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Original Article

Pharmacognostic Specifications, Quercetin and Quercitrin Quantification in *Bauhinia malabarica* Leaf

Paphitchaya Thetsana[1], Chayanon Chaowuttikul[1], Chanida Palanuvej[1], Nijsiri Ruangrungsi[1,2,*]

ABSTRACT

Introduction: *Bauhinia malabarica* Roxb. is a tropical tree that found throughout Thailand. Leaves have a sour taste and have been used in Thai remedies for wound healing, diuretic, dysentery and emmenagogue. **Objective:** This study aimed to focus on pharmacognostic specification and quantitative analysis of quercetin and quercitrin in *B. malabarica* leaves. **Methods:** Various methods such as macroscopic and microscopic evaluations of *B. malabarica* leaf were studied along with physico-chemical parameters and quantitated quercetin and quercitrin using RP-HPLC. **Results and Conclusion:** Whole plant, structures of dried powder crude drug, cross section of midrib and leaf measurement were established. Paracytic stomata and multicellular trichome were found on lower epidermis. *B. malabarica* leaves from 15 sources throughout Thailand were examined the pharmacognostic specification according to WHO guideline. Physico-chemical parameters showed that loss on drying, total ash, acid insoluble ash and water content should not be more than 8.00, 7.08, 1.79 and 8.28 % of dry weight while ethanol and water soluble extractive values should not be less than 13.78 and 16.47 % of dry weight respectively. Quercetin and quercitrin were the markers for quantitative analysis using RP-HPLC with diode array detector in *B. malabarica* ethanolic extract. Quercetin and quercitrin contents were found to be 0.18 g and 0.38 g in 100 g of dried crude drug. Method validation was determined according to ICH guideline. All results were in the acceptable range and could be used for identity, safety, efficacy and quality of *B. malabarica* leaves in Thailand.

Key words: *Bauhinia malabarica* Roxb, Leaf measurement, Pharmacognostic specification, HPLC, Quercetin, Quercitrin.

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INTRODUCTION

Bauhinia is a large genus of the family Leguminosae that approximately 300 species of trees, shrubs and climbers. This genus is native in tropical zones such as Africa, Asia and South America. They are commonly known as cow's paw or cow's hoof because of leaf shape.[1] Mostly *Bauhinia* species are planted for showy flowers and ornamental shrubs. *Bauhinia malabarica* is a tropical tree that found throughout Thailand. Young leaves and flowers are edible. Leaves have a sour taste and have been used for wound healing, diuretic, dysentery and emmenagogue. Previous study, seven flavonol derivatives have been purified from *B. malabarica* leaves using chromatographic techniques such as 6,8-di-C-methylkaempferol 3 methyl ether, kaempferol, afzelin, quercetin, isoquercitrin, quercitrin and hyperoside.[2] Quercetin is a flavonoid normally found in many fruits, vegetables, leaves and grains. The primary benefits of quercetin are possesses strongly antioxidant and anticancer.[3] Quercitrin is a glycoside formed from quercetin and deoxy sugar rhamnose. Quercitrin has been demonstrated biological activities such as anti-inflammatory effect, preventing diarrhea and strongly anti-leishmanial activity.[4] High performance liquid chromatography (HPLC) is a popular analytical technique used to separate, identify and quantify chemical compounds in medicinal plants. Pharmacognostic specification is the standard method to identify medicinal plants. Although, *B. malabarica* has been used to treat many diseases for a long time, but still lack of pharmacognostic study. Therefore, this study is focused on pharmacognostic specification and quantitative analysis of quercetin and quercitrin in *B. malabarica* leaves.

MATERIALS AND METHODS

Plant collection

B. malabarica fresh leaves were collected from Chulalongkorn University and dried leaves of *B. malabarica* were purchased from 15 traditional drugstores throughout Thailand. All plant samples were authenticated by one of the authors (Ruangrungsi, N.). Dried leaves were ground to fine powder and stored at College of Public Health Sciences, Chulalongkorn University.

