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Development and validation of a HPTLC method for simultaneous estimation of quercetin, Chlorogenic acid and trigonelline in polyherbal antibacterial formulation

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Abstract
The overall aim of the present study is development of simultaneous quantification of Quercetin QC, Chlorogenic Acid (CA) and Trigonelline (TN). The literature reports wide array of analytical techniques for the qualitative and quantitative determination of these biomarkers individually and also in combination with other compounds, no reports are available till date for the simultaneous quantification of QC, CA and TN. The UV absorption spectra of the three biomarkers at different wavelengths were recorded by multi-wavelength detector, the QC, CA and TN showed λ_{max} of 290 nm, 330 nm, 270 nm in absorbance mode, respectively. The simultaneous detection and co-quantification of all three compounds was successfully achieved for the first time. The developed and validated HPTLC method for simultaneous determination of QC, CA and TN is rapid, reliable and low cost.

Keywords: HPTLC, quercetin, chlorogenic acid, trigonelline

Introduction
Over the past decade, interest in herbal medicine has increased tremendously [1]. According to the World Health Organization (WHO), 60% - 80% population in developing countries depends essentially on plants for primary health care needs [2]. The revival of herbal medicines has amplified the international trade immensely and worldwide herbal therapeutics market is anticipated to reach about USD 117 billion by 2024 [3]. The variability in constituents of plant material, variety of extraction and processing techniques used by different manufacturers, results in distinct inconsistency in the quality of herbal products. Furthermore, various hypersensitivity reactions, interactions with herbal drugs and effects from adulterants have been reported, drawing the attention of regulatory agencies for the standardization of plant based drugs. Specific guidelines on plant based drugs and to study their prospective safety, efficacy and quality, as a prerequisite for global harmonization have been recently established by The World Health Organization (WHO) [4].
Design of analytical process which can dependably determine quantitative evaluation of bioactive compounds called as 'Markers' and other key components, is a challenging task for scientists [5]. However, technological advancements which take place in the processes of isolation, extraction, purification and structural determination of natural compounds have made it possible to generate apt strategies for the standardization of plant based medicines [6].
A wide array of analytical techniques such as high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS) atomic absorption spectroscopy (AAS) and capillary electrophoresis (CE) and molecular biomarkers in fingerprints are presently employed for quantification and standardization of the plant based medicines [7]. Among these techniques, HPTLC is most commonly employed fingerprint method for identification, evaluation and standardization of herbal and polyherbal formulations. It is rapid, simple, reliable and reproducible analytical technique and is useful for analyzing large number of samples in a short duration with minimum solvent and minimum sample clean up [8]. In addition, it allows direct application of suspensions or turbid samples. Nonetheless, it permits simultaneous analysis of multiple components in polyherbal formulations.
The overall aim of the present study is development of polyherbal antibacterial (PHA) formulation composed of blend of extracts of *Azadirachta indica* (AI), *Cichorium intybus* (CI)

and *Trigonella foenum-graecum* (TFG) for the treatment of vaginal infections. Literature suggest that quercetin (QC), chlorogenic acid (CA) and trigonelline (TN) are common phytoconstituents present in AI, CI and TFG, respectively (Fig 1). QC, CA and TN are reported to possess antimicrobial activity [9, 10, 11] and are used as herb-specific biomarkers. Thus, it was thought that quantification of QC, CA and TN could be helpful in evaluation and standardization of PHA formulation developed in the present study. Although, literature reports wide array of analytical techniques for the qualitative and quantitative determination of these biomarkers individually and also in combination with other compounds, no reports are available till date for the simultaneous quantification of QC, CA and TN [12, 13, 14]. Thus, the present work aimed at development of accurate, specific, repeatable and robust HPTLC method for the simultaneous determination of QC, CA and TN in herbal extracts and developed PHA formulation. The proposed method was validated in compliance with ICH guidelines for accuracy, precision, recovery, robustness, ruggedness, limit of detection (LOD) and limit of quantification (LOQ).

