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ORIGINAL RESEARCH PAPER

Development and validation of a high-performance thin-layer chromatographic method for the simultaneous estimation of berberine, gallic acid, mangiferin, and quercetin in Amritamehari churnam

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Abstract
Herbal drugs need to be standardized for their worldwide acceptance as it ensures the therapeutic efficacy and safety of the drugs. The phytopharmaceutical guidelines of the Central Drugs Standards Control Organization for botanical products suggest selecting a variety of compounds, i.e., a minimum of four phytoconstituents as the index for quality control of these products. Hence, for the quality control of multicomponent herbal formulations, simultaneous estimation of phytoconstituents would be a wise choice. The present work aimed to develop a simple and precise high-performance thin-layer chromatographic (HPTLC) method for the simultaneous estimation of berberine, mangiferin, gallic acid, and quercetin in Amritamehari churnam. Separation of phytoconstituents was carried out on silica gel $60F_{254}$ plates via a linear ascending technique using toluene−ethyl acetate−formic acid−methanol (5:4:1:1, *V/V*) as the mobile phase. Densitometric scanning was performed at 254 nm to quantify the spots. The R_F values of mangiferin, berberine, gallic acid, and quercetin were found to be 0.134, 0.325, 0.493, and 0.643, respectively. The proposed HPTLC method was validated as per the International Council for Harmonisation guidelines for linearity, accuracy, precision, limits of detection and quantification, robustness, and specificity. The calibration curves were linear with the correlation coefficients 0.99617, 0.99529, 0.99741, and 0.99845 for mangiferin, berberine, gallic acid, and quercetin, respectively. The developed method was successfully applied for the simultaneous determination of berberine, mangiferin, gallic acid, and quercetin in the commercial polyherbal formulation. The present HPTLC method, which can be used for the standardization of Amritamehari churnam, is being reported for the first time with these four phytoconstituents.

Keywords Berberine · Mangiferin · Gallic acid · Quercetin · High-performance thin-layer chromatography (HPTLC)

1 Introduction

Diabetes is an important human ailment affecting many people in different countries. In India, it is proving to be a major health issue, especially in urban areas. It is estimated that more than 300 million people in the world will have type 2 diabetes by the year 2025 [1, 2]. Among the numerous approaches to reduce the ill effects of diabetes and its secondary complications, herbal formulations are favored due to lesser side effects and low cost [3]. The traditional systems of medicine are effective against diabetes, but they lack standardization. These formulations need to be controlled for quality for ensuring their safety and efficacy. Rigorous quality control methods are mandatory for confirming the quality of products. The major challenge in the quality control of herbal formulations is to develop authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of bio-active/marker compounds and other major constituents. Since curative effects of medicinal herbs and their preparations are principally based on the synergic effect of their multi-ingredient, using one or two components as markers for the quality control of herbs is fundamentally flawed [4–9]. In India, the Central Drugs Standards Control Organization

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(CDSCO) has created separate guidelines for botanical products called "phytopharmaceutical guidelines"—2013 which defines "phytopharmaceutical drug as purified and standardized fraction with defined minimum four bio-active or phytochemical compounds (qualitatively and quantitatively assessed) of an extract of a medicinal plant or its part, for internal or external use of human beings or animals for diagnosis, treatment, mitigation, or prevention of any disease or disorder but does not include administration by parenteral route" [10, 11]. Therefore, for complex systems, selecting a variety of compounds as the index of quality control and using an effective method to simultaneously detect them would be a wise choice.

Chromatographic fingerprinting has been demonstrated to be an efficient and practical method for the standardization of various traditional medicines. Chromatographic methods like high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) are useful techniques for the qualitative and quantitative determination of drugs [12, 13].

