



## Determination of Ellagic acid in *Lagerstroemia speciosa* L. Leaf Extract

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### Introduction

*Lagerstroemia speciosa* L., known as Banaba (Family Lythraceae) is widely grown in Thailand and Southeast Asian countries.<sup>1</sup> The plant is traditionally used as an herbal medicine for diabetics. Currently, ellagic acid, one of the major active compounds in Banaba leaves,<sup>2</sup> has been reported to have a variety of biological activities such as antioxidant, anti-inflammatory and anti-arthritis properties.<sup>3-6</sup> Interestingly, these properties suggested that ellagic acid could possibly be used for protection of arthritis. Bioactive compounds of Banaba leaves have been quantified by using nuclear magnetic resonance spectroscopy, high performance liquid chromatography (HPLC) and high performance thin layer chromatography.<sup>7-9</sup> The complex mixture of compounds in Banaba leaf powdered extract are difficult to separate. Although there are several studies on chemical constituent of Banaba leaf, there is no report about the determination of ellagic acid in Banaba leaf powdered extract. The determination of ellagic acid content is important for quality control of Banaba leaf powdered extract and its related products. Hence, this study aimed to develop HPLC analytical method for quantification of ellagic acid in Banaba leaf powdered extract.

### Methods

#### Chemicals

Ellagic acid was purchased from Sigma-Aldrich. (St. Louis, MO, USA). Acetonitrile, hexane and methanol were bought from Burdick & Jackson (Muskegon, MI, USA). *o*-Phosphoric acid (85%) was obtained from ACI Labscan (Bangkok, TH) and sterile water used in HPLC analysis was purchased from A.N.B (Bangkok, TH). All organic solvents were analytical grade and filtered through a 0.2  $\mu$ m nylon membrane filter from National Scientific (Rockaood, UK) before use.

#### Plant extract preparation and spray drying of the extract

The Banaba leaves were collected from Chonburi province, Thailand. The plants were authenticated by Prof. Dr. Wongsatit Chuakul (Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University). The dried leaves of Banaba were grounded to a coarse powder and stored in well-sealed plastic container. The powder (50 g) was extracted with 1 L of hot water, between 75-95°C, for 20-30 minutes, after that the aqueous extract was filtered through a 0.45  $\mu$ m membrane filter and concentrated *in vacuo*. The concentrate was spray dried using Buchi mini spray dryer B-290 (Buchi, Switzerland) with inlet temperature at 150°C, outlet temperature at 100°C and 20% pump to obtain the powdered extract. Then it was kept at -20°C in the refrigerator for further analysis.

#### Loss on drying

Physical test of the powdered extract was determined by loss on drying according to the USP monograph.<sup>10</sup> Two grams of powdered extract was accurately weighed in aluminum weighing dish and heated at 105°C in hot air oven for one hour. The dish was left in a desiccator at room temperature before weighing. The second weighing was recorded after an hour of additional period of drying. The process was continued until two consecutive weights did not differ by more than 0.5 mg/g of powdered extract taken.

#### Sample preparation for thin layer chromatography (TLC) analysis

Ten milligrams of Banaba leaf powdered extract was accurately weighed, dissolved in 1 mL of water in the presence of a small quantity of 1 N NaOH in water (0.6% v/v) and filtered through a 0.2  $\mu$ m nylon

syringe filter. Pre-concentration of the samples was performed using Waters Oasis HLB (3 mL, 60 mg) solid phase extraction cartridges (Guyancourt, France). Before sample loading, the solid phase adsorbent was pre-conditioned with 1 mL of methanol followed by 1 mL of water. After sample loading, the solid phase was washed with 1 mL methanol followed by 2×1 mL of hexane. The extract was eluted with 2×500 µL of mixture of acetonitrile and 0.1% phosphoric acid in water (75:25 v/v). A standard solution of 500 µg/mL was prepared by dissolving accurately weighed quantity of ellagic acid in water in the presence of a small quantity of 1 N NaOH in water (0.6% v/v).

### **TLC analysis**

The samples and ellagic acid were subjected to TLC analysis using silica gel 60 F<sub>254</sub> aluminum sheet as stationary phase. Ten microliters of sample was spotted onto TLC plate and developed with chloroform-methanol-formic acid (9:1:2). After drying the plate in a fume hood, TLC plate was sprayed with anilidehydro-sulfuric acid reagent and heated at 105°C. TLC chromatogram was observed under visible and UV light at 254 and 366 nm.

