ESTABLISHMENT OF A REFERENCE STANDARD FOR EPIMEDIN C ISOLATED FROM HERBA EPIMEDII BY MASS-BALANCE

***NGUYEN LAM HONG**[1], ✉, **LE THI TRANG**[2], **TRAN VIET HUNG**[3]*
[1]Faculty of Analytical Chemistry and Drug Quality Control, Hanoi University of Pharmacy,
[2]National Institute of Drug Quality Control,
[3]Institute of Drug Quality Control Ho Chi Minh City,
✉Corresponding author: hongnl@hup.edu.vn

Received June 05th, 2026; Accepted June 15th, 2026

***Abstract:** Herba Epimedii comprises the dried aerial parts of various Epimedium species (family Berberidaceae). This medicinal material is widely recognized for its pharmacological activities, including sexual function enhancement, anti-osteoporotic, cardiovascular protective, anti-inflammatory, antioxidant, and immunomodulatory effects. Epimedin C is a primary bioactive constituent and is mandated as a reference standard for the qualitative and quantitative analysis of Herba Epimedii in the Vietnamese Pharmacopoeia V. Consequently, the development of a cost-effective, domestic Epimedin C reference standard is critical for the rigorous quality control of this botanical drug. In this study, 6.5 g of Epimedin C was isolated and purified from 5 kg of Herba Epimedii raw material. The structural integrity was confirmed via spectroscopic techniques (^{1}H and ^{13}C-NMR, IR) and HPLC-DAD profiling. The chromatographic purity was determined to be 98.2% using the area normalization method at a test concentration of 1.0 mg/mL, while the total volatile content was measured at 5.0% via Thermogravimetric Analysis (TGA). Finally, by applying the mass balance approach to account for all impurities, the label-assigned content for 125 vials of the Epimedin C reference standard was established as 93.3 ± 0.44% (k = 2).*

***Keywords:** Epimedin C, Herba Epimedii, isolation, mass-balance, reference standard.*

1. INTRODUCTION

Herba Epimedii is a medicinal material consisting of the dried aerial parts of several species belonging to the genus *Epimedium* (Berberidaceae). The herb is characterized by a high content of prenylated flavonoids, among which icariin and epimedin C are considered the major bioactive constituents. Numerous pharmacological studies have demonstrated that *Herba Epimedii* exhibits a wide range of biological activities, including enhancement of sexual function, anti-osteoporotic, cardioprotective, anti-inflammatory, antioxidant, and immunomodulatory effects. Owing to these diverse pharmacological properties, the herb has been extensively used in traditional medicine and modern therapeutic research for the supportive treatment of sexual dysfunction, osteoporosis, osteoarticular disorders, hypertension, coronary heart disease, and other chronic diseases. The characteristic chemical constituents of *Herba Epimedii* are prenylated flavonoids, among which icariin and epimedin C are regarded as the principal bioactive markers [1, 2].

Epimedin C is a prenylated flavonol glycoside with a kaempferol-derived aglycone skeleton. Its molecular structure is characterized by the presence of a prenyl group (3-methylbut-2-en-1-yl) attached at the C-8 position of the flavonol nucleus. In addition, the hydroxyl groups at C-3 and C-7 are glycosylated with characteristic sugar moieties, whereas the hydroxyl group at C-4′ is methylated to form a methoxy substituent. Structurally, epimedin C has been identified as an 8-prenylkaempferol derivative bearing an α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside moiety at C-3 and a β-D-glucopyranoside moiety at C-7. Epimedin C is readily soluble in polar aprotic solvents such as dimethyl sulfoxide (DMSO) and dimethylformamide (DMF), soluble in methanol and ethanol, sparingly soluble in acetone, chloroform, and diethyl ether, and practically insoluble in water [2, 3].

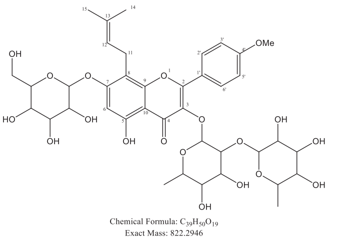


***Figure 1**. The chemical structure of epimedin C*

According to the Vietnamese Pharmacopoeia V (VP V) and the Chinese Pharmacopoeia 2020 (CP 2020) [4, 5], identification of *Herba Epimedii* is performed by thin-layer chromatography, while quantitative determination of icariin and epimedin C is conducted using HPLC–DAD with reference standards of icariin and epimedin C, respectively. In our previous work, a procedure for the establishment of an icariin reference standard isolated from *Herba Epimedii* was successfully reported [6]. In continuation of this effort, the present study describes an alternative approach for the establishment of an epimedin C reference standard based on the mass balance method [7]. The proposed strategy was developed to ensure a sustainable and independent supply of phytochemical reference standards isolated from medicinal materials, thereby supporting routine quality control and post-market surveillance of *Herba Epimedii* preparations in Vietnam.

