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HPTLC Method Development and Validation for Simultaneous Determination of Beta-Sitosterol and Oleanolic Acid in *Eclipta alba*

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ABSTRACT: *Eclipta alba*, a medicinal plant of the Asteraceae family, is widely recognized and extensively used in traditional medical systems such as Ayurveda, Siddha, Homeopathy, Unani, Chinese, and folk medicine. It contains significant phytochemical components like triterpenes, flavonoids, coumestans, steroids, saponins, and polypeptides. *Eclipta alba* is a key ingredient in various herbal and Ayurvedic formulations, including Indulekha Bringha oil and Liv.52 Gnx pill. The objective of this study was to develop a validated HPTLC method for the simultaneous quantification of beta-sitosterol and oleanolic acid in *E. alba*. Challenges may include optimizing separation and detection parameters. The method was validated following ICH guidelines. In the developed method, the stationary phase employed was silica gel 60 F_{254}, while the mobile phase consisted of a mixture of ethyl acetate, toluene, and formic acid (6:5:0.1 v/v/v). Derivatization with anisaldehyde sulfuric acid resulted in the formation of compact bands. The calibration curves for beta-sitosterol and oleanolic acid exhibited high correlation coefficients (r^2) of 0.9966 and 0.9945, respectively, indicating a good linear relationship within the concentration range of 450-1800 ng/spot and 400-1850 ng/spot based on the area. The method's precision, accuracy, robustness, and selectivity were evaluated. The LOD and LOQ for β-sitosterol were 107.94 ng/spot and 327.12 ng/spot, respectively. For oleanolic acid, the LOD was 128.81 ng/spot and the LOQ was 356.64 ng/spot. Overall, this HPTLC approach proved to be effective, straightforward, accurate, and reproducible. The study provides a reliable method for measuring the levels of oleanolic acid and beta-sitosterol in *Eclipta alba*. This method can be used to ensure the quality of *Eclipta alba* products and to guide their therapeutic use.

Keywords: *Eclipta alba*, β-sitosterol, Oleanolic acid, HPTLC, Validation.

INTRODUCTION

Growing demand for plant-based products drives their use in medicine, nutraceuticals, and cosmetics in developed countries (Patil *et al.*, 2011). The WHO emphasizes employing advanced analytical techniques and establishing physicochemical parameters to ensure the quality of crude drugs and addresses their complex composition through chemical and instrumental methods (Ali *et al*., 2016).

Eclipta alba (EA or *E. alba*), commonly known as bhumiraj, bhringraj, and aali jhar, is a medium-sized, branching annual herb with white flowers. It is also referred to as "False Daisy" in English (Timalsina and Devkota 2021). This plant is native to tropical and subtropical regions worldwide (Uddin *et al*., 2010; Baskaran and Jayabalan 2005). *E. alba* has a long history of traditional use in treating various skin disorders, including dermatitis, preventing baldness, and healing wounds. Honey and leaf juice derived from *E. alba* are used to treat infants with catarrh (Bakht *et al*., 2011; Jayathirtha and Mishra 2004). The juice of *E. alba* is consumed orally or applied topically to promote hair growth (Datta *et al*., 2009). In Nepal, the shoots and leaves of this plant are utilized to treat and prevent wound infections (Adhikari *et al*., 2019; Gautam, 2013). It is also employed by various ethnic communities in South American countries to treat snakebites (Diogo *et al*., 2009). In Ayurveda, *E. alba* is recognized for its rejuvenating and anti-ageing effects (Puri, 2003). In Bangladesh, it is used by different ethnicities to treat jaundice (Rai, 1970; Badgujar and Patil 2008). Additionally, the plant juice is employed to prevent the growth of disease-carrying insects such as mosquitoes (Govindarajan and Karuppannan 2011; Rajith and Ramachandran 2010). It is also used for the treatment of various ailments, including baldness, acidity (Roy *et al*., 2008), as well as gingivitis, bronchitis, asthma, wounds, burns, constipation, high temperature, body aches, wrinkles, acne, and other skin

conditions (Khan and Khan, 2008; Kumari *et al*., 2006; Tewtrakul *et al*., 2007; Neeraja and Margaret 2012).

