Revista Brasileira de Farmacognosia 27 (2017) 691–697

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Simultaneous HPLC analysis of crebanine, dicentrine, stephanine and tetrahydropalmatine in *Stephania venosa*

Sumet Kongkiatpaiboon[a,*], Nongnaphat Duangdee[a], Saisuree Prateeptongkum[b], Ngampuk Tayana[a], Wichayasith Inthakusol[a]

[a] *Drug Discovery and Development Center, Thammasat University, Rangsit Campus, Pathumthani, Thailand*
[b] *Department of Chemistry, Faculty of Science and Technology, Thammasat University, Rangsit Campus, Pathumthani, Thailand*

ARTICLE INFO

Article history:
Received 5 September 2017
Accepted 24 October 2017
Available online 15 November 2017

Keywords:
Alkaloids
Standardization
Menispermaceae
HPLC
Quantitative analysis

ABSTRACT

Stephania venosa (Blume) Spreng., Menispermaceae, has been traditionally used as tonic drug and treatment of various diseases in South East Asian countries. In order to evaluate the quality and standardization of *S. venosa* roots, the HPLC method for quantification of the content of major components in *S. venosa* was developed and validated. The chromatographic separation was performed on a Hypersil BDS C_{18} column using gradient system of 100 mM ammonium acetate in water and methanol with flow rate 1 ml/min. Detection wavelength was set at 210 nm for tetrahydropalmatine, 280 nm for dicentrine and crebanine, and 270 nm for stephanine. The validated method showed good sensitivity, linearity, precision, and accuracy. The suitable solvent that yielded highest alkaloids contents from the matrix was optimized. *S. venosa* samples collected from various locations were analyzed. The present study provided comprehensive overview of major components in *S. venosa*. A remarkable variation in the accumulation of alkaloids in each population and the between individual in the same population could be observed. Our results showed the heterogeneity of *S. venosa* in Thailand which would need a further study for species delimitations.

Introduction

Stephania venosa (Blume) Spreng., vernacularly named in Thai as "Sa-Bu-Leud", belongs to the Menispermaceae family (Forman, 1991). This plant is a vine indigenous medicinal herb distributed in South East Asian countries. The prominent red sap in its stem is a characteristic key for the species identification. It has been traditionally used as a tonic drug, for treatment of cancer and diabetes, aphrodisiac, and for various indications (Ingkaninan et al., 2006; Kongkiatpaiboon et al., 2016). Important biological activities have been reported including antimalarial (Likhitwitayawuid et al., 1999), cytotoxicity against cancer cell lines (Makarasen et al., 2011), antimicrobial (Makarasen et al., 2011), and acetylcholinesterase inhibition (Ingkaninan et al., 2006; Kongkiatpaiboon et al., 2016). Some cellular mechanisms have been explored. Crebanine, a major component of *S. venosa*, exerts anti-proliferative effects on human cancer cells through the induction of cell cycle arrest at the G1 phases and apoptosis (Wongsirisin et al., 2012). Stephanine, the alkaloid from *S. venosa*, could induce the reverse of mitotic exit, eventually leading to cell death by apoptosis (Le et al., 2017). L-Tetrahydropalmatine, the alkaloid from *S. venosa* which acts as a dopamine receptor agonist, could attenuate cocaine and methamphetamine self-administration and cocaine- and methamphetamine-induced reinstatement in rats (Mantsch et al., 2007; Gong et al., 2016), modulate methamphetamine reward behavior (Su et al., 2013), and inhibit the acquisition of ketamine-induced conditioned place preference by regulating the expression of extracellular signal-regulated kinases (ERK) and cAMP response element-binding protein (CREB) regulation in rats (Du et al., 2017). Dicentrine, a known alpha 1-adrenoceptor antagonist, could have a therapeutic potential to develop as antihypertensive, antihyperlipidemic and other cardiovascular drugs (Su et al., 1994; Yu et al., 1994a,b).

The local widespread uses of *Stephania* for medical properties has led to an increasing interest in this plant. The reported data showed that alkaloids are the main phytochemical constituents of this genus (Semwal et al., 2010). Although, isolation and elucidation of phytochemical constituents have been extensively done and some analytical methods have been developed in another *Stephania* species (Dary et al., 2017; He et al., 2016), there are the needed

* Corresponding author.
E-mail: sumet_k@tu.ac.th (S. Kongkiatpaiboon).

https://doi.org/10.1016/j.bjp.2017.10.004

responses of rapid and simultaneous analytical method for evaluating the quality and standardization of *S. venosa* roots. Therefore, HPLC method for quantification of the content of major components in *S. venosa* was developed and validated. Extraction efficiency caused by different solvent polarity was studied and optimized. Various sources of *S. venosa* samples were analyzed. The present paper should provide a basis for quality assessment and standardization of this plant for the development of phytopharmaceutical products.

