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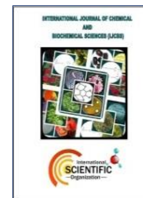

Quantitative Analysis of *Andrographolide* in Sambiloto (*Andrographis Paniculata*) Herbal Preparation Using UV-Vis, HPLC, And ^{1}H-NMR With Dibenzyl Ether as Internal Standard

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Abstract

The therapeutic potential of Sambiloto (*Andrographis paniculata*), particularly its bioactive compounds with antiviral and anti-inflammatory properties, has led to increased demand. This study aimed to quantify the *Andrographolide* content in Sambiloto herbal preparations using UV-Vis Spectrophotometry, HPLC, and qHNMR, with dibenzyl ether as an internal standard. The UV-Vis analysis showed *Andrographolide* content of 2.68% and 2.39% using external and internal standards, respectively. HPLC analysis, under optimal conditions (Acetonitrile: 0.1% o-phosphate, 40:60), revealed a content of 0.1925%. qHNMR analysis, using dibenzyl ether, indicated a content of 0.233%. Dibenzyl ether demonstrated potential as an innovative internal standard, offering a cost-effective alternative to expensive pure standards in qHNMR. This study enhances cost-effective and accurate methods for quantifying bioactive compounds in herbal preparations.

Keywords: qHNMR, External standard, Innovative, Quantification, Pure standard

Full-length article **Corresponding Author*, e-mail: cahyono@live.undip.ac.id Doi # https://doi.org/10.62877/90-IJCBS-24-25-19-90

1. Introduction

The Sambiloto plant (*Andrographis paniculata*) is traditionally used to alleviate cold symptoms and has gained attention as a potential antiviral agent against SARS-CoV-2 due to its active compound, *Andrographolide*. This compound is notable for its antiviral and anti-inflammatory properties. Initial studies suggest that Sambiloto extract can aid in the recovery of COVID-19 patients and offer protection to those at risk [1-3]. Given its rich phytochemical content, particularly *Andrographolide*, Sambiloto could play a significant role in public health strategies [4]. The quality of herbal medicines represent all factors, directly or indirectly, that influence their safety, effectiveness, and overall acceptability of the product [5]. Accurate quantification of *Andrographolide* in herbal preparations is therefore essential. Various analytical methods have been employed for this purpose, including UV-Vis spectrophotometry [6], RP-HPLC and HPTLC [7-8].

A validated TLC method has also been successful in quantifying *Andrographolide* in polyherbal tablets [9]. Typically, these techniques require a pure compound as a reference standard. The qHNMR method, using internal standards, offers benefits such as shorter analysis time, easier sample preparation, and the elimination of calibration curves [10]. The proportionality between signal intensity and the number of protons in the internal standard and analyte peaks makes qHNMR a versatile method for determining active compound content [11]. This study investigates the use of dibenzyl ether as a novel internal standard for qHNMR, providing a cost-effective alternative for *Andrographolide* quantification in Sambiloto herbal preparations. The selection of an internal standard is critical due to its chemical stability (non-reactive with the analyte), high boiling point, simple ^{1}H-NMR proton signals as a singlet peak with good intensity that doesn't overlap with those of the analyte [12].

This research aims to evaluate and compare *Andrographolide* (AND) content in *Andrographis paniculata* (AP) herbal preparation using these three analytical methods, providing new insights into the use of dibenzyl ether (DBE) as an innovative internal standard especially in ^{1}H-NMR spectroscopy for quantifying various compounds in plant extracts and herbal products without any pure compound. The results of this research expected to contribute to the development of accurate and reliable quantification methods for herbal products and enhance consumer confidence in the use of herbal medicines.

2. Materials and Methods

2.1 Materials

Andrographolide 98% was purchased from Sigma-Aldrich as a reference standard material, Sambiloto (*Andrographis paniculata*) capsules herbal preparation were obtained from online marketplace (manufacturer is not mentioned). Acetonitrile and O-phosphate 0.1% (HPLC

grade) as a mobile phase solvent; methanol; ethanol; and dibenzyl ether 98% as internal standard were purchased from Merck, $CDCl_3$ for NMR solvent from Sigma-Aldrich.

2.2 Methods
2.2.1 UV Analysis
2.2.1.1 Sample Solution Preparation

Dissolved *Andrographis paniculata* herbal preparation powder in ethanol to prepare a 1000 ppm solution. Pipetted and diluted the solution to achieve a final concentration of 200 ppm.

