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HPLC method for simultaneous quantitation of Luteolin and Quercitin from plant powder of *Acacia Catechu and Inula Viscosa*

Hina Shaikh*

Department of chemistry, Wilson College, Mumbai, India

***Corresponding author:** Dr.Hina Shaikh, Department of chemistry, Wilson College, Mumbai, India, **E-mail**: heenashaikhq@yahoo.in ,*

***Mobile:** +91 8097303246*

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ABSTRACT

A simple reversed phase high performance liquid chromatographic (RP-HPLC) method was established for simultaneous quantification of luteolin and quercitin in the two medicinally important plants, Acacia catechu and Inula viscosa.

Linear response was found in the concentration range of 1.0-500 µg/mL for Acacia catechu and 0.5-500 µg/mL for Inula viscosa respectively. The relative standard deviation for inter-day and intra-day precision was found to be <2%. Precision, specificity and robustness studies were carried out, values less than two (% R.S.D) were observed. Recovery values from 98.53 to 98.93 indicated that the accuracy of the method was good.

The proposed developed method was found to be precise, accurate and reproducible and can be used for routine quality control analysis and for the quantitation of quercitin and luteolin in herbal raw materials as well as in their formulations.

Keywords: HPLC; Luteolin; Quercitin; Acacia Catechu; Inula Viscosa

INTRODUCTION

Validation of analytical methods is mandatory in implementing a quality control system in any analytical laboratory. It provides an assurance of reliability during normal use and can be referred as a process of providing documented evidence of quality for several herbal and traditional drugs. Separation techniques such as chromatography and electrophoresis have been extensively used for quality control of herbal medicine because of their high efficiency and speed [1].

The medicinal plants (Rasayana) are the plants whose parts (leaves, seeds, stems, roots, fruit, foliage etc.) Extracts, infusion, decoctions, powders have been extensively used in the Indian traditional (Ayurveda) system of medicine for the treatment of different diseases of human [2]

Acacia catechu has been used traditionally against different diseases, especially gastrointestinal and stomach-related ailments, leprosy, and skin

diseases. In Ayurveda, it is used for mouth and mucous problems, cough, diarrhea, and skin diseases. An Ayurvedic skin tonic called "Khadirarishta" is prepared from *A. catechu* [2].

Inula viscosa (L.) is popular medicinal plant in the family Asteraceae. It has been used in traditional medicine in the treatment of cancer, diabetes, hypertension, bronchitis, tuberculosis, wounds, skin diseases, infertility, lung and gastro-duodenal disorders. *I. viscosa* showed promising antibacterial, antioxidant and anticancer activities [3-7].

Literature survey revealed that HPLC methods have been reported for estimation of luteolin and quercetin from different plant sources such as Beagle Dog [8], Vegetables, Fruits, and Teas [9]' Tibetan medicine *Meconopsis quintuplinervia* [10]. However, no HPLC method has been reported for simultaneous quantitation of luteolin and quercitin from methanolic extracts of dried whole plant powder of *Acacia catechu* and *Inula viscosa*

The present developed RP-HPLC method is advantageous compared to all the methods reported above because it uses a simple isocratic mobile phase comprising of 0.2% ortho-phosphoric acid and acetonitrile (60:40, v/v) to resolve luteolin and quercitin from other phytochemicals present in both the selected plant materials with significantly lower retention times for luteolin and quercitin than those observed in the reported methods. Thus, the overall time required for analysis is reduced.

The developed HPLC method was validated using ICH guidelines [11]. The developed and validated HPLC method is simple and fast as compared to the HPLC methods reported in the literature.

MATERIALS AND METHODS

Chemicals (2.1)

Acetonitrile (purity- 99.8 %) and trifluoro acetic acid (purity- 99.0 %) used in the present research work were of HPLC grade and were procured from Merck, India. Distilled water for HPLC was procured from LiChrosolv Merck, India. The reference standards, luteolin (purity ≥ 98%) and quercitin (purity ≥ 95%), were purchased from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany).

Plant material (2.2)

Both the plants *Acacia catechu* and *Inula viscosa* were collected from Keshav Shrishti, Maharashtra. The plant material of *Acacia catechu* was authenticated from Agharkar Research Institute, Pune, India (Auth.15-193). Herbarium of *Inula viscosa* was authenticated from Botanical Survey of India, Pune, India (Certificate No. BSI/WRC/Cert./2014) and collection number HSQ 01. Both plant materials were washed with water to remove soil particles, dried in shade, finely powdered and then sieved through BSS mesh size 85 and stored in an airtight container at room temperature (25 ± 2° C).

