Pharmacognostic specifications and quantification of (+)-catechin and (-)-epicatechin in *Pentace burmanica* stem bark

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ABSTRACT

Background: According to Thai traditional medicine, *Pentace burmanica* Kurz. stem bark has been used as crude drug for treating diarrhea. However, the crude drug is also found susceptible to adulteration. **Objectives:** To develop specific standardization parameters of *P. burmanica* stem bark in Thailand and to determine the (+)-catechin and (-)-epicatechin contents of *P. burmanica* stem bark by HPLC analysis. **Materials and Methods:** *P. burmanica* stem barks from various sources throughout Thailand were investigated according to WHO guideline of the pharmacognostic specification. High performance liquid chromatography (HPLC) was performed for (+)-catechin and (-)-epicatechin quantification. **Results:** Macroscopic evaluation was demonstrated as whole plant drawing. Microscopic evaluation of stem bark powdered drug showed fragment of fibers, resin masses, tannin masses, starch grain, calcium oxalate, and fragment of parenchyma. Physico-chemical parameters revealed that total ash, acid insoluble ash, loss on drying, and water content should be not more than 3.58, 0.50, 8.40, and 9.70% of dry weight respectively; while ethanol and water soluble extractive values should not be less than 21.90 and 19.06% of dry weight respectively. Both (+)-catechin and (-)-epicatechin were existed in *P. burmanica* ethanolic extract. Owing to the small amount of (+)-catechin, quantitation of its content was omitted. However, (-)-epicatechin contents was found as 59.74 ± 1.69μg/mg of crude extract. **Conclusion:** The pharmacognostic investigations can be used to set the standard parameters of *P. burmanica* stem bark in Thailand. HPLC method can be applied to determine (+)-catechin and (-)-epicatechin content in plant materials.

Key words: (-)-Epicatechin, (+)-Catechin, high performance liquid chromatography, *Pentace burmanica*, pharmacognostic specification

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INTRODUCTION

Pentace burmanica Kurz. (Tiliaceae) also known in Thai as Si-siad-pleuak has been used to treat several diseases. The *P. burmanica* stem bark is one of the plants use for treatment of diarrhea in Thai traditional medicine. The elders of Laos and Northeast Thailand use this stem bark as an ingredient in chewing betel (nuts of *Areca catechu*). Previous study revealed that water extract and 50% ethanol extract of *P. burmanica* inhibited growth of 3 bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus mutans*). The extract consists of about 9.93% tannin.[1,2] Therefore, (+)-catechin and (-)-epicatechin were selected as marker compounds in the present study.

According to market survey, it was observed that *P. burmanica* stem bark could be adulterated with other plant. Therefore; to control the quality of raw medicinal plants, establishment of standardization parameter is needed. The standardization is an essential measurement for quality, purity, and authentication of plant drugs.[3] Hence, this present study aimed to provide specific standardization parameters of *P. burmanica* stem bark in Thailand. The determination of the (+)-catechin and (-)-epicatechin contents of *P. burmanica* stem bark by HPLC analysis was also carried out.

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MATERIALS AND METHODS

Plant collection and extraction

P. burmanica stem barks were collected from 12 different Thai traditional drug stores or markets throughout Thailand. All sets of crude drugs were authenticated by Associate Professor Dr. Nijsiri Ruangrungsi. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. Five grams of ground sample of *P. burmanica* stem bark was exhaustively extracted with 95% ethanol using a Soxhlet apparatus. The ethanol extract was filtered through Whatman No. 4 and evaporated *in vacuo*. The extract yield was weighed, recorded and stored at -20ºC to avoid the possibility of degradation of active compounds.

Macroscopic and microscopic evaluation

Macroscopic evaluation of *P. burmanica* was visually examined of physical properties such as size, color, texture, and other visual inspection. The powder of *P. burmanica* stem bark was examined for histological characters under microscope with 10X, 20X, and 40X objective lens magnifications and 10X eyepiece lens. The results were illustrated by hand drawing in proportional scale related to the original size.