Material and methods

Chemical and reagents

HPLC grade methanol was obtained from Labscan (Thailand). Deionized water was purified by Ultra Clear™ system (Siemens Water Technologies Corp.). Ammonium acetate and all reagents were of analytical grade if not state otherwise.

Plant materials

Samples representing *Stephania venosa* (Blume) Spreng., Menispermaceae, were obtained from various location in Thailand (Table 1). Identification was done based on the key to species described in Flora of Thailand (Forman, 1991). Voucher specimens were deposited at Drug Discovery and Development Center, Thammasat University, Thailand. Each sample was thoroughly cleaned by tap water, cut into small pieces and dried in a hot air oven at 50 °C for 72 h. Each dried sample was ground into fine powder and kept in an air-tight container until used.

Extraction and isolation of major components

Dicentrine (**1**), tetrahydropalmatine (**2**), and crebanine (**3**) were isolated in our previous work (Kongkiatpaiboon et al., 2016). 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 dichloromethane: EtOAc:MeOH (70:25:5, v/v/v) as mobile phase. Fractions were monitored using TLC (silica gel 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 on a Sephadex LH-20 column eluted using methanol as eluent.

For the isolation of stephanine (**4**), the dried tuberous roots (500 g) of *S. venosa* were extracted by methanol (3 × 500 ml) for 3 × 72 h with occasional shaking at room temperature. The combined extract was filtered and evaporated under reduced pressure using a rotary evaporator to yield the methanolic crude extract (193.55 g). A portion of crude extract (5 g) was subjected to CC (Merck silica gel 60, 70–230 mesh) using mobile phase with increasing the polarity of ethyl acetate-dichloromethane (10:90) to ethyl acetate. The fractions were monitored using TLC (silica gel 60 F_{254}) sprayed with Dragendorff's reagent. Further purification was made with CC (Merck silica gel 60, 70–230 mesh) using EtOAc:hexane:MeOH (3:7:0.1, v/v/v) as the mobile phase to yield stephanine (**4**) 100 mg. The purity was assessed by TLC and HPLC. The isolated pure compound was characterized by comparing the melting point, ^{1}H NMR, ^{13}C NMR, and mass spectra with reported data (Blanchfield et al., 2003).

HPLC apparatus and conditions

HPLC was performed on an Agilent 1260 Series (Agilent Technologies) equipped with a 1260 Quat pump VL quaternary pump, 1260 ALS autosampler, 1260 TCC column thermostat, and 1260 DAD VL diode array detector. The separation was done on a Hypersil BDS C_{18} column (4.6 × 100 mm i.d., 3.5 μm) with a C_{18} guard column. The mobile phases were (A) 100 mM ammonium acetate in water and (B) methanol. Gradient elution was used from 50% B to 70% B in A for 20 min, 100% B for 10 min. The column was equilibrated with 50% B in A for 10 min prior to each analysis. The flow rate was set at 1 ml/min with controlled temperature at 25 °C. DAD detector was set at the wavelength of 210 nm for detection of tetrahydropalmatine, 280 nm for dicentrine and crebanine, and 270 nm for stephanine. The injection volume was 10 μl for every sample and standard.

Stock and working solutions standard compounds

Stock standard solution of tetrahydropalmatine, dicentrine, crebanine, and stephanine with purity more than 90% determined by HPLC was prepared by dissolving each standard compound in methanol to obtain the concentration of 1000 μg/ml. Working standard solutions were obtained by appropriate dilution of the stock solutions with methanol to obtained the desired concentration.

Investigation of suitable solvent for Stephania venosa *extraction*

To obtain the highest alkaloids content from *S. venosa* extraction, various solvents, i.e. water, methanol, ethanol, acetonitrile, and

Table 1
Percentage of dicentrine (**1**), tetrahydropalmatine (**2**), crebanine (**3**), and stephanine (**4**) in *Stephania venosa* collected from various locations of Thailand.

Location[a]	Content[b] (mg/g)			
	Dicentrine **(1)**	Tetrahydropalmatine **(2)**	Crebanine **(3)**	Stephanine **(4)**
Chiang Mai, Doi Ang Khang (N), Sample 1	17.08 ± 0.25	0.32 ± 0.03	12.33 ± 0.15	3.81 ± 0.06
Chiang Mai, Doi Ang Khang (N), Sample 2	–	1.48 ± 0.03	18.30 ± 0.21	0.49 ± 0.01
Chiang Mai, Doi Ang Khang (N), Sample 3	1.20 ± 0.01	–	16.52 ± 0.12	6.80 ± 0.06
Lampang, Muang (N)	–	1.31 ± 0.11	12.00 ± 0.65	<0.05
Uttaradit, Nampad (N)	–	1.78 ± 0.08	21.69 ± 0.19	0.38 ± 0.01
Kanchanaburi, Saiyok (SW)	–	0.87 ± 0.05	15.33 ± 0.16	0.32 ± 0.01
Prachuap Khiri Khan, Muang (SW)	–	6.10 ± 0.22	10.41 ± 0.21	<0.05
Roi-Et, Muang (NE)	–	5.47 ± 0.04	23.37 ± 0.14	0.05 ± 0.02
Udonthani, Phen (NE)	3.20 ± 0.04	0.61 ± 0.02	5.73 ± 0.05	1.00 ± 0.01
Udonthani, Nong Wua So (NE)	20.38 ± 0.72	1.61 ± 0.06	0.16 ± 0.02	–
Nakhon Ratchasima, Wangnumkheo (E)	–	9.19 ± 0.30	30.27 ± 0.90	<0.05

