ISOLATION, PURIFICATION AND STANDARDZATION OF RAW MATERIAL OF LOBETYOLIN EXTRACTED FROM *RADIX CODONOPSIS JAVANICAE* FOR REFERENCE STANDARD ESTABLISHMENT

PHAN VU THU HA[1,2]***, PHAM THI PHUONG***[2]***, LE QUANG THAO***[3,4]***, DANG THI VAN ANH***[5]
TONG THI THANH VUONG[2]***, LE THI TRANG***[3]***, LE DINH CHI***[2]***,*** ✉
[1] *Phu Tho Department of Health,*
[2] *Hanoi University of Pharmacy,*
[3] *National Institute of Drug Quality Control,*
[4] *Vietnam National University - University of Medicine and Pharmacy*
[5] *Vinh Medical University.*
✉ *Corresponding author: ledinhchi@gmail.com*

Received May 20th, 2026; Accepted June 16th, 2026

Abstract: *Lobetyolin is a compound widely used as a marker for the quality control of medicinal materials derived from several species of the Codonopsis genus in the monographs of various pharmacopoeias. In Vietnam, research on the establishment of a lobetyolin reference substance remains limited, and such standards are mostly purchased from foreign sources. This study developed an efficient process for the isolation and purification of lobetyolin from Codonopsis javanica using liquid-liquid extraction, normal-phase column chromatography, and preparative reversed-phase HPLC. The overall yield for the isolation and purification process was 24.65%. After purification, the lobetyolin raw material was characterized and standardized using the following parameters: identification via IR and UV-Vis spectroscopy, loss on drying, melting point, specific optical rotation, related substances, and chromatographic purity. The analytical procedure for the determination of related substances and chromatographic purity was fully validated in compliance with ICH and AOAC International guidelines. With a lobetyolin content of 98.96% (w/w), the purified material obtained in this study is suitable for the establishment of a lobetyolin reference substance for quality control purposes.*

Keywords: *Codonopsis javanica, lobetyolin*

1. INTRODUCTION

Lobetyolin ((2R,3R,4S,5S,6R)-2-[(4E,6R,7R,12E)-1,7-dihydroxytetradeca-4,12-dien-8,10-diyn-6-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol) is a polyacetylenic glucoside widely distributed in the genus *Codonopsis*, including *Codonopsis javanica* (Blume) Hook. F. [1]. Recognized as a crucial quality marker for both northern *Codonopsis* (e.g., *C. pilosula* or *C. tangshen*) and *C. javanica*, this compound has not yet been produced for commercial purposes by any local entity in Vietnam [1]. While previous research by Huynh Tran Quoc Dung et al. [2] and Nguyen Truong Huy et al. [3] has successfully established protocols for the isolation, standardization, and HPLC quantification of lobetyolin in *C. javanica* roots, these studies did not fully optimize the extraction parameters, such as solvent selection and extraction time [2]. Moreover, given the relatively high polarity of lobetyolin, its limited solubility in ethyl acetate – a solvent frequently employed in previous isolation procedures – suggests that a significant amount of the compound remains in the aqueous phase. This limitation underscores the need for a more efficient extraction and purification strategy to enhance production yield.

Therefore, this study aims to develop an optimized extraction, isolation, and purification process for lobetyolin, thereby enhancing the production efficiency of the material required for reference standard preparation. Concurrently, the study seeks to establish quality specifications and analytical procedures to standardize the purified lobetyolin, ultimately facilitating its application as a reference material for pharmaceutical quality control.

2. EXPERIMENTAL

2.1. Plant material

The roots of *Codonopsis javanica* (Blume) Hook. F. were collected in Mang Ri Commune, Tu Mo Rong District, Kon Tum Province, in October 2024. The botanical identity of the species was authenticated by the

Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The root samples were air-dried, ground into a fine powder, and determined to have a moisture content of 5.83%.

2.2. Equipment, instruments, and chemicals

Equipment: All equipments met the ISO/IEC 17025 standards and included the following: an ElmaSonic S100 ultrasonic cleaner, a Mettler Toledo MS105 analytical balance, Shimadzu HPLC/DAD systems, preparative HPLC system, Waters Acquity ARC HPLC/DAD system, a Büchi rotary evaporator, and a Memmert vacuum drying oven (all located at the National Institute of Drug Quality Control, NIDQC). Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker 600 MHz and 500 MHz spectrometers (Institute of Chemistry, Vietnam Academy of Science and Technology). Chromatographic separation was performed using an Inertsil C18 analytical column (250 × 4.6 mm, 5 µm; GL Sciences, Japan) and a YMC-Pack Prep C18 column (250 × 20 mm, 5 µm; YMC, Japan). Thin-layer chromatography (TLC) was carried out using silica gel 60 F254 plates (Merck, Germany).

Reagents and chemicals: Dichloromethane (Merck, Germany), methanol (Merck, Germany), acetonitrile (Merck, Germany), and acetone (Scharlau, Spain) were of analytical or HPLC grade. Normal-phase silica gel 60 (40 – 63 µm, Merck, Germany) was used for column chromatography.

