Identification of *Sida rhombifolia* from Its Related Plants Using Thin-Layer Chromatographic Analysis

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Abstract: Sida rhombifolia *belongs to the Malvaceae family and is often used to treat gout in Indonesia.* S. rhombifolia *has many efficacies and contains many different chemical components. The abundance and variation of chemical content and chemical compounds in this medicinal plant are challenging factors in ensuring medicinal plants' safety and quality control. Thin-layer chromatography (TLC) fingerprint analysis derived from* S. rhombifolia *extract can also be used for the medicinal plant's quality control. This research aimed to develop the optimum condition for the chemical fingerprint analysis of* S. rhombifolia *using a TLC fingerprint analysis. A total of 11 bands were produced with optimum separation using silica gel 60 F_{254} TLC plate, a mixed mobile phase condition with chloroform, ethyl acetate, and methanol (6.5:2:1.5). This fingerprint analysis performed an excellent separation in the TLC plate at 366 nm with sulfuric acid as reagent derivatization. In general, the results of the analysis validation, including stability, specificity, precision, and robustness of TLC fingerprints, met the acceptance criteria. The TLC fingerprint of* S. rhombifolia *can be distinguished from 2 related plants with similar leaf shapes,* Turnera ulmifolia *L. and* Hibiscus rosa-sinensis. *The developed method was validated, so it could be used to control* S. rhombifolia *quality.*

Keywords: *fingerprint analysis; identification;* Sida rhombifolia*; thin-layer chromatography*

■ INTRODUCTION

Sida rhombifolia, known as arrowleaf sida or *sidaguri* in Indonesia belongs to the Malvaceae family. *S. rhombifolia* is one of the 200 species in Sida distributed throughout tropical and subtropical regions worldwide. *S. rhombifolia* is known for its wide range of medicinal uses, for instance, treating stings and bites of a scorpion,

snakes, and wasps (its flower), skin diseases and sores (stem), treating stomach disorders, stomach pain, digestion problems (roots and leaves), diabetes (leaves), chickenpox, blood cleaning, and fatigue [1]. *S. rhombifolia* is also widely used in Indonesia to treat gout [2]. Several natural flavonoid compounds in *S. rhombifolia* have been reported as xanthine oxidase inhibitors and promise greater use to reduce the excess uric acid production in the human body.

Many commercial herbal products in Indonesia or other countries use plant extracts in their compositions. However, there are some problems with variability and the bioactive compounds' concentration level from plant extracts in herbal medicines' production. Medicinal plant quality also depends on several factors, such as environmental growth, harvest, and postharvest, which cause its consistency in its biological activity [3]. Adulteration is another source of the medicinal plant's biological activity's inconsistency and becomes one of the most significant drawbacks in promoting herbal products. This can occur intentionally by substituting partially or entirely a herbal drug with other inferior products due to morphological resemblance to the authentic herb. An unintentional adulteration can be caused by herbal suppliers' carelessness, such as the inconsistency in the herb collection process and the confusion of common names for different plants [4]. The adulteration process in herbal medicine would lead to an increased risk for consumer health due to the differences in the chemical composition that could affect its biological and toxicity activity [5]. In contrast with the component-based method that needs to measure the marker compound's content, fingerprint analysis offers an alternative way to evaluate all the detectable compounds without the necessity to characterize all the compounds.

Identifying accurate marker compounds is very difficult for all traditionally used plant materials. The thin-layer chromatography (TLC) fingerprints can accurately certify and identify plant materials, even if the amount of active material is not the same for different samples. Therefore, it is essential to obtain a reliable TLC fingerprint representing the bioactive compounds and chemically distinct components of plant materials [6]. The fingerprint analysis evaluation could compare the similarity or dissimilarity of the plant sample with a certain reference and present it as a fingerprint pattern. This could be used to identify the actual herbal plant and the false one. TLC fingerprint pattern analysis is an effective way to evaluate and control the quality of raw materials and their preparation process [7]. This method offers an advantage such as ease of sample preparation and could provide fundamental data on the herbal product's consistency, stability, and quality control [8]. The fingerprint analysis with TLC has been performed to identify and authenticate herbal plants such as *Wedelia chinensis*, *Psidium guajava*, *Curcuma mangga*, *Eugenia uniflora*, *Orthosiphon stamineus*, *Melastoma malabathricum* [9-14] and also differentiation of *C. longa*, *C. xanthorrhiza*, and *Zingiber cassumunar* [15].

Several papers have used TLC to analyze *S. rhombifolia* [1,16-18]. However, the profile information presented in the previously cited paper was not validated in terms of specificity according to the validation guidelines. Method validation needs to be done so that the developed method can evaluate the quality and stability of *S. rhombifolia*. Method validation which includes analyte stability, specificity, precision, and ruggedness, was also carried out to validate the developed Sidaguri TLC fingerprint method. The focus is on validating the qualitative TLC method, namely the R_f value, fingerprint profile, and band zone color. This paper describes our new cost-effective and simple TLC fingerprint analysis of *S. rhombifolia* to identify and authenticate its originality. Our result showed that the developed method could identify and discriminate *S. rhombifolia* from its related plants with the same leaf morphology, such as *Turnera ulmifolia* L. and *Hibiscus rosa-sinensis*.

