Original Article

Thai Journal of Pharmaceutical Sciences

HPTLC-densitometric and TLC-image analysis method for determination of alkaloids crebanine and dicentrine in *Stephania venosa* tuber and their commercial products

Savita Chewchinda[1], Sumet Kongkiatpaiboon[2]

[1]Department of Food Chemistry, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand, [2]Drug Discovery and Development Center, Office of Advanced Science and Technology, Thammasat University (Rangsit Campus), Pathum Thani, Thailand

Corresponding Author:
Sumet Kongkiatpaiboon, Drug Discovery and Development Center, Office of Advanced Science and Technology, Thammasat University (Rangsit Campus), 99 Moo 18 Phahonyothin Road, Klong Neung, Klong Luang, Pathumthani, Thailand.
Tel: +66-2-5643089 ext 77040,
E-mail: s_u_m_e_t@hotmail.com; sumet_k@tu.ac.th

Received: Jan 02, 2020
Accepted: Oct 25, 2020
Published: Feb 10, 2021

ABSTRACT

This study was aimed to develop and validate HPTLC densitometric and image analysis method for the quantification of crebanine and dicentrine in *S. venosa* tuber and their commercial products in Thailand. HPTLC analysis was performed on an aluminum sheet of silica gel using dichloromethane:ethyl acetate:methanol (21:6:3, *v/v/v*) as a mobile phase. The densitometric scanning was performed at the wavelength 290 nm, while image analysis was visualized after post-derivatization with Dragendorff's reagent and processed by the ImageJ processing software. The proposed methods showed acceptable validation parameters. The content of crebanine and dicentrine in *S. venosa* tuber and their commercial product was in the ranges of <0.31–1.64 ± 0.18 % and <0.25–1.31 ± 0.11 % w/w for HPTLC densitometric and <0.17–1.61 ± 0.07 % and <0.17–1.64 ± 0.03 % w/w for TLC image analysis method, respectively. The result suggested no significant differences ($P > 0.05$) between the mean contents analyzed by HPTLC densitometric and TLC image analysis method. HPTLC and image analysis method showed several advantages such as simple, fast data acquisition, and cost efficiency. Both methods are considered as alternative method for product quality assurance and can be used in the routine quality control analysis.

Keywords: Crebanine, dicentrine, HPTLC, image analysis, *Stephania venosa*

INTRODUCTION

Stephania venosa (Blume) Spreng. (Menispermaceae) is an indigenous herb distributed in South East Asian countries. It is locally known in Thai as "Sa-Bu-Leud" or blood soap due to its prominent characteristic red sap in the stem.[1,2] In Thai traditional medicine, *S. venosa* tuber has been widely used for the treatment of diabetes mellitus, cancer, and used as tonic drug.[3] Various biological activities of the tuber have been reported such as antimalarial, cytotoxic, antimicrobial, and acetylcholinesterase inhibitory activities.[4-7] The previous phytochemical studies on this plant have revealed the presence of isoquinoline alkaloids as major active compound. The tuber has been isolated and reported to contain crebanine, dicentrine, stephanine, tetrahydropalmatine, sukhodianine, dehydrostephanine, dehydrocrebanine, jatrorrhizine, and stepharine.[5,8]

The variation of chemical constituents in herbal extract possesses a great challenge to control the quality of herbal products. The qualitative and quantitative analyses are useful techniques to assess the safety and efficacy of plant material which mostly related to the content of marker compounds present in the plant. In general, the foremost characteristic red sap is absent where it provides in dry form or powdered drug. Thus, the analytical of active chemical markers is crucial for quality assessment of this plant. Crebanine and dicentrine are major characteristic components of this plant that also possesses various pharmacological activities[4-7] related to traditional uses. Thus, they were considered as chemical markers for quality assessment of *S. venosa*.

Various analytical methods were previously reported to isolate and determine alkaloid content in *S. venosa* tuber. TLC-densitometric method was developed to quantification of crebanine in *S. venosa* tuber.[9] Recently, HPLC-diode array detector (HPLC-DAD) was successfully developed for determination of crebanine, dicentrine, stephanine, and tetrahydropalmatine content in *S. venosa* tuber.[8] HPLC method is the most widely used to isolate, identify, and quantify the compounds from plant extracts and biological samples. Although HPLC provides rapid, reliable result, high sensitivity, and high reproducibility, HPLC seems to have some disadvantages over other analytical methods such as complexity, large quantities of solvents, and high cost. An alternative method is required to facilitate and reduces time of analysis with relatively few costs for routine analysis.

High-performance thin-layer chromatography (HPTLC) is a widely used technique for qualitative and quantitative

analysis of active compound in herbal products. It is also an ideal tool for identification of herbal materials. HPTLC which is simple, fast data acquisition, and cost efficiency, is considered as an alternative method for product quality assurance.[10] HPTLC can be couple with densitometric scanner or visualizing with optical scanner. The quantity of each analytes will be determined through their density using the processing software. Therefore, this study was aimed to develop and validate HPTLC densitometric and image analysis method for the quantification of crebanine and dicentrine in *S. venosa* tuber and their commercial products in Thailand.

MATERIALS AND METHODS

Chemicals and Reagents

Regents used in this study were of analytical grade. Dichloromethane, ethyl acetate, methanol, and ethanol were purchased from Labscan (Thailand). Deionized water was purified by Ultra Clear™ system (Siemens Water Technologies Corp.).

