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Validation of High-Performance Liquid Chromatography (HPLC) Method for Quantification of Ethyl p-Methoxycinnamate in *Kaempferia galanga* Extract

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ABSTRACT

Kaempferia galanga has the potential to be developed as a raw material for traditional medicine because it has pharmacological activities such as analgesic and anti-inflammatory. Ethyl p-methoxycinnamate (EPMC) is one of the marker compounds for determining the dosage of traditional medicine. Determination of EPMC content in the *Kaempferia galanga* extract is important for the standardization of the extract. This study aims to validate the high-performance liquid chromatography (HPLC) method for determining EPMC content in *Kaempferia galanga* extract. Separation was performed on the C18 column (5 µm, 4.6 mm x 150 mm), using an isocratic mobile phase consisting of water: acetonitrile (40:60) at 30°C temperature. The flow rate was 1 mL/min with an injection volume of 10 µL. Detection was performed using a photodiode array (PDA) set at 308 nm. The method was validated as per the guidelines given by International Conference on Harmonization guidelines. The retention time of EPMC on standard EPMC and *Kaempferia galanga* was 6.22 and 6.25 minutes, respectively. The linearity calibration curve was 10-60 µg/mL (r=0.9988) with the limit of detection (LOD) of 0.0011 µg/mL and limit of quantification (LOQ) of 0.0037 µg/mL. Adequate results were obtained for precision with a relative standard deviation (RSD) of 1.19%-2.37% and accuracy with a recovery of 94.07%-113.82%. The average EPMC content in *Kaempferia galanga* was 54.13%. Therefore, this finding demonstrated that the method was proved selective for the quantification of EPMC in *Kaempferia galanga* extract.

***Keywords*:** Ethyl p-methoxycinnamate, *Kaempferia galanga*, High-performance liquid chromatography, Method validation.

Introduction

Kaempferia galanga is a plant from *Zingiberaceae* family which is also known by different names in several regions, such as aromatic ginger, sand ginger, galanga, maraba, kencur, Sha jiang, Teu dau, Ban-u-kon, Krachai, Chandramulika, cekur[1,2]. *Kaempferia galanga* is found widely in tropical and subtropical regions, especially in Africa and Southeast Asia such as Vietnam, Myanmar, Bangladesh, Malaysia, Thailand, Taiwan, China, India, Japan, Sri Lanka, Laos, South Africa, and Indonesia[3,4]. *Kaempferia galanga* is a traditional medicinal plant that has numerous pharmacological activities, such as antioxidant, antimicrobial, vasorelaxant, antineoplastic, antidiabetic, analgesic, and anti-inflammatory activities[5,6]. The pharmacological activity of *Kaempferia galanga* comes from the content of its bioactive compounds. *Kaempferia galanga* contains various bioactive compounds, including ethyl p-methoxy cinnamate (EPMC) (61.46%-79.8%) as the main secondary metabolite followed by pentadecane (0.25%-14.55%), (Z)-ethyl cinnamate (1.47%-10.01%)[7].
EPMC is the main secondary metabolite compound in *Kaempferia galanga* that has been widely utilized and developed as herbal medicine in the form of jamu (Indonesian herbal medicine), capsules, sachets, and drug delivery systems[8,9].

