AMERICAN JOURNAL OF PHARMTECH RESEARCH
**Journal home page:** http://www.ajptr.com/

# Development and validation of an HPTLC method for quantitative estimation of quercetin and its application in *Bauhinia variegata* Bark Extract

**Trupesh Pethani[1]*, Mayuri Thumar[2]**
*1.Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, India*
*2.B. K. Mody Government Pharmacy College, Polytechnique campus, Rajkot, Gujarat, India*

## ABSTRACT

*Bauhinia variegata* Linn. (Fabaceae) is a widely used medicinal plant whose bark exhibits antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, and anticancer activities, largely attributed to its flavonoid content, of which quercetin is a key bioactive marker. Since no validated method previously existed for quantifying quercetin specifically in *B. variegata* bark, the present study aimed to develop and validate a simple, sensitive, and reproducible HPTLC method for this purpose, in accordance with ICH Q2(R1) guidelines. Chromatographic separation was achieved on silica gel 60 $F_{254}$ plates using a mobile phase of ethyl acetate: toluene: methanol: formic acid (3.5: 6: 0.5: 0.5, v/v/v/v), with densitometric detection at 383 nm, giving a well-resolved band at Rf 0.54. The method showed good linearity over 100–700 ng/band ($y = 23.063x - 430.75$, $R^2 = 0.991$), with an LOD of 20 ng/band and LOQ of 80 ng/band. Precision (%RSD < 2% for repeatability, intraday, and interday studies), accuracy (mean recovery 98.57–100.65%), robustness, and specificity all met acceptance criteria, confirming the reliability of the method. Application to the methanolic bark extract of *B. variegata* revealed a quercetin content of 0.012% w/w, with consistent results across replicate analyses. This validated HPTLC method offers a rapid, cost-effective, and reliable tool for the routine quality control, standardization, and phytochemical evaluation of *B. variegata* bark and its extracts.

**Keywords**: Quercetin; HPTLC; *Bauhinia variegata*; method validation; ICH Q2(R1); phytochemical standardization

*Corresponding Author Email: tmpethani@sauuni.ac.in
Received 26 February 2022, Accepted 04 April 2022

Please cite this article as: Pethani *T et al*., Development and validation of an HPTLC method for quantitative estimation of quercetin and its application in *Bauhinia variegata* Bark Extract. American Journal of PharmTech Research 2022.

# INTRODUCTION

Medicinal plants have long served as an important source of bioactive compounds for the development of traditional and modern therapeutic agents[1]. The quality, safety, and efficacy of herbal medicines largely depend on the presence and consistency of their phytochemical constituents; therefore, reliable analytical methods are essential for their standardization and quality control[2]. *Bauhinia variegata* Linn. (Family: Fabaceae) is a well-known medicinal plant extensively used in Ayurvedic and traditional systems of medicine[3]. Different parts of the plant, particularly the bark, possess diverse pharmacological activities including antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, hypolipidemic, immunomodulatory, and anticancer effects, which are primarily attributed to its rich flavonoid content[4]. Among the flavonoids present in *B. variegata*, quercetin is considered one of the most significant bioactive markers due to its potent antioxidant and therapeutic properties. Quantitative determination of quercetin is important not only for assessing the phytochemical quality of the plant but also for ensuring batch to batch consistency of herbal raw materials and formulations. However, variations in geographical origin, environmental conditions, harvesting season, and extraction procedures may considerably influence the quercetin content of the plant material, necessitating a validated analytical method for its reliable estimation [5]. High-Performance Thin Layer Chromatography (HPTLC) is widely recognized as a rapid, economical, sensitive, and reproducible technique for the qualitative and quantitative analysis of phytoconstituents[6]. The technique requires minimal sample preparation, allows simultaneous analysis of multiple samples, and is particularly suitable for routine quality control of herbal products. Therefore, the present study was undertaken to develop and validate a robust HPTLC method for the quantitative estimation of quercetin in accordance with ICH Q2(R1) guidelines. Furthermore, the validated method was applied for the determination of quercetin content in the methanolic bark extract of *B. variegata*, demonstrating its applicability for phytochemical standardization and routine quality evaluation.

