HPTLC METHOD FOR QUANTITATIVE DETERMINATION OF QUERCETIN IN HYDROALCOHOLIC EXTRACT OF DRIED FLOWER OF NYMPHAEA STELLATA WILLD.

Sachin U. Rakesh*, P. R. Patil, V. R. Salunkhe, P. N. Dhabale, K. B. Burade

Govt. College of Pharmacy, Vidyanagar,
Tal. Karad- 415 124, Dist. Satara, M. S.India.
*Corres.author:sachinrakesh@rediffmail.com
Phone no. 09823140715, 09975762933

ABSTRACT: A sensitive and reliable high performance thin layer chromatographic method has been developed for quantitation of quercetin in the dried flowers of *Nymphaea stellata* willd. The hydroalcoholic extract of *N. stellata* was chromatographed on silica gel 60 F_{254} plates with toluene: ethyl acetate: formic acid, 5: 4: 0.2 (v/v/v), as mobile phase. Detection and quantitation were performed by densitrometric scanning at λ= 380 nm, by using deuterium lamp. The accuracy of the method was checked by conducting recovery studies at three different levels, using the standard addition method and the average recovery of quercetin was found to be 99.33%. The proposed HPTLC method provide a good resolution of quercetin from other constituents present in hydroalcoholic extract of dried flowers of *N. stellata* and can be used for quantitation of quercetin present in the dried flowers of *N. stellata*. The method is rapid, simple and precise.
KEYWORDS: HPTLC, quercetin, *Nymphaea stellata* willd.

INTRODUCTION

Nymphaea stellata willd. (Nymphaceae), a medicinal plant has been mentioned for the treatment of liver disorders in Ayurveda, an ancient system of medicine. The leaves, roots and flowers have a wide range of pharmacological activities and are used for diabetes, eruptive fevers and as cardiotonic, emollient, diuretic, narcotic and aphrodisiac[1,2]. The plant also has antihepatotoxic[3], antidiabetic[4], antihyperglycaemic and antihyperlipidaemic[5] activities. The flowers of plant contain[6] flavanoids, gallic acid, astragalin, quercetin and kaempferol. The seeds also contain[7] proteins, pentosan, mucilage etc. A TLC method for detection of flavanoids has been reported in literature.[8] However HPTLC method for quantitation of quercetin from *N. stellata*, has not been reported in literature. Densitometric HPTLC has been widely used for the phytochemical evaluation of the herbal drugs, due to its simplicity and minimum sample clean up requirement. Hence a densitometric HPTLC method has been developed in the present work for quantitation of quercetin from hydroalcoholic extract of dried flowers of *N. stellata*. The extract was prepared by using soxhlet extraction method. The normal phase HPTLC method established in this research work uses aluminum backed silica gel 60 F_{254} plates which are less expensive than reversed-phase, preparative plates.

MATERIAL AND METHODS
Reagents and Standards
Toluene, ethyl acetate and formic acid were procured from Merck, India and acetone of analytical reagent grade was obtained from Qualigens Fine Chemicals (Mumbai, India). Quercetin was procured from Sigma Aldrich, Bangalore, India.

Plant Material
The herbarium of Nymphea stellata Willd. was identified and authenticated from Satara Ayurvedic Arkshala, Satara having voucher no. 648/A dated 30- 06- 2008. The sample was dried in the shade[9], finely powdered and the powder was passed through 80 mesh sieve and stored in airtight container at room temperature (30 ± 2^0 C). About 300 gm of the powder was taken in a Soxhlet extractor and extracted with hydroalcohol. The solvent recovered by distillation. The residue was concentrated, dried and stored in the desiccator for further experiment and analysis.

PREPARATION OF STOCK SOLUTIONS
Preparation of quercetin standard solution
A stock solution of standard quercetin (20 µg/mL) was prepared by transferring 2 mg of quercetin, accurately weighed, into a 100 mL volumetric flask, dissolving in 50 mL methanol. It was then sonicated for 10 minutes and the final volume of the solutions was made up to 100 mL

with methanol to get stock solutions containing 20 µg/mL.

