JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2024/35980

SHORT COMMUNICATION

# Mixture Design-driven Statistical Optimization and Method Validation in HPTLC: Targeting Rutin, Quercetin, and Gallic Acid

**Jitesh K. Jariwala[1,2], Ghanshyam R. Parmar[1*], Vinodkumar D. Ramani[2], Devendra J. Vaishnav[2], Naishad I. Solanki[2] and Dhiren P. Shah[2]**

[1]Department of Pharmacy, Sumandeep Vidyapeeth Deemed to be University, Piparia, Vadodara - 391760, Gujarat, India; ghanstaurus22@gmail.com

[2]C. K. Pithawalla Institute of Pharmaceutical Science and Research, Surat - 395007, Gujarat, India

## Abstract

Rutin (RT), Quercetin (QT), and Gallic Acid (GA) are recognized for their potent antioxidant and anticancer properties, prevalent across numerous plant species. The precise quantification of RT, QT, and GA is pivotal for evaluating the therapeutic potential of plant-based substances. In response, a new, straightforward, cost-effective, and reliable method using High-Performance Thin-Layer Chromatography (HPTLC) has been developed and validated for the quantification of these compounds. The mobile phase optimization employed a mixture design approach, achieving chromatographic separation with a mobile phase mixture of toluene, ethyl acetate, menthol, and formic acid at specific ratios (3.56:3.70:0.94:1.80 v/v/v/v/v/v/v). Silica gel 60 F254 HPTLC plates were utilized for the analysis. The retention factors (Rf) observed for RT, GA, and QT were 0.21, 0.58, and 0.74, respectively. This method demonstrated a robust linear relationship for concentrations ranging from 400 to 2000 ng per band, with correlation coefficients (R2) of 0.9921 for RT, 0.9936 for QT, and 0.9912 for GA. The Limits of Detection (LOD) and quantification (LOQ) were established at 100.84, 102.38, and 84.54 ng per band for LOD, and 305.58, 310.25, and 256.18 ng per band for LOQ, respectively, for RT, QT, and GA. This validated HPTLC method developed through a Design of Experiment (DoE) approach was successfully employed for the quantification of GA, QT, and RT from the fruits of *Adansonia digitata* and leaves of *Grewia asiatica* ethanolic extracts.

**Keywords:** *Adansonia digitata*, Gallic Acid, *Grewia asiatica,* HPTLC, Quercetin, Rutin

## Abbreviations

GA: Gallic acid, ICH: International Council for Harmonization, LOD: Limit of Detection, LOQ: Limit of Quantification, $R^2$: Correlation coefficient, RSD: Relative Standard Deviation, RT: Rutin, QT: Quercetin, SD: Standard Deviation.

## 1. Introduction

Following the outbreak of the COVID-19 pandemic, there has been a marked increase in the utilization of plant-based medicinal products, highlighting a worldwide trend toward natural healing solutions[1]. The primary factor behind this trend is the increasing recognition of the health advantages linked to plant-derived antioxidants, immunomodulatory substances, and anticancer agents. These natural chemicals have shown encouraging outcomes in augmenting immune function and offering defensive actions against many illnesses, including cancer. Nevertheless, the growing use of these plant-derived products has emphasized the pressing need for standardization to guarantee their safety, effectiveness, and quality[2,3].

The process of standardizing plant products is essential to provide consistent medicinal effects and guarantee the safety of consumers. HPTLC is the predominant approach employed by researchers for this objective, in contrast to alternative chromatographic methods[4,5]. HPTLC is used for standardizing plant products because of its cost-effectiveness, simplicity,

*Author for correspondence

Article Received on: 25.12.2023 Revised on: 24.02.2024 Accepted on: 28.02.2024

and ability to handle several samples at once. This makes it an ideal method for analyzing the qualitative and quantitative composition of plant elements[6].

Crucial phytochemicals from the flavonoid and phenolic acid groups include Rutin (RT), Quercetin (QT), and Gallic acid (GA), respectively (Figure 1). RT, a flavonol glycoside, helps scavenge free radicals and is well-known for its powerful antioxidant[7-9] and anticancer[10-12] capabilities. Another flavonoid, QT, has been the focus of many oncology study[13-16] and antioxidant potential[14,17-19]. Equally noteworthy is GA, a trihydroxybenzoic acid, which has a powerful antioxidant capacity[20-22] and, according to new research, might be used to prevent and treat cancer[23-25]. These chemicals have medicinal value, and the simultaneous HPTLC approach helps comprehend their combined effects, especially in antioxidant and anticancer applications. Improving their use in nutraceuticals and pharmacotherapy, this analytical breakthrough shows great potential for studying and efficiently controlling the quality of these beneficial chemicals.

Despite several pharmacological effects of these substances having been extensively studied, researchers investigating their simultaneous analytical testing approach are noticeably lacking. This lack of research emphasizes the importance of our technique (HPTLC), which provides a simplified and cost-effective way to evaluate these phytochemicals in different herbal formulations both qualitatively and quantitatively. Not only does our work address a significant gap in phytochemical analysis, but it also lays the groundwork for future studies to investigate their synergistic effects, which might improve their use in the creation of nutraceuticals and pharmaceuticals.

## 2. Materials and Methodology

### 2.1 Materials

Rutin (RT), Quercetin (QT), and Gallic acid (GA) were procured from Sigma-Aldrich, USA. Pre-coated TLC plates (Silica gel 60 F254, 10×10 cm, 2mm) from Merck, Germany and other analytical reagents were procured from S.D. Fine Chemicals Limited, Mumbai, India. *Grewia asiatica* leaves and *Adansonia digitata* fruits were collected from the Surat district, Gujarat, India.

### 2.2 Sample Preparation

#### *2.2.1 Standard Solution Preparation*

For this study, we produced separate solutions for analyzing RT, QT, and GA. Each compound was precisely measured to 5 mg and carefully transferred into individual 10 mL volumetric flasks. At first, these compounds were dissolved in 5 mL of AR-grade methanol. To achieve thorough dissolution and uniformity, the solutions underwent sonication for a period of 5 to 10 minutes. Following this process, each flask was filled to the mark with methanol to achieve the desired volume.

