ORIGINAL RESEARCH ARTICLE

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF QUERCETIN IN URTICA DIOICA EXTRACT AND ITS MARKETED FORMULATION BY RP-HPLC

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ABSTRACT
A validated reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed for the determination of Quercetin in Urtica dioica extract and its marketed formulation.The analysis was performed on an Agilent 1120 compact LC system using a Kromasil C18 column (4.6 × 250 mm, 5µL) at 30°C. A gradient mobile phase was employed at a flow rate of 1.0 mL/min, with UV detection at 256 nm and an injection volume of 20µL.The method was validated according to ICH Q2(R1) guidelines for specificity, linearity, accuracy, precision, LOD, LOQ, and solution stability. The limit of detection for impurities was below 2.4µg/mL. Calibration curve regression analysis showed excellent linearity (r^2 > 0.9995), and recoveries ranged from 80% to 120%, confirming accuracy. This RP-HPLC method efficiently quantified Quercetin in both natural and commercial samples, proving to be accurate, precise, specific, and robustness making it suitable for routine quality control applications.
Keywords: Urtica dioica extract, Quercetin, HPLC, Validation, Method development

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1. INTRODUCTION

Urtica dioica L., stinging nettle, belongs to the Urticaceae family. Among its by products are dry extract, infusion (herbal tea), crude dried powder, Fresh juice, decoction, or both are unquestionably significant in phytotherapy (Krystofova O;et.al,2010) Nettle contains a variety of biochemicals like histamine, acetylcholine, and formic acid as well as beneficial substances like proteins, amino acids, flavonoids, tannins, phytosterols, and saponins (Khare CP;et.al, 2007).Nettle contains flavonoids such as flavonols, flavonones, and flavonoid glycosides, The existence of flavonoids (rutin, and other quercetin, and isorhamnetin) is documented in scientific literature (Tucakov J; et.al,1997). Vitamins like thiamine, riboflavin, pyridoxine, folic acid, nicotinic acid, and ascorbic acid are present in nettle aerial parts' water and alcoholic extracts (Wetherilt H;et.al, 1992). Nettle has been used as a natural remedy for ages, dating back more than 2000 years. All plant parts seeds, leaves, and roots are utilized medicinally.

Fig no. 1 Urtica dioica extract leaf

According to the literature review, techniques such as spectrophotometric 6-7, high-performance thin layer chromatography (HPTLC), and reverse-phase high performance liquid chromatography (RP-HPLC) have been documented for the analysis of these medications both by themselves and in conjunction with other medications. Nevertheless, no HPLC technique for the quantitative measurement of quercetin in any capsule formulation has been documented. As a result, an effort was made to create a new, quick, and sensitive technique for determining Quercetin in a extract and Diagnose to Cure capsule formulation and to validate it in accordance with International Council on Harmonization (ICH) guidelines (Rafajlovska V;et.al,2013). The goal of the current study is to validate a quick, easy, affordable, and sensitive RP-HPLC method for quercetin quantitative quantification. The goal of the current study is to validate a quick, easy, affordable, and sensitive RP-HPLC method for quercetin quantitative quantification (Kataki MS;et.al) The established method was used to determine the quercetin concentrations in bulk, formulation, and plant extract. (Bone K, Mills S et.al)

2. EXPERIMENT

2.1 Determination of solubility:

Solubility was determined by taking 1 mg of quercetin and nettle leaves extract in a test tube. Required quantity of solvent was added at room temperature and Shaked for few minutes. solubility was checked in same manner as solvent such as methanol, water, ACN, ethanol etc (Pinelli Pet. al. 2008)

2.2 Determination λmax by using UV:

To find the λmax, we took 10mg of the quercetin component and dissolved it in 10ml of methanol and then determined separate aliquots of 10µg/ml in methanol for λmax. At this point, we are able to tell what the maximum absorbance (λmax) of flavonoid (Quercetin) is at a different wavelength on the different concentration. Quercetin show maximum UV absorbance at 256 nm wavelength; Hance, it was selected throughout the HPLC-UV (Batool R;et. al, 2017)

