Development and validation of HPLC method for determination of β-ecdysone from *Callisia fragrans* (Lindl.) woodson

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ABSTRACT
The HPLC method for the determination of β-ecdysone from Callisia fragrans was developed and validated. Thereby, crushed herbal powder of stem and leafs of Callisia fragrans (Lindl.) Woodson was extracted with MeOH by sonification. The MeOH extract was evaporated at low pressure to obtain the residue of total extract. This MeOH fraction was carried out liquid - liquid extracting method with solvents of increasing polarity: n-hexane, EtOAc. The fraction EtOAc containing β-ecdysone was performed by using the HPLC method. The simple chromatographic separation condition was carried out on a C18 column using isocratic mobile phase consisting of 60% (v/v) methanol and 40% (v/v) water with flow rate of 1.0 mL/min. The eluted process was monitored by a diode-array detector at wavelength of 242 nm. Results obtained in validation demonstrated an linear relationship between the corresponding peak areas and the concentration of β-ecdysone: y = 164896x - 42687, as confirmed by the correlation coefficient of 0,998. The concentration of β-ecdysone in Callisia fragrans (Lindl.) Woodson was determined by using the HPLC with 0.052% of dry powder.

Keywords: *Callisia fragrans , β-ecdysone , HPLC*

1. INTRODUCTION

Callisia fragrans (Lindl.) Woodson, Commelinaceae, is originated from Central America, also distributed to other places. It's a medicinal herbal remedy for treatment from *Callisia fragrans* (Lindl.) Woodson, many studies detected a lot of different compounds such as phenolic, flavonoids, coumarins, anthraquinone, triterpenic compounds [1], which have tremendous value in medicine and the other purposes. Ecdysteroids are considered as the steroid hormones, a large group of compounds, similar in structure, exist in *Callisia fragrans* [2]. These compound has promising applications in medicine, decreasing the blood cholesterol and glucose level, increasing protein anabolism. The major compound of ecdysteroids was β-ecdysone, which is a mainly important active compound using for treatment several of diseases: angiocardiopathy, herpes zoster, diabetic, inflammatory…[3]. β -ecdysone was detected in both animals and plants species. However, β-ecdysone in plants often have higher concentrations than in the arthropods, should be more isolated [4]. To identify and determine some ecdysteroids, highperformance liquid chromatography with mass selective detection (HPLC-MS) has successfully been employed [5]. The quantitative method for determination of β-ecdysone from plants has been still limited. The aim of this study is to set up the efficient and simple HPLC-DAD method for deter-mination of the β-ecdysone content,

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an important ecdysteroid from *Callisia fragrans.*, a species of Commelinaceae.

2. MATERIALS AND METHODS

2.1. Chemicals, reagents and plant materials

β-ecdysone (>= 99,5%, HPLC) was purchased from Sigma-Aldrich and used to prepare the stock solution of 1.5 mg/10 mL in methanol. Methanol (CH_3OH, HPLC grade) purchased from Merck, was use for mobile phase and sample preparation.

Callisia fragrans (Lindl.) Woodson, Commelinaceae, was collected in Ho Chi Minh city, Viet Nam in May 2020. The plant was identified by Dr. Ly Ngoc Sam, Institute of Tropical Biology, Viet Nam. Fresh sample was dried, powdered to give crushed sample with the moisture content range 10 - 12%.

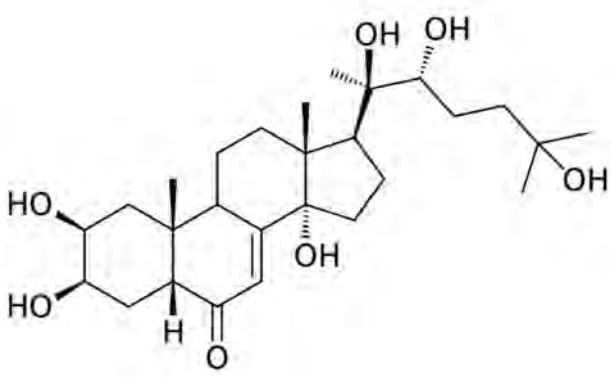

Figure 1. The structure of β-ecdysone

2.2. Instrumentation and chromatographic conditions

The Shimadzu LC-20 HPLC system was use for analytical method, equipped with quaternary pump (LC-20 AD), auto sampler injector loop 20 L (Rheodyne), degasser (DGU-14A), thermostatted column compartment (CTO-10A), and a detector (DAD-20A), a HiQ sil C18HS (C18) columm, 5 µm, 250 mm; 4,6 mm i.d., was used in the chromatographic analysis.

