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Simple TLC-Densitometric Method for the Quantification of Asiaticoside in *Centella asiatica* from Different Origins for Its Standardization

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Abstract. Gotu kola (*Centella asiatica* (L.) Urb.) is one of the “Jamu Saintifik” ingredients that contains various active compounds, including asiaticoside. Asiaticoside content depends on several factors, such as the plant’s origin, age, and harvesting season. This study was conducted to analyze the concentration of asiaticoside in gotu kola crude drug obtained from nine different planting locations using a TLC-densitometer. The TLC used silica gel 60 F₂₅₄ as the stationary phase, ethyl acetate-methanol-water (10:2.5:1) as the mobile phase, and anisaldehyde sulfuric acid as a derivatizing agent. The developed method met the specificity parameters, as characterized by the identical visible spectrum of the asiaticoside in the sample extract to that of the asiaticoside standard, with a maximum wavelength of 594 nm. The standard curve showed good linearity in the concentration range of 1.05–10.5 µg/band with an r-value of 0.989437. The limits of detection (LOD) and quantification (LOQ) were 0.48820 and 1.47939 µg/band, respectively. This method was repeatable and precise, with relative standard deviations between 2.72 and 7.16% for intraday precision and 2.82% for interday precision. Except for two samples, all the asiaticoside concentrations were quantifiable and ranged between 0.53933 and 3.46866 mg/g of crude drugs. In conclusion, they vary across the *C. asiatica* samples collected from different origins in Indonesia. The gotu kola crude drugs obtained from Lombok Barat, Magelang, and Hulu Sungai Selatan have the highest asiaticoside content among the nine samples studied.

Keywords: Asiaticoside, *Centella asiatica*, Gotu Kola, TLC-Densitometer

INTRODUCTION

Centella asiatica (L.) Urban, also known as Gotu Kola, is a plant with herbaceous habitus belonging to the Apiaceae family. In Indonesia, it is known by various regional names, including *pegagan*, *kaki kuda*, and *antan gede*. *C. asiatica* grows wild throughout the country and other tropical regions in general (India, Sri Lanka, China, Malaysia, Thailand, etc.) at varying altitudes, from lowlands to 2500 m above sea level [1]. *C. asiatica* is a perennial herb that is stemless but has short rhizomes and creeping stolons, 10–80 cm long, and has 2–10 single leaves arranged in a rosette.

Traditionally, *C. asiatica* is used to treat problems related to skin, nerves, and metabolic processes [2]. Previous research at the clinical trials level has also confirmed the effectiveness of *C. asiatica* and its compounds as anti-inflammatory, antioxidant, anti-apoptotic, and in improving mitochondrial function [3].

C. asiatica contains mainly pentacyclic triterpene compounds, such as asiatic acid, asiaticoside, madecassic acid, and madecassoside. It also contains flavonoids (quercetin and kaempferol), phytosterols (campesterol, sitosterol, and

stigmasterol), gulonic acid, ferulic acid, and chlorogenic acid. However, the pharmacological activity of the herb and its derivative products is particularly ascribed to triterpenes, especially asiaticoside. Various standardized *C. asiatica* extracts traded in the market are also standardized with the concentrations of this marker compound [2]. Monograph of *C. asiatica* in various herbal pharmacopoeias (Ayurvedic Pharmacopoeia of India, European Pharmacopoeia 9.0, Hong Kong Chinese Materia Medica Vol. 7, Indian Pharmacopoeia 2018, Malaysian Herbal Monograph 2009 Vol. 2, Pharmeuropa, Thai Herbal Pharmacopoeia 2018, USP41-NF36 S1, Vietnamese Pharmacopoeia 2017, and Indonesian Herbal Pharmacopoeia 2017) also use asiaticoside as a marker for analytical purposes.

