ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH
INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 18, Issue 5, 2025

Online - 2455-3891
Print - 0974-2441

Research Article

EVALUATING AND QUANTIFYING THE BIOACTIVE POTENTIAL OF *HYGROPHILA SPINOSA*

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Received: 02 March 2025, Revised and Accepted: 13 April 2025

ABSTRACT

Objectives: The quantification of Quercetin (QR) and Lupeol (LU) in the seed extract of *Hygrophila spinosa* has been accomplished using a highly efficient, rapid, and precise high-performance thin-layer chromatographic (HPTLC) method.

Methods: This advanced technique employed a carefully optimized mobile phase consisting of toluene, ethyl acetate, methanol, and formic acid in a precise ratio of 5:4:2:0.5 (v/v/v/v) to meticulously elute the QR and LU markers from the extract on silica gel 60 F254 HPTLC plates, measuring 10×10 cm. Detection was conducted at a wavelength of 236 nm, ensuring accurate identification of the compounds.

Results: Results revealed that the hydroalcoholic extract of *H. spinosa* contains substantial quantities of QR and LU, quantified at 17.0 mg/100 g and 17.4 mg/100 g, respectively. The robustness of the method has been thoroughly validated, demonstrating exceptional linearity, accuracy, precision, and specificity, as well as acceptable limits for both the limit of detection (LOD) and the limit of quantification. The calibration curve displayed remarkable linearity between 100 and 600 ng/band for both QR and LU, with a limit of quantitation of 4.97 ng/band for QR and 3.49 ng/band for LU, and LODs of 1.64 ng/band for QR and 1.15 ng/band for LU.

Conclusion: This innovative method represents a significant advancement in the quantification of valuable bioactive compounds. It can be confidently applied to measure QR and LU levels in *H. spinosa* seed extracts and related formulations, thereby contributing to the advancement of research and applications in phytochemistry and natural product development.

Keywords: Phytochemical, Validation, High-performance thin-layer chromatographic, Quercetin, Lupeol, *Hygrophila spinosa*.

DOI: http://dx.doi.org/10.22159/ajpcr.2025v18i5.54283. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

The plant *Hygrophila spinosa*, also known as Ikshura and Kokilasha, is scientifically classified as *H. spinosa* T. Anders. It is sometimes referred to as *Asteracantha longifolia* (L.) Nees or *Hygrophila auriculata* [1,2]. In Hindi, it is commonly called Gokshur and Gorimitaku [3,4]. The plant's leaves are soft, tasteless, and slightly bitter, arranged in whorls of six per node, with the outer two being larger than the inner ones. The stem has a sparse subhispid hairy texture and is subquadrangular at the thick nodes. The plant flowers and bears fruit between August and March [5,6].

Seeds of *H. spinosa* contain essential fatty acids such as oleic, linoleic, palmitic, uronic, and stearic acids. The whole plant extract has been found to contain aliphatic esters such as 25-oxo-hentriacotyl acetate and methyl-8-n-hexyltetracosanoate [7]. In addition, a hot ethanol extract from the aerial parts of the plant produces compounds such as 3-acetoxyurs-18-ene and 16-hydroxyl-26-methyleptacosan-2-one [8]. The leaves contain several bioactive compounds, including phenolics, glycosides, proteins, alkaloids, steroids, tannins, carbohydrates, oils, and lipids. The flowers also feature compounds such as 7-O-glucoside and 7-O-glucuronide [9].

A study by Patra A. analyzed the phytochemical composition of *H. spinosa* leaves using different extraction methods [10]. The plant is recognized for its high linoleic acid and glycoside content, which are believed to aid in treating various medical conditions [11,12]. One key bioactive compound, Quercetin (QR), identified as 3,3',4',5,7-pentahydroxyflavone, is widely studied for its potential to manage inflammatory and metabolic diseases [13], as well as its effects on neurological disorders [14], cancer [15,16], obesity, diabetes [17], calcium homeostasis [18], and asthma [19]. Another significant compound, Lupeol (LU), has been linked to potential benefits in treating neurological and cardiovascular disorders [20-22], along with exhibiting anti-inflammatory [23], antidiabetic [24], and antiviral properties [25].

Several researchers have worked on isolating and quantifying QR and LU using analytical techniques such as high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC). Shailajan S. and colleagues developed and validated an HPTLC method to quantify four pharmacologically active markers – QR, kaempferol, β-sitosterol, and LU – using a mobile phase of cyclohexane: ethyl acetate: formic acid (6:4.5:0.2 v/v/v) [26]. Similarly, HPTLC was employed to analyze the ethanolic extract of *Ficus glomerata* root for QR and LU using a mobile phase of toluene: methanol (9:1 % v/v) at 525 nm [27]. Another study examined the quantification of LU in *Andrographis echioides* leaf extract with a mobile phase of n-hexane: ethyl acetate (70:30 v/v) at an Rf of 0.55, using a 20-min saturation time [28].

