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Isolation and establishment of *trans*-cinnamic acid as a reference standard from Radix *Scrophularia buergeriana* Miq

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ABSTRACT

Scrophularia buergeriana Miq., belonging to the family Scophulariaceae, is a potential medicinal herb that has been used in traditional oriental medicine since ancient times. However, studies of *S. buergeriana* are still at a relatively early stage. Above all, there is still no established reference standard to control the quality of medicinal herbs and their products from *S. buergeriana* in Vietnam. Therefore, we present the isolation of *trans*-cinnamic acid from the root of *S. buergeriana* which was subsequently established as a chemical reference standard in this study. The isolation was conducted by recrystallization and CC on silica gel using different eluents. The purity and quality of the isolated compound were evaluated by spectroscopic methods and high-performance liquid chromatography (HPLC). Furthermore, the compound was evaluated for homogeneity and assigned value to establish reference standards according to General requirements for the competence of reference material producer's guideline (TCVN ISO 17034:2017), Appendix 3 of World Health Organization (WHO technical report No. 943:2006) and Statistical methods for use in proficiency testing by interlaboratory comparison (ISO 13528:2015). The research successfully demonstrated the isolation and establishment of *trans*-cinnamic acid as a chemical reference standard for *S. buergeriana* which was herein reported for the first time.

INTRODUCTION

Scrophularia buergeriana Miq., belonging to the genus *Scrophularia* (Scrophulariaceae), is also known as figwort, bei xuan shen, hac sam (black ginseng), or nguyen sam because of its bitter taste as ginseng and black color [1]. This species is mainly found in Northern China, Korea, Manchuria, and Japan. It is noted as a strongly fragrant perennial or biennial plant that plays an important role in traditional medicine in several Eastern country cultures because of its diverse biological activities [2]. The flowers of *S. buergeriana* Miq. are pale yellow–white in color, which is different from *S. ningpoensis* Hemsl., whose flowers grow in umbels and are purple. While extensive research has been conducted on *S. ningpoensis* Hemsl., studies on *S. buergeriana* Miq. are still in their early stages. *Scrophularia buergeriana* Miq. is known for its slightly cold taste and has been utilized as an anti-inflammatory agent in the treatment of tonsillitis, pharyngitis, swelling, and laryngitis. In addition, it exhibits antibiotic properties against various strains of skin bacteria and is applied for the alleviation of constipation [1,3]. Besides, it also helps cure fever, lower blood sugar, neuritis, and reduce beta-amyloid-induced neurotoxicity to improve memory impairment and dilate blood vessels [4].

Counterfeit products and poor-quality pharmaceuticals have taken advantage of and caused negative effects on consumers both in terms of health and finance. To prevent the manufacture and trade of counterfeit products and low-quality medicinal materials, it is necessary to conduct inspections and quality control on medicinal herbs and herbal preparations. One of the most accurate and effective methods currently used to control the quality of medicinal herbs is the use of reference standards, both isolated from natural sources and synthesized in laboratories. They have played critical roles in the quality system and trace analysis of products containing medicinal

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plants. Harpagide and harpagoside are well-known as reference standards for *S. buergeriana.* However, they are purchased from global companies (such as International Pharmacopeia, United States Pharmacopeia, European Pharmacopeia, British Pharmacopoeia, Sigma, Chromadex reference standards, and so on) at high costs with delivery time lasting weeks or months. In addition, there is also the risk of not finding suitable reference standards, potentially causing delays and errors in the analysis and testing of medicinal materials. Remarkably, we identified a significant proportion of *trans*-cinnamic acid in this species which could be established as the reference standard.

In this study, we described the isolation and structural elucidation of *trans*-cinnamic acid from the roots of *S. buergeriana*. The purity of the compound was determined by spectroscopic and high-performance liquid chromatography (HPLC) methods. *trans*-Cinnamic acid was subsequently established as the reference standard to control the quality of medicinal herbs and pharmaceuticals following standard references [5,6]. The homogeneity and assigned values were also evaluated.

MATERIALS AND METHODS

General experimental procedures

Nuclear magnetic resonance (NMR) was recorded on a Bruker Avance III 500 [500 MHz (^{1}H) and 125 MHz (^{13}C)] with $CDCl_3$ as the solvent. The HPLC method was developed on Shimadzu CBM20A (Japan). The IR and Ultraviolet spectrum (UV) spectra were performed by using iS50FT-IR and Shimadzu UV-2700, respectively. The melting point was defined by Wagner and Munz Polytherm A.

