CODEN (USA): IJPB07 ISSN: 2320-9267

Indian Journal of Pharmaceutical and Biological Research (IJPBR)

Journal homepage: www.ijpbr.in

Research Article

HPTLC method for isolation, identification and quantification of quercetin from *in vivo* and *in vitro* samples of *Naringi crenulata*

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ARTICLE INFO:

Article history:

Received: 28 March 2018
Received in revised form:
5 May 2018
Accepted: 10 May 2018
Available online: 30June 2018

Keywords:

Naringi crenulata,
callus,
Quercetin,
Leaf,
HPTLC.

ABSTRACT

Naringi crenulata (Roxb.) Nicolson, is a rare medicinal plant belonging to the family Rutaceae. It is a spinous tree and has great medicinal value. During the present study a rapid, simple, accurate and specific HPTLC method for quantitative estimation of quercetin present in the dried leaf powder and callus of *N. crenulata* has been developed .The method used in this work resulted in good peak shape and enabled good resolution of quercetin from *N. crenulata* samples. Quercetin was identified in *in vivo* (leaf) and *in vitro* (six weeks old callus) tissues. Presence of isolated quercetin was further confirmed by superimposable IR spectra of isolated and authentic samples of quercetin and NMR spectra of isolated quercetin. Variation in quercetin content in *in vivo* and *in vitro* samples in *N. crenulata* was observed. *In vivo* leaf had maximum amount of quercetin (0.13%) while minimum amount was found in *in vitro* callus (0.032%). High content of quercetin in leaf shows its potential of synthesizing quercetin. This study is also of practical importance because flavonoid quercetin is the most active of all flavonoids. Many medicinal plants owe their activity to their high quercetin content. Several studies revealed quercetin's significant anti-inflammatory activity due to direct inhibition of initial processes of inflammation. It also has potent antitumor and antioxidant properties including the inhibition of cancer cell proliferation and migration. This study is of practical importance because compound quercetin was firstly reported to be isolated from callus of *N. crenulata.*

Introduction

Flavonoids are a group of polyphenolic compounds possessing low molecular weight that exhibit a common benzo-y-pyrone structure. Flavonoids are synthesized by the polypropanoid pathway with phenylalanine as startup molecule. They are categorized into various subclasses including flavones, flavonols, flavanones, isoflavanones, isoflavanoids, anthocyanidins and catechins[1].

Flavonoids are a broad group of secondary metabolites with varied and important roles in plant physiology as well as they have gained recent interest because of their broad pharmacological activity. One of the prominent and medically most useful properties of many flavonoids is their ability to scavenge free radicals[2]. Flavonoids and other plant phenolics are reported, in addition to their free radical scavenging activity, to have multiple biological activities including vasodilatory, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, antiallergic, antiviral and estrogenic effects[3]. It was reported that flavonoids can improve the blood circulation and lower the blood pressure[4].

Plant flavonoids usually occur in plants as glycosides, although in some circumstances they may occur as free aglycones. They have been used extensively as chemotaxonomic markers. They provide pigmentation for fruits, flowers and seeds to attract pollinators and seeds dispersers. It is well known that antioxidant activity in higher plants has often been associated with phenolic compounds[5]. They assist in plant defence against pathogenic microorganism[6]. The number of flavonoids is constantly increasing due to the structural variation associated with these compounds. Flavonoids have recently been extracted from *Citrullus* colocynthis, *Tabernaemontana heyneana* Wall. and ginger[7-9].

Flavonoid quercetin is the most active of all flavonoids. Quercetin has the same basic chemical structure as all other flavonoids, being a 3-ringed molecule with hydroxyl (–OH) groups attached. Many medicinal plants owe their activity to their high quercetin content. Several studies revealed quercetin's significant anti-inflammatory activity due to direct

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inhibition of initial processes of inflammation. Potent antioxidant activity of quercetin is demonstrated too. Some tests showed antitumor properties of quercetin including the inhibition of cancer cell proliferation and migration[10].

Quercetin has been reported from many plant species viz. *Nymphaea stellata*. The isolation and identification of quercetin by HPTLC, HPLC and TLC studies have also been reviewed by many authors[11-13].

Quantitative estimation of quercetin is important for current research. Consequently, the present study was undertaken for isolation, identification and quantitative estimation of quercetin content from leaf and callus tissues (six weeks old callus) of *Naringi crenulata*.

