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VALIDATION OF SPECTROPHOTOMETRY METHOD FOR DETERMINATION OF (+)-CATECHIN IN ETHYL ACETATE FRACTION OF GAMBIR EXTRACT (*Uncaria gambir* Roxb.)

Validasi Metode Spektrofotometri untuk Penetapan Kadar (+)-Katekin dalam Fraksi Etil Asetat Ekstrak Gambir (Uncaria gambir Roxb.)

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ABSTRACT

Gambir leaves (Uncaria gambir Roxb.) is a plant that has been widely used by people for traditional medicine. The main compound of gambir extract is a (+)-catechin that has been proven as anti-dental plaque, antioxidant, antibacterial, and antihyperlipidemic. This study aims to validate a method for the quantitative determination of catechin in ethyl acetate fraction of gambir extract based on spectrophotometry. The validation was conducted by measuring the linearity, accuracy, and ruggedness of the method that fulfills the requirements along with the limit of detection (LoD) and limit of quantification (LoQ) determination. Determining the precision (based on %RSD and CV), 100 ppm of catechin were made to 7 replicates while accuracy was evaluated by calculating the recovery. The ruggedness of the method is determined by analyzing it on a different day. The linearity was determined by assessing the r-value on the relation between the absorbance curve and standard series concentration. The LoD and LoQ could be calculated based on the standard curve equation. The results showed the method fulfilled the linearity requirement with R 0.9996; had precision in 1.23% RSD; had accuracy in the range of 100.80% to 101.64%; the method ruggedness was not significantly different; had 3.85 ppm of LoD and 12.84 ppm of LoQ. The validation method showed a valid result, and the method can be used for routine analysis on the measurement of catechin in ethyl acetate fraction of gambir extract.

***Keywords**: Catechin, Uncaria gambir, Method Validation, Spectrophotometry*

ABSTRAK

Daun gambir (*Uncaria gambir* Roxb.) merupakan tanaman yang telah banyak dimanfaatkan masyarakat dalam pengobatan tradisional. Senyawa utama ekstrak gambir adalah (+)-katekin yang telah terbukti sebagai anti plak gigi, antioksidan, antibakteri, dan antihiperlipidemia. Penelitian ini bertujuan untuk memvalidasi suatu metode penentuan kuantitatif katekin dalam fraksi etil asetat ekstrak gambir secara spektrofotometri. Validasi dilakukan dengan mengukur linearitas, akurasi, dan kekasaran metode yang memenuhi persyaratan beserta penetapan *Limit of Detection* (*LoD*) dan *Limit of Quantification* (*LoQ*). Penentuan presisi (berdasarkan %RSD dan CV), 100 ppm katekin dibuat 7 ulangan sedangkan akurasi dievaluasi dengan menghitung perolehan kembali. Kekasaran metode ditentukan dengan menganalisisnya pada hari yang berbeda. Linearitas ditentukan dengan mengukur nilai r pada hubungan antara kurva absorbansi dan konsentrasi seri standar. Berdasarkan persamaan kurva standar, maka *LoD* dan *LoQ* dapat dihitung. Hasil penelitian menunjukkan metode memenuhi syarat linearitas dengan R 0,9996; memiliki

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presisi di 1,23% RSD; memiliki akurasi pada kisaran 100,80% sampai dengan 101,64%; kekasaran metode tidak berbeda nyata; memiliki 3,85 ppm *LoD*; dan 12,84 ppm *LoQ*. Validasi menunjukkan hasil yang valid, dan metode tersebut dapat digunakan untuk analisis rutin pada pengukuran katekin dalam fraksi etil asetat ekstrak gambir.

Kata kunci: Catechin, *Uncaria gambir*, Validasi Metode, Spektrofotometri

INTRODUCTION

As a tropical and archipelago country, Indonesia is endowed with rich and unique biodiversity. Indonesia's tropical forest covers about 143 million hectares and is covered by about 80% of the world's medicinal plants, and it's estimated that 28,000 plant species grow in Indonesia's tropical forests (Elfahmi *et al.*, 2014). One of the potential medicinal plants grown in Indonesia is gambir (*Uncaria gambir* Roxb). Gambir is a typical plant from West Sumatra, North Sumatra, Riau and South Sumatera (Nasution *et al.*, 2016). Gambir is a plant commodity that has high economic value for Indonesia. As the biggest gambir supplier, Indonesia supplies 80% of world needs while 90% of the supply is produced in West Sumatra (Evalia *et al.*, 2012).

