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TLC and HPTLC Finger Printing Analysis of *Cyperus rotundus* (Linn.)

Divya Jain [1], Rashmi Upadhyay [1], Sonika Jain [2], Anand Prakash [1], Pracheta Janmeda [1,*]

[1] Department of Bioscience and Biotechnology, Banasthali Vidyapith, Rajasthan-304022, India; divyajain31011996@gmail.com (D.J.), rashmiup432@gmail.com (R.U.); anandpkh@gmail.com (A.P.); pracheta@banasthali.in (P.J.);
[2] Department of Chemistry, Banasthali Vidyapith, Rajasthan-304022, India; sonikajain85@gmail.com (S.J.);
* Correspondence: pracheta@banasthali.in (P.J.);

Scopus Author ID 55443238700

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Abstract: *Cyperus rotundus* (Linn.) is a versatile plant belonging to the family Cyperaceae, used in herbal medicines worldwide to cure various human ailments. The present study attempts to analyze the profiles of flavonoids in different extracts of *C. rotundus* with the help of thin-layer chromatography (TLC) and high-performance thin-layer chromatographic (HPTLC) fingerprint. Flower and stem extracts of *C. rotundus* were screens out with the help of TLC, and the R_f values were determined. HPTLC was used to quantify the flavonoid from flower extract of a plant at a 1.0 mg/mL concentration. It revealed the occurrence of flavonoids, especially quercetin in the ethanolic extract of *C. rotundus* flower, by using mobile phase toluene-ethyl acetate-formic acid (3:4:2.5 *v*/*v*), on a pre-coated plate of silica gel and quantified the amount of quercetin by densitometry absorbance mode at 257 nm. The limits of detection and quantification were 30.08 & 91.16 ng/mL, and the relative standard deviation ranged between 1.03 to 1.48 for intra-day and inter-day for HPTLC. The calibration range was 200-700 ng per band (r^2 = 0.99321). Quercetin quantity in the ethanolic extract of the flower was found to be 0.011 mg/mL of the extract with an average recovery of 99.01–100.00%. Such fingerprinting is valuable in quality control and checking adulterants of natural drugs. Therefore, it can be helpful for the assessment of different marketable pharmaceuticals preparations.

Keywords: chemical profile; flavonoid; HPTLC; isolation; quercetin; TLC.

1. Introduction

Cyperus rotundus Linn. (Cyperaceae) is commonly known as “Nutgrass” in English, “Mustaka” in Sanskrit, and “Motha” in Hindi. It is a perennial, erect, smooth herb with scaly, wiry, creeping, slender, persistent, and dark rhizomes. It is reportedly native to India, extensively cultivated in the tropical regions in Sri Lanka, and widely spread in the tropical and subtropical areas around the world [1-3].

It is about 10-75 cm tall, and leaves are 10-18 cm long [4]. *In traditional Chinese medicine, C. rotundus rhizomes known as Xiang-Fu* is used to treat women’s hormone, menstrual irregulations, dysmenorrheal, and gastrointestinal problems [5-7]. In the Indian system of medicine, it helps in treating dysentery, amenorrhea, fever, bronchitis, leprosy, arthritis, blood disorders, dysmenorrhea, and diarrhea, etc. [8, 9]. It is a therapeutic herb and has been recognized in the Ayurvedic concepts. Alkaloids, polyphenol, sesquiterpenoids, essential oils, flavonol glycosides, and saponins were identified in *C. rotundus* rhizomes

phytochemical investigations [10, 11]. Despite its invasiveness, many pharmacological properties of this perennial plant, like antiemetic, anti-hypotensive, anti-diabetic, anti-obesity, anti-analgesic, anti-inflammatory, antimicrobial, and anti-malarial, etc. have been reported [12-17].

The available literature on the pharmacological benefits of the plant urges for the analytical study of its parts and to identify different bioactive constituents present in this plant by using various approaches, including TLC and HPTLC analysis. These chromatography techniques are still the most versatile and prominent methods used in qualitative analysis and separation applications. To estimate chemical and biochemical markers, HPTLC has developed into an efficient way to link the chemical compound profile of medicinal plants [18].

