ORIGINAL ARTICLE

Standardization of *Leonurus sibiricus* L. aerial part and capillary electrophoresis quantitative analysis of its leonurine content

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ABSTRACT

The quality parameters of *Leonurus sibiricus* L. aerial part crude drugs were evaluated. Fifteen crude drugs were collected from various locations throughout Thailand. The transverse section of the stem of *L. sibiricus* showed quadrangular character highlighted the ribs with angular collenchyma. The epidermis was uniseriate with abundant glandular trichomes distribution. Prismatic calcium oxalate prisms were found in the stem medullary parenchyma. The histological character of crude drug powder showed bordered pitted vessel, fragment of fiber, glandular trichome, prism crystal, spiral vessel, starch granule, and stomata. The loss on drying, total ash, acid-insoluble ash, and moisture contents should be not more than 8.18, 15.28, 4.04, and 8.91 g/100 g dry weight, whereas ethanol and water-soluble extractive values should be not less than 7.67, and 17.21 g/100 g of dry weight, respectively. Leonurine in the crude drugs were analyzed by capillary electrophoresis (CE) with photodiode array detector. The ethanolic extraction performed by Soxhlet apparatus yielded 18.86 ± 4.09 g/100 g dry weight. The electropherogram detected at 277 nm showed the migration time of leonurine at 6.2 min. The developed CE was found to be valid for leonurine quantification in *L. sibiricus* ethanolic extract. The contents of leonurine in 15 crude drugs ranged from 0.79 to 4.23 mg/g with the average of 2.38 ± 1.10 mg/g dry weight. This study established the pharmacognostic specification of *L. sibiricus* crude drug in Thailand with special reference to a bioactive compound, leonurine. CE was beneficial technique for the analysis of leonurine in *L. sibiricus* aerial parts.

Key words: Capillary electrophoresis, leonurine, *Leonurus sibiricus* L., standardization

INTRODUCTION

Leonurus sibiricus L. or Siberian motherwort, locally known in Thailand as "Khanchaa thet" or "Saa saa, Saa nam," is an herbaceous plant belonging to the Labiatae family. It is an annual or biennial herb with stems erect, three lobes pinnately divided leaves, lobules of leaves 1–3 mm wide, flower sessile, corolla white or reddish to purple-red , calyx densely pilose, especially at the middle. *L. sibiricus* is confined to stony or sandy grasslands and *Pinus* forests in its original native distribution (Russia, Mongolia, and China).[1] *L. sibiricus* has been used in traditional Thai herbal medicine, especially in women remedies for menstrual induction, preventing postpartum hemorrhage and improving blood circulation.

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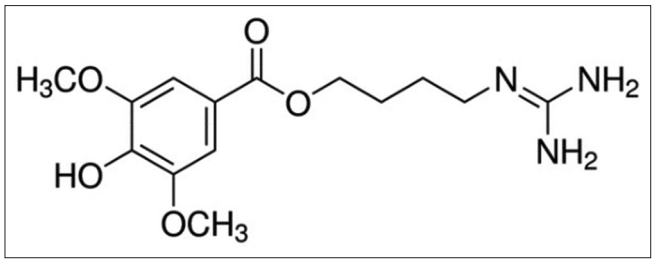

