RESEARCH ARTICLE

Quantitative Estimation of Polyphenolic Biomarker Quercetin and Chlorogenic Acid in *Moringa oleifera* Leaves by Hyphenated High-Performance Thin-layer Chromatography (HPTLC) Techniques

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ABSTRACT

Objective: *Moringa oleifera* possesses a wide range of biological activities and treats various ailments. A specific, sensitive, selective, precise, and reproducible High-performance thin-layer chromatography (HPTLC) -UV_{290nm} method has been established and validated for instantaneous assessment of the two biomarker phytoconstituents quercetin (QC) and chlorogenic acid (CGAs) from ethanolic extract of *M. oleifera* (MO) leaves.

Methods: Chromatography was performed on aluminum foil-backed silica gel 60 F254 HPTLC plates with the binary mobile phase Toluene: Ethyl acetate: Formic acid: Methanol (5.0:4.0:1.0.5.:0.5,v/v/v/v). Ultraviolet detection was performed densitometrically at the maximum absorbance wavelength, 290nm. The method was validated for precision, recovery, robustness, specificity, and detection and quantification limits according to ICH guidelines.

Results: The HPTLC method was used to detect spots for quercetin (QC) and chlorogenic acid (CGAs) (Rf value of 0.56 and 0.37, respectively). The limit of detection (24 and 39 ng band^{-1}), limit of quantification (72 and 107 ng band^{-1}), recovery (99.5–99.1 and 98.9–99.6%), and precision (≤1.98 and 1.97) were satisfactory for quercetin (QC) and chlorogenic acid (CGAs) respectively. Linearity range for QC and CGAs were 100–1000 (r^2= 0.9995) and 150–900 ng band^{-1} (r^2= 0.9967) and the contents estimated as 0.87 ± 0.01% and 0.63 ± 0.01% w/w respectively.

Conclusion: This simple, precise and accurate method resolved the other constituents present in the extract. The method has been successfully applied in the analysis and routine quality control of herbal material and formulations containing *M. oleifera* (MO).

Keywords: Chlorogenic acid, HPTLC, *Moringa oleifera,* Quercetin, Validation.

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INTRODUCTION

Plant-derived products supported by scientific confirmation have acquired user assurance as complementary and alternative medicine (CAM) for prevention and cure disease. Traditional medicines have been used from prehistoric periods, but they have not been standardized or validated for their efficacy.[1] Trade of herbal products is rising rapidly due to the popularization of CAM, but they lag in the knowledge on active principles, validation, etc. Herbs and herbal medicines are most susceptible to variation in their phytochemical profile because of variability in atmospheric conditions, maturity, post-harvest processing, storage, stability etc.[2] It is extremely important to standardize the formulation and its ingredients, supported marker compounds specific to every plant followed by validation for their efficacy. Phytochemical evaluation is one of the tackles for the standard quality assessment, which incorporates preliminary phytochemical screening, chemo profiling and marker compound analysis using modern analytical techniques.[3] Within the last twenty years, HPTLC method has emerged as a vital role for the qualitative and quantitative phytochemical investigation of herbal drugs and formulations. HPTLC is an acquiescent and commonly used

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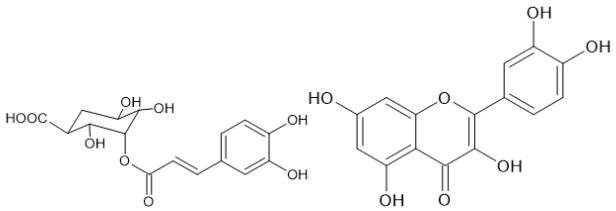
technique for qualitative and quantitative analysis of chemical markers in herbal raw materials. HPTLC has advantages of simplicity, sensitivity, accuracy and is one the foremost approached technique for developing fingerprint and marker-based standardization of herbal drugs and is typically applied not just for the identification, assay and testing for purity but also for stability, dissolution or content uniformity of raw materials and formulated products.[4]