Physico-chemical evaluation

Loss on drying, total ash, acid insoluble ash, water content, and extractive values parameters were performed to evaluate the pharmacognostic specification of *P. burmanica* according to WHO guideline for quality control methods for medicinal plant materials as briefly described below:[4]

Three grams of ground sample was dried at 105ºC to constant weight for determine loss on drying. Then, 3 grams of ground sample was ignited by gradually increasing the heat to 500ºC until white to observe the carbonless ash for total ash measurement. The ash was boiled with 25 ml of 2N HCl; insoluble matter was burned at 500ºC for 5 h. After that the amount of silica presented and siliceous earth were measured to obtain the percentage of acid insoluble ash. Water content was conducted by azeotropic distillation method using water saturated toluene. Determinations of extractive values were carried out with 95% ethanol and distilled water as solvents. Five grams of ground sample was macerated with 70 ml of solvent under shaking for 6 h and standing for 18 h before filtration. The extract was filtrated through Whatman No. 4 and adjusted to 100 ml by washing the residue. Twenty milliliters of the filtrate was evaporated to dryness on a water bath. Then, the sample was dried at 105ºC until constant weight was obtained.

(+)-Catechin and (-)-epicatechin quantification

Chemicals and materials

(+)-Catechin (CAS no. 154-23-4, purity ≥ 99%) and (-)-epicatechin (CAS no. 490-46-0, purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were of HPLC grade (RCI Labscan, Bangkok, Thailand). Formic acid was purchased from Fisher Scientific (Leicestershire, UK). Ultra-pure water was prepared by SNW ultra-pure water system (NW20VF, Heal Force). The filters were 46 mm × 0.45 μm nylon membrane filters (National Scientific, TN) and 13 mm × 0.45 μm PTFE membrane syringe filters (ANPEL Scientific Instrument, China).

Chromatographic conditions

Shimadzu DGU-20A3 HPLC (Shimadzu, Japan) consisted of a binary solvent delivery system, an auto-sampler, a column temperature controller, and a photo diode array detector (Shimadzu SPD-M20A, Shimadzu, Japan). System control and data analysis were processed with Shimadzu LC Solution software. The separation was performed with Inersil ODS-3 column (5 μm × 4.6 × 250 mm) using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as mobile phases at a flow rate of 1ml/min. The mobile phases were filtrated through 0.45 μm nylon membrane filters and degassed before analysis. The isocratic program was set at 20% B for 15 min. The column temperature was maintained at 40ºC and the injection volume was 1 μl. The detection wavelength was set at 280nm.

Preparation of standard solution

The stock solution of (+)-catechin and (-)-epicatechin were prepared by dissolving 1 mg of each compound in 1 ml of methanol. Then, the solution was filtered through a 0.45 μm PTFE membrane syringe filter.

Preparation of sample

One miligram of *P. burmanica* stem bark extract was dissolved in 1ml of methanol. The extract solution was filtered through a 0.45μm PTFE membrane syringe filter before chromatographic analysis.

Method validation

ICH guideline was employed for validation of analytical method.[5]

Linearity

Linearity was determined by the calibration curves that obtained from the HPLC analysis of (+)-catechin and (-)-epicatechin. The stock solutions of (+)-catechin and (-)-epicatechin were dissolved in methanol to give concentrations of 5, 10, 50, 100 and 200 μg/ml for evaluate the calibration curves. The calibration curves of these two compounds were fitted by linear regression.

Limit of detection (LOD) and limit of Quantitation (LOQ)

LOD and LOQ were calculated based on the residual standard deviation of a regression lines (σ) and the slope of the calibration curve (S) as follows:

$LOD = 3.3(\sigma)/S$

$LOQ = 10(\sigma)/S$

Precision

The precision of *P. burmanica* stem bark extract was evaluated at 2 levels including repeatability and intermediate precision. The relative standard deviation (RSD) of 9 determinations covering the specific range (3 concentrations and 3 replicates each) was evaluated and analyzed on one day and three consecutive days.

Accuracy

The accuracy of *P. burmanica* stem bark extract was determined by recovery method. The crude extract was spiked with (+)-catechin (50, 100, and 150 μg/ml) and (-)-epicatechin (50, 100, and 150 μg/ml) then percent recoveries were calculated by comparing the measured amount of those standards with the amount added.

Specificity

The specificity was evaluated by peak purity test.

Robustness

The robustness was determined for variations in flow rates (0.995 and 1.005 ml/min) and variations in column temperature (39 and 41°C). The percentage of RSD was calculated to evaluate whether the flow rate and temperature variations altered the results of HPLC analysis.

RESULTS AND DISCUSSION

Macroscopic and microscopic evaluation

Macroscopic and microscopic method are the simplest and cheapest method to establish the correct identification of plant materials.[4] The macroscopic and microscopic investigations were illustrated in Figure 1. The figure showed reddish brown to brown color in dried stem bark of *P. burmanica*. The anatomical and histological investigations of dried *P. burmanica* stem bark were demonstrated in the figure. Several histological characters including fragment of fibers, resin masses, tannin masses, starch grain, calcium oxalate, and fragment of parenchyma were found in powders of *P. burmanica* stem bark.

