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Design expert-supported development and validation of HPTLC method: An application in simultaneous estimation of quercetin and rutin in *Punica granatum, Tamarindus indica* and *Prunus domestica*

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ABSTRACT

Aim: A simple, precise and accurate high performance thin layer chromatographic method has been developed for the simultaneous estimation of quercetin and rutin in *Punica granatum*, *Tamarindus indica* and *Prunus domestica*.

Methods: The separation was carried out on Merck TLC aluminium sheets of silica gel 60 F_{254}, (20 × 10 cm) with 250 μm thickness using toluene:ethyl acetate:formic acid (5:4:0.5, *v/v/v*) as a mobile phase.

Results: The detection of quercetin and rutin was carried out at 297 nm. The drugs were satisfactorily resolved with R_f values of 0.44 ± 0.02 and 0.59 ± 0.02 for quercetin and rutin, respectively. The accuracy and reliability of the method was assessed by evaluation of linearity (100–1000 ng/spot for quercetin and 500–3000 ng/spot for rutin). Intra- and inter-day precision measured as coefficient of variation were less than 2% for both analytes. The limits of detection and quantification were found to be 33.54 and 101.66 ng/spot, respectively for quercetin and 367.21 and 1112.75 ng/spot, respectively for rutin.

Conclusion: The proposed method was found to be accurate, precise, reproducible and specific and can be applicable for the simultaneous determination of quercetin and rutin in samples.

1. Introduction

Herbal medicines are known the test of time for their safety, efficacy, cultural acceptability and lesser side effects. Due to their natural nature, there are believed that it will show better compatibility with biological system. From ancient year a lot of herbal plants were used in formulations as antidiabetic, antioxidant.[1] It is found that flavonoids are a group of polyphenolic compounds, which have been found widely throughout the plant kingdom. Out of which 300 varieties of flavonoids are known till date.[2] Most of them have low toxicity in mammals and some of them are largely used in medicine for maintenance of capillary integrity.[3,4] When these compounds used by humans, flavonoids have been found to a reduction in the occurrence of diseases such as cancer and heart disease.[5–10]

Rutin and quercetin both are flavonoids and are herbal origin. They possess antioxidant activity, antidiabetic effect and oxidation of low-density lipoproteins [LDL].[11] It was reported that enzymes such as bradykinin,[12] tyrosine kinase,[13] and 5′- nucleotidase are inhibited with quercetin in combination of other flavonoids.[14] Rutin and quercetin are known to act in regulation of hormone like transport, metabolism and action of thyroid hormones.

Pomegranate (*Punica granatum* L., Lythraceae); Tamarind (*Tamarindus indica* L., Leguminosae); and Plum (*Prunus domestica* L., Rosaceae), commonly used as nutritional supplements, are a major source of antioxidants[15–17] and contribute to the daily intake of a significant amount of these molecules.

Nowadays high performance thin layer chromatography (HPTLC) is a routine, accurate and precise analytical technique because of its several advantages like low operating cost; high sample throughput and minimum sample clean up requirement. The major benefits of HPTLC is that number of samples can be run simultaneously using a small quantity of mobile phase which is not possible in case of high performance liquid chromatography (HPLC), leads to lowering of analysis time and cost per analysis.[18–22]

This study reports the quantitative estimation of quercetin and rutin in *P. granatum*, *T. indica* and *P. domestica* for the first time using

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an HPTLC method which is suitable for routine use. Although quantitative estimation of quercetin and rutin has been reported by HPTLC in a variety of herbal extracts, this is the first time that such a method has been reported for *P. granatum*, *T. indica* and *P. domestica* with some modification of existing methods.

The proposed method for analysis of quercetin and rutin simultaneously in *P. granatum*, *T. indica* and *P. domestica* was validated as per the ICH guidelines[23] (International Conference on Harmonization, 1996) similar to the other methods reported by laboratory,[24–26] which are in use for the standardization of herbal drugs.

2. Material and methods

2.1. Plant collection and identification

The fresh fruits of *P. granatum*, *T. indica* and *P. domestica* were collected from Delhi, India and were authenticated at botany department, Jamia Hamdard, New Delhi, India, with a voucher specimen (PG/FP-367, TI/FP-368, PD/FP-369) which were deposited in the herbarium.

