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Abstract. Medicinal plants are widely used for the preparation of various pharmaceutical forms. *Eleutherine palmifolia* is a typical plant in Central Borneo in Indonesian. 1,4 naphthoquinone is a bioactive compound from E. palmifolia which has potential as a medicinal ingredient, one of which is anticancer. This study aimed to determine the levels of 1.4 naphthoquinone as a marker compound for the standardization of standardized herbal medicine (OHT) formulas of E. palmifolia extract. The separation was carried out with Thermo Fisher Scientific UHPLC Ultimate 3000 RS coupled with a diode array detector, and the C18 column was employed. The UV detection was performed at 254 nm, and the run time of 0.8 minutes. The mobile phase consists of an isocratic method, 95% methanol (A) and 0.5% chloroform (B). The results showed that 1,4 naphthoquinone was eluted at 3.260 min. The response of the standard 1,4 naphthoquinone linear in the concentration range of 3.0-21.0 µg/mL with r^2 = 0.9951. The accuracy of this method, which was 99.95% with RSD value ≤ 2%. UHPLC method was developed, which has been validated and shown applies to the determination and quantification of 1,4 naphthoquinone in E. palmifolia. Analysis of 1.4 naphthoquinone levels contained in E. palmifolia 12 µg/mL was 7.79 µg/mL ± 0.01.

1. Introduction

Eleutherine palmifolia (L.) Merr is a typical plant of Kalimantan in Indonesian. The people of known as a Dayak Onion [1]. The plant is belonging to the *Iridaceae* family [2]. According to the literatures, this plants contains many chemicals that can be used as traditional medicine. The presence of the content which needs to be further studied in *E. palmifolia* is naphthoquinone. Naphthoquinone derivatives have significant pharmacological properties. It has cytotoxic, antibacterial, antifungal, antiviral, insectidal, anti-inflammatory, and antipyretic properties [3]. 1,4 naphthoquinone or para-naphthoquinone is an organic compound derived from naphthalene. It forms volatile yellow triclinic crystals and has a sharp odour similar to benzoquinone. Naphthoquinone is a lipophilic compound with a log P value of 3.933 [4].

Several studies have shown that naphthoquinone compounds and their derivatives have potential activities as anticancer drugs [5-9]. Naphthoquinone is able to inhibit cell proliferation and induce apoptosis of melanoma cells. Cancer therapy approach through apoptotic induction mechanism has been known to be able to prevent the promotion, progression and re-emergence of cancer [9].

Various methods for quantification of naphthoquinone have been described, but quantitative analysis of naphtoquinone in *E. palmifolia* has not been reported. Studies have since revealed that this species has medicinal properties [10]. Lack of more comprehensive, systematic data on the phytochemical composition of the plant prompted the authors to undertake such studies. Results from preliminary UHPLC evaluation of the 1.4 naphthoquinone occurring in the tuber of *E. palmifolia* are presented in the paper.

2. Experimental

2.1. Sample Preparation

E. palmifolia was extracted three times with 500 mL of ethanol by sonication (10 min each, at ambient temperature). The sample of 25 grams was dissolved in ethanol 96% 500 mL (ratio 1:20). The collected filtrate is then separated by its solvent using a rotary evaporator.

2.2. Standard and Materials

2.2.1. Standards. The marker is 1,4 naphthoquinone compound used in the study purchased from Sigma Aldrich (Darmstadt, Germany).

2.2.2. Herbal material. The samples of E. palmifolia were purchased from vendors in Central Borneo, Indonesia. The samples were identified at the Materia Medica in Batu, East Java, Indonesia, with the accession number 074/342A/102.7/2018. The specimens are stored in the pharmacognosy Laboratory of the Pharmacy Department, Maulana Malik Ibrahim, State Islamic University of Malang. At Figure 1 was shown the image of *Eleutherine palmifolia* (A), Simplicia of *Eleutherine palmifolia* (B) and 1,4 naphtoquinone structure (C).