2.2.1.2 Preparation of Stock Solution

Dissolved 1 mg of 98% *Andrographolide* in 10 mL ethanol to make a 100 ppm stock solution.

2.2.1.3 Preparation of External Standard Solutions

Diluted the 100 ppm stock solution to obtain calibration series concentrations of 4, 8, 12, 16, and 20 ppm. Analyzed absorbance values using UV spectroscopy to create a calibration curve (concentration on x-axis, absorbance on y-axis).

2.2.1.4 Preparation of Internal Standard Solution

Mixed each calibration solution (4, 8, 12, 16, and 20 ppm) with 100 ppm Dibenzyl Ether in ethanol at a 1:1 volume ratio. Analyzed absorption to construct an internal standard calibration curve, using linear regression values to determine the *Andrographis paniculata* sample concentration.

2.2.2 HPLC Analysis

The test sample solution made by weighing 0.0035 grams and dissolving it in 2 cc of methanol. Likewise, the standard stock solution made by dissolving 0.0014 grams in 2 cc of methanol, varying the concentrations to 20, 40, 50, 60, and 80% of the standard stock solution injection volume. The chromatographic condition successfully developed for sample and standard solutions of *Andrographolide* analysis at 227 nm, using isocratic mobile phase mode of Acetonitrile: 0.1% O-phosphate (40:60); injection volume of 20 µL; and a flow rate of 1 mL/minute. The results obtained are retention time (minutes) and peak area of each concentrations by Empower-software version-3, and then the amount of *Andrographolide* per unit dose of herbal preparation capsules is calculated.

2.2.3 ^{1}H-NMR Analysis

Dibenzyl ether, *Andrographis paniculata* herbal preparation powder, and *Andrographolide* standard powder each analyzed to confirm peak selectivity. Subsequently, an internal standard method analysis was performed on a mixture containing dibenzyl ether and *Andrographis paniculata* powder in 3 mL of $CDCl_3$. Non-overlapping signals were identified at δ 4.5 ppm for dibenzyl ether and δ 1.25 ppm for *Andrographis paniculata*. Quantitative analysis of *Andrographolide* content in the mixtures conducted by comparing the signal intensities and the number of protons.

3. Results and Discussions

3.1 External and Internal UV-Vis Standards Quantification

UV absorbance measured using a DLAB SP-UV1100. The determination of *Andrographolide* (AND) content in *Andrographis paniculata* was performed by substituting the absorbance value of the 200 ppm sample solution, 0.390337, into the linear regression equation of the external standard calibration curve, $y=0.0379x+0.0206$, with a correlation coefficient (R^2) of <0.99. This equation derived from plotting absorbance versus concentration (ppm) at a maximum wavelength of 227 nm. The *Andrographolide* content was calculated to be 9.76 ppm or 4.87% in the 200 ppm sample solution. In one herbal preparation capsule containing 550 mg of powder, the content was 2.68%. A calibration curve is a regression model used to estimate unknown analyte concentrations by analyzing the instrument's response to known standards. Utilizing an internal standard helps correct for analyte loss during sample preparation and analysis, as long as it is appropriately chosen [13]. The internal standard calibration curve made with Andrographolide external solutions (4 to 20 ppm) with a constant dibenzyl ether concentration (100 ppm) in a 1:1 (v/v) ratio. *Andrographolide*'s wavelength is 227 nm, and dibenzyl ether's is 258 nm.

The calibration curve was derived from the absorbance ratio of AND/DBE versus AND concentrations per 100 ppm DBE. Figure 1 indicates potential overlapping spectra due to additional absorption at 227 nm by dibenzyl ether as internal standard (DBE absorption is not equal to zero at this wavelength). Internal standard calibration curve without correction showed low linearity ($R^2=0.8669$), produced significantly higher absorbance data than the initial absorbance. This increase is due to the absence of a correction or reduction of 50% absorbance at 227 and 258 nm caused by the absorption of dibenzyl ether. As a result, the absorbance data exceeded the measurable range, leading the t-test results to show values less than 0.05 (indicates strong evidence against the null hypothesis). To overcome this problem, we corrected the DBE absorption at this wavelength by reducing the absorbance of *Andrographolide* at 258 nm according to the respective standard concentration (4 - 20 ppm). The corrected absorbance data of the 200 ppm + 100 ppm dibenzyl ether (1:1 v/v) sample solution was substituted into the linear regression equation of the internal standard calibration curve $y=0,0931x + 0,0789$ with a correlation coefficient (R^2) of >0.99.

