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Developing a Validated HPTLC Methodology to Measure Eclipta alba's Linoleic and Oleanolic Acid

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ABSTRACT

A well-known medicinal plant found in tropical and subtropical areas of the globe is Eclipta alba (Asteraceae family). It is one among the herbs most often used in traditional medicine, such as folk medicine, Ayurveda, Siddha, homeopathy, and Unani. Every part of this therapeutic plant has a multitude of important phytochemical components, such as triterpenes, flavonoids, coumestans, steroids, saponins, and polypeptides. E. alba is a key medicinal component in many herbal and ayurvedic preparations, such as Liv.52 Gnx tablet and Indulekha brengha oil. Developing a reliable and consistent HPTLC method for measuring oleanolic and linoleic acid in E. alba simultaneously was the goal of the present study. The process yielded compact bands upon derivatization with anisaldehyde-sulfuric acid reagent. The stationary phase of the technique was silica gel 60 F254, while the mobile phase consisted of ethyl acetate, toluene, and formic acid at a ratio of 4:7:0.2 (v/v/v). The linear regression data for the standard linoleic and oleanolic acid calibration curves had correlation coefficients (r2) of 0.9966 and 0.9964, respectively. These values showed a strong linear relationship over a range of concentrations between 300 and 1500 ng/spot and 450 and 1600 ng/spot, respectively, with respect to the area. We evaluated the selectivity, robustness, accuracy, and precision of the technique.

Introduction

The world has become more aware of herbal treatments as a result of inadequate drug controls.[1] The WHO has underlined the need of developing physicochemical characteristics and using state-of-the-art analytical procedures to guarantee quality in crude pharmaceuticals. The complex and variable composition of the material must be considered in analytical control, and methods including chemistry, physicochemistry, and instrumentation must be used to provide a sufficient standard.[2] Eclipta alba is referred to by many local names, including bhumiraj, bhringraj, and aali jhar, in addition to the common English name "False Daisy."[3] E. alba is a medium-sized, branching annual plant native to tropical and subtropical regions of the globe that has white blooms.[4,5] It is historically used to heal wounds, dermatitis, and prevention of baldness, among other skin diseases.Babies with diarrhea may be treated with leaf juice and honey.[6,7] E. alba juice is used either topically or as a rally to promote hair growth.[8] The leaves and shoots are used in Nepal to treat wounds and stop infections.Many ethnic populations in South American countries utilize it to treat snakebite injuries [9, 10].Because of its anti-aging and revitalizing properties, it is employed in Ayurveda.[12] Several ethnic groups in Bangladesh utilize it to treat jaundice.[13, 14] This plant juice has been used to stop the spread of illness and eliminate insects that transmit it, such as mosquitoes.[15, 16] Additionally, it is used to treat a wide range of ailments, such as acidity, baldness, gingivitis, bronchitis, asthma, burns, wounds, constipation, fever, body pains, wrinkles, acne, and other skin issues [17].[18–21]

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Linoleic acid (LA) is a polyunsaturated omega-6 fatty acid. Numerous advantageous physiological attributes have been reported for it, including hepatoprotective, anti-atherosclerotic, anticancer, antimenorrhagic, and immunomodulatory qualities.[22] Oleanolic acid (OA) is a pentacyclic terpenoid generated from plants that exhibits a wide range of pharmacological characteristics, such as antioxidant, anti-inflammatory, hepatoprotective, and anticancer effects. There are several uses for its derivatives.[23] In E. alba, LA[24] and OA[25] were discovered. The goal of this work is to develop a validated HPTLC method for measuring oleanolic and linoleic acids simultaneously. We have developed a method using silica gel 60 F254 TLC plates that uses ethyl acetate, toluene, and formic acid (4:7:0.2 v/v/v) as the mobile phase. The quantitative estimate was performed by densitometric scanning at 540 nm wavelength after derivatization.