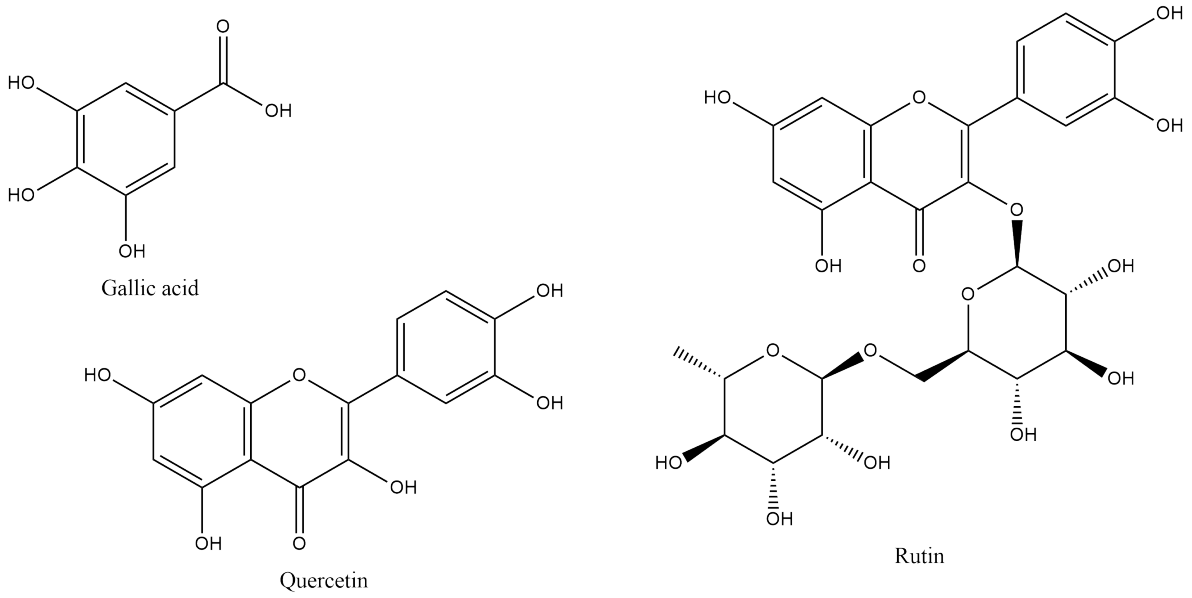


**Figure 1.** Chemical structure of Rutin, Quercetin, and Gallic acid.

### 2.2.2 Sample Preparation

The freshly harvested leaves of *G. asiatica* were meticulously collected and subsequently dried beneath a sunshade. At the same time, the fruits of *A. digitata* were delicately opened, and the pulp was carefully extracted and dried. Ethanolic (99.9%v/v) extract was prepared by immersing in the powder plant material for 72 hours at room temperature. The mixture was occasionally agitated to ensure complete extraction. After the maceration process, the extracts were filtrated using Whatman filter paper. The remaining mass was further treated with fresh ethanol to ensure full extraction. At last, the extracted substance was concentrated under reduced pressure using a rotary evaporator.

## 2.3 Instrumentation of HPTLC

The standard RT, QT, and GA solution were applied using a Linomat 5 device (CAMAG, Muttenz, Switzerland), with a Hamilton syringe (Bonaduz, Switzerland). TLC plates were developed into a twin-trough developing chamber. Following the successful development of Thin-Layer Chromatography (TLC), a UV cabinet was employed to observe the resulting TLC plates. The plates were scanned at 254 nm using the CAMAG TLC Scanner 3, which was equipped with a D2 lamp along with WinCATS 4 software (V 1.4.8).

## 2.4 Mobile Phase Selection

In the optimization of the mobile phase for HPTLC analysis, initial trials utilized a range of solvents including acetic acid, dioxane, formic acid, ethyl acetate, methanol, n-butanol, isopropanol, and toluene in various proportions. These first investigations uncovered several difficulties, including the low retention factor ($R_f$) values for RT, the issue of overlapping peaks between QT and GA, and the extremely high $R_f$ values for QT. Modifying the methanol concentration in the mobile phase considerably enhanced the Rf values for RT and QT. However, $R_f$ values were adversely impacted when the methanol percentage fell beyond the range of 2-3 %.

## 2.5 Statistical Optimization

After extensive testing with different combinations, a mixture of methanol, toluene, ethyl acetate, and formic acid was used for optimization, as it provided better separation of RT, QT, and GA. The optimization was done by I-optimal Mixture design, considering the total volume of 10 mL, with the mixture components represented as pseudo-components X1 to X4, corresponding to toluene, ethyl acetate, methanol, and formic acid, respectively. The optimization focused on the proportions of these solvents, considering their impact on the peak area and $R_f$ value for each analyte, with each mixture composition being tested once in random order and the centre point run replicated for thorough analysis. Table 1 describes the level of toluene, ethyl acetate, formic acid, and methanol used in statistical optimization. A total of 12 runs as suggested in the design were tested and results were analyzed in design expert software (V. 11.00.0) (Table 2).

## 2.6 Method Validation

The analytical method used to measure RT, QT, and GA underwent a thorough validation process. This process included tests to ensure accuracy, linearity, precision, repeatability, robustness, sensitivity, and specificity. The validation was performed using the most recent criteria Q2 (R1) established by the International Conference on Harmonization (ICH). The thorough validation process ensured that the method was reliable and met international standards[26].

### 2.6.1 Linearity

The linearity of the association was assessed by examining the correlation between the peak area and marker concentration. This was done by making six consecutive measurements around the concentration range between 400–2000 ng/band for GA, QT, and RT. The resulting correlation coefficient was determined.

