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Original Article

A Validated HPTLC Method for Simultaneous Estimation of Berberine and Trigonelline in Polyherbal Mixture: A Tool for Quality Control and Standardization

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ABSTRACT

Introduction: *Berberis aristata* and *Trigonella foenumgrecum* have been used in traditional medicine for various purposes due to their diverse therapeutic benefits such as anti-inflammatory, antidiabetic, antihypertensive and digestive properties. *Berberis aristata* and *Trigonella foenumgrecum* consists of bioactive markers Berberine and Trigonelline respectively. **Aim:** This study aimed to develop a simple, rapid reliable and robust HPTLC method for the simultaneous quantification of biomarkers in polyherbal mixture. **Materials and Methods:** The separation of these active compounds from the polyherbal mixture was achieved using a mobile phase consisting of toluene: ethyl acetate: formic acid: methanol (6:3:0.3:1v/v/v/v) on pre-coated Silica G 60 F^{254} plates. Validation of the method encompassed assessments of linearity, accuracy, precision, limits of detection and quantification, robustness and specificity. **Results:** Berberine and Trigonelline displayed distinct R_f values of 0.88 and 0.29 respectively. The method demonstrated excellent linearity across the concentration range of 2-10 µg/band, with correlation coefficients of 0.9966 and 0.9973 for Berberine and Trigonelline respectively. **Conclusion:** Percentage recovery was found within the range of 98.84-102.92%. The proposed HPTLC method can be implemented for the concurrent estimation of Berberine and Trigonelline for routine quality control of herbal formulations containing these herbs.

Keywords: Berberine, Trigonelline, Polyherbal mixture, HPTLC, Analytical method validation, Quality control.

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INTRODUCTION

The recent revival of interest in natural medicines, particularly those derived from plants are largely referable to the common perception that herbal remedies are better than synthetic medicines. From the centuries Ayurveda is the most widely used medicinal system.[1] Polyherbal mixture is one such traditional remedy which includes combination of plant materials to give synergistic effect and treat various health complications.[2,3] But Standardization of herbal ingredients and polyherbal formulations is critical for ensuring the quality and efficacy globally. The World Health Organization (WHO) collaborates with health ministry's to develop standardized methods for evaluating herbal products.[4] However, this can only be accomplished through rigorous evaluation and analysis of herbal products using advanced modern standardization techniques such as Thin-Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), High-Performance Thin-Layer Chromatography (HPTLC), Gas Chromatography (GC), Ultraviolet (UV) spectroscopy and Gas Chromatography-Mass Spectrometry (GC-MS).[5,6]

High-Performance Thin-Layer Chromatography (HPTLC) is a highly effective analytical technique for separating, identifying and quantifying compounds in complex mixtures and polyherbal formulations.[7] Simultaneous estimation using HPTLC is the process of analysing multiple components of a sample at once. Its simplicity, versatility and cost-effectiveness make it a desirable choice for quality control and standardization of herbal medicines.[8]

Berberine (Figure 1a) and Trigonelline (Figure 1b) are important phytoconstituents that have garnered attention for their potential antidiabetic properties. Trigonelline, a derivative of niacin found in fenugreek seeds, has been reported to exhibit hypoglycemic effects by enhancing insulin sensitivity and glucose uptake.[9,10] Berberine, a bioactive alkaloid presents in Druharidra has demonstrated promising antidiabetic effects through

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mechanisms including inhibition of hepatic glucose production and enhancement of insulin sensitivity in peripheral tissues.[11,12]

In a study conducted by Zeeshan A. S., *et al.*, two biomarkers from Entoban polyherbal syrup quantified gallic acid and Berberine using the HPTLC method in which authors utilized the two sets of mobile phases such as ethyl acetate: formic acid: methanol (12:9:4:0.5 v/v/v/v) and ethanol: water: formic acid (90:9:1 v/v/v). This study confirms the quantitative presence of Berberine with R_f value 0.76 employing ethanol: water: formic acid 90:9:1 (v/v/v) as a solvent system at a wavelength of 366 nm.[13] Sunita S. *et al.*, quantified Berberine and quercetine using HPTLC with an optimised mobile phase of Toluene: ethylacetate: methanol: formic acid (6:6:2:1 v/v/v/v). The optimized method resolved the biomarkers with R_f values of 0.24 for Berberine and 0.61 for quercetin.[14]

In a study by Shrikrishna M N *et al.*, HPTLC simultaneous method was developed for quercetin, gallic acid, curcumin and Trigonelline. Chromatographic analysis was performed using a silica gel 60 F254 TLC plate and a solvent system of isopropyl alcohol: ammonia: acetone 1:1:1(v/v/v) spots were identified at 254 and 366 nm. The R_f values for quercetin, gallic Acid, curcumin and Trigonelline were 0.66, 0.42, 0.81 and 0.34, respectively.[15] Omi Laila *et al.*, developed and validated, sensitive, fast and reproducible High-Performance Thin-Layer Chromatography (HPTLC) method for detecting Trigonelline in fenugreek seeds using (TLC) aluminium plates pre-coated with silica gel G60 F 254 and using n-propanol, methanol, water and ammonia 10:1.5:15:0.25 (v/v v/v). The method produced compact spot with a R_f value of 0.40 for Trigonelline.[16]

According to the literature found, till date reproducible, accurate and affordable HPTLC method has not been reported for the simultaneous estimation of Berberine and Trigonelline. Hence in this study, we propose a novel simultaneous approach using HPTLC for the analysis of Berberine and Trigonelline in an enriched polyherbal mixture derived from antidiabetic Ayurvedic herbs namely *Berberis aristata* and *Trigonella foenumgrecum*. By developing a robust analytical method, we aim to contribute the quality assessment and standardization of herbal formulations targeted for diabetes management, thereby promoting the integration of traditional medicine into modern healthcare practices.

