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QUALITY EVALUATION OF *PIPER BETLE* L. CULTIVATED IN VIETNAM BY HPLC-DAD METHOD

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Summary

Quality Evaluation of *Piper betle* L. Cultivated in Vietnam by HPLC-DAD Method

Piper betle L. is a popular traditional herbal medicine. This plant has several potential health benefits, including antioxidant, anti-inflammatory, and antimicrobial properties. The main active ingredient in *Piper betle* is hydroxychavicol. In this study, the HPLC-DAD method was applied to quantify hydroxychavicol in leaves and branches of *Piper betle*. This method uses a C_{18} column (250 x 4.6 mm, 5 µm), mobile phase includes acetonitrile and 0.05% phosphoric acid (30:70, v/v), flow rate of 0.5 mL/min, and sample injection volume of 10 µL. The HPLC-DAD method was validated according to ICH guidelines, the results showed that this method has high specificity, achieves linearity, achieves repeatability with RSD = 1.29%, and achieves accuracy with recovery rates of 97-103%. The method was applied to evaluate the hydroxychavicol content in *Piper betle* samples collected in several regions in Vietnam. The results showed that the hydroxychavicol content in *Piper betle* leaves was higher than in *Piper betle* branches. *Piper betle* leaves samples contain approximately 4.55-6.88% hydroxychavicol: highest in the sample collected in Thanh Hoa (6.88%) and lowest in the sample collected in Nam Dinh (4.55%). *Piper betle* branches contain only 0.57-1.68% hydroxychavicol. The results obtained are intended to contribute to standardizing the quality of *Piper betle* in Vietnam.

Keywords: *Piper betle* L., *Hydroxychavicol, HPLC-DAD, Quality control.*

1. Introduction

Piper betle is a well-known medicinal plant cultivated primarily in Southeast Asia. This plant comprises many bioactive compounds such as tannins, flavonoids, eugenol, hydroxychavicol, and chavibetol, representing the major components of the plant. This plant has been extensively studied for its pharmacological properties such as antibacterial, anticancer, antioxidant, antidiabetic, and anticancer [1],[2]. In particular, the antifungal and antibacterial effects of *Piper betle* have been published by many studies [3],[4],[5]. Ethanol extract from *Piper betle* leaves (with the main ingredient hydroxychavicol) have antifungal effects against *Malassezia furfur* and *Candida spp*. In addition, the hot water extract from *Piper betle* leaves has antibacterial effects against *C. albican*, *E. coli*, and *S. aureus*. Many studies confirm that the biologically active ingredient in *Piper betle* is hydroxychavicol [6]. In Vietnam, *Piper betle* is currently often used in decoction or extract form.

However, Vietnam Pharmacopoeia V, Chinese Pharmacopoeia 2020, and Hong Kong Chinese Materia Medica Standards have not specified quality standards for this herb [7],[8],[9]. In 2022, Phan Thi Thanh Thuy developed an HPLC-UV method to quantify hydroxychavicol in dichloromethane extract from *Piper betle* [10]. This study aims to develop the HPLC-DAD method to quantify hydroxychavicol in leaves and branches of *Piper betle* and evaluate the hydroxychavicol content in some *Piper betle* samples collected in different regions in Vietnam.

2. Experimental

2.1. Materials

The research object is *Piper betle* samples collected from several regions in Vietnam such as Nam Dinh, Thai Nguyen, Thanh Hoa, Hanoi, and Hung Yen provinces. In Thai Nguyen, *Piper betle* samples were collected in four different seasons of the year. Samples were collected from the aboveground part of the *Piper betle* plant, then washed and removed impurities, and divided into separate parts (leaves and branches). After being collected, the samples were dried at 50°C, then about 30 g of dry sample was taken and ground into a fine powder to obtain a test sample for research. Sample information is presented in Table 1.

Table 1. Information on *Piper betle* samples

No.	Sample code	Origin
1	TK-1	Nam Dinh
2	TK-2	Quang Thanh, Thanh Hoa
3	TK-3	Thanh Tri, Ha Noi
4	TK-4	Khoai Chau, Hung Yen
5	TK-5-Spring	Thai Nguyen
6	TK-5-Summer	Thai Nguyen
7	TK-5-Autumn	Thai Nguyen
8	TK-5-Winter	Thai Nguyen

2.2. Chemicals

Hydroxychavicol (CAS 1126-61-0, Lot#F2301077, 98%, 50 mg) was purchased from Alladin (China). Methanol and acetonitrile of HPLC grade were purchased from Merck (Germany). All other solvents were of analytical grade.

