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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF RUTIN, LUTEOLIN AND SCOPOLETIN IN AYURVEDIC FORMULATION

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ABSTRACT

The current study was aimed to develop a simple, accurate and reproducible Reverse Phase High Performance Liquid Chromatography (RP-HPLC) analytical method for simultaneous estimation of Rutin, Scopoletin and Luteolin in Ayurvedic formulation. The analytical method was developed using Oyster Base Deactivated Silica C18 (250mm X 4.6mm, 5µ) column in tertiary mode with mobile phase comprising of 0.3% Orthophosphoric acid in Water: Methanol: Acetonitrile (65:10:25), at flow rate of 1 ml/min and detection was carried out at 254nm using a UV detector. The retention time for rutin, scopoletin and luteolin were found to be 3.61min, 4.59min and 8.73min respectively. Linearity of all three phytomarkers was found to be in the range of 400-1200 ppm. The correlation coefficient for rutin, scopoletin and luteolin were 0.9988, 0.9994 and 0.9994 respectively. Limit of Detection and Limit of Quantification values of rutin, scopoletin and luteolin were found to be 24.25µg/ml and 74.50µg/ml, 17.38µg/ml and 52.69µg/ml and 5.44µg/ml and 16.48µg/ml respectively. Precision studies were carried out using intra-day and inter-day intervals which showed relative standard deviation values less than

2.00%. The accuracy study was determined by standard recovery method. The recovery values of the rutin (100.94% - 101.20%), scopoletin (100.13% - 100.62%) and luteolin (100.87% - 101.13%) indicate the method to be accurate. The developed method can be used as standardization tool for evaluation of formulation containing rutin, scopoletin and luteolin as marker compounds.

Keywords: Rutin, Scopoletin, Luteolin, Ayurvedic formulation, RP-HPLC, Validation

1. INTRODUCTION

Humans have been using natural products such as plants, livestock, microorganisms and marine organisms as medicines since prehistoric times. Humans have been using plats as medicines for at least 60,000 years, according to the records contained in fossils **[1]**.

Plants containing active ingredients that have therapeutic effect have been used for centuries for the treatment of disease and it are now being embraced in developing countries for the restoration of people's good health. Modern pharmacopeia has included 25% of monographs of drugs that are plant based as well as many other synthetic substitutes synthesized utilizing prototype compounds extracted from plant products. Due to increasing cost of prescription medications for personal health and well being, there is resurgence of interest in medicinal plants as a re-emerging health aid as well as bioprospecting for new plant derived products **[2]**.

WHO emphasizes the significance of standardization and quality control of plant based herbal medicines using modern apparatus and appropriate standards. Quality control of herbal medications is an efficient procedure for pharmaceuticals and is specified as something which should be accepted by all to ensure the purity and uniformity of marketed pharmaceutical products **[3]**.

Due to dynamic nature of the chemical constituents of plant-based medications, the quality of herbal preparations i.e., the profile of active constituents in the final product has implications in safety and effectiveness. Modern analytical techniques are increasing to overcome these problems of separation, detection and determination of active constituents for Polyherbal formulations. Advances in chromatographic and spectroscopic techniques have made it possible to evaluate the active constituents in a mixture with relatively little clean up. Methods involving High performance liquid

chromatography (HPLC) with a reverse phase column are best suited for analysing multiple constituents present in herbal preparations **[4]**.

A Polyherbal formulation consists of *Boerhavia diffusa* (Nyctaginaceae), *Aegle marmelos* (Rutaceae) *Macrotyloma uniflorum* (Fabaceae), *Ricinus communis* (Euphorbiaceae), *Barleria prionitis* (Acanthaceae), *Zingiber officinale* (Zingiberaceae) and *Premna Serratifolia* (Lamiaceae) for the treatment of pain in the lower back and hip, lower abdominal pain, menstrual cramps and its associated complications. In our present investigation we have developed a simple optimized and validated RP-HPLC method for the standardization of this Polyherbal formulation **[5, 6]**.

