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Research Article

Design of a Validated HPTLC Methodology for the Measurement of Linoleic and Oleanolic Acid in *Eclipta alba*

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ABSTRACT

Eclipta alba (Asteraceae family) is a widely recognized medicinal plant found in tropical and subtropical regions of the world. It is one of the plants that is most frequently utilized in traditional medical systems, including Ayurveda, Siddha, Homeopathy, Unani, and folk medicine. Many significant phytochemical components, including triterpenes, flavonoids, coumestans, steroids, saponins, and polypeptides, are present in each portion of this medicinal plant. Several herbal and ayurvedic formulations, like Indulekha bringha oil and Liv.52 Gnx pill, contain *E. alba* as an important therapeutic ingredient. The objective of the current work was to create a validated and consistent HPTLC technique for the simultaneous measurement of linoleic acid and oleanolic acid in *E. alba*. The procedure used silica gel 60 F_{254} as the stationary phase and ethyl acetate, toluene, and formic acid at a ratio of 4:7:0.2 (v/v/v) as the mobile phase, which produced compact bands upon derivatization with anisaldehyde-sulfuric acid reagent. The correlation coefficient (r^2) for the linear regression data for the standard linoleic and oleanolic acids calibration curves was 0.9966 and 0.9964, respectively, and it demonstrated a good linear relation over a range of concentrations of 300-1500 ng/spot and 450–1600 ng/spot with respect to the area. The approach's precision, accuracy, robustness, and selectivity were all assessed. LoD and LoQ for linoleic and oleanolic acids were measured to be 108.47 and 182.33 ng/spot and 258.30 and 327.54 ng/spot, respectively. We came to the conclusion that this approach, which uses HPTLC to quantify linoleic and oleanolic acids, is effective, straightforward, accurate, and reproducible.

Introduction

Due to insufficient drug regulations, awareness of herbal remedies throughout the world has increased.[1] The WHO has emphasized the need to ensure quality in crude drugs by adopting cutting-edge analytical techniques and establishing physicochemical parameters. Analytical control must take into account the material's complex and inconsistent composition, and chemical, physicochemical, and instrumental techniques must be used to create an adequate standard.[2]

Local names of *Eclipta alba*, often known as bhumiraj, bhringraj, and aali jhar, in addition to the popular name "False Daisy" in English.[3] Medium-sized, branching annual herbs with white flowers named *E. alba* are endemic to tropical and subtropical parts of the world.[4,5] It is traditionally used to treat a variety of skin disorders, including dermatitis, baldness prevention, and wounds. Honey and leaf juice are used to treat infants with catarrh.[6,7] *E. alba* juice is consumed orally or applied topically to encourage hair development.[8] In Nepal, the shoots and leaves are used to cure and prevent wound infections.[9, 10] It is used by several ethnic communities in South American nations to cure snakebites.[11] It is used in ayurveda for its rejuvenating and anti-aging effects.[12]

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It is used to cure jaundice by several ethnicities in Bangladesh.[13,14] This plant juice has been employed to prevent growth and kill disease-carrying insects like mosquitoes.[15, 16] Also, it is employed to cure a variety of illnesses, including baldness, acidity,[17] and others like gingivitis, bronchitis, asthma, wounds, burns, constipation, high temperature, body aches, wrinkles, acne, and other skin conditions.[18-21]

Many studies have been conducted on the elemental analysis and biological functions of various *E. alba* plant parts. A thorough knowledge of the potential uses of *E. alba* as a drug, cosmetic, and other formulations would be made possible via proper chemical characterization and standardization of plant materials.

One polyunsaturated omega-6 fatty acid is linoleic acid (LA). According to reports, it has a variety of beneficial physiological properties, such as hepatoprotective, anti-atherosclerotic, anticancer, anti-menorrhagic, and immunomodulatory properties.[22] Oleanolic acid (OA) is a plant-derived pentacyclic terpenoid with numerous pharmacological properties, including hepatoprotective, antioxidative, antiinflammatory, and anticancer activities. Its derivatives have a wide range of applications.[23] LA[24] and OA[25] were found in *E. alba*.

The present study's objective is to create an HPTLC technique that is verified for the simultaneous measurement of linoleic and oleanolic acids. Using silica gel 60 F_{254} TLC plates, we have established a technique employing ethyl acetate: toluene: formic acid (4:7:0.2 v/v/v) as the mobile phase. Densitometric scanning using a wavelength of 540 nm after derivatization was used to carry out the quantitative estimation.