In Indonesia, gambir is commonly used by people for chewing. The usage of gambir still exceeds and its development is often used for drugs such as burns, headaches, diarrhea, dysentery, mouthwash, mouth sores, skin pain, to expedite the process of digestion in the stomach, and intestines (Lucida & Rustini, 2010; Rauf & Siregar, 2015). Several pharmacological studies of gambir have been proved that its leaves have antioxidants, antibacterials, and hyperlipidemia properties (Ningsih & Rahayuningsih, 2019; Melia *et al.*, 2015; Yunarto *et al.*, 2015).

Catechins are the main bioactive compounds and have been used as metabolite biomarkers to determine the quality of gambir (Nurliayana *et al.*, 2016). A comprehensive study by Sazwi *et al.* (2013) stated that (+)-catechin, (+)-epicatechin, and seven dimer flavans known as protoanosianidin (gambirin A1, A2, B1, B2), procyanidin B1, procyanidin B3, and gambirin C have been isolated from aqueous extracts of gambir leaves. Andasuryani *et al.* (2014) showed that catechin was the most abundant constituent of gambir in addition to epicatechin and gambirin.

The demand of medicinal plants, dietary supplements, and cosmetics in developed or developing countries are increasing presently. This is due to the recognition that products from natural ingredients are non-toxic, have few side effects, are easy to obtain and affordable. In addition, it is also known that natural products also have a broader biological activity and a higher safety limit than synthetic drugs (Kataky & Handique, 2010).

The development of traditional medicine from plants requires the fractionation, isolation, and purification of active compounds from complex multi-component mixtures to produce high purity products. The use of catechin from gambir leaves as raw material for medicine has not been performed, due to less study of catechin from gambir leaves. Eventhought there are many studies about its pharmacological activities in tea leaves. Moreover, the purity of the catechin fraction is also needed to ensure its pharmacological effect. A method that can be used in the quantitative assay in a relatively short time, cheaper, and suit to the required standard in Herbal Pharmacopeia is spectrophotometry. For those reasons, catechin levels of gambir leaves are determined using validated spectrophotometry. The validation of the analytical method should fulfill all the requirements of the analytical application to ensure the reliability of the analysis

results. The validation encompasses specificity, linearity, precision, sensitivity, accuracy, quantitation limits, and ruggedness (Rohman, 2014).

METHOD

Time and Place.

The research was conducted at the Pharmacy Laboratory, Center for Research and Development of Biomedical and Basic Health Technology in January-April 2021.

Tools and Materials.

The tools used in this study were rotary evaporator (Buchi R-300), spectrophotometer (Pharo 300), HPTLC (Camag-4), digital balance (Metler Toledo HR-200), column chromatography, erlenmeyer 2.000 mL (Pyrex), 1.000 mL beaker glass (Duran), measure glass 500 mL (Pyrex), reagent bottle (Normax), desiccator, oven (Memmert). The materials used were gambir leaves extract, catechin standard 98% (Sigma Aldrich), ethyl acetate (Merck), distilled water (Brataco), formic acid (Merck), and methanol (Merck), non-fibre fabrics, and glass wool.

Fractionation.

Five hundred grams of gambir extract from Limapuluh Kota District was crushed with a mortar until smooth. It was put into a chromatography column then soaked with ethyl acetate and distilled water for 6 hours. The lower valve of the column is opened so that the distilled water comes out first, then the ethyl acetate gambir phase drips slowly and is collected into the erlenmeyer. During the process, ethyl acetate is added through the top of the column. The fraction results were concentrated with a rotary evaporator at a temperature of 40°C, then dried using a vacuum oven at 40°C to a fixed weight (Yunarto *et al.*, 2017; Kurniatri *et al.*, 2019). The obtained fraction was calculated to yield fraction, organoleptic testing, moisture content, and ash content.

Identification of catechin by HPTLC.

The qualitative identification of catechin in ethyl acetate fraction of gambir extract used chloroform: ethyl acetate: formic acid (5:4:1) as the mobile phase and silica gel F254 as the stationary phase. The test solution was 0.5% (+)-catechin standard solution, equivalent to 1 gram of the ethyl acetate fraction of gambir leaves in methanol. Standard solution: 0.5% catechin in methanol. The volume was dropped by 10 μL. The TLC process using HPTLC (Camag-4) was started with spot sampling and catechin standard on the TLC plate. The TLC plate was eluted in the chamber (saturated with the mobile phase). After the elution process reaches the upper limit, the plates are dried (Yunarto *et al.*, 2015).

Determination of the maximum wavelength.

Three ml concentration of 60 ppm (+)-catechin standard solution was measured by a spectrophotometer (Pharo 300) at a wavelength of 200-350 nm using 2% formic acid in methanol as the blank.