Three chemical markers were selected for quantification, Rutin for *Ricinus communis*, Luteolin for *Premna Serratifolia* and Scopoletin for *Aegle marmelos*. Literature review reports that there are various reported analytical method for quantification of Rutin **[6, 7, 16–21, 8–15]**, Scopoletin **[22–26]** and Luteolin **[27–30]** independently and in combination with other drug. On the contrary, no RP-HPLC technique for simultaneous estimation of mentioned combined phytomarkers in any dosage form has been documented. As consequences, the aim of the present work was to develop and validated RP-HPLC method for determining Rutin, Luteolin and Scopoletin in Ayurvedic formulation.

2. MATERIAL AND METHODS

2.1 Chemicals, Reagents and Materials

Phytomarkers – Rutin, Luteolin and Scopoletin were purchased from Yucca Enterprises. HPLC grade methanol, water and Acetonitrile were obtained from Merck India Pvt. Ltd. And HPLC grade of Orthophosphoric acid from Loba Chemie Pvt. Ltd.

2.2 Apparatus and Chromatographic conditions

Shimadzu LC 10 series system with a quaternary pump (LC-10 AT), a degasser (DGU-14AL), Auto sampler (SIL-10AD), controller (CBM-20A) with UV detector (SPD-20A) was used for analysis. The column used was Oyster BDS Premium (250×4.6 mm (5µm)) at ambient temperature. The detection was carried out at wavelength of 254nm. 0.3% Orthophosphoric acid in Water: Methanol: Acetonitrile (65:10:25% v/v/v) as a mobile phase with the flow rate of 1ml/min.

2.3 Preparation of reference standard solution (Rutin, Luteolin and Scopoletin)

Weigh 10 mg of each standard and transfer to 10 ml of volumetric flask, add approximate 5ml methanol to volumetric flask and dissolve the content by intermittent sonication and mixing. Make up the volume with methanol to prepare 1000µg/ml solution. Inject in HPLC after filter the solution with 0.2µ nylon syringe filter.

2.4 Preparation of sample solution

After shaking the syrup, accurately measure the 1 ml of the syrup formulation was taken in 10 ml volumetric flask and adds approximately 5 ml of methanol to it. Sonicate and mix properly to dissolve the all content. Mack up the volume with methanol and filter the solution with 0.2µ nylon syringe filter.

2.5 Selection of Wavelength

Standard solution of Rutin, scopoletin and luteolin were prepared and scanned using a UV spectrophotometer. The detection range was kept from 200-400 nm and the overlay spectra of rutin, scopoletin and luteolin obtained in **Figure 1**. The detection wavelength was selected as 254nm for the analysis of rutin, scopoletin and luteolin, as the all markers showed appropriate absorbance at this wavelength.

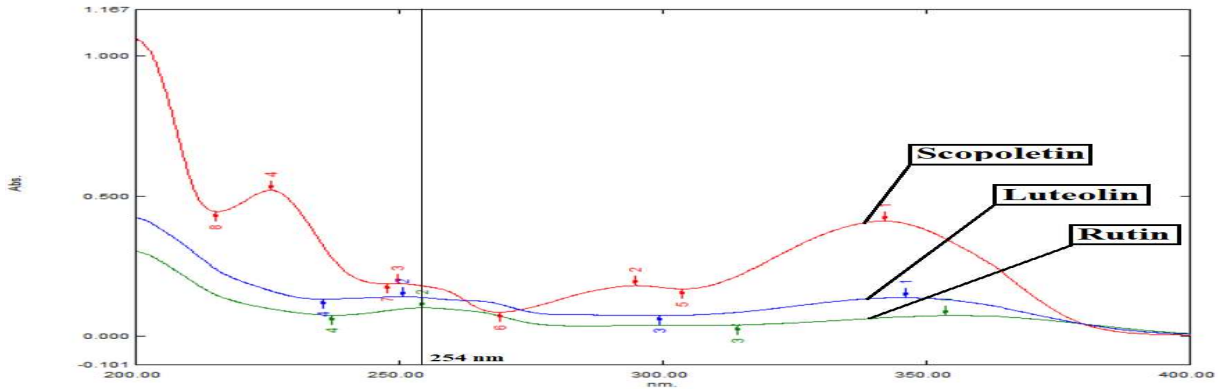

Figure 1: UV overlay spectrum of rutin, scopoletin and luteolin at 254 nm

2.6 Analytical Method Development

Several steps in the analytical method development were streamlined to ensure that selected phytomarkers are separated accurately and precisely. Several parameters were investigated

