ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 18, Issue 7, 2025

Online - 2455-3891
Print - 0974-2441

Research Article

RP-HPTLC PROFILING OF SECONDARY METABOLITES FROM *FICUS BENJAMINA* AND *FICUS KRISHNAE*

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Received: 14 April 2025, Revised and Accepted: 31 May 2025

ABSTRACT

Objective: This study was carried out to measure the amounts of quercetin and rutin in the hydroalcoholic extracts of *Ficus benjamina* and *Ficus krishnae* leaves using high-performance thin layer chromatography (HPTLC). The results showed a clear separation of these two compounds from other substances in the extracts. The method used is fast, easy to perform, and provides accurate and reliable results.

Methods: TLC plates coated with silica gel 60 F254 were used for the test, and each sample was applied at a speed of 150 nanoL/s. A CAMAG TLC Scanner III was used to scan the plates, with quercetin detected at 254 nm and rutin at 366 nm. The presence of these compounds in the samples was confirmed by comparing their UV spectra with those of the standard compounds. Quercetin and rutin were identified by their specific Rf values, 0.31 and 0.73, respectively.

Results: HPTLC was utilized to differentiate *F. benjamina* and *F. krishnae* by analyzing their phytochemical profiles. Using silica gel 60 F254 plates, rutin and quercetin were separated with solvent systems toluene: ethyl acetate: formic acid (5:3.5:0.5) and toluene: chloroform:methanol (7:2.5:0.5), respectively. The compounds exhibited Rf values of approximately 0.31 for rutin and 0.73 for quercetin, facilitating their identification and quantification in the plant extracts.

Conclusion: This study introduces a rapid, sensitive, and cost-effective HPTLC method for the routine quality assessment of herbal products. By quantifying quercetin and rutin in the leaves of *F. benjamina* and *F. krishnae*, as well as in related herbal formulations, the method supports the standardization and ensures the consistency of these plant-based preparations.

Keywords: *Ficus benjamina, Ficus krishnae*, Phytochemicals, Overlay spectra, HPTLC.

 DOI: http://dx.doi.org/10.22159/ajpcr.2025v18i7.55053. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Recently, there has been a growing interest in natural compounds found in plants because of their many uses and health benefits. Plants contain a wide variety of active chemicals that help improve human health in different ways. These natural substances work together to create the healing effects that many medicinal plants are known for. Some of the most important of these compounds are polyphenols, flavonoids, tannins, sterols, and alkaloids [1-3]. Various studies have shown that many of these active constituents of the plants are responsible for antitumor, anti-inflammatory, antiatherosclerosis, antimutagenic, antibacterial, anticarcinogenic, and antiviral activities [4].

Ficus benjamina L., locally named as weeping fig, is a broadly dispersed tree that grows in several European nations as well as Asia, the America, and Australia. The evergreen tree *F. benjamina* can reach up to a height of 60 ft and a width of 60–70 feet [5]. The plant becomes a huge, drooping shrub having thin twigs that grow up to 8 m tall and spread like a crown, which is nearly 10 m broad [6]. The plant is also investigated for the presence of various phytoconstituents and shows the presence of flavonoids, carbohydrates, tannins, phenolics, fats, oils, and saponins. Different parts of plants possess' medicinal properties. The plant is used for the treatment of certain skin disorders and exhibits antimicrobial activity, antioxidant activity, hepatoprotective activity, antitumor activity, antimycobacterial activity, antinociceptive activity, anti-diarrheal activity, and antiviral activity [7-9].

Ficus krishnae, commonly known by the names Makkhan Katori, Krishnae fig, or Krishnae's butter cup mostly distributed in Tropical Africa, India, and Sri Lanka. It is a rapidly growing tree having a height of 10 m and having spreading branches and aerial roots. Pocket-like folds at the base of the leaf make this tree a unique one. Treatment of ulcers, vomiting, fever, leprosy, and inflammation are some of the uses of the plant. Photochemical screening indicates the presence of various active constituents such as saponins, flavonoids, phenols, and alkaloids [10].