# MATERIALS AND METHOD

## Chemicals and reagents

Quercetin working standard was procured from Loba Chemie. Methanol, ethyl acetate, toluene, and formic acid of analytical or HPLC grade were used throughout the study.

## Instrumentation

HPTLC analysis was carried out using a CAMAG Linomat 5 sample applicator, CAMAG TLC Scanner, twin-trough developing chamber, and 100 µL Hamilton syringe. Analytical weighing was performed using a Shimadzu analytical balance.

**Preparation of standard solution**

Ten milligrams of quercetin was dissolved in methanol and diluted to obtain a stock solution of 1000 µg/mL. A working standard solution of 50 µg/mL was prepared by appropriate dilution with methanol. Calibration standards corresponding to 100–700 ng/band were applied onto HPTLC plates for densitometric analysis.

**Preparation of plant extract**

The powdered bark of *B. variegata* was successively extracted by Soxhlet extraction using solvents of increasing polarity like n-hexane, dichloromethane, chloroform, ethyl acetate, methanol, and hydro alcoholic[7]. Preliminary phytochemical screening using the Shinoda test revealed the presence of flavonoids predominantly in the methanolic extract; therefore, the methanolic extract was selected for quantitative estimation.

**HPTLC method development**

Chromatographic conditions were systematically optimized to obtain satisfactory separation, peak symmetry, and reproducible densitometric response for quercetin. Method development was carried out by evaluating different solvent systems composed of ethyl acetate, toluene, methanol, chloroform, acetone, n-hexane, and formic acid in various proportions. The effects of mobile phase composition, chamber saturation time, migration distance, and detection wavelength were investigated to achieve optimum chromatographic performance. Standard quercetin solutions were applied to silica gel 60 $F_{254}$ HPTLC plates, and chromatograms were developed under controlled conditions. The optimized chromatographic conditions selected from these experimental trials were subsequently used for method validation.

**Method validation**

The developed HPTLC method was validated according to ICH Q2(R1) guidelines with respect to linearity, precision, accuracy, specificity, robustness, LOD, and LOQ [8].

**Linearity**

Linearity was evaluated over the concentration range of 100–700 ng/band. Different concentrations of the standard solution were applied to the HPTLC plate, and the corresponding peak areas were recorded. A calibration curve was prepared by plotting peak area against the amount of analyte applied. The regression equation and correlation coefficient ($R^2$) were determined.

**LOD and LOQ**

LOD and LOQ were calculated from the standard deviation of the response and slope of the calibration curve using the equations:

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

where σ is the standard deviation of the response and S is the slope of the calibration curve. The obtained values were used to establish the sensitivity of the developed HPTLC method.

**Precision**

Precision was expressed as %RSD. Repeatability was assessed by repeated application and analysis of the same standard concentration under identical conditions. Intraday precision was evaluated by analyzing the selected concentrations repeatedly on the same day, while interday precision was determined by repeating the analysis on different days. The mean peak area, SD, and %RSD were calculated.

**Accuracy and Recovery**

Accuracy was assessed by the standard addition method at 80%, 100%, and 120% of the nominal concentration. Known quantities of the reference standard were added to the pre-analyzed sample, followed by HPTLC analysis. The percentage recovery and %RSD were calculated.

**Specificity**

Specificity was established by comparing the chromatograms of the blank, standard, and sample solutions. The analyte peak was identified based on its Rf value, and peak purity was assessed by comparing the spectra at the start, apex, and end of the peak. The absence of interfering peaks at the analyte Rf confirmed the specificity of the method.

**Robustness**

Robustness was evaluated by making small deliberate changes in the selected chromatographic conditions, including mobile-phase composition, chamber saturation time, development distance, and detection wavelength. The effect of these changes on the Rf value and peak area was evaluated, and the results were expressed as %RSD.

**Application of the validated method**

The validated calibration equation was used for quantitative estimation of quercetin in five replicate methanolic bark extract samples. The quercetin content was calculated from the corresponding peak areas.

