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Original Research Article

Development and Validation of an HPLC/PDA Methodology for Assessing the Quality of *Folium Perilla Frutescens* under Diverse Soil Conditions and Growth Stages Based on the Bioactive Compound Rosmarinic Acid

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ABSTRACT

Perilla frutescens (L.), renowned for its medicinal properties such as lowering blood uric acid, anti-inflammatory effects, and anti-allergy effects, contains a variety of beneficial compounds including phenolics, flavonoids, and essential oils. Among these, rosmarinic acid stands out for its remarkable ability to reduce blood uric acid levels. The study has introduced an innovative approach to extract and quantify rosmarinic acid from *Perilla* leaves, employing a straightforward extraction method with quick analysis. The optimized extraction method involves using ultrasound for 10 minutes with 70% ethanol. For chromatographic analysis, a Phenomenex Luna C18 column (250 mm x 4.6 mm, 5 µm) was utilized with a mobile phase composed of acetonitrile and 0.1% formic acid in a ratio of 27:73 (v/v), flowing at 1.2 mL/min for 7 minutes. This methodology underwent thorough validation following the Association Of Official Analytical Collaboration guidelines, demonstrating a limit of quantification of 0.1 µg/mL. Furthermore, it exhibits excellent linearity within the concentration range of 10-100 µg/mL, supported by a high correlation coefficient of 0.9988. The procedure is applied to quantitatively determine the rosmarinic acid contents in *Perilla* leaves across 17 provinces in Vietnam, revealing Ho Chi Minh City to have the most abundant concentration of this compound. Research on the growth stages of *Perilla frutescens* has revealed that the flowering stage exhibits the highest levels of rosmarinic acid. These findings are pivotal for evaluating the quality of raw materials and optimizing the use of *Perilla frutescens* as a medicinal herb.

Keywords: *Perilla frutescens* (L.) Rosmarinic acid, Uric acid, High-Performance Liquid Chromatography, Photodiode Array Detector.

Introduction

Perilla frutescens (L.) is a readily available source of raw material, found in most provinces and cities of Vietnam as well as in other Asian countries.[1] Furthermore, it is a medicinal herb that has been widely used among the Vietnamese population for a long time due to its antibacterial properties, aiding in the treatment of coughs, colds, seafood poisoning, stomach aches, and vomiting.[2]

Among the varied chemical constituents such as phenolics, flavonoids, essential oils, and triterpenes, the phenolic compounds are synthesized and accumulated in all parts of the plant, it plays crucial roles in plants, such as protecting against ultraviolet radiation and pathogens.[3] Contemporary studies consistently recognize rosmarinic acid as the primary phenolic compound present in *Perilla* leaves.[4,5]

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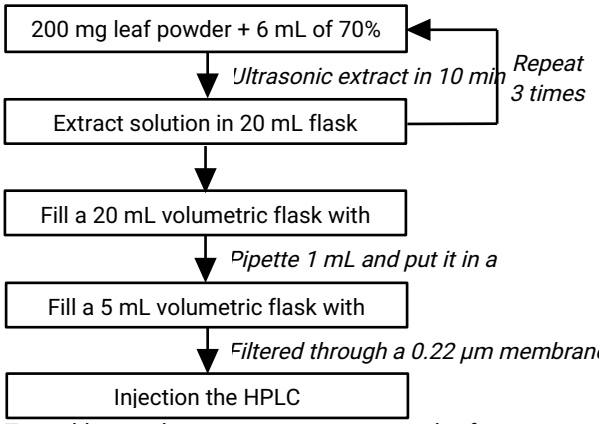
This compound has garnered attention for its diverse biological activities, including its role in reducing blood uric acid levels, exerting anti-inflammatory and anti-allergic effects, displaying antioxidant properties, and demonstrating the potential to inhibit the proliferation of cancer cells.[4-12]

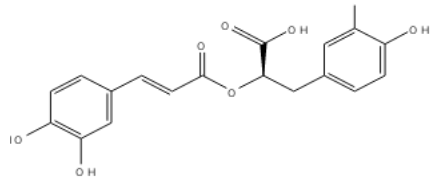
These findings highlight the diverse therapeutic potential of rosmarinic acid, establishing its significance as a valuable component in both traditional and contemporary medicinal applications.