Standard crebanine and dicentrine were isolated in our previous study.[7,8] As described, the sample was macerated with methanol for 3 × 72 h with occasional shaking. The combined extract was filtered and concentrated using a rotary evaporator. The methanol crude extract was then partitioned with dichloromethane and water. The lipophilic layer, which contains alkaloids, was roughly separated by column chromatography (CC) (Merck silica gel 60, 70–230 mesh) with dichlomethane:ethyl acetate:methanol (70:25:5, v/v/v) as mobile phase. Fractions were monitored using TLC (silica get 60 F_{254}) sprayed with Dragendorff's reagent. Further, purification was made by CC (Merck silica gel 60, 230–400 mesh). The final cleaning up was carried out using a Sephadex LH-20 column eluted with methanol. The purity was assessed by TLC and HPLC.

Plant Materials

The tubers of *S. venosa* were collected from different provinces in Thailand as follows: (1) Kanchanaburi; (2) Nakhon Ratchasima; (3) Prachuap Khirikhan; (4) Roi Et; (5) Lampang; (6) Phen district, Udon Thani; (7) Nong Wua So district, Udon Thani; and (8) Uttaradit. Identification was done based on the key to species described in Flora of Thailand.[2] Voucher specimens (Voucher no. SK-SV001-008) were deposited at Drug Discovery and Development Center, Thammasat University, Thailand. Each sample was washed with tap water, cut into small pieces, and dried in a hot air oven (Memmert, Germany) at 50 °C for 72 h. The dried samples were ground into fine powder and kept at −20°C for further studies.

For commercial products, six samples of *S. venosa* tuber capsule were purchased from local herbal drug stores in Bangkok in September, 2018.

Brand I: *S. venosa* capsule: Each capsule contained 350 mg of *S. venosa*. (Mfg date: 05/01/2018 Exp date: 05/01/2021)

Brand II: *S. venosa* capsule: Each capsule contained 500 mg of *S. venosa*. (Exp date: July 1, 2019)

Brand III: *S. venosa* capsule: Each capsule contained 500 mg of *S. venosa*. (Mfg date: July, 7 2018)

Brand IV: *S. venosa* capsule: Each capsule contained 350 mg of *S. venosa*. (Exp date: February 25, 2019)

Brand V: *S. venosa* capsule: Each capsule contained 300 mg of *S. venosa*. (Mfg date: February 17, 2017 Exp date: February 17, 2019)

Brand VI: *S. venosa* capsule: Each capsule contained 350 mg of *S. venosa*. (Exp date: January 19, 2020).

Preparation of Sample Solution

Optimization the extracting solvent for the highest alkaloids recovery from the plant matrix was done in our previous study.[8] The mixture of water-methanol at ratio of 30:70 (v/v) yielded the highest alkaloids was chosen as a suitable solvent for extraction. Sonication was used as an extraction method due to its simplicity and rapidly.

For HPTLC densitometric and TLC image analysis method, two portions of each sample were accurately weighed 0.1 g and 0.2 g, respectively, and then extracted by sonication for 30 min with 70% methanol and adjusted to 10 ml in volumetric flask. Each solution was filtered through a 0.45 μm nylon membrane filter before being applied to the HPTLC plate, respectively. For commercial products, each sample was ground to pass through a 0.5 mm sieve, and then extracted with 70% methanol as described above.

Preparation of Standard Solution

Standards of crebanine and dicentrine were accurately weighed and dissolved in methanol in a volumetric flask for the preparation of stock solutions (1 mg/ml). Working standard solutions of crebanine and dicentrine were prepared to obtain final concentration of 100 μg/ml for HPTLC densitometric and 500 μg/ml for TLC image analysis method, respectively.

Instrument and Chromatographic Condition

HPTLC was performed on an aluminum sheet of silica gel 60 F254 (20 cm x 10 cm, Cat. No. 1.05548.0001, Merck). Sample and standard solutions were applied to the plate as 7 mm bands with a Linomat V automatic sample spotter (Camag, Switzerland) under nitrogen flow, positioned at 10 mm from the bottom of the plate. The constant application rate was 150 nl/s. The mobile phase consisted of dichloromethane:ethyl acetate:methanol (21: 6: 3, *v/v/v*). The Camag twin through chamber was saturated with mobile phase about 30 min before development, then the plate was developed to a distance of 8 cm. Densitometric scanning was performed using a TLC scanner-3 (Camag, Switzerland) with WinCATs software. The wavelength of detection was set at 290 nm. The dimension of the slit was set at 6.0 × 0.45 mm.

For image analysis method, the thin-layer chromatogram was performed on an aluminum sheet of silica gel 60 F254 (20 cm × 10 cm, Cat. No. 1.05554.0001, Merck). TLC plate was carried out under the chromatographic condition described above. The developed TLC plate was dipped into Dragendorff's reagent and heat-dried on the plate heater (Camag, Switzerland) at 110°C for 5 min. Then, the TLC plate was optical scanned by a flatbed scanner (EPSON ME OFFICE 620F) at a resolution of 300 dpi. The image was saved as a

joint photographic expert group (JPEG) file for further image processing with ImageJ 1.52h image processing program (National Institutes of Health, USA). After preprocessing step, manual region of interest (ROI) of an image was selected, then followed by gel analyze option to create line profile plots. The area of the peak corresponding to the concentration of crebanine and dicentrine standard was integrated and used for quantification.