Materials And Methods

Instrumentation

The samples that were produced and aliquots of the standard stock solution were applied utilizing a sample applicator (Camag Linomat V) that was located in Munich, Switzerland. The plates were saturated with parameters for a 6 by 0.45 mm slit width, 10 s/L spraying rate, 20 mm/s scanning speed, 20 nm monochromator bandwidth, and 100 mm/step data resolution in a twin trough chamber. The Camag TLC Scanner III densitometer, which was controlled by Win CATS software, was used to measure zones. A deuterium source and a filter with a wavelength of 540 nm were used. Chromatographic plates (20 x 20 cm, 0.25 mm) were coated with aluminum using Silica Gel 60 F254 (E. Merck, Germany).

Plant Material: The whole E. alba plant was obtained in Kolkata (W.B.) in the month of February. The taxonomist identified and described the E. alba plant in its entirety. After being finely ground and allowed to air dry, the plant material was run through filter number 10.

Reagents and Chemicals

Innovative Chemical Interchange Pvt. Ltd (Carbino) and Sisco Research Laboratories Pvt. Ltd. provided the reference standards for oleanolic acid (96%) and linoleic acid (97%) respectively. All of the solvents used in this investigation have spectroscopic grades. Furthermore, all of the chemicals used—such as methanol, petroleum ether, anisaldehyde, acetic acid, sulfuric acid, ethyl acetate, toluene, and sulphuric acid—were of analytical quality and came from Merck in Mumbai, India.

Customary Setting Up

For measurement, methanol was used to dissolve reference standards of oleanolic acid and lindeic acid, yielding a 0.5 mg/mL solution. The International Conference on Harmonization (ICH) criteria were followed in the construction of the calibration curve.[26]

Sample Set-Up

utilizing a soxhlet apparatus, the dried coarse powder of E. alba was continuously heated for two days, exposing it to temperatures between 60 and 80°C for eight hours while utilizing petroleum ether. It was filtered, then evaporated at lower pressure in a vacuum. Using powdered crude material that has been air-dried, the yield was calculated. After measuring 100 mg of the extract, adding petroleum ether (10 mg/mL) to correct the volume, and refrigerating the mixture, the sample solution was created.

Adjustment

The ICH guidelines were followed while creating the standard curve. On a plate measuring 20 by 10 cm, each concentration was sprayed in triplicate using bands of 6 mm in width and 11.2 mm apart. The distance between the plate's side and bottom borders was 8 and 12 mm, respectively.

After saturation for 20 minutes, the bands were developed using ethyl acetate, toluene, and formic acid (4:7:0.2 v/v/v) at an application rate of 10 µL/s. Following development and air drying, the plate was immersed for two seconds in a TLC plate dipping chamber in an anisaldehyde-sulfuric acid reagent solution (anisaldehyde 0.5 mL, acetic acid 10.0 mL, methanol 85 mL, and sulfuric acid 4.5 mL). The plate was then taken out of the reaction and heated in a hot air oven set to 110°C for five minutes. Using the wavelength with the maximum sensitivity, 540 nm, the Camag TLC scanner III densitometer was used to measure standard zones while it was in the absorbance mode. The radiation source was a deuterium lamp. The assessment method was linear regression analysis with peak area.

Results and Discussion

A ratio of 4:7:0.2 (v/v/v) demonstrated adequate resolution when ethyl acetate, toluene, and formic acid were tested in various ratios using silica gel TLC plates as the mobile phase. Under ideal circumstances, derivatization produced a well-resolved symmetrical band for oleanolic and linoleic acids in the extract (Fig. 1). Linoleic and oleanolic acids were derivatized using anisaldehyde sulfuric acid reagent, which resulted in blue and pink color patches. Both the HPTLC chromatogram of the E.

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alba extract at 540 nm (Fig. 1) and the HPTLC chromatogram of standard linoleic acid (Rf = 0.61) and oleanolic acid (Rf = 0.72) showed a single strong peak. An optimal scanning result was obtained at a wavelength of 540 nm. It was found that the selected mobile phase solvent remained compatible for the whole testing time after a 24-hour mobile phase solvent compatibility test. Table 1 lists the ideal chromatographic parameters for the HPTLC analysis of LA and OA.

