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Research Article

RP-HPLC Bioanalytical Simultaneous Estimation of Kaempferol and Quercetin in Mice Plasma

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Abstract

Background: Kachnardi yoga a traditional medicine used to treat several bacterial infection and serious diseases, had also proven beneficial for the usage in breast cancer due to the presence of Kaempferol and Quercetin.

Purpose: The purpose of the study is to developed a bioanalytical technique for concurrently estimating KML and QRN from ethanolic extract of kachnardi yoga in mice plasma using RP-HPLC using Naringenin as internal standard.

Material and Method: The Gradient elution technique was developed to separate KML, QRN, and NGN within 20 minutes of total run time, where the mobile phase consisted of acetate buffer pH 5.35 and Acetonitrile in a Linear gradient pattern. The flow rate of 1.0 mL/min, and the injection volume of 50 µL. Detection was achieved at a wavelength of 375 nm, and the retention times for KML, QRN, and NGN were found to be 5.2, 7.2, and 10.2 minutes, respectively. Mice Plasma samples were processed using a protein precipitation technique to extract the analytes.

Results: The method demonstrated linearity with correlation coefficients (R^2) of 0.9994 for KML and 0.9992 for QRN, over a concentration of 75-1200 ng/mL and 35-1000 ng/mL, respectively. The results of all the validation parameters performed as per ICH guidelines M10 were within the accepted limits with less than 3 %RSD. The % recovery from plasma was more than 95%. Freeze- thaw, Bench-top, short-term, and long-term stability studies were also achieved at LQC, MQC, and HQC levels.

Conclusion: The established bioanalytical technique can simultaneously effectually estimate the KML and QRN in kachnardi yoga from plasma samples and is suitable for further pharmacokinetic studies.

Keywords: Kaempferol; Quercetin; Simultaneous Estimation Bioanalytical Method; RP-HPLC; Mice Plasma; Stability Studies; Bioanalytical Method Validation

Abbreviations

RP-HPLC: Reversed Phase High Performance Liquid Chromatography; KML: Kaempferol; NGN: Naringenin; QRN: Quercetin; IS: Internal Standard; ICH: International Council for Harmonization; LQC: Lower Quality Control; MQC: Mid Quality Control; HQC: High Quality Control; ULOQ: Upper Limit of Quantification; LLOQ: Lower Limit of Quantification.

Introduction

Cancer is one of the leading causes of mortality, affecting the health of many humans. Modernization and changing lifestyles of humans lead to increased cancer cases [1,2]. Breast cancer is among the most occurring known diseases worldwide. Due to increase in modernization, there is also increase in the treatment via herbal routes.

One such herbal ayurvedic extract showed promising effects against breast cancer. Kachnardi yoga (Bauhinia variegata, Bauhinia Racemose, Bauhinia purpurea), a folklore medicine basically used for infections, inflammation, diabetes, goiter, lymphadenopathy, hepatoprotection, nephroprotection, benign prostate hyperplasia and cough. The chemoprevention along with hepatoprotection was proven against N- nitroso-diethylamine induced human cancerous cells and hepatocarcinogenesis. The use of hydroalcoholic mixture of Bauhinia variegata is validated in-vivo in model of melanoma with beneficial effect on metastasis.

The ethanolic extract of plant stem consisting of Quercetin and Kaempferol, is successfully investigated in mice tumour model of triple negative breast cancer where it shows advantageous effect in tumour regression and mechanistic pathway in metastasis. The chemical structure of Kaempferol and Quercetin is depicted in Figure 1(a) and Figure 1(b) respectively.

An attempt was made to develop a simple, precise, and robust method for simultaneous estimation of Kaempferol and Quercetin from the ethanolic extract of Kachnardi yoga with the utmost precision and accuracy in a shorter duration of time at a very minute quantity from the mice plasma. The developed method should be capable of quantifying even subtle levels in the Kachnardi yoga ethanolic extract.

The literature review examines various analytical practices, including Liquid Chromatography, densitometric method and UV Spectrophotometric method for quantifying Kaempferol [3-5] and Quercetin [6-8] from the extract.

According to a literature review, several attempts have been made to develop an individual bio-analytical method for Kaempferol and Quercetin. Still, none have tried to develop a technique simultaneously capable of estimating Kaempferol and Quercetin in less time with the most precise and accurate data from the mice's plasma. The current article aims to develop an HPLC-UV bioanalytical method that can quickly, precisely, and robustly estimate Kaempferol and Quercetin simultaneously in APIs and from the extract of Kachnardi yoga from the mice plasma for the pharmacokinetic assessment and validate the method according to ICH guidelines ICH M10 [12]. The effectiveness and practicality of the suggested method were assessed with a focus on quality control research.

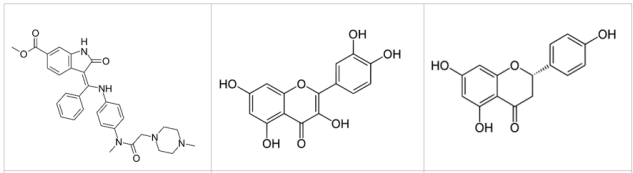


Figure 1(a): Chemical structure of Kaempferol [9].