Method Validation

The method was validated by evaluation of linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ) according to the International Conference on Harmonization guideline.[11]

Linearity

From working standard solutions of crebanine and dicentrine (100 and 500 µg/ml), 1–5 µl of each solution was applied to the HPTLC and TLC plate, corresponding to concentrations of 100–500 and 500–2500 ng/band for HPTLC densitometric and TLC image analysis method. Calibration curves were obtained by plotting the peak area versus the concentration of each standard.

Precision

The precision of the analytical method for each compound was determined by analyzing 6 replicates of sample from Phen district, Udonthani province after applying to HPTLC and TLC plate on the same day for repeatability and on three different days for intermediate precision. The precision was expressed as percent relative standard deviation (% RSD).

Accuracy

The accuracy of the method for each compound was evaluated by determination of recovery. The recovery of crebanine and dicentrine was performed on a sample spiked with three concentration levels of standards (approximately 50%, 100%, and 150% of the determined content of sample) (n = 3). The recovery was calculated as recovery (%) = 100 x (amount found–original amount)/amount spiked.

Limit of detection (LOD) and limit of quantitation (LOQ)

For HPTLC densitometric method, LOD and LOQ were determined based on the standard deviation of y-intercepts of regression lines (SD) and the slope of the calibration curve (S) of the sample in the range of LOD, LOQ according to the formula: LOD = 3.3(SD/S) and LOQ = 10(SD/S). For TLC image analysis method, LOD and LOQ were determined based on visual evaluation of the orange spot on the TLC plate after dipped into Dragendorff's reagent.

Quantitative Analysis of Crebanine and Dicentrine Content in *S. venosa* Tuber

Two microliters of each HPTLC samples (10 mg/ml) and 15 µl of each image analysis method samples (20 mg/ml) were applied to a HPTLC and TLC plate, respectively. In order to obtain approximately 20 μg/b and and 300 μg/band of the sample, two microliters of each HPTLC samples (10 mg/ml) and 15 μl of each image analysis method samples (20 mg/ml) were applied to a HPTLC and TLC plate, respectively, solution. The samples were analyzed by the proposed HPTLC densitometric and TLC image analysis method as described above. The sample was analyzed in triplicate and expressed as mean ± SD (n = 3).

Statistical Evaluation

The mean value of crebanine and dicentrine content in *S. venosa* tuber determined by HPTLC and image analysis method was tested by paired *t*-test at 95% confident level.

RESULTS AND DISCUSSION

A HPTLC densitometric and TLC image analysis were developed for analysis the major alkaloids, crebanine, and dicentrine in *S. venosa* roots. In our previous paper,[8] we reported the quantitative analysis of dicentrine, crebanine, tetrahydropalmatine, and stephanine using HPLC technique. However, tetrahydropalmatine and stephanine were presented in small amounts and could not simultaneously quantify together with their major compounds using TLC technique. Therefore, in this study, only the major alkaloids, crebanine and dicentrine in *S. venosa* tubers were demonstrated.

HPTLC was performed on an aluminum sheet of silica gel 60 F254 (20 cm × 10 cm, Cat. No. 1.05548.0001, Merck). Optimization of the mobile phase compositions was done. The mobile phase consisted of dichloromethane:ethyl acetate:methanol (21:6:3, *v/v/v*) gave the best peak resolution. The band of crebanine (Rf = 0.36) and dicentrine (Rf = 0.29) were found in the HPTLC chromatogram and separated from other components. The specificity of the bands of crebanine and dicentrine in *S. venosa* tuber was confirmed by overlaying the absorption spectra of the samples with crebanine and dicentrine reference standards [Figure 1]. The specificity of the analyzed peaks was checked at three different peak levels, that is, start, apex, and end positions of the peak corresponding to crebanine and dicentrine. The maximum absorbance of crebanine and dicentrine was 290 and 315 nm, respectively. Thus, the wavelength at 290 nm was chosen for the analysis.

Quantification of crebanine and dicentrine was done using UV-densitometric scanner and image analysis after post-derivatization with Dragendorff's reagent. UV-densitometric scanning was performed using a TLC scanner 3 (Camag, Switzerland) with WinCATs software, while image analysis was performed using ImageJ software which is a public domain Java image processing software that was developed by the National Institutes of Health, USA. This software can be freely downloaded through http://rsb.info.nih.gov/ij/. Numerous studies using image processing software as an application to determine the content of marker compound of herbal and pharmaceutical products have been reported.[12-16] This method is simple, inexpensive, and convenient quantitation method with good accuracy and precision for bioactive components in herbal and crude drugs.[17]

Linearity, precision, accuracy, LOD, and LOQ were analyzed for method validation parameters.[11] The proposed HPTLC and image analysis method showed acceptable validation parameters [Table 1]. Comparing the validation parameters of HPTLC and image analysis methods, both methods showed good linear relationship, high accuracy, and acceptable % RSD values of

