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Original Article

Simultaneous Determination of Gallic Acid, Berberine and Trigonelline in Polyherbal Churna by HPTLC Method

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ABSTRACT

Background: Ayurvedic medicines have a lot of potential because of their comprehensive approach to illness care, yet there is a need for sufficient standardization before they can be used in mainstream medicine. Nevertheless, there is a lack of work efforts directed towards the concurrent assessment of biomarkers in polyherbal churna using HPTLC fingerprinting. The objective of this study is to explore bioactive principles such as Gallic acid, Berberine, and Trigonelline in polyherbal churna using HPTLC fingerprinting. **Materials and Methods:** The in-house polyherbal churna is a blend of Amla, Methi, and Daruharidra. The formulation underwent a standardization process based on multiple quality control parameters. Using HPTLC technique, pharmacologically active biomarkers including gallic acid, trigonelline, and berberine were successfully identified. The mobile phase used for the HPTLC analysis was a mixture of Toluene: Ethylacetate: Glacial acetic acid: Water in the ratio of 2:3:4:1 v/v/v/v. **Results:** The method developed exhibited linearity, specificity, reproducibility, robustness, and accuracy, ultimately redefining the characteristics of the approach and cost-effective. The examination confirmed the presence of three biomarkers in the polyherbal churna, with corresponding R_f values of 0.66 for gallic acid, 0.12 for trigonelline, and 0.61 for berberine, respectively. **Conclusion:** The proposed HPTLC approach, which was developed, utilizes a more recent technique for simultaneously fingerprinting biomarkers from polyherbal formulations containing Amla, Methi, and Daruharidra. The method can be used for standardization of different herbal formulation containing these plant species.

Keywords: HPTLC, Gallic acid, Berberine, Trigonelline, Polyherbal churna.

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INTRODUCTION

Herbs and herbal medicines have long been utilized for the treatment of various ailments since ancient times. The utilization of herbal medicines, herbal materials, herbal preparations, and finished herbal products has experienced a notable surge in significance across global healthcare systems. As per the World Health Organization, around 80% of the global population depends on herbs and other traditional therapies, citing their safety, effectiveness, cultural acceptance, and minimal side effects. The therapeutic effectiveness of plant-based drugs relies on the authenticity and purity of the plant material.[1,2]

The concept of polyherbalism, which involves the formulation of drug combinations containing two or more drugs to achieve enhanced pharmacological effects, has been emphasized in the Sharangdhar Samhita, an ayurvedic text from 1300 AD. Polyherbal formulations have been found to possess superior and prolonged therapeutic potential compared to single herbs, as they enhance therapeutic activity while reducing the concentrations of individual herbs.[3]

Ensuring the efficacy of herbal products is inherently linked to guaranteeing their safety and quality. Therefore, the need for phytochemical standardization and evaluation is of utmost importance. The World Health Organization (WHO) and other regulatory systems are concerned with the effectiveness and safety of herbal medicines. Consequently, there is a constant demand for simple, reproducible, and cost-effective methods.[4] Various analytical approaches, such as TLC, HPTLC, HPLC, LC/MS/MS, GC/MS, and GC/MS/MS, have been employed for the determination and standardization of active phytoconstituents. Unlike HPLC and GC, HPTLC allows for the simultaneous identification and quantification of numerous constituents in various samples using a single plate. As a result, HPTLC has emerged as a rapid, straightforward, and reliable analytical technique in numerous phytochemical investigations and standardization processes.[5,6]

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This study aimed to formulate and assess the quality of polyherbal churna, incorporating a remarkable combination of herbs traditionally utilized for treating various diseases over several decades. The formulated churna is the combination of Amla (*Phyllanthus emblica*),[7] Methi (*Tigonellafoenum-graecum*)[8] and Daruharidra (*Berberis aristata*).[9]

In this study, we endeavored to conduct routine quality control of herbal formulations by fingerprinting major biomarkers. Therefore, our focus was on the simultaneous estimation of Gallic acid, Trigonelline, and Berberine (Figure 1) using HPTLC fingerprinting, accompanied by method validation in accordance with ICH guidelines.

