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GREEN AQBD-OPTIMIZED AND VALIDATED RP-HPLC METHOD FOR SIMULTANEOUS QUANTIFICATION OF QUERCETIN AND RUTIN IN *Ervatamia heyneana* LEAF EXTRACT

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ABSTRACT
By developing a straightforward, quick, and environmentally friendly green AQbD optimised RP-HPLC technique for simultaneous and accurate quantification of Quercetin and Rutin in *Ervatamia heyneana*— plant with significant ethnomedicinal relevance but little phytochemical standardisation—this study significantly advances analytical science and natural product research. The work fills a long-standing analytical gap produced by the structural similarity and overlapping UV spectra of several important flavonoids in the species by combining phytochemical screening, FT-IR, TLC, and LC–MS characterization. The study shows how AQbD can turn method development into a methodical, statistically guided process rather than an empirical trial-and-error approach. Strong model prediction (R^2 = 0.84–0.91) and the identification of flow rate as the most important parameter demonstrate the importance of design-based optimization to attain reproducibility and robustness. The validated approach provides a dependable platform for routine quality control in phytopharmaceutical, herbal, and nutraceutical applications, demonstrating outstanding specificity, linearity, precision, and accuracy. The work significantly advances the concepts of green analytical chemistry, as evidenced by the favourable MoGAPI and AGREE scores. The work supports safer, cleaner, and more sustainable laboratory operations in line with Sustainable Development Goal (SDG) 12-indicated responsible usage and development by lowering solvent usage, increasing chromatographic efficiency, and integrating environmental evaluation tools. All things considered, this study not only advances scientific knowledge of *E. heyneana* but also offers a transferable, environmentally friendly analytical framework that can direct future research on bioactive marker quantification, medicinal plants, and sustainable chromatographic method development.

Keywords: Assessment of Greenness, *Ervatamia heyneana.,* ICH Q2R1, LC-MS, Method validation, Phytochemical profiling.

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INTRODUCTION

Ervatamia heyneana (Wall.) T.Cooke (Apocynaceae) is an unexplored medicinal shrub, a native to the Western Ghats of peninsular India, despite its traditional use and rich phytochemical profile. Its leaves contain therapeutically important flavonoids, particularly Quercetin as well as Rutin, which are well-known for their antioxidant, anti-inflammatory, cardioprotective and chemopreventive activities.[1] However, to date, no validated analytical method has been reported for their simultaneous estimation in this species. Existing literature studies highlighted persistent analytical challenges arising due to structural similarity and overlapping UV spectra of these flavonoids, which often lead to inaccurate quantification and low resolution.[2] This gap limits phytochemical profiling and quality control of *E. heyneana*. This study aims to develop as well as validate a novel AQbD-based RP-HPLC method, which offers an effective technique with high selectivity, sensitivity, and reproducibility. Implementation of chemometric tools ensures robustness through systematic risk assessment and experimental design, enabling long-standing challenges associated with empirical, trial-and-error chromatographic optimization.

Additionally, its environmental sustainability was evaluated using AGREE and MoGAPI techniques, supporting its suitability for modern green analytical practices.[3-8] This study followed the United Nations Sustainable Development Goal (SDG) 12, which is aimed at promoting responsible consumption and production, and developing environmentally sensitive analytical practices. Through reduced solvent use, selecting optimised chromatographic conditions, and the application of green assessment tools, the method advances sustainable approaches in pharmaceutical and nutraceutical research while minimising laboratory-generated waste.

EXPERIMENTAL

Material and Method

Quercetin and Rutin standards were obtained from Sigma-Aldrich, and solvents from Merck Life Sciences Pvt. Ltd. HPLC analysis has been performed using Shimadzu LC-2030 Plus by UV detection at 248 nm, employing Shimpack C18/C8 columns. Solevent use was HPLC grade. Method optimisation was done using JMP software, and LC–MS characterization utilized a Waters XBridge C18 column.