Quercetin (QC) (3,3′,4′,5,7-pentahydroxyflavone), a common flavonol, is present as a glycoside in high fruits and vegetable apples concentrations berries, onions, and capers. QC accounts for nearly 50% of the stimulate cellular defenses.[5,6] It is one of the most widespread QC glycosides and probably occurs in up to 25% of any given local flora. It has a wide spectrum of pharmacological benefits such as antimicrobial, antifungal, antiallergic, anti-cancer, antidiabetic, and antihypertensive agent.[7] Chlorogenic acids (CGAs) are classified consistent with the character and number of cinnamic substituents and to the esterification position within the cyclohexane ring of the quinic acid.[8] 5-Caffeoylquinic acid is that the most generally occurring and most studied CGAs. CGAs have antioxidant properties, which play a vital role in protecting food, cells, and any organ from oxidative degeneration[9] and the coffee plant against microorganisms, insects, and UV radiation.[10] Moreover, CGAs contribute to stopping various diseases related to oxidative stress, further as cancer, aging, and cardiovascular and neurodegenerative diseases; besides, these substances have promising effects in modulating lipid and glucose metabolism.[11,12]

M. oleifera Lam. or munga is one of the foremost important plants widely cultivated in India. It belongs to the family Moringaceae. This plant is widely used as a nutritional herb. It contains valuable pharmacological action like anti-asthmatic, antidiabetic, hepatoprotective, anti-inflammatory, anti-fertility, anti-cancer, antimicrobial, antioxidant, cardiovascular, anti-ulcer, CNS activity, antiallergic, wound healing, analgesic, and antipyretic activity, *M. oleifera* Lam. The plant is additionally called as Horse-radish tree, Drumstick tree. Every part of this plant contains a valuable medicinal feature. It contains rich source of fat-soluble anti-ophthalmic factor, a water-soluble vitamin, and milk protein. Differing active phytoconstituents like alkaloids, protein, quinine, saponins, flavonoids, tannin, steroids, glycosides, fixed and fatty oil are present. Vital minerals in the *M. oleifera* include iron, potassium, calcium, copper, zinc, magnesium, manganese etc. Recently, a rare combination of zeatin, quercetin, β-sitosterol, kaempferol, caffeoylguinic acid isoquercetin, astragalin, and crypto-chlorogenic acid were reported to be major active components in *M. oleifera* leaves.[13,14] Isoquercetin is a strong robust natural antioxidant that possesses several potential therapeutic effects including antiasthma and anti-hypertension.[15-17] Astragalin is also a natural antioxidant agent exhibiting biological properties such as inflammation attenuation, dermatitis inhibition, and cellular protective effects.[18-20] This plant is additionally found within the tropical regions. Other constituents are niazinin A, niazinin B, and niazimicin A, niaziminin B. Presently, the literature indicates very limited methods reported for the quantitative estimation of these biomarkers present in herbs/herbal preparations. Recently, Željan *et al.,* 2013[21] reported an HPTLC method for simultaneous estimating quercitrin, isoquercitrin, hyperoside, and chlorogenic acid in herbs. Yadav *et al*., 2018[22] reported an HPTLC method employing chloroform: ethyl acetate: methanol: formic acid (5:3:1.5:0.5, v/v/v/v) as the mobile phase for simultaneous determination of quercetin, chlorogenic acid, and trigonelline in polyherbal antibacterial formulation. Hussain *et al*.,[23] also reported normal phase-HPTLC for estimation of crypto-chlorogenic acid, isoquercetin, and astragalin in extracts of *M. oleifera*. To date, there is no reported HPTLC method employing a design of experiments (DoE) approach for the simultaneous estimation of these three biomarkers in combination. Considering the non-existence of a routine analysis method, a simple HPTLC method for quantitative estimation of quercetin and chlorogenic acid simultaneously is proposed for *M. oleifera*. According to the ICH guidelines, the proposed method was validated by specificity, range, linearity, accuracy, precision, detection limit, quantitative limit, and robustness (ICH, 1996, 2005).[24]



Chlorogenic acid **Quercetin**

Figure 1 A & B: Chemical structure of QC and CGAs

MATERIALS AND METHODS

Plant Material and Chemicals

The fresh leaves of the plant of *M. oleifera* Lam. were collected from the field area of Moradabaad, UP, India in the month of January 2020; and the specimens (voucher no: NICAIR/RHMD/Consult/2020/3600-01) were authenticated by Dr. Sunita Garg (Emeritus Scientist), NICAIR New Delhi. India. Standard Chlorogenic acid (Purity: 98.7% w/w) and quercetin (purity: 98% w/w) were purchased from Natural Remedies Pvt. Ltd, Bangalore, India. All the solvents used were of chromatography grade and other chemicals used were of analytical reagent (AR) grade. Precoated silica gel 60 F_{254} HPTLC plates were purchased from E. Merck, Germany.

