METHOD DEVELOPMENT AND VALIDATION OF KAEMPFEROL BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Reena N. Autade[1], Syed Shoaib Ali[2]

[1] *M.Pharm Quality Assurance, Dr.Vedprakash Patil Pharmacy College, Maharashtra, India*
[2] *Associate Professor, Quality Assurance, Dr.Vedprakash Patil Pharmacy College, Maharashtra, India*

ABSTRACT

Kaempferol, a natural flavonoid compound found in various plant sources, possesses numerous pharmacological properties, including antioxidant, anti-inflammatory, and anticancer activities. In this study, a novel analytical method for the quantitative determination of kaempferol using Reverse-phase high-performance liquid chromatography (RP-HPLC) was developed and validated. The chromatographic separation was achieved on a C18 column using a mobile phase consisting of Acetonitrile: Water with 0.1 % formic acid 50:50 with isocratic elution. The detection was performed at a wavelength of 265 nm. The developed method demonstrated excellent linearity over the concentration range of 10-60 µg/mL with a correlation coefficient (R_2) of 0.9989. The method exhibited satisfactory precision with intra-day and inter-day relative standard deviations (RSD) below 2%. The accuracy of the method was confirmed by recovery studies, with mean recovery values ranging from 99.96% to 100.17%. Furthermore, the robustness and stability of the method were evaluated under various experimental conditions, demonstrating its reliability and reproducibility. The developed HPLC method provides a rapid, sensitive, and reliable means for the quantitative analysis of kaempferol in plant extracts, dietary supplements, and pharmaceutical formulations. This validated method can be effectively employed for routine quality control and pharmacokinetic studies of kaempferol-containing products.

Keyword: - *Kaempferol1 , Flavanoid2, Stability3, and Robustness4 etc….*

1. Introduction

The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice. Validation of analytical methods is also required by most regulations and quality standards that impact laboratories. "Validation is the process of collecting documented evidence that the method performs according to its intended purpose". This is based on analytical experiments performed according to the validation protocols that comply with the international guidelines i.e. ICH guidelines on method validation. The International Conference on

Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is a unique project that brings together the regulatory authorities of Europe, Japan and the United States and experts from the pharmaceutical industry in the three regions to discuss scientific and technical aspects of product registration. Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B) The US Food and Drug Administration (FDA) and US Pharmacopoeia (USP) both refer to ICH guidelines, i.e. these methods should be validated.

1.1 Validation Parameters

Table 1: Important validation parameters suggested by regulatory agencies

Parameter	ICH	USP	ISO 17025
Specificity	**X**	**X**	-
Selectivity	-	-	**X**
Precision	**X**	-	-
Repeatability	**X**	-	**X**
Intermediate precision	**X**	-	-
Reproducibility	**X**	**X**	**X**
Accuracy	**X**	**X**	**X**
Linearity	**X**	**X**	**X**
Range	**X**	**X**	-
Limit of detection	**X**	**X**	**X**
Limit of quantitation	**X**	**X**	**X**
Robustness	**X**	**X**	**X**
Ruggedness	**X**	**X**	-

2. Materials and Methods

Table 2: List of Materials

Sr.No.	Name of Materials	Supplier
1	Kaempferol	Tokyo Chemical Industry (TCI)
2	HPLC Grade Water	S D Fine-Chem Limited
3	0.1 % Formic acid	Dipa chemical Industry
4	Acetonitrile	Dipa chemical Industry
5	Methanol	Dipa chemical Industry

Table 3: List of Equipment's

Sr. No.	Name of Equipment's	Make	Model	Calibration due date
1	Analytical Balance	Wensar	PGB 301	Daily calibration
2	pH Meter	Ri	152-R	Daily calibration
3	Ultra Sonicator	Life care equipment's	3210	5th July 24
4	Filter	Ecotest	NY 0.45µm	-
5	FT-IR	JASCO	FT/IR-4600	-
6	UV Spectrophotometer	Lasany	LI-2702	17 th Aug 24
7	Column	Khromasil 100-5-C8	250X4.6mm, 5µm	-
8	HPLC	Shimandzu	LC-2010 AHT	26 th June 24

2.1 UV Method Development

2.1.1 Preliminary Studies and Spectral Studies of Kaempferol [1]

FT-IR Studies and UV Spectrometry studies of Kaempferol

The FT-IR spectra for both the drugs were recorded by using FT–IR (Brukers Alpha) to confirm the identity of the drugs. Solubility of both the drugs was determined by dissolving the drugs in various solvents varying in their polarity.

Identification by IR Spectroscopy

10 mg of Kaempferol and KBr was mixed properly then carefully triturated in a mortar pestle. Make thin plate, place in IR chamber and IR Spectrum was scanned.

2.2.2 Optimization of UV Spectrometry Conditions and Method Development

Various Solvents like water, Methanol, ethanol and phosphate buffer were used for the optimization of diluents for the Uv method development of Kaempferol. Optimized diluents were used for the preparation of standard solution and further dilutions.

2.2.3 Preparation of Kaempferol Standard Solution

Standard solution was prepared by accurately weighed 10 mg of Kaempferol working standard into a 10 ml volumetric flask, added 10 ml of Methanol: Water (8:2), shake and sonicated to dissolve the content, made up the volume with solvent mixture and filtered through 0.45 micron membrane filter. The solution was further diluted with solvent mixture to obtain the required concentration of standard concentrations (2-12 µg/ml) for Kaempferol.

2.2.4 Determination of λ max (Selection of Wavelength)

The standard solution of Kaempferol was scanned in the wavelength range of 200-400 nm on a UV-Visible Spectrophotometer from this, wavelength corresponding to maximum absorbance (λ max) was found to be 265 nm for Kaempferol

2.2.5 Development of standard curve for the Kaempferol

Various dilutions of Kaempferol from the standard solutions were prepared for the Kaempferol 2 PPM, 4 PPM, 6 PPM,8 PPM,10 PPM and 12 PPM were prepared whereas, by using optimized solvent mixture at the fixed wavelength 265 nm.

2.2.6 UV Method Validation [2]

Developed UV method for estimation of Kaempferol was validated as per ICH guideline for evaluating different parameters like Linearity, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantification (LOQ).

2.3.1 Linearity

Linearity of Kaempferol was established using estimation of absorbance of six different calibration standards and the calibration curve plot.

2.3.2 Accuracy

The solutions prepared i.e., 80%, 100% and 120% solutions were prepared and amounts added and amounts estimated for Kaempferol and the individual recovery and mean recovery values were calculated.

Following formula was used to calculate percent recovery.

% RC= [SPS-S/SP] × 100

Where, SPS= Amount found in the spiked sample

S= Amount found in the sample

SP= Amount added to the sample

% RC= Percent recovery

2.3.3 Precision

Intra- and inter-day precision of the method was established at three concentration levels. Intra-day precision was established by preparing nine different solutions of Kaempferol with concentrations of 2 µg/ml, 6 µg/ml and 10 µg/ml and its analysis at morning, afternoon and evening time. Deviation in results in terms of % relative standard deviation (% RSD) was calculated. Inter-day precision of Kaempferol was established by analyzing the above mentioned solutions at three consecutive days.

2.3.4 Robustness

Robustness of the method was evaluated by changing the solvents. Three different solvents viz. Ethanol, methanol and distilled Acetonitrile were used for dissolving Kaempferol and the absorbance of each was determined. Kaempferol levels in each sample were estimated using pre-defined calibration curve. Results were represented in terms of % RSD.

2.3.5 Ruggedness

Ruggedness of the method was determined by carrying out the analysis of Kaempferol solutions (2 PPM, 6 PPM and 10 PPM) at three different (25°C, 37°C and 60°C) temperatures for 24 hrs and absorbance were noted and % RSD was calculated.

2.3.6 Limit of Detection (LOD)

The LOD of the developed UV method for Kaempferol was calculated using the following formula

LOD=3.3×SD/S

Where, SD= standard deviation of Y- intercepts

S=Slope

2.3.7 Limit of Quantitation (LOQ)

The LOQ of the developed UV method for Kaempferol was calculated using following formula

LOQ=10×SD/S

Where, SD= standard deviation of Y- intercepts

S=Slope

2.4 Method Development by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) [03-05]

The objective of this experiment was to developed simple, accurate, sensitive, reproducible validated HPLC method for estimation of Kaempferol in *Convolvulus prostrates* herb by RP-HPLC.

2.4.1 Preparation of Mobile Phase:

Prepared a mixture of Water and Acetonitrile in the ratio of 70:30 v/v mixed well and degassed it.

2.4.2 Preparation of Standard Stock Solution:

Kaempferol (10 mg) were accurately weighed and transferred into a 10 ml clean, dry volumetric flask and about 7ml of diluent was added and sonicated to dissolve the drugs completely and the volume was made up to the mark with the same solvent. (Stock solution I) Later, 5ml of solution was pippetted out from the above stock solution into a 25 ml volumetric flask and the volume was made upto the mark with the diluent (stock solution II) Further 1.5 ml of solution was pippetted out from stock solution II into a 10 ml volumetric flask and diluted to the mark with diluent (stock solution III)

2.4.3 Optimization of Chromatographic Conditions and Method Development

Several chromatographic runs for mixture of Kaempferol were taken in various combinations of mobile phase. Proper selection of the method depends upon the nature of the sample (ionic/ionizable/neutral molecule, its molecular weight and solubility). Here, the reverse phase HPLC method was selected for the initial separation owingto its simplicity, suitability, ruggedness and its wider usage. Various mobile phases such as Acetonitrile and water(30:70), Acetonitrile and water (40:60), were tried. Finally, Water and Acetonitrile in the ratio of 50:50 was selected as mobile phase for further chromatographic study.

2.5 Method Validation

Validation study was intended to show that the method is suitable for assay and stability studies of Kaempferol. The method validation was carried out as per ICH guidelines for specificity, forced degradation, precision, linearity, accuracy and stability in analytical solution (ICH 1996, Q2 (R1) ICH, 2005).

2.5.1 System Suitability Study

20 µl of standard preparations in six replicates previously prepared were injected. The chromatograms and the peak responses were measured for Kaempferol. System suitability of the method was evaluated in terms of Retention time (RT), peak area, tailing factor, resolution and theoretical plate.

2.5.2 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Blank solution, individual standard solution and mixed standard solution of Kaempferol (10 µg/ml) were injected into the HPLC system. The peak purity data of Kaempferol was compared there should not be any interference at the retention time of the main peaks.

2.5.3 Precision

- System Precision

Six replicates of the mixed standard solution containing the Kaempferol (30 µg/ml) were injected into HPLC system. Prepared solutions were analyzed as per the proposed method. The mean, SD and % RSD were calculated.

- Method Precision

Six samples containing the known amounts of Kaempferol (30 µg/ml) were analyzed as per test method and the % assay and % RSD for both the drugs was calculated.

- intraday and Inter-day Precision

The intraday precision of the assay method for Kaempferol was evaluated at three concentration levels prepared from the sample stock solution (20 µg/ml, 30 µg/ml and 40 µg/ml) by performing analysis at an interval of two hrs and 12 hrs. The inter-day precision study was also performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels as used for intraday study.

2.5.4 Accuracy (Recovery Study)

Accuracy study of pre-optimized method was calculated using recovery studies by performing the standard addition method. Three levels of percent i.e. 80, 100 and 120 % amount was added externally to the solutions with predefined amount of (20 µg/ml, 30 µg/ml and 40 µg/ml) and the % recovery was calculated.

% Recovery = A/B+C x 100

A = Total drug estimated (mg)

B = Wt. (mg) of drug contributed by tablet powder
C = Amount of pure drug added (mg)

2.5.5 Linearity and Range

Linearity for the Kaempferol was determined by preparing the standard solutions at five concentrations in six replicates levels in the range of 10-60 µg/ml from the stock solutions. 20 µl of each solution was injected into the HPLC system and the peak area of the chromatogram obtained was noted. The mean area with its standard deviation and % relative standard deviation of peak areas were calculated. Mean AUC was plotted against concentration to obtain the calibration curve. Regression equations, correlation coefficients were computed from calibration curves

2.5.6 Stability in Analytical Solution

Stability of Kaempferol in analytical solution was verified by analyzing the sample (20 µg/ml, 40 µg/ml) in six replicates before and after 24 hrs by storing in refrigerator (8 °C) and at room condition. The % assay was calculated from the peak areas of Kaempferol.

2.5.7 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ for Kaempferol were calculated from slope and standard deviation of the response for Kaempferol. The LOD and LOQ were determined using equations.

$$LOD=3.3\times SD/S$$

$$LOQ=10\times SD/S$$

Where; σ = Standard deviation of response,

S = Slope of calibration curve

2.5.8 Robustness

Pre-analyzed sample solution containing mixture of 10 µg/ml of Kaempferol was prepared and analyzed as per proposed method by changing the flow rate to 1.2 ml/min and 0.8 ml/min. The system suitability parameters and

peak areas (or % assay) was evaluated in each condition and the results were compared with method precision results.

2.5.9 Ruggedness

Ruggedness of the method was determined by carrying out the analysis of Kaempferol (20 PPM, 30 PPM and 40 PPM) at three different (25°C, 37°C and 60°C) temperatures and area were noted and % RSD was calculated.

3.0 Results and Discussions

3.1.1 Preliminary Studies and Spectral Studies of Kaempferol

FT-IR Studies and UV Spectrometry studies of Kaempferol

Table 5: Observed Group Frequencies of Kaempferol by FT-IR

Name of Drug	Standard IR Range (cm^{-1})	Observed Frequencies in Kaempferol	Functional group Present
Kaempferol	3200-3600	3321.56	Hydroxyl (OH)
	1680-1750	1690.49	Carbonyl (C=O)
	3200-3000	3152.03	Aromatic C-H Stretching
	1300-1000	1283.81	Allylic C-H Bending

3.1.2 Optimization of UV Spectrometry Conditions and Method Development

Kaempferol: Observations

It is found that Kaempferol is soluble in Methanol, Ethanol, and Acetonitrile so, we had tried the solvent mixture of Methanol: water (50:50) found to be stable system so for Uv method development the mixture used.

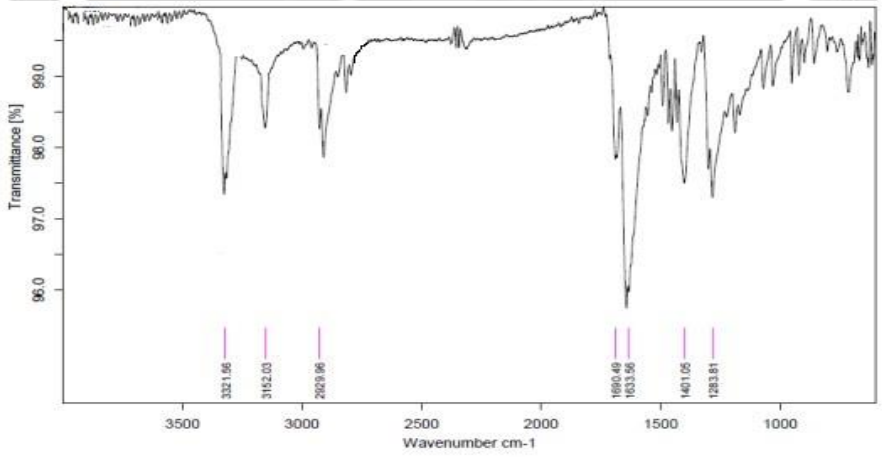

Figure 6: FT-IR Spectra of Kaempferol

The preliminary identification was carried out by recording the FTIR spectrum for Kaempferol. The observed group frequencies are tabulated in table. From the overlain spectrum Kaempferol 265 nm was selected as wavelength for chromatographic method development.

Determination of λ max (Selection of Wavelength) of Kaempferol

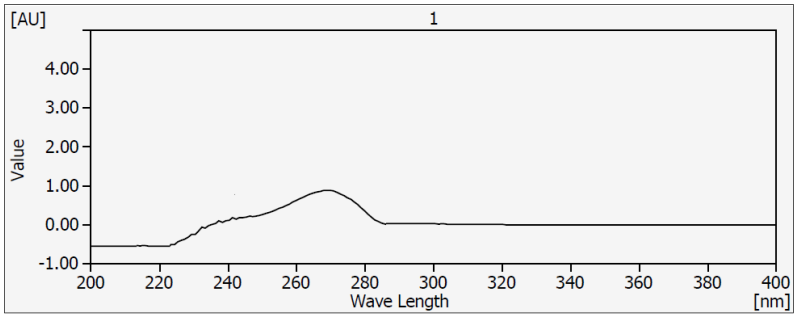

Figure 7: Absorbance maxima of Kaempferol

Absorbance maxima of Kaempferol were found to be on 265. The calibration curve of the drug was developed by using these maxima as fixed wavelength.