Preparation of sample solution

Accurately weighed 250 mg of dried hydroalcoholic extract of *N. stellata* was transferred to a 100 mL volumetric flask dissolving in 80 mL of methanol. It was then sonicated for 10 minutes and the contents of the flask were filtered through Whatman No. 1 paper (Merck, Mumbai, India). The final volume of the solution was made up to 100 mL with methanol to get stock solution containing 2.50 mg/ mL.

Instrumentation and chromatographic conditions

HPTLC was performed on 20 cm × 10 cm aluminum backed plates coated with silica gel $60F_{254}$ (Merck, Mumbai, India). Standard solution of quercetin and sample solution were applied to the plates as bands 8.0 mm wide, 30.0 mm apart, and 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100-µL Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28 ± 2°C), with toluene: ethyl acetate: formic acid, 5 : 4: 0.2 (v/v/v), as mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried with a hair dryer and then scanned at 380 nm with a Camag TLC Scanner with WINCAT software, using the deuterium lamp. The method was validated according to the ICH guidelines[11].

Calibration curve of quercetin

A stock solution of standard quercetin (20µg/mL) was prepared in methanol. Different volume of stock solution 2, 4, 6, 8 and 10 µL, were spotted on to TLC plate to obtained concentration 40, 80, 120, 160, 180 and 200 ng/spot of quercetin, respectively. The data of peak areas plotted against the corresponding concentration (Figure 2).

METHOD VALIDATION
Precision

Instrumental precision, intra-assay precision, and intermediate precision of the method were determined. Instrumental precision was measured by replicate (n = 10) application of the same quercetin standard solution (concentration 80 ng). Intra assay precision was evaluated by analysis of three replicate applications of freshly prepared standard solutions of same concentration, on the same day. Intermediate precision was evaluated by analysis of three replicate applications of standard solution of same concentration on three different days.
Robustness of the method

By introducing small changes in the mobile phase composition, mobile phase volume and duration of mobile phase saturation, the effects on the results were examined. Robustness of the method was done in triplicate at a concentration level of 160 ng/spot and the % R.S.D peak area was calculated.

Ruggedness

A solution of concentration 160 ng/spot was prepared and analyzed on day 0 and after 6, 12, 24, 48 and 72 h. Data were treated for % R.S.D. to assess ruggedness of the method.

Limits of Detection and Limit of Quantitation

The limits of detection (LOD) and (LOQ) were determined as the amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively.

Recovery studies

The accuracy of the method was established by performing recovery experiments at three different levels using the standard addition method. In 2 µL (2.50 mg/ mL) of sample of *N. stellata*, known amounts of quercetin standard (80, 120 and 160 ng per spot) were added by spiking. The values of percent recovery and average value of percent recovery for quercetin were calculated, which is given in Table 2.

Specificity

The specificity of the method was ascertained by analyzing the standard drug and extract. The spot for quercetin in the sample was confirmed by comparing the Rf values and spectra of the spot with that of the standard. The peak purity of the quercetin was assessed by comparing the spectra at three different levels, viz. peak start, peak apex and peak end positions of the spot.

System Suitability

System suitability tests are performed to verify whether resolution and repeatability were adequate for the analysis. System suitability was determined by applying freshly prepared standard solution of quercetin, concentration 160 ng/spot, five times to the same chromatographic plate. The plate was developed under the optimized chromatographic conditions then scanned and the densitograms were recorded. The measured peak areas for quercetin and their retention factors were noted for each concentration of quercetin and values of the mean peak area, the standard deviation (SD) and the relative standard deviation (%RSD) were calculated.

RESULTS AND DISCUSSION
Development of the optimum mobile phase

The TLC procedure was optimized with a view to quantify the herbal extract. Initially toluene: ethyl acetate: formic acid in varying ratios was tried. The mobile phase toluene: ethyl acetate: formic acid (5: 4: 0.5, v/v/v) gave good resolution with Rf = 0.26 for quercetin but neckless shape separation was occurred. Finally, the mobile phase consisting of (5: 4: 0.2, v/v/v) gave a sharp and well-defined peak at Rf = 0.29 (Fig. 1). Well-defined spots were obtained when the chamber was

saturated with mobile phase for 20 min at room temperature.