Figure 1: Chemical structure of leonurine

[2-4] The pharmacological study of *L. sibiricus* showed that the ethanolic extract of leaves and stems had promising activity on uterine contraction in Wistar rats.[2] In addition, *L. sibiricus* is one of the main ingredients in Prapchompoothaweep remedy, which is listed in Thailand National List of Essential Medicines (NLEM), for traditional treatment of common cold and allergic rhinitis symptoms. The clinical trials of this remedy on allergic rhinitis treatment revealed significant improvement of the symptoms and better quality of life related to rhinoconjunctivitis.[5,6] Drugs listed in NLEM must be essential to prevent and resolve health problems, the quality of crude drug including the active chemical composition is essential for medicinal efficacy. Various types of chemical compounds were found in the aerial parts of *L. sibiricus*, for example, alkaloid (leonurine),[7,8] labdane diterpenoid (sibiricinones,[9] leojaponin,[8] isoleoheterin,[10] leosibiric acids),[10] diterpene lactone (leonotinin, leonotin, dubiin, and nepetaefuran),[11] flavonoid (genkwanin[8] and rutin),[12] polyphenol (chlorogenic acid and caffeic acid),[12] iridoid glycoside (acetylharpagide and ajugoside),[12] phenylpropanoid glycoside (lavandulifolioside and verbascoside),[12] sterol (β-sitosterol and β-sitosterol glucoside),[12] etc., Leonurine [Figure 1] is a highlighted compound in *L. sibiricus* regarding its pharmacological properties such as neuroprotective effects and cardiovascular protective effects.[13-15] Leonurine or 4-guanidino-n-butyl syringate is a pseudoalkaloid which is acylguanidino derivative of syringic acid and 4-guanidino-n-butanol.[16] To identify the active constituents in medicinal plants, chromatographic techniques are world-widely used in various studies. High performance liquid chromatography (HPLC) has been previously reported for the analysis of leonurine in *L. japonicus* Houtt.[17-19] In addition, capillary electrophoresis (CE) is accepted as a separation technique for plant secondary metabolite analysis, for example, iridoids, phenylpropanoids, flavonoids,[20] lignans,[21] and alkaloids.[22,23] CE use high-voltage direct current electric field as driving force for analyte separation performed in capillaries and the analysis time or migration time is usually <30 min, faster than HPLC.[23,24] At present, leonurine quantification in *L. sibiricus* in Thailand, particularly with the CE technique, has not been reported. Therefore, this study presented the quality parameters of *L. sibiricus* aerial part crude drug in Thailand with special reference to its leonurine content analyzed by CE.

MATERIALS AND METHODS

Sample collection

L. sibiricus dried aerial parts were provided from 15 traditional drugstores located in four regions of Thailand [Table 1]. All crude drugs were authenticated by the expert taxonomist (Nijsiri Ruangrungsi) as well as comparison with the identified herbarium specimens at the Forest and Plant Conservation Research Office, Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Thailand. Microscopic anatomy of the stem was also compared to previous report.[25] Voucher specimens and numbers were deposited at the research institution.

Instruments and chemicals

CE system (Agilent 7100, USA), muffle furnace (Carbolite Gero, UK), digital microscope (Carl Zeiss, Germany), water purification system (Heal Force, China), TLC silica gel 60 GF254 plate (Merck, Germany), leonurine (SML0670, Sigma-Aldrich, USA), and AR grade solvents (RCI Labscan, Thailand).

Determination of pharmacognostic specification

Plant material identification

L. sibiricus flowering branches were visually inspected. The transverse section of the stem was examined under a digital microscope for the anatomical characteristics. The crude drug was pulverized and microscopically investigated for histological characteristics. Those characters were botanically illustrated by hand drawing.

Determination of loss on drying, total ash, and acid-insoluble ash

Three grams of the powdered crude drug were spread in an even layer in a pre-weighed crucible and heated at 105°C for the loss on drying content. After that, the crucible was placed in a muffle furnace for incineration at 550°C for 6–8 h. The crucible containing the total ash was weighed after cooling in a desiccator. Then, 25 ml of hydrochloric acid (70 g/l) was added and boiled gently for 5 min. The insoluble matters were collected on an ashless filter paper and transferred to the original crucible, dried on a hot plate, and incinerated again. After cooling in a desiccator, the crucible containing the acid-insoluble ash was weighed.[26]

Determination of ethanol- and water-soluble extractive matters

Five grams of the powdered crude drug was macerated in 70 ml of 95% ethanol or water for 6 h under shaking then 18 h under standing at room temperature. After filtration, the marc was washed and the volume was adjusted to 100 ml with the solvent used. Twenty milliliters of the filtrate were aliquoted into a pre-weighed small beaker, heated to dryness at 105°C, and weighed. The extractive matters were calculated.[26]

Table 1: *Leonurus sibiricus* sample location and leonurine content

Number	Sample collection area	Voucher specimen number	Yield of ethanolic extract (%)	Leonurine content (mg/g)*
1	Ubon Ratchathani	KCT_01	14.42	1.40
2	Phuket	KCT_02	16.68	2.49
3	Rayong	KCT_03	19.39	4.17
4	Ang Thong	KCT_04	23.14	4.23
5	Phetchabun	KCT_05	20.32	1.47
6	Bangkok_1	KCT_06	22.47	3.31
7	Udon Thani	KCT_07	11.92	1.56
8	Nakhon Sawan	KCT_08	21.86	2.36
9	Chanthaburi_1	KCT_09	19.31	1.47
10	Chanthaburi _2	KCT_10	23.05	1.68
11	Khon Kaen	KCT_11	24.82	2.83
12	Ranong	KCT_12	17.90	1.53
13	Phrae	KCT_13	16.23	3.88
14	Bangkok_2	KCT_14	20.04	2.46
15	Surat Thani	KCT_15	11.36	0.79
	Mean±SD		18.86±4.09	2.38±1.10