2.3 Chromatographic condition:

Analytes were separated using chromatography using an Agilent 1120 compact LC Shimadzu UV-detector HPLC system equipped with a UV detector. EZ-Chromlite software is pre-installed on the device. Kromasil C-18 column 4.6 * 250 mm 5 microliter (Manufacturer: Krom Asil) was used to finish the chromatographic separation. The temperature of the column oven was kept at 25°C. The mobile phase was made up of water as mobile phase B and CAN (Acetonitrile) as mobile phase A, with the pH adjusted to 2.1 ACN and water are mixed 70:30 v/v to create the preparation diluent. A flow rate of 1.0 mL/min was established. The wavelength that guarantees accurate identification of the drug in question and any associated contaminants is ideal. Here, we utilized 256 nm as the wavelength for the specified technique. In calibration, we prepared the standard solution of flavonoids (quercetin) with quantities 20-160µg/ml with methanol as solvent. The standard sample was ran three times, and we took an average detector response. The Urtica dioica extract and marketed formulation were also assayed three times and corresponding peak areas for flavonoids was compared with the calibration curve and amount of flavonoid (quercetin) was determined (Chang SK;et.al,1995)

2.4. Analytical method development using HPLC:

2.4.1 Preparation of solvent:

400 ml of acetonitrile and 400 ml of water first 0.22 micro membrane-vaccum filtered then ultra sonicated 10 mins both solvent after sonication fill up the in mobile phase chamber.

2.4.2 Preparation of stock standard solutions of Quercetin:

Quercetin was purchase from yucca enterprises at 2 to 8 °C. 10mg of quercetin standard was weighed and dissolved methanol in 10 ml volumetric to get 1000ppm (1000µg /ml/ml) solution and sonicated for 10 minutes. Furthermore, as per the need, dilutions it was prepared and used for the determination of repeatability, precision and robustness. (Mohammadi A; et.al,2016, Ramaswamy S; et.al, 2021)

2.4.3 Preparation of Urtica dioica extract solution:

Urtica dioica extract was purchased from Zyrex Ayurveda and dried at room temperature. In order to achieve a 1000 µg/ml concentration, 500 mg of Urtica dioica extract samples were placed in a 10 ml volumetric flask, diluted with methanol to the appropriate level, filtered through Whatman filter paper, and a final volume was prepared using a similar solvent. After passing through Whatman filter paper no. 0.45µm, the finished solution was sonicated for ten minutes. A 20µL volume was introduced into the HPLC apparatus. The quercetin standard's retention time was used to identify quercetin (Mohammadi A; et.al,2016)

2.4.4 Preparation of marketed formulation:

Marketed formulation Diagnose to Cure 500mg Capsules were purchased from Tirupati Medical & General Store.20 capsules were weighed accurately. A 10 mg powder quantity was weighed out and added to a 10 mL volumetric flask along with 7 mL of methanol. After a few minutes, it underwent ultrasonography treatment, and the remaining volume was made up. Ultrasonically treated methanol solution, Than it was passed through a 0.45 micro filter to make sure there was no particle matter (Malviya R; et.al,2010)

2.4.5 Procedure:

In the chromatograph, approximately 20µLof the sample solution were added for each measurement. The chromatograms were noted. and measurements were made of the replies. Theamount and assayof Quercetin was determined. (Mohammadi A; et.al,2016, Chan C; et.al,2008).

2.4.6 Mobile phase selection and optimization

When selecting the mobile phase, a number of experiments were carried out and the peak purity index, optimal separation, and other factors were taken into account. For HPLC, the analytes must dissolve in a mobile phase. For the majority of HPLC separations, the reverse phase separation technique is employed. In this system, analytes with higher polarity elute faster than those with lower polarity. These cause highly polar analytes to be sufficiently separated and nonpolar analytes to have an excessively long retention period, and vice versa. We use gradient elution, where the content of the mobile phase varies, and isocratic elution, where the composition of the mobile phase stays constant, when we talk about separations. The mobile phase was sonicated after passing through a membrane filter with a 0.45 µm pore size.

Table no. 1 Chromatographic conditions for HPLC

Column	**Kromasil C18, [100x4.6mm, 5µm]**
Detector	UV
Wavelength	256nm
Temperature	25oC
Injectionvolume	20µL
Run time	30min.