2. MATERIALS AND METHODS

2.1. Materials

The medicinal material, (*Epimedium brevicornum* Maxim.), was purchased from the Institute of Medicinal Materials, Ministry of Health.

2.2. Chemicals

Reference standard: Epimedin C reference standard ($C_{39}H_{50}O_{19}$), exhibited an assigned content of 98.0%, calculated on the as-is basis, batch number CFN99941, Chemfaces.

Chemicals and solvents: dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), methanol (MeOH), analytical grade ethanol; acetonitrile: chromatographic grade.

2.3. Instruments

All analytical instruments and equipment used in this study were calibrated and qualified in compliance with GLP and ISO/IEC 17025 requirements at the National Institute of Drug Quality Control and the Ho Chi Minh City Institute of Drug Quality Control. The instrumentation included an HPLC–DAD system (Hitachi, Japan), a SCIEX QTRAP 6500+ hybrid triple quadrupole/linear ion trap mass spectrometer (USA), a Nicolet iS50 FT-IR spectrometer (Thermo Scientific, USA), and a Thermogravimetric Analyzer (Mettler Toledo, Switzerland). Gravimetric measurements were performed using Mettler Toledo precision balances (d = 1 mg) and analytical balances (d = 0.1 mg). Chromatographic materials included silica gel 60 F254 TLC plates, silica gel 60 (40 – 63 µm and 63 – 230 µm; Merck), and RP-18 reversed-phase silica gel (150 µm). Additional laboratory apparatus comprised a Büchi rotary vacuum evaporator, glass chromatography columns, calibrated micropipettes, and standard laboratory glassware. Structural elucidation was carried out using 500 and 600 MHz NMR spectrometers (Bruker, Germany) at the Institute of Chemistry, Vietnam Academy of Science and Technology.

2.4. Methods

2.4.1. Extraction

To obtain an epimedin C-enriched fraction, 5.0 kg of *Herba Epimedii* was initially subjected to mild drying at 60°C for 12 h to reduce residual moisture while minimizing thermal degradation of flavonoid glycosides. After equilibration to ambient temperature, the material was pulverized to a coarse particle size and homogenized thoroughly. The powdered drug was subsequently extracted by maceration with 50% ethanol (3 × 20 L), each extraction cycle being maintained for 7 days to ensure exhaustive diffusion of the target constituents into the hydroalcoholic solvent system. The combined percolates were filtered and concentrated under reduced pressure using rotary vacuum evaporation at 60°C, yielding 70 g of a viscous concentrated extract enriched in epimedin C-related constituents [7].

2.4.2. Isolation

The sample was dissolved in methanol, then mixed with silica gel, vacuum evaporated to dryness, and the dried sample was placed on a silica gel-packed column and eluted with a $CHCl_3$: MeOH : H_2O (80 : 20 : 1) solvent system to isolate epimedin C [7]. The presence of Epimedin C in the collected fractions was monitored by TLC on silica gel plates in comparison with an epimedin C reference standard [4].

2.4.3. Purification

Epimedin C was purified by column chromatography using reversed-phase silica gel RP-18 (150 µm) (Column 3 – RP1), and the elution solvent system was investigated by a reversed-phase silica gel RP-18 thin layer plate.

2.4.4. Structural confirmation of isolated epimedin C

1D-NMR, IR, and HPLC/DAD spectra of the isolated and standard epimedin C were measured and compared with publications [3, 6, 8] confirming that the purified substance was epimedin C.

2.4.5. The chromatographic purity determination of isolated epimedin C.

HPLC/DAD method to determine the chromatographic purity of isolated epimedin C.

Quantitative analysis of epimedin C was carried out according to the monographic procedures specified in the Vietnamese Pharmacopoeia V and the Chinese Pharmacopoeia 2020 using HPLC coupled with diode-array detection [4, 5]. Chromatographic separation was performed on a reversed-phase C18 column (250 × 4.6 mm, 5 µm). Elution was conducted with a binary mobile phase composed of acetonitrile and water under gradient conditions, in which the acetonitrile content was maintained at 30% during the first 5 min and subsequently adjusted from 30% to 27% over the 5 – 30 min interval. The injection volume was 10 µL, and chromatographic monitoring was carried out at 270 nm.

Chromatographic purity was evaluated by the area normalization method using a test solution prepared at a concentration of 1.0 mg/mL

System suitability requirements: The resolution between the epimedin C peak and the peaks eluting immediately before and after it shall not be less than 1.5. The tailing factor of the epimedin C peak shall be within the range of 0.8 – 2.0, and the number of theoretical plates (N) shall be not less than 2000.

Test solution: A solution of isolated epimedin C at a concentration of 1 mg/mL in methanol.

Reference solution: A standard epimedin C solution at a concentration of 1 mg/mL in methanol.

System suitability criteria: The %RSD of the major peak area obtained from the system suitability solution shall not exceed 5.0%.