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As an herbal medicine, standardization is carried out to ensure quality, safety, and efficacy because each plant contains various chemical compounds that can affect the bioactivity and side effects of the plant. Several parameters for standardizing herbal medicines refer to World Health Organization (WHO) guidelines, including botanical parameters (macroscopic and microscopic), physicochemical (chromatographic fingerprint, loss on drying, ash value, acid insoluble ash, extractive value, moisture content, volatile matter), pharmacological (bitterness value, hemolytic property, astringent property, swelling index, foaming index), and toxicological parameters (arsenic and heavy metals, pesticide residues, microbial contamination)[10,11]. The chromatographic fingerprint is one of the important parameters in the standardization of herbal medicines, which is a method for identifying and determining the content of marker compounds in each plant or herbal medicine to ensure quality and pharmacological activity[12,13]. Marker compounds are compounds or groups of compounds present in plants with or without pharmacological activity[14]. An ideal marker compound should be specific for one plant or available in sufficient quantity for assay. EPMC is a marker compound contained in *Kaempferia galanga*[7,15]. Therefore, it is necessary to determine the EPMC content in the *Kaempferia galanga* extract to ensure that the EPMC content meets the requirements stated in the Indonesian Herbal Pharmacopoeia (IHP) II[16]. In previous studies, the EPMC content in *Kaempferia galanga* extract was analyzed using gas chromatography-mass spectrometry (GC-MS)[17]. However, GC-MS has several disadvantages in analyzing a compound, including it is only limited to volatile compounds hence non-volatile compounds must be derivatized; cannot be used for the analysis of compounds that are unstable at high temperatures because they will decompose at the start of separation; the range of compounds that can be analyzed is limited; low reproducibility; and poor quantification[18,19]. In another study, the EPMC content in *Kaempferia galanga* extract was also analyzed using thin layer chromatography (TLC)[20]. However, TLC also has several disadvantages, which are it has

poor sensitivity and resolution, limited sample capacity, poor quantification, low accuracy, not automatic, only as a qualitative analysis (not quantitative), time-consuming, affected by humidity and temperature, and the thin film cannot be reused[21,22,23]. The HPLC method can overcome these problems because it offers several advantages, including being a quantitative analysis method that works automatically; has high separating power, resolution, precision, and sensitivity; reproducibility; can be used for various types of samples; reusable columns; may use impure samples; and only required small sample volumes[21,23]. This method has been used in various research. Phattanaphakdee et al[25] using HPLC to quantify quercetin in hydrogel transdermal patches in red onion extract. Another research from Yunarto et al[26] also uses HPLC for catechin quantification in gambir (*Uncaria gambir* Roxb) leaves. Both of those studies demonstrate that the HPLC method has been developed until it met the requirement for linearity, accuracy, precision, and system suitability. Thus, the method was implemented to analyze the marker compound from sample[25,26].
Under conditions of development and differences in the analytical procedures of each method, it is necessary to validate the method to ensure that the HPLC analysis method is suitable for analyzing EPMC compounds[24]. In this research, we have developed a simple and specific HPLC method with a photodiode array (PDA) detector to determine EPMC in *Kaempferia galanga* extract. The method developed was validated according to *International Council for Harmonization* (ICH) guidelines through testing system suitability, testing sample content, selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision.

Material and Methods

Materials
The *Kaempferia galanga* rhizome (*Kaempferia galanga* L.) was obtained from Blitar, East Java, Indonesia, in June 2022. The plant was cultivated using an agroforestry system (GPS: 8°08'44.3"S 112°20'13.2"E). The plant sample was identified at the UPT Herbal Laboratory Balai Materia Medica, East Java, Indonesia (voucher no: 074/400/102.20-A/2022 by Achmad Mabrur, S.KM., M.Kes).

Ethyl p-methoxycinnamate (EPMC) standard (>98% purity, product number M1204) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Methanol (Cat. No. A4524) and acetonitrile (Cat. No. A998-4) of HPLC grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water for Injection (WFI) was purchased from PT. Ikapharmindo Putramas (Jakarta, Indonesia). A 0.2 µm membrane filter (Cat. No. A351860101) was purchased from Meissner Filtration Products, Inc. (Camarillo, CA, USA), and a nylon syringe filter 0.45 µm x 13 mm (Cat. No. S13NY045E) was purchased from Microlab Scientific Co., Ltd. (Yueqing, China).

Extraction procedure
The extract of *Kaempferia galanga* rhizome was prepared by maceration using 96% ethanol for 72 hours with stirring for 30 minutes using an overhead stirrer (RW 20 digital, IKA, Germany) and then filtered through fine filter paper using a vacuum pump (model DOA-P504-BN, GAST, USA). The filtrate was concentrated using a Rotary evaporator (RV 10 basic, IKA, Germany) with a chiller (MC 350, Lauda, Germany) at 40◦C in a vacuum (MZ 2C NT, Vacuubrand, Germany) and then dried using an oven (ED 53, Binder, Germany) at 50◦C for three days.

