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Validated thin-layer chromatography-densitometric method for simultaneous determination of piperine and plumbagin in "Benjakul" Thai Polyherbal formulation and its antioxidant activities

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ABSTRACT

Objective: The purpose of this study was to evaluate antioxidant activity and quantitative analysis of the piperine and plumbagin content in "Benjakul (BEN)," a Thai polyherbal formulation using the thin-layer chromatography (TLC)-densitometric method. BEN has been recognized in the national list of herbal medicinal products of Thailand as a carminative and adaptogenic drug. The formula consists, in equal proportions, of the following ingredients: Root of *Plumbago indica* Linn., root of *Piper sarmentosum* Roxb., stem of *Piper interruptum* Opiz., fruit of *Piper longum* Linn., and rhizome of *Zingiber officinale* Roscoe. **Materials and Methods:** The TLC densitometric method was validated and developed for the simultaneous determination of piperine and plumbagin in the BEN product. **Results:** The proposed TLC-densitometric method showed acceptable validation parameters. The content of piperine and plumbagin in the in-house preparation and four commercial BEN products was found in the ranges of 0.98 ± 0.0007–1.71 ± 0.0011% w/w and not detected - 0.61 ± 0.0009% w/w, respectively. The total phenolic contents were in the range of 5.8 ± 0.4–45.4 ± 1.3 g gallic acid equivalent/100 g extract. For the 2,2-diphenyl,1-picrylhydrazine (DPPH) scavenging activity, the effective concentration$_{50}$ values were in the range of 59.1 ± 8.5–478.0 ± 13.7 μg/ml, while the 2,2'-Azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging activities yielded results in the range of 22.0 ± 1.2–90.3 ± 3.8 mg trolox equivalents antioxidant capacity/g extract. **Conclusion:** BEN exhibited a potent antioxidant activity which may be related to the % content of piperine and plumbagin. Moreover, this proposed TLC densitometric method is rapid, reliable, sensitive, and economical for routine analysis of the piperine and plumbagin content in BEN raw materials and its commercial products.

INTRODUCTION

Benjakul (BEN) is a Thai polyherbal formulation, which consists of 5 ingredients, root of *Plumbago indica* Linn. (Plumbaginaceae), root of *Piper sarmentosum* Roxb. (Piperaceae), stem of *Piper interruptum* Opiz. (Piperaceae), fruit of *Piper longum* Linn. (Piperaceae), and the rhizome of *Zingiber officinale* Roscoe, (Zingiberaceae) in equal proportions. BEN has been indicated in the list of herbal medicinal products of the national list of essential medicines of Thailand 2016, in the traditional medicine group, as a carminative and adaptogenic drug.[1] Adaptogenic herbs have been used extensively in folk medicine to support the body in achieving a state of balance or homeostasis.

Adaptogenic drugs are believed to possess strong antioxidant activities; therefore, the therapeutic indication of these drugs can include diseases relating to many disorders such as cardiovascular diseases, neurodegenerative diseases, cancer, and aging.[2] In the southern part of Thailand, BEN has been administered as an adjuvant therapy to patients before receiving chemotherapy to improve their immune

functions. BEN promotes benefits to patients by decreasing chemotherapy side effects such as hair loss and pain.[3] Many pharmacological activities of BEN have also been reported including cytotoxicity, anti-inflammatory, and antibacterial activity.[4-6]

Piperine, an alkaloid which found in many species of *Piper*, has been identified as the major component of this polyherbal formulation. It has been found to exhibit antioxidant, anticancer activities, and the reduction of nausea and vomiting in patients with cancer cachexia.[7-9] In addition, plumbagin in *P. indica* has been shown to possess antioxidant and cytotoxic activities.[10,11] Thus, piperine and plumbagin could be regarded as marker compounds for the quality assessment of a BEN formulation [Figure 1]. Several analytical methods have been reported for the quantitative analysis of piperine and plumbagin, including high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), and the ultraviolet (UV)-visible spectrophotometric method.[12-14]

The quality control of herbal medicinal products, including qualitative and quantitative analysis, is an important process for ensuring safety and efficacy. The chromatographic fingerprint and methods of determination of the active ingredients are considered as key strategies in quality control. Among others, HPLC is an analytical method of choice for pharmaceutical analysis because of its wide range of applications. Meanwhile, the TLC-densitometric method, which is simple and has fast data acquisition and cost efficiency, is considered to be an alternative method for product quality assurance. Therefore, the aim of this study was to evaluate in the *in vitro* antioxidant activities and validate a TLC-densitometric method for the quantification of piperine and plumbagin content in BEN for the quality control of the raw materials and phytopharmaceutical products.

