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Original Research Article

Validation of HPLC Method for Quantifying 1,4-Naphthoquinone in Ethanol and Water Extracts of *Eleutherine bulbosa*

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ABSTRACT

Eleutherine bulbosa (*E. bulbosa*) is a traditional medicinal plant known to contain 1,4-naphthoquinone as the active compound responsible for its pharmacological effects. Ensuring consistent pharmacological effects in pharmaceutical preparations necessitates the quantitative measurement of the active compound content in the extract. This study aims to establish a method for quantifying the levels of 1,4-naphthoquinone in both ethanol and water extracts of *E. bulbosa* and to determine the optimal levels in both extracts. The High-Performance Liquid Chromatography (HPLC) method was employed to assess the 1,4-naphthoquinone levels. Method validation was conducted to evaluate the accuracy, precision, linearity, and selectivity of the method. The validation results indicate that this method has met the standards, with the linearity test yielding an "r" value of 0.9939, the selectivity test indicating selectivity at 3.507 minutes, the precision test resulting in a %RSD of 0.7159%, and the accuracy test showing a % recovery of 99.53%. These method validation parameters satisfy the required criteria, affirming the reliability of the method employed. The average levels of 1,4-naphthoquinone in the ethanol extract were determined to be 4.5797 ppm, while in the water extract, it measured 3.2314 ppm. These findings imply that the ethanol extract contains a higher concentration of 1,4-naphthoquinone compared to the water extract. Consequently, it can be inferred that the HPLC method for quantifying 1,4-naphthoquinone levels is valid, and the ethanol extract is recommended as a superior raw material for the development of traditional medicines due to its higher content of active compounds.

Keywords: Method validation, accuracy, precision, linearity, selectivity

Introduction

Eleutherine bulbosa (E. bulbosa) is a traditional medicinal plant that has been utilized by communities to treat various ailments. Previous studies have been conducted to uncover the pharmacological effects and active compound contents of *E. bulbosa* [1] with several findings indicating that *E. bulbosa* contains active compounds with antioxidant activity, capable of combating free radicals and protecting cells from oxidative damage. Additionally, it exhibits anti-inflammatory, antimicrobial, anticancer, and antidiabetic properties.[2,3] Furthermore, previous studies have identified several active compounds in *E. bulbosa*, including 1,4-naphthoquinone, which plays a significant role in its pharmacological effects. Other compounds found include flavonoids (quercetin, kaempferol, apigenin), alkaloids (eleutherine, palmatine), polyphenols (gallic acid and chlorogenic acid), naphthalenes (Eleuthroside A-D, Eleutherinol, B-sitosterol), and naphthoquinones (Eleutherine, Eleuthraquinone A-B, 1.4 naphthoquinone).[4] This information provides crucial knowledge for understanding the potential use of *E. bulbosa* in traditional medicine and the development of standardized herbal medicines and phytopharmaceuticals.[5] In the development of standardized herbal medicines or phytopharmaceuticals, the determination of levels is an important standardization parameter.[6]

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Standardization is a process that involves determining the quality and quantity of active components in drugs or herbal products. The determination of levels aims to ascertain the amount or concentration of active substances contained in herbal medicines. Proper determination ensures that standardized herbal medicines have concentrations in accordance with established safety limits. [7]Additionally, excessively high levels can cause side effects or toxicity, while excessively low levels may not provide the expected therapeutic effects. [8] On the other hand, with consistent determination of levels, standardized herbal medicines can maintain a stable therapeutic effect from one batch to another. This is essential for maintaining the quality and consistency of the resulting products.[9]

High-Performance Liquid Chromatography (HPLC) plays a crucial role in the quality control of natural products and enables the identification and analysis of specific compounds present in them. By utilizing sensitive detectors such as UV or mass detectors, HPLC can separate and identify active compounds, contaminants, or other hazardous substances in the products.[10] Furthermore, HPLC enables the quantitative measurement of active components in natural products. This is essential to ensure that the products meet the specified standards. With the use of appropriate calibration curves, HPLC can accurately and precisely measure the concentration of active components. [11] Therefore, by employing HPLC method, manufacturers can ensure consistent, safe, and quality products that comply with established requirements. This method contributes to consumer protection and fosters trust within the natural product industry.[12 13]

