Quantification of Andrographolide Isolated from Andrographis paniculata Nees Obtained from Traditional Market in Yogyakarta Using Validated HPLC

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ABSTRACT

This research was aimed to quantification of andrographolide isolated from A. paniculata Nees found in a traditional market in Yogyakarta using validated HPLC to obtain a high level content of andrographolide. The extraction of andrographolide from A. paniculata was carried out using ethanol as the solvent. Fractionation and isolation were continued using a non-polar solvent. Next, the extracts were recrystallized to obtain isolated andrographolide. The identity of the compound was confirmed by an analysis of the melting point, IR spectra, and TLC. The purity of the compound was confirmed by the validated HPLC. The data obtained were then compared using an analytical grade of andrographolide as the standard. The isolated andrographolide confirmed the melting point, IR spectra and TLC analysis were similar to the standard andrographolide. The method to determine the content of isolated andrographolide showed an adequate precision, with a relative standard deviation (RSD) smaller than 1%. The accuracy showed good recovery values were obtained for all concentrations used. The HPLC method in this study showed specificity and selectivity with linearity in the working range and good precision and accuracy, making it very suitable for the quantification of andrographolide isolated in A. paniculata. When compared to the standard, the purity of the isolated andrographolide was 95.74 ± 0.29%.

Keywords: andrographolide; extraction; isolation; validation

INTRODUCTION

Andrographis paniculata Nees (A. paniculata), a family of acanthaceae, can be widely found in Asia’s tropical regions [1]. Its indigenous populations grow across South India and Sri Lanka with a broad range of diversity. The species is also found in the northern areas of Java, Malaysia (covering Penang, Malacca, and Pangkor Island of Borneo), India, Indonesia, the West Indies (Jamaica, Barbados, and the Bahamas), and in different places, as an introduced species, in the Americas [2].

In Asian countries, it has long been used as a medicinal herb due to its main bioactive constituent andrographolide, more than 2% of which is accumulated in the leaves. The constituent is extremely

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bitter, colorless and has a crystalline \textit{bicyclic} compound \cite{3}. Active compounds extracted with ethanol or methanol from the whole plant, leaf and stem of \textit{A. paniculata} include more than 20 diterpenoids and ten flavonoids or more. The main diterpenoid in \textit{A. paniculata} is andrographolide (C\textsubscript{20}H\textsubscript{30}O\textsubscript{5}) found approximately 4\% in dried whole-plant extract, 0.8–1.2\% in stem extract, and 0.5–6\% in leaf extract. Deoxy andrographolide, neo andrographolide, 14-deoxy-11,12-didehydroandrographide, and isoandrographolide are its other major diterpenoids \cite{4}. In addition, the pharmacological activities of andrographolide include analgesic, anti-pyretic, anti-inflammatory, hepatoprotectant, anti-viral, antithrombotic, anti-cancer, hypoglycemic, and anti-hyperlipidemic \cite{5-7}. The molecular structure of andrographolide is shown in Fig. 1.

Meanwhile, in developing as well as developed countries, plant-based medicines, including pharmaceuticals, health products, cosmetics, and food supplement, have been in great demand since natural products are well known for their non-toxicity, less side effect, availability, and affordability. In India, for example, the demand for herbal medicine has been increasing because they have a wide range of biological activities, cost less, and offer a higher safety margin than synthetic drugs \cite{8}.

The thousands of islands of Indonesian archipelago are full of unique biodiversity, and their 143 million hectares of tropical forests have 28,000 plant species where 80\% of which become the medicinal plants of the world \cite{9}. One of these species, \textit{A. paniculata}, or \textit{Sambiloto} in Indonesian, has the potential to be grown in Java region. This herb can be well cultivated in slightly damp areas that have high rainfall and tropical temperatures, such as hill slopes, wastelands, plains, dry or wetlands, farms, seashores, and even roadsides \cite{10-11}.

In addition, to produce high-purity products from natural plants, the isolation and purification of the target compound from a complex multi-component mixture are needed. Until today, the use of isolated andrographolide as a raw material for medicine is very rare although its pharmacological activity has been extensively studied. Phytochemical profile of medicinal plants was influenced some factor such as the age of the plant, harvesting time, and growth location \cite{12}. The previous study has been reported to show variations in the secondary metabolites to \textit{Stevia rebaudiana} and \textit{Andrographis paniculata} \cite{13-14}. The andrographolide content in the plant samples of three accessions with no morphological differences of \textit{A. paniculata} was also reported to have variation in the andrographolide content \cite{15}. Therefore, it is necessary to study the techniques for extraction and isolation of andrographolide in \textit{A. paniculata}, especially in the area of Yogyakarta, to obtain a high level of isolated andrographolide. The purity of isolated andrographolide is important to ensure its pharmacological effects. So, the determination of the content of isolated andrographolide must be assessed by validated HPLC.