Numerous studies have examined the elemental analysis and biological properties of different parts of *E. alba*, highlighting the importance of chemical characterization and standardization for its potential medicinal, cosmetic, and formulation applications.

β-sitosterol (BS) is a notable phytosterol known for its beneficial physiological effects, including anorectic, anti-inflammatory, antiandrogenic, anticancer, antigonadotropic, antibacterial, and antilymphomic properties (Chakraborty *et al*., 2016). Oleanolic acid (OA) is a plant-derived pentacyclic terpenoid that has been shown to have numerous pharmacological properties, including hepatoprotective, anti-oxidative, anti-inflammatory, and anticancer activities. Its derivatives have a wide range of applications (Ayeleso *et al*., 2017). BS (Bhattacharjee *et al*., 2017) and OA (Jahan *et al*., 2014) were found in *E. alba*.

E. alba is a crucial ingredient in herbal remedies used for treating various ailments, with its essential components BS and OA being present in many formulations. Lack of standardization in herbal products raises safety concerns, highlighting the importance of regulatory compliance and promoting safe usage. A review of the literature identified the use of HPLC (Sriraman *et al*., 2015; Valerian *et al*., 2022), HPTLC (Dhruv and Tatke 2013; Mallick and Dighe 2014; Bhattacharjee *et al*., 2017), and GC (Sorenson and Sullivan, 2006) for quantifying beta-sitosterol. For oleanolic acid quantification, HPLC (Tian *et al*., 2010; Zhang *et al*., 2013), HPTLC (Wójciak-Kosior *et al*., 2005; Iram and Mohammed, 2010), and GC (Domingues *et al*., 2010) methods have been employed. A comprehensive analysis revealed that, HPTLC offers advantages over HPLC and GC, with minimal sample preparation, reduced solvent usage, and superior resolution and sensitivity, making it a more efficient and cost-effective analytical method. HPTLC's non-destructive nature allows further analysis of separated compounds, unlike GC, which destroys the sample, making HPTLC efficient, versatile, and capable of providing comprehensive results (Aslam, 2023). This study validates the first HPTLC method for simultaneous quantification of OA and BS in *E. alba*, providing a simpler approach.

The aim of this study was to develop and validate an HPTLC method for the simultaneous quantification of β-sitosterol and oleanolic acid. Silica gel 60 F_{254} TLC plates were used, with a mobile phase composed of ethyl acetate:toluene:formic acid (6:5:0.1 v/v/v). Quantitative estimation was performed using densitometric scanning at a wavelength of 530 nm after derivatization.

MATERIALS AND METHODS

A. Plant Material

In February, the entire *E. alba* plant was collected in Kolkata, West Bengal. The plant underwent characterization and authentication by a taxonomist. The material was finely pulverized, air dried, and filtered using a number 10 filter.

B. Chemicals and Reagents

The β-sitosterol (97%) reference standard was supplied by Sisco Research Laboratories Pvt. Ltd., while the oleanolic acid (96.00%) reference standard was obtained from Innovative Chemical Interchange Pvt. Ltd (Carbino). Spectroscopic grade solvents were used in the study. All chemicals, including ethyl acetate, toluene, sulphuric acid, acetic acid, formic acid, methanol, petroleum ether, and anisaldehyde, were of analytical grade and sourced from Merck in Mumbai, India.

C. Instrumentation

The instrumentation and settings used for the high-performance thin-layer chromatography analysis of β-sitsterol and oleanolic acid were documented in the Table 1 provided below, comprising various parameters and specifications essential for the experiment's execution.

D. Standard Preparation

To quantify β-sitosterol and oleanolic acid, reference standards were dissolved in methanol to create a 0.5 mg/mL solution. The calibration curve was constructed following the specifications set by the International Conference on Harmonization (ICH) (Panchal *et al*., 2017).

E. Sample Preparation

The dried coarse powder of *E. alba* was extracted using continuous heat extraction in a soxhlet apparatus for two days. The temperature ranged from 60 to 80°C for eight hours, using petroleum ether as the solvent. The extract was filtered and then evaporated under reduced pressure in a vacuum. The yield was determined using air-dried powdered crude material. To create the sample solution, 100 mg of the extract was measured and adjusted to a volume of 50 mg/mL with petroleum ether. The solution was stored in the refrigerator.

