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Development of the HPLC-DAD method for chromone determination and standardization in *Harrisonia perforata* (Blanco) Merr. and Thai herbal Ya-Ha-Rak formulation

Supawan Wechprakhon[a], Bungorn Sripanidkulchai[a], Naphapak Jaipakdee[a,b], Peera Tabboon[a,b,*]

[a] Center for Research and Development of Herbal Health Products, Khon Kaen University, Khon Kaen 40002 Thailand
[b] Division of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002 Thailand

[*]Corresponding author, e-mail: peerata@kku.ac.th

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ABSTRACT: *Harrisonia perforata* (Blanco) Merr. (*H. perforata*) is a plant material used in the Ya-Ha-Rak formula for the treatment of fever in COVID-19 patients in Thailand and contains two chromones as major active compounds, namely O-methylalloptaeroxylin (OML) and peucinin-7-methyl ether (P7ME). In Thailand, however, there is currently no standard quality control method available for this plant. This study aims to develop and validate analytical methods to measure levels of OML and P7ME in samples including branches, roots, and extracts of *H. perforata*, and Ya-Ha-Rak formulations. In the development process, a number of columns and elution conditions to determine OML and P7ME in the Ya-Ha-Rak formulations and *H. perforata* raw materials were investigated. Ideal conditions, including C18, high-purity silica at 4.6 × 150 mm, 3.5 µm particle size, controlling the column oven at 40 °C, and eluting with an acidic mobile phase, significantly improved the separation of OML and P7ME from other elutes in the chromatogram with good suitability parameters. The validation method gave good values for the correlation coefficient ($R^2 = 0.9999$) for both compounds. The limits of detection (LOD) and quantification (LOQ) were in the ranges of 1.31 and 1.03 µg/ml and 4.37 and 3.45 µg/ml, respectively. The recovery percentage fell between 99.97 and 109.75%, and the RSD value of inter-day and intra-day determinations was under 2.0%. The validated method was successfully applied to the analysis of real Ya-Ha-Rak and *H. perforata* raw material samples from various areas in Thailand.

KEYWORDS: HPLC, *Harrisonia perforata* (Blanco) Merr., Ya-Ha-Rak formulation, peucinin-7-methyl ether, O-methylalloptaeroxylin

INTRODUCTION

The global impact of the COVID-19 pandemic caused by the SARS-CoV-2 virus has resulted in significant human and economic losses worldwide. At present, there is no targeted antiviral medication that is advised for the treatment of COVID-19, a condition that has the potential to result in acute respiratory distress syndrome, multiple organ dysfunction syndrome, and fatality. The combined efforts of traditional medicines and conventional treatments have demonstrated a noteworthy reduction in mortality rates, a shortened duration of fever, a decrease in chest radiograph abnormalities, and relief from secondary fungal infections in patients undergoing glucocorticoid therapy for severe acute respiratory illnesses [1, 2]. With these functions, the consumption of herbs and natural products increased significantly on a global level [3]. However, potential low quality and untrustworthiness of many herbs and traditional medicines have led to a worsened situation. Therefore, standardization is the recommendation from many organizations such as the WHO guidelines and the American Herbal Product Association [4].

Harrisonia perforata (Blanco) Merr. (*H. perforata*) is a flowering plant in the Rutaceae family [5]. According to the National Main Medicine Act of 2556 [6], this plant is used as folk medicine by boiling its roots or shells to treat ailments such as diarrhea, fever, and aphthous ulcers [7]. Moreover, the *H. perforata* root is one of five plant materials used in the Ya-Ha-Rak formulation, which is prescribed as traditional Thai medicine used to relieve the symptoms of fever in COVID-19 patients in Thailand [8, 9]. O-methylalloptaeroxylin (OML) and peucinin-7-methyl ether (P7ME), 2 chromone substances, have been reported as the major active compounds in the roots, branches, leaves, and stems of *H. perforata* [10–12] with high potential of anti-inflammatory activity. In addition, chromone compounds may be used as adjunctive therapy for SARS-CoV-2 infection, according to several reports [13–15].

Standardization is crucial to ensure the authenticity, quality, strength, and purity of traditional medicine. Thus, to standardize herbal medicine as a safe drug, establishing various parameters using modern techniques for analysis is very important. Generally, chromatographic techniques such as High-performance liquid chromatography (HPLC) are used for standardization. It is well known that chromatography has very powerful separation abilities, suggesting the separation of complex systems such as traditional medicine. There is currently no information available, however, regarding the creation of a critical quantity analysis

system for *H. perforata* that could be used to monitor the quality of raw materials and finished goods that contain *H. perforata* as a component. Therefore, the objective of this study is to develop and validate analytical methods that will enable rapid, accurate measurements of OML and P7ME in samples including branches and roots and extracts of *H. perforata* and Ya-Ha-Rak formulations. HPLC, an appreciated qualitative and quantitative analysis method for complex components in herbal remedies, will be used in the present research.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile (ACN) was purchased from RCI LabScan, Thailand. Ultrapure water was obtained from Milli-Q system (Millipore, Bedford, USA). These solvents were used for mobile phase preparation. Peucinin-7-methyl ether (P7ME) (HPLC analytical reagent, 98.5%) and O-methylalloptaeroxylin (OML) (HPLC analytical reagent, 98.11%) were purchased from BioCrick (Sichuan, China). Methanol (analytical grade, Fisher Scientific, UK) was used as a solvent for standard and sample preparations. Analytical-grade acetic acid and formic acid were purchased from RCI LabScan and Kemaus, Cherrybrook, Australia, respectively. Branches of *H. perforata* were collected from Sakon Nakhon province, and roots were purchased from a local traditional herbal drug store in Khon Kaen province, Thailand.