HPLC instrumentation
Method development and validation were carried out with the HPLC (LC-2030C 3D Plus, Shimadzu, Japan) system equipped with a pump, autosampler, column oven, and photodiode array (PDA) detector. Separations and analyzes were carried out in a C18 column (4.6 mm x 150 mm, 5 µm). The HPLC system is controlled by Lab Solution software.

Preparation of standard solution
EPMC standard stock solution (1000 µg/mL) was prepared by weighing 10 mg using analytical balance (OHAUS Pioneer™, USA), then dissolved in 5 mL HPLC-grade methanol in a 10 mL volumetric flask. The solution was sonicated using a sonicator (M2800, Branson Ultrasonic, Emerson, Japan) for 5 minutes at room temperature, and then the solvent was added until the desired final volume. The stock solution was diluted with HPLC-grade methanol to obtain a standard solution of 100 µg/mL. Then, the standard solution (100 µg/mL) was diluted with HPLC-grade methanol to obtain working standard solutions with concentrations of 10, 20, 30, 40, 50, and 60 µg/mL. The solution was filtered using a 0.45 µm nylon syringe filter before being analyzed by HPLC.

Preparation of sample solution
Ten milligrams of *Kaempferia galanga* rhizome extract was weighed using an analytical balance (OHAUS Pioneer™, USA) and dissolved in 10 mL HPLC-grade methanol in a beaker glass, then transferred into a 25 mL volumetric flask. The solution was sonicated using a sonicator (M2800, Branson Ultrasonic, Emerson, Japan) for 5 minutes at room temperature, and then the solvent was added until the desired final volume. The solution was diluted with HPLC-grade methanol and filtered using a 0.45 µm nylon syringe filter. During the determination of EPMC content in *Kaempferia galanga* rhizome extract, 14 mg of *Kaempferia galanga* extract was weighed and dissolved in 10 mL of HPLC-grade methanol in a beaker glass and then put into a 25 mL volumetric flask. The solution was sonicated for 5 minutes at room temperature, and then the solvent was added until the desired final volume. 1 mL of the solution was diluted with HPLC-grade methanol to the final volume in a 10 mL volumetric flask and then filtered using a 0.45 µm nylon syringe filter.

HPLC conditions
Chromatographic separation was performed on a C18 column (4.6 mm x 150 mm x 5µm). The mobile phase consisted of water: acetonitrile (40:60), with isocratic elution at a flow rate of 1.0 mL/min, an injection volume of 10 µL, and a column temperature of 30ºC. Detection was performed at a wavelength of 308 nm using a PDA.

Method validation
According to ICH guidelines, the developed HPLC method was validated by determining the following parameters: such as system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and selectivity[24].

Selectivity
The solution used was a sample solution of *Kaempferia galanga* extract and EPMC standard (30 µg/mL) and then injected into the HPLC system for analysis. Selectivity was determined by evaluating the interference of other compounds in the *Kaempferia galanga* extract and the mobile phase components[24].

System suitability Test
The solution used in the system suitability test was EPMC standard solution (20 µg/mL). The solution was injected six times into the HPLC system with an injection volume of 20 µL. System suitability parameters include peak area, retention time (Rt), resolution (Rs), tailing factor (Tf), height equivalent to theoretical plates (HETP), and percent relative standard deviation (%RSD). The value and %RSD of each parameter are measured to test the suitability of the system[27].

Linearity
Linearity was determined by constructing a six-point calibration curve using six EPMC standard solutions with different concentrations, namely 10, 20, 30, 40, 50, and 60 µg/mL. The calibration curve was obtained by plotting the response of the peak area or area under the curve (AUC) to the concentration (µg/mL) of EPMC standard solution at a wavelength of 308 nm. Peak area values are plotted against each EPMC concentration to obtain a calibration curve. Linearity was determined by correlation coefficient (r) using least squares linear regression analysis[24].