MATERIALS AND METHODS

Chemicals and Reagents

Analytical grade Toluene, ethyl acetate, formic acid and methanol were purchased from M/s Merck Ltd., Mumbai, India. Trigonelline and Berberine reference standard were received as gift sample from Natural remedies Bangalore, Karnataka, India. Silica gel, G60 F254 coated HPTLC plates were purchased from Merck, India.

Plant Materials

Dried bark of Daruharidra (*Berberis aristata*) and Seeds of Methi (*Trigonella foenumgrecum*) were procured from KLE B. M. Kankanwadi Ayurvedic Maha Vidhylaya Belagavi, Karnataka, India.

Pharmacognostic Evaluation of Plant Materials

As a basic requirement authors checked the quality of procured raw plant materials by subjecting for various quality control parameters like Microscopical, Chemical and Physical as per standard monographs.[17,18]

Preparation of Polyherbal Mixture

Both the plant materials Daruharidra (*Berberis aristata*) and Methi (*Trigonella foenumgrecum*) were subjected for maceration using hydroalcoholic solvent (70:30 v/v), after subjecting for solvent evaporation using rotary evaporator crude extracts were obtained. Polyherbal mixture was prepared as per the AFI by thoroughly mixing an equal proportion of (1:1) of both the extracts.[19,20]

LCMS Analysis for Determination of Berberine and Trigonelline in Polyherbal Mixture

For the identification of bioactive compounds plant extracts were subjected for LC MS/MS (LTQ XL, Thermo Electron Corporation, USA) with an electrospray ion interface and a peak

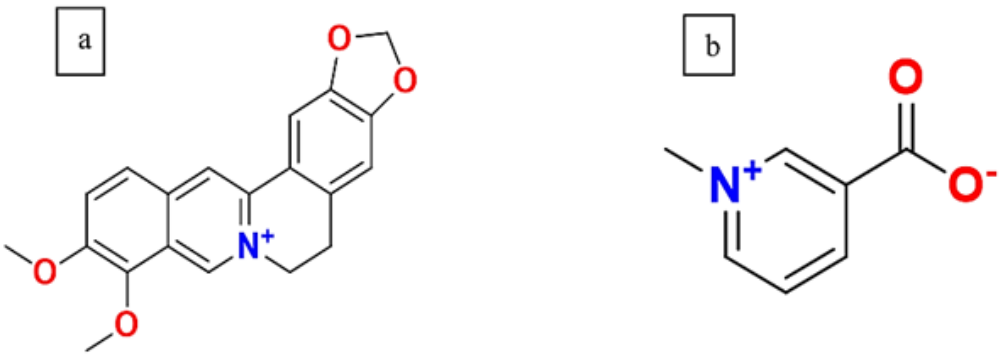

Figure 1: a: Berberine, b: Trigonelline.

scientific-nitrogen generator working in positive ion polarity. Helium was used as the carrier gas, with a flow rate of 1.0 mL/min. The injector was run at 250°C and the oven temperature was programmed as follows: 60°C for 15 min, then gradually raised to 280°C for 30 min. Component identification was depended on the Willey and NIST libraries, as well as a comparison of retention indices. The constituents were identified after comparison to those available in the computer library (NIST and Willey) attached to the LC-MS instrument and the results obtained have been tabulated.[21,22]

HPTLC Method Development

Preparation of Sample and Standard Solutions

Separate 10 mL volumetric flasks were used to prepare individual stock solutions of standard Berberine, Trigonelline and samples (extract and polyherbal mixture). Each flask contained precisely weighed 10 mg of the respective bioactive compound and samples. Methanol was then added to each flask, up to the mark, resulting in a final concentration of 1000 µg/mL for both solutions. To obtain working standard solutions at a concentration of 100 µg/mL, the individual stock solutions of Berberine, Trigonelline and samples were diluted with methanol. A combination of Berberine and Trigonelline stock solutions were prepared in a 10 mL volumetric flask filled with methanol up to the mark. The stock solution was subsequently diluted in a sequential manner to produce various working standards with concentrations ranging from 2 to 10 µg/per band.[23]

HPTLC Method Validation

The established analytical technique was verified in accordance with the recommendations of ICH Q2 (R1) using the following standards: linearity, range, the Limit of Detection (LOD), Limit of Quantification (LOQ), precision, specificity and robustness.[24,25]

Linearity and Range

Berberine and Trigonelline standard stock solutions of various volumes (2, 4, 6, 8 and 10 µL) were spotted onto an HPTLC plate (20×10 cm). Subsequently, sample stock solutions of 5 µL for both extracts and polyherbal mixture were spotted using a CAMAG Linomat V applicator equipped with a 100 µL test syringe. A mobile phase consisting of toluene: ethyl acetate: formic acid: methanol (6:3:0.3:1 v/v/v/v) was employed. Horizontal elution was carried out for 20 min during chromatography. Post-development, the plates were dried and analyzed using a CAMAG TLC visualizer at 254 nm. Subsequently, the developed plate was scanned at 254 nm using a CAMAG TLC densitometric scanner 3, which was integrated with Vision CAT programming.

Limit of Detection and Limit of Quantification

The limit of detection and Limit of quantification was determined using the formula below, which is based on the slope obtained from the linearity and the standard deviation.

$$LOD = 3.3 \times \frac{\sigma}{s} \ldots . (1)$$

$$LOQ = 10 \times \frac{\sigma}{s} \ldots . . (2)$$

Accuracy

To assess the credibility of the proposed method, recovery studies were conducted using the standard addition method across three concentration levels (80%, 100% and 120% of the final concentration). Pre-analyzed samples were augmented with a precise quantity of standard pure markers, Berberine and Trigonelline. The analyzed samples were then spiked with 80%, 100% and 120%.