Materials And Methods

Instrumentation

The prepared samples and aliquots of said standard stock solution were applied using a Camag Linomat V as sample applicator (Muttenz, Switzerland). In a twin trough chamber, the plates were saturated with settings for slit dimensions of 6 by 0.45 mm, 10 s/L spraying rate, 20 mm/s scanning speed, 20 nm monochromator bandwidth, and 100 mm/step data resolution. Zones were measured using the Camag TLC Scanner III densitometer, which was operated using Win CATS software. The filter used had a wavelength of 540 nm, and a deuterium source was used. Aluminum plates precoated using Silica Gel 60 F_{254} (20 × 20 cm, 0.25 mm) were used as the chromatographic plates (E. Merck, Germany).

Plant Material

In the month of February, the whole *E. alba* plant was collected in Kolkata (W.B.). The taxonomist characterized and authenticated the entire plant, *E. alba*. The plant material was finely pulverized, air-dried, and passed through filter number 10.

Chemicals and Reagents

The reference standards for linoleic acid (97%) and oleanolic acid (96%) were supplied by Sisco Research Laboratories Pvt. Ltd. and Innovative Chemical Interchange Pvt. Ltd (Carbino), respectively. The solvents employed in this study are all of spectroscopic grade. Additionally, every chemical used, including ethyl acetate, toluene, sulphuric acid, acetic acid, formic acid, methanol, petroleum ether, and anisaldehyde, was of analytical grade and sourced from Merck in Mumbai, India.

Standard Preparation

Linoleic acid and oleanolic acid reference standards were dissolved in methanol to create a 0.5 mg/mL solution for quantification purposes. The calibration curve was designed to comply with the ICH (International Conference on Harmonization) specifications.[26]

Sample Preparation

The dried coarse powder of *E. alba* was subjected to continuous heat extraction for two days using a soxhlet apparatus, exposing it to temperatures ranging from 60 to 80°C for eight hours with petroleum ether. It was filtered and evaporated in a vacuum under reduced pressure. The yield was determined using air-dried powdered crude material. The sample solution was produced by measuring 100 mg of the extract, adjusting the volume with petroleum ether (10 mg/mL), and storing it in the refrigerator.

Calibration

The standard curve was created in accordance with the ICH recommendations. Each concentration was sprayed on a plate (20 x 10 cm) in triplicate with bands that were each 6 mm wide and separated by 11.2 mm. The plate's bottom and side edges were 12 and 8 mm away, respectively. The application rate was 10 µL/s, and the bands were developed using ethyl acetate, toluene, and formic acid (4:7:0.2 v/v/v) after saturation for 20 minutes. After development and air drying, the plate was dipped in an anisaldehyde-sulfuric acid reagent (anisaldehyde 0.5 mL, acetic acid 10.0 mL, methanol 85 mL, and sulphuric acid 4.5 mL) solution for two seconds in a TLC plate dipping chamber. Afterward, the plate was removed from the reagent and allowed to heat for 5 minutes in a hot air oven at 110°C. Standard zones were measured using Camag TLC scanner III densitometer operating in the absorbance mode at a wavelength of 540 nm, which corresponds to the wavelength with the highest sensitivity. A deuterium lamp was used as a radiation source. Using peak area, linear regression analysis was used for evaluation.

Sample Assay Preparation

Sample and standard solutions were prepared as previously described, then spotted in three replicates on a plate and developed under the same circumstances as specified for the standard. During quantification, the sample solution

was spotted on the TLC plate in four different volumes: 5, 8, 12, and 15 µL. The analyte was discovered to be entirely separated from other components during the plates' development, derivatization, and drying in a hot air oven. As a result, the linear and compact zones were scanned at 540 nm, and peak areas for linoleic and oleanolic acids were observed.

Results and Discussion

Using silica gel TLC plates, several ratios of ethyl acetate, toluene, and formic acid were tested as the mobile phase, and a ratio of 4:7:0.2 (v/v/v) showed satisfactory resolution. After derivatization, a well-resolved symmetrical band for linoleic and oleanolic acids in the extract was found under optimum conditions (Fig. 1). Anisaldehyde sulphuric acid reagent produced blue and pink color spots when linoleic and oleanolic acids were derivatized. Standard linoleic acid (R_f = 0.61) and oleanolic acid (R_f = 0.72) both displayed a single sharp peak in the HPTLC chromatogram (Fig. 1), as well as the HPTLC chromatogram of the *E. alba* extract at 540 nm (Fig. 1). The most favorable scanning outcome was achieved at a wavelength of 540 nm. The mobile phase solvent suitability test was conducted for a duration of 24 hours, and it was determined that the chosen mobile phase solvent remained suitable throughout the testing period. The optimized chromatographic parameters for the analysis of LA and OA using HPTLC are provided in Table 1.

