VALIDATION OF NEW LIQUID CHROMATOGRAPHIC METHOD FOR NATURALLY ISOLATED QUERCETIN AND ITS COMMERCIAL APPLICATION TO AYURVEDIC FORMULATION

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ABSTRACT

Quercetin was isolated from *Aerva lanata* (L) and investigated for method development. A simple, accurate, reproducible method was developed using RP-HPLC. Commercial formulation of Quercetin 250mg capsules (Quercetin Complex-Solgar, USA) was used followed by an Ayurvedic proprietary medicine for the study. The chromatographic separation was performed on Shiseido Capcell Pak C_{18} column (250 X 4.6 mm, 5 µm), mobile phase developed was 0.3% formic acid:acetonitrile:methanol (40:20:40) at the flow rate of 10µL/min at 22^{0}C. The detector used was PDA with the detection wavelength of 370 nm. R_T was 4.9mn with LOD-0.816 µg/ml and LOQ-2.473 µg/mL. The correlation coefficient was 0.992%. The linearity range was observed in the concentration range of 2-20 µg/mL and the recovery was found to be 99.91 to 102.65 %. Finally, the method applied to KACHCHNAR Ayurvedic formulation justified with characteristic peak for identifying quercetin in it.

Keywords-Analytical method, *Aerva lanata*, Quercetin, UPLC, ICH guidelines, KACHCHNAR

INTRODUCTION

Pashanabheda is a category of medicinal plants available in all parts of India and approximately 12 medicinal plants are categorized into this category. *Pashanabheda* means stone breaking and these plants are used for Antiurolithiatic activity by Ayurvedic practioners in India[1-2].

Quercetin, chemically 2-(3,4-dihydroxy phenyl)-3,5,7- trihydroxy-4H-chromen-4-one, is a plant-derived flavonoid found in fruits, vegetables, leaves and grains. It is used as an ingredient of supplements, beverages and foods. Quercetin is frequently used therapeutically for various diseases including gout, pancreatitis and prostatitis and other inflammatory conditions. It is also used for treating conditions of the heart and blood vessels including hardening of the arteries (atherosclerosis), high cholesterol, heart diseases, circulation problems, chronic fatigue syndrome (CFS), and cancer and for treating chronic infections of the prostate[2-3].

Literature survey revealed that several methods for quercetin using U.V., HPLC, HPTLC and electrochemical determination have been reported. However, not a single analytical method is reported so far for quercetin isolated from natural sources like *Aerva lanata* (L) from Western Ghats. Hence, an analytical method has been developed here for quercetin isolated from *Aerva lanata* of Western ghats and validated as per ICH guidelines.

MATERIALS AND METHODS

Collection of plant material and isolation

Aerva lanata (L) available in Western ghats of Khanapur region from Belagavi district was collected and the plant was identified by a taxonomist, Dr. Harsha Hegde, RMRC, Belagavi (specification no-RMRC-507). Later, the powdered plant material was subjected to extraction with ethnol and water (80-20V/V), followed by fractionation with different organic solvents based on their polarities, such as dichloromethane, n-butanol and ethyl acetate. Based on the preliminary phytochemical study and spectral study results of UV and IR, *n*-butanol fraction was subjected to column chromatographic technique for isolation of active compound followed by purification and characterization by spectral techniques like IR, NMR and LCMS as quercetin[2].

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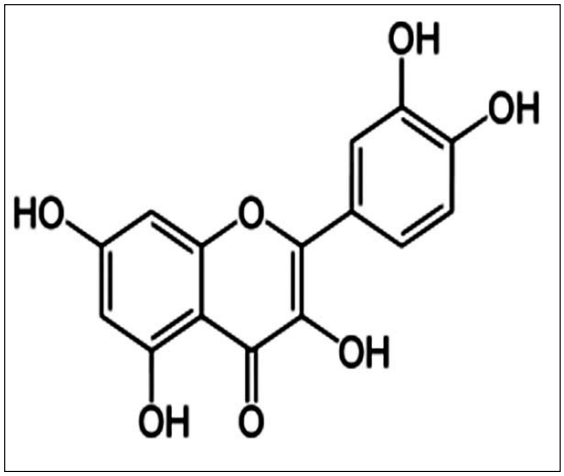
Natural quercetin was subjected to analytical method which was developed using HPLC-PDA at KAHER's BSRC, Nehrunagar, Belagavi-Karnataka (India) and the method was validated as per ICH Guidelines. The HPLC data of natural quercetin was compared with commercial formulation quercetin 250 capsules which were purchased from Solgar USA with a label claim of 250 mg[3-4].

