science, engineering and health studies

Research Article

Science, Engineering and Health Studies
https://lio1.tci-thaijo.org/index.php/sehs
ISSN (Online): 2630-0087

Development and validation of TLC-densitometry method for quantitation of 1′-acetoxychavicol acetate in *Alpinia galanga* (L.) Willd. rhizome green solvent extracts

Nitikan Thipunkaew[1], Piyawat Chaivichacharn[2], Pattarida Chanachai[3], Paveena Wongtrakul[3,4], and Sunee Channarong[3,4]*

[1] Department of Pharmacognosy and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Huachiew Chalermprakiet University, Samut Prakan 10540, Thailand
[2] Department of Pharmacology and Pharmaceutical Care, Faculty of Pharmaceutical Sciences, Huachiew Chalermprakiet University, Samut Prakan 10540, Thailand
[3] Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Huachiew Chalermprakiet University, Samut Prakan 10540, Thailand
[4] Cosmetic and Herbal Innovation Center (CHIC), Faculty of Pharmaceutical Sciences, Huachiew Chalermprakiet University, Samut Prakan 10540, Thailand

***Corresponding author**:
Sunee Channarong
Sunee.cha@hcu.ac.th

***Received**: 23 April 2024*
***Revised**: 9 July 2024*
***Accepted**: 14 July 2024*
***Published**: 26 December 2024*

***Citation**:
Thipunkaew, N., Chaivichacharn, P., Chanachai, P., Wongtrakul, P., and Channarong, S. (2024). Development and validation of TLC-densitometry method for quantitation of 1′-acetoxychavicol acetate in Alpinia galanga (L.) Willd. rhizome green solvent extracts. Science, Engineering and Health Studies, 18, 24050024.

ABSTRACT

Alpinia galanga (L.) Willd. has been used as a remedy for gastrointestinal diseases in traditional medicine in several countries. 1′-Acetoxychavicol acetate (ACA) is a major phenylpropanoid extracted from the galanga rhizomes and possesses many biological activities. In this study, galanga rhizome was extracted and projected for use in cosmetic and topical formulations. The thin-layer chromatographic densitometry method was developed for quantitation of ACA in the galanga extracts. The method was validated per AOAC guidelines. The ACA bands were analyzed at 219 nm. The calibration curve's regression line was a quadratic function in the range of about 2.062–6.186 μg/band. The equation was $-169.85x^2 + 3069.6x + 286.6$, with a correlation coefficient (R^2) of 0.9995. The relative standard deviations for the precision study were 1.62%–2.54%. The recovery was performed by spiking known amounts of ACA in the sample matrixes. After removing ethanol, the extracts, namely, soft extract, glycerol liquid extract and propylene glycol liquid extract were used to determine the percent recovery. The average recovery was 94.59 ± 2.33% for soft extract, 96.16 ± 2.44% for glycerol liquid extract, and 95.75 ± 3.04% for propylene glycol liquid extract. In conclusion, a simple and reproducible semi-automated TLC-densitometry was validated successfully. This method could be used for the estimation of ACA in galangal extracts containing glycerol and propylene glycol, both in markedly available samples and in the laboratory.

Keywords: *Alpinia galanga*; liquid extract; TLC-densitometry; 1′-acetoxychavicol acetate; method validation

1. INTRODUCTION

Alpinia galanga (L.) Willd., typically mentioned as greater galanga, Siamese ginger, or blue ginger, belongs to the family Zingiberaceae. The exact origin is not known, though it has grown widespread in Indonesia, South Asia, and Southeast Asia. In Thailand, *A. galanga* is called Kha. Galanga is a rhizomatous, perennial plant that attains a height of about 2.1–3.0 m. The galanga rhizome is externally reddish brown and internally yellowish white with a strong aromatic flavor. The plant leaves are about 26–53 cm long and 8–10 cm wide, shiny on both surfaces (Trimanto et al., 2021). Galanga rhizomes have long been used as flavoring ingredients and spices in Asia. It is also a very important medicinal plant used widely in Ayurvedic and Thai traditional medicine as a remedy for gastrointestinal diseases such as stomachache, dyspepsia, and vomiting (Zhou et al., 2018). Various studies of *A. galanga* rhizomes extracted with a variety of solvents found that they contained a lot of phenolic compounds and flavonoids (Tungmunnithum et al., 2020). The major compound isolated from *A. galanga* that has various biological functions is 1′-acetoxychavicol acetate (ACA) (Chudiwal et al., 2010; Kojima-Yuasa and Matsui-Yuasa, 2020; Akbarzadeh et al., 2023). Figure 1 shows the chemical structure of ACA, a semi-volatile phenylpropanoid and responsible for the pungent characteristic in galanga. Currently, ACA possesses great interest (Baradwaj et al., 2017). Many reports have revealed that ACA possesses anti-inflammatory, antioxidant, and antiviral activity and is also observed to exhibit anticancer activities (Verma et al., 2011). *A. galanga* is very popular in a variety of research areas due to its valuable components as a bioactive, both in the pharmaceutical and cosmetic fields. Most reports about activities in galanga extracts are based on the weight of the crude extract, not the ACA amount. The extraction methods using different solvents and different techniques from a particular laboratory revealed that the concentration of ACA in the extract varied and could not be compared. The source of the galanga, the harvesting time of the plant, the weather, the water, and the planting area have a real impact on the amount of ACA in the extract. The high-performance liquid chromatography (HPLC) technique alone or in combination with other instruments was usually used to ensure the characteristics and amount of ACA (Awang et al., 2010). The resulting yield and the solvent used for extraction play significant roles in research work and product development. Specific analytical methods were very important to the quantitation of ACA from these plants to improve the extraction effectiveness and quality control of the extract, as a raw material product as well as the final finished product. However, published HPLC techniques for quantitation of ACA are scarce, and analytical method validation has not been widely reported (Ramanunny et al., 2022; Subramaniam et al., 2022). HPLC has advantages over thin layer chromatography (TLC) in all characteristic features, but it has certain limitations like any other instrument. The column chromatography is costly and requires high pressure for operation. The reliability of the technique depends on the pump process, cleanliness of the sample, and mobile phase; thus, it is more complicated and difficult to develop a new method. The instrument cannot provide a universal detector (Gupta et. al., 2022). TLC-densitometry is not the competitor but is a complementary method (Badri et al., 2023). Fully automated high-performance thin layer chromatography (HPTLC) is now a powerful analytical tool suitable for quantitative studies. The method is fast and easy; it can be used for herbal extract screening prior to any other evaluation. HPTLC provides suitable applications for herbal and food supplements. The technique can produce more data from chromatograms including inspection from UV absorbance, fluorescence, images in short and long UV wavelengths, and visible color after staining with derivatization reagents (Gupta et. al., 2022). TLC-densitometry includes these features too. However, both HPLC and HPTLC are needed to conduct analytical method validation to ensure the specificity, accuracy, precision, and reproducibility of results.