[a] Floristic regions of Thailand: N = northern, NE = northeastern, E = eastern, SW = southwestern.
[b] Expressed as mean ± SD.

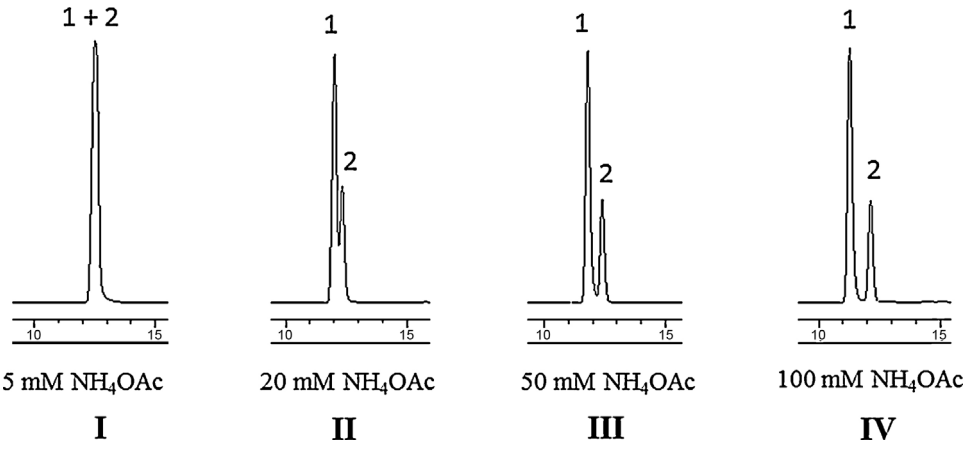


Fig. 1. Influence of ammonium acetate concentrations in the separation of dicentrine (**1**) and tetrahydropalmatine (**2**) in reversed-phase HPLC. Column: Hypersil BDS C_{18} (4.6 mm i.d. × 10 cm, 3.5 μm). Mobile phase: (A) ammonium acetate in water and (B) methanol. Gradient elution: 50% B in A to 70% B in A for 20 min; then 100% B for 10 min. Concentration of ammonium acetate solution: (I) 5 mM ammonium acetate solution, (II) 20 mM ammonium acetate solution, (III) 50 mM ammonium acetate solution, and (IV) 100 mM ammonium acetate solution; flow rate 1 ml/min, detection at 280 nm.

mixtures of water and methanol at different ratio, were evaluated as extracting solvents. Crude drug powder of *S. venosa* (50 mg) was accurately weighed and separately extracted with 5 ml of these solvents by sonication at the ambient temperature. Each extract was triplicately prepared and analyzed by the HPLC. The solvent yielding the highest content of alkaloids in the extract was chosen as the appropriate solvent for extraction.

Sample preparation

Each powdered sample of *S. venosa* roots was accurately weighed and extracted with water–methanol (30:70, v/v) at concentration of 10 mg/ml in an ultrasonic bath for each 30 min. Each sample was done in triplicate. Prior to injection, each solution was filtered through a 0.2 μm nylon membrane filter and analyzed with HPLC.

Method validation

Validation of the method was done according to the International Conference on Harmonization guideline (ICH, 1996/2005). The method was validated for linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ).

Linearity

Linearity of the method was studied by injecting seven known concentrations of the analytes in the range of 1.9–250 μg/ml in triplicate. The calibration curves were obtained by plotting the peak area versus the amount of the standard.

Precision

The measurement of intra- and inter-day precision was done by analyzing 50 μg/ml standard solution. The intra-day precision was determined by analyzing seven times within 1 day, while the inter-day precision was examined for three consecutive days by the proposed method. The precision was expressed as percent relative standard deviation (%RSD).

Accuracy

Recovery was used to evaluate the accuracy of the method. Standard addition was performed with pre-analyzed standard solution. Three different levels of standard mixtures were added to the sample extract. Spike samples were prepared in triplicate. The recovery was calculated as follows: recovery (%) = 100 × (amount found − original amount)/amount spiked.

