DEVELOPMENT AND VALIDATION OF UPLC-TQD/MS METHOD TO DETERMINE ACTIVE COMPOUND IN THAI HERBAL HARAK FORMULA

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ABSTRACT

This study aimed to develop an analytical method for determining four anti-inflammatory active compounds in the Harak herbal formula, an herbal formula in Thailand's national list of essential medicines with antipyretic properties. The targeted analytes were pectolinarigenin, gallic acid, ferulic acid, and (+)-catechin. The analysis utilized UPLC-TQD/MS, and chromatographic separation was achieved on an HSS T3 column (1.8 μm, 2.1 × 100 mm) using a gradient elution with 0.1% formic acid in water and 0.1% formic acid in acetonitrile. This method completes the separation of all four active compounds within 8 minutes. The method was validated by assessing linearity, precision, accuracy, recovery, LOD, and LLOQ. The linearity range for pectolinarigenin and gallic acid was 1.5-200 ng/mL, while for ferulic acid and (+)-catechin, it was 1-200 ng/mL. All active compounds exhibited excellent linearity, with coefficients of determination (R^2) ≥ 0.999. Precision, accuracy, and recovery were within the acceptance criteria for all active compounds. Finally, the validated method was successfully applied to assess the quality of 14 marketed Harak herbal formula samples. Due to the variations in the active compounds found, quality control of herbal raw materials is crucial for ensuring the quality of herbal medicines.

Keywords: Harak Formula, UPLC-TQD/MS, Validation, Quality Control

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INTRODUCTION

Thai herbal Harak formula (HRF) has various names, including Ben-Cha-Lo-Ka-Wi-Chian, Kaew Ha Duang, and Petch-Sawang, a traditional Thai herbal remedy used to treat fever for a long time. HRF is documented in the Tak-Ka-Si-la scripture (Foundation of Restoration and Promotion of Thai Traditional Medicine & Ayurved Thamrong School Center of Applied Thai Traditional Medicine, 2007). Currently, HRF has been included in Thailand's national list of essential medicines. Its efficacy is to reduce fever symptoms. The formula comprises the roots of five herbal ingredients, including *Ficus racemosa* L., *Capparis micracantha* DC., *Harrisonia perforata* Merr., *Tiliacora triandra* (Colebr.). Diels., and *Clerodendrum indicum* L (National Drug System Development Committee, 2023). In addition, HRF is an herbal formula with a cold property, making it suitable for external application to skin rash, reducing burning and irritation.

HRF has been previously pharmacological activities studied, such as antipyretic (Jongchanapong et al., 2010), antinociceptive (Jongchanapong et al., 2010), anti-inflammation (Juckmeta & Itharat, 2012; Juckmeta et al., 2019; Palo et al., 2017), anti-allergic (Juckmeta et al., 2014), antiplasmodial (Nutmakul et al., 2016), anti-dengue virus type 2 (Klangbud et al., 2022), and anti-melanogenesis (Lohakul et al., 2021; Onkoksoong et al., 2018; Pluemsamran et al., 2013). Regarding safety, studies have investigated platelet aggregation and LPS-induced inflammation in rats for oral administration. The results indicated no significant adverse effects (Booranasubkajorn et al., 2017; Chandranipapongse, 2017). In addition, skin irritation tests for external use have been conducted, and the HRF does not irritate the skin (Srichaipor et al., 2020).

Previous studies have reported various quality control measures, such as assessments of 19 marketed samples of HRF capsules. These assessments included physical properties analysis, microbial contamination testing, and chemical properties. High-performance liquid chromatography with a photodiode array detector (HPLC-PDA) was employed to identify the bioactive compound pectolinarigenin (Chunthorng-Orn et al., 2019). Another study investigated the quality of lupeol in 12 marketed samples of HRF using high-performance liquid chromatography with a photodiode array detector (HPLC-PDA) (Somwong & Chuchote, 2021) . Additionally, phenolic compounds (gallic acid, protocatechuic acid, vanillic acid, caffeic acid, ferulic acid, and sinapic acid) and flavonoids (catechin, rutin, and quercetin) were analyzed in both aqueous and ethanolic extracts of HRF using high-performance liquid chromatography with a UV-diode array detector (HPLC-UV) (Klangbud et al., 2022).

