ORIGINAL ARTICLE

Estimation of quercetin by high-performance thin-layer chromatography and *in vitro* anti-inflammatory activity of *Leptadenia reticulata* (Wight & Arn.) from different parts of India

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Abstract

Background: *Leptadenia reticulata* (Wight & Arn.) has made important contributions to both traditional and modern medicine. **Aims and Objectives:** The study aims to develop and validate an high-performance thin-layer chromatography (HPTLC) method for the determination of quercetin content in *L. reticulata* collected from various parts of the country and to assess its anti-inflammatory activity using the bovine serum albumin (BSA) inhibition method. **Materials and Methods:** Five samples were obtained from different geographical locations in India, and no significant variation was found in the morpho-anatomical study. HPTLC method developed using a toluene: chloroform: ethyl acetate: formic acid (2.5: 2: 4.5: 1) mobile phase at a wavelength of 300 nm. Second, anti-inflammatory activity of *L. reticulata* extract was assessed using the BSA inhibition approach. **Results:** For the standard and sample, R_f value of quercetin (0.49) was observed. Concentration range of quercetin (60–160 ng/spot) was determined to be linear for the linearity approach, with a regression coefficient for quercetin of 0.999 and an optimal recovery of 101.70%. The limits of detection and limits of quantification were found to be 0.78 and 2.36 ng, respectively. Study revealed quercetin content differed among *L. reticulata* collected from different geographical regions. The highest and lowest quercetin contents in *L. reticulata* were detected in LR-103 and LR-099, respectively. From the results obtained, sample LR-103 showed effective anti-inflammatory activity compared to LR-099 at certain concentrations. **Conclusion:** The developed method is suitable for the detection and quantification of the lowest content of quercetin in *L. reticulata*. The obtained data indicate that geographic origin and climatic conditions may be major factors in determining the phytocompounds and biological activities of *L. reticulata* plant species.

Key words: Anti-inflammation, high-performance thin layer chromatography, India, *Leptadenia reticulata*, Quercetin

INTRODUCTION

Herbal remedies have endured for a long time because of their safety, efficacy, folklore practice, and lack of adverse reactions. Herbal plants showed compatibility with human physiological mechanisms due to their natural properties. Most of the herbal plants have been employed in formulations as antidiabetic and antioxidant agents since ancient

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times.[1] Flavonoids are a type of polyphenolic chemical present throughout the plant kingdom and are identified in approximately 300 different subtypes of flavonoids.[2] Most of them are non-toxic to humans, and most of them are widely utilized in medicine to maintain endothelial integrity.[3,4] Flavonoids have been found to reduce the prevalence of diseases such as cancer and heart disease when these substances are consumed by people.[5,6] Bioactive compound quercetin (3,3',4',5,7-pentahydroxyflavone) is a yellow pigment extracted from different edible plants.[7] Quercetin compounds have an active role in *Leptadenia reticulata* (Apocynaceae) and are used to treat different ailments such as cancer, CVS, allergies, diabetes, immunomodulatory, and anti-infective properties.[8-10] *L. reticulata*, traditionally known as "Jivanti", is a popular plant that is used as a drug in the Indian ayurvedic system of medicine and folklore practice. It is well documented in Ayurveda for its revitalizing, renewing, and lactogenic properties.[11] The availability of numerous phytocomponents in *L. reticulata*, such as quercetin, α-amyrin, β-amyrin, diosmetin, rutin, β-sitosterol, stigmasterol, triterpene alcohol, simiarenol, and apigenin, contributes to its therapeutic action.[11] A few phytocompounds such as β-sitosterol and stigmasterol compounds play effective role in milk secretions.[12] *L. reticulata*is basically found in the northern region of India and in the southern part as well. Several high-performance thin-layer chromatography (HPTLC) methods were previously reported for the detection and quantification of quercetin compounds, and different linearity range, limits of detection (LOD), and limits of quantification (LOQ) using different mobile phases (solvents and ratios).[13] However, these methods have the disadvantage of a narrow linearity range with low sensitivity. Despite the fact that earlier research has reported that quercetin was assessed and measured using analytical techniques such as HPLC and HPTLC.[8,14] However, validation parameters were not done for quercetin in *L. reticulata* using the HPTLC method.[15] Secondary metabolites are not stable in geographically collected herbal plants, according to previous studies.[15-17] However, there are limited scientific reports available on *L. reticulata* in geographical regions. Previous studies found that extrinsic factors such as climatic conditions and soil types can affect the stability of chemical contents in plants.[15,18] Method development was done based on reports in literature[19-21], which are used for herbal drug standardization. *In vivo* anti-inflammatory activity of *L. reticulata* has been shown in previous studies; however, *in vitro* assays have not been carried out.[8]