Figure 1(b): Chemical structure of Quercetin [10].

Figure 1(c): Chemical structure of Naringenin [11].

Figure 1: Pyrolysis products from microwave pyrolysis of agro-residue.

Materials and Methods

Chemicals and reagents

All solvents used for the mobile phase were HPLC grade, Kaempferol, Quercetin, and Naringenin were purchased from sigma Aldrich.

Instruments and apparatus

A HPLC system (Agilent 1220 Infinity II), a compact Binary solvent delivery pump module, and a manual rheodyne injector with a 50 µL fixed loop with UV/Vis detector. Microsoft Excel (PK Solver) was used for statistical calculations using bioanalytical method validation.

Isosbestic point determination for KML and QRN (λ)

10-50 µg/mL and 20-100 µg/mL of KML and QRN working solutions were prepared to determine the wavelength. The scan was performed in the range of 200-400 nm. A detection wavelength of 375 nm was selected for further analysis of KML and QRN. The scan for wavelength determination of the KML and QRN standard solution is depicted in Figure 2.

Chromatographic conditions

Preparation of standard and resolution solution

The chromatographic conditions were optimized by different means (different buffers and different organic phases). Early chromatographic work was performed stepwise with various combinations of buffer phase with pH ranging from 5.20 to 5.50 and organic phases (acetonitrile (ACN) and/or methanol). The wavelength for monitoring the eluent was selected by scanning a standard so-

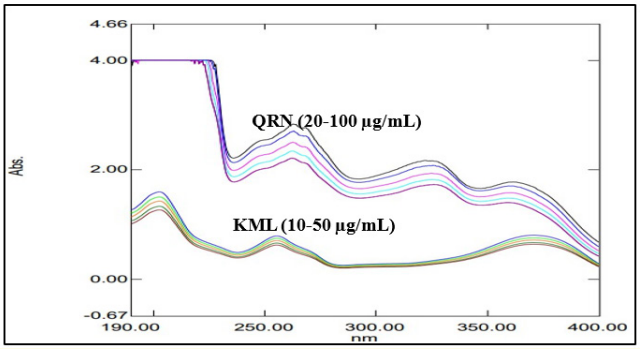


Figure 2: Overlay UV spectrum of KML (10-50 µg/mL) and QRN standard (20-100 µg/mL) with the isosbestic point (Optimal Wavelength) for both the drugs at 375 nm.

lution of KML and QRN within 200 to 400 nm using a double- beam UV/Vis spectrophotometer (Shimadzu Spectrophotometer UV 1800, Japan).

Trials were initiated to achieve the optimum separation by varying the concentrations of buffer agent on different peak parameters was evaluated, viz. mobile phase Ammonium Acetate (pH 5.20 to 5.50) in a range 5-20 mM concentration with ACN at 1.0 mL/min flow rate in a buffer to organic ratio noted from isocratic elution to Linear gradient elution. However, optimum effective peak symmetry was obtained in Linear gradient elution. Moreover, the effects of different levels of all these factors were systematically addressed on system suitability parameters such as %RSD of peak area, retention time, capacity factor, asymmetry, resolution, and peak width.

All noted measurements were performed with an injection volume of 50 µL and UV detection at 375 nm of samples dissolved in a diluent of water and Acetonitrile in the ratio of 1:1, respectively.

Preparation of standard and resolution solution

Diluted standard solutions of each analyte representing 10 µg/mL concentration were prepared with diluent. Naringenin (NGN, Figure 1c) was used as an internal standard (IS) for KML and QRN. Resolution solution containing 75 ng/mL and 35 ng/mL each of KML and QRN with 50 ng/mL of NGN was prepared from respective stock solutions.

For optimization purposes, 50 µL of resolution solution was injected into the chromatograph and system suitability parameters. %RSD of peak area for six injections of all analytes, %RSD of retention time for six injections of all analytes, and peak asymmetry factor at 10% peak height and resolution were studied.

Sample preparation and extraction

The protein Precipitation method was employed to separate KML and QRN from the plasma matrix. 5.0 mg each of KML, QRN, and Naringenin (NGN) were weighed, transferred into a 10 mL volumetric flask, dissolved, and made up to the mark using a diluent (500 µg/mL). 5 µg/mL was made from the solutions mentioned above. 500 ng/mL of NGN was used as the internal standard in mice plasma. 700 ng/mL of KML and 800 ng/mL of QRN were added. 0.8 mL of mice plasma was added to a 2 mL Eppendorf. After that, acetonitrile was used to bring the volume up to 2 mL for plasma precipitation. To separate the proteins from the prepared samples, they were vortexed for 5 minutes and centrifuged at 10000 RPM for 15 minutes at 4 ± 5 °C. The supernatant was carefully pipetted out and injected into the chromatographic system.

Bioanalytical method validation [12]

The developed bioanalytical method by HPLC were validated according to ICH M10 guidelines.