3. METHOD VALIDATION OF HPLC

Using the guidelines from the International Conference of Harmonization, the developed quercetin RP-HPLC method was validated. The following parameters were used in the validation of the method (Malviya R;et.al,2010).

3.1 Specificity:

A specificity analysis was then performed to show that the developed method could measure analytes even when there were possible interferences. The analysis process was performed in standard solutions and diluents, and the notes collected from this analysis are described.

3.2 Evaluating Linearity and Analytical Range:

The method's linearity was demonstrated by generating quercetin solutions with concentrations ranging from 20 to 160µg/ml. The mix standard solution was diluted in various amounts. During the HPLC injection of these solutions, the peak area was measured. By calculating the slope, intercept, and correlation coefficient of the calibration curves (peak area vs. concentration), the linearity of the analytical method was granted (Z Liu; et.al,2024).

3.3 Assay:

To calculate the percentage of drug in the formulation, assaying is performed. The assay was performed on 20 capsule that contained of Urtica dioica. Remove the powder in capsule; Then powdered sample was accurately weighed to determine 10 mg Quercetin. which was then added to a 100 ml volumetric flask. A 0.45 µm membrane filter was used to filter this solution after the methanol had been brought up to volume. After filtering, the solution was subjected to HPLC analysis in order to identify the peak region and estimate the percentage of active ingredient in the capsule formulation.

3.4 Precision:

The intermediate precision of the suggested analytical approach was ascertained by examining variation between the day, within the day, and between the instruments. For three days (three times, n = 3), three distinct Quercetin concentrations (10, 20, and 40 µg/mL) were examined to examine intra-day variance. For three distinct days, the same process was used to examine the inter-day variation (n = 10) (IM Savic; et.al,2013).

3.5 Accuracy:

In order to assess the precision of the suggested approach, ten independent stock solutions containing varying concentrations of Quercetin (80, 100, and 120µg/mL) were produced and examined. Accuracy was evaluated using mean recovery percentage and accuracy percentage. Using the standard addition method, the new assay method's accuracy was further supported. Various amounts of pure quercetin are added in this process (4, 8 and 10 µg/mL) to a pre-analyzed, known sample (Kumar R; et.al,2021)

3.6 Limit of detection (LOD) and limit of quantitation (LOQ):

Calibration standards were used to determine Quercetin LOD and LOQ. LOD and LOQ were determined to be 0.4µg/ml /S and 2.4µg/ml, respectively, where S is the calibration curve's slope and y is the regression equation's standard deviation of the y-intercept (Kumar R; et.al,2021).

3.7 Robustness:

Robustness is assessed by purposefully making little adjustments as the analytical process is being developed and analyzing the impact on a certain performance element, usually accuracy. Experimental design can be used to test the method's robustness. This methodology makes it possible to examine how independent variables affect the response value.

Temperature and mobile phase flow rate were included as independent variables in a full factorial design for the robustness study. The corresponding flow rates were 0.5, 0.8, and 1 mL/min. Nine experimental runs were needed, and they were carried out three time (N Sanghavi;et.al,2014)

4. RESULTS AND DISCUSSION

4.1 Preliminary Analysis of Quercetin and Urtica dioica extract

4.1.2 Identification of functional groups drug by IR:

- **Quercetin:**

Fig no.2 FTIR spectrum of Quercetin

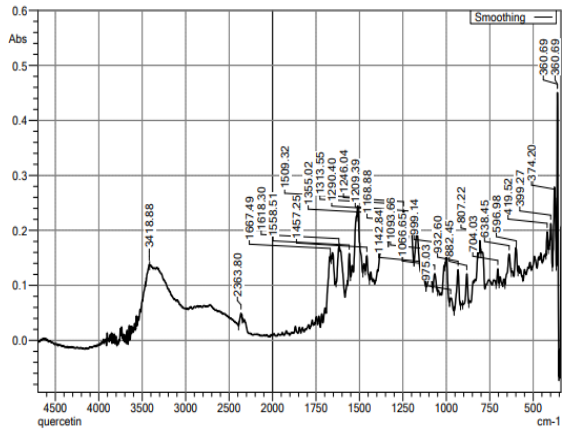


Table no. II Peak, functional group of Quercetin

Sr. no	Peak (cm^{-1})	Functional Group	Assignment
1	3418.88	O–H Stretching (broad)	Phenolic OH group
2	1657.24	C=O stretching	Aldehyde/ ketone
3	1276.04	C–O stretching	Phenol or ether
4	1045.52	C–O–C stretching	Ether linkage
5	879.52	Aromatic C–H	plane bending