For cosmetic purposes, galanga extract is used as a skin conditioner; its INCI name is *Alpinia galanga* rhizome extract (CAS No. 84625-26-3). The galanga extracts are applicable to cleansing products and skin care due to their antioxidant, antimicrobial, and anti-inflammatory activities. The marketed available products are usually in liquid form using skin friendly solvents such as butylene glycol or water base, and most of them are claimed to be imported products. To find out the best and most economical production method, maceration with agitation was investigated using ethanol, glycerol, and propylene glycol as the three green solvents. These green solvents have gained more attention due to their environmentally friendly nature and enhanced quality, safety, and stability of the extracts. Under hydrolytic conditions containing water or aqueous ethanol in conjunction with high temperature, ACA may convert to 1′-hydroxychavicol acetate and/or *p*-acetoxycinnamic alcohol and/or *p*-coumaryl diacetate (Jakobsen and Giversen, 2020). Simon et al. (2022) reported a stability study of various compounds isolated from *A. galanga* and found that ACA was unstable in aqueous media. ACA may degrade during the drying process as well as the extraction process. Thus, the extraction method should be carefully considered to maintain the amount of ACA. A dry or anhydrous solvent is a good choice in this circumstance. Ethanol is the most frequently used solvent in herbal remedies. It has superior extractive and preservative actions. If the formulation cannot contain ethanol, but requires a liquid extract, alternative green solvents such as glycerol or propylene glycol can be explored. In this study, both glycerol and propylene glycol were used to prepare galanga liquid extracts by maceration with ethanol, which was finally removed using a rotary evaporating apparatus. The ACA in the green solvent extracts was estimated by the TLC-densitometry method.

TLC-densitometry is a semi-automated analytical technique based on TLC principles in combination with a densitometer that provides superior evaluation of the chromatogram by a detector at a specific wavelength (UV/VIS range or fluorescence), and corresponding data can be used for quantitative purposes. In HPTLC, the HPTLC plate is coated with small particles of the stationary phase (typically 4–8 μm) with a layer thickness of 200 nm, so that much better separations are obtained than using a conventional TLC aluminum plate with the particle size of the silica at 5–20 μm. HPTLC is usually

automated including sample application, derivatization, plate running, and chromatogram evaluation with instrumentation allowing very precise sample detection. TLC-densitometry has emerged in recent years as a simple and rapid tool for analyzing mixed compounds. TLC contributes extensive screening tests and detects adulteration of herbal preparations. More advantageously, a TLC plate is about half the price of a HPTLC plate, and it is possible to perform multidimension separation using the same or different mobile phase systems (Waksmundzka-Hajnos et al., 2022). To date, TLC and HPTLC play a role in routine quality control of herbal extraction (Rojsanga and Sithisarn, 2016; Susanti et al., 2022), and active pharmaceutical ingredients in single or fixed combination formulations (Dołowy et al., 2014; Mohamed et al., 2015; Parys and Pyka-Pająk, 2022).

The present study was aimed at developing and validating a TLC-densitometry method for the estimation of ACA in galanga green solvent extracts prepared in our laboratory. The analytical method was developed and validated using parameters and acceptance criteria according to the AOAC Appendix K guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals (Latimer, 2023). The assessed parameters were specificity, linearity, accuracy, and precision to propose a simple, rapid, and efficient method to be used for routine quality control of the extract products using ACA as a biomarker.

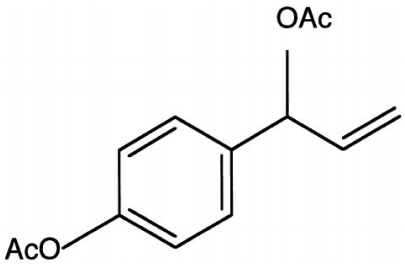

Figure 1. Structure of 1′-acetoxychavicol acetate (ACA)

2. MATERIALS AND METHODS

2.1 Materials

All chemicals and solvents used in the study were of analytical grade. D, L-1′-acetoxychavicol acetate (ACA) was purchased from LKT Laboratory, Inc., USA. TLC aluminum plates precoated with silica gel 60 F254 (Merck, Darmstadt, Germany) were used.

2.2 Plant preparation and extraction

Fresh *Alpinia galanga* (L.) Willd. rhizomes were purchased from a local farm in Samut Prakan province, Thailand. The rhizomes were cleaned, cut, and dried at 60°C using a hot air oven. The pulverized galanga powder was weighed (100 g) and placed in a 1000-mL Erlenmeyer flask. The powder was soaked with 500 mL of three different types of green solvents, which were ethanol, glycerol:ethanol (1:1), and propylene glycol:ethanol (1:1). The flasks were continuously shaken for six hours using an orbital shaker (Stuart SSL1, United Kingdom), and then separated by filtration using Whatman filter paper No. 1. Ethanol in all batches was finally removed using a rotary evaporator (Rotavapor R300, Buchi, Switzerland). Galanga extract using ethanol yielded a soft and viscous mass, so-called soft extract. The extracts using glycerol:ethanol (1:1) and propylene glycol:ethanol (1:1) were named glycerol liquid extract and PG liquid extract, respectively. The extracts were kept in amber glass bottles and stored in a refrigerator (4–8°C) until used.