Precision

The intraday and inter day precision parameters are determined independently. Inter day precision is assessed by repeating the same protocol over three subsequent days. Intraday precision is evaluated by repeating the same procedure three times within the same day. The calibration curve with the highest concentration at the center is selected for the precision study. According to the ICH criteria, the precision study calculations must have a Relative Standard Deviation (RSD) of less than 2%.

System Suitability

The system precision was assessed by determination of six different concentrations of standards each applied in triplicate.

Specificity

The method's specificity was assessed by applying standard and test samples on a single plate, then confirming the peak for the test sample by comparing its R_f and spectrum with those of the standard on the plate.

Robustness

The method's robustness was assessed by making slight alterations to the mobile phase composition, mobile phase volume and duration of mobile phase saturation and observing their effects on the results. This analysis was conducted in triplicate at a concentration level of 10 µg/band for Berberine and Trigonelline.

Quantification of Berberine and Trigonelline in Plant Extracts and Mixture

To quantify the Berberine and Trigonelline in extract and polyherbal mixture, final concentration of 10.0 mg/mL was prepared. 10 µL of sample was applied on HPTLC plates and developed. The quantities of Berberine and Trigonelline in the sample solution were determined from the calibration curve using peak areas of the compounds recorded.[26,27]

RESULTS

Pharmacognostic Evaluation

Collected plant materials were studied for Microscopical characters (Figures 2 and 3) indicates the presence of different microscopical features in *Berberis aristat*a bark such as Tracheids, Fibres, Stone cells and Cork cells. Whereas *Trigonella foenum grecum* seed reported with Epidermis, Endosperm, Tracheids and Aleurone layer. Phytochemical tests are used to detect the presence of various secondary metabolites in plant materials. Both the plants show the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, glycosides and phenolic compounds, among others. The physicochemical evaluation of *Berberis aristata* bark and Trigonella foenum-graecum seeds yields the following results. *Trigonella foenum-graecum* seeds have a total ash content of 3.86±0.389% W/W, acid-insoluble ash of 1.428±0.032% W/W, water-soluble ash of 1.33±1.274% W/W, moisture content of 5.5±0.581% W/W, alcohol-soluble extractive content of 6.38±0.524% W/W, water-soluble extractive content of 12.4±0.588%W/W, and ether-soluble extractive value of 5.3±0.216%W/W. *Berberis aristata* bark had total ash values of 9.33±0.540% W/W, with acid-insoluble ash of4.5±0.354% W/W and water-soluble ash of3.6±0.454% W/W, and moisture content of3.83±0.540%W/W. Extractive content: 7.72±0.795% W/W for alcohol and 8.81±0.368% W/W for water. The ether-soluble extractive value was 3.99±0.252% W/W.

Characterization of Berberine and Trigonelline by LCMS Studies

Total ion chromatogram of LCMS represented a clean peak for Berberine and Trigonelline standard at 2.95 min and another peak at 4.30 min (Figure 4). Berberine and Trigonelline both also observed in polyherbal mixture at 2.95 and 4.30 min respectively (Figure 5). In mass spectrometer mode a mass of m/z 336.1363 g/mol [M+H+] was obtained for the Berberine and 138.0738 g/mol [M+H+] for Trigonelline (Figures 6a, 6b).

Development of HPTLC Method

HPTLC method was developed using multiple mobile phases ultimately, the developed mobile phase comprising of toluene: ethyl acetate: formic acid: methanol (6:3:0.3:1 v/v/v/v) demonstrated superior performance by presenting sharper and well-defined peak resolution for both Berberine and Trigonelline standards (Figure 7). The optimized HPTLC method achieved a resolution of the standard compounds at R_f values of approximately 0.88 for Berberine and 0.29 for Trigonelline. Subsequent scanning of the TLC plate at 254 nm and examination of the 3D chromatogram obtained after densitometric scanning confirmed the identity of Berberine and Trigonelline bands in the sample chromatogram also. This developed method offers a unified analytical approach for both Berberine and Trigonelline standards in a single run using the similar mobile phase, hence enabling the simultaneous analysis of multiple standards in herbal formulations, providing a distinct advantage.

In the concentration range of 2-10.0 µg/band, both standards calibration plots were linear (Figure 8) for Berberine (R^2=0.9966) and for Trigonelline (R^2=0.9973). The Limit of Detection (LOD)