However, the lack of standard and control over rosmarinic acid content, crucial for the reduction of uric acid levels in *Perilla* leaf extract, has resulted in inconsistent product quality and diminished efficacy. So far, there has been relatively limited research using high-performance liquid chromatography coupled with the photodiode array detector (HPLC/PDA) technique to quantify rosmarinic acid in *Perilla* leaves for

evaluating the quality of herbal materials.



To address these gaps, our research focuses on the development and validation of a process for quantifying rosmarinic acid in *Folium Perilla frutescens* by the HPLC-PDA



method with rapid, simple, and sensitive properties. Concurrently, applying this process will offer insights into the rosmarinic acid content variation across different provinces in Vietnam. By undertaking this project, our research aims to enhance the quality and efficacy of medicinal herbs, promote standardized practices in product development, and contribute to the optimization of healthcare resources in our country.

Materials and Methods

Chemicals and reagents

Rosmarinic acid (shown in Fig 1) (98%) was supplied by Sigma-Aldrich® (Sigma-Aldrich Co., Germany). Acetonitrile, methanol, ethanol absolute, and formic acid were of HPLC grade (Merck, Germany) (purity > 99%). All the chemicals and solvents employed in sample preparation met analytical standards.

Fig 1: The chemical structure of rosmarinic acid.

Plant materials

In October 2023, mature leaves of *Perilla frutescens* (L.) Britt were harvested in Ninh Kieu, Can Tho (10°02'13.60" N, 105°47'17.70" E), Vietnam. The voucher specimen was identified by PCR (Polymerase Chain Reaction) and gene sequencing techniques at the molecular biology laboratory, Biotechnology Research and Development Institute (Can Tho University, Can Tho, Vietnam).

After undergoing cleaning and drying at temperatures ranging between 40-50°C, greater rosmarinic acid content can be extracted compared to fresh leaves.[13] The leaves were ground into coarse powder and carefully stored in a light-proof sealed container to preserve their quality. Samples were placed in sealed plastic bags and allowed to reach the required humidity levels following the guidelines outlined in the Vietnamese Pharmacopoeia V (2018).[14]

Preparation of stock and working standard solutions

A stock solution of rosmarinic acid was prepared by precisely weighing and dissolving it in methano to achieve a concentration of 1000 µg/mL. Consequently, working solutions were generated by diluting the stock solution with methanol to achieve the desired concentrations. An appropriate amount of each stock solution was mixed and diluted with methanol to obtain six different working standard solutions in the range of 10 - 100 µg/mL to construct the relevant calibration curve.

Preparation of sample solution

The sample solution was prepared following the optimized extraction procedure determined after the survey. Using the ultrasonic extraction method, 200 mg of powdered *Perilla* leaves were extracted for 10 minutes with 6 mL of 70% ethanol. The extraction procedure was repeated three times using three saparate samples. The resulting extracts were combined and then diluted with methanol in a 20 mL volumetric flask. Subsequently, 1 mL of this solution was pipetted into a 5 mL volumetric flask and further diluted with methanol. Finally, this solution was filtered through a 0.22 µm membrane into a vial. The process of extracting rosmarinic acid from *Perilla* leaves is presented in Fig 2.

Fig 2: Optimal rosmarinic acid extraction process

Instruments and analytical conditions

The HPLC analysis was conducted using a Hitachi L-2000 system from Tokyo, Japan equipped with an L-2455 photodiode array detector, an autosampler, a quaternary pump, a degasser, and a column thermostat. Data were analyzed using the OpenLAB CDS software.

We conducted a survey of optimal chromatography conditions with a Phenomenex Luna C18 column (250 mm x 4.6 mm, 5 µm) from Phenomenex, USA. The mobile phase was investigated with various types of solvent and ratio, including ACN, MeOH, acetic acid, and formic acid. Change the type of solvent, the ratio of solvent to the concentration of acetic acid, and formic acid. Isocratic or gradient elution program with 332 nm detection wavelength. The flow rate surveyed increased gradually from 0.8 - 1.5 mL/min, 20 µL sample injection volume and column kept at room temperature (20 – 25^{o}C).