Sensitivity
The sensitivity of the method is expressed in the limit of detection (LOD) and limit of quantitation (LOQ). LOD is the lowest concentration in a sample that can be detected but not necessarily measured, whereas LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy. In this study, the system LOD and LOQ were determined from the calibration curve. LOD and LOQ are determined using equations (1) and (2).

$$\text{LOD} = \frac{3x\ standard\ deviation\ of\ peak\ area}{slope\ of\ the\ calibration\ curve} \quad \ldots(1)[24]$$

$$\text{LOQ} = \frac{10x\ standard\ deviation\ of\ peak\ area}{slope\ of\ the\ calibration\ curve} \quad \ldots(2)\ [24]$$

Accuracy and Precision
Accuracy is the closeness of the test results obtained from these results to the true value. Precision is the degree of agreement between test results when the method is repeated several times on multiple sampling of homogeneous samples. Accuracy and precision were determined using standard addition methods. The sample solution was prepared by weighing 10 mg of *Kaempferia galanga* extract and dissolved in a 25 mL volumetric flask with HPLC grade methanol up to the desired final volume and then diluted in a 10 mL volumetric flask. Samples that were not added standard were used as unfortified samples. Before the sample was diluted, fortified samples were prepared by adding standard sample solutions at three different concentration levels (80%, 100%, 120%). The unfortified and fortified samples were replicated three times each. Accuracy is represented by percent recovery, while %RSD is calculated to represent precision using equations (3) and (4)[24].

$$\%\text{recovery} = \frac{\text{fortified concentration (Cf)}}{\text{amount added (Ca)+unfortified concentration (Cu)}} \text{ x } 100\% \quad \ldots(3)[24]$$

$$\%\text{RSD} = \frac{standard\ deviation}{mean} x\ 100\% \quad \ldots(4)[24]$$

Determination of EPMC content in Kaempferia galanga rhizome extract
The *Kaempferia galanga* sample solution, as described in "Preparation of sample solution" was injected into the HPLC and replicated seven times. The chromatograms were observed and the peak areas that appeared were entered into the regression equation. Then, the EPMC content was calculated in the *Kaempferia galanga* extract sample.

Statistical analysis
The results of this study were expressed by mean, standard deviation (SD), and percent relative standard deviation (%RSD). The regression analysis of calibration curves was performed by using Microsoft Office 365 Excel.

Results and Discussion

Optimization of HPLC analysis conditions
Optimization of HPLC conditions was carried out to obtain a good separation of EPMC compounds in the shortest possible time even though there is interference from other compounds in *Kaempferia galanga* rhizome. In this method, several adjustments are made such as column size and wavelength. This study used an isocratic elution of water as the mobile phase: acetonitrile (40:60), with C18 column (4.6 mm x 150 mm x 5µm), flow rate 1 mL/min, injection volume 10 µL, and at temperature column 30ºC. The maximum absorption wavelength for EPMC is 308 nm. This study obtained good peak separation results with symmetrical peaks and shorter retention times, which is 6.28 minutes compared to the results obtained by Srivastava et al[28] which is 9.32 minutes. The difference between the HPLC method in this study and the research conducted by Srivastava et al[28] was the wavelength of the detector and the length of the column used. In addition, the retention time of this study was also shorter compared to the results obtained by Winingsih et al[29], which was 10,046 minutes. The differences between the HPLC method in this study and the research conducted by Winingsih et al[29] were the column temperature, the injection volume, and the mobile phase used which was methanol:water (70:30) containing 0.1% trifluoroacetic acid (TFA). This difference become one of the factor that influence the retention time of this study to be shorter compared to that of Winingsih et al[29].

Selectivity
Selectivity was carried out by comparing the shape of the spectrum and the retention time of the EPMC standard solution with the *Kaempferia galanga* sample solution. Comparison of the retention time of EPMC in standard and sample solutions showed almost the same results, 6.220 and 6.247 minutes, respectively. The chromatogram showed no interference from the peaks of other compounds on the EPMC peaks (Figure 1) and had the same spectral shape between the *Kaempferia galanga* sample solution and the EPMC standard solution (Figure 2). Therefore, this method is declared selective for the determination of EPMC in pharmaceutical dosage forms.