The specificity of the method was performed by injecting blank plasma, spiked plasma samples, and plasma samples spiked with frequently prescribed medication, which were analyzed. Selectivity of the method was performed by injecting six samples at the LLOQ level along with six blank plasma samples, which were tested for interference by comparing the mean peak response obtained by injecting blank plasma samples to the mean peak response of LLOQ (75 ng/mL KML and 35 ng/mL QRN). Representative chromatograms were generated to show no interference of the plasma components or sample matrix in the presence of the main analyte peak.

Calibration curve of KML and QRN

The standard curve was determined on each day of the six-day validation period; the slope, intercept, and correlation coefficient were determined. Each run consisted of a double control, system suitability sample, blank samples (a plasma sample processed wit-

hout an IS), a control sample (a plasma processed with IS), and a calibration curve consisting of twelve non-zero samples covering the total range (LLOQ to 75 ng/mL for KML and 35 ng/mL QRN) and QC samples at three concentrations (n = 6, at each concentration). Such runs were generated on six consecutive days. Calibration samples were analyzed from low to high at the beginning of each run, and other samples were distributed randomly throughout the run. For the calculation of the standard curve, plots of peak area ratios against concentration were used.

Sensitivity

The sensitivity (LLOQ) was determined by signal-to-noise ratio. The resolution solution was serially diluted and spiked to the rat plasma, and injections were made to obtain a chromatogram. Similarly, blank plasma samples were also processed and injected into chromatographs. The LLOQ was expressed for the analyte concentration having a response at least 5 times more than a blank response.

Precision and accuracy

Precision and Accuracy for the developed method for KML were employed at LQC (112.5n g/mL), MQC (350 ng/mL), HQC (865 ng/mL), and ULOQ (1200 ng/mL). P&A for QRN were employed at LQC (52.5 ng/mL), MQC (400 ng/mL), HQC (850 ng/mL), and ULOQ (1000 ng/mL). All were performed in triplicate and analyzed using the HPLC method. Precision was expressed as the coefficient of variation (%CV). Precision and accuracy values (%CV) less than or equal to 15% for QC samples, whereas less than or equal to 20% for LLOQ and ULOQ were acceptable.

Recovery studies

Recovery was executed by injecting 5 replicates of aqueous LQC and 3 replicates of extracted QC samples at the LQC level. The extracted and unextracted areas of analyte and IS were injected, and % recovery was calculated.

Stability studies

- **Freeze–thaw stability study [12]:** Freeze-thaw stability (3 cycles) was assessed by injecting six freshly prepared samples and six stability samples at the LQC and HQC levels for both KML and QRN.
- **Bench-top stability study [12]:** The spiked plasma sample at HQC Level for KML and QRN was performed at room temperature (Bench-top) for 8 hours by injecting 6 sample sets at the MQC level.
- **Short-term Stability and Long-term Stability [12]:** The short-term stability (8 hr. Room Temperature) and long-term stability (7 days at -20˚C) were performed for KML and QRN by injecting 6 sample sets at the MQC level.

Matrix effect [12]:

The matrix effect was performed by the post-extraction addition method. This method was performed at two different concentration levels, i.e., LQC and HQC level samples.

The LQC and HQC samples in each blank biological matrix source were injected by external spiking of the extracted blank matrix.

Results and Discussion

Wavelength selection and optimization trials

This HPLC method provides precise quantification of the analytes at low plasma concentrations. To avoid plasma interference, the retention factor of the first analyte was kept at more than 3 minutes. The optimal wavelength of 375 nm was selected for the analysis when KML and QRN were scanned at 200-400 nm (Figure 2). The optimum response for KML and QRN was selected for working standard and sample at 375 nm. For the effective separation of KML and QRN with Internal Standard, different trials for mobile phase optimization with ACN and ammonium acetate (5.3 ± 0.05) as buffer were designed simultaneously, as mentioned below in Table No.1.

The optimized chromatographic conditions with gradient elution mode are mentioned in Table No.2, with SST parameters for resolution solution is noted in Table No.3. To select an internal standard (IS), NGN was chosen due to its similar structure and formula ratio. NGN was eluted under the optimized chromatographic conditions developed for the analysis of KML and QRN. The resolution between KML and QRN was found to be 3.00, and that of plasma protein was found to be 2.65. A representative system suitability chromatogram without plasma is given in Figure 3.

Table No.1: Optimization Trials Summary of HPLC conditions for KML and QRN Estimation.