The ultrasonic extraction method was chosen from the beginning to survey parameters related to the extraction process, including extraction solvent (70% ethanol, 90% ethanol, methanol), sample-solvent ratio (1:20, 1:30, 1:40), extraction time (7 min, 10 min, 15 min) and the number of extractions.

Method validation

As per the guidelines set forth by the Association Of Official Analytical Collaboration (AOAC) in 2023. Each sample was measured at least three times, and the results were calculated based on these experimental average values, the method for determination of rosmarinic acid (RA) was validated in terms of system suitability, specificity, linearity of calibration curve, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy.[15]

Applications

Perilla leaf samples were taken from 17 provinces in Vietnam for application. Information on the samples is presented in Table 1. Samples to evaluate rosmarinic acid content according to each growth stage were collected in An Giang (10°30'0.00" N, 105°10'0.01" E), Vietnam. The stages of sample collecting included vegetative growth, flowering, maturation, and senescence.

Table 1: Sample data of *Folium Perilla frutescens*

No.	Sample code	Source	GPS location	Collection time
1	FBF-01	An Giang	10°30'0.00" N, 105°10'0.01" E	Oct, 2023
2	FBF-02	Binh Duong	11°10'0.01" N, 106°40'0.01" E	Oct, 2023

3	FBF-03	Bac Lieu	9°16'60.00" N, 105°42'59.99" E	Oct, 2023
4	FBF-04	Ben Tre	10°10'0.01" N, 106°30'0.00" E	Oct, 2023
5	FBF-05	Ca Mau	9°10'36.55" N, 105°09'8.71" E	Oct, 2023
6	FBF-06	Can Tho	10°02'13.60" N, 105°47'17.70" E	Oct, 2023
7	FBF-07	Dong Nai	11°00'0.00" N, 107°10'0.01" E	Oct, 2023
8	FBF-08	Dong Thap	10°34'59.99" N, 105°37'59.99" E	Oct, 2023
9	FBF-09	Ho Chi Minh	10°46'5.99" N, 106°40'32.99" E	Oct, 2023
10	FBF-10	Hau Giang	9°46'33.78" N, 105°27'50.83" E	Oct, 2023
11	FBF-11	Long An	10°41'60.00" N, 106°10'0.01" E	Oct, 2023
12	FBF-12	Soc Trang	9°35'59.82" N, 105°58'18.95" E	Oct, 2023
13	FBF-13	Tay Ninh	11°18'36.14" N, 106°05'53.81" E	Oct, 2023
14	FBF-14	Tien Giang	10°24'0.00" N, 106°17'60.00" E	Oct, 2023
15	FBF-15	Tra Vinh	9°56'49.88" N, 106°20'32.10" E	Oct, 2023
16	FBF-16	Vinh Long	10°15'13.28" N, 105°58'19.92" E	Oct, 2023
17	FBF-17	Vung Tau	10°20'45.56" N, 107°05'3.34" E	Oct, 2023

FPF: Folium Perilla frutescens

Results and discussion

Optimization of HPLC conditions

RA with a logP of 1.82 is well-suited for reversed-phase chromatography. To enhance the separation of components, chromatographic conditions such as the mobile phase, isocratic elution process, and flow rate were optimized. Various ratios of different mobile phases were tested to evaluate their efficacy. Acetonitrile is preferred over methanol for eluting moderately polar substances from a C18 column due to its higher efficiency. Methanol's tendency to interact with water can cause heat generation and pressure fluctuations. Since rosmarinic acid has a pKa of 3.57, adding acid to the mobile phase is critical for improving chromatographic performance. The addition of acid can significantly improve resolution, regulate ionization of the analytes, and mitigate tailing effects, which are essential for achieving accurate and reproducible results. Numerous studies have demonstrated the effectiveness of using 0.1% formic acid in the mobile phase for these purposes.[5,16,17] Therefore, we decided to conduct a comprehensive investigation into the optimal mobile phase ratio, utilizing a mixture of acetonitrile and 0.1% formic acid.