3.1.3 Development of standard curve for the Kaempferol

Kaempferol

The calibration curve of Kaempferol was performed and graph plotted concentration vs. absorbance. The absorbance values of different concentration were noted. The regression equation was found to be y = 0.0983x-0.0015, with R^2 value of 0.9993. The graph was found to be linear.

Table 6: Concentration range and respective absorbance of Kaempferol

Sr No.	Concentration (ppm)	Absorbance
1.	2	0.184
2.	4	0.395
3.	6	0.597
4.	8	0.787
5.	10	0.991
6.	12	1.164

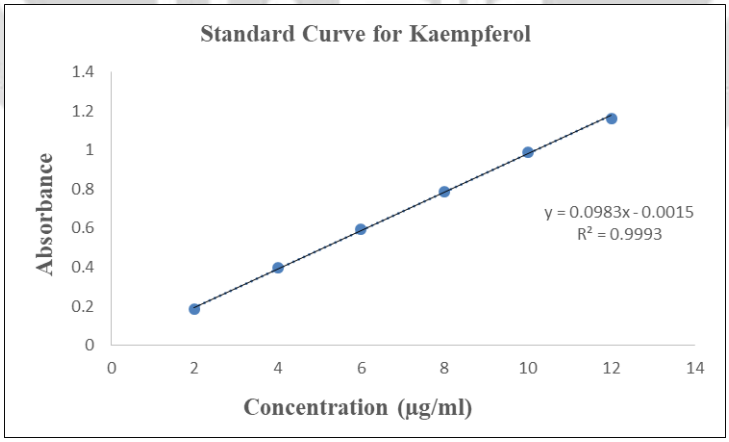

Figure 8: Standard Curve for Kaempferol

3.1.4 Method Validation for UV method development

Linearity

For the linearity of the Kaempferol six point calibrations curve were plotted in a concentration range of 2-12 (µg/ml). From the linearity study it was observed that the drug was found to be linear in the concentration range and the linear regression equation was y = 0.0983x-0.0015with correlation coefficient 0.9993.

Accuracy

Accuracy of the proposed UV method for Kaempferol was verified by conducting the recovery studies by using standard addition method. Standard drug concentration at three different percent levels was added to known amount of Kaempferol. The percent recovery of added standards was calculated (Table 7). The results showed better % mean recovery for respective percent levels. The % mean recovery values are closer to 100% showed high accuracy of the proposed UV analytical method.

Table 7: Evaluation data of Accuracy study of Kaempferol

	Kaempferol				
Levels (%)	**Origin Concentrations (µg/ml)**	**Amount added (µg/ml)**	**% Recovery**	**Mean % Recovery**	**% RSD**
80	6	4.8	98.99	100.00	0.886
100	6	6	100.34		
120	6	7.2	100.67		
80	8	6.4	100.25	100.51	0.790
100	8	8	99.87		
120	8	9.6	101.40		
80	10	8	99.80	99.23	0.716
100	10	10	99.29		
120	10	12	100.71		

3.1.5 Precision

Intra-day and inter-day precision study of Kaempferol were evaluated for the 6 µg/ml, 8 µg/ml and 10 µg/ml. Absorbance mean, percent assay and percent RSD were calculated for the intra-day as well as inter-day precision study (Table 8).

Table 8: Evaluation data for Intra-day and Inter-day study of Kaempferol

Intra-day	**Morning**			**Afternoon**			**Evening**		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
6	0.589	99.32	0.772	0.590	100.65	0.715	0.588	99.45	0.846
8	0.592	100.00	0.253	0.587	99.64	0.264	0.591	100.45	0.267
10	0.598	100.40	0.403	0.592	100.84	0.432	0.592	99.87	0.327
Inter-day	**Day 1**			**Day 2**			**Day 3**		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
6	0.591	99.32	0.676	0.589	99.43	0.489	0.591	99.54	0.640
8	0.788	99.61	0.380	0.789	9974	0.253	0.787	99.40	0.529
10	0.990	100.06	0.408	0.991	100.80	0.924	0.993	99.79	0.266

3.1.6 Robustness

Robustness study was evaluated by using three different solvent. The method was found to be robust as indicated by the % RSD values which are less than 2%. (Table 09)

Table 9: Evaluation data for Robustness of Kaempferol

Kaempferol			
Concentration (µg/ml)	**Solvents**	**Absorbance**	**% RSD**
8	Ethanol	0.786	0.319
8	Methanol	0.791	0.412
8	Methanol: Water (50:50)	0.788	0.328

3.1.7 Ruggedness

Ruggedness study of drug was carried out at the three different temperature levels. From the results it was found that the method was rugged showing the % RSD value less than 2%. (Table 10)

Table 10: Evaluation data for Ruggedness of Kaempferol

Kaempferol			
Concentration (µg/ml)	**Temperature (°C)**	**Absorbance**	**% RSD**
40	25	0.791	0.570
40	37	0.790	0.387
40	60	0.788	1.152

3.1.8 Limit of Detection (LOD) & Limit of Quantification (LOQ)

Form the results it was found that LOD & LOQ are in the sub-microgram level, which indicates the sensitivity of the method. (Table 12)

Table 11: Evaluation data for LOD & LOQ of Kaempferol

Kaempferol	
LOD	0.357 PPM
LOQ	1.154 PPM

3.2 Method Development by Reverse Phase High Performance Liquid Chromatography

Optimization of Chromatographic Conditions and Method Development

In order to achieve the optimized chromatographic conditions to separate and quantify Kaempferol one or two parameters were modified at each trial and chromatograms were recorded with all specified chromatographic conditions. Various trials [figure 8-11] were carried out to finalize the optimized chromatographic conditions mentioned in the Table 13. Poor resolution, bad peak shapes, disturbances in base line were the few reasons of the rejections of the trials.

Table 12: Various Trials and Optimization of Chromatographic Conditions

Trial No	HPLC System	Chromatographic Conditions	Observation	Remarks
1	HPLC (Shimazdu LC 2010 with Uv detector)	**Mobile Phase-**Acetonitrile:Water 30:70 **Column -** Inertsil C18 (4.6 x 250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-** Isocratic **Column temperature-** Ambient **Wavelength-** 265 nm	Peak shape was not good. Base line isnot clear.	**Rejected**

2	HPLC (Shimazdu LC 2010 with Uv detector)	**Mobile Phase**-Acetonitrile:Water 40:60 **Column -** Inertsil C18 (4.6 x 250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-** Isocratic **Column temperature-** Ambient **Wavelength-** 265 nm	Peak shape was not good. Base line isnot clear.	**Rejected**
3	HPLC (Shimazdu LC 2010 with Uv detector)	**Mobile Phase**-Acetonitrile:Water 50:50 **Column -** Inertsil C18 (4.6 x 250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-** Isocratic **Column temperature-** Ambient **Wavelength-** 265 nm	Peaks shape not much good	**Rejected**
4	HPLC (Shimazdu LC 2010 with Uv detector)	**Mobile Phase**-Acetonitrile:Water with 0.1 % formic acid 50:50 **Column -** Inertsil C18 (4.6 x 250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-** Isocratic **Column temperature-** Ambient **Wavelength-** 265 nm	Peaks shape were good, with good resolution and intensity	**Accepted**

Blank Chromatogram

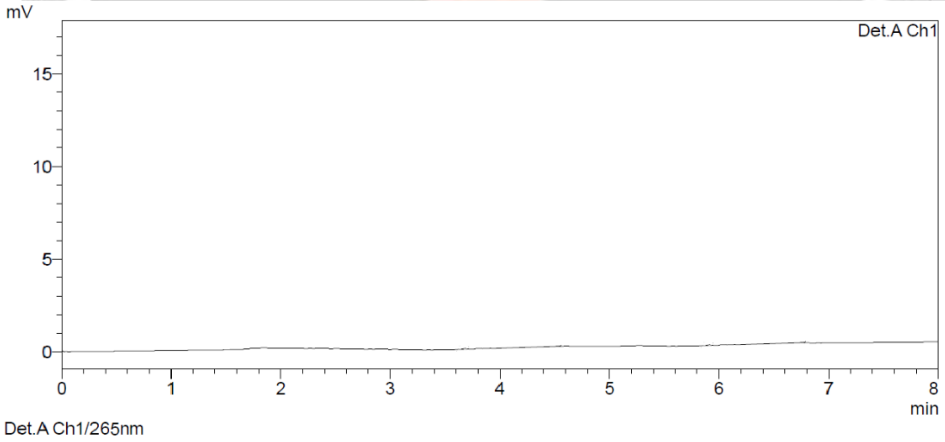

Figure 09: Blank Chromatogram

Trial 1

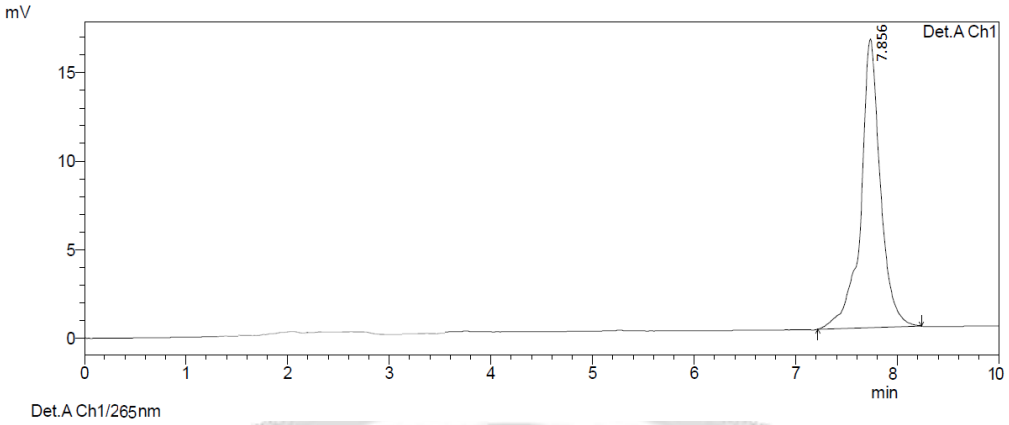

Figure 10: HPLC Chromatogram of Kaempferol for trial 1

Table 13: Evaluation parameter of trial 1

Sr. No.	Name	Retention Time (min)	Area (µV*sec)	Height (µV)
1	**Kaempferol**	7.856	219026	16283

Trial 2

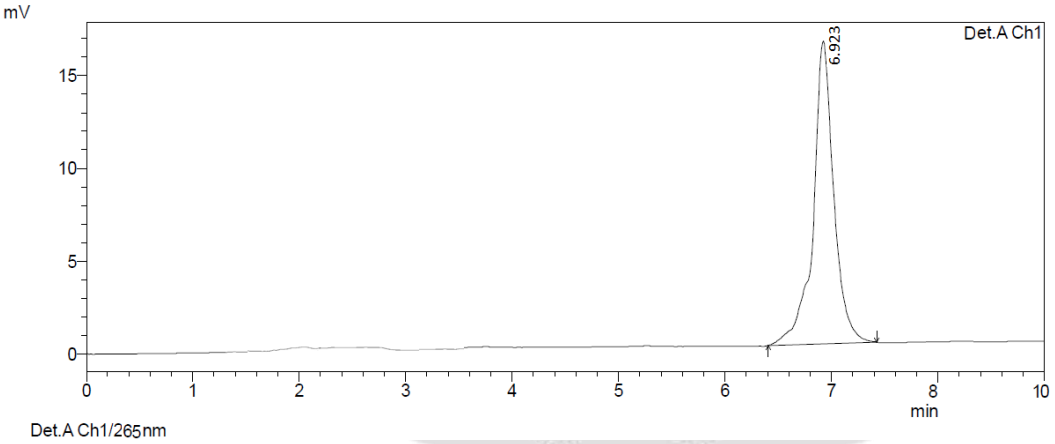

Figure 11: HPLC Fingerprinting of Kaempferol for trial 2

Table 14: Evaluation parameter of trial 2

Sr. No.	Name	Retention Time (min)	Area (µV*sec)	Height (µV)
1	**Kaempferol**	6.923	219146	16345

Trial 3

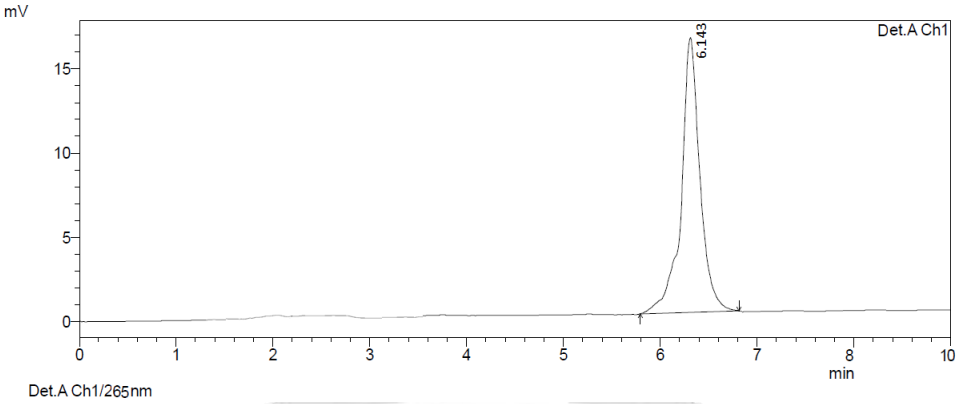

Figure 12: HPLC Fingerprinting of Kaempferol for trial 3

Table 15: Evaluation parameter of trial 3

Sr. No.	Name	Retention Time (min)	Area (µV*sec)	Height (µV)
1	**Kaempferol**	6.143	219264	16314

Trial 4

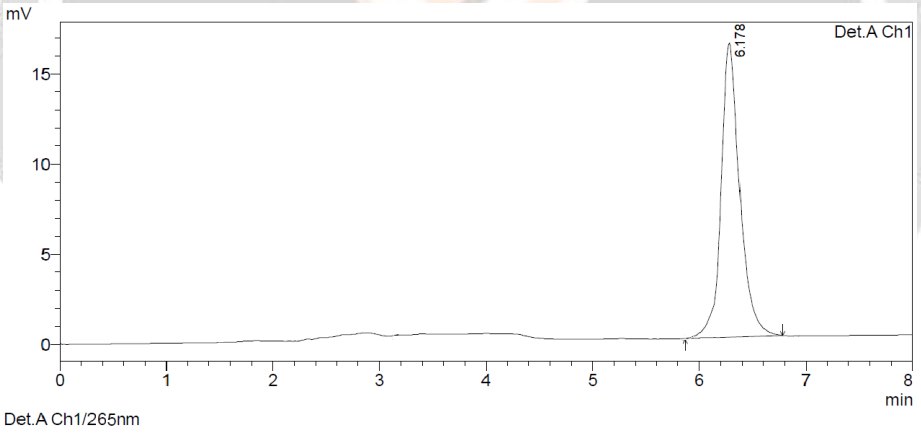

Figure 13: Optimized trial for HPLC Fingerprinting of Kaempferol

Table 16: Evaluation parameter of optimized trial

Sr. No.	Name	Retention Time (min)	Area (µV*sec)	Height (µV)
1	**Kaempferol**	6.178	219078	16234

3.3.1 Method Validation

The following parameters were considered for the analytical method validation of optimized method:

- System Suitability

- Specificity
- Linearity and Range
- Precision
    i) System Precision
    ii) Method Precision
    iii) Inter-day Precision
    iv) Intraday Precision
- Ruggedness
- Accuracy (Recovery)
- Robustness
- Limit Of Detection(LOD)
- Limit Of Quantitation (LOQ)
- Solution Stability

3.3.2 System Suitability

The HPLC method has been developed for the determination of the percentage assay of Kaempferol. The Mobile phase was used, Acetonitrile: Water with 0.1 % formic acid 50:50 with Column Inertsil C18 (4.6 x 250mm, 5µm) at Flow rate of 1 ml/min, Injection Volume was 20µl at 265nm. The chromatograms of standard and blank are shown in figure 16 and 17. The Retention time for Kaempferol was found to be 6.178 min respectively and other parameters like, resolution, tailing factor, and theoretical plates were found to be within acceptable limit.