Plants commonly found in various antidiabetic formulations include *Tinospora cordifolia, Gymnema sylvestre, Eugenia jambolana, Berberis aristate, Emblica officinalis, Terminalia chebula, Terminalia bellirica, Pterocarpus marsupium, Salacia reticulata, and Azadirachta indica* [3, 14–16]. There are several medicines in Ayurveda for the treatment of diabetes manufactured by standard pharmacies, like Amritamehari churnam (Kottakkal Arya Vaidya sala), Tribangshila (Zandu), Madhumehari granules (Baidyanath), Hyponidd (Charak), Diabecon (Himalaya), Glucomap (Maharshi), Debix tablets (Sandu), etc. Amritamehari churnam, also known as Amritadi churnam, was selected for development of simultaneous estimation of phytoconstituents present in it by HPTLC. Amritamehari churnam mainly consists of the following ingredients: Amrita *(T. cordifolia),* Meharimula *(S. fructicosa),* Dhatri—Amalaki (*E. officinalis*), Ratri—turmeric (*Curcuma longa*). These plants contain phytoconstituents like berberine, mangiferin, gallic acid, and quercetin. *Queen* et al. reported the gas chromatography–mass spectrometry (GC–MS) analysis of Amritamehari churnam to find the type of biomolecules present in it [17]. The study reported GC–MS peaks for various biomolecules, but no quantitative analysis was done.

There are methods available in the literature for determining berberine, mangiferin, gallic acid, and quercetin independently or in combination with other phytomakers. But there is no method documented for the simultaneous estimation of the mentioned combined phytomarkers in any dosage forms. Thus, the objective of the study was to develop a validated HPTLC method for the simultaneous estimation of berberine, mangiferin, gallic acid, and quercetin (Fig. 1) in the polyherbal formulation Amritamehari churnam, which can be used as a quality control method.

2 Experimental

2.1 Chemicals and reagents

The standards berberine HCl (96.3% purity), mangiferin (96% purity), and gallic acid (98.4% purity) were procured from Yucca Enterprises Pvt Ltd. (Mumbai, India). Standard

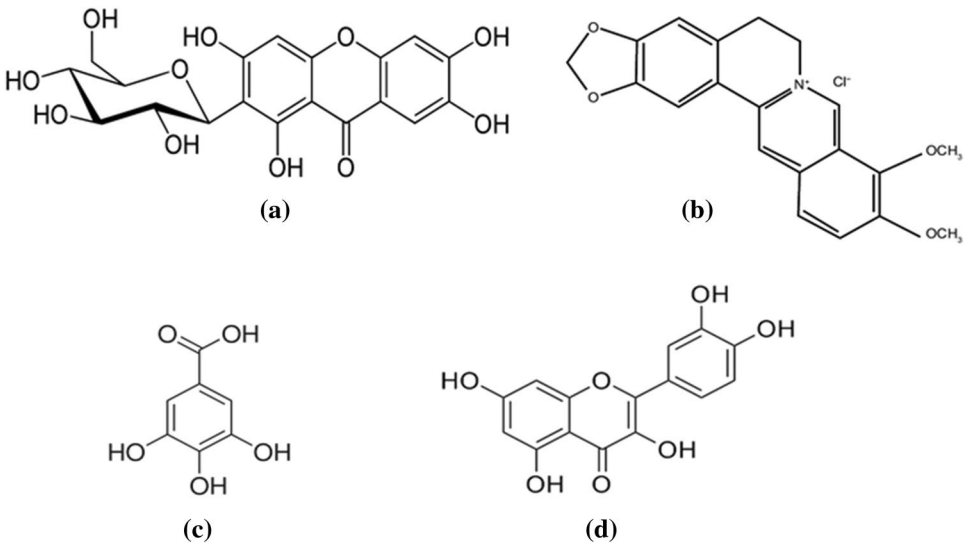

Fig. 1 The chemical structures of four phytoconstituents: **a** mangiferin, **b** berberine, **c** gallic acid, and **d** quercetin

quercetin (96% purity) was procured from Sigma Aldrich (Mumbai, India). The herbal drug formulation (Amritamehari churnam) was procured from Kottakkal Arya Vaidya sala (Mumbai, India). All chemicals used were of analytical grade and purchased from SD Fine-Chem (Mumbai, India).

2.2 Preparation of solutions

Standard stock solutions: a standard stock solution of berberine, gallic acid, mangiferin, and quercetin was prepared by dissolving 10 mg of standards in methanol yielding 10 mL of stock solution (1000 µg mL^{-1}).