2.2. Chemicals and reagents

Rutin (98%) and quercetin (99%) were purchased from Sigma–Aldrich (USA). All chemicals and reagents were of AR grade. Methanol and Aluminium-backed TLC plates pre-coated with 0.2 mm layer of silica gel 60 F_{254} (20 cm × 10 cm) were purchased from E. Merck (Germany); supplied by Anchrom Technologists, Mumbai.

2.3. Standard preparation

A stock solution of quercetin and rutin (1 mg/mL) were prepared by dissolving 10 mg of accurately weighed rutin and quercetin in water:methanol (1:1, *v/v*) and diluted to 10 mL with methanol in a standard volumetric flask. The stock solutions were filtered by membrane filter and applied on a TLC plate.

2.4. Sample preparation

The fruits of *P. granatum*, *T. indica and P. domestica* were dried in hot air oven at 40–50 °C for a week. The dried fruits were powdered using mixer grinder. The accurately weighed samples (5 g) were dissolved in 25 mL of extraction solvent water:methanol (1:1, *v/v*) and sonicated for 20 min at 27 ± 3 °C in ultra sonicator water bath. The stock solutions were filtered by membrane filter and applied on a TLC plate.

2.5. TLC instrumentation and conditions

The samples were spotted in the form of bands of width 5 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60 F_{254} (20 cm × 10 cm with 0.2 mm thickness, E. Merck, Germany) using a Camag Linomat V (Switzerland). A constant rate of application of 150 nL/s was employed and space between two bands was maintained 11.6 mm. The slit dimension was kept at 4 mm × 0.45 mm, and scanning speed was maintained at 20 mm/s. The mobile phase was composed of toluene:ethyl acetate:formic acid (5:4:0.5, *v/v/v*). Linear ascending development was carried out in twin trough glass chamber which was saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 20 min at room temperature and the chromatogram was developed up to the length of 80 mm. The developed TLC plates were dried in current air with the help of an air-dryer. Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 297 nm. The source of radiation which was utilized for it was deuterium and tungsten lamp.

2.6. Linearity (calibration curve)

Standard solutions of rutin and quercetin were prepared in methanol to get a concentration of 1 mg/mL. Different volumes of standard solution were spotted on the TLC plate in triplicate to obtain a final concentration of 100–1000 ng/spot for quercetin and 500–3000 ng/spot for rutin. The plate was then developed and the data of peak area versus drug concentration were treated by linear least squares regression analysis to get a regression equation.

2.7. Validation of the proposed method

The Proposed method was validated as per ICH guidelines.

2.7.1. Accuracy (%recovery)

The accuracy of the method was determined by calculating recoveries of quercetin and rutin by the standard addition method. Known amounts of standard solutions of quercetin and rutin were added at 50, 100 and 150% level to pre-quantified sample solution of quercetin and rutin. The amount of quercetin and rutin was estimated by applying obtained values to the respective regression line equations.

2.7.2. Precision

The precision of the system was determined by measuring repeatability of sample application and measurement of peak areas for six replicates of the same band. To evaluate inter-day precision, six samples at three different concentrations (400, 600 and 800 ng per spot for quercetin and 1000, 2000 and 3000 ng per spot for rutin) were prepared and analyzed on three different days. The intra-day precision (intermediate precision) was studied by comparing assays performed on the same day. The precision of the system and method were expressed as relative standard deviation (RSD %) and the standard deviation (SD) of peak area.

2.7.3. Robustness of the method

Robustness of the method was performed by introducing very small changes in the analytical methodology at a single concentration level. Robustness of the proposed method was determined by changing the composition of the mobile phase. The mobile phase, toluene (A), ethyl acetate (B), and formic acid (C) are used as solvent system. In the present study, the robustness was evaluated by using the Box–Behnken response surface design.[27] The design simultaneously evaluated the effects of the independent parameters on peak area: toluene, ethyl acetate, and formic acid. Design-Expert 8.0 software, (Stat-Ease, Inc., Minneapolis, MN, USA) was used to evaluate the result. Three-dimensional graphs represented peak area dependence on solvent system. Effects of the selected factors were evaluated over a range of conditions by determining the maximum area response of the quercetin and rutin peaks.

2.7.4. Limit of detection and limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were determined by the blank determination method. In order to estimate the LOD and LOQ, blank methanol was spotted six times and the area calculated. The signal-to-noise level was determined. LOD was considered as 3:1 and LOQ as 10:1.