Sr. No.	Mobile Phase	Elution method	Observation	Result
1	Acetonitrile – 10 mM Ammonium acetate	Gradient	Good elution strength, with better resolution but with low response.	Rejected
2	Acetonitrile – 10 mM Ammonium acetate	Gradient	Good elution strength, with better resolution but with high response.	Rejected
3	Acetonitrile – 10 mM Ammonium acetate	Gradient	Good elution strength, with better resolution and response. Interference of main peak and IS Peak.	Rejected
4	Acetonitrile – 10 mM Ammonium acetate	Gradient	Good elution strength, with better resolution and response. But there is less resolution with IS.	Rejected
5	Acetonitrile – 10 mM Ammonium acetate	Gradient	Good elution strength, with better resolution. Well resolved peaks for KML, QRN and NGN (IS).	Accepted

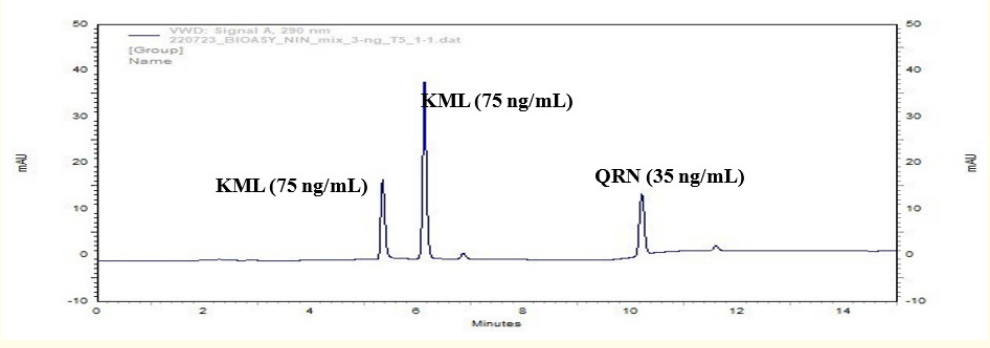


Figure 3: SST Chromatogram, SST Chromatogram representing KML, QRN, and NGN with optimized chromatogram without plasma.

Table No.2: Optimized chromatographic condition for KML and QRN Estimation.

Column	Agilent Zorbax C18 column (250 × 4.6 mm) 5 µm i.d.							
Mobile Phase	Buffer: 10mM ammonium acetate pH 5.35 ± 0.05 and Acetonitrile MP A (%v/v): Buffer: ACN: 80:20 MP B (%v/v): Buffer: ACN: 20:80							
Gradient Program	As mentioned below (Run Time: 20 min)							
	Time (min)	0.00	3.50	7.50	10.00	13.00	15.00	20.00
	MP A (%v/v)	72	35	35	12	12	72	72
	MP B (%v/v)	28	65	65	88	88	28	28
Mobile phase flow rate	1 mL/min							
Column oven temperature	40°C							
Injection volume	50 *µL*							
KML, QRN and NGN RT	5.60 ± 0.5 for KML		7.80 ± 0.5 for QRN		9.80 ± 0.5 for NGN (IS)			
Wavelength (nm)	375 nm							

Table No.3: System Suitability Studies for Resolution Solution.

SST Parameters	ICH Limits	KML	QRN
Retention time	NA	5.24 ± 0.04	7.81 ± 0.01
Resolution	≥ 2	NA	3.66 ± 0.03
Tailing Factor	≤ 2	1.23 ± 0.02	1.32 ± 0.02
Theoretical Plates	≥ 2000	113486.00 ± 1309.39	107653.33 ± 1315.10

The protein Precipitation method was preferred for extracting KML and QRN from the mice plasma because of minimum, easy, and reproducible extraction steps. The technique used cold ACN and MeOH. Trials were attempted to minimize the matrix effect and increase the extraction rate. The recovery of KML and QRN with IS was near about 60% and had a non- symmetrical peak shape with MeOH; therefore, ACN was chosen as a precipitating agent as using ACN did not alter the peak shape and had maximum recovery with minimal matrix effect. Figure 4 represents the plasma spiked chromatogram with sample and blank plasma. In the last precipitation step, the supernatant was mixed with diluent (20: 80: Water: ACN). The % mean recoveries for all the analytes ranged from 95-102% in the currently developed method for both the analytes.

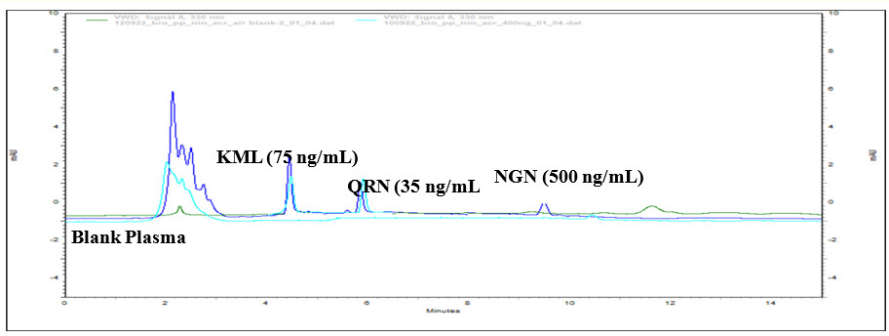


Figure 4: Chromatograms of Blank and Spiked Samples with Plasma at LLOQ Level Chromatogram representing Processed plasma Spiked with KML, QRN and NGN.

Validation parameters

The developed bioanalytical HPLC method was validated according to ICH M10 guidelines for the specificity and Selectivity, Precision and Accuracy, Calibration range, Recovery studies, Matrix effect, and Stability.

Specificity and selectivity

Selectivity of the method was performed at LLOQ level, i.e., for KML (75 ng/mL) and QRN (35 ng/mL), the maximum percentage interference for analyte was found to be 0.13% and 0.58% for KML and QRN respectively. In contrast, for Internal Standard, it was 1.10%.

