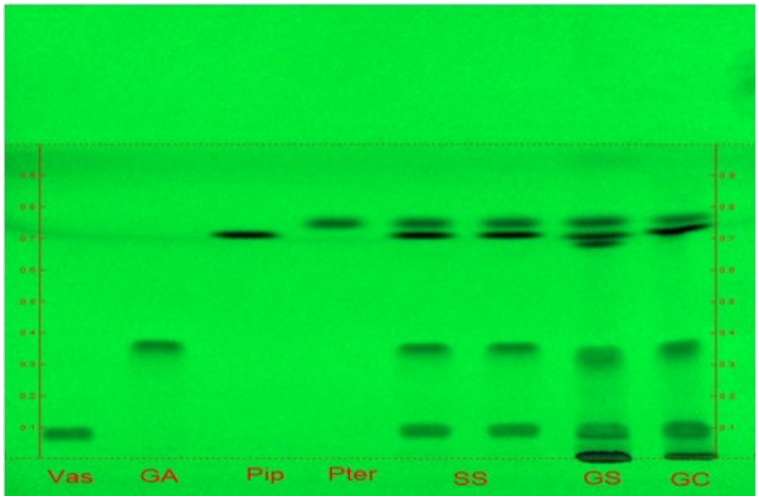

Figure 8: Photo documentation of individual standards, Standards Solution (SS), Amarantha Gynorite Syrup (GS) and Amarantha Gynorite capsule (GC) at 254 nm.

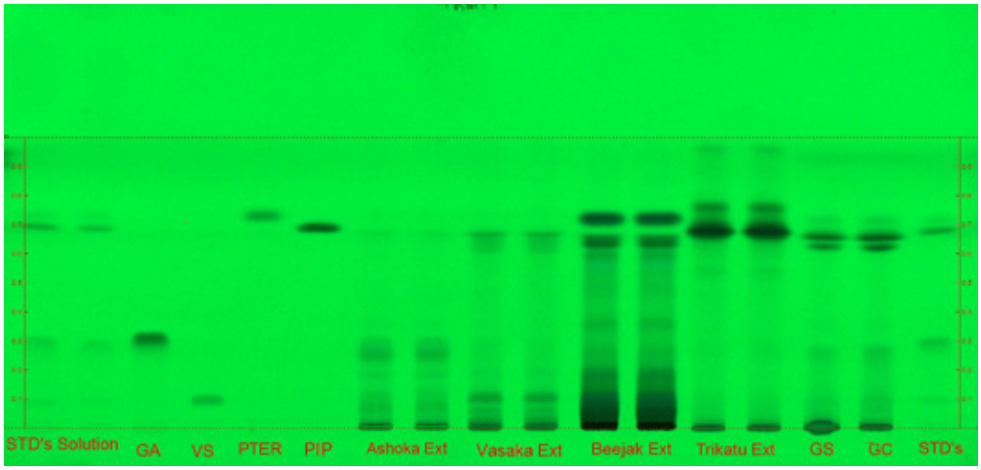

Figure 9: Photo document of HPTLC fingerprint of standards, standard solutions, herbal extracts and Ayurvedic Formulations.

Table 13: Results of robustness.

Parameters	VS		GA		PP		PTS	
	% RSD							
	Rf	% RSD	R_f	% RSD	R_f	% RSD	R_f	% RSD
Mobile Phase composition								
Tol: EA: MeOH: GAA (6:3:1:1.5)	0.12	0.5199	0.34	0.685	0.73	0.78	0.76	1.12
Tol: EA: MeOH: GAA (7:3:1:1.5)	0.09	1.28	0.32	0.82	0.70	1.10	0.74	0.78
Tol: EA: MeOH: GAA (7:4:1:1.5)	0.10	0.536	0.33	1.18	0.71	0.82	0.75	0.84
Saturation time (min)								
28	0.10	1.36	0.32	1.14	0.70	1.12	0.74	0.85
30	0.10	1.28	0.31	0.82	0.70	1.10	0.74	0.78
32	0.10	0.35	0.32	1.34	0.69	0.64	0.75	1.35

