RP-HPLC and HPTLC Identification and Quantification of Flavonoids in the Leaves Extract of Bauhinia acuminata Linn. (Caesalpiniaceae)

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Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i44A32622

Editor(s):
(1) Ana Cláudia Coelho, University of Trás-os-Montes and Alto Douro, Portugal.

Reviewers:
(1) Ashwani K. Dhingra, Guru Gobind Singh College of Pharmacy, India.
(2) Udaya Sankar Kadimi, CSIR-Central Food Technological Research Institute, India.

Complete Peer review History: https://www.sdiarticle4.com/review-history/74112

Received 06 July 2021
Accepted 16 September 2021
Published 18 September 2021

ABSTRACT

Aims: The present investigation is aimed at the identification and quantification of flavonoids in the methanol extract of leaves of Bauhinia acuminata Linn. (Caesalpiniaceae) by a validated HPTLC method and confirmation of the same by RP-HPLC.

Methodology: CAMAG HPTLC system with VisionCATS software and HPLC Agilent Infinity 1260 were employed for the study. Pre-coated silica gel 60 F₂₅₄ plates were used as stationary phase and Toluene : Ethyl acetate : Formic acid (5:4:0.2, v/v/v) was used as mobile phase in HPTLC while in HPLC Thermo hypersil BDS C18 column and Methanol and 0.4% Phosphoric acid (65 : 35) were used as stationary and mobile phase respectively.

Results: Among different standards used in HPTLC, only quercetin was found to be present and further quantitatively analyzed. The method shows good correlation coefficient (R² ≥ 0.99) and linearity in the range of 0.5 to 5.0 µg/band. The concentration of quercetin in the test extract was found to be 0.514 µg/mg of the extract. The LOD and LOQ values were 0.84 µg/band and 2.59 µg/band respectively. The RP-HPLC study also represented good % RSD of Retention time (0.025) and Area (0.09) which signifies the high precision and
repeatability. The assay result (4.99 %) also indicated good presence of the quercetin in the extract.

Conclusion: The methods were rapid, cost effective and may be employed for quality-control and standardization of quercetin in different plant extracts.

Keywords: HPTLC; HPLC; quercetin; Bauhinia acuminata.

1. INTRODUCTION

From the time immemorial plants find their use in medicinal fields and even today, in the modern age, they find extensive application in pharmaceutical industry [1]. But owing to the complex chemical nature of secondary plant metabolites, these types of formulations suffer huge problems for their standardization and subsequently quality control parameters which are chiefly dependent on the bioactive compounds [2-4].

Modern analytical techniques such as HPLC and HPTLC can be employed for separation and identification followed by both qualitative and quantitative analysis of different constituents in the plant extract or formulations [4-7]. Over the past decades HPLC has proved its analytical excellence due to its high sensitivity and accuracy and therefore it has been a method of choice for estimation of phytoconstituents [5] and on the other side, HPTLC being a very popular tool for quality-control of herbal drugs, enjoys some important advantages such as high sample-throughput, minimum sample clean-up and less time consumption which makes it highly cost effective and even low volume of mobile phase is sufficient for simultaneous analysis of mixture of different compounds [8-10].

Flavonoids are the very important phytoconstituents and owing to its antioxidant activities, they play key role in the modern treatment regime of the many of chronic degenerative diseases [11]. During past few decades these phytoconstituents along with many other polyphenols are extensively explored for their healing properties [12,13]. Quercetin, Kaempferol, Rutin etc. are very common flavonoids and considered as important marker compounds in this class which are precisely analyzed for their presence, identification and quantification in the different phyto extracts by HPTLC or HPLC [14-17].

Bauhinia acuminata Linn. (Caesalpiniaaceae), commonly known as Dwarf white bauhinia in English, is an erect shrub bearing bi-lobed leaves, white fragrant flowers and pods as fruits. It is distributed throughout India [18]. Different parts of the plant have been reported to have antibacterial [19], anti diabetic [20], antioxidant [13], anti-nociceptive and anti-cancer activity [21]. Chemically, methanol extract of Bauhinia acuminata leaves shows strong presence of flavonoids, other phenolic compounds, alkaloids and sterols [13, 21].

From the literature survey it is clear that no modern analytical study employing HPTLC and HPLC has been done on this species. Here in the present study an attempt has been made for identification of important flavonoid compounds present in the methanol extract of the leaves of Bauhinia acuminata Linn. (Caesalpiniaaceae) and their quantitative estimation by HPTLC; followed by further confirmation by HPLC.