- **Urtica dioica extract**

Fig no.3 FTIR spectrum of Urtica dioica extract

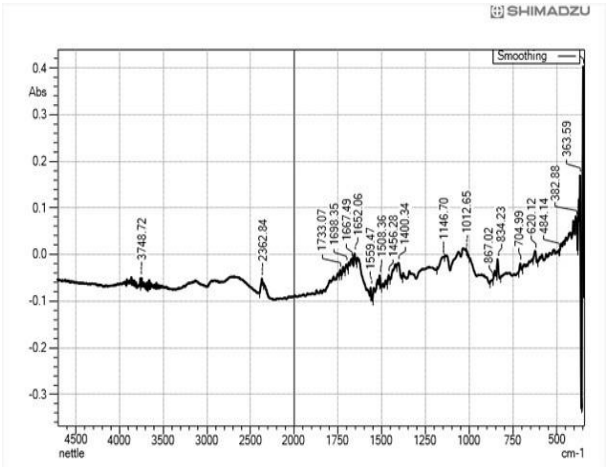
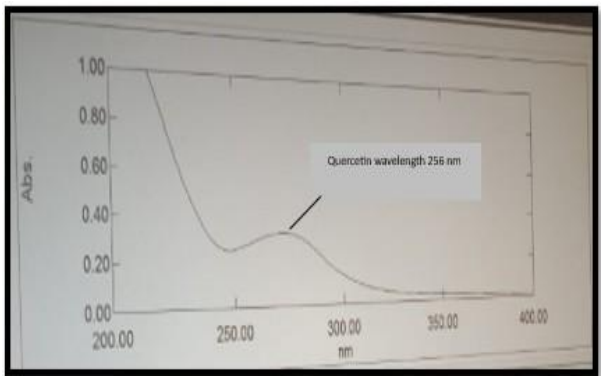


Table no. III Interpretation of Urtica dioica extract

Sr. no	Peak (cm^{-1})	Functional Group	Assignment
1	3418.72	–OH (broad)	Alcohols, phenols, water
2	1733.01	C=O	Aldehyde/ ketone
3	1459.26	C=C in aromatic ring	Aromatic skeletal vibrations
4	1269.83	C–O stretching	Phenol or ester
5	867.02	Aromatic C–H	Plane bending

4.1.2 Determination of λmax by UV:

Fig no.4 UV-spectrum of Quercetin



4.1.3 HPLC method optimization

The amount of quercetin in the plant extract was determined using an RP-HPLC method that was created and verified. To ensure a decent test performance, the chromatographic conditions were tuned. Several stationary phases, such as C18, various mobile phases, and organic modifiers, such as acetonitrile in the mobile phase, were tested throughout the method's optimization. Methanol was used as a mobile phase during the analysis of the quercetin standard, and the separation was satisfactorily obtained on an EZ-Chromlite C18 (4.6 × 250 mm, 5µm). The extract's ability to separate quercetin was inadequate if acetonitrile and water were combined. The wavelength selected for quercetin determination was 256 nm, where the highest absorption of quercetin was observed (Garg P;et.al,2021)

4.1.4 Identification of marker compound

The content of quercetin in the Urtica dioica extract and its formulation were determined by chromatographing the plant extract sample solution and applying the regression equation. The flavanoids (Quercetin) component of Urtica dioica extract and its formulation are being studied and quantified in the current study using the HPLC procedure.

