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UHPLC-UV method validation for simultaneous quantification of vitexin and isovitexin from *Santalum album* L. leaves

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Abstract. *Santalum album* L. is a precious medicinal herb with high economic value and has been extensively cultivated in Vietnam in recent years. Studies have revealed that the leaves contain two main active ingredients vitexin and isovitexin, which have demonstrated significant potential in treating diabetes, cancer, and inflammation. To contribute to the standardization of the title medicinal herb and its formula, a simple, fast precise and selective method for the simultaneous quantification of vitexin and isovitexin using ultra-performance liquid chromatography (UHPLC) has been developed and validated. The quantification procedure was performed on a Hypersil GOLD aQ Column (3 μm; 150 × 2.1 mm) at 35°C, with a mobile phase of acetonitrile (A) and and water with 0.1% formic acid (B), a flow rate of 0.3 mL/min, a detection wavelength of 336 nm, and an injection volume of 3 μL. The gradient program was set to 0.0-15.0 minutes, transitioning from 5% to 35% A, and 15.0-20.0 minutes, transitioning from 35% to 5% A. Validation of the quantification procedure, following ICH Q2 (R2) guidelines, demonstrated that the method achieved specificity, accuracy, precision, and linearity, with a high correlation between the peak area and the concentrations of vitexin and isovitexin (R^2 values of 0.9998, respectively). Thus, the developed method can be utilized to determine the content of vitexin and isovitexin in *Santalum album* L. leaves, contributing to the standardization of medicinal herbs.

Keywords: Vitexin, Isovitexin, *Santalum album* L., UHPLC, ICH guidelines

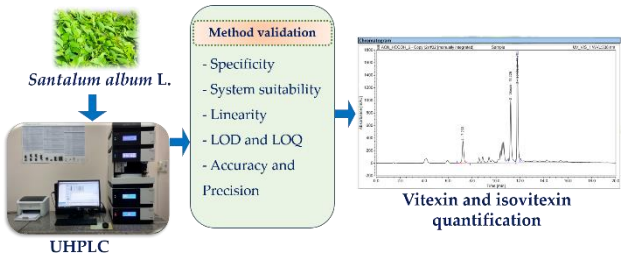
1 Introduction

White sandalwood (*Santalum album* L.) is a precious tree species with a valuable woody trunk, primarily distributed in countries such as India, Sri Lanka, Indonesia, Australia, Timor, Hawaii, and others [1]. In recent years, white sandalwood trees have been selectively bred and cultivated in various mountainous provinces of South Central and the Central Highlands in Vietnam. Presently, the primary purpose of cultivating white sandalwood is for harvesting wood and extracting essential oil from trees aged between 10 and 30 years. Moreover, young leaves from trees aged 3 years and older are utilized to make tea. However, in Vietnam, there has been limited research focusing on utilizing the leaves to extract active ingredients for medicinal applications. Notably, previous studies have revealed that the leaves contain two highly valuable active compounds: vitexin and isovitexin [2]. These two substances have demonstrated significant potential in the treatment of diabetes, cancer, and inflammation [1], [3].

Diabetes mellitus is a chronic, metabolic disease characterized by elevated levels of blood glucose. This ailment can result in severe complications impacting diverse bodily organs and systems, including cardiovascular disorders such as heart attacks and strokes, kidney impairment known as nephropathy, vision-

related issues termed retinopathy, nerve damage referred to as neuropathy, and complications involving the feet [4]. Given the rising global prevalence of diabetes [5], there is an urgent need for research into drugs and functional foods to support diabetes treatment. Interestingly, vitexin and isovitexin have the capacity to lower blood sugar levels, prevent damage to pancreatic β-cells, reduce weight gain, mitigate cardiovascular complications, and expedite wound healing [6]. Researchers also indicate that vitexin and isovitexin extracted from plants exhibit blood glucose-lowering effects with no signs of toxicity at their effective or highest evaluated doses [7]. Consequently, with its primary active ingredients being vitexin and isovitexin, SAL represent a promising source of medicinal components, aligning with the trend of harnessing natural compounds for pharmaceutical development. To utilize these components as medicine, it is essential to determine the precise content of active ingredients in medicinal herbs. Up to date, there has no procedure for simultaneous quantification of vitexin and isovitexin in *Santalum album* L. Leaves (SAL) both domestically and internationally yet.