MATERIALS AND METHODS

Pharmacognostical evaluation of raw plant materials

All the raw plant materials were assessed for quality parameters such as organoleptic parameters, physico and phytochemical evaluation as per standard procedures.[10,11]

Formulation and Evaluation of Polyherbal Churna

Authenticated raw plant materials were dried, finely powdered and assessed for quality control parameters such as morphological and physicochemical evaluation then passed through sieve no.18 and stored in airtight container separately. In-house polyherbal churna was prepared as per the composition consisting of Amla, Methi and Daruharidra in the ratio of (2:2:1). The mixture was passed through sieve no. 44 and stored in an airtight container. Formulation was subjected for various Pharmacognostical evaluation such as organoleptic parameters, physicochemical and Physical evaluation as per standard procedures.[12,13]

Chromatographic standardization of formulated churna

Formulated polyherbal churna was subjected for quality assessment by HPTLC fingerprinting. Initially, a simultaneous method was devised to analyze all the three biomarkers. Subsequently, this method was subjected to validation according to the ICH guidelines, as detailed below.

Instrumentation

The study utilized various tools and equipment, including the Linomat 5 sample applicator (Camag, Switzerland), a Twin trough chamber measuring 20×10 cm (Camag, Switzerland), a UV chamber (Camag, Switzerland), a Hamilton microliter syringe (Linomat syringe, Hamilton-Bonaduz Schweiz, Camag, Switzerland), pre-coated silica gel aluminum plates (60 F254) sized 20×10 cm with a thickness of 1 mm (E. Merck, Darmstadt, Germany), and the TLC scanner 4 (Camag, Switzerland). The entire process was managed and analyzed using the vision CATS version 3 software developed by Camag, Switzerland.

Preparation of standard solution

10 mg of Gallic acid, Trigonelline, and Berberine reference standards were individually weighed and placed into separate 10 mL volumetric flasks. Each standard was dissolved in methanol to attain a concentration of 1000 µg/mL for every reference standard.

Preparation of Sample Solution

10 g of in house churna was refluxed in 200 mL of 70% methanol. The solution was filtered and filtrate was evaporated under reduced pressure in rotary evaporator to get crude extract further sample solution was prepared similarly as of standard solution to get 1000 µg/mL in methanol.[15]

HPTLC Method development

Different concentrations of standard and sample solutions were prepared and applied on pre-coated HPTLC plates in the form of thin bands. Samples were sprayed with nitrogen gas drying steam continuously at a rate of 100 nL/sec. The mobile phase, consisting of Toluene: Ethyl acetate: Glacial acetic acid: Water in a ratio of 2:3:4:1 (v/v/v/v) was saturated for 20 min. Subsequently, the plate was allowed to develop for approximately 70 mm. Later the plate was dried using an air dryer. The TLC scanner 4 with win CATS Software has been used to analyze the plates.

HPTLC Method Validation

Following the guidelines set forth in ICH Q2 (R1), the validation of the HPTLC method developed in this study was evaluated through a comprehensive examination of parameters such as linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), intra-day precision, inter-day precision, accuracy, and robustness.

Linearity and range

The degree of linearity was ascertained by subjecting samples of concentrations (0.2 to 1.0 µg/mL). The specific wavelength for parameter application was selected.

Determination of the Limit of Detection (LOD) and Limit of Quantification (LOQ)

The determination of the Limit of Detection (LOD) and Limit of Quantification (LOQ) involved utilizing the slope and standard deviation in this study. The formula utilized is as follows, wherein σ represents the standard deviation, and S signifies the slope of the calibration curve.

$$LOD = 3.3 * \sigma / S$$

$$LOQ = 10 * \sigma / S$$

Specificity

Specificity of the method was confirmed by comparing the samples bands with those of the corresponding reference standards in terms of R_f and colour in fluorescence mode.

Accuracy

Accuracy was assessed using three different concentrations. To obtain additional concentrations of 50, 100 and 150 µg/mL, sample solutions were prepared. The areas obtained from analyzing these solutions were compared to those obtained from separately analyzing the formulation and standard at the same levels (50, 100, and 150%).

Precision

The methodology employed entailed a thorough evaluation of method variability via intraday and interday precision assessments. In the intraday precision, each concentration underwent three applications within a 24-hr timeframe. Conversely, for interday precision, each concentration underwent three applications with three-day intervals, and the results were expressed as %RSD.