2.3. Instrument and Chromatographic Conditions

The HPLC system (Shimadzu, Japan) used for the analysis consisted of a binary pump LC-30AD, a SIL-30AC autosampler, a CTO-10AS column oven, and a diode-array detector (SPD-M20A). Quantitative estimation was performed with LabSolutions software programs. The separation of compounds was performed on an Agilent C_{18} column (250 x 4.6 mm, 5 µm). The mobile phase was a mixture of acetonitrile and 0.05% phosphoric acid (30 - 70 %, v/v). The solvent flow rate was kept at 0.5 mL/min. The injection volume was 10 µL. Detection was set at a wavelength of 281 nm [10].

2.4. Preparation of standard solutions

Standard solutions of hydroxychavicol were prepared by dissolving the primary standard in ethanol. The stock solution was then diluted to establish a calibration curve in the range of 0.12 - 4.8 mg/mL for hydroxychavicol. The stock and working solutions were stored at 4°C.

2.5. Preparation of sample solution

Weigh accurately 1.000 g of the powdered sample and extract with 50 mL of ethanol by maceration method for 30 min. The solution was further cooled and filtered to get ethanolic extract. Filter through a 0.45 µm filter to get the sample solution and use for further analysis [12].

2.6. Validation method

The analytical method was developed and validated for system suitability, specificity, calibration curve, accuracy, precision, detection limit, and quantitation limit following the current ICH guidelines [11].

2.7. Calculation

The content of hydroxychavicol in the *Piper betle* sample was calculated as follows:

$$X(\%) = \frac{C_t \times V \times 100}{m \times (100 - a)} \times 100$$

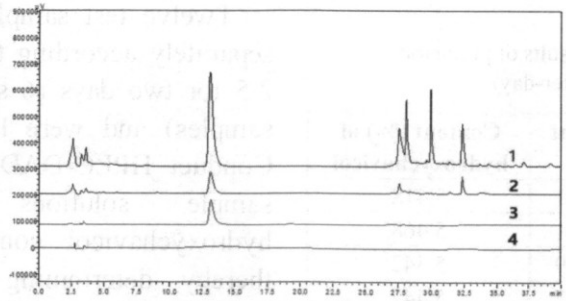
where C_t: the concentration of analyzed compounds in the sample solution from the calibration curve equation (mg/mL); V: volume of the sample solution (mL); m: weight of sample taken to prepare the sample solution (mg); a: the moisture of sample (%).

3. Results and Discussion

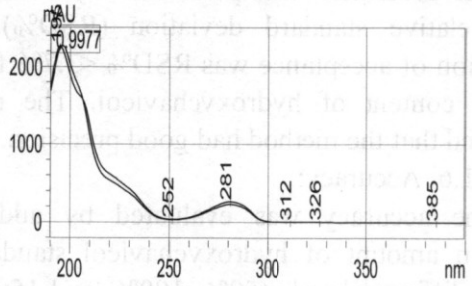
3.1. Validation HPLC-DAD method for quantification of hydroxychavicol in Piper betle samples

3.1.1. Specificity:

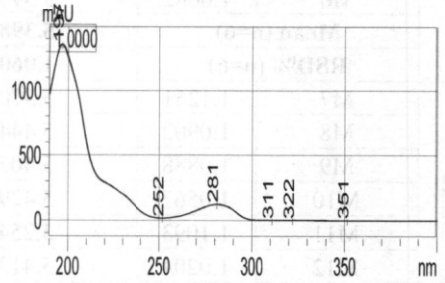
The experiment was performed with the blank, standard, and *Piper betle* sample solutions according to the analytical procedure. The retention time of hydroxychavicol was 13.1 min. The peak of the analyzed compound was confirmed by comparing the retention time and UV spectrum of hydroxychavicol in standard and sample solutions. The obtained results are shown in Fig. 1 and Table 1.



HPLC-DAD chromatograms of the blank, standard, and *Piper betle* sample solutions
(1-Piper betle leaves; 2-Piper betle branches; 3-hydroxychavicol; 4-blank sample)



UV spectrum of hydroxychavicol peak in standard and *Piper betle* leaves samples



UV spectrum of hydroxychavicol peak in standard and *Piper betle* branches samples

Fig. 1. The results of specificity

Table 1. Parameters of analyzed peak (hydroxychavicol)

	hydroxychavicol
Retention time (t_R, min)	13.15
Resolution (Rs)	2.567
Tailing factor (T_f)	1.124
Number of Theoretical Plates (N)	42989
Purity of peak	0.999