Ultra High-Performance Liquid Chromatography (UHPLC) employs columns with small particles and operates at elevated pressures, significantly reducing retention times compared to High-Performance Liquid Chromatography. Although the high pressures involved, separation efficiency is maintained or improved. The technique's ability to significantly shorten analysis times while maintaining quality makes it a valuable tool in analytical chemistry [8]. In light of this, the present study will take advantage of UHPLC-UV for developing and validating a simple, rapid, sensitive and reliable method for the simultaneous quantification of vitexin and isovitexin in SAL. To our knowledge, this is the first report on the simultaneous analysis of those compounds in SAL by UHPLC-UV. This research contributes to the standardization of medicinal herbs for pharmaceutical applications.



Scheme 1. A scheme of this study

2 Materials and Methods

2.1 Materials and chemicals

Santalum album leaves were collected in Buon Don district, Dak Lak province in September 2022. The collected samples were dried to a constant weight, packed in polyethylene bags, and stored at temperatures ranging from 0 to 4 °C until the extraction process.

The extraction solvent utilized was methanol (MeOH), meeting analytical purity standards. The solvents and chemicals employed in the UHPLC-UV analysis included methanol (MeOH), acetonitrile (ACN), formic acid (Merck, Germany), and double-distilled water. The vitexin and isovitexin standards used were sourced from Sigma-Aldrich, USA, with a purity exceeding 99.8%.

2.2 Sample preparation

To maximize the liberation of vitexin and isovitexin derivatives, an ultrasound-assisted extraction method using methanol solvent was employed [9], [10]. The dried samples were first pulverized and sieved to obtain uniformly sized SAL powders. Next, 5 g of powder was soaked in methanol for 2 minutes. Subsequently, it was sonicated for 20 minutes at 28 kHz (Vietsonic, VS28H, Vietnam) using a micro-tipped probe (10

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mm in diameter) immersed 1 cm into the solution. After sonication, the extracts were centrifuged at 15,000 × g for 15 minutes, and the supernatants were filtered through Whatman filter paper [11]. Following this, the extract was concentrated using a rotary evaporator system (Laborota 4000, Heidolph, Germany). The resulting residue was then dissolved in methanol and filtered through a 0.45 μm Millipore membrane filter before being injected into the UHPLC system.

2.3 Vitexin and isovitexin standard solutions

Accurately weigh 2.2 mg of the standard substance, then, mix the weighed standard substance with MeOH in a 10 ml volumetric flask. This mixture will yield a stock standard solution with a concentration of 220 μg/mL. Prepare working standard solutions by diluting the stock standard solution with MeOH. This dilution will create a concentration range suitable for establishing the standard curve in the UHPLC-UV method.

2.4 Development of UHPLC-UV Method

The samples were analyzed using a UHPLC-UV system (Thermo Ultimate 3000, USA) with a a Hypersil GOLD aQ Column (3 μm; 150 mm L × 2.1 mm ID). The analytical method was developed to select best chromatographic conditions for simultaneous quantification of vitexin and isovitexin. Based on several pieces of literature, the factors including mobile phase, flow rate, and column temperature were investigated.

Mobile phase

An investigation was conducted into different mobile phases, including methanol and acetonitrile (as Channel A solvents), and formic acid 0.1%, acetic acid 0.1%, and phosphoric acid 0.1% (as Channel B solvents). The flow rate remained at 0.3 mL/min, with each injection volume at 3 μL. The column temperature was maintained at 35 °C and detection occurred at awavelength of 336 nm. The objective was to select the most suitable mobile phase conditions based on specific criteria: achieving good separation peaks (Rs ≥ 1.5), obtaining balanced and minimal tailing effects (0.8 ≤ As ≤ 1.5), and ensuring a short total analysis time.

