



Research Article

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RP-HPLC method development and validation of Quercetin isolated from the plant *Tridax procumbens* L.

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Abstract

A simple, precise reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for the flavonoid quercetin, isolated from *Tridax procumbens* L. A simple, cost effective mobile phase consisting of Methanol: 0.1% ortho phosphoric acid (65:35 v/v) was optimized for the experiment. The detection was carried out using variable wavelength UV-VIS detector set at 369 nm. Method was validated according to ICH guidelines. Linearity for the developed method was found over the concentration range 5-30 µg/ml with a correlation coefficient of 0.999. Recovery of 80, 100 and 120% sample was found to be 99.804%. This method can be used for standardization of quercetin from various extracts and preparations.

Keywords: *Tridax procumbens*, quercetin, HPLC, Validation, Method development.

Introduction

Herbal drugs are used extensively in traditional folk medicine under developed area like African continent and developing countries in Asian region like India and China. Biologically active compounds from herbal sources have always been a great interest for scientists working on infectious and non-infectious diseases.¹ *Tridax procumbens* L. (family – Asteraceae) commonly known as *Jayanthi* in Sanskrit. It is native of America and is widely distributed throughout the tropics and sub tropical countries such as India, Brazil, Mexico, Australia, Indonesia, Sri Lanka, Bangladesh, Africa etc.² The plant has number of chemical constituents like alkaloids, tannins, flavonoids like luteolin, quercetin, keampherol, saponins, carotenoids, β-Sitosterol, n-hexane, and various acids like fumaric, lauric, myristic, palmitic, stearic, arachidic, benenic, palmitoic, linoleic acid etc.³ It is known for a number of pharmacological activities like antidiabetic, anti-inflammatory, wound healing, hepatoprotective and antioxidant activity. It shows anticancer activity and is being studied for prostate cancer and skin cancer.^{4, 5} Flavonoids are a group of naturally occurring polyphenolic compounds. They are usually subdivided according to their substituent into flavanols (kaempferol, quercetin), anthocyanins, flavones, flavonones and chalcones.⁶ Quercetin is one such flavonoid which is known to have antioxidant, anticancer, anti-inflammatory and antiviral activity. It is also useful for a variety of cardiovascular diseases.^{7, 8}

Material and Methods

Reagents

Quercetin standard was procured from Yucca Enterprises (Mumbai). HPLC grade Methanol along with AR grade ortho phosphoric acid were procured from SD Fine chemicals (India).

Instrument

HPLC studies were carried out using HPLC binary system with, two *PU2080plus* intelligent HPLC pumps, *UV2075plus* intelligent UV detector, Solvent Mixing module MX-2080-31,

Rheodyne® manual injector system, LcNet II / ADC system interface and Borwin® Chromatography Software. Jasco Corporation, Japan.

Isolation of Quercetin from *Tridax procumbens*

The flowers of *Tridax procumbens* L. were shade dried and grinded to obtain a coarse powder. It was extracted by Soxhlet extraction process with petroleum ether followed by successive extraction with chloroform and methanol. The methanolic fraction so obtained was successively extracted with petroleum ether, diethyl ether and ethyl acetate respectively with the help of a separating funnel. The ethyl acetate fraction was concentrated and hydrolyzed using 7% H₂SO₄ for 5 hrs. The hydrolyzed fraction was filtered and extracted with ethyl acetate by using a separating funnel. It was then concentrated to get the crude quercetin which was later crystallized by dilute ethanol.⁹

Determination of melting point of standard quercetin

Melting point of standard quercetin was found to confirm the purity of the standard.

Preparation of standard Stock Solution

Accurately weighed 25 mg of standard quercetin was transferred to a 25 ml volumetric flask and dissolved in Methanol. Volume was made with methanol to obtain standard stock solution of concentration 1000 µg/ml. This stock solution was further diluted for the studies.

Preparation of sample Stock Solution

Accurately weighed 25 mg of isolated quercetin was transferred to a 25 ml volumetric flask and dissolved in Methanol. Volume was made with methanol to obtain sample stock solution of concentration 1000 µg/ml. This stock solution was further diluted for the studies.