3.1.2. Linearity:

Table 2. Linearity study data

No.	**Concentration (mg/mL)**	**Peak area (mAU.s)**	**The bias (%)**
1	4.8	46553201	3.14
2	2.4	23791292	1.13
3	1.2	12112845	-0.42
4	0.48	4496766	7.63
5	0.24	2255218	8.66
6	0.12	1123485	11.62

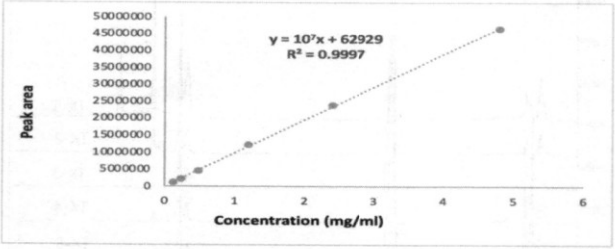


Fig. 2. The calibration curve of hydroxychavicol

The calibration curve of hydroxychavicol was established by using the peak area (y) as the vertical axis and the concentration of the analyte (x) as abscissa, respectively. Linear correlation coefficient (R^2) values were above 0.999, as well as each calculated concentration of each calibration point was within ± 15% of the true value (Table 2 and Fig. 2). Therefore, it can be concluded that this developed method conforms to the linearity.

3.1.3. LOD and LOQ:

The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were calculated by diluting sample solutions until signal-to-noise ratios of 3 and 10, respectively.

LOD and LOQ were estimated to be 0.11 and 0.33 µg/mL for hydroxychavicol.

3.1.4. System suitability:

Table 3. System suitability testing (n = 6)

No.	**Retention time, t_R (min)**	**Peak area (mAu.s)**
1	13.168	1123485
2	13.126	1062842
3	13.201	1090751
4	13.192	1083189
5	13.181	1083635
6	13.178	1108659
Mean	**13.17**	**1092093.5**
RSD	**0.20**	**1.95**

HPLC-DAD analyses were repeated six times with the same standard solution of hydroxychavicol 0.12 mg/mL. It can be observed from Table 3 that the RSD% of retention time and peak area were all under 2%. These indicate that the developed method conforms to system suitability criteria.

3.1.5. Precision:

Table 4. The evaluated results of precision (intra-day and inter-day)

Time	code	Weight (g)	Content (%) of hydroxychavicol
Day 1	M1	1.0501	5.418
	M2	1.0001	5.468
	M3	1.1709	5.342
	M4	1.0562	5.447
	M5	1.0712	5.324
	M6	1.0082	5.390
	Mean (n=6)		**5.398**
	RSD% (n=6)		**1.060**
Day 2	M7	1.1251	5.370
	M8	1.0902	5.444
	M9	1.0888	5.407
	M10	1.0567	5.429
	M11	1.1093	5.254
	M12	1.0204	5.413
	Mean (n=12)		**5.386**
	RSD% (n=12)		**1.283**

Twelve test sample solutions were prepared separately according to the procedure in section 2.5 for two days (6 samples/day x 2 days = 12 samples) and were labeled from M1 to M12. Conduct HPLC-DAD analysis of these 12 test sample solutions and calculate the hydroxychavicol content in the test sample, thereby determining intra-day and inter-day accuracy. The results are presented in Table 4.

The evaluation was performed by calculating the relative standard deviation (RSD%). The criterion of acceptance was RSD% <3.7% for the mean content of hydroxychavicol. The results showed that the method had good precision.

3.1.6. Accuracy:

The accuracy was evaluated by adding a known amount of hydroxychavicol standard at three different levels (50%, 100%, and 150%) to the sample.

Table 5. The results of evaluating the accuracy (n=3)

No.	Code sample	Weight (g)	The added concentration of hydroxychavicol (mg/mL)	Peak area (mAu.s)	The recovered concentration of hydroxychavicol (mg/mL)	Recovery rate (%)	Mean ±SD (%)
1	Non-spiked sample	1.0501	0	11440965			
2	50%-spiked sample (1)	1.0887	0.55	17669657	**0.548**	99.7	98.5 ± 1.1
3	50%-spiked sample (2)	1.0662	0.55	17236587	**0.541**	98.4	
4	50%-spiked sample (3)	1.0988	0.55	17703053	**0.536**	97.4	
5	100%-spiked sample (1)	1.0078	1.10	21374010	**1.071**	97.3	97.8 ± 1.2
6	100%-spiked sample (2)	1.0151	1.10	21484240	**1.066**	96.9	
7	100%-spiked sample (3)	1.0047	1.10	21493387	**1.090**	99.1	
8	150%-spiked sample (1)	1.0759	1.65	28991369	**1.673**	101.4	101.1 ± 0.8
9	150%-spiked sample (2)	1.0736	1.65	28732155	**1.654**	100.3	
10	150%-spiked sample (3)	1.0217	1.65	27572282	**1.677**	101.7	

The results in Table 5 showed that the recovery rates of hydroxychavicol were in the range of 97% - 103%. The results indicated that the accuracy of the developed method was acceptable.

