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Dot-blot assay with $AlCl_3$ reagent for rapid screening of total flavonoid content in food and herbal medicine materials

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ABSTRACT

The determination of total flavonoid content (TFC) through the aluminum chloride ($AlCl_3$) colorimetric test utilizing spectrophotometry is commonly used as a parameter for evaluating the quality of processes as well as food and herbal medicines materials. Nonetheless, it is laborious, lengthy, and necessitates substantial amounts of chemicals. This study aims to develop and validate the dot-blot assay with $AlCl_3$ reagent for determining TFC in plant materials. In this method, the samples were not subjected to chromatography; instead, the plates were derivatized using $AlCl_3$ in ethanol immediately after the application of samples and evaporation the solvent. The Al(III)-flavonoid complex was scanned (421 nm) using a TLC-scanner. The validation of the method was conducted on stability, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness. The TFC of samples determined by dot-blot assay and spectrophotometry was subsequently analyzed using linear regression and Pearson correlation. The dot-blot assay for determining TFC met the stability parameter (RSD = 1.31–8.25%), intraday precision (RSD = 2.20–6.11%), inter day precision (RSD = 1.56%), linearity (r^2 = 0.9928), LOD (251.03 ng/spot), LOQ (760.71 ng/spot), accuracy (recovery = 70.81–75.61%), and robustness (RSD = 2.55 and 7.60%). There was a very strong positive linear relationship between the dot-blot assay method and spectrophotometry, with a linear regression coefficient of 0.9078 and a Pearson correlation coefficient of 0.911. The proposed method proved to be simpler, faster, and more economical for determining TFC. Thus, the method was beneficial in providing references for enhancing the quality control of food and herbal medicines.

KEYWORDS

aluminum chloride colorimetric assay, dot-blot assay, functional foods, medicinal plants, total flavonoids content

INTRODUCTION

Medicinal plants are extensively utilized as active components in functional foods and herbal medicines. They consist of metabolites with various roles and functions, one of which is flavonoids. Flavonoids are part of the extensive family of phenolic chemicals, or polyphenols, containing approximately 6,000 distinct structures. They have a main 15-carbon flavone skeleton (C6–C3–C6), including two benzene rings (A and B) interconnected with a three-carbon pyran ring (C). Flavonoids are classified into two primary types based on the saturation of the core heterocyclic ring, e.g., anthocyanidins, flavones, flavonols, and isoflavones exhibit unsaturated C2=C3, while flavanones, dihydroflavonols, and flavan-3-ols include into those of saturated ones [1–3]. Flavonoids can be found in several crops and medicinal plants. Fruits, leaves, flowers, and seeds provide the primary sources of natural flavonoids. These secondary metabolites contribute to color, fragrance, and flavor characteristics. In humans, flavonoids highly contribute to numerous health benefits because of their bioactive properties, including antioxidant, antibacterial, anticancer, antiparasitic, antidiabetic, antifungal, antiviral,

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anti-aging, anti-inflammatory, cardio-protective, neuro-protective, and immunomodulatory effects [1, 4–6].

One plant usually contains more than one type of bioactive flavonoid, as seen in celery and Indonesian bay leaf. Celery contains apigenin, apiin, luteolin, luteolin-7-O-β-D-glucopyranoside, kaempferol, and chrysoeriol 7-glucosides [7–9]. Bay leaf contains quercetin, rutin, 5,3′,4′-trihydroxyflavone-3-C-glycoside, 5,4′,5′-trihydroxyflavone-3-C-glycoside, 5,6,3′,4′-tetrahydroxyflavone, 5,6,4′,5′-tetrahydroxyflavone, and 5,3′,4′-trihydroxyflavone or 5,4′,5′-trihydroxyflavone [10, 11]. Although the individual flavonoids levels can be determined, the total flavonoid content (TFC) is more often used as a parameter to evaluate process quality (e.g., selection of plant material sources, drying methods, extraction methods, formulation methods) as well as the quality of food and herbal medicines materials [12–20].

The determination of TFC is preferred because it offers several advantages, including not requiring the separation and purification of flavonoids from the sample, the ability to use various standard compounds (such as quercetin, rutin, and catechin), and the use of simple reagents. The aluminum chloride colorimetric test is often used to validate the TFC in plants, employing Al(III) as an intermediary. This test was initially introduced in 1960 by Christ and Muller for determining flavonols derivatives in pharmaceuticals [21, 22].

Nevertheless, this conventional approach is laborious, lengthy, and necessitates substantial amounts of reagents [23]. The development of a reliable large-scale assay could serve as a crucial tool to address these drawbacks. Hence, the advancement of this method is imperative. In this study, a so-called dot-blot assay with $AlCl_3$ reagent was developed for rapid screening of TFC in food and herbal medicine materials. In the dot-blot assay, the samples and standards are not subjected to chromatography; instead, the plates are directly derivatized with a reagent after the sample or standard solution is spotted and the solvent is evaporated [24, 25]. The rationale for this method is that the samples and standards are spotted with several microliters on a TLC plate and then derivatized with $AlCl_3$ solution to form an Al(III)-flavonoid complex which area is then measured with a densitometer at the maximum wavelength of the complex. From a literature survey, it was found that the dot-blot assay with $AlCl_3$ reagent has never before been applied to determine the TFC in plant species. As regards this background, this study aims to create and examine the dot-blot assay with $AlCl_3$ reagent in contrast to conventional ones, and to determine whether there is a linear correlation between the developed method and the conventional counterparts. It is hoped that the results of this research can provide an alternative method for determining TFC that is simpler, faster, and more economical.