Having a high-quality and uniform supply of raw materials is a problem faced by herbal medicine industries worldwide, including in Indonesia. It is in part because medicinal plants are grown or cultivated in various geographies across which temperature, humidity, rainfall, sunlight intensity, soil types, and other ecological factors may differ. These factors result in diverse biomass and concentration of chemical compounds in a plant. For instance, variations in asiaticoside contents of *C. asiatica* samples collected from locations with different geographical conditions have been previously reported [4–6]. Also, there have been accounts of variations in biomass due to different cultivation treatments [7]. However, variations in asiaticoside contents of *C. asiatica* samples from various locations in Indonesia remain under researched [8].

Several methods for detecting and measuring asiaticoside contents are TLC-densitometry [8–10], HPTLC-densitometry [4,11,12], and HPLC [13–15]. TLC is a separation method that has been used to analyze medicinal plants qualitatively and quantitatively for many years. Although other more sophisticated techniques have been developed, TLC remains the method of choice for herbal analysis and is adopted by many pharmacopoeias because of its many advantages: multiple samples, including comparison samples, that can be analyzed simultaneously, selection of stationary and mobile phases that can be performed flexibly, simple equipment, ease of use, high reproducibility, and relatively low cost of analysis [16].

Considering the high industrial demand for raw *C. asiatica*, this research was conducted to develop and validate the TLC-densitometric method for identifying and measuring asiaticoside concentration of *C. asiatica* (i) and to determine the asiaticoside contents of *C. asiatica* samples from various locations in Indonesia using the validated TLC-densitometry (ii). It is expected that with a simple analytical method, as derived in this study, the standardization or quality control of *C. asiatica* as raw materials and their derivative products can be conducted easily.

MATERIAL AND METHODS

Chemicals and Reagents

The asiaticoside standard was obtained from Sigma-Aldrich (USA). Precoated TLC Si Gel 60 F₂₅₄ and pro-analytical grade solvent were from Merck (Darmstadt, Germany).

Collection of Samples

Centella asiatica herbs were collected in July-September 2020 from four islands in Indonesia: Jawa (Tawangmangu, Batu, Probolinggo, and Magelang), Bali (Bangli), Lombok (Mataram, Lombok Barat, and Lombok Timur), and Kalimantan (Hulu Sungai Selatan). Details of the sampling locations are provided in Table 1. All the samples have been authenticated by the Center for Information and Development of Traditional Medicines (PIPOT), University of Surabaya.

TABLE 1. Locations of the *Centella asiatica* samples

Codes	Cities	Coordinates (Latitude, Longitude)	Elevation (masl)
a	Tawangmangu	7°39'49" S, 111°08'06" E	1,123
b	Batu	7°52'02" S, 112°31'11" E	895
c	Probolinggo	7°57'38" S, 113°23'00" E	550
d	Magelang	7°29'44" S, 110°08'10" E	714
e	Mataram	8°33'58" S, 116°07'27" E	33
f	Lombok Barat	8°34'17" S, 116°13'31" E	193
g	Lombok Timur	8°22'16" S, 116°32'09" E	1,166
h	Bangli	8°21'53" S, 115°20'44" E	850
i	Hulu Sungai Selatan	2°47'05" S, 115°15'37" E	23

Extraction

One gram of *C. asiatica* powder was first extracted with 7 ml of methanol using the ultrasonication method for 15 minutes at room temperature. Then, the extraction results were filtered and put into a 10 ml volumetric flask. The residue was rinsed with 3 ml of methanol and put into the same volumetric flask while filtered, then methanol was added until 10 ml.

Preparation of the Standard Solutions

To prepare the standard solutions, approximately 1.00 mg of asiaticoside standard was weighed, then put into a 1.5 ml microtube and added with 1000 μ l of methanol using a micropipette.