3.2. Analysis of samples

Quantitative analysis of hydroxychavicol in *Piper betle* samples according to the HPLC-DAD method presented above, the results are presented in Fig. 3, Fig. 4 and Table 6, Table 7.

The results showed that the hydroxychavicol content in *Piper betle* leaves was significantly higher than that in *Piper betle* branches. *Piper betle* leaves samples contain approximately 4.55-6.88% hydroxychavicol: highest in the sample collected in Thanh Hoa (6.88%) and lowest in the sample collected in Nam Dinh (4.55%). *Piper betle* branches contain only 0.57 - 1.68% hydroxychavicol.

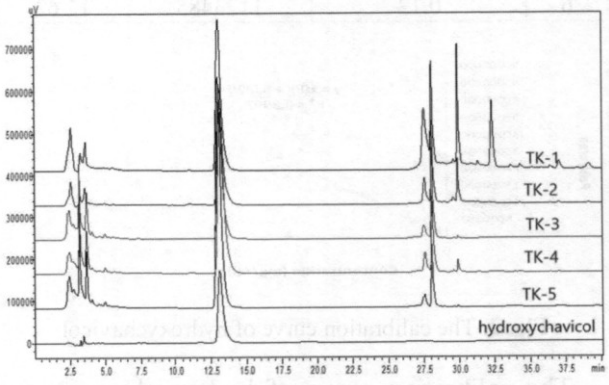


Fig. 3. HPLC chromatogram of *Piper betle* leaves samples collected in different regions

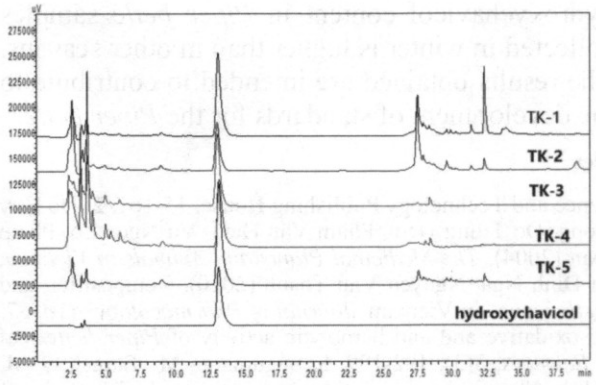


Fig. 4. HPLC chromatogram of *Piper betle* branches samples collected in different regions

Table 6. Hydroxychavicol content in *Piper betle* samples collected in different regions

Sample code	Origin	Content (%) of hydroxychavicol	
		***Piper betle* leaves**	***Piper betle* branches**
TK-1	Nam Dinh	4.55 ± 0.03	1.03 ± 0.05
TK-2	Thanh Hoa	6.88 ± 0.05	0.57 ± 0.06
TK-3	Ha Noi	5.27 ± 0.02	1.05 ± 0.02
TK-4	Hung Yen	4.91 ± 0.05	0.83 ± 0.05
TK-5	Thai Nguyen	6.22 ± 0.06	1.68 ± 0.04

Table 7. Hydroxychavicol content in *Piper betle* samples collected in four different seasons

Sample code	Content (%) of hydroxychavicol	
	***Piper betle* leaves**	***Piper betle* branches**
TK-5-Spring	5.61 ± 0.03	1.12 ± 0.02
TK-5-Summer	6.07 ± 0.02	1.40 ± 0.03
TK-5-Autumn	5.98 ± 0.04	1.46 ± 0.03
TK-5-Winter	6.21 ± 0.06	1.68 ± 0.04

The results showed that hydroxychavicol content in *Piper betle* samples collected in Thai Nguyen according to four seasons in a year was different, in which *Piper betle* samples collected in spring had the lowest hydroxychavicol content (5.61% in leaves and 1.12% in branches), *Piper betle* samples collected in winter had the highest hydroxychavicol content (6.21% in leaves and 1.68% in branches).

