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Thin Layer Chromatography (TLC) Fingerprint Analysis of *Moringa oleifera* Leaves Extract

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ABSTRACT: *Moringa oleifera is known to be rich in nutrients and phytochemicals. M.oleifera is also widely used for herbal medicine and functional food. Fingerprint profiling is one of the methods used to determine the quality and authenticity of M.oleifera leaves and prevent counterfeiting. The study aimed was to determine optimal chromatographic parameters (stationary and mobile phases, blot volume, and detection method) capable to generating the highest number of bands with good separation. The method used was Thin Layer Chromatography (TLC), accompanied by stability testing and precision testing. The results showed that fingerprint profile of M. oleifera leaves, created using the mobile phase toluene: ethyl acetate: methanol: formic acid (7:2:1:0.2), stationary phase TLC plate with silica gel 60 F_{254}. Test solution was 70% ethanol viscous extract of M. oleifera leaves, dissolved in methanol with a blotting volume of 5µl, observed in UV 366 nm and obtained eight bands. Stability and precision testing of the TLC condition optimization method showed that the analyte was stable on the TLC plate and in solution. The analyte also showed stability results during the chromatography process, and the repeatability and intermediate precision were met the criteria. The TLC fingerprint analysis method with chromatographic conditions optimized in this study can be used as one of the quality control methods of M. oleifera leaf raw materials.*

Keywords: *chromatography; Moringa oleifera; TLC-Fingerprint; quality control*

1. Introduction

Moringa oleifera (*M. oleifera*), the "miracle tree", thrives in almost all tropical and subtropical regions. Research shows that all parts of the plant (leaves, seeds, pods, flowers) of *M. oleifera* have good nutritional content and have been widely developed into alternative food nutrition [1]. *Moringa oleifera* is recognized not only for its high nutritional value but also for its rich profile of bioactive phytochemicals. Its leaves contain essential nutrients-such as vitamins, minerals, and proteins-alongside a diverse array of secondary metabolites, including carotenoids, alkaloids, flavonoids, glycosides, anthocyanins, anthraquinones, saponins, steroids, tannins, and terpenoids. The presence of these phytochemicals directly contributes to the plants pharmacological properties, which encompass anti-cancer, antidiabetic, cardioprotective, anti-aging, anti-arthritic, and anti-inflammatory activities [2]. The therapeutic potential of *M. oleifera*, particularly its leaves, has been extensively explored in countries such as India and Nigeria, supporting the development of numerous leaf extract-based products. As its use becomes more widespread, ensuring the quality, efficacy, and standardization of herbal raw materials has become increasingly important. Fingerprint profiling is therefore employed as a reliable analytical approach to assess the quality and authenticity of *M. oleifera* leaves and to prevent adulteration in commercial herbal products [3].

An efficient way to monitor and assess the quality of herbal medicine products and raw materials is fingerprint analysis. Due to its ability to display the chemical components, overall composition and relative concentrations. This method can also be used to assess the quality of medicinal plants. Chromatographic methods provide separation and detailed profiling, which confirms the authenticity and quality of herbal materials. The most utilized method for quality control is fingerprint analysis [4]. Fingerprint analysis using Thin Layer Chromatography (TLC) is a commonly used method. The benefits of using TLC method are easy sample preparation, simple operation, lower cost, less solvent volume used, time efficiency in sample processing, selective and sensitive and possible to observe the chromatogram visually [5]. Several methods for fingerprint analysis have been reported in the literature for detecting *M. oleifera* leaf quality, including Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) [6], and High-Performance Thin Layer Chromatography (HPTLC) [7–11]. This study analyzed the fingerprint profile of *M. oleifera* plants using the TLC method. The purpose of this study is to determine optimal chromatographic parameters (stationary and mobile phases, blot volume, and detection method) capable in generating the highest number of bands with good separation.

2. Material and methods

2.1. Chemicals and equipment

Some of the chemicals used were obtained from Merck (Darmstadt, Germany): n-hexane, diethyl ether, methanol, ethanol, acetic acid, formic acid, dichloromethane, ethyl acetate, toluene, chloroform, and TLC plates with silica gel 60 F_{254}. The equipment used in this study were a twin through chamber (Camag®, Muttenz, Switzerland), a sample applicator (Linomat 5, Camag®), a a TLC-Visualizer equipped with a 12-bit CCD camera, and Camag VideoScan 1.02 software.

