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DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR QUANTIFICATION OF QUERCETIN AND GALLIC ACID IN THE SEEDS OF *TAMARINDUS INDICA* AND *PURSLANE OLERACEA*

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ABSTRACT

A sensitive and reliable high performance thin layer chromatography method has been developed for the simultaneous estimation of quercetin and gallic acid in the seeds of Tamarindus indica and Purslane oleracea. Aqueous extracts prepared were applied on silica gel G 60 F254 plate. Toluene: ethyl acetate: formic acid in a combination of 5:4:1 v/v/v and 6:4:1 v/v/v were found the best mobile phase for gallic acid and quercetin standards separation. The plate was developed using suitable mobile phase and detection and quantification were performed by densitometric scanning at 254 nm / 366 nm. The accuracy of the method was confirmed by conducting recovery studies at different levels using the standard addition method. The average recovery of quercetin and gallic acid was found close to 99% suggesting the accurateness of the method. The proposed validated high performance thin layer chromatographic method offers a new, sensitive, specific and precise gauge for quantification of quercetin and gallic acid in in the seeds of Tamarindus indica and Purslane oleracea.

Keywords *– Quercetin; HPTLC; Gallic acid; Tamarindus indica; Purslane oleracea*

1. INTRODUCTION

Medicinal plants often contain a variety of bioactive compounds with diverse therapeutic and nutritional benefits. The presence and concentration of these bioactive constituents in herbal medicines and botanicals are influenced by factors such as the method of plant collection, geographical location, seasonal variations, and extraction techniques. Changes in the levels of key bioactive compounds can significantly impact the therapeutic effects of herbal preparations. Therefore, it is essential to standardize herbal drugs and functional foods by profiling the bioactive compounds, also known as marker compounds.[1]

In the context of industrial applications, the development of cost-effective, simple, and precise chemo-profiling methods is crucial. High Performance Thin Layer Chromatography (HPTLC) has emerged as a preferred analytical technique for fingerprinting and quantifying marker compounds in herbal drugs due to its suitability for high-throughput screening, sensitivity, and reliability in quantification at nanogram levels. However, HPTLC has not been effectively utilized for precise quantification of phytochemicals, with limited reports available, particularly in plants like *Tamarindus indica* and *Tamarindus indica* and *Purslane oleracea*.

Tamarindus indica L. (Fabaceae), or tamarind, is a tropical plant native to Africa. The plant has long been used as a food and herbal medicine. Its fruit pulp is well-known as a good source of vitamins, minerals, and organic acids. Tamarind fruit possesses several pharmacological activities, such as antifungal, antiasthmatic, hepatoprotective, and wound healing activities [2-5]. Moreover, other parts of this plant, such as its leaf, stem bark, root bark, and seed, have also been reported as medicaments, e.g., antibacterial, antihyperlipidemic, antiulcer, anticancer, antifungal, wound healing, hepatoprotective and immunopotentiation agents [5-11].

There are various chemical constituents in *T. indica*. The fruit pulps contain furan derivatives, carboxylic acid, phlobatannin, grape acid, apple acid, flavonoids, pectin, sugars, and the like [12-13]. The seeds contain campesterol, β-amyrin, β-sitosterol, fatty acids, tannins, sugars, mucilage and polysaccharides, cardiac glycosides, and phenolics, among others [14,15]. The components of the bark include tannins, saponins, glycosides, peroxidase, and lipids [12]. The leaves contain orientin, iso-orientin, vitexin, iso-vitexin, glycosides, peroxidase, vitamin B_3, and vitamin C [12,15]. Polyphenols, e.g., flavonoids and phenolics, are present in almost every part of the plant, making *T. indica* an up-and-coming source of antioxidative agents.

Purslane *(Portulaca oleracea* L.), belonging to the Portulacaceae family, is commonly known as purslane in English and Ma-Chi-Xian in Chinese. It is a warm-climate, herbaceous succulent annual plant with a cosmopolitan distribution. It is eaten extensively as a potherb and added in soups and salads around the Mediterranean and tropical Asian countries and has been used as a folk medicine in many countries. Diverse compounds have been isolated from *Portulaca oleracea*, such as flavonoids, alkaloids, polysaccharides, fatty acids, terpenoids, sterols, proteins vitamins and minerals. *Portulaca oleracea* possesses a wide spectrum of pharmacological properties such as neuroprotective, antimicrobial, antidiabetic, antioxidant, anti-inflammatory, antiulcerogenic, and anticancer activities. However, few molecular mechanisms of action are known [16].