EXPERIMENTAL

Chemicals and plant materials

The model plants used in this research include the herb of *Phyllanthus niruri* (“meniran”), leaves of *Apium graveolens* (celery), leaves of *Sonchus arvensis* (“tempuyung”), leaves of *Plantago major* (great plantain), leaves of *Blumea balsamifera* (“sembung”), leaves of *Coleus scutellarioides* (coleus), stems of *Tinospora crispa* (“brotowali”), bark of *Parameria laevigata* (“kayu rapat”), leaves of *Graptophyllum pictum* (“daun wungu”), and leaves of *Syzygium polyanthum* (Indonesian bay leaf). These plant materials listed in the Indonesian Herbal Pharmacopoeia monograph and they were chosen for their documented traditional uses as well as their standardization based on total flavonoid content, making them suitable representatives for this research. The plant samples were purchased in dried form from “UPF Pelayanan Kesehatan Tradisional Tawangmangu”, Indonesia. The crude drugs from these model plants are shown in Fig. 1. Plant samples were collected in December 2023, and voucher specimens were stored at “UPF Pelayanan Kesehatan Tradisional Tawangmangu”. Before use, the crude drugs were ground using a blender (Philips HR 2222, Amsterdam, the Netherlands) and sieved with a mesh size of 30. The powdered crude drugs were then stored in a tightly closed container and kept away from light. Standard quercetin was obtained from Sigma Aldrich Co. (St. Louis, MO, USA), pre-coated silica gel plates 60 F_{254} 20 × 20 cm, aluminum chloride ($AlCl_3$), sodium acetate, and ethanol were gained from Merck KGaA (Darmstadt, Germany).

Extraction protocol

The extraction procedures were selected based on the Indonesian Herbal Pharmacopoeia, 2nd Edition, taking into account the specific characteristics of each plant part (leaves, stems, or bark) and the expected content of total flavonoids [26]. These tailored methods were employed to ensure optimal recovery of bioactive compounds from each sample. *P. niruri*, *S. arvensis*, *P. major*, and *S. polyanthum* were extracted using the same method, employing Stirring-Assisted Extraction (SAE). A gram of crude drugs powder was combined with 25 mL of ethanol and stirred with a magnetic stirrer (Cimarec from Thermo Fisher Scientific, Waltham, MA, USA) at 300 rpm for 1 h. The extract was then strained into a volumetric flask until it reached exactly 25.0 mL. The other six samples were extracted using Ultrasound-Assisted Extraction (UAE) with an ultrasonic bath (Branson 1510, Branson, Brookfield, Connecticut, USA) operating at an ultrasonic power (P) of 150 W at 42 kHz at a temperature of 50 °C for varying durations. A gram of *A. graveolens* powder was combined with 25 mL of 70% ethanol and sonicated for 60 min. The extract was then filtered into a volumetric flask until a liquid extract volume of 25.0 mL was obtained. The extracts of *B. balsamifera* and *C. scutellarioides* were prepared in the same manner, with each using 1 g of crude drugs powder and 25 mL of ethanol, followed by sonication for 60 min. The mixture was then filtered into a volumetric flask until a liquid extract volume of 25.0 mL was obtained. The extract of *T. crispa* was prepared as follows: 10 g of crude drugs powder was combined with 50 mL of 70% ethanol and sonicated for 15 min. The mixture

Fig. 1. Physical characteristics of the model plant samples: *Phyllanthus niruri* (a), *Apium graveolens* (b), *Sonchus arvensis* (c), *Plantago major* (d), *Blumea balsamifera* (e), *Coleus scutellarioides* (f), *Tinospora crispa* (g), *Parameria laevigata* (h), *Graptophyllum pictum* (i), and *Syzygium polyanthum* (j)

was then filtered into a volumetric flask until a liquid extract volume of 50.0 mL was obtained. For the preparation of the *P. laevigata* extract, 0.5 g of sample was weighed. After the addition of 10 mL of ethanol, sonication was carried out for 60 min, and the extract was subsequently filtered to an end result of 10.0 mL. To prepare the *G. pictum* extract, 2.5 g of plant sample was added with 25 mL of 70% ethanol and sonicated over an hour, followed by filtration into a volumetric flask until a volume of 25.0 mL was obtained.

Dot-blot assay

Standard solution of quercetin and model plants extracts were each applied spot-wise (5 mm diameter) onto TLC plates using a 5 µL glass capillary tube (CAMAG, Muttenz, Switzerland). Spotting was performed using a Nanomat4 TLC applicator (CAMAG). The "dot-blot" assay was performed as follows: The samples were initially spotted onto the chromatographic plates, maintaining a 15 mm distance between each spot and 15 mm from the left, right, and bottom edges of the plate. The plates were then placed in a hood and dried for 5 min to allow the solvent to evaporate. Afterward, the TLC plates were sprayed with a 1% $AlCl_3$ solution in ethanol. After solvent evaporation, the spots of Al(III)-flavonoid complex were then observed under visible light and UV light (254 and 366 nm) using a UV Cabinet 4 (CAMAG). Subsequently, the plate of TLC was inspected using a TLC Scanner 4 (CAMAG). The generated densitogram was further examined using winCATS software (CAMAG).

Validation of the dot-blot assay method

The validation of the method was performed to assess the suitability of the dot-blot assay method for determining TFC in herbal samples. It was verified for stability, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness in accordance with ICH guidelines [27].

Standard solution preparation. The preparation of the quercetin stock solution began by weighing 25 mg of quercetin and dissolving it in ethanol to a final volume of 25.0 mL. Serial dilutions of the stock solution (1,000 µg mL^{-1}) were then made to achieve concentrations of 25, 50, 100, 150, 200, 250, 300, 350, and 400 µg mL^{-1}.