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Sample extraction

Five grams of *B. malabarica* fine powdered were exhaustively extracted with 95% ethanol using Soxhlet apparatus, filtered, the filtrate were evaporated to dryness and stored in refrigerator for quantitative quercetin and quercitrin using reverse phase high performance liquid chromatography (HPLC).

Morphological examination

B. malabarica fresh mature leaves were macroscopically and microscopically observed. Fine powder of *B. malabarica* leaves was mixed with a few drops of water on the slide and observed under microscope with the magnification of 10x to 40x. The middle part of fresh mature leaf was cut into 0.5x0.5 cm, soaked in 50% sodium hypochlorite in water until clear, washed and boiled in chloral hydrate solution (4:1, chloral hydrate: water) until translucent, examined under microscope for palisade, trichome and epidermal cell numbers. Palisade cells in four epidermal cells were counted and divided by 4 for calculation palisade ratio. Trichome index and stomatal index were obtained from this formula (S or T×100)/(S+E+T) whereas S=stomatal number, E=number of epidermal cells and T=trichome number. The midrib of *B. malabarica* fresh mature leaf was thinly cross-section with blade by hand and observed the anatomy under microscope with the magnification of 10x to 40x.

Physico-chemical characteristics

Fine powder of *B. malabarica* dried leaves was examined for water content, loss on drying, ashes, extractive matters and volatile oil content according to "Quality control methods for medicinal plants materials" that established by World Health Organization (WHO).[5] All samples were analyzed in triplicate. Grand mean and pooled standard deviation were calculated.

Thin layer chromatographic fingerprint

Three microliters of crude ethanolic extract solution were spotted onto the silica gel GF_{254} TLC plate. TLC plate was developed in saturated TLC chamber with toluene: acetone: chloroform: formic acid (2: 8: 8: 2). After development, the plate was observed under day light, UV 254 nm, UV 365 nm, stained with p-anisaldehyde and 1% aluminium chloride in ethanol and detected under UV 365 nm.

HPLC analysis

Shimadzu HPLC LC-20A system (Shimadzu, Japan) consists of system controller (CMB-20A), two solvent delivery units (LC-20A), an on-line degassing unit (DGU-20A^3), an auto-sample (SIL-20A), a column oven (CTO-20A) and a photo-diode array detector (SPD-M20A). System control and data analysis were processed with Shimadzu LC Solution software. The chromatographic separation was performed with an Inertsil® ODS-3 5µm C18 column (4.6 × 250 mm) coupled with ReproSil®-Pur ODS-3 C18 guard column (4.0 × 10 mm). Solvent A was 0.5% phosphoric acid and solvent B was methanol (for RP-HPLC), filtered through 0.45 µm nylon membrane filters and degasses before analysis. Isocratic mode was set as 50% solvent B for 30 min, using flow rate 1.0 ml/min, column temperature was set at 35°C. Each *B. malabarica* ethanolic extract and standards (quercetin and quercitrin) were prepared in methanol, filtered through 0.45 µm PTFE membrane syringe filter and injected 5 µl. Peak areas were observed under 255 nm and calculated using linear equations from calibration range of quercetin and quercitrin (20, 40, 60, 80 and 100 µg/ml).

Method validation

Calibration range, specificity, accuracy, repeatability, intermediate precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness were examined following to the ICH guideline.[6]

RESULTS AND DISCUSSION

Morphological examination

B. malabarica Robx. leaves are green color on both sides. The leaf shape was is bilobed, rough surface, thick leaf, 6 – 10 cm wide, 5 – 7 cm long (Figure 1) and sour taste. Flowers are small white or light green.