Materials and Methods

Chemicals

All the chemicals used in the experiments were of analytical grade procured from Merck. Reference standard quercetin was purchased from Sigma Chemicals.

Plant material

The leaf samples and callus (six weeks old callus) of *N. crenulata* used in the present investigation were collected and dried. The dried samples viz *in vivo* leaf and *in vitro* callus (six weeks old callus) were then powdered and analyzed separately for their quercetin contents. Five replicates were taken in each case.

Extraction procedure

Each of the dried and powdered samples viz. *in vivo* leaf and *in vitro* callus was soxhlet extracted in 80% methanol (100 ml/g dry wt) on a water bath for 24 hrs. The solvent was recovered by distillation. The residue was concentrated, dried and stored in the desiccators for further experiment and analysis. The dried residue was taken up in methanol for further analysis. Isolation, identification and quantification of quercetin was carried out by HPTLC, IR and NMR spectral studies in the plant species.

HPTLC Chromatographic conditions

Stationary Phase: Precoated silica gel plates Merck 60 F_{254} (10 x 10, 0.2 mm thickness)

Mobile Phase : Toluene : ethyl acetate: formic acid, 5:4:0.2 (v/v/v)

Spotting device :Linomat V Automatic sample spotter, CAMAG (Switzerland).

Development Mode: CAMAG twin trough chamber, CAMAG Densitometer: TLC Scanner III, CATS software, CAMAG.

Preparation of standard solution

The stock solution of 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 a solution of appropriate range of quercetin.

Instrumentation and chromatographic conditions

HPTLC was performed on 20 cm×10 cm aluminium packed plates coated with silica gel 60 F254 (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 10.0mm 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 ± 2^0c), 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 minutes. After development, the plates were dried in air and sprayed with 5% ethanolic $FeCl_3$ solution and subsequently heated at 120^0 C for derivatization. These plates were scanned and visualized under visible light at 525 nm and UV light at 254 nm and 366nm absorbance/reflection mode using reflection mode by CAMAG Scanner III and CATS software and deuterium lamp was used to analyze the plates.

Calibration curve of the standard

A stock solution of standard quercetin (20µg/ml) was prepared in methanol. Different volumes of stock solution 2,4,6,8 and 10 µl, were spotted on to TLC plate to obtain concentration 20, 40, 60, 80 and 100 µg/spot of quercetin respectively. The working standard was applied on precoated silica gel F_{254} HPTLC plates and the plates were developed as described earlier. The peak areas were recorded. The calibration curve of the standard concentration (X-axis) over the average peak height / area (Y-axis) was prepared to get a regression equation by Win Cats software, which was used for the estimation of quercetin.

HPTLC Quantification of the extracts:

The quercetin content of various extracts was determined by comparing the area of chromatogram with the calibration curve of concentration of standards. The R_f value of standard quercetin (0.65) was compared with the R_f value of the extracts. Quantitative estimation of the plate was performed in the remission/absorption mode at 254 nm, with the following conditions slit width 6.00x0.30mm, micro scanning speed 20mm/s and data resolution 100 µm step. Calibration parameters were as follows: calibration mode- single level, statistics mode-cv, evolution mode- peak height. The average content of the quercetin in different extracts was expressed in percentage.

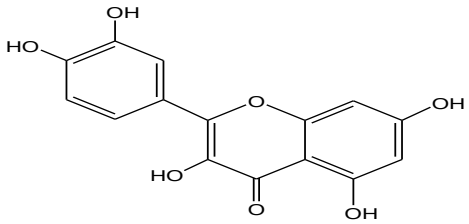
IR and NMR Spectral studies

Each of the fluorescent spots coinciding with those of standard reference compound of quercetin were marked, scrapped and collected separately with the adsorbent from plates. The bands were then eluted with methanol, elutes dried *in vacuo* and crystallized separately with acetone and methanol. Each of the crystallized isolates from all the samples tested were subjected to Infra-red spectrophotometric (Perkin-Elmer 337 Grating, Infra-red spectrophotometer using nujol or potassium bromide pellets) and NMR spectral studies along with respective standard compound of quercetin. The ^{1}H NMR spectra were recorded using Bruker AMX-400 (400 MHz) instruments.