### **Sample preparation for HPLC analysis**

Four milligrams of Banaba leaf powdered extract was accurately weighed, dissolved in 1 mL of water in the presence of a small quantity of 1 N NaOH in water (0.6% v/v) and filtered through 0.2 µm nylon syringe filter. Pre-concentration of the samples was performed using Waters Oasis HLB (3 mL, 60 mg) solid phase extraction cartridges (Guyancourt, France). Before sample loading, the solid phase adsorbent was pre-conditioned with 1 mL of methanol followed by 1 mL of water. After sample loading, the solid phase was washed with 1 mL methanol followed by 3×1 mL of hexane. The extract was eluted with 2×500 µL of mixture of acetonitrile and 0.1% phosphoric acid in water (75:25 v/v). Finally, the volume of eluate was adjusted to 2 mL by mixture of acetonitrile and 0.1% phosphoric acid in water (75:25 v/v) and 10 µL of the final eluate was injected into HPLC system for analysis.

### **HPLC analysis**

The analysis was performed on a Surveyor HPLC system (Thermo Finnigan, Germany) with photodiode array detector (PDA), auto-sampler and the Xcalibur software. Separation was carried out using LiChrospher® 100 RP-18, 5 µm, (250 X 4.6 mm) column (Merck, Darmstadt, Germany) at ambient temperature. The mobile phase consisted of acetonitrile (eluent A) and 0.1% v/v phosphoric acid in water (eluent B). The gradient program was started with 65% of eluent B and changed to 60, 55 and 50% of eluent B at 4, 10 and 16 minutes, respectively. It was held for 4 minutes and then percentage of eluent B was increased to 65% again for the following 5 minutes. The injection volume for all samples was 10 µL at flow rate 500 µL/min and total run time was 25 minutes. The spectra were recorded from 200 to 600 nm and the specific detection wavelength for ellagic acid was 254 and 366 nm.

### **Validation of the HPLC method**

The developed HPLC method was validated according to ICH guidelines.<sup>11</sup> Specificity was determined by comparing the HPLC retention time and absorption spectra of target peak from the analyzed samples with those of the reference compound. Calibration curve of reference standard was obtained from the five different concentrations of ellagic acid in the range of 10-50 µg/mL. Linear regression was obtained by plotting the mean peak area versus concentration. The calibration curve equation and the corresponding correlation coefficients (R<sup>2</sup>) were calculated. Repeatability and intermediate precision were evaluated by the repeated injection. The repeatability experiment was assessed using six determinations at 100% of the test concentration for a day, and intermediate precision was determined by six injections for three different days. The precision was expressed as % relative standard deviation (%RSD). Accuracy was evaluated through recovery study. Analyzed samples were spiked with 14, 28 and 42 µg/mL of ellagic acid. The amount of each analyte was determined in triplicate and the percent recovery of ellagic acid from spiked samples was calculated as follows:

