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Research Article

Quantitative Analysis of Hispidulin Content in *Clerodendrum petasites* Roots Distributed in Thailand

Thanyathorn Tangsongcharoen[1], Somchai Issaravanich[1], Chanida Palanuvej[1,*], Nijsiri Ruangrungsi[1,2]

ABSTRACT

Introduction: *Clerodendrum petasites* (Lour.) S. Moore (locally known as Mai-Thao-Yaai-Mom), belonging to the Verbenaceae family, is widely formulated into multi-herb remedy, Ben-Cha-Lo-Ka-Wi-Chian remedy, possessing antipyretic activity. *C. petasites* exhibits many biological activities, such as antioxidant, anti-inflammatory, antipyretic, etc. The flavonoid hispidulin is one of the main active compounds present in *C. petasites*, containing anti-atheromatous, antitumor and antispasmodic effects. **Objective:** The present study aimed to determine the hispidulin content in the dried roots of *C. petasites* using HPLC technique. **Methods:** *C. petasites* dried roots, collected from twelve different areas, were extracted with ethanol using Soxhlet apparatus, and then subjected to HPLC-PDA to quantify hispidulin content. The quantitative method using HPLC-PDA technique was validated. **Results:** The optimized HPLC coupling with PDA detector (HPLC-PDA) was validated for the quantitative analysis of hispidulin content in *C. petasites* roots in terms of linearity (y = 210,200,536.6667x – 448,756.2667; R^2 = 0.9997), accuracy (88.82-107.69% recovery), precision (0.66% RSD for repeatability precision; 1.17% RSD for intermediate precision), limit of detection (2.30 µg/mL), limit of quantitation (7.00 µg/mL), specificity (peak purity index = 1.0000) and robustness (% RSD < 1). The amount of hispidulin content in the extracts of *C. petasites* roots conducted from the validated method was found to be 0.0182 ± 0.0109 g/100 g crude drug. **Conclusion:** The HPLC-PDA analysis was able to effectively determine hispidulin in *C. petasites* roots. The hispidulin contents in *C. petasites* dried roots from various areas in Thailand were revealed which could be used for the specification of this crude drug with reference to its chemical marker.

Key words: *Clerodendrum petasites*, Ben-Cha-Lo-Ka-Wi-Chian remedy, hispidulin, HPLC-PDA.

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INTRODUCTION

For decades, herbal plants have been used medicinally all around the world, being an important aspect of various traditional medicine systems. In Thai traditional system, *Clerodendrum petasites* (Lour.) S. Moore, locally known as Mai-Thao-Yaai-Mom, belonging to the Verbenaceae family, is widely formulated into multi-herb remedy. The most famous remedy is Ben-Cha-Lo-Ka-Wi-Chian (or so-called Ya-Ha-Rak, Kaew-Ha-Dueng or Phed-Sa-Wang) remedy, which has been registered by the Thai Food and Drug Administration (FDA) for antipyretic activity.[1,2]

Clerodendrum petasites exhibits many biological activities, including antioxidant, anti-inflammatory, antimicrobial, antipyretic, antispasmodic and antibacterial activities.[2,3-7] The biological activities have been scholarly informed to be associated with the chemical constituents. The flavonoid hispidulin (also known as 4',5,7-Trihydroxy-6-methoxyflavone) (Figure 1) is one of the main active compounds present in *C. petasites*.[2,5] Hispidulin has been reported not only for its anti-atheromatous effect[8] and antitumor potential[9] but also for relaxation of the tracheal smooth muscles.[5]

To identify and isolate active constituents in medicinal plants, chromatographic techniques are

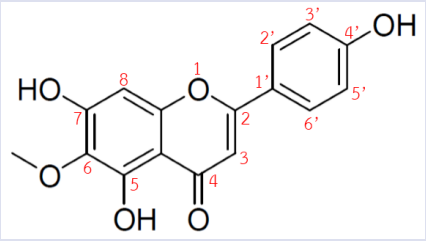

Figure 1: Chemical structure of hispidulin.

world-widely used in various studies. Such technique is high performance liquid chromatography (HPLC) that is commonly used for analytical purposes, both quantitatively and qualitatively.[10] HPLC is characterized by the use of high pressure to force a mobile phase solution through a column of stationary phase, allowing separation of complex mixture with high resolution by the differences in their partition and adsorption behaviors between the mobile phase and the stationary phase.[11,12]

Practically, the method is conducted on reverse-phased column (i.e., C_{18}) with more polar mobile phase (e.g., water, methanol, acetonitrile).[7,13,14] Although hispidulin has been highlighted for different biological properties, no report has been made on its quantification in *C. petasites*, particularly

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on its roots. As a consequence, the present study aimed to determine the hispidulin content in the dried roots of *C. petasites* using HPLC technique.