Robustness

The robustness of an analytical technique gauges its resilience to minor, deliberately introduced changes in method parameters, offering insights into its reliability under standard conditions**.** A method's robustness is assessed by changing the mobile phase and analysing the impact (if any) on the method's results and the alterations obtained must be in the criteria of the %RSD that is <2%.

RESULTS

Pharmacognostical evaluation of raw plant materials

Raw plant ingredients were evaluated for organoleptic parameters such as color, odor, taste, and texture (Table 1). Furthermore, the plant materials were meticulously analyzed for physico-phytochemical attributes encompassing extractive values, ash content, loss on drying, and chemical composition. It was observed that each individual plant component exhibited total ash values ranging between 5 to 10% w/w, water soluble ash values ranging between 3 to 5% w/w, and acid insoluble ash values up to 1% w/w. detailed physicochemical parameters are shown in Tables 2. Based on the findings of the phytochemical screening, the unprocessed plant materials were found to encompass a range of phytoconstituents, including alkaloids, glycosides, carbohydrates, flavonoids, tannins, and saponins (Table 3) represents the presence of various phytoconstituents. These results signify a noteworthy level of purity, indicating minimal contamination in the plant material

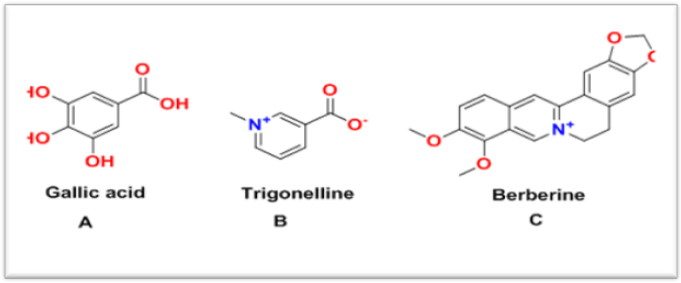

Figure 1: Chemical structure of Biomarkers.

Table 1: Organoleptic profile of plant ingredients.

Compound	Organoleptic parameters			
	Colour	Odour	Taste	Texture
Amla	Brown	Aromatic	Sour	Rough
Methi	Light brown	Odourless	Bitter	Rough
Daruharidra	Yellow	Odourless	Bitter	Irregular

Table 2: Physiochemical parameters.

Compound	Physiochemical parameters						
	Ash value (% w/w)			Extractive value (% w/w)			Moisture content (% w/w)
	Total ash	Acid insoluble ash	Water soluble ash	Alcohol	Water	Ether	
Amla	10%	1%	5%	40%	58%	33%	5%
Methi	9.8%	0.3%	3.5%	40%	24%	33%	5%
Daruharidra	5%	1%	3%	20%	14%	33%	6.5%

Table 3: Phytochemical screening evaluation.

Test	Amla	Methi	Daruharidra
Alkaloids	+	+	+
Glycosides	+	+	+
Carbohydrates	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Saponins	-	+	-
Proteins/amino acids	+	+	-

Table 4: Formulation of Churna.

Drug	Weight before sieving (gm)	Weight obtained [after sieving] (gm)	Weight taken for churna (gm)
Amla	100	50	20
Methi	100	70	20
Daruharidra	50	30	10

Table 5: Evaluation parameters of formulated Churna.

Organoleptic evaluation			
Colour	**Odour**	**Taste**	**Texture**
Light brown	Characteristic	Bitter	Smooth fine powder
Physical Evaluation			
Bulk density		0.45±0.02	
Tap density		0.55±0.01	
Angle of repose		40.2±0.03	
Hausner's ratio		1.22±0.017	
Carr's index		18.1±1.082	

Formulation of Churna

Raw plant materials were dried, weighed separately and subjected for size reduction, further sieved to get fine powder. 50 g of churna was prepared by weighing 2 parts of amla, 2 parts of methi and 1 part of Daruharidra (Table 4 and Figure 2) represents the prepared churna.