Consistency

a range of reference oleanolic and linoleic acid concentrations, each administered in duplicate, for examination. A acceptable linear connection was shown by the linear regression findings for the standard curve of standard linoleic acid across the concentration range of 300–1500 ng/spot with regard to the area. The correlation coefficient (r2) for the linear regression equation y = 23.94 x + 325.1, where x is the analyte concentration and y is the spot area, was 0.9966 (Fig. 2). Oleanolic acid, represented by the equation y = 4.296 x - 345.352 with a r2 of 0.9964 (Fig. 2), exhibited a linear relationship with area across concentration ranges of 450–1600 ng/spot. The plate scanning procedure included scanning the spotted reference oleanolic and linoleic acids. For linoleic acid, the range of the coefficient of variation (CV) was 0.0024 to 0.0188, while for oleanolic acid, it was 0.0034 to 0.175. Less unpredictability and a better degree of accuracy are indicated by lower CV values.

Moderate Precision (Acceptability)

The intraday and interday precisions of the suggested procedures may be ascertained by testing mixed standard solutions of linoleic and oleanolic acids for two different concentrations (400, 700 ng/spot for LA and 500, 800 ng/spot times on the same day and following day). Table 2 displays the results as a relative standard deviation (RSD). It was discovered that the intraday precision of oleanolic and linoleic acids fell between 0.72 and 0.74 and 0.64 and 0.89, respectively. Table 1: Optimized Chromatographic Parameter for Linoleic and Oleanolic Acids in HPTLC, on the other hand

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

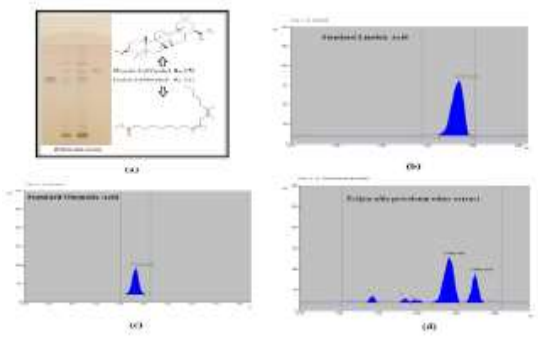

Fig. 1: (a) Following derivatization, the following chromatograms were obtained: (b) a standard linoleic acid HPTLC chromatogram; (c) a standard oleanolic acid HPTLC chromatogram; and (d) an HPTLC chromatogram of the petroleum ether extract of Eclipta alba.

The correlation coefficient (r2), where area and x represent the analyte concentration, was 0.9966 (Fig. 2). Oleanolic acid, represented by the equation y = 4.296 x - 345.352 with a r2 of 0.9964 (Fig. 2), exhibited a linear relationship with area across concentration ranges of 450–1600 ng/spot. The plate scanning procedure included scanning the spotted reference oleanolic and linoleic acids. For linoleic acid, the range of the coefficient of variation (CV) was 0.0024 to 0.0188, while for oleanolic acid, it was 0.0034 to 0.175. Less unpredictability and a better degree of accuracy are indicated by lower CV values.

Moderate Precision (Acceptability)

The intraday and interday precisions of the suggested procedures may be ascertained by testing mixed standard solutions of linoleic and oleanolic acids for two different concentrations (400, 700 ng/spot for LA and 500, 800 ng/spot for OA) three times on the same day and the following day. Table 2 displays the results as a relative standard deviation (RSD). It was

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discovered that the intraday precision of oleanolic and linoleic acids fell between 0.72 and 0.74 and 0.64 and 0.89, respectively. Conversely, Table 2: Intermediate accuracy of oleanolic acid (OA) and linoleic acid (LA)

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)	Recovery% (%)	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	800 ± 3.26	100.25	
	400	600	1004 ± 4.54	100.40	