2.2. Plant materials and preparation of extracts

This study used fine powder of *M. oleifera* leaves with a 500 mesh size (PT. Moringa Organik, Blora, Central Java, Indonesia). *M. oleifera* leaves crude drug powder was macerated with 70% ethanol for 3x24 hours, with occasional stirring for the first 6 hours. The filtrate from the maceration was concentrated using a rotary evaporator at 40°C - 50°C until a thick extract was obtained. The thick 70% ethanol extract of *M. oleifera* leaves was dissolved in methanol prior to blotting on a TLC plate [12].

2.3. Method development and validation

2.3.1. Preparation of stationary phase

A TLC plate with silica gel 60 F_{254} was used as the stationary phase. The thickness of the silica layer was 175-225 µm on an aluminum plate with a particle size distribution of 9.5-11.5 µm. Samples were blotted using a sample applicator (Linomat 5, Camag®) with the conditions (6 mm band, 10 mm from the left and right edges, 15 mm from the bottom edge, and 80 mm track spacing), using a 100 µL syringe (Camag). A sample volume of 5 µL was used to generate a fingerprint profile of the *M. oleifera* leaves extract.

2.3.2. Selection of mobile phase

This study used a modified mobile phase from the study developed by Elangovan [9] .

2.3.3. Detection and visualization

The eluted TLC plates were dried at room temperature, then the TLC plates were illuminated sequentially under UV 254 nm, UV 366 nm and white light using a TLC Visualizer equipped with a 12-bit CCD camera and documented using Camag VideoScan 1.02 software.

2.3.4. Stability of chromatogram

The sample was left at room temperature for three hours on the TLC plate and in a solution to assess the analyte's stability. The extracts were applied to tracks 1-6, while tracks 1-2 for extracts blotted onto the TLC plate and allowed to stand for 3 hours, tracks 3-4 for freshly prepared extracts blotted before elution, and tracks 5-6 for extract solutions allowed to stand for 3 hours and blotted just before elution. The analyte was stable when the difference between the Rf values in tracks 1 to 6 did not exceed 0.05. Two-dimensional (2D) elution was also used in this study to evaluate analyte stability during chromatography. An analyte was said to be stable during the chromatographic process if all zones lie on the diagonal line connecting the starting position and the intersection of two mobile phases [13].

2.3.5. Precision of chromatogram

Samples were extracted in the same procedure and then blotted three times on different plates, the plates were eluted with a new mobile phase on the same day. If the Rf value of each band zone was less than 0.02, the repeatability result was acceptable. The intermediate precision was done by extracting the sample in the same way and then blotted three times on different plates, then elute the plate with a new mobile phase on different days. If the Rf value in each band zone was less than 0.05, the intermediate precision result was acceptable [13].

3. Result and discussion

3.1. Mobile phase optimization

Fingerprint analysis is necessary to determine the chromatogram pattern of *M. oleifera* leaf extract. The chromatographic fingerprint of herbal medicines is a set of chromatographic patterns generated from several common chemical components that may contribute to the pharmacological activity or have some chemical properties. Chromatographic Fingerprint can show specific similarities and differences between various samples and for authentication of herbal samples and can prevent herbal counterfeiting [14,15]. Chemical fingerprint can be obtained through various separation-based techniques e.g., liquid chromatography (LC) and thin layer chromatography (TLC) or non-separation-based techniques e.g., Fourier transform infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR) [16]. In this study, fingerprint analysis of Moringa leaf extract was performed using TLC. TLC serves as an initial screening method utilizing semi-quantitative evaluation, which is simpler than other chromatographic techniques. The benefits of the TLC technique consist of straightforward sample preparation, simpler operation, lower cost, lower volume of solvent used, time efficiency in sample processing, selectivity and sensitivity [17].