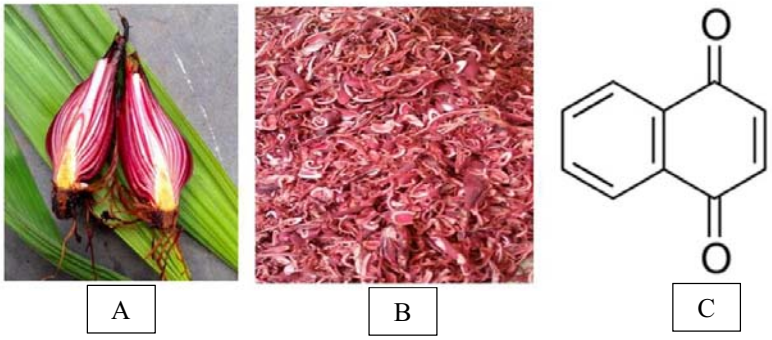

Figure 1. The image of (A) *Eleutherine palmifolia* (B) Simplicia of *Eleutherine palmifolia* (C) 1,4 naphtoquinone

2.3. *Ultra-High Performance Liquid Chromatography* (*UHPLC*)

2.3.1. Chromatographic conditions. The 1,4 naphthoquinone standard and E. palmifolia extract were analyzed by UHPLC. In the development of the UHPLC method, the selection of an appropriate mobile phase is a matter that needs attention. The compounds in E. palmifolia extract tend to be polar, so with the use of gradient elution at the beginning of elution time, there will be separation with polar compounds. The separation of the species was carried out with Thermo Fisher Scientific UHPLC Ultimate 3000 RS (Thermo Fisher Scientific, USA) coupled with a diode array detector (DAD), C18 column (5 µm, 4.6 mm × 250 mm) as employed. The UV detection was performed at 254 nm, and the run time of 0.8 minutes. The mobile phase consists of the isocratic method, 95% methanol (pump A) and 0.5% chloroform (pump B). The flow rate was 1.0 mL min^{-1}, and the injection volume was 10 µL. The temperature of the column was set at 25 °C.

2.3.2. Analytical validation. We evaluated parameters in the validation of the analytical methods such as selectivity, accuracy, precision, linearity.

a. Selectivity
Method selectivity was determined by comparing retention time (t_R) of *E. palmifolia* extract added with 1,4 naphthoquinone with t_R of 1.4 naphthoquinone.

b. Accuracy
Accuracy was evaluated by addition (spiking method) of the standard solution 1,4 naphthoquinone with concentrations 12 µg/mL. The analysis was performed in triplicate. The precision of the method is determined by the method of adding and expressed as a percent of inventiveness behind. Rally behind several standards that can be obtained after added to the sample. Determining the accuracy of 1.4 naphthoquinone in extracts made by adding a standard solution of 3.0 µg/mL as much as 2 mL into the extract *E.palmifolia*. Content standards are measured in a sample using a method of adding raw can be calculated by the formula (1) [11]:

$$C = S\left(\frac{R_1}{R_2 - R_1}\right) \quad (1)$$

Description:
C = concentration standards are measured in samples
S = concentration standards were added to the sample
R_1 = responses were given samples
R_2 = responses were given a mixture of the sample with an additional standard

c. Precision
The repeatability of the analytical method was evaluated by injecting six independent preparations of an *E. palmifolia* solution, on the same day by the same analyst. Obtaining the results, we calculated the mean and standard deviation (SD) with the formula (2) [12,13]:

$$SD = \sqrt{\frac{\sum_{i=1}^{n}(x_i - x)}{n-1}} \quad (2)$$

Description :
x_i = the acquisition back in replay to-*i*
x = average gain back
n = number of repetitions

d. Linearity
A calibration curve was prepared with volumetric flasks of 10 mL calibrated at the concentrations (µg/mL): 3.0; 6.0; 9.0; 15.0; 18.0; and 21.0 from standard stock solutions with a concentration of 100 mg/mL of 1,4 naphthoquinone in water ultrapure. The linearity of the method is determined by way of making the curve relationship between the concentration of the standard on the axis x and area on the axis y. The relation between 1,4 naphthoquinone concentration and mean response is implemented in a linear regression equation (y = a+bx) and the correlation coefficient (*r*). The average is implemented in a linear regression equation (y = a+bx) and the correlation coefficient (*r*) [12,13].