MATERIALS AND METHODS

Chemicals and Reagents

All of the following chemicals were of analytical grade: Folin–Ciocalteu reagent, 2,2-diphenyl,1-picrylhydrazyl (DPPH), 2,2'-Azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS), standard piperine, and plumbagin and were acquired from Sigma Chemical Co. (USA).

Plant Materials

For the in-house preparation, the following ingredients were procured from a local drugstore in Bangkok, Thailand, in September 2016: Root of *P. indica,* root of *P. sarmentosum,* stem of *P. interruptum,* fruit of *P. Longum,* and rhizome of *Z. officinale*.

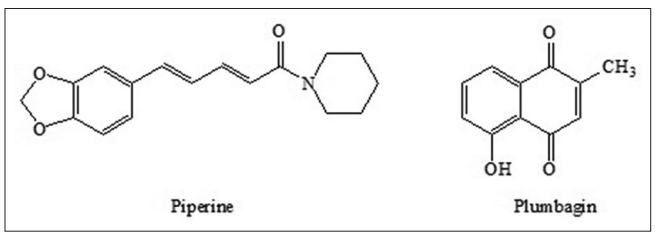

Figure 1: Chemical structure of piperine and plumbagin

The voucher specimens (SCFC081601-05) were deposited at the Department of Food Chemistry, Faculty of Pharmacy, Mahidol University. Dried plant samples were ground into coarse powders using an electric grinder and sieved through a #20 mesh and mixed in equal proportions according to the BEN formula. The powdered drug was kept in an airtight container and protected from light until used.

- BEN commercial product: Four samples of BEN commercial products were purchased from local herbal drug stores in Bangkok, Thailand, in September 2016.
- Brand A: Each capsule contained 500 mg of BEN formulation (Lot No. 0101402 Mfg date: 07/07/2014 Exp date: 07/07/2017).
- Brand B: Each capsule contained 300 mg of BEN formulation (Lot no. 15097DPD20301 Mfg date: 25/08/2015 Exp date: 25/08/2017).
- Brand C: Each capsule contained 500 mg of BEN formulation (Lot no. CA7-59001 Mfg date: 16/07/2016 Exp date: 16/07/2018).
- Brand D: Each capsule contained 500 mg of BEN formulation (Mfg date: 19/11/2015).

Antioxidant Activities

Preparation of BEN extracts

The powdered in-house preparation and the BEN commercial products were accurately weighed (500 mg) and dissolved in methanol, sonicated for 60 min, and then filled up to volume in a 50 ml volumetric flask. Each solution was filtered through a Whatman No. 1 filter paper and diluted 1:10 with methanol to use as the stock solution (1 mg/ml). Sample solutions were stored at 4°C in amber bottles for subsequent analyses.

Determination of total phenolic content using Folin–Ciocalteu method[15]

Each sample (in-house preparation and the BEN commercial products at a 0.2 ml of 1 mg/ml solution) was mixed with 0.5 ml of Folin–Ciocalteu reagent (diluted 1:10 with deionized water) and 0.8 ml of 7.5% sodium carbonate solution. The mixture was allowed to stand at room temperature for 30 min with intermittent shaking. The absorbance of the mixture was measured at 765 nm using a UV-visible spectrophotometer. Gallic acid was used for the standard curve in the range of 6.25–100 μg/ml. The content of total phenolic compounds is calculated as mean ± standard deviation (SD) (n = 3) and expressed as grams of gallic acid equivalents (GAE)/100 g of the extract.

DPPH radical scavenging assay

DPPH radical scavenging assay was modified from Pothitirat *et al.*[15] A total of 100 μl of in-house preparation and BEN commercial products were added to 100 μl of DPPH methanolic solution (152 μM). After the reaction, solutions were kept in the dark at room temperature for 15 min, and the absorbance of each solution was determined at a wavelength of 517 nm using a microplate reader (Tecan, USA). L-ascorbic acid was used as the standard and treated under the same condition as the samples.

The percentage of inhibition was calculated using the formula % inhibition = $([A_c-A_s]/A_c) \times 100$; where A_c is

the absorbance of the control solution at 517 nm, and A_s is the absorbance of the sample solution at 517 nm. Then, the effective concentration (EC_{50}) value, the concentration of sample required for 50% scavenging of DPPH free radicals, was determined from the plot between % inhibition and sample concentration. Each sample was done in triplicate, and the average EC_{50} value was reported as mean ± SD.