**Table 1.** Mixture design set up for optimization of mobile phase

| Factor Code | Factor name | Level | | |
|---|---|---|---|---|
| | | Low (-1) | Medium (0) | High (+1) |
| X1 | Toluene | 3.2 | 3.5 | 3.8 |
| X2 | Ethyl acetate | 3.7 | 4 | 4.3 |
| X3 | Formic acid | 0.7 | 1 | 1.3 |
| X4 | Methanol | 1.2 | 1.5 | 1.8 |

**Table 2.** Design Metrix with responses

| Run | Independent factors | | | | Dependent Factors | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | X1 | X2 | X3 | X4 | Y1 | Y2 | Y3 | Y4 | Y5 | Y6 |
| | TOL | EA | FA | MeOH | Area-RTN | Area-QUC | Area-GA | RF-RTN | RF-QUC | RF-GA |
| 1 | 3.2 | 4.3 | 1.3 | 1.2 | 6310.8 | 10698.7 | 9078.1 | 0.14 | 0.7 | 0.54 |
| 2 | 3.70 | 4.01 | 1.09 | 1.20 | 5524.9 | 10280.2 | 9341.8 | 0.08 | 0.64 | 0.46 |
| 3 | 3.80 | 4.30 | 0.70 | 1.20 | 6186.1 | 10949.5 | 9964 | 0.01 | 0.6 | 0.38 |
| 4 | 3.61 | 3.70 | 1.30 | 1.39 | 5956.4 | 9396 | 8831.3 | 0.14 | 0.68 | 0.5 |
| 5 | 3.41 | 4.26 | 0.94 | 1.40 | 6472 | 10551.9 | 4650.4 | 0.15 | 0.71 | 0.55 |
| 6 | 3.41 | 4.26 | 0.94 | 1.40 | 6472 | 10551.9 | 4650.4 | 0.15 | 0.71 | 0.55 |
| 7 | 3.54 | 3.93 | 1.02 | 1.52 | 7410.4 | 9261.5 | 9196 | 0.2 | 0.75 | 0.58 |
| 8 | 3.20 | 3.99 | 1.21 | 1.60 | 7374.2 | 10549.5 | 9941.4 | 0.23 | 0.76 | 0.62 |
| 9 | 3.80 | 3.71 | 0.88 | 1.61 | 5236.5 | 9766.6 | 8471 | 0.12 | 0.69 | 0.51 |
| 10 | 3.57 | 4.04 | 0.70 | 1.69 | 7941.8 | 10648.5 | 10534.6 | 0.1 | 0.77 | 0.6 |
| 11 | 3.20 | 4.30 | 0.70 | 1.80 | 5507.6 | 10663.5 | 9101.7 | 0.16 | 0.73 | 0.59 |
| 12 | 3.41 | 3.70 | 1.09 | 1.80 | 7737.5 | 10078.9 | 9327.4 | 0.24 | 0.71 | 0.57 |

### *2.6.2 Precision*

The interday and intraday study was done to evaluate precision. The variability of the peak area was measured and shown as a percentage Relative Standard Deviation (% RSD). The experiment's intraday precision was evaluated by carrying out three repetitions of three distinct concentrations on the same day. Similarly, the precision study was undertaken on various days.

### *2.6.3 Accuracy*

Recovery tests were done to evaluate the accuracy of the method. The experiment entailed introducing a pre-established quantity of a reference substance to samples at three distinct concentrations (50%, 100%, and 150%) and subsequently examining them using the proposed method. The analysis was conducted three times for optimal accuracy.

### *2.6.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)*

The sensitivity of the developed method was tested using LOD and LOQ which were calculated from the standard deviation of the response and the slope of the calibration curve of the marker using the following formula:

Where "σ" is the standard deviation of the response and "S" is the slope of the calibration curve.

### *2.6.5 Specificity*

The specificity of the method was ascertained by standard markers and sample comparison. Through the comparison of their $R_f$ values to the standard, the presence of biomarkers in the extract was established. It was found that RT, QT, and GA were detected.

### *2.6.6 Robustness*

The proposed method's robustness was tested by making slight modifications to the methodology, such as altering the wavelength, saturation time, and mobile phase composition, all at the same concentration. The method's robustness was determined by calculating the %RSD, which indicated its ability to maintain accuracy and precision.

## 2.7 Analyzing Rutin, Quercetin, and Gallic Acid in Ethanol Extracts of *Grewia asiatica* and *Adansonia digitata*

To quantify the concentrations of RT, QT and GA in the ethanolic extracts of *G. asiatica* and *A. digitata*, a concentrated solution (10 mg/mL) was applied

on a precoated HPTLC plate. This was followed by chromatographic separation under an optimized solvent system. The quantification of RT, QT and GA was calculated using peak area. Results, expressed in grams per 100 grams of extract, were derived from triple replicate analyses for accuracy and reliability.

## 3. Results

### 3.1 Primary Study

In the development of an optimized HPTLC methodology for the detection of RT, QT, and GA, a comprehensive exploration of mobile phase combinations was undertaken to refine the chromatographic resolution of RT, QT, and GA peaks. Drawing on insights from prior research, which has established the HPTLC technique as a viable method for analyzing these compounds individually or in synergy with additional biomarkers, our experimental design included a diverse array of solvents - ranging from toluene, n-hexane, ethyl acetate, cyclohexane, methanol, formic acid, and glacial acetic acid. This initial phase involved varying solvent combinations and ratios to identify the most effective mobile phase. Ultimately, a mobile phase composition of toluene, ethyl acetate, menthol, and formic acid was identified as superior, yielding clear, well-defined peaks for each marker compound. The optimal conditions were further validated by the distinct $R_f$ values observed for RT (0.21), QT (0.58), and GA (0.74), indicating successful separation. Further refinement of the chromatographic parameters was achieved through a mixture design approach, enhancing the method's efficacy and precision.

### 3.2 Advancing Chromatography: A Mixture Design Approach

#### *3.2.1 Model Fitting and Regression Analysis*

The incorporation of a mixture design approach in this study was predicated on its adaptability and its proficiency in refining HPTLC separations, permitting a detailed examination of both primary and interactive effects exerted by various factors. Initial experimental observations guided the selection of critical parameters for this optimization endeavour, focusing on the volumetric contributions of toluene, ethyl acetate, menthol, and formic acid. The optimization criteria were centred around the chromatographic metrics of peak area (area under the curve) and retention factor. To mitigate the influence of extraneous variables and ensure the integrity of the outcomes, all experimental runs were randomized. Analytical assessment of the results employed diverse mixture models, enabling the determination of the most suitable model based on the minimization of residual errors and the overall quality of fit. A favourable outcome was indicated by a positive value, whereas a negative value suggested a relationship that went against optimization. The experimental design and outcomes are delineated in Table 2, with the model's validation conducted through Analysis of Variance (ANOVA). Selection criteria for the optimal model included a high coefficient of determination ($R^2$), minimal discrepancy between predicted and adjusted $R^2$ values, and a low Predicted Residual Sum of Squares (PRESS), as detailed in Table 3. The model's robustness was further assessed by examining changes in $R^2$ upon the exclusion of regression variables, with the refined model's validity confirmed via F-statistics analysis (Tables 4 and 5).