Validation of Analytical Methods

Linearity. Linearity is determined by making a standard curve to assess the ability of an analytical method to obtain proportional results to the concentration in the sample (Syukri *et al.*, 2015). Linearity is evaluated by making a series of (+)-catechin standard solutions at a concentration of 20, 40, 60, 80, 100, and 120 ppm. The absorbance value is recorded and used as one variable for the standard curve in the relation between absorbance and the concentration of the standard solution. The r-value of the curve represents linearity and is expressed as linear if the value of $R > 0.995$ (Wardani, 2012).

Precision. The precision test was carried out by making levels of 100 ppm in 7 replicates and determining the levels with a spectrophotometer. The level is determined based on the standard curve that has been obtained. The mean and %RSD levels were calculated. The method is valid if %RSD ≤ 2% (Pertiwi, 2015).

Accuracy. The accuracy test is carried out by adding the analyte to the solvent. This test is carried out at concentrations of 80, 100, and 120 ppm. Furthermore, the mixture is analyzed and compared with the actual content of the added analyte (Sugihartini *et al.*, 2014). The accuracy test is accurate if the results had a recoverable value between 98-102% (Pertiwi, 2015).

LoD and LoQ. The limit of Detection (LoD) and limit of Quantification (LoQ) could be calculated statistically through the linear regression line of the calibration curve. The measurement value will be the same as the b value in the linear line equation y=a+bx, while the blank standard deviation is the same as the residual standard deviation (sy/x). LoD and LoQ could be calculated with the following formula (Bertil & Ornemark, 2014) :

$$LoD = \frac{3\ Sy/x}{Sl} \qquad LoQ = \frac{10\ Sy/x}{Sl}$$

Information:

Sy/x	=	residual standard deviation
Sl	=	the linear line direction (directional sensitivity) of the curve between responses to
Concentration	=	slope (b in the line equation of y = a + bx)

Ruggedness. Ruggedness evaluates the degree of reproducibility of test result obtained by analyzing the same sample under day variation. The ruggedness result is analyzed with T-test. The method is declared tough if the results had no significant difference (Alwi, 2017).

RESULT AND DISCUSSION

Fraction Characterization

The fractionation process was carried out using columns and obtained 392.2 g of ethyl acetate fraction from 500 g of gambir leaves extract (Figure 1).

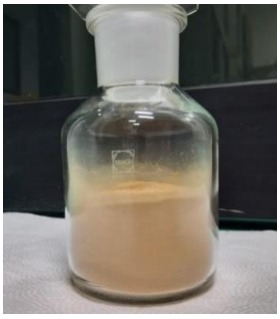

Figure 1. Ethyl acetate fraction of gambir extract

This result showed that catechin easily dissolved in ethyl acetate solvent, which is in line with previous research conducted by Kassim *et al.* (2011). The research stated that gambir extract was more soluble in ethyl acetate. The results of the characterization of the ethyl acetate fraction of gambir extract are shown in Table 1.

Table 1. Ethyl acetate fraction of gambir extract character

Characterization	Result
Shape	Solid, Powder
Color	Yellowish brown
Yield	78.44%
Moisture Content	4.9%
Total Ash Content	0.12%

The characterization of ethyl acetate fraction of gambir extract ensured that the extract had a constant value of certain parameters. Based on the characterization results, the water content of the fraction obtained was 4.9%, which means that it has fulfilled the requirements of the Herbal Pharmacopoeia, which is determined not more than 14%. The total ash content was 0.12% as required in the Herbal Pharmacopoeia, less than 0.5% (Ministry of Health, 2017). The less ash content of gambir extract indicated that the remaining material included physiological and non-physiological ash from the plant tissue, which was a residue of foreign material that stuck to plant surfaces such as sand and soil was very limited. The smaller the ash content, the smaller impurities in the ethyl acetate fraction (WHO, 2011).

Identification of catechin by HPTLC

TLC determination was conducted to identify and rapidly separate substances based on polarity differences between the sample and the solvent. Identification of catechin was carried out by comparing the catechin in ethyl acetate fraction to (+)- catechin standard on the TLC plate with an assumption that if the fraction had the same Rf as the catechin standard, it was concluded that the ethyl acetate soluble portion contained catechin. The ethyl acetate fraction of gambir extract TLC results can be seen in Figure 2. The TLC results in Figure 2 showed that the catechin of ethyl acetate fraction spots had the same Rf calculation as the catechin standard value 0.270.