Sample stock solution: an aliquot of 2 g of Amritamehari churnam was transferred to a 50 mL volumetric flask containing 30 mL methanol and was kept in sonicator for 2 h at room temperature (25 ± 2 °C); the volume was made up to 50 mL. The solution, thus, obtained was filtered through Whatman filter paper and used for analysis. A constant application of 5.0 µL $spot^{-1}$ was used.

2.3 Chromatographic conditions

Chromatography was performed on 20 cm × 10 cm precoated silica gel aluminum $60F_{254}$ plates (Merck, Darmstadt, Germany). The sample and standards were applied separately to the plate as 7 mm wide bands with a 100 µL syringe (Hamilton, Bonaduz, Switzerland) under a controlled nitrogen stream using Linomat V sample applicator (CAMAG, Muttenz, Switzerland) at a dosage speed of 150 nL s^{-1}. Method development was executed in a CAMAG twin-trough glass chamber (20 cm × 10 cm) saturated with the mobile phase toluene−ethyl acetate−formic acid−methanol (5:4:1:1, *V/V*) for 25 min at room temperature (25 ± 2 °C) before study. The length of the chromatogram run was 8 cm. After development, the plates were air-dried. The plates were photographed under ultraviolet (UV) 254 nm by CAMAG TLC Visualizer. Densitometric scanning was performed with CAMAG TLC Scanner 3 in the reflectance−absorbance mode at 254 nm and operated by winCATS software. The slit dimension was kept at 6 mm × 0.45 mm, and 10 mm s^{-1} scanning speed was employed. The identification of mangiferin, berberine, gallic acid, and quercetin was confirmed by comparing the retention factor (R_F) values and superimposing the absorption spectra of the samples and standards.

2.4 Validation of the method

The proposed analytical method was validated as per the International Council for Harmonisation (ICH) guidelines Q2 (R1) [18].

2.4.1 Linearity

Linearity was evaluated in the range of 50−1400 ng $spot^{-1}$ for berberine, mangiferin, quercetin and 100−2800 ng $spot^{-1}$ for gallic acid. Peak area versus concentration was subjected to least-square linear regression analysis and the slope, intercept and correlation coefficient for the calibration curve were determined.

2.4.2 Precision

The precision of the method was evaluated by repeatability (intra-day) and intermediate precision (inter-day) precision. Each level of precision was assessed by using a minimum of nine determinations covering the specified range for the procedure. Intra-day precision was performed three times on the same day, while inter-day precision was performed on three different days. The three concentrations used for precision studies were 400, 500 and 600 ng $spot^{-1}$ for mangiferin, berberine and quercetin and 800, 1000 and 1200 ng $spot^{-1}$ for gallic acid.

2.4.3 Accuracy

Accuracy was determined as the percentage recoveries of known amounts of mangiferin, berberine, gallic acid, and quercetin added to sample solutions. The analyzed samples were spiked with 80, 100, and 120% of median concentrations of standards (400 ng $spot^{-1}$ of mangiferin, berberine, and quercetin, 800 ng $spot^{-1}$ gallic acid). Accuracy was calculated by comparing the area before and after the addition of the standard drug.

2.4.4 Limit of detection and limit of quantification

The limit of detection (LOD) is the lowest level of analyte that can be detected in a sample but not necessarily quantified, under the stated experimental conditions. The limit of quantification (LOQ) is identified as the lowest

Table 1 Optimization of mobile phase

Sr. no.	Mobile phase	Composition (*V/V*)	Result
1	Toluene–ethyl acetate–formic acid	6:6:1	Low R_F value, low resolution
2	Toluene–ethyl acetate–formic acid	5:4:1	Mangiferin and berberine spots were not completely separated
3	Toluene–ethyl acetate–formic acid–methanol	5:4:1:0.5	Good resolution, R_F value of mangiferin less than 0.1
4	Toluene–ethyl acetate–formic acid–methanol	5:4:1:1	Well resolved compact bands for all 4 marker compounds

Table 2 Marker compounds and their respective R_F values

Sr. no.	Marker compound	R_F value
1	Mangiferin	0.134 ± 0.005
2	Berberine	0.325 ± 0.009
3	Gallic acid	0.493 ± 0.004
4	Quercetin	0.643 ± 0.007

amount of analyte that can be detected and quantified with acceptable accuracy, precision, and variability. LOD and LOQ were determined by the following equations: LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the residual standard deviation of the regression line and S is the slope of the calibration.