Thin layer chromatography (TLC) was performed on silica gel F_{254} (250 µm, Merck) or Rp-18 F_{254}s (Merck). Components on TLC were detected using an ultraviolet lamp (wavelength 254/365 nm, CN-15-LC-Vilber Loumart. Column chromatography (CC) was conducted by using silica gel 60 (40-63 µm, Merck) and silica gel Rp-18 (40-63 µm, Merck).

n-Hexane, acetone, and ethyl acetate were provided by J. T. Baker, ethanol was obtained from Merck. Methanol for HPLC standards was purchased from Supelco.

Plant material

Dried roots of *S. buergeriana* Miq. were purchased from OPC Pharmaceutical Joint Stock Company with a net weight of 10 kg. The medicinal sample was tested according to Vietnamese Pharmacopoeia V standards [7].

Extraction and isolation

The ground-dried roots of *S. buergeriana* (10 kg) were homogenized in 30 l of ethanol at room temperature. The solution was then filtered and evaporated under reduced pressure to give an ethanolic crude extract. The crude extract was suspended in water and extracted with ethyl acetate using a liquid–liquid extraction method, followed by rotary evaporation to yield an ethyl acetate extract (154.7 g).

The ethyl acetate extract was subjected to silica gel CC using gradient elution with *n*-hexane-acetone (0%–100%) to give 12 fractions (RSE1-12). Fraction RSE5 (10.05 g) was chromatographed on silica gel with *n*-hexane-acetone (0%–100%) as eluent to obtain 13 fractions (RSE5.1-13). By recrystallization technique, fraction RSE5.5 (7.29 g) was dissolved in acetone to yield solution RSE 5.5.1 (1.61 g) and RSE5.5.2 (5.76 g) precipitated in acetone. Fraction RSE5.5.2 was recrystallized and further purified by CC using RP-18 with acetone-isopropanol-MeOH-H_2O (1:1:3:5) to obtain *trans*-cinnamic acid (1.196 g).

Method validation and establishment of reference substance

The purity of *trans*-cinnamic acid was verified by TLC with three different mobile phases on silica gel F254 and Rp-18 plates. The compound is pure when the plates appear only in a single spot.

The compound was tested for its purity by the HPLC technique which was determined based on the % peak area of the analyte obtained in the chromatography. Mobile phase: phosphoric acid solution 0.1%—acetonitrile (linear gradient from 0% to 90% acetonitrile in 60 minutes), Gemini column C18 (250 × 4.6 mm, 5 µm), Photodiode array detection detector with detection wavelength set up at 272 nm, flow rate: 1.0 ml/minute, and injection volume: 20 µl, analysis time: 70 minutes. The test solution was prepared by dissolving 5 mg of the examination sample with 25 ml of methanol (MeOH) in a 50 ml volumetric flask, sonicating the sample completely, adding MeOH to the volumetric mark, and shaking to obtain a solution which the corresponding concentration was 0.1 mg/ml. Blank solution: MeOH solvent which was filtered through a 0.45 µm filtration membrane.

The test and blank solution sequentially were injected into the chromatography system and recorded by the chromatogram. Ignore all secondary peaks in the test solution with retention times corresponding to the peaks in the blank solution and peaks with areas smaller than the limit of quantity (LOQ) in the method validation when viewing the chromatogram at the detection wavelength of refined products. A compound was considered pure when the ratio of the main peak area of the test solution in the chromatogram reached > 95%.

Check the quality of raw materials according to established standards

Conduct experiments on sensory properties, measuring IR, UV, NMR spectrum, moisture, sulfate ash, and determining percent (%) purity HPLC calculated on anhydrous products.

Packing

Distribute a certain amount of sample into brown glass vials in an inert gas environment (nitrogen gas) with humidity < 30%RH using a Glove-box device, then seal the lid.

Determine the homogeneity of the vial filling process (ISO 17034) [5]

Samples were randomly taken from 10 to 30 units using Excel software. Determination of the purity of the substances was carried out according to the developed and validated HPLC method, each vial was determined twice. The homogeneity of the vial filling process was confirmed based on ISO 13528 guidance [6].

The homogeneity value of interlaboratory consistency

This experiment was performed by one-factor analysis of variance ANOVA (p = 0.95). Test samples that fulfill requirements from the above process randomly and conduct HPLC purity determination at three independent laboratories conformed to GLP (good laboratory practice) or ISO/IEC 17025 main principles [6].

