Original Article

THE DEVELOPMENT AND VALIDATION OF HPLC AND HPTLC-DENSITOMETRY METHODS FOR THE DETERMINATION OF 1, 4-NAPHTHOQUINONE CONTENT IN *ELEUTHERINE BULBOSA* EXTRACT

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ABSTRACT

Objective: The aim of this research was to develop and validate identification and Quantitation methods for 1,4-naphthoquinone in the extract of *Eleutherine bulbosa*.

Methods: High-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography with densitometric detection (HPTLC-densitometry) were employed as analytical techniques. HPTLC-densitometry was performed at a wavelength of 249 nm, while UHPLC was conducted at a wavelength of 254 nm. Both methods were utilized to analyze 1,4-naphthoquinone in 96% ethanol extract of *E. bulbosa* as a Quantitation parameter in the standardization of the formulation. HPTLC separation was carried out on a 20 cm × 20 cm HPTLC glass plate coated with silica gel 60 F254 using a mobile phase of chloroform: methanol (8:2, v/v). For HPLC analysis, a C18 column with an isocratic method was employed using a mobile phase of 95% methanol in pump A and 0.5% chloroform in pump B. The calibration curve of peak area against concentration showed linearity within the range of 2500-15000 ppm/spot–1 and 3–21 µg/ml for HPTLC and HPLC, respectively. Both methods were validated for accuracy, precision, linearity, selectivity, LOD, and LOQ.

Results: The results showed that both methods exhibited linearity that met the standards, as they produced correlation coefficients (r) greater than 0.9900. Furthermore, both methods demonstrated good accuracy, with consecutive recovery values of 99.53% and 101.89%. On the other hand, the methods fulfilled the precision requirements, with respective values of 0.7159% and 2.884% (in compliance with the requirement of<5%). Additionally, to meet the LOD and LOQ requirements in HPTLC, the LOD value obtained was 163 ppm, and the LOQ value was 495 ppm. In HPLC, the retention time of the standard 1,4-naphthoquinone and the analyte compound in the extract of *E. bulbosa* were the same, at 3.507 min. The selectivity test on HPTLC indicated that the 1,4-naphthoquinone compound was at an RF value of 0.81, which was also detected in the extract of *E. bulbosa* at the same RF value.

Conclusion: In conclusion, our findings demonstrate that HPLC and HPTLC methods for the determination of 1,4-naphthoquinone content have met the standards for linearity, accuracy, precision, selectivity, LOD, and LOQ. Therefore, these methods can be recommended for the quality control of raw materials of *E. bulbosa* extract.

Keywords: HPTLC, HPLC, 1.4-naphthoquinone, Method validation, *Eleutherine bulbosa*

INTRODUCTION

Eleutherine bulbosa (*E. bulbosa*) is a herbal plant that has been traditionally used for diabetes, breast cancer, stroke, hypertension, and sexual dysfunction, as well as for boosting breast milk production. It is also used for treating cardiovascular disease and has diuretic, emetic, purgative, antifertility, antihypertensive, and wound-healing properties [1].

In vitro studies have reported that the compound Naphthoquinone inhibits the proliferation of K562 cells [1], leukemia cells L1210 [2], and the transcription of TCF/β-catenin in SW480 colon cancer cells in a dose-dependent manner [3]. It has also been found to inhibit HeLa Cervical Cancer Cell Line [4]. *In vivo* studies of *E. bulbosa* extract have demonstrated its ability to induce colon cancer apoptosis [5] and inhibit ulcerative colitis [6].

1,4-naphthoquinone is an active compound found in high concentrations in *E. bulbosa* and is believed to play a crucial role in its pharmacological effects [7]. Therefore, it is important to develop a method for determining the content of 1,4-naphthoquinone in *E. bulbosa* extract as a reference for the quality control of products.

Accurate and consistent determination of the compound's concentration is also a specific parameter in the standardization of *E. bulbosa* extract, facilitating comparison between products and ensuring the appropriate dosage in clinical use [8]. Furthermore, information on the concentration of 1,4-naphthoquinone in the extract is important for strict quality control during the production process and to ensure that the resulting products are safe and compliant with applicable regulations. Thus, determining the concentration of 1,4-naphthoquinone in 70% ethanol extract of *E. bulbosa* is a crucial step in maintaining the quality, safety, and consistency of herbal products.