Design-Expert software was utilized to generate 3D surface and contour plots (Figure 2), providing a deeper insight into the impact of the variables (Table 6). An overlay plot was created to meet the specified targets for the optimal response variables, which are indicated by the yellow zone. (Figure 3) After statistical optimization, checkpoint analysis was done by selecting three different compositions and results were verified against the predicted $R_f$ value and AUC (Table 7). Final optimized mobile phase composition X1:X2X3:X4 [(TOL:EA:FA:MeOH) (3.56:3.70:0.94:1.80)].

### 3.3 Method Validation

The validation of the method was rigorously conducted by the International Conference on Harmonization (ICH) Q2 (R1) guidelines, evaluating various parameters such as accuracy, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), precision, and robustness.

#### *3.3.1 Linearity*

The developed HPTLC method demonstrated a significant correlation for RT, QT, and GA within a concentration range of 400-2000 ng/band, illustrated in Figure 4. Linearity for each compound was established

**Table 3. Model selection summery**

| Response | Source | SD | $R^2$ | Adj. $R^2$ | Pred. $R^2$ | PRESS |
|---|---|---|---|---|---|---|
| **Y1: Area-RTN** | Linear | 967.84 | 0.182 | -0.124 | -1.387 | 21872689 |
| | Quadratic | 236.84 | 0.988 | 0.933 | -17.898 | 173193934 |
| | Special Cubic | 7.78 | 1.000 | 1.000 | | + |
| **Y2: Area-QUC** | Linear | 383.680 | 0.6388 | 0.503 | 0.320 | 2218000 |
| | 2FI | 567.200 | 0.8027 | -0.085 | -303.698 | 993500000 |
| | Quadratic | 14.140 | 0.9999 | 0.999 | | + |
| **Y3: Area-GA** | Linear | 2135.577 | 0.1005 | -0.237 | -0.868 | 75769789 |
| | 2FI | 1155.160 | 0.9342 | 0.638 | -100.627 | 4122143337 |
| | Quadratic | 2.121 | 1.0000 | 1.000 | | + |
| **Y4: RF-RTN** | Linear | 0.03 | 0.866 | 0.816 | 0.733 | 0.0 |
| | 2FI | 0.02 | 0.972 | 0.847 | -40.335 | 1.8 |
| | Quadratic | 0.01 | 0.999 | 0.988 | | + |
| **Y5: RF-QUC** | Linear | 0.035 | 0.6260 | 0.486 | 0.033 | 0.025 |
| | 2FI | 0.010 | 0.9930 | 0.961 | -6.954 | 0.209 |
| | Quadratic | 0.007 | 0.9981 | 0.979 | | + |
| **Y6: RF-GA** | Linear | 0.035 | 0.7981 | 0.722 | 0.452 | 0.027 |
| | 2FI | 0.007 | 0.9980 | 0.989 | -0.503 | 0.074 |
| | Quadratic | 0.007 | 0.9990 | 0.989 | | + |

with regression equations of y = 4.8221x + 2443 ($r^2$ = 0.9921) for RT, y = 5.366x + 3957 ($r^2$ = 0.9936) for QT, and y = 5.554x + 3594 ($r^2$ = 0.9912) for GA, indicating precise analytical performance.

### *3.3.2 Precision*

The inter-day precision for RT, QT, and GA was determined to have mean % RSD values of 1.02, 0.83, and 1.01 respectively where n = 3. The intraday precision for RT, QT, and GA was measured to be 1.6, 1.41, and 1.59 (mean % RSD, n = 3) respectively.

### *3.3.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)*

The Limits of Detection (LOD) for RT, QT, and GA were 100.84 ng/band, 102.38 ng/band, and 84.54 ng/band, respectively. Correspondingly, the Limits of Quantification (LOQ) for these compounds were 305.58 ng/band for RT, 310.25 ng/band for QT, and 256.18 ng/band for GA, showcasing the method's sensitivity.

### *3.3.4 % Recovery*

The recovery studies aimed at evaluating the accuracy of the established HPTLC technique yielded average percentage recoveries for RT, QT, and GA, with values of 100.79%, 100.72%, and 105.09% across three trials, respectively.

## 3.4 Quantification of RT, QT and GA in Plant Extract

In the analysis of the ethanolic extracts from *G. asiatica* and *A. digitata*, chromatography identified three prominent peaks at $R_f$ values of 0.74, 0.58, and 0.21, which corresponded to QT, GA, and RT which is aligned perfectly with the standards, indicating no detectable interference from other constituents within

**Table 4.** Regression analysis

| Model term | Y1: Area-RTN | | | Y2: Area-QUC | | |
|---|---|---|---|---|---|---|
| | Full model | | Reduced Model | Full model | | Reduced Model |
| | Coefficient | p-value | Coefficient | Coefficient | p-value | Coefficient |
| X1 | -5282.98 | 0.0929 | -5604.38 | 10642.6 | 0.3322 | 9623.38 |
| X2 | -148.37 | | 336.18 | 10970.3 | | 11793.88 |
| X3 | 11789.4 | | 12192.44 | 10752.48 | | 9441.51 |
| X4 | 7983.34 | | 7806 | 12561.36 | | 9994.77 |
| X1X2 | 35423.5 | 0.0113 | 35200.44 | 1011.98 | 0.9216 | - |
| X1X3 | 101.72 | 0.9804 | - | -5605.38 | 0.5881 | - |
| X1X4 | 20766.69 | 0.0335 | 21718 | -5495.25 | 0.6157 | - |
| X2X3 | 1754.8 | 0.6865 | 5598.34 | -156.54 | 0.9877 | - |
| X2X4 | 6165.58 | 0.2344 | - | -3942.54 | 0.6971 | - |
| X3X4 | -6065.89 | 0.2602 | -6374.2 | -4878.33 | 0.6536 | - |
| | Y3: Area-GA | | | Y4: RF-RTN | | |
| X1 | 20688.55 | 0.5302 | 27921.22 | -0.1056 | 0.0464 | -0.06 |
| X2 | -13148.44 | | -8302.84 | 0.0714 | | 0.08 |
| X3 | 30775.56 | | 26291 | -0.0551 | | -0.09 |
| X4 | 28132.8 | | 26958.98 | 0.1578 | | 0.24 |
| X1X2 | 23879.97 | 0.3263 | - | 0.0894 | 0.8439 | - |
| X1X3 | -59637.44 | 0.0792 | -62340.1 | 0.5788 | 0.2722 | 0.6 |
| X1X4 | -47356.85 | 0.1304 | -58361.8 | 0.227 | 0.6358 | - |
| X2X3 | 51.48 | 0.998 | - | 0.5061 | 0.3293 | 0.56 |
| X2X4 | 5487.3 | 0.7878 | - | 0.1614 | 0.7163 | - |
| X3X4 | -66484.14 | 0.073 | -55428.2 | 0.8914 | 0.1622 | 0.82 |
| | Y5: RF-QUC | | | Y6: RF-GA | | |
| X1 | 0.391 | 0.0167 | 0.355 | 0.16 | 0.0037 | 0.168 |
| X2 | 0.411 | | 0.396 | 0.19 | | 0.166 |
| X3 | 0.792 | | 0.827 | 0.57 | | 0.637 |
| X4 | 0.524 | | 0.586 | 0.49 | | 0.518 |
| X1X2 | 0.79 | 0.0361 | 0.889 | 0.82 | 0.0184 | 0.858 |
| X1X3 | -0.017 | 0.9195 | - | 0.12 | 0.3687 | - |
| X1X4 | 1.047 | 0.0222 | 1.02 | 0.88 | 0.0167 | 0.84 |
| X2X3 | 0.386 | 0.1273 | 0.36 | 0.63 | 0.0297 | 0.57 |
| X2X4 | 1.042 | 0.0198 | 0.96 | 1 | 0.0114 | 0.99 |
| X3X4 | 0.196 | 0.3411 | - | 0.17 | 0.2854 | - |