Determination of the maximum wavelength of Al(III)-flavonoid complex. Determination of the maximum wavelength of the Al(III)-flavonoid complex was carried out to identify the optimal wavelength for detecting the complex, ensuring accurate and reliable measurements. This was performed using a single concentration of the working solution and the celery extract. Five microliters of the standard and sample were spotted onto the TLC plate, then derivatized with 1% $AlCl_3$ in ethanol. The plate was further dried in a fume hood, and the λ_{max} of Al(III)-flavonoid complex was determined by scanning in the range of 200–600 nm using a densitometer.

Determination of method stability. Method stability was determined using all concentrations of the quercetin working solution. Five microliters of the solutions were applied to the TLC plate and then derivatized. The area of Al(III)-quercetin complex was read with a densitometer at the λ_{max} determined in the previous stage. Measurements were taken at 10, 20, and 30 min after derivatization. The area's relative standard deviation (%RSD) at different reading times indicated the stability of the method.

Determination of linearity, limit of detection (LOD), and limit of quantification (LOQ). This study carried out the linearity testing to verify the linear correlation between quercetin content and the area of the formed complex compound. This testing was performed by spotting the quercetin working solution onto the TLC plate, which was then derivatized. The area of the formed Al(III)-quercetin complex was measured using a densitometer at the λ_{max}. Linear regression generated a standard curve by correlating concentration (x) and area (y). Linearity was determined using the determination coefficient (r^2) of the regression equation formed (y = bx + a). The LOD and the LOQ were computed using the equations 3.3σ/S and 10σ/S, here σ is the standard deviation of the response, and S is the slope of the calibration curve. In this research, σ was measured as the standard error (SE) of the calibration curve [27].

Determination of precision. Intraday precision evaluation was performed by spotting 5 µL of celery extract in 6 spots on the TLC plate, with 3 replicates on 3 different plates. The plates were then derivatized, and the formed Al(III)-flavonoid complex's area was read with a densitometer at the λ_{max}. For inter day precision determination, spotting was performed on three different TLC plates throughout a span of three days. The area's relative standard deviation (%RSD) on the same day and across the three distinct days indicated intraday and inter day precision, respectively [28].

Determination of accuracy. The standard addition method was used to determine the method's accuracy. Two concentration levels of quercetin standard solution (300 and 400 µg mL^{-1}) were added to celery extract (hereafter referred to as the spiked sample). A corresponding celery extract was also prepared and added with solvent (hereafter referred to as the un-spiked sample). Each solution was then spotted three times on a TLC plate with a spotting volume of 5 µL. Next, the Al(III)-flavonoid complex's area formed was measured with a densitometer at λ_{max}. The accuracy was determined by calculating the percentage recovery, which is the amount of quercetin obtained compared to the actual amount.

Determination of robustness. The robustness of the dot-blot assay method was assessed to evaluate the impact of minor intentional changes in testing conditions during the determination of TFC. The small changes made in this study were the concentration of the derivatizing agent, $AlCl_3$ (1% and 1.5%), and the analysis time (10, 20, and 30 min after derivatization).

TFC determination using dot-blot assay method

The total flavonoid content in 10 model plant samples was measured using the dot-blot assay method. The analysis was performed by spotting 5 µL of each sample extract onto a TLC plate, which was further derivatized. The Al(III)-flavonoid complex's area formed in each sample was analyzed using a densitometer at λ_{max}. The TFC (mg QE/g crude drugs) for each model plant was then determined through the equation of linear regression of the standard curve (y = bx + a), where the area read is represented by y and x represents the obtained TFC.

TFC determination by UV–Vis spectrophotometry

The TFC determination by UV–Vis spectrophotometry refers to the Indonesian Herbal Pharmacopoeia, 2nd Edition, using quercetin as a standard. A total of 0.5 mL of extract was combined with 1.5 mL of ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M CH_3COONa, and 2.8 mL of water. The mixture was further shaken and left to stand over 30 min at ambient temperature. The absorbance was subsequently measured at a wavelength of 425 nm. A series of quercetin standards (40, 60, 80, 100, 110, 120, 140, 160, 180, and 200 µg mL^{-1}) were made and evaluated identically to the extract samples to generate a standard curve (y = bx + a). The TFC (mg QE/g crude drugs) for each model plant was calculated using the quercetin standard curve.

Data analysis

To determine whether there is a correlation between the dot-blot assay method and the UV–Vis spectrophotometry method in determining TFC, a linear regression analysis was conducted. The correlation coefficient (r) from the equation of linear regression (y = bx + a) was used to assess whether there is a relationship between the two methods. Furthermore, to evaluate the strength of the relationship between these methods, Pearson correlation analysis (Product Moment) was performed, with the Pearson correlation coefficient indicating the strength of the association. The data were analyzed using SPSS software version 29.0.

RESULTS AND DISCUSSION

The total flavonoid content is a valuable parameter for assessing the quality of various raw plant materials, particularly in the context of further processing. One of the most commonly employed methods for its measurement involves the reaction of flavonoids with Al(III) ions, forming a complex as a result of their interaction with an $AlCl_3$ ethanolic solution. Despite its limitations, this method is extensively utilized and incorporated into numerous pharmacopoeias, making it an appropriate foundation for the present study. The dot-blot assay is a simple technique where non-developed plates with applied samples are recorded under UV–Vis light both before and after derivatization with a specific reagent. This method has been extensively applied in various studies to determine total antioxidant activity using DPPH or ABTS free radicals, antibacterial activity, and enzyme inhibition activity [24, 29–32]. Additionally, dot-blot assay has also been reported for determining total polyphenol content using the NP/PEG reagent, further showcasing its versatility in quantifying different bioactive compounds in a variety of samples [25]. However, no studies to date have explored the use of the dot-blot assay with $AlCl_3$ reagent to quantify total

flavonoid content in herbal materials. In this method, samples or standards are applied directly onto a TLC plate, which is then dried in a hood for 5 min to evaporate the solvent. The plate is subsequently stained with $AlCl_3$ reagent to detect flavonoid compounds. The remaining steps follow the principles of classical TLC-densitometry [33, 34], allowing for efficient and reliable analysis.