2.3 Mobile phase optimization

The mobile phase was trialed with hexane, chloroform, dichloromethane, and ethyl acetate in various mixed proportions. The chamber saturation time was 20 min. The standard solution of ACA (0.365 mg/mL) was prepared in absolute ethanol. The galanga soft extract, glycerol liquid extract, and PG liquid extract were diluted with absolute ethanol to the final concentrations of 0.01 g/mL, 0.3 g/mL, and 0.1 g/mL, respectively. Five microliters of each solution were spotted on a TLC plate and developed in a tank saturated with each mix of mobile phase. Ascending development was performed and allowed the solvent to rise to a height of 10–13 cm. After development, plates were dried in a current of air for 20 min. The TLC plate was visualized under UV light at 254 nm using a visualizing cabinet. The retention factor (R_f) value of the sample from each solvent was compared to the R_f value of the standard ACA. Then, the best resolution result of the mobile phase was selected. For case staining visualization, the plate was sprayed with 1% vanillin in ethanol and 10% sulfuric acid in ethanol, respectively. The plate was dried at 110°C for 10 min in a preheated oven.

2.4 Instruments and chromatographic conditions

Chromatography was performed on 20 × 10 cm precoated TLC silica gel 60 F254 aluminum plates (Merck, Darmstadt, Germany). Aliquots of standard solution and of the extracts were separately applied to the plate as a 6-mm wide band using the semi-automatic TLC applicator Linomat-V with nitrogen flow. Ascending development was carried out in a twin-trough glass chamber previously equilibrated with the mobile phase for 20 min and allowed to migrate up to about 8 cm from the starting line. Then, the plate was removed and air-dried for exactly 20 min in the hood. Densitometric quantification was performed at 219 nm in the reflectance-absorption mode, using densitometer; CAMAG TLC Scanner 3 linked to winCATS software (CAMAG, Muttenz, Switzerland) with a slit dimension of 5 mm x 0.45 mm, a scanning speed of 20 mm/s, and a data resolution of 100 μm/step. The quantity of ACA in the different samples was calculated using a quadratic polynomial regression line analysis of peak areas.

2.5 Preparation of ACA standard stock solution

Standard stock solution was prepared by transferring 0.00515 g of ACA to a 10 mL volumetric flask, dissolved, and made up to volume with absolute ethanol. This ACA stock solution (515 μg/mL) was used for studying linearity and precision.

2.6 Analytical method validation

The developed method for quantifying ACA in various green solvent extracts was validated for specificity, linearity, accuracy, and precision according to the AOAC Appendix K guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals (Latimer, 2023).

2.6.1 Specificity

Specificity in a method ensures that a peak response is due to a single component only (Rashmin et al., 2012). The specificity of the method was ascertained by analyzing the standard and the galanga extracts. The bands of ACA in the samples were confirmed by comparing their R_f values with the standards. The peak purity of the ACA component in galanga extracts was determined by spectra comparison at three different points, which were the peak start, peak apex, and peak end positions of the targeted band with those of the standard.

2.6.2 Linearity and range

The ACA standard solution (515 µg/mL in absolute ethanol) was applied on a TLC plate with a volume ranging from 4 µL to 12 µL in a 6 mm band length to create a calibration curve. The plate was developed in a solvent system of dichloromethane and ethyl acetate (8:2), as described above. Densitometric scanning was performed at 219 nm. The standard curve was determined in triplicate. The calibration graph was constructed by plotting the area under the peak (AU) vs. the corresponding concentration of ACA.

2.6.3 Accuracy

The accuracy of the method was measured through the recovery of ACA added to sample matrixes by spiking with three concentrations of standard solution, approximately 80%, 100%, and 120% of the determined content of the sample (n = 3). The three types of test samples were determined, i.e., 0.05 mg/10 mL soft extract, 0.5 mg/10 mL glycerol liquid extract, and 0.5 mg/10 mL PG liquid extract, using absolute ethanol as solvent. The ACA standard solution (507 µg/mL in absolute ethanol) was spiked in an individual test sample with a volume of 3 µL, 4 µL, and 5 µl, consisting of 2.028, 3.042, and 4.056 µg/band. The spots were analyzed by the proposed chromatographic method. The amounts of ACA in the samples were calculated using the regression equation derived from the calibration plot. The recovery was calculated as Equation 1.

$$Recovery\ (\%) = \left(\frac{amount\ found - original\ amount}{amount\ added}\right) \times 100 \quad (1)$$

2.6.4 Precision

Precision was achieved by performing repeatability and intermediate precision. The repeatability of the method was tested by applying 4.124 µg of ACA standard solution (515 µg/mL) to six bands. The intermediate precision was evaluated by analyzing three concentrations (2.062 µg, 4.124 µg, and 5.155 µg) of the ACA standard solution (515 µg/mL) on two different days. Each concentration was spotted in triplicate. The precision was expressed as a percent relative standard deviation (% RSD).

2.7 Quantitative analysis of ACA in galanga extracts

To estimate the concentration of ACA in the galanga extracts, each extract was accurately weighed, dissolved with absolute ethanol, and filtered through a 0.45-µm syringe filter. Four microliters of soft extract sample (7.3 mg/mL), 6 µL of glycerol liquid extract (501 mg/mL), and PG liquid extract (504 mg/mL) samples were applied onto a TLC plate, respectively. The samples were analyzed under the proposed chromatographic conditions and scanned for densitometric chromatograms. The amounts of ACA in the samples were calculated using the regression equation derived from the calibration plot. Each sample was analyzed in triplicate and expressed as the mean ± SD.

2.8 Statistical analysis

All data were reported as the mean and standard deviation of triplicates or six-fold. This allowed the expression of the data as average values with calculated relative standard deviations (% RSD). The statistical evaluation of the regression model was done using statistical tool, STATA software v.14.0. (StataCorp, USA).

3. RESULTS AND DISCUSSION

3.1 Mobile phase optimization

Various mobile phases were investigated for the separation of ACA in galanga extracts from other compounds. The results are summarized in Table 1. Both the mixture of chloroform:ethyl acetate (8:2 v/v) and dichloromethane:ethyl acetate (8:2 v/v) yielded satisfactory resolution, but the system composed of dichloromethane and ethyl acetate was selected because dichloromethane is less toxic and more environmentally friendly than chloroform.

