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Original Article

HPLC Method to Standardize Secondary Metabolites of Plant Extract: Application to *Ziziphus nummularia* and *Eclipta prostrata*

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Abstract

Investigating intricate plant extracts poses a formidable task in unearthing new bioactive compounds with therapeutic potential. Thus, the need for a rapid and sensitive method is crucial to ensure the quality and efficacy of herbal products in the botanical industry. A robust RP-HPLC technique was created, validated, and employed to separate, detect, and measure phytoconstituents. This method was then utilized for the analysis of chloroform extracts from the leaves of *Eclipta prostrata* and hydroethanolic extracts from *Ziziphus nummularia.* The RP-HPLC method involved employing a mobile/solvent phase of acetonitrile: methanol (7:3 v/v) at a constant flow rate of 1 mL/min, and the analysis was conducted at a wavelength of 208 nm. Eluted peaks were detected at retention times of 2.11±0.023 min, 3.233±0.045 min, 3.437±0.126 min, and 4.120±0.137 min, corresponding to rutin, stigmasterol, β-sitosterol, and quercetin, respectively. The response exhibited linearity in the range of 0.2-0.8 µg/ml, with a regression coefficient exceeding 0.991. The limits of detection (LOD) and quantification (LOQ) for these components ranged from 0.692-1.92 ng/ml and 2.10-10.45 ng/ml, respectively. This developed method demonstrated precision, specificity, reproducibility, and accuracy. It was subsequently applied to assess the content of dried leaf powder from *E. prostrata* and *Z. nummularia*. The RP-HPLC method has the potential to be beneficial for assessing both the qualitative and quantitative aspects of the components found in plant extracts and herbal products.

Keywords: E. Prostrate; Quercetin; RP-HPLC; Rutin; Sensitive; Stigmasterol; Z. Nummularia.

1. Introduction

India's traditional herbal medicine systems, including Ayurveda, Siddha, and Unani, offer natural remedies derived from plants. While these remedies have deep cultural significance, efforts to ensure safety, efficacy, and quality control are increasingly important for integrating them into modern healthcare practices. Plants have traditionally been a rich source of diverse secondary metabolites with potential therapeutic properties. Ensuring the safety and quality of phytomedicine aligns with

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the WHO's traditional medicine strategy, necessitating the development of specific strategies and methodologies to ensure the quality of these herbal medicines. RP-HPLC is the most employed technique for characterizing complex herbal plant extracts [1, 2].

Eclipta prostrata, a member of the Asteraceae family, goes by various common names such as false daisy, yerba de tago, guntagalagara aaku, Karisalankanni, and bhringraj. This small, branching herbaceous plant holds significant therapeutic value, offering remedies for a variety of health issues. It is widely used to address conditions like fever, hair loss, skin disorders, digestive and respiratory problems, as well as ailments affecting the liver and gastrointestinal system. *E. prostrata* contains a range of bioactive compounds, including alkaloids, triterpenoids, cumestans, flavonoids, and glycosides, distributed throughout different parts of the plant. Its pharmacological profile underscores its efficacy in areas such as antimicrobial, antioxidant, anti-inflammatory, anti-tumor, immunostimulatory, hepatoprotective, and hypolipidemic activities, among others [3, 4].

Ziziphus nummularia, belonging to the Rhamnaceae family, often referred to as wild jujube or jharberi, is a prevalent shrub in dry deciduous forests and scrub jungles, thriving in a wide range of habitats, except for saline patches and sand dunes. *Z. nummularia* exhibits diverse pharmacological properties, including cytoprotective, antioxidant, and anti-inflammatory activities. Phytochemical analysis has confirmed the presence of alkaloids, flavonoids, saponins, glycosides, and tannins in this plant [5, 6].