TLC System

Samples of the *C. asiatica* extract and asiaticoside standard were spotted on the silica gel 60 F₂₅₄ TLC plate in the form of 6 mm bands using a CAMAG 100-microliter sample syringe (Hamilton, Switzerland). The spotting was conducted automatically using a Linomat 5 sample applicator (CAMAG, Switzerland) with the help of N₂ gas. Then, elution was performed in a twin-through chamber (CAMAG, Switzerland) saturated with the mobile phase (ethyl acetate-methanol-water = 10:2.5:1 v/v/v) for 30 minutes. It was set in an ascending mode, with an elution distance of 80 mm from the spotting site. Afterward, the plate was dried in a fume hood, sprayed with anisaldehyde sulfuric acid reagent, and heated in an oven at 110°C for 10 minutes. Then, the TLC plate was documented in UV 254 nm, UV 365 nm, visible light and scanned using the TLC Scanner 4 (CAMAG, Switzerland) equipped with winCATS software, a scanning slit of 4 mm \times 0.3 mm, and a data resolution of 1 nm/step at a scanning speed of 100 nm/s.

Method Validation

Specificity Determination

First, 4 μ l of the standard asiaticoside solution and 6 μ l of the *C. asiatica* extract sample were spotted on the TLC plate and then eluted and documented according to the designed TLC system. Then, to determine the specificity of the method, the bands of the asiaticoside standard and sample were compared to observe their R_f values, the shape of the visible spectrum, and maximum wavelength value using a densitometer. In addition, the densitometer was also used to determine if the asiaticoside band contained only one compound or more by measuring the visible spectrum at the beginning, apex, and end of the peak [17].

Determination of Linearity, Limit of Detection (LOD), and Limit of Quantification (LOQ)

Linearity was determined by spotting the standard asiaticoside solution at various volumes (1, 2, 4, 6, 8, 10 μ l). For each volume, spotting was replicated three times on different plates. After elution and derivatization, the asiaticoside band was scanned with a TLC-scanner at the maximum wavelength obtained in the previous step, specificity determination. Afterward, the derived asiaticoside area (as *y*) was plotted against the mass of the spotted

asiaticoside (as x) to create a standard curve. The curve's linearity was determined from the correlation coefficient (r -value) of the formed linear regression equation ($y = bx + a$). The limit of quantification (LOQ) and detection (LOD) was each calculated from the standard deviation (SD) of the mean intercept and slope (\bar{x}) in the linear regression equation of the standard curve. The equations used were $LOD = 3.3 (SD/\bar{x})$ and $LOQ = 10 (SD/\bar{x})$ [12].

Precision Determination

Intraday precision was determined by spotting 4 μ l of the standard asiaticoside solution onto a plate six times, creating six replicates. The eluted TLC plate was analyzed using a densitometer, then the mean, standard deviation, and relative standard deviation (%RSD) of the asiaticoside area were calculated. To determine the interday precision, 4 μ l of the standard asiaticoside solution was spotted onto three different plates on three days. Afterward, the eluted plate was analyzed using a densitometer, then the mean, standard deviation, and relative standard deviation (%RSD) of the detected asiaticoside area were calculated [17].

Determination of the Asiaticoside Contents of *C. asiatica* Samples

To calculate the asiaticoside concentration in the *C. asiatica* herbs from nine (9) different locations, 8 μ l of each extract sample was spotted onto a TLC plate and analyzed by the validated method. The analysis was performed in three replications. For each sample, the asiaticoside area detected by the densitometer was substituted into the standard curve's regression equation, and the asiaticoside content was measured in mg/g dry weight.

Data Analysis

Statistical analyses were conducted to distinguish the asiaticoside concentration of the *C. asiatica* herbs obtained from various locations. One-way ANOVA ($\alpha = 0.05$) and a post hoc Tukey test were processed in GraphPad Prism Version 5.01.

RESULTS AND DISCUSSION

Physical Characteristics of the Sample Powder

The *Centella asiatica* herbs investigated in this study were collected from four major islands in Indonesia to represent areas with varying geographical conditions at different elevations, from 33 to 1,166 masl. The crude drugs produced from the samples shared similar organoleptic properties: greyish-green, dry, and brittle leaves (easily crumbled when crushed). Pictures of the nine powdered crude drugs are shown in Fig. 1.