■ EXPERIMENTAL SECTION

Materials

Materials used in this study involved *S. rhombifolia*, *T. ulmifolia*, and *H. rosa-sinensis* leaves obtained and identified by Mr. Taopik Ridwan from the medicinal plant garden Tropical Biopharmaca Research

Center LPPM IPB University. Sulfuric acid, methanol, ethanol, ethyl acetate, *n*-hexane, chloroform, dichloromethane, and 1-butanol were purchased from Merck (Darmstadt, Germany) and used without further purification.

Instrumentation

The instruments used in this study involved TLC semiautomatic CAMAG Linomat 5 (Muttenz, Switzerland), Densitometer CAMAG Reprostar 3 (Muttenz, Switzerland), CAMAG WinCATS software (Muttenz, Switzerland), ultrasonicator Branson 1510 (Dietzenbach, Germany), TLC plate silica gel F254 (Merck, Darmstadt, Germany), and analytical balance XT 220A (Precisa Gravimetrics, Switzerland).

Procedure

Sample preparation and extraction

All the sample leaves (100 g) were dried in the oven at 50 °C for 3 d, then pulverized into a powder, and then sieved with 100 mesh particle size. About 1 g of samples were macerated with 10 mL methanol using an ultrasonicator at 42 kHz for 30 min. After the extraction process was completed, the solution was filtered and diluted with methanol to obtain a 10% w/v concentration.

TLC separation condition

The extract of *S. rhombifolia* leaves was applied to the silica gel F_{254} plate as a band 8 mm in length with TLC semiautomatic Camag Linomat 5 equipped with WinCATS software. The sample (20 µL) was applied at a speed of 70 nL/s, and the distance between the band was 4 mm. Then, the TLC plate was placed into a chromatography chamber with the mobile phase to obtain the separation process. After the separation was completed, the TLC plate's chromatogram was documented using a Densitometer Camag Reprostar 3.

The selection of the TLC mobile phase

About 10 mL of each solvent (methanol, ethanol, ethyl acetate, chloroform, dichloromethane, and *n*-hexane) was added into the chromatography chamber and saturated for 30 min. First, we eluted the *S. rhombifolia* extract with a single solvent for TLC separation. Then the selected solvent (2–3 solvents) was mixed with another solvent to obtain the mobile phase mixture's best for the chromatography separation. After the separation process, the staining procedure at the TLC plate was performed using sulfuric acid to derivatize components in the extract of *S. rhombifolia* leaves. The sulfuric acid reagent was prepared by mixing 10 mL concentrated sulfuric acid with 90 mL cold methanol. Component detection was performed by exposing the TLC plate to 254 and 366 nm UV light. The TLC plate was then dried in the oven at 110 °C for 10 min to obtain a noticeable band color.

Validation method of TLC fingerprint

The validation method used to evaluate the TLC fingerprint of *S. rhombifolia* leaves extract involves several parameters: stability, specificity, precision, and robustness. The validation method has been developed following the previous procedure from Reich and Schibli [19]. The stability was evaluated by observing analyte separation during the separation process on the TLC plate and visualized by taking a photograph at 2^{nd}, 5^{th}, 10^{th}, 30^{th}, and 60^{th} min. The specificity was checked by comparing the TLC fingerprint derived from the leaves extract of *T. ulmifolia* and *H. rosa-sinensis* with the TLC fingerprint extract of *S. rhombifolia* leaves. The robustness was investigated by observing the two distances of solvent development at the TLC plate (7 and 8 cm) in two chambers (twin-through and flat-bottom). The precision was evaluated by repeating a similar procedure three times a day and performing it on three different days.

Analyte stability during two-dimensional solvent development. About 4 µL of the leaves extract was applied as a spot with 0.6 mm width using an automatic TLC sampler at the bottom right corner of the 10 × 10 cm TLC plate. Then, the solvent was developed at the TLC plate using a twin-through chamber, and the TLC plate was dried afterward. Next, the TLC plate was rotated 90° clockwise, and the separation was repeated using a fresh solvent for the development process. The TLC plate obtained from the two-step solvent development process was then documented before and after the derivatization. The analyte stability at the TLC plate can be evaluated when all components in the

extract emerge in a diagonal line in two-dimensional solvent development.

