

Original Article

DEVELOPMENT AND QUANTITATIVE DETERMINATION OF BARAKOL IN *SENNA SIAMEA* LEAF EXTRACT BY TLC-IMAGE ANALYSIS METHOD

APIRAK SAKUNPAK*, JIRAPORNCHAI SUKSAEREE, CHAOWALIT MONTON AND PATHAMAPORN PATHOMPAK

Faculty of Pharmacy and Sino-Thai Traditional Medicine Research Center (Cooperation between Rangsit University, Harbin Institute of Technology, and Heilongjiang University of Chinese Medicine), Rangsit University, Pathum Thani 12000, Thailand.
Email: apiraks@rsu.ac.th

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ABSTRACT

Objective: The aim of this study was to develop and validate an image analysis method for quantitative analysis of barakol content in *Senna siamea* leaf extract.

Methods: TLC-densitometric and TLC-image analysis methods were developed, validated, and used for quantitative analysis of *Senna siamea* leaf extract. The results obtained by these two different quantification methods were compared by paired *t*-test.

Results: Both assays provided good linearity, accuracy, reproducibility, and selectivity for determination of barakol in *Senna siamea* leaf extract.

Conclusions: The TLC-densitometric and TLC-image analysis methods provided a good reproducibility, accuracy and selectivity for the quantitative determination of barakol in *Senna siamea* leaf extract. A statistical comparison of the quantitative analysis of barakol in samples did not show any statistically significant difference between two analysis methods. As both methods were found to be equal, they therefore can be used for the analysis of barakol content in *Senna siamea* leaf extract.

Keyword: Quantitative, Barakol, *Senna siamea*, Image-analysis, TLC-densitometric

INTRODUCTION

Senna siamea Lam. is a plant belonging to the Leguminosae family. It is a small to medium sized tree, in Thai called "Khi-Lek." The fresh young leaves and flowers of Khi-Lek are used in Thai cuisine. They are edible when cooked as a curry and can also consumed as a health food as the plant has medicinal value. A decoction of the bark is given as a diabetic treatment; the roots are used as an antipyretic and the leaves for treating constipation, hypertension and insomnia [1]. It contains many compounds including emodin, luteolin, chromone alkaloids, flavanoid glycoside and barakol [2]. Barakol (3,4-dihydroxy-2,5-dimethyl-1,4-dioxypyphenalene) is the major compound was found in *S. siamea* leaves and is biological active. Pharmacological studies imply that barakol possessed anxiolytic activity on the elevated plus maze behavioral model and decreased spontaneous locomotor action in rats [3].

According to solid scientific support with regard to therapeutic efficacy, commercially available tablets of *S. siamea* leaf powder have been very popular in Thailand for producing natural sleep. However use for this purpose is now discouraged due to hepatotoxicity [4]. Acute hepatitis was reported in patients after taking a daily dose of 2-4 *S. siamea* tablets, containing 20-40 mg of barakol or approximately 0.3-0.6 mg/kg/day of barakol. This might imply that barakol at the recommended dose, which is at least 40-fold lower than the toxic dose in rat model, could induce toxicity. Consumption of *S. siamea* as an herbal medicine should therefore be wary of its barakol content in order to avoid any risk of toxicity [5]. Therefore, the amount of barakol in *S. siamea* leaves should be detected and controlled.

There are few methods for the quantification of barakol content from *S. siamea* such as HPLC and HPTLC method [6]. Although, the crucial advantages of these methods are highly sensitive and specific, the analytical instruments are quite costly and expertise is usually required [6, 7, 8]. Recently, scanning densitometers have become commonly used for quantitation of thin-layer chromatography (TLC), but the equipment does not cost less than that of HPLC. Commercial digital imaging and analyzing systems have developed software for quantitative analysis in gel electrophoresis, which also applies to the quantitation of TLC [9, 10]. The cost is less than that of the TLC scanning densitometer,

so a combination of TLC and image analysis software has been developed and applied for quantitative analysis. Thus, the aim of this study was to develop and validate this image analysis system for quantitative analysis of barakol content in *S. siamea* leaf extract.