Preparation of stock solutions (2.3)

Stock solution of 1000µg/mL of luteolin was prepared by dissolving 50.0mg of accurately weighed luteolin in 10.0mL of methanol in a 50.0mL standard volumetric flask. The standard volumetric flask was then sonicated in an ultrasonic bath (Model: TRANS-O-SONIC, Frequency: 50 Hz) for 5.0 minutes for complete dissolution of luteolin. The contents were then diluted up to the mark with methanol to obtain a solution of 1000µg/mL of luteolin. 5.0mL of the above stock solution of luteolin was then transferred to 50.0mL volumetric flask and the contents were diluted to 50.0mL using methanol to obtain working solution of standard luteolin with concentration 100µg/ml. A similar procedure was followed for preparation of standard stock and working solution of quercitin.

Sample Preparation (2.4)

About 1.000 g of dried whole plant powder of *Acacia catechu* was accurately weighed and transferred to a stoppered conical flask and 10.0 mL of methanol was then added to it. The flask was then shaken at 50 rpm, on a conical flask shaker overnight at room temperature (25°C + 2°C). The contents of the flask were filtered through Whatman No.41 filter paper (E. Merck, Mumbai, India). The filtrate was further used as sample solution for the assay experiment. The sample solution was filtered through 0.45µm filter paper before analysis. The same procedure was followed for preparation of extract of whole plant powder of *Inula viscosa.*

Chromatography (2.5)

Chromatographic analysis was performed using Shimadzu UFLC Prominence chromatograph, equipped with binary gradient pump (LC-20AD), having auto sampler (SIL-20 AC HT), oven (CTO-20 AC) and PDA detector (SPD-M20A).

A reversed-phase Dionex C18 (250mm x 4.6mm, 5µm) column was used for chromatographic separation. LC solution software was used for data acquisition. Different compositions of solvents were tried as mobile phase. Finally 0.01% trifluoroacetic acid in acetonitrile and 0.01% of trifluoroacetic acid in water in the volume ratio of 45:55 was selected which gave a good resolution between the sample components. The flow rate was maintained at 0.5 mL/min and the separated components were detected at 254 nm (luteolin) and 287 nm (quercitin).

METHOD VALIDATION

Validation of the Method

ICH harmonized tripartite guidelines were followed for the validation of the developed analytical method [11]

Linearity (3.1)

Each standard solution of luteolin and quercitin in the concentration range of 0.1 µg/mL to 500.0 µg/mL were injected in triplicates into the chromatographic system, under optimized chromatographic condition. The peak areas were recorded for each injected concentration of standards. The calibration curves of each of the two standards luteolin and quercitin were obtained by plotting graphs of mean peak areas vs. corresponding concentrations. The results listed in **Table 1.**

Limit of detection (LOD) and Limit of quantitation (LOQ) (3.2)

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined at signal to noise ratios of 3:1 and 10:1 respectively. The LOD and LOQ values obtained for both the components are listed in **Table 1.**

Table 1. Method validation.

Standards	Luteolin	Quercitin
Linear range (µg/mL) (n=3)	1.0 to 500.00	0.50 to 500.00
Correlation coefficient (r)	0.999	0.999
LOD (µg/mL)	0.3	0.16
LOQ (µg/mL)	1.0	0.5

Precision (3.3)

The method was validated in terms of repeatability and intermediate precision. Repeatability was carried out in the same laboratory, on the same day, by analyzing sample solution of dried whole plant powder of *Acacia catechu* and *Inula viscosa* in triplicate. The intermediate precision was carried out in same laboratory, but on three successive days. The values of % R.S.D. for both the plant was less than 2 indicating that the method is precise and reproducible.

System suitability (3.4)

System suitability experiment was performed by injecting six consecutive injections (10µg/mL) of each bioactive marker, namely luteolin and quercitin, during the start of the method validation. Values with % RSD of ≤ 2% were accepted.