Specificity

Specificity was determined by comparing similarities in color and R_f value between the bands of the asiaticoside standard and sample. In addition, their visible spectra were also overlaid to see any shared similarities. In the specificity analysis, the maximum wavelength of the asiaticoside standard was scanned within the range of 200–700 nm.

The chromatograms of the asiaticoside standard and sample shows that the band of the suspected asiaticoside in the sample had the same color as that of the asiaticoside standard, i.e., greenish blue in visible light (Fig. 2). The position of the two compound bands was also parallel.

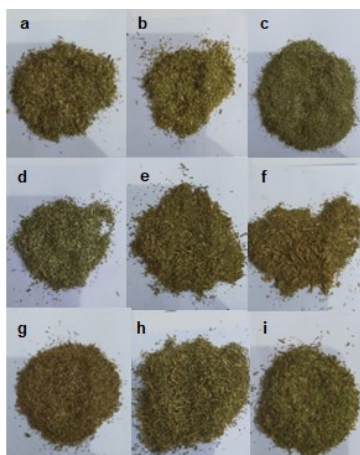


FIGURE 1. Organoleptic characteristics of the *Centella asiatica* powder from Tawangmangu (a), Batu (b), Probolinggo (c), Magelang (d), Mataram (e), Lombok Barat (f), Lombok Timur (g), Bangli (h), and Hulu Sungai Selatan (i)

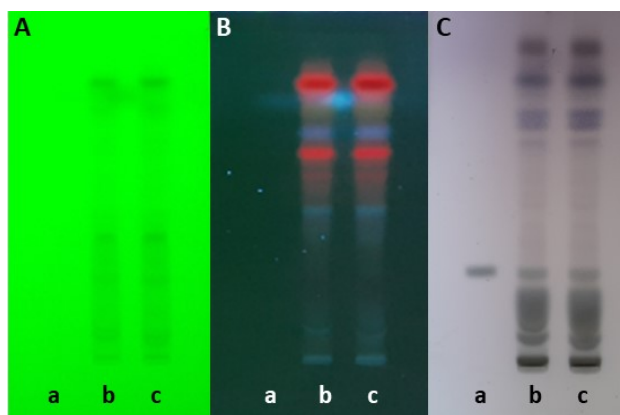


FIGURE 2. TLC chromatograms of the asiaticoside standard (a) and the *Centella asiatica* extract (b, c) with anisaldehyde sulfuric acid as the derivatization reagent, detected at 254 nm (A), 365 nm (B), and in visible light (C)

Results of specificity analysis shows the overlaid UV-vis spectra of the asiaticoside standard and the sample (Fig. 3). It indicates that the spectrum profile of the sample coincided with that of the standard, with a maximum wavelength of 594 nm. This result corresponds to a previous study that used maximum wavelengths of 595 nm [12] and 600 nm [4] for asiaticoside analysis.

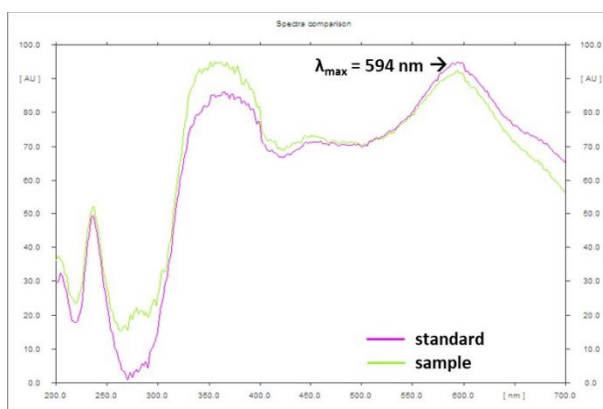


FIGURE 3. Overlaid UV-Vis spectra of the asiaticoside standard and the asiaticoside sample

Densitograms of the asiaticoside standard and sample shows that both of them had the same Rf value, 0.34 (Fig. 4). The peak purity of the sample (Table 2) is indicated by the values of r (s, m) and r (m, e), i.e., 0.998946 and 0.995208; both were > 0.99 [12]. From these data, it can be inferred that the TLC method designed for measuring the asiaticoside contents of *C. asiatica* has good specificity.