Powdered of *B. malabarica* leaves consist of multicellular trichome, paracytic stomata, epidermis, spiral vessel, prism of calcium oxalate and fibers (Figure 2). Microscopic leaf constant numbers including epidermal cell area, stomatal number, stomatal index, trichome number, trichome index and palisade ratio were shown in Table 1. Anatomical characteristic of *B. malabarica* leaf (midrib part cross section) was shown in Figure 3. Physico-chemical properties of *B. malabarica* dried leaves including water content, ethanol soluble extractive, water soluble extractive, loss on drying, total ash, acid insoluble ash and volatile oil content were demonstrated in Table 2.

Plants extracts can provide a chromatographic "fingerprint" used for identification purposes. The colors of the separated spots and their position relative to standard substances are important characteristics for the plant extract identification.[7] In this study, TLC fingerprint was the preliminary identification of *B. malabarica* (Figure 4).

Pharmacognostic parameters were shown the specific characteristics of *B. malabarica* leaf constant numbers. In morphological examination part, the specific character of *B. malabarica* leaves was sour taste, only this species has the sour taste, so, unknown sample which looks like *B. malabarica* needs to be tasted in the first step. Another specific character was not only multicellular trichome and paracytic stomata on lower epidermis, but also the average of palisade ratio. Palisade ratio is the diagnostic value in differentiating the species and dose not based on geographical variations.[8]

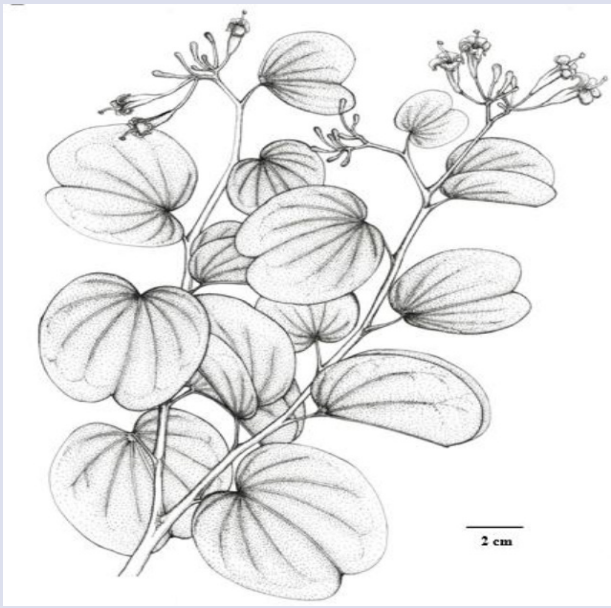

Figure 1: Branch of *B. malabarica* Roxb.

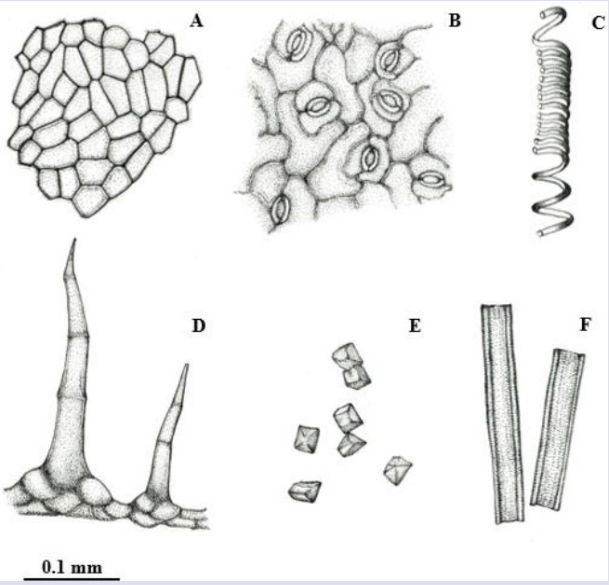

Figure 2: Histological characteristics of *B. malabarica* leaf powder consists of A=epidermis, B=paracytic stomata, C=spiral vessel, D=multicellular trichome, E=prism of calcium oxalate, F=fiber.

Table 1: Microscopic leaf constant numbers of *B. malabarica* leaves.