The development of sensitive, accurate, and precise methods is highly necessary for determining the concentration of this active compound [9, 10]. Therefore, this research aims to develop a new method for the determination of 1,4-naphthoquinone in *E. bulbosa* extract. Additionally, this study involves the validation of HPLC and HPTLC-densitometry methods for the Quantitation of this compound.

MATERIALS AND METHODS

Materials

The solvents used for preparing the mobile phase in HPLC analysis were ultrapure water, methanol (HPLC grade, Merck), ethanol (HPLC grade, Merck), and chloroform (HPLC grade, Merck). For HPTLC analysis, acetone (HPLC grade), n-hexane, and ammonia (analytical grade, Merck) were used for preparing the mobile phase. 1,4-naphthoquinone (Sigma, Germany) and PTFE membrane filters

(0.45 μm pore size, Sartorius) were used for filtering the mobile phase in HPLC analysis and samples, respectively.

Instrumentation and analytical conditions for HPTLC

The HPTLC analysis method was performed using HPTLC glass plates (20 cm × 10 cm) coated with silica gel 60 F254 (E. Merck, Darmstadt, Germany). The samples were applied to the plates as bands with a width of 8 mm and separated by a distance of 20 mm, using a Linomat V sample applicator from Camag (Switzerland) equipped with a 100 μl syringe from Hamilton (Bonaduz, Switzerland), and with a constant application rate of 0.2 μl/s. The plates were developed up to a distance of 9 cm in a twin trough glass chamber (20 cm × 10 cm) from Camag (Muttenz, Switzerland) filled with the vapor of the mobile phase (chloroform: methanol 8:2 v/v). The optimized saturation time for the chamber was 1 h and 15 min at room temperature [11].

Densitometric scanning was performed at a wavelength of 249 nm using the Camag TLC Scanner III in reflectance-absorbance mode and operated with CATS software (1.4.4 6337, Camag). A deuterium lamp was used as the radiation source, producing a continuous UV spectrum between 200 nm and 400 nm. The slit dimensions were 8 mm × 0.4 mm, and the scanning speed was set at 10 mm/s. The scattered light intensity was used to determine the concentration of the chromatographed substance. Peak areas were determined using linear regression [12].

Instrumentation and analytical conditions for HPLC

The analysis of the standard 1,4-naphthoquinone and the *E. bulbosa* extract in this study was conducted using the Thermo Fisher Scientific Ultimate 3000 RS UHPLC system (Thermo Fisher Scientific, United States) coupled with a diode array detector (DAD) and a C18 column (5 μm, 4.6 mm × 250 mm). Gradient elution was employed to separate the polar compounds in the *E. bulbosa* extract. The sample separation was performed with UV detection at a wavelength of 254 nm, with a total analysis time of 0.8 min. The mobile phase consisted of an isocratic method, comprising 95% methanol (pump A) and 0.5% chloroform (pump B). The flow rate used was 1.0 ml/min, and the injection volume was 10 μl. The column temperature was set at 25 °C.

Sample preparation for HPLC

Dry *E. bulbosa* extract was weighed 1 mg and dissolved in its respective solvent in a 10 ml volumetric flask to prepare a stock sample solution of 100 μg/ml. UAE samples were dissolved in ethanol solvent. To prepare the samples, 1,200 μL of the stock solution was transferred into a 10 ml volumetric flask and then filled with the solvent up to the mark [7].

Standard preparation for HPLC

The 1,4-naphthoquinone standard was weighed 100 mg and dissolved in water for injection in a 10 ml volumetric flask to prepare a stock standard solution of 10,000 μg/ml. Serial dilutions were performed to obtain a stock standard solution of 100 μg/ml. Standard solutions of 1,4-naphthoquinone were prepared with concentrations of 3.0, 6.0, 9.0, 12.0, 15.0, 18.0, and 21.0 μg/ml [7].

Method validation-linearity

For HPLC

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 stock standard solution with a concentration of 100 μg/ml. These solutions were then tested using the UHPLC instrument. A calibration curve was constructed, and the linear regression equation and correlation coefficient (r) were determined [13].

For HPTLC

Naphthoquinone standard powder was weighed at 2.5 mg, 5 mg, 7.5 mg, 10 mg, 12.5 mg, and 15 mg, and dissolved in 96% ethanol. This resulted in stock solution concentrations of 2500 ppm, 5000 ppm, 7500 ppm, 10000 ppm, 12500 ppm, and 15000 ppm. Subsequently, 2 μl of each solution was applied to an HPTLC plate using a capillary

pipette. The HPTLC plate was developed with the selected mobile phase, and the obtained spots were visualized under UV light at 254 nm and observed with a densitometer at a wavelength of 249 nm. The regression equation (y = bx+a) and correlation coefficient (r) were calculated [14].

