Quantitative Analysis of Barakol Content in *Senna siamea* Leaves and Flowers by TLC-Densitometry

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Key Words

Thin-layer chromatography-densitometry · Barakol · *Senna siamea*

Abstract

Objective: To develop a TLC-densitometric method for the determination of barakol content in *Senna siamea* leaf and flower extracts, and to compare the barakol content in mature leaves, young leaves and young flowers of the plant which are consumed as a vegetable in curry. **Materials and Methods:** The extraction of pure barakol was performed by boiling the fresh young leaves of *S. siamea* with 0.5% sulfuric acid followed by chloroform extraction. The extract was further purified and recrystallized from absolute ethanol. Authentic sample of barakol was used for the validation of the TLC-densitometric method. Chromatography was performed on a TLC aluminium plate precoated with silica gel 60 F₂₅₄ as a stationary phase and chloroform-methanol (85:15 v/v) as a solvent system. Fifteen percent ethanolic extracts of mature leaves, young leaves and flowers of *S. siamea* were analyzed and compared for barakol content using the validated TLC-densitometric method. Both the validation and analysis of barakol by TLC-densitometry were carried out at the absorbance mode of 366 nm. **Results:** Barakol was extracted as pure lemon-yellow crystals from young *S. siamea* leaves with 0.1% yield. Linearity was found over the range of 200–900 ng/spot ($r^2 = 0.997$). The developed meth-

od gave high precision (%RSD < 0.50) and accuracy (average 101.12%). The limit of detection and limit of quantitation were 8 and 50 ng, respectively. Barakol content in young leaves, mature leaves and young flowers were 1.67, 0.78 and 1.43% dry weight, respectively. R_f value of the barakol in young leaves, young flowers and authentic sample was the same: 0.45 ± 0.03 . **Conclusion:** The TLC-densitometric method was simple, precise and convenient; hence it is an effective procedure for the simultaneous determination of barakol in plant extracts.

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Introduction

Senna siamea (Lam.) Irwin and Barneby is indigenously found in Thailand and locally called 'Khi-Lek'. It has a long history of use as a folk medicine and its therapeutic efficacy is well recognized. Different parts of *S. siamea* can be used for various medical purposes [1–3]. Fresh young leaves and fresh young flowers of *S. siamea* have been used as vegetable in Thailand by boiling in water for half an hour, and then the boiled water is discarded. The marc is reboiled for another 1–2 times as before and the boiled leaves are then mixed with coconut milk and curry paste and cooked as a curry. Khi-lek curry is a well-known dish of Thailand and recommended to consume as a health food for a mild laxative and sleeping aid.

Fig. 1. The conversion reaction of barakol and anhydrobarakol.

An aqueous extract of fresh or dried leaves of *S. siamea* has also been recommended for treatment of insomnia [4]. For the past few decades, barakol, a major and biologically active constituent of the extract of *S. siamea*, especially from young leaves, has been extensively studied for its anxiolytic activity [5–7].

Barakol (3,4-dihydroxy-2,5-dimethyl-1,4-dioxyphenylene, $C_{13}H_{12}O_4$, melting point 166–170°C: decomposed) contains a tricyclic ring structure [8]. It is converted to anhydrobarakol ($C_{13}H_{10}O_3$, melting point 163°C) by losing a molecule of water (fig 1). Barakol occurs as pale lemon-yellow needle crystals.

There are few methods for the quantitation of barakol in plant extracts. Siripunya [9] reported an HPLC method for the determination of barakol in alcoholic solution and in syrup formulation containing *S. siamea* extract. Thongsard et al. [10] reported the application of an HPLC method with electrochemical detection to determine the extent of purity and stability of extracted barakol solution. So far, there is no report related to thin-layer chromatography (TLC)-densitometry for the determination of barakol in the extracts from different parts of *S. siamea*. Thus, the aim of this study was to develop and validate a TLC-densitometric method for the determination of barakol in *S. siamea* extracts and to compare barakol content in mature leaves, young leaves and young flowers of the plant, which are consumed as a vegetable in curry.

Materials and Methods

Plant Materials and Reagents

Samples of mature leaves, young leaves and flowers of *S. siamea* were collected from the Bangkok area, Thailand in June 2003 and were identified by comparing them with herbariums (BKF No. 65023, BKF No. 086142) at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry

of Natural Resources and Environment, Bangkok, Thailand. The voucher specimens were kept at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The samples were dried in a hot air oven at 60°C and the dried samples were ground by an electronic hammer mill to produce powders. All reagents and solvents used for analysis were of analytical grade. Barakol was extracted and purified by the method modified from Thongsard et al. [5].