Laboratory glassware: Standard laboratory glassware, including volumetric flasks, pipettes, chromatographic columns, graduated cylinders, and round-bottom flasks, were utilized throughout the experiment.

2.3. Methods

2.3.1. Extraction, isolation, and purification procedures

The powdered root of *C. javanica* was extracted using ethanol – water or methanol – water mixtures at diffferent volume ratios (The final extract solvent was ethanol – water (50 : 50, v/v), detailed results for solvent selection were summarized in 3.1.1). The crude extracts were concentrated under reduced pressure with a rotary evaporator to remove the solvents. Subsequently, impurities were removed via liquid-liquid partition using immiscible solvents of varying polarities. Lobetyolin was then isolated and purified using normal-phase and/or reversed-phase liquid chromatography.

2.3.2. Monitoring the presence and purity of lobetyolin throughout the isolation and purification process

At each stage of extraction and purification process, the presence and purity of lobetyolin were monitored using an HPLC-DAD system at wavelengths of 215 nm and 266 nm, utilizing a Phenomenex C18 column (250 × 4.6 mm, 5 µm). Samples were dissolved in an appropriate amount of methanol, and a 5 µL aliquot was injected into the chromatography system. The mobile phase was a mixture of acetonitrile-water whose composition varying according to a gradient program, in which the volume content of acetonitril was increased from 10% to 80% over 40 minutes, maintained at 80% for 15 minutes, then returned to 10% for system equilibration. The flow rate was set at 1.2 mL/min. Fractions exhibiting the highest peak area percentage of lobetyolin on sum of areas of all detected peaks were selected for subsequent processing.

2.3.3. Structural confirmation

The chemical structure of the isolated lobetyolin were confirmed using Nuclear Magnetic Resonance (NMR) spectroscopy and Electrospray Ionization Mass Spectrometry (ESI-MS).

2.3.4. Quality control specifications for bulk material of isolated lobetyolin

**** Identification***

- **UV-Vis spectra:** The UV-Vis spectrum of the lobetyolin peak in the chromatogram of the reference solution in Impurities test must exhibit absorption maxima at approximately 215 nm and 266 nm.

- **Infrared (IR) spectroscopy:** Performed according to the *Vietnamese Pharmacopoeia V*, Appendix 4.2 [1]. The IR spectrum of the bulk material must correspond to that of the reference standard.

**** Loss on drying***

Approximately 0.1 g of the material was dried under vacuum at 60°C in the presence of P_2O_5 as a desiccant until a constant weight was achieved (*Vietnamese Pharmacopoeia V*, Appendix 9.6) [1]. Weight measurements were performed using an analytical balance with a precision of 0.01 mg. The assay was conducted in triplicate, and the mean value of volatile impurities was calculated.

**** Melting point***

Determined in accordance with the *Vietnamese Pharmacopoeia V*, Appendix 6.7 [1].

* Specific optical rotation

A lobetyolin solution was prepared in methanol at exact concentration about 4 mg/mL. The optical rotation was measured at 25°C.

**** Impurities***

- *Chromatographic conditions:* The impurities in bulk material of lobetylolin were detected using the method in *Taiwanese Pharmacopoeia* [4]. The analysis was performed on a C18 column (250 × 4.6 mm, 5 µm). The mobile phase was a mixture of acetonitrile and 0.2% aqueous solution of acetic acid in gradient elution with volume percentage of acetonitrile increased from 15% to 30% (0 – 20 min), then from 30% to 95% (20 – 25 min), and returned to 15% for system re-equilibration. The flow rate was 1.0 mL/min, the injection volume was 10 µL, and the detection wavelength was set at 266 nm.

- *Sample preparation:* The test solution was prepared by dissolving the bulk material in methanol to a concentration of approximately 1.0 mg/mL. The reference solution was prepared by accurately diluting the test solution 100 times with methanol.

- *System suitability and acceptance criteria:* For system suitability, the lobetyolin peak on chromatogram of the reference solution must have the number of theoretical plates not less than 8000, tailing factor not exceeding 2.0, and relative standard deviation (RSD) of the peak area for 6 consecutive injections not exceeding 2.0%. The area of any idividual secondary peak on chromatogram of test solution should not exceed 2 times (2.0%) and the total area of all secondary peaks (if any) on chromatogram of test solution must not exceed 3 times (3.0%) the peak area of lobetyolin on chromatogram of the reference solution. Disregard all secondary peaks less than 0.05 times the peak area of lobetyolin on chromatogram of reference solution (0.05%).

The analytical procedure was fully validated in accordance with the guidelines of *AOAC International* [5].

**** Content of lobetyolin in bulk material***

The content (%, w/w) of lobetyolin in bulk material was calculated from the results of lost on drying and impurities using following formula:

$$X\ (\%) = (100\% - TI) * [100 - LD)]/100$$

in which:

X (%, w/w): mass content of lobetyolin in bulk material

TI (%, w/w): total impurities of bulk material determined by HPLC.

LD (%, w/w): lost on drying of bulk material.