The analyte signal at the LLOQ was more than five times the noise level, well exceeding the acceptance criteria (Table 4). No interference was observed at the retention times of the analyte and internal standard (IS) in the chromatogram at the LLOQ for KML and QRN (Figure 4). The peak purity plots for KML (Figure 5(a)), QRN (Figure 5(b)), and NGN (Figure 5(c)) demonstrated no interaction between the analytes, IS, and plasma, confirming the spectral purity of the peaks and the specificity of the developed method.

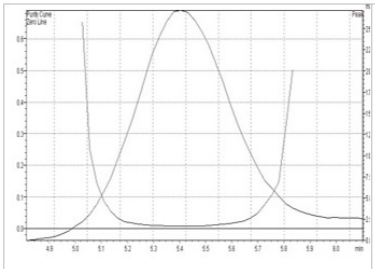


Figure 5(a): Bioanalytical Peak Purity Plot of KML.

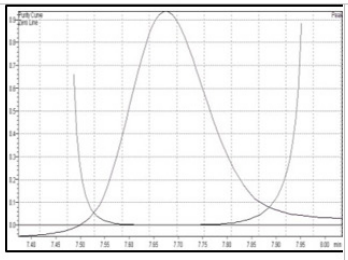


Figure 5(b): Bioanalytical Peak Purity Plot of QRN.

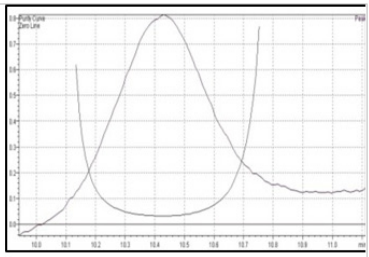


Figure 5(c): Bioanalytical Peak Purity Plot of NGN.

Table No.4: Selectivity of the method for KML and QRN.

Analyte (n = 6)	Sequence	% intefernce for analyte	% interference for IS
KML	Plasma Blank	0.13 ± 0.01	1.1 ± 0.10
	LLOQ (75ng/mL)		
QRN	Plasma Blank	0.58 ± 0.03	
	LLOQ (35ng/mL)		

Precision and accuracy

The intraday Precision and accuracy (Table No.5(a)) for KML and QRN were found in the range of 91.72% to 95.42% and 93.28% to 95.87%, respectively. The precision (%CV) for intraday was between 2.87-3.56 and 1.87-3.68 for KML and QRN, respectively.

Analyte (n = 12)	Quality Control	Mean Conc Found	SD	% CV	% Accuracy
KML	LQC (112.5 ng/mL)	107.35	3.82	3.56	95.42
	MQC (350 ng/mL)	321.70	9.22	2.87	91.92
	HQC (865 ng/mL)	807.06	26.87	3.33	93.30
	ULOQ (1200 ng/mL)	1100.69	34.53	3.14	91.72
QRN	LQC (52.5 ng/mL)	48.97	1.80	3.68	93.28
	MQC (400 ng/mL)	372.99	11.35	3.04	93.25
	HQC (850 ng/mL)	814.90	15.31	1.88	95.87
	ULOQ (1000 ng/mL)	934.69	22.57	2.42	93.47

Table No.5(a): Intraday Precision and Accuracy for KML and QRN.

The Interday precision and accuracy (Table No.5(b)) for KML and QRN were 90.21% to 90.85% and 91.86% to 92.79%, respectively. The (%CV) value for Interday precision was between 1.72-3.57 and 1.21- 6.02 for KML and QRN, respectively. All the accuracy and precision values met the acceptance criteria according to ICH guideline M10.

Analyte (n = 12)	Quality Control	Mean Conc Found	SD	% CV	% Accuracy
KM L	LQC (112.5 ng/mL)	106.56	3.80	3.57	94.72
	MQC (350ng/mL)	317.96	8.98	2.82	90.85
	HQC (865 ng/mL)	780.31	24.21	3.11	90.21
	ULOQ (1200 ng/mL)	1089.63	18.74	1.72	90.80
QRN	LQC (52.5 ng/mL)	48.62	0.59	1.21	92.60
	MQC (400 ng/mL)	367.46	9.53	2.59	91.86
	HQC (850 ng/mL)	788.69	24.80	3.14	92.79
	ULOQ (1000 ng/mL)	926.92	21.02	2.27	92.69

Table No.5(b): Interday Precision and Accuracy for KML and QRN.

Calibration curve and linearity

The 8-point calibration curve and linearity (Figure 6) were performed by spiking precise amounts of working solution into the blank Plasma to get final concentrations of 75-1200 ng/mL (Table No.6) for the KML, 35-1000 ng/mL for QRN (Table No.6), and 500 ng/mL for NGN, respectively. The calibration curve was prepared by plotting the peak area ratio of the KML (Figure 7(a)) and QRN (Figure 7(b)) transition pairs relative to the internal standard (NGN) against the nominal concentrations of the calibration standards. The % recovery from linearity of KML and QRN was found in the range of 97.16- 101.53% (Table No.7(a)) and 95.07 - 100.28% (Table No.7(b)), respectively.