Analyte stability in the sample solution. Evaluation of analyte stability in the sample solution was performed by applying 20 µL of the sample extract in 4 lines at the TLC plate. The first line consists of the fresh solution extract solution of *S. rhombifolia* leaves was applied to the TLC plate and left for 3 h. Then, the fresh extract of *S. rhombifolia* leaves was kept for 3 h and applied to the TLC plate as a third line. The second and fourth lines consist of the fresh extract of *S. rhombifolia* leaves and are applied to the TLC plate before the elution. After that, the TLC plate was placed into the twin-through chamber to separate the components. The documentation was performed before and after using sulfuric acid's sample derivatization process. If the difference of R_f value ≤ 0.05 between the spot at the TLC plate, it can be concluded that the extract of *S. rhombifolia* leaves was stable at both the stationary phase and the extract solution.

Analyte stability with visualization. The analyte's visualization stability was evaluated by applying 20 µL of *S. rhombifolia* leaves extract into the TLC plate and then placed into the twin-through chamber. After the solvent development, the TLC plate was then derivatized using sulfuric acid and visualized by visible and UV light at 366 nm. The observation was performed on the 2^{nd}, 5^{th}, 10^{th}, 20^{th}, 30^{th}, 60^{th} min.

Specificity test. Specificity was evaluated by applying 20 µL of the extract of *S. rhombifolia*, *T. ulmifolia*, and *H. rosa-sinensis* into the TLC plate as lines 1, 2, and 3 consecutively. Then, the TLC plate was eluted with a solvent using a twin-through chamber. After the solvent development process, the TLC plate was then documented before and after the sulfuric acid's derivatization process.

Precision evaluation. The sample of *S. rhombifolia* leaves was extracted by sonication process in triplicate, with each 20 µL extract applied to the TLC plate. The assessment was performed using three TLC plates in a twin-through chamber on a similar day. The acceptance criteria for TLC fingerprint derived from *S. rhombifolia* extract were given identical parameters in terms of number, position, color, intensity, and R_f value ≤ 0.02. Intermediate precision has also been evaluated using three different TLC plates on three days (one TLC plate per day).

Robustness of chamber type. For the robustness evaluation of chamber type, 20 µL of the leaves extracts of *S. rhombifolia*, *T. ulmifolia*, and *H. rosa-sinensis* were applied into a 5.5 × 10 cm TLC plate with the extract concentration of 10% w/v. Each extract's solvent development was performed using two chambers: twin-through and flat-bottom. After the solvent development was completed, the TLC plate was documented before and after the sulfuric acid's derivatization. The acceptance criteria for the TLC fingerprint derived from each extract gave identical parameters in terms of number, position, color, intensity, and R_f value ≤ 0.05.

Distance robustness of the solvent development. Evaluation of the robustness by measuring the distance in the solvent development was performed by 20 µL of *S. rhombifolia*, *T. ulmifolia*, and *H. rosa-sinensis* leaves extracts were applied to a 5.5 × 10 cm TLC plate with the extract concentration of 10% w/v. Then, each extract's solvent development was performed using a twin-through chamber with two different solvent development distances of 7 and 8 cm. After the solvent development was completed, the TLC plate was documented before and after the sulfuric acid's derivatization process. The acceptance criteria for the TLC fingerprint derived from each extract gave identical parameters in terms of number, position, color, intensity, and R_f value ≤ 0.05.

■ RESULTS AND DISCUSSION

TLC Fingerprint Mobile Phase

TLC fingerprint analysis is an analytical technique used to identify a medicinal plant. TLC will generally consist of a stationary and mobile phase, a medium for separating chemical components in a sample. In this study, silica gel 60 F_{254} was used as the stationary phase and optimizing the mobile phase composition to obtain maximum separation of components. Generally, optimizing the mobile phase in TLC is mostly by trial and error. We used eight solvents with different polarity levels ranging from nonpolar, semipolar, and polar to

get an optimum mobile phase that could be mixed to maximize separation. Besides the mobile phase, the number of bands produced is also influenced by the detection type. This study used sulfuric acid as a derivatized reagent because it could react with almost all chemical components. After derivatization with sulfuric acid, a separate band of the components can be seen under 366 nm UV light with more apparent in the band's color.

The mobile phase optimization is an important parameter for obtaining the optimum separation pattern of the TLC fingerprint obtained from the extract of *S. rhombifolia* leaves. In the first step, eight single mobile phases were used, and chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH) were the best mobile phase. These single mobile phases exhibited the most significant number of bands and the best separation of zones on the TLC plate (Fig. 1). About 6, 5, and 4 bands were produced by $CHCl_3$, EA, and MeOH, respectively. The detection of bands on the TLC plate was performed using UV light at 366 nm. These solvents were mixed in different ratio compositions. Eight types of combinations in the different ratios were deduced, and $CHCl_3$:EtOAc:MeOH in both compositions of (6.5:2.1:1.5) and (7:1.5:1.5) showed a suitable solvent system producing 11 bands on the TLC plate (Fig. 2). However, after a further investigation of both ratios of the solvent system, it was obtained that the (6.5:2:1.5) composition gave a better resolution factor rather than the (7:1.5:1.5) composition (Table 1). Therefore, $CHCl_3$:EtOAc:MeOH in the ratio of (6.5:2:1.5) was then selected as a solvent system for developing the TLC fingerprint from the extract of *S. rhombifolia* leaves. This solvent ratio composition exhibited 11 bands after derivatized with sulfuric acid reagent under 366 nm UV light.

