Research Paper

Estimation of Aloe-emodin Content in *Cassia grandis* and *Cassia garrettiana* Leaves Using TLC Densitometric Method and TLC Image Analysis

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Plants in genus *Cassia* contain anthraquinones that possess stimulant laxative property. *Cassia grandis* L.f. and *Cassia garrettiana* Craib have been widely distributed throughout Thailand as *Thai* herbal medicine and ornamental plant. The objective of this study was to develop and validate TLC densitometry and TLC image analysis for quantitative analysis of aloe-emodin content in *Cassia grandis* and *Cassia garrettiana* leaves. The fresh mature leaves of *Cassia grandis* and *Cassia garrettiana*, collected from 15 locations throughout Thailand were successively extracted with dichloromethane. Silica gel 60 GF254 was used as a stationary phase and hexane-ethyl acetate (1:1, v/v) was used as the mobile phase. Aloe-emodin contents estimated using TLC densitometry and TLC image analysis in *Cassia grandis* dried crude drug were 0.4122±0.0668 and 0.4130±0.0751 g % and *Cassia garrettiana* dried crude drug were 0.0346±0.0067 and 0.0351±0.0056 g %, respectively. The method was validated in terms of calibration range, accuracy, repeatability and intermediate precision, limit of detection, limit of quantitation, specificity and robustness. A statistical comparison of the quantitative analysis using TLC densitometry and TLC image analysis of aloe-emodin in *Cassia grandis* and *Cassia garrettiana* leaves were not statistically significant (p>0.05). Both validated methods can be used for evaluation of aloe-emodin contents in *Cassia grandis* and *Cassia garrettiana* leaves.

Key words: *Cassia grandis, Cassia garrettiana,* TLC densitometry, TLC image analysis, aloe-emodin contents

Cassia is a genus of tropical flowering plant belonging to the family Caesalpiniaceae. There are many *Cassia* species worldwide used in herbal medicine. Plants in genus *Cassia* contain mainly anthraquinone compounds, which are the largest group of natural quinones used as laxatives and antifungal drug for skin diseases[1]. Several anthraquinones and their derivatives from *Cassia* species have been documented such as rhein, emodin, sennosides and aloe-emodin. Aloe-emodin (fig. 1) is an anthraquinone found in plants (*Aloe* sp., *Rhamnus* sp. and *Cassia* sp.), fungi, lichens and insects[2] has interesting biological activities such as antiviral, antimicrobial, anticancer and hepatoprotective activities[3-6]. In Thailand, *C. grandis* is known as "*Kanlaphruek*" or "*Kalapaphruek*" in *Thai* language. This plant is wildly distributed as ornamental plant throughout the country. *C. garrettiana* is also a traditional *Thai* medicine called "*Sa mae san*" which has been used as emmenagogue, blood tonic for women and used to treat herpes zoster, leukaemia, and constipation[7,8]. Previous study identified aloe-emodin as the main anthraquinone isolated from *C. grandis* and *C. garrettiana* leaves[9-10].

Thin-layer chromatography (TLC) is a fast screening method for compound separation and identification of herbal extracts. This method is frequently used as

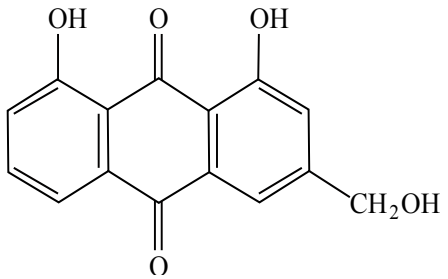

Fig. 1: Structure of aloe-emodin

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a qualitative and quantitative analysis as low cost of instrumentation, short time for analysis and easy to use[11,12]. Quantitative analysis can be performed using TLC densitometry and TLC image analysis. TLC densitometry is one of the suitable methods wildly used for quantitative analysis due to its accurate, precise and reliable procedure[13]. TLC image analysis using computer software technology has been in consideration as a simple, inexpensive and convenient quantitation method with good accuracy and precision for chemical compounds analysis in crude drug and medicinal plants[14]. Both methods successfully applied for quantification of chemical compounds in various herbal plants such as *Artocarpus lakoocha*, *C. fistula*, *Senna siamia* and *Chromolaena odorata*[15-18]. The quantification of aloe-emodin in *C. grandis* and *C. garrettiana* leaf extracts using TLC densitometry and TLC image analysis has not been reported. Thus, the objective of this study was to develop and validate TLC densitometry with winCATS software and TLC image analysis with ImageJ software for quantification of aloe-emodin contents in *C. grandis* and *C. garrettiana* leaves collected from different locations in Thailand.