in order to determine the most effective conditions for selecting the extracting solvent, stationary phase (column), mobile phase and detection wavelength. Various combination of mobile phase were tried to get the most effective separation of the phytomarkers as described in **Table 1**. After several trials, the optimal chromatography method for simultaneous estimation of phytomarkers was found to have better peak resolution and symmetry.

Table 1: Trials for optimization of Mobile phase for HPLC method

Sr. No.	Mobile phase	Ratio	Reason
1	Waste: Acetonitrile	50:50	Rutin and scopoletin were not separated and tailing was found in luteolin
2	0.1% formic acid Water: Methanol: Acetonitrile	50:50:20	Rutin, scopoletin and luteolin were on same retention time
3	0.1% formic acid Water: Methanol: Acetonitrile	60:20:20	Rutin and scopoletin were not separated
4	0.3% Ortho phosphoric acid in water : Methanol: Acetonitrile	85:15:20	Luteolin was eluted at a higher retention time.
5	0.3% Ortho phosphoric acid in water : Methanol: Acetonitrile	60:10:30	No resolution between peaks of Rutin and Scopoletin
6.	0.3% Ortho phosphoric acid in water : Methanol: Acetonitrile	65:10:25	Rutin scopoletin and luteolin were separated properly with good resolution.

Table 2: Chromatographic condition of developed method

Parameters	Value
Injection volume	10µl
Column temperature	Ambient
Detection wavelength	254 nm
Flow rate	1 ml/min
Mobile phase	0.3% Orthophosphoric acid in Water : Methanol : Acetonitrile (65:10:25)
Mode of Pump	Low pressure gradient
Run time	12 min.

2.7 Validation of developed method

Validation of the developed method was performed as per ICH guidelines Q2 (R1). Specificity, precision, linearity, consistency, robustness, limit of detection, limit of quantitation and system suitability were all accessed as part of the validation of the proposed method **[31]**.

2.7.1 Specificity

Specificity is the ability to evaluate the analyte unequivocally in the presence of components that may be expected to be present. To access the methods specificity, two samples

were analysed, one with formulation (unspiked) and the other with a phytomarkers spiked with formulation. The specificity was determined by comparing the chromatogram of unspiked samples to those of samples spiked with standard.

2.7.2 Linearity

The ability of an analytical technique to produce test results that are directly proportional to the concentration of analyte in the sample (within a given range) is known as linearity. For determination of linearity, 20 mg of standard was taken in 10 ml volumetric flask and diluted up to the mark with methanol to get the linearity stock solution (2000µg/ml). The linearity stock solution was serially diluted to get the concentration ranging from 400-1200 µg/ml.

2.7.3 Limit of detection (LOD) and Limit of quantitation (LOQ)

The lowest amount of analyte in a sample that can be detected but not generally quantified as an exact value is the limit of detection (LOD) of an individual analytical technique. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The established LOQ for all three markers above was 90% accuracy and below 2% RSD.

2.7.4 Precision

The degree of closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the specified conditions is expressed as the precision of an analytical method. Precision was evaluated in terms of intra-day precision and inter-day precision. The intra-day precision was determined by measuring the same concentration at six times on a same day. The inter-day precision was determined by measuring same concentration at six times on a three different days. And the mean, standard deviation and percent of relative standard deviation was calculated.