We found a mixture of chloroform, ethyl acetate, and methanol solvents with a ratio (of 6.5:2:1.5) as the optimum mobile phase for separating *S. rhombifolia* components. Using this mobile phase, about 11 bands

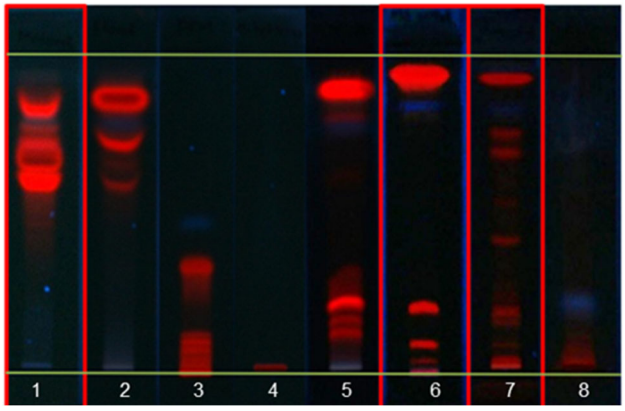

Fig 1. TLC chromatogram of *Sida rhombifolia* extract using single mobile phase: (1) methanol, (2) ethanol, (3) dichloromethane, (4) *n*-hexane, (5) *n*-butanol, (6) ethyl acetate, (7) chloroform, (8) water. The documentation was taken using UV light at 366 nm

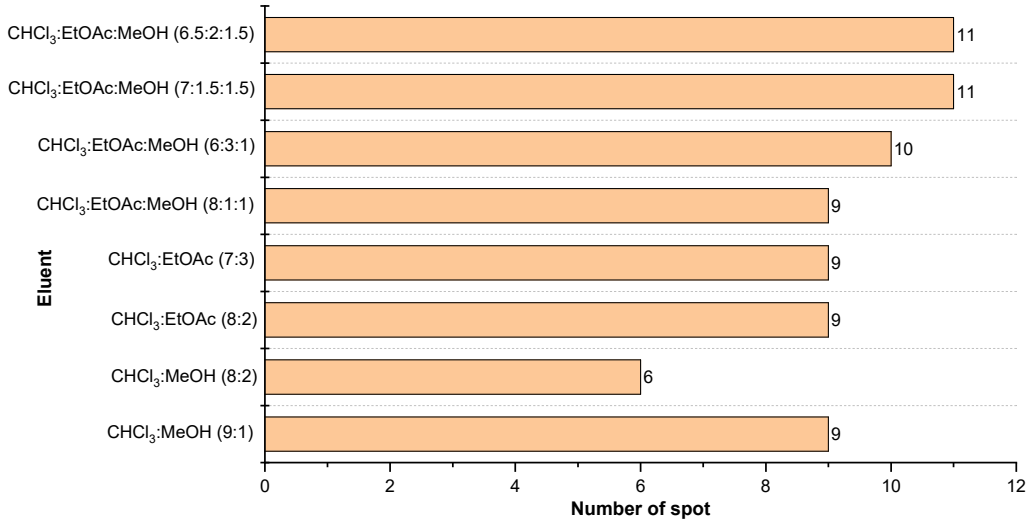

Fig 2. The number of bands obtained from the TLC fingerprint of *Sida rhombifolia* leaves extract with the solvent mixture and its visualization using UV 366 nm

Table 1. The resolution obtained from *S. rhombifolia* leaves extract using a mobile phase mixture of chloroform:ethyl acetate:methanol in two different compositions ratio (7:1.5:1.5) and (6.5:2.1:1.5) using UV 366 nm as detection wavelength

Chloroform:ethyl acetate:methanol (7 :1.5:1.5)					Chloroform:ethyl acetate:methanol (6.5:2:1.5)				
Band	Band distance (cm)	Band width (cm)	Rf	Rs	Band	Band distance (cm)	Band width (cm)	Rf	Rs
1	7.68	0.3	0.96		1	7.68	0.3	0.96	
				2.8					2.8
2	7.12	0.1	0.89		2	7.12	0.1	0.89	
				17.6					18.4
3	5.36	0.1	0.67		3	5.28	0.1	0.66	
				5.6					4.0
4	4.80	0.1	0.60		4	4.88	0.1	0.61	
				2.4					3.2
5	4.56	0.1	0.57		5	4.56	0.1	0.57	
				2.4					2.4
6	4.32	0.1	0.54		6	4.32	0.1	0.54	
				3.2					2.6
7	4.00	0.1	0.50		7	4.00	0.1	0.50	
				1.1					1.6
8	3.84	0.2	0.48		8	3.92	0.2	0.49	
				4.8					4.2
9	2.64	0.3	0.33		9	2.72	0.3	0.34	
				5.6					5.6
10	1.52	0.1	0.19		10	1.60	0.1	0.20	
				10.4					11.2
11	0.48	0.1	0.06		11	0.48	0.1	0.06	