Therefore, this study aims to develop a validated method for quantifying 1,4-naphthoquinone in ethanol and water extracts of *E. bulbosa* using HPLC. This novel research has not been reported in previous studies. Additionally, this study aims to determine the extract with the highest level of 1,4-naphthoquinone within the *E. bulbosa* extracts.

Materials and Methods

Preparation of Herbal Powder
Fresh *E. bulbosa* cultivated in South Malang, Indonesia, was washed and sliced for the drying process using an oven (BINDER, Germany). The dried herbal material was then ground into a fine powder using a hammermill and sieved to obtain a fine powder. The herbal powder was tested for moisture content using a moisture instrument (Mettler Toledo, Swiss) before being used in the extraction process. The moisture content was determined by placing 10 grams of herbal powder in a sample pan and drying it at a temperature of 105°C. [14]

Extraction of E. bulbosa using Ultrasonic-Assisted Extraction
Extraction using the UAE (Ultrasound-Assisted Extraction) method was performed to obtain ethanol extract. Ultrasonic-Assisted Extraction is a method of extracting compounds from natural materials using ultrasonic waves to enhance the efficiency and speed of the extraction process. 50 mg of *E. bulbosa* herbal powder was dissolved in 500 mL of 96% ethanol. The sample was extracted using the UAE method for three (3) rounds of 10 minutes each. The solvent from the filtrate was evaporated using a rotary evaporator (temperature: 50°C) and an oven (temperature: 40°C). [15]

Extraction of E. bulbosa using Freeze Drying
Extraction using the freeze-drying method was performed to obtain water extract. Each 122 grams of *E. bulbosa* was crushed and extracted using the maceration method with hot water solvent. Extraction was done in a ratio of 1:5, which is 1 part *E. bulbosa* with 5 parts hot water. The *E. bulbosa* mixture was filtered using paper filter, vacuum pump (Biobase, China) and Buchner flask (Schott, Germany) to obtain the filtrate, which was then dried using the freeze-drying method. In the freeze dryer (Christ, Germany), the liquid filtrate was first frozen at a temperature of -40ºC and then dried. During the drying process, the pressure was maintained at around 0.036 psi (0.0025 bar) while the temperature was gradually increased to approximately 38ºC. [16]

Preparation of Stock Solution
Dry *E. bulbosa* extract was weighed at 1 mg and then dissolved in its respective solvent, either ethanol, or water, for injection in a 10 mL volumetric flask to create a stock solution of 100 µg/mL. UAE samples were prepared using ethanol as the solvent, while freeze-drying samples were prepared using water for injection as the solvent. To prepare the samples, 1200 µL of the stock solution was taken and transferred into a 10 mL volumetric flask, then the solvent was added up to the mark. [17]

Standard and Materials
Standard
The standard used in the research is 1,4-naphthoquinone, which was purchased from Sigma Aldrich in Darmstadt, Germany. It was weighed at 100 mg and dissolved in water for injection in a 10 mL volumetric flask to create a stock solution of 10,000 µg/mL. Stepwise dilution was performed to obtain a stock solution of 100 µg/mL. Standard solutions of 1,4-naphthoquinone with various concentrations of 3.0, 6.0, 9.0, 12.0, 15.0, 18.0, and 21.0 µg/mL were prepared. [17]

Herbal Material
E. bulbosa was purchased from the South Malang region in January 2022. The herb was identified at the UPT Laboratory of Herbal Materia Medica with determination letter number 074/212/102.20-a/2022.