4.1.5 Preparation of the calibration curve of the Quercetin

Using methanol as the solvent system, standard solutions of quercetin were prepared for the calibration curve by injecting them into the column three times, calculating the mean of the standard drug's peak area, and plotting the graph against various drug concentrations (20-160µg/ml). The curve served as the foundation for determining the regression equations. The peak of plant extracts and formulation were compared using the reference solution's calibration curve. The flavonoid calibration curve showed a linear association between 20 to160 µg/ml (R2=0.9995) of standard concentration. The curve's linear regression equation was Y = 719791x - 5E+06, where x is the flavonoid concentration (µg/ml) and y is the ratio of the flavonoid peak area.[21]

Table no. IV Regression analysis of Quercetin calibration curves

Parameter	Standard Quercetin
Linearity range	20-160µg/ml
Regression equation	719791x - 5E+06
Correlation coefficient (r2)	0.9995
Slope	719791

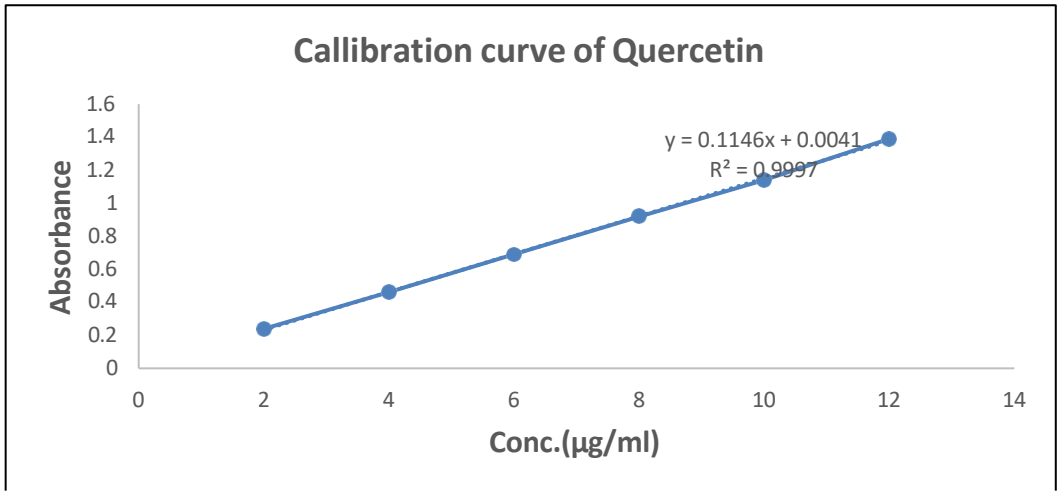


Fig no. 5 Calibration Curve of Quercetin

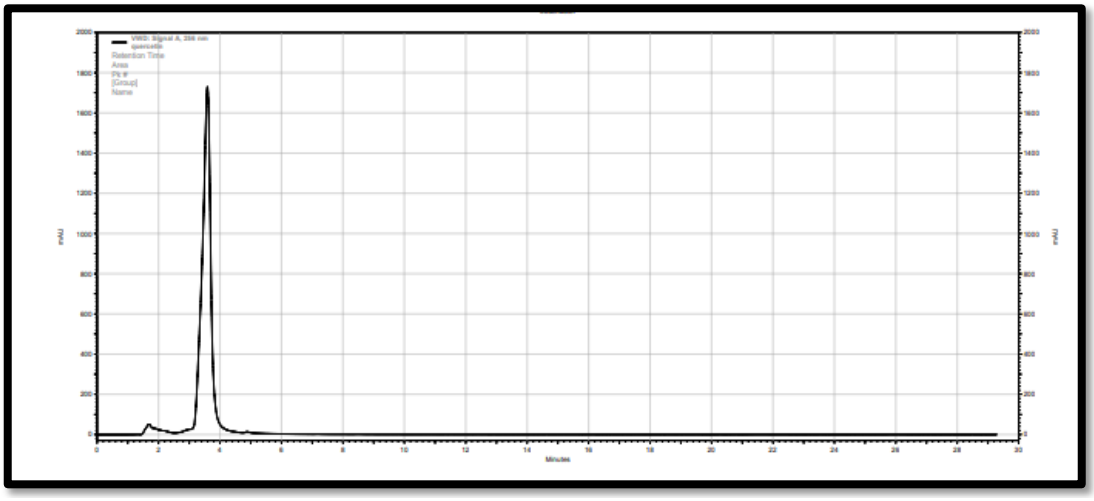


Fig no.6 The chromatogram of the Quercetin standard solution.