2.4.5 Robustness

Robustness was evaluated by introducing deliberate changes in the method parameters, and their effect on peak area and retention factor was observed. Only one parameter was altered at a time keeping the other parameters constant. The robustness of the method was evaluated by changing mobile phase composition and chamber saturation time. The mobile phase composition was altered by ± 5% changes in the composition of methanol. The original mobile phase toluene–ethyl acetate–formic acid–methanol (5:4:1:1, *V/V*) was changed to toluene–ethyl acetate–formic acid–methanol (5:4:1:0.9, *V/V*) and toluene–ethyl acetate–formic acid–methanol (5:4:1:1.1, *V/V*). The chamber saturation time was altered from 20 to 30 min.

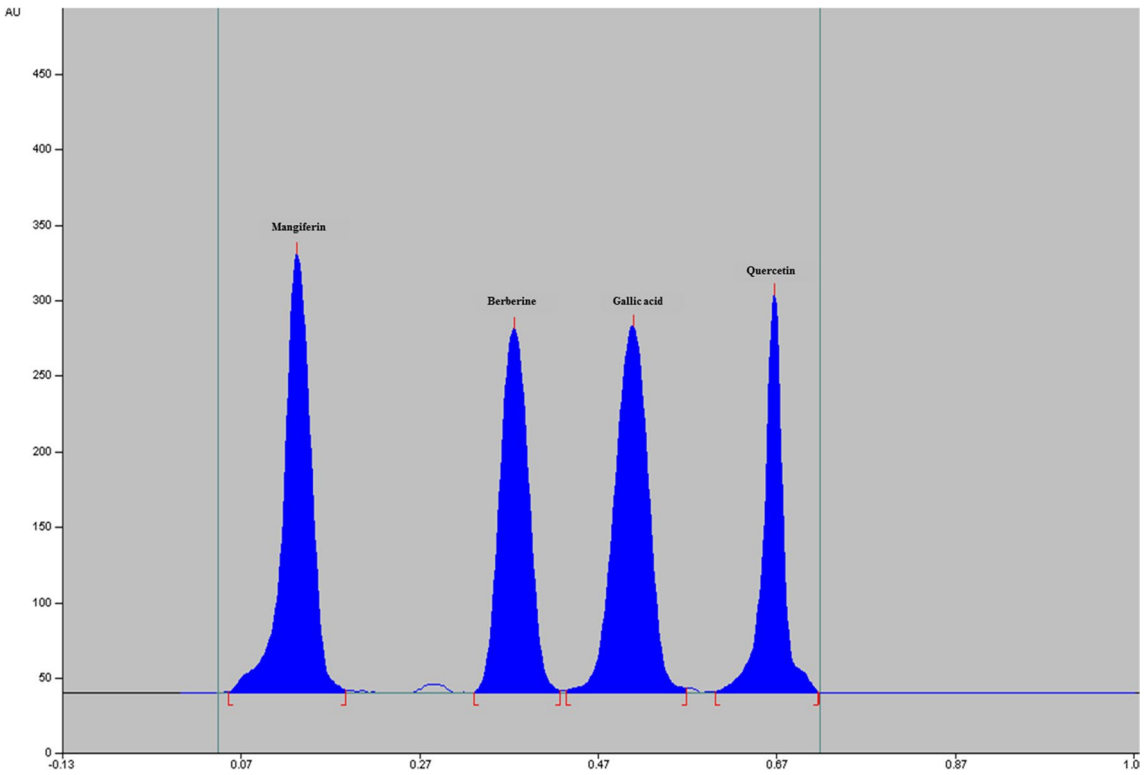

Fig. 2 HPTLC chromatogram of mangiferin, berberine, gallic acid and quercetin using optimized parameters

2.5 Analysis of polyherbal formulation

The validated HPTLC method was used to analyze the commercial formulation Amritamehari churnam containing these phytoconstituents.