The accurate identification of phytoconstituents in medicinal plants relies heavily on marker profiling and standardization. A primary concern in herbal preparations is ensuring quality, safety, and quantification of phytoconstituents in herbal formulations. In this regard, HPLC stands out as one of the most practical and comprehensive analytical techniques for the isolation, identification, and standardization of secondary metabolites in plant extracts. The HPLC method holds considerable promise for verifying the authenticity, creating a unique fingerprint, quantifying, and controlling the quality of herbal products. RP-HPLC remains the preferred tool for herbal analysis.

Quercetin, rutin (also known as Vitamin P), Beta-sitosterol, and stigmasterol are prevalent natural antioxidants found in various fruits, vegetables, and medicinal plants. They serve multiple purposes such as colorants, antioxidants, preservatives, stabilizers, and UV absorbers in various applications. Despite the numerous pharmacological and medicinal applications of both plants, a thorough review of the literature has revealed a notable absence of studies focusing on the standardization of β-sitosterol, stigmasterol, quercetin, and rutin in the leaves of *E. prostrata* and *Z. nummularia*. In a previous investigation, it was demonstrated that chloroform extracts from *E. prostrata* exhibited the highest therapeutic potential for Rheumatoid Arthritis, while hydroalcoholic extract from *Z. nummularia* demonstrated significant gastroprotective effects.

Therefore, an effort has been made to develop a method that can effectively separate,

identify, and quantify these secondary metabolites or phytoconstituents using the RP-HPLC method. This developmental approach was rigorously validated in accordance with the standards of the International Conference on Harmonization [7] and subsequently applied to quantify and standardize the chloroform extract of *E. prostrata* leaves and the hydroethanolic extracts of *Z. nummulari*a [8-10].

2. Materials and Methods

2.1. Plants Material Collection and Authentication

The collection of leaves of *Z. nummularia* was done in the month of September 2021 from Meera Nagar in state Jodhpur, India, and leaves of *E. prostrata* Linn. Was done in the month of October 2021 from Chandigarh Botanical Garden and Nature Park, Sarangpur, Chandigarh, India. The plant was authenticated by CSIR-NISCAIR, New Delhi by Dr. Sunita Garg (Emeritus Scientist). For any future reference, plant herbarium was prepared and submitted under the voucher specimen number NISCAIR/RHMD/Consult/2018/3301-02-1 and NISCAIR/RHMD/Consult/2018/3301-02-2.

2.2. Chemicals and Reagents

HPLC grade acetonitrile ACN, and water were used. All the chemicals and reagents are of analytical grade (AR) and were purchased from Merck Ltd. (Mumbai, India). Standards β-sitosterol, stigmasterol, quercetin, and rutin was purchased from Sigma Aldrich.

2.3. RP-HPLC Instrumentation

The HPLC system, which includes an Agilent HPLC with a rheodyne injector (loop size of 20 L), is fitted with a multi-wavelength UV-Visible detector and a Phenomenex-Luna C18 column. The mobile phase was filtered using membrane filters with 0.45 mm pore sizes (Millipore), and the sample was filtered using Whatman's syringe filters (NYL 0.45 mm).

2.4. Preparation of Extract

The leaves of the plants were collected, carefully washed, and then subjected to shade-drying. Afterward, the dried material was coarsely ground using a mechanical grinder. To obtain the extracts, a Soxhlet extraction method was employed. These extracts (chloroform extract of *E. prostrata* and hydroalcoholic (8:2) extract of *Z. nummularia*) were subsequently processed by evaporation, reduction, and drying using a rotary evaporator, and the resulting yields, as well as the color and texture of the extracts, were determined. The dried extract was stored at 4°C for future use.

2.5. Standardization of Extracts Using HPLC

In its essence, it represents a significantly improved version of column liquid chromatography. Instead of relying on the gravitational flow of a solvent through a column, it exerts high pressures, often reaching up to 400 atmospheres, to propel the solvent through the system. The analysis was performed utilizing an HPLC system with a Phenomenex-Luna C18 column, and a rheodyne injector (with a 20 µL loop) was employed. Wavelength 208 nm was the chosen wavelength for the analysis. The mobile phase used for the analysis consisted of a mixture of Acetonitrile and Methanol in a 7:3 volume/volume ratio, flowing at a rate of 1.0 ml/min.