The UV–Vis spectrum of Al(III)-flavonoid complex

The initial step in the dot-blot assay with $AlCl_3$ reagent is to determine the standard compound. Quercetin is a pentahydroxyflavone characterized by five hydroxy groups located at the 3-, 3'-, 4'-, 5-, and 7-positions. It is among the most prevalent flavonoids found in consumable vegetables, fruits, and medicinal plants [35]. Due to the existence of an o-dihydroxy group on the B ring and hydroxy-keto groups on the A and C rings, this compound can react with $AlCl_3$ to form a yellow-colored complex. As a result, quercetin is often used as a reference standard in the determination of TFC by UV–Vis spectrophotometry [21, 22]. Therefore, in this dot-blot assay method, quercetin is also used as a reference standard. The Al(III)-quercetin complex formation in the dot-blot assay method developed in this study is indicated by the compound's UV–Vis spectrum, which shows a maximum absorbance at 421 nm (Fig. 2). This wavelength experiences a bathochromic shift compared to the UV–Vis spectrum of quercetin in ethanol, which has a maximum absorbance at 375 nm [36].

These findings align with prior research using UV–Vis spectrophotometry, where quercetin exhibited a red shift from 367 to 428 nm [21]. Apigenin, luteolin, quercetin, and kaempferol are the main flavonoids found in celery leaves [7, 37]. The UV–Vis spectrum of apigenin in ethanol shows a maximum absorbance at 329 nm [38]. In this study, the reaction of celery extract with $AlCl_3$ produced a maximum absorbance at 413 nm (Fig. 2). This indicates the successful formation of Al(III)-flavonoid complex. Thus, the dot-blot assay method can be used for the determination of TFC, with the subsequent λ_{max} set at 421 nm. In previous studies using UV–Vis spectrophotometry, the absorbance of the Al(III)-flavonoid complex was measured over a fairly wide wavelength range of 404–430 nm [22] and 410–440 nm [21].

Stability

After reacting with $AlCl_3$, flavonoids typically form complexes that produce a color, the intensity of which depends on the flavonoid concentration and reaction time. Insufficient incubation time may not allow the complex to form completely, while excessive time can lead to degradation or instability of the complex, potentially compromising measurement accuracy. In spectrophotometric determination of TFC, analysis is generally conducted 2–60 min after $AlCl_3$ is added [22]. Similarly, in the dot-blot assay using DPPH reagent, the observation time after derivatization (up to 60 min) has been shown to influence the free radical scavenging activity of a compound [24]. Therefore, in the dot-blot assay

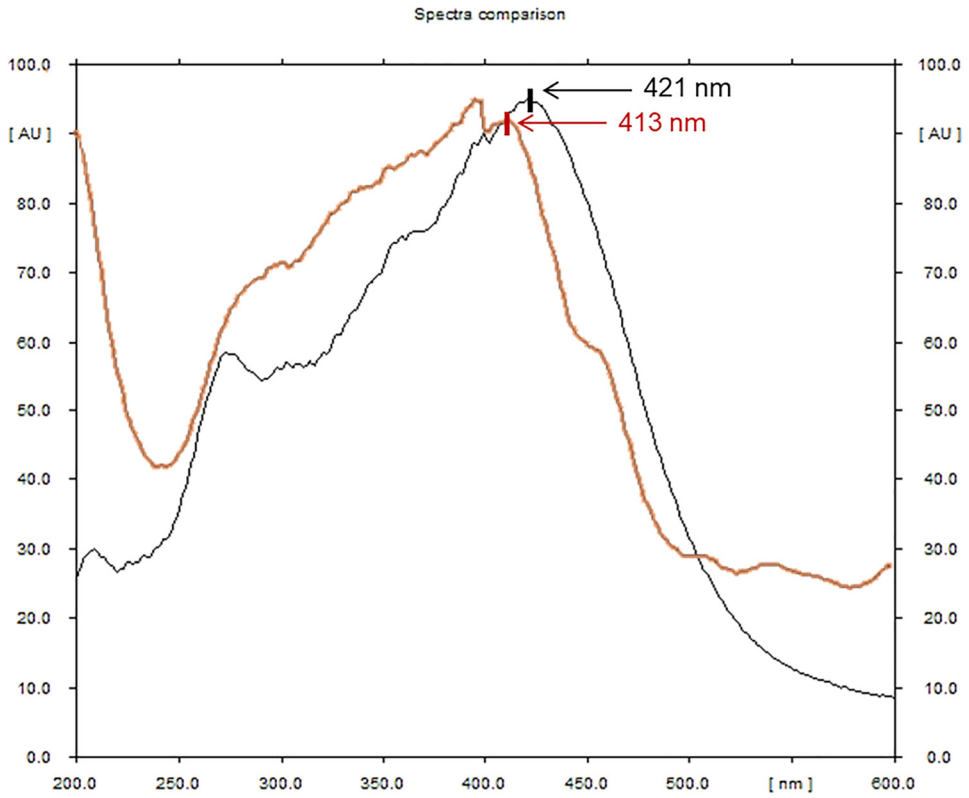

Fig. 2. UV–Vis spectra comparison of complex Al(III)-quercetin (λ_{max} = 421 nm) and Al(III)-celery flavonoid (λ_{max} = 413 nm)

with $AlCl_3$ reagent, TLC plates were documented at 10-min intervals over 30 min. This approach ensures the reaction reaches a stable state before measurement with a densitometer is performed.