## RESULTS AND DISCUSSION

**HPTLC method development**

Selection of the mobile phase was the most critical step in developing the HPTLC method, since it directly determines how well the analyte migrates and resolves on the plate. Seventeen solvent systems were screened, spanning single solvents (methanol, acetone, ethyl acetate, chloroform,

toluene, n-hexane, benzene) through to formic acid modified multi component mixtures. Non-polar single solvents (toluene, n-hexane, acetone, benzene) failed to mobilize quercetin at all, pointing to a need for intermediate to high polarity. Binary ethyl acetate mixtures (with methanol, chloroform, or toluene) did move the analyte but gave poor Rf values with spreading bands a sign of unresolved interaction with the silica surface. Adding formic acid as a fourth component fixed this, it sharpened the bands considerably by suppressing tailing, consistent with its known role in taming polyphenolic analytes like flavonoids [9]. The formic acid systems tested, ethyl acetate: toluene: methanol: formic acid (3.5: 6: 0.5: 0.5, v/v/v/v) gave the best result overall a well resolved, symmetric, non-tailing band at an optimum Rf of 0.54 and was selected for the final method. Chamber saturation time was optimized separately at 10, 15, and 20 min, with 15 min giving the most reproducible migration. Finalized chromatographic conditions used for method validation and sample analysis were summarizes in Table 1.

**Table 1: Optimized HPTLC chromatographic conditions for quercetin**

| Parameter | Optimized condition |
|---|---|
| Stationary phase | Silica gel 60 F254 |
| Mobile phase | Ethyl acetate: Toluene: Methanol: Formic acid (3.5:6:0.5:0.5, v/v/v/v) |
| Chamber saturation | 15 min |
| Detection wavelength | 383 nm |
| Development time | 15 min |
| Rf value | 0.54 |

**Method validation**

Validation of the HPTLC method against linearity, sensitivity, precision, accuracy, robustness, and specificity followed ICH Q2(R1) guidance, and each parameter is discussed below in relation to its practical significance for quercetin analysis.

**Linearity**

The calibration curve spanning 100–700 ng/band gave the regression equation $y = 23.063x - 430.75$ with $R^2 = 0.991$ (Figure 1). An $R^2$ this close to unity means the detector response tracks concentration almost proportionally across the entire range tested (Figure 2). The fact that the response held steady from the lowest to the highest band also suggests the detector wasn't saturating or losing sensitivity at either extreme a common failure point in densitometric HPTLC that can otherwise force a narrower validated range (Figure 3).