3.2 Method validation

3.2.1 Specificity

The specificity of the method was confirmed by comparing the R_f values and spectra of the spot with those of the standard. It revealed that the R_f values of the ACA was 0.78 and the R_f values of the soft extract, glycerol liquid extract, and PG liquid extract were 0.79, 0.78 and 0.77, respectively. Good correlations between standard ACA and sample spectra showed the specificity of the method. As shown in Figure 2A, the highest absorbances of the UV spectra of both the standard ACA and the tested component in the samples were at 219 nm. Figure 2B illustrates the densitometric chromatograms of the test samples and standard ACA. However, it was visualized only under 219 nm, so it is possible that some impurities were not detected by this wavelength. The peak purity was checked by comparing the spectra at three different levels, i.e., peak start (s), peak apex (m), and peak end (e) positions of the spot. It was found that the sample spots exhibited a good correlation ($r_{(s,m)}$ and $r_{(m,e)}$) to those of standard ACA, as shown in Table 2.

The tailing factor was calculated at 5% of peak height. The value close to 1.0 indicated complete symmetry, and the experiment system was suitable to separate the component (ACA) in the extracts.

The TLC chromatogram in Figure 3 demonstrates that the stationary phase and the optimized mobile phase could separate the substance of interest at a distance far from the other adjacent components. Thus, the optimized method was specific.

Table 1. Different solvent mixtures which were optimized for mobile phase

Mobile phase	Volume ratio	Estimated R_f value				Observed resolution
		ACA	Soft extract	Glycerol liquid extract	PG liquid extract	
Hexane:ethyl acetate	8:2	0.57	0.56	0.58	0.58	Fair
Hexane:ethyl acetate:ethanol	8:2:1	0.42	0.39	0.40	0.40	Fair
Chloroform:ethyl acetate	8:2	0.65	0.65	0.65	0.65	Good
Dichloromethane:ethyl acetate	8:2	0.75	0.76	0.76	0.76	Good

Table 2. Specificity results

Standard ACA and tested component in sample	R_f	Peak purity		Tailing factor
		$r_{(s,m)}$	$r_{(m,e)}$	
Standard ACA	0.78	-	-	1.0
Soft extract	0.79	0.9999	0.9997	1.0
Glycerol liquid extract	0.78	0.9997	0.9997	1.0
PG liquid extract	0.77	0.9999	0.9998	1.0

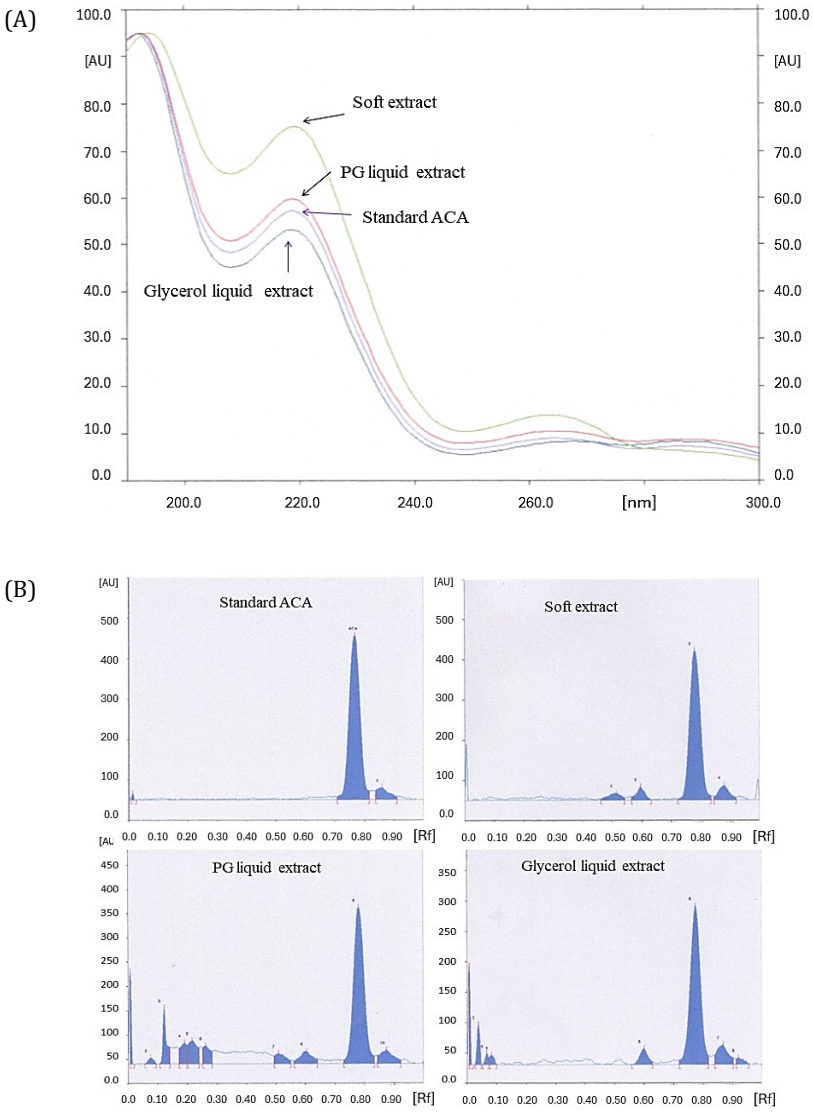

Figure 2. (A) UV spectra (190–300 nm) of standard ACA and various test samples, and (B) densitometric chromatograms of standard ACA and test samples (λ_{max} = 219 nm)