2.7.5 Accuracy

The closeness of agreement between the value accepted as a standard true value or an accepted reference value and the value found is expressed as the accuracy of an analytical method. In this study the accuracy study was done by spiking standard in placebo and sample with known quantity**.**

2.7.6 Robustness

The robustness of an analytical technique is a measure of its ability to remain unaffected by minor yet deliberate changes in system parameters and it indicates its reliability in regular use. Robustness of the developed method was evaluated by deliberately changing the flowrate from 1 ml/min to 0.8 ml/min and 1.2 ml/min and wavelength from 254 to 252 and 256 to evaluate robustness of the method.

3. RESULTS

3.1 Optimization of HPLC conditions

For the analysis initially different combinations of the mobile phase in the gradient and isocratic phases was done for obtaining the best separation and resolution of three phytomarkers. The mobile phase consisting of 0.3% Orthophosphoric acid in Water: Methanol: Acetonitrile (65:10:25%v/v/v) was finalized which gave good resolution and proper peak shape and eluted Rutin, Scopoletin and Luteolin at 3.6, 4.5 and 8.7 minutes respectively.

3.2 Quantification of markers

Under the optimized chromatographic conditions, the chromatogram of standard mixture of Rutin, Scopoletin and Luteolin is shown in Fig.2 which reveals good separation of the phytomarkers. Chromatogram obtained from Ayurvedic formulation (Sapthasaram kashayam decoction) having Rutin, Scopoletin and Luteolin as phytomarkers is shown in **Figure 3**. The % assay of individual markers in the formulation was calculated based on calibration curve of individual markers and summarized in **Table 3**.

3.3 Validation of developed analytical method

3.3.1 Specificity:

The specificity was demonstrated by comparing chromatogram of standard and sample solution

(Figure 4). From this study, it is clear that all three phytomarkers were clearly resolved, with no interference from the sample matrix.

3.3.2 Linearity

The linearity of calibration curve for Rutin, Scopoletin and Luteolin was established in the concentration range of 400-1200 µg/ml **(Figure 5)**. The calibration curve was plotted against area of peak verses concentration of injected linearity standard. The graph is shown in **Figure 6** and correlation co-efficient and recreation line equation were determined **(Table 4)**.

3.3.3 Precision

The intraday precision was measured six times a day and % RSD ranged from 0.1018% - 1.8106% and interday precision measured on six consecutive days and % RSD ranged from 0.7153% - 1.7789%. The results of precision are shown in **Table 5**.

3.3.4 Accuracy

The accuracy of the assay method was measured as relative recovery at three concentration levels. The % recovery for 80%, 100% and 120% concentration level for all the three phytomarkers is reported in **Table 6**.

3.3.5 Limit of detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined and the results are reported in **Table 7**.

3.3.6 System suitability

The system suitability of the developed method was confirmed by calculating various chromatographic conditions such as number of theoretical plates (N), tailing factor (T_f) and Retention time (R_t) from the chromatogram of the standard solutions. Results obtained are shown in **Table 8**.

3.3.7 Robustness

Robustness of the developed method was evaluated by deliberately changing the flowrate from 1 ml/min to 0.8 ml/min and 1.2 ml/min and Wavelength varied from 254 to 252 and 256 to evaluate robustness of the method. The results of the robustness are reported in the **Table 9**.

Table 3: Content of markers in formulation

Sr. No	Phytomarkers	Assay results
1	Rutin	5.15 mg/ml
2	Scopoletin	6.90 mg/ml
3	Luteolin	1.49 mg/ml