Flow rate

Different flow rates (0.2, 0.3, and 0.4 mL/min) were investigated while keeping the sample and mobile phase conditions constant. The purpose was to reduce the analysis time while still meeting the specified criteria.

Column temperature

The test sample was analyzed under the same mobile phase conditions, and selected flow rates at three different column temperatures: 30°C, 35°C, and 40°C. This step aimed to identify the most appropriate column temperature based on predefined criteria.

2.5 Validation of UHPLC-UV Method

The analytical method was evaluated for specificity, system suitability, linear range, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision according to the instructions of the ICH Q2 (R2) guideline [12].

Specificity

Conduct spectral scanning of various solutions, including blank solutions, vitexin standard samples, isovitexin standard samples, and sandalwood leaf test samples. This helps verify that the chromatographic method can differentiate between different compounds and provides evidence of the analytes' presence in the samples.

System suitability

System suitability was assessed by conducting six replicates of chromatography under the chosen chromatographic conditions. This step assesses the stability and consistency of the chromatographic system, columns, and conditions via assessing parameters like retention time, peak area, tailing coefficient, number of theoretical plates

Linearity

Establish the linearity by preparing a series of standard solutions with known concentrations of vitexin and isovitexin. Perform UHPLC-UV analysis to generate chromatograms. Calculate peak areas (y) and establish linear regression equations, demonstrating the relationship between peak area and analyte concentration (x). Linearity was found acceptable by demonstrating the correlation coefficient (R^2) was greater than 0.998.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Dilute samples and conduct chromatographic analysis. Record the response signal of the analyzed sample (S-Signal). Perform blank sample chromatography and record the signal (N-Noise). Calculate the signal-to-noise ratio (S/N). The LOD is considered reached when S/N = 3, and the LOQ is considered reached when S/N = 10. Determine the LOD and LOQ for vitexin and isovitexin. These values represent the lowest concentrations of the compounds that can be reliably detected and quantified, respectively.

Accuracy and Precision

Spike known amounts of vitexin and isovitexin into plant samples at different concentrations (low, medium and high) and analyze them using the UHPLC-UV method. At each level, repeat the analysis three times and calculate the average recovery (Eqs. 1) for all three levels to assess accuracy. Similarly, calculate the standard deviation (RSD) for intra-day precision, which can be computed using Eqs. 2.

$$\text{Recovery\%} = \frac{\text{Amount found} - \text{Amount sample}}{\text{Amount standard spiked}} \times 100 \quad (1)$$

$$\text{RSD\%} = \frac{\text{Standard deviation}}{Mean} \times 100 \quad (2)$$

For inter-day precision, inject three replicates of each concentration level of the standards for two consecutive days, maintaining the same concentration levels of vitexin and isovitexin, then, calculate RSD for each precision level.

Statistical Analysis

The values were presented as the mean ± standard deviation, and Microsoft Excel was utilized to process the data.

3 Results and Discussion

3.1 Developed UHPLC-UV Method

The optimized conditions were chosen based on the best chromatographic conditions, ensuring that vitexin and isovitexin eluted with symmetrical peak shapes, suitable resolution, and analysis time. The results were showed in Table 1.

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Table 1. Result of method optimazation

No.	Mobile phase	Flow rate (mL/min)	Column temperature (°C)	Observation	Result
1	ACN : H_3PO_4 0.1%	0.3	35	Poor resolution	Method rejected
2	ACN : HCOOH 0.1%	0.3	35	Good resolution	Method accepted
4	ACN : CH_3COOH 0.1%	0.3	35	Poor resolution	Method rejected
5	ACN : HCOOH 0.1%	0.2	35	Poor resolution	Method rejected
6	ACN : HCOOH 0.1%	0.4	35	Poor resolution	Method rejected
7	ACN : HCOOH 0.1%	0.3	30	Poor resolution	Method rejected
8	ACN : HCOOH 0.1%	0.3	40	Poor resolution	Method rejected

