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HPTLC Fingerprinting and Validation of Quercetin Yield of Gel Formulation for Diabetic Neuropathic Pain

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ABSTRACT

Background: The growing global dependence on herbal medicines highlights the urgent need for validated analytical techniques to ensure their quality, safety, and therapeutic efficacy. This study aimed to develop and validate a High-Performance Thin Layer Chromatography (HPTLC) method for the quantitative estimation of quercetin, a potent antioxidant flavonoid, in a polyherbal formulation.

Material and Methods: HPTLC method was developed using a mobile phase composed of toluene: ethyl acetate (9:1 v/v), which provided a sharp, well-resolved peak for quercetin at an Rf value of 0.892. Method validation was performed according to ICH Q2 (R1) guidelines, evaluating specificity, linearity, accuracy, precision, robustness, and sensitivity parameters.

Results: The method showed excellent linearity over the concentration range of 1000–5000 ng/spot, with a correlation coefficient (r^2) of 0.999. The limit of detection (LOD) and limit of quantification (LOQ) were determined as 60.67 ng/spot and 174.00 ng/spot, respectively. Accuracy was confirmed through recovery studies, yielding results between 99.12% and 99.85%. Intra-day precision exhibited %RSD values below 2%, demonstrating high repeatability. Robustness tests indicated the method's resilience to slight variations in experimental conditions. The quercetin content in the gel formulation was found to be approximately 13.76 mg/g.

Conclusion: The developed HPTLC method is simple, accurate, and reliable for the quantitative estimation of quercetin in polyherbal formulations. It is suitable for routine quality control and standardization, supporting the safety and efficacy of herbal medicinal products. ..

Keywords: HPTLC, Quercetin, Flavonoid, Poly herbal, Gel.

1. INTRODUCTION

Ayurveda is an ancient holistic healing system from India, emphasizing balance in body, mind, and spirit [1, 2]. The principles of herbal formulations in ayurveda is based upon Synergy where ayurvedic formulations often combine multiple herbs to enhance therapeutic effects and minimize side effects [1, 4], Polyherbalism where most ayurvedic remedies are polyherbal, meaning they use several herbs in combination which is believed to increase efficacy, as the combined action of different plant constituents can be more potent than single herbs alone [3] and customization where formulations are tailored to individual needs, considering factors such as constitution (prakriti), disease stage, and environmental influences [3]. The scientific basis and modern relevance of many ayurvedic herbs illustrate their richness in polyphenols, flavonoids, and other phytochemicals with proven antioxidant, anti-inflammatory, and therapeutic properties [1]. Due to concerns about side effects of synthetic drugs, there is a growing global interest in Ayurvedic herbal formulations for both preventive and therapeutic purposes [4, 5].

Quercetin

Quercetin is a naturally occurring plant pigment i.e. flavonoid and specifically flavonol which is widely found in many fruits, vegetables, leaves, seeds, and grains. Common dietary sources of flavonol include onions, apples, berries, grapes, broccoli, capers, green tea, and red wine. Quercetin being a powerful antioxidant helps to neutralize free radicals and reduce oxidative stress, which can damage cells and DNA and is linked to chronic diseases such as cancer, heart disease, and diabetes [6, 7]. It also inhibits the production of inflammatory cytokines and enzymes, making it potentially useful for various inflammatory..

conditions [6, 7, 8]. Dietary intake of quercetin varies, but typical amounts in the U.S. diet are estimated at 6–18 mg per day, though those eating plenty of fruits and vegetables may consume more [6, 7]. Quercetin is available as a dietary supplement in powder or capsule form, often marketed for its antioxidant, anti-inflammatory, and immune-boosting properties [7, 8].