TLC Instrumentation and Conditions

Chromatography was performed on a 20 cm × 10 cm aluminum Lichrosphere HPTLC plate precoated with a 200 µm layer of silica gel 60F254 (E. Merck, Darmstadt, Germany) as previously described.[24-29] The samples were applied as strips 6 mm wide and 10 mm in the distance using the Camag (Muttenz, Switzerland) sample applicator Linomat V with a 100 µL syringe. The constant application rate was 160 nLs-1. Linear ascending development using Toluene: Ethyl acetate:

Formic acid: Methanol (5.0:4.0:0.5:0.5,v/v/v/v) as mobile phase was performed in a 20 cm × 10 cm double trough glass chamber (Camag). Saturation in the mobile phase was minimal at room temperature (25 ± 2°C) and relative humidity 60 ± 5%. The development distance was 8 cm (development time 10 minutes), and 20 ml of mobile phase was used. The plate was air-dried at room temperature and heated (75°C. for 5 minutes) to identify a compact band. Densitometry analysis was performed at 290 nm in reflection mode using a CAMAG TLC Scanner III running on WinCATS software (version 1.2.0). The gap dimensions were 5 mm x 0.45 mm and the scan speed was 20 mm s^{-1}.

Preparation of Standard and Quality Control

A stock solution of quercetin and chlorogenic acid (10 $mg.mL^{-1}$), prepared in ethanol and diluted appropriately to a standard in the concentration range of 0.1-1.0 mg mL^{-1}. For calibration, QC standard solution (1 - 10 μL) was applied to the HPTLC plate to provide quantities in the 100-1000 ng band $^{-1}$. However, a quality control standard solution (0.5–5 µL) CGAs was used to provide an amount in the range of 150–900 ng band $^{-1}$. The peak area and plot amount were processed by least squares linear regression. Each amount was applied 6 times. Low, medium, and high quality control samples with 50, 100, and 200 ng band $^{-1}$ concentrations were taken with CGAs, and 100, 200 and 400 were examined with CGAs to validate the method.

Extraction of Plant Material for Analysis

Leaves of the plant MO were air-dried and crushed. 500 g of the pulverized material was packed in a muslin cloth, separately subjected to a Soxhlet extractor, and continuously heat-extracted with ethanol for 72 hours. Then, the ethanolic extracts of leaves of MO filterate through Whatman Paper No. 42 and the resulting filtrate were concentrated under reduced pressure and finally vacuum dried. The yields of ethanol extract were 11.5% w / w, respectively. The sample solution preparation protocol has been optimized for high-quality fingerprints and efficient extraction of marker compounds. Since the marker compound is soluble in ethanol, ethanol was extracted. Fingerprinting of ethanol extracts of MO was performed by spotting 10 µl of a sample solution of the appropriately diluted ethanolic extract on HPTLC plates. Each amount was applied 6 times. The peak area and plot amount were processed by least squares linear regression. The plates were developed and scanned as described above. The peak area was recorded and the amount of QC and CGAs was calculated from the calibration curve.

Method Validation

Validation of the developed method has been carried out as per ICH guidelines for linearity range, precision, accuracy, limits of detection (LOD) and quantification (LOQ), and recovery.

Precision and Accuracy

Precision (inter and intraday) and accuracy of repeated analysis of quality control samples (n = 6) with QC and 50, 100, and 200 ng $band^{-1}$ at low, medium, and high concentrations of 100, 200 and 400 ng $band^{-1}$ for CGAs evaluated respectively. Inter-day precision and accuracy were determined by repeating the intra-day assay on three different days. Precision is expressed as the coefficient of variation (CV,%) of concentration measured at each calibration level, and accuracy is expressed as % recovery [(drug detected/applied drug) x 100].

