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Determination of the barakol content of mature leaves, young flowers of *Senna siamea* (Lam.) Irwin and Barneby and in the herbal recipes

Chaowalit Monton[1,2*], Laksana Charoenchai[1,2*], Poj Kulvanich[1], Jirapornchai Suksaeree[1,2] and Krisana Kraisintu[2,3]

Abstract

Background: Barakol, a major substance from *Senna siamea* (Lam.) Irwin and Barneby, is found in variable amount depending upon the source of the plant. Scientific evidence to characterize the traditional preparation of *S. siamea* is needed. The aim of this study was to characterize the content of barakol in various plant sources and to validate the high-performance liquid chromatography (HPLC) method for the quantification of barakol content from plants harvested from five locations in the northeast of Thailand and in several herbal recipes in which *S. siamea* is one of the constituents.

Methods: Barakol was extracted from fresh young leaves of *S. siamea* and identified by chromatographic and spectroscopic techniques. Barakol content in mature leaves and young flowers of *S. siamea* harvested from five northeastern provinces in Thailand and its content in herbal recipes were quantified by validated HPLC. Separation was performed using C18 column and gradient elution of ultrapure water and methanol. Three herbal recipes containing *S. siamea* were studied to select the suitable solvent with which to extract barakol.

Results: Barakol was verified and its content determined using a validated HPLC method with a good linearity ($R^2 = 0.9999$), accuracy (%recovery = 99.25 to 101.59%), and precision (%RSD < 2%) including limit of detection (LOD) and limit of quantitation (LOQ) (16.80 and 5.04 ng/mL, respectively). The average barakol content in mature *S. siamea* leaves and young flowers was 0.300 and 0.279% *w/w*, respectively. Boiling water extracts yielded the highest barakol content in three herbal recipes (0.077, 0.123, and 0.085% *w/w*).

Conclusions: Boiling water was an appropriate solvent to maximize barakol yield from herbal recipes containing *S. siamea* from Surin mature leaves and Yasothorn young flowers.

Keywords: Barakol; *Senna siamea*; Herbal recipe; Insomnia

Background

Many people around the world suffer from sleeping disorders of which insomnia is the most common. Insomnia is divided into three categories: difficulty falling asleep (sleep onset disturbance), difficulty remaining asleep (sleep maintenance disturbance), and poor quality (nonrestorative) sleep (Walsh 2004). This problem adversely affects daytime functioning and may lead to anxiety, depression, pain, and increased risk of cardiovascular events, especially myocardial infarction (Morphy *et al.* 2007; Westerlund *et al.* 2013). Furthermore, home-, work-, and auto-related accidents are associated with sleep disturbance (Leger *et al.* 2014).

Senna siamea (Lam.) Irwin and Barneby is widely cultivated in Southeast Asia, including Thailand. Barakol has been recognized as a major biologically active component of flowers and young leaves of *S. siamea*. Fresh, young leaves of *S. siamea* contain approximately 0.40% *w/w* barakol (2,5-dimethyl-3aH-pyrano[2,3,4-de]-1-benzopyran-3a,8-diol, Mw = 232) (Padumanonda and Gritsanapan 2006). Barakol is an unstable, tricyclic ring structure that is converted to anhydrobarakol (Mw = 214) by loss of one molecule of water (Thongsaard *et al.* 2001). The conversion

* Correspondence: chaowalit@rsu.ac.th; laksana39@hotmail.com
[1]Faculty of Pharmacy, Rangsit University, Muang, Pathum Thani 12000, Thailand
[2]Sino-Thai Traditional Medicine Research Center (cooperation between Rangsit University, Harbin Institute of Technology, and Heilongjiang University of Chinese Medicine), Rangsit University, Muang, Pathum Thani 12000, Thailand
Full list of author information is available at the end of the article