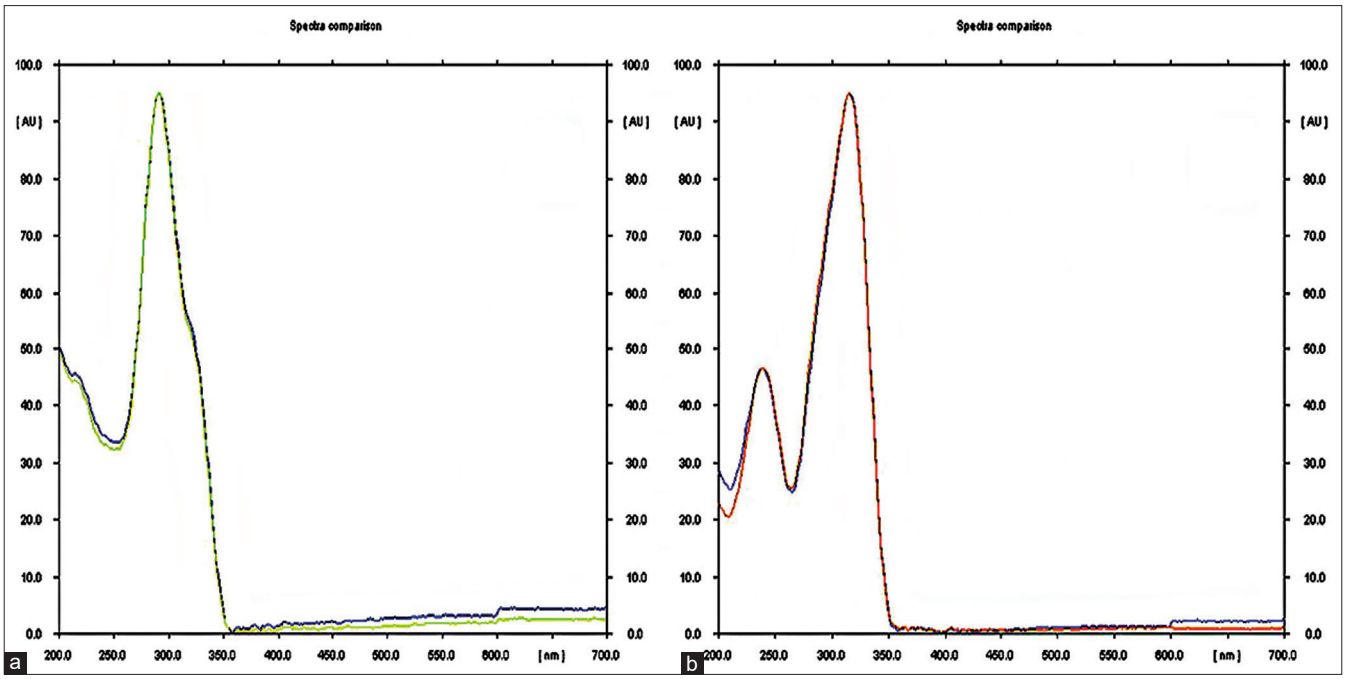

Figure 1: Overlay UV spectra scanning from 200 to 700 nm by HPTLC method of A) crebanine reference standard and sample and B) dicentrine reference standard and sample

Table 1: Validation parameters by the proposed HPTLC and image analysis method

Parameter	HPTLC		Image analysis	
	Crebanine	Dicentrine	Crebanine	Dicentrine
Range of linearity	100–500 ng/band	100–500 ng/band	500–2500 ng/band	500–2500 ng/band
Regression equation (n=5)	Y = 17.48X+1359.41	Y = 11.40X + 907.46	Y = 8.03X - 1063.34	Y = 8.51X - 1462.39
Coefficient of Determination (r^2)	0.9960±0.0010	0.9964±0.0019	0.9985±0.0009	0.9985±0.0008
% Recovery	100.27%±2.65 –101.30% ± 0.91	98.37%±2.28 – 101.33%±3.86	101.34%±1.57 – 101.92%±1.00	99.87%±0.65 – 102.83%±2.38
Repeatability (% RSD)	1.89%	1.35%	3.83%	3.55%
Intermediate precision (%RSD)	3.89%	3.44%	7.09%	8.45%
Limit of detection (LOD)	20.77 ng/band	16.82 ng/band	500 ng/band	500 ng/band
Limit of quantitation (LOQ)	62.93 ng/band	50.96 ng/band	500 ng/band	500 ng/band

X = concentration of crebanine and dicentrine in ng/μL, Y = peak area

repeatability and intermediate precisions. The sensitivity of measurement was estimated in terms of LOD and LOQ. For HPTLC method, LOD and LOQ were determined based on the standard deviation of y-intercepts of regression lines (SD) and the slope of the calibration curve (S) of the sample in the range of LOD and LOQ and found to be 20.77 and 62.93 ng/band for crebanine and 16.82 and 50.96 ng/band for dicentrine, respectively. For image analysis method, LOD and LOQ were determined based on visual evaluation of the orange spot on the TLC plate after dipped into Dragendorff's reagent, which the lowest concentration of 500 ng/band could be detected with the naked eye. LOD and LOQ of image analysis method were about 25 and 10 times higher than those of HPTLC parameters. Thus, it revealed lower sensitivity of image analysis method than HPTLC method.