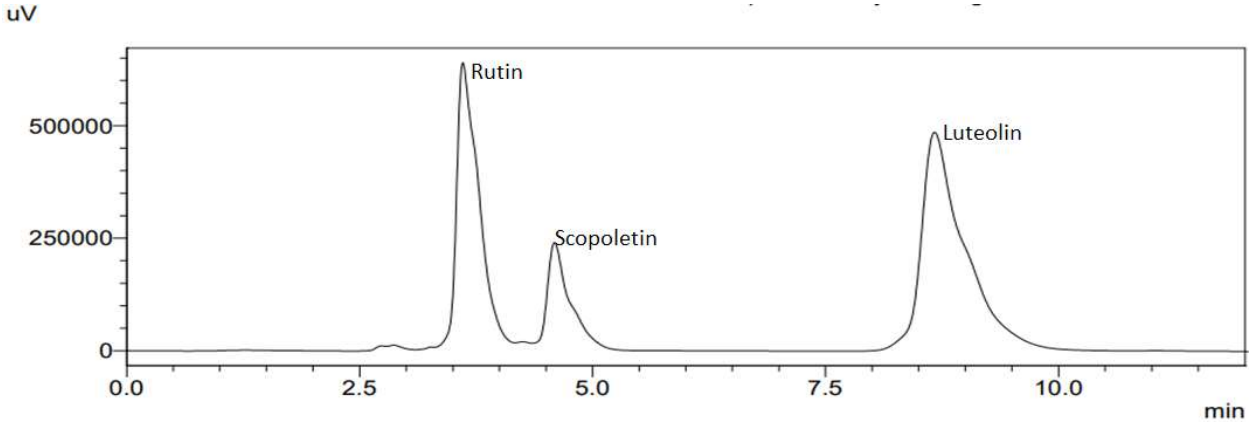

Figure 2: HPLC chromatogram of standard mixture of Rutin, Scopoletin and Luteolin

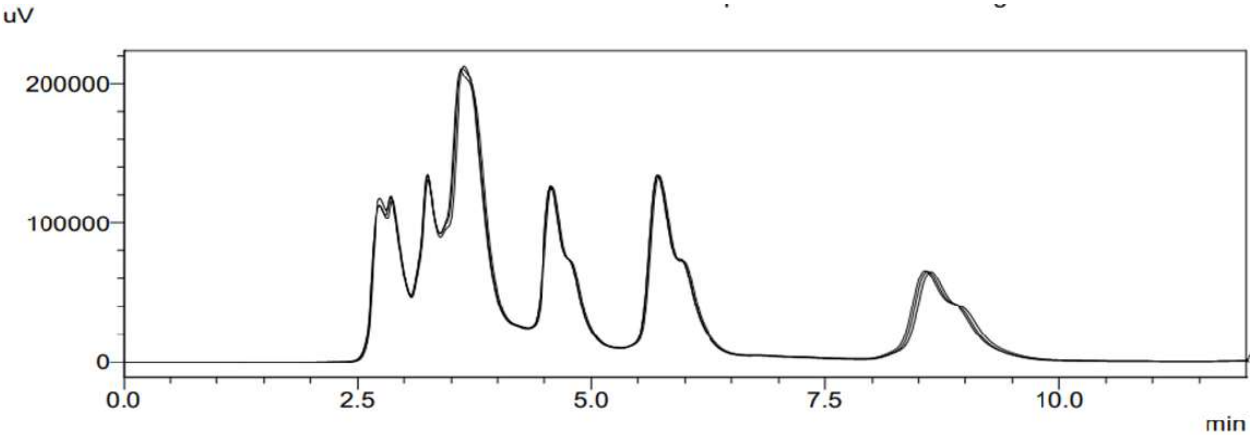

Figure 3: HPLC chromatogram of Ayurvedic formulation -Sapthasaram kashayam decoction