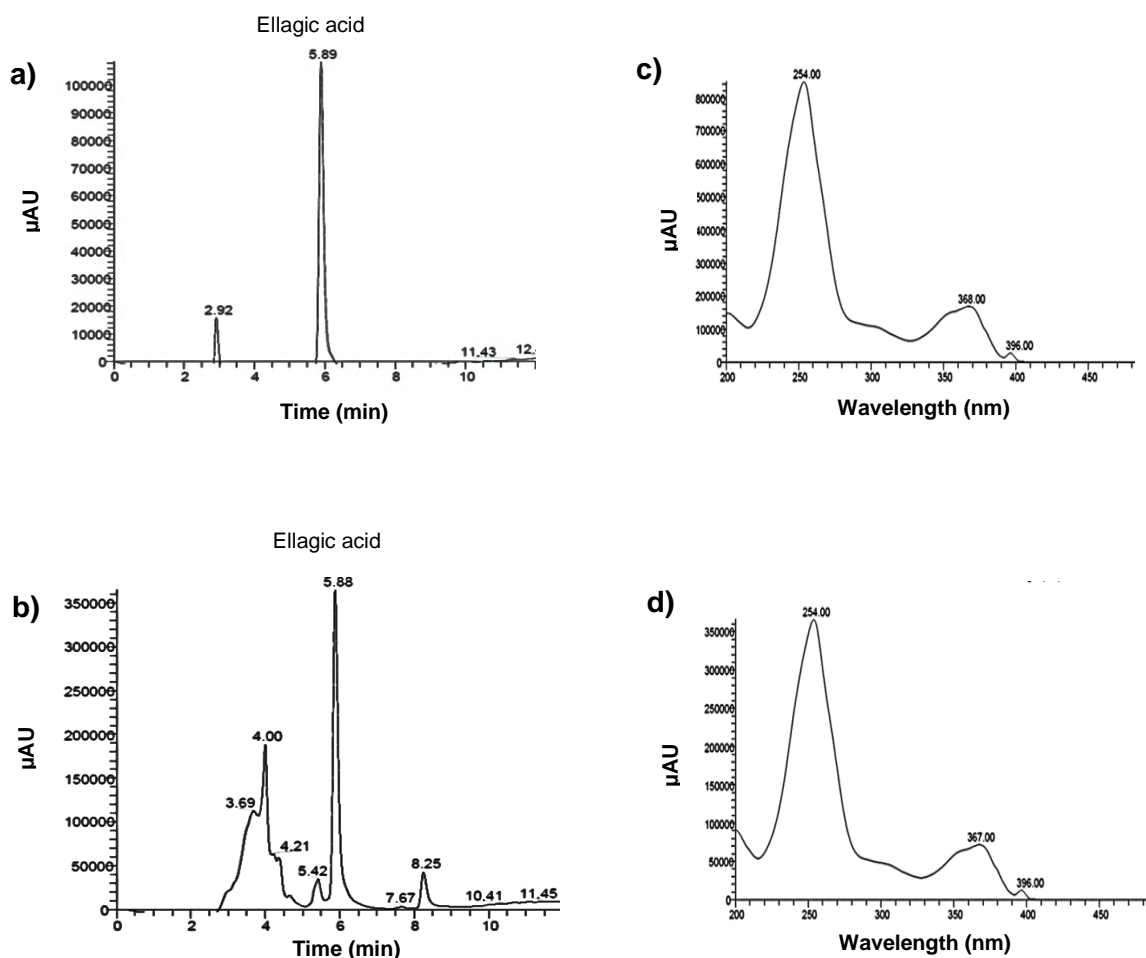
$$\% \text{ Recovery} = \frac{(\text{concentration of ellagic acid after spiking} - \text{concentration of ellagic acid in sample}) \times 100}{\text{calculated concentration of ellagic acid}}$$

## **Results and Discussion**

Banaba leaf powdered extract obtained from spray drying was a fine powder with light yellowish brown color. The powder had low hygroscopicity and low solubility in water. Loss on drying which is used to measure the amount of volatile matter of the powdered extract was determined for quality control. For the obtained spray dried powder, loss on drying was 3.27% which is within the acceptance criterion ( $\leq 8\%$ ), according to USP monograph.<sup>10</sup>

The chemical constituents of the powdered extract were identified by TLC analysis. Banaba leaf powdered extract was found to contain ellagic acid as the major compound (R<sub>f</sub> = 0.09). Sample preparation by using solid phase extraction successfully cleaned up Banaba leaf powdered extract. The content of ellagic

acid in Banaba leaf powdered extract was quantified using developed HPLC method. The chromatograms of ellagic acid standard and Banaba leaf powdered extract were shown in Figure 2a and 2b, respectively. The peak corresponding to ellagic acid in the extract was confirmed by comparing the UV spectrum with that of the reference standard (Figure 2c and 2d).



**Figure 2** HPLC chromatograms of a) ellagic acid standard and b) Banaba leaf powdered extract and the UV spectra of c) ellagic acid standard and d) Banaba leaf powdered extract

In this study, the HPLC method was developed to quantify ellagic acid content in Banaba leaf powdered extract. The HPLC method was validated for specificity, linearity, precision and accuracy. The retention time for ellagic acid was found to be 5.8 minutes. The specificity for ellagic acid was confirmed by comparing the retention time and UV spectra of the corresponding peak in the extract with those of ellagic acid standard (Figure 2). The calibration curve for ellagic acid was linear with a correlation coefficient greater than 0.995 over the range of 10-50  $\mu\text{g/mL}$ . Repeatability and intermediate precision for ellagic acid were less than 2% RSD. The accuracy for ellagic acid in Banaba leaf powdered extract was ranged from 97.4-100.5%. The determination of ellagic acid content in Banaba leaf powdered extract was found to be 1.4% w/w. The developed HPLC method was successfully validated and allowed the complete resolution of ellagic acid within an appropriate run time.

## Conclusion

A simple, rapid, accurate and reliable method by using a reversed-phase HPLC with PDA was developed to determine ellagic acid content in Banaba leaf powdered extract. The developed method showed good linearity, good reproducibility and high recovery rate. This method can be used for determination of ellagic acid in Banaba leaf extract and its related products containing ellagic acid as a biomarker. The result from this study confirmed that the developed HPLC method is suitable for quality control of Banaba leaf powdered extract and its nutraceutical products. In addition, the prepared Banaba leaf extract was high quality and possible to be used as raw material for nutraceutical products.

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