3 Results and discussion

3.1 Method optimization

To optimize the mobile phase, toluene−ethyl acetate−formic acid mixtures in various proportions were investigated. The various mobile phases tried are shown in Table 1. The resolution among the bands increased when methanol was introduced. Finally, the mobile phase toluene−ethyl acetate−formic acid−methanol (5:4:1:1, *V/V*) gave well resolved compact bands with an R_F value as shown in Table 2 and Fig. 2. The optimized chamber saturation time for the given mobile phase was found as 25 min at room temperature (25 ± 2 °C). The total run length of the chromatogram run was 80 mm.

3.2 Method validation

3.2.1 Specificity

The specificity of the method was ascertained by comparing the R_F values and spectrum of the band with those of the bands from standards. It can be assumed from the peak purity spectra (Fig. 3) that the method is specific for these components.

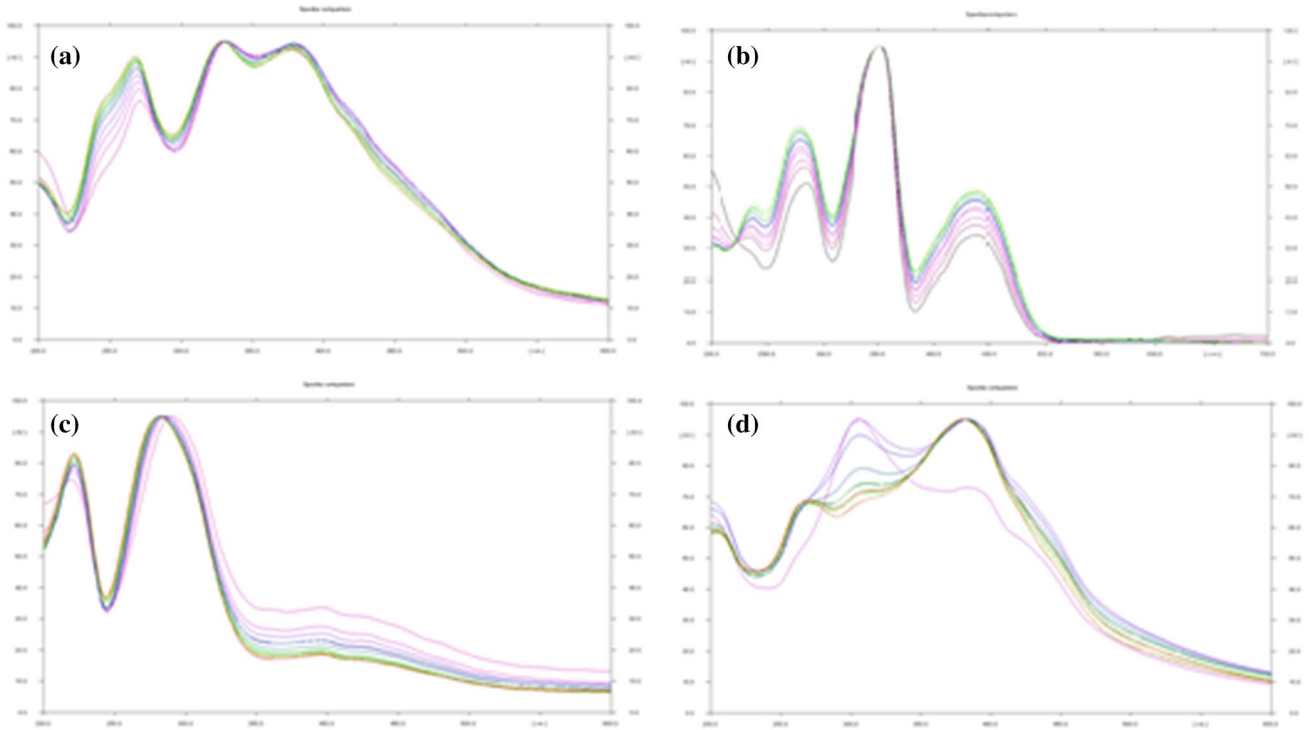

Fig. 3 Peak purity spectra of **a** mangiferin, **b** berberine, **c** gallic acid, and **d** quercetin

