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Original Research Article

Development and Validation of a High-Performance Liquid Chromatography-Based Method for Catechin Isolated from the Leaves of Gambir (*Uncaria gambir* Roxb)

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ABSTRACT

Gambir (*Uncaria gambir* Roxb) has long been thought to provide various health benefits due to the main secondary metabolite, catechin. The high catechin content makes gambir a strong antioxidant. The present study was conducted to develop and validate the quantification of catechin in gambir leaves by high-performance liquid chromatography (HPLC). The method was performed using a 100 mL sample with dilution. The separation process employed a Sunfire C18 (150 mm × 4.6 mm) column, the mobile phase was composed of solvent A: 0.03% trifluoroacetic acid in acetonitrile-water (5:95) and solvent B: 0.1% trifluoroacetic acid in acetonitrile at a 0.45 mL/min flow rate. The validation was conducted by measuring the accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), and robustness. The results showed that the percent recovery was 98.30-99.82% with a precision value of 0.28% RSD, good linearity with a 0.9996 R-value, 2.7 µg/mL of LOD, and 8.3 µg/mL of LOQ. There was no significant difference between changes in the mobile phase of 4% (comparison of area and retention time), and 6% (comparison of area). However, the results also demonstrated that the mobile phase was not resistant to changes in the mobile phase of 6% in the ratio of retention time because of the shift in retention time that was faster than the catechin standard. The findings of this study reveal that this method satisfies the validation requirements for accuracy, precision, linearity, and robustness tests, therefore, the HPLC-based method can be applied to analyze catechin in gambir leaves.

Keywords: Catechin, HPLC, Validation method, *Uncaria gambir*

Introduction

Gambir (*Uncaria gambir* Roxb) is one species from the Rubiaceae family that is widely grown in Indonesia and has the potential to be a medicine.[1] Gambir is a typical plant produced in four provinces in Indonesia, namely West Sumatra, North Sumatra, Riau, and South Sumatra.[2] However, as much as 90% of the gambir in Indonesia is produced in West Sumatra. The primary component of gambir extract is made up of 28–54% catechins, which are secondary metabolites of the flavonoid class.[3] The high content of catechin in gambir leaves makes them one of the plants that have the potential to be used as medicinal raw materials.[4] As a potent antioxidant and antihyperlipidemic, catechin compounds isolated from gambir leaves may have pharmacological benefits.[5,6] However, Yunarto, *et al*. (2021) highlighted in their research that, due to a lack of gambir leaf catechin isolate research, the use of gambir leaf catechin isolates as medicinal raw materials has not been widely accepted.[6]

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Standardized herbal medicinal raw materials are required to enable the analysis of the catechin isolate present in gambir leaves, which is necessary to promote the development of traditional medicines into medicinal products with innovations. The maximum extent of the development of new techniques and terms is urgently required.

The separation and identification of gambir leaf catechin isolate must be improved, and one analytical technique that can be employed is the high performance liquid chromatography (HPLC) method. Compared to other separation methods, HPLC has several advantages. Its advantages include analytical accuracy, high sensitivity, and the ability to use very small particles with a wider surface area to make the separation system better.[7] A new analytical method must be validated to prove that its performance parameters are sufficient to overcome certain analytical problems and to ensure that the analytical method used is accurate, specific, reproducible, and resistant to the range of compounds to be analyzed.[8] To ensure that the approach is practical for analyzing the compound of gambir leaf catechin isolates, validation of the analytical method for gambir leaf catechin isolate using HPLC is required.

This study was aimed at developing and validating a high-performance liquid chromatography-based method for catechin isolate from *Uncaria gambir* leaves.

Materials and Methods

Source of plant material

The plant materials used were fresh gambir leaves obtained from Lima Puluh Kota District, West Sumatera. The collection was done between September 30 and October 2, 2022. Dr. Nuraianas, Head of Herbarium Laboratory, Department of Biology, Andalas University, Padang,

identified and authenticated the plant leaves with ID No. 024/ANDA/II/2022.