HPTLC chromatograms of *S. venosa* tuber from different provenances and crebanine and dicentrine standard are shown in Figure 2. The content of crebanine and dicentrine of *S. venosa* tuber from different provinces was in the ranges of <0.31 to 1.64 ± 0.18% and <0.25 to 1.31 ± 0.11 % w/w, respectively [Table 2]. From the results, *S. venosa* tuber from Udon Thani Province contained both crebanine and dicentrine, while the others contained crebanine as a major compound. The result was in accordance with our previous HPLC study,[8] there was a remarkable variation in the accumulation of alkaloids in each population and the between individual in the same population. From the results, HPTLC has much lower plate counts and therefore separation power than that of HPLC. Particularly for complex sample like herbal product which is difficult to achieve sufficient resolution for all components.[18]

HPTLC chromatograms of *S. venosa* commercial products are shown in Figure 3. For *S. venosa* commercial products, the contents of crebanine and dicentrine were in the ranges of <0.31–1.20±0.21 %w/w, and <0.25 %w/w, respectively [Table 2]. From the results, Brand I and V showed identical

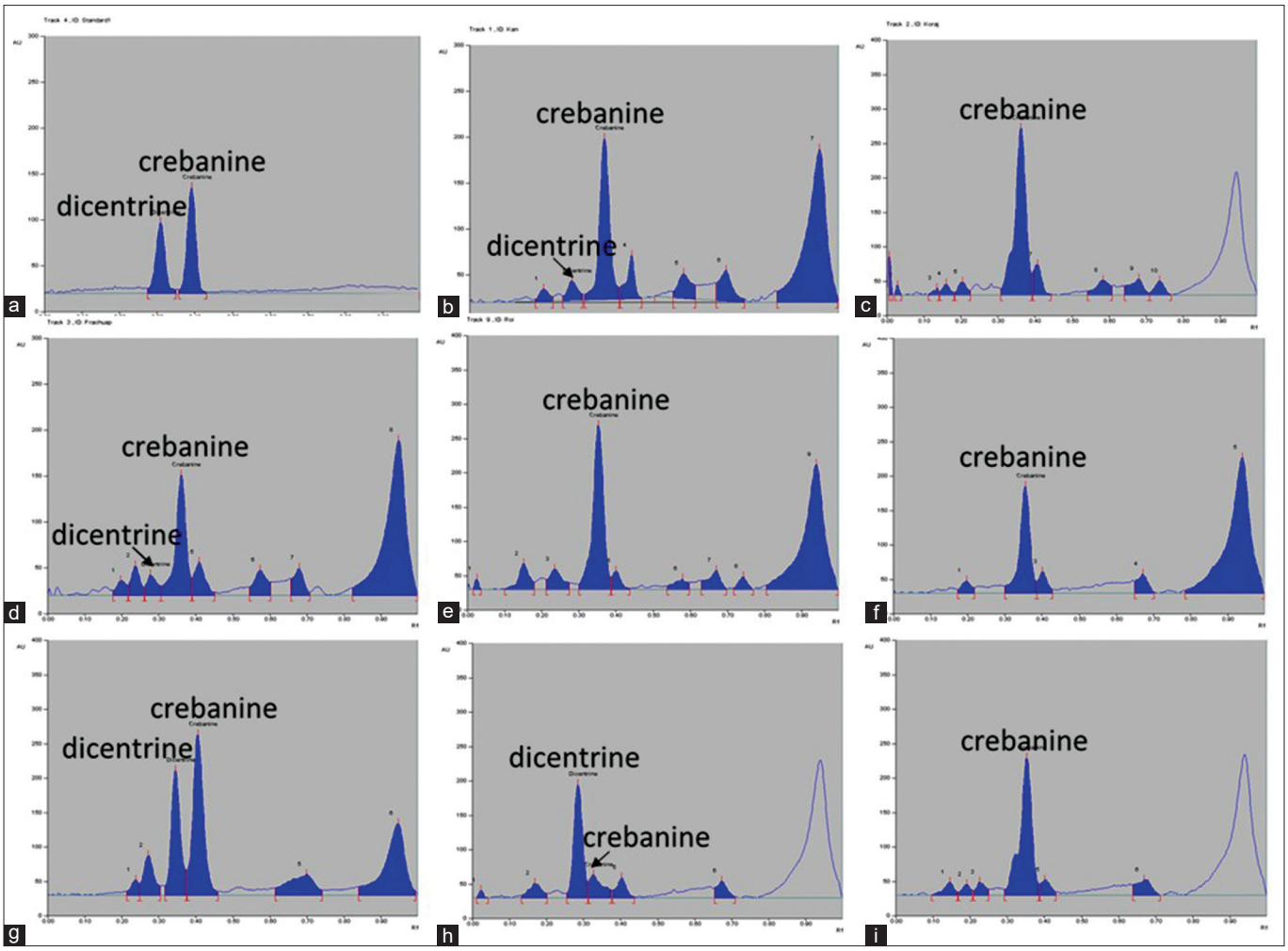

Figure 2: HPTLC chromatograms of *S. venosa* tuber from different provinces, A) crebanine and dicentrine reference standard, B) Kanchanaburi, C) Nakhon Ratchasima, D) Prachuap Khirikhan, E) Roi Et, F) Lampang, G) Phen district, Udon Thani, H) Nong Wua So district, Udon Thani, and I) Uttaradit

Table 2: The content of crebanine and dicentrine in *S. venosa* samples determined by validated HPTLC and image analysis method

