Pharmacogn. Res., 2025; 17(1):201-206.
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Original Article

A HPTLC Method for the Quantification of Stigmasterol Isolated from *Adenoon indicum* Dalz: Development and Validation

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ABSTRACT

Background: *Adenoon indicum* Dalz, a member of the *Asteraceae* family, is a substantial source of phytosterol chemicals, including stigmasterol. The plant traditionally used in Maharashtra, India, for ulcers, gastrointestinal irritations, and wounds, lacks a documented phytochemical profile. **Objectives:** The absence of standardization for *Adenoon indicum* Dalz leaves and extracts complicates quality monitoring. This study aims to develop a straight forward, novel, dependable, and precise HPTLC technique for quantifying stigmasterol in *Adenoon indicum* Dalz leaf extract. **Materials and Methods:** The isolation and measurement of stigmasterol were performed using HPTLC plates covered with Silica gel 60 F_{254} as the stationary phase. Toluene, ethyl acetate, methanol, and formic acid were combined in the mobile phase in a 3:3:2:1 volumetric ratio. The detection and quantification were performed by densitometric scanning at 265 nm. **Results:** An HPTLC technique was established using toluene, ethyl acetate, methanol, and formic acid in a ratio of 3:3:2:1 (v/v/v/v) as the optimal mobile phase. TLC plates at 490 nm revealed a singular phytochemical with an Rf value of 0.894±2.21. The approach demonstrated selectivity for stigmasterol in the extract of *Adenoon indicum* Dalz, as shown by the overlay of UV spectra. The technique was verified for linearity, precision, accuracy, Limit of Detection (LOD), and Limit of Quantification (LOQ). It exhibited linear calibration curves (400-1200 ng/band, r^2>0.995), % RSD < 2%, LOD and LOQ of 889.823 and 2696.433 ng/band, respectively, and a recovery rate of 98.2-100%. The methodology demonstrated robustness, with a relative standard deviation percentage of under 5%. **Conclusion:** A straightforward and sensitive HPTLC approach was validated for the detection of stigmasterol in *Adenoon indicum* Dalz, providing advantages in terms of time and cost-effectiveness.

Keywords: *Adenoon indicum* Dalz, High-performance thin-layerchromatography Stigmasterol.

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Received: 22-10-2024;
Revised: 15-11-2024;
Accepted: 03-12-2024.

INTRODUCTION

A significant number of contemporary research investigations are focused on the phytochemical analysis of higher plants and their ethnopharmacological significance. Phytochemical methods have progressed significantly and are essential for the identification of phytoconstituents. Plant systematics is a novel discipline focused on the discovery and characterisation of secondary metabolites in plants, including flavonoids and phytosterols.[1,2]

The leaves of this medicinal plant, which is extensively used in traditional medicine in tropical locations such as Africa, India, and China, contain active compounds such as triterpenoids, glycosides, and flavonoids. These compounds contribute to the plant's many medicinal properties, which include antibacterial, anti-inflammatory, wound healing, and antihypertensive effects. The herb has long been used to cure a wide range of ailments, including skin conditions, kidney stones, and allergies. It is also well known for its immunomodulatory, analgesic, and sedative effects.[3,4]

Chromatography is an important analytical method for identifying herbal drugs because it can separate, detect, and analyze complex phytochemical combinations. It is particularly helpful for identifying active compounds, ensuring quality control, and determining the legitimacy of herbal products, making it an essential tool in the study and development of herbal treatments.[5,6]

Nowadays, High-Performance Thin-Layer Chromatography (HPTLC) is a useful method for detecting herbal medications since it enables for precise separation and quantification of complex plant extracts. It is often used to fingerprint and

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DOI: 10.5530/pres.20251992

standardize herbal remedies, assuring the purity, validity, and uniformity of active components, which is essential for safe therapeutic uses.[7,8]