ABTS radical scavenging assay

An ABTS radical scavenging assay was modified from Mayur *et al.*[16] The ABTS radical solution was prepared by mixing 0.0768 g of ABTS and 0.0132 g of potassium persulfate in 20 ml of distilled water. The mixture was allowed to stand for 12–16 h at room temperature in the dark until reaching a stable oxidative state. Then, the solution was diluted by mixing ABTS radical solution with methanol to obtain an absorbance of 0.700 ± 0.020 unit at the wavelength of 734 nm using a microplate reader. The sample solution of in-house preparation and the BEN commercial products (10 μl of 1 mg/ml) was mixed with 200 μl of freshly prepared ABTS radical solution. After 6 min of initial mixing, the absorbance was measured at a wavelength of 734 nm using a microplate reader. All tests were carried out on three replicates. Trolox was used for the standard curve. Results were expressed in mg Trolox equivalents antioxidant capacity (TEAC)/g extract.

TLC-Densitometric Analysis

Instrument and chromatographic condition

The samples of in-house preparation and BEN commercial products were spotted as 7 mm bands on a TLC plate silica gel 60 F_{254} (20 cm × 10 cm with 0.2 mm thickness; Merck, Darmstadt, Germany), using a Camag Linomat 5 automatic sample spotter (CAMAG, Switzerland) with a constant rate of 100 nL/s. The mobile phase consisted of toluene:ethyl acetate:formic acid (8:2:0.5, v/v/v) was put in a twin through glass chamber (20 cm × 10 cm) at room temperature and left for saturation for 30 min before use. The linear ascending development was performed in the chamber saturated with the mobile phase. The length of the chromatogram run was 80 mm from the point of application. Densitometric scanning was performed using a Camag TLC scanner 3 at a wavelength of 254 nm. The slit dimension was 6.00 mm × 0.45 mm with a scanning speed of 20 mm/s.

Preparation of sample solution

In-house preparation: The powdered drug was accurately weighed (500 mg) and dissolved in methanol (20 ml), sonicated for 30 min, and then filled up to volume in a 25 ml volumetric flask. Each solution was filtered through a 0.45 μm nylon membrane filter and analyzed in triplicate. The sample solution of 5 μl/spot was applied.

BEN commercial products: 20 capsules of each BEN commercial product were accurately weighed. The amount of powdered capsule, theoretically equivalent to one capsule (500 mg), was dissolved in methanol (20 ml), sonicated for 30 min, and then adjusted to 25 ml in a volumetric flask. Each solution was filtered through a 0.45 μm nylon membrane filter and analyzed in triplicate. The sample solution 5 μl/spot was applied.

Preparation of standard solution

Piperine and plumbagin reference standards were separately and accurately weighed and dissolved in methanol in a volumetric flask for the preparation of the stock solution (1 mg/ml). Piperine and plumbagin working standard solutions were prepared to obtain a final concentration of 100 μg/ml and 50 μg/ml, respectively.

Method validation

The method was validated by the evaluation of linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness according to the International Conference on Harmonization Guidelines.[17]

Linearity

From the working standard solutions of piperine and plumbagin, 1–6 μl of each standard solution was applied as bands on the TLC plate, corresponding to a concentration of 100–600 ng/band for piperine and 50–300 ng/band for plumbagin, respectively. Their calibration curves were obtained by plotting the peak area versus the concentration of the standard solution of each standard.

Precision

The precision of the analytical method for each compound was determined by analyzing 100, 150, and 200 ng/band of piperine and 50, 75, and 100 ng/band of plumbagin standard solution after the application on a TLC plate (n = 3) on the same day, for intraday precision, and on 3 different days for interday precision. The precision was expressed as percent relative SD (% RSD).

Accuracy

The accuracy of the analytical method for each compound was evaluated by determination of recovery. The recovery of piperine and plumbagin was performed on a sample spiked with three concentration levels of standards (approximately 50%, 100%, and 150% of the determined content of BEN formulation) (n = 3).

LOD and LOQ

LOD and LOQ of the analytical method were determined based on the SD of the response and the slope (S) of each calibration curve of piperine and plumbagin according to the formula: LOD = 3.3 (SD/S) and LOQ = 10 (SD/S).

Statistical Analysis

Results are expressed as the mean values ± SD (n = 3). Data were analyzed by one-way analysis of variance (ANOVA) and Scheffe's test using SPSS Software. Differences at the 95% level were considered to be statistically significant.