Given the lack of validated HPTLC protocols for quantifying Gallic acid and Quercetin in *T. indica* and *P. oleracea*, there was a need to develop specific protocols for these biomarkers in these plants. The mobile phase composition plays a crucial role in TLC protocol development. Therefore, this study focused on developing a suitable mobile phase for the separation of marker compounds. The present study introduces simple, accurate, and reproducible HPTLC protocols for quantifying Gallic acid and Quercetin in *T. indica* and *P. oleracea*.

2. MATERIALS AND METHODS

2.1 Sample Preparation

The seeds of *T. indica* and *P. oleracea* were dried, powdered and stored in air tight jars until use. 2 gm of dried powdered drug was taken in to 100 mL round bottom flask and charged with 50 mL of water by using refluxed condenser. The process was done in triplicate by adding fresh solvent. The extract was concentrated and dried residue was reconstituted using water in a 10 mL volumetric flask and used for HPTLC analysis (Amir *et al*., 2013).

2.2 Estimation of Gallic acid

2.2.1 HPTLC instrumentation

Precoated HPTLC plates were used for application of samples. Calculated quantities of every extracts were mixed together in their relevant solvent. Linomat V applicator was used in sample application. The selected and optimized solvent system for TLC study was chosen for HPTLC analysis.

2.2.2 Chromatographic conditions

The chromatographic conditions necessary to acquire an efficient resolution by selected mobile phase discussed underneath.

Stationary phase: HPTLC precoated, silica gel G 60 F254 (Merck, Germany) Size: 10x 20 cm (0.2 µm thickness)

Developing chamber: Twin trough glass chamber Mode of application: Band B and size: 4 mm

Separation technique: Linear ascending Temperature: 20 ± 50°CSaturation time: 30 min

Scanning wavelength: 254 nm / 366 nm Scanning mode: Absorbance / Reflectance

The samples were applied using syringe on precoated silica aluminum plate G60F254. The band width of 3.0 mm, space between two bands 5.0 mm, application rate of 120 nL sec^{-1} and slit 3.0 × 0.30 mm and 20 mm sec^{-1} scanning speed was employed for the analysis. The mobile phase toluene: ethyl acetate: formic acid (5: 4: 1, *v/v/v*) and 20 mL of mobile phase was used for chromatography. A current of hot air was used with the help of an air dryer to dry the TLC plate.

2.3 Estimation of Quercetin

2.3.1 HPTLC instrumentation

Precoated HPTLC plates were used for application of samples. Calculated amount of individual extracts were mixed in their respective solvent and samples were applied with the help of Linomat V applicator. The selected and optimized solvent system forTLC study was chosen for HPTLC analysis.

2.3.2 Chromatographic conditions

Following are the chromatographic conditions required to get an effective resolutions by selected mobile phase.

Stationary phase: HPTLC precoated, silica gel G 60 F254 (Merck, Germany) Size: 10x 20 cm (0.2 µm thickness)

Developing chamber: Twin trough glass chamber Mode of application: BandBand size: 4 mm

Separation technique: Linear ascending Temperature: 20 ± 50°CSaturation time: 30 min

Scanning wavelength: 254 nm / 366 nm

The samples were applied using an Automated Camag TLC applicator, Linomat V (Camag, Multenz, Switzerland) on precoated silica aluminum plate G 60 F 254. The band width of 4.0 mm, space between two bands 5.0 mm, application rate of 120 nL sec-1 and slit 3.0 × 0.30 mm and 20 mm sec^{-1} scanning speed was employed for the analysis and the positioned 15 mm from the bottom of the plate and 20 mm from side of the plate.