The TLC plate was visualized under UV light at 254 nm,[10] without derivatization. A photograph of a TLC plate after chromatography of quercetin standard and a hydroalcoholic extract of the dried flowers of *N. stellata* are shown in Figure 1. The identity of the quercetin bands in sample chromatograms was confirmed by the chromatogram obtained from the sample with that obtained from the reference standard solution (Figure 3 and 4) and by comparing retention factors of quercetin from sample and standard solutions. The peak corresponding to quercetin from the sample solution had same retention factor as that from the quercetin standard (Rf 0.29) (Figure 3 and 4). A preparative TLC method reported in the literature[8] was developed for isolation of quercetin.

Method validation

The calibration plot shown in Figure 2 indicates the response is a linear function of concentration in the range 20 to 200 ng quercetin. The correlation coefficient, intercept and the slope were 0.9998, -1505 and 75.3 respectively.

The measurement of the peak area at three different concentration levels showed low values of % R.S.D. (< 2%) for inter- and intra-day variation, which suggested an excellent precision of the method (Table 1).

The low values of S.D. and % R.S.D obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method (Table 3).

The limits of detection (LOD) and quantification (LOQ) were 2.38 ng/μL and 7.14 ng/μL respectively, which indicate the adequate sensitivity of the method (Table 1).

Results from recovery studies, listed in Table 2, were within acceptable limits (98.0 to 102.0%), indicating the accuracy of the method was good.

Low % R.S.D. value of 0.23 between the peak area values proved the ruggedness of the method indicating that quercetin is stable during the extraction procedure as well as during analysis.

The peak purity of quercetin was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot. Good correlation ($r = 0.9998$) was obtained between the standard and the sample of quercetin (Fig. 3, 4).

In system suitability, the relative standard deviations of the quercetin peak areas and retention factors were 1.32 and 100%, respectively, because these values are < 2. Thus, the method is suitable for purpose.

CONCLUSION

A rapid, simple, accurate and specific HPTLC method for quantitative estimation of quercetin present in the dried flower of *N. stellata* has been developed and validated. The data could be used as a QC standard. The method used in this work resulted in good peak shape and enabled good resolution of quercetin from other constituents of the plant material. Because recovery (99.33%) was close to 100%, there was no interference with the quercetin peak from other constituents present in the plant.

Table 1: Method validation data for HPTLC quantification of quercetin in the dried flowers of *Nymphaea stellata*, willd.

Method property	Value
Linear range [ng/ spot]	20- 200
Correlation coefficient (r)	0.9998
Limit of detection (LOD) [ng/ spot]	2.38
Limit of quantitation (LOQ) [ng/ spot]	7.14
Instrumental precision (RSD [%] n = 10)	0.45
Intra assay precision (RSD [%] n = 3) on the same day	0.31
Intermediate precision (RSD [%] n = 3) on three successive days	0.23

Table 2: Recovery of quercetin from the extract of *N. stellata*.

Level	0	1	2	3
Amount of sample [ng]	250	250	250	250
Amount of standard added [ng]	0.00	80	120	160
Overall average recovery [%]	99.33			

Table 3: Robustness of the HTLC method (n=3, 160 ng/ spot)

Parameter	S. D. of peak area	% RSD
Mobile phase composition (toluene: ethyl acetate: formic acid)	1.27	0.20
Mobile phase volume (18, 20 and 22 mL)	1.24	0.19
Duration of saturation (20, 30 and 40 min)	1.18	0.18