Further studies on the methanolic fraction of *Ichnocarpus frutescens* extract identified ursolic acid, β-sitosterol, LU, and QR using toluene: ethyl acetate: formic acid (8:2:0.1, v/v) mobile phase. The compounds were detected at 650 nm and 310 nm, with concentrations of 0.27% for lupeol and 0.26% for quercetin [29]. The present study utilized HPTLC to quantitatively assess QR and LU in the hydroalcoholic extract of *H. spinosa* seeds. The method was validated following the guidelines set by the International Council for Harmonisation (ICH), assessing system specificity, limit of detection (LOD), and limit of quantification (LOQ).

HPTLC is preferable to HPLC because of a minimum amount of solvent consumption, low operational costs, easy sample preparation, and flexibility in the saturation phase, while HPLC has some limitations such as cost intensity for equipment, solvents, columns, and regular maintenance. For quality control purposes, the described method for quantifying QR and LU can be applied across various formulations to ensure consistent and effective dosages of these bioactive compounds. This approach enables the precise measurement of these constituents in herbal products, dietary supplements, and pharmaceutical formulations containing *H. spinosa* or other QR and LU sources. By adhering to rigorous HPTLC standards, manufacturers can guarantee product efficacy and safety while providing reliable data to consumers and regulatory bodies. Furthermore, this method is valuable for evaluating the stability and purity of formulations over time for knowing the shelf life and storage condition of formulations. Also, *in-vivo* study of the quantification of quercetin and lupeol will help determine the dose for exerting therapeutic effects and their pharmacological studies, which evaluate combined effects of quercetin, lupeol, and other phytochemical compounds. In this study, the content of these compounds alone may not exert therapeutic effects, as suggested by comparisons with the literature. However, combining these compounds or increasing their doses could enhance their biological activity.

Historically, the simultaneous quantification of QR and LU in *H. spinosa* hydroalcoholic seed extract has not been extensively documented. This study introduces a novel HPTLC method designed to identify and quantify both compounds within the extract. The findings revealed variations in the concentration of these phytoconstituents across different extracts, highlighting the method's effectiveness and potential for further research. These advancements enhance our understanding of the therapeutic potential of *H. spinosa* and pave the way for future studies exploring its health benefits and applications.

METHODS

Chemicals and reagents

QR and LU were sourced from Sigma-Aldrich (USA) and used as reference standards. Methanol, toluene, formic acid, and ethyl acetate were procured from Merck Chemicals, Mumbai, India. All other chemicals employed in the study were of analytical grade. Aluminum-backed TLC plates were sourced from E. Merck (Germany) and were pre-coated with a 0.2 mm layer of silica gel 60 F254, measuring 10×10 cm.

Preliminary studies of seed extract

Kisalaya Herbal Ltd. in Indore, India, provided a gift sample of *HS* seed extract (HSE). This extract was used as it is for this work. A preliminary pharmacognostic analysis of the seed extract was conducted to detect the presence of alkaloids, glycosides, saponins, phytosterols, phenols, flavonoids, and diterpenes [30].

Determination of total phenolic and flavonoid content

Determination of total phenolic content

To determine the total phenolic content in HSE, gallic acid was used as the standard. A stock solution of gallic acid was prepared in distilled water at a concentration of 100 μg/mL. Appropriate dilutions of gallic acid were then made, ranging from 5 to 30 μg/mL, and handled in the same manner as the sample. The sample stock solution was prepared at a concentration of 1000 μg/mL in distilled water. For quantifying polyphenols, 1 mL of Folin–Ciocalteu reagent (diluted with distilled water at a ratio of 10:100 v/v) was added to 0.2 mL of the standard or sample extract, followed by the addition of 3 mL of a 10% sodium carbonate solution in a test tube. The mixture was shaken vigorously and then allowed to stand undisturbed at room temperature for 30 min to complete the reaction. The absorbance was recorded at 765 nm using a spectrophotometer. A calibration curve was plotted based on the absorbance of the standard gallic acid across the concentration range, and the total phenolic content in the extract was calculated in terms of gallic acid equivalent (GAE) [31].

Determination of flavonoids

In this method, a rutin stock solution was prepared in methanol at a concentration of 1000 μg/mL. Dilutions were then made to achieve concentrations in the range of 100–600 μg/mL, along with a sample stock solution of 1000 μg/mL. In a test tube, 0.3 mL of a 10% $AlCl_3$ methanolic solution was mixed with 1 mL of the diluted standard solutions of rutin at various concentrations, as well as with the sample stock solution. The mixture was allowed to stand for 60 min at room temperature. The absorbance was measured at 570 nm, and a calibration curve was utilized to calculate the total flavonoid content in terms of mg/g of rutin equivalent to the dry extract sample, where y represents the absorbance and x denotes the concentration of rutin [32].