The mobile phase toluene: ethyl acetate: formic acid (5: 4: 1, *v/v/v*) and 20 mL of mobile phase was used for chromatography. After development, the plate was removed and dried up in a flow of hot air by means of an air dryer. Spots were visualized in Camag UV chamber (366 nm) and scanning was carried out using Camag TLC scanner III operated by WINCATS software equipped with tungsten lamp as a source of radiation.

2.4 Method Validation

ICH guidelines were used for method validation for its linearity, precision, accuracy, robustness, specificity, LOQ and LOD.

2.4.1 Specificity (Selectivity)

A superimposed UV spectrum of gallic acid and quercetin were primarily obtained from developed plate and λmax 297 nm and 366 nm were chosen as detection wavelength. In composite chromatograms, a UV spectrum of gallic acid was obtained to verify peak purity.

2.4.2 Calibration curve

A stock solution of gallic acid and quercetin (1mg/mL) was prepared in HPLC grade water and different volumes (1–20 µL) of stock solution were spotted in duplicate on TLC plate to get concentrations of 50, 100, 150, 200, 250 and 300 ng /spot of gallic acid. The developed plate was dehydrated with dryer and scanned at 297 nm 366 nm respectively. The data of peak height/area versus drug concentration were used for linear least square regression analysis and curve generation.

2.4.3 Precision

Repeatability of sample was determined by sample application and measurement of peak area for evaluating the coefficient of variability (C.V. %) for each sample. Repeatability was further carried out from the C.V. % values of the standard addition method at three different concentration levels. The intra-day and inter-day precision for the determination of gallic acid was carried out at three concentration levels of 150 and200 and 250 ng per spot and 2000, 2500 and 3000 ng per spot for quercetin.

2.4.4 Recovery studies

On the HPTLC plate the preanalyzed samples were spiked with additional 50, 100 and 150% of the standard gallic acid and in same manner for quercetin were applied in duplicates. This was done to confirm for the recovery of the gallic acid and quercetin at different levels.

2.4.5 Accuracy

The accuracy of the developed method was tested through performing recovery study by means of the assay of known added amounts of sample at four different levels (0, 50, 100 and 150%) of the standard gallic acid and quercetin. The mixtures were re-analyzed by the developed method and applied in duplicates.

2.4.6 Robustness of the method

Robustness of the method was observed by making small changes in the solvent system composition. Solvent system having different volume and composition of toluene: ethyl acetate: formic acid (5:4:1 and 6:4:1 *v/v/v*) for gallic acid and quercetin were tried. The selection of mobile phase was there for observed by considering the retention time, peak response, sensitivity, peak sharpness as well broadening and separation efficiencies. Toluene: ethyl acetate: formic acid in the combination of 5:6:1 *v/v/v* was not completely separated the analytes, while toluene: ethyl acetate: formic acid in the combination of 6:7:1 *v/v/v* produces tailing. Toluene: ethyl acetate: formic acid in a combination of 5:4:1 *v/v/v* and 6:4:1 *v/v/v* were found the best mobile phase for gallic acid and quercetin standards separation.

2.4.7 Specificity

Specificity of the proposed method was set by analyzing standard drug and sample. The spots and the peak of gallic acid in sample were recognized by comparing the *R*f and spectra of the spot along with that of standard.

2.4.8 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD is defined as the limit of detection of marker in a sample that can be analysed and LOQ is defined as limit of quantification at lowest concentration of analyte in the sample that can be determined by the proposed method under the stated experimental conditions. LOD was considered as 3:1 and LOQ as 10:1.

3. RESULTS AND DISCUSSION

3.1 Optimization of Chromatographic Conditions for HPTLC

Various solvent systems were tried for TLC analysis of gallic acid. The selection of mobile phase was therefore based by modification of TLC systems for the analysis of gallic acid. The solvent systems consisting of toluene: ethyl acetate: formic acid in ratios (5:4:1, *v/v/v*) gave dense and sharp spots with well separation. Twenty five milliliters of the mobile phase was consumed to reach the chromatogram up to 50 mm length. The optimized time for mobile phase saturation was 15 min at room temperature. Quantification of gallic acid was carried out in the absorbance mode at (A) 254 nm and (B) 255 nm at *Rf* (0.55) using deuterium and tungsten lamp (Fig. 1).