The *Andrographolide* content was determined to be 8.694 ppm or 4.347% in the 200 ppm sample solution, and 2.39% in one herbal preparation capsule (550 mg). A T-test comparing the absorbance data from external and internal standards showed a P-value of 0.076, which greater than 0.05 (5%), indicating no significant difference between the two methods [14]. UV spectroscopy alone not recommended for extract standardization due to potential interference from other compounds absorbing at the same wavelength. This occurs when multiple chemical species in a sample absorb light at the same wavelength, leading to overlapping spectra and complicating the analysis [15]. To accurately quantify *Andrographolide* in a drug preparation sample, it is necessary to separate each chemical species from the sample and estimate the content using the HPLC method. This ensures a more reliable measurement of *Andrographolide* [16].

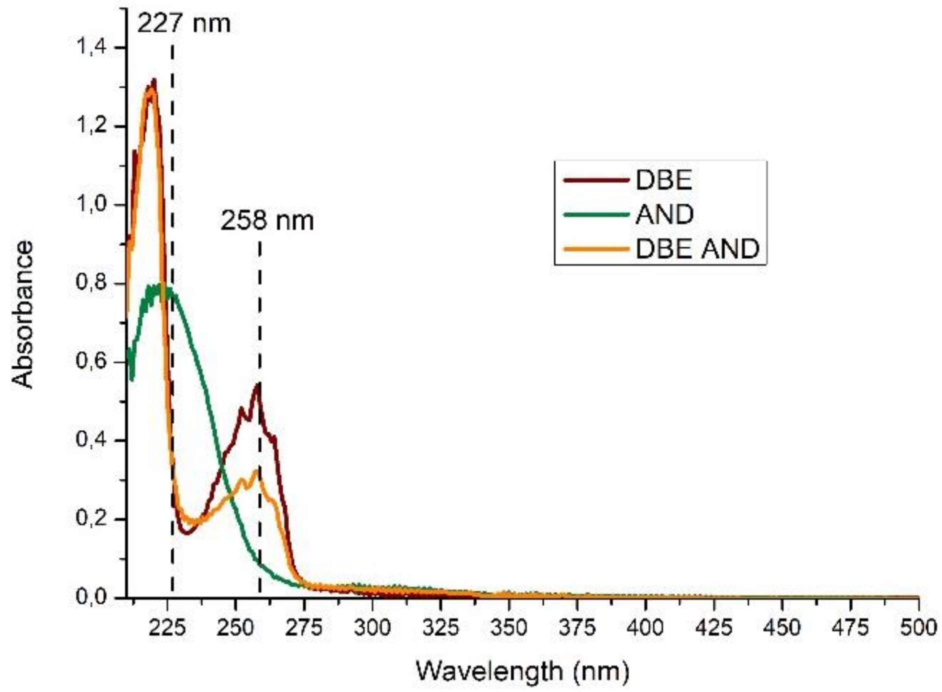

Figure 1. UV overlapping wavelength at 227 nm and 258 nm spectrum of AND, DBE, and DBE AND Internal Solution.