2.6. Standard Solution Preparation

An Eppendorf tube containing 1 mg of standard was dissolved in 1 mL of methanol, well mixed in a vortex. Solution was diluted with methanol to create a standard stock solution with a concentration of 1000 g/mL. Further dilutions were made as needed to create the calibration curve. Different concentrations, including 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1 µg/ml, were made from the stock solution.

2.7. Sample Solution Preparation

An Eppendorf tube containing 1 mg of *E. prostrata/ Z. nummularia* extract was taken, and 1 mL of chloroform/ ethanol was added to dissolve it. Following 15 minutes of vertexing, the sample was exposed to 30 minutes of ultrasonication. The solution was then filtered using a 0.45 syringe filter. The sample solution's 1000 µg/mL concentration was attained.

2.8. Mobile Phase Preparation

ACN and water were mixed in a 7:3 (v/v) ratio to prepare the mobile phase, which was then filtered through a 0.45 mm Millipore membrane filter and ultrasonically de-gassed.

2.9. Calibration Curve

Calibration curve was prepared for β-sitosterol, Stigmasterol, Rutin, and Quercetin by plotting graph between concentration vs area of peaks generated in the HPLC chromatogram. Linear regression equation & Regression coefficient (r^2) were determined. Further % content of β-sitosterol, Stigmasterol, Rutin, and Quercetin was determined in the extract & finally in the leaves part of the plants [11, 12].

2.10. Method Validation

In accordance with ICH recommendations, the method's accuracy, precision, limit of detection (LOD), linearity, and specificity were all validated [7, 11, 12].

2.11. Linearity

The linearity range of β-sitosterol, Stigmasterol, Rutin, and Quercetin was examined (n = 6) using standard solutions containing these compounds at concentrations ranging from 0.1 to 0.8 mg/mL under optimal chromatographic conditions. A linear regression analysis was used to determine the linearity of the calibration curve, which was created by graphing the peak area on the Y-axis vs the concentration on the X-axis.

2.12. Specificity

Comparing the retention times of the standard and extract samples allowed researchers to assess the method's specificity. This mostly serves to ensure the analyte's identity and purity as well as to reduce result inaccuracy.

2.13. LOD and LOQ

By calculating the SD of the response (s) and the slope of the linear equation (S), the LOD and LOQ were computed in accordance with the ICH guidelines. Equation was used to determine the LOD and LOQ. LOQ = 10 s/S and LOD = 3.3 s/S.

2.14. Precision and Accuracy

For each analyte, the assay precision for intra- and inter-day was assessed. Comparing the results from one run (n=6) allowed for data evaluation. Six replicates of the reference compounds were injected at three different concentrations to

analyse intra-day and inter-day variance and determine the precision of the approach. Values were displayed as % RSD. Standard addition methodology was used to calculate the method's accuracy, which was then expressed as a percentage of RSD. A known amount of (80, 100, and 120%) of standard solution was added to the pre-analysed solution with three repetitions of each concentration. The resultant % RSD for this study was found to be < 2.0 %.

2.15. Robustness

Robustness research was carried out to see how varying mobile phase compositions, flow rates, and wavelength detection affected the retention time. The results of the statistical study were reported as the mean ± % RSD using Graph Pad Prism Version 5.0.

3. Results and Discussion

3.1. Preparation of Extract

The extract was prepared using Soxhlet Extractor. The yield, colour and consistency of the extracts were found to be semisolid, dark brown color, 3.80 % w/w and 6.25 % w/w extractive yield of *E. prostrata* and *Z. nummularia* respectively.

3.2. Phytochemical Screening

Phytochemical analysis of the chloroform extract from *E. prostrata* leaves (CEEP) unveil the presence of various constituents that could account for the plant's medicinal and physiological properties. The phytochemical screening results indicated the existence of alkaloids, carbohydrates, saponins, phytosterols, flavonoids, tannins, proteins, and amino acids.