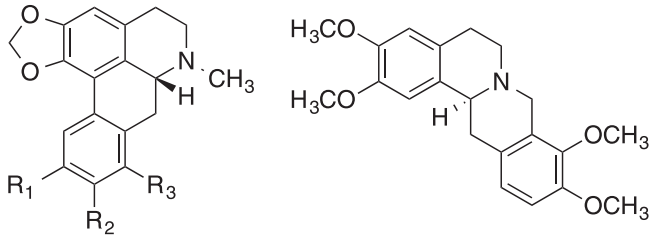
Limit of detection and limit of quantitation

Determination of signal-to-noise ratio was calculated under the proposed chromatographic condition. LOD was consider as 3:1 and LOQ as 10:1.

Results

HPLC method development

A HPLC method was developed for analysis the contents of major alkaloids, dicentrine (**1**), tetrahydropalmatine (**2**), crebanine (**3**), and stephanine (**4**) in *S. venosa* roots. Optimization of the mobile phase compositions was done. Reversed-phase C-18 column which is broadly used in pharmaceutical separation, was used in this study. The critical separation of **1** and **2** affected by ammonium acetate concentrations are shown in Fig. 1. From various mobile phases trialed, the system containing gradient solvent system using 100 mM ammonium acetate and water gave the symmetric peaks and provided the most efficient separation and speed (Fig. 1). The wavelength at 210 nm which gave high absorbance capacity was used for detecting tetrahydropalmatine (**2**), at 280 nm for dicentrine (**1**) and crebanine (**3**), and 270 nm for stephanine (**4**).



1 R_1=R_2=OCH_3; R_2=H
3 R_1=H; R_2=R_3=OCH_3
4 R_1=R_2=H; R_3=OCH_3

2

Suitable solvent for extracting alkaloids from Stephania venosa

In the course of optimizing the extracting solvent for the highest alkaloids recovery from a complex plant matrix, various solvents, i.e. water, methanol, ethanol, acetonitrile, and mixtures of water and methanol at different ratio, were evaluated as extracting sol-

Table 2
Alkaloids contents in *Stephania venosa* from Nampad district of Uttaradit province of Thailand (northern floristic regions of Thailand) from various extracting solvents.

Extracting solvents	Content[a] (mg/g)		
	Tetrahydropalmatine (**2**)	Crebanine (**3**)	Stephanine (**4**)
Water	1.48 ± 0.02	14.15 ± 0.40	0.11 ± 0.01
Water–methanol (70:30, v/v)	1.76 ± 0.07	19.28 ± 0.24	0.27 ± 0.01
Water–methanol (50:50, v/v)	1.77 ± 0.03	21.21 ± 0.25	0.35 ± 0.01
Water–methanol (30:70, v/v)	1.81 ± 0.06	21.69 ± 0.19	0.38 ± 0.01
Methanol	1.59 ± 0.06	17.67 ± 0.47	0.28 ± 0.01
Ethanol	0.95 ± 0.02	12.52 ± 0.19	0.11 ± 0.01
Acetonitrile	0.53 ± 0.03	8.65 ± 0.26	<0.05

[a] Expressed as mean ± SD ($n = 3$).

Table 3
Method validation parameters for the quantification of dicentrine (**1**), tetrahydropalmatine (**2**), crebanine (**3**), and stephanine (**4**).

Parameters	Results			
	Dicentrine (**1**)	Tetrahydropalmatine (**2**)	Crebanine (**3**)	Stephanine (**4**)
Regression equation[a]	$Y = 26.626X + 46.607$	$Y = 70.037X + 146.33$	$Y = 32.897X + 65.959$	$Y = 22.38 + 43.673$
Correlation coefficient (r^2)	0.9998	0.9981	0.9998	0.9998
Linear range, μg/ml	1.9–250	1.9–125	1.9–250	1.9–250
LOD, μg/ml	0.1	0.3	0.1	0.1
LOQ, μg/ml	0.3	1	0.3	0.3

[a] X is the concentration of each standard in μg/ml; Y is the peak area at 280 nm for dicentrine (**1**) and crebanine (**3**), 210 for tetrahydropalmatine (**2**), and 270 for stephanine (**4**).

vents. Sonication was chosen as an extraction method due to its simplicity, rapidity and compatibility with various solvents. After quantification by HPLC, the highest alkaloids content was found in the water-methanol (30:70, v/v) extract as shown in Table 2. Although, tetrahydropalmatine (**2**) and crebanine (**3**) extracted from mixture of water–methanol at ratio of 50:50 and 30:70 (v/v) were not significantly different. Mixture of water–methanol at ratio of 30:70 (v/v) could extract the highest stephanine (**4**) content compared to the other solvents. Thus, mixture of water–methanol at ratio of 30:70 (v/v) was chosen as a suitable solvent for extraction.