MATERIALS AND METHODS

Chemicals and reagents

Hispidulin (Sigma-Aldirch, USA, SML0582, ≥ 98%), acetic acid glacial (BDH Chemicals, UK), AR grade ethanol, HPLC grade acetonitrile and methanol (RCI Labscan Ltd., Thailand), Ultrapure water obtained from water purification system (Brinkmann, USA).

Instrumentation and equipment

Shimadzu HPLC LC-20A, consists of auto-sampler (SIL-20A HT), HPLC column oven (CTO-20AC), HPLC degasser (DGU-20A$_3$), HPLC LC-20A system, HPLC photodiode array (PDA) detector (SPD-M20A), HPLC system controller (CBM-20A), HPLC two-solvent delivery unit (LC-20AD) (Shimadzu, Japan), HPLC guard column (5 µm, 2.1 × 50 mm) (CN-3) and HPLC reversed-phased C_{18} column (5 µm, 2.1 × 250 mm) (ODS-3) (Inertsil®, GL Sciences Inc., Japan), water bath, water purification system (Brinkmann, USA).

Plant collection

Clerodendrum petasites roots from twelve different areas throughout Thailand were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi (Table 1). The voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand.

Plant extraction

Each root sample was cleaned, dried and grounded to powder (sieve number 60). The powder (5 g) was then exhaustively extracted with 95% ethanol (300 mL) by Soxhlet apparatus. After filtration, the filtrate was evaporated at 55-60°C using water bath till dryness. The extract was weighed to calculate the % yield, and then dissolved in methanol (1 mL) to obtain a final concentration. The extract in methanol was diluted to various concentrations and filtered through 0.45 µm PTFE membrane syringe filter for further HPLC analysis.

Standard preparation

The stock solution of hispidulin was prepared by dissolving the standard hispidulin (1 mg) in methanol (1 mL). The solution was filtered through 0.45 µm PTFE membrane syringe filter.

Chromatographic condition of HPLC

HPLC system control and data analysis were carried out by Shimadzu LC Solution software. The chromatographic separation was conducted using the reversed-phased C_{18} column coupled with HPLC guard column. The chromatographic condition was demonstrated as shown in Table 2. Each sample was analyzed in triplicate.

Method validation

Linearity, accuracy, precision, limit of detection, limit of quantitation, specificity and robustness were evaluated according to the Validation of Analytical Procedures.[15]

Linearity

The linearity of the method was expressed as a calibration range generated by plotting peak areas versus concentrations of standard hispidulin. The coefficient of determination (R^2) was calculated using Excel software.

Table 1: Areas and voucher specimen numbers of *Clerodendrum petasites* root collection.

No.	Area of *C. petasites* root collection	Voucher specimen number
1	Yasothon	CPTT01/2017
2	Lopburi	CPTT02/2017
3	Nakhon Nayok	CPTT03/2017
4	Phuket	CPTT04/2017
5	Lampang	CPTT05/2017
6	Nong Khai	CPTT06/2017
7	Phetchabun 1	CPTT07/2017
8	Phetchabun 2	CPTT08/2017
9	Chachoengsao	CPTT09/2017
10	Chaiyaphum	CPTT10/2017
11	Rayong	CPTT11/2017
12	Nakhon Pathom	CPTT12/2017

Table 2: Chromatographic condition of HPLC.

Parameter	Condition		
Column (Stationary phase)	Reversed phase: C_{18}		
Mobile phase	Solvent (A) - acetonitrile Solvent (B) - 0.1% aqueous acetic acid : acetonitrile (v/v, 80:20) Mode: Gradient elution		
	(A)	(B)	Time
	0%	100%	9 min
	0→50%	100→50%	6 min
	50%	50%	5 min
	50→94%	50→6%	10 min
	94%	6%	5 min
	0%	100%	20 min
	Total		55 min
Flow rate	0.5 mL/min		
Injection volume	20 µL		
Column oven temp.	35 °C		
Detector	PDA detector, λ_{max} 337 nm		