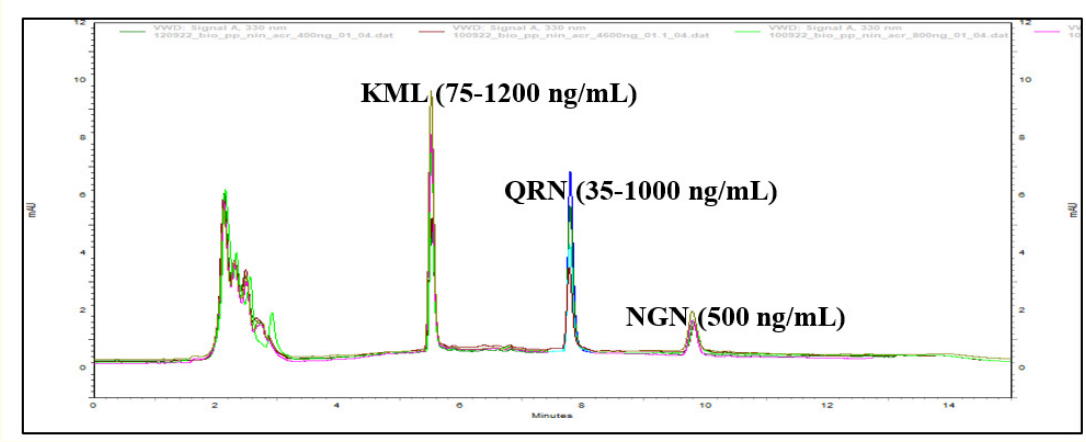
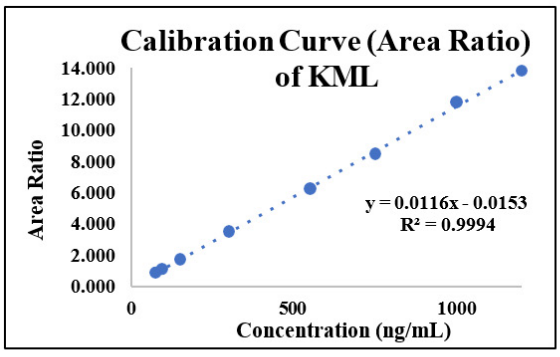


Figure 6: Bioanalytical Overlay plot for KML (75-1200 ng/mL) and QRN (35-1000 ng/mL) with IS NGN (500 ng/mL).



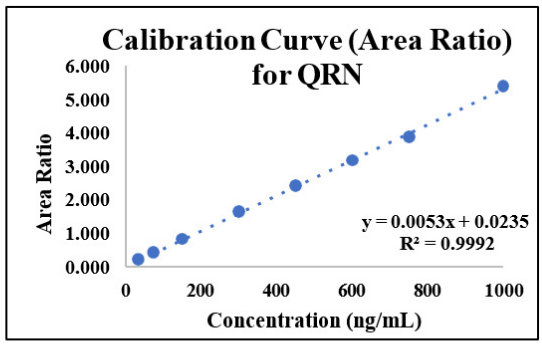


Figure 7 (a): Bioanalytical Calibration curve for KML.

Figure 7 (b): Bioanalytical Calibration curve for QRN.

Table No.6: Bioanalytical Linearity of KML and QRN.

KML (75-1200 ng/mL)			QRN (35-1000 ng/mL)		
Conc (ng/mL)	**Avg Area Ratio ± *SD* (n = 6)**	**RSD**	**Conc (ng/mL)**	**Avg Area Ratio ± *SD* (n = 6)**	**RSD**
75	0.856 ± 0.017	1.98	35	0.213 ± 0.008	3.66
95	1.099 ± 0.013	1.16	75	0.414 ± 0.010	2.31
150	1.739 ± 0.012	0.67	150	0.820 ± 0.021	2.56
300	3.522 ± 0.069	1.95	300	1.647 ± 0.021	1.28
550	6.245 ± 0.066	1.05	450	2.416 ± 0.038	1.57
750	8.494 ± 0.156	1.84	600	3.178 ± 0.077	2.42
1000	11.809 ± 0.416	3.52	750	3.880 ± 0.079	2.05
1200	13.804 ± 0.221	1.60	1000	5.386 ± 0.084	1.56