The World Health Organization (WHO) has emphasized the importance of standardizing herbal medicines to ensure they are both safe and effective. Studies have shown that using genuine and high-quality raw plant materials plays a key role in the therapeutic success of these remedies. As concerns about the safety, purity, and effectiveness of herbal products continue to grow, the need for proper standardization of plant materials has become increasingly essential [11]. In recent years, the advancement of spectral fingerprinting and chromatographic methods has significantly contributed to ensuring the quality control of complex herbal formulations. These modern analytical tools are now widely applied to detect and measure active compounds in plants, supporting the standardization of herbal medicines [12].

High-performance thin layer chromatography (HPTLC) fingerprinting is a useful tool for identifying medicinal plants and plays a key role in the routine quality control and standardization of herbal formulations. HPTLC is recognized for its speed, efficiency, and ability to produce consistent and reliable results [13]. When integrated with digital scanning, this technique enhances accuracy and precision in the analysis of plant-based compounds [14]. HPTLC methods are regarded as a viable alternative due to their significance as a key tool in routine drug analysis. HPTLC employs a minimal amount of mobile phase

for analysis, thereby reducing both the time and cost associated with analysis. In addition, it also reduces the risk of exposure and disposal problems of toxic effluents, which will ultimately reduce the chances of pollution in the environment. Thin layer chromatography (TLC) serves as a vital tool for compound identification. Despite its relatively lower resolution compared to HPLC, TLC offers distinct advantages, particularly in detecting a broader range of compounds. This method enables the detection of uneluted compounds, and even those lacking UV absorption, can be identified through reagent spraying. TLC-generated chromatograms yield favourable results for the fingerprinting of active compounds within extracts of plant, ensuring the extraction of active constituents. Through optimized conditions and data analysis systems, HPTLC emerges as a robust technique for chromatographic fingerprinting, akin to HPLC and GC, particularly in identifying complex extracts. In addition, HPTLC images provide additional intuitive parameters such as visible fluorescence while enabling the simultaneous determination of different samples on the same plate. While overcoming restrictions on plate development distance and efficiency, the HPTLC approach retains its inherent advantage [15,16]. The literature search has uncovered the absence of alternative methods for quantifying Quercetin and Rutin in leaf extracts of *F. benjamina* and *F. krishnae*. Utilizing a densitometric HPTLC approach, plant material can be efficiently screened for quantitative evaluation, eliminating the need for additional sample preparation. This study presents the development of a densitometric HPTLC method tailored for quantifying Quercetin and Rutin in hydroalcoholic extracts of dried leaves from *F. benjamina* and *F. krishnae*. The method demonstrates suitability for rapid quantitative screening of plant material without necessitating any specialized sample pre-treatment.

METHODS

Reagents and standards

To ensure accurate and reliable HPTLC analysis for quality control and standardization of herbal products, only analytical-grade solvents and chemicals were used. All chemicals and solvents were obtained from E-Merck. Fresh standard stock solutions (mg/ml) were prepared in ethanol before use. A Linomat applicator was employed to apply the sample solutions onto pre-coated TLC plates (5×10 cm, 0.2 mm thick) with silica gel 60 F254. These TLC plates were purchased from Merck Ltd., Mumbai, India.

Instruments and equipment's

HPTLC CAMAG Switzerland, applicator Linomat-V, CAMAG Linomat Syringe, CAMAG UV cabinet, CAMAG TLC Visualizer, CAMAG TLC scanner IIIwin-CATS-V 1.2.3 software, precoated TLC Silica gel 60 F254 plate (E-Merck, Darmstadt, Germany), CAMAG twin trough glass chamber.

Plant materials

In June 2021, the plant was acquired from the Botanical Garden, Sarangpur in Chandigarh. Scientist Dr. Sunita Garg of the Raw Materials Herbarium and Museum (RHMD), NISCAIR, New Delhi, verified the plant's authenticity. For *F. benjamina* and Ficus benghalensis (C. DC.) Corner 'Krishnae, respectively, the authentication numbers were NISCAIR/RHMD/consult/2021/3828-29-1 and NISCAIR/RHMD/consult/2021/3828-29-2. The leaves were separated manually and then cut, dried, powdered, sieved, weighed, and for further use, preserved in airtight containers.