RESULTS AND DISCUSSION

Antioxidant Activities

From the results, the total phenolic content of BEN was in the range of 5.8 ± 0.4–45.4 ± 1.3 g GAE/100 g extract. BEN commercial product (Brand A) showed the highest content

of total phenolic compound among all the samples. From the statistical evaluation, the one-way ANOVA showed a significant difference between the means of the total phenolic content in the in-house preparation and the 4 commercial products. For DPPH scavenging activity, the results showed that the EC_{50} value of BEN was in the range of 59.1 ± 8.5–478.0 ± 13.7 μg/ml. For ABTS radical-scavenging activities, the results from the BEN samples were in the range of 22.1 ± 1.2–90.3 ± 3.8 mg TEAC/g sample [Table 1]. Corresponding to the results of the DPPH assay, Brand A exhibited the strongest ABTS radical scavenging activity. The one-way ANOVA showed a significant difference between the means of the EC_{50} value and the TEAC value in all samples. Phenolic compounds are very important plant constituents as their hydroxyl groups confer scavenging ability. Free radical scavenging activities of BEN were directly proportional to the phenolic content present in the product.

TLC Densitometric Method

Toluene:ethyl acetate:formic acid (8:2:0.5, v/v/v) gave the best TLC-densitogram peak resolution of piperine and plumbagin out of the various mobile phases that were trialed [Figure 2]. The good specificity of the bands of piperine (R_f = 0.44) and plumbagin (R_f = 0.85) in the BEN formulations was confirmed by overlaying the absorption spectra of the samples with piperine and plumbagin reference compounds [Figure 3]. The specificity of the analyzed peaks was checked at three different peak levels, i.e., start, apex, and end positions of the peak corresponding to piperine and plumbagin.

The proposed TLC-densitometric method showed acceptable validation parameters [Table 2]. The calibration curve of piperine and plumbagin was linear over the range of 100–600 ng/band and in the 50–300 ng/band, respectively. The correlation coefficient value was ≥0.995, confirming the linearity of the method. The % RSD value of intra- and inter-day precision was <5%. The LOD of piperine and plumbagin was found to be 5.0 and 2.8 ng/band, while the LOQ of piperine and plumbagin was found to be in the 15.3 and 7.5 ng/band, respectively. The average recoveries of piperine and plumbagin were 99.9 ± 4.3% and 107.5 ± 8.5%, respectively. The validation parameters indicated both good precision and accuracy of the analytical method.

The densitograms of piperine and plumbagin in the sample of BEN are shown in Figure 4. The content of piperine and plumbagin in the in-house preparation and the 4 commercial products was in the ranges of 0.98 ± 0.0007–1.71 ± 0.0011% w/w and not detected - 0.61 ± 0.0009% w/w, respectively [Table 3]. Both piperine and plumbagin were found in the in-house preparation and Brand A product, whereas only piperine was found in Brands B, C, and D, with no plumbagin detected in these samples. A previous report on stability studies of BEN extract tablets showed that the tablets could be kept in a closed amber glass container and stored under accelerated conditions (45°C ± 2°C with 75 ± 5% RH) for up to 4 months.[18] The remaining percentages of pipierine and plumbagin were reanalyzed by HPLC at 4 weeks' intervals and it proved that piperine was stable, whereas plumbagin decomposed rapidly and could not be detected after 4 months. A decrease in the amount of plumbagin may be due to its low melting point (78–79°C). Therefore, the temperature should be control throughout each stage of production to avoid the overheating and degradation of plumbagin in the final product. In addition, the amount of piperine and plumbagin in the plant raw materials could vary depending on many influencing factors, such as geographical origin or the area of plantation, growth conditions, and environmental variations.

The strong antioxidant activities may relate to the amount of piperine, and particularly, plumbagin found only in the in-house preparation and Brand A. Previous studies on the antioxidant activities of plumbagin have revealed its antioxidant activities including DPPH scavenging activity, ferric reducing power, total antioxidant capacity, and inhibit lipid peroxidation.[19,20] The presence of both quinone and phenol groups in plumbagin structure is regarded as the main active radical displaying antioxidant capacity. Plumbagin scavenges oxidizing free radicals, takes part in redox cycling, followed by the oxidation of the reaction product and the concomitant generation of reactive oxygen species.[21] Therefore, plumbagin could be a potential source of a natural antioxidant that may possibly explain some of the reported therapeutic effects.