General Procedure Extraction Process

Powdered leaf sample 10 grams was extracted sequentially with benzene and 100 millilitres of methanol using Soxhlet extraction for 36 h at 65 °C. The extract has been filtered and concentrated to dryness under pressure by employing a rotary evaporator. The obtained residue was stored at a refrigerated temperature.[9]

Preliminary Examination

Standard qualitative assays were used for phytochemical screening of the leaf extract**,** which confirmed that alkaloids, flavonoids, tannins, phenolics, terpenoids, and carbohydrates were detected in the leaf extract using standard qualitative tests, with identification based on characteristic colour changes and precipitate formation.[10]

Characterisation of (LC-MS)

Analysis has been accomplished on a Waters XBridge C18 column using gradient elution of 0.1% formic acid and acetonitrile solvent at the flow rate of 1.2 mL/ min, with Ultraviolet detection, followed by MS analysis using a Waters Micromass Quattro micro triple quadrupole system for sensitive and selective quantification.[11]

Analytical Quality by Design (AQbD)

AQbD refers to a methodical, scientific strategy utilised in pharmaceutical and other industries to develop and optimise analytical techniques. It emphasises a proactive and risk-based methodology focusing on understanding and controlling the factors that affect the performance of analytical methods.[12]

Important Components of AQbD

Analytical Target Profile (ATP)

The stock solutions (1 mg/mL) of Quercetin and Rutin were prepared in methanol. Separation was performed utilising gradient Acetonitrile containing 0.1% formic acid, with UV detection at 258 and 360 nm for HPLC/LC–MS analysis.

Critical Performance Attributes (CPAs)

Analytical characteristics confirm method reliability. Accuracy, specificity, and linearity relate to system variability; precision, LOD, and LOQ indicate random error, while range and robustness are supportive.

Critical Analytical Attributes (CAAs)

The attributes affecting the separation techniques, like retention time, flow rate and a column temperature, was optimised, ensuring detection limits of plate count>2500, tailing <2, and peak area for quantification.

Critical Method Attributes (CMAs)

Shim-pack column,10 µL injection,40 ± 2 °C, and 1–1.4 mL/min flow were used. Detection at 258 nm with gradient acetonitrile, formic acid, and methanol enabled separation.

Study on Risk Assessment

Ishikawa and REM were used to identify root causes and classify low-, medium-, and high-risk factors affecting critical analytical attributes.[13]

Method Development for Quercetin and Rutin

Standard Stock Solution Preparation

Weighed exactly 10 mg each of Rutin and Quercetin standards were prepared into a 10-millilitre volumetric flask, added methanol, and sonicated until complete dissolution, and further made up the volume to 10mL with methanol. [14]

Method Development Trials

Simultaneous quantitative estimation of quercetin as well as rutin trials method was run by RP-HPLC using Ultra Violet detection at 258 nanometer,10 µL injection, 10 min run time, and 40 °C column temperature (Table-1).[15]

Table-1: Method Trials for Quercetin and Rutin

Trials no:	M.P Composition Methanol: 0.01% KH2PO4(v/v)	Column	Flow rate(ml/min)
1	40:60	Shim pack C-18 Solar Column (150×4.6mm, 5µm)	1.0mL/min
2	25:75	Shim pack C-18 Solar Column (150×4.6mm, 5µm)	1.2mL/min
3	30:70	Shim pack C-18 Solar Column (150×4.6mm, 5µm)	1.4mL/min
4	30:70	Shim pack C-8 Solar Column (150×4.6mm, 5µm)	1.4mL/min
5	30:70	Shim pack C-8 Solar Column (150×4.6mm, 5µm)	1.2mL/min
6	35:65	Shim pack C-8 Solar Column (150×4.6mm, 5µm)	1.0 mL/min

Detection Method

Method optimisation by DOE

Custom Design was applied for screening and optimisation under constraints. Factor limits were defined; 5 design iterations were generated for three responses in (Table-2).[16]