Method validation

The HPLC method was validated for analysis of the dicentrine (**1**), tetrahydropalmatine (**2**), crebanine (**3**), and stephanine (**4**) in *S. venosa* roots. Linearity, precision, accuracy, LOD, and LOQ were analyzed for method validation parameters (ICH, 1996/2005). Linearity was evaluated by using standard solutions dissolved in methanol at concentrations in the range of 1.9–250 μg/ml for dicentrine (**1**), crebanine (**3**), and stephanine (**4**) while of 1.9–125 μg/ml for tetrahydropalmatine (**2**). Each concentration was analyzed in triplicate. The plot of the peak areas versus the concentrations of all compounds provided a linear of this method with good correlation coefficient (Table 3). The investigation of intra-day precision by seven times injection of 125 μg/ml standard solutions within one day showed the result that the percentage of relative standard deviation was lower than 1% RSD. While the measurement of inter-day precision by three consecutive days with the same standard provided the percent relative standard deviation less than 3.8% (Table 4). The results gave an acceptable precision of the method. The accuracy of the method was determined by the recovery values. The results reported the recovery of dicentrine (**1**), tetrahydropalmatine (**2**), crebanine (**3**), and stephanine (**4**) in the ranged of 96.30–98.82% (average 97.78%), 95.36–100.76% (average 98.17%), 98.23–99.07% (average 98.79%), and 95.25–100.17% (average 98.42%), respectively, as shown in Table 5. The LOD and LOQ, at signal to noise ratio as 3:1 for LOD, and 10:1 for LOQ, were 0.1 and 0.3, 0.3 and 1, 0.1 and 0.3, and 0.1 and 0.3 μg/ml, for dicentrine (**1**), tetrahydropalmatine (**2**), crebanine (**3**), and stephanine (**4**), respectively (Table 3).

Table 4
Intraday and interday precision of dicentrine (**1**), tetrahydropalmatine (**2**), crebanine (**3**), and stephanine (**4**); results are shown as %RSD.

Compound	Intra-day			Inter-day
	Day 1	Day 2	Day 3	
Dicentrine (**1**)	0.38	0.39	0.25	3.14
Tetrahydropalmatine (**2**)	0.54	0.40	0.18	3.56
Crebanine (**3**)	0.67	0.38	0.36	3.80
Stephanine (**4**)	0.33	0.41	0.27	3.71

Table 5
Recovery study of dicentrine (**1**), tetrahydropalmatine (**2**), crebanine (**3**) and stephanine (**4**).

Level	Compound	Theoretical[a] (μg/ml)	Found[b] (μg/ml)	Recovery[b] (%)
1	Dicentrine (**1**)	46.32	45.49 ± 0.53	98.22 ± 1.15
	Tetrahydropalmatine (**2**)	45.53	43.42 ± 1.95	95.36 ± 4.28
	Crebanine (**3**)	87.96	86.40 ± 0.79	98.23 ± 0.90
	Stephanine (**4**)	14.77	14.07 ± 0.35	95.25 ± 2.35
2	Dicentrine (**1**)	62.13	61.40 ± 0.69	98.82 ± 1.10
	Tetrahydropalmatine (**2**)	65.98	64.92 ± 1.76	98.39 ± 2.40
	Crebanine (**3**)	121.36	120.23 ± 1.10	99.07 ± 0.91
	Stephanine (**4**)	21.13	21.17 ± 0.17	100.17 ± 0.81
3	Dicentrine (**1**)	80.88	77.89 ± 0.70	96.30 ± 0.87
	Tetrahydropalmatine (**2**)	88.66	89.33 ± 0.84	100.76 ± 0.96
	Crebanine (**3**)	160.63	158.70 ±	98.79 ± 0.93
	Stephanine (**4**)	24.63	24.60 ± 0.14	99.84 ± 0.80
Average	Dicentrine (**1**)			97.78
	Tetrahydropalmatine (**2**)			98.17
	Crebanine (**3**)			98.70
	Stephanine (**4**)			98.42

[a] Theoretical value is the amount calculated by original amount plus amount spiked.

[b] Expressed as mean ± SD ($n = 3$).