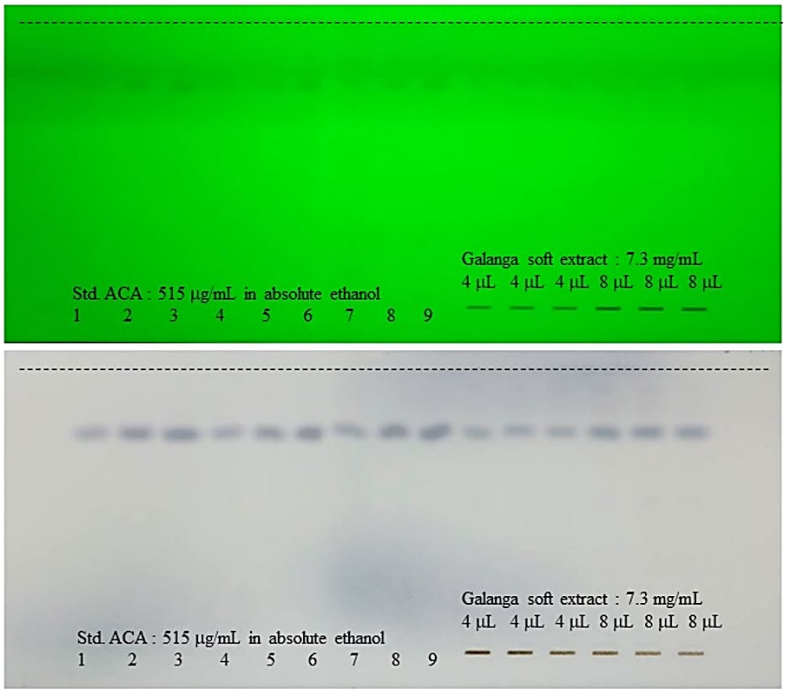

Figure 3. TLC chromatograms of standard ACA (n = 3); (lane 1–3 = 2.062 µg, lane 4–6 = 3.093 µg, lane 7–9 = 4.124 µg), and galanga soft extract (n = 3); under 254 nm [above] and after spraying with staining reagent [below]

3.2.2 Linearity and range

The TLC-densitometry principle is to measure the reflectance of separated compounds in absorption or fluorescence mode. Thus, most calibration functions are non-linear. Table 3 shows the calibration plots of peak area (AU) against concentration 2.062–6.186 µg/band of ACA. The linear regression equation was $y = 1642.8x + 2868.7$.

The correlation coefficient (R^2) was 0.9817. When the equation was used, the calculated result was bias. Nonetheless, it was found that such a large R^2 does not fit the data well. The equation still could not provide meaningful results. In general, if there is no significantly better non-linear regression equation, a three-point calibration model must be used.

Table 3. Calibration plot data of ACA in the linear and range study

Spotted volume (µL)	Concentration (µg/band)	Peak area			Mean	SD
		Run 1	Run 2	Run 3		
4	2.062	5870.78	5744.1	6060.47	5902.29	159.23
6	3.093	8358.38	7739.17	8467.16	8103.17	392.69
8	4.124	9980.4	9798.51	10137.7	9968.12	169.76
10	5.155	11743.6	11434.2	11853.5	11643.9	217.38
12	6.186	13046.9	12383.2	12817.9	12600.5	337.10
Linear regression equation					$Y = 1642.8x + 2868.7$	
(R^2)					0.9817	

To ensure that the model fitted to the data, it was done as described by Chen and Chen (2022). They examined the modeling of many previously reported chemical calibration curves using criteria other than the linear equation's R^2 value for fitting agreement. Nonlinear functions such as power equations, exponential rise to maximum, and higher-order polynomials were examined, and they found that nonlinear equations worked well with most of the tested data sets.

In this study, the parameters in the models were estimated via the assumption of the least-squares approach, performed by STATA software v.14.0. After estimation, the predicted response in each observed response (y) was calculated from each individual equation for evaluation and selection. The model selection was determined by Akaike information criteria (AIC), the coefficient of determination (R^2) and error analysis.

Table 4 exhibits the determination of model fit. The AIC in each model was calculated; the lowest AIC was the most fit. Although the lower AIC was obtained from the more complex model, the simpler model would be selected if the difference in AIC was less than 2 units. For R^2 determination, the model providing the value of R^2 nearest 1.0000 was selected. The error analysis was determined by calculating the root mean square error (RMSE) which measured the average difference between the statistically predicted values and the observed values. The model providing the lower RMSE had the trend for selection.

Table 4. Comparison for the determination of model fit

Type of model		R^2	RMSE	AIC
Linear model	$y = \theta_2 x + \theta_1$	0.9763	407.01	224.6871
Quadratic polynomial model	$y = \theta_3 x^2 + \theta_2 x + \theta_1$	0.9914	**255.70**	**211.5415**
Cubic polynomial model	$y = \theta_4 x^3 + \theta_3 x^2 + \theta_2 x + \theta_1$	0.9914	266.33	213.4584
Quartic polynomial model	$y = \theta_5 x^4 + \theta_4 x^3 + \theta_3 x^2 + \theta_2 x + \theta_1$	0.9919	271.76	214.6337
Exponential model	$y = \theta_1 e^{\theta 2x}$	0.9965	638.01	238.2172
Exponential model with one asymptote	$y = \theta_1 \theta_2^x$	0.9965	638.01	238.1723
Power model	$y = \theta_1 x^{\theta 2}$	0.9991	315.25	217.0230

All models of nonlinear functions provided values of R^2 greater than 0.99, whereas the linear model was deemed unacceptable. The quadratic polynomial model was the most-fit model, which was determined by the lowest AIC, and the lowest RMSE. The higher degree of polynomial model did not increase the model fit via larger RMSE. Thus, the quadratic polynomial model produces the best fitting agreement. The calibration equation for quantitation of ACA in this study was $y = -169.85x^2 + 3069.6x + 286.6$.

Figure 4 and Figure 5 demonstrate the fitted plot and residual plot of both linear and quadratic polynomial models. The fitted plot of the linear model showed the overprediction at the upper and lower borderlines and underprediction around the middle of the standard curve. Moreover, the residual plot demonstrated the systematic trend of the nonlinear function, illustrating an inverse U-shape pattern. For the quadratic polynomial model, the fitted plot demonstrated a better relationship between the observed AU and the predicted AU from its equation. The residual plot demonstrated the scattering allocation around the zero line of residuals and exhibited no trend.