4. Discussion

In Vietnam, *Piper betle* is grown across the country from North to South, so the origin and quality of this medicinal herb are also very diverse. Vietnam Pharmacopoeia V does not have a monograph on this medicinal herb. However, many studies have been published on the chemical composition and biological effects of *Piper betle*, of which some studies have confirmed that hydroxychavicol is the main ingredient and related to the specific pharmacological effects of *Piper betle*. Therefore, evaluating the hydroxychavicol content in *Piper betle* is necessary and important to standardize the quality of *Piper betle*.

Phan Thi Thanh Thuy developed an HPLC-DAD method to quantify hydroxychavicol in dichloromethane fractional extracts from *Piper betle* leaves and applied the method to quantify hydroxychavicol in six *Piper betle* extract samples [10]. This method cannot be applied to quantify hydroxychavicol in *Piper betle* leaves because of differences in sample matrices. In this study, chromatographic conditions were referenced from this study [10] including mobile phase and flow rate. The column HPLC C_{18} (250 x 4.6 mm, 5 µm) was chosen because this column was more popular than the column HPLC C_{18} (250 x 2.1 mm, 5 µm). The selected wavelength was 281 nm which was more specific to hydroxychavicol than the wavelength of 210 nm (Fig. 1). The retention time of hydroxychavicol is 13.17 minutes and is suitable for application in the analysis of medicinal sample matrices. In particular, the HPLC-DAD method was validated on *Piper betle* leaf samples, so this method is suitable and can be applied to evaluate hydroxychavicol content in *Piper betle* samples collected in Vietnam. Currently, there is no published research on hydroxychavicol content in leaves and branches of *Piper betle* collected in Vietnam. The results of this study ensure novelty and provide a lot of useful information to develop quality standards for *Piper betle* in Vietnam.

The leaves and branches of *Piper betle* contain large amounts of hydroxychavicol and the leaves contain higher hydroxychavicol content than the branches. Hydroxychavicol is the main ingredient in *Piper betle* and meets the requirements for use as a marker according to WHO guidelines [13]. This result suggests the selection of the leaves as raw material for developing products from *Piper betle*. Initial survey results show that the hydroxychavicol content in *Piper betle* samples collected in winter is higher than that in other seasons. However, it is necessary to survey several more growth cycles and collect samples in several different regions to have accurate conclusions about this trend.

5. Conclusion

In this study, we used the HPLC-DAD method to evaluate the hydroxychavicol content in leaves and branches of *Piper betle* collected in some regions in Vietnam. The results obtained show

that hydroxychavicol is the main ingredient in *Piper betle*, with a wide range of content ranging from 4.55-6.88% in *Piper betle* leaves and 0.57-1.68% in *Piper betle* branches. In addition, the hydroxychavicol content in *Piper betle* samples collected in winter is higher than in other seasons. The results obtained are intended to contribute to the development of standards for the *Piper betle*.

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PRELIMINARY RESEARCH ON ESTABLISHING THE QUALITY STANDARDS FOR ALCOHOL-PROCESSED *RADIX ACHYRANTHIS BIDENTATAE*

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Summary

Preliminary Research on Establishing the Quality Standards for Alcohol-Processed *Radix Achyranthis bidentatae*

In this study, a foundational standard for the herbal medicine *Achyranthes bidentata* Blume, commonly known as "Hoai nguu tat," processed with alcohol, is developed. The evaluation criteria encompass qualitative organoleptic assessment, moisture content, total ash content, and extractable substances in the herbal material, which collectively contribute to establishing a quality benchmark for *Achyranthes bidentata* products available in the market. The study results also provide recommendations for upgrading the monograph of *Radix Achyranthes bidentata* in the Vietnamese Pharmacopoeia. The quantitative analysis specifies that the content should be no less than 2% for oleanolic acid and 0.038% for β-ecdysterone, with a moisture content not exceeding 7%, total ash content not exceeding 8%, and extractable substances not less than 7%.

Keywords: *Oleanolic acid, Achyranthes bidentata* Blume, *β-ecdysterone.*

1. Introduction

Traditional medicine is currently experiencing significant growth and being favored due to its proven therapeutic efficacy. The processing methods of medicinal herbs and traditional medicines greatly influence their chemical compositions and therapeutic effects. Thus, applying standards meant for unprocessed herbs to traditionally processed medicines is not entirely appropriate. Consequently, standardization of traditional medicinal materials has been addressed in several national pharmacopoeias. However, in the Vietnamese Pharmacopoeia V, only specific processed herbal monographs like "Dang sam" and "Che thuc dia" are presented, while others are lacking. Therefore, standardizing the raw materials is the most fundamental step.

Achyranthes bidentata Blume, also known as "hoai nguu tat" in traditional medicine, is characterized by its neutral property and bitter-sour