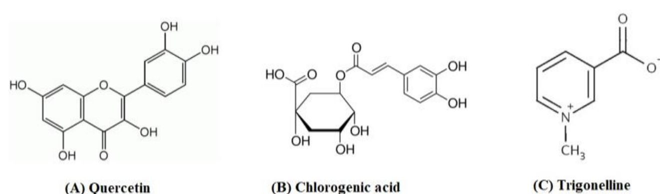

Fig 1: Structure of biomarkers

Materials and Methods

Materials

AI, CI and TFG were procured from Green Earth products International Exporters, New Delhi. All the solvents used in the study were of analytical grade and were purchased from Merck Ltd. (Mumbai, India).

Preparation of PHA Formulation

Thermosensitive bioadhesive PHA formulation was prepared by adding 1.2% chitosan lactate (equimolar) solution to 16% poloxamer solution with continuous stirring. The developed formulation was characterized for thermosensitive and bioadhesive behavior.

Instrumentation and chromatographic conditions

The samples were spotted with a Camag microlitre syringe on pre-coated silica gel HPTLC aluminium plates 60F-254 (20 cm × 20 cm) with 200 µm thickness using Camag Linomat V semi-automatic sample applicator. The HPTLC plates were washed with methanol and dried for 30 min at 90°C. Standard solutions were applied using the following settings: band length 6 mm, distance from left plate edge 10 mm and from the lower plate edge 15 mm, resulting in 21 tracks per plate with space between two bands 9 mm, application rate 150 nL s^{-1}. The slit dimension was kept at 5 mm x 0.45 mm and 20 mm s^{-1} scanning speed was employed. Linear ascending development was carried out in a twin trough glass chamber saturated with mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25°C ± 2°C) at relative humidity of 55% ± 5%. The length of chromatogram run was 80 mm. Based on the previously published data various combinations of solvents were tried to attain sharp and well-defined spots of individual components with resolution factor >1.

Densitometric scanning and UV detection

The detection of the compounds was done using UV spectrophotometry since all the three biomarkers were UV active. Densitometric scanning was performed on Camag TLC scanner III in multi-wavelength detection mode spanning the whole range of short-wave UV light (254 nm) and long-wave UV light (366 nm) of the developed air dried HPTLC plates. Relative heights and area of peaks in chromatograms were recorded within the range of 210-480 nm with scanning done in linear increments of 30 nm. The UV absorption spectra of the three biomarkers at different wavelengths were recorded and further screening was done in linear increments of 10 nm to select the maximum wavelength of absorption (λ_{max}) for individual biomarkers.

Preparation of standard solutions of biomarkers

Accurately weighed QC (50 mg), CA (50 mg) and TN (25 mg) were separately transferred into 25 mL volumetric flask containing 10 mL methanol, sonicated for 10 min and volume made up to 25 mL with methanol to obtain stock solution. The three stock solutions were then suitably diluted with methanol to get the standard solutions of 200 µg mL^{-1} of QC, 200 µg mL^{-1} of CA and 100 µg mL^{-1} of TN.

Calibration curve preparation for QT, CA and TGF

Different volumes of stock solutions ranging from 0.25 to 12 µL were spotted on the HPTLC plates to obtain concentrations ranging from 50 to 2400 ng $spot^{-1}$ of different biomarkers. All the above studies were carried out in triplicate (n=3), data of peak areas were plotted against the corresponding concentrations and were treated by linear and polynomial least square regression analysis.

Method validation

The developed HPTLC method for co-quantification of three biomarkers were validated in terms of linearity, accuracy, precision, recovery, reproducibility, specificity, LOD, LOQ, ruggedness and robustness in accordance with ICH guidelines.

Results and Discussion

Development of HPTLC method

Analytical method plays a pivotal role in the drug discovery and formulation development. The developed analytical method is used to ensure the identity, purity, potency and performance of drug product. As the primary aim of the present work was to standardize and develop a novel PHA formulation for broad spectrum antimicrobial activity, there was a need to develop and validate a suitable analytical method for simultaneous co-quantification of the herbal extracts and quality control of the PHA formulation. Although different TLC and HPTLC methods are previously reported for the determination of individual biomarkers, using different kind of plates and gradients, the present method offers an isocratic separation for all three compounds simultaneously on a simple silica gel plate using common solvents. Main focus was laid on development of a high-throughput method that could be conducted at laboratory with basic TLC facility. As a first step towards method development, isocratic HPTLCs were developed for determination of individual biomarkers. The UV absorption spectra of the three biomarkers at different wavelengths were recorded by multi-wavelength detector to select the maximum wavelength of absorption (λ_{max}) for individual biomarkers. Use of multi-