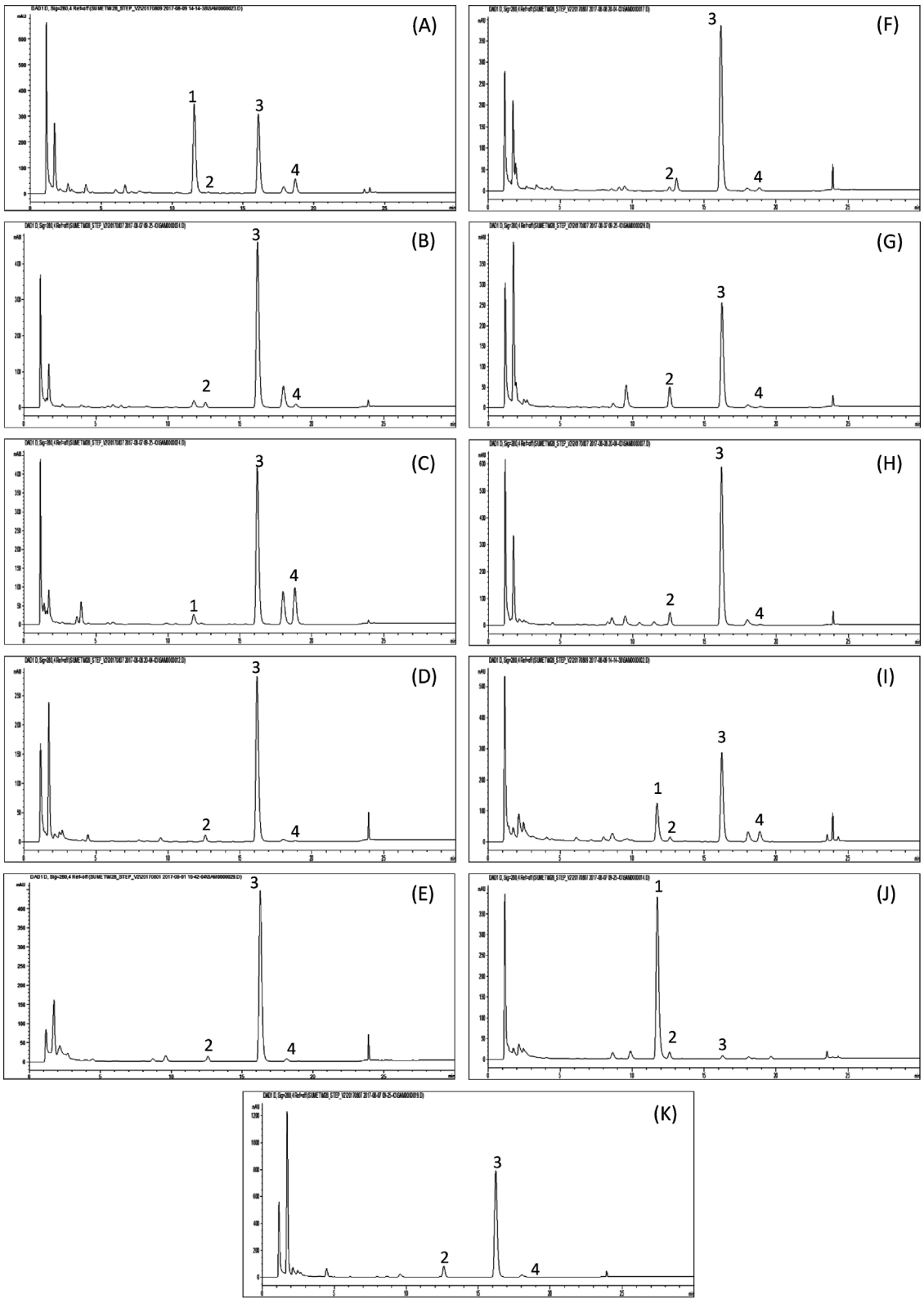


Fig. 2. HPLC chromatogram of hydromethanolic extract of *Stephania venosa* roots sample collected from various locations of Thailand (detection at 280 nm). (A) Chiang Mai, Doi Ang Khang, Sample 1, (B) Chiang Mai, Doi Ang Khang, Sample 2, (C) Chiang Mai, Doi Ang Khang, Sample 3, (D) Lampang, Muang, (E) Uttaradit, Nampad, (F) Kanchanaburi, Saiyok, (G) Prachuap Khiri Khan, Muang, (H) Roi-Et, Muang, (I) Udonthani, Phen, (J) Udonthani, Nong Wua So, (K) Nakhon Ratchasima, Wangnumkheo; Peak identification, **1** = dicentrine (t_R 11.6 min), **2** = tetrahydropalmatine (t_R 12.6 min), **3** = crebanine (t_R 16.2 min), **4** = stephanine (t_R 18.7 min).

Analysis of alkaloids contents in Stephania venosa *derived from various locations in Thailand*

To demonstrate the applicably of the method, *S. venosa* samples collected from various locations in Thailand were quantitatively analyzed after extraction with methanol–water (70:30, v/v). Triplicate of each sample was evaluated and the results are shown in Table 1 and Fig. 2. Dicentrine (**1**), tetrahydropalmatine (**2**), crebanine (**3**), and stephanine (**4**) were varied from not detected to 20.38, not detected to 9.19, 0.16 to 30.27, and not detected to 6.80 mg/g, respectively.

Discussion

Standardization of phytopharmaceutical products aims to control the consistency of the component product for safety and biological activity for reproducible products quality. HPLC, a method of choice for pharmaceutical analysis, is considered efficient and stringent for qualitative and quantitative analysis of plant chemical compounds. In this study, the HPLC method was developed for analysis the contents of major alkaloids, dicentrine (**1**), tetrahydropalmatine (**2**), crebanine (**3**), and stephanine (**4**) in *S. venosa* roots. Optimization of the mobile phase compositions and detection wavelength were done in order to maximize the efficiency and sensitivity of the method. Validation has been performed to ensure the linearity, precision, accuracy, and sensitivity of the method according to the ICH guideline (ICH, 1996/2005) and proved that the method is suitable for its intended use.