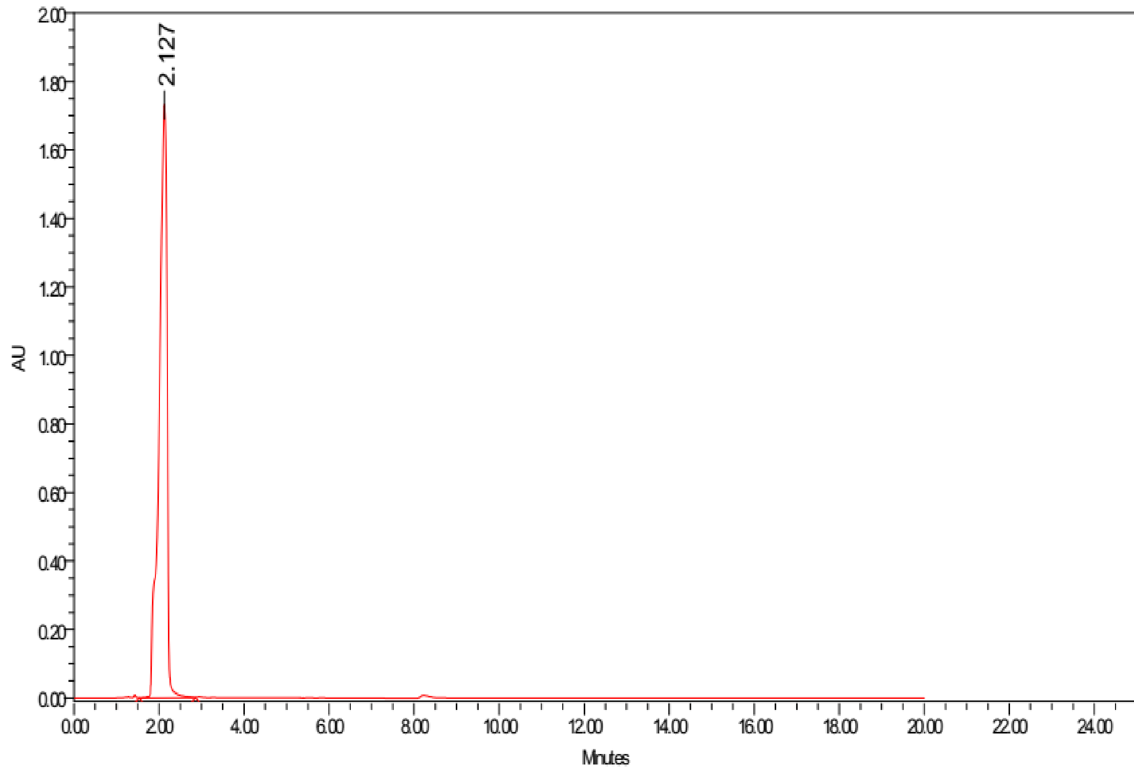

Figure 2. HPLC retention time chromatogram confirmation of *Andrographolide*.

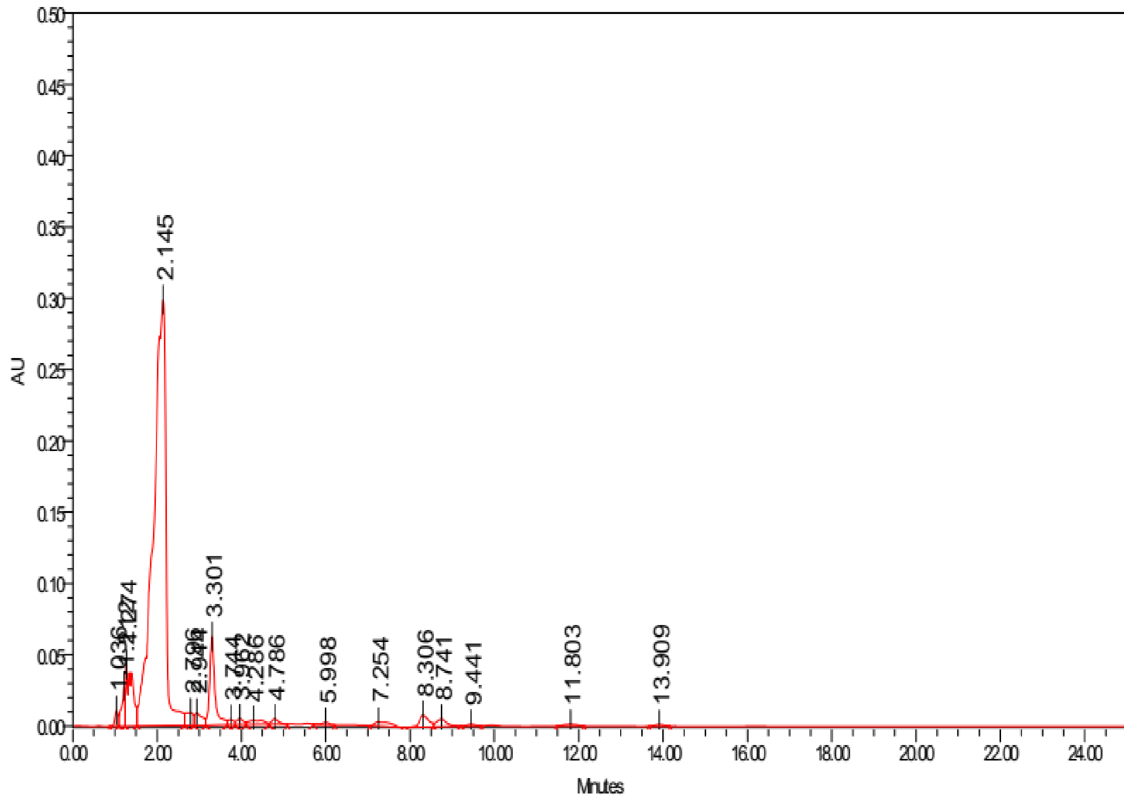

Figure 3. HPLC retention time chromatogram of *Andrographis paniculata* herbal preparation.