Table-2: Method Runs by Custom Design

S. No.	FACTORS			RESPONSE		
	Column	Composition	Flow rate	Retention time (1)	Retention time (2)	Tailing Factor (1)
1	ShimpackC8	40:60	1.5	1.778	3.562	1.672
2	ShimpackC18	25:75	1.5	1.778	2.701	1.554
3	ShimpackC8	25:75	1.5	1.775	3.46	1.625
4	ShimpackC8	40:60	1	2.184	4.11	1.808
5	ShimpackC18	40:60	1.25	1.642	3.811	1.58
6	ShimpackC18	40:60	1.5	1.778	3.264	1.499
7	ShimpackC8	25:75	1	2.382	4.11	1.783
8	ShimpackC8	40:60	1	2.072	4.589	1.85
9	ShimpackC18	25:75	1	2.141	3.873	1.632
10	ShimpackC18	25:75	1	2.135	3.773	1.588
11	ShimpackC8	25:75	1.5	1.757	2.776	1.561
12	ShimpackC18	40:60	1.5	1.678	3.342	1.478

Statistical Optimisation of Selected Responses of Method Development

Method effectiveness was improved by optimising temperature, wavelength, flow rate, column type, and solvent composition using graphical and numerical approaches.[17]

Validation of Method - Standard Stock Solution Preparation

Preparation for Quercetin and Rutin

10 milligrams of each Rutin as well as Quercetin was dissolved in methanol in a 10-millilitre volumetric flask, and diluted to 10 mL and sonicated.[18]

Blank

Mobile phase was injected to ensure that no peaks appeared in the chromatogram.[19]

System Suitability

The purpose of this test was to confirm the system's suitability and effectiveness for routine analysis.

Specificity

Specificity is the ability to distinguish analytes from related compounds; blank injection confirms the absence of interfering peaks. [20,21]

Accuracy

Accuracy was evaluated by % recovery using 50, 100, and 150 µg/mL solutions corresponding to 50%, 100%, and 150% levels injected into HPLC. [22]

Precision

The concentration of 100µg/ml is ready. Construct six vials and inject one at a time.[23]

Linearity and Concentration Range

Vials were filled with various concentrations, ranging from 50% to 150%, such as 50, 75, 100, 125, and 150 (µg/mL) injection. [24]

LOD and LOQ

From a 100 (µg/mL) stock, 0.2 and 0.6 mL were diluted to 10 mL; LOD and LOQ was established at (S/N) Ratio of signal to noise corresponding to 3:1 and 10:1, respectively. [25]

Robustness

The method's robustness was evaluated by varying the flow rate (±0.1 mL/min) and pH (±0.1), and changes in the mobile phase composition (±5%).[26]

RESULTS AND DISCUSSION

Extraction Process

The methanolic extraction of *Ervatamia heyneana* leaves yielded 72.93% w/w, indicating efficient recovery of phytoconstituents. The high yield suggests that methanol is a suitable solvent for the extraction of polar and non-polar bioactive compounds. This supports its applicability in phytopharmaceutical investigations and aligns with previously reported solvent efficiency trends.

Phytochemical Test and Screening of Leaf Extract

The phytochemical screening confirmed the existence of alkaloids, flavonoids, phenols, steroids, terpenoids and tannins (Table-3), which are associated with diverse biological activities, including antioxidant and therapeutic effects.

Table-3: Phytochemical Screening of *Ervatamia heyneana* Leaf Extract

Phytochemical Class	Test Performed	Observation	Inference
Alkaloids	Mayer's Test	Cream precipitate	+
Alkaloids	Wagner's Test	Reddish-brown precipitate	+
Alkaloids	Dragendorff's Test	Reddish-brown precipitate	+
Flavonoids	Ammonia Test	Yellow colour	+
Flavonoids	Sodium Hydroxide Test	Yellow colour disappears with acid	+
Tannins and Phenols	Ferric Chloride Test	Blue/green coloration	+
Tannins and Phenols	Lead Acetate Test	White precipitate	+
Steroids and Terpenoids	Liebermann- Burchard Test	Blue/green colour	+

Thin-layer chromatography (TLC) analysis result revealed the spots Rf = 0.61 (Quercetin), Rf = 0.20 (Rutin) under 365 nm UV light, confirming their presence through co-migration with standards. This establishes preliminary evidence for key flavonoids in the extract.