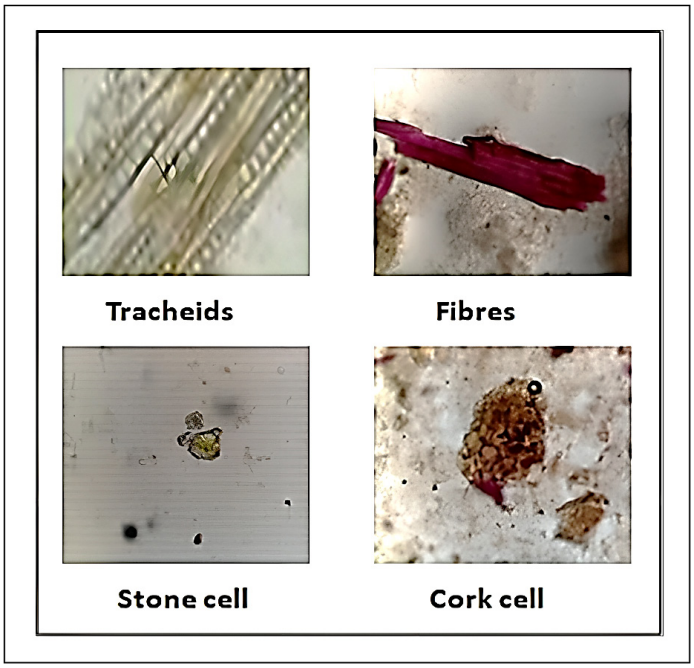

Figure 2: Microscopical characters of *Berberis aristata.*

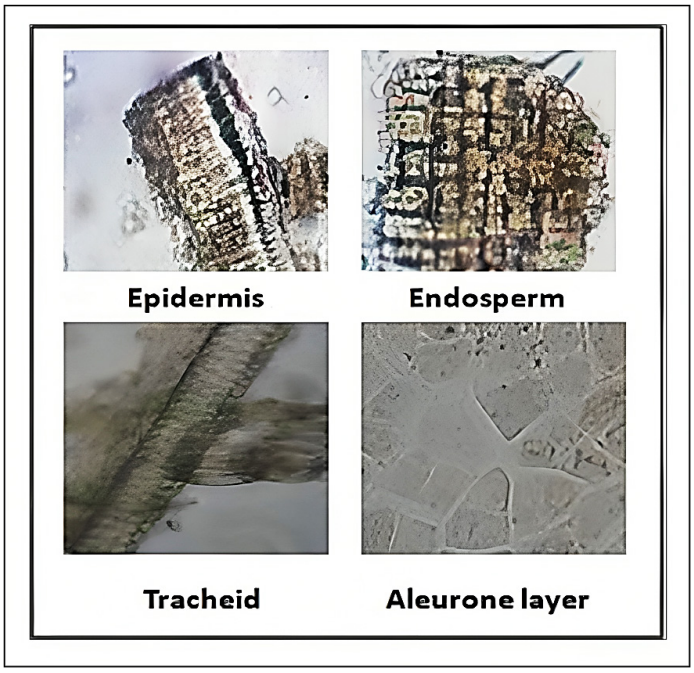

Figure 3: Microscopical character of *Trigonella foenumgrecum.*

and Limit of Quantification (LOQ) were determined using formulas based on the standard deviation of the response and the slope of the calibration curve. For Berberine and Trigonelline, the LOD values 0.48 and 1.47 and the LOQ was found to be 0.53 and 1.61 Berberine and Trigonelline respectively.

After spiking all the samples with known amounts of standard, the percent ratio between the recovered and expected concentrations was determined. Favourable recoveries were achieved through sample enrichment at three distinct concentration levels. The percent recoveries obtained following sample processing and application were notably encouraging, falling within the range of 101.145±0.34% for Berberine and 101.1±0.26% for Trigonelline.

The method's precision was assessed through both intra-day and inter-day measurements by analyzing three sets of samples with varying concentrations and the %RSD was calculated for each set. The results are listed in Table 1. The intra-day and inter-day precision of Berberine were 1.92 and 0.94, respectively and for Trigonelline 1.26 and 1.29 respectively.

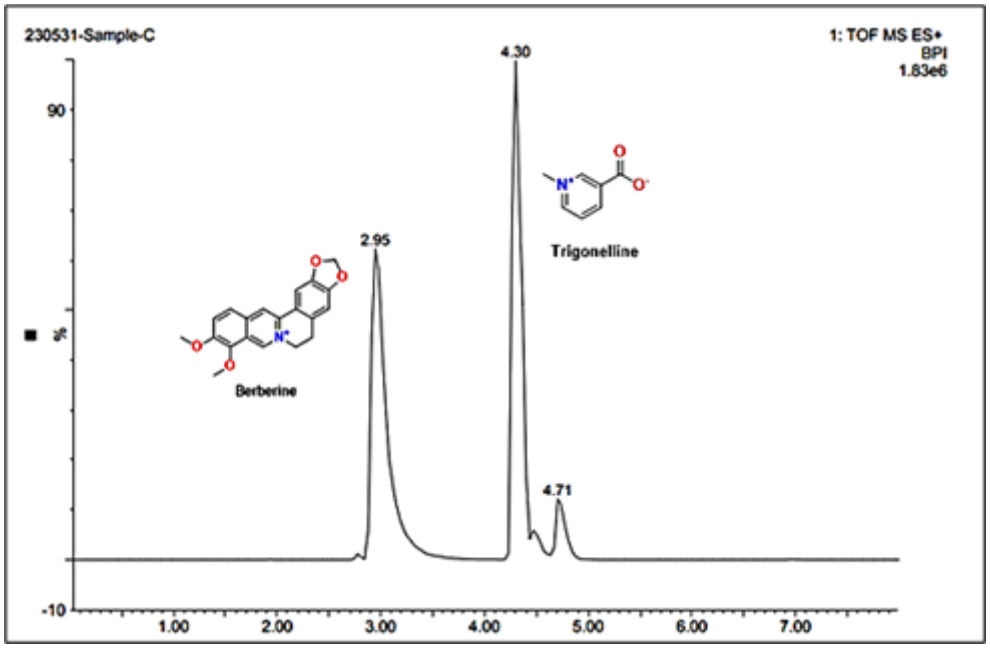

Figure 4: Total ion chromatogram obtained for standard Berberine and Trigonelline.