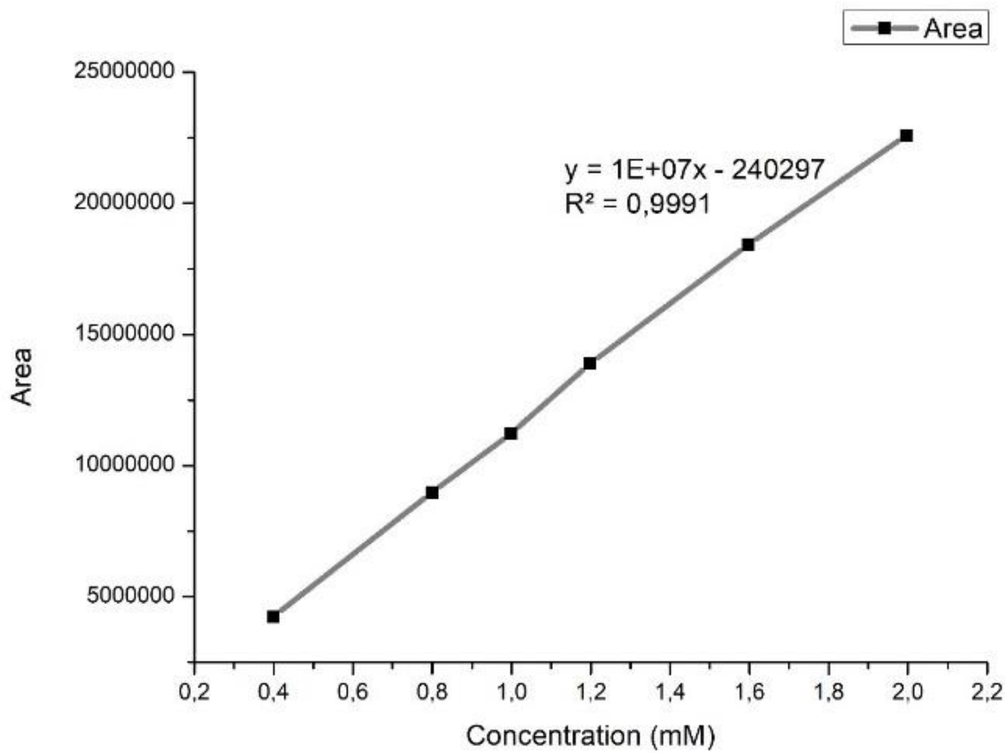

Figure 4. HPLC calibration curve.