Increasing the acetonitrile ratio from 20% to 30% resulted in significant changes in the chromatographic analysis of rosmarinic acid. Firstly, the rosmarinic acid peak appeared earlier in the chromatogram, leading to a shorter analysis time and saving time. Additionally, the peak became more symmetrical, and the peak area increased; however, the elution coefficient gradually decreased. At acetonitrile - 0.1% formic acid (30:70, v/v), this ratio resulted in rosmarinic acid eluting prematurely and closely to the preceding peak. Consequently, the acetonitrile - 0.1% formic acid ratio of 27:73 was selected for further analysis. This ratio provided a suitable retention time, and the parameters including the number of theoretical plates (N), capacity factor (k'), peak area asymmetry (As), and resolution (Rs) met the analytical requirements.

The pH of the mobile phase significantly influences chromatographic results by affecting analyte ionization and solubility.[7,18] To investigate the effect of pH, the research examined formic acid concentrations ranging from 0.1% to 2%. The results represented that increasing the formic acid concentration from 0.1% to 2% did not substantially alter the analysis time. However, a 1% formic acid concentration produced the most symmetric peak shape and the highest peak area. Therefore, a mobile phase consisting of acetonitrile and 1% formic acid in a 27:73 (v/v) ratio was selected.

Increasing the flow rate to 1.5 mL/min improved peak symmetry but shortened the analysis time too much, potentially causing overlap with impurity peaks. Thus, a flow rate of 1.2 mL/min was chosen to maintain a balance between peak shape and analysis time (shown in Fig 3).

In short, to separate RA from other peaks and meet the requirements for chromatographic parameters, chromatographic separation was carried out on a Phenomenex Luna C18 column (250 mm x 4.6 mm, 5 µm) from Phenomenex, USA. The mobile phase, comprised of 27% acetonitrile and 73% formic acid 0.1% (v/v), flowed at a rate of 1.2 mL/min under isocratic conditions, with detection occurring at a wavelength of 322 nm. The total run time was 7 minutes, utilizing an injection volume of 20 µL, and maintaining the analytical temperature at 25°C.

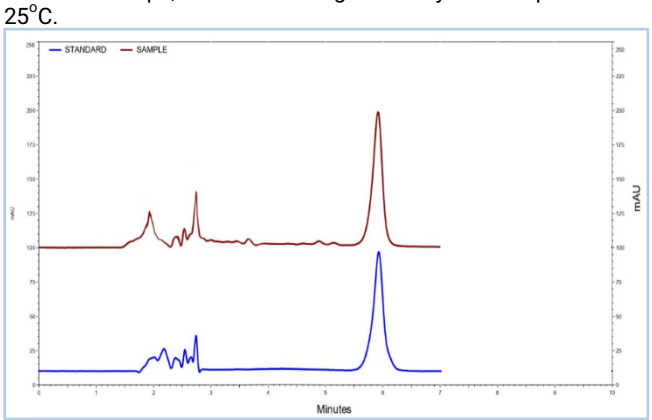


Fig 3: The chromatogram of rosmarinic acid in the optimal HPLC condition
(1) RA standard solution; (2) Perilla leaves extract with 70% ethanol

Optimization of the extraction process

In the pursuit of extracting RA from *Perilla* optimally, 70% ethanol proved superior to other solvents tested, such as 96% ethanol, 70% ethanol, and methanol, this is similar to the study of Hong et al.[19] Notably, ethanol/water mixtures showed enhanced efficacy compared to single-component solvents for phenolic extraction.[20,21] The water content in these mixtures expands plant tissue, thereby increasing the diffusion of polyphenols from plant cells.[22]

The appropriate ratio of medicinal herbs and extraction solvent volume contributes to increasing extraction efficiency as well as cost savings. When investigating different ratios of 1:20, 1:30, and 1:40 and based on the results of the total peak area of rosmarinic acid, the most appropriate ratio was chosen to be 1:30 even though the ratio. At a ratio of 1:40, the total peak area of rosmarinic acid is higher, but this difference is not significant, because the concentration of rosmarinic acid in the solvent had almost reached the saturation value (shown in Fig 4).

Another important factor is the extraction time and number of extractions. If the extraction time is prolonged, the number of extractions will decrease, but the proportion of the substance in the extract will not increase because the concentration of rosmarinic acid is nearly saturated, and free OH- radicals can be form and decompose quickly. Simultaneously, prolonging the duration of the ultrasound can facilitate absorption, dissolution, and diffusion.[23] Some other phenolic acids and impurities will dissolve more into the extract. The best extraction time of the process is 10 minutes and the extraction is repeated 3 times to extract all the rosmarinic acid from the plant.