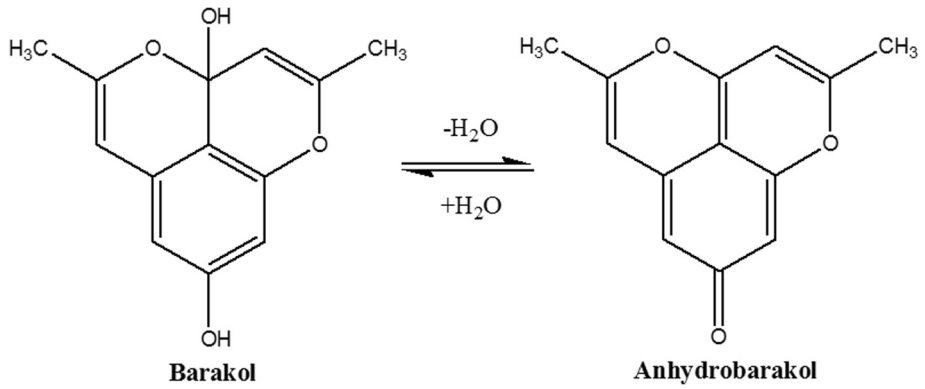

Figure 1 Chemical structure and conversion of barakol and anhydrobarakol (reproduced with permission) (Thongsaard et al. 2001).

from barakol to anhydrobarakol is shown in Figure 1. Barakol exhibits hypnotic effect in both animals and human volunteers and has been used as a medicinal agent by insomniacs. But, the Food and Drug Administration of Thailand removed the products containing *S. siamea* leaves from the market due to their hepatotoxicity (Chantong *et al*. 2009).

Many Chinese medicines comprised of herb combination are purported to offer synergism of therapeutic efficacy while lessening adverse effects (Yang *et al*. 2014). Thai traditional medicines often include a combination of herbs to theoretically optimize efficacy and minimize side effects. Whether such benefits, particularly with regard to hepatotoxicity of barakol, are derived from preparations containing *S. siamea* have not been scientifically demonstrated in controlled clinical trials. In order to ultimately enable *in vivo* investigation of the efficacy and safety of such preparations, we sought to standardize the mythology for characterization of barakol content of *S. siamea* and several herbal preparations. This paper reports on the characterization of two herbs containing *S. siamea* leaves. Seeds of *Senna tora* (Linn.) Roxb. and aerial parts of *Leonurus sibiricus* Linn were used. Firstly, extraction and quantification of barakol from fresh young leaves of *S. siamea* served as a standard. Secondly, comparisons were made of the barakol content of mature leaves and young flowers of *S. siamea* harvested from five different locations in Northeast Thailand using a validated high-performance liquid chromatography (HPLC) method. We then determined the solvent that yielded the greatest extraction of barakol from three *S. siamea* containing herbal recipes.

Methods

Plant materials and reagents

Mature leaves and young flowers of *S. siamea* were harvested from five locations in Northeast Thailand: (1) Muang district, Nakorn Ratchasima province; (2) Prakonchai district, Buriram province; (3) Buached district, Surin province; (4) Kamkuankaew district, Yasothorn province; and (5) Panomprai district, Roi Et province. Samples were harvested on 9 to 10 November 2013. The leaves and flowers of each source were harvested from the same tree. Samples from different locations were coded as NR, BR, SR, YS, and RE, respectively. Seeds of *S. tora* and aerial part of *L. sibiricus* were purchased from Chareonsuk Osod, Nakon Pathom province, Thailand. Water was purified by Puris, Expe-UP water system (model: Expe-UP series, Korea). All organic solvents were analytical grade obtained from Burdick and Jackson, Korea.

Extraction of barakol from *S. siamea* leaves

Barakol was extracted from fresh young leaves of *S. siamea* as previously reported (Wongtongtair *et al*. 2011). The fresh young leaves of *S. siamea* (1,000 g) were chopped into small pieces and boiled in 2,000 mL of 0.5% sulfuric acid for 30 min, then filtered with filter cloth. The residue was reboiled using the same method. The filtrates were combined, filtered, and alkalinized with 10% sodium hydrogen carbonate. The solution was partitioned three times with dichloromethane. The dichloromethane part was concentrated with rotary evaporator (Buchi, Thailand). An equal volume of cold water was gently added and the product cooled in a refrigerator for 30 min. Crystallized greenish-yellow needles of crude barakol were obtained. They were collected by vacuum filtration, washed with little amount of dichloromethane and cold water, dried, and kept in a desiccator until used.

Identification of isolated barakol

Five different methods including thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and ultraviolet (UV) spectrophotometry, infrared (IR) spectrometry, nuclear magnetic resonance (NMR), and mass spectrometry (MS) were used to identify the isolated barakol.

TLC

Chromatography was performed on a TLC precoated silica gel 60 GF_{254} plate 20 × 10 cm (Merck, Germany).