HPLC method development

Standard and the isolated fraction of quercetin were analyzed by HPLC technique using the following conditions (Table 1)

Table 1: Optimized Chromatographic conditions

Parameters	Description
Column	HiQ Sil C18HS
Column size	4.6 mm × 250 mm × 5µ
Mobile phase	Methanol: 0.1% ortho phosphoric acid (65:35%)
Flow rate	1 ml/min
Detector and wavelength	UV detector, 369 nm
Injection loop capacity	20 µl
Concentration of Samples	10 pm (standard and isolated fraction of quercetin)
Retention time	8.4 min
Run time	13 min

HPLC method validation

The developed RP-HPLC method was validated by determination of selectivity, linearity, limit of quantitation and detection, precision, accuracy, recovery, robustness and stability as per the ICH guidelines.¹⁰

a) System suitability studies

System suitability was established by injecting six replicate injections of standard solution of quercetin and the % relative standard deviation (% RSD) of peak areas, resolution factor, tailing factor and theoretical plates were determined.

b) Calibration curve (Linearity)

Linearity was established by triplicate injections of solutions containing standard quercetin.

c) LOD and LOQ (Limit of Detection and Limit of Quantitation)

The LOD and LOQ values were calculated from the calibration curves as $k \text{ SD}/b$ where $k=3$ for LOD and 10 for LOQ. SD is the standard deviation of the response of the minimum detectable drug concentration and b is the slope of calibration curve.

d) Accuracy (Recovery)

Accuracy of the method was studied by recovery experiments using the standard addition method at three different levels (80, 100, and 120%). Known amounts of standard solutions containing quercetin (8, 10, and 12 µg) were added to pre-quantified sample solutions (isolated quercetin) to reach the 80, 100, and 120% levels. These samples were analyzed in triplicate and recovery was calculated. Then the difference between the spiked and unspiked sample was determined for different recovery levels.

e) Precision (Repeatability)

Precision of the assay method was demonstrated by analyzing six different sample solutions containing isolated quercetin equivalent to 10µg/mL and from the area obtained, concentration was calculated, and the results were expressed as %RSD (Relative Standard Deviation).

f) Intermediate precision (Ruggedness)

Intermediate precision of the method was demonstrated by carrying out the experiment on different days, by different analysts, and on different C18 columns.

g) Robustness

Robustness of the method was demonstrated by deliberately varying the chromatographic conditions. The flow rate of the mobile phase was changed from 1.0 to 0.9 mL/min and from 1.0 to 1.1 mL/min. The composition of mobile phase was changed from 65:35 (Methanol: 0.1% ortho phosphoric acid) to 61.1:38.9

(Methanol: 0.1% ortho phosphoric acid) and from 65:35 (Methanol: 0.1% ortho phosphoric acid) to 68.2: 31.8 (Methanol: 0.1% ortho phosphoric acid) (i. e. 5% change). The sample solution for the robustness study was applied onto the column in triplicate, and the responses were determined.

Results and Discussion

Melting point of standard quercetin found to be 316°C (Literature value: 316°C).¹¹ Several mobile phase compositions were tried and a satisfactory separation and good peak symmetry were obtained by using the mobile phase composition methanol: 0.1% ortho phosphoric acid, (65:35 v/v). HPLC profile for both standard and isolated quercetin indicated a single peak at retention time of 8.4 min (figure 1 and figure 2). System suitability tests were carried out on freshly prepared standard solutions (n = 6) quercetin. System suitability parameters obtained with 20 µL injection volumes are summarized in Table 2. Linearity regression data, summarized in Table 3, show a good linear relationship between concentration and peak areas over a concentration range of 5-30 µg for quercetin (Figure 3). The correlation coefficient (R²) was found to be 0.999. LOD and LOQ were found to be 0.203µg/mL, 0.616µg/mL respectively. These values indicate that the method is sensitive. Accuracy studies indicated that the mean recovery of the added standard drug was 99.80% for isolated quercetin. In the precision studies, RSD of mean assay values was found to be 1.02%. The value indicates satisfactory repeatability of the method. The intermediate precision study revealed that the method is rugged with RSD values of 1.00%, 1.87%, 1.37% on different days, by different analysts, and on different column respectively. Specificity studies indicated no interference from impurities, or degradation products, and ensured that the peak response was due to quercetin only. Robustness studies signified that the results of the method remained unaffected by small, deliberate changes in the flow rate and column temperature. The RSD of mean assay values was found to be 1.09% with a flow rate of 0.9 mL/min. and 1.43% with a flow rate of 1.1 mL/min. Also, RSD of mean assay values was found to be 0.27% and 1.36% for the mobile phase compositions 61.1:38.9 v/v and 68.2: 31.8 v/v, respectively. The validation data is summarized in Table 4.