We chose the ultrasonic extraction method and did not survey other extraction methods because many researches have shown that the ultrasonic extraction method has better RA extraction efficiency.[24,25,26] The results revealed that the optimal extraction process utilized the ultrasonic method with 70% ethanol as the extraction solvent, a sample-solvent ratio of 1:30, and extraction completed after three 10-minute cycles.

Method validation

System suitability

Validation of the method was conducted following AOAC guidelines. System suitability tests were employed to ensure the chromatographic system's resolution and repeatability met the necessary criteria for analysis. Six consecutive injections of a standard solution containing 50 µg/mL RA and sample solution were made. The results of the RA system compatibility test are displayed in Table 2, where it is evident that every quality index complies with the requirements outlined (RSD < 2%).

Specificity

According to the findings, neither the mobile phase solvent nor the sample solvent exhibited peaks at the same retention time as the standard solution, sample solution, or spiked sample. In the spiked sample, there was a notable increase in the peak height and peak area of RA. When comparing the test sample's RA peak to the reference sample, both peak purity and UV-Vis compatibility were ≥ 99%. As a result, the method's specificity was acknowledged (shown in Fig 5 and Fig 6).

Linearity of the calibration curve

The linearity test was performed to elucidate the relationship between peak area and the concentration of rosmarinic acid. A correlation coefficient (R^2) nearing one indicates strong linearity. With an R^2 value of 0.9988, the method's linearity was confirmed. In the calibration curve, a robust linear regression was observed between the peak area and the standard concentration in the range of 10-100 µg/mL. (Table 3).

Table 2: The results of the system suitability test of standard and sample solution on the analytical technique

Analyte	Standard solution	Sample solution
Retention time (min)		
Mean	5.94	6.00
RSD%	0.01	0.8
Peak area		
Mean	4438583	4774634
RSD%	0.64	0.54
Capacity factor		
Mean	4.94	5.00
RSD%	0.01	0.95
Resolution		
Mean	15.52	3.08
RSD%	0.22	1.92
Asymmetry		
Mean	0.94	0.87
RSD%	0.01	1.33
Theoretical plate number		
Mean	5343	5707
RSD%	1.95	1.38

Table 3: Linearity, LOD, and LOQ for the HPLC/PDA method validation

Linearity, LOD, and LOQ		RA
Concentration (µg/mL)		10 - 100
R^2		0.9988
Calibration curve		Y = 120220X - 244637
LOD (n =6)	Conc (µg/mL)	0.035
	S/N	3.30
LOQ (n =6)	Conc (µg/mL)	0.100
	S/N	10.21

Limit of detection – Limit of quantification

The LOD and LOQ are key parameters indicating the lowest concentration that is reliably detectable and quantifiable by the analytical method. Using signal-to-noise (S/N) analysis, the LOD and LOQ values were determined to be 0.035 µg/mL and 0.1 µg/mL, respectively. It's noteworthy that this method exhibits high sensitivity, capable of detecting rosmarinic acid concentrations lower than those reported in previous research (shown in Table 3).[27,28]

Table 4: Precision in inter-day and intra-day for the HPLC/PDA method validation

		Intra-day	Inter-day		
			Day 1	Day 2	Day 3
Concentrati	1	35.72	36.83	36.25	34.38
on (µg/mL)	2	36.36	37.20	36.80	35.08

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3	36.13	36.32	35.97	35.79
4	36.47	36.32	36.38	36.70
5	36.46	37.42	37.32	34.36
6	36.49	36.33	37.28	35.85
Mean ± SD	36.27 ± 0.30		36.25 ± 0.66	
RSD (%)	0.83		1.82	

Precision and accuracy

Six samples were analyzed in an intra-day (n = 6) and inter-day (n = 18). The RSD% values were less than those recommended by the AOAC. For inter-day precision, the RSD% was 1.82%. For intra-day precision, the RSD% was 0.83%, which was likewise less than the 5.3% threshold (shown in Table 4). To confirm accuracy, we conducted three measurements at concentrations of 29.0, 36.0, and 43.0 µg/mL, (80%, 100%, and 120%) each analyzed three times. The RSD ranged from 1.04% to 1.95%, meeting the AOAC guideline standard of 5.0%. Additionally, the recovery %, ranging from 93.95% to 97.56%, is shown in Table 5. To ensure the accuracy and repeatability of the method before evaluating application samples, we conducted validation according to AOAC guidelines, which yielded results with higher sensitivity than existing studies. After meeting the validation requirements, *Perilla* leaf samples from 17 provinces of Vietnam and samples at different growth stages were quantified and compared for RA content.