Sample	HPTLC (% w/w)*		Image analysis (% w/w)*	
	Crebanine	Dicentrine	Crebanine	Dicentrine
Kanchanaburi	0.92±0.04	<0.25	0.87±0.01	-
Nakhon Ratchasima	1.61±0.09	-	1.61±0.07	-
Prachuap Khirikhan	0.53±0.01	<0.25	0.57±0.01	-
Roi Et	1.64±0.18	-	1.28±0.03	-
Lampang	0.80±0.01	-	0.77±0.02	-
Udon Thani (Phen)	0.71±0.01	0.48±0.01	0.70±0.02	0.45±0.01
Udon Thani (Nong Wua So)	<0.31	1.31±0.11	<0.17	1.64±0.03
Uttaradit	1.37±0.08	-	1.38±0.06	-
Brand I	<0.31	<0.25	-	-
Brand II	-	-	-	-
Brand III	-	-	-	-
Brand IV	-	-	-	-
Brand V	1.20±0.21	-	0.93±0.01	-
Brand VI	-	-	-	-

*Expressed as mean±SD (n=3)

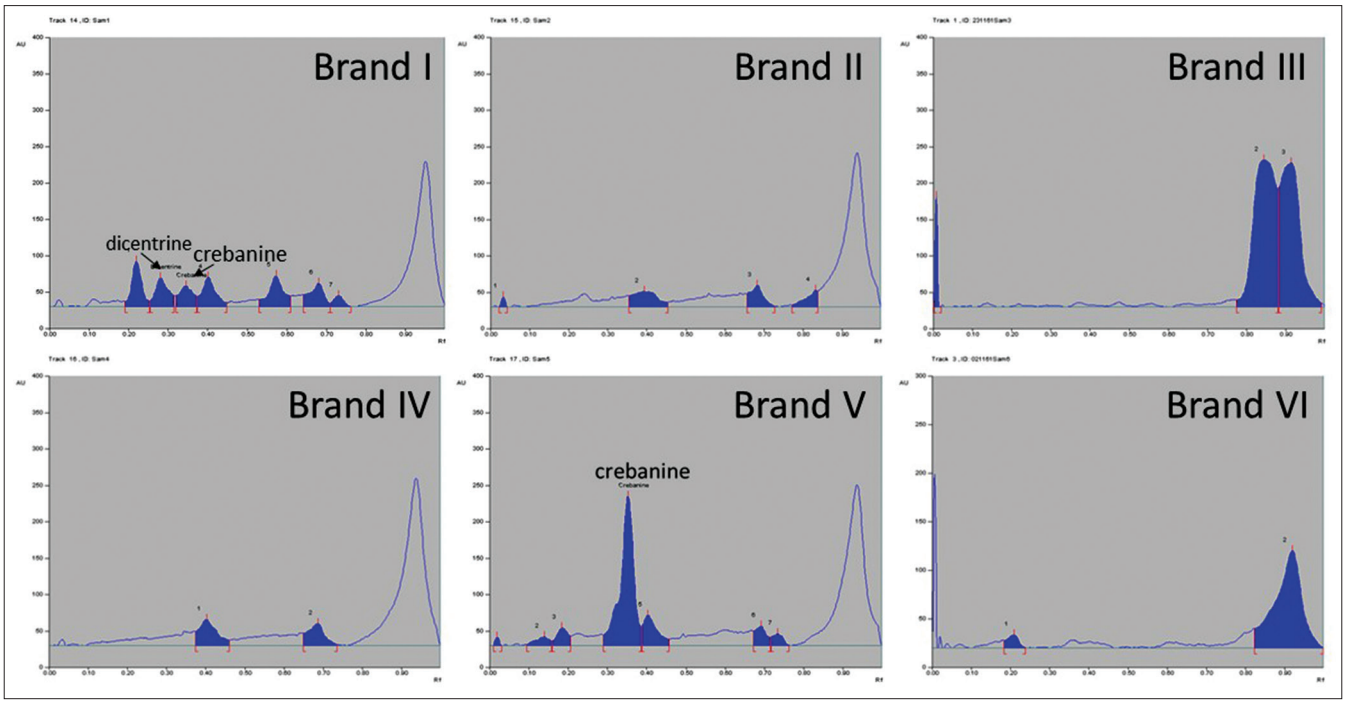

Figure 3: HPTLC chromatograms of *S. venosa* commercial products

HPTLC chromatogram with major spot of crebanine, while the chromatogram of Brand II, III, IV, and VI did not show the peak of crebanine and dicentrine. Figure 4 showed the HPTLC fingerprints of *S. venosa* tuber and their commercial products. HPTLC fingerprints provide the characteristic chromatographic pattern of chemical constituents present in the plant samples. Visual comparison of HPTLC fingerprint is a convenient method of initial screening and can be generated primarily for identification.[19] Moreover, after derivatization the plate with Dragendorff's reagent, the orange spot confirmed the presence of alkaloids in the samples. In Thailand, *S. venosa* has many vernacular names in various regions of the country. This may lead to wrong plant identification. This HPTLC fingerprint identified *S. venosa* tuber and discriminates the tuber of *Stephania pierrei* Diels and *Jatropha gossypifolia* L. (Euphorbiaceae) which have similar vernacular name in Thai. The HPTLC fingerprint could ensure the quality and prevent adulteration of its raw material. Other possible reasons of wrong plant identification may due to lack of authentic raw plant material or similarity of the characteristic of the plants.[20]

In the closure look of TLC image analysis method, the TLC chromatogram of *S. venosa* tuber exhibited an orange spot with similar *Rf* and color as crebanine standard spot after derivatization with Dragendorff's reagent. A minor orange spot, just below crebanine spot, was correspond to dicentrine standard [Figure 5]. The content of crebanine and dicentrine of *S. venosa* tuber from different provinces and commercial products analyzed by image analysis method is shown in Table 2. The results of paired *t* test suggested that there were no significant differences ($P > 0.05$) between the mean contents of crebanine and dicentrine analyzed by HPTLC and image analysis method.