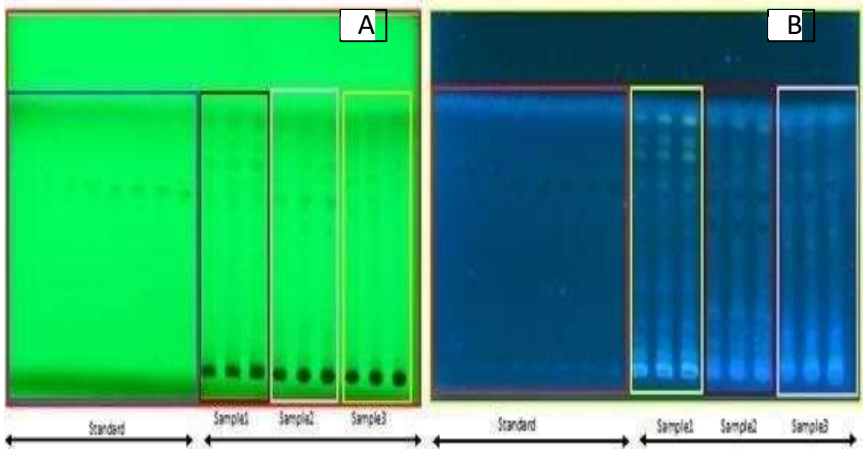

Fig. 1. Photograph of developed HPTLC plate of standard and samples; Standard: gallic acid; Sample1: *Tamarindus indica*; Sample 2 *Purslane* at (A) 254nm and (B) 255nm *Rf* (0.55) with mobile phase, toluene: ethyl acetate: formic acid (5:4:1, *v/v/v*)

3.2 Method validation Linearity of the method

The linearity of the calibration plot was in the range of 500-4000 ngspot^{-1} for the analysis of gallic acid by HPTLC method with r^2=0.552, the calibration curve obtained during analysis could be described by the linear equation y = 4.04 X + 5555, where Y is peak area and X is the concentration Table 1.

Table 1: Linear regression data for the calibration plots (n = 3) for gallic acid

Component	Concentration range ngspot^{-1}	Regression equation	r2
Gallic acid	500-4000	4.04 X + 5555	0.552

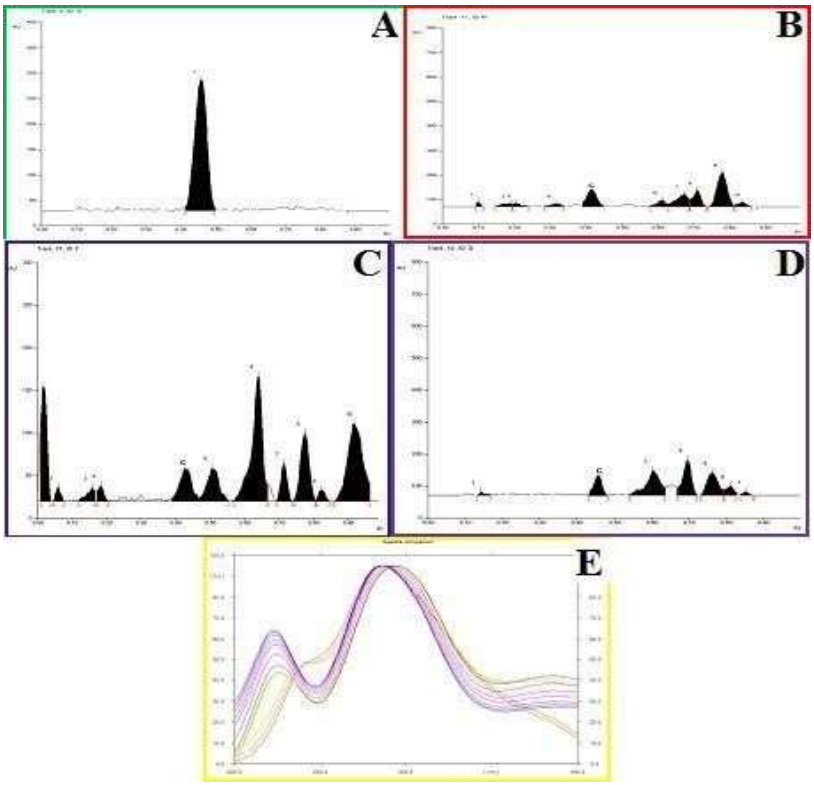

Fig. 2: HPTLC chromatogram of samples showing Galic acid peak in ethanolic extract of (A): *T.indica*(B) *Purslane* at 255 nm and *Rf* (0.55), E; Superimpose UV spectra of standard (gallic acid) with different samples at 255nm