To the best of our knowledge, there is no recognized approach in the literature for detecting stigmasterol in the methanolic extract of *Adenoon indicum* Dalz. As a result, the purpose of this study is to develop a previously unpublished HPTLC technique for measuring stigmasterol in *Adenoon indicum* Dalz. Recent advances in chromatographic and spectral fingerprinting are critical for ensuring the quality of complicated herbal medications. High-Performance Thin Layer Chromatography (HPTLC) has become a popular analytical method because to its low cost, high sample throughput, and capacity to quantify analytes at the micro and nanogram level. HPTLC provides quick analysis, little sample clean-up, and simultaneous evaluation of several samples, making it an excellent tool for fingerprinting active chemicals in herbal extracts, complementing procedures such as HPLC and GC.[6]

The created approach was improved and verified in compliance with the requirements of the International Conference on Harmonization (ICH). We developed a simple, High-Performance Thin-Layer Chromatography (HPTLC) approach for rapidly detecting stigmasterol in the *Adenoon indicum* Dalz. This approach worked well for quickly screening plant materials for genotypic assessment without the requirement for extra sample processing.

MATERIALS AND METHODS

Plant material

In November and December, the fresh foliage of the *Adenoon indicum* Dalz plant were collected from the Western ghat Amboli in the Kolhapur district of Maharashtra, India. The specimen, with the identification number BSI / WRC / Iden. Cer. / 2022 / 0209220020684, was obtained from the Botanical Survey of India, Pune, western regional center. The plant leaves were desiccated in the shade, stored at room temperature, and subsequently pulverized to a 40 mesh as required.

Preparation of extract

In a Soxhlet apparatus, 100 g of dried leaf powder was extensively defatted with petroleum ether for 24 hr at 40°C. After the solvent was extracted under pressure, a dark greenish material known as petroleum ether extract was produced. Before being extracted using solvents of increasing polarity concentrated at lower pressure and properly labeled, the resulting marc was allowed to dry at room temperature in the shade. The top layer of liquid was filtered through Whatman filter paper No. 1 after the petroleum ether extract was saponified with 1M alcoholic KOH to exclude fatty particles. A rotary evaporator was used to evaporate the solvent, producing 2 g of methanol extract, which was then obtained as an HPTLC sample.[9]

Standard preparation

A stock solution of stigmasterol (1 mg/mL) was prepared by dissolving 10 mg of the accurately weighed compound in a 1:1 (v/v) mixture of methanol and water, followed by dilution to 10 mL in a standard volumetric flask. The dilution of the stock solution produced a viable solution with a final drug concentration of 300 ng/mL. The working solution was filtered through a 0.45 µm membrane filter (Millipore, USA) prior to application on a TLC plate.

Experimental

HPTLC Analysis

The TLC study utilized pre-coated silica gel $60F_{254}$ plates with dimensions of 20 × 10 cm and 10 × 10 cm. The TLC plates were developed utilizing a mobile phase composed of toluene, ethyl acetate, methanol, and formic acid in a volumetric ratio of 3:3:2:1. A glass Thin-Layer Chromatography (TLC) chamber measuring 20 × 10 cm was utilized to saturate the mobile phase for a duration of 30 min. Samples were deposited onto TLC plates utilizing a sample applicator equipped with a 100 µL microliter Syringe (ILS) and controlled nitrogen flow. The plates underwent densitometric scanning with a CAMAG TLC Scanner, followed by data analysis utilizing WINCATS software.

Preparation of stock solution

The stock solutions of standard stigmasterol (1 mg/mL) were meticulously prepared utilizing Analytical Reagent (AR) grade methanol.

Preparation of test sample

Analytical reagent-grade methanol broke down 1 mg/mL *Adenoon indicum* Dalz extracts.