In the case of the hydro-alcoholic extract of *Z. nummularia* (HAZN), initial phytochemical tests demonstrated the presence of glycosides, alkaloids, saponins, sterols, tannins, and flavonoids, which could contribute to the plant's diverse range of pharmacological effects. These findings align with previously reported phytoconstituents of the plant in an earlier study [13].

The ability of *E. prostrata* and *Z. nummularia* to protect cells may be attributed to the presence of flavonoid constituents in their extracts. Flavonoids and saponins are known to exhibit cytoprotective effects through their antioxidant and anti-inflammatory properties. These extracts are abundant sources of antioxidants. Considering all the evidence, it is proposed that the anti-ulcer activity initiated by HAZN could potentially stem from the synergistic effects of flavonoids, saponins, and alkaloids.

3.3. Standardization of Plant Extract Using HPLC

RP-HPLC was conducted on a C18 column in reversed phase mode, maintaining the column temperature at an optimal 25°C. The mobile phase consisted of acetonitrile and water in a 7:3 volume/volume ratio, flowing at a rate of 1.0 ml/min. A wavelength of 208 nm was selected for constructing a linear calibration curve. Various dilutions were prepared using a standard stock solution of quercetin, beta-sitosterol, stigmasterol, and rutin.

The retention times recorded for rutin, stigmasterol, beta-sitosterol, and quercetin were 2.11±0.023 min, 3.233±0.045 min, 3.437±0.126 min, and 4.120±0.137 min, respectively (**Fig. 1** and **2**).

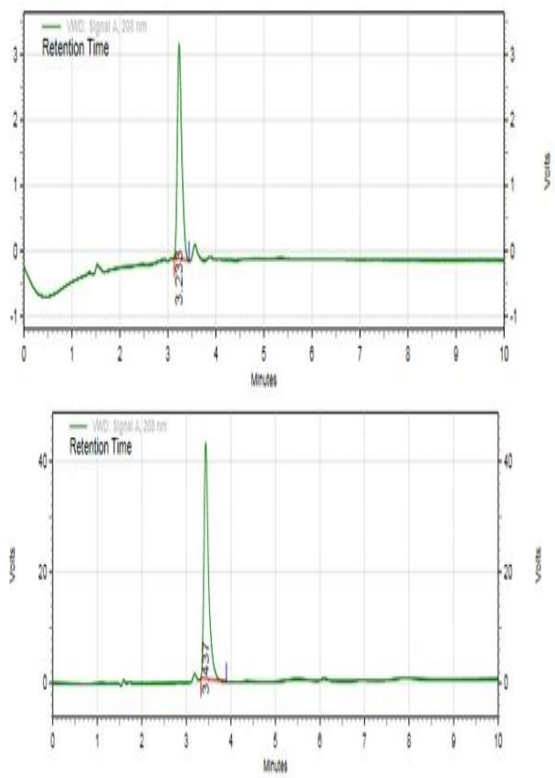

Figure 1. Chromatogram of β sitosterol and Stigmasterol.