Structure of Quercetin



2-(3,4 – dihydroxy phenyl) -3,5,7-trihydroxy-4H-chromen-4-one (Quercetin)

Preparation of standard solution

A stock solution was prepared by dissolving 10 mg of quercetin (pure drug) in 10 mL of methanol (1 mg/mL) and the volume was made up to 100 mL and filtered with 0.45 micron filter. The aliquots of the above stock solution was diluted further with methanol to get concentrations of2, 4, 8, 16, 20 µg/mL.

Preparation of sample (1) solutions

The sample solution was prepared by dissolving 10 mg of isolated quercetin in 10 mL of mobile phase (1mg/mL) and the volume was made up to 100 mL. The solution was sonicated, later filtered through the 0.45 micron filter and injected to give the final solution (10µg /mL).

Preparation of sample (2)-commercial Ayurvedic formulation

The sample solution (2) was prepared by dissolving 50 mg powder of the ayurvedic tablets in 10 mL (5 mg/1 mL) of mobile phase, diluted to make up the mark. The solution was sonicated and filtered through the 0.45 micron filter and 10µL was injected for the identification of quercetin in it using the above validated method. For this purpose, Ayurvedic formulation (KANCHANARGULGULU.DS) from AVN, Ayurveda Formulations Pvt Ltd Madurai was procured from market.

Instrumentation and chromatographic conditions

The analytical method was developed for quercetin using HPLC-PDA. The estimation of quercetin was performed on a Shimadzu liquid chromatographic system equipped with LC-2010AT VP solvent delivery system (pump) and Rheodyne 7725i injector with 20 µL loop volume. The Shiseido Capcell Pak C_{18}column (250 X 4.6 mm, 5 µm) was used to get a sharp peak of quercetin at room temperature and mobile phase was 0.3% formic acid: acetonitrile:methanol (40:20:40V/V/V) was filtered through 0.45 µm Millipore membrane (Germany) and degassed by ultra sonicator, pumped at the flow rate of 10 µL at 22°C and Rt was found to be 4.956 sec. The detection wavelength was 370 nm.

Validation of the method

The new method developed was validated as per ICH guidelines in terms of accuracy, precision, linearity, limit of detection, limit of quantification and robustness[5-12].

RESULTS AND DISCUSSION

Method development and validation

The main objective of this study was to develop a new HPLC-PDA method for naturally isolated quercetin from *Aerva lanata* and validate it as per ICH guidelines such as accuracy, precision, linearity, limit of detection, limit of quantification and robustness. In order to develop a new method for the Quercetin, preliminary study for the analysis of the drug in terms of parameters like detection wavelength, suitable mobile phase selection, optimum pH and concentration of the Quercetin to be taken was carried out. The method was optimized to get good peak, resolution and other parameters like theoretical plates. It was initially tried with different mobile phases like sulphuric acid: methanol, formic acid: methanol, formic acid: acetonitrile in different concentrations and finally the mobile phase was optimised based on separation efficiency achieved with 0.3% formic acid:acetonitrile:methanol (40:20:40V/V/V) by suitably adjusting the pH, pumped at the flow rate of 10 mL/min at 22°C. The mobile phases were run at different pH and finally the optimum pH was adjusted to 3.25± 0.03 with formic acid (0.3%).The retention time was 4.956 min and the recovery was found to be 99.91 to 102.65%. All solutions were prepared in a mixture of mobile phase. The tailing factor, resolution and theoretical plates were found to be in compliance with the ICH guidelines. The method was found to be economic as it utilizes comparatively less

volumes of mobile phase. The theoretical plates were 4929 and tailing factor was 1.217(□2) which signifies the efficiency of the column in Table II. The peaks obtained by injecting standard solution and sample solution are given in Fig. 2-4.

Table I: Calibration results for Quercetin

Concentrations	Peak Area
2	132463
4	226817
8	325003
16	436152
20	546061

Table II: System suitability parameters

System suitability	Results
% RSD*	0.88%
Theoretical plates	4929
Tailing factor	1.217
Retention time (RT in min)	4.956
Area	1530

Table III: Linear Regression analysis Results

Linearity range	2-20 µg/ml
Correlation coefficient (r^2)	0.992 %
Regression Equation	Y=0.51759 X +22653
LOD	0.816 µg/mL
LOQ	2.473 µg/mL

Table IV: Validation parameters (Precision and Robustness) and Results for Quercetin

Parameter Observations	Observations
Precision (%RSD*)	
Intra-day	0.43 - 0.66
Inter-day	0.01 - 0.17
Accuracy (% recovery)	
Level I (50%)	102.654
Level II (100%)	102.440
Level III (150%)	99.91
Robustness	
Flow rate 0.8 mL/min (% RSD*)	0.70
Flow rate 1.2 mL/min (% RSD*)	0.97
Assay (%)	99.91

The details of validated method as per ICH guidelines are discussed below[13-22].