Stability testing aims to determine how long the Al(III)-flavonoid complex on the TLC plate can be analyzed without showing significant changes. The peak area data and the stability graph of the reaction results at 10, 20, and 30 min after derivatization are presented in Table 1 and Fig. 3, respectively. From the table and figure, it is evident that the peak area does not experience significant changes from 10 to 30 min (RSD values ≤10%). Considering time efficiency, 20 min after derivatization was chosen as the analysis time for this dot-blot assay method.

Table 1. The peak area of the Al(III)-quercetin complex at various analysis times

Quercetin (ng/spot)	Peak area at minute			Mean ± SD	RSD (%)
	10	20	30		
125	1,603	1,424	1,372	1,466 ± 121	8.25
250	3,829	3,646	3,485	3,653 ± 172	4.71
750	9,516	8,205	8,640	8,787 ± 668	7.60
1,500	15,049	13,967	14,241	14,419 ± 562	3.90
1,750	17,581	17,404	17,006	17,330 ± 294	1.70
2,000	18,473	18,442	18,044	18,320 ± 239	1.31

Linearity, limit of detection, and limit of quantification

Linearity was determined using the determination coefficient (r^2) from the equation of linear regression of the standard curve. The curve was generated by applying (n = 3) quercetin standards at various concentrations, each consisting of 5 µL, equivalent to 125, 250, 750, 1,500, 1,750, and 2,000 ng/spot. The visual appearance of the Al(III)-quercetin complex is shown in Fig. 4, captured under visible and UV light at 366 nm to visually clarify the reaction results between flavonoids and $AlCl_3$. This reaction produces a yellow fluorescence (Fig. 4B), which is a characteristic feature of this interaction [28]. Meanwhile, the peaks of the complex compound, as measured by the densitometer, are displayed in Fig. 5. These figures illustrate that as the mass of quercetin spotted increases, the yellow fluorescence becomes more intense, and the peak area also increases.

There is a linear correlation between the mass of quercetin, ranging from 125 to 2,000 ng/spot and the area of the measured complex compound (y = 8.9642x + 990.34), with

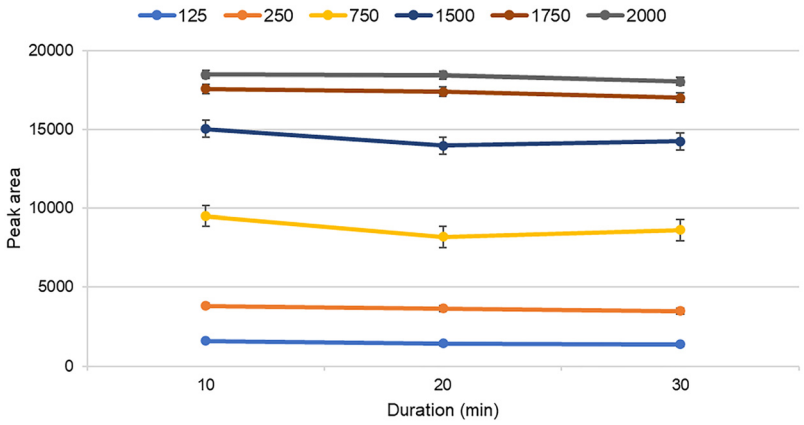

Fig. 3. Stability graph of the Al(III)-quercetin complex

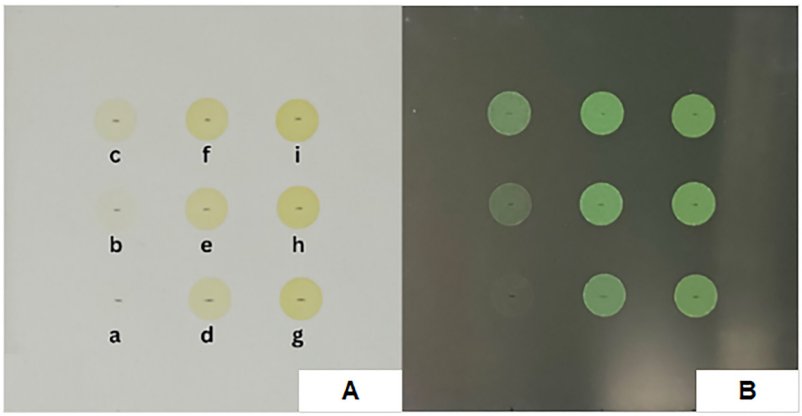

Fig. 4. Visual appearance of the Al(III)-quercetin complex under visible light (A) and UV light at 366 nm (B). The labels a-h correspond to quercetin concentrations of 125, 250, 500, 750, 1,000, 1,250, 1,500, 1,750, and 2,000 ng/spot, respectively