Method validation-selectivity for HPLC

Selectivity is determined by comparing the retention time (tR) of the Dayak onion extract with the retention time (tR) of the 1,4-naphthoquinone standard.

Limit of detection (LOD) and limit of quantitation (LOQ)

The determination of LOD and LOQ aims to establish the detection and quantitation limits. This is done by preparing 12 standard solutions starting from the lowest linear range value obtained from the linearity test (Harmita, 2004). Thus, based on the linearity test, standard stock solutions are prepared with concentrations of 2500 ppm, 2000 ppm, 1700 ppm, 1500 ppm, 1000 ppm, 700 ppm, 500 ppm, 300 ppm, 100 ppm, and 80 ppm [15].

Method validation-precision

For HPLC

Precision is evaluated by injecting the Dayak onion extract sample six times. The results are obtained by calculating the mean, standard deviation (SD), and % RSD.

For HPTLC

A sample solution with a concentration of 20000 ppm is spotted six times with a 2 μl capillary pipette on an HPTLC plate. It is then scanned with a densitometer, and the relative standard deviation (RSD %) is determined. The obtained RSD% value should be less than 5% to meet the requirements.

Method validation-accuracy

For HPLC

The determination of accuracy of 1,4-naphthoquinone in the extract is performed by adding 2 ml of a 3.0 μg/ml standard solution to the Dayak onion extract sample solution. The results are obtained by calculating the % recovery.

For HPTLC

Standard solutions are prepared with concentrations of 90% (9 mg), 100% (10 mg), and 110% (11 mg). They are then diluted with 1 ml of 96% ethanol. The Dayak onion extract is also diluted to a concentration of 20000 ppm, with 1 ml used for the preparation of the standard addition solution. The standard addition solution is prepared by taking 0.5 ml from each standard solution and mixing it with 0.5 ml of the sample solution, resulting in a total volume of 1 ml. Subsequently, the standard addition solution is spotted on the HPTLC plate using a 2 μl capillary pipette, with each solution spotted three times, yielding a total of nine spots. The plate is then observed with a densitometer, and the percentage of recovery (%recovery) is determined.

Determination of 1,4-naphthoquinone content by HPLC method

Approximately 12 μg/ml of the extract is used to measure the content of 1,4-naphthoquinone. It is filtered into vials using a syringe and a 0.45 μm filter. The calculation of 1,4-naphthoquinone content in the extract solution is performed using Chromeleon software version 7.2 for each sample with three replicates.

Determination of 1,4-naphthoquinone content by HPTLC method

The extract is diluted to a concentration of 20000 ppm and spotted onto an HPTLC plate with a volume of 2 μl. Then, it is developed using the selected mobile phase, and the obtained spots are observed under UV light at 254 nm and analyzed with a densitometer at a wavelength of 249 nm. The regression equation (y = bx+a) is calculated, along with the correlation coefficient (r). Subsequently, the content (b/b) is determined.

RESULTS

HPTLC analysis and validation

The optimized HPTLC method was validated for linearity, precision, accuracy, and detection and Quantitation limits.

Linearity and range

The results of the linearity test in HPTLC were used to evaluate the concentration linearity with respect to the area under the densitometer response. To accomplish this, standard solutions with concentrations of 2500 ppm, 5000 ppm, 7500 ppm, 10,000 ppm, 12,500 ppm, and 15,000 ppm were prepared. These solutions were then spotted onto the HPTLC plate using a capillary pipette with a volume of 2 μl. The HPTLC plate was developed using the selected mobile phase, and the resulting spots were observed under UV light with a wavelength of 254 nm. Subsequently, the scanned area using a densitometer was compared to the mass of the standard 1,4-Naphthoquinone.

From the comparison, the obtained equation for the calibration curve was $y = 1.195x + 18467$, with a coefficient of determination (r2) value of 0.9954 and a correlation coefficient (r) value of 0.9976. These values meet the AOAC requirements set at 0.9900 [16].

Precision and accuracy

Accuracy and precision tests were conducted to determine the extent to which the test results approach the true value, with the aim of obtaining good precision and accuracy values. In the accuracy test, standard solutions were prepared with concentrations of 90% at 9 mg, 100% at 10 mg, and 110% at 11 mg. These solutions were then diluted with 1 ml of 96% ethanol. Each concentration was spotted three times, resulting in three measurements.