**Table 5.** Model summery statistics

| Model | Model | Df | F-value | P-value (model) | $R^2$ | SSE | MSE | No. of term omitted | p-value (Lack of Fit) | Fcalculated | Fcritical (α=0.05) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Y1: Area-RTN | 1 | 9 | 17.93 | 0.0539 | 0.98775 | 112191 | 56095.4 | 2 | 0.058 | 0.117 | 3.677 |
| | 2 | 7 | 41.21 | 0.0014 | 0.9863 | 125300 | 31332.3 | | | | |
| Y2: Area-QUC | 1 | 9 | 0.9 | 0.6281 | 0.8027 | 643441 | 321720 | 6 | 0.057 | 0.277 | 8.812 |
| | 2 | 3 | 4.72 | 0.0353 | 0.6388 | 1178000 | 147207 | | | | |
| Y3: Area-GA | 1 | 9 | 3.16 | 0.2638 | 0.9342 | 2669000 | 1334000 | 3 | 0.501 | 0.576 | 4.099 |
| | 2 | 6 | 5.96 | 0.0345 | 0.8774 | 4973138 | 994628 | | | | |
| Y4: RF-RTN | 1 | 9 | 7.75 | 0.1195 | 0.9721 | 0.0012 | 0.0006 | 3 | 0.272 | 0.132 | 4.099 |
| | 2 | 6 | 24.13 | 0.0015 | 0.9666 | 0.0015 | 0.0003 | | | | |
| Y5: RF-QUC | 1 | 9 | 31.31 | 0.0313 | 0.993 | 0.00019 | 0.00009 | 2 | 0.478 | 0.818 | 3.677 |
| | 2 | 7 | 44.03 | 0.0013 | 0.9872 | 0.00034 | 0.00008 | | | | |
| Y6: RF-GA | 1 | 9 | 111.9 | 0.0089 | 0.998 | 9.8E-05 | 4.9E-05 | 2 | 0.557 | 1.475 | 3.677 |
| | 2 | 7 | 115.91 | 0.0002 | 0.9951 | 0.00024 | 0.00006 | | | | |
| 1=Full Model, 2=Reduced Model | | | | | | | | | | | |

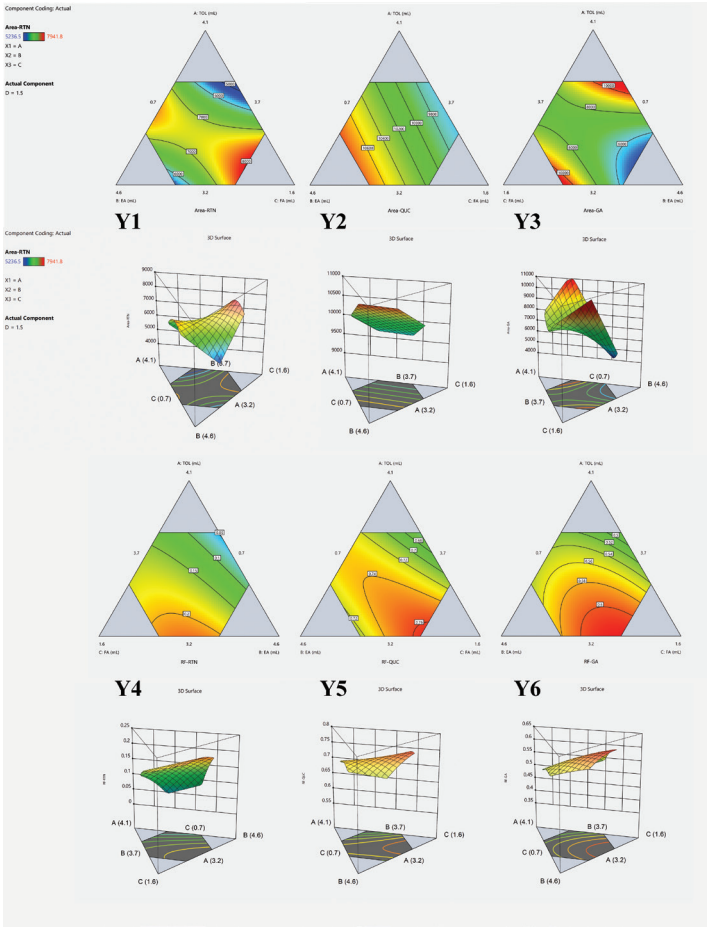


**Figure 2.** Contour and 3D surface plots for responses (Y1-Y6).

**Table 6.** Response target for optimization

| Response | Response | Lower limit | Upper limit | Target set for optimization |
|---|---|---|---|---|
| Y1 | Area-RTN | 5236.5 | 7941.8 | maximize |
| Y2 | Area-QUC | 9261.5 | 10949.5 | maximize |
| Y3 | Area-GA | 4650.4 | 10534.6 | maximize |
| Y4 | RF-RTN | 0.01 | 0.24 | 0.24 |
| Y5 | RF-QUC | 0.6 | 0.77 | 0.7 |
| Y6 | RF-GA | 0.38 | 0.62 | 0.5 |