2. Materials and Methods

2.1. Collection and preparation of extracts.

Mature flower and stem of *C. rotundus* were collected from Krishi Vigyan Kendra (KVK), Banasthali Vidyapith, Rajasthan, India, in October 2018. They were morphologically recognized with the help of available literature and authenticated by the botanist of KVK. The plant parts were washed thoroughly and then shaded, dried, and ground using a grinder. Fifty grams of powdered plant materials were placed in the soxhlet apparatus having solvents ranging from non-polar to polar [19-21]. Based on phytochemical screening, ethanol extract of flower and stem were selected, and 1 mg/mL concentration is prepared in HPLC grade solvent for further analysis [22].

2.2. Mobile phase selection.

Thin-layer chromatography (TLC) of ethanolic extracts of flower and stem of *C. rotundus* were carried out in various solvents compositions (formic acid, ethyl acetate, toluene, methanol, chloroform, butanol, acetic acid, and pyridine, etc.) by using various proportions at 30°C using silica gel Gas adsorbent. The solvent system which showed the best retention factor and resolution was selected out for further analysis.

2.3. Sample solution preparations.

1.0 mg/mL solution was prepared by diluting ethanol extract of flower (CREF), and stem (CRES) of *C. rotundus.* This solution was further investigated after being filtered through the 0.2 µm pore size syringe filter.

2.4. Standard solution for TLC and HPTLC.

One milligram of quercetin, rutin, and catechin was measured accurately and diluted in the methanol and filtered using a 0.45 µm syringe filter.

2.5. Standard preparations for calibration curve for HPTLC.

Quercetin solution was prepared and spotted on HPTLC plates at 2 µL, 3 µL, 4 µL, 5 µL, 6 µL, and 7 µL to obtain the concentrations of standards per band of 200, 300, 400, 500, 600, and 700 ng, respectively. The standards calibration curve has been constructed by modeling a peak [23-25].

2.6. Reagents and standards.

All analytical grade reagents and standards were procured from SRL and Merck, India.

2.7. Instrumentation and chromatographic conditions.

A comparative plate was prepared of standards with 3 µL spots of each, *i.e.*, quercetin, rutin, catechin, and 6 µL of ethanol extract of flower and stem, applied on a 10×10 HPTLC plate. The HPTLC method specifications are given in Table 1.

Table 1. Instrument conditions for HPTLC of flavonoid standard and CREF extract.

S. No.	Parameters	Description
Chromatographic parameters		
1	Stationary phase	Pre-coated silica gel aluminium plate $60F_{254}$ (10 cm x 10 cm and 20 cm x 10 cm with 250 µm thickness)
2	Spray gas	Inert gas
3	Band length	6.0 mm
4	Application rate	100 µm/step
5	Developing solvent	Toluene- ethyl acetate-formic acid (3:4:2.5)
6	Chromatogram runlength	55 mm
Scanning parameters		
1	Dimension of slit	4.00 x 0.30 mm, Micro
2	Scanning speed	20 mm/s
3	Densitometric scanning wavelength	257 nm; 366 nm
4	Lamp	Deuterium
5	Measurement mode	Absorption
6	Software	WinCATS
7	Data resolution	100 /step

2.8. Method validation.

The method was determined by specifying the given parameters according to the guidelines of the International Conference on Harmonization (ICH) and the U.S. Food and Drug Administration (FDA) [26-28].

2.8.1. Linearity and range.

The calibration curve per quercetin was developed from a range of 100 to 700 ng. It shows good linearity, suggesting the likelihood of using this approach to measure low and high quercetin concentrations [29].

2.8.2. Accuracy.

Quercetin at three different levels (80, 100, and 120%) was applied to check the method accuracy, to analyze the average and recovery percentage. For each level, it was performed in triplicates.

2.8.3. Limit of detection (LOD) and limit of quantification (LOQ).

To calculate the LOD and LOQ, different concentrations of standard quercetin were applied [30].

$$DL = 3.3\ \sigma/S \quad (1)$$

$$QL = 10\ \sigma/S \quad (2)$$

Where, σ is the standard deviation response, and *S* is the corresponding calibration curve's slope.

2.8.4. System suitability (robustness).

System suitability was measured as robustness to observe any effect on method condition by a small variation in process condition. Instrumental parameters like chamber saturation (15 min), developing TLC distance (8.5 cm), temperature, chamber dimensions (10 cm × 10 cm), humidity, and composition of mobile phase (toluene-ethyl acetate-formic acid; 5:4:1) changed to 3:4:2.5, were studied.

2.8.5. Instrument precision and repeatability.

Method repeatability was expressed as *%RSD*, and quercetin was confirmed by repeated scanning (n = 5) of the same band (150 ng per band) [23].