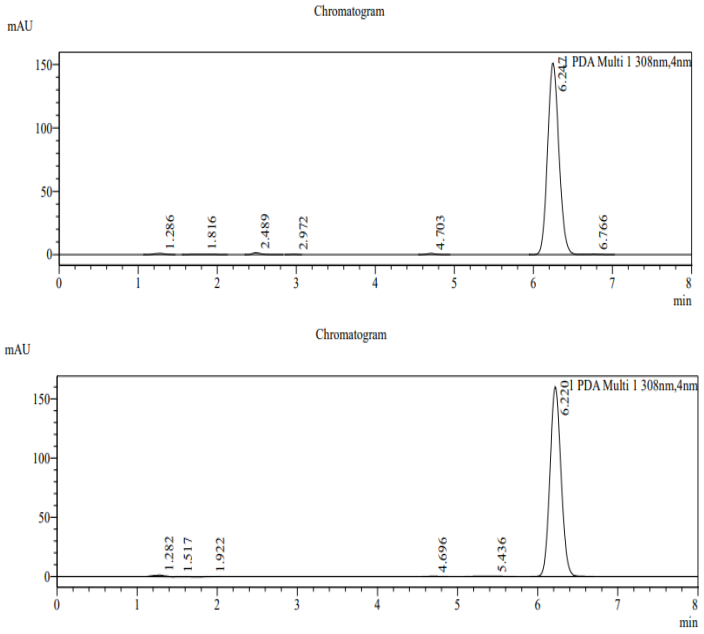

Figure 1: Chromatogram of the EPMC compound in; (A) sample *Kaempferia galanga* extract; (B) standard EPMC

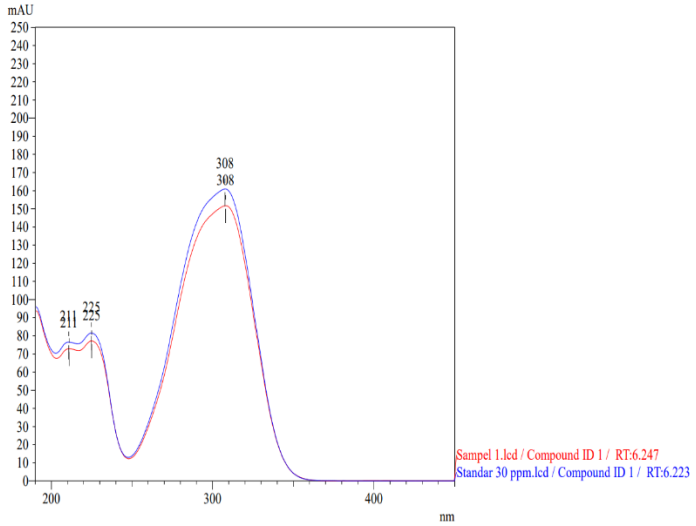

Figure 2: Comparison of the spectrum of EPMC standard and EPMC in the sample *Kaempferia galanga*

Table 1: System Suitability Test (SST) Results

Replication[a]	Area	Retention time (min)	HETP	Tailing Factor	Resolution
1	2503457	6.240	20.280	1.108	5.808
2	2482830	6.240	20.322	1.108	5.815
3	2485594	6.241	20.287	1.107	5.805
4	2486654	6.238	20.330	1.109	5.813
5	2503425	6.238	20.313	1.109	5.783
6	2502683	6.235	20.418	1.110	5.788
Average	2494107	6.239	20.325	1.109	5.802
SD	10029.80	2.160	49.590	1.049	13.357
%RSD	0.402	0.035	0.244	0.095	0.230

[a] The solution was EPMC standard solution (20 µg/mL)