Henceforth, the purpose of this study was to develop and validate a highly sensitive HPTLC method for the determination of quercetin in herbal plant extract. The optimized HPTLC method was applied to determine the lowest quantity of quercetin compound in *L. reticulata* plant material collected from different geographical locations in India and assess the *in vitro* anti-inflammatory activity.

MATERIALS AND METHODS

Sample Collection and Identification

L. reticulata (LR) was collected from different geographical parts of India with GPS coordinates [Table 1 and Figure 1]. Certified and authenticated plant material by a taxonomist. A further voucher specimen was deposited in the ICMR-NITM herbarium having voucher number: RMRC 1156.

Materials

Fluka provided the quercetin (98% w/w). Required analytical grade chemicals such as toluene, chloroform, ethyl acetate, formic acid, and methanol were procured (Merck, Life Science). Aluminum thin-layer chromatography (TLC) plate (Merck) having a coating of 0.2 mm layer of silica 60 F_{254} (20 × 20 cm) was purchased from a local vendor (Hubli, India). Phosphate buffered saline (PBS) composition (NaCl, KCl, Na_2HPO_4 and KH_2PO_4) and bovine serum albumin (BSA) protein were procured from Hi-media (Mumbai) and Genei (Bangalore).

Standard and Sample Preparation

A stock solution of quercetin (standard) is prepared by diluting 1 mg/mL in a volumetric flask. Prepared solutions were filtered through a Whatman filter paper before using on a TLC plate. Plant leaves of *L. reticulata* were dried at 40–50°C for 7 days. Next using a mixer grinder, the dried leaves were powdered. In the second step, sample powder of *L. reticulata* (15 g) was dissolved in 255 mL of extraction solvent 80% methanol and kept in rotary shaker (New Brunswick™, USA) for 72 h at normal temperature (37 ± 3°C). Likewise, prepared

Table 1: Sample (LR) collection details

Accession number	Cultivation region	Geographical region	Latitude (N)	Longitude (E)
LR-099	Hyderabad (Telangana)	South-central	17.3850°	78.4867°
LR-111	Deodar (Gujarat)	Western	24.1095°	71.7771°
LR-129	Belagavi (Karnataka)	South-western	15.8497°	74.4977°
LR-103	Bathinda (Punjab)	North-western	30.2110°	74.9455°
LR-133	Baran (Rajasthan)	Northern	25.2474°	77.1528°

LR: *L. reticulata, L. reticulata: Leptadenia reticulata*

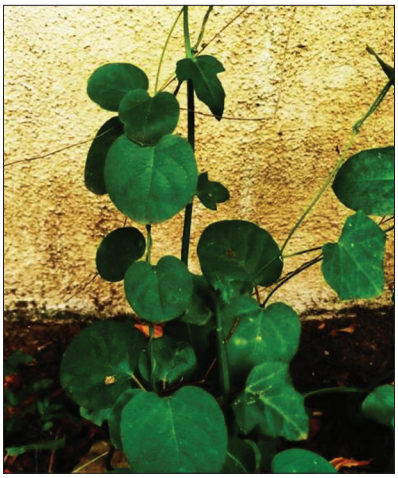

Figure 1: Fresh leaves of *Leptadenia reticulata*

stock solutions were filtered using a Whatman filter paper and applied on a TLC plate. For further analysis, sticky methanolic extract (10 mg/mL) was dissolved in Methanol.