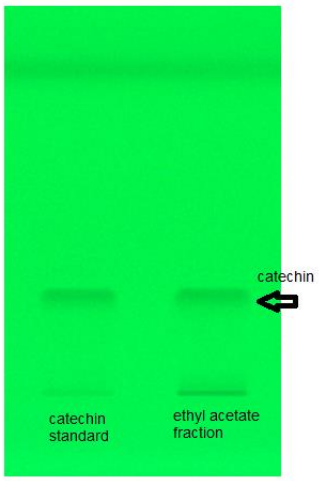

Figure 2. Chromatogram of catechin in ethyl acetate fraction of gambir extract

Validation of Analytical Methods

Validation of the analysis method becomes an essential factor since it could be used as a basis for further calculations if the validity of the analysis method had been proven. The parameters that were tested in the method validation encompass linearity, precision, accuracy,

the Limit of Detection (LoD), and Limit of Quantification (LoQ), and the ruggedness of the method (Bertil & Ornemark, 2014).

Maximum wavelength

The determination of catechin in ethyl acetate fraction of gambir extract was carried out by a UV-Vis spectrophotometry method at a wavelength of 200-350 nm. Based on Figure 3, the catechin standard had a maximum absorbance at a wavelength of 278 nm, so that the absorbance measurement of the sample solution was carried out at that wavelength.

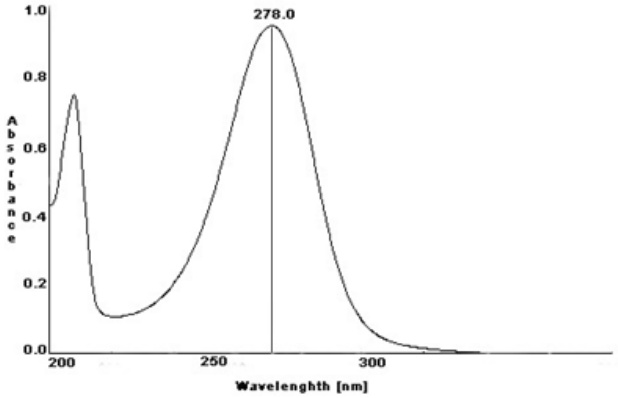

Figure 3. Maximum wavelength measurement

Linearity

Linearity is the ability of a method to keep the obtained test results directly proportional to the concentration of the analytic sample in a range (Gandjar & Rohman, 2007). A linearity parameter was used to determine the standard capability to prove a linear relationship between the sample concentration and the detector response. The correlation coefficient value is expected to be close to 1 or above 0.9900, indicating a suitable analysis method (Wardani, 2012). Linearity and range tests were carried out by making a standard curve to determine the relationship between catechin concentration and absorbance. The results of linear regression analysis can be seen in Figure 4, obtained by the linear equation of y = 0.0123x–0.047 with a correlation coefficient (R) = 0.9996.

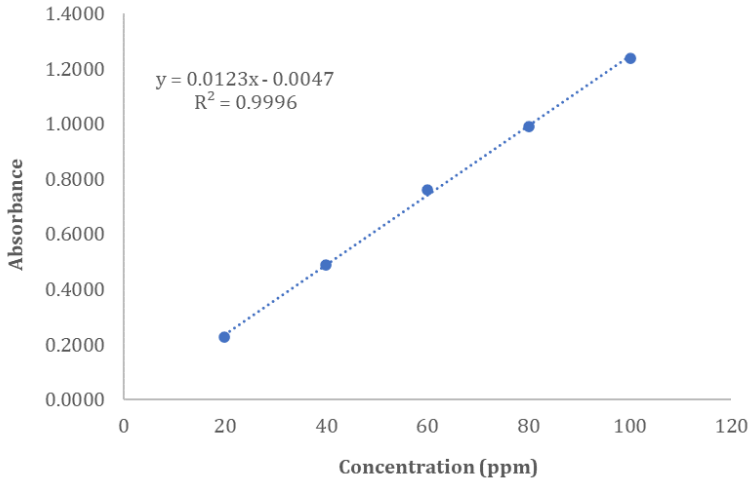

Figure 4. Standard curve relationship between standard catechin concentration and absorbance

The correlation coefficient gave linear results because it fulfilled the acceptance criteria. This method was declared linear based on the 0.9996 as R-value. The correlation coefficient

close to 1 indicates an increasingly linear relation between the concentration and the absorbance. According to Feldsine *et al.* (2002), linearity fulfills the stipulated requirements if the R-value is equal to or more than 0.9900. High correlation coefficient values indicated a linear relationship between the measured detector signal and the number of catechins. Thus it could be said that the determination of catechin levels using UV-Vis spectrophotometry had good linearity. The analytical method is stated as precise if it simultaneously provides consistent results on sample measurements. Precision is expressed as Standard Deviation (SD) or Relative.