Equipment and chemical materials
The types of equipment used include an HPLC (Waters), a rotary evaporator (Buchi), a fume hood (Esco), an oven (Memmert), an ashing furnace (Thermo Scientific), a moisture analyzer (Sartorius), an analytical balance (Metler Toledo), a micropipette (Socorex), a sonicator (GB-928 Ultrasonic Cleaner), C18 column (X-Bridge, 150 x 4.6 mm), a desiccator, and a vacuum liquid chromatography (VLC). The chemical materials used were catechin standard (Sigma), trifluoroacetic acid (Merck), ethyl acetate (Merck), methanol (Merck), acetonitrile (Merck), and aquabidest.

Extraction of gambir leaf extract
Gambir leaf extract was prepared by harvesting fresh gambir leaves and then steamed with boiling water. After steaming, it was removed and then compressed using a hydraulic press until the gambir sap was obtained. After that, the gambir sap was drained, stored in a baking dish, and chilled for two days until it became hard. Gambir sap was sliced into squares after hardening. To reduce the moisture content, the next step was to dry the product in an oven for a day at a temperature of 40 to 50°C.[9]

Fractionation of gambir leaf extract
Fractionation was carried out by macerating gambir leaf extract powder in a chromatographic column using ethyl acetate as solvent. After 24 hours, the faucet on the column was slightly opened, and the ethyl acetate fraction of gambir leaf extract was collected using an Erlenmeyer flask. The resulting fraction was concentrated using a rotary evaporator and dried in an oven at a temperature of 40-50ºC for 20-30 minutes.[10] After obtaining the products of the fraction, characterization, moisture content, and ash content were determined.

Isolation of catechins
The isolation of catechin was carried out using the vacuum liquid chromatography (VLC) method, which employed a gradient of hexane-ethyl acetate with a ratio of 80% hexane to 15% ethyl acetate plus 5% methanol. Following that, the presence of catechin compounds in the VLC process was monitored using HPLC.[11]

High-performance liquid chromatography conditions
The stationary phase used in the HPLC analysis was Sunfire C-18 (150 × 4.6 mm, 5 µm). The mobile phase used was 0.03% trifluoroacetic acid in a mixture of acetonitrile (ACN): distilled water with a ratio of 5:95 (A) and 0.1% trifluoroacetic acid in acetonitrile (B). The gradient conditions of the mobile phase were used, including 0-1.2 minutes (100% A), 1.2-12 minutes (71.5 A; 28.5 B), and 18-20 minutes. A sonicator was used to homogenize the mobile phase for 30 minutes. The column temperature was maintained at 30 °C with a Sunfire C18 column sized at 4.6 × 150 mm, 0.45 mL/min flow rate, a 1.0 µL injection volume, and UV detection at 280 nm using a PDA detector (Waters).[9]

Preparation of standard solutions
Standard stock solutions of catechin were prepared by weighing 25 mg (25,000 µg) of standard catechin and then dissolving it in mobile phase B up to 25 mL (1000 µg/mL). The standard stock solution was diluted with acetonitrile to obtain the desired serial concentrations (50, 75, 125, 150, and 200 µg/mL).

Sample preparation
Catechin isolate was prepared by dissolving 100 mg of catechin in 10 mL of acetonitrile. The solution was then diluted 20 times. The catechin sample solution was then put into the HPLC system after being filtered using a 0.45 m filter.

Methods of validation
Accuracy
The accuracy of the HPLC method was evaluated using a sample of catechin isolate with a known concentration added to each standard solution of catechin with a concentration of 80, 100, and 120% of the target analyte concentration. The accuracy of the HPLC method was assessed, and then the percentage recovery value was calculated using the equation:

% recovery = Amount recovered/Amount injected×100.

