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Identification of *Strobilanthes crispa* from its Related Plant using Thin-layer Chromatography Fingerprint Analysis

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ABSTRACT

Strobilanthes crispa known as *keji beling* in Indonesia, belong to the *Acanthaceae* family. Thin layer chromatography (TLC) fingerprint pattern analysis is an excellent technique for evaluating and controlling the quality of raw materials containing *Strobilanthes crispa*. TLC fingerprints can be used to analyze the fingerprint profiles of medicinal plants for identification, authentication, and discrimination from related plants. This study aimed to develop a TLC fingerprint analysis method to identify *S. crispa* for quality control. Eleven bands were effectively separated using a silica gel 60 F_{254} TLC plate with a mobile phase of dichloromethane, ethyl acetate, and chloroform at 16:3.2:0.8. The derivatization reagent used to detect the separated bands was 10% sulfuric acid at a wavelength of 366 nm. Validation of the TLC fingerprint analysis, which evaluated the robustness, stability, specificity, and precision, met these requirements. *S. crispa* can be distinguished by its TLC fingerprint from *sirih hutan* (*Piper aduncum*), a related plant with similar leaf morphology. This approach could be used to identify and authenticate of *S. crispa* from *P. aduncum*.
Keywords: Fingerprint analysis; *Strobilanthes crispa*; TLC; quality control

INTRODUCTION

Strobilanthes crispa is a member of the *Acanthaceae* family. Typically, as a traditional remedy to relieve kidney stones, people in Indonesia boil the stems and leaves of *S. crispa*. This plant is extensively utilized in herbal medicine owing to its diverse pharmacological activities, such as antioxidant, antimicrobial, antibacterial, and anticancer (Ban et al., 2022; Baraya et al., 2021; Nurraihana & Hanoon et al., 2013). Ghasemzadeh et al. (2015) state that, the antioxidant activity of *S. crispa* can be attributed to its phenolic and flavonoid constituents. *S. crispa* is rich in phenolic compounds such as ferulic acid, gallic acid, cinnamic acid, chlorogenic acid, and caffeic acid. It also contains flavonoids such as luteolin, scutellarin, catechin, and apigenin.

The development of quality control is crucial because of the increasing use of *S. crispa* in pharmaceutical goods, especially when dealing with high-quality raw materials. Quality control is essential to ensure that the product is suitable, consistent, effective, and safe, and to guarantee the pharmacological activity of the products (Chen et al., 2021). The quality of medicinal plants is influenced by various factors, including geographical location, soil fertility, temperature, climatic conditions, growth conditions, harvesting processes, and postharvest handling (Ncube et al., 2012). These factors contribute to the consistency of the biological activities of a plant.

Adulteration is another major cause of irregularities in the biological activity of medicinal plants and is a significant disadvantage when advertising herbal products. The intentional substitution of an herbal drug with inferior goods can occur when it partially or wholly bears a morphological resemblance to the actual medicinal plant. Herbal suppliers' lack of attention to detail, such as inconsistent herb-gathering procedures and misinterpretation of common names for distinct species, can lead to inadvertent adulteration (Hernadi et al., 2019). The adulteration of medicinal plants as the raw materials of herbal medicines can significantly elevate the risk to consumer health, as it alters the chemical makeup and impacts its biological and toxicological properties (Ekar et al., 2019).

Identifying precise marker chemicals is challenging for all plant materials that are traditionally used. Fingerprints using thin layer chromatography (TLC) can be used to authenticate and distinguish plant materials regardless of

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variations in the number of active constituents across multiple samples. Therefore, it is essential to obtain a trustworthy TLC fingerprint that accurately captures bioactive and chemically distinct components of plant materials (Parys et al., 2022). Fingerprint analysis presents a plant sample as a distinct fingerprint pattern and assesses its similarity or difference from a particular reference. This method can be used to distinguish between actual and fraudulent herbal plants. TLC fingerprint analysis is a useful tool for assessing and managing the quality of raw materials and the methods used to produce them. This method facilitates the preparation of samples and provides important information regarding the consistency, robustness, and quality control of herbal products (Rafi & Septaningsih, 2017). Fingerprint analysis using TLC. was conducted to identify and verify the authenticity of various herbal plants, including *Wedelia chinensis* (Banu & Nagarajan, 2014), *Psidium guajava* (Astuti et al., 2017), *Eugenia uniflora* (Bezerra et al., 2018), *Curcuma mangga* (Syafi'i et al., 2018), *Orthosiphon stamineus* (Kartini et al., 2020), *Melastoma malabathricum* (Mayasari et al., 2022), and *Sonchus arvensis* L. (Fauziah et al., 2023). Additionally, earlier studies have distinguished *Curcuma* and *Zingiber* (Rafi et al., 2011), and *Sida rhombifolia* from *Turnera ulmifolia* L. and *Hibiscus rosa-sinensis* (Rafi et al., 2023).