The accuracy calculation results showed a recovery value of 101.89%, which meets the requirement as it falls within the range of 80-120%. According to the United States Pharmacopeia (USP), the recovery value for non-synthetic substances or unregulated products is acceptable if it falls within the range of 80-120% [17]. As an indicator of precision, the RSD% value was used in this study, which had a value of 2.884, complying with the requirement of<5% [18].
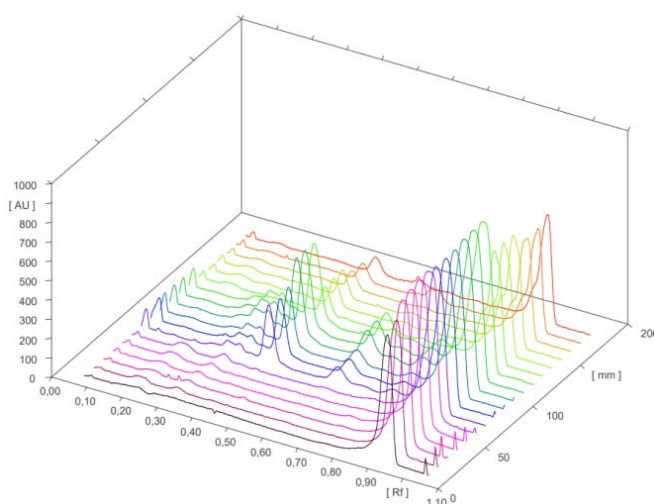

Fig. 1: Densitogram of HPTLC 1,4 naphthoquinone and *E. bulbosa* extract using TLC Scanner-4 (Camag) at UV 249 nm, with a scanning speed of 20 mm/s, data resolution of 100 μm/step, the position of the first track X 10.0 mm, the distance between tracks 10.0 mm, starting scan position Y 15.0 mm, and the last scan position Y 90.0 mm

Limit of detection (LOD) and limit of quantitation (LOQ)

The determination of LOD and LOQ aims to establish the specified limits for detection and Quantitation. To calculate the LOD and LOQ values, 12 standard solutions were prepared using the smallest linearity value obtained from the previous linearity test [19].

Based on the linearity test results, standard stock solutions were prepared with the following concentrations: 2500 ppm, 2000 ppm, 1700 ppm, 1500 ppm, 1000 ppm, 700 ppm, 500 ppm, 300 ppm, 100 ppm, 80 ppm, 50 ppm, and 20 ppm. After performing calculations using Microsoft Excel software, the LOD value was determined to be 163 ppm, and the LOQ value was determined to be 495 ppm.

Table 1: Validation results of HPLC and HPTLC methods for the determination of 1,4-naphthoquinone content in *E. bulbosa* extract

Methods	Concentration range	Linearity equation	Regression coefficient	Accuracy		Precisions
				Recovery %	RE %[a]	% RSD[a]
HPLC	3 ppm-21 ppm	Y=2.0434x+0.4969	0.9939	99.53	0.7159	0.7159
HPTLC	2500-15000 ppm	Y=1.195x+18467	0.9954	101.89	2.884	2.884

[a]Mean of six determination, RSD%, Percentege relative Standard Deviation; RE%, percentage relative error

HPLC analysis and validation

In the development of the HPLC method for the determination of 1,4-naphthoquinone content in *E. bulbosa* extract, method validation was conducted for linearity, selectivity, accuracy, and precision. The test results are presented in table 1.

Linearity

From the linearity test results of the HPLC method, a calibration curve was obtained with a linear regression equation of y=2.0434x+0.4969 and an R-value of 0.9939. An r-value ≥0.98 is the acceptance criterion for linearity according to BPOM in 2001, as stated in the research by Mulyati and Apriyani [20]. This indicates that the linearity test results meet the specified acceptance criteria.