Preparation of extracts

After the plant material was dried and ground into a fine powder, extraction was performed using a Soxhlet apparatus [17]. The extraction was carried out with a hydroalcoholic solvent (ethanol and water, 80:20 v/v). The extract was concentrated under reduced pressure at a temperature of 50–60°C, and the crude extract was stored properly. For HPTLC analysis, the extract was dissolved in 10 mL of methanol.

Chromatography conditions

Chromatography was carried out using a pre-activated, pre-coated 5×10, TLC Silica gel 60 F254 plate (E-Merck, Darmstadt, Germany). Each standard concentration and extract was applied separately (Standard and Extract) to the plate as a 6 mm wide band using a TLC applicator Linomat-V with N2 flow fitted with a 100 µL syringe (CAMAG, Switzerland), which is 8 mm from the bottom. The application dosage speed was 150 nL/s. CAMAG TLC scanner III was utilized for scanning at 254 nm and 366 nm for quercetin and rutin, respectively. Prewashing of the plates was done using methanol and then activated at 60°C for 5 min. A scanning speed of 20 mm/s and slit dimension of 5×0.30 mm was kept. Mobile phase (10 mL), toluene: ethyl acetate: formic acid (5:3.5:0.5), and toluene: chloroform: methanol (7:2.5:0.5) were used for rutin and quercetin, respectively. A twin glass chamber of 5×10 cm, saturated with mobile phase, was used for developing in a linear ascending manner.

RESULTS

Pre-saturating the CAMAG twin trough tank with 10 ml of the mobile phase for the required time before developing the TLC plate is an important step in HPTLC analysis. This helps ensure consistent conditions, better separation of compounds, and more accurate results when analyzing rutin and quercetin. The mobile phases were toluene: ethyl acetate: formic acid (5:3.5:0.5) and toluene: chloroform: methanol (7:2.5:0.5). A CAMAG horizontal developing chamber (10×10 cm) is used at room temperature up to 8 cm. TLC development was done by the ascending mode. Plates were dried using a hair dryer after development. The developed chromatogram was derivatized using spraying reagent 5% sulphuric acid, and anisaldehyde. It is then heated at 105°C for 5 min on a hot plate. A CAMAG UV cabinet (254 and 366 nm) was used for the observation of the plate. Scanning of the plates was done for quantitative analysis at 366nm utilizing CAMAG TLC scanner-III win-CATS-V 1.2.3 software (CAMAG).

The overlay of UV spectra from both the standard and the sample at the same Rf values confirmed the presence of specific compounds in the extracts, with Rf values of 0.31 for quercetin and 0.73 for rutin (Figs. 1-6 and Table 1). The fingerprint profile was performed by densitometry HPTLC analysis, which may be utilized for identification and quantification of herbal extracts.

Calibration curve

The concentrations of quercetin and rutin were determined using a linear calibration curve, which was validated within a concentration range of 40–180 ng/spot. Methanol was used to prepare the standard stock solution of quercetin at a concentration of 10 µg/mL. Spotting of stock solution with different volumes of 4, 8,10,12,14, and 18 µL was done to get concentrations 40, 80, 100, 120, 140, and 180 ng/spot, respectively with a bandwidth of 6 mm and the distance between tracks is 12 mm. Spotting was done using an automatic sample applicator. The peak area corresponding to each concentration was plotted against a standard spot or injection. The linear regression coefficients of the standard curve were measured with R^2±SD=0.9944±5.05& R^2±SD=0.9972, and the linear regression equation was y=57.52x+19201 and y=9.2868x+31047for quercetin and rutin, respectively (Figs. 7 and 8). Regression analysis has revealed a linear relation over a range of 40–180 ng/spot. The linear curve indicates adherence to Beer's Law is confirmed by an excessive correlation coefficient (r^2) and the % SD value for intercept values was <2% (RSD 0.38%). Slopes of the standard curves have not shown any significant difference (ANOVA; $p<0.05$).