HPTLC Apparatus and Chromatographic Conditions

HPTLC chromatography method was established following the method previously described for extracts.[22] Next, the samples were applied in 8-mm wide bands (using Hamilton microliter syringe) on a pre-coated TLC silica gel aluminum plate (E. Merck, Darmstadt, Germany) with the help of a Linomat V (*CAMAG*, Muttenz, Switzerland). For further processing, the slit dimension was kept constant at 6 × 0.45 mm, and the scan speed was adjusted (20 mm/s), said parameters were kept constant throughout the procedure. The mobile phase consisted of toluene: chloroform: ethyl acetate: formic acid in different ratios (2.5: 2: 4.5: 1, v/v/v/v). Further plates were prepared in mounting order using a CAMAG twin trough chamber that had been initially saturated with the mobile phase for about 20 min. Next, further samples were run for 7 cm, and TLC was done at a temperature of 25 ± 2°C, basically maintained at a relative humidity of 60%. Further plate was normally air-dried; next, the spectrum was monitored at 300 nm using a *CAMAG* TLC scanner IV and vision cats-software (version 3.1). Deuterium light was used as a radiation source. Using various solvents with polarity, the composition of the TLC mobile phase was optimized, and toluene: chloroform: ethyl acetate: formic acid (2.5: 2: 4.5: 1, v/v/v/v) was used as the mobile phase to achieve the high resolution. R_f value of quercetin was determined and found to be 0.49. The selected wave length was 300 nm, which corresponded to the absorption maxima of the quercetin spot.

HPTLC Method Validation

The established approach has been validated as per guidelines.[23] Further validation is performed to ensure that it is appropriate for its intended purpose. The method validation is done to assess the method's quality, reliability, and consistency. Furthermore, the method parameters, including linearity, precision, accuracy, LOD, LOQ, and recovery, were all determined.[24-28]

Linearity

For linearity, a standard methanol solution of quercetin was prepared with a concentration of 40 µg/mL. To achieve a final concentration of 60–160 ng/spot for quercetin, different volumes of standard solution in the range of 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 µL (n = 6) were applied to the TLC plate in triplicate. One plate was prepared, and the data of peak area versus drug concentration were analyzed using linear least squares regression to generate a regression equation.[29]

LOD and LOQ

LOD and LOQ were calculated using the following formula:
LOD = 3×N/B;
LOQ = 10×N/B
(N = Standard deviation of area of standard; n = 6, used to calculate noise; B = Slope of calibration plot).

Precision and Accuracy

Intra-day precision and accuracy were evaluated using triplicate analyses (n = 6) of QC samples (80, 100, and 120 ng/spot). Likewise, inter-day precision and accuracy were assessed by repeating the intra-day procedure in triplicate. For each calibration level, precision was measured by confirming the coefficient variation (CV, %) of different concentrations, while accuracy was determined and expressed as the percent recovery.

Specificity

Established method specificity was determined by analyzing the band of a standard quercetin solution. Comparison of the R_f values and spectra of the band with standard solution quercetin confirms the presence of quercetin in the samples. Further developed chromatograms and plates are scanned and checked for overlap in the spectra and R_f value.

Robustness

The robustness of the method was estimated after making minor changes in chromatographic conditions such as mobile phase, saturation time, composition, and volume. In the precise method, adjustments must be <2% of the % relative standard deviation (RSD).