Table 3 Linear regression parameters ($n = 3$)

Parameters	Mangiferin	Berberine	Gallic acid	Quercetin
Linear range (ng spot^{-1})	50−1000	100—1000	1200–2600	100—800
Correlation coefficient	0.99617	0.99529	0.99741	0.99845
Linear regression equation	$y = 9.1854x + 561$	$y = 8.1388x + 1104.2$	$y = 3.5417x + 4992$	$y = 7.2291x + 590.24$
Standard deviation of slope	0.2687	0.2802	0.1042	0.1643
Standard deviation of intercept	159.05	173.88	203.81	83.01

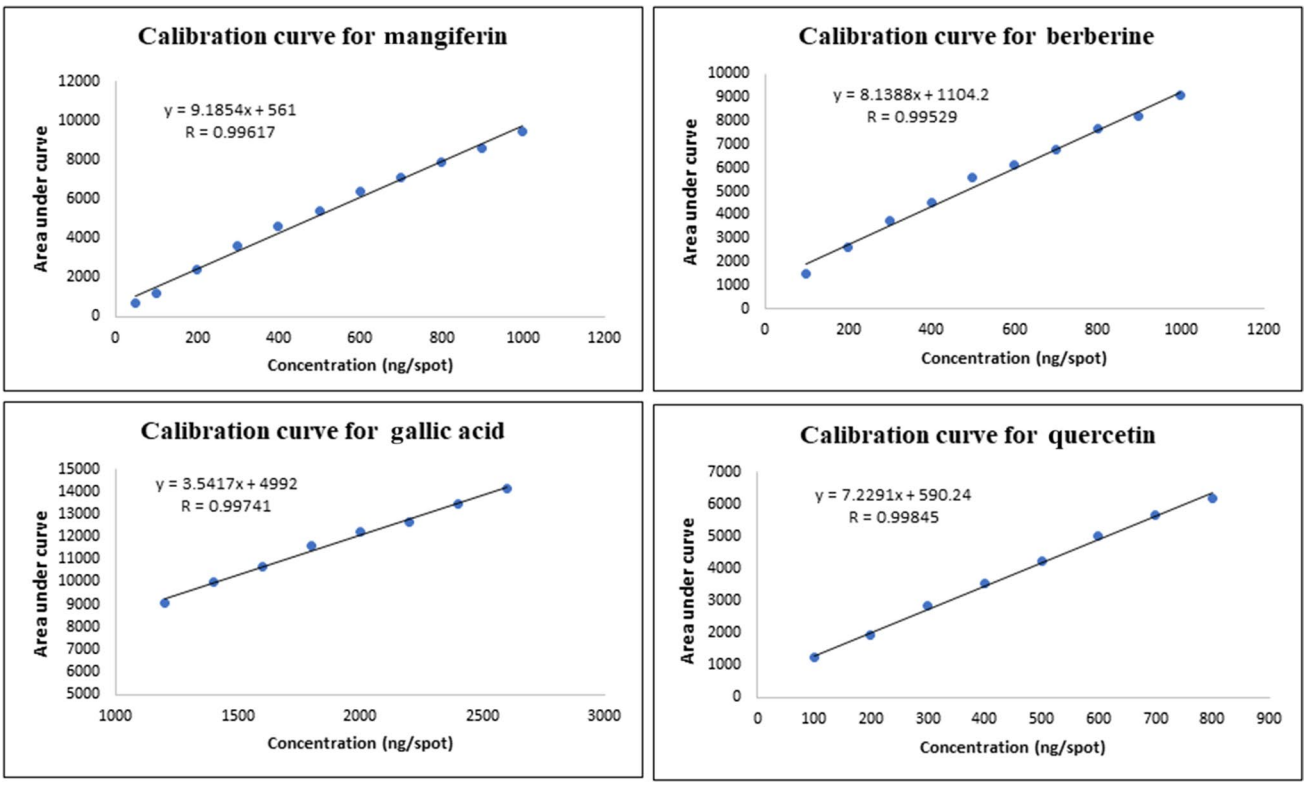

Fig. 4 Calibration curves of mangiferin, berberine, gallic acid, and quercetin

Table 4 Precision studies of mangiferin, berberine, gallic acid, and quercetin

Markers	Concentration (ng/spot)	Intra-day precision[a]	Inter-day precision[a]
Mangiferin	400	0.499	0.858
	500	0.305	0.781
	600	0.210	1.469
Berberine	400	1.490	2.102
	500	1.440	2.106
	600	0.658	3.246
Gallic acid	800	0.511	1.594
	1000	0.636	0.977
	1200	0.159	0.159
Quercetin	400	1.195	0.744
	500	0.728	0.728
	600	0.827	0.723

[a]*%RSD* mean ($n = 3$)

Table 5 Accuracy studies of mangiferin, berberine, gallic acid and quercetin

Phytoconstituents	Level (%)	Amount added (ng)	Recovery (%) ($n = 3$)	Mean recovery (%)
Mangiferin	80	320	95.01	93.73
	100	400	95.60	
	120	480	90.59	
Berberine	80	320	91.09	91.21
	100	400	89.59	
	120	480	92.95	
Gallic acid	80	640	91.71	94.46
	100	800	91.61	
	120	960	100.05	
Quercetin	80	320	105.97	99.02
	100	400	99.37	
	120	480	91.72	

3.2.2 Linearity