The percent recovery value was considered to have met the requirements if it had a 98–102% recovery value.[12]

Precision
Catechin isolate was tested for precision by dissolving it in mobile phase solvent B and replicated 6 times.[13] Then the concentration was determined using the HPLC.[13] From the data obtained, the standard deviation (SD) and relative standard deviation (RSD) were then calculated as the percentage of RSD (%RSD). The criteria can be considered accurate if the method used provides a relative standard deviation value or a %RSD value of 2% or less.[14]

Linearity
The linearity test was carried out with a standard solution of catechin consisting of 6 different concentrations (50, 75, 100, 125, 150, and 200 µg/mL). After that, the data were processed using linear regression to obtain a linear response to the concentration of the standard solution, which was expected to have a correlation coefficient value close to 1 or above 0.995, so that the analytical method could be considered good.[13]

Sensitivity
Sensitivity refers to the ability of the method to detect an analyte and estimate its concentration. The limit of detection (LOD) and limit of quantitation (LOQ) can be determined by the linear regression line of the calibration curve. The measurement value will be identical to the value of b in the linear equation y = b x a, and the blank standard deviation will be the same as the residual standard deviation (SD).[15] LOD and LOQ can be calculated using the following formula.[15]

$$\text{LOD} = \frac{3.3\,SD}{S} \quad \ldots\ldots (1) \qquad \text{LOQ} = \frac{10\,SD}{S} \quad \ldots\ldots (2)$$

Where SD is the standard deviation and S is the slope of the calibration curve.

Robustness
Robustness is evaluated by examining the clarity of a sample after making changes or variations in the composition of the mobile phase. The change in the composition of the mobile phase was carried out in mobile phase A, which included 0.03% trifluoroacetic acid in a 5:95 mixture of acetonitrile and aquabidest. Variations of changes in the mobile phase are changes in the concentration of 5% acetonitrile to 4% and 6% acetonitrile. To determine the effect of the change, the average result of the area read and the retention time of the variation of the mobile phase change was compared using the T-test, and the F value was calculated.[8]

Results and Discussion

The process of extraction was carried out using 10,000 grams of gambir leaves, yielding 630 grams of dried gambir leaf extract powder with a yellowish-brown color. From the extraction data, the extract yield was calculated to be 6.3%. The yield percentage result meets the requirements for the yield of gambir extract listed in the Indonesian Herbal Pharmacopoeia of a yield value not less than 2.9%.[16] The fractionation process was carried out by maceration using ethyl acetate as a solvent to bind catechin compounds from gambir leaf extract. Based on the results of a previous study, it was found that catechin dissolves best in ethyl acetate.[10] The characterization of the gambir leaf extract catechin was carried out to ensure that the isolate had a constant value for several parameters.[6] Based on the results of characterization (Table 1), catechin meets the specifications outlined in the Indonesian Herbal Pharmacopoeia in terms of shape, color, and odor. The catechin's water content was 3.08%, which implies it complies with the Indonesian

Herbal Pharmacopoeia's restriction of not exceeding 14%. The ash content was determined to be 0.34%, which satisfies the Indonesian Herbal Pharmacopoeia's criterion that the ash content not exceed 0.5%.[16]

Validation of analytical methods is the process of determining if a certain parameter has met the standards for use based on laboratory experiments.[8] According to the HPLC results, the catechin isolate sample from gambir leaves had a chromatogram with the same pattern and retention time as the catechin standard chromatogram (Figure 1), with a peak appearing at 14.20 minutes.[10] This indicates that the isolated sample contains catechin compounds. The accuracy was used to determine how closely the analysis of an analyte's levels matched the measured value and the real value as determined by the recovery value. The results of the calculations (Table 2) showed that the % recovery value obtained was 98.30 – 99.82% and that this value satisfies the criteria for the percent recovery value of 98–102%.[13] The previous validation study of the catechin analytical method conducted by Yunarto *et al*. (2021) resulted in a slightly higher % recovery value when compared to the results obtained in the present study, which is in the range of 100.80-101.64%.[17] This study used HPLC, which has better sensitivity than the spectrophotometric approach, although prior studies validated the catechin analytical method using a different technique, namely spectrophotometry. This could explain why the results are different.[18] Based on these values, it is clear that the HPLC method used to analyze gambir leaf catechin isolates satisfies the accuracy requirements and may produce reliable results.