Procedures

The TLC plates underwent pre-washing with methanol and were activated at 110°C in a dry heat oven for 10 min prior to utilization. The extracts of *Adenoon indicum* Dalz and markers were applied to pre-washed, activated Thin-Layer Chromatography (TLC) plates using a sample applicator. The TLC plates were subsequently developed using a mobile phase consisting of toluene, ethyl acetate, methanol, and formic acid in a 3:3:2:1 volume ratio. The development distance on the TLC plates was maintained at 7 cm. The bands on the plates were scanned at a wavelength of 265 nm, and the R_f values for each band were recorded.

Methods validation

For optimal chromatographic separation, it is essential to validate the analytical method. The optimized HPTLC method underwent validation in accordance with ICH recommendations Q2 (R1).[10]

Specificity

Markers and *Adenoon indicum* Dalz extract were applied to a TLC plate to establish the method's specificity. Furthermore, in order to ascertain the purity of stigmasterol peaks, the beginning, middle, and ending points of each chromatogram were examined.

Linearity

The stigmasterol and *Adenoon indicum* Dalz samples were applied to a TLC plate in distinct working solutions, with each band ranging from 400 to 1200 ng. The peak area was plotted against the marker concentration to determine the slope and correlation coefficient (R^2).

Limit of Detection and Limit of Quantification

The slope of the calibration curve and the Standard Deviation (SD) of the peak areas for each marker were used to calculate the limits of detection and quantification.

Precision

Intraday precision was assessed by applying six replicates of each marker at three distinct concentrations on an HPTLC plate. This study of intraday precision involves testing each concentration six times across various days.

Robustness

The robustness of the optimized HPTLC method was evaluated by implementing minor modifications in the mobile phase volume (±2 mL) and saturation time (±2 min). The results were expressed as a percentage of the Relative Standard Deviation (% RSD).

Accuracy

The recovery research using the stigmasterol standard addition methodology was used to assess the accuracy of the procedure. Stigmasterol was included in three distinct quantities (400 ng/band, 600 ng/band, and 800 ng/band) into the extract of *Adenoon indicum* Dalz. The mixes were examined for peak regions, and the % recovery was determined.

Quantification

Extracts from *Adenoon indicum* Dalz were administered in triplicate to pre-washed TLC plates using a sample applicator. The TLC plates were then prepared using an optimized mobile phase, as indicated by det *Adenoon indicum* Dalz in the preceding section. The peak areas for the marker were quantified, and the marker's concentration was determined by linear regression applied to the calibration curves.

Statistical analysis

All statistical data were calculated with Microsoft Excel.

RESULTS

Method development

The phytochemical components of *Adenoon indicum* Dalz extract were separated by HPTLC analysis on *silica* gel TLC plates. After evaluating many solvent systems, a mobile phase consisting of toluene, ethyl acetate, methanol, and formic acid (3:3:2:1 v/v/v/v) yielded the best separation. At 490 nm, the TLC plates showed discrete bands, indicating the presence of different phytochemicals (Figure 1). A key band with an R_f value of 0.894±2.21 suggested effective separation (Figures 2 and 3). A densitometric HPTLC method was devised to quantify stigmasterol, with a UV spectrum overlay indicating peak purity at 490 nm, which corresponded to the stigmasterol reference. This approach enabled precise measurement without interference from other substances in the 400-490 nm range.

Method validation

The established HPTLC method effectively quantified stigmasterol in *Adenoon indicum* Dalz extract, matching important validation criteria outlined in the ICH standards. The calibration curves for stigmasterol were linear between 400-1200 ng/band, with a good correlation coefficient (r^2>0.995), indicating the method's linearity (Table 1). Precision was strong, with % RSD values less than 2% for both repeatability and intermediate precision, indicating consistent results. Sensitivity was demonstrated by LOD and LOQ values of 889.823 ng/band and 2696.433 ng/band. High accuracy was demonstrated, with stigmasterol recovery ranging from 98.2±5.24% to 100.00±5.49%. Method robustness was maintained, as % RSD values remained below 5% despite slight changes in circumstances (Table 2), ensuring consistent performance across a variety of scenarios.