2.8.6. Inter-day and intra-day variation.

The method variability was performed by analyzing quercetin aliquots on the same day at 100, 150, and 200 ng per band in triplicates (intra-day precision) and on 3 successive days (inter-day precision). Chromatographic optimization conditions were used to evaluate HPTLC plates, and the peak was recorded with *%RSD* [30-32].

2.8.7. Specificity.

Analysis of samples and standard compounds determined the specificity of the method. Quercetin band from sample solution was defined by comparing R_f and band spectra against the standards. There are 3 different levels of bands, i.e., start, middle, and end positions, where compound peak quality was analyzed. It shows the admirable efficiency and selectivity of the chromatographic process.

2.8.8. Quercetin quantification in plant.

On HPTLC plate, 20 µL sample was applied to determine the quercetin amount (n = 5). It was developed using various optimized chromatographic conditions, and *%RSD* was calculated using the reported compound peak area from the calibration curve.

2.8.9. Stability of standard quercetin solution.

The standardized solutions determined standard stability in high areas at different time intervals: 8, 12, 24, 36, and 72 hours (h). The findings showed that there was no additional impurity in the chromatograms of the test solution was reported after 72 hours.

3. Results and Discussion

TLC is used as a separation method, and HPTLC is suitable for qualitative and quantitative analysis. In addition, this method provides a rapid screening technique with a relatively shorter examination and samples comparison on a single plate for analytical studies, showing the simplicity of the preparation, especially in herbal medicines [33].

3.1. Thin layer chromatography: identification and isolation of bioactive constituents from different parts extracts of C. rotundus.

According to separation and movement, toluene-ethyl acetate-formic acid (3:4:2.5, *v/v*) showed the best separation result for CREF and CRES. The ethanol extract of the flower separated into 2 clear peaks with R_f of 0.18 and 0.26 in 3:4:2.5 fraction of the mobile phase, which was visible in the iodine (Figure 1 and 2) and UV chamber (Figure 3). Stem ethanol extract also exhibited good separation in this fraction (Table 2). The separation of the different analytical compounds results from the competing intermolecular forces of the stationary and mobile phases [30]. Thus, the developed chromatogram fingerprint will be specific to the selected solvent system and serves effective standardization method for the particular plant species.

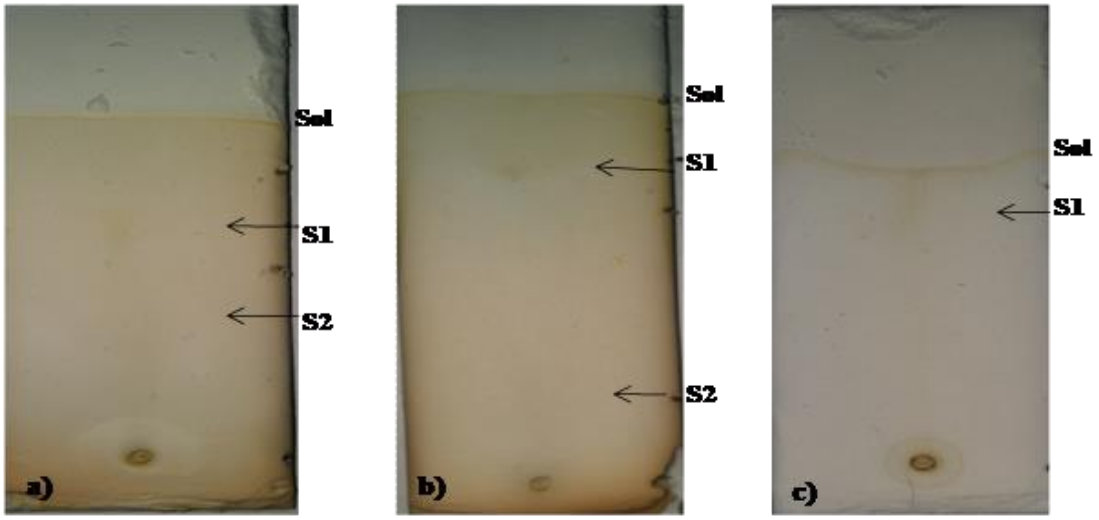

Figure 1. (a-c) TLC chromatograms of flower ethanolic extract of *C. rotundus*
Spot (S); Solvent (Sol).