The safety, efficacy, and quality of herbal products also depend on the quality of the raw materials used. Requirements and analytical methods for quality control of finished herbal products

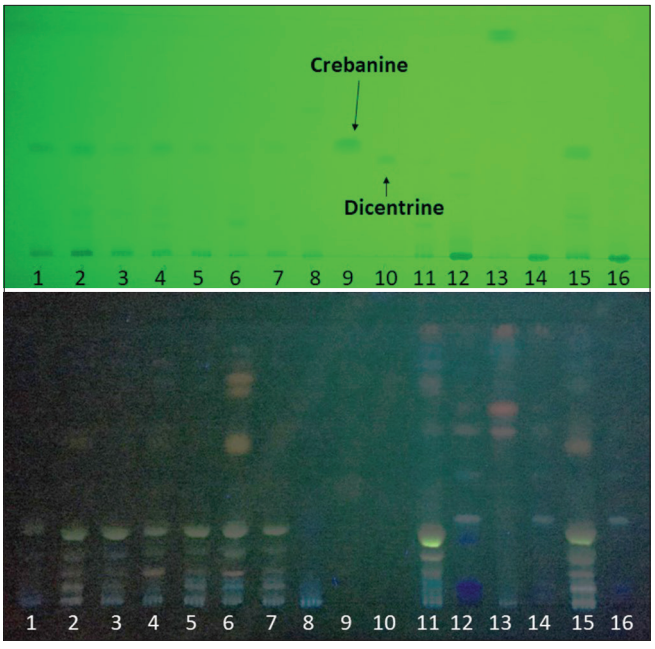

Figure 4: HPTLC fingerprints of S. venosa tuber from different provinces and commercial products (mobile phase:dichloromethane:ethyl acetate:methanol, 21:6:3 v/v/v); track 1 Kanchanaburi, track 2 Nakhon Ratchasima, track 3 Prachuap Khirikhan, track 4 Roi Et, track 5 Lampang, track 6 Phen district, Udon Thani, track 7 Nong Wua So district, Udon Thani, track 8 Uttaradit, track 9 crebanine, track 10 dicentrine, and track 11-16 commercial products Brand I-VI, A) detected under UV 254 nm, B) detected under UV 366 nm

are still in need. Due to simplicity and low operation cost of both analytical methods, they provide product quality assessment capability for small herbal product manufacturers. Both HPTLC and image analysis methods have specificity and suitable to

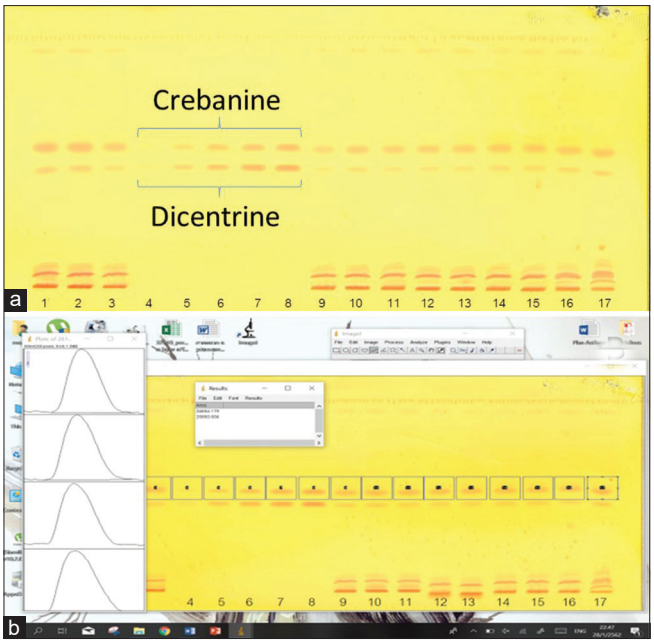

Figure 5: A) HPTLC fingerprints of S. venosa tuber from Phen district, Udon Thani (mobile phase: dichloromethane : ethyl acetate : methanol, 21 : 6 : 3 v/v/v) after derivatization with Dragendorff's reagent. Track 1-3: sample # 1, track 4-8: crebanine and dicentrine reference standard, track 9-11: sample #2, track 12-14: sample #3, and track 15-17: sample #4. B) ImageJ processing method by Image J 1.52h software

discriminate right from wrong plant. It has sufficient sensitivity for detecting small amounts of adulterant. HPTLC is a simple, rapid, high sample throughput, and cost-effective analytical techniques. Image analysis method takes an advantage of using visual evaluation and low-cost equipment for quantification.