Parameter	Mean ± SD
Epidermal cell area (μm²)	685.912 ± 8.592
Stomatal number (/mm²)	683.867 ± 18.179
Stomatal index	18.066 ± 0.431
Trichome number (/mm²)	12.800 ± 3.547
Trichome index	0.338 ± 0.092
Palisade ratio	3.767 ± 0.236

Table 2: Physico-chemical parameters of *B. malabarica* dried leaves.

Parameter	Content (% by weight)
Water content	8.280 ± 0.407
Ethanol soluble extractive	13.781 ± 0.197
Water soluble extractive	16.474 ± 0.389
Loss on drying	7.998 ± 0.046
Total ash	7.079 ± 0.047
Acid insoluble ash	1.788 ± 0.184
Volatile oil content	0

*The parameters were shown as grand mean ± pooled standard deviation. Samples were from 15 different places throughout Thailand and each sample was done in triplicate.

HPLC analysis

The content of quercetin and quercitrin in *B. malabarica* dried leaves were shown in Table 3. The content values were calculated and shown as grams of quercetin or quercitrin per 100 grams of dried crude drug.

The different values of quercetin or quercitrin in *B. malabarica* leaf ethanolic extract were depended on the geographic variation that affected to secondary metabolite of plants.[9]

Method validation

The method validation parameters of reversed phase high performance liquid chromatography were optimized by performing the effect of the mobile phase and detection wavelength on resolution and sensitivity. An isocratic elution method was shown good separation in both standard and *B. malabarica* ethanolic extract. In this study, the range of absorbance (200 – 800 nm) was screened in both quercetin and quercitrin, the

Table 3: The contents of quercetin and quercitrin in *B. malabarica* leaves.

No.	% yield (g/100g)	Content (g/100g of dried crude drug)	
		Quercetin	Quercitrin
1	27.382	0.1783 ± 0.0005	0.6002 ± 0.0012
2	26.691	0.1630 ± 0.0003	0.6932 ± 0.0006
3	29.267	0.1330 ± 0.0002	0.2904 ± 0.0003
4	26.862	0.1141 ± 0.0000	0.1436 ± 0.0002
5	30.579	0.0988 ± 0.0003	0.1089 ± 0.0001
6	25.115	0.1501 ± 0.0004	0.2454 ± 0.0005
7	18.471	0.0715 ± 0.0001	0.7512 ± 0.0007
8	23.262	0.1517 ± 0.0003	0.2642 ± 0.0008
9	29.887	0.2724 ± 0.0004	0.3053 ± 0.0002
10	26.199	0.1918 ± 0.0006	0.3740 ± 0.0002
11	26.657	0.2214 ± 0.0004	0.2125 ± 0.0003
12	29.908	0.2844 ± 0.0005	0.2787 ± 0.0001
13	25.429	0.1535 ± 0.0008	0.7062 ± 0.0002
14	24.184	0.2100 ± 0.0008	0.2528 ± 0.0000
15	29.302	0.3004 ± 0.0002	0.5233 ± 0.0005

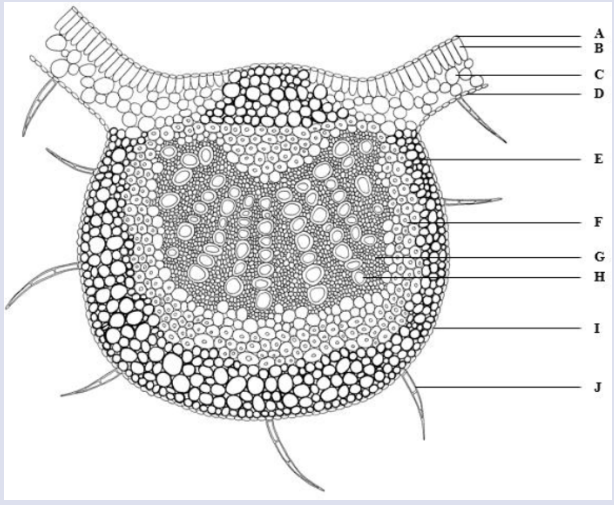

Figure 3: Anatomical characteristics of *B. malabarica* leaf (midrib part cross section) consists of A=upper epidermis, B=palisade cells, C=sponge cells, D=lower epidermis, E=collenchyma, F=scherenchyma cells, G=phloem, H=xylem, I=stomata, J=multicellular trichome.