Table 1: Chromatographic data for HPTLC of Quercetin

Sample	Rf	Maximum height	Area	Content (%)
Standard	0.65	708.2	36701.8	100%
Leaf	0.66	71.5	1750.8	0.13%
Callus	0.66	10.8	242.4	0.032%

Structure of Quercetin



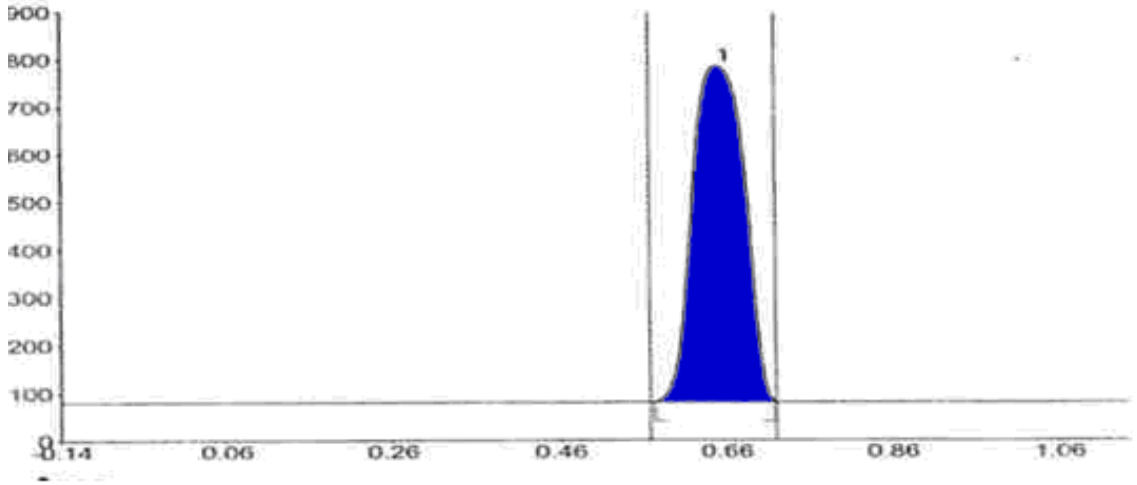


Fig. A: HPTLC chromatogram of standard quercetin

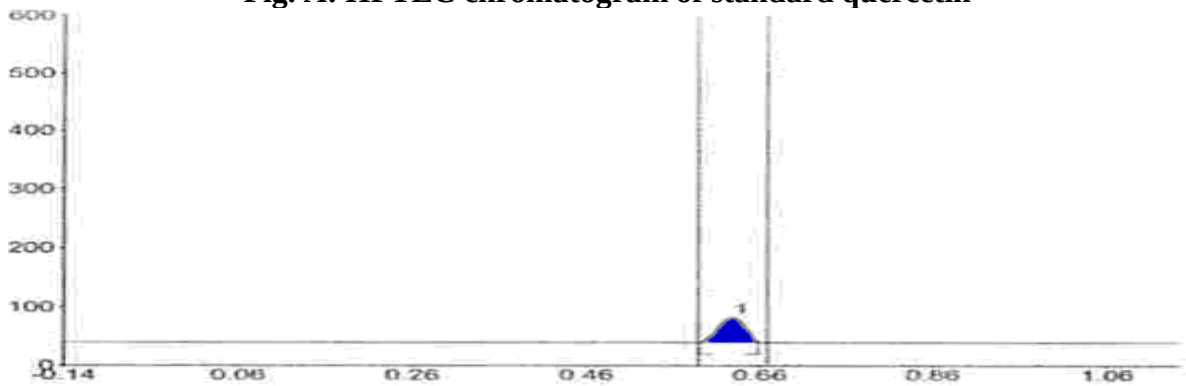


Fig. B: HPTLC chromatogram of quercetin from leaf extract

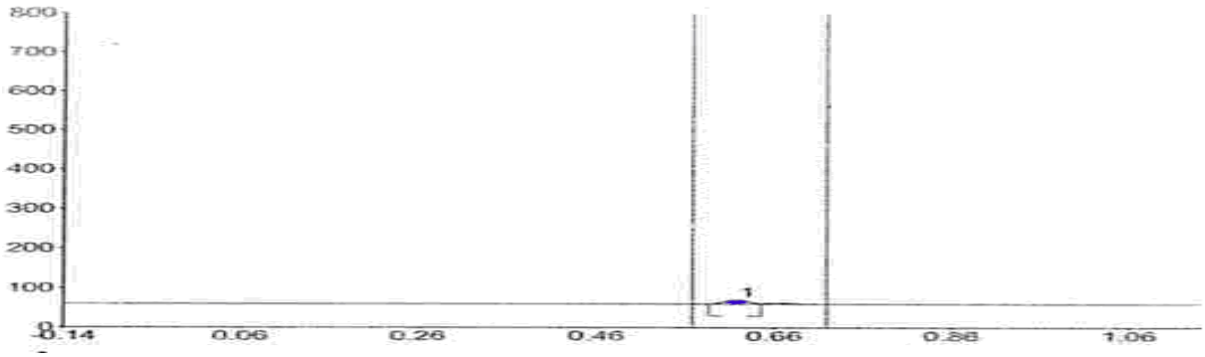


Fig. C: HPTLC chromatogram of quercetin from callus extract

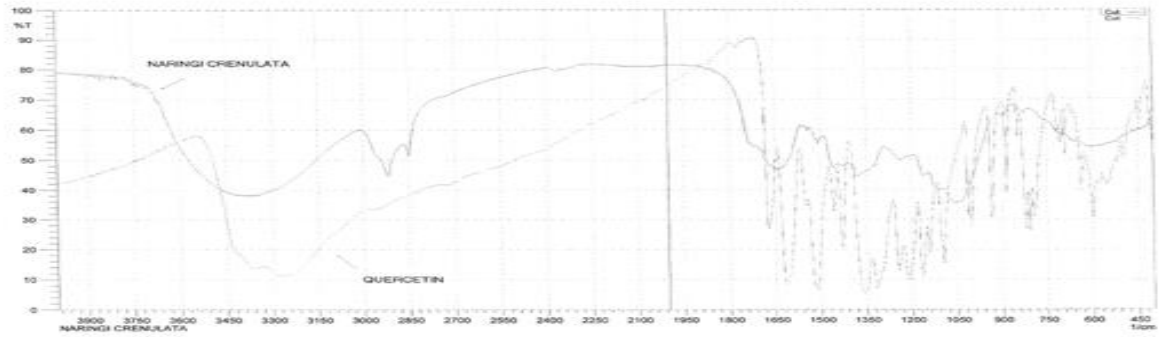


Fig. D: Superimposed IR spectra of standard quercetin and isolated quercetin

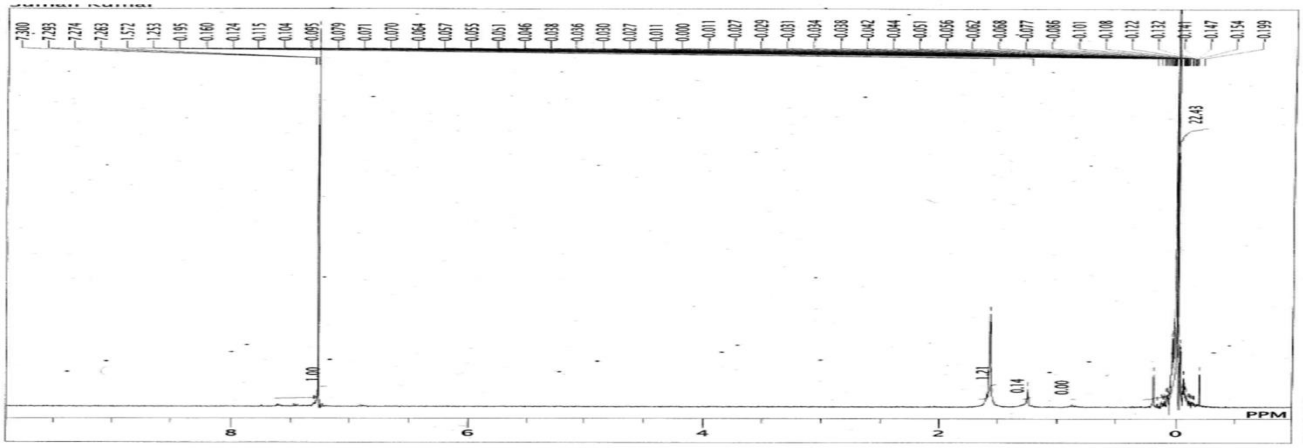


Fig. E: NMR spectra of isolated quercetin

Results

The HPTLC procedure was optimized with a view to quantify the samples extract. Initially toluene: ethyl acetate: formic acid in varying ratio was tried. The mobile phase toluene: ethyl acetate: formic acid, 5:4:0.2 (v/v/v) gave good resolution with R_f = 0.65. Well defined spots were obtained when the chamber was saturated with mobile phase for 20 min. at room temperature. The TLC plates were visualized under UV light at 254 nm after derivatization. A photograph of TLC plate after chromatography of quercetin standard and a methanolic extract of the samples of *N. crenulata* is shown in Figs. A& B. The identity of the quercetin bands in the sample chromatogram was confirmed by the chromatogram obtained from the sample with that obtained from the reference

standard solution. The R_f's obtained for the said plant extracts closely replicated the R_f's found for standard quercetin, thus making it a significant fingerprint parameter. The chromatogram of standard quercetin is shown in Fig A and that of quercetin identified in *Naringi crenulata* leaf and callus sample are shown in Figs. B & C. The respective R_f's obtained for each sample are