2. MATERIALS AND METHODS

2.1 Collection and Identification Plant

Leaves of Bauhinia acuminata Linn. (Caesalpiniaaceae) were collected from local area of Barasat, West Bengal, India and was identified and authenticated from Botanical Survey of India, Howrah, India. A sample herbarium is deposited in the Dept. of Pharmacognosy for future reference.

2.2 Preparation of the Leaves Extract

The plant material was shade dried and powdered. 200 g of powdered material was extracted using methanol by maceration method. The extract was filtered and dried under reduced pressure to get the concentrated extract. This extract was kept in desiccator for 3 days to get fully dried extract powder.

2.3 Reagents and Standards

All chemicals and solvents of analytical and HPLC grade and were purchased from Merck (Germany). Quercetin and other standards used in the study were obtained from Sigma-Aldrich.
(Bangalore, India) and precoated TLC plates were obtained from Merck.

2.4 HPTLC Study [8-10]

2.4.1 Preparation of Standard Solutions

Five different standard compounds from flavonoid class namely Kaempferol, Rutin, Quercetin, Gallic acid and Myricetin were used in the study. Stock solutions of standards were prepared in methanol just before use. Final concentration of each standard solution was 1 mg/ml.

2.4.2 Instrumentation and chromatographic conditions

The whole study was divided into two parts, first determination of the presence of marker compounds such as Kaempferol, Rutin, Quercetin, Gallic acid and Myricetin in the plant extract. The second part was based on the findings of the first part that is the quantification of the present or identified biomarker/s in the same extract.

2.4.3 Determination of flavonoids by HPTLC fingerprinting

HPTLC study was done by CAMAG HPTLC system (Muttenz, Switzerland). 2 µl of each of standard markers (1 mg/ml) and 7 µl and 10 µl of sample extract were applied as bands (dosage speed 150 nl/s using compressed air; 11.4 mm apart, distance from the bottom edge 8 mm and from plate side at least 15 mm) at 25 ± 5 °C temperature. The chromatograms were documented using TLC UV Cabinet 4 (CAMAG) after drying and the evaluation of chromatograms were done at different wavelengths such as 254 nm (Deuterium lamp), 366 nm (Mercury lamp) and 416 nm (Tungsten lamp) in a TLC scanner 4 controlled through visionCATS software (CAMAG; slit width 6 x 0.45 mm, scanning speed 100 mm/s). All the specifications are as shown in Tables 1 and 2.

2.4.4 HPTLC system

HPTLC is done by CAMAG HPTLC system (Muttenz, Switzerland) having Linomat 5 applicator with Hamilton (100µl) syringe. Twin trough development chamber and TLC scanner-4 were employed for the study. The system software was VisionCATS (Version 3.0.20154.1). Silica gel 60 F254 pre-coated plates (10 x 10 cm) were used as stationary phase to apply standards and tests as bands (8 mm) for 9 cm of solvent front. Toluene : Ethyl acetate : Formic acid (5:4:0.2, v/v/v) was used as mobile phase for which the saturation time of the chamber was 20 minutes. Post development drying was done at room temperature for 5 minutes.

2.4.5 Quantification of flavonoid – Quercetin in the extract

This HPTLC study was also studies as describes above with some change or modification. Here different volumes of standard Quercetin (0.5, 1, 2, 3, 4 and 5 µl) and 10 µl of sample extract were applied as bands (dosage speed 150 nl/s using compressed air; 11.4 mm apart, distance from the bottom edge 8 mm and from plate side at least 15 mm) at 25 ± 5 °C temperature. The chromatograms were documented using TLC UV Cabinet 4 (CAMAG) after drying and the evaluation of chromatograms were done at different wavelengths such as 254 nm (Deuterium lamp), 366 nm (Mercury lamp) and 371 nm (Tungsten lamp) in a TLC scanner 4 controlled through visionCATS software (CAMAG; slit width 6 x 0.45 mm, scanning speed 100 mm/s). All the specifications are as shown in Tables 1 and 2.

2.4.6 Calibration curve of quercetin

Different volumes of stock solution (1000 µg/ml) were spotted on the TLC plate to obtain concentrations 0.5, 1, 2, 3, 4 and 5 µg /spot of quercetin, respectively. The data of peak areas plotted against the corresponding concentration.

2.4.7 Method validation

The method was validated as per ICH guidelines [22].

2.4.7.1 Linearity

It was determined by analyzing 6 independent doses of standard quercetin ranging from 0.5 to 5 µg/band. The calibration curve was plotted and correlation coefficient and regression line equations were determined. Linearity is expressed in terms of correlation coefficient of linear regression analysis.