Calibration curve

A calibration curve was obtained for quercetin standard by injecting 2, 4, 8, 16 and 20 µL of the standard solution. Linearity was tested and regression equation was obtained (Y=0.51759 X +22653) and correlation coefficient (r-0.992) was calculated as shown in Fig. 1.

Linearity: The linearity was evaluated by analyzing different concentrations of the standard solutions of quercetin and it was found to be linear in the range of 2-20 µg/mL and correlation coefficient was 0.992. Calibration standards were prepared by diluting the stock solutions to obtain the concentrations. The linear calibration curve was obtained and the regression analysis (r^2) for Quercetin was 0.992. The regression of quercetin concentration over its peak area was found to be Y=0.51759 X +22653 where Y =peak area and X = concentration of Quercetin as shown in Table III.

Precision: Repeatability was studied by calculating the relative standard deviation (RSD) for six determinations of the concentration of about 1 mg/mL, performed on the same day and under same experimental conditions. The results of quercetin determinations in the standard solution with the relative standard deviation were calculated. Intermediate precision studies include the estimation of variations in analysis when a method is used within laboratories, on different days. The RSD values obtained for quercetin was 0.88%. The intraday precision was 0.43-0.66 and interday precision was 0.01-0.17 as shown in Table IV.

Accuracy: Accuracy was evaluated by % recovery. Standard addition (known amount of the drug added to sample solution and spike solution (known amount added to placebo) were used for accuracy determinations at three levels of concentrations. Recovery studies were performed with three different samples of quercetin spiked at 50% (50 µg/mL^{-1}), 100% (100 µg/mL^{-1}) and 150% (150 µg/mL^{-1}) , levels with all stock solutions prepared. The recovery range for quercetin was found to be 99.91 to 102.65% as shown in Table IV.

Robustness: A method is said to be robust when the alterations in the study produce no significant changes in the results obtained. The developed method is a robust method because no significant changes were observed in recovery percentage and in the retention time of the compounds. The results showed that varying the chromatographic conditions had no appreciable

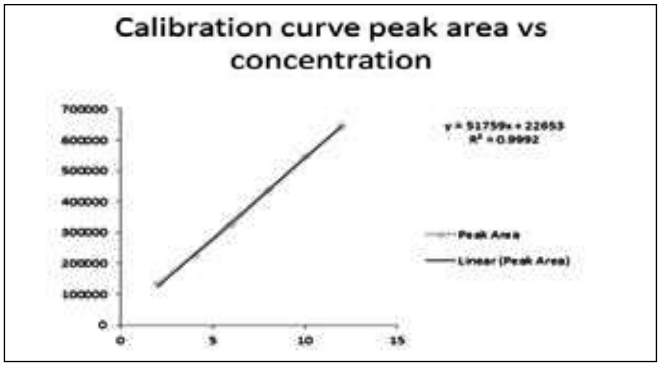


Fig.1: Calibration curve of standard Quercetin content

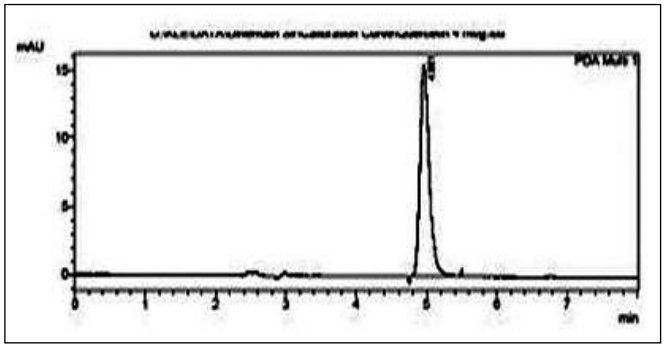


Fig. 2: Chromatogram of pure Quercetin (Querectin Complex-Solgar, USA)