appeared with a good resolution. The optimum mobile phase obtained is between semipolar and polar solvents. The semipolar solvent (chloroform) used has a large ratio to polar solvents, namely ethyl acetate and methanol. This aims to reduce the solvent's polarity and balance the resulting band position. The more polar a solvent or solvent mixture is, the farther the solvent moves the polar compound up from the starting point of the dotting while the nonpolar compound is held down.

Most components were separated between semipolar and nonpolar solvents (chloroform-ethyl acetate-methanol). The developed method of TLC fingerprint analysis of *S. rhombifolia* L. leaves is for a qualitative purpose only, and we could not precisely know the name of the separated components. However, from a previous study using chloroform and methanol as eluent, it is known that *S. rhombifolia* leaves have an alkaloid compound at an R_f value of 0.92 with a brownish-yellow color [20]. Flavonoid compounds usually appear as fluorescent zone under UV 366 nm. So maybe the TLC fingerprint chromatogram of *S. rhombifolia* contains some alkaloid or flavonoid compound because the Rf value is near the previous study.

Validation of the Developed TLC Fingerprint Method

According to Reich and Schibli [19], we must do a validation method as formal evidence that a method is suitable for analysis. The TLC fingerprint's developed method was validated by determining the analyte stability on the TLC plate in the extract solution and during the chromatography process, the analyte stability of derivatized zones, specificity, repeatability,

intermediate precision, and robustness. The focus on validating the developed HPTLC qualitative method for identifying *S. rhombifolia* leaves was fingerprint profiles, such as R_f value and bands color. The results obtained from the validation step are evaluated according to the criteria described by Reich and Schibli [19].

Stability

Due to the TLC system's nature, studying the analytes' stability before and during the chromatography process is crucial. Before the chromatography process, the analyte stability was determined by developing the extract of *S. rhombifolia* leaves prepared at different times. The expected results should not differ in position and band color. Fig. 3 shows *S. rhombifolia* extract's stability in the solution and on the TLC plate visualized with 366 nm UV light. The R_f values of the 3 bands were observed to see the stability of the TLC results represented by the upper (X) R_f 0.71, middle (Y) R_f 0.55, and lower (Z) R_f 0.21 in the leaf extract of *S. rhombifolia*. There is no distinct difference in position and color bands between the four lanes on the TLC plate. Therefore, it can be concluded that the sample of *S. rhombifolia* extract is considered stable for at least 3 h in solution and the TLC plate.

During the chromatography process, the sample stability of *S. rhombifolia* extract was also investigated by 2-D solvent development on the TLC plate. Two-dimensional (2D) solvent development was carried out to determine the analyte's stability during the chromatography process. If the sample is stable during chromatography separation, all components should be detected on the diagonal line indicating spot movement in two-dimensional solvent development on the TLC plate. Fig. 4 shows no difference in two-dimensional solvent development on TLC fingerprint from the extract of *S. rhombifolia* leaves visualized with 366 nm UV light. This result indicated that the analytes remained stable for 3 h before the chromatography separation began, both on the TLC plate and the extract solution. In terms of sample derivatization, the chromatographic results' stability was determined by observing the zones' color for 60 min. Fig. 5 shows that the number and color of the zones were stable for 60 min. Therefore, it can be concluded that the

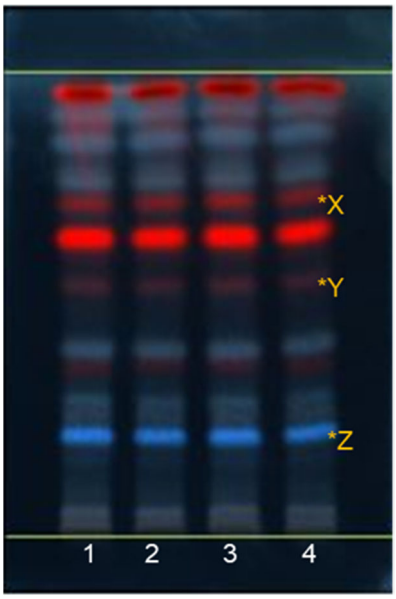

Fig 3. The TLC chromatogram of *S. rhombifolia* leaves extract for evaluation of analyte stability for 3 h and visualized with UV light 366 nm. (1) the extract of *Sida rhombifolia* leaves was left for 3 h, (2 and 4) the fresh extract of *S. rhombifolia* leaves was applied immediately to the TLC plate before the separation process, (3) the fresh extract of *S. rhombifolia* leaves was kept for 3 h and then applied to the TLC plate