wavelength detector permits a wavelength to be chosen where the solute absorbance is maximal and little interference is observed from additional solutes or the mobile phase. Upon scanning of the developed chromatograms the QC, CA and TN showed λ_{max} of 290 nm, 330 nm, 270 nm in absorbance mode, respectively (Fig. 2). The HPTLC chromatograms obtained for the individual biomarkers by isocratic HPTLC method for mono-component detection in absorbance mode is presented in (Fig. 3).

The analog chromatograms obtained after multi-wavelength detection of the three biomarkers by simultaneous HPTLC method is shown in Fig. 4. The final selection of the detection wavelength and absorbance mode was based on the relative signal intensity i.e. peak height and/or peak area of the biomarkers obtained at different wavelengths and the resolution factor of more than 1 for the two closely eluting compounds.

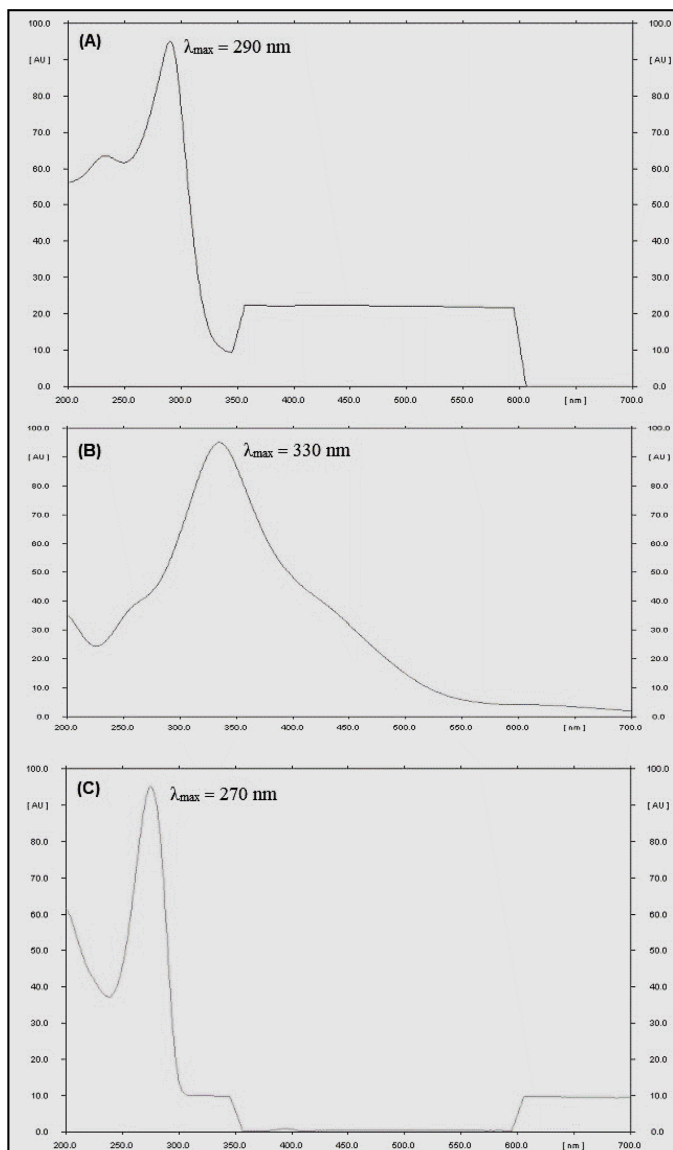

Fig 2: Spectra of (A) QC, (B) CA and (C) TN in absorbance mode

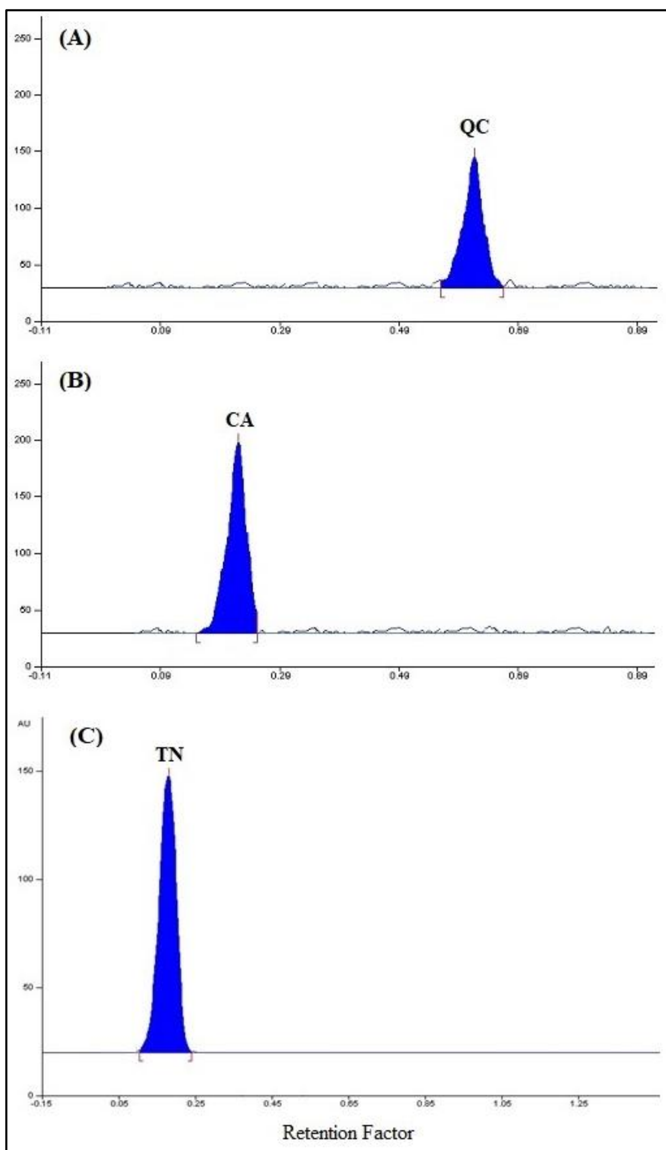

Fig 3: HPTLC mono component chromatogram for (A) QC detection at 290 nm, (B) CA detection at 330 nm and (C) TN detection at 270 nm