In the early stages of TLC, mobile phase optimization was performed. The choice of mobile phase Toluene:Ethyl acetate:Methanol in a combination of 7:2:1, was based on the findings of Elangovan's study [9]. The researchers modified the mobile phase by adding a solvent of formic acid. Formic acid was added to the mobile phase mixture to make it more polar. In previous studies on moringa leaf fingerprint patterns, the addition of small amounts of formic acid has been commonly utilized. Niranjan employed a mobile phase combination of toluene-ethyl acetate-formic acid (14:10:1) [18], Thomas used toluene-ethyl acetate-methanol-formic acid (4.9:4.1:2:0.5) [11], while Alam used toluene-ethyl acetate-formic acid-methanol (5.0:4.0:1.0.5:0.5) [7]. Toluene, ethyl acetate, methanol, and formic acid (in a ratio of 7:2:1:0.2) were utilized as the preferred mobile phase and produced 8 bands observed under UV 366 nm. In this study, a slight modification was made by adding formic acid at concentrations of 0.2, 0.4, and 0.5. Figure 1 and Table 1 display the chromatogram pattern of the optimization results using the modified mobile phase.

In optimizing the modified mobile phase through the addition of formic acid, a clear improvement in band separation was observed. Formic acid possesses a relatively high dielectric constant (approximately 58) [19], which contributes to an increase in the overall polarity of the mobile phase when it is used as a modifier [20]. In TLC, Adding small percentages of formic acid—typically in the range of 1–10%—can enhance chromatographic resolution by suppressing analyte ionization, minimizing tailing, and improving the mobility of polar compounds on silica gel [21–24]. As shown in Figure 1 and Table 1, the mobile phase of Toluene, Ethyl acetate, Methanol, and Formic acid (7:2:1:0.2) produces the highest number of bands (8 bands) with the best separation.

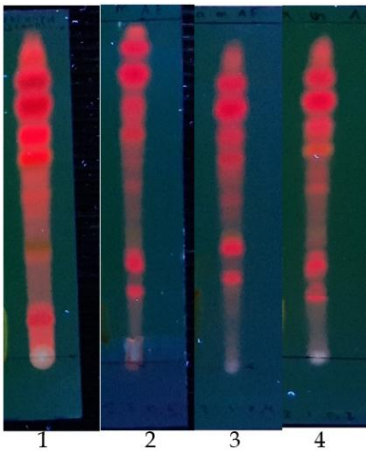

Figure 1. Mobile phase optimization (1) Toluene:Ethyl acetate:Methanol (7:2:1), (2) modified mobile phase optimization Toluene: Ethyl acetate: Methanol: Formic acid (7:2:1:0.5), (3) Toluene: Ethyl acetate:Methanol:Formic acid (7:2:1:0.4), (4) Toluene:Ethyl acetate:Methanol:Formic acid (7:2:1:0.2)

Table 1. Number of band of modified mobile phase optimization results

Quantity comparison mobile phase	Number of bands
Toluene:Ethyl acetate:Methanol (7:2:1)	7
Toluene:Ethyl acetate:Methanol: Formic acid (7:2:1:0.5)	7
Toluene:Ethyl acetate:Methanol: Formic acid (7:2:1:0.4)	7
Toluene:Ethyl acetate:Methanol: Formic acid (7:2:1:0.2)	8

3.2. M. oleifera leaf fingerprint profile

Fingerprint profiles of *M. oleifera* leaves were created using the following TLC conditions: The mobile phase comprised Toluene:Ethyl acetate: Methanol:Formic acid (7:2:1:0.2); stationary phase: TLC plate with silica gel 60 F_{254}, test solution: 5% solution of 70% ethanol condensed extract of *M. oleifera* leaves dissolved in methanol, blotting volume: 5µl; visualization: UV 254 nm, UV 366 nm, and white light. Figure 2 displays the TLC fingerprint profile results of *M. oleifera* leaf extract. In the white light observation, 7 bands were observed; in the UV 254 nm, 6 bands were observed; while in the UV 366 nm, 8 bands were observed. Observation with Camag VideoScan 1.02 software reveals 8 peaks as shown in Figure 3.

The results of videodensitogram observation (Figure 3, Table 2) show the retention factor (Rf) maximum values 1-8, indicating the values of 0.254, 0.319, 0.511, 0.563, 0.634, 0.689, 0.758, and 0.849, respectively.