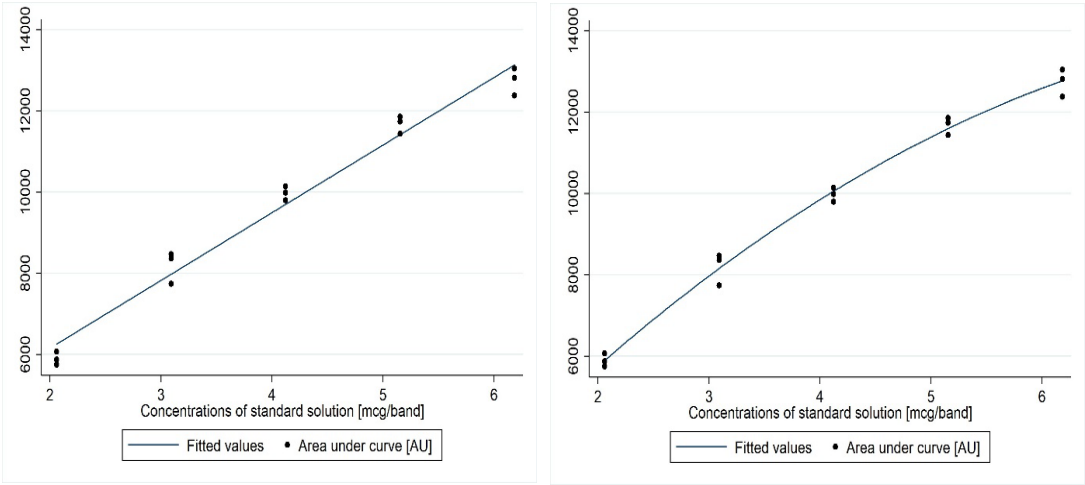

Figure 4. The fitted plot of linear model (Left) and quadratic polynomial model (Right)

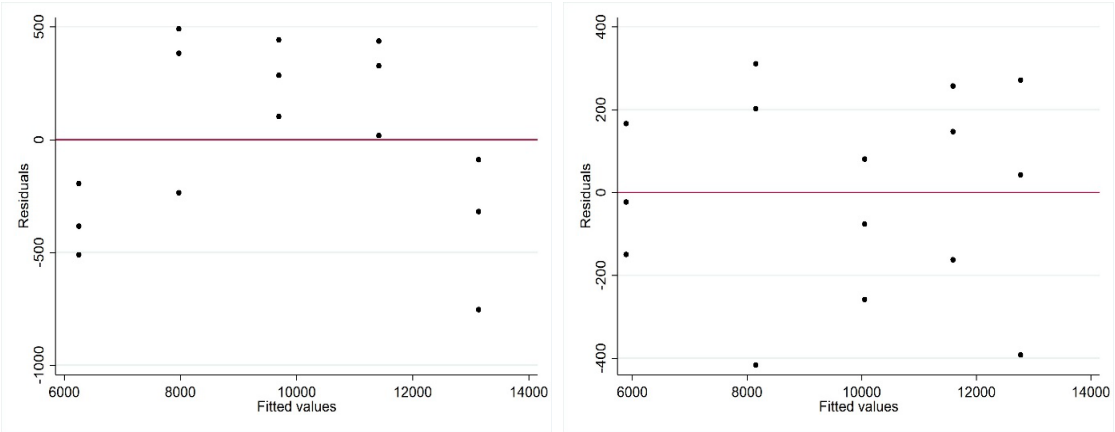

Figure 5. The residual plot of linear model (Left) and quadratic polynomial model (Right)

3.2.3 Accuracy

To evaluate the closeness of the test results to their true value, this experiment was carried out by standard spiking at three different levels in the three sample matrixes: soft extract, glycerol liquid extract, and propylene glycol liquid extract. Standard solutions consisting of 2.020 µg/band, 3.042 µg/band, and 4.056 µg/band, respectively, were spiked in an individual test sample. As shown in Table 5, the %recovery was found to be within the AOAC guidance limits since the concentration of the analyte was less than 0.1%; therefore, acceptance ranges for percent recovery fell within 90–108. The results showed that the method was accurate.

Table 5. Recovery study of ACA at low, medium and high levels by TLC densitometry

Extract	Concentration of ACA in sample (µg)	Concentration of ACA added (µg)	Concentration of ACA found (µg)			%Recovery (n = 3)
			Run 1	Run 2	Run 3	
Soft extract	0.704	2.028	1.920	1.915	1.946	95.02 ± 0.82
		3.042	2.949	2.981	2.893	96.68 ± 1.46
		4.056	3.705	3.799	3.700	92.08 ± 1.28
Glycerol liquid extract	0.305	2.028	1.974	1.877	1.828	93.34 ± 3.66
		3.042	2.937	3.009	2.951	97.49 ± 1.25
		4.056	3.913	3.979	3.991	97.66 ± 1.04
PG liquid extract	0.804	2.028	1.939	1.874	1.898	93.87 ± 1.62
		3.042	3.017	3.058	2.984	99.27 ± 1.22
		4.056	3.798	3.837	3.818	94.12 ± 0.48

3.2.4 Precision

3.2.4.1 Repeatability

Repeatability refers to the degree of agreement of results when the method is maintained as tightly as possible within the laboratory over a short period of time using the same operator and the same instruments. Repeatability was tested to ensure the accuracy of the semi-automatic applicator device by spotting, and the densitometric scanner by analyzing the same amount (4.124 µg/band) of standard solution in six replicates. The RSD of the repeatability study was 1.62%, as shown in Table 6.

Table 6. Repeatability study (n = 6)

ACA (µg)	Peak area	Measured ACA (µg)	Mean	SD	% RSD
4.124	10268.50	4.252	4.299	0.076	1.62
	10413.24	4.342			
	10514.90	4.406			
	10345.87	4.300			
	10372.44	4.316			
	10154.86	4.183			

3.2.4.2 Intermediate precision

Intermediate precision refers to a precision obtained from replicate measurements under routine variation within the laboratory, such as different analysts, different days, and different equipments. The acceptance criteria for the precision study were less than 3% as per the concentration of the analyte according to AOAC Appendix K guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals (Latimer, 2023). The concentration of ACA used in the experiment was less than 0.1%w/v. For all occurrences, the average %RSD values of repeatability and intermediate precision ranged from 1.62% to 2.54% (Table 6 and Table 7), confirming that the proposed method provided good precision.