3. RESULTS AND DISCUSSION

3.1. Development of extraction and purification procedures

3.1.1. Optimization of isolation and purification conditions

Ultrasound-assisted extraction (UAE) employs mechanical waves to accelerate the release of bioactive compounds from the plant matrix. Given its efficiency and suitability for laboratory-scale isolation of compounds for reference standard preparation, UAE was selected for this study.

To optimize the extraction solvent, approximately 2.5 g of *C. javanica* root powder was accurately weighed into six 100 mL stoppered conical flasks. Each flask was treated with 50 mL of one of the following solvents: mixtures of ethanol and water (30 : 70, 50 : 50, 70 : 30, 96 : 4, v/v) or of methanol and water (30 : 70, 50 : 50, 70 : 30, v/v) and methanol. The root powder was extracted with solvents by sonicationin 30 minutes. The obtained liquids were filtered through 0.45 µm membrane filters, and analyzed by HPLC according to the protocol described in Section 2.3.2.

The yield of lobetyolin was evaluated based on the peak area relative to the mass of the raw material. The results indicated that the mixtures of ethanol and water (50 : 50, v/v) and of methanol and water (50 : 50, v/v) provided the highest extraction yields (see also Figure 1). Because ethanol was cheaper and less toxic than methanol, the mixture ethanol – water (50 : 50, v/v) was employed as extraction solvent.

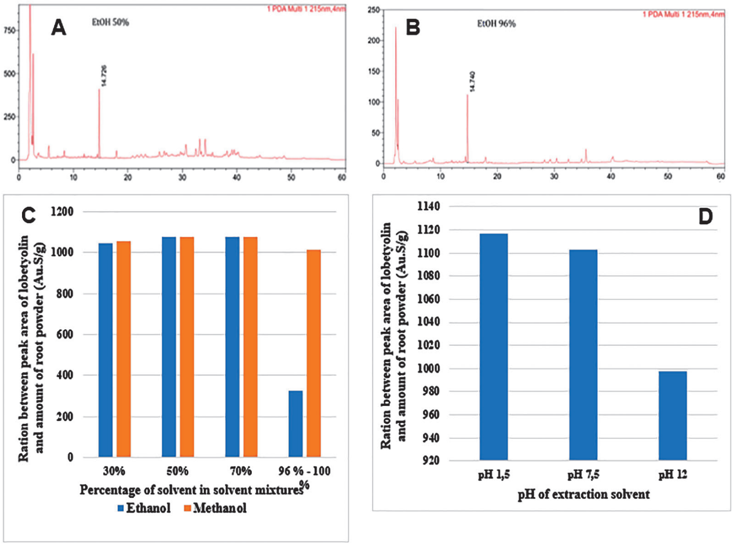

Figure 1. *HPLC chromatograms of the lobetyolin extracts from Vietnamese C. javanica roots obtained via ultrasound-assisted extraction using ethanol – water (50 : 50, v/v) (A), (90 : 10, v/v) (B) and comparison of extraction yields obtained with different solvent mixtures (C) and at different pH of the mixture ethanol – water (50 : 50, v/v) (D).*

To evaluate the effect of extraction duration, samples were subjected to ultrasound-assisted extraction (UAE) for 30 and 60 minutes using ethanol – water (50 : 50, v/v) as extraction solvent. The results showed 60-minute extraction did not provide significant improvement on extraction yield comparing to 30-minute extraction. Therefore, extraction time was fixed at 30 minutes. Furthermore, it was determined that performing three consecutive extraction cycles with ethanol – water (50 : 50, v/v) was sufficient to recover the bulk of lobetyolin from the *C. javanica* root powder.

To assess the influence of pH on extraction efficiency, the pH of ethanol – water (50 : 50, v/v) mixture were adjusted by mixing at volume ratio 9 : 1 with 0.5 N NaOH (actual pH about 12) or 0.5 N HCl (actual pH about 1.5). These alkalinized and acidified mixture were then used fot extraction of lobetyolin and comparing to unadjusted ethanol – water (50 : 50) (actual pH about 7.5). The results demonstrated that alkalinizing the solven mixture reduce the extraction efficiency, whereas acidifying the solvent mixture increased but not significantly the efficiency, therefore undjusted ethanol – water (50 : 50, v/v) was selected as the most suitable extraction solvent.

For the scale-up process, 5.08 kg of powdered *C. javanica* root was subjected to three rounds of UAE, each using 50 L of ethanol – water (50 : 50). The combined extracts were evaporated under reduced pressure to approximately one-fifth of the initial volume. The resulting condensed extract was resuspended in water and subjected to liquid-liquid partition using an equal volume of dichloromethane (3 times) and ethyl acetate (3 times), respectively. The distribution of lobetyolin in the dichloromethane, ethyl acetate, and aqueous phases was monitored (Figure 2).