HPTLC method development for QR and LU

Preparation of standard solution

A precise amount of 10 mg of both QR and LU was weighed and placed in separate 10 mL volumetric flasks. The volume was then increased to 5 mL with methanol by adding 5 mL of methanol and sonicating the mixture for 10 min, resulting in a solution concentration of 1000 μg/mL. From this stock solution, further dilutions were performed to obtain a concentration of 100 μg/mL, which was used for analysis.

Sample preparations

HSE extract, weighing precisely 50 mg, was dissolved in a 100 mL volumetric flask. To this, 25 mL of water was added, and the mixture was sonicated for 10 min. After sonication, an additional 75 mL of water was added to the mixture to achieve a final volume of 100 mL, resulting in a stock solution with a concentration of 500 μg/mL. The solution was then filtered using Whatman filter paper with a 0.45 μm pore size.

HPTLC instrumentation and chromatographic conditions

To optimize the HPTLC method, the TLC plate was pre-conditioned by placing it in methanol within a twin trough chamber and allowing it to air dry. The equipment used included a Camag HPTLC system, which consisted of a UV chamber, a twin trough chamber (measuring either 10×20 cm or 20×20 cm), a saturation pad, and a TLC Scanner 3 equipped with WinCATS 1.2.2 software from Camag, based in Muttenz, Switzerland. For the analysis, standards and samples were applied as bands measuring 6 mm in width onto pretreated silica gel 60 F254 (E. Merck, Germany) because It is more versatile stationary phase which is suitable for separating compounds like polar, nonpolar and partially polar substance and also it is used by previously reported results, using a Camag Linomat and a Camag microliter syringe. The plates were then placed in the twin trough glass chamber, where separation was allowed to occur through linear ascending development, covering a distance of up to 80 mm. A mixture of different solvents was employed to separate QR and LU. After development, the layers were dried and analyzed with a densitometer, maintaining a scanning speed of 20 mm/s and a constant slit size of 5×0.45 mm at wavelengths of 236, 330, and 344 nm. Both active compounds were well resolved, producing sharp peaks when analysed using a mobile phase composed of toluene, ethyl acetate, methanol, and formic acid in a ratio of 5:4:2:0.5 (v/v/v/v) at a detection wavelength of 236 nm with saturation time 15 min at room temperature (25°C±2°C) and 50±5% relative humidity.

Calibration curve for standard QR and LU

A standard solution of QR and LU, with concentrations ranging from 100 to 600 ng/band, was applied in triplicate on a pre-conditioned HPTLC plate. The plate was then developed and scanned under optimized chromatographic conditions. The peak areas were recorded, and calibration curves for QR and LU were prepared by plotting the peak area against the respective concentrations of QR and LU.

Validation of the method

According to the ICH Q2 (R1) guideline, the HPTLC method has been validated for accuracy, precision, LOD, LOQ, and specificity [33].

RESULTS AND DISCUSSION

Primary phytochemical assessment

The different parts of the plant contain various constituents, and the hydroalcoholic seed extract was qualitatively analyzed for the presence of flavonoids, phenolics, alkaloids, glycosides, saponins, phytosterols, and diterpenes.

Total phenol content

The total phenolic content of the seed extracts is quantified in relation to GAEs. A calibration graph for gallic acid was prepared using standard dilutions, and the linear regression equation Y = 0.0075x + 0.0849 (where r^2 = 0.999) was derived from the graph. In this equation, Y represents absorbance, and X represents the amount of gallic acid in micrograms. The hydroalcoholic extract demonstrated a phenolic content of 70 mg/g of GAE.

Total flavonoid content

The total flavonoid content of the seed extracts was determined using rutin as the standard. A linear regression equation was established: Y=0.0003x+0.0143, with an r^2 value of 0.997, based on the standard plot of rutin. In this equation, Y represents absorbance, and X represents the amount of rutin in micrograms. The flavonoid content found in the hydroalcoholic extract was 442 mg of Rutin Equivalent per gram.

Optimization of HPTLC method

Trial for mobile phase

The initial attempts at high-performance thin-layer chromatography utilized various solvents in different ratios for the mobile phase. These solvents included toluene, n-butanol, methanol, ethyl acetate, formic acid, and acetic acid. Problems arose, such as poor peak symmetry (Fig. 1a) when using a mobile phase composed of toluene, ethyl acetate, and formic acid in a ratio of 6:3:1 (v/v/v). Although the peaks were better resolved in Figs. 1b and c, the two active components did not yield sharp or symmetrical peaks. Ultimately, a combination of toluene, ethyl acetate, methanol, and formic acid in the ratio of 5:4:2:0.5 (v/v/v) was selected as the mobile phase. This combination provided well-separated and sharp, symmetrical peaks for QR and LU.