Extraction and Purification of Barakol for Using as a Standard

Fresh young leaves of *S. siamea* (6.0 kg) were sliced into small pieces and boiled with 12 liters of 0.5% sulfuric acid for 15 min; the mixture was then cooled and filtered. The marc was reboiled with 0.5% sulfuric acid using the same process as before. The filtrates were combined and made basic with an adequate amount of sodium carbonate to pH 8. The basic solution was extracted with chloroform (3 × 4 liters). The chloroform extracts were combined and shaken twice with deionized water. It was then filtered and concentrated under reduced pressure until the volume was one fourth of the starting volume. An equal amount of deionized water was added and the mixture was cooled for 30 min to get crude barakol, which was recrystallized from absolute ethanol to get green yellow crystal of barakol. Purified barakol was identified by comparing its UV, IR, 1H -NMR spectra and melting point with those of the authentic sample supplied by Dr. Chaichantipyuth C., Chulalongkorn University, Thailand.

Preparation of Standard Solutions

A stock solution of barakol was prepared by dissolving 10 mg of standard barakol in 10 ml of methanol in a volumetric flask. A standard solution of 100 ppm barakol was prepared by diluting the standard barakol with methanol.

TLC-Densitometry Instrumentation

A Camag TLC system (Switzerland) composed of an automatic TLC sampler (Linomat IV, Switzerland), TLC scanner and CATS 4 software was used for sample application and quantitative evaluation.

Chromatography was performed on a TLC precoated silica gel 60 F₂₅₄ plate 20 × 10 cm using chloroform-methanol (85:15) as a mobile phase. Total volume of solvent mixture was 30 ml. Sample bands were applied (6 mm length) at 9.4 mm intervals under nitrogen stream. A constant application rate of 0.1 μ l/s was employed. The slit dimension was kept at 6 mm × 0.45 mm and the scanning speed was 10 mm/s. Plates were developed in the Camag TLC chamber and equilibrated with the mobile phase for 40 min before inserting the plate. Chromatograms were evaluated using the peak area after scanning in absorbance mode at 366 nm for barakol.

After development, the plate was dried in air for 5 min, after which sample and standard zones were quantified by linear scanning at 366 nm by the use of a Camag TLC scanner III with a deuterium source and tungsten source. The WINCATS software controlling the densitometer produced a calibration plot by linear regression analysis relating standard zone weights to their scan areas, and the content of barakol in samples was automatically interpolated from the calibration curve.

In addition to these components, a Camag video documentation system in conjunction with the Reprostar 3 was used for imaging and archiving the TLC.

Fig. 2. UV spectra of standard barakol overlayed with barakol in the extracts (maximum absorption at 247 and 375 nm).

Method Validation

The HPTLC method was validated in terms of linearity, accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ). Linearity was determined over the range of 100–1,000 ng. Different volumes of stock solution of barakol (100 ppm), 1–10 μ l, were spotted on the TLC plate to obtain amounts of 100–1,000 ng of barakol. TLC plates were developed and analyzed as described previously. The data of peak area versus barakol concentration was plotted by linear regression analysis. Accuracy was performed by recovery studies, which were carried out by the standard addition method. The preanalyzed sample was the 15% alcoholic extract of young leaf powders of *S. siamea*. The sample solutions were spiked with 50, 75 and 100% of standard barakol and the mixtures were reanalyzed by the TLC-densitometric method. At each level of the concentration, five determinations were performed. Intraday precision was performed by analyzing barakol at three different concentrations (300, 400, 500 ng/spot) on the same day ($n = 5$). Interday precision was carried out in the same manner as intraday precision but on 6 different days ($n = 6$). In order to obtain an estimate of the LOD/LOQ, a series of concentrations of the barakol (range 1–100 ng) were spotted on the TLC plate and analyzed to determine LOD and LOQ by considering the signal-to-noise (S/N) ratio. LOD was considered as $S/N = 3:1$, while LOQ was $S/N = 10:1$.

Preparation of Samples for Estimation and Comparison of Barakol Content

An accurately weighed 0.1 g of powder of the mature leaves, young leaves and young flowers of *S. siamea* (from the same source) was separately extracted with 10 ml of 15% ethanol. Each sample was sonicated for 1 h and centrifuged at 3,500 rpm for 10 min and then filtered. The filtrate was adjusted to the original volume in a volumetric flask with 15% ethanol. The solution was analyzed for barakol content by applying 3 μ l of the solution on the TLC plate, followed by developing and scanning as described

previously. The concentration of barakol (mg/ml) in each sample was then calculated and the barakol content (% dry weight) was then determined.

Results

Identification of Extracted Barakol

The detailed characteristics of the extracted barakol including melting point, UV spectrography, details of absorption peaks and NMR chemical shifts are given in table 1. Ultraviolet spectra of the isolated barakol at the start, middle and end positions of the band completely overlapped with the band of the standard barakol (fig. 2). The amounts of barakol in young leaves, mature leaves and young flowers were found to be 1.67, 0.78, and 1.43% of dried powder, respectively. The purity of the isolated barakol was established as a single band from the TLC-densitometric method (fig. 3). The chromatograms of authentic barakol, mature leaves, young leaves and young flower extracts with an approximate range of R_f value 0.42–0.48 are given in figure 4. The band of barakol was confirmed by comparing the UV spectrum of a standard band with the corresponding sample band on the Camag TLC scanner.