*Dry weight. SD: Standard deviation

Determination of moisture content

Fifty grams of the powdered crude drug was transferred to the flask containing 200 ml of water-saturated toluene. The flask was connected to the apparatus for azeotropic distillation of water in crude drug and toluene. The volume of water in the receiving tube was then recorded.[26]

Thin-layer chromatographic fingerprint identification

Another one of 20-ml aliquot of the ethanolic filtrate from the extractive matter determination was evaporated and re-dissolved in 0.5 ml of methanol. Five microliters of the extract were applied to TLC plate, developed with the mobile phase of ethyl acetate: methanol: water: formic acid (8.4: 0.6: 0.5: 0.5) and observed under 254 and 366 nm ultraviolet light as well as by spraying with p-anisaldehyde's reagent.

Quantitative analysis of leonurine by capillary electrophoresis

Plant extraction

Five grams of each cleaned, dried, and pulverized sample was exhaustively extracted with 95% ethanol (200 mL) by the Soxhlet apparatus (6 h). After filtration, the filtrate was evaporated to dryness. The extract was weighed to calculate the % yield and then dissolved in methanol to obtain the final concentration (1 mg/1 mL). The extract in methanol was diluted to various concentrations and filtered through a 0.45 µm PTFE membrane syringe filter for further CE analysis.

Standard preparation and calibration curve of leonurine

The stock standard solution of leonurine was prepared by dissolving the leonurine (1 mg) in methanol (1 mL). The solution was filtered through a 0.45 µm PTFE membrane syringe filter. The standard stock solutions of leonurine (20-100 µg/mL) were applied in triplicate on a CE column. The resolved peak area was recorded for each of the standard concentration. The calibration curve of leonurine was plotted by taking peak area versus concentrations of standard.

Capillary electrophoresis conditions

CE system comprised of Agilent 7100 CE equipped with auto-sampler and photodiode array (PDA) detector. The condition was set at 20°C, and applied voltage of 25 kV. The detection was performed at 277 nm. All filtered samples and standard were injected 5 s from each vial. The buffer solution was 20 mM sodium phosphate buffer, pH 7.9. Capillary column was uncoated fused silica capillary 64.5 cm length (56 cm to the detector) × 50 µm i. d. The leonurine content of each sample was determined by comparing the peak area of standard leonurine with a calibration curve. All data acquisition was performed using HP ChemStation software, version B.04.03.

Method validation

According to ICH guidelines,[27] specificity, linearity, limit of detection and limit of quantification, precision, accuracy, and robustness were checked for confirming method validation. All the parameters were performed in triplicates.

Specificity

The specificity of the method was determined by analyzing the absorbance spectra of standard leonurine and samples. Peak purity was evaluated by comparing its peak at peak start, peak apex, and peakend position.

Linearity

The linearity of the method was expressed as a calibration range generated by plotting peak areas versus concentrations of standard leonurine. The coefficient of determination (R^2) was calculated using Excel software.

Limit of detection and limit of quantification

LOD, the lowest amount that can be detected, and LOQ, the lowest amount that can be quantitated, were calculated from the calibration curve using the following formula:

$$\text{LOD} = \frac{3.3(\text{residual SD})}{\text{S}}, \qquad \text{LOD} = \frac{10(\text{residual SD})}{\text{S}}$$

Where residual SD = the residual standard deviation of the regression line, S = the slope of the regression line.

Precision

The repeatability and intermediate precision were assessed by analyzing the sample solution with three concentrations (low, medium, and high) (each in triplicate) on the same day and three different days, respectively. The precision of leonurine content analysis was determined in terms of percent relative standard deviation (% RSD) by the following equation:

$$\%\text{RSD} = \frac{\text{SD}}{\text{MEAN}} \times 100$$

Accuracy

The percent recovery was determined by adding different level concentrations of standard leonurine to a pre-analyzed sample. The analysis was done by the proposed CE method and the analysis was carried out in triplicate.

$$\%\text{Recovery} = \frac{\text{A}}{\text{B}+\text{C}} \times 100$$

Where A = the amount of leonurine found in the spiked extracted sample

B = the amount of leonurine found in the un-spiked extracted sample

C = the amount of standard leonurine was added to the extracted sample

Robustness

A small variation on the pH buffer solution from 7.8 to 8.0 was applied for the robustness. The result was expressed as % RSD.