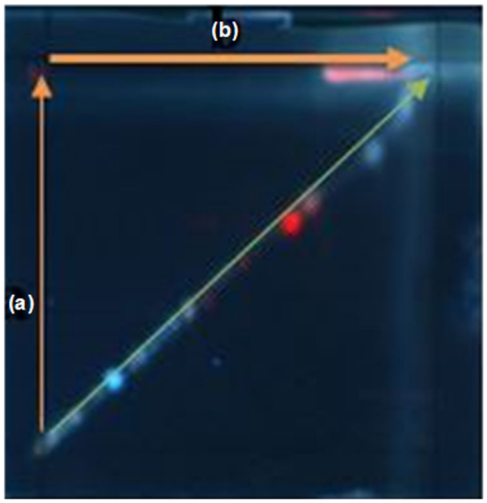

Fig 4. The TLC chromatogram derived from the extract of *S. rhombifolia* leaves extract with two-dimensional solvent development processes. (a) First solvent development process and (b) second development process and documented using UV light at 366 nm with the sulfuric acid reagent

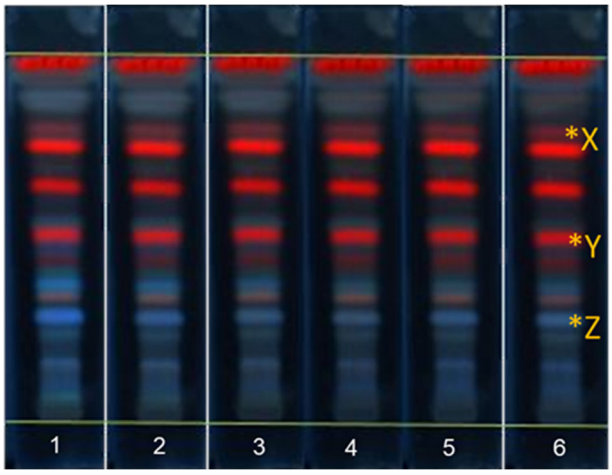

Fig 5. The TLC chromatogram of *S. rhombifolia* leaves extract after (1) 2nd, (2) 5th, (3) 10th, (4) 20th, (5) 30th, (6) 60th min using UV light 366 nm with the sulfuric acid derivatization reagent

chromatogram was stable for at least 1 h after sample derivatization.

Specificity

The specificity of the TLC fingerprint analysis was evaluated by comparing the separation profile of *S. rhombifolia* and its related plants, like *T. ulmifolia* and *H. rosa-sinensis*, due to their leaves' physical and morphological similarities. Herbal medicinal products are generally used raw materials in powdered. So, it will make difficult to distinguish between the three plants if they are present in powdered form. We choose *T. ulmifolia* and *H. rosa-sinensis* as potential adulteration of *S. rhombifolia*. The specificity was performed by applying each plant extract on the TLC plates in different lanes. We can compare TLC fingerprint similarity during the specificity tests according to the number of bands, color, intensity, and band position.

The chromatogram results identifying *S. rhombifolia* extracts compared with the extract from *T. ulmifolia* and *H. rosa-sinensis* showed different patterns detected with sulfuric acid derivatization under 366 nm (Fig. 6). Based on the specificity evaluation, *S. rhombifolia* leaves are considered specific because they have different patterns, positions, quantities, colors, and intensities of the resulting bands. The extract from *S. rhombifolia*, *T. ulmifolia*, and *H. rosa-sinensis* produced 11, 10, and 12 bands on the TLC plate, respectively. However, these extracts have band similarities in terms of R_f values at 0.95, 0.89, 0.81, and 0.69 (Table 2). The TLC fingerprint of *S. rhombifolia* extracts had a marker band (blue color) at 0.28, which can be used to distinguish and specify only

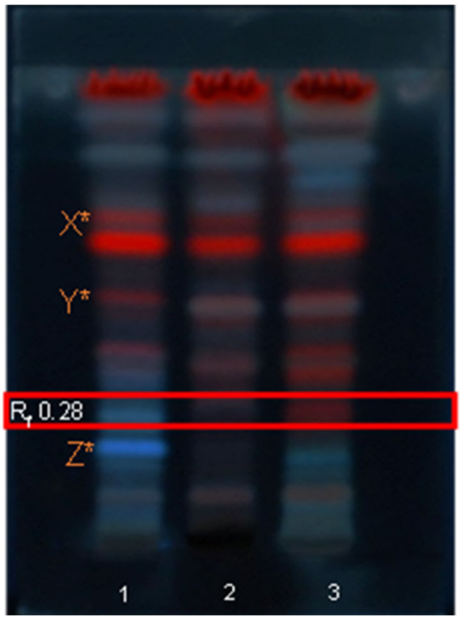

Fig 6. The TLC chromatogram obtained from the leaves extract of (1) *S. rhombifolia*, (2) *T. ulmifolia,* (3) *H. rosa-sinensis* using UV light at 366 nm with sulfuric acid as derivatization reagent

