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# Quantification of β-Sitosterol by Validated High Performance Thin Layer Chromatography (HPTLC) Densitometric Method in the Flowering Buds of *Mesua ferrea* Linn

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**Abstract**

The medium-sized to large-sized tree *Mesua ferrea* Linn, commonly known as Nagakesara in India, can grow around 20 to 30 meters high and has incredibly durable wood. The buds are globular, 2 mm diagonally, and have scales in the axillary buds. It includes a significant amount of β-sitosterol amides, which are important from a biological perspective. In the current study, we used high-performance thin-layer chromatography (HPTLC) in association with densitometry to quantify these marker chemicals from *Mesua ferrea* Linn flowering buds. The technique was found to be accurate. For intra-day analysis, the relative standard deviation (RSD) values of β-sitosterol ranged from 1.16 to 1.60 %, while for inter-day analyses, the values ranged from 0.68 to 1.77 %. By performing a recovery study at three different levels for the standard chemicals, the accuracy of the approach was assessed, and average recoveries were determined to be 99.98 %. This TLC-densitometric approach was observed to be accurate, specific, particular, sensitive, and easy. It can be employed in regular quality control for the simultaneous analysis of β-sitosterol from flowering buds of Mesua ferrea Linn.

*Keywords*: *Mesua ferrea* Linn*;* High-performance thin-layer chromatography; β-sitosterol; Densitometry.


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## 1. Introduction

Mesua ferrea (family: Calophyllaceae) is a medium- to large-sized tree. Its wood is quite durable, and its bark is reddish-brown to grey. Simple, lanceolate, sharp, leathery leaves with waxy blooms on the underside. Red, oppositely oriented, immature leaves are between 7 and 13 cm long and 2 and 4 cm broad. The flowers are white with a floral fragrance, up to 7.5 cm in diameter, with many golden-colored stamens shorter than the petals. The style, which is borne alone or in pairs, axillary or terminal, is twice as long as the stamens. The fruits are 2.5 to 5 cm long, oval with a conical apex, and have a woody pericarp that holds one to four seeds [1,2].

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In tropical Asia and India, the entire plant is widely used as a medicine [3]. It is traditionally used as a diuretic, antipyretic, expectorant, cardiotonic, carminative, and antibacterial treatment for colds and asthma, as well as for its antiemetic, anthelminthic, aphrodisiac, and antidote properties. Antioxidant, estrogenic and progestational activity, analgesic activity, hepatoprotective, antimicrobial, anticancer, antiulcer, antivenom, antispasmodic, anti-inflammatory, antiasthmatic, immunomodulatory, tyrosinase and elastase inhibitory activity, and antioxidant are the reported validated effects of this medicinal plant [4,5]. Phytochemical analysis of the plant reveals the presence of flavanone glycosides, xanthones, coumarins, and flavones [6].

Phytosterols, or plant sterols, are essential parts of the lipid bilayer that helps to stabilize cell membranes. There are around 44 different sterols known to exist in plants. β-Sitosterol is one of the most prevalent and prolific phytosterols (Fig.1). There have been reports of 500 plants containing β-sitosterol [7,8]. They are a significant part of the human diet and are structurally and physiologically related to cholesterol [9].

The earlier investigation on *Mesua ferrea* L revealed that the heartwood [10], root bark extracts [11], and stem parts [12] of the plant contain the steroid β-sitosterol. Very effective analyzing tools for herbal products involve HPTLC techniques, which are popular methods for ensuring quality control for equally herbal finished products and raw materials [13]. The HPTLC analyzed β-sitosterol, which is present in n-hexane, petroleum ether, ethyl acetate, acetone, and ethanol extract from flowering buds of *Mesua ferrea* Linn. We report our work on the simultaneous quantitation of β-sitosterol from *Mesua ferrea* Linn flowering buds by HPTLC densitometry.

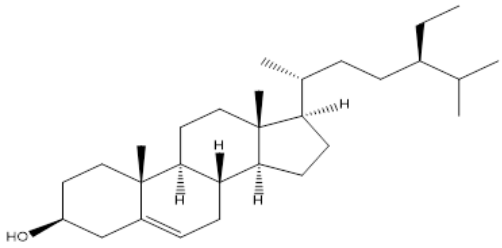


Fig. 1. The structure of β-sitosterol.

## 2. Experimental

### 2.1. *Plant material*

The medicinal plant, *Mesua ferrea* Linn's flowering buds, was collected from the University of North Bengal in India, and Griffin's Herbarium of the plant was authenticated at Banaras Hindu University, and also identifies the plant from Rajiv Gandhi Centre for Biotechnology (RGCB) in Kerala, India, through a universal DNA barcoding technique. The *Mesua ferrea* Linn's flowering buds were dried in the shade for up to two months, crushed into small pieces, and passed through a 60-number sieve. The

cold maceration method was used to extract the powder sample, and a low-polar to high-polar solvent was used.