Plant description

P. burmanica is a 5 to 15 m tall tree and steep extensive buttresses. Its grey outer bark is 2 cm thick, and the inner bark is reddish with a sticky red sap. Branches and young parts are reddish brown and hairy. The soft oval leaves measure 8 to 15 × 4 to 8 cm, with a white-green lower blade and jagged edges. The leaf stalk petiole and leaf nerves are hairy. Inflorescences consist of a 5 to 10 cm long cluster of white, hairy, bell-shaped flowers, each about 5 mm long. The fruit is a five-winged green capsule, 4 to 5 cm long and 5 to 5.5 cm wide with a hairy seed, 1 to 1.5 cm long.[6]

Physico-chemical evaluation

The physico-chemical evaluation of plant drugs is an important for detecting adulteration and quality of the drug. The ash investigation is helpful to determine the quality and purity of powdered crude drug. A high ash value is indicative of contamination, substitution, adulteration, and carelessness in preparing the crude drug for marketing purpose.[7-9] Table 1 demonstrated the pharmacognostic parameters of *P. burmanica* stem bark from 12 different sources throughout Thailand. The total ash, acid insoluble ash, loss on drying, and water content should be not more than 3.58, 0.50, 8.40, and 9.70% of dry weight respectively; while ethanol and water soluble extractive values should not be less than 21.90 and 19.06% of dry weight respectively.

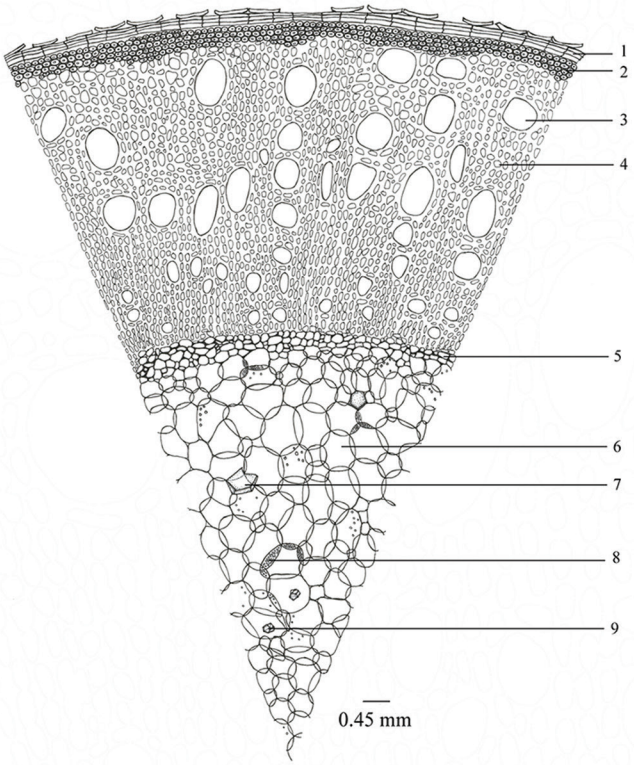
(+)-Catechin and (-)-epicatechin quantification

HPLC chromatogram of *P. burmanica* stem bark extract showed several chemical compounds containing in the extract [Figure 2]. (+)-Catechin and (-)-epicatechin were identified by comparing the retention time and UV spectrum of each peak with those of standard compounds. The quantitation of catechins was evaluated by comparing the area under peak with the calibration curve. (+)-Catechinin was detected but it cannot be determined quantitatively due to low concentration (< LOQ); whereas (-)-epicatechin was found to be 59.74 ± 1.69 μg/mg of crude extract. The maximum content of (-)-epicatechin was 91.55 μg/mg of crude extract whereas the minimum was 10.66 μg/mg of crude extract. Varied concentration of (-)-epicatechin might be due to the different of geographical areas and the age of *P. burmanica*. Previous study reported that the age and height of *P. burmanica* were related with a quantity of tannin extract.[1]

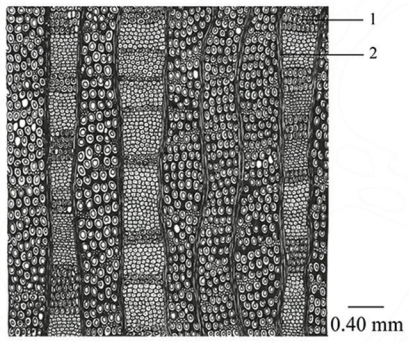
Table 1: Physico-chemical parameters (% by weight) of *P. burmanica* stem bark