Specificity (3.5)

The specificity of the proposed HPLC method was ascertained by injecting 10.0µL of blank solution to observe for interference, if any, with the peaks of interest in the chromatogram of the sample solution. It was observed that there is no interference from the blank solution. Methanol was taken as blank solution since standard and sample solutions were prepared in methanol.

The chromatograms were compared by overlay. Good correlation was observed between chromatograms obtained from luteolin and quercitin

standards and samples at all R_f positions. The peaks of luteolin and quercitin standard we're not masked by any peaks arising due to other components present in the sample solution. Thus, it can be concluded that the developed method is specific.

Ruggedness (3.6)

Ruggedness of the method was studied by determining the effects of small variations of mobile phase composition (±2%), and flow rate (1.00 ± 0.05 mL/min). Effect of these deliberate changes on the response (area) and retention time of QC samples of luteolin and quercitin was observed during the analysis. The results were expressed in terms of % mean difference. Values within a difference range of ±5% were accepted.

The amount of luteolin and quercitin from dried whole plant powders of *Acacia catechu* and *Inula viscosa* obtained by altered method to that obtained by normal method did not show any significant differences. Thus, it can be concluded that the developed method is robust.

Stability of standard luteolin and quercitin (3.7)

Standard solution containing the mixture of luteolin and quercitin with concentration of 10.0μg/mL each was injected in the chromatographic system, under the specified chromatographic conditions at time intervals of 0, 12, 24, 48 hours. The chromatograms were recorded and values of peak areas of standard luteolin and standard quercitin were noted for each injected solution, drawn at regular time intervals.

The values of mean peak area, standard deviation (S.D.) and percent relative standard deviations (%R.S.D) were calculated for standard luteolin and quercitin. No significant degradation was observed within the given time intervals, thus indicating that standard solutions of luteolin and quercitin of concentrations 10.0μg/mL each are stable for a period of minimum 48 hours and are thus sufficiently stable to perform the method under normal laboratory conditions.

Regression analysis (3.8)

The regression analysis of the calibration data was carried out to determine the relationship between the dependent variable (peak area) and the independent variable (concentration). The regression equation for the above data was found to be

$$\text{Luteolin: } y = 47357x - 9145$$

$$\text{Quercitin: } y = 59856x - 58539$$

The values of the correlation coefficient, intercept and slope were determined for the graph of mean peak area against corresponding concentration is given in **Table 2.**

Table 2. Method Validation.

Standards	Luteolin	Quercitin
Slope (m)	47357	59856
Intercept (c)	-9145	-58539
Correlation coefficient (r)	0.999	0.999

Recovery (3.9)

Recovery tests were carried out to further investigate the accuracy of the method by adding three different concentration levels of the mixed standard solutions to known amounts of *Acacia catechu* and *Inula viscosa*. The resultant samples were then extracted and analyzed with the described method. The mean percentage recoveries were calculated using the formula:

Recovery (%) = [(amount found – original amount) / amount added] x 100

Values within the range of 85 – 115% were accepted. Results obtained are tabulated in **Table 3.**

Application of validated method for the simultaneous quantitation of luteolin and quercitin from dried whole plant powders of *Acacia catechu* and *Inula viscosa* (4.0)

The developed and validated HPLC method was used for quantitation of luteolin and quercitin from dried whole plant powders of Acacia catechu and Inula viscosa. 10μL of the sample solution was injected into the chromatographic system. The identities of peaks of luteolin and quercitin in the sample solution were confirmed by comparing the chromatogram of the sample **Figure 2** and **Figure 3** with that of the standard solution of luteolin and quercitin **Figure 1**. Retention times of standards of luteolin and quercitin standards were 4.455 minutes and 6.422 minutes respectively. The retention times of luteolin and quercitin from plant powder of Acacia catechu were found to be 4.453 and 6.411 minutes respectively. The retention times of luteolin and quercitin from plant powder of Inula viscosa were found to be 4.440 and 6.426 minutes respectively.