Selection of wavelength for detection of QR and LU

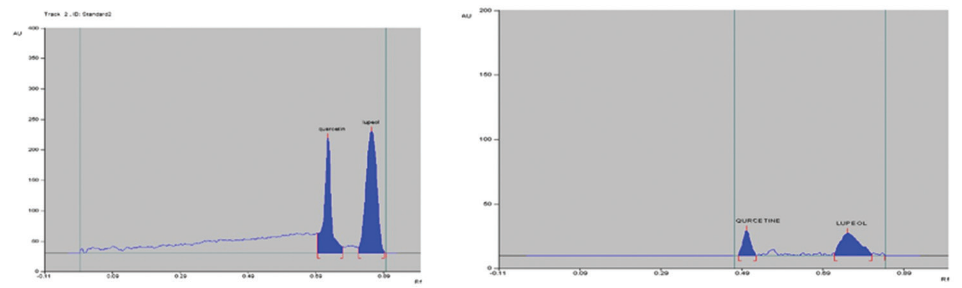
The optimized mobile phase consisted of toluene, ethyl acetate, methanol, and formic acid in a ratio of 5:4:2:0.5 (v/v/v). This mobile phase was used to determine the absorbance maxima for the quantification of QR and LU. The presence of spots with varying Rf values was observed by scanning TLC plates at UV wavelengths of 236, 330, and 344 nm. The documentation of TLC results is illustrated in Fig. 2a-c [28,29]. Among these wavelengths, the isosbestic point at 236 nm was selected for the HPTLC method, where the absorbance measured followed the Beer–Lambert law.

Optimization of chromatographic condition for HPTLC method

According to previous literature, LU and stigmasterol have been identified and quantified from a whole plant methanol extract of *H. auriculata* at a wavelength of 530 nm. The mobile phase used for this analysis was a mixture of toluene, ethyl acetate, and methanol in a ratio of 15:3:1.5 (v/v/v), with a linearity range of 100–1000 ng/band for LU and 50–500 ng/band for stigmasterol. In this literature, lupeol and stigmasterol were found to be at 0.19% and 0.47% w/w [34]. Another study focused on the analysis of QR and gallic acid isolated from the same plant using a methanol extract. This analysis was performed at 270 nm with a mobile phase of toluene, methanol, and formic acid in a ratio of 7.0:2.7:0.3 (v/v/v), achieving linearity ranges of 150–900 ng/band for QR and 100–1000 ng/band for gallic acid. In this study, 0.41% quercetin and 0.28 % gallic acid were found [35]. In addition, LU was studied in the roots and aerial parts of *Hygrophila schulli* plants using a petroleum ether extract with a mobile phase composed of benzene, chloroform, and ethanol in a ratio of 93:5.75:1.25 (v/v/v). In this study, the linearity range for LU was determined to be 200–1000 ng/band. The amount of lupeol was found in roots 5.02% w/w and aerial part 0.39% w/w [36].

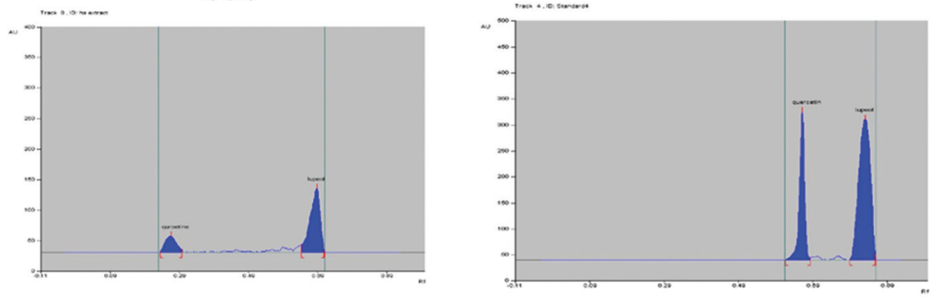
A novel method was developed for the separation of QR and LU from a hydroalcoholic seed extract. The mobile phase used for this separation comprised toluene, ethyl acetate, methanol, and formic acid in a ratio of 5:4:2:0.5 (v/v/v), with a saturation time of 15 min. Both QR and LU demonstrated linearity ranges of 100–600 ng/band at 236 nm. This mobile phase achieved the highest resolution for effective separation, yielding Rf values of 0.68 for QR and 0.83 for LU. The correlation coefficients were found to be 0.9925 for QR and 0.9844 for LU.

Table 1 presents the optimized conditions, linear regression equations, and correlation coefficients for this method. The composition of the mobile phase was strategically selected to optimize chromatographic



a Toluene: ethyl acetate: formic acid (6:3:1 v/v/v)

b n-butanol: methanol: ethyl acetate (6:3:4:1 v/v/v/)



c Toluene: ethyl acetate: acetic acid (5:3: 2 v/v/v)

d HPTLC chromatograms of standard Quercetin and Lupeol.