Accuracy

The spike method was performed for accuracy. Different levels of standard hispidulin (low, medium and high) were spiked into the extracted sample in triplicate and the % recovery was calculated by the following formula:

$$\% \text{ Recovery} = \frac{A}{B + C} \times 100$$

Where, A = the amount of hispidulin found in spiked extracted sample

B = the amount of hispidulin found in un-spiked extracted sample

C = the amount of standard hispidulin actually added to the extracted sample

Precision

The repeatability (intra-day) and intermediate (inter-day) precision examinations were assessed by analyzing the sample solution with three concentrations (low, medium and high) (each in triplicate) on the same

day and three different days, respectively. The precision of hispidulin content analysis was determined in terms of percent relative standard deviation (% RSD) by the following equation:

$$\% \text{ RSD} = \frac{\text{SD}}{\text{Mean}} \times 100$$

Limit of detection

The limit of detection (LOD), the lowest concentration that can be detected but not necessarily quantitated as an exact value, was determined from the calibration curve using the following formula:

$$\text{LOD} = \frac{3.3\ (\text{residual SD})}{\text{S}}$$

Where, residual SD = the residual standard deviation of regression line

S = the slope of regression line

Limit of quantitation

The limit of quantitation (LOQ), the lowest concentration that can be quantitated as an exact value, was calculated from the calibration curve using the following formula:

$$\text{LOQ} = \frac{10\ (\text{residual SD})}{\text{S}}$$

Where, residual SD = the residual standard deviation of regression line

S = the slope of regression line

Specificity

The specificity of the method was determined by analyzing absorbance spectra of standard hispidulin and samples. Peak purity was evaluated by comparing its peak at peak start, peak apex and peak end position.

Robustness

A small variation on the column oven temperature, from 34-36°C, was applied for the robustness. The result was expressed as % RSD.

Data analysis

The data were assessed by comparing the area under peak chromatogram with the calibration curve. The area under peak was analyzed using Shimadzu LC Solution software.

RESULTS AND DISCUSSION

According to the European Medicines Agency (EMA), the phytochemical compounds are commonly considered as the chemical marker, and their quantification usually indicates the quality of the plant materials due to the therapeutic activities.[16] For the analysis of flavonoid using HPLC technique, binary elution system is widely performed. An aqueous acidified polar solvent (e.g., aqueous acetic acid, perchloric acid, phosphoric acid or formic acid) is used as the first solvent, while another solvent is a less polar organic solvent (e.g., acetonitrile or methanol).[13] The condition of solvent system used in this study was practically consistent with the previous reports.[7,13,17]

The optimized HPLC coupling with PDA detector (HPLC-PDA) was validated for the quantitative analysis of hispidulin content in *C. petasites* roots in terms of linearity, accuracy, precision, limit of detection, limit of quantitation, specificity and robustness. The range of absorbance (200-800 nm) was screened for detection of hispidulin. The maximum wavelength of hispidulin was found to be at 337 nm. For linearity, the calibration curve of standard hispidulin in the range of 20-100 µg/mL was generated with the regression equation of y = 210,200,536.6667x – 448,756.2667 (Figure 2). The coefficient of determination (R^2) of standard hispidulin was found to be 0.9997, indicating that the analytical method is acceptable.[17,18] The peak of standard hispidulin was well separated with no interferences at the retention time of 28.18 min (Figure 3). The HPLC chromatogram of hispidulin in *C. petasites* root extract was shown in Figure 4. The peak purity index of hispidulin in *C. petasites* root extract was 1.0000 (Figure 5). This revealed that the method is selective and specific in this study.

The accuracy was demonstrated by recovery method through the spike of standard hispidulin at three different concentrations (20, 40 and 60 µg/mL) into *C. petasites* root extracted sample. The result was found to be admissible (88.82-107.69% recovery) (Table 3) as the percent recovery that is said to be acceptable lies between 80-120%.[15] Using the same amount of spiked concentrations, the average values of repeatability precision and intermediate precision were 0.66 and 1.17% RSD, respectively. The obtained % RSD in this study was agreeable because it was not more than 15% RSD, meeting the requirement reported by FDA.[19]

The limit of detection and the limit of quantitation, calculated from the residual standard deviation of regression line and the slope of calibration curve, displayed 2.30 and 7.00 µg/mL, respectively. By