2.4.7.2 Repeatability and Precision

Standard quercetin (3µg/band) was spotted six times, the peak area was measured and it was
expressed as % relative standard deviation (%RSD).

2.4.7.3 Limits of detection and limit of quantization

These were calculated using the standard deviation of the response (SD) and the slope (S) of the calibration curve using the following formula.

\[
\text{LOD} = 3.3 \times (\text{SD}/\text{Slope}) \\
\text{LOQ} = 10 \times (\text{SD}/\text{Slope})
\]

2.4.7.4 Specificity

By analyzing the standard compound and the test extract, the method specificity was ascertained. It involved the analyzing of \(R_f\) values, spectra and peak values of standard compounds and the test extract. Spectra were analyzed in three levels such as start, apex and end to assess the peak purity.

2.5 RP-HPLC Study [5]

The presence of quercetin was further confirmed by RP-HPLC.

2.5.1 Standard and sample preparations

10.08 mg of standard quercetin was weighed and dissolved in 50 ml of methanol, from which 5 ml was taken and the volume was made up to 50 ml. which produced the final concentration of 0.02016 mg/ml. 10.26 mg of dried extract was weighed and dissolved in 50 ml of methanol to produce final concentration of 0.2052 mg/ml. Finally 0.45 μm millipore filter was used to filter both the solutions prior to HPLC analysis.

2.5.2 HPLC Instrumentation and chromatographic conditions

The study was performed using HPLC Agilent Infinity 1260 (Karlsruhe, Germany) instrument with Quaternary Pump G7111B and VWD G7114A detector. The column (stationary phase) used was Thermo hypersil BDS C18 and mobile phase was Methanol and 0.4% Phosphoric acid (65: 35). Injection volume was 20 μl following Isocratic elution at flow rate of 1 ml/min. The detection was done at 190 – 400 nm, (369 nm).

3. RESULTS AND DISCUSSION

3.1 HPTLC Study

3.1.1 Optimization of mobile phase

Various combinations of solvents were tried to select and optimize mobile phase based on the literatures and trial and error methods. The aim was to run and obtain good \(R_f\) values for all the five standard flavonoids in a single mobile phase. Finally the combination of toluene, ethyl acetate and formic acid at the ratio of 5:4:0.2 v/v/v respectively resulted in the desired separation and good resolution. So this was selected as mobile phase for the further study.

3.1.2 Determination of flavonoids by HPTLC fingerprinting

\(R_f\) values were scanned at 254 nm, 366 nm and 416 nm. Here 254 nm scanned results are given in the Table 1. The 3D chromatogram and the plate of the same are given in Fig.1 and Fig. 2.

| Track | Particulars | \(R_f\) value |
|-------|-------------|---------------|
| 1     | Kaempferol  | 0.515         |
| 2     | Rutin       | 0.006         |
| 3     | Quercetin   | 0.35          |
| 4     | Gallic Acid | 0.135         |
| 5     | Myricetin   | 0.21          |
| 6 and 7 | Test extract | 0.004 |
|       | (Bauhinia acuminata) | 0.043 |
|       |             | 0.343         |
|       |             | 0.413         |
|       |             | 0.872         |
|       |             | 0.932         |
|       |             | 0.967         |
Fig. 1. 3D chromatogram of all standards (track 1 to 5) and test extract (track 6 & 7) scanned at 254 nm

Fig. 2. Pictogram of developed HPTLC plate of all standards (track 1 to 5) and test extract (track 6 and 7) scanned at 254 nm
The \( R_f \) values of all the standard compounds were matched with that of obtained in the test extract. 7 major peaks were found in the test extract when scanned at 254 nm and out these only two had similar \( R_f \) values when compared to the standards used. Rutin and Quercetin were found to be present in the test extract by comparing their \( R_f \) values. The values were 0.006 (standard) and 0.004 (test extract) for Rutin and 0.350 (Standard) and 0.343 (test extract) for Quercetin. But as the resolution for Rutin was not very good and conclusive so this was neglected and the further work for quantification was carried out with quercetin only.

### 3.1.3 Quantification of flavonoid – quercetin in the extract

Presence of quercetin in the test extract was again confirmed by comparing the \( R_f \) values of standard quercetin and test extract scanned at 254 nm, 366 nm and 371 nm in the same plate. The value for the standard quercetin was found to be 0.403833 ± 0.006555 and in the test extract similar value corresponding to quercetin was obtained as 0.405.

All the findings are shown in the Table 2 which shows that it possessed good linearity in the range of 0.5 to 5.0 µg/band and the correlation coefficient ≥ 0.99. The concentration of quercetin in the test extract of *Bauhinia acuminata* was found to be 0.514 µg/mg of the extract. Fig. 3 shows the calibration curve of standard quercetin. Different chromatograms are given in Figs. 4 to Figs. 7.