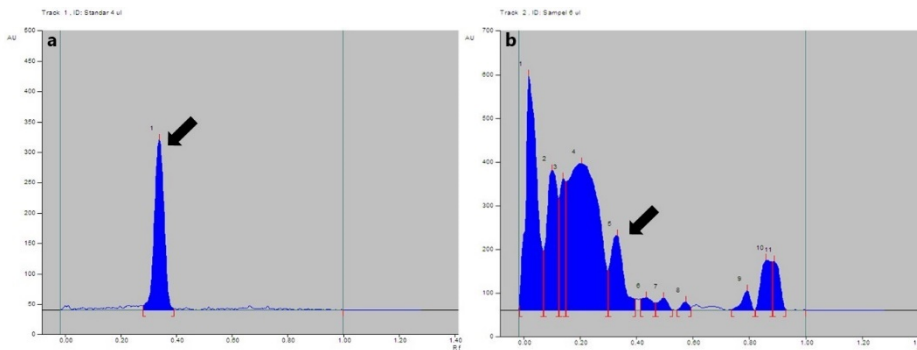


FIGURE 4. Densitograms of the asiaticoside standard (a) and *Centella asiatica* extract (b)

TABLE 2. Rf and peak purity parameter values of the asiaticoside standard and sample

Tracks	Identities	Assigned substances	Rf	r (s, m)	r (m, e)
1	Standard	Asiaticoside	0.34	0.999556	0.999006
2	Samples	Asiaticoside	0.34	0.998946	0.995208

Linearity, Limit of Detection (LOD), and Limit of Quantification (LOQ)

Linearity was determined by calculating the correlation coefficient (r) of the standard curve's linear regression equation. The standard curve was obtained from spotting different volumes of the standard asiaticoside solution (1.05 $\mu\text{g}/\mu\text{l}$), i.e., 1, 2, 4, 6, 8, and 10 μl or equivalent to 1.05, 2.1, 4.2, 6.3, 8.4, and 10.5 $\mu\text{g}/\text{band}$. The TLC chromatogram and two-dimensional densitogram of the standard asiaticoside solution are shown in Fig. 5, while the standard curve formed is in Fig. 6.

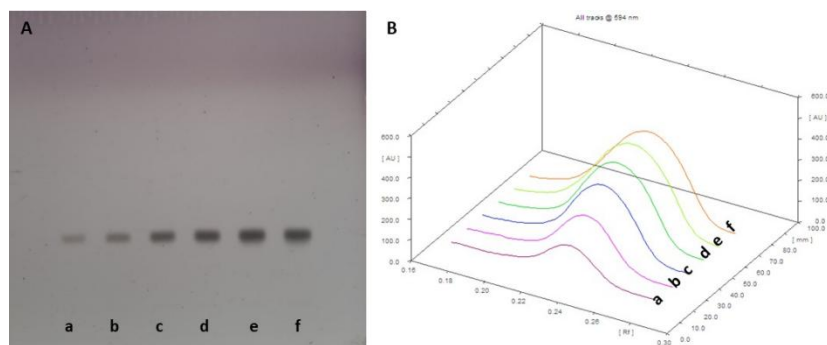


FIGURE 5. TLC chromatogram (A) and 2D densitogram (B) of the standard asiaticoside solution

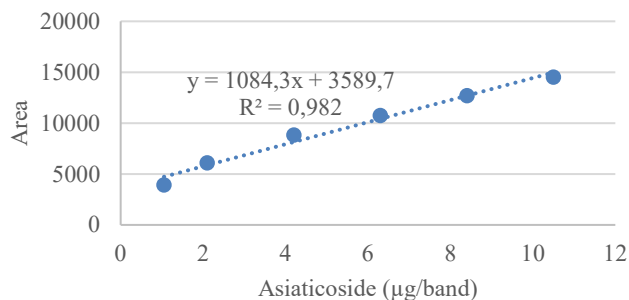


FIGURE 6. The curve of the linear regression equation between asiaticoside concentration and area