Procedure: Inject the reference solution and the test solution into the chromatographic system and record the chromatograms.

$$\begin{array}{c}\text{\% Epimedin C's}\\ \text{chromatographic purity}\end{array} = \frac{\text{Speak epimedin C}}{(\Sigma S_{\text{impurities}} + S_{\text{peak epimedin C}})} \text{ x } 100\% \qquad (1)$$

Chromatographic purity was determined by the peak area normalization method, with an acceptance criterion of not less than 95.0%.

2.4.6. Method for determination of volatile impurities by thermogravimetric analysis (TGA).

The percentage of volatile impurities was determined by thermogravimetric analysis (TGA): Approximately 5 – 7 mg of the reference material was accurately weighed and subjected to thermal analysis under a nitrogen atmosphere at a flow rate of 40 mL/min. The temperature program was conducted from ambient temperature to 125°C at a heating rate of 10°C/min, followed by a total analysis time of 3 h. The cumulative mass loss between ambient temperature and the plateau region of the thermogram was subsequently calculated to estimate the volatile content.

2.4.7. Establishment of the epimedin C reference standard

- Vial filling and sealing:

A predetermined quantity of the material was dispensed into amber glass vials under an inert atmosphere at a relative humidity corresponding to the residual moisture content of the purified substance as determined by TGA. The vials were subsequently sealed with rubber stoppers and crimped with aluminum caps.

- Assessment of vial-to-vial homogeneity in accordance with ISO Guide 35:2017.

Ten units were randomly selected for homogeneity assessment. The content of each vial (calculated on the as-is basis) was determined in duplicate by HPLC under the chromatographic conditions described in the epimedin C chromatographic purity section. One vial was arbitrarily assigned a content value of 100.0%, and the contents of the remaining vials were calculated based on the relative peak areas with reference to the assigned vial. Vial-to-vial homogeneity was subsequently evaluated in accordance with ISO Guide 35:2017 using one-way ANOVA analysis.

- Assignment of the certified value for the Epimedin C reference standard by mass-balance.

Following vial filling and confirmation of satisfactory homogeneity, the reference material was subjected to characterization as follows:

- Chromatographic purity of Epimedin C was determined by HPLC–DAD independently at two separate drug quality control laboratories and calculated using the peak area normalization approach according to Equation (1).
- Water content and residual solvents, which are volatile impurities, were determined by thermogravimetric analysis (TGA).
- The assigned value of the reference standard was established using the mass-balance approach [10], according to Equation (2), as follows:

$$\text{X (\%, as-is basis)} = \text{Chromatographic purity (\%)} \times [100\% - \text{water (\%)} - \text{residual solvents (\%)} - \text{inorganic impurities (\%)}]/100 \qquad (2)$$

where : represents the assigned content of the reference standard calculated on the as-is basis.

Water content and residual solvents, which are volatile impurities, were determined by thermogravimetric analysis (TGA).

- Assessment of the uncertainty associated with the assigned value.

- Measurement uncertainty associated with the determination of related substances by HPLC–DAD and volatile impurities by TGA was evaluated.

Twelve vials of the candidate reference material were distributed to two independent quality control laboratories, with six vials analyzed by each laboratory. The participating laboratories performed the determination of chromatographic purity and volatile impurity content under the established analytical conditions.

- The standard uncertainty associated with each participating laboratory.

$$U_S = \frac{t \times S}{\sqrt{N}} \quad (3)$$

where: is the number of experimental replicates; is the Student's *t*-value obtained from the statistical table at the corresponding confidence level; and the standard deviation.

- Combined uncertainty of the two participating laboratories (U combined-Uc)

$$U_c = \sqrt{u_{s1}^2 + u_{s2}^2} \quad (4)$$

- Expanded measurement uncertainty.

The expanded uncertainty was calculated by combining the uncertainties associated with the two analytical procedures, namely the determination of related substances and volatile impurities, according to the following equation:

$$U_{mr} = \sqrt{u_{c1}^2 + u_{c2}^2} \quad (5)$$

- Value assigned on the certificate of analysis (CoA): X ± 2. Umr

3. RESULTS AND DISCUSSION

3.1. Isolation and purification of epimedin C:

3.1.1. Isolation of epimedin C

With reference to the procedures described in the Vietnamese Pharmacopoeia V and the previously reported method for icariin isolation and purification [4, 6], crude epimedin C was further isolated from the concentrated ethanol extract remaining after removal of icariin on the first silica gel column (63 – 230 µm) using a $CHCl_3 : MeOH : H_2O$ (85 : 15 : 1) mobile phase system. Subsequent enrichment of epimedin C was achieved on the first silica gel column (63 – 230 µm) using the solvent system $CHCl_3 : MeOH : H_2O$ (80 : 20 : 1), followed by final purification on a second silica gel column packed with finer particles (40 – 63 µm) and eluted with the same solvent composition to obtain purified epimedin C. Isolation of epimedin C was therefore carried out sequentially on two silica gel-packed chromatographic columns as follows:

Column 1: Seventy grams of concentrated *Herba Epimedii* extract was dissolved in approximately 100 mL of methanol and subsequently adsorbed onto 150 g of silica gel (63 – 230 µm). The mixture was evaporated to dryness under reduced pressure to obtain a free-flowing powder suitable for dry loading. The prepared sample was then applied onto a glass chromatographic column (8 cm i.d.) packed with 800 g of silica gel (63 – 230 µm) and eluted with $CHCl_3 : MeOH : H_2O$ (85 : 15 : 1) to (80 : 20 : 1). The presence of Epimedin C in the collected fractions was monitored by TLC on silica gel plates in comparison with an epimedin C reference standard. Fractions containing epimedin C were combined and concentrated under reduced pressure to remove the solvent, yielding fraction 2 enriched in epimedin C (23 g).