Fig. 1: Optimization of mobile phase for (a-c) and optimized condition (d) for standard Quercetin and Lupeol

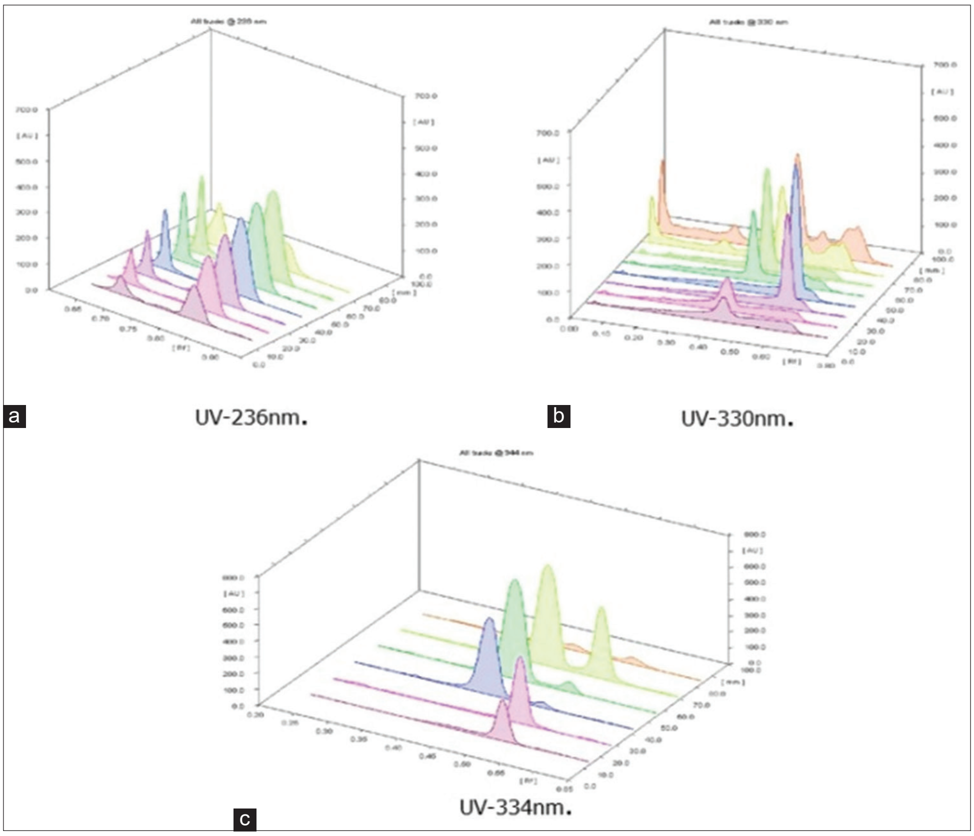


Fig. 2: (a-c) Densitograms of Quercetin and Lupeol at 236,330,334 nm

performance in liquid chromatography. This specific ratio effectively balances the polarity and solubility of the analytes, thereby enhancing separation efficiency and sensitivity.

Specificity

The specificity done by the standard solution before spiking and spiked extract solution revealed no significant interference of blank with the recovery of quercetin and lupeol; interfering with that method was specific. (Fig. 3).

Linearity

Linearity was evaluated by conducting three replicate measurements across the concentration ranges of both QR and LU, specifically from 100 to 600 ng/band. The relationship between the peak area and the concentration of the markers was thoroughly examined across this concentration range. Calibration curves were plotted, which were subsequently analyzed using standard linear regression methods to ensure accuracy and reliability.

Precision studies

To assess the precision of the developed method, standard solutions of QR and LU were carefully prepared and applied to the HPTLC plates at a concentration of 400 ng/band. The quantification of QR and LU was conducted using the calibration curve equation. In addition, the method's precision was evaluated by applying the sample solution at a concentration of 2000 ng/band, facilitating the determination of the active compounds present in the sample under optimized conditions (Tables 2 and 3).

Precision (repeatability and reproducibility)

To rigorously assess the repeatability of the method, a thorough statistical analysis was conducted. A precise aliquot of a standard solution of QR and LU was applied to the HPTLC plates at 2-h intervals on the same day (intra-precision) and again the following day (inter-precision). The samples were analyzed under optimized conditions,