Ultra-High Performance Liquid Chromatography (UHPLC)
Chromatographic Conditions
Standard 1,4-naphthoquinone and *E. bulbosa* extract were analyzed using the Thermo Fisher Scientific UHPLC Ultimate 3000 RS (Thermo Fisher Scientific, USA) with a diode array detector (DAD) and a C18 column. The isocratic method was employed with a mobile phase consisting of 95% methanol for pump A and 0.5% chloroform for pump B. The sample was injected with a volume of 10 µL at a flow rate of 1.0 µL/min and detected using a UV detector at a wavelength of 254 nm for 5 minutes. [18]

Method Validation
Linearity
Calibrated 10 mL volumetric flasks were prepared with concentrations of 3.0, 6.0, 9.0, 12.0, 15.0, 18.0, and 21.0 µg/mL from the standard stock solution with a concentration of 100 mg/mL, then analyzed using the UHPLC instrument. A calibration curve is constructed, and the linear regression equation and correlation coefficient (r) were determined. [19]

Selectivity
Selectivity was determined by comparing the retention time (RT) of the *E. bulbosa* extract with the retention time (RT) of the standard 1,4-naphthoquinone. [20]

Precision
Precision was evaluated by injecting the *E. bulbosa* extract sample six times. The results were obtained by calculating the mean, standard deviation (SD), and %RSD. Standard solutions were prepared with concentrations of 90% at 9 mg, 100% at 10 mg, and 110% at 11 mg. Then, they were diluted with 1 mL of 96% ethanol. The *E. bulbosa* extract was diluted to a concentration of 20,000 ppm with 1 mL. To prepare the addition solution, 0.5 mL was taken from each standard solution and mixed with 0.5 mL of the sample solution to obtain a 1 mL addition solution. Thus, three additional solutions were prepared, each with a volume of 1 mL. Then, spotting was performed on an HPTLC plate using a 2 µL capillary pipette, three times for each solution, resulting in a total of nine spots. Subsequently, observation was carried out using a densitometer, and the percentage of recovery (%recovery) determined. [21]

Accuracy
The accuracy of the determination of 1,4-naphthoquinone in the extract was assessed by adding 2 mL of a standard solution at a concentration of 3.0 µg/mL into the *E. bulbosa* extract sample solution. The result was obtained by calculating the %recovery value [22] using the formula:

$$\%\ recovery = \frac{(additional\ yield - actual\ yield)}{actual\ yield} \times 100$$

Determination of 1,4-Naphthoquinone Content in E. bulbosa
Approximately 12 µg/mL of the extract was utilized to measure the content of 1,4-naphthoquinone. It was filtered into a vial using a syringe and a 0.45 µm filter. The calculation of 1,4-naphthoquinone content in the extract solution was performed using Chromeleon software version 7.2 for each sample with three repetitions.

Statistical analysis
The analysis of the results was conducted using the independent sample t-test analysis with SPSS version 16 (Statistical Product and Service Solutions). The independent sample t-test was performed to compare two unrelated samples by observing the value of Sig. (2-tailed). If the value of Sig. (2-tailed) >0.05, it indicates that there is no significant difference. If the value of Sig. (2-tailed) <0.05, it indicates that there is a significant difference.[23]

Result and Discussion

Method Validation and HPLC Analysis
Method validation was conducted before determining the content of 1,4-naphthoquinone in *E. bulbosa* extract. Method validation is an essential process in which specific parameters of a laboratory experiment are evaluated to demonstrate that they meet the required standards. Validation is performed to assess the influence of equipment, reagents, and personnel involved in the analysis. The method validation conducted in this study included the parameters of linearity, selectivity, precision, and accuracy.