Figure 1: Photograph of chromatograms obtained, at 380 nm, from quercetin standard (A) and a hydroalcoholic extract (B) of the dried flower of *N. stellata* Wild.
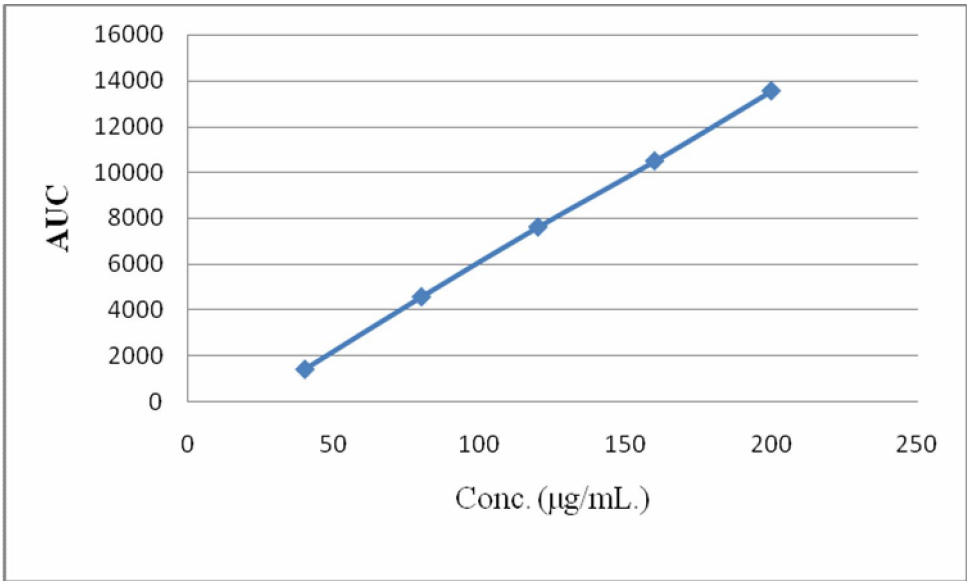

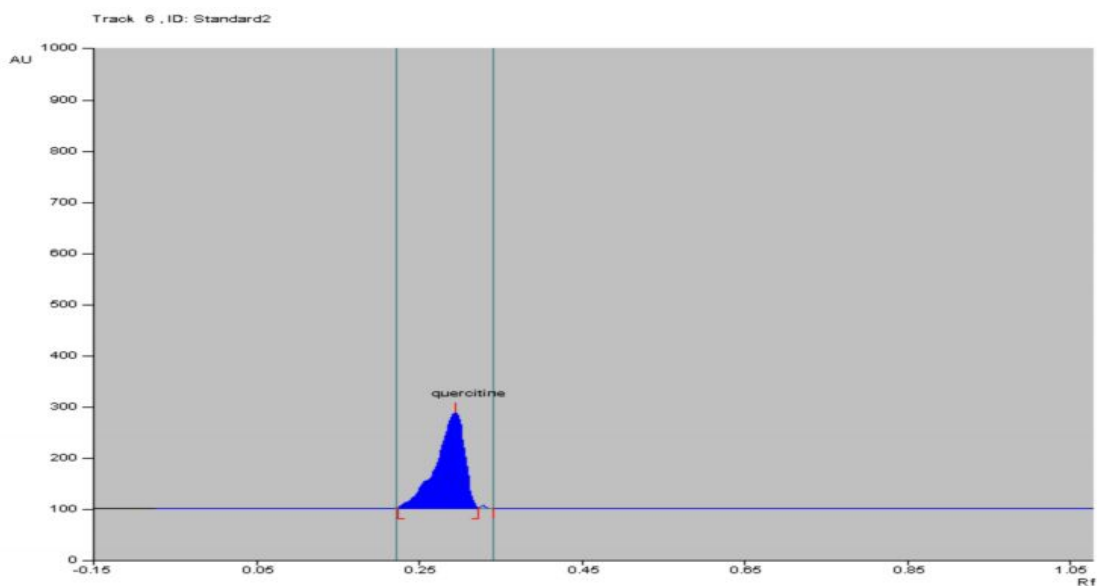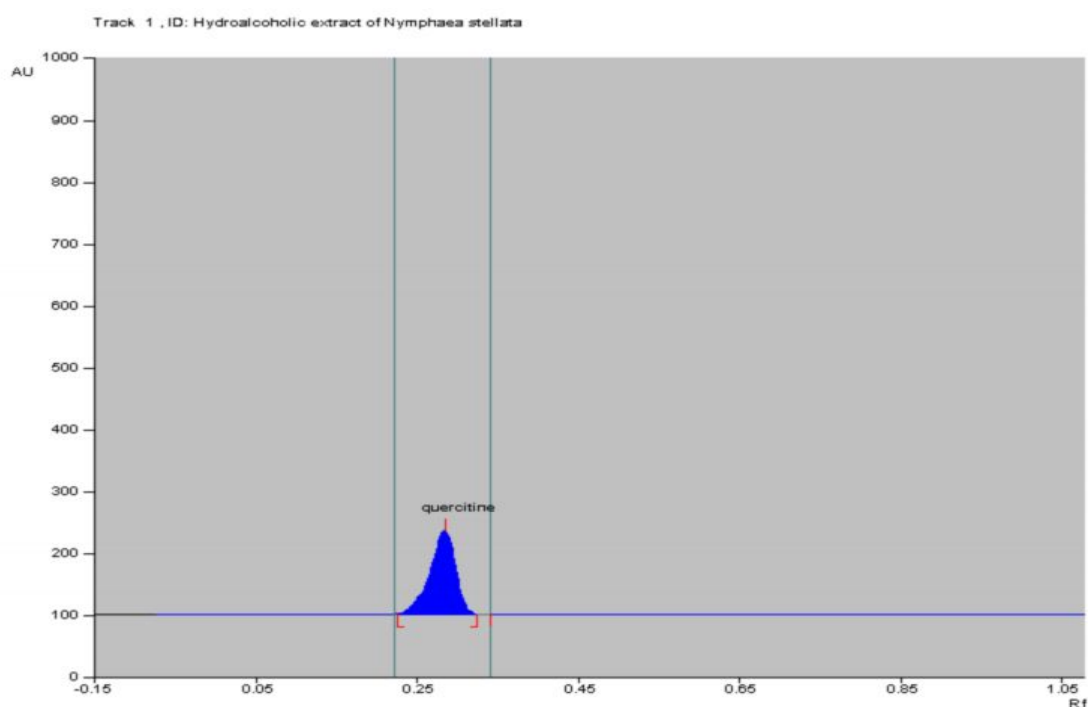**Figure 2: Calibration curve plot for quercetin.**