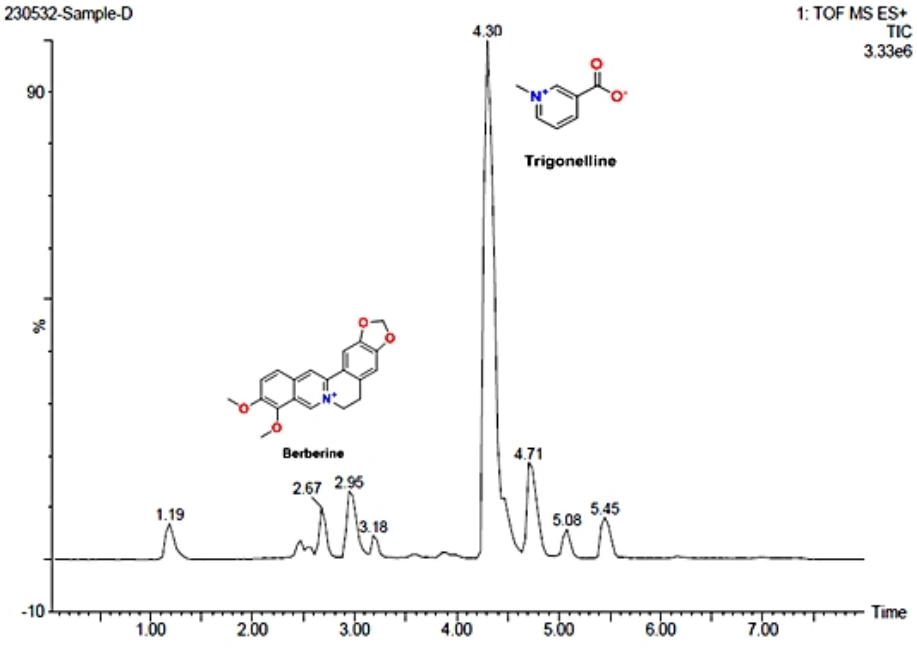

Figure 5: Total ion chromatogram for Berberine and Trigonelline in polyherbal mixture.

Table 1: Inter day and intraday precision for Berberine and Trigonelline.

Parameters	Concentration (µg/mL)		%RSD	
	Trigonelline	Berberine	Trigonelline	Berberine
Inter day precision	2.0	2.0	0.565	2.03
	6.0	6.0	1.64	1.99
	10	10	1.6	1.75
Mean			1.26	1.92
Intraday precision	2.0	2.0	0.824	0.46
	6.0	6.0	1.3	1.08
	10	10	1.77	1.28
Mean			1.29	0.94

Table 2: System suitability for Berberine and Trigonelline.

Conc. µg/mL	Berberine				Trigonelline			
	R_f	Area	%RSD		R_f	Area	%RSD	
			R_f	Area			R_f	Area
6	0.882	0.03189			0.293	0.02584		
6	0.874	0.03167			0.295	0.02562		
6	0.881	0.03179	0.464	0.445	0.293	0.02579	0.481	1.02
6	0.885	0.03204			0.296	0.02527		
6	0.882	0.03194			0.293	0.02594		
Mean	0.8808	0.03186			0.294	0.02569		

Table 3: Robustness analysisfor Berberine and Trigonelline.

Factors	Changes incorporated in chromatography		
	Level	R_f value	
Mobile phase composition Toluene: Ethyl acetate: Formic acid: Methanol		Berberine	Trigonelline
6.1:3.1:0.3:1.1	+0.1	0.895	0.298
6:3:0.3:1	0	0.881	0.293
5.9:2.9:0.3:0.9	-0.1	0.879	0.289
%RSD and mean		0.985 and 0.885	1.54 and 0.293
Mobile phase volume			
21	+1	0.889	0.318
20	0	0.882	0.294
19	-1	0.873	0.287
%RSD and mean		0.91 and 0.881	2.05 and 0.293
Duration of chamber			
15	+5%	0.875	0.289
20	0	0.881	0.293
25	-5%	0.896	0.297
%RSD and mean		1.22 and 0.8834	1.37 and 0.293

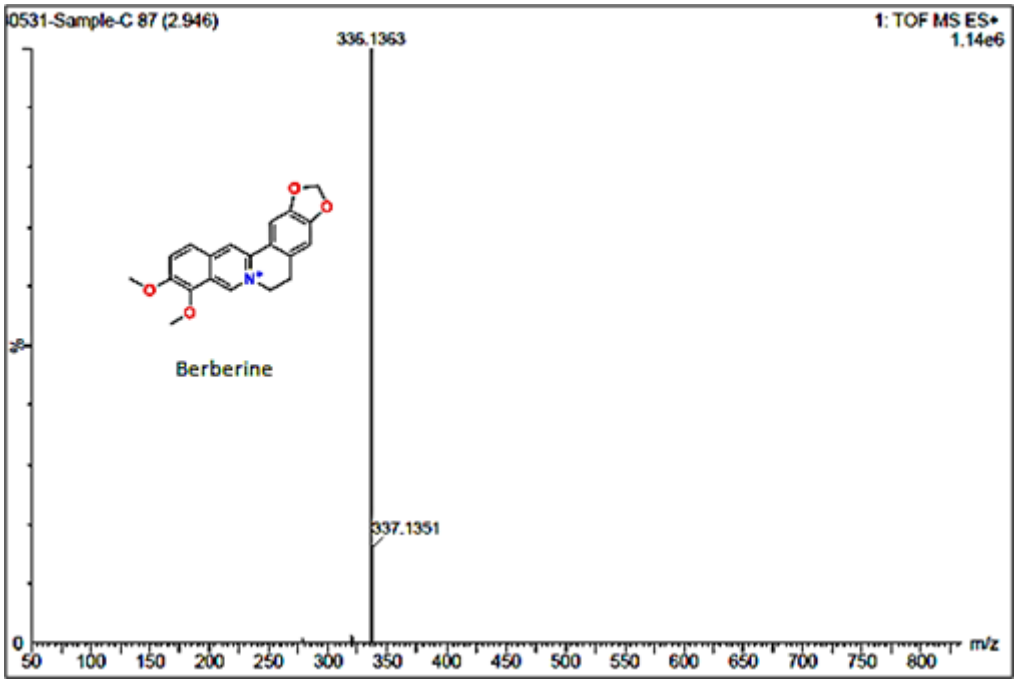

Figure 6a: MS spectra for Berberine.