F. Calibration

The standard curve was prepared following the ICH recommendations. Each concentration was sprayed in triplicate on a plate measuring 20 × 10 cm. The bands were 6 mm wide and spaced 11.2 mm apart. The plate's bottom and side edges were 12 and 8 millimeters away, respectively. The application rate was 10 µL/s, and the bands were developed by saturating them with a mixture of ethyl acetate, toluene, and formic acid (in a ratio of 6:5:0.1 v/v/v) for 20 minutes. After development and air drying, the plate was immersed in a TLC plate dipping chamber containing an anisaldehyde-sulfuric acid reagent solution for two seconds. Subsequently, the plate was taken out and heated for 5 minutes at 110 °C in a hot air oven. The standard zones were measured using Camag TLC scanner III densitometer operating in absorbance mode at a wavelength of 530 nm, which offers the highest sensitivity. A deuterium lamp served as the radiation source. Linear regression analysis was employed for evaluation based on peak area.

G. Sample Assay Preparation

The sample and standard solutions were prepared as described earlier and spotted in triplicate on a plate,

following the same development conditions as the standard. For quantification, the sample solution was spotted on the TLC plate in different volumes. The analyte was found to be well separated from other components during development, derivatization, and plate drying in a hot air oven. The linear and compact zones containing β-sitosterol and oleanolic acid were then scanned at 530 nm, and the respective peak areas were recorded.

RESULTS AND DISCUSSION

Silica gel TLC plates were utilized to evaluate different ratios of ethyl acetate, toluene, and formic acid as the mobile phase. A ratio of 6:5:0.1 (v/v/v) yielded a satisfactory resolution. Following derivatization, the extract exhibited a well-resolved and symmetrical band for β-sitosterol and oleanolic acid under optimized conditions. The application of anisaldehyde-sulfuric acid reagent resulted in colored spots for the derivatized β-sitosterol and oleanolic acid (Fig. 1). The standard compounds, β-sitosterol (R_f = 0.83) and oleanolic acid (R_f = 0.62), showed sharp peaks in the HPTLC chromatogram (Fig. 3). The most favorable scanning was achieved at a wavelength of 530 nm. The mobile phase solvent suitability test was conducted for 24 hours, confirming the continued suitability of the selected mobile phase solvent. Table 2 provides the optimized chromatographic parameters for the analysis of β-sitosterol and oleanolic acid using HPTLC. Fig. 2 exhibits the spectra scanning of β-sitosterol and oleanolic acid.

A. Linearity

A series of reference β-sitosterol and oleanolic acid concentrations were applied in triplicate for analysis. The standard curve for β-sitosterol exhibited a satisfactory linear relationship across the concentration range of 450–1800 ng/spot, with the linear regression equation y = 19.80x–2904, where y represents the spot area and x represents the analyte concentration. The correlation coefficient (r^2) was 0.9966 (Fig. 4). Similarly, oleanolic acid demonstrated a linear relationship with the spot area over the concentration range of 400–1850 ng/spot, with the equation y = 3.309x – 339.352 and an r^2 value of 0.99456 (Fig. 5). During plate scanning, the spotted reference compounds of β-sitosterol and oleanolic acid were analyzed. The coefficient of variation (CV) for β-sitosterol ranged from 0.082 to 0.122, while for oleanolic acid, it ranged from 0.014 to 0.082. Smaller CV values indicate higher precision and less variability in the measurements.

B. Intermediate Precision (Reproducibility)

To assess the precision of the proposed method, mixed standard solutions of β-sitosterol and oleanolic acid were evaluated at two different concentrations (600 and 1100 ng/spot for β-sitosterol, and 500 and 900 ng/spot for oleanolic acid). The analysis was conducted three times on the same day and repeated on the next day to determine intraday and interday precisions. The results, presented as relative standard deviations (RSD) in Table 3, revealed that the intraday precision for β-sitosterol ranged from 0.42 to 0.56, while for oleanolic acid it ranged from 0.31 to 0.36. Regarding interday precision, which assesses variability across different days, β-sitosterol exhibited a range of 0.32 to 0.70, while oleanolic acid ranged from 0.35 to 0.38. The RSD values below 2 percent indicate consistent and reproducible results, indicating good precision in the analysis.