Meanwhile, an analytical method must be validated to ensure the fulfillment of all the analytical application requirements and the result reliability. Consequently, linearity, precision, specificity, accuracy, sensitivity, and quantification limit must be demonstrated in the test for analysis adequacy \cite{16,17}. The objective of present work is to quantify of andrographolide isolated from \textit{A. paniculata} Ness found in a traditional market in Yogyakarta using validated HPLC to obtain high-level content of andrographolide.

**EXPERIMENTAL SECTION**

**Materials**

\textit{Andrographis paniculata} Nees plant was collected from traditional markets in Yogyakarta in May 2015. The standard andrographolide compound with 98\% purity was supplied by Sigma-Aldrich. The thin layer chromatographic (TLC) plates and silica gels were obtained from Brataco Chemica Indonesia. Such solvents as methanol, ethanol, ethyl acetate, chloroform, \textit{n}-hexane were produced by Brataco Chemica Indonesia.

**Instrumentation**

Rotavapor R-100 (Buchi), melting point (Stuart Scientific, UK), FTIR spectroscopy (Nicolet Avatar 360), TLC scanner 3 (Camag), High Performance Liquid Chromatography (WATERS e2695, with 2486 UV-Vis detector and WATERS 1525, with detector PDA 2998).
Procedure

Isolation of andrographolide of Andrographis paniculata (Burm. f.) Nees

Dried ground A. paniculata (Burm. f.) Nees was extracted for 24 h with ethanol. When the filtrate was gained, the sediment was re-extracted for another 24 h with the same solvent twice. The collected extract was then evaporated under reduced pressure to gain viscous extract. The result was then fractionated using n-hexane. Next, the insoluble fraction of n-hexane was fractionated with ethyl acetate, collected, and concentrated using a rotary vacuum evaporator to attain viscous extract. Then, hot water was used to wash this insoluble fraction, which was then diluted using ethanol to produce a purified extract. A chromatography process was done to the purified extract of Andrographis paniculata (Burm. f.) Nees using silica gel GF 254 in a column chromatography. Then, the extract was eluted using a mixture of methanol and chloroform (1:9) as a mobile phase. After the clear solution was collected, it was left for 24 h at 4 °C to produce andrographolide crystals, which were then collected and washed using cold methanol and cold n-hexane respectively [7].

Identification of isolated crystals

Melting point. A melting point apparatus was used to determine the melting point, and a test was then performed to isolate the andrographolide and the standard andrographolide.

FTIR spectroscopy. FTIR spectroscopy was utilized to study the samples molecular structures in the wave range of 400–4000 cm⁻¹. The isolated andrographolide and standard andrographolide were diluted using KBr mixing powder at 1% and pressed into transparent slices to be analyzed.

Determination of active compound using Thin Layer Chromatography (TLC). Using Microlitser syringe, the samples were spotted 1 cm from bottom with 8 cm elusion distance on pre-coated silica gel aluminium plate 60F-254 (10 x 10 cm) (E. Merck, Germany) and a Linomat 3 sample applicator. The isolated andrographolide was then subjected to TLC analysis together with the reference standard of andrographolide where a mixture of chloroform and methanol at a ratio of 9:1 was utilized. To ensure the purity of andrographolide, three different mobile phases, including ethyl acetate and acetone 7:3, chloroform and methanol 9:1, and chloroform and acetone 7:3 were used [18].

Purity test. The sample was dissolved in methanol and injected into the HPLC with detector PDA, then the chromatogram is read at a wavelength of 200–400 nm.

Quantitative analysis of andrographolide

Chromatographic conditions. The separation was acquired on Sunfire C18 column (150 mm x 4.6 mm, 5 μm) with an isocratic mixture of methanol and water at a ratio of 6:4, v/v as a mobile phase. The mobile phase’s flow rate was 0.8 mL/min, while the injection volume was 20 μL, and the wavelength of the detector was controlled at 229 nm [3].

Preparation of standard solutions. The reference andrographolide in HPLC grade methanol was used to prepare the standard stock solution of 200 μg/mL concentration through shaking and sonication. This stock solution was then utilized to prepare a series of mixed working standard solution in the range concentration.

System suitability. To guarantee that the chromatographic assay was appropriate for the analysis, a system suitability test was performed by measuring such chromatographic parameters as peak area, retention time, theoretical plates as well as tailing factor and by determining the standard deviation (RSD) for each parameter [19-20].

Validation parameters. The method was authenticated according to ICH guideline for its linearity, precision, accuracy, selectivity, limit of detection, and limit of quantification. To calculate the slope, intercept, and coefficient of determination/regression coefficient (r²) for calibration plot, a linear regression analysis was used. An evaluation was conducted based on the peak area.