High-precision analytical methods will always produce consistent measurement results from the same sample. The precision in this study was tested using catechin isolate with 6 replications. Relative standard deviation (RSD) or percent relative standard deviation (%RSD) are two ways to express the precision value.[13] As shown in Table 3, it was observed that the catechin content was 98.75% with a %RSD value of 0.28%. This value satisfies the %RSD requirement in the analyte with a concentration of 100%, which is 2%. The RSD result also has a lower value when compared to the previous validation study of the catechin analytical method conducted with spectrophotometry. The RSD value from the previous validation was 1.23%.[17] The HPLC method for the measurement of gambir leaf catechin isolates has a high level of precision and an acceptable level of repeatability, according to the %RSD value that satisfies the accuracy test results in this validation. The level of the linear relationship between analyte levels and the peak area can be expressed as the value of the correlation coefficient (R).[13] The linear regression analysis with HPLC yielded the equation y = 14234x - 88344 with an R-value of 0.9996 (Figure 2). The R-value gives linear results because it meets the acceptance criteria, which is close to 1 or above 0.995.[14] The determination of catechin isolate levels with HPLC has good linearity, according to the results of this linearity test, and may be utilized for analysis with reliable test results.

Table 1: Characterization of catechin from gambir leaves.

Characteristics	Result	Requirement
Shape	Solid, powder	Solid, powder
Colour	Yellowish brown	Light brown to yellowish brown
Smell	Unique	Unique
Flavour	Chelate, a little sweet ending	Chelate, a little sweet ending
Moisture Content	3.08%	≤ 14.0%
Total Ash Content	0.34%	≤ 0.5%

Table 2: Accuracy result of catechin.

Concentration	Real concentration (µg/mL)	Measured concentration (µg/mL)	% Recovery
80 % + sample	452.6	451.8	99.82
80 % + sample	452.6	447.7	98.91
80 % + sample	452.6	447.8	98.94
100 % + sample	464.1	457.1	98.50
100 % + sample	464.1	456.2	98.30
100 % + sample	464.1	456.8	98.43
120 % + sample	475.5	468.2	98.46
120 % + sample	475.5	468.2	98.45
120 % + sample	475.5	470.9	99.02

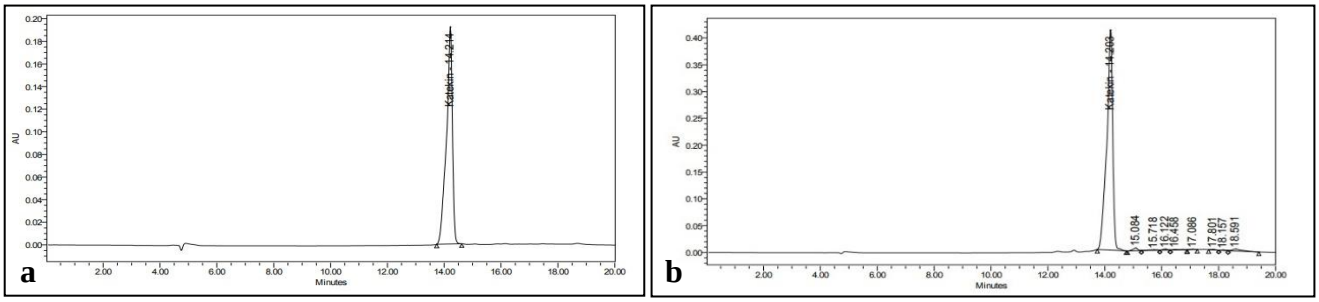

Figure 1: Chromatogram of (a) standard catechin, (b) catechin isolated from gambir leaf

The limit of detection (LOD) is the lowest concentration of an analyte in a sample that can still be detected, while the limit of quantity (LOQ) is the lowest concentration of an analyte in a sample that can be measured quantitatively using accuracy and precision.[13] The LOD and LOQ formulas can be used to determine the LOD and LOQ values based on the response standard deviation (SD) and slope (S) of the catechin standard curve. Based on the calculations presented in Table 4, the LOD value was 2.7 µg/mL and the LOQ value was 8.3 µg/mL. The LOD and LOQ values from the validation of the HPLC-based analytical method of catechin isolates have a slightly smaller value compared to the ones obtained for the previous spectrophotometric-based method, which had values of 3.85 and 12.84 µg/mL, respectively.[17]