The linear regression data showed a good linear relationship over the concentration range of 100−1000 ng spot^{-1} for berberine, 50−1000 ng spot^{-1} for mangiferin, 1200−2600 ng spot^{-1} for gallic acid, and 100−800 ng spot^{-1} for quercetin, respectively (Table 3; Fig. 4). The correlation coefficients of mangiferin, berberine, gallic acid, and quercetin were found to be 0.99617, 0.99529, 0.99741, and 0.99845, respectively. The peak area (y) is proportional to the concentration of

Table 6 Limit of detection (LOD) and limit of quantification (LOQ) of the four markers

Markers	LOD (ng)	LOQ (ng)
Mangiferin	21.79	66.02
Berberine	32.95	99.86
Gallic acid	156.10	473.02
Quercetin	29.78	90.25

the respective marker and the regression equations are as follows:

- for mangiferin, $y = 9.1854x + 561$;
- for berberine, $y = 8.1388x + 1104.2$;
- for gallic acid, $y = 3.5417x + 4992$;
- for quercetin, $y = 7.2291x + 590.24$.

3.2.3 Precision

The precision data on repeatability (intra-day) and intermediate precision (inter-day) for three different concentration levels are summarized in Table 4. The percent relative standard deviation (*%RSD*) was found to be within the acceptable limit in all cases, indicating that the proposed method was precise and reproducible.

3.2.4 Accuracy

The accuracy of the developed method was assessed by recovery study at three levels: 80%, 100%, and 120%. The results from accuracy studies (Table 5) were found to be in the range of 91.21–99.01%, indicating that the recovery of the proposed method was good.

3.2.5 Limit of detection and limit of quantification

The LOD values for mangiferin, berberine, gallic acid, and quercetin were 21.79, 32.95, 156.10, and 29.78 ng, respectively, and the LOQ values were 66.02, 99.86, 473.02, and 90.25 ng, respectively, which shows the adequate sensitivity of the method (Table 6).