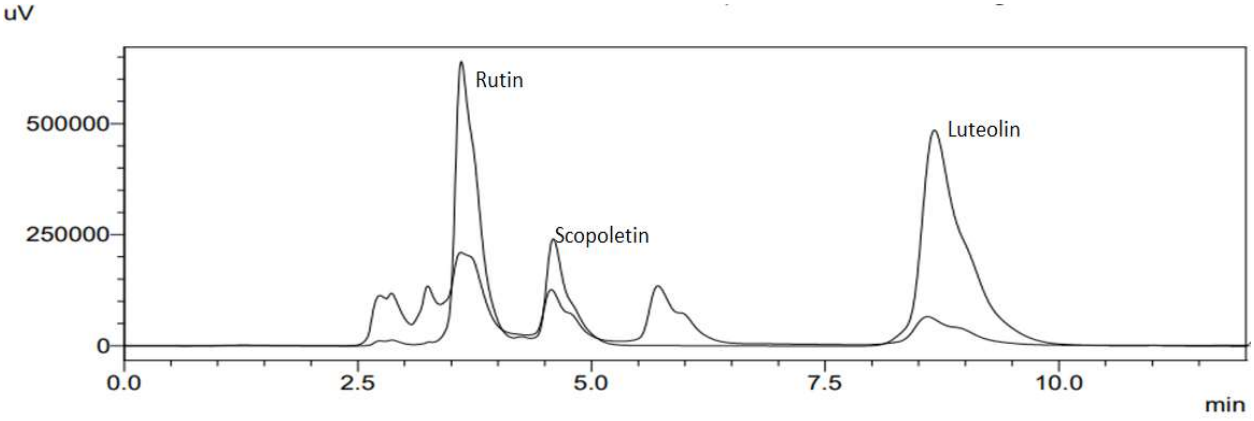

Figure 4: Comparison of HPLC chromatogram of standard with sample solution

Table 4: Linearity data of phytomarkers and their R^2 value

Sr. No	Phytomarkers	R^2 value	Equation
1	Rutin	0.9988	y= 10828x + 152466
2	Scopoletin	0.9994	y = 4198x + 28364
3	Luteolin	0.9994	y= 15402x + 179104

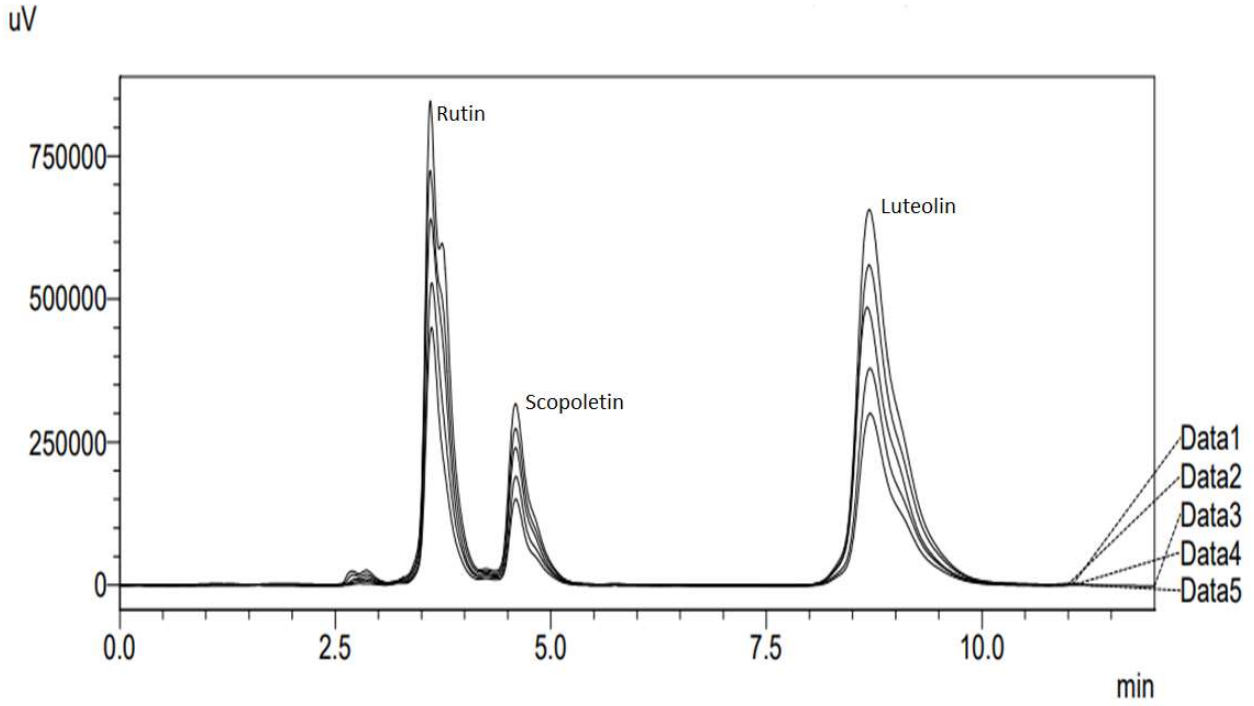

Figure 5: Overlay HPLC chromatogram for different linearity concentration of rutin, scopoletin and luteolin