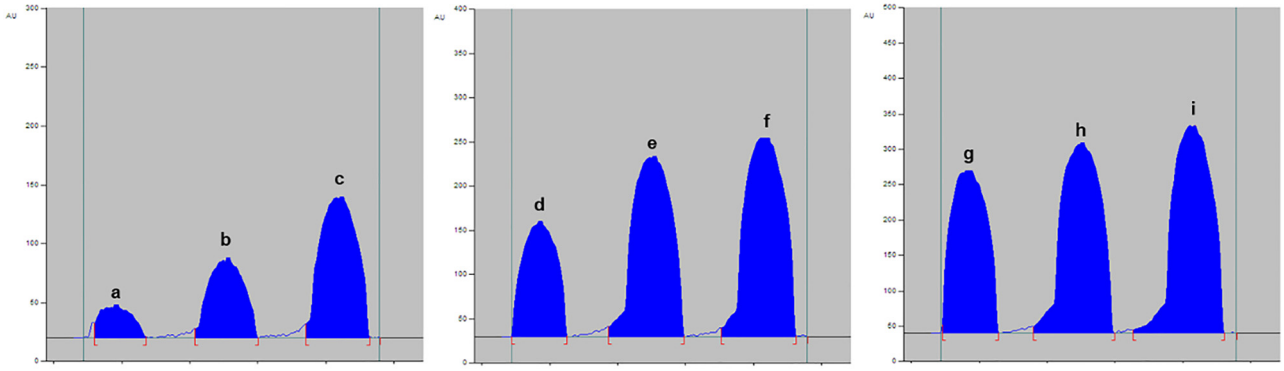

Fig. 5. Peak area of the Al(III)-quercetin complex at 421 nm. The labels a-i correspond to quercetin concentrations of 125, 250, 500, 750, 1,000, 1,250, 1,500, 1,750, and 2,000 ng/spot, respectively

an r^2 value of 0.9928. From this standard curve ($n = 3$), the limits of detection (LOD) and limits of quantification (LOQ) for the dot-blot assay with $AlCl_3$ reagent were calculated to be 251.03 and 760.71 ng/spot, respectively. From the literature review, no determination of TFC in herbal samples or any other samples using the dot-blot assay with $AlCl_3$ reagent has been found. Therefore, the LOD and LOQ obtained in this study cannot be compared with the results of other studies.

Precision

The results of the area readings for the Al(III)-flavonoid complex in celery leaves during the determination of intraday and inter day precision are shown in Table 2. The developed dot-blot assay method exhibits good intraday and inter day precision, evidenced by the low RSD values, which are 2.20–6.11% for intraday and 1.56% for inter day precision. The precision results for the developed dot-blot assay method, indicate its reliability for the determination of the Al(III)-flavonoid complex in herbal samples. The low RSD values observed for both intraday and inter day precision confirm the method's reproducibility and consistency. An RSD value below 10% is generally considered acceptable for analytical methods, demonstrating that the assay can produce stable and repeatable results over short and extended timeframes [25]. These findings suggest that the method is robust and suitable for screening as well as routine analysis of flavonoid content in herbal materials.

Table 2. The area of the Al(III)-flavonoid complex in the determination of precision

TLC plate number	Area (AU)		
	Day 1	Day 2	Day 3
1	8,822 ± 769	9,020 ± 788	9,221 ± 869
2	9,576 ± 847	8,991 ± 927	9,528 ± 709
3	9,957 ± 885	9,475 ± 839	9,141 ± 715
Mean ± SD	9,451 ± 577	9,162 ± 271	9,297 ± 204
RSD (%)	6.11	2.96	2.20
Intraday precision (%RSD, $n = 3$)	2.20–6.11		
Interday precision (%RSD, $n = 3$)	1.56		

Accuracy

The standard addition method was used to analyze the accuracy through two standard concentration levels (300 and 400 μg mL^{-1}). Both spiked samples and un-spiked samples were each spotted with 5 μL. Accuracy was determined and represented as % recovery, indicating the discrepancy between the standard values obtained after spotting on the TLC plate and the actual values of the prepared standards, presented in a percentage. The recoveries obtained from each standard concentration level were 70.81% and 75.61% (Table 3), indicating that the dot-blot assay method can be reproduced with fairly good accuracy. While the recoveries are not perfect, they indicate that the method can be reliably reproduced, offering a suitable level of precision for determining flavonoid content in samples.

Robustness

The experimental conditions for the Al(III)-flavonoid complexation reaction reported in the literature vary widely, including the concentration of $AlCl_3$ used (1–10%), the

Table 3. Results of the accuracy testing for the dot-blot assay method

Level	Sample	Area	Calculated TFC (ng/spot)	Theoritical TFC (ng/spot)	Recovery (%)
1	Un-spiked sample	5,437 ± 239			
	Spiked sample	11,188 ± 252	531.08 ± 35.12	750	70.81 ± 4.48
2	Un-spiked sample	5,881 ± 202			
	Spiked sample	13,649 ± 349	756.08 ± 45.15	1,000	75.61 ± 4.52

reaction medium (acidic or acetate solution, alcohol, water), the measurement time after the addition of $AlCl_3$ (2–60 min), the wavelength for reading (404–430 nm), and the standard compounds used (quercetin, rutin, galangin, and catechin) [21, 22]. This variability highlights the importance of optimizing these parameters to ensure consistent and accurate results in flavonoid determination. In this study, the robustness of the dot-blot assay method was tested by varying two key factors: the $AlCl_3$ concentration (1% and 1.5%) and the reading time of the plate with the densitometer (10, 20, and 30 min after derivatization). The results (Table 4) demonstrate that the developed method is reliable, as evidenced by the low RSD values (<10%) of 2.55% and 7.60%, confirming the method's precision and consistency under the tested conditions.

TFC in model plants using the dot-blot assay method

So as to quantify the TFC in model plants, 5 µL of each sample extract was spotted on a TLC plate and evaluated using the developed and verified method (Fig. 6). The TFC (Table 5) of the model plants was obtained by inserting the area values of each replication of sample as the y-value in the linear regression equation of the standard curve (y = 8.9642x + 990.34). The range of RSD values with this method is 0.68–4.76%, indicating that the TFC determination by means of the dot-blot assay method for all model samples has commendable precision. This shows that the method can consistently provide reliable results across different plant samples. The TFC of the extracts varied widely, with the lowest value of 0.37 mg QE/g crude drugs found in the stems of *T. crispa*, and the highest value of 9.95 mg QE/g crude drugs found in the leaves of *C. scutellarioides*. This range of values reflects the variation in flavonoid content among different plant species, underscoring the sensitivity and versatility of the dot-blot assay in analyzing plant extracts.