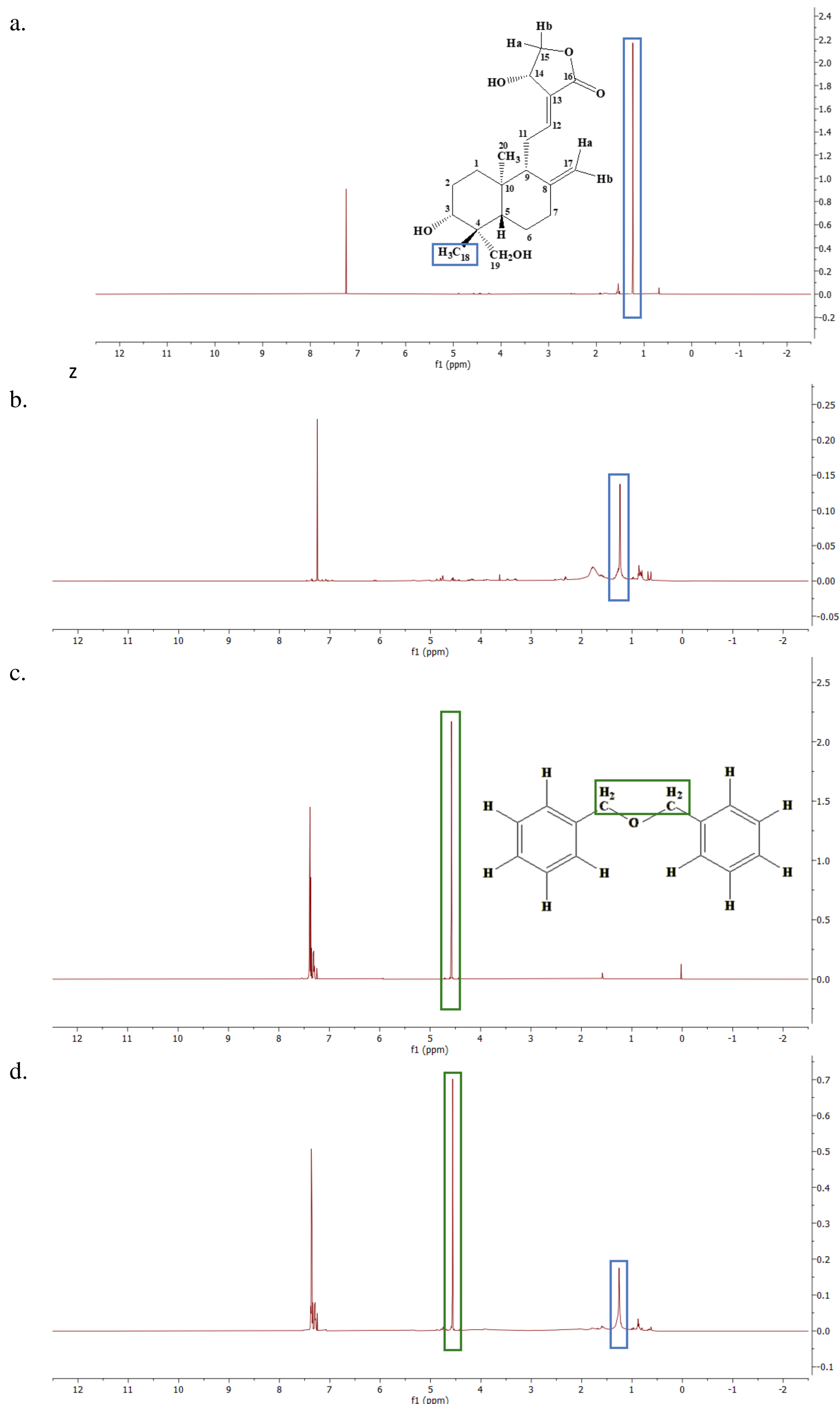

Figure 5. ^{1}H-NMR spectra of (a) *Andrographolide* in $CDCl_3$, (b) *Andrographis paniculata* herbal preparations in $CDCl_3$, (c) Dibenzyl Ether in $CDCl_3$, (d) Internal standard solution mixture of AP and DBE in $CDCl_3$.

Table 1. HPLC Chromatogram *peak areas*.

C (mM)	Area
0	0
0.3994	4251033
0.7988	8986827
0.9985	11225822
1.1982	13889864
1.5976	18437791
1.997	22600481

3.2 HPLC Quantitative Analysis Method

Solutions of the herbal preparation *Andrographis paniculata* and standard *Andrographolide* analyzed using a Perkin Elmer Altus A10 HPLC with an Inertsil 5µm ODS-3 column (4.6x150 mm) to determine the retention time (RT) and peak area. The *Andrographis paniculata* sample showed a retention time of 2.145 minutes with an area of 6228601, as detected by the Altus A-10 UV Detector. This retention time was consistent with the *Andrographolide* retention time shown in Figure 2. Quantitative analysis of *Andrographolide* content in *Andrographis paniculata* herbal preparations was determined using peak area data from the chromatogram and the calibration standard equation y=1E+07x – 240297 with R^2 0,999. The *Andrographolide* content per unit dose (550 mg powder per capsule) calculated to be 0.1925%.