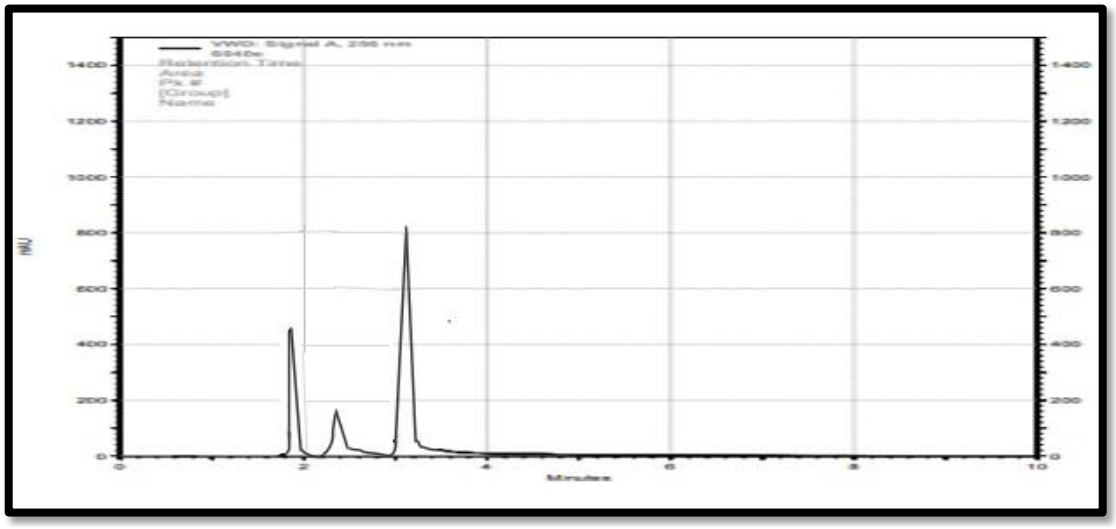


Fig no. 7 Chromatogram of the Quercetin in Urtica dioica extract

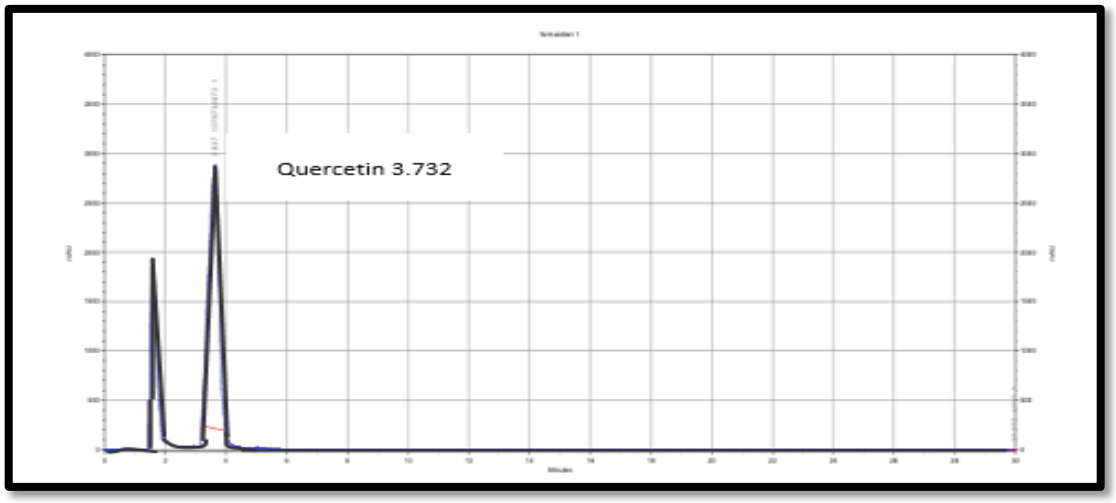


Fig no. 8 Chromatogram of the Quercetin in marketed formulation.

In the standard solution (100µg/mL), Quercetin had a retention time of 3.693 minutes.