C. Accuracy (Percentage Recovery)

The accuracy of the methods was assessed by calculating the recovery of β-sitosterol and oleanolic acid using the standard addition approach. Three sets of recovery trials were conducted, including 50%, 100%, and 125% additions of β-sitosterol and oleanolic acid. The peak area values were used in the regression equations of the calibration curves to determine the levels of the analytes (Table 4). The mean percentage recovery of β-sitosterol was 100.39%, while for oleanolic acid, it was 100.06%. These results indicate the accuracy and reliability of the analytical method.

D. Method Precision (Repeatability)

To evaluate the precision of the equipment, reference standard solutions of β-sitosterol and oleanolic acid at different concentrations were injected repeatedly (n = 6). The repeatability of the HPTLC instrument was assessed by applying the same sample solution six times to a plate using an automatic spotter with the same syringe. The sample spot was scanned six times for both β-sitosterol and oleanolic acid without changing the plate's position. The coefficient of variation (CV) range for β-sitosterol was found to be 0.017–0.058, while for oleanolic acid, it was 0.011–0.029. These results indicate the high precision of the equipment in generating consistent measurements for both analytes.

E. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The following equations were used to calculate the limit of detection (LOD) with a S/N of 3:1 and the limit of quantification (LOQ) with a S/N of 10:1 for both compounds in accordance with ICH regulations.

$LOQ = 3.3 \times \sigma/SD$

$LOQ = 10 \times \sigma/SD$

where the response's standard deviation is σ and SD stands for the standard deviation of the y-intercept of the regression line. β-sitosterol had an LOD of 107.94 ng/spot and an LOQ of 327.12 ng/spot. Similarly, oleanolic acid had an LOD of 128.81 ng/spot and an LOQ of 356.64 ng/spot.

F. Percentage concentration of β-sitosterol and Oleanolic acid in Eclipta alba extract

Under optimal conditions, using silica gel TLC plates and a mobile phase consisting of ethyl acetate, toluene, and formic acid in a ratio of 6:5:0.1 (v/v/v), satisfactory results were obtained. After derivatization, a distinct and symmetrical band for β-sitosterol and oleanolic acid was observed in the *E. alba* extract. The concentration of β-sitosterol in the extract was determined to be 4.72% w/w, while the concentration of oleanolic acid was found to be 0.56% w/w. The

calibration curve equations mentioned earlier, with x representing the amount of biomarkers and y representing the area under the curve, were used to determine these amounts. In the HPTLC chromatogram, both the standard β-sitosterol (R_f = 0.83 ± 0.2) and oleanolic acid (R_f = 0.62 ± 0.1) exhibited a single sharp peak, which was also found in the extract (Fig. 6). To ensure specificity, the R_f values of the standard and sample were compared.

The HPTLC method was validated following the guidelines of the International Conference on Harmonisation (ICH) (Ngamkhae *et al*., 2022). Precision studies, as presented in Table 3, showed % RSD values within acceptable limits (≤2), indicating good intra-day and inter-day variations for the simultaneous determination of OA and BS (Rahman *et al*., 2014). The correlation coefficients (r^2) obtained for the calibration curves ≥ 0.995, meeting the requirement set by the USP for linearity (Rahman *et al*., 2014). The linearity curves are illustrated in Fig. 4 and 5. Table 4 displays the percent recovery for OA and BS, falling within the acceptable accuracy range (97.0-103.0%) (Rahman *et al*., 2014). Moreover, additional validation parameters such as the LOQ and LOD were determined. Overall, the results from these validation parameters confirm the reliability and suitability of the analytical method for the accurate analysis of both compounds. In the study conducted by Bhattacharjee *et al*. (2017), the HPTLC analysis determined the β-sitosterol content in *E. alba* petroleum ether extract to be 4.65% w/w, while the HPLC analysis found it to be slightly higher at 4.67% w/w for *E. alba* petroleum ether extract. According to Jahan *et al*. (2014), the presence of OA was reported in *E. alba.* The HPTLC method was successfully employed to confirm the concentrations of β-sitosterol and oleanolic acid in the petroleum ether extract of *E. alba*. These chemical markers play a vital role in quality control and standardization of the herbal extract and its formulations. The developed HPTLC method accurately quantified these two major compounds in the extract, offering a versatile and time-saving approach for their analysis. This method holds significant importance in ensuring the quality and consistency of *E. alba* extract in various applications.