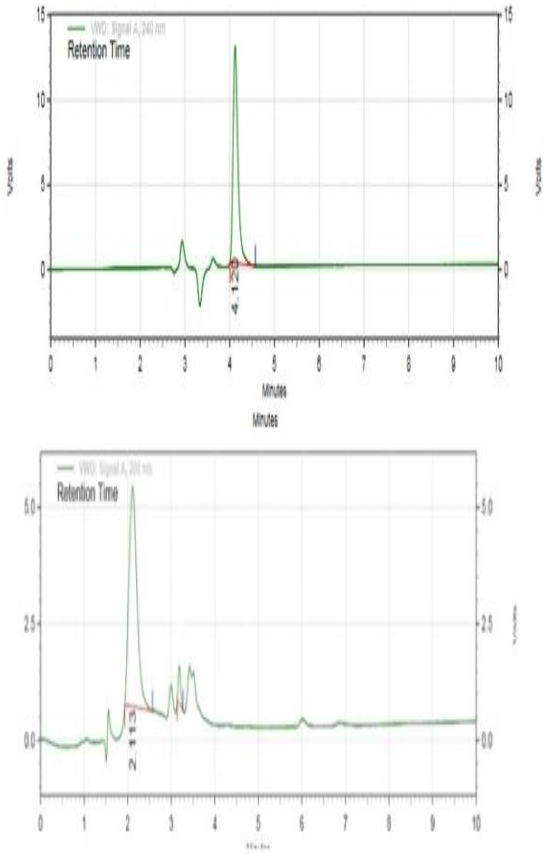

Figure 2. Chromatogram of Quercetin and Rutin.

A standard curve was generated by plotting the mean peak areas against concentration, and the linear regression equation and regression coefficient were determined, as outlined in the table 1. This method was both developed and validated, and subsequently employed for the quantification of constituents present in the plant extracts.

3.3.1. Linearity

The calibration ranges for rutin, stigmasterol, beta-sitosterol, and quercetin was identified as 0.1-0.8 µg/mL. The obtained results indicated coefficient of determination (r^2) values of 0.999, 0.998, 0.992, and 0.992 for beta-sitosterol, quercetin, stigmasterol, and rutin, respectively (**Table 1**).

Table 1. Analysis of compounds in the plant extracts using HPLC.

Standard	β- sitosterol (Mean ± SD)	Quercetin (Mean ± SD)	Stigmasterol (Mean ± SD)	Rutin (Mean ± SD)
Retention Time	3.437±0.126min	4.120±0.137min	3.233±0.045min	2.11±0.023min
Linear regression equation Y=mx+c	Y=329230x+983900	Y=192187x+199541	Y=402817x+173353	Y=489709x+17793
Regression coefficient (r^2)	0.999	0.998	0.992	0.992
Content in HAZN (µg/ml)	0.690±0.021	--	0.348±0.016	0.126±0.056
Content in CEEP (µg/ml)	1.779±0.035	0.107±0.054	1.578±0.027	0.215±0.015

3.3.2. Specificity

The well-shaped peak revealed by the specificity test that there were more extract constituents does not interfere with the main peaks.

3.3.3. LOD and LOQ

The method LOD and LOQ were found varied between 0.692- 1.92 ng/ml and 2.10-10.45 ng/ml, respectively.

3.3.4. Accuracy and Precision

The method's accuracy was indicated by the high recovery values between 97.20 and 100.56%. The method's repeatability is confirmed by the fact that the intra-day and inter-day precision's percent RSD was found to be <2% (**Table 2**).

3.3.5. Robustness

The robustness was assessed by examining (n=6) the standard solution under the smallest changes (±2).

In the ideal circumstances, such as flow rate and wavelength detection. The retention time, peak area, and recovery studies, however, showed no discernible differences.

3.4. Estimation of Content in the Extracts

Using the linear curve and the linear regression equation, the amount of rutin, stigmasterol, beta-sitosterol, and quercetin content in the extracts was calculated (as depicted in **Fig. 1-4**). In the case of CEEP, the calculated percentage content was as follows: 1.601% w/w for beta-sitosterol, 0.096% w/w for quercetin, 1.42% w/w for stigmasterol, and 0.193% w/w for rutin within the extract. Furthermore, the content in the dried leaf powder of *E. prostrata* was determined to be 0.061% for beta-sitosterol, 0.004% for quercetin, 0.054% for stigmasterol, and 0.007% for rutin, all on a weight/weight basis.

Table 2. Results of Accuracy study by Recovery method, Intraday and Interday precision values.