The first parameter investigated was the combination of different mobile phase compositions. A combination of Acetonitrile (A) and water with 0.1% formic acid (B) was determined to be the most suitable mobile phase. Therefore, this combination was selected for the next stage. At flow rates of 0.2 and 0.4 mL/min, it was not possible to effectively separate the vitexin and isovitexin peaks. By contrast, a flow rate of 0.3 mL/min provided good separation capabilities for vitexin and isovitexin peaks. Additionally, various chromatographic column temperatures, including 30°C, 35°C, and 40°C, were tested to improve chromatographic resolution. As a result, a column temperature of 35°C produced peaks with more optimal parameters in terms of resolution.

In conclusion, the optimal conditions involve a mobile phase consisting of two components: Acetonitrile (A) and water with 0.1% formic acid (B). The flow rate and injection volume were set at 0.3 mL/min and 3 μL, respectively. The total analysis time is 20 minutes, the column temperature was maintained at 35°C, and detection was conducted at a wavelength of 336 nm. The gradient program was set to 0.0-15.0 minutes, transitioning from 5% to 35% A, and 15.0-20.0 minutes, transitioning from 35% to 5% A. Under these conditions, the obtained peaks have good resolution, high, stable and well-balanced shapes, and require reasonable analysis time.

3.2 Validation of Developed UHPLC-UV Method

Specificity

The blank solutions, vitexin standard sample, isovitexin standard sample, and SAL test sample were analyzed using the specified chromatographic conditions. The resulting chromatograms are presented in Figure 1 and Figure 2.