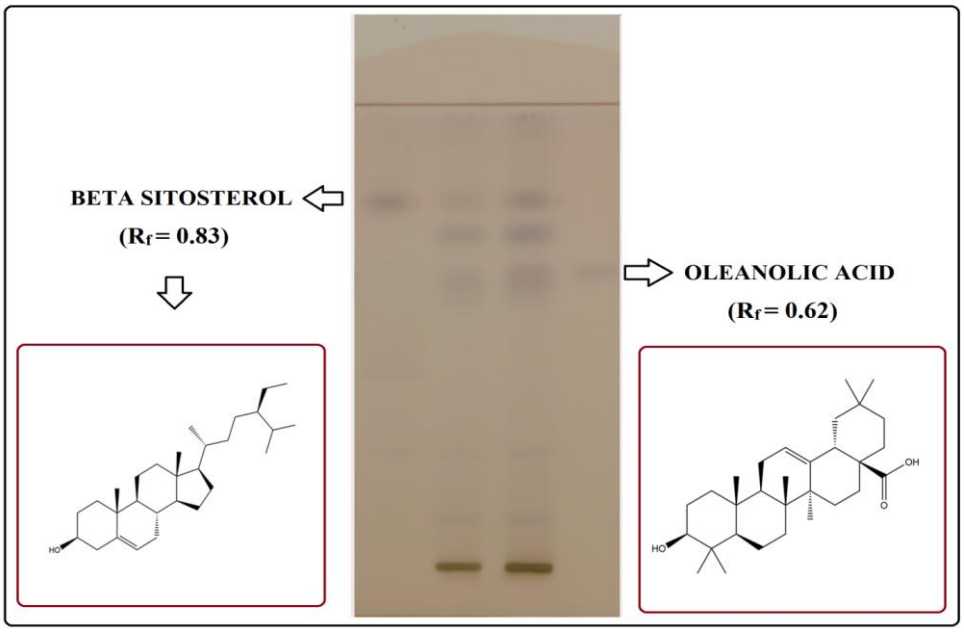

Fig. 1. After derivatization standard β-sitosterol, petroleum ether extract of *Eclipta alba*, oleanolic acid.

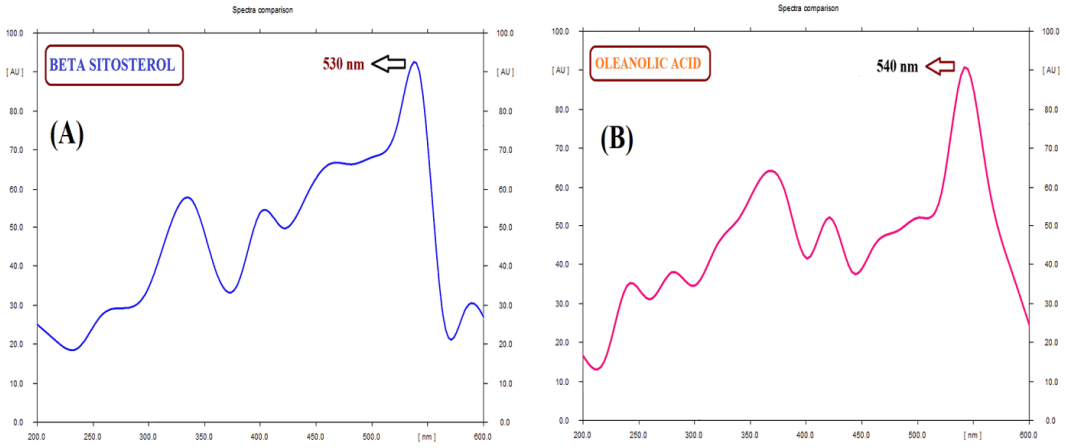

Fig. 2. Spectrum scans of standard (A) β-sitosterol, and (B) oleanolic acid.