Ya-Ha-Rak formulations were received from 3 hospitals, including source 1 from Pra Archan Phun Ajaro hospital (Sakon Nakhon province, Thailand), source 2 from Huai Thab Than hospital (Sisaket province, Thailand), and source 3 from Sangkha hospital (Surin province, Thailand). The commercial products of Ya-Ha-Rak were purchased from drug stores, and they were manufactured from various places in Thailand, including source 1 from Samutprakarn province (G308/53), source 2 from Roi Et province (G589/53), and source 3 from Nonthaburi province (G440/53).

Instrumentation

Analytical procedures were operated through an HPLC system (Agilent® 1260 series, Santa Clara, CA, USA), equipped with quaternary pump (Part No. G1311C), auto-sampler (Part No. G1329A), column oven (Part No. G1316A), diode array detector (Part No. G1315D), and integrated by Agilent Chemstation software. Two reversed-phase LC columns with different pore sizes (4.6×150 mm, 5 µm and 4.6×150 mm, 3.5 µm, Agilent C18 Zorbax Eclipse Plus, USA) were used in the preliminary study for the separation of chromones from other compounds. Gradient elution systems were developed using different mobile phases and additives for good separation. The developed and validated mobile phase system consisted of acetonitrile as mobile phase A and water as mobile phase B, containing 0.1% formic acid in both phases. The gradient system was programed as follows: 0–10 min: gradient from 30% to 45% solution A; 10–15 min: gradient from 45% to 70% solution A; 15–20 min: gradient from 70% to 95% solution A; and 20–25 min: gradient from 95% to 30% solution A. The detection wavelength was set at 250 nm.

Sample extraction and preparation

H. perforata extracts were separately prepared by maceration with 50% ethanol and 95% ethanol in the ratio 1:10 for 24 h. The solvent was filtered with filter paper (Whatman™, Buckinghamshire, UK), evaporated by using rotary evaporator (IKA RV 10 Basic, IKA, Japan), and dried with freeze-dryer (Gamma 1-16 LS, Martin Christ, Germany). Dried extract was kept at −20 °C until use. For determination of bioactive in Ya-Ha-Rak remedy, single step methanolic extraction was applied by diluting each sample with methanol (10 ml) and sonicating for 15 min at room temperature. The collected methanolic extracts were then filtered with 0.45 µm membrane filters into amber auto sampler vials for future analysis.

Optimization of HPLC analytical conditions

1. System suitability

The assessment of HPLC analytical system suitability was evaluated including capacity of factor (k'), resolutions (R_s), tailing factor (T_f), number of theoretical plates (N), and Height equivalent to theoretical plates (HETP) following the FDA reviewer guidance (validation of chromatographic methods) [16] to test the capability of the proposed method. Various factors were investigated such as the effects of solvent, pH, or acid additives as well as the effects of different column oven temperatures (25, 30, 35, and 40 °C). System suitability parameters were calculated, as explained in the following equations.

Capacity factor (k'):

$$k' = \frac{t_r - t_0}{t_0} \tag{1}$$

Resolution (R_s):

$$R_s = \frac{R_{t2} - R_{t1}}{0.5(\text{width of peak 1} + \text{width of peak 2})} \tag{2}$$

Tailing factor (T_f):

$$T_f = \frac{a+b}{2a} \tag{3}$$

Number of theoretical plates (N):

$$N = 16\left(\frac{T_r}{\text{peak width}}\right) \tag{4}$$

Height equivalent to theoretical plates (HETP):

$$\text{HETP} = \frac{L}{N} \tag{5}$$

Where t_r = retention time, t_0 = solvent front, R_{t1} = retention times of peaks 1, R_{t2} = retention times of peaks 2, a = the distance, measured at 5% of the peak height, between the peak leading edge and peak midpoint (perpendicular to the peak highest point), b = the distance, measured at 5% of the peak height, between the peak trailing edge and its midway, which is perpendicular to the peak highest point, and L = length of column.