Calibration Curve for KML																
Sequence No.	**Cali. Set-1**		**Cali. Set-2**		**Cali. Set-3**		**Cali. Set-4**		**Cali. Set-5**		**Cali. Set-6**		**Cali. Set-7**		**Cali. Set-8**	
	75 ng/mL		**95 ng/mL**		**150 ng/mL**		**300 ng/mL**		**550 ng/mL**		**750 ng/mL**		**1000 ng/mL**		**1200 ng/mL**	
	Area Ratio	**Actual Concentration**	**Area Ratio**	**Actual Concentration**	**Area Ratio**	**Actual Concentration**	**Area Ratio**	**Actual Concentration**	**Area Ratio**	**Actual Concentration**	**Area Ratio**	**Actual Concentration**	**Area Ratio**	**Actual Concentration**	**Area Ratio**	**Actual Concentration**
PA-1	0.828	72.87	1.079	94.55	1.731	150.88	3.431	297.76	6.272	543.19	8.669	750.24	11.645	1007.32	14.087	1218.32
PA-2	0.872	76.62	1.113	97.46	1.723	150.18	3.502	303.85	6.319	547.22	8.381	725.34	12.033	1040.83	13.674	1182.66
PA-3	0.863	75.91	1.094	95.86	1.750	152.53	3.532	306.47	6.198	536.79	8.549	739.84	12.174	1053.06	13.853	1198.09
PA-4	0.855	75.23	1.102	96.49	1.740	151.60	3.623	314.28	6.158	533.33	8.290	717.52	11.158	965.28	13.510	1168.43
PA-5	0.865	76.05	1.105	96.83	1.749	152.41	3.521	305.47	6.281	543.96	8.591	743.49	12.049	1042.21	13.905	1202.59
Mean	0.86	75.34	1.10	96.24	1.74	151.52	3.52	305.57	6.25	540.90	8.50	735.28	11.81	1021.74	13.81	1194.02
SD	0.02	1.47	0.01	1.11	0.01	1.00	0.07	5.93	0.07	5.67	0.16	13.47	0.42	35.92	0.22	19.13
%CV	1.98	1.95	1.16	1.15	0.67	0.66	1.95	1.94	1.05	1.05	1.84	1.83	3.52	3.52	1.60	1.60
% Accuracy	97.16%		99.53%		100.59%		99.25%		98.76%		100.03%		100.73%		101.53%	

Table No.7(a): Calibration curve and Linearity of KML.

Calibration Curve for QRN																
Sequence No.	Cali. Set-1		Cali. Set-2		Cali. Set-3		Cali. Set-4		Cali. Set-5		Cali. Set-6		Cali. Set-7		Cali. Set-8	
	35 ng/mL		75 ng/mL		150 ng/mL		300 ng/mL		450 ng/mL		600 ng/mL		750 ng/mL		1000 ng/mL	
	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration	Area Ratio	Actual Concentration
PA-1	0.199	33.27	0.412	73.53	0.811	148.96	1.617	301.28	2.396	448.65	3.205	601.71	3.866	726.62	5.242	986.87
PA-2	0.213	35.74	0.418	74.63	0.850	156.31	1.662	309.87	2.463	461.36	3.182	597.34	3.999	751.95	5.414	1019.57
PA-3	0.218	36.76	0.414	73.94	0.835	153.39	1.633	304.33	2.429	454.86	3.290	617.83	3.884	730.10	5.424	1021.36
PA-4	0.217	36.68	0.399	71.09	0.806	147.89	1.666	310.56	2.428	454.79	3.092	580.32	3.875	728.32	5.392	1015.31
PA-5	0.217	36.59	0.426	76.04	0.801	147.04	1.656	308.66	2.363	442.46	3.124	586.34	3.776	709.76	5.458	1027.81
mean	0.21	35.81	0.41	73.85	0.82	150.72	1.65	306.94	2.42	452.42	3.18	596.71	3.88	729.35	5.39	1014.18
SD	0.01	1.48	0.01	1.81	0.02	3.97	0.02	3.98	0.04	7.16	0.08	14.55	0.08	15.03	0.08	15.92
%CV	3.66	4.12	2.31	2.45	2.56	2.63	1.28	1.30	1.57	1.58	2.42	2.44	2.05	2.06	1.56	1.57
% Accuracy	95.07%		98.03%		99.31%		100.43%		99.70%		100.28%		96.88%		98.69%	

Table No.7(b): Calibration curve and Linearity of QRN.

% recovery studies

The % recovery performed at the LQC level by preparing six sample sets: KML (Table No.8(a)) was found to be 91.16% for recovery in solvent (without Plasma) and 90.54% in Plasma QRN (Table No.8(b)) was found to be 91.83% for recovery in solvent (without Plasma) and 89.53% in Plasma.

Table No.8(a): Recovery data for KML.

Set Sequence	Absolute recovery– In solvent		Relative recovery- In Plasma	
	Analyte LQC-A (112.5 ng/mL)		Analyte LQC-A (112.5 ng/mL)	
	Unextracted sample	Extracted Sample	Unextracted sample	Extracted Sample
Set-1	149324	141273	149324	141273
Set-2	149365	136736	149365	136736
Set-3	149524	132709	149524	132709
Set-4	145001	131736	145001	131736
Set-5	152635	137465	152635	137465
Mean	149169.80	135983.80	149169.80	135983.80
SD	2719.09	3856.93	2719.09	3856.93
% CV	1.82	2.84	1.82	2.84
% Recovery	91.16%		90.54%	

Table No.8(b): Recovery data for QRN.