Linearity
Linearity is a test conducted to determine the ability of a method to provide results that are directly proportional to the concentration of the analyte within a specified range. This test ensures a linear relationship between the analyte concentration and detector response (Figure 1). [24]
The results of linearity test in the method validation for the determination of 1,4-naphthoquinone content using HPLC method

indicate that a calibration curve can be generated with the linear regression equation y = 2.0434x + 0.4969, where "y" represents the concentration of 1,4-naphthoquinone in µg/mL and "x" represents the response generated by the HPLC method.
The obtained correlation coefficient (r) value is 0.9939, indicating a very strong relationship between the response generated by the HPLC method and the concentration of 1,4-naphthoquinone. According to the Indonesian Food and Drug Administration, the value of "r" approaching 1 (> 0.98), indicates that this method exhibits excellent linearity, making it reliable for the determination of 1,4-naphthoquinone content in the sample. [19]
With this linear regression equation, the concentration of 1,4-naphthoquinone in the sample can be predicted based on the response generated by the HPLC method. The higher the response, the higher the concentration of 1,4-naphthoquinone in the sample.
In the context of method validation, these results indicate that the HPLC method used for the determination of 1,4-naphthoquinone content meets the linearity requirements set by the Indonesian Food and Drug Administration with a high correlation coefficient value. This indicates that the method can provide accurate and reliable results for the quantitative analysis of 1,4-naphthoquinone. [25]

Selectivity
Selectivity is a test that demonstrates the ability of a method to accurately and precisely measure a specific substance in an extract while taking into account the presence of other components in the sample matrix[26]. Good selectivity is indicated by a clear separation between the analyte and other components. Additionally, to determine the identity of a peak, the data from the standard chromatogram can be compared to the analyte's chromatogram (Figure 2). [24]
In the selectivity test of 1,4-naphthoquinone compound in *E. bulbosa* extract, the results showed that the peak of 1,4-naphthoquinone appeared in the chromatogram of the *E. bulbosa* extract. Furthermore, both peaks had the same retention time, specifically at 3.507 minutes.
The conclusion that can be drawn from these results is that the method used in the analysis has demonstrated good selectivity, where the method has been able to separate and identify the target compound (1,4-naphthoquinone) in a complex mixture (*E. bulbosa* extract).
The specific appearance of the 1,4-naphthoquinone peak in the chromatogram of the *E. bulbosa* extract, along with the same retention time, suggests that the method is capable of distinguishing the target compound from other components in the extract. This indicates that the method has produced a specific response to 1,4-naphthoquinone and is not affected by interference from other components in the extract.
In the context of method validation, these results indicate that the method used has sufficient selectivity for the analysis of 1,4-naphthoquinone in *E. bulbosa* extract. The success of the method in separating and identifying the target compound specifically provides confidence that the obtained analysis results are accurate and reliable.

Precision
The precision test results in the method validation for determining of 1,4-naphthoquinone content in *E. bulbosa* extract using the HPLC method revealed a precision value represented by %RSD (Relative Standard Deviation) of 0.7159% (Table 1).
%RSD is employed to quantify the level of variation or dispersion of data in an experiment. In the precision test, %RSD is used to assess the repeatability of measurements performed under identical conditions. A low %RSD value indicates good precision, signifying consistent and closely clustered measurement results. [17 27]
In this study, the %RSD value of 0.7159% signifies that there is minimal variation among the six measurements of *E. bulbosa* extract samples for the determination of 1,4-naphthoquinone content. Since the acceptable %RSD value for the precision test is ≤ 2%, [28] the obtained precision result (0.7159%) is significantly below the maximum limit set. This demonstrates that the HPLC method employed exhibits a very high level of precision, yielding stable and consistent measurement outcomes.
With such high precision, the analysis results are dependable and offer a high degree of accuracy in determining the content of 1,4-naphthoquinone in *E. bulbosa* extract. The low level of precision also indicates that factors capable of affecting the variation in measurement results, such as measurement errors or instrumental fluctuations, have been effectively controlled in the analytical method. [29]