3.3 Accuracy

Methods accuracy was based on recovery studies which were carried out by adding 0% (250 ng), 50% (420ng), 100% (550ng) and 150% (500ng) of standard drug to the pre-analysed sample and the mixtures were re-analyzed by the optimized proposed methods. The recovery was found to be within the limit of 55.25-102.15% for proposed HPTLC method. The data of % recovery and % RSD are shown in Table 2.

Table 2: Accuracy of the HPTLC method (n=3) for gallic acid

% of standard spiked to the sample	Theoretical content (ng)	Amount of drug recovered ng ± SD	% of drug recovered	% RSD
0	250	255.02 ± 2.55	102.15	0.55
50	420	415.45 ± 1.43	55.25	0.34
100	550	555.53 ± 2.55	101.23	0.50
150	500	513.55 ± 1.54	101.55	0.24

3.4 Precision

Precision study of the proposed method was carried out in accordance with the ICH recommendations. Six different spots of standard sample (at three concentrations 150, 200 and 250 $ngmL^{-1}$) were applied from a single standard solution. % RSD was calculated for HPTLC method and inter-day and intra-day precisions study were done by applying three similar concentrations of gallic acid in the same day and different days, respectively. There results were shown in Table 3.

Table 3: Inter-day and intra-day precision of the HPTLC method (n=5) for gallic acid

Amount ($ngspot^{-1}$)	Inter-day precision		Intra-day precision	
	Mean peak area ± SD	%RSD	Mean peak area ± SD	%RSD
150	2553.04 ± 15.04	0.55	3033.03 ± 12.45	0.41
200	3554.20 ± 51.35	1.34	4225.40 ± 10.55	0.25
250	4545.55 ± 44.52	0.53	5054.02 ± 5.55	0.15

SD, standard deviation; RSD, relative standard deviation

3.5 Specificity

The comparison between sample and standard peak for its *Rf* and UV spectra was made to determine the specificity of the proposed methods. Three points peak purity i.e. Peak start, peak apex, and peak end was compared and found superimposed. This indicated that the standard gallic acid peaks and sample peaks were not merging with any other components or impurities.

3.6 Robustness

A small change in the composition of mobile phase and the detection wavelength was made to check the robustness of the method. The mobile phase consists of toluene: ethyl acetate: formic acid (5:4:1*v/v/v*), hexane: ethyl acetate: formic acid (5:4:1 *v/v/v*) and toluene: ethyl acetate: formic acid (5:4:1 *v/v/v*) respectively. Chromatograms were developed at altered detection wavelength and results were observed. Good chromatogram was developed with toluene: ethyl acetate: formic acid (5:4:1 *v/v/v*) at *Rf* 0.55 and 255nm showing that the proposed chromatographic methods is robust.

3.7 LOD and LOQ

The LOD and LOQ were determined as per the formula LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve. The LOD and LOQ for developed methods were found as 5.55μgmL^{-1} and 22.55μgmL^{-1} respectively.

3.8 Estimation of gallic acid

The newly developed and validated HPTLC methods were applied for the analysis of quercetin in plants extract. The gallic acid content was found to 1.53% w/w, 1.55% w/wand 0.124% w/w in *T.indica, Purslane* respectively by using HPTLC (Fig. 3).