From our results in primary survey of mobile phase conditions, the most suitable chromatographic conditions was applied with the isocratic program as a mobile phase mixture including methanol:water (60:40, v:v). The detection wavelength was 242 nm and flow rate of 1 mL/min.

2.3. Standard and working solutions

Accurately weighed appropriate 1.5 mg of β-ecdysone and dissolved with methanol in a 10.0 mL volumetric flask to a final concentration of 150 g/mL. The solution was sonicated to have the absolute solution. Working solutions (5.0 to 150.0 µg/mL) were prepared by dilution of aliquots of the standard solution with methanol (150 µg/mL) in a 5.0 mL volumetric flask.

2.4. Sample preparation

1 g powder of stem and leafs was extracted with 10 mL x 6 times methanol (MeOH) under sonification, the MeOH extracts were pooled together and evaporated under vacuum pressure to give the crude extract. The crude extract was continously partitionated with n-hexane (10 mL x 6 times) and ethyl acetate (EtOAc, 10 mL x 6 times), respectively. The crude EtOAc fraction was dissolved with the minimum volume MeOH, fixed in a 10 mL volumetric flask with mobile phase. Afterward, the resulting solution was filter through 4.5 m membrane (Milipore) to obtain the sample solution for HPLC quantitative analysis of β-ecdysone.

Formula for calculating β-ecdysone content ©%) was followed by:

$$C\ (\%) = [(A \times d)/m] \times 100 \times 10^{-6}$$

Where:
A: Concentration of β-ecdysone calculated from the linear regression.
d: Dilution (10 times).
m: Weight of sample (1 g).
100: Convert to percentage.
10^{-6}:Convert g to g unit.

2.5. Validation of analytical method for β-ecdysone

The analytical method for determining β-ecdysone contents in *Callisia fragrans* was validated by Appendix F of 2016 AOAC guidelines [6], with following parameters were determined: specificity, linearity, accuracy, precision, sensitivity.

2.5.1. Specificity

The specificity was demonstrated by running a procedural blank, where 2 mL of methanol were transferred to a suitable volumetric flask and diluted to 10 mL with mobile phase. In addition, the peaks of the β-ecdysone that could be found in EtOAC extracts of *Callisia fragrans* was determined by analysis of chromatograms of the standard solution and the sample solution and the standard spiked sample solution.

2.5.2. Linearity

The linearity of the calibration curve for the β-ecdysone was established by the external standard method, based on five concentrations: 5, 15, 40, 100 and 150 µg/mL. Three determinations were carried out for each solution. The calibration curves were obtained by plotting the peak area of the β-ecdysone versus the concentration of the standard solutions. The statistical parameters of the calibration curve as slope, intercept, and correlation coefficient were calculated by linear regression analysis.

2.5.3. Accuracy

The accuracy was evaluated by means of recovery assays carried out by adding known amounts of the standard to the sample, at three different levels (80%, 100% and 120%) of the initial concentration of the sample. For evaluation of recovery, β-ecdysone standard was added to the sample whose final concentration were 40, 50 and 60 µg/mL of this compound. Each solution was injected in triplicate. Average recoveries were calibrated by the formula recovery (%) = {(amount found – original amount)/amount spiked} x 100.

2.5.4. Precision

The repeatability of the method was evaluated for intra-day. The standard solutions were analyzed at three concentrations (50, 100 and 150 µg/mL). Three determinations were carried out for each solution. The relative standard deviation (R.S.D.%) within the measurements of the concentrations of β-ecdysone was used to evaluate the repeatability and intermediate precision.

2.5.5. Sensitivity

Limits of Detection (LOD) and Quantitation (LOQ) were determined using a calibration curve method according to ICH recommendations. The response standard deviation (RSD) was taken as the SD of y-intercepts of the regression lines of the three calibration curves. The LOD were calculated as follows:

$$LOQ = 10 \times \sigma / S$$
$$LOD = 3.3 \times \sigma / S$$

where σ is the standard deviation of the regression line, and S is the slope of the calibration curve.

2.6. Statistical analysis

All the experiments was independently performed 3 times, and the data of values of average, standard deviation (SD), and relative standard deviation (RSD) and charts were analyzed by Microsoft Office Excel 2016.