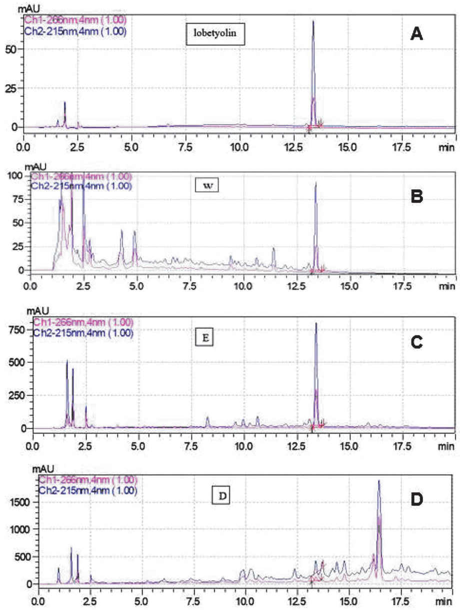

Figure 2. *HPLC chromatograms of the extract fractions after liquid-liquid partition: A: lobetyolin reference standard, B: aqueous phase, C: ethyl acetate phase, and D: dichloromethane phase*

Based on the results shown in Figure 2, lobetyolin was detected in both the aqueous and ethyl acetate phases. These phases were combined and concentrated under reduced pressure, yielding 28.0 g of crude extract. The crude extract was subjected to silica gel column chromatography and eluted with a dichloromethane – methanol gradient mixture at ratios of 40 : 1, 20 : 1, 10 : 1, and 5 : 1 (v/v). After solvent removal, four fractions were obtained (**W1**, **W2**, **W3**, and **W4**) and analyzed by HPLC. The results indicated that both **W3** and **W4** contained lobetyolin; therefore, these fractions were combined, designated as **W3**, and yielded a mass of 6.82 g.

The **W3** fraction was further purified on a silica gel column using a dichloromethane – methanol – water mixture (5 : 1 : 0.1, v/v/v) as the mobile phase. The presence of lobetyolin was monitored during elution. Fractions containing lobetyolin were pooled and concentrated under reduced pressure to obtain 1.54 g of a lobetyolin-enriched fraction, designated as **W3C** (Figure 3-A).

Subsequently, W3C was dissolved in 10 mL of methanol and purified using preparative liquid chromatography. After evaluating purification conditions with a YMC C18 column (250 × 20 mm, 5 µm), the optimal conditions were established as an isocratic elution with 25% acetonitrile in water, a flow rate of 8 mL/min, and an injection volume of 0.3 mL. The preparative chromatogram is shown in Figure 3-B. The fractions containing lobetyolin from multiple injections were pooled and concentrated under reduced pressure to obtain 1.06 g of compound **L**, which was identified as lobetyolin.

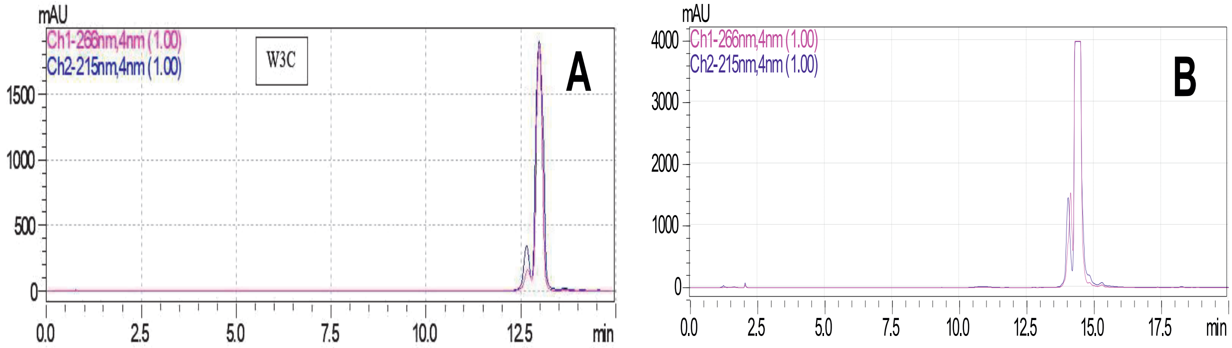

Figure 3. *Chromatograms of the lobetyolin-containing fractions: A: W3C fraction and B: Preparative HPLC chromatogram of during the purification of W3C.*

Comparing our findings with the recently published study by Huynh Tran Quoc Dung et al. [2], and based on our solvent optimization results, it is evident that 96% ethanol yields significantly less lobetyolin compared to 50% ethanol. Furthermore, we implemented a preliminary impurity removal step using dichloromethane via liquid-liquid partition before further partitioning with ethyl acetate. By monitoring the distribution of lobetyolin across fractions using HPLC, we observed that the compound was present in substantial amounts in both the ethyl acetate and aqueous phases. Consequently, combining both phases for subsequent purification was necessary to prevent significant loss of the target compound. Therefore, our modified extraction and purification protocol offers distinct improvements that substantially enhance the yield of lobetyolin compared to the previously reported method [2].

3.1.2. Structural confirmation of the purified lobetyolin

The structure of the purified lobetyolin in bulk material was confirmed using 1H-NMR, 13C-NMR spectroscopy (measured in CD_3OD at 600 MHz), mass spectrometry (MS) and by comparing IR spectrum of the bulk material with that of lobetyolin reference standard. The mass spectrum of the compound exhibited a pseudo-molecular ion peak at m/z = 431.1478 $[M+Cl]^-$, which is consistent with the molecular formula of lobetyolin ($C_{20}H_{28}O_8$, M = 396 g/mol).