Determination of rosmarinic acid

To find the average quantity of RA, each sample was examined three times. The range of RA content is 0.20% - 2.14%, which is expressed as mg RA per 200 mg of dry material. Ho Chi Minh City has the greatest levels of RA, whereas Long An has the lowest. Certainly, Ho Chi Minh City and Vung Tau could be suitable locations with red soil rich in nutrients and appropriate pH levels for the robust growth of basil and the significant increase in rosmarinic acid content. The variations in soil and climate between provinces might be the cause of the variations in RA content. Moreover, variations in RA content may result from different cultivation techniques, timing, and harvesting processes, which is similar to some previous studies (shown in Fig 7).[29]

The concentration of rosmarinic acid undergoes significant changes throughout the plant's developmental stages, reaching its highest peak when the plant is in the flowering stage (1.35% ± 0.11), and gradually decreasing as the plant ages (0.40% ± 0.06). This correlation is further supported by prior research suggesting that concentrations peak during the flowering stage, a period marked by the plant's robust synthesis of polyphenols, which serve as potent antioxidants safeguarding the plant against insects (shown in Fig 8).[30]

The biological effects of rosmarinic acid are varied, including anti-oxidation, anti-diabetic, anti-tumor, and anti-viral, etc.[31] Among them, antioxidant properties have been linked to a decrease in control over the mobility of phenolic hydrogen atoms, which enables human cells to protect themselves from damage caused by oxygen.[32] The anti-diabetic effect is known due to RA's ability to regulate glucose homeostasis.[31]

Table 5: Accuracy for the HPLC/PDA method validation

Compound	Level	Spiked Conc (µg/mL)	Measured Conc (Mean ± SD, µg/mL)	RSD%	Recovery (Mean ± RSD%)
RA	80%	29	27.94 ± 0.42	1.52	96.37 ± 1.51
	100%	36	35.12 ± 0.69	1.95	97.56 ± 1.96
	120%	43	40.4 ± 0.42	1.04	93.95 ± 1.04