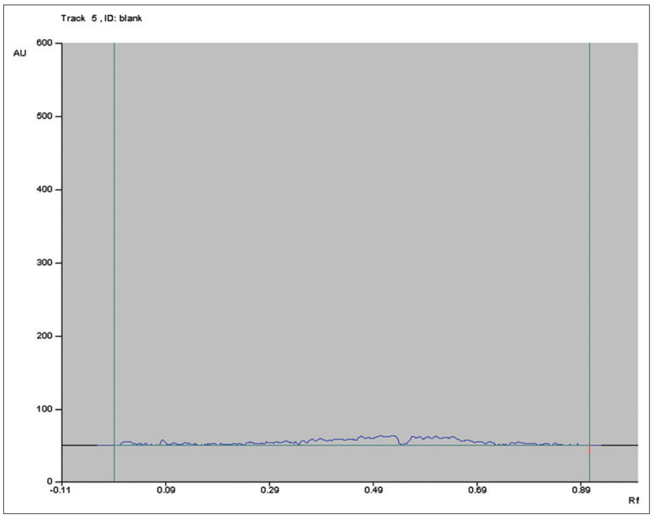


Fig. 3: Blank solvent chromatograms

Table 1: Method validation parameters for estimation of Quercetin and Lupeol

Parameters	Quercetin	Lupeol
Wavelength (nm)	236	236
Linearity (ng/band)	100–600	100–600
Regression equation	Y=7.7394x+212.2	Y=16.318x+3088.9
Correlation coefficient	0.9925	0.9844
Limit of detection (ng/band)	1.64	1.15
Limit of quantification (ng/band)	4.97	3.49

ensuring accuracy and reliability. The content was determined using the calibration curve equation, with the standard deviation and percentage relative standard deviation (%RSD) confidently falling within acceptable limits (Tables 4 and 5).

Table 2: Statistical evaluation for system precision and method precision of Quercetin (n=6)

S. No.	Standard (ng/band)	System precision			Method precision			
		Peak area	Amount found (ng/band)	% Content QR	Extract (ng/band)	Peak area	Content extract	% Content extract
1	400	3440	394.0	98.50	2000	2910	338.3	16.91
2	400	3457	386.2	96.50	2000	2946	340.8	17.04
3	400	3410	390.1	97.52	2000	2951	346.1	17.30
4	400	3462	396.9	99.22	2000	2940	339.6	16.98
5	400	3450	386.3	96.57	2000	2935	340.2	17.01
6	400	3460	397.2	99.30	2000	2930	338.7	16.90
	Mean	3446.5	391.8	97.93	Mean	2935	340.6	17.02
	S. D.	17.8	4.5	1.1	S. D.	13.2	2.59	0.13
	%R.S.D.	0.51	1.16	1.1	%R.S.D.	0.45	0.76	0.78

Table 3: Statistical evaluation for system precision and method precision Lupeol (n=6)

S. No.	Standard (ng/band)	System precision			Method precision			
		Peak area	Amount found (ng/band)	% Content LU	Extract (ng/band)	Peak area	Content extract (ng/band)	% Content extract
1	400	9920	397.7	99.42	2000	8680	350.00	17.50
2	400	9929	395.4	98.85	2000	8672	349.30	17.46
3	400	9925	391.9	97.97	2000	8670	349.40	17.47
4	400	9931	392.3	98.07	2000	8675	349.40	17.47
5	400	9937	387.2	96.80	2000	8681	349.44	17.47
6	400	9940	392.0	98.00	2000	8687	349.57	17.47
	Mean	9930.33	392.8	98.19	Mean	8678	349.5	17.47
	S. D.	6.7	3.26	0.81	S. D.	5.7	0.23	0.01
	% R.S.D.	0.06	0.83	0.82	% R.S.D.	0.06	0.06	0.07

Table 4: Studies for repeatability of Quercetin (n=6)

S. No.	Standard (ng/band)	Intra-day			Inter-day		
		Peak area	Amount found (ng/band)	% Content QR	Peak area	Amount found (ng/band)	% Content QR
1	400	3445.75	397.80	99.45	3438.97	395.9	98.97
2	400	3478.96	392.08	98.02	3352.90	392.8	98.20
3	400	3455.79	389.00	97.25	3358.10	386.4	96.60
4	400	3441.12	387.20	96.80	3426.28	385.2	96.30
5	400	3462.28	385.94	96.48	3345.75	387.8	96.90
6	400	3440.35	391.10	97.77	3340.00	390.4	97.60
	Mean	3454.04	390.50	97.62	3377.00	389.8	97.42
	S.D.	13.6	3.8	0.96	39.90	3.72	0.93
	% R.S.D.	0.39	0.97	0.99	1.18	0.95	0.95

Table 5: Studies for repeatability of Lupeol (n=6)

S. No.	Standard (ng/band)	Intra-day			Inter-day		
		Peak area	Amount found (ng/band)	% Content LU	Peak area	Amount found (ng/band)	% Content LU
1	400	9998.1	397.80	99.45	9938.0	395.9	98.90
2	400	9975.1	392.21	98.05	9952.9	393.8	98.45
3	400	9825.5	389.08	97.27	9858.1	386.4	96.60
4	400	9960.4	387.20	96.80	9826.2	385.2	96.30
5	400	9945.2	385.94	96.40	9985.7	387.8	96.90
6	400	9819.1	391.10	97.70	9840.0	390.4	97.60
	Mean	9920.5	390.55	97.61	9900.15	389.9	97.46
	S. D.	71.3	3.87	0.98	61.0	3.8	0.95
	% R.S.D.	0.71	0.99	1.0	0.61	0.9	0.98