Table 2: Accuracy and Precision Test Results

EPMC Concentration in Sample Cu[a] (mg)	Standard Concentration Ca[b] (mg)	Content Cf[c] (mg)	% *Recovery* total[d]	%RSD
0.22	0	0.22	98.38	
0.22	0	0.21	96.49	2.25
0.22	0	0.21	94.07	
0.22	0.18	0.44	109.84	
0.22	0.18	0.45	112.36	2.37
0.22	0.18	0.43	107.17	
0.22	0.22	0.49	110.31	1.50
0.22	0.22	0.50	113.65	
0.22	0.22	0.49	112.34	
0.22	0.27	0.55	111.84	1.19
0.22	0.27	0.56	113.82	
0.22	0.27	0.55	111.27	

[a] Cu is the unfortified concentration; [b] Ca is the calculated concentration of EPMC standard added to the test sample; [c] Cf is the fortified concentration; [d] % recovery

Table 3: Validation of Analysis Methods

Parameter	EPMC Analysis Results
Correlation coefficient (r)	0.9988
Recovery (%)	94.07-113.82%
RSD (%)	1.19-2.37%
LOD (µg/mL)	0.0011
LOQ (µg/mL)	0.0037

System suitability

System suitability aims to check the suitability of the system for analysis and its reproducibility. The %RSD value was <2.00%, as shown in Table 1. These results show excellent repeatability and thus the HPLC system is suitable for further analysis. The retention times of the research system suitability tests were well separated in the range of 6.235 – 6.241 minutes. The parameters of resolution, tailing factor, and HETP in this study have values that are by the requirements of the ICH, namely resolution (Rs) > 1.5, tailing factor (T) 1.0 or in the range 1.0-1.5, and the value of HETP is getting smaller the higher the efficiency of the column[24,27,30]. Resolution indicates the column's ability to separate two adjacent components into two single peaks. The valley between the two peaks must touch the baseline with a value of Rs 1.5, but generally, the preferred resolution value is Rs ≥ 2. The tailing factor or also known as the asymmetry factor indicates that the chromatographic peaks have a symmetrical or Gaussian shape. The value of T = 1 indicates a symmetrical peak or a perfect Gaussian shape. The value of T > 1 indicates the chromatogram peak is tailing, while T < 1 indicates the chromatogram peak is fronting. T values are usually in the range of 1.0-1.5. Height equivalent to theoretical plates (HETP) indicates column efficiency. The smaller the HETP and the higher the theoretical plate, the more efficient the column[27,31]. The results of the system suitability test demonstrate that the parameters tested met the requirements of the ICH guidelines. Therefore, the system is suitable for the proposed analysis.

Research conducted by Srivastava et al[28] did not show results from the parameters Rs, T, and HETP. However, this study has a better resolution value (5.802) compared to research by Winingsih et al[29], which has a smaller resolution value with a better tailing factor value (2.06929).

Linearity

The determination of linearity is carried out by constructing a standard curve to assess the ability of an analytical method to obtain results that are proportional to the concentration of EPMC in the sample. The calibration curve is shown in Figure 3. The regression line equation obtained for EPMC, y = 51256x + 19839 with a correlation coefficient (r) = 0.9988. The correlation coefficient (r) requirement in AOAC is 0.99, thus, it can be concluded that this linearity test meets the requirements[32]. The results showed a good correlation between peak area response and concentration.

Table 4: Results of Determination of EPMC Content

Replication[a]	Amount of Sample Taken[b] (mg)	Area	Concentration in 25 mL (µg/mL)	Content in 25 mL (mg)	%w/w	%RSD
1	13.4	1820102	289.76	7.24	54.06	3.57
2	13.5	1765104	281.08	7.03	52.05	
3	13.4	1912701	304.37	7.61	56.79	
4	13.4	1858673	295.84	7.40	55.19	
5	13.5	1782469	283.82	7.10	52.56	
Average				7.27	54.13	

[a] The solution was *Kaempferia galanga* extract solution; [b] The weighing error is ±0.05