2. Validation parameters

The current investigation applied to the validation of an analytical technique, which implied the assessment of several factors such as linearity, sensitivity (comprising the limit of detection (LOD), the limit of quantification (LOQ), precision, accuracy, and specificity) to comply with the criteria indicated by AOAC guideline [17]. The developed analytical method specificity was verified through the analysis of both extract and raw material samples of *H. perforata*. After conducting a linearity test, the working standard mixtures were prepared at varying concentrations of 5, 10, 25, 50, and 100 µg/ml. The correlation of determination (R^2) was found by plotting the space between the peaks and the amounts. The determination of LOD and LOQ involved the computation of the standard deviation of the response. This was achieved by integrating the response using the STEYX function in the MS Excel software. The standard error of the predicted y-value for each x in the regression was then determined, along with the slope (S) method, using the appropriate formulae:

$$\text{LOD} = \frac{\text{STEYX}}{\text{Slope}} \times 3.3 \quad \text{and} \quad \text{LOQ} = \frac{\text{STEYX}}{\text{Slope}} \times 10$$

The precision of an analysis was determined by conducting 6 repeated measurements within a single day and 3 consecutive days of 3 different concentrations of the working standard mixtures (5, 25, and 100 µg/ml) for the intra-day and inter-day assays, respectively. The resultant precision was thus established. The percent relative standard deviation (percent RSD) was computed. The accuracy of an analysis was established by spike recovery experiments. The standard solution of OML and P7ME was spiked with 95% ethanol extract. The final concentration of OML and P7ME in the mixture was 10 µg/ml, while the concentration of the 95% ethanol was varied to 100, 250, and 500 µg/ml, respectively. The accuracy was expressed as percentage recovery of spiked 95% EtOH extract through the utilization of a minimum of 3 concentrations and 6 repeated measurements, as determined by the following equation:

$$\%\,\text{recovery} = \frac{C2 - C1}{C3} \times 100$$

where $C1$ = un-spiked sample concentration, $C2$ = spiked sample concentration, and $C3$ = concentration of added standard mixture.

RESULTS AND DISCUSSION

In the herbal medicinal manufacturing process, one of the most important parts is the analytical procedure for both raw materials and finished products. Good capacity and accuracy of an analytical method is the ultimate goal for development to quantify the bioactive compounds in herbal matrices. In this century, more research focuses on plants and traditional formulations with the potential to relieve the symptoms of COVID-19 [8, 9]. *H. perforata* is one of the major plants used in Ya-Ha-Rak (Ben-Cha-Lo-Ka-Wi-Chian), which has been incorporated into the National List of Essential Medicines of Thailand and contains many chromones as major compounds such as OML [13]. Numerous reports also suggest that chromone compounds can be used as adjunctive treatment for SARS-CoV-2 infection [13–15]. Therefore, this simple method for routine use in quality control was developed.

Development and optimization of the proposed HPLC-DAD method

The HPLC chromatographic condition for qualitative fingerprint analysis should meet the requirements of providing adequate chemical information, good separation, and repeatability of the assay. So many factors, involving the quality of the separation and resolution, were performed. For the preliminary study, the different pore sizes of the RP-C18 HPLC columns as mentioned above were tested for their ability to separate P7ME and OML from background peaks, and finally using a column with a smaller pore size (3.5 µm) could provide better separation efficiency. After selection of the reverse phase column, the effect of the mobile phase composition on chromatographic separation was first investigated. Methanol and acetonitrile were compared as elution solvents. It was observed that the resolution of OML and P7ME peaks with neighboring peak were unsatisfactory when using methanol-water system (chromatogram not shown), while an acetonitrile-water system provided a better resolution 2.53 ± 0.00 and 1.13 ± 0.03 for OML and P7ME, respectively. Although acetonitrile was deemed more toxic and higher cost than methanol, meeting acceptable limits should be considered. Next, a simple buffer-free acetonitrile and water-based mobile phase was used in the selection of acid additive study. For the investigation of the effects of acid and pH on the separation of OML and P7ME from backgrounds, formic

Table 1 Effects of pH and acid on system suitability of the HPLC system.

Parameter	ACN-DI (pH 4.8)		ACN-DI (pH 4.0) 0.1% formic acid		ACN-DI (pH 2.3) 0.2% formic acid		ACN-DI (pH 3.2) 0.5% acetic acid	
	OML	P7ME	OML	P7ME	OML	P7ME	OML	P7ME
Capacity factor	13.00 ± 0.01	20.88 ± 0.00	9.28 ± 0.04	18.43 ± 0.04	9.35 ± 0.02	19.03 ± 0.01	9.77 ± 0.15	18.58 ± 0.08
Resolution	2.53 ± 0.00	1.13 ± 0.03	4.42 ± 0.18	14.60 ± 0.34	4.79 ± 0.12	16.46 ± 0.26	4.50 ± 0.79	13.89 ± 1.73
Tailing factor	1.07 ± 0.00	1.33 ± 0.00	1.08 ± 0.00	0.88 ± 0.00	1.00 ± 0.00	0.88 ± 0.00	1.00 ± 0.00	0.88 ± 0.00
Column efficiency	15,076.20	60,259.60	8,473.20	60,581.81	10,365.03	60,341.20	10,931.92	62,812.26
HETP ($\times 10^{-2}$ mm)	0.99 ± 0.00	2.26 ± 0.00	0.17 ± 0.00	2.47 ± 0.01	0.14 ± 0.00	2.48 ± 0.02	0.13 ± 0.03	2.38 ± 0.02
Retention time (min)	13.02 ± 0.01	20.35 ± 0.00	9.76 ± 0.04	18.46 ± 0.04	9.52 ± 0.02	18.42 ± 0.01	10.34 ± 0.14	18.80 ± 0.08