The limitation of this study was no standard compounds used as comparison in creating the fingerprint pattern of Moringa leaf extract. A band was selected that showed perfect bands, no tailing, sharp color intensity, and a good separation. The method of optimizing chromatographic conditions as validated. The purpose of method validation is verifying the feasibility for its intended use [13,25].

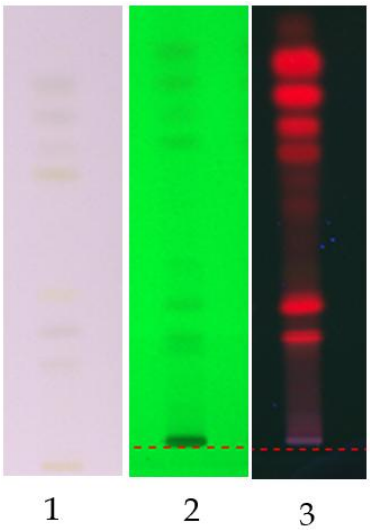

Figure 2. Fingerprint profile of *M. oleifera* leaf extract (1) under white light (7 bands), (2) UV light 254 nm (6 bands), (3) UV light 366 nm (8 bands)

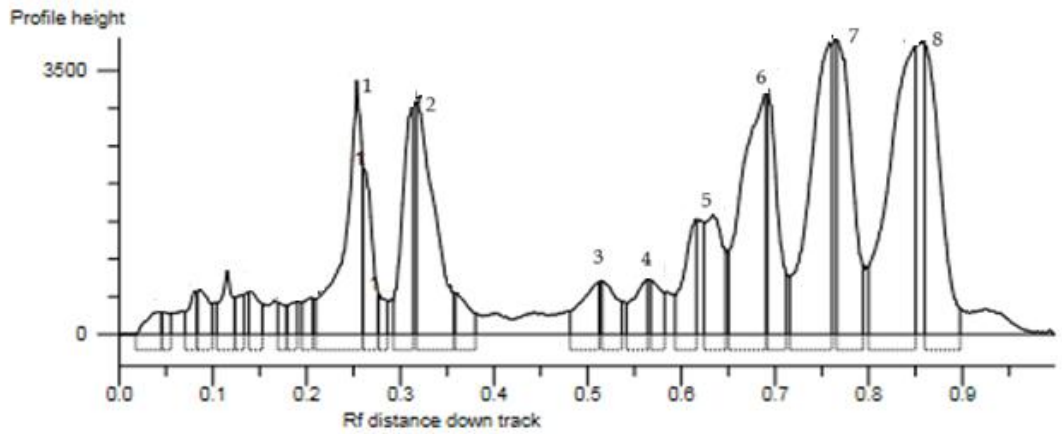

Figure 3. Videodensitogram of *M.oleifera*

Table 2. Rf value and area of *M.oleifera* peak

Peak	Start		Max.			End		Area	
#	Rf	H	Rf	H	[%]	Rf	H	A	[%]
1	0.210	466.2	0.254	3367.4	7.36	0.260	2181.1	41180.5	7.60
2	0.317	3047.1	0.319	3094.0	6.76	0.358	540.5	41601.9	7.68
3	0.481	305.2	0.511	688.5	1.50	0.512	688.4	9361.2	1.73
4	0.541	412.7	0.563	731.0	1.60	0.564	721.3	7768.1	1.43
5	0.624	1484.4	0.634	1591.0	3.48	0.647	1093.3	20039.6	3.70
6	0.650	1083.5	0.689	3235.3	7.07	0.691	3192.6	57535.1	10.62
7	0.715	752.7	0.758	3874.1	8.47	0.761	3841.6	63327.9	11.69
8	0.800	891.0	0.849	3824.4	8.36	0.850	3820.3	75736.4	13.98