Chloroform-methanol mixture (85:15, *v*/*v*) was used as a mobile phase (Padumanonda *et al*. 2007) with 20-mL total volume. Three barakol tracks were applied using capillary tube and kept dry under ambient temperature. The TLC plate was developed in the TLC chamber and equilibrated with the mobile phase for 45 min before use. After the plate was developed, it was dried under ambient temperature and read at 366 nm UV light with a TLC visualizer (Camag, Muttenz, Switzerland). The test was performed in triplicate and the retention factor (R_f) was calculated.

HPLC chromatogram and UV spectrum

Barakol was prepared in methanol in concentration of 20 μg/mL. Samples were analyzed by HPLC as describe below. One peak of barakol should be found. UV spectra of barakol were scanned between 200 and 400 nm by diode-array detector. The maximum absorbance was collected.

IR spectrometry

Approximately 200 mg of potassium bromide powder was ground. Two milligrams of barakol was blended with potassium bromide powder. The mixture was then compressed into pellet by hydraulic press with 8 tons force. FT-IR spectra were collected between 4000 and 400 cm^{-1} by accumulating 32 scans with the resolution of 16 cm^{-1} on the Nicolet 6700 spectrometer (Thermo Scientific, Waltham, MA, USA). Spectra were processed with the OMNIC 8.0 software (Thermo Scientific, USA).

NMR spectrometry

NMR data of barakol was recorded in $CDCl_3$ on a Fourier Transform NMR spectrometer (Unity Inova, Varian, Darmstadt, Germany), operating at 500 MHz for both protons and carbons.

Mass spectrometry

The LC-MS studies were performed by coupling the LC system with a Dionex Ultimate 3000. A Luna C18 column (250 × 4.60 mm i.d., 5 μm) equipped with 10 × 4.6 mm i.d. guard column was controlled at 25°C. The injection volume was 5 μL. The flow rate was 0.4 mL/min. The mass determinations were made in alternative ESI mode in the mass range of 100 to 2000 amu (Bruker Amazon SL, Münster, Germany).

HPLC conditions

HPLC analyses were performed on an Agilent 1260 series equipped with photodiode array detector and autosampler. Data analysis was carried out using OpenLab CDS EZChrom software (Agilent, USA). Separation was performed at 25°C on a Luna C18(2) column (250 × 4.60 mm i.d., 5 μm). The mobile phase consisted of water (A) and methanol (B). The gradient program went from 100% to 95% A in 5 min and holding for 2 min, decreased to 90% A in 3 min, decreased to 60% A in 15 min, and then decreased to 30% A in 30 min and holding for 3 min. The post run was set to decrease to 0% A and equilibrate for 10 min before the next injection. The mobile phase flow rate was 1 mL/min. The injection volume was 10 μL. The quantitation wavelength was set at 245 nm.

Standard barakol preparation

Standard barakol stock solution was prepared in methanol at concentration of 0.4 mg/mL. Standard barakol should be freshly prepared to avoid chemical degradation.

Method validation

Linearity was determined at six concentrations of standard barakol between 0.25 and 40.0 μg/mL in methanol. Precision (intra-day and inter-day) was studied at three concentrations of barakol, 2.50, 5.00, and 10.00 μg/mL, and reported as percentage relative standard deviation (%RSD). Accuracy was evaluated by addition of three barakol concentrations (2.50, 5.00, and 10.00 μg/mL) with known amount of barakol to sample solutions. Percentage recovery of barakol was reported. The limit of quantitation (LOQ) and limit of detection (LOD) were studied based on visual evaluation between signal and noise. The standard barakol solution was diluted with methanol until signal-to-noise ratios were 10 and 3, respectively. Furthermore, specificity, robustness, and system suitability were evaluated. In the case of robustness, changing the flow rate by ±0.2 mL/min, column temperature by ±2°C, and wavelength by ±2 nm were performed.

Quantification of barakol in samples

Dried mature leaves and young flowers of *S. siamea* were ground and sieved through 40-mesh sieve. The samples were individually extracted in 95% ethanol. Two grams of sample were extracted with 50 mL of 95% ethanol by sonication for 1 h. The sample was filtered through filter paper to obtain 95% ethanol extract solution. Then, the solvent was eliminated from the extract by evaporation under vacuum condition. Percentage yield was collected and extracts were kept in desiccator until used. Absolute ethanol was used as solvent when samples were prepared for HPLC analysis to obtain 500 μg/mL of sample.