Column 2: Fraction 2 was dissolved in approximately 20 mL of methanol and blended with 50 g of silica gel (40 – 63 µm). The solvent was subsequently removed under reduced pressure to afford a dry, free-flowing adsorbed powder suitable for column application, which was subsequently applied onto a glass column (6 cm i.d.) packed with 180 g of silica gel (40 – 63 µm). Elution was carried out using $CHCl_3 : MeOH : H_2O$ (85 : 15 : 1) to (80 : 20 : 1) as the mobile phase. Fractions exhibiting the characteristic epimedin C spot were pooled and concentrated to dryness under reduced pressure, affording fraction 2.1 (11.1 g).

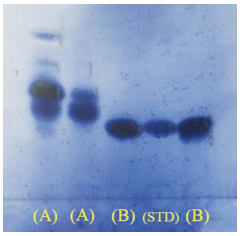


Figure 2. *TLC chromatograms of the fractions obtained during epimedin C separation from the ethanolic extract on the second chromatographic column using the mobile phase systems (A) $CHCl_3 : MeOH : H_2O$ (85 : 15 : 1) and (B) $CHCl_3 : MeOH : H_2O$ (80 : 20 : 1), in comparison with the epimedin C reference standard.*

3.1.2. Purification of epimedin C on a reversed-phase column

A 11.1 g portion of fraction 2.1 was subjected to further purification by reversed-phase column chromatography using 300 g of RP-18 silica gel (150 µm) (Column 3 – RP1). Elution was performed with a methanol – water system (60 : 40). Fractions containing epimedin C were combined and concentrated under reduced pressure to remove the solvent, yielding 6.5 g of epimedin C material suitable for reference standard establishment.

3.2. Structural confirmation and the chromatographic purity evaluation of epimedin C.

3.2.1. Structural confirmation of epimedin C

The isolated compound was obtained as a yellow amorphous powder. The ^{1}H-NMR spectrum exhibited characteristic singlet and doublet resonances attributable to four methyl groups at δ_H 0.94 (3H, d, J = 6.0 Hz), 1.23 (3H, d, J = 6.0 Hz), 1.66 (3H, s), and 1.74 (3H, s), together with a methoxy signal at δ_H 3.91 (3H, s, OMe). In the downfield region, aromatic proton signals were observed at δ_H 6.67 (1H, s, H-6), along with an A_2B_2 aromatic spin system at δ_H 7.11 (2H, d, J = 8.4 Hz, H-3′, H-5′) and 7.90 (2H, d, J = 9.0 Hz, H-2′, H-6′). Signals corresponding to anomeric protons appeared at δ_H 5.02 (1H, d, J = 1.8 Hz), 5.08 (1H, d, J = 7.8 Hz), and 5.56 (1H, d, J = 1.2 Hz), in addition to multiple aliphatic proton resonances distributed between δ_H 3.34 – 4.30.

The ^{13}C-NMR spectrum revealed the presence of 39 carbon signals, including a conjugated carbonyl carbon at δ_C 180.0, fourteen aromatic carbons resonating within δ_C 110.6 – 163.6, and three anomeric carbons at δ_C 102.3, 101.95, and 103.7. The remaining carbon resonances were distributed over the range δ_C 17.8 – 99.4. The coupling constants of the anomeric protons together with the chemical shifts of the corresponding carbons suggested the presence of glucose and rhamnose moieties in the molecule. Comprehensive interpretation of the 1D-NMR spectroscopic data, supported by comparison with published literature values, led to identification of the isolated compound as epimedin C.