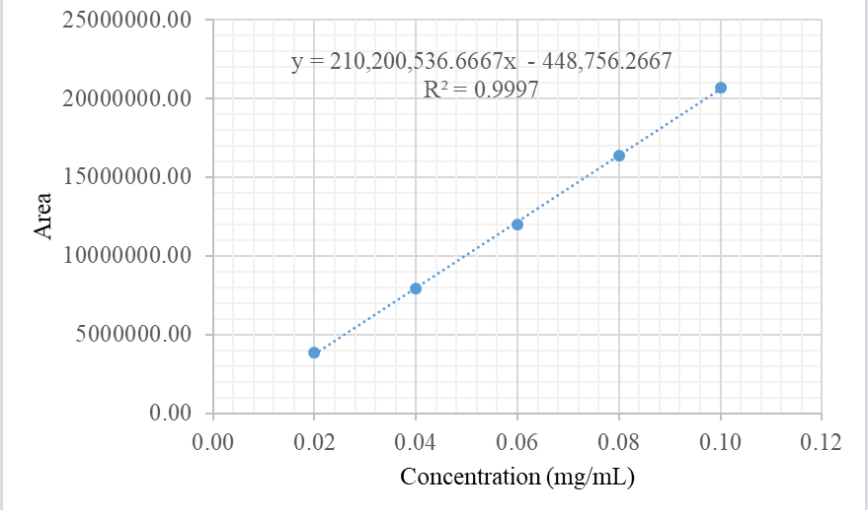

Figure 2: The calibration curve of standard hispidulin.

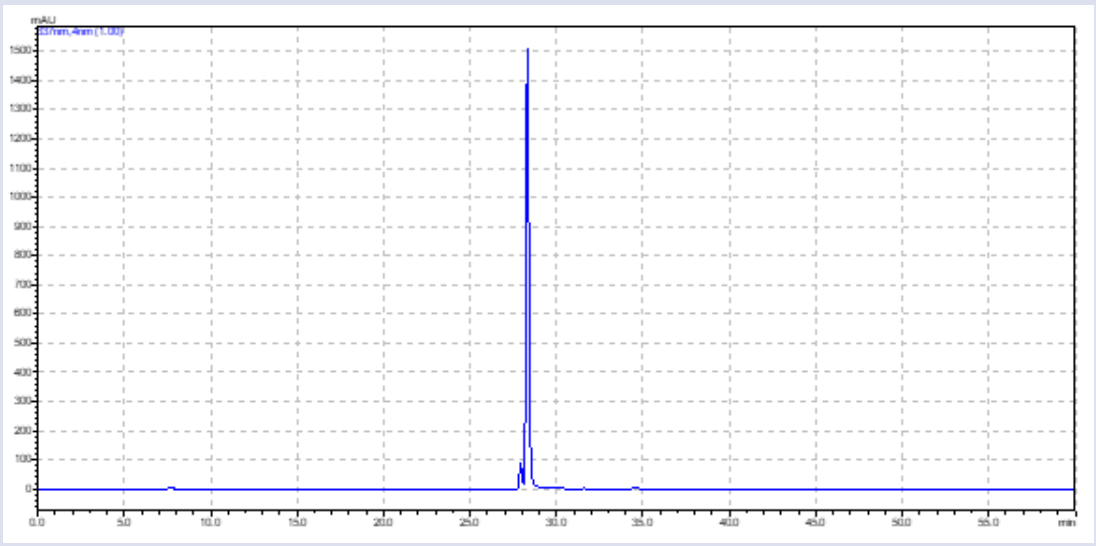

Figure 3: HPLC chromatogram of standard hispidulin at 337 nm.