Selection and Optimization of Mobile Phase (Robustness)

Robustness was tested 3 times on a 400 ng $band^{-1}$ with minor changes to mobile phase composition, mobile phase volume, mobile phase saturation time, and TLC plate activation. This will affect the results. I calculated and examined the peak area's RSD (%) and SE. Toluene: Ethyl acetate: Formic acid: Methanol (5.0:4.0:0.5:0.5, v/v/v/v).to various ratios (4.7:4.0:1.3.:1.0, v/v/v/v, 4.8:4.0:1.1.:1.1, v/v/v/v, 4.5:4.5:0.5.:0.5, v/v/v/v and 4.8:4.0:0.6.:0.6, v/v/v/v) mobile phase were used for chromatography. The amount and duration of mobile phase saturation investigated was 20 ± 2 mL (18, 20, 22 mL) and 20 ± 10 minutes (10, 20, 30 minutes), respectively. Before chromatography, the plate was activated at 60 ± 5°C for 2, 5 and 7 minutes.

Sensitivity

To estimate the detection limit (LOD) and quantification limit (LOQ), blank methanol values were plotted 6 times to determine the standard deviation (σ) of the analytical response. LOD was expressed as the slope of the 3σ / calibration graph and LOQ was expressed as 10σ/slope of the calibration plot.

Recovery Studies

Recovery was investigated by applying this method to drug samples supplemented with known amounts of markers corresponding to 50, 100, and 150% of QC and GGAs. Each level was analyzed 3 times. It was used to confirm QC and GGAs recovery at various levels of the extract. Recovery rates of markers of various concentrations in the sample were measured.

RESULTS

Chromatography

The chromatogram uses toluene: ethyl acetate: formic acid: methanol (5.0:4.0:0.5:0.5, v/v/v/v) as the mobile phase or solvent system for QC and GGAs under chamber saturation conditions (Figure 2 & 3). The same mobile phase was also used to separate ethanolic extracts of MO (Figure 4). We found that the optimized saturation time was 10 minutes. The UV spectrum measured for the spot showed maximum absorbance at about 290 nm, so densitometry analysis at 290 nm in reflection mode was performed as HPTLC. For gallic acid and quercetin, R_F 0.56 ± 0.02 and 0.37 ± 0.01 yielded sharp, symmetric, high-resolution compact bands (Figure 5).

As far as we know, there is no HPTLC method for simultaneously quantifying v/v/v/v) in ethanolic extract of *M. oleifera* herbs or extracts. Therefore, to quantify the bioactivity marker component of this herb, we attempted to develop and verify an inexpensive, simple and sober HPTLC technology with a hyphen. QC and GGAs were well resolved at R_F 0.56 and 0.37 from MO ethanolic extract samples in the solvent system, respectively, as in the standard case (Figures 2 and 3).