Methods validation

Precision

The development and validation of the analytical methods followed ICH guidelines to ensure their accuracy, precision, and consistency [18,19]. The precision of the instrument, as well as intra-day and inter-day variations, was assessed to confirm the reliability of the results [20]. Replicating the sample solution (n=6) was done to measure the instrument's precision. Applying a newly made standard solution with a concentration of 40–180 ng/spot on the same day allowed the study to assess intra-day assay accuracy. Six replicate applications of the same solution on six distinct days allowed for the analysis of intermediate precision, which was expressed as a percentage of CV.

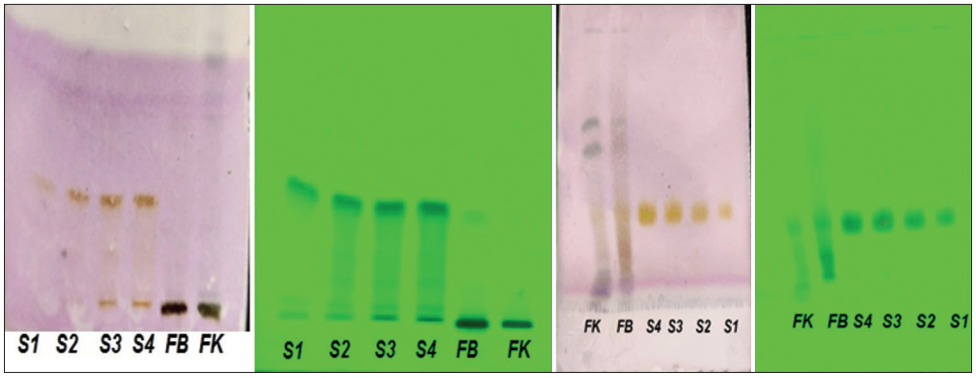

Fig. 1: TLC profiles of *Ficus benjamina* and *Ficus krishnae* along with standard rutin and Quercetin at 254nm and 366nm. Track S1: Standard 1; S2: Standard 2; S3: Standard 3, S4: Standard 4, FB: *Ficus benjamina* extract, FK: *Ficus krishnae* extract

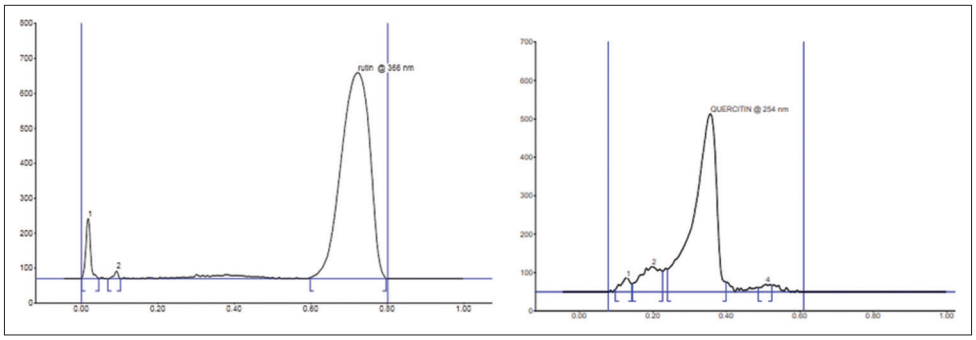

Fig. 2: Chromatogram of the standard Rutin and Quercetin

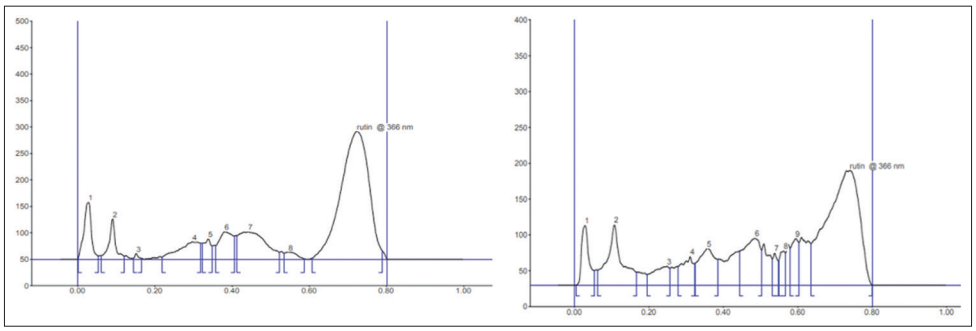

Fig. 3: Chromatogram of hydroalcoholic extracts *Ficus benjamin*a and *Ficus krishna*e showing the presence of rutin at Rf 0.73