2.3.3. Quantification of 1.4 Naphthoquinone in E.palmifolia. About 12 µg/mL of *E.palmifolia* was used to quantify the 1,4 naphthoquinone content. Filtered to vial using a syringe and a 0.45 µm filter. The calculation of the 1,4 naphthoquinone content in the solution *E.palmifolia* was made in validation Chromeleon software version 7.2 three vials from each sample was prepared and injected in triplicate.

3. Results and discussion

Research on traditional medicine, especially those in the form of medicinal plants, continues to increase even the number increases lately. Even so until now, not much results of research into medicinal plants which is used as medicine in-service health. Drugs that can be used at the community must meet the safety requirements, useful and standardized. *E.palmifolia* is often used in the community as an anti-tumour in the digestive organs including in oral cavity, but there is no scientific research, and active ingredients have no role yet Based on the approach ethnopharmacology *E.palmifolia* is proven to be anti-cancer. Ethnopharmacology is a theoretical approach with utilizing empirical indications of the use of ingredients plants as medicine.

3.1. Analytical validation

Validation is carried out to guarantee that the results obtained can be justified to calculate 1,4 naphthoquinone levels in *E.palmifolia* extract. The concentration of 1,4 naphthoquinone in *E.palmifolia* was evaluated through an analytical method which is presented in this work. Some analytical validation parameters were assessed.

The selectivity is the ability of a process to quantify the analyte accurately in the presence of interferences existing in the sample, such as impurities, degradation products, and excipients. Concerning a herbal, selectivity is an essential parameter because it can show that the marker or markers can be detected unequivocally. As those herbals are complex matrices, and cannot compare with placebo, this parameter is indispensable. Figure 2 shows the chromatograms of standard and extract *E.palmifolia* to the selectivity evaluation. The solvent chromatogram did not show the appearance of a peak. In this study, the analytical method developed for the quantification of 1,4 naphthoquinone, was selective because no peak appears in the default retention time, taking into account the race time of the mobile phase, the standard and the extract *E.palmifolia*. Several analytical methods have been proposed for the determination and quantification of 1,4 naphthoquinone from different sources. Analytical method where the separation was done in the isocratic mode, using methanol: chloroform (95:0,5 v/v) at a flow rate of 1 ml min^{-1}.

The developed method has been validated and showed excellent accuracy, precision, and linearity. The first parameter of the validation method is accuracy. Accuracy is a measure that shows the degree of closeness of the analysis results with the actual level of the analyte. Accuracy is stated as a percentage of recovery of added analytes. Accuracy can be determined in two ways, namely, spiked-placebo recovery and standard addition methods [11]. In this study, using the addition method, where the sample is analyzed, then concentration variations in the regression equation are added to the sample, mixed and analyzed again. The difference between the two results is compared with the actual level. Based on the calculation of the accuracy parameter to analyze 1.4 naphthoquinone, it shows the value of precentage recovery of 99.95% with the addition of the standard 2.0 µg/mL. A reasonable percentage recovery requirement is 98-102% [11].

Precision is a measure of the closeness of the results of the analysis obtained from a series of repeat measurements of the same size. The precision value is represented by the standard deviation value and% relative standard deviation (% RSD) of the repeatability of each 1,4 naphthoquinone standard series measured at a concentration. Precision criteria are given if the method gives a value of % RSD ≤ 2%. This criterion is very flexible, depending on the concentration of the analyte analyzed, the number of samples and laboratory conditions [6]. RSD of variation increase with decreasing levels of analyte analyzed [11].

The 1,4 naphthoquinone precision test results in *E.palmifolia* extract at an analyte concentration of 0.3-21.0 µg/mL (table 1) showed that the RSD value met the precision requirements, so that in this study the more significant the concentration of the analytes added, the better precision would be. This shows that the 1,4 naphthoquinone analysis method in *E.palmifolia* extract will give precise results if the analytes added are getting bigger.