For the quercetin standard, chromatographic factors like peak asymmetry and column efficiency were reexamined. Based on the Number of Theoretical Plates (N = 4984) that was acquired.

Acetonitrile and water 70:30 (V/V) solutions were employed as the mobile phase in the chromatographic analysis of Urtica dioica extract and its commercial formulation. The absorbance was measured at 256 nm and the flow rate was 1 ml per minute. At RT 3.693min, Quercetin showed good separation. The commercial formulation (Diagnose to Cure Capsules) showed a larger peak area at RT 3.732, while the HPLC chromatogram of Urtica dioica extract showed a peak area at RT 3.685.The quercetin peak in the formulation and plant extract chromatograms was compared to the RT values from the standard quercetin chromatogram under comparable circumstances. As seen in the preceding figures, standard quercetin produced a strong peak area at RT 3.693 min, while plant extracts and commercial formulations produced distinct peak areas at the same retention durations using HPLC. There were no discernible differences between conventional Quercetin and the results of the formulation and peaks of Urtica dioica extracts. It has been demonstrated by comparative chromatographic analysis that quercetin is present in both the commercial formulation (Diagnose to Cure Capsules) and the Urtica dioica extract.

4.1.6 Method validation of HPLC: Analytical method validation is the process of determining through laboratory tests that the technique's performance characteristics satisfy the needs for the intended analytical application. In compliance with the ICH criteria for analytical procedure validation, the designed HPLC method was verified. The method's robustness, linearity, accuracy, system precision, method precision, limit of detection (LOD), and limit of quantitation (LOQ) were all evaluated.

4.1.6.1 Specificity:

As blank has no peak at the quercetin retention time 3.693, Fig.6 for standard drug solutions and sample chromatogram shows that the peaks found in the standard solutions and sample solution at working concentrations are solely due to theUrtica dioica extract and its marketed formulation.

4.1.6.2 System Suitability Parameter:

Table no. V: System Suitability Parameter

Parameters	Quercetin
Resolution	1.39
Theoretical plates	4984.36
Tailing Factor	1.10
% RSD	0.23

4.1.6.3 Linearity and range:

Quercetin standard solutions were made at various concentrations. Plotting the concentration level against matching peak areas for Quercetin allowed calibration curves to be created. Within the studied concentration range of 20–160 µg/ml, Since Quercetin correlation coefficient was higher than 0.9995, the approach is considered linear since it satisfies the acceptance criteria for method validation.

Table no.VI: Linearity study

Compound	Linearity Range Conc. (µg/ml)	Correlation coefficient (r2)	Regression equation
Quercetin	20-160	0.9995	y = 719791x - 5E+06

4.1.6.4 Assay:

The % assay was determined and reported for analytes. According to reports, it was less than or equal to 99.8% for both medications and greater than or equal to 98.6%.

Table no.VII: Assay of Quercetin

Label Claim (mg/tab)	AUC standard (mean)	ACU sample (mean)	% Assay	% RSD	SD
10	59265256	59265267	99.20	0.415	0.412

4.1.6.5 Intra-day precision and Inter-day of Quercetin:

The RSD of the values in the intra -day precision of Quercetin was 0.51% in Table no. 8. The value shows that the approach is satisfactorily repeatable. The method is robust, according to the intermediate precision research, with RSD values of 0.248%, 0.272%, and 0.440 % on various days Table no .9 by various analysts, and on various columns, respectively.

Table no. VIII: Intra-day precision of Quercetin

Conc.(µg/ml)	Mean (AUC)	Concentration found (µg/ml)	SD	Intra-day %RSD
10	59617490	9.94	0.51	0.51%

Table no. IX Inter-day precision of Quercetin

Day	Conc. (µg/ml)	Mean (AUC)	Conc. Found mean (µg/ml)	Mean (% Assay)	SD	Inter-day % RSD
Day-1	10	59453832	9.93	99.32	0.248	0.248
Day-2	10	59480663	9.92	99.47	0.272	0.272
Day-3	10	59227510	9.89	99.03	0.440	0.440

4.1.6.6 Accuracy:

Through recovery trials, the percentage mean recovery of each chemical in the formulation at three different levels (80%, 100%, and 120%) was used to determine accuracy. All observed data fell between the not less than 99% and was not more than 100%, approved limits of mean recovery, indicating good recovery values and confirming the accuracy of the established approach.