CONCLUSION

High-performance thin-layer chromatography (HPTLC) densitometric and thin-layer chromatography (TLC) image analysis method have been developed and validated for the quantification of crebanine and dicentrine in *Stephania venosa* tuber and their commercial products. Both HPTLC and image analysis method have specificity and suitable to discriminate right from wrong plant and can be used as an alternative analytical method in the routine quality control analysis of *S. venosa* tuber commercial products and its raw material.

ACKNOWLEDGMENTS

The authors thank Faculty of Pharmacy, Mahidol University, Thailand, for the laboratory facilities. Partial of this work was supported by Thammasat University under Thai Government Research Fund (Contract No. 56/2559).

REFERENCES

1. Charles B, Bruneton J, Pharadai K, Tantisewie B, Guinaudeau H, Shamma M. Some unusual proaporphine and aporphine alkaloids from *Stephania venosa*. J Nat Prod 1987;50:1113-7.
2. Forman LL. Menispermaceae. Fl Thai 1991;5:300-65.
3. Chuakul W, Saralamp P, Paonil W, Temsiririrkkul R, Clayton T. Medicinal Plants in Thailand. Vol. 2. Bangkok: Department of Pharmaceutical Botany Faculty of Pharmacy Mahidol University; 1997. p. 206.
4. Ingkaninan K, Phengpa P, Yuenyongsawad S, Khorana N. Acetylcholinesterase inhibitors from *Stephania venosa* tuber. J Pharm Pharmacol 2006;58:695-700.
5. Likhitwitayawuid K, Dej-adisai S, Jongbunprasert V, Krungkrai J. Antimalarials from *Stephania venosa*, *Prismatomeris sessiliflora*, *Diospyros montana* and *Murraya siamensis*. Planta Med 1999;65:754-6.
6. Makarasen A, Sirithana W, Mogkhuntod S, Khunnawutmanotham N, Chimnoi N, Techasakul S. Cytotoxic and antimicrobial activities of aporphine alkaloids isolated from *Stephania venosa* (Blume) Spreng. Planta Med 2011;77:1519-24.
7. Kongkiatpaiboon S, Duangdee N, Prateeptongkum S, Chaijaroenkul W. Acetylcholinesterase inhibitory activity of alkaloids isolated from *Stephania venosa*. Nat Prod Commun 2016;11:1805-6.
8. Kongkiatpaiboon S, Duangdee N, Prateeptongkum S, Tayanaa N, Inthakusola W. Simultaneous HPLC analysis of crebanine, dicentine, stephanine and tetrahydropalmatine in *Stephania venosa*. Rev Bras Farmacogn 2017;27:691-7.
9. Bongkod W, Vallisuta O, Ruangwises N, Mitrevej A. TLC-Densitometric method for the quantification of crebanine in *Stephania venosa* (Bl.) Spreng. J Planar Chromatogr 2011;24:264-7.
10. Urakova IN, Pozharitskaya ON, Shikov AN, Kosman VM, Makarov VG. Comparison of high-performance TLC and HPLC for separation and quantification of chlorogenic acid in green coffee bean extracts. J Sep Sci 2008;31:237-41.
11. ICH. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. In: Validation of Analytical Procedures: Text and Methodology. Geneva: ICH; 2005.
12. Phattanawasin P, Sotanaphun U, Sriphong L. Validated TLC-image analysis method for simultaneous quantification of curcuminoids in *Curcuma longa*. Chromatographia 2009;69:397-400.
13. Soponar F, Cătălin A, Sârbu C. Quantitative evaluation of paracetamol and caffeine from pharmaceutical preparations using image analysis and RP-TLC. Chromatographia 2009;69:151-5.
14. Kerr E, West C, Hartwell SK. Quantitative TLC-image analysis of urinary creatinine using iodine staining and RGB values. J Chromatogr Sci 2016;54:639-46.
15. Changwichit K, Girard C, Temkitthawon P, Khorana N, Ingkaninan K. Quantitative analysis of a phenanthrene from *Eulophia* species by TLC-image analysis method. Songklanakarin J Sci Tech 2018;40:1324-8.
16. Kongkiatpaiboon S, Keeratinijakal V, Gritsanapan W. TLC-image analysis of non-chromophoric tuberostemonine alkaloid derivatives in *Stemona* species. Nat Prod Commun 2013;8:1065-8.
17. Ketmongkhonsit P, Chaichantipyuth C, Palanuvej C, Thitikornpong W, Sukrong S. A validated TLC-image analysis method for detecting and quantifying bioactive phyllanthin in *Phyllanthus amarus* and commercial herbal drugs. Songklanakarin J Sci Tech 2015;37:319-26.
18. Kaale E, Risha P, Layoff T. TLC for pharmaceutical analysis in resource limited countries. J Chromatogr A 2011;1218:2732-6.
19. Ram M, Abdin MZ, Khan MA, Jha P. HPTLC fingerprint analysis: A quality control for authentication of herbal phytochemicals. In: Srivastava M, editor. High-Performance Thin-layer Chromatography (HPTLC). Berlin: Springer; 2011. p. 105-16.
20. Seethapathy GS, Ganesh D, Kumar JV, Senthikumar U, Newmaster SG, Ragupathy S, *et al*. Assessing product adulteration in natural health products for laxative yielding plants, *Cassia*, *Senna*, and *Chamaecrista*, in Southern India using DNA barcoding. Int J Legal Med 2015;129:693-700.