3. RESULTS AND DISCUSSION

3.1. Validation

To develop the HPLC for determination of β-ecdysone, the chromatographic conditions such as eluent solvents, separative C18 column were evaluted and compared. For quan-tifying β-ecdysone of *Pfaffi a glomerata* (Amaranthaceae, known as Brazilian ginseng), the analysis were carried out a Phenomenex Column C18, 5 µm part size, 250 mm; 4,6mm i.d., gradient system using as mobile phase a mixture of methanol and water ranged from 10:90 (v/v) to 70:30 (v/v), flow rate 1,0 mL and detection at 245 nm [8]. In the other study, β-ecdysone of *Serratula komarovii* (Asteraceae), Acetonitrile-water (20:80) was used as eluent at a rate of 1 mL/min with Hypersil ODS column, 250 mm; 4.0 mm i.d. and dectection at 248 nm [9]. The HPLC method carried out in this study was to develop a simple and efficient chromatographic method, capable of eluting

and resolving β-ecdysone in *Callisia fragrans*. Solvent systems (methanol-water, acetonitrile-water in several different ratio) and separation columns HiQ sil C18HS column (250 x 4.6 mm i.d., 5 µm particle size), Supelco C18 (250 x 4.6 mm i.d., 5 µm particle size) were evaluated and compared. The optimization of the chromatographic conditions used MeOH and water as a isocratic mobile phase consisting of 60% (v/v) methanol and 40% (v/v) water for determining content in *Callisia fragrans* and the HiQ sil C18HS (C18) columm, 5 µm part size, 250 mm; 4,6 mm i.d., was used in the chromatographic analysis.

3.1.1. Specificity

Specificity is the ability of a method to discriminate between the study analytes and other components in the sample. Specificity of the HPLC method is demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients [10].

The specificity of the method was evaluated by analysis of the blank, standard, sample and standard spiked sample solution chromatograms (Figure 1). It had the markable different between of blank, standard, sample and standard spiked sample solution. the peak of β-ecdysone in the sample and standard spiked sample solution had the responsible height and area changes with the content of spiked standard. Furthermore, the chromatogrphy conditions also had the good separation between the peaks of β-ecdysone and the other components with the retention time 6.5 min, the tailing factor between 0.9 to 1.1.

3.1.2. Linearity

Results obtained in validation demonstrated an excellent linear relationship between the corresponding peak areas and the concentration of β-ecdysone in the range 5-150 µg/mL was achieved, as confirmed by the correlation coefficient of 0.9996.

The validating parameters of the calibration curve, including the linearity range, slope, intercepts, and correlation coefficients obtained by linear regression analysis are described in Table 1.