During the COVID-19 pandemic, the government promoted various herbal medicines, including HRF, to relieve symptoms of COVID-19. As a result, HRF is increasingly used because it effectively reduces fever in patients of COVID-19 with mild illness (Sitthipatsarin et al., 2023). Therefore, as HRF becomes more widely known, research on its efficacy, safety, and quality control is essential to provide evidence to support its use and increase consumer confidence.

Based on the information above, HRF has been extensively studied for its effectiveness and safety. However, the current quality control methods are limited; they often focus on quantifying a single marker compound, which is not enough to control the quality of the formulation, or analyzing phenolic and flavonoid compounds, as some of the compounds may not be directly relevant to HRF's therapeutic activities. Additionally, no method exists for simultaneously analyzing multiple active compounds in a single analysis. Therefore, this study aims to develop a method for quantifying the four active compounds in HRF using ultra-performance liquid chromatography coupled with a triple quadrupole mass spectrometer (UPLC-TQD/MS). The target compounds with anti-inflammatory activity are pectolinarigenin, gallic acid, ferulic acid, and (+)-catechin. Furthermore, this research will analyze the quality of HRF samples obtained from drugstores and online shops.

LITERATURE REVIEWS

The identified metabolites in herbal medicine can be categorized into two main approaches: untargeted and targeted analysis. In untargeted analysis, the goal is to simultaneously detect as many metabolites as possible in a given sample. This approach often employs techniques like liquid or gas chromatography (LC/GC) coupled with mass spectrometry (MS) or nuclear magnetic resonance (NMR). Targeted analysis focuses on analyzing specific metabolites of interest or confirming findings from an initial untargeted analysis (Khoomrung et al., 2017).

Untargeted Analysis of HRF: Chemical profiling of HRF and all five components, using liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (LC-QTOF-MS), revealed nineteen chemical constituents. These constituents, including phenolics and flavonoids known for their anti-fungal and anti-cancer activities, were detected in positive and negative ESI modes. Among these constituents, hesperetin (HSP) (or the (2S)-5,7-dihydroxy[1]2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydrochromen-4-one) was identified as a putative active compound of HRF (Seubnooch et al., 2018). Another study investigated the aqueous and ethanolic extracts of authentic HRF using Gas Chromatography-Mass Spectrometry (GC-MS). The analysis of the aqueous extract found 18 compounds, 14 of which were identified and 4 unknowns. 35 compounds were detected in the ethanolic extract, 31 of which were identified and 4 unknowns (Chunthorng-Orn et al., 2019).

Targeted Analysis of HRF: Quality control using Ultra Performance Liquid Chromatography (UPLC) targeted six phenolic compounds: caffeic acid, ferulic acid, gallic acid, kojic acid, p-coumaric acid, and vanillic acid. The analysis confirmed the presence of caffeic acid, ferulic acid, gallic acid, p-coumaric acid, and vanillic acid in HRF. This study further investigated the anti-melanogenesis activity of ferulic acid (FA) and gallic acid (GA) compared to HRF extracts. The results showed that HRF extracts, FA, and GA inhibited UVA-induced melanogenesis, likely through the upregulation of Nrf2-regulated antioxidant defenses in B16F10 cells (Onkoksoong et al., 2018). Method validation for determined pectolinagenin in HRF extract by a reversed-phase high-performance liquid chromatography (RP-HPLC) was examined, and the pectolinarigenin concentration was 18.50 mg/g of extract (Sakpakdeejaroen et al., 2014). Following method development, 19 HRF samples were analyzed to assess their quality. Authentic HRF contained 25.3 ± 0.31 mg/g of pectolinarigenin. Only 3 of the 19 samples showed levels similar to the authentic sample (Chunthorng-Orn et al., 2019). Previous research indicates that no method exists for simultaneously analyzing multiple active compounds in a single analysis.

Active compound

1) Pectolinarigenin is a dimethoxyflavone classified as a flavonoid. A study investigating the anti-inflammatory activity of pectolinarigenin found that it strongly inhibited COX-2-mediated PGE_2 synthesis in LPS-treated RAW 264.7 cells. Pectolinarigenin, isolated from HRF extract, exhibited higher anti-allergic activity than the positive control, ketotifen. The IC_{50} values were 6.30 μg/mL for pectolinarigenin and 40.39 μg/mL for ketotifen, respectively (Lim et al., 2008).