shown in Table 1. The peak corresponding to quercetin (0.66, 0.66) from the sample solution had almost same retention factor as that of standard quercetin (0.65). The characteristics IR spectral peaks were found to be superimposable with those of their respective standard reference of quercetin [Fig D].The linearity regression for the calibration showed correlation coefficient of 0.99 with respect to height and area in the range of 2.0-10.0 µl and the content of quercetin in methanolic extract of leaf and callus was found to be 0.13% and 0.032 % respectively.In ^{1}H NMR spectra quercetin can be identified by appropriate signals in characteristic range. All phenolic proton (-OH) of quercetin appeared as singlet at δ 4.7 ppm while enolic proton took place out of scale δ 15-17 ppm. All aromatic protons gave unresolved multiplet in expected aromatic region at δ 7.64 ppm [Fig E].

Discussion

During the present study a rapid, simple, accurate and specific HPTLC method for quantitative estimation of quercetin present in the dried leaf powder and callus of *N. crenulata* has been developed .Quantitative analysis is an important tool to provide information of the composition and level of the active components contained in a plant material, in which the major one are generally responsible for some particular pharmacological effects including antioxidant effect [14-15].The result indicated that the method used in this work resulted in good peak shape and enabled good resolution of quercetin from *N. crenulata* samples. The present HPTLC method was sufficient to generate linear regressions with good linearity. Quercetin was identified in *in vivo* (leaf) and *in vitro* (six weeks old callus) tissues. Presence of isolated quercetin was further confirmed by superimposable IR spectra of isolated and authentic samples of quercetin and NMR spectra of isolated