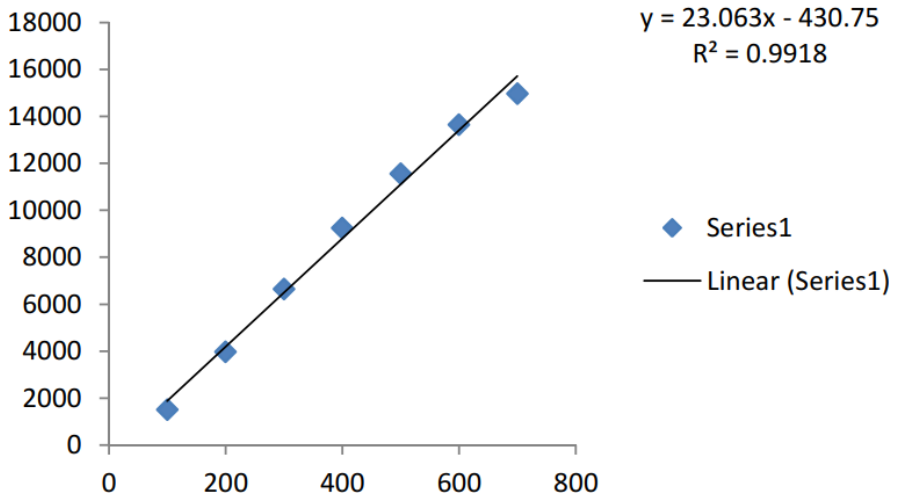


**Figure 1: Calibration curve of Quercetin (Concentration Vs peak area)**

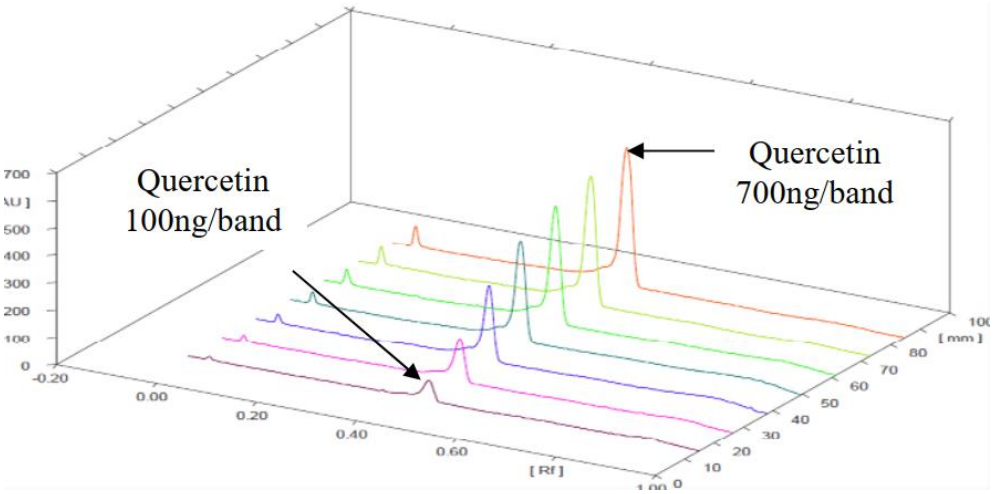


**Figure 2**: **Linearity Chromatograms of Quercetin**

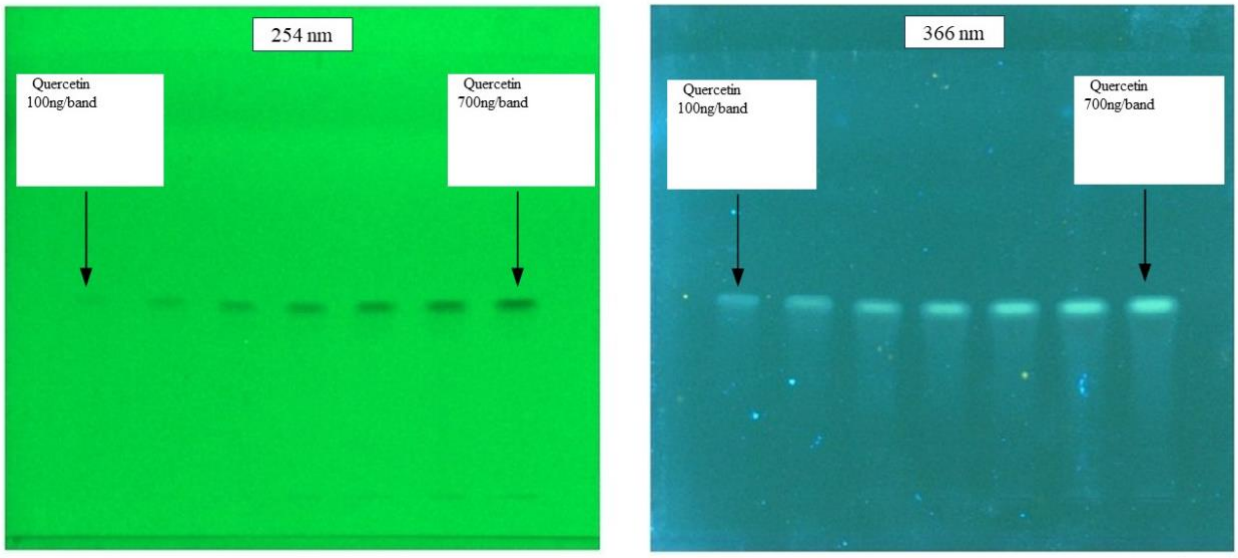


**Figure 3: HPTLC plate of quercetin at different concentrations under 254 and 366 nm.**

**LOD and LOQ**

An LOD of 20 ng/band and LOQ of 80 ng/band sitting below the linearity floor (100 ng/band) isn't just a formality it means the method has headroom below its working range, so trace level quercetin (for instance, in a dilute plant extract or a formulation with a low labelled quercetin content) is still catchable without needing to reconcentrate the sample. This is particularly relevant for a plant matrix application like this one, where the marker compound is rarely present at high absolute concentrations relative to the bulk of co-extracted material.