Parameters	Mean±SD*	Range**
Total ash content	3.58±0.07	3.37-3.78
Acid insoluble ash content	0.50±0.02	0.43-0.57
Loss on drying content	8.40±0.12	8.04-8.76
Water content	9.70±1.29	5.84-13.57
Ethanol extractive value	21.90±2.73	13.70-30.09
Water extractive value	19.06±2.58	11.31-26.81

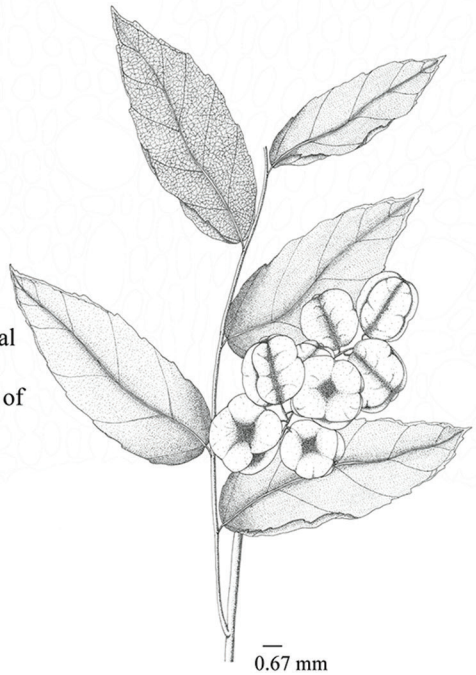
*The parameters were shown as grand mean±pooled SD. **mean±3SD, SD: Standard deviation



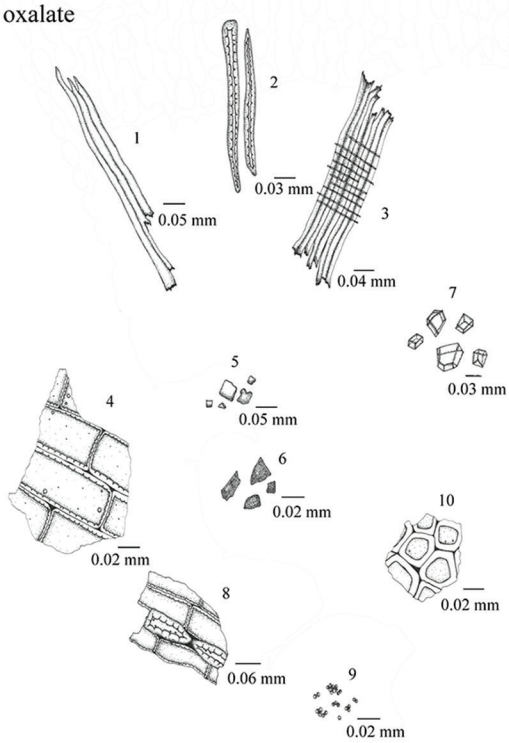
Transverse section of *Pentace burmanica* stem: 1. Periderm 2. Cortical fiber 3. Xylem 4. Cortical parenchyma 5. Endodermis 6. Parenchyma 7. Resin mass 8. Parenchyma containing starch grain 9. Prism crystal of calcium oxalate



Transverse section of *Pentace burmanica* stem bark: 1. Wood fiber 2. Wood parenchyma



Whole plant of *Pentace burmanica*



Dried stem bark of *Pentace burmanica*

Powdered *Pentace burmanica* stem bark: 1.Fragment of fiber 2. Sclerenchyma scleried 3. Fragment of xylem ray in radial longitudinal view 4. Parenchyma in longitudinal view 5. Resin masses 6. Tannin masses 7. Prism crystals of calcium oxalate 8. Parenchyma with sclerieds in longitudinal view 9. Starch grain 10. Cork in surface view

Figure 1: Macroscopic and microscopic characteristics of *Pentace burmanica* Kurz