Evaluation of churna

The formulated churna underwent a comprehensive assessment encompassing diverse parameters. Organoleptic attributes such as color, odor, taste, and texture were evaluated, while physical characteristics including bulk density, tap density, angle of repose, Hausner's ratio, particle size determination, and Carr's index were also examined. The findings, as presented in Table 5. Demonstrate that all observed parameters conform to established standards. Consequently, the formulated churna successfully fulfils all quality assessment criteria.

Standardization of formulated churna by HPTLC fingerprinting

Initially, a simultaneous method was developed utilizing biomarkers such as gallic acid, trigonelline, and berberine. This method employed a mobile phase composed of Toluene, Ethyl acetate, Glacial acetic acid, and Water (in a ratio of 2:3:4:1 v/v/v/v). The analysis exhibited favorable outcomes, demonstrating distinct and well-separated peaks with corresponding R_f values of 0.66, 0.12, and 0.61 for gallic acid, trigonelline, and berberine, respectively. The effectiveness of this approach was subsequently demonstrated through its successful application to a prepared churna formulation. The outcomes of this application revealed a consistent alignment between the R_f values of the biomarkers and the churna formulation.

Method validation

Linearity

Linearity of biomarkers (GA, TRG and BA) was validated by applying bandson the prewashed TLC plates (n=5) from the stock solutions of 1 mg/mL (200-1000 ng/band). Calibration curves for all standards were generated by plotting graphs illustrating the mean peak areas of each standard against their corresponding concentrations. The results, presented in Table 6, demonstrate a strong correlation between the mean peak area and the concentration of standards within the indicated concentration range.

Sensitivity

The sensitivity of the proposed method was evaluated through the determination of Limit of Detection (LOD) and Limit of Quantification (LOQ). Specifically, the LOD values for Gallic acid, Trigonelline, and Berberine were 6.81, 8.32, and 8.14 ng/band, respectively. Correspondingly, the LOQ values were 19.6, 25.21, and 24.61 ng/band.

Specificity

Upon assessing the specificity parameter, it was noted that the standard and sample exhibited similar R_f values and displayed overlapping conditions. Table 7 represents the R_f values and

Table 6: Results of Linearity.

Parameters	Gallic acid	Trigonelline	Berberine
Wavelength	309 nm	310 nm	310nm
Regression equation	Y=0.0432x+0.0011	Y=0.0177x+0.0066	Y=0.0091x+0.0092
Slope	0.0432	0.0177	0.0091
y-intercept	0.0011	0.0066	0.0092
Correlation coefficient(R^2)	0.9959	0.996	0.9956
R_f	0.66	0.12	0.61

Table 7: Specificity parameters.

Track ID	R_f value	Compound name
3	0.668	Gallic acid
14	0.668	Churna Sample
7	0.12	Trigonelline
14	0.12	Churna Sample
11	0.611	Berberine
14	0.611	Churna sample

Table 8: Percentage recovery of standard markers.

% Level	AUC for formulation and standard applied on different bands (ng/band)	AUC for formulation and standard applied on same band (ng/band)	% Recovery
Gallic acid			
50	10.1	10.19	99.11
100	12.5	12.41	100.80
150	24.12	24.35	99.05
Trigonelline			
50	11.92	12.03	99.08
100	13.32	13.06	101.99
150	16.23	16.6	101.31
Berberine			
50	11.6	11.3	102.65
100	12.28	12.29	99.91
150	14.46	14.32	100.97

chromatograms for the standard and samples shown in Figures 3a and 3b, 4a and 4b, 5a and 5b and 6.

Accuracy

The accuracy parameter was evaluated at different levels (50%, 100%, and 150%). Both the individual recovery percentages and the average recovery percentage surpassed 98%. This outcome underscores the method's precision and the extent of recovery achieved and results are shown in Table 8.

Precision

Precision in the method was illustrated through both intra-day and inter-day variation studies. In intra-day precision, standard solutions containing gallic acid, trigonelline, and berberine at a concentration level of 0.6 µg/band were analyzed thrice on the same day, with five replicates for each. The repeatability results were then expressed as the Relative Standard Deviation (%RSD). Meanwhile, inter-day precision involved replicating the procedure on three distinct days, and the corresponding results were tabulated in Table 9.

Robustness

After minor adjustments to sample preparation, solvent composition, band/band alignments, separation, assessment, and solvent front, no appreciable changes in R_f value were discovered. Thus, the developed method was found to be robust, and results are depicted in Table 10.