Table 2. Specific R_f value of *S. rhombifolia*, *T. ulmifolia*, *H. rosa-sinensis* extract using sulfuric acid as derivatization reagent followed by detection at UV light 366 nm

Band	R_f		
	S. rhombifolia	*T. ulmifolia*	*H. rosa-sinensis*
1	0.95	0.95	0.95
2	0.89	0.89	0.89
3	0.81	0.81	0.81
4	0.72	0.72	0.76
5	0.69	0.69	0.69
6	0.52	0.51	0.51
7	0.41	0.38	0.41
8	0.36	0.32	0.37
9	0.28	0.22	0.28
10	0.21	0.11	0.26
11	0.11		0.20
12			0.11

to this plant. Therefore, it can be concluded that the developed method can be used to differentiate *S. rhombifolia* from its related plant used in this study.

Precision and intermediate precision

Precision was performed on the same day using similar laboratory equipment, while intermediate precision was done in a similar laboratory using different equipment (tools and reagents) [21]. The precision was evaluated using plant extracts with three different TLC plates on a similar day, while intermediate precision was performed consecutively on three different days. Each extract was applied to the TLC plate, and its TLC chromatogram pattern was observed after separating the components. TLC fingerprint pattern criteria for the precision test were the number of bands, the band's position, color intensity, and the difference of R_f value between 3 bands on the TLC plate maximum at 0.02 ($\Delta R_f \leq 0.02$). For intermediate precision, the difference in R_f value from 3 bands at the TLC plate should be no more than 0.05 ($\Delta R_f \leq 0.05$) [19]. The intermediate precision requirement is higher than the precision parameter because we cannot maintain the laboratory's stable environment during the experiment.

Fig. 7 shows the pattern of the TLC fingerprint from *S. rhombifolia* extracts for precision evaluation, while Fig. 8 shows the TLC pattern for intermediate precision. Based on this observation, it can be seen from Fig. 7 that $\Delta R_f \leq 0.02$ was obtained from 3 bands at the TLC plate for precision evaluation, while the TLC fingerprint (Fig. 8) has $\Delta R_f \leq 0.05$ at the TLC plate for intermediate precision evaluation. R_f value's difference due to high humidity and temperature causes poor separation and produces a low-resolution value. The resolution factor's low value is affected by the mobile phase's saturation and causes poor separation on the TLC plate. Therefore, it can be concluded that the criteria for precision and intermediate precision can be accepted based on the chromatogram pattern in Fig. 7 and 8.

Robustness

Robustness was evaluated by the chromatography chamber type and the solvent development distance on the TLC plate during the separation of components. The accepted criteria of robustness parameter include the constant TLC fingerprint profile in terms of number, position, bands' color, and R_f value difference of no more than 0.05 [19]. This evaluation was performed by developing the extract of *S. rhombifolia*, *T. ulmifolia*, and

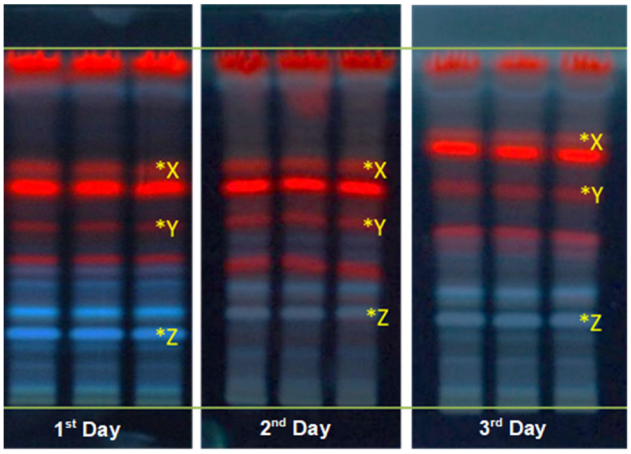

Fig 8. The three different TLC chromatograms obtained from the extract of *S. rhombifolia* leaves on (1) the first day, (2) the second day, and (3) the third day

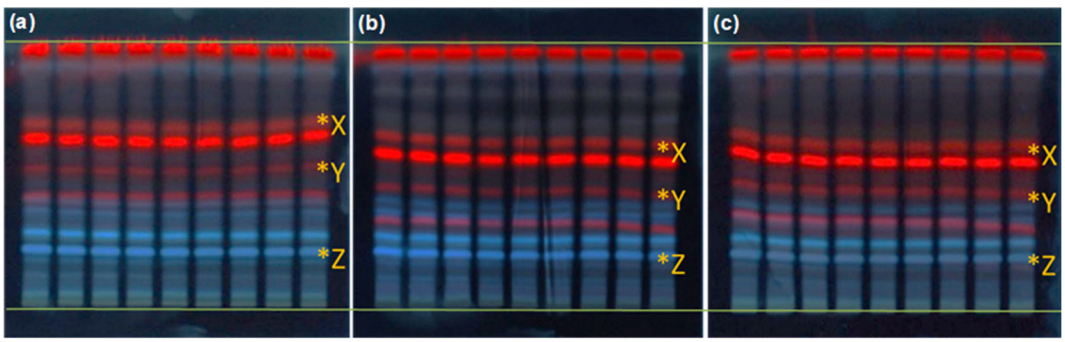

Fig 7. The three different TLC chromatograms obtained from the extract of *S. rhombifolia* leaves (a) first plate, (b) second plate, and (c) third plate