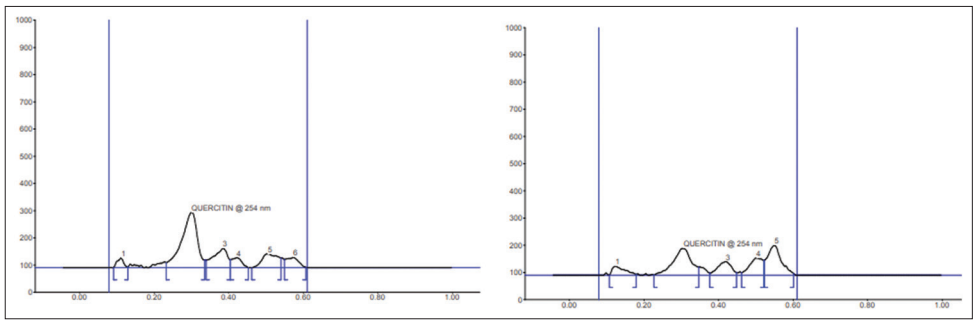

Fig. 4: Chromatogram of hydroalcoholic extracts *Ficus benjamin*a and *Ficus krishna*e showing the presence of Quercetin at Rf 0.31

LOD and LOQ

Methanol was used as a blank to calculate the limits of detection (LOD) and quantification (LOQ). Standard solutions of quercetin and rutin were employed, and the LOD and LOQ were determined based on the signal-to-noise ratio. The LOD was established at a S/N ratio of 3:1, while the LOQ was determined at a S/N ratio of 10:1.

Specificity

The specificity of the method was confirmed by analysing both the plant extracts and standard quercetin and rutin. By comparing the Rf values of the extracts with those of the standards, the presence of the compounds in the extracts was validated. By comparing spectra at three distinct levels of the peak/bands, namely the start, mid, and

Table 1: Method performance parameters for quantification of Quercetin and Rutin by proposed TLC densitometry method

Parameters	Method (Rutin)	Method (Quercetin)	Acceptance criteria (maximum acceptable)
Selectivity	Selective	Selective	
Specificity	Specific	Specific	No interference observed
Linear range (ng/spot)	40–180	40–180	Linearity, accuracy, and precision over the range
Correlation coefficient (r^2)	0.9972	0.9944	Within 0.9–1.1
Linear regression equation Y=mX+c (n=6)	Y=9.2868x+31047	Y=57.52x+19201	
LOD (ng) (n=3)	11.98	11.82	
LOQ (ng) (n=3)	35.94	35.47	
% Recovery (n=3)	98.64	95.89	Within 90–110%
Repeatability (%RSD, n=6)	0.38	0.68	%RSD≤2%
Precision (%CV)	1.05	1.23	%CV≤2%
Intraday Precision (n=6) (%CV)	0.44–1.8	0.65-1.15	%CV≤2%
Interday (n=6) (%CV)	0.46–1.75	0.71-1.25	%CV≤2%

LOD; Limit of detection, LOQ; Limit of quantification: RSD; Relative Standard Deviation