The developed method was found to be selective, accurate, and precise. The summary of validation parameters is shown in Table 8.

3.3 Estimation of ACA in the galanga extracts

The optimized mobile phase system could separate ACA from other components present in the galanga soft extract and liquid extracts. There was no interference in the chromatogram, and the visualized resolution was good. The R_f values of ACA in the samples matched those of the standard ACA. The ACA content in the galanga soft extract was found to be 19.45 ± 2.52% w/w. The ACA contents in the glycerol liquid extract and propylene glycol liquid extract were 0.67 ± 0.01% w/w and 0.87 ± 0.02% w/w, respectively.

To investigate the extraction power of the three solvents, the maceration time was fixed to 6 h. It was proved that the kinetic maceration method resulted in satisfactory yields. Extraction with pure ethanol has been reported to be very effective for galanga extraction. Accordingly, the galanga soft extract revealed the highest ACA concentration, while glycerol liquid extract and propylene glycol liquid extract resulted in 29 and 22 times lower concentrations than ACA, respectively. The result did not exceed expectations since, after removing ethanol from the solvent mixture, the remaining non-volatile liquid, which was theoretically about 250 mL, made the liquid

extract diluted. Ethanol was utilized to lower the viscosity of the two pure green solvents, increase the effectiveness of the extraction process, and provide antiseptic action during maceration. These liquid extracts could be useful in cosmetic formulations where low amounts of the extract is required. The liquid form is more ready to use compared to the soft or viscous extracts, which usually take time to dissolve and may harden after storing. Both glycerol and propylene glycol are normal ingredients in cosmetic and topical formulations. In this research, a safe, economical, and efficient method was investigated to produce galanga extract and the quality was controlled by determining ACA content. If a higher final concentration of the extract is needed, the ratio of glycerol or propylene glycol in the solvent mixture must be lowered. Many factors involved in increasing the extraction effectiveness should be optimized, such as the particle size of the galanga powder, the maceration time, and the temperature. The known amount of ACA in the extracts might be useful to explain the correlation with its biological activities in forthcoming studies as well as to control the quality of the crude extract for the intended formulations and the finished products.

Table 7. Intermediate precision study (n = 3)

Day	ACA (µg)	Measured ACA (µg)			Mean	SD	% RSD	% RSD (n = 3)	% RSD Total (n = 6)
		Run 1	Run 2	Run 3					
1	2.062	2.052	1.999	2.133	2.061	0.067	3.26	2.65	2.54
	4.124	4.078	3.972	4.173	4.074	0.101	2.47		
	5.155	5.268	5.034	5.355	5.219	0.166	3.18		
2	2.062	2.153	2.129	2.052	2.111	0.053	2.50	2.11	
	4.124	3.995	4.013	4.189	4.066	0.107	2.64		
	5.155	5.007	4.894	4.984	4.962	0.060	1.20		

Table 8. Summary of validation data for quantitation of ACA in galanga extracts

Parameters	Results
Selectivity	Selective
Analytical range	2.062–6.186 µg/band
Quadratic polynomial regression equation	$y = -169.85x^2 + 3069.6x + 286.6$
-Correlation coefficient	0.9995
Accuracy	
-Average % recovery (n = 9)	94.59 ± 2.33% for soft extract 96.16 ± 2.44% for glycerol liquid extract 95.75 ± 3.04% for propylene glycol liquid extract
Precision (% RSD)	
- Repeatability (n = 6)	1.62
- Intermediate precision (n = 6)	2.54

4. CONCLUSION

This study contains the first report the TLC-densitometry method for quantitation of ACA in galanga green solvent extracts using a quadratic polynomial regression line. Good separation was achieved using TLC aluminum plate coating with silica gel 60 F254 using dichloromethane and ethyl acetate (8:2) as a mobile phase. Quantitation was performed by densitometric scanning at 219 nm in reflectance-absorption mode. TLC is the only separation technique that can analyze different samples at the same time. This technique can be used to analyze crude extracts without the need for prior purification. To analyze the already-known phytochemicals, TLC is rapid and easy. Semi-automated TLC-densitometry was used to analyze ACA in galanga green solvent extracts, concomitantly developing the extraction process to improve the cost-effectiveness of commercial aspects. The developed method is simple, selective, accurate, precise, and reproducible for the estimation of ACA in galanga extract containing glycerol and propylene glycol, both in markedly available samples and in the laboratory. This method is economical and suitable for rapid routine quantitation of ACA for standardization purposes, not only for the two liquid extracts but for other galanga soft extracts using volatile organic solvents that were finally removed.

ACKNOWLEDGMENT

This research was financially supported by the Faculty of Pharmaceutical Sciences, Huachiew Chalermprakiet University. The authors thankful to Department of Pharmacognosy and Pharmaceutical Chemistry for providing the facilities to complete this research successfully.

REFERENCES

Akbarzadeh, F., Eslamzadeh, M., Behravan, G., Ebrahimi, A., Emami, S. A., Gilan, A., and Hoseinian, N. S. (2023). Assessing the effect of *Alpinia galanga* extract on the treatment of SSRI-induced erectile dysfunction: A

randomized triple-blind clinical trial. *Frontiers in Psychiatry*, 14, 1105828.

Awang, K., Azmi, M. N., Aun, L. I. L., Aziz, A. N., Ibrahim, H., and Nagoor, N. H. (2010). The apoptotic effect of 1'S-1'-acetoxychavicol acetate from *Alpinia conchigera* on human cancer cells. *Molecules*, 15(11), 8048–8059.

Badri, S., Ravi Kumar, J., Siva, P., Pavan, M., Basheer, K., Ashok, E., and Lavanya, B. (2023). A review on comparision of HPLC and HPTLC. *Journal of Innovations in Applied Pharmaceutical Science*, 8(3), 18–23.

Baradwaj, R. G., Rao, M. V., and Senthil Kumar, T. (2017). Novel purification of 1'S-1'-acetoxychavicol acetate from *Alpinia galanga* and its cytotoxic plus antiproliferative activity in colorectal adenocarcinoma cell line SW480. *Biomedicine & Pharmacotherapy*, 91, 485–493.