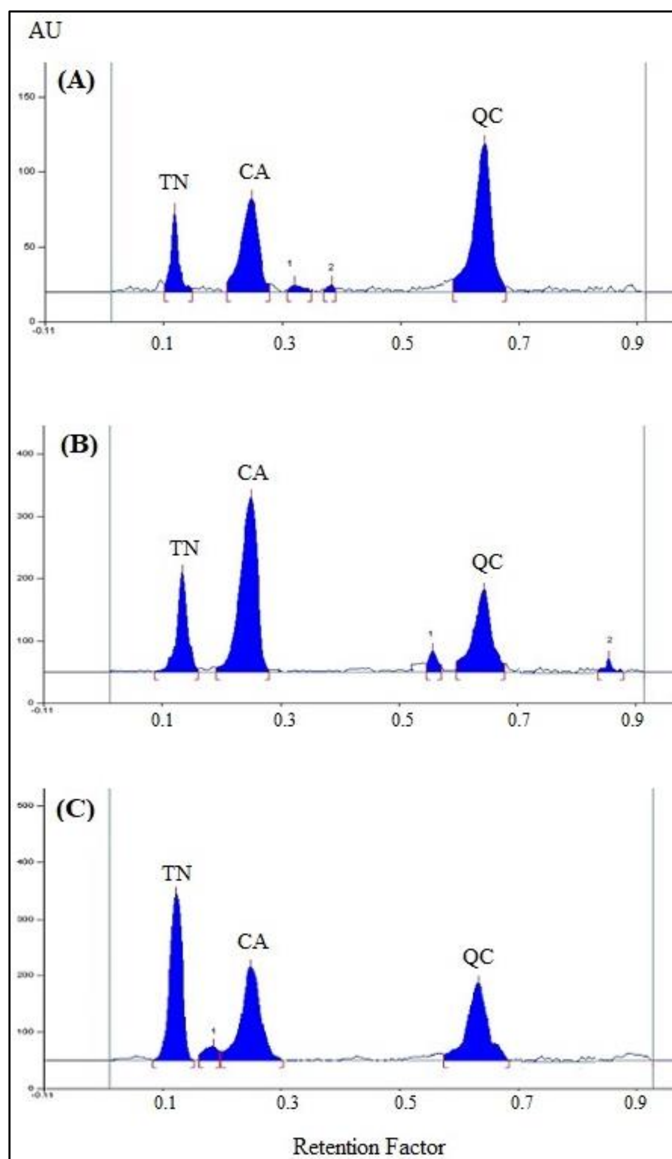

Fig 4: Simultaneous HPTLC detection for (A) QC, 290 nm; (B) CA, 330 nm; (C) TN, 270 nm

Manipulation of mobile phase composition is undoubtedly the most powerful means for adjusting both absolute and relative retentions of analytes in HPTLC. Solubility, polarity and stability of analytes were considered for the selection of mobile phase for determination of individual biomarkers. Based on previous literature, ethyl acetate-methanol-water (5:1:1, v/v/v) allows separation of QC and CA and n-propanol-methanol-water (4:1:4, v/v/v) allows chromatographic separation of TN. However, simultaneous separation of QC, CA and TN was not obtained with these mobile phases. Based on literature and experience gained from HPTLC methods, various combinations of chloroform (CH), ethyl acetate (EA), methanol (ME) and formic acid (FA) were tried following systematic mobile phase optimization method. The mobile phase composition and the corresponding retention factor (R_f value) for the three biomarkers are reported in (Table 1).

Table 1: Mobile phase optimization for simultaneous HPTLC method

Mobile phase	*Rf* value			Comments
CH:EA:ME:FA	TN	CA	QC	
5:3:1:0	0.09	0.18	0.87	Poorly defined solvent front
5:3:0:1	0.03	0.11	0.42	Peak barely resolved from solvent front
5:3:1:0.25	0.09	0.18	0.82	Solvent front as wavy line
5:3:1:0.50	0.12	0.13	0.79	Low resolution between TN & CA peaks
5:3:1.25:0.75	0.12	0.13	0.61	Low resolution between TN & CA peaks
5:3:1.5:0.5	0.13	0.24	0.62	Well separated spots with resolution > 1

CH: EA: ME: FA in a ratio of 5: 3: 1.5: 0.5 v/v/v/v showed well-separated spots at R_f values of 0.13, 0.24 and 0.62 for TN, CA and QC, respectively. Resolution factor between the peaks was found to be more than 1. The sample application volumes for simultaneous co-quantification were 0.5-12 µL for QC, 0.5-9 µL for CA and 0.5-15 µL for TN.

Method Validation

The linearity of the method should be tested in order to demonstrate a proportional relationship of response versus analyte concentration over the working range. Acceptability of linearity data is often judged by examining the correlation coefficient and the regression coefficient (R^2) > 0.998 is generally considered as evidence of acceptable fit of the data to the regression line. The standard solutions of the three biomarkers were spotted on HPTLC plates in the range of 50-2400 ng $spot^{-1}$ by varying the application volume from 0.5-12 µL. The calibration curve data for the simultaneous analysis of all the three biomarkers is presented in Table. The linearity range along with fitting of calibration data to linear/polynomial equations is shown in (Table 2). The high values of $R^2 > 0.998$ for all the biomarkers vis-à-vis - QC, CA and TN, clearly suggested the good fitness of the calibration data to the regression equations.