quercetin. Variation in quercetin content in *in vivo* and *in vitro* samples in *N. crenulata* was observed. *In vivo* leaf had maximum amount of quercetin (0.13%) while minimum amount was found in *in vitro* callus (0.032%). High content of quercetin in leaf shows its potential of synthesizing quercetin. Similarly the isolation and identification of quercetin by HPTLC, HPLC and TLC studies have also been reviewed by many authors[16-18].

Conclusion

On the basis of the results of the present study, it was concluded that the quercetin was successfully isolated from *N. crenulata*. A rapid, simple, accurate and specific HPTLC method for quantitative estimation of quercetin present in the dried leaf powder and callus of *N. crenulata* has been developed. The method used in this work resulted in good peak shape and enabled good resolution of quercetin from *N. crenulata* samples. The HPTLC fingerprinting is ideal which involves comparison between a standard and a sample. The use of markers like quercetin ensures that the concentration and ratio of components in the plant species are present in reproducible levels.Therefore HPTLC fingerprinting is proved to be a linear, precise, accurate method for herbal formulation and can be further used in quality control of not established herbals. This study is of practical importance because compound quercetin was firstly reported to be isolated from callus of *N. crenulata.*

Acknowledgement

The first and second author acknowledges financial support in the form of UGC-Post Doctoral Fellow for the present research work.

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Cite this article as: **Neelam Singh, Mukesh Kumar Meena , Vidya Patni.** HPTLC method for isolation, identification and quantification of quercetin from *in vivo* and *in vitro* samples of *Naringi crenulata*. **Indian J. Pharm. Biol. Res.2018; 6(2):5-9.**