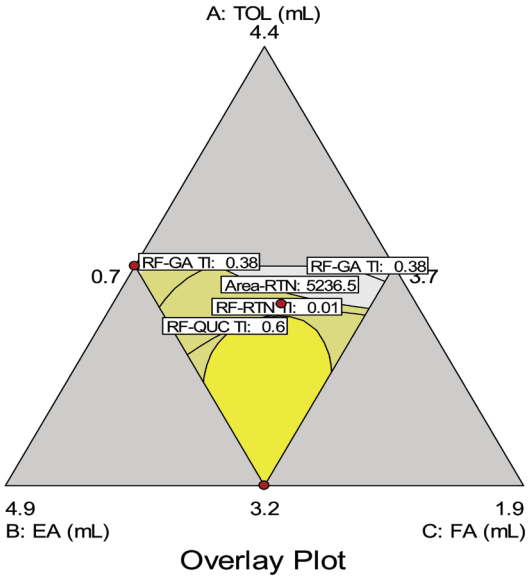


**Figure 3.** Design space (Yellow region) in overlay plot.

**Table 7.** Checkpoint analysis

| Suggested solutions | TOL:EA:FA:MeOH (X1:X2X3:X4) | Y1: Area-RTN | Y2: Area-QUC | Y3: Area-GA | Y4: RF-RTN | Y5: RF-QUC | Y6: RF-GA |
|---|---|---|---|---|---|---|---|
| **Predicted responses** | | | | | | | |
| 1* | 3.56:3.70:0.94:1.80 | 7278 | 9774 | 9084 | 0.200 | 0.719 | 0.563 |
| 2 | 3.31:3.70:1.30:1.69 | 8275 | 9684 | 10317 | 0.240 | 0.723 | 0.573 |
| 3 | 3.34:3.70:1.26:1.69 | 8098 | 9690 | 9687 | 0.239 | 0.722 | 0.571 |
| **Observed responses** | | | | | | | |
| 1* | 3.56:3.70:0.94:1.80 | 7337 | 10282 | 9699 | 0.21 | 0.74 | 0.58 |
| 2 | 3.31:3.70:1.30:1.69 | 7894 | 9626 | 9462 | 0.26 | 0.76 | 0.62 |
| 3 | 3.34:3.70:1.26:1.69 | 7648 | 10402 | 10724 | 0.21 | 0.67 | 0.53 |
| **% Prediction error** | | | | | | | |
| 1* | 3.56:3.70:0.94:1.80 | -0.81 | -5.20 | -6.77 | -5.00 | -2.94 | -2.94 |
| 2 | 3.31:3.70:1.30:1.69 | 4.61 | 0.60 | 8.28 | -8.33 | -5.06 | -8.22 |
| 3 | 3.34:3.70:1.26:1.69 | 5.56 | -7.34 | -10.70 | 12.23 | 7.16 | 7.26 |

*Final optimized mobile phase composition as there was least perdition error for all responses

the extracts. Results described in Table 8 showed that the ethanolic extracts of *G. asiatica* leaves contained higher levels of RT, QT, and GA compared to *A. digitata* fruits.

## 4. Discussion

Biomarker study is the most crucial part of herbal drugs. The development and validation of analytical

**Table 8.** Estimated content of RT, QT, and GA in ethanolic extracts of *Adansonia digitata* fruits and *Grewia asiatia* leaves

| Biomarker | Ethanolic extract of AD | Ethanolic extract of GA |
|---|---|---|
| RT | 832.56 ng/spot | 1614.06 ng/spot |
| QT | 2586.65 ng/spot | 3411.85 ng/spot |
| GA | 1743.4 ng/spot | 1865.71 ng/spot |