The final standard curve's equation, formulated from three replications, was $y = 1084.3x + 3589.7$. The LOD and LOQ calculated using the equation were $0.48820 \mu\text{g/band}$ and $1.47939 \mu\text{g/band}$, respectively. The r -value obtained was 0.989437 , indicating a linear relationship between concentrations in the range of $1.05\text{--}10.5 \mu\text{g/band}$ and each of the asiaticoside areas [4,12]. Previous research using a combination of TLC and densitometry also found LOD and LOQ nearly identical to the findings in this study, i.e., $0.72 \mu\text{g}$ and $1.2 \mu\text{g}$ [9]. However, in another study using HPTLC-densitometer, the derived LOD and LOQ of asiaticoside are lower [4,12,18]. LOD and LOQ are defined as the smallest amount of analyte that can be detected or quantified with reasonable statistical certainty, depending on the analytical method used. HPTLC is distinguished from TLC in that the former uses a stationary phase with a smaller particle size than the latter, thus providing better analytical performance, e.g., a smaller volume of the mobile phase, better precision, shorter analysis time, and increased sensitivity [19,20].

Precision

To determine intra- and interday precisions, the spotting of the asiaticoside standard onto the TLC plate was replicated six times and repeated on three different days. The asiaticoside areas read after the TLC-densitometer analysis are shown in Table 3.

TABLE 3. Intraday precision and interday precision test results

Replication	Areas		
	Day 1	Day 2	Day 3
1	13300.32	14416.70	13458.23
2	13683.41	13861.42	13748.83
3	13962.02	13588.83	13539.71
4	13327.32	13305.22	13683.28
5	12444.93	13085.16	13725.45
6	11433.20	12655.40	14505.11
Mean±SD	13025.20±932.63	13485.46±616.60	13776.77±374.25
RSD (%)	7.16	4.57	2.72
Intraday precision (%RSD, n = 6) = 2.72–7.16			
Interday precision (%RSD, n = 3) = 2.82			

Based on Table 3, it can be concluded that the TLC-densitometry method designed for detecting and measuring the asiaticoside contents of *C. asiatica* has good intra- and interday precision, with %RSD of 2.72–7.16 and 2.82, respectively [4,12].

Determination of Asiaticoside Concentration in *Centella asiatica* Samples

As seen in Table 4, the samples obtained from Lombok Timur and Bangli had minute asiaticoside contents (below the LOQ) that were not quantifiable. On the contrary, the other seven samples had varying concentration of asiaticoside, from 0.5393 to 3.4687 mg/g. Figure 8 shows that samples from Magelang, Lombok Barat, and Hulu Sungai Selatan had the highest asiaticoside content, while those from Batu, Probolinggo, and Mataram had the second-highest. The asiaticoside of the Tawangmangu sample was significantly lower than the *C. asiatica* collected from other regions ($p < 0.05$), making it the smallest concentration detected in the study. However, although the samples from Tawangmangu, Lombok Timur, and Bangli had low asiaticoside, they contained other metabolites in a quite substantial or even higher amount than the other samples. These results are evident in the TLC profile shown in Fig. 7.