Linearity

A series of various concentrations of the reference linoleic and oleanolic acids, each applied in triplicate, for analysis. Across the concentration range of 300–1500 ng/spot with respect to the area, linear regression results for the standard curve of standard linoleic acid demonstrated a satisfactory linear relationship. In the linear regression equation, y = 23.94 x + 325.1, where y indicates the spot area and x is the analyte concentration, the correlation coefficient (r^2) was 0.9966 (Fig. 2). Oleanolic acid had a linear relationship with area over concentration levels of 450–1600 ng/spot, with an equation of y = 4.296 x - 345.352 with a r^2 of 0.9964 (Fig. 2). The spotted reference linoleic and oleanolic acids were scanned during the plate scanning process. The coefficient of variation (CV) for linoleic acid ranged from 0.0024 to 0.0188, while for oleanolic acid, it ranged from 0.0034 to 0.175. The smaller CV values indicate a higher level of precision and less variability.

Table 1: Optimized chromatographic parameter for linoleic and oleanolic acids in HPTLC

Parameter		*Conditions*
Mobile phase		Ethyl acetate, toluene and formic acid at the ratio of (4:7:0.2 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
Speed of scanning (mm/s)		20
Measuring wavelength (nm)		540
Retention factor (R_f)	linoleic acid	0.61
	oleanolic acid	0.72
Diluent		Methanol

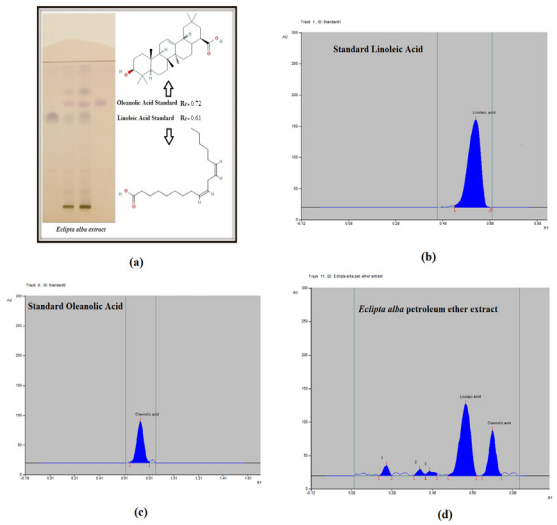

Fig. 1: (a) After derivatization standard linoleic acid, petroleum ether extract of *Eclipta alba*, oleanolic acid; (b) Standard linoleic acid HPTLC chromatogram; (c) Standard oleanolic acid HPTLC chromatogram; (d) HPTLC chromatogram of *Eclipta alba* petroleum ether extract.

Intermediate Precision (Reproducibility)

Evaluating mixed standard solutions of linoleic and oleanolic acids for two distinct concentrations (400, 700 ng/spot for LA and 500, 800 ng/spot for OA) three times on the same day and next day allowed to determine the intraday and interday precisions of the proposed methods. The outcomes are shown as a relative standard deviation (RSD) (Table 2). The intraday precision of linoleic and oleanolic acids was found to be in the range of 0.64–0.89 and 0.72–0.74, respectively. On the other hand,

Table 2: Intermediate precision of linoleic acid (LA) and oleanolic acid (OA)

Marker	*Conc. (ng / spot)*	*Intra-day (n=3)*		*Inter-day (n=3)*	
		*Conc. ± SD**	*RSD (%)*	*Conc. ± SD*	*RSD (%)*
LA	400	405 ± 3.60	0.89	406 ± 3.00	0.74
	700	707.66 ± 4.50	0.64	703.33 ± 5.03	0.72
OA	500	502.66 ± 1.52	0.30	500.33 ± 3.21	0.64
	800	802.33 ± 4.04	0.50	798.66 ± 3.05	0.38

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

Table 3: Recovery data of linoleic acid (LA) and oleanolic acid (OA)

Marker	*Conc. of marker present (ng)*	*Conc. of marker added (ng)*	*Conc. of marker found (ng)*	*Recoveryb (%)*	*Mean recovery (%)*
LA	300	150	457.66 ± 3.68	101.70	100.73
	300	300	603.66 ± 4.18	100.61	
	300	375	674.33 ± 3.29	99.90	
OA	400	200	601.33 ± 4.10	100.22	100.29
	400	400	802 ± 3.26	100.25	
	400	600	1004 ± 4.54	100.40	