In-vitro Anti-inflammatory Activity

Inhibition of protein denaturation

The effect of a methanolic extract of *L. reticulata* on inflammation was described using the following protocol.[30]

A volume of 250 µL of methanolic extracts or quercetin (standard) at different concentrations (12.5, 25, 50, 100, 200, and 400 µg/mL) was homogenized with 250 µL of PBS (pH 6.4) including BSA (1%) and incubated at 37°C for 15 min. A mixture of PBS and BSA was used as a control tube. The mixture was placed in a water bath at 70°C for 10 min, which caused the proteins to become denatured. The combination was cooled at room temperature, and each activity of the mixtures was measured at 660 nm using a microtiter reader (Epoch, Bio Tek, USA). Three attempts were made at each test. Inhibition percentage was obtained using the formula below.

% Inhibition = (Absorbance of control − Absorbance of sample)/Absorbance of control × 100

RESULTS AND DISCUSSION

Method Development and Validation

After scanning the plate using ultraviolet light, 300 nm was identified as the spectrum. Several trails later, the mobile phase was thoughtfully chosen to be toluene: chloroform: ethyl acetate: formic acid (2.5: 2: 4.5: 1, v/v/v/v), which showed clear separation for the standard and sample, and spectrum was recorded, which is shown in Figure 2. Further, developed HPTLC method was validated for the following parameter at a wavelength of 300 nm.

Calibration Curve

The linearity was determined to be in the 60–160 ng/band range, with a correlation value of 0.999 and a coefficient of variation of 0.40%. Data for linear regression are described in Table 2, and the calibrated curve is shown in Figure 3.

LOD and LOQ

LOD and LOQ were determined to be 0.78 ng/band and 2.36 ng/band, respectively, using the method described in the prior procedure. The findings showed that the new method was better than existing methods on its lowest LOD and LOQ and proved to be very sensitive.[31-33]

Precision and Accuracy

The coefficient of variation (CV, %) was used to measure the repeatability and intermediate precision and accuracy described at quercetin concentrations of 80, 100, and 120 ng/spot in Table 3. It was observed that the intra-day and inter-day precisions were, respectively, 1.87% and 1.98%. Six samples of quercetin at the same concentration and under the same experimental circumstances were tested

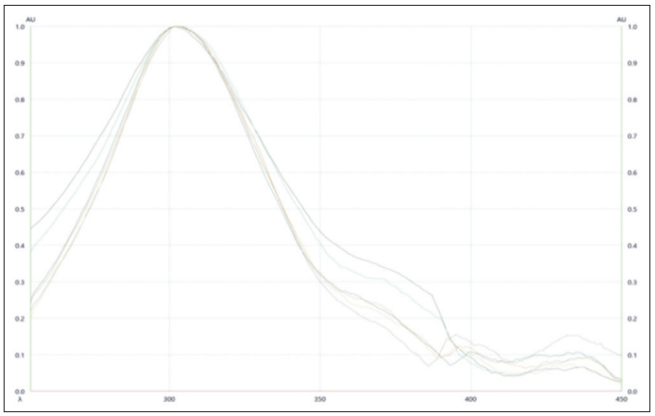

Figure 2: Absorption spectra of quercetin and plant samples of *Leptadenia reticulata*

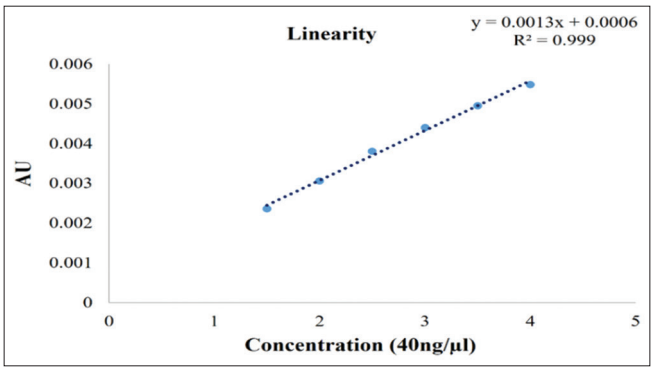

Figure 3: Calibration curve for quercetin

Table 2: Analytical method validation parameters for quercetin (*N*=6)

Parameters	Results
Linearity range (ng/spot)	60–160
Retention factor (R_f)	0.49
Correlation coefficient (R^2)	0.999
Regression equation	y=0.0013x+0.0006
Slope	0.0013
SD of intercept	0.000307
LOD (ng/spot)	0.78
LOQ (ng/spot)	2.36

SD: Standard deviation, LOD: Limits of detection, LOQ: Limits of quantification

to determine the repeatability method. The values were found to be within the acceptable range, which led to the conclusion that the method was accurate, dependable, and reproducible [Table 3].