The three herbal recipes were coded as F1, F2, and F3. The ingredients, part used, and weight ratio of herbal recipes are shown in Table 1. Each herb was individually ground and sieved through 40-mesh sieve. Then, herbs were mixed together by the geometric dilution method to obtain herbal recipes. In F1 formula, 3 g were dissolved in 50 mL of four solvent systems: boiling water, water at ambient temperature, 60% ethanol, and 95% ethanol. F2 and F3 were extracted in two solvent systems: boiling water

Table 1 Ingredients, part used, and weight ratio of herbal recipes

Formula	Ingredients	Part used	Weight ratio
F1	*S. siamea* (Lam.) Irwin and Barneby	Leaves	1
	S. tora (Linn.) Roxb.	Seeds	1
	L. sibiricus Linn.	Aerial parts	2
F2	*S. siamea* (Lam.) Irwin and Barneby	Leaves	1
	S. tora (Linn.) Roxb.	Seeds	3
F3	*S. siamea* (Lam.) Irwin and Barneby	Leaves	1
	L. sibiricus Linn.	Aerial parts	3

and 95% ethanol. All formulas were extracted by sonication at ambient temperature for 1 h. The sample was filtered through filter paper to obtain extract solution. The solvent was eliminated from the extract by evaporation under vacuum condition for ethanol-based extract and freeze-dried condition for water-based extract. Percentage yield was collected and extracts were kept in desiccator until used. Water-based extracts were dissolved in water while ethanol-based extract were dissolved in absolute ethanol to obtain 500 μg/mL of sample. All samples were filtered through a 0.2-μm nylon membrane before injection into the HPLC instrument. Peak area-under-the-curve of barakol was compared to the calibration curve and calculated into barakol content in dry plant per weight basis.

Results and discussion

Identification of extracted barakol

The five methods of identification of extracted barakol are reported. From TLC technique, the extracted barakol showed a single band with $R_f = 0.48 \pm 0.01$. UV spectrum with maximum wavelengths at 242 and 372 nm revealed that some chromophores were related to the benzene ring and conjugated double bonds of barakol. The HPLC chromatogram showed that a sharp peak of barakol eluted at retention time of 30.9 min. HPLC chromatogram of barakol is shown in Figure 2a.

The IR spectrum (Figure 3) of isolated barakol exhibited a broad band of hydroxyl group (-OH) at 2,800 to 3,600 cm^{-1}. A sharp peak at 1,676.75 cm^{-1} represented stretching vibration of -C-C = C-O-. A strong peak at 1,578.79 cm^{-1} and another at 1,564.91 cm^{-1} were characteristic of aromatic rings. The last strong peak at 1,465.55 cm^{-1} represented C-H stretching.

^{1}H-NMR spectrum showed two chemical shifts at 1.534 and 2.391 ppm indicating two hydroxyl protons. Two chemical shifts at 2.206 and 2.335 ppm indicated two methyl protons. Four chemical shifts at 5.954, 6.222, 6.260, and 6.368 ppm indicated four methylene protons. ^{13}C-NMR spectrum showed two chemical shifts at 19.200 and 20.640 ppm indicating methyl carbons. Eleven chemical shifts at 98.303, 104.422, 106.627, 107.762, 114.808, 130.921, 153.484, 158.245, 159.208, 166.115, and 183.620 ppm indicated 11 aromatic carbons. The ^{1}H-NMR and ^{13}C-NMR spectra are shown in Figures 4 and 5, respectively.

MS spectra showed mass-to-charge ratio (*m/z*) of 233 in positive ESI mode representing the molecular weight of barakol (232). All identification data were similar to standard barakol reported in previous publications (Padumanonda *et al*. 2007; Chantong *et al*. 2009). The identification results indicated that the extracted chemical constituent from fresh young leaves of *S. siamea* was barakol.

HPLC method validation

A linear correlation between peak area-under-the-curve and barakol concentration was in the range of 0.25 to 40.0 μg/mL. The linear equation of standard curve of barakol $y = 123274x + 14155$ exhibited good linearity ($R^2 = 0.9999$). The specificity was tested by observing UV spectrum of extracted barakol in the beginning, middle, and end of the barakol peak. The data revealed that all three points of the spectrum of barakol peak had the same pattern. In addition, a blank, the standard, and the plant extract were injected into HPLC and their chromatograms were confirmed. The results showed that the barakol peak was no interference of blank solution peak at the retention time of barakol indicating that the analytical method was valid. For intra- and inter-day precision, %RSD was less than 2%. The analytical method exhibited good accuracy with percent recovery of 99.25 to 101.59%. The precision and accuracy are shown in Table 2. The concentration of barakol was diluted until the signal-to-noise ratios were 10:1 and 3:1 indicating that LOQ and LOD were 16.80 and 5.04 ng/mL, respectively.