TABLE 4. Asiaticoside concentrations of the *Centella asiatica* samples obtained from nine locations

Sample codes	Sample origins	Asiaticoside concentration (mg/g)
a	Tawangmangu	0.5393±0.1172
b	Batu	1.0295±0.6675
c	Probolinggo	1.2104±0.3513
d	Magelang	3.2774±1.0630
e	Mataram	1.5700±0.6121
f	Lombok Barat	3.4687±0.9992
g	Lombok Timur	*
h	Bangli	*
i	Hulu Sungai Selatan	2.3390±1.2291

*: asiaticoside concentrations are not quantifiable (<LOQ); mean±SD (n = 3)

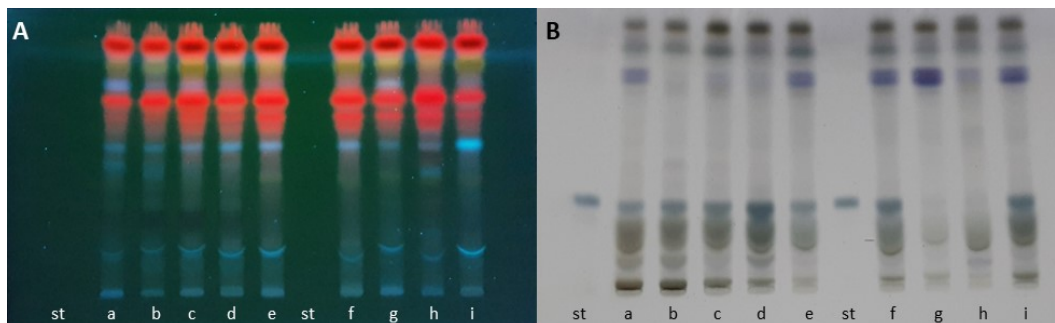


FIGURE 7. TLC chromatograms of the asiaticoside standard (st) and *Centella asiatica* samples from nine different locations (a-i), with anisaldehyde sulfuric acid as the derivatization reagent, detected in UV light at 365 nm (A) and visible light (B)

As seen in Table 4, the samples obtained from Lombok Timur and Bangli had minute asiaticoside contents (below the LOQ) that were not quantifiable. On the contrary, the other seven samples had varying concentration of asiaticoside, from 0.5393 to 3.4687 mg/g. Figure 8 shows that samples from Magelang, Lombok Barat, and Hulu Sungai Selatan had the highest asiaticoside content, while those from Batu, Probolinggo, and Mataram had the second-highest. The asiaticoside of the Tawangmangu sample was significantly lower than the *C. asiatica* collected from other regions ($p < 0.05$), making it the smallest concentration detected in the study. However, although the samples from Tawangmangu, Lombok Timur, and Bangli had low asiaticoside, they contained other metabolites in a quite substantial or even higher amount than the other samples. These results are evident in the TLC profile shown in Fig. 7.

The study results proved that the asiaticoside concentration of the sampled *C. asiatica* are influenced by the plant's geographical location. However, whether the difference is due to the location's elevation or other factors remains unknown and, thus, requires further investigation. Therefore, before cultivating *C. asiatica*, it is necessary to consider the local geographical conditions so as to achieve harvests with a standard and consistent asiaticoside content.

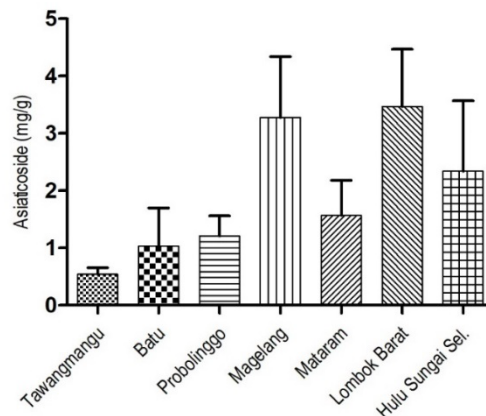


FIGURE 8. Asiaticoside concentrations of seven samples of *Centella asiatica*

CONCLUSION

The TLC-densitometry method designed is relatively simple but meets all the validation parameters. As a result, it can be used for analyzing asiaticoside in *Centella asiatica* both qualitatively and quantitatively. Furthermore, this study has found that the extracts of the *C. asiatica* collected from various locations and geographic characteristics in Indonesia contain different concentration of asiaticoside. In the future, the research can be developed to obtain data from unexplored regions in the country as well as other factors affected the different content of asiaticoside.

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