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Original Article

System optimization and validation to improve thin-layer chromatography of roselle calyces (*Hibiscus sabdariffa* L.) required by the Indonesian Herbal Pharmacopoeia Edition II

[Optimización y validación del sistema para mejorar la cromatografía en capa fina de cálices de rosa mosqueta (*Hibiscus sabdariffa* L.) requerida por la Farmacopea Herbal Indonesia Edición II]

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Abstract

Context: Roselle (*Hibiscus sabdariffa* L.) is one of the traditional crude drugs monographed in the Indonesian Herbal Pharmacopoeia Edition II (IHP II). As per IHP II requirements, a TLC pattern should be used in its authentication. However, the TLC system described for this purpose does not produce a clear reference chromatogram, often leading to inconclusive results.

Aims: To develop and validate TLC systems of *H. sabdariffa* calyces for a better-quality chromatogram than those listed in the IHP II, thereby facilitating crude drugs authentication.

Methods: To optimize TLC for *H. sabdariffa*, sixteen systems, differing in stationary phase, mobile phase composition, and/or visualization reagent were tested. The TLC system was then validated using several parameters such as analyte stability during chromatography, analyte stability in the extract solution and on the TLC plate, stability of the derivatization result, and precision on a plate as well as intermediate precision.

Results: Two systems (I and II) were successfully designed and applied to evaluate *H. sabdariffa* quality. System I used Si gel 60 F_{254} for the stationary phase, ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:2) for the mobile phase, *H. sabdariffa*'s ethanol extract (5%, 20 µL) for the test solution, cyanidin 3-O-glucoside (100 ppm, 4 µL) as a reference, no derivatization, and detection with visible light. System II combined Si gel 60 F_{254} for the stationary phase, ethyl acetate-formic acid-glacial acetic acid-toluene-water (80:11:11:20:19), *H. sabdariffa*'s ethanol extract (5%, 20 µL) for the test solution, chlorogenic acid (1000 ppm, 2 µL) and caffeic acid (50 ppm, 2 µL) as references, the visualizing reagent NP/PEG, and investigation under 366 nm UV light.

Conclusions: Both systems are simple but have good stability and precision, thus facilitating *H. sabdariffa* calyx authentication and paving the way for developing content analysis methods for *H. sabdariffa* markers.

Keywords: authenticity; herbal pharmacopoeia; quality evaluation; roselle; TLC pattern.

Resumen

Contexto: La rosa mosqueta (*Hibiscus sabdariffa* L.) es una de las drogas crudas tradicionales monografiadas en la segunda edición de la Farmacopea Herbal Indonesia (PHI II). Según los requisitos de la PHI II, se debe utilizar un patrón de TLC para su autentificación. Sin embargo, el sistema de TLC descrito para este fin no produce un cromatograma de referencia claro, lo que a menudo conduce a resultados no concluyentes.

Objetivos: Desarrollar y validar sistemas de TLC de cálices de *H. sabdariffa* para obtener un cromatograma de mejor calidad que los listados en el PHI II, facilitando así la autentificación de drogas crudas.

Métodos: Para optimizar la TLC para *H. sabdariffa*, se probaron dieciséis sistemas, que diferían en la fase estacionaria, la composición de la fase móvil y/o el reactivo de visualización. A continuación, se validó el sistema de TLC utilizando varios parámetros, como la estabilidad del analito durante la cromatografía, la estabilidad del analito en la solución del extracto y en la placa de TLC, la estabilidad del resultado de la derivatización y la precisión en una placa, así como la precisión intermedia.

Resultados: Se diseñaron y aplicaron con éxito dos sistemas (I y II) para evaluar la calidad de *H. sabdariffa*. El sistema I utilizó Si gel 60 F254 como fase estacionaria, acetato de etilo-ácido fórmico-ácido acético glacial-agua (100:11:11:2) como fase móvil, extracto de etanol de H. sabdariffa (5%, 20 µL) como solución de prueba, cianidina 3-O-glucósido (100 ppm, 4 µL) como referencia, sin derivatización, y detección con luz visible. El sistema II combinó Si gel 60 F254 para la fase estacionaria, acetato de etilo-ácido fórmico-ácido acético glacial-tolueno-agua (80:11:11:20:19), extracto de etanol de H. sabdariffa (5%, 20 µL) para la solución de prueba, ácido clorogénico (1000 ppm, 2 µL) y ácido cafeico (50 ppm, 2 µL) como referencias, el reactivo de visualización NP/PEG, y la investigación bajo luz UV de 366 nm.

Conclusiones: Ambos sistemas son sencillos pero tienen buena estabilidad y precisión, facilitando así la autentificación del cáliz de *H. sabdariffa* y allanando el camino para desarrollar métodos de análisis de contenido para marcadores de *H. sabdariffa*.

Palabras Clave: autenticidad; farmacopea herbal; evaluación de la calidad; rosa mosqueta; patrón TLC.

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INTRODUCTION

Roselle (*Hibiscus sabdariffa* L., family *Malvaceae*) is a flowering plant ubiquitously used as a medicinal and food plant in many countries in Central Africa, West Africa, South Africa, and Southeast Asia, including Indonesia, Thailand, Malaysia, and Vietnam (Ali et al., 2005; Antonia et al., 2019; Chumsri et al., 2008; Pham et al., 2019). The calyx contains various beneficial therapeutic compounds, especially organic acid, phenolic acid, flavonoid, and polysaccharide groups, making it the plant's most important part (Hapsari and Setyaningsih, 2021; Izquierdo-Vega et al., 2020). Anthocyanins are flavonoids responsible for the red sepals, and organic acids (citric acid, malic acid, tartaric acid, and hibiscus acid) for the sour taste. In addition to these typical organoleptic features, the compounds are known to exhibit numerous biological activities; for instance, antioxidative, antipyretic, antinociceptive, anti-inflammatory, antibacterial, antifungal, antiparasitic, anticancer, antidiabetic, antihypertensive, anti-cholesterol, and hepatoprotective (Ali et al., 2005; Izquierdo-Vega et al., 2020).