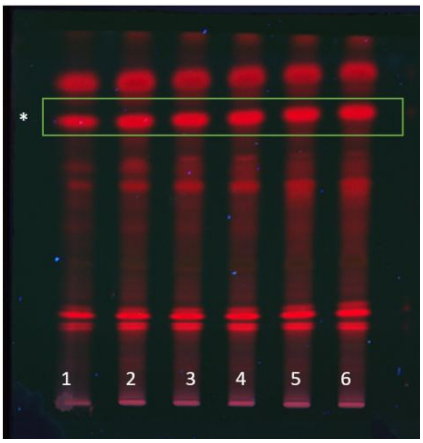

Figure 4. The stability of the analyte in both the plate and solution was analyzed using UV light at 366 nm. Tracks 1 and 2 (extract in a 3-hour TLC plate), tracks 3 and 4 (new extract as a comparator that was blotted just before elution), and tracks 5 and 6 (extract in a 3-hour solution), (*) marker

3.3. Method validation

3.3.1. Chromatogram stability

The stability of the analyte on the plate, in solution, and during chromatography, as well as the stability of the observed chromatogram, must be examined before validation [13]. It is necessary to ascertain the analyte stability throughout analysis, due to the nature of the process, which is carried out on an open system. The sample deposited on a highly active adsorbent surface can be altered by air, light, smoke, dust, temperature, and other variables. The analysis is generally unaffected by this; however, the process may result in sample degradation [19]. Testing the stability of the optimum method in TLC was started with testing the stability of the analyte on the plate and in solution. The sample was left at room temperature for three hours on the TLC plate and in solution to assess the analyte stability. Figure 4 displays the results of stability testing of analyte in TLC plate and solution. The results include the chromatogram, which is then presented as a videodensitogram. This enables viewing of the Rf value, color, area, and % area of the chosen band in each track. The videodensitogram analysis results, comprising Rf values, colors, selected band areas, and % areas, are presented in Table 3.

The Rf values of the selected bands (marked with stars) in Table 3 demonstrate the difference in blotting time of extract samples left for 3 hours

Table 3. Rf value, color, area, and % area of six tracks in the stability test of the analyte in plate and solution

Parameters	Track 1	Track 2	Track 3	Track 4	Track 5	Track 6
Rf	0.794	0.791	0.254	3367.4	0.809	0.815
Color	Red	Red	Red	Red	Red	Red
Area	65966.0	34670.0	38715.0	38715.0	36958.9	36496.4
% Area	21.37	9.11	9.11	10.72	10.45	10.88
Average Rf	0.793		0.803		0.812	
Δ Rr	0.010		0.000		0.009	

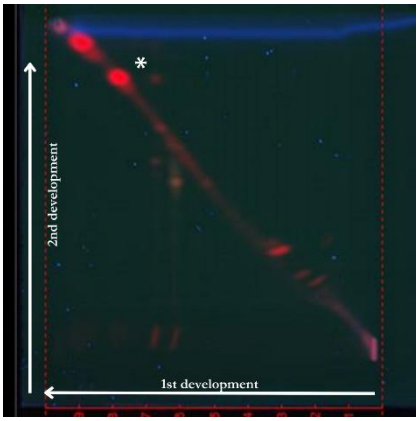

Figure 5. Analyte stability test during chromatography documented using a TLC visualizer with a 366 nm UV light (*) marker

on the plate (Tracks 1 and 2), fresh extract applied immediately (Tracks 3 and 4), and extract applied after being left for 3 hours in solution (Tracks 5 and 6). The Rf values for these bands are 0.794, 0.791, 0.800, 0.806, 0.809 and 0.815, respectively, with Δ Rf of 0.010, 0.000, and 0.009. According to Reich & Schbli, the Δ Rf acceptance criterion should not exceed 0.05 (Δ Rf ≥ 0,05), suggesting that the analyte is stable in both solution and on the plate [20].

Furthermore, stability testing of the analyte during chromatography was conducted using the two-dimensional TLC method. The sample was blotted on the right boundary of the plate, and then eluted to its limit. After drying the plate, it was rotated 90° to the left and eluted to the limit again, the second development was carried out in the same chamber, with the same mobile phase (Toluene:Ethyl acetate:Methanol:Formic acid (7:2:1:0.2)). The chromatogram showed analyte stability results, as shown in Figure 5. It is evident that the analyte resides at a single point on the diagonal line. The sample is deemed stable if the band creates a straight line that crosses both elution directions [20].

The emergence of diagonal lines on the 2D chromatogram during stability testing during chromatography indicates that the chemical compounds are stable during the chromatography procedure. Based on the presumption that a stable material have the same Rf value in both developments, stability testing using 2D chromatography was conducted. As a result, a line connecting the application location to the meeting point of two mobile phases has formed [20,26].