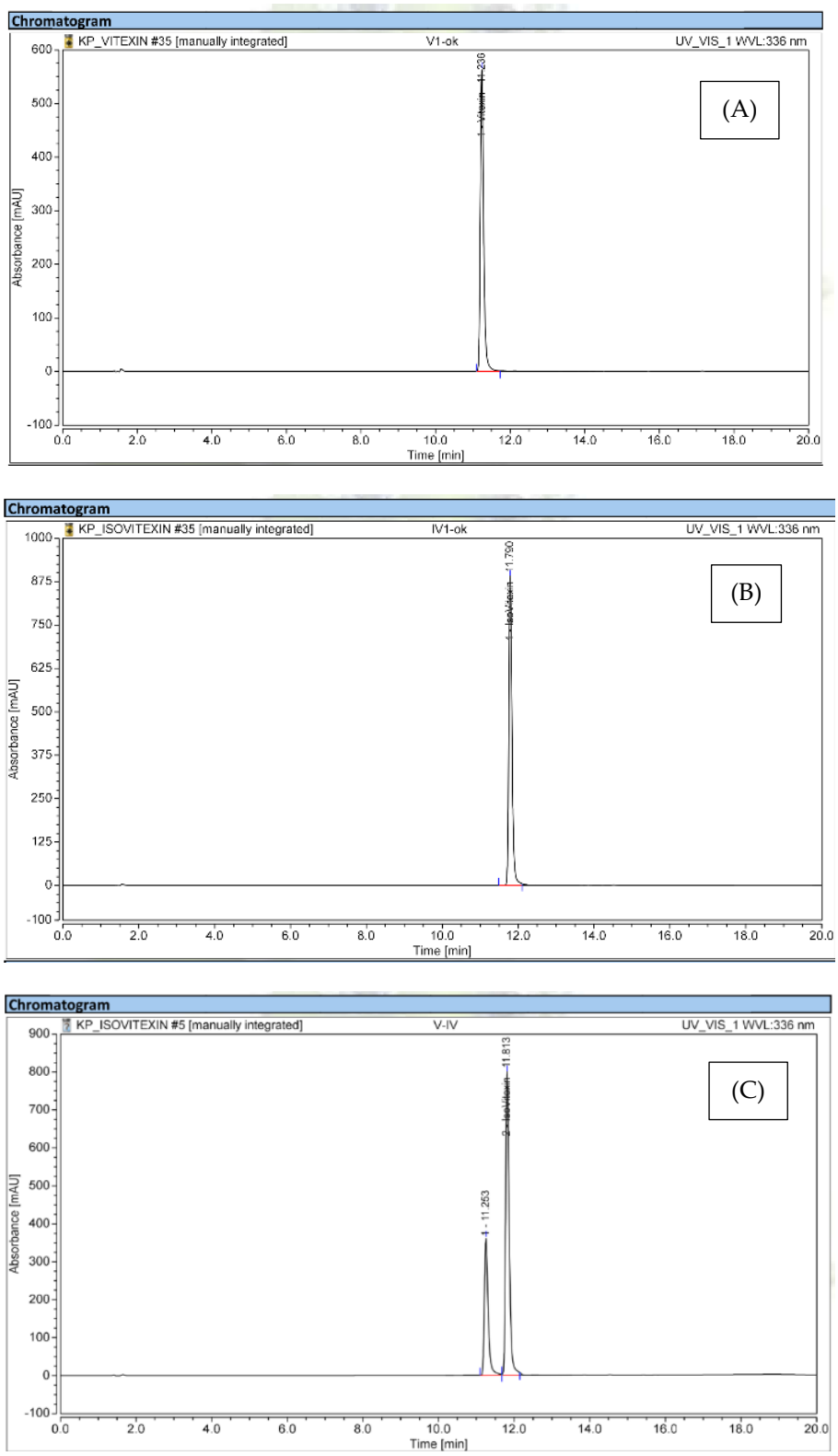

Fig. 1. UHPLC-UV chromatogram of commercial standards recorded at 336 nm: (**A**) vitexin, (**B**) isovitexin, (**C**) mix standard solution of vitexin (**1**) and isovitexin (**2**)

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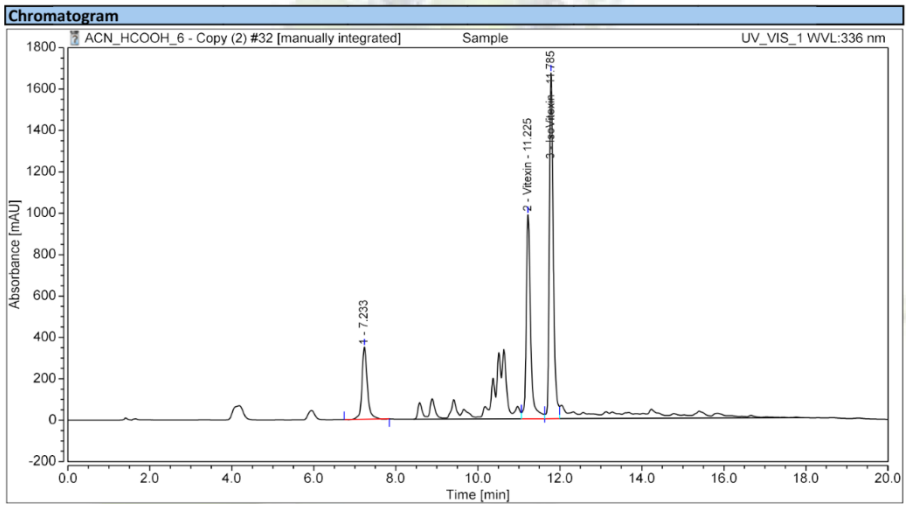

Fig. 2. Typical chromatogram of extracts at 336 nm. Peak identified: vitexin (**1**) and isovitexin (**2**)

The results indicate that the peak signals corresponding to standard vitexin and isovitexin appear at retention times of 11.225 minutes and 11.785 minutes, respectively. In the blank chromatogram, no peak signal appears at these retention times. Conversely, in the sample chromatogram, distinct peak signals are observed at these times and are well-separated from other signals on the chromatogram. This provides strong evidence that the quantitative method is specific and suitable for the simultaneous quantification of vitexin and isovitexin in SAL.

System suitability

The system suitability results, as summarized in Table 2, demonstrate the suitability of the chromatographic conditions for analyzing vitexin and isovitexin.