QT: Quercetin, RT: Rutin, GA: Gallic acid

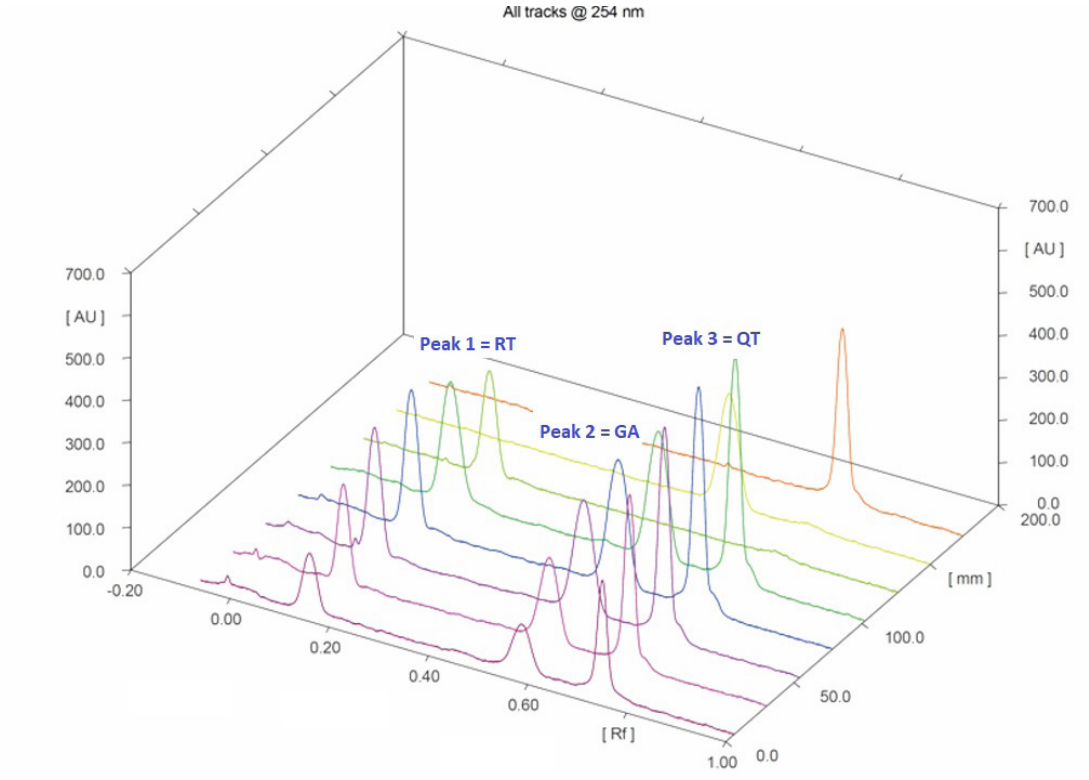


**Figure 4.** 3D chromatogram of Rutin, Quercetin, and Gallic acid.

methods are crucial for ensuring the quality of herbal medicines. Herbal industries utilize a range of sophisticated instrumental techniques, such as High-Performance Liquid Chromatography (HPLC), Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), Gas Chromatography (GC), Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS), and High-Performance Thin-Layer Chromatography (HPTLC), to quantify various phytochemicals in herbal formulations. RT, QT, and GA are significant phytochemicals whose simultaneous quantification is essential for the standardization of herbal drugs. Therefore, there is a need for an economical and user-friendly method that facilitates this process. Several HPTLC methods for simultaneous estimation of selected phytochemicals are available in the literature. In a study conducted by Kumar, and Lakshman[27], the concentration levels of RT, QT, and GA in *Terminalia chebula* were quantified using HPTLC. The author devised two separate analytical methods for the quantification of three compounds. For RT and QT, a mobile phase consisting of ethyl acetate, dichloromethane, formic acid, glacial acetic acid, and water in the ratio of 10:2.5:1:1:0.1 (v/v/v/v/v/v/v) was employed. In contrast, a mixture of toluene, acetone, and glacial acetic acid in the ratio of 3:1:2 (v/v/v) was utilized specifically for the quantification of GA.

In another study, Alam *et al.,* also developed two distinct methods for analyzing RT, QT, and GA in an anti-HBV active extract of *Guiera senegalensis*. They used acetonitrile: water (4:6, v/v) and Toluene: Ethyl acetate: Formic acid (6:4:0.8, v/v/v/v) as solvents for the analysis of rutin, quercetin, and gallic acid, respectively. There are two distinct methods for measuring RT, QT and GA, which require more time and involve higher costs for analysis. Several other methods were also reported by various researchers[28-30]. Compared to reported studies, our method offers a fast easy to perform and cost-effective HPTLC method of analysis.

The mobile phase plays a crucial role in the method development. To develop a precise, sensitive, easy-to-operate and cost-effective HPTLC method, various mobile phases were experimented with to enhance the resolution between the compounds. Through our investigation, we discovered that the combination of toluene, ethyl acetate, formic acid, and methanol

provides superior resolution when compared to other solvents. Design mixture assists in identifying the optimal combination of solvents to achieve the desired $R_f$ value within the design space.

An optimized mobile phase composition was achieved using a simple centroid design with axial points in a pseudo-component representation. The simple centroid design is a design that lies on the boundary. By conducting additional experiments within the simplex, we can accurately describe the properties of the mixture. Therefore, the typical simplex design was enhanced by the axial runs. In addition, the centroid was adjusted due to it not being a design point[31].

The present study resolves the issue of resolution of all three phytochemicals in HPTLC analysis. The method successfully quantifies RT, QT, and GA levels in ethanolic extracts of *A. digitata* and *G. asiatica*. Further testing of the method in other plant extracts is needed to confirm the applicability of the designed method.

## 5. Conclusion

The study's results highlighted that the separation resolution of RT, QT, and GA is significantly affected by the mobile phase's composition. An effective optimization of the mobile phase was achieved through the application of a simplex centroid mixture design coupled with a desirability function approach. Furthermore, this process led to the development of a new analytical method characterized by its efficiency and user-friendliness, particularly for the quantification of RT, QT, and GA in ethanolic extracts of *A. digitata* and *G. asiatica*. This method offers time savings and presents a practical solution for routine analysis. Future directions include extensive testing and validation across various plant extracts to broaden its applicability for all extracts containing RT, QT and GA, thereby underscoring its potential value to the herbal pharmaceutical industry.

## 6. Acknowledgments

The authors are thankful to Dr. Pintu Prajapti, Department of Pharmaceutical Quality Assurance and Dr Shailesh Shah, Principal of Maliba Pharmacy College for providing the resources for the work.

## 7. References

1. Garcia S. Pandemics and traditional plant-based remedies. A historical-botanical review in the era of COVID-19. Front Plant Sci. 2020; p. 1353. https://doi.org/10.3389/fpls.2020.571042 PMid:32983220 PMCid: PMC7485289
2. Dhami N, Mishra AD. Phytochemical variation: How to resolve the quality controversies of herbal medicinal products? J Herb Med. 2015; 5(2):118-27. https://doi.org/10.1016/j.hermed.2015.04.002
3. Shukla S, Saraf S, Saraf S. Approaches towards standardization and quality assessment of herbals. J Res Educ Indian Med. 2009; 15(1):25-32.
4. Beressa A, Wariyo A, Chala G, Tefera Y. Analytical method development for quality control and standardization of medicinal plants: A critical review. J Herbal Sci. 2021; 10(1):9-20.
5. Gunjal Sanket B, Dighe P. Analysis of herbal drugs by HPTLC: A review. Asian J Pharm Res Dev. 2022; 10(2):125-8. https://doi.org/10.22270/ajprd.v10i2.1056
6. Kumari R, Kotecha M. A review on the standardization of herbal medicines. Int J Pharma Sci Res. 2016; 7(2):97-106.
7. Koval'Skii I, Krasnyuk I, Krasnyuk I, Nikulina O, Belyatskaya A, Kharitonov YY, *et al.* Mechanisms of rutin pharmacological action. Pharm Chem J. 2014; 48:73-6. https://doi.org/10.1007/s11094-014-1050-6
8. Yang J, Guo J, Yuan J. *In vitro* antioxidant properties of rutin. LWT-Food Sci and Technol. 2008; 41(6):1060-6. https://doi.org/10.1016/j.lwt.2007.06.010
9. Enogieru AB, Haylett W, Hiss DC, Bardien S, Ekpo OE. Rutin as a potent antioxidant: Implications for neurodegenerative disorders. Oxid Med Cell Longev. 2018; 2018:6241017. https://doi.org/10.1155/2018/6241017 PMid:30050657 PMCid: PMC6040293
10. Imani A, Maleki N, Bohlouli S, Kouhsoltani M, Sharifi S, Maleki Dizaj S. Molecular mechanisms of anticancer effect of rutin. Phytother Res. 2021; 35(5):2500-13. https://doi.org/10.1002/ptr.6977 PMid:33295678
11. Farha AK, Gan R-Y, Li H-B, Wu D-T, Atanasov AG, Gul K, *et al.* The anticancer potential of the dietary polyphenol rutin: Status, challenges, and perspectives. Crit Rev Food Sci Nutr. 2022; 62(3):832-59. https://doi.org/10.1080/10408398.2020.1829541 PMid:33054344
12. Satari A, Ghasemi S, Habtemariam S, Asgharian S, Lorigooini Z. Rutin: a flavonoid as an effective sensitizer for anticancer therapy; insights into multifaceted mechanisms and applicability for combination therapy. Evidence-based complementary and alternative medicine. 2021; 2021:9913179. https://doi.org/10.1155/2021/9913179 PMid:34484407 PMCid: PMC8416379
13. Rauf A, Imran M, Khan IA, ur-Rehman M, Gilani SA, Mehmood Z, *et al.* Anticancer potential of quercetin: A