Data expressed as mean ± SD, $n = 3$.

acid and acetic acid in various concentrations were added to both acetonitrile and water by comparing the system suitability parameters to verify the performance of the chromatographic system for the intended separation. In Table 1, the results are displayed in set of parameters (capacity factor, resolution, tailing factor, column efficiency, and theoretical plates), all of which meet the FDA reviewer guidance (validation of chromatographic methods) value recommendations [16]. The capacity factor, column efficacy (number of theoretical plate), and HETP of all analytes were considerably stabled by changing the pH levels of the mobile phase. While the pH levels of the mobile phase decreased from 4.8, the resolution and tailing factor of OML and P7ME were found to be improved. The declining tailing factor of OML and P7ME in an acidic pH mobile phase is similar to the finding of a prior investigation that established the analytical technique for chromones in *Dysophylla stellate* [18]. The data previously published indicates that silanols on the silica surface are crucial in separating the analytes. They can enhance analyte retention through silanophilic interactions, as seen in our findings, where the retention time of OML and P7ME was shorter in the acidic condition (9 min and 18 min, respectively) compared to the non-acidic mobile phase (13 min and 20 min, respectively). Thus, the strong hydrogen bonding interaction occurred when deprotonated analyte and protonated silanol interacted. This, in consequence, leads to significant peak tailing. At lower pH of the mobile phase, less interaction happened between the 2 species due to the protonation of both analytes and silanols. Therefore, acidic condition was needed to further suppress the deprotonation of silanol and analyte to reduce peak tailing [19–21].

The elution system composed of 0.1% formic acid adjusted to pH 4.0 in the gradient elution program is the optimum system for the separation of OML and P7ME from background peaks in the chromatogram. The typical HPLC chromatogram for the determination of OML and P7ME is shown in Fig. 1. As mentioned by Lee et al [22], the true identity and purity of the peak can be evaluated by comparing the UV spectral scan of the peak with same retention time. The results found that all scans (standard mixture, *H. perforata* root sample, *H. perforata* branch sample, and Ya-Ha-Rak sample) were found to be in the same pattern. This supports the fact that the separated peak is pure and free of co-eluting impurities (Fig. 1ab).

Finally, after the development of the optimal separation and elution systems, the effects of different column oven temperatures were compared and expressed in Table 2. Usually, the column temperature is the critical parameter for the separation of analyte. Changes in temperature can affect the favorable interaction of an analyte with the stationary phase or the mobile phase. By comparison, the optimal conditions for OML and P7ME determination in *H. perforata* raw materials, extracts, and Ya-Ha-Rak formulations in this study were operated with Column name and properties, with a column temperature of 40 °C, and gradient elution of the ACN as mobile phase A and water as mobile phase B, containing 0.1% formic acid in both phases. The gradient system was programed as described earlier in the Instrumentation part. Both chromone compounds, OML and P7ME, were eluted separately within 25 min without interference from background peaks (Fig. 2).

Method validation

To meet the routine quality control analytical procedure, the developed HPLC was validated in the agreement of AOAC guideline. The evaluation consisted of linearity, LOD, LOQ, specificity, accuracy, and precision for both chromone compounds. Validation results were presented in Table 3. For both OML and P7ME, the correlation coefficient (R^2) displays good linearity (0.9999). The calculation, based on regression equation of LOD and LOQ value for both OML and P7ME, were 1.31 and 1.03 µg/ml and 4.37 and 3.45 µg/ml for LOD and LOQ values, respectively. The chromatograms of the STD at the LOD and LOQ concentrations demonstrate that the Area under the Curve (AUC) of the OML and P7ME peaks can be measured and integrated (Fig. S1). Intra-day and inter-day precision was expressed in Table 4 with respect to the RSD. The analytical results met the acceptance criteria of AOAC guideline for both analytes in low to high concentrations within the linear range of analysis. Therefore, it can be confirmed as a good precision