**Precision**

RSD values stayed under 2% at every level tested repeatability (1.79%), intraday (1.57–1.68% across 1, 2, and 3 h), and interday (0.96–1.42% across three days). The pattern here is worth noting, interday RSDs were actually slightly lower than intraday and repeatability figures, which suggests the day-to-day variability introduced by fresh mobile phase preparation and plate development wasn't a major source of error the method's precision is dominated more by short term instrumental application variability than by longer term drift. That's a reassuring sign for a method intended for routine, repeated use over time rather than a single analytical campaign.

**Accuracy& Recovery**

The accuracy of the developed HPTLC method was evaluated by a standard-addition recovery study at 80%, 100%, and 120% of the target concentration (Figure 4). The mean percentage recoveries were 100.24%, 98.57%, and 100.65%, with corresponding %RSD values of 0.52%, 0.37%, and 1.65%, respectively (Table 2). All recovery values were within the predefined acceptance range of 98–102%, with low %RSD values indicating good consistency among replicate measurements. Because this recovery study spikes standard into the actual sample matrix rather than testing the standard in isolation, it speaks directly to whether co-extracted plant constituents (tannins, other flavonoids, etc.) interfere with quantification and the near 100% recovery across all three levels indicates they largely don't, at least not in a way that biases the result systematically high or low.

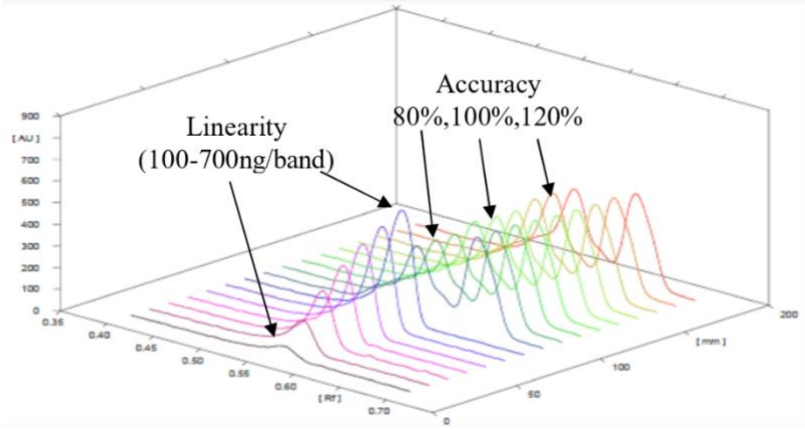


**Figure 4**: **Overlaid HPTLC chromatogram of the quercetin recovery study**

**Table 2: Accuracy and recovery study for quercetin**

| Spiking level | Mean % recovery | % RSD |
|---|---|---|
| 80% | 100.24 | 0.52 |
| 100% | 98.57 | 0.37 |
| 120% | 100.65 | 1.65 |

**Robustness**

Robustness was tested by deliberately varying four chromatographic parameters around their optimized values: chamber saturation time (12–18 min), detection wavelength (378–388 nm), mobile-phase ethyl acetate ratio, and plate development temperature (25–32 °C). Across all these perturbations, %RSD stayed within 1.22–1.96% (Table 3) never crossing the 2% threshold confirming the method holds up under the kind of small, everyday variations a lab is likely to introduce unintentionally. Looking at the spread more closely, temperature was the parameter with the least margin, reaching an RSD of 1.96% at its extreme close enough to the 2% ceiling to flag it as the variable most worth controlling carefully in routine use. Saturation time, by contrast, showed the tightest RSDs (1.22–1.71%), suggesting the method is comparatively forgiving of timing inconsistencies in chamber equilibration. Wavelength and mobile-phase ratio fell in between, both showing modest sensitivity but no cause for concern within the tested bounds. Overall, robustness confirms that the method's precision doesn't hinge on tightly controlled conditions it tolerates realistic day to day variability in saturation time, detection wavelength, mobile phase mixing, and ambient temperature, which supports its practical use for repeated, routine analysis rather than requiring lab specific fine tuning each time it's run.