Table 4. Robustness of the dot-blot assay method

Reaction condition	Variation	Peak area	RSD (%)
$AlCl_3$ concentration (%)	1	9,162 ± 271	2.55
	1.5	8,837 ± 793	
Reading time (minutes)	10	9,516 ± 713	7.60
	20	8,205 ± 1,176	
	30	8,640 ± 864	

TFC in model plants using the UV–Vis spectrophotometry method

The TFC (Table 5) of the model plants was calculated by inserting the absorbance values of each replication of sample as the y-value in the linear regression equation of the standard curve (y = 0.08x – 0.0367). The range of RSD values with the UV–Vis spectrophotometry method is 0.43–8.56%, indicating that the TFC determination through this method for all model samples also has commendable precision. This suggests that the UV–Vis spectrophotometry method is reliable for quantifying TFC across different plant samples. The TFC of the extracts ranged from 0.15 mg QE/g crude drugs, found in the bark of *P. laevigata*, to 2.96 mg QE/g crude drugs, found in the leaf of *C. scutellarioides*. These results highlight the variation in flavonoid content among the plant species tested and further support the precision of the UV–Vis method in determining TFC.

Correlation between dot-blot assay method and UV–Vis spectrophotometry

The TFC of model plants determined by the dot-blot assay and UV–Vis spectrophotometry was subsequently analyzed using linear regression to assess the relationship between

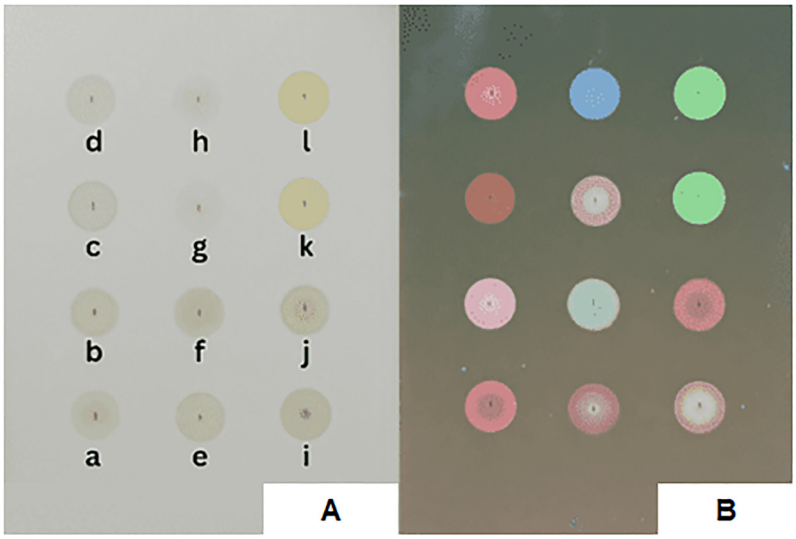

Fig. 6. Visual representation of the Al(III)-flavonoid complex under visible light (A) and UV light at 366 nm (B). The labels a-l correspond to *P. niruri*, *A. graveolens*, *S. arvensis*, *P. major*, *B. balsamifera*, *C. scutellarioides*, *T. crispa*, *P. laevigata*, *G. pictum*, *S. polyanthum,* quercetin 250 µg mL^{-1} and 300 µg mL^{-1}

Table 5. TFC of model plants determined by dot-blot assay and UV–Vis spectrophotometry

Model plant	TFC (mg QE/g crude drugs)			
	Dot-blot assay		UV–Vis Spectrophotometry	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
P. niruri	3.55 ± 0.11	3.12	0.56 ± 0.02	3.25
A. graveolens	9.74 ± 0.09	0.97	2.68 ± 0.23	8.56
S. arvensis	5.45 ± 0.14	2.64	0.42 ± 0.01	3.20
P. major	5.60 ± 0.27	4.76	0.87 ± 0.04	5.19
B. balsamivera	7.46 ± 0.11	1.54	1.49 ± 0.07	4.48
C. scutellarioides	9.95 ± 0.07	0.68	2.96 ± 0.05	1.57
T. crispa	0.37 ± 0.01	2.23	0.19 ± 0.01	5.05
P. laevigata	1.21 ± 0.04	3.67	0.15 ± 0.01	5.70
G. pictum	8.43 ± 0.37	4.37	1.67 ± 0.01	0.43
S. polyanthum	6.09 ± 0.11	1.75	1.61 ± 0.06	3.71

these two methods. The linear regression (Fig. 7) yielded an r value of 0.9078. This indicates a positive linear relationship between the dot-blot assay method and UV–Vis spectrophotometry. As the TFC measured by the dot-blot assay increases, the TFC measured by the UV–Vis spectrophotometry also increases. Subsequently, this study employed Pearson correlation analysis to determine the strength of the relationship between the dot-blot assay method and UV–Vis spectrophotometry for determining TFC in plant samples. A Pearson correlation coefficient (Product Moment) of 0.911 was obtained, which indicates that the two methods have a very strong positive linear correlation.

Table 5 also shows that the TFC for each model plant determined by the dot-blot assay method is higher than that obtained by UV–Vis spectrophotometry. These findings align with prior research indicating that the levels of α-mangostin in blood plasma measured by TLC-densitometry were higher compared to those measured by UV–Vis spectrophotometry [39]. It further indicates that the dot-blot assay with $AlCl_3$ reagent is more precise and sensitive in measuring TFC compared to the UV–Vis spectrophotometry method.