Selectivity

Selectivity is a test that evaluates the ability of a method to accurately and precisely measure the concentration of a specific substance in an extract [12]. A good selectivity test is characterized by an effective

separation between the analyte and other components. Additionally, to determine the identity of a peak, it can be compared with standard and analyte chromatogram data [21]. In the chromatogram of the

standard 1,4-naphthoquinone test and the chromatogram of the *E. bulbosa* extract sample, the same retention time of 3.507 min is observed, indicating that the method used has good selectivity.
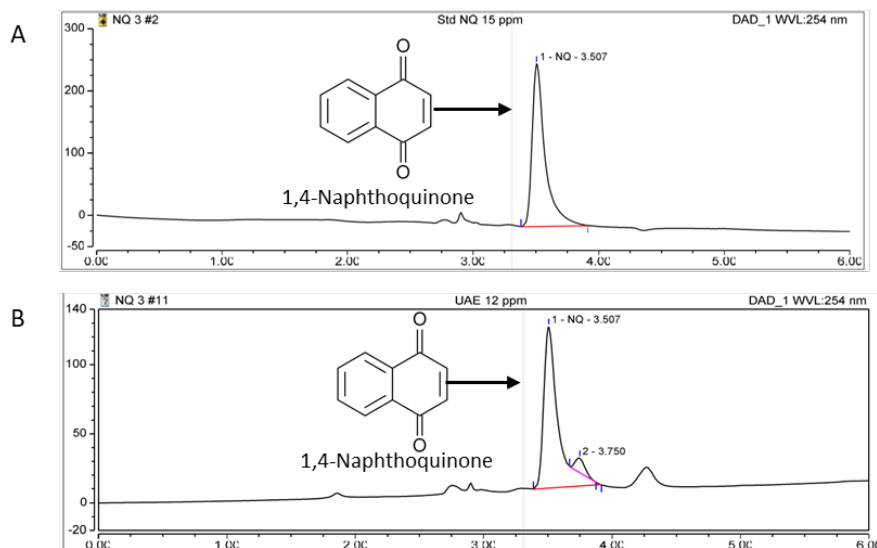

Fig. 2: HPLC Chromatogram: A. 1.4-Naphthoquinone; B. Ethanol extract of *E. bulbosa*. The retention time (tR) of 1.4-naphthoquinone compound in the *E. bulbosa* extract is the same as the retention time (tR) of the standard 1.4-naphthoquinone (pure compound), which is at 3.507 min

Precision

Precision test in this HPLC method is used to measure the level of random errors that may occur in a method. In this study, the precision method used is the repeatability method. Repeatability refers to the level of similarity influenced by the use of the same laboratory, the same analyst, the same equipment, and performed on the same day [22]. From table 1, the %RSD (Relative Standard Deviation) value obtained from the precision test is 0.7159%. The accepted standard for %RSD value in the precision test is ≤2%. This means that the measurements conducted in the precision test should provide consistent results and have a relatively low variation, not exceeding 2% [20]. Therefore, a smaller %RSD value indicates a higher level of precision in measuring the analyte within the sample matrix.

Accuracy

From table 1, it can be observed that the % recovery value for the accuracy test in the HPLC method is 99.53%. Based on previous

research, it has been reported that the acceptance standard for method validation in accuracy testing of analytes in sample matrices is within the range of 98%-102% [20]. Thus, it can be concluded that the obtained data results meet the requirements and demonstrate a good level of accuracy.

Determination of concentration using HPLC and HPTLC methods

The determination of 1,4 naphthoquinone concentration in *E. bulbosa* extract has been performed using both validated methods. In the HPLC method, the concentration was determined by inserting the area under the curve (AUC) value as the y-value in the regression equation $y=2.0434x+0.4969$. Meanwhile, in the HPTLC method, the concentration of 1,4 naphthoquinone was obtained from the equation $y=1.195x+18467$.

From the analysis results of 1.4 naphthoquinone concentration in *E. bulbosa* (table 2), it was found that the concentrations obtained from both methods were not significantly different (P>0.005).

Table 2: Results of 1.4 naphthoquinone concentration analysis in *E. bulbosa* using HPLC and HPTLC methods

Method	Average Area*	Average concentration* (ppm)	%w/w concentration (mg/100 mg)
HPLC	9.853±0.01	4.5797±0.01	0.380 %
HPTLC	33712,17±2.8	12757,46±1.2	0.318%