H. sabdariffa var. Sabdariffa and *H. sabdariffa* var. Altissimac Wester are the two most traded varieties. Their varied phenotypes and genotypes have also been reported (Antonia et al., 2019; Hapsari and Setyaningsih, 2021; Sukkhaeng et al., 2018; Torres-Morán et al., 2011). In Indonesia alone, there are two known variants: red roselle and purple roselle (Aryanti et al., 2019). As an ingredient in food, beverages, and traditional medicine, dried *H. sabdariffa* is traded in at least three forms: whole calyx, flakes, and powder. The dried calyx has a purplish to blackish-red color.

As part of traditional remedies, *H. sabdariffa* is monographed in several herbal pharmacopoeias, including Indonesian Herbal Pharmacopoeia Edition II (IHP II), Malaysian Herbal Monograph, and European Pharmacopoeia 7.0 (Committee MHM, 2015; EDQM, 2008; Health IMO, 2017). The monographs contain botanical characteristics to be used in the authentication of a suspected *H. sabdariffa* specimen and quality control. Aside from organoleptic and microscopic identifications, IHP II also includes a TLC system and its associated pattern for a more sensitive authentication method. In this case, users can compare their results with the provided pattern to determine similarities (or differences) in the number, color, and Rf or Rx values of spots produced by a particular TLC system. Therefore, the reference chromatogram should have a clear pattern and separation. This is, however, not seen in the one used for *H. sabdariffa* authentication in IHP II (Fig. 1), where the five different compound spots show indistinct colors and are separated poorly—hindering its use as a reference in related analyses.

For the above reasons, the research was intended to develop and validate TLC systems for a better-quality chromatogram, where the compound spots are not overlapping and streaking but distinguishable (their amount and color are easily identifiable). This research is essential in fostering the use of *H. sabdariffa* as a traditional crude drug with standardized quality and, thus, maintaining its consistent efficacy and safety.

MATERIAL AND METHODS

Chemicals

Chemicals used were TLC silica gel 60 F_{254} plates, TLC cellulose plates, n-hexane, toluene, ethyl acetate, chloroform, n-butanol, acetone, acetic acid, formic acid, ethanol, methanol, and polyethylene glycol (PEG); all from Merck KGaA (Darmstadt, Germany). In addition, the analyses also used 2-aminoethyl diphenylborinate, quercetin, gallic acid, rutin, chlorogenic acid, caffeic acid, and p-coumaric acid from Sigma Aldrich Co. (St. Louis, MO, USA). Cyanidin 3-O-glucoside was obtained from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany), and demineralized water was from the Pharmaceutical Chemistry Laboratory, Faculty of Pharmacy, University of Surabaya, Indonesia.

Plant material

The plant material used in this research was purple *H. sabdariffa* calyces (Fig. 2) harvested in Magetan Regency, Jawa Timur Province, Indonesia, in April 2021 (7°37'22"S; 111°17'4"E). Botanical identification was conducted by the Center for Traditional Drug Information and Development, the University of Surabaya, with the plant authentication certificate No. 1476/D.T/VI/2022. Whole dried *H. sabdariffa* calyces were ground in a blender (Philips HR 2222) and sifted through a 20 mesh sieve. The powder was then stored in a dark, airtight container until further use.

Extract preparation

The method described in IHP II was adopted to extract the calyces. First, calyx powder weighing 0.5 g was added with 10 mL of 95% ethanol, then shaken in a water bath (MemmertGmbH + Co. KG, Schwabach, Germany) for ten minutes at 60°C to improve the extraction process. Afterward, the derived extract was passed through a sieve into a volumetric flask and

Figure 1. TLC pattern for *H. sabdariffa* authentication according to the Indonesian Herbal Pharmacopoeia Edition II.

S: *H. sabdariffa* calyx extract, P: cyanidin 3-O-glucoside.

Figure 2. Purple *H. sabdariffa* calyces.

poured with demineralized water until the volume was 10.0 mL.

TLC system optimization

To optimize TLC for *H. sabdariffa*, sixteen systems differing in stationary phase, mobile phase composition, and/or visualization reagent derived from the literature study were tested. The two stationary phases used in this optimization stage were silica gel 60 F_{254} and cellulose. The sample and reference solution were each dotted onto the plate 1.5 cm from the bottom, 1 cm from the right and left side, and 1 cm from the adjacent spots. Dotting or spotting was conducted manually using a 2 µL capillary pipe (CAMAG, Muttenz, Switzerland). Elution was allowed until 8 cm from the lower edge of the plate in a twin-trough chamber (CAMAG, Switzerland). The visualizing agents included $FeCl_3$ reagent, ammonia vapor, and NP/PEG reagent. After selecting the optimal mobile phase, stationary phase, and visualization reagent, the optimization stage continued with testing the volume of the spotted sample and the reference solution type and volume. The reference solutions optimized at this stage were quercetin, cyanidin 3-O-glucoside, gallic acid, rutin, chlorogenic acid, caffeic acid, p-coumaric acid, and vanillic acid. All tests were conducted in three replicates.

TLC system validation

The TLC system for *H. sabdariffa* was validated using several parameters: analyte stability during chromatography, analyte stability in the extract solution and on the TLC plate, stability of the derivatization result, and precision on a plate as well as intermediate precision. In the validation process, samples and reference solutions were spotted using a CAMAG 100 µL-sample syringe (Hamilton, Switzerland) automatically with a Linomat 5 applicator (CAMAG, Switzerland) under N_2 gas flow.