3.3.2. Precision of chromatogram

After the stability test, repeatability and intermediate precision tests were performed. In quantitative analysis, precision is conveniently ex-

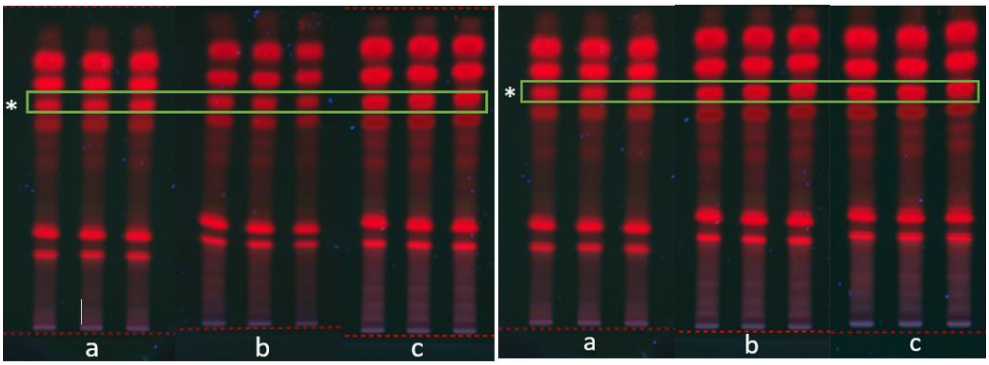

Figure 6. (1) Repeatability videoscan on 366 nm UV light, (a) analyte on the first plate, (b) analyte on the second plate, (c) analyte on the third plate. (2) Intermediate precision videoscan on 366 UV light, (a) analyte on the first day's elution, (b) analyte on the second day's elution, (c) analyte on the third day's elution

Table 4. Rf value on repeatability and intermediate precision tests

Parameters	Repeatability			Intermediate precision		
	Plate A	Plate B	Plate C	Plate A	Plate B	Plate C
Average Rf	0.644	0.673	0.684	0.694	0.697	0.696
Δ Rf	0.00	0.00	0.02	0.00	0.00	0.00

pressed in terms of the standard deviation or the coefficient of variance of the result (peak or high area). In qualitative analysis, however, precision can be related to the Rf value of the separated compound. The similarity of the fingerprint sequence (no missing or extra zones), background color, and separated zones can be compared to qualitatively assessing the precision. Repeatability must be achieved with the same equipment, the same analyte, and the same day, whether it is a repeat or not. Intermediate precision aims to ensure that different equipment, analyte, and days are not repeated [20]. In this study, *M. oleifera* leaf extract samples have met the repeatability precision and intermediate precision. The test results also showed the color of the band at the same location and there were no missing bands or additional bands during the test.

Repeatability chromatogram precision is achieved when identical fingerprint profiles are produced with respect to the number, location of colors, and color intensity. The difference in Rf value among 3 different TLC plates where samples were eluted under the same conditions on the same day was less than 0.02 [20]. Figure 6 (1) shows the repeatability results. Intermediate precision that meets requirements involves an identical fingerprint profile regarding the number, color placement, and color intensity. The differences between the Rf values of 3 different plates, in which samples were eluted under the same conditions on 3 different days, were not exceed 0.05 [20]. Figure 6 (2) shows the results of the interval precision.

Table 4 displays the average Rf and ΔRf in the results of the repeatability and intermediate precision. It is evident that the ΔRf values are not exceed 0.02 on any of the three TLC plates during the repeatability precision test. The *M. oleifera* leaf extract samples met the criteria for repeatability precision. In the intermediate precision test, the ΔRf value is not exceed 0.05, so the intermediate precision parameters are meet the requirement.

4. Conclusion

The TLC fingerprint analysis method with chromatographic conditions that have been optimized in this study can be used as one of the quality control methods for *M. oleifera* leaf raw material. The addition of small amounts of formic acid improves analyte separation. The best mobile phase for the TLC fingerprint of *M. oleifera* was toluene:ethyl acetate:methanol:formic acid (7:2:1:0.2).

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