Multiple studies have used chromatography to analyze *S. crispa* (Cheong et al., 2022; Reich & Schibli, 2007; Muslim et al., 2010). However, no prior study has employed the TLC method for fingerprint analysis of *S. crispa* plants, especially as an identification and authentication method. Method validation is necessary to assess the quality and stability of *S. crispa* using this approach. The developed *S. crispa* TLC fingerprint method was validated by considering various variables such as analyte stability, specificity, accuracy, and ruggedness. The principal aim was to validate the precision and dependability of the qualitative TLC technique by evaluating the fingerprint profile, band zone color, and retardation factor (R_f) value. This study presents a unique and cost-effective TLC fingerprint analysis technique to determine and verify the authenticity of *S. crispa*. The findings of our study demonstrated that the devised technique successfully distinguished *S. crispa* from other plants with similar leaf morphologies, such as *Piper aduncum.*

MATERIALS AND METHODS

Materials and Instrument

S. crispa and *P. aduncum* leaves were collected from the Tropical Biopharmaca Research Center Medicinal Plant Garden at IPB University. Merck (Darmstadt, Germany) provided *n*-hexane, sulfuric acid, diethyl ether, acetonitrile, acetone, dichloromethane, chloroform, ethyl acetate, ethanol, and methanol.

Several instruments were used in the study: ultrasonicator Branson 1510 (Dietzenbach, Germany), TLC semiautomatic CAMAG Linomat 5 (Muttenz, Switzerland), densitometer CAMAG Reprostar 3 (Muttenz, Switzerland), analytical balance XT 220A (Precisa Gravimetrics, Switzerland), ultrasonicator Branson 1510 (Dietzenbach, Germany), and TLC plate silica gel F_{254} (Merck, Darmstadt, Germany).

Methods

Sample Preparation and Extraction

The entire set of leaf samples, weighing 100 g, was dried in an oven at 50 °C for 3 days. Subsequently, the dried leaves were crushed into a fine powder and passed through an 80 mesh particle-size filter. Approximately 1 g of each sample was macerated using an ultrasonicator operating at a frequency of 42 kHz for 30 min in 10 mL of ethanol. Following the extraction, the mixture was filtered and methanol was added to dilute it to a 10% (w/v) concentration.

TLC Separation Condition

The concentrated essence of *S. crispa* leaves was applied to a silica gel F_{254} plate. WinCATS software-equipped semiautomatic TLC Camag Linomat 5 was used for this procedure. Twenty microliters of the sample were added at 70 nL/s, with a 4 mm gap between each band. Separation was accomplished by placing the TLC plate and mobile phase in a chromatography chamber. A Camag Reprostar 3 was used to record the chromatograms of the TLC plates after separation.

Selection of the TLC Mobile Phase

The chromatography chamber for the 20 cm × 10 cm plates was filled with 10 mL of the mobile phase, which included *n*-hexane, diethyl ether, acetonitrile, acetone, dichloromethane, chloroform, ethyl acetate, ethanol, and methanol. The mobile phase was allowed to become saturated for 30 min. Initially, we used a single solvent to separate the *S. crispa* extract for TLC elution. Combining two or three selected solvents with another solvent created an ideal mobile-phase mixture for chromatographic separation. After separation, the components of the *S. crispa* extract were chemically altered on a TLC plate by staining with 10% sulfuric acid. The components were identified by exposing the TLC plate to ultraviolet light at 254 and 366 nm. In order to

obtain a visible color band, the TLC plate was dried for 10 min at 110 °C.

Validation Method of TLC Fingerprint

TLC fingerprint of *S. crispa* leaf extract was evaluated using a validation approach, considering its characteristics, such as robustness, accuracy, stability, and specificity (Reich & Schibli, 2007). A validation strategy was developed using the process described by Reich and Schibli (2007). The analyte separation on the TLC plate was observed, and pictures were taken to visualize the process at specific intervals (2^{nd}, 5^{th}, 10^{th}, 30^{th}, and 60^{th} min) to evaluate the stability. Specificity was evaluated by comparing the TLC fingerprints of *S. crispa* and *P. aduncum* leaf extracts. The experiment focused on robustness by monitoring two solvent growth distances on a TLC plate (7 and 8 cm) in two different types of chambers (flat-bottom and twin-through). Three duplicate daily operations on three different days were used to evaluate the precision.

Analyte Stability Throughout Solvent Development in Two Dimensions

Using an automatic TLC sampler, 4 µL of the *S. crispa* extract was placed as a 0.6 mm wide area at the bottom right corner of the 10 cm × 10 cm TLC plate. The TLC plate was eluted with the mobile phase for first development using a twin-through chamber and dried after that. Subsequently, fresh solvent was used for the second development phase, and the TLC plate was rotated 90° clockwise to repeat the separation. Next, the TLC plate produced by the two-step solvent development procedure was recorded before and after derivatization. Analyte stability on the TLC plate was assessed when every extract component appeared in a diagonal line during two-dimensional solvent development.