MATERIALS AND METHODS

The fresh mature leaves of *C. grandis* and *C. garrettiana* were collected from 15 locations in Thailand. Plant specimens were authenticated at the College of Public Health Sciences, Chulalongkorn University and Faculty of Pharmacy, Rangsit University, Thailand. Plant specimens were compared to the herbarium specimens at the Botanical Garden Organization, Ministry of Natural Resource. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. Each authentic sample was dried in hot air oven at 45° and ground to a powder for phytochemical screening of anthraquinones.

Phytochemical screening of anthraquinones:

Dilute hydrochloric acid (2 M) was added to the sample and the mixture was heated on a hot water bath for 15 min, then cooled and filtered. The filtrate was then extracted with dichloromethane. The dichloromethane layer was separated and shaken with ammonium hydroxide. Pink to red colour was developed in alkali layer[19].

Preparation of standard solutions:

A stock solution containing 0.5 mg/ml of standard aloe-emodin (Sigma-Aldrich, USA) was prepared in dichloromethane containing 10 % methanol and diluted to obtain a series dilution of 0.04, 0.08, 0.12, 0.16, and 0.20 mg/ml of standard solutions and then stored in refrigerator at 4°.

Preparation of dichloromethane extracts of *C. grandis* and *C. garrettiana*:

Six grams of the leaf powder of *C. grandis* and *C. garrettiana* was extracted with dichloromethane using a Soxhlet apparatus. The extract was filtered and the solvent was evaporated by rotary evaporator. The yield of each plant sample was calculated and recorded. The extract was dissolved in dichloromethane containing 10 % methanol to make the final concentration 5 mg/ml of *C. grandis* and 20 mg/ml of *C. garrettiana*.

TLC densitometry method:

Three microliters of 15 dichloromethane extracted samples of *C. grandis* and *C. garrettiana* and aloe-emodin standard solutions were applied on the TLC-silica gel 60 GF_{254} 20×10 cm plate (E. Merck, Germany) using a Camag Linomat 5 automatic sample spotter (Camag, Switzerland) under a flow of nitrogen gas. Each sample band was set at 10 mm and distance between bands was 8.9 mm. The TLC plates were developed in a Camag glass twin-through chamber (20×10 cm), which was pre-saturated in mobile phase of hexane-ethyl acetate (1:1 v/v) for 1 h at room temperature. The plate was scanned under wavelength at 434 nm using TLC scanner 3 (Camag, Switzerland) with winCATS software. Aloe-emodin contents in *C. grandis* and *C. garrettiana* leaves extract were quantitated by peak area. The test was done in triplicate.

TLC image analysis using ImageJ software:

TLC plate was photographed under ultraviolet light at 254 nm by a digital camera. Quantitative analysis of the aloe-emodin contents in *C. grandis* and *C. garrettiana* leaves extract and the colour intensity of spot on TLC plates were observed using ImageJ software (Department of Health and Human Services, National Institutes of Health, United State). The test was done in triplicate.

Method validation:

TLC densitometry and TLC image analysis of aloe-emodin contents in *C. grandis* and *C. garrettiana* leaves extracts were validated in terms of calibration range, accuracy, repeatability, intermediate precision, limit of detection (LOD), limit of quantitation (LOQ), specificity and robustness according to the International Council for Harmonisation guidelines (ICH Q2R1)[20].