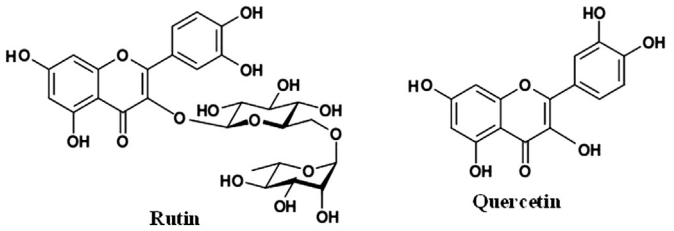

Fig. 1. Structure of quercetin and rutin.

2.8. Determination of quercetin and rutin in P. granatum, T. indica and P. domestica

2 μL of each sample as prepared above was applied in triplicates on TLC plates. It was developed and scanned as per the method described. The results of peak area obtained corresponding to quercetin and rutin were used for quantification in samples using regression equation. The results of triplicate analysis were expressed as average amount of quercetin and rutin in % w/w (Fig. 1).

3. Results

3.1. Development of the optimum mobile phase

The developed TLC procedure was optimized for analysis and quantification of quercetin and rutin in *P. granatum*, *T. indica* and *P. domestica*. TLC procedure was optimized with varying mobile phase ratios toluene:ethyl acetate:formic acid. The mobile phase toluene:ethyl acetate:formic acid with ratio (5:4:0.5, *v/v/v*) gave excellent resolution, dense, compact and well separated spots of quercetin and rutin as well as a sound peak at R_f were found to be 0.59 and 0.44 for quercetin and rutin, respectively (Fig. 2 and Fig. 3). Other chromatographic conditions like sample application rate and volume, run length, chamber saturation time, sample application positions, detection wavelength and distance between tracks were optimized for precise and reproducible R_f values, better resolution, and symmetrical peak shape for the compounds.

3.2. Calibration curves

The linear regression data obtained for the calibration curves ($n = 3$) showed an excellent linear relationship over wide concentration range 100–1000 ng per spot for quercetin and 500–3000 ng per spot for rutin with respect to peak area as shown in Table 1.

3.3. Validation of the method

The method was validated with respect to parameters including precision, robustness, accuracy, LOD, LOQ and specificity.

3.3.1. Precision

The measurement of peak area six times inter-day and intra-day showed %R.S.D (<2%) which suggested precision of the method as shown in Table 2.

3.3.2. Robustness

By introducing deliberate changes in solvent system and the effects on the results were examined (Fig. 4). There were very slight changes in the peak area, R_f and resolution. The Design Expert software proposed the following polynomial equation for peak area of quercetin and rutin.

Quercetin (Y1):

Peak Area +2870.13 − 0.24*A-0.87*B − 3.66*C+4.49*A*B − 0.38*A*C+9.84*B*C − 15.28*A2 − 13.16*B23.25*C2.

Where A is the toluene (*v/v*), B is ethyl acetate (*v/v*) and C is the formic acid (*v/v*). According to the above equation,

As per the polynomial equation toluene, ethyl acetate and formic acid have negative effect on the peak area of quercetin. Formic acid has more effect on the peak area than toluene and ethyl acetate. As the concentration of formic acid increases, decreased the peak area of quercetin, as indicated by the negative coefficient value in the polynomial equation. However, the lower magnitude of