Meanwhile, a recovery study help to determine the accuracy of the method by putting a known amount of standards at 80, 100, and 120% level in the placebo and by repeating the quantitative analyses using the proposed method.

To determine the selectivity of the method, a comparison between the retention time and the reflectance/absorbance spectrum of the standard compound and the corresponding peak attained from the andrographolide isolated was conducted. A comparison between the UV-visible spectra of marker compound and its counterpart in formulation was also done at three different positions, including the peak start, peak center, and peak end.

Based on the response at a signal to noise ratio of 3:1 and 10:1, the LOD and LOQ were calculated.
then experimentally verified by diluting the standard concentration until the average response was obtained after six repetitions [21-23].

RESULT AND DISCUSSION

Identification Isolated Crystal

Isolated crystals from A. paniculata are presented in Fig. 2. The figure shows that the andrographolide isolated from A. paniculata was a crystalline shape and colorless. The melting points of standard andrographolide and isolated andrographolide were found to be between 227–233 °C and 227–232 °C respectively. This indicates that the isolated andrographolide has similar properties to the standard andrographolide.

FT-IR Analysis

This analysis was aimed to prove that the spectra of the isolated andrographolide were similar to the standard andrographolide to confirm the functional groups in the determination of the molecular structure. The FT-IR spectra of the standard andrographolide and the andrographolide isolated from AP are illustrated in Fig. 3. The figure described the peak position and interatomic bonding from the molecular structure of andrographolide. The peak at 3100–3500, 2800–3000, 1727, 1458, and 1220 cm\(^{-1}\) may be due to presence of O-H, C-H, C=O, C=C stretching, and C-O-C of lactone ring respectively, present in the molecular structure of andrographolide. The above picture also shows that the standard andrographolide and isolated andrographolide attributed the similar spectra. This proves that the isolated andrographolide content is similar to that of the standard andrographolide.

Determination of Active Compound Using TLC

**TLC test with single mobile phase**

This test was intended to ensure the presence of andrographolide in the isolated crystals by comparing its chromatographic profile to that of the standard andrographolide. The mobile phase used was in line with the data of Indonesia's Herbal Pharmacopoeia for A. paniculata extract using a mobile phase of chloroform : methanol (9:1). The results of TLC test for isolated andrographolide and standard andrographolide using a mobile phase of chloroform : methanol (9:1) are presented in Fig. 4.

The figure shows that the isolated and standard andrographolide had similar \(R_t\) which was 0.56 for the standard and 0.57 for the isolated. Therefore, it can be claimed that the isolated crystals contained andrographolide due to the identical \(R_t\) value.

The two-dimensional chromatogram shows that the standard andrographolide and the isolated andrographolide had the same \(R_t\) value. The following figure ensures the obtained \(R_t\) value (Fig. 5).

**TLC test using three mixtures of mobile phase with different polarities**

The test using three mixtures of mobile phase with different polarities was aimed to ensure the purity of
the isolated andrographolide. The chromatograms are expected to have a single point with different \( R_f \) values. The results can be seen in Fig. 6.

The figure shows that, in the three mixtures of mobile phase with different levels of polarity, a single point with different \( R_f \) values was obtained. As the mobile phase, ethyl acetate : acetone (7:3), chloroform : methanol (9:1), and chloroform : acetone (7:3) showed 0.86, 0.57, and 0.42 \( R_f \) values, respectively. Therefore, by using three mixtures of mobile phase with different levels of polarity, different \( R_f \) values were attained at 254 nm of wavelength. This purity test using TLC led to a conclusion that the isolated crystals contained andrographolide.

**Purity test using HPLC with PDA detector**

This test was aimed to observe the three-dimensional spectrum of the isolated andrographolide to determine the purity of the isolated at a wavelength of 200–400 nm. The test results can be seen in Fig. 7.

The figure illustrates the presence of a single peak in the wavelength range of 220 nm without the existence of other peaks beside or behind. Therefore, it can be concluded that regarding HPLC the isolated andrographolide was pure.