FT-IR Characterisation

The characteristic absorption bands of characteristic functional group O–H stretching (3366 cm^{-1})-indicative of phenolic groups, C–H stretching (2853–2919 cm^{-1}), Aromatic C=C (1605, 1444 cm^{-1}) and C–O stretching (1249 cm^{-1}). These findings confirm the presence of phenolic and flavonoid functional groups, consistent with compounds such as Quercetin and Rutin.

Characterisation of LC–MS

The study confirmed Rutin Rt = 3.903 min, m/z = 611.47 and Quercetin: Rt = 6.255 min, m/z = 303.26. The results matched with standard peaks (Fig.-1 and Fig.-2) confirmed these bioactive flavonoids, hence strengthening the analytical reliability of the method.

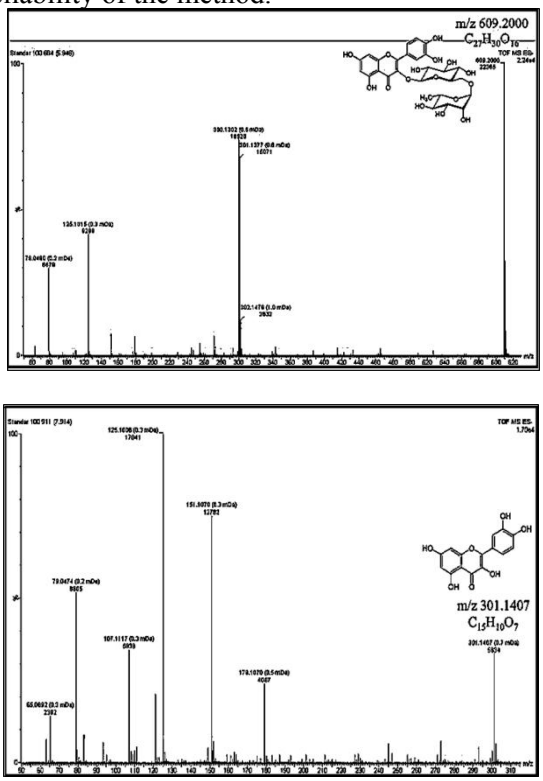

Fig.-1: LCMS Chromatogram of Standard Rutin and Quercetin

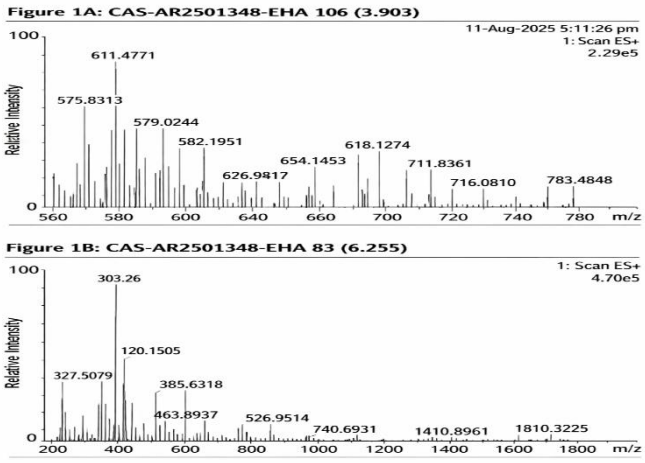

Fig.-2: LCMS Chromatogram of *Ervatamia Heyneana* Leaf Extract

Method Development

The optimised RP-HPLC method using KH_2PO_4: Methanol (35:65 v/v) on Shimpack C18/C8 columns at 258 nm achieved effective separation of Rutin Rt 3.202 min and Quercetin Rt 6.131 min in standard and leaf extract sample. The chromatogram showed good resolution, symmetry, and reproducibility (Fig.-3 and Fig.-4), indicating suitability for routine analysis.