Analyte Stability in The Sample Solution

The analyte stability in the sample solution was assessed by putting 20 µL of the sample extract in four lines at the TLC plate. The TLC plate was first coated with a fresh extract solution of *S. crispa* leaves in the first solution and left for 3 h. After 3 h, fresh *S. crispa* leaf extract was added as the third line to the TLC plate. The fresh *S. crispa* leaf extract was placed on a TLC plate before elution for the second and fourth lines. The TLC plate was inserted into a twin-throughput chamber to separate the components. Sample derivatization with sulfuric acid was performed before and after documentation. The extract of *S. crispa* leaves was considered stable in the stationary phase and in the extract solution if the difference in the R_f values between the spots on the TLC plate was less than 0.05.

Analyte Stability Visualization

The stability of the analyte visualization was evaluated once the TLC plate was filled with 20 µL of *S. crispa* leaf extract and placed within the twin-through chamber. After separation using the mobile phase, sulfuric acid was used to derivatize the TLC plate, and 366 nm was used to view the separated band obtained in the TLC plate. It was observed at the 2^{nd}, 5^{th}, 10^{th}, 20^{th}, 30^{th}, and 60^{th} min.

Specificity

The specificity evaluation was made possible by applying 20 µL of the extracts of *S. crispa* and *P. aduncum*, respectively, to the TLC plate as lines 1 and 2. The TLC plate was then eluted using the mobile phase in a twin-through chamber. The TLC plate was visualized before and after sulfuric acid derivatization.

Precision

The *S. crispa* leaf sample was extracted thrice using a sonication method; 20 µL of each extract was put on a TLC plate. The three TLC plates were placed in a twin-through chamber on the same day for examination. The TLC fingerprint obtained from *S. crispa* extract was subjected to the same approval criteria: number, position, color, intensity, and R_f value ≤ 0.02. Additionally, three distinct TLC plates were used over 3 days (one TLC plate per day) to assess intermediate precision.

Robustness

Different chamber types

The developed method also evaluated for its robustness by using different chamber types. A total of 20 µL of the leaf extracts of *S. crispa* and *P. aduncum* were put on a 5.5 cm × 10 cm TLC plate at a 10% w/v extract concentration. The mobile phase developed each extract in two chambers: twin-through and flat-bottom. The TLC plate was recorded before and after sulfuric acid derivatization. Each TLC fingerprint of the extract was evaluated in terms of quantity, location, color, intensity, and R_f value ≤ 0.05 to meet the acceptance criteria of robustness.

Solvent Development

A total o**f** 20 µL of *S. crispa* and *P. aduncum* leaf extracts with a concentration of 10% w/v were applied to a 5.5 cm × 10 cm TLC plate to assess the robustness of the solvent development by measuring the distance. Subsequently, a twin-through chamber was utilized for the solvent development of each extract, including two distinct

solvent development distances of 7 and 8 cm. Documentation of the TLC plates was recorded before and during the derivatization with sulfuric acid. The TLC fingerprint of each extract satisfies the same acceptance standards in terms of quantity, location, color, intensity, and R_f value ≤ 0.05.

RESULTS

Determination of Optimum TLC Mobile Phase

An optimal mobile phase was selected to achieve the highest degree of separation. Tests were conducted using a range of solvents with varying polarities, as depicted in Figure 1. This selection aimed to optimize the separation of analytes according to their distinct polarity levels. The resolution and number of bands observed were important indicators of the effectiveness of the thin-layer chromatography (TLC) profile. The reference standard suggests that a minimum resolution value of 1 is acceptable, while an optimal resolution exceeds 1.5. Maximum visibility of the TLC profile was achieved using UV lamps at a wavelength of 366 nm, which enhanced the separation process by combining solvents with varying polarity indices.

The optimal separation of analytes was achieved using a solvent derived from chloroform (CHCl3), dichloromethane (DCM), ethyl acetate (EtOAc), or methanol (MeOH), yielding four distinct bands with high resolution and maximum clarity. These four solvents were mixed in varying ratios to achieve the best separation outcomes. The mobile phase ratios used in these tests were: (A) 14:0.2:5.8, (B) 14:0.3:5.7, (C) 13.5:0.2:6.3, (D) 13.5:0.5:6 for $CHCl_3$:MeOH: DCM; (E) 14:0.4:5.4:0.1 for $CHCl_3$:MeOH: DCM:EtOAc; (F) 16:3:1, (G) 16:2.5:1.5, (H) 15.5:2.5:2, (I) 16:3.2:0.8 for $CHCl_3$:DCM:EtOAc. Figure 2 shows the TLC profiles obtained from these solvent combinations.

Significant variations in the separation of flavonoids from *S. crispa* were noted, dependent on the polarity of the mobile phase. This was observed in differences in band separation, number of bands, and resolution. The number of bands generated from each mobile phase composition is shown in Figure 3. Eluent combination I produced the highest number of bands, yielding 11 bands with a resolution greater than 1.5, which was the best outcome.