For the repeatability study quercetin was applied 6 times (3 µg/band) and the area was found to be 0.02730333 ± 0.00020666. The % RSD for the repeatability study was found to be 0.75.

![Calibration curve of standard Quercetin](image)

**Fig. 3. Calibration curve of quercetin**

| Parameters                  | Values                     |
|-----------------------------|----------------------------|
| \( R_f \)                   | 0.403833 ± 0.006555        |
| Regression equation         | \( y = 0.0074x + 0.0047 \) |
| Correlation coefficient (\( R^2 \)) | 0.9919                   |
| Linearity range             | 0.5 – 5 µg/band           |
| LOD                         | 0.85 µg/band              |
| LOQ                         | 2.59 µg/band              |

**Table 2. Method validation data**
3.2 RP-HPLC study

20 μl of standard quercetin solution was injected five times and the retention time and area was noted. The % RSD was found to be 0.025 and 0.09 respectively for Retention time and Area which signifies the high precision and repeatability. The Retention time and Area were found to be 4.587 ± 0.001 (Mean ± SD) and 983.78 ± 0.93 (Mean ± SD) respectively.

For the assay, 20 μl of standard quercetin solution was injected the peak retention time was noted as 4.592 and the peak area was found to be 982.973. The same method was repeated for the test extract of Bauhinia acuminata which resulted in the confirmation of presence of quercetin at retention time 4.594 which was identical with that of the standard. The area of the same was calculated as 500.127. The assay result was found to be 4.99 % which indicates good presence of the quercetin in the leaves extract of Bauhinia acuminata. HPLC chromatograms of standard quercetin and the test extract are shown in Figs. 8 and 9 respectively.
Fig. 6. 3D chromatogram of standard quercetin (track 1 to 6, 0.5 – 5 µg/band) and test extract (track 7, 10 µg/band) scanned at 254 nm

Fig. 7. Pictogram of developed HPTLC plate of standard quercetin (all tracks) and test extract (scanned at 254 nm)
The HPTLC investigation shows good separation of standard kaempferol, quercetin, gallic acid and myricitin, whereas rutin didn’t run properly in the mobile phase tested. Among these, presence of quercetin has only been identified by comparing the Rf values of test extract with that of the standard which was further confirmed by the RP-HPLC.

Quercetin and few other flavonoids are considered biomarkers for many of plant species since very long. Different multifaceted therapeutic effects are now very common for quercetin. Some of the mentions worthy activities are antioxidative, anti-inflammatory, cardio protective, hepatoprotective and even anticancer activities [10].
Different combinations and ratios of solvents, both for HPLC and HPTLC, have been reported for the detection of various flavonoids in the different types of plant extract [4,5,10,23,24]. Therefore, in this study, the major peaks obtained in the chromatogram of test extract may be of different flavonoids, phytochemically presence of which has been already reported [13,21].

Though few therapeutically potential activities of *Bauhinia acuminata* is recently being recognized but these are in primary level only and needed to be recognized by research with scientific rationale. Here in this study, to the best of our knowledge, a HPTLC method has been developed for identification and quantification of few possible important biomarkers of flavonoid class among which presence of quercetin is successfully detected and quantified, further the presence of the same is also confirmed by the RP-HPLC study.

4. CONCLUSION

The quantitative analytical methods of HPTLC and HPLC have revealed the presence of quercetin, a well-known flavonoid, in the leaves of *Bauhinia acuminata* Linn. (Caesalpiniaceae) when extracted employing methanol as solvent. Simultaneously the study also indicated the absence of compounds like gallic acid and myricitin. The quantitative analysis of quercetin present in the extract also makes it eligible to be called as a biomarker for the leaves of *Bauhinia acuminata* Linn. (Caesalpiniaceae). In addition, the findings from this study scientifically endorse the traditional applications in folk medicines and also some of the reported activities of this plant. Further research towards the identification, isolation and characterization of the other biomarkers present in the plant and application of such modern chromatographic analytical method to standardize them may lead to the integration of this plant into the field of mainstream medicine.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ACKNOWLEDGEMENT

The authors express their sincere gratitude to the authorities of BCDA College of Pharmacy & Technology, Hridayapur, Kolkata, West Bengal, India, Netaji Subhas Chandra Bose Institute of Pharmacy, Chakdaha, Nadia, West Bengal, India and Department of Pharmaceutical Technology, NBU, Darjeeling, West Bengal, India.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle4.com/review-history/74112