Table 17: System Suitability Parameters for Kaempferol

Sr. No.	Name	Retention Time*	Area*	USP Tailing*	USP Plate Count*
1	Kaempferol	6.178	219078	1.63	2146

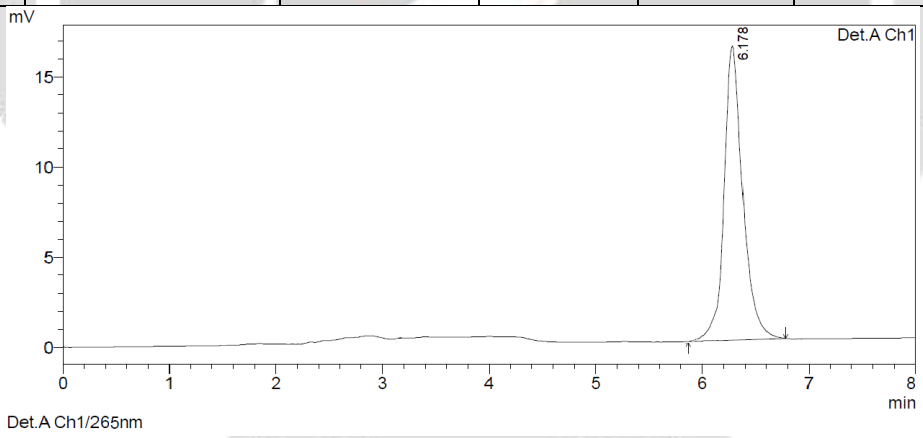

Figure 14: Standard Chromatogram of Kaempferol

Specificity

There was no interference from the blank at the retention time of analyte peaks. The peak purity data of blank solution and standard solution was examined. The peak purity plots are shown in figure 18-22 which reveals the homogenous peaks.

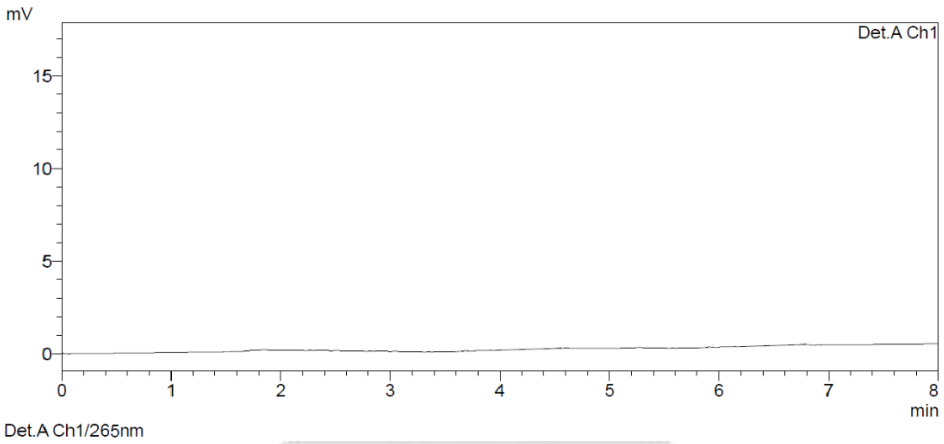

Figure 15: Blank Chromatogram

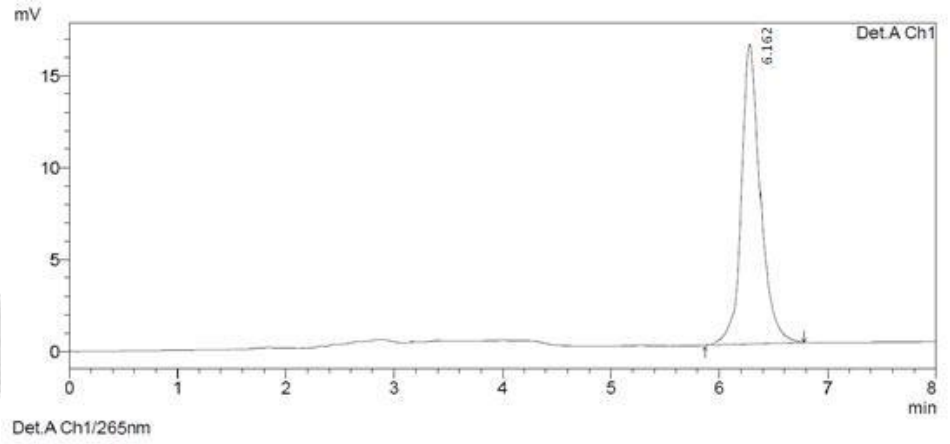

Figure 16: Purity Chromatogram of Standard 1 of Kaempferol

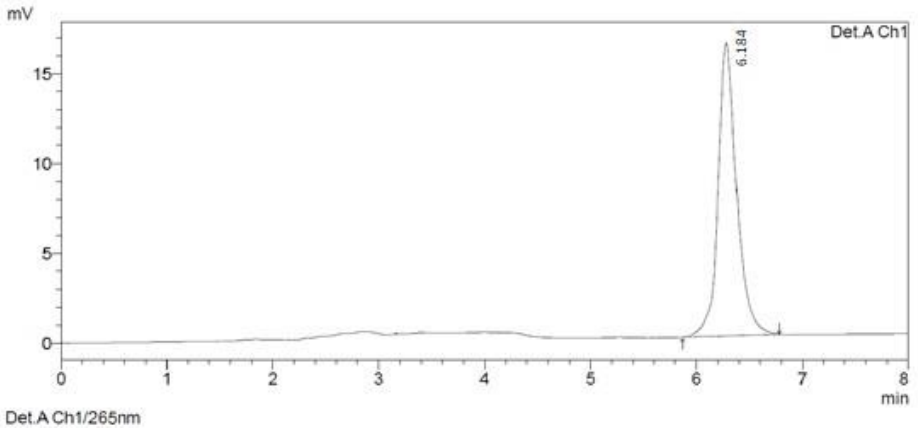

Figure 17: Purity Chromatogram of Standard 2 of Kaempferol

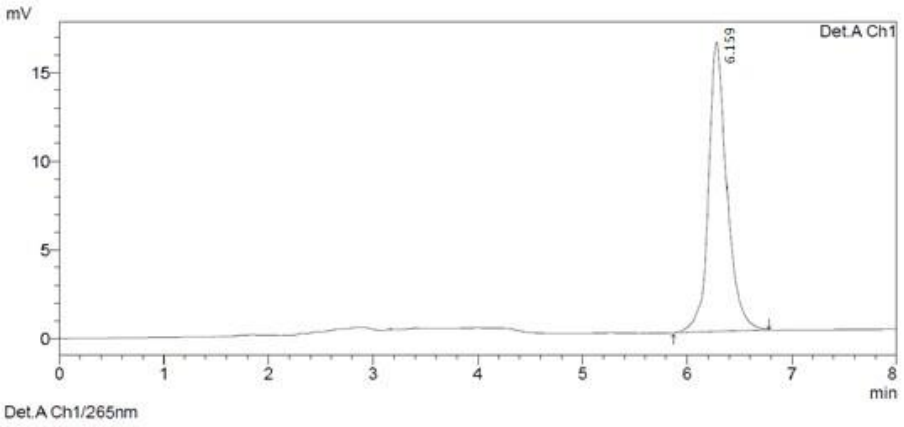

Figure 18: Purity Chromatogram of Standard 3 of Kaempferol

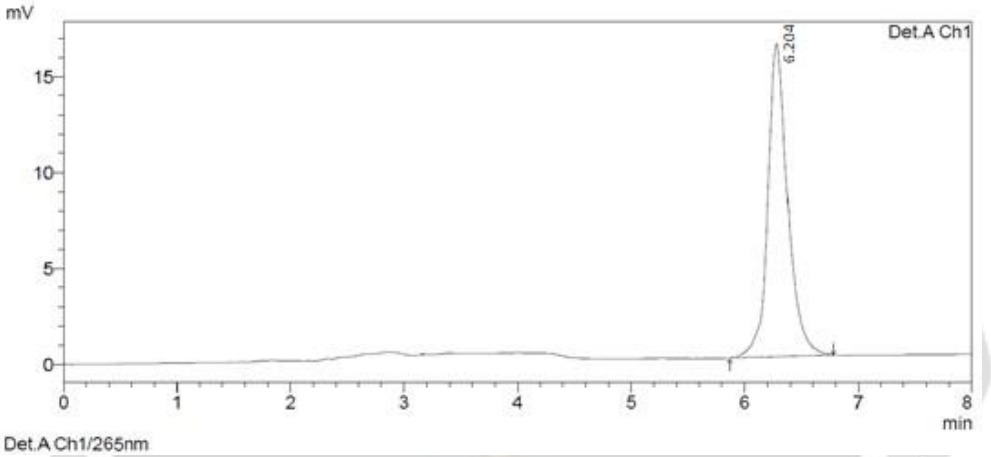

Figure 19: Purity Chromatogram of Standard 4 of Kaempferol

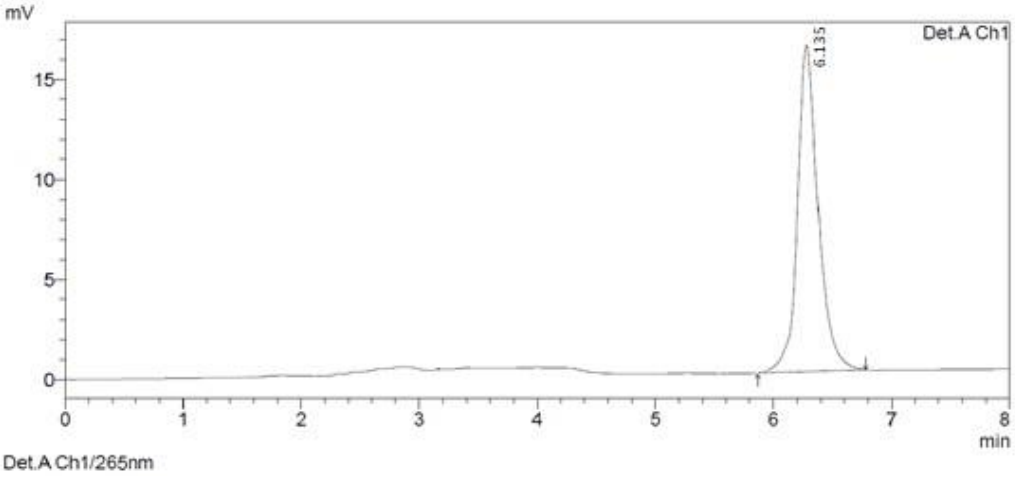

Figure 20: Purity Chromatogram of Standard 5 of Kaempferol

Table 18: System Precision Data of Kaempferol

Sr. No.	Peak areas of Kaempferol
1	219043
2	219148
3	219267
4	219056
5	219171
Mean	**219137**
SD (±)	**91.616**
RSD (%)	**0.0418**
Retention Time (min)	**6.16**
Theoretical Plate (Number)	**2467.6**
Tailing Factor	**0.965**

Precision

a) System Precision

The system precision was performed by measuring the peak response for standard drugs solutions (30 µg/ml) in six replicates. Peak responses, mean, and % relative standard deviation (%RSD) for Kaempferol was found to be 0.0810 %. The results are shown in table 19 and were found well within the acceptable criteria.

Table 19: System Precision Data of Kaempferol

Sr. No.	Peak areas of Kaempferol
1.	745308
2.	745876
3.	745987
4.	746542
5.	746987
Mean	**746140**
SD (±)	**645.047**
RSD (%)	**0.0810**

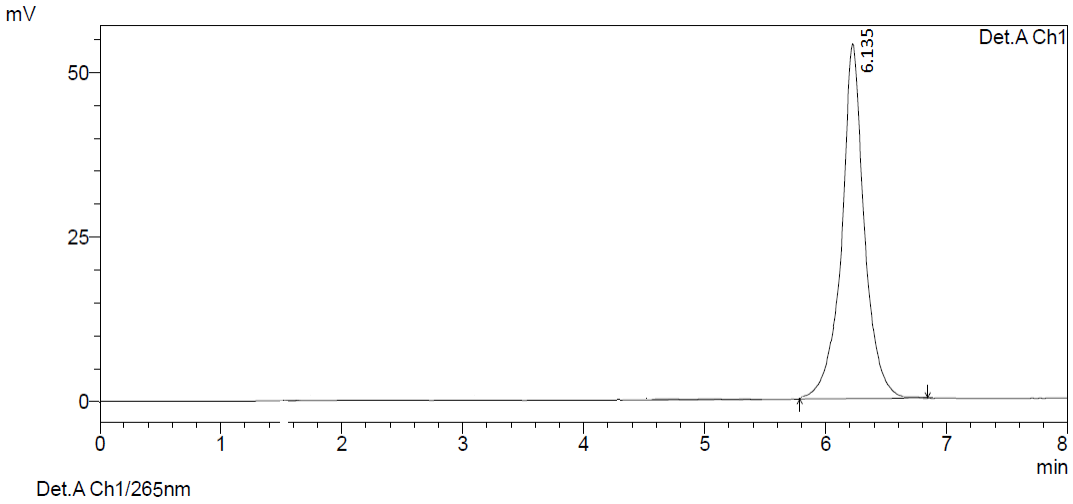

Figure 21: Chromatogram of System precision 1 (30 µg/ml)

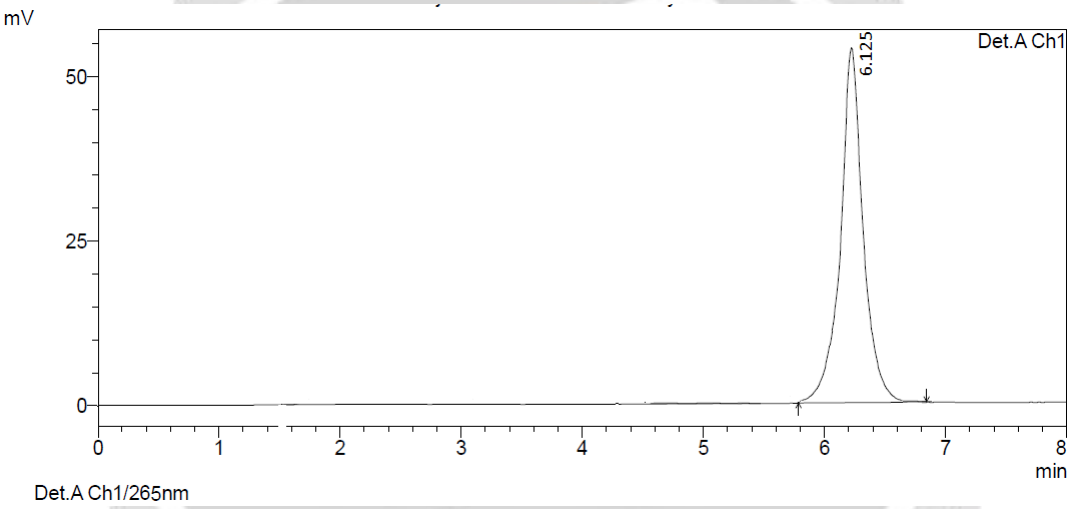

Figure 22: Chromatogram of System precision 2 (30 µg/ml)

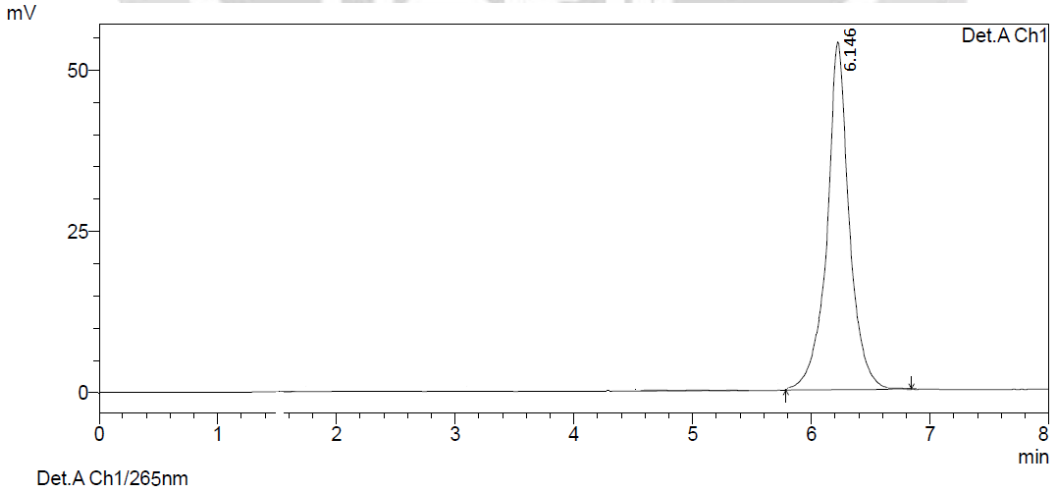

Figure 23: Chromatogram of System precision 3 (30 µg/ml)