The detailed NMR spectral data are summarized as follows:

^{1}H-NMR (*CD_3OD, 600 MHz*): 1.68 (3H, m, H-13), 1.83 (3H, dd, 6.6 Hz, 2.4 Hz, H13), 2.21 (2H, q, 6.6 Hz, H-12), 3.28 (1H, dd, 9.0 Hz, 7.8 Hz, H-2′), 3.22 (1H, m, H-5′), 3.31 (1H, t, 9.0 Hz, H-4′), 3.35 (1H, t, 9.0 Hz, H-3′) 3.61 (2H, t, 6.6Hz, H-14), 3.68 (1H, dd, 12.0 Hz, 6.0 Hz, H-6′a), 3.88 (1H, dd, 12.0 Hz, 2.4 Hz, H-6′b), 4.29 (1H, dd, 6.0 Hz, 7.8 Hz, H-9), 4.34 (1H, d, 7.8 Hz, H-1′), 4.45 (1H, d, 6.0 Hz, H-8), 5.48 (1H, dd, 15.6 Hz, 7.8 Hz, H-10), 5.60 (1H, dd, 15.6 Hz, 2.4 Hz, H-3); 5.93 (1H, dt, 15.6 Hz, 6.6 Hz, H-11), 6.36 (1H, dd, 15.6 Hz, 6.6 Hz, H-2).

^{13}C-NMR (*CD_3OD, 150 MHz*): 18.8 (C-1), 29.8 (C-12), 32.9 (C-13), 62.3 (C-14), 62.7 (C-6′), 66.6 (C-8), 71.2 (C-6), 71.6 (C-4′), 72.5 (C-5), 74.8 (C-2′), 78.0 (C-3′), 78.0 (C-5′), 78.1 (C-4), 81.2 (C-7), 81.9 (C-9), 100.7 (C-1′), 110.5 (C-3), 126.5 (C-10), 138.9 (C-11), 145.3 (C-2).

The 1H-NMR and 13C-NMR spectra of compound **L** were consistent with those previously reported for lobetyolin by Masamichi Yuda et al. (1989), recorded in the same solvent [6].

Consequently, the purified compound was confirmed to be lobetyolin.

3.1.3. Evaluation of process reproducibility

To evaluate the reproducibility of the extraction and purification procedure, the experiment was repeated twice, each time using approximately 5 kg of powdered *C. javanica* root. The yields of the purified lobetyolin obtained were 1.31 g and 1.17 g, respectively. These results demonstrate that the isolation and purification process of lobetyolin from *C. javanica* exhibits satisfactory reproducibility.

3.1.4. Extraction, isolation, and purification procedure for lobetyolin from C. javanica

Based on the results obtained, the optimized extraction, isolation, and purification procedure for lobetyolin from *C. javanica* is summarized as follows:

Extraction

Approximately 5 kg of powdered *C. javanica* root was subjected to ultrasound-assisted extraction (UAE) for 30 minutes, repeated three times, using 50 L of 50% ethanol per extraction at a temperature not exceeding 50°C. The combined extracts were concentrated under reduced pressure to one-fifth of the initial volume. Water was added, and the resulting mixture was subjected to liquid-liquid partition with equal volumes of dichloromethane and ethyl acetate (repeated 3 times each). The ethyl acetate and aqueous phases were pooled and concentrated under reduced pressure to yield fraction **W**.

Isolation and purification of lobetyolin

Fraction W was subjected to silica gel column chromatography and eluted with a dichloromethane – methanol gradient (40 : 1, 20 : 1, 10 : 1, and 5 : 1, v/v) to yield four sub-fractions: **W1-W4**. Fractions **W3** and **W4** were combined to form fraction **W3**.

Fraction **W3** was further purified via silica gel column chromatography using a dichloromethane – methanol – water mixture (5 : 1 : 0.1, v/v/v) to obtain three sub-fractions: **W3A**, **W3B**, and **W3C**. Finally, fraction **W3C** was purified using preparative HPLC on a YMC C18 column (250 × 20 mm, 5 µm) with an isocratic mobile phase of 25% acetonitrile in water (flow rate: 8 mL/min; injection volume: 0.3 mL). Fractions corresponding to the retention time of lobetyolin were collected and concentrated under reduced pressure to obtain the pure compound **W3C1**.

3.2. Validation of the HPLC method for determination of impurities

3.2.1.Specificity

The specificity of the method was evaluated by comparing the chromatograms of the blank (methanol) and the test samples prepared according to the analytical procedure. Forced degradation studies were conducted under various stress conditions. Separate test samples were treated as follows: addition of 1 mL of 5 N HCl, 1 mL of 5 N NaOH, or 1 mL of 30% H_2O_2 (hydrogen peroxide), with each mixture left at room temperature for 30 minutes. For thermal degradation, the sample was heated at 80°C for 2 hours.