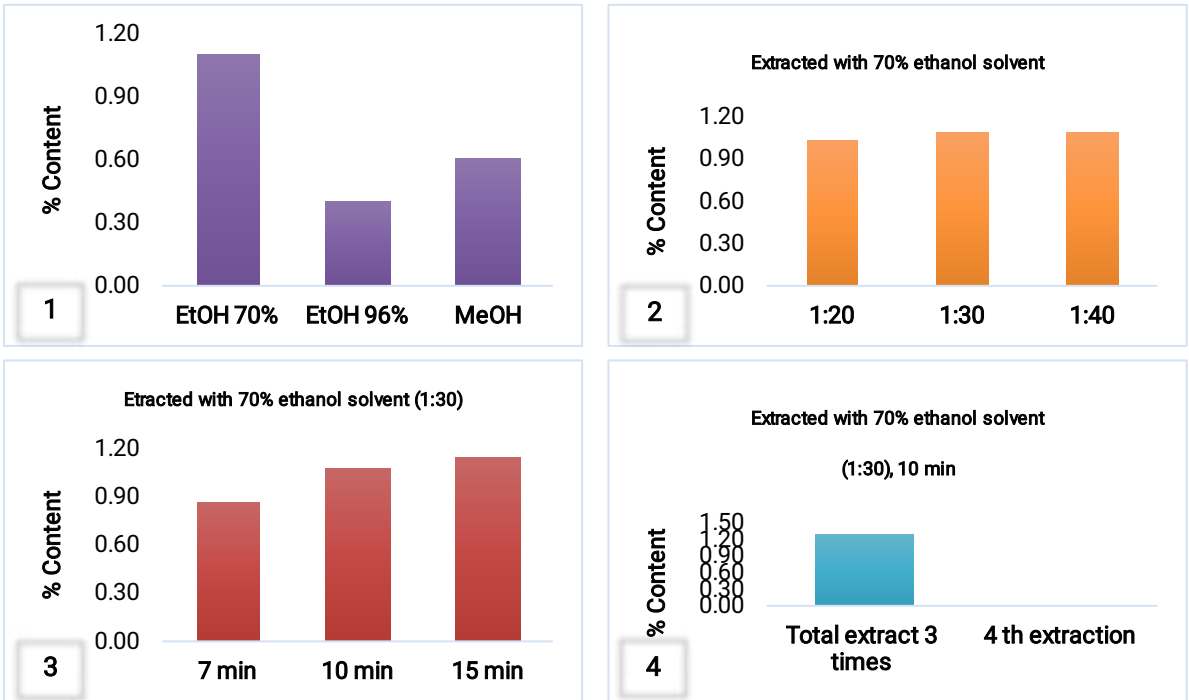

Fig 4: Rosmarinic content was obtained during the optimization of extraction conditions
(1) Extraction solvent; (2) Sample-solvent ratio; (3) Extraction time; (4) Number of extractions

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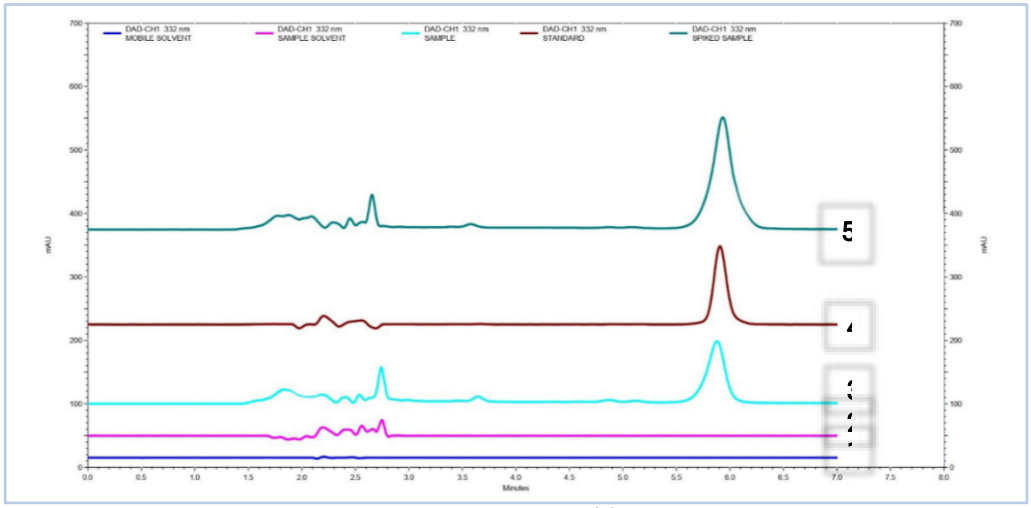

Fig 5: The chromatogram of five samples
(1) Mobile phase solvent; (2) Sample solvent; (3) *Perilla* leaves extract with 70% ethanol
(4) RA standard solution; (5) Spiked sample