MATERIALS AND METHODS

Plant material

S. siamea leaves were collected from Pathum Thani Province, Thailand, in January 2013. The plant was authenticated at the Herbarium of the Southern Center of Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, where herbarium specimen (Voucher No. SKP 098 19 19 01) is kept. These plants were dried at 50 °C for 24 h in a hot air oven and were reduced to coarse powders using a grinder.

Chemicals

All solvents use for chromatography were methanol (HPLC grade), water (HPLC grade), hexane (analytical grade), ethyl acetate (analytical grade) obtained from Merck (Darmstadt, Germany). Membrane filter (0.45 µm pore size) form Millipore were used for filtration of the mobile phases and sample.

Purification of barakol from *S. siamea* leaves

Barakol was isolated from *S. siamea* leaves as previously described [4]. The young fresh leaves of *S. siamea* (300 g) were reduce into small pieces and boiled with 3% sulfuric acid in water 600 mL for 15 min. The extract was filtered through filter paper. The marcs were subsequently extracted again under the same conditions. The filtered were combined and alkalized with sodium carbonate to pH 8. The basic filtered was partition with chloroform. The chloroform extract was washed with deionized water. The chloroform phase was filtered and concentrated under reduced pressure until the volume was one fourth of the starting volume. After that an equal volume of cold deionized water was added and the mixture was cooled for 30 min in refrigerator to get crude barakol and the crude product is purified by recrystallization from ethanol. Purified barakol was identified by comparing its spectroscopic data with an authentic sample.

Preparations of plant extracts

The dried leaf powder of *S. siamea* (200 mg) was extracted with methanol (20 mL) using sonication method for 1 hr. The extract was then filtered and adjusted to 50 mL with methanol. Samples were filtered through a 0.45 µm membrane filter and analyzed immediately after extraction in order to avoid possible chemical degradation. All assays of samples were performed in triplicate.

Standard solutions

A stock solution of the reference standard, barakol was made in methanol, and subsequently diluted to provide a series of the standard ranging from 84-2.625 ng/band for use in constructing a calibration curve for barakol.

TLC-densitometric method

A TLC precoated silica gel 60 F₂₅₄ plate measuring 20 × 10 cm (Merck, Darmstadt, Germany) was used. Samples were applied with a 100 µL sample syringe using the Linomat V system (Camag, Muttenz, Switzerland). CAT 4 software and TLC scanner were used for sample application and quantitative evaluation. 10 µL of sample solution was applied as 8 mm bands with a 15 mm distance between the bands. Chromatography was developed in a pre-saturated state for 30 min in a vertical twin trough glass chamber (Camag, Muttenz, Switzerland). Ethyl acetate, hexane and methanol (40:40:20 v/v/v) were used as mobile phases. After development, the plate was dried at room temperature for 10 min. Barakol was quantified by direct densitometric scanning of a developed plate at 254 nm without derivatization.

TLC-image analysis method

TLC conditions for determination of barakol content used are described as above. After the TLC plate was developed in a twin trough glass chamber, the plate was dried at room temperature. A photo to document the TLC plate was taken with a TLC visualiser (Camag, Muttenz, Switzerland) at 366 nm. The image was saved in joint photographic experts group (JPEG) format. The image was opened with the Adobe Photoshop program, converting the digital color photo into black and white. It was then resized and cropped according to the plate dimension at 20 × 10 cm, and saved at a resolution of 50 pixels cm⁻¹ for image analysis of barakol content with ImageJ (National Institute of Mental Health, USA).

Method validation

Various amounts of the stock solution (84-2.625 ng/band) were analyzed by TLC- densitometric method as described above, and calibration curves were made by plotting peak areas against concentration. The repeatability of the scanning method was tested by replicating the standard barakol six times after application to a TLC plate; then the % RSD (relative standard deviation percentage) was calculated. The variability of the method was studied by analyzing aliquots of different concentrations of standard solutions of barakol (42, 21, and 10.5 ng/band) on the same day (intraday-precision) and on different days (interday-precision) and % RSD values were calculated. Accuracy was evaluated by means of recovery assays carried out by adding known amounts of the reference compounds to the sample solutions. In order to obtain estimates of LOD and LOQ a series of concentrations of barakol were spotted on TLC plates. LOD and LOQ were determined by considering the signal to noise ratio (S/N). LOD was considered as S/N 3:1, while LOQ was S/N 10:1.