Critical separation of dicentrine (**1**) and tetrahydropalmatine (**2**) affected by ammonium acetate concentrations (Fig. 1) repetitiously showed the interaction of alkaloids with the residual silanol group of the column as described in our previous paper (Kongkiatpaiboon and Gritsanapan, 2012). It was probably due to the competitive interaction of the buffer cation with residual silanols as described by the Langmuir isotherm (Langmuir, 1916; Flieger and Czajkowska-Zelazko, 2011). However, slightly variation of batch-to-batch in HPLC column production was observed. Therefore, in practical application, optimal condition may need different ammonium acetate concentration.

Solvents used during the extraction are a crucial role in the quality and quantity of extracted compounds. Sonication, which is simple, rapid and has no limitation on any solvent type, was performed to determine the suitable solvent. With various types of solvent trialed (Table 2), the mixture of water–methanol (30:70, v/v) was the most suitable solvent that yielded highest alkaloids contents from the matrix. We also performed the study effect of solvent polarity to the dicentrine (**1**) extracting yields. The result was appeared in the same manner (data not shown). Mixture of water–methanol (30:70, v/v) was also the efficient solvent for extracting dicentrine (**1**) compared to the other solvents. Thus, it was chosen as extracting solvent in the sample preparation.

S. venosa samples collected from various locations were analyzed using the developed HPLC method which could be used for routine analysis. Besides the reported isolated components in each individual *S. venosa* study (Likhitwitayawuid et al., 1999; Ingkaninan et al., 2006; Yodkeeree et al., 2013; Kitisripanya et al., 2013; Le et al., 2017), the present study provided comprehensive overview of major components in *S. venosa*. A remarkable variation in the accumulation of alkaloids in each population and the between individual in the same population could be observed. Due to incomplete data on the occurrence of these alkaloids, no further geographic segregation can be deduced. Morphological data on each individual has been recorded and will be find out for the relationship with their phytochemical components in further study. Our results showed the heterogeneity of *S. venosa* in Thailand which would need a further study for species delimitations.

Conclusion

The HPLC method was developed for analysis the contents of major alkaloids, dicentrine, tetrahydropalmatine, crebanine, and stephanine in *S. venosa* roots. Validation has been performed to ensure the linearity, precision, accuracy, and sensitivity of the method. Critical separation was observed in peak separation of dicentrine and tetrahydropalmatine which was affected by ammonium acetate concentrations in mobile phase. The suitable solvent that yielded highest alkaloids contents from the matrix was optimized. *S. venosa* samples collected from various locations were analyzed. The method could be used for routine analysis of *S. venosa* raw materials in practical application. The present study provided comprehensive overview of major components in *S. venosa*. A remarkable variation in the accumulation of alkaloids in each population and the between individual in the same population could be observed. The heterogeneity of *S. venosa* in Thailand suggests a further study for species delimitations.

Authors' contribution

SK contribution included collecting samples, designing and performing laboratory work, analyzing the results, and preparing the paper. NT and WI contribution included isolation and purification of the compounds. ND and SP contribution included data interpretation and identification of the compounds. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors gratefully acknowledge the financial support provided by Thammasat University under Thai Government Research Fund (Contract No. 56/2559). We thank Ms. Pajaree Inthachub for discussion and identification of plant materials. We also acknowledge Chulabhorn Research Institute for the NMR measurement.

References

Blanchfield, J.T., Sands, D.P.A., Kennard, C.H.L., Byriel, K.A., Kitching, W., 2003. Characterisation of alkaloids from some Australian *Stephania* (Menispermaceae) species. Phytochemistry 63, 711–720.

Dary, C., Bun, S.S., Herbette, G., Mabrouki, F., Bun, H., Kim, S., Jabbour, F., Hul, S., Baghdikian, B., Ollivier, E., 2017. Chemical profiling of the tuber of *Stephania cambodica* Gagnep. (Menispermaceae) and analytical control by UHPLC-DAD. Nat. Prod. Res. 31, 802–809.

Du, Y., Du, L., Cao, J., Holscher, C., Feng, Y., Su, H., Wang, Y., Yun, K.M., 2017. Levo-tetrahydropalmatine inhibits the acquisition of ketamine-induced conditioned place preference by regulating the expression of ERK and CREB phosphorylation in rats. Behav. Brain Res. 317, 367–373.

Flieger, J., Czajkowska-Zelazko, A., 2011. Comparison of chaotropic salt and ionic lipid as mobile phase additives in reversed-phase high-performance liquid chromatography of biogenic amine. J. Sci. Sep. 34, 733–739.

Forman, L.L., 1991. Menispermaceae. Flora Thailand 5, 300–365.

Gong, X., Yue, K., Ma, B., Xing, J., Gan, Y., Wang, D., Jin, G., Li, C., 2016. Levo-tetrahydropalmatine, a natural mixed dopamined receptor antagonists, inhibits methamphetamine self-administration and methamphetamine-induced reinstatement. Pharmacol. Biochem. Behav. 144, 67–72.