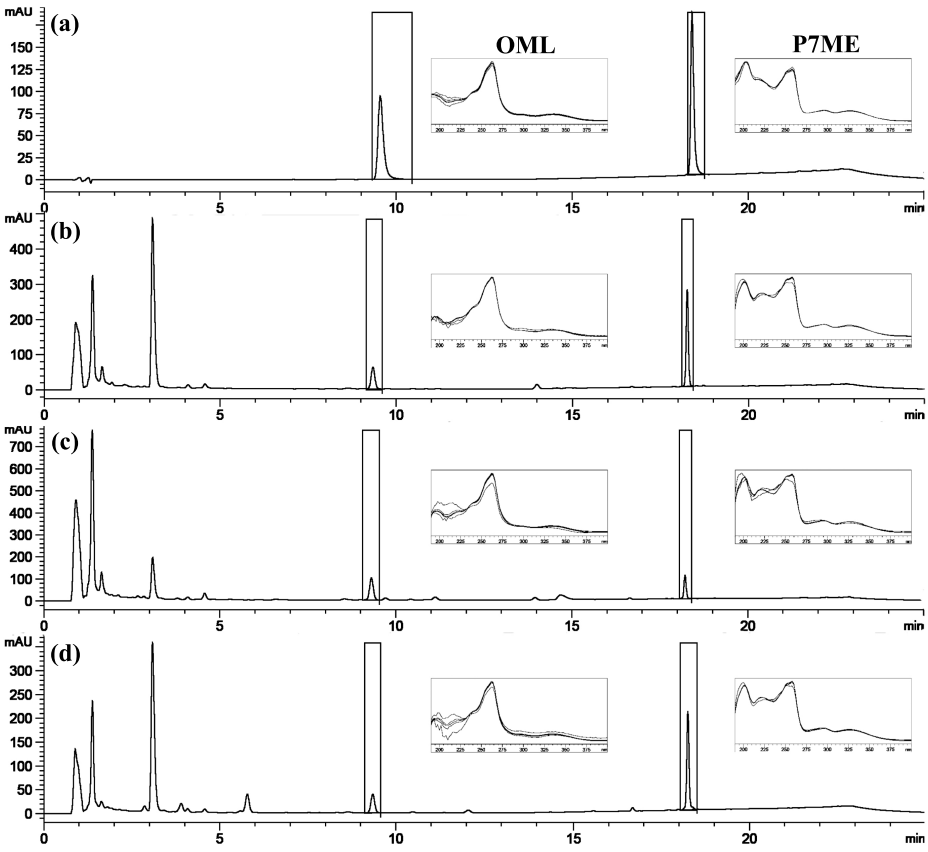

Fig. 1 UV scanning of OML and P7ME chromatographic peak of Standard mixture of (a) OML and P7ME, (b) HP root, (c) HP branch, and (d) Ya-Ha-Rak-formulation.

Table 2 Effects of temperature on system suitability of HPLC system.

Parameter	20 °C		25 °C		30 °C	
	OML	P7ME	OML	P7ME	OML	P7ME
Capacity factor	8.77 ± 0.12	18.60 ± 0.02	9.24 ± 0.09	18.60 ± 0.01	9.28 ± 0.04	18.43 ± 0.04
Resolution	1.76 ± 0.30	16.14 ± 0.16	3.10 ± 0.14	15.37 ± 0.32	4.42 ± 0.18	13.87 ± 0.32
Tailing factor	1.00 ± 0.00	0.88 ± 0.00	0.94 ± 0.00	0.80 ± 0.00	1.08 ± 0.00	0.80 ± 0.00
Column efficiency	8,612.67	61,636.35	7,704.62	61,636.35	8,473.20	54,232.63
HETP ($\times 10^{-2}$ mm)	1.74 ± 0.04	0.24 ± 0.00	1.94 ± 0.02	0.24 ± 0.00	1.77 ± 0.01	0.27 ± 0.00
Retention time (min)	9.33 ± 0.10	18.25 ± 0.10	9.33 ± 0.10	18.25 ± 0.10	9.33 ± 0.10	18.25 ± 0.10
Parameter	35 °C		40 °C			
	OML	P7ME	OML	P7ME		
Capacity factor	8.91 ± 0.04	18.21 ± 0.01	8.99 ± 0.11	18.47 ± 0.17		
Resolution	2.53 ± 0.07	13.47 ± 0.39	1.61 ± 0.14	13.56 ± 0.51		
Tailing factor	1.00 ± 0.00	1.00 ± 0.00	1.24 ± 0.04	1.13 ± 0.00		
Column efficiency	8,339.95	47,117.45	8,143.20	48,731.47		
HETP ($\times 10^{-2}$ mm)	1.79 ± 0.00	0.31 ± 0.00	1.84 ± 0.09	0.30 ± 0.00		
Retention time (min)	9.33 ± 0.10	18.25 ± 0.10	9.33 ± 0.10	18.25 ± 0.10		

Data expressed as mean ± SD, $n = 3$.