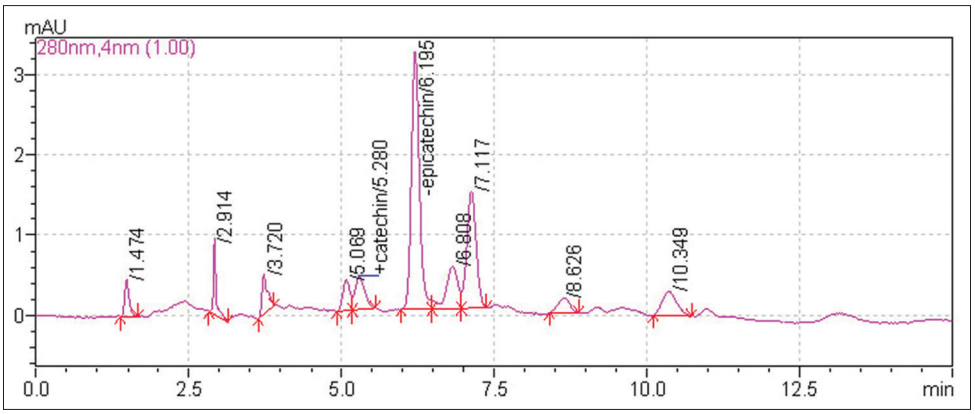


Figure 2: HPLC chromatogram of *Pentace burmanica* stem bark extract

According to ICH guideline, the tests of linearity, LOD, LOQ, precision, accuracy, specificity, and robustness should be performed for the validation of an analytical method.

(+)-Catechin and (-)-epicatechin at 5 concentration levels were investigated for linearity of the HPLC method. The calibration curves of both catechins were linear in the range of 5-200 μg/ml. The regression equation of (+)-catechin and (-)-epicatechin were $y = 746.29x - 2203.3$ and $y = 517.61x - 652.07$ respectively. Good correlation coefficient (r^2) was obtained ($r^2 \geq 0.99$) in this study.

The LOD values, taken as the lowest concentration of analyte in a sample which can be detected were found to be 4.80 μg/ml for (+)-catechin and 5.14 μg/ml for (-)-epicatechin. The LOQ values, taken as the lowest concentration of analyte in a sample which can be quantitively determined were 14.54 μg/ml for (+)-catechin and 15.57 μg/ml for (-)-epicatechin.

The precision of *P. burmanica* extract was conducted as% RSD of 9 determinations covering the specific range. The accuracy was determined by recovery test. The results of precision and accuracy of (+)-catechin and (-)-epicatechin in *P. burmanica* extract were illustrated in Table 2. The percent RSD of repeatability and intermediate precision were found to be less than 3 which revealed that the HPLC method was precise. The average recoveries of both (+)-catechin and (-)-epicatechin were obtained with good recovery in the range of 91.11 to 97.02% and 88.53 to 93.78% respectively with% RSD less than 2. According to ICH guideline, good agreement of recovery was ranged from 80 to 120% with the required for complex matrices.[5] Hence, the results indicated that this method was accurate for (+)-catechin and (-)-epicatechin quantification in *P. burmanica* stem bark.

The specificity was performed by peak purity checking. The peak purity test is useful to show that the analyte

Table 2: Precision and accuracy of (+)-catechin and (−)-epicatechin in *P. burmanica* extract

Compound	Spike concentration (μg/ml)	%RSD		% recovery (*n*=3)
		Repeatability precision (*n*=9)	Intermediate precision (*n*=3)	
(+)-Catechin	50	0.42	1.66	93.07
	100	0.37	2.07	97.02
	150	0.27	2.93	91.11
(−)-Epicatechin	50	0.33	0.97	87.12
	100	0.31	0.76	93.78
	150	0.62	1.13	88.53

RSD: Relative standard deviation

chromatographic peak is not attributable to more than one component. The results showed peak purity index of both catechins were more than 0.99 which can be suggested that no impurity detected in those peaks.

The robustness should be investigated during the analysis of HPLC method, and it should demonstrate the reliability of analysis with the respect to deliberate variation in the parameters of the method.[5] This present study revealed that there were no differences (% RSD < 5) in the area of the curve and retention time of (+)-catechin and (-)-epicatechin when the flow rate of mobile phase was varied from 0.995 to 1.005 ml/min and the column temperature was varied from 39 to 41°C. The results suggested that the HPLC method proved to be robust for (+)-catechin and (-)-epicatechin analyzed, under the condition evaluated.

CONCLUSION

This study reported for the first time the pharmacognostic specification parameters as well as the content of (-)-epicatechin in a standardized *P. burmanica* stem bark. The pharmacognostic investigations complimented with HPLC results is useful to set the standard parameters of *P. burmanica* stem bark in Thailand and able to be applied for the authentication and

quality control of this crude drug. Moreover, HPLC method can be applied to determine (+)-catechin and (-)-epicatechin content in plant materials.

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