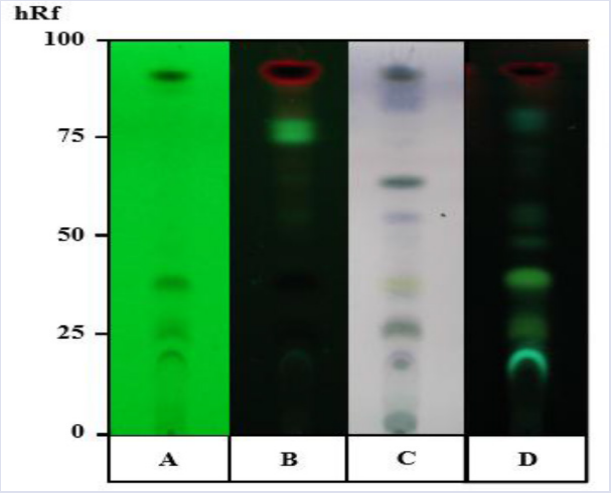

Figure 4: TLC fingerprint of *B. malabarica* leaf ethanolic extract; A=detection under UV 254 nm, B=detection under UV 365 nm, C=stained with p-anisaldehyde and D=stained with 1% aluminium chloride and detected under UV 365 nm.

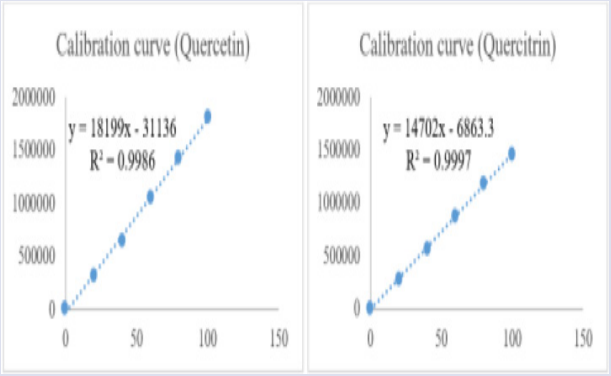

Figure 5: The calibration curve of quercetin and quercitrin.

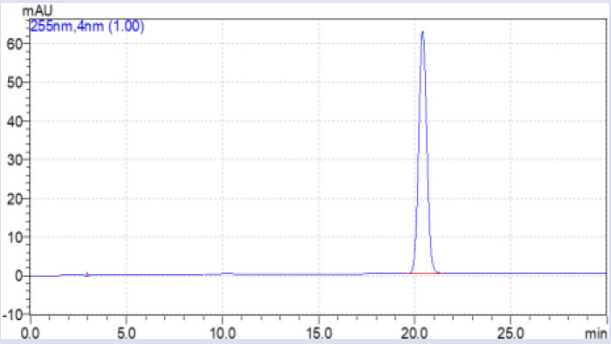

Figure 6: HPLC chromatograms of standard quercetin at 255 nm by HPLC-DAD.