Table 2: Calibration data of simultaneous HPTLC method for determination of QT

S. No	Concentration ng/ spot	Area ± S.D.*		
		QC	CA	TN
1	50	-	-	310.75 ± 17.28
2	100	1371.93 ± 105.02	546.19 ± 29.59	457.03 ± 19.25
3	200	2844.32 ± 333.60	908.88 ± 59.72	845.48 ± 23.64
4	400	5670.54 ± 339.09	1533.13 ± 46.38	1559.40 ± 11.38
5	600	7510.41 ± 218.81	2348.73 ± 57.33	2263.19 ± 29.13
6	1000	10700.90 ± 275.22	3779.25 ± 72.82	3401.91 ± 74.00
7	1200	12475.81 ± 248.91	4362.23 ± 83.53	3954.48 ± 64.91
8	1500	14469.23 ± 313.57	5251.61 ± 101.95	4608.66 ± 42.83
9	1800	15628.24 ± 149.29	5947.57 ± 193.50	-

*Area is represented as average of three experiments (n=3) ± S.D.

Polynomial Regression (second order) is used if the amounts of substance cover a wider range of concentrations (1:50 to 1:100) or if large amounts are calibrated. Linear Regression is used for calibration in a limited concentration range (1:5 to 1:10) or for low amounts (within the linear range of the detector). It was observed that the calibration data could be best fitted to linear model for CA and TN, whereas the polynomial model was the choice for calibration data of QC (Table 3).

Table 3: Linear and polynomial calibration by peak areas (n=6)

Compound	Range (ng/ spot)	Equation	R2
CA	100 – 1800	Y = (3.26 ± 0.09) x + (309.73 ± 49.34) Linear	0.9991
TN	50 – 1500	Y = (3.03 ± 0.04) x + (261.92 ± 19.49) Linear	0.9995
QC	100 – 2400	Y = - 0.0033 x^2 + 14.31 x + 157.26 Polynomial	0.9983

* Slope ± standard deviation and Intercept ± standard deviation

Accuracy and precision

Accuracy is the measure of exactness of an analytical method and is defined as closeness of a result to the true value. Accuracy can be applied to a single measurement, but is more commonly applied to the mean value of several repeated measurements. Precision quantifies the variability of an analytical result as a function of operator and day-to-day environment and is defined as degree of closeness of one measurement to the next. It is expressed as percent relative standard deviation (% RSD) and standard error (SE) for a statistically significant samples. Desirable % RSD and SE values should be less than 2 for repeatability of application and recovery should be between 98-102%. Table 4 shows the values of % RSD and SE for intra- and inter-day precision at each of the three standard levels of all the three biomarkers. In the present study, the % RSD values obtained for the recovery of QC was found to be less than 0.1 and for CA and TN were found to be less than 0.5. The SE values for all the biomarkers were observed to be less than 2 demonstrating excellent precision of the developed method (Table 4).

Table 4: Intra- and inter-day precision data of simultaneous HPTLC method

Amount (ng $spot^{-1}$)	Intra-day Precision				Inter-day Precision			
	Mean Area	SD	% RSD	SE	Mean Area	SD	% RSD	SE
QC								
500	6561.83	3.97	0.061	1.62	6564.99	2.52	0.038	1.03
1000	12647.28	3.85	0.030	1.57	12658.94	4.83	0.041	1.98
1500	14491.01	4.72	0.033	1.93	14492.51	3.02	0.021	1.23
CA								
400	1621.25	4.77	0.294	1.95	1604.59	4.56	0.315	1.87
800	2924.79	3.31	0.113	1.35	2916.13	3.07	0.105	1.25
1600	5526.25	3.66	0.066	1.49	5552.91	4.71	0.085	1.92
TN								
500	1173.31	2.63	0.224	1.07	1170.64	3.08	0.263	1.26
1000	2074.60	2.87	0.138	1.17	2086.77	4.28	0.205	1.75
1500	3894.59	3.26	0.084	1.33	3891.09	2.79	0.072	1.14

*Area is presented as an average of six experiments (n=6)

Robustness

For robustness, the effect on results were examined by introducing small changes in the mobile phase volume, mobile phase composition, mobile-phase saturation duration and activation of pre-washed TLC plates with methanol. Robustness of the method was done in triplicate (n=3) and the % RSD and SE of peak areas were calculated. Table 5 shows the robustness data for developed simultaneous HPTLC method and presents the values of SD, % RSD and SE for each biomarker upon deliberate changes. The low values of % RSD and

SE obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method.