Method Validation

Stability Test

The stability of the analytes during the thin-layer chromatography (TLC) process was assessed due to the inherent variability in open systems, where time elapses between procedural steps. Stability testing is essential as external factors can influence the separation outcomes. Two-dimensional chromatography was utilized to evaluate the stability of the analytes throughout the TLC procedure. As depicted in Figure 4, the TLC chromatogram consistently presented the analytes' stains along a diagonal line connecting the application point and the intersection of the solvent fronts in both development directions. This indicates stable behavior throughout the process.

To assess the stability of the chromatographic data post-derivatization, the color of the zones was monitored for 60 minutes. Figure 5 illustrates that the number and color of the chromatographic zones remained unchanged during this period, suggesting that the chromatogram was stable for at least 60 minutes following derivatization. The stability of the *S. crispa* extract was also evaluated before the chromatography process. Extracts prepared at different times were tested to ensure analyte consistency in terms of spectral hue and location on the TLC plate. As shown in Figure 6, the extracts exhibited consistent color bands and positions in the four lanes under UV light at 366 nm. The lack of significant change in the bands' location and color across the lanes indicates that the *S. crispa* extract remained stable in solution and on the TLC plate for at least 3 hours. For sample derivatization, the color of the zones was observed for 60 min to evaluate the durability of the chromatographic data. As Figure 5 illustrates, the number and color of the zones did not change throughout the 60 min. Therefore, the chromatogram remained steady for at least 60 min after the sample was derivatized.

Specificity

The specificity of the TLC fingerprint analysis was evaluated by comparing the separation patterns of *S. crispa* with those of its botanical counterpart, *Piper aduncum* (Figure 7). Given the physical and morphological similarities between the leaves of these two species, the ability to distinguish their TLC profiles was critical, especially when using powdered forms of the plants, which are commonly utilized in herbal medicine. Powdered raw materials from these plants would be difficult to differentiate visually, making accurate chromatographic fingerprinting essential for their identification.

Distinct lanes on the TLC plates were employed to analyze the plant extracts, allowing for direct comparison. The specificity of the TLC fingerprints was determined by assessing the color, intensity, number of bands, and band placement in each sample. The analysis revealed significant differences in the TLC separation

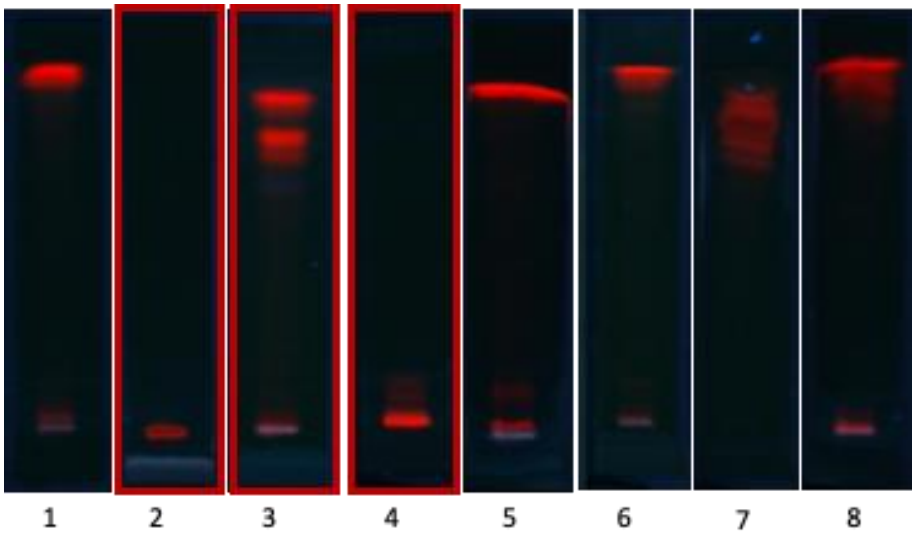

Figure 1. TLC profile of *S. crispa* under UV 366 nm with single mobile phase 1) *n*-hexane, 2) diethyl ether, 3) dichloromethane, 4) chloroform, 5) ethyl acetate, 6) acetone, 7) methanol, 8) acetonitrile