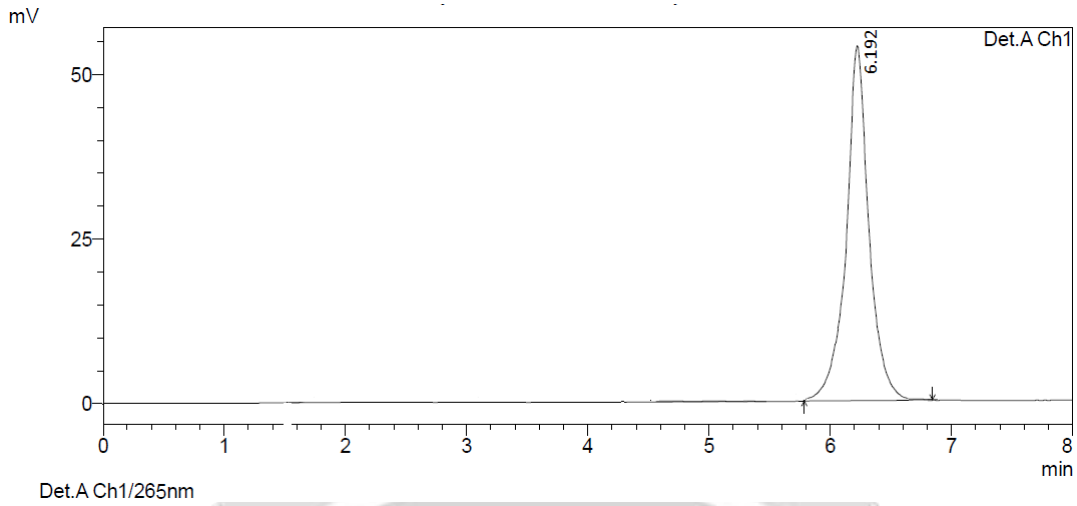

Figure 24: Chromatogram of System precision 4 (30 µg/ml)

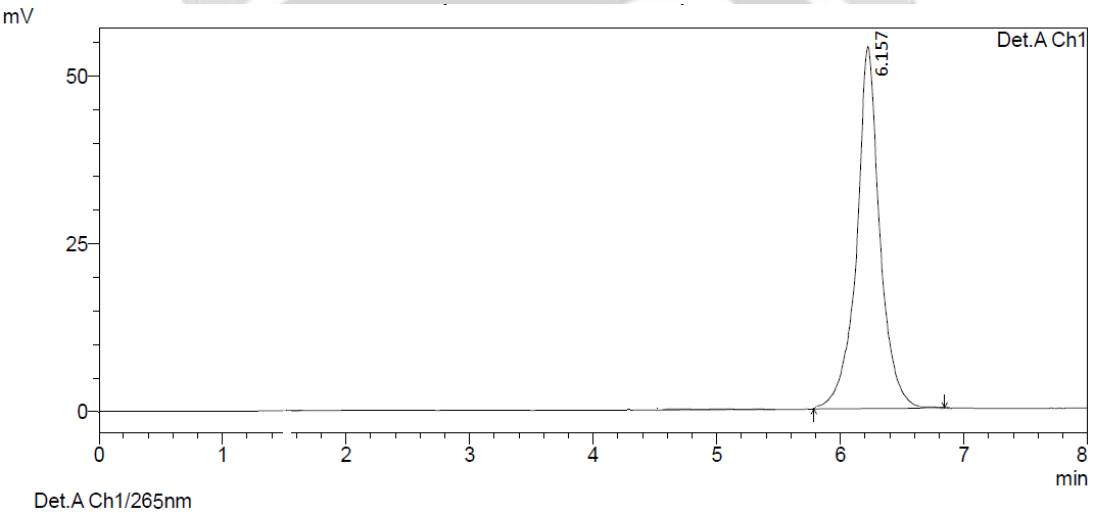

Figure 25: Chromatogram of System precision 5 (30 µg/ml)

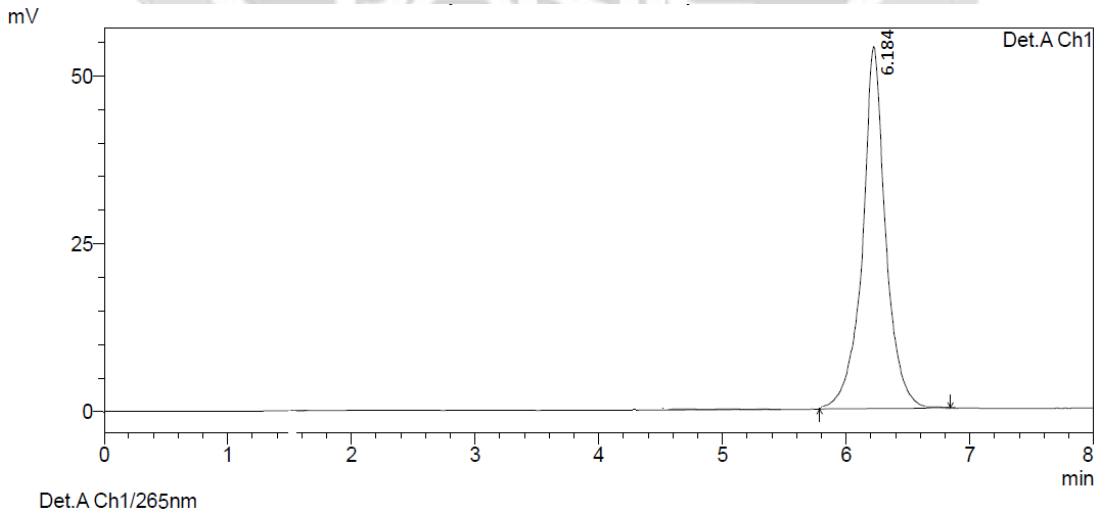

Figure 26: Chromatogram of System precision 6 (30 µg/ml)

b) Intraday and Inter-day Precision

The % RSD in intraday precision for Kaempferol (20, 30, 40 µg/ml) was found to be 0.012, 0.039, 0.0112 % in inter-day precision % RSD for Kaempferol (20, 30, 40 µg/ml) was found to be 0.144, 0.235, 0.106 %. Percent RSD in intraday and inter-day studies were found well within the acceptable limits. The results obtained are mentioned in the table 20 and 21.

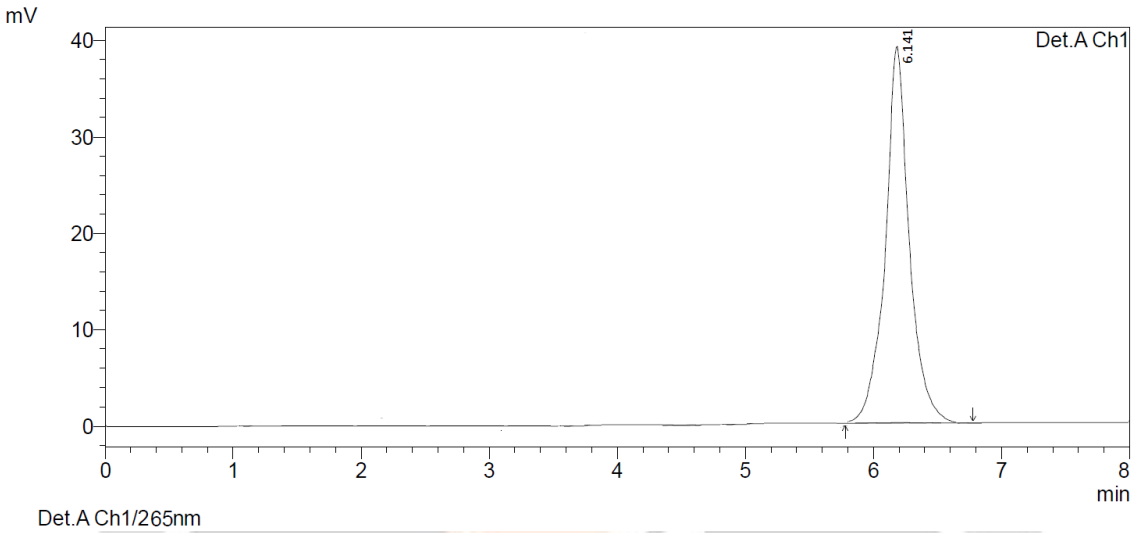

Figure 27: Chromatogram of Intraday precision at 20 µg/ml (Morning)

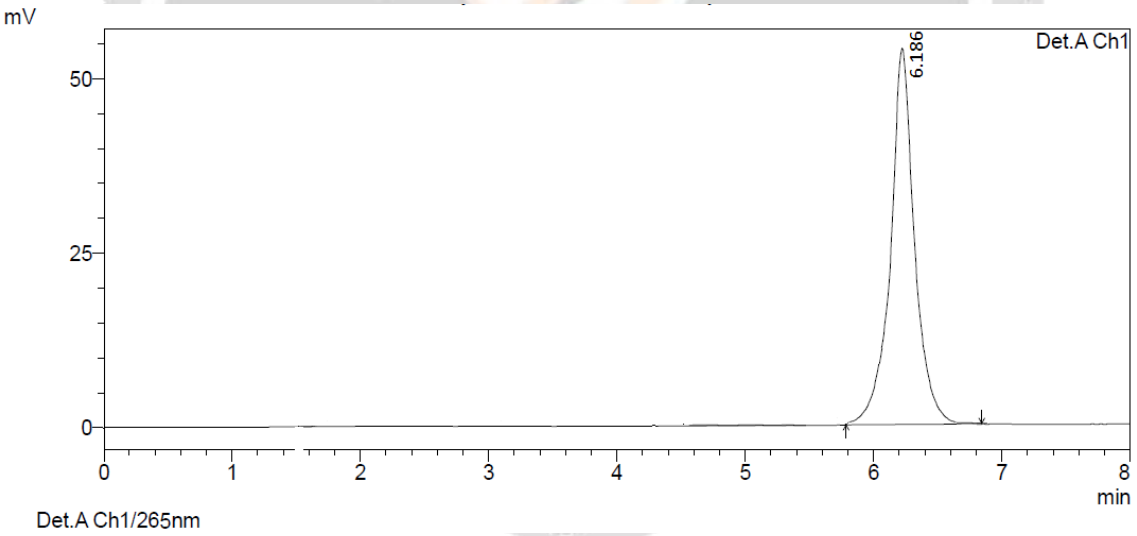

Figure 28: Chromatogram of Intraday precision at 30 µg/ml (Morning)

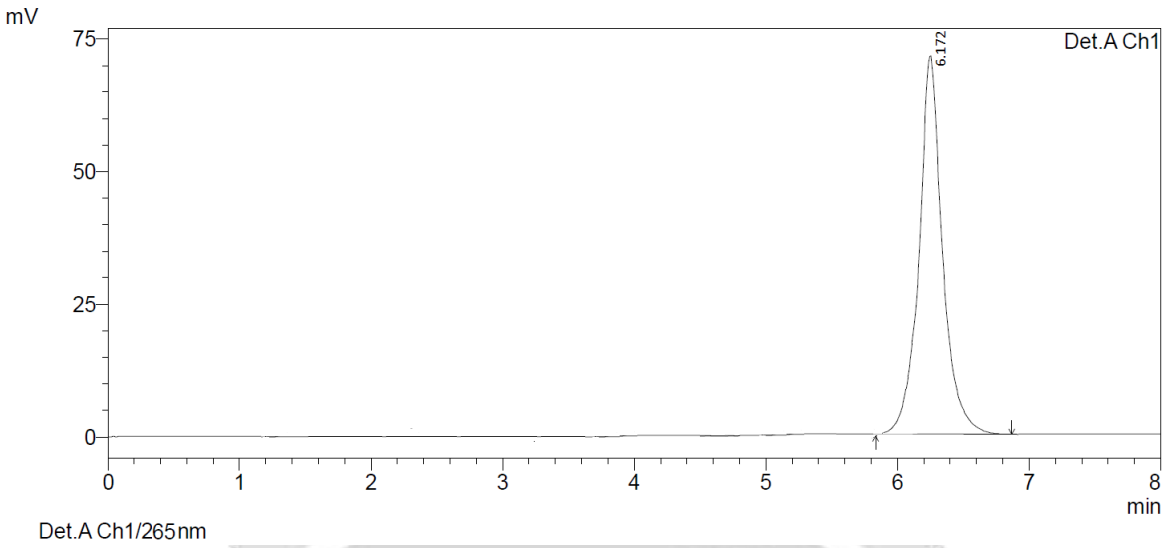

Figure 29: Chromatogram of Intraday precision at 40 µg/ml (Morning)

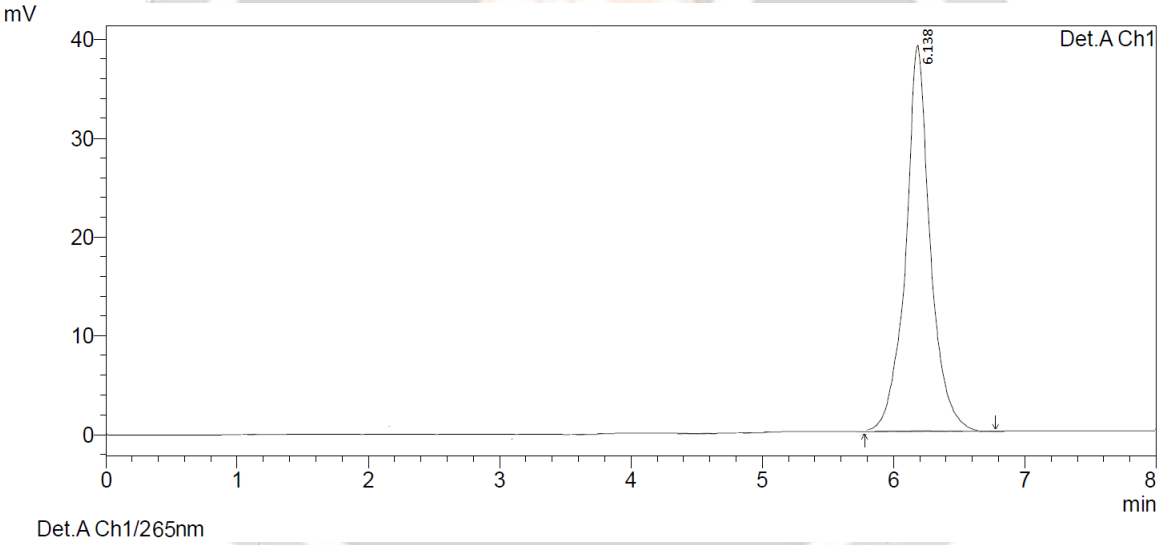

Figure 30: Chromatogram of Intraday precision at 20 µg/ml (Afternoon)

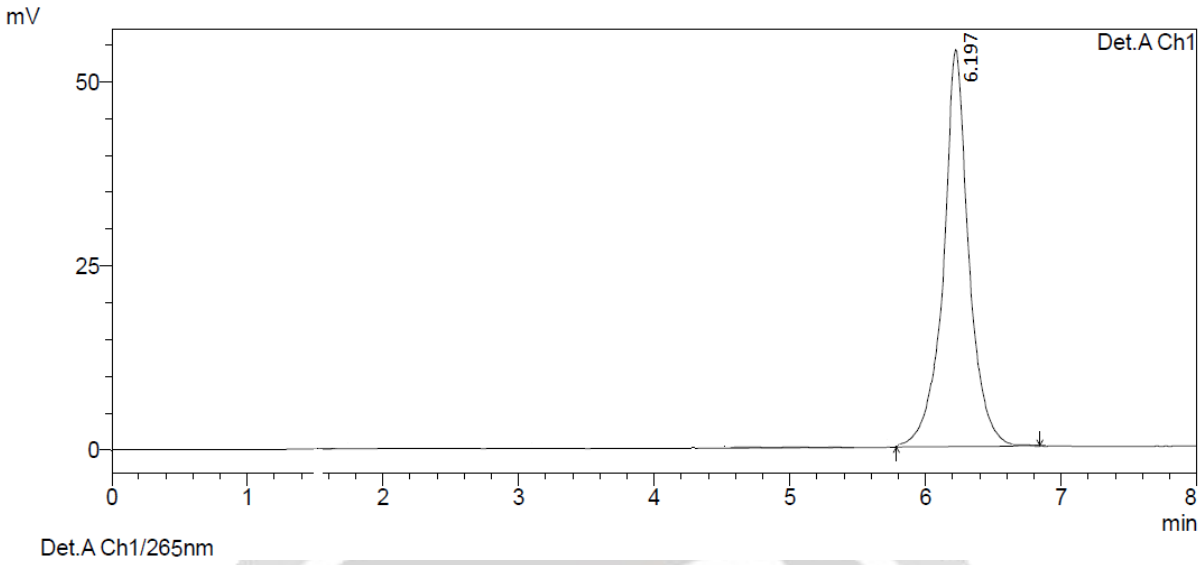

Figure 31: Chromatogram of Intraday precision at 30 µg/ml (Afternoon)