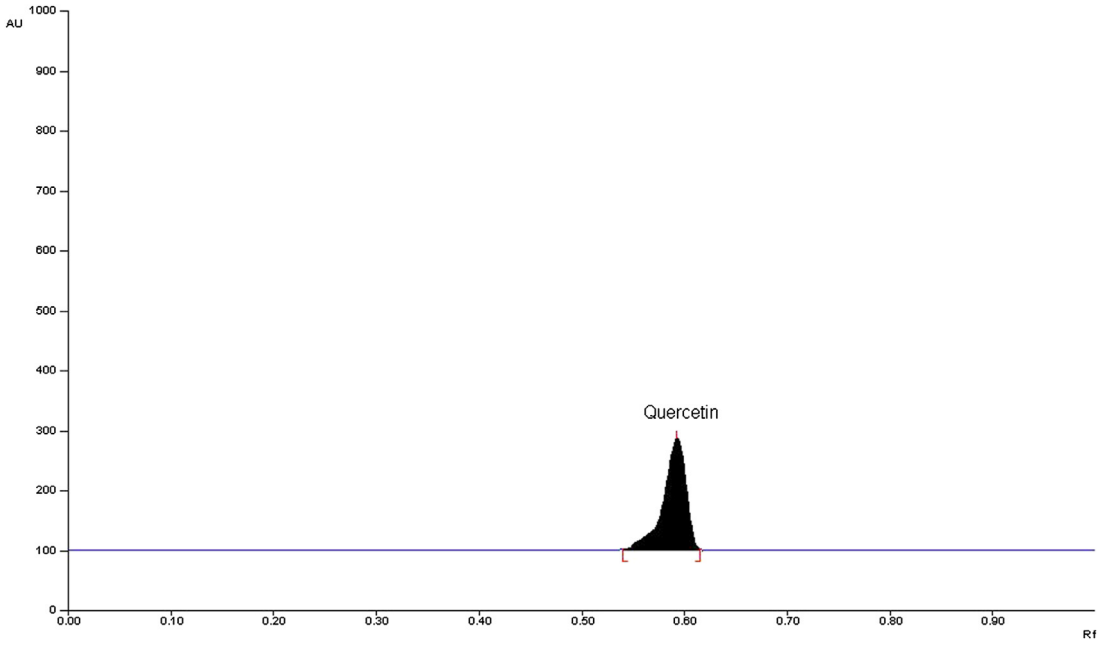

Fig. 2. A typical TLC chromatogram of quercetin ($R_f = 0.59$).

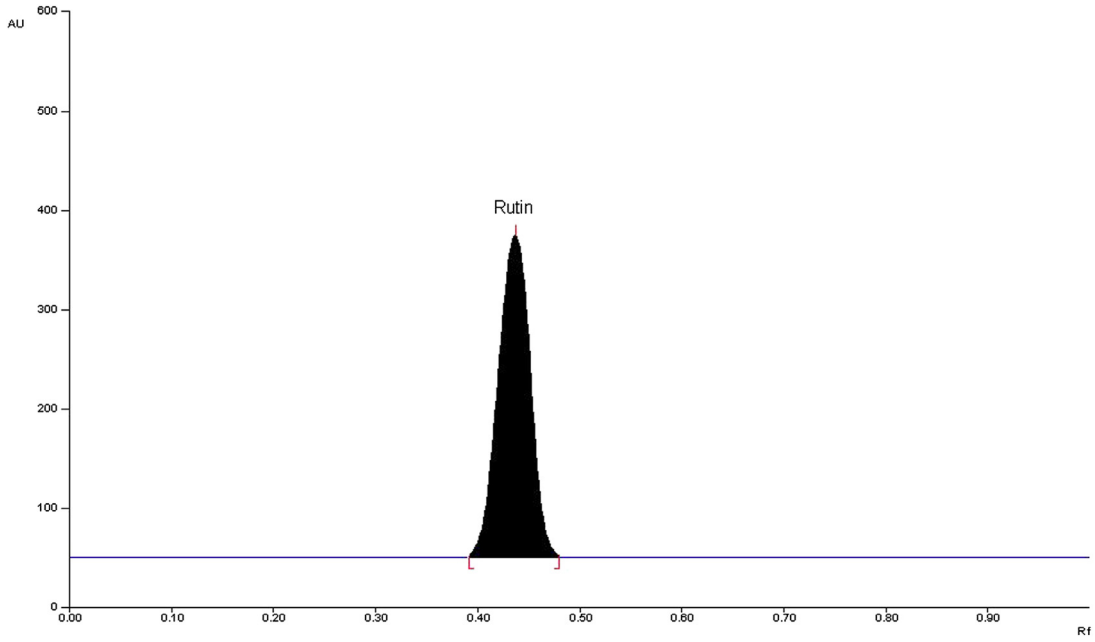

Fig. 3. A typical TLC chromatogram of rutin ($R_f = 0.44$).

the coefficient indicated that the effect of toluene and ethyl acetate has less negative effect on peak area, means increase the concentration, decrease the peak area.

Rutin (Y2):

Peak area + 1568.97 + 2.51*A + 1.27*B−2.31*C + 3.18*A*B + 0.022*A*C + 4.12*B*C − 0.89*A2 − 6.54*B2 + 0.35*C2.