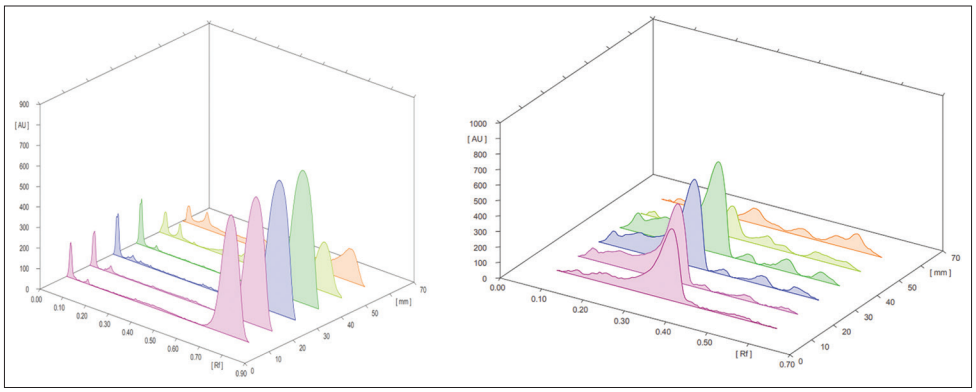

Fig. 5: 3D plot of Thin Layer Chromatogram of hydroalcoholic extracts of *Ficus benjamina* and *Ficus krishnae* with standard Rutin and Quercetin

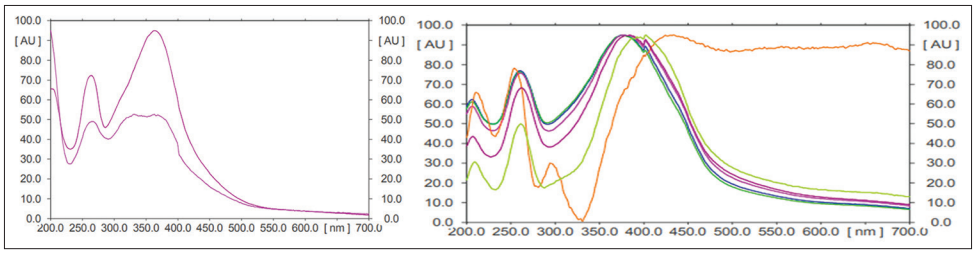

Fig. 6: Overlay spectra of standard rutin and quercetin corresponding Rf band of *Ficus benjamina* and *Ficus Krishnae*

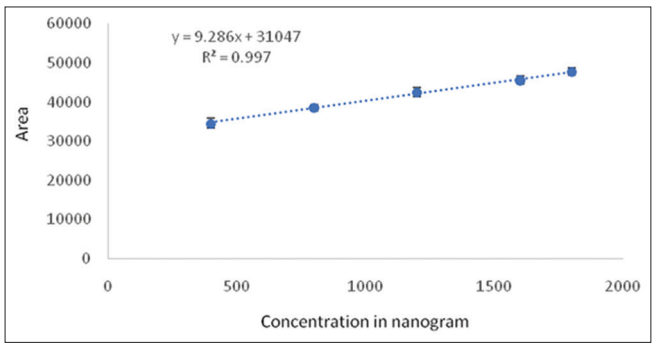

Fig. 7: Calibration curve concentration versus area calibration plot of standard rutin (n=6)

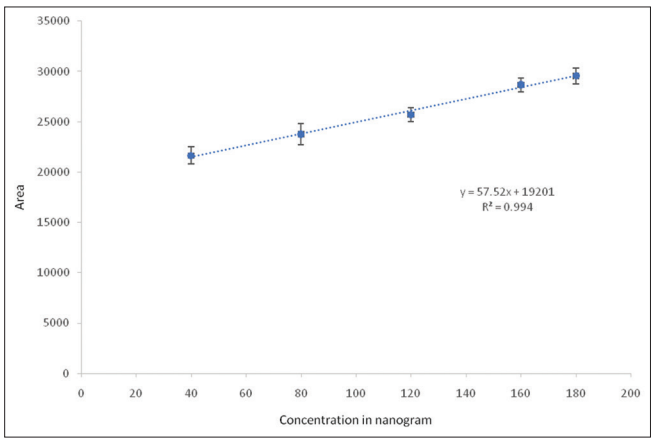

Fig. 8: Calibration curve concentration versus area calibration plot of standard quercetin (n=6)

end positions of the bands, the peak purity of quercetin and rutin was assessed.

Robustness

The study estimated the performance of a method by varying parameters like composition of mobile phase, volume, and saturation duration within a certain limit (±10%), without significant changes in results. The overall results were expressed as %RSD between the data at each variable condition.

Accuracy

The accuracy of the method was assessed by analysing recoveries, which were conducted in triplicate. The recovery studies were performed by spiking the standard concentration of quercetin and rutin (100 mg/spot), with the addition of 50%, 100%, and 150% of quercetin and rutin by using the standard addition technique. The calculation of the value of average % recovery for quercetin and rutin was determined.