The linearity for the response of barakol was assessed in a range of 200–900 ng per spot of standard barakol. The calibration lines were represented by linear equation: $y = 17.022x + 7352.6$ ($r^2 = 0.997$). Accuracy was determined by percentage recovery. The estimation of barakol

Table 1. Method of identification and characteristics of barakol

Method	Characteristic
1 Melting point	167°C (decomposed)
2 Molecular weight	232
3 Solubility	soluble in methanol, ethanol and acetone moderately soluble in chloroform and dichloromethane readily soluble in benzene, carbon tetrachloride and water
4 R _f value	0.45 ± 0.03 (silica GF ₂₅₄ , chloroform:methanol 85:15)
5 Color	pale yellow
6 UV	λ _{max} (MeOH) at 247 and 375 nm
7 IR	–3,453.7 cm ^{–1} and 3,295.8 cm ^{–1} (broad) [characteristic peak of hydroxyl group (–OH)] –1,683.4 cm ^{–1} (sharp) [characteristic peak of carboxyl group (C=O)] –1,591.2 cm ^{–1} and 1,564.9 cm ^{–1} (sharp) [characteristic peak of aromatic rings] –1,472.8 cm ^{–1} (sharp) [characteristic peak of C-H stretching]
8 ¹ H-NMR	
Position (numbering as in fig. 1)	chemical shift (ppm)
3	6.34 (1H, s)
4	6.46 (1H, d, J _{meta})
6	6.57 (1H, d, J _{meta})
9	6.10 (1H, s)
2-Me	2.25 (3H, s)
8-Me	2.42 (1H, s)

Fig. 3. Single chromatographic band of isolated barakol indicating its purity.

in the ethanolic solution after spiking with 50, 75 and 100% of additional standard barakol afforded recoveries of 101.53, 99.80, and 101.78%, respectively, as shown in table 2. Average accuracy was 101.12%. The relative standard deviation precision for intra- and interday analysis ranged from 0.18 to 0.26% and from 0.25 to 0.49%. The LOD with S/N ratio of 3:1 was found to be 8 ng/spot. The LOQ ratio of 10:1 was found to be 50 ng/spot.

Discussion

The TLC-densitometric method was fast, convenient and required small amounts of solvent and sample. The 15% ethanol was exhaustively used in extracting barakol from young leaves, young flowers and mature leaves of *S. siamea* as had successfully been applied to the analysis of pharmaceutical formulations of zolpidem hemitartrate and melatonin [11, 12], and to analysis of natural com-

Fig. 4. Comparison of chromatograms of standard barakol and scan area with barakol content in mature leaf, young leaf and young flower extracts (15% ethanol) of *S. siamea*. Chromatograms of standard barakol ($R_f = 0.44$, scan area: 10,974.55–15,010.32, $n = 5$, **a**), barakol of mature leaf extracts ($R_f = 0.44$, average scan area: 11,458.11, $n = 5$, **b**), barakol of young leaf extracts ($R_f = 0.44$, average scan area: 16,211.43, $n = 5$, **c**), barakol of young flower extracts ($R_f = 0.44$, average scan area: 14,992.04, $n = 5$, **d**).

Table 2. Accuracy of barakol by TLC-densitometric method ($n = 5$)

Excess barakol added to the matrix, %	Theoretical content, ng/spot	Experimental content, ng/spot	Accuracy %	RSD %	DEV %
50	620.76	630.29	101.53	1.99	1.53
75	720.76	719.35	99.80	1.03	0.19
100	820.76	835.34	101.78 (101.12) ^a	2.02	0.12

RSD = Relative standard deviation; DEV = deviation from the theoretical value.

^a Average accuracy.

pounds such as diospyrin and ginsenosides in plant extracts [13, 14]. Equally important, with a single plate (20 × 10 cm), TLC-densitometry can be used to develop up to 24 chromatographic runs and therefore, several samples can be compared simultaneously as was done in this study. The R_f value, UV, IR and $^1\text{H-NMR}$ spectral data, and melting point of the purified barakol were similar to those of the authentic sample and also previously reported values [15–17]. Compared to HPLC-ECD, which had been used to determine the purity of pure barakol [10], TLC-dosimetric method is less expensive and therefore offers an alternative method for quantitative analysis of barakol content in *S. siamea* leaves and flowers.

Conclusion

The TLC-densitometric method was simple, precise, convenient, and hence it was an effective procedure for the determination of barakol in plant extracts. This technique may also be used for the estimation of barakol in other alcoholic plant extracts.

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