Chen, H.-Y., and Chen, C. (2022). Evaluation of calibration equations by using regression analysis: An example of chemical analysis. *Sensors*, 22(2), 447.

Chudiwal, A. K., Jane, D. P., and Somani, R. S. (2010). *Alpinia galanga* Willd.—An overview on phyto-pharmacological properties. *Indian Journal of Natural Products and Resources*, 1(2), 143–149.

Dołowy, M., Kulpińska-Kucia, K., and Pyka, A. (2014). Validation of a thin-layer chromatography for the determination of hydrocortisone acetate and lidocaine in a pharmaceutical preparation. *The Scientific World Journal*, 2014(1), 107879.

Gupta, M. K., Ghuge, A., Parab, M., Al-Refaei, Y., Khandare, A., Dand, N., and Waghmare, N. (2022). A comparative review on high-performance liquid chromatography (HPLC), ultra performance liquid chromatography (UPLC) & high-performance thin layer chromatography (HPTLC) with current updates. *Current Issues in Pharmacy and Medical Sciences*, 35(4), 224–228.

Jakobsen, H. B., and Giversen, I. (2020). Method for preparing pharmaceutical compositions of rhizomes from *Alpinia galanga* or *Alpinia conchigera* having a high content of 1'S-1'-acetoxychavicol acetate (ACA) (U.S. Patent No. 10603349B2). United States Patent and Trademark Office.

Kojima-Yuasa, A., and Matsui-Yuasa, I. (2020). Pharmacological effects of 1'-acetoxychavicol acetate, a major constituent in the rhizomes of *Alpinia galanga* and *Alpinia conchigera*. *Journal of Medicinal Food*, 23(5), 465–475.

Latimer, G. W., Jr. (2023). Appendix K guidelines for dietary supplements and botanicals. In *Official Methods of Analysis of AOAC International* (Latimer, G. W., Jr. Ed.), 22nd ed., New York, NY: Oxford University Press.

Mohamed, A. I., Omar, M. A., Hammad, M. A., and Mohamed, A. A. (2015). Development and validation of TLC–densitometric method for simultaneous determination of two binary antihypertensive mixtures containing felodipine in fixed dose combinations. *Biomedical Chromatography*, 30(2), 200–207.

Parys, W., and Pyka-Pająk, A. (2022). Use of TLC-densitometric method for determination of valproic acid in capsules. *Molecules*, 27(3), 752.

Ramanunny, A. K., Wadhwa, S., Gulati, M., Gupta, S., Porwal, O., Jha, N. K., Gupta, P. K., Kumar, D., Prasher, P., Dua, K., Al Saqr, A., Almawash, S., and Singh, S. K. (2022). Development and validation of RP-HPLC method for 1′-acetoxychavicol acetate (ACA) and its application in optimizing the yield of ACA during its isolation from *Alpinia galanga* extract as well as its quantification in nanoemulsion. *South African Journal of Botany*, 149, 887–898.

Rashmin, P., Mrunali, P., Nitin, D., Nidhi, D., and Bharat, P. (2012). HPTLC method development and validation: Strategy to minimize methodological failures. *Journal of Food and Drug Analysis*, 20(4), 794–804.

Rojsanga, P., and Sithisarn, P. (2016). Validated TLC-densitometric method for determination of oxyresveratrol contents in ma-haad (*Artocarpus lakoocha*) heartwood extracts. *Mahidol University Journal of Pharmaceutical Sciences*, 43(2), 91–96.

Simon, A., Nghiem, K. S., Gampe, N., Garádi, Z., Boldizsár, I., Backlund, A., Darcsi, A., Nedves, A. N., and Riethmüller, E. (2022). Stability study of *Alpinia galanga* constituents and investigation of their membrane permeability by ChemGPS-NP and the parallel artificial membrane permeability assay. *Pharmaceutics*, 14(9), 1967.

Subramaniam, B., Siddik, Z. H., and Nagoor, N. H. (2022). Development and validation of a reversed-phase HPLC method for quantification of 1′acetoxychavicol acetate content in a nanostructured lipid carrier formulation. *Brazilian Journal of Pharmaceutical Sciences*, 58, e20627.

Susanti, M., Pratama, A. R., Suryani, M. I., Suryati, and Dachriyanus. (2022). Development and validation of TLC-densitometry method for quantification of tetraprenyltoluquinone in the stem bark hexane extract of *Garcinia cowa* roxb. *Heliyon*, 8(9), e10437.

Trimanto, T., Hapsari, L., and Dwiyanti, D. (2021). *Alpinia galanga* (L.) willd: Plant morphological characteristic, histochemical analysis and review on pharmacological. *AIP Conference Proceedings*, 2353, 030021.

Tungmunnithum, D., Tanaka, N., Uehara, A., and Iwashina, T. (2020). Flavonoids profile, taxonomic data, history of cosmetic uses, anti-oxidant and anti-aging potential of *Alpinia galanga* (L.) Willd. *Cosmetics*, 7(4), 89.

Verma, R. K., Mishra, G., Singh, P., Jha, K. K., and Khosa, R. L. (2011). *Alpinia galanga*-An important medicinal plant: A review. *Der Pharmacia Sinica*, 2(1), 142–154.

Waksmundzka-Hajnos, M., Hawrył, M., Hawrył, A., and Jóźwiak, G. (2022). Thin layer chromatography in phytochemical analysis. In *Handbook of Bioanalytics* (Buszewski, B., and Baranowska, I., Eds.), pp. 565–596. Cham: Springer.

Zhou, Y.-Q., Liu, H., He, M.-X., Wang, R., Zeng, Q.-Q., Wang, Y., Ye, W.-C., and Zhang, Q.-W. (2018). A review of the botany, phytochemical, and pharmacological properties of galangal. In *Handbook of Food Bioengineering Volume 7, Natural and Artificial Flavoring Agents and Food Dyes* (Grumezescu, M. A., and Holban, A. M., Eds.), pp. 351–396. Cambridge, MA: Academic Press.