He, J., Liu, Y., Kang, Y., Yang, P., Wang, Y., Guo, J., Huang, J., 2016. Identification of alkaloids in *Stephania hainannensis* by liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. Phytochem. Anal. 27, 206–216.

ICH, 1996/2005. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Validation of Analytical Procedures: Text and Methodology. ICH, Geneva.

Kitisripanya, T., Komaikul, J., Tawinkan, N., Atsawinkowit, C., Putalun, W., 2013. Dicentrine production in callus and cell suspension cultures of *Stephania venosa*. Nat. Prod. Commun. 8, 443–445.

Kongkiatpaiboon, S., Gritsanapan, W., 2012. HPLC quantitative analysis of insecticidal didehydrostemofoline and stemofoline in *Stemona collinsiae* root extracts. Phytochem. Anal. 23, 554–558.

Kongkiatpaiboon, S., Duangdee, N., Prateeptongkum, S., Chaijaroenkul, W., 2016. Acetylcholinesterase inhibitory activity of alkaloids isolated from *Stephania venosa*. Nat. Prod. Commun. 11, 1805–1806.

Ingkaninan, K., Phengpa, P., Yuenyongsawad, S., Khorana, N., 2006. Acetylcholinesterase inhibitors from *Stephania venosa* tuber. J. Pharm. Pharmacol. 58, 695–700.

Langmuir, I., 1916. The constitution and fundamental properties of solids and liquids. J. Am. Chem. Soc. 38, 2221–2295.

Le, P.M., Srivastava, V., Nguyen, T.T., Pradines, B., Madamet, M., Mosnier, J., Trinh, T.T., Lee, H., 2017. Stephanine from *Stephania venosa* (Blume) Spreng showed effective antiplasmodial and anticancer activities, the latter by inducing apoptosis through the reverse of mitotic exit. Phytother. Res. 31, 1357–1368.

Likhitwitayawuid, K., Dej-adisai, S., Jongbunprasert, V., Krungkrai, J., 1999. Antimalarials from *Stephania venosa*, *Prismatomeris sessiliflora*, *Diospyros montana* and *Murraya siamensis*. Planta Med. 65, 754–756.

Makarasen, A., Sirithana, W., Mogkhuntod, S., Khunnawutmanotham, N., Chimnoi, N., Techasakul, S., 2011. Cytotoxic and antimicrobial activities of aporphine alkaloids isolated from Stephania venosa (Blume) Spreng. Planta Med. 77, 1519–1524.

Mantsch, J.R., Li, S.J., Risinger, R., Awad, S., Katz, E., Baker, D.A., Yang, Z., 2007. Levo-tetrahydropamaltine attenuates cocaine self-administration and cocaine-induced reinstatement in rats. Psychopharmacology (Berlin) 192, 581–591.

Semwal, D.K., Badoni, R., Semwal, R., Kothiyal, S.K., Singh, G.J.P., Rawat, U., 2010. The genus *Stephania* (Menispermaceae): chemical and pharmacological perspectives. J. Ethnopharmacol. 132, 369–383.

Su, H.L., Zhu, J., Chen, Y.J., Zhao, N., Han, W., Dang, Y.H., Xu, M., Chen, T., 2013. Roles of levo-tetrahydropalmatine in modulating methamphetamine reward behavior. Physiol. Behav. 118, 195–200.

Su, M.J., Nieh, Y.C., Huang, H.W., Chen, C.C., 1994. Dicentrine, an alpha-adrenoceptor antagonist with sodium and potassium channel blocking activities. Naunyn. Schmiedebergs Arch. Pharmacol. 349, 42–49.

Wongsirisin, P., Yodkeeree, S., Pompimon, W., Limtrakul, P., 2012. Induction of G1 arrest and apoptosis in human cancer cells by crebanine, an alkaloid from *Stephanai venosa*. Chem. Pharm. Bull. 60, 1283–1289.

Yodkeeree, S., Wongsirisin, P., Pompimon, W., Limtrakul, P., 2013. Anti-invasion effect of crebanine and O-methylbulbocapnine from *Stephania venosa* via down-regulated matrix metalloproteinases and urokinase plasminogen activator. Chem. Pharm. Bull. 61, 1156–1165.

Yu, S.M., Ko, F.N., Chueh, S.C., Chen, J., Chen, S.C., Chen, C.C., Teng, C.M., 1994a. Effect of dicentrine, a novel alpha 1-adrenoceptor antagonist, on human hyperplastic prostates. Eur. J. Pharmacol. 252, 29–34.

Yu, S.M., Kang, Y.F., Chen, C.C., Teng, C.M., 1994b. Effects of dicentrine on haemodynamic plasma lipid, lipoprotein level and vascular reactivity in hyperlipidaemic rats. Br. J. Pharmacol 108, 1055–1061.