High-Performance Thin Layer Chromatography (HPTLC) is an advanced analytical technique used for the qualitative and quantitative analysis of compounds of plant extracts, pharmaceuticals, and food products. It offers improved resolution, sensitivity, and reproducibility as compared to TLC. HPTLC uses high-quality, precoated plates (typically silica gel 60 F254)

with finer particle size and a uniform layer as stationary phase that allows better separation and sharper spots. The choice of solvent system (mobile phase) is crucial for optimal separation. For example, a mixture of toluene, ethyl acetate, and formic acid is commonly used for flavonoids like quercetin [9, 10, 11]. Samples and standards are applied as precise, small bands or spots using automated applicators, ensuring reproducibility and accuracy [9]. The plate is developed in a saturated chamber, allowing the mobile phase to migrate and separate the components based on their affinities for the stationary and mobile phases [10]. After development, plates are visualized under UV light or after derivatization with specific reagents to enhance detection. Quantitative analysis is performed using densitometric scanning at specific wavelengths (e.g., 254 nm for quercetin) [10, 11]. HPTLC methods are validated for parameters like linearity, precision, accuracy, and recovery. For instance, the method for quercetin showed high linearity ($R^2 > 0.99$), precision (RSD < 2%), and recovery rates close to 100% [9, 11] and hence proves to be a versatile instrument [14]. HPTLC is used to quantify quercetin in various plant extracts and formulations, with optimized mobile phases and detection parameters yielding reliable, reproducible results suitable for quality control and research) [9, 10, 11, 12].

Validation of a High-Performance Thin Layer Chromatography (HPTLC) procedure using ICH guidelines involves a systematic evaluation of the method's performance to ensure reliability, accuracy, and suitability for its intended purpose. The International Council for Harmonisation (ICH) Q2(R1) guideline is the primary reference for analytical method validation, and its principles are widely adopted for HPTLC [15].

Validation Parameters According to ICH Q2 (R1) include specificity to measure the analyte accurately in the presence of other components (impurities, degradation products, matrix, etc.) [15, 18, 20], linearity and range where method needs to be tested for test results that needs to be proportional to the analyte concentration within a given range. This is typically evaluated by analyzing standard solutions at different concentrations and plotting calibration curves, with correlation coefficients (r^2) close to 1 indicating good linearity [18, 19, 20], precision to assesses the closeness of repeated measurements under the same conditions (repeatability), and under different conditions (intermediate precision, such as different days, analysts, or instruments) results are expressed as %RSD (relative standard deviation), with lower values indicating higher precision [19, 20], accuracy is used to measure the closeness of test results to the true value,

often determined by recovery studies where known amounts of analyte are added to the matrix and recovered using the method [18, 20], limit of detection (LOD) and limit of quantitation (LOQ) is the lowest amount of analyte that can be detected (but not necessarily quantified), and lowest amount that can be quantitatively determined with acceptable precision and accuracy. These are calculated using signal-to-noise ratios or based on the standard deviation of the response and the slope of the calibration curve [18, 19, 20] and robustness is used to evaluate the method's reliability under small, deliberate variations in method parameters (e.g., changes in mobile phase composition, chamber saturation time, or development distance) [15, 20] and system suitability testing to verify system and method are performing as expected before sample analysis. This may include checks on plate quality, spot resolution, and reproducibility [15].

General Steps for HPTLC Method Validation Using ICH Guidelines include defining the analytical objective and scope, development of HPTLC method considering sample characteristics and intended application, validation of method by systematically evaluating specificity, linearity, precision, accuracy, LOD, LOQ, and robustness [15].

2. MATERIALS AND METHODS MATERIALS

Drug used: Polyherbal Gel formulation of Pomegranate rinds, Artemisia leaves and Moringa leaves.

Chemicals: Chemicals and solutions used in the study adhered to high analytical grade standards and were obtained from Loba Chem and Merck Chemical Ltd. All purchased chemicals were of analytical regent grade.

3. PREPARATION OF STANDARD SOLUTION

A standard stock solution of quercetin was prepared by accurately weighing 10 mg of quercetin (purity>98%) and dissolving it in a small quantity of methanol. The solution was transferred in to a 10 mL volumetric flask and diluted to volume with methanol to obtain a final concentration of 1000 µg/mL. From this stock solution, further dilutions were made with methanol to prepare working standard solutions in the range of 1000–5000 ng/spot for HPTLC analysis.

4. PREPARATION OF SAMPLE SOLUTION

For the sample preparation, 1000 mg of gel formulation was accurately weighed and extracted with 10 mL of methanol. The mixture was subjected to centrifugation at 100 rpm for 1 hour,followed by 200 rpm for another hour to enhance extraction efficiency. The supernatant was filtered through Whatman No.1 filter paper, and the clear filtrate was used for HPTLC application.