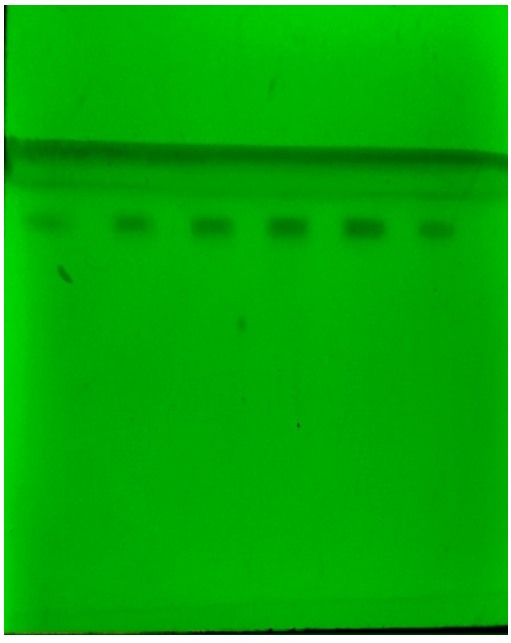

Figure 1: TLC Photo recorded plate.

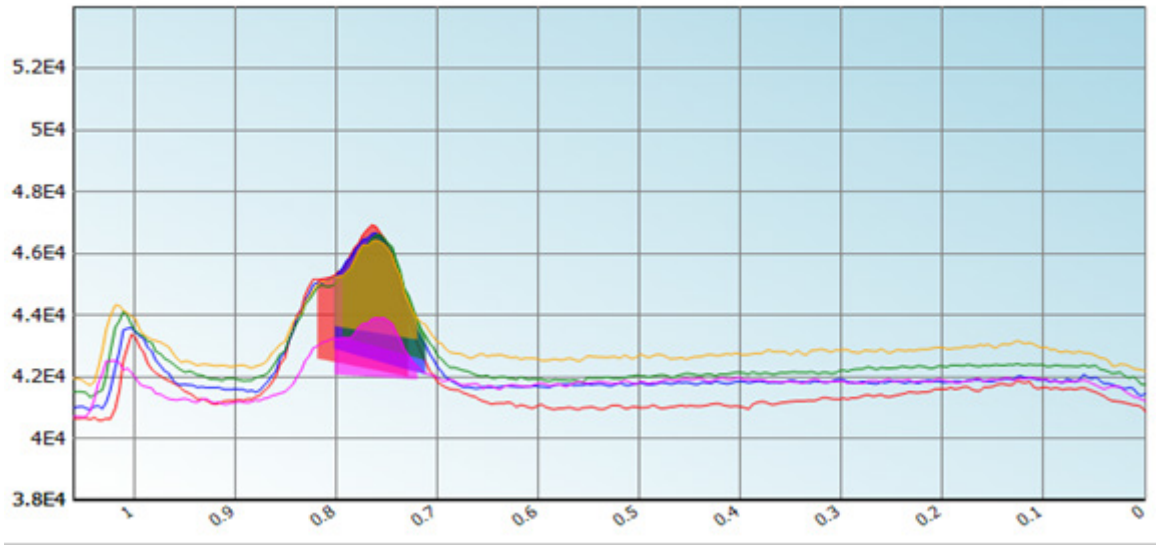

Figure 2: Standard overlay chromatogramspectra of stigmasterol reference standard and *Adenoon indicum* Dalz extract scanning from 200 to 700 nm.