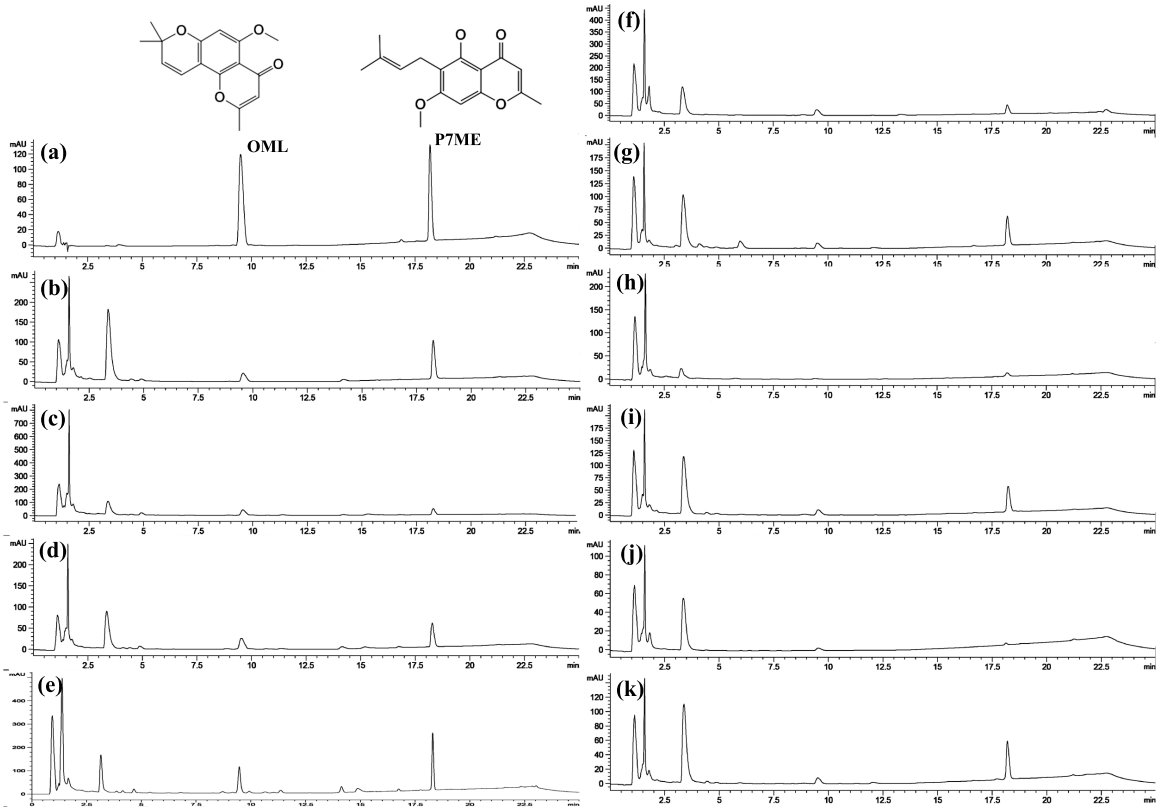

Fig. 2 Chromatogram of standard mixture of (a) OML and P7ME, (b) HP root, (c) HP branch, (d) 50% ethanolic extract, (e) 95% ethanolic extract, (f) Ya-Ha-Rak-hospital 1, (g) Ya-Ha-Rak-hospital 2, (h) Ya-Ha-Rak-hospital 3, (i) Ya-Ha-Rak-drug store 1, (j) Ya-Ha-Rak-drug store 2, and (k) Ya-Ha-Rak-drug store 3.

Table 3 Linear range, regression equation, R^2, LOD, LOQ, and retention time of OML and P7ME.

Analyte	Linear range (μg/ml)	Regression equation ($y = ax + b$)	R^2	LOD (μg/ml)	LOQ (μg/ml)	Retention time[a] (min)
OML	5–100	$y = 35.85x + 58.29$	0.9999	1.31	4.37	9.33 ± 0.10
P7ME	5–100	$y = 35.30x + 71.89$	0.9999	1.03	3.45	18.25 ± 0.10

[a] Data expressed as mean ± SD, $n = 3$.

Table 4 Accuracy and inter- and intra-day precision of OML and P7ME.

Analyte	Concentration	Precision (%RSD)		Concentration	Accuracy[a]
	(μg/ml)	Inter-day	Intra-day	(μg/ml)	(% recovery)
OML	5	0.27	0.32	5	99.97 ± 2.01
	25	0.78	0.82	25	101.00 ± 6.00
	100	1.23	0.69	100	102.01 ± 5.04
P7ME	5	0.50	0.64	5	109.75 ± 6.43
	25	0.61	1.12	25	100.10 ± 5.11
	100	0.59	1.32	100	100.98 ± 7.34

[a] Data expressed as mean ± SD, $n = 3$.

Table 5 OML and P7ME contents in raw material and finished products.

Analyte	Raw material		Extract		Ya-Ha-Rak from hospital			Ya-Ha-Rak from drug store		
(mg/g)	Root	Branch	95% EtOH	50% EtOH	Source 1	Source 2	Source 3	Source 1	Source 2	Source 3
OML	0.41 ± 0.03	0.67 ± 0.10	17.78 ± 0.68	13.35 ± 0.45	0.02 ± 0.00	0.16 ± 0.01	0.01 ± 0.00	0.36 ± 0.02	0.02 ± 0.00	0.08 ± 0.01
P7ME	1.83 ± 0.04	0.73 ± 0.04	32.04 ± 1.71	4.48 ± 0.38	0.00 ± 0.00	0.98 ± 0.03	0.13 ± 0.01	0.71 ± 0.07	0.00 ± 0.00	0.84 ± 0.01

Data expressed as mean ± SD, $n = 3$.

method with RSD value lower than 2.0% (AOAC guideline) [17]. Recovery values (%) of OML and P7ME were in the range 99.97–109.75% in high, medium, and low analytical concentrations. This also met the requirements of AOAC criteria in terms of analytical accuracy (AOAC guidelines). From these results, it can be confirmed that the developed HPLC method in this study is appropriate for use as an assay for the quality control of both chromones in both raw materials of *H. perforata* and the finished product of Ya-Ha-Rak.