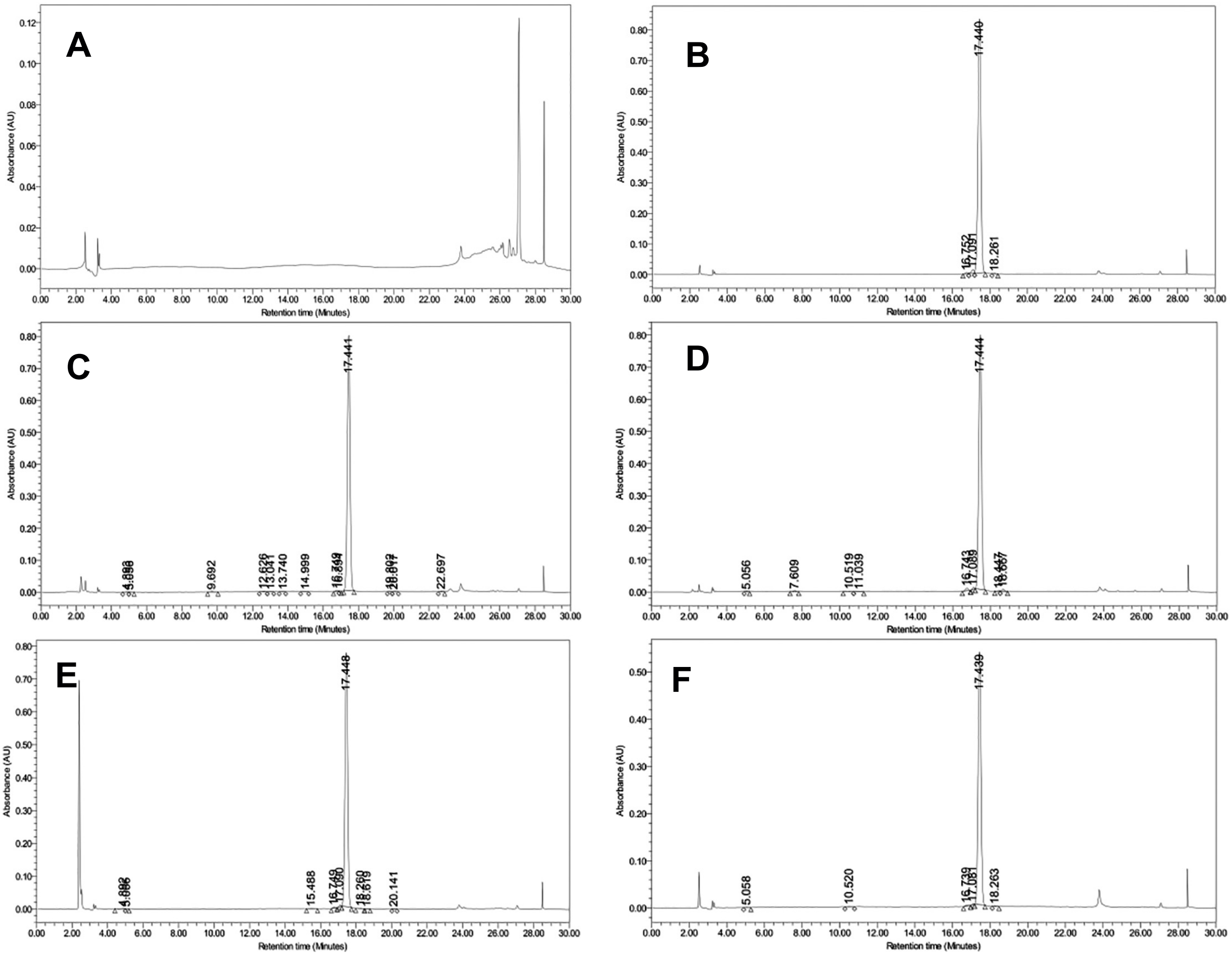

***Figure 4**. Typical chromatograms for specificity assessment (A: methanol, B: bulk material of lobetyolin, C – F: forced degradation of bulk material by different agents (C: acidic stress with HCl, D: alkaline stress with NaOH, E: oxydative stress with H2O2, F: thermal strees)).*

The specificity validation results indicated that the blank sample showed no interfering peaks near the retention time of the main peak (Figure 4-A). The forced degradation samples under all stress conditions exhibited significant degradation, with the closest impurity peak being adequately resolved from the main peak (minimum resolution = 1.51 > 1.5) (Figure 4, C-F). Additionally, the main peaks in all degraded samples met the requirements for peak purity. Therefore, the method demonstrates sufficient specificity [1].

3.2.2. Other criteria

The method was also assessed in terms of system suitability, linearity and range, limit of quantitation, limit of detection and precision. The results summarized in Table 1 proved that the method was suitable and reliable for determination of impurities in the bulk material of lobetyolin.