The above polynomial equation of rutin shows that toluene, ethyl acetate and formic acid effect on the peak area of rutin. Toluene has more positive prominent effect on the peak area of rutin as compared to ethyl acetate and formic acid, and formic acid has more negative effect than the ethyl acetate. As concentration of toluene increases, the peak area also decreases. On the other hand formic acid concentration increases, the peak area decreases individually, but this negative effect was balanced by the toluene because they have more prominent positive effect on the peak area.

3.3.3. Recovery studies

The proposed TLC method used for extraction and subsequent estimation of quercetin and rutin in samples after spiking with 50, 100 and 150% of additional drug, which demonstrates good recovery of 99.75–102.4922% as shown in Table 3.

3.3.4. LOD and LOQ

The LOD with S/N ratio of 3:1 was found to be 33.54 ng/spot and 367.21 ng/spot for quercetin and rutin, respectively. LOQ with S/N ratio of 10:1 was found to be 101.66 ng/spot and 1112.75 ng/spot quercetin and rutin, respectively.

3.3.5. Specificity

The peak purity of rutin and quercetin was accessed by comparing the spectra at peak start, peak apex and peak end position of the spot. Good correlation (0.991) and (0.993) was obtained between the standard and sample of quercetin and rutin.

Table 1
Linear regression data for the calibration plots ($n = 3$) for quercetin and rutin.

Component	Concentration range (ng/spot)	Regression equation	r^2
Quercetin	100–1000	3.047 X+475.40	0.991
Rutin	1000–3500	0.585X+267.90	0.993

3.4. Determination of quercetin and rutin in samples

A well-resolved single spots of quercetin and rutin were observed at R_f 0.44 and 0.59, respectively in the chromatograms of the samples. It was also observed from the superimposed UV spectra that there was no interference from the other components present in the extracts. The results are analyzed in Table 4, with low %RSD value indicating the suitability of this method for routine analysis of quercetin and rutin in *P. granatum*, *T. indica* and *P. domestica*.

4. Discussion

HPTLC is a novel technique primarily used in identification, qualitative, quantitative and visual analysis of drug samples.[28] Current manuscript showed that the HPTLC techniques using these solvent is very useful for quercetin and rutin analysis. It was reported that the technique could be used to solve many qualitative and quantitative analytical problems in a wide range of pharmaceutical fields, including medicines, biological sciences, food analysis, toxicology and environmental analysis.[29]

Table 2
Inter-day and intra-day precision of the HPTLC method ($n = 6$) for quercetin and rutin.

Amount (ng/spot)	Inter-day precision		Intra-day precision	
	Mean peak area ± SD	%RSD	Mean peak area ± SD	%RSD
Quercetin				
400	849.88 ± 4.96	0.584	887.39 ± 13.61	1.534
600	1725.92 ± 23.94	1.387	1735.98 ± 8.84	0.509
800	2822.74 ± 26.39	0.935	2841.71 ± 15.18	0.534
Rutin				
1000	389.95 ± 4.45	1.143	401.09 ± 7.81	1.948
2000	856.36 ± 6.26	0.731	863.16 ± 8.35	0.968
3000	1551.48 ± 5.88	0.379	1558.50 ± 8.29	0.531

SD, standard deviation; RSD, relative standard deviation.