For robustness test, the three parameters were studied: flow rate, column temperature, and detection wavelength. The %RSD of peak area and %RSD of retention time were less than 2% (Table 3), indicating the analytical method was robust. In addition, the system suitability data showed that %RSD of all parameters from six replicate injections was less than 2%. The theoretical plates were 102,425 (more than 2000) and asymmetry or tailing factor was 1.74 (less than 2) for the barakol peak. The system suitability results are shown in Table 4.

Quantification of barakol content

The mature leaves and young flowers of *S. siamea* were harvested from five locations in Northeast Thailand and coded as NR, BR, SR, YS, and RE. Percent yields of these plants, which were extracted by 95% ethanol, were in the range of 10 to 16%. The extraction yields of *S. siamea* leaves were approximately 11 to 14% (average 12.32%) in comparison to the flower extract of 10 to 16% (average

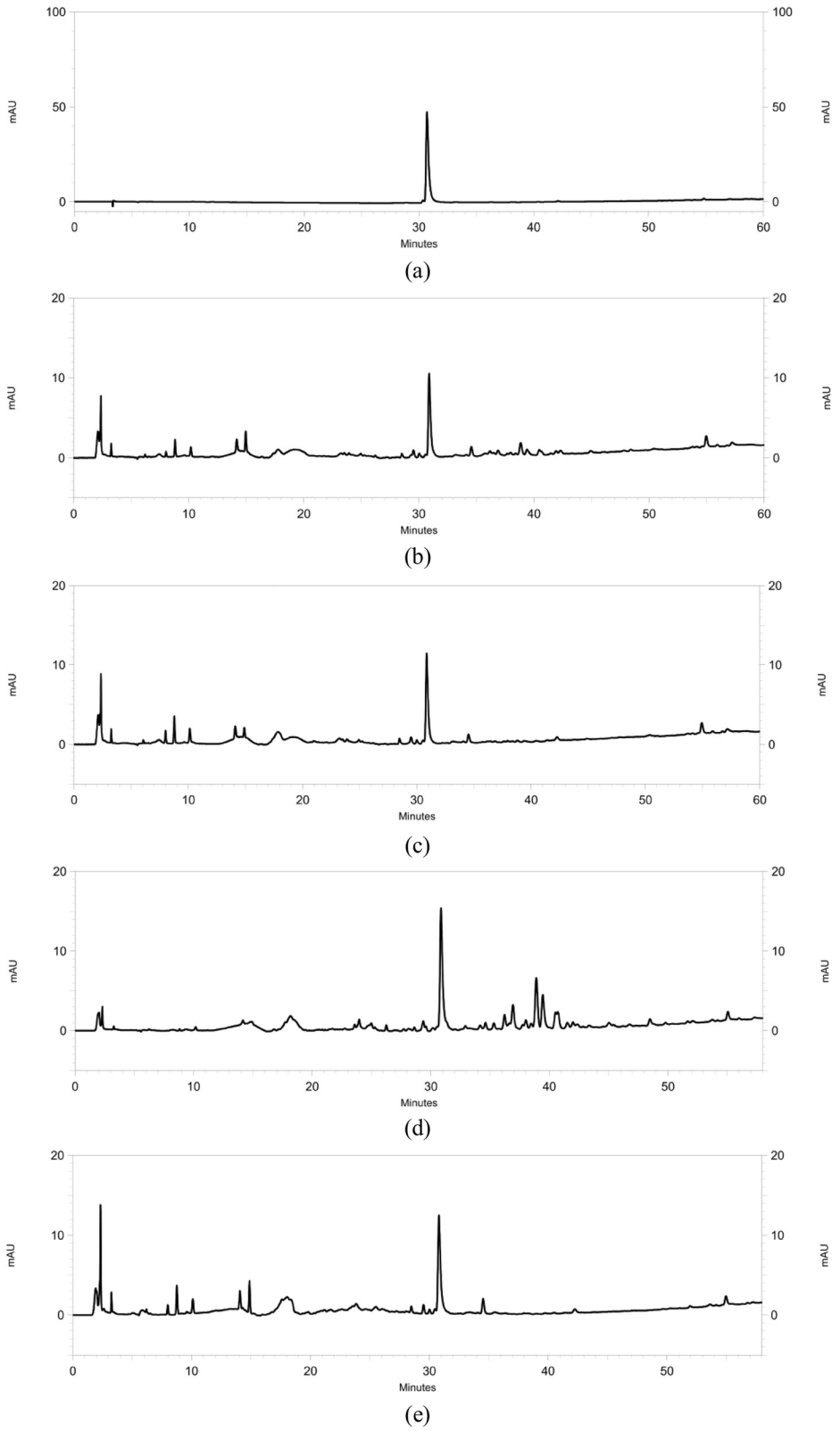

Figure 2 HPLC chromatogram of barakol (a), boiling water extract (b) and water extract of F1 (c), boiling water extract of F2 (d) and F3 (e).

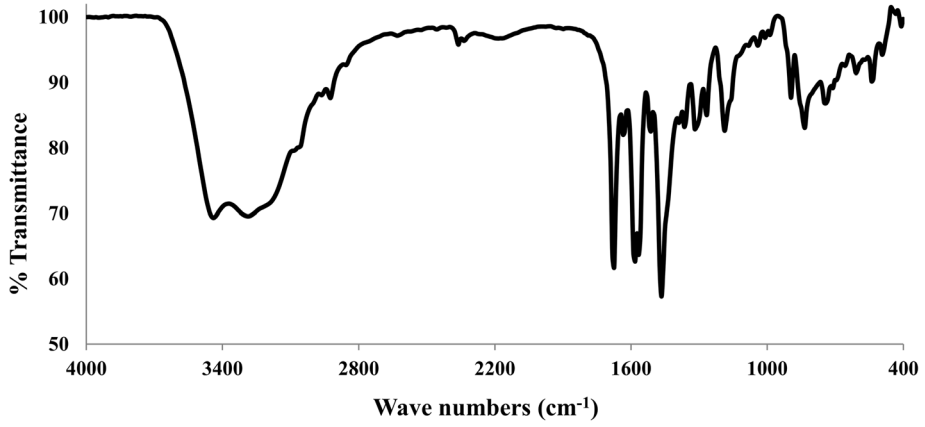

Figure 3 IR spectrum of barakol.

12.64%). Barakol content in leaves of *S. siamea* on dry weight basis ranged from 0.043 to 0.617% *w*/*w*. The lowest barakol content was found in mature leaves of NR (0.043 ± 0.005% *w*/*w*). The highest barakol content was found in SR mature leaves (0.617 ± 0.072% *w*/*w*) and YS young flowers (0.398 ± 0.026% *w*/*w*). Ranking of barakol content in *S. siamea* mature leaves was SR > RE > YS > BR > NR. The average barakol content in *S. siamea* mature leaves was 0.30% *w*/*w*, which was slightly less (approximately 0.40% *w*/*w*) than in fresh young leaves reported

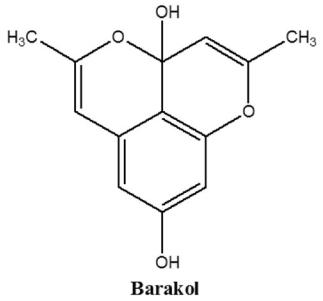

Figure 4 ^{1}H-NMR spectrum of barakol.