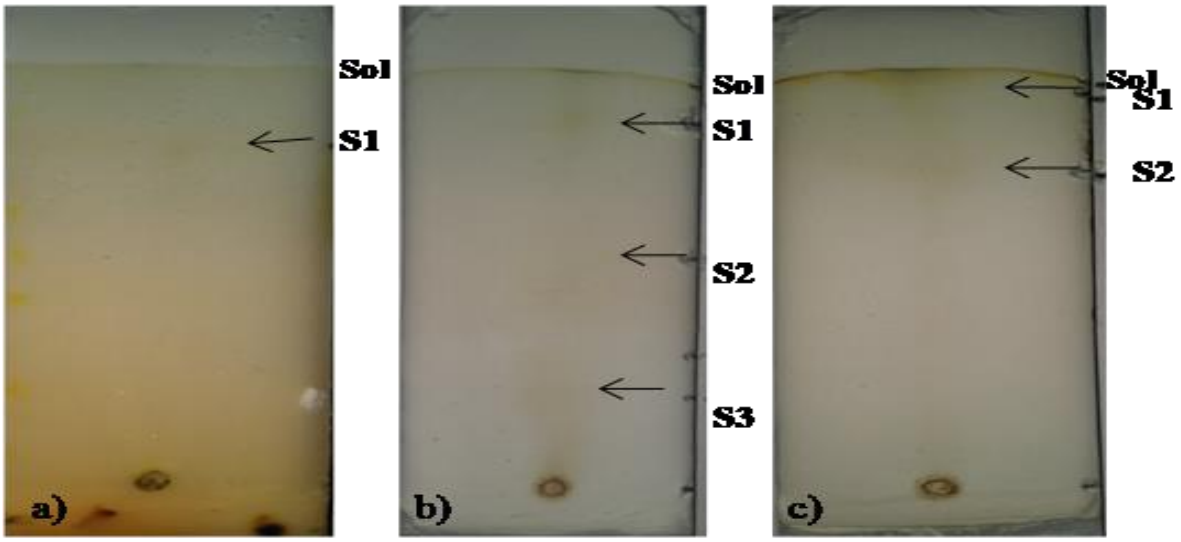

Figure 2. **(a-c)** TLC chromatograms of stem ethanolic extract of *C. rotundus*
Spot (S); Solvent (Sol).

3.2. High-performance thin-layer chromatography (HPTLC).

Based on TLC observations, a comparison of 3 flavonoid standards, i.e., quercetin, rutin, and catechin, with the stem and flower ethanol extract was carried out using HPTLC

(Figure 4). Peak with similar R_f (0.77) of quercetin (Table 3) was identified in ethanol extract of the flower. The peak obtained in stem ethanol extract R_f (0.32) did not correspond to either of the standards as rutin exhibited R_f (0.50) and catechin R_f (0.75). Based on the above results, HPTLC of quercetin with the ethanol extract flower (CREF) was carried out using (Figure 5). Peak with similar R_f 0.77 of quercetin (Table 4) was identified and found to be 57.75 ng, i.e., 0.011 mg/mL of extract.