Analyte stability during chromatography

A sample solution was applied to the lower-right side of a 10 × 10 cm plate in the form of a spot. The plate was eluted until the mobile phase reached about 8 cm from the start line, then lifted and allowed to dry. Next, it was rotated 90° clockwise, placed in the chamber, and re-eluted with a new mobile phase. After drying, it was derivatized with NP/PEG reagent and documented under 366 nm UV light. An analyte is deemed stable if the compound spots form a diagonal line connecting the spotting position with the intersection of the two mobile-phase fronts (Reich and Schibli, 2007). Analysis was performed in three replicates on three different TLC plates.

Analyte stability in the extract solution and on the plate

On a TLC plate, the *H. sabdariffa* calyx extract was deposited on lanes 1 and 2. The extract and the recently spotted plate were left to stand for three hours. Then, the 3-hour-old extract was applied to lanes 5 and 6. A freshly prepared extract was also spotted onto lanes 3 and 4 right before elution with the optimized system. The chromatogram before and after derivatization using NP/PEG reagent was documented with a TLC visualizer (CAMAG, Muttenz, Switzerland). This process also generates other data, including Rf value, peak height, and peak area of each compound spot appearing on the chromatogram. Of all

Table 1. Optimal thin-layer chromatography systems for *H. sabdariffa*.

System	Stationary phase	Mobile phase	Test solution	Reference solution	Derivatization*	Detection
I	Si gel 60 F_{254}	Ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:2)	Ethanol extract of roselle calyx (5%, 20 µL)	Cyanidin 3-O-glucoside (100 ppm, 4 µL)	N/A	Visible light
II	Si gel 60 F_{254}	Ethyl acetate-formic acid-glacial acetic acid-toluene-water (80:11:11:20:19)	Ethanol extract of roselle calyx (5%, 20 µL)	Chlorogenic acid (1000 ppm, 2 µL) Caffeic acid (50 ppm, 2 µL)	NP/PEG	366 nm UV light

*N/A: not applicable

the spots, one to a few were selected as probable markers, and the average, standard deviation, and relative standard deviation (RSD) of their peak heights were calculated. RSD was calculated from the marker spots on lanes 1, 2, 3, and 4 to determine analyte stability on the plate and from those on lanes 3, 4, 5, and 6 to assess the stability in the extract solution (Reich and Schibli, 2007). Analysis was performed in three replicates on three different TLC plates.

Precision on a plate and intermediate precision

The *H. sabdariffa* extract solution was deposited on a plate in five replicates for the precision on a plate test and on three consecutive days for the intermediate precision test. Each plate was eluted with the optimized mobile phase. Then, the chromatogram before and after derivatization with a visualizing reagent was documented using a TLC visualizer. Rf values were then compared and peak heights of the marker spots were used to calculate their average, standard deviation, and RSD. RSD of the marker spots from one plate was calculated to determine precision on a plate, while the one from the different-day application was for intermediate precision (Spangenberg et al., 2011).

TLC pattern generation for *H. sabdariffa*

The validated TLC systems for *H. sabdariffa* calyx were each used to create a TLC pattern. It was then compared with the pattern depicted in IHP II. Observations were made to see similarities or differences in these parameters: the number, color, Rf or Rx value, and separation of the spots.

Statistical analysis

All data were reported as mean ± SD (n = 3-5), then the relative standard deviations (RSD) were calculated and analyzed using descriptive statistics to determine whether the developed method satisfies the validation parameters.

RESULTS AND DISCUSSION

Optimized TLC system

Two of the 16 TLC systems tested in the research are deemed suitable for creating a clear chromatogram for *H. sabdariffa* calyces, namely systems I and II (Table 1). Figs. 3 and 4 show the chromatograms derived from the two systems.

Under visible light, the *H. sabdariffa* calyx extract tested with the system I was separated into two red spots suspected as anthocyanins (see spots a and b in Fig. 3A). While they shared a similar red chroma with cyanidin 3-O-glucoside, the Rf values differed. Therefore, both spots were proposed as markers for system I and referred to as 'anthocyanin 1' and 'anthocyanin 2'. These compounds could be delphinidin-3-glucoside, delphinidin-3-sambubioside, or cyanidin-3-sambubioside, which are also red to purple in color (Santos et al., 2013; Committee MHM, 2015; Lago et al., 2014). The system I produced a better TLC pattern than IHP II, where only one anthocyanin spot is not separated from other compound spots (Fig. 1). Fig. 3A also shows that 20 µL of extract (lane 2) and 4 µL of reference solution (lane 5) produced a good pattern. For these reasons, it is recommended that the *H. sabdariffa*'s chromatogram be made with system I, combining Si gel 60 F_{254} as the stationary phase, ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:2) as the mobile phase, *H. sabdariffa*'s ethanol extract (5%, 20 µL) as the test solution, cyanidin 3-O-glucoside (100 ppm, 4 µL) as the reference solution, no derivatization, and investigation under visible light.