Application to sample

Real samples from many sources around Thailand were applied to determine OML and P7ME contents, and the results were presented in Table 5. Preferable chromatographic separation was achieved when analyzed both raw materials and finished goods, as shown in Fig. 2. According to the study, P7ME is the major compound in roots and branches. In addition, the P7ME content in roots is more than twice as high as that found in branches. Extraction with 95% ethanol gives higher chromone content (higher than that with 50% ethanol), especially P7ME. This may be related to the lipophilicity of the P7ME, which was eluted and separated with ethyl acetate in the isolation process, as reported previously [23]. The Ya-Ha-Rak formulations, containing roots from 5 plant species including *Ficus racemosa* Linn., *Capparis micracantha* DC., *Clerodendrum petasites* S. Moore., *Tiliacora triandra* Diels., and *H. perforata*, were collected from many areas in Thailand and are available both at hospitals and drug stores. The developed HPLC method was successfully applied to determine the content of OML and P7ME. There was virtually no interference peak in both chromone peaks as shown in Fig. 2f-k. However, it was surprising that the amount of OML and P7ME from different sources varied greatly. It is similar to a previous study [24], which suggested that the reason for the variation in the amount of active compounds is possibly due to inaccurate taxonomic assignments of cultivars. Toxicity or low effectiveness can occur if patients are subjected to too high or too low amounts of active ingredients of herbal formulations. Therefore, the development of qualitative and quantitative analytical methods is necessary to apply in many steps of the manufacturing process such as quality control of raw materials and finished products.

CONCLUSION

In this study, a rapid, reliable, and accurate separation system was developed and applied to determine chromones in many parts of *H. perforata* and various commercial products. This is the first report on the quantification of OML and P7ME in various types of products such as raw materials, extracts, and commercial products. All validation parameters (linearity, LOD, LOQ, recovery, and precision) met the requirements of the AOAC criteria and were satisfactorily applied to the real samples. Evidently, this validation method can be successfully applied for future routine analysis and the quality control of *H. perforata* raw materials as well as Ya-Ha-Rak formulations from batch to batch.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.052.

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REFERENCES

1. Lyu B, Xu M, Lu L, Zhang X (2023) Burnout syndrome, doctor-patient relationship and family support of pediatric medical staff during a COVID-19 Local outbreak in Shanghai China: A cross-sectional survey study. *Front Pediatr* **11**, 1093444.
2. Sanachai K, Wilasluck P, Deetanya P, Wangkanont K, Rungrotmongkol T, Hannongbua S (2023) Targeting the main protease for COVID-19 treatment: A comprehensive review of computational screening and experimental studies. *ScienceAsia* **49**, 477.
3. Aljowaie RM, Andleeb S, Kangal A, Al-Ghamdi AA, Rehman KU, Javed R, Mahmood A, Eisa YH (2023) Prospect of herbal medication as prevention against COVID variants. *J King Saud Univ Sci* **35**, 102360.
4. Kushwaha A, Pathak D, Saha S, Singh K (2022) Standardization of some ayurvedic formulation used in Covid-19. *Ymer* **21**, 2756–2766.
5. Philipp (1912) *Harrisonia perforata* (Blanco) Merr. the plant list. Available at: https://shorturl.asia/BvTp3.
6. National drug information national list of essential medicine. Available at: https://shorturl.asia/0UMoa.