2) Gallic acid, or 3,4,5-trihydroxybenzoic acid, is a trihydroxybenzoic acid classified as a phenolic compound. An in vitro study investigating the anti-inflammatory activity of gallic acid utilized LPS-stimulated RAW 264.7 macrophages. The results demonstrated that gallic acid significantly inhibited PGE_2 production versus the control group (Seo et al., 2016).

3) Ferulic acid is a hydroxycinnamic acid derivative classified as a phenolic compound. A study investigating the anti-inflammatory effects of ferulic acid in LPS-stimulated RAW 264.7 cells demonstrated that ferulic acid reduced PGE_2 production and suppressed COX-2 expression in a concentration-dependent manner. Therefore, ferulic acid inhibits PGE_2 production by suppressing COX-2 protein expression (Kim et al., 2019).

4) Catechin is a flavonoid in two steric forms: (+)-catechin and its enantiomer. Its chemical structure is 3,3',4',5,7-pentahydroxyflavan. An in vitro study, catechin inhibited COX-2

enzyme activity in LPS-stimulated RAW 264.7 cells. The results indicated a significant decrease in NO production and PGE_2 levels compared to the control group (Varia et al., 2021).

RESEARCH METHODOLOGY

Herbal materials

Fourteen HRF samples were obtained from various commercial sources, including drugstores and online shops. The samples were labeled as No. 1 to 14 and stored at room temperature until analysis.

Instrument and Analytical Condition

The Waters ACQUITY UPLC®system (Waters Corp., USA) is equipped with a binary pump solvent management system, an auto-sample, a column compartment, and a Waters Xevo® TQ-XS triple quadrupole mass spectrometer (Waters Corp., USA) with an electrospray ionization (ESI)source used for the analysis and method validation. Active compounds were separated on a Water ACQUITY UPLC® HSS T3 column (1.8 µm, 2.1 × 100 mm) protected by ACQUITY UPLC® HSS T3 VanGuard Pre-column (2.1 × 5 mm) at 40 °C with autosampler temperature at 10 °C. The mobile phase was a mixture of 0.1% formic acid in water (A) and 0.1 formic acid in acetonitrile (B). The gradient elution was as follows: 0-0.5 min, 2-30 %B; 0.50-1.00 min, 35 %B; 1.00-2.00 min, 50 %B; 2.00-3.00 min, 98 %B; 3.00-5.50 min,100 %B; 5.50-5.80 min, 35 %B; 5.80-8.00 min 2 %B. The flow rate was 0.4 ml/min, and the injection volume was 2 µL. Data was collected and processed using the MassLynxTMV4.2 software. The mass spectrometer settings used were capillary voltage 2.9/3.10 kV for negative and positive ionization modes, collision gas (Ar, 99.9%), source temperature 150 °C, desolvation temperature 500 °C, desolvation gas (N2) flow 1000 l/h, and cone gas flow 150 l/h.

Standard stock and Sample Preparation

Four standard stock solutions were prepared using methanol at a 1.0 mg/mL concentration and stored at -80 °C until use.

Sample preparation was performed according to Seubnooch et al (Seubnooch et al., 2018). 100 mg of each powdered sample was extracted with 1 mL of methanol using ultrasonication for 60 minutes to identify the chemical constituents. After centrifugation at 15,000 rpm for 20 minutes at 4°C, the supernatant was filtered through a 0.2 µm PVDF syringe filter. The final concentration is 1.5 mg/mL. Before analysis, 10 ng/mL of the internal standards Caffeine (3-Methyl^{13}C) and Cholic acid (2,2,4,4-D4) were added to these solutions.

Method validations

The method validation followed the guidelines outlined in the US FDA's Bioanalytical Method Validation guidance document and AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (AOAC International, 2002; U.S. Department of Health and Human Services Food and Drug Administration, May 2018). The developed LC-MS method was validated in terms of linearity, precision and accuracy (intra-day and inter-day), the limit of detection (LOD), the lower limit of quantification (LLOQ), and recovery.

The limit of detection (LOD) was defined as the concentration at which the signal-to-noise ratio (S/N) ≥ 3, while the lower limit of quantification (LLOQ) was defined as the concentration with an S/N ≥ 10 (n = 6). At the LLOQ, the acceptable criteria of precision (CV% within ±20%) and accuracy (80-120%) were required.

Linearity was assessed using calibration curves constructed from the analysis of mixed standard solutions at various concentrations (n = 3). Linearity was deemed acceptable when the coefficient of determination (R^2) exceeded 0.995. The allowable deviation was ±20% for the LLOQ and ±15% for other concentrations.