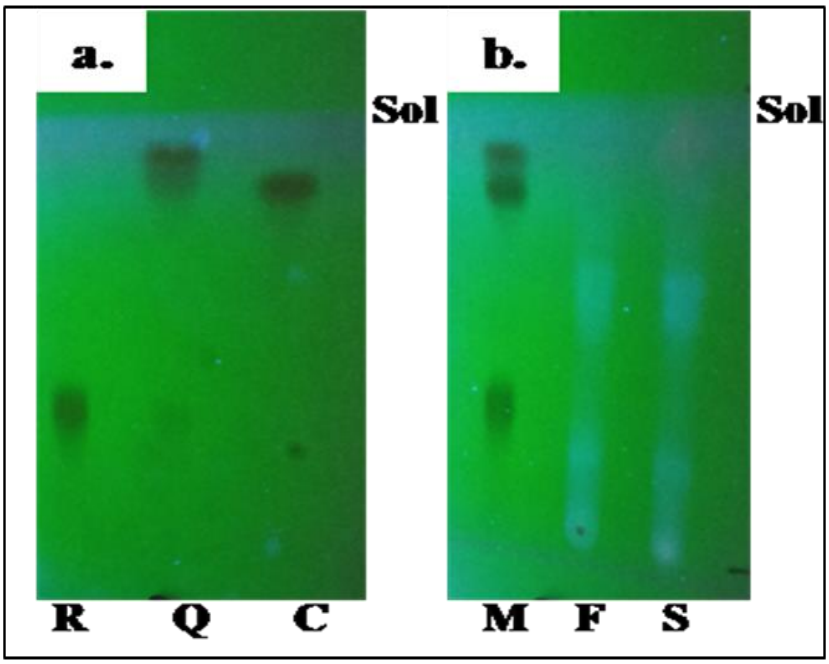

Figure 3. **(a-b)** TLC Plates after derivatization in UV. Rutin (R); Quercetin (Q); Catechin (C); Mixture of standards (M); Flower ethanol extract (F); Stem ethanol extract (S); Solvent (Sol)

Table 2. TLC separations of flavonoids from CREF and CRES of *C.rotundus.*

Mobile phase		**Solvent run**	**No of spots**			**Distance of spots**			**Total spots**	**R_f value**	**Observation**
Solvents	Ratio		Vis	IC	UV	Vis	IC	UV			
Flower ethanol extract (CREF)											
T:E:F	3:4:2.5	4.5	0	0	2	-	-	3.2; 1.3	2	0.71; 0.26	Not complete run, separation occurred
Stem ethanol extract (CRES)											
T:E:F	3:4:2.5	5	0	0	2	-	-	2.5; 0.9	2	0.5; 0.18	Not complete run, separation occurred

TEF: Toluene: ethyl acetate: formic acid; *Rf*: Retention factor.

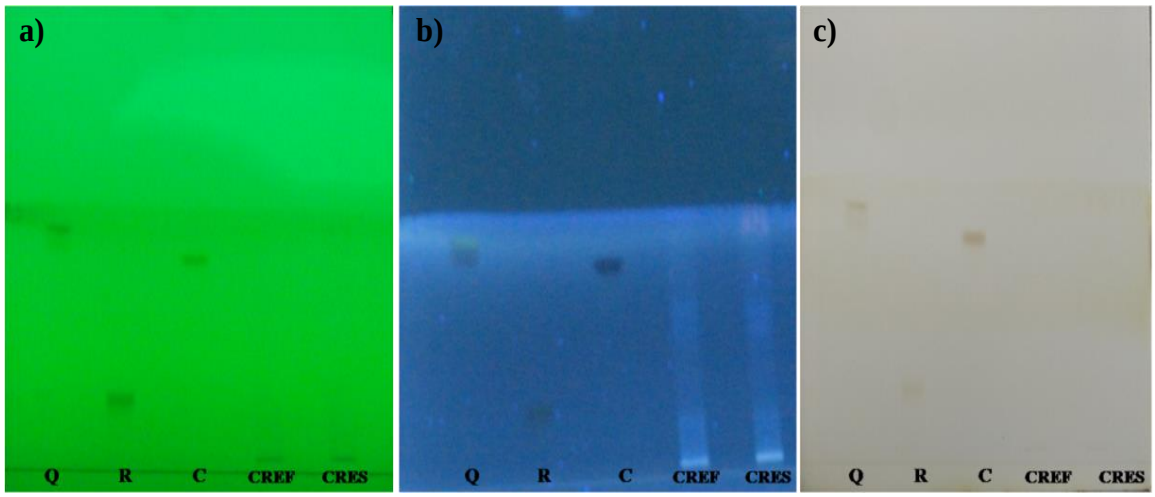

Figure 4. (a-c) HPTLC fingerprints of quercetin, rutin, and catechin, ethanol extract of flower, and ethanol extract of stem visualized under 254 nm, 366 nm, and after iodine derivatization.

Table 3. HPTLC profile of the three standards (quercetin, rutin, and catechin), ethanol extract of flower, and stem of *C. rotundus.*

Track	Sample	Mobile phase	Amount applied	Max R_f	Area	Area %	Assigned substance
1	Quercetin	T:E:F (3:4:2.5)	1 µl	0.77	2371.6	100	Standard
2	Rutin		1 µl	0.32	662.8	100	Standard
3	Catechin		1 µl	0.75	553.7	100	Standard
4	CREF		6 µl	0.76	472.6	100	Quercetin
5	CRES		6 µl	0.68	437.9	100	Unknown