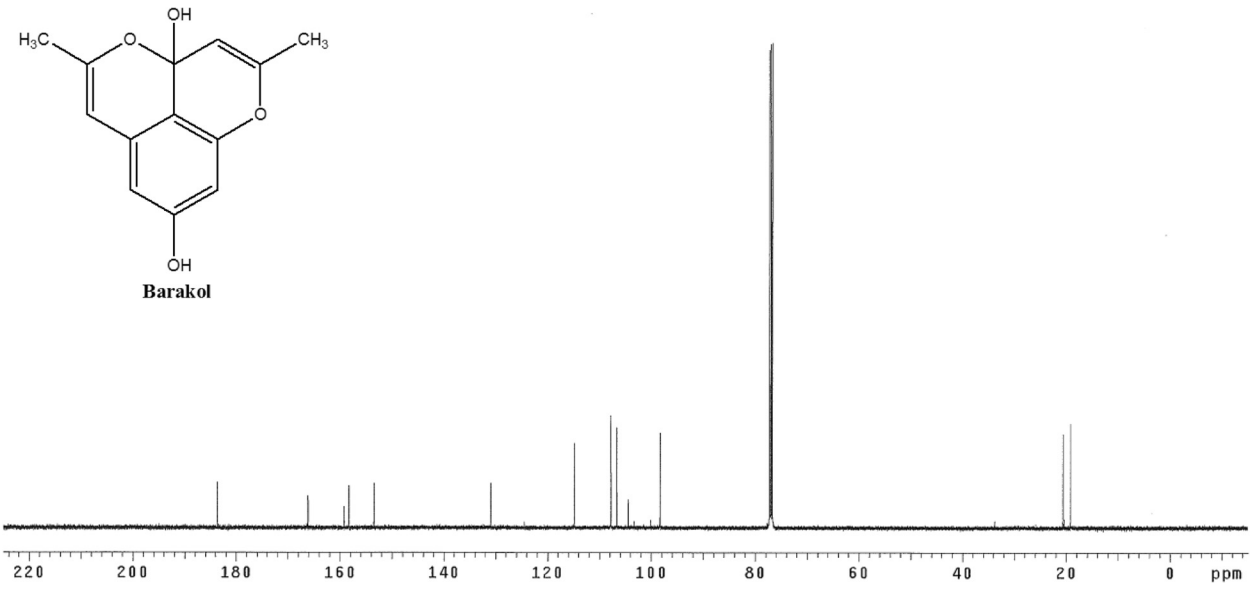

Figure 5 ^{13}C-NMR spectrum of barakol.

previously (Padumanonda and Gritsanapan 2006). For *S. siamea* young flowers, barakol content ranged between 0.189 and 0.398% *w*/*w* with an average value of 0.279% *w*/*w*. Ranking of barakol content in young flowers of *S. siamea* was YS > SR > RE > BR > NR (Table 5). The barakol content of *S. siamea* mature leaves (0.30 ± 0.23% *w*/*w*) and young flowers (0.28 ± 0.08% *w*/*w*) was slightly higher than the previous report (0.10% *w*/*w*) (Padumanonda et al. 2007). The results suggest that the mature *S. siamea* leaves harvested from Surin province are the best source for herbal recipes. Besides *S. siamea* young leaves, the young flowers from Surin, Yasothorn, and Roi Et provinces are good raw materials providing high barakol content.

In our study, the barakol content was determined in three herbal recipes referred to as F1, F2, and F3. Our preliminary study showed that 95% ethanol extract of *S. siamea* gave higher barakol yield than boiling water extract, 0.727 ± 0.121% *w*/*w* versus 0.599 ± 0.041% *w*/*w*, respectively. However, in three herbal recipes, boiling water extracts gave higher percent yields (14.67 to 15.44%) and barakol content (0.077 to 0.123% *w*/*w*) than did 95% ethanol extracts (8.78 to 11.58% and 0.019 to 0.025% *w*/*w*) as shown in Table 6. For the F1 recipe, 60% ethanol extract resulted in the highest percent yield (16.45%) but the lowest barakol content (0.018% *w*/*w*). Boiling water extract of F1 showed similar barakol content to water extract at ambient temperature (0.077 and 0.075% *w*/*w*), but relatively higher than 95% ethanol extract (0.019% *w*/*w*). Thus, boiling water was selected to compare with 95% ethanol for extraction of the other two recipes, F2 and F3. The F2 recipe showed higher barakol content than the F3 recipe, 0.123 and 0.085% *w*/*w*, respectively, in boiling water extracts. In contrast, the F2 recipe showed quite low barakol content (0.025% *w*/*w*) and could not be determined in F3 recipe for 95% ethanol extract. This occurrence may be caused by higher solubility

Table 2 Result of precision and accuracy studies ($n = 3$)

Concentration (μg/mL)	Precision (%RSD)		Spike amount (μg/mL)	Accuracy
	Intra-day	Inter-day		Recovery (%)
2.5	1.15	1.90	2.5	101.59 ± 0.47
5.0	1.02	0.99	5.0	100.45 ± 0.45
10.0	0.85	1.40	10.0	99.25 ± 0.09

Table 3 Robustness results

Condition	Variation	%RSD of peak area	%RSD of retention time
Flow rate (mL/min)	0.8	0.32	0.29
	1.0	0.11	0.02
	1.2	1.13	0.04
Column temp. (°C)	23	0.98	0.02
	25	0.11	0.02
	27	0.98	0.04
Wavelength (nm)	243	0.06	0.02
	245	0.11	0.02
	247	0.02	0.02

Table 4 System suitability results ($n = 6$)

Parameters	Retention time	Peak area	Theoretical plates (USP)	Asymmetry
Mean ± SD	30.87 ± 0.03	1682935 ± 12944	102425 ± 831	1.74 ± 0.01
%RSD	0.11	0.77	0.81	0.77

of some chemical constituents in herbal recipes than that of barakol, makes less barakol extracted by 95% ethanol.