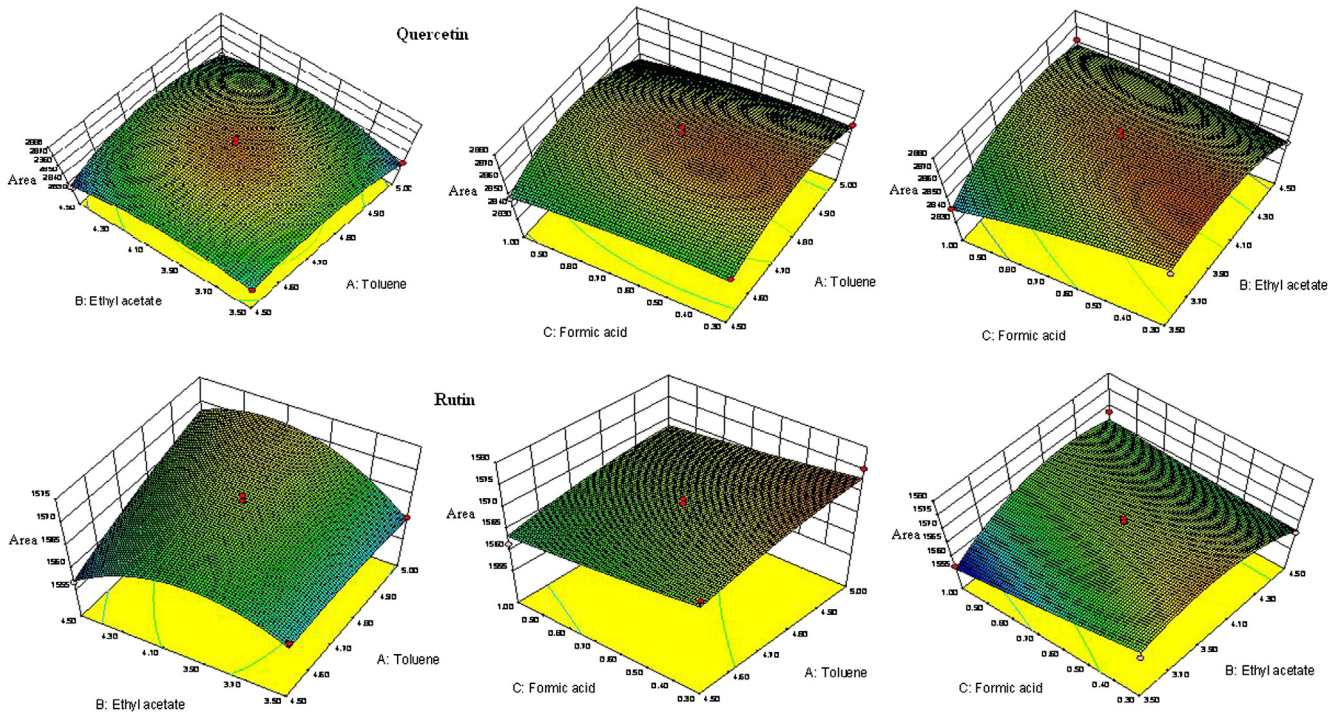

Fig. 4. Three-dimensional graphs of quercetin and rutin by using Box-Behnken experimental design.

Table 3
Accuracy of the HPTLC method ($n = 3$) for quercetin and rutin.

% of standard spiked to the sample	Theoretical content (ng)	Amount of drug recovered ng ± SD	% of drug recovered	% RSD
Quercetin				
0	300	307.47 ± 1.96	102.4922	0.639644
50	450	448.89 ± 4.94	99.75333	1.101466
100	600	606.74 ± 3.78	101.1244	0.624019
150	750	752.38 ± 9.00	100.3178	1.197337
Rutin				
0	2500	2527.36 ± 13.08	101.0945	0.517785
50	3750	3746.14 ± 39.94	99.89707	1.066339
100	5000	5035.89 ± 42.17	100.7178	0.8375
150	6250	6269.917 ± 16.95	100.3187	0.270423

HPTLC method is considered useful due to the development of forced flow TLC methods, improved stationary and mobile phase selection, as well as new methods of quantification methods.[30] A validated stability indicating HPTLC analytical method has been developed for the determination of quercetin and rutin in *P. granatum*, *T. indica* and *P. domestica*. The proposed method is specific, accurate, simple, precise, less time consuming, cost effective and has the ability to separate the drugs from its other constituents. Statistical analysis proved that the method is suitable for the routine analysis of quercetin and rutin. Although the Design-Expert 8.0 software predicted that various factors could affect the peak area and the values of peak area were in an acceptable range to demonstrate sufficient robustness of the analytical method. The proposed method is superior as compared to other TLC densitometric methods, because it has been properly optimized and well validated. It also has better linearity range and limits of determination. The statistical analysis of data obtained reveals that the developed method is reproducible and selective and can be used for routine qualitative and quantitative analysis of quercetin and rutin in *P. granatum*, *T. indica* and *P. domestica* and its marketed formulation.

Table 4
Quantification of quercetin and rutin in tested herbs by HPTLC.

Samples	Quercetin (% w/w)	Rutin (% w/w)
Punica granatum	0.0892	0.124
Tamarindus indica	0.0628	0.0941
Prunus domestica	0.0571	0.153

5. Conclusion

The developed TLC technique is precise, specific and accurate. Statistical analysis proves that the method is suitable for quercetin and rutin in *P. granatum*, *T. indica* and *P. domestica*. The proposed TLC method is less expensive, simpler, rapid, and more flexible than LC.

Conflicts of interest

All authors have none to declare.

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