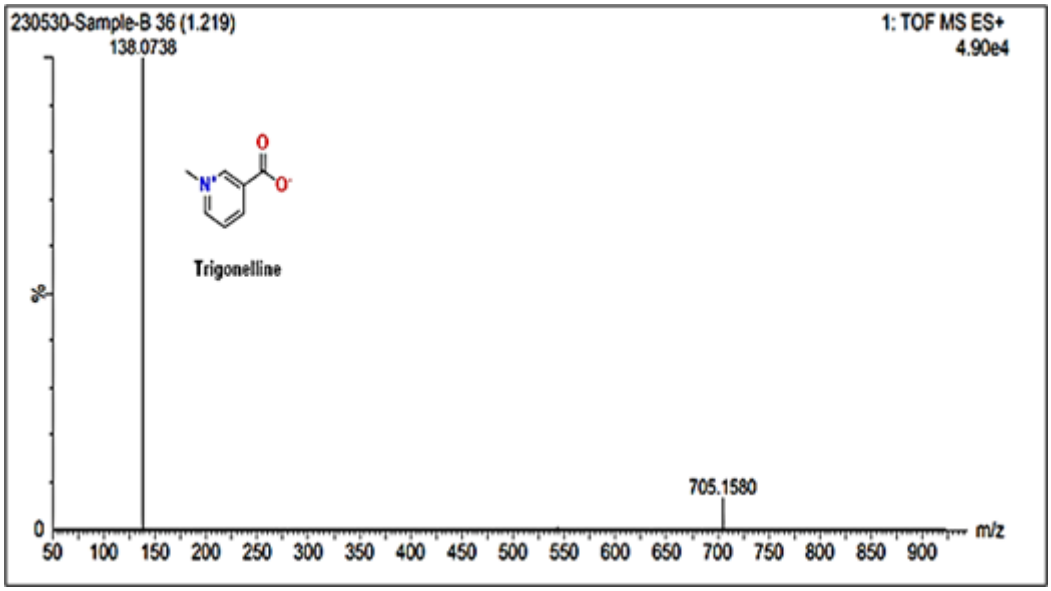

Figure 6b: MS spectra for Trigonelline.

To assess system suitability 5 µL of 6µg/mL working standard of Berberine and Trigonelline solution were applied under optimized chromatographic conditions. The values of parameters were presented in Table 2.

The analytical method's specificity refers to its capability to accurately target the analyte of interest while minimizing interference from other expected components. The developed HPTLC method demonstrated specificity, with distinct and separate peaks observed (Figures 9a-9d). Variation in mobile phase composition resulted in a %RSD of 0.985 and 1.54 changes in mobile phase volume showed a %RSD of 0.91 and 2.05 and changes in the duration of chamber saturation exhibited a %RSD of 1.22 and 1.37 for Berberine and Trigonelline respectively. These values fall within the acceptable range of ≤2%, confirming that the developed method was robust. The data of results of developed method has been presented in Table 3.

Quantification of Trigonelline content in *Trigonella foenum graecum* (Seeds) extract

186.9 ng/mL of Trigonelline was found to be present in hydro-alcoholic extract of *Trigonella foenum graecum*.

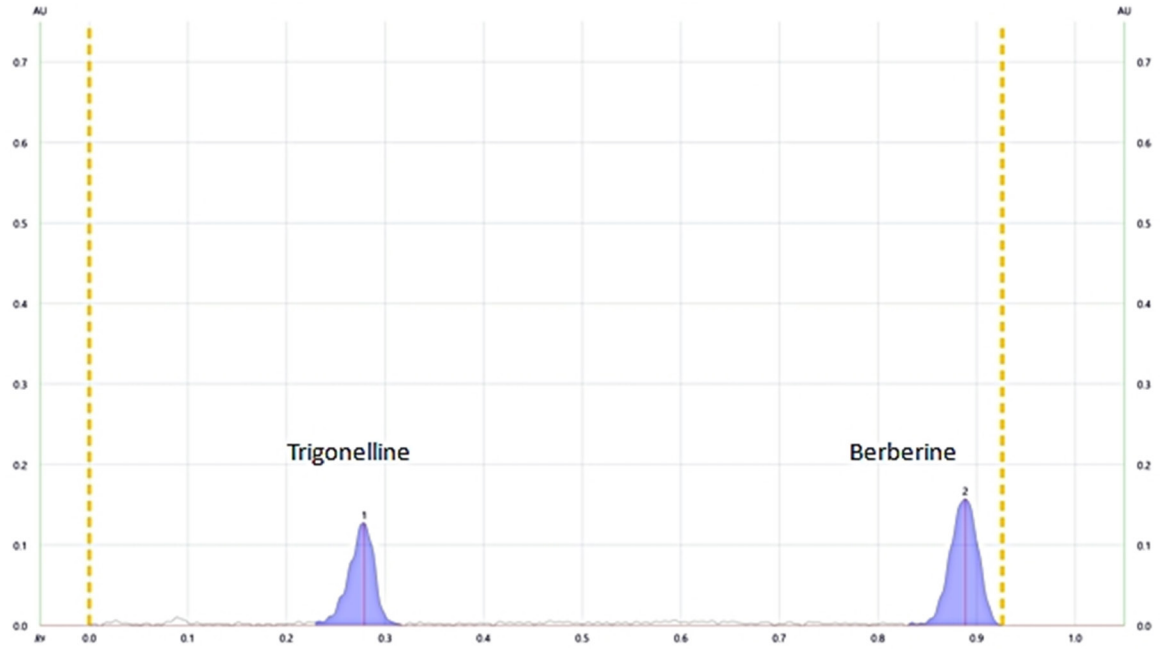

Figure 7: Simultaneous chromatograms of Trigonelline (0.29) and Berberine (0.88).