In addition to anthocyanins, *H. sabdariffa* is a good source of flavonoids from other subclasses like quercetin-3-glucoside, methyl epigallocatechin, myricetin, quercetin, and kaempferol. No less than 95 flavonoids have been discerned from the calyces. Besides, the plant is rich in other phenolic compounds, such as neochlorogenic acid, chlorogenic acid, cryptochloro-

genic acid, methyl chlorogenate, coumaroylquinic acid, dihydroferulic acid-4-O-glucuronide, ethyl chlorogenate, and 5-O-caffeoyl shikimic acid (Hapsari and Setyaningsih, 2021). Therefore, this research also experimented with reference solutions other than the one used in IHP II (cyanidin 3-O-glucoside), namely flavonoids (quercetin and rutin) and phenolic acid (gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, and vanillic acid). From the varying TLC systems tested for optimization, chlorogenic acid (Fig. 4C, spot a) and caffeic acid (Fig. 4C, spot b) were readily detectable, while other compounds were not. To ensure that spots a and b (Fig. 4C) were chlorogenic acid and caffeic acid, their UV spectrum was scanned and compared with their respective reference compounds. Fig. 5A-B shows an overlap between the former and the latter with a maximum wavelength of 320-330 nm, indicating that spots a and b are indeed chlorogenic acid and caffeic acid (Kim et al., 2015; Orfali et al., 2021). Therefore, both acids can be used as alternative analytical markers for *H. sabdariffa* quality analysis, especially based on TLC patterns.

Chlorogenic and caffeic acids are the two most common phenolic acids found in *H. sabdariffa*, which also possess anti-diabetic and anti-hyperlipidemic properties. Previous research confirmed the presence of chlorogenic acid (67.12 mg/g) and caffeic acid (15.38 mg/g) in *H. sabdariffa* extract and identified their neuroprotective activity by testing it against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), monoamine oxidase (MAO), and ecto-5'-nucleotidase (E-NTDase) (Hapsari and Setyaningsih, 2021). Furthermore, because of their high contents and biological activities, chlorogenic and caffeic acids are suitable as not only analytical but also active markers for *H. sabdariffa* calyces (Chan et al., 2007).

Fig. 4C also shows that 2 µL of chlorogenic acid (lane 1), 2 µL of caffeic acid (lane 4), and 20 µL of the extract (lane 8) produced interpretable chromatograms. Thus, aside from system I, it is also suggested that the *H. sabdariffa*'s TLC pattern be made using system II, consisting of Si gel 60 F_{254} as the stationary phase, ethyl acetate-formic acid-glacial acetic acid-toluene-water (80:11:11:20:19) as the mobile phase, *H. sabdariffa*'s ethanol extract (5%, 20 µL) as the test solution, chlorogenic acid (1000 ppm, 2 µL) and caffeic acid (50 ppm, 2 µL) as the reference solutions, derivatization with the visualizing reagent NP/PEG, and detection with 366 nm UV light.

Analyte stability during chromatography

To validate the optimized systems I and II, analyte stability in the extract during chromatography was tested with a two-dimensional elution. Fig. 6A-B shows that both systems produced good analyte stability. This is evident in the plate's diagonal line in the elution results (Kartini et al., 2021; Reich and Schibli, 2007).

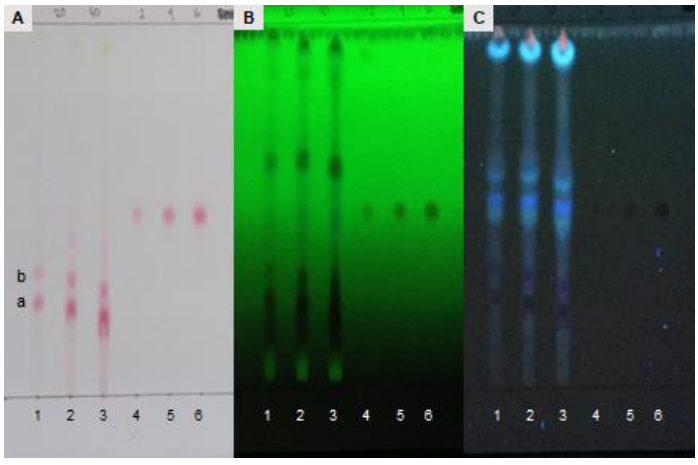

Figure 3. TLC patterns of *H. sabdariffa* calyx extract developed using system I under visible light **(A)**, 254 nm UV light **(B)**, and 366 nm UV light **(C)**.

Different lanes from left to right: 10, 20, and 30 µL of purple *H. sabdariffa* extract and 2, 4, and 6 µL of cyanidin 3-O-glucoside (100 ppm).

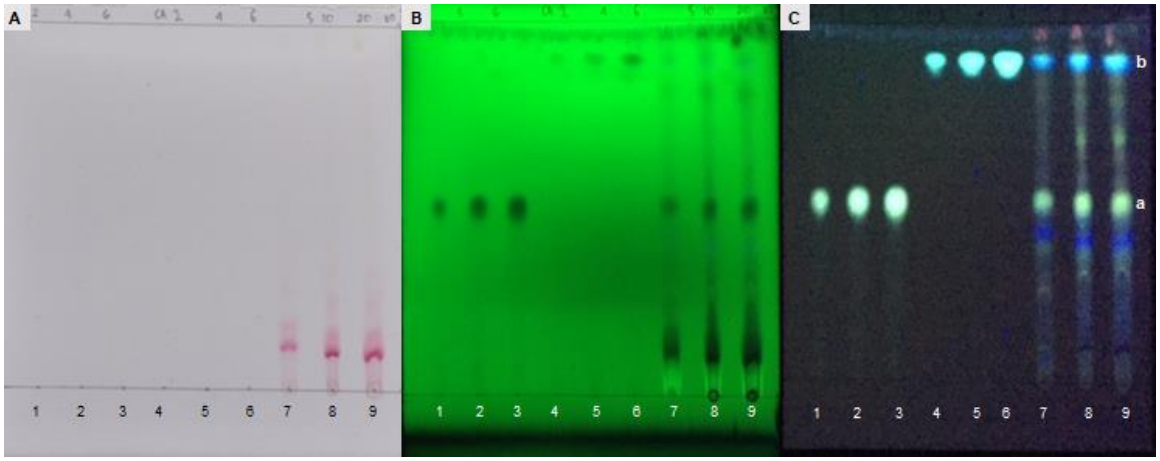

Figure 4. TLC patterns of *H. sabdariffa* calyx with system II observed under visible light **(A)**, 254 nm UV light **(B)**, and 366 nm UV light after derivatization with NP/PEG **(C)**.

