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

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.
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. Nisiri 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 -20°C 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:

Three grams of ground sample was dried at 105°C to constant weight for determine loss on drying. Then, 3 grams of ground sample was ignited by gradually increasing the heat to 500°C 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 500°C 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 105°C until constant weight was obtained.

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 0.45 µm PTFE membrane syringe filter before chromatographic analysis.

Method validation

ICH guideline was employed for validation of analytical method.

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:

\[
LOD = \frac{3.3(\sigma)}{S}
\]

\[
LOQ = \frac{10(\sigma)}{S}
\]

**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 41ºC). 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. (+)-Catechin 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.[10]

| 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±SD, SD: Standard deviation
Figure 1: Macroscopic and microscopic characteristics of *Pentace burmanica* Kurz

Transverse section of *Pentace burmanica* stem bark: 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.

Whole plant of *Pentace burmanica*

Dried stem bark of *Pentace burmanica*

Powdered *Pentace burmanica* stem bark: 1. Fragment of fiber 2. Sclerenchyma 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.
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 quantitatively 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.[9] 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 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.[9] 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 41ºC. 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 identification of Pentace burmarnica stem bark.

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

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

RSD: Relative standard deviation
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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