The assigned value and measurement uncertainty

Follow the instructions of ISO 13528:2005 based on the valuation of laboratory results [6].

RESULTS AND DISCUSSION

Structure elucidation of *trans*-cinnamic acid

From the ethyl acetate of the roots of *S. buergeriana*, a purified compound was isolated as a white flake crystal. Mp. 124°C–128°C. The UV spectrum (in MeOH) showed maxima at λ_{max} 274, 215, and 204 nm. The infrared spectrum (IR) exhibited characteristic absorption at ν_{max} 3064 (O-H), 2525 and 2343 (C-H), 1693 (C = O), and 1622 (C = C) cm^{-1}. The ^{1}H NMR spectrum (Table 1) revealed signals corresponding to two *trans*-olefinic protons [δ_H 7.81 (1H, d, J= 16.0 Hz, H-3) and 6.47 (1H, d, J= 16.0 Hz, H-2)] and a monosubstituted benzene ring [δ_H 7.56 (2H, m, H-2′, H-6′) and 7.41 (3H, m, H-3′, H-4′, H-5′)]. The ^{13}C NMR spectrum (Table 1) showed resonances for a carbonyl carbon [δ_C 172.3 (s, C-1)], two olefinic carbons [(δ_C 147.1 (d, C-3) and 117.3 (d, C-2)] and six aromatic carbons [δ_C 134.1 (s, C-1′), 130.8 (d, C-4′), 129.0 (d, C-3′, C-5′) and 128.4 (d, C-2′, C-6′)]. Based on the elucidation of the spectral data and comparison with relevant literature [8], the isolated compound was identified as *trans*-cinnamic acid (also called 3-phenylacrylic acid) (Fig. 1).

Method validation and establishment of *trans*-cinnamic acid as reference standard

Purity determination

Thin-layer chromatography: Each test sample in three different conditions gives exactly one single spot at a wavelength of 254 nm on the TLC. It can be concluded the purity of *trans*-cinnamic acid sample on TLC (Fig. 2).

Table 1. ^{1}H and ^{13}C NMR ($CDCl_3$) data of *trans*-cinnamic acid.

Position	δ_H	δ_C
1		172.3
2	6.47 d (16.0)	117.3
3	7.81 d (16.0)	147.1
1′		134.1
2′	7.56 m	128.4
3′	7.41 m	129.0
4′	7.41 m	130.8
5′	7.41 m	129.0
6′	7.56 m	128.4

Condition 1: Silica gel, chloroform – EtOAc (8:2), R_f = 0.25 (Fig. 2a);

Condition 2: Silica gel, *n*-hexane – acetone (7:3), R_f = 0.5 (Fig. 2b);

Condition 3: Silica gel Rp-18, acetone – H_2O (8:2), R_f = 0.75 (Fig. 2c).

HPLC method: *trans*-Cinnamic acid achieved the standard requirement of purity standard more than 95%, according to ASEAN standards, so it was chosen as the material to establish the reference standard (Fig. 3).

System suitability

Verified by six repeated injections of a standard solution with a concentration of approximately 0.1 mg/ml. The results showed the suitability for analyzing *trans*-cinnamic acid compounds (Table 2).

Selectivity

Determined by comparing the chromatogram of the blank sample, the test sample, along with the 10 ml test samples separately added with 1 ml NaOH 1N, HCl 1N, and H_2O_2 10 % solutions, and the test sample was incubated at 70°C for 24 hours. The chromatogram of the blank did not show a peak with a retention time corresponding to the analytical peak. Based on the chromatogram superposition results, the *trans*-cinnamic acid peak is completely separated from other impurity peaks that were tested under four different stress conditions. This procedure achieved the requirements of specificity. The results of spectral superposition are shown in Figure 4.

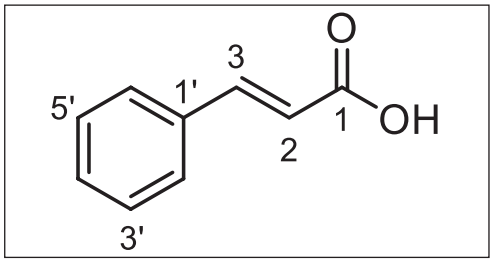

Figure 1. The chemical structure of *trans*-cinnamic acid.