The calibration range was constructed by analysis of five concentrations of standard aloe-emodin in the range 0.12, 0.24, 0.36, 0.48 and 0.60 µg/spot. A plot of average area under curve versus concentration was obtained. The calibration range was expressed as the correlation coefficient (r^2). The accuracy of the analytical procedure was performed by recovery of spiking known three concentrations of standard aloe-emodin in the sample. The accuracy was determined as recovery of aloe-emodin in percent. The repeatability and intermediate precision were determined in the same day and in the three different days and expressed as percent relative standard deviation (% RSD). LOD and LOQ were calculated from the calibration curve as 3.3 (SD/*S*) and 10 (SD/*S*), respectively where, SD was the standard deviation of regression line and *S* was the slope of regression line.

The specificity of the method was ascertained by analysing the standard solutions and sample extracts. The bands for aloe-emodin in samples were confirmed by comparing the R_f value and UV absorbance spectra of the bands with those of standard. The peak purity of sample was assessed by comparing the overlay spectra of standard aloe-emodin and sample extracts at three different positions, peak start, peak apex and peak end positions of the spot detected at 434 nm. The robustness of the method was done by slightly changing the suitable mobile phase ratio in the experiment and interpreted as % RSD of peak areas.

Data analysis of *C. grandis* and *C. garrettiana*:

The aloe-emodin contents obtained using TLC densitometry and TLC image analysis were compared by paired *t*-test statistical analysis.

RESULTS AND DISCUSSION

The chromatographic condition for quantitating aloe-emodin contents was examined using silica gel 60 GF_{254}. The selected mobile phase, hexane-ethyl acetate (1:1 v/v) demonstrated the best separation of aloe-emodin in *C. grandis* and *C. garrettiana* leaves with R_f value 0.50±0.007 and 0.50±0.009, respectively. The aloe-emodin band of the plant samples was confirmed by comparing an R_f value with standard aloe-emodin.

The yield of dichloromethane extract by Soxhlet extraction and aloe-emodin contents determination using TLC densitometry and TLC image analysis of *C. grandis* and *C. garrettiana* leaves were presented in Tables 1 and 2, respectively. TLC chromatogram of *C. grandis* and *C. garrettiana* under UV 254 was shown in fig. 2A and B, respectively. TLC densitogram of *C. grandis* and *C. garrettiana* scanned in the range of 200-700 nm was shown in fig. 3A and B, respectively. Aloe-emodin contents using TLC densitometry and TLC image analysis in *C. grandis* dried crude drug were 0.4122±0.0668 and 0.4130±0.0751 g %. The highest and lowest aloe-emodin contents were observed in the samples from Prachin Buri and Nakhon Ratchasima province, respectively. The aloe-emodin contents in *C. garrettiana* dried crude drug using TLC densitometry and TLC image analysis were 0.0346±0.0067 and 0.0351±0.0056 g %. The highest aloe-emodin content was observed in the samples from Phetchabun province, whereas the lowest content was

TABLE 1: EXTRACT YIELD AND ALOE-EMODIN CONTENTS IN *C. GRANDIS* LEAVES

Source (Province in Thailand)	Extract yield (%)	Aloe-emodin contents (g % of dried crude drug)	
		TLC densitometry	TLC image analysis
Ubon Ratchathani	13.5253	0.3738±0.0120	0.3891±0.0097
Surin	13.7510	0.3858±0.0066	0.4196±0.0134
Si Sa Ket	13.9805	0.3931±0.0076	0.4059±0.0055
Chaiyaphum	14.8775	0.4124±0.0073	0.4420±0.0158
Nakhon Ratchasima	12.0755	0.2663±0.0052	0.2592±0.0146
Nakhon Sawan	13.6750	0.4321±0.0137	0.3905±0.0127
Phichit	13.6764	0.4670±0.0125	0.4428±0.0168
Phitsanulok	14.6409	0.4400±0.0154	0.4456±0.0114
Sukhothai	14.3488	0.4272±0.0071	0.4318±0.0275
Uttaradit	14.2157	0.4548±0.0119	0.4431±0.0138
Pathum Thani	12.2809	0.3286±0.0067	0.3071±0.0128
Nakhon Pathom	15.1001	0.4669±0.0238	0.4778±0.0159
Prachin Buri	15.7777	0.5228±0.0160	0.5192±0.0070
Phra Nakhon Si Ayutthaya	15.2663	0.4776±0.0142	0.5182±0.0266
Bangkok	12.2121	0.3349±0.0054	0.3023±0.0139
Average	13.9602±1.1229	0.4122±0.0668	0.4130±0.0751