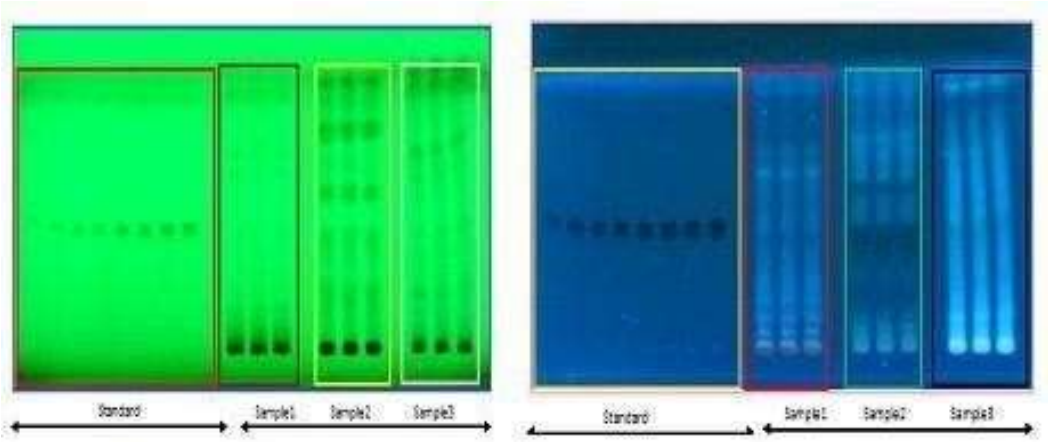

Fig. 3. Photograph of developed HPTLC plate of standard and samples; Standard: gallic acid; Sample1: *Tamarindus indica*; Sample2 : *Purslane* at (A) 254nm and (B) 355nm Rf (0.55) with mobile phase , toluene: ethyl acetate: formic acid (5:4:1, *v/v/v*)

3.9 Method validation Linearity of the method

The linearity of the calibration plot was in the range of 500-4000 ng spot^{-1} with r^2=0.550and the linearity equation was y = 2.155X + 145.350, where Y is peak area and X is theconcentration.

3.10 Accuracy

Methods accuracy was carried out by adding 0% (2000 ng), 50% (3000 ng), 100% (4000 ng) and 150% (5000 ng) of standard drug to the preanalysed sample and the mixtures were re- analyzed by the optimized proposed methods. The recovery was found to be within the limit of 55.52-100.05% for proposed HPTLC method. The dataof % recovery and % RSD are shown in (Table 4).

Table 4: Linear regression data for the calibration plots (n = 3) for quercetin

Component	Concentration range (ng/spot)	Regression equation	r2
Quercetin	500-4000	2.155X + 145.350	0.550

Table 5: Accuracy of the HPTLC method (n=3) for quercetin

% of standard spiked to the sample	Theoretical content (ng)	Amount of drug recovered ng ± SD	% of drug recovered	% RSD
0	2000	2005.33 ± 4.15	100.3	0.20
50	3000	2555.55 ± 3.05	55.52	0.10
100	4000	3555.35 ± 5.452	55.55	0.23
150	5000	5002.55 ± 10.11	100.05	0.20