Different lanes from left to right: 2, 4, 6 µL of chlorogenic acid (1000 ppm), 2, 4, 6 µL of caffeic acid (50 ppm), and 10, 20, 30 µL of the purple *H. sabdariffa*.

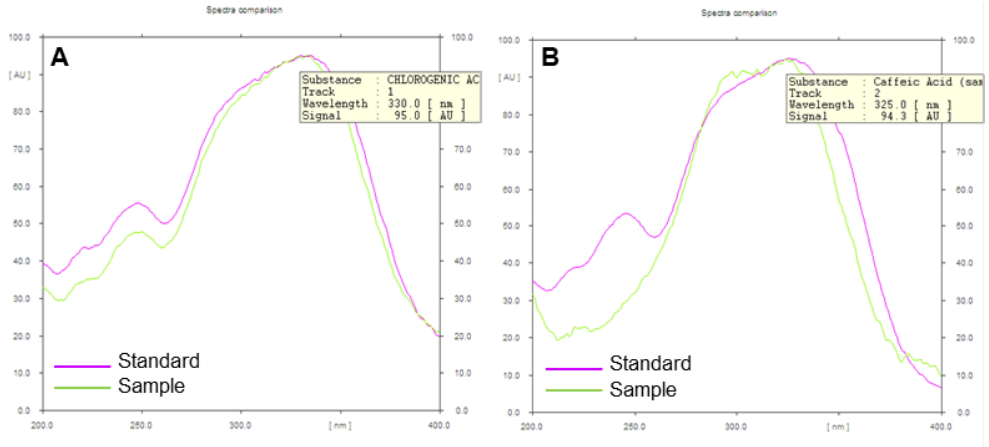

Figure 5. Spectral comparison between chlorogenic acid standard and sample **(A)** and caffeic acid standard and sample **(B)**.

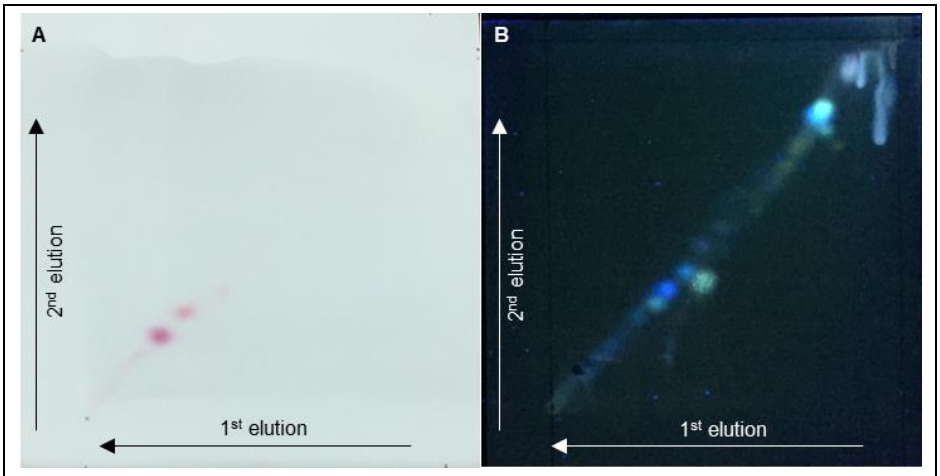

Figure 6. Analyte stability during chromatography to validate TLC system I, observed under visible light **(A)**, and system II under 366 nm UV light **(B)**.

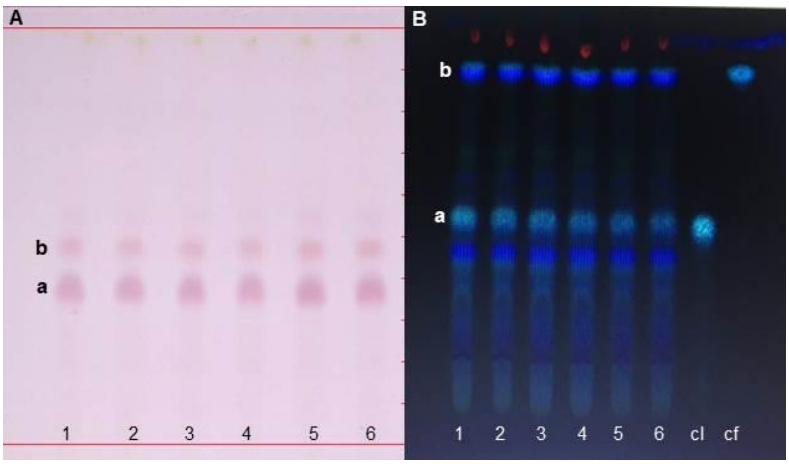

Figure 7. Chromatograms derived from testing the analyte stability in the extract solution with TLC systems I **(A)** and II **(B)**.

Lanes 1 and 2: the extract left for three hours after application to the TLC plate; lanes 3 and 4: freshly prepared extract; lanes 5 and 6: the extract left for three hours in the solution; cl: chlorogenic acid, cf: caffeic acid.

Table 2. Peak heights of the marker compounds obtained from testing the analyte stability in the extract solution and on the plate.