TABLE 2: EXTRACT YIELD AND ALOE-EMODIN CONTENTS IN *C. GARRETTIANA* LEAVES

Source (Province in Thailand)	Extract yield (%)	Aloe-emodin contents (g % of dried crude drug)	
		TLC densitometry	TLC image analysis
Bangkok	9.4392	0.0429±0.0013	0.0442±0.0014
Prachin Buri	9.1868	0.0406±0.0016	0.0409±0.0009
Nakhon Pathom	8.9384	0.0387±0.0021	0.0386±0.0018
Kanchanaburi	7.9699	0.0346±0.0015	0.0307±0.0009
Chon Buri	7.0660	0.0244±0.0007	0.0267±0.0013
Nonthaburi	8.9855	0.0346±0.0023	0.0400±0.0028
Prachuap Khiri Khan	9.5884	0.0415±0.0031	0.0375±0.0029
Lop Buri	8.5373	0.0356±0.0015	0.0355±0.0020
Phetchabun	9.8464	0.0460±0.0009	0.0424±0.0018
Uttaradit	7.9638	0.0326±0.0016	0.0365±0.0014
Maha Sarakham	8.1402	0.0328±0.0008	0.0336±0.0016
Khon Kaen	7.2331	0.0240±0.0024	0.0279±0.0024
Nakhon Ratchasima	7.9457	0.0272±0.0020	0.0285±0.0021
Rayong	8.0455	0.0278±0.0030	0.0290±0.0012
Sukhothai	9.6732	0.0356±0.0015	0.0343±0.0006
Average	8.5706±0.8846	0.0346±0.0067	0.0351±0.0056