Set Sequence	Absolute recovery– In solvent		Relative recovery- In Plasma	
	Analyte LQC-A (52.5 ng/mL)		Analyte LQC-A (52.5 ng/mL)	
	Unextracted sample	Extracted Sample	Unextracted sample	Extracted Sample
Set-1	36373	33530	36373	33530
Set-2	35388	32189	35388	32189
Set-3	35012	32602	35012	32602
Set-4	35701	33290	35701	33290
Set-5	36176	32443	36176	32443
Mean	35730.00	32810.80	35730.00	32810.80
SD	558.05	572.80	558.05	572.80
% CV	1.56	1.75	1.56	1.75
% Recovery	91.83%		89.53%	

The results indicate that the plasma extraction procedure developed for KML and QRN is acceptable. Nearly a 1% difference from the extraction procedure from solvent to plasma suggests that the developed extraction procedure can be used for analytical purposes.

Matrix effect

The matrix effect was performed by the post- extraction addition method. This method was performed at LQC level samples by injecting 6 sample sets of MML and QRN (Table No. 9).

Sequence	LQC MF Factor		IS MF Factor	IS Normalized Matrix Factor	
	KML	QRN		KML	QRN
Set-1	1.0560	1.0907	1.2103	0.8726	0.9012
Set-2	1.0539	1.0500	1.1494	0.9169	0.9135
Set-3	1.0517	1.0881	1.2214	0.8610	0.8908
Set-4	1.0326	1.0960	1.1598	0.8904	0.9450
Set-5	0.9997	1.0967	1.1981	0.8344	0.9153
Set-6	1.0294	1.0533	1.1453	0.8988	0.9197
Mean	1.04	1.079	1.18	0.88	0.914
SD	0.02	0.022	0.03	0.03	0.018
% CV	2.08	2.00	2.81	3.34	2.02

Table No.9: Matrix effect of KML and QRN.

The LQC samples in each blank biological matrix source were injected by external spiking of the extracted blank matrix.

Stability studies

Stability studies were accomplished for freeze-thaw and Bench-top at LQC and HQC levels for KML and QRN. Long- and short- term stability was performed at MQC levels for KML and QRN, respectively.

Table No.10(a) and Table No.10(b) depict KML and QRN's stability data at different condition.

Table No.10(a): Solution Stability data for KML.

Stability (n = 6)	Quality Control	Mean (ng/mL) ± SD	% CV	% Accuracy ± SD
Fresh LQC (112.5 ng/mL)	LQC	104.76 ± 3.27	3.12	93.12 ± 2.91
Fresh HQC (865 ng/mL)	HQC	792.22 ± 19.17	2.42	91.59 ± 2.22
Freeze-thaw (3 Cycle)	LQC	103.91 ± 1.42	1.36	92.36 ± 1.26
	HQC	786.29 ± 20.14	2.56	90.90 ± 2.33
Benchtop Stability (8hr Room Temp)	LQC	101.31 ± 1.45	1.46	90.06 ± 1.32
	HQC	785.30 ± 13.58	1.73	90.79 ± 1.57
Short-term Stability (MQC = 350 ng/mL)	MQC (Fresh)	324.72 ± 6.63	2.04	92.78 ± 1.89
	MQC	319.85 ± 6.71	2.10	91.38 ± 1.92
Long-term Stability (7 days at -20˚C) (MQC = 350 ng/mL)	MQC (Fresh)	324.72 ± 6.63	2.04	92.78 ± 1.89
	MQC	317.28 ± 6.58	2.07	90.65 ± 1.88

Stability (n = 6)	Quality Control	Mean (ng/mL) ± SD	% CV	% Accuracy ± SD
Fresh LQC (52.5 ng/mL)	LQC	48.25 ± 1.17	2.42	91.91 ± 2.22
Fresh HQC (850 ng/mL)	HQC	794.78 ± 11.23	1.41	93.50 ± 1.32
Freeze-thaw (3 Cycle)	LQC	47.70 ± 0.86	1.80	90.85 ± 1.63
	HQC	780.31 ± 20.24	2.59	91.65 ± 2.38
Benchtop Stability (8hr Room Temp)	LQC	47.37 ± 0.80	1.69	90.23 ± 1.53
	HQC	764.26 ± 10.72	1.40	89.91 ± 1.26
Short term Stability (MQC = 400 ng/mL)	MQC (Fresh)	364.21 ± 5.63	1.89	91.05 ± 1.72
	MQC	363.47 ± 5.63	1.55	90.87 ± 1.41
Long-term Stability (7 days at -20˚C) (MQC = 400ng/mL)	MQC (Fresh)	364.21 ± 5.63	1.89	91.05 ± 1.72
	MQC	361.73 ± 3.38	0.93	90.43 ± 1.85

Table No.10(b): Solution Stability data for QRN.

Conclusion

The developed method is simple, rapid, accurate, and reliable for the analysis of Kaempferol and Quercetin in mice plasma from Kachnardi Yoga, fulfilling all criteria for method validation as outlined in the ICH guideline M10.

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Conflicts of Interest

There are no conflicts of interest.

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Summary

The study aimed to develop and validate a simultaneous RP-HPLC method for the estimation of Kaempferol and Quercetin in the ethanolic extract of Kachnardi Yoga, using mice plasma as a biological matrix.

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