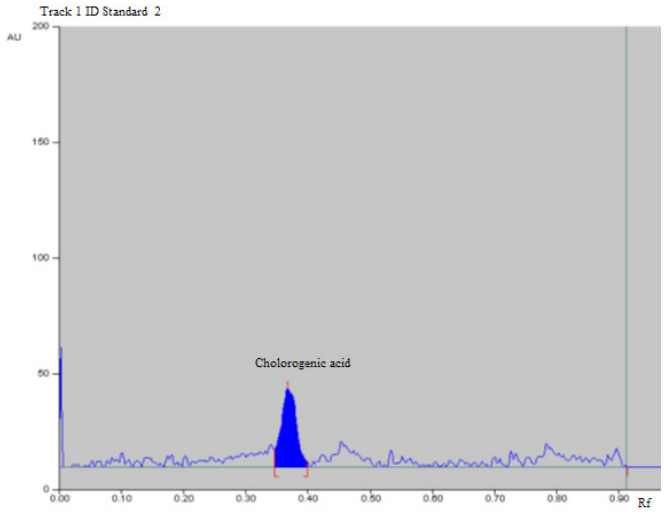


Figure 2: HPTLC chromatogram of standard Chlorogenic acid at R_F 0.37

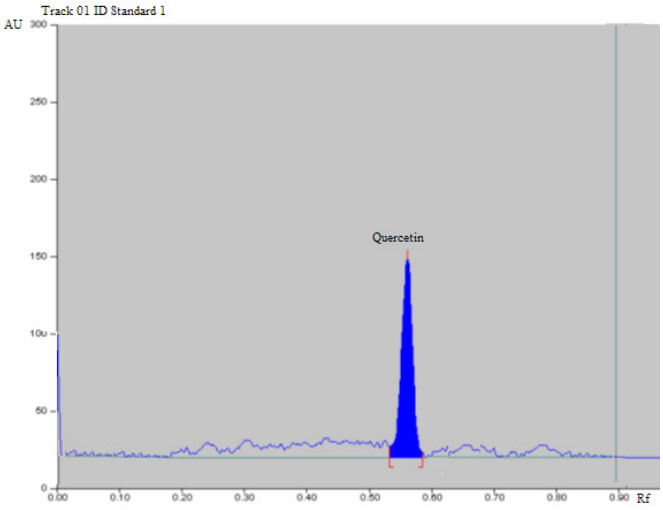


Figure 3: HPTLC chromatogram of standard Quercetin at R_F 0.56

The plate was visualized at two different wavelengths, 254 nm and 290 nm, as the compound was found to absorb in a variable range of spectra. In addition, this helped generate better fingerprint data, making it possible to better distinguish species by improving the visual identification of individual compounds. The method developed here proved to be highly selective with good baseline resolution for each compound. The sample extract's band identity of compounds 1-11 was confirmed by superimposing their UV absorption spectra on the standard spectrum at 290 nm (Table 1).

Calibration

The linearity of compounds (quercetin and chlorogenic acid) was validated by the linear regression equation and correlation coefficient. The six-point calibration curves for QC and GGAs were linear in the range of 100–1000 ng band^{-1} and 150–900 ng band^{-1}. The reference compound's regression equation and correlation coefficient were: Y=0.0049X+0.016 (r2=0.9995) for quercetin and Y=0.043-0.019 for chlorogenic acid (r2=0

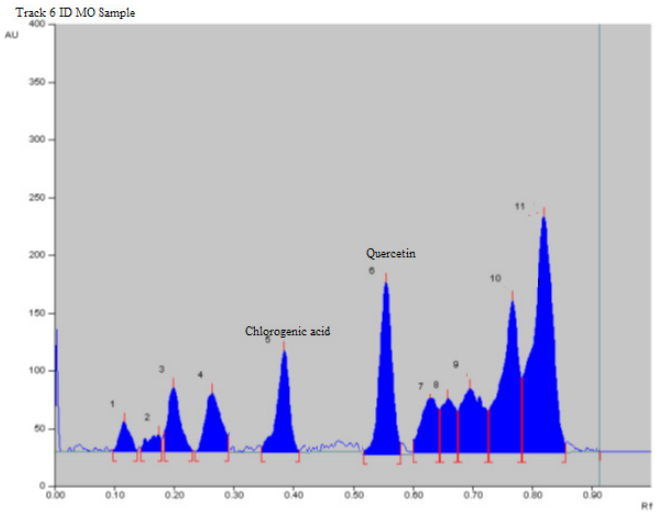


Figure 4: HPTLC chromatogram of ethanolic extract of *M. oleifera* scanned at 290 nm [peak 1-11; QC (0.56) and CGAs (0.37)]