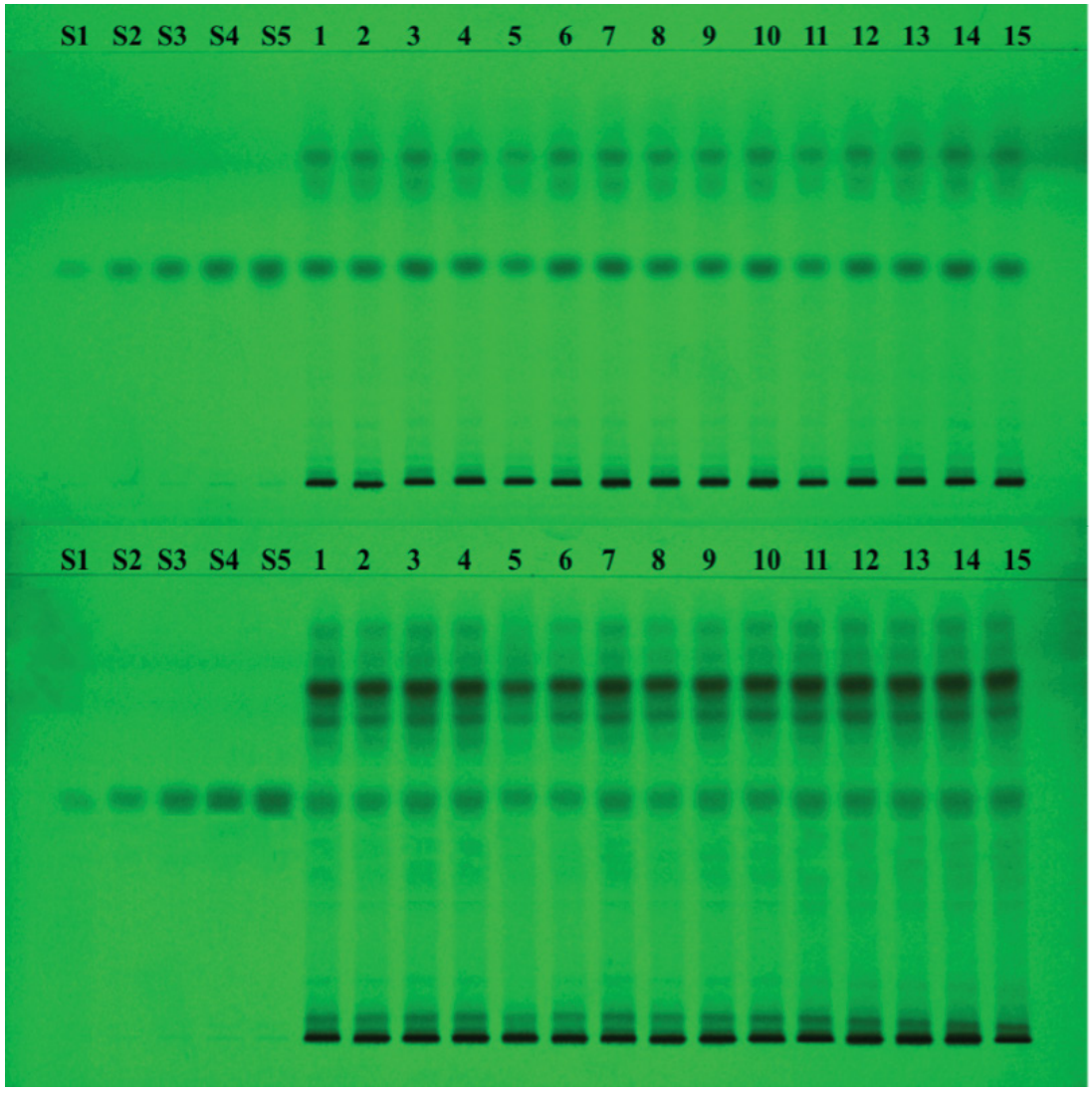

Fig. 2: The TLC plates under UV 254 nm
A. Standard aloe-emodin (S1 to S5) and *C. grandis* leaf extracts from 15 various locations in Thailand; B. standard aloe-emodin (S1 to S5) and *C. garrettiana* leaf extracts from 15 various locations in Thailand