Table 2. Results of surveying the system suitability of vitexin and isovitexin

Index	Analyte			
	Vitexin		Isovitexin	
	Medium	RSD (%)	Medium	RSD (%)
Retention time (min)	11.232 ± 0.003	0.03	11.79 ± 0.01	0.08
Peak area (mAu.s)	17.85 ± 0.10	0.56	17.83 ± 0.10	0.56
Tailing Coefficient	1.38 ± 0.01	0.72	1.32 ± 0.01	0.76
Number of Theoretical Plates	85072 ± 504.08	0.59	100483.57 ± 433.36	0.43

The results indicate that both vitexin and isovitexin have tailing coefficients within the range of 0.8-1.5, and the number of theoretical plates exceeds 2500. The relative standard deviations (RSD) for retention time and peak area of vitexin are 0.03% and 0.56%, respectively, while for isovitexin, they are 0.08% and 0.53%. All these values are well below 2%. Therefore, the selected chromatographic conditions and the UHPLC-UV system employed are suitable, ensuring the stability and reliability of the simultaneous quantitative analysis of vitexin and isovitexin. Compare to other study simutanous phenolic compound in cluding vitexin and isovitexin, this method took shorter time [13].

Linearity

The results of the standard curve and regression equation indicate a linear correlation between analyte concentration (x) and peak area (y) within specific concentration ranges. The linearity results are shown in Figure 3.

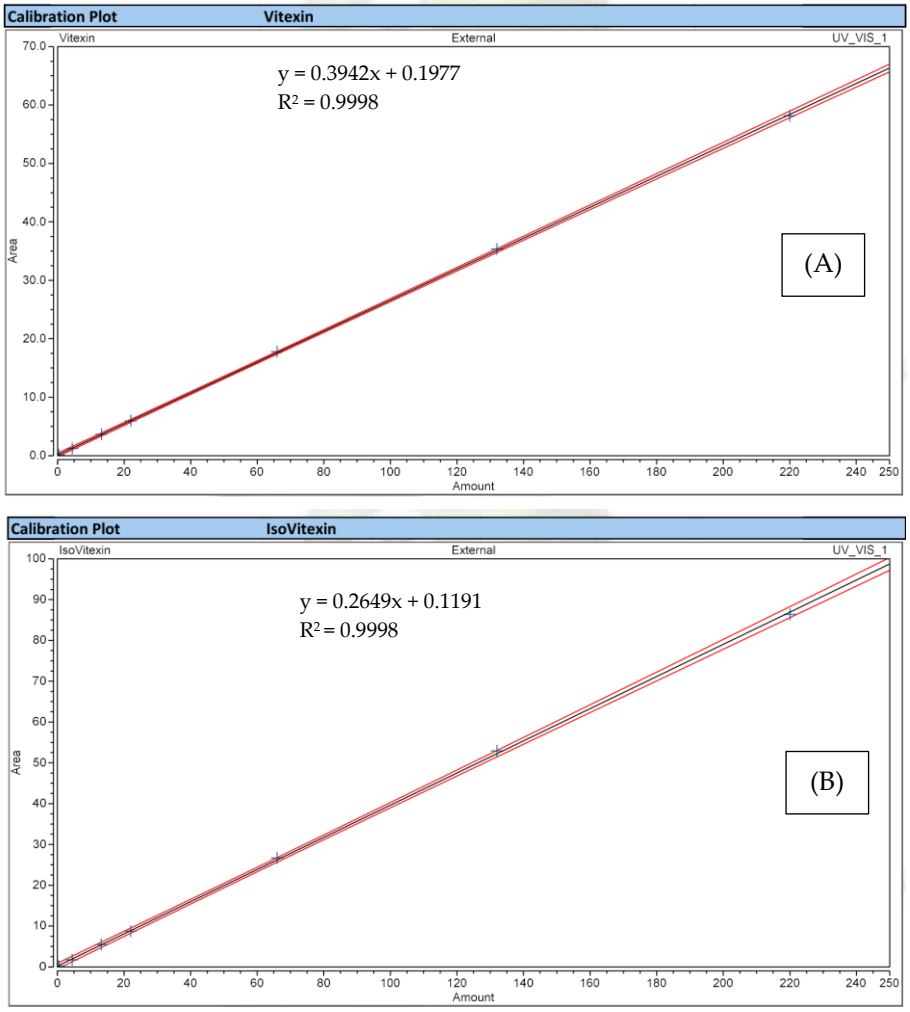

Fig. 3. The correlation between concentration and peak area for (**A**) vitexin and (**B**) isovitexin