The precision and accuracy were determined using five replicates on the same day (intra-day), and three independent runs were performed over three consecutive days (inter-day) by

analyzing mixed standard concentrations at LLOQ, low, medium, and high of the linear range of each calibration curve. Acceptable precision (CV%) was defined as within ±20% at the LLOQ and ±15% at other concentrations. The acceptable accuracy range was 80-120% for the LLOQ and 85-115% for other concentrations.
To evaluate the method's recovery, known amounts of standard solutions were added to the sample extracts. Each standard was spiked into the sample solution at three concentrations, covering the specific range in triplicate analyses. The acceptable range is 75-120%.

RESEARCH RESULTS

Optimization of UPLC-MS Conditions

The study aimed to develop a reliable method for quality assessment of HRF using specific active compounds. Using LC-MS, the first optimized the conditions for simultaneous detection and quantification of four markers (pectolinarigenin, gallic acid, ferulic acid, and (+)-catechin). The markers were separated using a gradient elution method with a specific mobile phase on an ACQUITY UPLC® HSS T3 column maintained at 40°C. The chromatogram showed sharp peaks, and the resolution was good. The optimized parameters and resulting chromatograms are presented in Table 1 and Figure 1.

Table 1 List of compound-dependent MRM parameters for each standard and internal standard.

Compound	Ion Mode	Parent ion (m/z)	Daughter ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
Pectolinarigenin	+	315.1300	300.0700	52	24
			167.9700	52	36
Gallic acid	-	169.0242	125.0538	30	14
			124.9440	8	18
Ferulic acid	-	192.9600	178.0200	12	12
			134.0100	12	16
(+)-Catechin	-	288.9800	122.9300	32	32
			108.9600	32	28
Caffeine (3-Methyl^{13}C)	+	196.0500	139.0600	54	22
			111.0500	54	20
Cholic acid (2,2,4,4-D4)	-	411.3300	290.4400	56	38
			328.4500	56	38

Note: Caffeine (3-Methyl-^{13}C) and Cholic acid (2,2,4,4-D4) are internal standard.

Method validations

The method demonstrated good linearity for all marker analytes, as evidenced by the high coefficients of determination (r^2) ranging from 0.999 to 0.9992 for the calibration curves. All calibration points were within the acceptable range. The LOD values ranged from 0.005 to 0.5 ng/mL, while the LLOQ values ranged from 1.0 to 1.5 /mL, as shown in Table 2. Precision and accuracy, intra-day and inter-day analyses were conducted for (+)-catechin and ferulic acid at 1, 3, 40, and 125 ng/mL, and for gallic acid and pectolinarigenin at 1.5, 4.5, 40, and 125 ng/mL. The %CV and %accuracy values were within ±15%, as shown in Table 3. Recovery was determined at three concentrations (low, medium, and high) and within the acceptance criteria, as shown in Table 4.