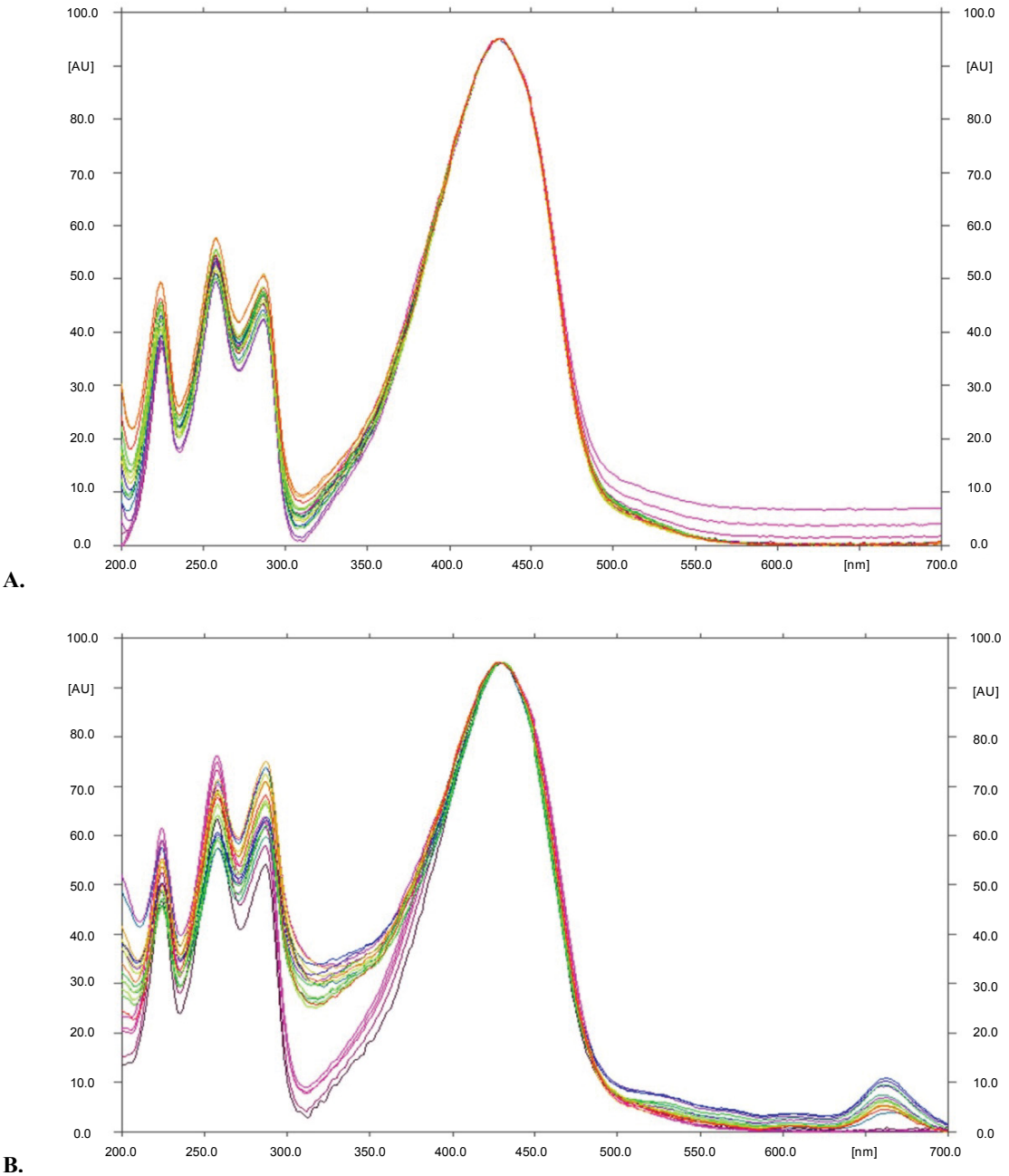

Fig. 3: The absorption spectra of standard aloe-emodin and samples

A. Absorption spectra of aloe-emodin in standard and sample bands of *C. grandis*; B. absorption spectra of aloe-emodin in standard and sample bands of *C. garrettiana*

obtained from the samples from Khon Kaen province. A statistical comparison of the quantitative analysis using TLC densitometry and TLC image analysis of aloe-emodin contents in *C. grandis* and *C. garrettiana* leaves were not statistically significant ($p>0.05$) using paired *t*-test.

The method was validated for its linearity, specificity, accuracy, precision, LOD, LOQ and robustness. The linear calibration curves of aloe-emodin contents were ranged from 0.12-0.60 µg/spot. The specificity was confirmed by comparing UV spectrum of the peak in standard aloe-emodin and all 15 samples. The result showed the maximum absorbance at the wavelength of 434 nm (fig. 2B). Good correlation of both samples was also obtained between standard and sample overlay spectra ($r^2>0.9950$). The recovery values of both methods within accepted limit (98.16-103.38 and 97.58-107.86 % for *C. grandis* and 98.17-105.53 and 97.35-105.94 % for *C. garrettiana*). The repeatability and the intermediate precision of both methods of *C. grandis* and *C. garrettiana* were less than 3 % RSD.

The LOD and LOQ of TLC densitometry and TLC image analysis were found to be 0.0198 and 0.0601 µg/spot and 0.0171 and 0.0517 µg/spot for *C. grandis* and 0.0214 and 0.0648 µg/spot and 0.0188 and 0.0568 µg/spot for *C. garrettiana*. The robustness was performed by slightly changing composition of mobile phase (hexane-ethyl acetate 1:1, 0.9:1.1, 1.1:0.9 v/v) showed the values of 0.28 % RSD in TLC densitometry and 0.50 % RSD in TLC image analysis of *C. grandis*, whereas these values were 0.56 and 0.58 % RSD in *C. garrettiana*. The validity of TLC densitometry and TLC image analysis of *C. grandis* and *C. garrettiana* was presented in Table 3, respectively.