Marker	Lanes 1–2	Lanes 3–4	Lanes 5–6	RSD (%) from lanes 1–4	RSD (%) from lanes 3–6
Anthocyanin 1	1632.1	1444.5	1247.6	12.0	9.2
Anthocyanin 2	1191.4	883.8	793.1	5.5	6.4
Chlorogenic acid	1632.1	1566.9	1421.2	11.5	8.1
Caffeic acid	4143.8	5157.4	4416.2	15.4	10.9

Analyte stability in the extract solution and on the plate

To assess stability more objectively (Fig. 7), the peak heights of the four markers (anthocyanin 1, anthocyanin 2, chlorogenic acid, and caffeic acid on lanes 1–2 and 5–6) were compared with those detected on lanes 3–4. There is no difference between the individual lane, indicated by the RSD calculated for each marker was ≤20 % (Table 2). It means the four analytes have good stability in the extract solution and on the TLC plate (Kartini et al., 2021; Reich and Schibli, 2007).

Stability of the chromatography results

Evaluating the stability of the chromatography results aimed to determine how long after elution or derivatization the chromatogram would still show the same results. Fig. 8B–F shows that anthocyanin 1 (spot a) and anthocyanin 2 (spot b) on lanes 1–6 appeared in the same color and intensity as those in Fig. 8A, indicating that the chromatogram made with the system I has good stability within 30 minutes after elution. Fig. 9B-F also show that chlorogenic acid (spot a) and caffeic acid (spot b) on lanes 1–6 had the same color and intensity as those in Fig. 9A, suggesting that the chromatogram made with system II has good stability until 30 minutes after derivatization.

Then, to achieve more objective results, the peak height average was calculated for each marker (anthocyanin 1, anthocyanin 2, chlorogenic acid, and caffeic acid on lanes 1–6) and was plotted against time of observation to make a curve (Fig. 10). The graph shows no significant decline on all the marker curves during the 30-minute observation, demonstrating good stability of the four analytes within 30 minutes after elution or derivatization. These results indicated that the chromatogram could be either documented immediately or delayed for not more than 30 minutes after elution or derivatization.

Precision on a plate and intermediate precision

Precision represents the closeness between chromatography results from multiple sample replicates and random errors in a method. For qualitative analyses, precision can be linked to the Rf values of the separated compounds (Reich and Schibli, 2007). However, in this current study RSD of the peak height was calculated as well to make the data more comprehensive. Tables 3 and 4 show the markers' Rf and peak heights from five application replicates eluted with systems I and II. These data indicated that

the two systems designed to determine the *H. sabdariffa*'s TLC pattern have good precision on a plate and intermediate precision, with a RSD of smaller than 20%.

Comparison between *H. sabdariffa* chromatograms made with the optimized and the IHP II systems

Fig. 11A-B shows the TLC patterns of *H. sabdariffa* made with the IHP II system. Investigations under visible light (Fig. 11A) revealed one red spot of the extract (spot 1, lane S) with a different Rf from the reference solution, cyanidin 3-O-glucoside (St). The spot was clear but showed tailing, and the rest of the sample did not seem to elute from the application site. When observed under 366 nm UV light (Fig. 11B), the extract (lane S) produced one dark blue fluorescent spot (spot 1) above the tailed bright one. Meanwhile, the reference cyanidin 3-O-glucoside (St) fluoresced a blackish-blue color with a relatively large area. It will be difficult to use TLC patterns with poor separation as a reference, making misidentification of a crude drug highly likely.

With the optimized TLC systems, the *H. sabdariffa* calyx extract was eluted into more spots with better shape and separation (Fig. 11C-D). The system I produced two well-separated red spots with different Rf values than the reference cyanidin 3-O-glucoside (St). Therefore, both spots are concluded as not cyanidin 3-O-glucoside but suspected as anthocyanins (1 and 2) because they appeared red in visible light. Therefore, anthocyanins 1 and 2 are proposed as analytical markers for *H. sabdariffa*. System II produced several spots, two of which were chlorogenic acid and caffeic acid with clear blue fluorescence, no tails, and were well-separated from other compounds. Because they were easily and clearly detected using TLC, chlorogenic and caffeic acids are also proposed as analytical markers for *H. sabdariffa* calyces.