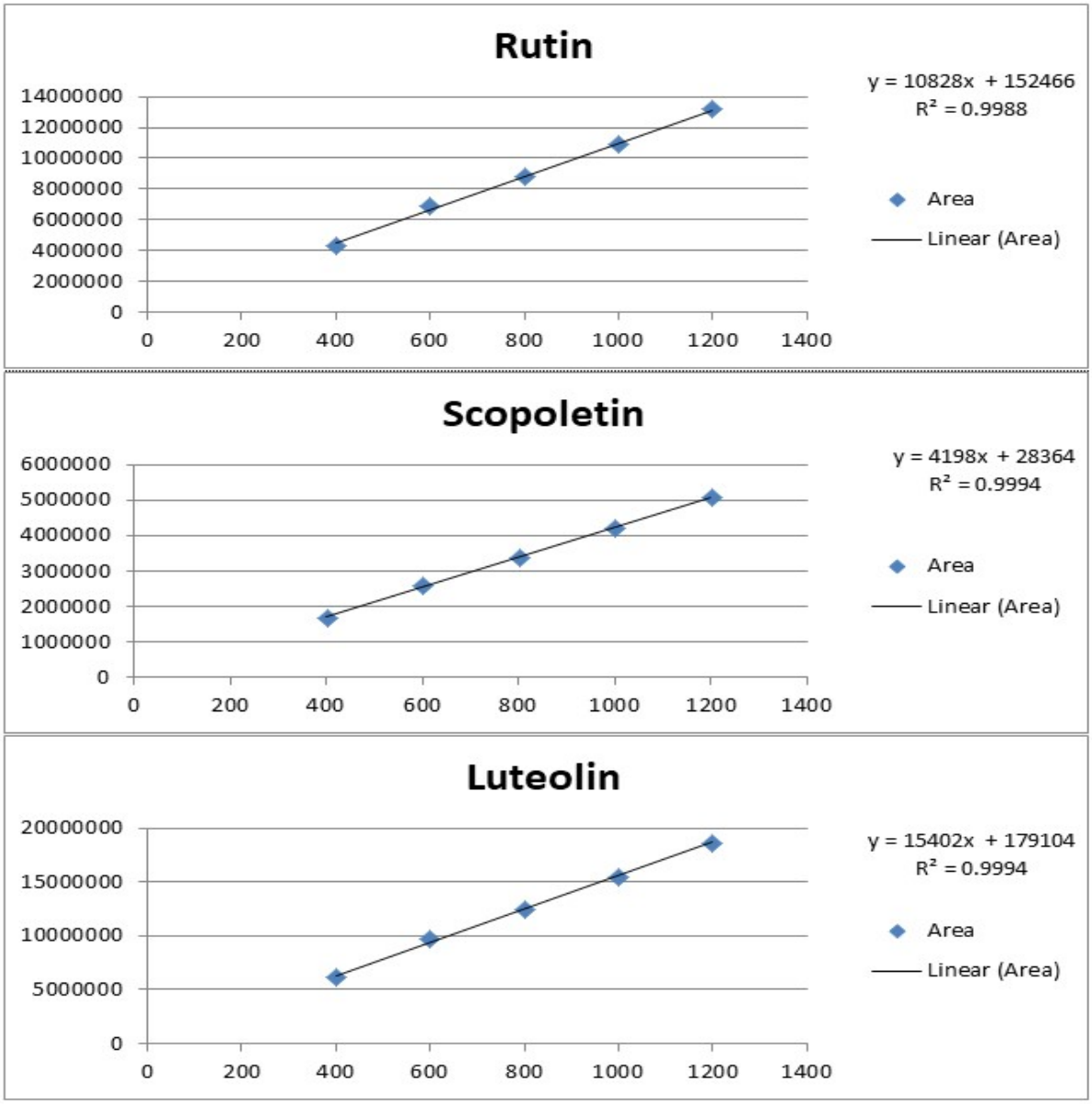

Figure 6: Calibration curve of rutin, scopoletin and luteolin

Table 5: Intra-day and Inter-day precision study

Sr. No	Phytomarkers	% RSD	
		Intraday (n=6)	Interday (n=6)
1	Rutin	0.883	1.072
2	Scopoletin	0.915	1.161
3	Luteolin	0.741	1.165

Table 6: Accuracy study of the developed method

Parameters	% Recovery		
	Rutin	Scopoletin	Luteolin
80%	100.94	100.62	101.13
100%	100.65	100.13	100.87
120%	101.20	100.31	101.04

Table 7: LOD and LOQ data of developed method

Parameters	Rutin	Scopoletin	Luteolin
LOD	24.25µg/ml	17.38µg/ml	5.44µg/ml
LOQ	74.50µg/ml	52.69µg/ml	16.48µg/ml

Table 8: System suitability parameter

Sr. No	Parameters	Values		
		Rutin	Scopoletin	Luteolin
1	Retention time (minutes)	3.61	4.59	8.73
2	Theoretical Plates	6849	17635	17179
4	Tailing factor	1.803	2.058	1.78

Table 9: Results of robustness study

Sr. No	Parameters	Deliberate changes	%RSD		
			Rutin	Scopoletin	Luteolin
1	Flowrate	1 ± 0.2 ml/min	1.1158	0.9987	0.8882
2	Wavelength	254 ± 2	1.0449	0.7659	0.7842

4. DISCUSSION

Various chromatographic criteria like stationary phase, selection of mobile phase, detection wavelength, flow rate, column temperature, and solubility of components are to be optimized for the successful development of analytical method. The present research work involves development and validation of RP-HPLC method for simultaneous estimation of Rutin, Scopoletin and Luteolin in Polyherbal formulation.

Various trials of different combination of the mobile phase in the isocratic and gradient phases was carried out in order to get the best conditions for the separation of the phytomarkers. The parameters like column chemistry, solvent for sample preparation were also optimized and reported.

Validation of the developed method was done by determining various parameters like linearity, accuracy, precision, limit of detection, limit of quantification according to ICH guidelines.