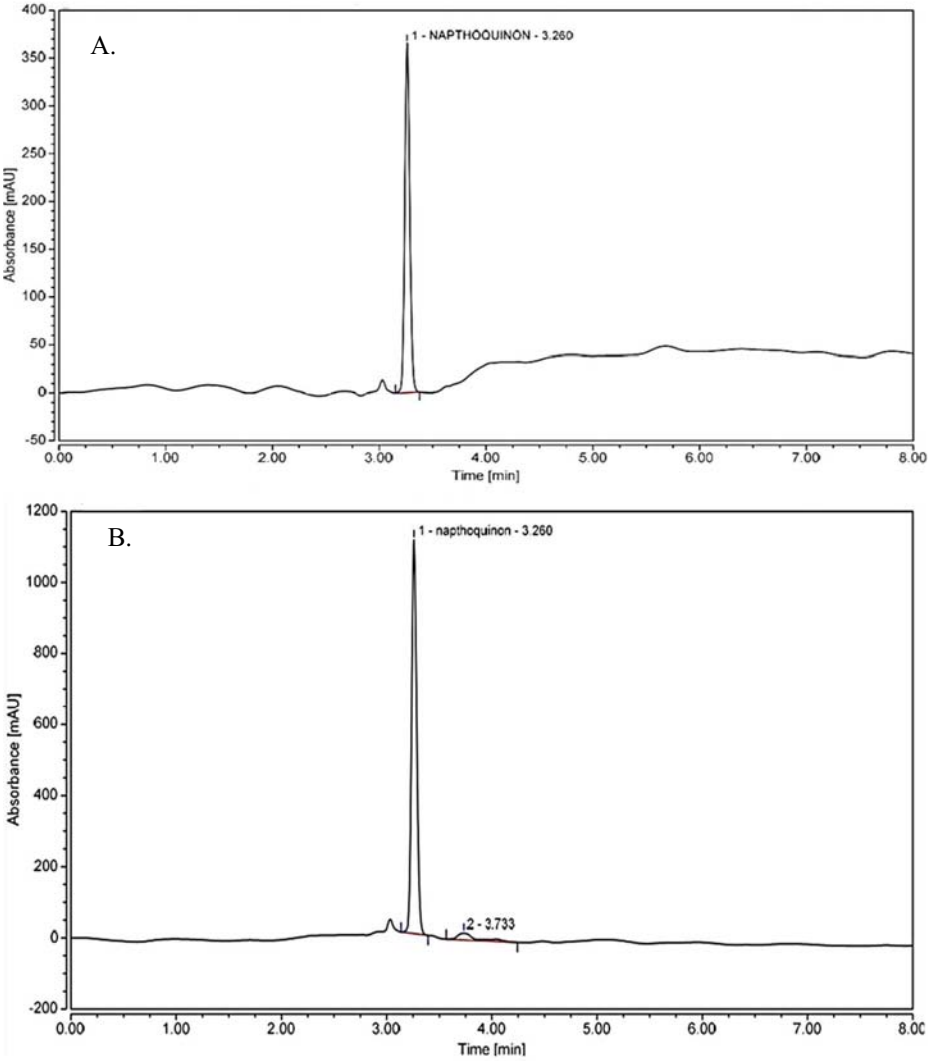

Figure 2. Chromatograms obtained in the selectivity parameter of the analytical method. (A) Chromatogram of 1,4 naphthoquinone in 3.260 min (B) 1.4 naphthoquinone in *E.palmifolia* in 3.260 min.

Table 1. Precision of 1,4 Napthoquinone at 3.0-21.0 µg/mL.

Concentration (µg/mL)	Mean concentration that is legible	SD	% RSD
3.00	1.24	0.00	0.57
6.00	5.28	0.00	0.05
9.00	7.22	0.00	0.02
15.00	12.09	0.00	0.00
18.00	17.29	0.00	0.00
21.00	18.89	0.00	0.00

Linearity test is an analytical method that illustrates the ability of a tool to obtain test results that are proportional to the levels of analytes in the extract at a certain range of levels [9]. Linearity data is obtained by making a curve of the relationship between the standard concentration of melamine on the x-axis and absorbance on the y-axis. Before obtaining linearity data, a standard standard curve of 1,4 naphthoquinone with a concentration of 3.0; 6.0; 9.0; 15.0; 18.0; and 21.0 µg/mL.