Table 5: Robustness of the simultaneous HPTLC method (n=6, 1000 ng $spot^{-1}$)

Parameter	Quercetin			Chlorogenic acid			Trigonelline		
Mobile Phase Composition (CH-EA-ME-FA, v/v/v/v)	SD	% RSD	SE	SD	% RSD	SE	SD	% RSD	SE
4.9 : 3.0 : 1.75 : 0.75	2.849	0.069	1.163	1.743	0.045	0.712	1.034	0.034	0.422
5.1 : 3.0 : 1.75 : 0.75	1.795	0.085	0.733	2.345	0.091	0.958	2.369	0.067	0.967
5.0 : 2.9 : 1.75 : 0.75	3.512	0.057	1.434	2.187	0.062	0.893	1.187	0.029	0.485
5.0 : 3.1 : 1.75 : 0.75	2.837	0.073	1.158	1.663	0.039	0.679	1.052	0.071	0.430
.9 : 2.9 : 1.75 : 0.75	3.148	0.046	1.285	1.981	0.074	0.809	2.084	0.038	0.851
4 5.1 : 3.1 : 1.75	3.954	0.028	1.615	2.043	0.052	0.834	1.332	0.041	0.544
Mobile Phase Volume (18, 20 and 22 ml)	1.543	0.022	0.630	1.237	0.078	0.505	1.119	0.028	0.457
Duration of Saturation (15, 30 and 45 min)	2.531	0.054	1.033	1.379	0.055	0.563	2.568	0.062	1.049
Activation of TLC plates (2, 5 and 7 min)	1.088	0.032	0.444	1.006	0.037	0.411	1.007	0.017	0.411

Ruggedness

As given in Table 6, standard solutions of different biomarkers at a given concentration level were applied in six replicates (n=6) and analyzed on day 0 and after 6, 12, 24, 48 and 72 h. Repeatabilities were determined by analyzing the same sample (n=6) on the same plate after 0, 6, 12, 24, 48 and

72 h. Low % RSD value (< 2) between peak area values obtained for all the biomarkers proved the ruggedness of the method.

Table 6: Ruggedness testing of the simultaneous HPTLC method (n=6)

Area (AU)	QC (500 ng spot^{-1})	CA (500 ng spot^{-1})	TN (250 ng spot^{-1})
Area 1	6568.19	1948.32	1019.96
Area 2	6561.34	1943.12	1017.28
Area 3	6559.27	1946.91	1022.13
Area 4	6565.42	1939.8	1024.36
Area 5	6563.12	1941.45	1020.29
Area 6	6567.39	1944.22	1021.37
Mean Area	6564.12	1943.97	1020.90
SD	3.50	3.23	2.37
% RSD	1.4286	1.3180	0.9674
SE	0.0533	0.1660	0.2321

As recommended by ICH guidelines, the detection and quantification limits were calculated based on the calibration curves and experimentally verified. Considering an application volume of 12 µL, the LOD and LOQ found with the developed method were: 3.0502 and 9.2429 ng spot^{-1} for QT; 18.5442 and 56.1947 ng spot^{-1} for CA; 6.5033 and 19.707 ng spot^{-1} for CU; and 21.2306 and 64.3352 ng spot^{-1} for TGF.

The pre-analyzed samples were spiked with extra 50, 100 and 300 % of the standard solution of different biomarkers (Table 7) and the mixtures were again analyzed by the proposed HPTLC method to check for the recovery of biomarkers at different levels in the formulations. The study was conducted in six replicates (n=6) and the % R.S.D. and S.E. values were recorded. Recovery of the four compounds was calculated for each compound at four different levels after spiking with 0, 50, 100 and 300%. The % R.S.D. value were found to be less than 0.1% (for QC) and less than 0.5% (for CA and TN) with S.E. was less than 2. The average % recovery for all the four compounds at all levels ranged from 99.35-101.88%.