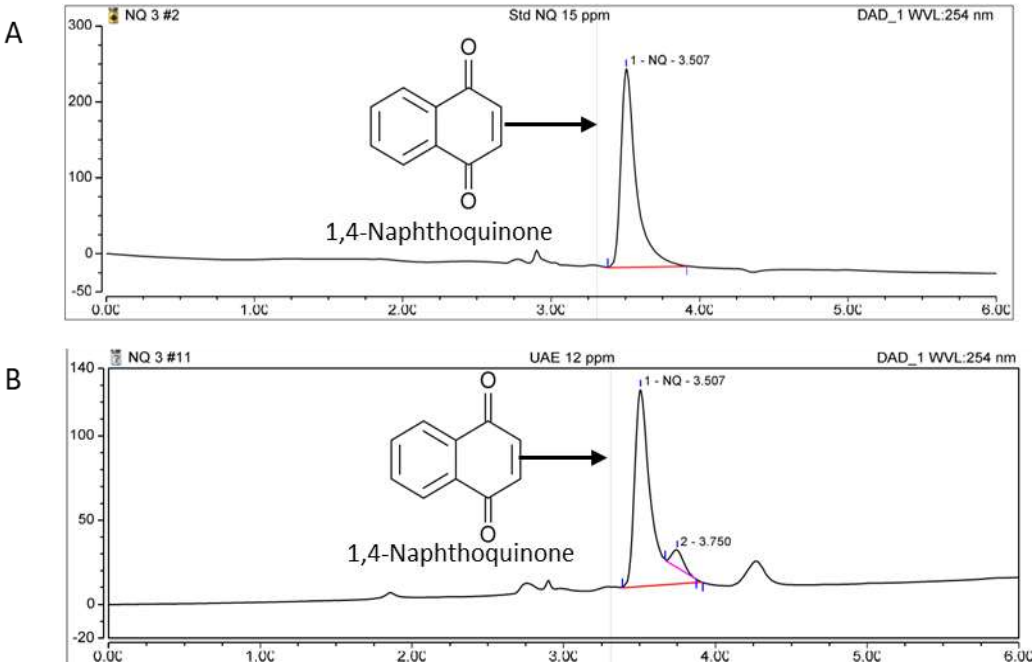

Figure 1: HPLC Chromatogram: A. 1.4-naphthoquinone; B. Ethanol extract of *E. bulbosa*. The retention time (RT) of 1.4-naphthoquinone compound in the *E. bulbosa* extract is the same as the retention time (RT) of the standard 1.4-naphthoquinone (pure compound), which is at 3.507 minutes.

Accuracy

The accuracy test conducted during the method validation for determining the 1,4-naphthoquinone content using the HPLC method yielded an accuracy value represented by %recovery of 99.53% (Table 2).

%Recovery is employed to assess the recovery rate or the ability of the analytical method to accurately retrieve the actual amount of the studied compound. A favorable %recovery value signifies that the analytical method possesses a high degree of accuracy, indicating its capability to closely approach or recover the actual quantity of the analyzed compound. [30]

In this study, the %recovery value of 99.53% indicates that the HPLC method utilized for the determining 1,4-naphthoquinone content boasts an exceptionally high level of accuracy. This value falls within the acceptable range for accuracy testing, typically between 98% and 102%.[31] This illustrates that the analytical method can closely retrieve the actual quantity of 1,4-naphthoquinone in the *E. bulbosa* extract, with minimal margin for error.

With this high level of accuracy, the analytical results can be relied upon to provide precise information regarding the content of 1,4-naphthoquinone in the *E. bulbosa* extract. The successful retrieval of the actual compound quantity indicates that the HPLC method has undergone thorough testing and validation, establishing its credibility for determining 1,4-naphthoquinone content in the *E. bulbosa* extract. [32]

Comparison of Content Determination

In this study, a comparison of content determination was conducted using the UHPLC instrument. The analysis results were presented in the form of chromatograms depicting peaks or curves with an associated area under the curve (AUC) value This AUC value serves as a quantitative measure for determining the content of a compound. [33 34]

To ascertain the content or concentration of 1.4-naphthoquinone in the *E. bulbosa* extract, the AUC value is employed as the y-value in the linear regression equation obtained during the validation of the linearity method, expressed as $y = 2.0434x + 0.4969$.