### 2.2. *Standards and chemicals*

Standard β-sitosterol was porches from Sigma-Aldrich Chemical Pvt. Limited (Invoice No. TI/2021-22/1028) and all solvents, reagents, and chemicals were collected from Sisco Research Laboratories Pvt. Ltd. and SD Fine Chemicals, India.

### 2.3. *Instrumentation and chromatographic conditions*

The samples were applied with a 100 µL syringe (Hamilton) through the CAMAG® Linomat 5 (Switzerland) HPTLC apparatus. Two glass twin-trough chambers 20 × 10 cm and 10 × 10 cm (CAMAG, Switzerland) were used to develop plates. TLC Silica gel 60 F254 (Merck, Invoice No ASM/140/21-22) was used for chromatographic separation as the stationary phase on aluminum plates. To establish the contingency of different fractions of phytoconstituents, an extract of *Mesua ferrea* Linn's flowering buds was prepared by using a mobile phase comprising toluene, ethyl acetate, and acetic acid (6:2:0.1) v/v**.** A developed chamber (CAMAG twin trough plate) was given the prepared solvent system with mobile phase (25 ml) and left for 20 min. The finished plate was then scanned after drying for five min at room temperature. To scan the produced spots, a TLC Scanner 4 (S/N: 270741) was used. Finally, each spot's Rf value was recorded on the plate using vision CAT software.

### 2.4. *Sample preparation*

*Mesua ferrea* Linn's flowering buds (150 g) were powdered and immersed continuously for seven days in a conical flask filled with 400 mL of n-hexane solvent before being filtered using Whatman No. 1 filter paper. The marc was then allowed to dry for 24 h before being utilized once more with the subsequent solvents, which were, in order, petroleum ether, ethyl acetate, acetone, ethanol, and water. Each filtrate sample was taken in a Petri dish that had been previously weighted, evaporated at 40°C through a water bath, stored in desiccators, and then transferred into a tightly closed container. Each sample was prepared with the methanol solvent, then spun for 10 min at 1000 rpm, and finally, 10 µL of each sample was applied through a Hamilton syringe for spots.

### 2.5. *Preparation of standard solutions*

A stock solution of β-sitosterol 100 µg/mL in chloroform was prepared as a sample purchased from Sigma-Aldrich in an ampoule and stored in a (-20 $^{o}$C) refrigerator. From this, directly collect the sample through a 100 µL Hamilton syringe and apply the automatic TLC applicator Linomat 5 in an aluminum silica gel 60 $F_{254}$ plates.

### 2.6. *Qualitative HPTLC exploration*

Using a mobile phase toluene: ethyl acetate: acetic acid (6:2:0.1) solvent solution, a qualitative HPTLC examination of each extract of *Mesua ferrea* Linn's blooming buds was performed against a standard β-sitosterol to ensure the presence of bioactive components.

Table 1. The beta-sitosterol content in all the extracts of the flowering buds of *Mesua ferrea* Linn.

| Sl No. | Extracts | 1 mg of extract contains (mg) |
|---|---|---|
| 1. | n-hexane | 0.161 |
| 2. | petroleum ether | 0.156 |
| 3. | ethyl acetate | 0.112 |
| 4. | acetone | 0.097 |
| 5. | ethanol | 0.081 |
| 6. | water | 0.072 |

### 2.7. *TLC–Densitometric quantification of β-sitosterol*

#### 2.7.1. *Preparation of calibration curve of β-sitosterol*

Amounts of 10, 15, 20, 25, and 30 µL of standard β-sitosterol solution were applied in triplicate on aluminum silica gel 60 F254 plates with band Position Y: 8.0 mm, length: 8.0 mm, and width: 0.0 mm with 1, 1.5, 2, 2.5, and 3 µg of β-sitosterol. The plates were developed in a twin trough chamber at 40% relative humidity and 25°C±2°C with 25 mL of the mobile phase (toluene, ethyl acetate, and acetic acid; 6:2:0.1 v/v) to a distance of up to 8 cm. The plates were dried at room temperature and then scanned in absorbance mode at 254 nm using the Tungsten lamp source. The peak areas were measured, and on graphing applied concentrations of β -sitosterol vs. peak areas, the calibration curve for β -sitosterol was created.