3.2.6 Robustness

Robustness studies of the method were determined by introducing small changes in chromatographic parameters such as mobile phase composition and chamber saturation time. Results of robustness study are shown in Table 7. The R_F values and the peak area were not significantly affected. The *%RSD* values in all robustness parameters were found to be within the acceptable limit. The resolution and the

Table 7 Robustness studies of the method

Parameter	*%RSD* of peak area ($n = 3$)			
	Mangiferin	Berberine	Gallic acid	Quercetin
Percentage of methanol in the mobile phase (±5%)	0.205	0.620	0.276	0.815
Chamber saturation time (±5 min)	0.784	1.094	0.514	1.407

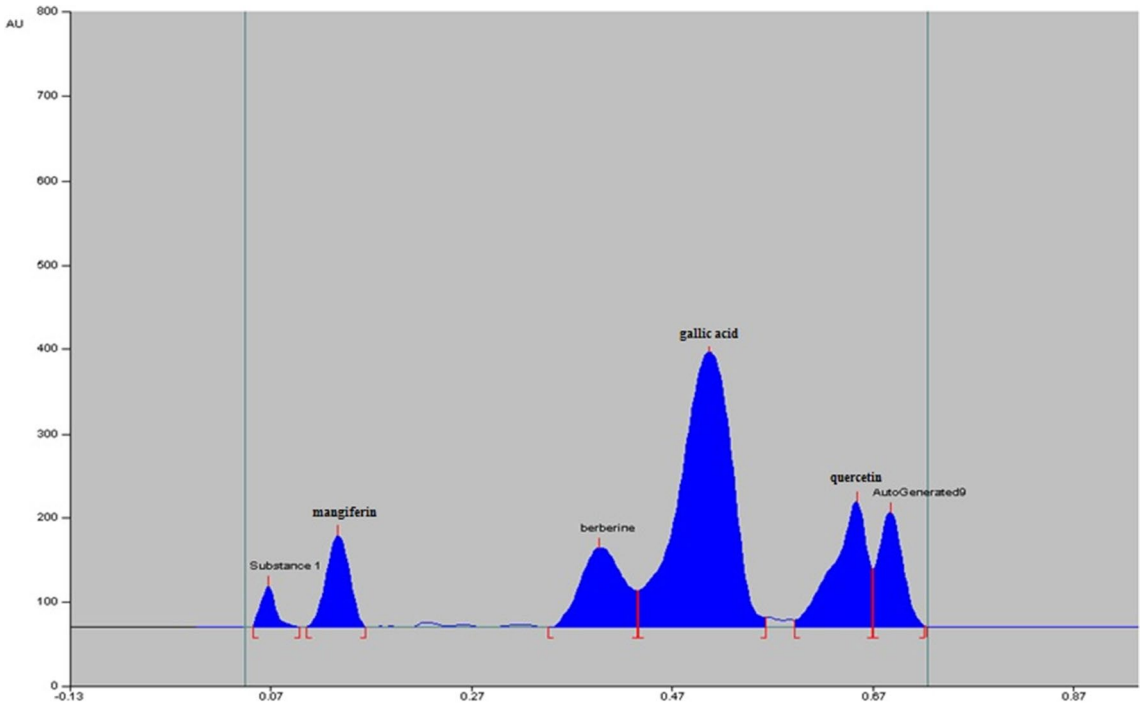

Fig. 5 HPTLC chromatogram of Amritamehari churnam showing all four markers using optimized parameters

Table 8 Contents of mangiferin, berberine, gallic acid, and quercetin in polyherbal formulations

Formulations	Content (mg) ± SD ($n = 3$)			
	Mangiferin	Berberine	Gallic acid	Quercetin
Amritamehari churnam (mg/g)	0.651 ± 0.010	0.941 ± 0.001	11.940 ± 0.170	1.159 ± 0.023

separation of markers were also unaltered. The developed method, thus, can be considered robust.

3.3 Analysis of polyherbal formulation

The developed HPTLC method was used to analyze a polyherbal formulation, Amritamehari churnam. All the four phytoconstituents were eluted at their specific R_F values (Fig. 5). No interfering peaks were observed from any of the inactive ingredients at the R_F of the four phytoconstituents. The contents of mangiferin, berberine, gallic acid, and quercetin found in Amritamehari churnam are as listed in Table 8.

4 Conclusion

The proposed method was found to be sensitive, precise, and accurate for analysis of berberine, mangiferin, gallic acid, and quercetin in Amritamehari churnam. Therefore, the application of this developed method commercially can uplift the global acceptance of herbal-based medicines like Amritamehari churnam.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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