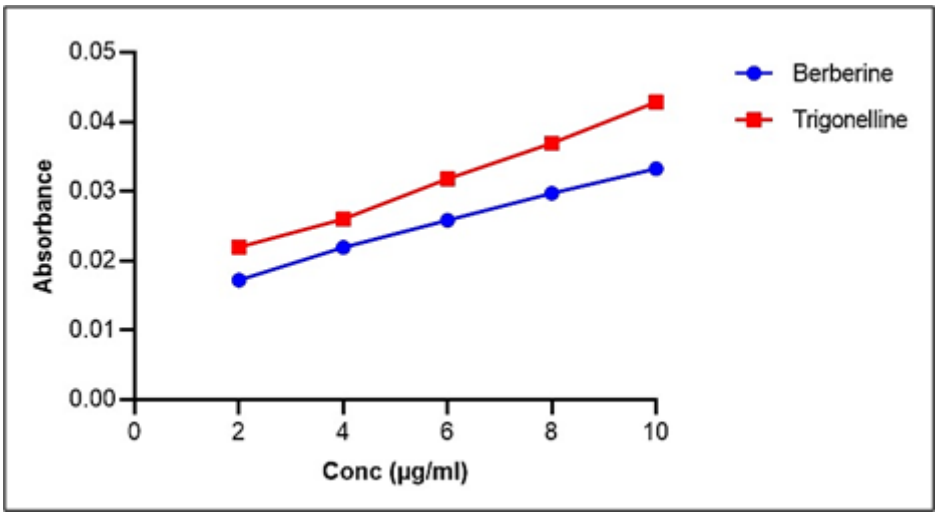

Figure 8: Calibration curve of Berberine and Trigonelline.

Quantification of Berberine content in *Berberis aristata* (Bark) extract

205.1 ng/mL of Trigonelline was found to be present in hydro-alcoholic extract of *Berberis aristata.*

Quantification of Trigonelline and Berberine content in polyherbal mixture

The quantification analysis revealed substantial levels of both Berberine and Trigonelline in the polyherbal mixture, suggesting the presence of these bioactive compounds. The analysis demonstrated the presence of Trigonelline at 178.3 ng/mL and Berberine at 217.6 ng/mL Figure 10.

DISCUSSION

The microscopical evaluation provided insights into the structural characteristics of *Berberis aristata* bark and *Trigonella foenum-graecum* seeds. Phytochemical analysis revealed that both plants contain a range of secondary metabolites such as alkaloids, flavonoids, tannins, saponins, terpenoids, glycosides and phenolic compounds. The physicochemical parameters were found to be within pharmacopeial standards. This indicates that the quality and purity of the plant materials are acceptable and consistent with established guideline. LCMS analysis provided valuable data on the presence of Berberine and Trigonelline. The total ion chromatograms showed clean and distinct peaks

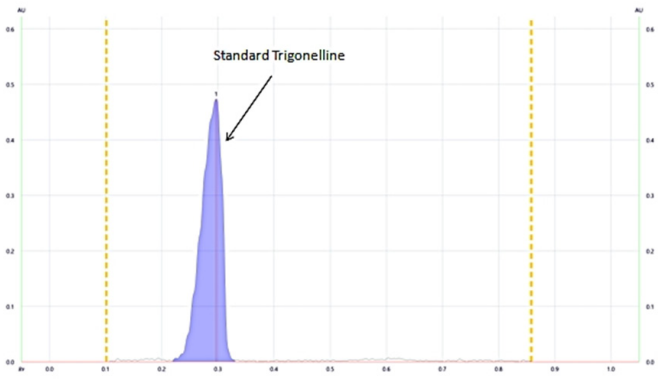

Figure 9a: Standard Trigonelline Spectra.

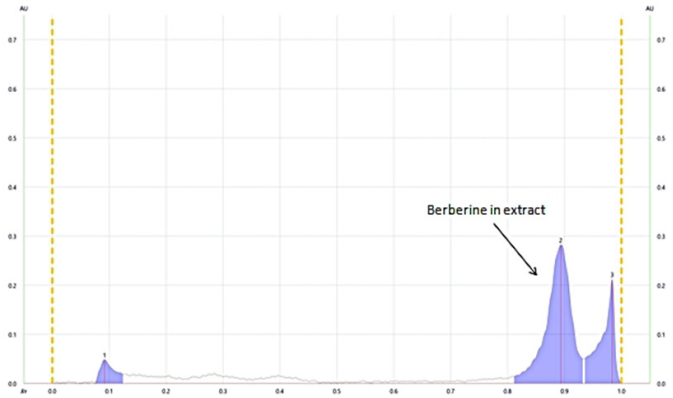

Figure 9c: Spectra of Berberine in BA extract.

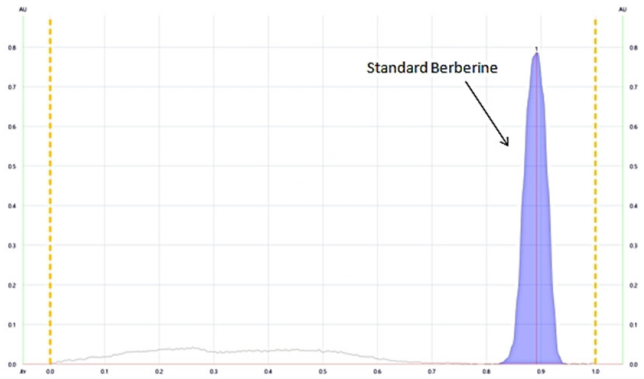

Figure 9b: Standard Berberine Spectra.