^{1}H-NMR (600 MHz, CD_3OD): δ_H (ppm): 0.94 (3H, d, J = 6.0 Hz, CH_3 (rha-2)); 1.23 (3H, d, J = 6.0 Hz, CH_3 (-3-O-rha)); 1.66 (3H, s, H-15); 1.74 (3H, s, H-14); 3.91 (3H, s, OMe); 5.02 (1H, d, J = 1.8 Hz, H-1(rha-2)); 5.08 (1H, d, J = 7.8 Hz, H-1(-Glc)); 5.21 (1H, t, J = 6,0 Hz, H-12); 5.56 (1H, d, J = 1.2 Hz, H-1(-3-O-rha)); 6.67 (1H, s, H-6); 7.11 (2H, d, J = 8.4 Hz, H-3′, H-5′); 7.90 (2H, d, J = 9.0 Hz, H-2′, H-6′);

^{13}C-NMR (150 MHz, CD_3OD), δ_C (ppm): Aglycon: [18.3 (C-15); 22.7 (C-11); 25.9 (C-14); 56.1 (OMe); 99.4 (C-6); 103.7 (C-10); 110.6 (C-8); 115.2 (C-3’, 5’); 123.6 (C-12); 123.9 (C-1’); 131.9 (C-2’, 6’); 132.7 (C-13); 136.6 (C-3); 155.0 (C-9); 159.2 (C-2); 161.0 (C-4’); 162.1 (C-5), 163.6 (C-7); 180.0 (C-4)]; Glc: [102.3 (C-1); 74.9 (C-2); 78.3 (C-3); 70.3 (C-4); 78.9 (C-5); 62.4 (C-6)]; 3-O-rha: [102.0 (C-1); 78.2 (C-2); 72.1 (C-3); 74.0 (C-4); 71.1 (C-5); 17.9 (C-6)]; Rha-2: [103.7 (C-1); 71.9 (C-2); 72.3 (C-3); 73.5 (C-4); 72.0 (C-5); 17.8 (C-6)].

Comparison of the 1D-NMR spectroscopic data of the purified compound with previously reported literature values [3, 6, 8] confirmed its structural identity as epimedin C.

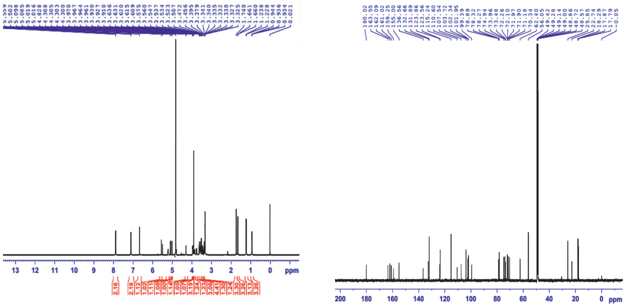
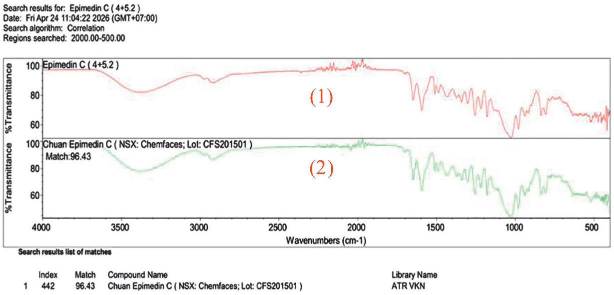


***Figure 3**. ^{1}H-NMR and ^{13}C-NMR spectra of the purified compound.*

The infrared spectrum (IR)

The infrared spectrum IR (KBr), ν_{max} (cm^{-1}) of the purified substance corresponded to that of the epimedin C standard (coefficient of similarity 96.43%) (Figure 4).


Search results for: Epimedin C (4+5.2)
Date: Fri Apr 24 11:04:22 2026 (GMT+07:00)
Search algorithm: Correlation
Regions searched: 2000.00-500.00

Search results list of matches

	Index	Match	Compound Name	Library Name
1	442	96.43	Chuan Epimedin C (NSX: Chemfaces; Lot: CFS201501)	ATR VKN

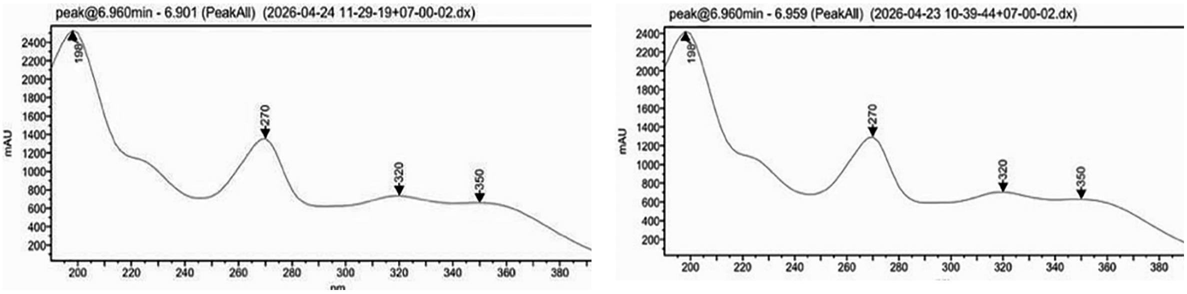
***Figure 4**. IR spectra of the purified compound (1) and the Epimedin C reference standard (2)*

HPLC/DAD

The retention time of the main peak on the chromatogram of the test solution (6.959 minutes) corresponds to the retention time of the epimedin C peak (6.901 minutes) on the chromatogram of the standard solution.