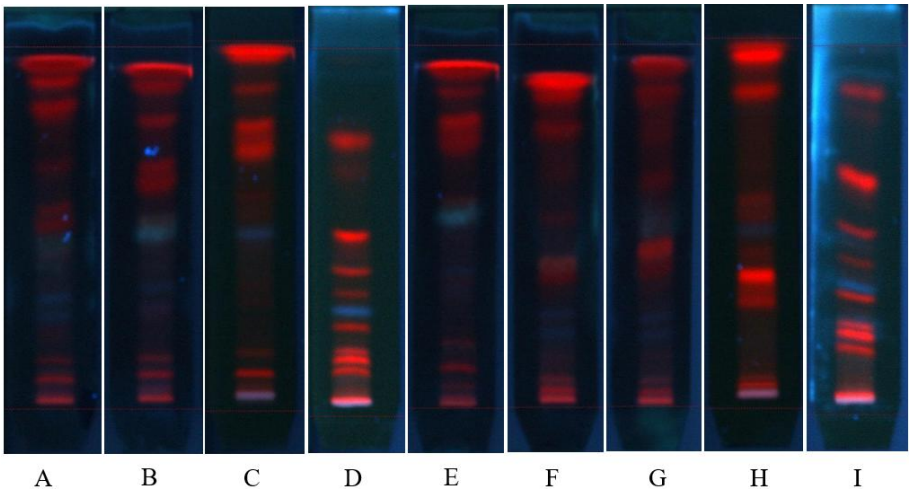

Figure 2. Number of mixed mobile phase bands

patterns of *S. crispa* and *P. aduncum*. Notably, at a retention factor (Rf) of 0.62, *P. aduncum* exhibited a vivid blue band, which was absent in *S. crispa*. These results demonstrated the clear distinction between the two species based on their TLC profiles.

Precision Test

The precision of the experiment was assessed using three different TLC plates applied with *S. crispa* extracts on the same day, while intermediate precision was evaluated on subsequent days with different instruments and materials. Chromatogram patterns were analyzed to determine the separation of the various components. The evaluation criteria included the number of bands, their positions, color intensity, and the maximum allowable variation in Rf values between bands. The acceptable deviation for precision was set at $\Delta Rf < 0.02$, while intermediate precision was acceptable with a deviation of $\Delta Rf < 0.05$.

The TLC fingerprint patterns of *S. crispa* used to assess precision are shown in Figure 8 (a, b, and c), while intermediate precision patterns are presented in Figure 8 (d, e, and f). In all cases, the observed ΔRf values for three bands on the TLC plates were less than 0.04 for precision and less than 0.05 for intermediate precision. These results indicate that the experiment met the required thresholds for both precision and intermediate precision.

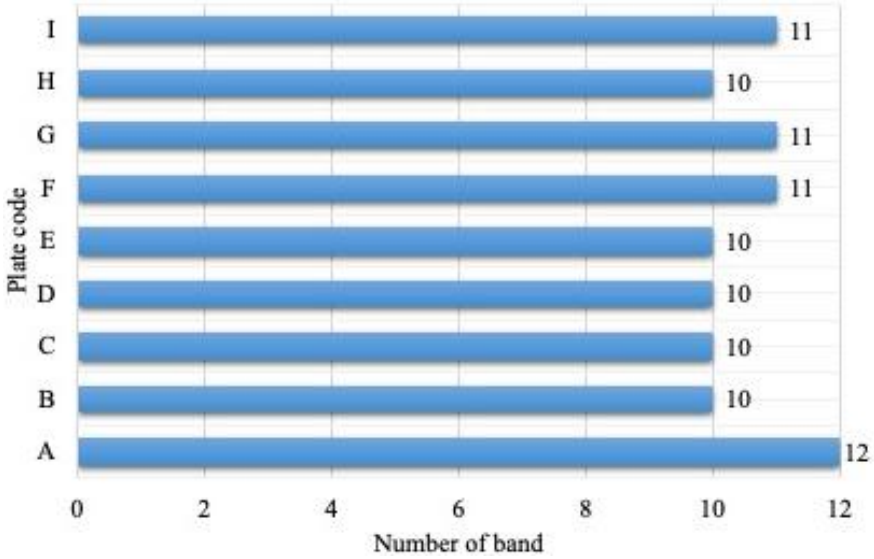

Figure 3. Number of bands derived from the TLC pattern of *S. crispa* leaves extract with the mobile phase combination and its visualization using UV light at a wavelength of 366 nm

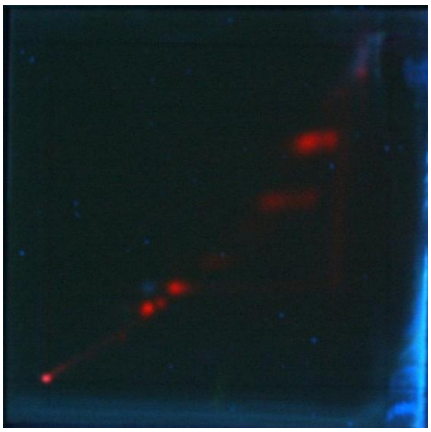

Figure 4. TLC chromatogram of the stability test for analytes under UV 366 nm derivatization