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

DISCUSSION

The development of chromatographic methods for the identification and Quantitation of active compounds in herbal products plays a crucial role in quality control. High-Performance Liquid Chromatography (HPLC) and High-Performance Thin-Layer Chromatography (HPTLC) are two commonly used methods in the quantitative analysis of active compounds in extracts or natural products [23]. The main difference between HPLC and HPTLC lies in the separation principle. HPLC utilizes a liquid mobile phase that flows through a stationary phase in the form of a chromatography column, while HPTLC uses a liquid mobile phase that is absorbed

into a stationary phase in the form of a thin layer on a chromatography plate [24]. Additionally, these methods differ in terms of analysis speed, where HPLC typically requires a longer analysis time due to the separation of compounds occurring within the chromatography column, whereas HPTLC has a shorter analysis time as the separation takes place on the thin layer of the chromatography plate [16]. Moreover, both methods exhibit differences in sensitivity. HPLC generally offers higher sensitivity compared to HPTLC, primarily because HPLC employs more sensitive detectors such as UV or fluorescence detectors [22]. Therefore, when selecting a quantitative analysis method for active compounds in extracts or natural products, specific requirements,

desired sensitivity, availability of equipment and resources, as well as the properties of the compounds to be analyzed, should be carefully considered.

The development and validation of a method for the determination of 1,4-naphthoquinone in *E. bulbosa* extract is crucial because this compound is a major component that plays a key role in the pharmacological effects of the plant. 1,4-naphthoquinone has been known to exhibit significant antioxidant and anticancer activities [4]. Therefore, in order to ensure the effectiveness and safety of using *E. bulbosa* in pharmacological applications, a method is needed to accurately and quantitatively determine the content of 1,4-naphthoquinone in the plant extract.

The development of a valid and accurate method for the analysis of 1,4-naphthoquinone is important for several reasons. First, by knowing the concentration of 1,4-naphthoquinone in the *E. bulbosa* extract, the appropriate dosage for therapeutic use can be determined. The precise quantity of this compound can influence the expected pharmacological effects and optimize treatment outcomes.

Second, having a valid and reliable method enables the quality monitoring and sustainability of *E. bulbosa* extract used in the pharmaceutical industry or herbal products.

Lastly, method validation is important in maintaining scientific integrity and confidence in research findings. Through proper validation, the reliability and accuracy of 1,4-naphthoquinone analysis data can be ensured, and the research findings can serve as a basis for further studies, drug formulation, or therapeutic product development.

From the validation results of the HPLC method (table 1) within the concentration range of 3 ppm to 21 ppm, a coefficient of determination (r2) value of 0.9954 and a correlation coefficient (r) value of 0.9976 were obtained. These values meet the AOAC requirement set at 0.9900, indicating that the method meets the linearity standard. Furthermore, the developed HPLC method also fulfills the requirements for selectivity, accuracy, and intraday precision. The selectivity test shows a similarity in retention time between the 1,4-naphthoquinone standard and the analyte compound in the *E. bulbosa* extract, occurring at 3.507 min. For accuracy, a % recovery of 99.53% falls within the accuracy standard range of 98%-102%. Therefore, it can be concluded that the obtained data meets the requirements and demonstrates good precision. Additionally, a %RSD (Relative Standard Deviation) value of 0.7159% was obtained, meeting the precision standard of≤2%. Overall, the HPLC method can be recommended for the determination of 1,4-naphthoquinone content due to its compliance with the requirements of linearity, selectivity, accuracy, and precision.

The results of the development and validation of the HPTLC method (table 1) for the determination of 1,4-naphthoquinone content lead to the conclusion that the method exhibits linearity that meets the standard, as it yields a correlation coefficient (r) of 0.9976, which is greater than the minimum requirement of r=0.9900. Additionally, the method demonstrates good accuracy, with a recovery value of 101.89%, satisfying the USP requirement of falling within the range of 80-120% [25]. On the other hand, the method validation results indicate compliance with the precision requirement, with a value of 2.884 (meeting the requirement of<5%) [26]. Moreover, the method has fulfilled the criteria for LOD and LOQ, with an LOD value of 163 ppm and an LOQ value of 495 ppm. The selectivity test results show that 1,4-naphthoquinone is found at an RF value of 0.81, which is consistent with its detection in the *E. bulbosa* extract at the same RF value. Overall, the validation of the HPTLC method has met the standards for linearity, accuracy, precision, selectivity, LOD, and LOQ.

CONCLUSION

In conclusion, both HPLC and HPTLC methods have proven to be effective in the quantitative analysis of 1,4 naphthoquinone. Validation studies have demonstrated that both methods exhibit high linearity, selectivity, accuracy, and precision. Both methods are reliable and suitable for routine analysis of 1,4 naphthoquinone. Therefore, they can be recommended for the determination of 1,4 naphthoquinone content in the quality control of raw materials of *E. bulbosa* extract.

AUTHORS CONTRIBUTIONS

All authors contributed equally to this work.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

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