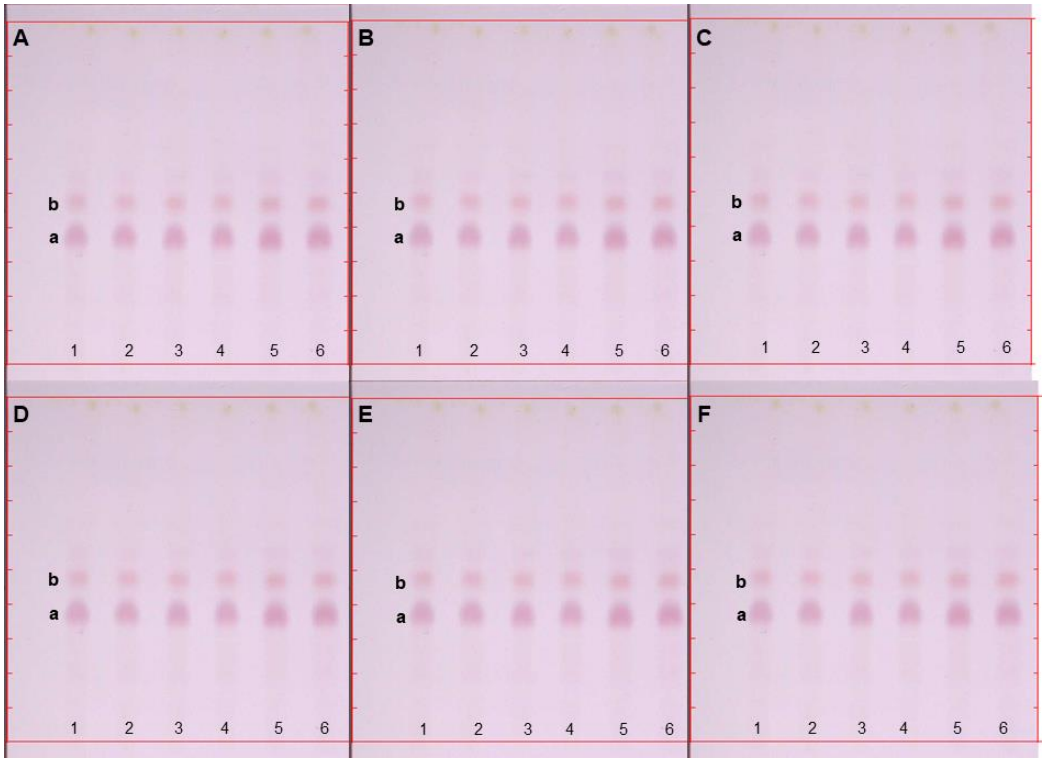

Figure 8. *H. sabdariffa* chromatograms made with TLC system I under visible light at different times of observation: 0 **(A)**, 5 (**B**), 10 **(C)**, 15 **(D)**, 20 **(E)**, and 30 minutes **(F)** after elution.

Different lanes from left to right: the first to the sixth replicate; a and b: anthocyanin 1 and anthocyanin 2.

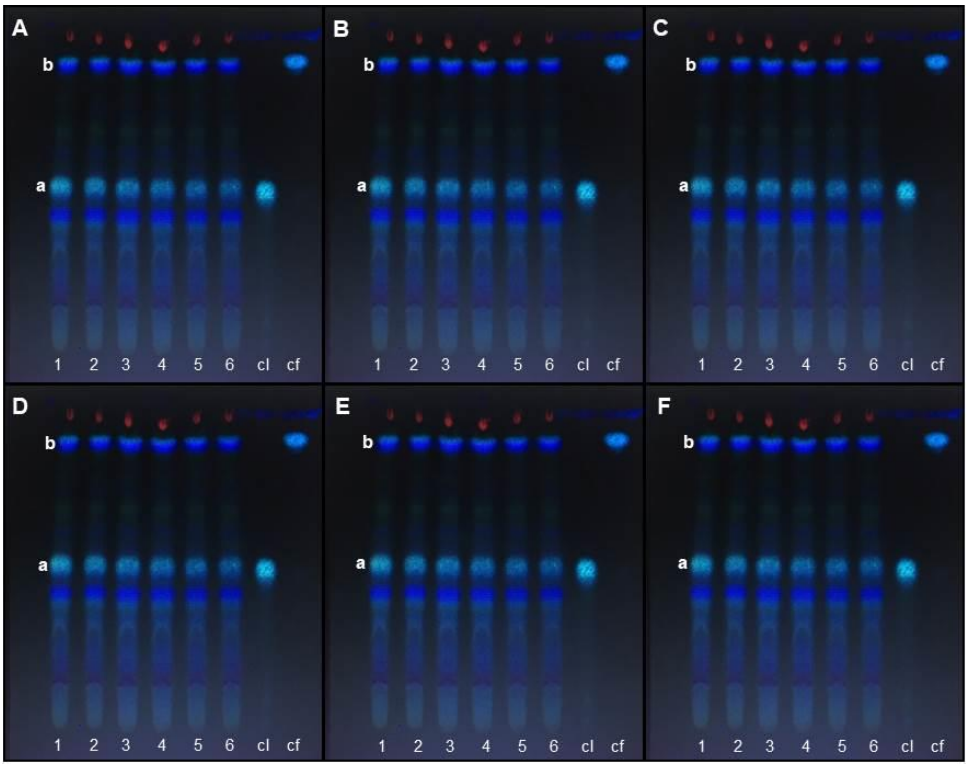

Figure 9. *H. sabdariffa* chromatograms made with system II under 366 nm UV light at different times of observation: 0 **(A)**, 5 **(B)**, 10 **(C)**, 15 **(D)**, 20 **(E)**, and 30 minutes **(F)** after derivatization with NP/PEG.
Different lanes from left to right: first to sixth replicates, a and b: chlorogenic acid and caffeic acid.

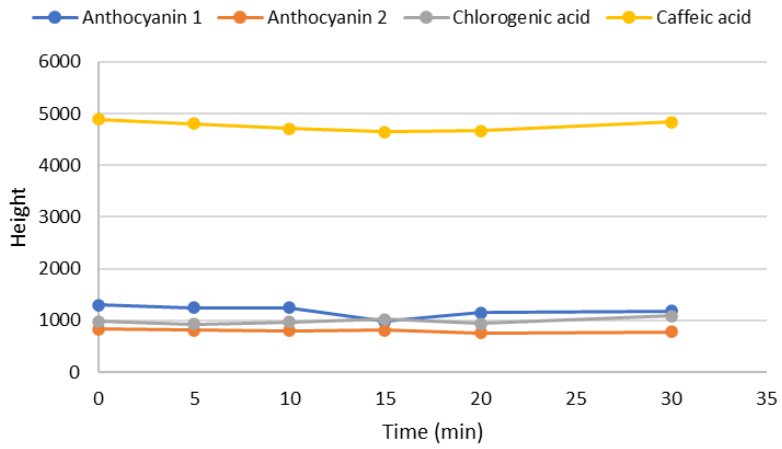

Figure 10. Stability graphs of anthocyanin 1, anthocyanin 2, chlorogenic acid, and caffeic acid after elution or derivatization.

Table 3. Rf values and peak heights of the marker anthocyanin during precision on a plate and intermediate precision.