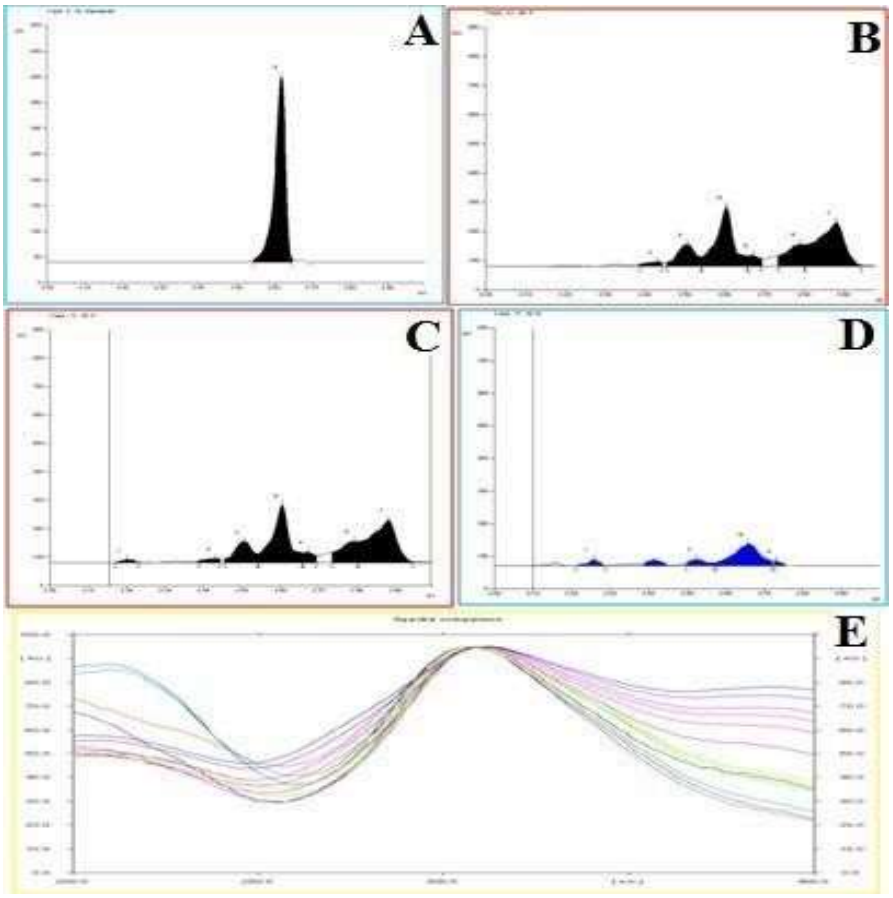

Fig. 4: HPTLC chromatogram of samples showing quercetin peak in ethanolic extract of (A): *Tamarindus indica*; (B): *Purslane* at 355nm and *Rf* (0.55), E; Superimpose UV spectra of standard (quercetin) with different samples at 355nm

3.11 Precision

Precision study was carried out in accordance with the ICH recommendations. Six different spots of standard sample (at three concentrations 2000, 2500 and 3000 $ngmL^{1}$) were applied from a single standard solution.. The inter-day and intra-day precisions study were done by applying three similar concentrations of quercetin in the same day and different days, respectively. The results were shown in Table 6.

Table 6: Inter-day and intra-day precision of the HPTLC method (n=5) for quercetin

Amount ($ngspot^{-1}$)	Inter-day precision		Intra-day precision	
	Mean peak area ± SD	%RSD	Mean peak area ± SD	%RSD
2000	15225.52 ± 20.554	0.12	15255.25 ±55.35	0.31
2500	15555.51 ± 52.15	0.32	21424.55 ±112.54	0.52
3000	20445.22 ± 33.52	0.15	22355.55 ± 55.21	0.35

SD, standard deviation; RSD, relative standard deviation

3.12 Specificity

It was determined by comparison between sample and standard peak for its *Rf* and UV spectra the specificity of the proposed methods. It indicated that the standard quercetin peaks and sample peaks were not merging with any other components or impurities.

3.13 Robustness

The robustness of the method was determined by making small changes in mobile phaseconsist of methanol: ethyl acetate: formic acid (5:5:1 *v/v/v*), hexane: ethyl acetate: formic acid (5:3:1 *v/v/v*) and toluene: ethyl acetate: formic acid (5:4:1 *v/v/v*) respectively. Good chromatogram was developed with toluene: ethyl acetate: formic acid (5:4:1 *v/v/v*) at *Rf* 0.55 and 355nm showing that the proposed chromatographic methods is robust.

3.14 LOD and LOQ

The LOD and LOQ for developed methods were found as 5.55 μgmL^{-1} and 22.55 μgmL^{-1} respectively.

3.15 Estimation of quercetin

The quercetin content was found to 1.52%, 0.143% and 0.055% w/w in *T.indica and Purslane* respectively by using HPTLC (Table 7).

Table 7: Quantification of gallic acid and quercetin in tested herbs by HPTLC

Samples	Gallic acid (%w/w)	Quercetin (% w/w)
Tamarindus indica	1.53%	1.52 %
Purslane	1.55%	0.143%

4. CONCLUSION

The methods developed in this study for the quantification of Gallic acid and Quercetin were found to be precise, specific, sensitive and accurate. These methods could be utilized for quality check of various herbal and nutraceutical preparations developed using *Tamarindus indica* and *Purslane oleracea*.

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