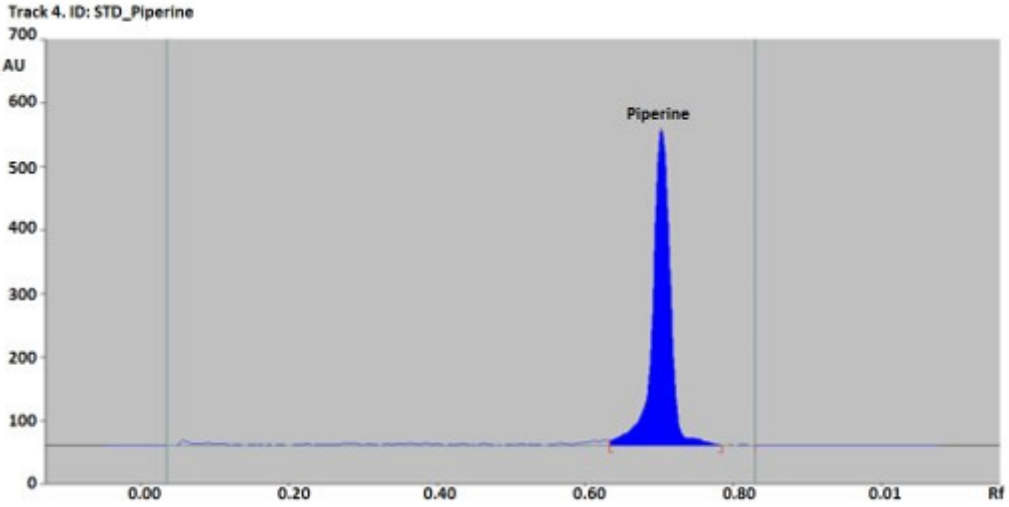

Figure 10: Densitogram of piperine Rf 0.68±0.03.

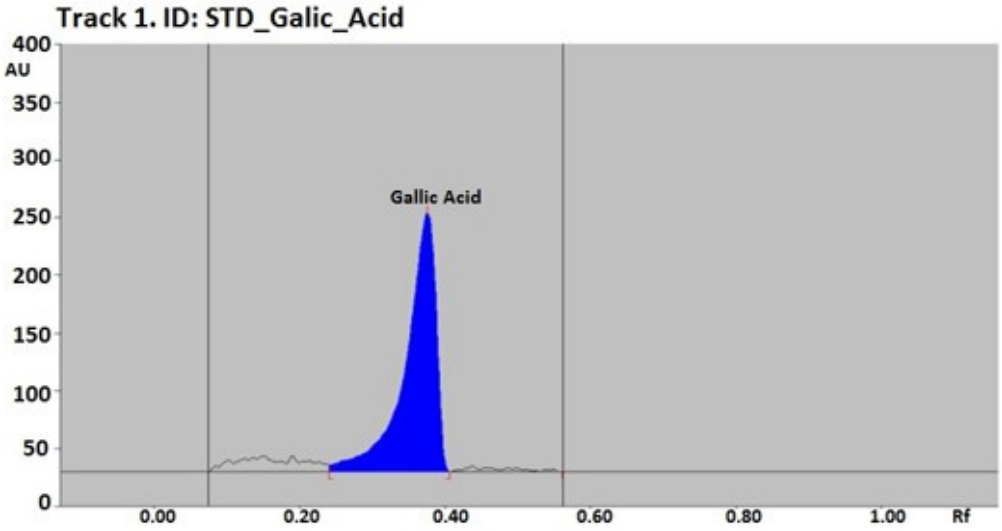

Figure 11: Densitogram of gallic acid Rf 0.34±0.03.