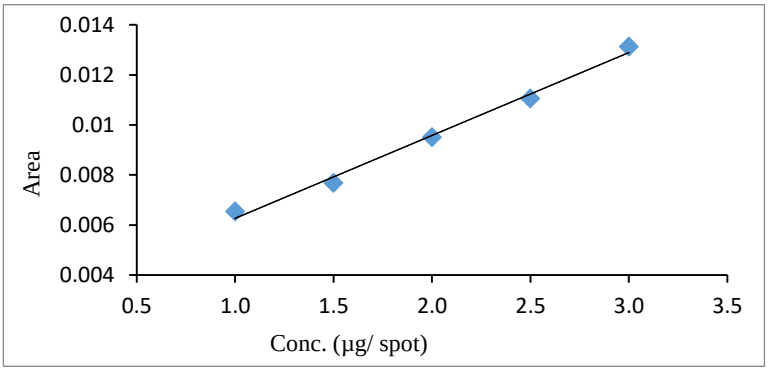


Fig. 2. Calibration curve of β-sitosterol.

Table 2. Linear regression data of calibration plot, limit of detection, and limit of quantification.

| Parameter | Results |
|---|---|
| Wavelength (nm) | 254 |
| Range, µg/ spot | 1.0-3.0 |
| Linearity (*r*) | 0.991 |
| Specificity | Specific |
| Limit of detection (LOD), µg/ spot | 0.40 |
| Limit of quantification (LOQ), µg/ spot | 1.22 |

2.7.2. *Simultaneous quantification of β-sitosterol in samples*

To obtain peak areas of β-sitosterol, 10 µL of the sample solution was applied to an HPTLC plate, which was then scanned at 254 nm. To identify the bands, absorption spectra were taken. The amount of β-sitosterol was determined in the sample through the calibration curves.

**2.8. *Validation of the method***

The analytical process validation was conducted by International Conference on Harmonization Regulations (CPMP/ICH/381/95C; PMP/ICH/281/95). The methods were validated in support of accuracy, precision, the limit of detection, limit of quantification, specificity, and recovery studies [14].

2.8.1. *Precision*

The precision was examined by evaluating the β-sitosterol standard solutions after application on an HPTLC plate (n = 6) and then expressing the results as % RSD, the precision of the method was established. The technique was investigated through the analysis three times of aliquots of standard solutions containing 1 µg/spot, 2 µg/spot, and 3 µg/spot of β-sitosterol on the same day (intra-day precision) and on different days (inter-day precision), with the results expressed as a percentage of RSD.

2.8.2. *Limit of detection and limit of quantification*

The limit of quantification (LOQ) and Limit of detection (LOD) was reviewed using dilutions of standard - β-sitosterol solutions in addition to the methanol used as blank. LOD and LOQ were then calculated from the correlating β-sitosterol calibration curves (y=mx + c) and using the formula LOD = 3.3*SD/b and LOQ = 10*SD/b, where SD stands for standard deviation and b for the slope of the calibration curves, respectively.

Table 3**.** The precision of the proposed HPTLC-densitometric method for β-sitosterol.

| Intra-day precision (repeatability, n=3 | | | |
|---|---|---|---|
| Amount of β-sitosterol | Measured peak area (3 days, morning, noon, afternoon) | Standard deviation (SD) | Relative standard deviation (%RSD) |
| 1 µg/spot | 0.010677 | 0.016468 | 1.60 |
| 2 µg/spot | 0.017399 | 0.000196 | 1.16 |
| 3 µg/spot | 0.024689 | 0.000290 | 1.18 |
| Inter-Day Precision (Intermediate Precision, *n* = 3) | | | |

| Amount of β-sitosterol | Measured peak area (3 days readings) | Standard deviation (SD) | Relative standard deviation (%RSD) |
|---|---|---|---|
| 1 µg/spot | 0.01061 | 0.0000818 | 0.77 |
| 2 µg/spot | 0.01680 | 0.0001153 | 0.68 |
| 3 µg/spot | 0.02473 | 0.0002837 | 1.14 |

2.8.3. *Specificity*

The developed method's specificity was evaluated by examining the reference standards and samples simultaneously. The spots of β-sitosterol were compared to the sample extract's Rf value; each standard's spectrum and corresponding band were superimposed in the sample track. Overlaying absorption spectra allowed researchers to determine the band's purity in the sample.

2.8.4. *Recovery studies*

Recovery experiments at three levels, such as 50, 100, and 125 % addition of β-sitosterol, were carried out to evaluate the method's accuracy. Calculations were made for the recoveries and average recoveries percentage. At each level, a determination was made in triplicate optimized chromatographic conditions.

Table 4. Recovery study of β-sitosterol by the proposed HPTLC–densitometric method.

| Concentration (µg /Spot) | 50 % | 100 % | 150 % |
|---|---|---|---|
| 10 µL | 5 | 10 | 15 |
| Final Conc. | 15 µL | 20 µL | 25 µL |

| Standard compound | Sample amount (µL) | Theoritical value (µg) | Average area (n-3) | Practical value (µg) | Recovery percentage | Average recovery percentage |
|---|---|---|---|---|---|---|
| β-sitosterol | 15 | 1.5 | 0.00748 | 1.49 | 99.33 | 99.98 |
| | | 2.0 | 0.00903 | 2.01 | 100.50 | |
| | 25 | 2.5 | 0.01051 | 2.50 | 100.13 | |

## 3. Results and Discussion

In the current investigation, we used HPTLC densitometry to quantify the marker chemicals β-sitosterol in different solvent extracts of *Mesua ferrea* Linn flowering buds. Small adjustments were made to optimize the mobile phase's composition. Using a TLC Scanner 4 (S/N: 270741) with vision CAT software, the identity of β-sitosterol in *Mesua*

*ferrea* Linn was established by projecting the absorption spectra at 254 nm, and the highest amount of β-sitosterol was found in n-hexane extracts and lowest in water Table 1.