System suitability

System suitability tests were conducted to ensure that repeatability and resolution were adequate for the analysis. A standard solution of quercetin and rutin, with a concentration of 200 mg/spot, was applied for the determination. The experiment was repeated 6 times, under the identical chromatographic conditions, and then scanned and measured densitogram results. Measurement of peak area and the Rf value for each concentration of quercetin and rutin was noted. The values of standard deviation (SD), mean peak area, and the % CV were calculated.

Estimation of quercetin & rutin in *F. benjamina* and *F. krishnae* extracts

To determine the amounts of quercetin and rutin in the extracts, 1 g of powdered plant material is first defatted, followed by extraction with an 80% hydroalcoholic solution, and then concentrated. For the analysis, a measured amount of the extract was dissolved and diluted with methanol. The solution was subjected to centrifugation at 3000 rpm for 15 min. The supernatant is used for quercetin and rutin content analysis after filtration and spotted on a TLC plate, followed by the development and scanning. Repetition of the method was done 6 times, and possible interference by other components in the extract was studied. Spot at Rf values 0.31 and 0.73 corresponding to quercetin and rutin was identified in the chromatogram of the extracts in addition to other components (Figs. 1-5).

Chromatograms provide essential insights into the chemical composition of herbal extracts. Chromatographic fingerprinting is an effective and logical method for verifying the authenticity of traditional medicines. Chromatography methods are employed to provide distinct patterns of identification for therapeutic herbs. The fingerprint patterns that are produced for the individual components can then be used to identify the ratio of all the detectable analytes as well as the presence or absence of interest markers. Due to the cost-effectiveness and simplicity of the method developed by HPTLC, it is a preferred tool over HPLC and GC for the evaluation of the quality of medicinal plants. Therefore, a distinctive feature providing a picture-like image of high performance for the purpose of creating an herbal chromatographic fingerprint, thin layer chromatography in conjunction with a digital scanning profile, is rather appealing. Two nearly related herbal medications were fully identified and differentiated using the parameters and information provided by the TLC. This technique of fingerprint patterns has been determined for the extracts of *F. benjamina* and *F. krishnae*. The standards of Quercetin & Rutin were quantified precisely using pre-coated silica gel F254 HPTLC plates with solvent system toluene: ethyl acetate: formic acid (5:3.5:0.5) and toluene: chloroform: methanol (7:2.5:0.5) for rutin and quercetin, respectively, the Rf value was about 0.31 and 0.73. Chromatographs of standards and extract of both plants are shown in Figs.1-5.

In addition to the HPTLC fingerprints, rutin analysis was conducted at a wavelength of 366 nm to examine the presence of various polyvalent active constituents. The Rf values for the two species were found to range from 0.11 to 0.57. Further from the table and the chromatogram, it has been found that out of 6 components in *F. benjamina*, the component with Rf value 0.30 was more predominant, as the percentage area is 52.22%. In the same way, out of 5 polyvalent compounds in *F. krishnae,* the compounds with Rf value 0.31 were found to be present in higher content, as the percentage area is 34.082% from this Rutin content was determined.

Similarly, HPTLC fingerprints and Quercetin analysis were observed at a wavelength of 254 nm for the existence of different active constituents. The range for Rf values was found to be between 0.03 and 0.74 for the two species. Further from the table and the chromatogram, it has been found that out of 9 components in *F. benjamina*, the component with Rf value 0.73 was more predominant, as the percentage area is 59.28%. In the same way, out of 10 polyvalent compounds in *F. krishnae,* the compounds with Rf value 0.73 were found to be present in higher content, as the percentage area is 47.72% from this quercetin content was determined.

DISCUSSION

A simple, accurate, and precise HPTLC method was developed to measure quercetin and rutin in the leaves of *F. benjamina* and *F. krishnae*, which has not been reported before. The TLC procedure has been optimized to accurately quantify herbal extracts. The Solvent system consisting of toluene: ethyl acetate: formic acid (5:3.5:0.5) and toluene: chloroform: methanol (7:2.5:0.5) for rutin and quercetin respectively, has shown better, sharper, and resolved peaks. With the help of a chromatogram of standard compound, spot at Rf values of 0.31 and 0.73 was identified as quercetin and rutin. A saturated chamber with a mobile phase was used for the development of the spotted plates at room temperature to obtain well-defined spots.