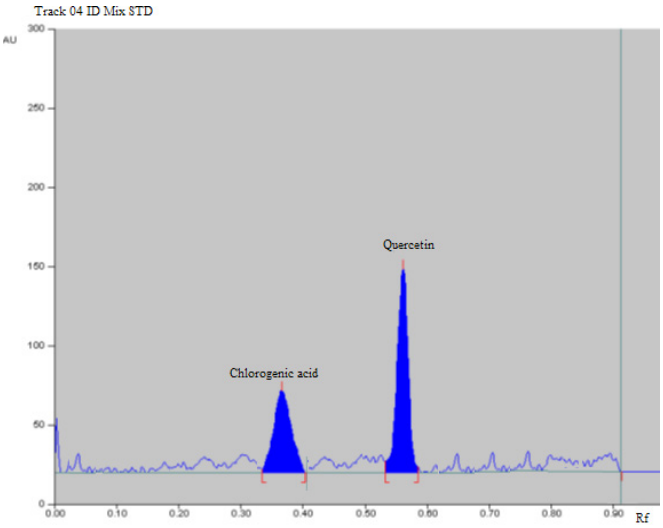


Figure 5: Chromatogram of QC and CGAs simultaneously determined in by using Toluene: Ethyl acetate: Formic acid: Methanol (5.0:4.0:1.0.5.:0.5,v/v/v/v).as solvent system scanned at 290 nm [GA (0.37) and QE (0.56)]

0.9967), which revealed a good linearity response for the developed method and are presented in Table 2. The mean values (± sd) of the slope were 0.0045 ± 0.0002 and 0.038 ± 0.005 and intercept were 0.016 ± 0.007and 0.018 ± 0.002 respectively for QC and GGAs. No significant difference was observed in the slopes of standard plots (ANOVA, $P > 0.05$).

METHOD VALIDATION

Precision and accuracy

Table 3 presents intra-day and inter-day precision (as a coefficient of variation, (%CV) and accuracy of the assay for QC and GGAs at three QC levels (150, 300, and 600 ng band^{-1}). Intra-day precisions (n = 6) for QC and GGAs were ≤1.78% and ≤1.91%. However, the inter-day precisions were ≤1.97% and ≤1.95%, respectively, which demonstrated the good precision of the proposed method. Intra-day accuracy

Table 1: TLC fingerprints of ethanolic extract of *M.oleifera* at 290 nm

Plants	*Solvent system*	*R_f value*
Moringa oleifera	Toluene: Ethyl acetate: Formic acid: Methanol (5.0:4.0:1.0.5.:0.5,v/v/v/v).	0.11, 0.16, 0.21, 0.27, 0.37, 0.56, 0.64, 0.68, 0.71, 0.76, 0.82.

Table 2 R_F, linear regression data for the calibration curve and sensitivity parameter for QC and CGAs

Parameter	*Quercetin*	*Chlorogenic acid*
R_F	0.56	0.37
Linearity range (ng band^{-1})	100 –1000	150 – 900
Regression equation	Y=0.0049X+0.016	Y=0.043-0.019
Correlation coefficient (r^2)	0.9995	0.9967
Slope ± sd	0.0045 ± 0.0002	0.038 ± 0.005
Intercept ± sd	0.016 ± 0.007	0.018 ± 0.002
Standard error of slope	0.00028	0.0036
Standard error of intercept	0.0040	0.0011
LOD	24	39
LOQ	72	107

Table 3 Precision and accuracy of the method

Quercetin				*Chlorogenic acid*			
Nominal concentration[a]	Obtained[a,b]	Precision[c]	Accuracy[d]	Nominal concentration[a]	Obtained[a,b]	Precision[c]	Accuracy[d]
Intraday batch							
150	148.3	1.78	98.9	200	197.5	1.73	98.3
300	299.1	1.65	99.2	400	398.6	1.91	99.6
600	600.4	1.55	99.7	800	801.3	1.37	100.1
Interday batch							
150	149.2	1.97	99.4	200	197.7	1.94	98.8
300	299.8	1.76	99.9	400	396.9	1.93	98.2
600	598.3	1.70	99.7	800	798.8	1.95	99.8

[a]Concentration in ng band^{-1}
[b]Mean from six determinations (n=6)
[c]Precision as coefficient of variation (CV, %) = [(standard deviation)/(concentration found)] × 100
[d]Accuracy (%) = [concentration found)/(nominal concentration)] × 100