**Table 3: Robustness study of the developed HPTLC method for quercetin**

| Parameter varied | Range investigated | % RSD range |
|---|---|---|
| Chamber saturation time | 12–18 min | 1.22–1.71 |
| Detection wavelength | 378–388 nm | 1.43–1.88 |
| Mobile-phase composition | Slight variation in ethyl acetate ratio | 1.32–1.81 |
| Plate development temperature | 25–32 °C | 1.68–1.96 |

**Specificity**

The specificity of the developed HPTLC method was evaluated by analyzing blank, methanol (solvent), mobile-phase, and quercetin standard tracks under the optimized chromatographic conditions. The chromatographic profiles were examined for any peaks that could interfere with the detection and quantification of quercetin at its characteristic Rf. No additional interfering peaks were observed at or around the Rf corresponding to quercetin in the blank, solvent, or mobile-phase tracks (Figure 5). The quercetin peak was therefore distinguishable from the components associated with the extraction solvent and mobile phase. These findings demonstrated that the developed HPTLC procedure provided adequate selectivity for quercetin under the established chromatographic conditions and was suitable for its quantitative determination in the analyzed samples.

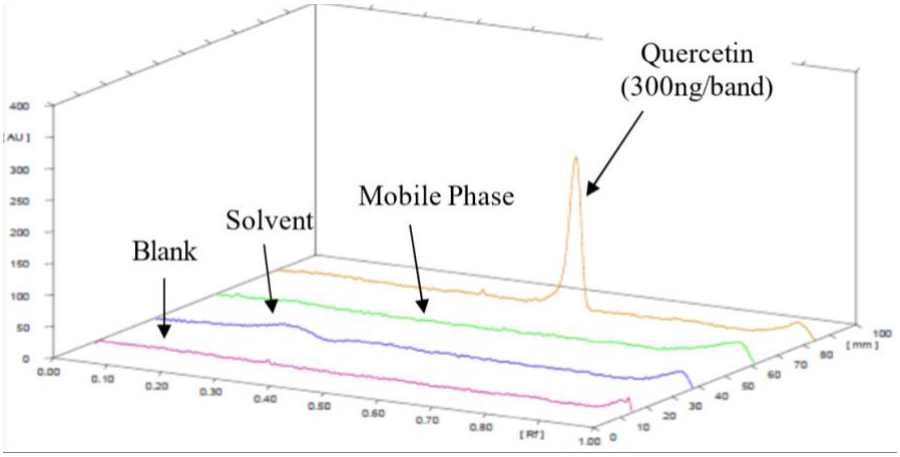


**Figure 5: Overlaid HPTLC chromatogram of the quercetin specificity study**

**Application of the validated HPTLC method**

The validated method was used to quantify quercetin in the methanolic bark extract of *B. variegata*. Five replicate samples were analyzed, giving a mean peak area of 5045.6 (Figure 6). Using the calibration equation, this corresponded to 117.48 ng of quercetin per 10 µL of extract, equivalent to a bark content of 0.012% w/w. The peak areas obtained across the five replicates were fairly consistent, indicating that the method gave reliable results even when applied to a real plant extract rather than a pure standard solution. This suggests that other compounds naturally

present in the bark, such as tannins and additional flavonoids, did not significantly interfere with the quantification of quercetin.

Since no earlier method had been reported for estimating quercetin specifically from *B. variegata* bark, this result provides useful baseline data for the plant. It also shows that the developed method is sensitive enough to detect quercetin even at low concentrations in a natural extract. Overall, these findings support the use of the validated HPTLC method for the routine quality assessment and standardization of *B. variegata* bark and its extracts.