RESULTS AND DISCUSSION

Pharmacognostic specification of *Leonurus sibiricus* aerial part

The drawing of a flowering branch and photograph of the dried crude drug (aerial parts) of *L. sibiricus* are shown in Figures 2 and 3. The transverse section of the stem of *L. sibiricus* showed anatomical characters of collenchyma, epidermis, glandular trichome, parenchyma, prism crystal, xylem fiber, and xylem vessel [Figure 4]. The quadrangular stem highlighted the ribs with angular collenchymatous cells was illustrated. The epidermis was uniseriate with abundant glandular trichomes distribution. Prismatic calcium oxalate prisms were found in the stem medullary parenchyma. The microscopic anatomy of *L. sibiricus* stem in Thailand was found to be unique as *L. sibiricus* stem reported in Brazil.[25] The histological evaluation of powdered crude drug showed a bordered pitted vessel, fragment of fiber, fragment of the lower epidermis, glandular trichome, prism crystal, parenchyma in longitudinal view, parenchyma in transverse view, spiral vessel, starch granule, and stomata [Figure 5]. The physicochemical parameters due to the quality of *L. sibiricus* are shown in Table 2. The loss on drying, total ash, acid-insoluble ash, and moisture contents should be not more than 8.18, 15.28, 4.04, and 8.91% by dry weight, whereas ethanol and water-soluble extractive values should be not <7.67 and 17.21 g/100 g of dry weight, respectively. TLC fingerprint iss shown in Figure 6. The total

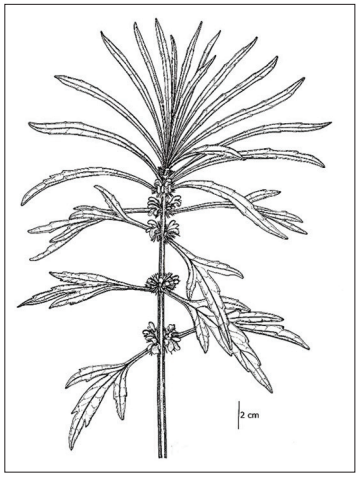

Figure 2: *Leonurus sibiricus* flowering branch

Figure 3: Crude drug of *Leonurus sibiricus* aerial parts

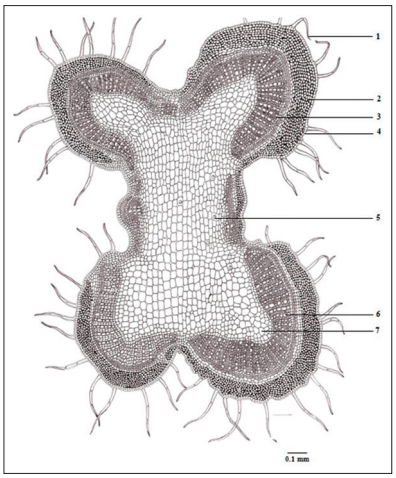

Figure 4: Transverse section of the stem of *Leonurus sibiricus*. (1) Glandular trichome, (2) collenchyma, (3) xylem fiber, (4) epidermis, (5) prism crystal, (6) xylem vessel, (7) parenchyma

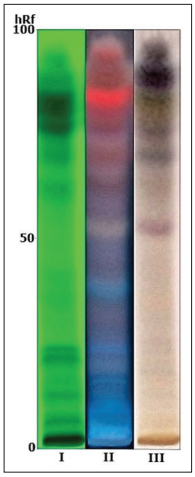

Figure 6: TLC of methanolic extract of *Leonurus sibiricus.* Solvent systems; ethyl acetate: methanol: water: formic acid 8.4:0.6:0.5:0.5. Detections; I = detection under UV light (254 nm). II = detection with UV light (366 nm). III = detection with p-anisaldehyde's reagent

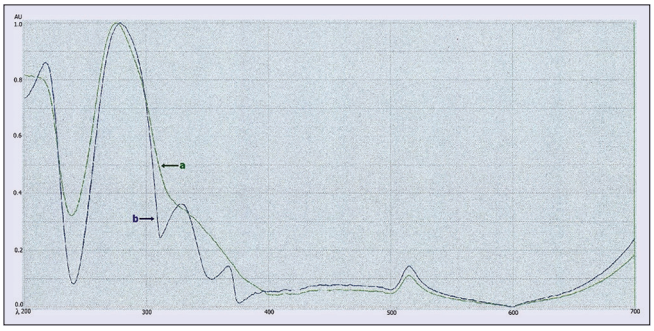

Figure 8: The absorption spectra of standard leonurine (a), and sample (b)

and acid-insoluble ashes were higher than previous study of *L. sibiricus* crude drugs in Korea in 2001 which reported loss on drying, total ash, and acid-insoluble ash as 7.94% ±0.63%, 8.51% ±2.1%, and 1.24% ±0.77%, respectively.[28]