Standard Deviation (RSD), or Coefficient of Variation (CV). In this method, precision was tested using a sample solution made into seven replicates for 100 ppm concentration. The calculation results are presented in Table 2, showing 1.23% of RSD, which means the method was declared valid because it met the requirements, since %RSD <⅔ CV Horwitz.

Table 2. Values of CV and %RSD in the precision test

Repetition	Absorbance	Concentration (%)
1	1.1750	93.99
2	1.1735	93.87
3	1.1935	95.47
4	1.2015	96.10
5	1.1880	95.03
6	1.2005	96.02
7	1.2140	97.10
Average		95.37
SD		1.17
% RSD		1.23
CV Horwitz		2.01
⅔ CV Horwitz		1.34

The precision parameter measures the repeatability of the analytical method and is usually expressed as the relative standard deviation of several samples that differ statistically significantly. In general, the accuracy value is calculated using a standard deviation (SD) to produce a Relative Standard Deviation (RSD) or Coefficient Variation (CV). The smaller the percent of RSD states good accuracy, the higher the value of precision (Wardani, 2012).

Accuracy

An accuracy test is made to obtain the recovery value (% recovery). The accuracy in this analysis used the addition method with three kinds of concentrations: 80 ppm, 100 ppm, and 120 ppm, which was made to 3 replicates. The results of catechin analytes percent recovery valued at 100.80%-101.64% can be seen in Table 3. These results were in line with the criteria for percent recovery of 98-102%. According to Pertiwi (2015), the calculation of accuracy uses the following formula, as shown in Table 3.

Table 3. Catechin in ethyl acetate fraction % recovery results

Concentration (ppm)	Real concentration (ppm)	Measured concentration (ppm)	Recovery (%)
80	87.73	88.43	100.80
80	87.73	88.67	101.08
80	87.73	89.16	101.64
100	93.61	95.02	101.51
100	93.61	95.10	101.59
100	93.61	94.77	101.25

Concentration (ppm)	Real concentration (ppm)	Measured concentration (ppm)	Recovery (%)
120	99.49	100.46	100.98
120	99.49	100.46	100.98
120	99.49	100.59	101.11

Accuracy is the closeness between the measured value and the accepted value, either the value of the convention, the actual value, or the reference value (Alwi, 2017). The accuracy is expressed as the percent return of the analyte added, and the accuracy value can be expressed as LoD and LoQ.

Limit of Detection (LoD) indicates the smallest analyte concentration in a sample that the instrument can still measure. At the same time, the Limit of Quantification (LoQ) shows the lowest concentration of analytes in the sample that can still be analyzed with precise precision and accuracy. The LoD value obtained in this test was 3.85 ppm, while the LoD value obtained was 12.84 ppm. LoD and LoQ values could be determined based on the standard curve obtained. LoD is the smallest number of analytes in the sample that can be detected which still gives a significant response compared to blanks. LoQ is the smallest number of analytes in the sample that can still carefully fulfill the criteria. It can be quantified with good accuracy and precision (Wardani, 2012).

Ruggedness

The same analyst tested the ruggedness of the method on different days. The test was carried out the same as precision testing, using a sample solution made into seven replicates of 100 ppm. In this test, the value of f arithmetic < f table and t value < t table was obtained, which means that the comparison of the two data was not significantly different. The method was declared ruggedness if the results were not significantly different. Ruggedness is defined as the absence of a different operating or working environment influencing the test results. The ruggedness of the method is the result of repeated testing obtained from analyzing the same sample under various conditions, such as laboratories, analysts, instruments, reagents, temperatures, at different times (Alwi, 2017).

Based on Table 4, the T-test value (1.07) is smaller than T-table (2.18) with a 95% confidence level. The result means that the data was not significantly different, so that the method was declared ruggedness.

Table 4. Results of the method ruggedness that conducted on different days

Repetition	Data of 1st day	Data of 2nd day	T-test (p)
1	93.99	96.97	
2	93.87	96.25	
3	95.47	95.69	
4	96.10	95.61	1.07
5	95.03	97.59	
6	96.02	94.40	
7	97.10	95.53	

CONCLUSION

The study concluded that the method of analysis in the determination of (+)- catechin level in ethyl acetate fraction of gambir extract by UV-Visible spectrophotometry is an excellent and valid method to be used. All validation parameters, i.e., linearity, precision, accuracy, and ruggedness, fulfills the standard requirements of Farmakope Herbal Indonesia.

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