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

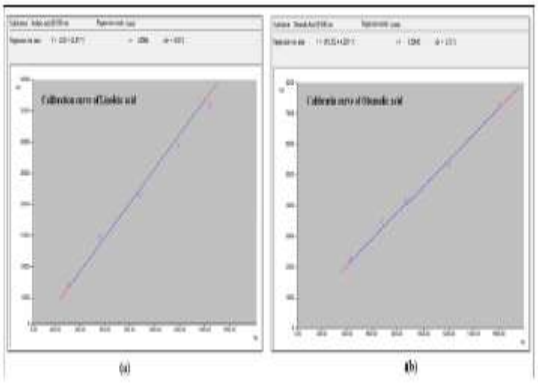

Fig. 2: Calibration plot of standard (a) Linoleic acid;N(b) Oleanolic acid

The linoleic and oleanolic acids' interday accuracy, which measures the variability across various days, varied from 0.38 to 0.64 and from 0.30 to 0.50, respectively. A reliable and consistent set of findings is indicated by an RSD value of less than 2%.

Accuracy (Recovery Percentage)

The accuracy of the procedures was evaluated by calculating the recovery of oleanolic and linoleic acids using the standard addition technique. Three distinct stages of the recovery experiment were conducted, with linoleic and oleanolic acids included at 50, 100, and 125% concentrations. Calculations were made for the overall mean recoveries as well as the recovery percentage. The amounts of oleanolic and linoleic acids were determined by plugging peak area values into the regression equations of the calibration curve (Table 3). For linoleic and oleanolic acids, the mean % recovery was 100.73 and 100.29, respectively. These numbers show how accurate and dependable the analytical process is.

Technique Accuracy (Replicability)

To check the accuracy of the apparatus, reference standard solutions (n = 6) with varying amounts of oleanolic and linoleic acid were repeatedly injected. The same sample solution was applied to a plate with the automated spotter six times using the same syringe, and the sample spot was repeatedly scanned for both oleanolic and linoleic acids six times without the plate being moved in order to evaluate the repeatability of the HPTLC apparatus. For linoleic and oleanolic acids, the range of coefficient of variation (CV) was found to be 0.021–0.052 and 0.016–0.026, respectively. Limit of Detection and Limit of Quantification 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 recommendations:

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

where SD is the standard deviation of the regression line's y-intercept and σ is the response's standard deviation. Linoleic acid was determined to have a LoD of 108.47 ng/spot and a LoQ of 258.30 ng/spot. In a similar vein, oleanolic acid's LoD was found to be 182.33 ng/spot, while its LoQ was quantified as 327.54 ng/spot. The percentage concentration of OA and LA in the extract of Eclipta alba Results were good when using silica gel TLC plates with a mobile phase containing formic acid, toluene, and ethyl acetate in the ratio of 4:7:0.2 v/v/v. After derivatization, the extract showed a clear, symmetrical band for linoleic and oleanolic acids. It was found that the E. alba extract contained 0.86 and

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0.52% w/w of LA and OA, respectively. This was found using the previously discussed calibration curve formulae for LA and OA, where x stands for the number of biomarkers and y for the area under the curve. The extract (Fig. 2b) showed a single strong peak in the HPTLC chromatogram for both the standard linoleic acid (Rf = 0.61 ± 0.2) and oleanolic acid (Rf = 0.72). The Rf of the standard and sample were compared to confirm specificity.

Conclusion

The whole plant material of E. alba was subjected to a quantitative investigation of LA and OA using this HPTLC approach. RSD readings of 2% show that the accuracy of the approach is reassuringly sufficient. The recovery of OA was 100.22 to 100.40% and that of LA was 99.90 to 101.70%, indicating the effectiveness and dependability of the procedure. Fingerprint profiling of chromatograms produced from E. alba extracts may be used for evaluation and comparison of commercial samples of the whole plant or a subset of it. Shorter processing durations, lower sample sizes, single-optimized extractions using reasonably priced chemicals, and reduced mobile phase volumes are the intrinsic advantages of this method over HPTLC.

This rapid, simple, sensitive HPTLC method may be used to evaluate the aerial part of E. alba as a quality control measure.

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