A robustness test was not conducted in a previous validation method using the spectrophotometer.[17] Hence, a robustness test was established to validate the analytical method for gambir leaf catechin isolates in this study. Robustness is the capacity of a method to remain unaffected by a variation of its input parameters.[18] In this study, the robustness was tested by injecting a standard catechin solution under the same flow rate conditions while employing variations in the mobile phase produced under normal conditions.[19] A robustness test should be considered in the development of a method given its capacity to remain unaffected by small, deliberate variations in its parameters.[20] Based on the results of the robustness test, T and F values were estimated (Table 5). The results indicate that there is no significant difference between the standard method and variations of the mobile phase ACN 4% and 6%, as well as between the standard method's retention time and variations of the mobile phase ACN 4%. This indicates that the analytical method is considered to have resistance to changes in parameter variations. While the shift in time is around one minute faster when compared to the mobile phase ACN 5%, the comparison of retention time with the standard method with variations in the mobile phase ACN 6% or ACN above 5% is regarded as having no resistance to changes in parameter variations. The high concentration of ACN in the mobile phase is what caused the shift in retention time. The concentration of the organic solvent used as the mobile phase will significantly affect the retention time in the HPLC system.[21] The use of ACN as the mobile phase is also beneficial for increasing the mobile phase's eluent strength. Eluent strength is the ability of organic solvents to elute analytes; the higher the eluent concentration, the greater the elution capacity, and the faster the retention time will be. [22] Based on the results of the robustness test carried out, it can be inferred that the greater the concentration of ACN, the faster the retention time detected. This is because the concentration of the mobile phase is one of the most important factors affecting the retention of analytes in the HPLC method.

Conclusion

The findings of this study reveal that the HPLC method for analyzing catechin from gambir leaves is valid. When the method was validated, it was found to be specific, accurate, precise, robust, and effective. Therefore, it is suitable for routine quality control of products containing catechin.

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.

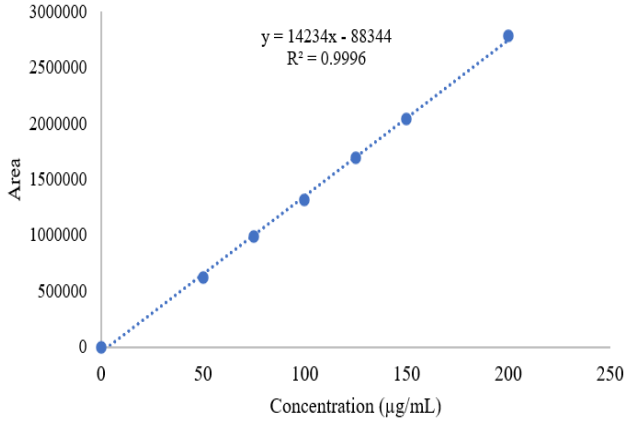

Figure 2: Catechin standard calibration curve

Table 3: Precision result of catechin.

Repetition	Area	µg/mL	Grade (%)
1	7100949	500.9	98.17
2	7060655	503.2	98.63
3	7091619	505.4	99.06
4	7088151	505.2	99.01
5	7083897	504.9	98.95
6	7062136	503.3	98.65
Mean (%)			**98.75**
SD			**0.27**
% RSD			**0.28**

Table 4: LOD and LOQ test results.

Concentration (µg/mL)	y	ŷ	y- ŷ	(y-ý)²
50	620112.5	616756	3356.5	11266092.3
75	988581.0	976306	12275.0	150675625.0
100	1321111.5	1335856	-14744.5	217400280.3
125	1697173.0	1695406	1767.0	3122289.0
150	2041376.0	2054956	-13580.0	184416400.0
200	2785253.0	2774056	11197.0	125372809.0
	Σ			692253495.5
	SD			11766.5
	LOD			**2.7**
	LOQ			**8.3**

LOD: Limit of detection; LOQ: Limit of quantification.

Table 5: Robustness test result.

Variation comparison	F-Test	T-Test	Description
Area of standard metode + ACN 4% method	1.01	1.09	No significance difference
Area of standard metode + ACN 6% method	1.29	0.31	No significance difference
RT of standard metode + ACN 4% method	0.002	0.58	No significance difference
RT of standard metode + ACN 6% method	11.80	69.90	Significance difference

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