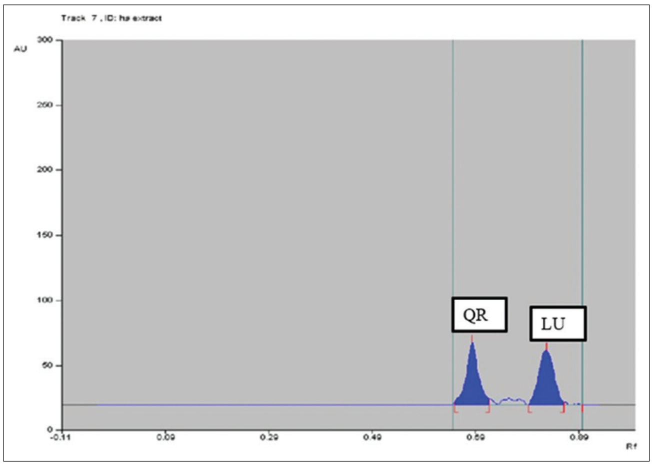


Fig. 4: High-performance thin-layer chromatographic chromatograms of standard Quercetin (R_f=0.68) and Lupeol (R_f=0.83)

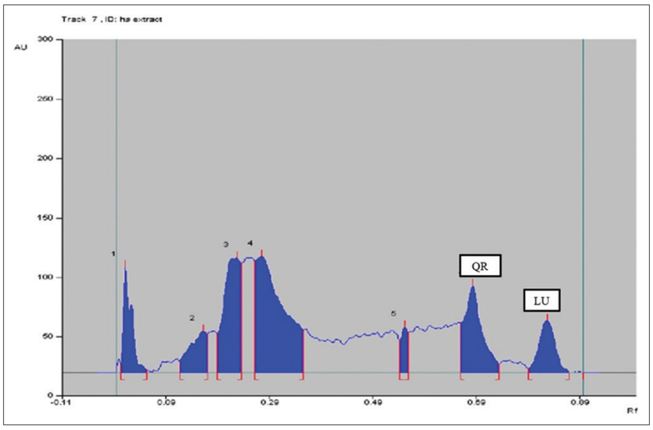


Fig. 5: Chromatograms of *Hygrophila spinosa* extract at 236 nm

Robustness

By introducing small changes in mobile phase ratio and saturation time, the effects on R_f and area were observed. The robustness of the method was done in triplicate at a concentration level of 400 ng/spot of QR and LU, and the results indicated that the method was robust.

Accuracy

The accuracy was evaluated by calculating the percentage content at concentrations of 80%, 100%, and 120%. Each sample extract was analyzed in triplicate at each concentration level. In the precision studies, the content of QR and LU was determined to be 100% at a concentration of 2000 ng/band. For the analysis, stock solutions were prepared, and applications of 1600 ng (80%), 2000 ng (100%), and 2400 ng (120%) were made on a TLC plate, with the analysis conducted as previously described (Tables 7 and 8).

Quantitative assay

To develop the assay method, we precisely weighed 50 mg of the extract and transferred it to a 10 mL volumetric flask. The solution was sonicated for 30 min and then filtered through Whatman 0.45 µm filter paper to ensure clarity and accuracy. From this solution, we pipetted out 0.1 mL and diluted it with 10 mL of methanol, achieving a concentration of 500 µg/mL. We then applied 4 µL of the extract to the chromatography plate, which was expertly developed and scanned under optimal chromatographic conditions. Following the scan, we integrated the peaks for both the standard and sample bands. The results indicate that the percentage content of QR was 17.00 mg, whereas LU was 17.48 mg, as accurately determined from the calibration curve.

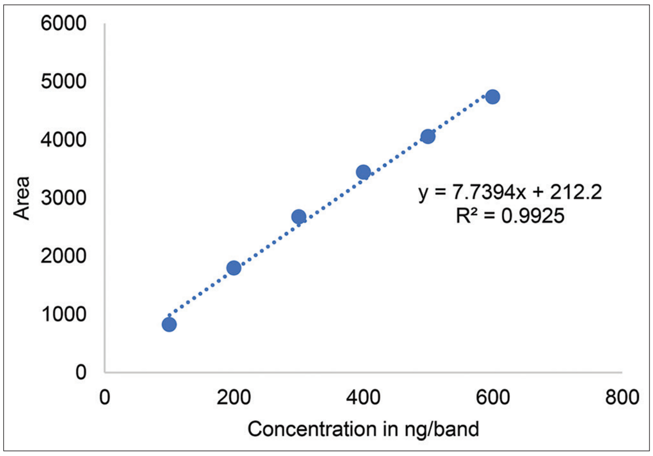


Fig. 6: Calibration curve of Quercetin in toluene: ethyl acetate: methanol:formic acid (5:4:2:0.5 v/v/v)