3.3 ¹H-NMR Analysis Results

3.3.1 ¹H-NMR spectra of Andrographolide and Andrographis paniculata preparations

All ^{1}H-NMR spectra were acquired using a JEOL JNM-ECZ500R/S1 operating at 500 MHz. The characteristic signals were identified by MestReNova-15.0 shows in a certain chemical shift of methine olefinic H-12 at δ 6.982-6.958 (td, 1H). The methylene protons (C-17) are respectively H-17a at δ 4.903 (s, 1H) and H-17b at δ 4.589 (s, 1H). Furthermore, the characteristic signals for doublets, each integrating one proton indicate the presence of a hydroxymethylene group, with the doublet of H-19a (d, 1H) at δ 4.203-4.181 and the doublet of H-19b (d, 1H) at δ 3.503 overlapping with signals from H-3 (dd, 1H). The ^{1}H-NMR spectrum shows a resonance at δ 0.702, which is a signal from the methyl group with three singlet protons belonging to H-20 (s, 3H). The spectrum of CH-15, which is two diastereotopic hydrogens (methylene), appears as a signal that integrates one proton each at δ 4,477-4,443 as H-15a (dd, 1H) and at δ 4,274-4,249 as H-15b (dd, 1H). Another singlet peak with good resolution integrates three protons of H-18 [17]. The integration signals from H-15a, H-19a, and H-18 were also present in the *Andrographis paniculata* sample. Dibenzyl ether (shown in Figure 5c above) produced distinct peaks at 4.58 ppm of 4H (two equivalent of CH_2 groups) and 7.43-7.34 ppm, indicating the presence of methylene and aromatic protons, respectively.

3.3.2 Quantitative ¹ H-NMR (qHNMR)

NMR inherently offers the benefit of delivering both qualitative and quantitative data simultaneously [18]. Quantitative analysis using ^{1}H-NMR requires at least one signal integration of the non-overlapping peaks of each compound [19]. Unlike chromatographic techniques, quantitative NMR does not require a pure target analyte as a reference sample for calibration. Since the signal intensity is directly proportional to the number of ^{1}H atoms present, different substances can serve as references, providing significant flexibility in reference substance selection. Additionally, qNMR offers the benefits of simple sample preparation and the ability to recover the analyte [20]. Quantitative NMR also allows the determination of several components in the analyzed sample using one standard reference material and one measurement setting. By simply weighing the dibenzyl ether and the herbal preparation, then dissolving both in the selected solvent, the reference material is incorporated into the solution for analysis [21]. The signal intensity (I), the weighted (m) value of the reference material and extract powder are included in the calculation, enabling the determination of the absolute amount using the equation:

$$m_x = \frac{I_x}{I_{std}} \times \frac{N_{std}}{N_x} \times \frac{Mx}{M_{std}} \times \frac{m_{std}}{m_{powder}} \times P_{std} \times T \qquad [11]$$

Where mx is the mass of the target compound x (*Andrographolide*) in the extract, I_x and I_{std} respectively show the signal intensity of the AP sample of 1.01 and the DBE internal standard of 221.74. N indicates the number of protons from each DBE signal, namely 4H and AP 3H; M is the relative molecular mass for DBE of 198.26 and *Andrographolide* 350.45. m_{std} is the weighing mass of the internal standard compound of 0.0248 grams, m_{powder} is the mass of *Andrographis paniculata* extract powder of 0.0617 grams, P_{std} is purity of the internal standard compound (98%), and T is the average powder mass of one dose/capsule (550 mg). It is known that the percentagemass of target compound x (*Andrographolide*) in one dose/ capsule is 0.233%. These qualitative (Figure 5) and quantitative results further show that DBE can be used as an internal standard providing unique signal chemical shift, sharp singlet peak, high purity, appropriateness solubility in NMR solvent, stable and non-reactive to towards the analyte.

4. Conclusions

Both results of quantitative UV-Vis analysis methods show no significant difference in average values: 2.68% for external standard and 2.39% for the internal standard method. This indicates that the absorbance observed at the maximum wavelength is not solely due to *Andrographolide*; other compounds also contribute to absorption at the 227 nm wavelength, resulting in higher percentage values compared to HPLC and qHNMR. Therefore, the use of UV spectroscopy alone not recommended for extract standardization due to suspected interference from other compounds, necessitating additional methods such as HPLC for analysis. Quantitative analysis using UV and HPLC generally requires a pure standard material suitable for determining a single compound, which is expensive. The qHNMR analysis shows that the percentage mass of the target compound x (*Andrographolide*) in one drug capsule is 0.233%, which is consistent with the percentage mass obtained using HPLC (0.1925%). This further indicates that DBE can be used as an internal standard in qHNMR, which is considered an alternative quantitation method due to its ease of sample preparation, requiring only the weighing of both materials and dissolving them in a suitable solvent, using a single measurement setup, and not requiring a pure standard material.

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