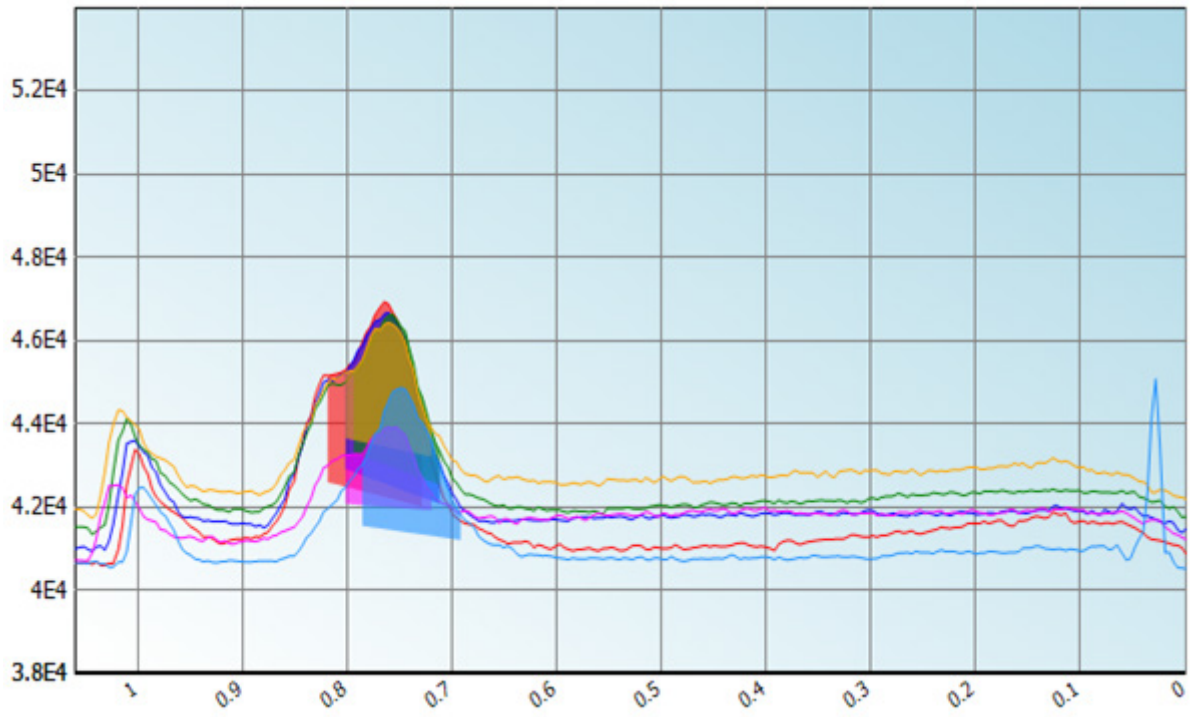

Figure 3: *Adenoon indicum* Dalz extract overlay chromatogramspectra of stigmasterol and stigmasterol reference scanning from 200 to 700 nm.

Method application

The developed HPTLC method was effectively employed to determine the stigmasterol content of *Adenoon indicum* Dalz extracts. The HPTLC densitogram (Figure 2) of the extract showed a distinct peak at an Rf value of 0.894±0.21, suggesting the presence of stigmasterol. This exact Rf value, combined with the sharpness and clarity of the peak, demonstrates the method's precision in identifying and quantifying stigmasterol in the *Adenoon indicum* Dalz sample.[11,12]

DISCUSSION

The researchers effectively optimized an HPTLC method for separating and quantifying stigmasterol in *Adenoon indicum* Dalz. The chosen solvent system (toluene, ethyl acetate, methanol, and formic acid in a 3:3:2:1 ratio) was successful in separating phytochemicals on silica gel plates. The R_f values and UV spectrum overlay supported the method's specificity, resulting in precise stigmasterol quantification without interference from other phytochemicals. Furthermore, the stability of the stigmasterol

Table 1: Validation parameters by the proposed HPTLC method.

Parameters	Stigmasterol
Range of linearity	400-1200 ng/band
Regression equation, (*n*=6)	$Y = 1.178X+621.4$
Correlation coefficient (*r*2)	0.993±0.0017
Repeatability (% RSD)	0.49%-1.46%
Intermediate precision (% RSD)	0.36%-1.90%
Recovery (%), (*n*=3)	(98.2±5.24)%-(100.00±5.49)%
Limit of Detection (LOD)	889.823 ng/band
Limit of Quantitation (LOQ)	2696.433 ng/band

X=concentration of morin in ng/mL, Y=peak area.