The determination of 1.4-naphthoquinone content in both the ethanol extract and water extract of *E. bulbosa* revealed a significant difference in the average content between the two types of extracts. The ethanol extract exhibited a higher content compared to the water extract, as depicted in Table 3.

Statistical analysis results indicated that this difference is highly significant, with a p-value less than 0.005. A p-value less than 0.005 denotes a very high level of significance, signifying that the observed difference between the two extracts types methods is not a result chance or random fluctuations but represents a genuine and statistically significant distinction. [18]

In this context, the statistical analysis results highlight a significant disparity in the 1.4-naphthoquinone content between the ethanol extract and water extract of *E. bulbosa*. This distinction may be attributed to variations in the compound's extraction properties in ethanol and water solvents, as well as potential variations in its solubility in the two types of extracts. [32]

This information can offer valuable guidance in selecting the most suitable extract for specific applications. Additionally, the results suggest that the analytical method employed ample sensitivity and accuracy to detect disparities in 1,4-naphthoquinone content between the two extract types.

However, it is crucial to note that further interpretation regarding these differences may necessitate additional analysis and comprehensive considerations. Other factors such as the quality of the raw material, extraction methods, and inherent variability in compound composition should also be taken into account to gain a more comprehensive understanding the observed differences in content between the ethanol extract and water extract of *E. bulbosa*.

Conclusion

Based on the results of method validation, which encompassed linearity, selectivity, precision, and accuracy, it can be concluded that the employed method has been substantiated as valid. The linearity test demonstrates a robust correlation between the response and the concentration of 1,4-naphthoquinone, while the selectivity test showcases the method's capability to discriminate the target compound from other constituents. The precision test reveals minimal variability in measurements, and the accuracy test indicates a recovery rate closely approximating the true quantity. Furthermore, a significant variance in the concentration of 1,4-naphthoquinone between the ethanolic extract (4.5797 ppm) and the water extract (3.2314 ppm) has been established through statistical analysis, with a p-value of less than 0.005. The method employed for the analysis of 1,4-naphthoquinone has been verified as valid and, thus, can be widely applied in practical scenarios. Moreover, when determining the concentration of 1,4-naphthoquinone, it is advisable to employ the ethanolic extract due to its higher concentrations compared to the water extract.

Table 1: Results of Precision Test for the Determination of 1,4-naphthoquinone Content in *E. bulbosa* Extract

Sample	Area	Average of area	SD*	%RSD**
Ethanol extract 12 ppm (1)	9.799	9.805	0.0702	0.7159 %
Ethanol extract 12 ppm (2)	9.759			
Ethanol extract 12 ppm (3)	9.712			
Ethanol extract 12 ppm (4)	9.92			
Ethanol extract 12 ppm (5)	9.829			
Ethanol extract 12 ppm (6)	9.811			

*SD : Standard Deviation; **%RSD: % Relative Standard Deviation

Table 2: Accuracy Test Results for the Determination of 1,4-naphthoquinone Content in *E. bulbosa* Extract

Sample	Concentration	Average of concentration	% *Recovery*
Ethanol extract 12 ppm	4.6115	4.6115	99.53 %
Ethanol extract 12 ppm + addition 1	9.6677	9.2013	
Ethanol extract 12 ppm + addition 2	8.6618		
Ethanol extract 12 ppm + addition 3	9.2743		

Table 3: Comparison of 1.4-naphthoquinone Content in Ethanol Extract and Water Extract of *E. bulbosa*

E. bulbosa Extract	Area Average*	Concentration±SD*(ppm)	% Concentration (w/w)
Ethanol Extract	9853 ± 20	4.5797 ± 0.02	0.045797%
Water Extract	7100 ± 15	3.2314 ± 0.01	0.032314%

*The data are presented as mean ± standard deviation, n=3.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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