Lane	Anthocyanin 1						Anthocyanin 2					
	Day 1		Day 2		Day 3		Day 1		Day 2		Day 3	
	Rf	Height	Rf	Height	Rf	Height	Rf	Height	Rf	Height	Rf	Height
1	0.392	2402.7	0.286	2166.3	0.286	2805.2	0.474	1558.3	0.350	1191.6	0.362	1316.1
2	0.384	2392.3	0.287	2192.0	0.294	2965.3	0.473	1509.0	0.356	1199.9	0.367	1461.1
3	0.386	2223.8	0.292	2156.0	0.295	2999.9	0.468	1376.1	0.359	1215.7	0.367	1518.4
4	0.383	2071.3	0.291	2159.7	0.297	2964.3	0.473	1074.8	0.359	1180.5	0.370	1530.1
5	0.381	2146.7	0.297	2140.8	0.298	2969.6	0.461	1306.9	0.362	1208.5	0.369	1544.0
Mean ± SD	2253.3 ± 169.4		2164.7 ± 21.2		2926.1 ± 80.6		1362.3 ± 220.3		1195.1 ± 11.9		1462.8 ± 104.3	
RSD (%)	7.5		1.0		2.8		16.2		1.0		7.1	
Precision on a plate, RSD (%)	1.0–7.5						1.0–16.2					
Intermediate precision, RSD (%)	17.0						10.1					

*Mean, SD, and RSD were calculated from the height of peaks.

Table 4. Rf values and peak heights of the markers chlorogenic acid and caffeic acid during precision on a plate and intermediate precision.

Lane	Chlorogenic acid						Caffeic acid					
	Day 1		Day 2		Day 3		Day 1		Day 2		Day 3	
	Rf	Height	Rf	Height	Rf	Height	Rf	Height	Rf	Height	Rf	Height
1	0.503	614.0	0.455	694.1	0.471	883.3	0.912	4507.9	0.879	4786.7	0.904	4646.8
2	0.503	597.6	0.460	596.8	0.476	741.7	0.905	4530.3	0.879	4848.4	0.907	4843.8
3	0.503	622.8	0.445	795.1	0.479	938.8	0.912	4808.8	0.879	4783.9	0.914	5004.8
4	0.508	613.8	0.447	768.8	0.479	1105.3	0.908	4833.8	0.875	5177.7	0.914	4987.2
5	0.503	661.7	0.449	715.8	0.482	856.3	0.912	4939.7	0.877	4829.6	0.914	5024.6
Mean ± SD	622.0 ± 24.0		714.1 ± 77.0		905.1 ± 133.0		4724.1 ± 193.6		4885.3 ± 165.8		4901.4 ± 159.2	
RSD (%)	3.9		10.8		14.7		4.1		3.4		3.3	
Precision on a plate, RSD (%)	3.9–14.7						3.3–4.1					
Intermediate precision, RSD (%)	19.3						2.0					

*Mean, SD, and RSD were calculated from the height of peaks

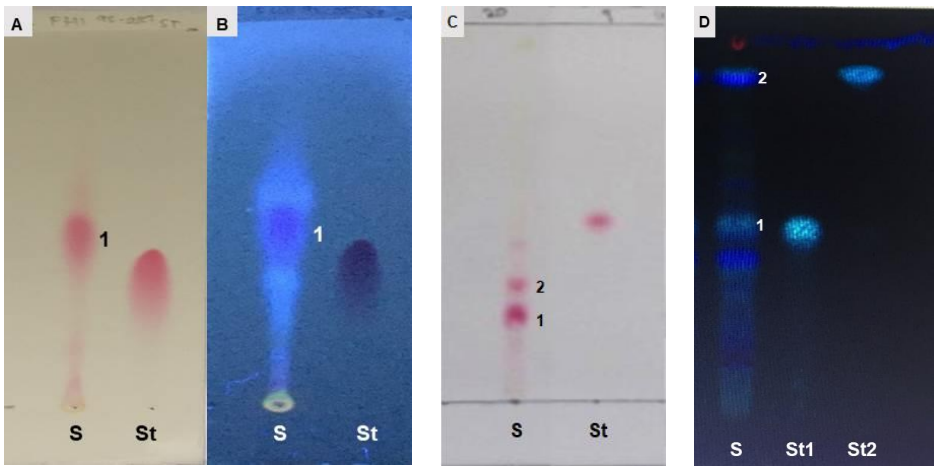

Figure 11. TLC patterns of *H. sabdariffa* extract separated with the standard TLC system in IHP II **(A, B)** and the developed systems I **(C)** and II **(D)**.

S: purple roselle extract, St: cyanidin 3-O-glucoside, St1: chlorogenic acid, St2: caffeic acid.

CONCLUSION

The research has successfully developed two TLC systems (I and II) for *H. sabdariffa* calyx authentication and quality control better than the one described in IHP II. System I uses Si gel 60 F_{254} for the stationary phase, ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:2) for the mobile phase, *H. sabdariffa*'s ethanol extract (5%, 20 µL) for the test solution, cyanidin 3-O-glucoside (100 ppm, 4 µL) for the reference solution, no derivatization, and visible light detection. System II combines Si gel 60 F_{254} as the stationary phase, ethyl acetate-formic acid-glacial acetic acid-toluene-water (80:11:11:20:19) as the mobile phase, *H. sabdariffa*'s ethanol extract (5%, 20 µL) as the test solution, chlorogenic acid (1000 ppm, 2 µL) and caffeic acid (50 ppm, 2 µL) as the reference solutions, derivatization with the visualization reagent NP/PEG, and detection with 366 nm UV light. These developed systems have good stability and precision, facilitating *H. sabdariffa* calyx authentication and paving the way for developing content analysis methods for *H. sabdariffa* markers.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Dewi BAASK	Kartini K
Concepts or ideas		x
Design		x
Definition of intellectual content		x
Literature search	x	x
Experimental studies	x	x
Data acquisition	x	x
Data analysis	x	x
Statistical analysis	x	x
Manuscript preparation	x	x
Manuscript editing		x
Manuscript review	x	x

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