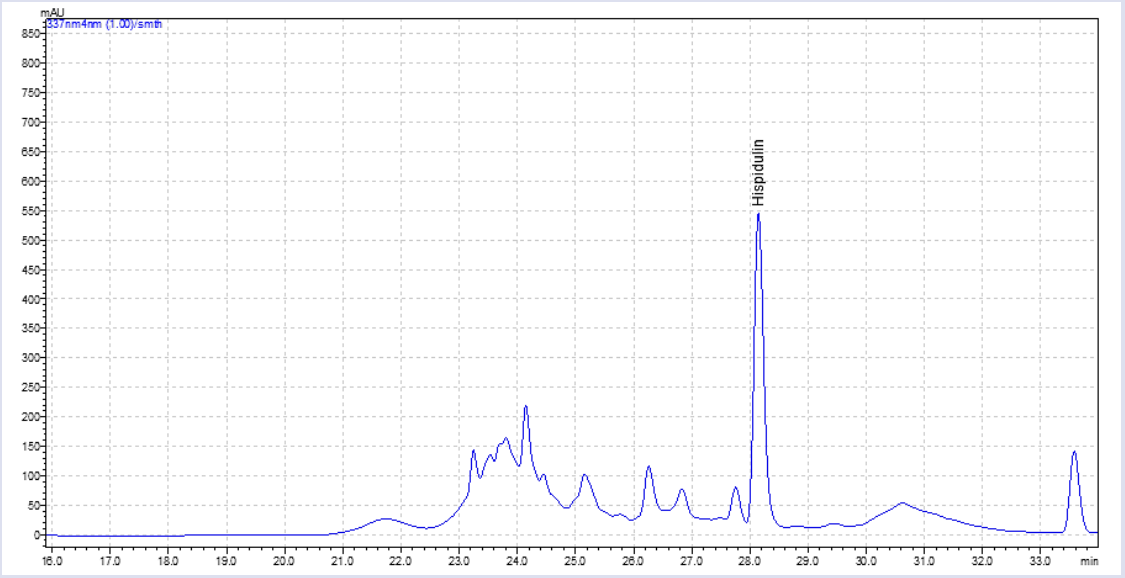

Figure 4: HPLC chromatogram of hispidulin in *Clerodendrum petasites* root extract.

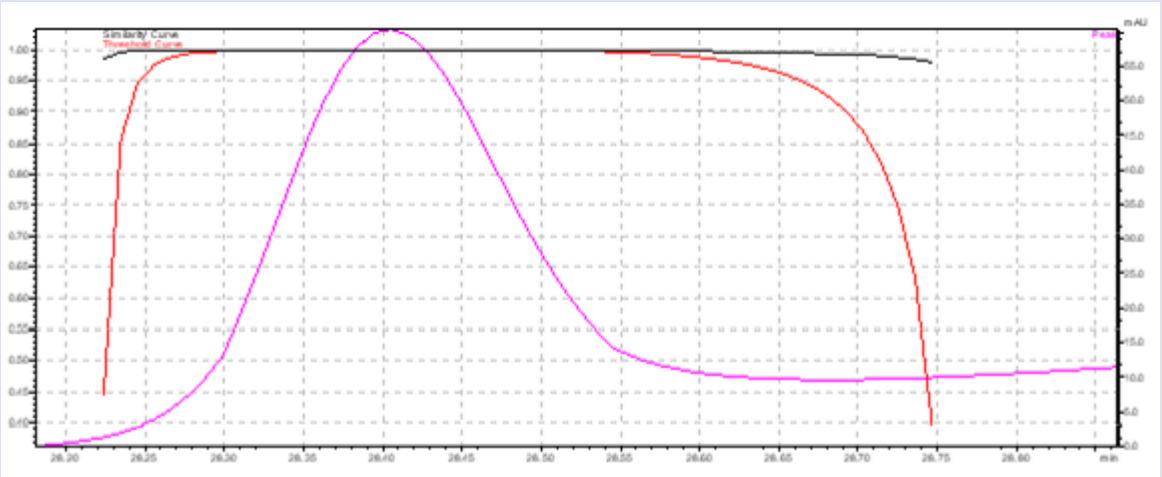

Figure 5: Peak purity of hispidulin in *Clerodendrum petasites* root extract (Peak purity index: 1.0000).

Table 3: Accuracy and precision of hispidulin in *Clerodendrum petasites* root extracts.

Level of spiked extract	Spiked extract (µg/mL) (mean ± SD)	% Recovery (n = 3)	% RSD	
			Repeatability precision (n = 3)	Intermediate precision (n = 3)
Unspiked	23.83 ± 0.51	-	2.12	1.52
Low	38.92 ± 0.09	88.82	0.22	2.05
Medium	66.84 ± 0.14	104.73	0.20	0.34
High	90.27 ± 0.08	107.69	0.09	0.75

Table 4: Robustness of hispidulin in *Clerodendrum petasites* root extracts.

Temperature	Area	Retention time
34 °C	13,372,766	28.25
35 °C	13,255,963	28.18
36 °C	13,445,963	28.13
Average	13,358,231 ± 95,830	28.19 ± 0.063
% RSD	0.72	0.22

Table 5: Hispidulin content in *Clerodendrum petasites* root extracts.