Determination of barakol content

Two hundred milligrams of *S. siamea* leaves was extracted with methanol 20 mL using sonication method for 1 hour. The extract was then filtered and adjusted to 50 mL with methanol. Samples were filtered through a 0.45 µm membrane filter and analyzed immediately after extraction. A 10 µL of each sample was spotted onto TLC plates and analyzed by the methods described above. The content of barakol in *S. siamea* leaf extract was analyzed by the TLC-

image analysis and TLC-densitometric methods. Each sample was analyzed in triplicate.

Statistic

Values are expressed as a mean ± SD. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Tukey's test (P<0.05).

RESULTS AND DISCUSSION

Method optimization and method validations

A mixture of ethyl acetate, hexane and methanol (40:40:20 v/v/v) used as mobile phase gave a good resolution of barakol. After the TLC plate was developed, the presence of barakol peak was clearly observed by TLC-densitometric and TLC-image analysis methods in the TLC chromatograms of samples with a R_f value of 0.17 (Fig. 1 and Fig. 2).

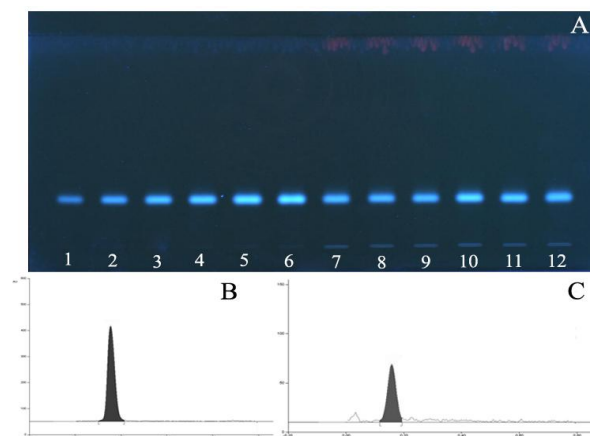


Fig. 1: (A) TLC photo-documentation at 366 nm (tracks 1-6 were standard barakol of 84 to 2.625 ng/band, and tracks 7-12 were methanol extract of *S. siamea* leaves, (B) TLC-chromatograms of standard barakol and (C) methanol extract of *S. siamea* leaves by TLC-densitometric method

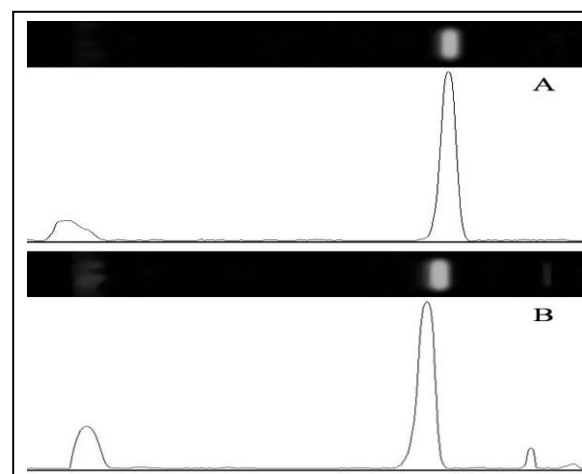


Fig. 2: TLC chromatograms obtained by ImageJ software: (A) Standard barakol and (B) methanol extract of *S. siamea* leaves

A multiple wavelength detector (MWD) was used to produce UV absorption spectra to identify the bands of barakol in *S. siamea* leaf extract, which were in good agreement with the spectrum of pure barakol (Fig. 3).