Recovery

After using the suggested approach to estimate quercetin after spiking with 80, 100, and 120% added drugs, a recovery for quercetin ranging from 95% to 105% was observed, as

shown in Table 4. The RSD for quercetin recovery ranged from 0.75 to 2.5 [Table 4].

Specificity

Following the application of the specificity parameter, it was discovered that the R_f value (0.49) and position of the quercetin (in standard and sample) were equivalent. The chromatograms for the standard and sample have been produced and are shown in Figure 4.

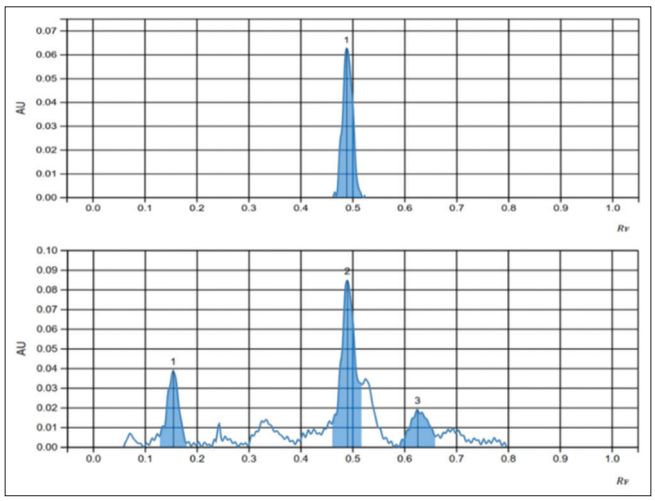

Figure 4: Thin-layer chromatography chromatogram of quercetin (A: Standard; B: Sample)

Robustness of the Method

The standard deviation of the peak portions for each parameter was calculated, and the RSD was found to be within an acceptable range. Lowest percentage RSD readings obtained after making slight deliberate adjustments to the established HPTLC method demonstrated the method's robustness [Table 5].

Quantification of Quercetin in *L. reticulata*

The validated method was used to explore the quercetin content in the leaves of *L. reticulata*. Quercetin content was measured and estimated in *L. reticulata* samples [Table 6 and Figure 5]. The quercetin content range identified was 6.71–13.86 mg/g among five *L. reticulata* accessions collected from five different divisions of India. The highest content of quercetin (13.86 mg/g) was observed in the Bathinda (Punjab) region (LR-103), and the lowest content (6.71 mg/g) was observed in the Hyderabad (Telangana) region (LR-103) [Table 6]. Despite the fact that quercetin was found in the *L. reticulata* fraction using HPTLC and HPLC approaches.[8,14] Nevertheless, method development for quercetin, which is found in *L. reticulata*, was performed; however, method validation parameters have yet to be defined. In this study, all validation parameters for quercetin, notably LOD, LOQ, accuracy, recovery, and robustness, were confirmed. In another scenario, variations in the chemical profiles of plants may be altered by differences in biotic

Table 3: Observed data of intra-day and inter-day variability data for the analysis of Quercetin

Standard marker	Concentration (ng)	Intra-day		Inter-day	
		Percentage RSD	Mean RSD	Percentage RSD	Mean RSD
Quercetin	80	1.87	1.63	1.98	1.87
	100	1.59		1.84	
	120	1.44		1.79	