From previous HPLC methods for the quantification of the BEN product ingredients,[11] a reversed-phase HPLC was performed with a gradient mobile phase composed of water and acetonitrile with a total runtime of 60 min. Piperine and plumbagin have also been regarded as marker compounds with a retention time of 28 and 25 min, respectively. The

Table 1: Total phenolic content, DPPH scavenging activity, and ABTS scavenging activity of BEN product

BEN product	Total phenolic content (g GAE/100 g extract)	DPPH scavenging activity EC50 value (μg/ml)	ABTS scavenging activity (mg TEAC/g extract)
In-house preparation	5.8±0.4[a]	239.3±5.7[b]	34.4±5.7[a]
Brand A	45.4±1.3[c]	59.1±8.5[a]	90.3±3.8[b]
Brand B	10.5±0.9[b]	448.8±38.1[c]	38.6±1.7[a]
Brand C	6.7±0.6[a]	478.0±13.7[c]	25.4±1.0[a]
Brand D	10.9±1.1[b]	466.3±15.2[c]	22.1±1.2[a]
L-ascorbic acid		4.3±1.1	

Mean±SD (n=3), GAE: Gallic acid equivalent, TEAC: Trolox equivalent antioxidant capacity, values in the column followed by a different letter (a-c) are significantly different (P<0.05) and values having same letter are not statistically significant. DPPH: 2,2-Diphenyl, 1-picrylhydrazyl, ABTS: 2,2'-Azino-bis (3-ethyl benzothiazoline-6-sulfonic acid), BEN: Benjakul, EC50: Effective concentration

results were revealed to be in accordance with our study where piperine has been identified as the major compound. The amounts of piperine and plumbagin in the BEN extract determined by the HPLC method were 47.61 and 2.46 mg/g, respectively. In comparison to the HPLC method, the simplicity and high sample throughput of the TLC densitometric method is suitable as an alternative method for the routine analysis and quality control of the BEN product.

Table 2: Method validation parameters by the proposed TLC-densitometric method

Parameter	Piperine	Plumbagin
Range of linearity	100–600 ng/band	50–300 ng/band
Regression equation (n=3)	Y=342.96+8.07X	Y=297.09+8.95X
Correlation coefficient (r^2)	0.99762±0.0016	0.99523±0.0020
% RSD intraday precision	0.4–4.1	0.6–3.5
% RSD interday precision	2.0–3.8	3.1–3.9%
% Recovery	99.9±4.3	107.5±8.5
LOD	5.0 ng/band	2.8 ng/band
LOQ	15.3 ng/band	7.5 ng/band

X: Concentration of piperine and plumbagin in ng/ml, Y: Peak area. TLC: Thin-layer chromatography, RSD: Relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantitation

Table 3: The content of piperine and plumbagin in BEN products determined by the validated TLC-densitometric method

BEN product	Content of major compound[a] (% w/w)	
	Piperine	Plumbagin
In-house preparation	0.98±0.0007	0.61±0.0009
Brand A	1.48±0.0003	0.51±0.0014
Brand B	1.31±0.0009	Not detected
Brand C	1.18±0.0011	Not detected
Brand D	1.71±0.0011	Not detected

[a]Expressed as mean±SD (n=3). BEN: Benjakul, TLC: Thin-layer chromatography, SD: Standard deviation

CONCLUSION

BEN obviously contains significant amounts of total phenolic compounds and exhibits *in vitro* antioxidant activities. The antioxidant activities of the BEN samples seem to correlate with the total phenolic contents. Moreover, they could also correlate to the amount of piperine and plumbagin in the products, which could be responsible for the wide range of traditional uses of BEN.

The proposed TLC-densitometric method showed several advantages over the alternatives such as several samples can be separated on the same plate, fewer organic solvents are used, no