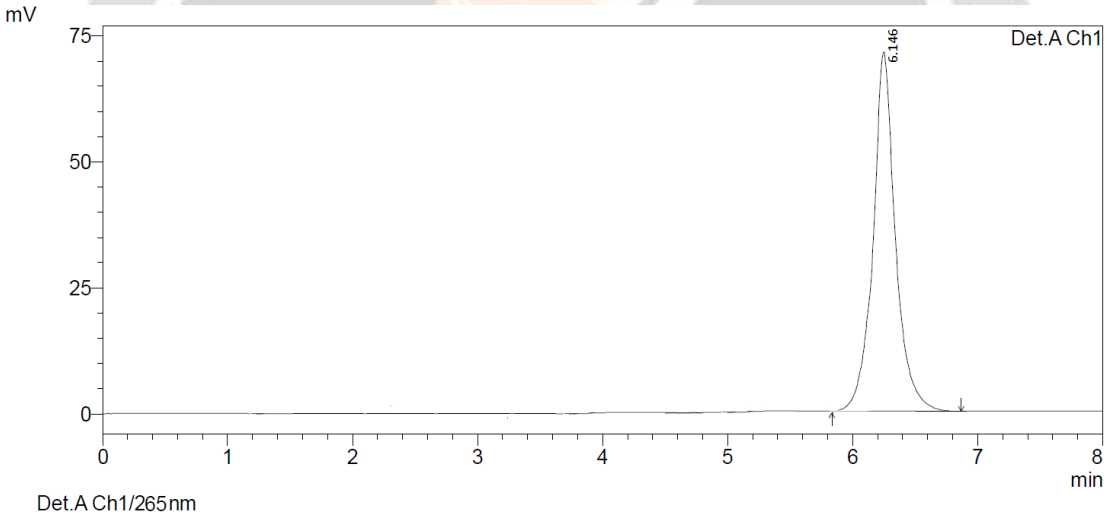

Figure 32: Chromatogram of Intraday precision at 40 µg/ml (Afternoon)

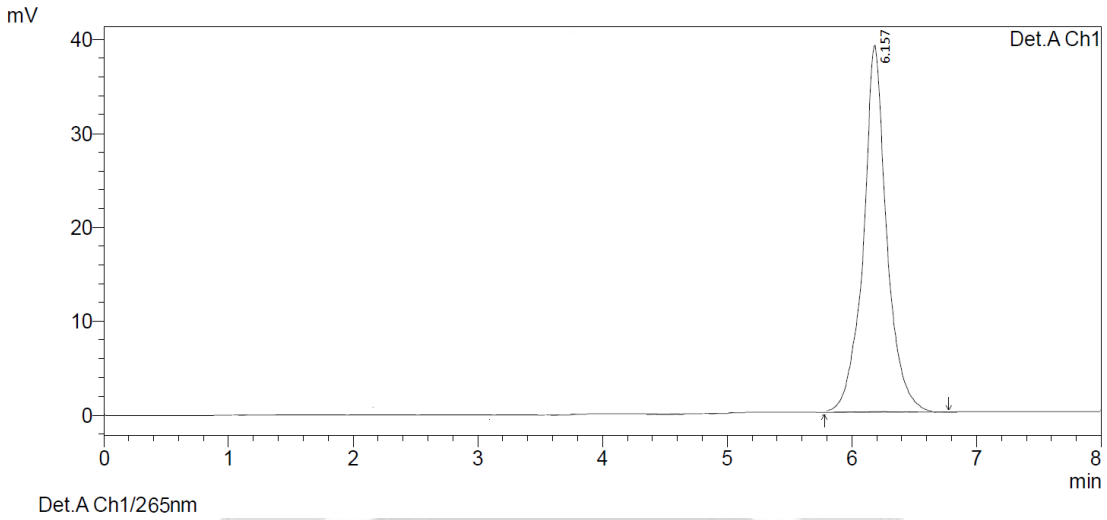

Figure 33: Chromatogram of Intraday precision at 20 µg/ml (Evening)

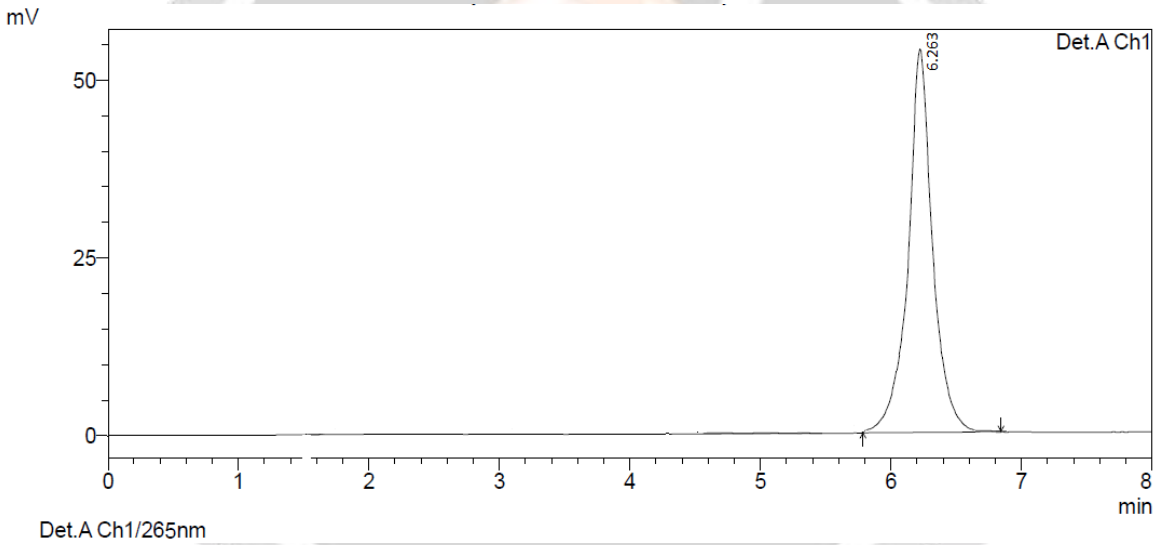

Figure 34: Chromatogram of Intraday precision at 30 µg/ml (Evening)

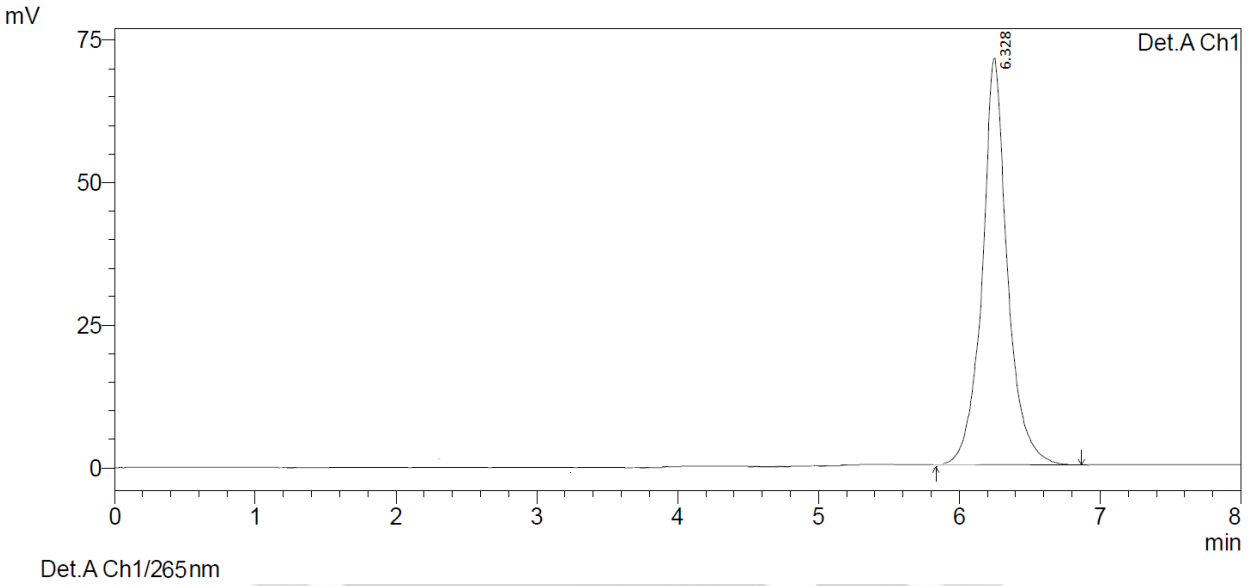

Figure 35: Chromatogram of Intraday precision at 40 µg/ml (Evening)

Table 20: Intraday Precision data of Kaempferol

Kaempferol					
Sr. no.	**Conc. (µg/ml)**	**Area**	**Meanpeak area**	**SD(±)**	**%RSD**
1	20	513560 (Morning) 513578 (Afternoon) 513649 (Evening)	513605	63.571	0.012
2	30	745316 (Morning) 745284 (Afternoon) 745347 (Evening)	745316	31.501	0.0039
3	40	987139 (Morning) 987201 (Afternoon) 987346 (Evening)	987229	106.237	0.0112

Interday Precision:

Interday precision study was performed by analyzing standard solution at three different concentration 40, 60, and 80 PPM on three different consecutive day. The chromatogram of interday precision studies are shown in figure 38 to 46 and results are shown in table 21.

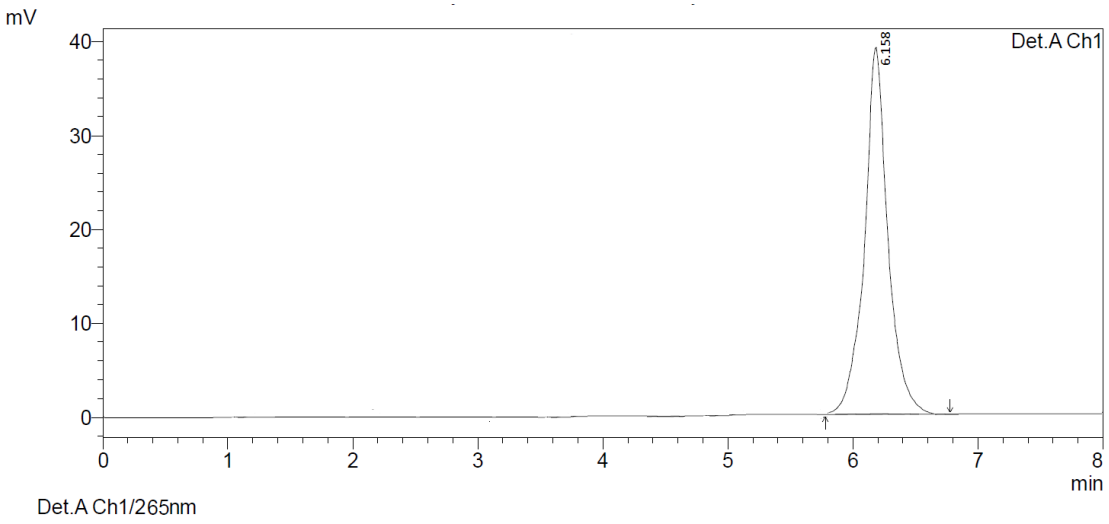

Figure 36: Chromatogram of Interday precision at 20 PPM (Day 1)

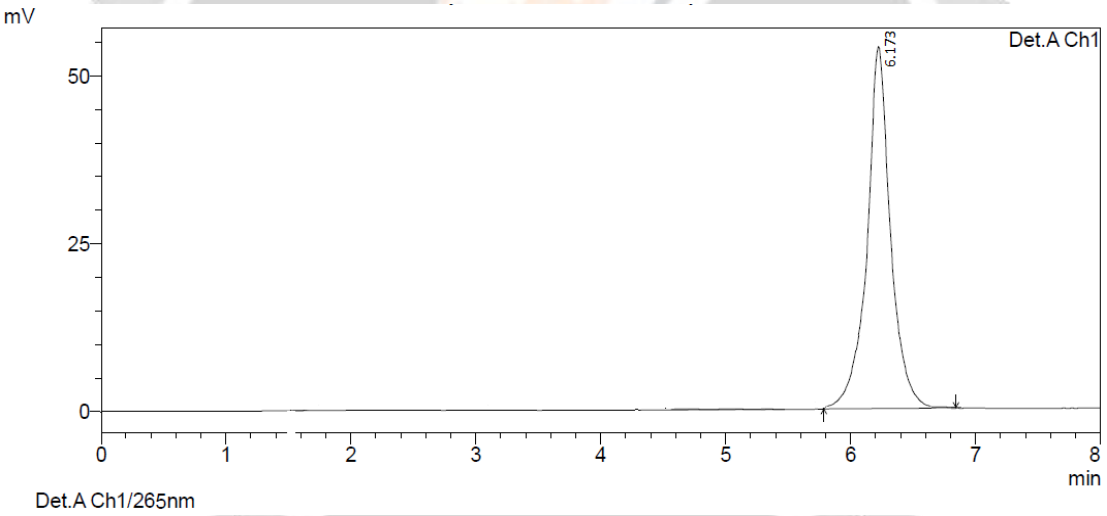

Figure 37: Chromatogram of Interday precision at 30 PPM (Day 1)

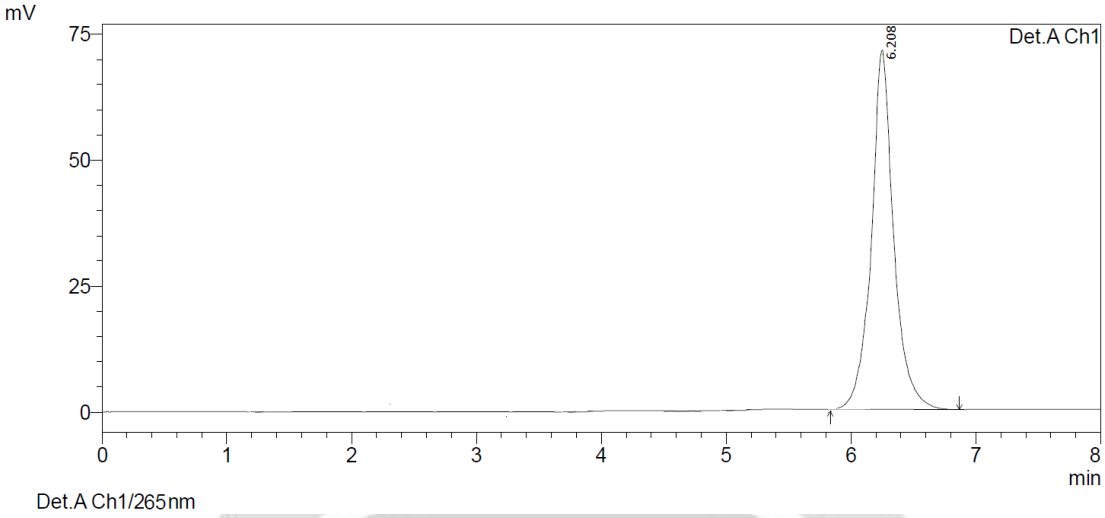

Figure 38: Chromatogram of Interday precision at 40 PPM (Day 1)

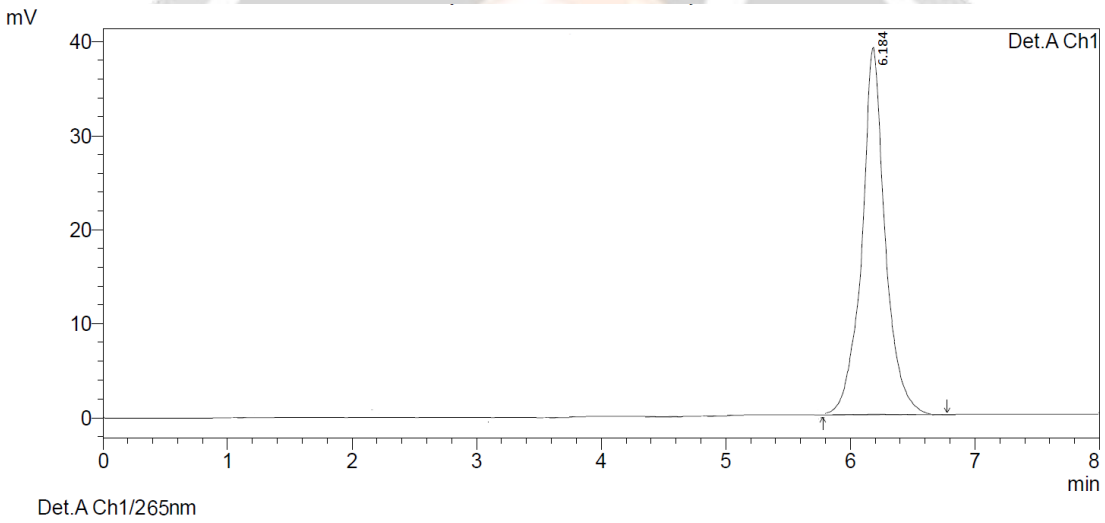

Figure 39: Chromatogram of Interday precision at 20 PPM (Day 2)