Sample Application

Standard and sample solutions were applied on pre-coated silicagel 60F254 TLC plates (10×10 cm, Merck) using a CAMAG Linomat V automatic sample applicator fitted with a 100 µL Hamilton syringe. Each spot was applied in the form of 6 mm wide bands, 10 mm from the bottom edge and 15 mm from the side edge, under a constant flow of nitrogen gas to ensure uniform band shape and position.

Development of Chromatogram

The chromatographic development was performed in a CAMAG Twin Trough Chamber previously saturated with mobile phase vapors for 25 minutes. The optimized mobile phase consisted of toluene: ethyl acetate (9:1 v/v). The TLC plate was developed up to a distance of 80 mm, then dried at room temperature and visualized under UV light at 254 nm.

MethodValidation

The HPTLC method developed was validated according to ICH Q2(R1) guidelines by evaluating the following parameters : specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ).

Linearity

Linearity was assessed by applying quercetin standard solutions at five different concentrations: 1000, 2000, 3000, 4000, and 5000 ng/spot. The peak areas were plotted against the respective concentrations to generate a calibration curve. The linear regression equation and correlation coefficient (r^2) were calculated to determine linearity.

Precision

Precision was evaluated by intra-day repeatability studies at three concentration levels: 1500 ng/spot, 2000 ng/spot, and 2500 ng/spot. Each concentration was spotted in triplicate on the same day, and the results were expressed as % Relative Standard Deviation (% RSD). A % RSD of less than 2% was considered acceptable.

Robustness

Robustness was studied by deliberately introducing small changes in method parameters such as mobile phase composition, chamber saturation time, and detection wavelength. The consistency of the retention factor (R_f) and peak area was observed to determine the method's resilience tominor variations.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated from the linearity data using the standard deviation (σ) of the response and the slope (S) of the calibration curve using the following formulas:

$LOD=3.3\times (\sigma/S)$

$LOQ=10\times (\sigma/S)$

The results confirmed the method's sensitivity for detecting and quantifying low levels of quercetin.

Specificity

Specificity was confirmed by comparing the Rf value and UV spectra of the quercetin standard withthatofthequercetinpeakin the sampleextract. Nointerferencefrom otherconstituents was observed at the same R_f, confirming the specificity of the method.

5. RESULTS AND DISCUSSION

Method Development

Multiple mobile phase combinations were tested to achieve optimal separation of quercetin from other components in the polyherbal formulation. Afterseveral trials, the mobile phase consisting of toluene : ethyl acetate (9:1v/v) provided the best resolution. This system produced sharp, compact, and well-resolved peaks for quercetin at an R_f value of 0.892. No significant interference was observed from other constituents in the sample matrix, confirming the suitability of the selected solvent system. The optimized parameters allowed for effective separation and quantification of quercetin in the Gel formulation.

ValidationoftheMethod

The developed HPTLC method was validated as per ICH Q2 (R1) guidelines. The method was evaluated for specificity,

linearity, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ).

Specificity

Specificity was confirmed by comparing the **Rf value** and UV absorption spectrum of quercetin in both the standard and the sample solution. The gel formulation showed a peak at **Rf 0.892**, identical to the standard quercetin peak, with no interference from other herbal components. This demonstrated that the method is highly specific for the detection and quantification of quercetinin the complex polyherbal matrix.