Table 4 Robustness of the method

	Quercetin		*Chlorogenic acid*	
Optimisation condition	*SD*	*%RSD*	*SD*	*%RSD*
Mobile phase (Toluene: Ethyl acetate: Formic acid: Methanol (5.0:4.0:0.5:0.5, v/v/v/v; 4.7:4.0:1.3.:1.0, v/v/v/v; 4.8:4.0:1.1.:1.1 v/v/v/v; 4.5:4.5:0.5.:0.5, v/v/v/v and 4.8:4.0:0.6.:0.6, v/v/v/v)	1.89	1.52	1.71	1.69
Mobile-phase volume (18, 20, and 22 mL)	1.21	1.67	1.42	0.73
Duration of saturation (10, 20, and 30 min)	1.89	1.73	1.17	1.11
Activation of TLC plates (2, 5, and 7 min)	1.88	1.58	1.52	1.12

QC and GGAs were 98.9–99.7% and 98.3–100.1%. However, inter-day accuracy for QC and GGAs were 99.4–99.7% and 98.8–99.8%, respectively. These values are acceptable, so the method was accurate, reliable, and reproducible.

Robustness

SD and %RSD were calculated for QC and GGAs. The low SD and %RSD values (<2) after making small deliberate changes to the method indicated that the method was robust (Table 4).

Sensitivity

The LOD values for QC and GGAs were 24 and 39 ng band^{-1}, respectively. However, the LOQ values were 72 and 107 ng band^{-1}, respectively (Table 2), indicating sufficient assay sensitivity. LOD and LOQ were determined from the slope t the bottom of the calibration plot. This showed that the proposed method had good sensitivity for quantifying the above compounds.

Table 5: Recovery studies of QC and CGAs

Concentration added to analyte (%)	*Theoretical (ng)*	*Added (ng)*	*Detected (ng)*	*Recovery (%)*	*RSD (%)*
Quercetin					
50		200	496.9	99.5	1.92
100	300	400	698.2	99.2	1.51
150		600	897.1	99.1	1.49
Cholorogenic acid					
50		100	197.5	98.9	1.74
100	100	200	298.2	98.8	1.92
150		300	397.4	99.6	1.27

Table 6: QC and CGAs contents estimated in ethanolic extract of *M. oleifera* by developed method

	Quercetin *		*Chlorogenic acid*	
Moringa oleifera (MO)	Content (ng spot^{-1})	%RSD	Content (ng spot^{-1})	%RSD
	87.0	1.08	63.0	1.14

*Volume applied in each replicate was ten microlitres

Recovery Studies

Good recoveries were achieved by concentrating the sample at three quality control levels, QC and GGAs. The results showed in Table 5, the recovery rates for both the sample treatment and post-application are in the range of 99.5-99.1% Quercetin) and 98.9-99.6% (Chlorogenic acid).

HPTLC-UV$_{290\ nm}$ Analysis of Bioactive CGAs and QC in Ethanolic Extract of MO

The contents of quercetin and Chlorogenic acid in the ethanolic extract of *M. oleifera* were estimated according to the proposed method, and the results obtained are summarized in Table 6. The proportions of quercetin and chlorogenic acid in the extract were 0.87 and 0.63 ng spot^{-1}. For the first time, a simple, accurate, and fast HPTLC method has been developed to simultaneously quantify two bioactive compounds in *Moringa Oleifera*.

DISCUSSION

Phytochemical analysis and fingerprinting are important steps in plant identification and impurity detection.[30] HPTLC successfully separates various constituents and provides reliable, accurate, economically useful qualitative and quantitative data for various applications such as quality control and standardization of food and commercial herbal formulations. HPTLC-based fingerprints of QC and CGAs have been previously reported.[31] Here, we report for the first time the validation of an HPTLC-based, robust, sensitive and accurate method for simultaneously measuring QC and CGAs at 290 nm using a well-defined Rf value. HPTLC fingerprints of ethanolic extract of SI and SX indicate the presence of QC and CGAs, which can be quantified by the developed method. This study reports a unique method for HPTLC-based simultaneous quantification of two polyphenols, such as QC and CGAs that were productively used to standardize SI and SX. For the first time, we report the QC and CGAs levels of the ground portion of *M. oleifera*. These were previously reported to be present throughout the plant.

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