DISCUSSION

The research endeavors revolve around the development and assessment of a polyherbal churna comprising Amla, Methi, and Daruharidra, which demonstrates considerable potential in offering comprehensive health benefits for overall well-being. The combination of these herbs may enhance their individual therapeutic effects, making the churna a valuable contribution to traditional medicine systems. The churna was subjected to various quality control parameters to ensure its quality. Additionally, a new validated HPTLC method was employed to identify pharmacologically active biomarkers, namely gallic acid, trigonelline, and berberine. The use of authentic ingredients in the preparation of polyherbal churna ensures the quality and effectiveness of the product. Amla, Methi, and Daruharidra are well-known for their medicinal properties, and their inclusion in the churna enhances its therapeutic benefits. Standardization of the churna through various quality control parameters ensures consistency in its composition and potency. This is particularly crucial in herbal medicines, as the efficacy of the product largely depends on the specific components present in it. By adhering

Figure 2: Churna.

Table 9: Results of Intra-day and Inter-dayprecision studies.

Precision (*n*=5)	Gallic acid	Trigonelline	Berberine
Inter-day precision (%RSD)	1.74±0.093	0.83±0.047	0.97±0.042
Intra-day precision (%RSD)	1.73±0.061	0.77±0.019	1.53±0.084

The values are mean±SEM.

Table 10: Robustness data.

Change in chromatographic conditions	Gallic acid	Trigonelline	Berberine
Mobile phase volume (%RSD)	0.476±0.047	0.87±0.041	0.77±0.041
Mobile phase composition (%RSD)	0.763±0.087	0.63±0.012	0.93±0.012
Duration of saturation (%RSD)	0.877±0.014	0.89±0.14	0.95±0.14

The values are mean±SEM.

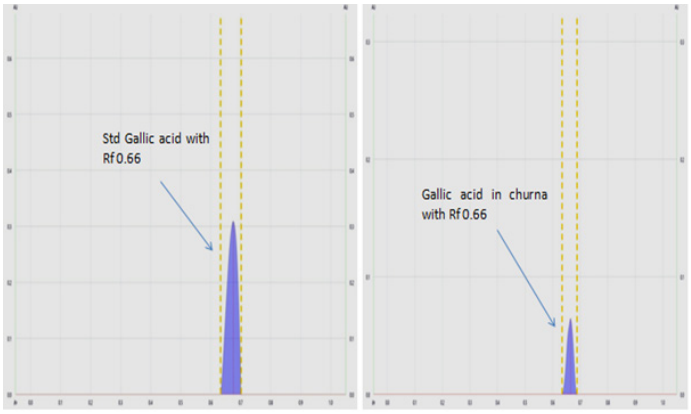

Figure 3: a) Standard Gallic acid, b) Gallic acid in churna.

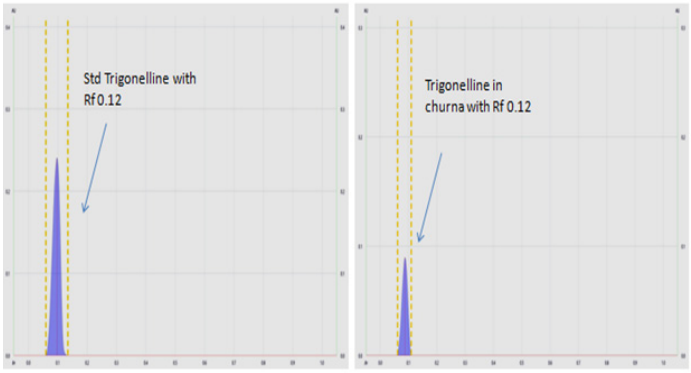

Figure 4: a) Standard Trigonelline, b) Trigonelline in churna.