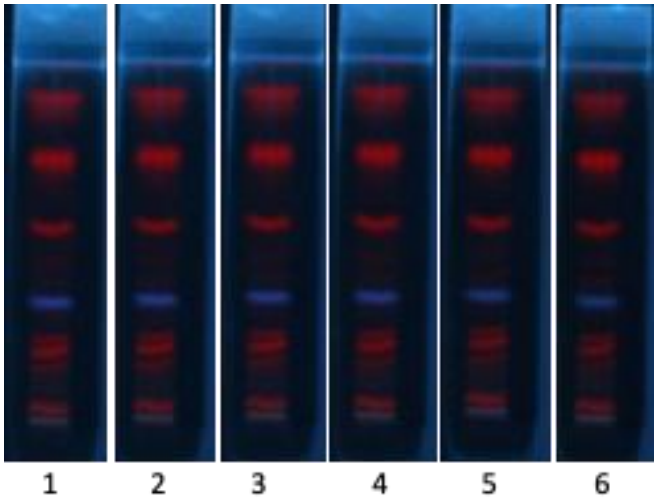

Figure 5. TLC chromatogram of the *S. crispa* plant was produced after an analyte stability test lasting (1) 2 min, (2) 5 min, (3) 5 min, (4) 5 min, (5) 10 min, (6) 20 min, (7) 30 minutes, (8) 30 minutes, and (9) 60 minutes

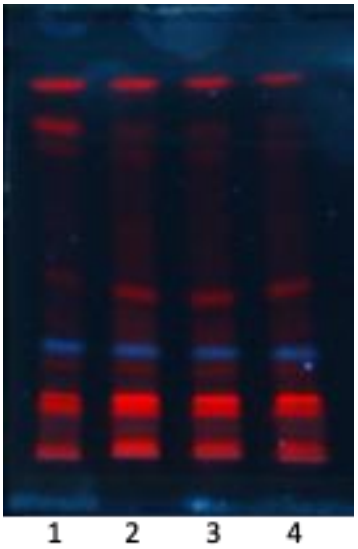

Figure 6. *S. crispa* leaf extract TLC chromatogram, observed at 366 nm UV light for 3 h to assess analyte stability. (1) *S. crispa* extract was left for 3 h; (2) and (4) fresh *S. crispa* extract was applied straight to the TLC plate prior to the separation process; and (3) fresh *S. crispa* extract was maintained for 3 h before being applied to the TLC plate

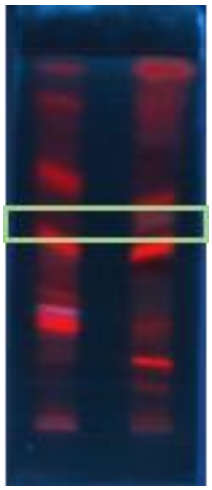

Figure 7. Fingerprint patterns of *S. crispa* with *P. aduncum* after derivatization at 366 nm UV detection

Robustness

The robustness of the component separation was assessed by evaluating the chromatography chamber type and the solvent migration distance on the TLC plate. The robustness parameter was defined by a maximum deviation of 0.05 in the number of bands, their location, color, and Rf values. Two distinct types of chromatography chambers, twin-throughput and flat-bottom, were used to analyze *S. crispa* and *P. aduncum* extracts (Figure 9a & b). The solvent development distances on the TLC plate were varied between 7 and 8 cm (Figure 9c & d) to further assess the robustness of the separation process.

The comparison of results from the two solvent migration distances showed no statistically significant differences in the fingerprint patterns, with a standard deviation in Rf values of less than 0.05. This confirmed that the robustness criteria were met across different development distances and chamber types.

DISCUSSION

Determination of Optimum TLC Mobile Phase

The selection of an optimal mobile phase is critical for effective TLC separation. The results showed that varying solvent combinations and ratios significantly influenced band separation and resolution , with eluent combination I producing