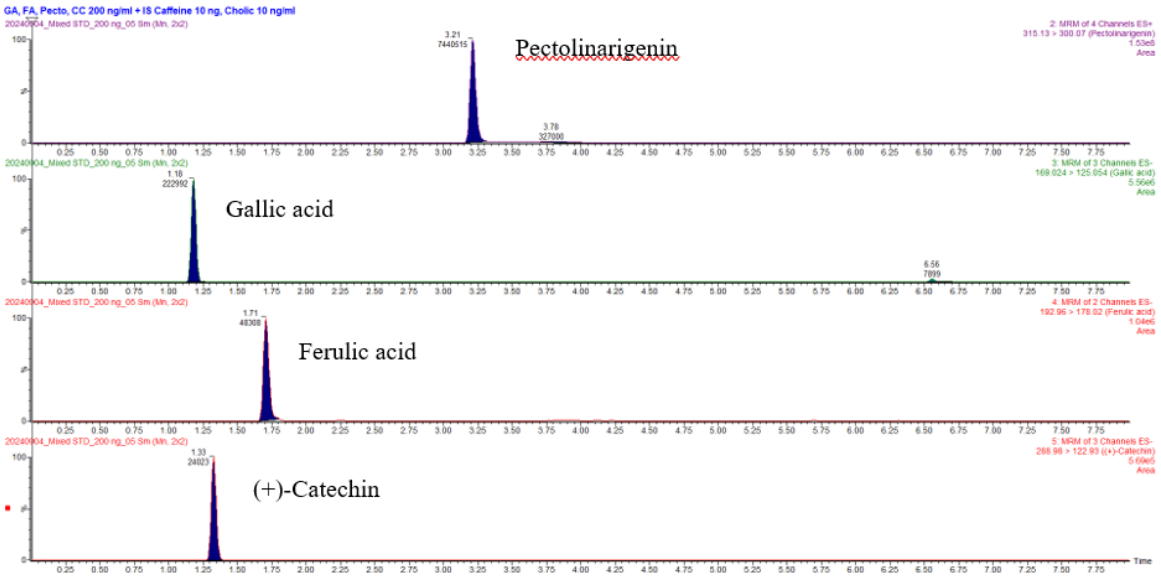

Figure 1 MRM chromatograms of four active compounds. (Pectolinarigenin, Gallic acid, Ferulic acid and (+)-Catechin)

Table 2 Various parameters for simultaneous determination of compound analytes in HRF using the UPLC-TQD/MS.

Analyte	Linear Range (ng/mL)	Regression Equation	R^2	LOD (ng/mL)	LLOQ (ng/mL)
Pectolinarigenin	1.5-200	y = 3.0768x + 3.8149	0.999	0.005	1.5
Gallic acid	1.5-200	y = 10.566x + 10.937	0.9991	0.5	1.5
Ferulic acid	1-200	y = 2.4112x + 2.6444	0.9992	0.1	1
(+)-Catechin	1-200	y = 1.1545x + 1.341	0.9992	0.5	1

Table 3 Precision and accuracy data of the four compound analytes in the developed UPLC-TQD/MS.

Analytes	Concentrations (ng/mL)	Intra-day (n = 5)		Inter-day (n = 5)	
		Precision CV (%)	Accuracy (%)	Precision CV (%)	Accuracy (%)
Pectolinarigenin	1.5	3.647	97.069	4.148	96.643
	4.5	2.828	95.934	3.331	95.122
	40	3.795	92.670	2.670	93.177
	125	1.600	97.775	1.154	97.957
Gallic acid	1.5	5.822	94.553	4.452	95.916
	4.5	4.559	96.146	5.352	95.628
	40	5.094	95.988	2.542	96.834
	125	4.963	96.561	2.720	97.771
Ferulic acid	1	7.793	93.106	6.165	94.479
	3	5.077	94.911	3.452	95.566
	40	5.021	96.202	4.126	96.068
	125	3.769	97.302	4.472	96.630
(+)-Catechin	1	8.351	93.183	6.804	94.224
	3	4.575	96.369	4.154	96.682
	40	3.529	96.797	2.453	96.545
	125	3.802	97.200	2.817	97.525

Table 4 Recovery data of the four compound analytes in the developed UPLC-TQD/MS.

Analytes	Concentration (ng/mL)	Mean Recovery (%) (N = 3)	CV (%)
Pectolinarigenin	4.5	102.907	3.860
	40	99.753	1.785
	125	105.262	3.805
Gallic acid	4.5	106.909	4.708
	40	113.216	5.163
	125	115.866	2.149
Ferulic acid	3	95.469	5.300
	40	101.240	1.211
	125	98.731	0.897
(+)-Catechin	3	97.547	6.726
	40	99.881	0.283
	125	98.011	2.350

Sample analysis

The developed analytical method for quantifying four active compounds was applied to assess the quality of 14 marketed HRF samples purchased from drugstores and online shops. The amounts of all four active compounds fell within the range of each established calibration curve. The average pectolinarigenin content ranged from 6.940 to 80.027 µg/g, gallic acid content ranged from 1.353 to 19.638 µg/g, ferulic acid content ranged from 3.709 to 15.785 µg/g, and (+)-catechin content ranged from 1.686 to 16.452 µg/g. Analysis of the 14 samples revealed variations in the quantities of all four compounds, as shown in Figure 2.