From the specificity study it is revealed that all the phytomarkers were clearly resolved without any interference from the mobile phase or baseline disturbances. Also, there are no significant peaks observed from the formulation matrix indicating no interference from the matrix of the formulation. In linearity study, the $r^2 > 0.99$ was obtained for all the phytomarkers which indicate that the method developed is linear for all the phytomarkers. LOD and LOQ were estimated which gave idea about the minimum concentration of the markers that can be detected and quantified.

Interday and Intraday precision study results indicate the %RSD value < 2% in accordance with the ICH guidelines. Accuracy was performed by recovery study which indicates that the method was able to recover the markers in the formulation matrix.

The results demonstrate that the developed method was linear, accurate and precise. Moreover, the method was rapid, selective and sensitive which can be used for the routine quality control analysis of the formulation and plant materials.

5. CONCLUSIONS

Due to the complex nature of the Ayurvedic and polyherbal formulation, it is important to ensure the quality of finished product based on reliable scientific method. The present research involves the development and validation of analytical method used in quality control of Ayurvedic formulation. The developed and validated RP-HPLC method will help in the standardization of polyherbal formulation by means of biologically active phytomarkers. The developed RP-HPLC method is accurate, precise, reproducible and repeatable. With the growing demand of the polyherbal formulation and increasing belief in the usage of herbal medicine, the method will be reliable standardization tool which will help in marinating the quality of important Polyherbal formulation.

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