The HPLC chromatograms of boiling water extract and water extract of F1 are shown in Figure 2b,c, respectively. Chromatograms of boiling water extract of F2 and F3 are shown in Figure 2d,e, respectively. In addition, HPLC chromatogram of 60% ethanol showed more peaks (data not shown) indicating presence of other chemical constituents in the herbal recipe. We found similar HPLC chromatogram patterns between boiling water and ambient temperature water extracts, but the different pattern was only between the retention times from 35 to 43 min. This retention time range was consistent with the presence of some chemical constituents of *S. tora*. The result implies that boiling water could extract some chemical constituents in herbal recipes to a greater extent than water at ambient temperature. Thus, in this study, boiling water was the most appropriate solvent for extraction of barakol and other chemical constituents from herbal recipes. Furthermore, Thai traditional medicine uses alcoholic solvent for extraction of the main components from *S. siamea* and uses boiling water for extraction of *S. siamea* containing formulae. These results indicate that boiling water is appropriate for Thai traditional herbal recipes.

Conclusions

Barakol, a major substance from *S. siamea* harvested from Northeast Thailand, was isolated and characterized. This study reported that the average barakol content in mature leaves and young flowers of *S. siamea* was 0.300% *w*/*w*. Samples from Surin and Yasothorn provinces yielded the highest barakol content for mature leaves and young flowers, respectively. Furthermore, boiling water was the best solvent for extraction of barakol from *S. siamea* containing recipes, thus, supporting its use in Thai traditional medicines preparations.

Table 5 Extraction yield and barakol content of mature leaves and young flowers of *S. siamea* harvested from different places in the northeast of Thailand

Code	Mature leaves		Young flowers	
	Extraction yield (%)	Barakol content (%)	Extraction yield (%)	Barakol content (%)
NR	10.84 ± 0.48	0.043 ± 0.005	10.21 ± 0.70	0.189 ± 0.016
BR	12.55 ± 0.63	0.135 ± 0.034	11.81 ± 0.48	0.221 ± 0.003
SR	12.57 ± 0.28	0.617 ± 0.072	9.97 ± 0.74	0.296 ± 0.020
YS	13.83 ± 0.73	0.301 ± 0.034	15.37 ± 1.03	0.398 ± 0.026
RE	11.79 ± 1.04	0.404 ± 0.057	15.86 ± 0.82	0.292 ± 0.042
Average	12.32 ± 1.10	0.300 ± 0.226	12.64 ± 2.81	0.279 ± 0.081

All data were represented in mean ± SD ($n = 3$).

Table 6 Effect of solvent types on barakol content of F1, F2, and F3 extract

Formula	Solvent type	Extraction yield (%)	Barakol content (%)
F1	Boiling water	14.67 ± 0.34	0.077 ± 0.006
	Water	15.67 ± 0.94	0.075 ± 0.010
	60% ethanol	16.45 ± 0.69	0.018 ± 0.001
	95% ethanol	8.78 ± 0.39	0.019 ± 0.004
F2	Boiling water	14.89 ± 0.19	0.123 ± 0.004
	95% ethanol	11.58 ± 0.31	0.025 ± 0.004
F3	Boiling water	15.44 ± 0.19	0.085 ± 0.001
	95% ethanol	10.51 ± 0.90	N.D.

All data were represented in mean ± SD ($n = 3$).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CM carried out the experiments, acquisition of the data, and drafting of the manuscript. LC and PK helped in the analysis of the results and revision of the manuscript. JS helped in the analysis and interpretation of the data and reviewed the manuscript. KK designed, gave advice on the project, and reviewed the manuscript. All authors read and approved the final manuscript.

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Author details

[1]Faculty of Pharmacy, Rangsit University, Muang, Pathum Thani 12000, Thailand. [2]Sino-Thai Traditional Medicine Research Center (cooperation between Rangsit University, Harbin Institute of Technology, and Heilongjiang University of Chinese Medicine), Rangsit University, Muang, Pathum Thani 12000, Thailand. [3]College of Oriental Medicine, Rangsit University, Muang, Pathum Thani 12000, Thailand.

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