Ultraviolet-Vis (UV-Vis) absorption spectrum

The UV absorption profile of the purified compound was recorded by HPLC-DAD over the wavelength range of 200 – 400 nm using a methanolic solution at a concentration of 1 mg/mL. The obtained spectrum was consistent with that of the Epimedin C reference standard, exhibiting a characteristic absorption maximum at 270 nm (Figure 5).



UV spectra of epimedin C standard (1)

UV spectra of purified substance (2)

Figure 5. *UV spectra of epimedin C standard and purified substance*

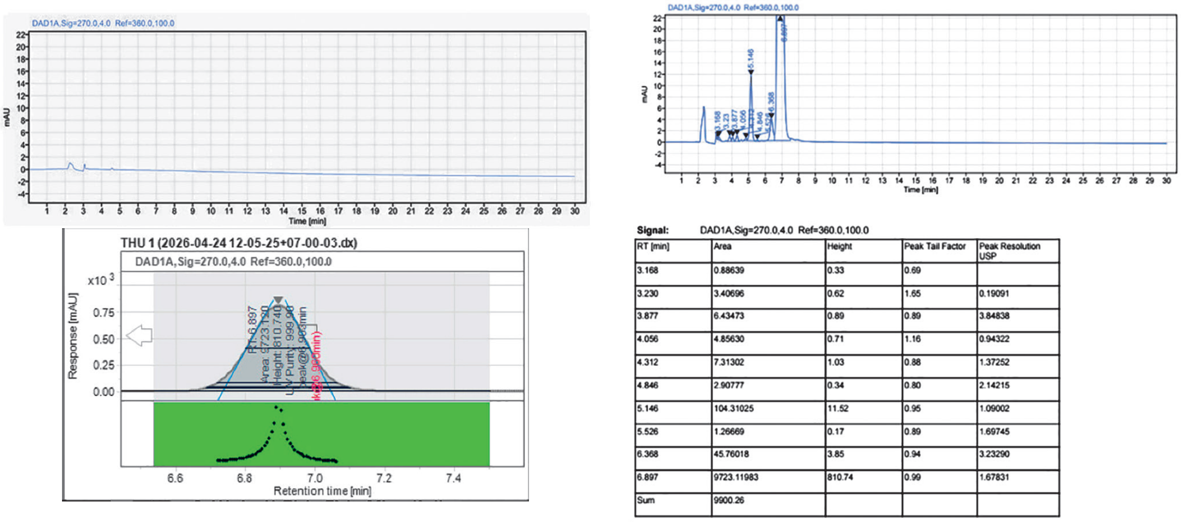
The ^{1}H-NMR spectral data, together with the IR spectral overlap, HPLC/DAD chromatographic behavior, and UV absorption profiles of both the isolated compound and the reference standard, collectively demonstrate a high degree of structural and physicochemical concordance, thereby confirming the identity of the purified substance as epimedin C.

3.2.2. Determination of the purity of the isolated epimedin C

3.2.2.1. Determination of the chromatographic purity of the isolated epimedin C by HPLC/DAD

The chromatographic purity of the isolated compound was determined using an area normalization approach in accordance with the established analytical method.

Chromatograms of the test solutions were recorded under validated HPLC conditions, and the retention time together with the integrated peak area corresponding to epimedin C was systematically evaluated. This procedure was performed in triplicate independent measurements to ensure analytical reliability and reproducibility. The resulting data indicated an average chromatographic purity of 98.2% for epimedin C, as illustrated in Figure 6.



Signal: DAD1A,Sig=270.0,4.0 Ref=360.0,100.0

RT [min]	Area	Height	Peak Tail Factor	Peak Resolution USP
3.168	0.88639	0.33	0.69	
3.230	3.40696	0.62	1.65	0.19091
3.877	6.43473	0.89	0.89	3.84838
4.056	4.85630	0.71	1.16	0.94322
4.312	7.31302	1.03	0.88	1.37252
4.846	2.90777	0.34	0.80	2.14215
5.146	104.31025	11.52	0.95	1.09002
5.526	1.26669	0.17	0.89	1.69745
6.368	45.76018	3.85	0.94	3.23290
6.897	9723.11983	810.74	0.99	1.67831
Sum	9900.26			

Figure 6. *Chromatogram of blank sample, test solution for determining chromatographic purity and epimedin C peak purity.*

3.2.2.2. Volatile impurities were determined by thermogravimetric analysis (TGA).

Approximately 6 mg of the reference standard was accurately weighed into a TGA crucible. Analyze the sample using the established thermogravimetric method to determine the volatile content (estimated at approximately 5.0%).

3.3. Establishment of the epimedin C reference standard

3.3.1. Vial filling and sealing:

The Epimedin C reference material was processed by dispensing 10 mg aliquots into individual 2 mL amber glass vials, yielding a total of 125 units. The filling and sealing operations were conducted under strictly controlled environmental conditions, maintaining a relative humidity of < 5% and a temperature of 25°C ± 2°C. To ensure long-term stability and prevent oxidative degradation, each vial was purged with ultra-high purity nitrogen (99.999% N_2) prior to hermetic sealing at the Institute of Drug Quality Control Ho Chi Minh City.