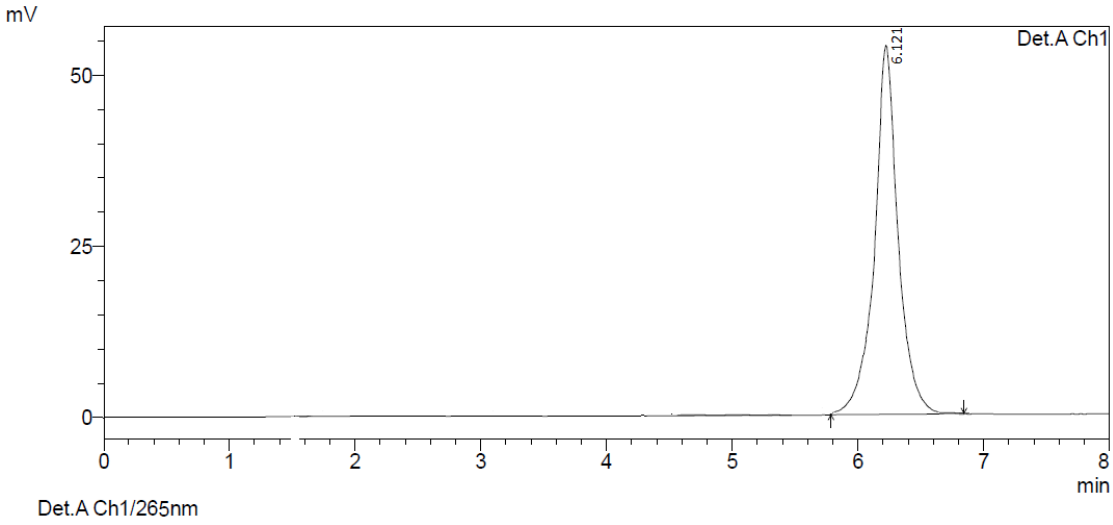

Figure 40: Chromatogram of Interday precision at 30 PPM (Day 2)

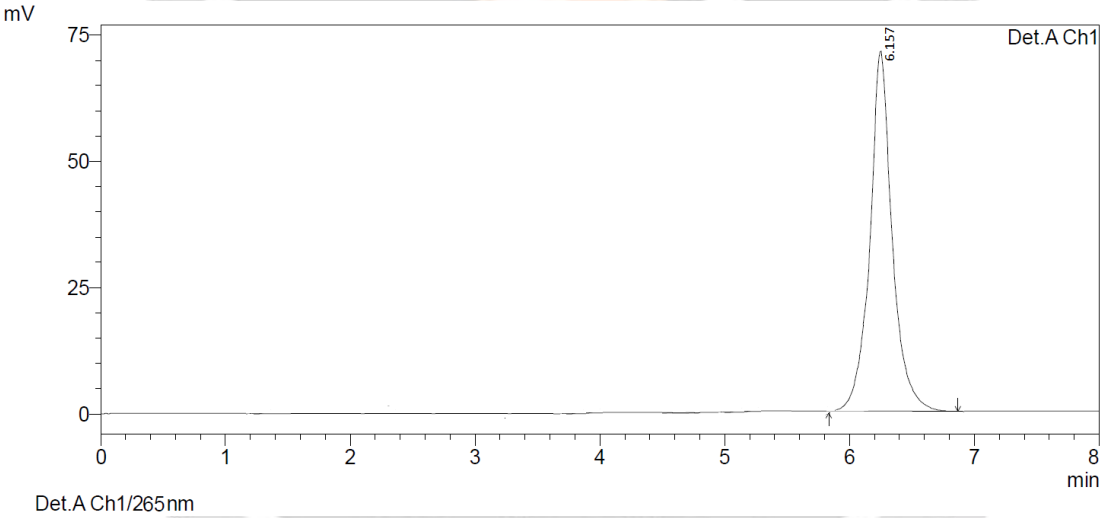

Figure 41: Chromatogram of Interday precision at 40 PPM (Day 2)

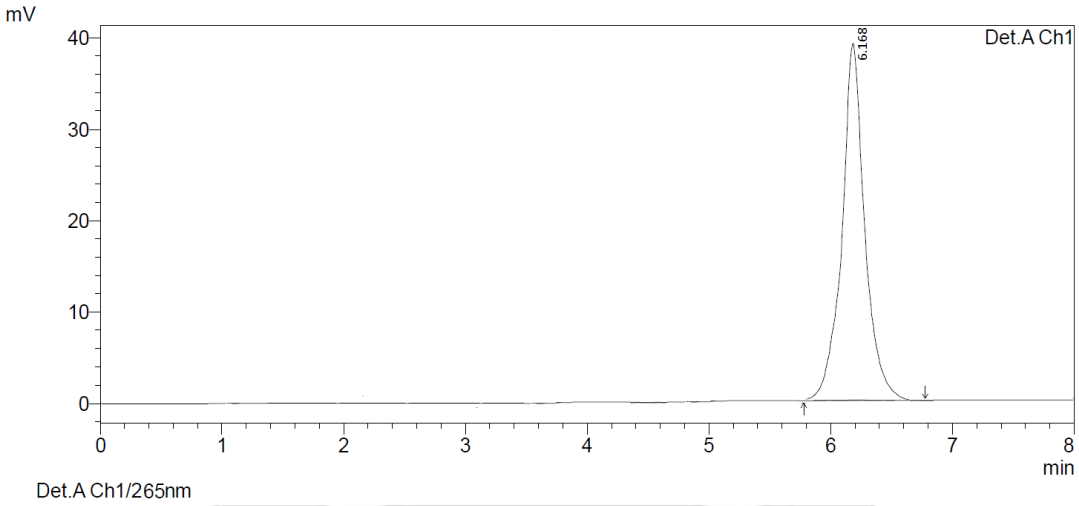

Figure 42: Chromatogram of Interday precision at 20 PPM (Day 3)

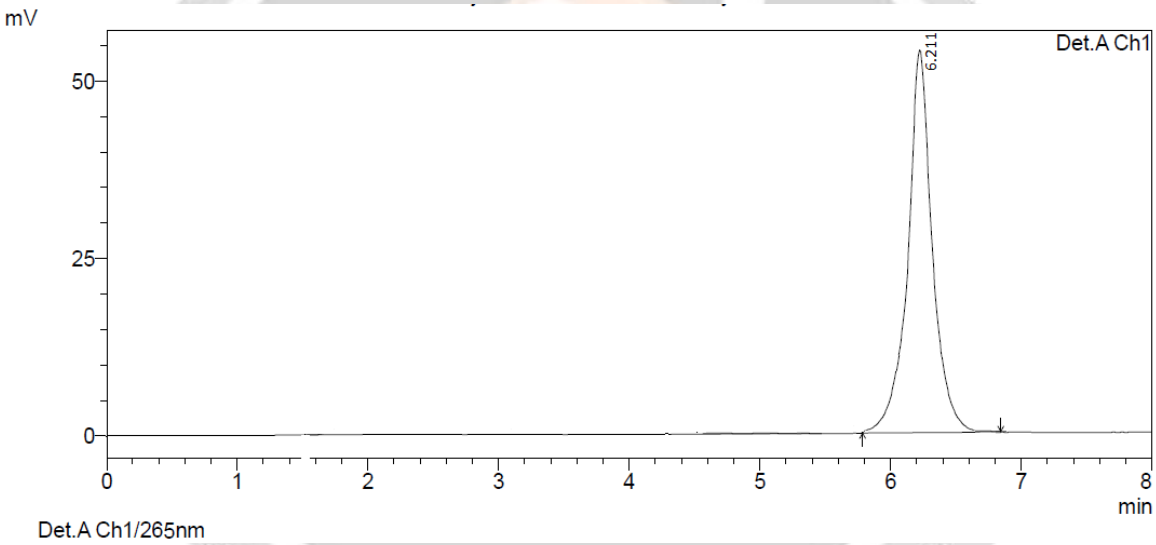

Figure 43: Chromatogram of Interday precision at 30 PPM (Day 3)

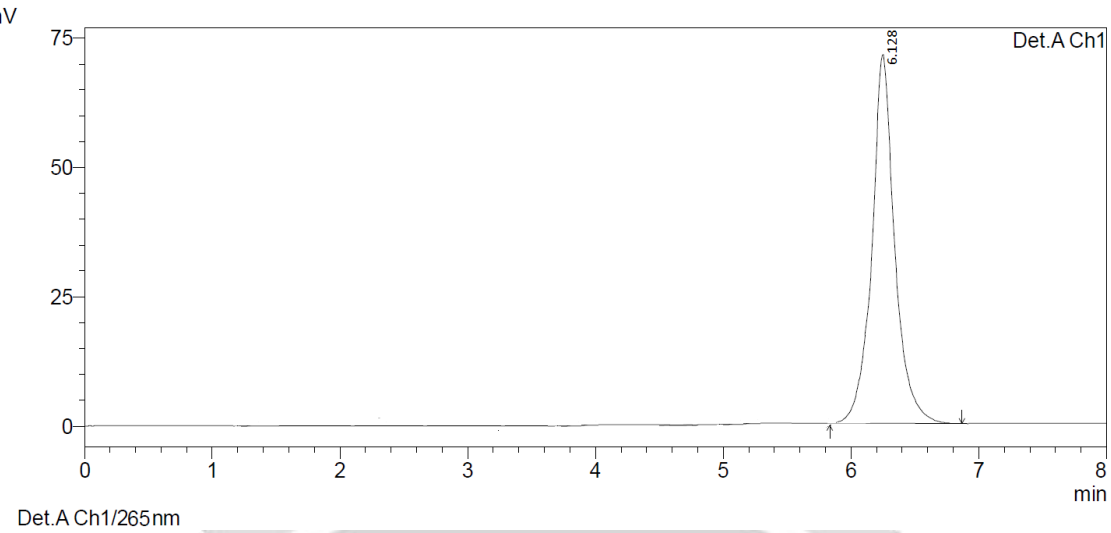

Figure 44: Chromatogram of Interday precision at 40 PPM (Day 3)

Table 21: Inter-day Precision data of Kaempferol

Kaempferol					
Sr. no.	**Conc. (PPM)**	**Area**	**Meanpeakarea**	**SD(±)**	**%RSD**
1	20	513548 (Day 1)	514307	739.353	0.144
		514348 (Day1)			
		515025 (Day1)			
2	30	745317(Day 2)	744322	1869.35	0.235
		745486(Day 2)			
		742167(Day 2)			
3	40	986808(Day 3)	987641	1012.68	0.106
		988768(Day 3)			
		987346(Day 3)			

Accuracy (Recovery Study)

The accuracy of the assay method was evaluated by standard addition method in triplicate at 80%, 100 % and 120% level of the labeled claim and the percentage recovery was calculated. The mean % recovery was found to be 99.96%, 99.82% and 100.17 % respectively for Kaempferol. The results of the recovery study are shown in the table22.

Table 22: Recovery study for Kaempferol

Kaempferol							
Level	**Set**	**Amount added(µg/ml)**	**Amount found(µg/ml)**	**%Recovery**	**Mean**	**SD**	**%RSD**
80%	1	32	32.06	100.19	99.96	0.234	0.234
	2	32	31.99	99.97			
	3	32	31.91	99.72			
100%	1	40	40.00	100.00	99.82	0.275	0.275
	2	40	39.80	99.50			
	3	40	39.98	99.95			
120%	1	48	48.02	100.04	100.17	0.150	0.150
	2	48	48.06	100.13			
	3	48	48.16	100.33			