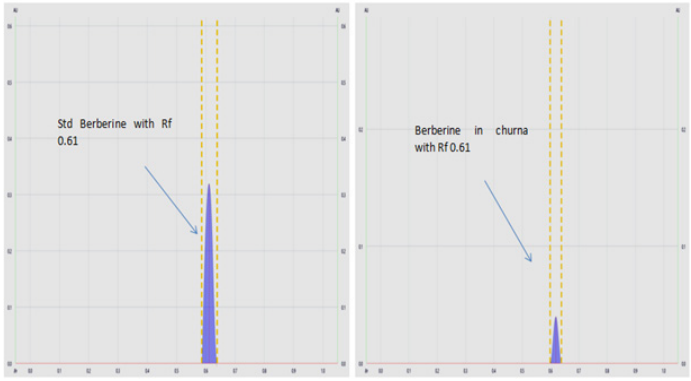

Figure 5: a) Standard Berberine, b) Berberine in churna.

to stringent quality control measures, the churna can provide consistent and reliable results to the users.

The choice of the mobile phase is crucial for the separation of the target compounds. In this study, a modified mobile phase composed of a Toluene: Ethyl acetate: Glacial acetic acid: Water [2:3:4:1 v/v] mixture was optimized to obtain a sharp and well-resolved separation of Gallic acid, Berberine, and Trigonelline. The ratio of these solvents was optimized to achieve the best separation with good resolution, adequate peak shape, and reasonable run time. The HPTLC analysis revealed the presence of the biomarkers in the polyherbal churna, with gallic

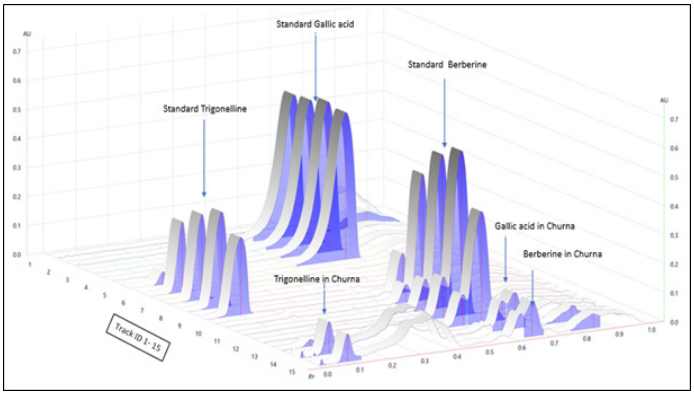

Figure 6: HPTLC chromatograms at 254 nm, 366 nm for standard compounds and churna sample (Track 1-15).

acid exhibiting an R_f value of 0.66, trigonelline with an R_f value of 0.12, and berberine with an R_f value of 0.61 respectively.

The validation of the developed method encompassed diverse parameters, including linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), precision, robustness, and accuracy. The calibration curves for each compound displayed excellent linearity within the tested concentration range, boasting correlation coefficients (R2) exceeding 0.99. Precision assessment involved calculating the percentage Relative Standard Deviation (%RSD) from replicate injections, revealing results indicative of acceptable precision. The outcomes affirmed the effectiveness of the proposed HPTLC method for simultaneously determining Gallic acid, Berberine, and Trigonelline in polyherbal churna. Overall, the meticulous preparation, standardization, and analysis of polyherbal churna using genuine ingredients and the HPTLC method underscore a commitment to quality and efficacy in herbal medicine. The inclusion of pharmacologically active biomarkers further strengthens the therapeutic potential of churna, making it a reliable and effective option for individuals seeking natural remedies.

CONCLUSION

A polyherbal churna formulation was prepared in this study, and the product was standardized using several pharmacognostical and HPTLC methods. Based on the findings, it is reasonable to assume that the proposed HPTLC method is a more recent technique for simultaneous fingerprinting of markers such as gallic acid, trigonelline, and berberine in polyherbal formulations containing amla, methi, and daruharidra because the results are visible and the method is less expensive when compared to HPLC. The analytical method is suitable for general use based on the validation parameters. The established HPTLC method is suitable for the routine quality control analysis of all marker compounds in commercial formulations. These results play a crucial role in shaping a biological approach for future research endeavors.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HPTLC: High Performance Thin Layer Chromatography; **TLC:** Thin Layer Chromatography; **ICH:** International Conference on Harmonisation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **R_f:** Retention factor; **RSD:** Percent Relative Standard Deviation; **AUC:** Area Under Curve; **GA:** Gallic acid; **TRG:** Trigonelline; **BA:** Berberine.

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