Concentration µg/ml	Recovery (n=3)		Intraday Precision (n=6)		Interday Precision (n=6)	
	Mean %	RSD %	Accuracy %	Precision RSD %	Accuracy %	Precision RSD %
			β-sitosterol			
0.8	100.05	1.24	99.95	0.14	100.15	0.26
0.6	99.75	1.35	100.06	0.59	98.4	0.87
0.4	98.68	1.25	98.54	1.14	104.2	2.21
			Stigmasterol			
0.8	100.03	0.84	99.15	0.78	99.67	1.24
0.6	99.55	0.63	98.34	1.15	98.22	2.11
0.4	98.05	0.95	97.45	1.56	97.35	1.98
			Rutin			
0.8	99.75	2.12	100.14	0.89	95.23	1.67
0.6	100.25	1.34	99.67	1.12	98.45	0.96
0.4	97.20	0.89	99.05	2.36	99.14	1.17
			Quercetin			
0.8	100.56	1.14	105.12	1.18	99.76	2.26
0.6	98.83	1.67	99.34	2.49	98.45	1.05
0.4	99.32	2.28	98.78	2.12	97.31	1.13

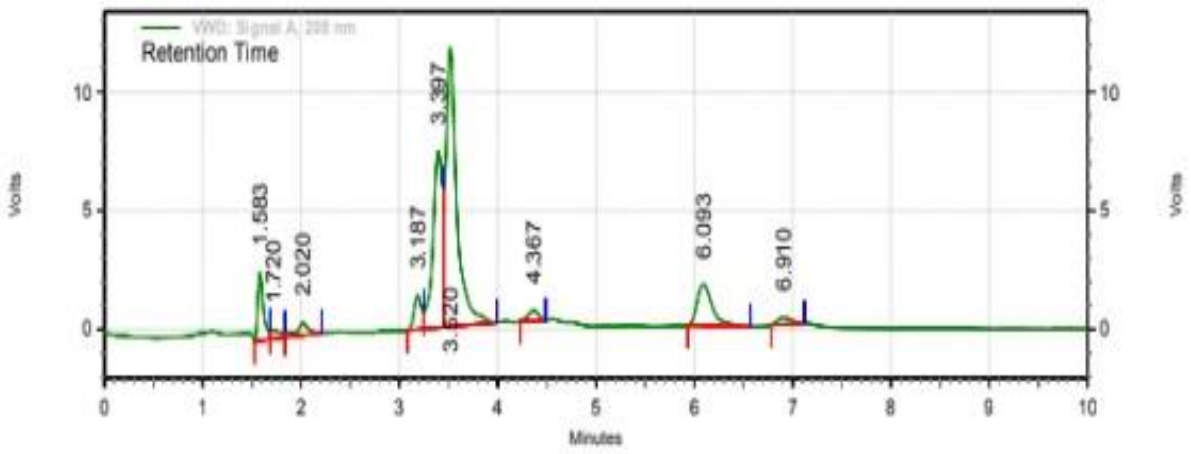

Figure 3. Chromatogram of *E. prostrata* Chloroform extract.