***Table 1**. Summary of validation results.*

Criteria	Requirements	Results
System suitability (n = 6)	RSD for retention time of lobetyolin: ≤ 1.0%	0.2%
	RSD for peak area of lobetyolin: ≤ 2.0%	0.8%
	Number of theoretical plates for peak of lobetyolin: ≥ 8000	60225 – 6571
	Tailing factor for peak of lobetyolin: ≤ 2.0	0.98 – 1.00
Linearity and range	Lower than impurity limit to 120% of impurity limit	0.50 – 50.0 µg/mL (2.5% of individual impurity limit to 167% of total impurity limit)
	$R^2 \geq 0.99$	$R^2 = 1.00$
	Accepted recovery range (%) at concentrations corresponding to impurity level (according to AOAC [5]):	Recovery range obtained with calibration curve:
	+ Less than 0.1%: 90 – 107%	97.5 – 97.8%
	+ From 0.1% to less than 1.0%: 95 – 105%	101.8%
	+ From 1.0%: 97 – 103%	99.3 – 100.7%
LOQ	Lower than individual impurity limit (2.0% or 10 µg/mL)	2.5% of individual impurity limit (0.05% or 0.50 µg/mL)
LOD		0.8% of individual impurity limit (0.016% or 0.16 µg/mL)
Precision	Repeatability (n = 6, one day, one analyst)	
	RSD values corresponding to total impurity level (if detected) + Less than 0.1%: ≤ 5.3% + From 0.1% to less than 1.0%: ≤ 3.7% + From 1.0%: ≤ 2.7%	Day 1: - Average total impurity: 1.02%, RSD = 0.6% Day 2: - Average total impurity: 1.02%, RSD = 0.6%
	Intermediate precision (n = 12, 2 days, 2 analysts)	
	RSD values corresponding to total impurity level (if detected) + Less than 0.1%: ≤ 5.3% + From 0.1% to less than 1.0%: ≤ 3.7% + From 1.0%: ≤ 2.7%	Day 1 + Day 2 - Average total impurity: 1.02%, RSD = 0.5%

3.3. Establishment of quality specifications for lobetyolin bulk material

Following the structural elucidation, the bulk material of purified lobetyolin was evaluated based on a comprehensive set of quality parameters, including identification (UV-Vis spectroscopy, IR spectroscopy, and melting point), loss on drying, specific optical rotation, and related substances, to establish a reference standard.

3.3.1. Identification

The UV-Vis spectrum, derived from the chromatogram of the reference solution in the impurities test provided two maxima at 214 nm and 266 nm (Figure 5), and the IR spectrum of bulk material gave also IR spectrum of lobetyolin (Figure 6). So the bulk material gave the expected UV-Vis and IR spectral responses of lobetyolin, and these identification tests were suitable for identify lobetyolin in bulk material.

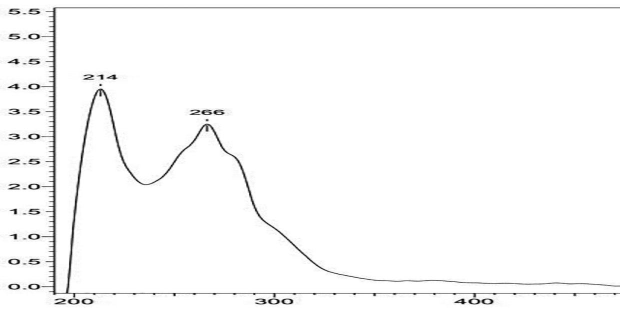

Figure 5. *UV-Vis spectrum of bulk material.*

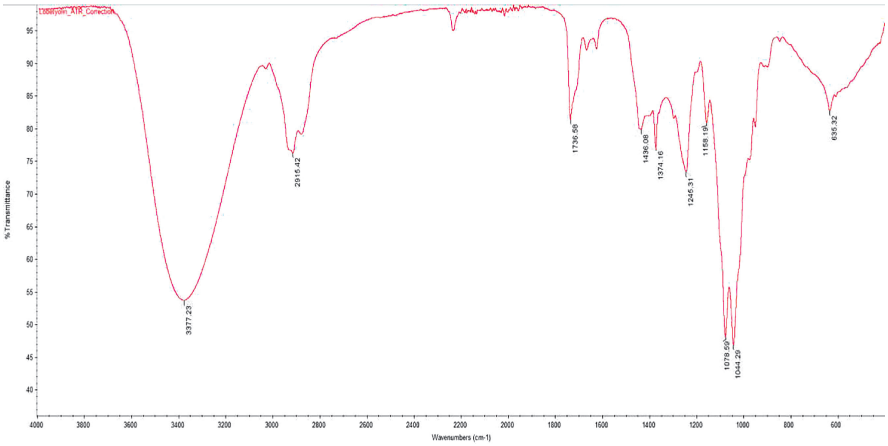

Figure 6. *IR spectra of bulk material*

3.3.2. Melting point

The melting point of bulk material of lobetyolin was determined using two different portions of the material, yielding values of 74.8°C and 75.0°C. Based on these results, the melting range from 74°C to 76°C was established as the acceptance criterion for the bulk material.

3.3.3. Loss on drying

Due to the relatively low melting point of lobetyolin (> 74°C), the previous use of Thermogravimetric Analysis (TGA) for loss on drying may have yielded unreliable results due to the potential influence of the sample melting phase. Given the improved extraction efficiency achieved in this study, we were able to obtain a sufficient quantity of the purified material to perform and establish a standardized loss on drying assay.