In the concentration range of 0.4 - 220.0 µg/ml, both vitexin (y = 0.2649x + 0.1191) and isovitexin (y = 0.3942x + 0.1977) exhibit a direct proportional relationship with the analyte concentration, supported by high correlation coefficients (R^2 = 0.9998), demonstrating a linear relationship between analyte concentration and peak area.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ are critical parameters that define the sensitivity and capability of an analytical method. They are essential for method validation and help determine the method's effectiveness in detecting and quantifying analytes. LOD represents the lowest concentration of an analyte that can be reliably detected but not necessarily quantified, while LOQ is the lowest concentration that can be both reliably detected and quantified with acceptable precision and accuracy [14]. The results showed the LOD values for vitexin and isovitexin were 0.09 µg/ml and 0.11 µg/ml, respectively. As the result, the LOQ values were derived as 0.27 µg/ml for vitexin and 0.30 µg/ml for isovitexin.

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Accuracy and Precision

To assess the accuracy and precision of the analysis, the standard addition method was employed. A sample of SAL was detected by the proposed UHPLC method. The contents of vitexin and isovitexin in this sample were 38.563 μg/mL and 37.956 μg/mL, respectively. Mixed this sample with vitexin and isovitexin at concentrations equivalent to 50%, 100%, and 200% of the test sample. Three separate samples were prepared for each concentration. These test solutions were sequentially injected into the chromatography system, and the results are presented in the Table 3.

Table 3. Accuracy and precision for different levels of vitexin and isovitexin standard addition

Accuracy and precision	Analyte	Level (%)	Cs (μg/mL)	Cx (μg/mL)	C (μg/mL)	Accuracy (Recovery (%))	Precision (% RSD)
Day 1 (n=3)	Vitexin	50%	38.563	19.001	56.90 ± 0.08	96.49	0.42
		100%	38.563	38.011	76.14 ± 0.16	98.87	0.42
		200%	38.563	76.013	114.76 ± 0.71	100.24	0.93
	Isovitexin	50%	37.956	20.112	57.09 ± 0.10	95.15	0.54
		100%	37.956	37.998	74.71 ± 0.33	96.72	0.89
		200%	37.956	76.505	115.43 ± 0.38	101.26	0.49
Day 2 (n=3)	Vitexin	50%	40.175	20.017	59.56 ± 0.09	96.83	0.47
		100%	40.175	40.114	79.52 ± 0.50	98.08	1.28
		200%	40.175	80.01	121.10 ± 1.10	101.15	1.36
	Isovitexin	50%	40.587	20.116	59.97 ± 0.13	96.33	0.66
		100%	40.587	39.898	79.93 ± 0.18	98.60	0.45
		200%	40.587	81.005	123.11 ± 0.20	101.87	0.24
Different days (n=6)	Vitexin	50%	38.26	19.557	56.99 ± 0.14	95.82	0.88
		100%	38.26	38.005	76.14 ± 0.82	97.79	1.35
		200%	38.26	76.259	114.76 ± 0.63	100.75	0.87
	Isovitexin	50%	40.381	20.067	59.76 ± 0.24	96.58	0.58
		100%	40.381	40.006	79.72 ± 0.40	98.34	0.91
		200%	40.381	80.508	122.11 ± 1.31	101.51	0.96

Cs: Sample Concentration; Cx: Added Concentration; C: Founded Concentration

The results indicate that the UHPLC-UV method achieved high accuracy for both vitexin and isovitexin in all samples, with an average recovery rate (Rev) falling within the range of 95.0% to 102.0%, RSD in range of 0.24 to 1.35. These recovery rates and RSD meet ICH Q2 (R2) guideline's requirements for the accuracy and precision of an analytical method.