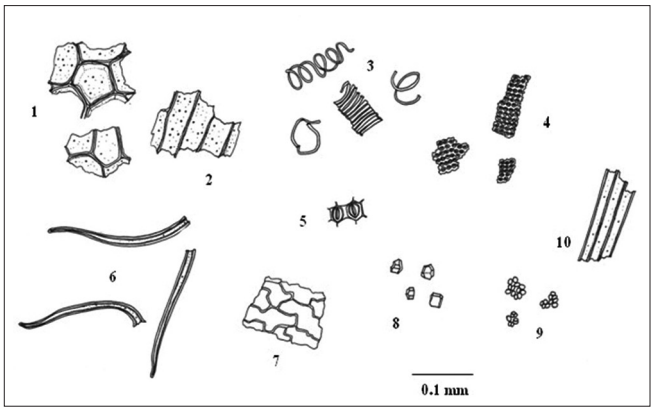

Figure 5: Histological characters of *Leonurus sibiricus* aerial part powder. (1) Parenchyma in transverse view, (2) parenchyma in longitudinal view, (3) spiral vessel, (4) bordered pitted vessel, (6) glandular trichome, (7) fragment of lower epidermis, (8) prism crystal, (9) starch granule, and (10) fragment of fiber

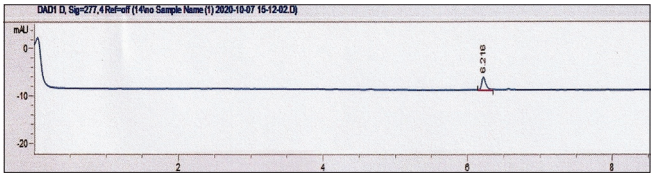

Figure 7: Capillary electrophoresis Chromatogram of ethanolic extract of *Leonurus sibiricus* L. at 277 nm

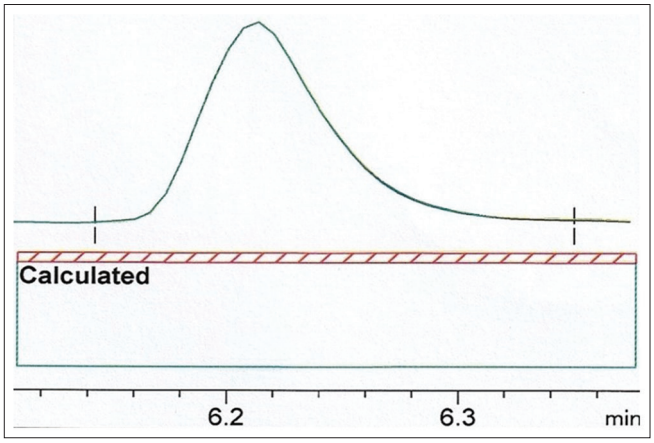

Figure 9: Peak purity of leonurine in *Leonurus sibiricus* extract (peak purity index: 0.9999, peak start at 6.143 min and end at 6.350 min)

Leonurine content in *Leonurus sibiricus* aerial part

The ethanolic extraction using Soxhlet apparatus of *L. sibiricus* crude drugs from 15 locations yielded 18.86 ± 4.09% dry weight [Table 1]. CE coupling with PDA detector was used for quantitative analysis of leonurine in the extracts in this study due to several advantages, i.e. high separation efficiency, short analysis times, low waste generation, and a diverse range of applications.[29] The optimized condition was developed and the method was validated in terms of linearity, accuracy, precision, LOD, LOQ, specificity, and robustness [Table 3].[27] The

Table 2: The physicochemical parameter due to quality of *Leonurus sibiricus*

Parameter	Content (percentage by dry weight)*
Total ash	15.28±0.16
Acid-insoluble ash	4.04±0.36
Ethanol-soluble extractive	7.67±0.21
Water-soluble extractive	17.21±1.20
Loss on drying	8.18±0.08
Moisture	8.91±0.65