Table 7: Recovery studies for QC, CA and TN by the simultaneous HPTLC method (n=6)

	Quercetin			Chlorogenic acid			Trigonelline		
Excess Drug	Recovery (%)	% RSD	SE	Recovery (%)	% RSD	SE	Recovery (%)	% RSD	SE
Level – 1	99.70	0.073	1.95	100.31	0.282	1.87	101.16	0.433	0.87
Level – 2	99.90	0.064	1.53	99.50	0.285	0.78	100.61	0.323	1.16
Level – 3	101.48	0.057	0.96	99.83	0.143	1.70	99.66	0.199	1.69
Level – 4	100.97	0.025	1.59	100.63	0.089	0.92	99.68	0.097	0.73

Specificity

A method is specific if it produces a response for only one single solute. Since it is almost impossible to develop a chromatographic assay for a drug in a matrix that will respond to only the compound of interest, the term selectivity is more appropriate. Selectivity describes the ability of an analytical method to differentiate various substances in the sample and is applicable to methods in which two or more components are separated and quantitated in a complex matrix. It is a measure of degree of interference from such things as other active ingredients, excipients, impurities and/or degradation products, ensuring that a characteristic peak at a given R_f value can be assigned to only single component, *i.e.,* no co-retention exists. The specificity of the method was ascertained by analyzing the standard solutions and extracts. The spot for biomarkers in the samples were confirmed by comparing their R_f values and spectra of the spot in samples with that of the standard solutions. The peak purity of biomarkers was assessed by comparing their spectra at three different levels, viz. peak start, peak apex and peak end positions of the spots. The video-densitometric image of the relative position of the 3 biomarkers on HPTLC plates and the corresponding resolution factor calculated for their relative peak purity is shown in Fig. 2.

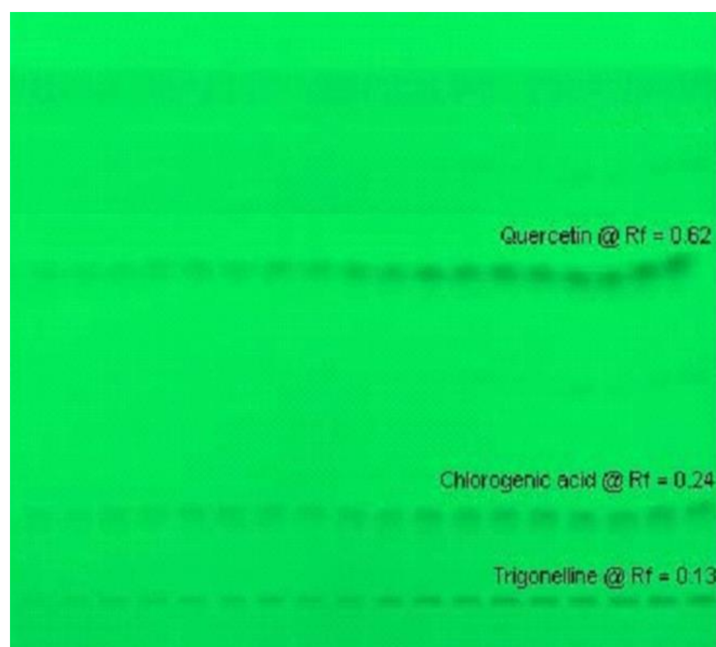

Fig 2: Video-densitometric image of developed HPTLC plate

Biomarker content in PHA formulation

The developed HPTLC method was then applied to analyze percentage content of biomarkers in PHA formulation. HPTLC determination showed the characteristic peaks of TN, CA and QC in chromatogram at R_f values of 0.13, 0.24 and 0.62, respectively. The % content of these biomarkers in PHA formulation, as analyzed by HPTLC, was found to be 99.93%, 100.15% and 99.56% for QC, CA and TN, respectively with respect to the labeled composition. Further, developed HPTLC method is sensitive enough to detect biomarkers in presence of polymers used in the development of the formulation.

Conclusion

To the best of our knowledge, the separation in one run and the simultaneous detection and co-quantification of all three compounds was successfully achieved for the first time. The developed and validated HPTLC method for simultaneous determination of QC, CA and TN is rapid, reliable and low cost. The sensitivity and specificity of the method allow it to utilize for standardization of AI, CI and TFG and the method was also applicable for determination of potency of polyherbal formulation developed through use of AI, CI and TFG.

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