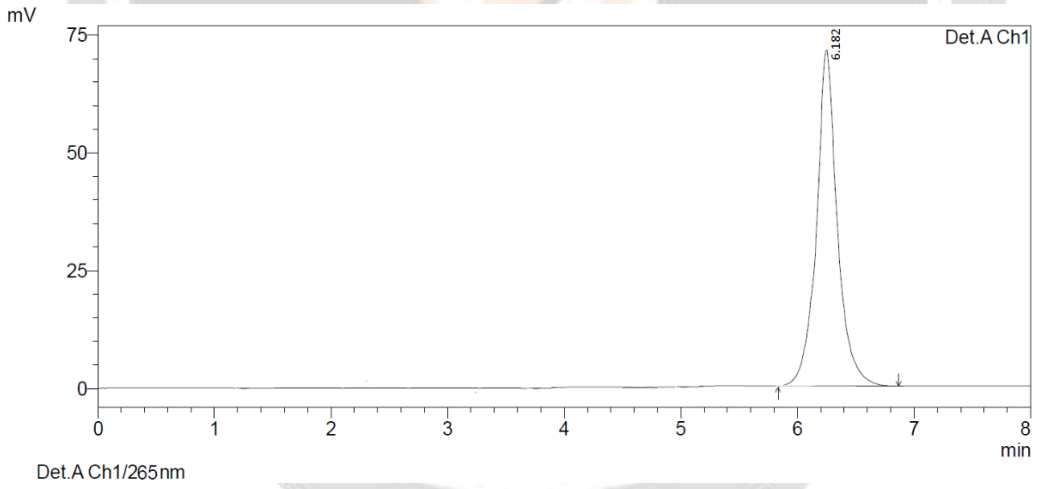

Figure 45: Chromatogram of Kaempferol accuracy at 80% level 1

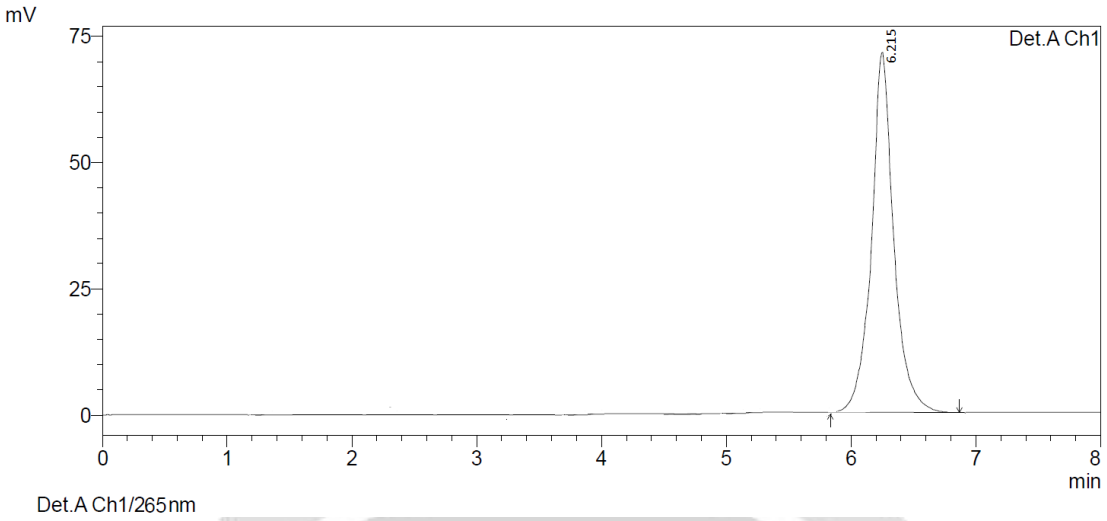

Figure 46: Chromatogram of Kaempferol accuracy at 80% level 2

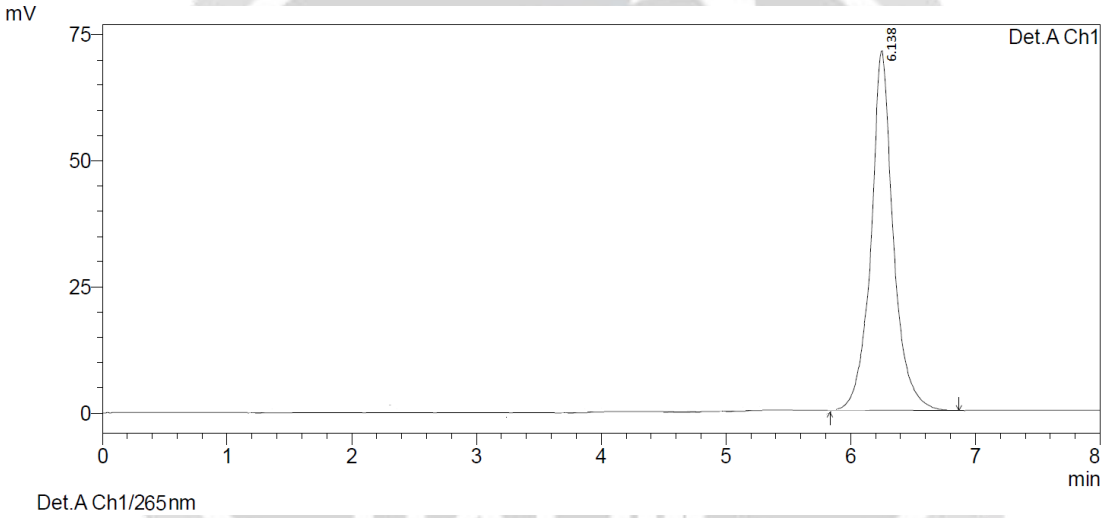

Figure 47: Chromatogram of Kaempferol accuracy at 80% level 3

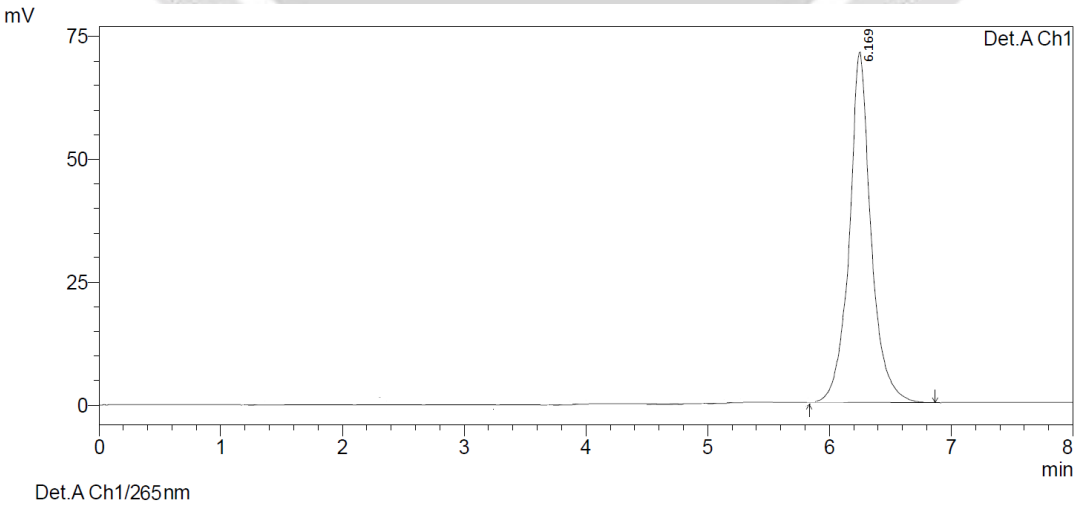

Figure 48: Chromatogram of Kaempferol accuracy at 100% level 1

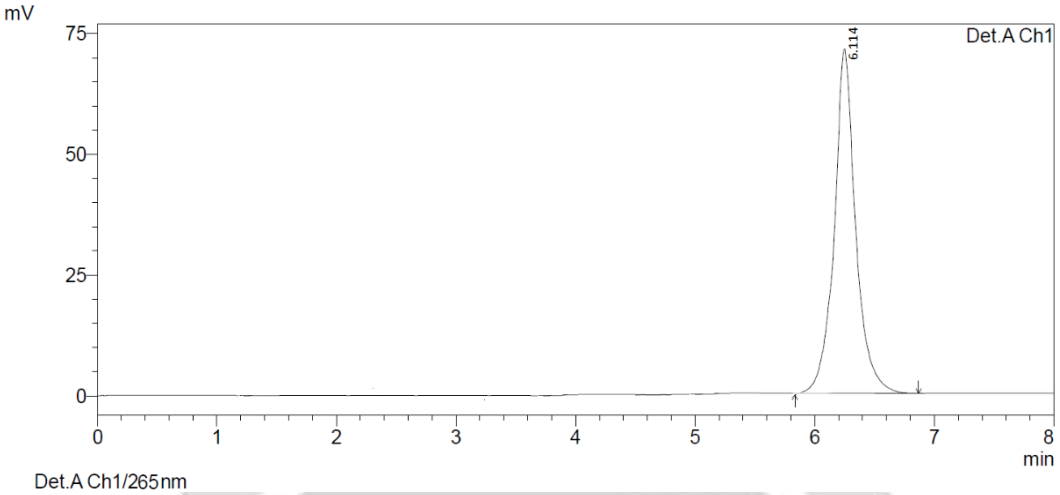

Figure 49: Chromatogram of Kaempferol accuracy at 100% level 2

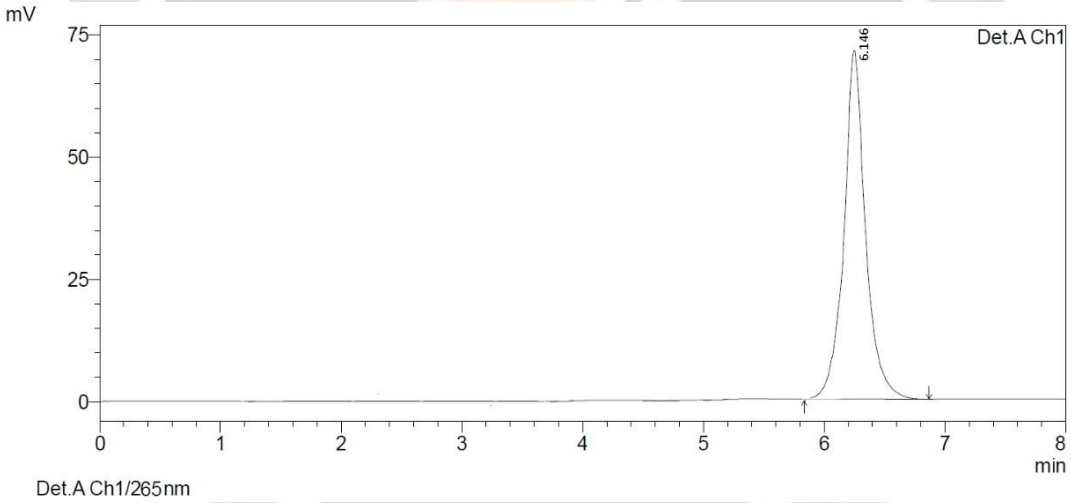

Figure 50: Chromatogram of Kaempferol accuracy at 100% level 3

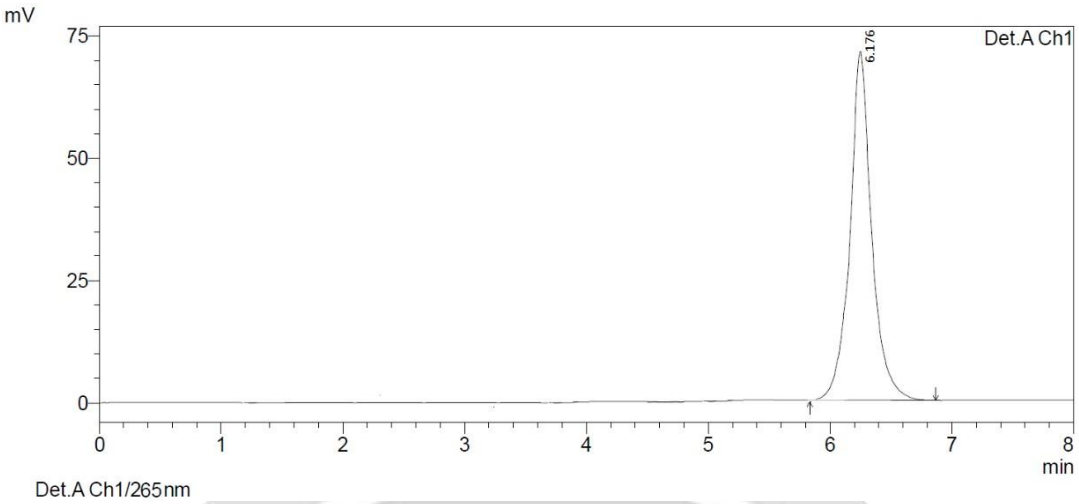

Figure 51: Chromatogram of Kaempferol accuracy at 120% level 1

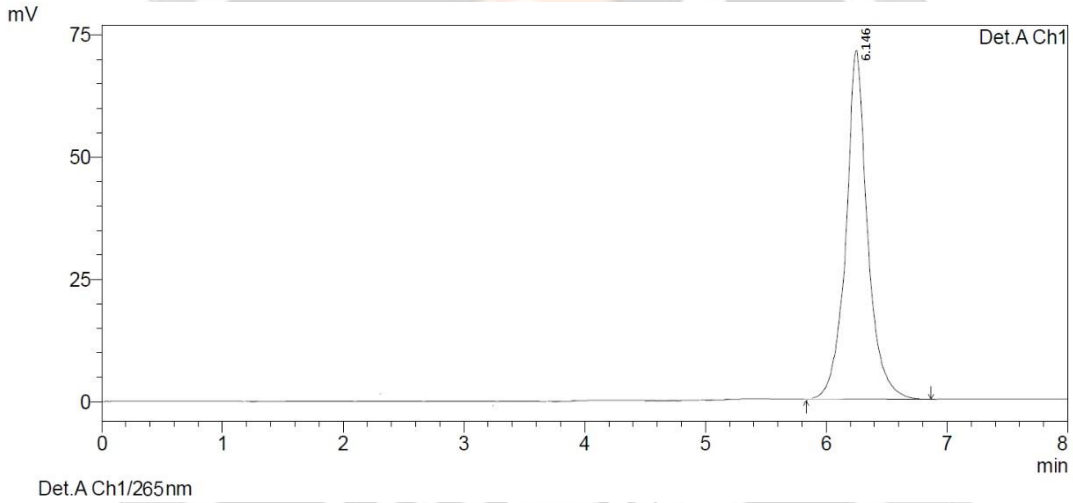

Figure 50: Chromatogram of Kaempferol accuracy at 100% level 3

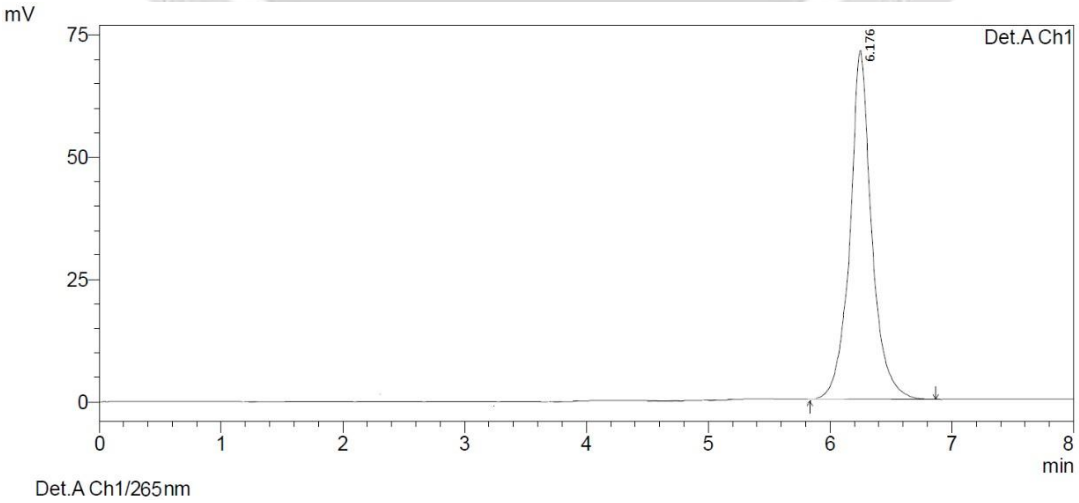

Figure 51: Chromatogram of Kaempferol accuracy at 120% level 1

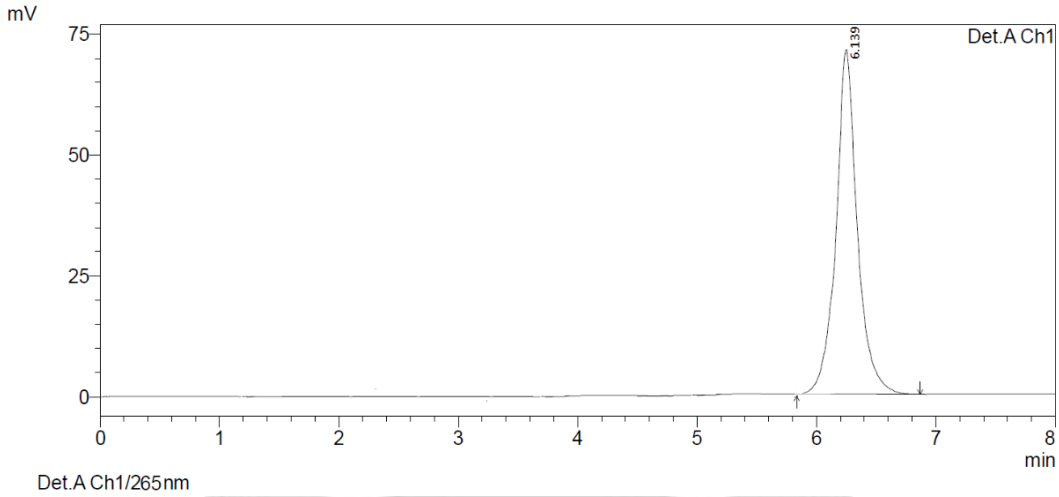

Figure 52: Chromatogram of Kaempferol accuracy at 120% level 2

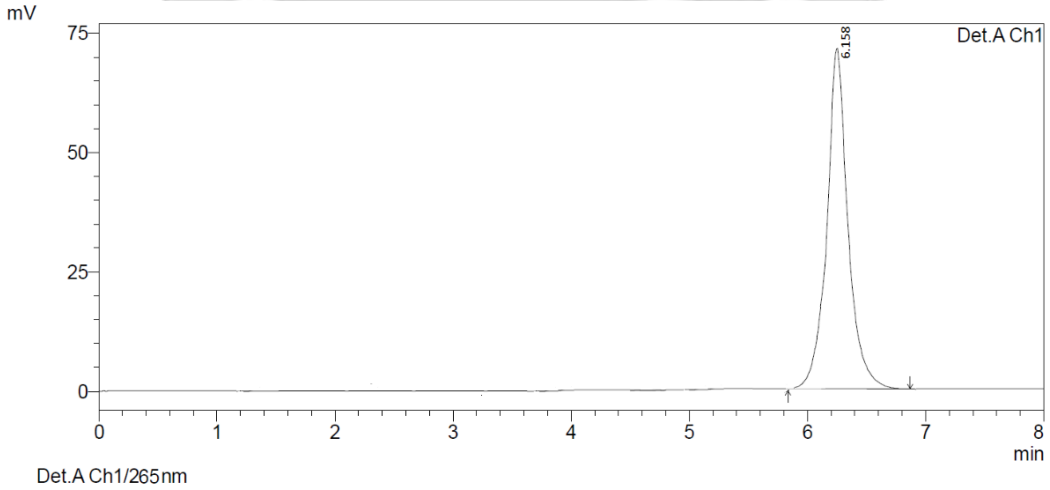

Figure 55: Chromatogram of Kaempferol accuracy at 120% level 3

Table 23: Test preparation data of accuracy

Level (%)	Set	Area
80	1	989068
	2	987562
	3	987063
100	1	987318
	2	982706
	3	986838
120	1	987568

	2	988764
	3	989367

Linearity and Range

Linearity for Kaempferol was found to be in the range of 10 - 60µg /ml with correlation coefficient value (r2) 0.9989. The results were tabulated in table 24 and graphically represented in figure 57-62.