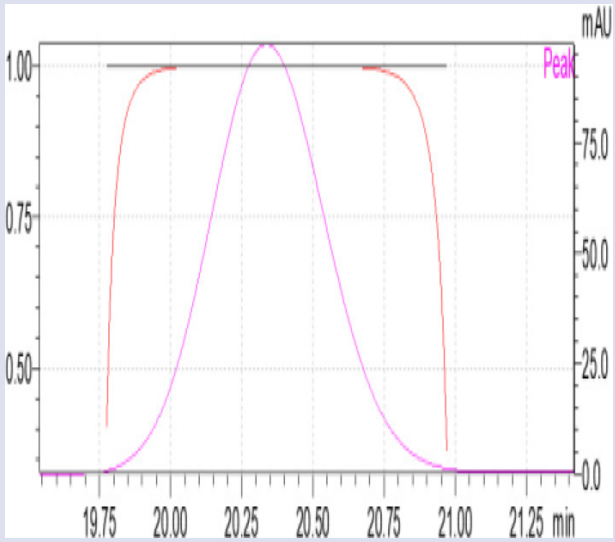

Figure 7: The peak purity of quercetin in *B. malabarica* leaf ethanolic extract.

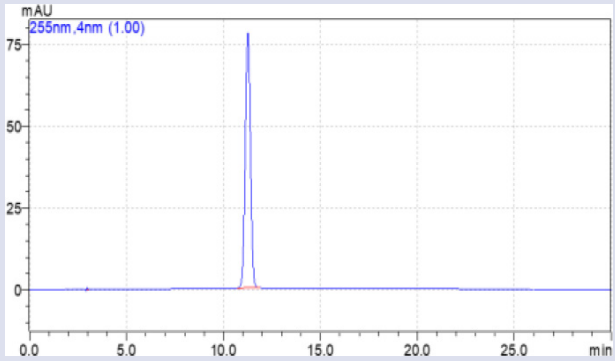

Figure 8: HPLC chromatograms of standard quercitrin at 255 nm by HPLC-DAD.

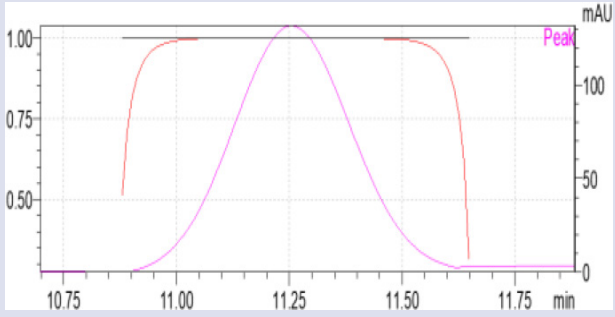

Figure 9: The peak purity of quercitrin in *B. malabarica* leaf ethanolic extract.

result was shown that the detection wavelength at 255 nm was presented the optimum absorbance wavelength.

Linear calibration curves in the range of 20, 40, 60, 80 and 100 µg/ml were created for each compound by plotting the peak area with the concentration. The regression equation of quercetin and quercitrin were y = 18199x – 31136 and y = 14702x - 6863.3. The linear demonstrated excellent correlation as 0.998 in quercetin and 0.999 in quercitrin respectively (Figure 5). The correlation of method (R^2) value was reached 0.99 which mean that this analytical technique is acceptable.[10]

The peaks of quercetin and quercitrin were well separated at different retention times with resolutions of 20.324 and 11.245, respectively. No interferences or excipient peaks co eluted with the analytes were observed, indicating the method is selective and specific in this study.[11]

Peak purity test was based on absorbance spectrum using diode array detector which confirmed the mobile phase was suitable for analyte chromatographic peak that showed a good separation from another compound. If all the individual spectra recorded during elution of a peak are identical even detected at any periods of peak, the peak is considered as absolutely pure.[12] Peak purity index of quercetin and quercitrin showed more than 0.999 (Figure 7, 8 and 9), it demonstrated that all peaks were pure.