Table no. X Accuracy study of Quercetin

Sample ID	Reps	Amount Added (µg/ml)	Amount of recovery (µg/ml)	% Recovery	% Mean recovered	% RSD
80 %	Reps1-Reps 3	8	7.97-7.96	99.63-99.50	99.66	0.194
100%	Reps1-Reps 3	10	9.80-9.98	98.00-99.80	99.10	0.973
120%	Reps1-Reps 3	12	11.89-11.88	99.08-99.0	99.30	0.46

4.1.6.7 LOD and LOQ :

The standard deviation of response and the calibration curve's slope were used to calculate the LOD and LOQ. The. For quercetin, the LOD was 0.2 and the LOQ was 2.4µg/ml.

Table no. XI: LOD and LOQ of Quercetin

Parameter	Conc. (µg/ml)
LOD	0.4µg/ml
LOQ	2.4µg/ml

4.1.6.8 Robustness:

The method's robustness to minor intentional modifications in terms of flow

rate and wavelength was confirmed by the lack of noticeable changes seen when certain of the chromatographic conditions' parameters were changed. Generally speaking, quercetin peak showed symmetry (Tailing factor<2) and were well-separated (Resolution>2).

Tablet no. XII: Robustness study of Quercetin

Changein flowrate (ml/min)						
Factors (Flow rate)	0.5	0.8	1	Mean	SD	%RSD
Retention Time	3.532	3.112	3.60	3.415	0.0264	0.87
Tailing factor	0.75	1.0	1.2	0.98	0.0023	0.23
No. of Theoretical plates	4984	3868.84	5184	4678.9	91.1	1.95
Area	58154320	58564298	58214156	5831092	221495	0.38
Change in wavelength (nm)						
Factors (wavelength)	253	254	256	Mean	SD	%RSD
Retention Time	3.412	3.530	3.625	3.52	0.01066	0.30
Tailing factor	1.21	1.23	1.30	1.25	0.0047	0.38
No. of theoretical plates	4656.69	4984.36	5253.35	4964.80	92.20	0.19
Area	58543142	58347610	58914752	5833517	437996	0.75

Table no.XIII: Summery of Method validation parameters for HPLC of Quercetin

Sr.No.	**Parameters**	**Results**
1.	Detection wavelength	256 nm
2.	Mobile phase	ACN: Water (pH2.1 adjusted using Triethylamine) 70:30
3.	Retention time	3.690 min.
4.	System suitability parameters Theoretical plates (N) Tailing factor	4984.36 1.10
5.	Specificity	Specific
6.	Linearity Range Slope Correlation coefficient (r^2)	20-160µg/ml 5E+06 0.9995
7.	Assay	99.8%
8.	Limit of detection (LOD)	0.04 µg/ml
9.	Limit of quantification (LOQ)	2.4µg/ml
10.	Intra-day precision (%RSD)	0.510
11	Inter-day precision (%RSD) Day-1 Day-2 Day-3	 0.248 0.272 0.440
12	Accuracy (% recovery) 80 % 100 % 120%	 99.66% 99.10 % 99.30 %
13	Robustness Change in flow rate (ml/min.) Change in wavelength (nm)	Passes the system suitability Passes the system suitability

CONCLUSION

This study attempts to create an RP-HPLC method for estimation of Quercetin. The approach met all method validation requirements, including linearity and range, accuracy, and precision, and was developed successfully. Furthermore, the new approach was successfully used to quantify the content of Quercetin in Urtica dioica extracts, commercial formulations, its selectivity and sensitivity.

DECLARATIONS

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Data availability: The data supporting the findings of this study are available from the corresponding author upon reasonable request. The accompanying author can provide the data that support the study's conclusions upon reasonable request.

Declaration of competing interest: The authors declare that there are no conflicts of interest.

Consent to Publish: The authors attest that ready give consent to publish the data and conclusions in this study. Bothco-authorshave given their approval for the paper to be submitted and consents to its publication.

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