No.	Area of collection	Yield of extract (g/100 g crude drug)	Hispidulin in extract (g/g) (n = 3)	Hispidulin in crude drug (g/100 g crude drug) (n = 3)
1	Yasothon	7.84	0.0036 ± 0.00010	0.0283 ± 0.0008
2	Lopburi	6.04	0.0065 ± 0.00021	0.0392 ± 0.0013
3	Nakhon Nayok	8.47	0.0034 ± 0.00003	0.0285 ± 0.0003
4	Phuket	6.94	0.0022 ± 0.00002	0.0151 ± 0.0001
5	Lampang	5.08	0.0022 ± 0.00007	0.0114 ± 0.0004
6	Nong Khai	13.73	0.0008 ± 0.00002	0.0105 ± 0.0002
7	Phetchabun 1	6.69	0.0008 ± 0.00001	0.0053 ± 0.0001
8	Phetchabun 2	5.46	0.0012 ± 0.00001	0.0066 ± 0.0001
9	Chachoengsao	4.74	0.0021 ± 0.00005	0.0098 ± 0.0002
10	Chaiyaphum	11.86	0.0011 ± 0.00001	0.0134 ± 0.0002
11	Rayong	5.37	0.0039 ± 0.00008	0.0207 ± 0.0004
12	Nakhon Pathom	14.48	0.0020 ± 0.00002	0.0297 ± 0.0003
	Average		0.0025 ± 0.00165	0.0182 ± 0.0109

varying the column temperature from 34-36°C, the results implied that there were insignificant differences (% RSD < 1) in the retention time, the area under standard curve of hispidulin, and the hispidulin in extracted samples (Table 4). The test for robustness proved the reliability of the method as it remained unaffected by small variations in the method parameters.[15]

The developed HPLC was valid for quantification of hispidulin in *C. petasites* dried root crude drug. As the geographical variation affected the plant secondary metabolites[20,21], the roots collected from twelve areas were revealed for the hispidulin contents ranged from 0.0053 – 0.0392 with the average of 0.0182 ± 0.0109 g/100 g by dry weight (Table 5). This finding could be beneficial for the specification of *C. petasites* dried root crude drug in Thailand. The previous study of *C. petasites* aerial part in Thailand demonstrated that hispidulin was the predominant compound (1.2% w/w in a dried ethanolic extract).[7] Another report showed that hispidulin was the active compound in *C. petasites* dried aerial part (2 kg), with the actual yield of 105.12 mg hispidulin.[5] Hispidulin in *C. indicum* root was also found but in less content than in *C. petasites*. It was reported the actual yield of 8.5 mg hispidulin from 1 kg of *C. indicum* dried root.[22,23]

CONCLUSION

The RP-HPLC with PDA detector performed in the study was valid for the determination of hispidulin in *C. petasites* dried roots. The hispidulin contents in *C. petasites* dried roots from various areas in Thailand were revealed which could be used for the specification of this crude drug with reference to its chemical marker.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENT

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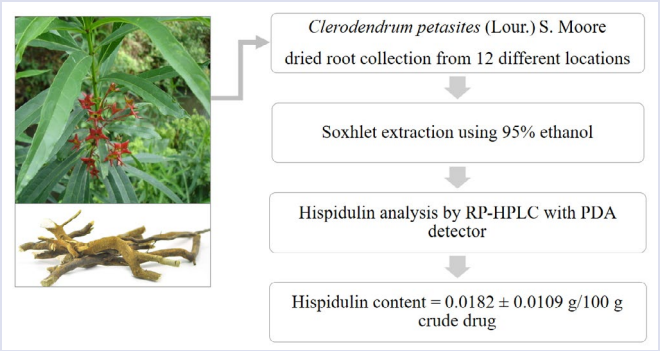
GRAPHICAL ABSTRACT



SUMMARY

- *Clerodendrum petasites* (Lour.) S. Moore (Mai-Thao-Yaai-Mom), belonging to the Verbenaceae family, is widely formulated into Ben-Cha-Lo-Ka-Wi-Chian remedy, possessing antipyretic activity. The flavonoid hispidulin is one of the main active compounds present in *C. petasites*.
- The present study aimed to determine the hispidulin content in the dried roots of *C. petasites*, collected from twelve different areas, using HPLC-PDA technique.
- The optimized HPLC coupling with PDA detector (HPLC-PDA) was successfully validated for the quantitative analysis of hispidulin content in *C. petasites* roots in terms of linearity, accuracy, precision, limit of detection, limit of quantitation, specificity and robustness.
- The amount of hispidulin was found to be 0.0182 ± 0.0109 g/100 g crude drug.
- The HPLC-PDA analysis was able to effectively determine hispidulin in *C. petasites* roots, which could be used for the specification of this crude drug with reference to its chemical marker.

ABOUT AUTHORS

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