Track 6 , ID: Standard2

Figure 3: Chromatogram of Standard quercetin.

Track 1 , ID: Hydroalcoholic extract of Nymphaea stellata

Figure 4: Chromatogram of extract of *N. stellata*.

REFERENCES

1. Nadakarni, K.M. vol. I, (3rd Eds), Indian Materia Medica, Popular Book Depot, Dhoot Papeshwar: Bombay, 1954, 860.
2. Kirtikar KR, Basu BD, vol. I, Indian medicinal plants Dehradun: International Book Distributors, 1999, 114.
3. Bhandarkar M. R. et al, Antihepatotoxic effect of *Nymphaea stellata* willd against carbon tetrachloride-

induced hepatic damage in albino rats, J. Ethnopharmacol, 2004, 91, 61–64.

4. Subbulakshmi G, Mridula Naik, Indigenous foods in the treatment of diabetes mellitus, Bombay Hosp J, 2001, 43, 548-561.

5. Rajagopal K, Sasikala K, Antihyperglycaemic and antihyperlipidaemic effects of *Nymphaea stellata* in alloxan-induced diabetic rats, Singapore Med J, 2008, 49 (2), 137.

6. Kizu H, Tomimori T, Phenolic Constituents from the Flowers of *Nymphaea stellata*, Nat Med, 2003, 57 (3), 118.

7. Kapoor, Khan V. P., Raina P. S. H., Farooqui, M. H., Chemical analysis of seeds from 40 non- leguminous species, Part III. Sci. Cult., 1975, 41, 336.

8. Quality Standards of Indian Medicinal Plants, Vol. 3, Published by Indian council of Medicinal Research, New Delhi, 2005, page no. 34.

9. Fried B., Sherma J., Practical Thin layer Chromatography: A multidisciplinary Approach, CRC Press, U. S. A., 1996, 41.

10. Vinatoru M., Toma M., Radu O. et al., The use of ultrasound for the extraction of bioactive principles from plant materials, Ultrason Sonochem, 1997, 4, 135.

11. I.C.H. Guidelines on Analytical Method Validation, In: Proc. Int. Convention on Quality for the Pharmaceutical Industry, Toronto, Canada, Sept., 2002.

*****