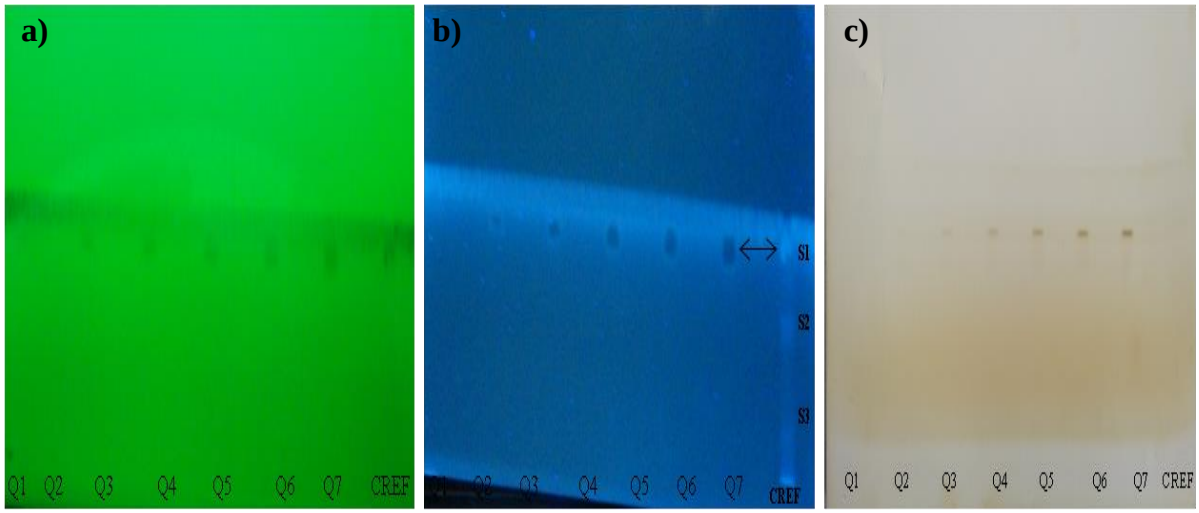

Figure 5. (a-c) HPTLC fingerprints of different concentrations of quercetin (Q1 – Q7) and ethanol extract of flower (CREF) visualized under 254 nm, 366 nm, and after iodine derivatization.

Table 4. HPTLC profiles of different concentrations of quercetin and CREF extract of *C. rotundus.*

Track	Sample	Mobile phase	Amount applied	Max R_f	Max height	Max %	Area	Area %	Assigned substance
1	Quercetin	T:E:F (3:4:2.5)	2 µl	0.80	95.0	100	1062.4	100	Standard
2	Quercetin		3 µl	0.80	176.3	100	2158.9	100	Standard
3	Quercetin		4 µl	0.79	246.7	100	3034.2	100	Standard
4	Quercetin		5 µl	0.78	314.9	100	3910.4	100	Standard
5	Quercetin		6 µl	0.77	372.2	100	4795.0	100	Standard
6	Quercetin		7 µl	0.77	408.4	100	5471.2	100	Standard
7	CREF		2 µl	0.77	19.8	100	291.7	100	Quercetin

CREF- *C. rotundus* ethanol extract of flower; T:E:F- Toluene: ethyl acetate: formic acid; *Rf* - retention factor

3.3. Method validation of quercetin by HPTLC.

The relationship between the concentration of quercetin and peak response was linear at 200-700 ng /band with an R^2 value of 0.99321. The quercetin DL and QL were found to be 30.08 ng per band and 91.16 ng per band, respectively (Figure 6 and 7). The three different levels of quercetin, i.e., 80, 100, and 120%, are the percentage mean recovery, which is found to be in the range of 98-99 % (Table 5).

The standard and the test solutions were applied on the HPTLC plates with slightly different combinations of the three solvents. In addition, the approach has also been checked for reliability, specificity, and repetitiveness. The quercetin presence in the ethanol extract of the flower was confirmed by comparing R_f values of the standard with the sample. The *%RSD* ranged from 0.93 to 1.05 for intra-day and inter-day accuracies. Method validation results have been summarized in Table 6.