RSD: Relative standard deviation

Table 4: Recovery data of quercetin and sample

Sample area	Standard area (%)	Spiked area	Recovery (%)	Average (%)	RSD (%)
0.00143	0.00034 (80)	0.00187	105	101.70	1.32
	0.00100 (100)	0.00254	104		0.75
	0.00194 (120)	0.00322	95		2.5

RSD: Relative standard deviation

Table 5: Robustness method (*N*=3) for quercetin

Quercetin Amount (ng/spot)	Mobile phase composition	
	Toluene: chloroform: ethyl acetate: formic acid (2.5:2:4.5:1) % RSD	Toluene: chloroform: ethyl acetate: formic acid (2:2:5:1) % RSD
100	1.59	1.92
120	1.44	1.01

RSD: Relative standard deviation

(soil microflora-associated species) and abiotic factors (soil micronutrients, temperature, soil pH, and so on).[17] In this research, the quercetin content assessed in *L. reticulata* and observed a significant difference in quercetin compound. Similar research was carried out for *Gloriosa superba* and identified the significant geographical differences of colchicine compound in *G. superba*. In addition, a study was executed on *Curcuma longa* and noted considerable topographical differences in curcumin.[15-16] According to the literature survey, the chemical disparity in *L .reticulata* from various regions of India has not yet been recorded. As a result, this research will encourage the use of *L. reticulata* in the production of herbal products.

Effect of Protein Denaturation

Methanolic extracts of *L. reticulata* samples and quercetin were able to inhibit protein denaturation in a concentration-dependent manner, and the inhibitory effect of different *L. reticulata* samples at different concentrations (12.5–400 µg/mL) on inhibition of protein denaturation is shown Figure 6. Inhibition % of protein denaturation of *L. reticulata* samples and quercetin were observed within the range from 7.8% to 85.46% and 41.35% to 95.56% at the concentration range of 12.5–400 µg/mL. Protein inhibition activity of quercetin was noticed in the effective range, and in *L. reticulata* samples, LR-103 (Bathinda) showed the highest activity compared to LR-099 (Hyderabad) [Tables 6 and Figure 6]. The study concluded that quercetin and *L. reticulata* both showed notable performance against inflammation [Table 7]. The result showed that plant activity can be affected by geographic origins and edaphic influences.

Table 6: Content of quercetin in LR from geographical regions

Accession number	Geographical cultivation region	Quercetin content (mg/g)
LR-099	Hyderabad (Telangana)	6.71
LR-111	Diyodar (Gujarat)	10.63
LR-129	Belagavi (Karnataka)	7.44
LR-103	Bathinda (Punjab)	13.86
LR-133	Baran (Rajasthan)	11.02

L. reticulata: Leptadenia reticulata, LR: *L. reticulata*

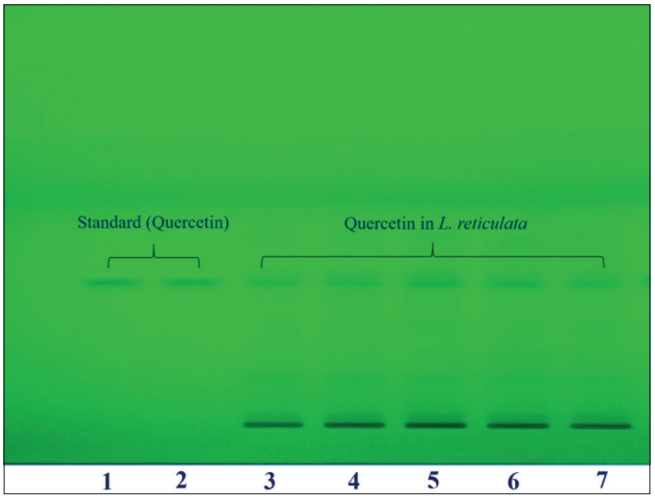

Figure 5: Developed high-performance thin-layer chromatography plate. Lanes 1-2: Standard (quercetin); Lanes 3-7: *Leptadenia reticulata* samples (LR-099, LR-111, LR-129, LR-103, and LR-133)