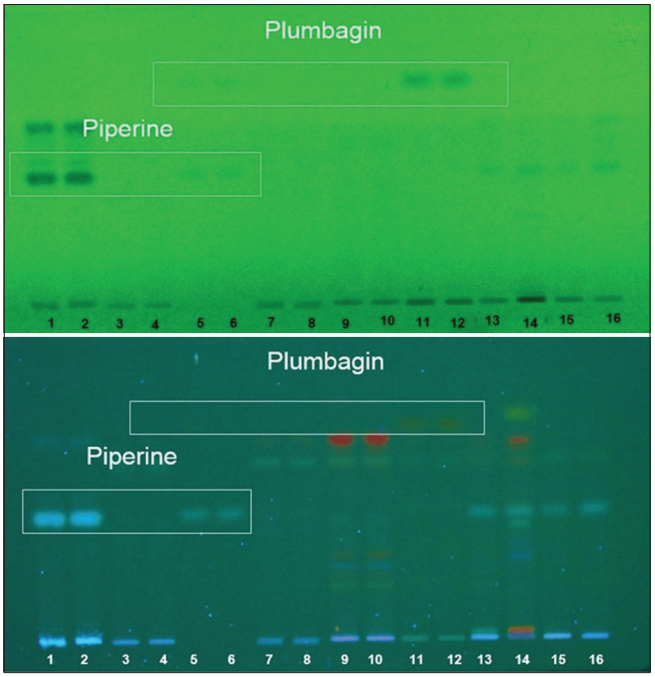

Figure 2: Thin-layer chromatography chromatogram of Benjakul extracts (silica gel F_{254}, Toluene: ethyl acetate: formic acid 8:2:0.5 v/v/v); track 1, 2 = fruit of *Piper longum*, track 3, 4 = rhizome of *Zingiber officinale*, track 5, 6 = standard piperine and plumbagin, track 7, 8 = stem of *Piper interruptum*, track 9, 10 = root of *Piper sarmentosum*, track 11, 12 = root of *Plumbago indica*, track 13 = Brand C, track 14 = Brand A, track 15 = Brand B, track 16 = Brand D (a) detected under ultraviolet (UV) 254 nm, (b) detected under UV 366 nm

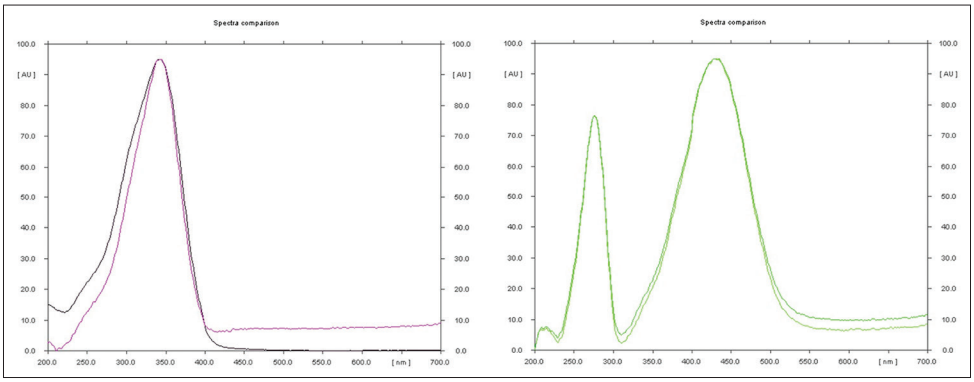

Figure 3: Overlay ultraviolet spectra scanning from 200 to 700 nm by thin-layer chromatography-densitometric method of (a) piperine reference standard and sample, (b) plumbagin reference standard and sample

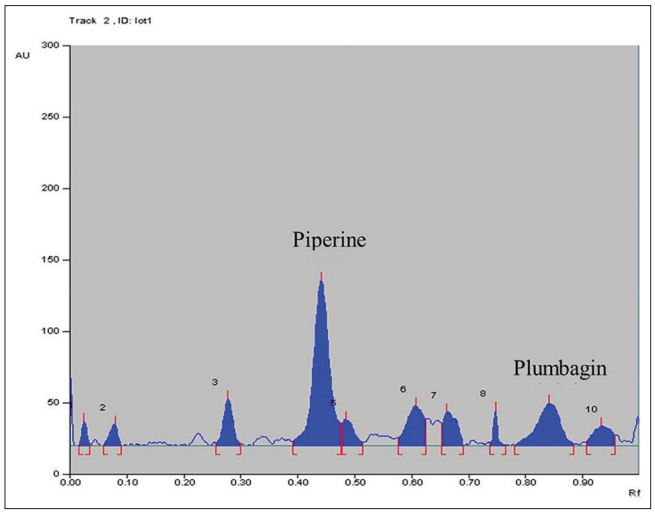

Figure 4: Densitogram of Benjakul commercial product (Brand A)

clean-up process is required, and it offers a rapid and low-cost analysis. Therefore, a densitometric TLC can be conveniently used for both the qualitative and quantitative analysis of piperine and plumbagin in BEN for quality assessment in both the raw materials and the finished product. This proposed method can be employed as a routine analysis of BEN, which could contribute to good quality phytopharmaceutical products.

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