7. *Prayawitsanuprasatwech Medical Studies Thai Medicine Part 1*, Pramotepublihing, Bangkok.
8. Wang D, Huang J, Yeung AWK, Tzvetkov NT, Horbańczuk JO, Willschke H, Gai Z, Atanasov AG (2020) The significance of natural product derivatives and traditional medicine for COVID-19. *Processes* **8**, 937.
9. Yimer G, Ekuadzi E, Fasinu P, Melo AC, Pillai G (Colin) (2021) Traditional medicines for COVID-19: Perspectives from clinical pharmacologists. *Br J Clin Pharmacol* **87**, 3455–3458.
10. Yuan W-J, Gao W-F, Zhang J-H, Cao P, Zhang Y, Chen D-Z, Li S-L, Di Y-T, et al (2017) (±)-Perforison A, a pair of new chromone enantiomers from *Harrisonia perforata*. *Nat Prod Commun* **12**, 1934578X1701200.
11. Yan X-H, Di Y-T, Fang X, Yang S-Y, He H-P, Li S-L, Lu Y, Hao X-J (2011) Chemical constituents from fruits of *Harrisonia perforata*. *Phytochem* **72**, 508–513.
12. Choodej S, Sommit D, Pudhom K (2013) Rearranged limonoids and chromones from *Harrisonia perforata* and their anti-inflammatory activity. *Bioorg Med Chem Lett* **23**, 3896–3900.
13. Sestili P, Stocchi V (2020) Repositioning chromones for early anti-inflammatory treatment of COVID-19. *Front Pharmacol* **11**, 854.
14. Sharma V, Panwar A, Sankhyan A, Ram G, Sharma AK (2022) Exploring the potential of chromones as inhibitors of novel coronavirus infection based on molecular docking and molecular dynamics simulation studies. *Biointerface Res Appl Chem* **13**, 104.
15. Shakibay Senobari Z, Masoumian Hosseini M, Teimouri MB, Rezayan AH, Samarghandian S, Hekmat A (2023) Chromone-embedded peptidomimetics and furopyrimidines as highly potent SARS-CoV-2 infection inhibitors: docking and MD simulation study. *BMC Res Notes* **16**, 224.
16. CDER Center for Drug Evaluation and Research (1994) Reviewer Guidance, Validation of Chromatographic Methods. Available at: https://shorturl.asia/wo8Q4.
17. AOAC Guidelines for Single Laboratory Validation of Chemical Method for Dietary Supplements and Botanicals. In: *AOAC Official Methods of Analysis*, vol 2002.
18. Gautam R, Srivastava A, Jachak SM (2010) Determination of chromones in *Dysophylla stellata* by HPLC: Method development, validation and comparison of different extraction methods. *Nat Prod Commun* **5**, 555–558.
19. Méndez A, Bosch E, Rosés M, Neue UD (2003) Comparison of the acidity of residual silanol groups in several liquid chromatography columns. *J Chromatogr A* **986**, 33–44.
20. Engelhardt H, Blay Ch, Saar J (2005) Reversed phase chromatography – the mystery of surface silanols. *Chroma* **62**, s19–s29.
21. Zhang J, Wang Q, Kleintop B, Raglione T (2014) Suppression of peak tailing of phosphate prodrugs in reversed-phase liquid chromatography. *J Pharm Biomed Anal* **98**, 247–52.
22. Lee C, Zang J, Cuff J, McGachy N, Natishan TK, Welch CJ, Helmy R, Bernardoni F (2013) Application of heart-cutting 2D-LC for the determination of peak purity for a chiral pharmaceutical compound by HPLC. *Chromatographia* **76**, 5–11.
23. Tuntiwachwuttikul P, Phansa P, Pootaeng-On Y (2006) Chromones from the branches of *Harrisonia perforata*. *Chem Pharm Bull* **54**, 44–47.
24. Tabboon P, Tuntiyasawasdikul S, Sripanidkulchai B (2019) Quality and stability assessment of commercial products containing phytoestrogen diaryheptanoids from *Curcuma comosa*. *Ind Crops Prod* **134**, 216–24.

Appendix A. Supplementary data

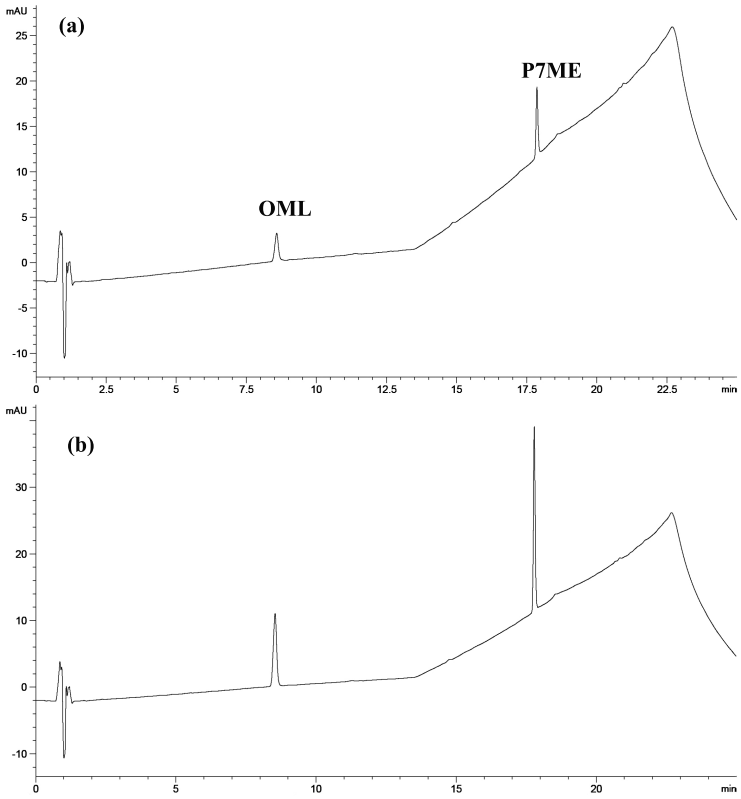

Fig. S1 Chromatogram of standard mixture of OML and P7ME at the concentration of (a) 1.0 µg/ml and (b) 3.0 µg/ml.