Linearity is expressed in the correlation coefficient (r^2). The correlation coefficient (r^2) in this study was 0.9951. This value meets the requirements set by the AOAC guideline, which is 0.9900. High correlation coefficient values indicate a linear relationship between the measured detector signal and the amount of 1,4 naphthoquinone in *E.palmifolia* extracts (Figure 3).

The intercept value (a) indicates the influence of the matrix on the analyzed solution. Intercept values that are farther from zero are affected by the matrix in the higher solution. This can interfere with the determination of the analyte in the sample to be analyzed. The slope value (b) expresses the sensitivity of a method. A considerable slope value indicates that a small change in concentration is very influential on the detector signal produced so that a method can be said to have excellent sensitivity [9]. The result of all validation step was tabulated in Table 2.

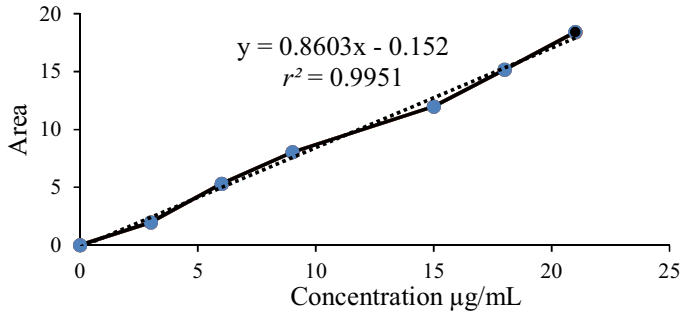

Figure 3. Linearity graph analytical validation pada 254 nm.

Table 2. Validation Parameters of 1,4 Naphthoquinone in *E.palmifolia*.

Validation Parameters	Result
Selectivity (Retention time t_R)	3.260
Accuracy %	99.95
Precision (%RSD, n=6)	≤ 2
Linearity (correlation coeficient)	0.9951

3.2. Quantification of 1.4 Naphthoquinone in E.palmifolia by UHPLC

High-performance liquid chromatography is a chromatographic technique based on the difference in the distribution of component molecules between two phases (stationary phase and stationary phase) which have different polarity. The HPLC technique is a liquid chromatography technique that can be used both for separation, identification, HPLC or high-performance liquid chromatography is one of the chromatographic techniques based on the difference in the distribution of component molecules between two phases (mobile and stationary phases) different polarity. The HPLC technique is a liquid chromatography technique that can be used both for the need for separation and identification.

The working principle of the HPLC tool is that when a sample to be tested is injected into a column, and the sample will then be decomposed and separated into chemical compounds according to differences in affinity. The results of the separation will then be detected by a detector (UV spectrophotometer) at a specific wavelength. The results that emerge from the detector are then recorded by a recorder which can usually be displayed using an integrator or using a personal computer (PC) connected online with the HPLC device. The calculation of the 1,4 Naphthoquinone content in the solution *E.palmifolia* was made in validation Chromeleon software version 7.2. The sample was

prepared and injected in triplicate. The research can be seen that the content of 1,4 naphthoquinone in *E.palmifolia* extracts. Data shows that *E.palmifolia* extracts contains a high amount of 1,4 naphthoquinone. Concerning the quantification of 1,4 naphthoquinone in *E.palmifolia*, it has been shown that the concentration of 12 µg/mL was 7.79 µg/ mL ± 0.01, which was calculated against the 1,4 naphthoquinone standard.

4. Conclusion

UHPLC method was developed, which has been validated and shown apply to the determination and quantification of 1,4 naphthoquinone in *E.palmifolia*. The process was approved according to standards showing selectivity, accuracy, precision, and linearity. Analysis of 1,4 naphthoquinone levels contained in E. palmifolia 12 µg/mL was 7.79 µg/mL ± 0.01

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