The TFC determination by means of the dot-blot assay method utilizes fewer resources and requires a shorter time compared to conventional methods (UV–Vis spectrophotometry). The dot-blot assay method reduces the duration and effort required for transferring reagent solutions into cuvettes for manual spectrophotometric analysis, enabling a greater number of replications on the same extract compared to conventional methods. The requirement for several cuvettes, pipettes, and test tubes is removed, as the sole consumable used is the TLC plate and capillary tubes. The amount of reagent required is also reduced. In conventional TFC testing, the total volume of $AlCl_3$ reagent used is 100 µL per reaction, whereas only a few sprays (drops) of the same reagent are needed per TLC plate for dot-blot assay. Another flexibility of the dot-blot assay with $AlCl_3$ reagent lies in the equipment used to capture images and the software for analyzing those images. In addition to using a densitometer, TLC plates derivatized with $AlCl_3$ can also be scanned with an

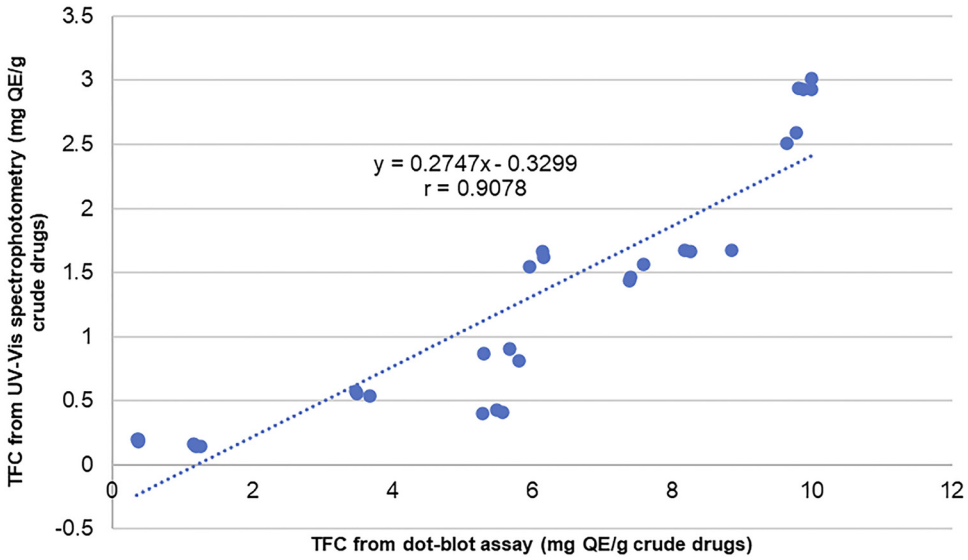

Fig. 7. Linear regression of TFC from dot-blot assay against UV–Vis spectrophotometry method

HP Deskjet Ink Advantage 2060 printer or a Canon Lide 50 flatbed scanner and then analyzed using ImageJ software. Image capture can also be performed with the Reprostar 3 or TLC Visualizer 3 from Camag, followed by analysis using Videodensitometer software [31]. Moreover, the standard curve for quercetin from the dot-blot assay method and UV–Vis spectrophotometry shows r^2 values of 0.9928 and 0.9967, respectively. The linearity is marginally greater for the conventional method compared to the dot-blot assay method. Nevertheless, the time savings, the increased number of samples processed daily, and the decreased solvent usage attributable to TLC plates significantly outweigh the somewhat greater variability, particularly in detecting variations among sample lots.

Nonetheless, in the dot-blot assay, several factors must be considered to reduce inaccuracies in the results, including the precision of the sampling technique or standard solution with capillary tubes used and the sample's solubility in the solvent. If the extract or sample exhibits partial insolubility in the used solvent, the obtained results may be less reliable, as particles inside the sample might disrupt the sample spotting process on the TLC plate. Therefore, the choice of sample solvent is crucial in this testing. Additionally, during the derivatization process, it is essential to ensure that the $AlCl_3$ reagent is sprayed evenly across the entire surface of the TLC plate. In the determination of TFC with $AlCl_3$ reagent, Al(III) reacts with the o-dihydroxy groups on the B ring and the hydroxy-keto groups on the A and C rings of flavonoids. The method is particularly tailored for flavones and flavonols, thus necessitating the development of dot-blot assay methods for total flavonoid determination using other techniques. This study used various model plants to exemplify distinct species and plant organs (leaves, stems, bark, and whole plant parts). This seeks to present an overview of the broad applicability of the dot-blot assay method when applied to plant materials containing phytochemical substances with diverse physical and chemical characteristics. The composition of these substances is affected by the type of raw material, since the use of leaf parts with chlorophyll or stems and bark with lignin may disrupt the analysis process.

As previously mentioned in the results of the statistical analysis with linear regression and Pearson correlation, this study concluded that the dot-blot assay method can serve as an alternative to UV–Vis spectrophotometry for determining TFC. The advantages of the dot-blot assay method developed in this study include greater sensitivity, speed, reduced consumption of samples and reagents, as well as its simplicity and cost-effectiveness.

CONCLUSION

To our best knowledge, this is the first report on the dot-blot assay with $AlCl_3$ reagent for determination of total flavonoid content (TFC) in food and herbal medicine materials. The developed method met the parameter of method validation, i.e., stability, intraday precision, inter day precision, linearity, LOD, LOQ, accuracy, and robustness. There was a very strong positive linear relationship between the dot-blot assay method and spectrophotometry. The proposed method proved to be simpler, faster, and more economical for determining TFC. Thus, the method was beneficial in providing references for enhancing the quality control of food and herbal medicines.

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SUPPLEMENTARY MATERIAL

Supplementary data to this article can be found online at https://doi.org/10.1556/1326.2025.01298.

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