comprehensive review. Phytother Res. 2018; 32(11):2109-30. https://doi.org/10.1002/ptr.6155 PMid:30039547
14. Baghel SS, Shrivastava N, Baghel RS, Agrawal P, Rajput S. A review of quercetin: antioxidant and anticancer properties. World J Pharm Pharmaceutical Sci. 2012; 1(1):146-60.
15. Massi A, Bortolini O, Ragno D, Bernardi T, Sacchetti G, Tacchini M, *et al.* Research progress in the modification of quercetin leading to anticancer agents. Molecules. 2017; 22(8): 1270. https://doi.org/10.3390/molecules22081270 PMid:28758919 PMCid: PMC6152094
16. Tang S-M, Deng X-T, Zhou J, Li Q-P, Ge X-X, Miao L. Pharmacological basis and new insights of quercetin action with respect to its anti-cancer effects. Biomed Pharmacother. 2020; 121:109604. https://doi.org/10.1016/j.biopha.2019.109604 PMid:31733570
17. Yang D, Wang T, Long M, Li P. Quercetin: Its main pharmacological activity and potential application in clinical medicine. Oxid Med Cell Longev. 2020; 2020:8825387. https://doi.org/10.1155/2020/8825387 PMid:33488935 PMCid: PMC7790550
18. Zhang M, Swarts SG, Yin L, Liu C, Tian Y, Cao Y, *et al.* Antioxidant properties of quercetin. Oxygen transport to tissue XXXII. 2011: Springer. https://doi.org/10.1007/978-1-4419-7756-4_38 PMid:21445799
19. Xu D, Hu M-J, Wang Y-Q, Cui Y-L. Antioxidant activities of quercetin and its complexes for medicinal application. Molecules. 2019; 24(6):1123. https://doi.org/10.3390/molecules24061123 PMid:30901869 PMCid: PMC6470739
20. Badhani B, Sharma N, Kakkar R. Gallic acid: A versatile antioxidant with promising therapeutic and industrial applications. RSC Advances. 2015; 5(35):27540-57. https://doi.org/10.1039/C5RA01911G
21. Yen G-C, Duh P-D, Tsai H-L. Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. Food Chem. 2002; 79(3):307-13. https://doi.org/10.1016/S0308-8146(02)00145-0
22. Aruoma OI, Murcia A, Butler J, Halliwell B. Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. J Agric Food Chem. 1993; 41(11):1880-5. https://doi.org/10.1021/jf00035a014
23. Kahkeshani N, Farzaei F, Fotouhi M, Alavi SS, Bahramsoltani R, Naseri R, *et al.* Pharmacological effects of gallic acid in health and diseases: A mechanistic review. Iran J Basic Med Sci. 2019; 22(3):225.
24. Subramanian A, John A, Vellayappan M, Balaji A, Jaganathan S, Supriyanto E, *et al.* Gallic acid: prospects and molecular mechanisms of its anticancer activity. RSC Advances. 2015; 5(45):35608-21. https://doi.org/10.1039/C5RA02727F
25. Jiang Y, Pei J, Zheng Y, Miao Y-j, Duan B-z, Huang L-f. Gallic acid: A potential anti-cancer agent. Chin J Integr Med. 2022; 28(7):661-71. https://doi.org/10.1007/s11655-021-3345-2 PMid:34755289
26. ICH. Validation of analytical procedures Q2 (R2). Geneva, Switzerland 2022.
27. Kumar A, Lakshman K, Jayaveera K, Tripathi SM, Satish K. Estimation of gallic acid, rutin and quercetin in *Terminalia chebula* by HPTLC. Jordan J Pharm Sci. 2010; 3(1):63-8.
28. Mehesare SS, Waghmare SP, Thorat MG, Hajore SW, Hatzade RI, Ingawale MV. Quantification of gallic acid, rutin and quercetin in the hydro-ethanolic extract of *Holarrhena antidysenterica* using High-Performance Thin Layer Chromatography (HPTLC). Acta Scientific Veterinary Sciences (ISSN: 2582-3183). 2022; 4(4). https://doi.org/10.31080/ASVS.2022.04.0350
29. Hussain J, Bassal M, Sarhan H, Aga MIH. Qualitative and quantitative comparison of rutin, quercetin and gallic acid concentrations in Syrian *Capparis spinosa*. L leaves. J Pharmacogn Phytochem. 2017; 6(4):407-15.
30. Biswas S, Harwansh RK, Kar A, Mukherjee PK. Validated high-performance thin-layer chromatographic method for the simultaneous determination of quercetin, rutin, and gallic acid in *Amaranthus tricolor* L. Journal of Planar Chromatogr. 2019; 32(2):121-6. https://doi.org/10.1556/1006.2019.32.2.7
31. Ramani VD, Jani GK, Sen AK, Sailor GU, Sutariya VB. Development, and validation of RP-HPLC method for pitavastatin calcium in bulk and formulation using experimental design. J Appl Pharm Sci. 2019; 9(10):075-83. https://doi.org/10.7324/JAPS.2019.91010