The assay was conducted in triplicate, yielding volatile impurity contents of 0.02%, 0.03%, and 0.02%. Consequently, an upper limit of not more than 0.2% was established for volatile impurities in the lobetyolin raw material.

3.3.4. Specific optical rotation

Lobetyolin possesses chiral centers at C8-C9, resulting in different stereoisomers (*erythro* and *threo* forms) with distinct optical rotation properties. Therefore, a supplementary method is essential for identification when NMR spectroscopy is not feasible. Recognizing the importance of differentiating these optical isomers, this study determined the specific optical rotation of the purified compound. This is a crucial supplementary criterion that was not evaluated or established in previous research [2].

The specific optical rotation was determined for three samples in methanol at a concentration of 4 g/L and a temperature of 25°C. The results yielded an average specific optical rotation of -28.12^0. Based on these findings, an acceptance criterion range of -26.7^0 to -29.5^0 was established for the specific optical rotation of lobetyolin (C = 4 g/L, methanol).

3.3.5. Impurities

The content of individual impurities and total impurities detected in the bulk material by using the

HPLC method validated in section 3.2 were summarized in Table 2. The results proved that the bulk material meet the proposed requirements on impurities as described in section 2.3.4.

Table 2. *Impurity determination in bulk material*

Date/Analyst	Individual impurity (%)	Average total impurities (%)
Day 1, analyst 1 (n = 6)	≤ 0.49	1.02
Day 2, analyst 2 (n = 6)	≤ 0.49	1.02

3.3.6. Lobetyolin content in the bulk material

Based on results of total impurities (1.02%, detailed results in section 3.3.5) and lost on drying (0.0233%, detailed results in section 3.3.3), the content of lobetyolin in bulk material was calculated as followed:

X (%) = (100% - 1.02%) * [100 - (0.0233)]/100 = 98.96%.

3.3.6. Proposed specifications of lobetyolin bulk material

From the results obtained in this study, the specification of bulk material of lobetyolin isolated and purified from Vietnamese *C. javanica* for establishment of lobetyolin reference standard was proposed as followed:

- *Appearance:* Yellow-brown powder.

- *Identification:*

+ *UV-Vis absorption spectrum:* In the wavelength range of 200 – 400 nm, absorption maxima are observed at 215 nm and 266 nm.

+ *Infrared (IR) spectrum:* The IR spectrum of the test sample must be concordant with that of the reference standard.

- *Loss on drying:* Not more than 0.2%.

- *Specific optical rotation:* From -26,7^{o} to -29,5^{o}.

- *Melting point:* từ 74^{o}C đến 76^{o}C.

- *Impurities:* Each individual impurity not more than 2.0%. Total impurities not more than 3.0%.

- *Content of lobetyolin:* not less than 95% (w/w).

4. CONCLUSION

In this study, an efficient procedure for the isolation and purification of lobetyolin from *C. javanica* was successfully developed and optimized, achieving a yield of 24.65% to 28.60%. By optimizing critical stages of the extraction process, this method ensures high reproducibility and scalability, rendering it suitable for the laboratory-scale production of lobetyolin reference material.

The purified compound was structurally characterized using UV-Vis and IR spectroscopy. Furthermore, a comprehensive set of quality specifications was established – encompassing related substances, volatile impurities, specific optical rotation, melting point, and chromatographic purity – thereby significantly upgrading the current quality control standards for lobetyolin raw materials. The purified product achieved a purity of 98.96 % (on an as-is basis), comfortably exceeding the purity threshold (> 95%) required for reference standard establishment. These findings provide a robust foundation for the future development and standardization of lobetyolin as a national reference standard.

REFERENCES

1. Ministry of Health (2019), *Vietnamese Pharmacopoeia V*, Vol. 2, Medical Publishing House, pp. 1154–1157.
2. Nguyen, T. H., Nguyen, T. M. T., Huynh, T. Q. D., Ngo, T. M. D., Tran, M. K., & Nguyen, M. D. (2021), HPLC analysis of lobetyolin in *Radix Codonopsis javanicae*, *Journal of Medicinal Materials*, 26(5), pp. 282–288.
3. Huynh, T. Q. D., Le, T. H. H., Phan, T. T. T., Nguyen, T. H., & Nguyen, M. D. (2021), Isolation and establishment of lobetyolin reference standard from *Radix Codonopsis javanicae*, *Journal of Medicinal Materials*, 26(3), pp. 154–160.
4. Ministry of Health and Welfare Taiwan (2022), *Taiwan Herbal Pharmacopeia*, 4th ed., English version, pp. 117–119.
5. Yuda, M., Ohtani, K., Mizutani, K., Kasai, R., Tanaka, O., Jia, M. R., Ling, Y. R., Pu, X. F., & Saruwatari, Y. (1990), “Neolignan glycosides from roots of *Codonopsis tangshen*”, *Phytochemistry*, 29(6), pp. 1989–1993.
6. AOAC (2016), *Official Methods of Analysis, Appendix F: Guidelines for Standard Method Performance Requirements*.