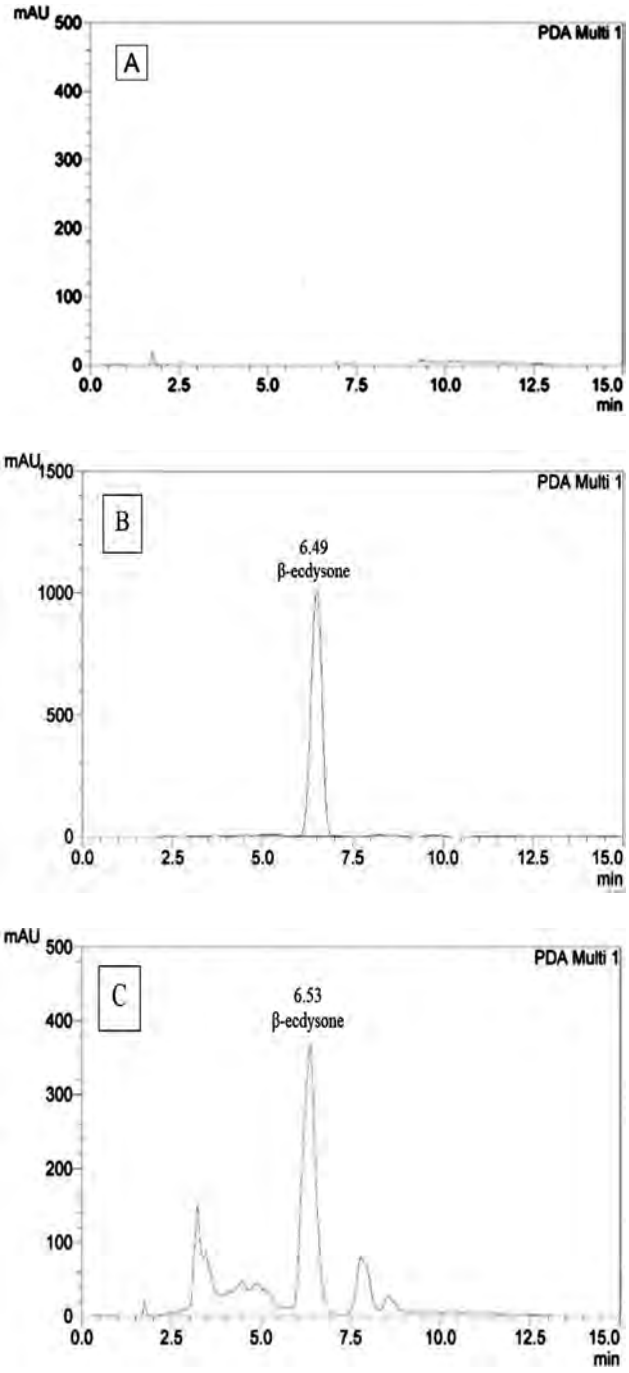

Figure 2. Chromatograms of the extract spiked β-ecdysone for the specificity of HPLC method. A: Blank solution; B: standard solution; C: standard spiked sample solution.

Table 1. Linearity parameters for the calibraion curve of β-ecdysone

β-ecdysone	Concentration (µg/mL)				
	150	100	40	15	5
Peak area	24491830	16773791	6511710	2368068	758860
Linerity range (µg/mL)	5 - 150				
Slope (a)	164896				
Intercept	42687				
r^2	0.9980				
Equation	$y = 164896x - 42687$				
RSD	1.91				