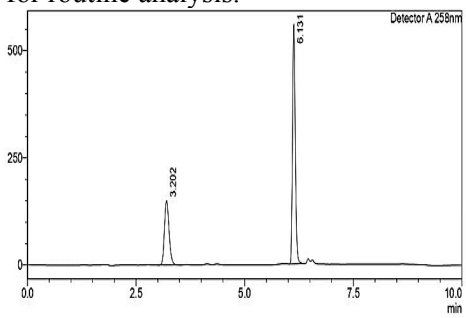

Fig.-3: HPLC Chromatogram of Standard Rutin and Quercetin

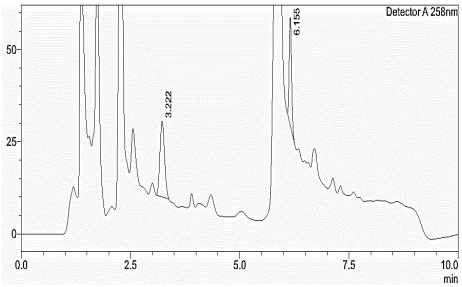

Fig.-4: HPLC Chromatogram of *Ervatamia Heyneana* Leaf Extract

Selected Optimal Chromatogram

The selected trial showed clear separation of Rutin (3.202 min) and Quercetin (6.131 min), confirming the method is robust and suitable for accurate quantification, as shown in (Fig.-5).

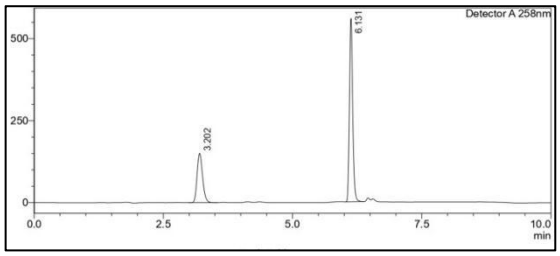

Fig.-5: Optimal Chromatogram of Simultaneous Estimation of Rutin and Quercetin in *Ervatamia Heyneana* Leaf Extract

Effect Summary

The Flow rate was the most significant factor affecting performance, followed by column type, while mobile phase had a lesser but significant effect, making flow rate the dominant parameter.

Prediction profiler of the optimised method and statistical optimisation of specific HPLC method responses

Numerical optimisation showed a strong correlation between parameters and responses, with a maximum desirability of 72.84%. The models were statistically significant ($R^2 = 0.84–0.91$, $p < 0.05$), confirming good model fit and method reliability.

Analytical Method Validation

Specificity- Interfering peaks were not observed in blank samples at Rutin and Quercetin retention times, confirming method specificity.

System Suitability

System suitability met acceptance criteria with the tailing factor of ≤2.0, %RSD ≤2.0, and theoretical plate count ≥2000 as shown in 9(Table-4).

Table-4: Studies on System Suitability

Peak No.	Area	Retention time (min)	Tailing Factor	Theoretical plates	Resolution
1. Rutin	559909	3.204	1.207	3978	---
%RSD of 5 replicates	0.196				
2. Quercetin	942599	6.140	1.312	40213	17.917

Accuracy

Accuracy at 50%, 100%, and 150% levels was within 97–103%, confirming acceptable method accuracy as shown in (Table-5 and Table-6).

Table-5: Accuracy Studies or %Recovery Studies (Rutin)

Rutin						
Level of accuracy	Initial drug peak area	Total peak area	Difference in peak area	Drug Recovery in (µg/mL)	% Recovery	Mean % recovery
50%	137138	205418	68280	3.98	99.58	Mean= 99.83 SD= 1.29 %RSD= 1.29%
	137138	204812	67674	3.95	98.69	
	137138	206548	69410	4.05	101.23	
100%	137138	275128	137990	8.05	100.62	Mean= 100.83 SD= 1.01 %RSD= 1.00%
	137138	276905	139767	8.15	101.92	
	137138	274210	137072	8.00	99.95	
150%	137138	341916	204778	11.95	99.55	Mean= 99.94 SD= 1.63 %RSD= 1.63%
	137138	346412	209274	12.21	101.73	
	137138	339807	202669	11.82	98.52	

Table-6: Accuracy Studies or %Recovery Studies (Quercetin)