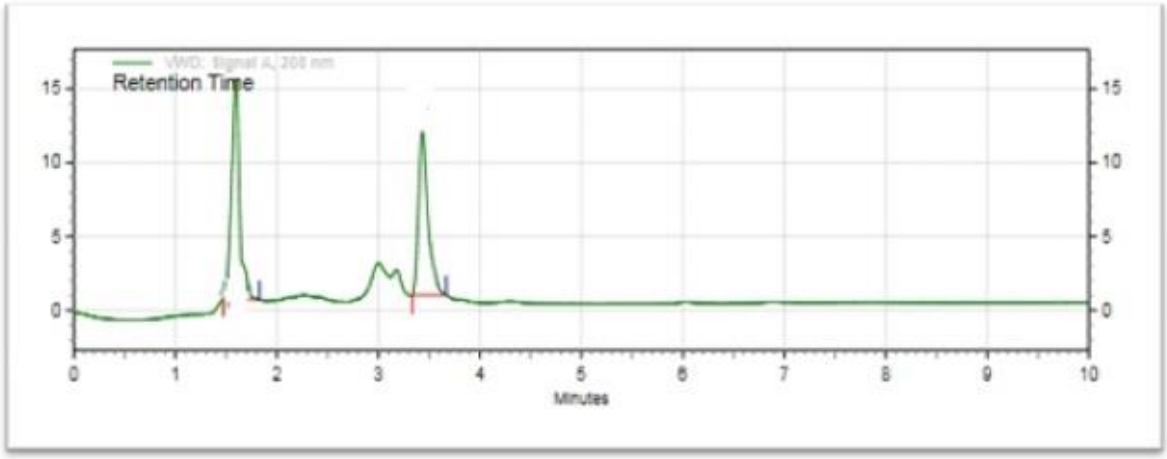

Figure 4. Chromatogram of *Z. nummularia* Hydro alcoholic extract.

In the case of HAZN, the calculated percentage content was as follows: 0.622% w/w for beta-sitosterol, 0.313% w/w for stigmasterol, and 0.113% w/w for rutin within the extract. Additionally, the content in the dried leaf powder of *Z. nummularia* was determined to be 0.038% for beta-sitosterol, 0.019% for stigmasterol, and 0.007% for rutin, all on a w/w basis.

The quality of herbal medicines can vary significantly depending on factors like harvest times, plant sources, drying methods, heavy metal contamination, microbial content, and other related aspects. Hence, it's crucial to identify most of the phytochemicals in medicinal plant products to ensure the reliability and consistency of pharmacological research and the effectiveness of these products [1, 2]. Chemical makers play a vital role in assuring the quality of medicinal plants and the derived products. However, the limited evidence of chemical markers remains a significant concern for ensuring the ongoing quality of herbal medicines.

Considering these challenges, our recent work has focused on validating the RP-HPLC

technique for Eclipta prostrata and *Z. nummularia* plant leaves. This validation is aimed at ensuring the presence of active phytochemicals and the reproducibility of the established method [3-5]. The developed method has been successfully utilized to quantify the concentrations of rutin, beta-sitosterol, quercetin, and stigmasterol in the leaf extracts of *E. prostrata* and *Z. nummularia*. This approach has demonstrated precision, accuracy, robustness, repeatability, and applicability within a specific linear range.

In *E. prostrata*, the content of beta-sitosterol, quercetin, stigmasterol, and rutin was determined to be 0.061%, 0.004%, 0.054%, and 0.007% w/w, respectively. In the case of *Z. nummularia* leaves, the content was found to be 0.038% for beta-sitosterol, 0.019% for stigmasterol, and 0.007% for rutin, all on a weight/weight basis. These components, including beta-sitosterol, quercetin, stigmasterol, and rutin, are likely responsible for the biological activities observed in *E. prostrata* and *Z. nummularia*.

4. Conclusion

The RP-HPLC method was developed and validated for the quantification of β-sitosterol, quercetin, stigmasterol, and rutin in CEEP and HAZN. Using the linear curve and linear regression equations, the quantities of these compounds in the extracts were determined. In CEEP, the calculated percentage content was as follows: 1.601% for beta-sitosterol, 0.096% for quercetin, 1.42% for stigmasterol, and 0.193% for rutin within the extract. Furthermore, the content in the dried leaf powder of *E. prostrata* was determined to be 0.061% for beta-sitosterol, 0.004% for quercetin, 0.054% for stigmasterol, and 0.007% for rutin, all on a weight/weight basis.

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This study has confirmed the presence of β-sitosterol, quercetin, stigmasterol, and rutin as antioxidant compounds in the chloroform extract of *E. prostrata* and the hydroalcoholic extract of *Z. nummularia*, which may account for their antioxidant, anti-inflammatory, and anti-ulcer properties. Therefore, the method can be effectively used for the standardization, quality assurance, and pharmacokinetic studies of herbal plant extracts.

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Conflict of interest

The authors declare to have no conflict of interest.

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