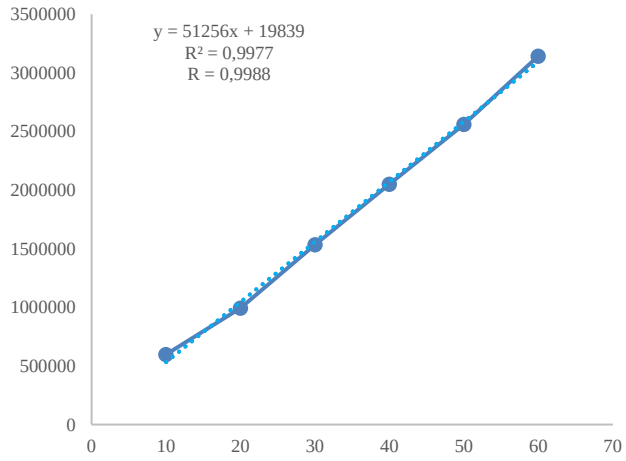

Figure 3: Calibration curve of EPMC

Sensitivity

The sensitivity of the method is determined by the limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ values for EPMC were obtained from the linear regression line of the calibration curve with the linear regression equation y = 51256x + 19839. Based on the calculation results, the LOD was 0.0011 µg/mL, and LOQ was 0.0037 µg/mL. Low LOD and LOQ values indicate good method sensitivity[33].

Accuracy and precision

Accuracy is expressed as %recovery, while precision results are expressed as %RSD. The %recovery and %RSD values obtained in this study are shown in Table 2. The results of accuracy and precision tests show results that meet the AOAC requirements for repeatability and closeness of the measurement results to the true value. The AOAC requirements were %recovery in the range of 90-107%, and %RSD <5.3% for analytes of 0.022%[32]. The precision and accuracy results are shown in Table 2. The results of the method validation are summarized in Table 3. The study on the validation of the EPMC analysis method that was carried out by Srivastava et al[28] showed a slightly better average %recovery value when compared to the results obtained in this study, which was 99.99% ± 2.12. In this study, the %RSD results also had a lower value when compared to the EPMC analysis method validation study that had been conducted by Srivastava et al[28], which were 1.86% and 2.02%[28].

Determination of EPMC content in Kaempferia galanga rhizome extract

The extraction yield of *Kaempferia galanga* was 10.89%. These results indicate that the percentage of yield meets the requirements for the extraction yield of *Kaempferia galanga* rhizome listed in the Indonesian Herbal Pharmacopoeia (IHP) II with a yield value of not less than 8.3%[16]. Based on the test results in Table 4, the percentage of EPMC content in the *Kaempferia galanga* rhizome extract was 54.13%. According to the IHP II, the requirement for EPMC content in *Kaempferia galanga* rhizome extract is not less than 4.3%, so this study fulfills this requirement[14]. Several previous studies have shown that there is a difference in the percentage of EPMC content in *Kaempferia galanga* rhizome extract. Research by Srivastava et al[34] showed that the EPMC content in the *Kaempferia galanga* rhizome extract was 2.15%, whereas Mukkasombut et al[35] showed 22.71%, and Winingsih et al[29] showed 78.74%. Differences in EPMC content in *Kaempferia galanga* rhizome extract can be caused by plant location, harvest time, solvent extraction, and production method[11]. Both *Kaempferia galanga* extract and EPMC have numerous pharmacological activities. EPMC isolated from *Kaempferia galanga* has anti-inflammatory activity by reducing the leukotriene B4 (LTB4) production in the rats[36]. EPMC also was found to be potential as anti-metastatic and cancer therapy by targeting NFκB[37]. *Kaempferia galanga* extract has been formulated into several dosage forms, such as hydrogel-containing extract emulsion, lozenge, sunscreen, and other pharmaceutical dosage forms[38,39,40]. Thus, the quantification of marker compound is important to evaluate the formulation process of the pharmaceutical dosage form.

Conclusion

The results of the HPLC method validation demonstrated that this method could be used for EPMC quantification in *Kaempferia galanga* rhizome extract. Thus, this method could be implemented to quantify the EPMC in a pharmaceutical dosage form containing *Kaempferia galanga* extract as one of the quality control parameters.

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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