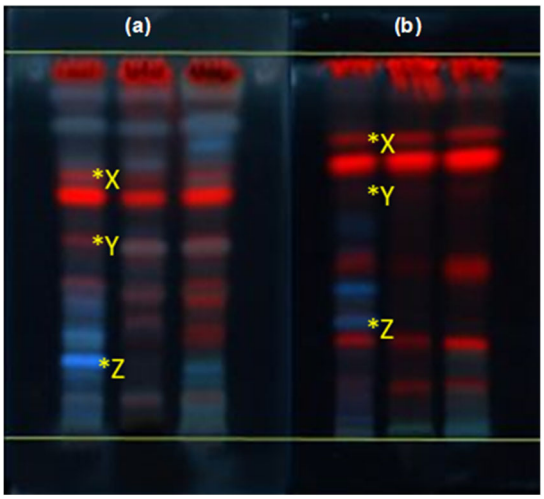

Fig 9. The TLC chromatogram of two different TLC plates using the extract of *S. rhombifolia* (line 1), *T. ulmifolia* (line 2), *H. rosa-sinensis* (line 3) from 2 different types of chamber chromatography (a) twin-through and (b) flat-bottom

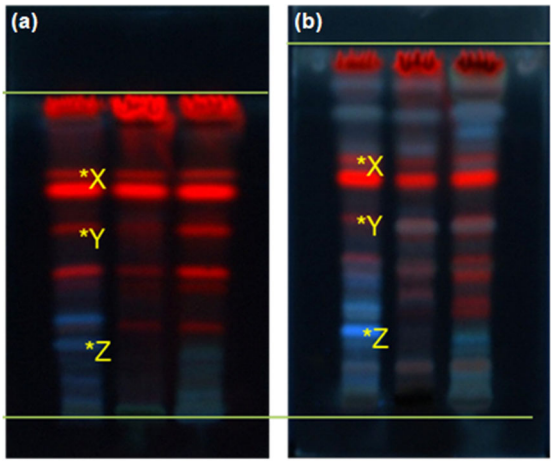

Fig 10. The TLC chromatogram of two different TLC plates from two different distances of solvent development at the twin-through chamber (a) 7 cm and (b) 8 cm

H. rosa-sinensis using the twin-through and flat-bottom with a different surface area and two different solvent development distances of 7 and 8 cm. Fig. 9 shows the chromatogram results from two chromatography chamber types that produce distinct differences between two TLC plates. The difference in R_f value was obtained more than 0.05 from 3 bands represented by top (X), middle (Y), and bottom (Z) in the extract of *S. rhombifolia* leaves. The chromatography chamber's flat bottom gave a higher R_f value (ΔR_f) difference than the twin-through chamber. Based on this result, the robustness parameter still does not meet its acceptance level regarding the chromatography chamber type. Fig. 10 shows the chromatogram with two distances of solvent development with different TLC fingerprint patterns obtained from the extract of *S. rhombifolia* leaves. The difference in R_f values from the X, Y, and Z bands was obtained at more than 0.05 and still did not meet the quality control standard.

Fig. 9 and 10 show a distinct difference in the number of bands, position, and R_f value. This is due to relative humidity and mobile phase saturation changes and causes an increase in R_f value. The mobile phase's development process using a flat-bottom and twin-through chamber needs different times regarding its surface area. Therefore, this method is specific only for the twin-through chamber with an 8 cm distance of solvent development. This method could not maintain the fingerprint profile's stability when it changes to different solvent development and chamber type.

■ CONCLUSION

In conclusion, the TLC fingerprint analysis of *S. rhombifolia* fingerprints showed that all chromatographic conditions were applied, the most suitable of which was an aluminum plate coated with silica gel 60 F_{254} as the stationary phase and a mixture of chloroform, ethyl acetate, and methanol (6.5:2:1.5) (v/v) as the mobile phase with sulfuric acid derivatization under UV visualization at 366 nm. The leaf fingerprint profile of *S. rhombifolia* produced 11 bands with good resolution. This fact confirms that the specificity of the proposed method is reliable with specificity, accuracy, precision, and robustness. This fingerprint analysis method has met the acceptance criteria for method validation and can be used to identify and control the quality of *S. rhombifolia* leaves.

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