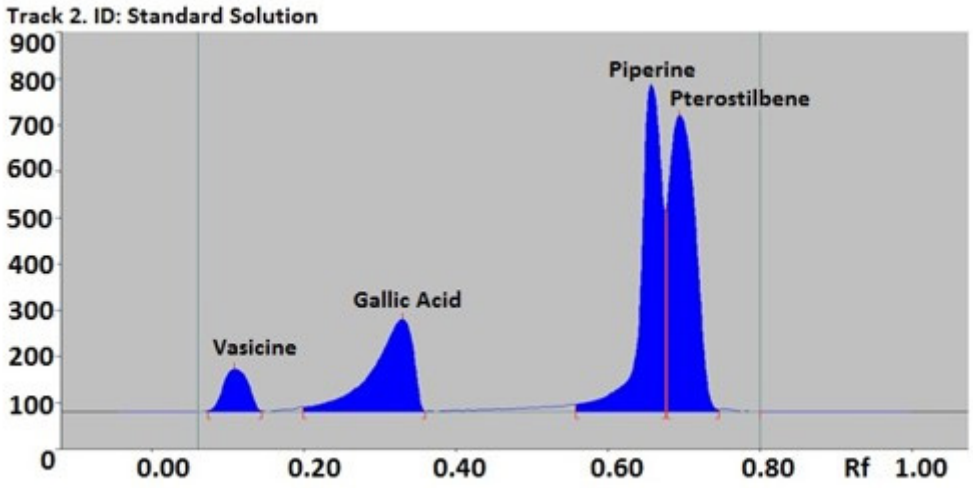

Figure 12: Densitogram of standards solution (vasicine, gallic acid, piperine & pterostilbene).

to be 100.21% for VS, 99.96% for GA, 100.66% for PP, and 99.28% for PTS. The analytical results underwent validation regarding accuracy and precision. The Limits of Detection (LOD) were determined to be 0.662 ng, 1.573 ng, 1.152 ng, and 1.688 ng, while the Limits of Quantification (LOQ) were established as 2.018 ng, 4.769 ng, 3.492 ng, and 5.115 ng for VS, GA, PP, and PTS, respectively.

The precision of the proposed HPTLC method was evaluated through system suitability studies, method precision, intermediate precision, and repeatability assessments, with the percentage Relative Standard Deviation (% RSD) remaining within acceptable criteria of less than 2.0%.

Vasicine, gallic acid, piperine, and pterostilbene were quantitatively measured in the Ayurvedic proprietary formulations, specifically Amarantha Gynorite syrup and Amarantha Gynorite capsules. The developed method proved to be effective for quantifying these marker compounds.

CONCLUSION

A simple and cost-effective method called isocratic HPTLC was created to analyze marker compounds like GA, VS, PTS, and PP from Amarantha Gynorite syrup and capsules. This method is accurate, precise, and reliable. It is also specific and can be easily reproduced.

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ABBREVIATIONS

GA: Gallic Acid; **GAA:** Glacial Acetic Acid; **HPLC:** High Performance Liquid Chromatography; **HPTLC:** High-Performance Thin Layer Chromatography; **HQC:** High Quality Control; **ICH:** International Council on Harmonization; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **LQC:** Low Quality Control; **MQC:** Medium Quality Control; **PCOS:** Polycystic Ovary Syndrome; **PP:** Piperine; **PTS:** Pterostilbene; **R_f:** Retention Factor; **R&D:** Research and Development; **RSD:** Relative Standard Deviation; **UV:** Ultraviolet; **VS:** Vasicine.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

A validated HPTLC method was developed for simultaneous estimation of vasicine, gallic acid, piperine, and pterostilbene in Amarantha Gynorite syrup and capsule. Separation was achieved on silica gel G60F254 plates using Toluene:Ethyl acetate:Formic acid:GAA (2:1:1:0.75 v/v) with detection at 280 nm. The method showed good linearity (5-50 µg/mL), precise R_f values, high recovery (~99-101%), and low LOD/LOQ values. Overall, the method proved accurate, precise, rapid, and cost-effective for routine quality control analysis.

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