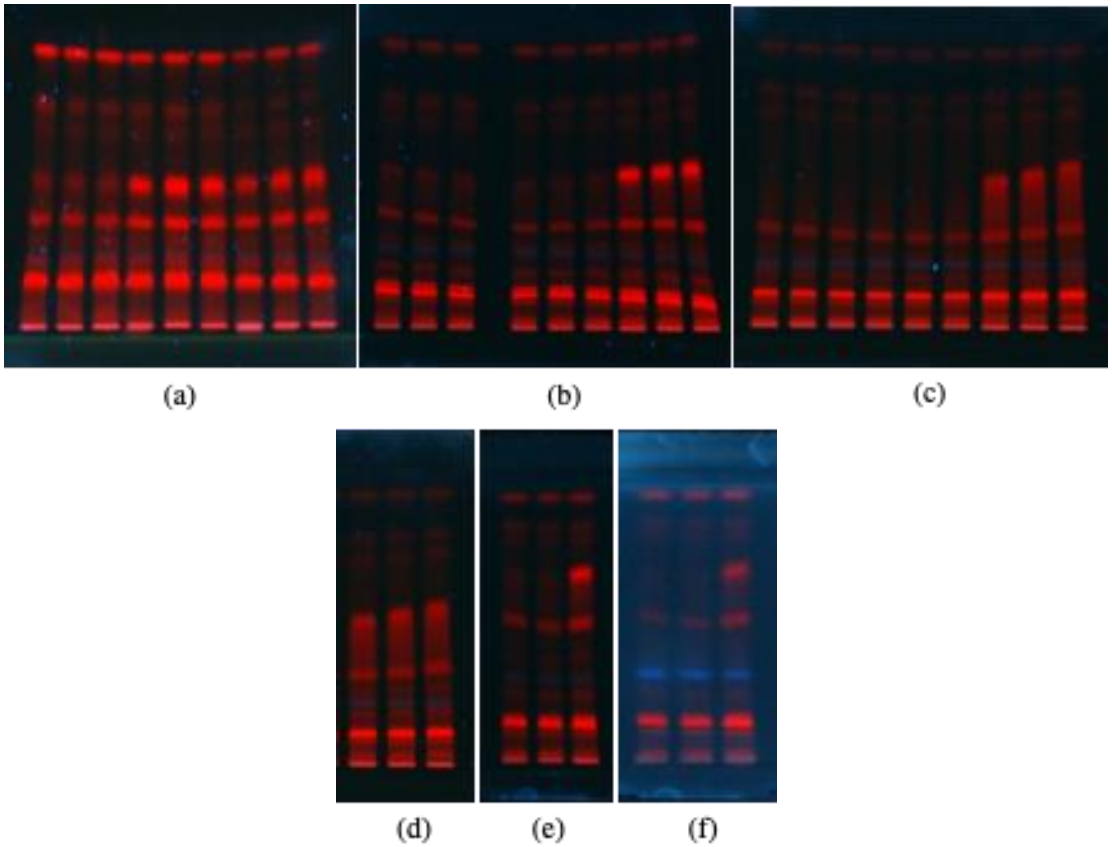

Figure 8. After derivatization and UV 366 nm detection, the TLC profiles of *S. crispa* precision measurements of (a) Plate 1, (b) Plate 2, (c) Plate 3, and the precision between (d) Day 1, (e) Day 2, and (f) Day 3

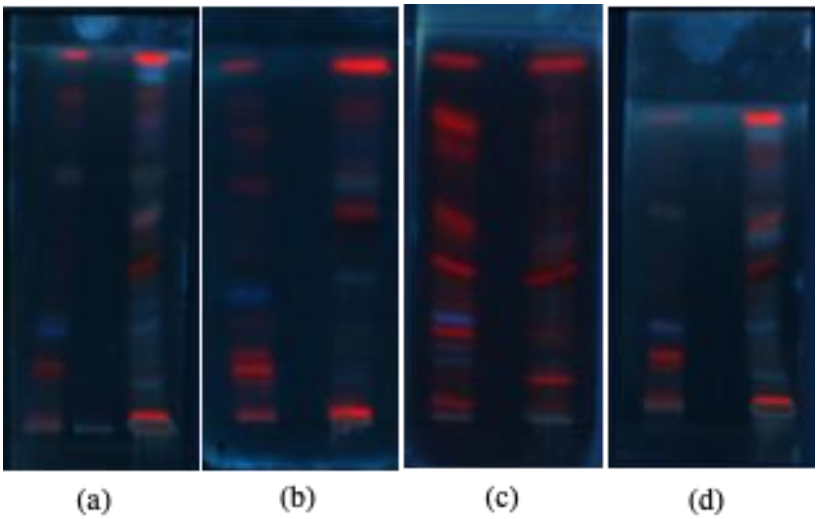

Figure 9. TLC profiles using the subsequent techniques in the following chamber configurations: a) twin-through, b) flat-bottom, c) 8 cm spacing, and d) 7 cm spacing, following derivatization and detection using UV 366 nm of development results

the best results. A solvent mixture generating 11 distinct bands, with a resolution value exceeding 1.5, was deemed most effective for flavonoid separation from *S. crispa*. Resolution is a key factor in determining mobile phase performance, as indicated by the mixture containing code A, which yielded a resolution value greater than 1.5. However, derivatization was necessary to enhance

Table I. Resolution of the *S. crispa* leaves extract was determined using a mobile phase combination consisting of $CHCl_3$:DCM:EtOAc (16:3.2:0.8) and $CHCl_3$:DCM:MeOH (13.5:6:0.5) UV 366 nm was used as the detection wavelength.

$CHCl_3$:DCM:EtOAc (16:3.2:0.8)					$CHCl_3$:DCM:MeOH (13.5:6:0.5)				
Band	Band distance (cm)	Band width (cm)	Rf	Rs	Band	Band distance (cm)	Band width (cm)	Rf	Rs
1	0.16	0.2	0.02		1	0.32	0.3	0.04	
				1.6					4.8
2	0.24	0.1	0.06		2	1.28	0.1	0.16	
				9.6					3.2
3	1.2	0.1	0.15		3	1.6	0.1	0.20	
				3.2					1.6
4	1.52	0.1	0.19		4	1.76	0.1	0.22	
				1.6					7.2
5	1.84	0.1	0.22		5	2.48	0.1	0.31	
				1.6					1.6
6	2.8	0.1	0.30		6	2.64	0.1	0.33	
				2.8					5.6
7	3.28	0.2	0.34		7	3.2	0.1	0.40	
				4					6.4
8	4.8	0.1	0.39		8	3.84	0.1	0.48	
				3.2					5.2
9	6.64	0.1	0.48		9	4.88	0.3	0.61	
				2.13					7.68
10	7.28	0.2	0.61		10	6.8	0.2	0.85	
				3.6					
11	7.52	0.2	0.86						

the separation since the initial number of bands produced was not optimal. The derivatization process, combined with UV irradiation at 366 nm, resulted in further improvements in separation quality, particularly for eluent combination I.