Table 24: Linearity and Range for Kaempferol

Concentration (µg/ml)	Average PeakArea*
10	219826
20	516860
30	749808
40	989339
50	1235854
60	1495637
Slope	**25073**
CC	**9669.1**

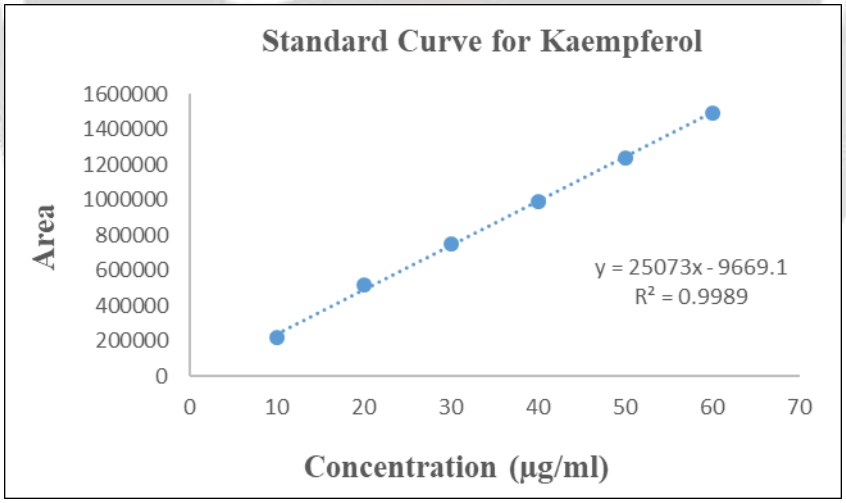

Figure 56: Standard Curve for Kaempferol

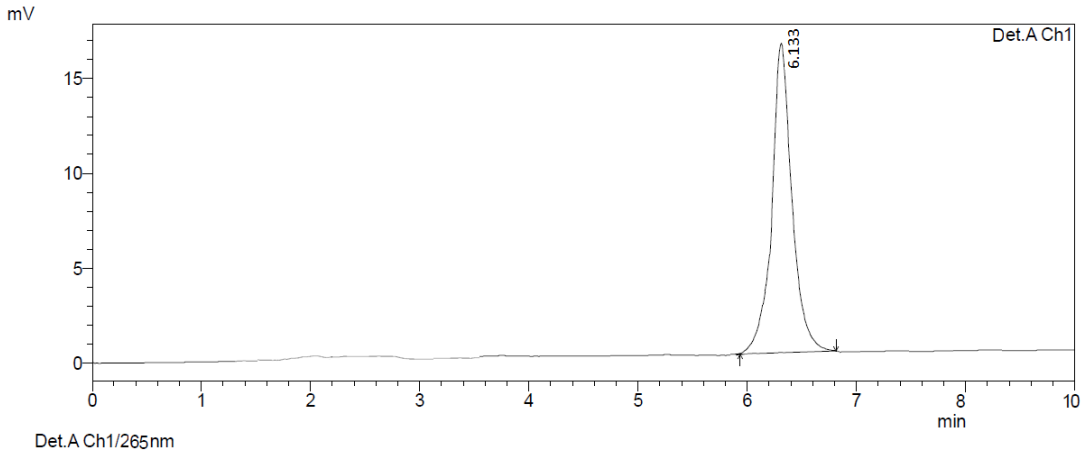

Figure 57: Standard Chromatogram for Linearity 10 µg/ml

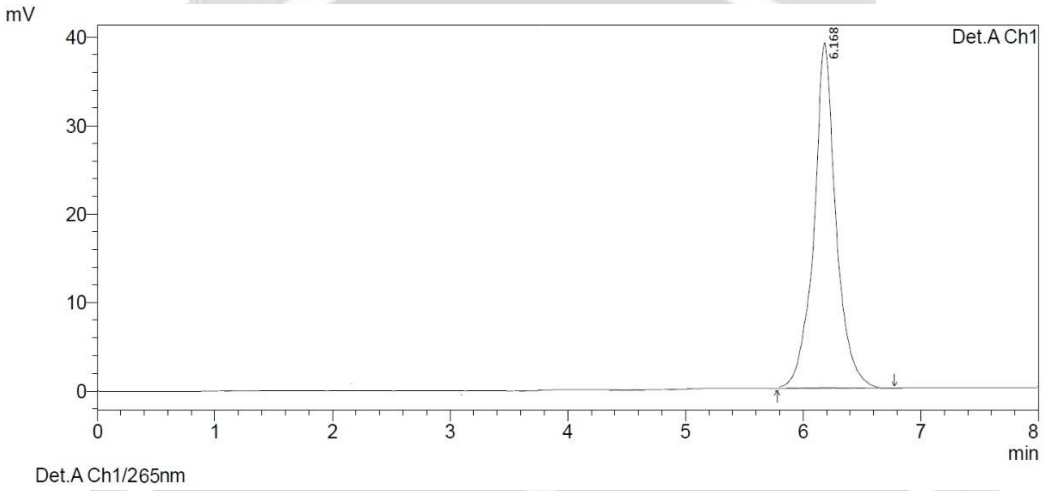

Figure 58: Standard Chromatogram for Linearity 20 µg/ml

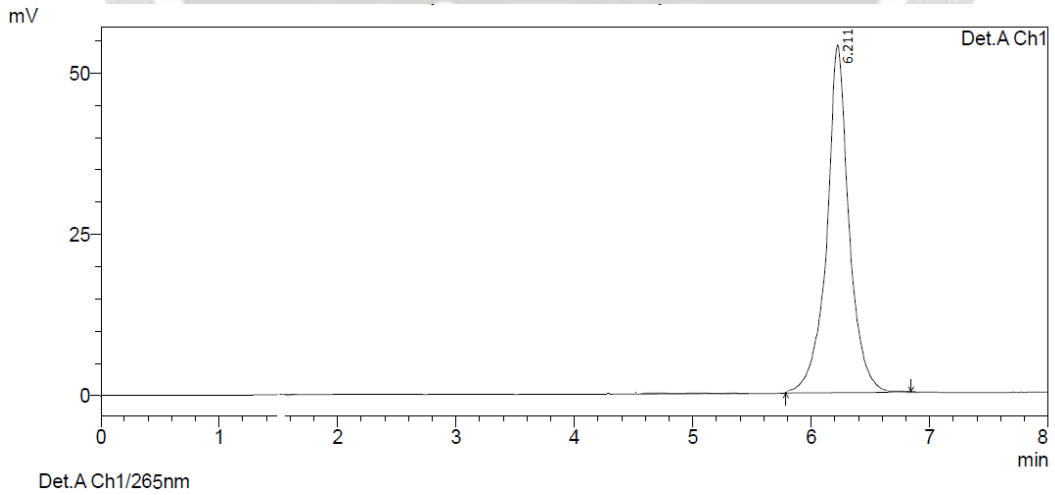

Figure 59: Standard Chromatogram for Linearity 30 µg/ml

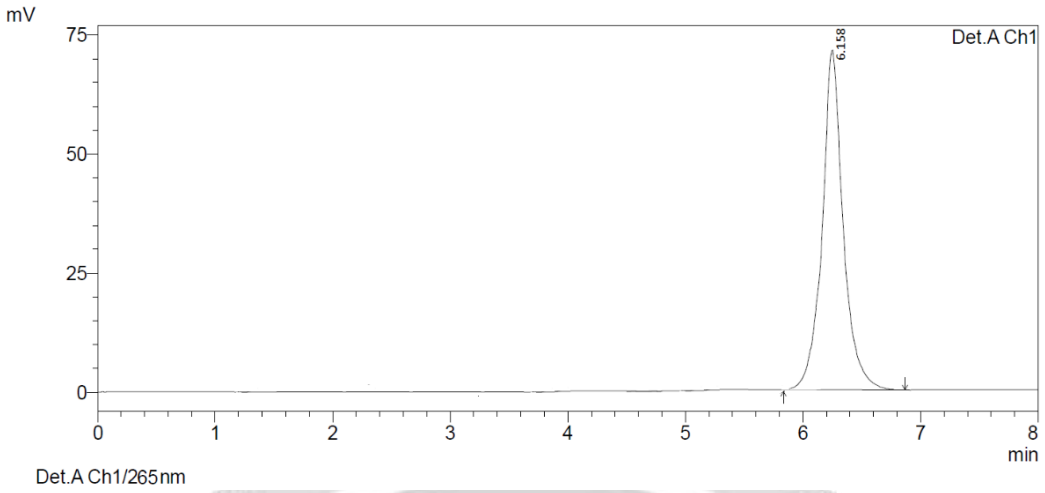

Figure 60: Standard Chromatogram for Linearity 40 µg/ml

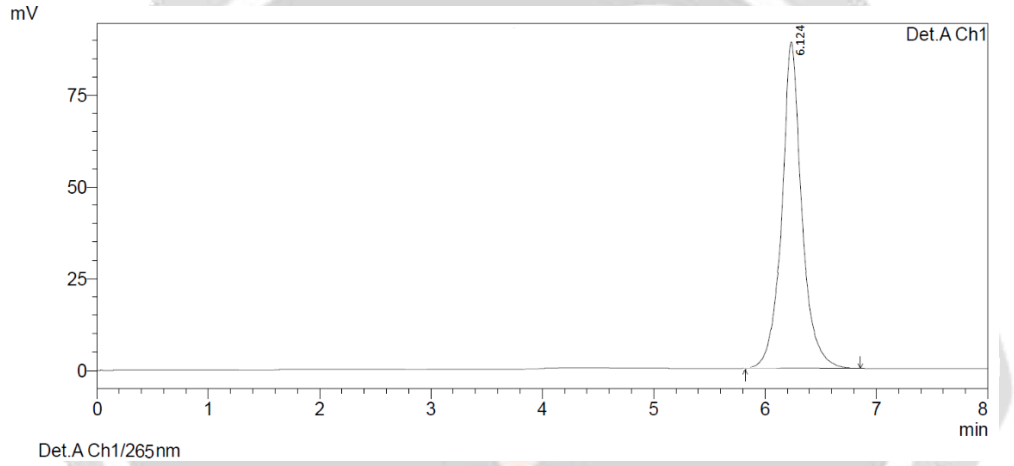

Figure 61: Standard Chromatogram for Linearity 50 µg/ml

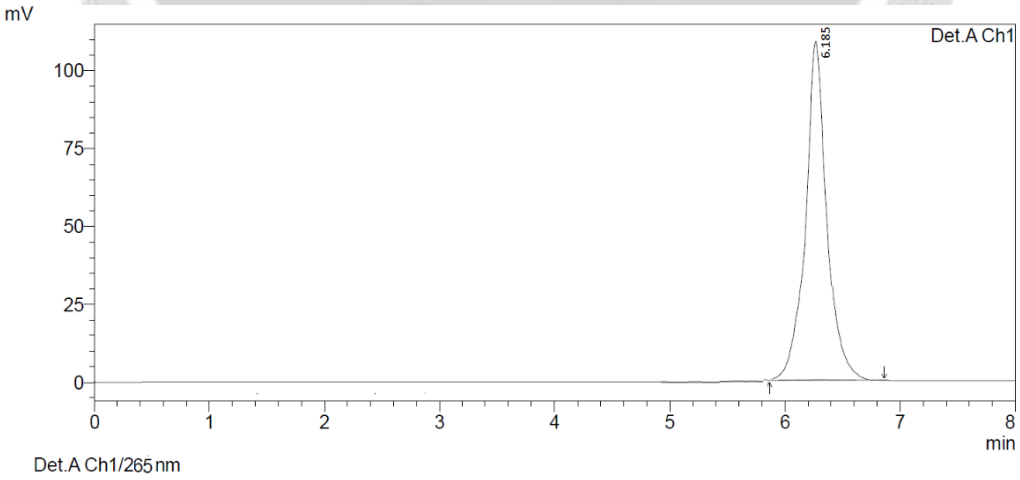

Figure 62: Standard Chromatogram for Linearity 60 µg/ml

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

For Piracetam the LOD and LOQ were found to be 0.163µg/ml and 1.172µg/ml respectively. For These values indicate that the method is suitable for the determination of the lower concentration and confirms that proposed method is sensitive for the determination.

Robustness

The system suitability parameters and peak areas were evaluated in each condition and the results were compared with method precision results. %RSD at each condition was found less than 2. This indicates the robustness of the method. The results are tabulated in table 26.

Table 26: Robustness data of Kaempferol

Flow Rate	Kaempferol			
	RT	**Area**	**Theoretical Plate**	**Tailing Factor**
0.8 ml/min	6.226	988637	2468	0.964
	6.105	978645	2349	0.897
	6.256	973486	2568	0.968
Average	**6.195**	**980256**	**2461.66**	**0.943**
SD	**0.079**	**7702.901**		
%RSD	**1.290**	**0.785**		
1.2 ml/min	6.056	985637	2367	0.968
	6.084	983489	2564	0.869
	6.124	972984	2148	0.944
Average	6.088	980703	2359.66	0.927
SD	0.0341	6770.861		
%RSD	0.561	0.690		

We are validating the robustness parameter for Kaempferol analysis, the optimized flow rate of the method was 1 ml/min so, we have taken the 0.2 % deviation in the system parameter i.e. flow rate and observed for the changes in RT, Area, Theoretical plate and Tailing factor. From the results it was concluded that, there was no such changes were observed indicating the developed method for Kaempferol is robust.

Robustness

The robustness parameter was determined by analyzing the different concentration at different temperature. The results were showed in table 27.

Table 27: Data of Robustness for Kaempferol

Kaempferol				
Change in Parameters	**Area of Standard**	**Mean**	**SD**	**%RSD**
25°C	981576	980195	3347.35	**0.341**
	976382			
	982637			
37°C	981738	982083	4126.81	**0.420**
	986371			
	978139			
60 °C	972681	981100	7449.36	**0.759**
	983782			
	986937			

4. CONCLUSIONS

The results clearly indicate that the RP-HPLC technique can be successfully applied for the estimation of Kaempferol in Convolvulus prostratus. The method was validated in accordance with ICH guidelines. The mobile phase is simple to prepare and economical and as the process is precise and accurate, drug is also stable for 24 hours. In addition, the main features of the developed method are short run time and retention time around 6.15 min. In the current research, the method shows good reproducibility, moreover the RP-HPLC method is accurate, precise, specific, reproducible, sensitive and cost effective for the analysis of Kaempferol. Hence this method can be easily and conveniently adopted for routine quality control analysis of Kaempferol

5. REFERENCES

[1]. Darshan R. Telange, Arun T. Patil, Amol Tatode, Bhushan Bhoyar. Development and Validation of UV Spectrophotometric Method for the Estimation of Kaempferol in Kaempferol: Hydrogenated Soy PhosphatidylCholine (HSPC) Complex. Pharmaceutical Methods, 5 (1), 2014: 34-38.

[2]. Ying Shi, Ya-Dan Guo, Yi-Duo Mi, Jia Cheng, Wen Dong, Gao-Feng Zhang & Yi Zeng. Potential of UV-Vis Spectroscopy for Determining the Mechanism of the Synergistic Antioxidant Process of Kaempferol with Three Other Flavonoids and β-Carotene. Journal of Applied Spectroscopy. 2023, 90; 883-892.

[3]. Om Prakash , Debarshi Kar Mahapatra, Ruchi Singh, Namrata Singh , Neelam Verma, Akash Ved. Development of a New Isolation Technique and Validated RP-HPLC method for Quercetin and Kaempferol from Azadirachta indica leaves. Asian J. Pharm. Ana. 2018; 8(3): 164-168.

[4]. YU, Jing-jing, LI, Feng-hua, Chen, Su-hong, Lü, Gui-yuan. Determination of quercetin and kaempferol in Pinus massoniana Lamb.from different areas and different species of pine needles by HPLC. Chinese Journal of Pharmaceutical Analysis, 2014, 34 (11) 1969-1974 (6).

[5]. Shalini K., Ilango K., Development, Evaluation and Rp-Hplc Method For Simultaneous Estimation of Quercetin, Ellagic Acid and Kaempferol In a Polyherbal Formulation. 2023, International Journal of Applied Pharmaceutics, 13(3), 183–192.

[6]. World Health Organization (WHO), "Substandard and Falsified Medical Products," 2017.

[7]. A. van Riet-Nales et al., "Toward Predicting Drug-Drug Interactions with Physiologically Based Pharmacokinetic Models," Drug Metabolism and Disposition, 2014.

[8]. A. van Riet-Nales et al., "Toward Predicting Drug-Drug Interactions with Physiologically Based Pharmacokinetic Models," Drug Metabolism and Disposition, 2014.

[9]. R.Y. Wang et al., "Imaging of Pharmacokinetic Rates and Hepatic Blood Flow in Human Subjects with Positron Emission Tomography," Nature Medicine, 1995.

[10]. F.P. Guengerich, "Cytochrome P450 and Chemical Toxicology," Chemical Research in Toxicology, 2008.

[11]. C. D'Mello et al., "Biomarkers of Renal Toxicity: An Overview," Analytical and Bioanalytical Chemistry, 2009.

[12]. R. Di L and A.J. Kerns, "Drug-Like Properties: Concepts, Structure Design and Methods," from Drug-Like Properties: Concepts, Structure Design and Methods, 2008.

[13]. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), "Guidance for Industry: Q1A (R2) Stability Testing of New Drug Substances and Products," 2003.

[14]. World Health Organization (WHO), "Substandard and Falsified Medical Products," 2017.

[15]. D. Hazell and M.L. Shakir, "Under-reporting of Adverse Drug Reactions: A Systematic Review," Drug Safety, 2006.

[16]. World Health Organization (WHO), "Substandard and Falsified Medical Products," 2017.

[17]. A. van Riet-Nales et al., "Toward Predicting Drug-Drug Interactions with Physiologically Based Pharmacokinetic Models," Drug Metabolism and Disposition, 2014.

[18]. A. van Riet-Nales et al., "Toward Predicting Drug-Drug Interactions with Physiologically Based Pharmacokinetic Models," Drug Metabolism and Disposition, 2014.