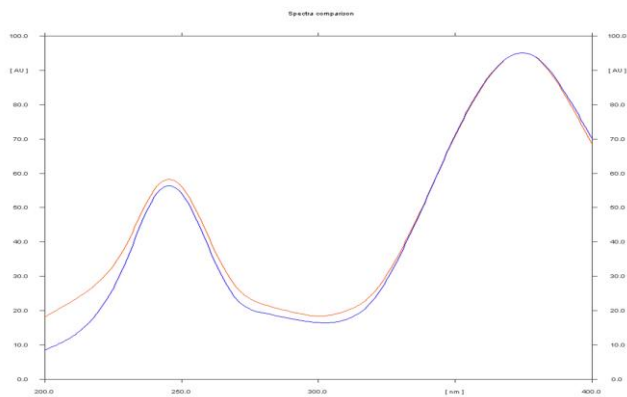


Fig. 3: UV spectra of standard barakol overlaid with barakol in methanol extract of *S. siamea* leaves (maximum absorption at 254 and 375 nm)

The calibration curves of TLC-densitometric and TLC-image analysis methods were found to be a straight line, and the polynomial regression data showed good linear relationship over the

concentration range of 84-2.625 ng/band from both methods. The regression equations were $Y = 119653X + 523.21$ and $Y = 66.97X + 6372.5$, respectively. The low value of relative standard deviation of the two methods showed that both were precise. The relative standard deviation values for both intraday and inter-day analysis of barakol were found to be less than 2%, ensuring repeatability and reproducibility of the procedure. The recovery rates were determined to be 95 to 105%. The LOD and LOQ were found to be 13 and 20 ng/band, and 7 and 10 ng/band, respectively. All of the validated data is shown in Table 1.

The result of the paired *t*-test ($P < 0.05$) indicated that there was no significant difference between the mean values of barakol content. Therefore, both TLC-densitometric and TLC-image analysis methods were found to be equal, and can be used for the determination of barakol content in methanol extract of *S. siamea* leaves.

Determination of barakol in methanol extract of *S. siamea* leaves

The TLC-densitometric and TLC-image analysis methods were used to determine the barakol content in methanol extract of *S. siamea* leaves. The contents of barakol are shown in Table 2.

Table 1: Validation data of TLC-densitometric and TLC-image analysis methods

Parameters	TLC-densitometric method		TLC-image analysis method	
Linear range	84-2.625 ng/band		84-2.625 ng/band	
Linear equations	$Y = 119653X + 523.21$		$Y = 66.97X + 6372.5$	
R ²	0.9995		0.9983	
Precision (%RSD)				
Intraday	0.72		0.58	
Interday	1.83		0.82	
LOQ	20 ng/band		10 ng/band	
LOD	13 ng/band		7 ng/band	
Accuracy reference value (ng/band)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
21	98.5	1.07	97.2	1.31
10.5	101.8	1.13	104.1	1.12
42	102.9	1.34	103.3	1.23
Average recovery	101.1		101.5	

Table 2: Barakol content in methanol extract of *S. siamea* leaves determined by TLC-densitometric and TLC-image analysis methods

Samples	Content of barakol (% w/w)	
	TLC- densitometric method (Mean ± SD)	TLC-image analysis method (Mean ± SD)
1	2.64 ± 0.11*	2.70 ± 0.21*
2	2.62 ± 0.10*	2.69 ± 0.18*
3	2.68 ± 0.08*	2.72 ± 0.11*
4	2.71 ± 0.12*	2.75 ± 0.15*
5	2.60 ± 0.13*	2.68 ± 0.21*

* No Significant difference ($P < 0.05$) when TLC-densitometric and TLC-image analysis methods were compared

DISCUSSION

This is the first report of the validated TLC-image analysis method using a common computer technology for detection and quantitation of barakol in methanol extract of *S. siamea* leaves. Both methods provided a good reproducibility, accuracy and selectivity for the quantitative determination of barakol in methanol extract of *S. siamea* leaves.

A statistical comparison of the quantitative determinations of barakol in samples did not show any statistical significant difference between TLC-densitometric and TLC-image analysis methods. The results indicate that this TLC-image analysis method can be used for quantitative analysis of the barakol content in methanol extract of *S. siamea* leaves.

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