3.3.2. Assessment of Between-Vial Homogeneity

A set of ten vials was randomly selected from the batch to assess content homogeneity, following the procedure detailed in section 2.4. A one-way Analysis of Variance (ANOVA) was performed to evaluate the between-vial variation. The results confirmed that the calculated F-statistic was less than the critical value (F < Fcrit), demonstrating no statistically significant difference in content among the sampled units, thereby confirming the homogeneity of the reference standard batch.

3.3.3. The assigned value of the Epimedin C reference standard was determined using the Mass Balance Approach

3.3.3.1. Determination of Chromatographic Purity of Epimedin C by HPLC-DAD

Twelve vials were randomly sampled from the qualified batch to confirm unit-to-unit consistency. The selected units were distributed equally between two independent laboratories for inter-laboratory assessment of chromatographic purity. Each laboratory determined the purity of Epimedin C by the area normalization method, as detailed in Section 2.4, by preparing a single test solution from each vial. The analytical results are summarized in Table 1, yielding a mean chromatographic purity of 98.2% for the Epimedin C reference standard batch.

Table 1. *Determination of Chromatographic Purity of Epimedin C by HPLC-DAD*

	Determination of Chromatographic Purity of Epimedin C (%)	
Order	**Laboratory 1**	**Laboratory 2**
1	98.18	98.21
2	98.14	98.21
3	98.14	98.22
4	98.17	98.21
5	98.15	98.17
6	98.17	98.17
Average (n = 6)	**98.16**	**98.20**
SD	0.016	0.022
Us	**± 0.017**	**± 0.023**
Average (n = 12)	98.2	
$Uc_{(HPLC)}$	$Uc_{(HPLC)} = \pm 0.03$	

3.3.3.2. Volatile impurities were determined by thermogravimetric analysis (TGA).

A test portion of approximately 6 mg of the Epimedin C reference standard was accurately weighed into a tared TGA crucible. Thermogravimetric Analysis (TGA) was performed according to the validated parameters specified in the research methodology. The total volatile content, comprising both moisture and residual solvent, was determined by calculating the mean mass loss from six replicate experiments (n = 6). As summarized in Table 2, the average total volatile impurity content was determined to be 5.0%.

Table 2. *Determination of Total Volatile Content by Thermogravimetric Analysis (TGA).*

Order	Weighed amount (mg)	Vial numbering sequence	Volatile impurities content (%)
1	5.70	19	4.53
2	6.50	37	4.59
3	6.20	55	5.51
4	5.90	64	4.79
5	6.30	78	5.33
6	6.10	104	5.28
Average			5.01
SD			0.42
Us (TGA)			0.44

3.3.3.3. The assigned value of the Epimedin C reference standard was determined by the Mass Balance Approach

- The content of Epimedin C (%, as-is) is determined according to the following formula:

X (% as-is) =(98.2) x [(100-5.0)/100]= 93.3%

- Expanded measurement uncertainty

Expanded measurement uncertainty Umr = $\sqrt{u^2_{c(HPLC)} + u^2_{c(TGA)}}$

$$U_{mr(epimedin\ C)} = \sqrt{0.03^2+0.44^2} = \pm 0.44\%$$

- The label-assigned content of the Epimedin C reference standard is 93.3 ± 0.44% (k= 2)

Discussion

The qualitative identification of Epimedin C was performed in accordance with the Epimedium monograph of the Vietnamese Pharmacopoeia V, utilizing Thin Layer Chromatography (TLC) with silica gel G as the stationary phase and a solvent system of $CHCl_3$: MeOH : H_2O (3 : 1 : 0.1, v/v/v) as the mobile phase [4]. The isolation of Epimedin C was optimized based on established protocols [6]. Initially, the crude extract underwent fractionation using two consecutive silica gel column chromatography stages, employing a $CHCl_3$: MeOH : H_2O (80 : 20 : 1, v/v/v) solvent system. To achieve the required purity for reference standard designation, the semi-purified residue was subjected to reversed-phase (RP-18, YMC) preparative chromatography using an isocratic methanol-water (60 : 40, w/w) mobile phase. This solvent system was selected as a cost-effective alternative to the acetonitrile-water gradient previously reported [4]. In comparison with the findings of Nguyen Van Hoang, Tran Viet Hung, et al. [7], a robust and reproducible procedure for the isolation and purification of Epimedin C was successfully established, affording 6.5 g of highly purified material, approximately three times the yield reported previously [7]. The isolated Epimedin C achieved a chromatographic purity of 98.2% by HPLC–DAD and was comprehensively characterized using 1D NMR, IR, and HPLC–DAD techniques, providing a reliable basis for its application as a reference standard.