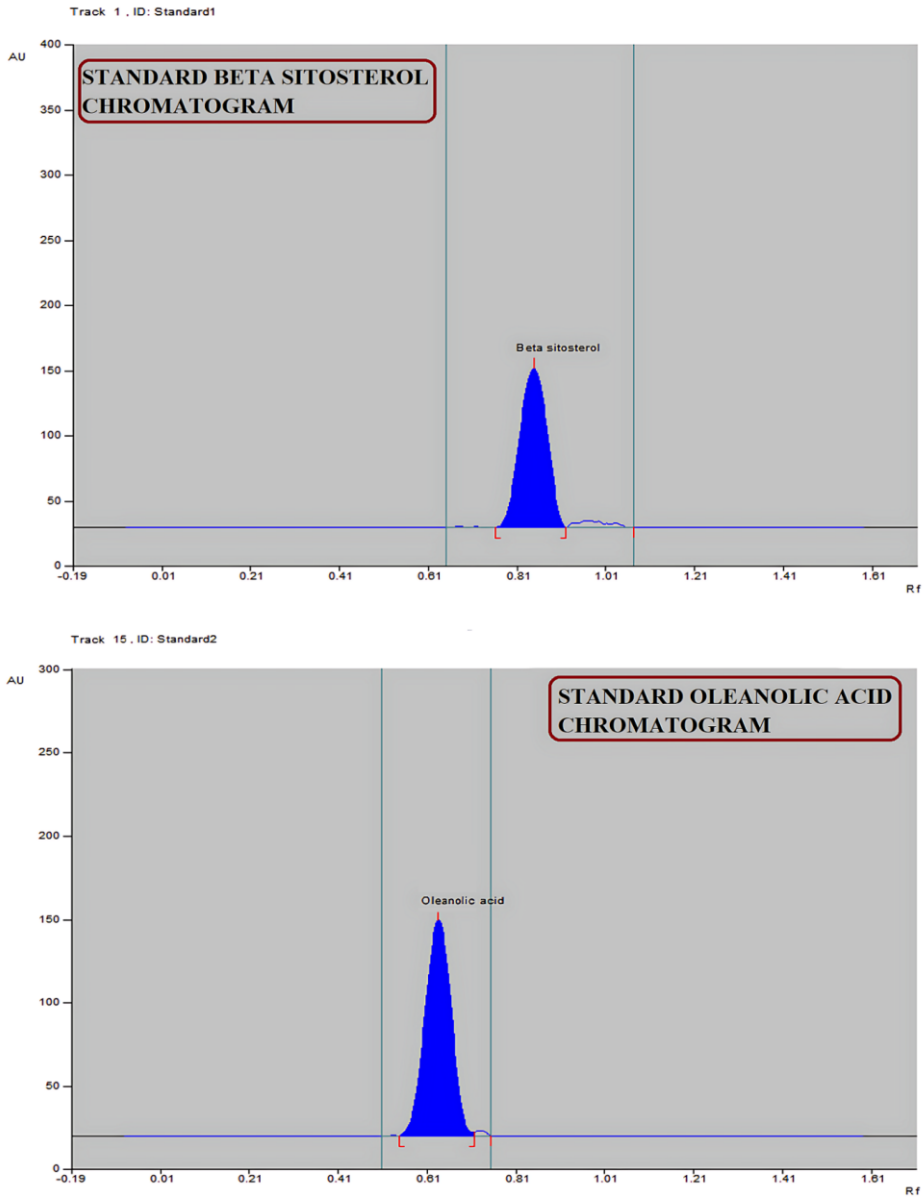

Fig. 3. Standard β-sitosterol and oleanolic acid HPTLC chromatogram.