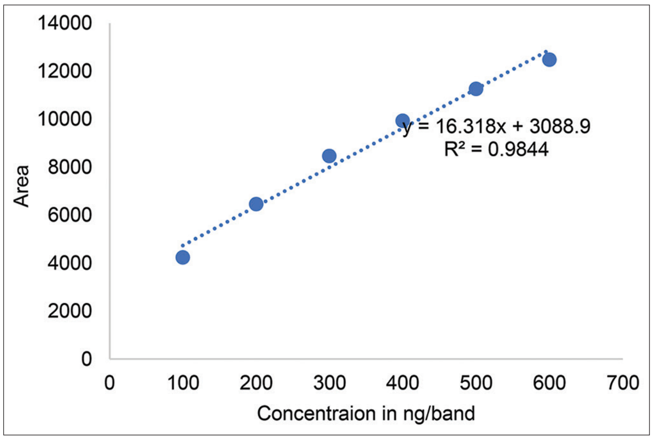


Fig. 7: Calibration curve of Lupeol in toluene: ethyl acetate: methanol:formic acid (5:4:2:0.5v/v/v)

Table 6: Robustness of method for Quercetin (n=3)

S. No.	Mobile phase (v/v/v/v)	%R.S.D. of QR	%R.S.D. of LU
1	Mobile phase composition (±0.1 mL)	0.16	0.12
2	Duration of saturation time (±5 min)	0.12	0.13

Table 7: Accuracy studies of Quercetin (n=3)

Level of %	Extract quantity applied (ng/band)	Peak area	Amount of content found (ng)	Percent of content	% Accuracy ±S.D.
80%	1600	2780	322.00	80.50	80.75±0.19
	1600	2790	323.85	80.96	
	1600	2785	323.27	80.81	
100%	2000	3475	403.00	100.84	101.08±0.18
	2000	3491	405.20	101.30	
	2000	3484	404.41	101.10	
120%	2400	4120	478.00	119.00	119.63±0.44
	2400	4135	479.00	119.90	
	2400	4150	481.00	120.00	

LOD and LOQ

The LOD and LOQ values were established following the guidelines of the ICH. The LOD was calculated using the formula: LOD=3.3*σ/(slope of the calibration curve), where σ represents the standard deviation of

Table 8: Accuracy studies of Lupeol (n=3)

Level of %	Extract quantity applied (ng/band)	Peak area	Amount of content found (ng)	Percent of content	% Accuracy ±SD
80%	1600	7954	320.40	80.1	80.2±0.14
	1600	7960	320.60	80.1	
	1600	7985	321.60	80.4	
100%	2000	9989	402.30	100.5	100.43±0.09
	2000	9980	402.00	100.5	
	2000	9960	401.40	100.3	
120%	2400	11967	482.05	120.5	120.13±0.38
	2400	11950	481.30	120.3	
	2400	11880	478.50	119.6	

the response. The LOQ is determined using the equation: LOQ=10*σ/(slope of the calibration curve).

CONCLUSION

The study results show considerable differences in the concentrations of QR and LU across various extracts, confirming the reliability and practical applications of the developed method. These findings enhance the understanding of *H. spinosa's* therapeutic potential and encourage further research into its medicinal benefits. This study will also help in the clinical research for their applications in pharmacokinetic and pharmacodynamic evaluation parameters.

A novel and precise HPTLC technique was successfully employed to detect and quantify QR and LU in the seed extract of *H. spinosa*. This method proved to be accurate and specific, allowing for the effective estimation of these bioactive compounds in the extract.

QR and LU are also present in several other plant species, including *Calendula officinalis, Sesbania grandiflora, Celtis occidentalis, Ficus religiosa, Strobilanthes ciliatus* Nees, *A. echioides*, *Crataeva nurvala*, and *Grewia titiaefolia*. The proposed method can, therefore, be adapted for the routine analysis of these compounds in a variety of medicinal plants. However, Limitations highlight areas for future research, including multi-solvent extraction, biological assays, and broader sampling strategies. Addressing these gaps could further substantiate the findings' therapeutic potential and analytical methods.

AUTHORS CONTRIBUTION

Smriti Gaikwad: Literature review, conceptualization, experimental work, and manuscript writing. Dr. Lata Kothapalli: Conceptualization and critical reviews. Dr. Asha Thomas: Data analysis.

ACKNOWLEDGMENT

For providing the essential infrastructural facilities, support, and direction to carry out this study, the authors are grateful to Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, Maharashtra, India.

FUNDING

Nil.

CONFLICTS OF INTERESTS

There are no conflicts of interest.

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