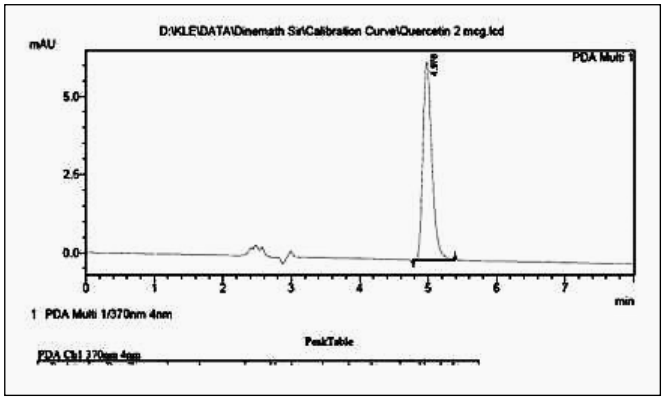


Fig.3: Chromatogram of Quercetin- 2 mcg- sample 1

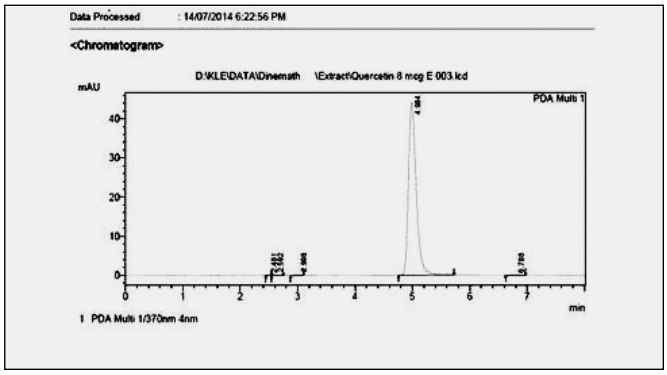


Fig.4: Chromatogram of Quercetin -8 mcg (sample 1)

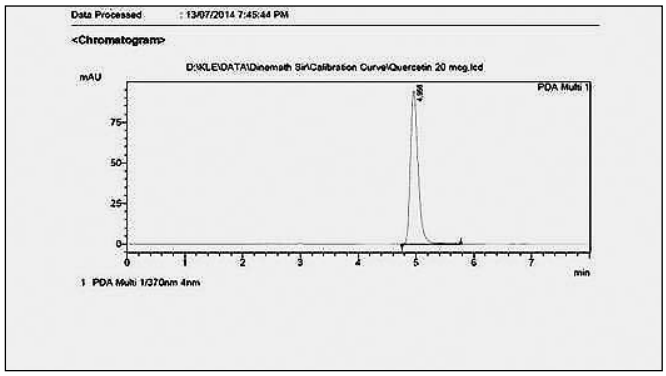


Fig.5: Chromatogram of Quercetin- 20 mcg (sample 1)

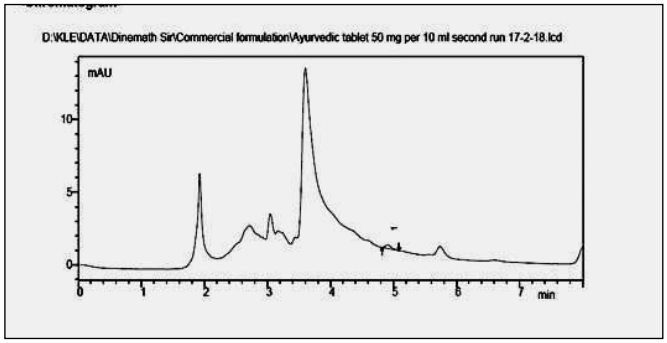


Fig.6: Chromatogram of Quercetin (5 mg)- CommecialAyurvedic formulation

effects on the chromatographic parameters as shown in Table IV.

Limit of detection and Limit of quantification: LOD in Table III was calculated by using the following equations.

LOD = 3.3 x SD/S and

LOQ = 10 x SD/S,

Where SD = the standard deviation of the response, S = Slope of the calibration curve

In the present work, these parameters were established from the SD of the response and the slope of the calibration curve. The LOD for signal to noise ratio was found to be 0.816µg/mL and the LOQ was found to be 2.473 µg/mL for the estimation of Quercetin. The peaks obtained by injecting different concentrations of standard solutions and sample solution indicate similarity of peak area and peak height. This justifies the separation and purity of quercetin from the method as shown in Fig.2-4[12-21].

This validated method was later used to find the presence of quercetin in a commercial Ayurvedic proprietary formulation (KANCHNAR) which has shown

the presence of quercetin in it. The peak height and peak areas of both sample and Ayurvedic drug were found to be almost matching, which justifies the method applicability to Ayurvedic formulations, as seen in Fig. 5[22-23].

CONCLUSION

The proposed analytical method for estimation of isolated quercetin from the extract of *Aerva lanata* (L) using HPLC-PDA was found to be accurate, precise, linear, robust, and reproducible. This method is unique since no reported method was available till date for natural quercetin content isolated from *Aerva lanata* plant and probably not applied to an ayurvedic proprietary medicine to show the content in it which substantiates the claims, both the content and pharmacological activity made by the Ayurvedic therapies in India. This method can be used for the estimation of quercetin in commercial herbal formulations and other Ayurvedic medicines for their quality control. The determined validation parameters for developed method are in the acceptable ranges for the analysis. The good percentage recovery indicates that method is accurate, while the concentration of quercetin can be accurately and precisely quantified with a very low limit of detection. The recovery was found to be 99.91 to 102.65 % which is a good indicator of the method.

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