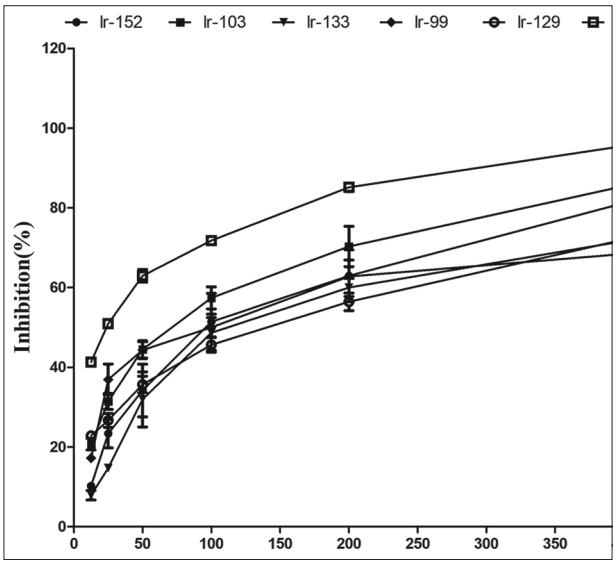

Figure 6: Effects of five *Leptadenia reticulata* samples and quercetin on protein denaturation

Table 7: Effect of methanolic extract of LR leaves and quercetin on protein denaturation

	Percentage inhibition (mean±SD)					
Concentration (µg/mL)	**LR-152**	**LR-103**	**LR-133**	**LR-99**	**LR-129**	**Quercetin**
400	81.22±21.75	85.46±3.67	71.7±13.75	68.45±1.81	72.06±2.28	95.56±1.38
200	62.94±11.29	70.31±8.78	60.03±3.9	62.78±7.18	56.43±3.84	85.16±1.79
100	51.42±12.40	57.43±4.79	48.64±8.14	50.04±4.23	45.63±3.16	71.79±1.31
50	34.17±11.44	44.50±3.74	31.96±11.98	44.32±3.7	35.69±3.61	62.95±2.79
25	23.39±6.26	31.44±3.74	14.73±1.35	36.97±6.7	26.69±3.03	50.96±1.27
12.5	10.25±1.05	20.69±2.51	7.89±2.08	17.21±1.08	22.84±0.4	41.35±1.38
IC_{50} (µg/mL)	126.64±15.47	66.23±7.18	137.71±10.43	93.1±9.75	118.4±10.11	22.89±1.98

LR: *L. reticulata*, *L. reticulata*: *Leptadenia reticulata*, SD: Standard deviation

A similar investigation was done for *Cyclocarya paliurus*, which revealed variations in activity.[34] The geographically determined plant can therefore be utilized to achieve more efficient performance.

CONCLUSION

A simple, sensitive, reliable, and accurate HPTLC method was developed and validated in accordance with ICH recommendations for quantifying quercetin in Indian wild accessions of *L. reticulata*. According to the findings of the HPTLC experiment, validated method was found to be superior to existing methods at its lowest LOD and LOQ. The variations in quercetin content obtained in *L. reticulata* collected from different regions of India may be attributed to its altitude, which is influenced by different factors, including climatic circumstances and other edaphic factors. Developed method might well be utilized to determine the lowest range of quercetin content in *L. reticulata*. According to protein denaturation assay, anti-inflammatory activity of *L. reticulata* was effective. The study's findings showed that climatic conditions may play an essential role in the characterization of phytochemicals and biological activities of *L. reticulata*. The study might be beneficial for traditional medicine.

AUTHORS' CONTRIBUTION

Plant sample collection, laboratory work, and the manuscript are done by Roshan Kumar Sharma. The interpretation, analysis, and review of the paper were assisted by Sunil S. Jalalpure, Rabinarayan Acharya, B. S. Prasad, Satisha Hegde, Bhaskar Kurangi, and Umesh Patil. The final manuscript was read and approved by all authors.

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