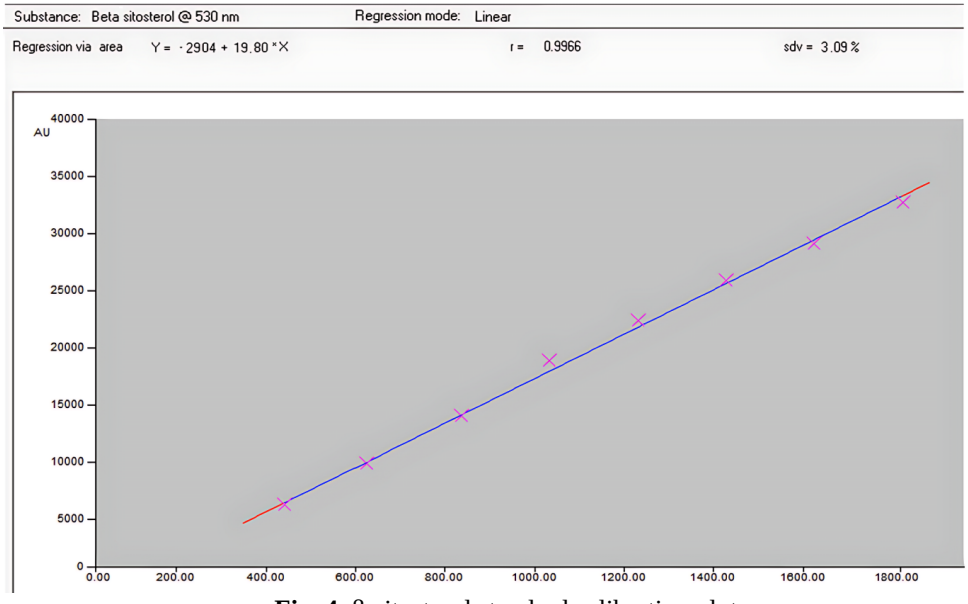

Fig. 4. β-sitosterol standard calibration plot.

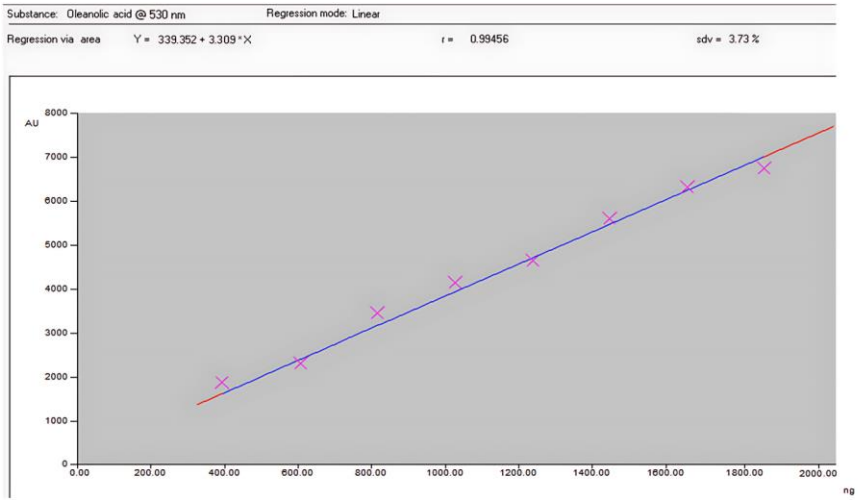

Fig. 5. Oleanolic acid standard calibration plot.

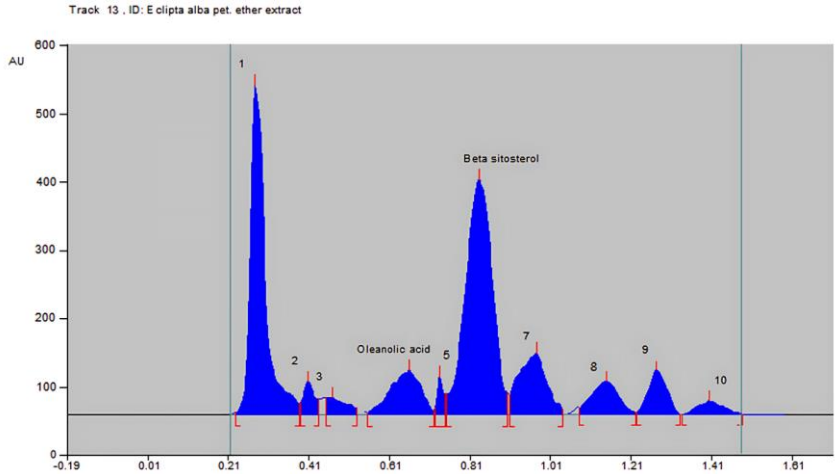

Fig. 6. HPTLC chromatogram of *E. alba* petroleum ether extract.

Table 1: Instrumental details and settings for the HPTLC analysis.