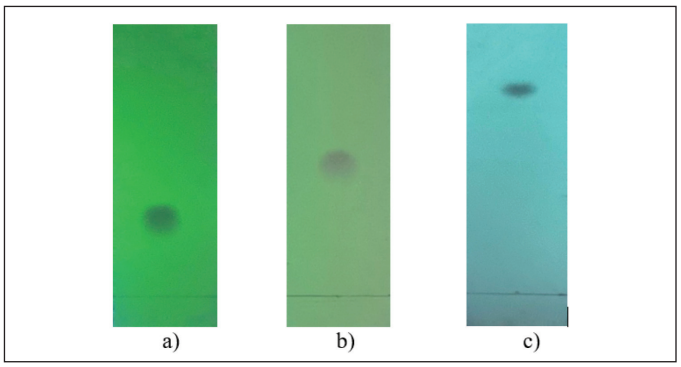

Figure 2. TLC of trans-cinnamic acid sample in three different conditions.

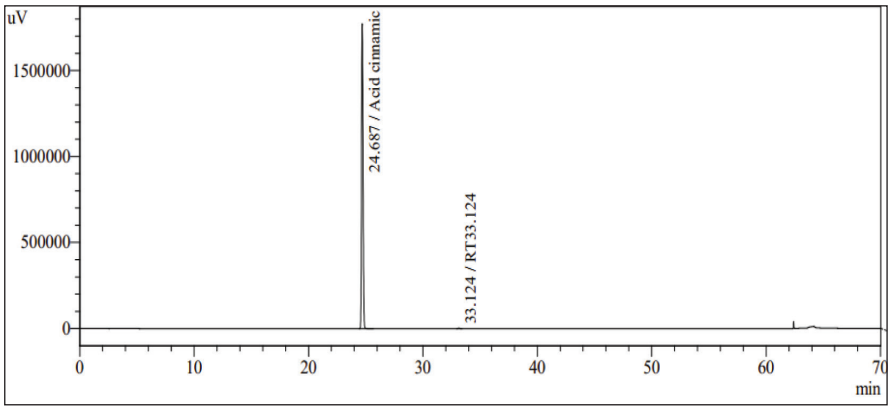

Figure 3. HPLC chromatography of *trans*-cinnamic acid.

Table 2. The parameters of system suitability testing.

No injects	Retention time (min)	Peak area	Theoretical plates	Symmetry coefficient
1	24.74	16056,079	143,408	1.33
2	24.79	15945,698	142,287	1.30
3	24.83	15911,486	141,543	1.28
4	24.78	15905,820	141,735	1.27
5	24.82	15908,582	142,382	1.24
6	24.66	15850,234	141,483	1.26
Average	24.77	15929,650	142,140	1.28
RSD (%)	0.25	0.43	-	-

Linearity

Investigating the correlation between x (concentration) and y (peak area) using the least squares method, the results showed a close correlation between concentration and peak area (R is in the range of 0.998—1,000). Based on the calibration curve, determine the regression equation y = 159685292 × + 221886 (R^2 = 0.9995), the limit of detection and LOQ values of the method were 0.005 mg/ml and 0.014 mg/ml, respectively (Fig. 5).

Precision (including repeatability and intermediate precision)

The method achieves repeatability relative standard deviation (RSD) (%) = 0.03% ≤ 2% with n = 6 (day 1) and intermediate precision RSD (%) = 0.05% ≤ 2% with n = 12 (for 2 days) (Table 3).

Accuracy

Accuracy demonstrates the recovery rate of the values measured based on the regression equation compared to the actual value of isolated *trans*-cinnamic acid at the corresponding investigation. The results showed that at all three different concentrations investigated, the recovery efficiency was in the range of 98%—102%. The method indicated the accuracy requirements (Table 4).

Establishment of *trans*-cinnamic acid as reference standard

Material evaluation

The isolated *trans*-cinnamic acid achieves the reference material requirements to establish a standard substance according to WHO TRS 943 (Table 5).

Homogeneity and interlaboratory comparison

Proceed packing 150 vials, each vial contains 5 mg. Take 10 random vials to check homogeneity to ensure the results are the same between vials. Each vial is analyzed twice to determine purity. Trial the homogeneity between vials using the statistical method of one-factor ANOVA analysis. Evaluating the homogeneity between vials according to the statistical method of one-factor ANOVA analysis gives the result $F_{experimental} = 0.13 < F_{theoretical} = 4.41$. Therefore, it is concluded that there is homogeneity between vials (Table 6).