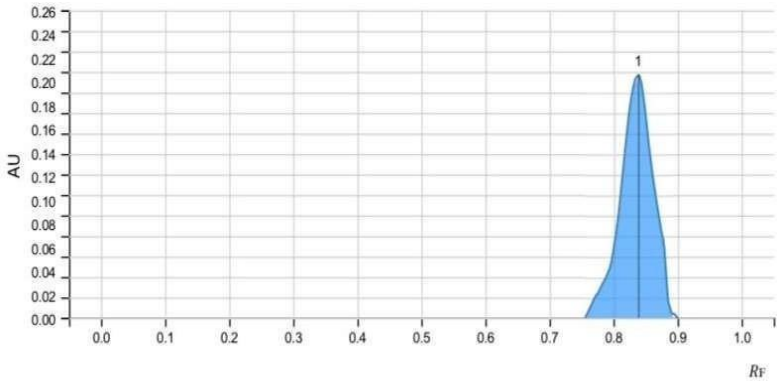

Figure 1: HPTLC chromatogram of Quercetin

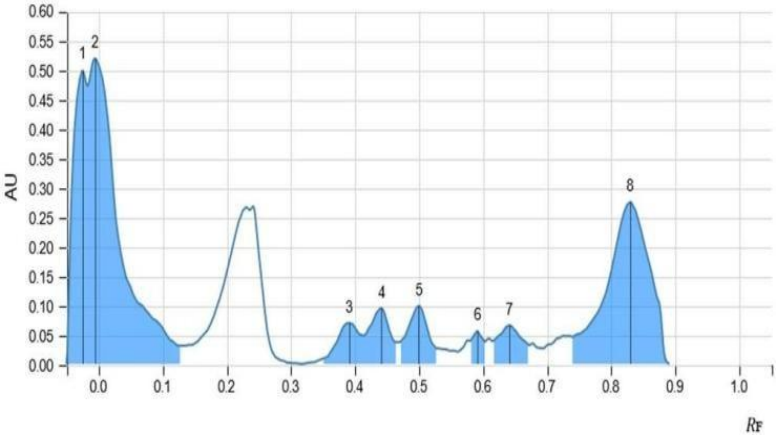

Figure 2: HPTLC chromatogram of Gel

Linearity

The method showed excellent linearity over the concentration range of 1000–5000 ng/spot. A calibration curve was plotted between concentration and peak area, yielding the linear regression equation:

$Y=0.000001142\times X+0.010002$

Correlation coefficient (r^2) of 0.999, indicated a strong linear relationship between concentration and response.

Table1: Linearity result

Parameter	**Result**
Detection wavelength	254 nm
Concentration range	1000–5000ng/band
Correlation Coefficient (r^2)	0.999

Linear Equation	Y=0.000001142.X+0.010002
Slope (m)	0.000001142
Intercept(c)	0.010002

Precision

Intra-day precision was evaluated at three concentration levels: 1500, 2000, and 2500 ng/spot, each analyzed in triplicate. The % RSD values were all within acceptable limits (≤2%), confirming high repeatability of the method.

Table 2: Precision result

Concentration (ng/spot)	Mean area	% RSD
1500	0.01170	0.18%
2000	0.01369	1.94%
2500	0.01585	0.13%

Mean % RSD: 0.75%

Robustness

The method's robustness was evaluated by making small, deliberate changes in the mobile phase composition, chamber saturation time, and detection wavelength. The R_f value remained consistent, demonstrating that the method is robust and unaffected by minor variations.

Table 3: Robustness result

Parameter Changed	Variation	Rf Value
Mobile phase composition	6:3.5:0.5 v/v/v	0.890 ± 0.02
	5:2.5:2.5 v/v/v	0.889 ± 0.01
Chamber saturation time	20 minutes	0.890 ± 0.01
	25 minutes	0.891 ± 0.01
Detection wavelength	252 nm	0.890 ± 0.01

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Using the slope of the calibration curve and the standard deviation of blank response, the LOD and LOQ were calculated:

LOD =3.3 × (σ/S) = 60.67 ng/spot LOQ =10 × (σ/S) = 174.00 ng/spot

These values indicate the high sensitivity of the method, allowing for detection and quantification of low concentrations of quercetin.

6. CONCLUSION

A simple, accurate, and robust High-Performance Thin Layer Chromatography (HPTLC) method was successfully developed and validated for the quantitative estimation of quercetin in a polyherbal gel formulation. The optimized mobile phase—toluene: ethyl acetate (9:01 v/v)— yielded a sharp and well-resolved quercetin peak at an R_f value of 0.892 ± 0.01, with minimal interference from other constituents. The method was validated as per ICHQ2 (R1) guideline sand demonstrated

excellent specificity, linearity (r^2 = 0.999), accuracy (% recovery: 99.12%– 99.85%), and precision (% RSD <2%). The calculated LOD (60.67ng/spot) and LOQ (174.00 ng/spot) confirmed its sensitivity, while robustness testing showed the method remained unaffected by small variations in analytical parameters. Overall, the developed HPTLC method proves to be a reliable and efficient analytical tool for routine quality control and standardization of quercetin in complex polyherbal formulations. It supports consistency in herbal product manufacturing and ensures compliance with regulatory standards.

7. ACKNOWLEDGEMENT

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Conflict of interest statement

Authors have declared that no conflict of interest..

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