The findings confirm that the selection of solvents with appropriate polarity indices and their precise ratios play a vital role in achieving optimal separation outcomes. Table I presents the Rf values and resolution metrics for mobile phase combinations A and I, providing quantitative confirmation of their effectiveness.

Method Validation

Stability Test

Stability testing is a crucial component of the TLC separation process, especially in open systems where multiple external factors can influence results. The stability of the analytes was confirmed through two-dimensional chromatography, where consistent band formation along a diagonal line demonstrated the robustness of the analytes during the separation process. The evaluation of the derivatized samples further supported this, with no observable changes in band color or count over 60 minutes.

Additionally, the assessment of the *S. crispa* extract prior to chromatography showed that both in solution and on the TLC plate, the analytes remained stable for up to 3 hours. This ensures the reliability of the separation process and demonstrates that *S. crispa* extract can be consistently analyzed without significant degradation or variation over time. The results from these stability assessments reinforce the effectiveness of TLC as a method for studying the components of *S. crispa* under controlled conditions.

Specificity

The specificity test, comparing *S. crispa* with *P. aduncum*, highlights the effectiveness of TLC fingerprinting for distinguishing between botanically similar species. Despite their physical and morphological resemblance, particularly when presented in powdered form, the TLC separation analysis revealed significant differences in their chromatographic profiles. The vivid blue band at Rf 0.62 in *P. aduncum*, absent in *S. crispa*, was

particularly noteworthy, serving as a clear marker for differentiation.

This finding underscores the importance of TLC in ensuring the quality and authenticity of herbal medicines, particularly in cases where species may be visually indistinguishable when ground into powder. The ability to differentiate these species through TLC fingerprinting not only prevents contamination but also ensures the accurate identification of herbal products, which is essential for both safety and efficacy in medicinal use. The results support the reliability of TLC as a tool for distinguishing closely related species in botanical studies and herbal medicine quality control.

Precision Test

The precision and intermediate precision evaluations of the TLC fingerprinting of *S. crispa* demonstrated consistency in the separation of the components, as evidenced by the low variation in Rf values across the tested plates. The ΔRf values remained within acceptable limits (less than 0.04 for precision and less than 0.05 for intermediate precision), confirming the experiment's reliability.

However, variations in temperature and humidity were identified as factors that could affect the Rf values, leading to reduced resolution and insufficient separation. In particular, saturated mobile phases were found to lower the resolution factor, resulting in inadequate TLC plate separation. These observations underscore the importance of controlling environmental conditions to ensure consistent outcomes. Despite these potential challenges, the results suggest that the experiment meets the criteria for precision and dependability in TLC fingerprinting. The findings highlight the robustness of the TLC method in determining precision, even under variable conditions, making it a valuable tool for the reliable analysis of plant extracts.

Robustness

The robustness of the TLC fingerprinting process was successfully demonstrated by varying both the chromatography chamber type and solvent migration distance. The results indicate that the separation of *S. crispa* and *P. aduncum* components remained consistent, as evidenced by the low deviation in the number of bands, their positions, color, and Rf values. The standard deviation of less than 0.05 in Rf values between the two developmental distances confirms the reliability of the method under different experimental conditions.

This robustness is a critical factor in ensuring the reliability and reproducibility of TLC fingerprinting, particularly in cases where variations in chromatography conditions are inevitable. The results suggest that the method is sufficiently stable to accommodate minor variations in experimental setup without compromising the quality of the separation. Thus, the study demonstrates that the TLC method for analyzing *S. crispa* and *P. aduncum* extracts is robust and dependable for practical applications, even under variable conditions.

CONCLUSION

Overall, the TLC fingerprint analysis of *S. crispa* demonstrated the application of various chromatographic conditions. The optimal conditions involved using an aluminum plate coated with silica gel 60 F_{254} as the stationary phase and a mobile phase consisting of a mixture of chloroform, dichloromethane, and ethyl acetate at a ratio of 16:3.2:0.8 (v/v). Additionally, 10% sulfuric acid derivatization was employed and visualization was performed under UV light at 366 nm. The *S. crispa* leaf fingerprint profile exhibited 11 distinct bands with excellent resolution. These results confirm the recommended approach's robustness, specificity, accuracy, and precision. The fingerprint analysis strategy was appropriate for identifying and quality controlling *S. crispa* leaves because it successfully met the acceptance requirements for method validation.

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