Equipment	Specifications
Sample applicator	Camag Linomat V (Muttenz, Switzerland)
Applicator syringe	Hamilton (100 µL)
Spraying rate	10 s/L
Saturation chamber	Twin trough chamber
Slit dimensions	6 mm × 0.45 mm
Scanning speed	20 mm/s
Monochromator bandwidth	20 nm
Data resolution	100 mm/step
Densitometer	Camag TLC Scanner III
Densitometer software	WinCATS
Wavelength	530 nm
Light source	Deuterium source
Chromatographic TLC plates	Aluminium plates precoated with silica gel 60 F_{254} (20 cm × 20 cm, 0.25 mm), E. Merck, Germany.

Table 2: Optimized chromatographic parameter for β-sitosterol and oleanolic acid in HPTLC

Parameter		Conditions
Mobile phase		Ethyl acetate, toluene and formic acid at the ratio of (6:5:0.1 v/v/v)
Stationary phase		Silica gel 60 F_{254} (20 cm × 10 mm)
Temperature		27 ± 0.5 °C
Distance travel (mm) by mobile phase		80
Duration of chamber saturation (min)		20
Derivatization		Anisaldehyde-sulfuric acid
Drying		5 minutes in a hot air oven at 110 °C
Speed of scanning (mm/s)		20
Measuring wavelength (nm)		530
Retention factor (R_f)	β-sitosterol	0.83
	Oleanolic acid	0.62
Diluent		Methanol

Table 3: Intermediate precision of β-sitosterol (BS) and oleanolic acid (OA).

Marker	Conc. (ng / spot)	Intra-day (n=3)		Inter-day (n=3)	
		Conc. ± SD*	RSD (%)	Conc. ± SD	RSD (%)
BS	600	599.33 ± 3.33	0.56	602.98 ± 4.25	0.70
	1100	1105.33 ± 4.67	0.42	1105.32 ± 3.60	0.32
OA	500	500.51 ± 1.78	0.36	503.26 ± 1.93	0.38
	900	901.31 ± 3.10	0.31	905.61 ± 3.17	0.35

* SD : standard deviation where n is number of times (n=3)

Table 4: Recovery data of β-sitosterol (BS) and oleanolic acid (OA).

Marker	Conc. of marker present (ng)	Conc. of marker added (ng)	Conc. of marker found ± SD* (ng)	Recovery (%)	Mean recovery (%)
BS	500	250	752.12 ±1.61	100.28	100.39
	500	500	1005.45±4.70	100.54	
	500	625	1129.12±3.63	100.36	
OA	450	225	675.78±2.46	100.11	100.06
	450	450	901.78±3.17	100.19	
	450	563	1011.78±2.97	99.87	

* SD: standard deviation where n is number of samples (n=3)

CONCLUSIONS

The developed HPTLC technique enables the quantitative analysis of β-sitosterol and oleanolic acid in *E. alba* entire plant material. With RSD values of 2%, the method demonstrates satisfactory accuracy. The recovery percentages for β-sitosterol (100.28–100.36%) and oleanolic acid (99.87–100.19%) further confirm the efficiency and reliability of the method. In addition, the fingerprint profiling of chromatograms obtained from *E. alba* extracts can be used for comparing and evaluating commercial samples of the whole plant or specific portions. Compared to HPLC, HPTLC offers advantages such as shorter processing times, lower sample requirements, optimized extractions using cost-effective chemicals, and smaller mobile phase volumes. This quick, easy, and sensitive HPTLC procedure serves as a valuable quality control tool for assessing the aerial portion of *E. alba*.

FUTURE SCOPE

Potential applications in quality control of herbal raw materials, and finished formulations, exploring synergistic effects with other compounds, and investigating the correlation between chemical composition and biological activity for optimized therapeutic use.

Author contributions. Conceived and designed the analysis: Arpan Chakraborty, Arka Bhattacharjee, Manas Chakraborty, Goutam Mukhopadhyay. Collected the data: Arpan Chakraborty, Arka Bhattacharjee, Baishakhi Mondal, Alpana Majumder. Contributed data or analysis tools: Arpan Chakraborty, Arka Bhattacharjee, Baishakhi Mondal, Alpana Majumder. Performed the analysis: Arpan Chakraborty, Arka Bhattacharjee, Baishakhi Mondal. Wrote the paper: Arpan Chakraborty, Arka Bhattacharjee, Baishakhi Mondal.

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Conflict of Interest. None.

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