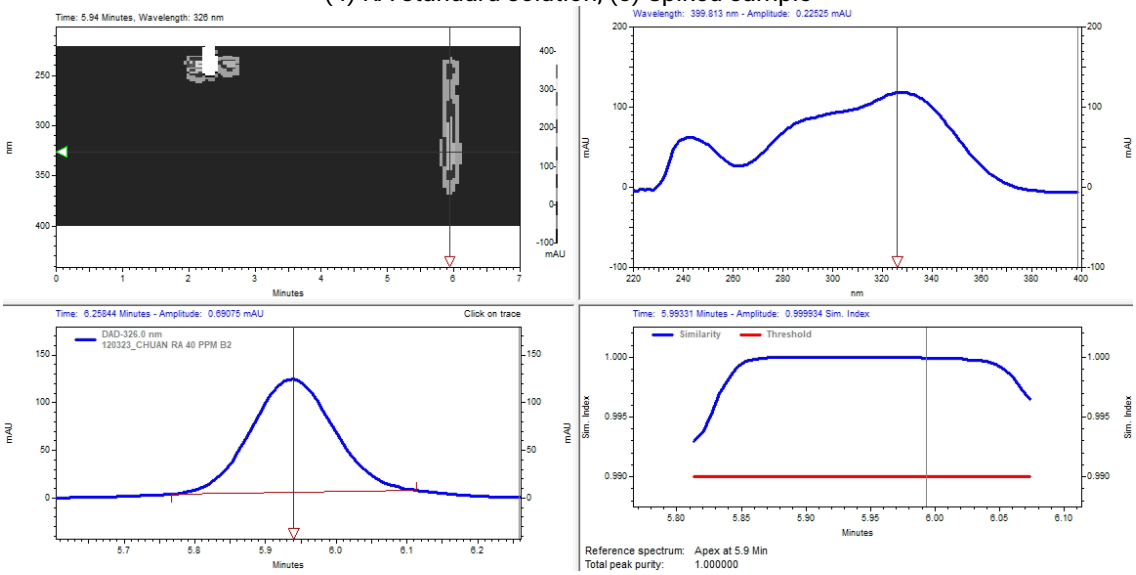

Fig 6: UV spectrum and peak purity of *Folium Perilla frutescens* extract

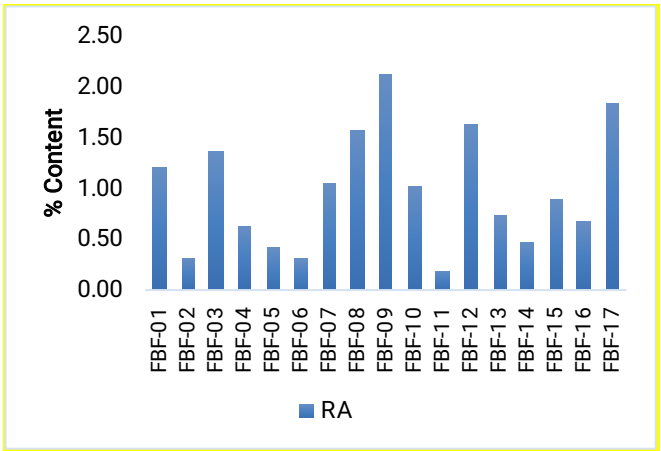

Fig 7: Content of rosmarinic acid in 17 samples from provinces in Vietnam

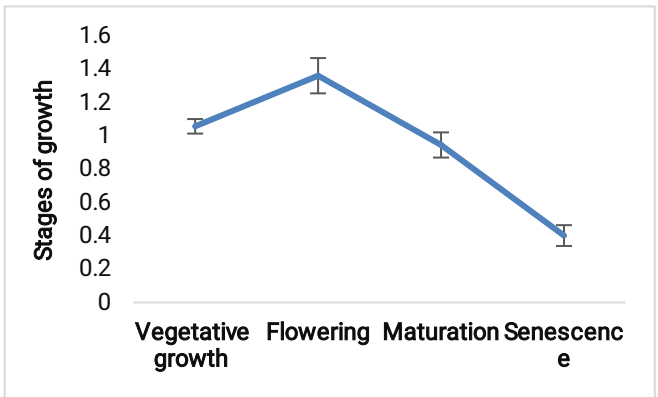

Fig 8: Content of rosmarinic acid in leaves based on stages of growth

Conclusion

The extraction process utilizes non-toxic organic solvents, with

each extraction lasting 10 minutes. The 70% ethanol, considered safer for human health compared to other organic solvents, is used. This extraction method is suitable for developing health products like standardized Perilla extract containing rosmarinic acid (RA). The method demonstrated system suitability, specificity, accuracy, precision, LOD, LOQ, and linearity across the 10-100 µg/mL concentration range. The method's key benefits include speed and ease of sample analysis, alongside compatibility with commonly used equipment. This method forms the foundation for creating new products and could effectively be used in the quality control of health supplements containing rosmarinic acid. Given its diverse biological effects, determining the RA content in different provinces and growth stages provides valuable information for selecting high-quality raw material sources. This, in turn, helps optimize the utilization of *Perilla* medicinal resources in developing products related to human health. Moreover, this method can be broadly applied to compare differences in RA content in *Perilla* leaves across different countries, or to explore other factors potentially influencing RA content such as leaf color, harvesting time of day, and storage conditions.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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