Randomly take 6 other vials and send them to 3 independent analytical laboratories to analyze trans-cinnamic acid. The assessment of vial homogeneity among the 3 laboratories was performed through quantitative results between the three laboratories. The homogeneity was tested by the statistical method of one-factor ANOVA analysis. Based on the results of the one-factor ANOVA statistical analysis method, a comparison between the two values showed that $F_{experimental} = 0.798 < F_{theoretical} = 3.682$, proving the quantitative results between the 3 other laboratories. The difference is not statistically significant. The procedure for analyzing *trans*-cinnamic acid content is highly repeatable, the analyte content does not depend on the laboratory participating in the evaluation (Table 7).

Assigned value

The value is determined from the percentage purity results of 3 laboratories. Conformed to ISO 13528 statistics, $x_{average}$, s* values, and calculation of the assigned value of *trans*-cinnamic acid content (n = 18) are confirmed by statistical processing. *Trans*-cinnamic acid is eligible to register as a national standard with the assigned value of 99.60% on anhydrous substance (n = 18, s = 0.06) and the measurement uncertainty is 0.13%.

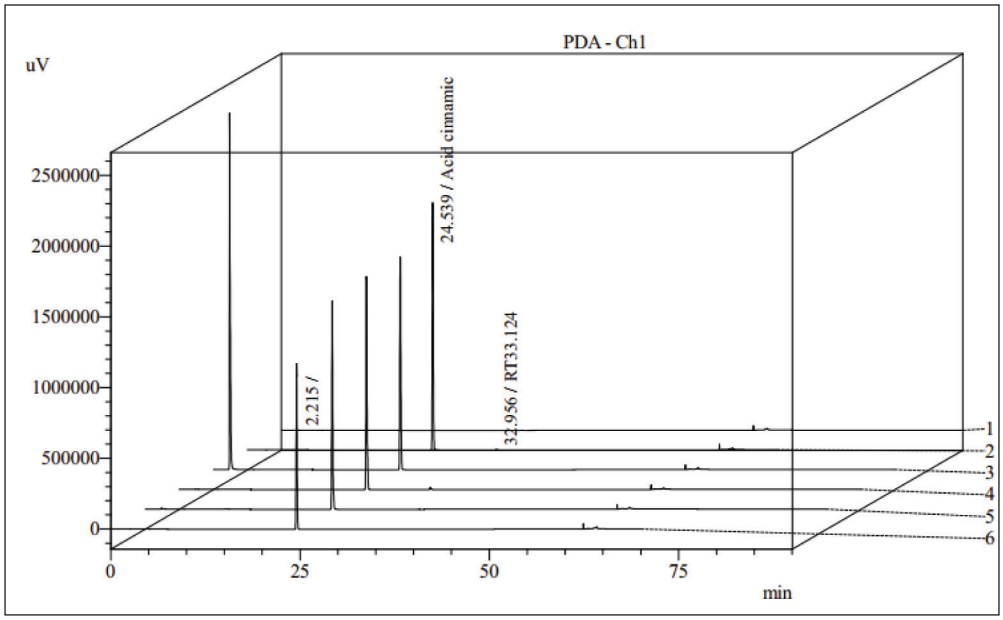

Figure 4. HPLC chromatogram of *trans*-cinnamic acid. Blank, 2, 3, 4, 5, 6. Test solution treated with H_2O_2, HCl, NaOH, and high temperature, respectively.

No.	Concentration (mg/ml)	Peak area
1	0.050	8080383
2	0.075	12403135
3	0.100	16082402
4	0.125	20287088
5	0.150	24099068

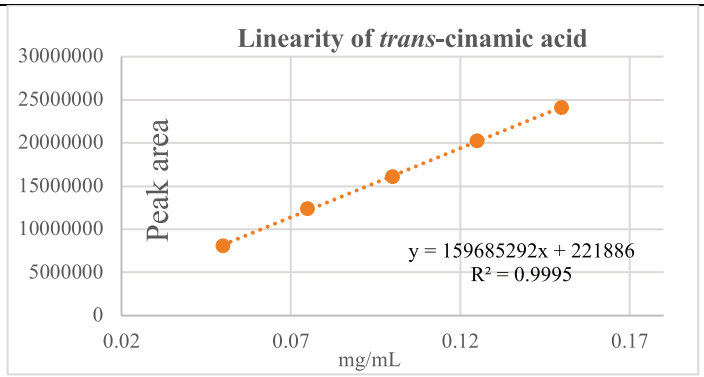

Figure 5. Calibration curve of *trans*-cinnamic acid.