The chromatographic purity of the Epimedin C reference standard was determined using the area normalization method. The test solution was prepared at a concentration of 1.0 mg/mL to optimize the detection of all impurities. The chromatographic conditions were adapted from the quantitative assay for *Epimedium* in the Vietnamese Pharmacopoeia V, utilizing a C18 column (250 x 4.6 mm, 5 µm). The elution was performed using a gradient system of acetonitrile and water (0 – 5 min: 30% acetonitrile; 5 – 30 min: 30% to 27% acetonitrile), with UV detection at 270 nm [4, 5]. Furthermore, the total volatile content, including moisture and residual solvents, was determined to be 5.0% using Thermogravimetric Analysis (TGA).

In this study, the determination of inorganic impurities via the sulfated ash test was excluded based on the following considerations. Firstly, the isolation process of Epimedin C employed solely organic solvents and chromatographic media, minimizing the risk of inorganic contamination; thus, the residual inorganic content in the purified material is expected to be negligible. Secondly, adherence to the Vietnamese Pharmacopoeia V (Appendix 9.9) necessitates a significant sample mass (0.5 – 1.0 g per test, in duplicate) for sulfated ash determination. Given the low extraction yield inherent to the isolation of secondary metabolites from natural sources, such sample consumption is unjustified. Consequently, the purity assessment focused on organic impurity profiling and volatile content determination, which are more indicative of the stability and composition of the isolated reference standard.

This study addresses the methodology for establishing primary reference standards using the mass balance approach, particularly when certified primary standards are unavailable, rare, or cost-prohibitive [10]. Furthermore, this work expands upon the foundational research by Nguyen Van Hoang, Tran Viet Hung, et al., which focused on the establishment of Epimedin C secondary standards traceable to a certified primary standard. Our approach provides an alternative strategy for reference standard characterization, independent of traditional secondary standard-to-primary standard traceability models [7].

4. CONCLUSION

This study describes the isolation, purification, and characterization of the Epimedin C reference standard. Epimedin C was initially isolated from Epimedium extract via multi-stage silica gel column chromatography, employing a mobile phase system of $CHCl_3$: MeOH : H_2O (80 : 20 : 1, v/v/v). Subsequent purification was achieved using reversed-phase (RP-18) preparative chromatography with an isocratic methanol – water (60 : 40, w/w) mobile phase, yielding 6.5 g of the purified compound. Structural identity was unambiguously confirmed using 1D-NMR (^{1}H and ^{13}C), IR spectroscopy, and HPLC-DAD analysis. The purified material was subdivided into 125 vials, each containing 10 mg of Epimedin C. Following a successful evaluation of between-vial homogeneity, the chromatographic purity was determined to be 98.2% via HPLC-DAD. The volatile content was quantified at 5.0% using Thermogravimetric Analysis (TGA). Based on the mass balance approach, the label-assigned content of the Epimedin C reference standard was determined to be 93.3%, with an expanded uncertainty of ±0.44% at an approximate 95% confidence level (k = 2).

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the conduct and reporting of this study.

REFERENCES

1. Do Tat Loi, Vietnamese medicinal plants and remedies, *Medical Publishing House;* 2015: 905-906.
2. Ziying Huang and et al (2026), *Epimedium*: Medicinal plant resources, chemical constituents, pharmacological actions, and biosynthesis of prenylated flavonol glycosides, *Industrial Crops and Products*, Volume 245, Article: 123320.
3. Fazila Zulfiqara and et al (2017), Prenylated flavonol glycosides from *Epimedium* 2 *grandiflorum*: cytotoxicity and evaluation against 3 inflammation and metabolic disorder, *Phytochemistry Letters* Volume 20, Pages: 160-167.
4. Vietnam Pharmacopoeia Council, Ministry of Health (2019), *Epimedium* monograph, Vietnam Pharmacopoeia V, Volume II, Medical Publishing House, Hanoi, 1134-1135.
5. Chinese Pharmacopoeia Commission-Ministry of Health, *The People of Republic of China Pharmacopoeia*, People's Medical Publishing House; Volumn Ia, 2020:184-186
6. Nguyen Lam Hong et al. (2026), Establishment of icariin standard isolated from *Herba epimedii* by mass balance method, Journal of Medicine and Pharmacy N°. 70, February 2026, 84-94.
7. Nguyen Van Hoang et al. (2017), Isolation and establishment of epimedin C standard from *Herba Epimedii*, Ho Chi Minh City Medical Research, Supplement Vol. 21, N°. 1, 2017, 395-400.
8. Juntao Li and et al (2022), Isolation, bioassay and 3D-QSAR analysis of 8-isopentenyl flavonoids from *Epimedium sagittatum maxim*. as PDE5A inhibitors, Chinese Medicine, Volume 17, article number 147.
9. International Organization for Standardization (2017), Guide 35: Reference materials–Guidance for characterization and assessment of homogeneity and stability, *International Organization for Standardization (ISO)*, Geneva, 114.
10. World Health Organization (2016), *The International Pharmacopoeia, Reference Substances and Reference Spectra: Reference substances and reference spectra,* World Health Organization.