Aloe-emodin is a major component in the leaf extracts of *C. grandis* and *C. garrettiana* and this compound was employed as marker for TLC densitometry and TLC image analysis in the present study. The amounts of aloe-emodin showed a variation quantities in plant materials collected from various locations in Thailand as the chemical constituent contents in herbal plant can be vary with the plant origin, harvest season, environmental factor and herbal preparation method[21]. This data will be useful as guidance for sample collection of *C. grandis* and *C. garrettiana* that contained high aloe-emodin contents in Thailand. The aloe-emodin contents of *C. grandis* and *C. garrettiana* leaves from 15 various locations in Thailand obtained from TLC densitometry and TLC image analysis were compared using paired *t*-test statistical analysis. It was indicated that the aloe-emodin contents in *C. grandis* and *C. garrettiana* leaves from both methods were not significantly different with $p>0.05$. Both methods could be used as an alternative method for routine quantitative analysis of major compounds in the *C. grandis* and *C. garrettiana* leaves extracts. According to ICH guidelines, the analytical method was validated to confirm that the analytical procedure employed reliable and accurate data. The linear calibration curves of aloe-emodin contents in *C. grandis* and *C. garrettiana* leaves using both methods showed good linearity relationships in range of 0.12-0.60 µg/spot with correlation coefficient (r^2) more than 0.99. The identical absorption spectrum of standard aloe-emodin in this study showed the maximum absorbance at 434 nm, which is in accordance to the previous study indicated that maximum UV absorption spectrum of aloe-emodin could be detected at 430 nm[22]. The recovery assay in both methods was accurate. Determination of aloe-emodin contents in *C. grandis* and *C. garrettiana* repeatedly within and between set of experiments by both methods revealed acceptable precisions. LOD and LOQ value from both methods of *C. grandis* and *C. garrettiana* confirmed that the lowest concentration of standard aloe-emodin (0.12 µg/spot) used in this study were suitable. The robustness studied by changing composition of mobile phase indicated that changing composition of mobile phase was not affected in both methods.

This is the first report of the validated TLC densitometric method and TLC image analysis using ImageJ software for quantitation of aloe-emodin contents in dichloromethane extract of *C. grandis* and *C. garrettiana* leaves collected from 15 different locations in Thailand. The data obtained from this study may be valuable for indicating alternative sources of aloe-emodin. Due to *C. grandis* and *C. garrettiana* distributed throughout Thailand, the supply of the leaves material will be easily available. Statistical

TABLE 3: METHOD VALIDITY OF TLC DENSITOMETRY AND TLC IMAGE ANALYSIS OF ALOE-EMODIN CONTENTS IN *C. GRANDIS* AND *C. GARRETTIANA* LEAVES

Parameter	TLC densitometry	TLC image analysis
C. Grandis		
Accuracy (% recovery)	98.16-103.38	97.58-107.86
Precision: repeatability (% RSD)	0.42-1.09	0.42-0.97
Precision: intermediate precision (% RSD)	0.84-2.20	0.91-2.40
Limit of detection (µg/spot)	0.0198	0.0171
Limit of quantitation (µg/spot)	0.0601	0.0517
Robustness (% RSD)	0.28	0.50
C. Garrettiana		
Accuracy (% recovery)	98.17-105.53	97.35-105.94
Precision: repeatability (% RSD)	0.55-1.08	0.30-0.50
Precision: intermediate precision (% RSD)	0.87-1.28	1.03-1.30
Limit of detection (µg/spot)	0.0214	0.0188
Limit of quantitation (µg/spot)	0.0648	0.0568
Robustness (% RSD)	0.56	0.58

analysis indicated that the aloe-emodin contents determined using TLC densitometric method and TLC image analysis showed no significantly different with $p>0.05$ hence TLC image analysis may be used as an alternative method for quantitative analysis of *C. grandis* and *C. garrettiana* leaves due to its rapid, simple, precise, accurate and cost effectiveness.

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Conflicts of interest:

There are no conflicts of interest.

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