Table 3. Comparison results of repeatability and intermediate precision.

	Purity (%)	
Test samples	**Day 1**	**Day 2**
1	99.703	99.790
2	99.777	99.752
3	99.699	99.709
4	99.729	99.676
5	99.746	99.729
6	99.690	99.625
Average	99.719	
SD	0.045	
RSD (%)	0.05	

Table 4. Results of the percentage recovery efficiency.

% Level concentration	**% Recovery**	**%RSD**
75%	100.1	0.93
	100.3	
	101.8	
100%	98.9	1.33
	99.8	
	101.5	
125%	99.8	0.37
	100.3	
	99.5	

Table 5. Results of materials evaluation according to reference standards.

No	Standard	Quality state	Result	Conclusion
1	Properties	White crystal	White crystal	Correct
2	Qualitative			
	IR	Characteristic oscillations were consistent with the references.	-OH (ν_{max} 3064 cm^{-1})	Correct
			-C-H (ν_{max} 2525 and 2343 cm^{-1})	
			-C=O (ν_{max} 1693 cm^{-1})	
			-C=C- (ν_{max} 1622 cm^{-1})	
	UV-VIS spectrum	Maximum absorption at λ_{max} 274, 215 and 204 nm	λ_{max} 274, 215 and 204 nm.	Correct
	NMR spectrum	Data are consistent with references	See Table 1	Correct
3	Humidity	Not higher than 0.5%	0.1%	Achieve
4	Sulfate ash	Not higher than 0.1%	0.02%	Achieve
5	Chromatographic purity	> 95%	99.69%	Achieve

Table 6. Measurement results for homogeneity test.

Vial number	Section 1 (%)	Section 2 (%)	Descriptive statistics
34	99.738	99.730	$N = 20$
96	99.624	99.654	Sample average = 99.635%
102	99.627	99.617	
54	99.588	99.579	%RSD = 0.04
8	99.624	99.615	(With $p = 0.95$)
77	99.653	99.641	
64	99.610	99.593	
85	99.622	99.611	
11	99.641	99.625	
26	99.653	99.647	

Table 7. Results of inter-laboratory vial homogeneity assessment.

Vial number	Results		
	Lab 1	Lab 2	Lab 3
1	99.254	99.568	99.643
2	99.264	99.587	99.597
3	99.288	99.548	99.657
4	99.647	99.618	99.151
5	99.653	99.599	99.625
6	99.610	99.693	99.640
Average	99.453	99.602	99.552
A_s	0.041	0.003	0.039
%RSD	0.204	0.051	0.198

CONCLUSION

From the ethyl acetate extract of the roots of *S. buergeriana* Miq., a pure compound was isolated and determined to be *trans*-cinnamic acid. The compound was analyzed by the HPLC method with purity > 95% and chosen to establish the reference standard. The construction process achieves the requirements for system compatibility, linearity, specificity, precision, repeatability, and intermediate precision. The *trans*-cinnamic acid standard derived from the medicinal herb *S. buergeriana* with a spectral dataset including UV, IR, NMR spectra, and basic standards that attain the requirements of national standards, has been established to contribute to the list of standard substances of the Institute of Drug Quality control of Ho Chi Minh City, Vietnam. In conclusion, the study presents the successful establishment of the chemical reference compound, *trans*-cinnamic acid, to serve the quality testing of medicinal products containing roots of *S. buergeriana* Miq. circulated. The discovery has shown a significant contribution to the quality control of medicinal herbs and their products on the market.

LIST OF ABBREVIATIONS

d	Doublet
HPLC	High-performance liquid chromatography
IR	Infrared spectrum
LOD	Limit of detection
LOQ	Limit of quantity
NMR	Nuclear magnetic resonance
PDA	Photodiode array detection
RSD	Relative standard deviation
s	Singlet
TLC	Thin-layer chromatography
UV	Ultraviolet spectrum

AUTHOR CONTRIBUTIONS

Ngan Nguyen Kim Luu: Isolated the compound, conducted experiments, and wrote the manuscript.

Ngan My Tran: Conducted experiments and acquired data.

Phuong Thu Tran: Organized research and revised the manuscript.

Duong Hoang Trinh: Designed experiments, acquired and interpreted data, and completed the manuscript.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

PUBLISHER'S NOTE

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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