r^2: determination coefficient

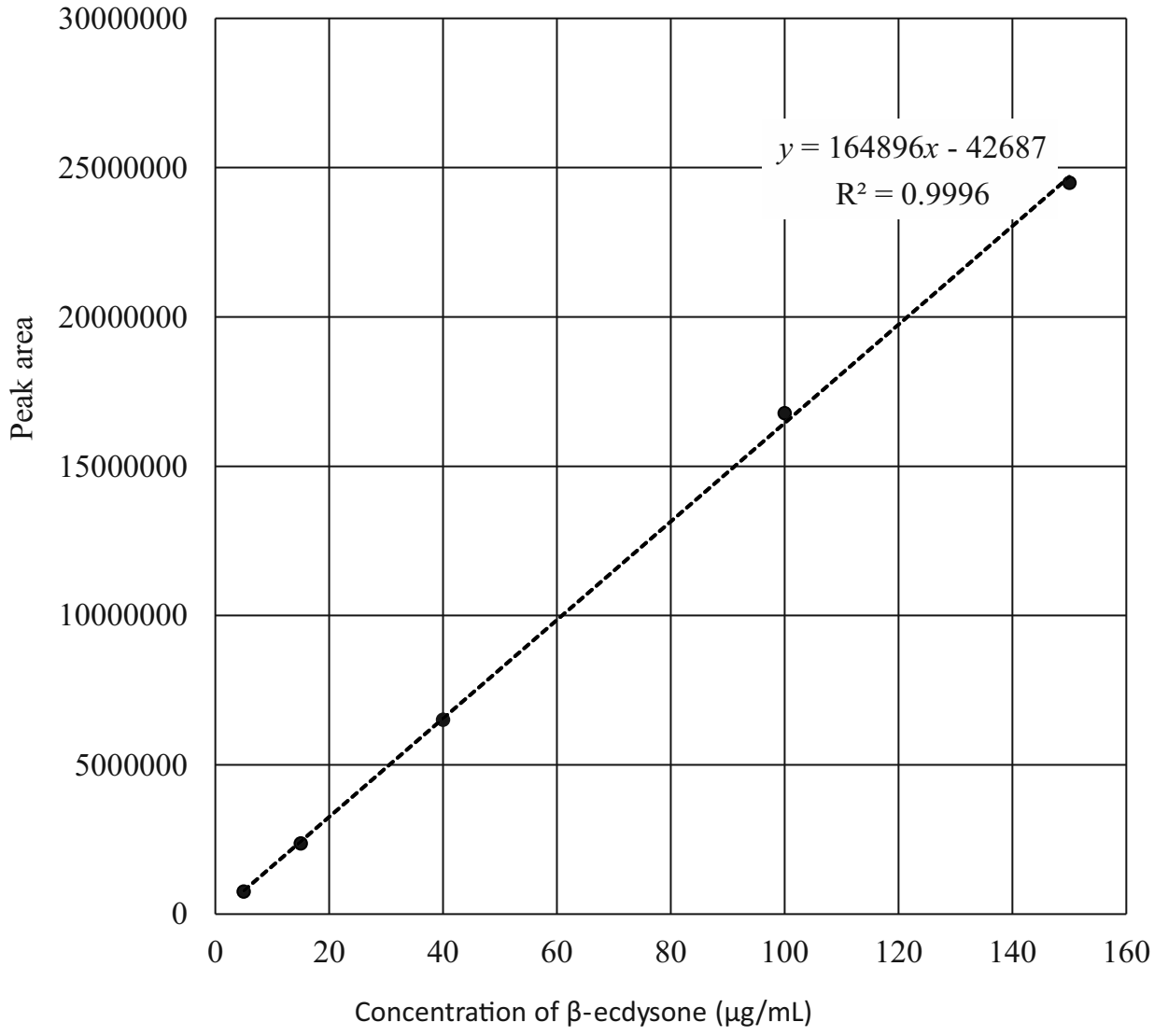

Figure 3. Calibration curve and equation of regression line of β-ecdysone

3.1.3. Accuracy and Precision

The accuracy of method was evaluated with recovery test by adding of β-ecdysone in known amounts to the crude extract of *Callisia fragrans* range 80%, 100%, 120% content in sample, the results showed a medium recovery of 99.07% with RSD below 1.15% for all analyzed concentrations, confirming the accuracy of the method. The precision of method was evaluated in terms of repeatability and showed RSD values lower than 2.0%. The results are described in Table 2.

Table 2. The precision parameters of the method

β-ecdysone	Sample							
	1	**2**	**3**	**4**	**5**	**6**	**Average**	**RSD (%)**
Rt	6.51	6.59	6.53	6.51	6.53	6.52	6.531667	0.458449
Peak area	8507780	8492784	8562349	8481295	8523197	8530442	8516308	0.34109
Tailing factor	1.01	1.02	1.02	1.06	1.03	1.04	1.03	1.736752

3.1.4. Sensitivity

The Limit of Detection (LOD) and Quantitation (LOQ) is defined as the smallest quantity of β-ecdysone that is detectable and quantifiable in a sample with acceptable precision and accuracy. There are a number of ways for the calculation of LOD, as discussed in the ICH guidelines on method validation, it was not necessarily quantified under the stated experimental conditions; it were determined as 5.79 µg/mL and 11.56 µg/mL, respectively, by the response standard deviation (RSD) was taken as the SD of the regression line (σ) of the three calibration curves.

3.2. Analysis of sample

Evaluation of chemical markers in herbal allows the assessment of the quality of the material. Quantification of β-ecdysone in HPLC is necessary to assess its presence in the sample of of *Callisia fragrans*. The results of β-ecdysone content in dried herbal *Callisia fragrans* is 0.0519 ± 0.0006% (100 g dried powder of *Callisia fragrans* contents 51.9 mg β-ecdysone).

4. CONCLUSIONS

This study validated an analytical method to determine the β-ecdysone content in *Callisia fragrans*. The analytical method was validated on Shimadzu HPLC system LC20 model, performing proper linearity (R^2 = 0.9995) from 5 to 150 µg, acceptable repeatability and reproducibility, and high recoveries, the data levels and ranges as well as the acceptance criteria according to ICH guidelines on method validation and AOAC: Guidelines for Standard Method Performance Requirements. This validated method was the simple, efficient and can be employed in determination of β-ecdysone from *Callisia fragrans* and products prepared from this herbal.

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Xây dựng và thẩm định phương pháp định lượng β-ecdysone của cây lược vàng *Callisia fragrans* (Lindl.) bằng kỹ thuật sắc ký lỏng hiệu năng cao

Bùi Thế Vinh và Phan Nguyễn Thu Xuân

TÓM TẮT

Nghiên cứu này xây dựng và thẩm định phương pháp HPLC để xác định β-ecdysone từ cây lược vàng (Callisia fragrans (Lindl.) Woodson. Bột cây lược vàng (Callisia fragrans) được chiết siêu âm với methanol (MeOH). Dịch chiết MeOH được làm bay hơi ở áp suất thấp để thu được cao chiết tổng. Phần MeOH này được chiết lỏng - lỏng với các dung môi có độ phân cực tăng dần: n-hexan, ethylacetate (EtOAc). Phân đoạn EtOAc chứa β-ecdysone được phân tích bằng phương pháp HPLC. Điều kiện sắc ký được thực hiện trên cột C18 sử dụng pha động đẳng dòng gồm metanol 60% (tt/tt) và nước 40% (tt/tt) với tốc độ dòng 1.0 mL/phút, ở bước sóng phát hiện 242 nm. Kết quả thẩm định quy trình phân tích đã cho thấy các thông số độ tuyến tính, độ đúng, độ chính xác, tính

đặc hiệu đều đạt yêu cầu của một phương pháp phân tích. Hàm lượng của β-ecdysone trong Callisia fragrans (Lindl.) Woodson được xác định bằng cách sử dụng HPLC là 0.0519 % bột dược liệu khô.

Từ khóa: *β-ecdysone, Callisia fragrans (Lindl.), HPLC*

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