Table 2: Results of robustness study.

Chromatographic conditions	RSD/%
Mobile phase composition ratio (toluene: ethyl acetate: methanol: formic acid, (3:3:2:1, v/v).	2.74
Time from spotting on the HPTLC plate to development (30 min).	2.10
Time from development to densitometric scanning (30 min).	1.75

peak at wavelengths above 400 nm demonstrated the method's robustness for further research. This HPTLC method offers a consistent and specific methodology for assessing stigmasterol in herbal extracts, making it a useful tool for quality control and phytochemical research.

The HPTLC method for stigmasterol quantification in *Adenoon indicum* Dalz extract was verified and shown to be extremely reliable, with strong linearity (r^2>0.995) across the concentration range, making it suitable for quantitative analysis. Low % RSD values imply good precision and reproducibility, but low LOD and LOQ values enable sensitive detection of tiny stigmasterol levels. High recovery rates ensure an accurate portrayal of stigmasterol content, making it suitable for quality control. Robustness testing revealed that the approach remains unaffected by slight alterations, making it appropriate for routine use. This dependable HPTLC approach improves the standardization and quality assessment of *Adenoon indicum* Dalz products in herbal analysis.

The use of HPTLC to quantify stigmasterol in *Adenoon indicum* Dalz extracts has various advantages over other analytical approaches. Thin Layer Chromatography (TLC) techniques, such as HPTLC, are frequently used to analyze pharmaceuticals, botanical products, foods, environmental samples, and clinical specimens. TLC's benefits, such as its simplicity, dependability, and adaptability for high-throughput analysis, make it an excellent choice for fingerprint profiling and quantification of important marker molecules in herbal medicines. As a result, the proposed HPTLC approach is ideal for regular analysis, quality control, and standardization of *Adenoon indicum* Dalz-based raw materials and polyherbal formulations.[13,14]

CONCLUSION

A simple and sensitive HPTLC technique was successfully developed and validated for detecting stigmasterol in *Adenoon indicum* Dalz. The suggested HPTLC technique has acceptable validation parameters. When compared to the HPLC technique, this technology had many benefits, including simplicity, speed, numerous sample handling, less solvent, quicker analysis time, and a cheaper cost per analysis. As a consequence, this validated HPTLC technique may be used to quantitatively assess stigmasterol levels in *Adenoon indicum* Dalz extracts and herbal formulations.

ACKNOWLEDGEMENT

The authors are gratefully acknowledged by BVCPK's Principal Dr. H. N. More and TKCP's Principal Dr. John D'Souza for constant support and encouragement. Also, we are thankful to Shivaji University, Kolhapur for providing analytical facilities.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ICH: International Conference on Harmonization; **r^2:** Regression Coefficient; **HPTLC:** High-Performance Thin Layer Chromatography; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **σ:** Standard Deviation; **RSD:** Relative Standard Deviation; **µg/mg:** Microgram per Milligram; **µL:** Microliter; **ng:** Nanogram; **S:** Slope; **y = mx + c:** Linearity Equation.

SUMMARY

In this present study, the quantitative estimation of stigmasterol in *Adenoon indicum* Dalz extracts and herbal preparations by HPTLC densitometric analysis has not been reported. This easy, sensitive, and cost-effective HPTLC method standardizes *Adenoon indicum* Dalz extract in herbal formulations and goods to ensure quality control. The scientific data helps with chemical profiling, quality control, and regulatory compliance for *Adenoon indicum* Dalz and its products.

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Cite this article: Patil AB, Shinde AJ. A HPTLC Method for the Quantification of Stigmasterol Isolated from *Adenoon indicum* Dalz: Development and Validation. Pharmacog Res. 2025;17(1):201-6.