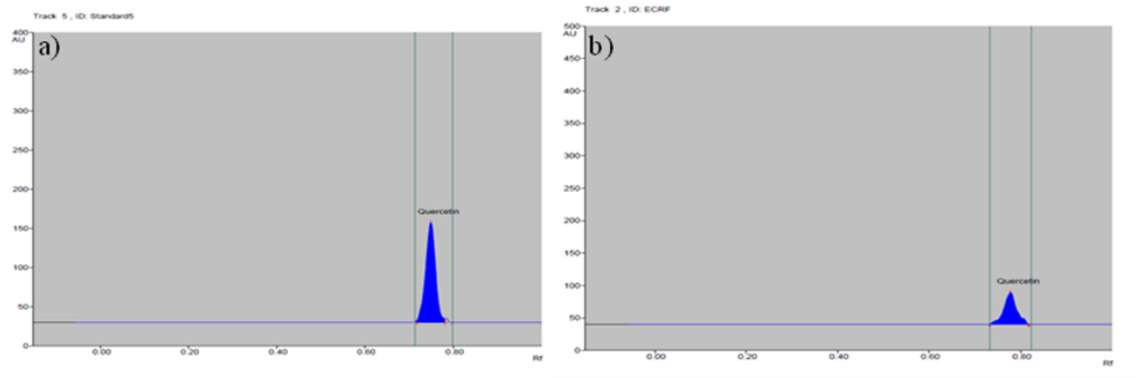

Figure 6. HPTLC spectrum a) Quercetin standard and b) ethanol extract of the flower.

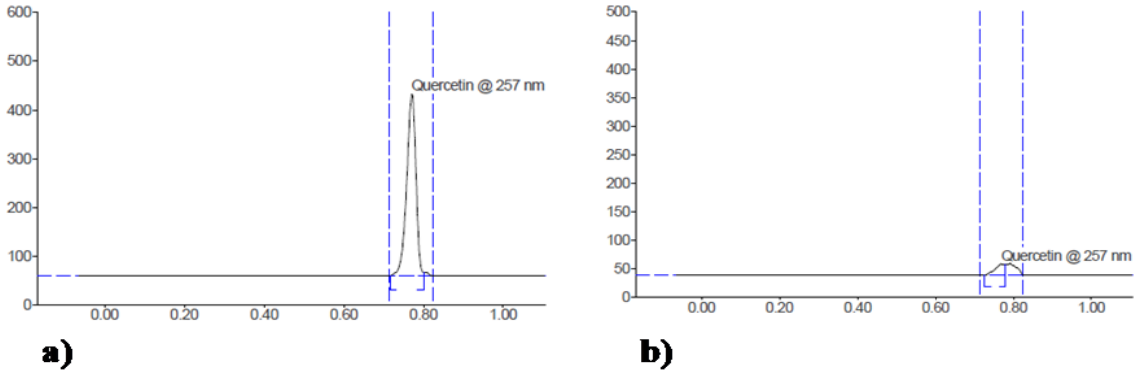

Figure 7. HPTLC chromatogram a) quercetin standard and b) ethanol extract of flower at 257 nm.

Table 5. Results of recovery analysis.

Compound	Amount present in the sample (ng per band)	Amount added (ng per band)	Total amount	Amount of compound detected (ng per band)	Percentage recovery (n = 3)	%RSD
Quercetin	57.75 ng	80 % (46.2)	103.95	102.91	98.99	0.79 %
		100 % (57.75)	115.5	114.5	99.13	0.98%
		120 % (69.3)	127.05	126.7	99.72	1.05%

Table 6. Method validation results.

Parameter	Value
R_f	0.80
Range of linearity	100-700 ng
Linear Regression equation	Y = -16.91 + 0.6351 * X
Correlation of determination (r^2)	0.99321
Instrument precision (n = 5)	*%RSD* = 0.94
Repeatability (n = 5)	*%RSD* = 1.05
Specificity	Specific
DL	30.08 ng /band
QL	91.16 ng / band

3.4. Stability of quercetin.

It was checked by *%RSD*, the values of percentage relative standard deviation within range (<1.05), which indicates that quercetin solution was constant as no major degradation were observed.

3.5. Quantification of standard quercetin in C. rotundus.

Quercetin is reported in the stem and flowers of *C. rotundus*. Literature evidence and these findings evoked us to quantify quercetin in the flowers of *C. rotundus*, as 0.011 mg/mL of extract.

4. Conclusions

TLC and HPTLC methods for detecting and quantifying active ingredients present in C. rotundus were carried out in the present work to separate and determine quercetin in ethanolic extracts of the flower of *C. rotundus*. The chromatographic condition provides tremendous selectivity, effectivity, and good peak parameters. TLC and HPTLC results of the present study firmly depicted that this plant contains a wide range of flavonoids, which is the reason behind its excellent pharmacological properties.

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Conflicts of Interest

The authors declare no conflict of interest.

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