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

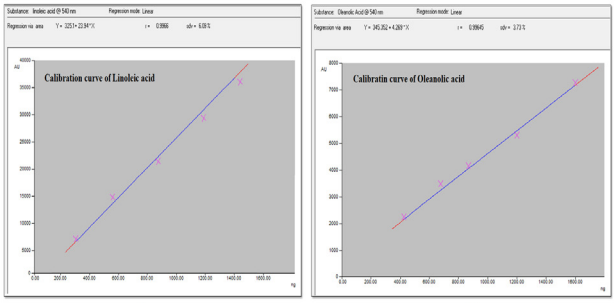

Fig. 2: Calibration plot of standard (a) Linoleic acid; (b) Oleanolic acid

the interday precision of linoleic and oleanolic acids, which evaluate the variability across different days, ranged from 0.30–0.50 and 0.38–0.64, respectively. The RSD value below 2% suggests that the results obtained are consistent and reproducible.

Accuracy (Percentage Recovery)

Calculating the recovery of linoleic and oleanolic acids using the standard addition approach was used to assess the methods' accuracy. Three separate phases of recovery trials (50, 100, and 125% inclusion of linoleic and oleanolic acids) were carried out. Both the percent recoveries and the total mean recoveries were calculated. By putting peak area values into the calibration curve's regression equations, levels of linoleic and oleanolic acids were calculated (Table 3). The mean percentage recovery of linoleic and oleanolic acids was 100.73 and 100.29, respectively. These values demonstrate the accuracy and reliability of the analytical method.

Method Precision (Repeatability)

Injecting reference standard solutions (n = 6) of different linoleic and oleanolic acid concentrations was repeatedly done to test the equipment's precision. The repeatability of the HPTLC instrument was assessed by applying the same sample solution six times on a plate with the automatic spotter using the same syringe and taking repeated scans of the sample spot six times for both linoleic and oleanolic acids without changing the position of the plate. The coefficient of variation (CV) range for linoleic and oleanolic acids was determined to be 0.021–0.052 and 0.016–0.026, respectively.

Limit of Detection and Limit of Quantification

According to the ICH recommendations, 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 were computed for both substances using the following equations:

$$LoQ = 3.3 \times \sigma/SD$$
$$LoQ = 10 \times \sigma/SD$$

where the response's standard deviation is σ and SD stands for the standard deviation of the y-intercept of the regression line. The LoD for linoleic acid was measured to be 108.47 ng/spot, while the LoQ was found to be 258.30 ng/spot. Similarly, the LoD for oleanolic acid was determined to be 182.33 ng/spot, and the LoQ was measured as 327.54 ng/spot.

Percentage Concentration of LA and OA in *Eclipta alba* Extract

Under optimal conditions, utilizing silica gel TLC plates and a mobile phase consisting of ethyl acetate, toluene, and formic acid in a ratio of (4:7:0.2 v/v/v) showed satisfactory results. Following derivatization, a distinct and symmetrical band for linoleic and oleanolic acids was observed in the extract. The amounts of LA and OA in the *E. alba* extract were determined to be 0.86 and 0.52% w/w, respectively. The calibration curve equations for LA determined this and OA mentioned previously, where x represents the amount of biomarkers and y represents the area under the curve. In the HPTLC chromatogram, both the standard linoleic acid (R_f = 0.61 ± 0.2) and oleanolic acid (R_f = 0.72) exhibited a single sharp peak, found in the extract (Fig. 2b). In order to verify specificity, the R_f of the standard and sample were compared.

Conclusion

This HPTLC technique was created for the quantitative study of LA and OA in *E. alba* entire plant material. RSD values were 2%, indicating that the method's accuracy is reassuringly adequate. The recovery of LA was 99.90 to 101.70% and that of OA was 100.22 to 100.40%, demonstrating the method's efficiency and reliability. For comparison and assessment of commercial samples of the whole plant or a portion of *E. alba*, fingerprint profiling

of chromatograms derived from extracts of *E. alba* may be employed. The inherent benefits of this approach over HPTLC are shorter processing times, smaller sample quantities, single-optimized extractions utilizing affordable chemicals, and smaller mobile phase volumes. This HPTLC procedure is quick, easy, and sensitive and may be employed as a quality control tool to assess the aerial portion of *E. alba*.

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None.

Conflicts of Interest

There are no conflicts of interest.

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