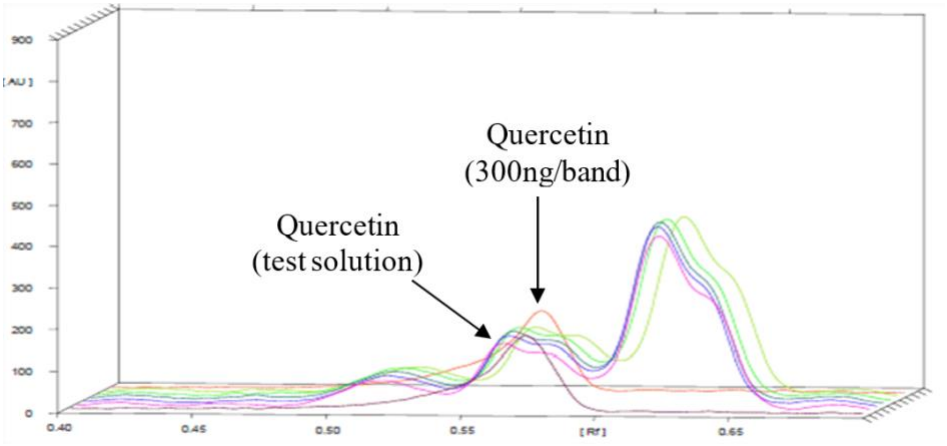


**Figure 6: Overlaid chromatograms of quercetin standard and bark extract of *B. variegata***

## CONCLUSION

A simple, sensitive, precise, and robust HPTLC method was successfully developed and validated for the quantitative estimation of quercetin. The method showed good linearity (100–700 ng/band; $R^2$ = 0.991), satisfactory sensitivity (LOD 20 ng/band; LOQ 80 ng/band), precision, accuracy, specificity, and robustness. Application of the method to *B. variegata* bark extract confirmed a quercetin content of 0.012% w/w. The developed method therefore provides a practical and cost-effective approach for the routine quality control, standardization, and phytochemical evaluation of *B. variegata* bark and its extracts.

## REFERENCES:

1. Atanasov AG, Zotchev SB, Dirsch VM, Orhan IE, Banach M, Rollinger JM, et al. Natural products in drug discovery: advances and opportunities. Nature Reviews Drug Discovery. 2021;20(3):200-16.
2. Muyumba NW, Mutombo SC, Sheridan H, Nachtergael A, Duez P. Quality control of herbal drugs and preparations: The methods of analysis, their relevance and applications. Talanta Open. 2021;4:100070.
3. Johnson P, Krishnan V, Loganathan C, Govindhan K, Raji V, Sakayanathan P, et al. Rapid biosynthesis of *Bauhinia variegata* flower extract-mediated silver nanoparticles: an

effective antioxidant scavenger and α-amylase inhibitor. Artificial Cells, Nanomedicine, and Biotechnology. 2018;46(7):1488-94.

4. Sharma K, Kumar V, Kumar S, Sharma R, Mehta CM. *Bauhinia variegata*: a comprehensive review on bioactive compounds, health benefits and utilization. Advances in Traditional Medicine. 2021;21(4):645-53.
5. Dhami N, Mishra AD. Phytochemical variation: How to resolve the quality controversies of herbal medicinal products? Journal of Herbal Medicine. 2015;5(2):118-27.
6. Morlock GE. High-performance thin-layer chromatography combined with effect-directed assays and high-resolution mass spectrometry as an emerging hyphenated technology: A tutorial review. Analytica Chimica Acta. 2021;1180:338644.
7. Trupesh Pethani MT, Devendra Vaishnav, Ashvin Dudhrejiya. A Concise Study of Pharmacognostic and Antioxidant Potential of Stem bark from *Bauhinia variegata* Linn. Research Journal of Pharmacy and Technology. 2018;11(8):3250-6.
8. Borman P, & Elder, D. validation of analytical procedures: text and methodology. . ICH quality guidelines: an implementation guide. 2017:127-66.
9. Patel RB, Patel MR, Batel BG. Experimental Aspects and Implementation of HPTLC. In: Srivastava M, editor. High-Performance Thin-Layer Chromatography (HPTLC). Berlin, Heidelberg: Springer Berlin Heidelberg; 2011. p. 41-54.