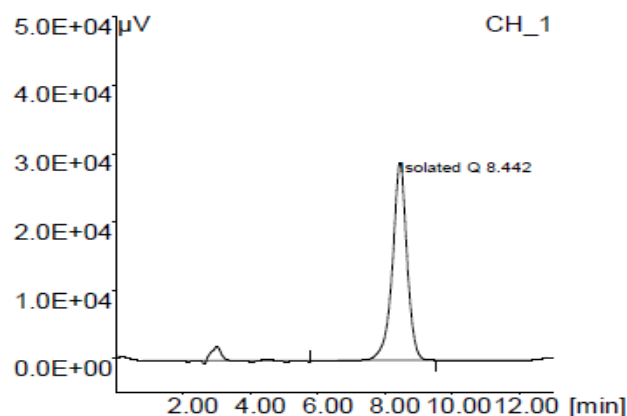


Figure 2: HPLC chromatogram for isolated quercetin

Table 2: System suitability test parameters for quercetin

Parameter	Quercetin (±%RSD)
Retention Time(min)	8.46 ±0.044
Theoretical plates	2284.99 ± 23.80
Asymmetry	0.910 ± 0.0048
Area(mcV/Sec)	937159.8 ± 9474.07

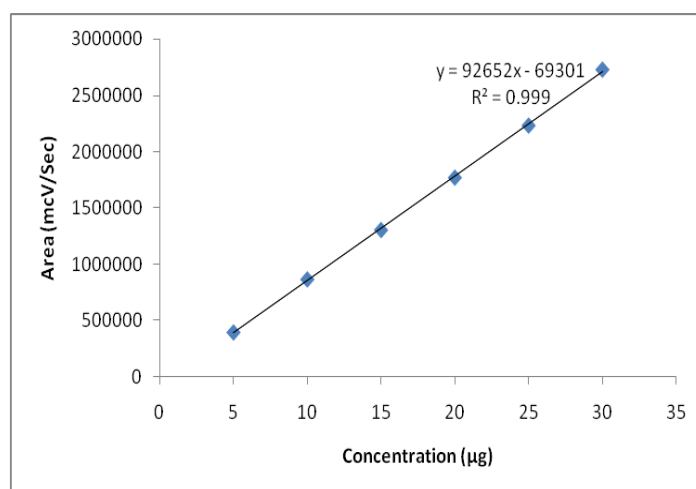


Figure 3: Linearity of quercetin

Table 3: Regression analysis of the calibration curves for quercetin

Parameter	Standard quercetin
Linearity range(µg/mL)	5-30
Regression equation	y = 92652x - 69301
Correlation coefficient (r ²)	0.999
Slope	92652
Intercept	16166

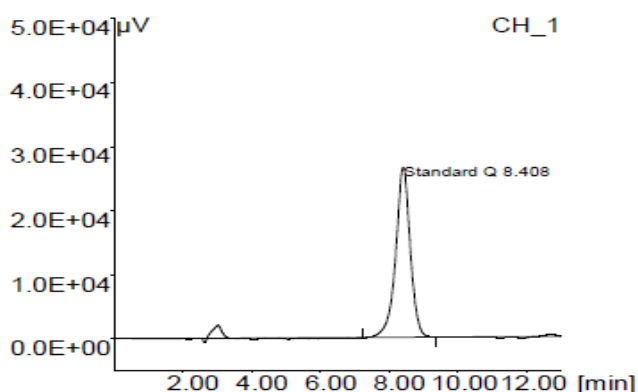


Figure 1: HPLC chromatogram for standard quercetin

Table 4: Summary of validation parameters for the proposed HPLC method for isolated Quercetin

Parameter	Isolated quercetin (\pm RSD)
LOD (μ g/ml)	0.203 μ g/mL
LOQ (μ g/ml)	0.616 μ g/mL
Accuracy(% recovery)	99.80%
Precision ^a	101.47 \pm 1.02
Intermediate precision ^a	
1. Different day	97.80 \pm 1.00
2. Different analyst	94.32 \pm 1.89
3. Different column	99.28 \pm 1.37
Robustness (61.1:38.9 v/v Mobile phase comp.) ^b	98.09 \pm 0.27
Robustness (68.2: 31.8 v/v Mobile phase comp.) ^b	101.83 \pm
Robustness (0.9ml/min Flow rate) ^b	98.36 \pm 1.09
Robustness (1.1ml/min Flow rate) ^b	99.76 \pm 1.43

Conclusion

The proposed HPLC method is simple, rapid, specific, accurate and precise for determination of the flavonoid quercetin. Because of the short chromatographic run time (13 min), the developed method can be adopted for the routine quantification and quality control of quercetin and in *in-vivo* animal studies. This method was found to be better than the reported HPLC method for quercetin.

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