The accuracy was determined by recovery method which spiked three concentrations (20, 40 and 60 µg/ml) of standard quercetin and quercitrin into *B. malabarica* leaves ethanolic extract, acceptable of %recovery is between 80 – 120 %,[6] the results ranged from 97.378 – 99.177 and 98.181 – 102.288 %recovery as shown in the Table 4. The precision, both repeatability and intermediate precision were done on spiked sample with three different concentrations in the same day and three different days. The result of %RSD was determined the error of the method, acceptable of %RSD was not more than 15 %RSD.[13] The result of repeatability and intermediate precision in quercetin and quercitrin were shown in Table 4. The residual standard deviation of a regression line and the slope of calibration range in both quercetin and quercitrin were used to calculate LOD and LOQ analysis. LOD and LOQ of quercetin were 4.755 and 14.408 µg/ml and 1.941 and 5.883 µg/ml in quercitrin, respectively.

Table 4: Accuracy and precision of quercetin and quercitrin in *B. malabarica* leaves.

Compounds	Spike concentration (µg/ml)	% recovery (n = 3)	%RSD	
			Repeatability precision (n = 3)	Intermediate precision (n = 3)
Quercetin	20	97.390	1.150	2.947
	40	97.378	1.496	2.522
	60	99.177	1.164	1.519
Quercitrin	20	98.610	1.432	0.813
	40	98.181	1.548	2.947
	60	102.288	1.432	1.130

*n = triplicated

Table 5: Robustness of quercetin and quercitrin in *B. malabarica* leaves.

Compounds	% RSD of sample					
	Flow rate		Temperature		Wavelength	
	Rt	Area	Rt	Area	Rt	Area
Quercetin	4.051	6.785	3.028	6.848	0.087	2.553
Quercitrin	4.011	7.043	2.602	6.643	0.128	1.863

*Rt= Retention time

The slightly changes of column temperature, flow rate and wavelength were calculated from one sample for robustness method. Column temperature was varied from 34 – 36°C, the flow rate was set in the range of 0.950 – 1.050 ml/min and the detection wavelength was defined at 252nm, 255nm and 258nm in both quercetin and quercitrin. The results were shown in the Table 5. The variations of column temperature, flow rate and detection wavelength were demonstrated that there were no differences (%RSD < 8) in the retention time and the area of the curve of quercetin and quercitrin. So, the robustness was showed reliably of the method.[10]

CONCLUSION

The characters of *B. malabarica* leaves according to macroscopic and microscopic evaluations, leaf measurement, physico-chemical parameters and RP-HPLC analysis shall be useful to identity and characterized the safety, efficacy and quality in the further use and studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

RP-HPLC: Reversed phase high performance liquid chromatography; **S:** Stomatal number; **E:** Number of epidermal cells; **T:** Trichome number; **µm²:** Square micrometer; **mm²:** Square millimeter; **Rt:** Retention time.

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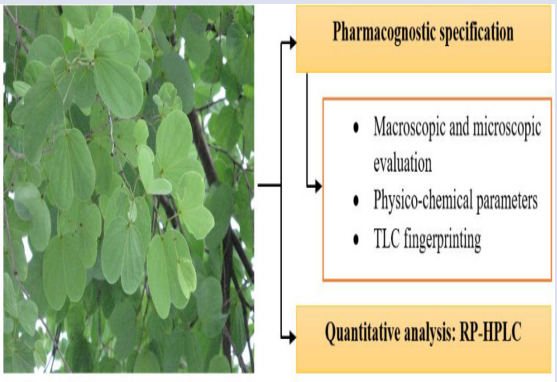
GRAPHICAL ABSTRACT



SUMMARY

- *Bauhinia malabarica* Roxb. is a species in family Leguminosae. It has been widely used as medicinal plants for a long time. In Thailand, *B. malabarica* leaves is locally used to treat wound healing, diuretic, dysentery and emmenagogue.
- This study focused on pharmacognostic specification and quantitative analysis of quercetin and quercitrin in *B. malabarica* leaves.
- The paracytic stomata and multicellular trichomes were found on lower epidermis of *B. malabarica*.
- Pharmacognostic parameters were shown the specific characteristics of *B. malabarica* leaf constant numbers.
- Quercetin and quercitrin contents were determined using RP-HPLC and method validation was in the acceptable range according to ICH guideline.

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