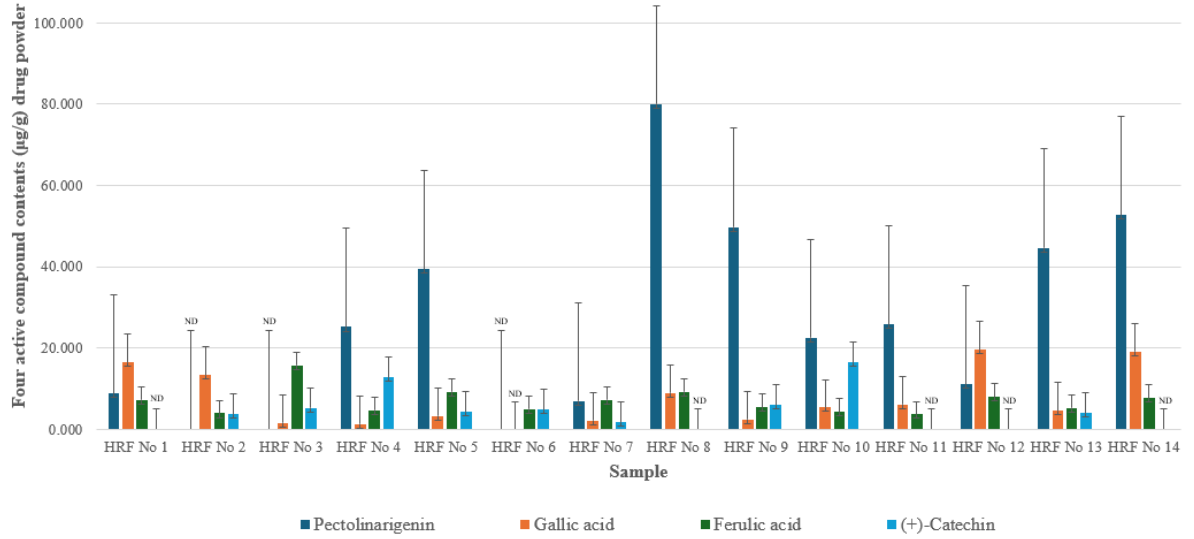

Figure 2 Four active compounds contents in marketed HRF from 14 samples. The amount of active compounds is shown as the mean. The standard deviation (SD) represents the variation in the amounts of the four active compounds among the 14 HRF samples. ND = not detected

DISCUSSION & CONCLUSION

Previous studies for quantifying compounds in HRF, such as pectolinarigenin and phenolic compounds using HPLC required lengthy analysis times (30-35 minutes) and high flow rates (1 mL/min) (Klangbud et al., 2022; Sakpakdeejaroen et al., 2014). In contrast, the present study developed a UPLC-TQD/MS method capable of quantifying four active compounds within 8

minutes, using a flow rate of 0.4 mL/min. The UPLC-TQD/MS system is highly sensitive and specific and is well-suited for targeted analysis. This approach offers significant advantages, including faster analysis times and reduced mobile phase consumption, leading to cost savings and a more environmentally friendly approach.

Fever is a physiological response to inflammation, characterized by the biosynthesis of prostaglandin E2 (PGE_2), a key inflammatory mediator, from arachidonic acid by the action of cyclooxygenase (COX) enzymes (Kim et al., 2019). The four standards used in this analysis, pectolinarigenin, gallic acid, ferulic acid, and (+)-catechin are suitable marker compounds for quality control due to their anti-inflammatory pharmacological activities. All four standards have been shown to inhibit PGE_2 production (Kim et al., 2019; Lim et al., 2008; Seo et al., 2016; Varia et al., 2021).

The analysis of fourteen HRF samples showed variations in the quantities of four active compounds. Pectolinarigenin exhibited the most significant variability and was not detected in samples HRF No. 2, 3, and 6. Gallic acid was absent in sample HRF No. 6, while (+)-catechin was not detected in samples HRF No. 1, 8, 11, 12, and 14. Several factors could contribute to this variability, including the age of the raw materials and the herbal medicine and adulteration with plant stems (Nutmakul et al., 2013). In addition, HRF samples Nos. 1, 2, 3, 6, and 8 do not have FDA-issued drug registration numbers, indicating that they are drugs that have not passed the FDA Thailand's quality inspection. This may affect the amount of active ingredients in the medicine. Therefore, to ensure the consistent quality of herbal medicines, quality control must be implemented at every production stage, from selecting raw materials to manufacturing in certified factories.

In conclusion, this study developed an LC-TQD/MS method for simultaneously quantifying four active compounds, pectolinarigenin, gallic acid, ferulic acid, and (+)-catechin, for the quality control of HRF. The developed method was validated by assessing linearity, LOD, LLOQ, precision, accuracy, and recovery. All assessed parameters were determined to be within the acceptance criteria. This method offers several advantages, including rapid analysis time, minimal mobile phase consumption, and high reproducibility, making it suitable for the quality control of HRF in industrial production.

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