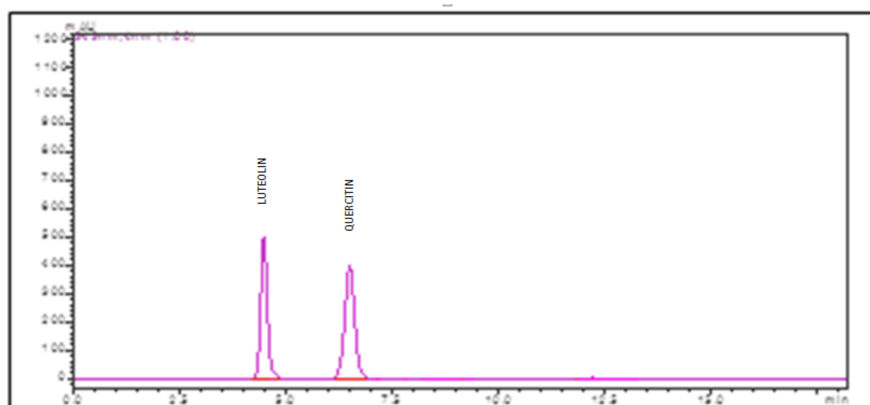

Figure 1. HPLC chromatogram obtained of Standard luteolin and quercetin.

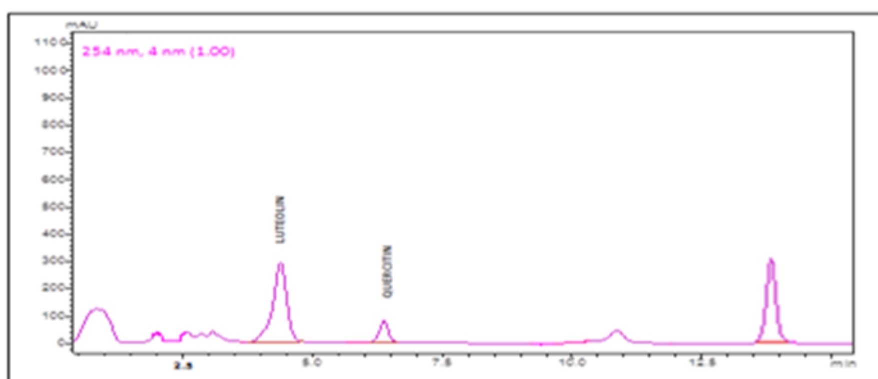

Figure 2. HPLC chromatogram obtained for methanolic extract of dried whole plant powder of *Acacia catechu.*

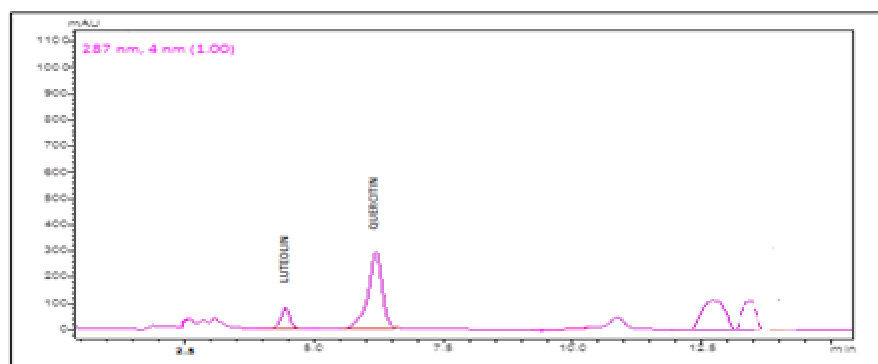

Figure 3. HPLC chromatogram obtained for methanolic extract of *Inula viscosa.*

RESULTS

The method developed to quantify luteolin and quercitin from dried whole plant powders of *Acacia catechu* and *Inula viscosa* was validated and the results are tabulated in **Table 3.**

Table 3. Results validation.

Parameters	Observations	
	Luteolin	Quercitin
Assay (mg/g)		
Plant powder of *Acacia catechu*	0.9235	0.0836
Plant powder of *Inula viscosa*	0.0528	0.2334
Percent Recovery (%)		
Plant powder of *Acacia catechu*	98.53	98.86
Plant powder of *Inula viscosa*	98.65	98.93

CONCLUSION

A reverse-phase High Performance Liquid Chromatographic method has been developed and validated for simultaneous quantitation of flavone aglycones, quercitin and luteolin from methanolic extracts of dried plant powder of *Acacia catechu* and *Inula viscosa*. The developed method is simple, precise and accurate and can also be used for routine quality control analysis and for the quantitation of quercitin and luteolin in herbal raw materials as well as in their formulations.

CONFLICT OF INTEREST

Conflict of interest declared none.

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