The linearity ranges 1 µg/spot to 3 µg/spot was determined to be for β-sitosterol, with a correlation coefficient of 0.991 (Fig. 2 and Table 2). The process was validated in particular aspects of the precision recovery studies represented in Tables 3 and 4, the limit of detection (LOD), and the limit of quantification (LOQ) in Table 2. The chromatogram of every extract's specificity was obtained using the described approach and had a peak that ranged in Rf value from (0.800 to 0.824) for the sample and was found to be close to Rf (0.837) for the reference β-sitosterol. Avoiding any mobile phase or another ingredient not interfering with the Rf values of the samples demonstrated the method's specificity in the presence of the reference standard and different extracts. At three different levels, the average percentage recoveries of β-sitosterol were shown to be 99.98% (Table 4).

## 4. Conclusion

According to ICH guidelines, the procedures were validated to support precision, accuracy, the limit of quantification, the limit of detection, recovery, and specificity assessments. Using samples of low polar to high polar solvent extracts and β-sitosterol, the developed techniques of specificity study have demonstrated specificity. The fact that the intra-day and inter-day RSD percentage is within the allowable range of 2% demonstrates the precision of the approach. This newly created approach for the simultaneous measurement of *Mesua ferrea* Linn's blooming buds extracts was found to be specific, precise, accurate, and also gave satisfactory results, according to the stated experimental results, statistical data, and parameters. The anticipated method can be further applied to the formulation and development of a novel herbal drug formulation.

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## References

1. K. Z. W. Myint, T. Kido, K. Kusakari, H. P. Devkota, T. Kawahara, and T. Watanabe, Nat. Prod. Res. **35**, 1024 (2021). https://doi.org/10.1080/14786419.2019.1613395
2. P. Rajalakshmi, V. Vadivel, N. Ravichandran, and P. Brindha. Pharmacogn J. **11,** 225 (2019). https://doi.org/10.5530/pj.2019.11.35
3. X. Zhang, R. Gao, Y. Liu, Y. Cong, et al. Drug. Discov. Ther. **13,** 222 (2019). https://doi.org/10.5582/ddt.2019.01053
4. M. K. Chahar, D. S. S. Kumar, T. Lokesh, and K. P. Manohara. Int. Immunopharmacol. **13**, 386 (2012). https://doi.org/10.1016/j.intimp.2012.05.006
5. P. Shirsat, A. R. Ziyaurrahman, R. Kashikar, M. Athavale et al., Drug. Chem. Toxicol. **45**, 1570 (2022). https://doi.org/10.1080/01480545.2020.1847134

6. S. Wang, Y. Guo, D. Yao, L. Liu, et al., Fitoterapia **138**, ID 104192 (2019). https://doi.org/10.1016/j.fitote.2019.104192
7. J. C. Lim and D. W. Chung, J. Appl. Polym. Sci. **125**, 888 (2012). https://doi.org/10.1002/app.36259
8. A. B. Awad, M. F. Chinnam, C. S. Fink, and P. G. Bradford. Phytomedicine **14**, 747 (2007). https://doi.org/10.1016/j.phymed.2007.01.003
9. S. D. Ambavade, A. V. Misar, and P. D. Ambavade. Orient Pharm. Exp. Med. **14**, 193 **(**2014). https://doi.org/10.1007/s13596-014-0151-9
10. Y. L. Chow and H. H. Quon. Phytochemistry **7,** 1871 (1968). https://doi.org/10.1016/S0031-9422(00)86662-5
11. S. S. Teh, G. C. Ee, M. Rahmani, Y. H. Taufiq-Yap, R. Go, and S. H. Mah, Molecules **16**, 5647 (2011). https://doi.org/10.3390/molecules16075647
12. S. Keawsa-Ard, B. Liawruangrath, and S. Kongtaweelert, Chiang Mai. J. Sci. **42**, 185 (2015).
13. M. Ram, M. Z. Abdin, M. A. Khan, and P. Jha, ed. M. Srivastava, HPTLC (Springer, Berlin, Heidelberg, 2011) pp. 05-116. https://doi.org/10.1007/978-3-642-14025-9_7
14. ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology, Q2(R1), ICH (2005).