All in all, vitexin and isovitexin represent a promising pair of pharmaceutical substances for potential drug applications. These compounds exhibit poor solubility in water but are soluble in methanol, making methanol an ideal solvent for sample treatment to minimize water impurities. The development and validation of UHPLC method for the simultaneous quantification of vitexin and isovitexin, following ICH Q2 (R2)

guideline criteria, have yielded positive results. The analysis method successfully meets the criteria, with the linear regression equation demonstrating a remarkably high coefficient of determination and correlation coefficient, indicating tight linearity and a broad linear range. Furthermore, the analytical method exhibits excellent repeatability, with straightforward sample processing and analysis procedures, rendering it suitable for sample analysis. These findings provide a reliable analytical method for their quantification in various applications.

3.3 Application of UHPLC-UV Method for Quantification of vitexin and isovitexin from SAL leaves

Using the validated analytical methods, we conducted a simultaneous analysis of vitexin and isovitexin content in SAL collected in Dak Lak. The calculated content according to the dry weight of the leaf samples is presented in Table 4.

Table 4. Results of Analysis of Vitexin and Isovitexin Contents in Real Samples

Analyte		Sample			
		Bud Leaves	**Young Leaves**	**Buds**	**Old Leaves**
Vitexin	Means (mg/g)	73.09 ± 0.01	50.61 ± 0.01	30.98 ± 0.01	19.84 ± 0.01
	RSD (%)	0.01	0.02	0.03	0.05
	1/2.RSDH (%)	1.48	1.57	1.69	1.80
Isovitexin	Means (mg/g)	84.57 ± 0.01	48.53 ± 0.01	27.09 ± 0.01	16.66 ± 0.01
	RSD (%)	0.01	0.02	0.04	0.06
	1/2.RSDH (%)	1.45	1.58	1.72	1.85

The analysis of the content of vitexin and isovitexin in leaf samples of varying ages reveals a notable trend: the content of both substances decreases rapidly with leaf age, progressing from bud leaves and young leaves to small cake leaves and finally to old leaves. This finding aligns with the current market practice of utilizing buds and young leaves for tea production. Furthermore, the analysis results emphasize that vitexin and isovitexin are the predominant compounds present in SAL, with significant ratios ranging from approximately 19.84 mg/g to 73.09 mg/g and 16.66 mg/g to 84.57 mg/g, respectively. This content surpasses that found in buckwheat which is considered to have high vitexin and isovitexin content at 3.548 mg/g and 5.579 mg/g, respectively [15]. Consequently, SAL is a promising source of raw materials for extracting vitexin and isovitexin. This finding not only bolsters the potential of SAL cultivation in various mountainous provinces of the South Central region but also contributes to the diversification of medicinal herb resources.

It's worth noting that while bud leaves and young leaves exhibit significantly higher content compared to banh te leaves, their raw material cost is approximately three times higher than that of banh te leaves. Therefore, to ensure a sustainable and cost-effective source of raw materials for production, further research and optimization of the extraction process may be explored, with the aim of obtaining standardized extracts rich in vitexin and isovitexin from a combination of leaves.

4 Conclusions

The study has developed and validated a procedure for simultaneously quantifying vitexin and isovitexin in SAL. The results demonstrated that, with the sample processing process and

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chromatography conditions outlined above, the chromatographic method meets the specificity, system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision criteria as per ICH Q2 (R2) guidelines.

The study also assessed the content of vitexin and isovitexin in SAL at different stages of growth. However, in order to utilize SAL as a source of medicinal ingredients or functional foods, it is essential to conduct research on factors that influence the content of vitexin and isovitexin in the leaves. These factors may include the timing of harvest and the harvest season, among others. Further investigation is needed to understand these influences.

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