*The parameters were shown as grand mean±pooled SD. The samples were from 15 different sources. Each sample was performed in triplicate. SD: Standard deviation

Table 3: Method validation parameters of leonurine in *Leonurus sibiricus* extract

Parameters	Validity
Linearity range (μg/mL)	20-100
Coefficient of determination (R^2)	0.9995
Linear regression equation (y)	0.0871x+0.9537
Precision: Repeatability (average % RSD)	2.97
Precision: Intermediate precision (average % RSD)	3.16
LOD (μg/mL)	1.59
LOQ (μg/mL)	4.82
Accuracy (average % recovery)	99.54
Robustness (average % RSD)	2.38

LOD: Limit of detection, LOQ: Limit of quantification, RSD: Relative standard deviation

electropherogram of the ethanolic extract of *L. sibiricus* is shown in Figure 7. Leonurine migration time was 6.2 min. The absorbance spectrum of leonurine scanned from 200 to 700 nm with the maximum absorption at 277 nm is shown in Figure 8. The identity of the spectra of standard and sample leonurine [Figure 8] as well as peak purity index of 0.9999 [Figure 9] represented the specificity of the method. The calibration curve showed linearity in the range of 20–100 μg/mL with the regression equation of y = 0.0871x + 0.9537 and the coefficient of determination (R^2) was 0.9995 [Figure 10]. The accuracy was confirmed by spiking standard leonurine at three different concentrations (10, 30, and 50 μg/mL) into *L. sibiricus* extracted sample. The result was found to be 96.39%–101.83% recovery (average 99.54% recovery) [Table 3]. The average values of repeatability and intermediate precisions were 2.97 and 3.16% RSD, respectively. The limit of detection and the limit of quantitation, calculated from the residual standard deviation of the regression line and the slope of the calibration curve, displayed 1.59 and 4.82 μg/mL, respectively. The robustness was performed by varying the pH buffer solution from 7.8–8.0, and the average result was found to be 2.38% RSD [Table 3]. The developed CE was valid for the quantification of leonurine in *L. sibiricus* ethanolic extract. Table 1 shows the contents of leonurine

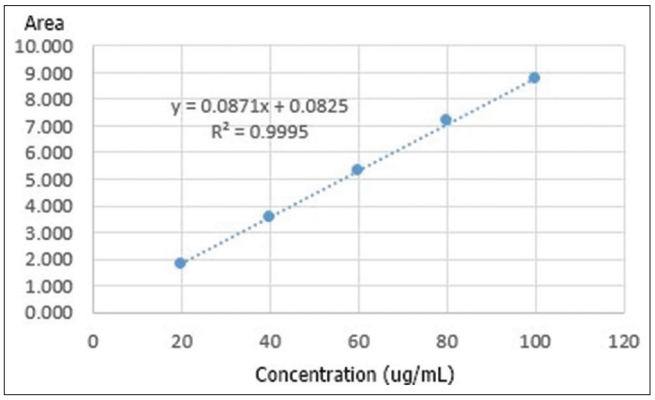

Figure 10: The calibration curve of standard leonurine

in 15 *L. sibiricus* crude drugs that ranged from 0.79 to 4.23 mg/g with the average of 2.38 ± 1.10 mg/g or 0.24% ±0.11%. The amount of leonurine in *L. sibiricus* crude drug in this study was higher than the previous report of *L. sibiricus* crude drug in Korea that demonstrated the leonurine content analyzed by HPLC as 0.124% ±0.066%.[28] In addition, leonurine found in this study seemed to be higher than leonurine in *L. japonicus* crude drug in China quantitated by HPLC-MS/MS that was found to be 0.73 to 2.33 mg/g.[19] This finding revealed the beneficial application of CE technique for quality evaluation of medicinal plant material with reference to its bioactive phytochemicals.

CONCLUSION

The pharmacognostic specification of *L. sibiricus* in Thailand was established. The microscopic characteristics, including the transverse section of the stem and the powder characters of *L. sibiricus*, were illustrated. The TLC fingerprint and physicochemical parameters were demonstrated by this study and could be useful for the identification of *L. sibiricus* dried crude drug. The CE equipped with a PDA detector was developed, validated, and performed for quantification of leonurine in *L. sibiricus*. The leonurine contents in *L. sibiricus* from various areas in Thailand were revealed which could be used for the specification of this crude drug concerning its chemical marker.

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College of Public Health Sciences, Chulalongkorn University.

Conflicts of interest

There are no conflicts of interest.

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