**Determination of andrographolide content in the isolated andrographolide**

**System suitability.** The analytical results obtained from the developed method are only valid if the defined
Fig 8. Chromatogram of standard andrographolide (A) and isolated andrographolide (B) at 229 nm

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Data obtained</th>
<th>RSD (%)</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak area</td>
<td>501675.500 ± 1337.985</td>
<td>0.267</td>
<td>RSD &lt; 1 %</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>4.337 ± 0.004</td>
<td>0.098</td>
<td>RSD &lt; 1 %</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.232 ± 0.023</td>
<td></td>
<td>T ≤ 2</td>
</tr>
<tr>
<td>Resolution</td>
<td>4.890 ± 0.157</td>
<td></td>
<td>Rs &gt; 2</td>
</tr>
<tr>
<td>Retention (Capacity) Factor</td>
<td>2.414 ± 0.004</td>
<td></td>
<td>k ≥ 2</td>
</tr>
</tbody>
</table>

*a = Mean of six repetitions*

System suitability criteria are fulfilled. In this investigation, the experimental result (Table 1) indicates that the chromatographic system was suitable for the intended analysis. A standard solution containing 10 μg mL⁻¹ of andrographolide was injected six times. The retention time for the reference compound was 4.337 min. Meanwhile, the RSD values for the peak area and retention time were 0.267% and 0.098%, respectively, which suggested the reproducibility for these parameters. The good peak symmetry of andrographolide was indicated by the value 1.232 for the tailing factor parameter. The resolution and retention (capacity) factor values reaching 4.890 and 2.414 suggested a good suitability.

Validation parameters for quantification of andrographolide. The proposed method was validated for the determination of andrographolide content of the isolated from A. paniculata as shown in Table 2.

Linearity. A linear regression analysis was used to calculate the slope, intercept, and coefficient of determination/regression coefficient (r²) for the calibration plot. The linearity was determined by using six concentrations of standard solution. The calibration curve was obtained by plotting the area versus the concentrations of standard solution. The response was
linear in the concentration ranges investigated (Fig. 9). Fig. 9 shows that the value of $R^2$ reached 0.9999, and this suggests that there was a good linearity.

**Precision.** Three different concentrations of isolated andrographolide solution in triplicates were injected at three different times on the same day. It was then repeated on three different days to record the intra-day and inter-day variations in the results. The low % RSD values of intra-day (0.267) and inter-day (0.558) for the isolated andrographolide reveals that the proposed method was precise (Table 2).

**Limit of Detection (LOD) and Limit of Quantification (LOQ).** To determine the limits of detection and quantification, different dilutions of the marker compound, which was andrographolide, were injected with a blank mobile phase and determined according to a signal to noise ratio of 3:1 and 10:1, respectively. The LOD and LOQ for the standard compound were found to be 0.09 and 0.31 μg mL$^{-1}$ respectively.

**Accuracy.** The recovery was determined using the standard addition method. The standard andrographolide was added to the formulation of two different concentrations, and then extraction and analysis were performed as previously described. The recovery was calculated for each standard at each concentration. It was determined that the method performs within acceptable limits between 80% and 120%. The accuracy test through recovery of the matrix is crucial to the development of methodologies for herbal products [24]. The results and the average mean of recovery data for each level of marker were within the accepted range as shown in Table 3. The recovery resulted in the value of 101.09–103.17% and revealed a good data accuracy since the value was within the range of 95–105%. The low value of relative standard deviation indicates that the proposed method was accurate.

**Quantification of isolated andrographolide.** The purity of andrographolide in the isolated andrographolide was 95.74 ± 0.29%.

**CONCLUSION**

The andrographolide isolated from *A. paniculata* Nees plant obtained from traditional markets in Yogyakarta had a similar melting point, IR spectra and.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Data obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity (correlation coefficient)</td>
<td>0.9999</td>
</tr>
<tr>
<td>Range (μg/mL)</td>
<td>0.8 - 32</td>
</tr>
<tr>
<td>Precision intra-day (% RSD, n = 6)</td>
<td>0.267</td>
</tr>
<tr>
<td>Precision intra-day (% RSD, n = 6)</td>
<td>0.558</td>
</tr>
<tr>
<td>Limit of detection (μg)</td>
<td>0.09</td>
</tr>
<tr>
<td>Limit of quantification (μg)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

**Table 3. Results from recovery determination**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Data obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial concentration in the isolated crystals (μg/mL)</td>
<td>3.97</td>
</tr>
<tr>
<td>Concentration added (μg/mL)</td>
<td>7.39</td>
</tr>
<tr>
<td>Total concentration (μg/mL)</td>
<td>11.36</td>
</tr>
<tr>
<td>Concentration found (μg/mL)</td>
<td>11.41</td>
</tr>
<tr>
<td>RSD (%) (n=3)</td>
<td>0.704</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>101.09</td>
</tr>
<tr>
<td>Mean recovery (%)</td>
<td>101.87</td>
</tr>
</tbody>
</table>

![Fig 9. Calibration curve of andrographolide at 229 nm](image)
TLC test to those of the standard andrographolide. The method for quantification andrographolide isolated from *A. paniculata* demonstrated that the method showed good linearity, recovery, reproducibility and low limits of detection and quantification. The purity of the isolated andrographolide compared to the standard and determined using the validated HPLC was 95.74 ± 0.29%.

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