The TLC plate was visualized at wavelengths of 254 nm and 366 nm. By examining the scanned chromatogram, the spots corresponding to rutin and quercetin in the extracts of dried leaves from both plants were identified. Identification of bands of Quercetin and Rutin in the chromatogram is further confirmed by comparing Rf values and superimposed scanned spectra of band at same Rf values in the standard and extracts (Fig. 6). Rf values were found to be identical in the standard and extracts (Rf 0.31 and 0.73) (Fig. 1). Validation of densitometric TLC method was done with respect to precision, repeatability, and accuracy (Table 1). Range of linearity for quercetin and rutin was found 40–180 mg/spot with the correlation coefficient, intercept, and the slope 0.9944 (SD 0.05), 57.52 and 19201, for quercetin (Y=57.52X +19201) and 0.9972 (SD 0.05), 9.2868 and 31047, for rutin (Y=9.2868X+31047). The % recovery was found in the range of 96.77–99.74%. Peak area measurements for varying concentrations of quercetin and rutin have shown low values of percentage CV (<2%) for the intra-day (0.44–1.8) and inter-day (0.46–1.75) variance. This suggests as good precision and reproducibility of the method. For quercetin and rutin, respectively, LOD and LOQ were 11.82 and 11.98 mg and 35.47 and 35.94 mg, respectively, indicating the proper sensitivity of the method (Table 1). Results obtained from the recovery study given in Table 1 were within tolerable ranges, demonstrating good accuracy of the procedure. The ruggedness of the method has been illustrated by low values of %RSD of 0.38%, which shows that quercetin and rutin are stable during the procedure of extraction and analysis. By in fluxing small variations in the formation of the mobile phase, the effect of operational parameters can examine the robustness.

Contents of Rutin were investigated by using the Thin Layer Chromatography densitometric technique and were found to be 1.594±0.038%w/w in *F. benjamina* extract and 1.259±0.028%w/w in *F. krishnae* extract. Similarly, using the calibration curve content of Quercetin in extract was found to be 1.238±0.013%w/w in *F. benjamina* extract and 0.698±0.025%w/w in *F. krishnae* extract.

CONCLUSION

Identifying and quantifying phytochemicals in plants is essential for standardization, ensuring the efficacy and safety of herbal drugs. Establishing quality standards through this process will enhance global acceptance and market expansion. Our study quantitatively estimated the levels of quercetin and rutin in dried leaves of *F. benjamina* and *F. krishnae* extract using a simple, precise, rapid, specific, and accurate method. This data serves as a valuable benchmark for quality assurance in herbal medicine production.

Good resolution from other plant material constituents was obtained through the optimization of chromatographic settings. No interference

from other constituents was found in the plant as the recovery results (96.77–99.74%) were found close to 100%. HPTLC images revealed that all the sample constituents were separated distinctly and showed no tailing and diffuseness. The amount of active components in the extract always has an impact on the plant activity. The developed method for estimation of quercetin and rutin is essential to standardize various herbs because they are used in a variety of different diseases. The reported method of HPTLC is for the simultaneous evaluation of Quercetin and Rutin. The study offers a straightforward, sensitive, cost-effective method suitable for quick and routine quality control analysis. In addition, it demonstrates the quantification of Quercetin and Rutin in *F. benjamina* and *F. krishnae* leaves, as well as in herbal preparations, for the purpose of standardization.

AUTHORS CONTRIBUTIONS

Puneet Kaur: Performed all experimental work and wrote the manuscript with support of Payal Mittal and Rajiv Sharma.

Payal Mittal and Rajiv Sharma: Supervised the practical work and conceived the original idea, helped in research, analysis and manuscript.

CONFLICT OF INTEREST

Nil.

AUTHOR FUNDING

Author did not get any funding or any financial assistance from any funding agencies.

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