Quercetin						
Level of accuracy	Initial drug peak area	Total peak area	Difference in peak area	Drug Recovery in (µg/mL)	% recovery	Mean % recovery
50%	222628	335422	112794	4.05	101.33	Mean= 99.83 SD= 1.29 %RSD= 1.29%
	222628	336104	113476	4.08	101.94	
	222628	332812	110184	3.96	98.98	
100%	222628	446214	223586	8.03	100.43	Mean= 100.83 SD= 1.01 %RSD= 1.00%
	222628	440916	218288	7.84	98.05	
	222628	443219	220591	7.93	99.09	
150%	222628	551412	328784	11.81	98.46	Mean= 99.94 SD= 1.63 %RSD= 1.63%
	222628	561603	338975	12.18	101.51	
	222628	560324	337696	12.13	101.12	

Precision

% RSD should be < 2% for repeatability; % RSD should be < 3% for intermediate precision / Reproducibility.

Linearity and range

Excellent linearity was observed with $R^2 = 1.000$ for Rutin and 0.9997 for Quercetin.

LOD and LOQ

The ratio of S/N used for the calculation of LOD and LOQ for Rutin and Quercetin has been determined to be 3.23 and 3.22, respectively. The low value complied with ICH Q2(R1) guidelines, confirming the method's acceptable sensitivity.

Robustness

The testing showed %RSD ≤2.0% under slight flow rate and temperature changes, confirming method robustness as shown in as shown in (Table-7 and Table-8).

Table-7: Study of Robustness by Changing Flow (Rutin and Quercetin)

Analyte	Flow Rate (mL/min)	Peak Area 1	Peak Area 2	Peak Area 3	Peak Area 4	Peak Area 5	Average	SD	%RSD
Rutin	0.9	618439	612753	618923	616140	615808	616412.600	2461.754	0.399
Rutin	1.1	503710	496966	495281	497670	497241	498173.600	3224.923	0.647
Quercetin	0.9	1,019,752	1,024,216	1,019,221	1,017,060	—	1,020,243.800	2632.979	0.258
Quercetin	1.1	841,533	826,749	832,623	831,344	—	832,179.000	5708.736	0.686

Table-8: Study of Robustness by Changing Column Temperature (Rutin and Quercetin)

Analyte	Temperature (°C)	Peak Area 1	Peak Area 2	Peak Area 3	Peak Area 4	Peak Area 5	Average	SD	%RSD
Quercetin	35	1,001,896	1,025,912	1,010,992	1,003,176	1,009,888	1,010,372.800	9,562.103	0.946
Quercetin	45	1,010,271	1,010,067	994,422	1,010,739	1,008,505	1,006,800.800	6,970.630	0.692
Rutin	35	586,138	592,036	592,471	582,416	585,751	587,762.400	4,350.373	0.740
Rutin	45	584,726	584,270	573,836	584,280	584,016	582,225.600	4,696.889	0.807

Assessment of Greenness

AGREE (0.59) and MoGAPI (70) scores indicated acceptable greenness, confirming the method has good environmental sustainability and was suitable for routine quality control.

CONCLUSION

The current study on AQbD optimized and validated RP-HPLC method development for simultaneous quantification of quercetin and rutin in *E. heyneana* leaf extract clearly showed the efficacy of the statistical design approaches. Risk assessment was done using Ishikawa and the risk estimation matrix to prevent method interactions. Using a Shimpack C18 and C8 column at 258 nm and a rate of flow of 1.0 mL per min, the method was developed by RP-HPLC and statististically optimized using an Custom design. By optimising the method for maximum desirability in accordance with ICH Q2R1, the prediction profiler verified the robustness of the method validation parameters. The greenness assessment tool AGREE and MoGAPI showed moderate compliance with green analytical chemistry principles. While the method involves minimal hazardous solvents and generates moderate waste, the use of low sample amounts, renewable plant material, minimal energy consumption, and safe handling practices contributes positively to its overall greenness. This work improves SDG-12 by providing a green, resource-efficient analytical approach that minimises solvent consumption and environmental impact. The designed experimental research facilitates sustainable phytopharmaceutical research to promote responsible laboratory procedures.

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CONFLICT OF INTERESTS

The Author declared there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-12: Responsible Consumption and Production*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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