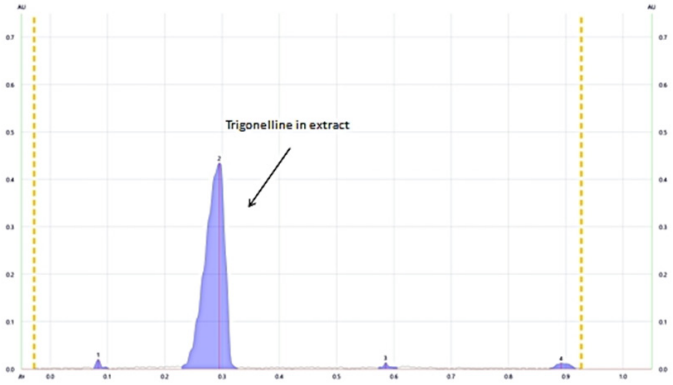

Figure 9d: Spectra of Trigonelline in TFG extract.

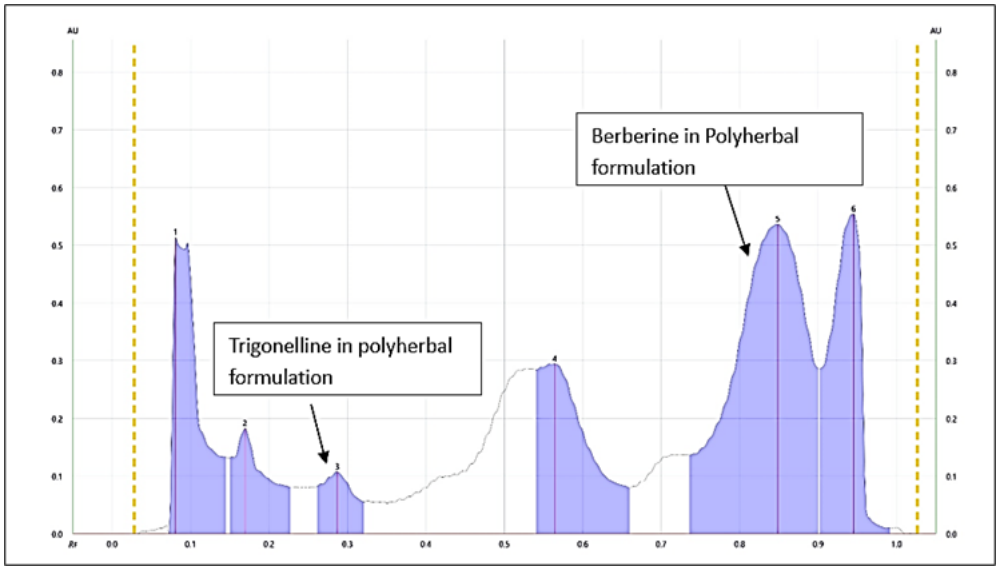

Figure 10: Spectrum of Berberine and Trigonelline in polyherbal mixture.

for both compounds, confirming their identities at 2.95 min and 4.30 min, respectively. The mass spectrometry data, with m/z values of 336.1363 g/mol for Berberine and 138.0738 g/mol for Trigonelline, further validated the presence and quantity of these compounds in both the standards and polyherbal mixture. The optimization of the HPTLC method, using a mobile phase of toluene: ethyl acetate: formic acid: methanol (6:3:0.3:1 v/v/v/v), demonstrated excellent resolution of Berberine and Trigonelline with R_f values of 0.88 and 0.29, respectively. This method allows for simultaneous analysis of both compounds in a single run, enhancing efficiency and accuracy in herbal formulation analysis. The method's robustness, confirmed by the low %RSD values, indicates its reliability for routine quality control. Its accuracy, precision, system suitability and specificity were confirmed with acceptable data. The quantification results indicated substantial levels of Berberine and Trigonelline the polyherbal mixture.

CONCLUSION

In recent years, there has been an increase in the demand for Ayurvedic formulations; however, the main barrier to accepting and using herbal ingredients and Ayurvedic formulations is poor quality. As a result, producing high-quality products for those in need is critical. Product quality can be achieved through proper standardization using various standard tools such as HPTLC and HPLC which are effective techniques for analyzing, identifying and assessing the quality of herbal products due to their versatility, accuracy, reliability and high throughput. And compare cost effectiveness. Therefore, this study aimed towards development and validation of HPTLC method for analysis of Trigonelline and Berberine simultaneously in enriched polyherbal extract derived from two antidiabetic Ayurvedic herbs. The method's validation demonstrates its suitability for routine quality control, with satisfactory results obtained for linearity, precision, accuracy, specificity and robustness. The significant concentrations of Trigonelline and Berberine detected in the extract underscore its potential as an effective antidiabetic formulation. This study contributes to the standardization and quality assurance of Ayurvedic formulations, facilitating their integration into modern healthcare practices.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HPTLC: High-Performance Thin-Layer Chromatography; **µg:** Microgram; **mL:** Milliliter; **µL:** Microliter; **nm:** Nanometer; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **RSD:** Relative Standard Deviation; **SD:** Standard Deviation; **ICH:** International Conference of Harmonization, **AFI:** Ayurvedic Formulary of India.

AUTHOR CONTRIBUTION

The development and validation of the HPTLC technique for Berberine and Trigonelline have been collaboratively achieved by all authors. The development and validation of the HPTLC technique was aided by Sneha B. Patil and Dr. Sunil S. Jalalpure provided guidance for the current research project, including advice on reviving the literature and producing the paperwork. Dr. Priya Shetti guided for the writing of manuscript and framing of the research paper.

SUMMARY

The Pharmacognostic evaluation of *Berberis aristata* and *Trigonella foenum-graecum* demonstrates the efficacy of the developed analytical methods in accurately characterizing and quantifying key bioactive compounds. The methods employed-microscopical, phytochemical, physicochemical, LCMS and HPTLC-provide comprehensive tools for quality control, ensuring the consistency, safety and efficacy of herbal products. The results underscore the importance of rigorous analytical standards in the development and assessment of herbal formulations.

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